Phycosynthesis of TiO_2 Nanoparticles from Dictyosphaerium sp. Strain HM1 (DHM1), Au and Ag Doped TiO_2 and its Antibacterial, Antibiofilm and Anticancer Activity

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Industrial Biotechnology

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Acknowledgment

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“Dedicated to My beloved Abu, Ammi & Aghajan

Abstract

The present study reports a green, cost effective and biological methodology to synthesis Titania nanoparticles from algal extracts of Dictyosphaerium sp. Strain HM1 (DHM1). This synthesis method was also optimized by different physiochemical parameters like temperature, reaction time, molar concentration and calcination temperature to obtain different crystalline form of TiO_2 nanoparticles. The results were initially characterized by UV-VIS spectrophotometry showing maximum absorbance at 450nm and information regarding size distribution and morphology was visualized through SEM which ranges from 30-50 nm. EDX analysis confirmed the elemental analysis of TiO_2 NP's and XRD confirmed the different crystalline forms of TiO_2 NP's (Anatase and Rutile). These TiO_2 NP's were than doped with gold and silver to obtain AuNP's and AgNP's in order to check its antibacterial, antibiofilm and anticancer activity. It was concluded than undoped TiO_2 had no pathogenic activity while doped nanoparticles showed pathogenic behavior with $AuTiO_2$ NP's having highest antibacterial and antibiofilm activity. Moreover, results from invitro toxicity suggest very exceptional anticancer activity of $AuTiO_2$ NP'S against colorectal cell line HCT116 and the triple negative human breast cancer cell lines HCC1954. This healing can also induced marked apoptosis leading to typical cell cycle arrest in G2/M phase. It is recommended to further characterize these nanoparticles by using advance characterization techniques like NMR, Raman spectroscopy and TEM analysis. In silico approach should be adapted to examine the interaction between doped nanoparticles and its cellular interaction with bacteria and human cell

Keywords: TiO_2, anticancer, antibacterial, Dictyosphaerium sp., nanoparticles, $AuTiO_2$, $AgTiO_2$

Abbreviations

%	Percent
°C	Degree Celsius
DI water	Deionized Water
E. coli	*Escherichia Coli*
EDX	Energy Dispersive X-ray
et al.	et alia
FTIR	Fourier Transform Infrared
g	Gram
hr	Hour
i.e.	id est means “that is”
IR	Infrared
Kb	Kilobasepair
KBr	Potassium Bromide
KeV	Kilo electron Volt
KV	Kilovolt
LB	Luria Bertani
M	Molar
min	Minute
ml	Milliliter
mM	Millimolar
ng	Nanogram
nm	nanometer
NMR	Nuclear magnetic resonance
PBS	Phosphate Buffer Saline
RPM	Rate Per Minute
SEM	Scanning Electron Microscope
SRB	Sulforhodamine B
UV-Vis	Ultra-violet-visible
V	Volt

XRD	X-ray Diffraction
μg	micro gram
μl	Microliter

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CHAPTER 1 : INTRODUCTION

Nanotechnology is an emerging interdisciplinary expertise pursuing to discover the unique technological advances of manipulating the structure of materials at a scale of individual atoms, molecules and their structured aggregates. The nano prefix in the word nanotechnology states reduction of mass, volume/weight by 1o raise to power 9 (Nanoscale) or time. One nanometre is actually "one billionth of a meter" or ten angstroms (Joachim, 2004).

According to the National Nanotechnology Initiative, "science of Nanotechnology is the control and understanding of matter at measurements of around 1 to 100 nm, where matter properties become different from those of individual atoms or molecules or bulk materials to understand materials to achieve this experience for different applications (Ruzicka, 2013). By manipulating the structural arrangement and bonding of atoms, novel materials can be designed with a vast range of physical, chemical and biological properties.

Generally, nano size particles can be categorized into two main sets: Organic nanoparticles and the second Inorganic nanoparticles. Examples of Organic nanoparticles are carbon nanoparticle while inorganic nanoparticles are semiconductor (TiO_2, ZnO), magnetic nanoparticle, noble nanoparticle (Au, Ag). Because of adaptable function and superior material properties Inorganic nanoparticles have created. Due to its small nano size feature these nanoparticles are having many applications like they can be used in drug delivery systems and chemical imaging. They are widely available because of its adaptable role and are used for the cellular transportation rich functionality and good biocompatibility.

Nanoparticles are also good exporter for drug delivery (targeted) and controlled release of drug. Nanoparticles are profitable material semiconductor nanoparticle (TiO_2 and ZnO) for medical science (Example is when molecular medicines are combined with mesoporous silica). Gold nanoparticle is used as a good carrier for use in cancer treatment of biological targets (Cheon and Underwood,

2009). Silver based nanoparticle is used in healing the wounds and infectious disease due to its good antimicrobial activity (Behrouz Ghorani, Sara Naji-Tabasi, Aram Bostan, 2018). The importance of nanoparticles synthesis in Nanobiology is because of its shapes variable size, controlled dispersity, chemical composition and their possible use in the technology of medicinal sciences for the better treatment of individual welfare.

Rutile, anatase, and Brookite are the three crystalline forms of TiO_2 (anatase and rutile structures are depicted in Figure 1.1) and these polymorphs are comprised of. octahedra TiO_6 which are bound in special methods (Ruzicka, 2013). Structure of anatase TiO_2 relates to the tetragonal system (with. Dipyramidal habit) and is composed of predominantly point-sharing octahedral. Anatase TiO_2 under UV.irradiation is mainly used in catalysis. Rutile polymorph of TiO2 is having prismatic habit with tetragonal crystalline structure. This type of TiO2 is one of the most stable forms towards temperature and can form long chains through octahedra edge-sharing and is commonly used in paint (white pigment). Brookite TiO_2 is having crystalline of orthorhombic symmetry. Brookite is difficult to synthesis, and is generally inactive photocatalytically. The most common and stable form of TiO2 is Rutile. This form of TiO2 is having band gap of 3.0 eV a direct-bandgap semiconductor. This band gap is smaller than other crystalline form of TiO2. Rutile form because of its increased rate of electron-hole recombination is generally not suitable for photocatalysis. (Ruzicka, 2013). Therefore, Titania nanoparticles is multipurpose, material having many applications in products like used as white pigment in paint, electrodes, capacitors, solar cells, food coloring agent, sunscreen lotions and in toothpastes as a whitener. TiO_2 possess photocatalytic activity Generally TiO_2 is selected in anatase form, because of higher potential energy of photogenerated electrons beside this anatase form is inexpensive environmental friendly, high and is stable photo chemically.

Morphologies of TiO_2 NP's largely comprised of nanostructures examples include nanowires, mesoporous structures, nanotubes and nanorods. For the synthesis of TiO_2 nanostructured variety of methods are used like hydrothermal, solvothermal, sonochemical method, microwave method, electrodeposition, direct oxidation, sol-gel and chemical vapor deposition (CVD) (Malekshahi Byranvand et al., 2013).

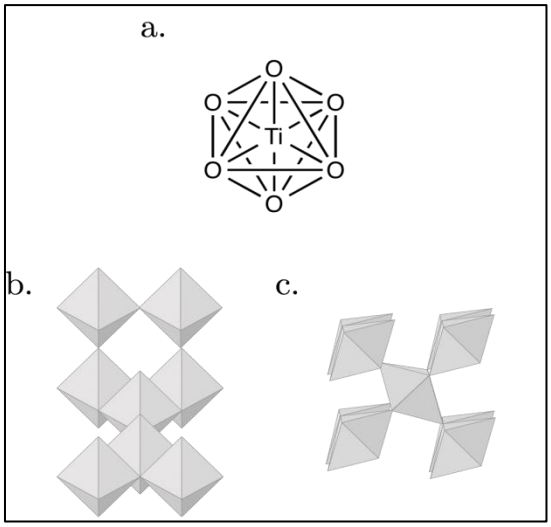


Figure 1.1: Arrangement of octahedra TiO_6

A) anatase B) rutile and C) Brookite.

Titania NP's having size around 100nm or less than 100 in diameter is of great interest in generation of advanced materials because of its versatile environmental applications like gas sensor in waste water treatment by photocatalytic degradation of various contaminants, degradation of carcinogenic dyes, solar cell. Beside environmental applications these nanoparticles are used in cosmetics prevent UV radiation. They can also be used in filters to clean water by removing odors and harmful bacteria as they possess strong germicidal properties. Photocatalysis activity of TiO_2 has been extensively studied for their growth inhibition and killing of harmful bacteria as they possess strong oxidation strength, chemical stability and nontoxicity. During the process of photocatalytic reactions, TiO_2 NP's generate reactive oxygen species which can cause rapid inactivation of microorganism. (Anandgaonker et al., 2015)

Titanium dioxide (TiO_2) is studied as a good semiconductor and is used as biocide. Because titania is having photocatalytic property and even cells having cancer. Titania NPs have significant application in medicines and hence used as antibacterial agents and for delivery of drugs to specific target. TiO_2 has also been

considered as a biologically stable in humans and animals because of its good compatibility towards cells and less toxicity. In order to increase the colloidal behaviour of titania NPs which is because of poor solubility or stablilty in water or biological systems surface topography of TiO_2 NPs should be studied. Current findings are on modification of TiO2 NPs surface with polymeric substance. This modification can be done to minimize aggregation and sedimentation. Toxicity should be also reduces because of this process. In this regard, polyethylene glycol is used as it is hydrophilic toward water (León et al., 2017).

Titania can be doped by the addition of metal or non-metals to increase its electronic character. By using this method band gap of titania is decreased and photo response of semiconductor is altered. One of the most briefly studied method for titania modification is doping in the literature and is widely spread as compared to applications (Ruzicka, 2013).

Antimicrobial agents are those which can inhibit or kills microorganism's growth. These agents are useful in many industrial and environmental applications. The activity of TiO_2 nanoparticles (1-100) nm in diameter is interesting because of specific properties as size, shape, and structure of TiO_2 nanomaterials. TiO_2 nano-materials known as glamour of resent medical research due to its microbicide effect to different diseases-causing organisms. TiO_2 nanoparticles also have bactericidal effect on bacteria which is extremely important due to ability of pathogenic .bacteria to enter in ecosystem food chain. The antimicrobial action of TiO_2 is due to the ability to activate free hydroxyl radicals (^{-}OH) by TiO_2 particles. The antimicrobial effect of TiO_2 nanoparticles against fungi and bacteria has been determined and communicating in modern research (Mahdy, Mohammed and Kareem, 2017).

1.1-Objectives of the Study

In the current research work, titania nanoparticles were synthesized by process of calcination from algae while silver nitrate and auric chloride were used as metallic doping to form gold and silver doped TiO_2 nanoparticles. These nanosized catalysts were characterized by the procedures like as X-ray diffraction (XRD), Fourier Transform, Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), UV-Vis Spectrophotometry and Energy Dispersive Spectroscopy (EDS). Antibacterial, antibiofilm and anticancer activity of both undoped and doped TiO_2 were studied.

Aims and objectives of the work

- Algal mediated synthesis of TiO_2 and its doping with gold and silver.
- Structural Characterization**.**
- Determination of therapeutic effect of synthesized nanoparticles.

CHAPTER 2 : LITERATURE REVIEW

Nanotechnology has modified the control of issue at the nanoscale by organizing and depicting of nanomaterials. Nano-level changes the properties of the materials. Wings of the butterfly are one instance of nanostructure usually which is a result of the pith of nanoparticle valuable stone. Our life can be furthermore changed with the help of these nanomaterials. Because of the interesting properties (optical, electrical and thermodynamic) nanomaterials has been known (Wu et al., 2013). An illustration of one interesting genius is surface plasma resonation seemed through metallic nanoparticles. whereas substance of quantum confinement shows fluorescent properties in semiconductors (Stroyuk et al., 2006). Wu et al. determine in his study that nanomaterials are quite superior over electrical warm and driving properties than considerable scale assessed materials that are just because of distant attainment surface to volume degree.

The uses of nanomaterials in life are called nanobiology. Because of fluorescent properties, nanomaterials are being used as biosensors and bioimaging, by their cytotoxic effect on life forms and tumor cells; these are used as antitumor and antimicrobial administrators, in photothermal treatment because of their appealing resonation imaging. considering their alluring properties and surface plasmon resonation in alluring hyperthermia (Nath and Banerjee, 2013;Stroyuk et al., 2005)

Titanium dioxide is a semiconductor metal oxide having experimental formula TiO_2. Like other metal oxides artificially resistant, thermal untiring and hard. It has been made economically as a shading and whitener since the mid of twentieth century and its pyroclastic advance was seen through “Chalking” at the end of that century, in which it may create photodegradation of laches in paints (Chen and Mao, 2007). In forming stage Titanium Dioxide seems the most known material as a photocatalyst, and become a useful material in different fields like sensors, characteristics toxic substance remediation water part, self-cleaning surface, and water part. Titania is naturally found in three special crystalls (brookite, anatase, rutile). All these crystallines are the products of TiO_6 bound in different ways. In these three

crystalline, Rutile is the one of the most temperature. stable and is formed by TiO6 edge sharing to outline the long chain, while brookite a blend of point-sharing and edge and anatase is made of dominatingly point-sharing octahedra. The most measured stages for pyroclastic applications are rutile and anatase, it is difficult to consolidate brookite constantly (Pelaez et al., 2012).

The most widely supposed and stable class of TiO_2 is rutile polymorph, semiconductor with direct band gap of approximately of 3.0 eV which is smaller than other polymorphs of Titania nanoparticles. Because of rutile polymorph unusually extended rate of electron-opening recombination, it is not a functional .competitor of photocatalysis. Anatase TiO_2 is metastable up to 600– 1000°C and as such despite the way that it is fewer unfaltering as compare to rutile notwithstanding it sees basic habit. Anatase's having 3.2 e.V band gap energy that forces its groth limit to Ultra Violet light. Semiconductor with indirect band-gap energy is Anatase because of which it can easily joined with holes getting on the surface of particle, exceptionally constructs the lifetime of excited electron-opening sets due to light .in the material. anatase having energized electron ($E^\circ = -0.66$ eV versus SHE) are pleasingly active to escalate the reduction of H+ to hydrogen, while holes ($E^\circ = 2.54$ eV versus. SHE) can oxidize water to O_2, and what's more help in the degradation of different common toxic substances (Yamamoto et al., 2011).

In TiO_2 Nanoscale titanium dioxide the sol-gel association may be organized by various procedures, such as microwave synthesis, arrange oxidation, sol-gel, solvothermal mix, creation or physical vapor oath, electrodeposition, and micellar. The sol-gel system is considered the most notorious methodology. in recurring pattern literature (MacWan, Dave and Chaturvedi, 2011). It is a flexible technique that .offers access to nearly nothing, all around described elements even at reduced temperatures. What's more, acceptably easy to acquaint new fragments with the reaction mix, which considers the amalgamation of further created TiO_2 - based materials. Of each a regular sol-gel association, an inorganic trailblazer TiR4 is separated in water or a mix of solvents. The direct hydrolyzation of trailblazer gives Ti(OH) xR4-x, that after encounters .advance through any extension or substitution reactions to form titanium dioxide. Whereas preliminary examinations of TiO2 connotation would all in all use titanium tetrachloride ($TiCl_4$) as a precursor (Xu, 2014).

In hydrolysis, this comprehended the course of action of chloride, which may provoke the contaminating effects in the product. The usage of titanium alkoxides as criers has engrossed in current strategies. These engineered mixtures give high-perfection oxides, because of rapid react, decently sensible and pH-objective. In seeing water both salt (halide and alkoxide) of Titanium hydrolyze rapidly. This can be performed without size, power over shape and crystallinity. of the entire product. The most notable part is the usage of acids as peptizing administrator, especially the properties of TiO_2 can be affected by its destructive usage. There has also been a significant examination .into the nonhydrolytic amalgamation of TiO_2. The rate of the TiO_2 plan can be tumble-down unquestionably, considering undeniably unmistakable control over the product by. replacing standard hydrolysis-development instrument with an elective reaction way (Mutin and Vioux, 2009).

Doping is the demonstration of including little measures of outside parts of a semiconductor with the true .objective to change its electronic property, by making slight gatherings (mid-opening states) in the band-gap. By the help of this technique, it is possible to modify semiconductor's photo response. Among the most comprehensive examined technique doping is the most acceptable by which TiO_2 has changed. in the composition, because of its application in the current photocatalyst portrayed as inevitable its preparation is infinite (Teoh, Scott and Amal, 2012).

Using different techniques such as magnetron sputtering, hardening in a gas including molecule implantation and sol-gel change Titanium dioxide material may be doped. Doping can occur in. either interstitial or substitutional structure. In the interstitial structure, it is arranged between existing network particles, while in substitutional structure the dopant particle replaces a cross segment atom. Introduction of doping may construct the photoresponse of the nanomaterial, it in like manner presents mass deformations, i.e. variations in the valuable stone structure enable electron-opening recombination. Studies showed that photoactivity rises with doping obsession to a point where activity lessens because of superfluous exciton recombination. TiO2 doping segments may be widely confined into three social events (cationic doping, anionic doping, and multi-part doping). Also, by methods for the age of anionic opening inside the TiO2 cross segment self-doping can occur (Joung et al., 2006;Wei et al., 2017).

To improve photoresponse of TiO_2 Cationic dopant is used to improve the photoresponse of TiO_2 Cationic doping has been broadly used. in undertakings., with continuous reports focusing on the usage of alkali, transitions, post-transition, and fair metals, and metalloids, carbon61 (as C^{4+}), sulfur62 (as S^{6+}), and exceptional earth. Result simply does not depend on kind and proportion of doping element, however also on the production for dopant and on the notice response. Some structures are fabulous, and the properties of dopant show by itself. That must be considered with the ultimate objective to manufacture a thorough copy of the Dopant system. Examples include Oropesa et al 2003 found that TiO_2 surface. doping with Sn(IV) examined a little expansion of opening band gap, upon decline to Sn(II) the dopant's electronic nature moved to give mid-band states lying imperceptibly over the TiO_2 valence band, achieving unquestionable light action. Not withstanding the sweeping grouping of effort on cationic dopant of TiO2. The general nonattendance of achievement in working up an unfaltering, discernible light unique TiO_2 photocatalyst as cation dopant main amid the 2000s to the examination of anionic and co-dopant TiO_2. Anionic dopant in 2001, Asahi et al. appropriated they give a record of the doping of TiO_2 using nitrogen 68. The focused previous examination on metal as dopants was an incredible departure (Ruzicka, 2013).

Asahi investigated the hypothetical and investigative congruity of nitrogen, carbon, sulphur, phosphorus as dopants basically, however past examinations took focused on substituting. Ti territories for doping, this examination rather observed O center. According to makers nitrogen was the best dopant and ranging the traceable light activity of TiO_2. The empty space left by an empty oxygen center can be easily included by phorus. The delivered mid-opening space by doping would in truth happen moreover distant into the band gap which makes them exciton recombination centers. Fluorine exhibited no penchant to incorporate mid-gap states in speculative figuring. Nitrogen-doped TiO_2 was much unique at debasing methylene blue than undoped in TiO_2 examined test examinations. This development depended on substitutionally-doped not on interstitially doped, shown by further work. In this paper, there has too much interest in the anionic-doping, however engrossed a lot of research. on the dopant nitrogen. There has moreover been excitement for other anionic dopants, comprising phosphorus, chlorine, iodine, fluorine, and sulphur.

While a bit of this examination has viably made materials with a development obvious light response, this is every now and again due to roaming impacts (e.g. oxygen openings caused by phosphorus doping, which themselves limited the. band gap). It is fascinating to perceive that nitrogen-doping of rutile TiO_2 obtained from either no variation or even a little expanding the band gap, regardless of what is seen by anatase. It is known, that rutile practices. a more clarified associate change following substitution doping of oxygen for nitrogen, which in this manner drops the most astounding purpose of the valence band edge. In rutile any delay in photo absorption because of nitrogen doping balance the change.in the band opening in the interpretation of rearrangement (Ruzicka, 2013).

The generating microbial limit opponent metal particles result in stream excitement for the expert, against to contamination machinists and the TiO_2 NPs have demonstrated colossal antibacterial activity the headway of safe strains Factory administrator Et al avowed that when TiO_2 is introduced to impressive radiation.it generates responsive oxygen species. In antibacterial coating .nanoparticulate TiO_2 is used and it has been examined that wastewater disinfection.is an adversary of threatening development expert. The biocidal polymer-functionalized TiO_2 NPs showed improved restriction of bacterial advancement against *E. coli* and *S. aureus* conversely with the unsullied TiO_2 NPs. Zhang and Chen exposed that minor Ag gather quantity and the exceptional structure of TiO_2 NPs.supporting extremely dispersed to be the sources of transcendent bactericidal execution of the room temperature ionic liquids decided Ag/TiO_2 (Chen and Mao, 2007). Through layer-by-layer assembly, a multilayered, multifunctional film containing TiO_2 NPs as contact dynamic antibacterial administrator and Nanosilver as a release dynamic antibacterial authority was produced. Marciano et al examined that the bactericidal activity of valuable stone like carbon films containing TiO_2 NPs and its action by oxidative mischief to the microorganism divider. In the engineered coordinated effort between E. coli and the motion pictures a reduction.in the interfacial essentialness of minute life forms bond results in an extension, it is an extra factor for budding .bactericidal development. Rajakumar et al declared that the biosynthesis of TiO_2 NPs was refined using *Aspergillus flavus* evacuate as a reducing and garnish administrator which ended up being a good antibacterial material against E. coli (Rai et al., 2012). Hassan

et al conferred the antibacterial development, joint effort segment against *S. aureus, E. coli, Sal. typhimurium* and *Kleb pneumoniae* association and depiction of Titania nanorods. by sol-gel electrospinning technique (Diebold, 2016). Cell support plays a vital role in consummation the oxidative rancidity in sustenance via looking through the free extraordinary which is .formed in the middle of the oxidation process. Period of free radicals or responsive oxygen species during integration and assorted actions past the cell fortification limit of a natural system offers climb to oxidative stress. In neurodegenerative afflictions, heart diseases, developing process and in the dangerous development oxidative weight plays a vigorous role. In heart infections, neurodegenerative ailments harm and in the developing process oxidative weight admit an occupation.

CHAPTER 3 : MATERIALS AND METHODS

The study was conducted in Atta-ur-Rahman School of Applied Biosciences (ASAB) and the analytical analysis of the samples were checked at US Pakistan centre for advance study and energy (USPCASE), School of Chemical and Materials Engineering (SCME) and school of natural sciences (SNS) at National University of Sciences and Technology (NUST), Islamabad, to synthesize titanium dioxide nanoparticles (Phycosynthesis) using algal biomass filtrate of Dictyosphaerium sp. Strain HM1 (DHM1), as reactant and titanium tetra isoproxide (TTIP) as substrate. This green synthesis procedure was opted to produce biologically active and nano sized Titanium dioxide nanoparticles by a reaction carried out using Dictyosphaerium sp. Strain HM1 (DHM1). The detail of materials used and analytical methods employed during this study is given below.

3.1 Phycosynthesis of Titanium Dioxide Nanoparticles

In 50mL ethanol, One gram of the dried algal Biomass was mixed and reflux was done for extraction at 50°C temperature for five hours. After 5 hrs the extract was filtered by using Whatmann filter paper. This extract is stored at 4°C or used directly for titania nanoparticle synthesis. In next step the Erlenmeyer flask having 0.4M of (TTIP) titanium tetraisoproxide (Aldrich-205273) and 50 ml of algal extract reaction was carried out under stirring at 50°C for approximately four hrs the formed titania nanoparticles were obtained by centrifugation (10000 rpm for 15 minutes). This step is followed by washing of these nanoparticles by using ethanol. After this the washed nanoparticles were dried in muffle furnace at 500°C (3 hrs). The calcinated titania nanoparticles were than further investigated by using different techniques.

3.2 Optimization of physiochemical parameters

Optimization of different physiochemical parameters involved in production of TiO2 was studied prior to complete characterization of TiO_2. Different reaction mixtures were set up before analysis by UV/Vis spectrophotometer (UVD-2950) for conformation of synthesized TiO_2. Wavelength region 300-600 nm was opted as a function for TiO_2 having sampling interval of 1nm for each reaction mixture. Ethanol

was used as a blank for base line correction of spectrophotometer. Spectral analysis of UV/Vis adsorption in each reaction mixture was recorded by software attached with the instrument and numerical data was plotted on graphs to compare spectral data of different samples.

Effects on size of TiO_2 nanoparticles were studied at various temperatures of 25, 50, and 75 °C. The Size distribution of Titanium dioxide nanoparticles was tuned by UV-Vis Spectrophotometer (UVD-4950). Different molar concentrations of TTIP used were 0.2mM, 0.4mM, and 0.8mM to check the effect on the rate of synthesis and size distribution. Effect of plant extract concentration on size of nanoparticles and its rate of synthesis was also checked by dissolving 30ml, 50ml and 70ml of extract in 6.67ml TTIP. Reaction with TTIP (0.4M) was set in concentrations of 6.67ml, 4.67ml, 8.67ml TTIP with 50ml plant extract. Different calcination temperatures (523°C, 773°C, 823°C and 973°C) were carried out to study different phase of titanium dioxide nanoparticles i.e. rutile and anatase by using X ray diffraction.

3.3 Characterization of Titanium Dioxide Nanoparticles

3.3.1 UV Spectrophotometry

Titanium Dioxide production was initially confirmed using UV-Vis Spectrophotometry. $3/4^{th}$ of cuvette was filled with nanotitania solution and was loaded in spectrophotometer (UVD-4950) where the nanotitania absorbed photons of particular wavelength dependent upon the size of particle distribution and the absorption spectra was recorded between 300-800 nm wavelengths. As a reference Ethanol was run before recording the SPR spectra of nanotitania solution.

3.3.2 X-Ray Crystallography (XRD)

The dried titania nanoparticles were coated on a XRD grid and then their spectra were evaluated and recorded using X-ray generation device, model no. IPDS II (STOE, Germany) was run at a voltage rating of 40kV and a constant current rating of 30 mA. This was used to generate Cu Kα1 radiation, the radiation produced was used to evaluate the spectra. Software WinXPOW connected to XRD instrument was used for generation and analysis of XRD peaks for TiO_2 samples.

3.3.4 Scanning Electron Microscopy (SEM)

Detailed information about surface topography and morphological analysis SEM is an efficient technique where scanning of electron beams was focused on sample. For SEM small amount of TiO_2 nanoparticle powder was added in 1000 ml of DI water and glass vial was placed in ultrasonicator (Cole-Parmer) for 1 hour to break the agglomerated Titania nanoparticles and to evenly distribute them throughout the dispersion. A drop of water from micropipette was then added on 1cm×1cm slide, and was dried under lamp. This was followed by sample coating with gold to make the surface conductive by sputter coater. After covering Specimen was positioned on stubs using conductive tape imaging was done at 20 keV at the amplification of 75,000X by Scanning electron microscope (Jeol JSM-6490LA).

3.3.5 Energy dispersive spectroscopy (EDS)

The presence of elemental phases was revealed through Energy Dispersive X-Ray attached with the SEM. The EDs spectra of TiO_2 NPs were performed by the SEM (JSM-6490LA- JEOL) machine. 20.0 k was set as accelerating voltage with 1 mA probe current. T3 PHA mode was chosen with 57.65 seconds real time and live time of 50.00 seconds for the analyzer 3334 counts was counting rate.

3.3.6 Fourier Transform Infrared Spectroscopy (FTIR)

This study utilized the FTIR spectrometer for scanning the samples for functional groups present in the wavelength region 450- 4000 cm^{-1}. Together algal extract and nanoparticle samples were analyzed for reduction of functional groups of algal extract and presence of functional groups on the exterior of nanoparticles. It is important to remove water from the samples before loading, so KBr was used for this purpose due to its hygroscopic properties. Hydraulic press was used to prepare the pellet of KBr and 2-3 drops of samples (algal cell filtrate and TiO_2 NPs) were added separately. IR waves passed through the sample to detect functional moieties on the exterior of titania nanoparticles. The transmission spectra were recorded and interpreted to identify the bond stretching in functional groups.

3.4 Doping of TiO_2 Nanoparticles with metal ion (Ag-TiO_2 and Au TiO_2)

Noble metals like Ag and Au possess unique electronic and catalytic properties. Silver is also known for its high antimicrobial activity in both metallic as well as ionic form(Hamal and Klabunde, 2007)(Zhang and Chen, 2009)(Rai *et al.*, 2012). The Ag-TiO2 and Au TiO2 were synthesized by adding 1 g TiO_2 to 100 ml DI water in a silver and gold doping 1% molar ratio of AgNO3 and $Aucl_3$ was also Addedto DI waterthe mixture was then stiired and allowed to settle down in beaker at dark for 24 hrs. The obtained slurry was then dried at 100°C for 24 hours in an oven. The obtained powder was calcined at 500°C for 3 hours in a muffle furnace. This resulted nano sized particles of silver-doped TiO_2 and gold doped TiO_2, herein after referred to as Ag-TiO_2 and Au-TiO_2. Newly synthesized Ag- TiO_2 and Au-TiO_2 were illustrated with UV-Vis Spectrophotometry, X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Energy dispersive X-Ray spectroscopy (EDS).

3.5 Modification of TiO_2 with PEG

In order to stop accumulation and sedimentation TiO2 NPs need to be modified. One of the most well-known methods is covering nanoparticles with polymer. In this work for modification of nanoparticles PEG 6000 (Sigma Aldrich) was used. In 200 ml beaker 100 ml of DI water is added after this 1 g of Tio2 is added and sonicated for 2 hrs then 200 mg of PEG was added and sonicated for 3hr. PEG–TiO_2 NPs were centrifuged at 5000 rpm for 30 min at 25 °C. PEG–TiO2 NPs after modification were dried at 60 °C for 1 h.

3.6 Antibacterial activity of synthesized TiO_2, Ag- TiO_2, Au- TiO_2

3.6.1 Culture Collection

The antimicrobial activity of synthesized TiO_2, TiO_2 Control, Ag- TiO_2 and Au- TiO_2 was examined against Gram+ve *Listeria monocytogenes* and Gram–ve *Pseudomonas aeruginosa, Escherichia coli* and *salmonella* bacterial cultures (ATCC strains). The microbial cultures were procured from UG Industrial (Dr abdurehman) Biotechnology lab ASAB, NUST.

3.6.2 Preparation of inoculum

Overnight bacterial cultures at 37°C from stock were inoculated on nutrient broth. Then they are transferred to nutrient agar to obtain isolated colony. Healthy cultures were used for antibacterial assay after suitable growth.

3.6.3 Agar well diffusion approach

The antimicrobial action of synthesized TiO_2, Ag- TiO_2, and Au- TiO_2 were confirmed using agar well diffusion approach. Overnight old bacterial culture on NA plates was used and one colony from each culture is taken and poured in 600ul normal saline. After this, each of the bacterial colony in saline is vortex and uniformly spreaded with the spreader on muller hington agar. Three wells of 10mm diameter each were made on using sterilized gel puncher/borer. Each well contains 80ul of synthesized Nanoparticles of different dilution i.e 1mg/ml, 10mg/ml and 20 mg/ml respectively. The test samples were placed in incubator at 37±°C for 24 hr subsequently the inhibitions zones was measured. Diameters of inhibition zone formed in all the three replicates were measured in mm using measuring scale. Gentamycin and D.I water was used as control.

3.8 In Vitro Cell Cytotoxic Assay:

For invitro cytotoxic assessment, Sulforhodamine B (SRB) proliferation assay was done. The mechanism is based on conjugation of protein dye Sulforhodamine B with amino acid residues of cellular proteins. Protein dye binds to the cellular protein under minor acidic environment and can be obtained from the cells or solubilize under minor basic environment for measurements (Stahl and Voigt, 2004).

3.8.1 Cell Lines

Cells from HCT 116 human colorectal cancer cells lines were seeded at optimized condition densities in 96-well plates and incubated for 24hrs at 37°C with 5% CO2 in a humidified incubator.

3.8.2 Sulforhodamine B (SRB) Assay

The inhibition of cell propagation is studied by using SRB proliferation assay. All cancerious cell lines (HCT 116 human colorectal cancer cells lines and triple breast cell lines) used were retained in their respective recommended culture media

(DMEM or RPMI) complemented with 10% FBS and 1 × Antibiotic-Antimycotic, in humidified incubators with 5% CO2 at 37 °C. All the cell lines used were passaged for less than 6 months before being substituted from initial passage frozen supplies.

All four nanoparticle samples, TiO2, AuTiO2, AgTiO2 and TiO2-Control, were prepared in autoclaved Milli-Q water and sonicated before treatment. HCT116 colorectal cells (seeding density 1500 cells/well) were seeded in culture plates 96-well. in DMEM media. Next day, cells were treated with 3-fold dilutions of all four samples starting from 5µM in triplicates. These plates were again incubated at 37^{o}C with 5% CO2. After 72-hr incubation, the cells were fixed with 10% trichloroacetic acid (TCA) for at least 2 hours, washed with water, dried and then stained with 0.06% SRB dye for 30 minutes. The plates were then washed again with 1% acetic acid, dried and 100µl of 10mM Tris pH 10.5 was supplemented per well using a Multidrop Dispenser, to solubilize the bound SRB. Plate reader was used to measure the optical density was measured by using platereader at 490nm a wavelength and the analysis was performed for measuring the GI50 using GraphPad PRISM software.

3.9 Western Blot Analysis for p53 and apoptosis induction

CAL-51 cells were seeded in 6-well culture plate in DMEM media added with FBS 10% and 1 × Antibiotic-Antimycotic (seeding density 0.5 million cells per well) and incubated overnight at 37^{o}C and 5% $CO_{2.}$. $AuTiO_2$ NPs were then added to the cells at 0.25, 0.5, 1.0 and 2.5µg/ml concentrations with autoclaved Milli-Q water as a negative control, for 24 hours. Following incubation, the cells from each respective well were collected and lysed in Triton X100-based lysis buffer (50 mmol/L NaCl, 25 mmol/L Tris-HCl, pH 7.5, 1% Triton X-100 supplemented with phosphatase and protease inhibitors). Bradford's Assay was carried out on the resulting supernatant to equilibrate the total protein concentrations of the samples before running in 12% SDS-PAGE gel for electrophoresis. Proteins were subsequently transferred onto the nitrocellulose membrane and then blocked with 5% skimmed milk and placed in incubator at 4^{o}C for 24 hrs with primary antibodies; anti-mouse p53 (Santa Cruz Biotechnology, #SC-126) at 1:1000 dilution, anti-rabbit cleaved PARP (Cell Signalling Technology, #5625) at 1:500 dilution and anti-mouse GAPDH (MerckMillipore, AB2302) at 1:1000 dilution. Following washing with PBST (PBS + 0.1% Tween), one-hour incubation with

secondary antibodies was carried using 1:1000 dilutions of Rabbit Anti-Mouse IgG H&L (Abcam, ab6728) and Goat Anti-Rabbit IgG H&L (Abcam, ab6721), respectively. The blots were then developed using ECL™ Prime Western Blotting Kit (GE Healthcare, Sigma-Aldrich) and imaging was performed with ChemiDoc™ Imaging Systems (Bio-Rad Laboratories).

3.10 Cell Cycle analysis using Flow Cytometry

Cells CAL-51 were seeded in 6-well plates in DMEM media added with 10% FBS and 1 × Antibiotic-Antimycotic (0.5 million cells per well) and incubated at 37^{o}C and 5% CO_2. Next day, $AuTiO_2$ NPs were added to the cells at 0.25, 0.5 and 1.0 µg/ml concentrations for 24 hours, with autoclaved Milli-Q water .as a solvent control. The cells from each respective well were trypsinized and collected in 15 ml falcon tubes, which were then centrifuged (3000rm, 5mins) and washed with IX PBS+1%FBS, followed by 1-hour incubation and fixation with ice-cold 75% ethanol. After washing cells were pelleted and resuspended in 1ml 1X PBS+1%FBS containing Propidium Iodide/ RNase H solution (10 µg/ml PI/0.5% RNase) for 1 hour at 37 ^{o}C, and analyzed using BD FACS Calibur.

CHAPTER 4 : RESULTS

4.1. Absorption spectroscopy of synthesized algal mediated TiO_2 nanoparticles

UV-Vis spectroscopy was used for indication of size distribution and shape of Titania nanoparticles. Single peak with maximum absorbance at 450-490 nm indicates the uniform shape of Titania nanoparticles as shown in figure 4.1. Size distribution of Nanoparticles was tuned by optimization of conditions during synthesis, different parameters of temperature, molar concentration, concentration of Algal extract, were chosen, for the optimization of size distribution of titania nanoparticles.

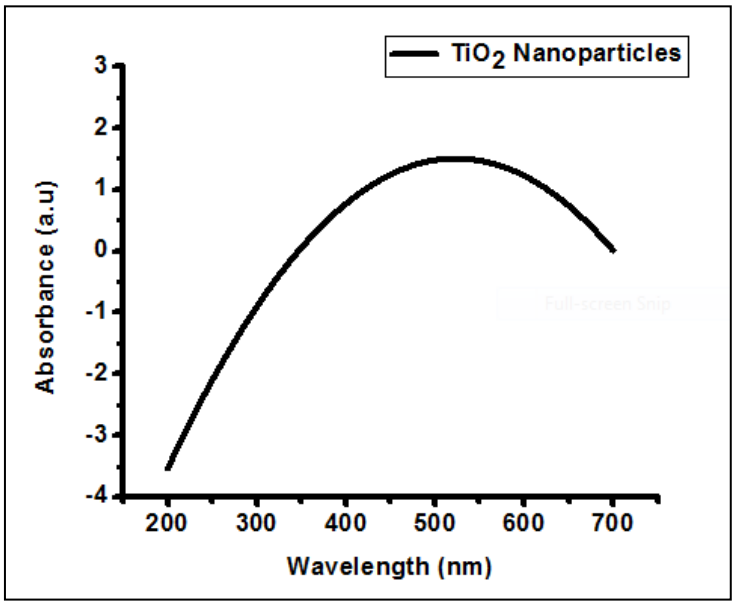


Figure 4.1: Characteristic Peak for indication of Titania nanoparticle synthesis

Maximum absorption at 450 nm indicates the synthesis of Titania nanoparticles.

4.2. Optimized physiochemical parameters for biosynthesis reaction

Different reaction mixtures were set up in different conditions like temperature, pH, molar concentration of TTIP, concentration of algal extract in order to evaluate the optimum condition for bio- synthesis reaction to produce TiO_2 NP's before analysis by UV/Vis spectrophotometry.

4.2.1. Effect of Temperature on reaction

Temperature variation play vital role in every catalytic reaction and biosynthesis of TiO_2 NPs by reduction with TTIP also require high temperature for their production in maximum number. At 25°C the rate of synthesis of TiO_2 NPs was comparatively low while. The rate of synthesis was highest at 50°C but the size distribution was also increased which was evident from broad absorption spectrum (Shown in pink line). Small Peak at region of Absorbance value of reaction mixture at different temperature indicates at optimal temperature for synthesis of TiO2 using algal extract is around 50 C as indicated in figure4.2.

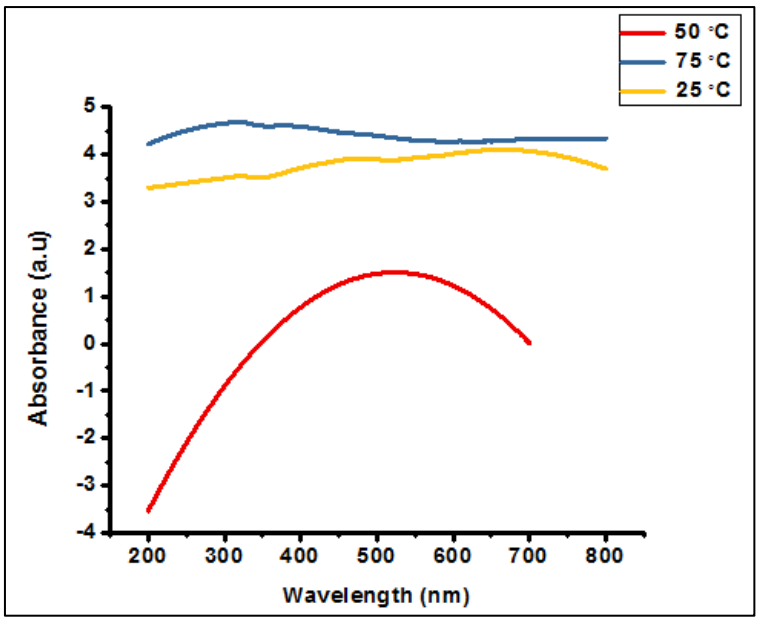


Figure 4.2: Temperature effect on the rate of synthesis of Titania NPs

Absorbance spectra of TiO_2 NPs Temperatures of 25°C, 50°C and 75°C showing the formation kinetics of Titania nanoparticles. 50°C is indicated as optimum temperature for synthesis of Titania Nanoparticles.

4.2.2. Effect of Reaction Time

Biosynthesis reaction for producing TiO_2 was monitored for 8hrs in term of change in absorbance Value of reaction mixture at 490 nm. After every one hour of interval UV-Vis analysis of reaction mixture is done in order to find the complete formation of titania nanoparticles. Maximum absorbance was recorded by sample reacted for 4 hrs and the absorbance value increases with further increase in the

heating time. Hence for complete formation of TiO_2 nanoparticles it is found that 4hr is the most effective time.

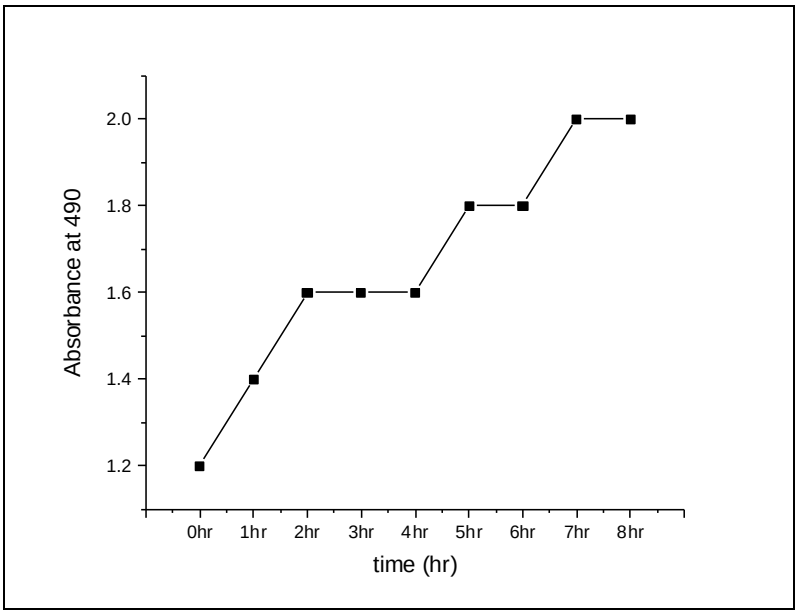


Figure 4.3: Reaction time effect on the rate of synthesis of Titania Nanoparticles

Comparison of absorbance values of reaction mixture at different time gaps (0hr, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr, 7hr, and 8hr) at 450 nm

4.2.3. Effect of Molar Concentration

Effect of molar concentrations was also studied by tuning the molar concentration of TTIP used in the reaction mixture. O.4 M TTIP showed optimum SPR spectra as shown in figure 4.4 as the peak shifted towards lower wavelength compared to high molar concentration. High concentration of TTIP showed broad size distribution of Nanoparticles as evident from peak shift towards higher wavelength. While molar concentration of 0.2M not only increased the rate of synthesis but also synthesize bigger sized nanoparticles.

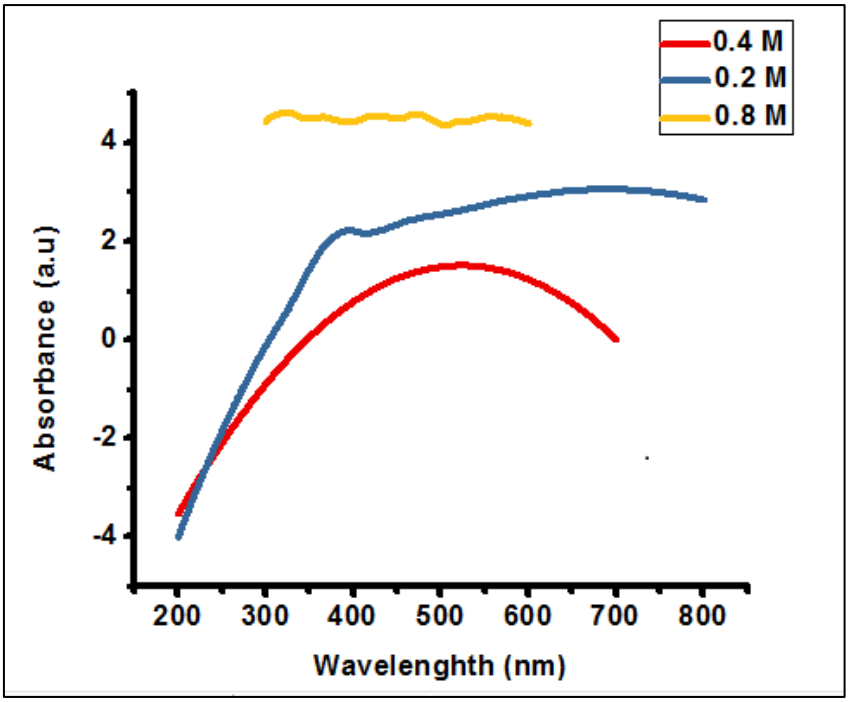


Figure 4.4: Effect of molar concentration of TTIP on formation kinetics

Graph shows the best optimised concentration for narrow size distribution is 0.4 M which is shown by spectrum shift towards lower wavelengths.

4.2.4. Effect of calcination Temperature on crystalline structure of TiO_2

The X- ray Diffration patterns of the samples. (Fig. 4.5) indicate that the found samples were nanocomposites of TiO_2 composed of anatase crystalline form and rutile crystalline nanoparticles. Figure 4.2.6 clearly shows the existence of tetragonal titania crystal, i.e., rutile (JCPDS No. 21-1276) and anatase (JCPDS No. 21-1272). The presence of rutile nanocomposites was confirmed by the presence of (110) diffraction peak detected at 2 theta of 27.4° in the XRD configuration and (101) peak located at 2 theta of 25.3°. Anatase peak of TiO_2 started to appear at 400°C and increased up to 700°C. Rutile phase began to appear at calcination temperature of 700°C at 900°C there is no anatase crystalline form. It was observed that anatase phase peak increase with increase in temperature and was totally disappeared and converted to rutile phase at 97°C. These peaks were constant with a previously reported study of synthesized titania nanoparticles by common sol-gel method. Transition of these phases changed at around 600°C or higher temperature. In conclusion crystallite size increase as calcination temperature increases showing significant increase between 700 and

800°C temperature range. Anatase crystallite size of the samples calcined at 700 and 800°C was determined to be 28.5 and 58.9 nm, respectively. These outcomes showed that phase transition and crystalline form is because of calcination temperature as compared to molar ratio of extract and TTIP).

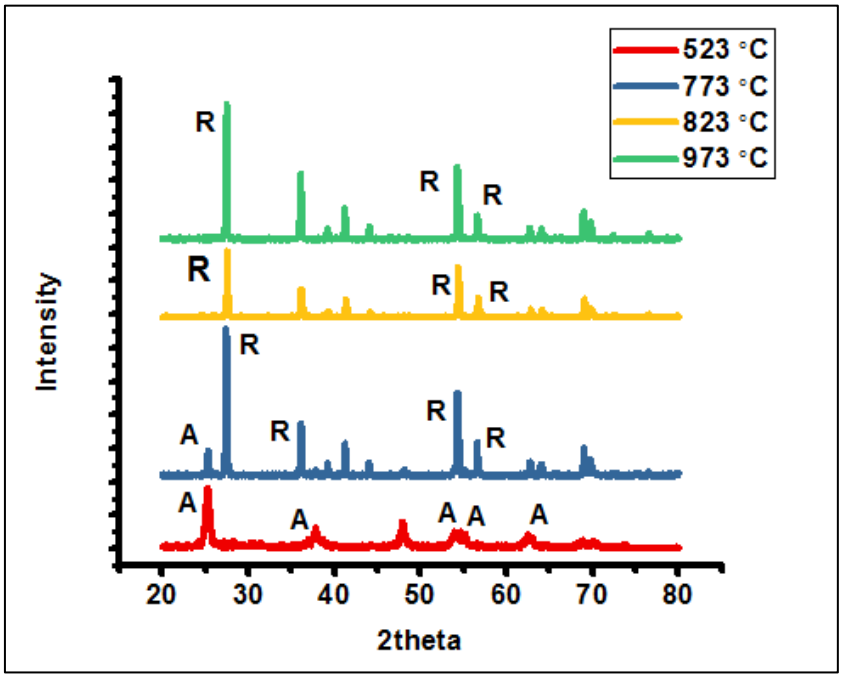


Figure 4.5: TiO2 nanoparticles synthesized with various calcination temperatures

XRD pattern represent anatase and rutile (A and R), respectively.

4.3. Characterization of TiO_2 Nanoparticles

4.3.1. Fourier Transform Infrared Spectroscopy

Investigation of functional groups and possible capping structure can be performed using Fourier Transform Infrared Spectroscopy; the possible reducing agent was identified by observing the band alteration from 598 cm^{-1} to 853 cm^{-1}, which indicates conversion of C-Br band into unsaturated band reducing TTIP to TiO_2. Extract shows peaks at position 3383 cm^{-1} (O–H stretching), 2913 cm^{-1} (C–H broadening), 1667 cm^{-1} (C=C broadening), 1451 cm^{-1} (C–O broadening) and 1056 cm^{-1} (C–O broadening).

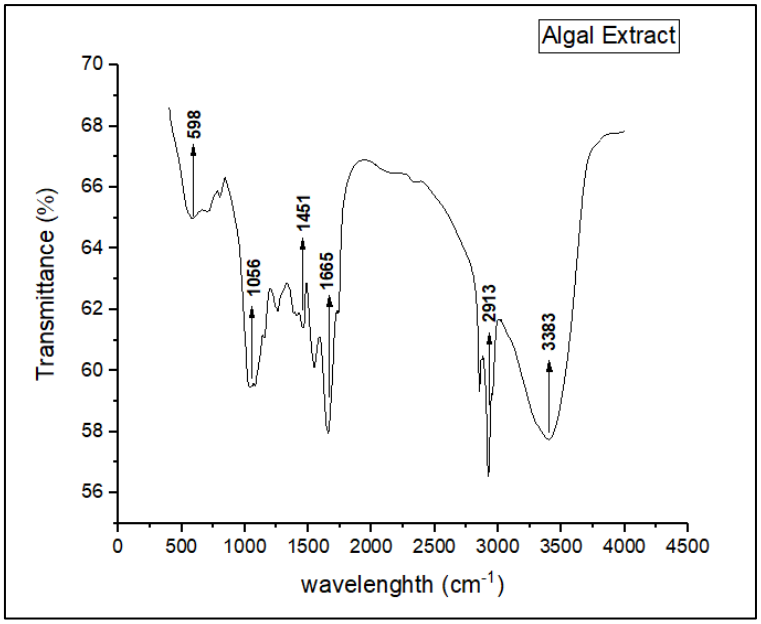


Figure 4.6: FTIR spectra of aqueous dispersion algal extract without any interaction with titanium tetra isoproxide.

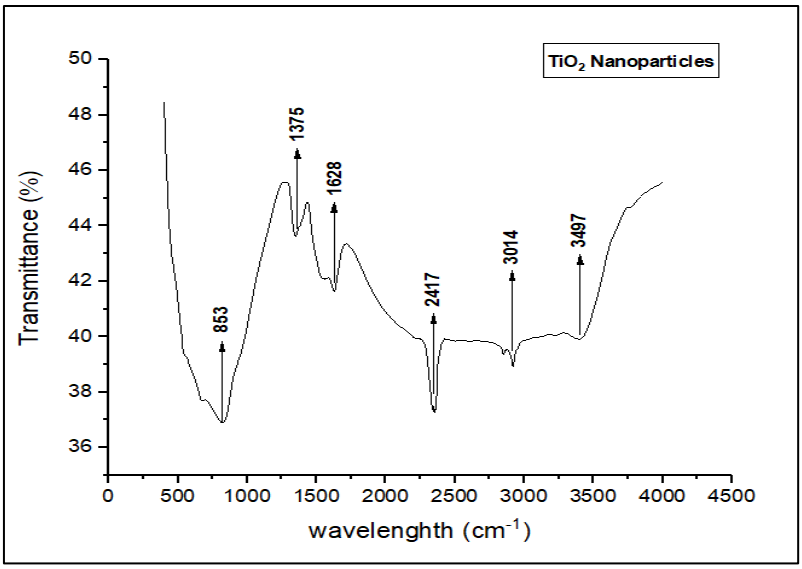


Figure 4.7: FTIR spectra of reaction mixture containing TiO2 nanoparticles synthesized from Algal extracts and titanium tetra isoproxide

4.3.2. X-Ray diffraction

In order to investigate the size of the nanoparticles and phase structure, XRD analysis was carried out. The XRD analysis results showed 2θ peaks at 25.08, 37.96, 48.02, and 54.8° that correspond to the anatase phase of TiO2 as per ICDD powder

diffraction data card no. 21-1272 (Peng et. al. 2005; Zhang et al. 2008). XRD of biologically synthesized TiO_2 show intense Bragg peaks at (101), (200) and (213) in the required range and agreed with the values that are reported for TiO2 nanoparticle, thereby confirming the purity and crystallinity of stable TiO_2.

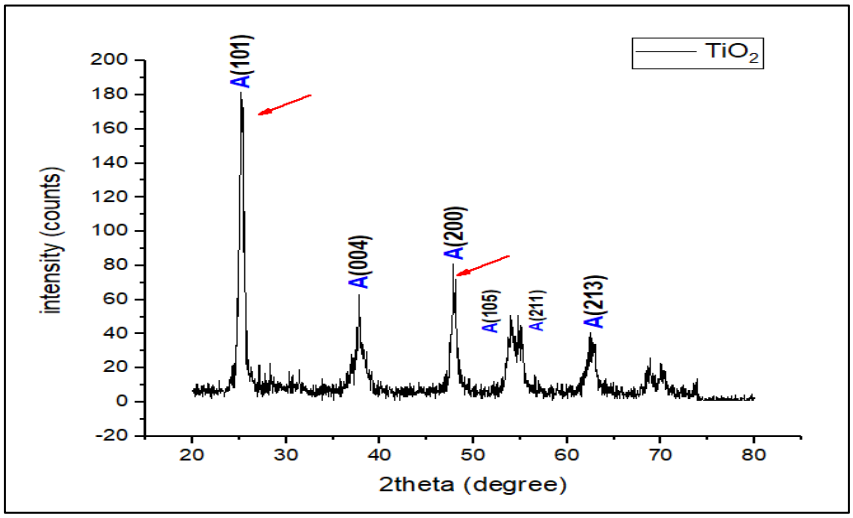


Figure 4.8: X-RD pattern of purified TiO2 synthesized from algal extract.

4.3.3. Scanning Electron Microscopy

TiO_2 Nanoparticles were illustrated for their size and morphology through SEM microscopy. Images taken at magnification of 75,000 X confirmed their uniform morphology an average size of 30 nm to 45 nm was observed.

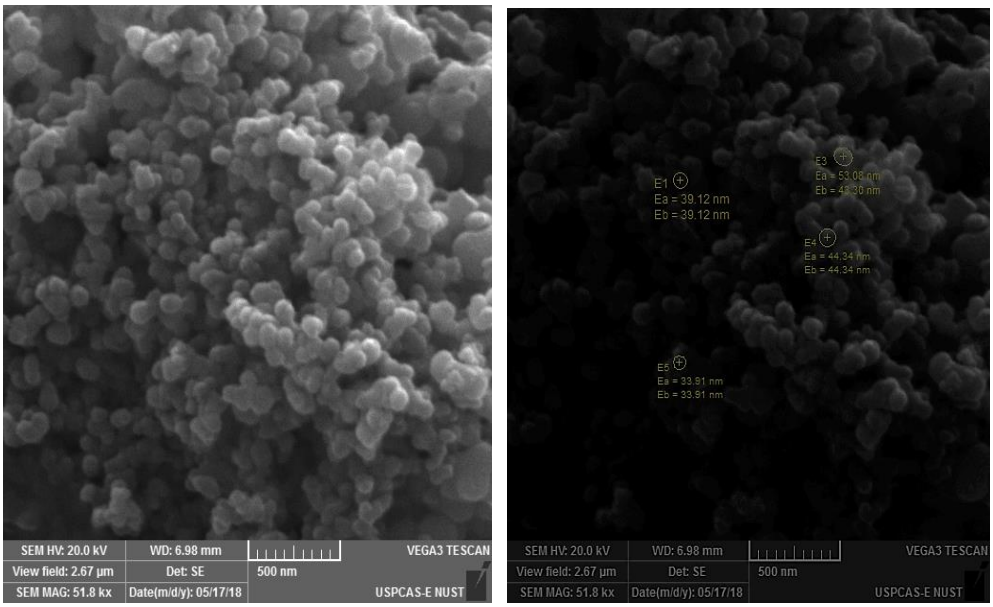
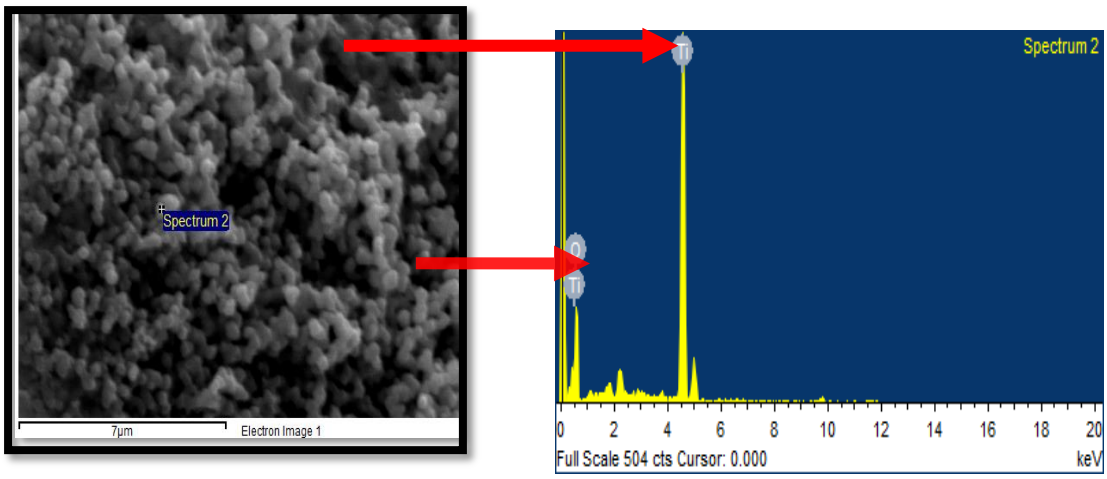


Figure 4.9: Scanning Electron Micrograph of Titania Nanoparticles.

This figure represents size range and particle size distribution of Titania Nanoparticles. SEM images show evenly distributed Nanoparticles with spherical morphology and an average size of 39 nm.

4.3.4. Energy Dispersive X-Ray Spectroscopy

Elemental analysis of SEM images was accomplished in order to check the composition of Nanoparticles. The EDS shows two peaks, which reveals presence of Titanium and Oxygen at 0.5 keV and 4.5 keV respectively. The atomic percentage was 48.70 and 52.29 of Ti and O respectively. The present arrangement of Ti and O indicates the formation of non-stoichiometry within detection limit of EDS No any other impurities could be seen.



Element	Weight %	Atomic %
O k	61.96	82.98
Ti k	38.04	17.02
Totals	100	

Figure 4.10: EDX analysis of TiO2 Nanoparticles Element titanium and oxygen confirms the presence of TiO2 Nanoparticles

4.4. Characterization of silver and gold doped TiO2 (Ag- TiO2 & Au- TiO_2)

4.4.1. UV-VIS Absorption Spectroscopy of Ag- TiO2 & Au- TiO_2

The synthesized doped Nanoparticles were illustrated by UV-Vis spectrrophotometry which revealed that Ag doped TiO_2 & Au doped TiO_2 possess strong absorption spectra as shown in (figure 4.11 and 4.12) peak at 430 showed the formation of Ag-TiO_2 nanoparticles while Single peak with maximum absorbance at 390nm indicates the uniform shape of Au- TiO_2 nanoparticles

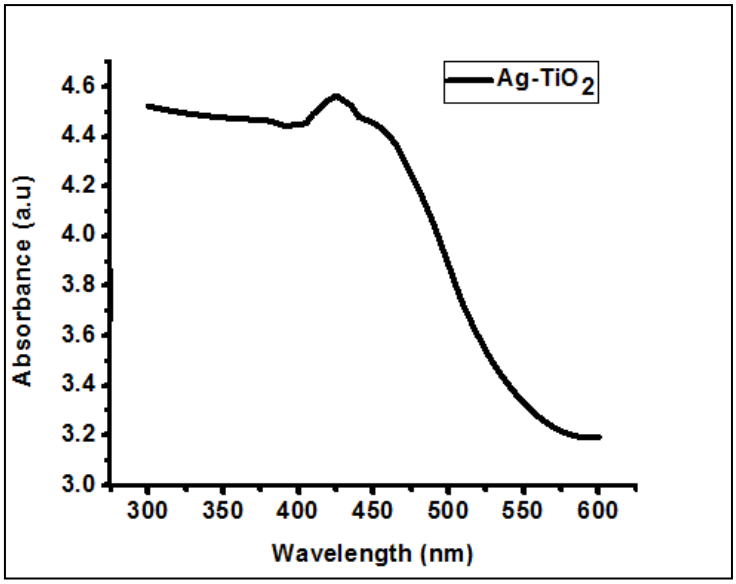


Figure 4.11: UV spectra of absorption of Ag doped TiO2

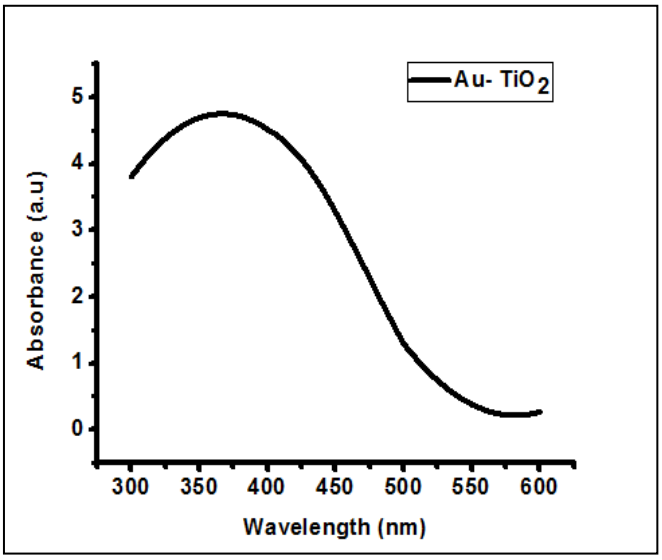


Figure 4.12: UV spectra of absorption of Au- TiO2

4.4.2. X-Ray crystallography of Ag- TiO_2 & Au- TiO_2

Figure 4.4.2 (a) and (b) shows X-ray crystallography patterns of crystalline Ag doped TiO_2 & Au doped TiO_2. Crystallography peaks coordinated well with the TiO_2 anatase phase (JCPDS Card No: No.89-4921). After the incorporation of Au and Ag onto the TiO_2 no other impurity peaks were observed, which indicates that integration of gold and silver does not transform the crystalline structure of the TiO_2. However peak intensities of gold and silver doped TiO_2 is less when we related it with the TiO2

pure sample. From results it is observed that there are no other peaks or impurities of other crystalline phases of titania nanoparticles in the sample.

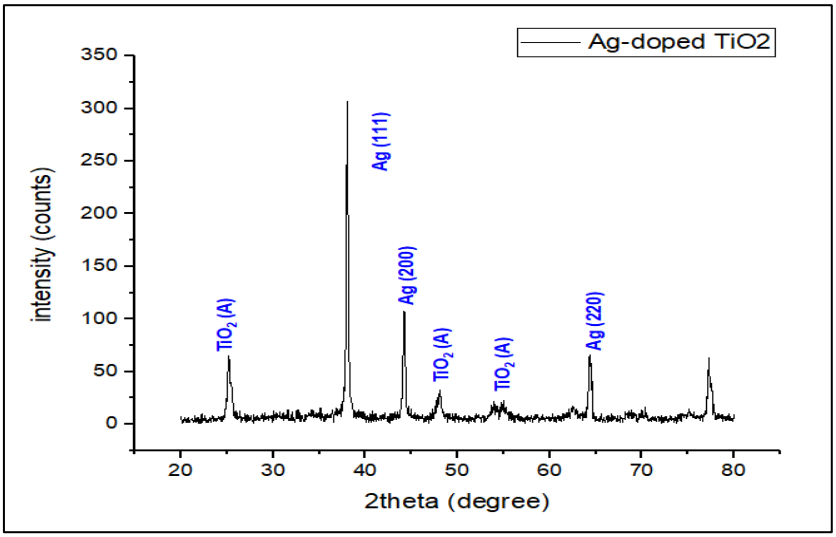


Figure 4.13: X-Ray crystallography pattern of Ag -TiO2

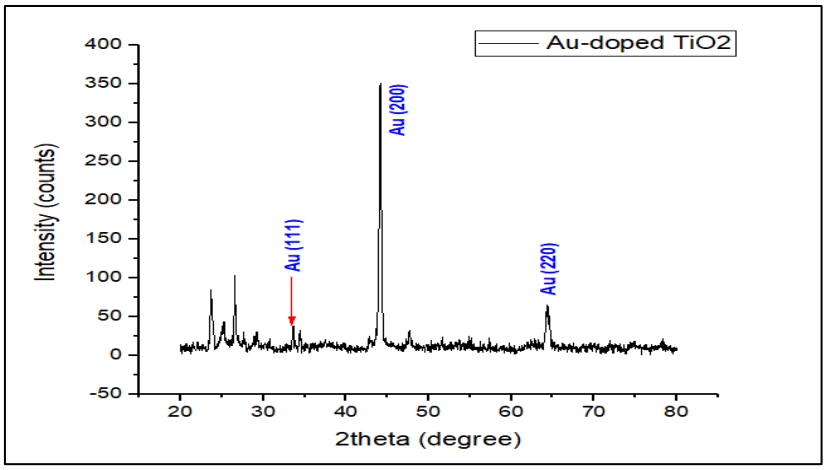


Figure 4.14: X-Ray crystallography pattern of Au doped TiO2

4.4.3. Fourier Transform Infrared Spectroscopy of Ag- TiO_2 & Au- TiO_2

For gold and silver doped TiO_2 nanoparticles FT-IR spectroscopy analysis was performed to investigate different functional moities. In FT-IR spectroscopy of Ag-TiO_2 (Fig. 4.15) show absorption peaks at 3417, 2923, 2314, 1647, 1437 and 643 cm_1 relates to the hydroxyl stretching, CH stretching, C=C broadening, C=C broadening, CH broadening and CCl broadening respectively and in the FT-IR spectra of Au- TiO_2 (Fig. 4.16) contain 3454, 2922, 1621, 1307, 966, 714 and 556 relates to OH broadening, CH stretching, C=C broadening, C-H broadening, =C-H bending, C-

Cl broadening and C-Br broadening correspondingly.

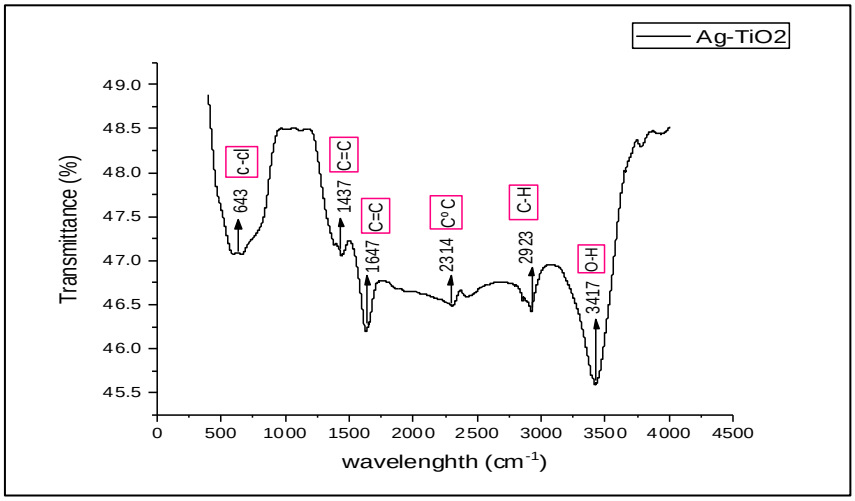


Figure 4.15: FT-IR spectra of Ag doped TiO2

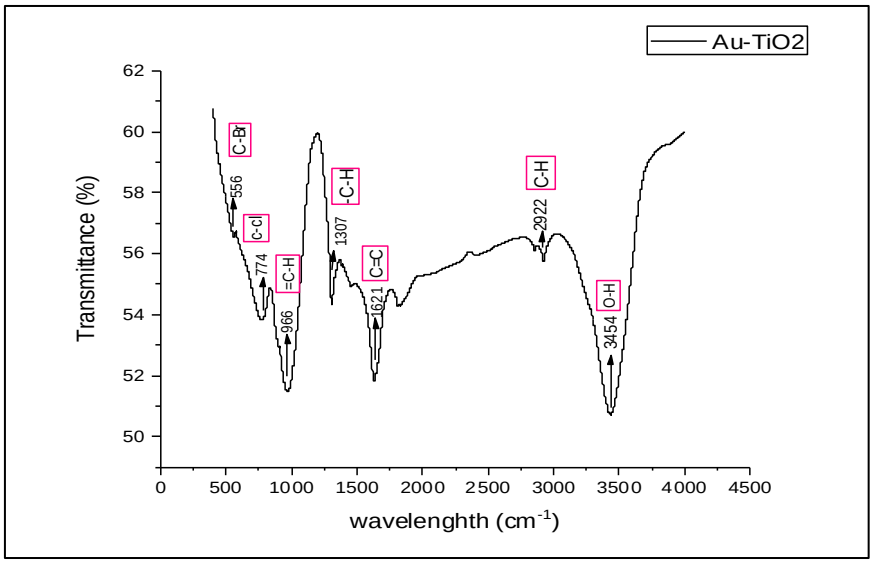


Figure 4.16: FT-IR spectra of Au doped TiO2

4.4.4. Morphology and elemental analysis of Ag- TiO_2 & Au- TiO_2

To determine the shape and size of the biosynthesized, Au doped TiO_2 and Ag doped TiO_2 calcined at 500°C SEM has been used (Fig. 4.17 a and b). According to SEM analysis doped TiO_2 is having nearly spherical morphology with homogenous distribution of nano sized particle. This homogeneous distribution showed that the particles are well interacted i.e. during synthesis of these doped nanoparticles there is well interaction between pure TiO_2, Au and Ag doped TiO_2 NPs s elemental analysis

of these doped nanotitania is done by EDS equipped with the SEM . EDS analysis confirmed the presence of Ti, O, Au and Ag elements as shown in Fig. 4.17 c and d. impurities like si and oxygen is because of glass slide as samples were prepared on glass slide.

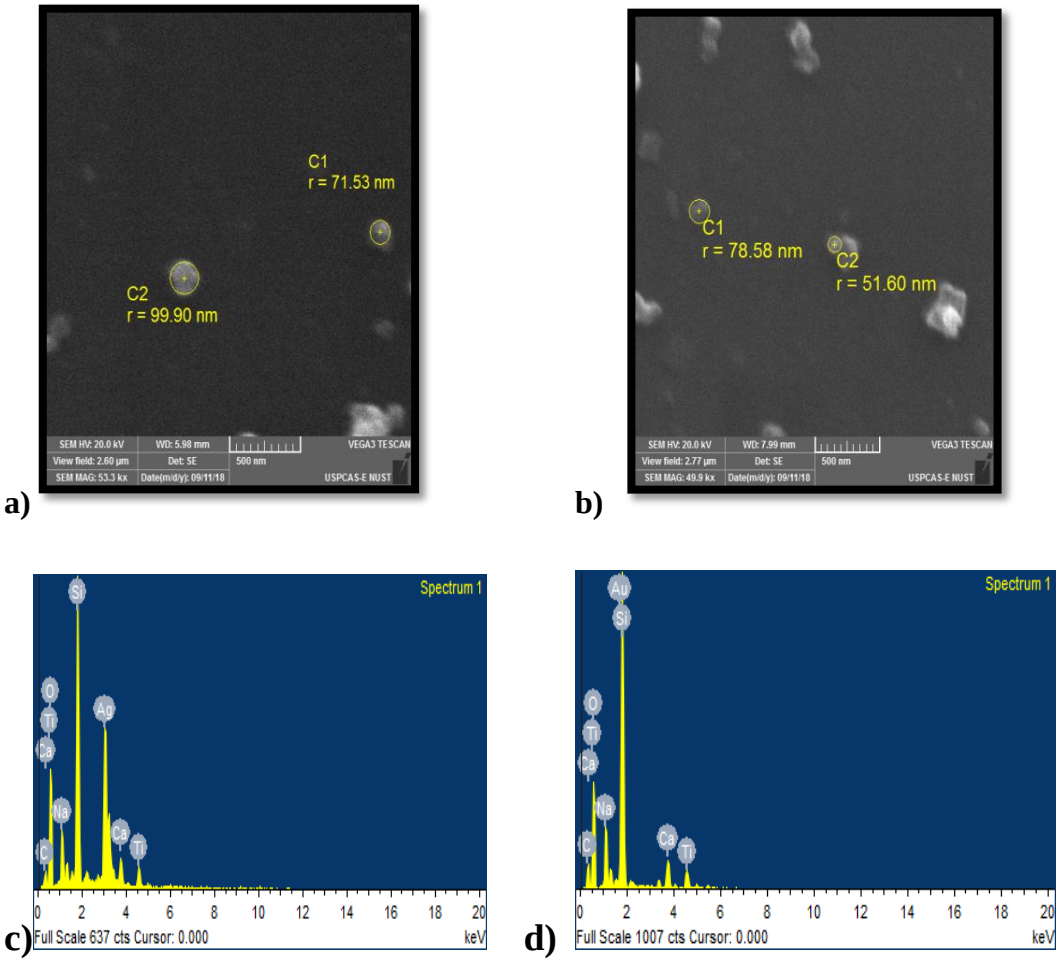


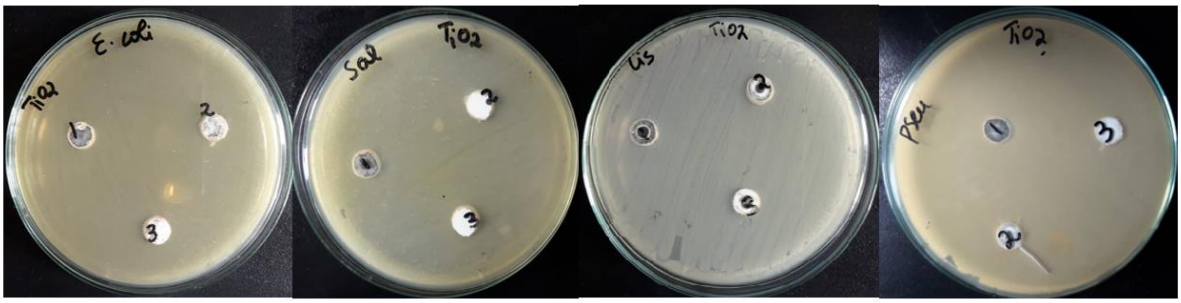
Figure 4.17: SEM analysis

Au -TiO2 (a) and Ag-TiO2 (b) EDX analysis of Au doped TiO2 (c) and Ag- doped TiO2 (d)

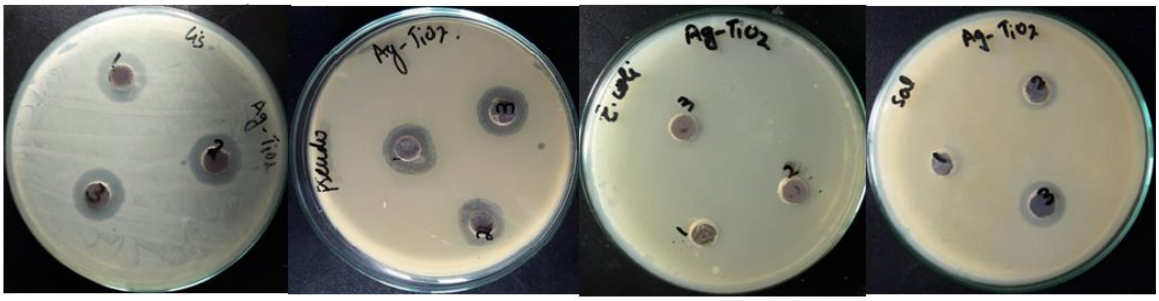
4.5 Antibacterial action of TiO_2, Ag- TiO2 and Au- TiO_2

The well diffusion method was performed against gram positive and gram negative bacterial pathogen the E. *coli*, *P. aeruginosa*, *L. monocytogen* and *Salmonella enterica*. The synthesized TiO2 NPs (ethanolic extract) displayed no antibacterial activity against pathogenic strains of *E. coli*, *P. aeruginosa*, *L. monocytogen* and *Salmonella enterica* while Ag- TiO_2 of Au TiO_2 showed

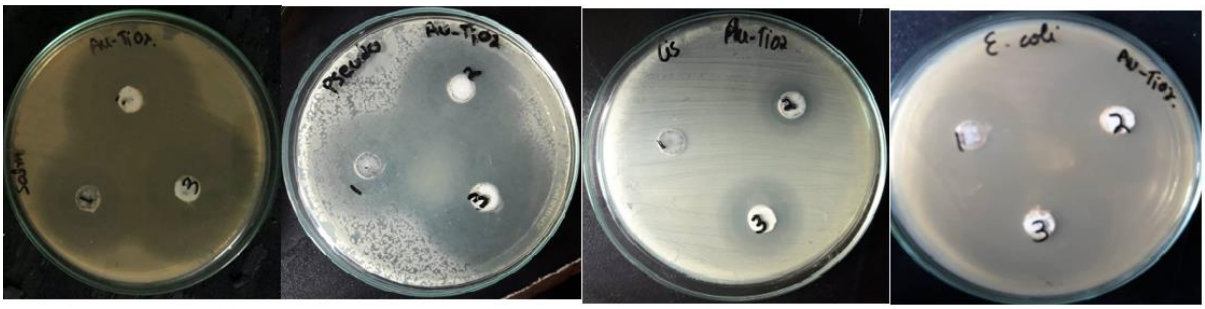
antibacterial action. The maximum ZOI was observed in the Au-TiO2 NPs against E. coli (Figure 4.18 c). However, in case of Ag-TiO_2 maximum inhibition zone measuresd was 32mm (figure 4.5.1b). Antibiotic disk bacitracin shows inhibition zone of 21 (*P. aeruginosa*), 19 (*E. coli*), 22 (*L. monocytogen*) and 19 (*salmonella enterica*)



a)



b)



c)

Figure 4.18: The antibacterial action

Pure a) TiO2, b) Ag and c) Au doped TiO2 NPs *against E. coli, P. aeruginosa, L. monocytogen* and *S.enterica*

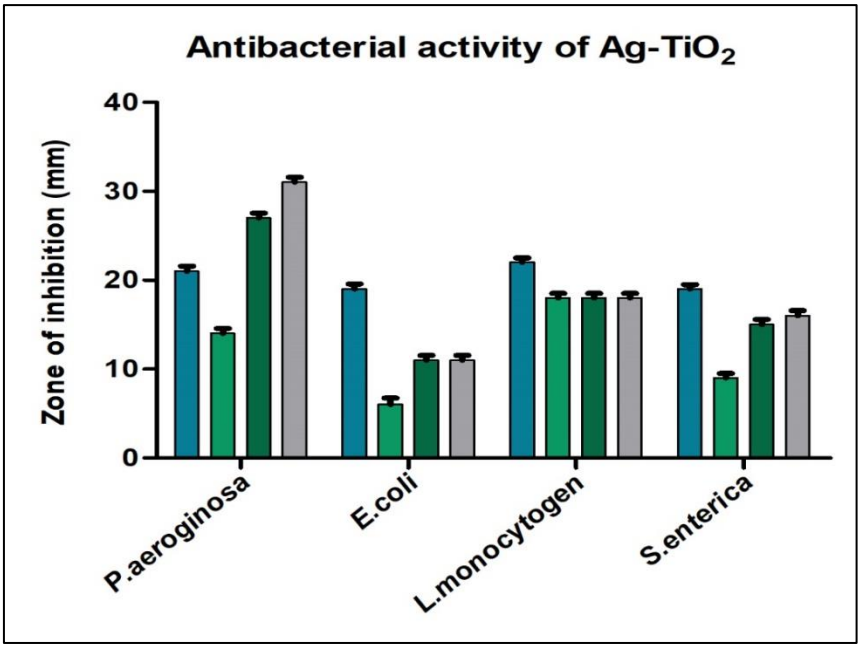


Figure 4.19: Antibacterial activity of synthesised Ag- TiO2 nanoparticles in comparison with antibiotic gentamycin

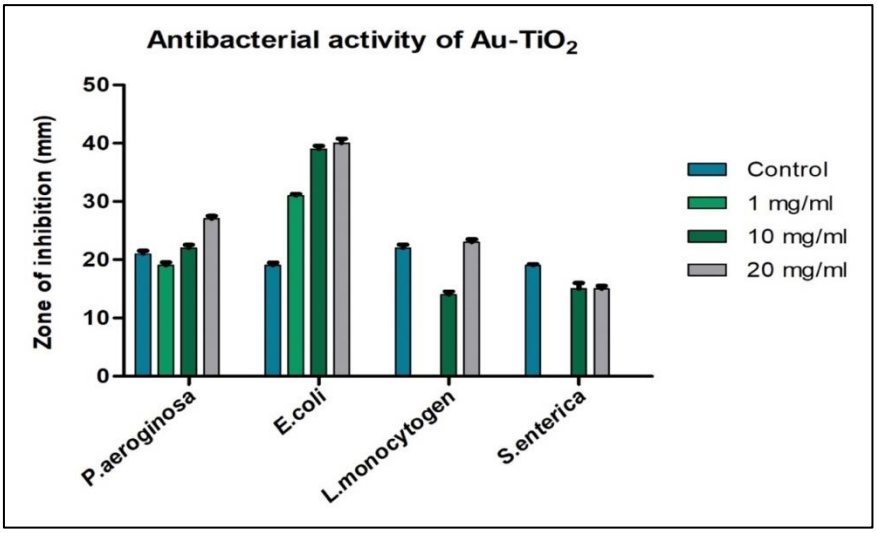


Figure 4.20: Antibacterial activity of synthesised Au- TiO2 nanoparticles in comparison with antibiotic gentamycin.

4.6 Antibiofilm activity of Ag-TiO_2 and Au-TiO_2

According to national institute of health (NIH) and center of disease control (CDC) microorganism that produce biofilm can cuuse approximately 65-80% of infection including both gram +ve and gram -ve bacteria mostly *E.coli, P.aeroginosa, S.aureus* (Joo & Otto, 2012). Biosynthesized Ag-TiO_2 and Au-TiO_2 were tested for

inhibition of biofilm against *E. coli, P.aeroginosa, l.monocytogen and S.enterica* having known for their ability of Biofilm formation. Test microorganisms were cultivated in 96 well microtiter plate with and without Ag-TiO_2 and Au-TiO_2. After 24 hours of incubation there is no significant decrease *in* cell free filtrate (positive control) did not show any significant decrease in Biofilm formation. However in case of *P.aeroginosa* AgNP's treatment results in decrease of almost 10% of biofilm formation at 60ul of 10mg/ml and about 20% decrease in 40ul and 60ulof 10mg/ml and 20mg/ml (Fig.4.21). In case of *E. coli and S.enterica* Ag-TiO_2 NP's show no decrease in antibiofilm formation except 60ul of 10mg/ml on *S.enterica* show decrease in antibiofilm formation. In *l.monocytogen* Ag-TiO_2 NP's show more than 60% of antibiofilm property. In case of $AuTiO_2$ NP's *E. coli, P.aeroginosa and S.enterica* there is no significant decrease in antibiofilm formation except S.enterica which show 20% of inhibition at concentration of 40ul of 10mg/ml. In *l.monocytogen* Au-TiO2 NP's show more than 60% of antibiofilm property.

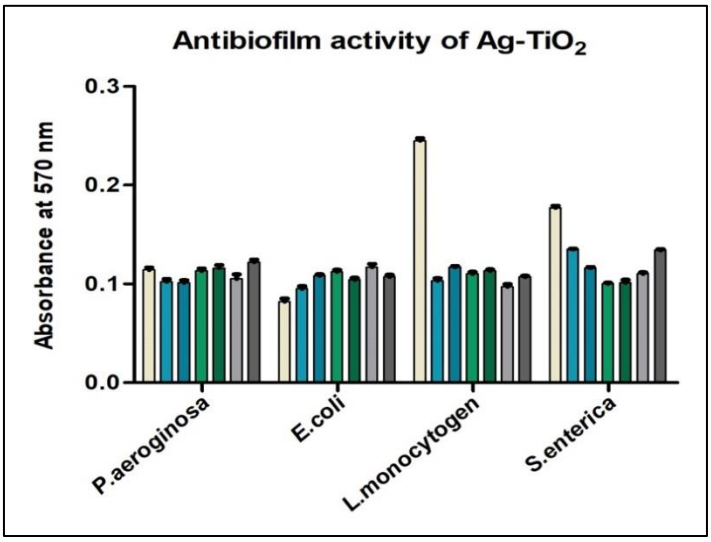


Figure 4.21: Antibiofilm activity of Ag-TiO2 and Au-TiO2 NPs at different concentrations on E.coli, P.aeroginosa, l.monocytogen and S.enterica.

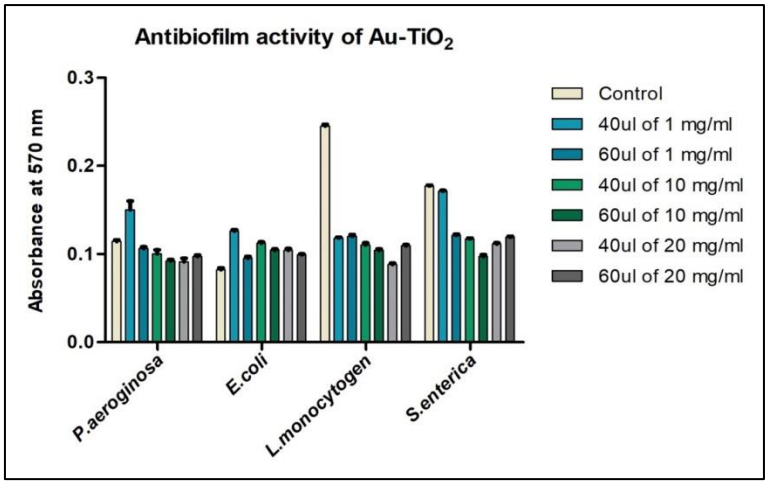


Figure 4.22: Antibiofilm activity of Ag-TiO2 and Au-TiO2 NPs at different concentrations on E.coli, P.aeroginosa, l.monocytogen and S.enterica.

4.7 Anticancer properties of TiO_2, Ag- TiO_2 and Au- TiO_2

To evalvate the antiproliferative effect of undoped TiO_2 and doped TiO_2 on HCT116 (colorectal cancer cell line) the effect of different concentration of NP's were measured on growth of HCT116 cells by sulphorhodamine B assay. As shown in figure, doped Au-TiO_2 NP's stop the growth of cells in dose-dependent fashion. After treatment with Concentration of 0.1 and above $AuTiO_2$ has IC 50 values as 0.1186 at the same time silver doped and undoped nanoparticles had no significant inhibitory effect on development of human colorectal cell lines.

$AuTiO_2$ NPs exhibited the most potent activity in these assays, and were then carried forward to investigate their anti-proliferative effect on the triple negative human breast cancer cell lines; HCC1954 (in RPMI media; seeding density 1000 cells/well), BT549 (in RPMI media; seeding density 1500 cells/well) and CAL-51 (in DMEM media; seeding density 2000 cells/well). Results showed a significant cytotoxic effect on these cell lines in a dose dependent manner with highest activity against Cal-51 (Fig 4.24).

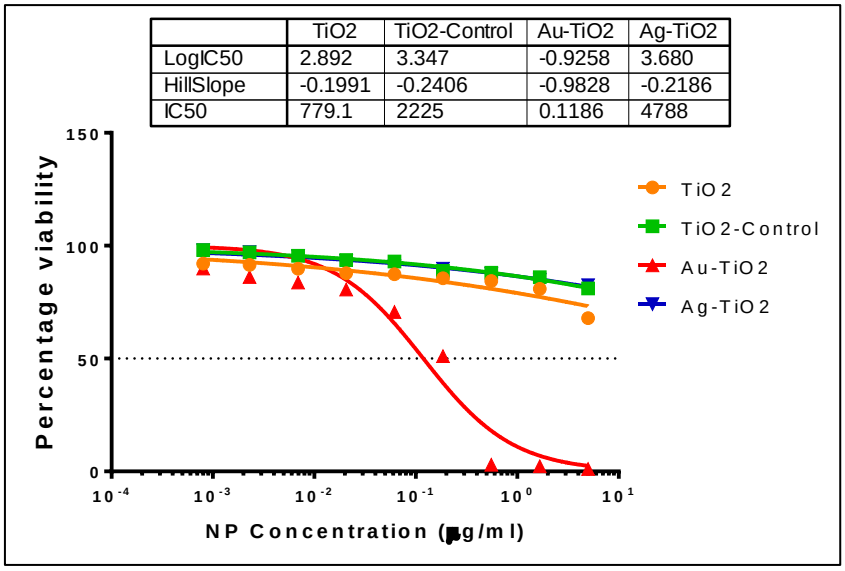


Figure 4.23: Cytotoxic effect of undoped and doped TiO2 nanoparticles by SRB assay

Anticancer activity of Au-TiO_2 (red) and Ag-TiO_2 (blue), TiO_2 (green) and TiO_2 control (orange) against HCT 116 cell lines.

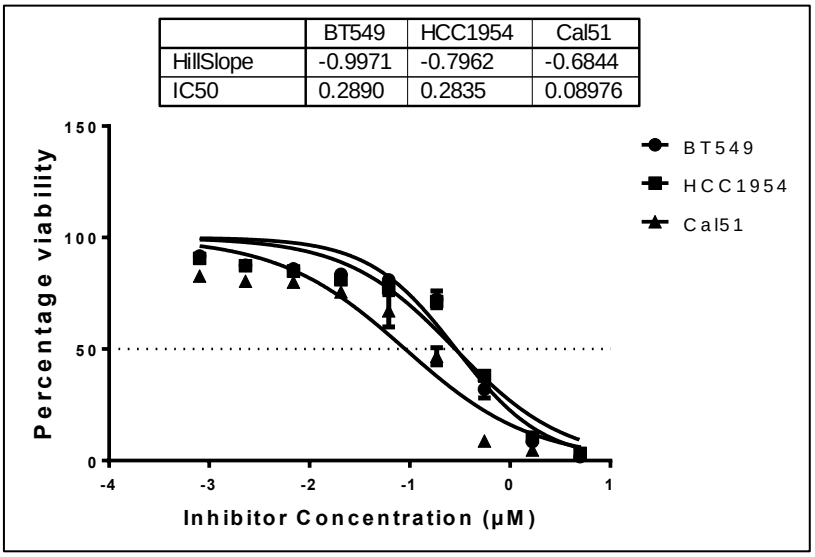


Figure 4.24: Anti proliferative effect on the triple negative human breast cancer cell lines

HCC1954 (in RPMI media; seeding density 1000 cells/well), BT549 (in RPMI media; seeding density 1500 cells/well) and CAL-51 (in DMEM media; seeding density 2000 cells/well).

4.8 Effect of Au- TiO_2 NP's on Cell Cycle

The nature of Au-TiO_2 NP's action was studied further by cell cycles flow cytometric analysis. DNA content of cell treated with three different concentrations of 0.25ug/ml, 0.5ug/ml and 1.0ug/ml Au-TiO_2 for 24 hrs. the experimental results show that untreated cell showed the normal cell cycle pattern for constantly growing cells, where as cells treated with Au-doped TiO_2 nanoparticles showed a gradual gathering and hence significantly accelerated cell cycle progression with increase in concentration of Au-TiO2 (1.0 ug/ml). The percentage of cell entering the S and G2/M phase is significantly higher than control (figure 4.25a). increase number of cell in G2/M phase of cell population demonstrating existence of dead cells. figure 4.25 illustrate the distribution of cells in different cell cycle phases

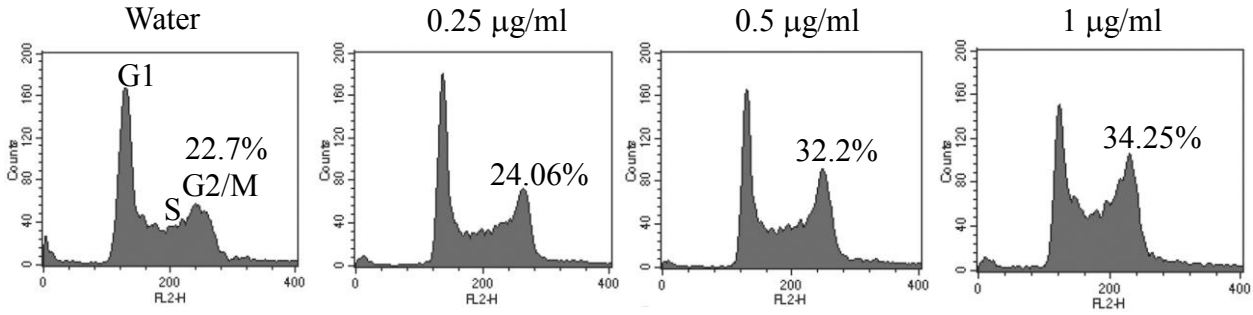


Figure 4.25: Anti proliferative effect on the triple negative human breast cancer cell lines

(a) cells without any treatment, (b) cells with 0.25 ug/ml of Au-TiO_2 (c) cells with 0.5 ug/ml of Au-TiO_2 and (d) cells with 1.0 ug/ml of Au-TiO_2.

4.9 P53 and apoptosis induction by AuTiO_2 NPs in Cal51 breast cancer cells

To determine cellular response P53 play important role as its having multiple function like protecting genome integrity, apoptotic induction, glycolytic cycle regulation, cell differentiation and even in self-eating of cells that is autophagy. The

process of cell death is primarily controlled by p53 which involve two main processes that is intrinsic pathway mitochondrial or extrinsic death receptor pathway. In this research work, the expression of p53 was studied to compare the DNA damaging potential of Au-TiO_2 NP's study showed that the increased number of p53-positive cells w examined by Au-TiO_2 NPs treated cells as compared with control. There is dose-dependent increase in the expression of p53 (tumor suppressor protein) in CAL51 breast cancer cells treated with Au-TiO_2 NPs at different concentrations of 0.25 μg/mL, 0.5 μg/mL, 1.0 μg/mL and 2.5 μg/mL for 24 h (Figure 4.26). These experiments confirmed that tumor suppressor gene expression is induced by Au-TiO_2 NP- which can inhibit propagation of CAL51 breast cancer cell unusually. This study provides new approach for cancer treatment. Small number of DNA strand breaks or single-stranded gaps is extremely sensitive to the p53 pathway. For early detection of DNA cuts in tumors p53 pathway can be considered as a factor and can play vital role many biologic processes are controlled by activation of p53 including growth detention, apoptosis and further activation of PARP. p53 protein expression profile was significant and hence supports the DNA damaging potential of the Au- TiO_2 NPs.

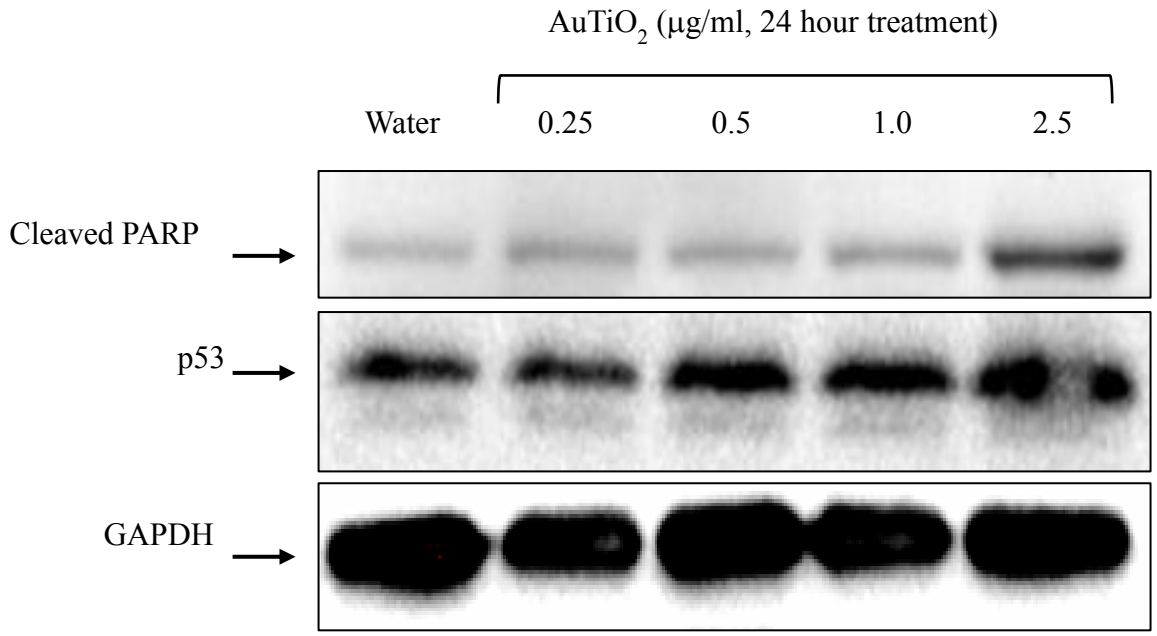


Figure 4.26: Western blot analysis of CAL51 breast cancer cells

Cancer cells were treated with indicated Au-TiO_2 NP's concentration for 24 h for indicated antibodies. GAPDH served as loading control

CHAPTER 5 : DISCUSSION

In modern society nanotechnology is an interdisciplinary approach that deal with very minute object which can be further used to eradicate the modern world problems. In twenty first century it has application in almost every field of life including nanomedicines which is subject of interest for all scientists around world. Nanomedicines is actually field of study where targeted approaches are used to design new drugs and therapeutics to decrease the side effects of conventional medicines (Wagener et.al., 2006).

Nanoparticles due to their characteristics are getting huge attention and can be utilized in nanomedicines. Titania nanoparticles due to their importance are at great hype because of temperature stability, photostability and antimicrobial property along with its photocatalytic properties in degradation of various azo dyes along with killing of various microbes in presence of UV light titanium dioxide doped with metal has been used for the degradation of many phenols in particular and organic pollutant for in general. Example of phenols are; chlorophenols which exhibit high toxicity and cancer-causing character and having low taste and odour thresholds. These compounds are not degraded effectively by biological methods directly because they are toxic and badly biodegradable. Their removal in wastewaters and drinking water is of great attention.

In order to overcome the short comings of chemical methods, biological methods are gaining more attention recently. One of the simple, cost effective, dependable and environmentally friendly approaches for nanoparticles synthesis is green approach. Green synthesis of NP's has shown much more attention because of its high yield production. Microorganisms, whole plants and extracts from various plant parts and algae have been used to produce nanoparticles. As compare to physicochemical methods of production biosynthesis methods can actually provide better morphology and defined size of NPs. But there is only a few study reported in antimicrobial activity of green synthesised TiO_2 Nanoparticles it was proposed to synthesise TiO_2 nanoparticle from Algal extracts of Dictyosphaerium sp. Strain HM1

(DHM1), extract was used to synthesis Titanium oxide nanoparticles and was tested for its antimicrobial, Antibiofilm and anticancer activities.

UV-visible analysis was done to monitor the completion of bioreduction of Titanium dioxide ions in aqueous solution. Reduction of Titanium IV isopropoxide to Titanium dioxide nanoparticles during exposure to Algal extracts is followed by a gradual increase in colour development from dark to green. The formation and completion of titania nanoparticles was characterized by depleting Shimadzu UV visible spectrophotometer. The UV Vis Spec analysis of the sample showed maximum absorbance at 440-450nm which confirmed the presence of TiO_2 NPs. This was in conformity with Valli and Geetha 2015. [16] During optimization ethanol was reacted with TTIP and was checked for UV spectra which show no absorbance peak this means that enzymes present in algal extract (ethanolic) are responsible for bioreduction of TTIP to form TiO_2 nanomaterials.

The functional groups of the Algal extract with titanium dioxide were identified by using FT-IR spectra of extract (Fig. 4.3.1 a and b) and titanium dioxide nanoparticle. In the present study, the stretching at the wave number 3200 to 3600 cm-1 shows the occurrence of O-H functional moities, alcohol, strong and broad peak. The sharp peak was observed in the range 2850-3000cm-1 shows the C-H stretch free. And a C=C stretch ranging from 1620-1680 cm1 shows the alkenes. A relationship of these outcomes with earlier reports showed that in the process of nanoparticle synthesis alcohols, alkanes and alkenes may be contributing. The results are similar to Ankita et al., 2016 peaks were observed at 853 cm-1 indicated O-Ti-O bond. The peak near 3000/cm was due to the -hydroxyl stretching and the Ti-oxygen stretching vibration was confirmed by the peak at the region of 786-853/cm.

Nanoparticle sample was diluted upto 1000 fold to prepare slide for SEM analysis. The resultant slide was further used for SEM and EDS. From the SEM images, it was detected that most of the TiO_2 nanoparticles were showing spherical shapes particles structure. The size was ranging from 40 to 60nm.

Concentration and size are two main factors affecting antimicrobial properties of nanoparticles during synthesis these nanoparticles may form agglomerates therefore ultrasonication and modification with polyethylene glycol m(PEG),

polyvinylpyrolidine (PVP) and bovine serum albumin (BSA) are used for the disintegration of agglomerates formed by nanoparticles (brayener et al., 2006). In this study, Antibacterial effect of different concentrations of synthesized nanoparticles were checked against gram positive, *L. monocytogen* and gram negative bacteria *Pseudomonas aeruginosa, Salmonella enterica and Escherichia coli,* by agar well diffusion method. The outcomes indicate that Au-TiO_2 nanoparticles significantly inhibit the growth of *E. coli* compared with the control gentamycin antibiotics.

Nosocomial infection is caused by *E. coli* main causative agents and resistant to maximum broad spectrum Antibiotics. Antibiotic resistance of microorganisms is highly increased because of misuse or overuse of antibiotics. Introduction of new anti-bacterial agents can control the mortality and morbidity rate of the infectious diseases. .nanomaterial shows strong inhibiting effect (broadened spectrum) against bacterial strain. Beside *E. coli* food borne pathogens like *listeria monocytogenes, salmonella enterica* and *pseudomonas aeruginosa* are also been used for their anti-bacterial action. In current study, different concentrations of Ag doped TiO_2 and Au doped TiO_2 could inhibit the growth of *E. coli, listeria monocytogenes, salmonella enterica* and *pseudomonas aeruginosa*. Au doped TiO_2 can be a suitable disinfectant and can be used in hospitals, cotton fabrics with antibacterial effect is developed by using the Nano materials, such as TiO_2 in textile industry, hence, wound bands could be possible to reduce the infection rate in patients.

The $AuTiO_2$ showed anticancer effect at a concentration of 10μg/ml on cell line HCT116 (colorectal cancer cell line). However the synthesized $AuTiO_2$ NPs showed a potent anticancer activity against HCC1954. The synthesized TiO_2 nano particles could inhibit the proliferation of cells HCC1954 till a concentration of 1μ/M. compared to that of positive control. Ankita chatterjee et al. (2016) carried out phytosynthesis of titaniia nanoparticles from green extract of V. radiata legumes and estimated the anticancer activity on osteosarcoma cell line. Cytotoxicity assay revealed that $AuTiO_2$ nanoparticles were capable of inhibiting proliferation of breast cancer cell lines.

After determining the cytotoxic potential of Au-TiO_2 NPs on Cal51, we calculated their potential to modulate the cell cycle progression. On treatment of these

cells with 0.25 μg/ml Au-TiO_2 NPs for overnight incubation, approximately 24.06 % of cells were detected in G2/M phases of cell cycle. While at a higher dose of 1 μg/ml Au-TiO_2 NPs, 34.25 % cells were found to be in G2/M phases as compared to untreated cells (control) where they are found to be nearby 22.7 % in G2/M (Fig. 4.8).

In conclusion TiO_2 nanoparticles were synthesized using cheap, faster, eco-friendly and easy procedure by using algal extract of Dictyosphaerium sp. Strain HM1 (DHM1). These nanoparticles are than doped with Ag and Au. Newly synthesized nanoparticles i.e. TiO_2, Ag- TiO_2 and Au-TiO_2 were characterized with UV-Vis Spectrophotometry, X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray spectroscopy (EDS). Antibacterial, antibiofilm and anticancer activity were checked with maximum activity shown by Au-TiO_2 nanoparticles. Au-TiO_2 NPs exhibit greater abilities to inhibit call51 breast cancer cells proliferation. Au- TiO_2 NPs increased the apoptotic cell population with dose-dependent manner (G2/M). This study revealed that the major mode of cell death was cell apoptosis on the underlying molecular mechanisms induced by Au- TiO_2 NPs. Further studies triggered by the Au- TiO_2 NPs on apoptotic signaling pathway in call51 breast cancer cells are p53 and cleaved PARP pathways. Our findings propose that Au- TiO_2 NP is a possible titania species having anticancer properties. However, synthesized TiO_2 show no antibacterial, antibiofilm and anticancer activity and further research is to be done to use these nanoparticles for degradation of different antibiotics and textile dyes and to compare their activity with doped TiO_2 nanoparticle.

CONCLUSION AND FUTURE RECOMMENDATION

Current study was established on focusing of Dictyosphaerium sp. Strain HM1 (DHM1) as a potential extracellular enzymatic system for production of TiO_2 nanoparticles. After physiochemical parameters optimization of biosynthetic reaction, these TiO_2 NP's were illustrated with UV-Vis Spectrophotometry, X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray spectroscopy (EDS). These nanoparticles were than co doped with gold and silver. When physical features of synthesized nanoparticles were characterized, they were further elucidated for potential bioactivities regarding food borne pathogen and potential health risks. These phycosynthesized TiO_2 nanoparticles along with doped nanoparticles were observed for antibacterial, antibiofilm and anticancer activities and Au doped TiO_2 have considerable features and can be used as potential disinfectants and opens a new area of research in cancer therapeutics.

After analyzing data and result depicted in this study, it is recommended to further characterize these nanoparticles by using advance characterization techniques like NMR, Raman spectroscopy and TEM analysis. In silico approach should be adapted to examine the interaction between doped nanoparticles and its cellular interaction with bacteria and human cells. These nanoparticles can also be used for drug delivery system to enhance its efficiency for therapeutics agents. These nanoparticles can also be used for photocatalytic degradation of different dyes related to textile industry along with degradation of certain antibiotics present in contaminants.

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