Green synthesis of silver nanoparticles using *Ocimum sanctum* and its antibacterial activity against multidrug resistant *Acinetobacter baumannii* (#76623)

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Green synthesis of silver nanoparticles using *Ocimum sanctum* and its antibacterial activity against multidrug resistant *Acinetobacter baumannii*

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The biosynthesis of nanoparticles employing the green route is an effective strategy in nanotechnology that provides a cost effective and environmentally friendly relation to physical and chemical methods**.** This study aims to prepare an aqueous extract of *Ocimum sanctum* based silver nanoparticles **(**AgNPs**)** through green route and test its antibacterial activity**.** The biosynthesized silver nanoparticles were characterized by color change, UV spectrometric analysis, FTIR and particle shape and size morphology by SEM and TEM images**.** The nanoparticles are almost spherical to oval and rod shape with smooth surfaces and with a mean particle size in the range of 55 nm with a zeta potential of-2.7 mV. The antibacterial activities of AgNPs evaluated against clinically isolated multidrug resistant *Acinetobacter baumannii* showed that the AgNPs from *O. sanctum* are effective in inhibiting *A. baumannii* with MIC and MBC of 32 and 64µg/mL and SEM images of *A. baumannii* treated with AgNPs revealed damage and rupture in bacterial cells. The time killing assay by spectrophotometry revealed the time and dose dependent killing action of

AgNPs against *A. baumannii* and the assay at various concentrations and time interval indicate a statistically significant result in comparison with the positive control colistin at 2µg/mL($P<0.05$). The cytotoxicity test using MTT assay protocol showed the prepared nanoparticles of *O. sanctum* is less toxic against human cell A549. This study opens up a ray of hope to explore the further research in this area and to improve the antimicrobial activities against multidrug resistant bacteria.

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Abstract

The biosynthesis of nanoparticles employing the green route is an effective strategy in nanotechnology that provides a cost effective and environmentally friendly relation to physical and chemical methods. This study aims to prepare an aqueous extract of *Ocimum sanctum* based silver nanoparticles (AgNPs) through green route and test its antibacterial activity. The biosynthesized silver nanoparticles were characterized by color change, UV spectrometric analysis, FTIR and particle shape and size morphology by SEM and TEM images. The nanoparticles are almost spherical to oval and rod shape with smooth surfaces and with a mean particle size in the range of 55 nm with a zeta potential of -2.7 mV. The antibacterial activities of AgNPs evaluated against clinically isolated multidrug resistant *Acinetobacter baumannii* showed that the AgNPs from *O. sanctum* are effective in inhibiting *A. baumannii* with MIC and MBC of 32 and 64µg/mL and SEM images of *A. baumannii* treated with AgNPs revealed damage and rupture in bacterial cells. The time killing assay by spectrophotometry revealed the time and dose dependent killing action of AgNPs against *A. baumannii* and the assay at various concentrations and time interval indicate a statistically significant result in comparison with the positive control colistin at 2µg/mL ($P<0.05$). The cytotoxicity test using MTT assay protocol showed the prepared nanoparticles of *O. sanctum* is less toxic against human cell A549. This study opens up a ray of hope to explore the further research in this area and to improve the antimicrobial activities against multidrug resistant bacteria.

Subjects: Bacteriology, Pharmacology, Nanotechnology

Key words: Antibacterial activity, biosynthesis, green nanotechnology, *Ocimum sanctum*, silver nanoparticles.

INTRODUCTION

The synthesis of nanoparticles through green route is a growing subject in nanotechnology that offers cost-effective and environmental friendly alternatives to traditional physical and chemical processes (*Yeo, Lee & Jeong, 2003*). Although, metal-based nanoparticles are the most promising emerging formulation designs, silver nanoparticles are outstanding owing to their all-around pharmacokinetic profiles, no human toxicities and specific antimicrobial properties (*Allen, Hunter & Agrawal, 1997; AshaRani, Hande & Valiyaveettil, 2009*). It is of paramount interest to the pharmaceutical manufacturers that overall process of producing nanoparticles systems is ecologically balanced while being cost-optimized (*Gadeet al., 2008; Ouda,2014*). Both physical and chemical methods have their own disadvantages in terms of energy consumptions and toxicities related to chemical processing (*Singh& Raja,2011; Wei Xet al.,2012*). Contrary to the traditional synthetic methods, biological methods of generating nanoparticles are quite adaptive to the environment vis-à-vis cost effective (*Govindaraju et al., 2010*). The most important merit of biologically synthesized nanoparticles is their non-toxic nature and easy biological metabolism. These advantages have made biologically-derived nanoparticles one of the most emerging formulation designs widely accepted in the pharmaceutical eco-system (*Das & Smita, 2018*).

In recent years, plants have been widely explored for finding active principles to treat complex ailments. Novel phytoconstituents derived from plant sources are spanning again around the pharmaceutical markets and one the mostly employed medicinal plant is the Holy Basil i.e., *Ocimum sanctum* L. with proved medicinal significance for anticancer, antimicrobial, cardio-protective, antidiabetic, analgesic, antispasmodic, antiemetic, hepatoprotective, antifertility, adaptogenic and diaphoretic actions. Leaves of the *Ocimum sanctum* L. contain eugenol as a major active chemical constituent and has been proved for its therapeutic efficacy in various ailments in modern clinical practice (*Hemaiswarya, Kruthiventi & Doble, 2008; Raseetha, Cheng & Chuah, 2009*). There are various studies done on synthesis of nanoparticles through green route using parts of plant extracts such as tea leaf, stem bark of *Callicarpa maingayi*, *Terminalia chebula*, *Papaver somniferum* and *Aloe vera*. Silver nanoparticles have been reported for anti-angiogenesis, anti-inflammatory, anti-platelet activity, anti-bacterial and anti-viral activity (*Bindhani & Panigrahi, 2015*).

Misuse of antimicrobials during last two decades increases the existence of antibiotic resistance in almost all the bacterial strains. This has not only made several anti-microbial drugs worthless but it has also compelled the researchers to explore alternative solutions for fighting against deadly microbial infections (*Nikaido, 2009*). Hence, some recent studies focused upon using silver nanoparticles (AgNPs) as one of the alternative and proven the antimicrobial property of silver nanoparticles against both Gram negative and positive bacteria without any cytotoxic signs (*Donlan & Costerton, 2002; Biel et al., 2011; Lazar, 2011*). *Acinetobacter baumannii* is a Gram negative, opportunistic bacterium which causes nearly 2-10% of all hospital associated infections, particularly among immunocompromised patients (*Karlowskyet al., 2003*). The major challenges

with *A. baumannii* is its extraordinary ability to quickly develop resistance against new drugs, to form biofilm on abiotic surfaces which helps them to survive on hospital equipment for long period and also to tolerate the harsh environment for survival (*Djeribi, 2012*). *A. baumannii* is considered as "Red Alert" human pathogen and is ranked number one critical pathogen with high antibiotic resistant by World Health Organization for research and new drug discovery (*WHO, 2017*).

This study describes the easy, fast and simple method for biosynthesis of AgNPs from *Ocimum sanctum* (*O. sanctum*) leaf extract. We attempted to characterize the biosynthesized nanoparticles and also evaluated the antibacterial activity against multidrug resistant *A. baumannii* (MDR-*A. baumannii*).

MATERIAL AND METHODS

O. sanctum leaves were collected from Delhi Pharmaceutical Sciences and Research University, New Delhi (Verified CSIR-National Institute of Science Communication and Policy Research, New Delhi, authentication no. NIScPR/RHMD/consult/2022/4040-41). Silver nitrate was purchased from LobaChemie Private Limited, Mumbai, India.Antimicrobial susceptibility test against multidrug resistant *A. baumannii* was performed at Sikkim Manipal Institute of Medical Sciences, Sikkim, India (EhticalClearenceNo. SMIMS/IEC/2019-29).

Plant collection and identification

O. sanctum is a relatively small, erect sub shrub that reaches up to 60 cm in height and has reverse green or purple leaves and a hairy stem. The leaves are ovate, measuring up to 5 cm long, toothed and have a petiole (*Pattanayak et al., 2010*). For the present study, fresh leaves were collected in September, 2021 and brought to the laboratory at Delhi Pharmaceutical Science and Research University, New Delhi, in air tight paper bags for further processing.

Preparation of *O. sanctum* leaves aqueous Extract

To prepare the aqueous extract of leaves, fresh leaves were collected and placed in a beaker and washed with distilled water many times to make it free from dust and finally washed with millipore water (MILLI-Q® HX 7000 SD, Merck**,** Australia). A total of 25g washed leaves were chopped into fine pieces and crushed in 100 mL millipore water using a mortar and pestle. The aqueous extract was ground and then boiled for 10 minutes at 80°C in a 250 mL beaker. The aqueous leave extract was then allowed to cool at room temperature (37°C) and then filtered with Whatman filter paper (GE Healthcare Life Science, Karnataka, India). The prepared leave extract was collected and stored at 4ºC for further use (*Rao et al., 2013*).

Preparation of 1 mM silver nitrate solution

The stock solution was prepared by weighing 170 mg of silver nitrate (LobaChemie Pvt. Ltd. Mumbai, India) and dissolved it into 1000 mL of millipore water (MILLI-Q® HX 7000 SD, Merck, Australia). 1ml solution was taken and further dissolved into 100 mL millipore water. This solution

was stored in amber coloured bottle to prevent the self-oxidation of silver nitrate solution (*Saifuddin, Wong & Yasumira, 2009*).

Green synthesis of silver nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) were prepared by a single step synthesis reported previously (*Ramteke et al., 2013*). In the process 90 mL solution of silver nitrate at 1 mM concentration was placed on a magnetic stirrer (Remi, Mumbai, India) at 400 rpm and 10 mL of aqueous extract of *O. sanctum* leaves was added drop-wise for half an hour in a beaker containing silver nitrate solution. The colour of the solution was turned from brown to hazy brown (Figure 1 A-D) indicating the formation of AgNPs. The preparation was kept at rest at room temperature (37°C) for 1 hour. All procedures were performed in a dark room due to presence of silver nitrate. The nanoparticles were separated by the process of centrifugation (Remi, Mumbai, India) and prepared sample was stored at refrigerated temperature.

Characterization of the synthesized AgNPs of *O. sanctum*

The biosynthesized AgNPs were characterized by various parameters (Figure 2). Absorption spectrum of the synthesized AgNPs was observed spectrophotometrically at room temperature using UV–Vis spectrophotometer (Shimadzu UV, 1800, Japan) at a resolution of 1 nm. In addition, the average particle size and zeta potential were determined by dynamic light scattering, using the Litesizer 500 (Anton Paar, Buchs, Switzerland). Furthermore, morphology of AgNPs of *O. Sanctum* were visualized using by Scanning Electron Microscopy (SEM, Leo 435 VP 501B, Philips, Austin,

Texas), its accelerated voltage is up to 30 kV and magnification efficacy ranges from 10x to 300,000x. Following this, prepared nanoparticles were also characterized by Transmission Electron Microscopy (TEM; JEOL, Tokyo, Japan) using a copper grid coated with carbon film and with phosphotungstic acid (1%; w/v) as a negative stain and then air dried, and allowed to rest at room temperature (37°C) to obtain the TEM images.

The samples were then dried, ground with KBr pellets, and examined by Fourier transform infrared spectroscopy (FTIR; PerkinElmer, United States) to recognize the functional groups and potential of bio-molecule probably causing the reduction of silver (Ag) ions and capping of AgNPs biosynthesized by *O. sanctum*.

Antibacterial studies of the biosynthesized AgNPs of *O. sanctum*

Bacterial broth preparation

Pure culture of MDR *Acinetobacter baumannii,* confirmed by RT-PCR was sub-cultured in a Muller Hinton broth medium (Hi-Media, Mumbai, India) at 37°C for 18-24 hours. The bacterial broth was diluted next day using Muller Hinton broth and adjusted to 0.5 MacFarland turbidity (10^8 CFU/mL) using Densicheck (Biomerieux, North Carolina, USA). This bacterial broth was further tested for susceptibility to AgNPs of *O. sanctum* by different methods (Figure 3).

Agar well diffusion method

The antimicrobial susceptibility was tested on a Muller Hinton Agar (MHA) plate (Hi Media, Mumbai, India). The 0.5 MacFarland turbid broth of *A. baumannii* was inoculated by the lawn

culture method using sterile cotton swab on a MHA plate and the plate was air dried. Three holes of 6 mm diameter were made in the plate with the help of a sterile borer. A volume of 100 µL of AgNPs of *O. sanctum*, aqueous extract of *O. sanctum* and sterile double distilled water were added to the respective holes. The plate was then incubated at 37°C for 18-24 hours. The zone of inhibition was measured with the help of scale, the next day against the respective holes (*Alzahrani et al., 2020*).

MIC and MBC determination

MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) were determined by the microdilution method (*Dash et al., 2012*). The *A. baumannii* having a concentration of 10^8 CFU/mL was prepared in Nutrient broth medium (Hi-media, Mumbai, India) and 100 µL of this broth was added to the wells of microtitre plate. The AgNPs of *O. sanctum* were diluted in deionized water by serial dilution ranging from 2-200 µg/mL and 100 µL of these different concentrations of AgNPs of *O. sanctum* were added to wells loaded with the bacterial broth. The microtitre plate was then incubated at 37°C for 24 hours. The MIC value was noted by observing the turbidity on microtitre wells due to the bacterial growth. The MIC value corresponded to the minimum concentration of AgNPs of *O. sanctum* that inhibited the 99% of bacterial growth.

The MBC was obtained by sub-culturing the bacteria on a sterile MHA plate from the microtitre wells without turbidity and incubated at 37°C for 24 hours. The minimum concentration of AgNPs

205 which completely killed and reflected no growth of bacteria on the MHA plate was considered as
206 a MBC value. The MBC value corresponded to the minimum concentration of AgNPs of *O.*
207 *sanctum* that restricted 100% bacterial growth.

208

209 **The killing kinetic assay**

210 The killing kinetic assay of *A. baumannii* against AgNPs was performed spectrophotometrically
211 (Shimadzu UV, 1800, Japan) at OD 600nm. A volume of 100 µL of 10^8 CFU/mL of bacterial broth
212 after treatment with the 100 µL of 32 µg/mL of MIC, 64µg/mL of MBC, 128µg/mL, 256µg/mL
213 and512µg/mL of AgNPs of *O. sanctum* were measured by quantifying the bacterial viability at 0,
214 2, 4, 8, 12, 18 and 24 hours of incubation. The negative control (bacterial cell without AgNPs and
215 antibiotic colistin) and positive control (bacterial cell treated with antibiotic colistin at MIC of 2
216 µg/mL) were included in the test. The percentage of inhibition of growth was calculated in
217 comparison with the negative control (*Das et al., 2017*) and statistical correlation was made with
218 positive control.

219

220 **Action of Silver nanoparticles on the structures of bacterial cells**

221 The 10 mL volume of *A. baumannii* in nutrient broth medium with a concentration of 10^8 CFU/mL
222 were treated with MIC value of AgNPs and incubated at 37°C with shaking at 198 rpm for 12
223 hours. A control experiment was performed in absence of AgNPs. After incubating for 12 hours the
224 bacterial culture tube was centrifuged and the supernantant was discarded.. The pellets formed were
225 fixed with 50 µL of 2.5% glutaraldehyde for 5 minutes at 37°C and washed three times with 1X

PBS. The pellets were finally suspended in a 50 µL PBS and used to take images by scanning electron microscopy (SEM, Leo 435 VP 501B, Philips, Austin, Texas) (*Das et al., 2017*).

Cytotoxicity test using MTT assay

Human lung adenocarcinoma cell line A549 was obtained from the NCCS cell repository (Pune, India). A549 cells were seeded in 96-well tissue culture plate at a density of 5000 cells per well. After 24 hours of growth, cells were treated for 24 and 72 hours with different concentrations of AgNPs. After treatment, the media with nanoparticles was discarded and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] at a final concentration of 0.5 mg/mL was added to each well. The plates were then incubated for two hours at 37°C in a CO_2incubator. After incubation, media with MTT was discarded and the formazan crystals formed were dissolved in DMSO at 37°C for 15-20 minutes. Absorbance of dissolved formazan was measured at 570 nm with a reference wavelength of 690 nm. Similarly, the test was also performed with *O. sanctum* extract. The control experiment was performed without AgNPs or *O. sanctum* extract. The resultant absorbance which is directly proportional to cell viability was converted into percent viability and viability of control cells was considered as 100%.

Statistical analysis

All data were recorded, edited, and entered using the statistical package software SPSS version 25 (SPSS, Chicago, IL, USA). The differences between mean values were tested for significance by one-way ANOVA analysis. P value < 0.05 was considered to be statistically significant.

RESULT

Color Change

The change in color of the prepared solution during the procedure is shown in Figure. 1, indicating the reduction of silver ions. However, the color of fresh *O. sanctum* extract changed from brown to hazy brown, when it was mixed with silver nitrate solution, after an hour the solution appears dense brown, it represents the reduction of silver ions (*Banerjee et al., 2014*).

UV-Visible (UV-Vis) Spectroscopy

To examine the optical properties of the synthesized AgNPs of *O. sanctum*, the UV-Vis spectroscopy (Shimadzu UV, 18000) measured the sample in every 20 minutes' time interval. It was observed that biosynthesized AgNPs occurs at 452 nm (Fig.4).

Particle shape, size and morphology

SEM images of prepared nanoparticles indicated that particles were, almost spherical and rod shaped with smooth surfaces having a size range of 73.24-87.89 nm. The SEM image shows agglomeration of individual silver nanoparticles (Fig. 5 a and b).

Morphological examination by TEM confirmed spherical shape of most of the nanoparticles with a size range from 29-54.9 nm (Fig. 6 a and b), while some oval and/or elliptical shaped nanoparticles were also formed which is the common feature of most of the biologically synthesized nanoparticles. Lighter edges with heavier center were also visible confirming the capping of protein biomolecules with AgNPs. The mean particle size of nanoparticles was found to be 55 nm, that

268 was fully concordant with the results from TEM and SEM analysis (Fig. 7 a). The particles showed
269 the zeta potential around -2.7 mV respectively and increase in negative values confirmed the
270 repulsion between the particles, which also verified the stability of the formulation (Fig. 7 b).
271
272 **Fourier Transform Infrared Spectroscopy (FTIR)**
273 To determine the potential interaction between *O. sanctum* and biosynthesized silver nanoparticles,
274 FTIR measurements were performed on biosynthesized silver nanoparticles. Figure 8a and 8b,
275 illustrate the FTIR spectra of aqueous extract of *O. sanctum* and the biosynthesized AgNPs,
276 respectively. Strong peaks for aqueous leaf extract at 3338.52cm^{-1}, 1634.59cm^{-1} and 666.49cm^{-1}
277 were clearly visible and biosynthesized nanoparticles showed the peak at 3339.30cm^{-1}
278 1634.70cm^{-1} and 666.89cm^{-1}.
279 Strong bands at 3338.52cm^{-1}and 3339.30cm^{-1} indicated the presence of phenols and alcohols with
280 free OH group. The vibrational peaks at 1634.59cm^{-1} and 1634.70cm^{-1} represent the presence of
281 amide I group. The absorption bands at 666.49cm^{-1} and 666.89cm^{-1} assigned to the aromatic C-H
282 bending.
283
284 **Antibacterial Activity**
285 **Agar well diffusion method, MIC and MBC determination**
286 The zone of inhibition determined by the agar well diffusion method after 24 hours of incubation
287 formed by AgNPs of *O. sanctum* against the MDR *A. baumannii* was 15 mm and no zones were
288 observed against distilled water and *O. sanctum* extract (Figure: 9). The MIC and MBC of AgNPs

289 of *O. sanctum* determined by microdilution method against the MDR *A. baumannii* were 32 µg/mL
290 and 64 µg/mL respectively.
291
292 **Killing Kinetic assay**
293 The bactericidal activity was observed gradually up to 12 hours of incubation with the 64 µg/ml
294 (MBC) and higher concentrations of AgNPs and the complete killing was observed within 24 hours.
295 The result showed a time-dependent and gradual, inhibitory and bactericidal activity against the
296 MDR *A. baumannii*. In comparison to the killing action of positive control (Colistin at 2µg/mL of
297 MIC) the AgNPs at its different concentration and at different time interval shows statistically
298 significant results ($P<0.05$). A 32 µg/mL of AgNPs at 4, 12 and 24 hours, 64 µg/mL at 4, 12 and 24
299 hours, 128 µg/mL at 4 hours, 256 µg/ml at 24 hours and 512 µg/mL at 4 and 12 hours indicate a
300 statistically significant result at P value <0.05 in comparison with positive control colistin to kill
301 the MDR *A. baumannii* (Table 1).
302
303 **Effects of silver nanoparticles on bacterial cells**
304 The electron micrographs by SEM of *A. baumannii* cells in case of untreated and treated with
305 AgNPs were shown in figure 10. The presence of AgNPs in the bacterial cell membrane and its
306 content were observed by electron microscopy. The SEM images of untreated *A. baumannii* showed
307 a typical clear surface structure having smooth and intact cell morphology whereas in case of *A.*
308 *baumannii* treated with AgNPs showed severely damaged cell structure with rupture, gaps,

irregular surface and presence of fragments. The result showed the penetration of AgNPs inside the bacterial cells and kills it by various mechanisms.

Cytotoxicity test using MTT assay

Cytotoxicity against human lung adenocarcinoma cell line A549 at concentrations of 500 µg/mL and 250 µg/mL of both AgNPs and *O. sanctum* extract showed that the cells did not remain viable after 24 hours and 72 hours of treatment. However, at all the other concentrations ranging from 0.97 µg/mL-125 µg/mL the A549 cells showed viability almost equivalent to untreated cells at both the time points (Figure 11).

DISCUSSION

Present study developed a strategy for single step synthesis of AgNPs using aqueous *O. sanctum* extract. The color change was mainly due to addition of extract in silver nitrate solution during the reaction (*Pirtarighat, Ghannadnia & Baghshahi, 2019*). The color intensity increased with respect to time of incubation (*Kumar, Selvi & Govindaraju, 2013; Fayaz et al., 2010*).

It is interesting that the ultraviolet and visible (UV-Vis) absorption spectrum of the prepared mixture confirmed the silver nanoparticles formation from silver ions, with a peak at 452 nm. This finding supported by the broad band of UV–Vis absorption is mainly due to the presence of organic metabolites in *O. sanctum* based aqueous extract (*Rao et al., 2013*). Furthermore, SEM and TEM analysis of biosynthesized nanoparticles (Fig. 3 and 4) represented that nanoparticles are almost spherical to oval and rod like shaped with smooth surfaces and having mean particle size of 55 nm

with -2.7 mV zeta potential showing stable particles. The morphological study revealed the agglomeration of individual silver nanoparticles. A previous study showed that the average size of silver nanoparticles biosynthesized using the leaf extract of *O. sanctum* was 42 nm (*Rao et al, 2013*). The FTIR spectra depicted some extent of shifting of AgNPs spectra then aqueous extract, which might be due to the presence of functional groups present in the biosynthesis of plant extract and capping of nanoparticles. It also exhibited that biosynthesized AgNPs and aqueous extract (Fig. 6) that differed very slightly in their absorption bands. This may be illustrated on the base that available biomolecules in plants play a crucial role for the reduction of metal ions and formation of small size nanoparticles (*Kandasamy et al, 2013*). In addition, peaks at 3339.30 cm^{-1} and 1634.70 cm^{-1} indicated the binding of proteins, carbohydrates, and nitrogenous compounds on the surface of nanoparticles (*Das & Smita, 2018*). Our finding further supported by a previous study demonstrated that proteins bind to the surface of metal nanoparticles through a free carboxylate group therefore stabilized the AgNPs (*Ajitha, Reddy & Reddy, 2014*). It is imperative that the *O. sanctum* extract played a dual role for a reducing agent as well as a stabilizing agent for AgNPs. This is therefore highly recommended for more comprehensive studies to justify this association to come up with the final conclusion.

The antimicrobial studies determined that there was no zone of inhibition found in tulsi extract and sterile distilled water but AgNPs from *O. sanctum* have a promising effective antibacterial activity on the MDR *A. baumannii* showing a zone of inhibition of 15mm. The MIC and MBC results also proved that the bio-reducing AgNPs were able to inhibit the growth of MDR *A. baumannii*. In addition, the SEM images of *A. baumannii* treated with AgNPs reveled the penetration of AgNPs

into the bacterial cells, causing damage and rupture. The antibacterial activity can be explained based on nanoparticles interaction with microorganisms (*Franci et al., 2015*) by the released silver ions can be attached to the cell wall of bacteria , modulating the cell membrane permeability, respiration blockage (*Dhaset al., 2014; Manjumeena et al., 2014; Muhsin & Hachim, 2014*), and destabilization of bacterial outer membrane and plasma membrane degradation followed by reduction of intracellular ATP (*Ajitha, Reddy & Reddy, 2014; Pirtarighat,Ghannadnia & Baghshahi, 2019*). Silver ions also have great affinity to interact with sulphur or phosphorus of cell biomolecules and ultimately ceasing the bacterial replication (*Umashankari et al., 2012*). The AgNPs might also have affected some of the cellular components and induced the damage of cell membrane, which finally results in cell decomposition and death. (*Li, Xie& Shi, 2010*).

The bactericidal activities of biosynthesized AgNPs from the extract of other *Ocimum* species are also reported. Tailor G et al. (2020) observed antibacterial activity of AgNPs prepared from *Ocimum canum* against *Escherichia coli*, with minimum zone of inhibition of 17mm at 10 ppm concentration of AgNPs while the maximum zone of inhibition of 24.5 mm was observed at 30 ppm concentration (*Tailor G et al., 2020*). The susceptibility of 15 mm, 13 mm and 12 mm was observed against *Bacillus vallisomortis, Bacillus subtilis* and *Escherichia coli* respectively, using AgNPs synthesized from *Ocimum bacilicum* (*Pirtarighat, Ghannadnia & Baghshahi,* 2019). Using the biosynthesized AgNPs from the extract of *O. gratissimum*, Das B et al. noted no zone of inhibition in silver nitrate solution alone but the bio-reduced AgNPs showed considerable growth inhibition against pathogenic *Escherichia coli* and *Staphylococcus aureus*. They observed the zone size of 8 mm and 12 mm against *Escherichia coli* using 4 µg/mL (MIC) and 8 µg/mL (MBC) of

AgNPs respectively. Similarly, the zone size of 10 mm and 16 mm were observed against *Staphylococcus aureus* using 8 µg/mL (MIC) and 32 µg/mL (MBC). The combined activity of phytochemical of *Ocimum gratissimum* and AgNPs had demonstrated beneficial role to reduce the dose required for total microbial growth inhibition (*Das B et al., 2017*).

The killing kinetic assay showed time and dose dependent action against MDR *A. baumannii*. The bactericidal activity was gradual and complete killing was observed within 24 hours using MBC and higher concentrations of AgNPs. Statistically significant result was observed in comparison to the killing action of antibiotic colistin. The time kill curve analysis by Das B et al. using AgNPs against *Escherichia coli* and *Staphylococcus aureus* showed bacterial killing activity which increases with time of exposure of the bacteria in AgNPs at their respective MBC concentration and complete bactericidal result were obtained. The bacterial exposure with AgNPs demonstrated a rapid dose and time dependent killing leading to early stationary phase (*Das B et al., 2017*). This rapid bactericidal activity of AgNPs could come up to significantly decrease the bacterial mechanism to induce resistance development . Therefore, the AgNPs might be a promising alternative to significantly reduce the development of drug resistance in bacteria and an effective antimicrobial agent for human use after the strong clinical trials (*Thammawithan et al., 2021*).

Various studies reported the bactericidal activity of AgNPs depends on its size and shape. The smaller the size higher would be the antibacterial property compare to the big size particles (*Panacek et al., 2006*). This result could be due to the higher penetration ability of smaller size nanoparticles (*Morones et al., 2005*). This result of small size nanoparticles showed good results against bacterial inhibition but studies also reported the adverse effect and health issues of

393 nanoparticles because of its nano size. (*Basavaraja et al., 2008*; *El-Ansary & Al-Daihan, 2009*). This
394 small size of nanoparticles makes them mobile both in the human body and the external
395 environment as well (*Braydich-Stolle et al., 2005*). Pal et al. demonstrated that truncated triangular
396 AgNPs reveled the highest bactericidal activity against *Escherichia coli*, when compared with rod
397 and spherical shaped nanoparticles (*Pal, Tak & Song, 2007*). The similar result was also shown by
398 Sharma et. Al (*Sharma, Yngard & Lin, 2009*).
399 Apart from the antibacterial efficacy against MDR *A. baumannii*, cytotoxicity testing to the
400 mammalian cell is also a crucial to develop novel antimicrobials. The optimum features which
401 supports the efficacy of new antimicrobial agent requires properties like it should have potent
402 antimicrobial activity and also the low cytotoxicity level, which was clearly observed in our
403 findings (*Thammawithan et al., 2021*). Our finding with the bio-synthesized AgNPs of *O. sanctum*
404 showing the antibacterial activity against MDR *A. baumannii* at a MBC of 64 µg/mL and
405 cytotoxicity against the human A549 cell only above the concentration of 250 µg/mL clearly
406 indicates that the AgNPs are less to moderately toxic against human cells compare to its effect on
407 bacterial cells. This study shows a good ray of hope to develop novel antibiotics using nanoparticles
408 with more intense research and clinical trials.
409
410 **CONCLUSION**
411 This study focused on the novel single step innovative green approach for the biosynthesis of silver
412 nanoparticles from aqueous leaf extract of *O. sanctum*. One of the most important benefits of this
413 method is that it is eco-friendly and reduces traces of organic solvents that are hazardous to human

health. Silver nanoparticles were successfully synthesized and confirmed by the colour change. The various evaluation parameters supported the nano-sized range with stable silver nanoparticles owing to the presence of biomolecules present in leaf extract that may be acted as the surface active stabilizing agents supporting the formulation of silver nanoparticles. The antibacterial studies revealed its efficacy against clinically isolated MDR *A. baumannii* and the cytotoxic activity of AgNPs and *O. sanctum* extract against mammalian cells showed moderate action. The method was a very innovative, cost effective approach and further studies would be performed to prove its efficacy more effectively.

This study is a part of PhD thesis entitled "Molecular characterization, detection of carbapenem resistance genes and effect of natural products using nanotechnology against multidrug resistant *Acinetobacter baumannii* isolated from various clinical specimens from Central Referral Hospital, Sikkim, India", Walailak University, Thailand.

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437

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Figure 1

Preparation of silver nanoparticles of *O. sanctum*

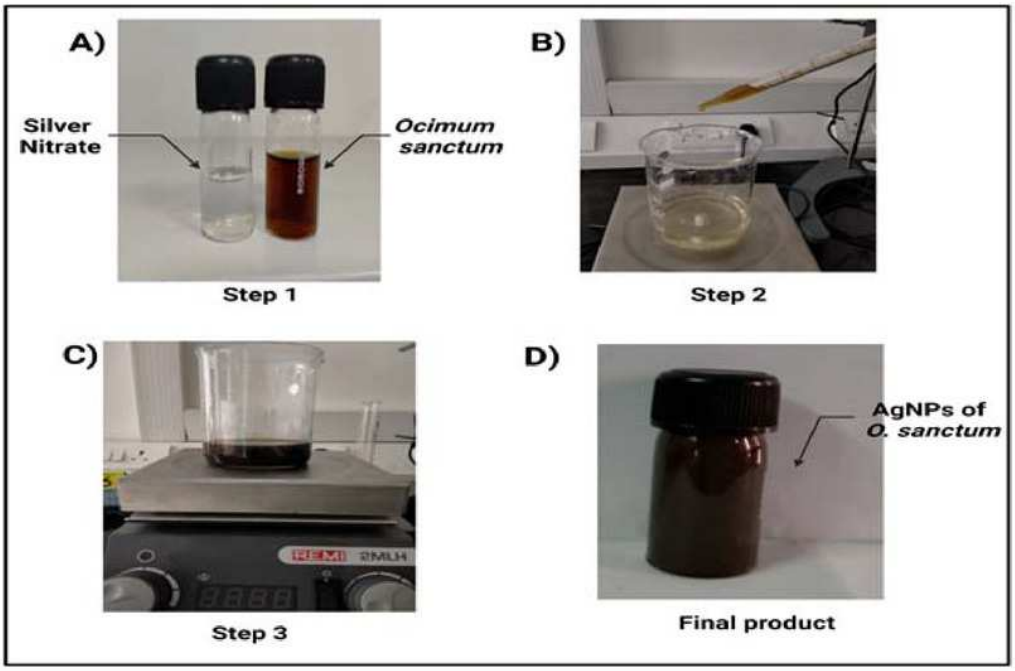

Figure 1: Preparation of silver nanoparticles of *O. sanctum*

Figure 2

Synthesis and characterization of AgNPs of *O. sanctum*

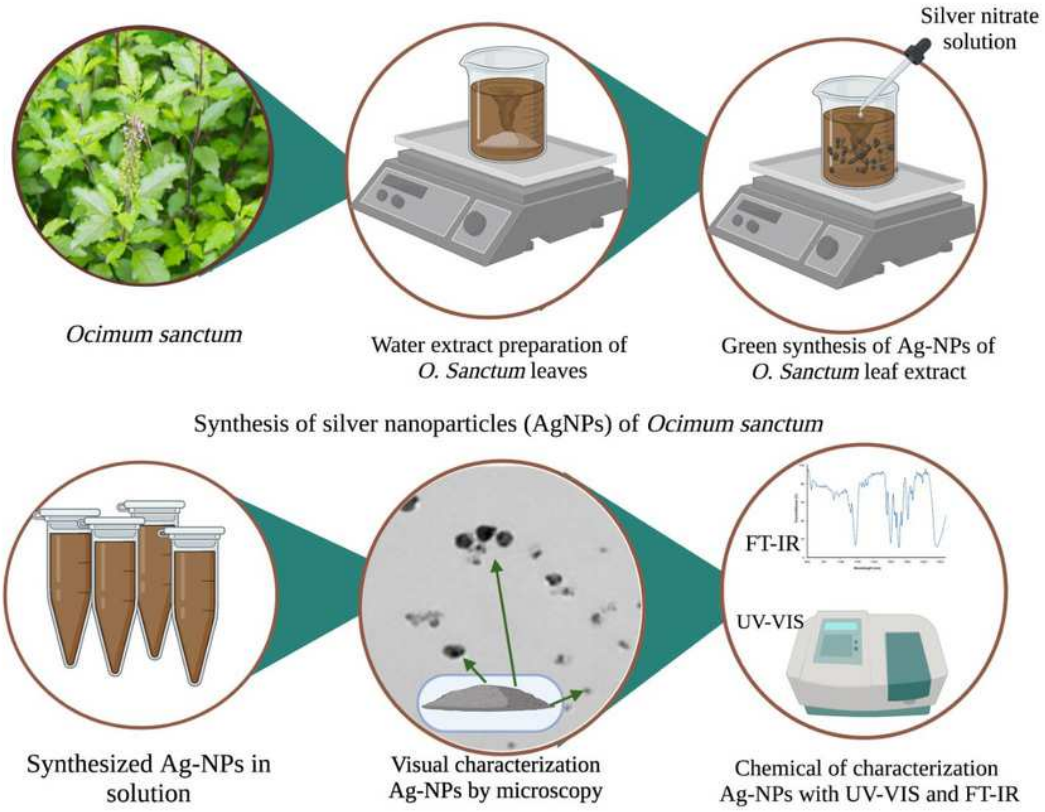

Figure 2: Synthesis and characterization of AgNPs of *O. sanctum*

Figure 3

Antibacterial analysis of AgNPs of *O. sanctum* against *A. baumannii* performed by different methods

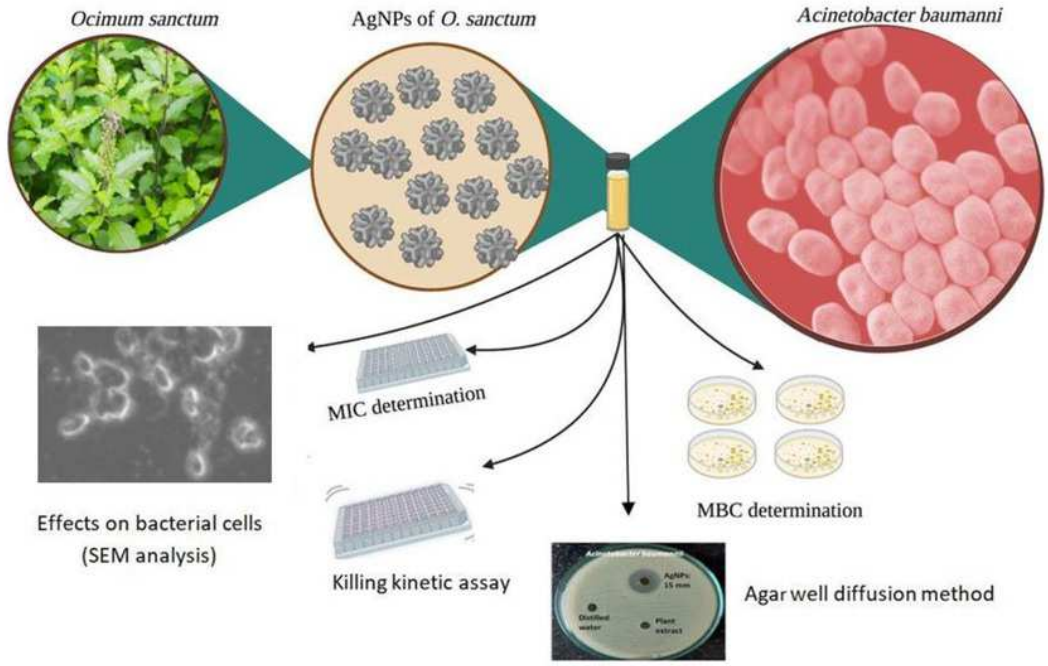

Figure 3: Antibacterial analysis of AgNPs of *O. sanctum* against *A. baumannii* performed by different methods

Figure 4

Graphical representation UV absorbance of AgNPs of *Ocimum sanctum*

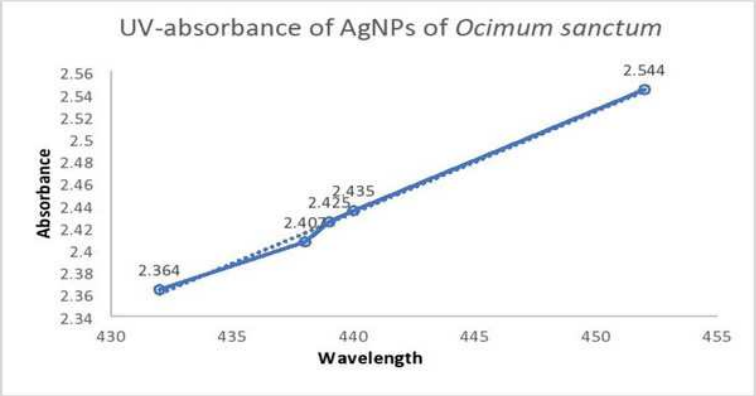

Figure 4: Graphical representation UV absorbance of AgNPs of *Ocimum sanctum*

Figure 5

SEM image represented the; (a) particle range under 20µm (2.00KX magnification with working distance (WD) 12.0mm), (b) particle range under 2µm (20.00KX magnification with working distance (WD) 12.0mm).

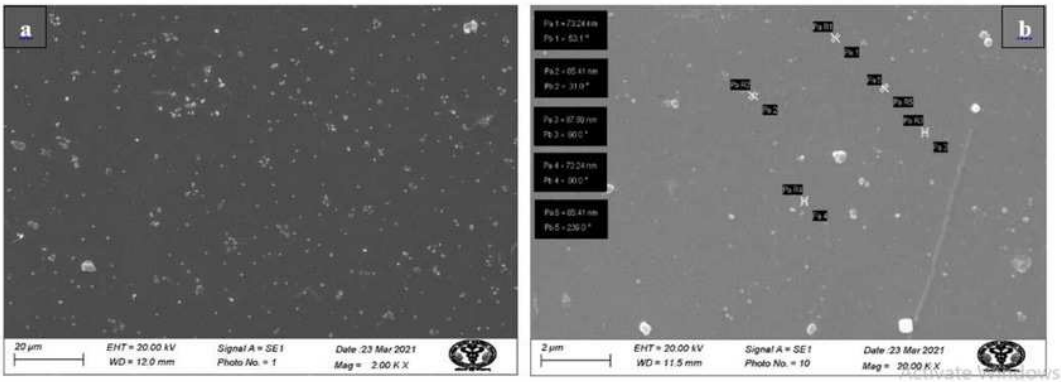

Figure 5: SEM image represented the; (a) particle range under 20µm (2.00KX magnification with working distance (WD) 12.0mm), (b) particle range under 2µm (20.00KX magnification with working distance (WD) 12.0mm).

Figure 6

TEM micrograph of AgNPs synthesized by the reaction of 0.001M silver nitrate with *O. sanctum* leaf extract

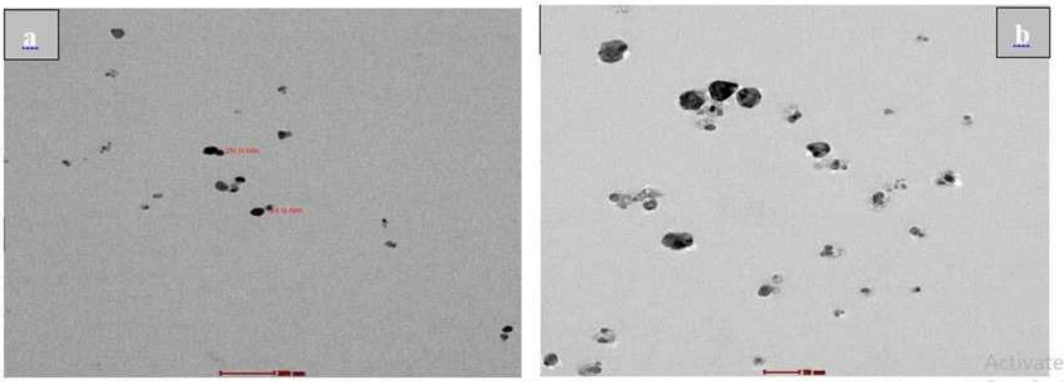

Figure 6: TEM micrograph of AgNPs synthesized by the reaction of 0.001M silver nitrate with *O. sanctum* leaf extract

Figure 7

Graphical Representation of (a) Particle Size Determination (b) Zeta Potential Measurement of biosynthesized AgNPs

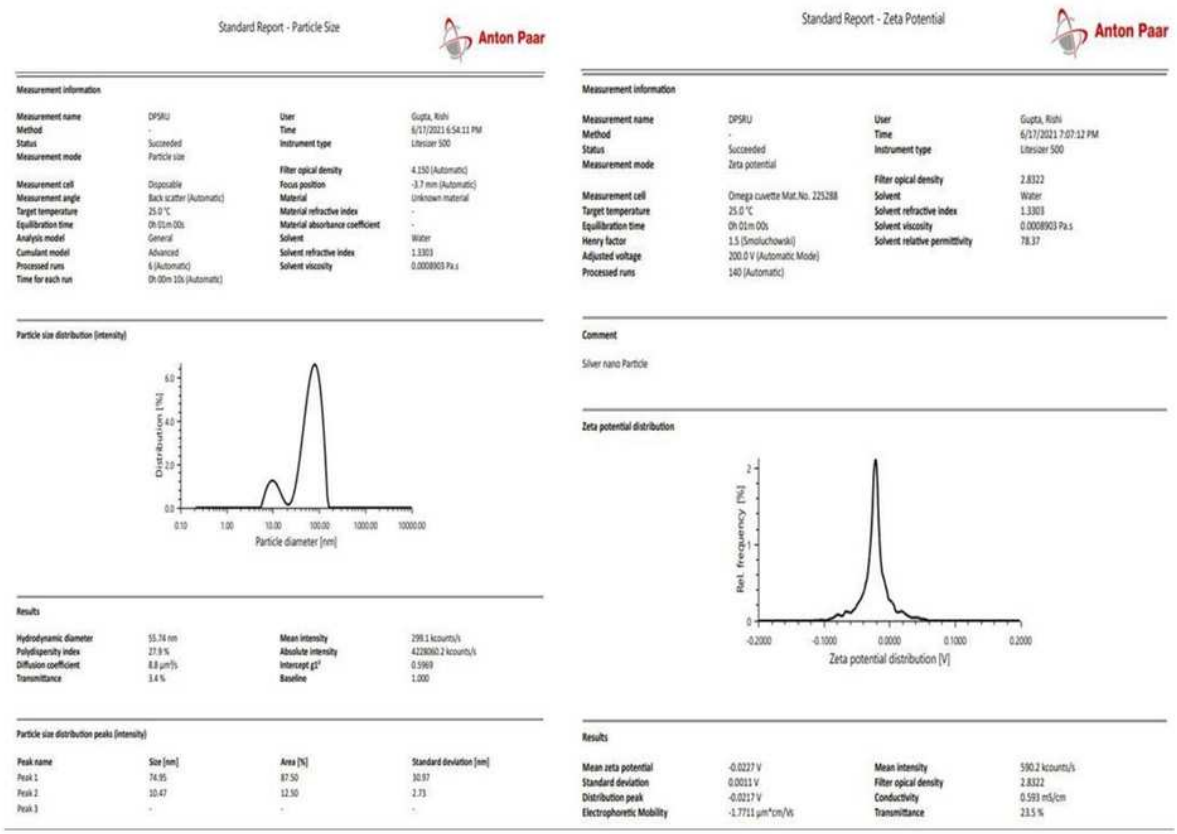

Figure 7: Graphical Representation of (a) Particle Size Determination (b) Zeta Potential Measurement of biosynthesized AgNPs

Figure 8

FTIR spectra of (a) Aqueous extract of *O. sanctum* leaf and (b) biosynthesized AgNPs of *O. sanctum*.

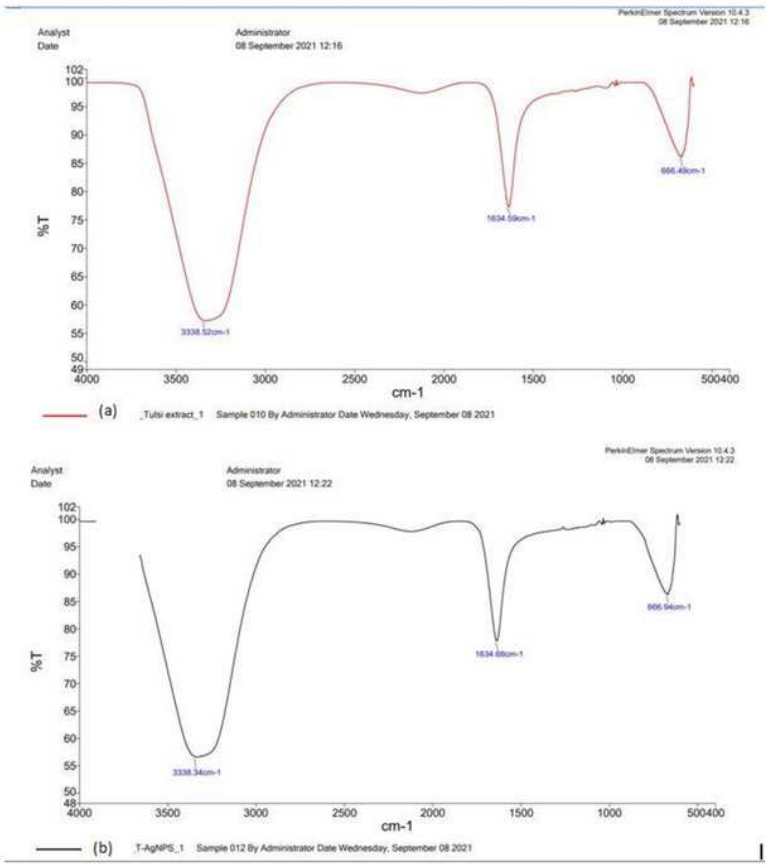

Figure 8: FTIR spectra of (a) Aqueous extract of *O. sanctum* leaf and (b) biosynthesized AgNPs of *O. sanctum*.

Figure 9

Agar well diffusion of AgNPs, plant extract and distilled water against *A. baumannii*

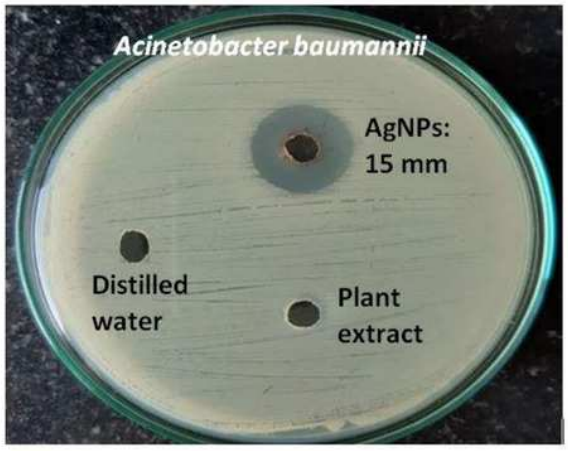

Figure 9: Agar well diffusion of AgNPs, plant extract and distilled water against *A. baumannii*

Figure 10

SEM micrograph of *A. baumannii* before and after treatment with AgNPs; (a, b) clear, smooth cells before treatment; (c, d) damaged, ruptured cells after treatment.

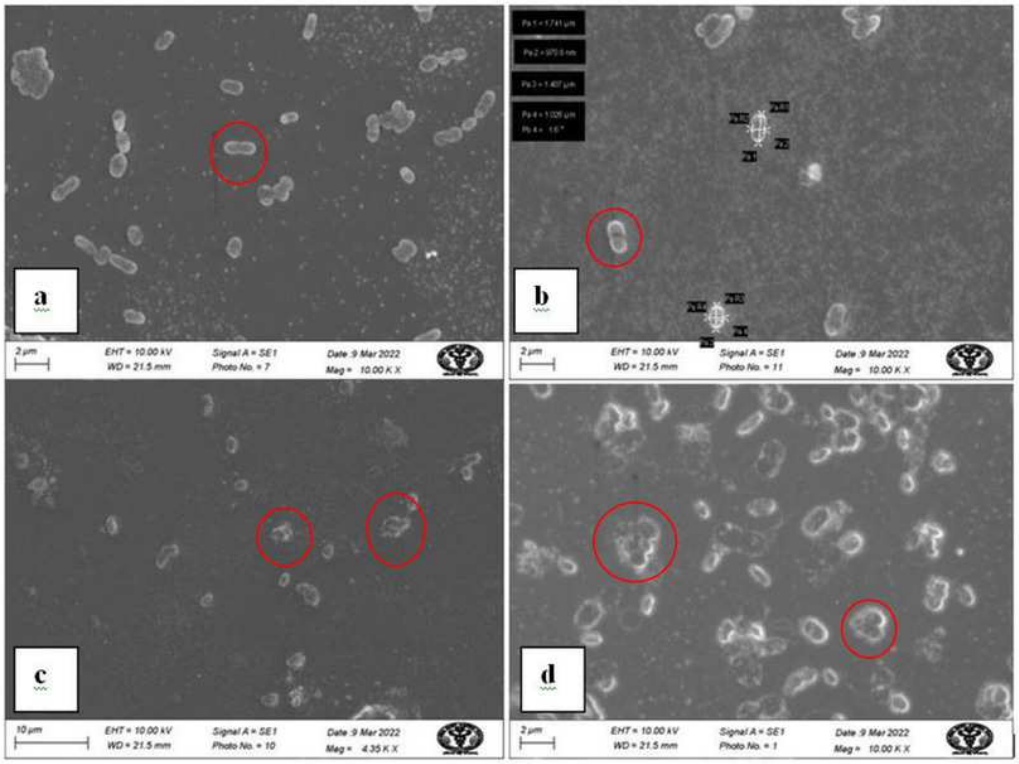

Figure 10: SEM micrograph of *A. baumannii* before and after treatment with AgNPs; (a, b) clear, smooth cells before treatment; (c, d) damaged, ruptured cells after treatment.

Figure 11

Graphical representation of Cytotoxicity test result by MTT assay.

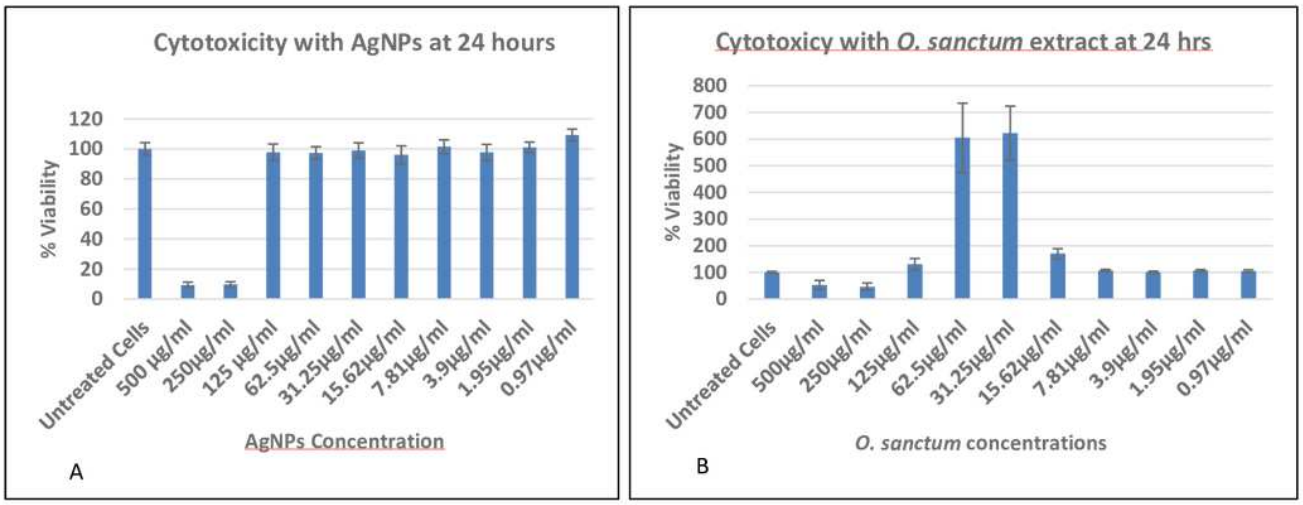

Figure 11: Graphical representation of Cytotoxicity test result by MTT assay.

Table 1(on next page)

Killing kinetic assay showing cell viability at different concentrations of AgNPs and time interval and statistical significance with positive control.

Table 1: Killing kinetic assay showing cell viability at different concentrations of AgNPs and time interval and statistical significance with positive control.

Ag NPs (µg/ml)	Duration of test (hour)							
	0 hr		4 hr		12 hr		24 hr	
	Abs±SD	PV (%)	Abs±SD	PV (%)	Abs±SD	PV (%)	Abs±SD	PV (%)
32	0.036 ±0.0	100	0.038 ±6.2	52.05*	0.105 ±1.16	49.06*	0.221 ±0.56	63.50*
64	0.042 ±7.7	104.24	0.034 ±0.28	43.03*	0.028 ±0.68	12.72*	0.019 ±0.37	5.36*
128	0.050 ±4.0	98.81	0.029 ±1.57	33.33	0.025 ±0.34	10.96	0.018 ±0.28	4.97
256	0.068 ±3	99.04	0.050 ±1.01	47.61	0.043 ±0.60	17.47	0.035 ±0.49	9.21*
512	0.112 ±7.1	100.19	0.095 ±1.24	63.75*	0.090 ±0.75	31.03*	0.078 ±0.36	19.9
Colistin (2ug/mL)	0.036 ±1.6	100.95	0.034 ±2.49	46.57	0.109 ±1.56	50.93	0.210 ±0.54	57.37

* Indicate significant at P value <0.05 in comparison with positive control colistin, PV=Percent viability, Abs= Mean Absorbance, SD=Standard Deviation