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PHYTOSYNTHESIS OF ZINC OXIDE NANOPARTICLES WITH ACETONIC EXTRACT OF FLOWERS OF *GERANIUM ROBERTIANUM* L. (*GERANIACEAE*)

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Abstract

Chemical and physical stability, high adsorption capacity and low-toxicological profile are very important features for application of nanoparticles (NPs) in the biomedical field. Physicochemical and biochemical characteristics of nanoparticles are very much dependent on the synthesis method. One of the promising, environmentally conscious method is the green synthesis of NPs using plant extracts. The aim of the this research was to present new plant extract for the synthesis of zinc oxide nanoparticles (ZnO NPs) and to test antimicrobial activity of in this way obtained ZnO NPs.

In this study acetonic extract of flowers of *Geranium robertianum* L. (*Geraniaceae*) was used for phytosynthesis of zinc oxide nanoparticles (ZnO NPs). *G. robertianum* aerial parts are a good source of plant phenolics, particularly flavonoids which have an important role in metal nanoparticle synthesis. Total flavonoid content (4.2%) of acetonic extract used in ZnO NPs synthesis was determined by aluminum chloride spectrophotometric assay on 425 nm. Characterization of ZnO nanoparticles obtained by phytosynthesis procedure was done using UV/VIS spectrophotometry and IC spectroscopy. Obtained ZnO NPs were also tested as potential antimicrobial agents, considering that microorganisms rarely develop antimicrobial resistance against NPs. The Muller-Hinton agar well diffusion method was used for evaluation of ZnO NPs antimicrobial activity against Gram negative (*Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*) and Gram positive (three clinical isolates of *Staphylococcus aureus*) bacteria.

The total flavonoid content of acetonic extract of flowers of G. *robertianum* used for ZnO NPs synthesis was 4.2%. The size of obtained ZnO NPs was approximately 40 nm. All three *S. aureus* isolates exhibited sensitivity (average value of inhibition zones around 15 mm), while Gram-negative bacteria were resistant to ZnO NPs activity.

The results from our study confirm that acetonic extract of flowers of *G. robertianum* can be used for synthesis of ZnO NPs with significant antimicrobial activity towards Gram-positive bacteria.

***Key words**: Nanoparticles, Green chemistry, Geranium robertianum L., Antimicrobial resistance.*

1. Introduction

Nanoparticles (NPs) have been produced for a long time, using various physical and chemical methods. One of the currently very promising method, which is under exploitation and serves as convenient substitute for physical and chemical methods is biosynthesis of nanoparticles [1]. Biosynthesis, or often called green chemistry method, implies the use of plants (plant extract or living plant). Green chemistry method (GCM) is very eco-friendly and cost-effective. Plant extract during production of nanoparticles may have serve as reducing or capping agents. There are so many reports on biosynthesis and useful applications of metallic NPs obtained by reduction process using plant extracts [2, 3]. Plant extract selection can affect morphology of NP and its size. Content of plants are crucial for NP production. Many molecules that

exist in plants, such as alkaloids, vitamins, phenolic compounds, polysaccharides, can serve as potential reducing agent, therefore reducing capacity of these compounds, as well as, reduction potential of metal ions are very important and have great impact on the amount of NP production [1].

G. robertianum is herbaceous plant with characteristic pink flowers and pungent smell, growing naturally in rocky woods, beside slopes and shores. On flowers opening, early in the morning five stigmas folded close together could be observed [4].

The plant belongs to family *Geraniaceae* and has well established role in traditional medicine as an: antihypertensive, antispasmodic, anti-inflammatory and antimicrobial remedy. Chemical characterization of extracts from different parts of plant have been previously published. The most abundant chemicals in polar extract of this herb are phenolics such as tannins, flavonoids and phenolic acids [5]. Content of flavonoids is directly correlated with its significance in therapeutical application. Beside its medicinal importance, content of flavonoids is important factor in nanoparticle phytosynthesis through the processes of metal ions bioreduction and chelate complexes formation which ultimately result in stabilization of nanoparticles [6, 7].

Nanoparticles are promising new antimicrobial agents and nowadays numerous studies are being conducted concerning their usage in the treatment of infections as well as in medical prevention. Although in today's science the invention of a universal antimicrobial substance is considered absolutely essential, we are still far away from reaching that goal. Worldwide spreading of multiresistant bacterial pathogens, indicated the need for development of new antimicrobial compounds. The various antibacterial mechanisms of nanomaterials are mostly attributed to their high specific surface area-to-volume ratios, and their distinctive physicochemical properties [8].

One of the promising and attractive metal oxide nanoparticles, which can be produced by GCM methods are ZnO NPs. ZnO belongs to the group of the n-semiconductor materials type IIb -VI, with energy band gap (≈ 3.4 eV) and high thermal and mechanical stability at room temperature. Due to its unique chemical and physical properties such as high chemical stability, high photostability, high thermal conductivity, high index of refraction, antibacterial activity etc. is a widely functional material [9]. Also, since ZnO is being non-toxic and used for UV light protection it is very common ingredient in many pharmaceutical and cosmetic products [10].

There are various chemical approaches for synthesis of ZnONPs, such as: sol gel [11], solvent free method [12], microemulsion route [13], sonochemical way [14], and chemical precipitation [15, 16].

From the GCM approach various plant extract were used. *Qu et al.*, [17], synthesized ZnO nanoparticles using *Physalis alkekengi* L. The ZnO NPs were polydispersed with mean dimeter of 72.5 nm and were recommended for the remediation of zinc-contaminated soils. In another paper GCM was used for fabrication of ZnO NPs with grapefruit (*Citrus paradisi*) peel extract. The particle size varied between 12 and 72 nm. The obtained ZnO NPs were highly stable and had significant antioxidant activity [18]. It was also reported that hyper-accumulator plants are very good choice for production of heavy metal NPs [19].

In this study we tested possibility of stable ZnO nanoparticle phytosynthesis with flavonoid content characterized acetonic extract of flowers of *G. robertianum*. Afterwards we evaluated antimicrobial activity of in this way synthesized ZnO nanoparticles.

2. Materials and Methods

2.1 Plant material

Plant material was collected in June 2019 in flowering phase on Mt. Manjača (N44°36'43.26'', E17°4'51.43"). Flowers of *G. robertianum* were separated from other aerial parts and dried on air in dry shady place. The voucher specimens are deposited in Dr Relja Suručić private herbarium.

2.2 Preparation of the herbal extract

Dried and powdered flowers parts (20 g) were extracted with acetone (2 × 100 mL) at room temperature and the combined acetonic extracts were concentrated under reduced pressure.

2.3 Total flavonoid content determination

The powdered plant material was extracted under reflux conditions (boiling T) with the mixture of 20 mL acetone 1 mL urotropine (5 g/L), and 2 mL HCL (25%) during 30 min. The extract was cooled to room temperature and filtered. The residue was twice re-extracted under same conditions. Both acetonic extract and re-extracts were combined, and the volume was filled up to 100 mL with acetone (stock solution1).

20mL of stock solution was transferred to separation funnel and mixed with 20 mL of water and 15 mL of ethyl acetate. Process of liquid-liquid extraction was repeated three times under same condition. Afterwards, ethyl acetate extract was washed twice with water and transferred to 50 mL volumetric flask

and made to volume with ethyl acetate, resulting in the stock solution 2. Aliquot of the stock solution 2 was transferred to 10 mL volumetric flask and made to volume with 5% acetic acid solution in methanol, resulting in blank solution. A second aliquot of the stock solution 2 was transferred to another 10 mL volumetric flask, 1 mL of 2% $AlCl_3$ was added and made to volume with 5% acetic acid solution in methanol (test solution). After 30 min. the absorbance of the test solution was measured at 425 nm against blank solution.

Total content of flavonoids was calculated according to the equation below (based on specific absorption coefficient of hyperoside $A_{1\%/1cm}$ = 500. The results represent the average of three determinations:

$$TFC = (A \times 1.25)/m$$

Where: TFC and A represent total flavonoid content and measured absorbance of analysed sample solution, respectively; m represents mass of herbal drug in grams used for sample solution preparation.

2.4 Synthesis of ZnO/geranium

50 mL of zinc acetate dihydrate (0.01 M) was mixed with 5 mL of geranium extract, dissolved in methanol (1 mg/mL). After adjusting the pH value at 12 with 2M NaOH, mixture was stirred for 2 hours at room temperature. Mixture was then centrifuged at 5,000 rpm for 5 minute and light-yellow powder was obtained. Powder was dried in an oven at 60 ^{0}C during 24 hours.

2.5 Instrumentation

Spectrophotometric measurements were performed on UV-VIS 1800 Shimadzu spectrophotometer (Shimadzu, Kyoto, Japan) equipped with 1 cm quartz cells.

Fourier transform infra-red spectroscopy (FTIR) was carried out by using Tensor 27 instrument with addition of platinum stand on which a very small amount of pure ZnO oxide and ZnO/germanium sample was placed (Bruker, USA).

The particle size and morphology of ZnO NP was observed by scanninig electron microscope (SEM) JEOL JSM-6390 LV.

2.6 Antimicrobial testing

Antimicrobial testing was performed using agar well diffusion method against three clinical isolates of Gram-negative bacteria (*Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*) and three clinical isolates of Gram-positive bacteria (one methicillin sensitive and two methicillin resistant strains of *Staphylococcus aureus*). Muller Hinton agar was inoculated with corresponding standardized bacterial inoculum (0.5 McFarlands) and afterwards punched aseptically with sterile cork borer. Into the well 50 µL of nanoparticles solution (5mg/mL) was introduced. After overnight incubation, diameter of inhibition zone around well was measured.

3. Results and Discussion

3.1 Total flavonoid content

The total flavonoids content in acetonic extract of *G. robertianum* flowers was 4.2%. This result fulfills the minimum requirements of the flavonoid content provided in *Betulae folium* monograph of the European Pharmacopoeia 7.0 [20]. *Betulae folium* is considered as a flavonoid-rich herbal drug. Considering that flavonoids as a chemical class contribute to the process of nanoparticle phytosynthesis, detailed chemical composition was not investigated in the present study. Nevertheless, Kartnig and Bucar-Stachel, [20], showed in their study that from aerial parts of *G. robertianum*: quercetin-3-O-galactoside (Isoquercitrin), quercetin-3-O-rhamnogalactoside, quercetin-3-O-rhamnoglucoside, quercetin-3-O-rhamnoside (quercitrin), quercetin-3-O-rutinoside (Rutin), quercetin-4′ -O-glucoside (spiraeoside), quercetin-7-O-glucoside (quercimeritrin), kaemferol-3-O-glucoside (astragalin), kaemferol-3-O-rhamnogalactoside, kaemferol-3-O-rhamnoglucoside, and kaemferol-3-O-rutinoside (nicotifloria) could be isolated.

3.2 Characterization of ZnO NPs

Characterization of ZnO NPs was done by FTIR spectroscopy and morphology and particle size was evaluated by SEM analysis. Figure 1 presents the FTIR spectrum of pure ZnO and ZnO obtained with methanolic extract of geranium.

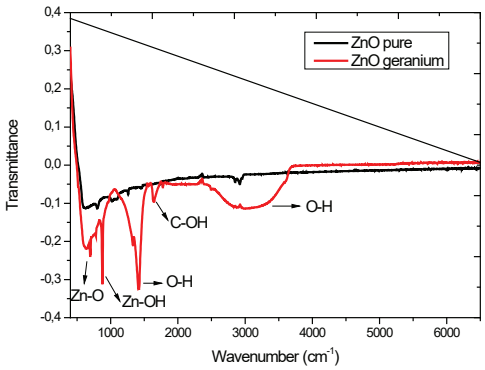

Figure 1. FTIR spectrum of pure ZnO and ZnO with methanolic extract of geranium

The FTIR spectrum was recorded in the range 400 - 6,500 cm^{-1}. From the FTIR spectrum, presence of metal oxide bonds and various functional groups was determined. Significant vibration band was recorded at 625 cm^{-1} and assigned the stretching Zn-O bond [21]. Band at 870 cm^{-1} proves the presence of Zn-OH bond. Broad peaks at 3036 cm^{-1} (stretching) and 1642 cm^{-1} (bending) indicate the presence of hydroxyl functional groups at the surface of materials, and they are especially visible at the ZnO/geranium sample [22, 23]. In ZnO/geranium sample we have also detected band at 1417 cm^{-1} which indicated the conformation of C-OH groups [3].

Figure 2 presents micrograph of ZnO NPs. Particles possess quite irregular shapes, with the mean particles size of 40 nm and were agglomerated.

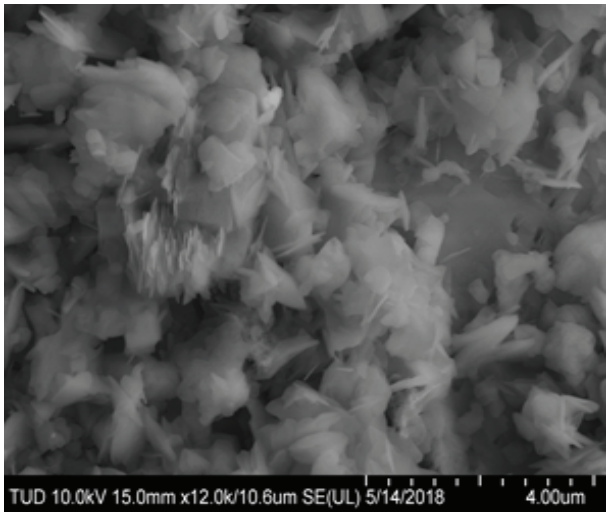

Figure 2. SEM micrograph of ZnO NPs

This is not unusual for the NPs in general, because morphology, shape and size of particles are quite dependent on experimental conditions and precursors used. Particles differ in size and shape (almost spherical, bullet-like, flower-like, etc.) [24]. Agglomeration could be associated with electrostatic interactions and polarity [25].

3.3 Antimicrobial analysis

Nanoparticles are very promising compounds, but, as our study showed, they often act selectively. All three Gram negative bacteria (*Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*) were completely resistant to ZnO/geranium nanoparticles. On the other hand, all three Gram-positive bacteria (one methicillin sensitive and two methicillin resistant strains of *Staphylococcus aureus*) were sensitive to ZnO/geranium nanoparticles, with average inhibition zone of 15 mm. Main reason for selectivity is various structures of bacteria, designed as Gram-positive and Gram-negative, based on differences in cell well arrangement. According to data, there are several distinctive mechanisms of ZnO nanoparticles action against bacteria: direct contact and disruption of cell wall, toxicity of liberated Zn ions and formation of reactive oxygen radicals, often with noxious activity [26]. All mechanisms are reliant to bacterial cell wall type, as a barrier that they need to overcome to achieve their effect. In this study, Gram negative bacteria were completely resistant to ZnO nanoparticles in contrast to sensitive Gram-positive bacteria. We supposed that nanoparticles could not penetrate through complex Gram-negative cell wall, consisting of thick impermeable phospholipid layer as outer leaflet placed over the thin peptidoglycan layer. In contrast, Gram-positive bacteria have simple cell wall consisting of thick permeable peptidoglycan, which enables nanoparticles to penetrate and achieve their effect. According to *Jesline et al*., [27], as well as our study, ZnO nanoparticles could be recommended as antistaphylococcal agent.

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4. Conclusions

- The results from our study confirm that acetonic extract of flowers of *G. robertianum* can be used for synthesis of ZnO NPs with significant antimicrobial activity towards Gram-positive bacteria.

- ZnO/geranium nanoparticles should be further developed for usage in treatment of human skin staphylococcal infections.

5. References

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