ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 6, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

IN VITRO ANTIBACTERIAL ACTIVITY OF BIOSYNTHESIZED SILVER NANOPARTICLES FROM ETHYL ACETATE EXTRACT OF *HYDROCOTYLE SIBTHORPIOIDES* AGAINST MULTIDRUG RESISTANT MICROBES

MANASHI GARG[1], BANASMITA DEVI[2], RASHNA DEVI[2]

[1]Department of Biotechonology, Assam downtown University, Panikhaiti, Guwahati, Assam, India. [2]Department of Biochemistry, Assam downtown University, Panikhaiti, Guwahati, Assam, India. Email: garg.mansi91@rediffmail.com

Received: 17 February 2016, Revised and Accepted: 24 March 2017

ABSTRACT

Objectives: *Hydrocotyle sibthorpiodes* is known to contain several phytoconstituents which are constantly involved in the formation of Silver nanoparticles that may affect several multi-drug resistant microbes. Therefore, the study was undertaken to evaluate the efficacy of different concentration of nano silver solution on three bacterial isolates. It was also aimed to qualitatively assess the different phytoconstituents responsible for the synthesis.

Methods: Three bacterial isolates of *Klebsiella pneumonia*, *Pseudomonas aeroginosa* and *Staphylococcus aureus* were identified. Synthesis of AgNPs with different concentration (2/4/6/8/10µl/ml) was done and applied to the selected isolates. The phytochemical compounds of the ethyl acetate extract were assayed by several colored reactions qualitatively.

Results: The size and stability biosynthesis of the metallic silver nanoparticles were confirmed by photophysical characterization as well as SEM (Scanning Electron Microscopy), XRD (XRay Diffraction), Zeta potential and DLS (Dynamic Light Scattering) with an average size of 13.37 ±10 nm. The increasing concentration of the particle solution showed significant inhibition zone for all the three isolates viz., *Klebsiella pneumonia*, *Pseudomonas aeroginosa* and *Staphylococcus*. *aureus showing the value of* 3.0±0.17, 2.7±0.32 and 3.6±0.57 respectively for 10 µl/ml concentration. Phytochemical screening of the whole plant extract also revealed an array of bioactive compounds which may have an effective role in the reduction process.

Conclusion: The study demonstrated a simple, efficient and eco-friendly synthesis of stable silver nanoparticles from the ethyl acetate extract of *Hydrocotyle sibthorpiodes* having fairly superior antimicrobial activity against human pathogens.

Keywords: Multidrug resistant, *Hydrocotyle sibthorpioides,* Antibacterial activity, Silver nanoparticles, Qualitative assay.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i6.17976

INTRODUCTION

The new arena of nanotechnology research finds extensive application in the development of nanomedicine that covers health care related areas of nano science and technology. The biocidal activity of metallic nanoparticles adversely affects the metabolism of the microbial domain [1] and well extends beyond its virotoxicity. However, the distinctive area of nanoresearch is often concerned with the global environment, and a great deal of effort has been put on that to provide a better platform for the biosynthesis of nanoparticles using plants that are more innocuous, inexpensive, and environmentally friendly as they do not leave hazardous residues to pollute the atmosphere compared to the chemical methods that are among the most important approaches in metallic nanoparticles synthesis. However, these methods use high cost and toxic reagents as reducing and stabilizing agents. The crude extract of plants is enriched with several novel secondary metabolites such as phenolics, flavonoids, alkaloids, and terpenoids that play a magnificent role in the bioconversion of silver ions to nanoparticles.

Considering the vast potentiality of plant sources, the recent study demonstrates the probable activity of the ethyl acetate fraction of the whole plant of *Hydrocotyle sibthorpioides* as a biological technique for metallic silver nanoparticle (AgNP) synthesis. The study also aims at exploring the antibacterial effect of the synthesized nanopaticles against three multidrug clinical isolates; *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* as well as the qualitative evaluation of the ethyl acetate fraction for practical evaluation of the bioactive classes of secondary metabolites. The process is simple, reliable, and has several advantages with low cost, compatibility, stability and also can be proved their medical application as wound dressing against skin infection pathogen.

Hydrocotyle sibthorpiodes as species of Apiaceae family was reported to be use traditionally for the efficacy of *H.sibthorpiodes* against opportunistic fungal infection, *Cryptococcus neoformans* and escalation of multi-drug resistance bacteria *Klebsiella pneumonia* has also been shown by Khoo et al 2016 [2]. It has also been used for the treatment of various medical conditions including Hepatitis B viral infection, influenza, jaundice and hepatom [3,4] .Silver nanoparticles can be synthesized at a possibly lower concentration of the obtained fraction of the plant source without leaving any harmful residues unlike chemical approach.

METHODS

Preparation of plant extract

The whole plant of *H. sibthorpioides* was collected locally from Panikhaiti area of Guwahati, Assam (India). The plant was authenticated with a standard herbarium specimen in the Department of Botany, Gauhati University, with an Acc No. 18135. The collected whole plant consisting of leaves, stem, and root was washed with water, dried for 2 week, and grinded into fine powder. About 500 g of the powdered plant sample was extracted with 1000 ml of ethyl acetate using Soxhlet apparatus for 5-6 hrs. The obtained fraction was then filtered using What man's No.1 filter paper, and the filtrate was concentrated using a rotary evaporator. The concentrated extract was stored at 4°C before use.

The percentage yield of the plant extract was calculated as:

$$\frac{\text{Dry weights of the plant extract (g)}}{\text{Initial weight of the plant extract}} \times 100$$

Qualitative analysis of the ethyl acetate extract [5]
The ethyl acetate extract thus obtained was qualitatively tested using several biochemical tests for the presence of different classes of phytochemical constituents.

Test for flavonoids

Sodium hydroxide (NaOH) test

Few drops of 10% NaOH was added to 2 ml of the test extract. Formation of intense yellow color indicated the presence of flavonoids which turns colorless on addition of dilute acid.

Test for steroids and triterpenoids

Libermann-burchard's test
A volume of 2 ml of the extract was treated with few drops of acetic anhydride, heated to boiling, cooled, and then 1 ml of concentrated sulfuric acid (H_2SO_4) was added along the side of the test tube. Brown ring at the junction of solution and appearance of green color at the upper layer indicated the presence of steroids.

Salkowski test
A volume of 1 ml of chloroform and few drops of concentrated H_2SO_4 were added to 2 ml of the test extract along the side of the test tube. A red-brown ring formed at the interface indicated the presence of triterpenoids.

Test for Alkaloids: Mayer's test

About 10 mg of the solvent-free extract was stirred with little quantity of dil. Hydrochloric acid and filtered. Few ml of this filtrate was treated with two drops of Mayer's reagent which indicated a creamy white precipitate for alkaloids.

Test for tannins

Ferric chloride ($FeCl_3$) test
The test extract was mixed with few drops of 5% $FeCl_3$. Formation of bluish-green color indicated the presence of hydrolysable tannins.

Test for carbohydrate

Molisch's test
A volume of 2 ml of the extract and 2 ml of the Molisch's reagent was mixed well.1ml of concentrated H_2SO_4 was added slowly along the side of the tube and was allowed to stand for few minutes. A violate ring indicated the presence of carbohydrate.

Green synthesis of AgNP
Typically, 5 ml of 0.1% *H. sibthorpioides* ethyl acetate extract was added to 10 ml of 10 mM silver nitrate ($AgNO_3$) and 85 ml of double distilled water so as to make the final concentration to 1 mM. To this reaction mixture, 50 µl of NaOH was added, and the reaction was carried out at 28±2°C for 24 hrs. The formation of AgNPs was observed by the appearance of yellowish-brown color which was considered as the production of a colloidal suspension of AgNPs.

Separation of biosynthesized AgNPs for characterization
The green synthesized nanoparticle solution was separation by subjecting to centrifugation at10, 000 rpm for 10 minutes. The sedimented AgNPs at the bottom of the centrifuge tube was removed and washed three times with distilled water. The finally obtained particles were dried and used for dynamic light scattering (DLS), zeta potential, X-ray diffraction (XRD), and scanning electron microscope (SEM) analysis.

Isolation of bacterial culture
The experimental organisms were collected and identified biochemically in Downtown Hospital, Assam, India. The pure cultures were maintained on nutrient agar medium. Each culture was further maintained by subculturing regularly on the same medium and stored at 4°C before use in the experiments.

Standardization of the bacterial isolates
A loopful of the test organisms were inoculated on nutrient agar tubes and incubated at 37°C for 18-24 hrs. A loop of discrete colonies of the organism obtained on the culture tubes was collected using a sterile wire and inoculated into 5 ml of peptone water for 30 minutes. The tubes were adjusted with 0.5 Mc far land standard tube by shaking thoroughly which was equivalent to 10^8 CFU/ml.

Assessment of antibacterial activity of the synthesized AgNPs
activity was done with minor modification by the method Schaad *et al,* 2001 [6] and Sarma *et al*, 2017 [7]. The Muller Hinton Agar plates were prepared by swabbing 100µl of the standardized culture of the test organisms with sterile cotton swabs and were allowed to dry for sometime in the laminar flowhood.

To evaluate the antibacterial activity of the developed AgNPs by well diffusion assay, the wells were made using sterile cork borer, and different concentration of nanosilver (2/4/6/8/10 µl/ml) solution was added to the respective wells. All the plates were further incubated at 37°c for 24 hrs in a bacteriological incubator (Model no-YSI-438) and then after, the diameter of inhibition area (cm) of the AgNPs as well as the plant extract was measured. The test was performed in triplicate for the entire test organism.

RESULTS

Yield percentage of the plant extract
From the extraction yield percentage depicted in Table 1, it was found that extraction of 500 g of the whole plant of *H. sibthorpioides* with 1000 ml of ethyl acetate gave a yield of 138.8%.

Qualitative analysis of the ethyl acetate extract
Table 2 derived positive results for the presence of the different tested phytoconstituents in the ethyl acetate extract of the whole plant of *H. sibthorpioides.* Apparently, these compounds may be responsible for the bio reduction of silver ions into nano sized particles[8,9].

Characterization of synthesized AgNPs
nanoparticles showed yellowish brown color in the aqueous solution due to the excitation of surface Plasmon resonance [10].The complete

Table 1: Yield percentage of *H. sibthorpioides* whole plant extract

Plant	Dry leaves (g)	Ethyl actetate extract (g)	Yield %
H. sibthorpioides	500	69.4	138.8

H. sibthorpioides: Hydrocotyle sibthorpioides

Table 2: Qualitative analysis of ethyl acetate extract of *H. sibthorpioides*

Test for	Observation	Result
Triterpenoids	A red-brown ring formed at the interface	Present
Flavanoids	Formation of intense yellow color	Present
Steroids	Brown ring at the junction of solution and appearance of green color at the upper layer	Present
Tannins	Formation of bluish green	Present
Carbohydrate	Appearance of a violate ring	Present
Alkaloids	Formation of a creamy white precipitate	Present

H. sibthorpioides: Hydrocotyle sibthorpioides

change in color took around 30 minutes; thereafter no further change in the reaction mixture was observed. This formation was also confirmed by obtaining a respective absorption spectrum [11, 12] that originated through SPR (Surface Plasmon Resonance) with a sharp characteristic peak in the range of 300-700nm (Fig. 1). The obtained data also affirmed the intake photo physical property of the nanoparticles as well as the size dependent effect of SPR [13].

XRD analysis

The XRD patterns of synthesized AgNPs showed several distinct peaks at 2θ values of 38°, 44.5°, 64.5°, 77.5°, and 82° representing the indexed peaks 111, 200, 220, 311, and 222 (Fig. 2). The Bragg peaks of fcc structure of silver reference pattern confirm the formation of AgNPs and the presence of metallic silver in the fcc lattice.

SEM analysis

SEM was used to study the surface morphology of the nanoparticles. The image revealed the particles were well dispersed and the predominantly spherical as shown in Fig. 3. The average particle size was calculated to be around 13.37±10 nm. The particle size was also calculated using DLS histogram which agreed the SEM data. Zeta potential, an important parameter to study the stability of the particles and, was found to be in the potentially stable value of −29.6 mV (Fig. 4).

Antibacterial assays

From the results obtained in table 3, it was found that the different prepared concentration of silver nanoparticle solution formed upon addition of different concentration of Silver nitrate were more effective than the corresponding amount of the plant extract alone. The result obtained was in consonance to the work performed by Kaviya *et al*., 2014 [14] reported the Minimum Inhibitory Concentration of AgNPs (Silver Nanoparticles) against P.aeroginosa. The difference in the sensitivity of the tested organism was due to the difference in the chemical constituents of the cell wall [15, 16].

Several studies have proposed the mechanism of action of the bactericidal effect of AgNPs (Silver Nanoparticles). Mostafa *et al*., 2014 [17] suggested the possible attachment of the particles to the bacterial surface through the sulfur containing proteins results in disturbing permeability and respiration functions of the bacterial cell causing death. The increasing bactericidal effect of the AgNPs (Silver Nanoparticles) could be related to the small surface area and the size of the particles formed as concentration effect of the plant extract used.

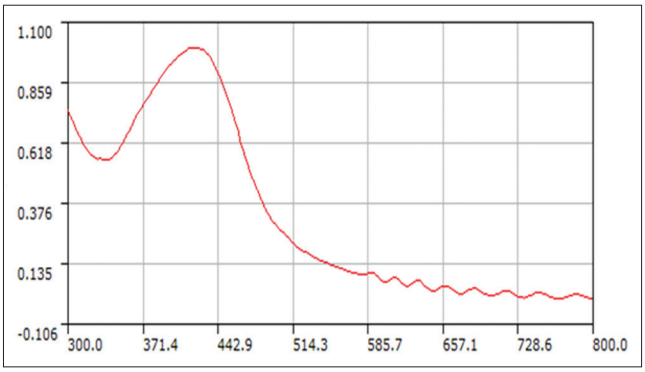

Fig. 1: Ultraviolet-visible spectra of silver nanoparticles

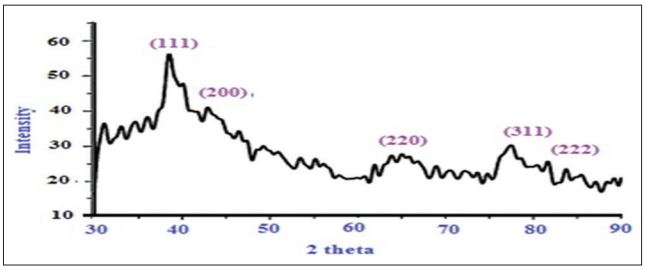

Fig. 2: X-ray diffractogram of synthesized silver nanoparticles

Statistical analysis

All the assays were performed in triplicates. The data obtained were expressed as mean ± Standard deviation and analysis were done by calculating analysis of variance. P≤0.05 were considered statistically significant.

DISCUSSION

The emerging scenario of the antibiotic resistant bacterial strains have become an important issue that is creating problems in treatment of many infectious diseases and therefore the search of an alternative therapy is necessary. The reported work of Patil *et al* 2012 [18] showed a uniform synthesis of nanoparticle found to be an effective approach for various applications. The microwave assisted green synthesis of AgNPs (Silver Nanoparticles) in the present study using the ethyl acetate fraction of *H. sibthorpiodes* was found to be an efficient way in terms of time and stability of the nanoparticles. The DLS (Dynamic Light Scattering) and stability study of the nanoparticle shown by Zeta potential analysis also divulged a constant size of 20 nm (nanometer) in Scanning Electron Microscopy with an effective surface area which was less than the Silver nanoparticles synthesized using gallic acid in an aqueous chemical reduction method with an average sizes of 7, 29, and 89 nm respectively by Castanon *et al* 2008 [19, 20]. However, the results were found to be in consonance with the findings by Yang *et al* 2016 [21] and Shankar *et al* 2004 [22] which showed the synthesis of small and stable silver nanoparticles with good antibacterial activities. The qualitative estimation of the plant extract showed the presence of different phytoconstituents which are constantly involved in reduction reaction to synthesize eco-friendly nanoparticles [23-27].

Efficient antimicrobial activity of the as-synthesized nanoparticles against *S. aureus*, *K. pneumonia* and *P. aeroginosa* was confirmed forming potential inhibitory zones satisfied the present aim of the study

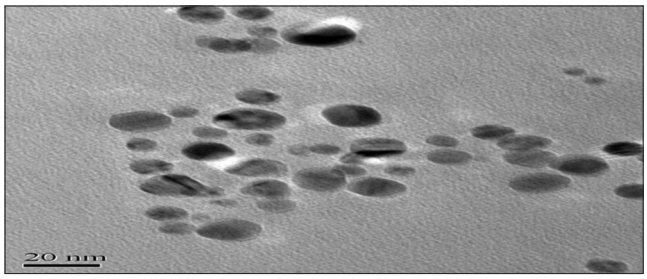

Fig. 3: Scanning electron microscope analysis of silver nanoparticles synthesized by *Hydrocotyle sibthorpioides* ethyl acetate extract

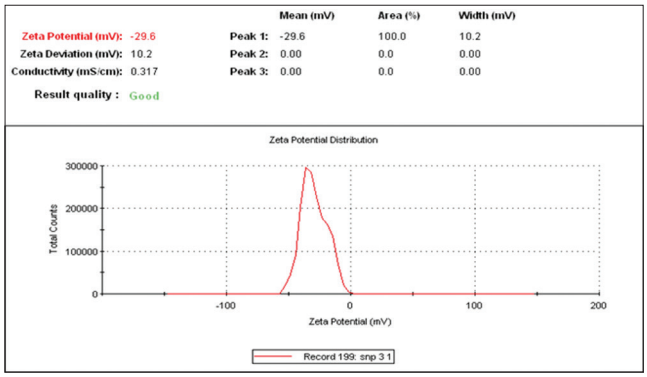

Fig. 4: Zeta potential analysis of silver nanoparticles

Table 3: Antibacterial activity of AgNPs (2-10 µl/ml) against three bacterial species

Bacterial strains	2 µl/ml (cm)	4 µl/ml (cm)	6 µl/ml (cm)	8 µl/ml (cm)	10 µl/m (cm)
S. aureus	1.3±0.08	1.7±0.06	2.0±0.16	2.1±0.53	3.6±0.57
P. aeroginosa	1.8±0.12	1.8±0.25	2.2±0.18	2.3±0.15	2.7±0.32
K. pneumoniae	1.3±0.26	1.5±0.11	1.8±0.24	2.8±0.05	3.0±0.17

S. aureus: Staphylococcus aureus, P. aeroginosa: Pseudomonas aeroginosa, K. pneumonia: Klebsiella pneumonia, AgNPs: Silver nanoparticles

to overcome the MDR (Multi Drug Resistant) strains of the pathogenic microbes. The findings however, support the previous reports of antibacterial activity of silver nanoparticles against *S. aureus* MRSA and *P. aeruginosa* [28-30].

CONCLUSION

The present study demonstrated a simple, efficient, and eco-friendly one-pot green synthesis of stable AgNPs from the ethyl acetate extract of *H. sibthorpioides* having fairly superior antimicrobial activity against human pathogens. This method serves a competitive alternative to the other conventional chemical methods. The synthesized agents may find potential applications in biomedical field using them as a base product in developing nanomaterials of potential concern. In addition, it is necessary to envision that green method of synthesis of these particles has number of substantial benefits in context to several parameters, including non-toxicity and cost-effectiveness.

ACKNOWLEDGMENT

The authors would like to acknowledge the support from Assam Downtown University for providing the facilities required as well as Institute of Advanced Studies in Science and Technology Boragaon, Guwahati, Assam, to get the characterization data.

REFERENCES

1. Mohanpuria P, Yadav PN, Yadav SK. Biosynthesis of nanoparticle, technological concepts and future application. J Nanopart 2007;7:9275.
2. Khoo KS, Gwee PS, Beh V, Chung TC, Tan LY, Sit NW, *et al*. Antifungal and antibacteral properties of three medicinal plants from Malaysia. Acta Hortic 2016;15:1023.
3. Rai SK. Medicinal plants used by Meche people of Jhapa district, Eastern Nepal. Our Nat 2004;2:27-32.
4. Yu F, Yu F, McGuire PM, Li R, Wang R. Effects of *Hydrocotyle sibthorpioides* extract on transplanted tumors and immune function in mice. Phytomedicine 2007;14(2-3):166-71.
5. Rajesh S, Patric DR, Rathi JM, Sahayaraj K. Biosynthesis of silver nanoparticles using *Ulva fasciata* (Delile) ethyl acetatextract and its activity against *Xanthomonas campestris* pv. *Malvacearum*. J Biopesticide 2012:119-28.
6. Schaad NW, Jones JB, Chun W. The bacteriology committee of the American phyto pathological society. Plant Pathogenic Bacteria. 3rd ed. USA: APS Press; 2001.
7. Sarma G, Nakuleswar JD, Prerna S, Suresh JC. Synthesis, characterization and antimicrobial activity of *Belia grandiflora* assisted AgNPs. J Microbiol Biochem Technol 2014;6:274-8.
8. Singh K, Manju P, Sangeeta K, Uma C, Prakash YJ. Antibacterial activity of synthesized silver nanoparticles from *Tinospora cordofolia* against multi drug resistant strains of *Pseudomonas aeroginosa* isolated from burn patients. J Nanomed Nanotechnol 2014;5:192.
9. Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ, *et al*. Antimicrobial effects of silver nanoparticles. Nanomedicine 2007;3(1):95-101.
10. Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. J Biomed Mater Res 2000;52(4):662-8.
11. Aromal SA, Philip D. Green synthesis of gold nanoparticles using *Trigonella foenum-graecum* and its size-dependent catalytic activity. Spectrochim Acta A Mol Biomol Spectrosc 2012;97:1-5.
12. Hussain JI, Kumar S, Hashmi AA, Khan Z. Silver nanoparticles: Preparation, characterization and kinetics. Adv Mater 2011;2(3):188-94.
13. Kumar GG, Sangita MS, Bappadity M, Kiran M. Phytochemical evaluation of methanolic extract of *Zizyphus xylopyrus*. Int J Drug Discov 2011;1:231-3.
14. Kaviya S, Santhanalakshmi J, Viswanathan B, Muthumary J, Srinivasan K. Biosynthesis of silver nanoparticles using citrus sinensis peel extract and its antibacterial activity. Spectrochim Acta A Mol Biomol Spectrosc 2011;79(3):594-8.
15. Dubey SP, Lahtinen M, Sillanpaa M. Green synthesis and characterizations of silver and gold nanoparticles using leaf extract of *Rosa rugos*. Process Biochem 2010;45:1065-71.
16. Othman AS, Ahmed NA. Antibacterial effect of the ethanol leaves extract of *Moringa oleifera* and *Camellia sinesis* against multi drug resistant bacteria. Int J Pharmacol 2016;13(2):156-65.
17. Mostafa MH, Ismaila EH, El-Baghdadyc KZ, Mohamed D. Green synthesis of silver nanoparticles using olive leaf extract and its antibacterial activity. Arabian J Chem 2014;7(6):1131-9.
18. Patil RS, Kokate MR, Kolekar SS. Bioinspired synthesis of highly stabilized silver nanoparticles using *Ocimum tenuiflorum* leaf extract and their antibacterial activity. Spectrochim Acta A Mol Biomol Spectrosc 2012;91:234-8.
19. Castañón GA, Martínez F, Gutierrez J, Facundo R. Synthesis and antibacterial activity of silver nanoparticles with different sizes. J Nanopart Res 2008;10:1343-8.
20. Mervat F, Zayed MF, Eisa WH, Shabaka AA. *Malva parviflora* extract assisted green synthesis of silver nanoparticles. Spectrochim Acta A Mol Biomol Spectrosc 2012;98:423-8.
21. Ren Y, Hui Y, Wang T, Wang C. Green synthesis and antimicrobial activity of monodisperse silver nanoparticles synthesized using Ginkgo biloba leaf extract. Phys Lett A 2016;380(45):3773-7.
22. Shankar SS, Rai A, Ahmad A, Sastry M. Rapid synthesis of Au, Ag, and bimetallic Au core-Ag shell nanoparticles using neem (*Azadirachta indica*) leaf broth. J Colloid Interface Sci 2004;275:496-502.
23. Amendola V, Bakr OM, Stellacci F. A study of the surface plasmon resonance of silver nanoparticles by the discrete dipole approximation method: Effect of shape, size, structure assembly. Plasmonics 2010;5(1):85-97.
24. Banothu V, Adepally U, Lingam J. *In vitro* total phenolics, flavonoids contents, antioxidant and antimicrobial activities of various solvent extracts from the medicinal plant *Physalis minima*. Int J Pharm Pharm Sci 2017;9(3):192-8.
25. Hendra P, Andreani PR, Pangestuti E, Julianus J. Evaluation of antihyperlipidemic, anti-inflammatory and analgesics activities of *Eurycoma longifolia* in animal models. Int J Pharm Pharm Sci 2017;9(3):166-9.
26. Jiale H, Guowu Z, Bingyun Z, Daohua S, Fenfen L, Yuan L, *et al*. Biogenic silver nanoparticles by *Cacumen platycladi* extract: Synthesis, formation mechanism, and antibacterial activity. Ind Eng Chem Res 2009;50:9095-106.
27. Chen D, Qiao X, Qiu X, Chen J. Synthesis and electrical properties of uniform silver nanoparticles for electronic applications. J Mater Sci 2009;44(4):1076-81.
28. Paredes D, Ortiz C, Torres, R. Synthesis, characterization, and evaluation of antibacterial effect of Ag nanoparticles against *Escherichia coli* O157:H7 and methicillin-resistant *Staphylococcus aureus* (MRSA). Int J Nanomed 2014;9:1717-29.
29. Nasim K, Asghar T. Antibacterial effect of silver nanoparticles on *Acinetobacter baumannii*. Iran J Public Health 2014;43:39.
30. Singh K, Panghal M, Kadyan S, Chaudhary U, Yadav JP. Antibacterial activity of synthesized silver nanoparticles from *Tinospora cordifolia* against multi drug resistant strains of *Pseudomonas aeruginosa* isolated from burn patients. J Nanomed Nanotechnol 2014;5:2.