

## Biosynthesis of Silver Nanoparticles Using *Juglans Regia* Green Husk (Walnut) Water Extract and evaluation Antibacterial activity

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### ABSTRACT

Biosynthesis of Ag-NPs at room temperature by using *Juglans regia* (*J. regia*) green husk extract which acts as reductant and stabilizer, simultaneously. The Ag/*J. regia* were characterized by using UV-visible, zeta potential, TEM, and AFM. Formation of Ag/*J. regia* was determined by UV-vis spectroscopy, where absorption maxima surface plasmon at 400-460 nm. The zeta potential analysis indicated that *J. regia* green husk extract was negative and increasing in Ag/*J. regia*. TEM images show the mean particle size was 31.37 nm with the standard deviation of 7.1 nm, where confirm by AFM measurements. The XRD study indicates the crystalline nature of the Ag-NPs. The antibacterial activity of Ag-NPs was investigated against Gram-positive and Gram-negative bacteria by the disc diffusion method were found to have high antibacterial activity. These results show that Ag-NPs can be useful in different biologic research and biomedical applications.

*Keywords:*

Silver nanoparticles, biosynthesis, *Juglans regia*, green husk, antimicrobial activity.

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### 1. Introduction

1 Researchers in the past decade have been paying great attention to the field of nanotechnology  
2 that involves reactions at molecular as well as atomic levels. Nanotechnology includes the  
3 characterization, synthesis, as well as device and material application of the tiniest parts that measure  
4 at least a dimension on scales of 100 nm and lower [1]. The field of nanotechnology provides a wide  
5

1 range of technological foundation for applications in various areas including antibacterial [2],  
2 biosensor [3] industrial bio-processing as well as molecular medicine[4] and biomedical applications  
3 [5].

4 Recently, nanotechnology, particularly metal nanoparticles, have emerged as a rapidly new field  
5 due to their unique chemical and physical properties in technological innovations and industry [6].  
6 Synthesis of nanoparticles using green methods has drawn a growing interest due to their  
7 environmentally friendly and low-cost aspects compared with current chemical and physical  
8 methods [7].

9 Nanoparticles that are metallic are particularly in demand in the engineering and biomedical  
10 science fields due to their great potential in the area of nanotechnology, thereby leading to a large  
11 amount of potential application in the separation of magnetic and biotechnology field [8]. In the  
12 synthesis of silver nanoparticles (Ag-NPs) via green method, a solution of silver salt is reduced using  
13 an extract of the plant. This process involves a chemical reaction which takes place through two steps:  
14 1. formation of small silver atoms nuclei as nucleation phase, 2. and growth phase containing  
15 grouping these small nuclei, in which leads to nanoparticles creation [9].

16 Silver ions can be reduced using several methods such as by using  $\gamma$ -rays[10], ultraviolet (UV)  
17 irradiation [11], reduction of electrochemical and heating[12], as well as by applying decreasing  
18 chemicals, involving sodium borohydride [13], hydrazine [14], N,N-dimethylformamide [15],  
19 polyethylene glycerol [16], glucose [17], formaldehyde [18], ethylene glycol [19], and sodium in  
20 ammonium liquid [20]. Nevertheless, further economic, financially suitable, and environmentally  
21 friendly synthesis path in synthesizing Ag-NPs is required. Ag-NPs' green synthesis includes three  
22 major phases that should be assessed according to the perspectives of green chemistry such as  
23 selecting the medium for solvent, agent for reducing, as well as a stabilizer that is non-toxic on Ag-  
24 NPs [21].

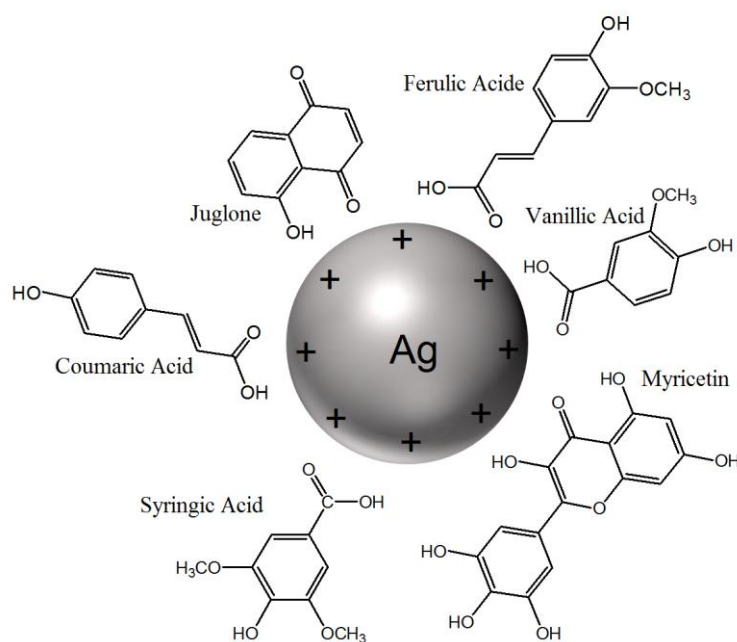
25 The nanoparticles' biosynthesis that involves the link between biotech and nanotech, has been  
26 getting growing attention given the increasing requirement for developing technologies that are  
27 environmentally friendly for the synthesis of materials. Search for the best biomaterial for  
28 nanoparticles' biosynthesis is continued via various methods that are synthetic. The method of  
29 biosynthetic utilizing extracts from plants has gained more interest compared to methods using  
30 chemicals and physical approaches including the utilization of microbes. The approach is appropriate  
31 for the metal synthesis at a nanoscale given the lack of any need for maintaining an environment that  
32 is aseptic [22].

33 Different parts of the walnut tree (*Juglans regia*) such as kernels, leaves, tree bark and also fruit  
34 green husk were utilized for both industries of pharmaceuticals and cosmetics [23]. The study by  
35 Carvalho et al. (2010) established the activity of the antioxidant in walnut leaves, seeds, and green  
36 husks, as well as in antimicrobial activities [24]. The *J. regia* aqueous extracts were examined by  
37 Ghasemiet al. (2011) studied the methanolic ones [25].

38 The findings of Carvalho et al. (2010) display the potential of these low-cost natural materials as  
39 the source of compounds that are phenolic with activities of antimicrobial and antiradical and it also  
40 reveals that green husk knowledge should be widened [26].

41 Based on past literature review, the phenols' content varies from the *J. regia*. The high-  
42 performance liquid chromatography approach utilized in determining the external standards has  
43 allowed the act of identifying six compounds that are phenolic including vanillic acid, myricetin,  
44 coumaric acid, syringic acid, juglone, and ferulic acid [27-28]. All the above results are matching with  
45 phenols as represented in Figure 1. According to this schematic illustration that we suggest, they  
46 could be involved closely in the reducing and stabilizing of  $\text{Ag}^+$  to  $\text{Ag}^0$  where the presence of electrons  
47 from oxygen atoms helped in the absorption of compounds on Ag-NPs [29].

1 In the present work, the walnuts extract was proposed to be a suitable and convenient plant to  
2 the green synthesis of Ag-NPs. The method used in this research is totally green and involves an easy  
3 single step process by combining the solution of AgNO<sub>3</sub> and walnuts aqueous extract. The silver  
4 nitrate is utilized as the silver precursor and walnuts extract due to the high contents of polyphenols  
5 compounds were utilized as the stabilizer and reducing agents, simultaneous. Furthermore, utilizing  
6 nontoxic and cheap compounds in the plant extract and reaction in moderate temperature are some  
7 of these procedures advantages. As far as the researchers are aware, this is the first time that walnut  
8 extracts are used in the Ag-NPs' synthesis.



9  
10  
11 **Figure 1.** Schematic of synthesized Ag-NPs interactions with activated functional groups of *J. regia*.

## 12 13 **2. Materials and Methods**

### 14 *2.1 Material*

15 The walnut green husk was collected from Sorkh-e-Hesar Tehran, Iran and specimen of this plant  
16 identified by Iranian Research Institute of Plant Protection (IRIPP). Silver nitrate was used as a silver  
17 precursor and provided by Bendosen Company (99.89%, C0721-2284551). Nutrient agar and nutrient  
18 broth were purchased from MERCK KGaA. All reagents in this effort were analytical grade and were  
19 used as received without further purification. All solutions were freshly prepared using double  
20 distilled water and kept in the dark to avoid any photochemical reactions. All glassware used in  
21 experimental procedures were cleaned in a fresh solution of HNO<sub>3</sub>/HCl (3:1, v/v), washed thoroughly  
22 with double distilled water, and dried before use.

### 23 *2.2 Extraction Preparation*

24 Walnut green husk (Fig. 2a) was washed and dried in an oven dryer at 40 °C for 48 h. The green  
25 husk of *J. regia* dry ground in a mill, stored in glass bottles and kept at room temperature for next  
26 analysis (Fig. 2b). The finely ground *J. regia* green husk (0.5 g) was added to the 100 ml of the boiling  
27 water for 30 min. It then was filtered through the filter paper. The concentrated extracts were kept in  
28 a dark place at 4 °C for future use (Fig. 2c).

1



2

3 **Figure 2.** *Juglans regia* green husk (a), powder of *J. regia* green husk (b) and aqueous extract of *J. regia*  
4 green husk (c).  
5

### 6 2.3 Synthesis of *J. regia* Ag-NPs

7 In a typical reaction procedure, 0.5 g extract of *J. regia* was added to 100 ml distilled deionized  
8 water with vigorous stirring for 30 min. A twenty-fivemilliliters of  $\text{AgNO}_3$  ( $5 \times 10^{-3}$  M) was added  
9 and homogenized by using a magnetic stirrer at room temperature ( $25^\circ\text{C}$ ) for 10 h. The Ag-NPs were  
10 gradually obtained during the incubation period.

### 11 2.4 Antibacterial activity

12 The in-vitro antibacterial activity of the new compounds was evaluated against two pathogenic  
13 Gram negative bacteria; *E. coli* (*Escherichia coli*), *P. aeruginosa* (*Pseudomonas aeruginosa*), and two  
14 Gram-positive bacteria, *S. aureus* (*Staphylococcus aureus*), and *B. cereus* (*Bacillus cereus*) via a disc  
15 diffusion method. The in-vitro antibacterial test was carried out in keeping with the recommended  
16 standards of the National Committee for Clinical Laboratory Standards based on the determination  
17 of the inhibition zone in millimeters (mm) in nutrient agar, [38, 39]. The microbe cultures were  
18 standardized to the 0.5 McFarland standard which is approximately  $10^8$  cells. Ampicillin was applied  
19 as a positive control ( $10 \mu\text{g mL}^{-1}$  concentrations). Briefly, test compounds previously sterilized with  
20 UV were inoculated with 6 mm diameter paper discs and then positioned on the nutrient agar surface  
21 of the microbial growth plate. The plates were inverted and incubated at  $37^\circ\text{C}$  for 18-24 h until  
22 sufficient growth was achieved. After incubation, the diameters of the zones around the specimens  
23 showing the inhibition amount were measured in millimeters from the back of the petri plates by the  
24 ruler (naked eye).  
25

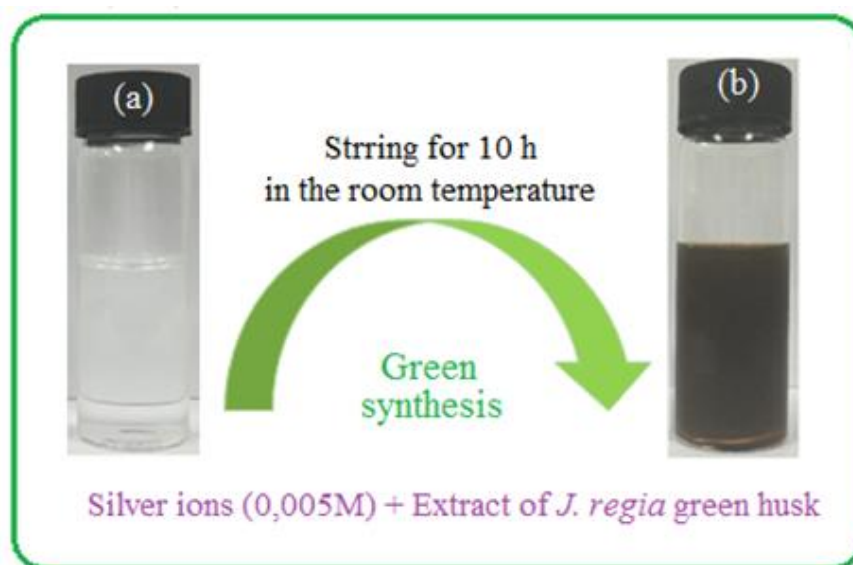
### 26 2.5 Characterization of the *J. regia* Ag-NPs

27 The XRD analysis was carried out to determine the phase crystallinity and purity of the  
28 synthesized Ag-NPs (studied by using PXRD in the small angle range of  $2\theta$  ( $10-90$  degrees). The scan  
29 speed of 2 degrees/minutes was applied to PXRD patterns recording). The Ag/*J. regia* preparations  
30 were characterized by the use of ultraviolet-visible (UV 1800, SHIMADZU) spectroscopy (UV-vis) in  
31 the range 400-800 nm. The image of TEM were characterized by applied a Hitachi H-71001 electron  
32 microscope (Hitachi High-Technologies Corporation, Tokyo, Japan), Japan transmission electron  
33 microscope (TEM). Zeta potential using the Zeta/Nano Particle Analyser, (Systems Nano-Plus,  
34 Japan), was utilized to measurement the charge of the droplet surface of solution which may cause

1 effects on the chemical and physical stability of the Ag-NPs[30]. Surface roughness measured by  
2 Atomic Force Microscopy (AFM, BRUKER: Innova USA) in noncontact mode as well as provides  
3 high-resolution two-dimensional and three-dimensional image information. Surface roughness  
4 determined by AFM in tapping mode. The tapping mode is that the probe does not touch the sample  
5 during scanning but oscillates above it.

### 6 3. Results and Discussion

7 Figure 3a and b represent the reduction of  $\text{Ag}^+$  into Ag-NPs during exposure to *J. regia* green husk  
8 extracts could be followed by the color change. The fresh suspension of *J. regia* green husk was pale  
9 yellow in color. However, after addition of  $\text{AgNO}_3$  and stirring for 10 h at room temperature, the  
10 emulsion turned to dark brownish color. The color changes in aqueous solutions are due to the surface  
11 plasmon resonance phenomenon. The result obtained in this investigation is interesting because it  
12 can serve as a foundation in terms of identification of potential forest plants for synthesizing Ag-NPs.  
13 The color change has evidenced the reduction of  $\text{Ag}^+$  ions to  $\text{Ag}^0$  by the green husk of *J. regia* extraction  
14 via aredox reaction. Inspection of the sample by UV-vis revealed that the optimum point after 10 h to  
15 the reaction at room temperature was obtained.

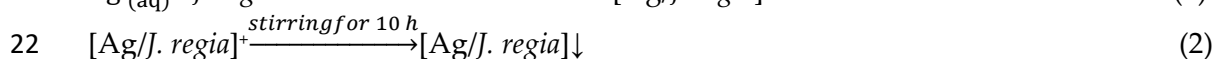
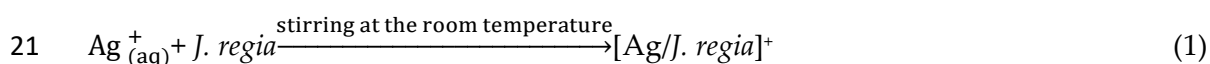


16

17 **Figure 3.** Photograph of color changing during of reducing Silver nitrate (a) to Ag-NPs after 10 h.

18

19 *Juglans regia* green husk as a source of carbonyl and phenolic groups can reduce silver ions to Ag-  
20 NPs. The possible chemical equations for preparing the Ag-NPs are:



23 After dispersion of silver ions in the *J. regia* aqueous solution matrix (Equation 1), the extract was  
24 reacted with the Ag to form  $[\text{Ag}/J. regia]^+$  complex, which reacted with functional groups in the  
25 molecular structure to form  $[\text{Ag}/J. regia]$  due to the reduction of silver ions through the oxidation  
26 process (Equation 2).

27



### 3.1 X-Ray Diffraction Analysis

The crystallinity of *J. regia*/Ag-NPs has been studied by the XRD pattern. Figure 3 shows the XRD pattern of *J. regia* and the *J. regia*/Ag-NPs. Figure 4(a) indicates the peak at 28.81 which corresponded to the *J. regia* structure. In addition, Figures 4 (b) shows the *J. regia*/Ag-NPs after 10 h. These two patterns show the peaks in 38.26, 44.47, 64.71, 77.74, and 81.91 that could be attributed to (111), (200), (220), (311), and (222) crystallographic planes of the face-centered cubic (fcc) structure. Based on reference database ICDD/ICSD from X'Pert High Score Plus (reference code: 01-087-0719), these peaks are aspecific compound to the crystalline of Ag. The particle size has been calculated using Debye-Scherrer equation (3):

$$D = \frac{k\lambda}{\beta \cos \theta} \quad (3)$$

where *D* is the average crystalline particles size of Ag-NPs, *K* is the Scherrer constant with value of 0.9,  $\lambda$  represents the X-ray wavelength (radiation/wavelength Cu *K*-alpha is 0.154 nm), and  $\theta$  is the Bragg angle which is  $2\theta = 38.30^\circ$ ,  $\theta = 19.15^\circ$ . Here in,  $\beta$  is the full width at half maximum of the diffraction peak. It can be found that the calculated average size is 33.81 nm which was almost the same under TEM observation.

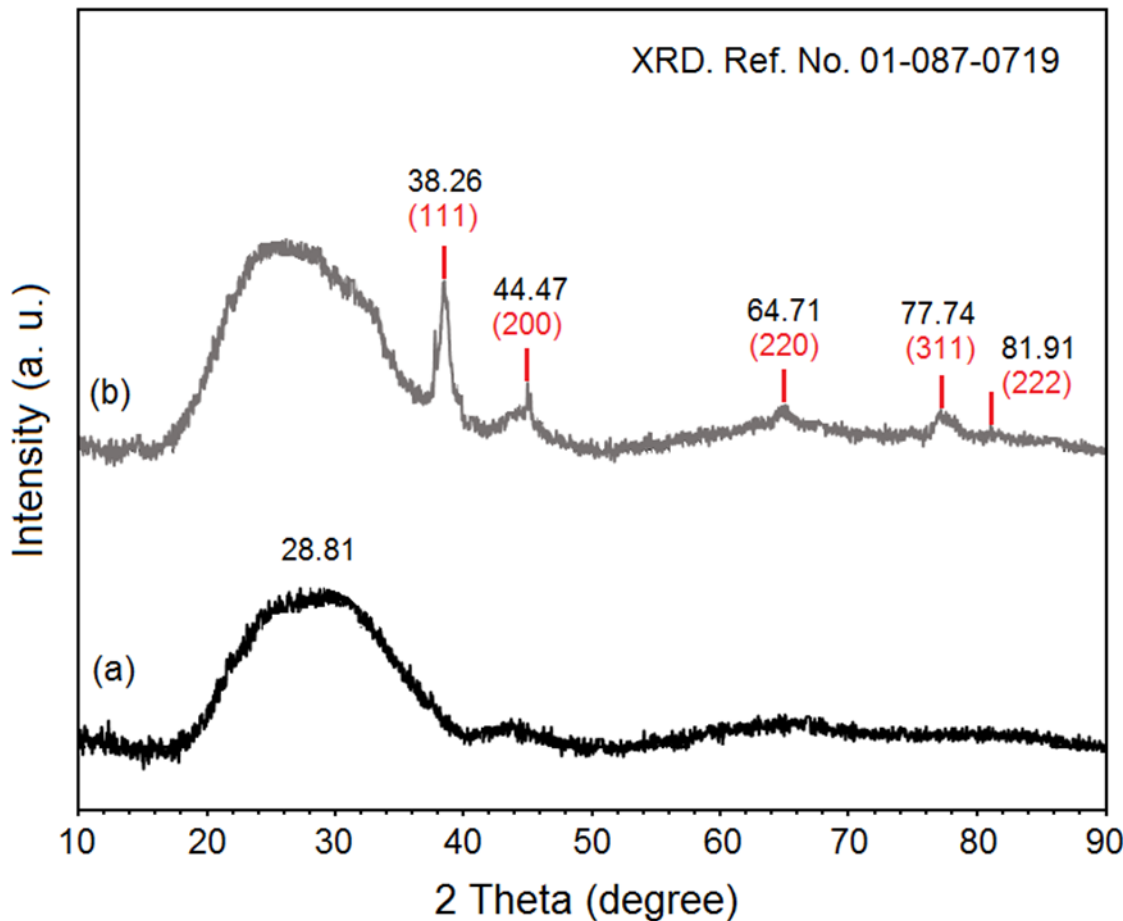
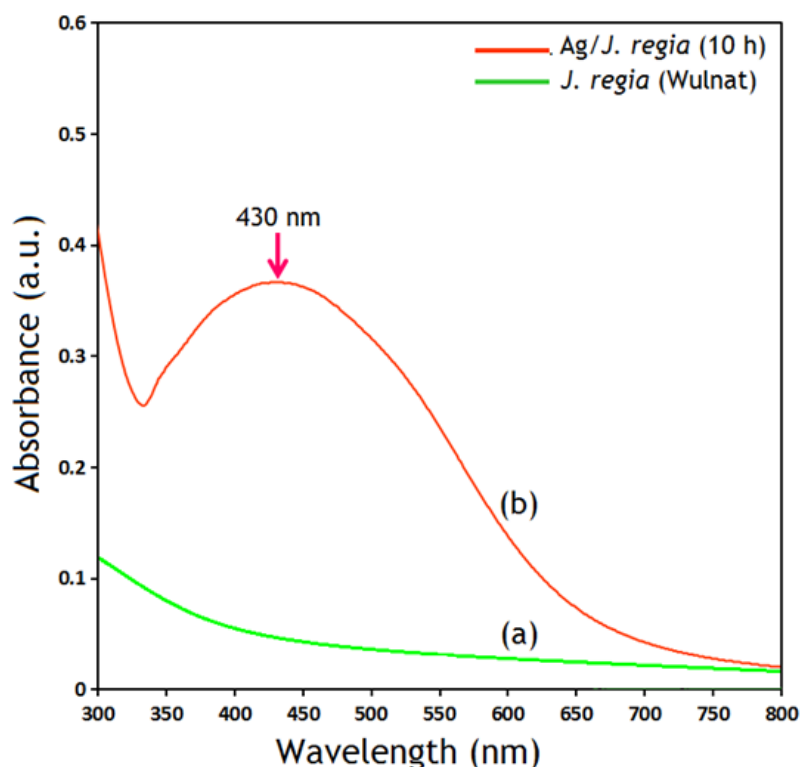


Figure 4. The XRD Pattern of *J. regia* (a) and *J. regia*/ iron oxide nanoparticles (b).

### 3.2 Ultraviolet-visible Spectroscopy Analysis

The aqueous extract of *J. regia* green husk and Ag/*J. regia* was measured by UV-Vis spectroscopy over the wavelength range from 300–800 nm was showed in Fig. 5a and b. there is no any absorption peak for aqueous extract of *J. regia* green husk (green). The surface plasmon resonance (SPR) bands

1 are influenced by the size, shape, morphology, composition and dielectric environment of the  
2 prepared nanoparticles. Previous studies have shown that the spherical Ag-NPs contribute to the  
3 absorption bands at around 420–450 nm in the UV-visible spectra. These absorption bands were  
4 assumed to correspond to the Ag-NPs with relatively small size (less than 40 nm) [31]. UV-Vis  
5 absorption spectra showed that the broad SPR band contained one peak at 430 nm. This peak  
6 illustrates the presence of a homogeneous distribution of hydrosol Ag-NPs after 10 h of stirring times  
7 and also indicates that the concentration of Ag-NPs has increased with the increase of absorbance.

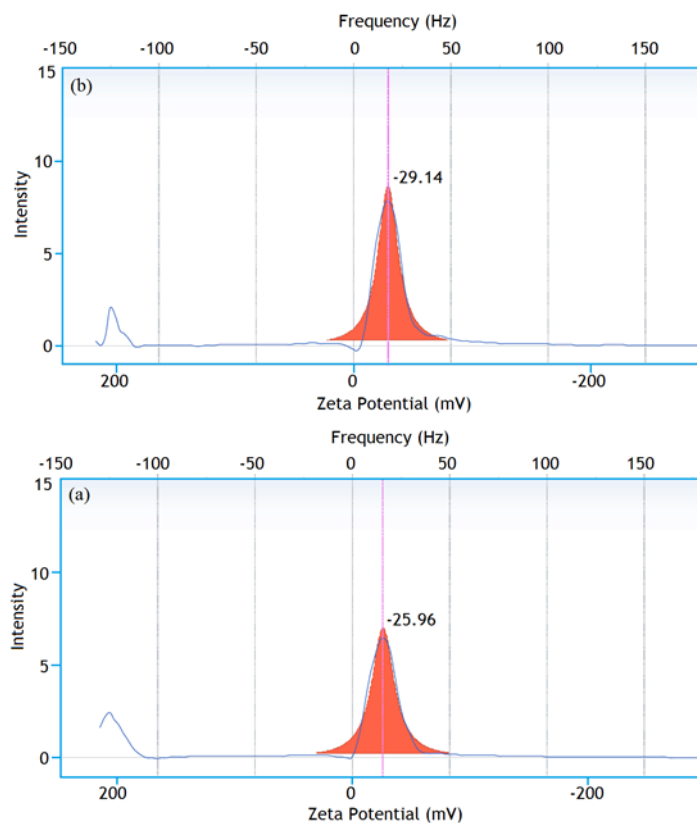


8

9 **Figure 5.** UV-vis absorption spectra of *J. regia* extract (a) and Synthesized Ag-NPs at room temperature (b).

### 10 3.3 Zeta potential analysis

11 The Zeta analysis was carried out to find the potential to gain information regarding the surface  
12 features of the nanoparticles. Long-term stability of particulate systems may be indicated by this  
13 equipment. A suspension that is stable for electrostatic repulsion that is physically stabilized using  
14 the value of zeta value of approximately  $\pm 30$  mV is needed. Furthermore, an integrated stabilization  
15 of electrostatic and steric at  $\pm 20$  mV is suitable [32]. The potential for zeta of *J. regia* contains a value  
16 of -25.96 mV, while the values of *Ag/J. regia* in room temperature transform to 29.14 mV (Fig6a and  
17 b). According to the appropriate value for solution stability ( $\pm 20$  mV), the *Ag/J. regia* demonstrate  
18 appropriate stability. The *Ag/J. regia* in room temperature is gradually lowered, but with suitable  
19 amounts of stable expression, thus resulting in the stable *Ag/J. regia* nanoparticles.



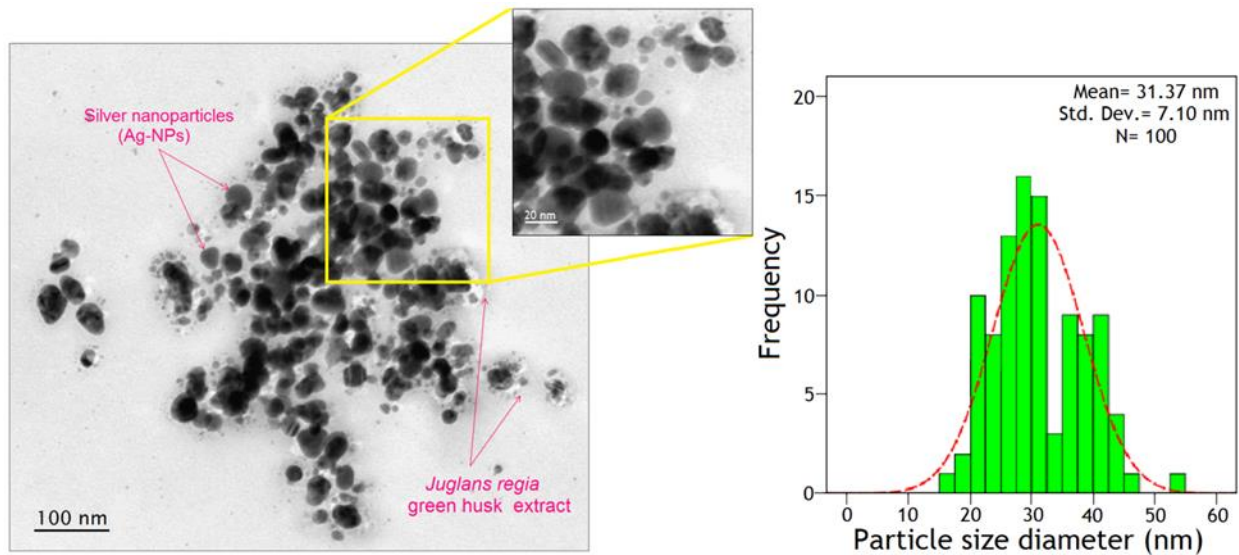
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2 **Figure 6.** Zeta potential results for (a) *J. regia* and (b) Ag/*J. regia* at room temperature respectively.

### 3 3.4 Morphology Study

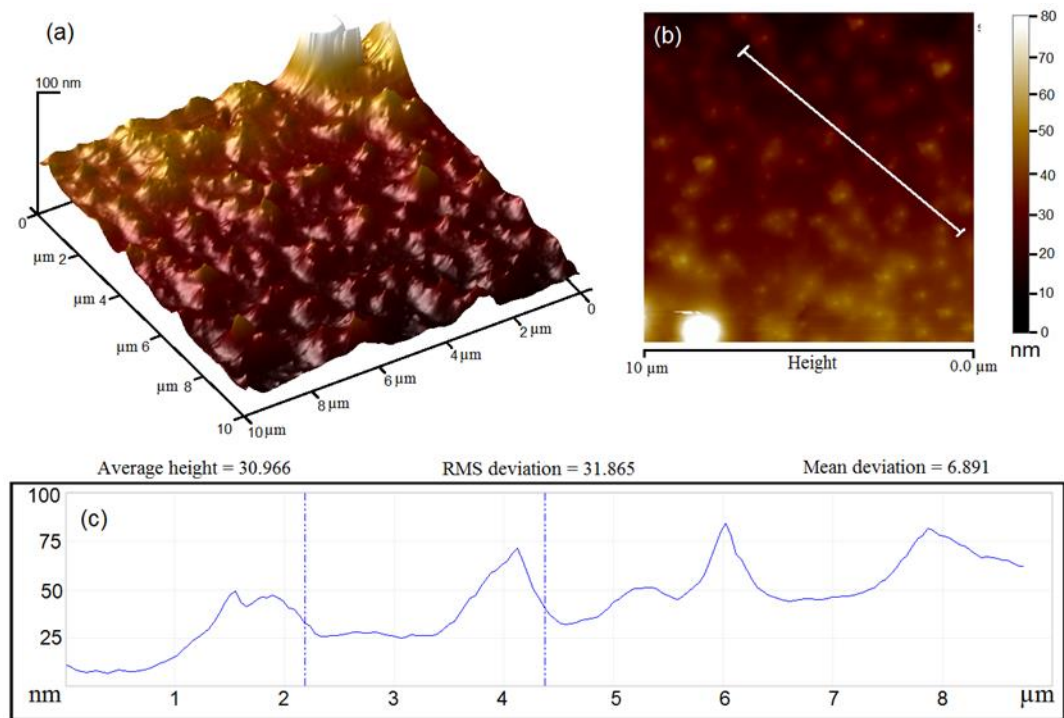
4 The image of the TEM was used to examine the shape and size of the Ag-NPs that are synthesized  
5 and it reveals that many of the nanoparticles are spherical. The image shows that most of the  
6 agglomerated particles are present because of the thickening traits of the green husk of *J. regia*[33]. In  
7 addition, the act of agglomeration is expected as the Ag-NPs that are synthesized are small and  
8 possess magnetic characteristics[34]. A histogram of the particle size distribution was carried out  
9 based on the sizes of the 100 nanoparticles. Also, TEM result can be observed clearly that Ag-NPs  
10 surrounded by the *J. regia* extract in the high magnification of TEM. Thus, the TEM image and their  
11 size distribution are shown in Figure 7, the result showed narrow particle size distributions, with  
12 diameters in the range of 24.27–38.47 nm. Moreover, the mean diameter and standard deviation of  
13 Ag-NPs is  $31.37 \pm 7.10$  nm.





**Figure 7.** TEM images and corresponding size distributions of Ag/*J. regia* after 10 h from the reaction time.

The result of the AFM reveals the surface morphology of the Ag-NPs that is formulated in the media for *J. regia* green husk. AFM's established value and close to the established TEM, and the *J. regia* green husk films having the Ag-NPs demonstrated a densely uniform and packed structure. Figure 8a and b represent the three diameters and two diameters of surface structure for Ag-NPs, while Figure 8c display transverse profiles depicting the looming and depth of the surface structure formed by the tapping mode. Therefore, the green husk films of Ag-NPs *J. regia* could offer a biocompatible and rough surface for unique applications of biology, including the immobilization of the cells.



**Figure 8.** Atomic force microscopy images of Ag/*J. regia* after 10 h and display transverse profiles depicted (a-c).

### 3.5 Antimicrobial tests

In this antibacterial study, inhibition zone values were obtained for the Ag-NPs against both Gram positive (*S. aureus* and *B. cereus*) and Gram negative (*E. coli* and *P. aeruginosa*) bacteria were examined using disc diffusion technique, and results are represented in Table 1. An average inhibition zone of 19(±1) and 13(±1) mm were determined for Ag-NPs against *S. aureus* and *B. cereus*, respectively. An average inhibition zone of 9(±1) mm was measured against *E. coli* for Ag-NPs. Also, no activity was detected against the *P. aeruginosa* bacteria. The inhibition activity of antibacterial agents depends on their permeability into the ribosomes of microorganisms or microbial cells [35]. Although no significant difference was determined between the antibacterial activities of Ag-NPs, based on our observations with naked eyes, both esters had greater inhibition zones against Gram-positive bacteria compared to Gram-negative ones. This is due to the fact that most Gram-negative bacteria have a thicker cell wall than Gram-positive bacteria and are thus more difficult to penetrate into their cells [36-37].

**Table 1.** Antibacterial inhibition zone (mm) of Ag-NPs.

Samples	Inhibition zone (mm)			
	Gram positive bacteria		Gram negative bacteria	
	<i>S. aureus</i>	<i>B. cereus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
Ampicillin	44	35	26	NA
<i>J. regia</i>	NA	NA	NA	NA
Ag-NPs	19	13	9	NA

NA\*: No activity found at the tested concentration.

## 4. Conclusions

In this research, silver nanoparticles were synthesized in room temperature by using simple and biosynthesis method. According to the results, the aqueous extract of *J. regia* green husk has a great effect in reducing and stabilizing of Ag<sup>+</sup> to Ag<sup>0</sup> at the room temperature. The Ag-NPs were subjected to characterized by UV-vis, XRD, zeta potential, TEM and AFM. The XRD represents crystalline of Ag-NPs without apparent impurities. According to the result obtained from zeta potential analysis the Ag-NPs were improved after synthesized Ag-NPs by using plant extract. According to the result mentioned above, AFM's established value and close to the established TEM result with an average size and standard deviation of 31.37 ± 7.1 nm at the room temperature. The zeta potential analysis indicated that *J. regia* green husk extract has negative charge and zeta potential value increasing by fabricate Ag-NPs. The use of *J. regia* green husk of the plant takes full advantage of the waste material in any desired is economically friendly, safe and efficient. Using green source as *J. regia* for the biosynthesis of paper Ag-NPs is a better alternative compared to the chemical or physical synthesis, since this is the free biosynthesis and ecological pollutants. Antibacterial activity of Ag-NPs was demonstrated, and showed strong antibacterial activity against Gram-positive more than Gram-negative bacteria. Needless to say, further studies are required to investigate the bactericidal effects of Ag-NPs on different types of bacteria for potential widening of this subject area, such as surgical devices or as drug delivery vehicles.

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6 Sorkh-e-Hesar Tehran (IRAN). This research and its development in nanoparticle biosynthesis and  
7 its application in medicine are inspired by his ideas.

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