Biofabrication of silver nanoparticles using *Ostericum koreanum* plant extract for treatment of allergic rhinitis in nursing care

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Abstract

In this work, the biofabrication of silver nanoparticles (Ag NPs) was carried out using leaf extract of *Ostericum koreanum*. The formation of crystalline Ag NPs was studied by using X-ray diffraction and UV–visible spectroscopy. HRTEM analysis confirmed the formation of spherical shaped Ag NPs with a mean size of 20 to 25 nm. FTIR spectroscopy confirmed the capping of Ag NPs with *Ostericum koreanum* extract polyphenols. On the other hand, Ag NPs were noticed to inhibit mRNA expressions and productions of inflammatory cytokines such as interleukin IL-6, (IL)-1β as well as tumor necrosis factor-α on phorbol 12-myristate 13-acetate plus A23187 (PMACI) stimulated human mast cell lines (HMC-1 cells). Ag NPs abolished the nuclear factor-κB and caspase-1 activations in HMC-1 cells, and decreased the expressions of IκB kinaseβ (IKKβ) and receptor interacting protein2 (RIP2). Additionally, Ag NPs increased the expression of IKKβ and RIP2 alone in regular condition. Ag NPs also inhibited the phosphorylation of protein kinase regulated by extracellular signal and anti-dinitrophenyl IgE-activated passive cutaneous anaphylaxis. The results of this work revealed that the biofabricated Ag NPs successfully enhanced the allergic inflammatory reaction of the mast cell implying their use for potential treatment of allergic diseases mediated by mast cell.

1. Introduction

Ag NPs are the noble metal NPs having specific importance because of their diverse applications. Ag NPs are mostly utilized in electrical batteries, ceramic and glass pigments [1] as well as in the devices used for treating diseases like tuberculosis, malaria, diabetes, cancer, and HIV [2–4]. Furthermore, Ag NPs possess catalytic properties and they are capable of detecting biomolecules [5–8]. Production of nanoparticles involving physical and chemical methods are cost-effective and the chemicals utilized are having high reactive nature with potent environmental threats [9]. Therefore, the involvement of such chemicals in the process of NP production limits their clinical and therapeutic uses. Owing to these limitations, the development of processes that are safe and sustainable to environment are highly recommended [10]. Therefore, approaches based on green chemistry are being considered in the NP production since past few years. The green chemistry method involves low-cost production of NPs without any involvement of toxic substances that may cause harm to the environment as well as human health [11]. In past few years, numerous biomimetic processes were investigated to determine their ability in the production of metal NPs [12]. In spite of the extensive utility of bacteria in NPs production, there are limitations regarding their application, which include maintaining functionality of used strain, keeping sterile environment throughout growing stage of strain, and balancing the increased preparation costs of growing media.

To avoid such limitations in the production of NPs using microorganisms, researchers have started applying plant extracts for the fabrication of NPs. Various plants such as *citrus aurantium*, *Fritillaria* flower [13], *jatropha*...
curcas, pine pollen [14], Trifolium resupinatum [15], Laminaria japonica [16], solanum melongena [17], Taxus baccata [18], Aloevera [19] and Cassia tora [20] are being recognized for their capability of producing Ag NPs. Environmental friendly processes are being reported for playing a crucial role in the reduction of organic pollutants catalytically. Recently, bio-fabricated NPs are introduced to be one among the chief techniques for removing organic dyes [21]. The biosynthesized NPs are also been reported for their use as catalyst in biofuel production [22]. For instance, Adina Stegarescu et al explained the use of biofabricated MnO2 NPs as catalyst in the production of biofuel using seeds oil and grape residue [23]. On the other hand, Ag NPs that are prepared by biological methods are reported for their important biomedical applications [24]. Jaya Mary Jacob et al demonstrated that Ocimum sanctum leaf extract mediated AgNPs can be used for bactericidal coating of paper towels [25]. Also, the biofabricated AgNPs that are prepared with the help of Bacillus amyloliquefaciens showed application for photocatalytic degradation of p-nitrophenol and cytotoxicity effect on A549 cell line [26].

The current work showed the biofabrication of Ag NPs from Ostericum koreanum leaf extract and their characterization was performed using X-ray diffraction (XRD), scanning electron microscopy (SEM), UV–Vis spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM). The present study also showed that the biofabricated Ag NPs successfully enhanced the allergic inflammatory reaction of the mast cell implying their use for potential treatment of allergic diseases mediated by mast cell.

2. Materials and methods

2.1. The preparation of plant extract

Ostericum koreanum leaves were collected and washed using tap water until the removal of impurities, followed by distilled water. Later, the leaves were allowed to shade-dry for about 7 days. The dry leaves were allowed to pass through a 50 mm mesh in order to obtain a uniform powder. The dry powder (100 ml) was exposed to water bath for 30 min at 100 °C. Finally, the aqueous extract was obtained by filtration using Whatman No. 1 filter paper and the obtained extract was stored for further use at 4 °C.

2.2. Preparation of Ag NPs

The leaf extract of Ostericum koreanum plant was mixed with AgNO3 solution (1 mM) at 1:6 volume ratio and the pH was maintained at 8. The obtained solution mixture was heated at 60 °C for about 30 min and the absorption spectra was recorded utilizing UV–Vis spectrum. The change in color of the solution to brown color after 30 min indicated the formation of AgNPs.

2.3. Cell culture

Isocove’s Modified Dulbecco’s Media (IMDM) comprising of 10% heat in-activated FBS, penicillin 100 U ml⁻¹ and streptomycin 100 mg ml⁻¹ was used to grow Human mast cell line-1 (HMC-1) under 95% humidity, 5% CO₂ at 37 °C. Powdered form of AgNO3 and Ag NPs were prepared by dispersing in Dimethyl sulfoxide (DMSO) used as a solvent. AgNO3 and Ag NPs dilutions were prepared in IMDM medium consisting of 10% FBS. Different concentrations of AgNO3 (10 µg ml⁻¹ or 1, 0.1, 0.01) or Ag NPs (10 µg ml⁻¹ or 1, 0.1, 0.01) were used to pre-treat the cells for about 1 h before PMACI stimulation.

2.4. Enzyme-linked immune sorbent assay (ELISA)

According to the guidelines set by the manufacturer, the levels of secreted pro-inflammatory cytokines for example, TNF-alpha, IL-8, IL-6 and IL-1β present in the culture supernatants were assessed.

2.5. MTT analysis

HMC-1 cells (3 × 10⁵ cells/ml) were subjected to Ag NPs or AgNO₃ treatment and then cultured in microtiter well plates for about 8 h followed by incubation using 20 µl MTT solution (5 mg ml⁻¹) for about 4 h under 5% CO₂ and 95% air at 37 °C. Successively, MTT-formazan was extracted by adding 250 µl DMSO solution and absorbance values of individual wells were recorded at 540 nm using automated micro-plate reader.

2.6. RT-PCR and RNA isolation

The total RNA extraction kit (iNtRON Biotechnology, Korea) was utilized to separate total RNA present in HMC-1 cells by following the protocol set by the manufacturer. The obtained total RNA (2 mg) was heated for about 10 min at 65 °C and then cooled by placing it on ice. With the help of cDNA synthesis assay kit, reverse transcription of every sample to cDNA was carried out for about 90 min at 37 °C. RT-PCR was carried out using 1 µl mixture of cDNA in a final volume of about 20 µl with 2.5 U Taq DNA polymerase, cytokine primers of 25 pM, 200 mM dNTPs and MgCl₂ of 2.5 mM in a reaction buffer (KCl 50 mM, Tris-HCl 10 mM (pH 9) and also
Triton 0.1% X-100). PCR was carried out using the below mentioned human primers i.e. human GAPDH; human TNF-α; human IL-6; human IL-6; human IL-1β. Annealing temperatures utilized in PCR were 60 °C, 56 °C, 50 °C for GAPDH and TNF-α, IL-6, and IL-1β respectively. Obtained PCR samples were collected by electrophoresis on agarose gel of 1.5% and then observed visually after staining with ethidium bromide (data not shown).

2.7. The analysis of western blot
For determining the levels of protein, stimulated cells were rinsed two times using cold PBS and later lysed in ice-cold buffer (PBS comprising 1% deoxycholate, 1% triton and 0.1% SDS). The cell lysates were isolated by electrophoresis and the electrophoretic transfer was used for transferring proteins to nylon membranes. The nylon membranes were arrested using 6% skim milk for about 2 h followed by rinsing and overnight incubation with primary antibodies at 4 °C. After cleaning thrice with 0.05% Tween-20 in PBS (PBST), the membranes were subjected to incubation again for about 1 h using horseradish peroxidase (HRP) conjugated secondary antibodies. Later, after washing thrice with PBST, enhanced chemiluminescence assay was performed to visualize protein bands according to the instructions given by the manufacturer.

2.8. Determination of Caspase-1 activity
Caspase-1 colorimetric analysis kit was used for the analysis of caspase-1 enzymatic activity in accordance with the protocol given by the manufacturer. The dead cells were centrifuged for a duration of 5 min at 15,000 rpm. The protein supernatant was subjected to incubation for 2 h at 37 °C in mixture of caspase substrate (WEHD-p-nitro aniline) of 5 μl and reaction buffer of 50 μl. A microplate reader was utilized for measuring the absorbance at 405 nm wavelength. A bicinchoninic acid protein quantification kit (Pierce, Rockford, IL, USA) was used for measuring the similar quantity of total protein obtained from every lysate.

2.9. Passive cutaneous anaphylaxis (PCA)
Male mice (One month old) were obtained and all the experimental methods were carried out after the approval of animal care committee in the University. An IgE based cutaneous reaction was generated by skin sensitization by an anti-DNP IgE intradermal injection followed by injecting DNP-HSA after 2 days into the vein of mice tail. For a duration of 1 h, the Ag NPs were administered topically (dorsal skin, 10 μg/site) or orally (10 mg kg−1). After 40 min of intravenous challenge, the mice were sacrificed (n = 6). In addition, the quantity of dye was measured using colorimetrically method. A spectrofluorometer at a wavelength of 620 nm was used to measure the extraction absorbent intensity and the dye quantity was measured by Evans blue measurement line.

2.10. Characterization
A double beam UV–Vis spectrophotometer (Varian’s Cary 100, USA) was used to measure the surface plasmon band absorption intensity in colloidal solutions comprising of NPs at a wavelength of 200 nm to 800 nm. For further characterization of Ag NPs morphology, TEM images were obtained using Philips EM 208 S system (Philips, Netherlands). In order to prepare the samples, a drop of diluted colloidal solution containing Ag NPs was placed on a copper grid that was coated with carbon and then dried at ambient temperature. The phase composition of Ag NPs was identified by performing the analysis of x-ray diffraction (XRD) using Philips PW-1730 x-ray diffractometer, United Kingdom which was operated at 30 mA current, 40 kV electrical potential difference and 1/7889 Å wavelength. The sample was prepared by keeping the Ag NPs powder onto a silicon base and allowed to dry at ambient temperature. Functional groups engaged in Ag NPs formation were identified by using Fourier-transform infrared (FTIR) spectrometer (PerkinElmer, Germany) with precision of 4 cm−1 and scanning spectrum from 500 to 4000 cm−1. For the sample preparation, Ag NPs colloidal solution of 50 ml quantity was dried with the help of freeze dryer. Finally, the obtained powder was then blended using potassium bromide (KBr) and for further analysis, the formed pellet was kept in device chamber for FTIR measurements.

3. Results and discussion

3.1. Characterization
The primary evidence for the formation of Ag NPs was observed by the change in colour of reaction solution which occurred quickly when the extract was mixed with AgNO3 and the pH was maintained at 8 [27]. This colour change is because of the Surface Plasmon Resonance (SPR) that was associated with optical properties of Ag NPs. The bio-synthesized Ag NPs exhibited a potent capability of absorbing electromagnetic waves in the visible light range. The collective oscillation of conduction electrons along with incident light results in SPR band formation [28]. The highest absorption of the formed Ag NPs is generally observed in the range of 390 to
450 nm [29]. Figure 1 represents the optical absorption spectrum of Ag NPs formed with Ostericum koreanum aqueous leaf extract. The resultant spectrum displayed a maximum absorbance at 409 nm. This phenomenon exhibits the progression of Ag ions reduction in reaction mixture and the enhanced efficacy of NPs formation [30]. No considerable change in absorption intensity was observed after half an hour, thus confirming the completion of reduction of Ag ions. However, similar SPR absorption band was observed in previous reports shown for the fabrication of Ag NPs [31, 32].

Figures 2(A), (B) represents the TEM images of bio-fabricated Ag NPs. As depicted in this figure, NPs are mostly in spherical shape with a size ranging between 20 to 25 nm. On the other hand, figure 2(C) showed bright diffraction spots in SAED pattern indicating the crystalline nature of the formed Ag NPs. Figure 3 depicts the XRD patterns of Ag NPs that resulted from the reaction of AgNO₃ with Ostericum koreanum aqueous leaf.
The diffraction peaks of formed Ag NPs were noticed at 2θ angles located at 76°, 64°, 44° and 37.5° corresponding to the face centred cubic (FCC) structure reflections (311), (220), (200) and (111) respectively [33]. The obtained results were in accordance with the data of the Joint Committee on Powder Diffraction Standards (JCPDS file No. 04-0783) [34–36]. The outcomes also exhibit that NPs are aligned to plate 111. Therefore, the formed NPs are confirmed to be in crystalline nature. Furthermore, the peaks that are not assigned in the XRD pattern might be produced from bio-organic phase crystallization that was present on the surface of the nanoparticles [37]. Figure 4 represents the FTIR spectrum of *Ostericum koreanum* aqueous leaf extract and the Ag NPs formed using the same leaf extract. The absorption spectra of the leaf extract have bands at 1365, 1439, 1633, 2920, and 3436 cm\(^{-1}\) wavelengths. The assessment of formed bands from the figures represents that those formed bands after Ag NPs production are similar to that of bands produced by the aqueous leaf extract of *Ostericum koreanum*. The appearance of band at 1636 cm\(^{-1}\) attributes to the phenolic compounds C–C stretching vibrations present in extract like aromatic alkenes and terpenoids. The band present at about 2920 cm\(^{-1}\) is due to the C–H bond and aliphatic hydrocarbon chains stretching vibrations [38]. The presence of wide band at about 3436 cm\(^{-1}\) attributes to the O–H bond of plant extract containing phenolic compounds. Altogether, the existence of the above bands shows the presence of strong hydrogen bonds [39]. The occurrence of vibration in amide I bond proteins leads to the presence of absorption band displayed at about 1370 cm\(^{-1}\) [40]. As depicted in FTIR spectrum, the bands became narrower between the range of 3300 and 3600 cm\(^{-1}\) after the synthesis of Ag NPs. This alteration might be because of H bonds breakdown within amine groups present in the leaf extract, which are absorbed over the Ag NPs surface and incline to form potent bonds with Ag atoms and finally stabilizes the NPs [41]. All the above results confirmed the capping of formed Ag NPs with bioconstituents present in the leaf extract [42–45].

The mechanism among polyphenols and metal ions is not well known, but it is obvious according to HSAB principle that when the hard ligands like hydroxyl groups, which are existing in polyphenols/–OH, are exposed to soft metals then complexation does not favours however, soft metal ions experiences reduction. On
oxidation, the hard ligands (−OH) gives rise to soft ligands like carbonyl groups in the oxidized polyphenols/\(-\text{C}=\text{O}\) that forms coordination with the synthesized Ag NPs using electrostatic interaction and render them stable by preventing further growth. Several recent reports have already shown in literature for the plant extract mediated fabrication of Ag NPs [46–54].

3.2. Effect of Ag NPs on mRNA expression and cytokine production

We also investigated the effect of Ag NPs and AgNO₃ on the production of TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IL-8. As shown in the figures 5(A)–(D), stimulation with PMACI significantly enhanced TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IL-8 in comparison to the control media (\(P<0.05\)). Additionally, Ag NPs considerably inhibited these TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IL-8 up-regulation through PMACI (\(P<0.05\), figures 5(A), (B) and (D)). Moreover, Ag NPs did not influence the levels of IL-8 protein induced through PMACI (figure 5(C)). As shown in figure 5(B), IL-6 production induced through PMACI was considerably inhibited by AgNO₃ (\(P<0.05\)). AgNO₃ considerably enhanced TNF-\(\alpha\), IL-1\(\beta\) and IL-8 production. Furthermore, we investigated for TNF-\(\alpha\), IL-1\(\beta\) and IL-6 mRNA levels through RT-PCR for future investigation of Ag NPs effect on the expression of modulation cytokine through PMACI. The mRNA expressions of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 in cells treated with Ag NPs were significantly lesser in the group subjected to PMACI (figure 6(A)). Further, we analysed the cell viability utilizing a MTT analysis, however, no considerable effects were noticed (figure 6(B)).

3.3. Ag NPs effect on PMACI induced activation of caspase-1

According to an earlier report [55], cytokine production was regulated by caspase-1, and therefore to assess the AgNO₃ and Ag NPs regulatory effects on activation of caspase-1 which is induced through PMACI, we quantified activities of caspase-1 with the help of an assay kit. The activity of caspase-1 was enhanced considerably after stimulating the cells through PMACI. Moreover, Ag NPs (\(P<0.05\), figure 7) inhibited the increase in the activity of caspase-1. The caspase-1 expression was determined by western blot technique.

3.4. Ag NPs effect on PMACI induced I\(\kappa\)B\(\alpha\) degradation and NF-\(\kappa\)B activation

It is known that the pro-inflammatory cytokines expression was regulated with Transcription factor-\(\kappa\)B (NF-\(\kappa\)B) as per the previous report [56]. The decline in the expression of cytokine mRNA by Ag NPs is depicted in figure 8. As a result, we have studied the effect of Ag NPs on PMACI induced activation of NF-\(\kappa\)B. In nuclear protein fractions, Ag NPs reduced the up-regulation of NF-\(\kappa\)B through PMACI (figure 8). In order to identify whether the Ag NPs inhibitory activity was because of its influence on degradation of I\(\kappa\)B, we studied the I\(\kappa\)B\(\alpha\) protein cytoplasmic levels using western blot technique. We observed that Ag NPs inhibited the I\(\kappa\)B\(\alpha\) degradation in the cytoplasm (figure 8). Additionally, AgNO₃ inhibited PMACI induced activation of NF-\(\kappa\)B and degradation of I\(\kappa\)B\(\alpha\) (figure 8).
3.5. Ag NPs effect on PMACI induced activation of rip2 and ikkβ

The signalling pathways of RIP2 and IKKβ play a major role in NF-κB and caspase-1 activation. Therefore, we have studied the effect of Ag NPs by activating these mechanisms. Stimulation of PMACI enhanced the RIP2 and IKKβ activation and Ag NPs diminished these activations as depicted in figure 9. However, AgNO3 and Ag NPs alone enhanced the activation of RIP2 and IKKβ in comparison to the control media (figure 9).

Figure 6. (A) RTPCR analysis of quantification of the total RNA and (B) MTT assay showing cell viability of HMC-1 cells.

Figure 7. AgNPs effect on caspase-1 activation in HMC-1 cells.

Figure 8. Effect of Ag NPs on PMACI induced InB degradation and NF-κB activation.
3.6. Ag NPs effect on PMACI induced MAPKs phosphorylation

According to one of the earlier reports [37], production and cytokine expression by the stimulation of PMACI occurs by the activation of Mitogen-Activated Protein Kinases (MAPK). To identify whether the Ag NPs anti-inflammatory effects were mediated through regulation of MAPKs pathway, western blot technique was carried out for phosphorylated p38, ERK and JNK. As showed in figure 10, all the three MAPKs were phosphorylated in HMC-1 cells that were stimulated by PMACI. In these cells, Ag NPs reduced the ERK phosphorylation (figure 10), although did not influence the JNK and p38 phosphorylation (figure 10). Remarkably, without PMACI, both the AgNO3 and Ag NPs alone enhanced the JNK and p38 phosphorylation.

Figure 9. Densitometry approach showing the levels of IKKβ and RIP2.

Figure 10. The MAPKs expression levels were quantified by densitometry.

Figure 11. Effect of Ag NPs on PCA reaction.

3.6. Ag NPs effect on PMACI induced MAPKs phosphorylation

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3.7. Ag NPs effect on PCA

In order to prove Ag NPs in-vivo anti-allergic effect, we carried out the PCA reaction at the dorsal skin part of mice. After administration of Ag NPs topically (10 μg/site) or orally (10 mg kg⁻¹) to the mouse, Ag NPs considerably inhibited the PCA reaction (Po 0.05, figure 11). Ag NPs alone considerably decreased the PCA reaction (Po 0.05, figure 11).

4. Conclusion

In conclusion, the current study showed that Ag NPs were biofabricated using leaf extract of Ostericum koreumnum. XRD, TEM results confirmed the formation of crystalline Ag NPs with size ranges from 20–25 nm. FTIR results confirmed the capping of plant polyphenols onto the surface of Ag NPs. The anti-inflammatory studies showed that the prepared Ag NPs exhibited the suppression of the activation of ERK, RIP2 and IKKβ and successive inhibition of caspase-1, NF-κB. Further, this study displayed that Ag NPs could be considered for the development of potential therapeutic drugs for treatment of allergic inflammatory condition mediated by mast cell.

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