International Journal of Agricultural Technology 2017 Vol. 13(7.3):2615-2622
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Biologically Synthesized Gold Nanoparticles (AuNP) using Pine (*Pinus kesiya*) Pollen Extract Show Antifungal Activity against *Candida albicans*

Somar Israel D. Fernando [*] , Khristina G. Judan-Cruz[1] and Arren Christian M. De Guia[2]

[1]Department of Biological Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines; [2]College of Veterinary Science and Medicine, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines.

Somar Israel D. Fernando Khristina G. Judan-Cruz and Arren Christian M. De Guia. (2017). Biologically Synthesized Gold Nanoparticles (AuNP) using Pine (Pinus kesiya) Pollen Extract show Antifungal Activity against Candida albicans. International Journal of Agricultural Technology 13(7.3): 2615-2622.

Pollen from pine tree species (*Pinus kesiya*) synthesized with gold nanoparticles (AuNP) was tested for its fungicidal activity against *Candida albicans,* a persistent fungus infecting humans. Overgrowth of *C. albicans* present in the gut are commonly triggered by various factors such as high sugar diet, antibiotics, chronic stress, contraceptive pills and diabetes that may lead to several candidiasis diseases. Three concentrations of 500, 300 and 100 ug/ml of synthesized pine pollen with nano gold particles (AuNP) were subjected to paper disc assay using corn meal agar against *C. albicans*. Synthesized extract of *P. kesiya* pollen at 500 ug/ml concentration showed the highest antifungal activity against *C. albicans.* The prevalent antifungal activity of the biological synthesized gold nanoparticles using *P. kesiya* can lead to its promising utilization as natural fungicidal agent in the field of nano biotechnology.

Keywords: Pine Pollen Extract, *Pinus kesiya, Candida albicans*

Introduction

Pinus kesiya of the family Pinaceae are common in northern Philippines, particularly in highland regions with an altitude of approximately 1,500 meters (4,900 feet) that provides optimum conditions for its growth such as average temperature ranges of 15 to 23 ℃ (59 to 73 ℉) . *P. kesiya* is a large tree that grows 45 m tall with a bole-free of branches for 15-20 m and approximately 100 cm in diameter for a matured tree. Slender and flexible needles in bundles of 3-4 can grow 14-25 cm long. Mature cones are pendulous, ovoid and around 4-10 cm. The naturally occurring pines inhabit a wide range of forest and savanna habitats.

[*] **Corresponding author:** Somar Israel D. Fernando; **Email:** marzoesomarfernando@yahoo.com

In recent years, rapid advances in collection, storage and processing techniques and the availability of pine pollen is no longer restricted to medical use but have become utilized in the food and cosmetic industry. Despite the long tradition of medical use of pine pollen, scientific validation on its activity is lacking. Reported biological activities of pine pollen include antimicrobial as well as cytotoxic properties that are attributed mainly to the flavonoids, pinocembrin, galangin and pinobanksin as well as prenylated coumeric and diterpenic acids. Caffeoylquinic acid derivatives in pine pollen also show immunomodulatory and hepatoprotective actions. Urofuran lignans contained in pine pollen also inhibit the growth of some bacteria (Castaldo and Capasso, 2002). There are evidences that inclusion of pine pollen in a rat diet may affect fecal dry matter, crude protein and crude ash concentration as well as nutrient digestibility. These data implicates the use of Pine pollen as a feed additive that may help enhance dietary fiber supply (Zhao and Bao, 2001).

Nanoparticles have gained increased use due to their specific performance in pharmacy, medicine and engineering. Nanoparticles have been applied to hygiene, dentistry and advances in the prevention, diagnosis, drug delivery and treatment of diseases. Biological synthesis of metal nanoparticles accounts a vital role in nano biotechnology, allowing production of non-toxic and eco-friendly particles (Khatami *et al*., 2017). Biological synthesis of gold nanoparticles (AuNPs) may be studied using pine pollen ethanol extract as a novel, cost-effective, modest and harmless bio reserve.

Synthesis of NPs using biological entities has generated great interest due to their unusual optical, chemical, photo electrochemical and electronic properties. Biological synthesis of gold nanoparticles using plant extracts has many advantages because phytochemical compounds present can act as both reducing and capping agents that result to a highly stable and biocompatible metallic nanoparticle (Mohanpuria *et al.*, 2008).

Pine pollen extract has essential phytochemical constituents that are responsible for the reduction of Au^{+3} present in gold chloride solution and, along with other high and low molecular weight proteins, these phytochemicals stabilize the synthesized AuNPs by capping

Materials and Methods

Pine Pollen Collection

The male cones of pine were collected in Baguio City, Philippines and placed in a sterile resealable plastic bag. Male cones were removed by manual

pounding to separate the pollen and stored in wide mouthed amber bottles and kept refrigerated until use.

Figure 1. Pine pollen from male catkins

Preparation of materials through Aqua regia

Aqua regia mixture was prepared by combining nitric acid and hydrochloric acid in a molar ratio of 1:4. All glasswares used in the synthesis of AuNPs were carefully washed by aqua regia solution and rinsed thrice by distilled Mili Q water before using. Thus, the noble metals gold and platinum were dissolved in the process. It is primarily used to produce chloroauric acid for refining the highest quality (99.99%) gold.

Aqua regia is also used in etching and in specific analytic procedures. It is also used in to clean glassware of organic compounds and metal particles. This method is preferred and pressingly a standard over the traditional chromic acid bath for cleaning NMR tubes, because no traces of paramagnetic chromium can remain to spoil spectra (Hoffman, 2005).

Pine pollen Extraction

Fifty grams of pine pollen were soaked in 80% ethanol (1:10 ratio) in a stoppered flask for 72 hours and filtered using filter paper disc. Rotary evaporator was used to remove ethanol from the extract. Extract obtained was lyophilized using Lyophilizer (Freeze Drying Unit Subliminator) and kept in a cool dry place until use.

Rehydration of Extract

Lyophilized pine pollen extract were rehydrated using sterile Mili Q distilled water then subjected to a shaking incubator for 1 hour of continuous shaking to fully rehydrate the solution and stored in a tightly stoppered sterile amber bottle and kept cool until use.

Biological Synthesis of Pine pollen extract

Gold chloride was prepared at the 10^{-3} M concentration with sterilized Milli Q water. Pine pollen extract was mixed with 10^{-3} M gold chloride for the synthesis of gold nanoparticles. Gold chloride was taken in similar quantities without adding pine pollen extracts to main respective controls. The containers were incubated under dark conditions at room temperature with constant stirring of magnetic stirrer and observations were recorded.

The resulting solution was kept for 60 minutes in that condition until it turned into pink red color indicating the formation of AuNPs and to ensure the stability of the nanoparticles. The change in color indicates the formation of AuNPs in the solution due to excitation of surface Plasmon vibration in the metal nano particles.

The AuNPs obtained from the solution were purified by centrifugation at 4000 rpm for 20 minutes followed by dispersion of the pellet thrice in deionized water to remove the water soluble biomolecules such as proteins and secondary metabolites. The water suspended NPs were frozen at 30 ◦C overnight and then kept under vacuum for 24 h to dry the NPs.

Characterization of synthesized AuNPs

Pine pollen extract was tested for potential reducing and capping agent for the synthesis of AuNPs. The synthesized AuNPs were analyzed by UV-vis Spectroscopy analysis using Nano drop spectrophotometer.

UV-visible Spectroscopy Analysis

The formation of AuNPs was monitored by UV-visible spectroscopy in nano drop spectrometer by analyzing the excitation due to the applied electromagnetic field of surface Plasmon resonance (SPR) and absorption values were recorded.

Stability of AuNPs

The synthesized AuNPs were kept stationary over 3 months in dark for all concentrations and evaluated visually and under UV-vis spectrophotometer every week for probable colloid, aggregate or precipitate formation to ensure that the synthesized AuNPs were stable.

Analysis of Antifungal activity

Three concentrations of 500, 300 and 100 ug/ml of synthesized pine pollen ethanol extracts were used. Sterile paper discs (5 mm) were soaked and air dried on sterile petri plates with synthesized pine pollen ethanol extract under a Biosafety laminar flow. Prepared media on petri plates of Corn Meal agar, a selective medium for the cultivation of *C. albicans*, swabbed with fungal culture were used. Air dried disc with three concentrations were individually seeded on plates in a three concentrations per plant manner, Ketoconazole served as positive control while sterile distilled water served as negative control. Plates were incubated at 37℃ for 3-5 days.

Results and Discussion

Visual Observation

Upon addition of pine pollen extract to $AuCl_3$, reduction of the gold ions to Au occurred and the reduction reaction was noticeable in mixtures through color change from yellowish to reddish pink. This reddish pink color is attributed to the Surface Plasmon Resonance (SPR) arising due to the collective oscillation of induced free conduction electrons in AuNPs.

UV-Vis Spectral Study

Biosynthesized pine pollen extract mediated AuNPs particles were confirmed using UV-vis spectrophotometer by analyzing the excitation due to the applied electromagnetic field of surface Plasmon resonance (SPR).

Surface Plasmon resonance (SPR) peaks attained in UV-vis spectroscopy are one of the versatile techniques that confirm the formation of metal nano particles. SPR was generated due to the coherence of electrons on the surface of AuNPs. The formation of AuNPs was monitored by UV-visible spectroscopy in nano drop spectrometer. Absorption values were recorded in a

wavelength of 300 nm. It clearly indicated the formation of AuNPs of the pine pollen extract.

Antifungal Activity of AuNPs

Synthesized gold nano particles exhibited fungicidal activity at its most effective concentration of 500 ug/ml against *Candida albicans*, a natural fauna on humans that becomes virulent when mucosal barriers are at stake. The results in this concentration is comparable with the effect of the positive control (ketoconazole). Other concentrations also exhibited significant antifungal activities against *C. albicans*. (Table 1, Fig.2).

Table 1. Zones of inhibition (in mm) of synthesized Gold nano particles using pine pollen extract.

Treatments	Zone of Inhibition (mm)
500 ug/ml	**17.51^{d}**
300 ug/ml	13.99^{c}
100 ug/ml	11.49^{b}
+ Control	17.87^{d}
- Control	0^{a}

*Superscripts of different letters indicate significant difference among the treatments.

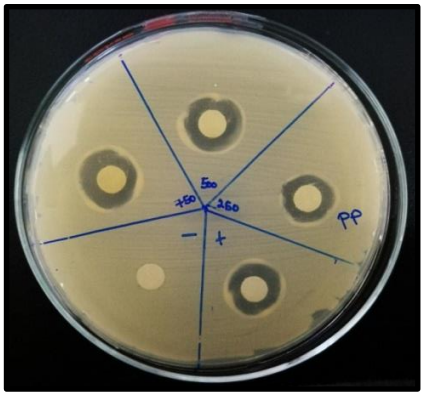

Figure 1. Disc diffusion assay of synthesized AuNPs against *Candida albicans*

AuNPs manifests inhibitory effect through different cellular mechanisms including binding to cytoplasmic membrane causing cell membrane destruction, forming depths on the cell surface and modifying cell wall permeability and

lastly the inhibition of major cellular functions such as respiration, DNA replication, and cell division, resulting in loss of cell integrity and viability (Ravishankar and Jamuna, 2011 and Sondi and Salopek-Sondi, 2004).

Pine pollen extract is a suitable reducing and stabilizing agent. The extract can be applied for the synthesis of other noble metal nanostructures. The results obtained gives strong evidence that could warrant the consideration of AuNPs as an antifungal agent that could evade the side and passive immune effects of other biocide medications.

Similar studies reported the same using AuNPs. The peel of banana (*Musa paradisiaca*) extract-mediated AuNPs also showed efficient antifungal activity towards the pathogenic fungi, *C. albicans* (BX and BH) (Bankar *et al*., 2010). Other reports on nanoparticles include the antifungal activity of silver nanoparticles in the size range of 7–20 nm synthesized by a proprietary bio stabilization process against *A. niger* and *C. albicans* (Jain *et al.,* 2009) as well as the significant antifungal activity of modified denture base acrylic combined with silver nanoparticles against *C. albicans* strain (Nam *et al*., 2012).

Biological synthesis provides progression over chemical and physical method as it is environment responsive, cost effective and certainly can accommodate large scale synthesis. Biological synthesis does not require the need to use high pressure, high energy, temperature and toxic chemicals.

Gold in its substance form has long been considered an inert, noble metal with some therapeutic and even medicinal value. AuNPs are also thought to be relatively non-cytotoxic (Connor *et al*., 2005). In biology, AuNPs are used for the development of biosensors, DNA labels (Groning *et al*., 2001; *Tang et al.,* 2006) and in medicine (Paciotti *et al.,* 2004). However, there are differing reports on the extent of the toxic nature of these particles owing to their different modifications, surface functional attachments, shape and size (Takahashi *et al*., 2006; Pan *et al.,* 2007).

The prevalent antifungal activity of the biologically synthesized gold nanoparticles using *P. kesiya* can lead to its promising utilization as natural fungicidal agent in the field of nano biotechnology. Moreover, this plant-mediated synthesis method represents a considerable improvement for the preparation of AuNPs for it allows better control over their nanostructures.

AuNPs are capable of rendering high antifungal efficacy and hence, has a great potential in the preparation of drugs that can be used against fungal diseases and can pave the way to development of techniques on preparing nanomedicines for fungal-related diseases but thorough investigations on the toxicity and mechanism of action at cellular level should be done.

Acknowledgement

The authors are grateful to Dr. Michelle N. Balbin, Dr. Daryl G. Dela Cruz and Shiela Marie D. Baluran and to the Animal Health and Biosafety Laboratory, Philippine Carabao Center General Headquarters and Gene pool, Science City of Munoz, Nueva Ecija, Philippines for the use of the laboratories.

References

Bankar, A., Joshi, B., Kumar, A.R., Zinjarde, S. (2010). Banana peel extract mediated synthesis of gold nanoparticles. Colloids Surf. B Biointerfaces 80, 45–50.

Castaldo, S. and Capasso, F. (2002). Propolis, an old remedy used in modern medicine. Fitoterapia, 73(1), 1–S6.

Connor, E.E., Mwamuka, J., Gole, A., Murphy, C.J., Wyatt, M.D. (2005). Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. Small 1, 325–327.

Groning, R., Breitkreutz, J., Baroth, V., Muller, R.S. (2001). Nanoparticles in plant extracts: factors which influence the formation of nanoparticles in black tea infusions. Pharmazie 56, 790–792.

Hoffman, R. (2005). How to make an NMR sample, Hebrew University.

Jain, J., Arora, S., Rajwade, J.M., Omray, P., Khandelwal, S., Paknikar, K.M. (2009). Silver nanoparticles in therapeutics: development of an antimicrobial gel formulation for topical use. Mol. Pharmacol. 6, 1388–1401.

Khatami M, Heli H, Jahani P.M, Azizi H, Lima Nobre M. (2017). Copper/copper oxide nanoparticles synthesis using *Stachys lavandulifolia* and its antibacterial activity. *IET Nanobiotech.* 2017; 11. DOI: 10.1049/iet-nbt.2016.0189.

Mohanpuria, P., Rana, N.K., Yadav, S.K. (2008). Biosynthesis of nanoparticles: technological concepts and future applications. J. Nanopart. Res. 10, 507–517.

Nam, K.Y., Lee, C.H., Lee, C.J. (2012). Antifungal and physical characteristics of modified denture base acrylic incorporated with silver nanoparticles. Gerodontology 29, e413–e419.

Paciotti, G.F., Myer, L., Weinreich, D., Goia, D., Pavel, N., McLaughlin, R.E., Tamarkin, L. (2004). Colloidal gold: a novel nanoparticle vector for tumor directed drug delivery. Drug Deliv. 11, 169–183.

Pan, Y., Neuss, S., Leifert, A., Fischler, M., Wen, F., Simon, U., Schmid, G., Brandau, W., Jahnen-Dechent, W. (2007). Size-dependent cytotoxicity of gold nanoparticles. Small 3, 1941–1949.

Ravishankar Rai V, Jamuna Bai A. (2011). Nanoparticles and their potential application as antimicrobials. In: Mendez-Vilas A, editor. Science against microbial pathogens: communicating current research and technological advances. *Format Res Cent*; 5(4). 197-209. DOI: 10.4236/anp.2016.54022

Sondi I. and Salopek-Sondi B. (2004). Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. *J Coll Inte Sci*.; 275(1):177-82. DOI:10.1016/j. jcis.2004.02.012.

Takahashi, H., Niidome, Y., Niidome, T., Kaneko, K., Kawasaki, H., Yamada, S. (2006). Modification of gold nanorods using phosphatidylcholineto reduce cytotoxicity. Langmuir 22, 2–5.

Tang, D., Yuan, R., Chai, Y. (2006). Ligand-functionalized core/shell Ag–Au nanoparticles label-free amperometric immun-biosensor. Biotechnol. Bioeng. 94, 996–1004.

Zhao, L. and S. F. Bao. (2001). The study on morphologic observation and nutritional components of natural and broken Masson Pine pollen. Acta. Nutr. Sin. 23, 153–156.

(Received 15 October 2017; accepted 25 November 2017)