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Original Research Article

Green synthesis of gold nanoparticles from waste macadamia nut shells and their antimicrobial activity against *Escherichia coli* and *Staphylococcus epidermis*

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ABSTRACT

Background: The study for the first time demonstrates an eco-friendly and room temperature procedure for biosynthesizing gold (Au) nanoparticles from waste Macadamia nut shells. Currently Australia contributes around 40% to the global market and generates around AUS $150 million of export revenue. However, a consequence of large nut production is the generation of large quantities of waste nut shells. The green chemistry-based method is clean, nontoxic and eco-friendly. The method presented in this study produced a variety of Au nanoparticle sizes and shapes.
Methods: The straightforward green chemistry-based technique used waste Macadamia nut shells to generate Au nanoparticles, which were subsequently studied using several advanced characterization techniques. Furthermore, the Kirby-Bauer sensitivity method was used to evaluate the antibacterial properties of the extracts and synthesized gold nanoparticles.
Results: Advanced characterisation revealed the Au nanoparticles were crystalline, ranged in size from 50nm up to 2µm, and had spherical, triangular and hexagonal morphology. The gram-negative bacteria *Escherichia coli* produced a maximum inhibition zone of 11mm, while *Staphylococcus epidermidis* produced a maximum inhibition zone of 9mm.
Conclusions: The study has shown that waste Macadamia nut shell extracts have no antibacterial activity, but the synthesised Au nanoparticles did display antibacterial activity to both *Escherichia coli* and *Staphylococcus epidermidis*. Thus, the present work has demonstrated a waste valorisation strategy that can be used to produce high-value Au nanoparticles with antimicrobial properties for use in future pharmaceuticals.

Keywords: Agricultural waste, Antibacterial, Gold nanoparticles, Green synthesis

INTRODUCTION

Using gold (Au) nanoparticles as a platform technology in several biomedical applications such as biosensors, fluorescent immunoassays, cancer treatment, targeted delivery of pharmaceuticals and antibacterial agents has attracted considerable interest in recent years.[1-4] Importantly, the size, shape and surface reactivity can influence the chemical, physical, optical and electronic properties of nanoparticles.[5,6] For example, Au nanoparticles display a strong surface plasmon resonance when exposed to electromagnetic radiation. From the biomedical perspective, living cells of most organisms range in size from 10µm to 50µm, and their internal

organelles are within the sub-micron scale. Thus, the size difference between nanoparticles and cells is very large and can lead to greater bio-physical interactions between both internal and external cellular molecules.[1,2] For example, studies have shown administered Au nanoparticles readily enter cancer cells and accumulate. Then during subsequent thermal therapy, the optical and chemical properties of the accumulated Au nanoparticles can promote the destruction of cancer cells.[7,8] Studies have also shown anticancer drugs attached to Au nanoparticles can be targeted towards tumour more effectively. Thus, improving payload delivery, reducing treatment durations and minimizing the side effects produced by the drugs.[1,3,9] Recent studies have also shown Au nanoparticles can be used as both antibacterial and antifungal agents.[10] The resulting interaction between Au nanoparticles and microorganisms can produce membrane damage via bio-sorption and toxicity via cellular uptake.[11,12] The mechanisms that produce the antimicrobial action are not fully understood. However, recent studies suggest nanoparticle size, shape and surface reactivity influence its antimicrobial properties.[13] Therefore, the antimicrobial properties of Au nanoparticles have an important and immediate health benefit. Since bacterial and fungal species are constantly developing immunity against many currently used antibiotics. There is a current need to develop new and more effective antimicrobial agents to fight antibiotic resistant strains of bacteria and fungal species. Recent studies have shown Au nanoparticles can be used as antimicrobial agents against many bacterial and fungal species.[14,15]

Generally, nanoparticles are manufactured using expensive chemical and physical processes that often use toxic chemicals and solvents. These toxic materials have associated hazards such as environmental toxicity, cytotoxicity and carcinogenicity.[16] However, in recent years an alternative to traditional manufacturing has emerged and produces nanoparticles by eco-friendly biological entities.[17] This green chemistry-based method can deliver a wide variety of nanoparticle types, sizes and shapes. The method begins by mixing an aqueous metal salt solution with a water-based extract derived from a suitable plant source. Biochemical reduction of the metal salt solution converts metal ions from their mono or divalent oxidation states to zero-valent states and initiates nanoparticle nucleation.[18] This is followed by smaller neighboring particles clustering to former larger thermodynamically stable nanoparticles.

During growth, the nanoparticles form their most energetically favorable and stable shapes that can include cubes, spheres, triangles, hexagons, pentagons, rods and wires.[19] Also during this stage biomolecules present in the extract act as capping agents to coat and stabilize the nanoparticles. During nanoparticle generation factors such as plant extract concentration, metal salt concentration, reaction time, reaction solution pH and temperature influence the size, shape and surface properties.[17,20]

The present study, reports for the first time, the use of waste Macadamia nut shells to manufacture high-value Au nanoparticles with antibacterial properties. The quantities of waste Macadamia nut shells produced annually offers a renewable and eco-friendly resource that can be utilized for the manufacture of high-value Au nanoparticles. The advantages of this green chemistry-based method include: (1) the nut shell acts as both stabilizing agent and reducing agent during nanoparticle manufacture; (2) the aqueous-based synthesis process is eco-friendly, and (3) the method is simple, straightforward and does not require specialized equipment. The Au nanoparticles produced were characterized using UV-visible spectroscopy, X-ray diffraction analysis, energy dispersive spectrometer (EDS) analysis and scanning electron microscopy (SEM). Additionally, the antibacterial properties of the Au nanoparticles towards *Escherichia coli* and *Staphylococcus epidermidis* were evaluated using the Kirby-Bauer sensitivity method.[21]

METHODS

Materials

Gold chloride [$AuCl^{4}$-, (99.99%)] was the source of Au+ ions and was supplied by Sigma-Aldrich (Castle Hill, NSW, Australia) and used without any further purification. Raw Macadamia nut shells were supplied by the Suncoast Gold Macadamia Company, Queensland Australia. The nut shells were manually cleaned to remove any remaining nut fragments and other contaminants before being fragmented. All aqueous solutions used in this study were produced from Milli-Q® water, which was generated by a Barnstead Ultrapure Water System D11931 (Thermo Scientific Dubuque IA: 18.3 M Ω cm^{-1}).

Synthesis of Au nanoparticles

A glass beaker containing 100 ml of Milli-Q® water was bought to the boil and then allowed to simmer at 95°C throughout the 2hrs soaking period. The extraction process started with the addition of cleaned Macadamia nut shells (10 g) to the simmering water. After 2hours, the Milli-Q® water had changed from its clear appearance to a milky brown colour.

The liquid was then allowed to cool down to room temperature. Following cooling, the liquid was filtered three times using 0.22 µm syringe filters. After filtration the extract had a pale-yellow appearance. Then varying amounts of extract (1,5,10,15,20 and 25 ml) were added to an aqueous 1ml solution of $AuCl^{4}$- (500 ppm) as seen in (Figure 1). The reaction mixtures were then left overnight (approximately 22 hours) for biosynthesis to occur.

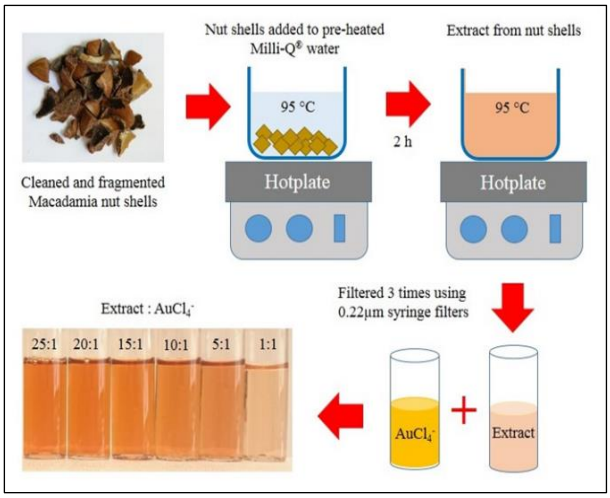

Figure 1: Schematic representation of experimental procedure and various extract volume ratios prepared.

Advanced characterization

All samples were evaluated using UV-visible spectroscopy, x-ray diffraction (XRD), energy dispersive spectroscopy (EDS) and scanning electron microscopy (SEM). UV-visible spectroscopy of the samples was carried out using a Varian Cary 50 series UV-visible spectrophotometer version 3, over a spectral range from 200 to 1100nm (spectral resolution of 1nm), and at room temperature of 24°C. Identification of metallic Au in the samples was done using a Bruker D8 series diffractometer (with flat plane geometry) over a 2 second acquisition period. The diffractometer collected data over a 2θ range (15° to 80° with an incremental step size of 0.04°) and operated at 40kV and 30mA (Cu Kα = 1.5406Å radiation source). Particle size and shape was determined from images generated by a JEOL JCM-6000, Neo Scope TM electron microscope. Prior to imaging, samples were dried, fitted to holders using carbon adhesive tape and sputter coater (Cressington 208HR) with a 2nm layer of platinum to prevent charge build up. The EDS attachment of the electron microscope was used identify the presence of metallic Au.

Antibacterial activity of Au nanoparticles

Antibacterial activity of the biosynthesized nanoparticles was evaluated using the sensitivity method of Kirby-Bauer.[21] In the present study, two bacterial strains (*Escherichia coli*; gram-negative and *Staphylococcus epidermidis*; gram-positive) were challenged. The sub-cultured bacteria were swabbed uniformly over a nutrient agar medium contained in several Petri dishes (90mm Dia.) using a sterile cotton swab. Nanoparticle solution samples (50µL) were deposited on sterile disks (6mm Whatman® AA 2017-006) using a micropipette. After air drying for 20minutes the disks were placed on respective bacteria treated agar plates using sterile forceps. Incubation of the plates was carried out at 37ºC for 48hours. Following incubation, inhibition zones present in the various Petri dishes were measured and the antibacterial performance determined.

RESULTS

After biosynthesis, the reaction mixtures had turned brown in colour. The resulting colour was the excitation of the surface plasmon resonance (SPR) of the Au nanoparticles present in the samples. The presence of nanoparticles in the samples was also revealed by the scattering of laser light as seen in (Figure 2) insert.

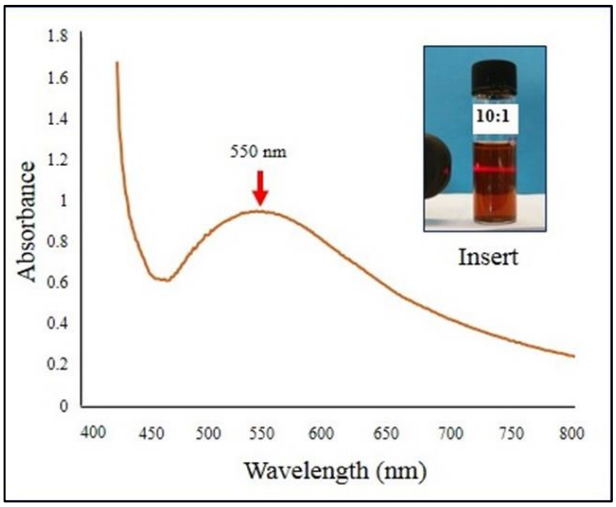

Figure 2: UV-visible spectroscopy analysis of au nanoparticles synthesized from a 10:1 ratio (macadamia nut shell and aucl4-) and a photograph showing au nanoparticles detected in the reaction mixture by laser light scattering.

The formation of Au nanoparticles was also confirmed by UV-Visible spectroscopy for the respective reaction mixtures. The SPR occurred at 550nm for the samples, with a representative UV-visible spectrum for the 10:1 ratio presented in (Figure 2). XRD spectroscopy was used to identify the presence of crystalline Au in the samples. The phases detected were consistent with phases incorporated in the ICDD (International center for diffraction data) databases. A representative XRD pattern for a 10:1 sample is presented in (Figure 3A).

Subsequent EDS analysis also confirmed the presence of metallic Au in the samples as seen in (Figure 3B). The relatively long reaction period results in many of the nanoparticles being able to grow and form micrometre scale plates. (Figure 3C) presents a representative aggregate of nanoparticles and micrometre scale particles and plates. The enlarged image presented in (Figure 3D) shows triangular and hexagonal crystalline shapes interspersed within the aggregate. These larger shapes are characterised by their smooth plate-like structure. The side lengths of the triangular shaped plates were found to be around 500nm, while the side lengths of the hexagonal shaped plates were between 400 and 500nm. The

thickness of both plate types was found to vary from 50 to 100nm. The images also revealed spherical particles with diameters ranging in size from 50nm up to 200nm. The antibacterial study evaluated the performance of the synthesized Au nanoparticles against *Escherichia coli* and *Staphylococcus epidermidis*. Initially, both bacterial strains were evaluated against test disks treated with Macadamia nut shell extract. The results indicated the extract had no antibacterial properties and produced a null result in the inhibition zone measurements as seen in the representative *Escherichia coli* result presented in (Figure 4A). The antibacterial challenge also found varying ratios of extract to AuCl4-produced differing degrees of bacterial susceptibility. The 10:1 (extract: AuCl4-) ratio produced the largest inhibition zone for *Escherichia coli* (11mm) and *Staphylococcus epidermidis* (9mm). A represent inhibition zone result is presented in (Figure 4B). The results for three ratios are shown in (Figure 1C) and reveal the 10:1 ratio gave the best inhibition zones for both bacterial strains.

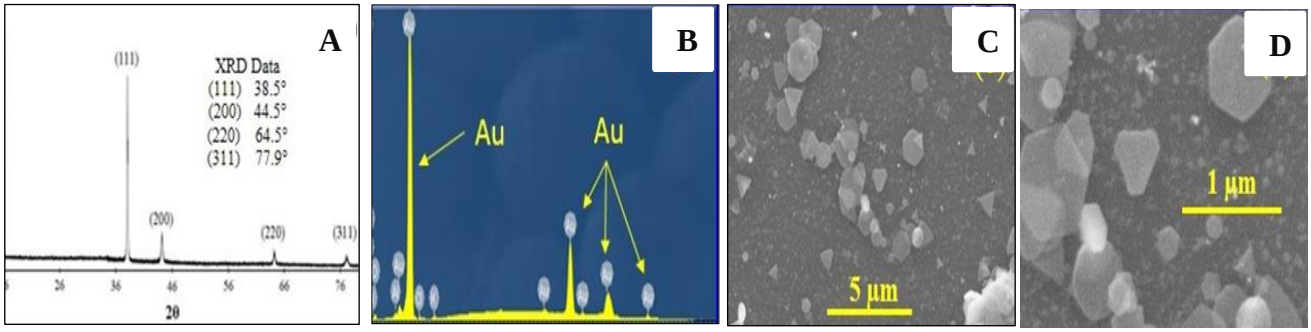

Figure 3: (A) XRD pattern showing the presence of crystalline Au; (B) EDS confirmation of metallic Au present in samples; (C) representative SEM image showing various Au nanoparticle shapes, and (D) enlarged image of triangular and hexagonal nanoparticles.

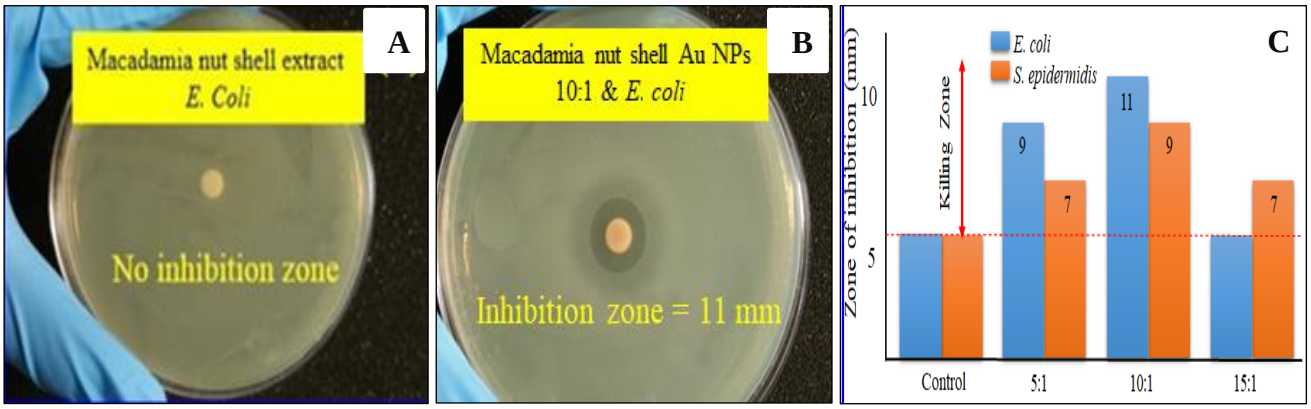

Figure 4: (A) A representative petri dish sample of sterile disk and extract only; (B) *Escherichia coli* being challenged by 10:1 reaction mixture mediated Au nanoparticles, and (C) representative results for the antibacterial evaluation.

DISCUSSION

In recent years, green chemistry-based methods that use plant extracts to produce nanoparticles have been extensively studied. Many of these studies have been reported and summarized in recent review articles.[22-24] In particular, the reduction of the Au (III) ions to their metallic form (Au^0) by natural compounds present in plant extracts has many advantages compared to traditional chemical reduction methods. These advantages include the purity, biocompatibility, and eco-friendly nature of the synthesized Au nanoparticles.[24,25] Furthermore, agricultural plant wastes have also been examined recently as a possible renewable source of biomass for producing high quality Au nanoparticles.[26-28] The present study has examined the viability of using a large horticultural waste, in this case Macadamia nut shells, to produce Au nanoparticles with antibacterial properties.

Natural water-soluble compounds extracted from waste Macadamia nut shells were found to be effective in reducing Au (III) ions to their metallic form (Au^0). The compounds were also found to act as efficient modelling and stabilizing agents that controlled the growth of the

Au nanoparticles. Initial UV-Visible spectroscopy revealed a maximum absorbance peak at 550nm (Figure 2), which is similar to the maximum absorbance of 560 nm reported by Singh et al using a ginger leaf extract.[29] The samples were also examined with XRD and EDS to detect the presence of Au nanoparticles. As shown in (Figure 3A), XRD analysis confirmed the presence of crystalline Au nanoparticles with a face centered cubic structure. The XRD analysis was found to be consist with the results of other researchers using plant extracts to produce Au nanoparticles.[30-32] Furthermore, the presence of elemental Au in the samples was also confirmed by EDS compositional analysis of the samples as shown in (Figure 3B). Samples were also examined with SEM to confirm the presence of Au nanoparticles. As shown in the representative SEM image (Figure 3C), a variety of particle sizes and shapes were generated during synthesis. The process not only produced smaller spherical nanoparticles, but also generated a large number of triangular and hexagonal micrometer scale plates. Similar biosynthesis studies using plant extracts have also shown a variety of shapes other than spherical can be generated as a result of the various biomolecules present.[33,34] For example, Narayanan and Sakthivel have generated Au nanoparticles from Coriandrum sativum (coriander) leaf extracts that ranged in size from 7 to 58nm, and had decahedral, spherical and triangular shapes.[35] However, in spite of the amount of research in this field, the biosynthesis process is not fully understood and remains to be explained. Several studies have suggested that biomolecules, like proteins and metabolites maybe involved. [36] While other studies have also suggested the synthesis process itself could also be influenced by the type of plant extract used to generate the Au nanoparticles.[37,38] Antibacterial testing revealed the Macadamia nut shell extract had no effect on the bacterial strains. However, bacterial strains challenged by Au nanoparticles generated by reaction mixtures composed of varying amounts of extract did have an antibacterial effect. Extract mixture ratios that did produced an antibacterial response were ratios of 5:1, 10:1 and 15:1. With the 10:1 ratio producing the largest inhibition zones of 11mm for *Escherichia coli* (gram-negative) and 9mm for *Staphylococcus epidermidis* (gram-positive) as shown in (Figure 4 C). The likely explanation for the difference in inhibition zones comes from the variation in cell wall composition. However, this aspect of the research needs further research to establish the exact mechanisms involved. But several studies have suggested the Au nanoparticles can induce cellular damage that ultimately leads to the death of various bacterial strains.[39,40] It is for this reason that Au nanoparticles are considered a new class of biomedical material capable of overcoming the immunity of several bacterial strains to conventional antibiotics. Consequently, there is a current need to develop new antibacterial agents to fight antibiotic resistant bacterial strains. Au nanoparticles have the potential to be effective antibacterial agents, especially if they can be synthesized using horticultural wastes like Macadamia nut shells and green chemistry routes. The green chemistry route ensures a clean, nontoxic and eco-friendly method for generating Au nanoparticles.

CONCLUSION

In the present work, aqueous extracts derived from waste Macadamia nut shells were evaluated for the bio-reduction of Au (III) ions to their metallic form (Au^0). The formation of stable Au nanoparticles was confirmed by the SPR peak (550nm) obtained from UV visible spectroscopy. The crystalline nature of the Au nanoparticles was revealed by the presence of peaks in the XRD data. SEM analysis revealed spherical particles with diameters ranging size from 50nm up to 200nm. Also present were larger triangular and hexagonal crystalline shapes, with side lengths around 500nm and thicknesses varying between 50 and 100nm. The Kirby-Bauer sensitivity method provided clear evidence of antibacterial properties being displayed by the Au nanoparticles towards *Escherichia coli* and *Staphylococcus epidermidis*. Moreover, the present work has demonstrated a waste valorisation strategy can be used to produce high-value Au nanoparticles with antimicrobial properties. It is anticipated that the results of this study will lay a good foundation for future industrial scale production of Au nanoparticles using a renewable resource such as Macadamia nut shells, possibly putting an end to the large-scale disposal of waste shells in landfill sites currently seen in Australia and around the world.

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