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ANTIDIABETIC EFFECT OF SILVER NANOPARTICLES SYNTHESIZED USING LEMONGRASS (*CYMBOPOGON CITRATUS*) THROUGH CONVENTIONAL HEATING AND MICROWAVE IRRADIATION APPROACH

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ABSTRACT

Biosynthesis of nanoparticles using plant extract is an eco- friendly approach which eliminates the need for using physical and chemical techniques. Nanoparticle use in medical field specially anti-diabetes and anti-cancer application has remained in recent studies owing to its large surface area to volume ratio, which imparts them the capability to interact with any kind of membrane and enzymes in a better manner. This paper presents the optimized synthesis of silver nanoparticles (Ag NPs) using lemongrass and visualizing its in-vitro anti-diabetic potential. This study involves the comparative analysis of Conventionally heated (CH) and Microwave irradiated (MI) technique for the synthesis of Ag NPs from lemongrass for the first time. The crystalline nature of the nanoparticles was confirmed using X-ray diffraction assay (XRD) with average size range of 75 nm, the size, and shape of the nanoparticles were confirmed as spherical shaped using scanning electron microscope (SEM), atomic force microscopy (AFM) demonstrated aggregate formation of size range 138 nm with mean average size of individual nanoparticle as 80 nm, elements present in the nanoparticles confirmed using elemental dispersive analysis of X-ray (EDAX) and the functional groups of plant extract responsible for nanoparticles synthesis have been confirmed using Fourier transform infrared spectroscopy (FT-IR). Surface morphology and the dispersity were observed by Transmission electron microscopy (TEM) analysis. The anti-diabetic potential of nanoparticle synthesized was studied using amylase activity inhibition Assay and glucose diffusion-inhibitory Assay.

Keywords: Green synthesis, Silver nanoparticles, Microwave irradiated, lemongrass, Anti-diabetic

INTRODUCTION

Nano-science manipulates atom or molecule at a sub-microscopic level for their better functioning **(Ahmed and Ikram, 2015)**. Designing of molecules at nanoscale level imparts better properties like magnetic, electronic, catalytic, optoelectronic properties. Shape, size and surface morphology of the particles highly influence the intrinsic properties of nanoparticles **(Ahmad *et al*., 2010; Ahmed and Ikram, 2015)**. Nanosciences is one of the most extensively studied areas of research where green sources are being used for the synthesis of new and noble nanoparticle **(Banerjee *et al*., 2014)**. Metal nanoparticles have withdrawn considerable attention from researchers because of their enhanced physicochemical and biological properties like biological tagging, photonics, catalysis, electronics, photography owing to their large surface area to volume ratio, reduced imperfections and surface plasmon resonance phenomenon **(Begum *et al*., 2009; Bobbu *et al*., 2016)**. Silver nanoparticles have other applications in the field of dentistry, food, and healthcare industries. Silver nanoparticles possess extraordinary antimicrobial properties which are clearly demonstrated by the background literature **(Elgorban *et al*., 2016; Gajbhiye *et al*., 2009)**. For example, silver nanoparticle synthesized using leaf extract of *Artemisia nilagirica* had a potent antibacterial effect on *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Proteus subtilis* **(Vijayakumar *et al*., 2013)**. Similarly, apple extract mediated synthesized nanoparticle had shown its anti-bacterial effect on *E.coli, S. aureus, P. aeruginosa* **(Zainal *et al*.,2016)**. These antimicrobial effects of silver nanoparticles have led to the development of nano-silver products like contraceptive devices, wound dressing, implants, surgical instruments coated with nano-silver. It is being used for the formulation of the antimicrobial paint coating, water treatment, food cans, textiles as well **(Christensen *et al*., 2011)**.

Silver nanoparticles have been synthesized using traditional approaches like solution reduction, thermal decomposition, reverse micellar chemical and photochemical reactions, radiation assisted, the sonochemical and electrochemical process **(Gavhane *et al*., 2012)**. These processes involved the use of toxic chemicals hazardous to nature and thus, the process of synthesizing metal nanoparticle shifted to more eco-friendly green approaches **(Ghaffari-Moghaddam and Hadi-Dabanlou, 2014)**. Literature study indicates the synthesis of silver nanoparticles from plant sources like *Vitis vinifera* **(Gnanajobitha et al., 2013)**, *Tragia involucrata, Cymbopogon citronella, Solanum verbascifolium* and *Tylophora ovata* **(Joy Prabu and Johnson, 2015)**, *Arbutus Unedo* **(Kouvaris *et al*., 2012)**, *Acalypha indica* **(Krishnaraj *et al*., 2010)**, *Clitoria ternatea* and *Solanum nigrum* (Krithiga *et al*., 2015) , *Syzygium cumini* **(Kumar *et al*., 2010)**, *Sesuvium portulacastrum* L. **(Nabikhan *et al*., 2010)**, *Piper nigrum* **(Paulkumar *et al*., 2014)**, *Kalanchoe pinnata* **(Phatak and Hendre, 2015)**, *Eclipta prostrata* **(Rajakumar and Abdul Rahuman, 2011)**, *Pongamia pinnata* **(Rajeshkumar, 2016)**.

Present study focuses on a comparative study of conventional heated and 2 minutes microwave irradiated boiled sample of lemongrass mediated nanoparticle synthesis and its further characterization using UV-visible spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), scanning electron microscope (SEM), Energy dispersive analysis of X-ray (EDAX), Transmission electron microscopy (TEM), Atomic force microscopy (AFM). Antidiabetic potential of the synthesized nanoparticles has been evaluated using Alpha-amylase inhibitory assay and Glucose diffusion retardation assay.

MATERIALS AND METHODS

Fresh leaf extracts preparation

Fresh leaves of lemongrass were collected from VIT, Vellore campus. Leaves were washed with running tap water and double washed with Milli-Q water. Leaves were left to dry at room temperature. Leaves were cut into small pieces using a sterile scalpel. 10 g of leaves were crushed using mortar and pestle and mixed with 100 mL of Milli-Q water. The solution was heated at 80°C and was later filtered using Whatman filter paper no.1. The solution was re-filtered using Whatman filter paper of smaller pore size. The filtrate was used as an extract.

Silver nanoparticle formation

10 mL of the extract was mixed with 90 mL of 1Mm $AgNO_3$ solution. The reaction mixture was microwave irradiated for 2 minutes for MI boiled sample and left unboiled for the formation of the conventionally heated sample (Figure. 1). The reaction mixture was kept in an open shaker at room temperature for continuous and efficient mixing of the extract with 1mM AgNO3 solution. MI heated flask showed immediate color change from yellow to dark brown while CH flask took some extra time for the color change. Visual color change and UV-visible analysis act as a confirmatory test for the synthesis of nanoparticles.

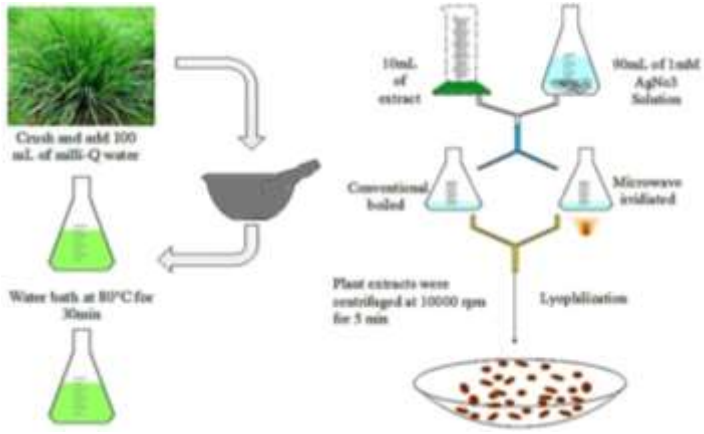


Figure 1 Green synthesis of silver nanoparticles

Characterization studies of nanoparticles

After visual confirmation and UV-visible analysis of nanoparticle synthesis, the reaction mixture was centrifuged at 10,000 RPM for 5 minutes and double washed with Milli-Q water followed by double centrifugation. The solution was lyophilized at -80°C. Pellet was collected and used for further characterization studies. UV-visible spectroscopy was performed at the range of 300-500 nm and absorbance was measured at the interval of 5 nm each. XRD spectra were obtained using XDL 3000 powder X-ray diffractometer operating at 30 mA and 40 kV with Cu Kα of 1.5405˚A. XRD analysis was carried out to identify crystallinity and size of nanoparticle. FTIR analysis was performed at wavelength range of 400-4000 cm^{-1} to identify the functional groups involved in the synthesis of nanoparticle. SEM analysis was carried out to analyze the morphology of nanoparticle and EDAX analysis was performed to further investigate the presence of elemental silver in nanoparticle. AFM analysis was carried out to visualize the topography of synthesized nanoparticle. TEM analysis was conducted to determine morphology, roughness and dispersity of nanoparticle.

In-vitro antidiabetic assay

The in-vitro anti-diabetic assay was performed using two different techniques: Alpha-amylase inhibitory assay and Glucose diffusion-inhibitory assay.

Alpha-amylase inhibitory assay

Alpha-amylase inhibition was determined by quantifying the amount of maltose liberated during the experiment. The method reported by Bhutkar and Bhise has been followed **(Bhutkar and Bhise, 2012)**. Different concentration of nanoparticles (20, 40, 60, 80, 100 µL) was pre-incubated with 100 µL of α-amylase solution (1 U/mL) at room temperature for 30 minutes. 100 µL of starch solution (1% w/v) was further added to it and the mixture was incubated at room temperature for 10 minutes. 100 µL of 96 mM (3, 5- dinitrosalicylic acid solution) DNSA reagent was added to it to stop the reaction and the solution was heated in a water bath for 5 minutes. Control was maintained where the equal quantity of enzyme extract was replaced by sodium phosphate buffer maintained at a pH value of 6.9. Reading was measured at 540 nm. The experiment was performed in triplicate. Acarbose was used as a positive control.
% inhibition was calculated using the formulae-

$$\%\ \text{inhibition} = \frac{C - T}{C} * 100$$

Where, C= control, T= test sample.

Glucose diffusion retardation assay

The method reported by Abideen and Vijaya Sankar has been followed **(Abideen S and Vijaya Sankar M, 2015)**. 1 mL of nanoparticle solution was poured inside a dialysis membrane along with 2 mL of 22 mM of D-glucose solution in 0.15 M NaCl. The dialysis membrane was tied at both the ends to prevent the release of incorporated fluid. The membrane was immersed in a beaker containing 40 mL of 0.15 M NaCl and 10 mL of Milli-Q water. Control was maintained where the equal quantity of nanoparticle solution was replaced by Milli-Q water. The beaker was kept on an orbital shaker at room temperature and movement of glucose solution into external solution was observed every half an hour for 3 hours. Glucose concentration (mg/mL) in the external solution was measured using standard glucose oxidation kit.
GDRI (glucose diffusion retardation index) was calculated using the below-mentioned formulae:

$$\textbf{GDRI} = \frac{(100 - \text{glucose concentration in external solution in presence of nanoparticle})}{\text{glucose concentration in external solution without addition of nanoparticle}}$$

RESULTS AND DISCUSSION

Visual identification

Visual identification is the preliminary confirmatory test for nanoparticle synthesis. The color change from white yellow to brown indicates the synthesis of nanoparticle **(Rajeshkumar, 2016)**. The conventionally heated sample did not show any color change initially but had shown light brown color after 24 hours of incubation. MI sample of lemongrass gave light brown color immediately after heating and turned to dark brown after 6 hours of incubation time. After 24 hours of incubation, particles started to settle at the bottom of the flask which indicated aggregation of nanoparticles in the flask and completion of the process of nanoparticle synthesis. Similar results were obtained for the synthesis of silver nanoparticle from the fresh bark of *Pongamia pinnata* **(Rajeshkumar, 2016)**. Figure 2(a) represents fresh leaf extract of lemongrass, 2(b) represents a color change in a conventionally heated sample of lemongrass after 24 hours, 2(c) represents a color change in 2 minutes MI sample of lemongrass.

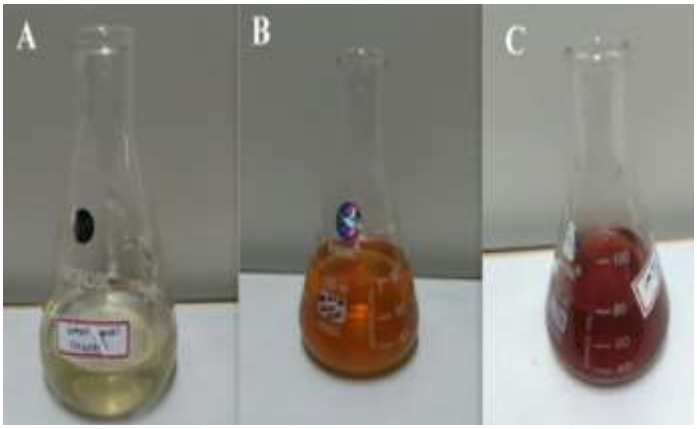


Figure 2 (a) Fresh leaf extract of lemongrass (b) fresh sample of lemongrass (c) 2 minutes boiled sample of lemongrass.

UV-Visible spectroscopic analysis

Silver nanoparticle show peak in a certain range due to surface plasmon resonance (SPR). Figure 3 (a) and (b) represents a peak in the range which demonstrates the presence of silver nanoparticle. For conventionally heated sample, the peak was measured at an interval of 1 hour for 24 hours and then at 53, 67, 92 hours. 24 hours peak observed at 435 nm indicated that the synthesis of silver nanoparticle has started. Peak intensity further got raised from 24 hours to 92 hours due to the effect of SPR effect in the mixture solution. MI sample showed no peak initially but a strong peak at 440 nm was observed after an hour of incubation which further got raised and a narrow peak at 435 nm was observed after 6 hours of incubation which strongly indicated the presence of silver nanoparticle. After 6 hours of incubation, peak started declining which suggested that the synthesis process of nanoparticle has been stopped and particles started aggregating.

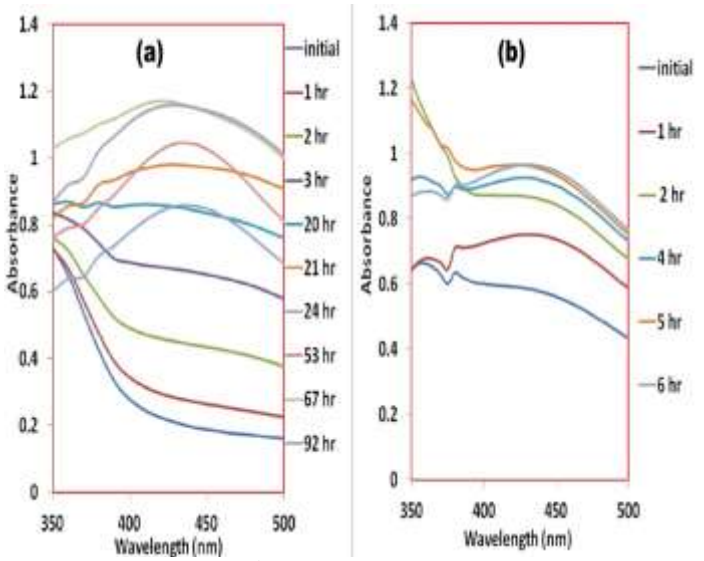


Figure 3 UV-Vis spectra of nanoparticles (a) unboiled sample (b) 2 minutes microwave heated the sample

X-ray diffraction analysis

XRD spectra are used as a confirmatory result of the crystalline nature of synthesized nanoparticle. Figure 4 (a) and (b) represents XRD spectra of 2 minutes MI sample of nanoparticle and a conventionally heated sample of nanoparticle respectively. Bragg's reflection peak observed at 38.02°, 46.01°, 67.37°, 77.26° in 2 minutes MI sample of nanoparticle and at 38.02°, 46.05°, 68.91° and 77.16° in conventionally heated sample corresponds to (1,1,1), (2,0,0), (2,2,0), (3,1,1) lattice planes respectively suggesting face-centered cubic (fcc) crystalline nature of silver nanoparticles (Rajkuberan *et al*., 2015) . Peak comparison of synthesized nanoparticle and pure crystal silver reported by Joint Committee on Powder Diffraction Standards (File no. 04-0783) has been made. The average size of the synthesized nanoparticle has been calculated using Debye-Scherrer equation (Rajeshkumar *et al*., 2014). Table 1 and 2 represents the calculation data used in the equation to calculate the particle size.

$$D = \frac{K\lambda}{\beta cos\theta}$$

Where,
D= average particle size (nm)
K= Shape factor of spherical crystal with cubic symmetry (0.94)
λ= X-ray wavelength (1.5406 Å)
β= full width at half maximum (FWHM)
θ= diffraction angle

Table 1 silver nanoparticles size analysis of the conventionally heated sample.

2θ	FWHM (β)	D (nm)
27.61	0.03	28.61
32.03	0.04	28.79
38.02	0.11	79.86
46.05	0.03	30.06
54.69	0.08	116.8
68.91	0.09	111.84
77.16	0.12	88.47

Table 2 Silver nanoparticle size analysis of 2 minutes MI sample.

2θ	FWHM (β)	D (nm)
27.65	0.03	28.53
32.03	0.03	97.61
38.02	0.13	67.51
46.01	0.03	20.97
54.69	0.055	169.9
67.37	0.07	142.49
77.26	0.09	118.04

Average size obtained using the above-mentioned equation was 92.15 nm for 2 min MI sample and 69.20 nm for the conventionally heated sample. The size obtained were similar to size derived using SEM and TEM analysis.

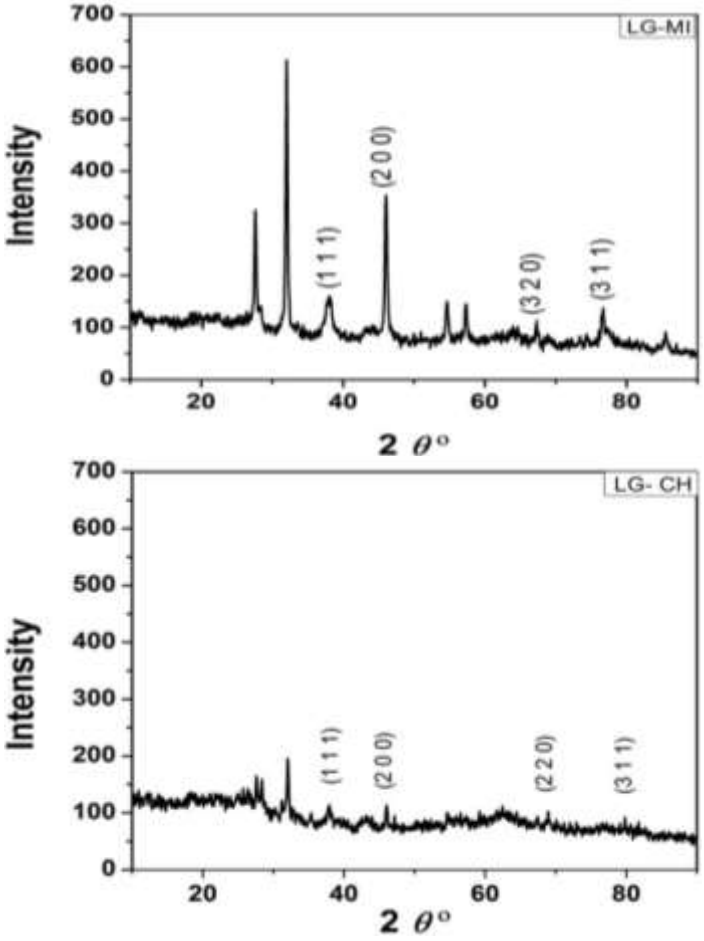


Figure 4 (a) XRD spectra of 2 minutes MI silver nanoparticles (b) XRD spectra of the conventionally heated silver nanoparticles

FT-IR Analysis

FT-IR Analysis gives us the idea about possible functional groups involved in the synthesis of silver nanoparticles. Figure 5 represents an FT-IR analysis of fresh leaf extract of lemongrass and lemongrass mediated synthesized nanoparticle. FT-IR analysis of leaf extract showed a broad peak at 3311.78 corresponding to O-H stretch of phenol, medium peak obtained at range of 2920.23 and 2848.86 corresponded to C-H stretch of alkane, peak at 1705.07 represented the presence of C=O stretch of conjugated aldehyde, medium peak obtained at 1602.85 and 1332.81 corresponded to C=C stretch of unsaturated ketone, O-H bend of alcohol respectively. FT-IR analysis of fresh leaves mediated synthesized nanoparticle represented a broad peak at 3304.06 corresponded to O-H stretch of phenol, medium peak obtained at 2916.37 and 2846.93 demonstrated the presence of C-H stretch of alkane, medium broad peak at 1595.13 corresponded to N-H bend of amine, weak peak at 1381.03 represented the C-H bend of alkane, medium peak at 1029.99 corresponded to C-O stretch of alkane. Weak peaks obtained at 572.86 and 526.57 correspond to C-H bend of an alkyne. The disappearance of C=O stretch of aldehyde in nanoparticle indicated the absorbance of this band in the nanoparticle. Thus, it could be concluded that aldehyde group has aided in nanoparticle synthesis along with another phytochemical constituent.

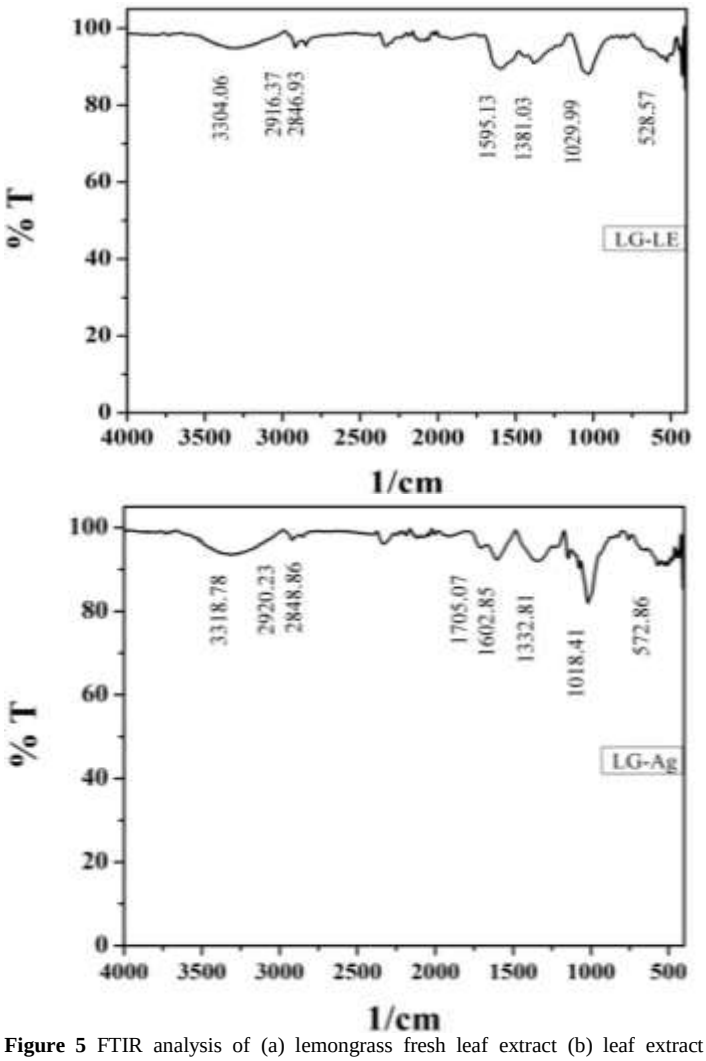
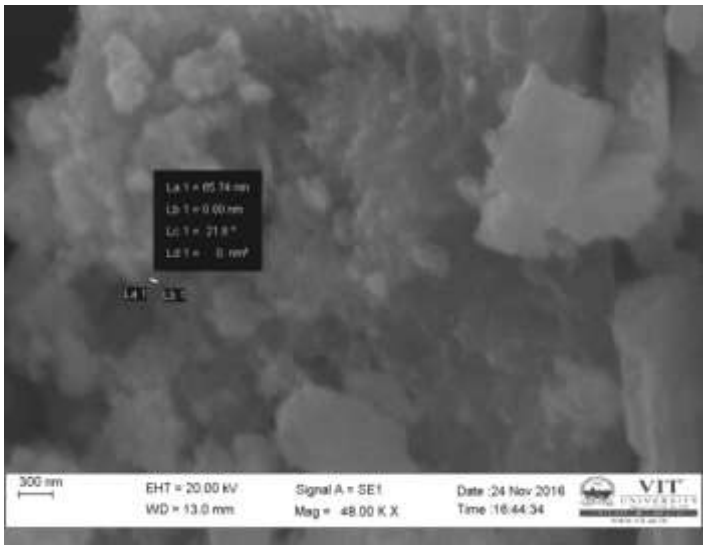


Figure 5 FTIR analysis of (a) lemongrass fresh leaf extract (b) leaf extract mediated silver nanoparticles

SEM and EDAX analysis

Scanning electron microscope is a powerful tool used to identify the morphology of nanoparticle. Figure 6 (a) represents spherical and irregular shaped nanoparticle with a size range of 65.74 nm. the presence of large-sized nanoparticle indicates the aggregation that might have occurred due to the long incubation period. Figure 6 (b) represents the EDAX analysis of nanoparticle. EDAX analysis demonstrates the elemental composition of the synthesized nanoparticle. The intense signal obtained at 3 keV demonstrates the presence of elemental silver in nanoparticle **(Paulkumar *et al*., 2014)**. Another element majority present in the nanoparticle is chlorine which indicated the role of phytochemicals in the fabrication of nanoparticle.



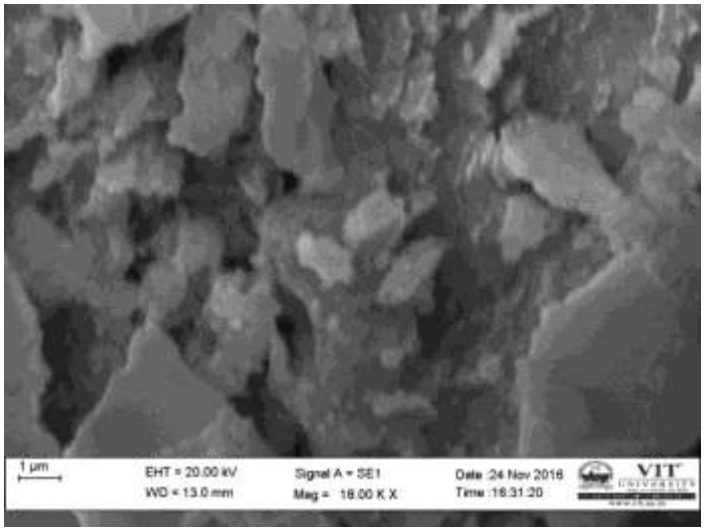


Figure 6 (a) SEM image of the silver nanoparticles

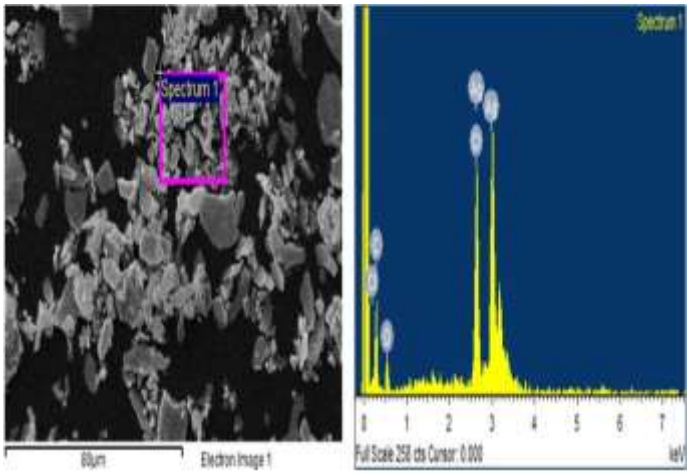


Figure 6 (b) EDAX analysis of silver nanoparticles

AFM Analysis

AFM analysis represents the topography of synthesized nanoparticles. Figure 7 represents the AFM image of *Cymbopogon citratus* fresh leaf extract mediated synthesized silver nanoparticles. Spherical shape nanoparticle with a size range of 138 nm was observed in the AFM imaging. This represents the aggregation of the nanoparticle. Individual nanoparticle with a mean average diameter of 80 nm was found. The result showed similarity with the previously reported silver nanoparticle, synthesized from *Ficus benghalensis* leaf extract having a size range of 75 nm (Saware *et al*., 2014).

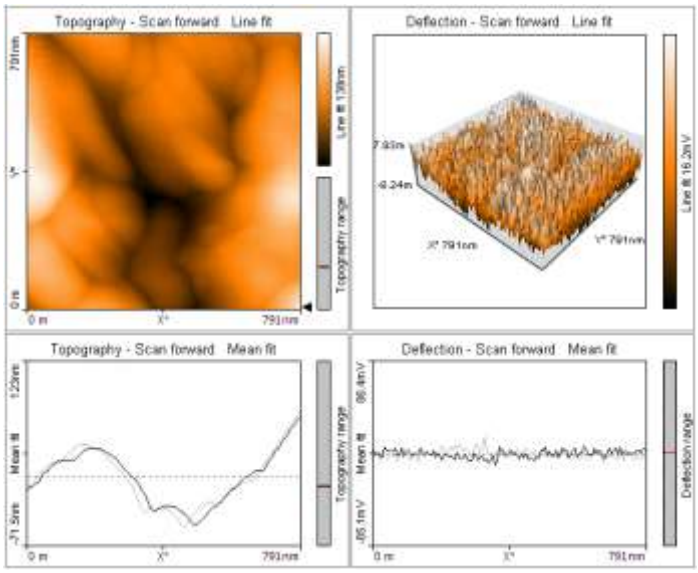


Figure 7 AFM image of *Cymbopogon citratus* mediated synthesized silver nanoparticles

TEM Analysis

TEM image of synthesized nanoparticle revealed the spherical and polydispersed nature of nanoparticle. Aggregates were also observed at some places. Figure 8 represented the TEM micrograph of silver nanoparticle synthesized using fresh leaf extract of *Cymbopogon citratus*. Nanoparticles were spherical in shape with an average size range of 50 nm.

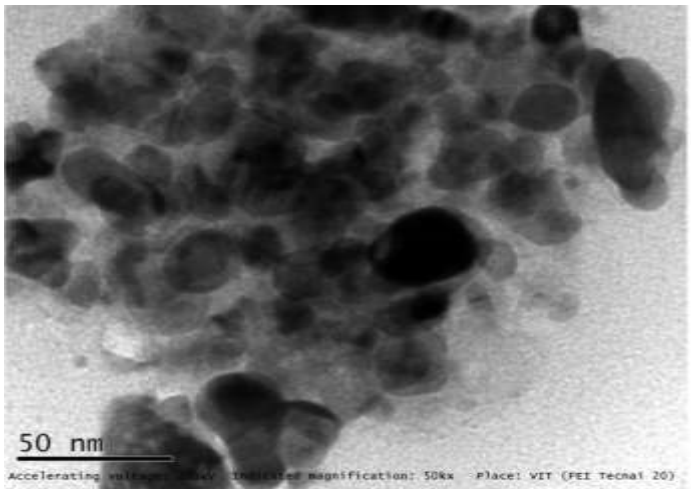


Figure 8 TEM Image of silver nanoparticles

Anti-diabetic assay of nanoparticles

Anti-diabetic assay of nanoparticles has been interpreted through alpha-amylase inhibitory assay and glucose diffusion retardation assay.

Alpha-amylase inhibitory assay

Alpha-amylase is the main enzyme involved in the breakdown of starch, carbohydrate and release of sugar into the main bloodstream which further leads to increased blood glucose level and ultimately diabetes. The inhibitory effect of this enzyme can prove to have a potential therapeutic effect on diabetes. Figure 9 represents the % inhibition effect of silver nanoparticle on the enzyme. Silver nanoparticle exhibited an inhibitory effect on the enzyme in a dose-dependent manner and when the dose was increased to 100µg/mL, it resembled the % inhibition exhibited by standard Acarbose. It could be interpreted from the results that silver nanoparticle could be used as an alternative to Acarbose because of the potential inhibitory effect that it has on the enzyme.

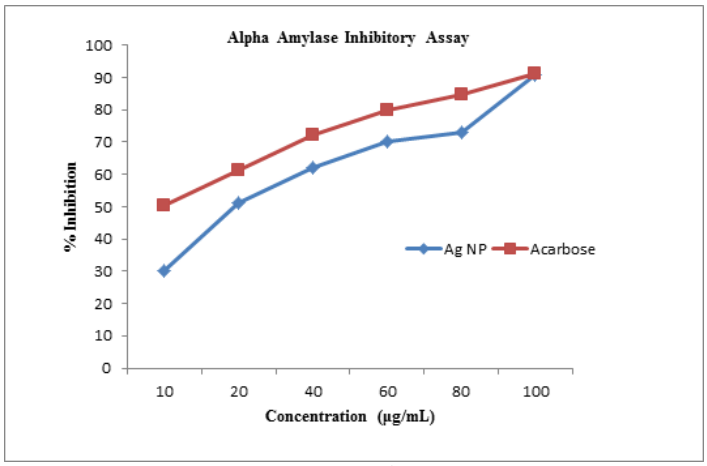


Figure 9 Alpha-amylase inhibitory assays of silver nanoparticles

Glucose diffusion retardation assay

Effect of silver nanoparticle on retardation of glucose into an external solution through dialysis membrane has been represented in Table 3. Biosynthesized silver nanoparticle did not allow the escape of glucose into the external solution by maybe binding or absorbing it and thus can act as a potential drug candidate for the treatment of non-insulin dependent diabetes.

Table 3 GDRI Index of synthesized silver nanoparticles

Time interval (min)	$O.D_{510}$ nm of external solution		% Inhibition
	In presence of Ag NPs	In absence of Ag NPs	
30	1.148	1.124	79.55
60	1.257	1.233	72.40
90	1.149	1.136	78.67
120	1.176	1.098	81.36

The results were similar to an experiment conducted by Bharti *et al*., 2013 which showed that the plant has anti-diabetic effect proven by a series of in-vivo experiments (Bharti *et al*., 2013).

CONCLUSION

The present study demonstrates the synthesis of silver nanoparticles using fresh leaf extract of lemongrass. Results have been compared to conventionally heated and 2 minutes microwave irradiated sample of the reaction mixture. The color change from pale yellow to brown demonstrated the synthesis of silver nanoparticles. UV-Vis readings depicted fast synthesis of Ag NPs in the case of MI heated sample as compared to the conventionally heated sample may be due to the fast interaction of phytochemicals with silver nitrate solution. XRD and SEM analysis confirmed the size range of synthesized nanoparticle to be 50-80 nm with an average particle diameter of 75 nm. Aggregate formation was visualized by AFM imaging and SEM analysis interpretations which might have occurred due to the longer incubation time than required for nanoparticle synthesis. TEM analysis reported average size range of nanoparticle as 50 nm, which was in accordance with SEM and XRD results. Spherical and irregular shaped nanoparticles were confirmed by SEM analysis. FTIR analysis demonstrated the adsorption of an aldehyde group on the surface of the nanoparticle. EDAX analysis confirmed the presence of elemental silver. Synthesized nanoparticle proved to have anti-diabetic activity clearly demonstrated by the results. Thus, this green approach of silver nanoparticle synthesis through microwave irradiated heating can be a fast, economic, eco-friendly approach and the synthesized silver nanoparticle can prove to be a potential anti-diabetic drug candidate.

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