

**Characterization and Antibacterial Efficacy of Silver
Nanoparticles from *Ageratina adenophora* Leaf Extract**

By

M.Durga

(13PBT003)

A Thesis Submitted to

Avinashilingam Institute for Home Science and Higher Education for Women

Coimbatore – 641043

In Partial Fulfilment of the Requirement of the Degree of

Master of Science in Biotechnology

March 2015

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Signature of the Head of the Department


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1.0 INTRODUCTION

Nanotechnology is an interdisciplinary area of science which has been developing interest across the globe with huge momentum to user in forming nano revolution. Nanotechnology deals with the synthesis of nanoparticles which has encountered immense progress due to innumerable applications (Cheon and Horace, 2009). Nanoparticles (NPs) are particles less than 100 nm in diameter which exhibit enhanced size-dependent properties compared to its bulk material (Baker and Satish, 2012).

For over 5,000 years ago, the use of nanomaterials for human health was reported in the Ayurveda medicine of Indian system, in which nanoscience technology was applied even before the term “nano” was coined (Dubey *et al.*, 2010). Nanoparticles show completely new and improved properties, such as size, morphology and distribution. Novel applications of nanomaterials and nanoparticles are emerging rapidly on various fields (Kaviya *et al.*, 2011).

Production of nanoparticles can be done through different methods such as, chemical and photochemical reactions in reverse micelles, reduction in solutions, thermal decomposition of silver compounds, electrochemical, sonochemical, radiation assisted, microwave assisted method and biological routes (Sulaiman *et al.*, 2013).

Nanoparticles synthesis from biological methods using microorganisms, enzyme and plant or plant extract offers numerous benefits over chemical and physical methods (Sunkar and Nachiyar, 2012). It is cost effective, environmental friendly, easily developed for large scale synthesis (Kouvaris *et al.*, 2012). In this method there is no need to use high pressure, temperature, energy and toxic chemical that may have adverse effect in the medical applications (Sivakumar *et al.*, 2012).

Metal nanoparticles have different properties because of their small sizes when compared to bulk metals and these materials can be employed in various photoelectronic, magnetic, catalytic, sensor and biomedical applications (Maria

et al., 2010). Metal nanoparticles have drawn a lot of attention due to their unusual physical and chemical properties, which largely differ from their bulk properties. They show unique properties such as chemical stability, excellent conductivity and catalytic activity, etc. which are dependent on the particle size, distribution and shape (Zhang *et al.*, 2010).

Silver has the highest electrical and thermal conductivity among all the metals. Silver materials with zero, one, or two-dimensional nanostructures such as nanoparticles, nanowires and nanocubes have great potential for applications in optics and catalysis (Ahn *et al.*, 2009; Takenobu *et al.*, 2009).

Silver (Ag) is a metal that inactivates the microbes by interacting with their enzymes, proteins, DNA to restrain cell proliferation or cell division. It also prevents the bacterial regeneration by binding to the negatively-charged bacterial cells which change the functionality of the cell membrane. When compared to other usual biocide, silver is a durable biocide with high temperature stability and very low volatility (Maria *et al.*, 2010).

Silver has been recognized as having an inhibitory effect towards many bacterial strains and micro organisms commonly present in medical and industrial processes (Mostafa *et al.*, 2011). Many attempts have been made in silver nanoparticles to use as an anti-cancer agent (Vaidyanathan *et al.*, 2009).

Silver nanoparticles (AgNPs) are widely used when compared to other metal nanoparticles, including spectrally selective coating for biolabeling, solar energy absorption, intercalation materials as optical receptors for electrical batteries, antibacterial agents, nonlinear optics and catalysts in chemical reactions (Shameli *et al.*, 2012).

Ag and AgNPs are widely used in medical industry as topical ointments that prevent infections in burns and open wounds (Pasupuleti *et al.*, 2013). The first commercialized NPs are AgNPs that are used as broad-spectrum antimicrobials in consumer products such as cosmetics, clothing, detergents, water filters, dietary supplements, electronics and children's toys (Bondarenko

et al., 2013). The silver nanoparticle can be used to fabricate flexible electronics by ink-jet printing due to their low sintering temperatures (Hsu and Wu, 2011).

Secondary metabolites are responsible for medicinal activity of plants. Qualitative phytochemical analysis of these plants confirm the presence of various phytochemicals like saponins, terpenoids, steroids, anthocyanins, fatty acids, tannins, leucoanthocyanins and emodins. The results suggest that the phytochemical property for curing various ailments leads to the isolation of new and novel compounds (Das *et al.*, 2010).

Research on plants has gone through the remarkable progress with current increase in plant research in synthesis of nanoparticles with controlled size and shape. Synthesis of nanoparticles through plants has become one of the popular alternatives for conventional methods. Heavy metal tolerance by plants is a well-known natural phenomenon which results in phytoremediation and phytomining (Iravani, 2011).

The silver nanoparticles are well-known for their antimicrobial and anti-inflammatory wound healing abilities. The antimicrobial properties of silver ions are widely used as bactericide in surgical prostheses and dental implants (Augustine and Rajarathinam, 2012).

The most widespread method of metallic nanoparticles synthesis is based on the chemical reduction of metal salt solution by a reducing agent (Szczepanowicz *et al.*, 2010). But the chemicals used in such processes are quite often toxic and flammable which is not suitable for many applications including biological applications like impregnation in wound dressings (Vivekanandhan *et al.*, 2012).

Plants have a major role in the synthesis of nanoparticles to overcome the problem of toxicity in synthesis. The different-shaped polyol and water-soluble heterocyclic components of plant biomolecules have both protective and reductive activity and are mainly responsible for the reduction of silver and gold ions using chemical and radiation methods (Arunachalam *et al.*, 2013).

Biological synthesis of nanoparticles is not only a good way to fabricate benign nanostructure materials, but also to reduce the use and generation of hazardous substances to human health and the environment (Jacob *et al.*, 2012).

Scientific classification	
Kingdom:	Plantae
Order:	Asterales
Family:	Asteraceae
Tribe:	Eupatorieae
Genus:	<i>Ageratina</i>
Species:	<i>A. adenophora</i>
Binomial name: <i>Ageratina adenophora</i>	
synonyms: <i>Eupatorium adenophorum</i> , <i>Eupatorium glandulosum</i>	

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, based on their use in traditional medicine. Plants are the basic source of knowledge of medicine. The basic molecular and active structures for synthetic fields are provided by rich natural sources (Preethi *et al.*, 2010).

The medicinal properties of plants have been investigated in the recent scientific development throughout the world, due to their potent antioxidant and antimicrobial activity, no side effects and economic viability (Panda *et al.*, 2011). Medicinal plants are the principle health care resources for the majority of the people all over the world (Kumar *et al.*, 2011). Medicinal plants are those that provide people with medicines to treat illness, maintain and promote health (Sivasankari, 2010). Plants used for traditional medicine contain a wide range of

substances that can be used to treat chronic as well as infectious diseases (Dash *et al.*, 2011).

Ageratina adenophora (Spreng) (Syn. *Eupatorium glandulosum*, *Eupatorium adenophorum*) is a profusely branching under shrub up to 90-120 cm in height with a few ascending branches, leaves simple, opposite, sessile, subentire, lanceolate and glabrous type which belongs to the family Asteraceae. *A. adenophora* is a very common weed and invasion of this weed have replaced the larger part of the vegetation in throughout our country and are considered as a major threat to native plants and animals. It is reported to possess diverse medicinal properties and finds use in traditional medicines. The leaves are used as astringent, thermogenic and stimulant in folklore medicine in India (Vasanthi and Gopalakrishnan, 2013).

A.adenophora leaf juice is used to stop bleeding of cut and wounds, forming clots. Root juice is used to treat fever. Pure juice of the leaf is dropped in the eye for insomnia treatment. A decoction of the plant has been recommended to treat jaundice and ulcers (Subba and Kandel, 2012). Inhibition of DHFR enzyme present in the plant has been found to be has found to be antiprotozoal, antimalarial and antimicrobial agents (Sharma and Chauhan, 2012).

The objectives of the present study were:

1. To analyse the phytochemical constituents
2. To confirm the analysed phytochemicals by TLC
3. To optimize the method for the synthesis of silver nanoparticles using *A.adenophora* leaf extract
4. To characterise the synthesized nanoparticles
5. To determine the antibacterial potential of the silver nanoparticles

The literature pertaining to the present study is briefly reviewed in the next chapter.

2.0 REVIEW OF LITERATURE

2.1 NANOTECHNOLOGY

One of the most active research areas in the modern material science is nanotechnology (Sriram and pandidurai, 2014). Nanotechnology is used to describe the utilization and creation of materials with structural features between the atoms and bulk materials with at least one dimension in the nano range (Song and Kiml, 2009).

The field of nanoscience has blossomed over the last twenty years and the need for nanotechnology will only increase as miniaturization becomes more important in areas such as computing, sensors and biomedical application. Advances in the field largely depend on the ability to synthesize nanoparticles of various materials, shapes and sizes, as well as to efficiently assemble them into complex architectures. Nanotechnology provides the ability to engineer the properties of materials by controlling their size and this has driven research towards a multitude of potential uses for nanomaterials (Das *et al.*, 2009).

Nanotechnology is a skyrocketing multidisciplinary field of research that interweaves physics, chemistry, bionanoscience and materials science. Recently, nanobiotechnology is becoming as an emerging and remarkable technology for the production of novel functional materials such as nano-sized particles (Duncan, 2011; Sharma *et al.*, 2009). Bionanotechnology integrates biotechnology and nanotechnology for developing biosynthetic and environmental- friendly technology for synthesis of nanomaterials (Kudle *et al.*, 2013).

2.2 NANOPARTICLES

Development of green nanotechnology is generating interest for researchers toward ecofriendly biosynthesis of nanoparticles (Singhal *et al.*,

2011). The application of nanoscale materials and structures, ranging from 1 to 100 nanometers (nm), is an emerging area of nano science and nanotechnology. Nanomaterials provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, water-treatment and medicine. The development of techniques for the controlled synthesis of nanoparticles of well defined size, shape and composition, to be used in the biomedical field and areas such as electronics and optics, has become a big challenge. Development of reliable and eco-friendly processes for synthesis of metallic nanoparticles is an important step in the field of application of nanotechnology. One of the options to achieve this objective is to use 'natural factories' such as biological systems (Gurunathan *et al.*, 2009).

Nanoparticle development has restored interest in the antimicrobial effects of metals, which declined following the widespread application of modern synthetic antibiotics (Kim *et al.*, 2009). Nanoparticles are considered to be the building blocks of next generation of electronics, optoelectronics and various chemical. Nanoparticles have been widely used in various real world biomedical, industrial and scientific applications. Several types of metal nanoparticles (MNPs) such as gold, silver, zinc and copper have been successfully synthesized with various anticipated applications that includes nutrition and nutraceuticals, medicine and diagnosis and electronics and optics (Duncan, 2011; Sharma *et al.*, 2009). Various physical and chemical methods have been utilized for the production of silver, gold and other nanoparticles successfully (Bankura *et al.*, 2012; Wei *et al.*, 2012).

2.3 APPLICATIONS OF NANOPARTICLES

Nanotechnology and engineered nanoparticles has become an emerging field in the area of materials science. Nanotechnologies manipulate matter at an atomic scale creating new nanoproducts with novel properties. The novel properties of those types of nanoparticles have been widely investigated for their use in medicine, cosmetics, technology and environment. One of the most

challenging fields of materials science is the one involving biomaterials. The research takes a lot of effort in order to provide new and improved biomaterials with specific applications in medicine (Hutchison, 2008).

The continuing appearance of antibiotic resistance in microorganisms challenges the scientific community to constantly develop new bioactive compounds and drug targets with high biocompatibility and antibacterial properties (Gutierrez et al., 2010; Jaiswal *et al.*, 2010). Nanoparticles are used as potential drug carriers in the treatment of cancer (Hojo *et al.*, 2011).

2.4 SILVER NANOPARTICLES

Metal nanoparticles have a high specific surface area and a high fraction of surface atoms. Because of the unique physicochemical characteristics of nanoparticles, including optical properties, catalytic activity, antibacterial properties, electronic properties, and magnetic properties, they are gaining the interest of scientist for their novel methods of synthesis.

Silver is well known for possessing an inhibitory effect toward many bacterial strains and microorganisms commonly present in medical and industrial processes. In medicines, silver and silver nanoparticles have a wide application including skin ointments and creams containing silver to prevent infection of burns and open wounds, medical devices and implants are prepared with silver-impregnated polymers. In textile industry, silver-embedded fabrics are now used in sporting equipment (Singhal *et al.*, 2011).

Silver nanoparticles play a tremendous role in the field of biological system, living organisms and medicine when compared with any other nanoparticles (Gurunathan *et al.*, 2009; Jain *et al.*, 2009; Parashar *et al.*, 2009). Silver nanoparticles are a good choice of inorganic nanomaterials in combination with various classes of antibiotics for use against pathogenic micro-organisms because of their surface chemistry and chemical stability of the silver nanoparticles, their appropriate size and the capacity to maintain a constant shape and size in solution (Ghosh *et al.*, 2012).

Ag nanoparticles (AgNPs) are the first commercialized NPs that are nowadays used as broad-spectrum antimicrobials in over 300 consumer products including clothing, cosmetics, detergents, water filters, electronics, dietary supplements and children's toys (Marambio-Jones and Hoek, 2010; Cerkez *et al.*, 2012). Actually, colloidal silver, e.g., protein-stabilized nanosized Ag particles, has been used for numerous medical purposes (Nowack *et al.*, 2011).

Silver nanoparticles play a significant role in the field of biological systems, living organisms and medicine (Gurunathan *et al.*, 2009; Jain *et al.*, 2009; Parashar *et al.*, 2009). In addition, silver-containing consumer products such as colloidal silver gel and silver-embedded fabrics are now used insporting equipment (Song and Kim, 2008).

2.5 TYPES OF NANOPARTICLES

Some examples are listed below for general and multi-functional classes of nanoparticles used in biotechnology and particularly in the area of nanomedicine.

2.5.1 METAL NANOPARTICLES

Nanoparticles of metallic origin have been shown to exhibit unusual properties that day normally will not display in their bulk form (Blackman, 2009). In biochemistry, for example, they are considered to be better catalysts (Astruc, 2008) and good biological and chemical sensors in medicine, their potential as drug delivery agents has been reported.

2.5.2 LIPOSOMES

Initially, liposomes were used as model systems to study biological membranes, but by 1970, had been developed into a medium for the transportation of drugs (Graff *et al.*, 2004).an interesting property of liposomes is their natural ability to target cancer. Liposomes of certain sizes, typically less than 200 nm enable the intracellular release of many macromolecules including

protein toxins, gene therapy vectors, and oligonucleotides into cancer cells in addition to the delivery of suitable concentration of drugs to the target tumor cell surface (Orive et al., 2004). They are very important tools in diagnostics as they are able to degrade in the cells once the delivery has been carried out (Salata, 2004).

2.5.3 QUANTUM DOTS

Quantum dots or nanocrystals are semiconductor nanoparticles that can emit light in all colours of the spectrum depending on their size. QDs are valuable tools in biotechnology, especially in cellular imaging and have broad potential in the management and treatment of cancer (Xing et al., 2006).

2.5.4 LIQUID CRYSTALS

Liquid crystals are pharmaceuticals made from liquid organic crystalline materials and are designed to mimic naturally-occurring biomolecules like proteins or lipids. They are regarded as very safe and specific vehicle of drug delivery as they can target a particular area of the body where tissues are inflamed; they are also capable of detecting tumors (<http://Biotech.about.com/od/nanotechnology/a/typesnanopart.htm>).

2.5.5 DENDRIMERS

Dendrimers are highly branched structures with uniform size, radial symmetry and assume a circular shape in solution (Graff et al., 2004). In nanomedicine, dendrimers have been found to be invaluable tools in attaching fluorescent dyes, enzymes, cell identification tags and other molecules because of the many molecular “hooks” present on their surface. Their high stability and the possibility of functionalizing them with biomolecules like antibodies and receptors, makes them very important medium in targeted drug delivery (Esfanda and Tomalia, 2001).

2.6 METHODS FOR SYNTHESIS OF SILVER NANOPARTICLES

Reducing the particle size of materials is an efficient tool for improving their bioactivity. A number of preparation methods have been described for the synthesis of metallic nanoparticles (Pal *et al.*, 2007) such as, reverse micelles process (Xie *et al.*, 2006), salt reduction (Pillai and Kamat, 2004), microwave dielectric heating reduction (Patel *et al.*, 2005), ultrasonic irradiation (Salkar *et al.*, 1999), radiolysis (Karim *et al.*, 2007; Remita *et al.*, 2007), solvothermal synthesis (Starowicz *et al.*, 2006), electrochemical synthesis (Liu *et al.*, 2001) and biological techniques (Naik *et al.*, 2002). The most widespread method of synthesis of metallic nanoparticles is based on the chemical reduction of metal salt solution by a reducing agent (Szczepanowicz *et al.*, 2010).

But the chemicals used in such processes are quite often toxic and flammable which make them unsuitable for many applications including biological applications like impregnation in wound dressings (Maneerung *et al.*, 2008; Vivekanandhan *et al.*, 2012) and sutures (Augustine and Rajarathinam, 2012). Biological synthesis of nanoparticles are not only a good way to fabricate benign nanostructure materials, but also to reduce the use or generation of hazardous substances to human health and the environment (Jacob *et al.*, 2012). These biological methods are regarded as safe, sustainable, cost-effective and environment friendly as well as they do not require any special culture preparation and isolation techniques (Gardea *et al.*, 2003). Biosynthesis of silver nanoparticles using microorganisms like bacteria (Saifuddin *et al.*, 2009), fungi (Duran *et al.*, 2009) and yeast (Kowshik *et al.*, 2003) are already reported. However, exploitation of the plant extracts as the potential agents for the biosynthesis of nanoparticles has opened a new way-the green synthesis of nanoparticles (Augustine *et al.*, 2013).

Nanoparticles can be synthesized using various approaches including chemical, physical, and biological. Although chemical method of synthesis requires short period of time for synthesis of large quantity of nanoparticles, this method requires capping agents for size and stabilization of the nanoparticles. Chemicals used for nanoparticles synthesis and stabilization are toxic and lead

to non-ecofriendly byproducts. The need for environmental non-toxic synthetic protocols for nanoparticles synthesis leads to the developing interest in biological approaches which are free from the use of toxic chemicals as byproducts. Thus, there is an increasing demand for “green nanotechnology”. Many biological approaches for both extracellular and intracellular nanoparticles synthesis have been reported till date using microorganisms including bacteria, fungi and plants (Singhal *et al.*, 2011).

Nanoparticles can be prepared using a variety of chemicals and physical methods, including chemical reduction, photochemical reduction, electrochemical reduction and heat vaporization. The reagents can be inorganic compounds, such as hydrazine, sodium/potassium borohydrate and salts of tartrate or organic compounds, like ascorbic acid, sodium citrate and amino acids, which are capable of being oxidized. Because noble metal nanoparticles are now widely used in areas of human contact, there is a need to develop environmentally friendly processes that do not use toxic chemicals in their synthesis. A quest for an environmentally sustainable synthetic process has led to several biometric approaches, one of the fundamental processes of which involves bioreduction (Arunachalam *et al.*, 2013).

There are various choices for the biological synthesis of nanoparticles. For example synthesis of particles using microorganisms, enzymes, fungicide plants or plant extracts (Dubey *et al.*, 2009; Jae and Beom, 2009) have been suggested as alternatives for chemical and physical methods. At times it is advantageous to synthesis nanoparticles using plants or parts of plants over other biological processes (Ponarulselvam *et al.*, 2012).

2.7 PLANT MEDIATED SILVER NANOPARTICLE SYNTHESIS

Plant mediated nanoparticles synthesis has led to a remarkable progress via unfolding a green synthesis protocol towards nanoparticles synthesis. It seems to have drawn quite an unequivocal attention with a view of reformulating the novel strategies as alternatives for popular conventional

methods. The production of nanoparticles by plants relies on various factors among which, type of processing with optimized parameters is very much essential towards synthesis of nanoparticles such as growing plant in a media incorporated with raw material for the synthesis of nanoparticles, use of dried powdered plant material which is employed in the synthesis of plant material, drying plant material and evaluating nanoparticles synthesis and employing fruits and flowers in the synthesis of nanoparticles (Baker *et al.*, 2013).

The recent development and implementation of advance technologies have emerged the nano-revolution which provides the tools and technology as platforms for the investigation of biological entities which offer inspiration models for bio-assembled components toward synthesis of nanoparticles. Biosynthesis of nanoparticles is a type of bottom up approach which employs a biological system or its components for the formation of nanoparticles, where the main reaction is reduction of raw metal into nanoparticles (Li *et al.*, 2007).

The production of nanoparticles by plants were known to be stable than the nanoparticles synthesized by microorganisms. The use of plants in synthesis of nanoparticles has become one of the popular alternatives for conventional methods. In recent years, epoch research on plants has gone through the remarkable progress with current upsurge in plant research in synthesis of nanoparticles with controlled size and shape. Thus, these unique properties of metal tolerance by plants have been exploited with respect to nanoparticles synthesis (Iravani, 2011).

Plant mediated synthesis of nanoparticles is conferred due to the presence of biomolecules such as proteins, amino acids, vitamins, polysaccharides, polyphenols, terpenoids, and organic acids such as citrates etc. present in the plants as their phytochemicals. Apart from mediating the synthesis, these molecules also stabilize the nanoparticles formed with desired size and shape (Baker *et al.*, 2013).

Green nanoparticle synthesis has evolved into an important branch of nanotechnology because of its potential application in the biomedical, magnetics, energy science and aerospace industries. Large amounts of nanoparticles can be easily synthesized from plants and the majority of these are nontoxic (Braditchote *et al.*, 2009). They have been used in pharmaceutical drug- based industries to treat B-chronic lymphocytic leukemia, for detecting DNA, to inhibit bacteria and fungi (Parashar *et al.*, 2009).

2.8 ANTIMICROBIAL ACTIVITY

The size and the specific surface area of the silver nanoparticle affect the antibacterial activity. Specifically, the toxicity of AgNPs to *E. coli* is proportional to the relative surface area of silver oxide monolayers, which dissolve and release Ag ions upon contact with water (Sotiriou *et al.*, 2012).

The application of silver nanoparticles as an antimicrobial agent was investigated and exhibited better antimicrobial activity against all human pathogens. Additionally, the silver nanoparticles showed good inhibition activity towards *C. albicans*. The mechanism of inhibitory action of silver nanoparticles on microorganisms is not very well known (Shameli *et al.*, 2012).

However, several mechanisms have been proposed to explain the inhibitory effect of silver nanoparticles on bacteria. It is assumed that the high affinity of silver towards sulfur and phosphorus is the key element of the antimicrobial effect. Due to the abundance of sulfur containing proteins on the bacterial cell membrane, silver nanoparticles can react with sulfur- containing amino acids inside or outside the cell membrane, which in turn affects bacterial cell viability. It was also suggested that silver ions (particularly Ag⁺) released from silver nanoparticles can interact with phosphorus moieties in DNA, resulting in inactivation of DNA replication, or reacting with sulfur containing proteins, leading to the inhibition of enzyme functions which results in loss of cell viability and eventually resulting in cell death (Kim *et al.*, 2011).

Escherichia coli, *Bacillus subtilis*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Syphilis typhus*, and *Staphylococcus aureus* are some of the organisms against which the silver nanoparticles show a potential antibacterial effect (Iravani and Zolfaghari, 2013).

2.8.1 EFFECT OF AgNP ON *Escherichia coli*

The growth inhibition of *C. albicans* was double in silver nanoparticles when compared with gold nanoparticles. The antibacterial effect was more pronounced in Gram-negative bacteria than Gram-positive ones. The antimicrobial activity of colloidal silver particles is influenced by the particle dimensions (Kaviya *et al.*, 2011). Silver has long been recognized as having an inhibitory effect on microbes present in medical and industrial processes. Gold nanoparticles are harmful to bacteria and fungi. They bind closely to the surface of microorganisms, causing visible damage to cells, with complete destruction of flagella, stimulated production of biofilm, and aggregation within the biofilm. Comparison of gold and silver nanoparticle sizes indicates the latter to be smaller than the former. The smaller particles have greater antimicrobial effects (Geethalakshmi and Sarada, 2012).

2.9 MICROORGANISM–CAUSATIVE AGENT OF INFECTIOUS DISEASES

The development of antimicrobial agents has been undeniably one of the greatest accomplishments of modern medicine. In recent years, multiple drug resistance in both human and plant pathogens has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases (Rai *et al.*, 2011).

Bacterial and fungal pathogens are the important cause of morbidity and mortality worldwide and have a major impact on public health. They are clinically important bacterial pathogens that cause food poisoning and gastroenteritis in millions of people worldwide every year (Vasudha *et al.*, 2011).

2.9.1 BACTERIAL STRAINS

Pseudomonas aeruginosa is a Gram negative, aerobic, rod shaped bacterium with unipolar motility. It is an opportunistic human pathogen (Bennasar, 2010).

Escherichia coli, a Gram negative, rod shaped bacterium that is commonly found in the lower intestine of warm – blooded organisms (Bradley and Williams, 2011). *E. coli* strain can be used as a probiotic agent in medicine, mainly for the treatment of various gastroenterological diseases (Berger and Sodha, 2010).

Staphylococcus aureus, a member of the family *Micrococcaceae*, is a Gram-positive bacterium that normally colonizes the epithelial surface in 30 to 40% of humans. Despite advances in antimicrobial therapy, *S. aureus* remains a major cause of infections in the hospital setting (Ingavale *et al.*, 2005).

Salmonella typhi belongs to the family *Enterobacteriaceae*. It is a motile, facultative anaerobe that is susceptible to various antibiotics. *Salmonella typhi* leads to the development of typhoid or enteric fever. It is characterized by the sudden onset of a sustained and systemic fever, severe headache, nausea and loss of appetite (Mandal *et al.*, 2011).

2.10 PLANTS AS A SOURCE OF MEDICINE

Researchers are increasingly turning their attention to natural products and looking for new leads to develop better drugs against cancer, as well as viral and microbial infections. Several synthetic antibiotics are employed in the treatment of infections and communicable diseases (Malar *et al.*, 2011).

Medicines have made large contributions to human health and well being. In our country we are using crude plants as medicine since Vedic period. A major part of the total population in developing countries still uses traditional folk medicine obtained from plant resources. In the present era, plant and herb

resources are abundant, but these resources are dwindling fast due to the onwardmarch of civilization (Molla *et al.*, 2010).

Medicinal plants represent a rich source from which antimicrobial agents obtained. The antimicrobial activities of plants may reside in a variety of different compounds. Antimicrobial drugs have caused a dramatic change not only for the treatment of infectious diseases but also for the fate of mankind. Antimicrobial chemotherapy made remarkable advances, resulting in the overly optimistic view that infectious diseases would be conquered in the near future (Saga and Yamaguchi, 2009).

India has great diversity of medicinal plants and these medicinal plants are used in Ayurvedic, Unani medicines and a number of pharmaceutical products (Jain *et al.*, 2013). Application of green chemistry to the synthesis of nanomaterials has much importance in basis of medicinal and technological aspects (Begum *et al.*, 2009; Mondal *et al.*, 2011).

Ageratina adenophora (Spreng.) is a perennial, herbaceous invasive plant which has invaded around 30 countries in tropical and subtropical zones of the world (Wang, 2005).

A.adenophora is seldom attacked by bacteria, fungi and insects, suggesting that rich bioactive secondary metabolites that might be defense related might exist in this plant. Previously, structurally diverse chemicals including (mono-, sesqui-, di-, and tri-) terpenoids, phenylpropanoids, flavonoids, coumarins, sterols and alkaloids were reported from this species some of which were shown to possess allelopathic, phytotoxic and antifeedant activities (Zhao *et al.*, 2009; Zheng *et al.*, 2012).

Presence of flavonoid glycosides in the leaves has been reported. Many flavonol glycosides: 4'- methylquercetagetin 7-O-(6''-E-caffeoyl- β -d-glucoside) and quercetagetin 7-O-(6''-acetyl- β - d-glucoside), 7-O-glucosides of 6-hydroxykaempferol, quercetin and quercetagetin, 3,5,7- Trihydroxy-6,4'- dimethoxyflavone (betuletol and its 3-galactoside) have been isolated and

characterized from the leaves of *A. adenophora* (Yang *et al.*, 2013). A new quinic acid derivative, 5-O-trans-o-coumaroylquinic acid, was isolated from the aerial parts of the invasive plant *A. adenophora* (Spreng.), along with chlorogenic acid methyl ester, macranthoin F and macranthoin G. All the compounds showed in vitro antibacterial activity towards the assayed bacterial strains (Zhang *et al.*, 2013).

The methodology adopted in the present study is presented in the next chapter.

3.0 METHODOLOGY

Plants have been an important source of medicine for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine (Preethi *et al.*, 2010). Plants are used in the treatment of bacterial and fungal infections for its wide range of bioactive molecule (Sajad *et al.*, 2011).

The present study was aimed to screen the effect of silver nanoparticles conjugated with *A. adenophora* leaf extract for their antimicrobial activity.

3.1 COLLECTION OF PLANT SAMPLE

The leaves of the plant sample were collected from in and around the Nilgiri district of Tamil Nadu. The plant sample is authenticated from Tamil Nadu Agricultural University (TNAU), Coimbatore.

3.2 PHYTOCHEMICAL ANALYSIS

To analyse the phytochemical from the methanolic extract of *Ageratina adenophora* leaves. The procedure is outlined in the **Appendix I**.

3.3 THIN LAYER CHROMATOGRAPHY

To characterize the thin layer chromatography for analysed phytochemicals. The procedure is outlined in the **Appendix II**.

3.4 PREPARATION OF METHANOLIC EXTRACT

Fresh leaves of *A. adenophora* were collected and cleaned to remove adhering dust particles, washed under running tap water, gently blotted dry between folds of tissue paper. 10g of leaf sample was weighed, cut into small pieces and added to 100ml of methanol. This was stored in dark with mild shaking for 24 hrs. The mixture was then filtered through Whatman No. 1 filter paper. The final extract was stored at 4°C for further experiments.

3.5 PREPARATION OF SILVER NANOPARTICLES

Silver nanoparticles were prepared from the methanolic extract of *A. adenophora* leaves. To 10 ml of the leaf extract 90 ml of 1mM silver nitrate solution was added (Donda *et al.*, 2013). The extent of nanoparticles synthesis was monitored by measuring the absorbance at 400-800nm.

3.5.1 ROOM TEMPERATURE

The samples and silver nitrate solution were incubated at room temperature for 72 hrs (Paulkumar *et al.*, 2014).

3.5.2 HEATING IN WATER BATH

The methanolic extract of *A. adenophora* leaves in the presence of silver nitrate was heated for various durations (5, 10, 15 and 20 min) in a water bath at a temperature of 60° C (Gulcin *et al.*, 2011; Mubarakali *et al.*, 2011).

3.5.3 HEATING BY MICROWAVE

The mixture of methanolic extract of leaves with silver nitrate solution was heated in microwave for various durations namely 10, 20, 30 and 40 seconds (Nooroozi *et al.*, 2012).

3.5.4 EXPOSURE TO SUNLIGHT

The methanolic extracts of *A. adenophora* with silver nitrate solution were exposed to sunlight for various durations (5, 10, 15 and 20 min) with silver nitrate solution (Sulaiman *et al.*, 2013).

Plate 1

Ageratina adenophora



3.5.5 SEPARATION OF SILVER NANOPARTICLES

To separate the synthesized silver nanoparticles, samples were centrifuged at 13,000 rpm for 20 min under refrigeration and washed 3 times with deionized water. A dried powder of the silver nanoparticles was obtained by freeze drying.

3.6 CHARACTERIZATION OF SILVER NANOPARTICLES

The synthesized silver nanoparticles were characterized as per the methods explained below.

3.6.1 UV-VISIBLE SPECTRA

The light nanoparticles like silver (Ag) exhibit unique and tunable optical properties on account of their Surface Plasmon Resonance (SPR). A volume of 100 μ l of synthesized nanoparticles sample was diluted with 900 μ l silver nitrate solution and subjected to spectral analysis using an UV-Visible nanophotometer (Optizen, Korea) in a scanning range of 400 nm to 600 nm.

3.6.2 X-RAY DIFFRACTION (XRD)

The XRD technique was used for the determination and confirmation of the crystal structure of silver nanoparticles. The sample for XRD analysis was prepared by depositing the centrifuged sample on a microscopic glass slide and it was air-dried overnight. The diffractogram was recorded from PAN analytical, XPERT- PRO diffractometer using CuK α ($\lambda=1.54060$) as X-ray source.

3.6.3 ZETA POTENTIAL

Zeta potential is another important parameter that is related to nanoparticle stability or aggregation in a dispersion, and can have significant implications on product performance.

Light scattering, especially dynamic light scattering, and electrophoretic light scattering are now the standard techniques for measuring the size and

zeta potential of dispersed nanoparticles. Two new techniques, Nanoparticle Tracking Analysis (NTA), and also Resonant Mass Measurement (RMM) can also be used for sizing nanoparticles as well as counting the number of particles present in a sample, and both are especially suited to polydisperse samples.

3.7 TEST MICROORGANISM

The bacterial strains used in the present study were the clinical isolates maintained in our laboratory. The bacterial strains used were *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

3.7.1 ANTIBACTERIAL ASSAY

Preparation of medium from Mueller Hinton Agar medium. This medium was prepared by dissolving 33.9g of the commercially available Mueller Hinton Agar Medium (Hi-Media) in 1000ml of distilled water. The effects of silver nanoparticles conjugated with *A.adenophora* leaf extract on the several bacterial strains were assayed by antibacterial method. The medium was autoclaved at 151 bs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto petri plates (25-30ml/plate) while still molten. Preparation of the test culture Inoculums of the microorganism were prepared from overnight culture grown in nutrient broth and the suspension was adjusted with a turbidity equivalent to that of 0.5 MacFarland standards. The procedure is outlined in the **Appendix III**.

4.0 RESULTS AND DISCUSSION

Nanotechnology is a rapidly growing multidisciplinary field which includes many branches of science like physics, material sciences, biotechnology, super molecular chemistry, chemical engineering, mechanical engineering and medicine (Donda *et al.*, 2013).

Nanotechnology manipulates matters and creates new nanoproducts with novel properties at an atomic scale (Ciobanu *et al.*, 2013). In the modern field of material science nanotechnology is one of the upcoming areas of research (Geoprincy *et al.*, 2013).

Medicinal plants represent a rich source of antimicrobial agents. Plants have a great potential for producing great benefit to mankind. There are many approaches to search for biologically active principles in plants (Malar *et al.*, 2011).

Medicinal plants are nature's gift to cure limitless number of diseases among human beings. The abundance of plants on the earth's surface has led to an increasing interest in the investigation of different extracts obtained from traditional medicinal plants as potential sources of new antimicrobial agents (Bushra and Ganga, 2011). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are flavanoids, alkaloids, phenolic compounds and tannins (Edeoga, 2011).

In this study, the methanolic extract of *A. adenophora* leaves were used to prepare silver nanoparticles. The study was formulated to optimize and compare the methods of synthesis of silver nanoparticles from the methanolic extract of *A. adenophora* leaves and the antimicrobial activity against clinical isolates was analysed. The bacterial strains used are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* were used.

The results and discussion were presented as follows.

4.1. Phytochemical analysis

4.2. Thin layer chromatography

4.3. Characterization of silver nanoparticles

4.3.1. UV-Visible spectroscopic studies

4.3.2. X-Ray Diffraction studies

4.3.3. Zeta potential analysis

4.4. Antibacterial activity

Phytochemical analysis of *Ageratina adenophora*

Table-1

S.No.	Parameters	Result
1	Alkaloids	+
2	Flavonoids	+
3	Steroids	+
4	Terpenoids	-
5	Tannins	-
6	Phenols	-
7	Saponins	-

4.1. PHYTOCHEMICAL ANALYSIS

The results of the phytochemical analysis of the leaves of *Ageratina adenophora* is presented in the **Table-1**. The methanolic extract of the leaves were analysed to detect the phytochemical constituents present.

The qualitative analysis results revealed the presence of flavonoid, alkaloid and steroid which are the high source of secondary metabolites. It is interesting to note the absence of terpenoids, tannins, phenols and saponins of

the above selected plant which needs further detailed scanning. The results of phytochemical qualitative study provides positive therapeutic potential present in *Ageratina adenophora*

The presence of alkaloids, flavanoids and steroids in the qualitative analysis emphasis that an attempt can be made to draw a relationship between the medicinal properties of *Ageratina adenophora*

Parekh *et al.*, (2006) suggested that the bark of *Bautinia variegata* L., a medicinal plant contains tannins, alkaloids and saponins.

Raghavendra *et al.*, (2006) reported that the different solvent extracts of *Oxalis corniculata* leaf sample revealed the presence of phytochemicals such as carbohydrates, glucosides, phytosterols, phenolic compounds/ tannins, flavonoids, proteins and amino acids.

4.2 THIN LAYER CHROMATOGRAPHY

The results of the TLC of the leaves of *Ageratina adenophora* is presented in the **Plate 2, 3 and 4**. Presence of alkaloids, flavonoids and steroids were further confirmed by TLC. Methanolic extract of *Ageratina adenophora* leaves was subjected to TLC with solvent system specific for alkaloid, flavonoid and steroid.

- The TLC chromatogram of alkaloid showed 6 bands with Rf values 0.05, 0.12, 0.17, 0.27, 0.43 and 0.61 respectively.
- The TLC chromatogram of flavonoid showed 4 bands with Rf values 0.05, 0.21, 0.32 and 0.40 respectively.
- The TLC chromatogram of steroid showed 6 bands with Rf values 0.09, 0.06, 0.14, 0.22, 0.28 and 0.32 respectively.

All the spots were visualized under 254 and 366 nm.

Verma *et al.*, (2012) revealed that the chloroform: methanol system was shown to be most suitable TLC system with the largest discriminating power.

Three bands with Rf values 0.40, 0.45 and 0.48 which indicate the presence of phenolic compounds were obtained.

TLC of methanolic extract of *Ageratina adenophora* leaves

**Plate 2
Alkaloid**



**Plate 3
Flavonoid**



**Plate 4
Steroid**

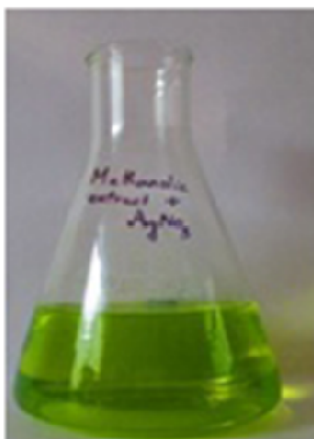


VISIBLE COLOR CHANGE OF AGNPS

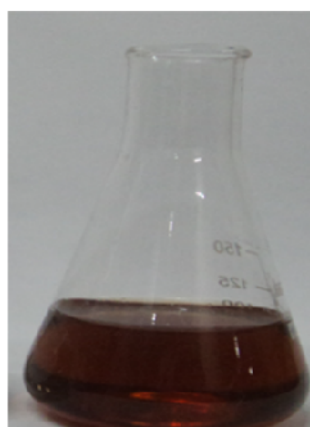
The silver nanoparticles were synthesized using four different methods, namely heating in water bath (60°C), microwave heating, room temperature and exposure to sunlight. The synthesis of nanoparticles was noticed by a change in color and the increase in absorbance. As per the results of the study, all the four methods, namely heating in water bath (60°C), microwave heating, room temperature and exposure to sunlight were efficient in the synthesis of AgNPs. But, there were some difference in the pattern of synthesis of particles between each method.

Visible colour change during the formation of AgNPs

Plate 5



Methanol Extract



Extract containing AgNPs

The silver nanoparticles (AgNPs) synthesized on exposure to sunlight for 20 minutes showed the maximum yield and efficiency after 24 hrs. From the visual color change, preliminary information regarding the formation of silver nanoparticles can be obtained. As the silver nanoparticles are formed, the color of the solution changes from pale green to brown which is an indication of the presence of silver nanoparticles. The variation of the color was due to the change in surface plasmon resonance of silver nanoparticles during the formation.

The fresh suspension of *E. chapmaniana* was initially yellow in colour and after addition of AgNO₃ and exposure to bright sunlight, the suspension turned reddish brown (Sulaiman *et al.*, 2013). (Khan *et al.*, 2013) reported that the addition of *P. glutinosa* plant extract into the aqueous solution of AgNO₃, the color of the solution gradually changed from light yellow to brown, which indicates the formation of AgNPs. Silver nanoparticles exhibited dark reddish-brown colour in aqueous solution due to the surface plasmon resonance phenomenon. Reduction of silver ion into silver nanoparticles during exposure to the plant extracts could be followed by color change (Krishnaraj *et al.*, 2010). (Augustine *et al.* 2013), revealed that the silver nanoparticles are formed, as the color of the solution changes from white to pale yellow to brick red which is an indication of the presence of silver nanoparticles. The variation of the color was due to the change in surface plasmon resonance during the formation of silver nanoparticles.

4.3 CHARACTERIZATION OF SILVER NANOPARTICLES

Characterization of nanoparticles is one of the important aspects which has to be carried out in order to find the structure, shape and charge of the nanoparticles. The fate of nanoparticles mainly depends on the characterization which reveals the size, shape and nature of the nanoparticles.

4.3.1 UV-VISIBLE SPECTROSCOPIC STUDY

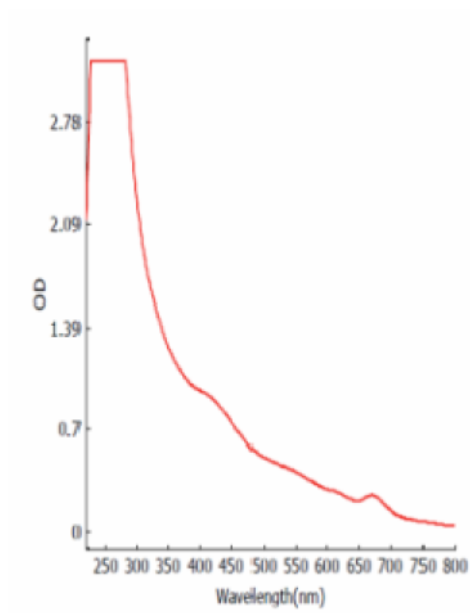
Ultraviolet-visible spectrometry was used to examine the size and shape of the nanoparticles in aqueous suspension. The synthesized silver nanoparticles mediated by *A. adenophora* leaf extract were subjected to optical measurements by UV-visible spectroscopy, showed absorbance peaks within the range 400-450 nm which indicates the presence of silver nanoparticle.

Extinction spectrum of AgNPs synthesized from *A. Adenophora*

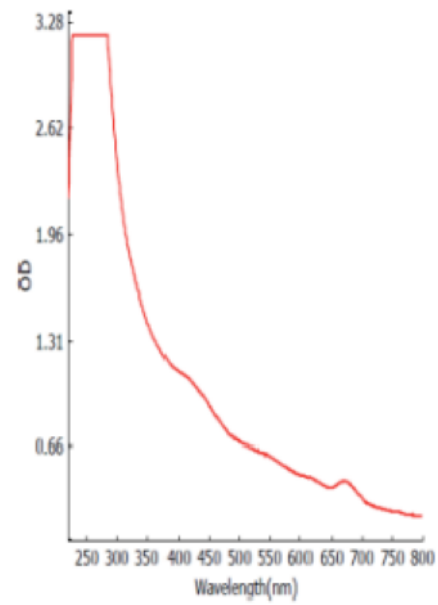
Heating in water bath (60°C)

Figure 1

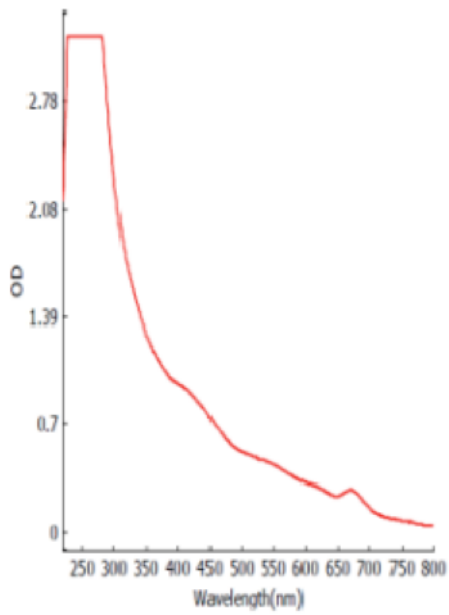
a) 5 minutes



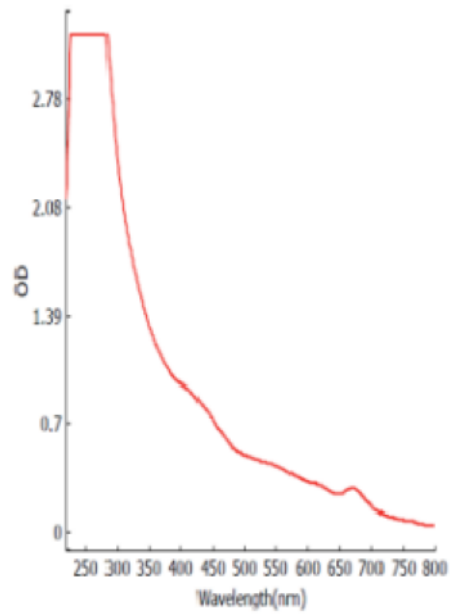
b) 10 minutes



c) 15 minutes

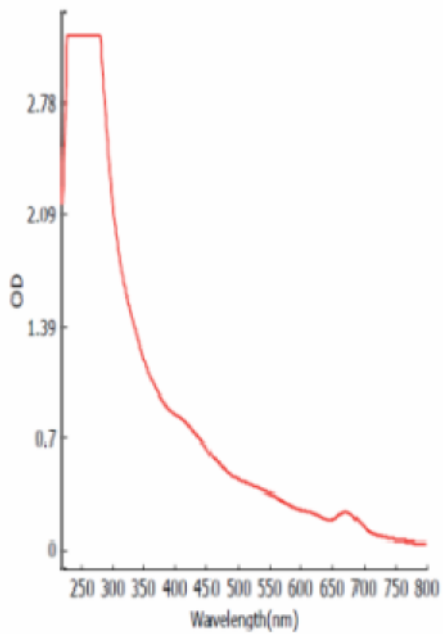


d) 20 minutes

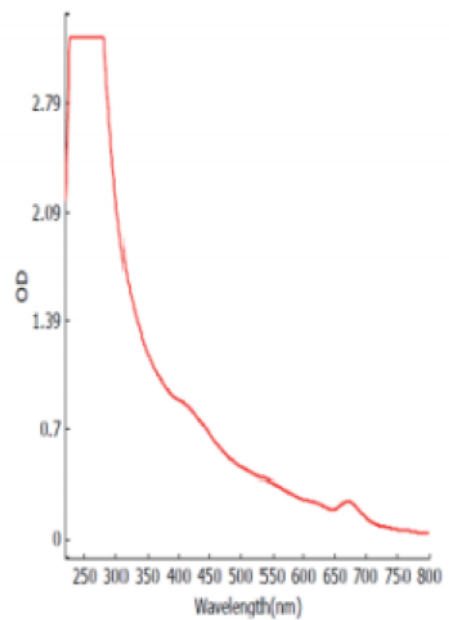


Microwave heating

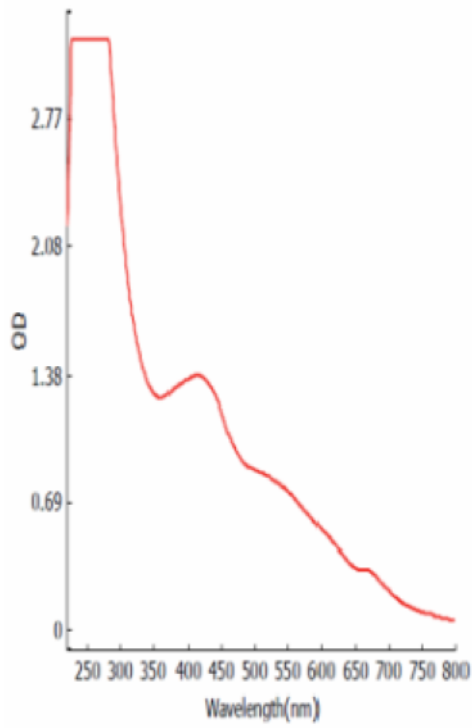
(a) 10 seconds



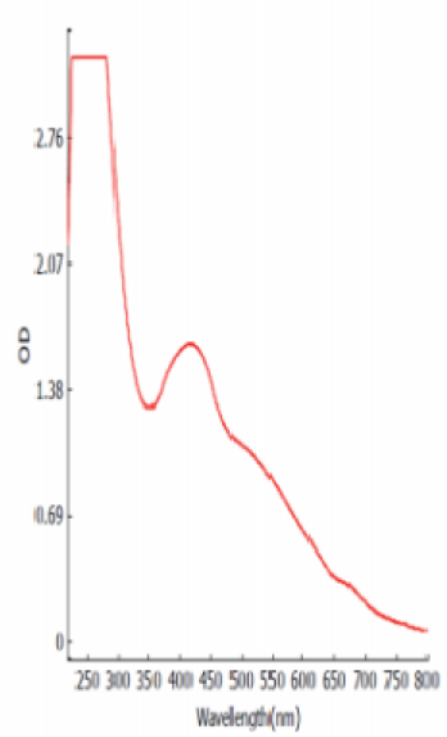
(b) 20 seconds



(c) 30 seconds

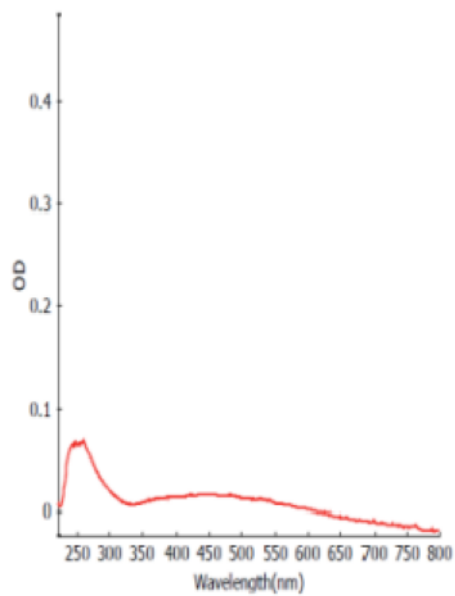


(d) 40 seconds



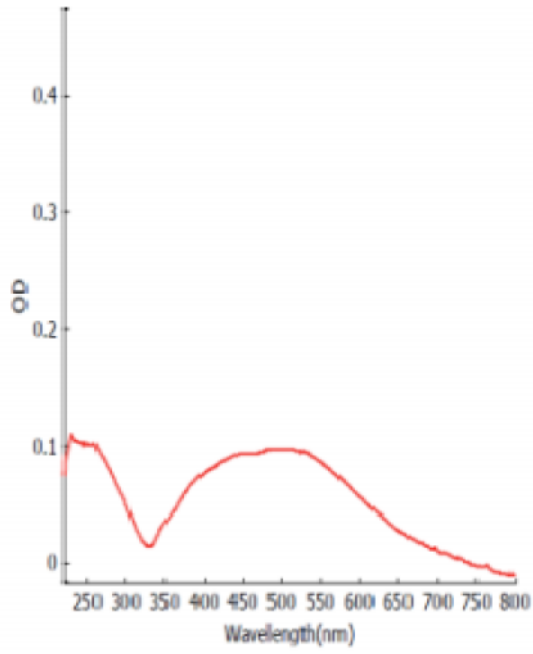
Incubation at room temperature

(a)72 hours

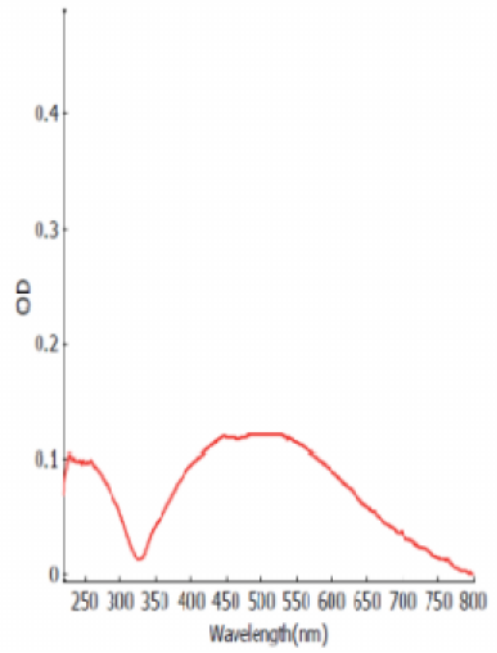


Exposure to sunlight

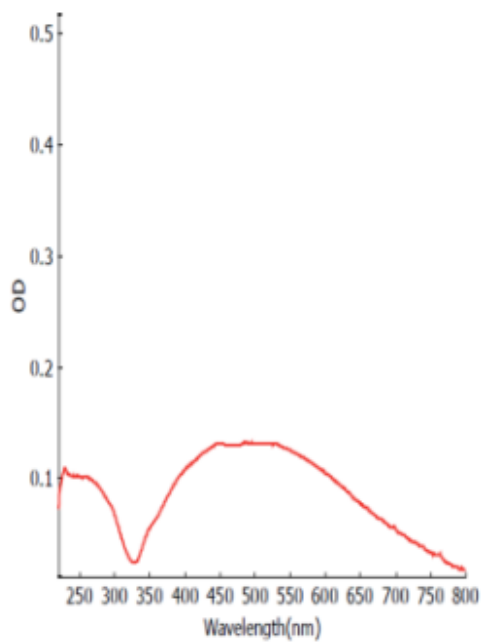
(a) 5 minutes



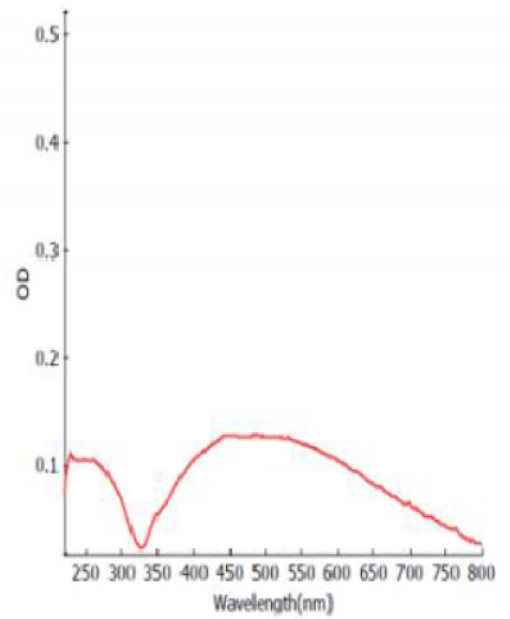
(a)10minutes



(c) 15minutes



(d) 20minutes



The production of the silver nanoparticles synthesized from the *E. chapmaniana* leaf extract was evaluated through spectrophotometry at a wavelength range of 250-800 nm which revealed a characteristic peak at 413 nm for *E. chapmaniana* AgNPs, which confirmed the formation of the silver nanoparticles (Sulaiman *et al.*, 2013). Depending upon the shape and size of the NPs, AgNPs exhibit absorption under a visible range of 380–450 nm (Kumar *et al.*, 2012). Dubey *et al.*, (2009) have reported that the silver surface plasmon resonance band occurs at 412 nm and steadily increases in intensity as a function of time of reaction is observed in the UV-visible spectra recorded from the aqueous silver nitrate and *Eucalyptus hybrida* leaf extract.

Bioreduction of Ag to AgNPs mediated by *D. bulbifera* tuber extract was examined by recording the absorption spectra as a function of time with an absorbance maximum at 450 nm, the yellowish-brown color of silver nanoparticles arises due to excitation of surface Plasmon vibrations (Ghosh *et al.*, 2012). (Karunakar *et al.*, 2013) reported that the UV spectral analysis gave maximum absorption spectra within the range of 450nm-470nm both for *Phyllanthus reticulatus* leaf and root mediated particles which stands as a strong proof to report them as silver nanoparticles.

Formation of stable silver nanoparticles using the seed extract of *Jatropha curcas* aqueous colloidal solution were confirmed using UV– visible spectral analysis with a characteristic SPR at 425nm (Bar *et al.*, 2009).

With reference to the above literature, it is evident that the characteristic increase in absorption in the range of 400- 450nm confirmed the synthesis of silver nanoparticles from *A. adenophora* leaf extract.

4.3.2 X-RAY DIFFRACTION STUDIES

The XRD analysis showed three major peaks at 2 values of 32.0755, 46.0912 and 27.6476 degrees, with intensities of 416, 280 and 177 respectively. Apart from these, significant minor peaks were also observed. The major peaks matched the Bragg's reflections and lattice planes reported for AgNPs of many medicinal plants by several workers (Gnandesigan *et al.*, 2011; Phylip, 2011;

Dipankar and Murugan, 2012; Mitra *et al.*, 2012; Phanjom *et al.*, 2012). The additional minor peaks observed in our study may be suggestive of the crystallization of bioorganic phase that occurs on the surface of the nanoparticles, as proposed by (Ponarulselvam *et al.*, 2012).

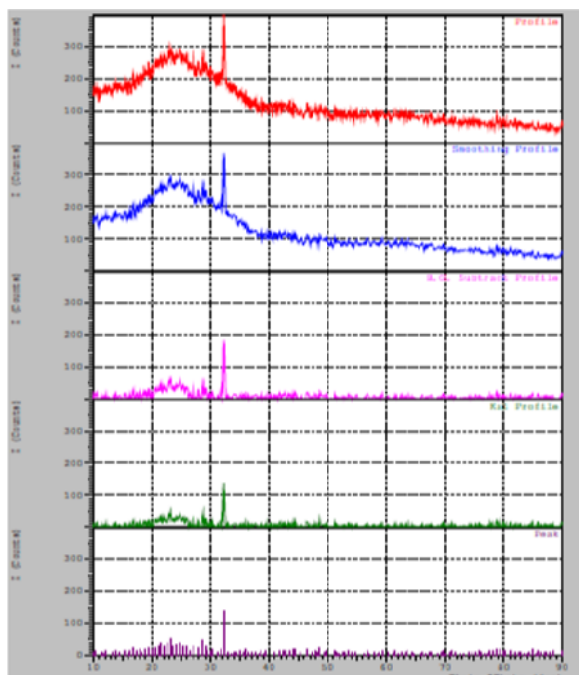
XRD is commonly used for determining the chemical composition and crystal structure of a material; therefore, detecting the presence of silver nanoparticles from the plant tissues can be achieved by using XRD to examine the diffraction peaks of the plant (Vidhu *et al.*, 2011).

The X-ray diffraction (XRD) is proven to be a valuable research tool to confirm the formation of Ag nanoparticles and calculating the crystalline particle size (Zayed *et al.*, 2012). (Baishya *et al.*, 2012) reported that the XRD pattern clearly showed that the silver nanoparticles formed by the reduction of Ag⁺ ions by the *Bryophyllum* leaf extract are crystalline in nature. The XRD pattern of silver nanoparticles synthesized from *Trianthema decandra* showed three intense peaks (Geethalakshmi and Sarada, 2010).

Thus, in the present study, all the physicochemical characteristics (absorption spectrum, zeta potential and XRD spectrum) confirmed the nature of the AgNPs synthesized from *A. Adenophora* leaf extract. Having characterized the AgNps, it was imperative to assay their bioactivity, and bioavailability of the medicinal plant components.

XRD patterns of silver nanoparticles of *Ageratina adenophora* leaf extract

Figure 2



4.3.3 ZETA POTENTIAL ANALYSIS

For the obtained nanoparticles, zeta values were measured and found to fall between -2.5 and -38.3 mV. Values of the zeta potentials of the citrate treated silver nanoparticles in addition to their narrow size distributions provide satisfactory evidence about their little tendency towards aggregation. This behavior unambiguously suggests the presence of strong electric charges on the particles' surfaces to hinder agglomeration.

These values were found to fall in the negative side which showed the efficiency of the capping materials in stabilizing the nanoparticles by providing intensive negative charges that keep all the particles away from Reddick threshold. Charged particles are able to interact across long distances via

electrostatic forces and the surface charge may be disrupted as a way to induce nanoparticles assembly by modifying the condition of the reaction.

In this work, colloidal silver nanoparticles were proven stable over a wide range of the pH with no tendency for aggregation or formation of self assembled aggregates. This observation is contrary to suggestions that surface modification is mandatory to the particles. It is universally accepted that charged particles are capable of interacting across a long distance scale via electrostatic repulsive forces.

Disruption of the surface charges plays a paramount role in the assembly of the nanoparticles. Therefore, using these nanoparticles as a drug may provide a new horizon in avoiding bacterial and fungal resistance.

In the studied series (S1) the electric charges were strong enough to hinder agglomeration and to provide stabilization of the nanoparticles. This suggests that the Ag NPs particles and thus their solution is stable, which is also in accordance with the result reported before for colloidal nanoparticles stability.

Kirthika, *et al.*, (2014) reported that the structure of the silver nanoparticle had an average diameter of 581-d nm and average zeta potential of -13.3 mV which suggested that the silver nanoparticles have a higher stability. The high negative value of zeta potential may be caused due to the various constituents present in the extract

Bhattacharya, *et al.*, (2012) reported that the nanoparticles synthesized from *Phytolacca decandra* had a size of 91 nm, polydispersity was found to be 0.119 and had a zeta potential of -1.56 mV. These nanoparticles exhibited anticancer and antibacterial properties.

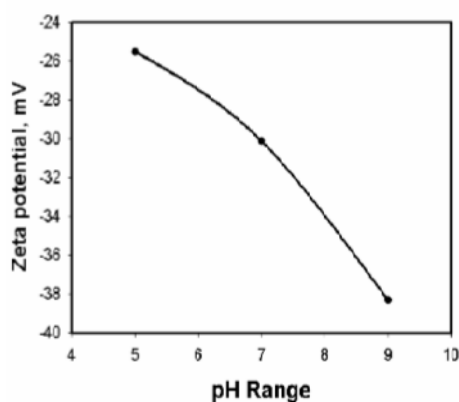
Zeta potential measurements using silver nanoparticles at different pH ranges

Table 2

S.No.	P ^H	Zeta potential
1.	5	-2.5±0.38
2.	7.4	-31.1± 0.41
3.	9	-38.3 ±0.33

Zeta potential of silver nanoparticles of *Ageratina adenophora* leaf extract.

Figure 3



4.4 ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES

In this study, the antimicrobial activity of silver nanoparticles synthesized from *A. adenophora* leaf extract against clinical isolates were analysed. The bacterial strains, used were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, The antibacterial activity

of the AgNPs were tested at 10µg concentration. The results were assessed on the basis of zone of inhibition. Based on the optimization of AgNPs synthesis, it was decided to analyze the AgNPs were synthesized only by exposure to sunlight for 20 minutes. Chloramphenicol was used as the standard antibiotic.

The AgNPs were evaluated for their antibacterial activity against above mentioned bacterial strains. All assays were done in duplicates, the diameter of zone of inhibition was measured in mm. The results obtained for antibacterial activity of AgNPs by antibacterial assay method are presented in **Table 3** and **Plate 6, 7, 8 and 9**. The AgNPs was found to be more effective against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Antimicrobial activity of biosynthesized silver nanoparticles against both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) microorganisms at different concentrations showed that they possessed a strong dose-dependent antimicrobial activity against both of the test microorganisms. The concentration of biosynthesized nanoparticles were increased with decrease in the microbial growth. Biosynthesized silver nanoparticles were observed to exhibit more antimicrobial activity on Gram-negative microorganism than Gram-positive ones (Singhal *et al.*, 2011).

Antibacterial activity of AgNPs

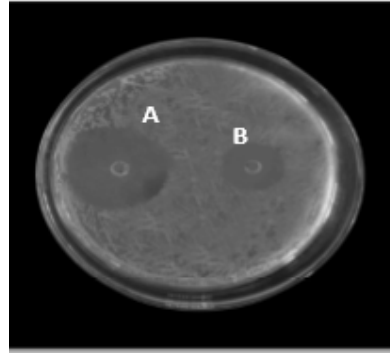
Table 3

S.No	Microorganisms	AgNPs	Control
1.	<i>Escherichia coli</i>	12	21
2.	<i>Staphylococcus aureus</i>	12	19
3.	<i>Pseudomonas aeruginosa</i>	6.0	10
4.	<i>Klebsiella pneumoniae</i>	5.0	15

Green synthesis of silver nanoparticles of *Psidium guajava* showed very strong inhibitory effects against *S. aureus* (Sriram and Pandidurai, 2014).

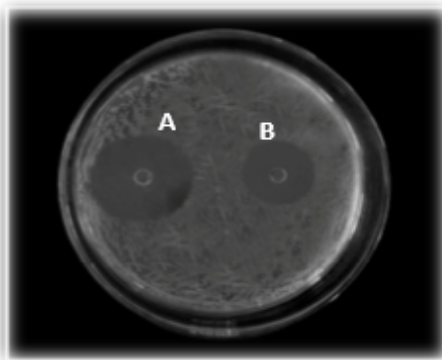
Antibacterial activity of silver nanoparticles

Plate 6



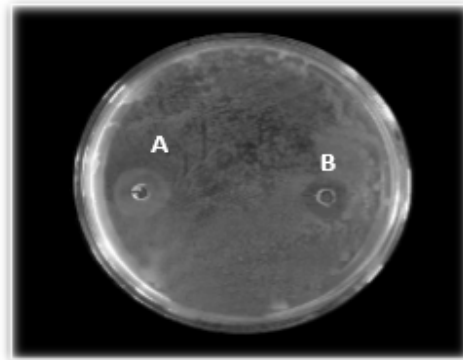
Staphylococcus aureus

Plate 7



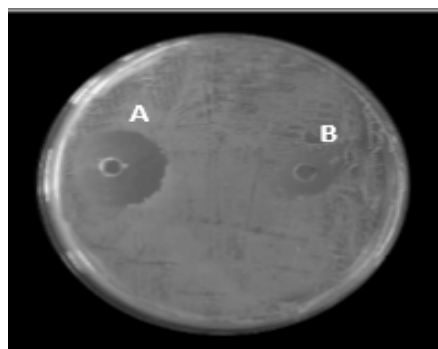
Escherichia coli

Plate 8



Pseudomonas aeruginosa

Plate 9



Klebsiella pneumoniae

Augustine *et al.* (2013) demonstrated that silver nanoparticles synthesized at all the silver nitrate concentrations displayed antibacterial activity against both *E. coli* and *S. aureus*. Nanoparticles prepared using 1 mM silver nitrate was the most efficient to inhibit both *E. coli* and *S. aureus*. There is a decrease in antibacterial activity as the concentration of silver nitrate increased.

With these reports, our study indicates that the AgNPs showed good antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*.

The present findings of this study are summarized and presented with the conclusions drawn, in the following chapter

5.0 SUMMARY AND CONCLUSION

The synthesis of nanoparticles is an emerging area of research due to their use in a variety of biological methods. Silver nanoparticles are widely synthesized due to its applications in various fields. Its applications have been seen mostly in products that come in direct contact with humans. Hence, there is a need to develop biological methods for the synthesis of silver nanoparticles.

In the present study, the synthesis of nanoparticles using the methanolic leaf extract of *Ageratina adenopora* was done to optimize the method for the synthesis of silver nanoparticles and to check their antibacterial activity.

Four different methods like heating at 60°C in a water bath, microwave, at room temperature and exposure to sunlight were used to synthesis the silver nanoparticles. It was found that all the four methods produced the AgNPs efficiently from *Ageratina adenopora* leaf extract. However, exposure to sunlight for 20 minutes was the most quick and efficient method for the synthesis of AgNPs from methanolic leaf extract of *Ageratina adenopora*.

Medicinal plants represent a rich source from which antimicrobial agents were obtained. The antimicrobial activities of plants may reside in a variety of different compounds. Antimicrobial drugs have caused a dramatic change for the treatment of infectious diseases. Antimicrobial chemotherapy made remarkable advances, resulting in the overly optimistic view that infectious diseases would be conquered in the near future.

The synthesis of a newer class of anti-bacterial agents is in need of time, especially against drug-resistant bacteria, such as Gram-positive and Gram-negative strains, which are responsible for a number of serious infections. The search of new antimicrobial agents with reduced toxicity and lower side effects is of continuous process.

The present study entitled "Phytochemical Characterisation and Thin layer Chromatography of *Ageratina adenophora*" was carried out for screening and separating the phytochemical constituents in the methanolic extract of *Ageratina adenophora* leaves. In our present study, the methanolic extract of *Ageratina adenophora* leaves were prepared and screened for the alkaloids, flavonoids,

tannins, saponins, terpenoids, phenolic compounds and steroids. The result comprehensively and conclusively validates that the effect of *Ageratina adenophora* holds a good store of secondary metabolites and found to be alkaloids, flavanoids and steroids. Hence, this interesting phytochemicals screening for *Ageratina adenophora* was performed to exhibit its therapeutic potential.

The AgNPs synthesized by various methods were subjected to absorption spectrum in both UV and visible range, and the spectra showed a characteristic peak between 400-450nm. The range indicates that synthesis of silver nanoparticles increased on increasing the time of exposure, confirming that the immediate colour change was due to the synthesis of AgNPs.

Thus, the present study confirms that the *A.adenophora* leaf extract readily forms AgNPs, by incubation with AgNO₃. Though all the methods tested yielded high amounts of AgNPs, the maximum bioactivity resided with the AgNPs synthesized by exposure to sunlight for 20 minutes. The AgNPs of *A.adenophora* leaf extract were characterized using physicochemical techniques (absorption spectrum, zeta potential, XRD pattern), which showed the definite characteristics of AgNPs. The AgNPs also possessed strong antibacterial activity and less hemolytic activity, making this medicinally important plant more efficient for medicinal uses.

It elicits on all the aspects of the herb and throws the attention to set the mind of the researchers to carry out the work for developing its various formulations, which can ultimately be beneficial for the human beings as well as animals

SUGGESTIONS FOR FUTURE RESEARCH

- Other than AgNPs, other metal NPs can be synthesised using leaves of *A.adenophora* and their bioactivity can be compared.
- The cytotoxic effects can be studied using *in vitro* models.
- The anticancer activity of the synthesised nanoparticles can be studied.

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APPENDIX I

PHYTOCHEMICAL ANALYSIS

(Santhi *et al.*, 2011)

PROCEDURE:

DETECTION OF ALKALOIDS

- a. **Mayer's test:** A fraction of the extract was treated with Mayer's reagent (1.36g of Mercuric chlorate and 5g of Potassium iodide in 100ml distilled water) and noted for a cream colored precipitate.
- b. **Dragendroff's test:** A fraction of the extract was treated with Dragendroff's reagent and observed for the formation of reddish orange precipitate.
- c. **Wagner's test:** A fraction of the extract was treated with Wagner's reagent (1.27g of Iodine and 2g of Potassium iodide in 100ml of distilled water) and observed for the formation of reddish brown precipitate.

DETECTION OF PHENOLIC COMPOUNDS

- a. **Ferric chloride test:** A fraction of the extract was treated with 5% FeCl₃ solution and observed for the formation of deep blue colour.
- b. **Lead acetate test:** A fraction of the extract was treated with 10% lead acetate solution and observed for the formation of white precipitate.

DETECTION OF FLAVONOIDS

- a. **Aqueous NaOH test:** To a fraction of the extract, 1N aqueous NaOH was added and observed for the formation of yellow-orange colour.
- b. **Concentrated H₂SO₄ test:** To a small fraction of the extract, concentrated H₂SO₄ was added and observed the orange colour formed.

c. **Schinodo's test:** To a small fraction of the extract, a piece of magnesium turnings was added, followed by concentrated HCL and then heated slightly and the formation of dark pink colour was observed.

DETECTION OF SAPONINS

a. **Froth test:** A fraction of the extract was vigorously shaken with water and observed for persistent foam which lasts for at least 15minutes.

b. **Emulsion test:** This was performed by adding two drops, of olive oil to the frothing solution and shaken vigorously. The formation of emulsion indicated the presence of saponins.

DETECTION OF TANNINS

a. **Braemer's test:** To 0.5g of methanolic extract added 10ml of water, boiled and filtered. To the filtrate few drops of 10%FeCl₃ was added. A dark green, blue or brown color.

DETECTION OF STEROIDS

a. **Libermann-Buchard test:** To the methanolic extract added 2ml of chloroform followed by 10 drops of acetic anhydride and 2 drops of Conc.H₂SO₄ and the rose red color developed quickly changed through blue to green indicated the presence of cholesterol.

b. **Salkowski test:** The methanolic extract was dissolved in chloroform and shaken with an equal volume of Conc.H₂SO₄ and the presence of red color in chloroform layer and green fluorescence in acid layer indicated the presence of sterols.

DETECTION OF TERPENOIDS

To the methanolic extract added 2ml of chloroform. To this chloroform extract added 5ml of Conc.H₂SO₄ along the sides of the test tube. A reddish brown coloration in the interphase indicated the presence of terpenoids.

Once the phytochemical present in the seeds were identified, we performed thin layer chromatography (TLC) to separate each fraction.

APPENDIX II

THIN LAYER CHROMATOGRAPHY

The methanolic extract of seeds were subjected to Thin Layer Chromatography on silica gel G60 F254 plates (EMerck) using different solvent systems specific for the phytochemical constituent and were developed by spraying 10% H₂SO₄.

S.No	Phytochemical constituent analyzed	Solvent system
1	Alkaloids	Ethyl acetate-Methanol-Water (10 : 1.35 : 1)
2	Flavanoids	Ethyl acetate-Butanone-Formic acid-Water (5 : 3 : 1 : 1)
3	Steroids	Toluene-Methanol (9: 1)

REQUIREMENTS

- ❖ Glass plate / TLC plate
- ❖ Glass tank with lid
- ❖ Spreader
- ❖ Adsorbent silica gel
- ❖ Mobile phase

PROCEDURE FOR TLC

Spot the sample to dry so that spotting can be done repeatedly for more concentrated sample spot. Poured the developing solvent into a tank to a depth of 1.5cm after equilibrium, removed the cover plate and placed the TLC plate (sample applied) vertically in the tank so that it stands in the solvent with the spotted end dipping in the solvent. The separation of the compounds occurs as the solvent moves upward. Once the solvent reaches the top of the plate, removed it from the tank, dried and sprayed with spraying reagent for the identification of separated compounds.

The R_f values of the spots were calculated as the ratio of the distance travelled by the solute to that by the solvent front

APPENDIX III

ANTIBACTERIAL ACTIVITY

(Chauhan *et al.*, 2010)

PRINCIPLE

This technique is employed to assay the antibacterial activity present in silver nanoparticles. The silver nanoparticles are allowed to diffuse into the well and interact in a plate freshly seeded with the test organisms. The diameter of the zone of inhibition can be measured in millimeters.

REAGENTS

1. Ampicillin (standard antibacterial agent)-concentration.
2. Nutrient agar.

Media composition:

30g – beef extract, 1.75g-agar made upto 100ml-distilled water.

The p^H was adjusted to neutral. The medium was autoclaved at 121±1°c for 15minutes. After cooling to 50°c, the solution was poured into sterile petri plates and allowed to solidify.

3. Test microorganisms: *Staphylococcus aureus*, *Escherchia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*

PROCEDURE

Petri plates containing 20ml nutrient agar medium were seeded with 24hours culture of bacterial strains. Wells were cut and various concentration of silver nanoparticles (10µg, 20µg, 30µg, 40µg, 50µg) were added. The plates were then incubated at 37°c for 24hours. Streptomycin was use as a positive control. The antibacterial activity was determined by measuring the diameter of the inhibition zone formed around the well.