

Red alga *Hypnea musciformis* (Wulf) Lamour mediated environmentally benign synthesis and antifungal activity of gold nano particles

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ABSTRACT

Stable spherical shaped gold nano particles were synthesized using red alga *Hypnea musciformis* (Wulf) Lamour extract as the capping agent as well as reducing agent. The synthesized gold nano particles were characterized by ultraviolet-visible (UV-Vis) spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy. The surface morphology of the synthesized nano particles was characterized using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray diffraction (XRD). The UV-Vis spectrum of the gold nano particles in aqueous medium showed a peak at 552 nm corresponding to the surface plasmon resonance band (SPR) of gold nano particles. TEM micrograph showed the formation of gold nano particles of size 6.25 to 33.33 nm. The XRD spectrum of the gold nano particles exhibited Bragg's pattern of reflection. The antifungal activity of the gold nano particles was tested against *Aspergillus niger* and *Mucor spp.*

KEYWORDS: Antifungal activity, Bio reduction, Gold nano particles, *Hypnea musciformis*, Red alga, TEM, UV-Vis spectrum, X-ray diffraction.

INTRODUCTION

Over the past few decades, extensive research has been done to understand the role and application of biological entities for the environmental remediation of toxic metals and radionuclides (Rai *et al.*, 1981; Whitton 1984; Stephen and Mcnaughton 1999). Among the lower organisms, algae have a tremendous role in bioremediation of toxic and precious metals and their bioconversion to different nontoxic forms (Mehta and Gaur 2005). They not only accumulate metals by chelation and chemical transformation, but are also reported to produce bio-mineral structures and metal nano particles.

Nanotechnology is an emerging area of opportunity that seeks to fuse nano/micro fabrication with bio systems to the benefit both the technologies (Manoj Singh *et al.*, 2011). Nanotechnology provides the broad knowledge of applied science and technology to control the matter from atomic to molecular scale (Natarajan Kannan and Selvaraj Subbalaxmi, 2011). Nanotechnology is an important and emerging technical tool for development of eco-friendly, reliable methodology for the synthesis of nanoscale materials using biological sources (Gilaki, 2010). Nano particles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology (Satyavani *et al.*, 2011).

Synthesis of nano particles using biological entities has great interest due to their unusual optical (Krolikowska *et al.*, 2003), chemical (Kumar *et al.*, 2003), photo electrochemical (Chandrasekharan and Kamat 2000) and electronic (Peto *et al.*, 2002) properties. By using biological entities as capping agents may reduce the toxicological properties of nano particles. A wide variety of physical, chemical and biological processes result in the synthesis of nano particles, some of these are novel and others are quite common. Nature has devised various processes for the synthesis of nano scaled materials which have contributed to the development of relatively new and largely unexplored areas of research. The synthesis and assembly of nanoparticles would benefit from the development of clean, nontoxic and environmentally acceptable “green chemistry” procedures, probably involving organisms ranging from bacteria to fungi and even, plants (Sastry *et al.*, 2004).

Though there are several physical and chemical methods available for the synthesis of metallic nano particles, researchers turned to use biological methods due to their eco-friendly nature. Variety of inorganic nano materials are synthesized by biological processes using bacteria, yeast, and fungi (Kluas *et al.*, 2001). A new biological methods to produce nano particles are constantly being studied and developed.

Various physical and chemical synthesis methods, aimed at controlling the physical properties of the particles, are currently employed in the production of metal nano particles. Most of the methods are still in the developmental stage and various problems are often experienced with the stability of the nano particle preparations, control of the crystals growth and aggregation of the particles (Brust and Liely, 2002). Gold nano particles have been synthesized in large numbers using biological reductive reagents (Ahmad *et al.*, 2003; Shankar *et al.*, 2004; Liu *et al.*, 2005; Gericke and Pinches, 2006; Luo, 2007; Dhamotharan *et al.*, 2010; Rajasulochana *et al.*, 2010; Bhuvaneswari *et al.*, 2011; Swaminathan *et al.*, 2011; Radhika *et al.*, 2012;

Dhamotharan *et al.*, 2012; Thennarasan and Murugesan, 2014; Vishnu Kiran and Murugesan, 2014), reacting with chloroaurate solutions to form colloidal suspensions.

It is well known that the shape of nano particles plays a crucial role in modulating their optical properties and therefore the possibility of developing biological processes to achieve such shape control is exciting. The development of techniques for the controlled synthesis of gold nano particles of well-defined size and shape is a big challenge and numerous chemical methods, aimed at controlling the physical properties of the particles are reported (Burda *et al.*, 2005).

Although the efforts directed towards the biosynthesis of nano materials are recent, the interactions between micro-organisms and metals have been well documented (Beveridge *et al.*, 1997) and the ability of microorganisms to extract and/or accumulate metals is employed in commercial biotechnological processes such as bioleaching and bioremediation. Although gold is the subject of one of the most ancient themes of investigation in science, its renaissance now leads to an exponentially increasing number of publications, especially in the context of emerging Nanoscience and nanotechnology with nano particles and self-assembled monolayers (SAMs).

The existing chemical production processes are regarded as having a relatively high environmental cost, which led to increasing pressure on developing clean, nontoxic, and environmentally benign synthetic technologies. Recently biosynthetic methods employing either microorganism (Mandal *et al.*, 2005) or plant extracts have emerged as sustainable replacements to chemical synthetic procedures. Sastry *et al.*, (2004) reported biological syntheses of gold and silver nanoparticles using microorganisms intracellularly and extracellularly (Ahmad, *et al.*, 2003). Xie *et al.*, (2007) have reported the extra cellular synthesis of gold nano plates in an extract of alga and identified that proteins acted as the primary reducing and shape-directing agent.

In this study, marine red alga *Hypnea musciformis*, recognized as one of the ecologically important alga, commonly existing in the natural environment was investigated for reducing gold ions at room temperature in a single step process to form the stable gold nano particles. The antifungal activity of the gold nano particles have been evaluated against *Aspergillus niger* and *Mucor spp.*

MATERIALS AND METHODS

Collection and preparation of Algal extract

Hypnea musciformis was collected from Mandapam camp on the South East Coast of Tamilnadu, India. They were brought to the laboratory cleaned thoroughly in freshwater followed by distilled water and then shade dried for 3–5 days. The alga was washed and dried in an oven at 60°C, ground with an agate mortar and sieved to a mesh size of <0.5 mm. Before experimentation, the biomass were washed thrice in deionized water to remove the unwanted materials.

Synthesis of Gold nano particles

For the synthesis of gold nano particles, chloroauric acid (HAuCl₄) was used. Double-distilled, deionized water was used for all the experiments. Gold nano particle

formations were carried out by taking 500 mg of dry *Hypnea musiformis* in a 250 mL Erlenmeyer flask with 10^{-3} M aqueous HAuCl_4 solution and incubated at room temperature. The pH was checked during the course of reaction and it was found to be 5.09.

Characterization

The gold nano particles were characterized by UV-Vis spectroscopy, Fourier transform infra-red spectroscopy (FT-IR), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray diffraction (XRD) measurement.

Antifungal Assay

Antifungal activity of gold nano particles was tested against Human pathogenic fungi namely *Aspergillus niger* and *Mucor sp.* The test organisms were the kind gift of the Department of Plant Biotechnology and Microbiology, Lady Dock College, Madurai 2, India.

The disc diffusion method was adopted for antifungal assay. Hi-Media sterile paper discs (SD 067) (6 mm diameter) were impregnated with gold nano particles. The test fungi were maintained on potato dextrose agar (PDA) plates. The medium had the following composition.

Potato Dextrose Agar Medium

Commercially obtained PDA MO 96 (Hi-media) was used. The medium contained Potato infusion from 200 g potatoes:

Dextrose	200 g
Agar	180 g
Sterile Distilled water	1000 mL

Prepared media was sterilized by autoclaving at 1.1 kg/cm^2 pressure (121°C). Seeding of PDA plates with the desired test fungi was done with actively growing cultures with the help of a sterile cotton swab. The impregnated discs were firmly placed on these seeded plates and incubated at $29 \pm 1^\circ\text{C}$ for 48 hrs and observed for zones of inhibition around the discs.

RESULTS AND DISCUSSION

Synthesis

Nano particles of noble metals are characterized by the presence of bright colours ascribed to the oscillations of the surface electron cloud of these particles. On subjecting the alga (*Hypnea musciformis*) to 10^{-3} M concentration of AuCl_4^- ions, visual observations of the biotransformation indicated the formation of nano particles extracellularly, which resulted in the solution turning pink-ruby red colour. This colour was very distinct as compared to the control, which was pale yellow in colour. Fig.1A shows the powder of marine alga with gold ions at the beginning of the

reaction. Fig.1B shows the colour change of the medium to pink-ruby red after 2 hrs of incubation. These colours arise due to excitation of surface plasmon vibrations in the metal nano particles.



Fig. 1 Picture showing the filtrate of *Hypnea musciformis* biomass in aqueous solution of 1×10^{-3} M chloroauric acid at the beginning of the reaction (A) and after 2 hr of reaction (B).

UV-Visible spectroscopic analysis

Figure 2 shows the UV-Vis spectra recorded from the aqueous chloroauric acid- the alga (*Hypnea musciformis*) reaction medium after 2 hrs of incubation. As shown in Figure 2, the solution showed a broad surface Plasmon resonance centered at 552 nm indicating the nano dimension gold nano particles. The Plasmon band was broad and less prominent because of the polydispersity present in the sample, but its presence confirmed the existence of gold nano particles. As indicated in the experimental section, the bioreduction was carried out in the dark and, clearly, the formation of gold nano particles is due to the algal biomass. It is also observed that the surface Plasmon band in the gold nanoparticle solution remains close to 552 nm throughout the reaction period, which indicates that the particles are dispersed in the aqueous solution with no evidence for aggregation. After completion of the reaction, it was observed that the nano particle solution was stable for more than 6 months with little signs of aggregation even at the end of this period.

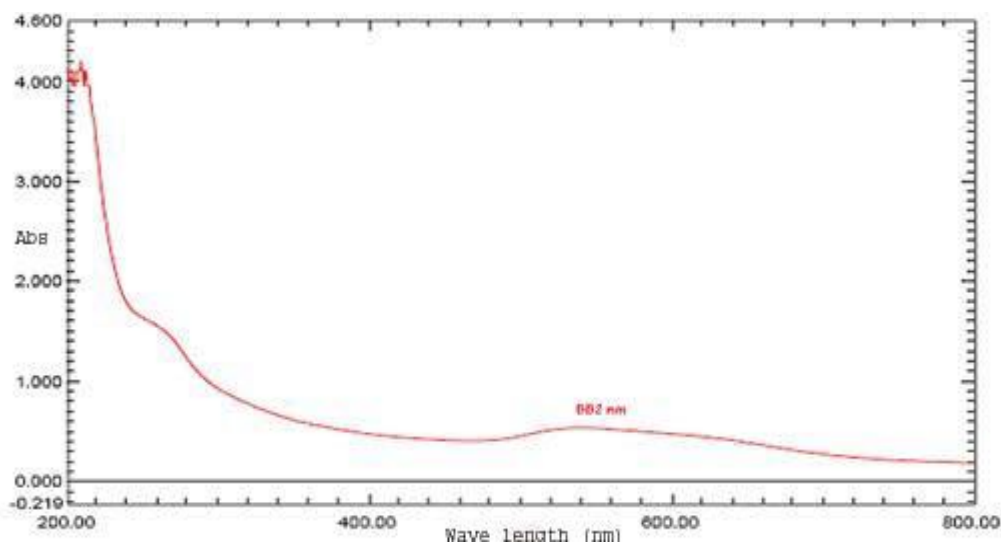


Fig. 2 UV-Visible absorbance spectrum of Gold nanoparticles showing the surface Plasmon band

FT-IR spectroscopic analysis

The FT-IR spectrum (Fig. 3) shows the presence of three bands at 3572.48, 2088.53 and 1646.91 cm^{-1} . The 3572.48 cm^{-1} band may be assigned to OH stretch vibrations, the 2088.53 cm^{-1} band may be assigned to C=C stretching vibrations and 1646.91 cm^{-1} band may be assigned to C=O stretching vibrations in the amide linkages of the proteins (Gole *et al.*, 2001). The positions of these bands are close to that reported for native proteins and are excellent agreeing with that observed in gold colloid: pepsin biconjugates (Kumar and Mc Lendon, 1997). It is well known that proteins can bind to gold nano particles either through free amine groups or cysteine residues in the proteins (Gole *et al.*, 2001) and therefore, the possibility of stabilization of the gold nano particles are by surface-bound proteins. The FT-IR spectroscopic study has gold nano particles confirmed that the carboxyl group of amino acid residues and peptides of proteins has the strong ability to bind metal, so that the proteins could possibly form a coat covering the metal nano particles to prevent agglomeration of the particles and stabilizing in the medium. This evidence suggests that the biological molecules could possibly perform the function, for the formation and stabilization of the gold nano particles in aqueous medium.

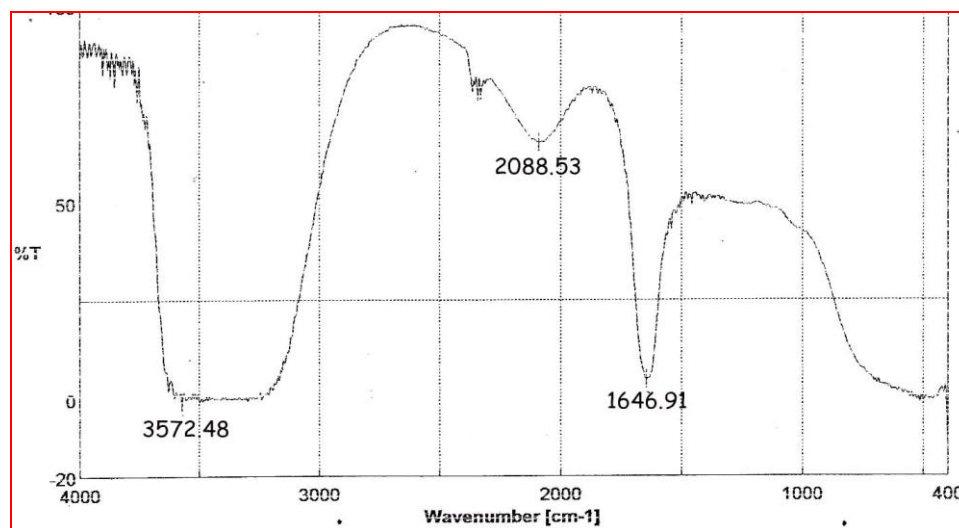


Fig. 3 FT-IR spectrum of gold nano particles synthesized in *Hypnea musciformis*.

Scanning Electron Microscopy (SEM)

SEM analysis of gold nano particles, besides being present in colloidal form in solution also precipitates on the surface of biomass of *Hypnea musciformis*, clearly indicating that the gold nano particles formed by the reduction of Au^+ ions bound to the surface of the cells (Fig.4). The brighter cubical areas of the backscattered electron image correspond to metallic gold indicating the cubic structure of gold. Kuyucak and Volesky (1990) and Ting *et al.*, (1995) have also reported elemental gold precipitation on the algal biomass of *Sargassum* and *Chlorella sp.*, respectively. The gold nano particles seen outside the cell wall of alga may be due to weakly bound, gold nano particles dislodged from the biomass during the preparation of films for SEM investigations. The SEM image showing algal biomass extract further confirmed the development of gold nano particles.

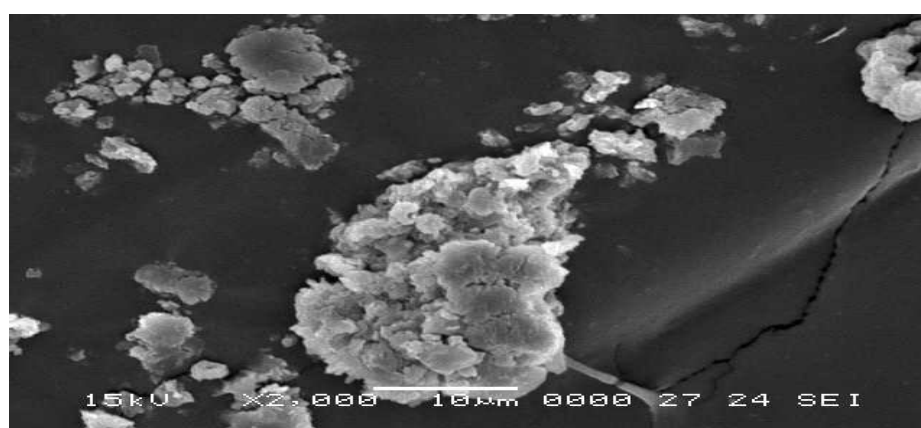


Fig. 4 Scanning electron micrograph of gold nano particles

Transmission Electron Microscopy (TEM)

The TEM images recorded from gold nano particle solutions are shown in Fig. 5. The gold nano particles formed were spherical with diameters ranging from 6.25 to 12.25 nm and 25 to 33.33 nm, respectively. As can be seen in the TEM image, a large number of nano particles were more than 6.25 nm in diameter. The higher magnification image indicates that the nano particles were generated on a selective surface of gold nano particles. This is caused by the formation of gold nano particles with a liquid fluid surface. Similar experimental results were also obtained when gold nano triangles were prepared using lemongrass extract (Shankar *et al.*, 2004). Figure.5 shows the TEM micrographs of the gold nano particles formed predominantly polydisperse with diameter ranging from 6.25 to 33.33 nm. On careful observation of various magnifications of TEM images of gold nanoparticles it is noted that the particles are of different size ca. around 11 nm, and also gold nano particles have an inclination of forming spherical structures.

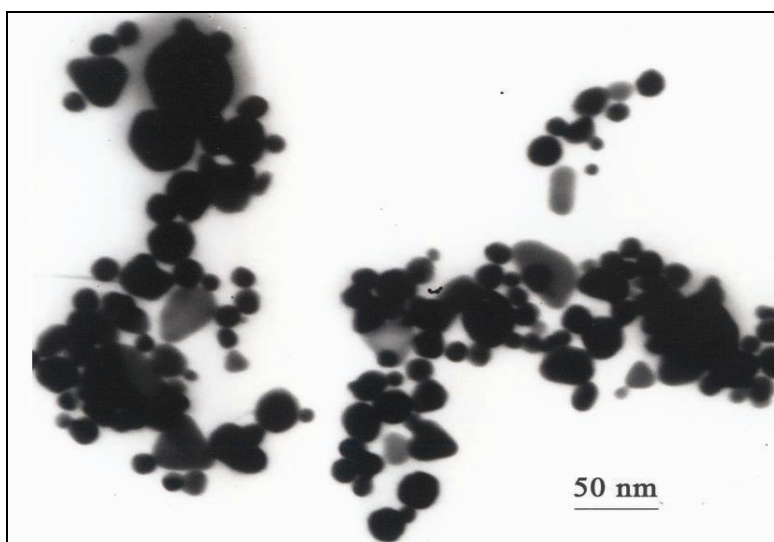


Fig. 5 TEM images of gold nanoparticles synthesized by using *Hypnea musciformis*. (Indicating bar refers the magnification of the TEM images)

Particle size distribution

Figure 6 shows the size distribution of gold nano particles ranging from 6.25 to 33.33 nm where maximum number of gold nano particle size was around 6.25 nm. From the analysis, it is observed that there is variation in the particle sizes with almost 44.68% of the particles in 6.25 nm ranges, 3.29% in the 8.3 nm ranges, 7.44% in 10 nm ranges, 34.04% in 12.5 nm ranges, 8.51% in 25 nm range and 2.12% in 33.3 nm range.

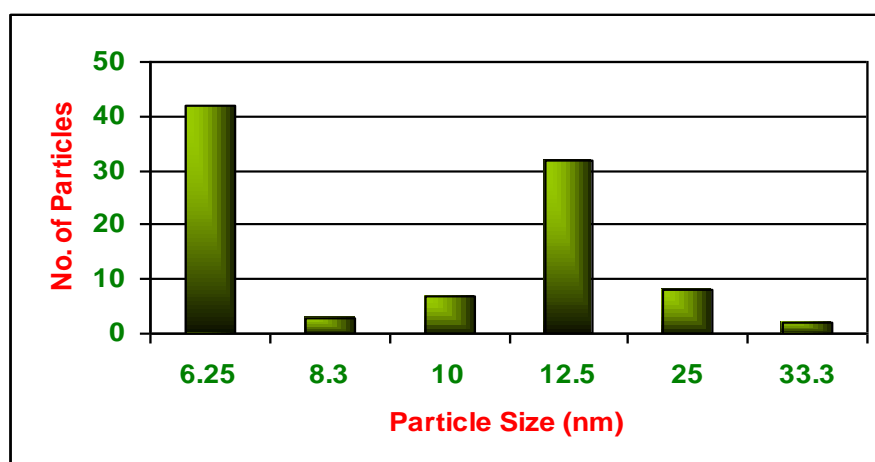


Fig. 6. A particle size distribution histogram of as synthesized gold nano particles determined from TEM images

X-Ray Diffraction pattern

The Figure.7 shows XRD pattern corresponds to the gold nano particles. Bragg reflections are present, which could be indexed on the basis of the face-centered cubic (fcc) gold structure. An overwhelmingly strong diffraction peak located at 38.09° is ascribed to the $\{1\ 1\ 1\}$ facets of face-centered cubic metal gold structures, while diffraction peaks of other four facets are much weaker. It is worth pointing that the ratio of intensity between the $\{2\ 0\ 0\}$ and $\{1\ 1\ 1\}$ peaks are much lower than the standard value. The ratio between the $\{2\ 2\ 0\}$ and $\{1\ 1\ 1\}$ peaks is also much lower than the standard value. These observations indicate that the gold nano particles formed by the reduction of Au (III) by alga *Hypnea musciformis* are dominated by the $\{111\}$ facets, and most of the $\{111\}$ planes parallel to the surface of the supporting substrate were sampled. The gold nano particles formed by the reduction of Au (III) by alga *Hypnea musciformis* are dominated by the $\{111\}$ facets, and most of the $\{111\}$ planes parallel to the surface of the supporting substrate. The XRD patterns, thus clearly show that the gold nano particles formed by the bioreduction of AuCl_4^- ions using *Hypnea musciformis*.

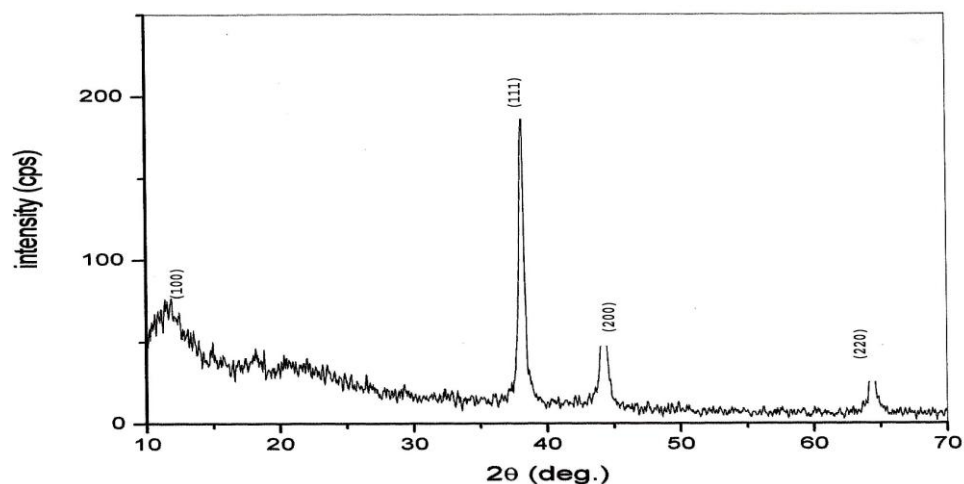


Fig. 7 X-ray diffraction pattern of the gold nano particles obtained from *Hypnea musciformis*

Antifungal activities of Gold nano particles

The antifungal activity of a control sample containing all the initial reaction substances except the reducing agent was very high because of the presence of free gold ions, which are very toxic to fungi. The antifungal properties of the composites were tested against *Aspergillus niger* and *Mucor* spp. It is necessary to emphasize that the tested gold nano particles have antifungal effects resulting not only in inhibition of microbial growth but also in killing of fungi (Fig.8).

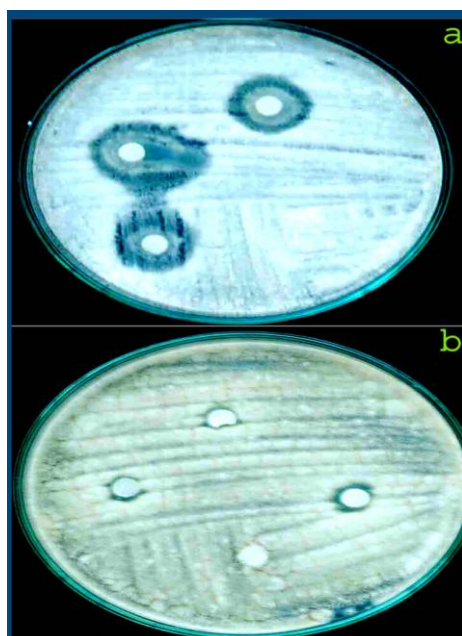


Fig. 8 Antifungal activity of gold nano particles of *Hypnea musciformis* against (a) *Aspergillus niger* (b) *Mucor* spp.

Previous studies looking at the interactions of gold (I) compounds with biological systems suggest that the major mode of anti-fungal activity involves the highly specific coordination of gold (I) to thiol groups present in the proteins, especially with L-cysteine, although other groups are thought to be involved.

Conclusion

In the present study, the synthesis of spherically shaped stable gold nano particles was achieved using *Hypnea musciformis* at room temperature and neutral pH. A biological process with the ability to strictly control the shape of the nano particles produced was confirmed using by UV-Visible spectroscopy, FT-IR, SEM, TEM and XRD analyses. It is speculated that proteins might have played an important role in the stabilization of gold nano particles. Interestingly, the extract played a dual role as a capping agent as well as reducing agent which led to the environmentally viable synthesis of nano particles. In future, it would be important to understand the biochemical and molecular mechanism of nano particle formation by the cell filtrate in order to achieve better control over the size and polydispersity of the nano particles. This eco-friendly approach for the nano particle synthesis is simple, amenable for large scale commercial production and technical applications. The gold nano particles showed anti-fungal activity against *Aspergillus niger*.

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