Synthesis and Characterization of Silver Nanoparticles Synthesized from Methanolic Extracts of Freshwater Algal Species

BY

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Synthesis and Characterization of Silver Nanoparticles Synthesized from Methanolic Extracts of Freshwater Algal Species

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MS THESIS WORK

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Dedicated to

Everyone who didn’t read this

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ABSTRACT

The present study was designed to find a simple and cost effective method of synthesis of silver nanoparticles with less cytotoxicity using the methanolic extract of algae *Dictyosphaerium 6-8* and *Chlorella vulgaris*. The synthesis of silver nanoparticles was first detected change on color of the reaction mixture. Further analysis via UV-Vis Spectroscopy confirmed the presence of silver nanoparticles which was indicated by maximum absorbance within 410-430 nm range. AFM and SEM study revealed the size range and morphology of the silver nanoparticles. Silver nanoparticles synthesized from methanolic extract of *Dictyosphaerium 6-8* had an average size of 25nm whereas silver nanoparticle synthesized from *Chlorella vulgaris* were of 46 nm average size. X-Ray Diffraction analysis showed that the silver nanoparticles synthesized from both algae were of crystalline nature. Fourier Transform Infrared Spectroscopy was performed to detect the functional group present on the silver nanoparticles. Presences of alkenes, alkynes, amides, halides and aromatic were present on the surface of silver nanoparticles. MTT assay was run on three different cell lines to determine the cytotoxicity of the silver nanoparticles. The result show that silver nanoparticles synthesized from methanolic extract of *Dictyosphaerium 6-8* and *Chlorella Vulgaris* had limited cytotoxic effect on cancerous cell lines Hep-2 and Huh-7 and had minimal cytotoxic effect on normal human cell line HCEC. The study concludes that silver nanoparticles synthesized from methanolic extract of *Dictyosphaerium 6-8* and *Chlorella Vulgaris* are biocompatiable and can be used for medical application like drug delivery and gene therapy in cancer and normal cells.

Chapter 1

INTRODUCTION

Nanotechnology is a rapidly emerging field which focuses on the, understanding, engineering and manipulation of matter at nanoscale (Bowman and Hodge, 2007). The term “Nanoparticles” encompasses all the natural and synthetic, organic and non-organic particles that range between 1-100 nanometers (Terrones *et al.,* 1999).Because of their very small and simple structure, nanoparticles have unique applications in many fields including but not limited to, Helthcare, Agriculture, Automobile, Electronics, Environment, Chemical Industry, Textile, Imaging and many more (Feynman, 1991).

Metallic nanoparticles are not only the most studied nanoparticles, but in recent years, their wide range of applications have proved them to be one of the most interesting and useful nanoparticles (Jena et al 2014). Which is because of the unique metallic property know as Surface Plasmon Resonance (SPR) (Zeng *et al*., 2001). Metallic nanoparticles can be of Silver, Iron, Zinc, Cobalt, Gold, Copper, Tungsten, Lithium, Nickel and many more (Pantidos and Horsfall, 2014). These nanoparticles have also been engineered into many different structures, each with their own unique properties, advantages and limitations (Tokonami *et al*., 2011). Of the most common nanoparticle structures are nanotubes, nanofilms, nanosheets and nanospheres, while other structures include nanoring, nanomesh, nanoflower, nanoflake, nanofoams, nanopillars, nanocages and many more (Bruda *et al.,* 2005). Non-Metallic nanoparticles have also attracted the attention of researchers in recent decades. These include Ceramic nanoparticles, Carbon nanoparticles, Semiconductor nanoparticles and Quantum dots (Juzenas *et al., 2008*).

Medical applications of nanoparticles are widespread and have seen great commercial advancement in recent years. The very small size of the nanoparticles makes their applications in the field of medicine. Nanoparticles are being used for Targeted drug delivery, fluorescent labelling, detection of biomolecules, probing of nucleic acids, tissue engineering, Magnetic Resonance Imaging, hyperthermia, Isolation of biomolecules,

signal transduction studies, targeted protein inhibition and many more (Salata, 2004). Furthermore biosynthesis of these nanoparticles have opened path for cheap and safe methods of synthesizing nanoparticles at industrial scale which was never possible with chemical synthesis.

Number of recent studies have identified some exceptional applications of nanoparticles in the field of agriculture and food sector. Nanoparticles have been used to deliver chemicals like fertilizer and growth regulators to the roots of the plant (Birner *et al.,* 2009). Nanoparticles help in refining field sensing systems to monitor the environmental stresses and crop condition and improve the plant traits against environmental stresses and diseases (Chen and Yada, 2011). Identification of microorganisms and food quality monitoring using biosensors, nano encapsulation of food compounds through nanocomposite, nanoemulsification, and nanoestructuration (Ezhilarasi *et al*., 2013), smart, active and intelligent food packaging systems are a few examples of ensuring improved and contamination free food and agriculture products (Sekhon, 2010). Edible coatings of silver nanoparticles can increase shelf life of fresh fruits and improve their export quality (Baldwin *et al.,* 2011; Costa *et al.,* 2012). Nanoencapsulation of probiotics can enable the delivery of designed bacterial population to targeted parts of the gastro-intestinal tract where they interact with specific receptors (Burgain *et al.,* 2011; Gbassi and Vandamme, 2012).

Silver nanoparticle have proved themselves the most beneficial type of nanoparticles. Silver nanoparticles have a wide range of size distribution and most of their physical and biological properties are effected by the size of these nanoparticles. The size of silver nanoparticles can be as low as 10 nm while mostly remaining in 20-40 nm range and can go as high as 80 nm. Silver nanoparticles show excellent antimicrobial activity the mechanism of which is not clearly understood, however recent advances have indicated that anti-microbial effect of silver is due to the attachment of Ag on the membrane that causes disturbance in cell's respiration and ion exchange mechanism (Rai*et al.,* 2009). The cell eventually loses control of its homeostasis that prevents to continue its normal

function. This antibacterial activity of silver nanoparticles has been utilized to design new water filters and air filters to avoid water born infections (Tiwari *et al., 2008*).

Researchers have worked on many different biological and chemical techniques to synthesize silver nanoparticles and recent years have seen a steady increase in number of methods and sources that can be used to produce silver nanoparticles. Silver nanoparticles have been produced from biological sources including, but not limited to, bacteria, algae, fungi, plants, insects and many more (Kaushik *et al.,* 2010). Biological synthesis of silver nanoparticles is not only simple and cost effective, it provides control over the final size and shape of the nanoparticles by altering and fine tuning different parameters like temperature, pH, Light intensity and source (Soni *et al.,* 2011).

The size of silver nano-particle greatly effects its activity and properties like antibacterial activity, optical properties, light scattering, catalytic activity, chemical stability and more. In general the smaller the size of nanoparticle, the greater is its antibacterial effect (Sivaraman *et al.,* 2010). This is because small particles have higher surface area to mass ratio than large size or bulk particles. Another reason why small size nanoparticles are more effective against bacteria is because they can easily cross the cell membrane and enter the cell disrupting its normal functions of the cell by releasing Reactive Oxygen Species that can damage the DNA and potentially cause apoptosis (Gutierrez*et al.,* 2010).

The cytotoxic effects of these silver nanoparticles have also been investigated greatly and it has been found that silver nanoparticles can be harmful for body at higher concentrations. Evidence suggests that silver nanoparticles synthesized from biological sources like yeast, bacteria, fungi, algae etc. are less harmful than that synthesized from chemical and physical sources (Reddy *et al.,* 2014). Due to this reason researchers have shifted their efforts to green synthesis of silver nanoparticles which are biocompatible, safer, cheaper and easier to produce (Veerasamy *et al*., 2010).

Although bacteria has been used for the synthesis of silver nanoparticles at large scale, they are not preferred over botanical sources (Vadlapudi *et al.,* 2014). Tough Experimental conditions and difficulty in handling are some of barriers that favor plants over microorganisms for the production of silver nanoparticles (Sastry *et al.,* 2003). Plants provide most ecofriendly, cost effective and productive methods of nanoparticles synthesis (Mittal *et al.,* 2013). Plants contain natural biomolecules such as enzymes/proteins, amino acids, polysaccharides and vitamins (Iravani, 2011), and phytochemicals like terpenoids, flavones and ketones, which are anticipated to have reducing and capping properties, that helps in the synthesis of silver nanoparticles (Jha *et al.,* 2009). Many medicinal plants like *Azadirachtaindica* (Neem) (Tripathy *et al.,* 2010), *Cinnamomumcamphora* (Yang *et al.,* 2010), *Ocimumsanctum* (Tulsi) (Singhal *et al.,* 2011) and *Artemisia nilagirica* (Vijayakumar *et al.,* 2013), have been used for the production of silver nanoparticles. Part of the plant that is being used also effects the properties of silver nanoparticles. For instance, stems and root of *Ocimum sanctum* (Tulsi) synthesized silver nanoparticles with average size of 10-12 nm (Ahmad *et al.,* 2010) and leaves of same plant produced silver nanoparticles with size range of 4-30 nm (Singhal *et al.,* 2011).

Algae and fungi have also been used toproduce silver nanoparticles. Fungi have great bioaccumulation ability and their resistance to external factors have made them a recent choice for the synthesis of Silver nanoparticles (Castro *et al.,* 2013). Fungi are also easy to scale up and relatively safe to handle making them a very good candidate for large scale synthesis of silver nanoparticles (Kaushik *et al.,* 2009). Silver nanoparticles have been produced from fungi *Verticillium* which produced Intracellular silver nanoparticles embedded within the cell wall of the fungus. (Mukherjee *et al.,* 2001). Extracellular silver nanoparticle synthesis has been achieved by using fungus *Aspergillus fumigatus* (Bhainsa *et al.*, 2006). The nanoparticles synthesized were of very small size, 5-25nm, and were formed immediately within 20-30minutes of reaction initiation. These nanoparticles also showed great stability at room temperature for more than 4 months.

Only a few reports of nanoparticles synthesized from algae exist. *Sargassumwightii*, a marine water algae was firstly used to demonstrate nanoparticle synthesis from algae (Singaravelu *et al.,* 2007). Other than that *Chlorella vulgaris*is shown to produce stable nanoparticles of very fine size of 16-20 nm (Jianping *et al.,* 2007).

The aim of this study is to synthesize silver nanoparticles from a cheap and environmental safe source. For this purpose algae was selected for the primary source of silver nanoparticles. Effort is made to synthesize silver nanoparticles from the methanolic extracts of two different algae strains, *Dictyosphaerium 6-8* and *Chlorella Vulgaris*. Both the strains isolated from Kallar Kahar Lake situated in Chakwal district, Pakistan. Methanolic extract of these algal strains were used to reduce silver nitrate into silver nanoparticles.

These nanoparticles will be characterized using different techniques to determine their size shape and physical properties such as, Atomic Force Microscopy (AFM), Fourier Transformed Infrared Spectroscopy (FTIR), X-Ray Diffraction (XRD), X-Ray Fluorescence (XRF), Scanning Electron Microscope (SEM) and Ultra Violet/Visible Spectroscopy (UV/Vis). Cytotoxicity of the synthesized nanoparticles was studied via MTT assay on normal and cancer cell lines. For normal cell HCEC cell lines was used and for cancer cells Huh-7 and Hep-2 were used.

Aims and Objective

This study is designed to test the following hypothesis

- Algae *Dictyosphaerium6-8* and *Chlorella Vulgaris*can be used as cheap alternative source for the synthesis of silver nanoparticles

The specific aims of this study includes

- Biosynthesis of silver nanoparticles from methanolic algal extracts of *Dictyosphaerium6-8* and *Chlorella Vulgaris*
- Characterization of these newly synthesized silver nanoparticles
- Cytotoxic evaluation of the synthesized nanoparticles on normal and cancer cell lines

Chapter 2

REVIEW OF LITERATURE

2.1 Nanotechnology

Nanotechnology deals with the study and manipulation of particles of size range less than 100 nm (Goddard 2007). Nanoscale particles have unique properties due to their small size and large surface area which large sized particles lack (Adams and Barbante, 2013). Nanotechnology makes can be used to make products smaller, lighter, cheaper, stronger, smarter, cleaner and more precise (Merkle, 2000). Another advantage that nano-sized material have over their counterpart bulk particles is that they have much greater magnetic, optical, photoctalytic, thermal and electrical properties (Basavaraj, 2012). Some of the fields that have benefited from nanotechnology are, medicine, optics, food, packaging, textiles, agriculture, cosmetics, optoelectronic devices, aerospace, construction, semiconductor devices, and catalysis (Kango *et al*., 2013).

Nanomaterials can be of different types including, carbon black and fumed silica to microgram quantities of fluorescent quantum dots (Hoet *et al*., 2004). Nanomaterials can exist in many different shapes such as nanocrystals, nanotubes, nanoclusters (Terrones *et al*., 2002) liposomes, micelles, nanoparticles and dendrimers (Gupta *et al.*, 2012). Core shell nanostructures occur in three variant forms nanoshells, nanoeggs and nanocups (Knight and Halas, 2008). Nanomaterials have novel properties which increases their important in commercial applications such as cosmetics, skincare sanitary (Wu et al., 2013). In medicine nanoparticles have made targeted drug delivery and tailored drug possible. (Hughes, 2005). Nanoparticles can be used in imaging technique to obtain high resolution observation for diagnosis of cancer and other diseases. (Torchilin 2007). Nano-sciences are experiencing massive investment worldwide and the future will see a rise in the use of nanoparticles in everyday consumer products (Paull et al., 2003).

2.2 Silver Nanoparticles

Silver nanoparticles are one of the most common and easy to produce metallic nanoparticles and recent years have seen lots of application of silver nanoparticles as they are much effective than silver ions (Lara *et al*., 2010). Silver ions have the potential to inhibit bacterial replication, by rupturing cell wall, binding to thiol group of protein, denaturing DNA and causing cell death (Russel and Hugo, 1994). Silver has been used for many centuries as antiseptic because of its bactericidal properties (Moyer *et al*., 1965). Silver has also been used for the treatment of burns and other skin ailments (Parikh *et al*., 2005). Silver nanoparticles are promising candidates for use as microbiocides due to their efficacy in small doses and minimal side effects (Sondi and Sondi, 2004). Silver nanoparticles can be entrapped in a matrix that can be used in drug carrier system where drug can be dissolved or attached to a nanoparticle matrix or dissolved in liquid medium that restricts their agglomeration ((Brigger *et al*., 2002).

The size and shape of nanoparticles determine many important properties like the in vivo circulation, cytotoxicity, biological providence, genotoxicity and the targeting ability of nanoparticles (Panyam and Labhasetwar, 2003). With the rapid development of nanotechnology, applications have been extended further and now silver is the engineered nanomaterial most commonly used in consumer products (Rejeski, 2009). Several varieties of silver compounds that are being used as antimicrobials for centuries include silver nitrate, silver zeolite, silver sulfadiazine, silver oxide, silver powder and silver chloride (Rai *et al.,* 2012).

2.3 Synthesis of Silver Nanoparticles

Silver nanoparticles have been synthesized by chemical, physical and biological methods. Physical methods used for the synthesis of silver nanoparticles are vapor condensation and arc discharge method (Sharma *et al.,* 2009). Chemical synthesis of silver nanoparticles is achieved by chemical reduction, photochemical reduction and

electrochemical synthesis (Khan *et al*., 2011). Silver nanoparticles have also been synthesized from different biological sources like bacteria, algae, fungi, insects, plants and other sources (Mukherjee *et al*., 2009). Different experimental conditions like, pH, temperature, reaction time, etc., have significant effect on the final size, shape, morphology and stability of the silver nanoparticles (Li *et al.,* 2011).

Chemical methods of synthesis of silver nanoparticles involves reduction of silver ions by using reducing agents like, hydrogen, hydrazine, Dextrose, ethylene glycol, citrate, ascorbate etc. (Hiramatsu *et al.,* 2004). In this process, various complexes get reduced with silver Ag^{+} ions that leads to the creation of silver atoms, which then agglomerate to form oligomeric clusters. These clusters then turn into the colloidal silver nanoparticles (Evanoff and Chumanov, 2004; Sondi *et al.,* 2003). Alteration in experimental conditions resulted in nanoparticle synthesis with modified size and shape and were synthesized in 5 minutes by Sodium Borohydrate with size distribution of 3-28nm at room temperature (Ghorbani *et al.,* 2011).

Physical methods used for the synthesis of silver nanoparticles involves condensation through evaporation in a tube furnace. This process requires lot of energy and time (Simchi et al., 2007). Another method is to use ceramic heater which involves heating the solution gradually and slowly to evaporate liquid and resulting in small sized nanoparticles at high concentration (Jung *et al.,* 2006). Lasers have also been used for the synthesis of silver nanoparticles. By laser ablation methods silver nanoparticles have been produced with different shape size and structure (Barcikowski *et al.,* 2009). By controlling different factors like, ablation time, laser pulse time, pulse frequency and the effective liquid medium influence the characteristics of the silver nanoparticles formed and the ablation efficiency (Mafune *et al.,* 2003).

Biological methods of silver nanoparticles are safe easy and less toxic as compared to chemical and physical methods (Krutyakov *et al.,* 2008) as Most of the chemical approaches used for the synthesis of silver nanoparticles are expensive and implicates the usage of hazardous chemicals that are responsible for various biotic risks (Geoprincy *et al.,* 2013). The biological method for the silver nanoparticles synthesis has three

major components; a solvent medium, non-toxic reducing agent and a stabilizing agent (Prabhu and Poulose, 2012). Microorganisms, like prokaryotic bacteria, are widely used for the synthesis of silver nanoparticles (Kaushik *et al.,* 2010). Yeast and other fungi have also been used to synthesize biocompatible silver nanoparticles (Kowshik et al., 2003). Different plant extracts have also been used to synthesize biocompatible silver nanoparticles. Using plant extract is a rapid and easy method to synthesize nanoparticles because maintaining the microbial culture is not required (Sastry *et al.,* 2004) and are widely distributed, easily available, much safer to handle and act as a source of several metabolites (Kumar and Yadav 2009). Plant extracts contain phytochemicals with reducing properties, such as terpenoids, aldehydes, amines, flavones, carboxylic acid and ketones that may be involved in biosynthesis of silver nanoparticles (Jha *et al.,* 2009). Using medicinal plants for the synthesis of silver nanoparticles can reduce the required dose and side effects and improve their biomedical activity (Logeswari *et al.,* 2013). Extracts of different algae have also been used for the synthesis of silver nanoparticles.

2.4 Synthesis of Silver Nanoparticles using Algae

In recent years researchers have turned their efforts to use algae for the synthesis of silver nanoparticles because it is greener and ecofriendly (Yousefzadi *et al.,* 2014). Biosynthesis of silver sustainable silver nanoparticles is carried out through bio reduction of silver ions from by different organic chemicals present in the algal extract (Salari *et al.,* 2014). Silver nanoparticles based on biological origin are highly appreciable because of less toxicity, high efficacy and biocompatibility and high rate of synthesis (Namasivayam *et al.,* 2014). To Date only few algaes have been used in the synthesis of silver nanoparticles these include, *Sargassum wightii, Kappophycus alvarezii, Gelididella acerosa, Spirulina platensis, Pterocladia capillacae* and *Spirogyra varians* (Singaravelu *et al.,* 2007). Some of the algaes are able to accumulate high concentration of heavy metals in their cells which makes them excellent tool for bioremediation and can be used for the synthesis of silver nanoparticles (Karez *et al.,* 1992).

It is reported that extract of *Chlorella vulgaris* has strong binding ability towards tetrachloroaurate ions to form algal-bound nanoparticles, 88% of which attained metallic state and the nanocrystals were accumulated in the inner and outer parts of cell surfaces with decahedral, tetrahedral, icosahedral structures (Luangpipat *et al.,* 2011). *Spirulina platensis* is an edible blue–green alga and the dried alga was used for the extracellular synthesis of gold, silver nanoparticles (Govindaraju *et al.,* 2009). *Sargassum wightii* and *Kappaphycus alvarezii* have recently been used for the synthesis of biocompatible nanoparticles of silver and gold (Rajasulochana *et al.,* 2010). Silver and gold nanoparticles of different sizes have also been synthesized from cellular extracts of algae *Tetraselmis kochinensis* and *Fucus vesiculosus* (Senapati *et al.,* 2012; Mata *et al.,* 2009).

2.5 Capping of Nanoparticles

Basic principle for preparation of silver nanoparticles are almost same, but biosynthesized silver nanoparticles from different sources imparts different properties by capping their surfaces with the capping agent (Badway 2011). Silver nanoparticles synthesized from anti-oxidant constituents of *R. dauricum* flower exhibited antioxidant properties. These properties were seen to be due to the presence of Phenolic compounds (Mittal *et al.,* 2012). This is how Silver nanoparticles were synthesized (Elechiguerra *et al.,* 2005) which showed antiviral activities against HIV-1. This methodology of preparing silver nanoparticles by selecting a source which can transfer a particular property to the surface of silver nanoparticles will not only make them biocompatible but also novel and unique at the same time (Allen *et al.,* 2010).

2.6 Fluorescence of Silver Nanoparticles

Synthesis of Silver nanoparticles not only enhance optical properties but also makes them fluorescent (Abdullah and Annapoorni 2005). As silver nanoparticles efficiently absorb and scatter efficient light due to a phenomenon of Surface Plasmon Resonance (Hung 2008) they significantly reduce the photobleaching phenomena. (Singh *et al.,* 2013). These inorganic fluorophores, known as Quantum dots, can be used for

biomedical application as bioimaging, biosensors in drug delivery. This property of luminescence is because of size ranges that are less than 10 nm (Valizadeh 2012). Here semiconductor nanoparticles which show fluorescent properties are also used in current biomedical research in bioimaging. Cadmium sulphide is one example, which is known for its fluorescent properties. Semiconductor Quantum dots have been reported for their cytotoxicity because of Cadmium core shell, which due to core oxidation releases cadmium ions within cells causing cytotoxicity. (Chan *et al.,* 2002; Selvan *et al.,* 2005) Metallic quantum dots on other hand Show not only Surface Pasmon Resonance but also show the fluorescent properties of quantum dots. (Lindberg and Hellsing 2005). Therefore, they can be used as an alternative to semiconductor based quantum dots (Govorov *et al.,* 2006). For biomedical applications, there is a need for synthesis of non-toxic metallic nanoparticles e.g. non-toxic fluorescent silver/gold nanoparticles. (Das *et al.,* 2010).

2.7 Mechanism of Silver Nanoparticle Action

There are many proposed mechanisms of activity of silver nanoparticles and it is suggested that size, mobility and composition of silver nanoparticles play an important role in antimicrobial activity (Quang *et al.,* 2013). It is also suggested that silver nanoparticles make specie independent anti-biofilm, hence it can be used to control the spread of resistant microbes (Kalishwaralal *et al.,* 2010). Changes in bacterial cell wall permeability, drug target site modification, excretion of antimicrobial drugs through efflux pumps of membranes and inactivation of antimicrobial agent are several factors that make microorganisms resistant to drugs (Giraud *et al.,* 2006). Silver nanoparticles can minimize the resistance because of active mechanisms that are specific to silver nanoparticles (Kim *et al.,* 2007).

There are many proposed theories on how silver nanoparticles may affect the cell. Silver nanoparticle may adhere to bacterial cell wall to induce conformational changes in its structure and result in subsequent damage to bacterial cell (Klasen, 2000). It is also thought that silver nanoparticles may alter cell membrane permeability both through direct interaction with phospholipid bilayer or through release of reactive oxygen

species which then alters membrane permeability and results in bactericidal effects (Fayaz *et al.,* 2010). Silver ions released in the presence of silver nanoparticles may interact with some thiol groups in enzymes to form Ag-S bonds, which then alters the function of the bacterial enzymes which are important in transmembrane energy generation and ion transport (Matsumura *et al.,* 2003). Another reason may be that silver nanoparticles internalize and accumulate within bacterial cell to induce the formation of "pits" on bacterial membranes which results in bacterial cell lysis (Nair *et al.,* 2009). Silver nanoparticle may also inhibit some enzymes via Ag-S and interfere with transmembrane energy generation inside bacterial cells which in turn induces the formation of ROS (Yamanaka *et al.,* 2005). Reactive Oxygen Species production is induced by both silver nanoparticles through oxidative stress which in turn alters cell membrane permeability (Raghupathi *et al.,* 2011). Ag ions may also induce bacterial apoptosis by binding with 30S ribosomal subunit, which would then deactivate the complex and halt protein translation (Yamanaka *et al.,* 2005).

2.8 Cytotoxicity of chemically synthesized Silver Nanoparticles

Chemically synthesized silver nanoparticles have been shown cytotoxic effects because of generation of reactive oxygen species (ROS) (Martindale and Holbrook 2002). Effects of silver nanoparticles have been seen on various cell lines including Huh7 cell lines, MCF 7, HepG2, NIH3T3 cell lines (Hsin *et al.,* 2010). The exact mechanism depends upon the size and capping agents of silver nanoparticles but chemically synthesized silver nanoparticles reduced the glutathione (GSH) levels and promotes the production of ROS (Dewanjee *et al.,* 2009). GSH is an important ROS scavenger, hence play an important role in minimizing oxidative stress by binding to ROS (Anderson *et al.,* 2004). Silver nanoparticles mediated oxidative stress is because of inhibition of GSH synthesizing enzyme thereby increasing oxidative stress within the cell. However, superoxide dismutase and catalases are also inhibited by silver nanoparticles. (Hsin *et al.,* 2010). Cell membrane undergo lipid peroxidation and DNA and mitochondrial damage occurs as a result of ROS generation. Cytochrome C released from mitochondria causes activation of Caspase 9 and caspase 3. This release is due to down regulation of Bcl-2

and up regulation of Bax leading to cellular apoptosis which is caused by silver nanoparticles (Hsin *et al.,* 2008). Biosynthesized nanoparticles, however, do not exhibit cytotoxic properties because of surface capping which potentially reduces their toxicity (Aziz *et al.,* 2013).

2.9 Antioxidant properties of biosynthesized silver nanoparticles

Total Phenolic content of a plant determines the mechanism of silver nanoparticles synthesis from Plant extract (Abdel-Aziz *et al.,* 2013). Phenols exhibit good antioxidant and reducing properties (Pietta 2000) which is required for synthesis of silver nanoparticles (Castanon *et al.,* 2008). As these phenol groups are oxidized quinoid compounds are produced which have the ability to bind to the surface of nanoparticles responsible for capping and stability of nanoparticles (Wang *et al.,* 2007). Hence, the synthesized nanoparticle will be capped with antioxidant capping agent thereby reducing the cytotoxicity of these nanoparticles. Combination of Fluorescent and antioxidant properties of silver nanoparticles may provide an efficient alternative for cytotoxic quantum dots for their biomedical applications including drug delivery and bioimaging (Selvan *et al.,* 2005).

2.10 Applications of Silver Nanoparticles

Silver nanoparticles have seen wide spread application in recent decades. Their most prominent application can be seen in medical field. The size of most cells of multicellular organisms is approximately 10µm (Feynman 1991). The cellular organelles are much smaller and are in the sub-micron size domain. Protein, Carbohydrates, nucleic acids and other biomolecules have typical size of just 5 nm, which is similar in size to average manmade nanoparticles (Murray *et al.,* 2000). This size comparison shows us the benifit of using nanoparticles as probes that would allow us to infiltrate the cellular machinery without introducing too much damage (Taton *et al.,* 2002). Advancement and development of nanobiotechnology depends relies heavily on our understanding of biological processes on the nanoscale level (Whitesides 2003). Following is a list of some of the applications of silver nanoparticles investigated in recent years.

- Drug delivery (Panatarotto *et al.,* 2003)
- BioImaging (Bruchez *et al.,* 1998)
- Protein detection (Nam *et al.,* 2003)
- DNA Probing (Mahtab *et al.,* 1995)
- Tissue Engineering (Ma *et al.,* 2003)
- Tumor Destruction (Yoshida *et al.,* 1991)

Food preservation is an acute problem because of increase in global demand and low food production. Maintaining the quality of food products is not an easy task due to increase in respiration rate and ethylene production which ultimately results in loss of quality (Aguilar *et al.,* 2010). Antimicrobial active packaging is promising food packaging for extending the shelf life of fresh food. Compounds of natural origin like plant essential oils prevents the growth microbes (Zivanovic *et al.,* 2005). The extraordinary antimicrobial properties of silver nanoparticles have made them applicable in food industry (Bosetti *et al.,* 2002). Some examples of application of silver nanoparticles to food are the use of nanoparticles to preserve vegetable and vegetables derived products (An *et al.,* 2008). Antimicrobial packaging of fresh cut vegetables can solve this problem of contamination of pathogens. Lettuce and paprika samples were treated with silver nanoparticles and polylactide (PLA) films by a solvent casting technique. The PLA-silver nanoparticles films showed strong antibacterial, antifungal and antiviral activity in vitro, with increasing effects at higher silver concentrations (Abad *et al.,* 2013). Different concentrations of silver montmorillonite embedded in agar were used as an antimicrobial packaging system to control quality deterioration of Fior di Latte cheese and mozzarella cheese (Incoronato *et al.,* 2011). Acidophilic microorganisms like lactic acid bacteria and yeast, are major contaminants of citrus juices because they are capable of growing in a wide pH range (Alwazeer *et al.,* 2003). This make them able to spoil minimally processed fruit juices and generating a “buttery” off-flavor and swelling of packages (Sampedro *et al.,* 2007). Antimicrobial food packaging materials extend the lag phase of microorganisms and reduce their growth rate. This extends the shelf life and maintain juice quality and safety (Suppakul *et al.,* 2003).

2.11 Anticancer Activity of silver nanoparticles

Traditionally drugs are administered orally and injected in blood circulation. This method of administration is not efficient especially for proteins, nucleic acids and other biological molecules that entail novel supply technologies. The efficiency of drug depends on particle size, nanoparticle conjugated with biomolecules can ensure targeted and timed release of drugs and can enhance bioavailability (Rodriguez *et al.,* 2005). The small size of nanoparticles allow them to cross the blood brain barrier, the tight epithelial junctions of the skin, the branching pathways of the pulmonary system, that normally impede delivery of drugs to the desired target. All these attributes are increasing the sale and development of drug delivery (Gain, 2003). Novel drug delivery methods will not only result in effective treatment but will provide more intellectual property shield to already existing drug formulations (McKinnie, 2006). Therapeutic compounds that failed to show potential effectiveness due to toxicity or inability to administer them may be reformulated in nanoparticle delivery system. For example, chemotherapeutic agents that are well known for their systematic side effects can be delivered directly to tumor cite and this will reduce their health risks (Emerich and Thanos, 2006).

The biomedical activities of silver nanoparticles have raised new possibility in diagnosis and targeted treatment of human cancer (Singh and Nalwa, 2011). The concept of nanomedicine involves the use of precision-engineered nanoparticles for the development of novel diagnostic and therapeutic system for cancer and other lethal diseases (Dubey *et al.,* 2010). Biologically synthesized silver nanoparticles are preferred over chemically and physically synthesized silver nanoparticles to generate an antitumor agent with low toxicity to normal cells (Park *et al.,* 2011). The size, concentration and dose of silver nanoparticles effects its activity against cancer. A number of studies report that silver nanoparticles have dose dependent effect on tumors. Silver nanoparticles intercalate between DNA strands, causes nuclear pigmentation and stop angiogenesis (Bhattacharya and Mukherjee, 2008). An in vivo study of silver nanoparticles on Dalton's Lymphoma Ascites (DLA) cell lines revealed that nanoparticles increased the survival

time in mouse model with tumor by about 50% as compared to mice which were not treated with silver nanoparticles. The mouse model under treatment of silver nanoparticles also maintained normal body weight because the ascitic fluid was decreased by 65% in comparison to other mice. Elevated white blood cells and platelets count in ascitic fluid was also decreased to normal range. The number of DLA cell count was also decreased in ascitic fluid which proved the antitumor activity of silver nanoparticle and suggested a cost effective treatment of cancer (Sariram *et al.,* 2010).

Chapter 3

MATERIALS AND METHODS

1. Algae collection and Identification

Two strains of algae identified through ITS sequence analysis as, *Dictyosphaerium 6-8* and *Chlorella Vulgaris,* were provided by Ph.D. student Muneeba Khalid, these strains were originally isolated by Dr Ehsan's lab (Centre of Energy Systems) from Kallar Kahar Lake which is situated near Chakwal district, Punjab. These strains were cultivated in lab for biomass production. Both strains were cultivated in 1 liter conical flasksin deionized water. Common air blowers were used to provide proper aeration and turbidity for the growth medium. pH of the medium was maintained at 7.5 with HCl and NaOH. Algal cells were harvested after 7-10 days of incubation to prepare methanolic extracts for the synthesis of nanoparticles. The algal cells were harvested after the growth and the new batch was prepared for algal biomass. One batch of algae was prepared in approximately 2-3 weeks.

2. Preparation of algal extract

Algal extracts were prepared in 99.99% methanol by dissolving 1g dried algae powder in 99 ml methanol resulting in 1% w/v algal solution. This solution was stirred vigorously at 60°c for 30 minutes using a magnetic stirrer on a hot plate. The homogenized solution was filtered into clean glass beakers using syringe filters of 20µm pore size. The extract was stored in refrigerator at 4°c and used subsequently for silver nanoparticle synthesis.

3. Silver nanoparticles synthesis using algal extract as reducing agent

1mM, 5mM and 10mM silver nitrate solutions were formed by dissolving silver nitrate in deionized water. The silver nitrate solutions reacted with algal extracts in 4:1 ratio i.e. for 100 ml final volume 20ml of extract was reacted with 80ml of 1mM, 5mM and

10mM silver nitrate solutions separately in a 100ml flask. In a control flask no silver nitrate was added and simple deionized water was added with the methanolic extract of algae. The flasks were incubated at 37^{o}C for 12-16 hours. The algal filtrate acted as a reducing and capping agent for Ag^{+} in $AgNO_3$ solution resulting in pure silver nanoparticles. Synthesis of silver nanoparticles was indicated by change in color from bright green to pale brown whereas no color change was observed in the control reaction.

4. Characterization of silver nanoparticles

4.1. Visual conformation of silver nanoparticle synthesis

Synthesis of silver nanoparticles in the reaction mixture was confirmed by visually comparing the color of reaction mixture with the control. In the reaction mixture the color was changed from bright green to pale brown after 1 day incubation. It was observed that the color of reaction mixture with 10mM silver nitrate solution added, was slightly darker than 1mM reaction mixture. This indicates that increase in silver nitrate concentration increased the rate of chemical reaction.

4.2. Ultraviolet visible spectroscopy (UV-Vis)

Spectroscopy is very powerful technique which is used to analyze the size, concentration and stability of silver nanoparticles quantitatively. Silver nanoparticles were characterized initially by observing the specific peak of silver nanoparticles via UV–visible spectra on Spectrophotometer (LABOMED, Inc. U.S.A, Model UVD-2950). Samples were diluted at 1:5 with deionized water, to avoid absorbance due to color change. Quartz cuvette was filled with diluted solution and loaded in spectrophotometer chamber where silver nanoparticles absorbed the photons of particular wavelength, depending upon the particle size distribution and the absorption spectra, was recorded between wavelengths ranging from 360-500 nm. Controls solution was also analyzed which contained no silver nitrate.

4.3. Fourier transform infrared spectroscopy (FTIR)

Functional groups attached to the silver nanoparticles were observed using FTIR. (Spectrum-100, Perkin-Elmer, U.S.A). The wavelength used for scanning ranged from 450nm – 5000 cm^{-1}. Potassium Bromide being hygroscopic was heated to 110^{o}C to remove the traces of water. Pallet of Potassium Bromide was prepared using hydraulic press. The solution was diluted 5 times and a drop of this diluted solution was added using micropipette which was loaded in the chamber where IR waves passed through the sample to detect functional groups on the surface of Silver nanoparticles. The transmission spectra was recorded and interpreted to identify the bond stretching in functional groups.

4.4. Scanning Electron Microscopy (SEM)

SEM is a useful technique used for analyzing surface topography, morphology, size and composition in which a beam of electrons is used to excite the target sample which forces the conducting sample to lose electrons from their own shell. These electrons are detected by the sensor which compiles this information into a mono chrome 2 dimensional image with a resolution of up to 1nm and magnification of 1,000,000 X, depending on the make and model of the machine. The samples in this research were analyzed at 200,000 X magnification.

For SEM analysis the silver nitrate solution was diluted 10 times with deioniozed water and the sample was homogenized using ultrasonicator (Cole-Parmer). Single drop of diluted sample was placed on clean glass slide and dried on a hotplate for 10 minutes. The slide was cut in a 1cm x 1cm and was then placed in carbon coater for carbon coating to make it conductive. The sample was placed in the vacuum chamber of the Scanning Electron Microscope (TESCAN VEGA 3). And images were obtained at different magnification ranging upto 100,000 X magnification. The built-in software of the SEM machine was used to measure the size of individual nanoparticles.

4.5 X-Ray Diffraction analysis (XRD)

X-Ray diffraction is used to study the atomic and molecular structure of silver nanoparticles crystals. The machine throws an intense beam of X-rays on the test sample and the sample will reflect these x-rays at different intensities and angle. This data is calculated by the machine and a 3D crystal structure is formed which can reveal the position of an atom in a crystal lattice and also its angle.

For XRD analysis the sample was highly concentrated via centrifuge. 1ml of the sample was put in the eppendorf tube and the tubes were centrifuged at 15,000 RPM for 15 minutes. The supernatant was carefully removed and more sample solution was added to centrifuge the sample again. This process was repeated 7-8 times until the pallet formed was large enough for XRD. The pallet was placed on a glass slide and dried under a lamp for 20 – 25 minutes. This deposited a thick layer of silver nanoparticles on the glass slide which was now ready for XRD analysis. The samples were carefully placed in the machine (STOE, model number theta_theta, Germany). The radiation source was copper K alpha and the voltage was set to 40 KV, current 40 mA and the angle used was 20-80 theta.

4.6 Atomic Force Microscopy (AFM)

Atomic Force Microscopy was performed to analyze the 3D structure of the nanoparticle which reveals information about shape, size and height of the nanoparticles at a very high resolution.

For AFM analysis the samples were diluted 10 times with deionized water and homogenized using ultrasonicator to get a uniform distribution of nanoparticles in the solution. One drop of this sample was placed on a clean glass slide and dried for 15-20 minutes on a hotplate. These samples were then loaded in Scanning Probe Microscope (Joel JSPM-5200). Scanning was performed in tapping mode (scan area: 3.50 x 3.50 µm,

Image height: 0.005 µm, Reference voltage: -4.27 V, Frequency: 183.598 [kHz]). 2D and 3D images of nanoparticles were taken at different resolution. Scanning probe imaging processor (SPIP) was used for AFM analysis. Detailed surface topography and size distribution measurement was performed using SPIP 6.2.8 for AFM image analysis, 3-D analysis and particle size distribution.

4.7 X-Ray Fluorescence Spectroscopy (XRF)

XRF is an elemental analysis technique which uses high energy X-rays bombarded on the inner shell electrons of element present in sample to eject them creating a hole which is filled by the electrons in the upper shell (Bambynek *et al.,* 1972). Characteristic X-rays are produced when electron in higher energy orbit jumps into low energy orbit the energy of which is equal to the energy difference between the 2 orbits. (Kalnicky and Dennis 2001).

Elemental analysis of silver nanoparticles was performed using X-Ray Fluorescence spectrophotometer (Element analyser). Sample was loaded in the XRF spectrophotometer (JEOL JSX3202 M) where X-rays passed the sample. Algal extracts of *Dictyosphaerium 6-8* and *Chlorella Vulgaris* were loaded in a chamber for the detection of Silver element. Measurements were performed at tuneable energy of 30KV and 1mA at 11522 counts per second.

4.8 Purification and concentration of silver nanoparticles

Silver nanoparticles were at 15,000 RPM in 1.5 ml eppendorf tubes. Supernatant was removed and the pallet was re-suspended, this process was repeated 5 times for a thick pallet with large mass. The condenser was used to evaporate the solvent from condensed nanoparticle solution and was left to dry. The dry mass of nanoparticles was weighed and used for other activites.

4.9 Cytotoxic evaluation of silver nanoparticles via MTT assay

To evaluate the cytotoxicity of the synthesized nanoparticles MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used on 3 different cell lines, Huh-7, Hep-2 and HCEC. This assay is used to evaluate the effect of different concentration of drugs and chemical on cells death rate.

For this assay 100 µl of cells, at $1x10^5$ cells per well, were first cultured on 96 well micro liter plate and were incubated at 37^oC for 24 hours in RPMI media which also included GPPS and FCS. After 24 hours different concentration of silver nanoparticle was added and a positive control was created in which Taxol drug was added instead of silver nanoparticles. After further incubation of 24 hours at 37^oC, 20µl MTT reagent was added in each well. The plate was incubated in a cell culture incubator for 3 hours until a purple precipitate were visible. Then 100µl of DMSO was added to all wells and the plate was left covered at room temperature in the dark for 1 hour. The absorbance of each well including the blanks were recorded at 570 nm in a microplate reader. The average values from triplicate readings were determined and the average value of the blank was subtracted from it. Cell viability was calculated and converted to percentage viability. Percentage viability was then plotted against increasing concentrations of 5%, 10%, 15%, 20% and 25%.

Chapter 4

RESULTS

4.1 Biosynthesis of silver nanoparticles

Synthesis of silver nanoparticles using methanolic extract of algae specie was indicated by color change in the reaction tubes compared with that of control reaction. The control contained the purified algal extract dissolved in methanol and deionized water without silver nitrate. After 24 hours the change in color was observed from green to different shades of brown for the methanolic extract of both algal species *Dictyosphaerium6-8* (Fig. 4.1) and *Chlorella Vulgaris* (Fig. 4.2) as both reaction tubes changed color from green to brown. Silver nitrate concentrations of 1mM, 5mM, 10mM were reacted with algal extracts of both species in different reaction tubes. 10 ml of silver nitrate solution was reacted with 40 ml of algal extract of both species individually.

The algal extract contained reducing enzymes which reacted with Ag^{+} present in the methanolic extract, reducing them to Ag^{0} nanoparticles which were stable at 37^{0}C. Synthesis of silver nanoparticles caused the color change from light green to dark brown the change in color continued and after 3-4 days, it was observed, that the reaction mixture changed to darker brown indicating that the reaction is still carrying on where as the color of control reaction mixture did not show any change even after 2-3 weeks of incubation at room temperature.

UV-Vis spectroscopy showed maximum absorbance at 420-430 nm as the silver nanoparticles had undergone phenomenon of resonance when frequency of incident photons corresponded to the natural frequency of oscillating electrons at surface of silver Nanoparticles producing absorption spectra (Ghorbani*et al* 2011). After 24 hours the color change from green to brown indicated synthesis of nanoscale silver structures showing Surface Plasmon Resonance (SPR) and maximum absorbance in 420-430 range which is characteristic property for Silver nanoparticles(Nestor *et al* 2008).

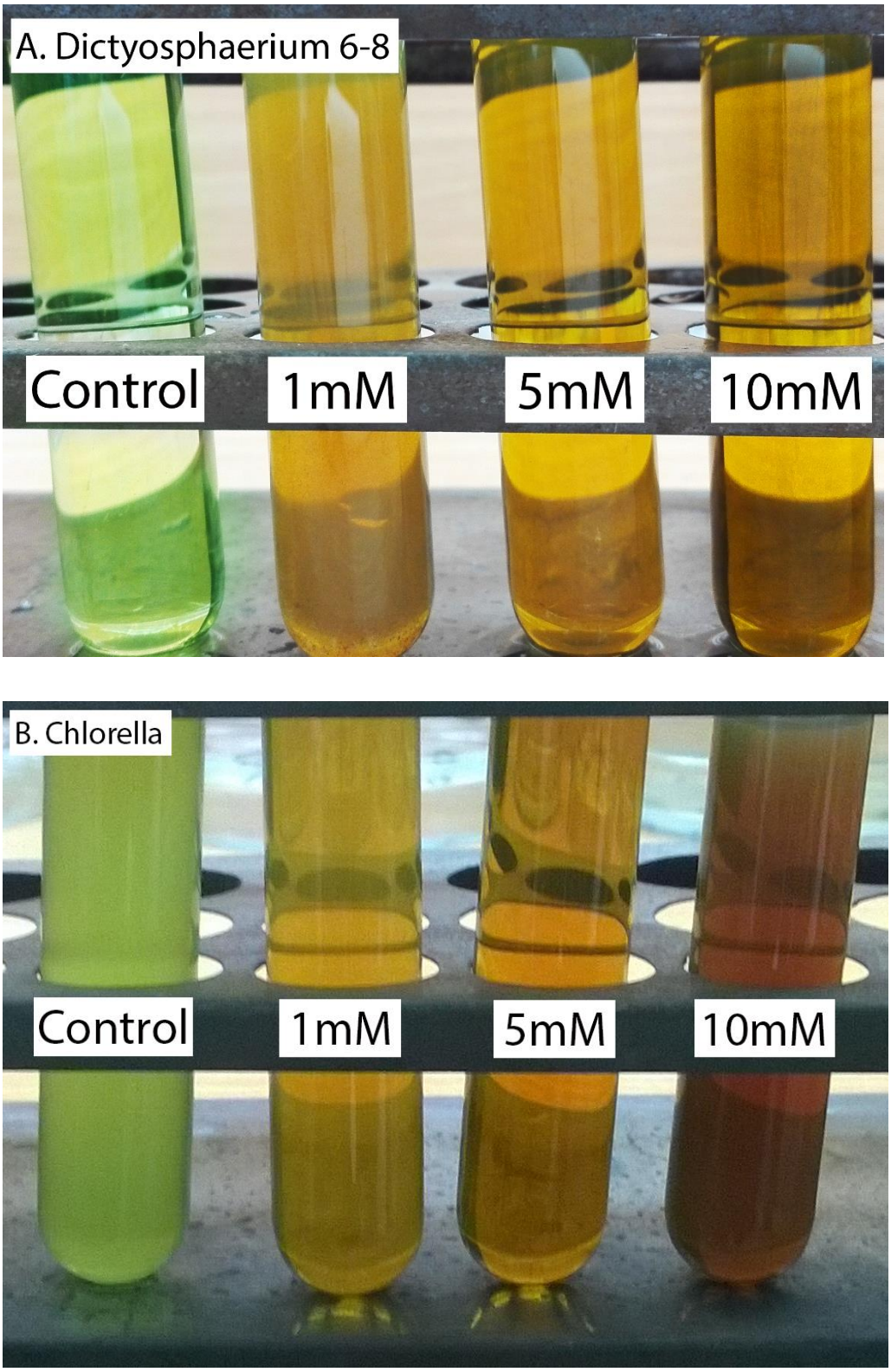


Fig. 4.1 Silver Nanoparticle synthesized from A. *Dictyosphaerium 6-8* extract. B. *Chlorella Vulgaris* extract. Change in color confirms the synthesis of silver nanoparticles.

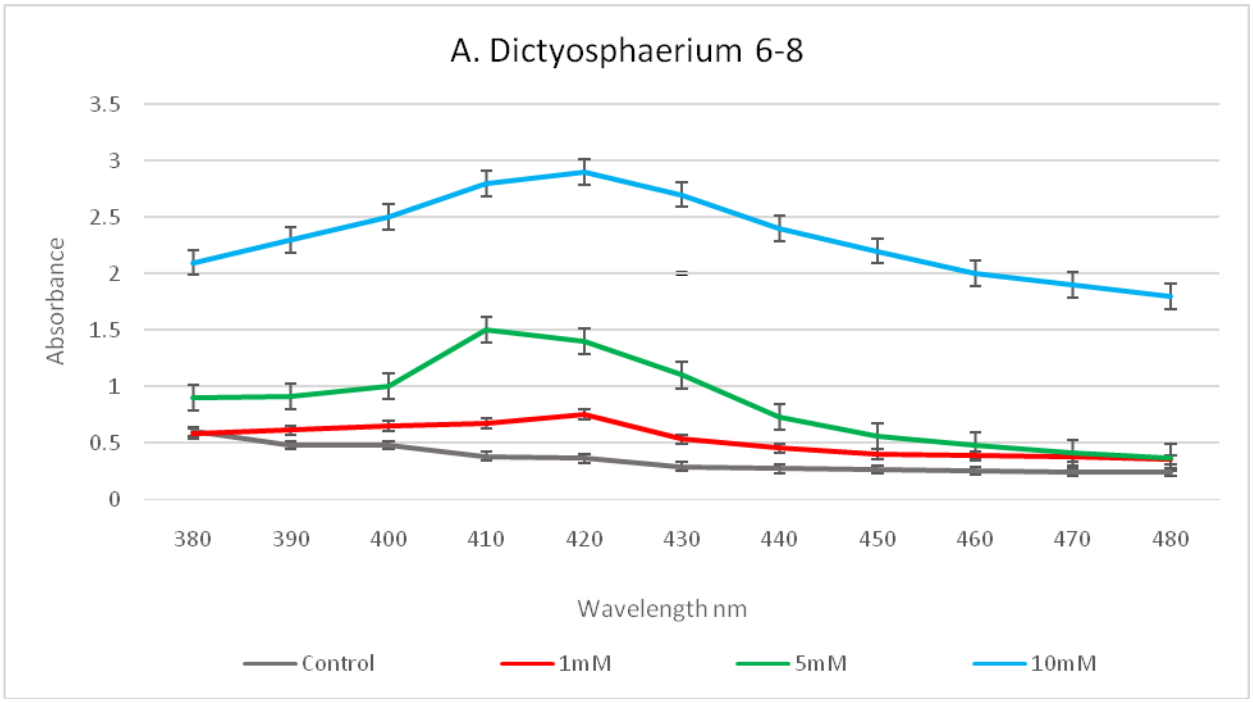
4.2 Characterization of Silver nanoparticles through UV-VIS Spectroscopy (UV-Vis)

UV-Vis spectroscopy confirms the prescence of silver in the solution for this analysis the sample was diluted 5 folds. The characterstic peak of silver was observed at approx. 410-430 nm as shown in the figure 4.2. This confirms the prescence of silver nanoparticles in the solution.

Absorbtion values on Y-axis indicates the amount or concentration of silver nanoparticles synthesized via the reaction. The values on X-axis represents the wavelength of light absorbed. Different elements and compounds absorb different wavelength of light which is their charasteristic property. This absorbance property can be used to detect specific element in a liquid solution. Silver nanoparticles show absorbance at 410-430 nm range. The results in figure 4.2 show high peaks at 410-430 nm range indicating the prescence of silver in the solution.

The result show that at different concentration of silver nitrate used in the reaction results in different concentration of silver nanoparticles synthesized. In general higher concentration of silver nitrate used result in higher concentration of silver nanoparticles produced. Figure 4.2a confirms silver nanoparticles produced from *Dictyospharium 6-8* at 1mM, 5mM, 10mM concentration of silver nitrate. It is observed that at 10mM (blue) silver nitrate concentration, the amount of silver nitrate produced is approximately 2 times higher than that produced at 5mM (green) silver nitrate concentration and approximately 4 times higher than produced at 1mM (red) silver nitrate concentration. The control (grey) rection mixture shows no prescence of silver nanoparticle.

Similar pattern can be observed in case of *Chlorella Vulgarius*(Fig. 4.2 b). Higher absorbance peaks with 10mM (blue) at 410-420 nm show high amount of silver nitrate produced compare to both 1mM and 5mM solution. These result are not surprising because high amount of silver nitrate in the solution means that the reducing enzymes present in the methanolic plant extract have high amount of substrate, i.e silver nitrate, to reduce it into product, i.e silver nanoparticles.



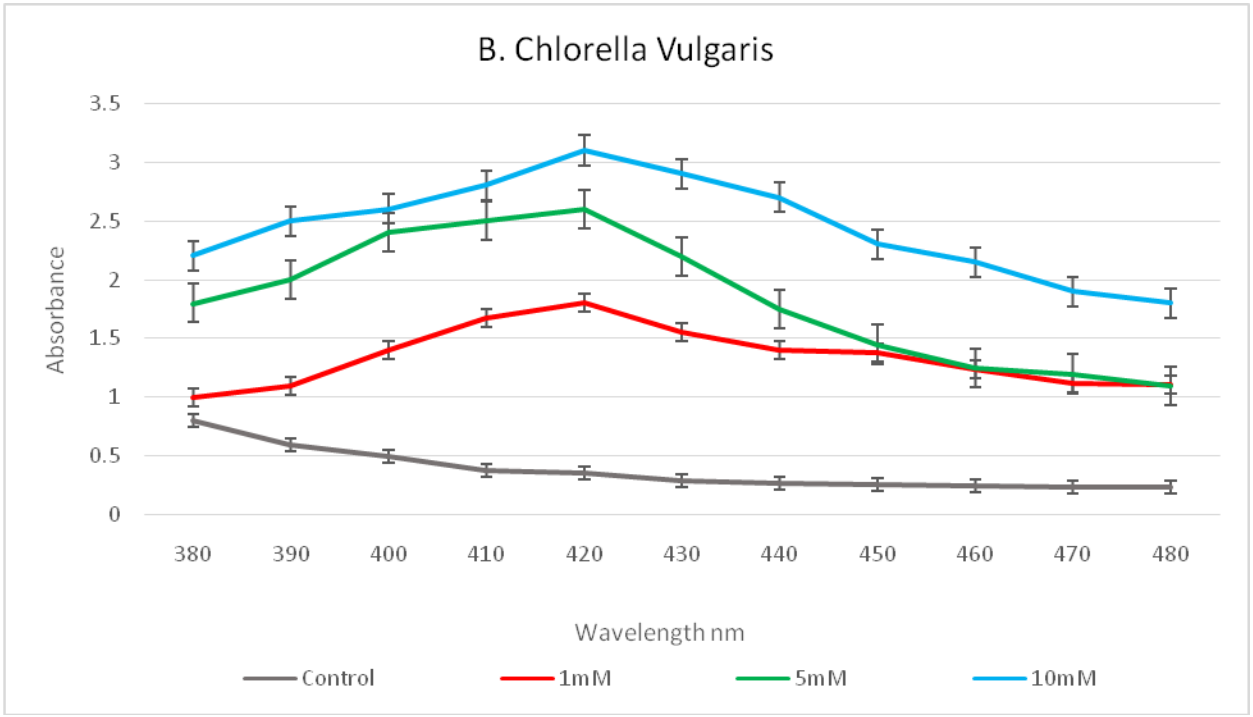


Fig. 4.2: Comparison of amount of silver Nanoparticles produced from methanolic extract of A. *Dictyosphaerium 6-8* and B. *Chlorella Vulgaris*, at 1mM, 5mM and 10mM silver nitrate concentration. At 10mM concentration of silver nitrate highest amount of silver nanoparticles were synthesized compared to 5mM and 1mM

4.3 Scanning Electron Microscopy of silver nanoparticles (SEM)

Scanning electron microscopy was used to determine the size and shape of silver nanoparticles. For this purpose 4 samples were analyzed to study the effect of concentration of 1mM and 10mM silver nitrate solution on methanolic extract of *Chlorella Vulgaris* and *Dictyosphaerium 6-8*. Samples were sonicated, diluted 10 folds and sonicated again to get a uniform and homogeneous distribution of silver nanoparticles throughout the solution. Results confirmed the synthesis of nano size silver nanoparticles of spherical geometry as observed in figure 4.3.

The results show that, when methanolic extract of *Dictyosphaerium 6-8* was reacted with 1mM (fig. 4.3 a) and 10mM (fig. 4.3 b) of silver nitrate solution, silver nanoparticles were produced with average diameter of 26 nm and 24 nm respectively. The particle size range was 22 nm – 32 nm for 1mM solution and 23 nm – 29 nm for 10mM solution. It can be observed that the difference in size of particles produced from both reactions is negligible. This indicates that concentration of silver nitrate solution in the reaction mixture has very minimal effect, if any, on the final size of silver nanoparticles.

Methanolic extract of *Chlorella vulgaris* produced nanoparticles of average size of 46 nm and 47 nm at 1mM and 10mM concentration (fig 4.3 c, d). The nanoparticle size ranged from 46 – 49 nm when 1mM silver nitrate solution was used and 46 – 48 nm size was observed when 10 nm concentration was used. The particles formed were of spherical nature and uniformly distributed. It can be observed from SEM micrographs that the concentration of silver nitrate used had no effect on final size of the silver nanoparticle.

The images showed that nanoparticles synthesized from *Dictyosphaerium 6-8* were much smaller in diameter than those synthesized from *Chlorella vulgaris* and were roughly half the size. The results also proved that concentration of silver nitrate had no effect on the final size of the silver nanoparticles.

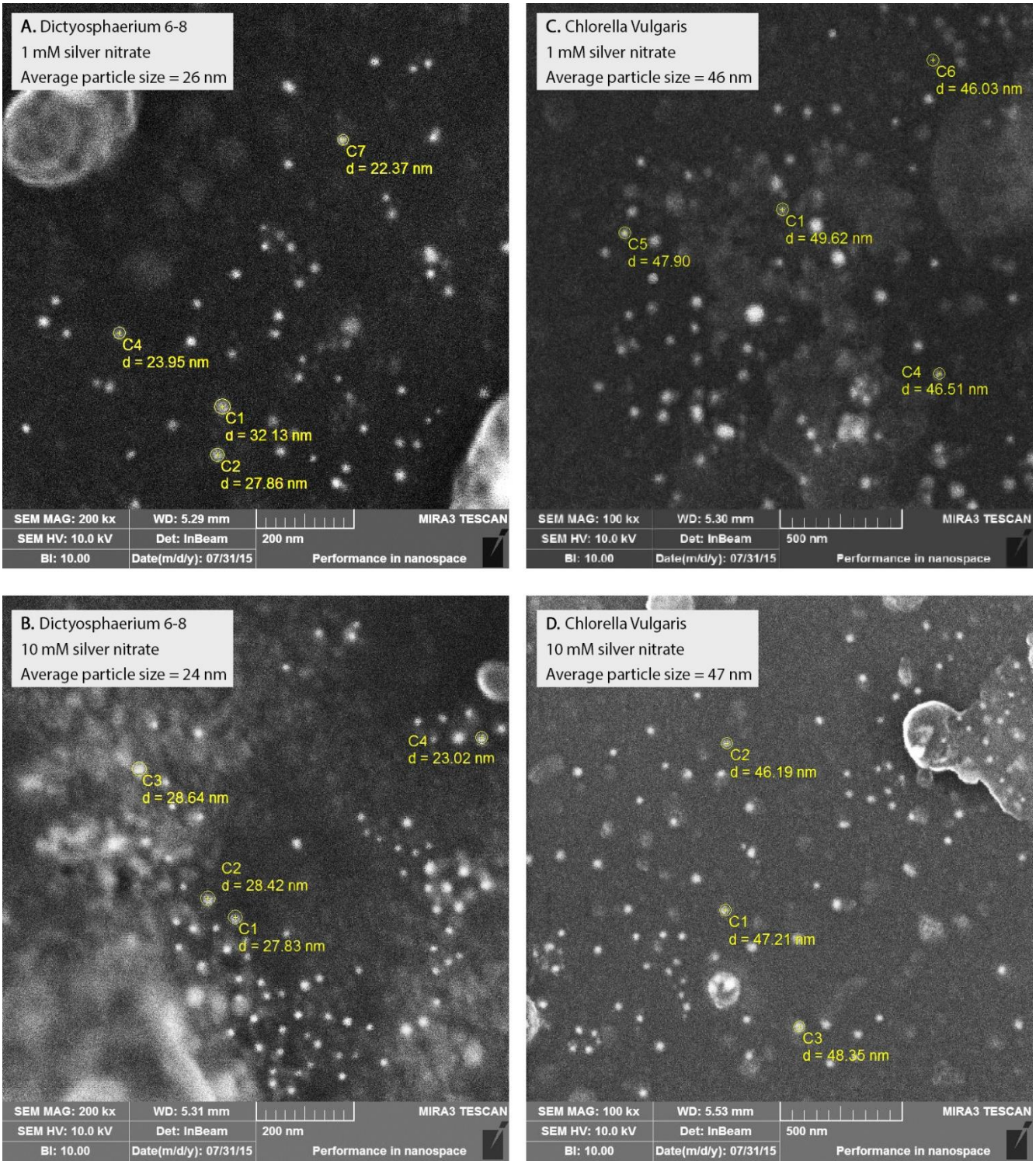


Fig. 4.3: Scanning Electron Microscope image of Silver Nanoparticles synthesized from (a) *Dictyosphaerium 6-8* at 1mM concentration (b) *Dictyosphaerium 6-8* at 10mM concentration (c) *Chlorella Vulgarius* at 1mM concentration and (d) *Chlorella Vulgarius* at 10mM concentration.

4.4 Atomic Force Microscopy of silver nanoparticles (AFM)

Atomic Force microscopy was employed to study the topographic feaures including size distribution, shape and morphology of the silver nanoparticles. Nanoparticles produced from both species of algae were scanned and compared in 3 differnet modes, area scan (fig 4.4), Line scan (fig 4.5) and 3-D scan (fig 4.6)

4.4.1 Area Scan of Silver Nanoparticles

In area scan the samples were scanned for particles and detail about size distribution of the quantum sized silver nanoparticles. The size of silver nanoparticles produced from algae *Dictyosphaerium 6-8* were compared with those produced from *Chlorella vulgaris* results are shown in figure 4.4. Results show that *Dictyosphaerium 6-8* (fig 4.4 a) produced nanoparticles within a very small range form 6-12 nm which can be confirmed via the histogram. The histogram shows the relative presence of nanoparticles of different sizes. The red peak shows a nanoparticle size range from 4 - 38.8 nm where most of them are within 6-12 nm range.

Nanoparticles produced from *Chlorella vulgaris* are shown in figure 4.4 b. It can be observed that *Chlorella vulgaris* produced relatively large sized of nanoparticles and broader range. The histogram goes up to 56.4 nm on X-axis revealing large size of silver nanoparticles produced. Most of the nanoparticles produced were within 5-12 nm range. Whereas the maximum size of nanoparticles was 56 nm.

Comparing both the images and histograms, proved that nanoparticles produced from *Chlorella vulgaris* were of relatively larger size as compared to nanoparticles produced from *Dictyosphaerium 6-8.*

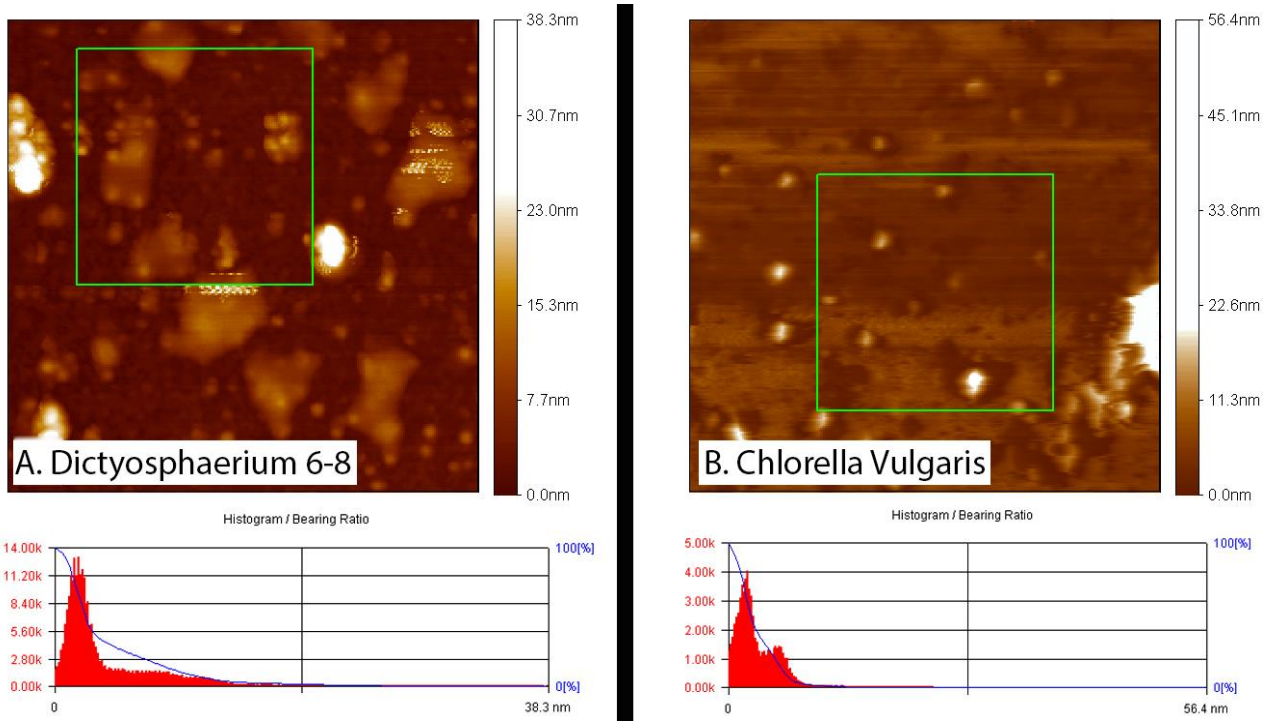


Fig. 4.4:2-D image and histogramfrom scanning electron microscope of:A. Nanoparticles synthesised from *Dictyosphaerium 6-8.* B. Nanoparticles synthesized from *Chlorella vulgaris.* The green box shows the area scanned for particle size.The histogram shows the size of nanoparticles in X-Axis and the number of numberparticles in Y-axis. The scale shows the height of the nanoparticles which is represented in different color and crossponds to the color in the image.

4.4.2 Line Scan of Silver Nanoparticles

Line scan is a different approach from area scan but is used for similar purpose. Fig 4.5 shows images and graph of silver nanoparticles produced from *Dictyosphaerium 6-8* (fig 4.5 a) and *Chlorella vulgaris* (fig 4.5 b). The graph show the size of nanoparticle present at a specific point in the image rather than whole area which was the case for area scan. The color of the line corresponds to the color of the line in the image. The height (Y-axis) reveals the size of nanoparticles and the X-axis value represents the distance of the nanoparticle from the origin.

A comparison of the both images reveals that the nanoparticles produced from *Chlorella Vulgaris are* of broader range compare to the nanoparticles produced from *Dictyosphaerium 6-8.* The table shows the smallest size of nanoparticle detected by the sensor was 3.22 nm in case of *Dictyosphaerium 6-8* and 2.31 nm in case of *Chlorella Vulgaris*. Whereas the maximum size of nanoparticles was 38.8 nm in case of *Dictyosphaerium 6-8* and 56.6 nm for *Chlorella Vulgaris.*

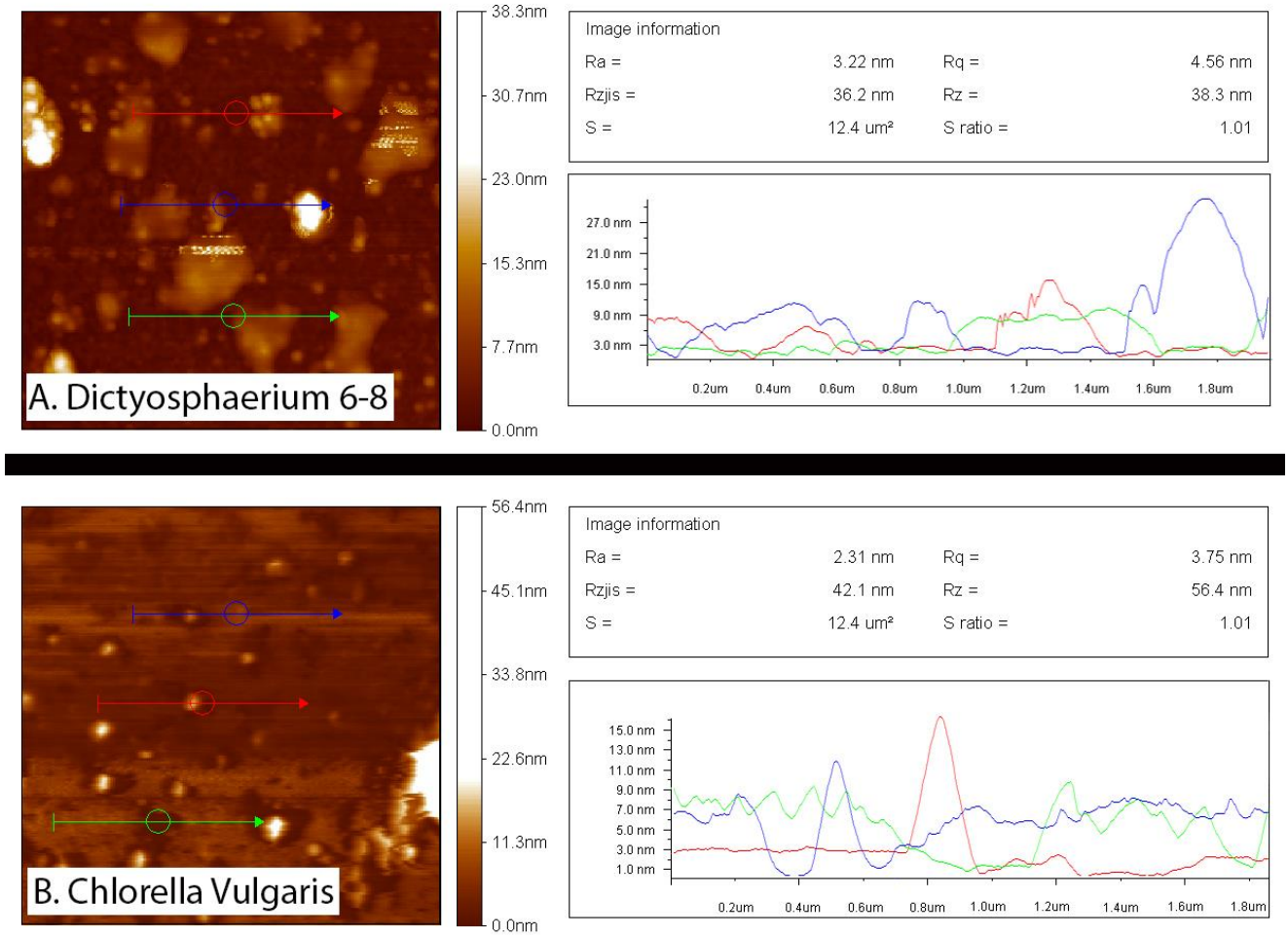


Fig. 4.5:Srface scanning of sample along a lineof A. Nanoparticles synthesised from *Dictyosphaerium 6-8.* B. Nanoparticles synthesized from *Chlorella vulgaris.*The values shown in the table show different roughness values where Rq =Root mean square roughness, Rz = 10 point average roughness, Rzjs = Average roughness, S = Surface area, S ratio = surface area ratio. The X-axis values on graph are the point on the respective line shown in red, green and blue color whereas the Y-axis value represents the size of nanoparticles. The color of line in graph represents the color of line in the image.

4.4.3 3-D Scan of Silver Nanoparticles

Scanning Probe Imaging Processor was used to employ a peak-and-valley method to study the surface topology of nanoparticles. This method is used to generate 3-D images which can reveal any roughness or damage on nanoparticle size (fig 4.6). An Area of 3.5 µm x 3.5 µmwas scanned for both algal sources, *Dictyosphaerium 6-8* (fig 4.6 a) and *Chlorella Vulgaris* (fig 4.6 b). 3-D topographical images revelead smooth surface of nanoparticles where no defects in the morphology and no cracks on particle sruface were observed.

Comparison of both images reveal that silver nanoparticles produced from *Dictyosphaerium 6-8* were more uniformly distributed compare to the nanoparticles produced from *Chlorella Vulgaris*.

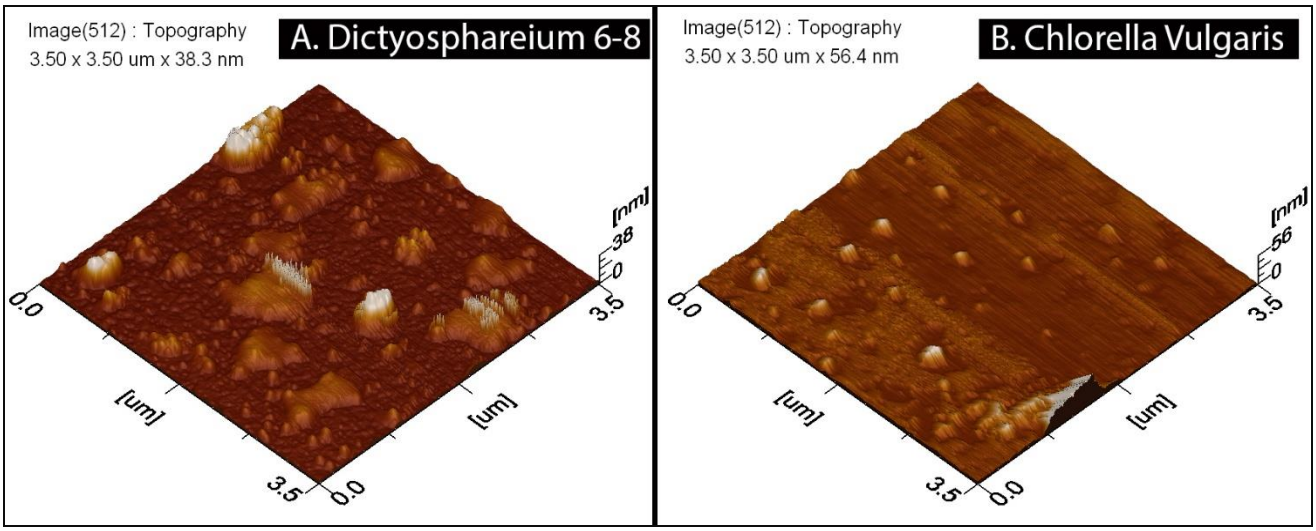


Fig 4.6: 3-D Atomic force microscopicimage of A. Nanoparticles synthesised from *Dictyosphaerium 6-8.* B. Nanoparticles synthesized from *Chlorella vulgaris.* 3-D image shows a silver nanoparticles in their perfect form revealing the topography. The height of nanoparticles can also be seen fig A shows maximum height of 38nm where as in figure B nanoparticles of size upto 82nm can be seen.

4.5 Fourier Transform Infrared Analysis (FTIR)

FTIR is used to detect functional groups present on the surface of silver nanopartilces. This technique also reveals how the atoms are bonded together, either via single, double or triple bond. Capping of the molecules can also be detected by this technique. Chemically active reducing agents present can also be detected by FTIR. FTIR analysis was carried out for silver nitrate produced from both *Dictyosphaerium 6-8* and *Chlorella Vulgaris*and compared with the control to detect possible functional groups and reducing agents (fig. 4.7). The graph is plotted in wavelength (x-axis) against percentage transmittance (y-axis). Results of the FTIR scan was compared with that of control for the respective solution. The control contained algal extract and deionized water but without silver nitrate.

The results obtained form reaction mixture of *Dictyosphaerium 6-8* show a stretch at 792 wavelngth which is a characterstic of many alkyl halides (fig 4.7 a). Comparing the results with the control, a significant shift at 1644m can be observed which indicates the prescence of amines specifically 1^{o} amines. A shallow change at 2102 indicates the prescence of C ≡ C stretch. This is due to the reason that after reduction many free carbon radicals are formed with different valances and they react with each other to form double and triple bond structures i.e alkenes and alkynes respectively.

Comparison of FTIR analysis of silver nanoparticles produced from *Chlorella Vulgaris* and the control created for the reaction, shows similar results with high amount of, alkyls, amides, amines, alkenes and other common organic compounds (fig. 4.7 b). The control was formed in a similar fasion with algal extract and deionized water and contained no silver nitrate. Presence of aromatic ether was observed in a reaction mixture from *Chlorella Vulgaris*. Ether is a class of compounds and are rarely found in free form in plants and algaes. 773 stretch shows presence of cyclic ether which is possiblly acting as a capping agent on silver nanoparticles.

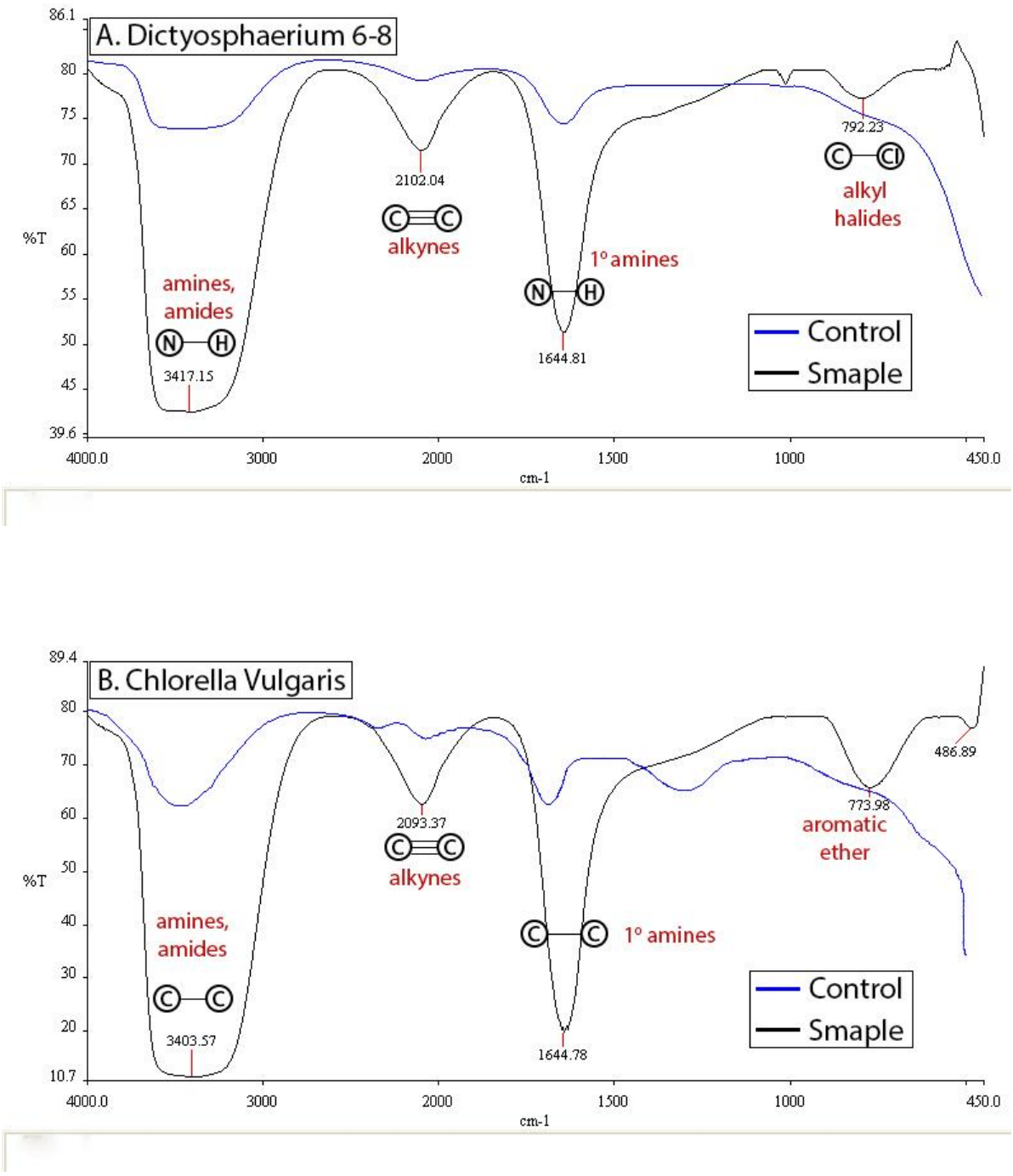


Fig. 4.7: FTIR comparison of control and reaction mixture of A. *Dictyosphaerium 6-8* B. *Chlorella Vulgaris*. The result show different in transmittance at different wavelengths. Points on X-axis are wavelength and Y-axis values show the percentage trancperency of a particular family or class of compound. A higher Y-value means higher tranparency means low absorbance of target compound and vice-versa. The blue line represents control and the black line represents the reaction mixture.

4.6 Elemental analysis via X-Ray Fluorescence Spectroscopy (XRF)

XRF was used to determine the elemental composition of the extracts to detect the presence of silver and its relative concentration in the solution. The algal extract of *Dictyosphaerium 6-8* and *Chlorella Vulgaris* was treated with 1mM and 10mM concentration of silver nitrate solution each. This mixture was diluted 5 times and analyzed for the presence of silver and compared to study the effect of silver nitrate concentration on the silver nanoparticles concentration in the mixture.

Result indicate that a significant concentration of silver was present in the solution. Comparison of the graphs revealed that the mixture with 10mM silver nitrate solution produced slightly large quantities of silver nanoparticles compared to the solution with 1mM silver nitrate solution. This can be confirmed by observing the peak of silver in figure 4.8 b, and comparing it with the peak of silver in figure 4.8 a. Presence of other elements including calcium, potassium and zinc was also detected which is expected as these elements are present in abundance in any biological extract. The graph shows the potential difference of the X-rays used on in, Kilo Electron volts unit (keV) plotted on the X-axis and the intensity of the diffracted X-rays plotted on y-axis, represented in Counts per Second unit (CPS).

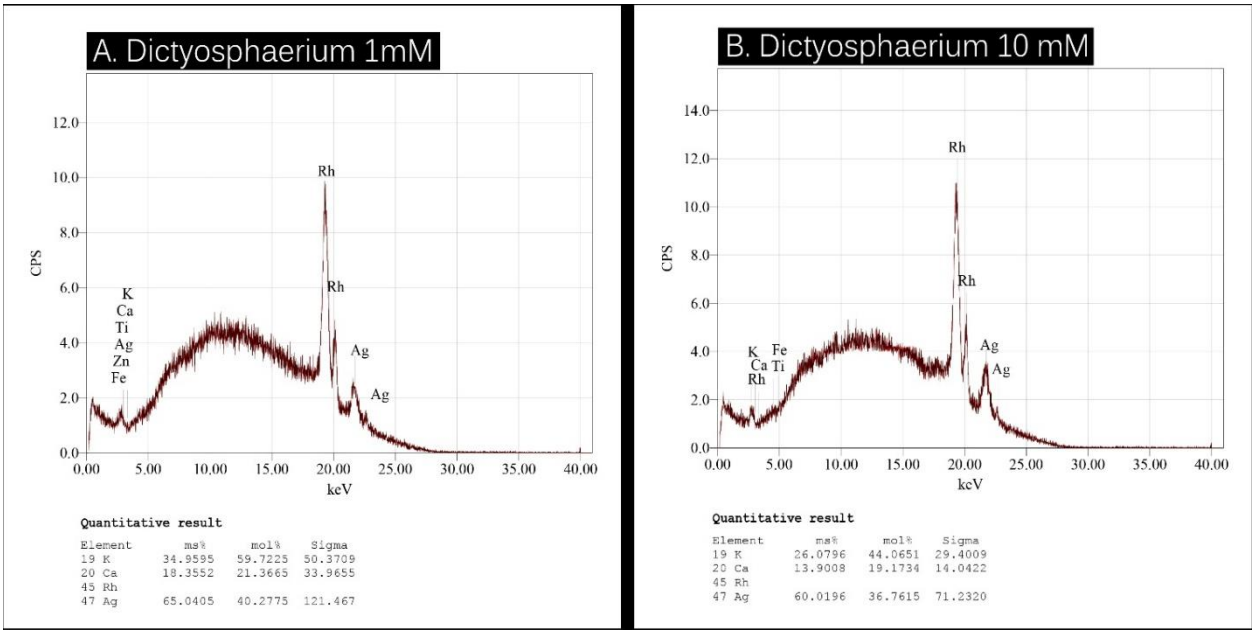


Fig. 4.8 Elemental analysis of Silver nanoparticles from *Dictyosphaerium 6-8* at A. 1mM silver nitrate concentration B. 10mM silver nitrate concentration. Peaks at different potential difference (x-axis) are charcterstics of specific elements. The height of the peak revelas the relative concentration of the labeled element.

Graph obtained from XRF analysis of *Chlorella Vulgaris* extract is presented in figure 4.9 for both 1mM (fig. 4.9 a) and 10mM (fig. 4.9 b) concentrations of silver nitrate. Comparison of silver peaks of both reactions revealed the same pattern i.e. silver nanoparticles produced from 10mM concentration of silver nitrate were of higher concentration than that produced from 1mM silver nitrate concentration. Similarly presence of other biological elements was also observed but are not a topic of interest in this research. Comparison of peaks of both the algae solutions revealed that, in case of both concentration mixtures, *Chlorella Vulgaris* formed slightly high amount of silver nanoparticles compare to *Dictyosphaerium 6-8.*

The table provide a detailed quantitative assessment of different parameters of detected elements. These values are approximated and have an error chance. It can be observed that the 1mM and 10mM mixtures are44% and 79% silver by mass respectively.

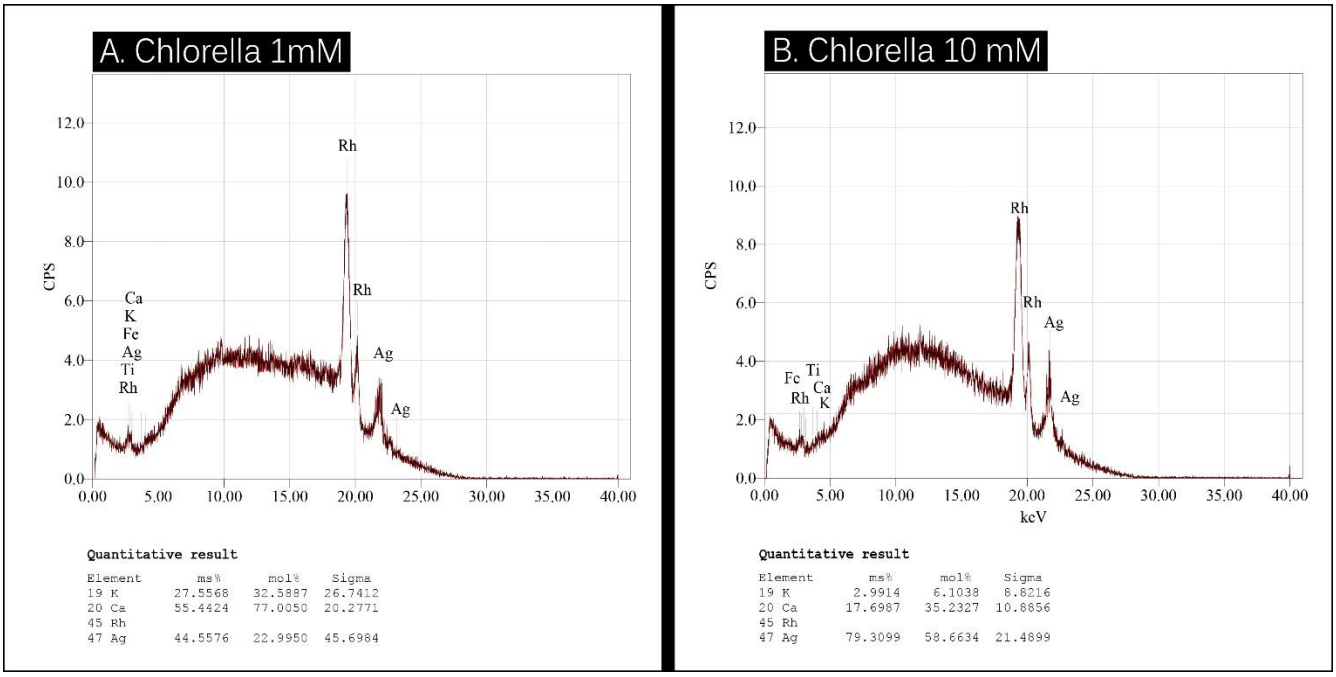


Fig. 4.9 Elemental analysis of Silver nanoparticles from *Chlorella Vulgaris* at A. 1mM silver nitrate concentration B. 10mM silver nitrate concentration. The table below shows approximate mass (ms%), approximate molarity (mol%) and the error coffecient (sigma)

4.7 X-ray Diffraction Analysis (XRD)

X-Ray Diffraction analysis is used to study the crystalline nature of the nanoparticles. XRD was performed for silver nanoparticles synthesized from *Dictyosphaerium 6-8* and *Chlorella Vulgaris*. The particles were purified and concentrated via centrifugation. The XRD scan is used to study the different facets of the crystals. Presence of different lattice planes can also be detected which is indicated by Miller Index. X-rays are bombarded on the sample at different angles, which is diffracted back by the sample at a different angle. The presence of Bravia lattice in the sample crystal dictates the angle at which the x-rays are refracted. This angle is plotted on X-axis in figure 4.10. The absorbance values plotted on Y-axis determines the concentration of that crystal in the sample.

Silver nanoparticles produced from *Dictyosphaerium 6-8* were monocrystalline in nature as can be seen in figure 4.10 a. This means that only one type of crystal lattice was observed in the sample. The peak at 38◦ reveals the presence of crystalline silver as this is the characteristic peak for silver. The miller index is a notation system used to categorize different lattices of a crystal. The miller index of the silver crystals observed is 111 which indicates the presence of 111 plane in the sample. High absorbance peak at only 1 refractive angle means that almost all the silver nanoparticles produced from *Dictyosphaerium 6-8*, were of same crystalline structure.

XRD scan of silver nanoparticles produced from *Chlorella Vulgaris* revealed different results (fig. 4.10 b). The crystals were of polycrystalline nature. This means that silver in the solution has formed more than one type of crystal. This is evident from the graph as three peaks at different locations can be observed. The peaks are observed at 28◦, 32◦ and 38◦ which corresponds to 121, 200 and 111 plane respectively. It can also be observed that these peaks have significantly low absorbance values compare to the absorbance value of peak shown in figure 4.10 a. This indicates that all three lattice of crystals are present in almost same quantity but none of them in large quantity.

The XRD results show that silver nanoparticles produced from *Chlorella Vulgaris* are of polycrystalline nature whereas silver nanoparticles produced from *Dictyosphaerium 6-8* were of monocrystalline nature.

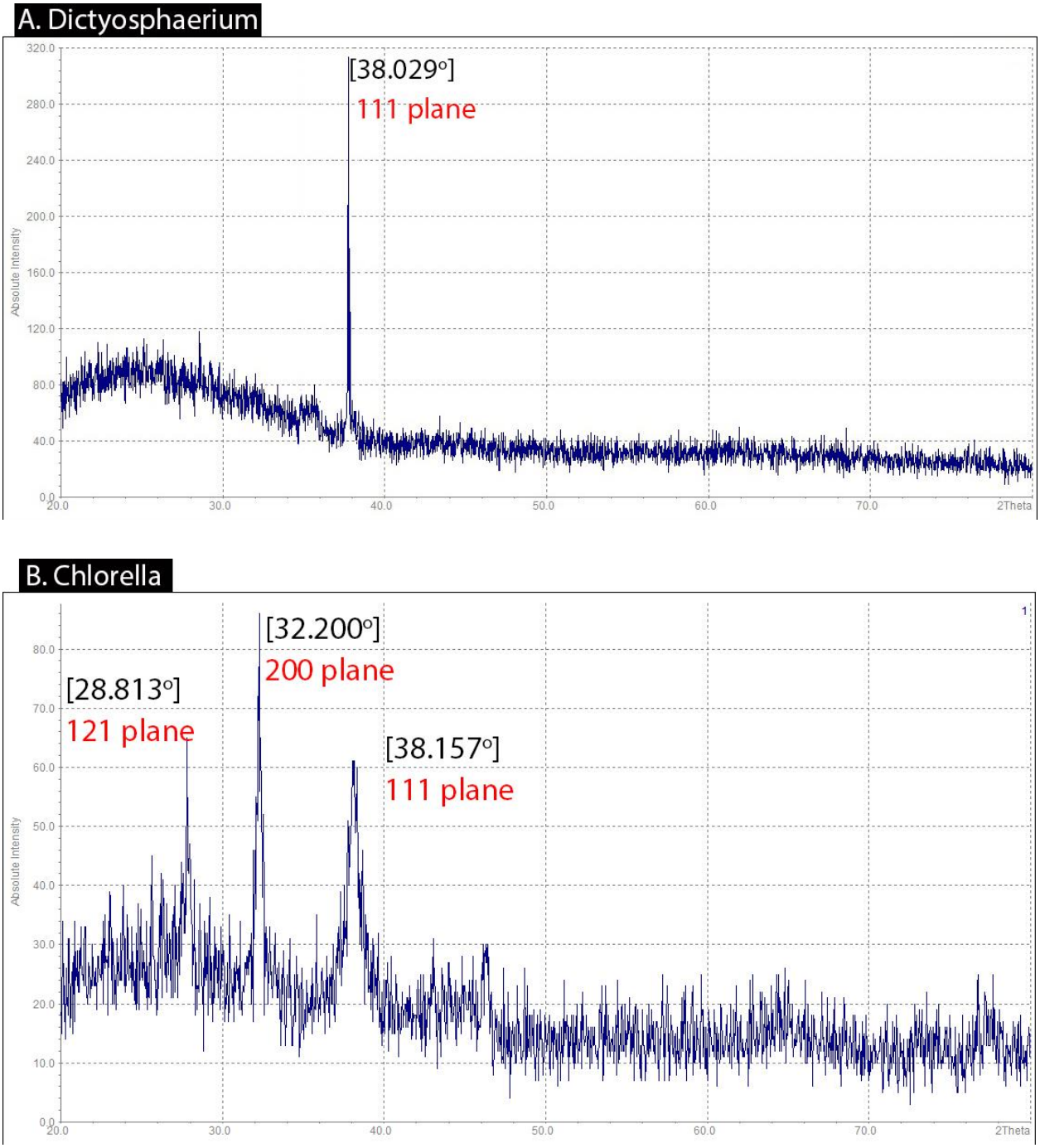


Fig 4.10 XRD analysis of silver nanoparticles synthesized from A. *Dictyosphaerium 6-8* B. *Chlorella Vulgaris.* The angle of refraction is shown in black color. The miller index of lattice observed is shown in red color. The height of peak represent the intensity of the diffracted x-rays translating the concentration of the respective crystal lattice present

4.8 Cytotoxic ecaluation of silver nanoparticles via MTT assay

4.8.1 Cytotoxic effect of silver nanoparticles on Huh-7 cell lines

To determine the cytotoxic effect of the silver nanoparticles synthesized from both algae MTT assay was run on Huh-7 cell lines. A positive control was formed in which Taxol was used instead of silver nanoparticles. Cytotoxic effects of silver nanoparticles were observed under microscope at different hours to observe the behavior of nanoparticles. Metabolic activity of the cell under different conditions can be observed via MTT assay.

The results are shown in graph 4.3. The graph is represented in cell survival rate on y-axis and algal species on X-axis. The color shows the concentration used along with the control. The results indicate high cell survival rate even at very high concentration of 4mg / ml. The result implies that silver nanoparticles prepared using this method are of very low risk to health and have potential to be used as a drug carrier by transporting the drug into the target cells.

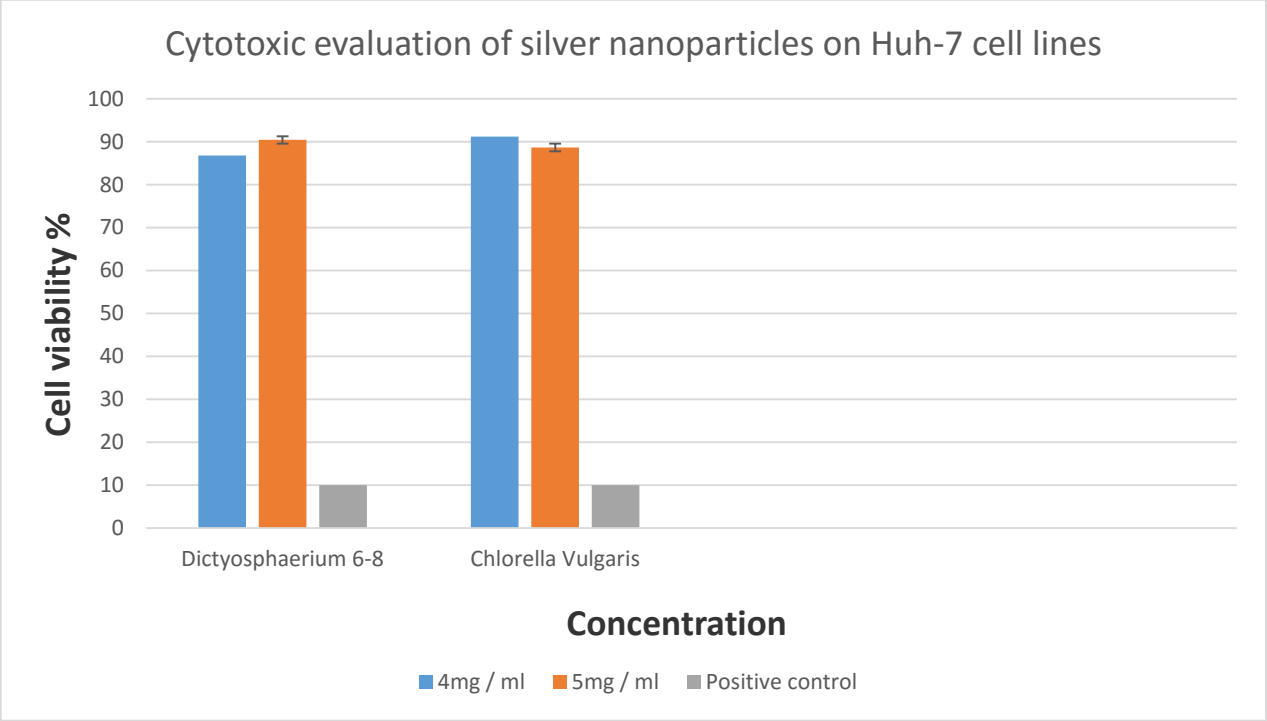


Fig. 4.11: Chart showing cytotoxic effects of the silver nanoparticles synthesized on the Huh-7 cell lines. The blue and orange bars shows concentration of silver nanopartilces at 4mg/ml and 5mg/ml respectively. The grey bar shows positive control which contains Taxol instead of silver nanoparticles

4.8.2 Cytotoxic effect of silver nanoparticles on Hep-2 cell lines

MTT assay was also performed on Hep-2 lines which is a HeLa derived Human carcenoma cell line. Different concentrations of silver nanoparticles were used on the cell lines to test the effect of silver nanoparticles on cell death rate. A positive control was formed which contained a cytotoxic drug, Taxol.

The result can be seen in figure 4.12. It can be observed that silver nanoparticles had no adverse effect on the Hep-2 cell line which is another cell line. When compared with positive control it can be seen that cells treated with silver nanoparticles have a high chance of survival. The result also show that silver nanoparticles produced from chlorella vulgaris had higher cell toxcicity but only by small margin.

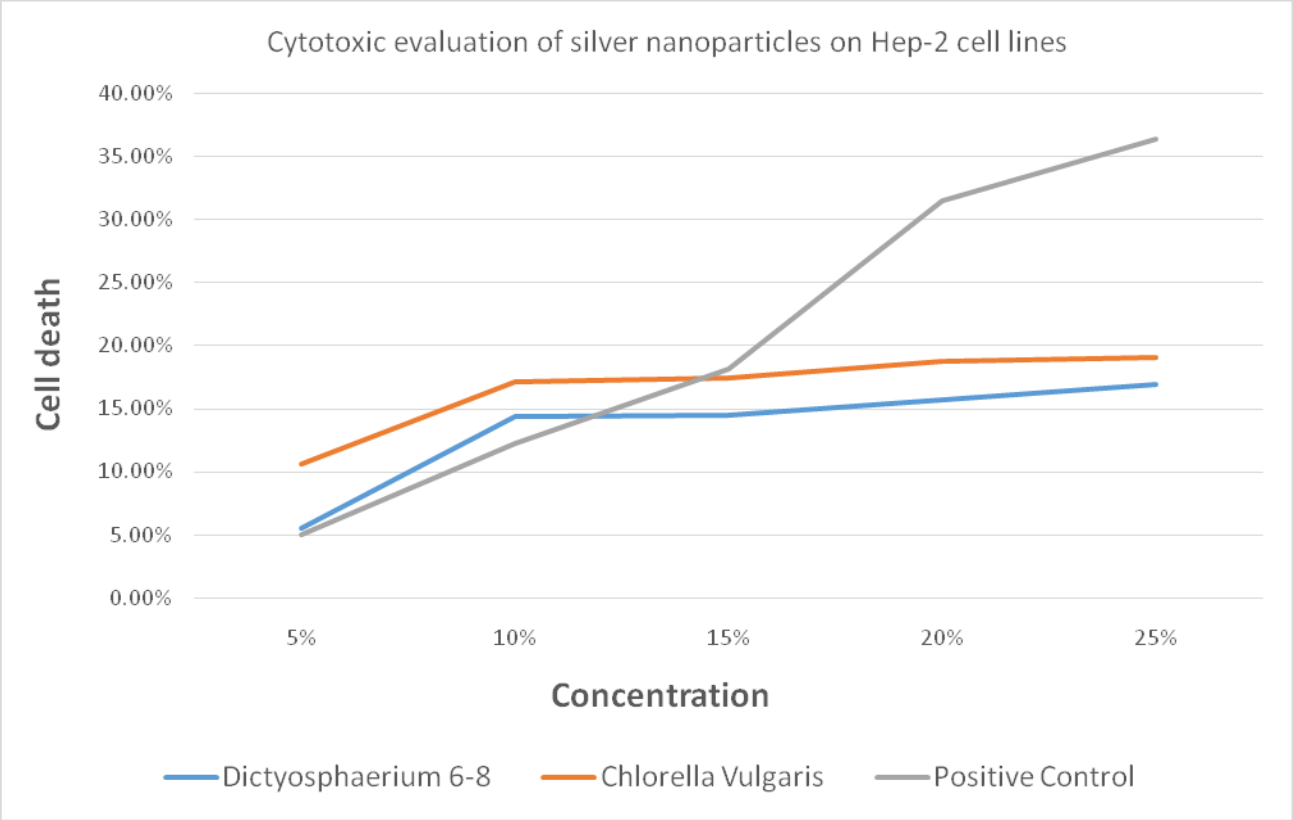


Fig. 4.12: Chart showing cytotoxic effects of the silver nanoparticles synthesized on the Hep-2 cell lines. Blue and Orange lines represent nanoparticles synthesized from *Dictyosphaerium 6-8* and *Chlorella Vulgaris* respectively. The grey line is positive control which contained Taxol but did not contain any silver nanoparticles.

4.8.3 Cytotoxic effect of silver nanoparticles on HCEC cell lines

Human Corneal Epithelia Cells (HCEC) were also treated with different doses of silver nanoparticles to study their cytotoxic effect on normal cell lines. Taxol was used as a cytotoxic drug in the positive control formed to compare the results against.

The results are plotted in figure 4.13. The reuslts idicate that silver nanoparticles synthesized from both alges have very limited cytotoxic effect on the normal cell lines. HCEC is a normal cell lines and low toxicity in normal human cells depicts the absense of general rule for cytotoxic potential of silver nanoparticles on human cells indicating that such nanoparticles can be used as a delivery vehicle in drug delivery systems.

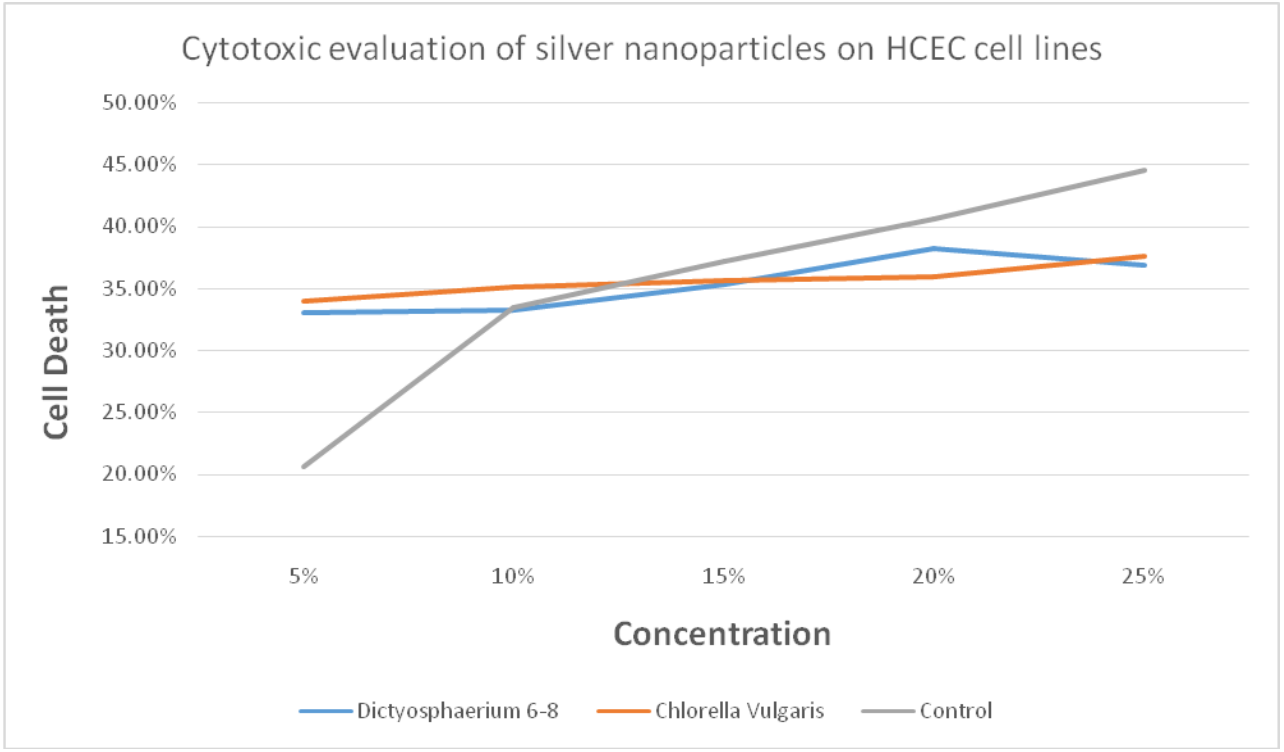


Fig. 4.13: Chart showing cytotoxic effects of the silver nanoparticles synthesized on the HCEC cell lines. Blue and Orange lines represent nanoparticles synthesized from *Dictyosphaerium 6-8* and *Chlorella Vulgaris* respectively. The grey line is positive control which contained Taxol but did not contain any silver nanoparticles. In the chart percentage cell death (Y-Axis) is plotted against concentration of silver nanoparticles (X-Axis) used.

Chapter 5

DISCUSSION

The present research was conducted to find a novel approach for the synthesis of silver nanoparticles using a cheap and environmental friendly source. For this purpose two species of algae, *Dicyosphaerium 6-8* and *Chlorella Vulgaris* were used. Methanolic extract of alga was treated with silver nitrate solution for the synthesis of silver nanoparticles. Silver nanoparticles synthesized from both these algal species have not yet been reported. The silver nanoparticles synthesized were characterized using different techniques for assessment of different properties including, shape, structure, size distribution, topography, stability, concentration and many more. Furthermore, cytotoxic effect of these silver nanoparticles were studied on cancer cell lines, Huh-7 and Hep-2, and normal cell line HCEC.

Extracellular extract of *Chlorella Vulgaris* has already been used for the synthesis of Gold nanoparticle of 9-20 nm size (Noruzi, 2015). *Spirulina platensis* has been used for the synthesis of stable silver nanoparticles of size range between 20-40nm (Vijayan *et al.*, 2014). *Kappaphycus alvarezii* and *Sargassum wighti* was used for extracellular synthesis of silver nanoparticles by, (Parial and Pal, 2014; Vijayan *et al.*, 2014), respecteively. *Tetraselmis kochinensis* was used for the synthesis of gold and silver nanoparticles (Azizi *et al.*, 2014). Extract of *Chondrus crispus* was used with chloroauric acid for the synthesis of stable nanoparticles of silver and gold (Castro *et al.,* 2009).

The first indication of silver nanoparticles synthesis was the change in color from green to brown (fig. 4.1). Further confirmation of silver nanoparticle synthesized was performed by UV-Vis Spectroscopy. This technique uses ultra violet and visible range of electromagnetic spectrum to determine the absorbance of the mixture at different wavelength which is a unique property of different elements. Silver shows maximum absorbance at 400-430 nm range which can be seen in figure 4.2. The concentration of silver nitrate used had a direct effect on the rate of silver nitrate synthesis as at higher concentration (10mM) the absorbance was maximum for

silver nanoparticles synthesized from *Dicyosphaerium 6-8* and *Chlorella Vulgaris*. This is due to the fact that the reaction is first order reaction (Kolla *et al.*, 2014) which means that the rate of reaction is dependent on one unimolecular reactant i.e. silver nitrate. Increasing the concentration of silver nitrate increases the chemical interaction that can take place per unit time resulting in faster reduction of the silver nitrate to nano size silver particles. The high concentration of silver also means high amount of silver will be produced because all the silver nitrate will eventually be converted to silver nanoparticles as this is an enzymatic (Walsh *et al.*, 2014). The presence of silver in our reaction mixture was further confirmed by Elemental Analyzer using X-Ray Fluorescence (XRF). The result show considerable amount of silver present in the reaction mixture (fig. 4.9).

To visualize the shape and size of the silver nanoparticles synthesized from both *Dicyosphaerium 6-8* and *Chlorella Vulgaris*, Scanning Electron Microscopy was performed. This provided a clear visual of the silver nanoparticles as can be seen in figure 4.3. The average size of silver nanoparticles produced from *Dicyosphaerium 6-8* was 25 nm whereas the average size of silver nanoparticles synthesized from *Chlorella Vulgaris* was 46 nm indicating that the silver nanoparticles synthesized from *Chlorella Vulgaris* were larger than those produced from *Dicyosphaerium 6-8.*

The result also show that concentration of silver nitrate used in the mixture had no effect on the size of the silver nanoparticles as it can be seen reaction containing 1mM and 10mM silver nitrate solution had same sized silver nanoparticles. It can be observed that the silver nanoparticles synthesized were of spherical shape and were uniformly distributed. The size of silver nanoparticles have great effect on their chemical and physical properties because as the size of the nanoparticles decreases the surface area increases. Cytotoxicity of silver nanoparticles is also greatly affected by size of the silver nanoparticles (Hsin *et al.,* 2010) as small sized nanoparticles can easily evade phagocytosis and hence excretion or degradation by cellular mechanisms.

Silver nanoparticles synthesized from different biological sources differ in size range. *Pseudomonas stutzeri* was used for synthesis of intracellular nanoparticles of upto 200nm (Klaus *et al.,* 1999). Silver nanoparticles between 20-30 nm were synthesized extracellularly

using *Morganella sp.* (Parikh *et al.,* 2008). Cynobacteria *Plectonema boryanum* was used to synthesize silver nanoparticles of size less than 10nm (Bonde and Pande, 2014).

For detailed study of the size range of the silver nanoparticles synthesized, Atomic Force Microscopy (AFM) was employed which is a type of Scanning Probe Microscope (SPM) detailed study on the different sizes of the nanoparticles present in the sample. AFM also allows 3-D visualization of the sample giving us information about the height of the silver nanoparticles. The results are displayed in figure 4.5 – 4.7. AFM has advantage over SEM because AFM is able to detect surface topography on nanometer scale (Ma *et al.*, 2014). AFM can show us 3-D images of the sample which is not possible through SEM.

Silver nanoparticles synthesized from biological sources are capped on the surface by some organic molecule which may cause the nanoparticle to lose its toxicity compare to the silver nanoparticles synthesized from chemical or physical sources. Surface of these nanoparticles can be modified by different capping agents including peptides, nucleic acids, enzymes and more. This makes them more compatible with biological systems and can be safely used with living cells (Aziz *et al.*, 2014).These nanoparticles may also be capped with any antioxidant compound that can be used for the treatment of high stress. The silver nanoparticles synthesized from *Dicyosphaerium 6-8* and *Chlorella Vulgaris* also had several functional groups attached on its surface as was detected by Fourier Transformed Infrared Spectroscopy (FTIR) (fig. 4.7).

Alkenes, alkynes, amines and other hydrocarbons were present on the surface of silver nanoparticles all of which are of organic nature. The presence of these organic molecules, such as alkenes, alkynes, amides and ether, indicated that silver nanoparticles synthesized from both these algal species should have low cytotoxicity for cells. Silver nanoparticle synthesized from *Chlorella Vulgaris* contained aromatic ether attached on its surface as can be seen by the 773 stretch. This can cause the silver nanoparticles to be soluble in water as ether is a polar compound and is soluble on water. These capping agents, identified by FTIR, makes these silver nanoparticles more stable and cause them to evade our body's immune system making them less toxic for living systems as compared to the silver nanoparticles synthesized from chemical methods (Mishra *et al.*, 2015). This nature of silver nanoparticles synthesized for algae

methanolic extracts make them ideal for applications such as drug delivery, imaging, probing and other applications, to be used for in vivo studies with minimum toxic effects.

Depending on the method used for synthesis of silver nanoparticles, they can be either of amorphous or crystalline nature. Using X-Ray Diffraction Crystallography (XRD) it was observed that the silver nanoparticles synthesized from algae *Dicyosphaerium 6-8* and *Chlorella Vulgaris* were of crystalline nature. The silver nanoparticle synthesized from *Dicyosphaerium 6-8* were of monocrystalline nature meaning all the crystals were of same type and contained 111 facets only. The silver nanoparticles synthesized from *Chlorella Vulgaris* were of polycrystalline nature as they had 3 different types of facets i.e. 111, 200 and 121 facets.

Cytotoxic activity of the synthesized silver nanoparticles were evaluated using MTT assay on cancer cells, Huh-7 and Hep-2 and normal cell line HCEC. Hep-2 and Huh-7 are cancerous cell line and are used extensively for research purposes (Sun and Nassal, 2006). Both these cell lines contain all the common characteristic of a typical cancer cell which was the reason for my choice of these cell lines. For positive control Taxol drug was used. The results show that silver nanoparticles synthesized from *Dicyosphaerium 6-8* and *Chlorella Vulgaris* were not cytotoxic.

Conclusion

The study concluded that methanolic extract of algae *dictyosphaerium 6-8* and *chlorella vulgaris* can be effectively use for synthesis of stable silver nanoparticles. The study showed that the silver nanoparticles synthesized from both these algae lie in a very narrow size range. Silver nanoparticles synthesized from *chlorella Vulgaris* were twice the size of those produced from *dictyosphaerium 6-8.* The study concluded that silver nanoparticles synthesized from algal species show no cytotoxicity in both cancer and Human cell lines. This study also concluded that silver nanoparticles with less cell cytotoxicity can be synthesized from algal extracts which is a cheap and rapid alternative to already used methods for the synthesis of silver nanoparticles.

Future Prospects

Nanobiotechnology has unlocked the potentials application of nanotechnology in medicine. Nanoparticles are the core of nanotechnology and they can be used in different aspect of biomedical application. Non-toxic fluorescent nanoparticles synthesized by this method can be employed in drug delivery, in bioimaging, in killing of cancer cells by hyperthermia and phtothermal therapy. They can be used as an alternative to carcinogenic ethidium bromide in gel electrophoresis. Zeta potential and NMR analysis can be performed for further characterization and confirmation of these Silver nanoparticles as quantum dots. DPPH assay can give an insight about their anti-oxidant nature. They can therefore be used in skin care products. Approaches to gene therapy can also be improved by using less cytotoxic silver nanoparticles as a gene delivering agent.

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