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BIOSYNTHESIS OF THE SILVER NANOPARTICLES USING TWO LEAVES EXTRACTS FROM *E.ODORATUM* (TBAGNPS) AND *C. CITRATUS* (LGAGNPS) AND ITS BIOLOGICAL ACTIVITIES

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Abstract

The fabrication of silver nanoparticles by using watery extracts of *Eupatorium odoratum* L. (Taw-Bizat) and *Cymbopogon citratus* Stapf (Lemon Grass) leaves was mainly conducted. Silver nitrate was used as a metal precursor and two selected leaves extracts were applied as biomimetic routes of reducing agent for synthesis of silver nanoparticles (AgNPs). The surface morphology of fabricated silver nanoparticles (AgNPs) were performed with FESM (Field Emission Scanning Electron Microscopy). From the result of XRD (X-ray diffraction), the average particle size of TBAgNPs (32 nm) from *E. odoratum* and LGAgNPs (38 nm) from *C. citratus* watery extracts were observed. The size distribution of each prepared (AgNPs) was analysed by using advanced techniques Zeta potential-DLS (Dynamic Light Scattering). The localized surface plasmon resonance bands for formation of TBAgNPs (440 nm) and LGAgNPs (435 nm) in the ratio of 1:3 and 1:5 were exhibited in UV-visible spectra. The role of selected stabilizing agent of *E. odoratum* and *C. citratus* leaves extracts and the effect of stirring time on synthesis of AgNPs was reported. Furthermore, the laser beam of reflected rays as observed in the fabrication of AgNPs by the Tyndall effect. In addition, the antioxidant activities of TBAgNPs and LGAgNPs (49.27 μg/mL, 94.32 μg/mL) were determined by DPPH radical scavenging assay method. The antimicrobial activities of two kinds of leaves extracts and the prepared TBAgNPs and LGAgNPS were discussed against different strains of microorganisms.

Keywords: fabrication, biosynthesis, morphology, average particle size, antioxidant, antimicrobial activity

Introduction

Nano-chemistry deals with synthesis of nanoscale building blocks with controlled size, shape, structure and their composition and their organization into functional architecture using self-assembly templating and lithographic techniques. A length scale is used by a nanometer, nanoscience and nanotechnology have been around for several decades, particularly in research, development and manufacturing in information technology, where film layers and lithographically defined features in the nanometer range are needed (Guzman, 2008). Nowadays, the nanomaterials become critically important because of their unique properties such as physical, magnetic, structural, thermal, mechanical, chemical and electronic properties. Nanoparticles can be divided into two groups: (i) organic nanoparticles and (ii) inorganic nanoparticles (Kumar and Yadav, 2009). Organic nanoparticles contain carbon nanoparticles. Inorganic nanoparticles involve magnetic nanoparticles, noble nanoparticles (like gold and silver), semiconductor nanoparticles (like titanium dioxide) possess optical properties (Albrecht, 2006). The synthesis of NPs are broadly divided into two main classes: (1) bottom-up approach and (2) top-down approach. The breakdown (top-down) method by which an external force is applied to a solid that leads to its break-up into smaller particles. The build-up (bottom-up) method that produces nanoparticles starting from atoms or gas or liquid based on atomic transformation or molecular condensation (Guzman , 2008; Veerasamy *et al.*, 2011). Silver

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nanoparticles are used in various fields, especially in biomedical industry or diagnosis, drug delivery, cell imaging, and implantation. Biosynthesis of silver nanoparticles could be advantages than photochemical reduction and chemical reduction methods (Saxena *et al.,* 2012). In biosynthesis including plant, bacteria, fungi and yeast have been used to prepare nanoparticles (Fatma and Nivien, 2015; Kumar *et al.*, 2014). Therefore, in this research proposal, biosynthesis of obtained silver nanoparticles (TBAgNPs, LGAgNPs) in the environmental friendly were conducted by using the two selected plants namely: *Eupatorium odoratum* Linn. and, *Cymbopogon citratus* Stapf. These two plants were widely distributed in the tropical and subtropical regions of Africa, Asia and America. In Myanmar, they are widely distributed throughout the country (Emmanuel and Anthony, 2017). Then, characterization of fabricated silver nanoparticles was studied by applying advanced modern techniques (Masurkar *et al.*, 2011). The antioxidant activity of fabricated AgNPs was also studied by using DPPH assay method. After that, the determination of inhibitory effect of reducing agent and prepared silver nanoparticles were studied against six kinds of microorganisms.

Materials and Methods

Sample Collection

The leaves of *E. odoratum* and *C. citratus* were collected from Hmone Pya village, Daw Oo Khu Quarter, Loikaw Township, Kayah State, Myanmar in the middle of November, 2017 [Figures 1 (a), (b)]. After cleaning, the leaves were air-dried at room temperature for three weeks and the dry sample were ground into powder and stored separately in air-tight containers to prevent moisture changes and other contamination. These selected plants were identified at the Department of Botany, University of Yangon.

(a) (b)

Figure 1 Plant of (a) *E. odoratum* L. (b) *C. citratus* Stapf

Preparation of Silver Nanoparticles (TBAgNPs, LGAgNPs)

Each of dried powdered sample of *E.odoratum* and *C. citratus* (30 g) was boiled in 150 mL of distilled water for 48 h to obtain the respective extract. This obtained extract was filtered through Whatman filter paper No.1. These filtrates were cooled down at 4 °C. Then, 0.017 g of $AgNO_3$ was dissolved in a volumetric flask with 10 mL of distilled water and made up to 100 mL of the solution to give 0.001 M of $AgNO_3$ solution. It was used in this biosynthesis of silver nanoparticles. 20 mL each of the prepared extract was added to the 0.001 M of $AgNO_3$ solution (60, 80,100 mL) as the different volume ratios of 1:3, 1:4, 1:5 v/v in each conical flask under aseptic condition. The flask was heated and stirred with magnetic stirrer at different temperatures 40 °C, 50 °C, 60 °C and different stirring times (20 min, 40 min, 60 min). Then, it was placed in a dark place over night. A change in the colour was observed indicating the formation of silver nanoparticles. The solution was centrifuged at 6000 rpm for 20 min to obtain silver nanoparticles and supernatant was discarded. Then, the obtained particles from *E.odoratum* (TBAgNPs) and *C. citratus* (LGAgNPS) were washed to purify and dried at 100°C in an oven for 24 h.

Characterization of the Prepared Silver Nanoparticles

The Field Emission Scanning Electron Microscopy (FESEM) technique was applied to analyze the surface morphology of fabricated TBAgNPs, LGAgNPs. The prepared AgNPs was characterized by XRD analysis and the particle size was calculated by using Debye- Scherrer equation. In addition, determination of particle size distribution of AgNPs was measured by using Zeta potential-DLS instruments at 25 °C with percent intensity. The localized surface plasmon resonance bands of AgNPs were studied by using Shimadzu UV-1800 spectrometer.

Screening of Antioxidant Activity of the Prepared Silver Nanoparticles

Antioxidant activity of the prepared AgNPs was determined UV-visible spectroscopic by using DPPH (1,1-diphenyl, 2-picryl, hydrazyl) radical scavenging assay (Halliwell, 2012).

Preparation of test sample solutions

4 mg of each of the prepared TBAGNPs and LGAgNCs was dissolved in 10 mL of 95 % ethanol and thoroughly mixed by vortex mixer. The mixture solution was filtered and filtrate was used as a stock solution, 4 μg/mL. Desired concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.125 μg/mL) of sample solutions were prepared from the stock solution by serial dilution with appropriate amount of 95 % ethanol.

Preparation of 60 μM DPPH solution

To get a 60 μM DPPH solution, 2.364 mg of DPPH was thoroughly dissolved in 100 mL of 95 % ethanol. This solution was freshly prepared in the brown coloured flask and kept in refrigerator for no longer than 24 h.

Preparation of blank solution

Blank solution was prepared by mixing 1.5 mL each of the test sample solution with 1.5 mL of 95 % ethanol.

Procedure

The control solution was prepared by mixing of 1.5 mL of 60 μM DPPH solution and 1.5 mL of 95% ethanol using vortex mixer, (Halliwell, 2012). The sample solution was also prepared by mixing thoroughly 1.5 mL of 60 μM DPPH solution and 1.5 mL of test sample solution. The solution was allowed to stand at room temperature for 30 min. After 30 min, the absorbance of these solutions were measured at 517 nm by UV-visible spectrophotometer. Absorbance measurements were done in triplicate for each solution and the mean values so obtained were used to calculated the percent inhibition of oxidation, (Kahlonene, 1999).

% oxidative inhibition of the sample = $\frac{A_{Control} - (A_{Sample} - A_{Blank})}{A_{Control}} \times 100$

$A_{Control}$ = absorbance of DPPH in 95 % EtOH solution

A_{Sample} = absorbance of sample and DPPH solution

A_{Blank} = absorbance of sample and 95 % EtOH solution

Average, $\bar{x}$ = $\frac{x_1 + x_2 + x_3 + \ldots + x_n}{n}$

Standard derivation (SD) = $\sqrt{\frac{(\bar{x}-x_1)^2+(\bar{x}-x_2)^2+(\bar{x}-x_3)^2+\ldots+(\bar{x}-x_n)^2}{n-1}}$

$\bar{x}$ = average % inhibition

$x_1+x_2+\ldots+x_n$ = % inhibition of test sample solution

n = number of times

Then, IC_{50} (50 % oxidative inhibitory concentration) values were also calculated by linear regressive excel program.

Screening of Antimicrobial Activity of the Prepared AgNPs

Agar well diffusion method (Balouiri *et al.*, 2016) was employed for determining antimicrobial activity of the plant extracts and prepared AgNPs: TBAgNPs and LGAgNPs against six pathogenic microorganisms namely *Bacillus subtilis*, *Staphylococus aureus*, *Pesudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli*, (Cruickshank, 1960) at PRD, Pharmaceutical Research Department, Ministry of Industry, Yangon, Myanmar.

Results and Discussion

Sample Collection and Preparation of Reducing Agent

Currently, the leaves of *E.odoratum* and *C. citratus* were collected from Hmone Pya village, Daw Oo Khu Quarter, Loikaw Township, Kayah state, Myanmar. The dried leaves powdered was extracted with distilled water and it was used as reducing agent as well as capping agent in the preparation of AgNPs.

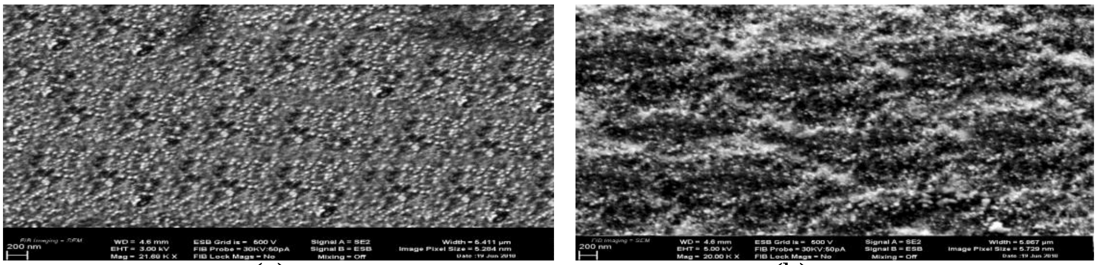
Synthesis of Silver Nanoparticles (AgNPs)

The solution of silver nitrate (0.001 M) was used as a metal precursor for this synthesis of silver nanoparticles. The fabricated silver nanoparticles (TBAgNPs, LGAgNPs) were observed under visual condition. AgNPs were formed with a colour change from yellow to brownish-black colour during the reaction period within 20 min. The colour change of brownish-black was observed in the formation of AgNPs and it was due to the effect of reducing agent as well as capping agent of *E. odoratum* and *C. citratus* leaves extracts of selected sample in this research. The extract (20 mL) from each of the *E. odoratum* and *C. citratus* leaves was added to different volumes 0.001 M of silver nitrate solution (60, 80,100 mL). The various ratios of leaves extracts and $AgNO_3$ (1:3, 1:4, 1:5 v/v) solutions were separately placed in a conical flask. The solution was mixed on a magnetic stirrer while heating at a temperature of about 40 °C, 50 °C, 60 °C. After stirring time for 20, 40, 60 min, it was kept in the dark place. Then, this reaction process was carried out in dark to avoid unnecessary photochemical reactions (Harris and Bali, 2008). A change in the colour was observed and it indicated the formation of silver nanoparticles. The solution was centrifuged at 6000 rpm for 20 min to obtain silver nanoparticles and supernatant was discovered. Among them, 1:5 v/v of leaves extracts and $AgNO_3$ solution at 60 °C and stirring time 60 min was observed not in the colloidal state and can be filtered easily than other conditions. The particles obtained were washed and dried at 100 °C in an oven for 24 h. The dried particles were observed in 1.816 g.

Characterization of the Prepared Silver Nanoparticles

FESEM analysis

The AgNPs (TBAgNPs and LGAgNPs) prepared by using 1:5, v/v ratio of each plant extract and 0.001 M $AgNO_3$ solution at 60 °C with 20 min were chosen for studying their characteristics. The surface morphology of prepared TBAgNPs and LGAgNPs were studied by the field emission scanning electron microscopy (FESEM). From these observation, the surface morphology of each prepared silver nanoparticles TBAgNPs was observed very smoothly and in more spherical nature than LGAgNPs (Figure 2).

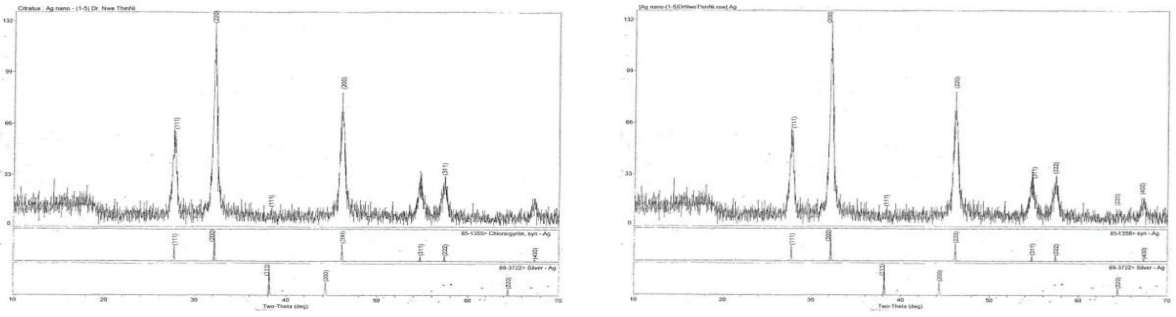


(a) (b)

Figure 2 FESM images of the prepared AgNps (a) TBAgNPs (b) LGAgNPs

XRD analysis

The average crystalline size of the prepared TBAgNP and LGAgNPs was determined by X Ray Diffratometer (XRD) analysis and calculated by using Debye-Scherrer's equation. From XRD diffractogram of TBAgNP, the four distinct diffraction peaks at 2θ values of 29.879, 30.128, 36.401 and 44.566 were respectively indexed to 111, 200, 220 and 311 reflection planes of face centered cubic structure of silver. In addition, the average particle size of LGAgNP was also calculated from three distant peaks of 111, 220, 200 with 2θ values of 35.138, 40.401, 37.495. The average particle size of TBAgNP (32 nm) occurred smaller than that of LGAgNPS (38 nm) (Figure 3).

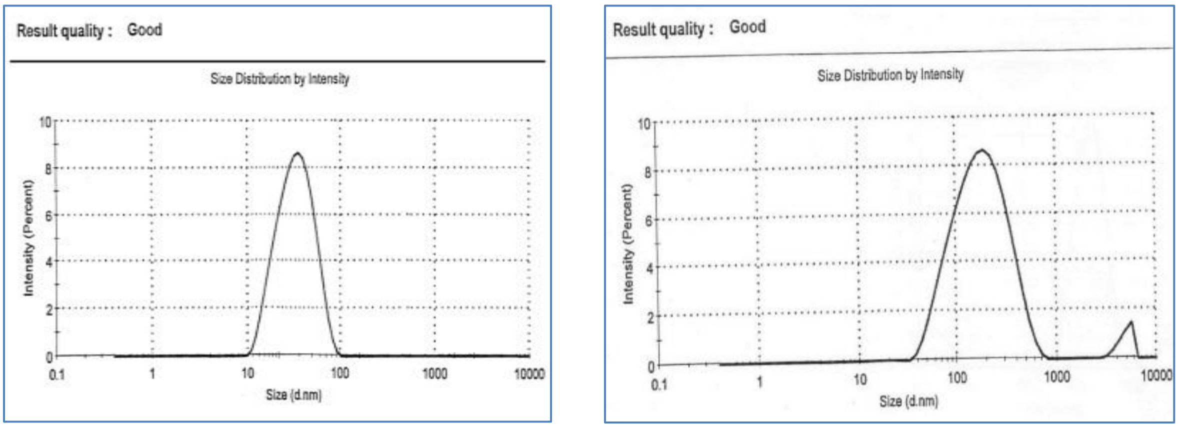


(a) (b)

Figure 3 XRD Diffractograms (a) TBAgNPs (b) LGAgNPs

Zeta potential-DLS analysis

The size distribution of prepared AgNPs was reported with intensity using zeta potential-DLS and shown in Figure 4. From this result, the size distribution of prepared TBAgNPs and LGAgNPs were observed between 1-100 nm range with peak intensity.

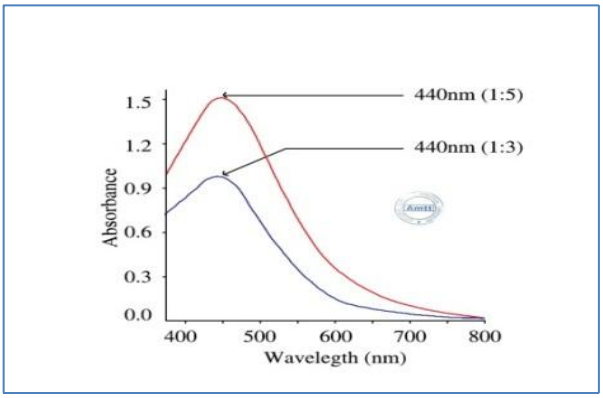


(a) (b)

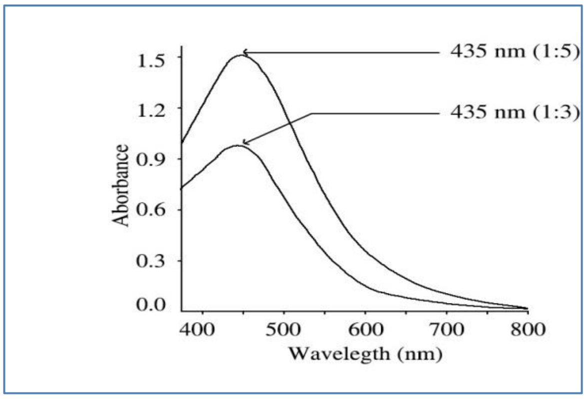
Figure 4 The Zeta potential- DLS spectra of the prepared (a) TBAgNPs (b) LGAgNPs

UV-visible spectral analysis

The formation of stability of metal nanoparticles in aqueous solution was determined by using the UV-visible spectroscopy and it is an important technique to exhibit UV-visible absorption maximum in the range of 300-360 nm due to the excitation of surface plasmon vibration. The localized surface plasmon resonance bands were observed at 440 nm for TBAgNPs and 435 nm for LGAgNPs. The increase in the concentration of the silver nitrate increased the absorbance intensity but the wavelength was not changed (Figure 5). The absorbance intensity of the AgNPs prepared by using 1:5, v/v ratio of leaves extract and $AgNO_3$ solution was observed to be lighter than the AgNPs prepared by using 1:3 v/v ratio of leaves extracts and $AgNO_3$ solution.



(a)

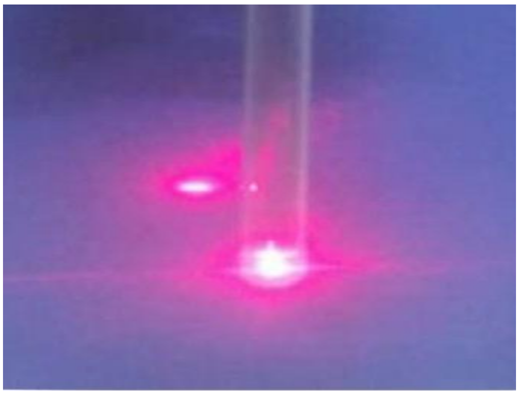


(b)

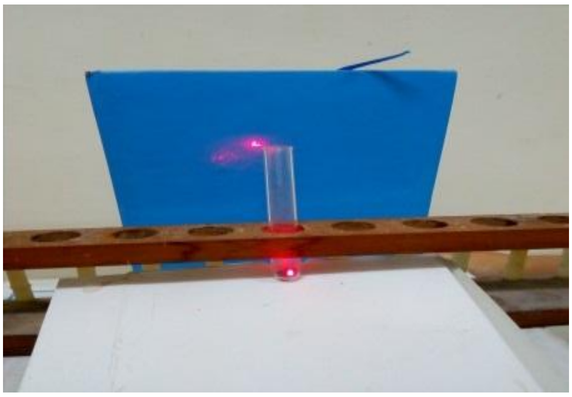
Figure 5 UV visible absorption spectra of the prepared AgNPs (a) TBAgNPs (b) LGAgNPs

Tyndall effect

The Tyndall effect, also known as Willis-Tyndall scattering is light scattering by particles in a colloid or in a very fine suspension. The particle even large enough that they can be scattered light, the Tyndall effect occurred (Saxena, 2012). Since the presence of a colloidal suspension can be monitored by the reflection of a laser beam from the particles because a laser pointer emitted that the polarized light, and the pointer can also be oriented that the beam appear to disappear. If the colloidal particles are present, the laser beam passed and if the particles are absent, the beam did not pass through it. In these experiments, the laser beam was completely observed to pass through the both of the prepared nanoparticles of TBAgNPs and LGAgNPs, (Figure 6).



(a)



(b)

Figure 6 Tyndall effect of light scattering the prepared AgNPs (a) TBAgNPs (b) LGAgNPs

Antioxidant Activity of the Prepared Silver Nanoparticles

The antioxidant activity was expressed as 50 % oxidative inhibitory concentration (IC_{50}). The lower the IC_{50} values, the higher the antioxidant activity of the sample. By using DPPH free radical scavenging assay, the IC_{50} values of the prepared TBAgNPs and LGAgNP were respectively observed to be 49.27 μg/mL and 94.32 μg/mL. The more potent antioxidant activity was observed in TBAgNPs than LGAgNPs, (Figure 7 and Table 1). Besides, the IC_{50} values of the standard ascorbic acid was found to be 7.28 μg/mL.

Antimicrobial Activity of Watery Extract of Plant Leaves and and Prepared Silver Nanoparticles

The antimicrobial activities of the plant leaves extracts and the prepared TBAgNPs and LGAgNPs were evaluated against six strains of microorganisms: *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans, Escherichia coli* by using agar well diffusion method (Table 2). Therefore, nanoparticles were generally more active than reducing agent (plant extract) against the selected microorganism. It is reasonable to state that the binding of the particles to the bacteria depends on the surface area available for interaction. This is because, the silver nanoparticles may attach to the surface of the cell membrane and disturb its power function such as permeability and respiration, (Balouiri *et al.*, 2016). The two plant extracts and prepared AgNPs showed moderately antimicrobial activities. Among them, more potent antimicrobial activity was observed in TBAgNPs (20 mm) than LGAgNPs (18 mm) against *Bacillus pumilus*, and *Escherichia coli*.

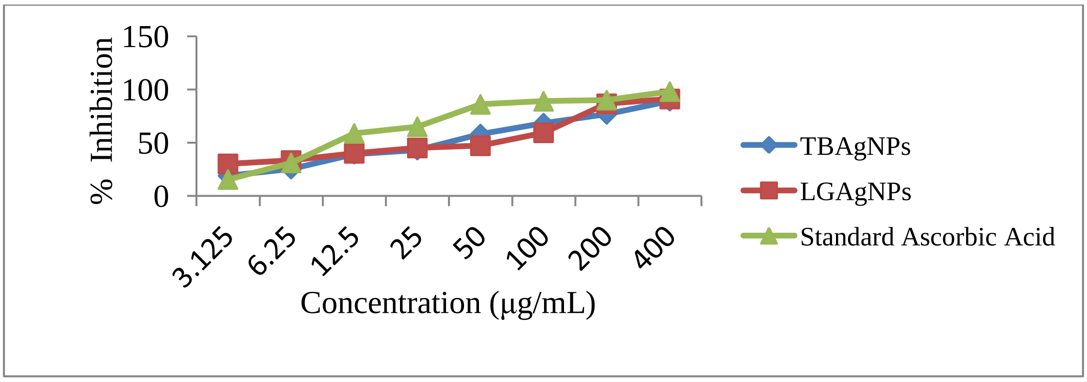


Figure 7 % inhibition of different concentrations of the prepared AgNPs and standard ascorbic acid

Table 1 Oxidative Percent Inhibitions and IC_{50} Values of Different Concentrations of Prepared Silver Nanoparticles and Standard Ascorbic Acid

Sample	**% Inhibitions (Mean ± SD) in various Concentrations (µg/ml)**								**IC_{50} (µg/mL)**
	3.125	**6.25**	**12.5**	**25**	**50**	**100**	**200**	**400**	
TBAgNP	19.134 ± 0.253	25.392 ± 0.267	39.421 ± 0.187	**43.239** ± 0.394	**58.344** ± 0.181	68.558 ± 0.258	76.788 ± 0.402	89.247 ± 0.597	49.27
LGAgNP	30.289 ± 0.503	33.421 ± 0.401	40.231 ± 1.502	45.238 ± 0.651	**47.342** ± 0.678	**59.518** ± 1.702	86.718 ± 0.501	91.238 ± 1.823	94.32
Standard Ascorbic acid	15.646 ± 0.542	**31.225** ± 0.472	**58.891** ± 0.723	65.221 ± 0.626	86.221 ± 0.792	89.128 ± 0.392	90.234 ± 1.143	98.245 ± 0.682	7.28

Table 2 Inhibition Zone Diameters of Watery Extract of Plant Leaves and AgNPs Against Six Microorganisms by Agar Well Diffusion Method

No.	Microorganisms	Inhibition zone diameter (mm)			
		Watery extract of Tawbizat	Watery extract of Lemongrass	Prepared TBAgNPs	Prepared LGAgNPs
1.	*Bacillus subtilis*	16 (++)	17 (++)	15 (++)	17 (++)
2.	*Staphylococcus aureus*	16 (++)	17 (++)	16 (++)	17 (++)
3.	*Pseudomonas aeruginosa*	15 (++)	16 (++)	17 (++)	15 (++)
4.	*Bacillus pumilus*	15 (++)	17 (++)	**20** (+++)	18 (+++)
5.	*Candida albicans*	16 (++)	16 (++)	18 (++)	16 (++)
6.	*Escherichia coli*	17 (++)	17 (++)	**20** (+++)	18 (+++)

(+) = low activity, (++) = medium activity, (+++) = high activity

Conclusion

From this research work, biosynthesis of silver nanoparticles is environmental friendly and non-toxic effect in environment. In the preparation of AgNPs (55.06 %) watery extracts of *E.odoratum* (Taw-Bizat) leaves and *C. citratus* (Lemon-Grass) were used as reducing agent as well as capping agent. The surface morphology of TBAgNPs was observed to show more spherical shape and to be more smooth than LGAgNPs according to result of FESEM. In addition, the particle size distribution of TBAgNPs and LGAgNPs showed with peak intensity in the range of (10-100) nm range under the zeta potential-DLS. The average particle size of TBAgNPs (32 nm) was slightly smaller than LGAgNPs (38 nm) determined by XRD analysis. The absorbance intensity of fabricated TBAgNPs (440 nm) and LGAgNPs (435 nm) were observed under the UV- visible spectrometer. In addition, if the nanoparticles were present, the light scattering passed through was observed. Therefore, Tyndall Effect of the laser beam passed through colloidal TBAgNPs and LGAgNPs. The antioxidant activity of prepared TBAgNPs (49.27μg/mL) was higher than that of LGAgNPs (76.78 μg/mL). Furthermore, the antimicrobial activity of prepared TBAgNPs (20 mm) was observed to be higher than that of leaves extracts of Taw-Bizat and Lemon-Grass and LGAgNPs against on *Escherichia coli* and *Bacillus pumilus.*

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