



A Simple Biosynthesis of Silver Nanoparticles from *Syzygium cumini* Stem Bark Aqueous Extract and their Spectrochemical and Antimicrobial Studies

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ABSTRACT

This paper describes a simple biosynthesis of silver nanoparticles (SNs) from aqueous stem bark extract of *Syzygium cumini* (SC), a natural product. The formation of SNs was confirmed and optimized by measuring surface plasmon resonance (SPR) peak around 420 nm by using UV-visible spectroscopy (UV-Vis). The possible functional groups of SC bark extract and their changes after treating with aqueous silver nitrate were evaluated by Fourier transform infrared spectroscopy (FTIR). The size of synthesized SNs was measured by dynamic light scattering (DLS) analysis, and morphology was examined by transmission electron microscopy (TEM) in nano range. The average size of SNs is ~14 nm and monodispersed. The effect of variation of bark extract amount, silver nitrate concentration, time and pH on the synthesis of SNs was investigated at different volumes. Antibacterial activity of SC-SNs was studied taking into account *Bacillus subtilis* (*bacillus*) and *Escherichia coli* (*E. coli*). The characteristics of the facial SC-SNs formed suggest for biomedical application as chemical sensors in the future.

INTRODUCTION

In recent years, an escalating percentage of nano-materials have emerged, providing advancement in diverse fields (Qu, 2016; Ahmad *et al.*, 2010; Sarkar *et al.*, 2010; Lird-prapamongkol *et al.*, 2010; Nune *et al.*, 2009). As nature makes optimum use of materials and space, many inorganic materials are produced in biological systems (Qu, 2016). Similar to such natural processes, plants (Ahmad *et al.*, 2010; Sarkar *et al.*, 2010), fungi (Binupriya *et al.*, 2010), and bacteria (Reddy *et al.*, 2010) are found to be of powerful sources for production of nano-sized metal particles (Sinha *et al.*, 2009). Biomolecules are the sources of reductants that are found to have a potential gain over their protecting group counterparts (Huang *et al.*,

2007). The highest challenge in the nanoparticles study is to get the desired size and shape of the metal nanoparticles using benign starting materials. To achieve these results, scientists made several efforts to develop beneficial and green chemistry compound inspection protocols for the synthesis of nanomaterials with tailor-made structural properties.

Currently there is a great need to derive an environmental friendly nanoparticle synthesis process that does not involve any toxic chemicals. However polymeric metal nanoparticles are synthesized by reducing agents such as hydrazine, 1, 2-hexadecanediol, NaBH₄ and ascorbic acid (Rao *et al.*, 2012; Praveena *et al.*, 2014; Rao *et al.*, 2010; Deepali *et al.*, 2015; Bilal *et al.*, 2017) for reduction of silver ions to SNs (Ag⁺ to Ag⁰), which are extremely toxic in nature. Also, natural plant extracts are used extensively as reducing agents as well as capping agents to inhibit the agglomeration and stability (Kharissova *et al.*, 2013; Malar-kodi *et al.*, 2014; Sharma *et al.*, 2009) of the synthesized nanoparticles. Interestingly the noble metallic nanomaterials are receiving

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more interest in recent research, due to their interesting properties exhibiting wholly innovative and improved characteristic properties compared to the voluminous particles of the bulk materials with specific characteristics, such as unique size distribution and required morphology (Ankanna *et al.*, 2010; Mahdieha *et al.*, 2012).

The potentiality of a plant system in biologically assisted synthesis of metal nanoparticles called “green synthesis” and has now achieved a great role in the field of nano science and technology (Jha *et al.*, 2009). Green synthesis is a chemistry which helps in the design, development and implementation of chemical products; also it facilitates in reduction or elimination of the substances that are hazardous to human health and the environment (Kumar *et al.*, 2008). Recently, there is a renewed interest in using a green chemistry approach to synthesize metal nanoparticles (Geethalakshmi *et al.*, 2010; Christensen *et al.*, 2011; Arokiyaraj *et al.*, 2014; Gandhi *et al.*, 2014). Sastry and coworkers (Ahmad *et al.*, 2003; Shankar *et al.*, 2004) employed an *in silico* approach to investigate the synthesis of stable Ag, Au and Ag-Au bimetallic nanoparticles using *Azadirachta indica* leaf broth and suggested that the flavanone and terpenoids are prime in responsibility for the stabilization of nanoparticles. Recent reports also suggested biosynthesis of Ag and Au nanoparticles from apiin (Kasthuri *et al.*, 2009), *Cinnamon zeylanicum* bark extract (Sathishkumar *et al.*, 2009), mushroom extract (Philip, 2009), *Acalypha indica* leaf extracts (Krishnaraj *et al.*, 2010), glucose and starch (Raveendran *et al.*, 2006). The biosynthesized nanoparticles such as Au, Ag, and Pt are widely used in medical and pharmaceutical applications (Singh *et al.*, 2013; Galdiero *et al.*, 2011) for catalysis (Burda *et al.*, 2005), anti-bacterial, antiviral agents (Singh *et al.*, 2013) and sensor applications (Nirlipta *et al.*, 2016).

Syzygium cumini (SC) is distributed on the Indian sub-continent, and in many others regions of South Asia and it is traditionally used ethno-medicinal plant of Myrtaceae family. The stem bark of SC is rich in many phyto-chemicals such as betulinic acid, friedelin, epi-friedelinol, β -sitosterol, eugenin and fatty acid ester of epi-friedelinol, β -sitosterol, quercetin kaempferol, myricetin, gallic acid and ellagic acid, bergensins, flavonoids and tannins (Ayyanar *et al.*, 2012). It is used as folk medicine. Also, the Ayurvedic Pharmacopoeia of India recommends the SC bark for diseases like acute diarrhea and haemorrhagic (<https://sites.google.com/site/blackberries7890/>). In addition to the above, SC bark is commonly used as a raw medicine for the treatment of sore throat, bronchitis, asthma, thirst, biliousness, dysentery and ulcers and it has a nature that is acrid, sweet, digestive, astringent to the bowels, anthelmintic (Kumar *et al.*, 2015).

In this paper, we report a simple biosynthesis of Ag metallic nanoparticles by the reduction of aqueous Ag^+ ions through a green approach using the bark extract of SC at room temperature. Due to the nutritional, medicinal and environmental properties (Prasad *et al.*, 2014; Rao *et al.*, 2015; Prabhakaran *et al.*, 2011; Sharma *et al.*, 2009) and also bioavailability of SC, it is chosen to produce nanoparticles with bark extracts. Rapid biosynthesis of nanoparticles using SC stem bark extract has been investigated. It is the easiest, most cost efficient, non-toxic, eco-friendly and efficient method for exploiting SC stem barks. So the bark of SC has advantages over the other reducing agents used in synthesis of metal nanoparticles. To the best of author's knowledge there

have been no reports on the biosynthesis of nano silver from the aqueous extracts of the SC bark, also their spectro-chemical and anti-bacterial studies.

MATERIALS AND METHODS

Materials

Silver nitrate (AgNO_3 ; 99.9%, Sigma Aldrich Chemie, Germany). *Syzygium cumini* (SC) stem bark was collected from the premises of Yogi Vemana University, Kadapa, India. All other chemicals used are analytical grade and received from Merck, India. Throughout the experiment double distilled water was used.

Extraction procedure

Aqueous *Syzygium cumini* stem bark extract was prepared by a green process technique, using the standard procedure described by Sekhar *et al.* (Sekhar *et al.*, 2016). Collected SC stem bark was washed several times with double distilled water to remove the dust and foreign particles on the surface of bark. 20 gms of bark samples were finely chopped and boiled in 100 mL double distilled water in 250 mL conical flask for 30 min. The SC extract was filtered with Whatmann grade No. 1 filter paper. The extract was centrifuged at 7500 rpm to remove the heavy molecular weight constituents. The pH of the SC aqueous extract was found to be 7.5. The collected extract was preserved at 4°C for further experimental analysis.

Biosynthesis of silver nanoparticles (SNs)

5 mL of aqueous SC stem bark extract was added to 100 mL of 1×10^{-3} aqueous solution of AgNO_3 at room temperature. The color change from pale yellow to dark brown was observed within 30 minutes, indicating the formation of SNs. UV-Visible spectra showed the plasmon resonance peak at around 420 nm.

Analytical characterization

To confirm the formation of SNs in the extract, absorption studies of developed SNs were carried out on a UV-visible spectrophotometer (LAB INDIA, UV-3092) for well-dispersed extract SNs solution in the wavelength range 200-800 nm. Fourier transforms infrared spectroscopy (FTIR) is only one of the superlative analytical tools, which provides the possibility to identify the functional groups in the aqueous bark extract and produced SNs. The IR spectra of the SC-SNs were measured using a FTIR spectrophotometer (Perkin Elmer Spectrum Two, UK). Mean diameter of the SNs was determined by a dynamic light scattering (DLS) method using a Brookhaven model BI-9000 AT (Brookhaven Instruments Corporation, USA). To measure the transmission electron microscopy (TEM) (HR-TEM, JEOL JEM-2010, accelerating voltage of 200 kV) samples of the green-synthesized SNs were prepared by placing a drop of the SN solution on carbon-coated copper grids and allowed the grid for dry prior to measurement.

Antibacterial activity

An antimicrobial activity of SNs was performed by Bauer-Kirby's disc diffusion method (Rahman *et al.*, 2014). The Muller Hinton Agar (M173Himedia, India) media was sterilized at 121°C and 15 lbs for 15 min in an autoclave. The cultured

plates were prepared with a depth of about 4 mm. The cultures were transferred to the center of an agar plate independently and homogeneously speeded the culture on surface of the plate with a sterile bent-glass rod (purveyor). 10 μ L of pure *Syzygium cumini* stem bark extract along with biosynthesized SNs solution and 1×10^{-3} M AgNO_3 were impregnated onto filter paper discs of \sim 5 mm diameter (which were prepared using Whatmann grade No. 1 filter paper) under aseptic conditions, then placed onto cultured plates using sterile forceps. The plates were then incubated for 24 h at 37°C in an incubation chamber. The antimicrobial activity was evaluated in replicates by quantifying the zone of inhibition (ZoI) for the test organisms viz. *Bacillus subtilis* and *Escherichia coli* respectively.

RESULTS AND DISCUSSIONS

The bioactive chemical constituents, which are present in *Syzygium cumini* (SC) bark extract and are responsible for biosynthesis SNs, are identified in this study. Reduction of Ag nanoparticles during exposure to bark extract could be detected by the color change. A color change from pale yellow to brown was observed within 5 min and for dark brown within 30 min. It may be due to the addition of aqueous AgNO_3 solution into SC bark extract, that the Ag^+ ions were attracted by the $-\text{O}^-$ group of biomolecules to form silver complex then after it was reduced to zero valence of silver (Ag^0) by free electrons which were produced in the reduction process. The SNs are produced and stabilized by carboxyl ($-\text{COO}^-$), hydroxyl (OH^-) groups present in the aqueous extract of bark (Pal *et al.*, 2007). This phenomenon is the result of the surface plasmon vibrations of SNs. It is due to the free electrons which are present at SNs. These surface plasmon vibrations of SNs produced a peak at 420 nm, which indicates the reduction of AgNO_3 into SNs. It is a well known fact that the optical properties of the metal nanoparticles are strongly dependent on their shape and size (Man *et al.*, 2007).

UV-visible spectra of band peaks produced by SNs with biosynthesizing bark extract clearly shows that there is no significant peak showing a sign of pure extract as shown in Figure 1C. However, soon after the addition of AgNO_3 solution to the SC bark extract, surface plasmon resonance (SPR) spectra of silver was obtained at 420 nm and increased the time of reaction in the peak wavelength devoid of any changes. Similarly after 100 min the increase in the number and size of the SNs gets completely stopped. This may be due to the depletion of the Ag^+ ions in the SC bark extract (Figure 1A). Interestingly Figure 1B and Figure 1C clearly depict the optimized concentrations of SC bark extract and volume of AgNO_3 solution for enhanced production of SNs. Based on the above data 500 μ L of SC bark extract would require 1.5 μ L of AgNO_3 solution, to reduce 1.5 μ L of aqueous AgNO_3 solution of Ag^+ complex. It is interesting to note that higher intensity peaks are obtained due to the depletion of silver ions in the extract. However variations in both AgNO_3 and SC bark extract confirmed that formation of SNs with the optimized concentrations showed superior plasmon resonance absorbance at 420 nm.

Kinetic studies of formation of SNs form SC bark extract

A kinetic study of the reduction process shows (Figure 1A) that the synthesis of SNs starts within a 30 sec and reaches the flat terrain after 30 min. In the initial 30 sec there is a gradual increase in absorbance followed by a slow increase in the

absorbance values which finally, become linear after 30 min thus suggesting very fast reaction kinetics of SNs synthesis from SC bark extract. A control solution with the same compositions was wrapped with aluminum foil and kept in a dark place for 2 weeks. The control did not show much color formation, suggesting that light plays a vital role in the biosynthesis of SNs. Similar results were observed in photochemical synthesis of SNs in the presence $-\text{NH}_2$ and $-\text{COO}^-$ terminated PAMAM dendrimers by keki *et al.*, (keki *et al.*, 2000).

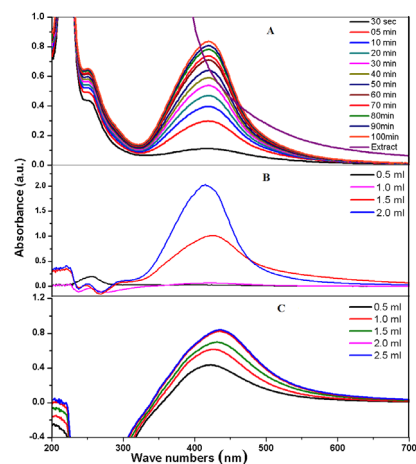


Fig. 1: UV-visible absorption spectra of SNs at different time intervals. (Visual observations and UV-visible Spectral Studies). A: Time variation, B: volume of AgNO_3 variation and C: volume of PE variation.

In the present study, it is proposed that after the addition of aqueous AgNO_3 into SC bark extract, the Ag^+ ions were attracted by the $-\text{O}^-$ group of biomolecules. The SNs thus formed are stabilized by $-\text{COO}^-$ groups present in the phytochemicals. The exposure of the reaction mixture to sunlight enhanced the speed in transfer of electrons in the reduction process i.e. the transfer of electrons from $-\text{O}^-$ to Ag^+ ion resulting in the complete reduction of Ag^+ ions to Ag^0 . A plot is drawn between the $\log(A_t - A_\infty)/(A_0 - A_\infty)$ vs. time t and a slope obtained as a pseudo first order rate constant (k_{obs}), the same is fitted in to the equation $A_t = A_\infty + (A_0 - A_\infty)\exp(-k_{\text{obs}}t)$, where A_0 and A_∞ are the initial and final absorbance of SNs respectively (Nadagouda *et al.*, 2008). The k_{obs} was found to be $4.79 \times 10^{-2} \text{ min}^{-1}$ with $R = 0.9908$ (Figure 2).

Dilute HCl and NaOH solutions were used to adjust the pH of reaction mixture of SC bark extract and AgNO_3 . The SNs did not form properly at acidic pH (pH-3.0) and increased with increasing of pH. However SNs were rapidly formed at basic pH (pH-9.0) rather than the acidic pH (Figure 3). Ag^+ ions more effectively interact with phytochemicals which are present in the bark extract at higher pH conditions ($>$ pH-7). At lower acidic pH the SPR band intensity and wavelength were low indicating the SNs are smaller in size and give a lesser yield. The basic pH-9.0 was the optimized pH for the production of SNs with SC bark extract at room temperature. SNs were showed a sharp SPR band with a wavelength (λ_{max}) 420 nm at basic pH-9.0 and thus formed more extensively. Finally, the results indicate that the pH-9.0 induces more nucleation and increasing growth of SNs, which is attributed to the activation of phytochemicals present in the SC bark extract involved in formation of SNs in the basic conditions.

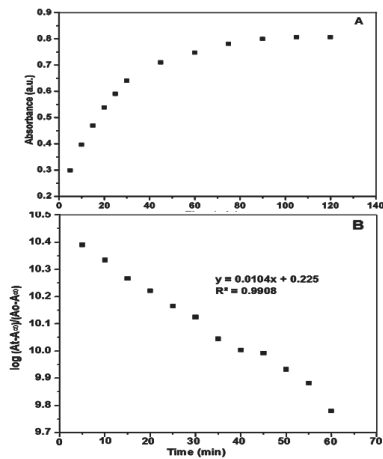


Fig. 2: Kinetic study of biosynthesized SNs.

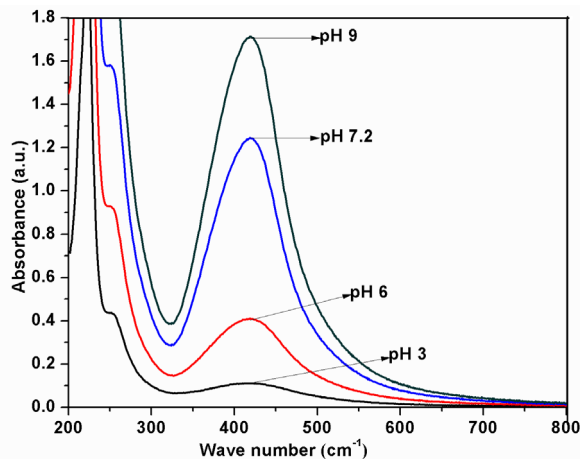


Fig. 3: UV-visible absorption spectra of SNs at different pH values.

FTIR analysis

Figure (4) shows that the FTIR analysis of bark aqueous SC bark extract was carried out to identify possible functional groups which may be attributed to the process of bio-reduction of (Ag^+) silver ions to silver nanoparticles (Ag^0). The analysis of SNs displayed several absorption peaks at 2931, 1519, and 1043 cm^{-1} in Figure (4.B). The peak shift of 3389 to 3415 cm^{-1} showed that the -OH stretching groups of a phenolic compound may be involved in biosynthesis of SNs. The absorption band at 2931 cm^{-1} indicated that $-\text{CH}_2-$ and $-\text{CH}_3$ symmetrical vibration of aliphatic hydrocarbons and the bands at 1727 cm^{-1} could be assigned to the $-\text{C}=\text{O}$ stretching of aliphatic esters and an intense band at 1043 cm^{-1} indicates the $-\text{C}-\text{N}-$ stretching vibrations of aliphatic amines. Figure (4.A) depicts a peak at around 1520 cm^{-1} which is N-O symmetrical stretching of $-\text{C}=\text{C}-\text{NO}_2$, that was observed whereas a total disappearance of this significant band in Figure (4.B) clearly shows that the functional groups are mainly involved in the bio-reduction of silver ions and formation of SNs in the green synthesis. The fingerprint information evidenced that the carbonyl groups were bound to silver ions and could act as capping agents to prevent the agglomeration and provides the stability to the nanoparticles.

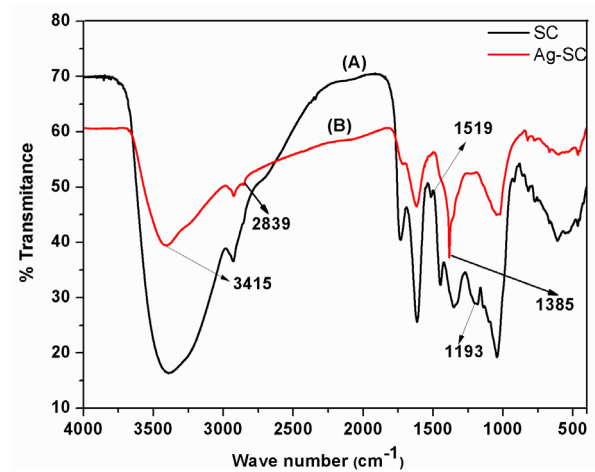


Fig. 4: FTIR spectra of (A) bark extract and (B) SNs.

The hydroxyl groups of phenolic compounds are present in the aqueous SC bark extract of SC chelates Ag^+ ions forming as ring (Nadagouda *et al.*, 2008). It is also interesting to note that the chelated groups were oxidized to quinones with the reduction of Ag^+ to Ag^0 , as a result of excitation of free electrons present in the phenolic compounds (Scheme I). The crash of the neighboring Ag^+ ion leads to the formation of SNs (Husen *et al.*, 2014). Hydroxyl and carboxyl groups present in the plant extract bind to metals like silver, aurum, platinum, iron (Zhu *et al.*, 2008; Link *et al.*, 2000; Sekhar *et al.*, 2016) etc. These hydroxyl and carboxyl groups inactivate the metal ions by chelating. When metal salts come in contact with the polyphenols present in the SC bark extract the phenolic hydroxyl and ester oxygen atoms form a conjugation effect. The esterification of the carboxyl and hydroxyl group of polyphenol makes the phenolic hydroxyl lose the hydrogen atoms easily forming a semi-Quinone structure. Thus scheme I explains that the quercetin has an easy electron losing capacity which results in the formation of hydrogen radicals, which produced the nanosized Ag particles.

Particle size distribution analysis of biosynthesized SNs

Particle size experiment of SNs was carried out by means of dynamic light scattering. Particle size ascertainment of the produced nanoparticles was shown under heading like size distribution by intensity. The size of SNs dispersed was drifted widely from 10 nm to 20 nm (Figure (5)), the average particle size is expected to be in the range of ~ 14 nm and the peaks width was found to be 2.5. Allocation of particle size by magnitude gives a spherical shaped group. In an earlier study, SNs size distributions were obtained in the range of 18.03 nm to 148.7 nm by using *Saururus chinensis* leaf extract (Morones *et al.*, 2005).

Transmission electron microscopic (TEM) analysis

TEM images are apprehended with a dry sample; before analysis of SNs using TEM and dynamic light scattering (DLS) was carried out to dictate the size of SNs in an aqueous solution using DLS. Size of SNs, size distribution, morphology of nano sized particles and surface morphology in solution are important factors in evaluating the biosynthesized SNs toxicity (Kreibig, 1995). Synthesized SNs showed with mean diameter of ~ 14 nm

(Figure 5), which accurately emulates TEM probations with different magnifications (Figure 6). TEM image reveals that the particles are in smaller size distribution, monodispersity, and are spherical in shape with an average size of ~15 nm.

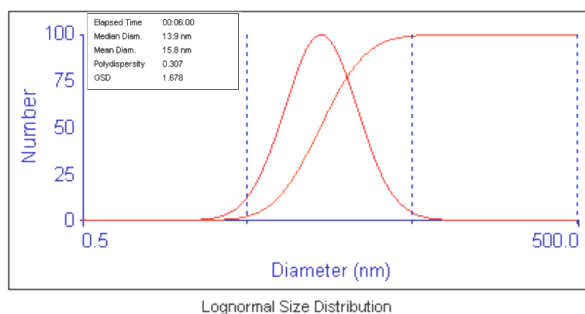


Fig. 5: Size distribution analysis of biosynthesized SNs.

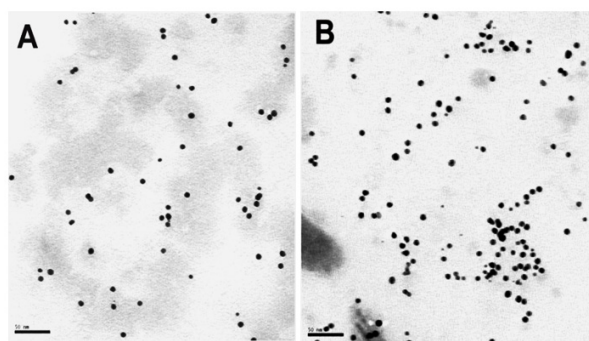


Fig. 6: TEM images of biosynthesized SNs.

Antimicrobial activity of SC-SNs

Among the noble metals (e.g., Ag, Pt, Au and Pd), silver (Ag) exhibits potential applications in the fields of biological systems and medicine (Mulvaney *et al.*, 1996). Metallic nanoparticles were prepared by biosynthesis of plant extracts with metal ions; surrounded by protein and metabolites having different functional groups of amines, alcohols, aldehydes and carboxylic acids (Dhermendra *et al.*, 2008). The SPR absorption peak plays a key role in the determination of optical absorption spectra of metal nanoparticles that shift to a longer wavelength with an increase in particle size (Rao *et al.*, 2017). The size of the SNs implies the possession of a large surface area to come in to contact with the bacterial cells, therefore it may have more interaction when compared to larger particles (Pal *et al.*, 2007; Rao *et al.*, 2015; Reddy *et al.*, 2014; Rao *et al.*, 2012; Mahitha *et al.*, 2013). The nanoparticles having a size less than 10 nm, interface with bacteria and produce electronic effects, which may apparently enhance the reactivity of SNs. Consequently, it may be corroborated that the bactericidal effect of SNs is size dependent (Ruparelia *et al.*, 2008; Azam *et al.*, 2009). The antimicrobial efficacy of the nanoparticles also depends on the shapes of nanoparticles. This attribute can be further confirmed by studying the inhibition of bacterial growth by using different shaped nanoparticles (Mahitha *et al.*, 2013). According to Pal *et al.*, (Pal *et al.*, 2007) the smaller size nanoparticles showed bacterial inhibition with silver ion concentration 1×10^{-3} M.

This indicates that, the SNs with different shapes have different effects on bacterial cells.

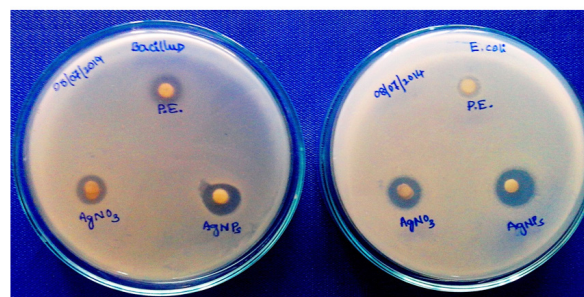


Fig. 7: Photographs showing bacterial colonies in Petri plates (A) and Quantification of the zone of inhibition (B) containing pure bark extract (P.E.), AgNO₃ (concentrations of 1×10^{-3} M), and biosynthesized SNs (SNs).

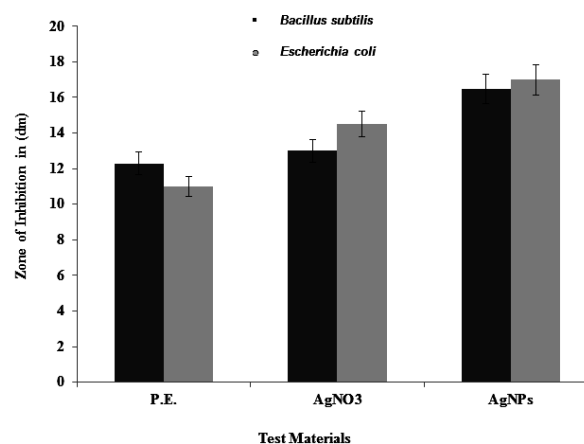


Fig. 8: Statistical analysis of antibacterial activity data of PE, AgNO₃ and biosynthesized SC-SNs.

In the present study, plant extract (P.E., SC aqueous stem bark extract); P.E. mediated SNs and pristine AgNO₃ were tested against *Escherichia coli*, *Bacillus subtilis*. The zone of inhibition (ZOI) in diameter was discerned by disk diffusion method and the results showed visible and clear zones (Figure 7A) for disks loaded with aqueous solutions of P.E.; pristine AgNO₃ and SNs colloids (each sample of 15 μ L). SNs exhibit effective ZOI against *Escherichia coli* species (17 mm) and moderate inhibition against *Bacillus subtilis* (16.5 mm) (Figure 7B). However, SNs showed better activities compared to pristine AgNO₃ and P.E. These zones were developed against both gram positive (*Bacillus subtilis*) and gram negative (*Escherichia coli*); it was interestingly note that *Escherichia coli* with thin cell wall are more susceptible to cell wall damage than *Bacillus subtilis* with thick cell wall by SNs (Ruparelia *et al.*, 2008). Similarly bacterial cell wall damage depends upon the strain of microorganisms used (Matela *et al.*, 2013). So, in the present case, *Escherichia coli* showed a better activity than *Bacillus subtilis* towards green synthesized SNs. ZOI assessment of antibacterial activity showed that the biosynthesized SNs have expansive antibacterial activity compared to plain plant extract (PE) and aqueous AgNO₃. The statistical analysis data is described in Figure 8 and it gives the variation of ZOI in

both *Escherichia coli*, *Bacillus subtilis*. The antibacterial activity is in all likelihood derived through the electrostatic attentiveness between negative charged cell membrane of microorganism and positive charged NPs (Dhermendra *et al.*, 2008; Matela *et al.*, 2013). The antibacterial potency of the biogenic SNs reported in the present retrospection may be attributed to the mechanism described above.

CONCLUSION

The present study demonstrates bio-reductive synthesis of nano sized silver particles using *Syzygium cumini* (SC) bark extract. The SC stem bark aqueous extracts appear to be environmentally friendly, so that this procedure could be used for rapid production of silver nanoparticles. Silver nanoparticles were synthesized by the green approach reported in this study using SC stem bark extracts. SC extract could act not only reducing the Ag ions but also controlling the size i.e. ~14 nm. Further the SNs formation conformed by UV-Visible, FTIR, DLS and TEM studies. In the future, the selection of such medicinal plants may create a new platform for recognizing the probable of natural medicines in nanoscience for biomedical applications. The antibacterial activity of SNs suspension showed enhancement of the activity towards *Escherichia coli* and *Bacillus subtilis*. Hence the developed procedure has several advantages such as controlling the size of metal nanoparticles being, eco-friendly and cost effective.

CONFLICT OF INTEREST

Nil.

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