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Antimicrobial efficacy of phyto-synthesized silver nanoparticles using aqueous leaves extract of *Rosamarinus officinalis* L.

Said Munir[1], Tanzeela Amanat[2], Maryam Arshad Raja[3], Khaloud Mohammed[4], Rabab Ahmed Rasheed[5], Dina S Hussein[6], Farah Shireen[1]*, Junaid Ahmad[7], Zeeshan Ahmad[8] and Shubana Hayat[9]

[1]Sarhad Institute of Allied Health Sciences, Sarhad University of Science & Technology, Peshawar, Pakistan
[2]Allama Iqbal Medical College, Lahore, Pakistan
[3]Fatima Jinnah Medical University Lahore, Pakistan
[4]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia
[5]Histology & Cell Biology Department, Faculty of Medicine, King Salman International University, South Sinai, Egypt
[6]Department of Chemistry, College of Sciences and Health, Cleveland State University, Cleveland, USA
[7]Department of Microbiology, Immunology, Infectious Diseases, Transplantation and Related Diseases, University of Rome, Tor Vergata, Italy
[9]Department of Microbiology, Hazara University, Mansehra, Pakistan

Abstract: The current research investigation demonstrated that the aqueous leaves extract of *Rosamarinus officinalis* possesses cardinal phyto-chemicals to fabricate AgNPs in an eco-friendly way. The phyto-synthesized AgNPs were characterized to be stable, monodispersed, polycrystalline and mostly spheroidal in conformation. The nano-spheriods were observed to be 25-75 nm in diameter, displaying λ_{max} peak at 430 nm. From the comparative antimicrobial investigations, it was observed that AgNPs manifested tremendous bactericidal properties against all test organisms particularly *S. epidermis* (89%), *S. aureus* (84%) and *K. pneumonia* (84%), owing least MIC values of 40 µL. The aced fungicidal activity was also exhibited by AgNPs against all fungal test species particularly *C. herbarum* (90%), *A. flavus* (85%), *R. stolonifer* (85%) and *C. jadinii* (85%). In contrast to AgNPs, all crude ethanolic, aqueous, methanolic and n-hexanoic extracts manifested less to moderate antimicrobial activity against all test micro-organisms with three-fold escalating MIC values i.e., 160µL.

Keywords: *Rosamarinus officinalis*, phyto-synthesis, silver nanoparticles, antimicrobial.

INTRODUCTION

Nano-biotechnology is a cardinal field of nano-science, which is the combination of biological aspects and nanotechnology (Lateef, Adelere, Gueguim-Kana, Asafa, & Beukes, 2015). The word "nano" is derived from a Greek word that means tiny materials, mostly in the size of 1-100nm (Patil & Kim, 2017). Among widely fabricated metal nanoparticles, silver nanoparticles (AgNPs) are gaining pre-eminent attention due to their dynamic characteristics peculiarly in the field of medicine. They are documented to possess excellent antimicrobial, antioxidant, anti-proliferative, anti-histamine and anti-inflammatory properties with aced biocompatibility and least toxicity (Chinnappan., 2018).

Phyto-synthesis of AgNPs using whole plant or plant parts is preferred over other practiced physical and chemical modus operand due to the omission of toxic chemicals, ease and economic upstreaming steps plus minimally tedious and eco-friendly down-streaming (Pontaza-Licona *et al*., 2019). *Rosmarinus officinalis* L. is commonly known as rosemary which belongs to the family, Lamiaceae. The herb is heavenly fragmented having needle-like leaves and varied bluish-purple and pinkish-white flowers (De Oliveira, Camargo, & De Oliveira, 2019). Leaves of rosemary are widely used in cooking, due to their appealing aroma. It also possesses excellent therapeutic potency such as hepato-protective, antimicrobial and anti-inflammatory properties. The leaf extracts possess all necessary phyto-chemicals, which act as natural food preservation, thus the herb is regarded as safe by European Union (Gonçalves *et al*., 2019).

Hence by reviewing the early manifested phyto-chemicals probed in leaves of *Rosmarinus officinalis* herb, the research investigation was blueprinted in order to greenly synthesize eco-friendly nanoparticles in an economic way using aqueous leaves extracts of rosemary. Further comparative antimicrobial investigation of phyto-synthesized AgNPs versus crude ethanolic, methanolic, n-hexane and aqueous extract will be conducted against pathogenic bacterial and fungal species.

MATERIALS AND METHODS

Collection of plant material

Shade-dried leaves of the test herb i.e. *Rosmarinus officinalis* were procured from a local market of Peshawar, Pakistan which was further identified by the

**Corresponding author:* e-mail: farah.biotech@suit.edu.pk

botanist Mr. Ghulam Jelani, Botany Department, University of Peshawar, Pakistan.

Preparation of ethanolic, methanolic and n-hexanoic extracts

The shade-dried leaves of *Rosamarinus officinalis* herb were finely pulverized utilizing an electric blender. Then, analytical grade organic solvents of variable polarity i.e. ethanol, methanol and n-hexane for extraction purposes were employed. To every 100mL selected solvent, 40g of powdered plant material was subjected to maceration and then incubated for 14days at room temperature. At the end of the incubation period, the extracts were filtered using Whatmann filter paper No. 40, thus collecting the filtrates. The filtrates evenly spread onto the sterile petri-plates were then concentrated by placing them on a hot plate at ≤50°C. Finally, the dried concentrated extracts were collected and stored at room temperature (Shireen *et al*., 2022).

Preparation of aqueous leaf extract

For the preparation of aqueous extract, 25g of dried and pulverized plant material was subjected to 500mL distilled water in a sterile conical flask. The concoction was then boiled for 30minutes. Further, the prepared aqueous extract in the form of the filtrate was cooled and filtered off using Whatmann filter paper No. 40. The filtrate was then similarly concentrated using a hot plate adjusted at ≤50°C. Finally, the dried aqueous extract was collected and stored in sterile vials at room temperature (Shireen *et al*., 2022).

Phytosynthesis of AgNP

10 mL leaves broth of *R. officinalis* was combined with 1mM $AgNO_3$ solution at ≤37°C, which was further incubated at 75°C for 6 minutes using a shaking water bath. Approaching climax of the incubation periods, the indication of precise phyto-synthesis of AgNPs was manifested by noticing the color change from greenish-yellow to dark brown as depicted in fig. 1. Finally, the phyto-reduced AgNPs were concentrated using a hot plate adjusted at ≤50°C following spreading drying method onto sterile petri-plates.

Purification of agnps

The synthesized AgNPs were further segregated from biological residues and NO_3 ions following ultra-centrifugation method at 12000rpm (Shireen *et al*., 2022). For this purpose, concentrated AgNPs were added to distilled water in sterile Eppendorf tubes. The tubes were then centrifuged at 12000rpm for 15minutes. The pelleted purified AgNPs were then collected and dried at room temperature while the supernatants were discarded.

Characterization of fabricated agnps

The purified AgNPs were then scrutinized for their physio-chemical and biological properties by probing parameters related to morphology, size, stability, bio-distribution and mode of action using various spectroscopic techniques which are mentioned as follows.

UV-Vis spectroscopy

R. officinalis AgNPs' optical characteristics were observed using a UV-VIS spectrophotometer (Shimadzu UV-1601, Japan) at a temperature of around 40°C. Within the range of 350-500 nm, the optical resolution was changed at a 10nm interval.

X-Ray diffraction measurements (Xrd)

By using an X-ray diffractometer equipment (JDX-3532, China), the crystallinity of the AgNPs was calculated. A nickel gauze filter was used to filter the copper radiation, which had a wavelength of 1.54187 nm. At 30 kV/30 mA, the power supply was in use.

Scanning electron microscopy (SEM)

SEM (JEOL-JSM-5910, USA) was operated at 150X, 500X, and 1000X magnifications to analyze the surface area and morphology of phyto-reduced AgNPs. Thin coats of AgNPs were then spread over copper grids, which were further dried using a mercuric lamp. The sample was then allowed to rest for 5–10 minutes prior to the investigation.

Energy-dispersive x-ray spectroscopy (EDX)

EDX (INCA-200, UK) model was to examine the elemental properties of greenly synthesized AgNPs. The purpose of the characterization was to assess the presence of silver along with other equally cardinal biological elements.

Transmission electron microscopy (TEM)

TEM (Techni-G2-300kV, USA) was operated to analyze the precise size and 2-D structure of AgNPs. The carbon-coated Al-stubs were precisely filmed with thin quantity of prepared AgNPs. Using aspectic blotting paper, the edges were scrubbed off. Finally, after drying, the AgNPs loaded Al-stubs, the samples were magnified at 150X, 500X and 1000X.

Antimicrobial activity of fabricated AgNPs

Antibacterial assay

For the antibacterial assay, agar well diffusion protocol was followed (Shireen *et al*., 2022). For this purpose, pure cultures of *Staphylococcus aureus, Staphylococcus epidermis, Escherichia coli, Klebsiella pneumonia* and *Salmonella typhi* were used as test bacterial species. At first, a sterile nutrient agar medium (Sigma-Aldrich, Germany) was prepared using aseptic petri-plates. Then the solidified media was punctured with a 6 mm flame-sterilized borer for well formation. Further, 3 mg/mL stock solution of phyto-synthesized AgNPs and crude ethanolic, methanolic, n-hexanoic and aqueous extracts were formulated in DMSO. From each stock solution, 100 uL of the working solution was subjected to each well. The plates were left undisturbed for 30 minutes and then the test bacterial species were seeded onto the medium. All of the experimental plates were then incubated at 37°C for 24 hours. The standard drug, Amoxicillin, was wielded as positive control while for the negative control, DMSO was used. After 24 hours of incubation, the responsive zone of inhibitions was measured in mm and the percentage inhibition against the test samples was computed by the following formula:

$$\text{Percent growth inhibition} = \frac{\text{Zone of inhibition of sample (mm)}}{\text{Zone of inhibition of standard (mm)}} \times 100$$

Minimum inhibitory concentration (MIC) assay

Ensuring the antibacterial assay, fabricated AgNPs and crude rosemary extract were scrutinized for their respective MICs at variable concentrations of 10, 20, 40, 80, 160, 320, and 640µL against the selected bacterial species (*Staphylococcus aureus, Staphylococcus epidermis, Escherichia coli, Klebsiella pneumonia* and *Salmonella typhi*) by following the protocol of Banso *et al*.(Banso & Adeyemo, 2000). For this purpose, sterile nutrient broth media (Sigma-Aldrich, Germany) was prepared in sterilized test tubes. To every 5mL volume of nutrient broth media, the test samples were pipetted according to the above-stated concentrations. Finally, the fresh bacterial cultures were inoculated and incubated at 37°C for 24 hours. The MIC results were assessed on the basis of turbidity.

Antifungal assay

For the antifungal assay, the tube dilution method was followed, which is documented by Ahmad *et al.* (2016) (Shireen *et al*., 2022). The test fungal species included *Rhizopus stolonifer*, *Cyberlinnera jadinii*, *Aspergallius flavus*, *Aspergallius terreus*, *Fusarium oxysporum* and *Cladosporium herbarum*. Firstly, sterilized Sabouraud dextrose agar (SDA) was prepared in sterilized test tubes in a volume of 5 mL. Then, 24 mg/mL stock solution of biogenic AgNPs and crude leaves extracts was prepared. From the stock solution, 67.6 µL working solution was added to each test tube respectively. The supplemented media was then solidified in a slanting position. The solidified slants were then inoculated with the test fungal species. The standard drug, Miconazole was wielded as a positive control, while for negative control, sterile DMSO was used. Further, all the experimental test tubes were incubated at ≤ 25°C for 5 - 7days. The linear mycelial growth was observed and percent growth inhibition was computed by the following formula;

$$\text{Percent growth inhibition} = 100 \frac{\text{Line growth in test sample(mm)}}{\text{Line growth in control (mm)}} \times 100$$

RESULTS

Characterization of phyto-synthesized AgNPs

Uv-vis spectroscopy

From UV-Vis spectroscopy, it was manifested that the λ_{max} for biosynthesized AgNPs was aligned at 430nm, which was recorded as 0.72. The result is depicted in fig. 2. In contrast to AgNPs, the irregular absorbance patterns observed by aqueous extracts manifested precise characterization of AgNPs.

Scanning electron microscopy (SEM)

From SEM analysis, it was manifested that the green AgNPs were efficiently purified and stabilized *via* bio-reducers and bio-cappers present in the leaves of *R. officinalis* plant as active phytochemicals. From the micrographs, it was analyzed that the AgNPs were mono-dispersed, stable and mostly spheroids in shape. Other varied conformations such as tubular, triangular and cuboidal, were also observed. The result is depicted in fig. 3.

Fig. 1: (a) Greenish-yellow color of $AgNO_3$ & aqueous extract reaction mixture (b): Blackish brown color of the reaction mixture indicates the reduction of Ag^+ to Ag^o

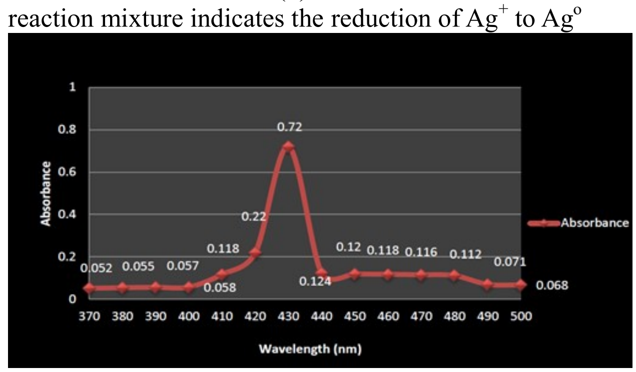

Fig. 2: λ_{max} of fabricated AgNPs at 430 nm *via* UV-Vis spectroscopy.

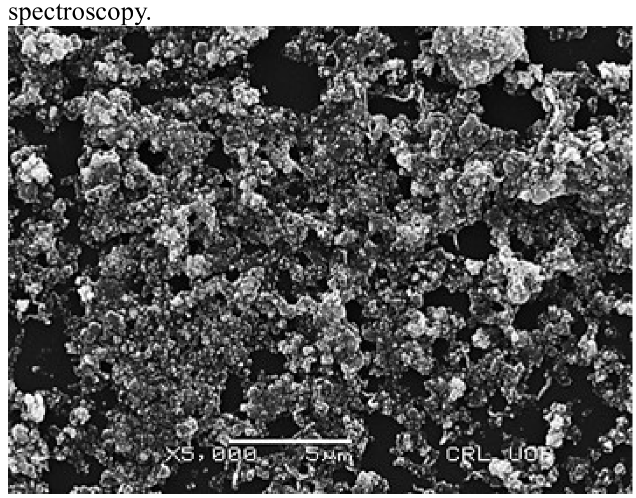

Fig. 3: 3-dimensional micrograph of fabricated AgNPs via SEM.

Transmission electron microscopy (tem)

From the 2-dimensional micrograph of AgNPs, it was manifested that the phyto-synthesized AgNPs were morphologically spheroidal, oval and triangular with diameter in the range of 25-75 nm. The result of the micrograph is depicted in fig. 4.

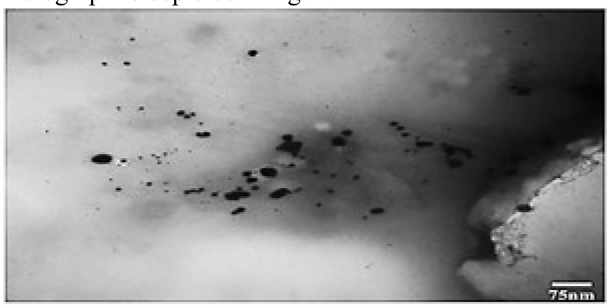

Fig. 4: 2-dimensional micrograph of fabricated AgNPs *via* TEM

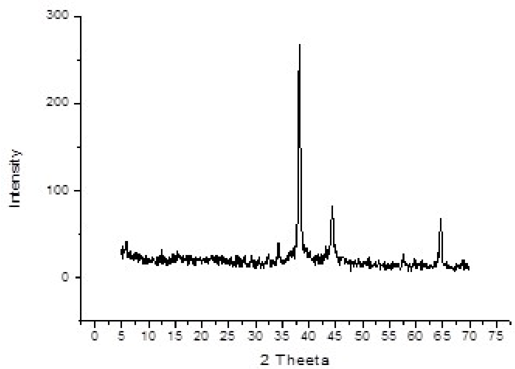

Fig. 4: Intense sharp peaks depicting polycrystallinity of fabricated AgNPs *via* XRD spectroscopy.

X-ray diffraction (XRD) analysis

From the XRD spectroscopy, the sharp peaks in the graph represent the polycrystalline property of fabricated AgNPs. By computing the 2θ angle and D-values at variable intensities *via* Debye-Scherrer's equation, it was estimated that the average size of R. officinalis mediated AgNPs diameter is 35nm respectively. The spectroscopic micrograph is depicted in fig. 5.

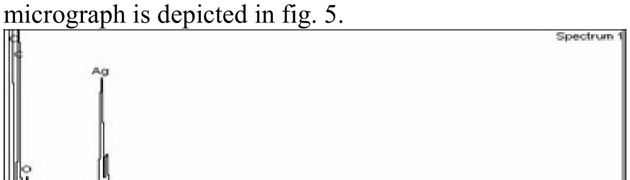

Fig. 5: Intense sharp peak of Ag^o (silver) element depicts the presence of silver particles in fabricated AgNPs *via* EDX spectroscopy.

Quercus semecarpifolia
Smith aqueous leaf extract
Quercus semecarpifolia
Smith aqueous leaf extract
Quercus semecarpifolia
Smith aqueous leaf extract

Quercus semecarpifolia
Smith aqueous leaf extract

Energy dispersive x-ray spectroscopy (EDX)

From the EDX spectroscopy, it was manifested that fabricated AgNPs possessed the biological elements i.e. carbon (43%), chlorine (0.78) and oxygen (25%) along with silver (30%), thus implying the precise formation of AgNPs. The spectroscopic micrograph is depicted in fig. 6.

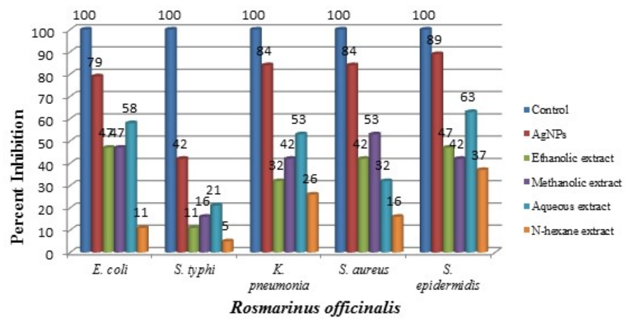

Fig. 6: Graphical illustration of comparative antibacterial assay of fabricated AgNPs and crude *R. officinalis* leaves extracts against selected bacterial species.

Antibacterial activity

From the antibacterial assay, it was manifested that phyto-synthesized AgNPs possessed tremendous activity against all pathogenic bacterial species. The aced activity was observed against *S. epidermidis* (89%), following *S. aureus* and *K. pneumonia* (84%) along with *E. coli* (79%). Moderate activities of AgNPs were observed against *S. typhi* (42%). In contrast to AgNPs, the aqueous extract was observed to be moderately active against *S. epidermidis* (63%), *E.coli* (58%) and *K. pneumonia* (53%). The rest of the crude extracts i.e., ethanolic, methanolic and n-hexanoic extracts were observed to be poorly active against all test samples, thus reckoning the destruction or absence of dynamic phytochemicals that have antibacterial properties of *R. officinalis* leaves. The results are summarized in fig. 7.

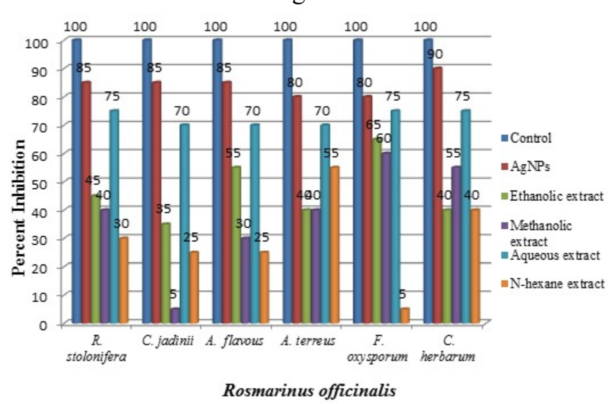

Fig. 7: Graphical illustration of comparative antifungal assay of fabricated AgNPs and crude *R. officinalis* leaves extracts against selected fungal species.

Minimum inhibitory concentration

From the MIC assay, it was manifested that the minimum inhibitory concentration for phyto-generated AgNPs was 40µL against *S. epidermidis, S. aureus* and *K. pneumonia.* 80µL sample concentration MIC values were recorded against *E. coli* and *S. typhi*. In contrast to the AgNPs, the MIC values for crude aqueous extract were recorded at 80µL for *S. epidermidis*, *K. pneumonia* and *E. coli*, respectively. Crude ethanolic extract and methanolic extracts possessed similar MIC readings, i.e. 80µL against all test bacterial species except for S. typhi i.e. 160µL for both extracts. Crude n-hexanoic extract possessed aced MIC values against *S. epidermis* i.e., 80µL while the rest of the bacterial species was potentially inhibited at 160µL.

Antifungal activity

From the antifungal assay, it was manifested that green AgNPs possessed remarkable anti-mycelial activity against all test fungal species peculiarly *Cladosporium herbarum* i.e., 90%. In comparison to AgNPs, aqueous extracts exhibited good results against all test fungal species. In contrast, phyto-synthesized AgNPs in lesser doses can manifest promising outcomes in the field of medicine due to their narrow size distribution and higher surface area, which have been testified to treat infections caused by *Candida albicans* and *Candida tropicalis.*

DISCUSSION

Nanotechnology is a relatively new field with many possibilities in pharmacy and other fields of sciences. Many problems of medicine can be solved by nanotechnology including Multi Drug Resistance and cancer vaccines, etc. A study documented by Yaqoob *et al.* (2020) manifested that variability in morphology, stability and assembly of green fabricated AgNPs depends on the wet lab conditions and phytochemicals that act as dynamic bio-reducers (Yaqoob 2020). It was also investigated that nanospheres in the range of 1–100 nm are desired for medicinal purposes due to their higher surface area, ease in cell penetration and aced activity (Liu *et al*., 2021). According to Markets study in 2018, it was documented that ultra-fine structures of AgNPs account for their efficacy in many healthcare, pharmaceutical, engineering and agricultural sectors (Markets, 2018). Morphology and size similarity of green AgNPs were reckoned with that of biosynthesized using leaves broth of *Q. incana* and *Q. brantii* plants. The AgNPs were about 80 nm in diameter, which were observed to be active against many plant pathogens including *Erwinia, Ralsontia, Pectobacterium and Xanthomonas* species (Chahardooli 2014). According to a study documented by Khattak *et al.* (2019), it was manifested that AgNPs produced by leaves extract of *Quercus semecarpifolia* had polycrystalline texture, with face-centered cubic structures in an estimated range of about 28.40 nm (Khattak *et al*., 2019). From the previously documented studies, it was suggested that greenly synthesized AgNPs possess all the organic elements i.e. carbon, nitrogen, calcium, hydrogen and oxygen along with reduced silver, which alludes to their precise reduction from Ag^+ to Ag^o (Nasar, Murtaza, Mehmood, Bhatti, & Raffi, 2019). Recent studies documented by Ahmad *et al.,* in 2020, testified that leaves-mediated AgNPs possessed efficient antibacterial properties by having large surface areas, which could easily be penetrated in the bacterial cells thus generating ROS, which can destabilize the functional proteins and nucleic acids that are required for their survival (Ahmad *et al.*, 2021). In another study, it was documented that the rosemary plant possesses many bactericidal phytochemicals such as polyphenols and alkaloids, which can be synergized with Ag^+, in order to ameliorate their activity (Fenfen 2014) According to a previous study, it was documented that gram-negative pathogens particularly *Escherichia coli, Klebsiella pneumoniae*, *Salmonella typhimurium* and *Salmonella enteritidis* were potentially inhibited at 3.9-7.8µg/mL AgNPs concentration, this could help to remediate foodborne illnesses in less concentration with aced medicinal properties (Behravan *et al*., 2019; Loo *et al*., 2018). Moderate antifungal activity was observed by polar methanolic, ethanolic and n-hexanoic extracts and could be reckoned as to study of Toledo *et al.* (2015), which suggests that alteration in polarity of organic solvents could alter the chemical nature of phyto-compounds in plants extracts, which as a consequence, have the potency to reduce / lose its antifungal property. An increase in extraction time along with the purification of phyto-compounds has the aptitude to directly upsurge the antifungal potential of crude extracts (Mendes de Toledo *et al*., 2015). In order to remediate the infection caused by *C. albicans* and *C. tropicalis,* 80µg of green AgNPs were proven to have similar activity to that of Amphotericin B (Mallmann *et al*., 2015)

CONCLUSION

From the research outcomes, it is concluded that phyto-synthesis provides a turbo-productive mechanism to synthesize silver nanoparticles (AgNPs) in upsurged quantity and quality. The method is effective because various phyto-chemicals, peculiarly reducing sugars and polyphenols, act as potential bio-reducing and bio-capping agents, which impart qualitative characteristics to these biogenic AgNPs such as higher surface area due to size range within 1-100nm accounting them as ultra-fine nanoparticles, which is preferred in the sphere of medicine, particularly as antimicrobial agent. The study also evinced that green AgNPs possess extra-ordinary antimicrobial activity against all pathogenic bacteria and fungal test species and can be exploited in medicine to formulate novel antibiotics, thus tackling the superbug issue. In contrast, all crude extracts possess less to moderate activities against the test micro-organisms imparting higher MIC values i.e., 160µL.

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