



## Green synthesis of silver nanoparticles using *Piper betle* exhibit high antimicrobial activity against *Salmonella enteritidis* and *E. coli*

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### Abstract

*Piper betle* (*P. betle*) is a popular medicinal plant in Asia. Plant leaves have been used as a traditional medicine to treat various health conditions. *P. betle* leaf has plentiful antimicrobial products. The extraction of leaves from *P. betle* is of high interest for industrial applications. The aqueous leaf extracts of *P. betle* provided the materials required to produce silver nanoparticles (AgNPs) and promoted their antimicrobial activity. UV/vis absorption spectroscopy, transmission electron microscopy (TEM), and X-ray diffraction (XRD) were used to analyze the AgNPs. This study investigated the antimicrobial AgNPs produced by *P. betle* against *S. enteritidis* and *E. coli*. The minimum biocidal concentration (MBC) and minimum inhibitory concentration (MIC) were the methods used to determine the antimicrobial activity of greenly synthesized silver nanoparticles (AgNPs). Plasmon absorbance was shown in correspondence to peak absorption values of 400–500 nm and the color change of the extract to dark brown. The MICs of *P. betle* and AgNPs were 4.6 mg/mL and 0.06 µg/mL against *S. enteritidis* and 3.7 mg/mL and 0.06 µg/mL against *E. coli*, respectively. AgNPs did not cause the hemolysis of RBCs *in vitro*. Findings reveal AgNPs have higher antimicrobial activity than aqueous leaf extracts of *P. betle* against *S. enteritidis* and *E. coli*.

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### Introduction

The enterohemorrhagic *E. coli* serotype is a human pathogen that causes outbreaks of bloody diarrhea and hemolytic uremic syndrome worldwide. In addition, *S. enteritidis* is one of the most commonly isolated serovars in humans, and any serovar is capable of causing gastrointestinal illness in humans of varying severity. Particles with a maximum size of 1–100 nm, which show strange chemical, physical, and optical features distinct from the bulk material, are called nanoparticles (NPs) (1-3). On the contrary, fewer toxic reactants and additives are utilized by biological methods (e.g., plant extracts). The reaction requires room temperature and takes place without harsh or rigorous conditions. The plant extracts exhibit low cytotoxicity when they are combined with NPs. For that

purpose, safety, eco-friendliness, and low cost are achieved using biological methods with plant extracts, which represent a viable substitute when applied in microbiology (4). Many industrial and medical experiments use silver as an antimicrobial due to its inhibitory effects on microbes. Most importantly, NPs are used as topical ointments, protecting the patient's open wounds from infection with bacteria and fungi (5,6). *Piper betle*, a member of the Piperaceae family, is high in polyphenols like acetyl eugenol, trans-isoegenol, chavicol, chavibetol, chavibetol acetate, and allyl pyrocatechol diacetate (7), which can act as reducing and stabilizing agents. The leaves of *P. betle* are more helpful to humans. Their beneficial effects include their antimicrobial activity, antioxidants, and anti-diabetic properties. The leaves are also known for their wound-healing properties and possess other beneficial properties

(7). However, the information about the antibacterial features of *P. betle* against *S. agalactiae* is not well known. AgNPs are the most competitive antimicrobial materials in the application of nanomedicine due to their properties that enable them to interact with the cell membrane of microorganisms and, in the process, generate reactive oxygen species that eventually cause cell death (8).

The current study used aqueous extracts of *P. betle* to synthesize AgNPs and characterize their crystal structure, morphology, mean size estimation, and distribution using XRD and TEM techniques. Therefore, this research investigated the antimicrobial activity of an aqueous extract of *P. betle* and AgNPs manufactured from *P. betle* extract against *S. enteritidis* and *E. coli* at the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentration (MBC). The study has some limitations that must be addressed. Because the zone of inhibition has not been reported, these parameters should be tested in future studies. Furthermore, data from the Fourier transform infrared spectra (FTIR) analysis for identified putative functional groups of biomolecules engaged in bioreduction and capping for effective stabilization of silver nanoparticles have not been reported.

## Materials and methods

### Ethical approve

The Ethical Committee's approval was obtained from the Ethical Committee of the Middle East University (No. 2022MEU015), Amman, Jordan, in March 2022.

### Bacterial cultures

The research used *E. coli* (ATCC 25922) as a preferred reference strain for evaluating antibiotic susceptibility, and *S. enteritidis* was used for vaccine production. Both were obtained from the Jordan Bio-industries Center (JOVAC, Amman, Jordan).

### Plant extraction and synthesis of AgNPs

*P. betle* aqueous extract was prepared by mixing 10 g of dry leaf sample with 100 ml of highly purified water in a flask. The flask was plugged with cotton wool and stored for 72 h at (8 °C). Filtration of the mixture involved using Whatman filter paper (25mm pore size) to eliminate filtered plant residues (9). The next step was drying in a vacuum drier, and the dried pellets were kept for analysis at normal room temperature conditions. The pellets are then stored at a temperature of -20 °C until they are ready for use. The AgNPs were manufactured biologically, as previously explained (5,10). In brief, 10 ml of aqueous leaf extract of the *P. betle* (10 g/100 ml) was mixed with 5 mM of silver nitrate solution and left at room temperature (25°C) for four hours, and the next step was centrifugation at 10,000 rpm for ten minutes. After discarding the supernatant, distilled water (7000 rpm, 2 min) was used to wash the residue, then dried

in a vacuum dryer. Room temperature was required when storing dried pellets before they could undergo various analyses (11). After removing the supernatant, the residue was washed with distilled water at 7000 rpm for 2 minutes before being dried in a vacuum dryer. Dried pellets had to be stored at room temperature before various analyses could be performed (11).

### Characterization of green silver nanoparticles (AgNPs)

The manufactured AgNPs could be analyzed with the help of ultraviolet/visible spectrophotometry. Absorption revealed that silver nanoparticles formed surface plasmon resonance at 420nm. We used a 40 kV X'Pert Pro x-ray diffractometer (XRD) (PANalatical Empyrean-2012) to analyze the AgNPs composite after it had dried. The structural composition of the dried AgNPs was determined using the GBC-Diffttech MMA model. Ni-filtered Cu Ka ( $k = 1.54 \text{ \AA}$ ) radiation was used at 34.2 mA and 35 kV. The sizes of AgNPs were also determined using transmission electron microscopy (TEM). To prepare samples, SNPs were drop-coated on carbon-coated copper TEM grids. After two minutes, the films on the TEM grid were blotted with paper towels to remove any excess solution. The grid had been dried before measuring.

### Determination of minimum inhibitory concentration and minimum biocidal concentration

Adopted guidelines to determine MICs and MBCs of the active peptides were referenced by the Clinical and Laboratory Standards Institute (CLSI) (12). Micro broth dilution was used using sterile 96-well polypropylene microtiter plates. To summarize, Mueller Hinton Broth (MHB) was used as the growth media for organisms. The growth media supported the growth of bacteria overnight and then diluted to  $10^6$  colony forming units (CFU)/ml in the media before use. However, different dilutions used included a concentration range of 1-10 mg/ml in the case of an aqueous extract of *betle*, and the concentration of silver nanoparticles was 0.02-1  $\mu\text{g/mL}$ . After that, a combination of a concentrated solution and a diluted bacterial suspension, both at 50  $\mu\text{l}$ , is added to a 96-well microtiter plate. The plates used contained six replicates that contained peptides at a concentration divided by six wells (12-14). However, incubation of the plate was carried out at 37°C for 18 hours. After that, bacterial growth was determined; it included measuring OD at  $\lambda = 570 \text{ nm}$  using an Enzyme-linked Immunosorbent Assay plate reader. The determination also showed that the lowest concentration required to inhibit bacterial growth was MIC. In addition, each plate contained a positive control to maintain bacterial activity and a negative control to maintain bacterial sterility. Each experiment was repeated three times to obtain accurate results.

### Erythrocyte hemolytic assay

Hemolytic assays were performed to determine the possibility of a conjugation process causing hemolysis in human blood. The process entailed centrifuging 2 ml of human blood at 3000 xg for 5 min. The supernatant was discarded, and the following procedure involved the suspension of the cell pellet in 48 ml of phosphate-buffered saline. The last step was centrifugation at 3000 x g for 5 minutes, which was repeated three times for accuracy (15).

### Results

The absorbance of the sample increases as the nanoparticles are generated within the reaction mixture. There was a color change, which turned dark brown. It was an indication that nanoparticles were produced (Figure 1). The particles (AgNPs) measured 5 and 30 nm in size, according to TEM analysis (Figure 2A). From the XRD pattern, AgNPs have cubic and hexagonal structures, with particle sizes ranging from 10 to 50 nm. The XRD pattern displayed three intense peaks in a spectrum of two values from 10 to 80. Bragg reflections were seen with three different values: 38.52°, 46.529°, and 64.53°. These three sets of lattice planes can be indexed to the 111, 200, and 220 facets of silver (Figure 2B).

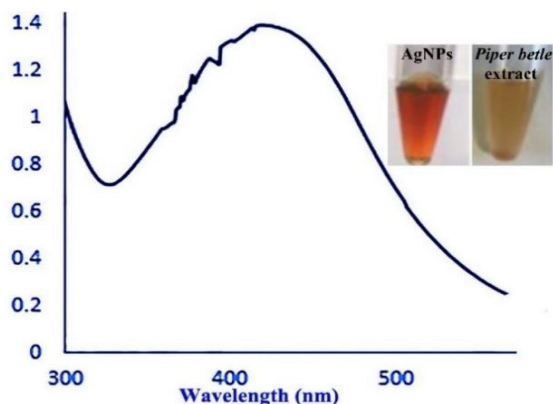


Figure 1: Absorption of silver nanoparticles surfaces plasmon resonance at 420 nm. The brown color indicates AgNPs formation.

Results obtained showed that AgNPs appeared to have high antimicrobial activity against both *S. enteritidis* and *E. coli* (ATCC 25922). The MIC and MBC values of the *P. betle* were 4.6 mg/ml, >6 mg/mL, and when conjugated with AgNPs, they were 0.06 µg/mL and 0.14 µg/mL, respectively, against *S. enteritidis*. Also, the MIC and MBC values of the *P. betle* were 3.7 mg/mL, >6 mg/mL, and when conjugated with AgNPs, were 0.06 µg/mL and 0.13 µg/mL, respectively, against *E. coli* (ATCC 25922) (Table 1). Finally, the hemolysis of RBCs was not observed due to the AgNPs effect (Table 2).

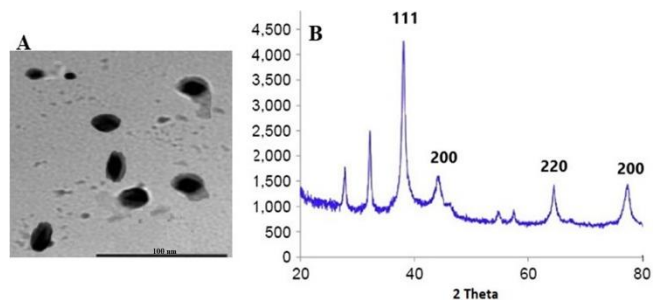


Figure 2: (A) TEM analysis of AgNPs (B) XRD pattern of synthesized AgNPs.

Table 1: The MIC and MBC values of *P. betle* extracts and AgNPs against *S. enteritidis* and *E. coli*

Antibiotics	<i>P. betle</i> extracts (mg/ml)		AgNPs conjugated with <i>P. betle</i> (µg/mL)	
	MIC	MBC	MIC	MBC
<i>S. enteritidis</i>	4.6	>6	0.06	0.14
<i>E. coli</i> (ATCC 25922)	3.7	>6	0.06	0.13

Table 2: The hemolysis activity of AgNPs in human erythrocytes *in vitro*

Concentration (µg/mL)	Hemolysis %
40	0
80	0
100	1

### Discussion

Nanotechnology holds great promise in the biomedical field, particularly for diagnostics and drug delivery. Nanotechnology can deliver therapeutic agents to specific cells and receptors (16). Many researchers worldwide were interested in developing new antibacterial agents that were eco-friendly. The reason that *P. betle* has antimicrobial activity is due to the tannin and flavonoids that are its products. The MIC for the *P. betle* against *S. aureus* was 0.78 mg/ml, which is more sensitive than the *Vibrio vulnificus* at 3.15 mg/ml. The concentration of *P. betle* required as an antibacterial differs between bacteria types (17).

AgNPs demonstrated effective antimicrobial activity against *Salmonella* spp. It inhibited *Salmonella* spp. with efficacy ranging from 84.81 to 99.92% at a concentration of 5 µg/mL, MIC range (≤0.002-0.313 µg/mL) with an average mean of 0.085±0.126 µg/mL, and the MBC range (0.078-1.250 µg/mL) with an average of 0.508±0.315 µg/mL (18). Also, AgNPs were shown to inhibit *E. coli* growth significantly. These findings imply that AgNPs could be employed as an antibacterial material (19). These findings

are consistent with the findings of our study; the MIC and MBC values of *P. betle* were 4.6 mg/mL and >6 mg/mL, and when conjugated with AgNPs, they were 0.06 µg/mL and 0.14 µg/mL, respectively, against *S. enteritidis*.

Several mechanisms can explain this result regarding the bactericidal effects of silver nanoparticles. Drug-binding nanoparticles have been developed to eradicate drug-resistant bacterial and viral infections (20-25). Our study found that the AgNPs antibacterial efficiency was higher than the aqueous extract of *P. betle* against *S. enteritidis* and *E. Coli*. AgNPs attaching to the cell membrane interrupts the cell's permeability and metabolic pathways (26). Also, silver nanoparticles can invade bacterial cell membranes (27-31). Besides, bacterial replication or interaction with other bacterial ribosomes can be inhibited by AgNPs, which generally can bond to the DNA of the cells (13,32). Another study showed that AgNPs could cause damage to the bacterial cell membrane and many membranous enzymes that make *E. coli* and *S. enteritidis* (33-36).

Other studies have found that AgNPs are toxic as they lead to malfunctions in the mitochondria and the formation of abnormal cells. Therefore, despite these findings, the concentrations of AgNPs needed in clinical trials are much lower than those used *in vitro*, and data on their long-term effects on health in animals and humans is still missing. Therefore, more research should be conducted to ensure the safety of AgNPs and their use in clinical studies (37-40).

## Conclusion

The study's findings support the use of AgNPs as a therapeutic agent. The conjugated *P. betle* with AgNPs expresses a consequential antimicrobial effect against *S. enteritidis* and *E. Coli* at lower concentrations than the aqueous extract of *P. betle*. This conjugated *P. betle* with AgNPs appears to inhibit the ability to adapt, resulting in the reduction of bacterial resistance. Regarding toxicity, AgNPs did not induce hemolysis of RBCs.

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## Competing interests

The authors declare that they have no competing interests.

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## تظهر جسيمات الفضة النانوية المحضرة باستخدام مستخلص أوراق التبوتول نشاطاً عالياً كمضادات ميكروبي ضد السالمونيلا المعوية والإيشريكية القولونية

محمد جمال سعادة

قسم العلوم الأساسية، كلية الصيدلة، جامعة الشرق الأوسط، الأردن

### الخلاصة

تحتوي أوراق التبوتول على العديد من المنتجات المضادة للميكروبات. ويستخدم الطب التقليدي أوراق النبات لعلاج الأمراض المختلفة. فأوراق التبوتول والتي تستخدم بكثرة من قبل شعوب جنوب آسيا وشبه القارة الهندية للمعالجة ولتحسين رائحة الفم وذلك لاحتوائها على مضادات الأكسدة والمعروفة باسم الفلافونويد والتي لها تأثير مضاد للالتهابات أيضاً. لذلك تم استخدام المستخلص المائي لأوراق نبتة التبوتول لاحتوائه على المواد اللازمة لإنتاج جسيمات الفضة النانوية والتي بدورها عززت نشاطها المضاد للميكروبات. تم استخدام التحليل الطيفي لامتصاص الأشعة فوق البنفسجية، والمجهر الإلكتروني، وحيود الأشعة السينية لتحليل جسيمات الفضة النانوية. هذه الدراسة بحثت عن فعالية جسيمات الفضة النانوية كمضادات للميكروبات ضد السالمونيلا المعوية والإيشريكية القولونية. كان الحد الأدنى من التركيز اللازم لقتل البكتيريا والحد الأدنى من التركيز الذي يمنع النمو المرئي للبكتيريا هما الطريقتان المستخدمتان لتحديد النشاط المضاد للميكروبات لجسيمات الفضة النانوية المصنعة. وجد ان التركيز الذي يمنع النمو المرئي لبكتيريا الإيشريكية القولونية عند استخدام المستخلص المائي لنبات التبوتول وعند استخدام جسيمات الفضة النانوية المحضرة بمستخلص مائي لأوراق التبوتول هو 3,7 و 0,6 ميكروجرام/مل على التوالي. وجد أن التركيز الذي يمنع النمو المرئي لبكتيريا السالمونيلا المعوية عند استخدام المستخلص المائي لنبات التبوتول وعند استخدام جسيمات الفضة النانوية المحضرة بمستخلص مائي لأوراق التبوتول هو 4,6 و 0,6 ميكروجرام/مل على التوالي. نستنتج ان فعالية جسيمات الفضة النانوية ضد السالمونيلا المعوية والإيشريكية القولونية أكبر من المستخلص المائي لأوراق نبتة التبوتول. كما لم تسبب جسيمات الفضة النانوية انحلال الدم لكريات الدم الحمراء في المختبر.