Bull. Mater. Sci. (2022)45:88
https://doi.org/10.1007/s12034-022-02665-w

Isoimperatorin-mediated green-synthesized silver nanoparticles: antibacterial, antioxidant, cytotoxicity, hemolytic and coagulation effects

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MS received 11 December 2021; accepted 29 December 2021

Abstract. The isolated and pure biological compounds have been widely used in natural-based nanoparticles, entailing applications in medicine, cosmetics and nutrition. In this study, the toxic effects (*in vitro*) and biomedical applications of isoimperatorin-mediated green synthesized silver nanoparticles (Iso-AgNPs) were investigated. The *in vitro* antioxidant, antibacterial and cytotoxic potential were determined by using standard protocols. Besides, blood compatibility was determined by hemolysis and coagulation tests. Based on the obtained results, Iso-AgNPs showed enhanced antioxidant capacity in comparison with the crude isoimperatorin and were found effective against the pathogenic bacterial strains. Cell viability study reveals the safety of Iso-AgNPs on H1299 and MCF-7 cell lines, and no significant cytotoxicity was observed. The non-hemolytic effect of Iso-AgNPs on erythrocyte cells (RBCs) and the absence of coagulation indicate high blood compatibility of these nanoparticles, which make them unique in comparison to the other silver nanoparticles. Altogether, our finding confirms the biological advantages of Iso-AgNPs as a cyto- and blood-compatible antibacterial and antioxidant nano-metal.

Keywords. Antibacterial; antioxidant; coagulation; hemolysis; isoimperatorin; silver nanoparticles.

1. Introduction

The unique properties of silver nanoparticles (AgNPs) like antibacterial, anticancer, antiviral, catalytic, chemical, photochemical and optoelectronic have attracted significant attention on bioelectronic, biosensing, detection and diagnosis applications of AgNPs [1–3]. The biological activity of AgNPs depends on their different physicochemical parameters such as shape, dimension, surface charge, surface coatings, concentration and colloidal state [4]. Due to the practical and beneficial antibacterial properties of AgNPs and their widespread application in wound therapy and the coating of medical devices, it is necessary to pay more attention to their safety and biocompatibility [5].

AgNPs can be synthesized by both chemical and biological procedures [6]; chemical processes are usually costly, harmful to the environment, and involve toxic materials [7], while the green synthesis of AgNPs is simple, inexpensive, eco-friendly and less time consuming [6]. Among different sources for the green synthesis of AgNPs, plant extracts are commonly used as a cheap and straightforward method for the fast production of AgNPs. They act as reducing agents to provide uniform nucleation and growth of nanoparticles and as a corona to stabilize nanoparticles [8]. However, the plant extract compositions and concentrations of constituents are extremely variable. Hence, the pure natural compounds with the well-defined concentration are preferred for the preparation of metallic nanoparticles; the nanoparticle properties, including shape, size and the main composition of nanosystem, can be under control and predicted, and the purification of nanoparticles is more facile [9,10]. Furthermore, isolated and pure biological compounds have been widely used in natural-based nanoparticles, entailing applications in medicine, cosmetics and nutrition [11].

Therefore, in this work, we evaluate the *in-vitro* toxic effects and biological activities of isoimperatorin-mediated AgNPs, including antioxidant, antibacterial, cytocompatibility and blood compatibility. Isoimperatorin, as a furanocoumarin with different biological properties such as anti-

Published online: 16 May 2022

inflammatory, antibacterial, spasmolytic, analgesic and anti-Alzheimer effects [12], can increase the properties of synthesized nanoparticles and improve its medical applications.

2. Experimental

2.1 *Characteristics of green-synthesized Iso-AgNPs using isoimperatorin*

As we explained in the previous article, Iso-AgNPs were synthesized using isoimperatorin as a reducing and capping agent, this pure compound was isolated from root parts of *Prangos ferulacea* in our laboratory [12]. In this method, the change in the colour of the reaction mixture containing isoimperatorin (2 mM) and $AgNO_3$ (1 mM) solutions (at ratio 6:12) to brown colour after exposure to sunlight confirmed the formation of Iso-AgNPs. The X-ray diffraction analysis and photography with transmission electron microscopy approved the formation and crystal structure of Iso-AgNPs with spherical shapes and sizes in the range of 79–200 nm (figure 1). More detailed information is available in our previous article [12].

2.2 *Evaluation of the anti-microbial activity of Iso-AgNPs*

2.2a Well-diffusion assay: Antibacterial effects of Iso-AgNPs and isoimperatorin on *Staphylococcus epidermidis* (ATCC 12228), *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 43300), *Escherichia coli* (ATCC 25922), *Serratia marcescens* (ATCC 13880) and *Pseudomonas aeruginosa* (ATCC 27253) were evaluated by well-diffusion method. The bacterial suspension at concentrations of 1.5×10^8 CFU ml^{-1} was swabbed onto Mueller Hinton Agar (MHA) plates. A quantity of 100 µl of Iso-AgNPs (300 µg ml^{-1}) was added to each well (6 mm diameter). An aliquot of 100 µl of isoimperatorin at the same concentration was used as a control. All plates were incubated at 37°C for 24 h, and after that, the inhibition zone was determined [13].

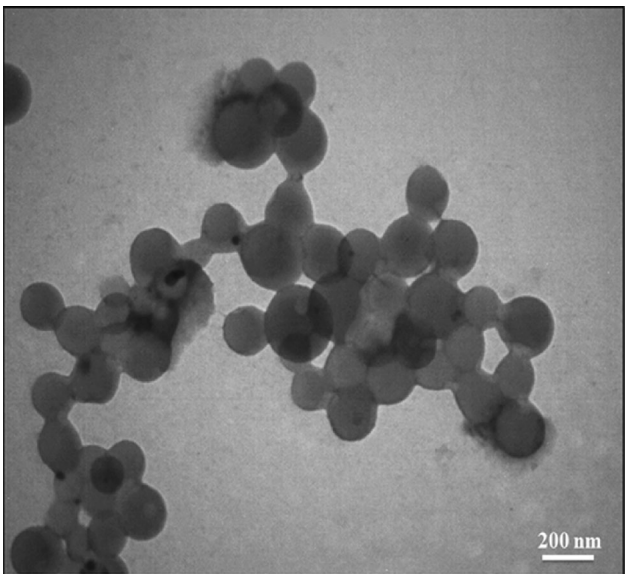

Figure 1. Transmission electron microscopy analysis for determination of shape and size of Iso-AgNPs.

2.2b Determination of minimum inhibitory concentration value: To measure minimum inhibitory concentration (MIC) values of the Iso-AgNPs, 100 µl of the tested bacterial strains was inoculated into 96-well plates followed by adding 100 µl of either the Iso-AgNPs or isoimperatorin at different concentrations. After incubation time at 37°C for 24 h, the concentration of bacteria in each well was measured based on the turbidity of the medium. A medium containing bacteria and the pure medium were placed as positive and negative controls, respectively. The lowest concentration of samples that inhibited bacterial growth was considered MIC of samples [13].

2.3 *Cell viability assay*

The effect of Iso-AgNPs on the human breast cancer cell line (MCF-7) and a human non-small cell lung cancer cell line (H1299) was estimated using the MTT test. An aliquot of 180 µl of the prepared cell suspension with a concentration of 50000 cells ml^{-1} was seeded in wells of 96-well plates. After 24 h, 20 µl of fresh medium containing either Iso-AgNPs suspensions or isoimperatorin solution was added to the wells to achieve different concentrations of testing subjects (10, 25, 50 and 100 µg ml^{-1}). After 48 h, with removing medium, the cells were rinsed. By adding MTT (0.5 mg ml^{-1}) to each well, the plate was incubated for 3 h in the incubator. After that, 150 ml of DMSO was added to each well to dissolve the formazan crystals. The absorbance was recorded by an ELISA reader (SynergyH1, Biotech) at a wavelength of 570 and 630 nm (reference wavelength) [14]. The following equation (1) was applied for the measurement of cell viability.

$$\text{Cell viability } (\%) = [(A570_S - A630_S)/(A570_C - A630_C)] \times 100, \quad (1)$$

where A_S and A_C are the mean absorbance of the sample and control-treated cells, respectively.

2.4 *DPPH-free radical scavenging assay*

To measure the antioxidant effect of isoimperatorin and Iso-AgNPs, 1 ml of 1,1-diphenyl-2-picrylhydrazyl (DPPH; 0.1 mM) fresh solution in methanol was poured into either the solution of isoimperatorin or suspension of Iso-AgNPs at concentrations of 5, 12.5, 25, 50 and 100 µg ml^{-1}. The

reaction mixture was incubated in the dark and stirred thoroughly for 30 min. Then, the absorbance of tested samples was recorded at 517 nm. DPPH in methanol was used as a control [15]. The following formula (2) was used to measure the percentage of free radicals scavenging:

$$\text{Percentage of free radicals scavenging } (\%) = [(A_C - A_S)/A_C] \times 100, \quad (2)$$

where A_S and A_C are the mean absorbances of the sample and control.

2.5 *Blood compatibility assay* (in vitro)

2.5a Assay of hemolytic activity of Iso-AgNPs: The *in-vitro* hemolytic activity of green-synthesized Iso-AgNPs was evaluated by Devi *et al* [16] method with a little modification. Briefly, a fresh human blood sample after taking from three healthy donors (24–30 years old) was prepared by centrifugation at 800g for 10 min and was washed three times using normal saline. The RBCs were suspended in normal saline (10% v/v) and then, 200 µl of this solution was mixed with 200 µl of each concentration of Iso-AgNPs, including 25, 50, 100, 200 and 400 µg ml^{-1} and was incubated at 37°C for 60 min. After incubation time, all prepared samples were centrifuged at 13,400 rpm for 5 min and then, 100 µg ml^{-1} of them were transferred to a 96-well plate for recording absorbance at 540 nm using a microplate reader (SynergyH1, Biotech). The mixture of RBCs with Triton-X-100 and normal saline was applied as a positive and negative control, respectively. Finally, the hemolytic activity of Iso-AgNPs is estimated by the following equation (3):

$$\text{Hemolysis } \% = (\text{absorbance of each concentration of NPs} - \text{absorbance of negative control})/(\text{absorbance of positive control} - \text{absorbance of negative control}) \times 100 \quad (3)$$

2.5b. Coagulation time assay: The effect of Iso-AgNPs on coagulation time was estimated based on aPTT (activated partial thromboplastin time) and PT (prothrombin time) assay [17]. In brief, the blood samples (freshly) were taken from healthy donors (24–30 years old) and centrifugated at 2,500g for 10 min to obtain platelet-poor plasma. To experiment, 100 µl of Iso-AgNPs at concentrations 50, 100, 200 and 400 µg ml^{-1} was dispersed in 900 µl of platelet-poor plasma and was incubated for 30 min at 37°C. Then, the suspension of each sample was centrifuged at 18000g for 5 min for the assay of aPTT and PT using the Coatron M2 Coagulometer (TECO, Germany). The results were expressed as mean ± SE and compared to the controls (normal saline).

3. Results and discussion

3.1 *Antibacterial activity of Iso-AgNPs*

The antibacterial activity of Iso-AgNPs was evaluated against Gram-positive bacteria consisting *S. epidermidis*, *B. subtilis* and *S. aureus*, and Gram-negative bacteria including *E. coli*, *S. marcescens* and *P. aeruginosa*. Among the Gram-negative strains, *S. marcescens*, *E. coli* and *P. aeruginosa* exhibited a maximum inhibition zone of 17, 15 and 13 mm, respectively, whereas *S. epidermidis* strain from Gram-positive bacteria showed a complete inhibition due to high sensitivity to Iso-AgNPs (table 1). The pure isoimperetorin efficacy was significantly lower than Iso-AgNPs on tested bacteria except on *S. epidermidis* that showed a similar inhibition zone.

MIC results are tabulated in table 1. After treating different bacterial strains with Iso-AgNPs, MIC values against Gram-negative strains of *P. aeruginosa* and *E. coli* were calculated as 18.75 µg ml^{-1}, and it was estimated as 37.5 µg ml^{-1} for *B. subtilis*. In comparison to similar studies, our AgNPs show significantly better MIC. The higher antibacterial efficacy of our AgNPs on these strains can be attributed to the synergistic effect of AgNPs and isoimperatorin. In a study conducted by Widelski *et al* [18], isoimperatorin isolated from *Angelica lucida* fruits showed appropriate antibacterial effects against *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli,* respectively [18]. As shown in table 1, the MIC of isoimperatorin against *B. subtilis* and *P. aeruginosa* is 150 µg ml^{-1}. The antibacterial activity of isoimperatorin is attributed to the coumarin ring that can inhibit bacterial nucleic acid synthesis. The lipophilicity of the molecule by the presence of an additional prenyl group in the furanocoumarin skeleton, which facilitates its passage from the thick bacterial membrane can also improve uptake of nanoparticles [18].

It is known that *S. aureus* and *P. aeruginosa* are the most important pathogens responsible for hospital-acquired infections that can cause serious problems; e.g., biofilm adhesion on the surface of various materials and implants, acute and chronic respiratory infections. They can also cause food poisoning in humans and animals [19–21]. *S. marcescens* may also cause bacteraemia and respiratory tract infections in intensive care units, particularly neonatal intensive care units, in the surgical site infections and meningitis after invasive methods in the central nervous system [22]. Furthermore, other kinds of bacteria used in this study can be food poisoning and critical opportunistic

Table 1. Antibacterial activity of Iso-AgNPs and isoimperatorin against various bacterial strains.

Bacterial strains	MIC		Well-diffusion	
	Iso-AgNPs (μg ml^{-1})	Isoimpratorin (μg ml^{-1})	Iso-AgNPs (mm)	Isoimpratorin (mm)
S. epidermidis	75	300	Full inhibition	Full inhibition
B. subtilis	37.5	150	6	6
S. aureus	150	300	6	6
P. aeruginosa	18.75	150	13	6
S. marcescens	75	300	17	6
E. coli	18.75	300	15	6

pathogens, which cause nosocomial infections such as wound among hospitalized patients. Because of these problems for patients and health services and the prevalence of antibiotic resistance, it is essential to find novel cost-effective antibacterial agents as alternative antibiotics, especially against MDR (multidrug-resistant) bacteria. As seen, synthesized Iso-AgNPs in this work can resolve a wide range of infections caused by these bacteria. Therefore, it can be used as a potent antibacterial agent in wound healing, food and cosmetic industries and health services.

3.2 *Cell viability study*

The biocompatibility, cytotoxicity and genotoxicity of AgNPs depend on various parameters, including size, solubility, concentration, surface coating, shape, surface charge, surface functionalization, growth media, cell type, exposure time, mode of action, mode of entry and distribution of particles [23]. The effects of Iso-AgNPs on MCF-7 and H1299 cell lines were evaluated by MTT assay. The cell viability of both cell lines was not significantly changed either by isoimperatorin or Iso-AgNPs at concentrations lower than 100 μg ml^{-1} (figure 2). Cell viability of MCF-7 and H1299 cells was 93–74 and 98–82% at concentrations of 10–100 μg ml^{-1} of isoimperatorin, respectively. The cell viability for Iso-AgNPs at the same concentration range as isoimperatorin was 90–69.5 and 85–70%, respectively (figure 2a and b). There is no correlation between the concentration of isoimpratorin and Iso-AgNPs and viability.

In our previous obtained results about the effects of spherical and small AgNPs and AuNPs synthesized using *Nigella arvensis* [13,15,24] and *Dracocephalum kotschyi* [14], on these both cell lines, the IC_{50} value calculated was approximately 10 μg ml^{-1}. It seems that nanoparticles with small size can be more cytotoxic on cancer cell lines due to its easy penetration to cell membrane and internalization. Besides, the surface chemistry, owing to the presence of phytochemical compounds of plant species on the surface of biosynthesized nanoparticles as capping or stabilizing agents is another factor that can be effective on the cytotoxicity effect of biogenic nanoparticles. Therefore, these

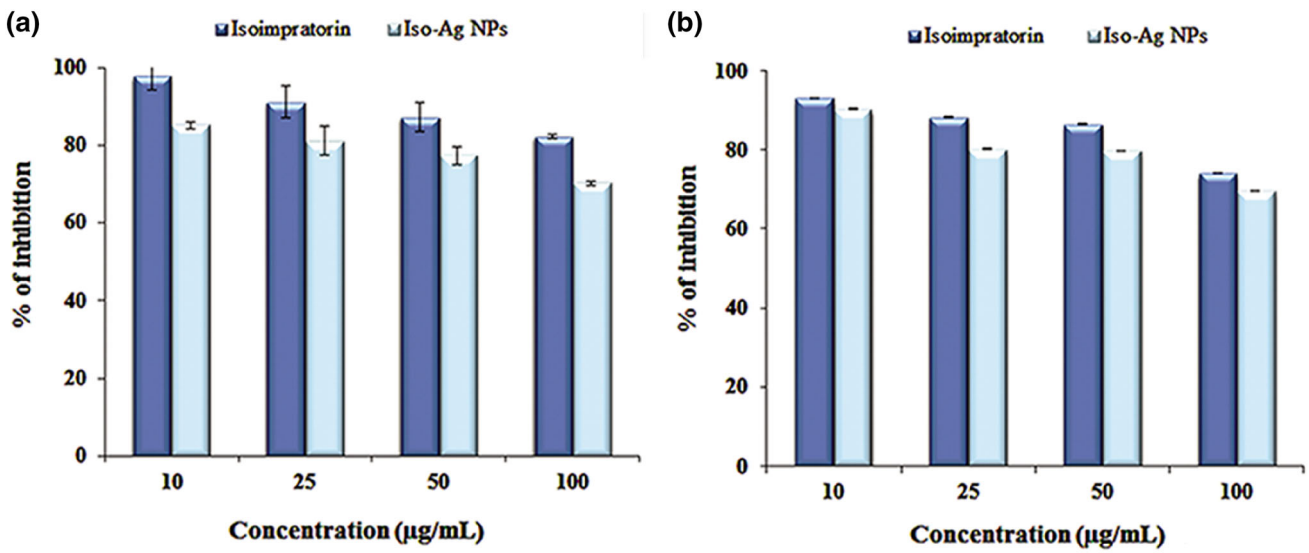

Figure 2. Cell viability of (**a**) H1299 and (**b**) MCF-7 cells exposed to different concentrations of isoimperatorin and Iso-AgNPs.

observed differences between Iso-AgNPs and other biogenic nanoparticles on both tested cells can be based on their surface chemistry, the type of reducing agents, surface coating due to the presence of isoimperatorin in the surface of synthesized nanoparticles as capping or coating agent along with their size and agglomeration state of nanoparticles.

One of the concerns about AgNPs is their cytotoxic effects on mammalian cells, such as liver, stem, lung epithelial and endothelial cells [23,25–32], which has limited their use as an antibacterial reagent or in other medicinal applications. Considering our results in the present study, green-synthesized Iso-AgNPs show no significant cytotoxic effects on tested cell lines below 200 μg ml^{-1}, indicating the advantage of these nanoparticles as a suitable and biocompatible option for medicinal and biological applications.

3.3 *Antioxidant activity of Iso-AgNPs*

DPPH assay provides an efficient technique to determine the primary radical scavenging activity of a compound [33]. The antioxidant activity in plants is usually due to the presence of phenolic compounds [34] and their redox attributes, which allow them to operate as singlet oxygen quenchers, hydrogen donors and reducing agents [35]. Polyphenolic compounds in plants have strong antioxidant activity and help in protecting cells against oxidative stress created by free radicals [36]. Removal of free radicals in cells inhibits pathological disruptions like heart attack, cancer, ageing, etc. [37].

The green-synthesized Iso-AgNPs exhibits a higher potential in scavenging DPPH than that of isoimperatorin (figure 3), particularly at lower concentrations. The inhibition activity of the Iso-AgNPs was increased in a dose-dependent manner. The most inhibition percentage of Iso-AgNPs was 38.3% at a concentration of 100 μg ml^{-1}, where this value for pure isoimperatorin was 32% in the same concentration, which is reasonable in comparison to similar studies [38]. Compared to the green-synthesized Iso-AgNPs, DPPH scavenging activity of commercial AgNPs (chemically synthesized with spherical shape and an average size of 8 nm) without any coating agent was decreased in a dose-dependent manner. The results demonstrated the oxidative activity of commercial AgNPs, which may be due to the lack of suitable capping agents on their surface or the high potential of these nanoparticles in the generation of radical species. Thus, the higher antioxidant activity of Iso-AgNPs can be due to the presence of isoimperatorin as a reducing and capping agent during the synthesis process. Therefore, Iso-AgNPs can be a good option for use in medicinal fields.

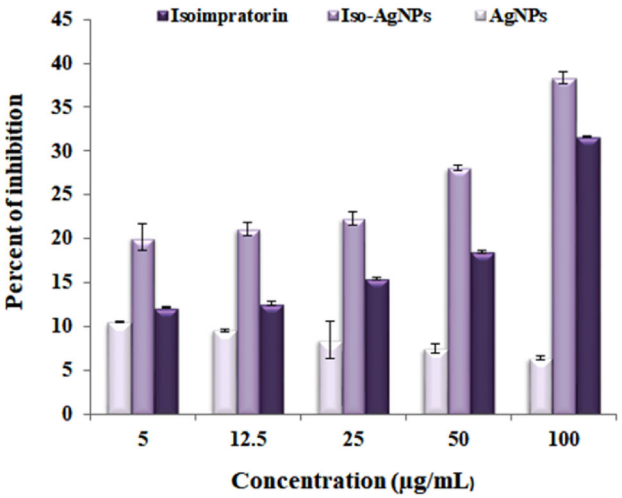

Figure 3. DPPH radical scavenging activity of isoimperatorin, Iso-AgNPs and commercial AgNPs at different concentrations of 5, 12.5, 25, 50 and 100 μg ml^{-1}.

3.4 *Blood compatibility*

3.4a Hemolytic activity of Iso-AgNPs: The determination of the toxicity effects of AgNPs on RBCs is very vital for their medicinal or clinical applications. The blood compatibility assessment of AgNPs is hugely important, and their interaction with RBCs may lead to considerable damages, including cell membrane injury, DNA damage and congenital malformations [39]. In this study, the toxicity effect of green-synthesized Iso-AgNPs on RBCs was determined using *in-vitro* hemolysis assay. The obtained results show that the green-synthesized Iso-AgNPs are non-toxic on RBCs. Figure 4a indicates the percentage of 3.4% and 7% of RBCs lysis at higher concentrations of 200 and 400 μg ml^{-1} of Iso-AgNPs, respectively, compared to the positive control (Triton X-100). The non-toxic effect of Iso-AgNPs on RBCs can be due to the presence of isoimperatorin as capping agents that surrounded these green-synthesized AgNPs; the previous study indicated isoimpratorin as a non-hemolytic compound [40]. In contrast to chemically synthesized AgNPs that are hemolytic even at low concentrations [41,42], green-synthesized Iso-AgNPs are hemocompatible and can be used in pharmacological and biomedical applications.

3.4b Coagulation effect of Iso-AgNPs: In the present study, the impact of Iso-AgNPs on plasma coagulation time was analysed using aPTT and PT assay. PT and aPTT are analysed for the activity of extrinsic and intrinsic pathways of the plasmatic coagulation, respectively [43]. According to our results, prepared Iso-AgNPs showed no change in PT value at different concentrations of 50–400 μg ml^{-1} in comparison to normal saline as a control (figure 4b). Also, at these concentrations, Iso-AgNPs show no change of PTT in contrast to the controls (figure 3b). Therefore, the Iso-AgNPs at different concentrations does not affect extrinsic

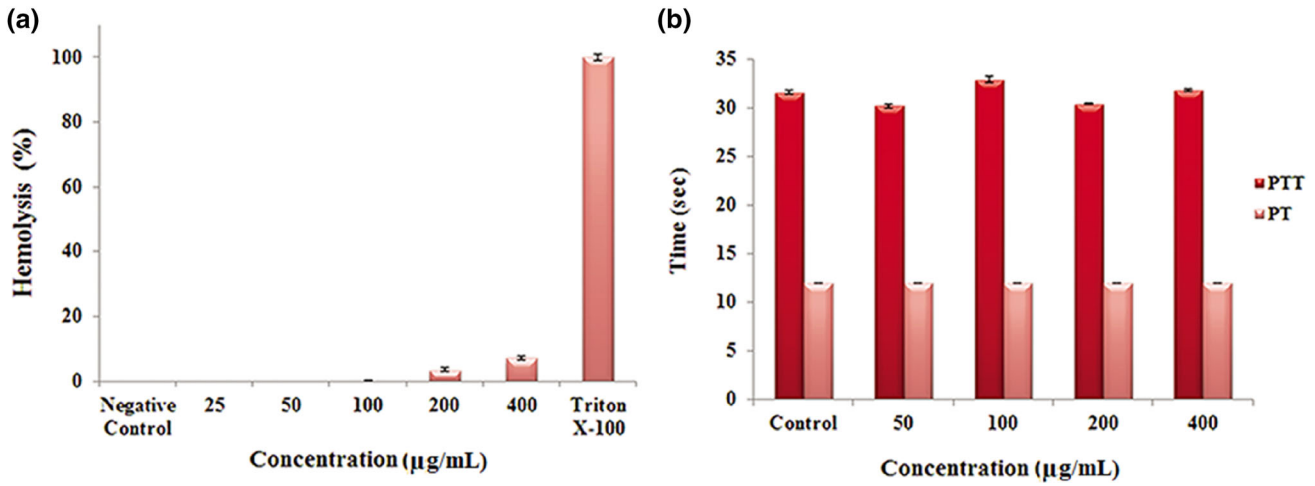

Figure 4. (**a**) Hemolytic activity (by assay of hemolysis percentage) and (**b**) coagulation effect (by assay of PT and PPT) of Iso-AgNPs at different concentrations of 25, 50, 100, 200 and 400 µg ml^{-1}.

and intrinsic pathways in blood coagulation. Based on coagulation time assays, these nanoparticles were in normal physiological levels for PT (9.4–12.5 s) and aPTT (25.1–36.5 s) [41].

4. Conclusion

Synthesized AgNPs using isoimperatorin as cost-effective and straightforward, precise and eco-friendly green methodologies were evaluated for their biological activities. Iso-AgNPs showed higher antioxidant and antibacterial actions in comparison to pure isoimpratorin. The green-synthesized Iso-AgNPs with no significant hemolytic effect did not affect coagulation time. The significances of this study demonstrate a broad range of medical applications for biocompatible and blood compatible Iso-AgNPs. Iso-AgNPs have potential therapeutic effects against human pathogens and also prevention of diseases caused by free radicals, e.g., cancer.

Acknowledgements

We gratefully acknowledge the Research Council of Kermanshah University of Medical Sciences and the National Institute for Medicinal Research Development (NIMAD) for financial support.

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