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Original Research Article

Immune-enhancing screening of fourteen plants on murine macrophage RAW 264.7 cells

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Abstract

Purpose: *To investigate the potential immune-enhancing effects of fourteen natural plant extracts on mouse macrophage RAW 264.7 cells.*
Methods: *Fourteen plant extracts from 7 different plants were tested on RAW 264.7 cells to determine their immunostimulant activities. Methylthiazolydiphenyltetrazolium bromide (MTT) and Griess assays were performed to evaluate cell viability and nitric oxide (NO) production, respectively. Then, immune related proteins were measured by western blot analysis, while cytokines and phagocytic activity were determined by enzyme-linked immunosorbent assay (ELISA) method.*
Results: *Among the 14 plant extracts, the hot water extract of Agastache rugose was selected based on the screening results on NO production. The hot water extract of A. rugose significantly increased NO production in a concentration-dependent manner without any cytotoxicity. In addition, the expression levels of proteins (iNOS and COX-2) and cytokines (TNF-α, IL-1β, IL-6 and IL-12) closely related to immune reaction were also significantly upregulated. Furthermore, phagocytic activity of RAW 264.7 cells significantly increased following treatment with A. rugosa.*
Conclusion: *The hot water extract of A. rugosa exhibits significant immune-stimulant activity. Therefore, A. rugosa can be used as a natural resource for immune enhancement or dietary supplement.*

Keywords: *Immune enhancing activity, Macrophage polarization, Natural plant extracts, Agastache rugosa, RAW 264.7*

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INTRODUCTION

The immune system is responsible for protection against infections of bacteria, viruses and fungi [1]. Macrophage is one of the representatives of innate immune cells which perform first line defense during infection initiates [2,3]. Macrophage is ubiquitously found in almost all tissues and plays an important role in immune reactions like phagocytosis [4,5]. In addition to phagocytosis, macrophage has various functions including the production of inflammatory mediators such as nitric oxide, prostaglandins

and cytokines in response to immune stimuli leading to immune cascades [6-9]. These immune players have beneficial effects on cancers by acting as guards against cancer, but sometimes could function as a helper for tumor development through weakening of the immune response [10]. Thus, it is necessary to control its functions when necessary.

Though overactivated immune system damages host's homeostasis, weakened immune system is also significantly harmful to human health. A proper immune reaction could prevent pathologic and cancerous assault. The causes of immune suppression include stress, environmental toxin, sedentary lifestyle, inadequate sleep, alcohol and tobacco abuse, cortisone, chemotherapy, antibiotics, other drug therapy and infection [11].

Therefore, the need for medicines and supplements to boost immune system are increasing, and many researches have been done or ongoing to search for immunostimulatory natural sources which have few side effects compared to chemical drugs [12-15]. Echinacea and ginseng are well-established examples of immunostimulatory natural products [15].

The aim of this study is to investigate the immune-enhancing effects of natural product extracts with a focus on the polarization of the rodent macrophage, RAW 264.7 cells. Seven plants were selected and extracted with two different methods. The first method used 80 % alcohol at room temperature and the other used hot water to extract the natural products.

A total of 14 plant extracts were employed in this research and screened using the expression level of nitric oxide (NO). The hot water extract of *Agastache rugosa* was selected for further studies because it gave the best activity on NO production. The effects of *A. rugosa* on immune related enzymes (iNOS, COX-2), cytokines (TNF-α, IL-1β, IL-6 and IL-12), and phagocytic activity were investigated.

EXPERIMENTAL

Materials and reagents

Griess reagent, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS, E. coli 0111:B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thiazoyl blue tetrazolium bromide (MTT) was acquired from biosesang (Seongnuam, Korea). The antibodies of cyclooxygenase-2 (COX-2), tubulin (TB, house keeper) (Santa Cruz Biotechnology, Dallas, TX, USA) and iNOS (BD Biosciences, San Jose, CA, USA) were used for Western blot. All antibodies of cytokines (TNFα, IL-1β, IL-6 and IL-12) were purchased from BD Biosciences. (San Jose, CA, USA). Ethanol for the extraction of plants was obtained from Samchun (Pyeongtaek, Korea). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Welgene (Gyeongsan, Korea).

Plant material collection and authentication

Seven dried plant materials (Table 1) were purchased from a commercial market (hanyakjae.net, Seoul, Korea) in 2017. Plant materials were confirmed and authenticated by Prof. S-Y Park and all voucher specimens were deposited in Pharmacognosy laboratory of College of Pharmacy, Dankook University.

Extraction

The dried and pulverized part for medical use of each plant was collected (100 g). It was extracted with 80% ethanol at room temperature and with hot water at a temperature that ranged between 80 – 90 °C. In general, the plant powder was 3 – 4 times extracted with enough volume of solvent soaking all powder. In case of 80 % ethanol extraction the exposal time was 6 hours and then the filtrate was evaporated *in vacuo* to yield the extract. For the hot water extraction, the end point was when the volume of the solvent was reduced by half and the filtrate was lyophilized to obtain the extract.

Table 1: Medicinal plants and their part used traditionally in oriental medicine

Plant name	Abbreviation	Medicinally useful part	Family
Buddleja officinalis Maximowicz	BO	Flower	Loganiaceae
Morus alba Linn	MA	Leaf	Moraceae
Cornus officinalis Sielbold et Zuccarini	CO	Fruit	Cornaceae
Paeonia lactiflora Pallas	PL	Root	Paeoniaceae
Agastache rugosa Fischer et Meyer O.Kuntze	AR	Aerial part	Labiatae
Angelica Kiusiana Maximowicz	AK	Aerial part	Unbelliferae
Forsythia suspensa Vahl	FS	Fruit	Oleaceae

Cell culture and MTT assay

Murine macrophage cells (RAW 264.7) were cultured in DMEM supplemented with 10% FBS. The cells were incubated in a humidified 5 % CO_2 atmosphere at 37 °C. The cytotoxicity of plant extracts was determined by MTT assay. RAW 264.7 cells (2.0 x 10^4 cells) were seeded and incubated for 24 h before they were treated with or without plant extracts at given concentrations (20 and 100 µg/mL) for 20 - 22 h. And then 5 mg/mL of MTT solution was added to each well for 3 h, followed with a removing the supernatants and DMSO was used for dissolving formazans. Absorbance was measured with a microplate reader (Biotek, Winooski, VT, USA) at 540 nm. Only DMEM treated group was considered as a negative control.

NO measurement

Cells were treated with plant extracts for 20 - 22 h and then the supernatants were reacted with same volume of Griess reagent (St. Louis, MO, USA) for 15 min. The absorbance was checked at 540 nm using microplate reader (Biotek, Winooski, VT, USA). LPS treated group was used as a positive control.

Western blotting analysis

Cells were seeded in 6-well plate at 2 x 10^6 cells/well and allowed to adhere to plate for 24 hours. And then they were treated with the hot water extract of AR selected from screening at given concentrations (10, 20, 50 and 100 µg/mL) for 20 - 22 h. For preparation of whole cell lysates, media was removed and cells were washed with phosphate-buffered saline (PBS) and lysed in Laemmli buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. The membranes were blocked in 5 % skim milk in PBS. The blots were incubated overnight at 4 °C with primary antibodies diluted in PBS followed by incubation with secondary antibodies conjugated to horseradish peroxidase in 5 % skim milk for 2 h. The target protein was detected by enhanced chemiluminescence (ChemiDoc XRS+, Biorad, Hercules, CA, USA). LPS (1 µg/mL) treated group was used as a positive control.

An enzyme-linked immunosorbent assay (ELISA)

After stimulation with the hot water extract of *A. rugosa* at given concentrations (10, 20, 50 and 100 µg/mL) for 20 - 22 h, the supernatant concentrations of TNF-α, IL-1β, IL-6, and IL-12 were measured by an ELISA. The 96-well plates were coated (overnight at 4 °C) with the capture antibodies. Each well was washed three times with 0.05 % Tween 20 in PBS (PBS-T), incubated with blocking solution for 1 hour at room temperature, and then washed four times with PBS-T. The samples and diluted standards were added, and the plate was incubated overnight at 4 °C. After four wash cycles, the detection antibody was added. After 45 min of incubation at room temperature, the wells were washed. Avidin-conjugated alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA, USA) was added, and the plates were incubated at room temperature for 30 min. After that, a substrate solution was added, and the plates were kept at room temperature for 5 – 30 min before the addition of stop buffer. Absorbance was read at 405 nm on a microplate reader (Emax, Molecular Devices, San Jose, CA, USA). LPS (1 µg/mL) treated group was used as a positive control.

Evaluation of phagocytic activity

The phagocytic ability of RAW 264.7 cells was determined by the CytoSelect™ 96-well phagocytosis assay kit (Cell Biolabs Inc., San Diego, CA, USA), following the manufacturer's instructions. Cells were seeded in 96-well plate at 1 x 10^5 cells/well and allowed to attach to the plate for 24 h. And then cells were treated with the hot water extract of *A. rugosa* (10, 20, 50 and 100 µg/mL), complete DMEM was used as a negative control and LPS (1 µg/mL) was for positive control. Subsequently, non-opsonized zymosan was addition and the amount of engulfed zymosan was measured at 405nm after 2 h incubation at 37 °C by microplate reader (Biotek, Winooski, VT, USA).

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was done by two-tailed Student t-test using Microsoft Excel 2016. Significant differences were considered at $p < 0.05$.

RESULTS

Effect of plant extracts on cell viability and NO production

All plant extracts except 20 and 100 µg/mL of hot water extract of *Angelica Kiusiana* and 100 µg/mL of 80 % ethanol extract of *A. rugosa* did not show any toxicity as relative cell viability was over 80% compared to vehicle treated group (Figure 1). Those three groups with cytotoxicity

were not included in NO production assay. To determine the effect of plant extracts on inducing NO in RAW 264.7, the medium supernatants were reacted with Griess reagent). As shown in Figure 2 A, the hot water extract of *Morus alba* at 100 µg/mL showed mild but statistically significant activity to increase the release of NO. Moreover, the how water extract of *A. rugosa* dramatically induced NO production up to 4 times more than vehicle treated group (Figure 2 A). However, no sign was found in groups treated with all 80 % ethanol extracts (Figure 2 B).

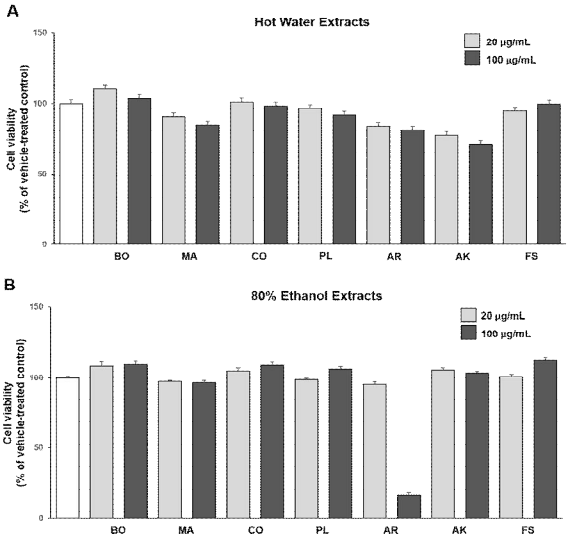

Figure 1: Effect of plant extracts on cell viability. The cell viability is seen in (A) cells treated with hot water extracts and (B) cells treated with 80 % ethanol extracts. The first left bar colored white is the vehicle-treated group. The cell viability below 80 % of vehicle treated group was considered cytotoxicity so 20 and 100 µg/mL of hot water extract of AK and 100 µg/mL of 80 % ethanol extract of AR were excluded from further assays. The values are presented as mean ± SD

Effect of hot water extract of AR on NO production and protein expression

To confirm the effect of hot water extract of AR on NO production, various concentrations (10, 20, 50 and 100 µg/mL) of *A. rugose* were applied to RAW 264.7 cells. The hot water extract of AR significantly increased NO release in a concentration-dependent manner (Figure 3). The treatment with 10 µg/mL (lowest concentration) also increased the production of NO up to 270% compared to vehicle treated group.

COX-2 and iNOS, immune related proteins which can be activated with macrophage polarization, were determined by Western blot.

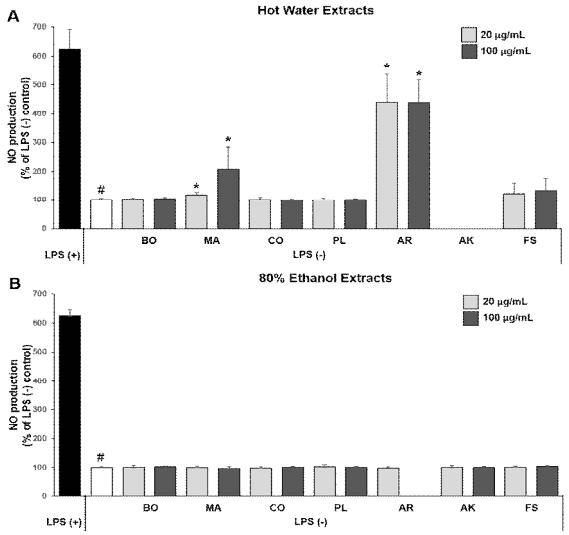

Figure 2: Effect of plant extracts on NO production. The NO expression was measured from media supernatants of (A) cells treated with hot water extracts and (B) cells treated with 80% ethanol extracts. The first left bar colored black is LPS treated group (LPS (+)) and the second left bar colored white is vehicle treated group (LPS (-)). The hot water extract of MA and AR enhanced NO production at both concentrations treated. The increase of NO production was analyzed by two-tailed Student t-test (#$p < 0.05$: LPS (+) vs. LPS (-) and *$p < 0.05$: LPS (-) vs each plant extract treated group). The values are presented as mean ± SD

The hot water extract of *A. rugosa* increased iNOS level in a concentration dependent manner though only 100 µg/mL treated group reached statistical significance (Figure 4 B). On the other hand, this extract elevated COX-2 expression more clearly, that is to say that statistical significance was observed in 10, 50 and 100 µg/mL of treatments compared to vehicle treated group (Figure 4 A).

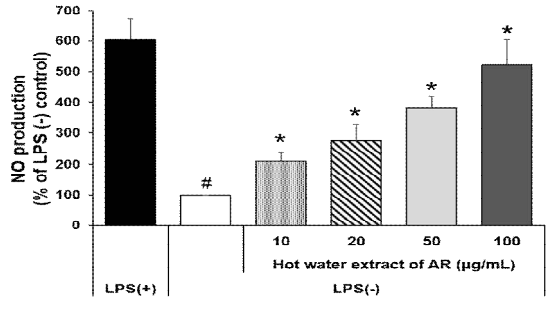

Figure 3: Effect of hot water extract of AR on NO production with a wider range of concentrations (10, 20, 50 and 100 µg/mL). The first left bar colored black is LPS treated group (LPS (+)) and the second left bar colored white is vehicle treated group (LPS (-)). The increase of NO production was analyzed by two-tailed Student t-test (#p < 0.05: LPS (+) vs. LPS (-) and *$p <$ 0.05: LPS (-) vs each concentration treated group). The values are presented as mean ± SD

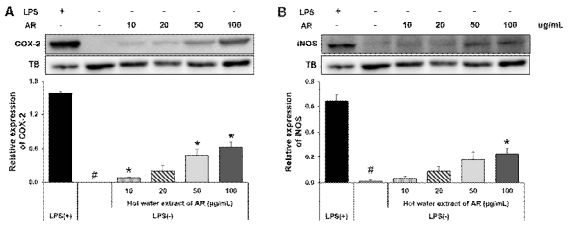

Figure 4: Effect of hot water extract of AR on relative expression of COX-2 (A) and iNOS (B) with given concentrations (10, 20, 50 and 100 μg/mL). The first left bar colored black is LPS treated group (LPS (+)) and the second left bar colored white is vehicle treated group (LPS (-)). The amount of the two enzymes was quantified according to the spot density of each group and followed by normalized by the house keeper protein, TB. The increase of relative expression of enzymes was analyzed by two-tailed Student t-test (#p < 0.05: LPS (+) vs. LPS (-) and *p < 0.05: LPS (-) vs each concentration treated group). The values are presented as mean ± SD

Effect of hot water extract of AR on cytokine expression

Cytokines such as TNF-α, IL-1β, IL-6 and IL-12, which are important in immune responses were measured by ELISA method. The concentration of cytokines was calculated by standard curve of each one. The hot water extract of *A. rugosa* upregulated the production of all cytokines known to be secreted from macrophage concentration-dependently compared to vehicle treated group (Figure 5). In case of TNF-α, the treatment of *A. rugosa* hot water extract increased TNF-α up to the level of LPS treated group.

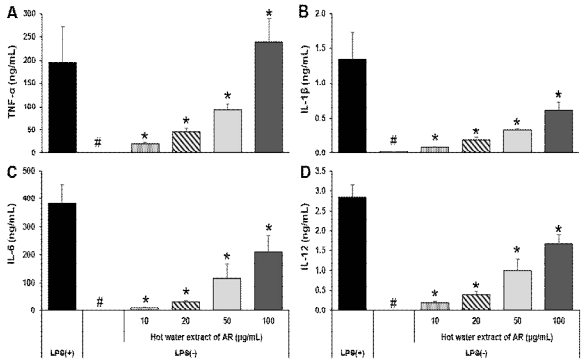

Figure 5: Effect of hot water extract of AR on production of proinflammatory cytokines such as TNF-α (A), IL-1β (B), IL-6 (C) and IL-12 (D) with given concentrations (10, 20, 50 and 100 μg/mL). The first left bar colored black is LPS treated group (LPS (+)) and the second left bar colored white is vehicle treated group (LPS (-)). The concentration of the four cytokines was quantified by ELISA using the standard curve of each one. The increase of relative expression of enzymes was analyzed by two-tailed Student t-test (#p < 0.05: LPS (+) vs. LPS (-) and *p < 0.05: LPS (-) vs each concentration treated group). The values are presented as mean ± SD

Effect of hot water extract of AR on phagocytic activity

Phagocytosis is one of crucial functions that the macrophage conducts for defending the host from xenobiotics. Phagocytic activity was evaluated with a commercial kit through checking the amount of engulfed zymosans isolated from yeasts. The hot water extract of *A. rugosa* significantly enhanced phagocytic ability compared to vehicle treated group in all concentrations employed (Figure 6). Even 10 μg/mL of the hot water extract of AR increased level of phagocytic activity close to the LPS treated group.

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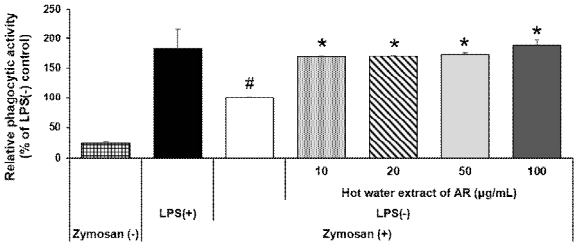

Figure 6: Effect of hot water extract of AR on phagocytic activity with given concentrations (10, 20, 50 and 100 μg/mL). The first left bar colored white with cross stripes is zymosan free group and the second left bar colored black and the third bar colored white are LPS treated (LPS (+)) and vehicle treated group (LPS (-)) respectively. The relative phagocytic activity was normalized by LPS (-) group. The increase of relative expression of enzymes was analyzed by two-tailed Student t-test (#p < 0.05: LPS (+) vs. LPS (-) and *p < 0.05: LPS (-) vs each concentration treated group). The values are presented as mean ± SD

DISCUSSION

In this study a total of 14 plant extracts from 7 plants were obtained using two different methods, 80% ethanol and hot water extraction. After screening for cytotoxicity and NO production in RAW 264.7 cells the hot water extract of *A. rugosa was* selected as the best candidate and used for further study. The hot water extract of *A. rugosa* successfully increased Immune associated proteins and cytokines and phagocytic activity.

Macrophage has two faces depending on the microenvironment in general. When macrophage is exposed to T1 cytokines, IFN-γ, TNFα and GM-CSF, or LPS, it can be polarized to become M1 type [16]. In contrast, M2 polarization of macrophage is initiated with cytokines IL-4 and 13 produced by Th2 cells, mast cells, and

basophil [17]. These two types M1 and M2 show totally different actions in the immune system. M1 macrophages produce proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 and IL-15and chemokine like CXCL10 [16]. In addition, it could express reactive oxygen intermediates (ROI) and NO and MHC class II found on the surface [18]. Consequently, M1 macrophage leads to immune activation that attacks xenobiotics or cancer.

On the other hand, M2 macrophage characterized by high expression of macrophage mannose receptor downregulates iNOS expression with upregulation of argnase-1 expression [19]. Furthermore, cytokines produced by M2 are anti-inflammatory cytokines such as TGF-β, IL-10 and IL-1 receptor antagonist [20]. Of course, M2 macrophage play a beneficial role in autoimmune disease but could be used as a survival strategy for tumor or some bacterial pathogens [21,22]. Thus, unless immune system is abnormally overactivated it is important to keep macrophages in the M1 phase as they are able to augment immune reaction from bacterial and cancerous invasions.

Also, in cases of some pathogen like Salmonella and pancreatic cancer they redirect the phenotype of macrophages from M1 phase to M2 phase for their survival in the hosts. [21]. Moreover, aging, medication like chemotherapy and virus infection such as AIDS cause the immune system to be compromised leading to exacerbation of the host's health [21]. Therefore, it is beneficial and necessary that macrophage should stay polarized in the M1 phenotype for the defense of infections or cancerous assault.

The hot water extract of *A. rugosa* has immunostimulant activity through macrophage polarization. The plant *A. rugosa* (Korean mint) is used as natural medicine traditionally in Asia in particular Korea, to treat colds, anorexia, cholera, vomiting, and miasma [23]. Various essential oils like eugenol and its derivatives, flavonoids such as tilianin and acacetin, and lignans like rosmarinic acid were isolated from *A. rugosa* [24]. Many researches about pharmacological activities of *A. rugosa* have been established including antimicrobial, antifungal, antioxidative [25,26]. The methanol extract of *A. rugosa* and its main component, tillianin showed inhibitory effect on cytokine induced cell adhesion molecule-1 leading to atherosclerosis, a chronic inflammatory disease [27]. Interestingly, Oh et al. reported that 90% methanol extract of AR decreased inflammatory level in rat osteosarcoma cell line stimulated with cytokine mixture [28]. Additionally, rosmarinic acid known as one of the representative components isolated from *A. rugosa* was reported to show anti-inflammatory effect [29]. However, the hot water extract of *A. rugosa* showed immunostimulant effect.

This result suggests that different extraction methods of the same medicinal plant could give completely opposite biological activities due to the different composition of chemical constituents extracted. Flavonoids and lignans such as tilianin and rosmarinic acid as well as some amounts of volatile oil could be obtained by alcohol extraction and they showed anti-inflammatory and antibiotic effect the constituents of hot water extraction might be different.

Most polysaccharides and hydrophilic compounds which have one or two even more sugars in their structures can be extracted in hot water. These compounds might boost the immune system through polarization of immune cells or they might be able to play a role like LPS without any toxic response. β-glucan isolated from mushrooms has been reported to be one of the famous natural immunostimulant polysaccharides and ginsenosides, triterpene glycosides with several sugars also are discovered to have immune enhancing effect [15,30]. Thus, natural resources already reported to have even anti-inflammatory effect could have immune-enhancing activity if the extraction method is changed.

CONCLUSION

The findings of this study show that out of a total of 14 plant extracts tested for immunostimulant activities, only the hot water extract of *A. rugosa* has been confirmed to boost immune reaction via macrophage polarization, based on the expression levels of immune related molecule (NO), proteins (iNOS and COX), cytokines (TNF-α, IL-1β, IL-6 and IL-12) and phagocytic activity. Therefore, the hot water extract of AR is a potential immune-enhancing natural agent and/or dietary supplement. However, these effects require confirmation via *in vivo* studies.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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