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Biogenic Gold Nanoparticles Fabricated from Withania coagulans Leaf Extract: Synthesis, Characterization, and Broad-Spectrum Antimicrobial Assessment

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Abstract

Aqueous extracts of *Withania coagulans* leaves were used to phytosynthesize green AuNPs. The AuNPs synthesized were found to be stable nano-crystalline sphericals with a size range of 20 to 100 nm. The AuNPs λmax peak was found at 550 nm, demonstrating the accurate production of AuNPs. The sharp Au^{o} peak in EDX analysis, together with other organic peaks, demonstrate that phytochemicals in plant extracts efficiently lowered the ionic Au^{+3} by serving as an efficient biocapping and bioreducing agent. In comparison to aqueous leaves extract, the in-vitro activities of these AuNPs show that they have excellent antifungal activity against *F. solani* (92%), *F. oxysporum* (91%), *M. furfur* (90%), *Penecillium* and *C. albicans* (89%). Antibacterial activity was shown to be moderate to good against *S. aureus* (63%), and *K. pnuemoniae* (54%).

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Introduction

The basic concept of nano-biotechnology comes from the combination of components that are (10^{-9}) nanometers in size and are used for the good of humanity. These tiny particles are now used in a variety of scientific disciplines, including engineering and medicine (Tayo, 2017). It is essentially referred to as an umbrella term that combines concepts from physics, chemistry, and engineering with those from biomedicine. These nano-devices have numerous uses, from chemotherapy to the field of cosmetics (Xiao *et al*., 2019). These nano-devices may be used in a variety of products, including biosensors, conductive inks, imaging devices, fertilizers, and pesticides (Khan *et al*. 2019).

Metal nanoparticles have been created chemically and mechanically in recent years. The most often targeted metals were copper, platinum, titanium, gold, and silver. Among the aforementioned, silver has undergone extensive research to determine whether it possesses a special antibacterial characteristics (Ramalingam *et al*., 2014). As gold ions operate as great antibacterial, antioxidant, anti-inflammatory, antiviral, and anti-cancer agents in the future, the availability of gold salts has drawn scientists' attention across the globe (Gurav *et al*., 2019). In order to expedite the treatment of rheumatoid arthritis, the gold salts are also conjugated with anti-inflammatory medications such aurothiomalate. Osteoporosis, wrinkles, and bone deformation in old age can be avoided by consuming colloidal gold within WHO permitted levels (Kean & Kean, 2008). In addition, laser equipment, pacemakers, antimicrobial stents, surgical scissors and bandages use gold alloys as laminations to treat and prevent multiple infections (Lin *et al*., 2009).

With the use of a chemical reducing agents, metallic gold salts could be efficiently converted into gold nanoparticles (AuNPs). The use of various reducing agents, such as sodium borohydride, sodium citrate, and ethylene diaminetetraacetic acid (EDTA), was extremely hazardous to human health and the environment. As a result, green synthesis of gold nanoparticles was developed to bio-reduce gold ions (Au^{+3}) to gold nanoparticles (Au^{o}) in order to address the issue (Slocik *et al*., 2005; Pissuwan *et al*., 2019).

The modern process known as "green synthesis" uses the naturally occurring reducing abilities of microorganisms (bacteria, fungi, and algae) and plants to transform the metal Au^{+3} into Au^{o}. Plant parts or complete plants are favored bio-reducing agents over microorganisms because of their wide availability, rapid conversion to nanoparticles, and simple purifying procedures (Ramalingam *et al*., 2019).

Phytosynthesis or plant mediated synthesis of AuNPs exploit plant natural phytochemicals particularly polyphenols to bio-reduce and bio-cap Au^{+3} to Au°.

According to recent reports (Ahmad *et al.*, 2017), the bio-manufactured AuNPs have unique therapeutic efficacy against microbial infections, ageing, malignancies, and aesthetic diseases. Because it avoids the use of hazardous chemicals and high energy inputs like heat and pressure, the manufacture of AuNPs from plant material has demonstrated to be a user and environment-friendly technology (Iravani, 2011). The combination of beneficial phytochemicals and gold ions, as well as the fabrication of nano-size structures that could easily penetrate biological membranes and impart their medicinal value, are thought to be the basis for the rationale behind the efficient therapeutic properties of these phyto-synthesized AuNPs (Nadeem *et al.*, 2017).

A promising avenue for medication distribution has been made possible in the realm of medicine by photosynthesized AuNPs (Klebowski *et al.*, 2018). The medicine can be transported to the desired places, such as nucleic acids and proteins, with the help of the monolayer of AuNPs. The AuNPs can also be chemically adsorbed to the surface utilizing ionic bonds or conjugated with the chosen medication using polyethylene glycol. According to Hu *et al.* (2020), the transport of the payloads is examined to take place in the presence of in-vivo (pH, cellular enzymes, and glutathione) and in-vitro (temperature) stimuli.

Pakistan is a country which is rich in medicinal plant flora. According to documented data, approximately 5000 plants possess medicinal properties. Out of 5000, 600 medicinal plants are exploited by herbalists to treat many diseases (Saleem *et al.*, 2018). As an under-developed country, the population mostly depends on the herbal medicine formulations to treat the affliction (Sulaiman *et al.*, 2020). Likewise, *Withania coagulans.*

Withania coagulans Dunal as shows in fig. 1.1, is trivially known as Paneer booti in Urdu while in English it is named as Indian rennet or vegetable rennet. The name is due to the presence of rennet-like protease which has the ability to coagulate milk protein in to cheese. The plant is widely distributed in Pakistan, Afghanistan and India (Beigomi *et al.*, 2014). The leaves, stem and fruit has been used in traditional medicine for treatment of many diseases. Extracts of the plant have been reported to manage diabetes and stomach discomforts (Kaarunya *et al.*, 2019). The aqueous leaves extracts of the plant was also reported to remediate recurrent leaf spot disease caused by *Alterneria alternata* (Sharma *et al.*, 2013).

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Fig.1.1. Showing whole plant of Withania coagulans

W. coagulans is a rigid, grey under shrub, 60-120 cm high, occurring in drier parts of the Punjab. Additionally, reports of it from the Kumaun, Garhwal, and Simla areas have been made. The shrub blooms from November through April, while the berries ripen from January through May. From the seed, the body naturally regenerates (Hemalatha *et al*., 2008).

According to Gupta *et al*. (2012), withanin, an enzyme found in the pulp and husk of berries, is responsible for the fruits' ability to cause milk to coagulate. The berries of *W. coagulans* are utilized in Punjab as a source of coagulating enzyme to coagulate milk, which is known as "paneer." Crushed plant berry pieces that have been knotted in a cloth are immersed in warm buffalo or sheep milk that has been heated to roughly 100 ^{o}F. Curdling of the milk takes 30 to 40 minutes (Hemalatha *et al*., 2008).

According to Maurya *et al*., (2010), *W. coagulans* has a variety of therapeutic qualities including antifungal, anticytotoxic, antidiabetic, hypolipidemic, neuroprotective, anti-inflammatory, anti-cancerous, anthelmintic, antioxidant activity, and wound healing activity. According to Khan *et al*., (2021), *Withania coagulans* has been used to treat acute and chronic hepatic diseases, aberrant cell growth, wasting disorders, neurological and physical issues, diabetes mellitus, and sleeplessness. As depicted in Fig. 1.2, the roots, leaves, and fruits of *W. coagulans* are responsible for a variety of pharmacological and therapeutic activities (Khan *et al*., 2021).

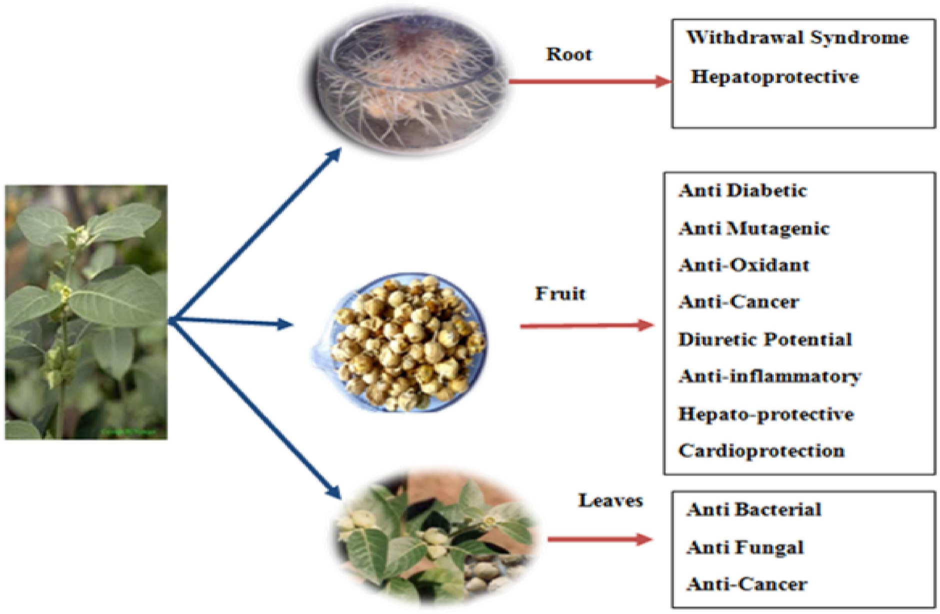

Fig. 1.2. Shows the therapeutic potential of Withania coagulans

Given the variety of uses for both AuNPs and *Withania coagulans*, a research project was created to bio-fabricate AuNPs utilizing the plant's aqueous extracts of leaf. The AuNPs would also be characterized in order to determine their specific functional makeup. Finally, the clinical investigation of the AuNPs' therapeutic potential will be conducted.

Materials and Methodology

Collection of Sample

Leaves of *Withania coagulans* plant were obtained from region of kohe Hassan khel FR Peshawar.

Preparation of aqueous leaves extract

Using distilled water, the *W. coagulans* leaves that had been gathered were cleaned and washed. It was then dried in the shade at room temperature. After that, an electric grinder was used to powder the dried leaves. The pulverized leaves weighed a total of 4 kg. 25 grams of the 4 kg of powdered leaves *were* cooked in 500 mL of distilled water in a sterile flask. The extract was taken out using Whattman filter paper No. 1 after 30 minutes of boiling. Finally, filtrate was obtained and stored in sterile bottles with blue caps for further use (Ahmad *et al.*, 2017).

Phyto-synthesis of AuNPs

In a sterile conical flask, 10 mL of an aqueous leaf extract was combined with 90 mL of an $AuCl_3$ solution (ImM). The reactant mixture was next placed in a water bath that was shaken for an hour at 75 °C. The accurate creation of AuNPs is indicated by the colour change from yellowish green to dark blackish brown at the end of the incubation period. The typical spread drying approach was used to dry the bio-reduced AuNPs, using a hot plate set at 50 °C. After then, using an ultra-centrifuge at 12.000 rpm, the dried AuNPs were scratched and filtered. The supernatants were then discarded, and pellets of pure AuNPs were collected and dried in hot air ovens at a temperature of 50 °C onto sterilized petri plates (Khan *et al.*, 2021).

Spectroscopic characterization of Phyto-synthesized AuNPs

Ultraviolet-Visible Spectrophotometery

Using an ultraviolet spectrophotometer, the maximum peak absorbance of Phyto-synthesized AuNPs was examined. The peak was seen between 400 and 600 nm in wavelength (Ahmad *et al.*, 2017).

Transmission Electron Microscope (TEM)

Using a TEM (Techni-G2-300 kV), the size of green AuNPs was examined. On the carbon-coated copper grid, a tiny layer of test AuNPs solution was applied before being vaporized for five minutes using a mercury vapour lamp. Last but not least, a 2D micrograph of test AuNPs displaying the size was noticed (Ahmad *et al.*, 2017).

In-vitro studies of AuNPs

Antibacterial Activity

The agar well diffusion assay was used to assess the antibacterial activity of AuNPs (Ahmad *et al.*, 2017). In sterile petri plates, autoclaved nutrient agar (Sigma-Aldrich, Germany) was added. Using an 18 – 24 hour-old culture and a sterile borer, a uniform bacterial lawn was created on petri plates. In each well, 100 µL of a stock solution (3 mg/mL) of AuNPs in dimethyl sulphoxide (DMSO, 1%) was added. Both DMSO and amoxicillin were utilized as controls, with DMSO serving as the positive control. Zones of inhibition (mm) were measured after 24 hours of incubation at 37 °C, and percent inhibition was computed using the provided formula.

$$\%\ \text{inhibition} = \frac{\text{Zone of inhibition of sample(mm)}}{\text{Zone of inhibition of positive control (mm)}} \times 100$$

Antifungal Activity

AuNPs' antifungal activity was assessed using the agar tube dilution assay (Ahmad *et al.*, 2017). AuNPs stock solution (24 mg/ml) was made in DMSO. Sigma-Aldrich Germany's Sabouraud dextrose agar, 4 ml, was placed in test tubes and autoclaved at 121°C for 15 minutes at 21 psi. After the tubes had cooled to room temperature (50 °C), 70 µL of stock solution was added, and it was left to harden in a slanting posture. Fresh cultures of test fungi were inoculated at designated slants using a sterile inoculating loop. As positive and negative controls, respectively, miconazole and DMSO were used. For 5 – 7 days, all the test tubes were incubated at 28 °C. The visible linear growth inhibitions of the fungus strains were calculated in comparison to controls after incubation.

$$\% \text{ inhibition} = \frac{\text{Linear growth of fungi in test sample(mm)}}{\text{Linear growth of fungi in standard (mm)}} \times 100$$

Results

Spectroscopic characterization of Phyto-synthesized AuNPs

Ultraviolet-Visible Spectrophotometery

According to UV-Vis spectrophotometer study, the λmax absorbance for AuNPs is at 550 nm, which supports the accurate synthesis of AuNPs. In comparison, the λmax absorbance peak was missing in the aqueous extract of *W. coagulans* leaves. Fig. 1 and Fig. 2 present a summary of the findings.

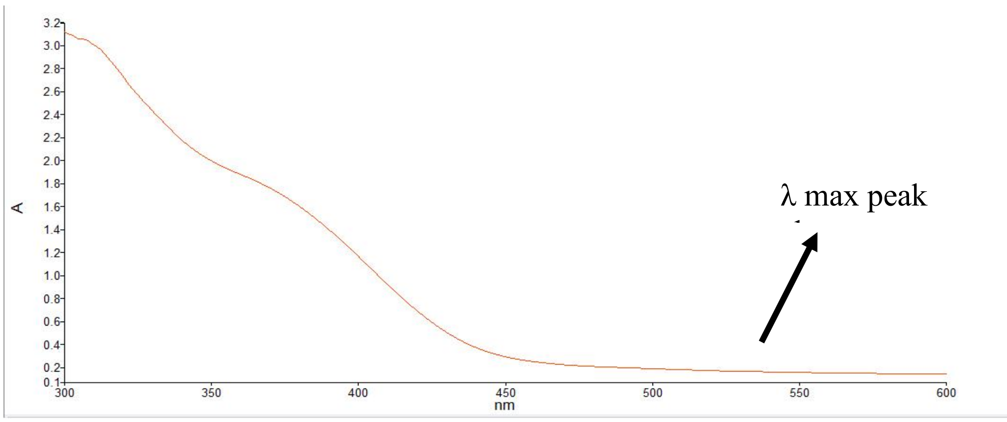

Fig. 1. UV-Vis analysis of aqueous leaves extract

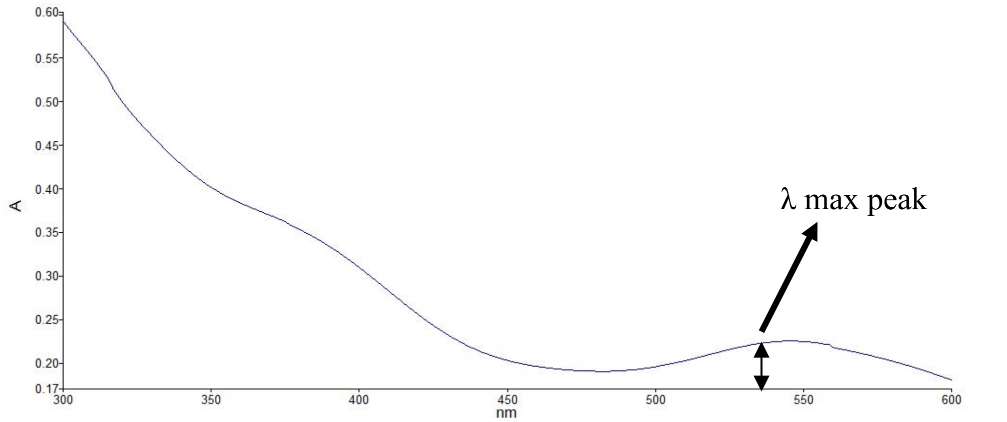

Fig. 2. UV-Vis analysis of green AuNPs

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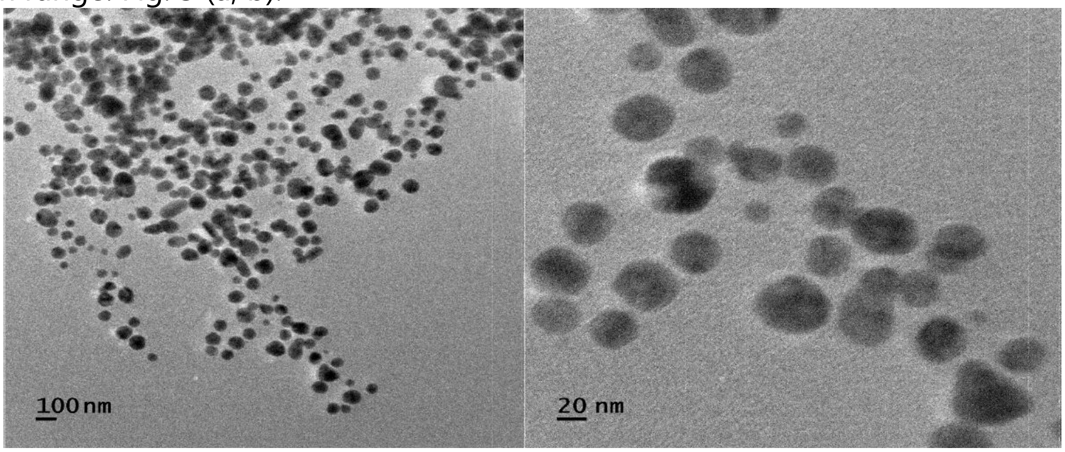
Transmission Electron Microscope (TEM)

According to TEM examination, the bio-reduced AuNPs are generally spherical in shape. A few aberrant morphologies were also discovered, which could be the result of nano-spheriods aggregation. The average size of these nano-spherical AuNPs was in the 20 – 100 nm range. Fig. 3 (a, b).



(a) _ 3 (a , b). TEM analysis of green A (b)

In-vitro studies of AuNPs

From the comparative in-vitro assays of AuNPs and aqueous leaves extracts, following results were observed;

Antibacterial Assay

The comparative antibacterial activity of aqueous extracts of leaves and AuNPs revealed that AuNPs have good bactericidal activity against *S. aureus* (63%), moderate activity against *E. coli* (40%), *S. pyogenes* (45%), and *K. pneumonia* (54%), and poor activity against *P. aeruginosa* (22%) and *S. typhi* (26%). Aqueous extract of leaves, on the other hand, displayed limited action against all of the test bacterial species except *P. aeruginosa*, which was resistant. The results are displayed in Fig. 4.4.

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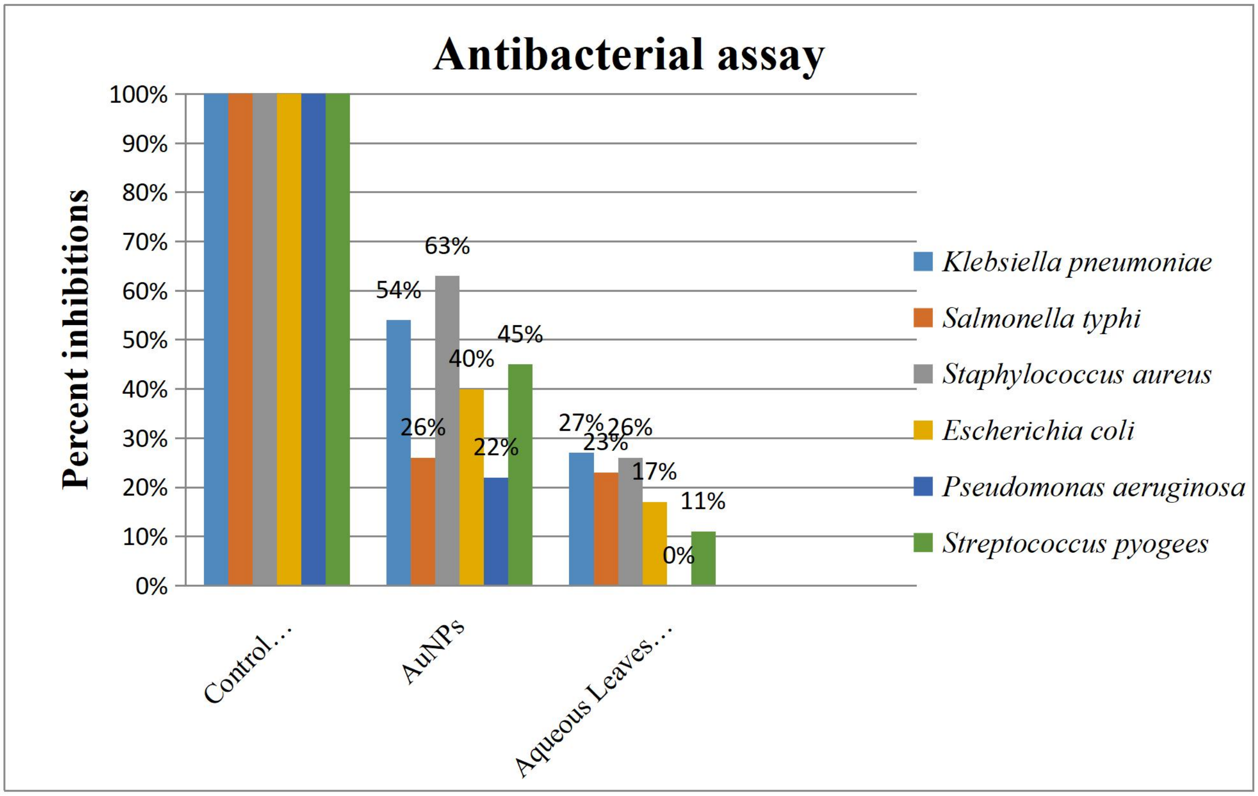

Fig. 4.4. Comparative antibacterial assay of AuNPs and crude aqueous extract from leaves of W. coagulans

Antifungal Assay

In the comparative antifungal testing, AuNPs effectively inhibited all of the test fungal growth. The highest levels of inhibition were seen in *F. solani* (92%), *F. oxysporum* (91%), and *M. furfur* (90%).Similar inhibition pattern was observed in *C. albicans* and *Penecillium spp*. showed a similar growth inhibition pattern, with 89% inhibition. *T. harizanum* (75%) and *A. flavus* (74%), both had high antifungal activity. The aqueous leaves extract, on the other hand, had moderate to good efficacy against all of the test fungus species. *Penecillium spp*. (69%) and *F. oxysporum* (67%), had the strongest inhibitory activity. While *A. flavus* was only 29% inhibited. Fig. 5.

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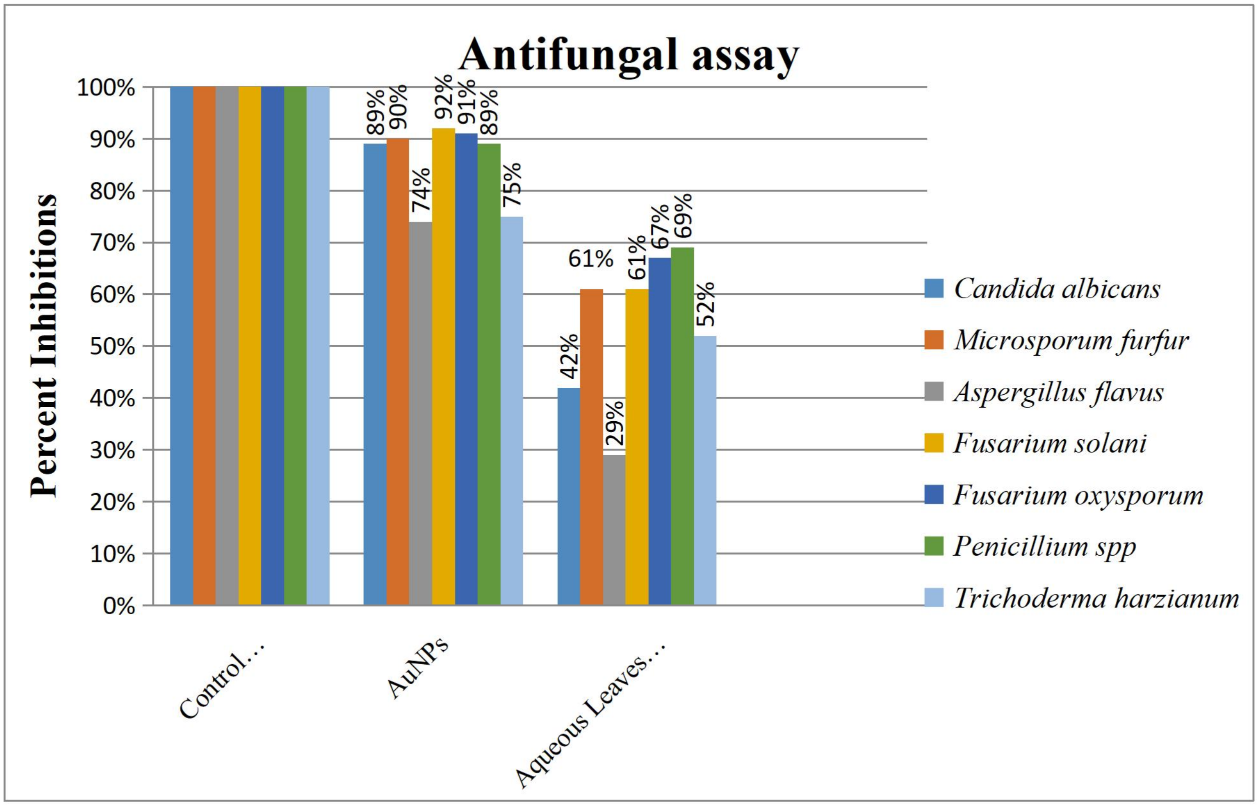

Fig. 5. Comparative antifungal assay of AuNPs and crude aqueous extract from leaves of W. coagulans

DISCUSSION

Nanobiotechnology, often known as nanobiology, is a trendy branch of science that focuses on manipulating atoms to shape into desirable products. Currently, nano-biologists have combined engineering, agriculture, and therapeutics with nanotechnology to create efficient commodities such as biofertilizers, tailored biosensors and medications, diagnostic tools, and even nano-infused coatings (Xiao *et al*., 2019). There are effective chemical and physical ways for mass-producing these nanostructures. However, the majority of these treatments are highly toxic, expensive, and not cost-effective (Khan *et al*., 2019). Switching to a green process that uses microorganisms, plants, and enzymes is a positive step forward for the industry (Rodriguez-Luis *et al*., 2016).

Metal nanoparticles bio-reduced from their ionic state to particle form utilizing plant extracts produced excellent results in past years. These phyto-synthesized metal nanoparticles, including silver, gold, zinc, and copper, have been used to assist mankind in a variety of nanodevices (Ramalingam *et al*., 2019). Gold nanoparticles synthesised

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from plant extracts are chosen over traditional methods because they are environmentally benign, cost effective, and have great therapeutic effects (Pissuwan *et al*., 2020). Many plants, including *Aloe vera*, *Bauhinia purpurea*, *Cinnamomum camphora*, *Dodonaea, Musa acuminata*, *Mentha longifolia*, and *Emblica officinalis*, have been studied for their ability to create AuNPs. These nano-gold particles have a solid crystalline structure and range in size from 10 to 100 nm.

The AuNPs had round and oval morphologies and may agglomerate during a prolonged incubation period (Rauf *et al*., 2021). Similarly to the current study, the leaves of *W. coagulans* contain phenolic chemicals and reducing sugars, which mediate the cost-effective and environmentally friendly synthesis of AuNPs. AuNPs have the same morphology as previously manufactured AuNPs, i.e. 100 nm. Few irregular objects were also typically spherical in shape with sizes ranging from 20 – 100 nm.

It has recently been shown that green AuNPs have enormous therapeutic effects ranging from cosmetic enhancement to the treatment of chronic malignancies. It was previously discovered that aqueous leaf extracts of *Opuntia ficus-indica*, *Anacardiium occidentale*, *Bauhinia tomentosa*, *Chenopodium album*, *Dracocephalum kotschyi* and *Camellia sinensis* have potentials to biosynthesize AuNPs. These AuNPs were observed to lie in the range between 10 – 200 nm. These fabricated nanoparticles were investigated to act in synergism with that of plant extracts to treat many bacterial infections. The AuNPs were found to be active against *E. coli*, *Staphylococcus aureus*, *Pseudomonas* and *Salmonella* species (Nadeem *et al*., 2017). Similar to current study, leaves mediated AuNPs of *W. coagulans* were observed to be active against *Staphylococcus aureus*, *Klebsiella pneumonia*, *E. coli* and *Streptococcus* species.

The green AuNPs were observed previously to efficiently possess antifungal properties. It was previously evidenced that AuNPs synthesized using aqueous leaves extract of *Punica granatum*, *Pistacia integerrima* and *Nepenthes khasiana* can inhibit the mycelial growth of *Candida albicans, Aspergillus flavus* and *Microsporum gypseum* (Amina *et al*., 2020). Similar like the current study, AuNPs fabricated using aqueous leaves extracts of *W. coagulans* can inhibited the fungal proliferation of *T. harizanum*, *F. solani*, *M. furfur*, *F. oxysporum*, *Penecillium* and *Aspergillus flavus*.

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