RESEARCH ARTICLE

The application of Spirulina platensis based green synthesized silver nanoparticles demonstrated potent anti Shigella flexneri effects by specifically targeting pathogenic gene expressions

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ABSTRACT

Background: The emergence of antibiotic-resistant strains of *Shigella flexneri*, an important cause of shigellosis, has led to extensive research to find alternative treatment approaches. Therefore, in the current study, the antibacterial effects of the green synthesized silver nanoparticles (AgNPs) using *Spirulina platensis* on *S. flexneri* and also the expression of pathogenic genes *ipaB*, *ipaD*, *ipaH* and *qnrS* were studied.

Methods: After the synthesis of AgNPs using *S. platensis*, its antibacterial effects on *S. flexneri* were studied using microdilution method with 96-well plate. Also, in order to determine the minimum bactericidal concentration (MBC), 10 µL of the contents of the MIC well and so on was swapped on nutrient agar medium. After RNA extraction, cDNA synthesis and primer design, expression levels of *ipaB*, *ipaD*, *ipaH* and *qnrS* genes were studied using Real-Time PCR technique. Data analysis was done in GraphPad Prism V.8 software.

Results: The MIC of the green synthesized AgNPs was measured as 0.0625 µg/ml and its MBC was 0.125 µg/ml. The results of RT-PCR analysis indicated a significant decrease in the expression levels of pathogenic genes *ipaB*, *ipaD*, *ipaH* and *qnrS* in AgNPs-treated *S. flexneri*.

Conclusion: The green synthesized AgNPs using *Spirulina platensis* has strong antibacterial effects on *S. flexneri* and the action mechanism was attributed to the downregulations of *ipaB*, *ipaD*, *ipaH* and *qnrS* genes. The in vivo and clinical studied are needed.

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INTRODUCTION

Infectious diseases are of the most important and common diseases in the world, which cause many problems to the health system of most countries, especially developing countries (1). One of these kinds of diseases is shigellosis, which is caused by *Shigella* sp. bacteria, especially *Shigella flexneri* (2*)*, and is an important cause of bacterial gastroenteritis and dysentery (3). 12.5% of deaths caused by diarrheal diseases are due to *Shigella* and its mortality rate is higher in children under 5 years of age (4). Clinical manifestations include diarrhea, dysentery, high fever, abdominal cramps, myalgia, and rectal tenesmus or spasm (5).

The ability of *S. flexneri* to penetrate into epithelial cells is due to the presence of its large invasive plasmid whose genes are responsible for the coding of invasive proteins (IPs) (6). These invasive proteins include *ipaA*, *ipaB*, *ipaC*, *ipaD*, and *ipaH* (7). After the bacteria contact the host cells, IpaB and IpaC inject inosines into the cytoplasm of the cell by creating a pore on the plasma membrane (8). IpaD provides bacteria with the ability to phagocytize (9), and IpaA causes depolymerization of F-actin by binding to vinculin (10). On the other hand, ipaH protects *Shigella* from macrophages (11), which can move to the nucleus of the host cell and stimulate the secretion of *Shigella* proteins (12). One of the acquired genes involved in creating relative resistance to quinolones in *Shigella* is *qnrS* which protects bacterial DNA by inhibiting the binding of quinolones to DNA gyrase and topoisomerase 4 (13).

The administration of water and electrolytes as well as prescribing antibiotics such as ampicillin, tetracycline, erythromycin, trimethoprim/sulfamethoxazole, and in severe cases ciprofloxacin are among the treatment approaches (14). However, the emergence of antibiotic-resistant strains of *S. flexneri* has reduced the effectiveness of treatments (15, 16). Therefore, there is a need for new approaches to the treatment of shigellosis.

Nanoparticles (NPs) have many applications in medicine due to their unique physicochemical and biological properties (17). The synthesis of NPs using chemical approaches is associated with side effects and environmental harms, which limit their application (18). To overcome these problems, NPs green synthesis methods using plant extracts have been introduced, which are cost-effective and environmentally friendly (18). One of the widely used NPs is silver nanoparticles (AgNPs), whose anticancer and antimicrobial properties have been studied in many studies (19-21). AgNPs have antibacterial properties against gram-positive and gram-negative bacteria, and this compound has shown antimicrobial effects against antibiotic-resistant bacteria (22). For example, AgNPs have shown antibacterial effects on *E. coli*, *S. Typhimurium*, *S. aureus* and *B. subtilis*, and it seems that the smaller size of this nanoparticle is associated with increased antimicrobial activity (23).

Therefore, the current research was aimed to investigate the antibacterial effects of green synthesized silver nanoparticles on *S. flexneri* and evaluate the pathogenic ipaA, ipaD, ipaH and qnrS genes' expressions.

EXPERIMENTAL

AgNPs synthesis

Ethanol extract of spirulina algae (Spirulife, Esfahan, Iran) was used for the synthesis of AgNPs. For this purpose, 20 g of dry spirulina powder was dissolved in 200 ml of 96% ethanol and placed on a shaker at 140 rpm for 35 min. Then, with Whatman filter paper, the solution was filtered and the obtained extract was centrifuged at 13000 rpm for 20 minutes. 340 mg of $AgNO_3$ (Merck, Germany) was mixed in 100 ml of distilled water and 100 ml of spirulina extract and placed on a shaker for 24 hours. After observing the color change of the solution and ensuring the complete reduction of silver ions to silver nanoparticles, the sediment was washed three times

using a centrifuge at 13000 rpm for 20 minutes. Finally, the final sediment was collected after drying at 40°C for 120 min.

Shigella flexneri culture

S. flexneri (ATCC 12022) was obtained from the Microbiology Department of Pasteur Institute of Iran and cultured in nutrient broth at 32°C for 24 hours. Then, the bacteria were separated by centrifugation at 4000 rpm and the McFarland method was used to determine the microbial population. The initial turbidity of the microbial suspension was determined using 0.5 McFarland solution. In order to prepare a microbial population equal to 1.5 $\times 10^6$ bacteria/mL, physiological serum was used .

Minimum inhibition and bactericidal concentration

Microdilution method based on CLSI 2017 standard was used to measure minimum inhibition concentration (MIC) of $AgNO_3$-NPs (19). Briefly, successive dilutions of $AgNO_3$-NPs in the concentration range of 0.063 to 32 mg/ml were poured into the wells of 96-well plates, and then 1 mL of nutrient broth was added to it along with 1 mL of microbial inoculum (1.5 $\times 10^6$ bacteria). The plates were incubated for 24 hours at 37°C. The well containing nutrient broth culture medium with bacteria and the well containing culture medium without bacteria were considered as positive and negative control, respectively.

To determine the minimum bactericidal concentration (MBC), 10 µL from the last well that did not show any bacterial growth were taken and cultured in MH agar medium. The plates were incubated for 24 hours at 37°C.

Gene expression

RNA was extracted using the RNX-PLUS method. Briefly, bacteria were trypsinized and separated by centrifugation for 48 hours after treatment with AgNPs. Then 500 µL of RNX-PLUS solution was added to the samples. Then, 200 µL of chloroform was added and incubated at 4 °C for 5 min and centrifuged at 12000 rpm for 15 min. cDNA synthesis was performed using a kit (BioFact, South Korea) according to the manufacturer's instructions.

Primer design was done using NCBI database and Primer 3 software. The sequences of the primers of *ipaD*, *ipaB*, *ipaH* and *qnrS* genes are given in Table 1. The expression levels of the studied genes were determined by RT-PCR technique using the Cyber green method (Q Rotor Gene, Qiagen). 16s rRNA gene was used as control. The reaction mixture included 7 µL of master mix, 0.5 µL of forward and reverse primers, 5 µL of deionized water, and 1 µL of cDNA. The time-temperature schedule of the RT-PCR machine is given in Table 2.

Statistical analysis

$2^{-\Delta\Delta Ct}$ method was used to analyze the expression levels of *ipaD*, *ipaB*, *ipaH* and *qnrS* genes. Also, the gene expressions between groups were analyzed by unpaired Pearson T-Test at probability levels of $P<0.05$.

RESULTS AND DISCUSSIONS

Results

MIC and MBC

Microdilution method was used to determine the AgNPs MIC against *S. flexneri* and the results showed that the growth of bacteria decreased with increasing concentration of AgNPs and no bacterial growth was observed at the concentration of 0.0625 µg/ml. Therefore, this concentration was considered as the MIC of AgNPs. Next, 10 µL of the wells containing 0.0312 µg/ml AgNPs and so on were removed and cultured in the nutrient agar medium, and after 48 hours, it was observed that the bacteria did not grow in the medium containing 0.125 µg/ml AgNPs and so on. Therefore, the MBC of AgNPs against *S. flexneri* was considered 0.125 µg/ml.

Table 1. The sequences of primers used for measuring the expression levels of *ipaD*, *ipaB*, *ipaH* and *qnrS* genes by RT-PCR technique

Gens	Sequence [5'-3']	GC%	TM (°C)
ipaB	Forward: ACGACTGCTGCAACTAGGAC Reverse: GGAACAAGCCCTGAATCCGA	55	60
ipaD	Forward: ACGGAGTTTCCGTCGTTACC Reverse: GAAGCCGAGCTTGATGGAGA	55	60
ipaH	Forward: ACGACTGCTGCAACTAGGAC Reverse: TGAGATGCTGGAGAATGAGTACC	50	59.6
qnrS	Forward: TCACACATATCGGCACCACA Reverse: TCGCAAGTTGGCATTGTTGG	55	59.97

Table 2. The time- temperature schedule of the RT-PCR

Steps	Temp. (°C)	Time
Denaturation & enzyme activation	95	10 min
Step 1: Denaturation	95	15s
Step 2: Annealing	59	25s
Step 3: Extension & Floresence acquiring	72	30s
Melting curve analysis	65-95	1°C each step

Gene expression analysis

ipaB & ipaH

Both *ipaB* (P=0.006) and *ipaH* (P=0.004) genes expressions in AgNPs-treated *S. flexneri* were decreased significantly compared to the control. The expression level of *ipaH* in control was measured 1.18±0.3, however, in AgNPs-treated *S. flexneri* was measured 0.33±0.08, indicating downregulation of *ipaH* in AgNPs-treated *S. flexneri*. The same was seen for *ipaB* gene, and the expression level was decreased ~3 times compared untreated *S. flexneri* (Control). ipaD & qnrS

Significant differences in terms of *ipaD* (P=0.005) and *qnrS* (P=0005) gene expression were observed in *S. flexneri* treated with MIC concentration of AgNPs compared to untreated bacteria (Figure 2). The expressions of both genes decreased in AgNPs-treated *S. flexneri*, which indicates the effect of AgNPs on reducing the expression of *S. flexneri* pathogenic

genes.

Discussion

The results of the present study showed that green synthesized silver nanoparticles have antibacterial effects against *S. flexneri* and the mechanism of antibacterial effects was attributed to the downregulation of pathogenic genes *ipaB*, *ipaD*, *ipaH* and *qnrS*.

We used *Spirulina platensis* to synthesize green AgNPs. Green synthesis of nanoparticles can reduce its side effects on organisms and the environment (24). This algae shows good anti-inflammatory and antioxidant properties due to having various beneficial compounds such as vitamins and amino acids (25) and is widely used in the synthesis of most metal nanoparticles (26, 27). For example, Gunasundari et al. (2017) used ultrasonic-assisted *S. platensis* to synthesize metal nanoparticles including Zn, Fe, and Ag and reported antimicrobial effects on Gram-positive and negative bacteria as well as *Aspergillus niger* (28). Also, Mahdieh et al. (2012) used this algae for the synthesis of crystallized silver nanoparticles (SNPs) (29). Therefore, the *S. platensis* has great potential in the green synthesis of metal nanoparticles due to convenient to handle, low toxicity and reduction of harmful effects on the environment (30), and the results of the present study confirm that this algae can be used in the green synthesis of AgNPs.

The synthesized green AgNPs showed antibacterial effects on *S. flexneri*, which showed the potential of its application in the treatment of diseases caused by this pathogen. Its MIC was calculated as 0.0625 µg/ml, which is lower than other studies investigating the anti-*Shigella* effects of silver nanoparticles. This difference can be attributed to the nanoparticle synthesis method and bacterial species. For example, Angamuthu et al. (2023) estimated the MIC of *M. indica* silver nanoparticles on the multi-drug-resistant strain *S. flexneri* to be 20 µg/ml (31), which is much higher than the present study. This difference can be attributed to the different strain and the method for AgNPs synthesis. In the study of Bagherzade et al. (2017), the MIC of green AgNPs synthesized by the aqueous extract of saffron plant on pathogenic bacteria was reported to be 250 µg/mL, which shows a very high value (32). It seems that synthesis factors, bacterial strains and toxicity criteria are important factors in this difference. In another study, Muthukrishnan et al. (2015) reported the highest inhibitory concentration of pathogenic bacteria by AgNPs synthesized with *Ceropegia thwaitesii* as 100 µg/mL (33). They used the disk diffusion method to investigate the antimicrobial effects of AgNPs, while in the present study, the microdilution method was used, which can explain the reason for this difference in the antibacterial concentration of this nanoparticle.

In the present study, it was observed that green synthesized AgNPs using spirulina caused changes in the expression of pathogenic genes such as *ipaB*, *ipaD*, *ipaH* and *qnrS* in *S. flexneri* bacteria and reduced their expressions. Therefore, in this research, it was found that the anti-*Shigella* mechanism of AgNPs is the effect on the expression of pathogenic genes. These genes play an important role in the penetration of bacteria to the epithelial cells and also protect the DNA against destructive factors (12). Therefore, the synthesized green AgNPs increase the sensitivity of Shigella flexneri to protective agents by reducing the expressions of *ipaB*, *ipaD*, *ipaH* and *qnrS* genes, thus exerting anti-*Shigella* effects.

CONCLUSION

The green synthesized AgNPs using *Spirulina platensis* has strong antibacterial effects on *S. flexneri* and the action mechanism was attributed to the downregulations of *ipaB*, *ipaD*, *ipaH* and *qnrS* genes. The *in vivo* and clinical studied are needed.

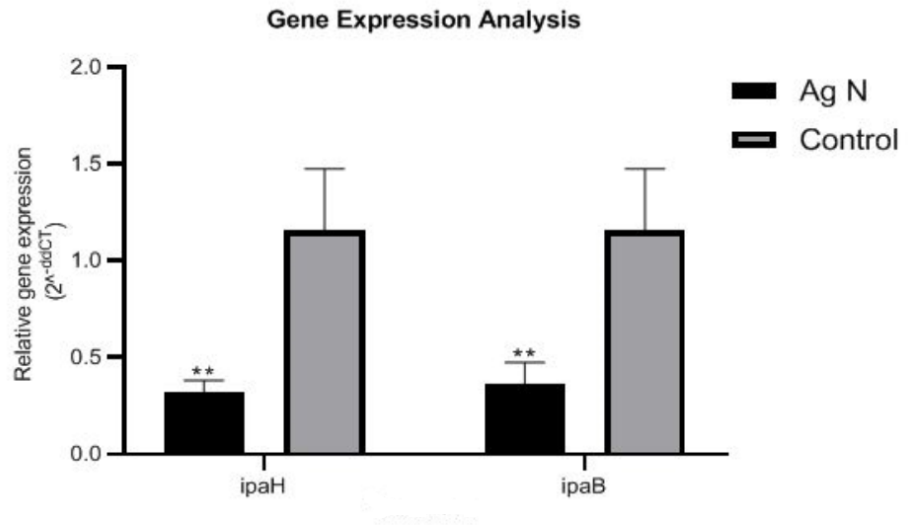

Figure 1. *ipaB* and *ipaH* genes expressions in AgNPs treated and untreated (control) *S. flexneri*. ** shows significant differences at probability level of P<0.01.

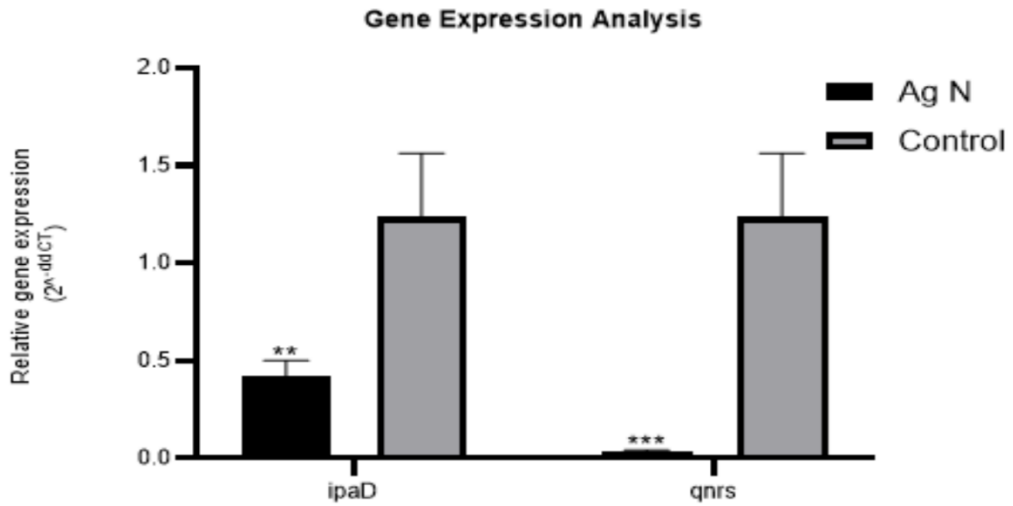

Figure 2. *ipaD* and *qnrS* genes expressions in AgNPs treated and untreated (control) *S. flexneri*. ** and *** show significant differences at probability level of P<0.01 and P<0.001, respectively.

REFERENCES

1. Barreto ML, Teixeira MG, Carmo EH. Infectious diseases epidemiology. Journal of Epidemiology & Community Health. 2006;60(3):192-5.

2. Kotloff KL, Riddle MS, Platts-Mills JA, Pavlinac P, Zaidi AK. Shigellosis. The Lancet. 2018;391(10122):801-12.

3. Barrett J, Fhogartaigh CN. Bacterial gastroenteritis. Medicine. 2017;45(11):683-9.

4. Feigin VL, Abajobir AA, Abate KH, Abd-Allah F, Abdulle AM, Abera SF, et al. Global, regional, and national burden of neurological disorders during 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. The Lancet Neurology. 2017;16(11):877-97.

5. Von Seidlein L, Kim DR, Ali M, Lee H, Wang X, Thiem VD, et al. A multicentre study of Shigella diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. PLoS medicine. 2006;3(9):e353.

6. Watarai M, Funato S, Sasakawa C. Interaction of Ipa proteins of Shigella flexneri with alpha5beta1 integrin promotes entry of the bacteria into mammalian cells. The Journal of experimental medicine. 1996;183(3):991-9.

7. Buysse JM, Stover CK, Oaks EV, Venkatesan M, Kopecko DJ. Molecular cloning of invasion plasmid antigen (ipa) genes from Shigella flexneri: analysis of ipa gene products and genetic mapping. Journal of bacteriology. 1987;169(6):2561-9.

8. Kotloff KL, Pasetti MF, Barry EM, Nataro JP, Wasserman SS, Sztein MB, et al. Deletion in the Shigella enterotoxin genes further attenuates Shigella flexneri 2a bearing guanine auxotrophy in a phase 1 trial of CVD 1204 and CVD 1208. The Journal of infectious diseases. 2004;190(10):1745-54.

9. Dickenson NE, Zhang L, Epler CR, Adam PR, Picking WL, Picking WD. Conformational changes in IpaD from Shigella flexneri upon binding bile salts provide insight into the second step of type III secretion. Biochemistry. 2011;50(2):172-80.

10. Bourdet-Sicard R, Rüdiger M, Jockusch BM, Gounon P, Sansonetti PJ, Van Nhieu GT. Binding of the Shigella protein IpaA to vinculin induces F-actin depolymerization. The EMBO journal. 1999;18(21):5853-62.

11. Fernandez-Prada CM, Hoover DL, Tall BD, Hartman AB, Kopelowitz J, Venkatesan MM. Shigella flexneri IpaH7. 8 facilitates escape of virulent bacteria from the endocytic vacuoles of mouse and human macrophages. Infection and immunity. 2000;68(6):3608-19.

12. Sethuvel DPM, Perumalla S, Anandan S, Michael JS, Ragupathi NKD, Gajendran R, et al. Antimicrobial resistance, virulence & plasmid profiles among clinical isolates of Shigella serogroups. Indian J Med Res. 2019;149(2):247-56.

13. Pu X-Y, Pan J-C, Wang H-Q, Zhang W, Huang Z-C, Gu Y-M. Characterization of fluoroquinolone-resistant Shigella flexneri in Hangzhou area of China. Journal of antimicrobial chemotherapy. 2009;63(5):917-20.

14. Williams PCM, Berkley JA. Guidelines for the treatment of dysentery (shigellosis): a systematic review of the evidence. Paediatrics and International Child Health. 2018;38(sup1):S50-S65.

15. Shen H, Chen J, Xu Y, Lai Z, Zhang J, Yang H, et al. An outbreak of shigellosis in a Children Welfare Institute caused by a multiple-antibiotic-resistant strain of Shigella flexneri 2a. Journal of Infection and Public Health. 2017;10(6):814-8.

16. Puzari M, Sharma M, Chetia P. Emergence of antibiotic resistant Shigella species: A matter of concern. Journal of Infection and Public Health. 2018;11(4):451-4.

17. Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC. Nanoparticles in Medicine: Therapeutic Applications and Developments. Clinical Pharmacology & Therapeutics. 2008;83(5):761-9.

18. Kharissova OV, Dias HVR, Kharisov BI, Pérez BO, Pérez VMJ. The greener synthesis of nanoparticles. Trends in Biotechnology. 2013;31(4):240-8.

19. Abass Sofi M, Sunitha S, Ashaq Sofi M, Khadheer Pasha SK, Choi D. An overview of antimicrobial and anticancer potential of silver nanoparticles. Journal of King Saud University - Science. 2022;34(2):101791.

20. Hembram KC, Kumar R, Kandha L, Parhi PK, Kundu CN, Bindhani BK. Therapeutic prospective of plant-induced silver nanoparticles: application as antimicrobial and anticancer agent. Artificial Cells, Nanomedicine, and Biotechnology. 2018;46(sup3):38-51.

21. Ghramh HA, Ibrahim EH, Kilany M. Study of anticancer, antimicrobial, immunomodulatory, and silver nanoparticles production by Sidr honey from three different sources. Food science & nutrition. 2020;8(1):445-55.

22. Rai MK, Deshmukh SD, Ingle AP, Gade AK. Silver nanoparticles: the powerful nanoweapon against multidrug-resistant bacteria. Journal of Applied Microbiology. 2012;112(5):841-52.

23. Cheon JY, Kim SJ, Rhee YH, Kwon OH, Park WH. Shape-dependent antimicrobial activities of silver nanoparticles. International Journal of Nanomedicine. 2019;14:2773-80.

24. Gour A, Jain NK. Advances in green synthesis of nanoparticles. Artificial cells, nanomedicine, and biotechnology. 2019;47(1):844-51.

25. Wu Q, Liu L, Miron A, Klímová B, Wan D, Kuča K. The antioxidant, immunomodulatory, and anti-inflammatory activities of Spirulina: an overview. Archives of Toxicology. 2016;90(8):1817-40.

26. Kalabegishvili T, Kirkesali E, Rcheulishvili A. Synthesis of gold nanoparticles by blue-green algae Spirulina platensis. Frank Lab. of Neutron Physics; 2012.

27. Muthusamy G, Thangasamy S, Raja M, Chinnappan S, Kandasamy S. Biosynthesis of silver nanoparticles from Spirulina microalgae and its antibacterial activity. Environmental Science and Pollution Research. 2017;24(23):19459-64.

28. Gunasundari E, Senthil Kumar P, Christopher FC, Arumugam T, Saravanan A. Green synthesis of metal nanoparticles loaded ultrasonic-assisted Spirulina platensis using algal extract and their antimicrobial activity: IET Nanobiotechnol. 2017 Jul 26;11(6):754-8. doi: 10.1049/iet-nbt.2016.0223. eCollection 2017 Sep.

29. Mahdieh M, Zolanvari A, Azimee AS, Mahdieh M. Green biosynthesis of silver nanoparticles by Spirulina platensis. Scientia Iranica. 2012;19(3):926-9.

30. Mukherjee A, Sarkar D, Sasmal S. A Review of Green Synthesis of Metal Nanoparticles Using Algae. Frontiers in Microbiology. 2021;12.

31. Angamuthu S, Thangaswamy S, Raju A, Husain FM, Ahmed B, Al-Shabib NA, et al. Biogenic Preparation and Characterization of Silver Nanoparticles from Seed Kernel of Mangifera indica and Their Antibacterial Potential against Shigella spp. Molecules [Internet]. 2023; 28(6).

32. Bagherzade G, Tavakoli MM, Namaei MH. Green synthesis of silver nanoparticles using aqueous extract of saffron (Crocus sativus L.) wastages and its antibacterial activity against six bacteria. Asian Pacific Journal of Tropical Biomedicine. 2017;7(3):227-33.

33. Muthukrishnan S, Bhakya S, Senthil Kumar T, Rao MV. Biosynthesis, characterization and antibacterial effect of plant-mediated silver nanoparticles using Ceropegia thwaitesii – An endemic species. Industrial Crops and Products. 2015;63:119-24.