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Biosynthesis and characterization of gold nanoparticles using leaves extract of *Piliostigma thonningii* and their antimicrobial activity

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Abstract

Synthesis and characterization of gold nano-particles using leaf extracts of *Piliostigma thonningii* and Estimation of antimicrobial and antifungal activity. Method: Green approach has been utilized for the synthesis of gold nano-particles. Different aqueous plant extracts has been prepared which was then utilized for the biosynthesis of gold nano-particles. Estimation for the synthesis of nano-particles were done using UV-Visible spectroscopy and Fourier- Transform infrared spectroscopy. Antimicrobial and antifungal activity of gold nano-particles prepared using aqueous extract was investigated using disc diffusion method. FTIR of collected nano-particles gave an idea about the type of bio-molecules which helped in the reduction of auric and silver salts into corresponding nano-particles. Antimicrobial and antifungal activity of gold nano-particles showed that the nano-particles have better anti-microbial activity (kept as standard) when experiments were performed under similar conditions.

Keywords: gold nano-particles, *Piliostigma thonningii*, antimicrobial and antifungal activity, FTIR.

Introduction

Natural products once served humankind as the source of all drugs, and higher plants provided most of these therapeutic Agents. Today, natural products (and their derivatives and analogs) still represent over 50% of all drugs in clinical use, with higher plant-derived natural products representing ca. 25% of the total (Balandrin et al, 1993). The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involves the use of plant extracts. This means that about 3.5 to 4 billion people in the world rely on plants as sources of drugs (Farnsworth 1988). In the United States plant-derived drugs represent about 25% of the prescription drugs market, and in 1991 this equated to a retail value of approximately $15.5 billion (Pezzuto 1997). From 1983 to 1994 39% of the New Approved Drugs were of natural origin, including original natural products, products derived semi synthetically from natural products, and synthetic products based on natural product models. (Cragg et al, 1997). Further evidence of the importance of natural products is provided by the fact that almost half of the world's 25 best selling pharmaceuticals in 1991 were either natural products or their derivatives (O'Neill and Lewis 1993). Conservative estimates suggest that there are more than 250,000 species of higher plants existing on this planet, and only a very small percentage of plants have been exhaustively studied for their potential value as a source of drugs. Obviously natural products will continue to be extremely important as sources of medicinal Agents.

In addition to the natural products which have found direct medicinal application as drug entities, many others can serve as chemical models or templates for the design, synthesis, and semi synthesis of novel substances for treating humankind's diseases. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer-based molecular modelling design, none of them can replaced the important role of natural products in drug discovery and development.

The term "nanotechnology" describes the field of developments in which size-dependent properties of materials in the nanometre regime play a dominant role, and where these properties can be used to generate new techniques and devices (Schmid et al, 2009). The materials can include nanoparticles with dimensions of less than 100nm as well as patterned surfaces and more sophisticated assemblies. Nanotechnology is an important field of modern research dealing with design, synthesis, and manipulation of particles structure ranging from approximately 1-100 nm in one dimension. Nanotechnology is an interdisciplinary Field with contributions from physics, chemistry, biology, materials science, medicine and other disciplines. Remarkable growth in this up-and-coming technology has opened novel fundamental and applied frontiers, including the synthesis of nanoscale materials and exploration or utilization of their exotic physicochemical and optoelectronic properties.

Nanotechnology is rapidly gaining importance in a number of areas such as health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, energy science, optoelectronics, catalysis, reprography, single electron transistors, light emitters, nonlinear optical devices, and photo electrochemical applications (Colvin and Alivisatos 1994; Wang 1991). Nanomaterials are seen as solution to many technological and environmental challenges in the field of solar energy conversion, catalysis, medicine, and water treatment. In the context of global efforts to reduce hazardous waste, the continuously increasing demand of nanomaterials must be accompanied by green synthesis methods. Nanotechnology is fundamentally changing the way in which materials are synthesized and devices are fabricated. Incorporation of nanoscale building blocks into functional assemblies and further into multifunctional devices can be achieved through a "bottom-up approach". Research on the synthesis of nanosized material is of great interest because of their unique properties like optoelectronic, magnetic, and mechanical, which differs from bulk (Atul et al, 2010). Nanoparticles can be broadly grouped into two, namely, organic nanoparticles which include carbon nanoparticles (fullerenes) while, some of the inorganic nanoparticles include magnetic nanoparticles, noble metal nanoparticles (like gold and gold) and semi- conductor nanoparticles (like titanium oxide and zinc oxide). There is a growing interest in inorganic nanoparticles i.e. Of noble metal nanoparticles (gold and silver) as they provide superior material properties with functional versatility. Due to their size features and advantages over available chemical imaging drug Agents and drugs, inorganic particles have been examined as potential tools for medical imaging as well as for treating diseases. Inorganic nonmaterial have been widely used for cellular delivery due to their versatile features like wide availability, rich functionality, good compatibility, and capability of targeted drug delivery and controlled release of drugs (Xu et al, 2006). In the present study synthisis and characterization of gold nano particles using leaf extract of *Piliostigma thonningii* and thier effects on microbes were reported.
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Materials and Methods

Plant extract preparation

Fresh leaves of *Piliostigma thonningii*, (Family-Fabaceae), were collected from in Tiruchirappalli district, Tamil nadu, and washed several times with water to remove the dust particles and then Shade dried to remove the residual moisture and grinded to form powder. Then plant extract was prepared by mixing 1% of plant extract with deionized water in a 250ml of (Borosil, India) conical flask. Then the solution was incubated for 30 min. and then subjected to centrifuge for 30 min at room temperature with 5000 rpm. The supernatant was separated and filtered with (mm filter paper) filter paper with the help of vacuum filter. Then the solution was used for the reduction of gold ions (Au+) to gold nanoparticles (Auo).

Figure 1. *Piliostigma thonningii* Plant leaf and their Powder form

Synthesis of gold nanoparticles

For the synthesis of Gold nanoparticles, gold chloride prepared at the concentration of 10^{-3} M with pre-sterilized Milli Q water was used. A quantity of 1.5 ml of each extract was mixed with 30 ml of 10^{-3} M of gold chloride for the synthesis of gold nanoparticles. Gold chloride was taken in similar quantities of 1.5 ml each without adding plant extracts to main respective controls. The saline bottles were tightly covered with aluminium foil in order to avoid photo reduction of gold ions, incubated at room temperature under dark condition and observations were recorded.

Characterization of gold nanoparticles

UV-vis analysis

The optical property of AuNPs was determined by UV-Vis spectrophotometer (Perkin-Elmer, Lamda 35, Germany). After the addition of $HAuCl_4$ to the plant extract, the spectra's were taken in different time intervals up to 24hrs between 450 nm to 540 nm. Then the spectrum was taken after 24hrs of $HAuCl_4$ addition.

FTIR analysis

The chemical composition of the synthesized Gold nanoparticles was studied by using FTIR spectrometer (perkin-Elmer LS-55- Luminescence spectrometer). The solutions were dried at 75^{o} C and the dried powders were characterized in the range 4000–400 cm-1 using KBr pellet method.

SEM analysis

The morphological features of synthesized gold nanoparticles from *P. thonningii* plant extract were studied by Scanning Electron Microscope (JSM-6480 LV). After 24Hrs of the addition of $HAuCl_4$ the SEM slides were prepared by making a smear of the solutions on slides. A thin layer of platinum was coated to make the samples conductive. Then the samples were characterized in the SEM at an accelerating voltage of 20 KV.

DLS & Zeta potential analysis

Dynamic light scattering (DLS) which is based on the laser diffraction method with multiple scattering techniques was employed to study the average particle size of gold nanoparticles. The prepared sample was dispersed in deionized water followed by ultra-sonication. Then solution was filtered and centrifuged for 15 min. at 25^{0}C with 5000 rpm and the supernatant was collected. The supernatant was diluted for 4 to 5 times and then the particles distribution in liquid was studied in a computer controlled particle size analyzer (ZETA sizer Nanoseries,Malvern instrument Nano Zs).

Testing of antimicrobial activity

The test strains were: *Aeromonas liquefaciens* MTCC 2645 (B1), *Enterococcus faecalis* MTCC 439 (B2), *Klebsiella pneumonia* NCIM 2883 (B3), *Micrococcus luteus* NCIM 2871 (B4), *Salmonella typhimurium* NCIM 2501 (B5), *Vibrio cholerae* MTCC 3906 (B6), *Candida albicans* MTCC 1637 (F1), *Cryptococcus* sp. MTCC 7076 (F2), *Microsporum canis* MTCC 3270 (F3), *Trichophyton rubrum* MTCC 3272 (F4). The cultures were obtained from MTCC, Chandigarh and NCIM, Pune, India.

Microbial strains were tested for antimicrobial sensitivity using the disc diffusion method (Bauer et al 1966). This method was used to evaluate in vitro antibacterial and antifungal activity of test sample against certain human pathogenic microorganisms on Muller Hinton agar (MHA) and potato dextrose agar (PDA), respectively. A sterile cotton swab was used to inoculate the standardized bacterial suspension on surface of agar plate. The 15 and 30 µL of test solutions were poured in each disc (6 mm diameter), separately. One separate disc was used for control study by taking sterile triple distilled water (without test sample). The plates were incubated at 37±1°C for 24–48 h (for bacteria) and 25 ±1°C for 48-72 h (for fungus). After incubation, the zone of inhibition was measured with ruler/HiAntibiotic ZoneScale-C. The assays were performed in triplicate and the average values are presented. Methicillin – 10mcg (for bacteria) and Itraconazole – 10mcg (for fungus) was used as positive control. All the media, standard discs and HiAntibiotic ZoneScale-C were purchased from Hi-Media (Mumbai, India).

Results and Discussion

Characterizations of AuNPs

UV-Vis spectrophotometer analysis

Reduction of gold salt into gold nanoparticles during exposure to plant extracts was observed as a result of the colour change. The colour change is due to the Surface Plasmon Resonance (SPR) phenomenon. The metal nanoparticles have free electrons, which give the SPR absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave. The sharp bands of gold nanoparticles were observed around 540 nm in case of *P. thonningii*. From different literatures it was found that the gold nanoparticles show SPR peak at around 540 nm. From our studies we found the SPR peak for *P. thonningii* at 540 nm.

So we confirmed that *P. thonningii* leaf extract has more potential to reduce Au ions into Au nanoparticles, which lead us for further research on synthesis of gold nanoparticles from *P. thonningii* leaf extracts. The intensity of absorption peak increases with increasing time period. This characteristic colour variation is due to the excitation of the SPR in the metal nanoparticles. The reduction of the metal ions occurs fairly rapidly; more than 90% of reduction of Au+ ions is complete within 2 Hrs. after addition of the metal ions to the plant extract. The metal particles were observed to be stable in solution even 4 weeks after their synthesis. By stability, we mean that there was no observable variation in the optical properties of the nanoparticles solutions with time.

On the behalf of UV-vis data it was cleared that reduces metal ions. So the further characterizations were carried out with *P. thonningii* (Figure. 2). The UV-Vis absorption spectroscopy is one of the main techniques followed to examine size and shape of the nanoparticles in the aqueous suspensions (Wiley et al. 2006). Huang et al. (2007) reported formation of gold nanoparticles when constant aqueous $HAuCl_4$ at 50 ml, 1 mM with 0.1 g biomass produced gold nanoparticles as indicated by sharp absorbance at around 540 nm in *Cinnamomum camphora.*

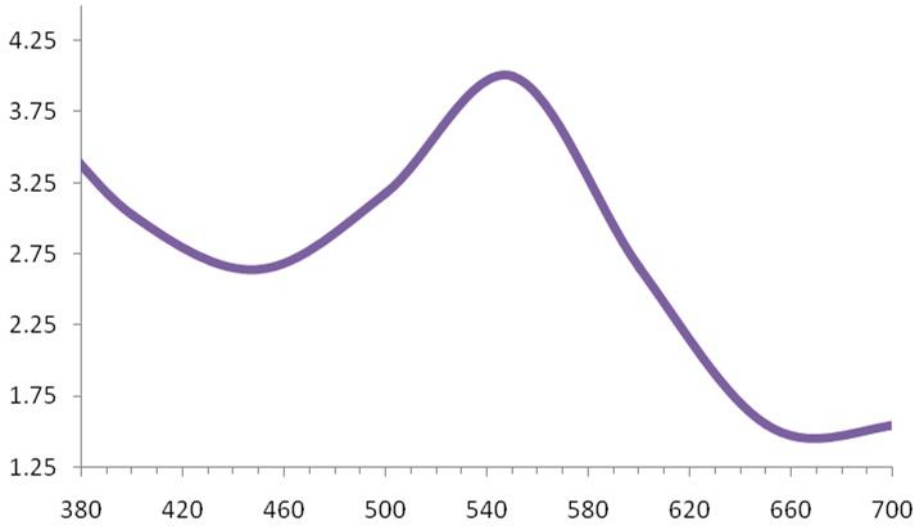


Figure 2. UV-VIS spectral analysis of Au nanoparticles

FTIR analysis

FTIR measurements were carried out to identify the biomolecules for capping and efficient stabilization of the metal nanoparticles synthesized. The FTIR spectrum of gold nanoparticles wherein some pronounced absorbance was recorded in the region between 4000 and 400 cm^{-1}. The FTIR spectra of aqueous *P. thonningii* extracts and AuNPs are shown in Figure 3 and 3a respectively. *P. thonningii* extract have shown in Figure. 3 the main peaks at around 3270, 2900, 1616, 1388, 766, 672 cm-1 whereas, the gold nanoparticles in the presence of *P. thonningii* extract shows Figure. 3a the major peaks at 3434, 2361, 2076, 1636, 1403.45, 1113.74 and 670.92 cm-1.

P. thonningii extract shows the peak at 3270 cm-1 can be assigned to O-H stretch and peak at 2900 cm-1 corresponds to C-H stretch. The band at 1616 cm-1 is assigned to C=O stretch. The band found at 1388 cm-1 can be assigned to C-O-C stretch. Another band at 766 cm-1 and 672 cm-1 assigned to C-H bend and C-H bond respectively. After synthesis the gold nanoparticles by the *P. thonningii* extracts have the broad peak at 3434 cm-1 assigned to O-H stretch and also peak at 2361 cm-1 assigned to O-H stretch. The band at 2076 cm-1 and 1636 cm-1 are assigned to C-H stretch and C=O stretch respectively. The band found at 1403.45 cm-1 and 113.74 cm-1 are assigned to C-O-C stretch. Another band found at 670.92 cm-1 assigned to C-H bond.

Therefore, the synthesized nanoparticles were surrounded by proteins and metabolites having functional groups. From the analysis of FTIR studies we confirmed that the carbonyl groups from the amino acid residues and proteins has the stronger ability to bind metal indicating that the proteins could possibly from the metal nanoparticles (i.e.; capping of gold nanoparticles) to prevent Agglomeration and thereby stabilize the medium. This suggests that the biological molecules could possibly perform dual functions of formation and stabilization of gold nanoparticles in the aqueous medium. Carbonyl groups proved that flavanones or terpenoids absorbed on the surface of metal nanoparticles.

Flavanones or terpenoids could be adsorbed on the surface of metal nanoparticles, possibly by interaction through carbonyl groups or π-electrons in the absence of other strong ligating Agents in sufficient concentration. The presence of reducing sugars in the solution could be responsible for the reduction of metal ions and formation of the corresponding metal nanoparticles. It is also possible that the terpenoids play a role in reduction of metal ions by oxidation of aldehydic groups in the molecules to carboxylic acids. These issues can be addressed once the various fractions of the plant extract are separated, identified and individually assayed for reduction of the metal ions. This rather elaborate study is currently underway.

Table 1. Vibration modes of synthesized NP's

Wave number (cm^{-1})	Vibration modes
3434	O-H Stretch
2361	O-H Stretch
2076	C-H Stretch
1636	C=O Stretch
1403.45	C-O-C Stretch
1113.74	C-O-C Stretch
670.92	C-H bond

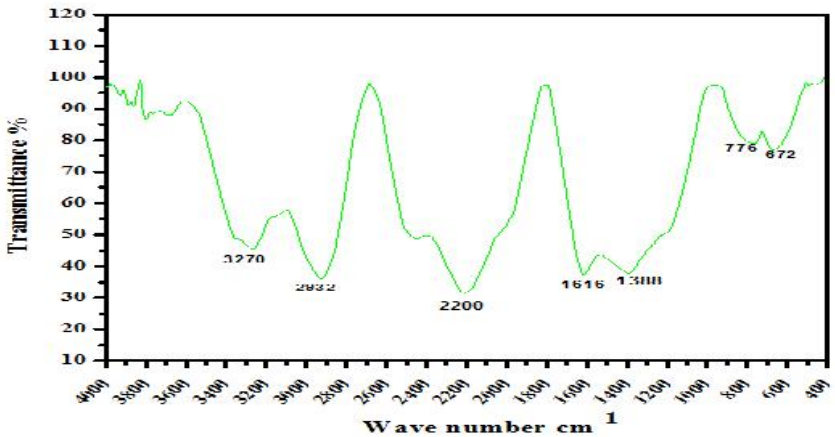


Figure 3. FTIR analysis of vibration modes and function groups of *P. thonningii*

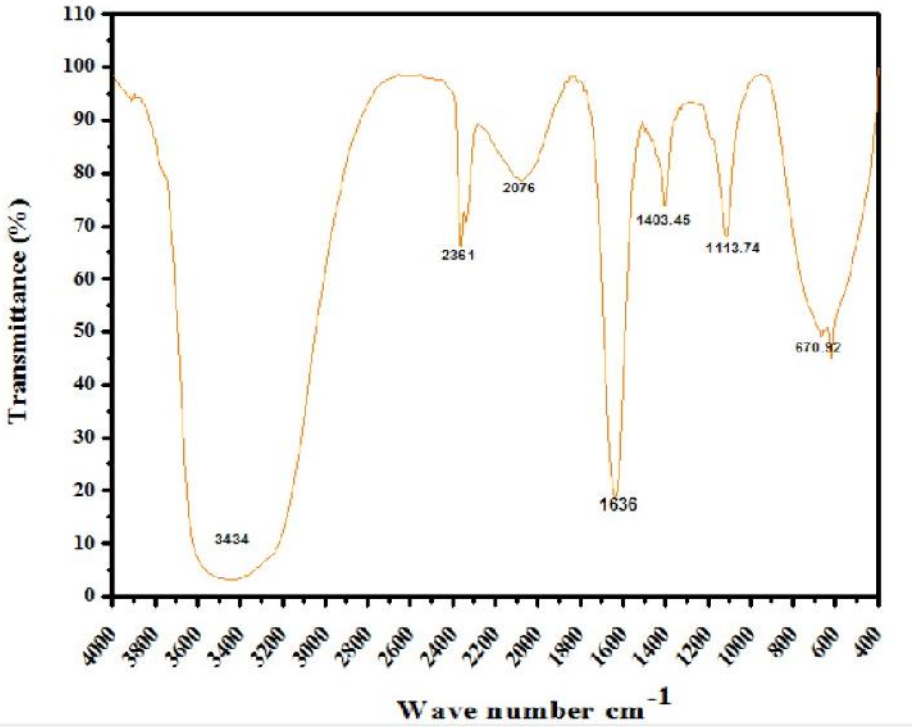


Figure 3a. FTIR analysis of vibration modes and function groups of AuNPs

SEM analysis

SEM provided further insight into the morphology and size details of the gold nanoparticles. Comparison of experimental results showed that the diameters of prepared nanoparticles in the solution have sizes several nano meters i.e. between 1-100 nm. The size was more than the desired size as a result of the proteins which were bound in the surface of the nanoparticles (Figure 4).

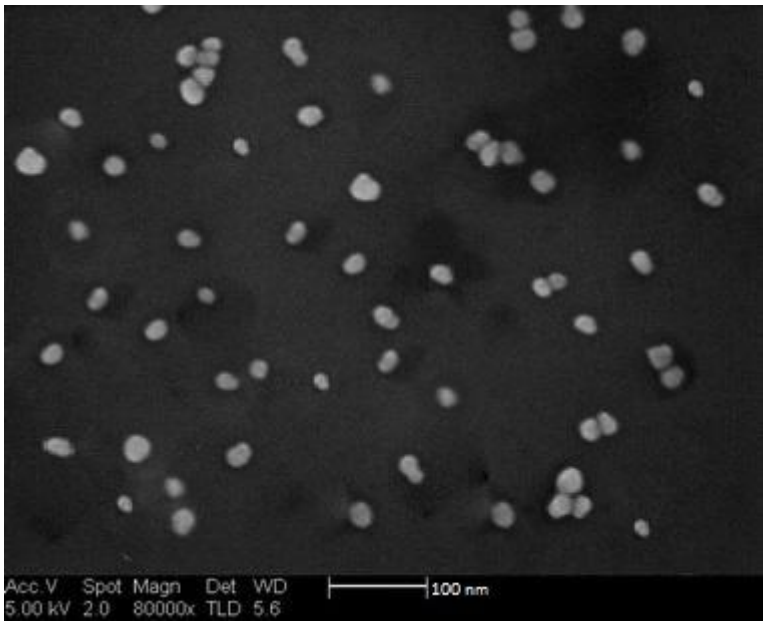
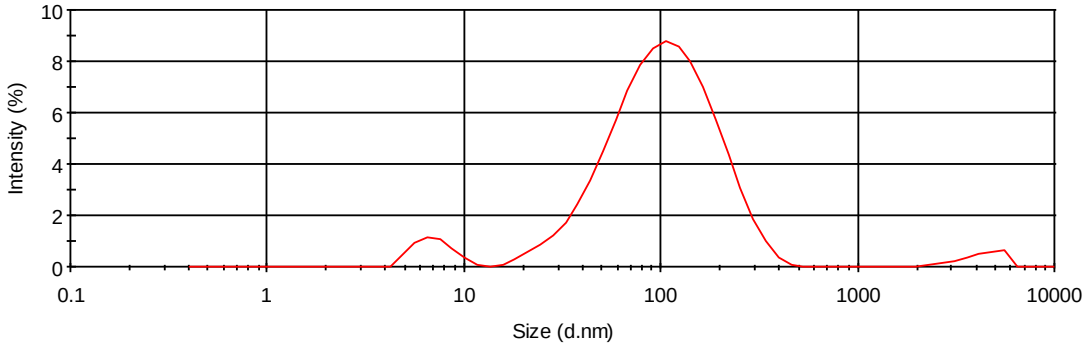


Figure 4. SEM –microscopic view of *P. thonningii* reduced gold nano particles

DLS analysis

The particle size distribution (PSD) of synthesized gold nanoparticles, it was found that Au nanoparticles size were in the range of 80-120nm. However, beyond 100 nm range the percentage of nanoparticles present is very less. The highest fraction of AuNPs present in the solution was of 73nm is very appropriate since it gives lowest average size of nanoparticles.



P. thonningii (Gold Nano particles); Z-Average (d.nm): 73.26

Figure 5. Dynamic Light Scattering of Particle Size Analyser of Au Nanoparticles

Zeta potential analysis

The Figure 6 shows the zeta potential (ζ) is a measure of the electrostatic potential on the surface of the nanoparticles and is related to the electrophoretic mobility and stability of the suspension of nanoparticles of the nanogold. The overall absorbance of Zeta Potential revealed the energetically very unstable. Therefore, the particles undergo Agglomeration/Aggregation to stabilize themselves. So there were some potential charges on the surface of the nanoparticles which makes them stable. These charge potential we got from this analysis. Zeta potential (Surface potential) has direct relation with the stability of a form/structure as mentioned below (Table 2; Figure 6).

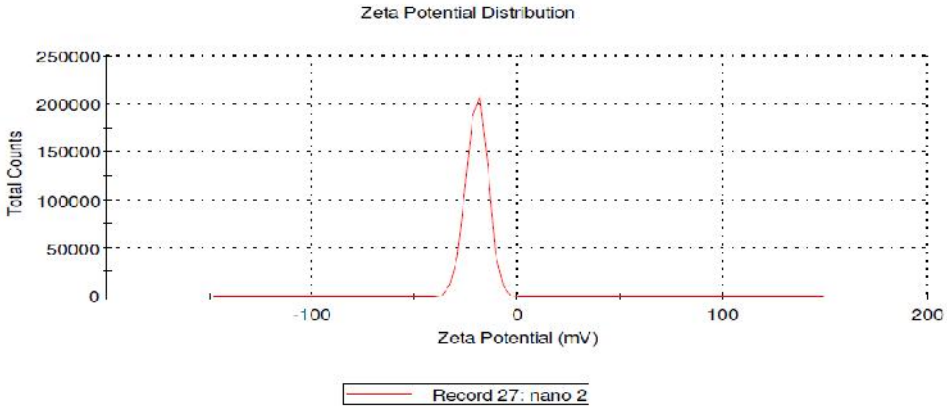


Figure 6. Zeta Potential Measurement of Au Nanoparticles

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -19.7	Peak 1:	-19.7	100.0	5.29
Zeta Deviation (mV): 5.29	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.820	Peak 3:	0.00	0.0	0.00
Result quality : Good				

Zeta Potential (mV): from ±10 to ±30 = Incipient instability

Table 2. A table showing the stability of the NPs according to the potential charge

Zeta potential [mV]	Stability behaviour of the colloid
from 0 to ±5	Rapid coagulation or flocculation
from ±10 to ±30	Incipient instability
from ±30 to ±40	Moderate stability
from ±40 to ±60	Good stability
more than ±61	Excellent stability

Antibacterial and antifungal screening

Gold nanoparticles were tested in triplicates for antimicrobial activity. The values were recorded and averaged (Table 3 and Plate 1). *P. thonningii* has tested and recorded the results for the gram-positive, gram-negative bacteria and fungi. The gram-positive were highly sensitive than gram-negative bacteria. Selected microorganisms were showed significant sensitivity against the biosynthesized nanoparticles. The antimicrobial activity of test sample was examined with various pathogenic microorganisms using the (measure the inhibition zone) Disc diffusion test.

Table 3. Antimicrobial screening of AuNPs derived by *P. thonningii* leaves

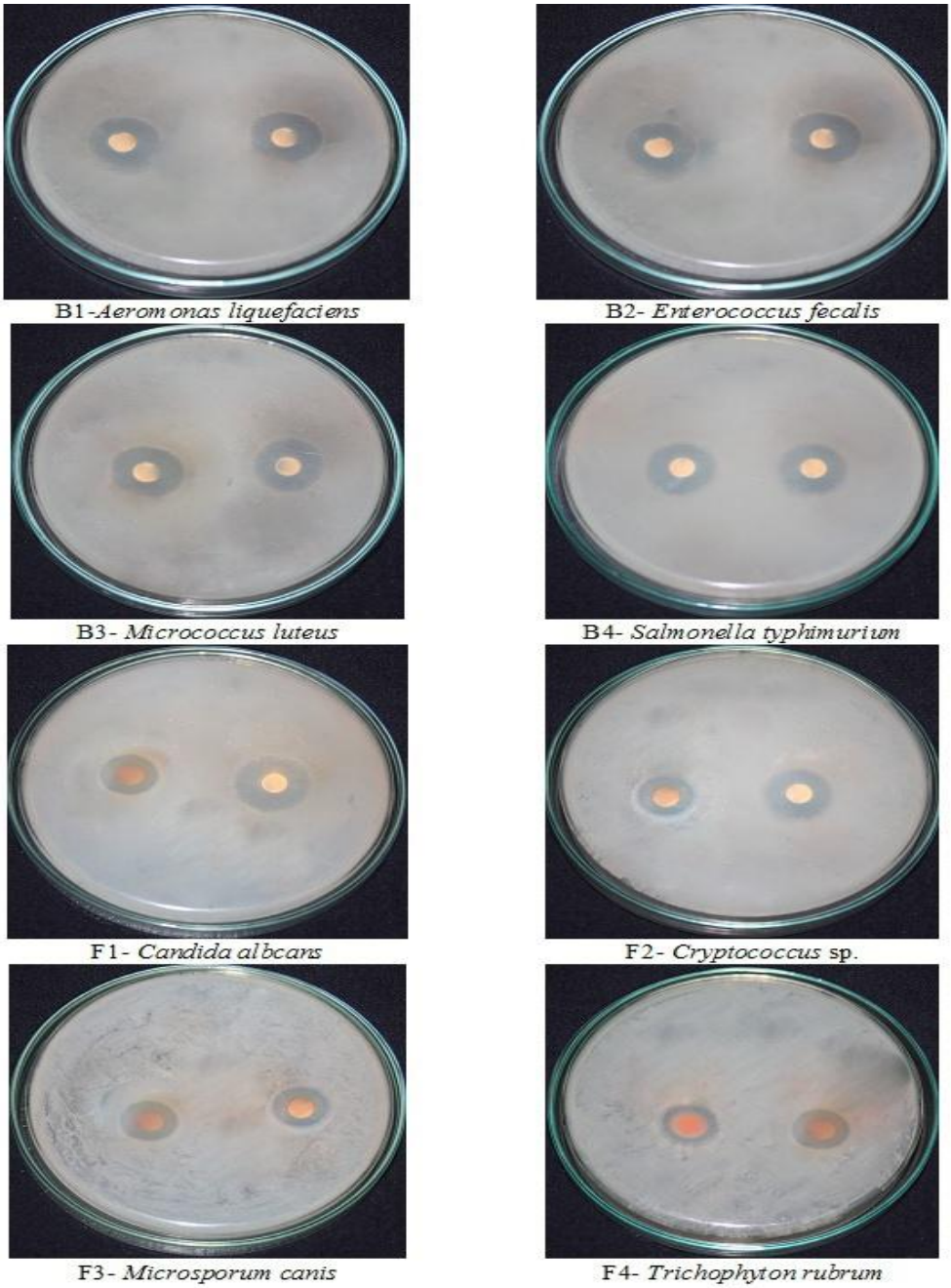
S.No	Test Microorganisms	Zone of inhibition (mm) Sample (15 & 30) µL / disc				Diseases	Route of Transmission
	Bacteria	**15 µL**	**30 µL**	**PC**	**Remarks**		
1	*Aeromonas liquefaciens* B1	16	18	14	> PC	Wound Infections / Gastroenteritis	Water / Food
2	*Enterococcus fecalis* B2	15	17	8	> PC	Endocarditis / Bladder, Prostate, and Epididymal Infections / Nervous system Infections	Water / Food
3	*Micrococcus luteus* B3	14	16	38	< *PC*	Skin & Pulmonary infections / Septic shock / Pneumonia endocarditis	Soil / Dust / Water / Airways / Food
4	*Salmonella typhimurium* B4	15	18	0	> PC	Typhoid	Water / Food
	Fungi						
5	*Candida albicans* F1	12	14	10	> PC	Skin (Integument) Infections / Gastrointestinal tract Infection	Airways / Wound / Soil / Water
6	*Cryptococcus* sp. F2	12	13	9	> PC	Cryptococcal disease / Bronchiectasis / Endophthalmitis.	Airways / Wound / Soil / Water
7	*Microsporum canis F3*	11	12	9	> PC	Tinea capitis /Ringworm	Airways / Wound / Soil / Water
8	*Trichophyton rubrum* F4	13	15	7	> PC	Tinea corporis / Tinea cruris / Tinea pedis / Onychomycosis	Airways / Wound / Soil / Water

PC - Positive Control (Using antibiotic disc; Bacteria – Methicillin (10mcg/disc) ; Fungi – Itraconazole (10mcg/disc) Samples - 15 µL / disc & 30 µL / disc; > PC– greater than positive control; < PC – less than positive control

The results of the antimicrobial activities are summarized in Plates 1. In the present study, higher (30 µL/disc) concentration of sample got greater sensitivity than (15 µL/disc) lower concentration in all the tested microorganisms. In this study, all the pathogens were fairly affected and nil effect was not observed in the test samples. The gold nanoparticles not only interact at the surface of cell membrane, but also enter inside the bacteria and cause damage of the cells by interacting with phosphorus/ sulfur containing DNA and its replication. In bacteria, the test sample was most effective against B5 while smaller effect was noticed from B4. In fungi, this was effective against F4 whereas smaller effect was observed in F2.

All the microbial strains depict higher sensitivity to the higher concentration (30 µL) and he concluded that the gold materials are an efficient alternative to antibiotics for the treatment. This nanoparticles release gold ions in the bacterial cells, which enhance their bactericidal activity. There is no antimicrobial activity in solution devoid of sample used as a vehicle control (sterile triple distilled water), reflecting that antimicrobial activity was directly related to the sample.

Plate 1. Antibacterial activity of biologically synthesized gold nanoparticles



The rapid biological synthesis of gold nanoparticles using *P. thonningii* leaves extract provides environmental friendly, simple and efficient route for synthesis of benign nanoparticles. The synthesized nanoparticles were of spherical and sheet shaped and the estimated sizes were 60-100 nm. The size was bigger as the nanoparticles were surrounded by a thin layer of proteins and metabolites such as terpenoids having functional groups of amines, alcohols, ketones, aldehydes, etc., which were found from the characterization using UV-vis spectrophotometer, SEM, DLS, Zeta Analyzer, XRD, and FTIR techniques. All these techniques it was proved that the concentration of plant extract to metal ion ratio plays an important role in the shape determination of the nanoparticles. The higher concentrated nanoparticles had sheet shaped appearance whereas the lower concentrations showed spherical shaped. The sizes of the nanoparticles in different concentration were also different which depend on the reduction of metal ions. From the data of DLS it was found that the 30:1 ratio solution had sharp nanoparticles of around 5 nm and some has around 180 nm and they had the potential of around 15.5 mV. Antimicrobial and anticancer studies show the excellent results in the test sample and also give the good results. From the technological point of view these obtained gold nanoparticles have potential applications in the biomedical field and this simple procedure has several advantages such as cost-effectiveness, compatibility for medical and pharmaceutical applications as well as large scale commercial production. Further detailed study will be needed to apply the AuNPs on large scale level production and applications

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