In Vitro study for hesperidin nanoparticles effect on phagocytic activity against *Staphylococcus aureus*

دراسة تأثير دقائق الهزبردين النانوية على فعالية البلعمة في المختبر ضد بكتيريا المكورات العنقودية

Saja H. Ali Ghassan M. Sulaiman Mohammad M. F. Al-Halbosiy*

Applied Science Department/ University of Technology

*Biotechnology Research Center/ University of AL- Nahrain

سجى حسين علي غسان محمد سليمان محمد محمود فرحان الحلبوسي*

قسم العلوم التطبيقية/ الجامعة التكنولوجية

* مركز بحوث التقنيات الاحيائية/ جامعة النهرين

E-mail: sajahussein264@yahoo.com

Abstract

Hesperidin is one of the flavonoids from citrus peels and it recognized to possess various biological activities such as, anti-inflammatory, anti-carcinogenic, antioxidant and antimicrobial potentials. The present investigation studies the immunological adjuvant influence of hesperidin nanoparticles. Hesperidin nanoparticles were prepared by nano-precipitation technique by using Poly (D, L-lactic-co-glycolic acid) (PLGA) polymer and Poloxamer 407 was used as a stabilizer. This method was used because of their advantage of low setup cost and simplicity. Hesperidin nanoparticles were characterized by fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), X-ray diffraction (XRD) and particle size analysis (PSA) analytical methods. The effect of hesperidin nanoparticles was higher than the effect of pure hesperidin, and there was an obvious increase in phagocytosis index (PI 82%) of hesperidin nanoparticles when compared with pure hesperidin (PI 56%) and in comparison with the control samples (PI 22%). In conclusion we need further studies about if nano-hesperidin has therapeutic effects.

Key words: Hesperidin nanoparticles, PLGA, Poloxamer 407, *Staphylococcus aureus*, phagocytic activity

الملخص

الهزبردين هو احد الفلاوفونيدات من قشور الحمضيات والمعروف بامتلاكه الكثير من الفعاليات البايولوجية مثل مضاد للالتهابات، مضاد للسرطان، مضاد للأكسدة ومضاد للمايكروبات. البحث الحالي يدرس التأثير المساعد المناعي لدقائق الهزبردين النانوية. حضرت دقائق الهزبردين النانوية باستخدام تقنية الترسيب النانوي باستخدام بوليمر Poly (D,L-lactic-co-glycolic acid (PLGA)وPoloxamer407 حيث تم استخدام بوليمر متوازن. واستخدمت هذه الطريقة نظرا لمميزاتها مثل انخفاض الكلفة و سهولتها. وقد تم توصيف دقائق الهزبردين النانوية باستخدام التقنيات التحليلية مثل التحليل الطيفي للأشعة تحت الحمراء، المجهر الألكتروني الماسح، حيود الاشعة السينية وتحليل حجم الجزينة. كان تأثير دقائق الهزبردين النانوية اعلى من تأثير الهزبردين النقي وقد كان هناك زيادة واضحة في فعالية البلعمة (مؤشر البلعمة 82 %) للهزبردين النانوي عند مقارنته مع الهزبردين النقي (مؤشر البلعمة 56 %) وبالمقارنة مع عينات السيطرة (مؤشر البلعمة 22%). نوصي بالمزيد من الدراسات حول الهزبردين النانوي فيما اذا كان يمتلك تأثيرات علاجية.

الكلمات الدالة: جزيئات الهزبردين النانوية، PLGA، Poloxamer 407, المكورات العنقودية، فعالية البلعمة

Introduction

Hesperidin is a flavanone (class of flavonoids), it is found plentiful in citrus fruits. The richest dietary sources of hesperidin are Sweet oranges (*Citrus sinensis*) and tangelos [1,2]. Hesperidin is a β-7-rutinoside of hesperitin because it consists of an aglycone, hesperitin and a disaccharide, rutinose [3]. It is reported that hesperidin possess diverse important biological functions such as anti-analgesic, anti-hypercholesterolemic activity, anti-carcinogenic activity and anti-inflammatory activity, in addition to have antimicrobial and antioxidant effects [4,5]. Hesperidin has low solubility in water and is a yellow to brown crystalline powder with melting point at 260°C. There were many factors limit their oral bioavailability, such as poor water solubility and precipitation in an acidic environment [6]. Thus, the reduction of particle size to nanometer range is a potent formulation approach that can increase the saturation solubility and dissolution rate [7]. Anti-solvent precipitation (nano-precipitation) is one of the methods was used to prepare microparticles and nano-sized particles and improve drug solubility, the precipitation method have the advantage of its low setup cost and simplicity. By this method, the drug is dissolved in a good solvent and precipitated as nanocrystals by quick addition of the drug solution to the anti-solvent solution [8]. The usage of biodegradable polymeric nanoparticles is highly preferred because they offer promise in drug delivery system. Such nanoparticles provide controlled/sustained release property, subcellular size and biocompatibility with tissue and cells. Apart from this, these nano-medicines are stable in blood, non-toxic, non-thrombogenic [9], non-immunogenic, non-inflammatory, do not activate neutrophils, biodegradable, avoid

reticuloendothelial system and applicable to various molecules such as drugs, proteins, peptides or nucleic acids [10,11]. PLGA (poly-d,l-lactide-co-glycolide) is one of the most successfully used biodegradable nano-system for the development of nano-medicines because it undergoes hydrolysis in the body to produce the biodegradable metabolite monomers, lactic acid and glycolic acid [12]. Since the body effectively deals with these two monomers, there is very minimal systemic toxicity associated by using PLGA for drug delivery or biomaterial applications [13]. Phagocytosis is defined as the ingestion of large particles by cells such as neutrophils, macrophages, and dendritic cells. This process involves the recognition and binding of foreign bodies such as bacteria or fungi by receptors on the cell surface and can be cleared from infection sites. So, Phagocytosis contributes to the first line of defense against infections [14]. The aim of this work was to evaluate the impact of hesperidin nanoparticle in enhancing the phagocytic activity of macrophage against bacterial infection.

Characterization of Fabricated Hesperidin Nanoparticles

Hesperidin nanoparticles were characterized by using FTIR, XRD, SEM and particle size analysis (PSA) as described in our previously study [15].

Materials and Methods

Hesperidin, D, L-lactide and glycolide (PLGA), and Poloxamer 407, were purchased by Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) (BDH, UK), Lishman stain (Sigma, USA). Bacterium *S. aureus* in concentration 1×10^6 cell/ ml was obtained from the Medical microbiology laboratory, Biotechnology division, Department of applied science, University of Technology, Baghdad, Iraq.

Samples Collection and Handling

Human blood samples were withdrawn freshly from 10 healthy volunteers in heparin containing tubes. The sample of bacteria was activated for 24 h in 37±°C by using nutrient and Brain Heart Infusion broth, and before use it was diluted with sterilized PBS to obtain 1×10^6 cell/ ml.

***In vitro* Phagocytic Activity Assay**

Phagocytic activity of macrophage was determined as the method mentioned by Abd AL-Rhman, *et.al* [16] with some changes. As the following, 200 µl of blood was added to many sterilized tubes and then 200 µl of pure hesperidin and hesperidin nanoparticles (20 µg/ ml) was added to these tubes except one which considered as control. All samples were incubated in water bath for 30 min at 37±°C. After that, 20 µl of activated bacterial suspension were added to all tubes and mixed carefully and incubated for 1 h in the incubator at 37±°C. After this period, one drop from each sample was used to make a blood smear, and left to dry at room temperature. After drying step the slides were stained with drops of Lishman stain for 10 min, and then washed with distilled water and left to dry. Finally the slides examined with light microscope under 100X, and about 100 cells were counted per side and the phagocytosis index was calculated using the next formula:

$$\textbf{phagocytosis index}\ (\%) = \textbf{No. of phagocytic cells} \div \textbf{No.}\ (\textbf{phagocytic} + \textbf{non phagocytic}) \times \mathbf{100}$$

Statistical Data Analysis

The collected data were statistically analyzed by utilizing ANOVA (analysis of variance) by using SPSS software (SPSS/16.0; SPSS Inc., Chicago, IL, USA). Results are representative as the mean ± S.D. of the three independent experiments.

Results and Discussion

Figure 1 represents the phagocytosis process of white blood cells (WBCs) induced by pure hesperidin and hesperidin nanoparticles against *Staphylococcus aureus* after treated with 20 µg/ ml of each compound for 30 min, the phagocytosis index (PI) of human blood samples was also represented. These results showed that hesperidin nanoparticles were enhanced the phagocytic activity of WBCs against *S. aureus* when compared to non-treated sample. There was considerable increase in PI of blood sample treated with hesperidin nanoparticles 82% in contrast with PI of pure hesperidin which was 56%, while PI of blood sample that indicates the control was 22%. These data provide the evidence about the impact of hesperidin nanoparticles on phagocytic cells and this effect promoted the phagocytic activity of these cells to ingest *S. aureus* bacterium. Importantly, nanoparticles exhibited their effect by two mechanisms, delivery and adhesion of nanoparticles onto the cells and uptake of nanoparticles by the cell by means of phagocytosis [16]. Upon phagocytosis, the mononuclear phagocytic system (MPS) induces the production of reactive oxygen intermediates (ROI) and cytokines, which are contribute in host defense against pathogenic microorganisms, in addition to activation of macrophage after ingestion of these particles. It is

well known that particle uptake by phagocytic cells is generally influenced by surface properties and particle size [17]. So, when macrophages are activated by a particulate stimulant (hesperidin nanoparticles in current investigation), cytokines and oxygen intermediates are produced and released, which take part in the antimicrobial activity of macrophages [18].

Table (1): Phagocytosis index against *Staphylococcus aureus*

Blood samples	Percent of phagocytosis index
Negative control	0%
Positive control	22%
Pure hesperidin	56%
Hesperidin nanoparticles	82%

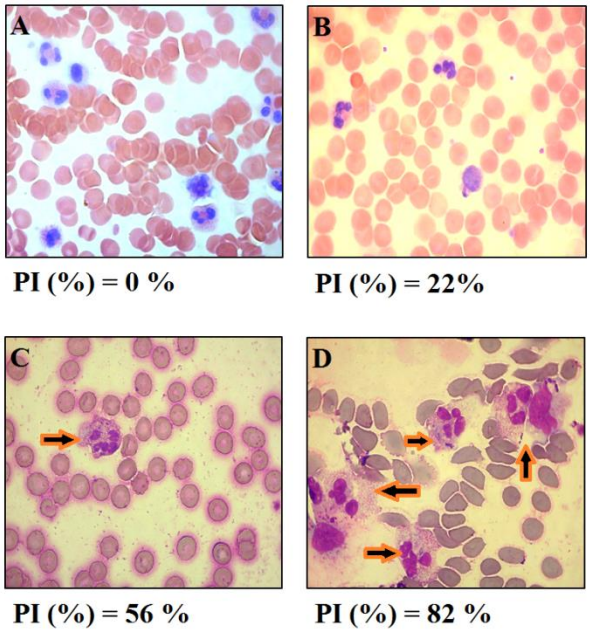

Figure. (1): The images represent the phagocytic activity against *Staphylococcus aureus* and as the follow: (A) negative control of blood sample without bacteria, (B) positive control of blood sample incubated only with *S. aureus*, (C) blood sample with bacteria after treated with 20 µg/ ml of pure hesperidin and (D) blood sample with bacteria after treated with nano-hesperidin. The phagocytic index (PI) is also presented and the photos represent the best one and not the number of phagocytic cells.

Conclusion

Summarizing, we have fabricated hesperidin nanoparticles and characterized them, and studied their effect on phagocytes. In conclusion, incubation of hesperidin/PLGA nanoparticles with human blood phagocytes resulted in particle uptake and cell activation. Our results demonstrated that high stimulation was induced by hesperidin/PLGA nanoparticles and have more potent effect than free hesperidin. Further investigations are needed to study the hesperidin nanoparticles potentials as an alternative therapeutic agent.

References

1. Wilmsen, P.K., Spada, D. S. and Salvador, M. (2005). Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. J. Agric. Food Chem. 53(12):4757-4761.
2. Kalpana, K. B., Srinivasan, M. and Menon, V. P. (2009). Evaluation of antioxidant activity of hesperidin and its protective effect on H_2O_2 induced oxidative damage on pBR322 DNA and RBC cellular membrane. Mol. Cell Biochem. 323(1-2): 21-29.
3. Roohbaksh, A., Parhiz, H., Soltani, F., Rezaee, R. and Iranshahi, M. (2015). Antioxidant and anti-inflammatory properties of the citrus flavonoids hesperidin and hesperetin: An updated review of their molecular mechanisms and experimental models. Phytother. Res. 29(3):323-331.
4. Natarajan, N., Thamaraiselvan, R., Lingaiah, H., Srinivasan, P. and Periyasamy, P. M. (2011). Effect of flavonone hesperidin on the apoptosis of human mammary carcinoma cell line MCF-7. Biomed. Preventive Nut. 1: 207-215.

5. Mishra, K. (2013). Structure-activity relationship of anti-oxidative property of hesperidin. Int. J. Pharm. Erudition. 2(4): 40-53.
6. Varghese, J. J. and Mallya, R. (2015). Formulation development and evaluation of antioxidant potential of hesperidin nanocrystals. World J. Pharm. Res. 4: 1149-1170.
7. Junyapraserta, V. B. and Morakula, B. (2015). Nanocrystals for enhancement of oral bioavailability of poorly water-soluble drugs. Asian J. Pharm. Sci. 10(1): 13-23.
8. Kakran, M., Sahoo, N. G., Li, L. and Judeh, Z. (2012). Fabrication of quercetin nanoparticles by anti-solvent precipitation method for enhanced dissolution. Powder Technol. 223: 59-64.
9. Bonifácio, B. V., da Silva, P. B., dos Santos Ramos, M. A., Negri, K. M., Bauab, T. M. and Chorilli, M. (2014). Nanotechnology-based drug delivery systems and herbal medicines: a review. Int. J. Nanomed. 9: 1-15.
10. Panyam, J. and Labhasetwar, V. (2003). Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv. Drug Deliv. Rev. 55(3): 329-347.
11. Des Rieux, A., Fievez, V., Garinot, M., Schneider, Y. J. and Préat, V. (2006). Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. J. Control Release. 116(1): 1-27.
12. Crotts, G., and Park, T. G. (1998). Protein delivery from poly (lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues. J. Microencapsul. 15(6): 699-713.
13. Kumari, A., Yadav, S. K. and Yadav, S. C. (2010). Biodegradable polymeric nanoparticles based drug delivery systems. Colloids Sur. B: Biointerfaces 75: 1-18.
14. Flannagan, R. S., Jaumouille,V. and Grinstein, S. (2012). The cell biology of phagocytosis. Annu. Rev. Pathol. 7: 61-98.
15. Sulaiman, G. M., Farhan, M. M. and Ali, S. H. (2018). Fabrication of hesperidin nanoparticles loaded by poly (lactic co-glycolic) acid for improved therapeutic efficiency and cytotoxicity. In press.
16. Abd AL-Rhman, R. M., Ibraheem, S. R. and AL-Ogaidi, I. (2016). The effect of silver nanoparticles on cellular and humoral immunity of mice *in vivo* and *in vitro*. Iraqi J. Biotechnol. 15(2): 21-29.
17. Prior, S., Gander, B., Blarer, N., Merkle, H. P., Subirá, M. L., Irache, J. M. and Gamazo, C. (2002). *In vitro* phagocytosis and monocyte-macrophage activation with poly (lactide) and poly (lactide-co-glycolide) microspheres. Eur. J. Pharm. Sci. 15(2):197-207.
18. Robinson, J. M. and Badwey, J. A. (1994). Production of active oxygen species by phagocytic leukocytes. In: Zwilling, B.S., Eisenstein, T.K. (Eds.), Macrophage–Pathogen Interactions. Immunology Series, (60): 159-178.