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In vitro antibacterial activity of biogenic gold nanoparticles from *Murraya koenigii* seed extract against pathogens associated with traumatic wound infections

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Abstract
Nosocomial infection is a serious health hazard worldwide and bacteria associated wound infection has been regarded as the most common cause of this infection. Multidrug resistance bacterial pathogens infecting the nonsurgical traumatic wounds could be life threatening due to therapeutic limitations. The present study focuses on the synthesis and characterization of the gold nanoparticles from the seed extract of *Murraya koenigii* and its *in vitro* antibacterial efficacy against human pathogens *Pseudomanas aeruginosa, Escherichia coli, Enterococcus spp and Staphylococcus aureus* isolated from wounds. Results from Disc diffusion method indicate that the biogenic gold nanoparticles from *Murraya koenigii* seeds exhibited strong antibacterial activity against gram positive bacteria and moderate activity against gram negative bacteria.

Keywords: *Murraya koenigii*, gold nanoparticles, antibacterial activity, wound pathogens, nosocomial infection

1. Introduction
Nosocomial infections occur worldwide and are prevalent in both developed and developing countries. Infections acquired in hospitals are among the major causes of death and morbidity. Surgical wounds, urinary tract infections and lower respiratory tract infections are the major and frequently occurring nosocomial infections. They cause burden both to the patients and to public health. WHO estimates about 8.7% of patients in hospitals acquire infections and over 1.4 million people suffer from nosocomial infectious complications [1]. The prevalence of nosocomial infections is high in intensive care units and acute surgical and orthopaedic wards. Susceptibility to infections is higher in patients with old age, underlying diseases or chemotherapy. These infections not only cause functional disabilities and emotional stress among patients but also lead to reduced quality of life [2]. Exposure of the subcutaneous tissue due to loss of skin integrity provides a warm, moist and nutritive environment favourable for colonization and proliferation of pathogens leading to infection [3]. Wounds range from acute surgical wounds, traumatic wounds such as those that occur during an accident, burns or chronic wounds in case of diabetic foot, leg and pressure ulcers [4]. These wound infection normally depend upon the pathogenicity and virulence of the microorganisms and the immune response of the host. The clinical symptoms of wound infections are erythema, pain, tenderness, heat, oedema, callulites and abscess/ plus [5]. Therefore, wound infection results in active disease that is likely to delay the wound healing process [6]. Wound healing involves the removal of damaged tissues or invaded pathogens from the site of injury to restore the normal architecture of cutaneous and/or visceral defects [7]. The common pathogenic bacteria that cause infections are *Staphylococci, Pseudomonas, Streptococci, Enterococci, Escherichia coli, Klebsiella, Enterobacter, Citrobacter, Acinetobacter, Proteus* and *Staphylococcus aureus* [8]. These bacterial pathogens infect the internal tissues and thus delaying the healing process, causing wound breakdown, herniation and dehiscence [9]. The increase in incidence of these infections is due to multidrug resistance strains that may cause morbidity and mortality [10] and antibiotics used for the treatment against these multidrug resistant strains leads to renal toxicity [11]. In order to combat the resistance developed by multidrug resistant pathogens novel combination therapy using antibiotics or antibiotics with natural compounds has been proposed [12]. Besides the development of modern medicine in health management and healing, 80% of world's population still rely on plant-based medicines for their primary health care [13].

As an alternative to the commercial antibiotics, different medicinal systems in India such as Ayurveda, Siddha, Unani and local health traditions uses plants and their products for the treatment of wounds and burns [14]. Herbal products play an important role in effective health management and to develop cheaper healthcare options [15-16] for the treatment of wounds and burns [17].

Murraiya koenigii, a native plant of Indian subcontinent is traditionally used as medicine in Indian medicinal systems. The plant is widely used as herb, spice, condiments and also used to treat various types of ailments. The whole plant is considered to be a tonic and stomachic [18]. The seeds were reported to possess anti diarrhoeal and cytotoxic activity [19-20]. Here we aimed to determine the *in vitro* antibacterial activity of gold nanoparticles (AuNPs) synthesized from *M. koenigii* seed extract against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococci* the common bacterial isolates associated with traumatic wound infections.

2. Materials and Methods

2.1 Preparation of *M. koenigii* seed aqueous extract for the synthesis of NPs

The young and healthy seeds of *M. koenigii* were collected from the field of Centre for Bioscience and Nanoscience Research (CBNR), Eachanari, Coimbatore, Tamilnadu, India. The collected seeds were washed with tap water and rinsed thrice with distilled water to remove any bound foreign materials. The fresh cleaned seeds were shade dried for 15 days at room temperature and pulverized with a sterile electrical blender to obtain seed powder. The powdered samples were stored in an air tight container until further use.

2.2 Synthesis of Gold nanoparticles

The broth solution of *M. koenigii* seed was prepared by taking 20 g of dried powder in a 500-mL Erlenmeyer flask along with 200 mL Milli-Q water and boiled for 10 min before finally decanting it. The extract was filtered with Whatmann filter paper no. 1, stored at −4 °C, and used within 1 week. The filtrate was treated separately with 1mM aqueous chloroauric acid (HAuCl4) solution in an Erlenmeyer flask and incubated at room temperature. The reaction mixture was checked for the development of colour change and absorbance spectra was monitored using UV–Visible spectroscopy. The reaction volume was quantified by measuring the absorbance of mixtures using UV–Visible spectrophotometer in the ranges of 300-700 nm. Ninety five-milliliter aqueous solution of 1 mM of HAuCl4 was reduced using 5 mL of *M. koenigii* seed extract at room temperature for 10 min resulting in a deep pink/red coloration of the solution indicating the formation of AuNPs.

2.3 Characterization of AuNPs

2.3.1 UV–Visible spectrophotometer

Reduction of gold ions by *M. koenigii* seed extract and the resulting formation of AuNPS were observed by UV–Vis spectroscopy. The synthesized AuNPs exhibit unique optical properties due to their Surface Plasmon Resonance (SPR) which depends on the shape, size and distribution of nanoparticles [21]. To determine the excitation of surface plasmonic vibration of the reduced gold ions, a small aliquot of the sample was diluted with distilled water and the absorption maxima was scanned by UV-Visible spectrophotometer in the range 300-700nm using Perkin-Elmer Lambda 2 UV198 Visible spectroscopy.

2.3.2 Fourier transform infrared and X-ray diffraction analysis

The synthesized nano solution was centrifuged at 60,000g for 40 min and the pellet were dissolved in deionized water and filtered through Millipore filter paper (0.45 μm). Fourier transforms infrared (FTIR) and X-ray diffraction (XRD) analysis were carried out using a small aliquot of the filtrate containing gold nanoparticles.

2.3.3 Fourier transform infrared spectroscopy

In order to identify the possible bio-reducing and capping agent involved in the synthesis of nanoparticles, the *M. koenigii* seed extract was subjected to FTIR spectroscopic analysis. The sample after freeze drying was mixed with potassium bromide (KBr) and pelletized. The wavelength spectrum of the pelletized sample was recorded by measuring the spectra in the diffuse reflectance mode using Perkin-Elmer spectrum RX1 (wavelength range between 4000 cm^{-1} and 400 cm^{-1}).

2.3.4 X-ray diffraction analysis

X-ray diffraction was performed to determine the dimension of biologically synthesized AuNPs with h, k, l values. The XRD diffractogram of synthesized AuNPs was carried out on a film of the solution drop-coated onto glass substrates on a Phillips PW 1830 instrument with operating conditions of voltage 40 kV and a current of 30 mA in Cu, Kα1 radiation. Particles size (L) of the Au was calculated using PAN analytical Xpert PRO Model instrument following Debye–Scherrrer's equation

$$L=0.9\lambda/\beta \cos h\, \theta$$

where, λ is the wavelength of the X-ray, β is full width and half maximum, and θ is the Bragg's angle.

2.3.5 Scanning Electron Microscopy (SEM) analysis

The reactions mixture is centrifuged at 6000 rpm for 10min and the pellet obtained was resuspended in small amount of sterilized double distilled water. A small amount of this suspension was sprayed on to glass slide to make a thin film and kept in hot air oven to dry. The thin film was used for the SEM analysis.

2.4 Screening of antibacterial property in synthesized nanoparticles

Antibacterial activity was analyzed with synthesized AuNPs by disc diffusion method against human pathogenic bacterial strains isolated from wounds. It was performed using an 18 h culture at 37 °C in 10 ml of Nutrient Broth. The cultures were adjusted to approximately 10^5CFU/ml with sterile saline solution. Five hundred microliters of the suspensions were spread over the plates containing Nutrient agar using a sterile cotton swab in order to get a uniform microbial growth. Under aseptic conditions, empty sterilized discs (Whatman no. 5, 6 mm dia) were impregnated with 50 μL of concentrations of the extract, standard antibiotic tetracycline, purified AuNPs and 50 and 75μl of crude AuNPs and placed on the agar surface.

2.5 Determination of Minimum inhibitory concentration (MIC)

To observe the minimum inhibitory concentration (MIC) of synthesized AuNPs, a simple procedure was followed. The effect of AuNPs on the kinetics of bacterial growth was examined by using enzyme-linked immunosorbent assay (ELISA) reader spectrophotometer. Dehydrated powder of nutrient broth was used to make the nutrient broth (NB) medium. Sterility of all glasswares and NB medium was performed by incubating at 37 °C for 24 h. The transparent NB media (50µl) was filled in all microtitre wells (A, B, C, D 1-5). The wound pathogens *Pseudomonas aeruginosa* (B w1-w5) and *Staphylococcus aureus* (DW1-5) were used to test the antibacterial activity of AuNPs. The experiment incorporated a positive control (microtitre wells (A W1-W5), (C W1-W5) having AuNPs and nutrient broth media, without inoculum) and a negative control (microtitre wells (B W1-W5), (D W1-W5) having inoculums and nutrient broth media, with AuNPs). The absorbance values for experimental test tubes (having nutrient broth media, inoculum and AuNPs) were corrected by deducting the corresponding absorbance values for the positive controls. All the experiments were performed in triplicates. The five doses ranging from 10µl to 50µl were designed for the AuNPs solution and the micro titre plate was incubated at 37 °C for 24 h. Then it was read by spectrophotometer. The optical density (OD 570nm) values from ELISA plate analyzer were taken in absorption mode which observed the bacterial growth in each sample. Five values of OD for each sample and their mean were calculated.

3. Results

3.1 Synthesis of AuNPs

AuNPs were synthesized using aqueous seed extracts of *M. koenigii*. The HAuCl4 solutions turned to deep pink/red colour with the addition of seed extract (Fig. 3). The nanoparticles were synthesized rapidly within 1 h of incubation period.

3.2 Characterization of AuNPs

UV–Visible spectrophotometer absorbance spectrum of the reaction mixture at different wavelengths ranging from 300 to 700 nm revealed a peak at (Fig. 4) 535 nm for AuNPs. The optimal condition to synthesize narrow-size range nanoparticles with high stability was fixed at 70 °C temperature, pH=7, 1 mM concentration of metal ion, stoichiometric ratio of reaction mixture=95 mL of HAuCl4 with 5 mL of seed extract and 1 h incubation time. Intensity of pinkish violet colour increased in direct proportion to the incubation period. It was due to the excitation of surface plasmon resonance (SPR) effect and reduction of metal ions into nanoscale particles. The gold (Fig. 4) surface plasmon resonance was observed at 535 nm, which steadily increased in intensity as a function of time of reaction (ranging from 10 min to 1 h) without showing any shift of the wavelength maximum.

3.3.1 FTIR and XRD analysis

FTIR spectroscopy analysis was performed to ascertain the involvement of possible seed bio compound responsible for reduction of Au^{+} ions and capping of AuNPs synthesized by using seed extract. Figure 5 shows the synthesized AuNPs using *M. koenigii* seed extract and the absorption spectrum manifests prominent transmittance located at 3678.95, 3670.12, 3104.87, 2378.65, 2301.85, 2204.98, 1789.63, 1172.63, 1048.96 and 887.26 in the region 500–4000 cm−1 (Fig. 5).

XRD peak corresponding to 2θ values in the range of 30-80^{o} were indexed as (1 1 1), (2 0 0), (2 2 0) reflection of fcc structure of metallic gold (JCPDS file no 04-0784) revealing that the synthesized AuNPs are pure crystalline gold. (Fig.6)

3.3.2 Scanning electron Microscopy

The morphology and the size of the AuNPs were determined using Scanning Electron Microscopy (SEM). The scanning electron micrograph reveals that the gold nanoparticles synthesized were slightly aggregated. The synthesized AuNPs were mostly spherical in shape ranging between 36 to 68 nm (Fig.7).

3.4 Antibacterial efficacy of synthesized nanoparticles

The antibacterial activity of *M. koenigii* seed extract and the synthesized AuNPs were investigated against the pathogenic bacterial strains isolated from wound infections using disc diffusion method. The synthesized AuNPs exhibited significant antibacterial activity that was observed by the zone of inhibition developed. *S. aureus and P. aeruginosa* was found to be more susceptible to aqueous seed extract and synthesized AuNPs with 5mm and 3mm zone of inhibition respectively. 8mm and 14 mm zone of inhibition was recorded against *Enterococci* by crude and AuNps respectively. The result suggest that *S. aureus. P. aeruginosa and Enterococci* were effectively inhibited compared to *E. coli*. Thus AuNPs synthesized from *M. koenigii* seed extract can be used as effective growth inhibitors against various microorganism isolated from wound infections (Fig. 8 & Table 1).

3.5 Minimum inhibitory concentration of AuNPs

The Minimal inhibitory concentration of AuNPs against the bacterial strains *S. aureus and P. aeruginosa* ranged from 0.436 to 1.954 µl indicating the bacteriostatic activity of AuNPs.. Bacterial growth was measured as increase in absorbance at 570nm. The MIC or cell death (%) for *P. aeruginosa and S. aureus* was high in 50µl dose and 40µl dose of 1mM AuNPs respectively (Fig. 9 & Table 2).

4. Discussion

The green synthesis of metallic nanoparticles is cost effective and the AuNPs are synthesized from a variety of biological sources such as proteins, flagella, bacteria and fungus [22-25]. The gold nanoparticles are of particular interest in biomedical application due to their unique optical properties and biocompatibility [26]. The AuNPs have a wide range of therapeutical applications including anti tumour activity [27], antibacterial activity, gene therapy, drug delivery and DNA and RNA analysis [28]. Plant extracts are widely used for the synthesis of AuNPs [29] due to the presence of numerous reducing and stabilizing phytochemicals such as proteins, amines, phenols, carboxylic acids, ketones, aldehydes etc [30].

4.1 Characterization of synthesized AuNPs

The reducing and stabilizing or capping properties of phytochemicals in *M. koenigii* seed extract in synthesis of AuNPs were examined. The size, shape, morphology and antibacterial activity of the AuNPs were also carried out.

The visual observation of colour change to deep pink/red colour indicates the formation of AuNPs. (Fig 1). Thus the

reduction of Au^{+3} to Au^0 by the plant bio molecules were confirmed. The change in colour developed is because of the Surface Plasmon Resonance which was due to the oscillation of free conduction of electrons [31]. The absorbance maximum was recorded at 535nm. Sourav Ghosh *et al*, [32] obtained UV-Vis absorbance spectra wavelength measuring at 535nm for the synthesized AuNPs from aqueous extract of Fenugreek seeds. The size and shape of the synthesized AuNPs play a crucial role in biological applications and hence the synthesis of nanoparticles with minimum particle size is of utmost importance. In our present investigation the size of the synthesized nanoparticles were in the range between 36 to 68 nm with spherical shaped structures with an average particle size of 21.89 ± 2nm. Philip *et al*., [33] synthesized gold nanoparticles in the size range 10 to 20nm from the leaf extracts of *M. koenigii* and Dhara Shukla and Padma Vankar [34] reported the synthesis of AuNPs with particle size of 30 to 130nm which was similar to the results obtained in the present study.

In order to confirm the results obtained from UV- Vis spectrophotometer and to investigate the composition and phase structure of synthesized AuNPs, XRD analysis of done. The broadening of peaks was due to synthesized AuNPs. The result indicates that Au^+ of chloro auric acid had been reduced to Au^o by *M. koenigii* seed extract. The diffraction peaks at 38.13º, 43.92º and 64.51º corresponding to (111), (200) and (222) facets of Au^o. The sharp bands of Bragg peaks indicate the capping of AuNPs by the bio molecules present in the *M. koenigii* seed extract [35].

The FTIR spectrum showed absorbance band at 3678 cm^{-1} which indicate the low concentration of hydroxyl group [36]. The band at 3740 cm^{-1} also indicates the presence of non hydrogen bonded OH group. Aromatic C-H stretching is confirmed by the band at 3104.87 cm^{-1}. The presence of strong bands at 1789.63 corresponds to simple aromatic compounds, 1172.63 cm^{-1} represents reactive carbonyl such as anhydride, acid halide, 1048.96 cm^{-1} represents simple hydroxyl compounds and 887.26 cm^{-1} indicates CH in plane deformation or the presence of aliphatic chloro compounds. The other bands formed may be due to noise vibrations [37].

4.2 Antibacterial efficacy of AuNPs from *M. koenigii*

The antibacterial efficacy of synthesized AuNPs from *M. koenigii* seed extract was examined against pathogenic bacteria isolated from wound infections. No previous studies are reported for the antibacterial activity of AuNPs synthesized from *M. koenigii* seed extract.

The antimicrobial activities of synthesized AuNPs were reported earlier [33]. AuNPs synthesized from natural honey exhibited strong antibacterial activity against multidrug resistant pathogens [38]. Antibacterial activity of AuNPs synthesised from *Salicornia brachiata* against *P. aeruginosa* and *E. coli* was reported by Ayaz Ahmed *et al* [39]. Similarly AuNPs from *Mentha piperita* against *S. aureus* and *E. coli* was also reported [40].

The most commonly occurring bacterial pathogens in wound infections were selected for its susceptibility against *M. koenigii* seed extract mediated AuNPs. In the present investigation two gram positive bacteria *Staphylococcus aureus* and *Enterococci* two gram negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* were selected to examine the antibacterial activity of synthesied AuNPs. Many studies reported that *P. aeruginosa* and *S. aureus* were the predominant bacterial isolates from different wound infections [41-45]. The virulence factors coagulase, catalase, clumping factor A and leucocidines in *S. aureus* and elastase produced by *P. aeruginosa* were associated to their pathogenicity. Shittu *et al*., [45] reported *S. aureus* and *E. coli* as the predominant bacterial species isolated from wound infection in two health institutions in ILE-IFE, Nigeria. Selvaraj *et al*., 2016 reported the wound infections by *Enterococci, Streptococci, Klebsiella, Pseudomonas, Staphylocci and S. aureus*. Jonathan *et al*., [44] also reported the isolation of *Enterococci*, *S. aureus*, *P. aeruginosa* and *E. coli* with other bacterial isolates associated with traumatic wound infections. The results obtained from the present study were also in agreement with the above findings.

Synthesized AuNPs from *M. koenigii* seed extract exhibited significant antibacterial activity that was measured by the zone of inhibition developed. The gram positive strains, *S. aureus* and *Enterococci* were effectively inhibited by AuNPs of *M. Koenigii* seed extract producing 5mm zone of inhibition for both the bacterial isolates. The AuNPs had moderate activity towards *E.coli* compared to the other selected bacterial species. A 3mm zone of inhibition was *P. aeruginosa* was inhibited effectively than *E. coli* and thus it could be inferred from the study that the synthesized AuNPs of *M. koenigii* was more effective in inhibiting gram positive than gram negative bacterial strains. Abu-Shanab *et al* [46] also reported that the gram negative bacterial species exhibited more resistance to plant extracts compared to gram positive bacteria and this may be due to the permeability barrier of cell membrane. Lee seong Wei *et al*., [47] reported that the *M. koenigii* aqueous and methanolic extract had no effect on the growth of *E. coli* isolated from aquatic animals.

4.3 Minimal Inhibitory Concentration (MIC) of AuNPs of *M. koenigii*

In order to determine the Minimal Inhibitory Concentration (MIC) of AuNPs of *M. koenigii* seed extract the two bacterial pathogens that were effectively inhibited by disc diffusion assay, *S. aureus* (gram negative) and *P. aeruginosa* (gram positive) were selected. The MIC assay of AuNPs against the selected bacterial strains ranged between 0.436 to 1.954 µl indicating the bacteriostatic activity of AuNPs. The absorbance values were measured at 570nm. Bacterial growth was measured as increase in absorbance. The MIC of aqueous extract of *M. koenigii* leaf extract against the bacterial pathogens *S. aureus*, *P. aeruginosa* and *E. coli* was also reported by Shruthi *et al*., [48].

5. Conclusion

The present study revealed that the aqueous extract and the synthesized AuNPS of *M. koenigii* exhibited antibacterial properties which reveals the basis for its use an alternative to synthetic drugs and also justifies its role in traditional medicinal system to treat skin infections. Thus the *M. koenigii* can be utilized for the preparation of effective drugs due to their various pharmacological properties reported earlier. Though, few reports are available for the antibacterial activity of *M. koenigii* leaf extract and leaf extracted silver and gold nanoparticles. Extensive research is needed to explore the pharmacological potential of AuNPs synthesized from seed extract of *M. koenigii*. Results obtained from the present investigation will form the basis for further studies in investigating potential medicinal value of the plant.

6. Conflict of interest statement

We declare that we have no conflict of interest

Fig 1: The young and healthy seeds of *Murraya koenigii*

Fig 2: Aqueous Seed extract of *Murraya koenigii*

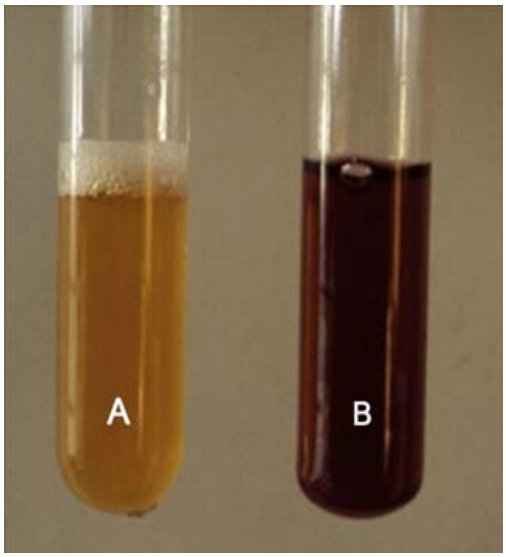

Fig 3: Synthesised AuNPs from the seed extract of *Murraya koenigii* (a) Control (b) 1mM seed extract AuNPs

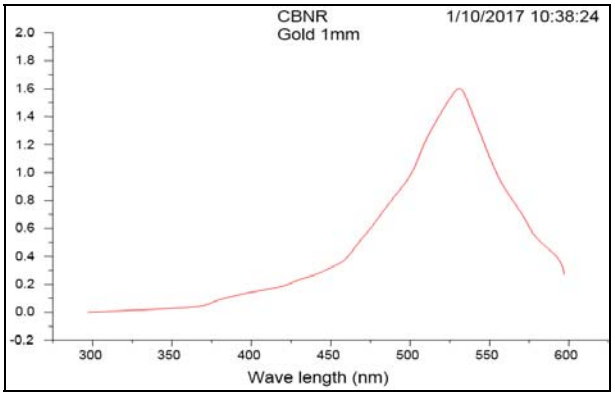

Fig 4: UV – Vis Spectrophotometer analysis

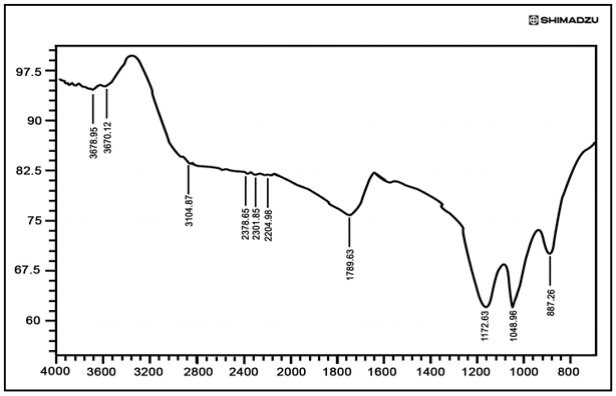

Fig 5: FTIR spectrum of synthesized AuNPs from seed extract of *Murraya koenigii*

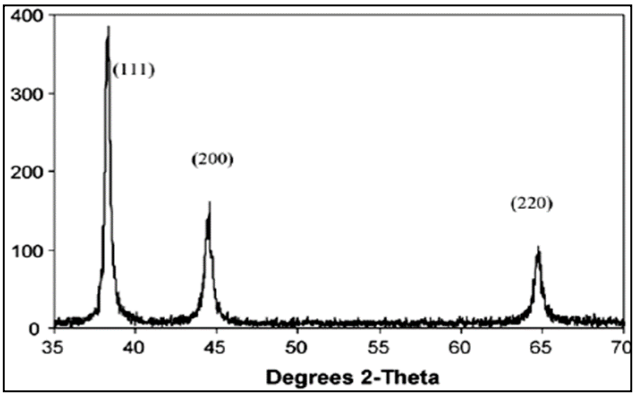

Fig 6: X-ray diffraction (XRD) pattern of synthesized gold nanoparticles from aqueous seed extract of *Murraya koenigii*

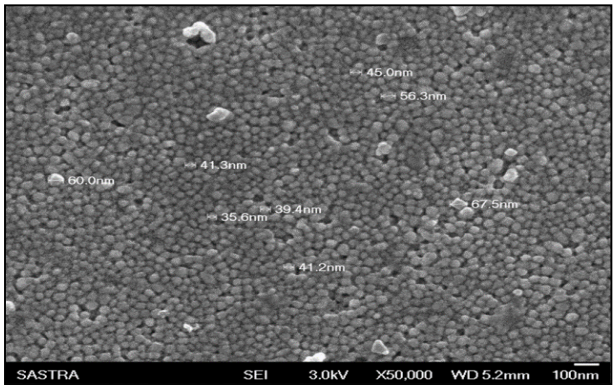

Fig 7: SEM micrograph of AuNPs from *M. koenigii* seed extract

Pseudomonas aeruginosa *Escherichia coli* *Staphylococcus aureus* *Enterococci*

Fig 8: Antibacterial activity of AuNPs from *M. koenigii* seed extract

A: Control **(**HAuCl4) **B:** Aqueous seed extract **C:** Crude AuNPs **D:** Purified AuNPs **E:** Standard antibiotic Tetracyclin

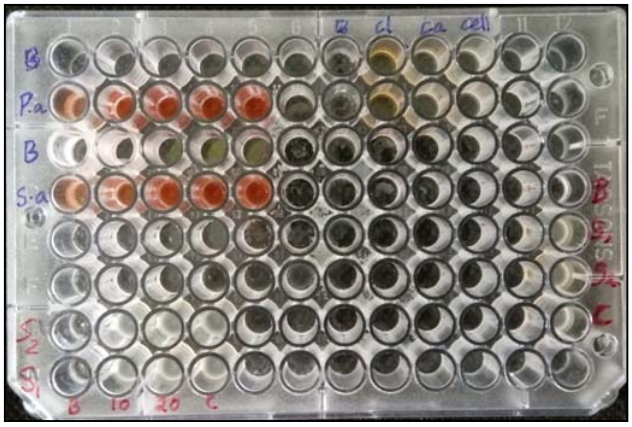

Fig 9: MIC of AuNPs from *M. koenigii* seed extract against selected bacterial strains (96 well plate method)

Table 1: Antibacterial activity of aqueous and synthesized AuNPs from *M. koenigii*

Bacterial isolates from wound infection	Zone of Inhibition (mm)		
	Standard antibiotic	Crude Seed Extract	1mM AuNPs (Purified)
Pseudomonas aeruginosa	5	2	3
Escherichia coli	2	2	2
Staphylococcus aureus	6	3	5
Enterococci	5	3	5

Table 2: Minimal Inhibitory Concentration (MIC) of aqueous and synthesized AuNPs from *M. koenigii*

S.NO	1mM AuNP Dose (µl)	*Pseudomonas aeruginosa* (Test)	Cell Death (%)	*Staphylococcus aureus* (Test)	Cell Death (%)
1.	10	0.523	23.66%	0.436	20.86%
2.	20	1.453	65.74%	0.826	39.52%
3.	30	1.559	70.54%	1.051	50.28%
4.	40	1.544	69.86%	1.954	93.49%
5	50	1.950	88.23%	1.540	73.68%

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