Biosynthesis and Antibacterial Activity of Silver and Gold Nanoparticles from the Leaf and Callus Extracts of *Amaranthus dubius, Gunnera perpensa, Ceratotheca triloba* and *Catharanthus roseus*

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REFERENCE DECLARATION

I, Miss Naazlene Patel – Student Number: 20513868 and Professor Bharti Odhav do hereby declare that in respect of the following dissertation:

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This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Professor Bharti Odhav.**

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Student's signature

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"All Glory to God"

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LIST OF ABBREVIATIONS

2,4-D	: 2,4-Dichlorophenoxyacetic acid
Ag	: Silver
$AgNO_3$	: Silver Nitrate
Au	: Gold
AU	: Relative Light Unit
BAP	: benzylaminopurine
CSIR	: Council for Scientific Industrial Research
DMSO	: Dimethylsulfoxide
ELISA	: Enzyme-Linked ImmunoSorbent Assay
FTIR	: Fourier Transform Infrared
$HAuCl_4$	: Hydrochloroauric Acid/ Gold Chloride
HIV	: Human Immunodeficiency Virus
INT	: *p*-Iodonitrotetrazolium Violet
MHB	: Mueller Hinton Broth
MIC	: Minimum Inhibitory Concentration
NAA	: α-naphthalene acetic acid
NAD	: Nicotinamide Adenine Dinucleotide
NADH	: Reduced Nicotinamide Adenine Dinucleotide
NIR	: Near Infrared
NNS	: National Nanotechnology Strategy
NP	: Nanoparticle
PCR	: Polymerase Chain Reaction
ROS	: Reactive Oxygen Species
SERS	: Surface Enhanced Raman Spectroscopy
SPR	: Surface Plasmon Resonance
TEM	: Transmission Electron Microscopy
USD	: United States of America Dollar
UV-vis	: Ultraviolet-visible

ABSTRACT

The biosynthesis of NPs has many advantages over the tedious, expensive and toxic physical and chemical methods of synthesis. Plants are stocked with valuable metabolites that are capable of reducing metal salts to form NPs. In this study, aqueous leaf extracts of *A. dubius*, *G. perpensa*, *C. roseus* and *C. triloba* were reacted with $AgNO_3$ and $HAuCl_4$ to determine the plants reducing abilities and hence synthesis of Ag and Au NPs capabilities. The synthesis reactions were carried out at different temperatures and extract concentrations for optimization. The goal was to form NPs within the specific wavelength range. Polar solvents: methanol and ethyl acetate extractions were carried out at the optimized conditions to evaluate the best solvent for the extraction of phytochemicals from the plants. The plant leaf extracts that were successful (*A. dubius*, *G. perpensa* and *C. triloba*) in synthesizing NPs were then micropropagated to form callus cultures. The reducing abilities of these callus cultures extracts were determined by varying temperature and concentration parameters. Characterization of the NPs formed by the different extracts was performed using UV-vis, TEM and FTIR. UV-vis spectrophotometry was used as a confirmatory as well as characterizing tool. TEM analysis was able to provide a description on the size and shape of the NPs whereas FTIR provided information on the biomolecules responsible for synthesis and capping of NPs. The stability of the NPs was determined by UV-vis scans over a period of 30 days which allowed observation of the alteration in peak shape and absorbance and hence condition of particles. Phytochemical tests were performed on the leaf extracts of the four plants to elucidate possible phytochemicals responsible for the reduction of metal salts. Antibacterial activity of the NPs was evaluated by using the disk diffusion assay and MICs were determined by the broth dilution method against pathogenic bacteria.

A. dubius, *G. perpensa* and *C. triloba* were capable of synthesizing Ag NPs and Au NPs which were indicated by yellowish orange and reddish purple colour changes respectively. *G. perpensa* was able to spontaneously form Ag and Au NPs without any addition of heat whereas *A. dubius* and *C. triloba* required heat to form Au NPs. As the temperature of the reactions increased, the absorbance and possibly the number of NPs produced, increased. When the concentration of the extract was doubled, the absorbance was seen to decrease. *C. roseus* did not produce any Ag or Au NPs with any of the leaf extracts. Only *A. dubius*

and *C. triloba* callus extracts were investigated for NP synthesis and it was found that *A. dubius* callus extracts were unsuccessful in synthesizing NPs and *C. triloba* callus extracts were able to form unstable Ag and Au NPs.

The spherical Ag NPs that were formed from aqueous extracts of *A. dubius* were slightly larger than the methanolic Ag NPs. The Ag NPs produced by *G. perpensa* were in the same size range for aqueous and methanolic extracts. *C. triloba* Ag NPs formed from the methanolic extract were closer in size to *A. dubius* aqueous Ag NPs but the *C. triloba* aqueous extract produced much larger Ag NPs than the other extracts. The Ag NPs produced from *A. dubius* aqueous and methanolic extracts as well as *C. triloba* methanolic extracts exhibited the longest stability of 30 days. Ag NPs from *G. perpensa* aqueous extracts had the least stability.

G. perpensa did not form any hexagonal Au NPs and the spherical and triangular Au NPs were smaller unlike in *A. dubius* and *C. triloba* Au NPs. The Au NPs formed by the aqueous extracts of *A. dubius* and *C. triloba* were larger in comparison to their methanolic counterparts. The Au NPs produced from *G. perpensa* aqueous and methanolic extracts as well as *A. dubius* and *C. triloba* methanolic extracts exhibited the longest stability of 30 days. Au NPs were stable for longer in comparison to Ag NPs. FTIR provided evidence that Ag and Au NPs have a chemical bond with the amide group in amino acids. However the intensities of biomolecules for Au NPs are more pronounced compared to the Ag NPs. It was also found that the Ag NPs synthesized by methanolic leaf extracts have slightly higher intensities than Ag NPs synthesized from aqueous leaf extracts. Phytochemical screening showed the absence of tannins in the *C. roseus* leaf, *A. dubius* and *C. triloba* callus extracts and presence in the other three plants.

C. triloba methanolic extract Ag NPs showed the highest activity against Gram-positive *S. aureus.* Aqueous and methanolic Ag NPs from *G. perpensa* and *C. triloba* as well as *A. dubius* methanolic Ag NPs had activity against all fourteen bacteria. *A. dubius* aqueous Ag NPs had no activity against *Enterobacter* spp. and a strain of *Klebsiella pneumoniae*. *G. perpensa* Ag NPs had better antibacterial activity and lower MICs against Gram-positive and Gram-negative pathogenic bacteria compared to *A. dubius* and *C. triloba*. There was no antibacterial activity seen with Au NPs.

The size and shape of NPs are the keys to their biomedical properties. Green synthesis of NPs is a feasible way for the future. This study showed that NPs can be synthesized very easily and economically. A key finding of this study is that different plants produce varying sizes and aggregation of NPs.

1. INTRODUCTION

Nanotechnology involves the manipulation of matter on an atomic and molecular scale which results in devices or materials that have at least one dimension in the 1-100 nm range (Allhoff *et al.*, 2010). Materials at nano-scale have properties that differ to their bulk counterparts. They are seen to be much more sensitive and enable a unique type of interaction (Singh *et al.*, 2010). The morphology, size and shape of nanoparticles (NPs), are the key players in nanotechnology that can be controlled by changing certain parameters in their synthesis procedures. These characteristics determine the properties of NPs and in turn their potential applications (Mohanpuria *et al.*, 2008). Electrical and optical properties of silver (Ag) and gold (Au) NPs have been utilized in various biological applications (Chamakura *et al.*, 2011). Ag and Au NPs are applied in various products such as shampoo; shoes; cosmetic products; along with medical and pharmaceutical applications. Silver nanocrystalline particles are also known to have antimicrobial activity. They have been used in catalysis, in micro-electronics and other therapeutic applications (Jain *et al.*, 2007). Au NPs have shown applications mainly in diagnostics and drug delivery systems (Song and Kim, 2009).

Nanoparticles have been produced by physical and chemical methods; however, there is a need for environmentally safe, low-cost, rapid, less laborious, easily scaled-up synthesis methods. The chemical processes that are used to synthesize NPs are expensive and also lead to toxic chemicals on the surface of the NPs that may have an adverse effect in medical applications. Biosynthesis has thus solved this problem by eliminating the need for high pressure, energy or toxic chemicals and is an area of research that is becoming quite popular.

The synthesis of Au NPs ranging from <1 to several hundred nanometers in size, and shapes has been shown in both aqueous and nonpolar organic solutions (Brust *et al.*, 1994; Daniel and Astruc, 2004). The usual synthetic route to prepare Au NPs involves the reduction of an Au salt (usually a halide) in solution by various reducing agents in the presence of a stabilizer. The use of rapid reductants results in bigger and generally spherical NPs whereas weak reducing agents result in smaller NPs and a slower reaction (Turkevich *et al.*, 1951; Daniel and Astruc, 2004). Many chemical reduction methods have

been applied to synthesize stable and various shapes of Ag NPs in water by the use of different reducing agents. The shape, size and the size distribution strongly depended on the strong and weak tendency of organic substrates to reduce the Ag salts. Polymeric materials are often used as stabilizers to prevent agglomeration of the NPs. Due to the growth process of NPs controlled by the stabilizers, it is possible to manipulate the shape and size of Ag NPs by choosing different agents (Hussain *et al.*, 2011).

Biological systems such as fungi, bacteria, algae and plant extracts are able to produce different NPs with varying behaviour patterns. These particles are produced extracellularly and are stable in solution (Gericke and Pinches, 2006; Mohanpuria *et al.,* 2008; Singh *et al.,* 2010). Many plants have been used to study the synthesis of NPs, most of which involve the use of leaf extracts (Shankar *et al.*, 2004; Ankamwar *et al.*, 2005; Chandran *et al.*, 2006; Huang *et al.*, 2007; Song *et al.*, 2009; Dwivedi and Gopal, 2010; Elumalai *et al.*, 2010; Nabikhan *et al.*, 2010; MubarakAli *et al.*, 2011; Sheny *et al.*, 2011). Successful tissue culture-derived callus synthesis is also an option, as seen with callus extracts of *Carica papaya* (Mude *et al.*, 2009) and *Sesuvium portulacastrum* (Nabikhan *et al.,* 2010). According to Huang *et al*., (2007), polyphenolic and water-soluble heterocyclic components found in plants are mainly responsible for the reduction of gold and silver salts and the stabilization of the NPs.

With regard to biosynthesis in South Africa, Au NPs were synthesized using different microorganisms (Gericke and Pinches, 2006). Platinum NPs have been synthesized from sulfate-reducing bacteria however, precise control over the particle morphology was not attained (Riddin, 2009). The formation of Ag NPs by the reduction of aqueous silver metal ions during exposure to both fresh and dry seaweed extracts of *Codium capitatum* is reported (Kannan *et al.*, 2013). The CSIR hosts one of the national facilities for the synthesis and characterization of nanomaterials in South Africa. Recent acquisition of modern facilities has enhanced the centre's capability for production a wide variety of nanomaterials both for research and industrial applications. These comprise of carbon nanotubes, nanocomposites, polymers, quantum dots and metallic-based NPs such as silicon and titanium dioxide (Maity and Ray, 2008).

In this thesis our aim is to investigate the synthesis and characterization of Ag and Au NPs from leaf and callus extracts of *A. dubius*, *G. perpensa*, *C. triloba* and *C. roseus* and determine their antibacterial activity on pathogenic bacteria.

Objectives

1. To synthesize Ag and Au NPs from aqueous, methanolic and ethyl acetate leaf extracts of *Amaranthus dubius; Gunnera perpensa; Ceratotheca triloba* and *Catharanthus roseus* using $HAuCl_4$ and $AgNO_3$ respectively
2. To determine the influence of temperature of the reaction and concentration of extracts on NPs synthesis.
3. To evaluate if callus produced by the plants can produce Ag and Au NPs
4. To characterize the synthesized Ag and Au NPs by UV-vis spectrophotometry, TEM and FTIR
5. To determine the stability of the synthesized NPs over a month
6. To identify the nature of the phytochemicals responsible for the reduction of $AgNO_3$ and $HAuCl_4$ respectively.
7. To investigate the antibacterial activity of the Ag and Au NPs against selected sensitive and resistant pathogenic bacteria

The objectives are achieved in five chapters. Chapter one and two provides background information and the literature review. This is followed by the methodology in chapter three, the results in chapter four and discussion and conclusion in chapters five and six. All the references cited in the thesis are presented in chapter seven. Figure 1 shows an overview of this thesis.

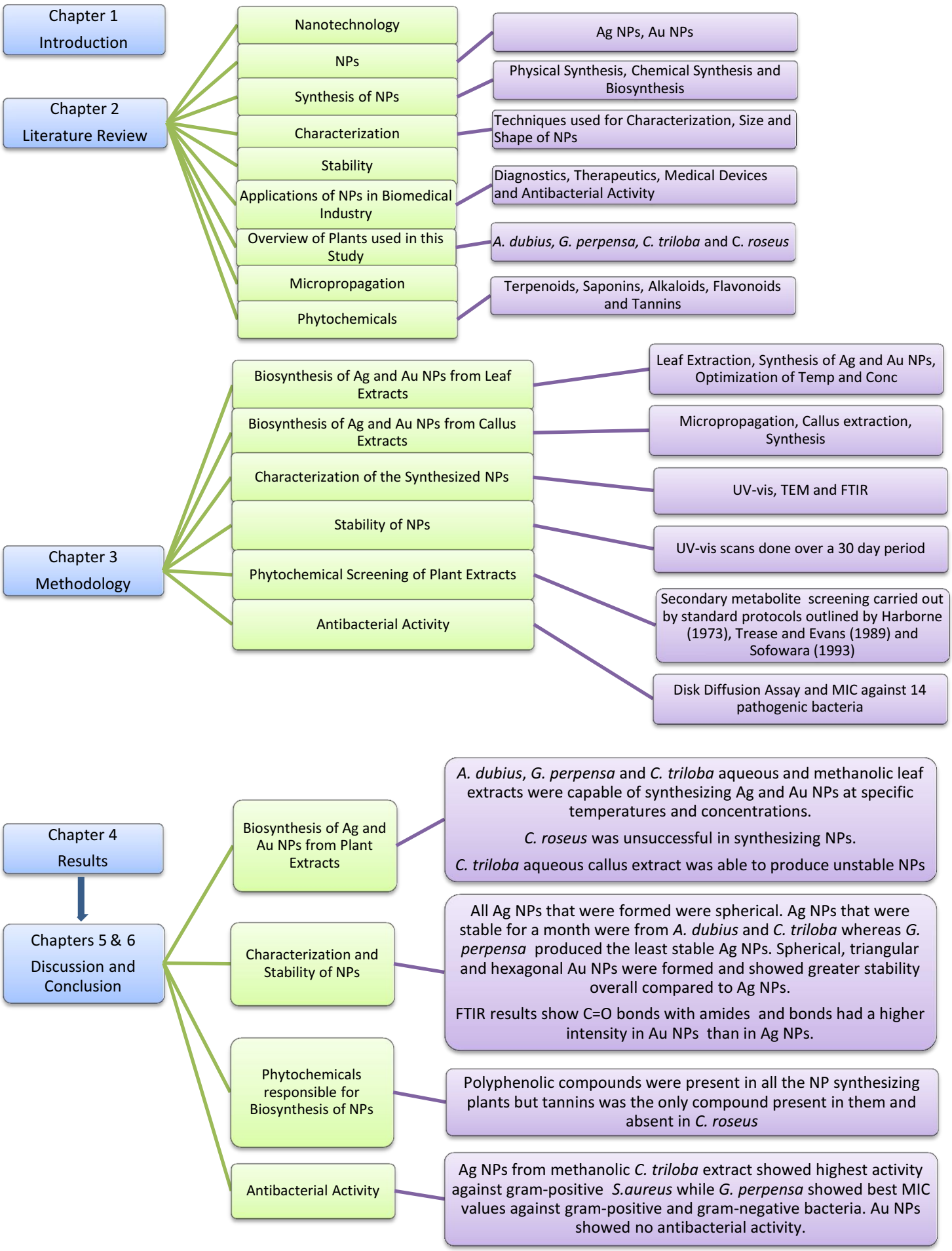

Figure 1: Overview of the thesis

2. LITERATURE REVIEW

2.1 Nanotechnology

Nanotechnology is the application of science to control matter at a molecular level and is an interdisciplinary field which involves physics, chemistry, biology and engineering. A nanometer refers to one billionth of a meter (Sahoo *et al.*, 2007). The physicist, Richard P. Feynman explained the concept of nanotechnology in 1959 through his talk "There's plenty of room at the bottom". It only became known as 'nanotechnology' in 1974 when Professor Norio Taniguchi devised the term. Although it seems like novel science, it has been used for aesthetic and curative purposes for centuries. (Singh *et al.,* 2010).

Nanomaterials offer greater permeability and sensitivity allowing more unique interactions than larger particles. The merging of nanotechnology and biotechnology seems logical considering that nanostructures are capable of interacting with molecular and DNA components which are at the nanometer scale (Singh *et al.,* 2010). "Bionanotechnology is a subset of nanotechnology where the biological world provides the inspiration and/or the end goal" (Goodsell, 2004). Exclusive interactions with similarly sized biological materials and the ability to traverse these materials allow for the discovery of new and intriguing biological applications.

South Africa has shown interest in nanotechnology with regard to the impact it will have on the mineral markets. In 2005, the National Nanotechnology Strategy (NNS) was launched and funding of around 600 million USD was allocated to research and development. India is currently leading in the trilateral nanotechnology development programme with Brazil and South Africa. There is a concern that natural resources might become superfluous due to the development of cheaper, stronger and more functionally rich materials. The focus areas of the National Nanotechnology Strategy (NNS) are: establishing characterization centres throughout the country enabling researchers with access to advanced machines; creation of Research and Innovation networks to enable collaborations between different institutes; initiatives to increase human capital resources and flagship projects highlighting the benefits of nanotechnology in the chemistry, water, energy, health and mineral sectors (Wetter, 2010).

2.2 Nanoparticles

A nanoparticle (NP) (10^{-9}m) refers to a small object that in terms of its properties, functions as an entire unit. The physical and chemical properties have a molecular and an atomic origin and thus behave differently in comparison to their bulk compounds. There are two groups of NPs, organic and inorganic: the organic group is comprised of carbon NPs and fullerenes whilst the inorganic group consists of noble metal (Ag and Au) and semiconductor (titanium dioxide and zinc oxide) NPs. Inorganic NPs provide better material properties with wide availability, good biocompatibility and functional adaptability (Singh *et al.*, 2010). Inorganic NPs show important novel chemical, physical and biological characteristics as well as good functionality owing to their nano-scale size. An example to prove this is the colour difference between metallic gold which is yellow whereas Au NPs that have a wine red hue (Daniel and Astruc, 2004; Bhattacharya and Mukherjee, 2008).

Properties of NPs are derived from the high quantity of surface area and the high surface area to volume ratio which allows them to have a significant surface energy. As their diameter decreases, the available surface area of the particle itself increases and thus has an influence on potential applications (Christian *et al.*, 2008; Singh *et al.,* 2010). NPs also offer additional advantages when they are chemically modified to form a suspension, emulsion or aerosol. In addition, some chemicals can also modify the function and indirectly stabilise against aggregation or coagulation by changing the surface layer of the particle (Singh *et al.,* 2010). The full potential of NPs depends on stability, biocompatibility and directed specificity to the target sites with regard to systemic administration (Mody *et al.*, 2010).

Due to their unique and tuneable properties, they are able to find applications in photothermal therapy (Jain *et al.,* 2007), catalysis (Xiao and Xia, 2010), optoelectronics (Mohanpuria *et al.,* 2008), water purification (Jain and Pradeep, 2005), quantum dot lasers for detection (Ledenstov *et al.*, 1996), Surface Enhanced Raman Spectroscopy (SERS) detection and biosensing (Stuart *et al.*, 2005).

2.2.1 Silver Nanoparticles

Ag ions have been used for the treatment of burns and wounds for centuries. In the year 1700, silver nitrate was used in its solid form to treat venereal diseases, abscesses and fistulae from salivary glands. In the 19th century, $AgNO_3$ was used in varying concentrations to treat fresh burn wounds. In 1881, eye inflammations of new-borns (opthalmia neonatorum) were cured using $AgNO_3$ eye drops. The use of silver slowly declined when penicillin came into use. Recently however, clinicians have gone back to the use of silver in wound dressings (Klasen, 2000; Lansdown, 2002).

Ag is inert in its metallic form but gets ionized when it comes into contact with the moisture of the wound. The ionized silver is very reactive which brings about binding to bacterial proteins which results in structural change of the cell and eventually leads to death. These ions are also responsible for inhibiting bacterial replication by denaturing bacterial DNA and RNA. The amount of silver and the rate at which it gets released determines its antimicrobial ability (Lansdown, 2002; Rai *et al.*, 2009).

Ag NPs are clusters of silver atoms that are metallically bonded together and are well known for their antibacterial activity. In aqueous solutions, they release Ag ions which accounts for this activity (Chaloupka *et al.*, 2010). Ag NPs are now being seen as an affordable and valuable method for enhancing water filtration in developing countries (Jain and Pradeep, 2005). In animal models there is also evidence of potent anti-inflammatory activity and better wound healing with a scar-less endpoint (Tian *et al.*, 2007). Ag NPs biosynthesized from fungi have also been incorporated into textile fabrics yielding a positive antibacterial effect (Duran *et al.*, 2007). The preservation of food by inhibition of microorganism growth has also been performed by integrating Ag NPs into plastics, food storage bags, chopping boards and refrigerator surfaces (Chaudhry *et al.*, 2008). Vegetable oil was used to make an environmentally safe metal NP embedded paint which showed extremely promising antibacterial activity against Gram-positive and Gram-negative bacteria. This could be the future of coating several surfaces to eliminate bacterial problems (Kumar *et al.*, 2008). Also, a promising future prospect for HIV therapeutics could lie in Ag NPs, as studies have shown the prevention of key interactions of $CD4^+$ cells with envelope proteins of HIV-1 by Ag NPs in *in vitro* assays (Lara *et al.*, 2010). Antibacterial activity against biofilm formation by *Pseudomonas aeruginosa* and

Staphylococcus epidermidis has been established. This is very important as pathogenic bacteria are often seen to form biofilms and the exopolysaccharide substance that is produced is able to reduce the susceptibility of the microorganism to drug administration. Antibiotics kill the microorganisms only, whereas the Ag NPs are able to inhibit the microorganisms as well as inhibit the ability to synthesize the exopolysaccharide (Kalishwaralal *et al.*, 2010). Another important aspect of Ag NPs is that they have been found to be non-toxic to humans in small concentrations (Rai *et al.,* 2009). Exposures to concentrations lower than 3 parts per million did not produce a significant immune response (Klippstein *et al.*, 2010). The European Protection Agency is in the process of determining environmental limits for Ag NPs since they are becoming more popular commercially. It is not seen as being harmful to the environment by some since the nature of the NPs become unstable rapidly and forms the harmless clumps of silver metal (Maass, 2008; Levard *et al.*, 2012).

2.2.2 Gold Nanoparticles

Au NPs are considered to be the most stable NPs and have applications in size-related optical and electronic properties in the field of biology. This colloidal gold as it is known has been used to stain glass and ceramic from around the 4^{th} or 5^{th} century B.C in China and Egypt (Daniel and Astruc, 2004). Au NPs were also used in the middle ages to diagnose syphilis, cure heart problems and dysentery. Jeremias Benjamin Richters in 1818 gave a possible reason as to why there is a difference in the colour of gold solutions in comparison to their bulk form which states "pink or purple solutions contain gold in the finest degree of subdivision, whereas yellow solutions are found when the fine particles have aggregated". In 1857, Faraday was responsible for producing gold colloidal solutions and observing their optical effects (Ostwald, 1909; Daniel and Astruc, 2004). Although bulk Au is known to be inert, Au NPs have very reactive cores that are especially useful in catalytic activities. Their combination of low inherent toxicity, high surface area and tuneable stability provide them with unique qualities that should enable new delivery strategies (Ghosh *et al.*, 2008).

Au NPs are now being considered as perfect anti-angiogenic compounds since they are easy to synthesize and characterize, biocompatible and able to bind easily to thiols which would in turn inhibit or denature the function of angiogenesis inducer proteins. Various

diseases such as cancer, arthritis, etc., depend on angiogenesis thus Au NPs could lead to new therapeutic measures for these diseases (Bhattacharya and Mukherjee, 2008). Selective killing of target bacteria by irradiating Au NP-attached bacterial surface with a laser has also been accomplished (Zharov *et al.*, 2006). Strategies are emerging that could allow Au NPs to become the next generation of diagnostic tools, as these NPs show great sensitivity and specificity that can easily replace conventional molecular methods such as PCR and PCR-based approaches. Future trends of Au NPs in diagnostics include their functionalization, allowing modification of the Au NP biomolecule interface and modulation of the properties of the biomolecule (Daniel and Astruc, 2004). There is not much research with regard to the toxicity of Au NPs to human cells and this needs to be taken into consideration when applying these particles for medical uses.

2.3 Synthesis of Nanoparticles

NPs can be synthesised physically, chemically and biosynthetically and are grouped into top-down and bottom-up approaches. According to Revaprasadu and Mlondo (2006) from South Africa, NPs synthesized by chemical routes will be the preferred method of choice in future applications here as well as internationally, due to the control of the size, shape, and ease of management of these NPs. Table 1 is a summary of the advantages and disadvantages of the different synthesis methods that are discussed in detail further below.

Table 1: Summary of advantages and disadvantages associated with each of the synthesis methods

	Advantages	**Disadvantages**
Physical Synthesis Method		
Co-precipitation	Simultaneous incidence of nucleation, growth, and/ or agglomeration processes	Products are scarcely soluble and produced under high supersaturation
Laser ablation	Enables colloidal NPs solutions to be formed in a variety of solutions	Produces NPs in small quantities and requires a high energy per unit
Vapour-phase	Can produce spherical particles	Requires supersaturated vapour and optimal condensation kinetics with high temperatures
Inert Gas Condensation	Ability to control the morphology of single state NPs	Ultra-high vacuum chambers are needed with different gases and high pressures

Chemical Synthesis Method		
Turkevich	Particle size can be controlled within a range of 10 to 100 nm with limited polydispersity	Uses organic solvents that are harmful to the environment and requires purification
Solvothermal	Choice among various solvents thereby increasing versatility of synthesis	High pressures and above boiling point temperatures required.
Sol-gel	Size and stability control has been achieved by some materials	Complexity of process and the synthesized precipitates are generally amorphous
Biosynthesis Method		
Microorganisms	Easy, low-cost, environmentally-friendly	Incubation periods to grow microorganisms
Fungi		Incubation periods to grow fungi
Algal biomass/cyanobacteria		Growth periods and conditions required
Plant extracts		Bio-sustainability
Callus cultures	Environmentally-friendly, ease of scale-up	Laborious process and specialized facilities required

2.3.1 Physical Synthesis

Physical synthesis follows the top-down method which involves NPs or organised assemblies being directly generated from bulk materials by way of generation of atoms through various distribution techniques. These distribution or removal techniques can be mechanical, chemical, electrochemical, etc. based on the bulk substrate and the desired outcome. Milling is one of the most widely used techniques in the manufacturing industry. There is a certain amount of control when it comes to the dimensions of the final product in that the travel path of the mill is precisely controlled by computer aided numerical systems (Singh *et al.,* 2010).

Co-precipitation is one of the earliest synthesis methods of NPs. It consists of less soluble products from aqueous solutions undergoing thermal decomposition to produce oxides. These reactions involve the simultaneous incidence of nucleation, growth, and/ or agglomeration processes. The products of precipitation reactions are scarcely soluble and produced under high supersaturation (Cushing *et al.*, 2004).

Laser ablation refers to utilization of a laser beam to irradiate solid surfaces to remove material. According to the laser wavelength specific to a certain material and optical properties, different amounts of the material will be removed by a single laser pulse and

laser energy will be absorbed over different depths. This method produces NPs in small quantities and requires a high energy per unit. Carbon nanotubes are often formed in this way (Singh *et al.* 2010).

In **vapour-phase** synthesis, the vapour phase mixture is thermodynamically volatile compared to the solid material formed. This consists of supersaturated vapour and if it is sufficient and if condensation kinetics allow, the particles will nucleate homogeneously. This happens rapidly in a relatively uncontrolled manner. Once particles form in the gas phase, they coagulate, and at high temperatures, particles combine faster than they coagulate which forms spherical particles (Swihart, 2003).

Inert gas condensation where ultra-high vacuum chambers are filled with argon or helium gas and at different high pressure values of a few hundred pascals, different metals evaporate. These evaporated metal atoms lose their kinetic energy and thus condense to form small crystals. Advances in this technique have been seen by being able to control the morphology of single state NPs by controlling coalescing after particle formation (Swihart, 2003; Singh *et al.*, 2010).

2.3.2 Chemical Synthesis

The bottom-up strategy is the basis of chemical synthesis methods. The foundation of this type of synthesis of NPs is that a salt of the metal is dissolved in a solvent and reduced to the zero valence state. Molecular components are the starting materials, followed by chemical reactions, nucleation and growth to form complex clusters (Brust *et al.,* 1994; Ju-Nam and Lead, 2008). Metal atoms that are produced by reduction in solutions are primarily insoluble, which leads to clusters called ‘embryos’ through slow aggregation. The moderately sized embryos will either dissociate or grow to become stable. Once the embryos have reached a certain size, they separate from the solution as solid particles and are referred to as nuclei. The nuclei grow to nanosize particles and thereafter diffusion of metal atoms onto the particles occurs and/ or the nanosize particles aggregate to form the final NPs. The density and the shape of the NPs depend on the dominant mechanism. NPs produced via aggregation will predominantly have a spherical shape and a lower density (Goia and Matijevic, 1998). If the surface is not protected by a capping agent, interactions between particles will generally occur so as to reduce their high surface energy which

causes the aggregation. Capping agents can be organic or biological molecules as well as polymers. They are responsible for charge or steric stabilization mechanisms which prevent further agglomeration making them integral components of NPs (Feldheim and Foss, 2002; Ju-Nam and Lead, 2008). Most physical methods have a disadvantage as they synthesize particles that are attached to a substrate or fixed in a matrix, which limits their potential in applications. The correct choice of organic moieties as capping agents has the potential to reduce or increase toxicity in environmental systems (Ju-Nam and Lead, 2008).

The conventional method for synthesizing Au NPs is by the **Turkevich** method. This method consists of the reduction of $HAuCl_4$ salt in a boiling solution of sodium citrate. The average particle size can be controlled to be in the range of 10 to 100 nm with limited polydispersity. Citrate molecules act as both reducing and capping/stabilizing agents and functionalization of the particles are possible from this step. The chemical reduction of Ag and Au NPs can be expressed in equations (1) and (2) respectively:

$$AgNO_3 + NaBH_4 \rightarrow Ag + \tfrac{1}{2}H_2 + \tfrac{1}{2}B_2H_6 + NaNO_3 \quad (1)$$ (Van Dong *et al.*, 2012)

$$2HAuCl_4 + 3C_6H_8O_7 \text{ (citric acid)} \rightarrow 2Au + 3C_5H_6O_5 \text{ (3-ketoglutaric acid)} + 8HCl + 3CO_2 \quad (2)$$ (Tabrizi *et al.*, 2009).

Solvothermal synthesis involves the use of polar solvents under high pressure and above boiling point temperatures. Under these conditions, the reactants' solubility increases dramatically which permits the reaction to occur at low temperatures. Examples of these include microwave-hydrothermal reactions and flow-solvothermal reactions.

Sol-gel technique is used to construct metal oxides from a chemical solution. The solution acts as a precursor solution and can be cast into an appropriate container with a certain shape or deposited on the substrate which forms a film. The pores of the film become distorted and organic species are released (Cushing *et al.,* 2004).

2.3.3 Biosynthesis

Biosynthesis like chemical synthesis methods uses the bottom-up strategy whereby the main reaction is the reduction of the metal salts to form NPs. Almost all of the NP synthesis methods involve the use of hazardous chemicals, low material conversions,

difficult and wasteful purifications and high energy requirements. Biosynthetic methods employing either biological microorganisms or plant extracts have emerged as a simple or viable alternative to chemical and physical synthesis methods. Green synthesis provides various advancements as it is cost effective, environmentally friendly, easily scaled up for large scale synthesis and there is no need to use high pressure, energy, temperature and toxic chemicals (Veerasamy *et al.*, 2011). Green synthesis generally has three main criteria: 1) solvent medium selection; 2) environmentally benign reducing agent selection; and 3) non-toxic substances for NPs stability selection (El-Shishtawy *et al.*, 2011). In biosynthesis, it is taken that the natural material extract acts as reducing agents for the generation of metal NPs (Dubey *et al.*, 2010). NPs are synthesized by bacteria, fungi, algae and plants. Since the noble metal NPs are in contact with humans in their applications, there is a growing need to develop eco-friendly processes to synthesize NPs to prevent the exposure of toxic chemicals (Song and Kim, 2009). However, most of the methods are still in the developmental stage and various problems are often experienced with the stability of NPs, control of crystal growth and aggregation of particles.

Microorganisms that range from simple prokaryotic bacterial cells to eukaryotic fungi have been involved in NP production. These organisms enzymatically reduce dissolved heavy metals and metalloids to produce insoluble NPs (Popescu *et al.*, 2010). Bacteria are unicellular microorganisms that are able to survive in stressful situations due to their mechanisms of resistance such as efflux pumps, inactivation of metals, changing of solubility and toxicity by redox states of the metal ions, extracellular precipitation of metals and volatilization of toxic metals enzymatically (Rouch *et al.*, 1995). Researchers have taken a closer look at exploring the mechanisms used by bacteria to form NP formation and metal reduction because of the application in biorecovery of important metals and the bioremediation of toxic metals. This led to the deduction that reducing agents like enzymes and biochemical pathways which are important for metal reduction have been linked to NP production. Proteins are also likely to bring about bioreduction (Korbekandi *et al.*, 2009). *Lactobacillus* strains usually found in buttermilk are capable of forming Ag and Au NPs when exposed to the respective metal salts. Nanocrystals with well-defined morphologies are formed. This shows that sugars and enzymes in cell walls bring about the nucleation of Au and Ag NPs. These metal nuclei move into the cell where they aggregate and become larger particles (Nair and Pradeep, 2002). The bacterium *Rhodopseudomonas capsulata* formed stable Au NPs extracellularly. The size and shape of

Au NPs could be changed by altering the pH of the reaction. The authors suggested that the bioreduction of the Au salt could have been due to NADH-dependent enzymes that are secreted by *R. capsulata* (He *et al.*, 2007).

Fungi are heterotrophic organisms and obtain their nutrients by absorption. They have the potential to secrete enzymes in large amounts. Yeasts have been shown to synthesize semiconductor NPs. *Schizosaccharomyces pombe* and *Candida glabrata* have intracellularly produced cadmium sulphide NPs. The yeast cells produce metal-chelating peptides that stabilize the NPs (Dameron *et al.*, 1989). Extracellular synthesis of stable Ag NPs using *Fusarium oxysporum* has also been studied (Ahmad *et al.*, 2003).

Live **algal biomass** and **cyanobacteria** can form Au NPs along with biorecovery and phytomining of Au cost effectively. *Spirulina subsalsa* and *Rhizoclonium heiroglyphicum* are some of the examples that have yielded positive results (Chakraborty *et al.*, 2009). *Spirulina platensis* biomass is able to extracellularly synthesize Ag, Au and bimetallic NPs in the range of 17 nm-25 nm and it was suggested that the a possible explanation for the reduction of the salts is due to proteins (Govindaraju *et al.*, 2008). Metal ions are trapped on the surface of the cells due to the electrostatic interaction between ions and negatively charged carboxylate groups present in the cells of the algal biomass. The enzymatic reduction of these ions leads to the formation of nuclei which continue to grow with the further reduction of metal ions (Mandal *et al.*, 2006).

Plant Biosynthesis - Plants as autotrophs have an advantage over other systems in terms of their morphological characterization, molecular distribution, primary and secondary metabolite systems which allows them adaptability in a range of conditions (Jha *et al.*, 2009). They have many advantages:

- they are found along a wide range of ecological boundaries making them easily available;
- they are safe to handle;
- they are stocked with valuable metabolites; and
- they allow any chemical protocol to be seen as ‘green’.

Their metabolites are seen as treasures which have not been used to its full capacity especially when relating it with metallic NP synthesis (Kalishwaralal *et al.,* 2010). Using plants for NP synthesis can be advantageous over other biological processes because:

- it is easily available;
- eliminates the need for expensive tissue culture facilities and expertise; and
- can be suitably scaled up for large scale synthesis of NPs under non-aseptic environment (Jha *et al.*, 2009).

Intracellular synthesis of Ag or Au NPs was found in living alfalfa plants. This was possible via the uptake of Au and Ag ions respectively, from solid media (Gardea-Torresday *et al.*, 2002; Gardea-Torresday *et al.*, 2003; Sharma *et al.*, 2007). Recently, the extracellular synthesis of NPs using leaf extracts has become a popular area of research due to its rapidity and rather than using whole plants, is more economical due to the easier downstream processing. Table 2 provides a summary of many of the studies undertaken with aqueous leaf extracts to synthesize Ag and Au NPs.

Another option is synthesis of NPs by micropropagation. The first report on such synthesis was on *Carica papaya* with the production of Ag NPs (Mude *et al.,* 2009). Callus cultures of salt marsh plant *Sesuvium portulacastram* have been induced and the aqueous extracts were able to successfully synthesize Ag NPs. Results of this study showed that the extract of leaf callus was actually more efficient in synthesis of more stable Ag NPs in comparison to the intact leaf extract. They suggested that this could have been due to the metabolically active mass of young cells with different ploidy present in the callus which was able to produce various types of metabolites owing to the reduction of silver ions (Nabikhan *et al.,* 2010). There are not many reports on tissue culture-derived callus extract synthesis of NPs. Industrial scale production of NPs would be possible by combining tissue culture systems with downstream processing procedures. Also, plant tissue culture represents a simple technology that allows for selection of various desirable traits from *in vitro* cultures (Richard *et al.*, 2006). A disadvantage of this method however, is the long and labour-intensive tissue culture method that needs to be set up.

Table 2: Leaf extracts used to synthesize Ag and Au NPs and the resultant shapes and size ranges

LEAF EXTRACT	NPs Synthesized and Shapes	Size Range (nm)	Reference
Azadirachta indica (Neem)	Ag NPs – Spherical and polydispersed Au NPs – Spherical, triangular, hexagonal Ag / Au Bimetallic NPs	5 -35 ~40 50 – 70	(Shankar *et al.*, 2004)
Tamarindus indica (Tamarind)	Au NPs – Triangular	100 – 500	(Ankamwar *et al.,* 2005)
Aloe vera	Ag NPs – Spherical Au NPs – Triangular	~15	(Chandran *et al.,* 2006)
Cinnamomum camphora	Ag NPs – Spherical Au NPs – Spherical, triangular	55-80	(Huang *et al.,* 2007)
Coriander	Au NPs – Spherical, triangular, truncated triangles	6.8 – 58	(Narayanan and Sakthivel, 2008)
Magnolia kobus and *Diopyros kaki*	Au NPs – Spherical, triangular, hexagonal, pentagonal	5 – 300	(Song *et al.,* 2009)
Rosa rugosa	Ag NPs – Spherical Au NPs – Spherical, triangular, hexagonal	12 11	(Dubey *et al.,* 2010)
Sesuvium portulacastrum	Ag NPs – Spherical	5 – 20	(Nabikhan *et al.,* 2010)
Euphorbia hirta	Ag NPs – Polydispersed	40 – 50	(Elumalai *et al.,* 2010)
Chenopodium album	Ag NPs – Spherical Au NPs – Spherical and triangular	10 – 30 10 – 30	(Dwivedi and Gopal, 2010)
Terminalia catappa	Ag NPs – Spherical	10 – 35	(Ankamwar, 2010)
Olive	Ag NPs – Spherical	13	(Khalil *et al.*, 2010)
Coriandum satrivum	Ag NPs – Spherical	8 – 75	(Sathyavathi *et al.*, 2010)
Murraya koenigii	Ag NPs – Spherical Au NPs – Nearly Spherical and triangular	10 ~20	(Philip *et al.*, 2011)
Moringa oleifera	Ag NPs – Spherical	57	(Prasad and Elumalai, 2011)
Coleus amboinicus	Ag NPs – Spherical and triangular	~26	(Narayanan and Sakthivel, 2011)
Garania mangostana (mangosteen)	Ag NPs – Spherical	6 – 57	(Veerasamy *et al.,* 2011)

Mentha piperita	Ag NPs – Spherical Au NPs – Spherical	90 150	(MubarakAli *et al.,* 2011)
Citrullus colocynthis	Ag NPs – Spherical	31	(Satyavani *et al.*, 2011)
Stevia rebaudiana	Ag NPs – Spherical and polydispersed	2 – 50	(Yilmaz *et al.*, 2011)
Catharanthus roseus	Ag NPs – Spherical and polydispersed	48 - 67	(Mukunthan *et al.*, 2011)
Anacardium occidentale	Ag NPs – Spherical Au NPs – Almost spherical Ag / Au Bimetallic NPs	19 8.5 8	(Sheny *et al.,* 2011)
Ficus benghalensis	Ag NPs – Spherical	16	(Saxena *et al.*, 2012)
Prosopis juliflora	Ag NPs – Triangular, pentagonal, hexagonal	35 - 60	(Raja *et al.*, 2012)
Iresine herbstil	Ag NPs – Spherical	44 – 64	(Dipankar and Murugan, 2012)
Amaranthus spinosus	Au NPs – Spherical and triangular	10.7	(Das *et al.*, 2012)
Amaranthus spinosus (ethanolic extract)	Au NPs – Spherical and triangular	10.7	(Das et al., 2012)
Pepper leaves	Ag NPs – Spherical	5 – 60	(Mallikarjuna *et al.*, 2012)
Cissus quadrangularis	Ag NPs – Spherical	50 – 60	(Valli and Vaseeharan, 2012)
Catharanthus roseus	Ag NPs – Spherical and polydispersed	35 – 55	(Ponarulselvam *et al.*, 2012)
Piper pedicellatum	Ag NPs – Spherical Au NPs – Spherical, triangular, hexagonal	2 – 30 2 – 40	(Tamuly *et al.*, 2012)
Ocinaum tenuiflorum	Ag NPs – Irregular	28	(Logeswari *et al.*, 2012)
Solanum tricobatum	Ag NPs – Irregular	22	(Logeswari *et al.*, 2012)
Syzygium cumini	Ag NPs – Irregular	26	(Logeswari *et al.*, 2012)
Centella asiatica	Ag NPs – Irregular	28	(Logeswari *et al.,* 2012)
Euphorbia prostate	Ag NPs – Rod shaped	28 – 80	(Zahir and Rahuman, 2012)
Carmellia sinensis	Ag NPs – Spherical	2 – 10	(Loo *et al.*, 2012)
Aloe barbadensis	Ag NPs – Spherical, quasi-spherical Au NPs – Quasi-spherical, triangular, rod shaped, hexagonal	25 25	(Elizondo *et al.*, 2012)
Artimisia nilagirica	Ag NPs – Square Spherical Triangular Hexagonal	70 – 90 10 – 45 45 – 60 10 – 25	(Vijayakumar *et al.*, 2013)

Mechanism of Action - The mechanisms of action for biologically synthesized NPs from bacteria, fungi, yeast, algae and cyanobacteria have been investigated with the conclusion being that a collection of enzymes, especially nitrate reductase is responsible for the reduction of the metal salts (Ahmad *et al.,* 2003; Ghorbani *et al.*, 2011). It is taken that in green synthesis, the natural material extract acts as a reducing agent for the synthesis of NPs (Dubey *et al.,* 2010). Water soluble phytochemicals like flavones, quinines and organic acids which are found in plant tissues are likely to result in reduction of the metal ions (Jha *et al.,* 2009). The amount of accumulation of NPs varies with reduction potential of ions and the plants reducing capacity which in turn depends on the polyphenols and water-soluble heterocyclic compounds present. These compounds were also said to be responsible for the stability of the NPs (Huang *et al.,* 2007). Free amino acids or cysteine residues in proteins are linked to the stabilization of the NPs (Gole *et al.*, 2001). Another study done on Neem leaf broth provided evidence that terpenoids were the surface-active molecules responsible for stabilizing NPs and along with reducing sugars were responsible for the bioreduction of the metal salts. This implies that many plant extracts could be used for synthesis of NPs due to the presence of terpenoids and reducing sugars in them. It was also concluded that there is a possibility that the capping species for Ag and Au NPs could be different and the nature of coordination with the metal surfaces could vary in both cases. An assumption that polyphenolic compounds could be adsorbed on the metal NPs surfaces by interaction through carbonyl groups or π-electrons in the absence of strong ligating agents was made. A possibility of terpenoids oxidizing aldehylic groups in the molecules to carboxylic acids was stated (Shankar *et al.*, 2004). According to FTIR results for the *Magnolia kobus* extract used to form Au NPs, the compounds found to be present were proteins and secondary metabolites such as terpenoids (Song *et al.,* 2009).

Different solvent extractions were carried out with Black Tea leaves to identify which types of biomolecules were important in reduction of the metal salts and it was found that extracts lacking polyphenols/flavonoids were unable to produce Ag and Au NPs. A naturally available hydroxyflavone, quercetin was also tested for efficiency in biosynthesis in comparison to the tea leaf extracts. Although the quercetin was able to synthesize Au NPs, it was unable to bring about the reduction of the Ag salt. It can be deduced from this that a synergistic relationship between other polyphenols in the plant extract had a higher potential for reduction of the metal salt rather than just the individual flavonoid. The Au NPs were also much larger in size and irregularly shaped compared to those Au NPs

produced by the leaf extract. It is also safe to say that different compounds and/or concentrations of these metabolites could be responsible for the synthesis of Ag and Au NPs respectively (Begum *et al.*, 2009). Various compounds such as alkaloids, flavones, steroids, polysaccharides, amino acids, oximes and menthol in the leaf extract of *Mentha piperita* has been proven to be responsible for the bioreduction of $AgNO_3$ (MubarakAli *et al.,* 2011). *Chenopodium album* leaf extract was found to have a high level of oxalic acid which was responsible for being a reducing agent as well as a ligand. Phenolic antioxidant compounds have the ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers possibly due to their metal chelation properties (Dwivedi and Gopal, 2010). Figure 2 below is a representation of a probable reduction mechanism of metabolites such as pinitol and allantoin found in the leaf extract of *Pisonia grandis* (Firdhouse *et al.,* 2012).

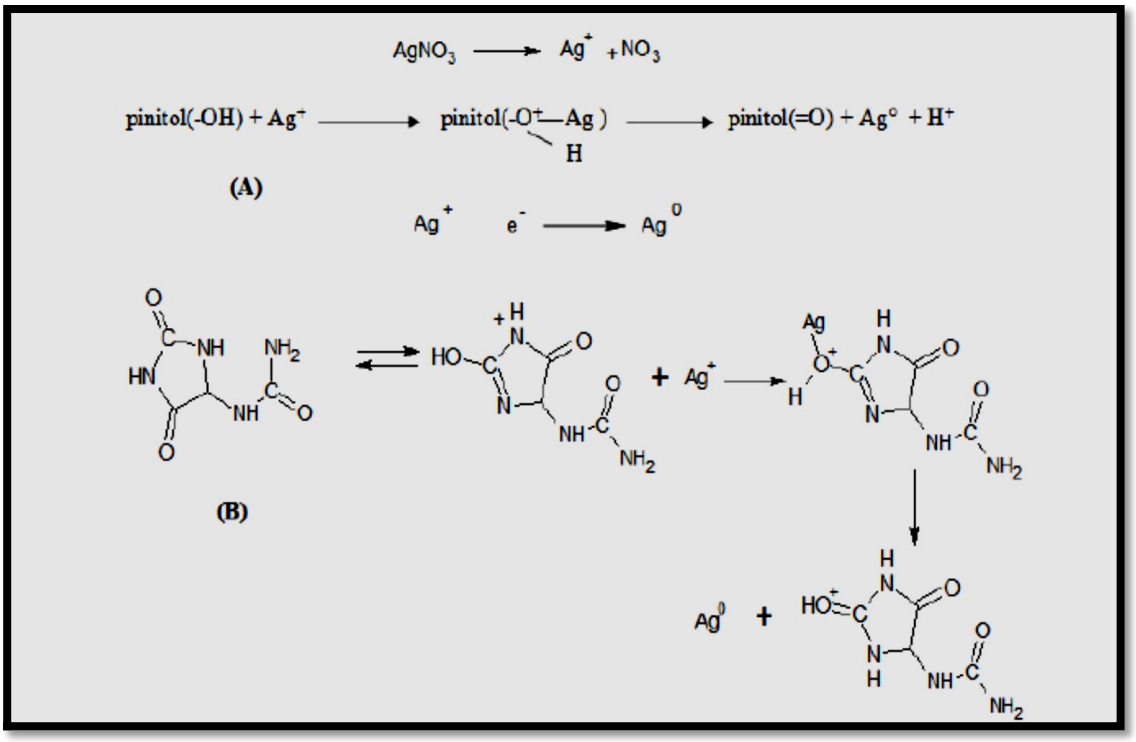

Figure 2: Probable mechanism of reduction by (A) pinitol and (B) allantoin biomolecules to form Ag NPs (Firdhouse *et al.,* 2012)

2.4 Characterization

2.4.1 Techniques used for Characterization

According to the Mie theory, metal NPs interaction with light results in the collective oscillation of the metal-free electrons with respect to the metal lattice in the presence of the electromagnetic field of the light known as Surface Plasmon Resonance (SPR) (Mie, 1908; Daniel and Astruc, 2004). NPs provide a range of photophysical properties which include enhanced light scattering, luminescence, absorption and SERS along with the option of tuning these properties to suit the need at hand (Jain *et al.,* 2007). Due to this SPR phenomenon, UV-vis spectroscopy allows us to confirm the synthesis of NPs and provides raw information on the size, shape and composition. It has been well established that the SPR of metallic Ag NPs exhibit a yellow hue and an absorption band between 395 nm - 425 nm whereas Au NPs exhibit a ruby red colour and gives rise to an absorption band at 510 nm - 540 nm (Richard, 1978; Ju-Nam and Lead, 2008). The band is influenced by the particles shape, the dielectric constant of the medium and temperature. Since all metal NPs need some stabilizing surface ligand, the band energy is also associated to that ligand composition (Daniel and Astruc, 2004).

Another reference technique for characterization of metal NPs is Transmission Electron Microscopy (TEM). The transmitted electrons and the scattering of the beam caused by its interaction with the matter are used to create an image of the sample. TEM analysis is able to ascertain the size and shape distribution of the particles. However, it is ineffective for samples that are composed of particles that are less than 1 nm in size (Pradeep, 2007).

Fourier Transform Infrared (FTIR) spectroscopy involves the absorption of electromagnetic radiation at frequencies that relate to the vibration of specific sets of chemical bonds from within a molecule. It is one of the techniques that focuses on the ligands found on the surface of the metal NPs. This analysis provides information on the organic and biological molecules that are responsible for capping of the NPs (Murray *et al.*, 2000). Figure 3 is a schematic representation of the basic principles of the three instruments used for characterization in this study in relation to NPs.

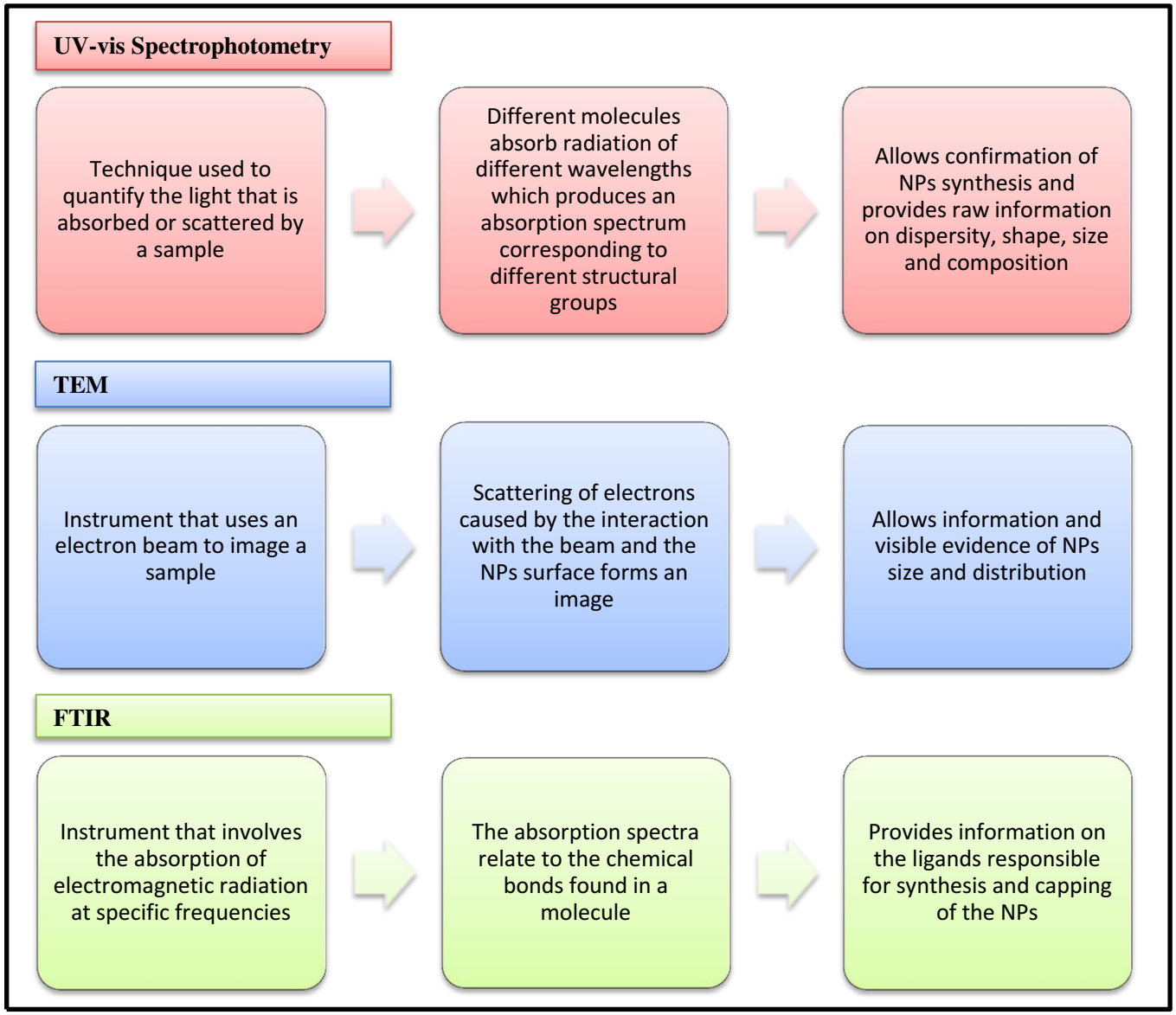

Figure 3: Schematic diagram of the basic principles behind the characterization methods of UV-vis spectrophotometry, TEM and FTIR analysis for NPs

2.4.2 Size and Shape of Nanoparticles

The intrinsic properties of a metal NP are mainly determined by its size, shape, composition, crystallinity and structure (Sun and Xia, 2002). Chemical reactivity is highly dependent on surface morphology. The number of edges and kink sites as well as the surface-area-to-volume ratio can dictate unique surface chemistries (Yang *et al.*, 2008).

Studies show that the interaction between the reducing or stabilizing agents and metallic ions strongly affect the size and shape of the particles. The size and shape of NPs could be controlled by the addition of various polymer molecules and organic salts. The reason for the various particle morphologies was the diversity of the interactions between the polar

groups of the stabilizing agents and the metal. The very strong interaction between the organic polar groups and Ag for example results in very thin and large plates as the particle shape. The addition of alkali salts to the reaction solution yielded particles, which were morphologically not uniform and show a very broad size range distribution (Widoniak *et al.*, 2005).

It was found that Ag NPs possess a more uniform spherical shape and Au NPs are able to form anisotropic shapes such as triangular and polygonal plates (Dubey *et al.,* 2010). The main difference of shape control between Ag and Au NPs are related to the comparative advantage of reductive and protective biomolecules (Armendariz *et al.*, 2004). It was found that the NP size and spherical structures could be controlled by changing the reaction temperature and leaf broth concentration with smaller sizes being formed at higher temperatures and leaf broth concentrations (Song *et al.,* 2009).

The main interest in anisotropic particles is due to their sharp edges, which lead to high local electric field gradients under illumination, making them attractive candidates for a number of applications such as optical biosensing and SERS (Grzelczak *et al.*, 2008). Au and Ag nanotriangles may have potential applications in the treatment of cancer hyperthermia and optical coatings (Chandran *et al.,* 2006).

2.5 Stability of Nanoparticles

Due to the high surface energy of NPs, any collision event between two particles results in agglomeration and precipitation out of solution. This makes it necessary to stabilize the NPs by providing a barrier to prevent the interaction of two NPs with one another. Usually the barriers (capping agents) are based on charge or steric stabilization of the NP. In the charge case, a charged surface has counter ions and some solvent molecules bound to it. These counter ions prevent aggregation by causing repulsion. In steric stabilization, a long chained molecule is attached to the surface of the particle and has a high affinity for the solvent. To separate the solvent molecule from the surface would require a lot of energy which would be unfavourable and in turn, provides a barrier against aggregation. Capping agents can be organic or biological molecules as well as polymers. They prevent further agglomeration making them integral components of NPs (Christian *et al.,* 2008; Ju-Nam and Lead, 2008).

2.6 Applications of Nanoparticles in the Biomedical Industry

2.6.1 Diagnostics

Despite their high sensitivity and reproducibility, conventional diagnostic methods for a microbial infection require cumbersome sample preparation and long readout times (Kaittanis *et al.*, 2010). Unique electrical, magnetic, luminescent and catalytic properties of nanomaterials enable fast, sensitive and cost effective diagnosis as well as rapid determination of the susceptibility and resistance of antibacterial drugs (Jain, 2007). This means that patient monitoring could be made easier with a smaller sample needed for diagnosis (Singh *et al.,* 2010). A great deal of effort has been invested in developing affordable, robust and reproducible nano-diagnostic assays to be globally accessible and applicable even in rural areas of developing countries (Kaittanis *et al.,* 2010). These rapid diagnostics will hopefully eventually be provided at point-of-care. This will enable faster screening and tailored therapeutic intervention, prevention and reduce the significant number of hospital admissions that result from therapeutic failure (Baptista *et al.*, 2008).

2.6.2 Therapeutics

NPs are used for *in vivo* drug delivery. They are able to traverse membrane boundaries and are readily absorbed into the bloodstream. Surface coatings may be adjusted to allow fast or slow release and even longer shelf-life. Tissue-specific delivery is a possible step with a stronger localized dose which would allow the patient to take a smaller concentration of the drug and in turn lower side-effects and toxicity to the patient. This will be helpful in infectious diseases and cancer therapeutics. NPs have the ability to cross the blood-brain barrier and could lead to new techniques to diagnose and treat neurodegenerative diseases. There is also the possibility of nanocapsules being used for cholesterol removal and osteoporosis treatment by nanostructured silicon (Roco, 2003; Singh *et al.,* 2010). It has been shown that Au NPs can act as carriers of insulin by stabilizing them with chitosan. This harmless biopolymer allows insulin adsorption on the surfaces of the NPs and transmucosal delivery (Bhumkar *et al.*, 2007).

The use of pharmacological agents is limited when it comes to efficacy and selectivity. Au NPs provide the drugs with improved stability, solubility and bio-distribution. Antibiotics

delivery using nanomaterials offer multiple advantages (Mansour *et al.*, 2009; Sosnik *et al.*, 2010):

- controllable and relatively uniform distribution in the target tissue;
- improved solubility;
- sustained and controlled release;
- improved patient-compliance;
- minimized side effects; and
- enhanced cellular internalization.

2.6.3 Medical Devices

Medical devices used for MRI and X-Ray contrast agents have insufficient tissue specificity and retention. NPs can offer a solution since they are able to diffuse slowly out of the bloodstream which allows for circulatory system imaging which would provide benefit for example to stroke cases. A future prospective is the possibility of retinal implant devices that uses solar technology on a nano-scale. NP probes could deliver a localized chemotherapy dose to a specific tissue in cancer patients and relieve the normal lengthy conventional protocol. NPs have also demonstrated the ability to assist with new bone matrix material which could be integrated into prostheses applications (Singh *et al.,* 2010).

2.6.4 Antibacterial Activity

Antibacterial activity via NPs is one of the most important applications. Antibacterial agents are able to locally destroy bacteria without causing toxicity to the surrounding tissue generally (von Nussbaum *et al.*, 2006). However, with the broad usage of antibiotics, bacterial resistance is on the rise particularly due to genetic mutations. These antibiotic-resistant strains are responsible for the production of new, more fatal diseases. The drug-resistant strains that cause these diseases bring about the need for high dosage treatment methods which produce adverse side effects and increased cost (Hajipour *et al.*, 2012). Certain classes of NPs have antimicrobial activity and the ability to be a carrier for antibiotic delivery *in vitro* and in animal models. The mechanisms of antibacterial activity of the NPs depends on the bacterial species as well as the intrinsic properties, surface modification and composition of NPs (Hajipour *et al.,* 2012). In NPs, the energy of the

system increases due to the increase in ratio of surface to bulk atoms which reduces the system stability and in turn enhances the antimicrobial properties in comparison with the bulk form. A distinct advantage of NPs lies in the avoidance of non-specific interaction between the drug and unaffected tissue (Sun and Xia, 2002).

Ag NPs have proved to be a successful broad spectrum antibacterial agent in many medical streams. Ag NPs are highly toxic to multidrug resistant bacteria, which in turn indicate the potential for use in biomedical applications (Jain *et al.*, 2009). The mechanism of the antimicrobial effect of Ag lies in it being released into solution and higher ratios of NPs to bacteria resulting in quicker bactericidal activity. The findings were related to previous studies and there are a number of methods by which Ag NPs inactivate *Escherichia coli* namely: direct effect of damaging the function of proteins and lipids in the cell wall and cytoplasmic membrane which affects: transport; respiration metabolism; indirect production of reactive oxygen species and possible contact with the DNA once the wall and membrane have been damaged (Figure 4). Some reports show that *Staphylococcus aureus* displays a higher resistance to the Ag NPs than *E. coli* which is probably due to the thicker peptidoglycan component in the cell wall thus requiring a longer treatment time (Chamakura *et al.,* 2011).

Studies conducted thus far on antibiotic properties of functionalized Au NPs have shown that they display effective antibacterial activity on certain species, such as *E. coli* and *Pseudomonas aeruginosa*. However, these results are variable and the NPs do not function with the same level of success on other bacterial species (Pissuwan *et al.*, 2010).

Microbes are unlikely to develop resistance against silver, as they do against conventional and narrow-target antibiotics, because the metal attacks a broad range of targets in the organisms which means that they would have to develop a host of mutations simultaneously to protect themselves (Pal *et al.*, 2007). Antimicrobial NPs offer many advantages and benefits that conventional agents cannot provide. Table 3 provides a summary of the advantages and disadvantages that antimicrobial NPs have over free antimicrobial agents.

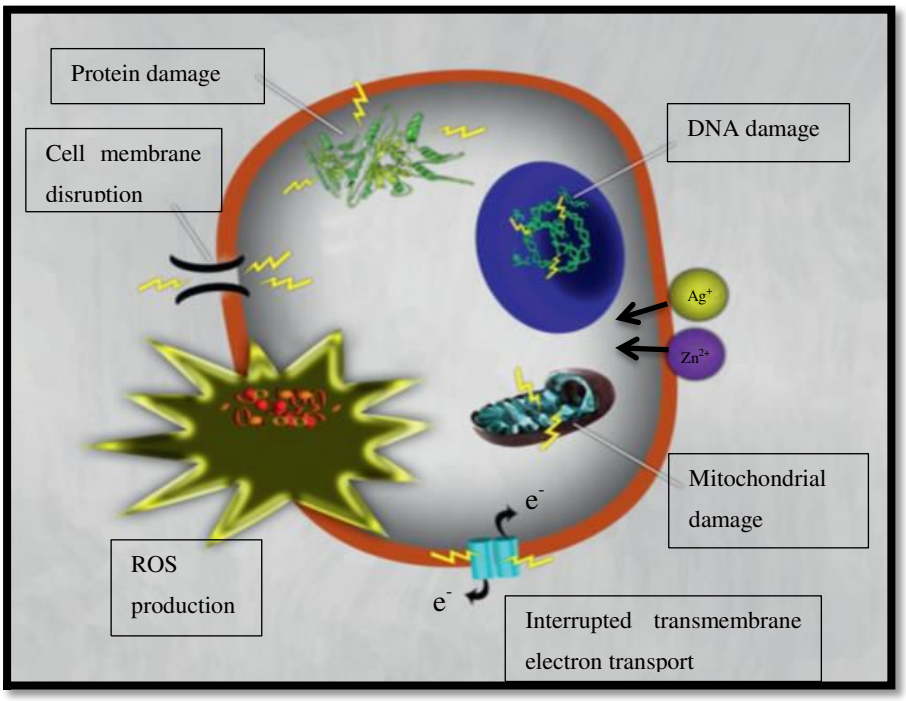

Figure 4: Mechanisms of toxicity of Ag NPs on bacterial cell (Hajipour *et al.,* 2012)

Table 3: A comparison of the advantages and disadvantages of antimicrobial NPs versus free antimicrobial agents (Huh and Kwon, 2011).

Antimicrobial NPs	**Free antimicrobial agents**
Advantage	**Disadvantage**
Targeted drug delivery via specific accumulation	No specific accumulation
Lowered side effects of chemical antimicrobials	High side effects of chemical antimicrobials
Low antimicrobial resistance	High antimicrobial resistance
Extended therapeutic lifetime due to slow elimination	Short half-life due to fast elimination
Controlled drug release	Usual pharmacokinetics of free drugs
Broad therapeutic index	Narrow therapeutic index
Improved solubility	Sometimes poor solubility
Low immunosuppression	Immunosuppression
Low cost	High cost
Disadvantage	**Advantage**
Accumulation of intravenously injected nanomaterials in tissues and organs	Absence of nanomaterials in the whole body
High systemic exposure to locally administrated drugs	Low systemic exposure to locally administrated drugs
Nanotoxicity (lung, kidney, liver, brain, germ cell, metabolic, etc.)	Absence of nanotoxicity
Lack of characterization techniques that are not affected by NPs' properties	Well-established characterization techniques

2.7 Overview of Plants

2.7.1 *Amaranthus dubius*

Figure 5: ***Amaranthus dubius***

A. dubius (Figure 5) belongs to the family Amaranthaceae and is commonly known as African spinach. In Zulu, it is known as Umfino imbuya (Hutchings *et al.*, 1996; Van Wyk and Gericke, 2000).

It is a weedy plant common throughout the humid lowland tropics and originated from tropical America. Immigrants have introduced it into several African and Central American countries. *A. dubius* is a protected weed used as a pot herb and a cultivated vegetable in African countries.

It is an annual herb up to 150 cm tall, branched, has slender stems with short to long hairs. The leaves are arranged spirally and are simple. Flowers are unisexual and subsessile. The vegetative development is rapid which means a high rate of photosynthesis at high temperature. While the older branches are blooming, the plants continue to produce new shoots. It is found often in waste places, roadsides, river banks and cleared forest areas because of their preference for fertile, well-drained soils that have a loose structure. They grow well at day temperatures above 25°C and at a minimum of 15°C at night. Pollination is effected by wind but due to the high pollen production, self-pollination is quite common (Grubben, 2004).

The main use of *A. dubius* is as a cooked leaf vegetable which is dark green and tender but its taste is neutral. It has medicinal properties for lactating mothers; children and patients that are experiencing constipation; fever; anaemia and kidney problems. In Tanzania, the whole plant is used to treat stomach ache (Grubben, 2004).

The leaves and stems contain nitrate and oxalate. The presence of hydrocyanic acid and oxalic acid at high levels makes amaranth less fitting for fresh consumption by humans and animals. Amaranthaceae is known to have betalain pigments. Phytochemical studies show several methylenedioxy, flavonols, saponins, triterpenoids, ecdysteroids and specific root-located carbohydrates (Muller and Borsch, 2005). The biomass of amaranth has high biological value, and is used as source of protein, amino acid and dietary fibre for fattening pigs, rabbits, and lambs. Gallic acid (GA), Caffeic acid (CA), Rutin (RU), Ferulic acid (FA) and Quercetin (QU) are phenolic compounds that have been found in this family. These phenolic groups serve as a source of readily available hydrogen atoms so that the subsequent radicals produced can be delocalized over the phenolic structure (Pasko *et al.*, 2005).

2.7.2 *Gunnera perpensa*

Figure 6: ***Gunnera perpensa***

G. perpensa belongs to the family of Gunneraceae. It is commonly referred to as river pumpkin (Bergman *et al.*, 1992). The Zulu name ughobo refers to the flowing of fluids and its use in traditional medicines to remove excess fluid from the body (Ngwenya *et al.*, 2003).

Gunnera occurs naturally in Africa, Madagascar, New Zealand, Indonesia, Hawaii, Mexico and central and South America. These plants are common in tropical Africa and extend down Eastern and Southern Africa as well as along the Western Cape. This genus has around 45-50 species with *G. perpensa* being the only species found in Africa (Mendes, 1978; Bergman *et al.,* 1992).

Gunnera has many characteristics unique to the genus, some of these indicate that these plants belong to one of the oldest angiosperm families and amongst the largest herbs (Bergman *et al.,* 1992). *G. perpensa* is a perennial, robust herb that stands up to 1 m tall and is always found near water. The roots are up to 300 mm thick and the leaves arise from a central tuft near the top of the apex. These leaves are large, kidney-shaped, dark bluish green and covered with hairs on both surfaces (Figure 6). The flowers are small, tiny reddish brown and protrude from a long spike which is taller than the leaves. It is an obligate wetland plant that is found in marshy areas and along streams. It is unable to tolerate frost and cold conditions (Mendes, 1978).

It is used by the rural population for the treatment of dysmenorrhea and aqueous extractions have been used to relieve rheumatoid pain, assist in childbirth and treat female infertility (Hutchings *et al.,* 1996). The rhizomes have been proven to have analgesic and anti-inflammatory properties (Nkomo *et al.*, 2010). In South Africa, a decoction of the roots is used to expel the placenta after birth and relieve menstrual pain (Van Wyk and Gericke, 2000).

Caffeic acid, quercetin, ellagic acid, and ellagitannins as well as two anthocyanins are present whereas alkaloids, cyanogenic glycosides, iridoids, proanthocyanidins, saponins, and sedoheptulose were not detected in certain *Gunnera* species (Doyle and Scogin, 1988). Derivatives of 1, 4 –benzoquinones and trans-phyt-2-enol were found in methanol extracts of leaves of *G. perpensa* (Drewes *et al.*, 2005).

2.7.3 *Ceratotheca triloba*

Figure 7: ***Ceratotheca triloba***

C. triloba belongs to the family Pedaliaceae and is popularly known as the wild foxglove. In Zulu, it is udonqabathwa and in Swazi, ludvonca (Van Wyk and Gericke, 2000).

These are opportunistic annuals found mainly in grasslands in the summer rainfall areas of South Africa. *C. triloba* is one of four species found in Southern Africa (Smithies, 2000).

It can grow up to 1.5- 2 m tall which varies in height depending on the amount of water it receives. The three-lobed leaves are soft and green with a bluntly serrated margin. Plants can either have pink or white flowers with dark red or yellow-green stems respectively. The leaves are found on long, thin stalks up the stems (Figure 7). The stems, leaves and flowers are covered in white fine mucilaginous hairs, which often give the stems and leaves a slimy feel. Each flower has 5 lobes and is around 50 mm long with delicate lines running into the throat. After a few weeks of flowering, the green fruits turn dry and brown splitting open to release pear-shaped seeds. They prefer rich, well-drained soils in full sunlight or semi-shade. They germinate best in disturbed areas like roadsides where they flower and seed before winter (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.,* 1996).

Medicinal uses include treatment for fever, painful menstruation, nausea, diarrhoea and stomach pain (Hutchings *et al.,* 1996).

There is not much work done on the phytochemical constituents of *C. triloba*. However anthraquinones are a class of natural compounds to have been isolated from *C. triloba*. These compounds display antibacterial activity and are applied in food, medicine and the dye industry. These compounds are known to be beneficial as they have shown antibacterial activities (Mohanlall *et al.*, 2011).

2.7.4 *Catharanthus roseus*

Figure 8: ***Catharanthus roseus***

C. roseus belongs to the family Apocynaceae. The common name for this plant is 'Madagascar periwinkle'. This plant is native to the Madagascar and is now widespread in many tropical and subtropical regions. It has now spread throughout the tropics and subtropics (including South Africa) by human activities. Self-pollination and a relatively high tolerance of disturbance have enabled this species to spread from cultivation and grow in many parts of the world. Due to this, the species is sometimes considered an invasive weed, although it does not normally proliferate amply to eradicate native vegetation (Jackson and McDonald, 1986; Hutchings *et al.,* 1996).

C. roseus is a perennial that can grow to 80 cm high. It produces dark green, oval, glossy leaves and flowers throughout summer (Figure 8). The leaves are oval to oblong, 2.5–9 cm long and 1–3.5 cm broad, glossy green and hairless. Their arrangement is opposite in pairs. The flowers are white to dark pink with a darker red centre, with a basal tube 2.5–3 cm long and a corolla 2–5 cm diameter with five petal-like lobes. It is appreciated for its long flowering period, throughout the year in tropical conditions, and from spring to late autumn, in warm temperate climates. Full sun and well-drained soil are preferred (Jackson and McDonald, 1986, Hutchings *et al.,* 1996).

In India, wasp stings have been treated with the juice from the leaves, whilst in Africa the leaves have been used for menorrhagia and rheumatism (Duke, 1985). There have also been reports on its anti-diabetic properties (Nammi *et al.*, 2003).

Extracts of the dried plant roots contain important alkaloids such as vinblastine; vincaleukoblastine and vincristine. Vinblastine has been used for treatment of neoplasms, generalized Hodgkin's disease and resistant choriocarcinoma. Vincristine is pharmacologically important and is used in the treatment of leukaemia in children. Vinblastine and vincristine are able to lower the number of white cells in the blood making them useful anti-cancer compounds (Jackson and McDonald, 1986, Hutchings *et al.,* 1996).

2.8 Micropropagation

Plants contribute largely to the integrity of the environment. Conservation of the components of biological diversity is vital since lack of renewable natural resources is leading to scarcity. Plant cell and tissue culture can be an alternative approach to maintain sustainable supply of plant materials for producing bioactive compounds continuously (Flick *et al.*, 1983). Cellular totipotency is the ability of living cells of plants to theoretically give rise to full plants. Isolated plant tissues may be induced to form an actively dividing and undifferentiated mass of plant cells called callus. It is considered a wound response that occurs in plant organs as well as specific plant tissues (Collin and Edwards, 1998). The pieces of plant tissues from the wilderness, known as explants, usually have contaminating microorganisms present and these have to be removed through a specific sterilization process due to their ability to kill the explants by overgrowth or

toxin release. Contamination can also be caused by instruments, transfer technique, culture medium, transferring environment and incubation facilities (Dodds and Roberts, 1995).

Plant growth regulators such as auxins and cytokinins are very important in tissue culture and their amounts in the media are responsible for the growth of plant tissues (Murashige and Skoog, 1962). Generally a high concentration of auxin and a low concentration of cytokinin would result in cell proliferation and the formation of callus. Finding the correct balance between auxin and cytokinin concentration is crucial in callus formation. Shoot or bud regeneration can result when part of the callus tissue is transferred to differentiation medium. Growth regulators such as auxins and cytokinins, the most common ones being 2,4-Dichlorophenoxy acetic acid (2,4-D), benzylaminopurine (BAP) and alpha-naphthalene acetic acid (NAA), are known to induce callus formation when used in appropriate concentrations (Misawa, 1994).

The application of tissue culture for large-scale plant production meant for commercial purposes is well demonstrated. Many varieties have grown well *in vitro*, allowing various kinds of experimental manipulations. It has solved many problems of plant growth, differentiation, morphogenesis and in the application of different growth substances (Ghatnekar and Kavian, 2000).

2.9 Phytochemicals

Plants contain a variety of compounds which vary according to family and species. The distinct distribution of these compounds is used as taxonomic markers and allows plants their characteristic colours, tastes and odours. There are primary metabolites that are required for the normal functioning of the plant and secondary metabolites that are produced for protecting the plant against pests and pathogens (Bennett and Wallsgrove, 1994). Figure 9 shows a schematic representation of the biosynthetic relationship between primary and secondary metabolites.

South Africa has an abundance of medicinal plants, used in the traditional treatment of various diseases and therefore assumed to be non-toxic to humans (Hutchings *et al.,* 1996). The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant.

In plants, these compounds are mostly alkaloids, steroids, tannins and phenol compounds, which are synthesized in specific parts or in all parts of the plant. These secondary metabolites may exert their action by resembling endogenous metabolites, ligands, hormones, signal transduction molecules or neurotransmitters and thus have beneficial medicinal effects on humans due to similarities in their potential target sites

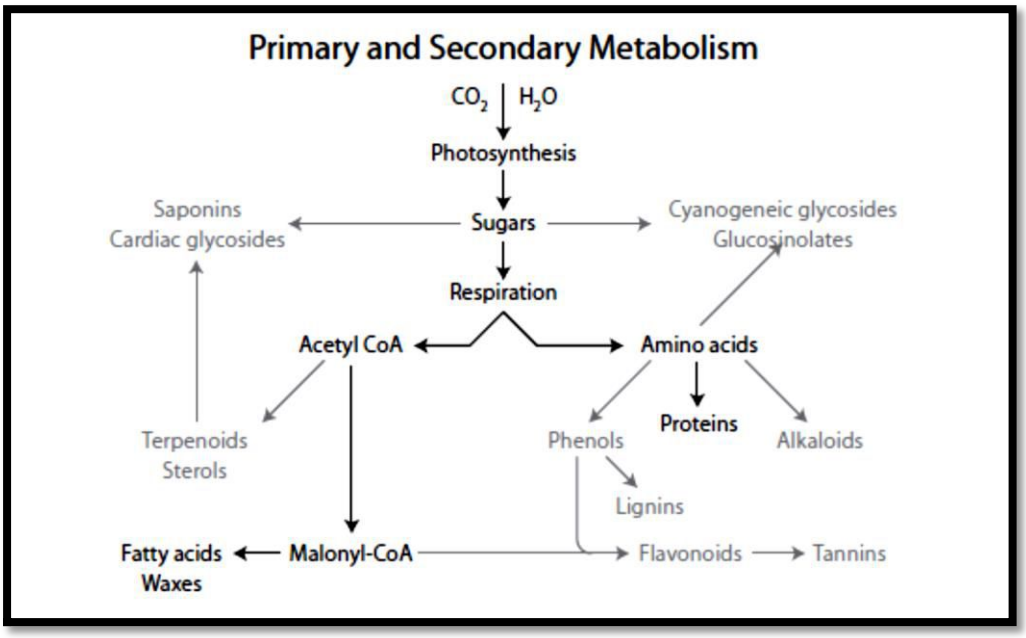

Figure 9: Plant primary and secondary metabolite relationship

The following is a summary of some of the secondary metabolites found in plants that have been associated with biosynthesis of NPs:

2.9.1 Terpenoids

It is the largest class of secondary metabolites and have a common biosynthetic origin from isoprene. Some of the terpenes found in this group include monoterpenes ($C_{10}H_{16}$), polyterpenes $(C_5H_8)n$, sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$) and triterpenes ($C_{30}H_{48}$). These compounds are mostly associated with defence as toxins and protection against pests and insects. Steroids fall under this category since they are terpenes with a particular ring structure (Mazid *et al.*, 2011).

2.9.2 Saponins

Saponins are low molecular weight glycosides that either belongs to the triterpenoid class or the steroidal class with one or more sugars that allow it to form a stable foam in aqueous solutions due to their amphiphilic nature. They are known to have a bitter taste and are able to hemolyze red blood cells. They are able to lower surface tension which makes them harmful to cold-blooded animals. They are also used medically in the treatment of excessive salivation, epilepsy and migraines. They are popular ingredients found in cleansers and shampoos. These compounds are also used in Ayurveda as treatment for eczema and other skin conditions (Vincken *et al.*, 2007).

2.9.3 Alkaloids

These are divided into 3 major classes depending on the precursors and the final structure. True alkaloids are derived from amino acids and contain nitrogen in a heterocyclic ring. These compounds are also basic in nature. They are responsible for defence against microorganisms and attack from herbivores. They are produced by a range of organisms, including bacteria, plants and animals. Some of these compounds are known to have a pharmacological effect and are used as medication or recreational drugs (Mazid *et al.,* 2011).

2.9.4 Flavonoids

This is one of the largest groups of phenolic compounds widely distributed in plants and responsible for defence and pigmentation. Flavonols are found in the epidermal layer of leaves to protect them from UV rays. Flavonols are polymerized by peroxidases and polyphenoloxidases. They are commonly known for their antioxidant activities. Flavonoids compared to other active compounds have a very low toxicity. They have also been seen to help the body in reaction to allergens, carcinogens and viruses (Bennett and Wallsgrove, 1994).

2.9.5 Tannins

These compounds are present in vegetable extracts that are responsible for converting animal skin to leather. In plants, they are composed of different molecular sizes and complexities. They contain a large number of hydroxyl functional groups and can form cross-linkages with proteins and other macromolecules. They are regarded as toxins responsible for reduced survival of herbivores and repellent to pests (Chung *et al.*, 1998).

3. METHODOLOGY

3.1 Biosynthesis of Silver and Gold Nanoparticles from Leaf Extracts

3.1.1 Collection and Preparation of Plant Materials

A. dubius, G. perpensa, C. triloba and *C. roseus* were identified using taxonomic keys and collected from around Durban, Kwa-Zulu Natal throughout the course of the project except in winter and autumn seasons. The details of the plants and the location from where they were collected are listed in Table 4. The plants were de-leafed and washed repeatedly with distilled water to remove all foreign materials. Material was stored in the cold room at 4°C and used within one week.

Table 4: Details of the plants used in this study

Scientific name	Family	Common name	Location collected
Amaranthus dubius	(Amaranthaceae)	African spinach, Indian spinach	Mamba Valley
Gunnera perpensa	(Gunneraceae)	River pumpkin	Caversham Glen
Ceratotheca triloba	(Pedaliaceae)	Wild foxglove	Pampally Way
Catharanthus roseus	(Apocynaceae)	Madagascar periwinkle	Pemilton Road

3.1.2 Preparation of Leaf Extracts

Aqueous extraction of leaves was carried out according to the method outlined by Dubey *et al.,* (2010) with minor modifications. For the aqueous extract, 50 g of thoroughly washed leaves was finely cut and then boiled in 250 ml autoclaved distilled water for 20 minutes. This was filtered with Whatman No. 1 filter paper and the filtrate was sterilized with a syringe filter through a 0.22 µm pore filter (Millipore). For the methanolic and ethyl acetate extracts, the leaves were treated as the aqueous extracts but instead of water, 250 ml methanol and 250 ml ethyl acetate were used respectively. These extracts were kept in the refrigerator at 4°C and were used within ± 5 days.

3.1.3 Synthesis of Silver Nanoparticles

1 M $AgNO_3$ (Sigma) was prepared in sterile distilled water. This was used to prepare a 60 ml of 1 mM $AgNO_3$ solution to which 2.5 ml of the aqueous leaf extract was added at room temperature (approx. 25° C). The colourless solution was mixed with a stirrer bar for five minutes and visually observed for a yellow-brown colour change indicative of the formation of Ag NPs (Dubey *et al.,* 2010). The solutions that produced a positive colour change were then stored at 4°C in glass bottles.

3.1.4 Synthesis of Gold Nanoparticles

0.1 M $HAuCl_4$ (Sigma) was prepared in sterile distilled water. This was used to prepare a 60 ml of 1 mM $HAuCl_4$ solution to which 2.5 ml of the aqueous leaf extract was added at room temperature (approx. 25° C).The pale yellow solution was mixed with a stirrer bar for five minutes and visually observed for a maroon-purple colour change indicative of the formation of Au NPs (Dubey *et al.,* 2010). The solutions that produced a positive colour change were then stored at 4°C in glass bottles.

3.1.5 Optimization of Parameters for Synthesis of Nanoparticles

According to Huang *et al.,* (2007), varying the temperature and concentration of extract in the reaction can produce NPs that are synthesized can be within a narrow size distribution.

3.1.5.1 Temperature

The synthesis procedures for aqueous extracts in sections 3.1.3 and 3.1.4 were carried out at four different temperatures: room temperature - 25°C, 40°C, 60°C and 95°C. This was done by heating the water in an Erlenmeyer flask up to the required temperature, which was measured by a thermometer and thereafter adding the metal salt ($AgNO_3$/$HAuCl_4$) solutions and 2.5 ml aqueous leaf extract to the flask. The colour change was used as an indication of NP synthesis which was further confirmed by UV-vis spectrophotometry as described in 3.3.1.

3.1.5.2 Concentration

Those temperatures at which a positive colour change and the best peaks formed were investigated with a quarter, half and double the initial concentration of 2.5 ml aqueous leaf extract (0.6 ml, 1.25 ml and 5 ml). The colour change was used as a visual result and further analysed by UV-vis spectrophotometry to determine the best peak and in turn the optimal synthesis conditions.

3.1.6 Effect of Solvent Extraction on Biosynthesis of Silver and Gold Nanoparticles

The rationale behind this was indirectly based on a study where different solvent extractions were prepared from Black Tea leaves and used to synthesize Ag and Au NPs (Begum *et al.,* 2009). These experiments showed that different solvents with different polarity indices were either capable or incapable of producing NPs. The polarity indices for water = 10.2; methanol = 5.1 and ethyl acetate = 4.4. The methanol and ethyl acetate solvents were chosen to provide a comparative evaluation based on their polar indices and their ability to dissolve particular polar phenolic compounds.

Thus the methanol and ethyl acetate leaf extracts were used to prepare Ag and Au NPs as per the method in sections 3.1.3 and 3.1.4 at temperatures and concentrations used are listed in Table 5.

Table 5: Optimized temperatures and concentrations for Ag and Au NPs for aqueous leaf extracts of *A. dubius*, *G. perpensa* and *C. triloba*

Plant	NPs	Temperature (°C)	Concentration (ml)
A. dubius	**Ag**	40	2.5
	Au	60	2.5
G. perpensa	**Ag**	25	0.32
	Au	40	1.25
C. triloba	**Ag**	95	0.63
	Au	60	5

3.2 Biosynthesis of Silver and Gold Nanoparticles from Callus Extracts

3.2.1 Micropropagation

The plant material for the plants that yielded positive results for the synthesis of Ag and Au NPs from their leaf extracts i.e. *A. dubius*; *G. perpensa* and *C. triloba* were collected. Leaves were washed repeatedly with tap water and then thrice with distilled water to remove impurities and foreign particles.

Sterilization:
The leaves of *A. dubius*, *G. perpensa* and *C. triloba* were disinfected by two sterilizing agents (mercury chloride and sodium hypochlorite) which are popular in tissue culture sterilization. Each plant underwent two sets of sterilization techniques to see which would be successful. The leaves were either disinfected for five minutes in 0.1% mercury chloride and 20 minutes in 40% sodium hypochlorite or for five minutes in 0.1% mercury chloride and 15 minutes in 30% sodium hypochlorite. The leaves were then thoroughly rinsed thrice with sterile distilled water under a laminar flow hood.

Callus Induction:
The MS medium (Murashige and Skoog, 1962) purchased from Sigma was used in this study. The basal MS media (42.2 g/L MS) was manipulated with auxin 2,4-D and cytokinin BAP in different concentration combinations of 0.5 µg/L and 1 µg/L. The pH of the media was adjusted to 5.8. Cefotaxamine 25 µg/L was used to prevent microbial contamination.

After sterilization the leaves were cut into square pieces (1 cm^2) using sterile scalpel blades. Four to five leaf squares were placed on each petri plate containing the modified basal salt MS medium. Aseptic conditions were sustained by performing all inoculations under the laminar flow and sterilizing instruments with 70% ethanol and flaming them. All cultures were maintained at 25°C in a 16 h photoperiod (16 h light/16 h dark) in a growth chamber.

Due to the results obtained for *G. perpensa*, two more sterilization methods were performed: 20 min in 75% sodium hypochlorite only or for 20 min in 25% sodium

hypochlorite only. The media preparation was also modified by changing the cytokinin to NAA instead of BAP at different concentrations of 0.5 µg/L and 1 µg/L.

3.2.2 Preparation of Callus Extracts

The method was adapted from Nabikhan *et al.,* (2010), massed calli (6 g) was collected over a 3 month period of sub-culturing of *A. dubius* and *C. triloba*. The calli mass was ground with a mortar and pestle and added to 30 ml autoclaved distilled water or methanol or ethyl acetate which was then boiled for 20 minutes and filtered with Whatman No. 1 filter paper. The filtrate was subsequently syringe filtered through a 0.22 µm pore filter (Millipore).

3.2.3 Synthesis of Silver and Gold Nanoparticles

The synthesis of Ag and Au NPs using the aqueous, methanolic and ethyl acetate extracts was carried out as per procedures as outlined in sections 3.1.3 and 3.1.4 with one modification in that twice the concentration (5 ml) of the callus extract was used (Nabikhan *et al.,* 2010).

3.3 Characterization of the Synthesized Nanoparticles

3.3.1 UV-visible Spectra Analysis

A scan in the wavelength range of 200 nm to 800 nm was carried out to confirm the synthesis and stability of the NPs. Samples were poured into quartz cuvettes, placed into the Varian Cary 100 UV-vis spectrophotometer and absorbance measurements in relative light units (AU) were taken. The Cary WinUV software was used to analyse results. A cuvette of distilled water was used as the blank. Ag NPs and Au NPs were expected to show a sharp peak in the ranges 395 nm - 425 nm and 520 nm - 600 nm respectively according to literature (Link and El-Sayed, 2000; Ju-Nam and Lead, 2008). The range with regard to this study was refined to a smaller range ie. 400 nm - 425 nm for Ag NPs and 520 nm - 550 nm for Au NPs so as to provide uniformity amongst the particles produced with respect to their size and shape.

3.3.2 Transmission Electron Microscopy (TEM) Analysis

The JEOL JEM-1010 Transmission Electron Microscope at the University of Kwa-Zulu Natal (Westville Campus) was used. The NPs samples were prepared by centrifuging the NPs solutions in the Eppendorf Centrifuge 5810 at 13000 rpm for 45 minutes. The supernatant was siphoned off and the resulting suspension was re-suspended in distilled water. Each specimen used for analysis was prepared by immersing a formva-coated copper grid into the respective solution, removing the grid and allowing it to dry for a few minutes before analysis. The size and morphology of the NPs were noted.

3.3.3 Fourier-Transform Infrared (FTIR) Spectroscopic Analysis

The FTIR measurement was used to identify if the same biomolecules were responsible for the synthesis and capping of NPs for the different extracts and between the Ag and Au NPs for each plant. NPs samples were centrifuged in the Eppendorf Centrifuge 5810 at 13000 rpm for 45 minutes. The resulting suspension was re-suspended in distilled water and repeated thrice to remove any free biological material that may interfere with the results (Jain *et al.,* 2009). The Perkin Elmer, Spectrum 100 FTIR spectrophotometer, was used to analyze the samples. A drop of distilled water was used as a blank and this background was removed from the sample scan. A drop of sample was then placed on the machine and a scan measuring transmittance percentage over the range of wavenumbers from 500 to 4000 cm^{-1} was taken. E-FTIR software was used to analyse the results and ascertain peak values which was then interpreted.

3.4 Stability of Nanoparticles

This study was done by analyzing the UV-vis spectrophotometer scans of each aqueous and methanolic NPs solution. The absorbance was read for the first two days, the fourth day and thereafter every three days until for thirty days. The aim was to investigate if these NPs were stable for thirty days as seen in previous studies (Shankar *et al.,* 2004). A change in absorbance values and the symmetry and structure of the peaks were used to determine the instability of NPs.

3.5 Phytochemical Screening of Plant Extracts

These were carried out according to methods outlined by Harborne (1973); Trease and Evans (1989); Sofowara (1993). Healthy leaves of *A. dubius*, *G. perpensa, C. triloba* and *C. roseus* were dried in an oven and ground to a fine powder using a Wareing blender. Phytochemical screening for the presence or absence of terpenoids, steroids, saponins, alkaloids, flavonoids, tannins and phlobatannins were carried out on powdered specimens of the plants. The aqueous extract was prepared by boiling 2 g of the powdered sample in 20 ml distilled water.

3.5.1 Terpenoids (Salkowski's Test)

For the presence of terpenoids, 5 ml of each extract was mixed with 2 ml of chloroform. Concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed showing a positive result for the presence of terpenoids.

3.5.2 Steroids Test

For the presence of steroids, 0.5 g powdered plant samples were boiled in 5 ml ethanol for five minutes. This was treated with 2 ml acetic anhydride. 2 ml H_2SO_4 was carefully added, drop-wise, to this and observed for a colour change from violet to blue/green indicating the presence of steroids.

3.5.3 Saponins Test

For the saponins test, 10 ml of the aqueous extracts were mixed with 5 ml distilled water and shaken vigorously to form a stable froth. The frothing was mixed with three drops of olive oil and shaken vigorously again. The solution was observed for the formation of an emulsion, a heavy emulsion indicating the presence of saponins.

3.5.4 Alkaloids (Dragendroff's Test)

For the presence of alkaloids, 2 ml of aqueous extracts were treated with a few drops of Dragendorff's reagent (Potassium Bismuth Iodide Solution) (Merck). Formation of a red precipitate indicated the presence of alkaloids.

3.5.5 Flavonoids Test

For the presence of flavonoids, 1 g of the powdered plant samples were heated with 10 ml ethyl acetate in a steam bath for three minutes. The mixtures were filtered and 4 ml of the filtrate of each was shaken with 1 ml diluted (1:10) ammonia solution. A positive result was indicated by a yellow colouration.

3.5.6 Tannins

For the presence of tannins, 0.5 g of the powdered plant samples were each boiled in 20 ml distilled water and a few drops of 0.1% ferric chloride was added to each. These were observed for a brownish green or black colouration to indicate a positive result.

3.5.7 Phlobatannins

For the presence of phlobatannins, 2 ml of aqueous extract of each of the plant samples was boiled with 2 ml 1% Hydrochloric acid. The presence of phlobatannins was indicated by the presence of a red precipitate at the base of the test tubes whereas a negative result was indicated by the absence of a precipitate.

3.6 Antibacterial Activity

The antibacterial activity of aqueous and methanolic Ag NPs and Au NPs were carried out on selected bacteria by agar disk diffusion assay method and the minimum inhibitory concentration (Eloff, 1998). MIC is defined as the lowest concentration of an antimicrobial agent that kills all the organisms in a given sample and is carried out generally by dilution methods. In this test, microorganisms are tested on their ability to produce visible growth in microplate wells containing broth and dilutions of the NPs. A growth indicator (p-iodonitrotetrazolium violet (INT)) is used because the NPs could cause precipitation and turbidity when mixed with microbial growth media.

3.6.1 Disk-Diffusion Antibacterial Assay

The fourteen bacteria used as test organisms were as follows: Enterococcus (V11967), Pseudomonas (S6125), *Enterobacter* spp. (P4177), *E. faecalis* (U11951), *E. faecalis* (U11394), *E. coli* (B3578), *E. coli* (U10948), *E. coli* (P4055), *K. pneumoniae* (P3811), *K. pneumoniae* (V258), *K. pneumoniae* (U10705), *S. aureus* (S6158), *S. aureus* (P4215), *S. aureus* (S5878). These strains are clinical pathogens obtained from Lancet Laboratories, SA and are differentiated by their reference codes following the species name designating their site of isolation from the host.

Stock cultures were prepared on Nutrient Agar plates (Biolab), sealed and stored at -70°C. When required the cultures were grown in Mueller Hinton Broth (MHB) (Biolab) for 24 h at 37°C. The absorbance of bacterial cells was adjusted to MacFarland Standard of 0.5 which corresponds to 10^8 CFU/ml (Matasyoh *et al.*, 2007). 100 µl of each bacterial sample was swabbed onto Mueller Hinton Agar (Biolab) plates. All the NP solutions were centrifuged at 13 000 rpm for 60 minutes, the supernatant was siphoned off and the NP pellet was weighed and re-suspended in DMSO (an important polar aprotic solvent that dissolves both polar and nonpolar compounds and is miscible in a wide range of organic solvents as well as water) to form 30 µg/ml solutions (Novak, 2002). 15 µl of all solutions was pipetted onto 5.5 mm sterile filter paper (Whatman No. 1) disks and air dried in a biological safety cabinet. The treated discs were placed on the surface of the inoculated bacterial plates and incubated at 37°C for 24 h. Control disks with DMSO served as the negative control, whilst $AgNO_3$ (1 µg/ml) and $HAuCl_4$ (1 µg/ml) were the positive controls and 1 mM ciprofloxacin was used as the reference drug control. All tests were carried out in triplicate. Ciprofloxacin is a fluoroquinolone synthetic antimicrobial agent that is active against Gram-positive and Gram-negative bacteria and thus regarded as a broad spectrum antibiotic. It has been used to treat urinary tract infections, sexually transmitted diseases as well as respiratory and gastrointestinal infections (Rosemary *et al.*, 2006).

3.6.2 Minimum Inhibitory Concentration Assay

Rows A, B, C, E, F and G of the microplate as shown in Figure 10 were filled with 100 µl sterile Mueller Hinton Broth (MHB) using a micropipette. In Row A, column 1, Ag NP aqueous solution was added to make a concentration of 50 µg/ml. From that well, 100 µl was transferred into column 2 and was taken up and released three times to ensure

adequate mixing. This was done until column 6 which was a concentration of 0.125 µg/ml. The final 100 µl from this well was discarded. The negative control was the solvent DMSO; one well filled with MHB served as a sterility control and a well filled with MHB plus the test organism was the growth control.

Bacterial culture (100 µl) was added to each well except the sterility controls. The microplates were sealed and incubated overnight at 37°C for 12 hours. Following this, 50 µl of 2 µg/ml indicator INT was added to each well and incubated for 30 minutes. Inhibition of growth was indicated by colourless suspensions whereas growth was seen as violet coloured suspensions.

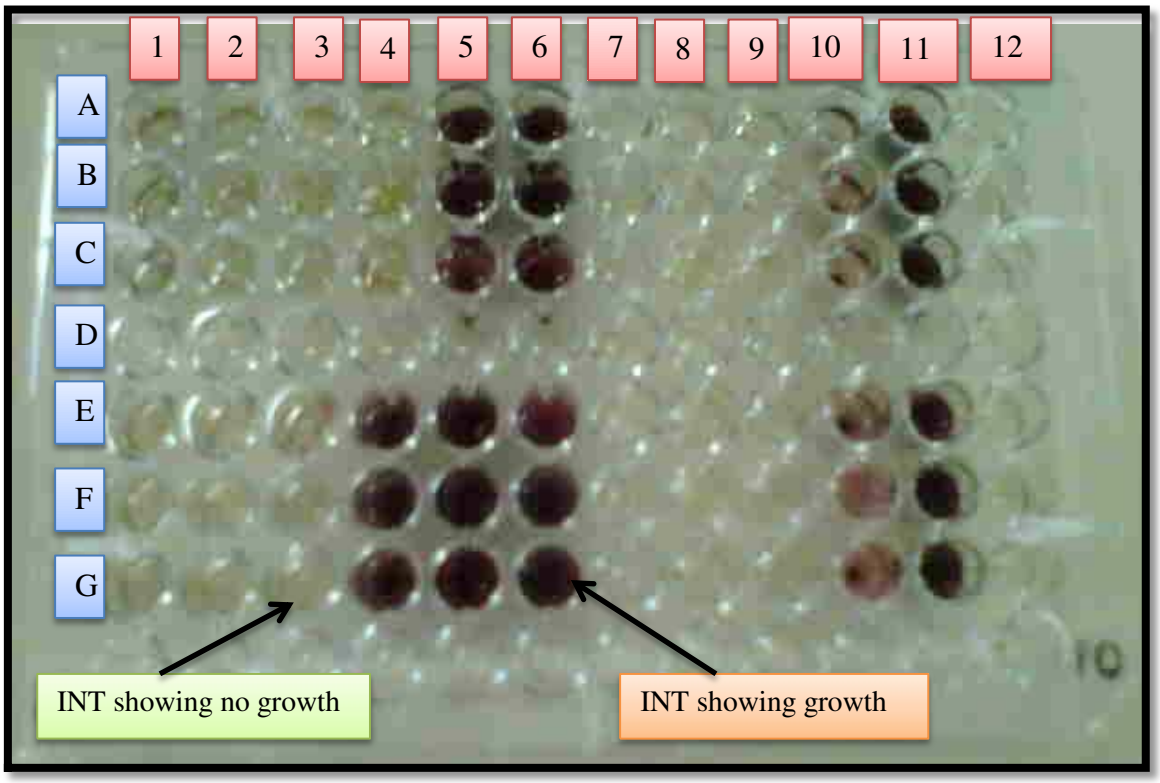

Figure 10: A 96 well plate showing an example of the broth dilution MIC assay

4. RESULTS

4.1 Biosynthesis of Silver and Gold Nanoparticles from Aqueous Leaf Extracts

Table 6 summarizes the results of the colour changes. The positive yellowish orange colour change when added to $AgNO_3$ can be seen in Figure 11a and the positive reddish purple colour change when mixed with $HAuCl_4$ is shown in Figure 11b. The colour change was almost instantaneous with *G. perpensa* for Ag and Au NPs formation whereas the *A. dubius* Ag NPs required stirring for ten minutes. It was noticed that the Ag NP solution that formed from *G. perpensa* turned from yellowish-orange to dark brown after ten minutes.

Table 6: Results of colour change with 2.5 ml aqueous leaf extracts when added to $AgNO_3$ and $HAuCl_4$ solutions at 25°C

NPs/Plant	*A. dubius*	*G. perpensa*	*C. triloba*	*C. roseus*
Ag	√	√	X	X
Au	X	√	X	X

√ - positive colour change

X - no colour change

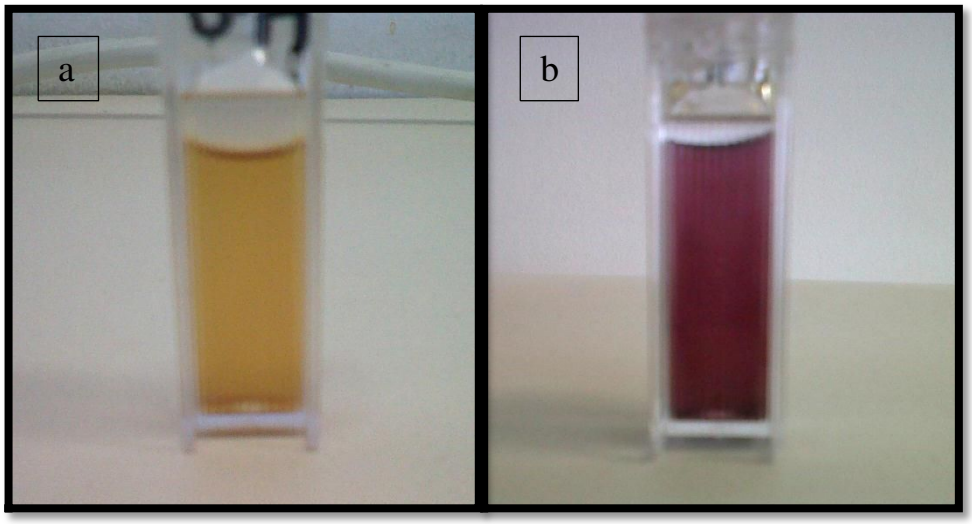

Figure 11: Positive colour change results for (a) *A. dubius* aqueous leaf extract reaction with 1 mM solution of $AgNO_3$ and (b) *G. perpensa* aqueous leaf extract with 1 mM $HAuCl_4$

4.2 Optimization of Nanoparticle Synthesis

4.2.1 Effect of Temperature

The reactions for *A. dubius* aqueous leaf extract at 25°C, 40°C, 60°C and 95°C produced positive colour changes when added to the $AgNO_3$ solution. This was confirmed with UV-vis spectroscopy in Figure 12 in which there was a peak at approximately 300 nm which corresponds to the plant extract and peaks in the 400 nm – 425 nm range which indicates the presence of Ag NPs. The wavelength and absorbance values for the best peaks (symmetric shape and highest absorbance) that were formed for *A. dubius* aqueous leaf extracts is shown in Table 7. The concentration optimization assay was carried out at these temperatures.

Table 7: Best peaks formed for 2.5 ml aqueous leaf extract of *A. dubius* Ag NPs at different temperatures

Temperature (°C)	Wavelength (nm)	Absorbance (AU)
25	421	2.825
40	419	2.969
60	427	2.981

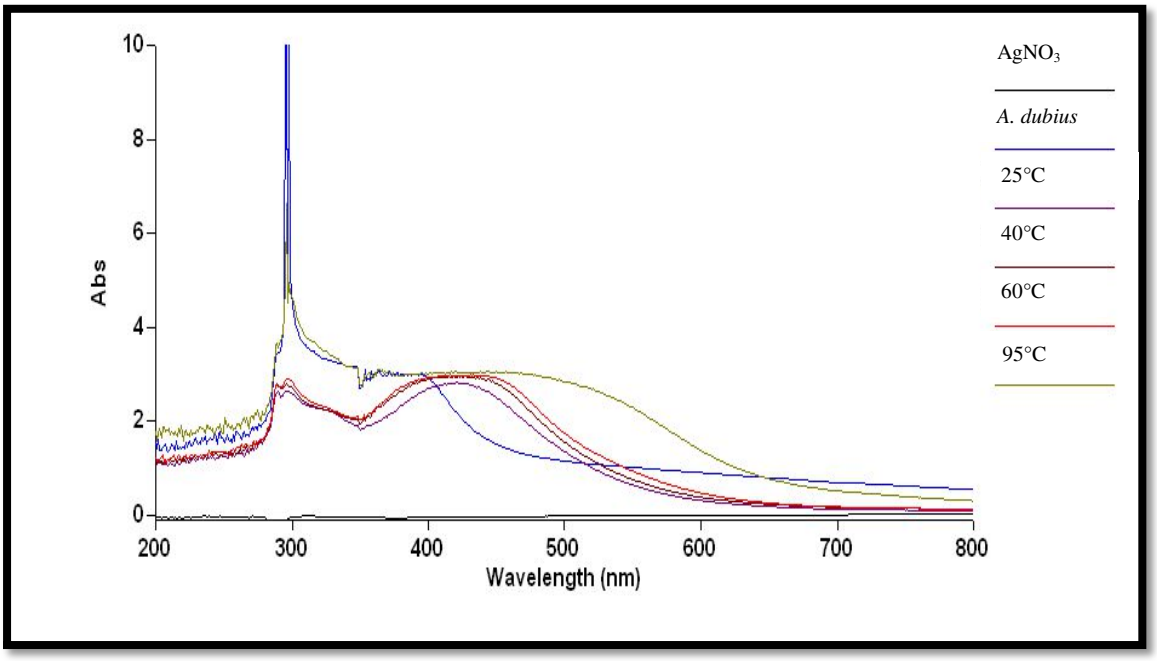

Figure 12: UV-vis spectra of 2.5 ml *A. dubius* aqueous leaf extract reaction with $AgNO_3$ at different temperatures

The reactions that produced positive colour changes when added to the $HAuCl_4$ solution for *A. dubius* aqueous leaf extract was at 60°C and 95°C. No NPs formed at 25°C and 40°C. The UV-vis results for the *A. dubius* aqueous leaf extracts at 60°C and 95°C are shown in Figure 13 shows the peak formation in the required range of 520 nm - 550 nm for Au NPs. The best peak was formed by the reaction that occurred at 60°C with the wavelength of 538 nm and absorbance of 2.967 AU.

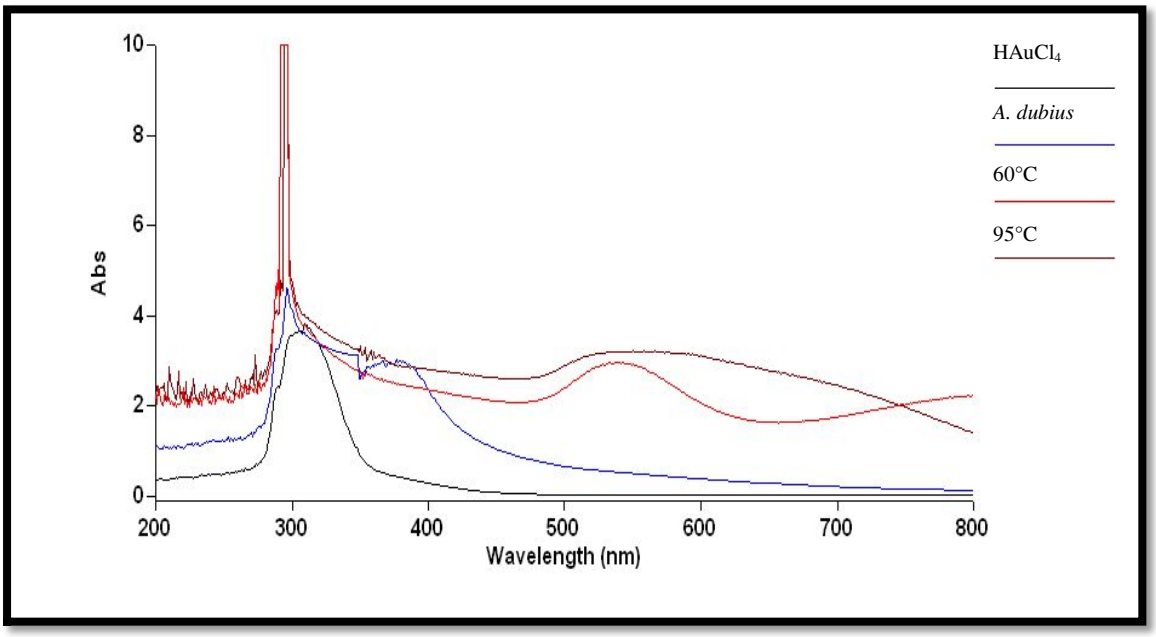

Figure 13: UV-vis spectra of 2.5 ml *A. dubius* aqueous leaf extract reaction with $HAuCl_4$ at different temperatures

G. perpensa aqueous leaf extract showed a positive colour change when added to the $AgNO_3$ solution at 25°C and 95°C but turned dark brown after ten minutes. The UV-vis results are shown in Figure 14 which shows a non-optimal peak formation. The peak formed at 25°C with a wavelength of 474 nm and absorbance of 2.907 AU was then tested with different extract concentrations since there was a reaction.

G. perpensa aqueous leaf extract at 25°C, 40°C, 60°C and 95°C produced positive colour changes when added to the $HAuCl_4$ solution. The UV-vis results are shown in Figure 15 which clearly shows the peak formation in the required range of 520 nm - 550 nm for Au NPs. The best peaks that were formed by the reactions that occurred are presented in Table 8.

Table 8: Best peaks formed for 2.5 ml aqueous leaf extract of *G. perpensa* Au NPs at different temperatures

Temperature (°C)	Wavelength (nm)	Absorbance (AU)
25	532	3.088
40	568	3.169
60	568	3.220

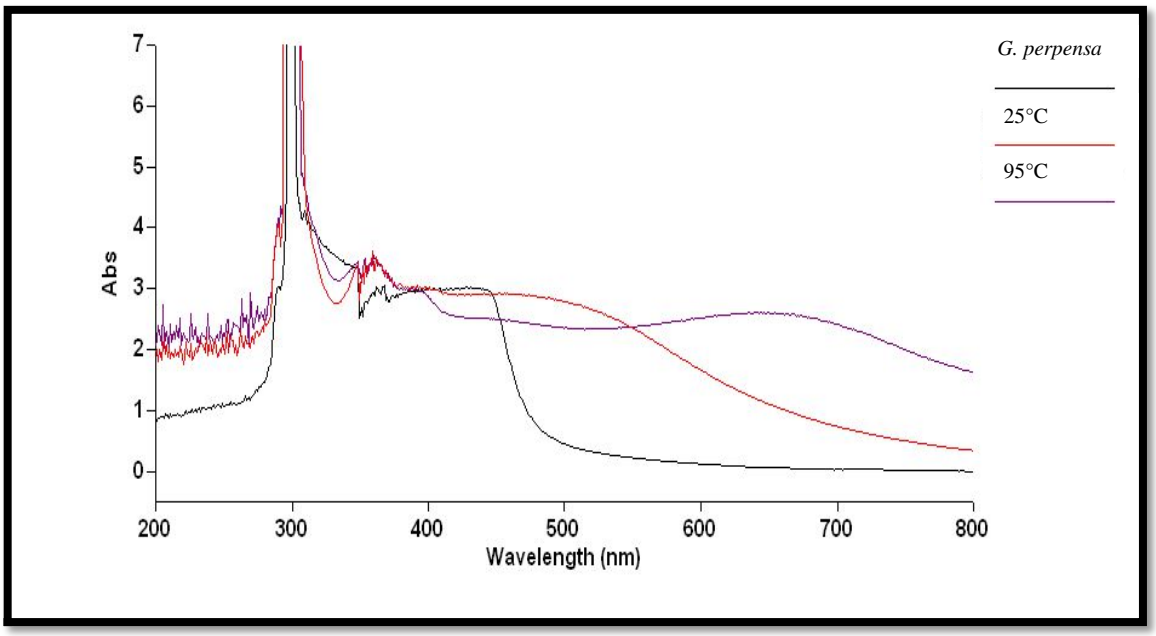

Figure 14: UV-vis spectra of 2.5 ml *G. perpensa* aqueous leaf extract reaction with $AgNO_3$ at different temperatures

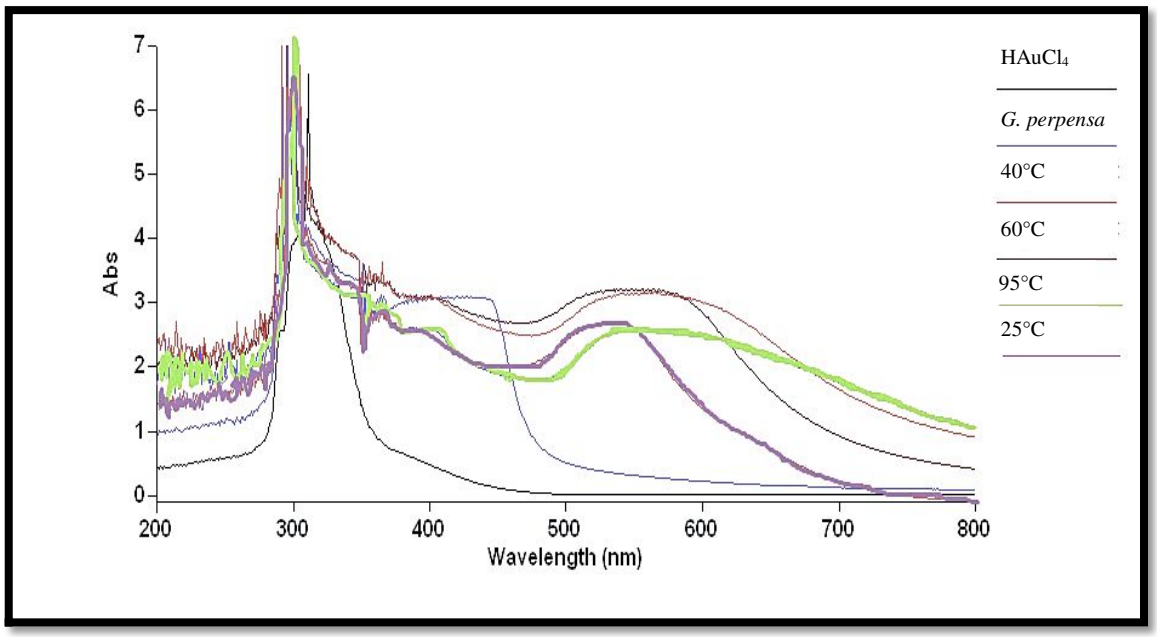

Figure 15: UV-vis spectra of 2.5 ml *G. perpensa* aqueous leaf extract reaction with $HAuCl_4$ at different temperatures

The reactions for *C. triloba* aqueous leaf extract showed a positive colour change when added to the $AgNO_3$ solution at 60°C and 95°C (Figure 16). The peak at 60°C had a wavelength of 451 nm and absorbance of 2.291 AU. The peak at 95°C had a wavelength of 448 nm and absorbance of 2.351 AU. The concentration optimization was performed at these two temperatures.

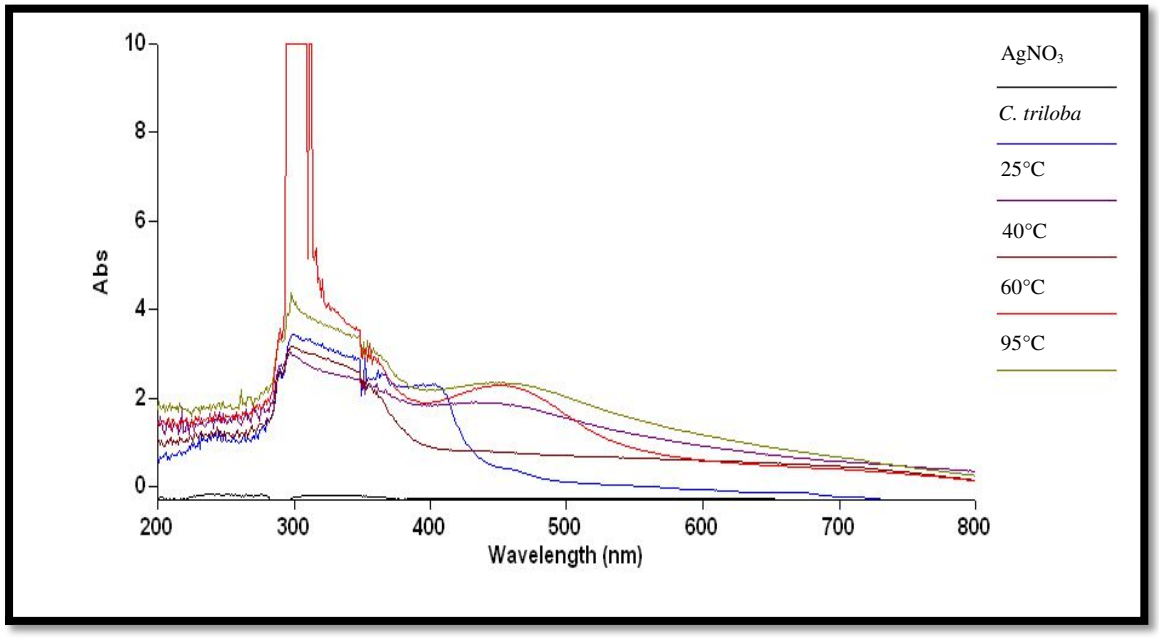

Figure 16: UV-vis spectra of 2.5 ml *C. triloba* aqueous leaf extract reaction with $AgNO_3$ at different temperatures

C. triloba aqueous leaf extract showed a positive colour change when added to the $HAuCl_4$ solution at 60°C and 95°C. The UV-vis results are shown in Figure 17 which shows the peak formation for these two temperatures. The peak formed at 60°C had a wavelength of 548 nm and absorbance of 1.354 AU. The peak formed at 95°C had a wavelength of 545 nm and absorbance of 2.535 AU. The concentration optimization was performed at these two temperatures.

Aqueous *C. roseus* leaf extract showed no positive colour change when added to the $AgNO_3$ or the $HAuCl_4$ solution. The UV-vis results are shown in Figure 18 and Figure 19 respectively. There was no peak formation.

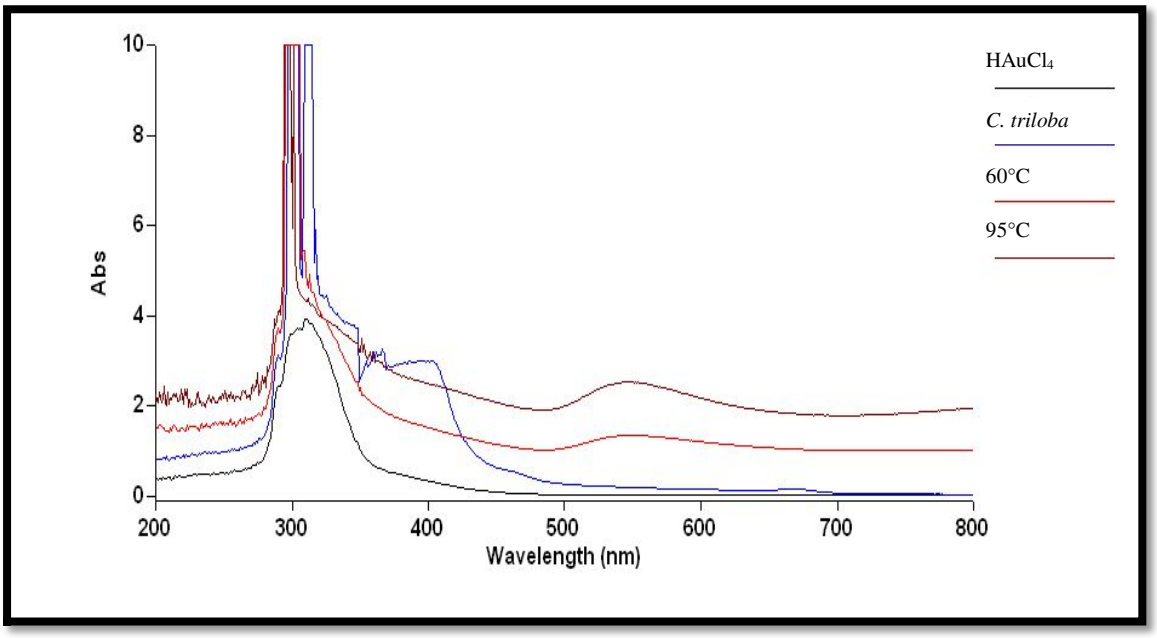

Figure 17: UV-vis spectra of 2.5 ml *C. triloba* aqueous leaf extract reaction with $HAuCl_4$ at different temperatures

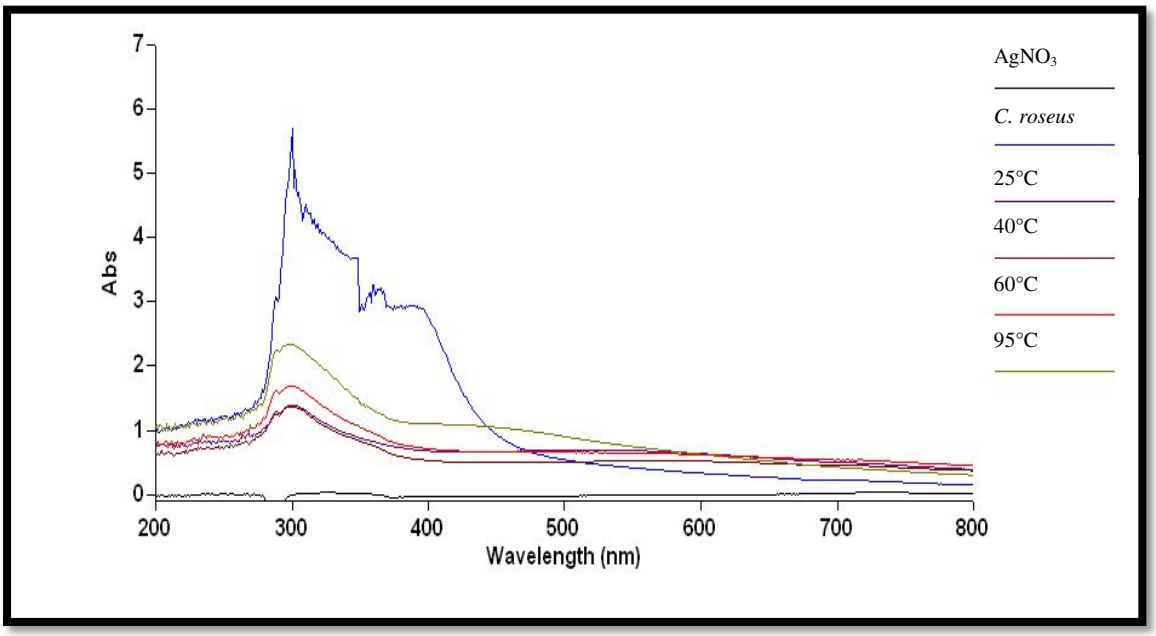

Figure 18: UV-vis spectra of 2.5 ml *C. roseus* aqueous leaf extract reaction with $AgNO_3$ at different temperatures

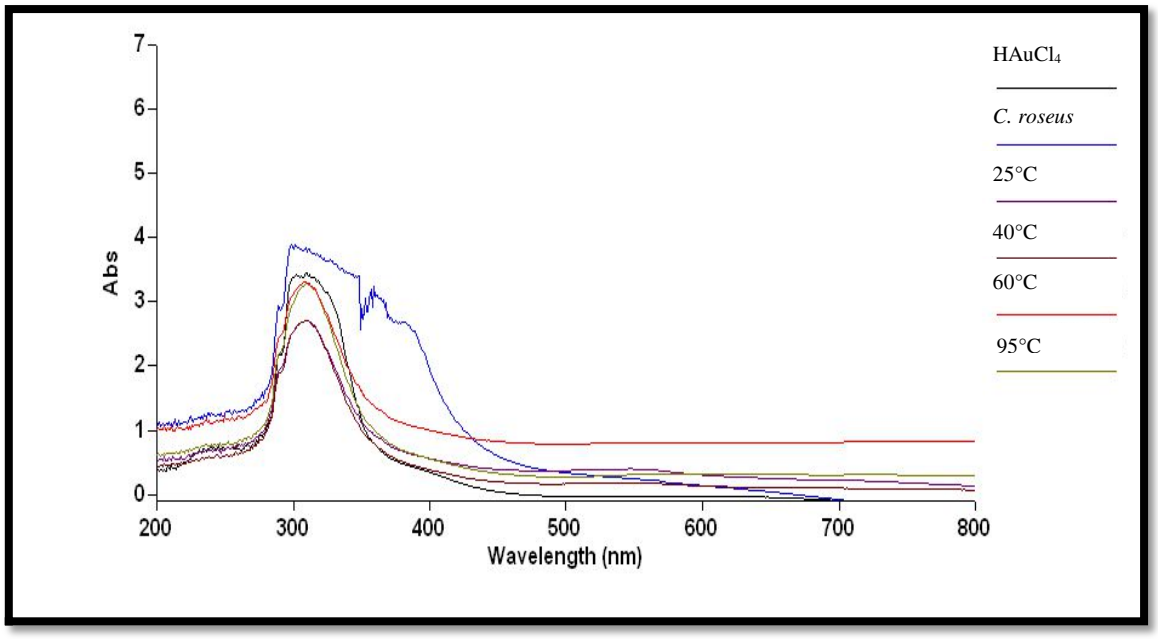

Figure 19: UV-vis spectra of 2.5 ml *C. roseus* aqueous leaf extract reaction with $HAuCl_4$ at different temperatures

4.2.2 Effect of Concentration of the Extract (Reducing Agent)

Ag NP synthesis by *A. dubius* extract was carried out at 25°C, 40°C and 60°C but with four different concentrations of the aqueous leaf extract. The results obtained are listed in Table 9. At 25°C, 40°C and 60°C, as the concentration increases, so too do the absorbance values with the exception of the 5 ml concentration where it decreases. For the 0.63 ml, 1.25 ml and 2.5 ml, it can also be seen that the wavelengths are in the range required for Ag NPs but for the 5 ml, it has a longer wavelength out of range. The optimum concentration is chosen based on the correct colour change, highest absorbance, in the correct range and a peak that is symmetrical. This is highlighted in Table 9.

Au NP synthesis by *A. dubius* extract was carried out at 60°C for all the concentrations. There was no colour change found for 0.63 ml and 1.25 ml aqueous leaf extract. The 5 ml extract concentration led to a broad peak which was out of the required range and a dark purple colour formed with a precipitate. The optimal concentration was 2.5 ml for Au NP synthesis.

Ag NP synthesis by *G. perpensa* extract was carried out by four concentrations of aqueous leaf extract that were reacted with $AgNO_3$ at 25°C. There was a positive colour change for

0.63 ml, 1.25 ml and 2.5 ml but none for 5ml. However, all these reactions turned a dark brown colour after ten minutes and wavelengths were out of the desired range. A final concentration of 0.32 ml was tried at 25°C and it produced a positive colour change as well as a symmetric peak with an average wavelength of 420 nm and absorbance of 1.468 ± 0.17 AU. The colour of solution also remained unchanged for 24 hours.

Au NPs synthesis by *G. perpensa* extract was carried out by four concentrations of aqueous leaf extract that were added to $HAuCl_4$ solutions at 25°C, 40°C, and 60°C. As can be seen in Table 9, the wavelength becomes longer as the concentration of the extract increases. The absorbance for the reactions at the three temperatures for concentrations 0.63 ml, 1.25 ml and 2.5 ml are approximately 3.0 AU, and they are around the same wavelength that falls in the desired range. For the 5 ml reactions at the different temperatures, there is a noticeable decrease and the wavelengths are out of range.

Ag NPs synthesis by *C. triloba* extract was carried out at 60°C and 95°C. In Table 9, it is shown that as the concentration of the extract increases, the wavelength increases and at 60°C, the peaks produced are broad and wavelengths are out of range. At 95°C, the optimum peak was produced with symmetry, a wavelength of 421 nm and an absorbance of 1.885 AU. The rest of the concentrations used at this temperature produce peaks out of range.

Au NPs synthesis by *C. triloba* extract showed no reaction for the 0.63 ml and 1.25 ml concentrations at both 60°C and 95°C. Both the 2.5 and 5 ml concentrations produce broad peaks out of range at 95°C. These two concentrations both produce Au NPs at 60°C but the best peak is produced with 5 ml extract resulting in a symmetric peak at 546 nm and the higher absorbance of 2.640 AU.

Extracts from *C. roseus* at different concentrations and at different temperatures did not produce any colour change after the addition of $AgNO_3$ and $HAuCl_4$. Thus indicating the synthesis of Ag and Au NPs was unsuccessful.

Table 9: Average wavelength (λ) and Absorbance (Abs) values for Ag and Au NPs at different concentrations at the optimized temperatures

Plant	NPs	Temp (°C)	Concentrations (ml)							
			0.63		1.25		2.5		5	
			λ (nm)	Abs (au)	λ (nm)	Abs (au)	λ (nm)	Abs (au)	λ (nm)	Abs (au)
A. dubius	Ag NPs	25	420	2.063 ± 0.20	417	2.671 ± 0.04	424	2.938 ± 0.10	431	2.870 ± 0.13
		40	422	1.875 ± 0.14	420	2.496 ± 0.08	423	2.986 ± 0.02	428	2.950 ± 0.13
		60	417	1.900 ± 0.13	422	2.504 ± 0.11	431	2.998 ± 0	437	2.927 ± 0.01
	Au NPs	60	No Reaction	No Reaction	No Reaction	No Reaction	539	2.949 ± 0.03	557	0.855 ± 0.13
G. perpensa	Ag NPs	25	436	2.991 ± 0.00	445	3.008 ± 0.10	452	2.954 ± 0.14	No Reaction	No Reaction
	Au NPs	25	535	3.028 ± 0.20	540	3.151 ± 0	533	3.094 ± 0.01	559	1.395 ± 0.05
		40	542	3.084 ± 0.07	546	3.165 ± 0.10	564	3.163 ± 0.01	570	1.873 ± 0.18
		60	538	3.033 ± 0.14	542	3.135 ± 0.16	556	3.226 ± 0.01	562	1.696 ± 0.26
C. triloba	Ag NPs	60	442	1.548 ± 0.22	449	1.217 ± 0.18	451	2.559 ± 0.3	455	1.548 ± 0.22
		95	421	1.885 ± 0.24	432	1.552 ± 0.25	447	2.144 ± 0.18	449	1.707 ± 0.51
	Au NPs	60	No Reaction	No Reaction	No Reaction	No Reaction	548	1.348 ± 0.05	546	2.640 ± 0.18
		95	No Reaction	No Reaction	No Reaction	No Reaction	552	2.258 ± 0.39	590	3.043 ± 0.01

N=3

- Optimal Ag NP synthesis conditions

- Optimal Au NP synthesis conditions

4.2.3 Effect of Methanolic and Ethyl Acetate Extracts

A. dubius, *G. perpensa* and *C. triloba* methanolic extracts showed the formation of Ag and Au NPs which was shown by a colour change and UV-vis scans. *A. dubius*, *G. perpensa* and *C. triloba* ethyl acetate extracts produced slight changes in colour after an hour of stirring and then precipitated in the solution.

4.3 Biosynthesis of Silver and Gold Nanoparticles from Callus Extracts

Since the leaf extracts of *A. dubius*, *G. perpensa* and *C. triloba* showed positive results for synthesis of Ag and Au NPs, these plants were chosen for callus formation.

4.3.1 Micropropagation

Sterilization:

To identify the ideal surface sterilization conditions of the explants, different combinations of chemical sterilizing agents i.e. mercury chloride and sodium hypochlorite were used. *A. dubius* and *C. triloba* were both successfully sterilized by the exposure of leaves to five minutes in 0.1% mercury chloride and fifteen minutes in 30% sodium hypochlorite. These plates showed no contamination and were used to sterilize leaves for further use. All four sterilization techniques were unsuccessful in sterilizing the leaves of *G. perpensa* (Figure 20) and thus were not used for NPs synthesis.

Callus Induction:

To determine the best callus induction response, various concentration and combinations of growth regulators were used. Callus initiation was observed on the surface or cut ends of the explants after two weeks of inoculation. The best callus induction response for leaf explants of *A. dubius* (Figure 21) and *C. triloba* (Figure 22) were observed on MS medium supplemented with 1 µg/L 2,4-D and 1 µg/L BAP. No callus induction was observed on the combination of 0.5 µg/L 2,4-D with 0.5 µg/L BAP or the 0.1 µg/L 2,4-D or 1 µg/L BAP only.

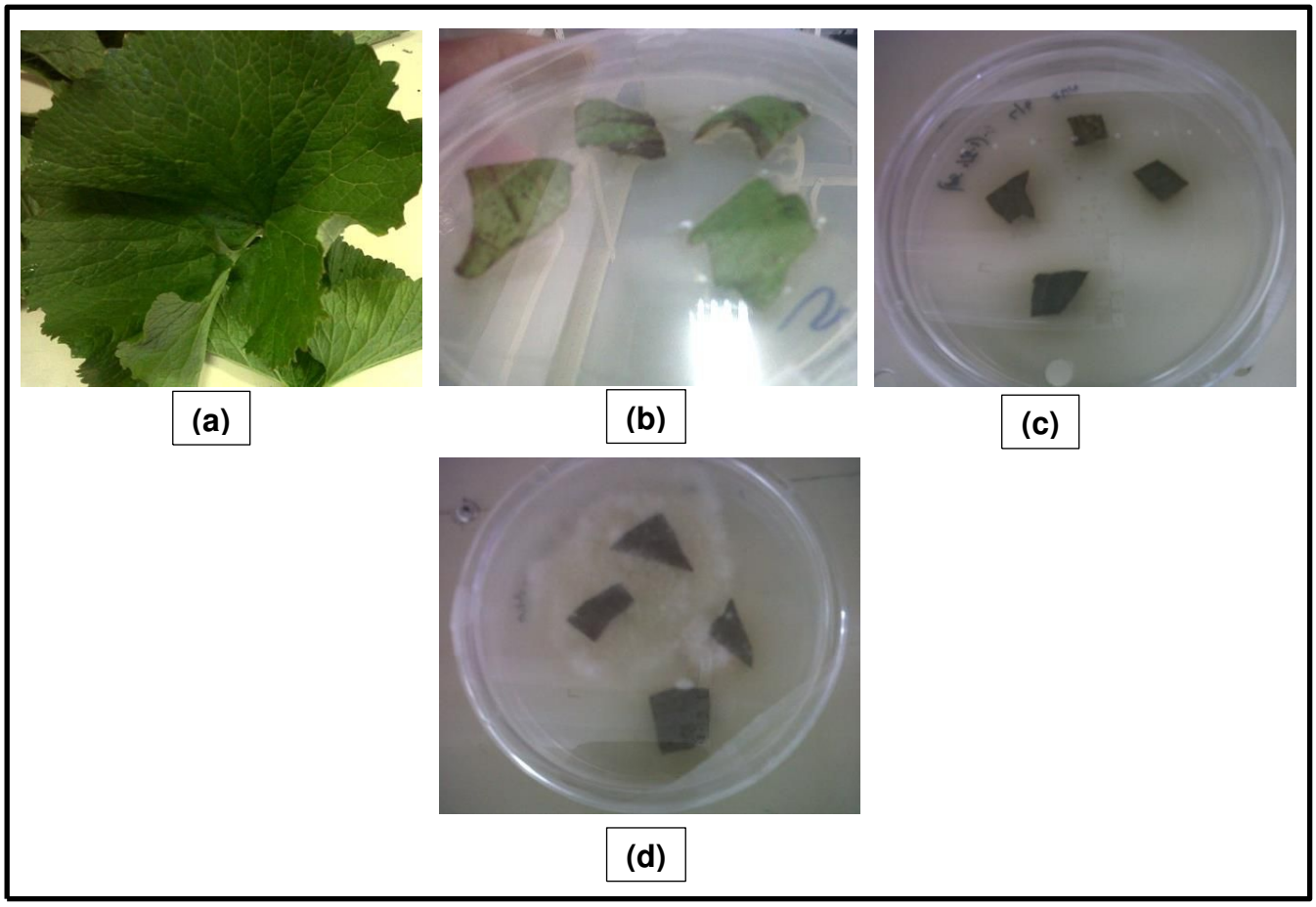

Figure 20: Micropropagation of (a) *G. perpensa* leaves, (b) leaf explants on modified MS media, (c) darkening of the leaves as well as media and (d) contamination of the explants.

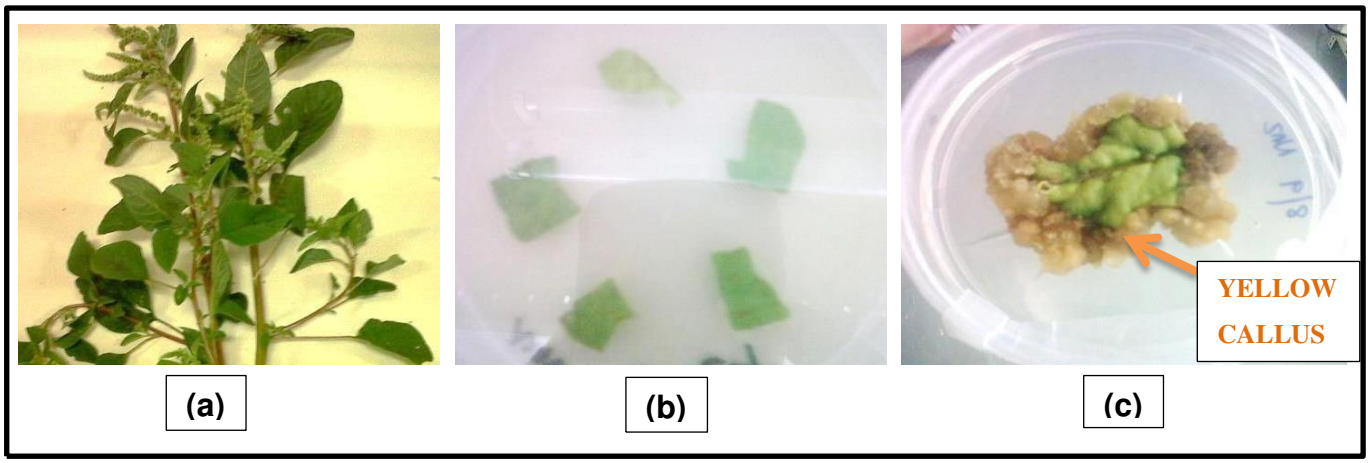

Figure 21: Micropropagation of (a) *A. dubius* leaves, (b) *A. dubius* leaf explants on modified MS media and (c) callus formation after four weeks

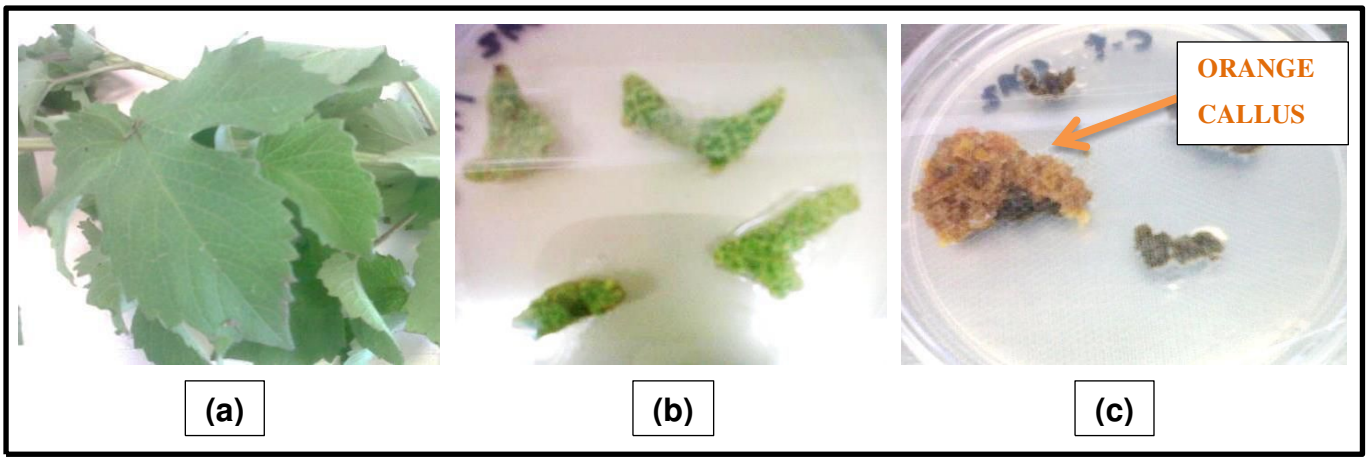

Figure 22: Micropropagation of (a) *C. triloba* leaves, (b) *C. triloba* leaf explants on modified MS media and (c) callus formation after four weeks

4.3.2 Synthesis of Silver and Gold Nanoparticles

Aqueous, methanolic and ethyl acetate callus extracts of *A. dubius* when added to $AgNO_3$ and $HAuCl_4$, at all the temperatures and concentrations, remained colourless and pale yellow respectively indicating no Ag and Au NPs synthesis.

No colour change occurred at any of the temperatures when 2.5 ml aqueous, methanolic and ethyl acetate callus extracts were tested. Whereas a positive colour change was seen after 15 minutes of stirring for 10 ml aqueous callus extracts of *C. triloba* at 95°C when added to $AgNO_3$ and $HAuCl_4$. The methanolic and ethyl acetate extracts did show a slight colour change for this temperature and concentration. However, within ten minutes of colour change, all the solutions showed a precipitate. These are considered unstable solutions and according to literature, many NPs lose their unique properties once they have aggregated and precipitated from suspension due to lack of surface functionalization, being a critical component in their application (Christian *et al.,* 2008). It was decided that no further analysis should be done on unstable NPs and trying to gain stability was beyond the scope of the project.

4.4 Characterization of the Synthesized Nanoparticles

4.4.1 UV-vis Spectra Analysis

The optimum synthesis conditions for aqueous leaf extracts were determined in the previous section 4.2. The following UV-vis scans show optimal peaks formed for biosynthesized Ag NPs and Au NPs from the aqueous leaf extract of *A. dubius* in Figure 23 and methanolic extract in Figure 24. The wavelengths of the Ag NPs formed by the aqueous and methanolic extracts are in the same range. The absorbance of the aqueous Ag NPs is higher than the methanolic Ag NPs by double the value and also has a sharper and more symmetrical peak in comparison to the methanolic Ag NPs. The wavelengths of the aqueous Au NPs and the methanolic Au NPs are in the same range. The absorbance values for aqueous and methanolic Au NPs are similar, ~2.9 AU and ~2.6 AU respectively. There is also not much difference in the symmetry and shape of the peaks.

The peaks formed by Ag NPs from the aqueous and methanolic leaf extract of *G. perpensa* (Figure 25) both fall in the same wavelength range, their absorbance values are ~2.9 AU and ~1.3 AU respectively which correlates with the difference in *A. dubius* Ag NPs. Here also, the peak is much sharper than in the aqueous than in the methanolic Ag NPs. The wavelength of methanolic Au NPs is shorter than the aqueous Au NPs but is still in the desired range. The absorbance of this peak is slightly lower (~2.9 AU compared to ~3.2 AU) than the aqueous Au NPs. The aqueous Au NPs form a broader peak with a shoulder at just after 700 nm compared to the methanolic Au NPs which have a symmetrical and sharp peak as visible in Figure 26.

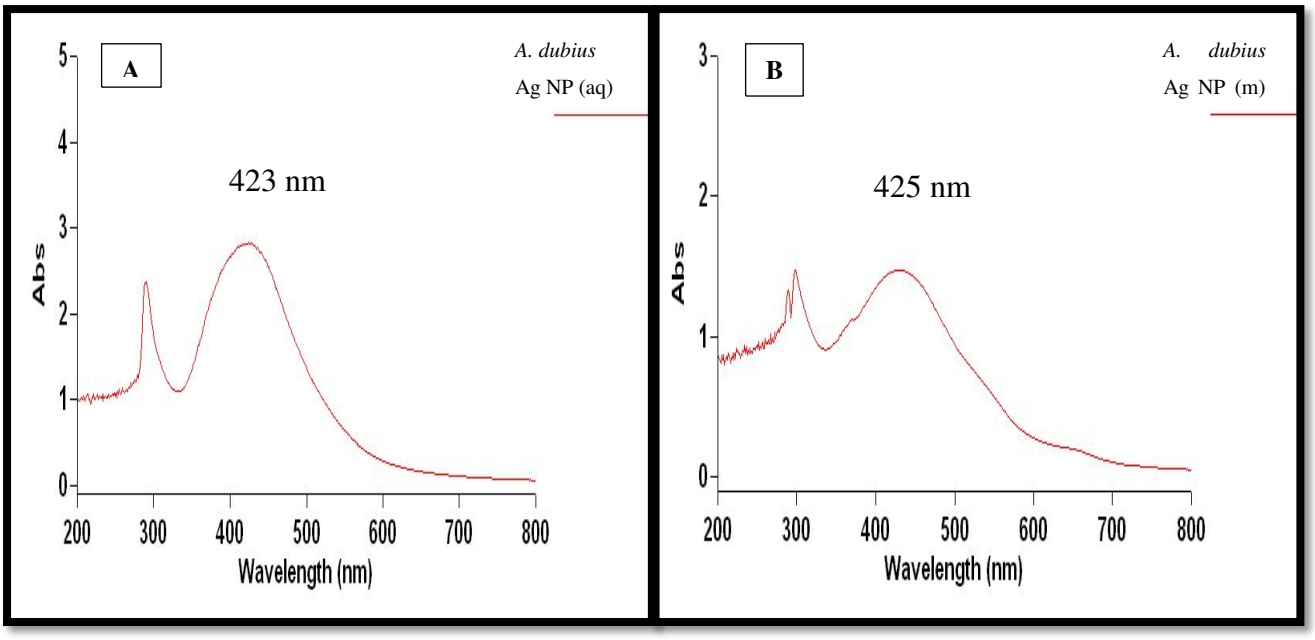

Figure 23: UV-vis spectra of the reaction between (A) aqueous and (B) methanolic leaf extracts of *A. dubius* with $AgNO_3$ at optimized conditions

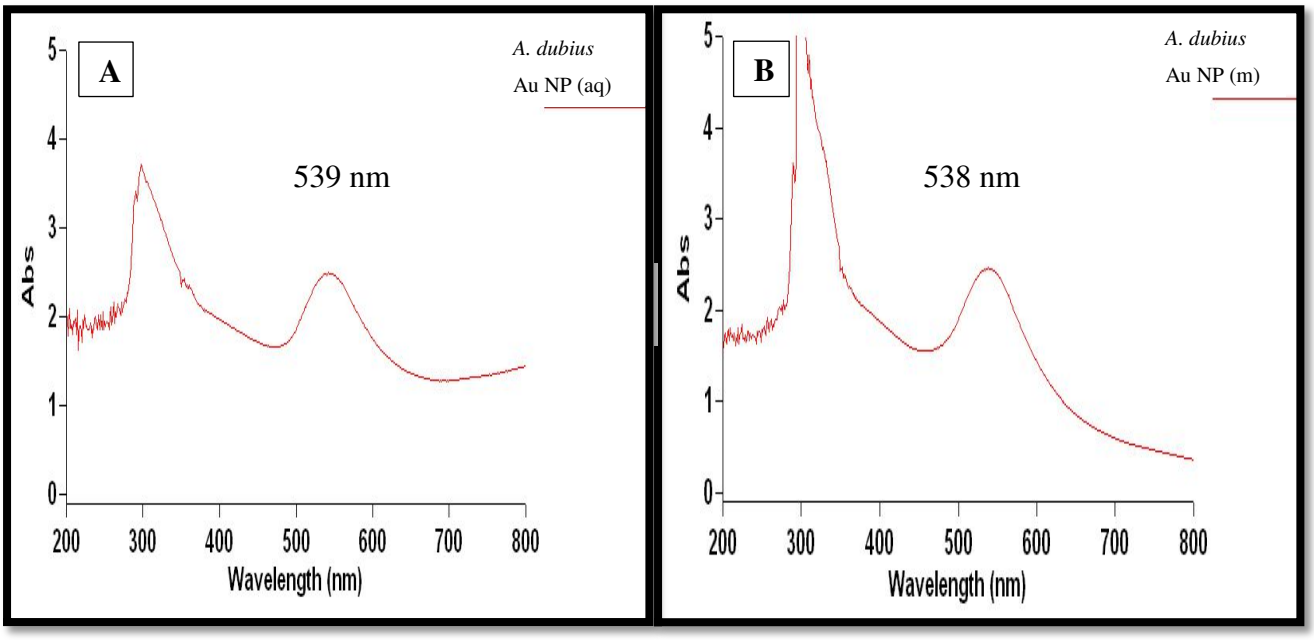

Figure 24: UV-vis spectra of the reaction between (A) aqueous and (B) methanolic leaf extracts of *A. dubius* with $HAuCl_4$ at optimized conditions

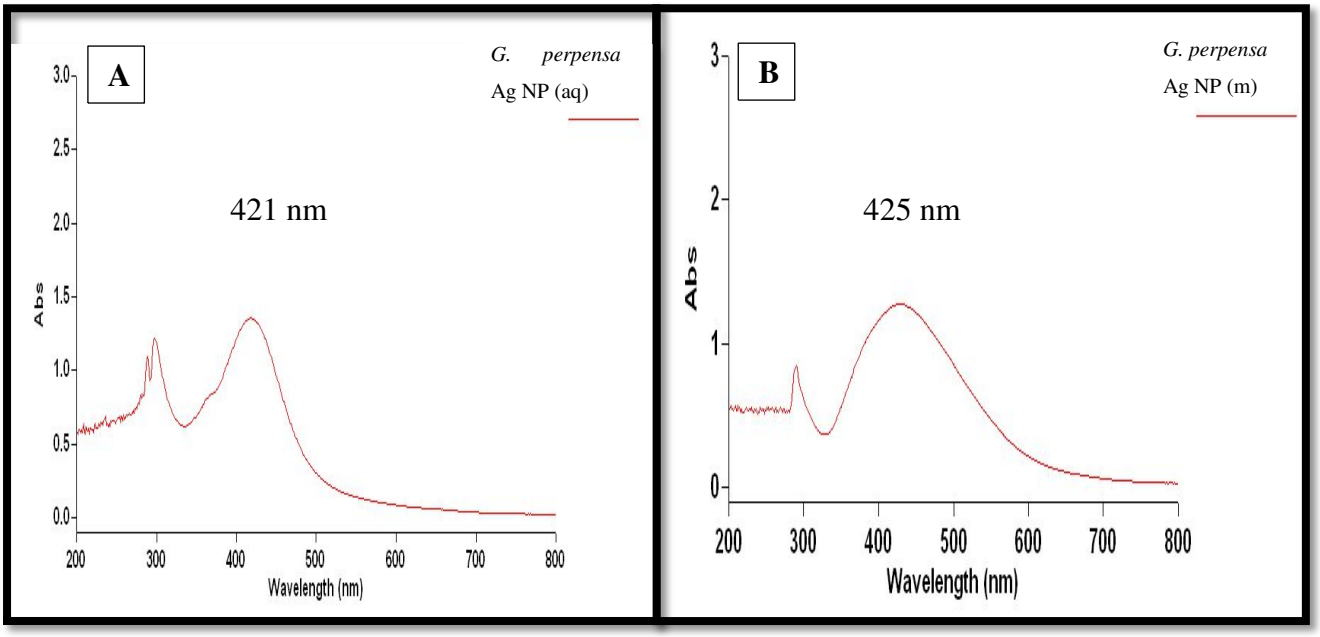

Figure 25: UV-vis spectra of the reaction between (A) aqueous and (B) methanolic leaf extracts of *G. perpensa* with $AgNO_3$ at optimized conditions

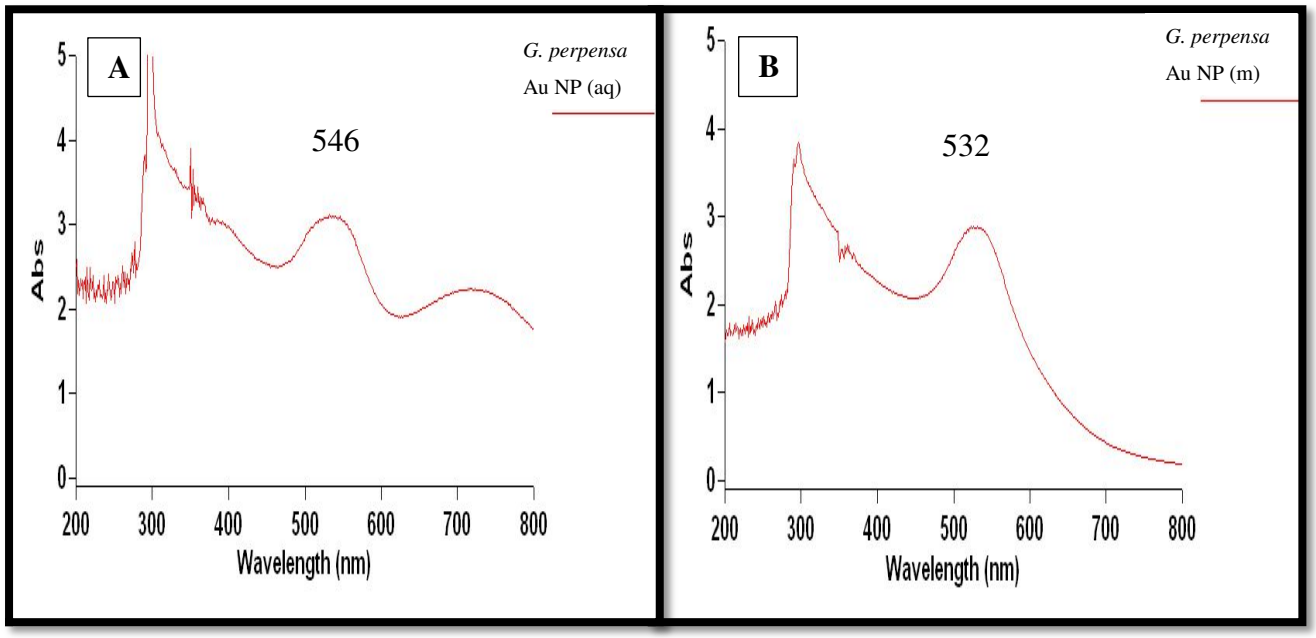

Figure 26: UV-vis spectra of the reaction between (A) aqueous and (B) methanolic leaf extracts of *G. perpensa* with $HAuCl_4$ at optimized conditions

Aqueous and methanolic leaf extracts of *C. triloba* form Ag NPs at wavelengths in the same range, with little difference between them as well as the absorbance values (~1.9 AU and ~1.2 AU respectively). The aqueous Ag NPs produce a slightly broader peak in comparison to the methanolic Ag NPs as can be seen in Figure 27.

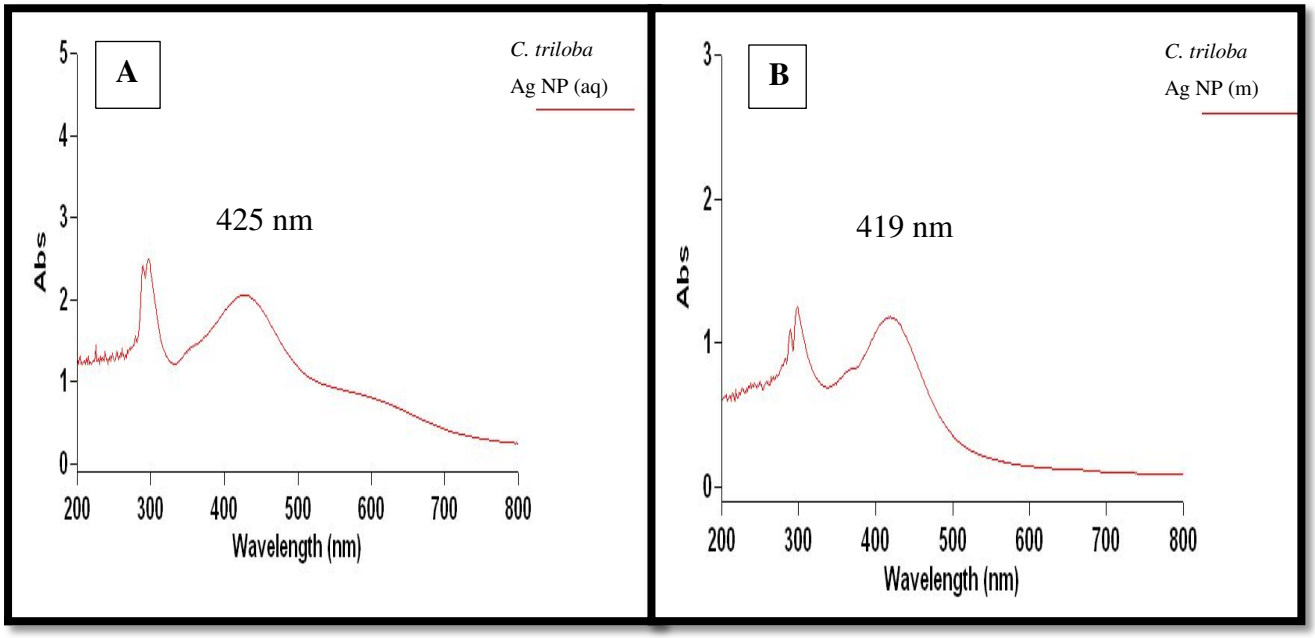

Figure 27: UV-vis spectra of the reaction between (A) aqueous and (B) methanolic leaf extracts of *C. triloba* with $AgNO_3$ at optimized conditions

The wavelength range, absorbance (~2.9 AU and ~2.7 AU) as well as the symmetry and shape of the peaks for Au NPs from aqueous and methanolic extracts are in close relation. There are only slight differences between the two Au NPs (Figure 28).

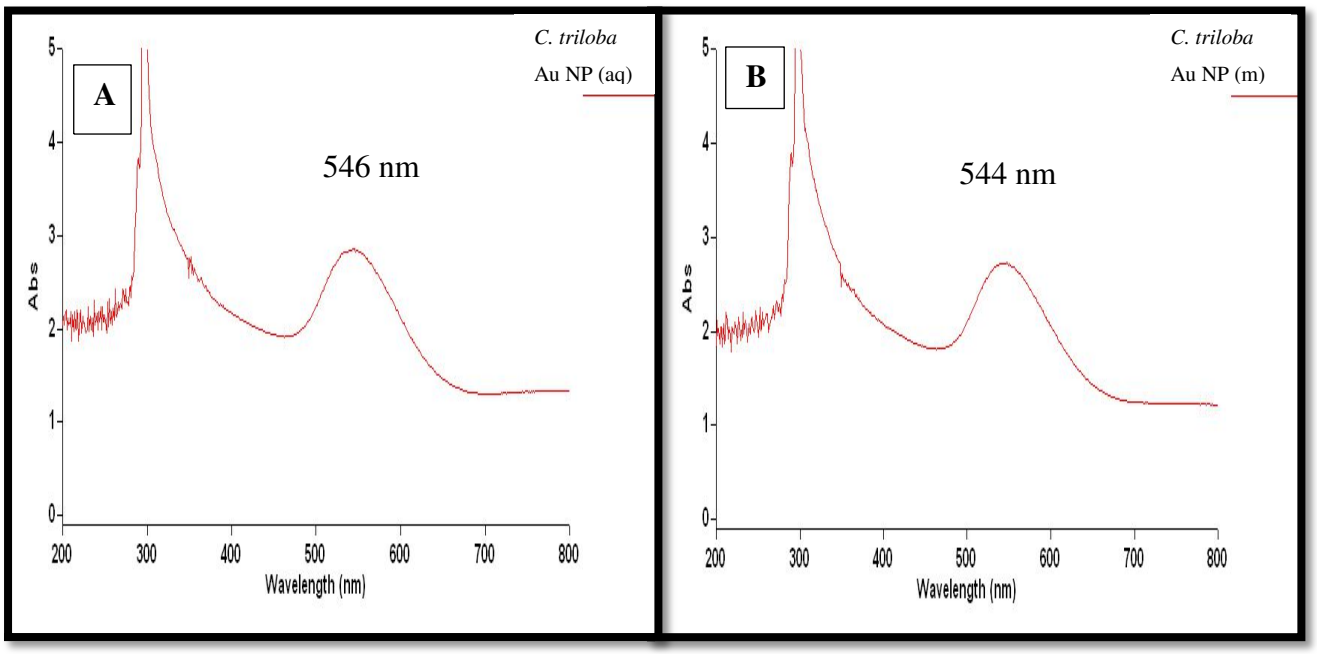

Figure 28: UV-vis spectra of the reaction between (A) aqueous and (B) methanolic leaf extracts of *C. triloba* with $HAuCl_4$ at optimized conditions

4.4.2 Transmission Electron Microscopy (TEM) Analysis

TEM analysis is able to determine the size and shape distribution of the particles. This is useful information which is needed for specific applications of particles.

A. dubius produced spherical shaped Ag NPs for both aqueous and methanolic leaf extracts as can be seen in Figure 29. The images also show that the particles produced by the methanolic extract are smaller in size compared to those produced by the aqueous extract. Aqueous Ag NPs range in size from 19 nm to 38 nm whereas methanolic Ag NPs sizes are between 7 nm to 16 nm. The NPs were found to be well dispersed from both extracts.

The Au NPs (Figure 30) synthesized from *A. dubius* were spherical and triangular. Most of the triangles were much larger in size (25 nm to 100 nm) in comparison to the smaller spherical (9 nm to 25 nm) Au NPs. The Au NPs from the methanolic extracts were spherical, triangular and a few were hexagonally shaped. The triangular shaped particles and the hexagonally shaped Au NPs are both large. Sizes range from 9 nm to 27 nm for spherical-, 9 nm to 45 nm for triangular- and 13 nm to 26 nm for hexagonal-shaped particles. Some of the bigger spherical particles are actually the clumping of one or two particles together thus giving it an oblong shape hence measurements of these were not taken into account.

G. perpensa aqueous and methanolic leaf extracts produced spherical and ellipsoidal shaped Ag NPs as shown in Figure 31. The size range of the particles is similar to that of *A. dubius*. However, unlike in *A. dubius*, the methanolic Ag NPs are not smaller than the aqueous Ag NPs and fall in the same range of ~13 nm to 24 nm.

Aqueous *G. perpensa* leaf extract formed spherical and also triangular Au NPs as shown in Figure 32. The methanolic sample of Au NPs forms the same shapes as the aqueous Au NPs. The aqueous spherical Au NPs are between 10 nm to 21 nm whereas the methanolic Au NPs are slightly smaller in the range of 9 nm to 16 nm. The triangular shaped Au NPs are much larger in the aqueous samples than in the methanolic (12 nm to 39 nm compared to 10 nm to 23 nm) samples. Dispersity in the aqueous sample is more apparent than in the methanolic sample.

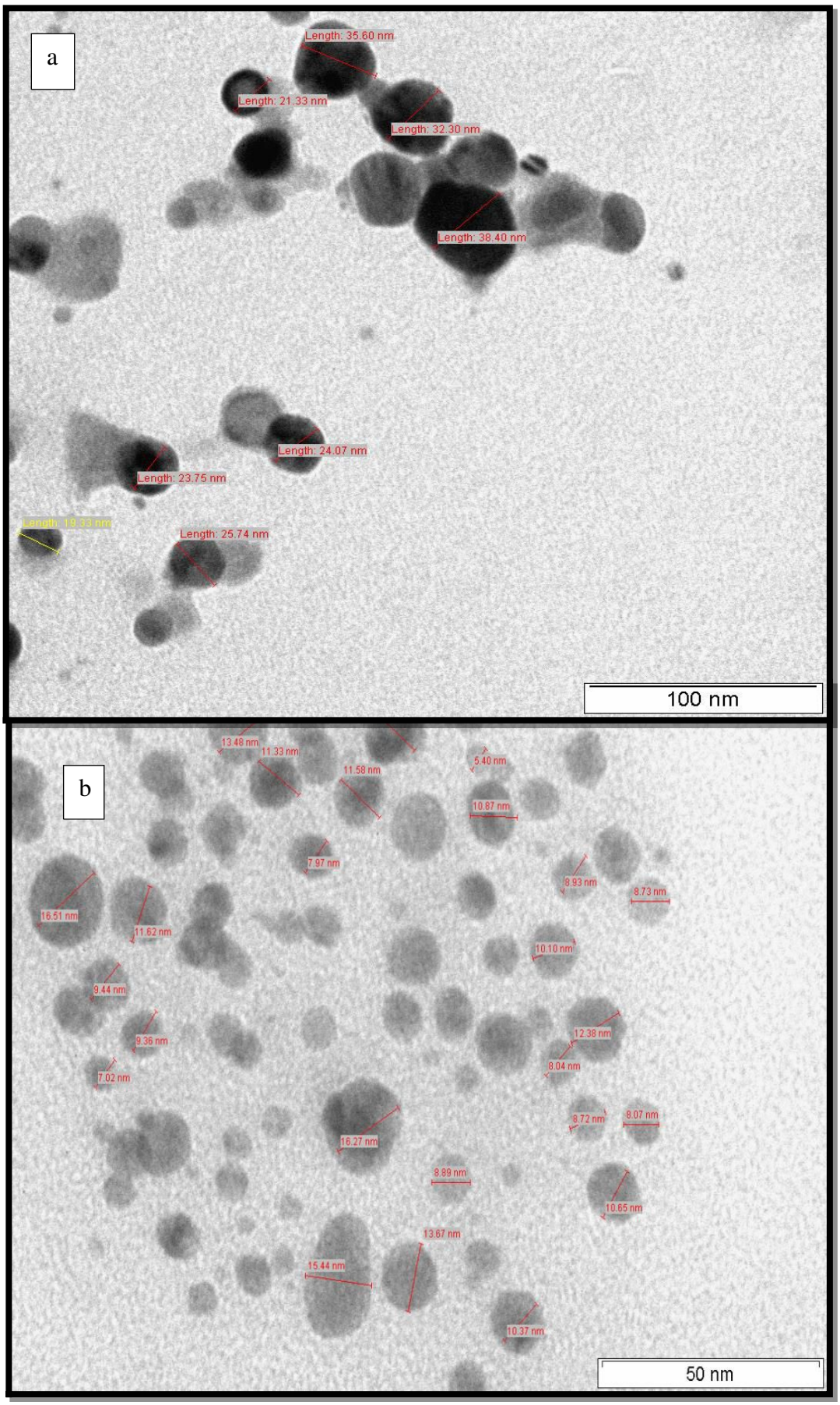

Figure 29: TEM images showing the range of sizes and shapes of the Ag NPs synthesized from (a) aqueous and (b) methanolic *A. dubius* leaf extracts

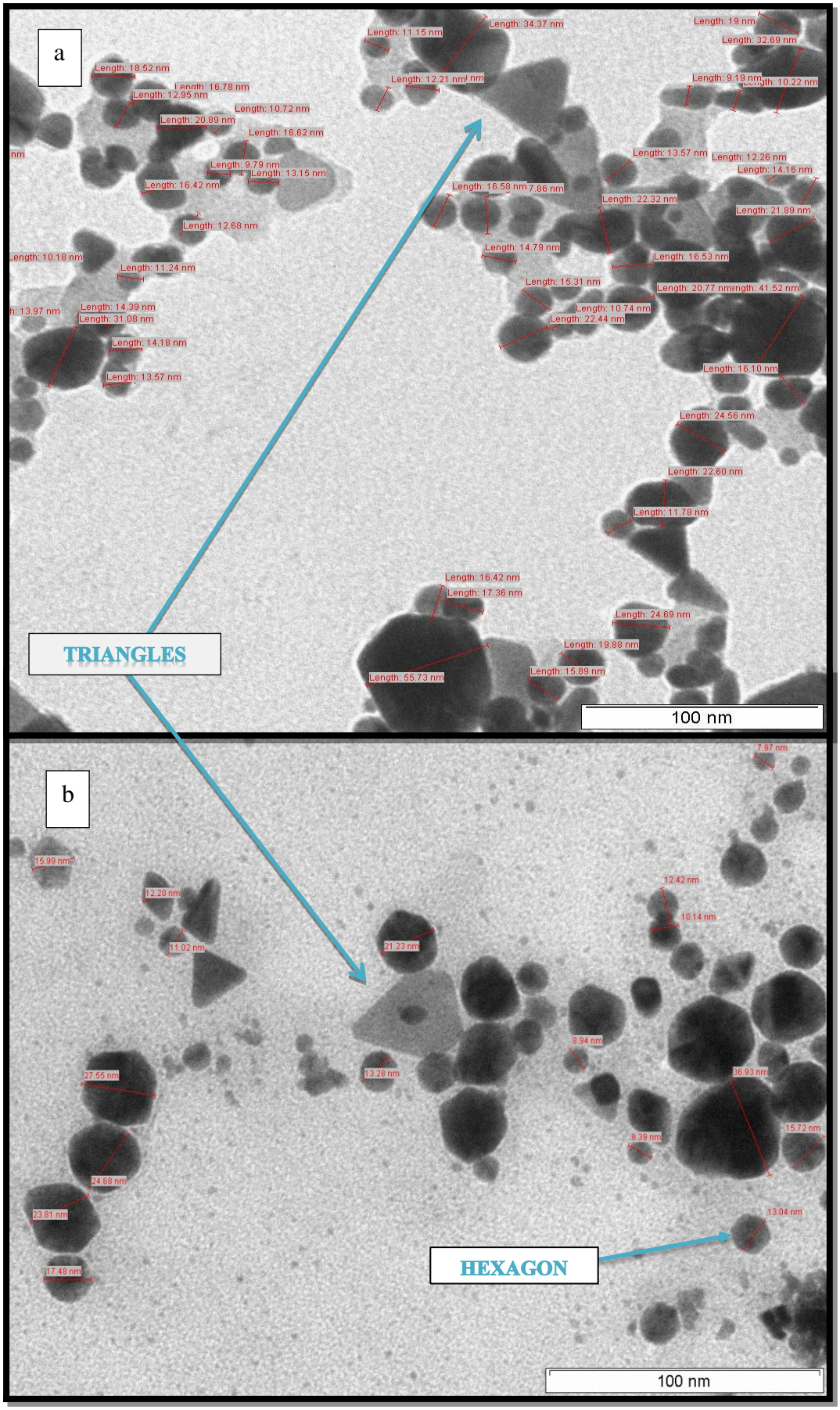

Figure 30: TEM images showing the range of sizes and shapes of the Au NPs synthesized from (a) aqueous and (b) methanolic *A. dubius* leaf extracts

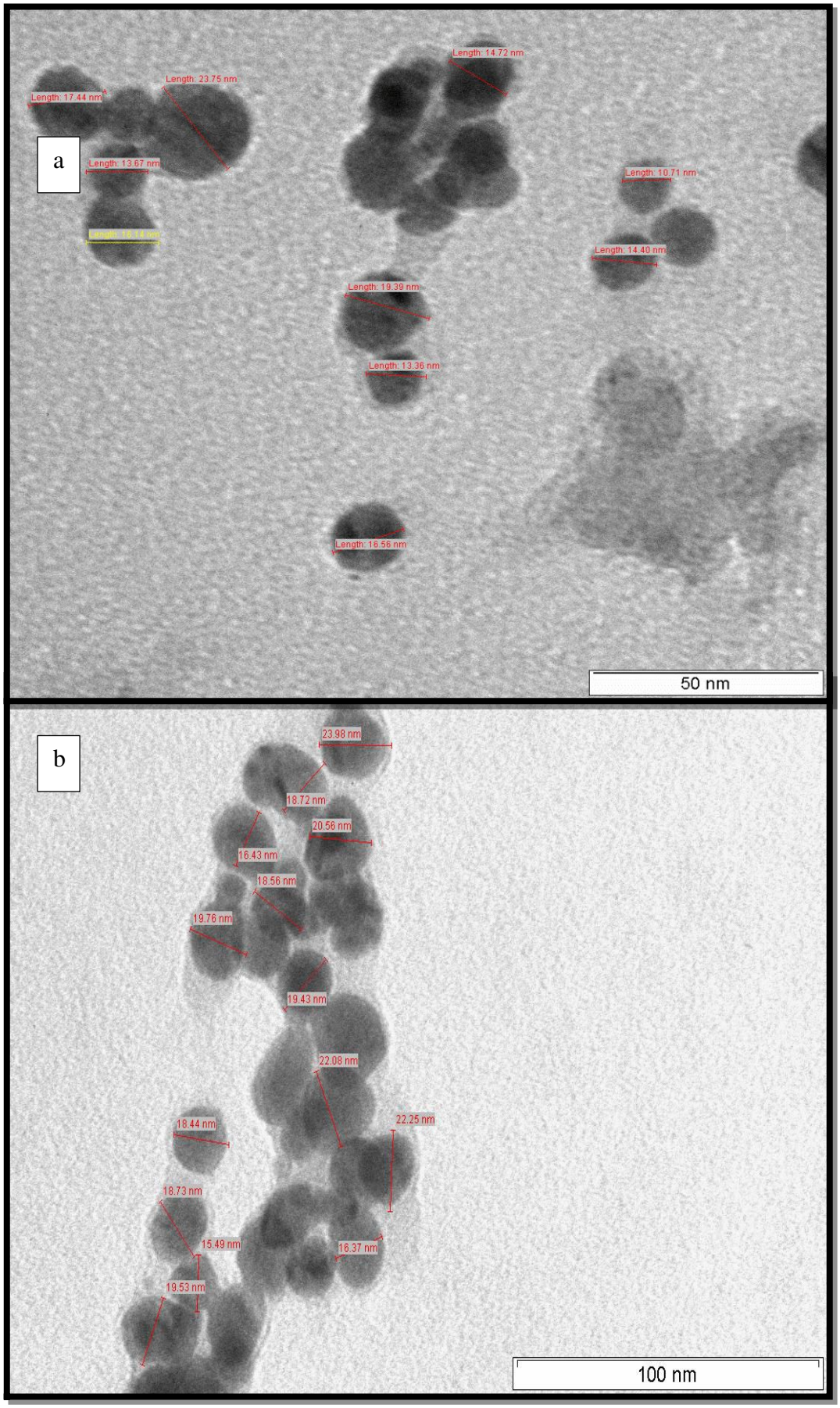

Figure 31: TEM images showing the range of sizes and shapes of the Ag NPs synthesized from (a) aqueous and (b) methanolic *G. perpensa* leaf extracts

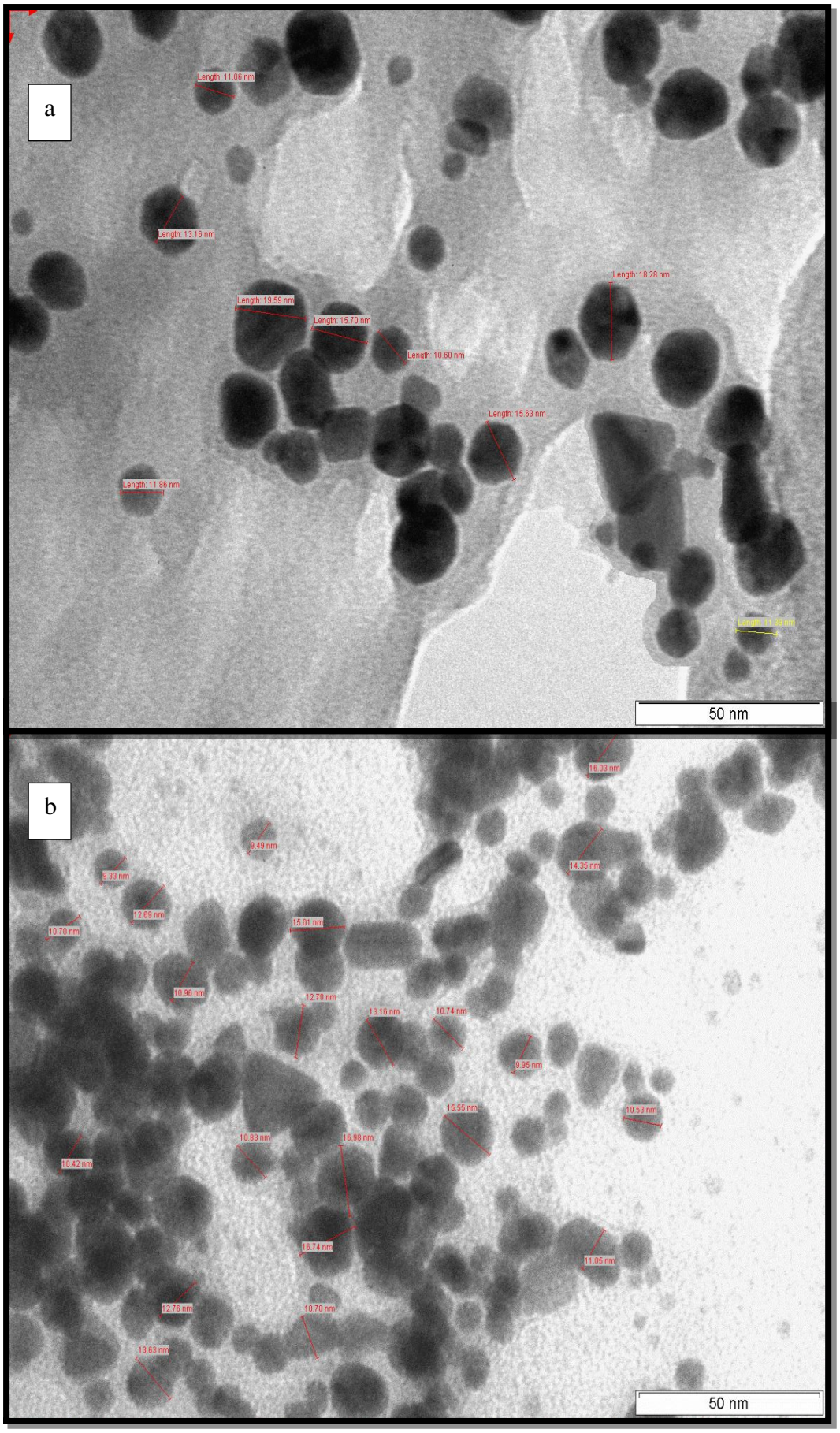

Figure 32: TEM images showing the range of sizes and shapes of the Au NPs synthesized from (a) aqueous and (b) methanolic *G. perpensa* leaf extracts

Figure 33 shows the synthesized spherical and ellipsoidal shaped Ag NPs of *C. triloba* formed from aqueous and methanolic extracts. The particles produced are much larger in comparison to those formed by *A. dubius* and *G. perpensa*. The aqueous Ag NPs range in size from 22 nm to 50 nm which are larger than the methanolic Ag NPs which are 10 nm to 42 nm in size. The Ag NPs were also dispersed in these samples.

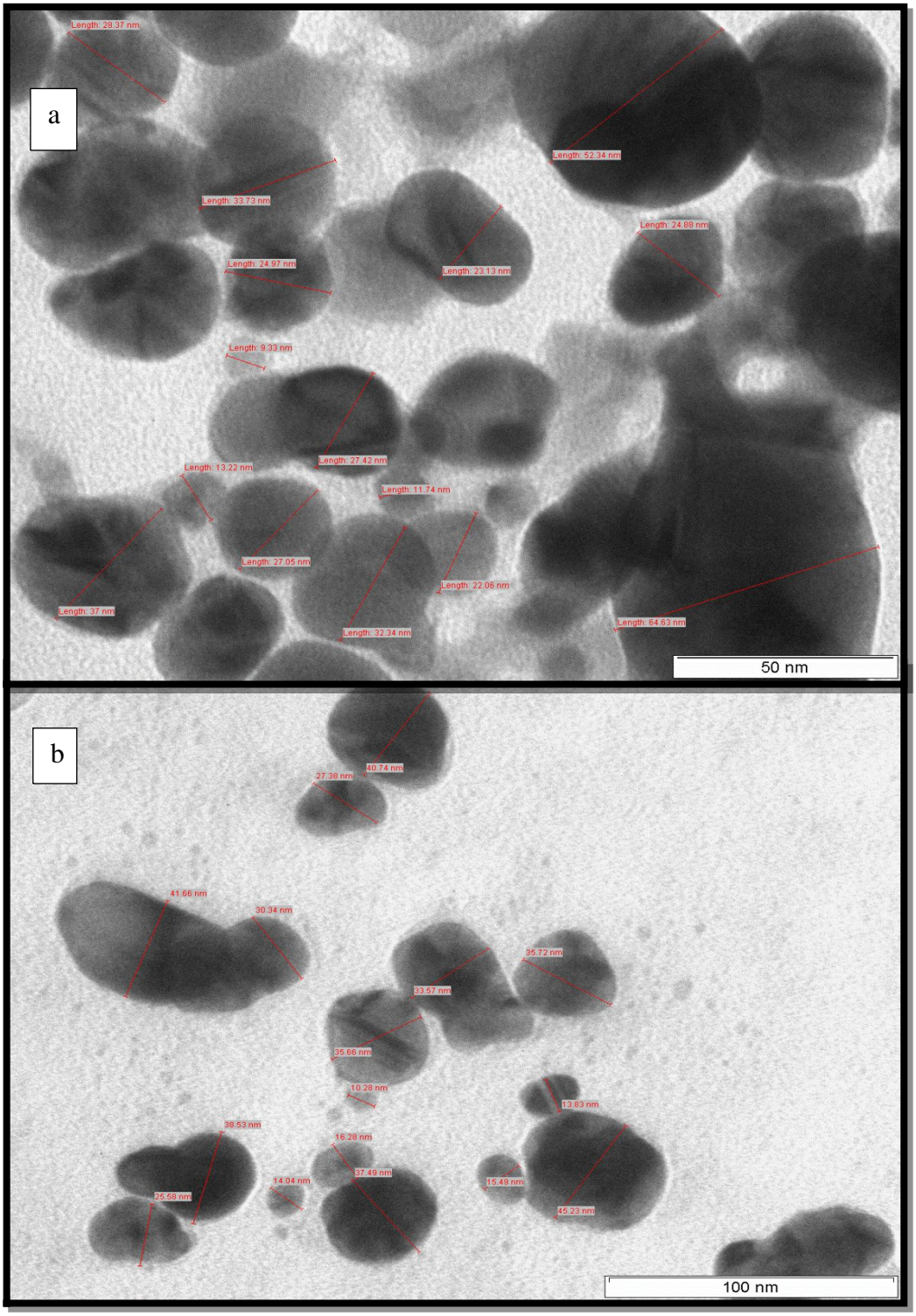

Figure 33: TEM images showing the range of sizes and shapes of the Ag NPs synthesized from (a) aqueous and (b) methanolic *C. triloba* leaf extracts

The aqueous and methanolic extracts of *C. triloba* produce a few spherical, rod and triangular shaped particles with majority being hexagonal Au NPs as can be seen in Figure 34. The spherical particles formed by the aqueous extract are smaller (17 nm to 32 nm) when compared to the larger triangular (20 nm to 190 nm) and hexagonal shapes (18 nm to 112 nm) formed. The triangular Au NPs (12 nm to 50 nm) produced from the methanolic extract are larger in comparison to the spherical (7 nm to 36 nm) and hexagonal (10 nm to 28 nm) particles that form. A few rods that were seen are thin and elongated in the aqueous Au NP sample but are much shorter in the methanolic samples. There are also more spherical Au NPs formed in the methanolic sample.

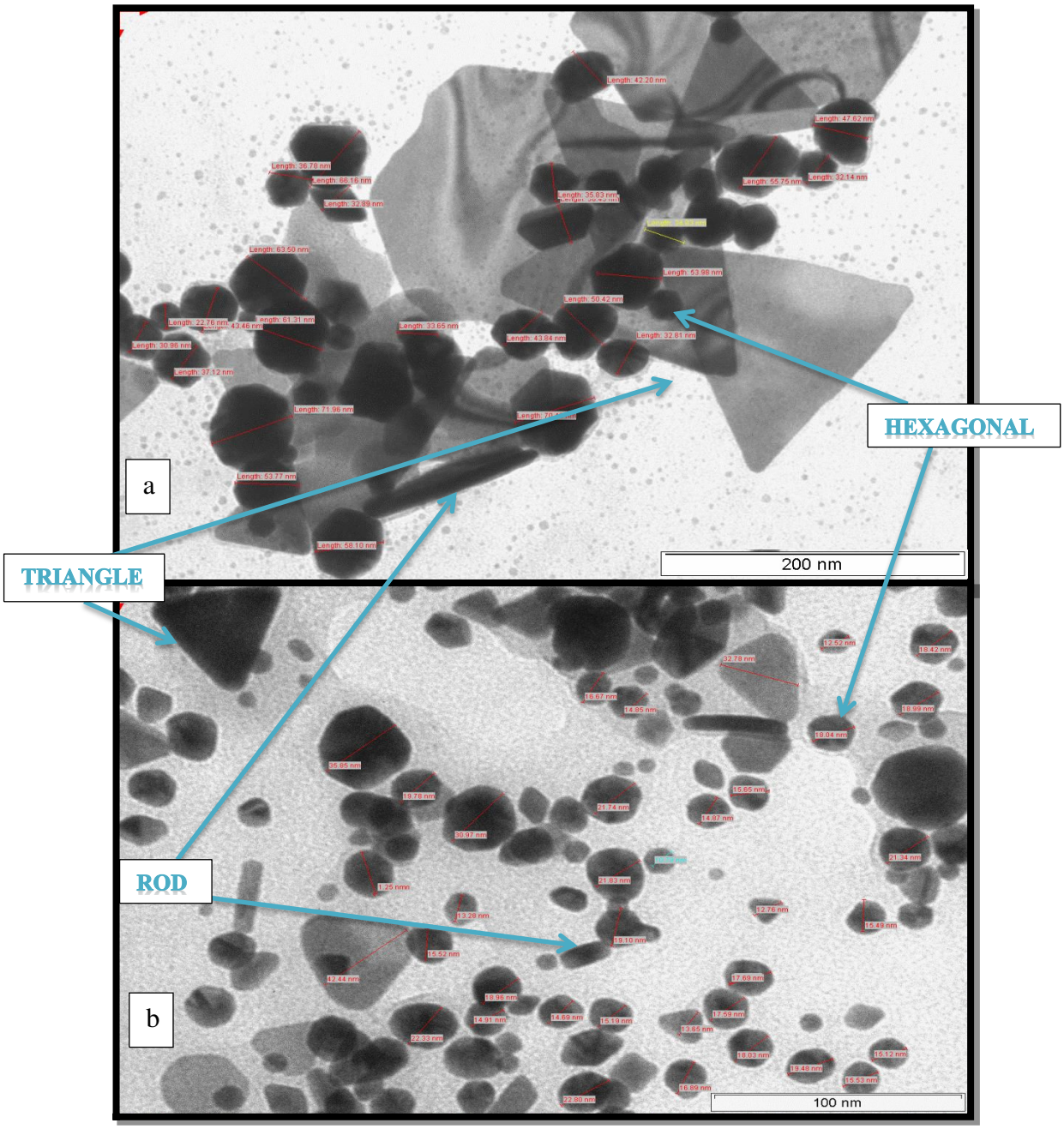

Figure 34: TEM images showing the range of sizes and shapes of the Au NPs synthesized from (a) aqueous and (b) methanolic *C. triloba* leaf extracts

4.4.3 Fourier-Transform Infrared (FTIR) Analysis

FTIR measurements were carried out to identify the possible biomolecules responsible for capping and stabilization of the metal NPs synthesized by each of the plants. It was also used to identify if there was any link between biomolecules between NPs formed from aqueous and methanolic extracts in terms of the Ag and Au NPs.

Figure 35 (a) and (b) shows the spectra of aqueous and methanolic extract of *A. dubius* formed Ag NPs. The aqueous extract formed Ag NPs sample shows intense peaks at 3302 cm^{-1} and 1635 cm^{-1}. The methanolic Ag NP sample shows intense peaks at a very similar range of 3298 cm^{-1} and 1635 cm^{-1}.

The Au NPs synthesized by aqueous and methanolic leaf extracts of *A. dubius* presented in Figure 35 (c) and (d) show intense peaks in the same range as the Ag NPs. The intense broad band at 3302, 3298, 3288 and 3299 cm^{-1} is due to N-H and O-H stretching mode in the linkage of proteins. The common peak of medium intensity at 1635 cm^{-1} in all four samples is attributed to the C=O stretching mode in amine I group which is commonly found in protein released from the leaves of the plant (Huang *et al.,* 2007), indicating the presence of proteins as capping agents for Ag NPs.

Figure 36 (a) and (b) represents Ag NPs from aqueous and methanolic extracts of *G. perpensa* and these show intense peaks at 3289, 1635 cm^{-1} and 3289, 1634 cm^{-1} respectively. This is similar to the peaks formed in *A. dubius*. The methanolic extract of Ag NPs also show other low intensity peaks at 2973, 1473, 1382, 1252, 1152, 1072 and 953 cm^{-1} compared to the aqueous Ag NPs. The band at 1382 cm^{-1} corresponds to C-N stretching vibrations of aromatic amine (Philip, 2009).

Figure 36 (c) and (d) shows the Au NPs and it can be seen that they are in close relation to the Ag NPs spectra. However, these have a higher intensity of those low intensity peaks formed with the Ag NPs. The bands at 1473, 1382, 1152 cm^{-1} are known to be associated with the stretching vibrations for C-C (aromatic), C-O-C (ethers) and C-O (-C-OH). The 1152 cm^{-1} band arises most probably from the C-O of aromatic-OH group normally in hydroxyflavones. The bands at 1388 cm^{-1} are representative of germinal methyls and 1078

cm^{-1} of ether linkages. The bands at 2973 and 2981 cm^{-1} show a C-H vibration suggesting the presence of flavonoids. The 1252 cm^{-1} band is most likely due to a simple hydroxy compound (Tamuly *et al.,* 2012).

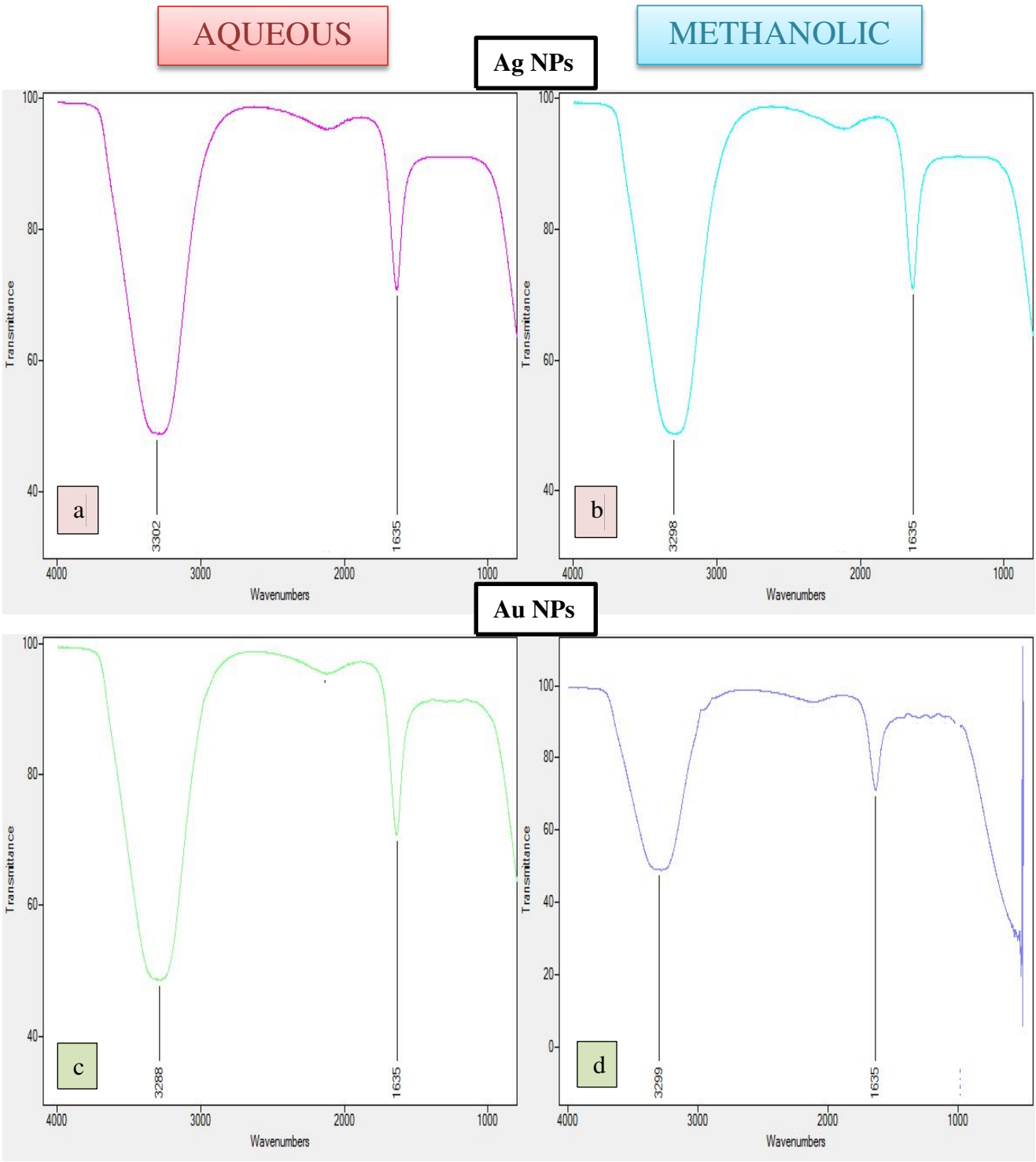

Figure 35: FTIR spectra of Ag NPs using (a) aqueous and (b) methanolic leaf extracts and Au NPs using (c) aqueous and (d) methanolic leaf extracts of *A. dubius*

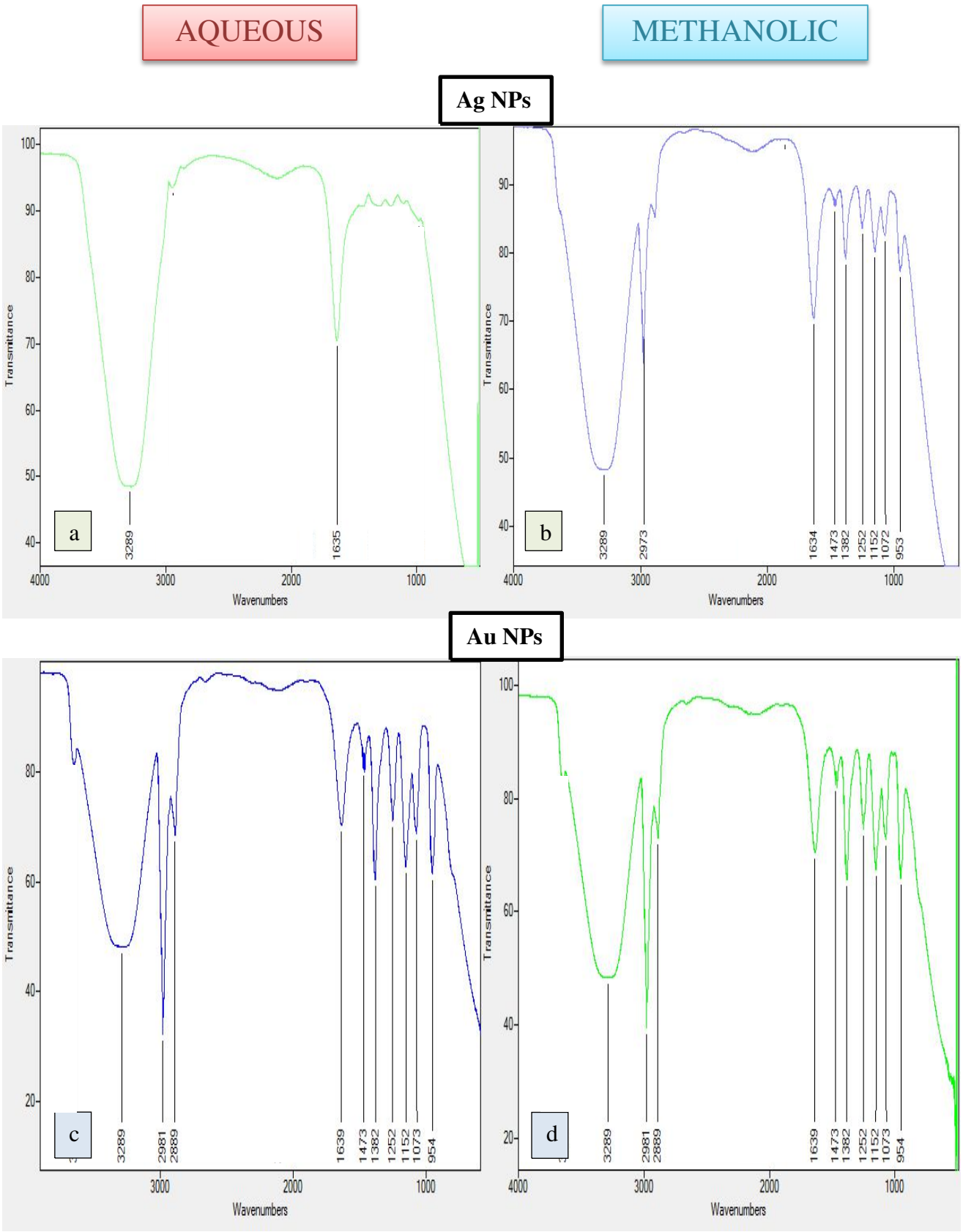

Figure 36: FTIR spectra of Ag NPs using (a) aqueous and (b) methanolic leaf extracts and Au NPs using (c) aqueous and (d) methanolic leaf extracts of *G. perpensa*

Figure 37 (a) and (b) shows the spectra of the Ag NPs produced from the aqueous and methanolic leaf extracts of *C. triloba*. The Ag NP samples as in the previous two plants show intense peaks at ~3290 cm^{-1} and 1636 cm^{-1}. The Ag NPs from the methanolic extract has one more low intensity peak at 2981 cm^{-1} as is the Ag NPs from the methanolic extract of *G. perpensa*. The aqueous Au NPs follow the same trend as in the Ag NPs but the methanolic Au NPs show slightly stronger intensities at 1382, 1251, 1153 and 1072 cm^{-1}, as those found in *G. perpensa* Au NPs as visible in Figure 37 (c) and (d).

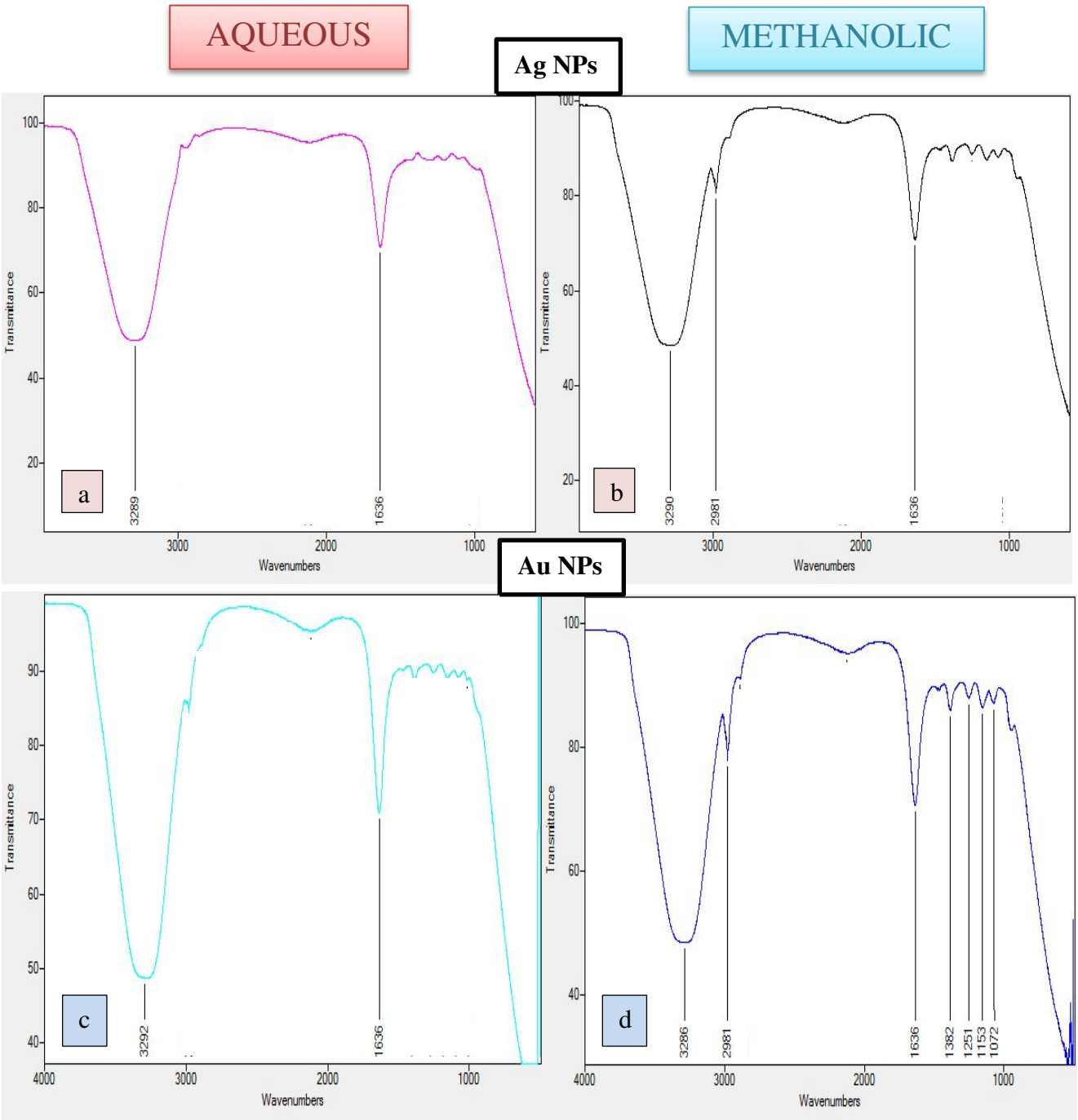

Figure 37: FTIR spectra of Ag NPs using (a) aqueous and (b) methanolic leaf extracts and Au NPs using (c) aqueous and (d) methanolic leaf extracts of *C. triloba*

4.5 Stability of the Synthesized Nanoparticles

Aggregation, precipitation and sedimentation are likely to change the shape and size of NPs and it might interfere with the suitable properties of nanostructured materials (Korbekandi *et al.,* 2009). It has been reported that NPs synthesized using plant extracts are surrounded by a thin layer of some capping organic material from the plant leaf broth and are thus stable in solution for up to 4 weeks. Stability of NPs was ascertained by observing deviation of the optical properties of the NPs solutions with time. This was determined by monitoring the UV-vis scans due to the surface plasmon resonance (SPR) and peak formed by the respective NPs. The peaks would show a decrease in absorbance and a noticeable alteration in symmetry indicative of instability (Shankar *et al.,* 2004; Korbekandi *et al.,* 2009).

4.5.1 *A. dubius* Silver Nanoparticles

Figure 38 shows that the Ag NPs synthesized from the aqueous leaf extracts of *A. dubius* were stable over 30 days. The wavelength did not move out of the range of 400 nm – 425 nm. The absorbance did increase slightly but the peak had become broader by the end of the month as seen in Figure 39, possibly indicating aggregation. Ag NPs synthesized from the methanolic extracts of *A. dubius* (Figures 40 and 41) show that these were also stable over 30 days.

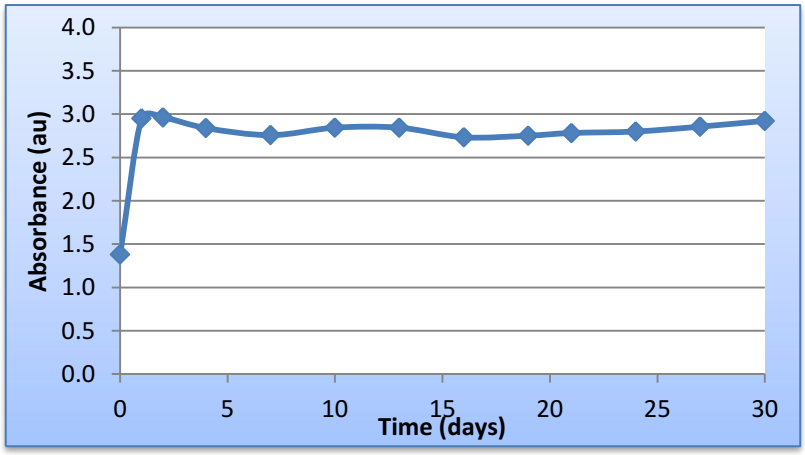

Figure 38: Stability assay of Ag NPs produced from aqueous leaf extracts of *A. dubius* according to the absorbance over 30 days

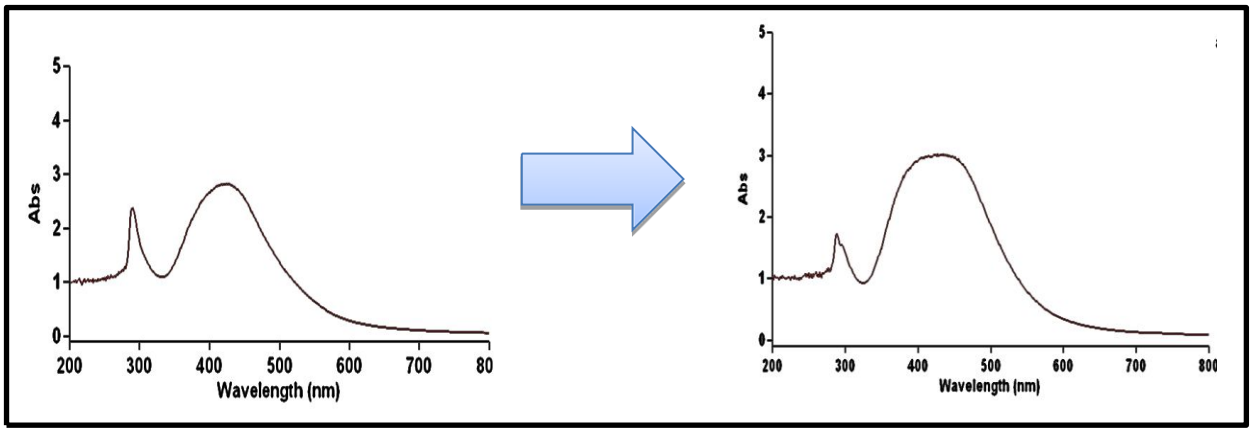

Figure 39: UV-vis spectra showing the difference in the shape of the peak of aqueous *A. dubius* Ag NPs at 30 days

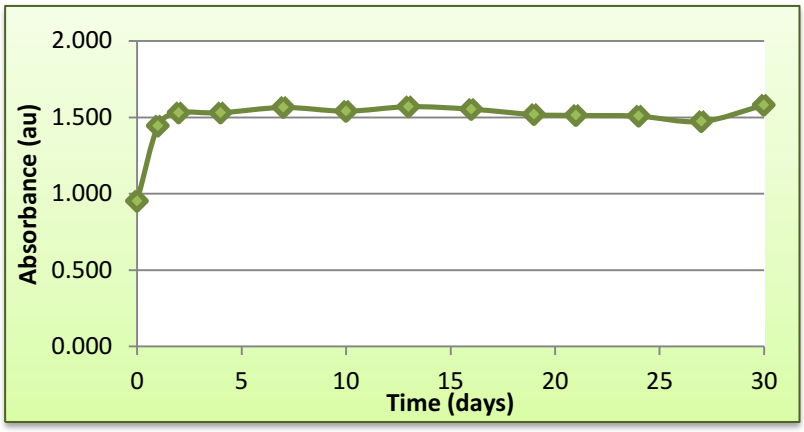

Figure 40: Stability assay of Ag NPs produced from methanolic leaf extracts of *A. dubius* according to the absorbance over 30 days

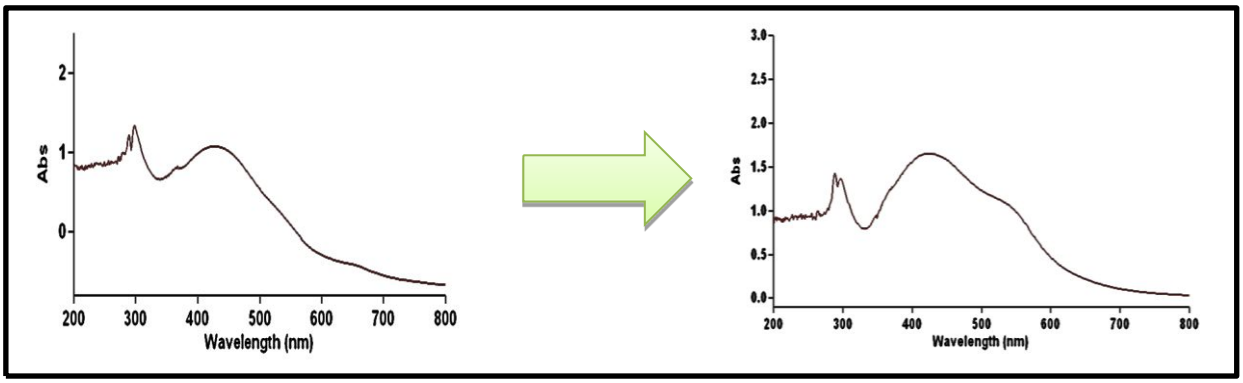

Figure 41: UV-vis spectra showing the difference in the shape of the peak of methanolic *A. dubius* Ag NPs at 30 days

4.5.2 *A. dubius* Gold Nanoparticles

Au NPs synthesized from the aqueous leaf extracts of *A. dubius* were stable for 21 days (Figure 42). The absorbance values were seen to decrease and by day 24, the wavelength moved out of the desired range of 520 nm - 550 nm and there was no longer a visible sharp peak as shown in Figure 43.

Au NPs synthesized from the methanolic leaf extracts of *A. dubius* were stable over 30 days (Figure 44). The wavelength was within the range of 520 nm – 550 nm and the peak remained symmetrical (Figure 45).

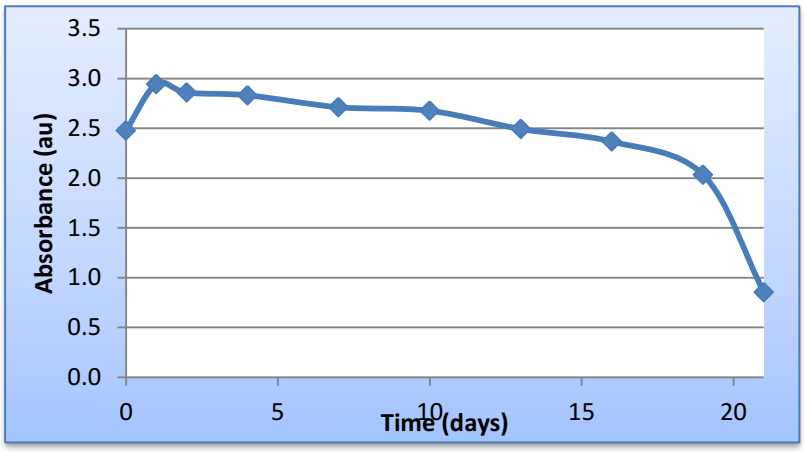

Figure 42: Stability assay of Au NPs produced from aqueous leaf extracts of *A. dubius* according to their absorbance over 21 days

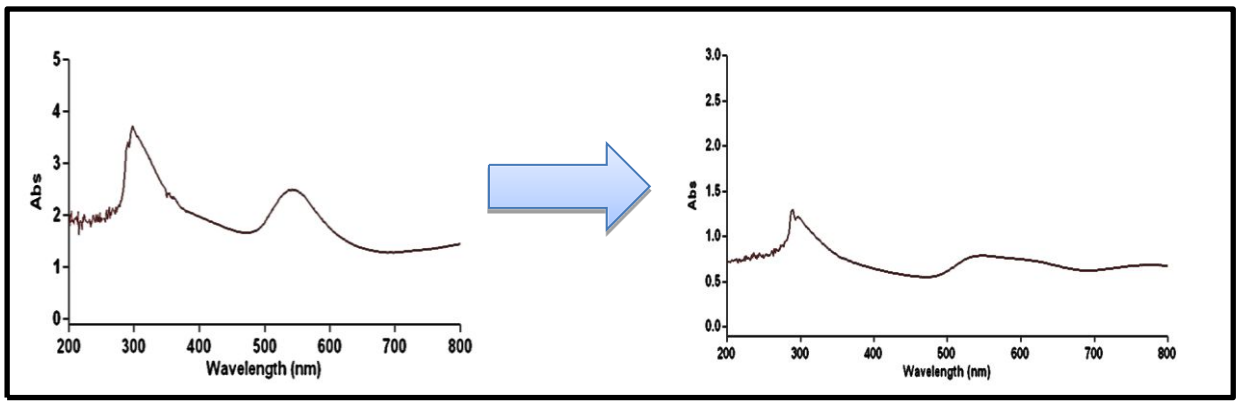

Figure 43: UV-vis spectra showing the difference in the shape of the peak of aqueous *A. dubius* Au NPs after 21 days

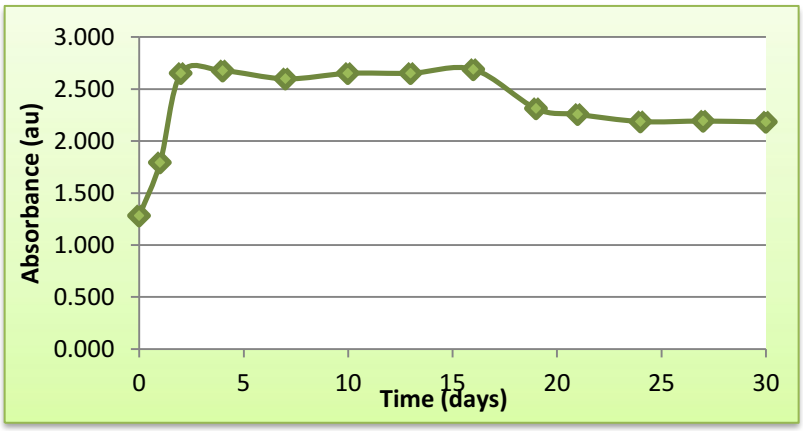

Figure 44: Stability assay of Au NPs produced from methanolic leaf extracts of *A. dubius* according to the absorbance over 30 days

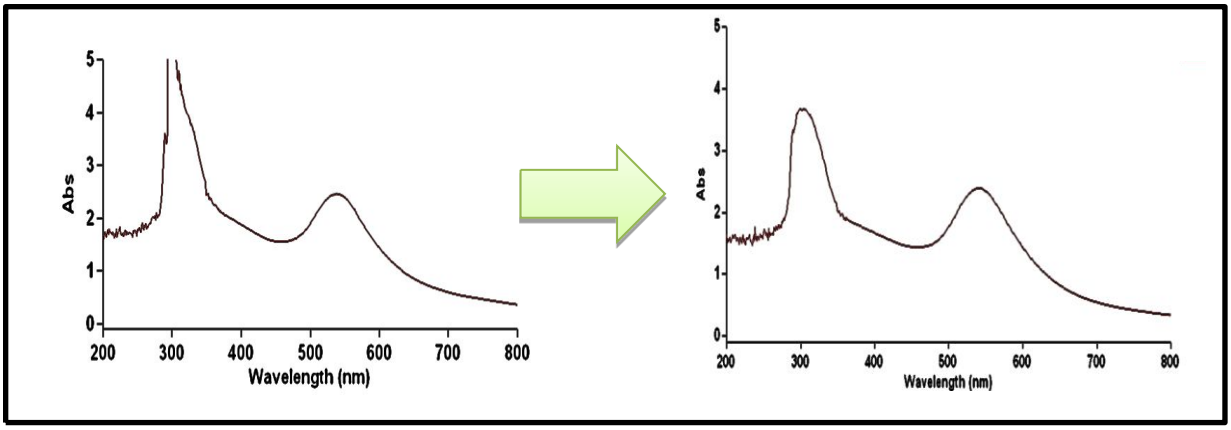

Figure 45: UV-vis spectra showing the difference in the shape of the peak of methanolic *A. dubius* Au NPs at 30 days

4.5.3 *G. perpensa* Silver Nanoparticles

Ag NPs synthesized from the aqueous leaf extracts of *G. perpensa* were only stable for two days (Figure 46). The absorbance slowly decreased and by day 4, the wavelength moved out of the desired range and there was no longer a visible peak as shown in Figure 47.

Ag NPs synthesized from the methanolic extracts of *G. perpensa* were stable over 10 days (Figure 48). By day 13, the wavelength moved out of the range and the absorbance increased with the peak produced much broader than at the beginning as shown in Figure 49.

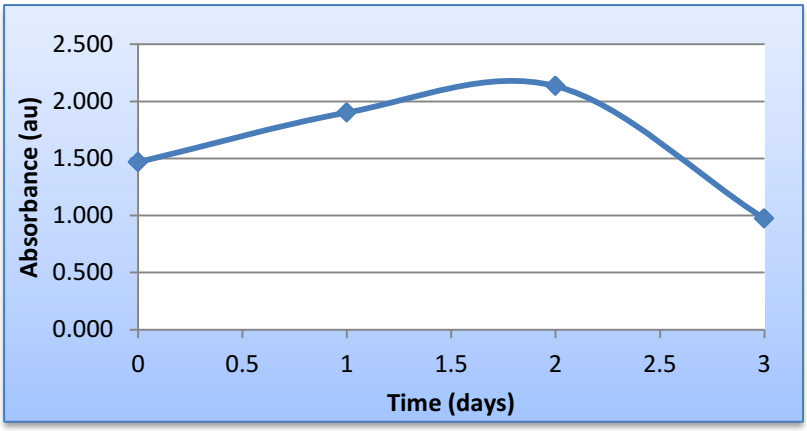

Figure 46: Stability assay of Ag NPs produced from aqueous leaf extracts of *G. perpensa* according to their absorbance over 2 days

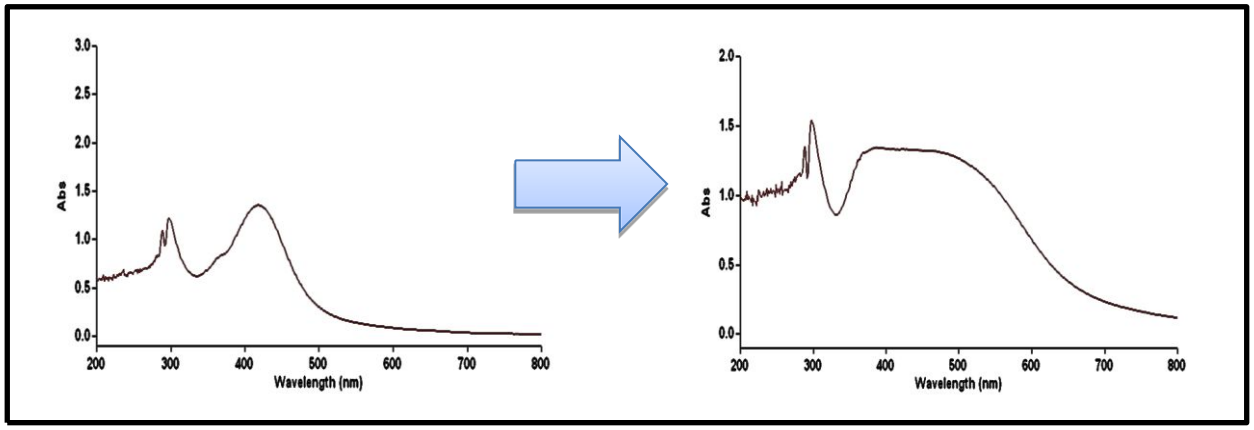

Figure 47: UV-vis spectra showing the difference in the shape of the peak of aqueous *G. perpensa* Ag NPs after 2 days

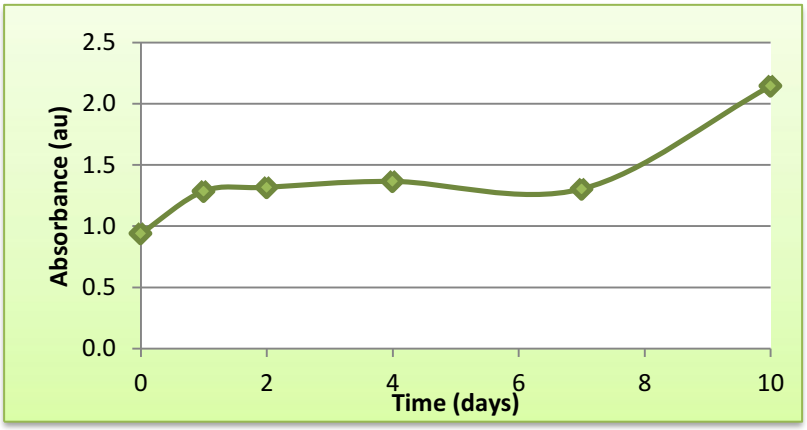

Figure 48: Stability assay of Ag NPs produced from methanolic leaf extracts of *G. perpensa* according to their absorbance over 10 days

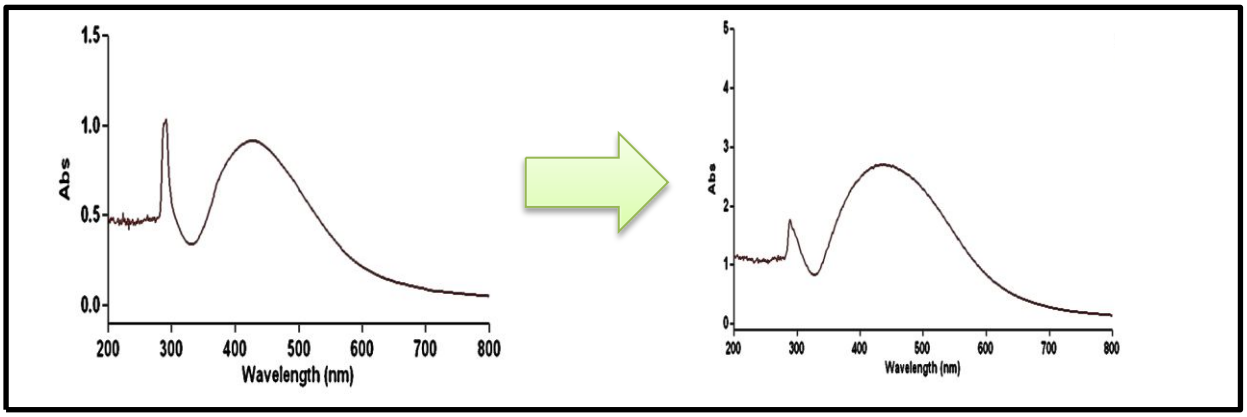

Figure 49: UV-vis spectra showing the difference in the shape of the peak of methanolic *G. perpensa* Ag NPs after 10 days

4.5.4 *G. perpensa* Gold Nanoparticles

Au NPs synthesized from the aqueous extracts of *G. perpensa* were stable over 30 days (Figure 50). The wavelength did not move out of the range of 530 nm- 550 nm. The peaks and absorbance did not change significantly as shown in Figure 51.

Au NPs synthesized from the methanolic extracts of *G. perpensa* were stable over 30 days (Figure 52). The wavelength was within the required range as well and the peak was stable over 30 day period (Figure 53).

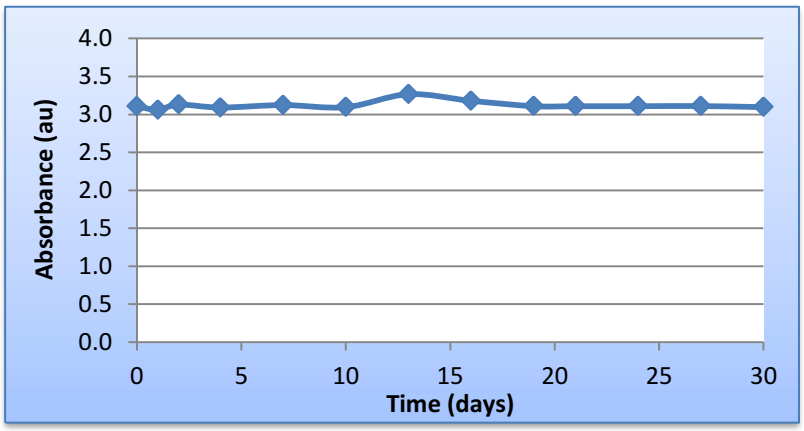

Figure 50: Stability assay of Au NPs produced from aqueous leaf extracts of *G. perpensa* according to the absorbance over 30 days

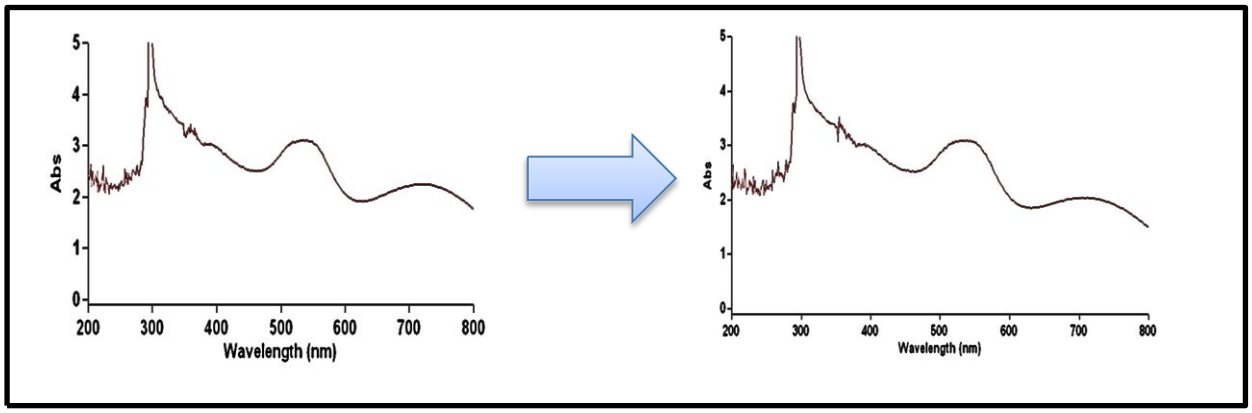

Figure 51: UV-vis spectra showing the difference in the shape of the peak of aqueous *G. perpensa* Au NPs at 30 days

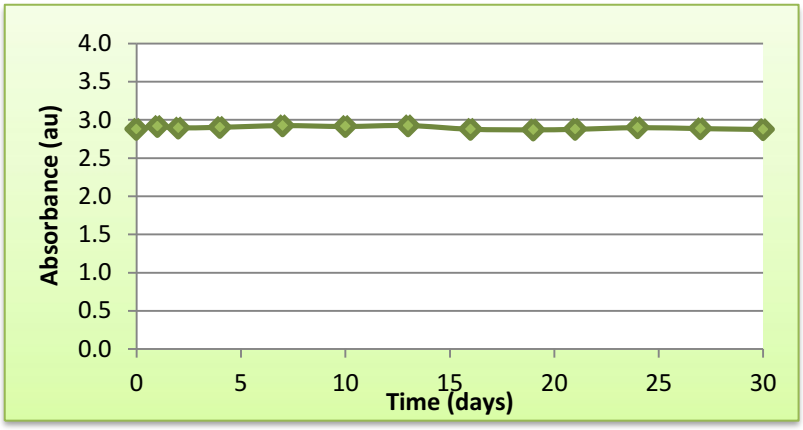

Figure 52: Stability assay of Au NPs produced from methanolic leaf extracts of *G. perpensa* according to the absorbance over 30 days

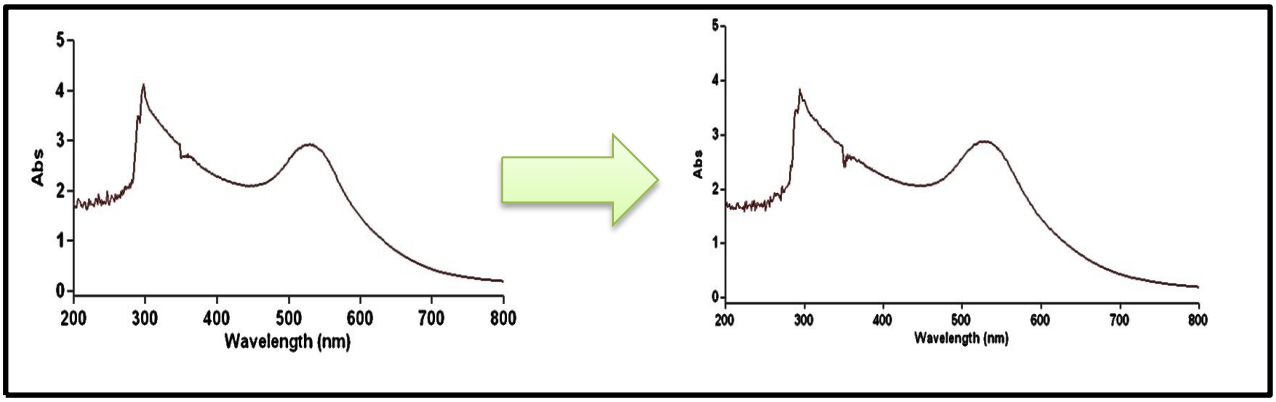

Figure 53: UV-vis spectra showing the difference in the shape of the peak of methanolic *G. perpensa* Au NPs after 30 days

4.5.5 *C. triloba* Silver Nanoparticles

Ag NPs synthesized from the aqueous leaf extracts of *C. triloba* were stable over 7 days (Figure 54). The absorbance had decreased and the wavelength moved out of the desired range of 400 nm to 425 nm after day 7. The resulting peak is also broader than the peak initially as shown in Figure 55.

Ag NPs synthesized from methanolic leaf extracts of *C. triloba* are stable over 30 days (Figure 56). The wavelength remained in the required range. There was a slight elevation in the absorbance of the peak at 30 days but not much difference in the peak structure as visible in Figure 57.

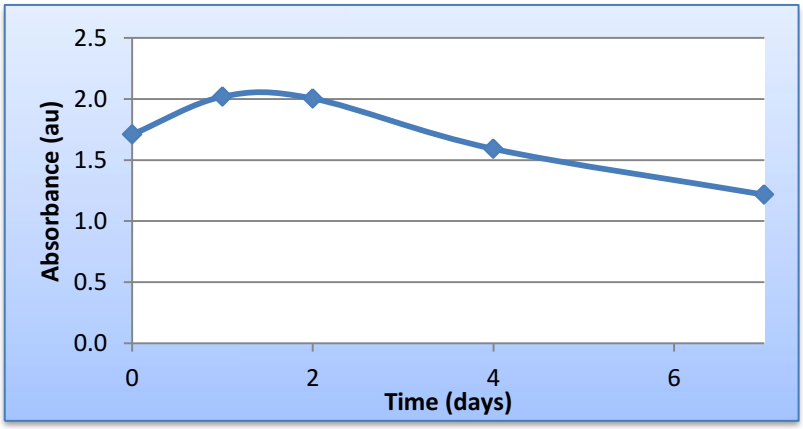

Figure 54: Stability assay of Ag NPs produced from aqueous leaf extracts of *C. triloba* according to the absorbance over 7 days

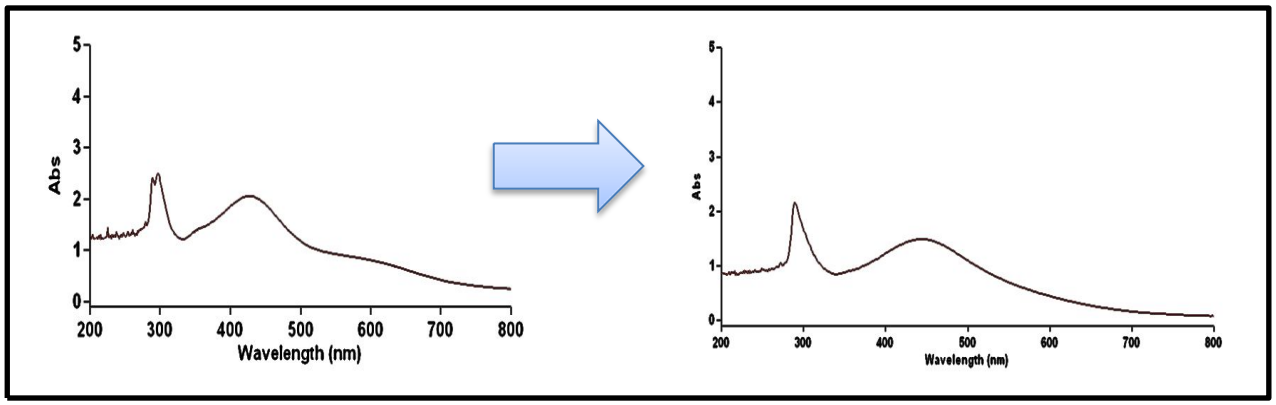

Figure 55: UV-vis spectra showing the difference in the shape of the peak of aqueous *C. triloba* Ag NPs after 7 days

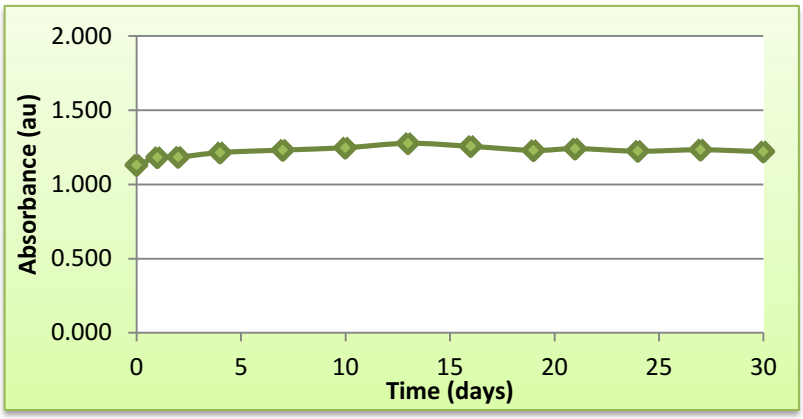

Figure 56: Stability assay of Ag NPs produced from methanolic leaf extracts of *C. triloba* according to the absorbance over 30 days

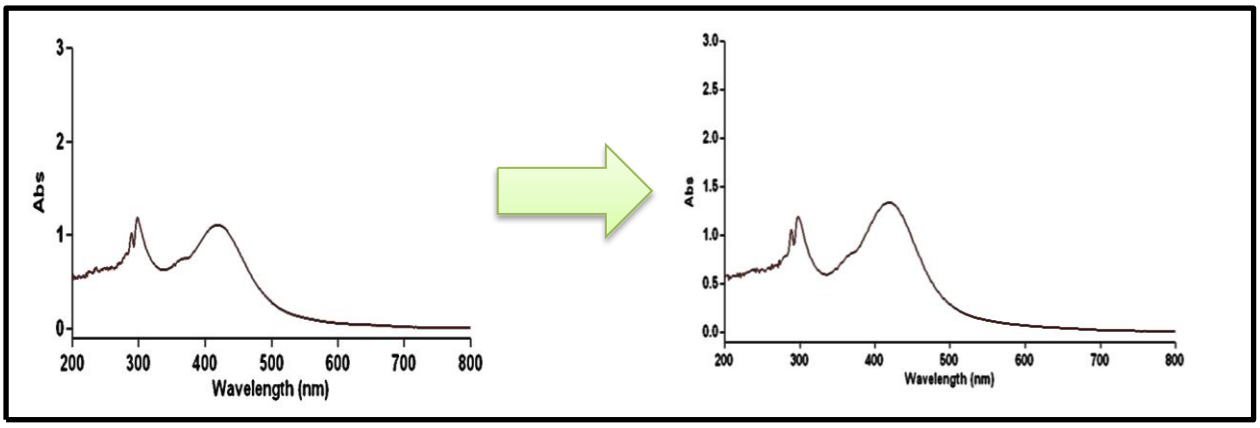

Figure 57: UV-vis spectra showing the difference in the shape of the peak of methanolic *C. triloba* Ag NPs after 30 days

4.5.6 *C. triloba* Gold Nanoparticles

Au NPs synthesized from aqueous leaf extracts of *C. triloba* were stable for 21 days (Figure 58). The absorbance gradually decreased and by day 24, the wavelength moved out of the desired range and there was no longer a sharp peak as shown in Figure 59.

Au NPs synthesized from methanolic leaf extracts of *C. triloba* were stable over 30 days (Figure 60). The wavelengths remained in the required range. There was an elevation in the absorbance of the peak at 30 days and the peak appeared sharper as can be seen in Figure 61.

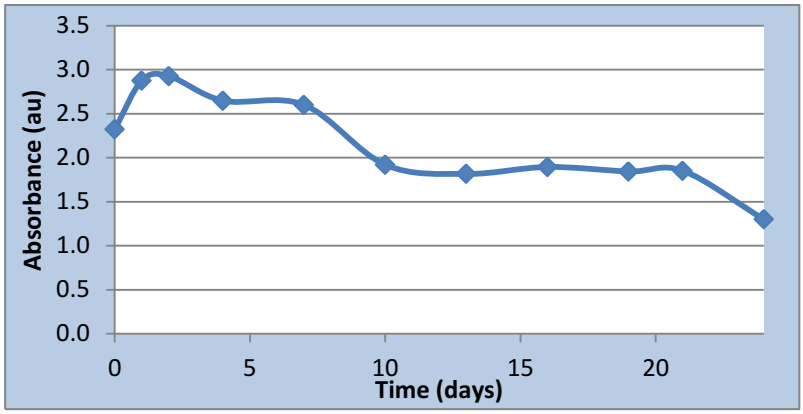

Figure 58: Stability assay of Au NPs produced from aqueous leaf extracts of *C. triloba* according to the absorbance over 24 days

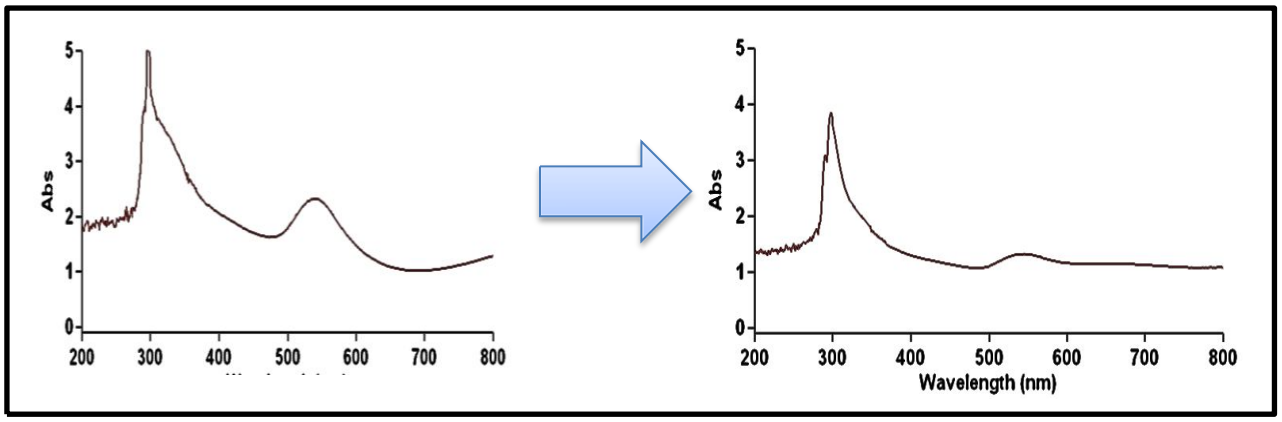

Figure 59: UV-vis spectra showing the difference in the shape of the peak of aqueous *C. triloba* Au NPs after 24 days

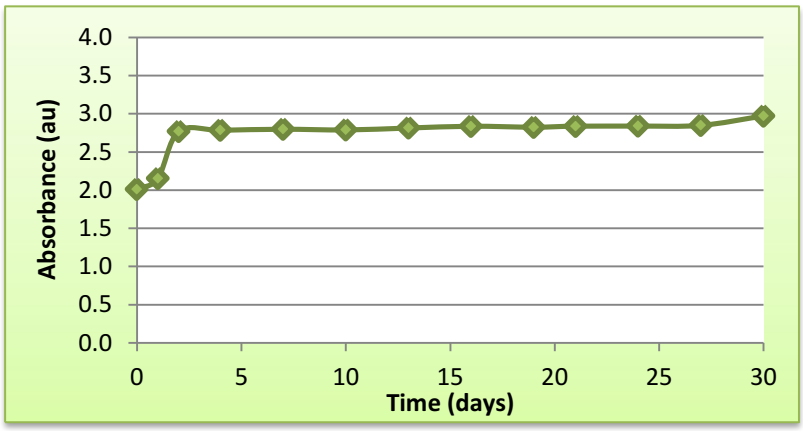

Figure 60: Stability assay of Au NPs produced from methanolic leaf extracts of *C. triloba* according to the absorbance over 30 days

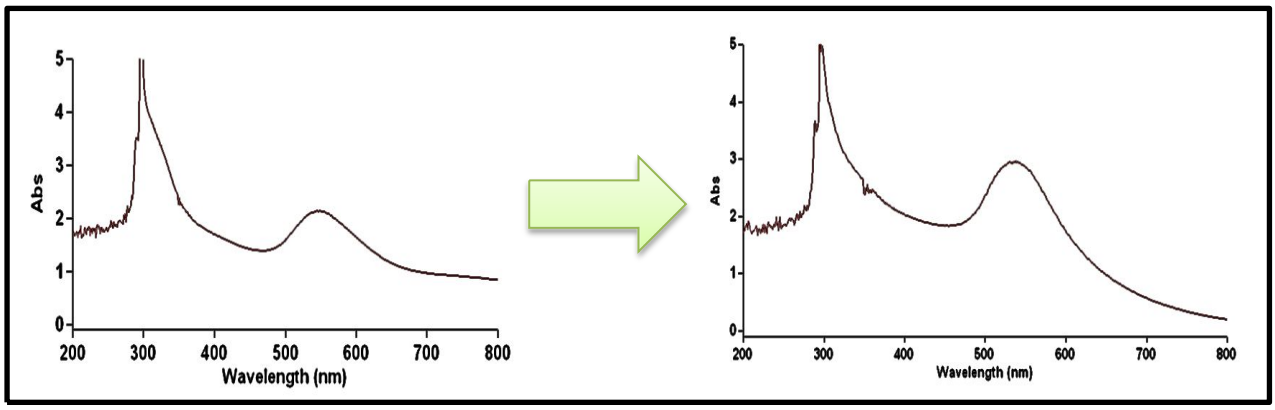

Figure 61: UV-vis spectra showing the difference in the shape of the peak of methanolic *C. triloba* Au NPs after 30 days

4.6 Phytochemical Screening of Plant Extracts

Major phytochemical classes were tested to establish a connection between the compounds present in the leaves and callus cultures of the plants, and their reducing ability of metal salts to form NPs. It is known that phenolic compounds are responsible for the reduction in biosynthesis (Huang *et al.,* 2007). The separation techniques used are based on the polarity of the phytochemicals. The results of the phytochemical screening found in the leaf and callus extracts of the plants are summarized in Table 10.

The leaf extracts of *A. dubius, G. perpensa, C. triloba* and *C. roseus* formed a blue-green interface between the layers which showed a positive result for steroids; formed a heavy emulsion owing to presence of saponins and produced a yellow colour change to indicate the presence of flavonoids.

The leaf extracts of *A. dubius, G. perpensa* and *C. triloba* produced a brownish-black colouration indicating the presence of tannins while *C. roseus* had no reaction.

The leaf extracts of *A. dubius* and *C. triloba* formed a red precipitate giving a positive result for the presence of alkaloids whereas *G. perpensa* and *C. roseus* had no precipitate.

The callus extracts of *A. dubius* and *C. triloba* were seen to have positive results for the presence of saponins and flavonoids.

Table 10: Phytochemical compounds present in leaf extracts of *A. dubius, G. perpensa, C. triloba* and *C. roseus* and callus extracts of *A. dubius* and *C. triloba*

PHYTOCHEMICAL TESTS	*A. dubius*	*G. perpensa*	*C. triloba*	*C. roseus*	*A. dubius* callus	*C. triloba* callus
Terpenoids	X	X	X	X	X	X
Steroids	√	√	√	√	X	X
Saponins	√	√	√	√	√	√
Alkaloids	√	X	√	X	X	X
Flavonoids	√	√	√	√	√	√
Tannins	√	√	√	X	X	X
Phlobatannins	X	X	X	X	X	X

(X = absent, √ = present)

4.7 Antibacterial Activity of Silver and Gold Nanoparticles

4.7.1 Disk Diffusion Antibacterial Assay

Table 11 provides a comprehensive summary of the average results of the disk diffusion zones with their respective standard deviations. The negative control DMSO gave a negative result with no inhibition against all the bacteria. Ciprofloxacin showed antibacterial activity with three species i.e. *E. faecalis* U11951, *K. pneumoniae* P3811 and *S. aureus* S6158 and had no activity on the rest of the bacteria. The 1 mM $AgNO_3$ solution exhibited activity against all the bacteria. The highest zone of inhibition against *K. pneumoniae* P3811 was by ciprofloxacin (8.2 ± 1 mm) in comparison to $AgNO_3$ and the aqueous and methanolic Ag NPs which showed smaller zones of inhibition except for Ag NPs from aqueous *A. dubius* extracts which showed no inhibition. For majority of the bacteria, the $AgNO_3$ zones were smaller than the zones produced by the Ag NPs. All the Au NP discs showed no antibacterial activity against any of the bacteria and therefore not included in the table or in the MIC assay.

Table 11: Antibacterial activity of Ag NPs from the aqueous and methanolic leaf extracts of *A. dubius*, *G. perpensa* and *C. triloba*

Bacteria	Zone of Inhibition (mm)								
	A. dubius		*G. perpensa*		*C. triloba*		Controls		
	aqueous	methanolic	aqueous	methanolic	aqueous	methanolic	DMSO	$AgNO_3$	Ciprofloxacin
1. Enterococcus V11967	7.8 ± 0.3	8.7 ± 0.3	7.7 ± 0.6	8.5 ± 0.5	7.3 ± 0.3	8 ± 0	0	6.7 ± 0.3	0
2. Pseudomonas S6125	7.8 ± 0.3	7.8 ± 0.3	8.8 ± 0.3	8.7 ± 0.3	7 ± 0.5	8.2 ± 0.3	0	8.5 ± 0.5	0
3. Enterobacter spp. P4177	0	6.5 ± 0.5	7.3 ± 0.3	6.8 ± 0.3	6.5 ± 0	7 ± 0	0	6.7 ± 0.3	0
4. *E. faecalis* U11951	6.7 ± 0.3	7.8 ± 0.3	8.2 ± 0.6	8.7 ± 0.3	7.5 ± 0	8.7 ± 0.3	0	8 ± 0.5	6.7 ± 0.3
5. *E. faecalis* U11394	7.7 ± 0.3	8.2 ± 0.3	7.5 ± 0.5	8.5 ± 0.5	8 ± 0	9 ± 0	0	8 ± 0	0
6. *E. coli* B3578	6.5 ± 0	7.7 ± 0.3	8.7 ± 0.3	8.5 ± 0.5	6.8 ± 0.3	7.7 ± 0.6	0	8 ± 0	0
7. *E. coli* U10948	6.2 ± 0.3	6.5 ± 0.5	8.0 ± 0.5	8.7 ± 0.6	6.8 ± 0.3	7.8 ± 0.3	0	7.5 ± 0.5	0
8. *E. coli* P4055	6 ± 0	6.3 ± 0.3	9.0 ± 0.5	8.3 ± 0.6	6.8 ± 0.3	7.8 ± 0.8	0	7 ± 0	0
9. *K. pneumoniae* P3811	0	6 ± 0	7.8 ± 0.3	8 ± 0	7 ± 0.5	7.7 ± 0.3	0	7.5 ± 0.5	8.2 ± 1.0
10. *K. pneumoniae* V258	6 ± 0	6.7 ± 0.3	8.0 ± 0	7.2 ± 0.3	6.5 ± 0	7.5 ± 0.5	0	8.2 ± 0.3	0
11. *K. pneumoniae* U10705	6.3 ± 0.3	6.7 ± 0.3	8.5 ± 0.5	7.8 ± 0.3	6.8 ± 0.3	7.3 ± 0.6	0	7.2 ± 0.3	0
12. *S. aureus* S6158	6.2 ± 0.3	6.8 ± 0.3	7.8 ± 0.3	7.3 ± 0.3	6.7 ± 0.3	6.8 ± 0.3	0	7.5 ± 0	7.7 ± 1.0
13. *S. aureus* P4215	7.8 ± 0.3	8.7 ± 0.3	8.8 ± 0.3	8.5 ± 0.5	8.3 ± 0.3	9.2 ± 0.3	0	8.7 ± 0.3	0
14. *S. aureus* S5878	6 ± 0	6 ± 0	7.0 ± 0.9	7.5 ± 0.5	6 ± 0	7.2 ± 0.3	0	7.3 ± 0.3	0

N = 3

- Highest zones of inhibition

A. dubius shows the least promising results with the lowest zones of inhibition found in both the aqueous and methanolic Ag NPs for Gram-negative *E. coli* U10948, *E. coli* P4055, *K. pneumoniae* P3811, *K. pneumoniae* V258 and Gram-positive *S. aureus* S5878. Aqueous Ag NPs showed no activity for Gram-negative *Enterobacter* spp. P4177 and *K. pneumoniae* P3811. The best activity for the aqueous Ag NPs (7.8 ± 0.3 mm) was seen against gram positive *Enterococcus* V11967, *S. aureus* P4215 and Gram-negative *Pseudomonas* S6125. The best activity for the methanolic Ag NPs (8.7 ± 0.3 mm) was seen against the former two Gram-positive microorganisms as well.

G. perpensa aqueous Ag NPs had its highest activity against *E. coli* P4055 with a zone of inhibition of 9.0 ± 0.5 mm. Its lowest activity was against *S. aureus* S5878 with a zone of 7 ± 0.9 mm. The methanolic Ag NPs showed its highest activity of ~8.7 mm against *Pseudomonas* S6125, *E. coli* U10948 and Gram-positive *E. faecalis* U11951. These NPs however, show good activity against all strains of *E. coli*. The lowest activity (6.8 ± 0.3 mm) is seen against *Enterobacter* spp. P4177.

C. triloba aqueous Ag NPs showed moderate antibacterial activity with its highest zone of inhibition of 8.3 ± 0.3 mm against *S. aureus* P4215 while the methanolic Ag NPs showed the highest of all activity (9.2 ± 0.3 mm) against this organism as well (Figure 62). This result is similar to that of *A. dubius* Ag NPs. The aqueous Ag NPs produced their lowest activity with zones of 6.5 mm against *Enterobacter* spp. P4177 (as was seen with *A. dubius* aqueous Ag NPs and *G. perpensa* methanolic Ag NPs) and *K. pneumonia*e V258. It was also noticed that these methanolic Ag NPs had moderate activity against all strains of Gram-negative *E. coli* and *K. pneumoniae*.

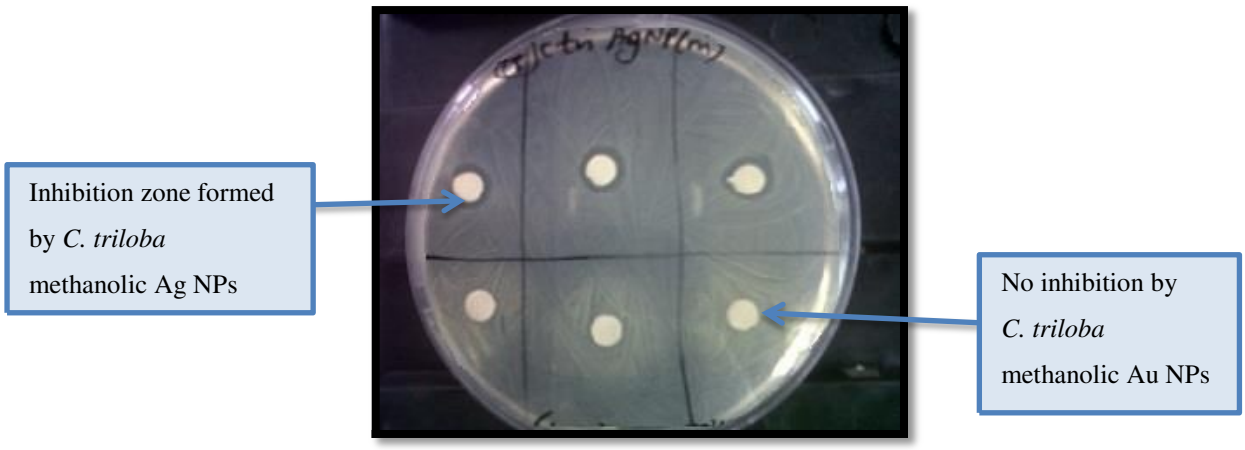

Figure 62: Disk diffusion of *C. triloba* methanolic Ag NPs against *S. aureus* P4215

4.7.2 Minimum Inhibitory Concentration Assay

The results of the MIC assay for the aqueous and methanolic Ag NPs of each plant against each of the bacteria can be seen in Table 12. DMSO which served as the negative control and the sterility control wells produced clear solutions when the indicator was added.

Ag NPs produced from the methanolic extract of *A. dubius* had one of the highest inhibitory effects on *Enterococcus* V11967 at a concentration of 3.2 µg/ml. Most of the bacteria were inhibited by aqueous and methanolic extract Ag NP concentrations at 12.5 µg/ml. The gram negative strains of *E. coli* B3578, *E. coli* U10948, *K. pneumoniae* P3811 and *K. pneumoniae* U10705 were inhibited at a slightly lower concentration of Ag NPs (6.3 µg/ml). The Ag NPs from the methanolic extract had the same MIC as the aqueous extract for the *E. coli* strains. There was no inhibitory effect seen on *Enterobacter* spp. P4177 but the Ag NPs from the methanolic extract had an MIC of 50 µg/ml.

G. perpensa aqueous and methanolic Ag NPs show the best MIC values in this assay. The Ag NPs formed from the aqueous extract had an MIC of 3.2 µg/ml for four of the bacteria: *E. coli* P4055, *K. pneumoniae* P3811, *K. pneumoniae* U10705 and *S. aureus* S5878. The Ag NPs from the methanolic extract also had a high MIC of 3.2 µg/ml against three bacteria namely: *Enterococcus* V11967, *E. faecalis* U11394 and *K. pneumoniae* U10705. Ag NPs from aqueous and methanolic extracts had MICs of 6.3 µg/ml against *Pseudomonas* S6125, *E. coli* B3578, *E. coli* U10948, *K. pneumoniae* V258 and *S. aureus* P4215. The lowest MIC for Ag NPs from aqueous and methanolic extracts of *G. perpensa* of 25 µg/ml was against *Enterobacter* spp. P4177.

C. triloba aqueous extract Ag NPs had its highest MIC of 6.3 µg/ml against *E. coli* P4055 and *K. pneumoniae* U10705. The NPs had an MIC of 12.5 µg/ml for the rest of the bacterial strains except *E. coli* B3578 where its MIC value is 25 µg/ml. The Ag NPs from the methanolic extract prove to be less potent in that it has an MIC of 25 µg/ml all the bacteria except *E. faecalis* U11951, *K. pneumoniae* V258 and *K. pneumoniae* U10705 for which it has its highest MIC of 12.5 µg/ml and against *Enterobacter* spp. P4177 where it has its lowest MIC of 50 µg/ml.

Table 12: Minimum Inhibitory Concentration results of Ag NPs synthesized from aqueous and methanolic leaf extracts of *A. dubius*, *G. perpensa* and *C. triloba*

Bacteria	Plant	MIC (µg/ml)	
		Aqueous Ag NPs	Methanolic Ag NPs
1. Enterococcus V11967	***A. dubius***	12.5	3.2
	G. perpensa	12.5	3.2
	C. triloba	12.5	25
2. Pseudomonas S6125	***A. dubius***	12.5	12.5
	G. perpensa	6.3	6.3
	C. triloba	12.5	25
3. Enterobacter spp. P4177	***A. dubius***	-	50
	G. perpensa	25	25
	C. triloba	12.5	50
4. *E. faecalis* U11951	***A. dubius***	12.5	12.5
	G. perpensa	6.3	12.5
	C. triloba	12.5	12.5
5. *E. faecalis* U11394	***A. dubius***	12.5	12.5
	G. perpensa	6.3	3.2
	C. triloba	12.5	25
6. *E. coli* B3578	***A. dubius***	6.3	6.3
	G. perpensa	6.3	6.3
	C. triloba	25	25
7. *E. coli* U10948	***A. dubius***	6.3	6.3
	G. perpensa	6.3	6.3
	C. triloba	12.5	25
8. *E. coli* P4055	***A. dubius***	12.5	12.5
	G. perpensa	3.2	6.3
	C. triloba	6.3	25
9. *K. pneumoniae* P3811	***A. dubius***	6.3	12.5
	G. perpensa	3.2	6.3
	C. triloba	12.5	25
10. *K. pneumoniae* V258	***A. dubius***	12.5	12.5
	G. perpensa	6.3	6.3
	C. triloba	12.5	12.5
11. *K. pneumoniae* U10705	***A. dubius***	6.3	12.5
	G. perpensa	3.2	3.2
	C. triloba	6.3	12.5
12. *S. aureus* S6158	***A. dubius***	12.5	12.5
	G. perpensa	12.5	6.3
	C. triloba	12.5	25
13. *S. aureus* P4215	***A. dubius***	12.5	12.5
	G. perpensa	6.3	6.3
	C. triloba	12.5	25
14. *S. aureus* S5878	***A. dubius***	12.5	12.5
	G. perpensa	3.2	6.3
	C. triloba	12.5	25

5. DISCUSSION

5.1 Biosynthesis of Silver and Gold Nanoparticles from Plant Extracts

A one step green synthesis of Ag and Au nanostructures is described as the usage of naturally occurring biodegradable plant-based surfactants, without any special reducing/ capping agent (Elizondo *et al.,* 2012). The green method in this study uses water and methanol as benign solvents and extracts of *A. dubius*, *G. perpensa* and *C. triloba* as the reducing agents.

The reduction of metallic ions into metallic particles is influenced by parameters such as temperature, pH, concentration, etc. As seen in the results, the temperature at which the reaction is carried out can affect the outcome since not all reactions take place at room temperature. It is also interesting to note that the results are plant specific, as each plant has its own different compounds and different concentrations of these compounds make for different synthetic reactions. It can also be stated that while some reduction reactions with the plant extract are spontaneous, others require extra energy in the form of temperature, as shown by Au NPs formation by *G. perpensa* leaf extract in comparison to those formed by extracts of *A. dubius* and *C. triloba*. It was found that as temperature increases, so too does the absorbance values. However, this does not necessarily mean that a high absorbance produces a monodisperse solution of nanoparticles as is seen from UV-vis scans which produced broad peaks. The broad peaks are mainly due to large anisotropic structures while the fairly sharp and symmetrical peaks are observed for small spherical particles (Philip *et al.,* 2011). When the concentration is doubled, the absorbance is seen to decrease, this is probably due to the excess biomolecules available thus decreasing the chance of positive reaction between the biomolecules and metal salts. Depending on the extract quantity, it can also lead to aggregation and unstable particles as in the case of *G. perpensa* which formed Ag NPs at a much lower concentration in comparison to the other plant extracts which is due to presence of sufficient biomolecules responsible for capping and efficient stabilization. Ag and Au salts are seen to react differently with each plant and at different parameters possibly due to their different reduction requirements and the variation in each plants phytochemistry. This leads to the conclusion that shape and size of

NPs can be controlled by varying the concentration of leaf extract and reaction temperature.

In previous studies it has been shown that *C. roseus* produces Ag NPs, however, the colour produced by the extracts when reducing the $AgNO_3$ is very different to what was achieved using our extract (Mukunthan *et al.,* 2011; Ponarulselvam *et al.,* 2012). Also, these studies were conducted in India and it is well known that based on the locality and environmental conditions in which the plant is found, the phytochemical constituents are adapted for that specific area. Seasonal changes are also able to affect the composition of leaf constituents which was discovered while conducting experiments at different times of the year with varying results. It is thus crucial to keep the location and seasonal collection of plant material constant.

The methanolic extracts of *A. dubius*, *G. perpensa* and *C. triloba* were capable of synthesizing Ag and Au NPs whereas the ethyl acetate extracts produced colour changes but these NPs were extremely unstable. This can be attributed to the fact that ethyl acetate extracts a far smaller concentration of the reducing biomolecules or that it does not extract the biomolecules responsible for capping the NPs.

Callus cultures of *A. dubius* and *C. triloba* have a different phytochemical make-up in comparison to their leaf counterparts. Although there was a visible reaction for the *C. triloba* callus extract with the metal salts, these unstable particles could be due to the mass of young cells with different ploidy in the callus which may be metabolically active to produce various types of chemicals responsible for the reduction of silver ions (Nabikhan *et al.,* 2010). Callus cultures are also grown in a laboratory environment which means that their need to produce secondary metabolites is decreased significantly because of the lack of environmental stresses. This however can be changed by inducing a specific stress into suspension cultures causing the over-production of a certain metabolite. This can be investigated as a biosynthetic option of NPs. It still is a laborious process in comparison to the use of fresh leaf extracts.

5.2 Characterization and Stability of Nanoparticles

Colour of silver and gold NPs is attributed to SPR arising due to the collective oscillation of free conduction electrons induced by an interacting electromagnetic field (Grzelczak *et al.,* 2008). According to Mie's theory, only a single SPR band is expected in the absorption spectra of spherical NPs, whereas anisotropic particles could give rise to two or more SPR bands depending on the shape of the particles. The analysis of SPR absorption band can also provide valuable information on the size, structure and aggregation properties of NPs which makes UV-vis spectrophotometry a vital characterization technique for NPs. However, the molar concentration of NPs is hard to establish, the main reason being that NPs are not monodispersed (Lui *et al.*, 2007). The characteristic peak of Ag NPs between 400 nm to 425 nm was shown by both aqueous and methanolic NPs by the three plants which produce only spherical particles. A peak at 520 nm to 550 nm is characteristic for the transverse plasmon resonance of Au NPs whereas the peak at above 650 nm is characteristic for the longitudinal plasmon resonance of either Au nanorods or triangular or hexagonal shaped Au NPs (Daniel and Astruc, 2004). This explains the shoulder that forms in the UV-vis scans of *G. perpensa* for Au NPs from the aqueous leaf extract.

Variation in shape and size of NPs synthesized by biological systems is common. Depending upon the extract concentration and temperature used for the preparation, Ag and Au crystallize in different shapes and sizes to form spherical, prisms and hexagonal structures. Sizes vary from the nanometre to micrometre scale level depending on the plant extract used for preparation. Au NPs synthesized by chemical methods are generally spherical, and nanostructures with a triangular morphology are rare which were produced by all three leaf extracts in this study. The high absorption coefficient of these Au nanotriangles in the NIR region might make them useful in fabricating photonic devices such as optical sensors. These shapes have the optical edge due their sharp edges in comparison to the spherical particles. Another exciting application based on the large NIR absorption of the particles could be in hyperthermia treatment of tumours (Hirsch *et al.*, 2003). Also, the sensitivity of chemiluminescent analysis of antibodies in a clinical sample could be improved by using irregularly shaped Au NPs, which have 100-fold greater catalytic activity in comparison with spherical Au NPs (Wang *et al.*, 2006). There have been reports on chemical and physical methods that are used to control the shape and size

of NPs synthesized using plant extracts which could help with monodispersity but still keep it environmentally friendly (Rai *et al.*, 2006).

The aqueous and methanolic extracts of *A. dubius* were able to produce peaks with the highest absorbance for Ag NPs when compared to the *G. perpensa* and *C. triloba* extracts. The spherical Ag NPs that were formed from aqueous extracts of *A. dubius* were slightly larger (19 nm to 38 nm) than the methanolic Ag NPs (7 nm to 16 nm). The Ag NPs produced by *G. perpensa* were in the same size range for aqueous and methanolic extracts (13 nm to 24 nm) which was similar to the *A. dubius* Ag NPs. *C. triloba* Ag NPs formed from the methanolic extract (10 nm to 42 nm) were closer in size to *A. dubius* aqueous Ag NPs however, *C. triloba* aqueous extract produced much larger particles (22 nm to 50 nm) than the other extracts. The Ag NPs produced from *A. dubius* aqueous and methanolic extracts as well as *C. triloba* methanolic extracts exhibited the longest stability of 30 days. *G. perpensa* aqueous Ag NPs showed the least stability.

The aqueous and methanolic extracts of *G. perpensa* were able to produce peaks with the highest absorbance for Au NPs when compared to *A. dubius* and *C. triloba* extracts. *G. perpensa* did not form any hexagonal Au NPs and the spherical and triangular Au NPs were smaller in comparison to those Au NPs formed from the other two plants. *A. dubius* aqueous and methanolic extracts produced triangular Au NPs in the size range of 25 nm to 100 nm and 9 nm to 45 nm respectively. *C. triloba* produced slightly larger particles than *A. dubius* Au NPs. The Au NPs formed by the aqueous extracts of *A. dubius* and *C. triloba* were larger in comparison to their methanolic counterparts. The Au NPs produced from *G. perpensa* aqueous and methanolic extracts as well as *A. dubius* and *C. triloba* methanolic extracts exhibited the longest stability of 30 days. Au NPs were stable for longer in comparison to Ag NPs.

The FTIR results presented direct evidence for the formation of a chemical bond between Ag and Au NPs and the nitrogen of the amide group present in the amino acids. It has been reported that either through free amino groups or cysteine residues, the protein is able to bind to the NPs that lead to the stabilization of NPs by surface bound protein (Gole et al., 2001; MubarakAli et al., 2011). There are not too many significant differences in the FTIR spectra between the aqueous and methanolic Ag NPs or Au NPs. The methanolic sample of Ag NPs synthesized from *G. perpensa* forms higher intensity peaks at 2973, 1473, 1382,

1152 and 1072 cm^{-1} compared to the aqueous Ag NPs. This leads to the deduction that methanolic samples are able to elute more polar compounds and hence provides a greater stability in methanolic Ag NPs than aqueous NPs. The larger size of the NPs that can be seen in this study might be due to the capping of NPs by groups of or larger proteins. FTIR results show that Au NPs have peaks that are characteristic of the presence of flavonoids with a higher intensity and could be responsible for longer stability seen by Au NPs. This reinforces the different capping abilities of Ag and Au NPs. When comparing aqueous and methanolic Ag NPs to each other, methanolic Ag NPs have a slightly higher intensity than aqueous Ag NPs which would explain the longer stability in most cases.

UV-vis spectroscopy is an important technique to ascertain the formation and stability of metal NPs in aqueous solution. NPs synthesized using plant extracts are surrounded by a thin layer of some capping organic material which allows the stability of NPs in solution. The nature of stabilizing agents plays an important role in size distribution and the shape of NPs which greatly determines the NPs functional properties (Widoniak *et al.,* 2005). Chemical materials and polymers have been used to stabilize NP solutions but these stabilizers are only thermally removable at extremely high temperatures of more than 200°C and this can be seen as a possible interference to the NPs themselves. Some chemical stabilizers like ethanol are only able to keep a NP solution stable for 48 hours before complete precipitation. Many physical and chemical synthetic methods have been used to synthesize NPs but there are still problems experienced with stability, aggregation, control of nanocrystal growth, shape and size. Green chemistry is showing promising results in order to combat these problems (Korbekandi *et al.,* 2009).

In this study, Ag NPs produced from leaf extracts of *G. perpensa* and *C. triloba* have a very short time period of stability and this is due to the low concentrations of extract being able to reduce the $AgNO_3$ salt but not protect the quasi-spherical NPs from aggregating because of the deficiency of biomolecules to act as protecting agents. It is observed that Au NPs on the whole, have a longer stability period than when compared to Ag NPs as a result of the different reduction and binding requirements between Ag and Au salts which was confirmed by FTIR analysis. Methanolic Ag and Au NPs have greater stability compared to the aqueous counterparts due to the difference in compounds eluted by each solvent. A longer study can be done in future to determine the exact length of time the NPs are stable, this was only carried out over a month for comparison against previous studies. NPs lose

their unique properties once they have aggregated and precipitated from suspension due to their surface functionalization being a critical component in their application (Christian *et al.,* 2008). This is the reason that the NPs synthesized need to be stable when using them in applications.

It is not easy to find systematic studies in literature that evaluate the stability of NPs for long periods of time. Most of the studies only report that particles are stable without precipitation for a short period of time. Industry is focused on looking for new solutions that would allow the development of new products to contend in a market with increasing competitiveness. However, the NPs available in the market are expensive and exist in limited amounts (Pinto *et al.*, 2010). In this way it is necessary to develop simple and low cost processes to produce large quantities of stable NPs. Aqueous and methanolic extracts of *A. dubius* along with the methanolic extracts of the other two plants have shown that it is possible for Ag NPs to be stable for 30 days. Aqueous and methanolic extracts of *G. perpensa* and the methanolic extracts of the other two plants were also successful in producing Au NPs that were stable for over 30 days without the addition of any other constituents. Further research and possibly refining the synthesis procedures could lead to an effective and marketable process.

5.3 Phytochemicals Responsible for Biosynthesis of Nanoparticles

It was deduced by Shankar *et al.,* (2004) that reducing sugars (aldoses) and terpenoids and polyphenolic compounds play a key role in the reduction of Ag and Au ions and the formation of corresponding NPs while ketones/ aldehydes bind to emerging spherical NPs to form large nanotriangles and hexagons. *Euphorbia prostate* is an annual herb in India that has been reported to possess phytochemicals such as anthraquinones, flavonoids, phenols, phlobatannins, polysaccaharides, saponins, tannins and terpenoids which was able to produce Ag NPs that were toxic against parasites (Kamgang *et al.*, 2007; Zahir and Rahuman, 2012). The results produced in this study show that the major component responsible for the reduction of the metal salts was tannins as this was the only phytochemical present in the three plant extracts and absent from the *C. roseus* leaf extract which was the plant incapable of synthesizing NPs. A naturally available hydroxyflavonoid called quercetin which is found in many plant extracts was used to produce Au NPs but unable to produce Ag NPs under similar reaction conditions. This led the researchers to the

conclusion that although one type of polyphenol could produce Au NPs, the generation of Ag NPs is probably due to other flavonoid/ polyphenols with a higher reduction potential (Begum *et al.,* 2009). Although phytochemicals such as saponins and flavonoids that are also said to be responsible for reduction were found in all the leaf and callus extracts, it could be that the concentrations of these phytochemicals were not sufficient in the extracts or that in other experiments, the synergistic behaviour of different phytochemicals were the reason for NP synthesis.

The mechanism behind biosynthesis of NPs lies in the reducing ability of biomolecules in the plant extracts. Since the exact mechanism is not known, many possible reasons have been assumed. Large amounts of H^+ ions are produced along with ATP. Nicotinamide adenine dinucleotide (NAD^+) is a coenzyme found in all living cells and is involved in redox reactions, carrying electrons from one reaction to another. NAD^+ is an oxidizing agent i.e. it accepts electrons from other molecules and becomes reduced. This reaction forms NADH, which can donate electrons. NAD^+ keeps on getting re-oxidized and gets constantly regenerated due to redox reactions. This might lead to the formation of Ag^0 and Au^0 (Ahmad *et al.*, 2011).

Plants contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components. Phenolic compounds possess hydroxyl and ketone groups which are able to bind to metals and show chelating effect. The general chelating ability of phenolic compounds is probably related to the high nucleophilic character of the aromatic rings rather than to specific chelating groups within the molecule. Flavonoids can also directly scavenge molecular species of active oxygen. Antioxidant action of flavonoids resides mainly in their ability to donate electrons or hydrogen atoms (Saxena *et al.,* 2012).

Phenolics and other chemicals within plant leaf extract do not only clearly reduce metal salts but also provide excellent tenacity against agglomeration. Proteins and enzymes present in the leaf extract are possibly facilitators of the formation of pure metallic NPs by reduction of the metal ions (Saxena *et al.,* 2012). Stability of over four months of Au NPs synthesized by *Terminalia catappa* leaf extract was attributed to the various acids and hydrolysable tannins present (Ankamwar, 2010). Methanolic extracts of plants were shown to yield the highest quantity of tannins whereas aqueous showed the weakest (Salminen,

2003). This would explain the longer stability periods seen by the methanolic NPs in this study. Leaf extracts of *G. perpensa* and *C. roseus* as well as callus extracts of *A. dubius* and *C. triloba* do not contain alkaloids which could be part of the reason that these extracts do not form NPs or produce unstable NPs.

A. cruentus and *A. hybridus* phytochemical testing was done, which showed presence of polyphenols, tannins, flavonoids, steroids and terpenoids, saponins, no alkaloids were found. Literature data demonstrated that the variance within the varieties of *Amaranthus* genotypes is, in general, highly influenced by environmental factors when the focus is on content of polyphenols (Steffenson *et al.*, 2011). *A. dubius* was found to possess compounds such as saponins, flavonoids, steroids and tannins along with the rest of the plants used in this study. Young leaves of *C. triloba* which have long and short trichomes present produced a yellow, oily secretory product. The presence of phenolic compounds was confirmed in the heads of the trichomes, however, negative results were produced for the presence of terpenoids and alkaloids (Naidoo *et al.*, 2012). Our phytochemical tests show the presence of alkaloids which could again be due to seasonal and location differences. The presence of anthraquinones in *C. triloba* has been reported (Mohanlall *et al.,* 2011). An anthraquinone is an aromatic organic compound and naturally occur in some plants, fungi, lichen and insects, wherein they serve as a basic skeleton for their pigments.

5.4 Antibacterial Activity

The increasing number of bacterial strains that are resistant to available pharmaceutical compounds due to genetic mutations is a vital issue for public health. Improved approaches will be required to improve the methods for both diagnosis and destruction of these organisms (Pissuwan *et al.,* 2010). Flavonoids, terpenoids, tannins and phlobatannins are phytochemicals that have been demonstrated to have antimicrobial activity. Medicinal plants are rich in these compounds and are assumed to be a non-toxic, perfect base for NP biosynthesis (Maiyo *et al.*, 2010).

Silver nitrate which is readily soluble in water has been exploited as an antiseptic agent for many decades. Dilute solutions have been used to treat wounds and burns. A literature survey reveals that the positive charge on the Ag^{+} ion is crucial for its antimicrobial activity. However, the Ag ions or salts have only limited usefulness as antimicrobial agents

for several reasons; the interfering effects of salts and the discontinuous release of inadequate concentrations of Ag ions from the metal. In contrast, these limitations can be overcome using Ag NPs because these are highly reactive species due to their large surface areas (Nabikhan *et al.,* 2010). The exact mechanism of the antibacterial effect of NPs is partially understood. The antibacterial activity is probably due to the electrostatic attraction between the negatively charged cell membrane of the microorganism and positively charged NPs (Dibrov *et al.*, 2002; Prasad and Elumalai, 2011). Zeta potential is generally used to calculate the electrokinetics of the NPs, this assay was not carried out in this study and thus there is no accurate surface charge evaluation that can be made (*Kim et al., 2005*).

The cell wall of a bacterium provides shape, rigidity, strength and protection for the cell against mechanical damage and osmotic rupture. There are two main classes of cell walls i.e. Gram-positive and Gram-negative. Gram-positives have a large peptidoglycan layer and the gram-negatives have a high lipid content. The gram-negative bacteria are often associated with resistance due to their negative surface charge making it difficult for hydrophobic compounds to take effect (Singleton, 1999). The toxic effect of NPs on *E. coli* are said to depend on various factors such as temperature, pH, concentration of both NPs and the bacteria and aeration. Lower agglomeration of the NPs increases the toxicity due to the larger surface area available for interaction with the bacterial membranes (Pramanik *et al.*, 2012). It is important to note that each bacterial strain has different properties and thus the mechanism of destroying them is specific to each strain (Hajipour *et al.,* 2012). This can be seen by *S. aureus* P4215 which exhibits little resistance to the aqueous and methanolic Ag NPs from the *A. dubius*, *G. perpensa* and *C. triloba* whereas *S. aureus* S5878 shows a high resistance to these NPs.

The antibacterial activity of Ag and Au NPs were investigated by Kim *et al.* (2007), while the Ag NPs with an average diameter of 13.5 nm showed positive results against *E. coli* and *S. aureus*, the Au NPs of approximately 30 nm displayed no activity. The best activity on average in this study was shown by *G. perpensa* Ag NPs which were in the size range of 13 nm to 24 nm and therefore is agreeable with the above investigation. The antimicrobial activity in terms of inhibition zones significantly varied with test microbes and types of NPs. This differential antimicrobial activity observed in this study can be attributed to the differential size range and shapes of Ag NPs in the same sample. There is a discrepancy when it comes to finding a correlation between the antibacterial activity of

NPs and specific bacteria by different researchers because experiments produce NPs that are poorly defined and characterized and the usage of different strains of bacteria. There is no standard to use and thus a lot of work needs to be done if a reference system is to be envisioned.

C. triloba methanolic Ag NPs showed the highest activity (9.2 ± 0.3 mm) against Gram-positive *S. aureus* P4215. It also showed high activity against Gram-positive *E. faecalis* U11951 while *G. perpensa* aqueous Ag NPs showed high activity against Gram-negative *E. coli* P4055. The lowest activity was shown by aqueous and methanolic Ag NPs of *A. dubius* against Gram-negative *E. coli* P4055 and *K. pneumoniae* P3811 and V258 strains as well as Gram-positive *S. aureus* S5878. The aqueous Ag NPs also had no activity against Gram-negative *Enterobacter* spp. P4177 and *K. pneumoniae* P3811.

There are contrasting reports when it comes to the antibacterial activity of Au NPs. Some studies show positive results for Au NP bactericidal activity and believe that it is initially mediated by strong electrostatic attractions to the negatively charged bilayer of the cell membrane due to cationic Au NPs being found to be moderately toxic while anionic particles were unreactive. Another study showed that Au NPs did not have any bactericidal activity on their own (Burygin *et al.*, 2009). Although the Au NPs synthesized by the plant leaf extracts in this study were biologically inert, there is the possibility of them being engineered to possess antibacterial functionality. The 4, 6-diaminopyrimidine thiol-modified Au NPs that were chemically synthesized had bactericidal activity against *E. coli* and did not produce any ROS-related processes which could be the cause of low toxicity of Au NPs to mammalian cells (Brust *et al.,* 1994).

The *G. perpensa* Ag NPs have also been shown to have the best MIC values amongst a range of bacteria. Although *A. dubius* Ag NPs showed inferior activity in the disc diffusion assay compared to *C. triloba*, here they had lower MICs. A lower MIC value exhibited by the Ag NPs against pathogenic bacteria is of great significance in the health care delivery system. The Ag NPs could be used as an alternative to orthodox antibiotics in the treatment of infections caused by the microorganisms (Dipankar and Murugan, 2012). A lower MIC would also provide the patient with a smaller dosage requirement thus limiting or even preventing harmful side effects.

6. CONCLUSION

The results of this study are summarized in Table 13. The aqueous and methanolic extracts of *A. dubius* are capable of synthesizing Ag NPs and Au NPs. Positive colour changes were produced at optimal parameters. The optimal leaf extracts concentration at which a symmetrical peak was formed was 2.5 ml at 40°C for Ag NP synthesis and 2.5 ml at 60°C for Au NPs. The aqueous and methanolic extracts of *A. dubius* were able to produce peaks with the highest absorbance for Ag NPs when compared to the *G. perpensa* and *C. triloba* extracts.

A. dubius required heat to form Au NPs whereas Ag NPs were able to form at room temperature. Ethyl acetate extracts of *A. dubius* produced slight colour changes but these NPs were extremely unstable. *A. dubius* was induced to form callus however these extracts were unsuccessful in synthesizing NPs. The spherical Ag NPs that were formed from aqueous extracts of *A. dubius* were larger than the Ag NPs from the methanolic extracts. The Au NPs formed by the aqueous extracts of *A. dubius* were larger in comparison to their methanolic counterparts. Ag NPs from *A. dubius* aqueous and methanolic extracts was stable for the 30 day period. The Au NPs produced from *A. dubius* aqueous and methanolic extracts were stable for 30 days. FTIR provided evidence that Ag and Au NPs have a chemical bond with the amide group in amino acids. FTIR intensities of the molecules are more pronounced for Au NPs than compared to the Ag NPs. This is possibly the reason for the longer stability found in Au NPs. The lowest activity was shown by aqueous and methanolic Ag NPs of *A. dubius* against Gram-negative *E. coli* and *K. pneumoniae* strains as well as Gram-positive *S. aureus* S5878. The aqueous Ag NPs also had no activity against Gram-negative *Enterobacter* spp. P4177 and *K. pneumoniae* P3811.

The aqueous and methanolic extracts of *G. perpensa* are capable of synthesizing Ag NPs and Au NPs. The optimal leaf extracts concentration at which a symmetrical peak was formed was 0.32 ml at 25°C for Ag NP synthesis and 1.25 ml at 40°C for Au NPs. *G. perpensa* was able to spontaneously form Ag and Au NPs without any addition of heat. Ethyl acetate extracts of *G. perpensa* produced slight colour changes but these NPs were extremely unstable. *G. perpensa* was unsuccessful in forming a callus culture. The Ag NPs

produced by *G. perpensa* were in the same size range for aqueous and methanolic extracts which was similar to the *A. dubius* Ag NPs.

The aqueous and methanolic extracts of *G. perpensa* were able to produce peaks with the highest absorbance for Au NPs when compared to *A. dubius* and *C. triloba* extracts. Unlike in *A. dubius* and *C. triloba* Au NPs, *G. perpensa* did not form any hexagonal Au NPs and the spherical and triangular Au NPs were smaller in comparison to those formed from the other two plants as well. *G. perpensa* aqueous Ag NPs showed the least stability. The Au NPs produced from *G. perpensa* aqueous and methanolic extracts were stable for 30 days. FTIR provided evidence that Ag and Au NPs have a C=O bond with the amine group in amino acids. Ag NPs from methanolic extracts of *G. perpensa* have a slightly higher intensity than aqueous Ag NPs. The intensities of the molecules are more pronounced for Au NPs than compared to the Ag NPs for G. perpensa extracts. *G. perpensa* aqueous extract Ag NPs showed high activity against Gram-negative *E. coli* P4055. According to this study, on average, the results show that *G. perpensa* Ag NPs have a better antibacterial activity against Gram-positive and Gram-negative pathogenic bacteria than compared to *A. dubius* and *C. triloba*. The *G. perpensa* Ag NPs have also been shown to have the best MIC values amongst a range of bacteria.

The aqueous and methanolic extracts of *C. triloba* are capable of synthesizing Ag NPs and Au NPs. The optimal leaf extracts concentration at which a symmetrical peak was formed was 0.63 ml at 95°C for Ag NP synthesis and 5 ml at 60°C for Au NPs. *C. triloba* required heat to form Ag and Au NPs. Ethyl acetate extracts of *C. triloba* produced slight colour changes but these NPs were extremely unstable. *C. triloba* was induced to form callus however these extracts formed unstable NPs. *C. triloba* Ag NPs formed from the methanolic extract were closer in size to *A. dubius* aqueous Ag NPs however, *C. triloba* aqueous extract produced much larger particles than the other plant extracts. The Au NPs formed by the aqueous extracts of *C. triloba* were larger in comparison to their methanolic counterparts. Ag NPs from *C. triloba* methanolic extracts was stable for the 30 day period. The Au NPs produced *C. triloba* methanolic extracts exhibited stability for 30 days. FTIR showed that Ag and Au NPs have a C=O bond with the amine group in amino acids. Ag NPs from methanolic extracts have a slightly higher intensity than aqueous Ag NPs. *C. triloba* methanolic extract Ag NPs showed the highest activity against Gram-positive *S. aureus* P4215 as well as high activity against Gram-positive *E. faecalis* U11951.

Table 13: Summary of results for *A. dubius*, *G. perpensa*, *C. triloba* and *C. roseus*

Synthesis	Extracts	*A.dubius*		*G. perpensa*		*C. triloba*		*C. roseus*	
		Ag NPs	Au NPs	Ag NPs	Au NPs	Ag NPs	Au NPs	Ag NPs	Au NPs
Leaf Extract Synthesis	A	√	√	√	√	√	√	X	X
	M	√	√	√	√	√	√	X	X
	E	*	*	*	*	*	*	X	X
Optimum Temperature (°C) for Leaf Extract Synthesis	A & M	40	60	25	40	95	60	X	X
Optimum Concentration (ml) for Leaf Extract Synthesis	A & M	2.5	2.5	0.32	1.25	0.63	5	X	X
Callus Micropropagation	p/p	√		X		√		X	
Callus Extract Synthesis of Ag NPs	A	X	X	X	X	*	*	X	X
	M	X	X	X	X	X	X	X	X
	E	X	X	X	X	X	X	X	X
Characterization									
UV-vis Peaks (AU)	A	~3	~2.9	~1.3	~3.2	~1.9	~2.9	X	X
	M	~1.5	~2.6	~1.3	~2.9	~1.2	~2.8	X	X
Shape of NPs	A	spherical	spherical,triangular	spherical	spherical,triangular	spherical	spherical,triangular, hexagonal	X	X
	M	spherical	spherical,triangular, hexagonal	spherical	spherical,triangular	spherical	spherical,triangular, hexagonal	X	X
Size Range (nm)	A	19 to 38	spherical- 9 to 25 triangular- 25 to 100	13 to 24	spherical- 10 to 21 triangular- 12 to 39	22 to 50	spherical- 17 to 32 triangular- 20 to 190 hexagonal- 18 to 112	X	X
	M	7 to 16	spherical- 9 to 27 triangular- 9 to 45 hexagonal- 13 to 26	13 to 24	spherical- 9 to 16 triangular-10 to 23	10 to 42	spherical- 7 to 36 triangular- 12 to 50 hexagonal- 10 to 28	X	X

FTIR Major Biomolecules	**A**	C=O stretching in amine I group	C=O stretching in amine I group, C-H vibration	C=O stretching in amine I group	C=O stretching in amine I group, C-H vibration	C=O stretching in amine I group	C=O stretching in amine I group, C-H vibration	X	X
	M	C=O stretching in amine I group	C=O stretching in amine I group, C-H vibration	C=O stretching in amine I group	C=O stretching in amine I group, C-H vibration	C=O stretching in amine I group	C=O stretching in amine I group, C-H vibration	X	X
Stability (days)	**A**	30	21	2	30	7	21	X	X
	M	30	30	10	30	30	30	X	X
Phytochemicals Present	**p/p**	Steroids, Saponins, Alkaloids, Flavonoids, Tannins		Steroids, Saponins, Flavonoids, Tannins		Steroids, Saponins, Alkaloids, Flavonoids, Tannins		Steroids, Saponins, Flavonoids	
Antibacterial Activity									
Disk Diffusion Inhibition Zones (mm)	**A**	Susceptible to *Enterobacter* spp. P4177 and *K.pneumoniae* P3811	X	Inhibited all gram-positive and gram-negative bacteria	X	Inhibited all gram-positive and gram-negative bacteria	X	X	X
	M	Inhibited all gram-positive and gram-negative bacteria	X	Inhibited all gram-positive and gram-negative bacteria	X	Inhibited all gram-positive and gram-negative bacteria and highest zone of 9.2 against gram-positive *S. aureus*	X	X	X
MIC (µg)	**A**	6.3 on gram-negative *E. coli* and *K. pneumoniae*	X	3.2 on gram-positive *S. aureus* and gram-negative *E. coli*and *K. pneumoniae*	X	6.3 on gram-negative *E. coli* and *K. pneumoniae*	X	X	X
	M	9.0 on gram-positive Enterococcus	X	3.2 on gram-positive Enterococcus, *E. faecalis* and gram-negative *K. pneumoniae*	X	12.5 on gram-positive *E. faecalis* and gram-negative *K. pneumoniae*	X	X	X

A-aqueous extract √-positive result/formed

M- methanolic extract **X**- negative result/did not form

E-ethyl acetate extract *****- unstable NPs formed

p/p- per plant

C. roseus did not produce a positive colour change or yield positive UV-vis results and was thus unable to synthesize Ag and Au NPs. The phytochemical screening revealed that the only compound that was missing in the *C. roseus* extract and present in the other three plants was tannins. This is possibly the most likely reason that *C. roseus* was not able to synthesize NPs since these were absent in the callus extracts of *A. dubius* and *C, triloba* as well. Although there were saponins and flavonoids present in *C. roseus*, which are known to be responsible for reduction, these were not in sufficient quantities to produce a reaction unlike in the callus extracts of *C. triloba* which were able to produce unstable NPs.

Green synthesis of NPs is a necessary technology that requires more research. The results of this study show that *A. dubius*, *G. perpensa* and *C. triloba* are plants that can produce useable NPs. Monodispersity and stability are problems with biosynthetic procedures that need to be resolved. Ag NPs have proven to have antibacterial activity, and as deduced from this study, depends on the plant. Although, Au NPs formed from the plants does not have antibacterial properties, these particles are still useful for other applications.

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