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Antibacterial activity of green silver nanoparticles synthesized from *Anogeissus acuminata* against multidrug resistant urinary tract infecting bacteria *in vitro* and host-toxicity testing

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ABSTRACT

Silver nanoparticles (AgNPs) with aqueous leaf-extract of the timber-yielding plant *Anogeissus acuminata* were synthesized for *in vitro* control of pathogenic bacteria. Characterization of AgNPs with ultraviolet-visible spectroscopy, scanning electron microscopy (SEM), energy dispersive X-ray (EDX) spectroscopy, X-Ray diffraction (XRD) study and Fourier transformed infrared spectroscopy (FTIR) study was done for a confirmation of the synthesis. The SEM analysis confirmed that the metal particles were below 100 nm size. The antibacterial activity of AgNPs was monitored by agar-well diffusion method against 11 multidrug resistant (MDR) urinary tract infection (UTI) causing pathogenic bacteria, isolated from clinical samples. At 15 μg/ml AgNPs, values of the zone of inhibition (ZI) ranged from 19 to 13 mm, while against the standard antibiotic, gentamicin 30 μg/ml ZI ranged from 28 to 20 mm. Host toxicity testing of AgNPs with cultured lymphocytes from human umbilical cord blood *in vitro* was done; at 3000 mg/l AgNPs, 25% of cell death occurred. Thus, the synthesized AgNPs with aqueous leaf extract of *A. acuminata* could control most MDR UTI bacteria without any toxicity to human lymphocytes.

Introduction

In developing and developed countries, today public health episodes with several infectious diseases demand due attention consistently. Urinary tract infection (UTI) is a grievous complication in women, progressing from cystitis through pyelonephritis and blood stream infection (BSI) to some intractable bacteraemia at innards and fatality at least in immunocompromised persons, without an emulative empiric check (Rath and Padhy, 2015). And UTI incidences account for nearly 25% infections, and around 50–60% women report UTIs at least once in the life (Al-Badr and Al-Shaikh, 2013). Furthermore, before the arrival of the urine culture report, an empiric treatment is undertaken upon for the control; but, failure of the employed antibiotic(s) causes the infection to proceed through BSI (Martinez and Wolk, 2016). In anticipation of a failure in control of pathogens, the physician prescribes a higher generation of antibiotics pre-emptively; and target and non-target bacteria too develop resistance to newly applied antibiotics (Hawkey, 2015). Progressively, the spectrum of antibiotics presently in use becomes selectively ineffective with eventual emergence of multidrug resistant (MDR) bacterial strains simultaneously resistant to several antibiotics (Mishra et al., 2013, 2017).

In this perspective, along with the administered antibiotic a concoction from traditional medicine when supplemented, the use of a higher level of antibiotic might be redundant. Moreover it would be prudent, if a formulation of silver green nanoparticles could be used in place of a crude plant extract for the control of bacteria, as silver ion is well-known for the antibacterial activity (Khalil et al., 2014). In continuation to the screening work with timber-yielding plants (Mishra et al., 2017), *Anogeissus acuminata* was selected for the synthesis of silver nanoparticles (AgNPs), in one-step green synthesis process, since this plant has ethnomedical uses in India against diarrhoea, dysentery, wound healing and skin diseases (Singh et al., 2016). In general, timber-yielding plants have an array of secondary phytochemicals that could help in the synthesis of green AgNPs with antibacterial efficacy, than those of a non-timber plant. *A. acuminata* was never reported for the synthesis of AgNPs, which is described here and AgNPs were characterized by UV–visible spectroscopy, scanning electron microscopy (SEM), energy dispersive X-ray (EDX) spectroscopy, X-ray diffraction (XRD) study and Fourier transformed infrared

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spectroscopy (FTIR) study. AgNPs were used for *in vitro* control of MDR strains of 11 UTI causing bacteria, isolated from clinical samples, and were tested *in vitro* with lymphocytes cultured from human umbilical cord blood for host toxicity.

Materials and methods

Synthesis of AgNPs

One hundred ml of 4 mM solution silver nitrate (Merck) was added to 100 ml of aqueous leaf extract of *Anogeissus acuminata* (Family Combretaceae), and the colloidal mixture was kept standing at room temperature for 24 h.

Characterization of AgNPs

Ultraviolet-visible spectroscopy study

The reduction of silver ions in the colloidal solution was confirmed by UV–vis spectroscopy with a Systronics double beam spectrophotometer 2203. A sample aliquot of the solution was observed for wavelength scanning at 300–700 nm against distilled water.

Scanning electron microscopy and energy dispersive X-ray spectroscopy

After 6 h of reaction, the colloidal sample of 15 ml was centrifuged at 14000 rpm for 4 min. The pellet was redispersed in 10 ml of deionized water and recentrifuged. The process was repeated three times and finally, the pellet of AgNPs was washed with acetone. The purified AgNPs (1 mg) were sonicated for 30 min for making a suspension from which, a drop of 25 μl was placed on the carbon coated copper grid and the sample lot was vacuum dried under a lamp. The prepared sample was subjected to SEM analysis by using Carl Zeiss SMT, Germany/EVOMA 15 Scanning Electron Microscope at Central Institute of Plastics Engineering Technology, Bhubaneswar. EDX spectroscopy was recorded with an EDX detector operated at 10 kV accelerating voltage.

X-ray diffraction study

The sample containing AgNPs was used for XRD study, and 25 μl AgNPs was loaded onto glass substrate as films of the solution, using a Shimadzu XRD 7000, X-ray Diffractometer, operating at a voltage 40 kV and a current of 30 mA with Cu Kα radiation.

Fourier transformed infrared spectroscopy study

Purified AgNPs were pulverized and analyzed using FTIR spectroscopy for which, AgNPs were mixed with KBr and pelletized and the spectra were recorded using Perkin Elmer Spectrum II FTIR Spectrophotometer, in the diffuse transmittance mode at a resolution of 4 cm^{-1}.

Antibacterial activity

Antibacterial activity of AgNPs was evaluated by agar-well diffusion method (Perez et al., 1990), against 11 MDR UTI bacteria, two Gram positive pathogenic bacteria, *S. aureus*, *Enterococcus faecalis* and 9 GNs, *A. baumannii*, *Citrobacter freundii*, *Enterobacter aerogenes, Escherichia coli, Klebsiella oxytoca, K. pneumoniae, Proteus mirabilis, P. vulgaris* and *Pseudomonas aeruginosa,* isolated from urine samples of UTI patients at the hospital, using an appropriate medium, specific for each bacterium. All bacterial strains were subjected to antibiotic sensitivity test by the Kirby–Bauer's disc diffusion method (Mishra et al., 2013). Different concentrations of AgNPs (5, 10 and 15 μg/ml) were placed in specified 8 mm size wells and taking 1 mM $AgNO_3$ solution and gentamicin 30 μg/ml as the reference. The activity of AgNPs against the UTI causing bacteria was determined by measuring the size of zone of inhibition in bacterial lawn.

In vitro *toxicity study of synthesized AgNPs*

In vitro toxicity study of synthesized AgNPs was done as described with umbilical cord blood (UCB) using acridine orange/ethidium bromide (AO/EB) staining (Patnaik and Padhy, 2017).

Results

Gradual change of the colour of the colloidal mixture of the leaf extract and $AgNO_3$ solution from yellow to dark brown indicated the formation of AgNPs in the mixture, confirmed by UV–vis spectroscopy, with a peak at 452 nm. From the FTIR analysis, the spectra displayed bands at, 3373, 1695, 1600, 1505 and 1336 cm^{-1} (Fig. 1). The band at 3373 cm^{-1} corresponds to —OH stretching of phenolic compounds, 1695 cm^{-1} refers to carboxylic —C=O

Fig. 1. FTIR spectra of synthesized AgNPs from leaf extract of *A acuminata.*

stretching, 1600 and 1505 cm^{-1} corresponds to —C=C stretching and band at 1336 cm^{-1} refers to —C—O stretching.

Crystal behaviour of the purified solid AgNPs was determined using powder XRD (Fig. 2). A number of strong diffraction peaks were seen at 2θ values of 38.01°, 44.15°, 64.35° and 77.32°, which correspond to the I/I_1 values as, 100, 27, 24 and 27, respectively. The lattice constants were in conformity with the database of Joint Committee on Powder Diffraction Standards, file no. JCPDS-04-0783, whereas, broadening in diffraction peaks indicated that small crystallite size was obtained. The SEM analysis was confirmed that the metal particles were in nano-size; those were spherical in shape and the diameter of particles was below 100 nm (Fig. 3). EDX spectroscopy results confirmed the significant presence of 100% silver with no other contaminants. The optical absorption peak at 3 keV, is typical for the absorption of metallic silver nanocrystallites due to the SPR (Fig. 4). Besides of Ag there are other bands for C, O and other elements peaks which appeared due to the scattering caused by the compounds that are bound to the surface of silver as cationic surfactants act as capping agents for AgNPs.

The antibiotic profile of each pathogenic bacterium was determined using specified antibiotic discs (Table 1). GP isolates, *E. faecalis* strains were resistant to 17 and *S. aureus* were resistant to 13 of 18 antibiotics used. Among the 9 GN isolates, *A. baumannii, E. aerogenes* and *E. coli* were resistant to 11, *C. freundii, K. pneumoniae, K. oxytoca* and *P. aeruginosa* were resistant to 12, *and P. mirabilis* and *P. vulgaris* were resistant to 10 antibiotics of the total 14 antibiotics used. The details of the individual antibiotic resistant profiles of individual bacteria were recorded (Table 1). Thus, the isolated bacterial strains were MDR. Phyto-synthesized AgNPs were studied for antibacterial activity against the above said 11 MDR UTI causing bacteria. The diameter of the zone of inhibition in mm around each well in different concentration levels of AgNPs (5, 10 and 15 μg/ml) and 1 mM/ml $AgNO_3$ solution, as the control were measured (Table 2). At the concentration level of 5 μg/ml AgNPs, values of the zone of inhibition ranged from 14 to 10 mm, at 10 μg/ml concentration the zone of inhibition ranged from 16 to 11 mm and at 15 μg/ml concentration values of zone of inhibition ranged from 19 to 13 mm against the standard antibiotic, gentamicin, 30 μg/ml ranged from 28 to 20 mm (Table 2). Thus, it was noted that the synthesized AgNPs had *in vitro* control against MDR UTI bacterial strains.

When observed under a fluorescent microscope (Magnus at 40×) green coloured cells were live lymphocytes, whereas cells with orange and red coloured cells were apoptotic and necrotic lymphocytes, respectively (Fig. 5). At 500 mg/l AgNPs, the percent lethality of cells counted by AO/EB staining was 3.43 ± 0.75, while 500 mg/l was the MIC value of synthesized AgNPs to human lymphocytes cultured from umbilical cord blood. The percent toxicity value gradually increased and was 38.2 ± 1.1 at 5000 mg/l level of synthesized AgNPs (Table 3). The lethal concentration 25 (LC_{25}) value of synthesized AgNPs toxicity to UCB was 2951.21 mg/l, determined by Finney's probit method; this is the antilog value of $\log_{10}$ concentration of 3000 mg/l. At 3000 mg/l AgNPs 25% cell death occurred. The LC_{25} value was calculated from the probit graph (Fig. 6).

Discussion

The antibacterial activity described herein validated the ethnic information of curative use of *A. acuminata* leaf in inflammation and skin diseases. Additionally, *A. acuminata* not being an edible plant even, its bark is used traditionally to treat diabetes suggesting absence of host toxicity, a fact corroborated by this work. Moreover during green synthesis of AgNPs, secondary metabolites of this plant are expected to have contributed to the recorded outlandish antibacterial activity. In literature, leaf extracts from *Chenopodium album* of the same family Combretaceae (Dwivedi and Gopal, 2010) and several other plants, *Acalypha indica*, *Aloe vera*, *Azadirachta indica*, *Bryophyllum* sp., *Cassia fistula*, *Cocos nucifera*, *Cycas*, *Cyperus* sp., *Enhydra fluctuans*, *Garcinia mangostana*, *Gliricidia sepium*, *Hibiscus rosa-sinensis*, *Hydrilla* sp., *Ipomoea aquatica*, *Ludwigia adscendens*, *Ocimum sanctum*, *Psidium guajava* and *Rosa rugosa* have been used for the synthesis of silver as well as, gold nanoparticles (Mittal et al., 2013). It had been suggested that plant metabolites were the stabilizing agents to protect AgNPs against possible aggregation. Green nanoparticles are used in the preparation of topical ointments to treat infection against burn and open wounds (Mittal et al., 2013; Reda et al., 2011). AgNPs are employed in textile fabrics, as food additives, in plastics and packages for the inherent antimicrobial activity. AgNPs are extensively used in other applications such as, biolabelling, sensors, electrodes and integrated circuits (Prakash et al., 2013).

Although the mode of antimicrobial action of AgNPs involves slow release of silver ions via oxidation within or outside the cell, affecting the permeability of cell membranes of microbial and other cells, during control (Li et al., 2010). In general, nanoparticles are known to inactivate proteins and interfere with the replication of DNA (Chaloupka et al., 2010). Green nanoparticles are useful in cosmetics, food and medicine (Parashar et al., 2009); and those have received a considerable attention due to the growing need to develop environmentally benign technologies in material synthesis.

Biological syntheses of nanoparticles, using extracts of fungi, bacteria or plants, are cost effective and environment friendly, in comparison to synthesis by physical or chemical methods (Talebi et al., 2010). Additionally, AgNPs are well known for antifungal and antibacterial activities that render uses in industries, medicines, textiles, etc. (Krishnaraj et al., 2010; Kumar et al., 2014a). Antibacterial effects of Ag salts have been noticed since antiquity,

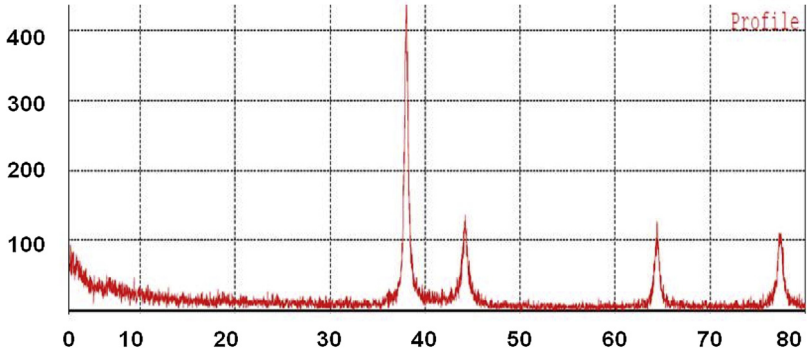

Fig. 2. XRD-analysis of AgNPs synthesized using leaf extract of *A. acuminata*.

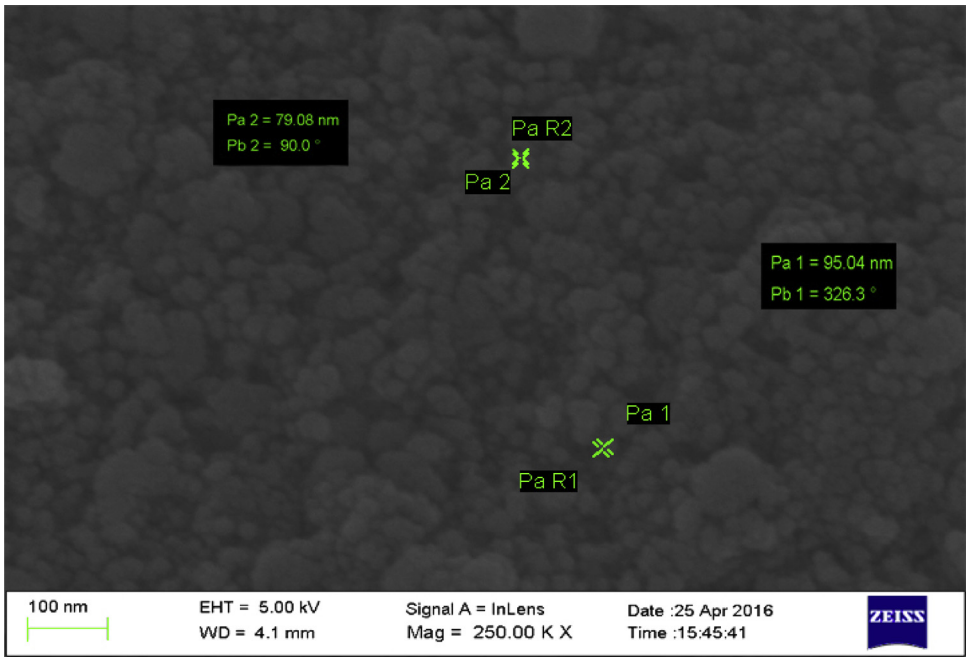

Fig. 3. SEM micrograph of AgNPs synthesized using leaf extract of *A. acuminata*.

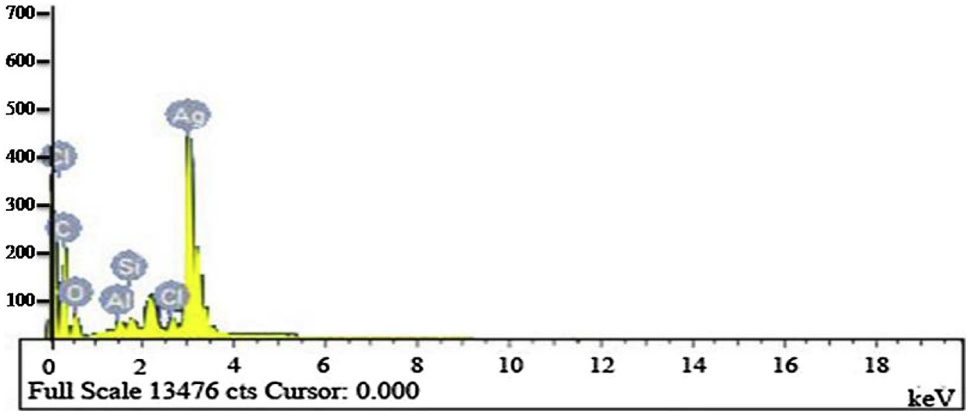

Fig. 4. EDX spectroscopic analysis of AgNPs synthesized using leaf extract of *A. acuminata*.

Table 1
Antibiotic susceptibility results of multidrug resistant Gram-positive and Gram-negative bacteria.

Bacterium	Susceptibility to prescribed antibiotics																	
	Amino-glycosides		β-lactams				Cephalo-sporins		Fluoroquinolones				Glyco-peptides		Lincosa-mide	Sulfon-amide	Stand alones	
	Ac	Ge	Ak	Am	Ox	Pt	Ce	Cf	Of	Le	Nx	Gt	Tei	Va	Cd	Cot	Ch	Lz
*E. faecalis**	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R
*S. aureus**	R	R	R	R	MS	R	R	R	R	R	R	R	MS	MS	MS	R	R	S
A. baumannii	R	R	R	R	ND	R	R	R	R	R	MS	S	ND	ND	ND	R	R	S
C. freundii	R	R	R	R	ND	R	R	R	R	R	R	MS	ND	ND	ND	R	R	S
E. aerogenes	R	R	R	R	ND	R	R	R	R	R	R	MS	ND	ND	ND	R	MS	S
E. coli	R	R	R	R	ND	S	R	R	R	R	R	R	ND	ND	ND	S	R	S
K. oxytoca	R	R	R	R	ND	R	R	R	R	R	R	MS	ND	ND	ND	S	R	R
K. pneumoniae	R	R	R	R	ND	R	R	R	R	R	R	S	ND	ND	ND	R	R	S
P. mirabilis	R	R	R	R	ND	S	R	R	S	R	S	MS	ND	ND	ND	R	R	R
P. vulgaris	R	R	R	S	ND	R	R	S	S	R	R	S	ND	ND	ND	R	R	R
P. aeruginosa	R	R	R	R	ND	R	R	R	R	R	R	MS	ND	ND	ND	R	R	S

Note: * marked bacteria are Gram-positives and the rest are Gram-negatives. R, Resistant; S, Sensitive; MS, moderately sensitive; ND, not done. Antibiotics (μg/disc), Ac: amikacin 30, Ak: amoxyclav 30, Am: ampicillin 10, Cd: clindamycin 2, Cf: cefpodoxime 10, Ch: chloramphenicol 30, Cot: co-trimoxazole 25, Ce: ceftriaxone 30, Ge: gentamicin 10, Gt: gatifloxacin 5, Nx: norfloxacin 10, Le: levofloxacin 5, Lz: linezolid 30, Of: ofloxacin 5, Ox: oxacillin 1, Pt: piperacillin/tazobactam 100/10, Tei: teicoplanin 5, Va: vancomycin 30.

Table 2
Antibacterial activity as size of zone of inhibition (mm) using agar-well diffusion method due to synthesized AgNPs in solution (μg/ml) and $AgNO_3$ (1 mM/ml) as the positive and gentamicin 30 μg/ml as the standard references against bacteria.

Bacteria	Size of zone of inhibition by synthesized AgNPs			$AgNO_3$ (1 mM/ml)	Gentamicin (30 μg/ml)
	5 μg/ml	10 μg/ml	15 μg/ml		
E. faecalis	13	15	18	17	25
S. aureus	14	16	19	15	28
A. baumannii	10	12	13	11	20
C. freundii	12	15	17	13	21
E. aerogenes	13	16	18	14	23
E. coli	12	15	17	11	26
K. oxytoca	12	14	15	13	22
K. pneumoniae	10	13	14	11	20
P. mirabilis	12	13	15	13	22
P. vulgaris	10	11	13	12	23
P. aeruginosa	13	15	16	13	26

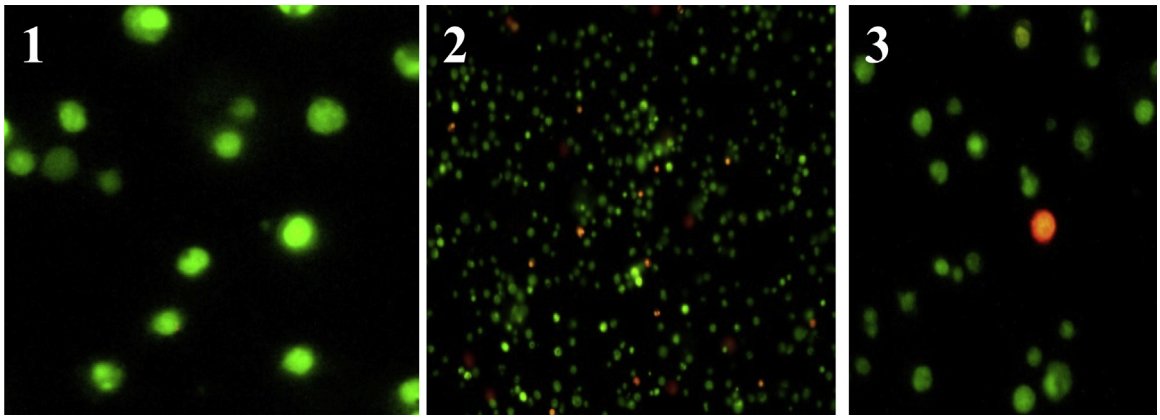

Fig. 5. Microscopic images of lymphocytes with AO/EB staining: (1) Control cells, 100× (2) Cells after growing with 3000 mg/l AgNPs, 10 × (3) Cells after growing with 5000 mg/l AgNPs 40×.

Table 3
Percent lethality values of synthesized green AgNPs to human lymphocytes growing in DMEM, assessed by AO/EB staining with probits.

Concentration of extract (mg/l)	Log_{10} concentrations	Lethality of cells by AO/EB staining with Mean ± SD	Probit functions of percent lethality by AO/EB staining
0	–	–	–
500	2.69	3.43 ± 0.75	3.17
1000	2.99	9.5 ± 1.3	3.68
1500	3.17	13.5 ± 0.7	3.89
2000	3.30	17.4 ± 1.1	4.06
2500	3.39	21.6 ± 1.1	4.21
3000	3.47	26.2 ± 0.8	4.36
3500	3.54	29.4 ± 0.6	4.45
4000	3.60	32.8 ± 1.4	4.55
4500	3.65	35.8 ± 0.5	4.63
5000	3.69	38.2 ± 1.1	4.69

Note: AO/EB, Acridine orange/ethidium bromide; DMEM, Dulbecco's modified Eagle's medium.

especially for burn wounds; in fact, it is well known that Ag ions and Ag-based compounds are highly toxic to microbes (Khalil et al., 2014). Acticoat™, a unique antimicrobial barrier dresser, with the silver sulfadiazine influences the respiratory chain at the cytochrome level, by disrupting the microbial electron transport system mediated by the silver ion, because of the slow release of silver ions from Acticoat™. This commercial formulation is very popular for the inherent strong and extended antimicrobial effect, compared to the other classical silver containing topical agents. Furthermore, Ag based agents are effective against opportunistic infections from aerobic, anaerobic bacteria and fungi on wound sites as well as, dermal viral irruptions (Ulkur et al., 2005). Because of increase of bacterial resistance to antibiotics, AgNPs have been acknowledged and prescribed constantly as antimicrobials in bacterial control (Mehmood et al., 2017). Moreover, the development of bacterial resistance has not been reported against AgNPs; eventually, it could be the coveted agent for a dependable everlasting therapeutic use in the control of most infections, often as topical ointments. The additional advantage with AgNPs for antimicrobial effect stems from relative non-toxicity to hosts and safety as antibacterial agents in smaller concentrations (Kumar et al., 2014b). In the present study, the AgNPs from *A. acuminata* also did not affect to the *in vitro* cultured human lymphocytes and were non-toxic.

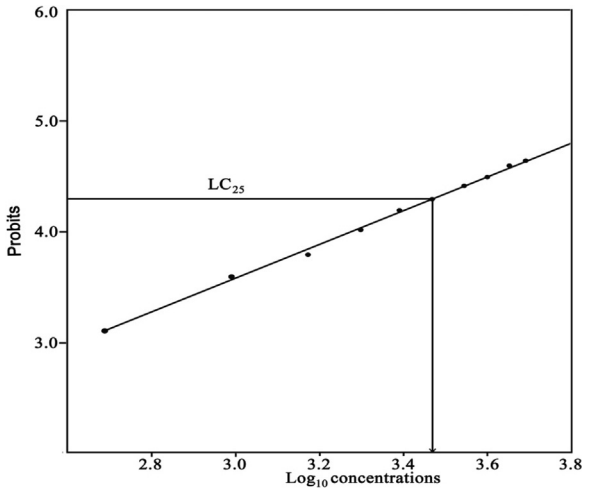

Fig. 6. Probit function of the percentage lethality values were plotted against the $\log_{10}$ concentration of synthesized AgNPs in the toxicity study of cultured human lymphocytes.

Conclusion

Synthesized with the leaf extract of the timber-yielding plant *A. acuminata*, AgNPs had antibacterial activity against MDR bacterial pathogens *in vitro*, isolated from clinical samples. Moreover, non-toxicity to man was assessed with lymphocytes cultured *in vitro* from human umbilical cord blood with the MIC value of 500 mg/l synthesized AgNPs and the LC_{25} value was 2951.21 mg/l. This could be used as an antibacterial agent against MDR pathogenic bacteria.

Conflict of interests

The authors declare no conflict of interests in this article.

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