The effect of Punica granatum nanoparticles in phagocytic cells in patients with Multiple Myeloma

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ABSTRACT

Multiple myeloma is a cancer that forms in a type of white blood cell called a plasma cell. Plasma cells help you fight infections by making antibodies that recognize and attack germs. This study aimed to investigate effect of aqueous extract and silver nanoparticles from Punica granatum peel on the ability of phagocytic cells in patients with Multiple myeloma. Chemical components of Punica granatum were investigated. It was found that they contained most of chemical compounds such as Glycosides, Flavonoids, Alkaloids, Saponins, Tanins, Resins, Terpenes, and Steroids. Our results showed the presence of a significant decrease ($p<0.001$) in ability of phagocytic cells in patients, when the results were compared to the control group which included healthy individuals. While, the results showed increase in the percentage of phagocytic cells after added Punica granatum peel silver nanoparticles by increases activity of NADPH enzyme. This increase indicates that the Punica granatum peel silver nanoparticles contain chemical components which act as immune modulators by increase of the percentage of phagocytic cells to engulf bacteria.

Keywords: Peel extract, nanoparticles, Silver nitrate, Phagocytic cells, Multiple myeloma

تأثيرالحبيبات النانوية لمستخلص قشور الرمان على الخلايا البلعمية لمرضى المايلومة المتعددة

الخلاصة

المايلومة المتعدده هو مرض سرطاني يصيب الخلايا البلازمية مما يؤدي الى تكاثر هذه الخلايا بشكل غير طبيعي وتكدسها في نخاع العظم ويشكل نسبة 10% من الاصابات بسرطان الدم. هيأت النباتات الطبية بمختلف انواعها قاعدة اساسية ومهمة لأنظمة الطب التقليدية اذ تتم الاستفادة منها في

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توفير بعض انواع العقاقير التي تتميز بخلوها من الجوانب السلبية المؤثرة على اعضاء جسم الانسان على العكس من العقاقير الكيميائية التي لاتخلو من بعض تلك الجوانب.
لذلك هدفت الدراسة الحالية الى أختبار تأثير الجزيئات النانووية لمستخلص قشور الرمان المائي في فعالية الخلايا البلعمية لمرضى المايلومة المتعددة Multiple myeloma ، اذ تم تحضير المستخلص المائي لقشور الرمان وكذلك تم تحضير الجزيئات النانووية بطريقة نترات الفضة وتم التحري عن المحتوى الكيميائي للمستخلص المائي لقشور الرمان فوجد احتواءه على مركبات كيميائية عديدة مثل الكلايكوسيدات والفلافونات والقلويدات والصابونيات والتانينات والتربينات والراتنجات والستيرويدات.
تم أختبار فعالية الخلايا البلعمية على الالتهام لمرضى المايلومة المتعددة ومجموعة السيطرة قبل وبعد اضافة المستخلص المائي والجزيئات النانووية لقشور الرمان. أظهرت نتائج الدراسة الحالية قبل اضافة المستخلص وجود انخفاض معنوي كبير $P<0.001$ في فعالية الخلايا البلعمية على الالتهام لمجاميع المرضى عند مقارنة النتائج بمجموعة السيطرة. أما بعد أضافة المستخلص المائي والجزيئات النانووية لقشور الرمان فقد أظهرت النتائج أرتفاعا ملحوظا في فعالية الخلايا البلعمية عن طريقة زيادة فعالية انزيم NADPH .هذه الزيادة قد تكون دليل على فعالية الجزيئات النانووية لقشور الرمان وعملها كمحورات مناعية من خلال زيادة النسبة المؤوية لقابلية الخلايا البلعمية على الالتهام.

INTRODUCTION

Multiple myeloma is a cancer of plasma cells that develops in the bone marrow [1]. Plasma cells are found in the bone marrow and they are playing important roles in immune system through produce humoral factors which is called antibodies. In multiple myeloma plasma cells produce large number of antibody called monoclonal (M) protein [2]. Cancer chemotherapy is the use of drugs to remove or control cancer cells. These drugs can be taken by many ways such as mouth or given in a I.V. or a I.M. They enter the bloodstream and reach all areas of the body, making this treatment useful for cancers such as multiple Punica granatum myeloma that often spread widely. Chemotherapy drugs destroy tumor cells but also can damage normal cells. They are given carefully to avoid or reduce the side effects of chemotherapy. The side effect of chemotherapy including reduce in white blood cells number and their function such as phagocytosis. These side effects depend on the type, dose of drugs given, the length of time they are taken, sex and age of patients [3]. Plant extract could be an alternative to traditional chemical methods for the production of metallic nanomaterials in a clean, nontoxic and ecologically sound manner [4]. Punica granatum is a wide fruit that cultivated throughout the Mediterranean regions. Previous studies were demonstrated on the antioxidant properties of Punica granatum [5]. It was proves that Punica granatum contains some species of flavonoids and anthocyanidins, and shows potent antioxidant activity [6]. In particularly, the most essential constituents of pomegranate peel are phenolic compunds; gallic acid and other fatty acids; flavonols; flavones, flavanones; and anthocyanidins [5]. Recently, biosynthesis of nanoparticles using plant extract has emerged an easy and viable alternative to traditional chemical and physical methods. Synthesis of nanoparticles using plants can provide more biocompatible nanoparticles than chemical synthesis. Whereas chemical synthesis may lead to the presence of some toxic chemical species on the surface of nanoparticles that may have undesirable effects in some biomedical applications [7]. Preparation of gold and silver nanoparticles by living plants were firstly reported by Gardea-Torresdey *et al.*, 1999[8]. The use of plant extract for the synthesis of nanoparticles could be advantageous over other environmentally benign biological processes by reducing the complicated methods of maintaining cell cultures [9].Previously it was reported that plant extracts that contain different constitutes like polysaccharides, antioxidant

metabolites, phenolic compounds and flavonoids [10] have been used for the biosynthesis of nanoparticles. We aimed to develop a rapid ecofriendly method for the synthesis of silver nanoparticles using Punica granatum peel extract. Our results demonstrated the silver nanoparticles of Punica granatum peel extract increases of phagocytic activity of patients with Multiple myeloma through induces of NADPH enzyme activity. The synthesis of silver nanoparticles by this method is extremely fast, ecofriendly and stable for several weeks with no additional surfactants.

Materials and Methods

Preparations of Punica granatum peel extract.

40 grams powder of dried Punica granatum peel was soaked in 300 ml of deionized water. In this process, extraction is done by incubating the flask on an orbital shaker (Genex. Cat.No. 60HZ, USA) at the room temperature and agitating at 100 rpm for 48 hours. After the extraction, the aqueous solution was filtrated twice by using Whatman filter paper No.1 (Whatman. Cat.No. Q/FML61-2004, England) [4].

Synthesis of Silver nanoparticles

9 ml of 2mM of silver nitrate solution was prepared. 1 ml of freshly prepared pomegranate peel extract was added separately to the silver nitrate solution. The bioreduction of AgNO3 ions occurred within 3hrs (figure1). Peel extract its yellow color solution which turned into the dark brown color slowly indicate the formation of silver nanoparticles [11].

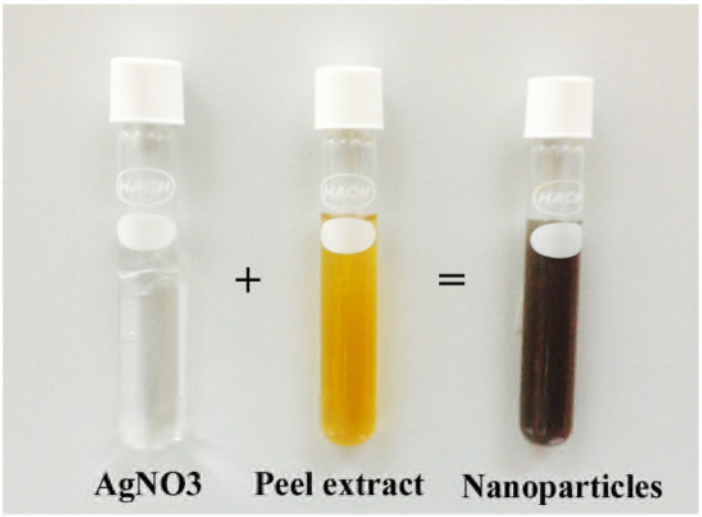

Figure (1)Synthesis of Silver nanoparticles.

Preparation of Staphylococcus aureus

Staphylococcus aureus were cultured overnight at 37°C in Nutrient Broth (HIMEDIA. Cat.No.M002-500G, India), harvested by centrifugation at 3000 rpm for 20 min and then washed three times with PBS (Sigma. Cat.No. 4417, USA) . For opsonization, $1x10^6$ bacteria were incubated for 30 min at 37°C under slow rotation (4 rpm) in 1 ml HBSS supplemented with 0.1% (wt/vol) HBSS (Sigma. Cat.No. H6648,USA) and 15% (vol/vol) heat-inactivated human serum from the blood of

healthy donors with blood group AB. After two washes with ice-cold HBSS, the bacteria were suspended in this medium at a concentration of $1x10^7$ bacteria/ml [12].

Phagocytosis of S. aureus by cells

Phagocytosis of opsonized *S. aureus* by cells was determined as described before (REF). Briefly, equal volumes of serum-opsonized *S. aureus* and blood samples were incubated at 37°C for 45 minutes. A sample was centrifuged for 5 minutes at 200 rpm, and the number of bacteria in the supernatant was determined
Microscopically after stained the phagocytic cells with Gimsa's stain. Phagocytic index is expressed as the percentage of phagocytic cells which calculated by this equation Phagocytic index =Phagocytic cells /Total cells number (Phagocytic cells + Non-phagocytic cells) $*100$ [12].

Isolation of BMDMs

Primary bone marrow macrophages BMDMs were isolated as described from C57/BL6 mice [13]. Briefly, mice were sacrificed by cervical dislocation and femurs and tibias were dissected out. Femurs and tibias were flushed with media using a 25G needle (BD. Cat.No.300600, USA) to obtain bone marrow mononuclear phagocytic precursor cells. To remove tissues and debris cell suspension was passed through a Nitex mesh (Cadisch Sons, London, UK). Cells were cultured in RPMI-1640 supplemented with 10% heat inactivated FCS, Streptomycin (100 µg/ml), Penicillin (100 IU/ml), L-glutamine (2mM) and 25 mM Hepes solution (Sigma.Cat.No.709-20,USA) pH 7.3 on 9 cm plastic Petri dishes (Sterlin. Cat.No.24998, UK) at a concentration of 3×10^6 cells/plate. Macrophages were selected by addition of M-CSF 10ng/ml (Peprotech. Cat.No.315-02, UK). Cells were cultured at 37˚C, 5% CO_2 and allowed to grow for between 6 – 9 days before use.

Cell viability assay

Cell viability was determined by exclusion of trypan blue. 50µl of the cells were incubated at room temperature for 3 minutes with 50µl of 0.04% (w/v) trypan blue (Sigma. Cat. No. T8154, USA) in PBS and viable cells excluding trypan blue counted in a hemocytometer (Superior. Germany). Cell viability in all assays was >85%. In addition, the viability of the cells remained comparable throughout all time pointes used in this study [13].

Bacterial cultures

P. areuginosa were cultured in LB broth (Invitrogen. Cat. No.12780-052, USA) to mid-log phase (OD 0.4-0.6) immediately prior to use. The bacteria were then centrifuged at 3500 rpm for 15 minutes at 4˚C, the pellet was washed twice in sterile PBS and then resuspended in the same Pen Strep -free media as the cells being infected to a concentration of approximately 1×10^6cfu/µl=(OD600/0.4)*1.8ml[14].

Internalization assay

Viable intracellular bacterial counts were determined by a gentamicin protection assay as described in [15].

Measurement of NADPH activity

NADPH Assay Kit (abcam. Cat.No.ab65349, UK) provides a convenient tool for sensitive detection of the intracellular nucleotide: NADPH. The enzymes in the

system specifically recognize NADPH in an enzyme cycling reaction. The reaction specifically detects NADPH. The enzyme cycling reaction significantly increases detection sensitivity. Results can be quantified using plate reader at OD450 nm. We used standard protocol described by manufactured.

Statistical analysis

Values are expressed as the mean ± SD. Two-tailed unpaired Student's *t*-test was used to compare means. $P<0.05$ was considered statistically significant. Analyses were processed using GraphPad Prism software for Windows (version 5.0, GraphPad Software, Inc., San Diego, CA).

Results and discussion

Characterization of silver nanoparticles

UV-visible spectrum analysis

Synthesized nanoparticles were confirm by UV-visible spectroscopy (Shimadzu, UV-1650 PC, Japan), operating in wave length (300-1100) nm (Figure 2). The peels extract its Yellow color solution which is changed into the dark brown color. Reduction of Silver ions in to silver nanoparticles during exposure to peel extract was observed as a result of color changes (Figure 1). The samples were observed by UV-visible spectrophotometer (Figure 2).The sharp bands of silver nanoparticles were observed around at (440) nm.

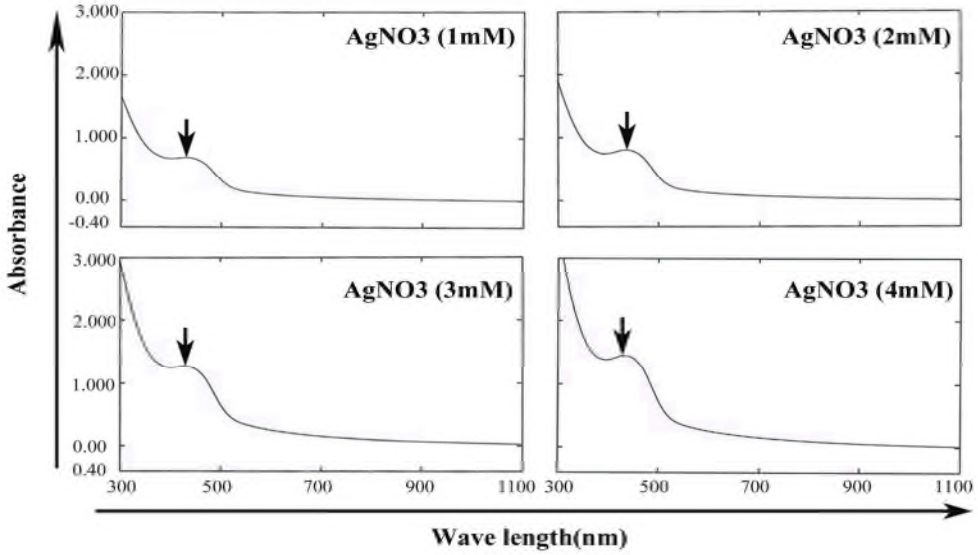

Figure (2) UV-visible spectra of silver nanoparticles.

Fourier transforms infrared spectroscopy

The FTIR measurements were carried out to identify the natural products for the reduction of silver ions to silver nanoparticles present in Punica granatum peel extract (Figure 3). The FTIR spectrum of peel extract peaks appear at 3421.83, 2929.97, 2362.88, 1735.99, 1618.33, 1452.45, 1055.10, 877.46, 775.41, 624.96 cm-1 respectively. The band at 3421.83 cm-1 indicates phenolic OH. The band 2929.97 cm-1 CH plane bends to alkenes. The bands at 1735.99 and 1618. 339 cm-1 corresponds to N-H bends to 1^{o} amines. The band at 1452.45 cm-1 is corresponds to

C-C stretching vibration to aromatics. The band at 1055.10 cm-1 corresponds to C-H Wag to alkyl halides. The band at 775.41cm-1 corresponding to C-H to aromatic group. The band at 877.46 cm-1 corresponding to C-CI stretching to alkyl halides. While the band at 624.96 cm-1 indicates C-H bending of alkynes

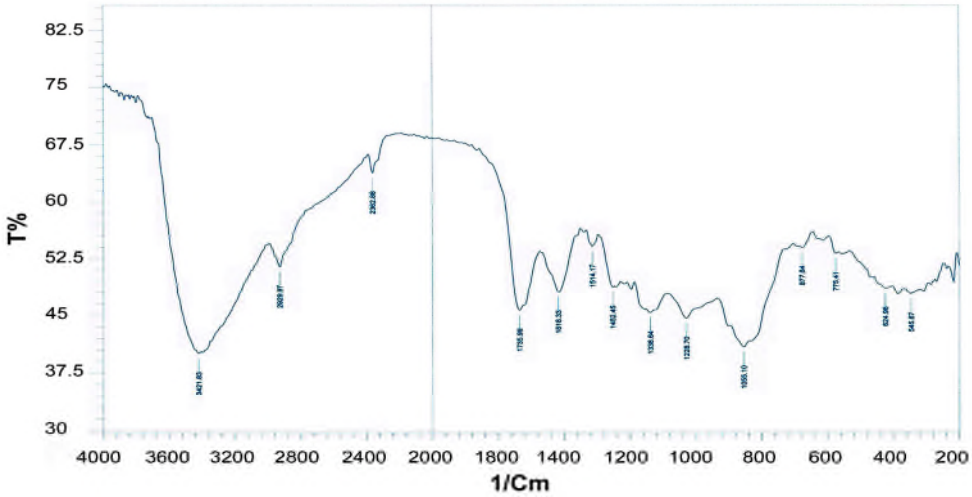

Figure (3) FTIR adsorption spectra of silver nanoparticles synthesized by Punica granatum peel extract.

X-Ray differentiation analysis

The XRD pattern thus clearly shows that the AgNO3 nanoparticles formed by the reduction of Ag ions by Punica granatum peel are crystalline in nature (Figure 4). Our results similar output was obtained by [11, 16].

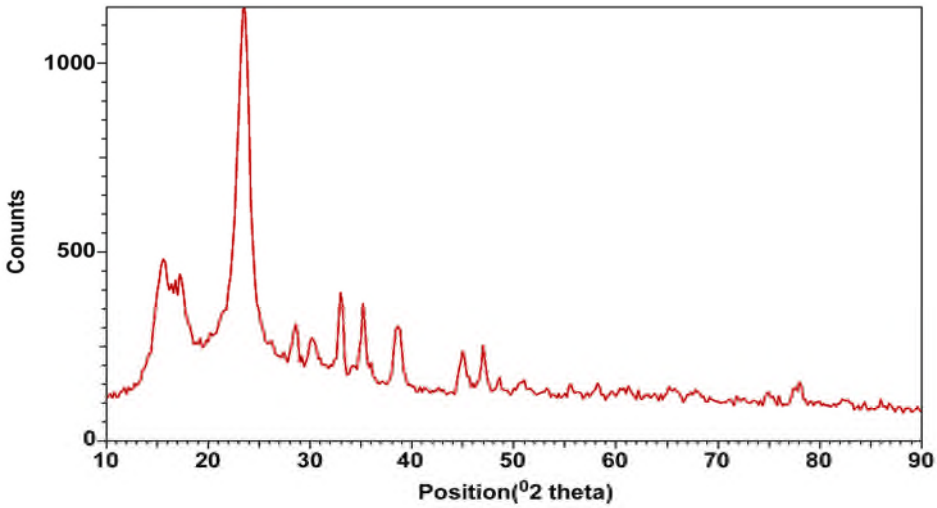

Figure (4) XRD of silver nanoparticles using Punica granatum peel extract.

Scanning electron microscopy (SEM)

SEM technique was employed to visualize the size and shape of Ag nanoparticles. Dried powder of the silver nanoparticles was placed on carbon-coated copper grid. The SEM characterizations of the synthesized Ag nanoparticles are shown in (Figure

5). Image of SEM showed relatively spherical shape nanoparticle formed with diameter range 15-70nm. The nanoparticles were examined under various magnifications power.

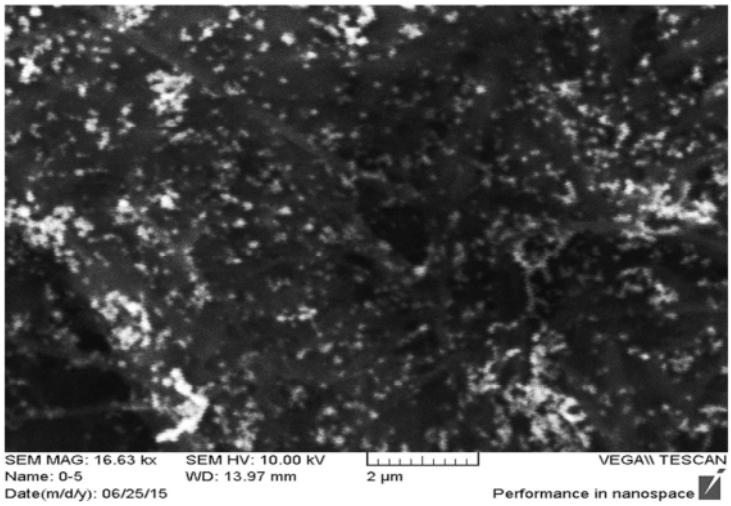

Figure (5) SEM image of silver nanoparticles.

Silver nanoparticles increases phagocytosis through induces NADPH activity

Phagocytosis is a process by which microorganisms or foreign bodies are engulfed, killed, and digested by phagocytic cells. Phagocytes, involving neutrophils, monocytes and macrophages, play an important role in the resistance against infections. These cells express several receptors involved in the antimicrobial functions, including receptors for the Fc part of immunoglobulin G (Fcg receptors, FcgR), complement components and specific glycosylated molecules [17]. To test role silver nanoparticles to increase phagocytosis process in patients Multiple myeloma. We did Intracellular killing of *S. aureus* by FcgRIIa expressing cells (Neutrophils and Monocytes). Our results showed significant increase in the ability of phagocytic cells in the presence of 10 µg/ml of silver nanoparticles when compare the results with patients samples in absence of peel extract silver nanoparticles (Table 1, 2), (Figure 6) respectively.

Table (1) Activity of phagocytic cells (Monocytes) in patients with Multiple in the presence and absence of silver nanoparticles (10µg/ml).

Groups	Control	Multiple myeloma	Multiple * myeloma	Multiple ** myeloma
Mean ± SEM	93.00 ± 2.082	35.00 ± 2.889	49.67 ± 1.453	60.33 ± 3.383

*** Punica granatum peel extrac**

****Punica granatum peel extrac nanoparticles**

Table (2)Activity of phagocytic cells (Neutrophils) in patients with Multiple in the presence and absence of silver nanoparticles (10µg/ml).

Groups	Control	Multiple myeloma	Multiple myeloma *	Multiple myeloma **
Mean ± SEM	90.67 ± 2.603	31.00 ± 1.528	44.33 ± 2.333	58.00 ± 3.606

*** Punica granatum peel extrac**

****Punica granatum peel extrac nanoparticles**

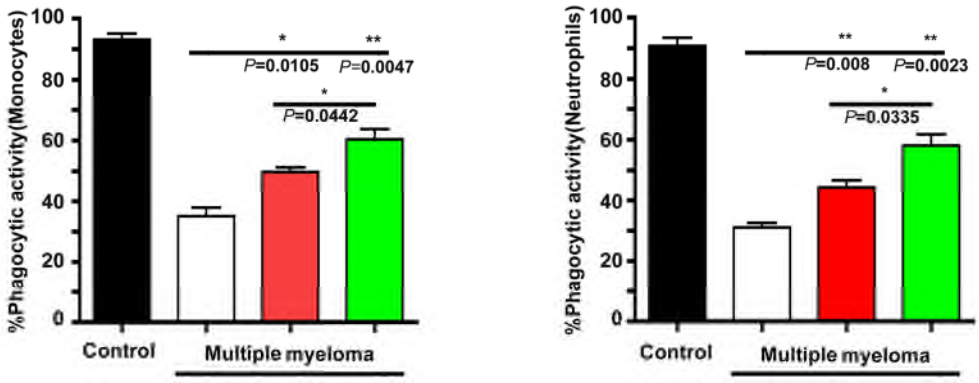

Figure (6) Silver nanoparticles increases phagocytosis in patients with Multiple myeloma.

Black column (Normal control) White column (Multiple myeloma samples), Red column (Multiple myeloma samples in the presence of Punica granatum peel extract 10µg/ml), Green column (Multiple myeloma samples in the presence of Punica granatum peel extract nanoparticles 10µg/ml).

To determine whether oxygen dependent microbial mechanisms are involved in the intracellular killing of Staphylococcus aureus by phagocytic cells. Next, we examined the net effect of these changes on the clearance of P. areuginosa exposed to BMDMs in the presence and absence Punica granatum peel extract silver nanoparticles at concentration 10µg/ml . Phagocytosis of the microbe will be followed by intracellular killing; we thus measured the numbers of viable intracellular bacteria present within macrophages as an indicator of the ability of these cells to clear the infection (Figure 7). In presence of peel extract the results showed reduced the numbers of viable intracellular bacteria; a significant reduction was achieved by pretreating the macrophages with silver nanoparticles 10µg/ml (Figure 7). Then, we tested activity of NADPH enzyme in BMDMs after infected them with Pseudomonas areuginosa in the presence and absence of Punica granatum peel extract nanoparticles. Our results showed a significant increase in activity of NADPH in cells pretreated with peel extract nanoparticles (Figure7).

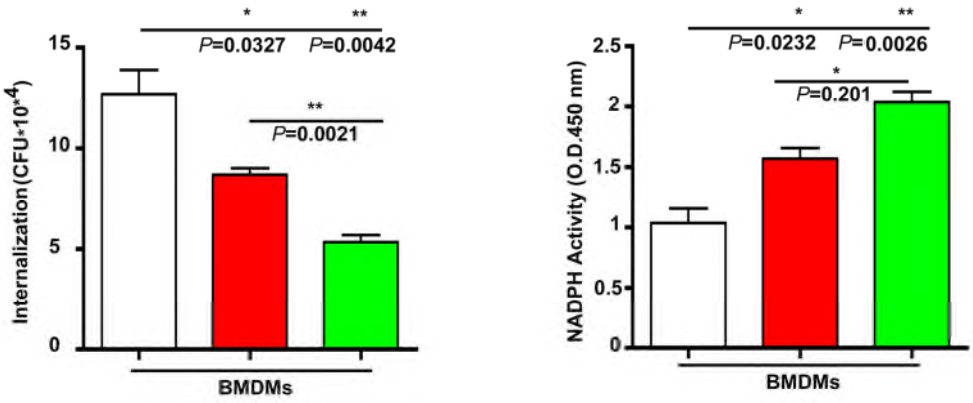

Figure (7)Silver nanoparticles induce activity of NADPH enzyme.White column (BMDMs alone samples), Red column (BMDMs samples in the presence of Punica granatum peel extract 10µg/ml), Green column (BMDMs samples in the presence of Punica granatum peel extract nanoparticles 10µg/ml).

Green methods for silver nanoparticles synthesis using eco-friendly and non-toxic compounds are possible. Bio-synthetic methods employing naturally occurring reducing agent as well as capping agent such as polyphenol, Tannic acids or plant extract. The systematic techniques such as UV-visible spectroscopy, FTIR, XRD, and SEM are applied to characterize the synthesized nanoparticles. The phase formation of Ag was confirmed by X-ray diffraction pattern. The nanoparticles size range of Ag was15-70nm measured by SEM. The silver nanoparticles synthesized using Punica granatum peel extract showed the ability to enhanced phagocytosis in patient with Multiple myeloma. From the technological point of view these obtained silver nanoparticles have potential applications in the biomedical field and simple procedure has several advantages such as cost effectiveness, compatibility for medical and pharmaceutical applications as well as large scale commercial production. The production of metallic nanoparticles from Punica granatum extract is a rapid, easy and clean way providing an innovative methodology to Nano biotechnology.

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