GREEN SYNTHESISED SILVER NANOPARTICLES FOR THE SUPPRESSION OF ALGAL GROWTH

by

B. L. BIJULA

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THESIS

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VELLAYANI, THIRUVANANTHAPURAM - 695 522

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DECLARATION

I, hereby declare that this thesis entitled **"GREEN SYNTHESISED SILVER NANOPARTICLES FOR THE SUPPRESSION OF ALGAL GROWTH"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani,

Date: 29/04/2019

B. L. Bijula

(2016-11-087)

CERTIFICATE

Certified that this thesis entitled **"GREEN SYNTHESISED SILVER NANOPARTICLES FOR THE SUPPRESSION OF ALGAL GROWTH"** is a record of research work done independently by Ms. B. L. Bijula (2016-11-087) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellayani,
Date: 29/4/2019

Swapna A

Dr. Swapna Alex
(Major Advisor, Advisory Committee)
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani

CERTIFICATE

We, the undersigned members of the advisory committee of Ms. B. L. Bijula (2016-11-087) a candidate for the degree of **Master of Science in Agriculture** with major in Plant Biotechnology, agree that the thesis entitled "**GREEN SYNTHESISED SILVER NANOPARTICLES FOR THE SUPPRESSION OF ALGAL GROWTH**" may be submitted by Ms. B. L. Bijula (2016-11-087), in partial fulfillment of the requirement for the degree.

Dr. Swapna Alex
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani

Dr. K.B.Soni
Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani

Dr. Deepa S. Nair
Assistant Professor and Head
Department of Plantation crops and Spices
College of Agriculture, Vellayani

Dr. Beena R.
Assistant Professor
Department of Plant Physiology
College of Agriculture, Vellayani

Dr. Joy M.
Associate Professor and Head
Department of Plant Pathology
College of Agriculture, Vellayani

(EXTERNAL EXAMINER)
Dr. Radhamany P. M.
Professor
Department of Botany
University of Kerala, Kariavattom

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LIST OF ABBREVIATIONS AND SYMBOLS USED

BBM	Bold's basal medium
AgNPs	Silver nanoparticles
$AgNO_3$	Silver Nitrate
°C	Degree Celsius
g	Gram
h	Hours
Fig.	Figure
µl	Micro litre
µg	Microgram
ml	Milli litre
mm	Milli metre
mg	Milligram
min	Minutes
viz.	Namely
%	Percent
et al.	And other co-workers
cfu	Colony forming units
No.	Number
sp or spp.	Species (Singular and Plural)
GS	Green synthesised
CS	Chemically synthesised

Introduction

1. INTRODUCTION

The term "nanotechnology" was coined by the Japanese scientist, Norio Taniguchi of the Tokyo University of Science. Nanotechnology is a branch of modern science that deals with particles having dimensions of nanoscale (1-100 nm). It provides the possibility of engineering the physical and chemical properties of a material by reducing its size which in turn attributes a wider range of applications of the material.

There are many conventional chemical and physical methods for the synthesis of nanoparticles. However, biological methods offer a much economical and eco-friendly route for synthesising nanoparticles (Feynman, 1959; Husen and Siddiqui, 2014). Biosynthesis is a bottom-up approach where the main reaction is oxidation/ reduction and it can be easily scaled up for large scale synthesis with very low energy, pressure, temperature and toxicity (Iravani *et al.*, 2014).

Green synthesis of nanoparticles using extracts of various plant parts has been reported (Ahmed *et al.*, 2016). It is an effective method for manipulating, controlling and stabilizing the size, characteristics and longevity of the nanoparticles produced. The characteristics of the green synthesised nanoparticles have been found to be much dependent on the metabolite composition of the plant used. Among the green synthesised nanomaterials, silver nanoparticles are the most extensively studied for antimicrobial activity (Henglein, 1989; Mulvaney, 1996). Although, there are reports on antibacterial and antifungal activity of green synthesised silver nanoparticles, antialgal activity of green synthesised silver nanoparticles have not been much explored.

Algae are inevitable component of the eco-system and form the very base of marine food chain. However, harmful algal blooms in natural and constructed water bodies are a threat for the environment as they for humans and other organisms. Moreover, invasion of algae on solar panel and roofs of polyhouses are a major

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problem that may lead to disruption of the proper functioning of the system. *Chlamydomonas reinhardtii* is a unicellular green alga, mostly used biological model organism, due to its ease of culturing and easiness in genetic manipulation.

In the present study, an attempt has been made to green synthesise silver nanoparticles using leaf extracts of plants having antialgal property *viz.*, *Tinospora cordifolia*, *Ricinus communis* and *Eichhornia crassipes*, and to evaluate their potential for the growth inhibition of model alga *C. reinhardtii*.

Review of Literature

2. REVIEW OF LITERATURE

Nanotechnology is a rapidly growing domain which has potential applications in the field of biotechnology, medicine, and information communication sector. It deals with synthesis, study and exploration of the possibilities of different types of nanomaterials derived from different sources (Rao *et al.*, 2004). Among the biological alternatives for synthesis of nanoparticles, plants and plant extracts seem to be the best option. The green synthesised silver nanoparticles have shown inhibitory effect on harmful bacteria, fungi, virus, etc. (Ahmed *et al.*, 2016: Tippayawat *et al.*, 2016).

2.1. ALGAE

2.1.1. Environmental Issues Caused by Algae

Algae form a major part of the environment being oxygen producers and occupying the base of food-chain in aquatic ecosystem. Besides, they are economically important sources of biofuel, pharmaceuticals, food and many other commercial products. However, recurrent and problematic harmful algal blooms (HABs) in marine recreational waters pose high health hazard to humans (Maso and Garcés, 2006).

According to Anderson and Garrison (1997), most of the coastal countries are affected by HABs, also known as "red tides" which refer to various phenomena constituting blooms of toxic micro algae that cause health hazards in humans and death in fish, marine mammals, sea birds and other ocean inhabitants. Besides, non-toxic HABs cause extensive ecological aftermath such as oxygen depletion in bottom waters, habitat alteration and deracination of indigenous species. Harmful algal blooms have been expanding their impact on environment and economy lately in parallel with escalating exploitation on coastal zone for food, recreation and shelter.

2.1.2. *Chlamydomonas*: Model Alga

Chlamydomonas reinhardtii, the single-celled, fresh water green alga is highly responsive to various genetic and molecular manipulations. The ability to adopt photoautotrophic and heterotrophic mode of nutrition depending on the external environment is a unique character of the alga. It grows photoautotrophically when light is provided as the single energy source and heterotrophically in dark if exposed to media containing acetate which makes it a better model organism to work on at the cellular or molecular levels (Grossman *et al.*, 2003).

Merchant *et al.* (2007) sequenced the complete nuclear genome of *C. reinhardtii* which is a lineage diverged from terrestrial plants about one billion years ago and the genome size was found to be nearly 120 mega bases. The study revealed various genes associated with photosynthetic and flagellar functions and paved way for better understanding of the ancestral eukaryotic cell.

The isolation of various proteins functioning as light-gated cation channels namely, Channelrhodopsin-1 and Channelrhodopsin-2 were initially isolated from *C. reinhardtii* (Nagel *et al.*, 2002).

Shrager *et al.* (2003) performed the genome project of *C. reinhardtii* with the funding of the National Science Foundation which comprised of construction and sequencing of cDNAs, construction of high-density cDNA microarray, generation of genomic contigs, generation of complete chloroplast genome sequence and creation of web-based resource for easy access of information.

Hiriart *et al.* (2006) evaluated the toxicity of silver on freshwater green algae, *C. reinhardtii* and *Pseudokirchneriella subcapitata* as influenced by the presence or absence of thiosulphate.

2.1.3. Characterization of Alga

Analysis of biochemical composition of micro algal biomass serves as a tool that provides an insight into adaptational response of the organism towards the dynamic environment and also reflects its metabolic and physiological status (Chen and Vaidyanathan, 2013).

Cunningham and Maas (1978) performed an experiment on strain 32a of *C. reinhardtii* by growing them in modified Bold's Basal Medium to study the variation in growth dynamics of the algae in continuous and batch culture techniques. It was observed that the growth kinetics of the unicellular alga was more complex in a rapidly fluctuating environment as compared to that of steady state cultures.

Sartory and Grobbelaar (1984) conducted an experiment for extraction of chlorophyll from three algal species *Scenedesmus quadricauda*, *Selenastrum capricornutum* and unicellular *Microcystis aeruginosa* using different solvents. Alcoholic solvents namely, methanol and ethanol, proved to be superior to acetone and acetone with DMSO in terms of efficiency of extraction.

Slocombe *et al.* (2013) estimated the protein content of six micro algal samples using Lowry's method of protein estimation in order to derive an agreeable small-scale extraction method for lipophilized micro-algae. Sequential single-tube extraction in Tri Chloro Acetic acid (TCA) and NaOH was performed for four hours which was followed by Lowry's protein extraction and estimation which required 30 min and this method ratified to be commonly ideal for the algal samples used.

Kazir *et al.* (2019) performed protein extraction and estimation from two marine macro algae *Ulva* sp. and *Gracilaria* sp. to derive an effective food-grade procedure which yielded high protein concentrate. Resultant algal protein

concentrates (APCs) were found to have protein contents of 70 and 86 per cent of dry weight respectively.

Phukan *et al.* (2011) conducted an experiment on *Chlorella* sp. to study the physical and chemical characteristics to evaluate its potential to be used as feedstocks in biofuel production. The carbohydrate content was estimated using Anthrone method and was observed to be 19.46 per cent of dry weight. Lipid was found to be 28.82 per cent and the alga was found to be a potential raw material to meet the biofuel requirement of the future generation.

Smith (1963) studied the carbohydrate content in the lichen *Peltigera polydactyla* using Anthrone method to test for any difference in carbohydrate in the "algal zone" and the medulla of the lichen. However, dissection experiments did not reveal any qualitative difference in the carbohydrate of both the zones.

Singh and Nikhil (2014) performed an experiment to estimate the lipid content of microalgae growing in wastewater of abandoned coal mining and open cast areas of Jharkhand, India. The lipid contents of dominating algal species isolated from the algal samples collected from municipality water bodies and coal mining areas were estimated using Floch's method of lipid extraction and estimation. The dominating algae in coal mine was found to be species of *Spirogyra* and *Oscillatoria*. It was observed that the lipid content of algal biomass obtained from coal mining areas was 16.3 per cent more as compared to those isolated from the pond water.

2.1.4. Plants with Antialgal Properties

Various allelochemicals obtained from plants were considered to be an effective, eco-friendly and economically feasible substance having algicidal properties. Water and methanol extracts of *Cinnamomum camphora* fresh leaves were tested for anti-algal properties on *Microcystis aeruginosa* and *C. reinhardtii* based on cell growth and photosynthetic abilities. Reduction of maximum quantum yield of

photosystem-II and degradation of photosynthetic pigments were observed in the algae on treatment with fresh leaf extract. Camphor, α-terpineol and linalool were found to be the three major algicidal agents out of twenty three compounds obtained from the water extract and nine new compounds obtained from the methanol extract of the leaf samples (Chen *et al.*, 2018).

Al-Haidari *et al.* (2016), evaluated the algicidal property of *Tinospora cordifolia* Willd against three algal isolates namely, *Anabaena circinalis*, *Scenedsmus quadricauda* and *Mougeotia scalaris*. It was found that *Anabaena circinalis* was most sensitive against the alkaloid extract of the plants producing an inhibition zone of 40 mm at 20 mg/ml concentration.

Zhang *et al.* (2013) conducted a study on the alga *Microcystis aeruginosa* for evaluating the algicidal property of a compound neo-przewaquinone A, obtained by column chromatography and bioassay-guided fractionation methods from the plant *Salvia miltiorrhiza* Bunge. The EC_{50} value of the compound on *M. aeruginosa* was found to be 4.68 mgl^{-1} whereas, on the algae *Chlorella pyrenoidosa* and *Scenedesmus obliquus*, the EC_{50} values were found to be as high as 14.78 and 10.37 mg l^{-1}, respectively. The compound caused morphologic damage, decreased the soluble protein content, total antioxidant and superoxide dismutase activity and increased the malondialdehyde content. Inhibition of photosynthesis-related genes (*psa*B, *psb*D, and *rbc*L) was also recorded.

Allelopathic inhibition of blue green alga *Microcystis aeruginosa* (PCC7806) by *Eichhornia crassipes* was evaluated by co-existence assay and distinct effects on growth was noticed under different initial algal densities. At lower initial algal densities (OD650) of 0.10 and 0.05, inhibition was found to be significant with a ratio of 95.6 per cent and 97.3 per cent respectively. The root exudate of the plant was found to be having the algicidal substance (Wu *et al.*, 2012).

2.1.5. *In Vitro* Assays for Toxicity Testing

Nam and An (2016) reported disc diffusion technique to be the best inhibition assay for soil algae while working on the soil alga *Chlorococcum infusionum* to test the toxicity caused by Cu^{2+} and Ni^{2+} metal ions. Mallison and Cannon (1984) performed toxicity assay for sixteen common pesticides on blue green alga *Plectonema boryanum* using spot on lawn assay. Agar well diffusion method is widely used to evaluate antimicrobial activity of plants or microbial extracts (Magaldi *et al.*, 2004). The work done by Perez *et al.* (1990) showed antibacterial activity of silver nanoparticles against *Staphylococcus warneri* when evaluated by agar well diffusion method. Silver nanoparticles synthesised from orange juice were tested against bacteria for their antibacterial activity by agar well diffusion method and was found to be an effective antibacterial agent (Hungund *et al.*, 2015). The antibiotic susceptibility of *Staphylococcus warneri* was evaluated by disc diffusion method (Dong *et al.*, 2017). The spot-on lawn method was used to confirm the antibacterial activity by using nanoparticles by Moodley *et al.* (2018).

2.2. NANOTECHNOLOGY

Nanotechnology is the branch of science that deals with matter at a dimension less than 100 nm and nanoparticles are clusters of atoms or molecules. A nanometer can be defined as one billionth of a meter (10^{-9}) and could be more precisely represented by the length of ten hydrogen atoms lined up in a row (Dhuper *et al.*, 2012). Nanomaterials have fantastic properties that may be efficiently exploited to provide eco-friendly as well as economically sustainable solutions to various technological and environmental challenges under diverse disciplines including agriculture, medicine, waste management and energy conservation (Sharma *et al.*, 2009).

The ability to manipulate matter at the atomic and molecular levels renders previously unforeseen possibilities in the field of modern science and technology.

Expeditious advances in nanotechnology by merging of nanotechnology with modern biotechnology offers an expansive new era of science (Sweeney *et al.*, 2003).

Nanotechnology has influenced almost all sectors of human life including agriculture, food and medicine and the use of plants and their extracts have paved way for the development of more eco-friendly and economical alternative route for synthesis of metal nanoparticles with well-defined size and shape (Husen and Siddiqi, 2014).

2.2.1. Synthesis of Nanoparticles

Nanoparticles of metals and semiconductors are synthesized basically by chemical methods such as electrochemical techniques, photochemical reactions and chemical reduction (Chandran *et al.*, 2006). The main drawback of chemical synthesis process is the extremely dangerous exposure hazards like carcinogenicity, cytotoxicity and genotoxicity (Mukherjee *et al.*, 2008). Due to these critical problems researchers are focusing more on 'green chemistry'. Green synthesis strategies help in using nontoxic chemicals, which are natural solvents and is renewable in nature. Methods of synthesis of nanoparticles are an expanding research area in which the research advancements have been rapidly carried out in sectors of electronics, chemistry, medicine, agriculture and energy.

Plant extracts have been used for the synthesis of nanoparticles for biomedical applications (Mittal *et al.*,2013).

2.2.1.1 Physical method

Gaurav *et al.* (1994) performed vapour condensation technique to produce ultrafine fullerene particles of Carbon (C_{60}) of size 30-40 nm by using continuous flow system. This technique was further standardized and used to produce metal nanoparticles and the method was widely accepted as evaporation condensation

method which required the use of high amount of electric power, large tube furnaces and labour (Natsuki *et al.*, 2015).

Nanoparticles of semi-conductor lead sulphate (PbS) of size 3-50 nm (Kruis *et al.*, 2000) and gold nanoparticles of size 20 nm with spherical shape (Magnusson *et al.*, 1999) were fabricated by evaporation condensation method. Another physical method of synthesis of nanoparticles was attempted by Mafuné *et al.* (2000) for synthesizing silver nanoparticles by laser ablation of silver plate in aqueous solution of sodium dodecyl sulphate.

2.2.1.2. *Chemical method*

Chemical method has been widely accepted due to the convenience and requirement of simpler equipments and nanoparticles like that of silver could be produced in large quantities at low cost and requires just three main progenitors namely, metal precursors, reducing agents and stabilizing /capping agents (Natsuki *et al.*, 2015).

Radziuk *et al.* (2007) performed chemical reduction of silver nitrate to silver nanoparticles in excess of aqueous sodium borohydroxide to obtain particles of size 23 nm. The aggregation nature of the particles when treated with NaCl solution in the presence of three different coating agents poly (diallyl dimethyl ammonium chloride), poly(ethylene glycol) and poly (allylamine hydrochloride) were studied and the former was found to render the least stability to the particles.

2.2.1.3. Biological method

The biosynthesis / green synthesis is an emerging branch in nanotechnology called "Nanobiotechnology" (Zakir *et al.*, 2014). It is a bottom-up approach involving reduction/oxidation reactions. The reaction takes place in one step, therefore molecules with dual characteristics *i.e.* reducing and capping agents are preferred (Mohanpuria *et al.*, 2008). Bacteria, fungi, plants, algae etc. are mostly involved in

the biosynthesis of silver nanoparticles (AgNPs). Several biological materials like honey (Philip, 2010), milk (Lee *et al.*, 2013), egg white (Lu *et al.*, 2012), coconut water (Elumalai *et al.*, 2014), lichen (Din *et al.*, 2015), faecal pellets (Karthika and Sevarkodiyone, 2015) and panchagavya (Govarthanan *et al.*, 2014) were also found effective in the production of AgNPs. During biosynthesis nanoparticles produced are immediately coated with protein caps to prevent aggregation. Natural capping obtained will provide more shelf life and stability than artificial.

Use of biological materials for the synthesis of nanoparticles is one of the novel routes in green chemistry which has several advantages over the physical and chemical methods. It is a cost effective and eco friendly method, and is easy to be scaled up for more synthesis. Also, it does not use high pressure, energy, temperature and toxic materials (Saxena *et al.*, 2010). Biosynthesis has been performed using a wide range of organisms and plants as in Zinc oxide nanoparticles from *Aeromonas hydrophyila* (Jayaseelan *et al.*, 2012), gold nanoparticles from *Epicoccum nigrum* (Sheikhloo *et al.*, 2011), AgNPs from onion extract (Saxena *et al.*, 2010), siliceous nanoparticles from diatoms (Mann, 2001) and magnetic nanoparticles from magnetotactic bacteria.

Biomaterials act as reducers as well as capping agents in synthesis of nanoparticles. Since it is a novel approach new advancement is seen in the synthesis of nanometal alloy and semiconductor nanoparticles. There are reports on the capability of the fungus *Fusarium semitectum* to produce gold-silver alloy nanoparticles (Basavaraja *et al.*, 2008). Some fungi are also reported to produce cadmium sulfide nanocrystals (Ahmad *et al.*, 2002).

2.2.1.3.1. Bacteria in AgNP synthesis

Bacteria and actinomycetes are widely used for the synthesis of AgNPs (Vaidyanathan *et al.*, 2010). *Pseudomonas stutzeri* AG259 was the first strain known to be used for synthesis of silver nanoparticles from silver mine (Haefeli *et al.*, 1984).

Escherichia coli (El-Shanshoury, 2011), *Actinobacteria rhodococcus* sp. (Otari, 2012) and *Pseudomonas* sp. (Thomas *et al.*, 2012) are also reported to be used for synthesis of silver nanoparticles. Nitrate reductase enzyme facilitates the mechanism of biosynthesis of AgNPs.

The mechanisms that help the bacteria to survive the metal ion concentration are through efflux systems, alteration of solubility and toxicity *via* reduction or oxidation, biosorption, bioaccumulation, extracellular complex formation or precipitation of metals, and lack of specific metal transport systems (Husseiny *et al.*, 2006). The optimization of the enzyme nitrate reductase in the bacterial cells was found to help in enhancing the reduction of Ag^+ ions to Ag^0 at a pH of 8 in the *in vitro* synthesis of nanosilver using bacteria *Bacillus licheniformis* (Vaidyanathan *et al.*, 2010).

2.2.1.3.2. Fungi in AgNP synthesis

Fungiare extensively used for the biosynthesis of AgNPs (Patil, 2014). They can produce large amount of nanoparticles since they secrete proteins in large quantities which directly results in the productivity of nanoparticles (Mohanpuria *et al.*, 2008). Fungus can trapthe Ag^+ ions at the surface of its cell and further reducethe silver ion by the cellular enzymes (Mukherjee *et al.*, 2001). The extracellular enzymes also play an important role in reduction like naphthoquinones and anthraquinones.

Ahmad (2003) in his study stated that NADPH-dependent nitrate reductase and a shuttle quinine extracellular process helps in the formation of nanoparticles. *Fusarium semitectum* is a fungus widely used for the biosynthesis of AgNPs. Similarly *Verticillium* sp. (Mukherjee *et al.*, 2001), the marine fungus *Penicillium fellutanum*a (Kathiresan *et al.*, 2009) and *Trichoderma asperellum* (Mukherjee *et al.*, 2008) have also showed efficiency in transforming silver nitrate into AgNPs. A major

drawback of using microbes for synthesis of silver nanoparticles is that they are very slow in processing compared to plant extracts.

2.2.1.3.3. Algae in AgNP synthesis

Algae have been reported to have extracellular synthesis of AgNPs. The extract of the unicellular green algae, *Chlorella vulgaris* is used to synthesise nanoparticles at room temperature. *Chaetomorpha linum* (Kannan, 2013), *Caulerpa resmosa* (Kathiraven 2014) and *Sargassum polycystum* (Kanimozhi *et al.,* 2015) are marine algae that synthesise AgNPs. The metal ion reduction was due to the action of carboxyl groups in aspartic or glutamine residues and the hydroxyl groups in tyrosine residues of the proteins (Xie *et al.*, 2007).

2.2.1.3.4. Plants in AgNP synthesis

Plant as a whole acts as a warehouse of green nanoparticles in the genesis of nanoparticles. Green synthesis of AgNPs also considered as nano-factories and their application in allied fields are major areas in which research is going on (Iravani 2011). Plant mediated AgNP synthesis is widely accepted for its rapid production of AgNPs, for meeting the excessive need and market demand of the product. Studies indicate that green synthesis of AgNPs using plants have advantages over others as they are easily available, safe, nontoxic, have plenty of metabolites that can contribute to the reduction of silver ions, is less time consuming and also shows a reduction in generation of hazardous waste matter which is a major problem in other methods (Reddy *et al.*, 2014).

Photobiological approach is a terminology used to express green synthesis of nanoparticles using plant extract as their reducing and capping agents in the synthesis of AgNPs. Microbes are known to be "bio-factories" for the synthesis of nanoparticles, while plants are known as "chemical factories" of the same, with more economic feasibility and minimal maintenance (Rupiasih *et al.*, 2013).

Plants possess the ability to detoxify metal and maintain homeostasis, which can be useful for many processes including phytoremediation (Abboud *et al.*, 2013). The ability to tolerate critically high levels of concentration of toxic metals is found in plants along with the ability to accumulate high concentrations of metals, both essential nutrients as well as non-essential metals (Sahayaraj *et al.*, 2012).

Plants and plant extracted materials have a wide range of metabolites with redox potentials that play a principle role as a reducing agent in the biosynthesis of nanoparticles. When compared with microbial synthesis of nanoparticles plant synthesised ones are more stable with higher rate of production (Ahmad and Sharma 2012).

Generally the biosynthesis using plant and plant extracts are of three main phases (Makarov *et al.*, 2014). These are the activation phase in which reduction of metal ion and nucleation of reduced metal atoms occur, the growth phase where small nanoparticles aggregate into a bigger one with increased thermodynamic stability and the termination phase in which final shape of the nanoparticles are formed.

Nakkala *et al.* (2014) used aqueous rhizome extract of *Acorus calamus*for synthesis of AgNPs which were found to possess an average size of 31.83 nm on analyzing with DLS particle size analyzer. The shape of the particles when viewed under Scanning Electron Microscopy (SEM), were found to be spherical. The green synthesized AgNPs revealed considerable antibacterial activity against *Escherichia coli* at log phase. Cytotoxicity assay using HeLa cells exhibited half maximal Inhibitory Concentration (IC_{50}) values of 92.48 and 69.44 µg/ml after 24 and 48 h respectively and the apoptotic cell death was analyzed by Acridine orange/ Ethidium bromide and AnnexinV-Cy3 staining techniques. The results emphasized on the application of green synthesised AgNPs in the discipline of nanomedicine.

Alex *et al.* (2012) reported the green biosynthesis of silver nanoparticles using the leaf extract of *Michelia champaca* and evaluated its antibacterial property against

Staphylococcus aureus and *Escherichia coli*. The particles were found to be of 30nm in size after TEM and exhibited antibacterial property at a concentration of 50ppm.

Green synthesis of AgNPs using *Alternanthera dentata* leaf extract was carried out for the first time by Kumar *et al.* (2014). The experiment was carried out at room temperature and AgNP formation was observed within 10 minutes and the particles were characterized using UV-visible spectroscopy, Fourier Transformed Infra Red (FT-IR) spectroscopy, X-ray diffraction (XRD), Scanning Electron Microscopy and TEM. The particles were found to be of spherical shape with average size ranging from 50-100 nm and exhibited antibacterial activity against *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Escherichia coli*.

Kumar *et al.* (2014) reported the synthesis of AgNPs from the whole plant extract of *Boerhaavia diffusa* which was observed to have face-centred cubic lattice with spherical shape and average size range of 25 nm after characterization using SEM, TEM, XRD and FT-IR spectroscopy techniques. UV-visible spectroscopy of the particles revealed the absorption maxima at 418 nm. The AgNPs were tested for antibacterial activity against three fish bacterial pathogens viz., *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Flavobacterium branchiophilum* and they demonstrated highest sensitivity toward *F. branchiophilum* when compared to other two bacterial pathogens.

Sun *et al.* (2014) performed the synthesis and characterization of AgNPs from tea leaf extract which yielded more or less spherical particles and the size ranged from 20-90 nm. Characterization of the particles was done by TEM, XRD, FT-IR, thermogravimetric analyzer and zeta potential analyzer. FT-IR spectral analysis revealed the role of tea extract as reducing and capping agent in the process of green synthesis. Antibacterial activity of green synthesized AgNPs was studied against *Escherichia coli* K12 strain MPAO1 by growth curve analysis and Kirby-Bauer disc

diffusion method at final concentrations of AgNPs at 50.0, 25.0, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.195 ppm. At a concentration as same as or more than 1.56 ppm, green synthesised AgNPs completely inhibited the growth of the bacteria.

Gopinath *et al.* (2012) explored the potential of *Tribulus terrestris* L. fruit extract in biosynthesis of AgNPs. Sun dried fruits were pulverized in 100 ml of de-ionized water to obtain fruit extract which was further treated with silver nitrate ($AgNO_3$) to obtain spherical particles of AgNPs which when exposed to TEM, was found to possess a size range of 16-28 nm. Antibacterial properties of the synthesized AgNPs were studied by Kirby-Bauer disc diffusion method, against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Streptococcus pyogens*, which were all multi drug resistant microorganisms. A volume of 15 µL of AgNP solution was added to the disc with fruit extract as control for inhibition assay. The inhibition zone was the highest for *Escherichia coli* (10.75 mm) and the lowest for *Pseudomonas aeruginosa* (9.25 mm).

Firdouse and Lalitha (2012) carried out synthesis of AgNPs from leaf extract of *Portulaca oleracea* using different methods namely, sonication, reaction at room temperature and reaction at 75°C (in water bath). The particle size of AgNPs was analysed using SEM and XRD techniques and was found to be less than 60 nm. The particles were stable because of the presence of functional groups attached to them which was revealed by FT-IR spectra.

Kumar *et al.*(2013) studied the synthesis of AgNPs with different shapes and sizes using different concentrations of *Coccinia indica* leaf extract in the reaction mixture. Leaf extract was taken in different volumes *viz.* 0, 2.5, 3.0 and 3.5 ml and were added to silver nitrate solution. The size of AgNPs was found to decrease with increase in concentration of *Coccinia indica* leaf extract added. The size range of the particles were analyzed to be 10-20 nm using TEM and XRD techniques and was found to have face centred cubic crystal lattice.

Rout *et al.* (2013) reported for the first time green synthesis of AgNPs by treating silver nitrate solution with aqueous extract of leaves of *Centella asiatica* L. which was obtained after treatment of dried leaf sample with 90 per cent methanol followed by dilution with dimethyl sulphoxide. The particles obtained were characterized by UV- visible spectroscopy, TEM, XRD, SEM and FT-IR techniques and was found to have spherical shape and size ranging between 30-50 nm. Antibacterial assay of AgNPs in combination with Streptomycin was carried out against human pathogenic bacteria *Staphylococcus aureus* (MTCC 9542) by agar well inhibition assay and the effect of Streptomycin-AgNP mixture was found to be as good as the antibiotic alone.

Kumar *et al.* (2013) carried out the green synthesis of AgNPs from *Premna herbacea*, a local herb of Bodoland, Assam, which acted as reducing as well as capping agent in the reaction, stabilizing the AgNPs formed. TEM analysis revealed the size of particles to be between 10-30 nm with spherical shape. UV- visible spectra of the particles exhibited a Surface Plasmon Resonance (SPR) peak at 425 nm. Different final concentrations of AgNPs from 0-100 µg was prepared in colloidal state and added on to tubes containing nutrient broth for determining the Minimum Inhibition Concentration (MIC). For human pathogenic gram negative bacteria *Shigella dysentrieae* and *E.coli* and it was found to be 55 and 70 µg/ml respectively. Antibacterial activity of AgNPs were further analysed by disc diffusion method using 0, 50 and 80 per cent dilutions of AgNP suspension. The zones of inhibition tend to increase with the increase in concentration of AgNP used.

Thombre *et al.* (2014) carried out the production of AgNPs using seed extract of *Argyeria nervosa* and were characterized using UV-Vis spectrophotometry, X-Ray Diffraction, SEM and FTIR. Analyses revealed the particle size to be 20-50 nm. Antibacterial and antifungal characteristics of the particles were studied using *Staphylococcus aureus*, *Bacillus subtilis* and *Aspergillus niger*.

Methanol extract of leaves of *Vitex negundo* L. was used to synthesise AgNPs which was further characterized by TEM, XRD and SEM to conclude that they possessed face-centred cubic structure and crystalline nature. The reaction was completed in 48 h. In UV- visible spectra two peaks, one at 422 nm and the other at 447 nm indicated the presence of two broad distribution of hydrosol silver nanoparticles and the particles were found to have an average diameter of 18.2 nm in case of smaller particles and the larger particles had their diameter in a range between 25-30 nm. The antibacterial efficiency of AgNPs was evaluated against two pathogenic bacteria, *Escherichia coli* and *Staphylococcus aureus* using the agar disc diffusion method and the inhibition zones were 12.0 and 11.0 mm respectively for AgNPs as against the control of silver nitrate which had inhibition zones of 9.0 and 9.5 mm respectively and the inhibition zones formed by the antibiotic control cefotaxime was found to be 28.0 and 21.0 mm respectively (Zargar *et al.*, 2011).

Green synthesised AgNPs obtained from *Calotropis procera* leaves and stem extracts were found to possess significant antimicrobial potential against *Salmonella typhi* and *Klebsiella pneumonia*, besides appreciable antioxidant property. UV-visible absorption peak was observed at 449.34 nm and 452.60 nm respectively for leaf and stem extracts. The antimicrobial activity of AgNPs was tested by disc-diffusion assay with concentrations of 100, 75 and 50 per cent and the zone of inhibition was found to be directly proportional to the concentration of AgNPs (Gondwal *et al.*, 2013).

Gnanajobitha *et al.* (2013) reported silver nanoparticles formed from *Vitis vinifera* fruit extract to have antibacterial property against *Bacillus subtilis* and *Klebsiella planticola.* They used agar disc diffusion method in which Ag nanoparticles of 10, 20, 30, 40, and 50 µl were impregnated into the disc and are placed above the LB plate containing bacterial strain and after a desired time, inhibition was assessed. Inhibition zone was measured to be 6, 7, 8, 9 and 10 mm respectively in diameter.

Silver nanoparticles made out of papaya fruit extract with a mixture of 1mM $AgNO_3$ solution were characterized using UV-vis absorption spectroscopy, FTIR, XRD and SEM by Jain *et al.* (2009). UV-vis spectroscopy readings showed an absorbance peak at 450 nm. The XRD pattern exhibited three intense peaks in the whole spectrum of 2θ value ranging from 10 to 80. The average size of the green synthesised particles was 15 nm both in cubical and hexagonal structures. The nanoparticles are found to be highly toxic at 50 ppm and they exhibited antibacterial activity against *E. coli* and *P. aeruginosa*.

2.2.2. Advantages of Green Synthesis of AgNPs

Biological method for nanoparticle synthesis acts as a boon to the field of nanotechnology. It involves bacteria, fungi, algae, actinomycetes (Narayanan and Sakthivel, 2010), plants or plant extracts for effective and economic production of NPs (Mohanpuria *et al.*, 2008; Rauwel *et al.*, 2015).

On comparison with the physical and chemical synthesis of AgNPs, biosynthesis of AgNP appears to be less toxic and extremely cost effective alternative. Physical and photochemical methods work in extreme high temperature in vacuum conditions making it highly expensive and the product generated to be comparatively very low in storage conditions (Kholoud *et al.*, 2010; Iravani *et al.*, 2014).

Environmental pollution at a highly irreversible rate is the main disadvantage of chemical process. The processes of extraction of the nanoparticles are also laborious with high-priced equipments and soaring energy consumption (Gudikandula and Maringanti, 2016).

Considering the demerits of the other methods for nanoparticle synthesis, biosynthesis of nanoparticles is comparatively a better option having less

environmental pollution with lesser consumption of energy (Husen and Siddiqui, 2014).

The nanoparticles produced from the biological systems have a longer shelf life and stability compared to other methods. They are cost effective, scaling up process is simple and the downstream processing and purification of the nanoparticles produced is easy (Annamalai *et al.*, 2011). Among all the merits of biosynthesis of nanoparticles, great importance is given to the natural capping of the nanoparticles solving the formation of nano aggregates which is a major problem. All other methods require an artificial capping as an extra protection to minimize aggregation (Singhal *et al.*, 2011). Use of microbial enzymes and plant extracts are more advantageous as they contain antioxidant or reducing properties which are responsible for the reduction of metal compounds into nanoparticles.

Shankar *et al.* (2003) reported the extracellular production of silver nanoparticles by the reaction of aqueous silver nitrate solution and geranium leaf extract which yielded highly stable, crystalline nanoparticles with size ranging from 16 to 40 nm that assembled into quasilinear superstructures.

Different methods of green synthesis of silver nanoparticles were studied during the past decade. Polysacharide method in which polysacharides were used as the reducing agent and stabilizing agent for production of nanoparticles of silver and gold and gold-silver alloy was conducted by Raveendran *et al.* (2006), in which starch served as the capping agent and β-D-glucose as the reducing agent and the resultant nanoparticles were found to be having size within the quantum size domain and were more responsive to size dependent variations in electronic properties.

Ahmed *et al.* (2016) reported a rapid, simple, eco-friendly, non-toxic and one-step approach for synthesis of silver nanoparticles from aqueous leaf extract of *Azadirachta indica* at room temperature within 15 min. The particles were found to have its UV-vis absorption spectra in the range of 436 to 446 nm and exhibited an

inhibition zone of about 9 mm when tested for inhibition against bacteria *Escherichia coli* and *Staphylococcus aureus*.

2.2.3. Properties of Nanoparticles

Nanoparticles are of immense importance in the present scenario because of its uniqueness and handling properties. Bulk materials have constant physical properties but their properties change when they are reduced to nanosizes. As compared to the bulk materials nanoparticles have large surface area to volume ratio. The surface area of nanoparticles determines its physical properties. Due to their size nanoparticles have quantum effects which help them to acquire more optical properties. Nanoparticles tend to have diffusion properties at high temperature because of their larger surface area. Nanoparticles can be integrated into polymer matrices so as to increase their reinforcements.

Nanotechnology has its application in medicine in which nanodrugs are used to cure diseases with lesser toxicity. Similarly, in the field of agriculture nanotechnology is used to control insects, weeds etc. Nanoparticles are also used in semiconductor devices, radiation therapy, solar cells, synthetic fabrics, fibers, gene therapy etc. They are best suited for using as anti-reflection coatings (Buzea *et al.*, 2007).

2.2.4. Antimicrobial Properties of Nanoparticles

Nanoparticles have been extensively studied so as to find out their antimicrobial properties against existing and emerging microbes. Several characteristics like high surface area to volume ratio make the nanoparticles a tough agent in fighting the target organism. They are advantageous in treating bacterial infections so they are used in coating for implantable devices and help in preventing infections (Wang *et al.*, 2017).

Nanoparticles are having effective multidrug resistance against deadly bacteria like *P. aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Enterococci* and other super bugs (Singh *et al.*, 2014).

Green synthesis of iron nanoparticles using leaf extract of *Mangifera indica* was carried out by reducing Fe^{3+} ions to $Fe^{0}$and the nanoparticles produced exhibited antimicrobial activity (Dhuper *et al.*, 2012).

The interaction of gold-superparamagnetic iron oxide NPs with bacterial protein tend to affect the redox system and metabolic activities of the bacterial cell. Activity of nanoparticles disrupts the integrity of cell membrane which is an important factor in maintaining cell morphology and transporting of ions and molecules across the cell (Niemirowicz *et al.*, 2014).

Superparamagnetic iron oxide has the ability to damage the macromolecules, including DNA, lipids, and proteins, thereby leading to bacterial death. Iron helps in increasing the formation of Reactive Oxygen Species by the mechanism of oxidative stress which stimulates the electron transport chain producing superoxide damaging the iron clusters (Leuba *et al*,. 2013).

2.2.4.1. Antimicrobial properties of silver nanoparticles

Colloidal silver has been gaining more scientific attention due to its unique properties like excellent electrical conductivity, chemical stability, catalytic activity and even more specific characteristics like antibacterial activity and non-linear optical activity (Henglein, 1989; Mulvaney, 1996). Metal nanoparticles exhibit physical properties that are much different from the metal in its bulk form, like enhanced catalytic activity, which can be attributed to its morphological changes with increased surface area to volume ratio (Yacaman *et al.*, 2001).

Sotiriou and Pratsinis (2010) reported that size of silver nanoparticles affected their antibacterial activity. Nanosilver with diameter less than 10 nm released more Ag^+ ions and thereby exhibited higher antibacterial activity as compared to those with relatively larger size. Microbicidal properties of the AgNPsare more dependent on their shape, size, concentration, and colloidal state (Pal *et al.*, 2007; Bhattacharya and Mukherjee, 2008; Rai *et al.*, 2012; Nateghi and Hajimirzababa, 2014; Raza *et al.*, 2016).

Silver nanoparticles act as a better option for treating the multidrug resistant microorganisms. Cavassin *et al.* (2015) have studied *in vitro* activity of silver nanoparticles against susceptible and multidrug resistant bacteria and came to a conclusion that bactericidal effect was dominant when coming to the antimicrobial-susceptible bacteria but also showed a strong inhibitory effect against multidrug resistant bacteria in agar diffusion method.

In a study conducted by Raza *et al.* (2016), two different types of silver nanoparticles were used to study the antibacterial property on *Pseudomonas aeruginosa* and *Escherichia coli*. It was observed that the smallest-sized spherical AgNPs (diameters in range of 15 to 90 nm) exhibited better antibacterial activity against both bacterial strains as compared to the triangular (edge length of 150 nm) and larger spherical shaped AgNPs.

Ruttkay *et al.* (2019) studied antibacterial activity of nine types of AgNPs against two bacterial strains *Staphylococcus aureus* and *Escherichia coli*. Out of the nine different types of silver nano synthesised, the two green synthesised AgNPs obtained by reduction using green tea and coffee extracts exhibited the highest antibacterial activity on both the bacterial strains over the other seven AgNPs of which three AgNPs were synthesised by citrate method and four by using gallic acid.

Panáček *et al.*(2009) tested the antifungal activity of silver nanoparticles produced by Tollens process which was tested on pathogenic yeast *Candida spp.* and

it was found that the AgNPs effectively inhibited the growth of the tested yeast at the concentrations below their cytotoxic limit of1 mg l^{-1}.

2.2.4.1.1. Antialgal properties of silver nanoparticles

Navarro *et al.* (2008) evaluated the short- term toxicity of silver nanoparticles and ionic silver on the photosynthesis of *C. reinhardtii*. The size range of the silver nanoparticles studied were from 10 to 200 nm and it was found that when compared as a function of the Ag^{+} concentration, toxicity of AgNP appeared to be much higher than that of $AgNO_3$.

Dash *et al.* (2012) reported for the first time the toxicity of AgNPs on eukaryotic algae *Pithophora oedogonia* and *Chara vulgaris* cultured in Bold's Basal Medium. The nanoparticles were of size range between 10 to 15 nm and found to reduce the chlorophyll content in both the algal species at significant levels. Greater reduction was found in *Chara vulgaris* as compared to that in *Pithophora oedogonia* and the reduction was more pronounced when the concentration of AgNPs used was 5mM.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Green synthesised silver nanoparticles for the suppression of algal growth" was done in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2016 to 2019. This chapter details the materials used and methods adopted for this experiment.

3.1 CULTURING OF CHLAMYDOMONAS

Pure culture of *Chlamydomonas reinhardtii*, was procured from Central Food Technological Research Institute (CFTRI), Mysore and grown in Bold's Basal Media (BBM) at 25 ± 3 °C and 3000 lux illumination in an incubator shaker maintained at 100 rpm (Plate 1).

3.1.1 Culture Medium

3.1.1.1 Chemicals

Chemicals (analytical grade) procured from Himedia Laboratories were used for the preparation of culture medium. Citrate stabilized silver nanoparticles (100 nm) from Sigma Aldrich, USA were used for the study.

3.1.1.2 Composition and preparation of medium

Bold's Basal Medium was prepared as specified in Appendix 1. The solid and broth media were prepared, autoclaved at a pressure of 1.06 kg cm^{-2} and a temperature of 121°C for 20 min. The media were then stored at 25 °C until use.

3.1.2 Growth Curve Analysis of *Chlamydomonas reinhardtii*

Single colony of *C. reinhardtii* was inoculated into 50 ml BBM broth and kept in rotary shaker (100 rpm) at 25 °C with an illumination of 3000 lux. After 24 h, 50 µl each of this culture broth was further inoculated into flasks containing fresh BBM broth. These were again incubated in a rotary shaker at the same conditions specified

above. At 24 h, 100 µl of the culture broth was serially diluted and plated on BBM agar plates and incubated in culture racks at the conditions specified above. The algal population was assessed in terms of colony forming units by counting number of colonies on the plates at an interval of 24 h for 17 days. The log cfu per ml was plotted on a graph to obtain the growth curve of the algae (Population vs Time).

3.2 GREEN SYNTHESIS OF SILVER NANOPARTICLES FROM PLANTS REPORTED WITH ANTI- ALGAL PROPERTIES

Green synthesis of silver nanoparticles was carried out from three plants reported to have anti-algal properties according to the protocol reported by Alex *et al.* (2012).

3.2.1 Plant material collection and preparation of plant leaf extract

Green and healthy leaves of *Tinospora cordifolia and Ricinus communis* were collected from the premises of College of Agriculture, Vellayani and the leaves of *Eichhornia crassipes* were obtained from the Vellayani Lake (Plate 2). Five gram each of the fresh leaves was thoroughly washed in Laboline and tap water, and then twice in sterile double distilled water. The leaves were then cut into fine pieces and boiled in 100 ml sterile distilled water and filtered through Whatman No.2 filter paper to get the leaf extracts.

3.2.2 Preparation of silver nitrate solution

1 mM silver nitrate ($AgNO_3$) solution was prepared by dissolving 15.2 mg of silver nitrate in sterile double distilled water and then making up the final volume to 90 ml.

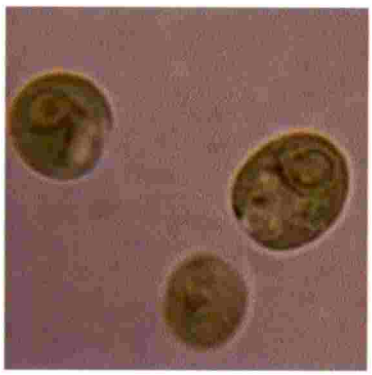


Plate 1. Microscopic image of *C. reinhardtii* (100x)

2A

2B

2C

Plate 2. Plants showing anti algal property

2A- *Ricinus communis*

2B- *Tinospora cordifolia*

2C- *Eichhornia crassipes*

3.2.3 Green synthesis of silver nanoparticles

Synthesis of silver nanoparticles was done by adding 10 ml of leaf extract of respective plants into 90 ml of 1mM silver nitrate solution and then heating for 40s in a microwave oven. The solutions were then kept at room temperature for the formation of silver nanoparticles. After the formation of silver nanoparticles by complete reduction, the solution was centrifuged (10,000 rpm for 15 min) and pelleted. The pellet was further resuspended in sterile double distilled water and centrifuged again at 10,000 rpm for 15 min. The pellet obtained was resuspended in 1ml of sterile distilled water.

3.2.4 Analysis of bioreduced silver nanoparticles

3.2.4.1. UV-Vis spectrophotometry

The formation of silver nanoparticles (AgNPs) *i.e.*, the reduction of Ag^+ ions to Ag^0 was monitored by scanning the bio-reduced silver nanoparticle solution at wavelength from 300 to 600 nm in a UV-Vis spectrophotometer (Bio-Rad) using a quartz cuvette with sterile double distilled water as reference.

3.2.4.2. Confirmation of conversion of silver nitrate to silver nanoparticles

The reduction of silver ions was confirmed by qualitative testing of the supernatant obtained after centrifugation of the bio-reduced silver nitrate solution with 0.1 M sodium chloride.

3.3 INHIBITION ASSAYS

The ability of green synthesized silver nanoparticles (5 ppm), filter sterilized leaf extract of the plant used for green synthesis and commercial silver nanoparticles (5 ppm) for inhibiting algal growth was studied by performing a series of inhibition assays namely agar well inhibition assay, filter paper disc assay and spot on lawn assay using *C. reinhardtii.* Sterile double distilled water served as absolute control.

The assays were done individually for each plant with its corresponding leaf extract and green synthesized AgNPs.

3.3.1 Agar well inhibition assay

Bold's Basal Medium agar plates were spread inoculated with *C. reinhardtii* and four wells (8 mm diameter) were cut out from four quadrants of the plate with a sterile cork borer. The base of the wells were sealed with 100 µl molten BBM agar. After solidification of the base of the wells, 200 µl each of the test solutions were added. The experiment was replicated four times. The diameter of inhibition zones around the wells were measured after an incubation period of eight days (Balouiri *et al.*, 2016).

3.3.2 Filter paper disc assay

C. reinhardtii was spread on BBM agar plates using an L rod. Sterile filter paper discs each of 5mm diameter were wetted with 10 µl each of the test solutions. The discs were dried inside the laminar air flow chamber and then placed at the four quadrants of the plate. Plates were further incubated for eight days at 25 °C under fluorescent white light of 3000lux. Four replications were maintained. The plates were observed for inhibition zones (Cunha *et al.*, 2016)

3.3.3 Spot on lawn assay

Bold Basal Medium agar plates were spread inoculated with *C. reinhardtii*. Once the spread was dry, 20 µl each of the test solutions were spotted on the plates at four quadrants. Four replications were maintained. Plates were incubated for eight days at 25 °C under fluorescent white light of 3000lux as described earlier (Moodley *et al.*, 2018).

3.4. POPULATION ASSAY OF *C. reinhardtii* TREATED WITH GREEN SYNTHESIZED AGNPs

Population assay was done by taking the viable plate count after treating with the green synthesised silver nanoparticles that showed maximum zone of inhibition in the above assays and the corresponding leaf extract. The treatments were done at two stages *viz.*, initial growth phase and start of exponential phase.

3.4.1 Viable plate count method

Single colony was taken from pure culture of *C. reinhardtii* maintained on BBM agar using sterile loop and was dispersed in 1 ml sterile distilled water. Fifty microlitre of the algal suspension was transferred into 50 ml BBM broth. The broth was then kept for 24 h for incubation. Fifty microlitre each of the algal culture was further transferred to 50 ml fresh BBM broth.

Green synthesised silver nanoparticles obtained from *Tinospora cordifolia* (*T.c.*AgNPs) were used for further studies as they exhibited higher anti algal property compared to the AgNPs synthesised using other two plant extracts. The effect of addition of green synthesized AgNPs oninitial growth phase of *C. reinhardtii* wastested by adding the AgNPs (*T. cordifolia)* to the medium on the same day of inoculation of *C. reinhardtii.* Effect of green synthesized AgNPs on *C. reinhardtii* duringearly exponential phase of growth was tested by adding AgNPs (*T. cordifolia)* at the beginning of exponential growth phase, ie. eighth day after inoculation of *C. reinhardtii*

Observations were taken on the eighth day after plating by counting the number of colonies.

3.5 BIOCHEMICAL ASSAYS OF *C. reinhardtii* CULTURE INOCULATED WITH GREEN SYNTHESISED SILVER NANOPARTICLES

Single colony of *C. reinhardtii* was taken from pure culture (maintained in BBM agar plates) and was dispersed into 1ml sterile double distilled water. Fifty microlitre of the algal suspension was transferred into 50ml BBM broth and incubated for 24 h. Fifty microlitre each of this culture was further transferred to 50 ml of fresh BBM broth. The culture was incubated for 12 days to attain exponential growth.

On the 12th day, the biochemical parameters of the culture *viz.*, chlorophyll, protein, lipid and carbohydrate contents were analysed. Effect of addition of green synthesized silver nanoparticles (5 ppm), filter sterilized leaf extract of the plant used for green synthesis and commercial silver nanoparticles (5 ppm) were tested by adding them on the 12th day to the growing algal culture and analysing the biochemical parameters at an interval of four days. Four replications were maintained.

3.5.1 Estimation of total chlorophyll content

Total chlorophyll content was estimated by warm extraction using methanol (Mckiney, 1941). The algal cells were pelleted from 10 ml of the algal culture by centrifugation. To the pellet, 10 ml of 96% methanol was added. The contents were shaken, covered and kept in a water bath at 60 °C for 30 min. After complete extraction, the samples were removed from the water bath and cooled to room temperature. The evaporation loss was made up with methanol. The samples were centrifuged at 5000 rpm for 15 min. The pigment was analyzed by comparing a sample of unknown transmission against a blank (96% methanol) of 100% transmission at wavelengths of 650 and 665 nm.

Calculation

Total cholorophyll (mg ml^{-1}) = 2.55 x 10^{-2} x E_{650} + 0.4 x 10^{-2} x E_{665}

3.5.2 Estimation of total carbohydrate content

Total carbohydrate content was estimated by Anthrone method (Hedge and Hofreiter, 1962).

3.5.2.1 Preparation of anthrone reagent

Anthrone reagent 100 mg and thiourea 1 g were dissolved in 100 ml of 75 per cent sulphuric acid. The mixture was then kept ina water bath at 85 °C for complete dissolution of the ingredients. The reagent was freshly prepared.

3.5.2.2 Preparation of standard glucose solution

Standard curve was prepared using graded concentrations of glucose stock solution prepared by dissolving one gram of glucose (AR grade) in 1000 ml of double distilled water to give 1000 µg ml^{-1} stock solution.

One ml of homogenous algal suspension from each treatment was taken in a test tube, washed with distilled water and resuspended in 1 ml distilled water. Four millilitre of anthrone reagent was added to each tube and shaken gently and thoroughly. The tubes were then covered and kept in boiling water bath for 15 min. The test tubes were then cooled in running tap water. The blank was set with 1 ml distilled water. The standards were also treated with anthrone reagent. Absorbance of each sample was read at 620 nm against the blank. The concentration of carbohydrate in each sample was determined by plotting the graph using the standard of glucose.

3.5.3 Estimation of total protein by Lowry's method

Total protein content of the algal culture was done by Lowry's method (Lowry *et al.*, 1951).

3.5.3.1 Preparation of reagents

(A) Alkaline sodium carbonate solution: Sodium carbonate 10 g was dissolved in little quantity of distilled water. After adding sodium hydroxide 2 g to it, the volume was made upto 500 ml.

(B) Copper sulphate: Copper sulphate 5 g was dissolved in 100 ml distilled water.

(C) Sodium potassium tartarate solution: Sodium potassium tartarate 10g was dissolved in 100 ml distilled water.

(D) Copper sulphate – sodium potassium tartarate solution: One part each of reagent B and C were mixed and diluted with 8 parts of distilled water. The solution was freshly prepared before each experiment.

(E) Alkaline reagent: 50 ml of reagent A and 1 ml of reagent D were mixed together and was prepared freshly before each experiment.

(F) Folin-Ciocalteau reagent: AR grade Folin- Ciocalteau reagent purchased from Himedia was diluted to 1N strength with distilled water.

(G) Trichloro acetic acid (TCA) solution (6 per cent w/v) was prepared by dissolving 6 g of TCA in 100 ml distilled water.

(H) Standard protein solution: Bovine Serum Albumin (BSA) solution at a concentration of 1000 ppm was prepared by dissolving 10 mg in 10 ml distilled water and was stored in freezer.

Ten ml of the algal culture was centrifuged and washed with double distilled water. TCA solution (6 per cent w/v) was heated to 60 °C and 10 ml of it was added to the harvested algal cells in a test tube and was allowed to stand for 1 min. Then,

TCA was removed using a micropipette and the residue precipitated at the bottom. Ten ml of reagent E was heated to a temperature of 55 °C and was added to the residue and kept aside for 3 min. so as to dissolve the protein that has been precipitated by TCA in the above step. The filtrate was collected using micropipette and the volume was made upto 5 ml with reagent C. Five millilitre of the reagent E was taken as blank. Graded concentration of BSA solution was prepared from the stock at concentrations ranging from 10 to 100 µg and the volume was made upto 5 ml using reagent E. Reagent F 0.5 ml was added to the test tubes and was mixed rapidly and was further allowed to stand for 30 min. at room temperature. Absorbance of each sample was read at 660 nm against the reagent blank.

The quantity of protein in the algal samples were determined from the graph plotted using the BSA standard solutions.

3.5.4 Estimation of total lipid

Estimation of lipids was done after extraction with perchloric acid and chloroform- methanol solution (Folch *et al.*, 1957).

3.5.4.1 Extraction of lipids

The reagents used were perchloric acid ($HClO_4$) and 0.2 N chloroform-methanol mixture (2:1 v/v).

Algal cells were collected from 10 ml of the culture by centrifugation and the supernatant was discarded. Tubes containing algal cell pellets were then placed on ice and 10 ml of ice-cold 0.2 N $HClO_4$was added. Contents inside the tubes were thoroughly vortexed and kept at 4 °C for 15 min. It was then centrifuged in a refrigerated centrifuge and the supernatant was removed carefully. The procedure was repeated with another aliquot of $HClO_4$ and the supernatant was discarded. Ten ml of chloroform- methanol mixture (2: 1 v/v) was added to the pellet and vortexed and kept aside for 5 min. at room temperature. The sample was centrifuged and the

supernatant was retained. To the combined chloroform- methanol extracts of the cells, 0.2 volumes of water were added. The solution was shaken for 5 min. and followed by centrifugation to separate the phases. The lower organic phase was collected and the rest was discarded. Chloroform-methanol extract was evaporated to a final volume of 2 ml inside the laminar air flow chamber.

Estimation

The following reagents were used for estimation:

(1) Palmitic acid standard solution-Palmitic acid was dissolved in chloroform to a final concentration of 1 mg ml^{-1}

(2) Dichromatic solution - Potassium dichromate ($K_2Cr_2O_7$) 0.125 g was dissolved in 50 ml H_2SO_4.

Using a micropipette, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 ml of the palmitic acid standard lipid solution was transferred to marked screw capped tubes. In another set of marked tubes, 0.1, 0.2 and 0.5 ml of the algal samples were taken. The contents were evaporated to dryness in laminar air flow chamber. Two millilitre of dichromate solution was added to all the tubes and was covered with heat resistant polyvinyl lined caps. The tubes were then placed in boiling water bath for 45 min. with intermittent shaking. The tubes were cooled; 1 ml of sample was removed from each tube and was further diluted to 100 ml with distilled water. Absorbance was read at 350 nm against distilled water blank. Standard curve was prepared with known concentrations and the values of unknown samples were determined graphically.

Assay was based on the disappearance of absorbance at 350 nm as the dichromate got reduced by increasing amount of lipids. Hence, the graph was plotted as the reciprocal of the absorbance against lipid concentration.

Results

4. RESULTS

The results of the study on "Green synthesised silver nanoparticles for the suppression of algal growth" are represented in this chapter.

4.1 GROWTH DYNAMICS OF *Chlamydomonas reinhardtii*

Single colony of *C. reinhardtii* was inoculated into BBM broth and the population was recorded after every 24 h as described in materials and methods 3.1.2. The result is shown in Table 1. The doubling time was recorded to be 24 h. The population increased from the first day to the twelfth day of inoculation and thereafter showed a decrease. The exponential growth phase was observed to begin on the eighth day of inoculation (Fig 1) and reached the maximum population growth on the 12th day. The population recorded on the 12th day of inoculation was 5.76log cfu ml^{-1}.

4.2 GREEN SYNTHESIS OF SILVER NANOPARTICLES FROM PLANTS REPORTED WITH ANTI-ALGAL PROPERTY

Silver nanoparticles were green synthesised from plants having antialgal property (as described in materials and methods 3.2) and were used to study their effect on inhibition of algal growth. Leaf extracts of *Tinospora cordifolia*, *Ricinus communis* and *Eichhornia crassipes* were prepared as described in materials and methods 3.2.1.

Different plant leaf extracts took different time periods for mediating complete conversion of $AgNO_3$ into AgNPs. The time duration taken for silver nanoparticle synthesis using leaf extracts of *T. cordifolia*, *R. communis* and *E. crassipes* were recorded to be 1 h, 2 h and 12 h respectively (Plate 3).

4.3 CHARACTERISATION OF GREEN SYNTHESISED SILVER NANOPARTICLES

Characterisation of AgNPs was done by analyzing the UV-visible absorption spectra of the green synthesised silver nanoparticle solutions under a range of wavelengths from 300-600 nm as described in materials and methods 3.2.4.1. The absorption maxima of the different

Table 1. Growth kinetics of *C. reinhardtii* in BBM broth

Time (days)	Population (log cfu/ml)	Population (cfu/ml)
1	2.10	1.2×10^{1}
2	2.28	1.8×10^{1}
3	3.40	2.5×10^{3}
4	3.61	4.1×10^{3}
5	4.18	1.5×10^{4}
6	4.37	2.3×10^{4}
7	4.41	2.5×10^{4}
8	4.50	3.1×10^{4}
9	4.60	4.0×10^{4}
10	4.78	6.0×10^{4}
11	4.96	9.0×10^{4}
12	5.76	5.7×10^{5}
13	5.41	2.5×10^{5}
14	5.48	3.0×10^{5}
15	5.30	2.0×10^{5}
16	5.00	1×10^{5}
17	5.00	1×10^{5}

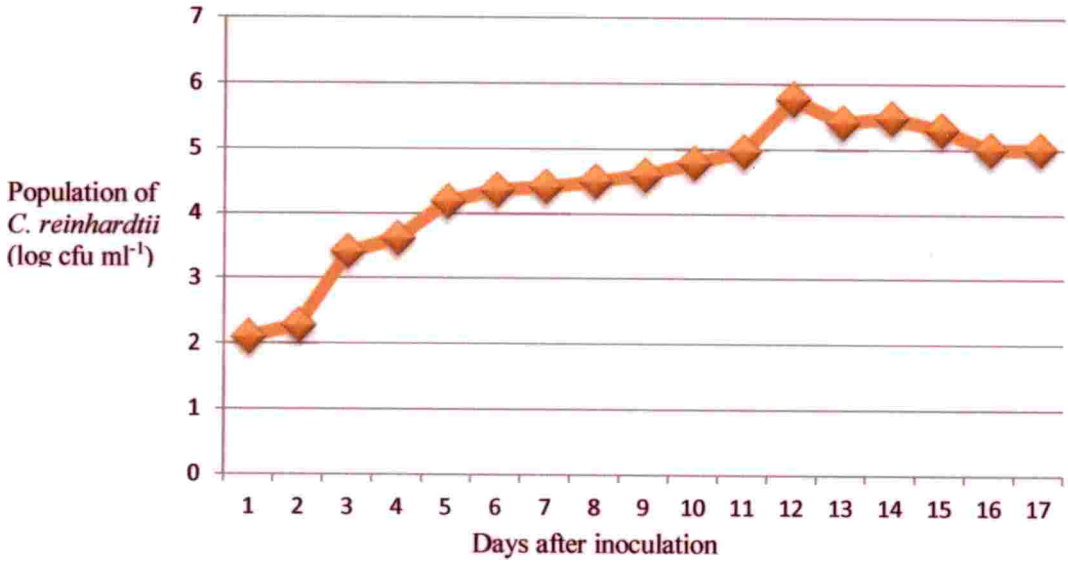


Fig. 1 Growth kinetics of *C. reinhardtii*

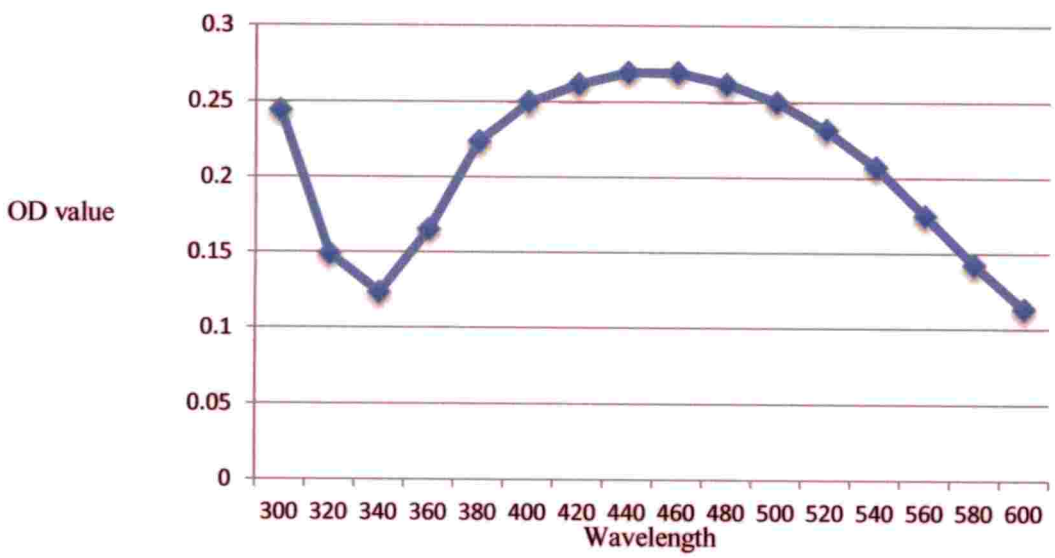


Fig. 2 UV-visible absorption spectra of green synthesised (*T. cordifolia*) silver nanoparticles

1 mM Silver nitrate solution

Heated in microwave oven for 40 seconds after adding leaf extract

Green synthesized AgNPs (*T. cordifolia)*

Green synthesized AgNPs (*R. communis)*

Green synthesized AgNPs (*E. crassipes)*

A – AgNPs (*T. cordifolia*)

B – AgNPs (*R. communis*)

C- AgNPs (*E. crassipes)*

A B C

Plate 3. Green synthesis of silver nanoparticles

solutions produced are presented in Table 2. The absorption spectra exhibited comparatively narrower peak in *T. cordifolia*and *E. crassipes* compared to *R. communis* (Fig. 2-4).

4.4 TESTING OF ANTI-ALGAL PROPERTIES OF GREEN SYNTHESISED SILVER NANOPARTICLES

4.4.1 Agar Well Inhibition Assay

In the assay, maximum growth inhibition of *C. reinhardtii* was obtained with AgNPs synthesised using *T. cordifolia* (Table 3). The inhibition of zone of AgNPs produced using *T. cordifolia* (7.3 mm) on *C. reinhardtii* was greater compared to the inhibition zone produced using *R. communis* (4.6mm) and *E. crassipes* (1.0 mm). Leaf extracts, chemically synthesised silver nanoparticles and absolute control did not exhibit conspicuous inhibition zones (Plate 4A).

4.4.2 Filter Paper Disc Assay

Filter paper disc assay was performed as described in section 3.3.2.In the assay, maximum growth inhibition of *C. reinhardtii* was obtained with AgNPs synthesised using *T. cordifolia*(Table 4).The inhibition zone of green synthesised silver nanoparticles using *T. cordifolia* (3.6 mm) was greater compared to those produced by *R. communis* (1.6 mm) and *E. crassipes* (1.0 mm). Leaf extracts, chemically synthesised silver nanoparticles and absolute control did not exhibit conspicuous inhibition zones (Plate 4B).

4.4.3 Spot-on Lawn Assay

In the spot-on lawn assay performed on *C. reinhardtii*, using green synthesised silver nanoparticles it was observed that maximum growth inhibition was obtained with AgNPs synthesized using *T. cordifolia* (Table 5). The AgNPs produced using *T. cordifolia* produced higher inhibition zone (6.0mm) compared to those produced by *R. communis* (3.3 mm) and *E. crassipes* (1.6 mm). Leaf extracts, chemically synthesised silver nanoparticles and absolute control did not exhibit any inhibition zone (Plate 4C).

All the three assays performed on *C. reinhardtii*, using green synthesized silver nanoparticles to study its anti-algal effect gave similar results. Among the three green systhesised

Table 2. Maximum absorption spectra of green synthesised silver nanoparticles from different plants

Plant	Maximum absorption spectra (nm)
Tinospora cordifolia	440- 460
Ricinus communis	360- 460
Eichhornia crassipes	440- 460

Table 3. Anti-algal assay of AgNPs on *C. reinhardtii* by agar well diffusion inhibition technique

Treatment	Inhibition zone (mm)*	Growth inhibition
Tinospora cordifolia AgNPs (5 ppm)	7.3	+++
Ricinus communis AgNPs (5 ppm)	4.6	++
Eichhornia crassipes AgNPs (5 ppm)	1.0	+
Tinospora cordifolia L.E.	Nil	-
Ricinus communis L.E.	Nil	-
Eichhornia crassipes L.E	Nil	-
Chemically synthesized AgNPs (5 ppm)	Nil	-
Absolute control	Nil	-

***Mean of three independent observations**

+ Presence of inhibition less than 2 mm

++ Presence of inhibition between 2-4 mm

+++ Presence of inhibition above 4 mm

- Absence of inhibition

Table 4. Anti-algal assay of AgNPs on *C. reinhardtii* by filter paper disc assay

AgNP	Inhibition zone (mm)*	Growth inhibition
Tinospora cordifolia AgNPs (5ppm)	3.6	++
Ricinus communis AgNPs (5ppm)	1.6	+
Eichhornia crassipes AgNPs (5ppm)	1.0	+
Tinospora cordifolia L.E.	Nil	-
Ricinus communis L.E.	Nil	-
Eichhornia crassipes L.E	Nil	-
Chemically synthesized AgNPs (5 ppm)	Nil	-
Absolute control	Nil	-

*Mean of three independent observations

+ Presence of inhibition less than 2 mm

++ Presence of inhibition between 2-4 mm

- Absence of inhibition

Table 5. Anti-algal assay by spot on lawn assay

AgNP	Inhibition zone (mm)*	Growth inhibition
Tinospora cordifolia AgNPs (5ppm)	6.0	+++
Ricinus communis AgNPs(5ppm)	3.3	++
Eichhornia crassipes AgNPs (5ppm)	1.6	+
Tinospora cordifolia L.E.	Nil	-
Ricinus communis L.E.	Nil	-
Eichhornia crassipes L.E	Nil	-
Chemically synthesized AgNPs (5 ppm)	Nil	-
Absolute control	Nil	-

***Mean of three independent observations**

+ Presence of inhibition less than 2 mm

++ Presence of inhibition between 2-4 mm

+++ Presence of inhibition above 4 mm

- Absence of inhibition

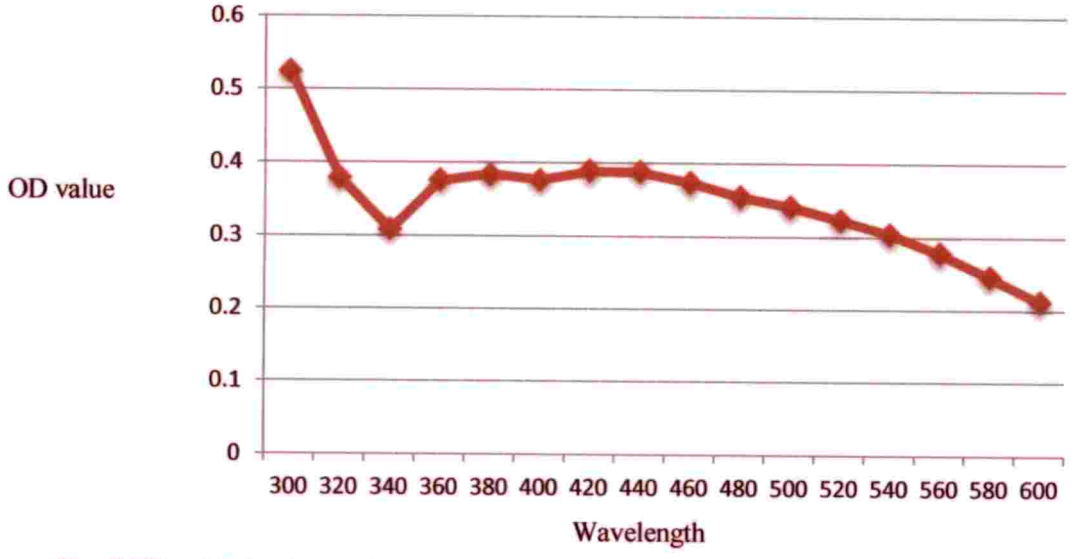


Fig. 3 UV-visible absorption spectra of green synthesised (*R. communis*) silver nanoparticles

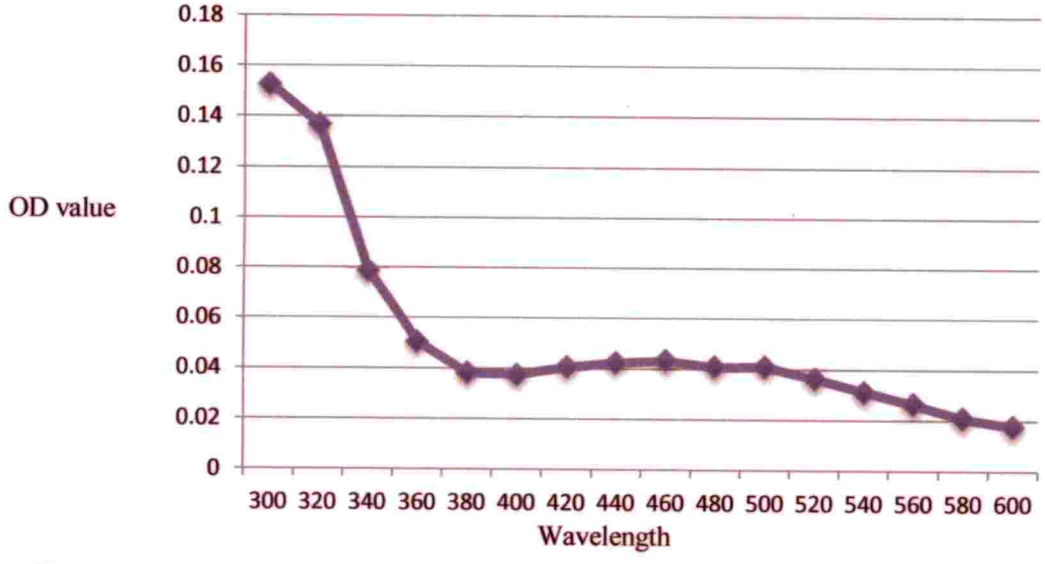
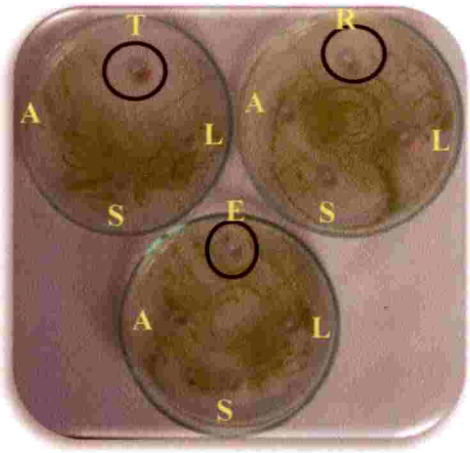
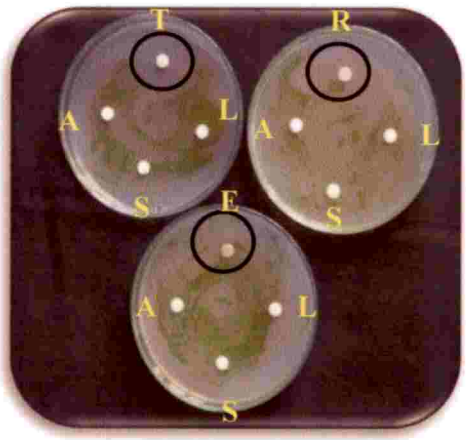


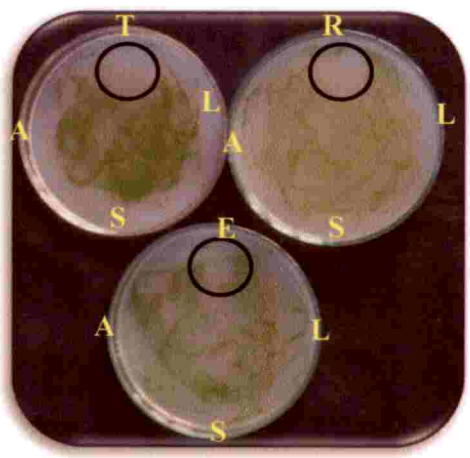
Fig. 4 UV-visible absorption spectra of green synthesised (*E. crassipes*) silver nanoparticles



4A



4B



4C

Plate 4. Inhibition assay of green synthesised silver nanoparticles on *C. reinhardtii* (4A- Agar well diffusion inhibition assay, 4B- Filter paper disc assay, 4C- Spot- on lawn assay .)

T- *Tinospora cordifolia* AgNPs

R- *Ricinus communis* AgNPs

E- *Eichhornia crassipes* AgNPs

L- leaf extract of the respective plant

A- Absolute control (Sterile water)

S- Chemically synthesised AgNP

silver nanoparticles, maximum antialgal property was exhibited by those sythesised using *T. cordifolia.*

4.5 POPULATION ASSAY OF *C. reinhardtii* WITH GREEN SYNTHESISED (*T. cordifolia*) SILVER NANOPARTICLES

The population assay of *C. reinhardtii* was carried out as described in materials and methods 3.4 using green synthesized AgNPs using *T. cordifolia,* as these AgNPs exhibited maximum inhibition zone in all the three inhibition assays. The population assay was carried out every fourth day, after giving treatments in the initial phase and at the beginning of exponential phase using green synthesised AgNPs from *T. cordifolia* (Plate 5).

4.5.1 Effect of Green Synthesised Silver Nanoparticles on *C. reinhardtii* in the Initial Growth Phase

Among the various treatments tried in the initial growth phase, the population count on the first day of the treatment did not show any significant variation. However, the population count significantly varied among the treatments on fourth, eighth and twelfth day of the treatment. The significantly higher inhibition in algal population was observed in green synthesized *T. cordifolia* AgNPs on all these days of treatment. The population count of *C. reinhardtii* was 3.12, 2.94 and 1.01 log cfu ml^{-1}, respectively on fourth, eighth and twelfth day of the treatment. The lowest inhibition was observed in absolute control on the 4^{th} day of treatment, which was on par with chemically synthesized AgNPs, with a higher population count of 3.94 and 3.92 log cfu ml^{-1}. On eighth day of treatment also, absolute control recorded minimum algal growth inhibition (4.12 log cfu ml^{-1}), which was on par with chemically synthesised AgNPs (4.06 log cfu ml^{-1}). However, on the twelfth day, minimum algal growth inhibition was obtained in the culture treated with chemically synthesised AgNPs with a population count of 6.19 log cfu ml^{-1}.

In the treatment with the green synthesized AgNPs, the population count was found to decline with every fourth day of observation until 12^{th} day of treatment. The green synthesized AgNPs using *T. cordifolia* caused a reduction in the population count (1.01 log cfu ml^{-1}) of *C. reinhardtii* on the twelfth day of treatment application compared to the initial population (2.09 log cfu ml^{-1}) in the algal culture.

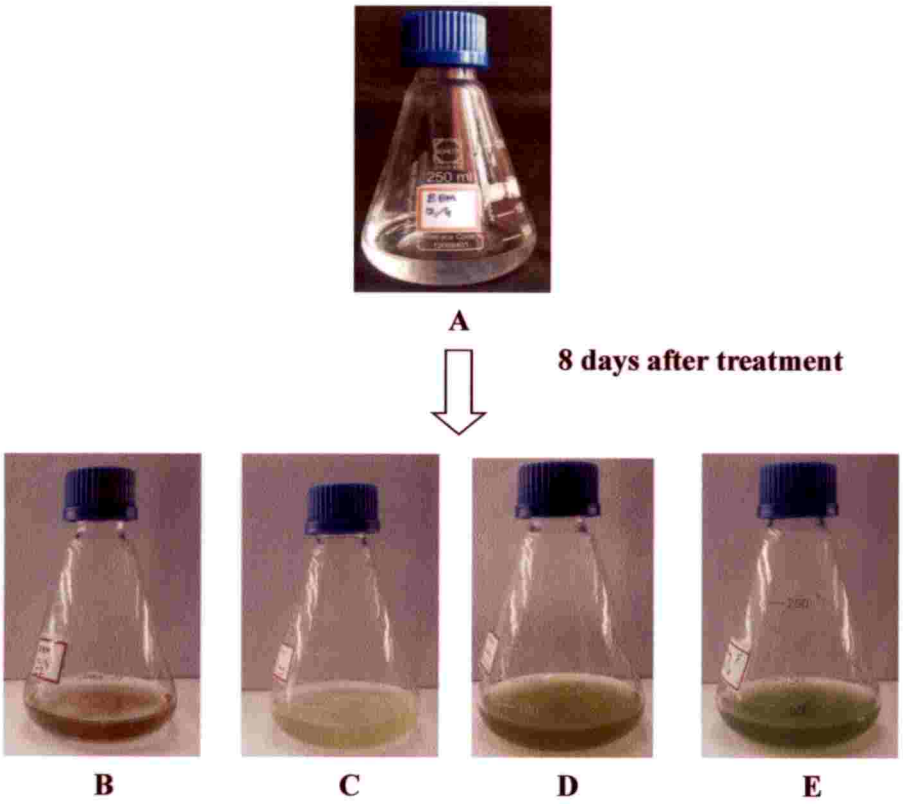


Plate 5. Effect of green synthesised (*T. cordifolia*) AgNPs on *C. reinhardtii* culture at initial growth phase

A. *C. reinhardtii* culture on the day of inoculation
B. *C. reinhardtii* culture treated with green synthesis (*T. cordifolia*) AgNps
C. *C. reinhardtii* culture treated with leaf extract (*T. cordifolia*)
D. *C. reinhardtii* culture treated with chemically synthesised AgNps
E. *C. reinhardtii* culture (absolute control)

The population count of *C. reinhartii* in culture showed an increasing trend with time, when the culture was exposed to *T. cordifolia* leaf extract, chemically synthesized Ag NPs and absolute control. The population count increased to 3.95 log cfu ml^{-1} on the twelfth day from an initial population of 2.03 log cfu ml^{-1} in the culture. The culture treated with chemically synthesised silver nanoparticles exhibited an increase in population to 6.19 log cfu ml^{-1} on the twelfth day from the initial population of 2.07 log cfu ml^{-1}. In the absolute control, the population increased to 5.94 log cfu ml^{-1} on the twelfth day compared to the initial population (2.08 log cfu ml^{-1}). However, it is observed in the study that the *C. reinhartii* culture treated with leaf extract gave a lower population count compared to those of chemically synthesized AgNPs and absolute control (Table 6, Fig. 5A, 5B).

4.5.2 Effect of Treatments Given at the Exponential Growth Phase on the Growth of *C. reinhardtii*

The treatments were given at the beginning of the exponential phase to study their effect on the population count of *C. reinhardtii* with time. The treatments were given on the 8th day (first day of the treatment) which marked the beginning of exponential phase and the population assay was carried out every fourth day upto the 20th day (12th day after the treatment) (Plate 6).

Among the various treatments tried, significant variation was observed with respect to population count on eighth, twelfth, sixteenth and twentieth day. The initial population count on the eighth day was 4.30, 4.60, 4.91 and 5.21 log cfu ml^{-1} respectively in cultures subjected to different treatments *viz.*, green synthesized *T. cordifolia* AgNPs, *T. cordifolia* leaf extract, chemically synthesized AgNPs and absolute control. The maximum population inhibition was obtained in cultures treated with green synthesized *T. cordifolia* AgNPs in all the different periods, of twelfth, sixteenth and twentieth day with a population count of 3.12, 2.94 and 1.84 log cfu ml^{-1}, respectively. The minimum inhibition of algal culture was obtained in the absolute control during all the time periods *ie.*, twelfth, sixteenth and twentieth day with a higher population count of 5.71, 5.82 and 6.11 log cfu ml^{-1} respectively.

The population assay of *C. reinhardtii*, on application of the treatments at the beginning of the exponential growth phase also exhibited a similar trend with time as with the treatments applied at the initial growth phase. Except in green synthesised AgNPs using *T.cordifolia,* all

Table 6. Effect of Green synthesised (*T. cordifolia)* AgNPs on *C. reinhardtii* during initial phase of growth

Treatment	Population of *C. reinhardtii* (log cfu ml $^{-1}$)			
	Day 1	Day 4	Day 8	Day 12
Leaf extract	2.03±0.02 (0.107)	3.74 ± 0.030 (5.49)	3.73 ± 0.025 (5.37)	3.95± 0.011 (8.91)
Green synthesized AgNPs	2.09 ± 0.015 (0.123)	3.12 ± 0.022 (1.318)	2.94 ± 0.020 (0.87)	1.01 ± 0.023 (0.01)
Chemically synthesis AgNPs	2.07± 0.038 (0.117)	3.92 ± 0.016 (8.3)	4.06 ± 0.024 (11.4)	6.19± 0.022 (1548.8)
Absolute control	2.08 ± 0.022 (0.120)	3.94± 0.020 (8.7)	4.12 ± 0.019 (13.18)	5.94 ± 0.021 (870.9)
C.D.	N/A	0.070	0.068	0.062
SE(m)	0.026	0.022	0.022	0.020

***Each reading is a mean of 4 independent observations**

**** Observations (plate counts) are taken 8 days after plating**

*****value in the parenthesis shows cfu x 10 ^{3} ml -1**

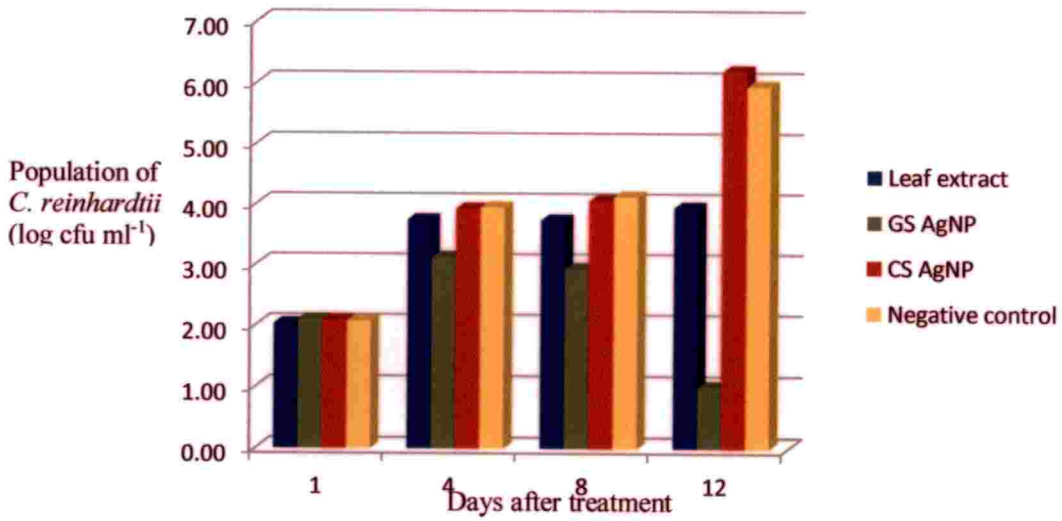


Fig. 5A Effect of green synthesised (*T. cordifolia*) AgNPs on *C. reinhardtii* during initial phase of growth

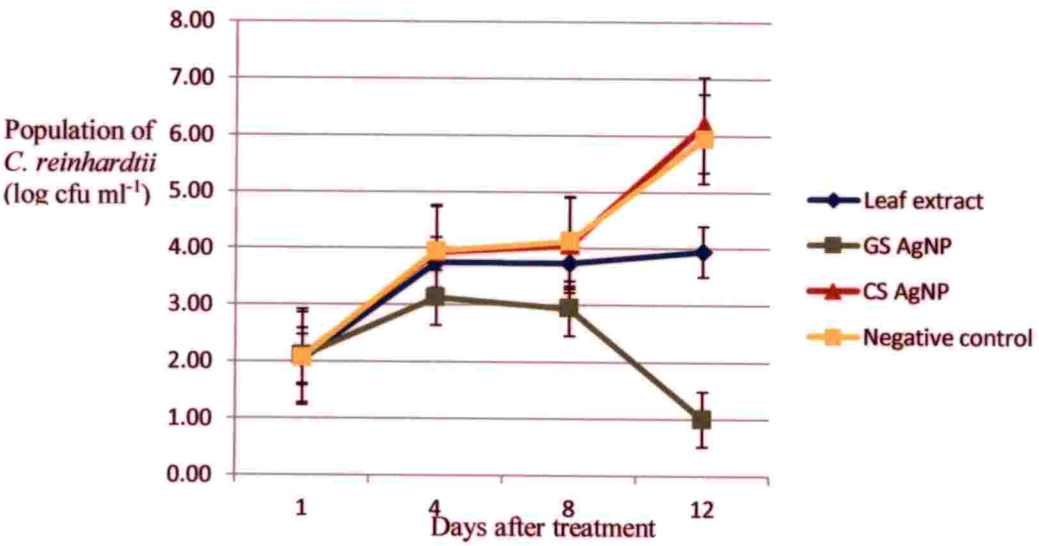


Fig. 5B Growth of *C. reinhardtii* treated with green synthesised (*T. cordifolia*) AgNPs during initial phase of growth

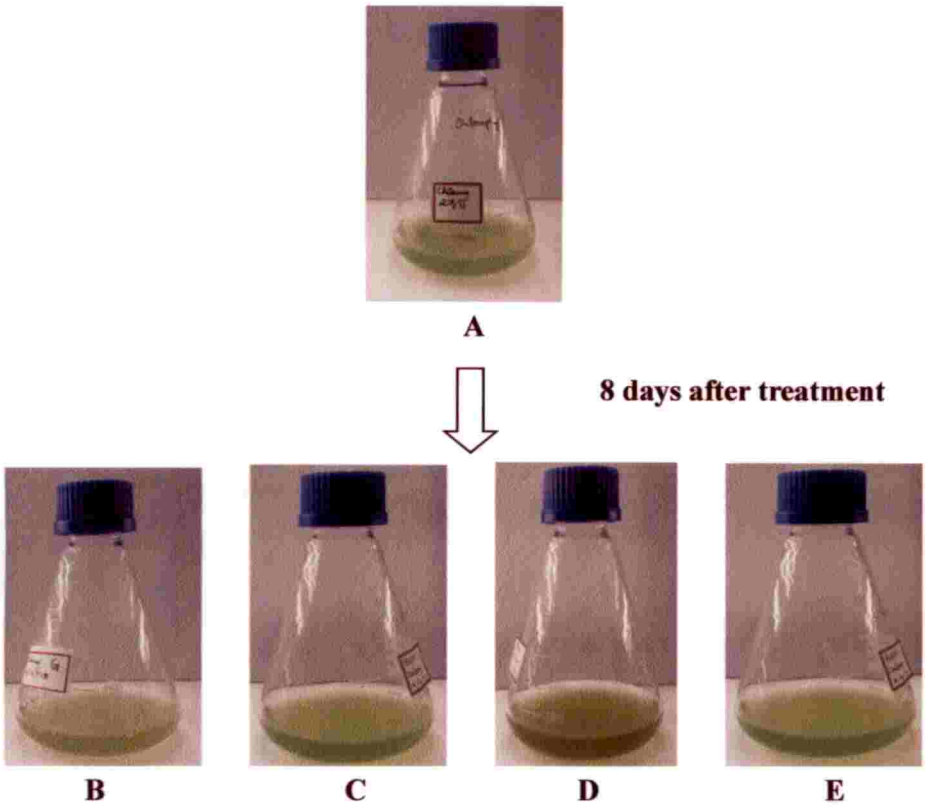


Plate 6. Effect of green synthesised (*T. cordifolia*) AgNPs on *C. reinhardtii* culture at early exponential growth phase

A. *C. reinhardtii* culture on the day of inoculation
B. *C. reinhardtii* culture treated with green synthesis (*T. cordifolia*) AgNps
C. *C. reinhardtii* culture treated with leaf extract (*T. cordifolia*)
D. *C. reinhardtii* culture treated with chemically synthesised AgNps
E. *C. reinhardtii* culture (absolute control)

other treatments *viz., T. cordifolia* leaf extract, chemically synthesised AgNPs and absolute control showed an increasing trend in population count with time.

The population count declined with time when *C. reinhartii* culture at the beginning of the exponential phase was exposed to green synthesised *T. cordifolia* AgNPs. The population in the cultures treated with green synthesized AgNPs on 20th day, ie. after 12 days of treatment, was found to be 1.84 log cfuml^{-1}compared to the first day of treatment (4.30 log cfu ml^{-1}).

The population of the culture treated with the leaf extract of *T. cordifolia* exhibited an increase in the population on the 20thday (5.77 log cfu ml^{-1}) from the first day of treatment (4.60 log cfu ml^{-1}). The population of the absolute control was found to increase from the first day of treatment (5.21 log cfu ml$^{-1)}$ to the 20thday of treatment (6.11log cfu ml^{-1}). The population of the culture treated with chemically synthesised silver nanoparticles was found to increase from 4.91 cfu ml^{-1} on the initial day of treatment to 6.02 log cfu ml^{-1}on the 20th day of treatment (Table 7, Fig. 6A, 6B).

The two different periods of application of the treatments, at the initial phase and at the beginning of the exponential phase gave differential values with respect to the reduction in population count over the time. At the initial phase, the reduction in population count in *C. reinhardtii* obtained after 12 days of the treatment with green synthesized *T. cordifolia* AgNPs was 51 per cent, while the reduction was 57 per cent, when the same treatment was applied at the beginning of the exponential phase. Thus green synthesised *T. cordifolia* AgNPs gave better algal growth inhibition when applied at the beginning of the exponential growth phase of the culture.

4.6 EFFECT OF GREEN SYNTHESISED SILVER NANOPARTICLES ON BIOCHEMICAL PARAMETERS OF *C. reinhardtii*

The treatments were applied on the 12th day of inoculation, when the culture reached the maximum growth stage. The biochemical parameters were estimated initially on the 12th day (before the treatment). After application of treatments on the twelfth day as described in materials and methods 3.5, the biochemical parameters were estimated at four days interval till eighth day of treatment application.

Table 7. Effect of Green synthesised (*T. cordifolia)* AgNPs on *C. reinhardtii* during early exponential phase of growth

Treatment	Population of *C. reinhardtii* (log cfu ml $^{-1}$)			
	Day 8	Day 12	Day 16	Day 20
Leaf extract	4.60 ± 0.025 (3.98)	5.28 ± 0.020 (19.05)	5.71 ± 0.046 (51.28)	5.77 ± 0.025 (58.8)
Green SynthesisedAgNP	4.30± 0.019 (1.99)	3.12 ± 0.020 (0.16)	2.94 ± 0.019 (0.08)	1.84 ± 0.020 (0.0069)
Chemically synthesisAgNP	4.91± 0.012 (81.2)	5.52± 0.025 (33.1)	5.71± 0.019 (51.2)	6.02 ± 0.020 (104.7)
Absolute control	5.21± 0.019 (16.2)	5.71 ± 0.019 (51.2)	5.82± 0.022 (66.06)	6.11± 0.016 (128.8)
C.D.	0.060	0.066	0.090	0.064
SE(m)	0.019	0.021	0.029	0.021

***Each reading is a mean of 4 independent observations**

**** Observations (plate counts) are taken 8 days after plating**

*****value in the parenthesis shows cfu x 10 ^{4} ml -1**

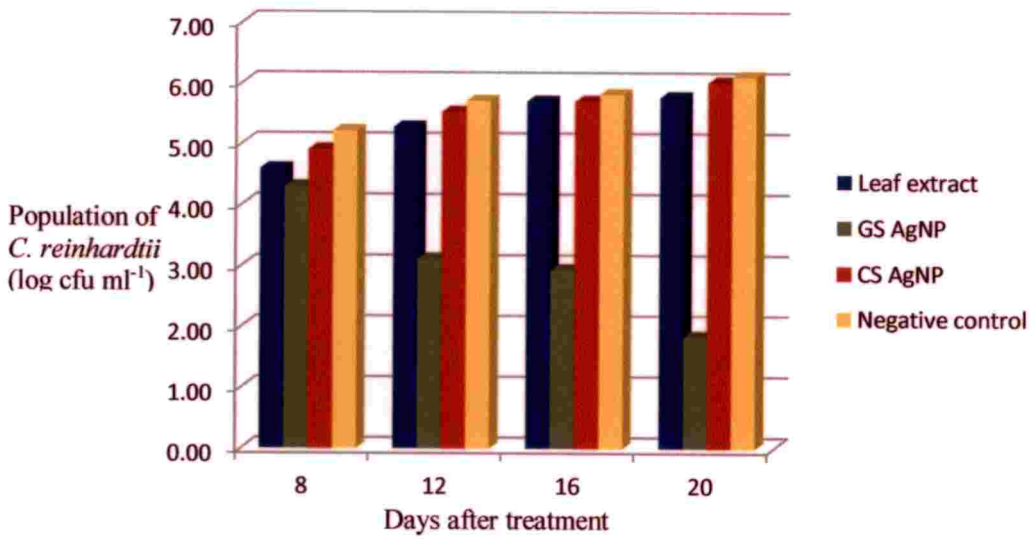


Fig. 6A Effect of green synthesised (*T. cordifolia*) AgNPs on *C. reinhardtii* during early exponential phase of growth

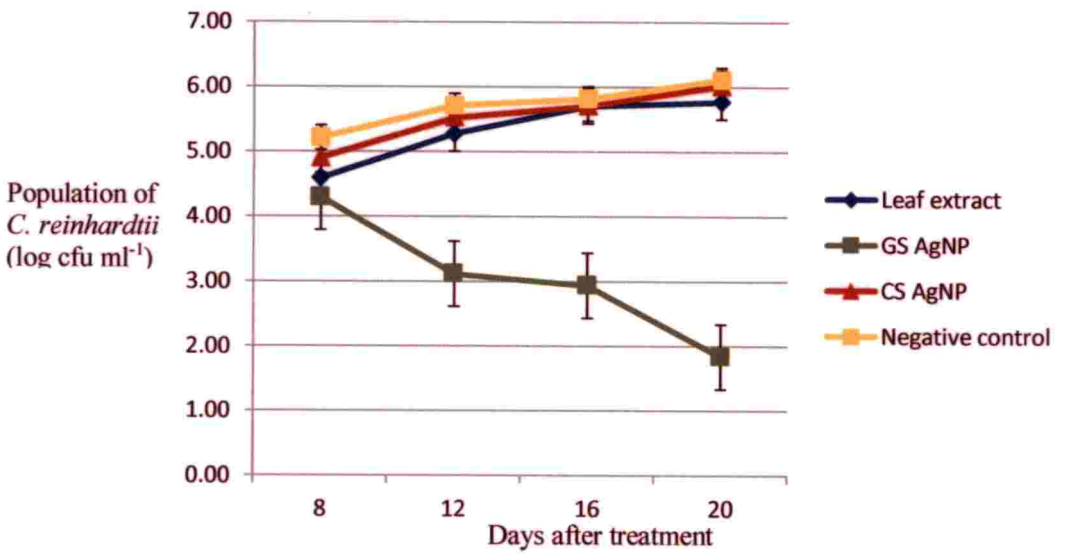


Fig. 6B Growth of *C. reinhardtii* treated with green synthesised (*T. cordifolia*) AgNPs during early exponential phase of growth

4.6.1 Total Chlorophyll Content

The initial chlorophyll content did not show any significant variation among the cultures subjected to different treatments. On the fourth and eighth day of treatment significant variation was observed in the chlorophyll content of the cultures exposed to various treatments *viz.*, green synthesised *T. cordifolia* AgNPs, *T. cordifolia* leaf extract, chemically synthesized AgNPs and absolute control. The maximum chlorophyll content (10.86 and 15.21 μg ml $^{-1}$, respectively) was observed in the absolute control on the fourth and eighth day of the treatment, indicating a low algal growth inhibition. The lowest chlorophyll content (6.08 and 5.96 μg ml $^{-1}$, respectively) was observed on the fourth day and eighth day in cultures treated with green synthesised *T. cordifolia* AgNPs (Table 8, Fig. 7A, 7B).

The chlorophyll content of the culture treated with green synthesised *T. cordifolia* AgNPs showed a decline with time. The chlorophyll content of the treated culture was found to decrease from 6.20 μg ml $^{-1}$ on the initial day of treatment to 5.96μg ml $^{-1}$ on the eighth day after the treatment application. The chlorophyll content of the culture treated with *T. cordifolia* leaf extract, chemically synthesised AgNPs and absolute control was found to increase over time. The chlorophyll content of the culture treated with the *T. cordifolia* leaf extract, increased from 6.21 to 11.73μg ml $^{-1}$while on treatment with the chemically synthesised AgNPs, the cholorophyll content increased from 6.23 to 13.06 μg ml $^{-1}$. In the absolute control, it increased from 6.06 μg ml $^{-1}$ to 15.21 μg ml $^{-1}$. However, it is observed that the rate of increase (88.88 per cent) in chlorophyll content in the culture treated with *T. cordifolia* leaf extract was much less compared to the increase on treatment with chemically synthesised AgNPs (109.63 per cent) and absolute control (150.99 per cent).

4.6.2 Carbohydrate Content

The initial carbohydrate content did not show any significant variation among the cultures subjected to different treatments *viz.*, green synthesized *T. cordifolia* AgNPs, *T. cordifolia* leaf extract, chemically synthesized AgNPs and absolute control. On the fourth and eighth day of treatment, significant variation was observed in the carbohydrate content of the cultures exposed to various treatments. The maximum carbohydrate content was observed in absolute control on the fourth and eighth day of the treatment (11.10 and 16.84μg ml $^{-1}$ respectively), indicating a

Table 8. Effect of green synthesized (*T. cordifolia*) silver nanoparticles on chlorophyll content of *C. reinhardtii* culture

Treatment	Chlorophyll content in the culture solution ($\mu g\ ml^{-1}$)		
	0^{th} day of treatment	4^{th} day of treatment	8^{th} day of treatment
Leaf extract	6.21 ± 0.198	9.61 ± 0.68	11.73 ± 0.51
Green synthesis AgNP	6.20 ± 0.223	6.08 ± 0.22	5.96 ± 0.24
Chemically synthesis AgNP	6.23 ± 0.276	10.86 ± 0.26	13.06 ± 0.30
Absolute control	6.06 ± 0.188	12.77 ± 0.91	15.21 ± 0.01
C D	NS*	1.85	0.996
SE (m)	0.224	0.593	0.32

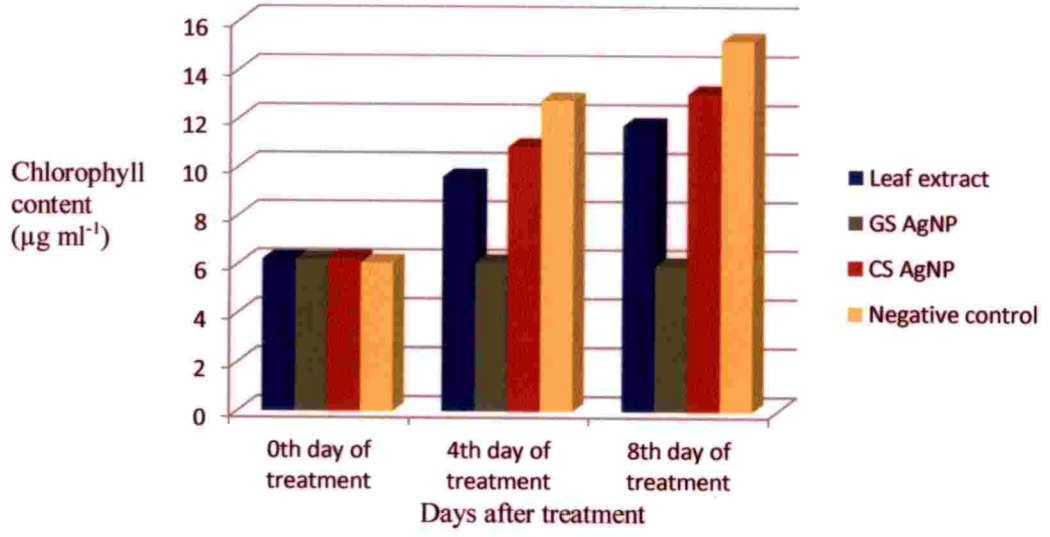


Fig. 7A Effect of green synthesised (*T. cordifolia*) AgNPs on Chlorophyll content of *C. reinhardtii* culture

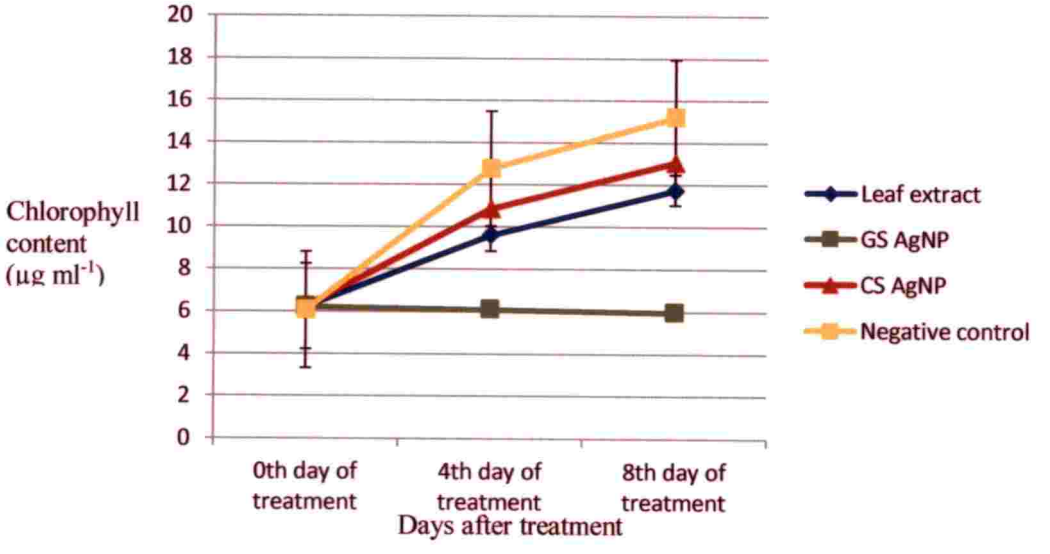


Fig. 7B Chlorophyll content of *C. reinhardtii* culture treated with green synthesized (*T.cordifolia*) AgNPs

low algal growth inhibition. The lowest carbohydrate content (4.46 and 2.46 μg ml $^{-1}$, respectively) was observed on the fourth day and eighth day in cultures treated with green synthesized *T. cordifolia* AgNPs (Table 9).

On analysing the variation in carbohydrate content after giving different treatments, it was observed that in the culture treated with green synthesised AgNPs there was a reduction from the initial value of 4.15 μg ml $^{-1}$ to 2.46 μg ml $^{-1}$ after eight days of treatment application. The carbohydrate content of absolute control increased from 4.14 μg ml $^{-1}$ on the initial day to 16.84 μg ml $^{-1}$ on the eighth day of treatment application. The carbohydrate contents of the cultures treated with leaf extract and chemically synthesised AgNPs were found to increase from initial values of 3.75 μg ml $^{-1}$ and 4.01 μg ml $^{-1}$ to 11.88 μg ml $^{-1}$ and 15.24 μg ml $^{-1}$respectively on the eighth day of treatment application. The carbohydrate content gave a declining trend with time only in algal cultures treated with green synthesised *T. cordifolia* AgNPs. In all the other treatments this parameter showed an increasing trend with time.The rate of increase in carbohydrate content was the highest in absolute control (306.76 per cent) followed by chemically synthesised AgNPs (280.05 per cent) and *T. cordifolia* leaf extract (216.80 per cent) (Fig. 8A, 8B).

4.6.3 Protein Content

The initial protein content showed significant variation among the cultures subjected to different treatments *viz.*, green synthesised *T. cordifolia* AgNPs, *T. cordifolia* leaf extract, chemically synthesised AgNPs and absolute control. The culture subjected to the treatment with *T. cordifolia* leaf extract recorded lowest protein content of 9.61 μg ml $^{-1}$.Maximum protein content (10.43 μg ml $^{-1}$) was observed with the culture subjected to treatment with green synthesised AgNPs, which was on par with that of the cultures subjected to chemically synthesized AgNPs and absolute control. On the fourth and eighth day of treatment significant variation was observed in the protein content of the cultures exposed to various treatments. The maximum protein content (18.08 and 25.93 μg ml $^{-1}$respectively) was observed in absolute control on the fourth and eighth day of the treatment, indicating a low algal growth inhibition. The lowest protein content (8.40 and 6.27μg ml $^{-1}$, respectively) was observed on the fourth day and eighth day in cultures treated with green synthesised *T. cordifolia* AgNPs (Table 10).

Table 9. Effect of green synthesized (*T. cordifolia*) silver nanoparticles on carbohydrate content of *C. reinhardtii* culture

Treatment	Carbohydrate content in culture solution (µg ml $^{-1}$)		
	0th day of treatment	4th day after treatment	8th day of treatment
Leaf extract	3.75 ±0.138	8.50 ±0.220	11.88 ±0.079
Green synthesised AgNP	4.15 ±0.284	4.46 ±0.177	2.46 ±0.198
Chemically synthesised AgNP	4.01 ±0.308	9.34 ±0.191	15.24 ±0.202
Absolute control	4.14 ±0.262	11.10 ±0.286	16.84 ±0.080
C D	NS*	0.69	0.47
SE (m)	0.26	0.22	0.15

Table 10. Effect of green synthesized (*T. cordifolia*) silver nanoparticles on protein content of *C. reinhardtii* culture

Treatment	Protein content in culture solution (µg / ml)		
	0th day of treatment	4th day after treatment	8th day of treatment
Leaf extract	9.61 ±0.175	21.19 ±0.112	24.46 ±0.160
Green synthesisedAgNP	10.43 ±0.121	8.40 ±0.035	6.27 ±0.022
Chemically synthesisAgNP	10.19 ± 0.131	16.64 ±0.078	22.31 ±0.009
Negative control	10.40 ±0.111	18.08 ±0.050	25.93 ±0.037
C D	0.42	0.23	0.25
SE (m)	0.14	0.08	0.08

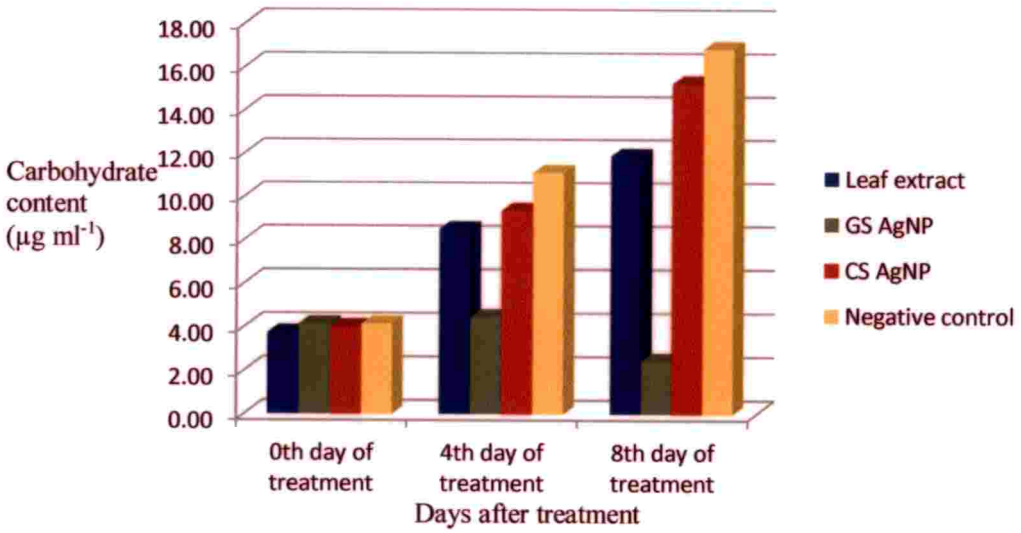


Fig.8A Effect of green synthesized (*T. cordifolia*) AgNPs on Carbohydrate content of *C. reinhardti* culture

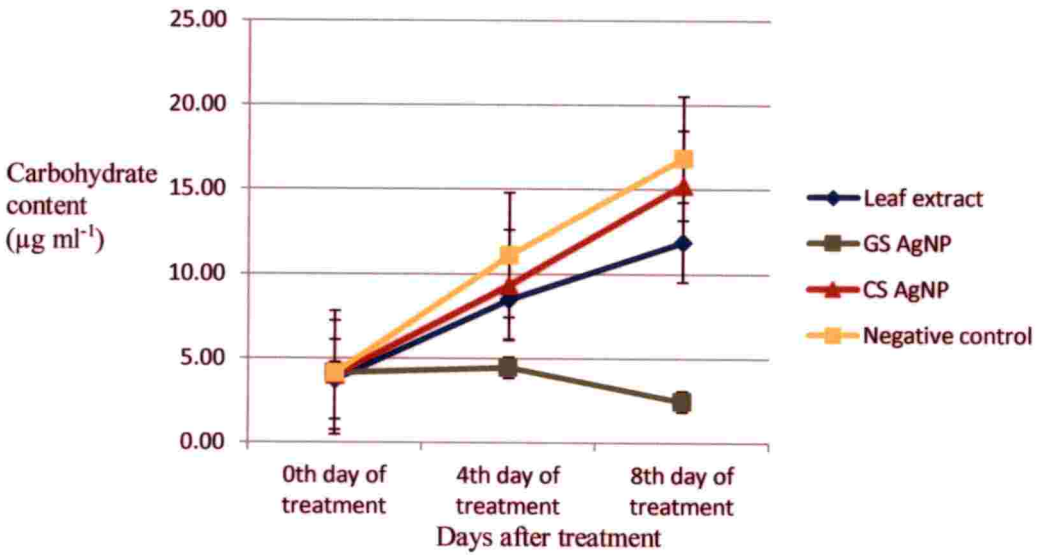


Fig. 8B Carbohydrate content of *C. reinhardtii* culture treated with green synthesised (*T.cordifolia*) AgNPs

The protein content of the algal culture subjected to different treatments also showed a similar trend over time as in chlorophyll content. The algal culture treated with green synthesized silver nanoparticles was found to decrease from 10.43µg ml $^{-1}$ on the initial day of treatment to 6.27µg ml $^{-1}$ on the eighth day after treatment application. Whereas, in other treatments, an increasing trend was observed with time. The protein content of cultures treated with *T. cordifolia* leaf extract was found to increase from 9.61 µg ml $^{-1}$ to 24.46 µg ml $^{-1}$ on the eighth day after the treatment. The protein content of cultures treated with chemically synthesised AgNPs were found to increase from 10.19 µg ml $^{-1}$ to 22.31 µg ml $^{-1}$. In the absolute control, the protein content increased from 10.40µg ml $^{-1}$on the initial day of treatment to25.93 µg ml $^{-1}$on the eighth day after the treatment. The rate of increase in protein content followed a different trend compared to chlorophyll content among the treatments *viz. T. cordifolia* leaf extract, chemically synthesised AgNPs and absolute control. The cholorophyll content in the culture treated with *T. cordifolia* leaf extract had a higher rate (154.53 per cent) of increase in protein content over time compared to chemically synthesised AgNPs (118.94 per cent) and absolute control (149.33 per cent) (Fig. 9A, 9B).

4.6.4 Lipid Content

Lipid content in the cultures subjected to different treatments *viz. T. cordifolia* leaf extract, green synthesised *T. cordifolia* AgNPs, chemically synthesised AgNPs and absolute control, were analysed using Floch's method. The lipid content in the cultures was below detectable levels even after eight days of treatment application.

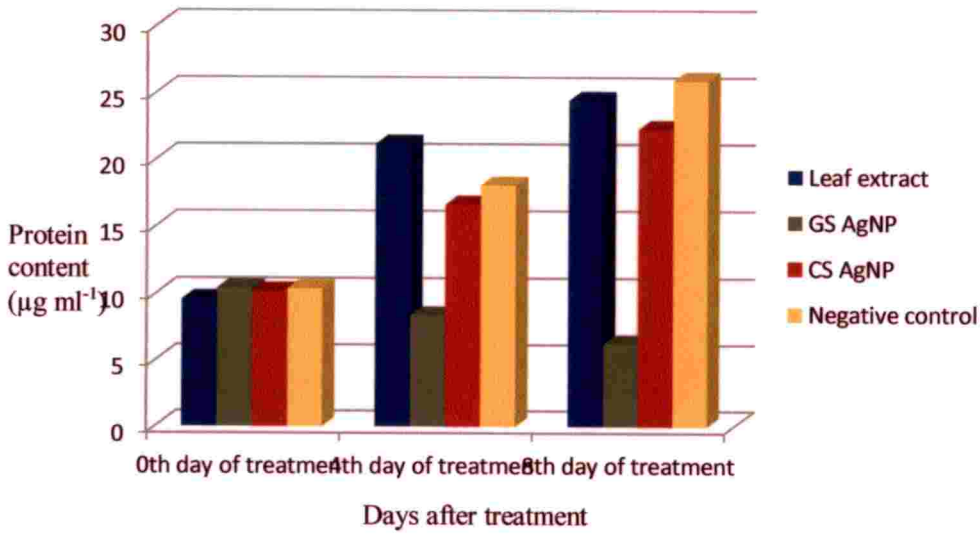


Fig. 9A Effect of green synthesised (*T. cordifolia*) AgNPs on Protein content of *C. reinhardtii* culture

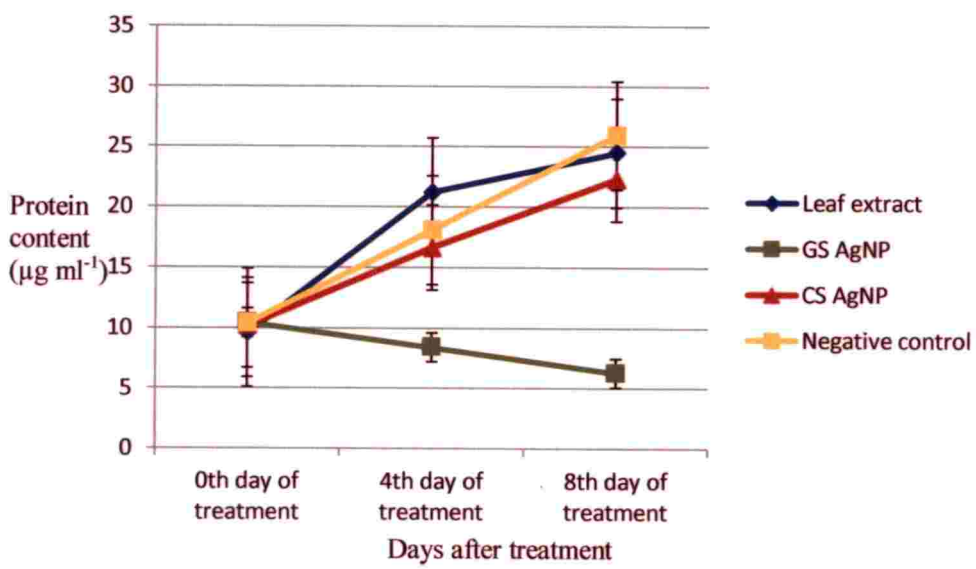


Fig. 9B Protein content of *C. reinhardtii* culture treated with green synthesized (*T.cordifolia* AgNPs)

Discussion

5. DISCUSSION

Silver nanoparticles exhibit powerful antimicrobial properties which make them an inevitable component of most of the antimicrobial preparations. "Bionanotechnology", the branch of science involved in developing biosynthetic and environmental-friendly technologies for synthesis of nanomaterials have been emphasizing more on evolving better protocols for synthesis of silver nanoparticles.

Algae form an important component of our ecosystem. They cause major problems in polyhouses, solar panels, fresh water bodies etc. as they reduce the amount of incident solar radiation. They are reported to cause diseases to plants and algal blooms in water bodies (Pozdnyakov *et al.*, 2017). There have been many reports on antibacterial and antifungal properties of silver nanoparticles (Le Ouay and Stellacci 2015; Mallmann *et al.*, 2015; Chien *et al.*, 2018; Balasubramanian *et al.*, 2019; Bocate *et al.*, 2019). However, reports on antialgal properties of silver nanoparticles especially that of green synthesised silver nanoparticles are very limited (Dhasarathan *et al.*, 2018). Hence, in the present study, it was attempted to green synthesise silver nanoparticles using leaf extracts of plants having antialgal property *viz.*, *Tinospora cordifolia, Ricinus communis* and *Eichhornia crassipes* (Wu *et al.*, 2012; Al-Haidari *et al.*, 2016; Ojha and Bora, 2017) and to evaluate their potential for the inhibition of growth of the model alga, *Chlamydomonas reinhardtii*.

Bolds Basal Medium is reported to facilitate the growth of many fresh water algal forms (Khan *et al.*, 2018; Marimuthu and Jayaraman, 2018). In the present study also both BBM agar medium and broth facilitated the growth of the fresh water alga, *C. reinhardtii* at 25 ± 3 °C and 3000 lux illumination.

C. reinhardtii has been worked on by various scientists in studies including molecular manipulation, analyzing phytotoxicity of various compounds, nitrogen scavenging, gene expression analysis, optimizing growth conditions etc.

(Blaby and Blaby-Haas 2018; Hong, 2018; Majewska *et al.*, 2018; Sasso *et al.*, 2018; Calatrava *et al.*, 2019). It is reported as the model alga due to its easiness in culturing and genetic manipulation, and comparatively reduced doubling time (Bonente *et al.*, 2012). In the present study also *C. reinhardtii* could be easily multiplied and the doubling time of the alga was found to be 24 h with maximum growth on twelfth day of inoculation. The growth pattern exhibited a nearly sigmoid curve with lag phase, log phase (exponential growth phase) and stationary phase. Bonente *et al.* (2012) have reported similar growth curve in *C. reinhardtii* acclimatised to varying light intensities.

In the present study when 1mM silver nitrate solution was treated with leaf extracts (5% w/v) of *Tinospora cordifolia, Ricinus communis* and *Eichhornia crassipes* in a microwave oven the colour changed to dark brown. Change in colour of the solution from yellowish to dark brown is reported to be an indication of conversion of Ag^+ ions in $AgNO_3$ to Ag^0 particles during green synthesis of AgNPs (Ahmed *et al.*, 2016, Kalishwaralal *et al.*, 2010, Sun *et al.*, 2014).The change in colour is attributed to the surface plasmon resonance which is one of the characteristic features of AgNPs. Visible colour change in the present study indicates the conversion of Ag^+ ions in $AgNO_3$ to Ag^0 particles.

In the present study the different plants tested exhibited different time duration for green synthesis of AgNPs. The minimum time duration taken was for *T. cordifolia* (1h) followed by *R. communis* (2h) and *E. crassipes* (12h). In the previous reports on green synthesis of silver nanoparticles, plants have exhibited variation in time taken for the formation of AgNPs (Velusamy *et al.*, 2015; Ahmed *et al.*, 2016). According to Ojha *et al.* (2017) the time duration taken for formation AgNPs using methanolic extract of *R. communis* at room temperature was 24h. In the present study, the time taken for the formation of silver nanoparticles using the leaf extract of *R. communis* could have reduced since it was heated for 40s in a microwave oven. Jayaseelan *et al.* (2011) have reported the time duration for green synthesis of AgNPs from *T. cordifolia* to be 2h when the reaction was carried out at room temperature. In the present study, the reaction

time was reduced, which may be due to the heating in microwave oven for 40s. Kiruba *et al.* (2012) reported the synthesis of AgNPs from *E. crassipes* in 5 min. at 70 ^{0}C. However, in the present study the time taken for completion of green synthesis was 12 h. The difference in the time taken may be due to the variation in the quantity of leaf sample used (20% w/v) and the temperature of incubation.

According to Alex *et al.* (2012), the complete conversion of AgNPs can be confirmed by the addition of 0.1 N NaCl to the supernatant obtained after centrifugation of the green synthesised AgNPs. Any free Ag^+ will react with Cl^- to form precipitate of silver chloride as a result of double displacement reaction between unreacted $AgNO_3$ in the suspension and NaCl to yield sodium nitrate ($NaNO_3$) and silver chloride (AgCl). In the present study, none of the samples tested yielded any precipitate, which is an indication that complete conversion has taken place in all the three samples.

According to Ahmed *et al.* (2016), the peak of UV-visible absorption spectra gives an indication of the uniformity of size of green synthesised AgNPs. According to them, increase in the concentration of the leaf extract and change in the reaction time caused a significant variation in the absorption maxima and its distribution on UV-visible spectrophotometric analysis. In the present study, the UV-visible absorption peak of *T. cordifolia* and *E. crassipes* have comparatively narrower range compared to that of *R. communis*. According to Singh *et al.* (2012), UV-visible absorption spectra of *R. communis* exhibited a absorption peak between 415 and 420 nm. In the present study, the peak of AgNPs produced using *R. communis* leaf extract was found to be between 360-460 nm. The variation may be due to the difference in the quantity of leaf sample used for green synthesis in both the studies and the ratio of leaf extract and $AgNO_3$ solution (1:1*vs* 1:9). According to Kiruba *et al.* (2012), the AgNPs produced using leaf extract of *E. crassipes* exhibited a UV-visible absorption peak between 420 nm to 440 nm at a pH of 5. In the present study, the maximum absorption range was between 440-460 nm. The slight variation may be due to the variation in pH and quantity of leaf sample used in both the studies. The narrowest UV-visible absorption peak

was obtained for *T. cordifolia* (440-460 nm) indicating better uniformity in the size of the green synthesised AgNPs. However, according to Singh *et al.* (2014), the UV-visible absorption peak was between 420-425 nm for green synthesised AgNPs using plant extract of *T. cordifolia*. This variation in the range of absorption peak may be due to the difference in the temperature of incubation and the quantity of leaf sample used for green synthesis in both the studies.

In the present study, the leaf extracts of *T. cordifolia, R. communis* and *E. crassipes* did not exhibit conspicuous inhibition zones in*in vitro* inhibition assays. However, according to Al-Haidari *et al.* (2016), *T. cordifolia,* exhibited antialgal property when tested against three algal isolates namely, *Anabaena circinalis*, *Scenedsmus quadricauda* and *Mougeotia scalaris.* Oil extracted from *R. communis* was reported to have antialgal property on three species of Cyanophyta *viz.*, *Nostoc carneum*, *Westillopesis prolifica*, and *Chroococcus turgidus* and one species of Chlorophyta *viz. Chlorella vulgaris* (Al-Husseinawi *et al.*, 2014). Acetone extract of root system of *E. crassipes* was found to have antialgal properties and the compounds responsible for the algicidal property were identified to be N-phenyl-2-naphthylamine, linoleic acid and glycerol-1,9-12(ZZ)- octadecadienoic ester (Xang *et al.*, 1992). The contradiction in the results in the present study and previous reports may be probably because in the reported works terpenes, alkaloids and phenols extracted from *T. cordifolia*, oil extracted from *R. communis* and acetone extracts of root system of *E. crassipes* have been used whereas in the present study water extracts of leaves of the corresponding plants have been used. Moreover, the concentration of the leaf extract used in the present study also differs from previous reports. The concentration of leaf extract was only 5% w/v *ie.*, the same concentration that has been used for the green synthesis.

In the present study, even though the leaf extracts of *T. cordifolia*, *R. communis* and *E. crassipes* exhibited no inhibition zones in case of *in vitro* inhibition assays, their potential antialgal property as reported earlier (Xang *et al.*, 1992; Al-Husseinawi *et al.*, 2014; Al-Haidari *et al.* 2016) was evident from the

population assays and biochemical assays. However, it was significantly less compared to that of the green synthesised AgNPs.

The green synthesised AgNPs of *T. cordifolia* exhibited the maximum inhibition zone in all the three inhibition assays *viz.*, agar well inhibition assay (7.3 mm), disc diffusion assay (3.6 mm) and spot-on lawn assay (6.0 mm). The uniformity in size may be a contributing factor for the increased inhibitory effect of green synthesised AgNPs of *T. cordifolia* compared to *R. communis* and *E. crassipes*.

The inhibition of algal growth exhibited by *T. cordifolia* in the *in vitro* assays was reconfirmed by the results obtained in population assays by treatment at the initial growth phase and at the beginning of the exponential growth phase of the algal culture. When green synthesised AgNPs were added on the same day of inoculation of *C. reinhardtii*, the population count on logarithmic basis (1.01 log cfu ml^{-1}) showed a reduction on the twelfth day compared to the untreated control (5.94 log cfu ml^{-1}). When green synthesised silver nanoparticles were added at the beginning of the exponential phase of growth of *C. reinhardtii*, the population count (1.84 log cfu ml^{-1}) showed a reduction on the eighth day compared to the untreated control (6.11 log cfu ml^{-1}). This indicates that green synthesised AgNPs from *T. cordifolia* were effective in inhibiting the growth of *C. reinhardtii* at different stages of growth of the algae. According to Oukarroum *et al.* (2014), using flow cytometry the viability of the cells of *Chlamydomonas acidophila* decreased significantly on treatment with AgNPs (50 nm) at different concentrations (0.1-100 mg l^{-1}) .

Variations in biochemical parameters *viz.*, total chlorophyll, carbohydrate, protein and lipid contents were recorded at three days interval, after treating the culture solution of *C. reinhardtii* at the exponential growth phase (Day 12) with green synthesised AgNPs of *T. cordifolia.* The carbohydrate content (2.46 μg ml^{-1}) showed a significant reduction in the treated cultures, but it was increased in the untreated control (16.84 μg ml^{-1}) on the eighth day of inoculation. There was also

a significant reduction in the protein content (6.27 μg ml^{-1}) in the treated culture compared to untreated control (25.93 μg ml^{-1}) on eighth day of inoculation. The chlorophyll content (5.96 μg ml^{-1}) remained stable on the eighth day in the treated culture whereas in the untreated control, approximately 2.5 times increase was recorded (15.21 μg ml^{-1}). Oukarroum *et al.* (2014) reported that there was significant reduction in cell viability and chlorophyll content accompanied by increased Reactive Oxygen Species in *Chlamydomonas acidophila* when exposed to AgNPs at different pH levels (4-7).

Higher surface area to volume ratio and unique physical and chemical properties may have contributed to the enhanced antialgal activity of the green synthesised AgNPs. The toxicity of the AgNPs is facilitated by their ability to form free radicals when in contact with the cell surface which eventually makes the cell wall porous, causing leakage of cell contents (Danilczuk *et al.*, 2006). Silver nanoparticles also interact with the phosphorous moiety of the DNA leading to interruptions in DNA replication which further leads to cessation of cell multiplication (Prabhu and Paulose, 2012).

Interestingly, the chemically synthesised AgNPs of size 100 nm (Sigma Aldrich, USA) that were purchased commercially did not exhibit antialgal property in the present study. Toxicity of AgNPs to a great extent depends on the size and the chemical nature of the stabilizing agents used to prevent aggregation. Transmission electron microscope analysis of the green synthesised AgNPsof the present study could reveal more details about their characteristics including their size. Navarro *et al.* (2008) have reported that chemically synthesised AgNPs of size 25 nm caused toxicity to the cells of *C. reinhardtii* and reduced their photosynthetic efficiency when the alga was exposed to varying concentrations of AgNPs (5-10 μmol l^{-1}). Lack of algal toxicity of the chemically synthesised AgNPs that were commercially purchased may be due to the difference in the size of the AgNPs in both the studies. Morones *et al.* (2005) reported that the microbicidal activity of silver nanoparticles was depended greatly on their surface area which would increase with decrease in the size of the particles. Moreover, the

chemically synthesised silver nanoparticles used in the present study were citrate-stabilised and this might have also affected the antialgal property. Dhasarathan *et al.* (2018) have reported the antialgal property of green synthesised AgNPs obtained using bamboo leaf extract on algae *Dictyosphaerium pulchellum*, and *Algoriphagus chordate*. The anti algal effect was found to be via cell wall breakage resulting in death of the algae.

The present study is the first report on green synthesised AgNPs using leaf extract of *T. cordifolia* exhibiting antialgal property against *C. reinhardtii*. Further studies can aid in developing an eco-friendly approach for control of invasive algae using green synthesised silver nanoparticles.

Summary

SUMMARY

The study entitled "Green synthesised silver nanoparticles for suppression of algal growth" was carried out during 2016-2019, in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to green synthesise silver nanoparticles using leaf extracts of plants having antialgal property and to evaluate their potential for the growth inhibition of model alga *Chlamydomonas* sp.

The study focused on producing green synthesised silver nanoparticles using leaf extracts (10% v/v) of giloy (*Tinospora cordifolia)*, castor (*Ricinus communis*) and water hyacinth (*Eichhornia crassipes).* The UV-vis absorption maxima of the green synthesised AgNPsusing leaf extracts of different plants showed variation. The peak of the UV-visible spectra of the silver nanoparticles produced using *T. cordifolia* (440-460nm) and *E. crassipes* (440-460nm) exhibited comparatively narrow peak compared to the absorption maxima of *R. communis* (360- 460nm).

Chlamydomonas reinhardtii (*C. reinhardtii*) was grown in Bold's Basal Medium. Growth kinetics was carried out based on functional relationship between time and population and the exponential growth phase was determined. The doubling time was recorded to be 24 h and the exponential phase of growth was found to be from eighth to twelfth day of inoculation. Maximum population was recorded on the twelfth day of inoculation (5.76 log cfu ml^{-1}).

Green synthesised silver nanoparticles were tested for their antialgal property against *C. reinhardtii* by agar well diffusion inhibition, disc diffusion and spot-on lawn assays. Silver nanoparticles (5 ppm) prepared using *T. cordifolia* leaf extract exhibited maximum inhibition of growth on *C. reinhardtii* followed by silver nanoparticles prepared using *Ricinus communis* and *Eichhornia crassipes* in all the three assays. The inhibition zone was maximum in agar well inhibition assay.

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Chemically synthesised silver nanoparticles of 100 nm size did not exhibit inhibition of growth on*C. reinhardtii*.

C. reinhardtii cultures were treated separately on the day of inoculation and at the beginning of the exponential growth phase using green synthesised silver nanoparticles of *T. cordifolia* and population assays were carried out to reconfirm the antialgal property. When green synthesised silver nanoparticles of *T. cordifolia* were added on the same day of inoculation of *C. reinhardtii*, the population count on logarithmic basis (1.01 log cfu ml^{-1}) showed a reduction on the twelfth day compared to the untreated control (5.94 log cfu ml^{-1}). The population in the culture treated with leaf extract of *T. cordifolia*was 3.95 log cfu ml^{-1}. The population of *C. reinhardtii* on the twelfth day after treatment with chemically synthesised silver nanoparticles was 6.19 log cfu ml^{-1}.

When green synthesised silver nanoparticles were added at the beginning of the exponential phase of growth of *C. reinhardtii*, the population count (1.84 log cfu ml^{-1}) showed a reduction after eight daysof treatment compared to the untreated control (6.11 log cfu ml^{-1}). The population count of *C. reinhardtii* culture treated with the leaf extract of *T. cordifolia* and chemically synthesised silver nanoparticles were 5.77 log cfu ml^{-1} and 6.02 log cfu ml^{-1} respectivelyon the twelfth day after treatment. Greeen synthesised silver nanoparticles from *T. cordifolia* were able to inhibit the growth of *C. reinhardtii* at different stages of growth of the alga.

Variations in biochemical parameters *viz*., total chlorophyll, carbohydrate, protein and lipid contents were recorded at three days interval, after treating the culture solution of *C. reinhardtii* at the exponential growth phase (Day 12) with green synthesized silver nanoparticles of *T. cordifolia*. The carbohydrate content (2.46 µg ml^{-1}) showed a significant reduction in the treated cultures but it increased in the untreated control (16.84 µg ml^{-1}) on the eighth day of inoculation. Protein content

(6.27 μg ml^{-1}) also significantly reduced in the treated culture compared to the untreated control (25.93 μg ml^{-1}) on the eighth day of inoculation. The chlorophyll content (5.96 μg ml^{-1}) remained stable on the eighth day in the treated culture whereas in the untreated control, approximately 2.5 times increase was recorded (15.21 μg ml^{-1}). Lipid content in treated and untreated culture solution was below detectable levels.

Results of the present study indicated that drastic inhibitory effect was exhibited by green synthesised silver nanoparticles using leaf extract of *T. cordifolia*against *C. reinhardtii.*

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Appendices

APPENDIX- I

COMPOSITION OF MEDIUM USED

1. Bold's Basal Medium

Components	Stock Solution ($g\ l^{-1}\ dH_2O$)	Quantity Used (to 1 litre)
$NaNO_3$	25	10 ml
$CaCl_2.2H_2O$	2.5	10 ml
$MgSO_4.7H_2O$	7.5	10 ml
K_2HPO_4	7.5	10 ml
KH_2PO_4	17.5	10 ml
NaCl	2.5	10 ml
EDTA solution	Detailed below	1 ml
Acidifed iron solution	Detailed below	1 ml
H_3BO_3	11.42 g	1 ml
Trace metals solution	-	1 ml

EDTA Solution

Components	Quantity Used (to 100 ml)
EDTA	50 g
KOH	31 g

Acidified Iron Solution

Components	Quantity Used (to 100 ml)
$FeSO_4.7H_2O$	0.498 g
H_2SO_4 (96%)	0.1 ml

Trace metals Solution

Components	Quantity Used (to 1 litre)
$ZnSO_4.7H_2O$	8.82 g
$MnCl_2.4H_2O$	1.44 g
MoO_3	0.71 g
$CuSO_4.5H_2O$	1.57 g
$Co(NO_3)_2.6H_2O$	0.49 g

GREEN SYNTHESISED SILVER NANOPARTICLES FOR THE SUPPRESSION OF ALGAL GROWTH

by

B. L. BIJULA

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ABSTRACT

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VELLAYANI, THIRUVANANTHAPURAM-695 522

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ABSTRACT

The study entitled "Green synthesised silver nanoparticles for suppression of algal growth" was carried out during 2016-2019, in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to green synthesise silver nanoparticles using leaf extracts of plants having antialgal property and to evaluate their potential for the growth inhibition of model alga *Chlamydomonas* sp.

In the present study, green synthesis of silver nanoparticles was carried out using leaf extracts of giloy (*Tinospora cordifolia)*, castor (*Ricinus communis*) and water hyacinth (*Eichhornia crassipes)*. The peak of the UV- visible spectra of the silver nitrate solution after treating with the leaf extracts (10% v/v) ranged from 360-460 nm indicating green synthesis of silver nanoparticles by biological reduction.

Growth kinetics of *Chlamydomonas reinhardtii* (*C. reinhardtii*) was carried out in Bold's Basal Medium (BBM) based on functional relationship between time and population for determining the exponential growth phase of *C. reinhardtii* for further treatment with green synthesised silver nanoparticles. The exponential phase of growth was found to be from eighth to twelfth day of inoculation, with maximum growth on the twelfth day.

Antialgal property of the green synthesised silver nanoparticles was determined by agar well diffusion inhibition, disc diffusion and spot-on lawn assays. Green synthesised silver nanoparticles (5 ppm) prepared using *Tinospora cordifolia* (*T. cordifolia*) leaf extract exhibited maximum inhibition of growth on *C. reinhardtii* followed by silver nanoparticles prepared using *Ricinus communis* and *Eichhornia crassipes* in all the three assays. Chemically synthesised silver nanoparticles of 100 nm size (Sigma Aldrich USA) did not exhibit inhibition of growth on *C. reinhardtii*.

C. reinhardtii cultures were treated separately on the day of inoculation and at the beginning of the exponential growth phase using green synthesised silver nanoparticles of *T. cordifolia* and population assays were carried out to reconfirm the antialgal property. When green synthesised silver nanoparticles were added on the same day of inoculation of *C. reinhardtii*, the population count on logarithmic basis (1.01 log cfu ml^{-1}) showed a reduction on the twelfth day compared to the untreated control (5.94 log cfu ml^{-1}). When green synthesised

silver nanoparticles were added at the beginning of the exponential phase of growth of *C. reinhardtii*, the population count (1.84 log cfu ml^{-1}) showed a reduction on the eighth day compared to the untreated control (6.11 log cfu ml^{-1}). This study showed that green synthesised silver nanoparticles from *T. cordifolia* were able to inhibit the growth of *C. reinhardtii* at different stages of growth of the algae, demonstrating its antialgal property.

Variations in biochemical parameters *viz.*, total chlorophyll, carbohydrate, protein and lipid contents were recorded at three days interval, after treating the culture solution of *C. reinhardtii* at the exponential growth phase (Day 12) with green synthesized silver nanoparticles of *T. cordifolia.* The carbohydrate content (2.46 µg ml^{-1}) showed a significant reduction in the treated cultures but it increased in the untreated control (16.84 µg ml^{-1}) on the eighth day of inoculation. Protein content (6.27 µg ml^{-1}) also significantly reduced in the treated culture compared to untreated control (25.93 µg ml^{-1}) on eighth day of inoculation. The chlorophyll content (5.96 µg ml^{-1}) remained stable on the eighth day in the treated culture whereas in the untreated control, approximately 2.5 times increase was recorded (15.21 µg ml^{-1}). Lipid content in treated and untreated culture solution was below detectable levels.

Results of the present study indicated that green synthesised silver nanoparticles using leaf extract of *T. cordifolia* was able to inhibit the growth of *C. reinhardtii*, thus exhibiting potential antialgal property. The study can be further extended to harmful algae for developing an ecofriendly approach for their control using green synthesised silver nanoparticles.