PHYTO-ASSISTED SYNTHESIS, PHYSICO-CHEMICAL CHARACTERIZATION AND CELLULAR EFFECTS OF SILVER NANOPARTICLES ON HUMAN NORMAL AND CANCER CELLS

Thesis submitted to University of Calicut
in partial fulfillment of the requirement for the award of

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By

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Certificate

This is to certify that the Thesis entitled **"Phyto-assisted synthesis, physico-chemical characterization and cellular effects of silver nanoparticles on human normal and cancer cells"** submitted to University of Calicut, as partial fulfillment of Ph.D. programme for the award of the degree of Doctor of Philosophy in Biotechnology by **Shaniba V. S.,** embodies the results of bonafide research work carried out by her under my guidance and supervision at the Department of Biotechnology, University of Calicut. This Thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition. The candidate has passed the course work of the Ph.D. programme in accordance with the UGC regulations.

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DECLARATION

I hereby declare that the work presented in the Thesis entitled **"Phyto-assisted synthesis, physico-chemical characterization and cellular effects of silver nanoparticles on human normal and cancer cells"** submitted to the University of Calicut, as partial fulfillment of Ph.D. programme for the award of the degree of Doctor of Philosophy in Biotechnology, is original and carried out by me under the supervision of Dr. P. R. Manish Kumar, Department of Biotechnology, University of Calicut. This has not been submitted earlier either in part or full for any degree or diploma of any university.

C.U Campus **Shaniba V. S**
Date :

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Shaniba V.S.

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ABBREVIATIONS

4-AP	:	4-aminophenol
4-NP	:	4-nitrophenol
µL	:	Microliter
µg	:	Microgram
ACT	:	Adoptive cell transfer
ADP	:	Adinosine di-phosphate
AFM	:	Atomic force microscopy
$AgNO_3$	:	Silver nitrate
AgNP	:	Silver nanoparticle(s)
AIDS	:	Aquired Immuno Deficiency Syndrome
ALP	:	Alkaline phosphatase
AMF	:	*Annona muricata* fruit
AMR	:	*Annona muricata* root
ANOVA	:	Analysis of variance
AO	:	Acridine orange
APAF1	:	Apoptotic protease activating factor 1
ATP	:	Adinosine tri-phosphate
BAK	:	Bcl_2 homologous antagonist/killer
BAX	:	Bcl_2 associated X protein
BCIP	:	5-Bromo- 4- chloro-3- indolyl phosphate
BCIP/NBT	:	5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium
Bcl-2	:	B-cell lymphoma 2
BCR/ABL	:	Breakpoint cluster region - Abelson
BER	:	Base excision repair
BH3	:	Bcl_2 homology 3
BPH	:	Benign prostatic hyperplasia

BSA	:	Bovine serum albumin
C	:	Celsius
CAR	:	Chimeric antigen receptor
CCl_4	:	Carbon tetrachloride
CDK	:	Cyclin dependent kinase
cDNA	:	Complementary DNA
cm	:	Centimeter
CRISPR	:	Clustered regularly interspaced short palindromic repeats
CYT	:	CytImmune Sciences
DCFH-DA	:	Dichloro-dihydro-flourescien diacetate
DISC	:	Death inducing signaling complex
DLS	:	Dynamic light scattering
DM	:	Dichloromethane
DMEM	:	Dulbecco's Modified Eagle's Medium
DMF	:	N,N-dimethylformamide
DMSO	:	Dimethyl sulfoxide
dNTP	:	Deoxy-nucleotide triphosphate
DNA	:	Deoxyribonucleic acid
DPPH	:	2, 2-diphenyl-1-picrylhydrazyl
DTT	:	Dithiothreitol
EA	:	Ethyl acetate
EB	:	ethidium bromide
EDTA	:	Ethylenediaminetetraacetic acid
EDX	:	Energy dispersive X-ray analysis
eIF	:	Eukaryotic initiation factor
ELISA	:	Enzyme-linked immune sorbent assay
EPR	:	Enhanced permeability and retention

ER	:	Endoplasmic reticulum
EtBr	:	Ethiduim Bromide
FACS	:	Fluorescence-activated cell sorting
FBS	:	Fetal bovine serum
$FeCl_2$	:	Ferrous chloride
FESEM	:	Field-emission scanning electron microscopy
$FeSO_4.7H_2O$	:	Ferrous sulfate heptahydrate
FITC	:	Fluorescein isothiocyanate
FTIR	:	Fourier-transform infrared spectroscopy
FWHM	:	Full width at half maximum
g	:	Gram(s)
GAE	:	Gallic acid equivalents
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
h	:	Hour(s)
H_2O_2	:	Hydrogen peroxide
HIV	:	Human influenza virus
hPBL	:	Human peripheral blood lymphocyte
IC	:	Inhibitory concentration
Ig	:	Immunoglobulin
IgG	:	Immunoglobulin G
JCPDS	:	Joint committee on powder diffraction standards
KBr	:	Potassium bromide
kDa	:	Kilodalton
kV	:	KiloVolt
LB	:	Luria Bertani
lncRNA	:	Long non-coding RNA
mA	:	Milli-Ampere

MBC	:	Minimum bactericidal concentration
MCF7	:	Michigan cancer foundation-7
Met	:	Methanol
mg	:	Microgram
MIC	:	Minimum inhibitory concentration
min	:	Minutes
miRNA	:	MicroRNA
mL	:	Milliliter
Mm	:	Millimeter
mM	:	Millimolar
M-MLV	:	Moloney murine leukemia virus
MMR	:	Mismatch repair
mRNA	:	Messenger RNA
MTT	:	3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MZL	:	*Manilkara zapota* leaf
Na_2CO_3	:	Sodium carbonate
$NaBH_4$	:	Sodium borohydride
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
NBT	:	Nitro-blue tetrazolium
NCCS	:	National Centre for Cell Sciences
ncRNA	:	Non-coding RNA
NER	:	Nucleotide excision repair
nm	:	Nanometer
nM	:	Nanomolar
NO	:	Nitric oxide
NP	:	Nanoparticle(s)

nsclc	:	Non-small cell lung cancer
OD	:	Optical density
PAGE	:	Polyacrylamide gel electrophoresis
PARP	:	Poly (ADP-ribose) polymerase
PBS	:	Phosphate buffered saline
PCR	:	Polymerase Chain Reaction
PE	:	Petroleum ether
PEG	:	Poly-ethylene glycol
PI	:	Propidium iodide
PUMA	:	p53 upregulated modulator of apoptosis
PVDF	:	Polyvinylidene fluoride or polyvinylidene difluoride
QE	:	Quercetin equivalents
RAF	:	Rapidly accelerated fibrosarcoma
RBC	:	Red blood cell
R_f value	:	Retardation factor
RF	:	Radio-frequency
RIPA	:	Radioimmunoprecipitation assay
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
RPMI	:	Roswell Park Memorial Institute medium
RTq-PCR	:	Real time PCR
SD	:	Standard deviation
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	:	Scanning electron microscope
siRNA	:	Short interfering RNA
SL	:	Synthetic lethality
SPR	:	Surface Plasmon Resonance

SPSS	:	Statistical package for the social sciences
TBE	:	Tris Borate EDTA
TBS	:	Tris-buffered saline
TBST	:	Tris-buffered saline containing Tween 20
TCA	:	Tri-chloro acetic acid
TLC	:	Thin layer chromatography
TRIS	:	2-amino-2 hydroxy methyl-propane-1-3- diol
US	:	United States
UV	:	Ultra violet
V	:	Volt
v/v	:	volume/volume
VEGF	:	Vascular endothelial growth factor
W	:	Watt
w/v	:	Weight/volume
WHO	:	World Health Organisation
XRD	:	X-ray diffraction

INTRODUCTION

1.1. Cancer : Conventional and emerging therapeutics

Cancer is the second leading cause of human death outnumbered only by heart disease related mortality worldwide. Of the 17.5 million cancer cases reported globally in 2015, 8.7 million succumbed to the disease. According to WHO (2018) fact sheets, lung cancer occurred in 1.8 million people and resulted in 1.69 million deaths followed by deaths due to liver (788000 cases), colorectal (774000 cases), stomach (754000 cases) and breast cancer (571000 cases). Particularly cancer causes one in seven deaths globally, causing more deaths than malaria, tuberculosis and AIDS (Jemal et al., 2011). Conventional cancer treatment strategies include surgery, radiotherapy and use of chemotherapeutic drugs, all of which lack specificity. Surgical treatment does not ensure complete elimination of cancer cells, can be traumatic with large tumors and becomes invalid in case of sub-clinical metastasis. Damage to healthy cells and tissues near the treated area, fatigue and development of secondary cancers are the risks associated with radiation therapy. The many harmful side effects of chemotherapeutic drugs, including the toxicity of their by-products adversely affect the non-targeted host cells. Further, development of intrinsic and acquired resistance against these drugs curtails success of chemotherapy (Peer et al., 2007). Targeted therapies involve use of inhibitors of angiogenesis, signal transduction pathways including immunotherapy with monoclonal antibodies which interfere with molecular pathways regulating tumor growth and progression (Topcul and Cetin, 2014; Ke and Shen, 2017). In 2017, the US Food and Drug Administration gave approval to ‘gene modification therapy’, also known as adoptive cell transfer (ACT) involving collection T cells from the patient engineered to express a gene for a special receptor - chimeric antigen receptor (CAR) - to target a specific protein on the patients cancer cell (Sharpe and Mount, 2015; Jackson et al., 2016; Levine et al., 2017). The development of CRISPR/Cas9 genome editing technologies have opened up new avenues in cancer and therapy (Zhan et al., 2018).

1.2. Nanotechnology and nanomedicine for cancer cure

Despite development and availability of several aggressive treatments modes, cancer-related death rates continue to rise (Jemal et al., 2011; Miller et al., 2016). Thus development of efficacious, biocompatible and cost effective methods for cancer cure becomes indispensable. Nanotechnology-based approaches have emerged as an exciting field with promises to overcome the limitations of conventional treatments due to their prolonged half-life and increased targeting efficiency (Alexis et al., 2010). Different parameters such as nanoparticle size allow them to enter intrinsically into tumors through enhanced permeability and retention (EPR) effect, their ability to evade the immune system and improve the drugs half-life; significantly reduce its effective dose. Nanoparticles also exhibit selective targeting due to their high surface density (Suri et al., 2007; Davis et al., 2008).

Nanoparticles (NPs) comprise of clusters of atoms in the size range of 1-100 nm. Nanomaterials have great importance as the physico-chemical properties of a metal are changed as it reaches the nano size capable of displaying properties which are different as compared to the bulk metal. Gold, silver, and platinum have been used mostly for the synthesis of stable dispersions of nanoparticles, which are useful in areas such as photography, catalysis, biological labeling, photonics and optoelectronics (Jacob et al., 2012). Chemical, physical and biological methods have been adopted for their synthesis. Fabrication of NPs through chemical and physical methods is most popular but requires use of toxic and hazardous chemicals, large amounts of energy and generates harmful wastes (Song and Kim, 2009; Rajasekharreddy et al., 2010).

Both chemical and physical methods adversely affect the environment in addition to being technically laborious and expensive. As an alternative, a ‘greener’ biogenic synthesis of nanoparticles employing natural products such as sugars, biopolymers, microorganisms and plant extracts containing phytochemicals are believed to play a key role as bioreductants in the conversion of metal ions to nanoparticles as well as provide capping agents to stabilize them. The use of plant extract has been found to be relatively more advantageous over microbial systems

for large scale production of biogenic nanoparticles since maintenance of aseptic conditions for microbial cultures is not industrially feasible (Ahmed et al., 2016)

A plethora of *in vitro* studies on different cancer cell lines have confirmed anticancer activities of biogenic gold, silver, copper, titanium, zinc and iron prepared from different bio-sources (Salunke et al., 2016; Barabadi et al., 2017). Among these, silver nanoparticles (AgNPs) have gained more popularity and acceptability due to their antibacterial, antifungal, larvicidal, anti-parasitic properties and industrial applications (Khatoon et al., 2017). AgNPs are cytotoxic to cancer cells and show excellent potential as an antitumor agent, in which AgNPs induce cytotoxicity. The exact mechanism of AgNPs anticancer activity is not fully understood yet. However, the proposed mechanisms involve generation of reactive oxygen species (ROS) causing mitochondrial damage, induction of sub-G_1 arrest in cell, (Ovais et al., 2016) upregulation of p53 protein, caspase-3 expression (Kovacs et al., 2016) and inhibition of VEGF-induced activities (Hackenberg et al., 2011). Cytoxicity is dependent on parameters such as particle size, surface area/reactivity, distribution pattern as well as cell type specificity (Xi Feng et al., 2016).

The physico-chemical and biological traits confer several advantages to these nanoparticles making them excellent drug-delivery vehicles which have increasingly become popular in clinical use in the recent past (Mudshinge et al., 2011; Safari and Zarnegar, 2014; Gong and Chen, 2016). They resolve several limitations of conventional drug-delivery systems such as non-specific biodistribution and targeting, lack of water solubility, poor oral availability and low therapeutic indices. Several stability and drug payload studies on the NP formulations have shown that they are highly stable with high carrier capacity, are feasible for incorporation of both hydrophilic and hydrophobic substances and can be administered by various routes including oral application and inhalation (Yousefi et al., 2009; Sharma et al., 2011). The use of surface-modified nano-scale liposomes (nanosomes) / nanocapsules / lipid-micelles to deliver hydrophilic and hydrophobic anticancer drugs as well as siRNA formulations is a promising development in cancer therapy. PEGylated liposomal doxorubicin was the first liposomal anticancer drug for the

treatment of ovarian cancers approved by the US Food and Drug Administration. Lipid-based nanocapsules provide a novel approach for encapsulation of poorly soluble drugs like cisplatin or paclitaxel. Further, 'active' and 'passive' targeted drug delivery is also being achieved by covalent coupling of nanoparticles with appropriate ligands such as lectins, antibodies, aptamers, folate and peptides (Bannerjee and Sengupta, 2011; Ranganathan et al., 2012; Pang et al., 2017; Liang et al., 2018). Researchers are developing "nanosponges" which are essentially polymer nanoparticles coated with a red blood cell membrane capable of absorbing toxins and remove them from the blood stream. A major nanomedicine research company has successfully completed Phase I clinical trial of CYT-6091 (gold nanoparticles for targeted delivery of drugs to tumors). CRLX 101 and nanotrain (connected DNA strands) are nano agents developed for targeted chemotherapy (Sebastian, 2017). DNA nanotechnology as represented by DNA based 2-3D nanostructures called DNA origami for drug delivery has recently emerged (Kumar et al., 2016). Thus, the knowledge and tools of nanotechnology offer tremendous potential for efficacious drug delivery, enhanced diagnosis-imaging for treatment of cancers and is sometimes referred to as Nano-oncology within the broader discipline termed as Nanomedicine. Cancer nanomedicine possesses the versatility to overcome some of the most challenging impediments to treatment success while using conventional chemotherapeutic drugs.

1.3. About this Thesis

1.3.1. Aims and Objectives

The present study embodifies a 'green', simple, nontoxic method for synthesis of silver nanoparticles with good monodispersity to be used for investigating their cellular effects and drug potential. The study was undertaken with the following aims and objectives:

(i) Laboratory scale production of silver nanoparticles in the presence of select medicinal plant extracts

(ii) Physico-chemical characterization of the biogenic AgNPs followed by

(iii) Evaluation of their bioactivities such as antioxidant, antimicrobial, antiproliferative including apoptogenic potential employing normal human and cancer cell lines.

Following a preliminary screening of a number of medicinal plant extracts, two were selected, namely, *Manikara zapota* (L.) P. Royen and *Annona muricata* (L.) based on successful biosynthesis of silver nanoparticles. The aqueous extracts from *M. zapota* leaves as well those prepared from fruits and roots of *Annona muricata* consistently yielded useful amounts of AgNPs. The size, nature and composition of biogenic silver nanoparticles so produced were then characterized by UV-vis spectroscopy, X-ray diffraction (XRD), Field-emission scanning electron microscopy (FESEM), Energy dispersive X-ray spectroscopy (EDX) and Fourier-transform infrared spectroscopy (FTIR). The antimicrobial activities of the biogenic nanoparticles were evaluated employing clinical bacterial isolates of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumonia*. The effects of the nanoparticles on eukaryotic cells were studied on normal human peripheral blood derived lymphocytes, erythrocytes, and cancerous colorectal, lung, cervical cell lines. Overall, the experimental studies can be grouped under the following subheads :

- *Optimization of physico-chemical parameters*: Ratio of plant extract, silver ion concentration, pH, temperature and time of incubation.
- *Identification of the morphology, size, crystalline nature and metal composition using standard techniques for characterization of the respective AgNPs* : UV-vis spectrometry, FESEM, XRD and EDX and FTIR for functional groups
- *Assessment of antibacterial* activities: Disc diffusion method, Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)
- *Assessment of antioxidants by non-enzymatic assays for*
  - DPPH radical scavenging
  - Nitric oxide scavenging
  - Hydroxyl radical scavenging
  - Total antioxidant capacity
  - Ferrous ion chelation capacity

- Reducing power
- Total phenolic and flavonoid content

- *Evaluation of cytotoxicity / antiproliferative activity*: assays for Trypan blue dye exclusion, time and dose-dependence MTT assay and clonogenicity
- *Assessment of cytomorphological changes by light, scanning and fluorescence microscopy:* observation of unstained cells and those stained with Hoechst 33258, ethidium bromide- acridine orange
- *Detailed cellular and molecular studies to elucidate the mode of action of biogenic silver nanoparticles enumerated below* :
  - Annexin V-FITC staining for detection of apoptotic cells
  - Detection of ROS and mitochondrial membrane potential
  - Evaluation of genotoxicity (comet assay)
  - Evaluation of cell migration (wound healing / scratch/cell migration assay)
  - Cell cycle distribution : flow cytometric analysis
  - Quantitative analysis of apoptosis-related gene expression: real-time polymerase chain reaction (RTq-PCR)
  - Western blot analysis: detection of key proteins involved in cell death
- *Cytotoxicity evaluation of biogenic AgNPs on normal human cells*: peripheral blood derived lymphocyte cultures (hPBLs) subjected to MTT and erythrocytes to hemolytic assay

1.3.2. Thesis layout

The thesis is divided into **five** chapters. The **first chapter** highlights the theme of the research work and also provides a flow chart summarizing the experimental study. The **second chapter** comprises of a review of literature, which includes (i) a brief description of nanotechnology, nanoparticles, silver nanopaticles and their methods of production, characterization and applications in various fields (ii) a brief overview of the molecularbiology of cancer, cell cycle deregulation, cancer critical genes and related information including the past, present and future of cancer therapy. The chapter also provides information on the plants/plant extracts and cancer cell lines employed for the study. The **third chapter** furnishes the details of methodology adopted for the experimental work. The **fourth chapter** presents the

results obtained along with relevant discussion under four subheads – (i) synthesis and characterization of the plant extract - mediated silver nanoparticles (ii) phytochemical, biochemical and antimicrobial evaluation of biogenic AgNPs (iii) cytotoxicity evaluation of these nanoparticles on normal and cancer cells and (iv) detailed cellular and molecular studies to elucidate mode of action of biogenic AgNPs. The **fifth chapter** entitled '**Summary** and **Conclusions**' highlights the total outcome of the doctoral research and inferences drawn. A bibliography of all citations and an appendix providing recipes for reagent preparation are provided at the end of the Thesis. *Pre-pages* include a list of tables, figures and abbreviations. An outline of the doctoral research work has been presented in Fig. 1.1.

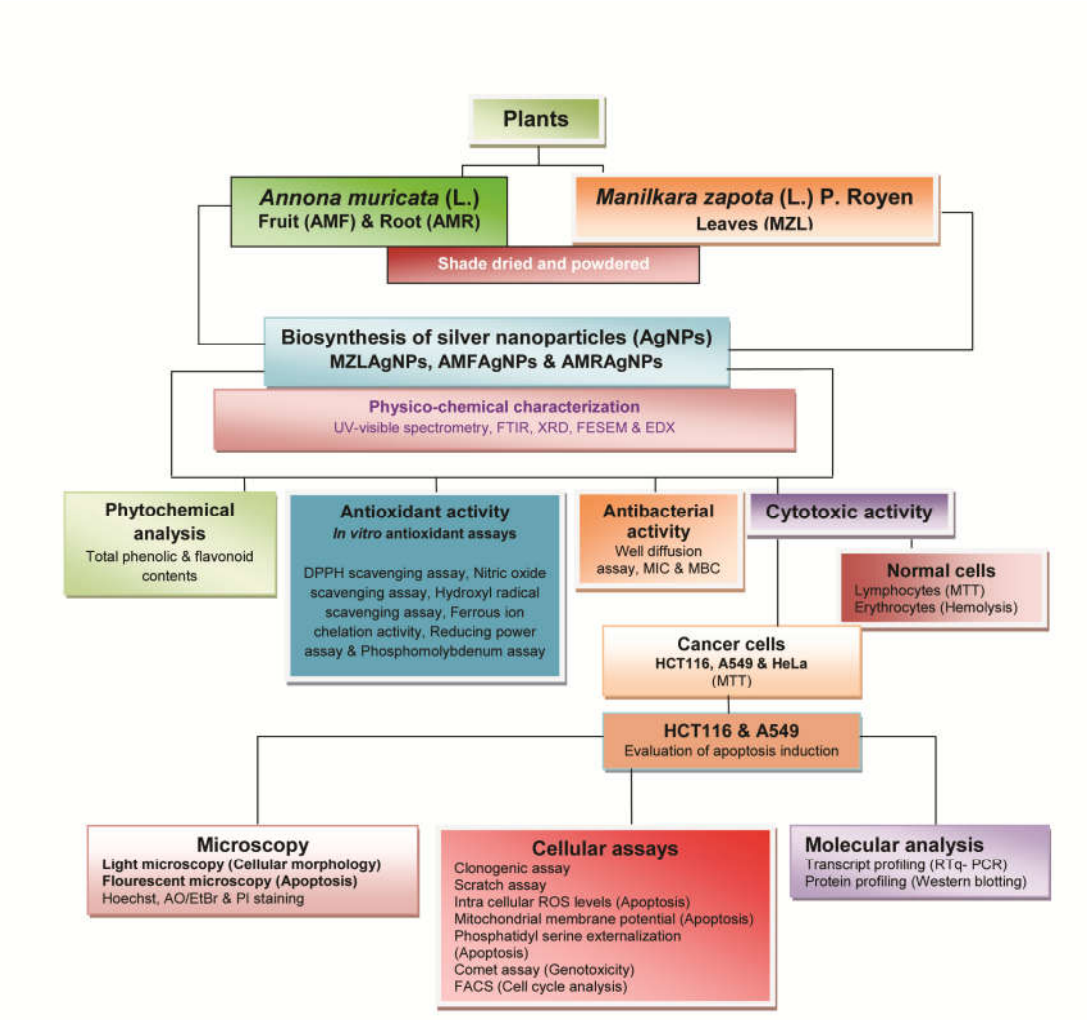

Fig. 1.1. ***Flowchart summarizing the doctoral research work presented in this Thesis.***

REVIEW OF LITERATURE

2.1. Nanotechnology and nanoparticles

Conceptually 'Nanotechnology' was first introduced by the famous physicist R.P. Feynman at the American Physical Society meeting at California Institute of Technology in 1959 when he described the probability of synthesis by the use of direct manipulation of atoms (Feynman, 1995). The term 'Nanotechnology' was coined by Norio Taniguchi of Tokyo University in 1974. Today the term signifies the latest field of modern research dealing with design, synthesis and applications of materials and devices whose size and shape have been engineered at the nanoscale. It is an area which includes almost all branches of science including biology, chemistry, physics, engineering, medicine, food and agriculture (Rejeski and Lekas, 2008) used to produce a broad range of products applicable to all these branches. Nanotechnologies are generally grouped as (i) *Wet nanotechnology* associated with living organisms such as enzymes, tissues, membranes and other cellular components (ii) *Dry nanotechnology* associated with physical chemistry and the production of inorganic compounds such as silicon and carbon and (iii) *Computational nanotechnology* associated with stimulations of nanometer-sized structures. These three dimensions correlate with each other for optimal functionality (Sinha et al., 2009). The synthesis of nanoscale materials with the preferred qualities is one of the most exciting aspects in recent nanoscience and nanotechnology. Nanotechnology supports diverse unique industries such as electronics, pesticide, medicines and parasitology, and thus provides a platform for collaboration (Bhattacharyya et al., 2010). Intense research in nanotechnology has opened up new avenues in medical technology for drug delivery, treatment and diagnosis for cancer, diabetes, pain, asthma, allergy, infections (Brannon-Peppas et al., 2004; Khan et al., 2017). Thus, introduction of the new term *nanomedicine* encompasses the use of nanotechnology in medicine by the manipulation of precisely engineered materials at nanoscale to develop novel therapeutic and diagnostic modalities (Zhang et al., 2008)

Nanoparticles are fundamental building blocks for nanotechnology. The prefix "nano" designates one billionth or 10^{-9} units. These are clusters of atoms in the size range of 1-100 nm (Williams, 2008) which are of great scientific interest as

they bridge the gap between bulk materials and atomic or molecular structures (Thakkar et al., 2010) falling in between microscopic and mesoscopic. They are considered as suitable diagnostic agents because of their shape, size and extraordinary optical and thermal characteristics (Mieszawska et al., 2013). Metallic nanoparticle synthesis (silver, gold, zinc etc) is the most fascinating area of investigation for researchers as they exhibit size and shape dependent properties that are of interest for applications in the fields of molecular biology, material sciences, biomedical engineering, electronics and medicine (Sudrik et al., 2006; Choi et al., 2007; Kanipandian et al., 2013).

2.2. Silver nanoparticles

Among the several noble metal nanoparticles, silver nanoparticles (AgNPs) stand out as the most used in all nanotechnology products (Doering and Nie, 2002). Silver at the nanoscale also has different properties from bulk silver. Interestingly, about 5000 years ago, many Greeks, Romans, Persians and Egyptians used bulk silver in one form or other to store food products (Grier, 1968). There are records about silver in literature as earlier as 300 BC. Until the discovery of antibiotics by Alexzander Flemming, silver was commonly used as an antimicrobial agent. AgNPs have gained boundless attention of the researchers due to their extraordinary defense against a wide range of microorganisms and also due to the appearance of drug resistance against common antibiotics (Sharma et al., 2009). These exceptional characteristics of AgNPs have spurred applications in diverse fields such as biomedical/ drug delivery (Chaloupka et al., 2010; Prow et al., 2011), water treatment (Dankovich and Grey, 2011) and agriculture (Nair et al., 2011). AgNPs form part of products such as inks, adhesives, electronic device and pastes due to high conductivity (Park et al., 2008). They also exhibit unique properties such as chemical stability, catalytic and more importantly anti-viral, antifungal in addition to anti-inflammatory activities, which can be incorporated into composite fibers, cryogenic superconducting materials, cosmetic products, antiseptic sprays, food industry and electronic components (Klaus-Joerger et al., 2001; Ahmad et al., 2003; Sharma et al., 2009). AgNPs are being successfully used in cancer diagnosis and treatment as well (Baruwati et al., 2009; Popescu et al., 2010).

2.3. Modes of synthesis of AgNPs

Generally there are two alternative approaches which are involved in the synthesis of metallic nanoparticles, the "bottom- up" approach and the "top- down" approach. Bottom-up or self assembly employs atom-by-atom, molecule-by-molecule or cluster-by-cluster construction of a structure. In this approach, initially the nanoparticles are formed and subsequently assembled into the final material using either chemical or biological procedures for synthesis. The bottom- up approach offers better flexibility and versatility with lesser defects in terms of material design. In the top-down approach, a suitable starting material is reduced in size using physical (mechanical) or chemical means. A major drawback of the top-down approach is the imperfection of the surface structure (Wiley et al., 2006; Thakkar et al., 2010; Natsuki et al., 2015). Top-down manufacturing of nanomaterials usually comprise mechanical energy, high energy lasers, thermal and lithographic methods. Both approaches apply for the synthesis AgNPs as depicted schematically in Fig. 2.1.

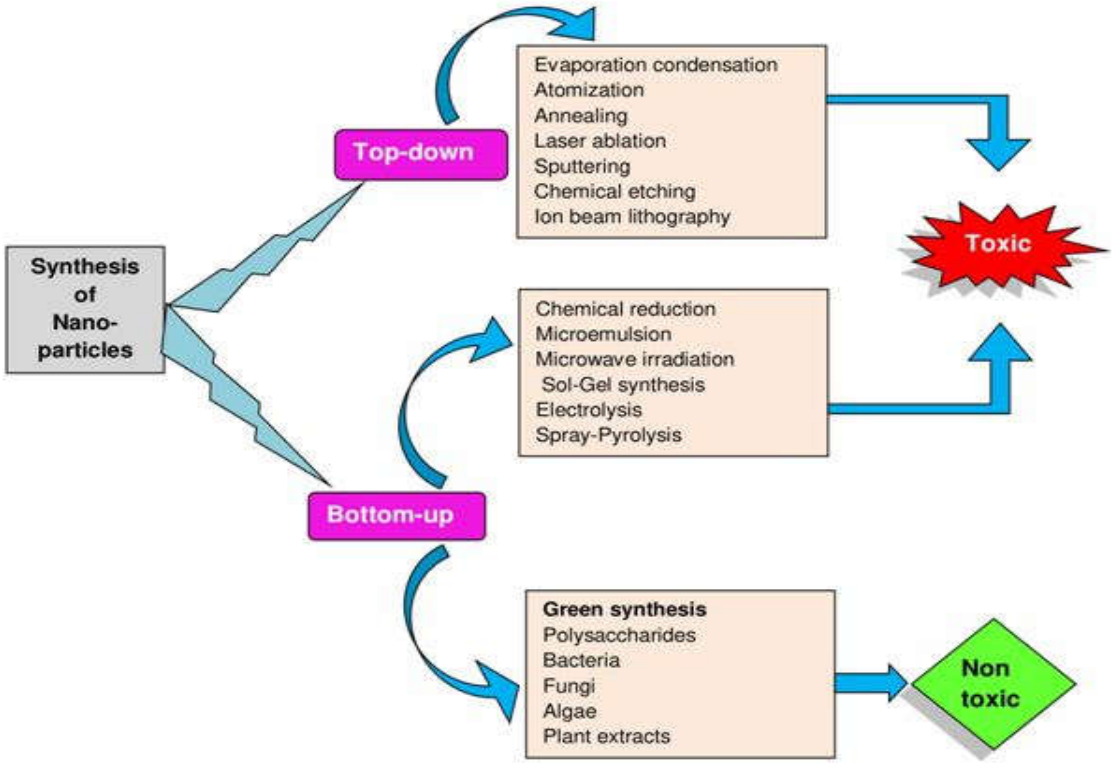

Fig. 2.1. ***Modes of synthesis of AgNps.***

2.3.1. Physical approaches

Nanoparticle synthesis by physical processes generally involves evaporation and condensation carried out using a tube furnace at atmospheric pressure. The source material within a boat centered at the furnace is vaporized into a carrier gas (Gurav et al., 1994; Magnusson et al., 1999). However, the tube furnace used for AgNPs synthesis has several disadvantages because as it occupies a large space, consumes a great deal of energy while raising the environmental temperature around the source material, and requires a long time to achieve thermal stability. Other methods in this category include atomization, annealing, arc discharge, laser ablation, radio frequency (RF), sputtering and focused ion beam lithography.

2.3.2. Chemical approaches

Chemical reduction is the most common method for the synthesis of AgNPs using organic and inorganic reducing agents. In general different reducing agents, such as sodium citrate, ascorbate, sodium borohydride ($NaBH_4$) hydroquinone, gallic acid, Tollens reagent, elemental hydrogen, polyol, N,N-dimethylformamide (DMF), polyethylene glycol, hydrazine, and ammonium formate are employed for reduction of silver ions (Ag+) in aqueous or nonaqueous solution (Pyatenko, 2010; Abbasi et al., 2014; Landage et al., 2014; Suriati et al., 2014). Microemulsion techniques and microwave-assisted synthesis are the other chemical methods implemented for AgNPs production (Malik et al., 2016) All these methods are widely used due to their straight forward nature and the potential to produce large quantities of the final product. However chemical approaches have certain limitations which include contamination from precursor chemicals, use of toxic solvents and generation of hazardous by-products.

2.3. 3. Biological methods

Biological methods have recently assumed predominance in the synthesis of metal nanoparticles. These methods are considered to be ‘green’ and more advanced than chemical and physical methods since they are environmentally benign, cost effective and easier to scale-up. Furthermore, these do not entail use of high temperature, pressure energy and toxic chemicals (Dhuper et al., 2012). A wide

range of biological reducing agents available in nature include polysaccharides, plant extracts, and an extensive range of microorganisms such as bacteria, actinomycetes, fungi, yeast and viruses which have been reported to be proficient in 'biogenic' AgNPs synthesis (Priester et al., 2014; Patra et al., 2014; Rahimi - Nasrabadi et al., 2014; Ovais et al., 2016). Recently microalgae were used for fabricating easily scalable, sustainable and environmentally safe nanoparticles (Dahoumane et al., 2017). The exact mechanism of biological synthesis of metal NPs is not yet understood well. It is believed that some enzymes play a role in reduction of metal ions to metal NPs (Honary et al., 2013; Subbaiya et al., 2017).

2.3.3.1. Synthesis using polysaccharides

In this method, AgNPs are synthesized using water as an ecofriendly solvent and polysaccharide as a capping agent, or sometimes, polysaccharide serves both as a reducing and capping agent. For example, starch-mediated synthesis was carried out with starch as capping and β-D-glucose as reducing agent in a mildly heated system (Raveendran et al., 2003) promoting weak attraction between starch and silver nanoparticles which can be revered at higher temperatures facilitating separation of the fabricated AgNPs (Mochochoko et al., 2013).

2.3.3.2. Microbe-mediated synthesis

Microorganisms as environmentally sustainable precursors have been shown to be important nanofactories due to their abundance and adaptation to extreme conditions, cost- effective cultivation, fast growth and ease of maintenance (Beyene et al., 2017). Microorganisms have the ability to acquire and depollute heavy metals due to various reductase enzymes, which are able to reduce metal salts to metal nanoparticles with a restricted size-distribution and polydispersity. Both *extracellular* and *intracellular* methods have been adopted for microorganism-mediated AgNPs synthesis. Among these, extracellular synthesis has achieved more attention because it eliminates the downstream processing steps essential for the release of nanoparticles. Intracellular synthesis, involves sonication to breakdown the cell wall, several centrifugation and washing steps for purification of nanoparticles (Singh et al., 2016).

The first report of silver nanoparticle synthesis involved utilization of a strain *Pseudomonas stutzeri AG259*, isolated from silver mine (Slawson et al., 1994). *Pseudomonas aeruginosa* (Kumar and Mamidyala, 2011; Das et al., 2016), *Escherichia coli* (Gandhi and Khan, 2016), *Weissella oryzae*, (Singh et al., 2015), *Bacillus methylotrophicus* (Wang et al., 2015), *Lactobacillus acidophilus* (Rajesh et al., 2015), *Klebsiella oxytoca* DSM 29614 (Buttacavoli et al., 2018), *Pseudomonas putida* MVP2 (Gopinath et al., 2015), *Bacillus licheniformis* (Elbeshehy, 2015), *Bacillus amyloliquefaciens* (Singh, 2011) have also been employed for AgNPs synthesis.

Fungus-mediated biosynthesis is advantageous over bacteria because it secretes high amount of proteins that amplifies production of metallic nanoparticles, is comparatively economical with simple downstream processing and biomass handling (Ingale and Chaudhari, 2013). Extracellular biosynthesis of silver nanoparticles using *Verticillium* AAT-TS-4 strain (Mukherjee et al., 2001), *Aspergillus Niger* (Gade et al., 2008), *Fusarium solani* (Ingle et al., 2009), *Pycnoporus* sp (Gudikandula and Maringanti, 2016) *Arthroderma fulvum* (Xue et al., 2016) *Trichoderma harzianum* (Guilger et al., 2017) and *Penicillium* sp (Naveen et al., 2010) have been employed. The extracellular AgNPs synthesis through silver-tolerant yeast strain MKY3 has also been reported (Kowshik et al., 2003). Further scope also exists for exploitation of other biological particles such as viruses, proteins, peptides and enzymes for nanoparticle synthesis.

2.3.3.3. Synthesis using plant extracts

'Phyto-nanotechnology' deals with green synthesis of metal nanoparticles through exploitation of plant resources. Plants have shown great potential in accumulation and detoxification of heavy metals and have attracted greater attention as a suitable alternative to physical approaches and chemical methods. They have proven to offer the best platform for nanoparticle synthesis since they can be cultivated free of toxic chemicals and are also capable of providing natural capping agents for rapid synthesis. A wide range of benefits over other biological systems include local availability, eco-friendliness, cost effectiveness, greater speed and non-

requirement of cell culture maintenance (Ahmed et al., 2016; Khatoon et al., 2017). Crude plant extracts contain a plethora of phytoconstituents such as phenolics, flavonoids, terpenoids, alkaloids, ketones, aldehydes, amides, sapogenins and carboxylic acids held responsible for bio-reduction of metal ions, conversion to metal NPs and also capping of NPs for stabilization (Chung et al., 2016).

Plant-based synthesis can be carried out either intracelluarly or extracellularly (Gardea-Torresdey et al., 2003; Harris and Bali, 2007; Haverkamp and Marshall, 2009; Dinesh et al., 2012; Renugadevi and Aswini, 2012; Hesgazy et al., 2015; Anjum et al., 2016). Intracellular synthesis takes place inside the plants growing in metal-rich media, soil and hydroponic solutions (Gardea-Torresdey et al., 2003; Haverkamp et al., 2006; Harris and Bali, 2007), whereas extracellular methods comprise use of leaf extracts / plant parts prepared by boiling or grinding in water or other solvents (Shankar et al., 2004; Ankamwar et al., 2005; Parashar et al., 2009). The latter method is considered better since it eliminates extraction and purification procedures (Makarov et al., 2014). Generally the solvent preferred for extraction of reducing agents from plants is water, but there are few reports on the use of organic solvents like methanol (Shafaghat et al., 2015; Sadeghi et al., 2015) and ethanol (Kulkarni et al., 2012; Logeswari et al., 2015) Despite differences in the solvents used for extraction, nanoparticle suspensions are generally made in aqueous media only. The bioreduction mechanism of metal nanoparticles in plant extracts is thought to occur in three main phases. The activation phase in which the reduction of metal ions and nucleation of the reduced metal atoms occur. The growth phase involves spontaneous coalescence of the small adjacent nanoparticles into particles of a larger size, accompanied by an increase in the thermodynamic stability of nanoparticles (a process referred to as Ostwald ripening) and the termination phase in which the nanoparticles are finally shaped (Keat et al., 2015).

The first report of a plant-based living system used for extracellular synthesis of nanoparticles is credited to *Medicago sativa* (alfalfa) endowed with the potential for synthesizing silver and gold nanoparticles exploiting its biochemical composition (Gardea-Torresdey et al., 2003). Alfala roots have the ability for

absorbing Ag from agar medium and transferring them into the shoots of the plant in the same oxidation state. In the shoots, these Ag atoms form nanoparticles by joining leading to larger arrangements. Rapid synthesis of AgNPs using geranium leaves taking around 9 hours at 90% reaction was shown by Shankar et al. (2004) compared to the 24-124 hours needed as per the earlier report mentioned above.

Nanoparticles are stable in solution even after four weeks of synthesis. Plant parts like leaf, bark, root, stem and fruits have been used for AgNPs synthesis (Table 2.1). The medicinally important plants such as *Tinospora cordifolia* (Anuj and Ishnava, 2013), *Boerhaavia diffusa* (Vijaykumar et al., 2014), *Aloe vera* (Chandran et al., 2006), *Terminalia chebula* (Edison et al., 2012), *Catharanthus roseus* (Mukunthan et al., 2011), *Ocimum sanctum* (Mallikarjuna et al., 2011), *Ocimum tenuiflorum* (Patil et al., 2012), *Azadirachta indica* (Tripathi et al., 2009; Ahmed et al., 2016), *Emblica officinalis* (Ankamwar et al., 2005), *Piper nigrum* (Shukla et al., 2010) and *Cinnamon zeylanicum* (Sathishkumar et al., 2009) have also been employed for AgNPs synthesis.

Table 2.1. Green synthesis of silver nanoparticles by different researchers using plant extracts

Sl No.	Plants	Plant parts	Size (nm)	Shape	References
1	*Abutilon indicum*	Leaves	7-17	Spherical	Ashokkumar et al., 2015
2	*Acalypha indica*	Leaves	20-30	Spherical	Krishnaraj et al., 2010
3	*Acorus calamus*	Rhizome	31.83	Spherical	Nakkala et al., 2014
4	*Aerva lanata*	Leaves	18.62	Spherical	Joseph and Mathew, 2014
5	*Allium sativum*	Leaves	4-22	Spherical	Ahamed et al., 2011
6	*Allophylus serratus*	Leaves, Calli	40-50	Spherical	Jemal et al., 2017
7	*Aloe vera*	Leaves	15-20	Sphericlal Triangualr	Chandran et al., 2006; Ashraf et al., 2015
8	*Alstonia scholaris*	Bark	50	Spherical	Shetty et al., 2014
9	*Alysicarpus monilifer*	Leaves	15	Spherical	Kasithevar et al., 2017
10	*Annona muricata*	Leaves	20-53	Spherical	Santhosh et al., 2015

11	*Annona squamosa*	Leaves	20-100	Spherical	Vivek et al., 2012
12	*Argyreia nervosa*	Seeds	20-50		Thombre et al., 2014
13	*Artocarpus heterophyllus*	Seed	10.78	Spherical, irragualr	Jagtap et al., 2013
14	*Averrhoa carambola*	Leaves	14	Spherical	Mishra et al., 2015
15	*Azadirachta indica*	Leaves	35-50	Spherical	Shankar et al., 2004
16	*Boerhaavia diffusa*	Plant	25	Spherical	Nakkala et al., 2014
17	*Boswellia ovalifoliolata*	Bark	30-40	Spherical	Ankanna et al., 2010
18	*Boswellia serrata*	Gum	7-11	-	Jyothi et al., 2012
19	*Budleja globosa*	Leaves	16	Spherical	Carmona et al., 2017
20	*Butea monosperma*	Bark	35	Spherical	Pattanayak et al., 2015
21	*Calotropis gigantean*	Latex	5-30	Spherical	Rajkuberan et al., 2015
22	*Calotropis procera*	Plant	19-45	Spherical	Gondwal et al., 2013
23	*Camellia sinensis*	Leaves	20-90	Spherical	Suna et al., 2014
24	*Catharanthus roseus*	Leaves	25-30	Spherical	Kotakadi et al., 2013
25	*Capparis spinosa*	Leaves	5-30	Spherical	Benakashani et al., 2016
26	*Carica papaya*	Leaves	50-250	Spherical	Banala et al., 2015
27	*Carica papaya*	Fruit	10-50	Cubic hexagoanal	Jain et al., 2009
28	*Centella asiatica*	Leaves	30-50	Spherical	Rout et al., 2013
29	*Chenopodium murale*	Leaves	35-50	-	Abdel-Aziz et al., 2014
30	*Cinnamon zeylanicum*	Bark	30-40	Quasi spherical	Sathish kumar et al., 2009
31	*Citrullus colocynthis*	Calli	31	Spherical	Satyavani et al., 2011
32	*Citrus sinensis*	Peal	48-60	Spherical	Barros et al., 2018
33	*Clerodendrum serratum*	Leaves	5-30	Spherical	Raman et al., 2015
34	*Cocos nucifera*	Inflorescence	22	Spherical	Mariselvam et al., 2014
35	*Cocos nucifera*	Coir	22	Spherical	Roopan et al., 2013

36	*Cola nitida*	Pod	12-80	Spherical	Lateef et al., 2016
37	*Couroupita guianensis*	Leaves, fruit	5-15	Cubic	Vimala et al., 2015
38	*Crataegus douglasii*	Fruit	29	Spherical	Moghaddam and Hadi-Dabanlou, 2014
39	*Datura stramonium*	Leaves	15-20	Spherical	Gomathi et al., 2017
40	*Delphinium denudatum*	Root	85	Spherical	Suresh et al., 2014
41	*Dillenia indica*	Fruit	40-100	-	Singh et al., 2013
42	*Dioscorea batatas*	Rhizome	10-880	Spherical	Sreekanth et al., 2012
43	*Eclipta prostrate*	Leaves	35-60	Triangles, pentagons	Rajakumar and Abdul Rahuman, 2011
44	*Elaeagnus indica*	Leaves	30	Spherical	Natarajan et al., 2013
45	*Emblica Officinalis*	Fruit	10-20	-	Ankamwar et al., 2005
46	*Emblica officinalis*	Leaves	15	Spherical	Ramesh et al., 2015
53	*Eucalyptus leucoxylon*	Leaves	55	Spherical	Nasrabadi et al., 2014
54	*Euphorbia helioscopia*	Leaves	2-14	Spherical	Nasrollahzadeh et al., 2015
55	*Ficus carica*	Leaves	21	Crystalline	Borase et al., 2015
56	*Garcinia mangostana*	Leaves	35	-	Veerasamy et al., 2010
57	*Gossypium hirsutum*	Leaves	30	Spherical	Kanipandian et al., 2014
59	*Grewia flaviscences*	Leaves	50-70	Spherical	Sana et al., 2015
60	*Helianthus annus*	Leaves		-	Leela and Vivekanandan, 2008
61	*Helicteres isora*	Root	16-95	Spherical	Bhakya et al., 2015
62	*Hemidesmus indicus*	Leaves	25.24	Spherical	Latha et al., 2015
63	*Hibiscus cannabinus*	Leaves	9	Spherical	Bindhu and Umadevi, 2013.
64	*Hypnea musciformis*	Leaves	40-65	Spherical	Roni et al., 2015
65	*Iresine herbstii*	Leaves	44-64	Spherical	Dipankar et al., 2012
66	*Ixora coccinea*	Leaves	13-57	Spherical	Karuppiah and Rajmohan, 2013

67	*Jatropha curcas*	Seed	15-50	Spherical	Bar et al., 2009
68	*Justicia adhatoda*	Leaves	5-50	Spherical	Bose and Chatterjee, 2015
69	*Lansium domesticum*	Fruit	10-30	Spherical	Shankar et al., 2014
70	*Lonicera japonica*	Leaves	36-72	Spherical	Kumar and Yadav, 2011
71	*Lycopersicon esculentum*	Fruit	10-40	Spherical	Maiti et al., 2014
72	*Macrotyloma uniflorum*	Seed	12	Anisotropic	Vidhu et al., 2011
73	Malus domestica	Fruit	30-40	Spherical	Roy, 2014
74	*Mangifera Indica*	Leaves	20	Triangular-hexagonal	Philip, 2011
75	*Mangifera indica*	Peel	7-27	Spherical	Yang et al., 2013
76	*Melia azedarach*	Leaves	78 34-48	Cubical, spherical	Sukirtha et al., 2012; Mehamood et al., 2013
77	*Melia dubia*	Leaves	35	Spherical	Kathiravan et al., 2014
78	*Memecylon edule*	Leaves	20-50	Triangular, circular	Elavazhagan et al., 2011
79	*Mentha piperita*	Leaves	90	Spherical	Mubarak Ali et al., 2011
80	*Momordica cymbalaria*	Fruit	155	Spherical	Swamy et al., 2015
81	*Morinda citrifolia*	Leaves	10-60	Spherical	Sathishkumar et al., 2012
82	*Morinda cirtifolia*	Root	30-55	Spherical	Suman et al., 2013
83	*Morinda tinctoria*	Leaves	79-76	Spherical, rod	Vanaja et al., 2014
84	*Moringa oleifera*	Leaf	11	Rectangle	Nayak et al., 2015
85	*Mukia maderaspatana*	Leaves	13-34	Spherical	Chitra et al., 2015
86	*Mukia scabrella*	Leaves	18-21	Spherical	Prabakar et al., 2013
87	*Musa paradisiacal*	Peel	23.7	Spherical	Ibrahim, 2015
88	Myrmecodia pendan	Plant	10-20	Spherical	Zuas et al., 2014
89	*Nelumbo nucifera*	Leaves	25-80	Spherical, triangular	Santhoshkumar et al., 2011
90	*Nelumbo nucifera*	Root	16.7	Polydispersed	Sreekanth et al., 2014

91	*Nothapodytes nimmoniana*	Fruit	44-64	Spherical	Mahendran et al., 2016
92	*Parthenium hysterophorous*	Root		Spherical	Mondal et al., 2014
93	*Pelargonium graveolens*	Leaves	16-20	Nearly Spherical	Shankar et al., 2003
94	*Piper betel*	Leaves	28-17	Spherical	Rani and Reddy, 2011
95	*Piper longum*	Fruit	46	Spherical	Reddy et al., 2014
96	*Pistacia atlantica*	Seeds	10-50	Spherical	Sadeghi et al., 2015
97	*Plukenetia volubilis*	Leaves	4-25	Optical	Kumar et al., 2015
98	*Pogostemon benghalensis*	Leaves	> 80	-	Gogoi et al., 2013
99	*Portulaca oleracea*	Leaves	< 60	-	Firdhouse et al., 2012
100	*Premna herbacea*	Leaves	10-30	Spherical	Kumar et al., 2013
110	*Prosopis farcta*	Leaves	10.8	Spherical	Miri et al., 2015
111	*Psoralea corylifolia*	Seeds	100-110	-	Sunita et al., 2014
112	*Quercus brantii*	Leaves	6	Spherical,polydispersed	Korbekandi et al., 2015
113	*Rizophora mucronata*	Leaves	60-95	Spherical	Gnanadesigan et al., 2011
114	*Rumex hymenosepalus*	Root	2-40	Tube, hexagonal	Rodriguez-Leon et al., 2013
115	*Saccharum officinarum*	Leaves	10-60	Spherical	Velu et al., 2017
116	*Saraca indica*	Leaves	23	Spherical	Perugu et al., 2016
117	*Sesbania grandiflora*	Leaves	22	Spherical	Jeyaraj et al., 2013
118	*Sesuvium portulacastrum*	Leaves	20-90	Spherical	Nabikhan et al., 2010
119	*Sinapis arvensis*	Seed	14	Spherical	Khatami et al., 2015
120	*Solanum nigrum*	Leaves	20-40	Spherical	Kumar et al., 2017
121	*Solanum torvum*	Leaves	14	Spherical	Govindaraju et al., 2010
122	*Swietenia mahogany*	Leaves	50	-	Mondal et al., 2011
123	*Tephrosia tinctoria*	Stem	73	Spherical	Rajaram et al., 2015
124	*Thuja occidentalis*	Plant	111	Spherical	Das et al., 2013

125	*Tinospora cordifolia*	Stem	60	Spherical	Anuj and Ishnava, 2013.
126	*Torreya nucifera*	Leaves	10-125	Spherical	Kalpana et al., 2014
127	*Trachyspermum ammi*	Seeds	87,99.8	-	Vijayaraghavan et al., 2012
128	*Trianthema decendra*	Leaves	17.9-59	Spherical	Geetha lakshmi and Sarada, 2013
130	*Tribulus terrestris*	Fruit	16-28	Spherical	Gopinath et al., 2012
131	*Trichodesma indicum*	Leaves	20-50	Spherical	Buhroo et al., 2017
132	*Viburnum lantana*	Leaves	20- 80	Spherical	Shafaghat et al., 2014
133	*Vitex negundo*	Leaves	≥20	Cubic	Zargar et al., 2014
134	*Vitis vinifera*	Fruit	30-40	-	Gnanajobitha et al., 2013
135	*Withania somnifera*	Leaves	5-30	Spherical	Raut et al., 2014
136	*Wrightia tinctoria*	Leaves	5-20.5	-	Rajathi and Sridhar, 2013
137	*Ziziphora tenuior*	Leaves	8-40	Spherical	Sadeghi and Gholamhoseinpoor 2015

2.3.3.3.1. Factors Affecting silver nanoparticle synthesis

The physico-chemical factors that affect the reduction process of silver ions to silver nanoparticles are (i) metal ion concentration (ii) extract concentration (iii) reaction temperature (iv) pH of the reaction mixture and (v) incubation time. These parameters are highly influential in determining the size, shape and morphology of the AgNPs.

Effect of silver nitrate concentration : Silver transforms into particles on addition of the plant extract, which results in color change. The solution turns dark yellow-brown due to the phenomenon of *surface plasmon resonance* (SPR) (Ankanna et al., 2010). Silver ion concentration greatly influences the biosynthesis process. Several researchers have studied about the impact of metal ion concentration in biogenic synthesis. Park (2014) reported that the variation of concentration of silver nitrate from 0.25 mM to 1.0 mM results in increased absorbance and sharper peaks.

Increase in metal ion concentration from 1-3 mM also reportedly results in larger particle size (Dubey et al., 2010; Ibrahim, 2015).

Effect of extract concentration : Changes in plant extract concentration also affect significantly the synthesis of AgNPs (Song and Kim, 2009; Bar et al., 2009). For instance, nanoparticles synthesis was found to vary with the use of different concentrations of garlic extract; higher concentrations lead to initiation of polydispersed AgNPs (White et al., 2012). Higher plant extract concentrations provide higher levels of bioconstituents for the metal reduction process characterized by a blue shift and sharp absorbance peak (Huang et al., 2007; Sathishkumar et al., 2009; Khalil et al., 2014) thereby reducing the mean diameter of NPs. The increased number of biomolecules results in nanoparticle agglomeration which reduces the absorbance of UV-vis spectrum (Krishnaraj et al., 2010).

Effect of reaction temperature : The reaction temperatures also influence greatly the process of silver reduction in terms of size and shape. This is due to increased kinetic energy at higher temperature leading to increased consumption of silver ions to produce smaller particles (Park et al., 2008; Kora et al., 2010; Singhal et al., 2011; Ibrahim, 2015; Verma and Mehata, 2016). The rate of synthesis and final conversion to AgNPs has also been observed to be faster with higher reaction temperatures (Song and Kim, 2009).

Effect of pH : The pH values in the range of 2-14 have been found to be critically important in determining the size, shape, production rate and stability of AgNPs (Gan and Li 2012; Vivek et al., 2012). Larger sized particles with ellipsoidal shape were mostly formed at lower pH values whereas significantly smaller spherical nanoparticles were obtained at higher pH conditions (Sathishkumar et al., 2009). The synthesis and development of nanoparticles is delayed by acidic conditions whereas basic conditions stimulate nanoparticle assembly (Klaus-Joerger et al., 2001; Korbekandi et al., 2009; Chung et al., 2016). In plants, depending on the species, synthesis occurs at different pH values. However, under extreme acidic conditions such as at pH 2.0, the reduction reactions are inactivated (Veerasamy et al., 2011).

Effect of incubation time : Exposure of metal ions to reducing agents also affects nanoparticle synthesis which is directly proportional to the incubation time (Krishnaraj et al., 2010; Gan and Li, 2012). SPR of AgNPs reportedly increases with time and becomes constant indicating completion of synthesis (Khatoon et al., 2017). Stabilization or completion of AgNP synthesis occurs at different incubation times dependent on the plant species used (Chandran et al., 2006; Vilchis- Nestor et al., 2008; Sulaiman et al., 2013; Thirunavoukkarasu et al., 2013).

2.3.3.4. Characterization of silver nanoparticles

Characterization of the metallic nanoparticle is critical not only for their applications but also provides necessary insights on factors controlling nanoparticles synthesis. Characterization is performed using a wide range of analytical techniques such as scanning / transmission electron microscopy (SEM/TEM)), powder x-ray diffractometry (XRD), energy dispersive X-ray spectroscopy (EDX), Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), atomic force microscopy (AFM), and UV-visible spectroscopy (Schaffer et al., 2009; Strasser et al., 2010; Shahverdi et al., 2011; Kim et al., 2014; Mehmood et al., 2014; Das et al., 2016). These techniques are used to resolve diverse parameters of the particle pertaining to their size, shape, crystallinity, fractal dimensions, surface area and surface plasmon resonance. Additionally these also provide information on orientation, intercalation, pore size, dispersion and stabilization of the particles with respect to the nano-composite materials (Ingale and Chaudhari, 2013). Preliminary confirmation of the production of nanoparticles is carried out by UV-Vis spectroscopy (Desai et al., 2012). For AgNPs, the characteristic absorbance values fall within the wavelength range between 410-460 nm (Huang and Yang, 2004) due to utilization of light in the visible and nearby IR region since. Absorption or reflectance in the visible range is a consequence of the apparent change in the color of the chemicals involved accompanying nanoparticle synthesis. The phase variety and crystal structure of synthesized AgNPs is analyzed by X-ray diffraction (XRD) spectroscopy, an important tool for identification of atomic and molecular structure of a crystal (Ramteke et al., 2013). The XRD for silver is authenticated by the 'Joint Committee on Powder Diffraction Standards' based JCPDS file. The presence of elemental silver is appraised by EDX technique. FTIR technique is used to evaluate the 'putative' biomolecules present in plant extracts involved in reduction and

capping of silver ions, leading to efficient stabilization of the synthesized AgNPs (Dipankar and Murugan, 2012). The morphology, particle size and size-distribution is evaluated by TEM, SEM and AFM; DLS is also utilized for determination of particle size distribution (Abou El-Nour et al., 2010).

2.3.3.5. Biomedical applications of silver nanoparticles

Due to their physical and chemical properties biogenic nanoparticles have been exploited enormously for applications in the areas of molecular biology and medicine often referred to as 'nanomedicine'. The excellent versatility combined with stability in different biological media have catapulted their use for numerous applications (Shukla et al., 2008; Ahmed et al., 2016) briefly reviewed below.

Antibacterial activity : The AgNPs exhibit excellent bactericidal action against both gram-positive and gram-negative bacteria (Jones and Hock., 2010; Nazeruddin et al., 2014) which includes numerous highly pathogenic bacterial strains. Antibacterial activity of AgNPs is influenced by different physical characteristics of the particle such as its shape, mass, size, exposure time, silver concentration, pH, associated macromolecules (Marambio-Jones and Hosk., 2010; Sadeghi et al., 2012) as well as the target microorganisms (Guzman et al., 2011). Smaller AgNPs, compared to larger, possess greater bactericidal property due to availability of increased binding surfaces with their shapes also contributing towards the same (Kvitek et al., 2008; Monteiro et al., 2009). Gram-positive and gram-negative bacteria have different sensitivities towards AgNPs due to variations in the thickness and molecular composition of the membrane structures of bacteria (Kim et al., 2007). Silver nanoparticles synthesized using *Abutilon indica* leaf extract have been shown to possess potent antibacterial activity on S*taphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi* and *E.coli* with an inhibition zone ranging from 14.5 - 18.3 mm (Ashokkumar et al., 2015). Likewise, bactericidal activity of AgNPs synthesized with *Morinda citrifolia* leaf extracts against a wide range of human pathogens, such as *E.coli, P. aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes* (gram-negative), *Bacillus cereus*, and *Enterococcus sp*. (gram-positive) has also been studied (Sathishkumar et al., 2012). AgNPs synthesized with *Origanum vulgare* leaf extracts have been reported with broad-spectrum antibacterial activity against nine different human pathogens. Higher than 10-mm zones of inhibition were observed against *E.coli* (enteropathogenic, EP), *Aeromonas hydrophila*, *Salmonella paratyphi*,

Salmonella sp., Shigella dysenteriae, and *Shigella sonnei* (Sankar et al., 2014). Bactericidal activity of AgNPs synthesized with *B. diffusa* plant extracts against three fish bacterial pathogens, *Aeromonas hydrophila, Pseudomonas fluorescens,* and *Flavobacterium branchiophilum* have also been investigated by Kumar et al. (2014). The antimicrobial activity of the medicinal plant *Onosma dichroantha* derived nanoparticles employing silver chloride instead of silver nitrate was a novel approach to the development of bactericides for a wide range of applications including treatment of burn wounds and injuries (Nezamdoost et al., 2014).

Antifungal activity : Fungal infections are most common in immune-compromised patients due to human immunodeficiency viral infections (Martin et al., 2003). One of the most common pathogens responsible for fungal infections is *Candida* species. The antifungal activity of AgNPs against 44 strains of six fungal species (*Candida albicans, C. tropicalis, C.glabrata, C. parapsilosis, C. krusei* and *Trichophyton mentagrophytes*) was reported earlier (Kim et al., 2009). Synthesis of AgNPs employing extracts of *Boswelliao valifoliolata, Shoreatum buggaia* and *Svensonia hyderobadensis*, their antifungal activity against *A. flavus, A. niger, Curvularia* sp*., Fusarium* sp. and *Rhizopus* sp. were evaluated (Savithramma et al., 2011). The results showed that all the three phyto-derived nanoparticles exhibited significant antifungal activity. Likewise chickpea seed - mediated AgNPs were also found displaying antifungal activities against *Rhizoctonia solani*, *Alternaria flavus*, and *Alternaria alternate* (Kaur et al., 2012). However, none of these reports offer a precise mechanism underlying the antifungal effect. One of plausible explanations put forth implicates destruction of membrane integrity of fungi and inhibition of the budding process in the case of yeast (Kim et al., 2009).

Antiviral activity : Viruses are acknowledged as one of the most eminent causes of disease and death worldwide (Rai et al., 2014). The use of AgNPs have evolved as one of the best options for management of viral infections (Galdiero et al., 2011) especially to prevent infections after surgery and as effective anti-HIV-1 agents (Elechiguerra et al., 2005). AgNPs reportedly interact preferentially with gp120 glycoprotein knobs of HIV-1 resulting in inhibition of viral binding to host cells (Lara et al., 2010). AgNPs have been shown to possess antiviral activity against a number of viruses infecting both prokaryotic and eukaryotic organisms (De-

Gusseme et al., 2010; Narasimha, 2012). Viral inhibition is dependent on the size of AgNPs; use of small sized AgNPs, 25nm or less, were found to be more effective against viral infections (Lara et al., 2010; Speshock et al., 2010).

Anti-parasitic activity : AgNPs have been shown to act as potent larvicidal agents against the dengue vectors - *Aedes aegypti* (Suresh et al., 2014) *Culex quinquefasciatus* (Mondal et al., 2014), malarial vectors - *Anopheles subpictus* (Rajakumar and Rahuman, 2011), *Aedes aegypti* (Kumar and Yadav, 2011) and other parasites (Marimuthu et al., 2011; Roopan et al., 2013). AgNPs of 25-30 nm size, synthesized using leaf extract of *Nerium oleander*, showed high mortality against both larvae and pupae of *Anopheles stephensi* (Roni et al., 2013). AgNPs synthesized using aqueous extracts of *Ashoka* and *Neem* leaves have been shown to possess appreciable antiplasmodial activity against *Plasmodium falciparum* with IC_{50} values of 8.0 and 30 μg/mL, respectively (Mishra et al., 2013).

Anti-diabetic activity : *Tephrosia tinctoria* stem extract - mediated AgNPs have been shown to scavenge free radicals. Further, they control blood sugar by decreasing levels of enzymes that hydrolyse complex carbohydrates (α-glucosidase and α-amylase) concomitant with boosted glucose uptake (Rajaram et al., 2015). *In vitro* and *in vivo* antidiabetic activity of *Pouteria sapota* leaf extract-mediated AgNPs have also been confirmed (Prabhu et al., 2018).

Anti-inflammatory and wound healing activities : AgNPs are known to possess strong anti-inflammatory activities (Paquet and Pierard, 1996; Ajuebor et al., 1999; Bhol and Schechter, 2007). Employing peritoneal adhesion and burn wound mice models, Wong et al. (2009) reported sustenance of higher mRNA levels of the anti-inflammatory cytokine, (IL-6) in the AgNPs treated mice group compared to that observed with other silver compounds, $AgNO_3$ and AgCl. Optimal dose of AgNPs (100 nm) induced heat shock protein (Hsp70) over expression and anti-inflammatory effect in Clone 9 cells (Ho et al., 2013). AgNPs dressings reportedly stimulated curing of stalled chronic leg ulcers in a human clinical study (Sibbald et al., 2007). Decreased strength of pathogenic bacteria in the wound resulted in lower neutrophil infiltration and inflammation. *Indigofera aspalathoides* and *Arnebia nobilis* extract

derived AgNPs stimulated wound healing activity in an excision animal model imparting new therapeutic direction for wound treatment in clinical practice (Arunachalam et al., 2013; Garg et al., 2014). AgNPs have been shown to affect differentiation of fibroblasts into myofibroblasts and promote wound contraction, thereby increasing the wound healing capability (Gunasekaran et al., 2011). Biogenic silver nanoparticles also play an important role in dermal contraction and re-epithelialisation during wound healing process resulting in increased rate of wound closure (Liu et al., 2010).

Anticancerous activity : Based on their origin, a variety of cancers exist such as thyroid, prostate, bladder, kidney, pancreatic, breast, melanoma, all types of leukemia, oral and colo-rectal. Conventional cancer treatments include chemotherapy, radiotherapy and surgery. Of these, the former two modes not only kill or damage cancer cells but are also notorious for non-selective adverse effects on normal healthy cells within the body besides being expensive (Fig. 2.2). Exploiting nanoparticles for cancer treatment overcomes such problems (Singh et al., 2017). Biogenic nanoparticles perform well as cytotoxic agents for cancer therapeutics since their cellular interactions generate reactive oxygen species (ROS) affect biomolecules like carbohydrates, proteins, lipids, DNA and RNA resulting in apoptotic and necrotic cell death (Asharani et al., 2009a; Asharani et al., 2009b). Cytotoxicity in turn depends on the cell type, interaction and distribution pattern of the nanoparticles. The size of the nanoparticle influences their binding to and activation of membrane receptors resulting in altered protein expression in cancer cells (Xi Feng et al., 2016). Depending on their size and concentration, AgNPs have been employed as sensitizers for the cure of radio-resistant glioblastoma malignant tumor (Xu et al., 2009). Biogenic AgNPs from *Sesbania grandiflora* leaf extracts induced free radical generation resulting in oxidative damage and caspase-mediated apoptosis (Das et al., 2013). Biosynthesized AgNPs from the leaf of *Suaeda monoica* exhibited dose-dependent toxicity against Hep-2 cells (Satyavani et al., 2012). Green synthesized silver nanoparticles from *Premna serratifolia* leaves reportedly displayed significant anticancer activity in carbon tetrachloride (CCl_4) induced liver cancer in Swiss albino (BALb/c) mice (Paul et al., 2015). Sre et al.

(2015) have reported on the cytotoxity of biogenic AgNPs from *Erythrina indica* against MCF-7 (breast) and HepG2 (hepatocellular carcinoma) cells. Green AgNPs from *Saccharina japonica* extract was found to be cytotoxic on cervical carcinoma cells (Sreekanth et al., 2016) whilst those derived from *Euphorbia nivulia* latex were cytotoxic against human lung carcinoma (A549) cells in a dose -dependent manner (Sukumar et al., 2013). Biogenic AgNPs from *vitex negundo* leaf extract showed antiproliferative effects on colon cancer cell line HCT 15 by arresting growth phase, decreasing DNA synthesis and inducing apoptosis (Prabhu et al., 2013). AgNPs derived from unripe fruit extract of *Solanum trilobatum* displayed mitochondria-mediated apoptosis in MCF-7 cells (Ramar et al., 2015). Biogenic AgNPs synthesized using green petals of *Rosa indica* were found to scavenge free radicals and induce apoptosis by generation of ROS in HCT-15 cells (Manikandan et al., 2015). AgNPs biosynthesized using *Ficus religiosa* leaf extract showed effective antitumor activity against DAL induced mice (Antony et al., 2013). Table 2.2 carries a summary of plant-mediated anticancer activities of silver nanoparticles against various types of human cancer cells encountered during literature survey.

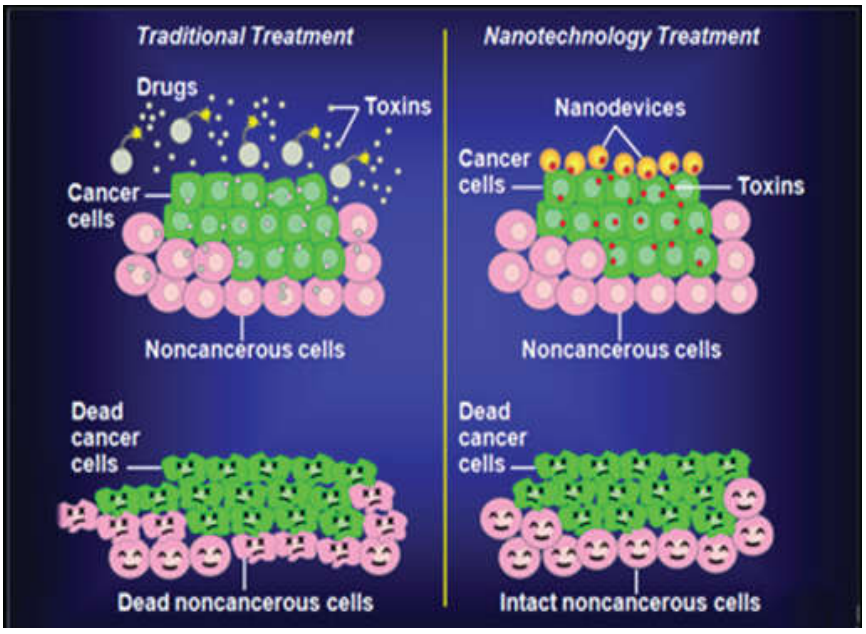

Fig. 2.2. ***Traditional versus nanotechnology-based treatment modes*** *(Singh et al., 2017).*

Antioxidant activity : Antioxidants are capable of deactivating free radicals before they attack cells and biological targets to cause various diseases (Hermans et al.,

2007). Previous reports of AgNPs synthesized from *Iresine herbstii* leaf extract showed good antioxidant activities (Dipankar and Murugan, 2012). Biogenic AgNPs from *Morinda pubescens* (Inbathamizh et al., 2013), *Gossypium hirsutum* (Kanipandian and Thirumurugan, 2014) and *Helicteres isora* (Bhakya et al., 2015) have shown *in vitro* antioxidant activities. Leptadenia *reticulate* leaf extract mediated AgNPs also exhibited high radical scavenging activity (Chung et al., 2016). Plant extracts stimulate DPPH radical scavenging activity of AgNPs in a dose-dependent manner. The ability of antioxidants to scavenge DPPH radicals is probably due to their ability to donate hydrogens and easily incorporate electrons; the latter is possible due to the presence of host lipophilic radicals (Nie et al., 2007).

2.3.3.6. Miscellaneous applications of AgNPs

Chrysanthemum morifolium extract derived AgNPs have been incorporated in clinical ultrasound gels for use with ultrasound probe since it exhibits bactericidal activity and contributes to the sterility of instrument (He et al., 2013). *Manilkara zapota* extract based AgNPs displayed acaricidal activity against *Rhipicephalus (Boophilus) microplus* (Rajakumar and Rahuman, 2012). AgNPs biosynthesized using *Acacia nilotica* pod extract have been employed to treat glassy carbon electrodes, which exhibited greater catalytic activity in reducing benzyl chloride compared to glassy carbon and metallic silver electrodes (Jebakumar et al., 2013). *Calliandra haematocephala* leaf extract-derived AgNPs showed hydrogen peroxide sensing capability (Raja et al., 2015). Phytofabricated AgNPs using *Artemisia tournefortiana* aerial part extract was found capable of degrading Coomassie Brilliant Blue 250-dye within 60 min demonstrating their photocatalytic property (Baghbani-Arani et al., 2017). *Jatropha gossypifolia* derived AgNPs exhibited higher amoebicidal activity against *Acanthamoeba castellanii trophozoites* (Borase et al., 2013). Using fresh leaf of *Anacardium occidentale* for synthesis of stable AgNPs used as a novel probe, toxic hexavalent chromium [Cr(VI)] in tap water could be sensed (Balavigneswaran et al., 2014). Ayurvedic medicine, *Guggulutiktham Kashayam* derived AgNPs were found to possess a size dependent catalytic activity based on the reduction of methylene blue in the presence of NaBH (Suvith and Philip, 2014). *Triticum aestivum* extract derived AgNPs proved to be an

excellent nanocatalyst for reduction of hydrogen peroxide (Waghmode et al., 2013). *Breynia rhamnoides* mediated AgNPs have been employed for degradation of 4-nitrophenol (4-NP) to 4-aminophenol (4-AP) (Gangula et al., 2011).

Table 2.2. Phyto-source for synthesis of AgNPs and their anticancer activity in various cell lines.

SI. No	Plant	Part used	Size (nm)	Morphology	Cancer cell lines	IC_{50} µg/ml	Reference
1	*Abelmoschus esculentus*	Pulp	6.7	Spherical	Jurkat	16.15	Mollick et al., 2015
2	*Acalypha indica*	Leaf	20-30		MDA-MB-231 (breast)	100	Krishnaraj et al., 2014
3	*Achillea bieberateinii*	Leaf	10-40	Spherical&Pentagonal	MCF-7(breast)	20	Baharara et al., 2015
4	*Adenium obesum*	Leaf	10-30	Spherical	MCF-7(breast)	217	Farah et al., 2016
5	*Ailanthus exelsa*	Leaf	22-30	Spherical	MCF-7 (breast)	265.5	Vinmathi and Jacob, 2015
6	*Alternanthera sessilis*	Leaf	30-50	Spherical	PC-3 (prostate)	6.85	Firdhous and Lalitha, 2013
7	*Alpinia katsumadai*	Seed	12.6	quasi-spherical	SGC-7901 (gastric carcinoma)	7.5	He et al., 2017
8	*Annona squamosa*	Leaf	20-100	Spherical	MCF-7 (breast)	30	Vivek et al. 2012
9	*Artemisia tournefortiana*	Aerial	22.89 ± 14.82	Spherical	HT29(colon adenocaricnoma)	40.71	Baghbani et al., 2017
10	*Butea monosperma*	bark	35	Nearly spherical	KG-1A (myeloid leukemia)	11.47	Pattanayak et al., 2015
11	*Citrullus colocynthis*	Calli	31	Spherical	HEp-2 (epidermoid larynx carcinoma)	125	Satyavani et al., 2011
12	*Commelina nudiflora*	Leaf	24-150	Spherical Triangular	HCT116 (colorectal carcinoma)	100	Kuppusamy et al., 2016
13	*Cymodocea serrulata*	Leaf	5-25	Spherical	A549 (lung carcinoma)	100	Palaniappan et al., 2015
14	*Dendropanax morbifera*	Leaf	100-150	Polygon	A549 (lung carcinoma)	100	Wang et al., 2016
15	*Dimocarpus longan*	Peel	9-32	Spherical	PC-3 (prostate)	5-10	He et al., 2016
16	*Elettaria cardamomum*	Seed			Hep-2	51	Krishnan et al., 2015
17	*Erythrina indica*	Root	20-118	Spherical	HepG2	2.5	Sre et al., 2015
18	*Eucalyptus*	Leaf	30-70		AGS (gastric adenocarcinoma)	9.01	Rashmezad et al., 2015
19	*Ficus benghalensis*	Bark	40	Spherical	MG-63 (osteosarcoma)	75.5	Nayak et al., 2016
20	*Helicteres isora*	Stem bark	16-95	Spherical	KB (oral)	70	Bhakya et al., 2016
22	*Iresine herbstii*	Leaf	44-64	Polydispersed	Hela (cervical)	51	Dipankar and Murugan, 2012
23	*Melia azedarach*	Leaf	78	Spherical	Hela (cervical)	300	Sukirtha et al., 2012

24	*Mentha arvensis*	Leaf	4–9	Spherical	MDA-MB-231 (breast)	6.25	Banerjee et at., 2017
26	*Morinda citrifolia*	Root	30-55	Spherical	Hela (cervical)	60	Suman et al., 2013
27	*Moringa oleifera*	Leaf	40	Spherical	Hela (cervical)	50	Vasanth et al., 2014
29	*Piper longum*	Leaf	17.6–41	Spherical	HEp2(epidermoid larynx carcinoma)	31.25	Jacob et al., 2012
30	*Podophyllum hexandrum*	Leaf	14	Spherical	Hela (cervical)	20	Jeyaraj et al., 2013
31	*Vitex negundo*	Leaf	5-47	Spherical	HCT 15 (colon)		Prabhu et al., 2013

2.4. Cancer, cell cycle and apoptosis

Cancer is a group of more than 100 distinct diseases characterized by uncontrolled growth of abnormal cells in the body. Tumours are usually of two types - benign and malignant. A benign tumour is localised, develops slowly and does not usually result in the patient's death. Malignant or cancerous tumours develop rapidly, are not localised and often are fatal for the patient. Cancer can be caused by environmental, occupational, life style, viral or genetic factors. Environmental carcinogens could be in the form of ionising and non-ionising radiations, plastic chemicals like vinyl chloride, polynuclear hydrocarbons like benzopyrene, food chemicals such as dioxins and chemical pollutants such as asbestos fibres (Fujiwara, 2018; Stec et al., 2018). In case of viruses, a large percentage of all human cancers worldwide are caused by viruses. Both DNA and RNA viruses have been shown to cause cancer.

Viral infection is generally not fully sufficient for causing cancer. Additional events and factors such as immunosuppression, somatic mutations, genetic predisposition, and exposure to carcinogens also play a role in cancer development (Liao, 2006**;** Casey et al., 2015; Tu et al., 2018). Figure 1 highlights the cellular properties or hallmarks associated with the transformation of a normal cell into a cancerous one (Fouad and Aanei, 2017)

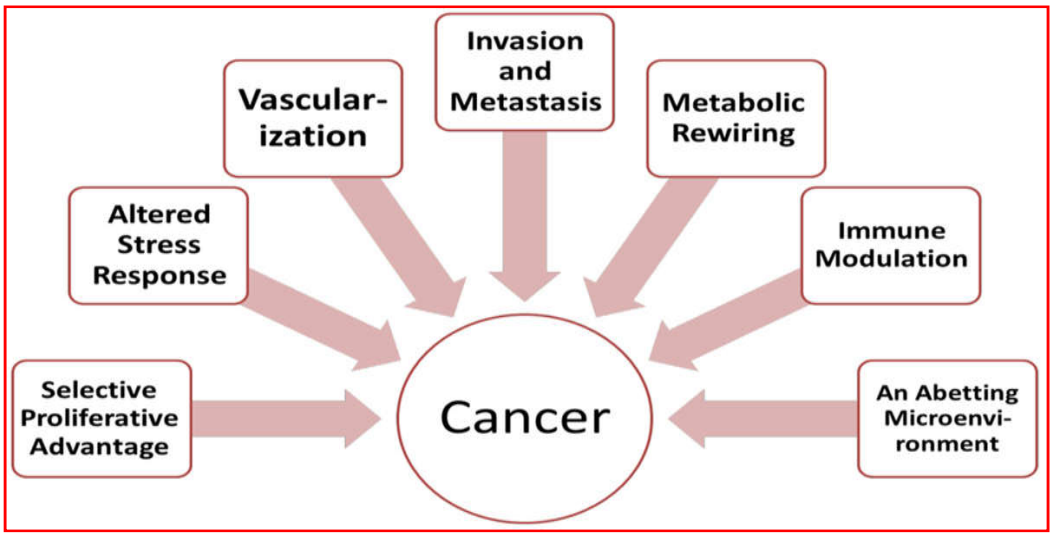

Fig. 2.3. ***Hallmarks of cancer*** *(Fouad and Aanei, 2017).*

Most of the mutations that cause cancer are not inherited but occur during the individual's lifetime. Cancer is a consequence of cumulative mutations that change proteins encoded by cancer-related genes. Based on the tissue of origin, the main types of cancers include (i) *carcinomas* – which starts in skin, or in tissues lining or covering internal organs (ii) *sarcomas* – develop in connective tissues like bone, cartilage, muscle, fat (iii) *leukemia* - forms in blood forming tissues such as bone marrows resulting in abnormal blood cells (iv) *lymphoma* and *myeloma* – originating in cells of immune system and (v) *brain and spinal cord* cancers of central nervous system (Kweon, 2018). The most common among women is breast cancer followed by colorectal cancer. Among men, lung and prostate cancer are on top of the list (Simon, 2018).

Cancer is considered as a disease of cell cycle and it continuously produces copies of itself. Normally, cells go through a process of growth, repair, division and regeneration in a cyclical manner as manifested through the cell division cycle which has four phases namely: G1, S, G2 and M. Signal transduction pathways called checkpoints function to carry out surveillance of cell cycle functioning which is driven mainly by protein complexes made up of a variety of cyclins and cyclin dependent kinases (CDK). CDK activity escalates at G1/S and G2/M transition points promoting replication and mitosis respectively in a synchronous manner (Fig.

2.3). But in cancer cells, exit from G phase and entry to S phase is deregulated and thus lead to uncontrolled proliferation.

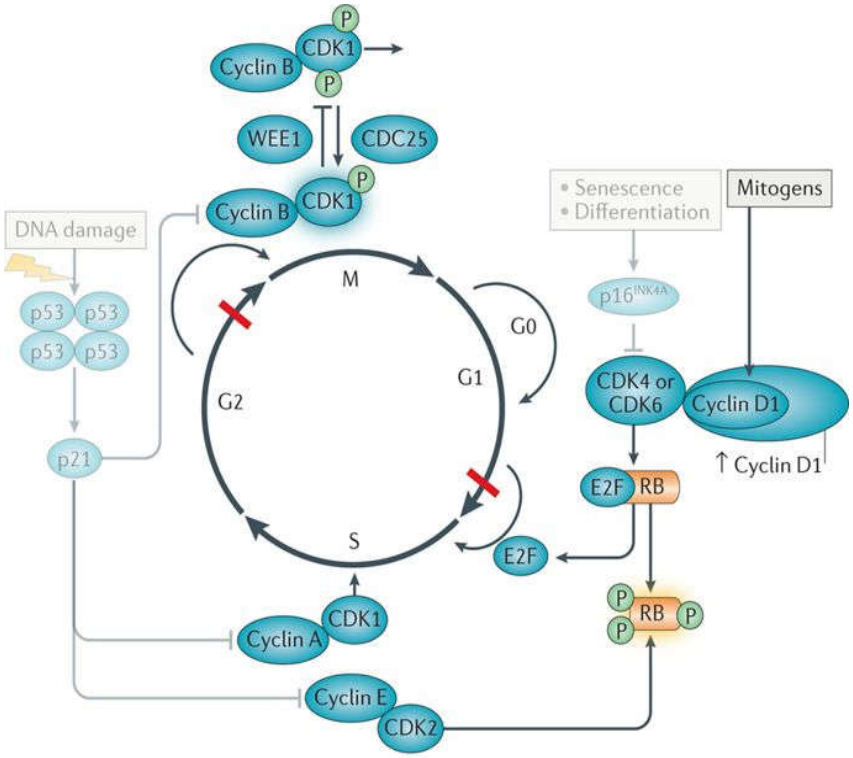

Fig. 2.4. ***Schematic representation of eukaryotic cell cycle*** *(Leemans et al., 2018).*

Protein products of cancer related genes directly or indirectly activate as well as inactivate specific cyclin-CDK complexes appropriate for either repair or arrest (WeinaCui et al., 2017). When an irrepairable damage is detected in a normal cell, the cell kills itself by a programmed suicidal process called apoptosis. Literature suggests that defects along the apoptotic pathway play a crucial role in carcinogenesis. Apoptosis is a highly conserved mechanism by which eukaryotic cells commit suicide thereby preventing cancer. Cancer cells have the tendency to proliferate without repairing damages incurred and fail to signal apoptosis (Pfeffer and Singh 2018; Oddo et al., 2018). Apoptosis occurs mainly through two pathways: intrinsic and extrinsic (Sarvothaman et al., 2015).

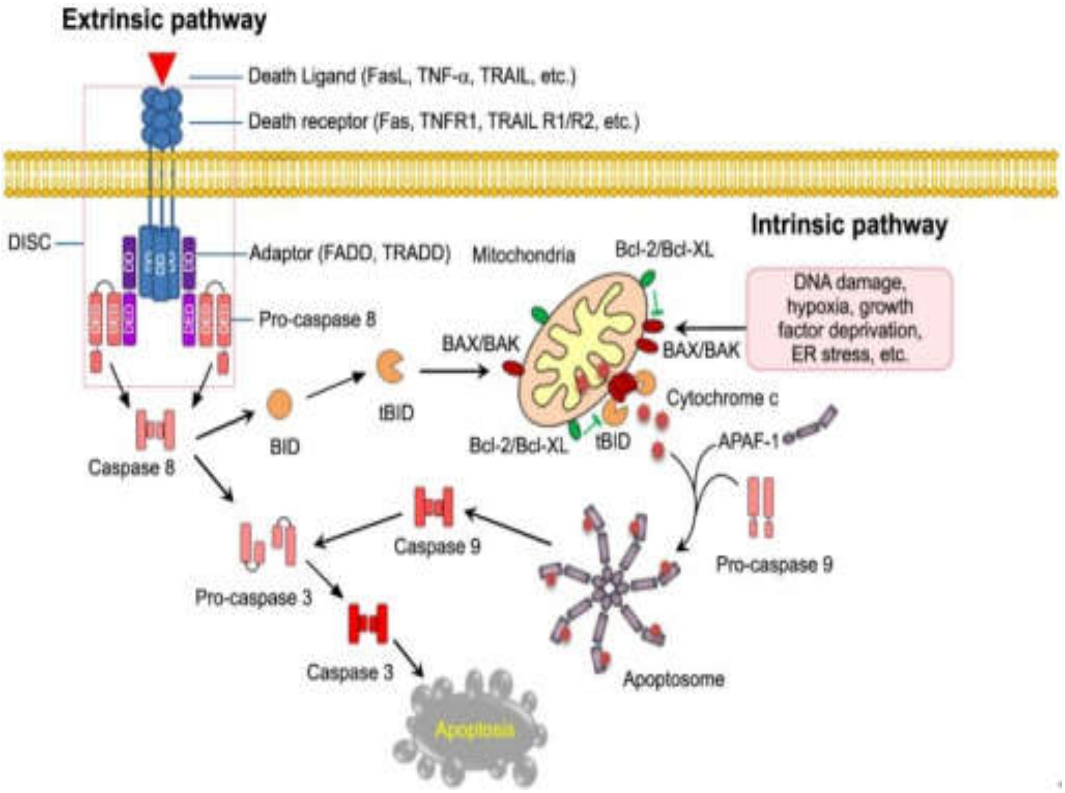

Fig.2.5. ***Schematic representation of intrinsic and extrinsic pathways of apoptosis*** *(Sarvothaman et al., 2015).*

The extrinsic pathway is initiated by ligation of death receptors with death ligands. This interaction results in the formation of the death inducing signaling complex (DISC), which contains the death receptor, an adaptor molecule and pro-caspase 8. Caspase 8 is auto-catalytically activated and subsequently transmits the death signal to effector executioner caspases, resulting in apoptotic cell death. The intrinsic pathway signaling cascade is triggered by a number of factors, including DNA damage, hypoxia, growth factor deprivation, and ER stress (Sendoel, and Hengartner, 2013). The death signal is sensed initially by the BH3-only protein, which then interacts with the downstream mediators of apoptosis (BAX and BAK). BAX and BAK undergo distinct conformational changes, which increase in permeability of the mitochondrial outer membrane, thereby releasing apoptogenic compounds. Released cytochrome-*c* binds to APAF-1 to facilitate formation of the apoptosome, a wheel-shaped heptameric complex, which can then recruit and

activate pro-caspase-9. As a consequence, caspase-9 activates effector caspases-3, -6, or -7 eventually leading to cell death (Sarvothaman et al., 2015; Mo et al., 2017).

2.4.1. Critical genes involved in cancer development

Alterations in three types of genes, namely, oncogenes, tumor-suppressor genes and stability genes by a combination of genetic and epigenetic accidents contribute to evolution of tumor (Weinberg, 2013).

Proto-oncogenes: The normal forms of genes which positively promotes the growth and proliferation of cells are called proto-oncogenes. Mutations in these genes render them constitutively active or active under conditions where the wild type genes are normally inactive. This activation can occur during chromosomal translocations, gene amplifications or subtle intragenic mutations (Lee and Muller, 2010; Vicente-Duenas et al., 2013). Examples include *Akt*, *bax*, *abl*, *cdk-2*, *cyclin D* among others.

Tumor suppressor genes: Mutations in tumor suppressor genes reduce the activity of its products. These mutations include missense mutations at residues that are essential for its activity, mutations that result in a trauncated protein, deletions or insertions of various sizes and epigenetic silencing. Mutations in both the maternal and paternal alleles of a tumor-suppressor genes are generally required to confer a selective advantage to the cell. Mutations in these genes introduce neoplastic process by increasing tumor cell number through stimulation of cell division or inhibition of cell cycle arrest or cell death (Zhao et al., 2012). p53 is the most studied tumour suppressor gene which plays a key role in suppressing malignant transformation. Wild type p53 suppresses tumor formation; its inactivated mutant form helps evasion of tumor cell death and tumor elimination (Engeland, 2018). Examples include p16, *p53*, *bcl2*, *rb*, *BRCA1*, *chk1/chk2* among others.

Stability genes: These genes (caretakers) when mutated promote tumorigenesis. They include mismatch repair, MMR genes (e.g., *MLH1, MSH2, MSH6,* and *PMS2*) nucleotide-excision repair, NER genes (e.g., *XPA, XPC, XPG, ERCC1, ERCC2*, and *ERCC4*) and base-excision repair, BER genes (e.g., *TDG*, MBD4, *SMUG1* and *MPG/AAG*) responsible for repairing mistakes made during normal DNA

replication. Other stability genes are large portions of chromosomes that control processes such as mitotic recombination and chromosomal segregation. Both alleles of stability genes generally must be inactivated to produce a physiologic effect (Jeggo et al., 2015; Chae et al., 2016; Zhao et al., 2018)

2.4.2. Driver and passenger gene mutations

Depending upon the loci of mutations occurring in the tumor genes, these mutations can be classified as 'driver' or 'passenger' mutations. Those that can drive cancer progression are called driver while others are called passenger mutations. These passenger mutations just hitchhike on the driver (Mirny, 2015). Cumulatively, these mutations result in deactivation of tumor suppressor genes followed by continuous activation of the cyclin-CDK complexes thereby leading to repeated cell divisions or uncontrolled proliferation. A typical tumor contains two to eight of driver gene mutations which promote a selective growth advantage while the remaining mutations are passenger that confer no such advantage. Some examples of driver genes are *MYC, KRAS, PIK3CA, ABLI, RBI, TP53,* and *WTI.* It is considered that 99.9% of all cancer related genes are passenger genes (Volgestein et al., 2013; Zhang and Zhang, 2017; Rajendran and Deng, 2017; Bailey et al., 2018).

2.4.3. Altered cellular housekeeping functions in cancer cells

Compared to normal cells, cancer cells usually lack proper differentian when. They have relatively less specialised structures and a high nuclear-cytoplasmic ratio. The genomic changes in cancer cells like gain or loss of whole chromosomes or chromosomal parts lead to substantial change in cellular protein composition. This genetic instability of cancer cells is due to the defect in repair mechanisms characteristic of such cells. They depend mainly on protein folding/degradation mechanisms. They also differ in their energy production mechanisms. Normal cells only use oxygen as a raw material if present but cancer cells prefer anaerobic glycolysis even in the presence or absence of oxygen due to speeding up of their energy needs (Hsu and Sabatini, 2008; Lodish et al., 2016).

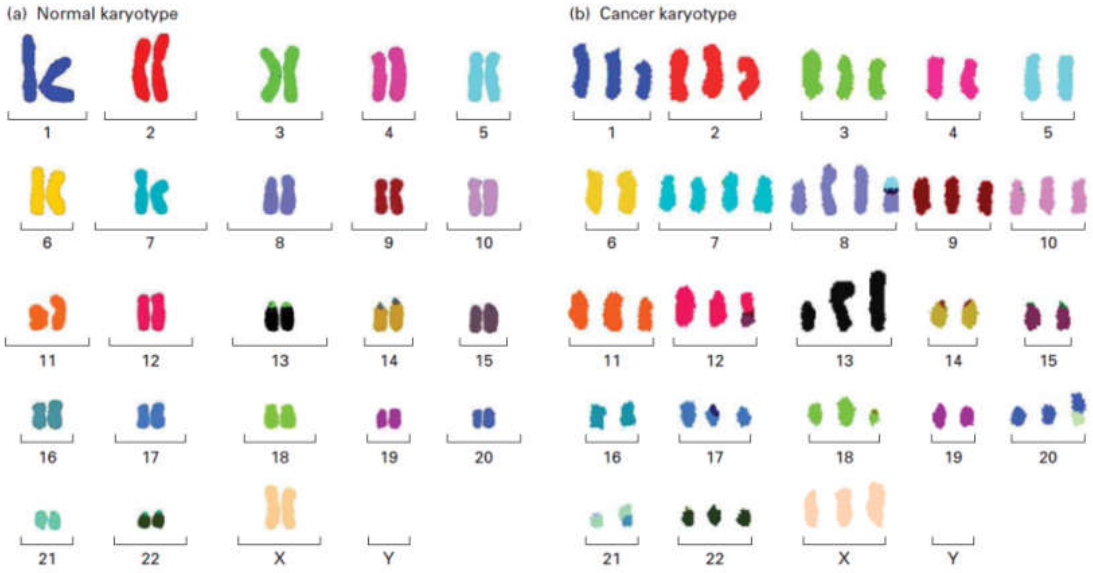

Fig. 2.6. ***Image of chromosomes from a normal human and a colorectal adenocarcinoma cell line with altered chromosome number and composites pieces from different chromosomes*** *(Lodish et al., 2016).*

2.4.4. Cancer epigenetics

Cancer is considered as a disease of the genome as well as epigenetics. The classic hallmarks of cancer can be achieved by epigenetic modifications alone. Aberrant proliferation can take place due to tumor suppressor silencing or insulator loss by DNA hypermethylation. In hypercalcemic type small cell carcinoma of the ovary, tumorigenesis is purely due to epigenetic modifications. Deregulation in gene-specific methylation in breast cancer clinical development and therapy resistance are well documented. Interestingly, triple negative breast cancer showed an enrichment of alterations in DNA repair genes, mainly BRCA1. Hypermethylation of this gene affected sensitivity to DNA-damaging chemotherapeutic agents, such as cisplatin. Hypermethylation-mediated silencing of several genes encoding Wnt-negative has also been reported (Stefansson et al., 2012; Stirzaker et al., 2015; Nebbioso et al., 2018).

2.4.5. Tumour stem cells

Studies revealing the presence of cells that carry stem-cell-like properties were conducted on breast cancer, colon cancer, melanoma and prostate cancer. These cells that possess the phenotype and function of a normal stem cell and

contain oncogenic mutations, can form new cells and lead to tumorigenesis. They are causative of cancer relapse and metastasis. Literature reports that bone marrow is an appealing site for tumor cells, particularly for cancer stem cells, which are able to resist cancer therapy by drugs and radiations. Once these cells reach bone marrow, they can remain dormant or cause bone metastasis long after the primary tumor has been surgically removed from patients (Klaus and Panabieres, 2014). Cancer stem cells have high ability for regeneration and self-renewal; they can modify themselves according to stress responses of the body and thus survive in highly hostile environments (Blanco et al., 2016; Roato and Ferracini, 2018).

2.5. Cancer prevention and treatment : Past, present and future

Progress in the development of highly sophisticated molecular techniques has enabled early detection, precise diagnosis and efficient treatment of cancer. For decades conventional anticancer treatment strategies have been surgery, chemotherapy and radiotherapy. While many of these therapies have offered substantial benefits for eradication of primary tumors as that of surgery for early diagnosed cancers, the incidence of disease relapse is still a commonly encountered problem that results from residual malignant cells or due to tumor metastases. Therefore, alternative treatment approaches to eliminate the resistant tumor cells are warranted.

Epidemiology reveals the possibility of prevention of many types of cancers. Cancer has a genetic basis and so mutations cannot be completely avoided as they are inescapable. Environmental factors also play an important role in determining the risk of cancer which makes some cancer more common in some places than others. Statistics predicts that of the 2% of cancers caused by UV/ionising radiations, given proper care, more than half are preventable. Likewise, of the 33% of cancers caused by cigarette smoking close to 25% is preventable. This is also true for the 5% cancers caused by occupational carcinogens. Though mutations are of primary concern, factors like food, hormones, infections and tissue damage play critical roles in cancer development. Tobacco smoke is the most important cause of cancer worldwide. Rise in pollution, enhanced use of processed foods and food

additives, lack of exercise and obesity have caused a very high probability of cancer incidence. Aflatoxin B1, one of the most common carcinogens produced by a fungus that contaminates foods, is an important cause of liver cancer in African-Asian countries (Alberts et al., 2015; American Cancer Society., 2017; Cancer Data and Statistics., 2018). Virus and other infections contribute to 15% of human cancers.

The search for cancer cure is difficult because of recurrence of tumor by metastasis. As already mentioned following surgery, some cells remain in the system by metastasis from which a new tumor arises. Cancer cells that survive radiation therapy mutate and evolve resistance to the poisons and irradiation used against them. In traditional cancer therapy, both drugs and radiations used also affect normal cells causing large number of side effects including fatigue, hair fall and nausea. In spite of all these difficulties, effective therapeutic strategies and drugs have been developed for combating various types of cancers (Doyle, 2018). A new generation of anticancer drugs designed to interfere with a specific molecular target - usually a protein with a critical role in tumor growth and progression – constitute 'targeted therapies (Meiyanto et al., 2012). For instance, Inhibitors of the poly (ADP-ribose) polymerases (PARPs) family of proteins increase the efficacy of DNA damaging agents and selectively target tumor cells with specific DNA repair defects (Papeo et al., 2013). Targeting hormones/hormone receptor is one of the important targeted therapies especially for breast cancer treatment. Aromatase inhibiting drugs, anastrozole, letrozole, and exemestane are included in this category. There has been acceleration in development of immunotherapies for cancer treatment in the past decade. Antibodies can be used that are specifically designed against cell surface proteins on the cancer cells that interact with and inactivate immune cells (Ruella et al., 2017). Monoclonal antibody therapy is used to specifically bind to target cells or antigens. This may stimulate the immune system of the patient to attack those cells or inhibit tumor growth (Guo et al., 2011). Tyrosine kinase imhibitors, proteasome inhibitors, protein kinase inhibitors, CDK inhibitors, RAF kinase inhibitors are also employed for targeted therapies. Epigenetic therapies aim to restore normal chromatin modification patterns through the inhibition of various components of the epigenetic machinery. Histone deacetylases and DNA

methyltransferase inhibitors represent the first putative epigenetic therapies (Popovic and Licht, 2012). A synthetic drug molecule called imatinab has been developed by the trade name *Gleevec* that targets the Bcr-Abl oncoprotein. Oncoproteins that are protein kinases are relatively easy to target using small molecules like imatinab (Quintás-Cardama et al., 2007; Zhang et al., 2018). There are other oncoproteins that are not protein kinases which are more difficult to target.

Non-coding RNAs (ncRNAs) have been identified as the key gene expression regulators. Only 2% of the entire human genomes represent protein coding genes containing 20,000 genes and the vast proportion of the genome is comprised of non coding RNA. These non-coding RNAs have significant role in the transcription of protein coding transcripts into functional proteins (Spizzo et al., 2012). lncRNAs can be found in many tissues, although brain and the central nervous system display the highest diversity of expressed IncRNAs (Ravasi et al., 2006). In cancer, IncRNA work through multiple mechanisms such as chromatin remodeling, chromatin interactions and as competing endogenous RNAs (Fang and Melissa, 2016). Additionally, IncRNA are implicated in several functions such as signal, decoy, scaffold, guide, enhancer RNAs and generation of short peptides (Moran et al., 2012; Li et al., 2014). Small non-coding RNA or micro RNA (miRNA) used as target agents are capable of reprogramming multiple oncogenic cascades. Researchers have conducted trials to diagnose different classes of cancers by using miRNA expression profiles and design miRNA biomarkers that help in cancer prognosis and diagnosis and therapeutics (Flemming, 2012; Lai, 2013; Rasool et al., 2016). The recent advent of the powerful genome editing CRISPR-Cas technology and RNA interference methods can be used to personally edit and correct the error in genome. CRISPR-Cas tools are easily programmable RNA-guided nucleases that enable rapid genome engineering. Further evolution of CRISPR–Cas9 may enable to cure Mendelian diseases in somatic tissues by directly correcting the underlying disease-causing mutations (Fellmann et al., 2016). In the future, pooled CRISPR screens will provide a set of essential genes across most cancer cell lines. It will require considerable experimental research to fully understand the biological mechanisms underlying the genetic interactions revealed

by CRISPR/Cas9 screens. The future use of CRISPR/Cas9 in translational medicine will largely depend on the ability to develop Cas9 variants with minimal or no side. Future improvements of viral and non-viral delivery methods will be necessary to improve the *in-vivo* application of CRISPR/Cas9 and will greatly accelerate cancer research in many areas (Kampmann, 2017; Zhan et al., 2017).

Cells exposed to one therapeutic sometimes evolves resistance against the same or related drugs – a phenomenon commonly known as multidrug resistance. A synergetic combination of two or more drugs thus become useful to enhance drug efficacy as well as for attacking multiple targets at a time. This approach is more practical in cancers which remain undetected in early stages and metastasise to multiple locations (Mokhtari et al., 2017). Though the search for such drug combinations is tiresome and time consuming, recent technological developments in genomics / personalized medicine with the associated new tools facilitate identification of genomic signatures or tumour-specific vulnerabilities from patient tumour samples to guide selection of precise therapeutic options. The recent concept of 'synthetic lethality' (SL) exploits relationship of two genes, which if inactivated individually result in viable phenotype with inactivation of both together becoming lethal (Lee et al., 2018)

2.6. Nanoparticles, nanomedicine and cancer

Nanotechnology-based approaches have emerged as an exciting field with promises to overcome the limitations of conventional treatments due to their prolonged half-life and increased targeting efficiency (Alexis et al., 2010). Cancer nanomedicine refers to the application of nanotechnology-based therapeutics and imaging agents for the diagnosis, monitoring, prevention and treatment of cancer. Many nanomaterials have been employed as delivery vehicles for drugs and/or imaging agents. These include liposomes, polymer carriers, such as micelles, hydrogels, polymerosomes, dendrimers and nanofibres; metallic nanoparticles (gold, silver, titanium), carbon nanostructures (nanotubes, nanodiamonds, grapheme) [Chow and Ho, 2013; Tong and Kohane, 2016]. The incorporation of chemotherapeutic agents in liposomal or polymeric nanoparticle delivery vehicles

has resulted in improved drug solubility, reduced drug clearance / resistance, and enhanced chemotherapeutic effectiveness. The use of surface-modified nano-scale liposomes (nanosomes) / nanocapsules / lipid-micelles to deliver hydrophilic and hydrophobic anticancer drugs is a promising development in cancer therapy. Doxil^{TM} (approximately 100 nm PEGylated liposome loaded with doxorubicin) and Abraxane^{TM} (approximately 130nm-albumin-bound paclitaxel NPs) have been approved by the the US Food and Drug Administration for the treatment of ovarian cancers and have shown improved pharmacokinetics and reduced adverse effects compared with their parent drugs. Other polymeric nanoparticles that deliver small molecule chemotherapeutics or small interference RNA (SiRNA) have also entered clinical trials. Targeted drug delivery by covalent coupling of nanoparticles with appropriate ligands such as lectins, antibodies, aptamers, folate and peptides have also been successfully demonstrated (Bannerjee and Sengupta, 2011; Renganathan et al., 2012; Hrkach et al., 2012; Pang et al., 2017; Liang et al., 2018). In addition metallic particles are promising therapeutic agents that convert light to heat (photothermal effect) to kill cancer cells. Small-sized inorganic nanoparticles (silica NPs) are in clinical trials as multimode imaging agents for lesion detection and cancer staging (Phillips et al., 2014; Chen et al., 2018). Nanomaterials can be employed as vehicles to deliver antigens or immune modulating therapeutics. Nanomaterials can enhance the efficacy of cancer immunotherapy by protecting their payload during circulation, promoting the delivery of antigen to antigen presenting cell, triggering the activation of antigen-specific T cell and regulating the immunosuppressive tumor microenvironment (Qian et al., 2018). Metallic nanoparticles enhance vaccine delivery by improving uptake of antigens by dendritic cells (and other antigen presenting cells) thereby improving the resulting anti-tumor cytotoxic T cell response (Almeida et al., 2014; Evans et al., 2018). Use of metallic nanoparticles have proven successful in a variety of immunotherapeutic applications, ranging from delivery of immunomodulating materials (antigens, adjuvants, cytokines, and checkpoint inhibitors) to induction of tumor antigen release upon local ablation.

2.7. Plants selected for silver nanoparticle synthesis: Botanical classification and medicinal uses

Two plants were selected following a preliminary screening carried using some of the medicinal plants to identify suitable plants - or parts thereof- for the generation of biogenic silver nanoparticles to be used for experimental studies. The details of their botanical classifications are given below:

2.7.1. *Manilkara zapota* (L.) P. Royen.

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Ericales
Family : Sapotaceae
Genus : Manilkara
Species : *M.zapota*

Fig.2.7. *Manilkara zapota* (L.) P. Royen

Manilkara zapota (L.) P. Royen or *Achras sapota*, commonly known as Sapodilla, Chickoo or Sapota, belongs to the family Sapotaceae including about 65

genera and 800 species (Milind and Preeti, 2015). It is an evergreen, glabrous tree of 8-15 m cultivated world over in tropical countries for various benefits like edible fruits, timber, latex etc. Sapodilla has its origin in Mexico and is native to Cenrtal America although it is cultivated in Asian countries including India (Anjaria et al. 2002). The members of this family can be easily recognized by the characteristic milky latex and alternate leathery leaves with parallel secondary and tertiary veins (Gentry, 1993). The plant parts have found wide use in traditional medicine. The leaves and flowers of *M.zapota* have been used against colds, cough, bronchitis, (Priya et al., 2014) and have good potential for analgesic, antihyperglycemic and hypocholesterolemic activities (Shivhare et al., 2011; Shazly et al. 2012). The flower has been traditionally used to relieve pulmonary complaints and fever and is also reported with antioxidant and antibacterial properties (Priya et al., 2014). The crushed seeds and bark have been used as aperients, diuretic tonic, astringent and febrifuge (Anjaria et. al 2002; Patricia et al. 2008). The seed paste is effective against stings and venomous bites; seed juice known to have diuretic and antihistamine qualities is effectual against anxiety/depression and removes bladder/kidney stones (Polska, 2015). The fruit is a natural energy booster containing fructose/sucrose,acids, proteins, phenolics, carotenoids and ascorbic acid (Siddappa and Bhatia, 1954; Mathew and Lakshminarayana, 1969; Selvaraj and Pal, 1984). It is a rich source of dietary fibre, minerals (potassium, copper, Iron) and vitamins (A,C, folate, niacin, pantothenic acid) and is recommended to prevent micronutrient malnutrition (Anand et al. 2007). The fruits also possesses antispasmodic property that helps in treatment of muscle spasms and pains. Besides flavonoids and polyphenols, the plant contains phytochemicals such as saponins, triterpenoids, dihydromyrecetin, myricitrin, quercitin, catchin, epicatchin, gallocatchin and gallic acid which are known to possess anti-inflammatory, antimicrobial, antioxidant, analgesic and spermicidal activities (Bhargava, et al., 1970; RB et al., 1979). Stem/bark extract showed antifungal activity against *Aspergillus flavus, Fusarium vasianfactum*. The antifungal activity may be due to the presence of terpenoids, flavonoids and glycosides (Osman et al., 2011). In an earlier study, Ma et al., 2003 reported isolation of two phenolic antioxidant compounds, methyl 4-O-galloylchlorogenate and 4-O-galloylchlorogenic acid, which were found to be cytotoxic against two colorectal cancer cell lines – HCT 116 and SW 480 with IC_{50} values ranging within 100-200 μM. Later Srivastava et al.,

2014 described the cytotoxicity of methanolic extract of chickoo against human and mouse breast cancer cell lines (EAC, MCF7 and T47D). All the three cell lines showed inhibition of cell viability at high doses. However cervical cancer cell line (HeLa) showed less sensitivity than breast cancer cell lines. Sumithra et al., 2014 reported that *M.zapota* flower extract showed strong cytotoxic effects against MCF7 breast cancer cell line while it possess very less toxicity towards non–Cancerous Vero cell line. The recent past also showed that methanolic extract from the fruit and an ethyl acetate extract (containing an aglycone – erythrodiol) prepared from leaves significantly inhibited tumour progression in mice (Srivastava et al. 2014; Rashid et al, 2014).

2.7.2. *Annona muricata* (L.)

Botanical Classification

Division : Angiosperms (Magnoliophyta)

Class : Magnolids

Order : Magnoliales

Family : Annonaceae

Genus : Annona

Species : *A.muricata* L.

Fig. 2.8. ***Annona muricata*** **(L.)**

Annonaceae is a very large plant family consisting of approximately130 genera and more than 2300 species (Leboeuf et al. 1980; Mishra et al. 2013). The medicinal uses of the family members have been known since more than a century

ago (Billon 1869) and this species has attracted human attention due to its bioactivity and toxicity. *Annona muricata* is known as Soursop (English), Graviola (Portuguese), Guan´abana (Latin American Spanish), is a member of the family.

A wide array of ethnomedicinal activities have been attributed to different parts of *A.muricata*. Indigenous communities in Africa and South America extensively use this plant in their folk medicine (Moghadamtousi et al., 2015). *A. muricata* is native to the warmest tropical areas in South and North America and is now widely distributed throughout tropical and subtropical parts of the world, including India, Malaysia and Nigeria (Adewole et al. 2006). It is an evergreen plant reaching heights mostly upto 5-8 m. It produces heart-shaped edible fruits of about 5-20 cm in diameter, green in color, with a white fleshy part inside which is used extensively to prepare syrups, candies, beverages, ice creams and shakes.

Former studies on this plant include those on important application-oriented bioactivities such as anticancer, antiparasitic and insecticidal (Patel and Patel, 2016). Parts of the tree are extensively used in traditional medicine as decoctions of bark, root, seed or leaf against an array of human ailments and diseases including cancer and parasitic infections. The fruit is employed as a natural medicine for arthritic pain, neuralgia, arthritis, diarrhea, dysentery, fever, malaria, rheumatism, parasitic infections, skin rushes and worms. The leaves are used for the treatment of cystitis, diabetes, headaches and insomnia (Adewole et al., 2006; Sousa and Vieira, 2010; Mishra et al., 2013). Moreover, ingestion of leaf decoction is used as analgesic and also used to treat colds, flu and asthma (Badrie and Schauss, 2009; Ross, 2010). Insecticidal activity has been reported from all parts including seeds, leaves, barks, stems, roots and flowers (Leatemia and Isma, 2004; Bobadilla et al., 2005; Pre´des et al., 2011). Plenty of studies report significant antiproliferative effects of different extracts including those on the polyketides known as acetogenins against various cell lines (Jaramillo et al., 2000; Arroyo et al., 2005; George et al., 2012; Astirin et al., 2013; Gavamukulya et al., 2014). Reports reveal the mechanism of action of ethyl acetate extract of *A.muricata* leaves against colon cancer cells (HT-29. HCT-116) and lung cancer cells (Moghadamtousi et al., 2015). Recent *in vitro* and *in vivo*

studies on the aqueous leaf extract of *A.muricta* reveal that it suppresses growth of benign prostatic hyperplasia (BPH-) cell line and reduces the size of rat prostate glands (Asare et al., 2015). A case study of metastatic breast cancer reported that consumption of the leaves in boiled water along with the anticancer drug Xeloda resulted in stabilization of the disease condition (Hansra et al. 2014). These significant anticancer and antitumor activities of *A.muricata* leaves have led to tablet formulations of the ethyl acetate-soluble fractions of the leaves containing acetogenins employable as adjuvant cancer therapy. Anti-inflammatory, hypoglycemic, sedative, smooth muscle relaxant, hypotensive, antisplasmodic, gastro-protective, molluscicidal and wound healing activities are also attributed to the leaves, barks and the roots of *A.muricata* (Santos and Sant'Ana, 2001; Bidla et al., 2004; Adewole et al., 2006; Hamid et al., 2012; Mishra et al., 2013).

Phytochemical studies have reported the presence of about two hundred and twelve bioactive compounds in *A.muricata* with the most dominant compounds being acetogenins. More than 120 acetogenins have been identified in ethanolic, methanolic and other organic extracts from the leaves, bark, stem, seeds, pulp, fruits and fruit peel of *A.muricata* (Alali et al., 1999; Li et al., 2001; Liaw et al., 2002; Chang et al., 2003; Ragasa et al., 2012). Alkaloids, flavonoids, carbohydrates, cardiac glycosides, saponins, tannins, phytosterols, terpenoids, proteins, essential oils are the other phytoconstituents present in *A.muricata*. Besides these, vitamins, carotenoids, amides, cyclopeptides and megastigmanes have also been identified (Coria-Te´ llez et al., 2016).

2.8. Cell lines used in the study

2.8.1. HCT 116 cells

HCT 116 is a human colorectal carcinoma cell line initiated from an adult male. The cells are adherent with an epithelial morphology and can metastasize in xenograft models. HCT cells have a mutation in codon 13 of the KRAS proto-onco gene and are appropriate transfection targets for gene therapy research (Rajput and Ashwani, 2008). The cells were carrying the p53 gene when it transducted with viral vectors, HCT cells remain arrested in the G_1 phase (Kaeser et al., 2003). The cells

were growing with a doubling time of approximately 18 hours. They are suitable for *in vitro* and *in* vivo experimentation. The proliferation of HCT 116 colonies was found to be inhibited by 5 Fu/P85 copolymer micelles (Zhu and Pengxi, 2016). These cells are widely used in biomedical studies involving colon cancer proliferation and corresponding inhibitors. The cell line has been employed in tumorigenicity studies. HCT 116 cell line was found to have two variations; one with a large expressions of the Insp8 gene, and the other without this gene expression. The Insp8 gene is a part of a cell's energy metabolism process, and it can then affect the cellular phenotype (Gu and Chunfang, 2016).

2.8.2. Hela Cells

Hela is a cell type in an immortal cell line used in scientific research. It is the oldest and most commonly used human cell line (Rahbari et al., 2009). This cell line was derived from cervical cancer cells taken on February 1951 (Scherer et al., 1953) from Henrietta Lacks a patient who died of cancer on October 1951. These were the first human cell grown in lab could be used for multitude of medical experiments. There are many strains of Hela cells as they continue to mutate in cell cultures, but all Hela cells were descended from the same tumor cells removed from Lacks. Hela cells were widely used in cancer research because it proliferate abnormally, rapidly, even compared to other cancer cells. It also employed in various types of investigations including disease research, gene mapping and effects of toxic substances of radiation on humans (Smith et al., 2017). Additionally Hela cells have been used to test human sensitivity to tape, glue, cosmetics and many other products (Batts, 2010). These cells have an active version of telomerase during cell division which prevents the incremental shortening of telomeres that is implicated in aging and eventual cell death. In this way the cells avoid the Hayflick limit, which is the limited number of cell divisions that most normal cells can undergo before becoming senescent (Ivanković et al. 2007). Hela cells are rapidly dividing cancer cells, and the number of chromes varied during cancer formation and cell culture. The current estimate is a hypertriploid chromosome number (3n+) 76 to 80 total chromosomes (rather than the normal diploid number of 46) with 20-25 clonally abnormal chromosomes, known as Hela signature chromosomes

(Bottomley et al., 1969; Macville et al., 1999; Landry et al., 2013; et al., Adey et al., 2013)

2.8.3. A549 cells

A549 cell line was first developed in 1972 by Giard et al. through the removal and culturing of cancerous lung tissue in the explanted tumor of 58-year-old Caucasian male. The cells produced were adeno adenocarcinomic human alveolar basal epithelial cells with a model chromosome number of 66. A549 cells, as found in the lung tissue of their origin, are squamous in nature and responsible for the diffusion of some substances, such as water and electrolytes across alveoli. The Cells are cultured *in vitro*, they grown as a monolayer, adherent or attaching to the culture flask. A549 cells are positive for keratin; it is evidenced by immunoperoxidase staining. The cells are able to synthesize lecithin and contain high levels of unsaturated fatty acids, which are utilized by the cytidine-diphosphocholine pathway and important for the maintenance of membrane phospholipids in cells. The cells are widely used as type II pulmonary epithelial cell model for drug metabolism and as transfection host (abcam.com 2012; ATCC.org 2012). The A549 non- small cell lung cancer (nsclc) cells have served as testing grounds for novel drugs - such as paclitaxel, docetaxel, and bevacizumab - both *in vitro* and *in vivo* through cell culture and xenografting, respectively (Franklin and Maryland, 2016). A549 has also been employed in viral research and associated protein expression changes as a consequence of viral infection (Thomas et al., 1998). Although A549 is a cancer cell line, it has also been studied for its response to tuberculosis, specifically the production of chemokines as it is induced by the invading bacteria (Lin et al., 1998).

MATERIALS AND METHODS

3.1. Chemicals and reagents

Silver nitrate, Tris base, glycine, acridine orange (AO), ethidium bromide (EtBr), potassium bromide, heparin, Folin-Ciocalteau's reagent, ascorbic acid, sodium nitroprusside, ferrozine, gallic acid, quercetin, hydrogen peroxide and media supplements were purchased from Sisco Research Laboratories (SRL,India). BCIP/NBT, DMSO, DCFH-DA, rhodamine 123, annexin V- FITC apoptosis detection kit, propidium iodide, TRI® reagent and crystal violet were purchased from Sigma-Aldrich (USA). Anti-caspase-3, -9, anti-PARP, anti- p^{53}, anti-p^{21} and anti-actin antibodies were purchased from Cell Signaling Technology, (USA). Goat anti-rabbit IgG-alkaline phosphatase (AP)-conjugate, goat anti-mouse IgG-ALP-conjugate, RNase inhibitor, oligo(dT)18 primer, DTT, reverse transcriptase enzyme, aluminium chloride, agarose were purchased from Bangalore Genei (Merck Life Sciences, India). SYBR Green PCR Master Mix was purchased from TAKARA (Japan); FBS and 0.25% Trypsin-EDTA were obtained from Gibco (Thermo Fisher Scientific, USA). DMEM, HiKaryo XL™ RPMI medium, MTT, penicillin, streptomycin, BSA and DPPH were procured from HiMedia Laboratories Pvt. Ltd, India. Cisplatin was purchased from Tokyo Chemical Industry Co. Ltd, (TCI) Japan. Positively-charged PVDF membrane was purchased from BDH laboratory supplies (England). All other chemicals and reagents used were of analytical grade.

3.2. Sample collection and extract preparation

Leaves of *M. zapota* (MZL) were collected from Malappuram district. *A .muricata* fruits (AMF) and roots (AMR) were collected from Thrissur and Malappuram districts. Young leaves of *M. zapota*, fresh mature fruits, and roots of *A.muricata* were then cut into pieces, shade dried and powered finely. For extract preparation, 10g of the powders were weighed and mixed with 200 mL milli Q water and boiled for 15 min. After cooling, the extracts were filtered through layers of muslin cloth and Whatman No.1 filter paper and stored at 4°C in the refrigerator.

3.3. Biosynthesis of silver nanoparticles

For green synthesis of AgNPs, different parameters such as volume of plant extracts (2.0, 4.0, 6.0, 8.0 and 10.0 mL), concentration of metal ion (0.5, 1.0, 2.0, 3.0 and 5.0 mM), temperature (37, 60, 70, 80, 90, 95, and 100 $^{\circ}$C), pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) and time (0, 30, 60, 90, 120, 150 min) were optimized. The reaction mixture was periodically monitored in the range of 200-700 nm using a UV-visible spectrophotometer (Perkin Elmer – Lambda 25) to detect the formation of silver nanoparticles as indicated by the appearance of brown color.

3.3.1. Characterization

The biosynthesized MZLAgNPs, AMFAgNPs and AMRAgNPs were initially characterized spectrophotometrically using a small aliquot of the sample diluted with distilled water. For further characterization, synthesized AgNPs were purified by repeated centrifugations at 12000 rpm for 20 min. The resultant pellet was re-suspended in milli-Q water and lyophilized (Scanvac, coolsafe). To characterize the bioactive constituents present in the extract, the freeze-dried powder was pelletized with potassium bromide (KBr) powder and subjected to FTIR analysis (Vivek et al., 2012). The spectra were recorded using a Jasco 4100 FTIR spectrophotometer in the wavelengths ranging from 4000 - 400 cm^{-1}. X-ray diffraction was performed to determine the crystal structure of nanoparticles (Bhakya et al., 2015). For this the nanoparticles were coated on a glass substrate and XRD measurements were carried out using a Rigaku miniflex X-ray diffractometer instrument with Cu Kα radiation (40 kV, 15 mA) in 2θ configuration ranging from 20-70°. The particle size was determined by Debye-Scherrer's formula

$$D = \frac{0.94\lambda}{\beta \cos\theta}$$

Where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM) and θ is the diffraction angle (Dipankar and Murugan, 2012). For morphological characterization, purified AgNPs were subjected to FESEM and EDX analysis using CARL-Zeiss Gemini 300 SEM instrument.

3.3.2. Quantitative analysis

Preparation of biogenic AgNP stock solution : The lyophilized powders of each of the three types of biogenic nanoparticles, namely, MZLAgNPs, AMFAgNPs and AMRAgNPs prepared as mentioned in section 3.3.1, were suspended in milli Q water was used as stock solutions at a concentration of 5.0 mg /mL. Aliquots of these were utilized for all experimental analyses.

Estimation of total phenolics and total flavonoids : Total phenolic content of all the three types of biogenic nanoparticles and their corresponding plant extracts were determined by Folin-Ciocalteau method using gallic acid as standard essentially as described by Gupta and Prakash (2009). Briefly, 200 µL of Folin-Ciocalteau's reagent was added to 2.0 mL of each reaction sample and kept for 10 min at room temperature. To this, 300 µL of 15% Na_2CO_3 was added. It was then mixed thoroughly and allowed to stand at 25 °C for 2 h. Absorbance was measured at 765 nm using UV-visible spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE).

The total flavonoid content of individual AgNPs and their respective extracts were determined as described by Fu et al. (2013). Briefly, 20 µL of reaction mixture (1 mg/mL) was mixed with 30 µL of 5 % sodium nitrite. After 6 min, 50 µL of 10 % aluminium chloride was added, and the mixture was allowed to stand for a further 5 min. Then, to the above mixture, 100µL of 10 % NaOH was added and its absorbance was measured at 510 nm. The total flavonoid content of each extract was expressed as Quercetin Equivalents (QE).

3.3.3. *In vitro* Antioxidant activity

DPPH free radical scavenging activity

The DPPH free radical scavenging activity of the biosynthesized AgNPs and plant extracts were evaluated based on the method of Chen et al. (2008). One milliliter of 100 mM DPPH (in methanol) was mixed with an equal volume of each type of nanoparticle suspension or the precursor plant extract to give concentrations ranging from 200-1000 µg/mL. The mixtures were then shaken and incubated in the dark for 30 min at 25ºC. Absorbance was recorded at 517 nm. Taking ascorbic

acid as the standard, free radical scavenging activity was calculated using the formula :

[(Absorbance of control − Absorbance of sample)/Absorbance of control] ×100

Nitric oxide scavenging activity

Nitric oxide (NO) radicals generated from sodium nitroprusside in an aqueous environment interact with oxygen to produce nitrite ions were quantified by using the Griess reagent assay with slight modifications (Awah et al., 2010). Briefly, separate aliquots (500µl) of AgNPs and extracts at varying concentrations (200 - 1000 µg/mL) were mixed with 2.0 mL of 10mM sodium nitroprusside followed by 0.5 mL of phosphate buffered saline (PBS). It was then incubated at 25°C for 2.5 h. From this reaction mixture, 0.5mL was added to 1.0 mL of sulfanilic acid followed by addition of 1.0 mL of naphthyl-ethylene-diamine dichloride (0.1%w/v) and incubation at 25°C for 30 minutes. Taking ascorbic acid as standard, absorbance was measured at 546nm. Nitric oxide radical scavenging activity was evaluated using the formula:

[(Absorbance of control − Absorbance of sample)/Absorbance of control] ×100

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined using the method described by Chung et al. (1997). The reaction mixture containing 200 µL of 10mM $FeSO_4 .7H_2O$, 200 µL of 10 mM EDTA and 200 µLof 2-deoxyribose was added to 1.2 mL of 0.1M phosphate buffer (Ph-7.4) containing varying concentrations (200-1000µg/mL) of each type of AgNPs/extracts. Ascorbic acid served as the standard. Freshly prepared 200 µL of 10 mM H_2O_2 was added to the mixture and incubated for 4 h at 37◦C. Later, 1.0 mL of 2.8% TCA and 1.0 mL of 1% TBA were added and placed in boiling water bath for 10 min. The mixture was brought to room temperature and centrifuged at 2000 rpm for 5 min and the absorbance was recorded at 532 nm. The percentage of hydroxyl radical scavenging activity was calculated as follows :

[(Absorbance of control − Absorbance of sample) /Absorbance of control] ×100

Ferrous ion chelating activity

Ferrous ion chelation effect was determined according to the method of Dinis et al. (1994). Briefly, 200 µL of each AgNPs/extracts (200-1000µL) were mixed with 100 µL of 2mM $FeCl_2$. The reaction, initiated by the addition of 200µL of 5mM ferrozine, made up to 5mL with methnol was mixed well and allowed to stand for 10 min at room temperature. The absorbance was read at 562 nm and percentage inhibition of ferrozine-Fe^{2+} complex was calculated by using the formula:

[1- (Absorbance of sample/ Absorbance of control)] × 100

Determination of reducing power

The reducing power of biogenic AgNPs was assessed essentially as described by Chen et al. (2008) with slight modifications. In brief, concentrations ranging from 200 - 1000µg/mL of the different nanoparticle preparations and the corresponding extracts were mixed individually with 0.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5mL potassium hexacyanoferrate (1%,w/v). The mixture was incubated at 50°C in a water bath for 20 min and then cooled immediately. To this, 0.5 mL of TCA (10%, w/v) was added and centrifuged at 3000 rpm for 10 min. From the supernatant, 1.0 mL was taken and mixed with an equal volume of distilled water. Finally 0.1 mL of freshly prepared 0.1% ferric chloride was added and the absorbance was recorded at 700nm. Ascorbic acid was used as standard. An increase in the absorbance of reaction mixture indicated stronger reducing power in a dose-dependent manner

Determination of total antioxidant capacity

The antioxidant capacity of biogenic AgNPs and plant extracts were evaluated by phosphomolybdenum method according to the procedure of Prieto et al. (1999). Varying concentrations of nanoparticles / extracts (200-1200 µg/mL) in a volume of 0.1 mL were combined with 1.0 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4.0 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 min. After cooling, the absorbance of each solution was measured at 695nm against a blank. Ascorbic acid was used as the

standard and the total antioxidant capacity was expressed as equivalents of ascorbic acid.

3.3.4. Antibacterial activity

Agar well diffusion assay

The antimicrobial activities of green synthesized AgNPs and the aqueous plant extracts (MZL, AMF, AMR) were tested against clinical isolates of gram negative (*E.coli*, *K. pneumoniae*, *P. aeruginosa*) and gram positive (*S. aureus*) bacteria employing Kirby-Bauer well diffusion method (Logeswari et al., 2015). The pure cultures of organisms were sub-cultured on LB broth at 37ºC on rotary shaker at 160 rpm. Each strain was swabbed uniformly on the individual plates using sterile cotton swab. Wells of size 7.0 mm were made on Muller-Hinton agar plates using gel puncture and 75 µL aliquots of AgNPs/plant extracts and distilled water (control) were poured into the wells. Following 18 h of incubation at 37ºC, inhibition zones were assessed.

Determination of MIC and MBC

The antimicrobial activities of silver biogenic AgNPs/plant extracts in terms of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was estimated. Individual bacterial cultures at 24 h growth were serially diluted with Luria Bertani (LB) growth medium and exposed to different biogenic AgNPs at varying concentrations ranging from 5-100 µg/mL. Following overnight growth at 37ºC, 0.1mL each of the test cultures and the corresponding untreated controls were spread on LB-agar plates for determination of MIC and MBC (Ibrahim, 2015).

3.3.5. Antiproliferative activity

Maintenance of cancer cell lines

The cytotoxicity was tested against three human cancer cell lines, colorectal carcinoma -HCT 116, cervical carcinoma - Hela and non-small lung alveolar carcinoma - A549 cells. These cells were obtained from the National Centre for Cell Sciences [NCCS, Pune (Govt. of India)], maintained in the laboratory. The cells were cultured in 25 cm^2 flask as a monolayer in DMEM medium supplemented with

10% FBS, 100U/mL each of penicillin-streptomycin and incubated at 37°C in a humidified 5% CO_2 atmosphere.

MTT assay

The cytotoxicity of biosynthesized AgNPs and plant extracts were evaluated by MTT assay. In viable cells, mitochondrial succinate dehydrogenase converts the water soluble yellow tetrazolium MTT salt to insoluble purple formazan crystals directly proportional to the number of metabolically active cells (Sabu et al., 2016). All three cell types were individually seeded (1×10^4 cells/well) into 96-well tissue culture plates and incubated for 24 h. The cells were then exposed to different concentrations of MZLAgNPs, AMFAgNPs, and AMRAgNPs for 24, 48 and 72 h; the standard chemotherapeutic drug, cisplatin, was used as a control drug for comparison of cytotoxicity. The untreated cells served as control. The toxicity of plant extracts *per se* was also evaluated at different concentrations (10-100 µg/mL) for 24 h. Following addition of MTT (500µg/mL), the cells were then incubated further for another 3 h at 37°C. The formazan crystals formed were then dissolved in 150µL of DMSO for optical density measurements at 620nm using an ELISA plate reader (Multiscan EX, Thermo Scientific, USA). The differences of absorbance between the treated and untreated control groups were used to determine cell viability (Mosmann, 1983) as follows:

$$\text{Percentage of cell viability} = \frac{(\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}})}{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}})} \times 100$$

Trypan blue dye exclusion assay

Cell viability was also determined by Trypan blue dye exclusion assay. Cells were seeded separately at a density of 1.5×10^5 cells/well into 12-well plate followed by MZLAgNPs / AMFAgNPs / AMRAgNPs treatment for 48 h. Following a PBS (pH 7.4) wash, the cells were stained with 4% trypan blue and counted using hemocytometer. Those cells which remained unstained due to dye exclusion, since they possessed intact membranes, were counted as live whilst the

blue stained ones were considered to be dead (He et al., 2016). Cisplatin treated cells were also used for comparative purposes as mentioned in the earlier section.

3.3.6. Clonogenic assay

Cells were plated separately at a density of 5×10^2 cells per well in six-well plates and treated with all three types of AgNPs / cisplatin; untreated cells were also maintained as controls. Following 48 h of incubation and a change of growth medium, cells were subjected to a further incubation period of 7 days. Colonies were then stained using 0.5 % (w/v) crystal violet and counted manually (Franken et al., 2006).

3.3.7. Cell migration assay

Cells were seeded in 35 mm culture dish and incubated until a monolayer was formed. Linear wounds were made by scratching with a microtip and the detached cells were removed with a PBS (ph 7.4) wash. Each culture dish was then treated individually the three different biogenic AgNPs / cisplatin. Cell movement into the wound area was then monitored by taking photographs at 0, 12 and 24 h intervals using a light microscope (Xie et al., 2015). The average migration rates were calculated as follows:

$$\text{Cell motility (\%)} = \frac{\text{The wound width at 0 h - the wound width at 12/24 h}}{\text{The wound width at 0 h}} \times 100$$

3.3.8. Cell cycle analysis

Assessment of cell cycle distribution and percentage apoptosis was carried out using a flow cytometer (BD FACS Aria^{TM}). Distribution of cells in different phases of cell cycle here is based on a univariate analysis of cellular DNA content following staining with propidium iodide (PI) at a single time point. This provides a snapshot of the DNA content variations within the three major phases of cell cycle within a population – G_0/G_1 (2n), S (2n → 4n), G_2/M (4n) – thereby revealing the overall cell cycle distribution. Interestingly, detection of cells undergoing apoptosis also becomes possible in this technique due to their fractional DNA content. Extensive DNA degradation occurs as part of the apoptotic process. It is to be noted

that fragmented low-molecular weight DNA undergoes extraction during the staining process in addition to loss of DNA during shedding of apoptotic bodies. Flow cytometric analysis software enable deconvolution of DNA content histograms for identification and estimation of the hypodiploid / sub-G_1 peak representing the apoptotic fraction in the population.

Cells were seeded at 1×10^5 cells/well into a six well plate. Following treatment with AgNPs /cisplatin for 48 h, cells were washed with PBS (pH 7.4) and fixed with 70% ethanol overnight at -20ºC, pelleted and stained with 20 µg/mL of propidium iodide containing 20µg/mL RNase for 30 min at 37°C (Fageria et al., 2017). The population of cells in G_0/G_1, S and G_2/M phases were then determined using BD FACS Diva software version 5.0.2 .

3.3.9. Microscopic analysis of cells

Light microscopy

Cells (1×10^5) were treated with different concentrations of biogenic AgNPs and cisplatin for 48 h. The control and treated cells were trypsinized, washed with ice-cold PBS and observed under phase-contrast inverted microscope to study the cytomorphological changes, if any, induced by the treatments.

Scanning electron microscopy

Following trypsinization, separate aliquots of biogenic AgNPs/cisplatin treated cells (1×10^5) were fixed in 4% glutaraldehyde followed by a PBS (pH 7.4) wash. It was then dehydrated by passing through an ascending graded acetone series (75-85-100%) and the dried cells were coated with gold (Sreekanth et al., 2007) and observed under a CARL-Zeiss Gemini 300 scanning electron microscope.

Hoechst 33258 staining

Cells (1.5×10^5) were seeded into 24-well plates and treated with various concentrations of biogenic AgNPs/cisplatin for 24 h. The cells were then harvested and washed with PBS (pH 7.4) and re-suspended in Hoechst 33258 solution (1mg/mL) for 10 min at room temperature in the dark. The nuclear morphology was examined under a fluorescence microscope.

Acridine orange-ethidium bromide dual staining

The morphological evidence of apoptosis in biogenic AgNPs treated cells was detected by dual staining with acridine orange and ethidium bromide. Acridine orange penetrates into living cells emitting green fluorescence after intercalation into DNA. The second dye ethidium bromide emits red fluorescence in the cells with an altered cell membrane. Viable cells have bright green nuclei with organized structure. Apototic cells have orange to red nuclei with condensed or fragmented chromatin, whilst necrotic cells have a uniformly orange to red nuclei with condensed structure. The control and treated cells were harvested and centrifuged at 800×g, washed with cold PBS (pH 7.4) and adjusted to a cell density of 1.5×10^5 cells/mL using PBS (pH 7.4) . The acridine orange/ ethidium bromide solution (1:1, v/v) was added to the cell suspension at a final concentration of 100 µg/mL (Thangam et al., 2012) prior to observation under a fluorescence microscope. Percentage of apoptotic cells was determined by formula:

$$\% \text{ of apoptotic cells} = \frac{\text{Total number of apoptotic cells}}{\text{Total number of normal and apoptotic cells}} \times 100$$

Assessment of intracellular reactive oxygen species

Cellular oxidative damage induced by biogenic AgNPs was studied using a cell permeable dye 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). This compound is oxidized by ROS into the fluorescent carboxy-dichlorofluorescein (DCF) inside the cells. Briefly, the control and treated cells were incubated with 10µM DCFH-DA at 37°C for 30 min in the dark and then washed twice with PBS (pH 7.4) prior to microscopic examination (Jeyaraj et al., 2015).

Determination of phosphatidylserine externalization

Translocation of phosphatidylserine (PS) from the inner to the outer phase of plasma membrane occurs during the early stage of apoptosis. Externalization of PS in control and treated cells was evaluated by staining cells with FITC-labeled annexin V-PI according to the manufacturer's protocol (Sigma, USA). The cells were mounted on slides and their images captured using fluorescence microscopy.

Assessment of mitochondrial membrane potential

Mitochondria are an integral part of apoptotic machinery. The depletion of mitochondrial membrane potential, an early marker of apoptosis leads to the release of its inter-membrane space proteins such as cytochrome *c* into the cytosol which then activates caspases. Mitochondrial membrane potential was evaluated by incubating the treated and control cells with lipophilic cationic dye Rhodamine 123 (10µg/mL) for 30 min (Zhen et al., 2011). Cells were then harvested, washed twice with PBS (pH 7.4), mounted on slides and their images captured using fluorescence microscopy.

3.3.10. Genotoxicity evaluation by comet assay

Alkaline gel electrophoresis, also known as comet assay, enables detection of DNA damage. It allows assessment of different types DNA damages such as single and double-strand breaks, crosslinks and base-pair damages in cells undergoing apoptosis. The control, AgNP and cisplatin-treated cell samples were washed with PBS (pH 7.4) and comet assay was carried out essentially as described by Singh et al. (1988). Following electrophoresis, the slides were washed with neutralization buffer, stained with ethidium bromide (10µg/mL) and observed under a fluorescence microscope.

3.3.11. Reverse transcription quantitative PCR (RTq-PCR) analysis

The relative levels of apoptosis-related gene expressions were evaluated by RT-qPCR employing six sets of gene-specific primers - PUMA, caspase-3, -8, -9, Bcl-2 and Bax (Kuppusamy et al., 2016; Jeyaraj et al., 2013) - the sequences of which are given in Table 3.1. Total RNA isolated from treated and control cells using TRI® Reagent was used to synthesize cDNA. Briefly, a 5 µl reaction containing 0.5 µl of oligo dT (100ng/ µl) and 1.0 µl of 0.5 µg/ µl RNA was incubated at 65 ^{o}C for 10 min and placed on ice. To this, added 1.0 µl of 10mM dNTP mix, 2.0 µl of 10x M-MLV reverse transcriptase buffer, 1.0 µl of M-MLV reverse transcriptase, 0.5 µl of RNasin, 0.5 µl DTT (20mM) and made upto 10 µl with sterile nuclease-free water. The reaction mixture was incubated at 37 ^{o}C for 1 h, heated to 95 ^{o}C for 10 min. and stored at -20^{o}C until use. The real-time PCR was

performed on an Illumina Eco^{TM} Real Time system (USA) using SYBR Green PCR Master Mix according to the manufacturer's protocol. Relative expression levels were calculated using $\Delta\Delta C_t$ method with GAPDH as internal reference gene.

Table 3.1. Primers used in RTq-PCR assay

Gene	Primer sequence
PUMA	Forward: 5'GACCTCAACGACAGTACGA3'
	Reverse: 5'GAGATTGTACAGGACCCTCCA3'
Caspase 3	Forward: 5'TGGCATACTCCACAGCACCTGGTTA3'
	Reverse: 5' CATGGCACACAAAGCGACTGGATGAA3'
Caspase 8	Forward: 5'CATCCAGTCACTTTGCCAGA3'
	Reverse: 5'GCATCTGTTTCCCCATGTTT3'
Caspase 9	Forward: 5'TTCCCAGGTTTTGTTTCCTG3'
	Reverse: 5'CCTTTCACCGAAACAGCATT3'
Bax	Forward: 5'GCCACCAGCCTGTTTGAG3'
	Reverse: 5'CTGCCACCCAGCCACCC3'
Bcl-2	Forward: 5' TATAAGCTGTCGCAGAGGGGCTA3'
	Reverse: 5'GTACTCAGTCATCCACAGGGCGAT3'
GAPDH	Forward: 5'AATCCCATCACCATCTTCCA3'
	Reverse: 5'CCTGCTTCACCACCTTCTTG3'

3.3.12. Western blot analysis

Western blotting was performed as previously described (Ausubel et al., 1992) to investigate expression levels of apoptotic and cell cycle-related proteins. The control and the treated cells were washed with cold PBS and lysed in RIPA buffer. Cells were carefully scraped and incubated on ice for 30 min. The total cellular lysate was centrifuged at 10,000 × g for 10 min to clear cell debris and protein concentration was measured using Bradford assay (Bradford, 1976). Protein samples (50μg) were electrophoresed on a 12.5% SDS-PAGE gel and then transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were

washed with Tris-buffered saline containing Tween-20 (TBST), blocked with 5% skimmed milk for I h at room temperature and incubated overnight at 4°C separately with rabbit-derived primary antibodies (dilution 1:1000) against caspase-3, -7, -9, PARP, p^{53} and p^{21} Wafi/Cip1 and a mouse monoclonal antibody for β actin acting as a loading control to detect house-keeping function. Following a wash with TBST, the membranes were incubated with ALP-conjugated secondary antibody (1: 2000 dilution) for 1h at room temperature. Following a subsequent wash with TBST, the blots were exposed to BCIP/NBT solution to visualize the immunostained polypeptides.

3.3.13. Assessment of AgNP cytotoxicity on normal human cells

Peripheral blood lymphocytes

Cellular toxicity of biogenic AgNPs/cisplatin was assessed employing human peripheral blood lymphocyte cultures (hPBLs). Briefly, isolated lymphocytes (10^5cells/mL) were added to 5.0 mL lymphocyte culture medium, HiKaryoXL^{TM}-RPMI, supplemented with phytohemagglutinin and incubated at 37°C for 48 h. Following treatment with different concentrations of biogenic AgNPs/cisplatin, MTT assay was carried out as described earlier (section 3.3.5.2.). The OD_{620nm} was measured and the difference of absorbance between the treated and untreated control cells was used to determine cell viability (Mosmann, 1983).

Red blood cells

Damage to plasma membrane of erythrocytes causes release of hemoglobin which can be assessed spectrophotometrically (Khan et al., 2015). To analyze hemolytic activity of the biogenic AgNPs/cisplatin, 5.0 mLof heparinized fresh human blood was taken and centrifuged at 1500 rpm for 10 min at 4°C. The plasma and buffy coat were removed and the erythrocyte pellet was washed thrice with PBS (pH 7.4). An aliquot of the resultant RBC suspension in buffer (0.6mL) was treated individually with varying concentrations (50-200 µg/mL) of AgNPs/cisplatin for 90 min at 37^{o}C. Distilled water and PBS served as positive and negative controls respectively. The samples were then centrifuged at 3000 rpm for 10 min and the

absorbance of the supernatants was measured at 543 nm. Percent hemolysis (H) was calculated by the formula,

$$\% \text{ hemolysis} = [OD_{\text{test sample}} - OD_{\text{PBS}} / OD_{\text{distilled water}} - OD_{\text{PBS}}] \times 100$$

3.4. Statistical analysis

Experimental data are expressed as the mean ± SD from three independent experiments. Results were analyzed for significance by one-way ANOVA using SPSS software version 20.0. Differences with $P < 0.05$ were considered significant. Asterisks (*) have used to identify the level of significance (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

3.5. Ethical Statement

The blood samples used for hemolysis assay and isolation of lymphocytes for lymphocyte culture and MTT assay was willingly self-donated. It may be noted that according to the Indian Council for Medical Research, New Delhi, India, Chapter-II, page no. 11-12, the ethical approval for this research was not deemed to be necessary. According to this guideline, proposals which present less than minimal risks are exempted from the ethical review process.

RESULTS AND DISCUSSION

I. Phyto-assisted synthesis and physico-chemical characterization of silver nanoparticles

4.1. Biosynthesis of nanoparticles – optimization of parameters

Plants offer one of the best platforms for 'green' synthesis of nanoparticles. As mentioned elsewhere in this Thesis (section 2.3.3.3.), this study exploited the bioreduction and capping potential of phytoconstituents present in medicinal plant extracts for biosynthesis of silver nanoparticles. Adopting such an extracellular method, biosynthesis of silver nanoparticles was achieved using extracts prepared from *Manilkara zapota* leaves, *Annona muricta* fruits and roots following a preliminary screening of several extracts derived from various medicinal plants.

A detailed study was conducted to ascertain the key factors involved in the biosynthesis of silver nanoparticles accompanying reduction of $AgNO_3$ to biogenic AgNPs in the presence of three specific extracts derived from *Manilkara zapota* leaf (MZL), *Annona muricata* fruit (AMF) and root (AMR). The reducing environment provided by the constituents present in each of these three plant extracts was found to trigger the biosynthetic process within a few minutes. This was discernible by the change in the color of the reaction mixture from colorless to yellowish brown, a clear visual clue to the formation of biogenic silver nanoparticles (Fig. 4.1).

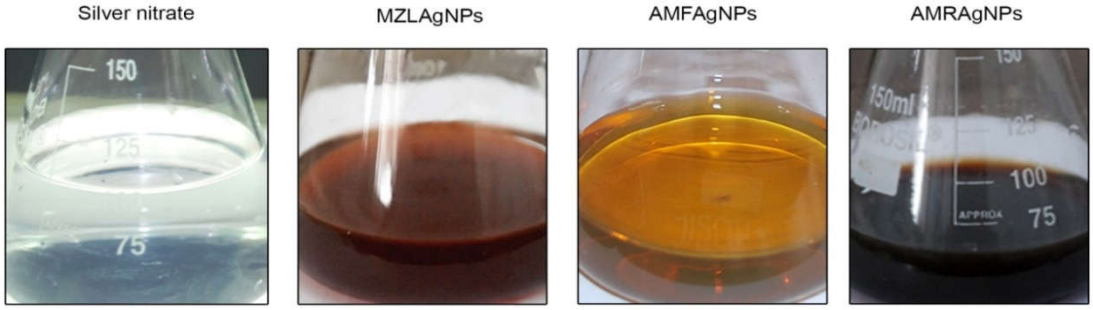

Fig. 4.1. ***Visual clue to synthesis of biogenic silver nanoparticles*** **:** *Aqueous solution of 1mM $AgNO_3$ changes color upon formation of M.zapota leaf-derived MZLAgNPs, A.muricata fruit-derived AMFAgNPs and root-derived AMRAgNPs.*

UV-vis spectroscopy was used to detect the presence of AgNPs. Absorbance, particularly in the range of 420-450 nm, was used as an indicator of reduction of Ag^+ to metallic Ag (Philip, 2011; Karuppiah and Rajmohan, 2013).

4.1.1. *Effect of extract concentration*

The concentration of plant extracts plays an important role in the formation of silver nanoparticles. Since the concentration appropriate for AgNPs biosynthesis is dependent on the plant species employed, optimization of the individual extract volumes was carried out. The change in absorbance is known to be directly correlated to the extract concentration apparently due to the presence of reducing phytoconstituents such as nitrate reductase, terpenoids, flavonoids and polysaccharides (Huang et al., 2007). Nanoparticles were successfully synthesized at all five concentrations namely, 2.0, 4.0, 6.0, 8.0 and 10.0 mL of the three individual extracts tested. However, extract concentrations beyond 10.0 mL led to decrease in the overall amount of nanoparticles produced, which was evident in the peak intensities measured by UV-vis spectorsopy. In the case of MZLAgNPs, good absorbance values / peak intensities were recorded at 2.0 mL of MZL extract per 98.0 mL of silver nitrate solution. Attempts to increase the extract concentration resulted in relatively small increments in nanoparticle yield which was attributable to an apparent dilution effect in respect of silver nitrate ions in the solution (Fig 4.2). In the case of AMFAgNPs and AMRAgNPs, relatively higher concentrations, 8.0 mL and 10.0 mL of the respective extracts were required to generate the characteristic absorption profiles (Fig. 4.2.).

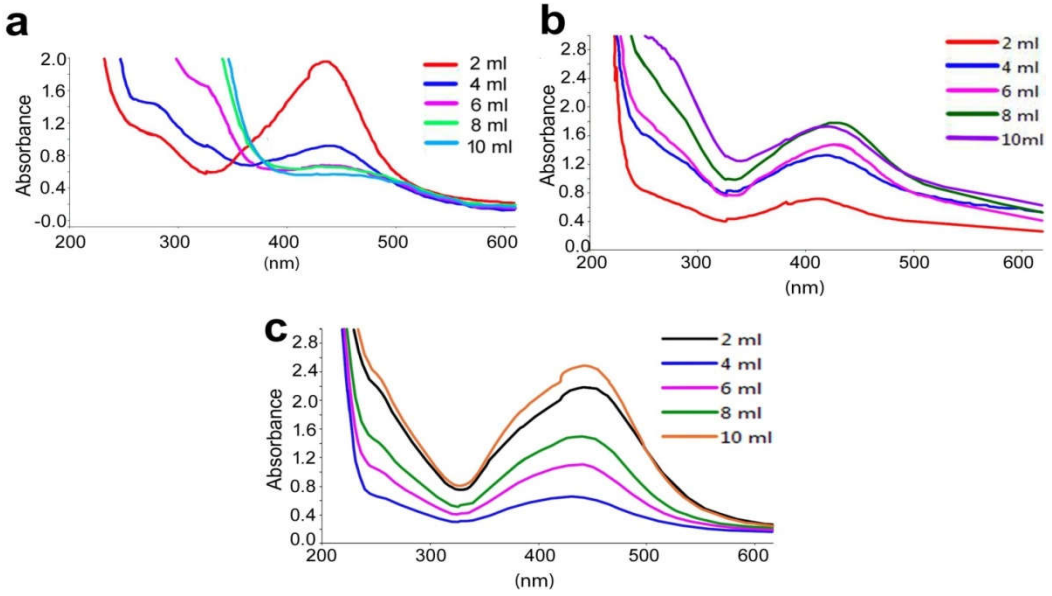

Fig. 4.2. ***Effect of plant extract concentration on synthesis of biogenic AgNPs derived from (a) MZL, (b) AMF and (c) AMR extracts.***

4.1.2. *Effect of precursor metal ion concentration*

Variation in the precursor metal ion concentration is known to influence nanoparticle biosynthesis (Bankar et al., 2010). The UV-vis spectra of the AgNPs obtained at different metal ion concentrations tested are shown in Fig. 4.3. The minimum concentration for effective synthesis was found to be 1.0 mM. Lower metal salt concentrations at 0.25mM and O.5mM failed to reveal the characteristic surface plasmon resonance (SPR) peak apparently due to the inadequacy of silver ions for the bioreduction process (Maria et al., 2015). Use of precursor concentrations beyond 1.0 mM resulted in agglomeration leading to formation of larger-sized particles.

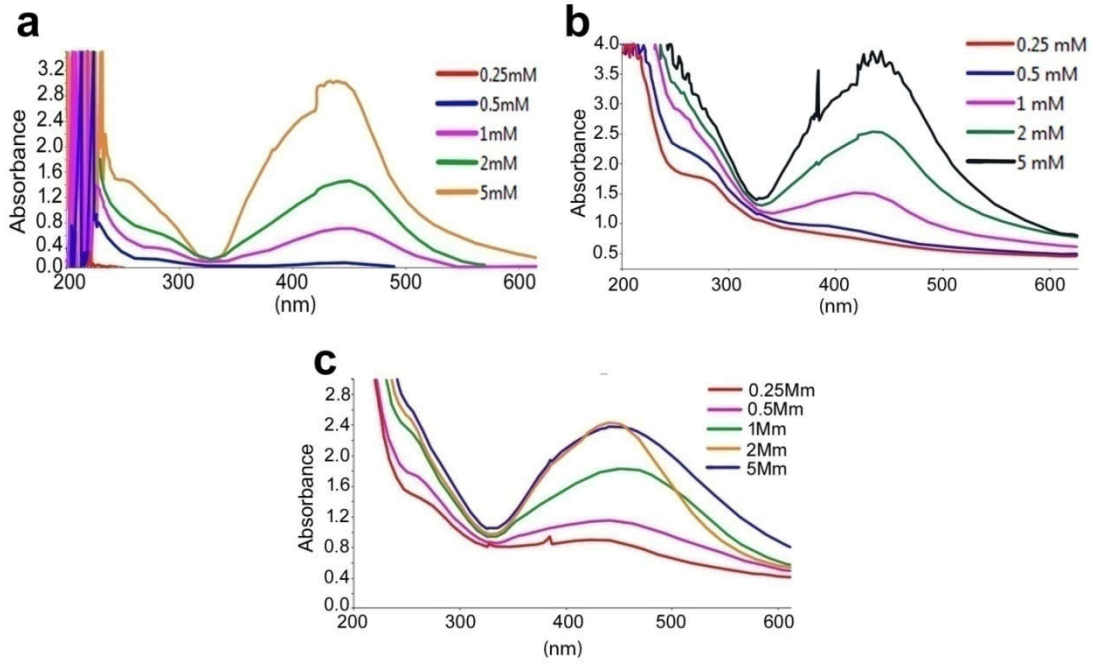

Fig. 4.3. ***Effect of silver nitrate concentration on synthesis of biogenic AgNPs derived from (a) MZL (b) AMF and (c) AMR extract.***

4.1.3. Effect *of temperature and period of incubation*

Temperature and time of incubation of the reaction mixture are well known factors, among others mentioned above, which influence biosynthesis of nanoparticles. In general, increase in temperature is reflected in higher intensity of SPR peaks indicating increased production of nanoparticles. Effective synthesis was

observed at 90°C compared to that at lower temperatures (Fig.4.4) within about 30 minutes of incubation with respect to MZLAgNPs and AMFAgNPs. In the case of AMRAgNPs, 95° C was found to be the optimum temperature for an incubation period of 30 min (Fig. 4.5). These observations are in line with previous reports on biogenic silver nanoparticles (Sathishkumar et al., 2012; Sukirtha et al., 2012).

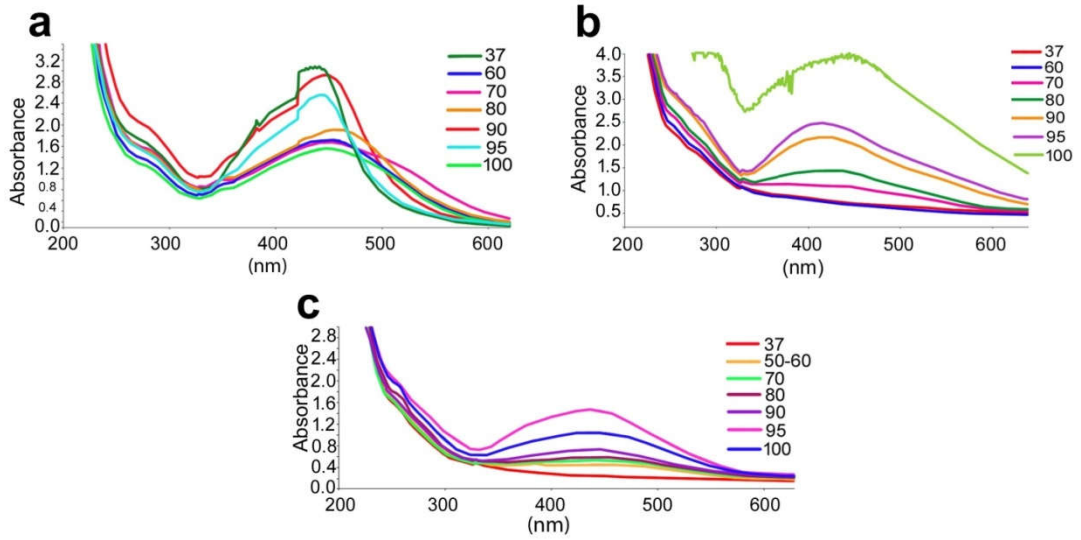

Fig. 4.4. ***Effect of temperature on synthesis of biogenic AgNPs derived from (a) MZL (b) AMF and (c) AMR extract.***

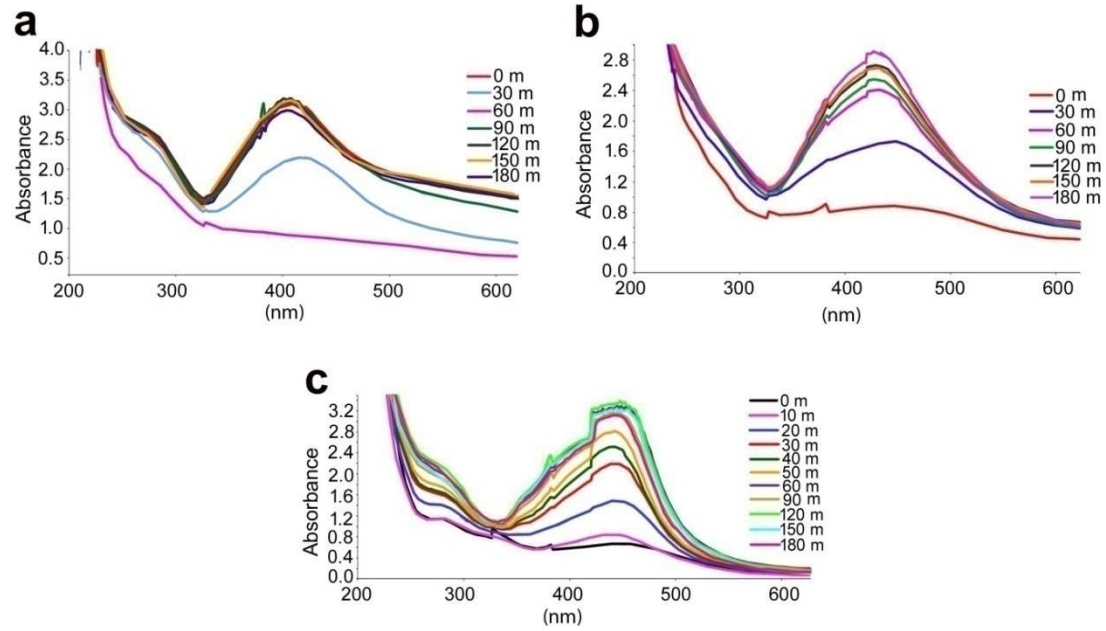

Fig. 4.5. ***Effect of period of incubation on synthesis of biogenic AgNPs derived from (a) MZL (b) AMF and (c) AMR extracts.***

4.1.4 *Effect of pH*

The effect of pH on biosynthesis of AgNPs was also evaluated by UV-vis spectroscopy. The results obtained showed that the synthetic reactions were also dependent on the pH of reaction medium. The absorbance values were found to increase gradually concomitant with the pH values increasing from 4.0 to 9.0. However, the effective synthesis of MZL, AMF and AMR derived AgNPs were found to occur at pH 4.0, 7.0 and 8.0 respectively, beyond which nanoparticle aggregation was observed to occur (Fig. 4.5). It is relevant to point out here that the pH of the reaction mixture is a key factor which also plays an additional role in the control of shape and size of nanoparticles (Sneha et al., 2010). Table 4.1 provides the complete set of optimized conditions for efficient synthesis of all three types of biogenic AgNPs – namely, MZLAgNPs, AMFAgNPs and AMRAgNPs in a volume of 100 mL.

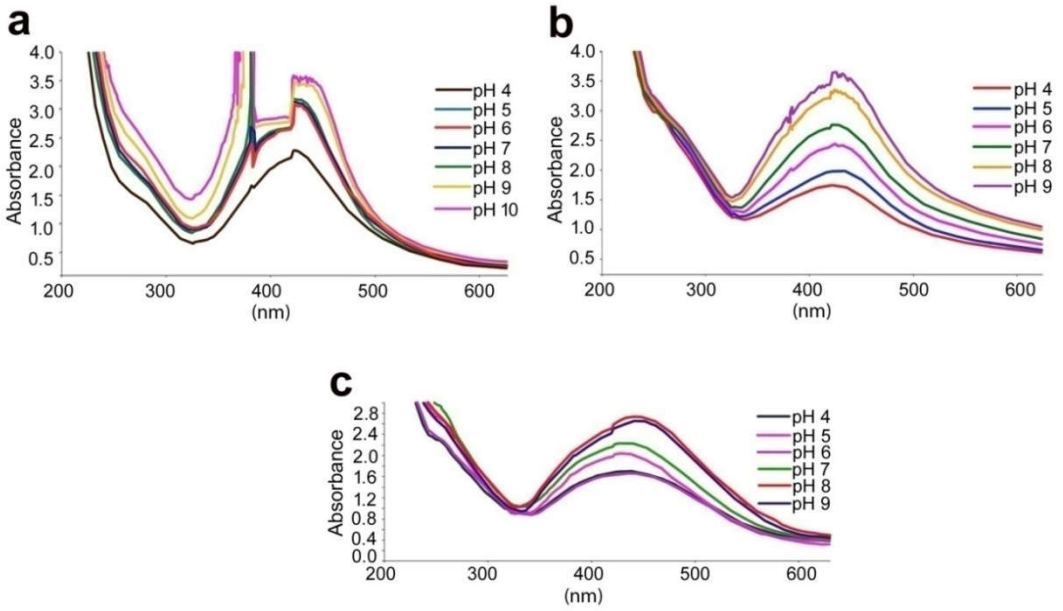

Fig. 4.6. ***Effect of pH on synthesis of biogenic AgNPs derived from (a) MZL (b) AMF and (c) AMR extracts.***

Table 4.1. Optimized conditions obtained for efficient synthesis of biogenic AgNPs

Plant part	Extract volume (mL)	Con. of $AgNO_3$ (mM)	pH of the mixture	Temperature (°C)	Period of Incubation (min)	Wavelength (nm)
M.zapota leaves	2	1	4	90	30	421
A.muricata fruits	8	1	7	90	30	430
A.nuricata roots	10	1	8	95	30	440

4.2. Characterization of Silver nanoparticles

4.2.1. *Fourier transform infrared spectroscopy* (*FTIR)*

FTIR spectroscopic analysis was carried out to check for the presence of possible bioreducing and capping phytoconstituents present in the extracts presumably adsorbed or associated with biogenic AgNPs. The spectra obtained for all three types of nanoparticles - MZLAgNPs, AMFAgNPs and AMRAgNPs - were found to exhibit a number of peaks reflecting the complex biochemical nature of the nanoparticle samples.

The IR bands of MZLAgNPs were observed at 3446 cm^{-1}, 2959 cm^{-1}, 2920 cm^{-1}, 2851 cm^{-1}, 1747 cm^{-1}, 1541 cm^{-1}, 1023 cm^{-1} and 692 cm^{-1} (Fig. 4.6). These IR peaks are known to be characteristic of flavonoids and terpenoids (amine, alcohol, ketone aldehyde and carboxylic acid) present in plant leaves (Philip, 2011). The sharp peaks obtained at 3446 cm^{-1} corresponded with O-H stretching of alcohols and phenols whilst peaks at 2959 cm^{-1}, 2920 cm^{-1} and 2851 cm^{-1} were representative of the stretching vibrations associated with $–CH_2$ and $–CH_3$ functional groups. Further, peaks at 1747 cm^{-1}, 1023 cm^{-1} corresponded to the carbonyl groups (C=O) and C-O bonds respectively. Peaks at 1541 cm^{-1} represented amide II N-H bending, those at 1451 cm^{-1} indicated N-O asymmetric stretching whilst peaks at 744 cm^{-1} and 692cm^{-1} represented C-Cl stretch.

AMFAgNPs displayed peaks located at 3650 cm^{-1}, 3422 cm^{-1}, 2923 cm^{-1}, 2853 cm^{-1}, 1747 cm^{-1}, 1457 cm^{-1}, 1023 cm^{-1} and 672 cm^{-1} (Fig.4.6). The peak at 3650 cm^{-1} corresponded to O-H stretching of alcohols and phenols. The sharp peak at 3422 cm^{-1} represented N-H group from peptide linkages of phytoconstituents within the extract (MubarakAli et al., 2011). Peaks at 2923 cm^{-1}, 2853 cm^{-1}, 1457 cm^{-1} and 672 cm^{-1} represented C-H functional groups whilst those at 1747 cm^{-1} and 1023 cm^{-1} represented carbonyl groups (C=O) and C-O bond respectively.

AMRAgNPs exhibited bands at 3760 cm^{-1}, 3429 cm^{-1}, 2922 cm^{-1}, 2852 cm^{-1}, 1744 cm^{-1}, 1629 cm^{-1}, 1384 cm^{-1} and 1019 cm^{-1} (Fig.4.6). The absorption peaks at 3760 cm^{-1} and 3429 cm^{-1} indicated the presence of phenol (O-H) groups. The C-H binding was represented by the peak at 2922 cm^{-1}, and 2852 cm^{-1}. The band appearing at 1744 cm^{-1} was attributed to carbonyl groups (C=O). The strong intense peak at 1629 cm^{-1} was assigned to C-C and C-N stretching normally associated with proteins (Kanipandian et al., 2014). Peak at 1384 cm^{-1} represented amide II groups whilst the peak at 1019 cm^{-1} indicated C-O single bonds.

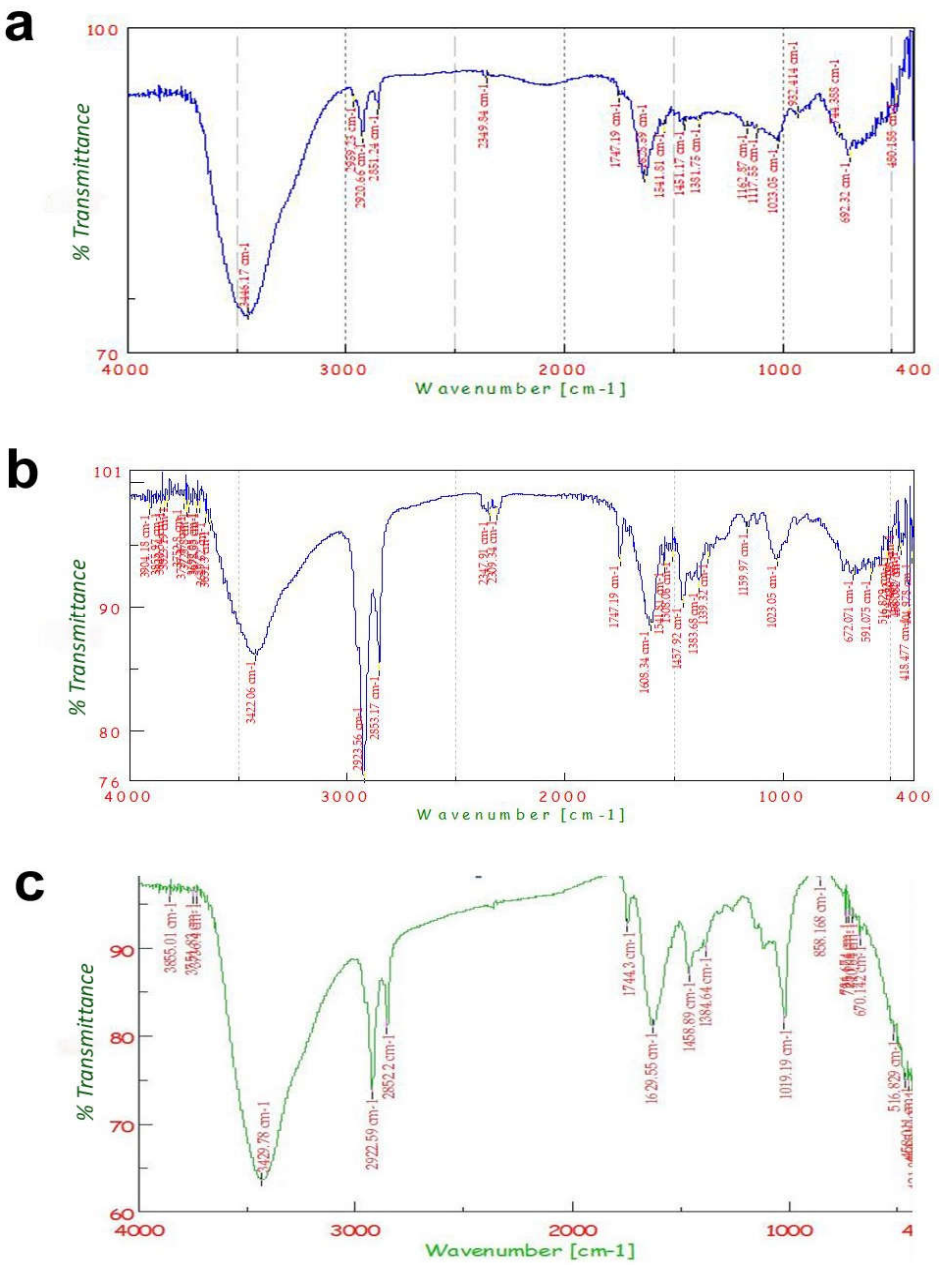

Fig. 4.7. ***FTIR spectra of (a) MZL (b) AMF and (c) AMR derived AgNPs.***

FTIR spectroscopic results have thus confirmed that the three extracts - MZL, AMF and AMR possess the biochemical components capable of performing dual functions of reduction and stabilization essential for biogenesis of silver nanoparticles. Hydroxyl, carbonyl and other functional groups like amines, alcohols, ketones, aldehydes and carboxylic acids present on secondary metabolites such as terpenoids have been implicated in nanoparticle generation (Jae and Beom,

2009). Interestingly, terpenoids have been reported to be the surface active molecules for nanoparticle synthesis in *Azadirachta indica* leaf extract (Shankar et al., 2004), whilst tannins in *A. muricata* have been shown to prevent aggregation and confer long term stability to biogenic AgNPs (Vivek et al., 2012). FTIR analysis also revealed the presence of protein-associated functional groups considered as one of the principle components involved in encapsulation and stabilization of biogenic AgNPs. The proteins have been shown to bind to silver nanoparticles via free amine groups or through cysteine residues in saponins and phenolics in plant extracts, thereby stabilizing the nanoparticles formed through the surface-bound proteins. Taken together, these results strongly agree with similar previous studies (Monali et al., 2009; Pant et al., 2012; Saranyaadevi et al., 2014).

4.2.2. *X-ray diffraction studies (XRD)*

The XRD patterns of all the three types of biogenic AgNPs obtained in the present study are given in Fig.4.8.

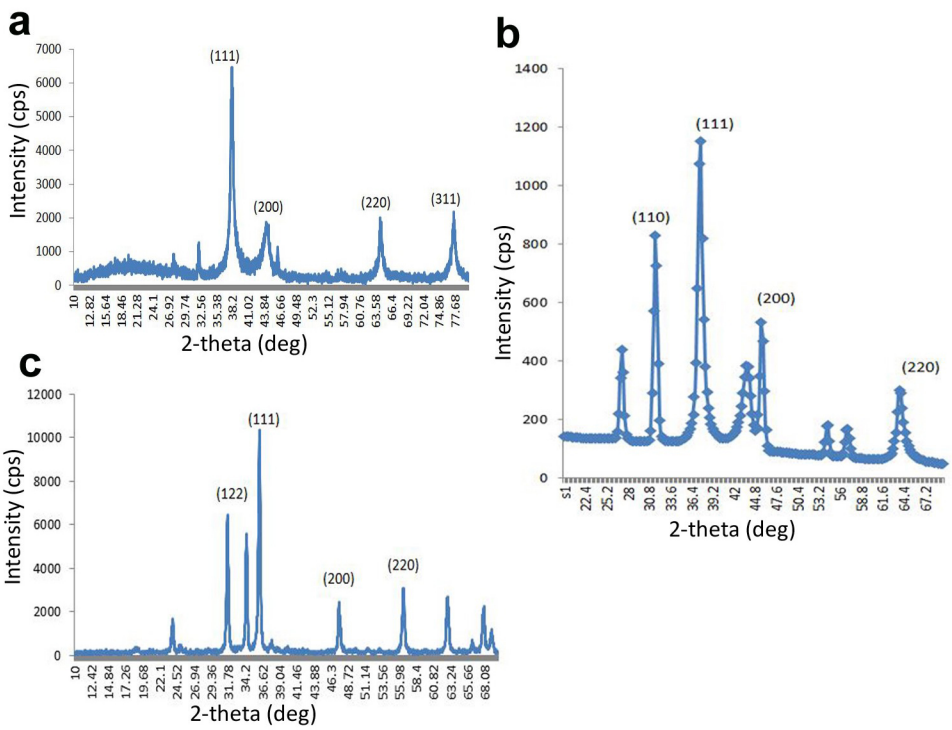

Fig. 4.8. ***X-ray diffraction patterns of (a) MZL (b) AMF and (c) AMR derived AgNPs.***

The appearance of multiple peaks in these samples confirmed the crystalline nature of biosynthesized AgNPs. The diffraction peaks at 2θ values of 38.05º, 44.2°, 64.39° and 77.26° for MZLAgNPs were found indexed to the (111), (200), (220) and (311) reflection planes of the face-centered-cubic (fcc) structure. The peaks identified at 2θ values of 27.6^{o}, 38^{o}, 46^{o} and 63.6^{o} for AMFAgNPs corresponded to (110), (111), (200) and (220) planes of face-centered-cubic phase. The peaks observed at 30.03$^{°}$, 38$^{°}$, 47.8^{o} and 63.1^{o}for AMRAgNPs were assigned to (122), (111) (200), and (220) reflection planes of face-centered-cubic phase structure (JCPDS card 89-3722). The sharp peaks clearly indicated the cubic crystalline nature of the biosynthesized nanoparticles within the nanoregime which was also consistent with the earlier reports (Satishkumar et al., 2009; Bankar et al., 2010; Bhakya et al., 2015). Some additional unassigned peaks were also observed in the XRD graph attributable to the presence of bioorganic matters and capping agents involved in AgNPs synthesis as mentioned in earlier reports (Shankar et al., 2003; Daizy, 2010; Sangiliyandi et al., 2013). The average size of MZL, AMF and AMR derived AgNPs were found to be 24 nm, 19 nm and 34 nm respectively, based on calculations using Debyee Scherrer's formula. Interestingly, size variations (19 versus 34 nm) of the particles obtained from two different parts (fruit versus root) of the same plant, *A.muricata*, is reflective of their biochemical compositional differences.

4.2.3. *Field emission scanning electron microscopy (FESEM)*

FESEM was employed to study the size, shape and morphologies of the biosynthesized AgNPs. The SEM images of the samples are displayed in Fig 4.9., which reveal the predominantly spherical and relatively uniform shape of nanoparticles.

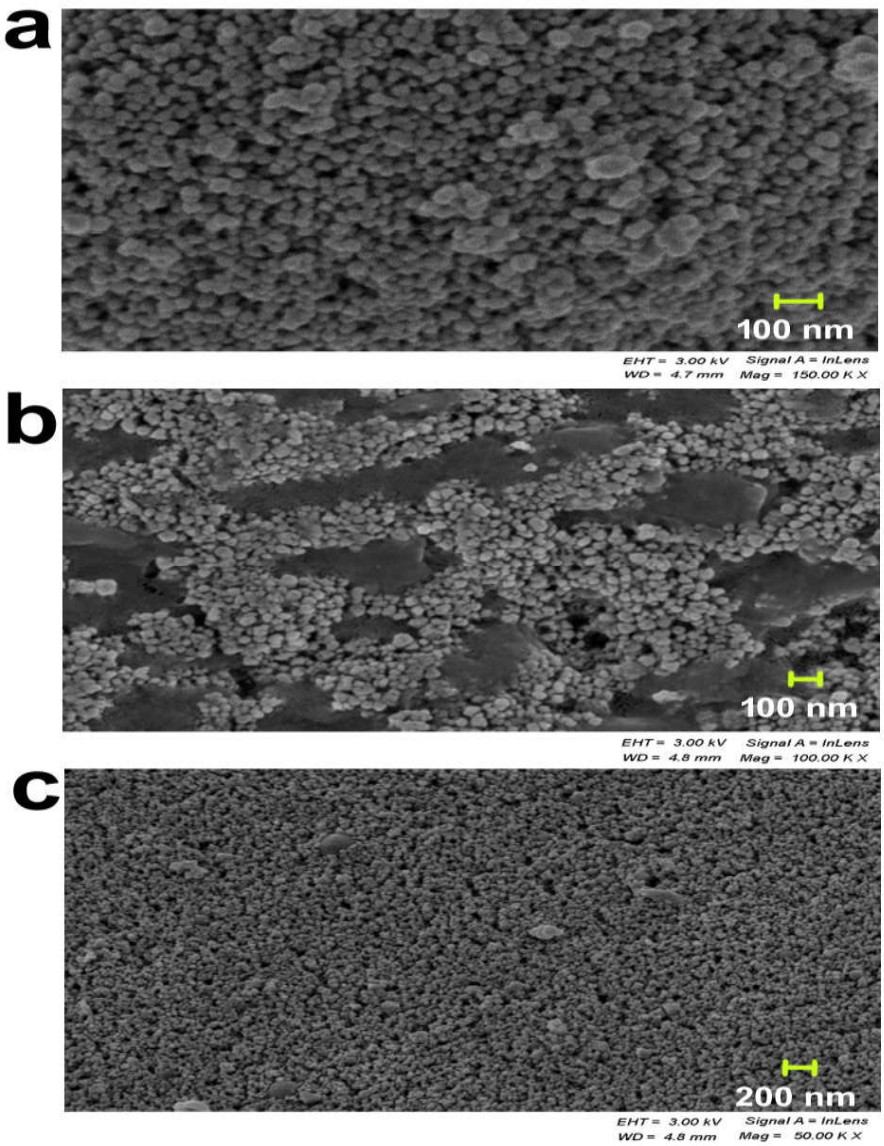

Fig. 4.9. ***FESEM images of (a) MZL (b) AMF and (c) AMR derived AgNPs.***

The average size of the silver nanoparticles as determined from the micrographic images was found to be 28.59±3.0 nm, 21.36 ± 3.4 nm and 31 ± 3.7 nm for MZLAgNPs, AMFAgNPs and AMRAgNPs respectively. Notably these values are close to those determined by the XRD technique (sub-section 4.2.2.).

4.2.4. *Energy dispersive X-ray spectroscopy* (*EDX)*

The occurrence of elemental silver was identified by EDX analysis (Fig. 4.10). The EDX spectrum illustrated the presence of strong signals for metallic silver. The dominant sharp signal peak was observed at approximately 3eV for

silver which was distinctive for the absorption of crystalline AgNPs (Ahamed et al., 2011; Vivek et al., 2012) and it strongly confirmed the reduction of silver nitrate to silver nanoparticles and quantification of elemental silver (Ramalingam et al., 2014; Muthukrishnan et al., 2015). The amount of silver present in MZLAgNPs (89.88%) was found to be higher than that present in AMR (81.07 %) and AMF derived AgNPs (71.54%). Low amounts of carbon, oxygen, phosphorous and chlorine observed were apparently attributable to plant constituents.

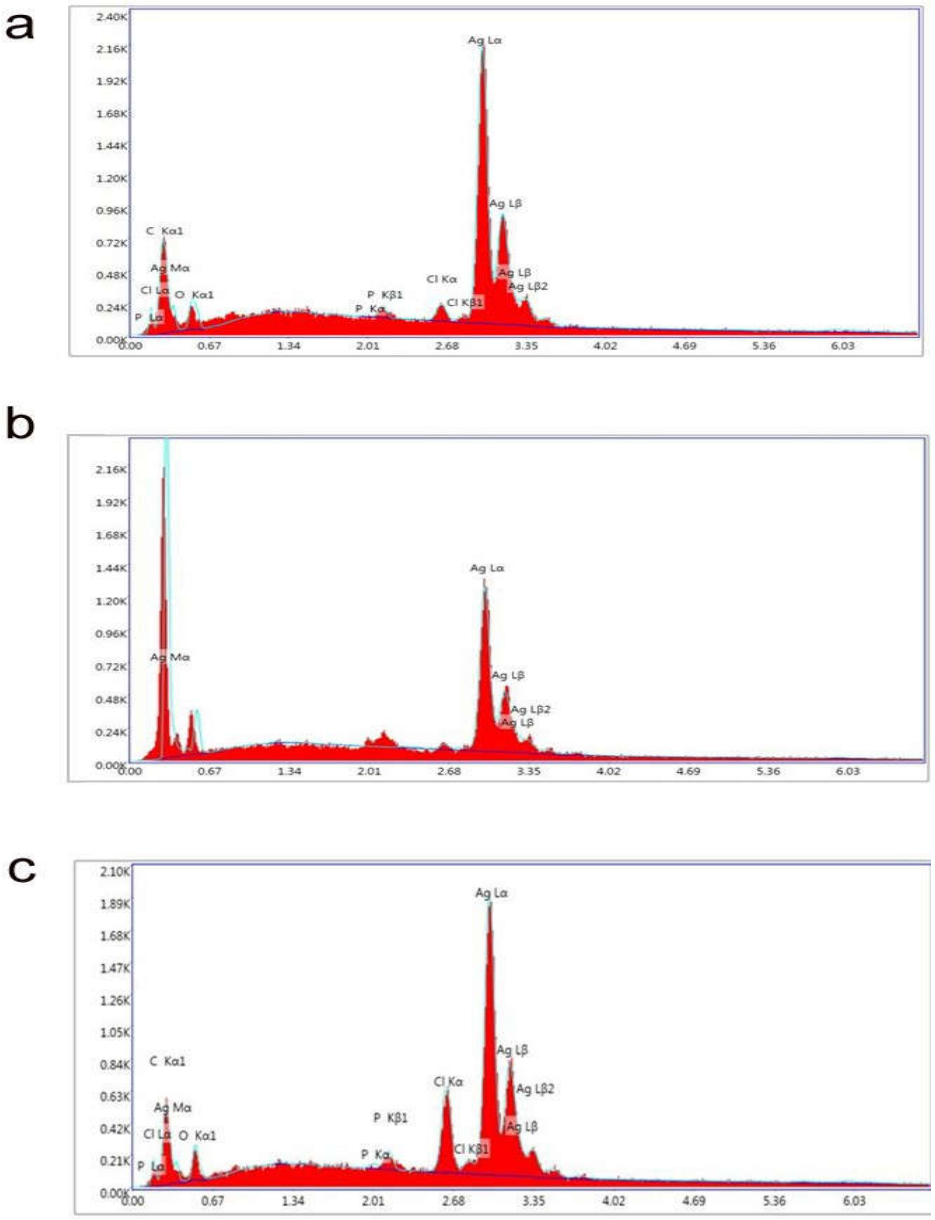

Fig. 4.10. ***The EDX spectrum of (a) MZL (b) AMF and (c) AMR derived AgNPs.***

Taken together, the combined data obtained from XRD and FESEM analysis of the three types of nanoparticles revealed that *A.muricata* fruit extract-derived AMFAgNPs were the smallest in size in comparison to the other biogenic particles.

II. Phytochemical, biochemical and antimicrobial evaluation of biogenic AgNPs

4.3. *Total phenolics and flavonoid content*

Phenolics are composed of one or more aromatic rings having single or multiple hydroxyl groups. These non-enzymatic compounds from natural sources have attracted attention due to their antioxidant capabilities (Javanmardi et al., 2003) of quenching free radicals by forming resonance-stabilized phenoxyl radical (Praveena and Pradeep, 2012) or prevent decomposition of hydroperoxide into its free radical (Baharum et al., 2014).

The total phenolic content of the three types of aforementioned nanoparticles and their corresponding plant extracts were evaluated by Folin-Ciocalteau method (Table.4.2). The highest total phenolic content of 100±1.0 mg GAE/g of the extract was obtained with MZL. Significantly large amounts, up to 80% of phenolics, was found associated with MZLAgNPs. Interestingly, despite the presence of much lower amounts of phenolics in the AMF extract (51%), upto 95% of it was found to be associated with AMFAgNPs. AMRAgNPs, on the other hand were found to contain the lowest amount of phenolic content (75%) compared to that found in the extract.

Table 4.2. Total phenolic and flavonoid contents of invidiual extracts versus biogenic silver nanoparticles

Samples	Total phenolic content (mg GAE/g sample)	Total flavonoid content (mg QE/g sample)
MZL Extract	100 ±1.00	310±0.58
MZLAgNPs	80.0 ±0.50	190±0.50
AMF Extract	51.0±1.52	300±2.00
AMFAgNPs	48.5±0.75	250±1.58
AMR Extract	65.0±0.58	450±0.005
AMRAgNPs	49.0±1.00	410±0.020

Flavonoids are widely distributed secondary metabolites with different metabolic functions in plants. They appear as glycosides and possess several phenolic hydroxyl groups on their structure. Many of them are strong antioxidants, capable of effectively scavenging different types of ROS such as hydroxyl and super oxide radicals (Jiménez-Estrada et al., 2013). In this study, total flavonoid content was evaluated by aluminium chloride colorimetric assay. The highest flavonoid content was found in AMRAgNPs with 410±0.02 mg QE/g of the nanoparticles followed by that in AMFAgNPs (250±1.58 mg QE/g) and MZLAgNPs (190±0.5 mg QE/g) [Table. 4.2].

4.4. *Antioxidant activity*

4.4.1. *In vitro antioxidant assays*

Generation of reactive oxygen and nitrogen species (ROS and RNS) causes irreversible damage to the cellular constituents such as lipids, proteins and DNA (Lopaczyski and Zeisel, 2001). An imbalance between free radical production and antioxidant defenses can lead to oxidative stress (Ardestani and Yazdanparast, 2007; Rajan et al., 2014), since antioxidants defend cells against free radical-mediated cell damages and protect humans from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms. These phytochemicals play a key role in conferring antioxidant properties to the plant extract derived AgNPs.

The antioxidant activities of the biogenic AgNPs and the corresponding plant extracts were analyzed employing different assays such as DPPH, nitric oxide, hydroxyl radical and ferric ion in addition to reducing power and phosphomolybdenum assay.

DPPH free radical scavenging activity

DPPH is an unstable free radical which reacts with free radical scavengers by accepting electrons / hydrogen to become a stable product, 2,2-diphenyl-1-picrylhydrazine, accompanied by a color change from purple to yellow (Fu et al., 2013). The results obtained with AgNPs and their corresponding plant precursors showed a dose dependent DPPH scavenging activity (Fig. $p<0.05$, 4.11). Of the three nanoparticle types, the highest activity was displayed by MZLAgNPs with

an EC_{50} value of 575 µg/mL. Overall, the activity profile decreased in the order of MZL Extract > MZLAgNPs > AMFAgNPs > AMF Extract > AMRAgNPs > AMR Extract. Similar results have been obtained with the beef steak plant, *Iresine herbstii*, leaf extract-mediated AgNPs (Dipankar and Murugan, 2012).

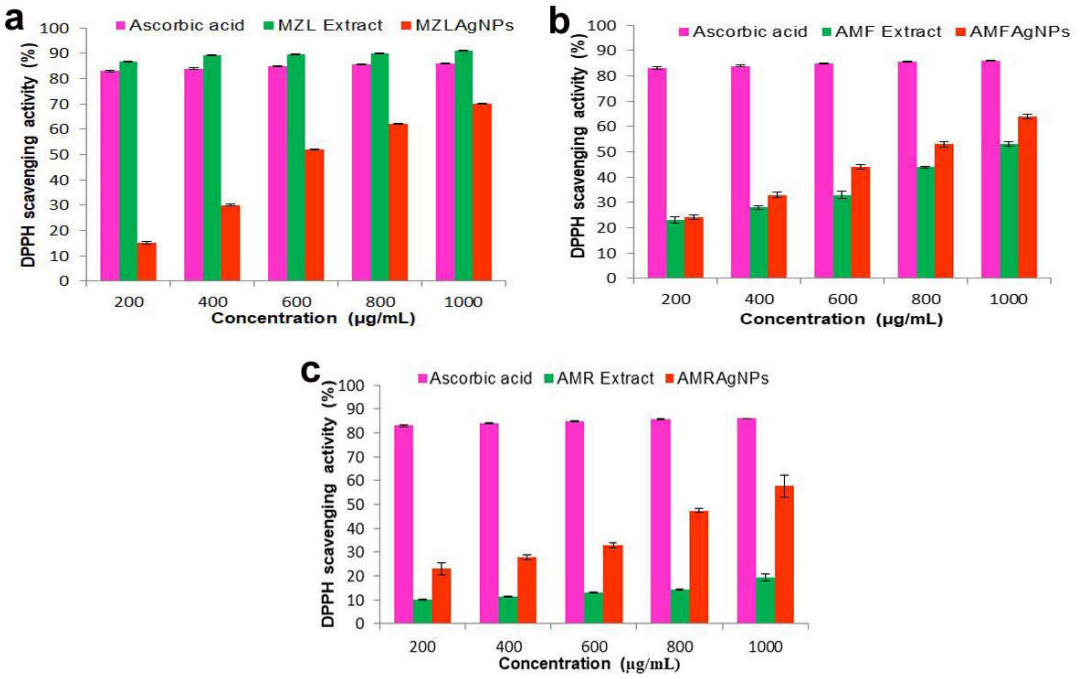

Fig. 4.11. ***DPPH free radical scavenging activity of (a) MZL (b) AMF and (c) AMR derived AgNPs, corresponding extracts and the standard, ascorbic acid*** (*Values represent mean ± S.D. of three experiments*).

Nitric oxide scavenging activity

Nitric oxide (NO) has important roles in various inflammatory processes and other pathological conditions. Chronic expression of cellular NO is associated with various carcinomas and inflammatory conditions such as juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (Hazra et al., 2008). NO radical scavenging activities of the three types of AgNPs were determined and found to be dose-dependent as observed earlier with the DPPH assay. Of the three, AMFAgNPs were found to display the highest NO-scavenging activity (80.02%) comparable to that of ascorbic acid taken as the standard. Such high NO scavenging activity has been reported in the case of silver nanoparticles biosynthesized employing root extracts of

the medicinal Indian screw tree, *Helicteres isora* in a previous study (Bhakya et al., 2015). The activity profile exhibited by the different samples were found to decrease in the order – AMFAgNPs > AMRAgNPs > AMF Extract > AMR Extract > MZLAgNPs > MZL extract. Interestingly, all the three types of biogenic AgNPs showed higher NO scavenging activities in comparison to the corresponding plant extracts (Fig. 4.12).

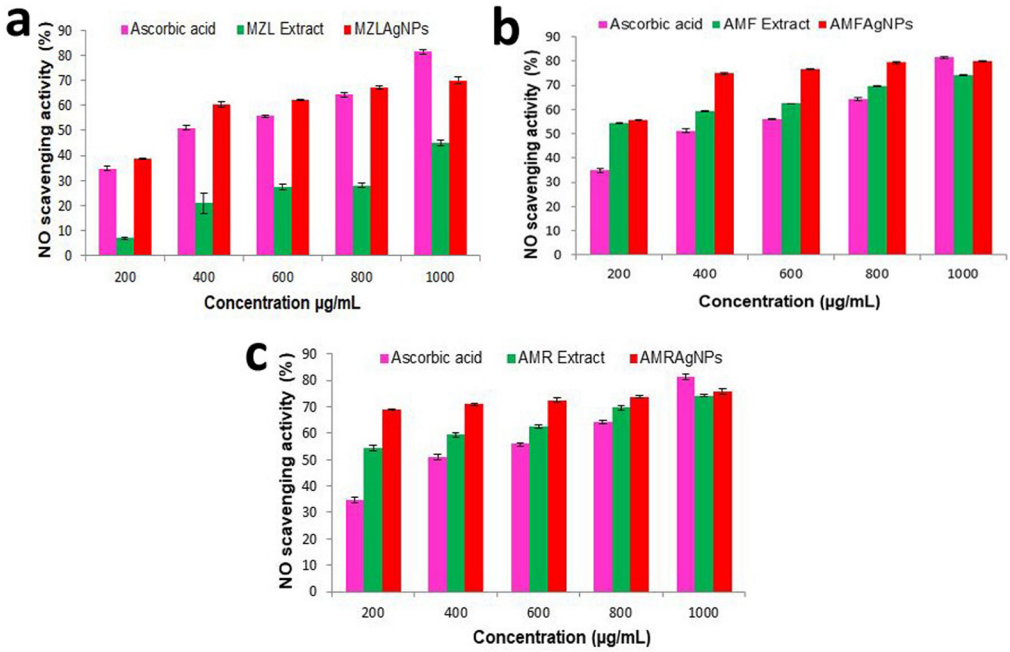

Fig. 4. 12. ***Nitric oxide scavenging activity of (a) MZL (b) AMF and (c) AMR derived AgNPs,*** **corresponding extracts and the standard, ascorbic acid** *(Values represent mean ± S.D. of three experiments; P<0.05).*

Hydroxyl radical scavenging activity

Hydroxyl radicals (OH) are one of the most potent reactive oxygen species causing DNA strand breakage contributing to carcinogenesis, mutagenesis and cytotoxicity (Hochestein and Atallah, 1988; George et al., 2014). These radicals also initiate the process of lipid peroxidation, extracting hydrogen atoms from unsaturated fatty acids (Kappus, 1991). In this study, OH radical scavenging activities of the extracts and the extract-derived AgNPs were determined. A concentration dependent OH radical scavenging activity was observed. The highest activity was associated with MZLAgNPs when compared to that of other

nanoparticles/extracts including ascorbic acid taken as the standard ($p<0.05$, Fig. 4.13.). The activity profile represented from high to low was observed to be – MZLAgNPs > AMRAgNPs >AMFAgNPs > AMR extract > AMF extract > MZL extract. Notably OH scavenging potential of the three types of AgNPs were superior to that of all extracts.

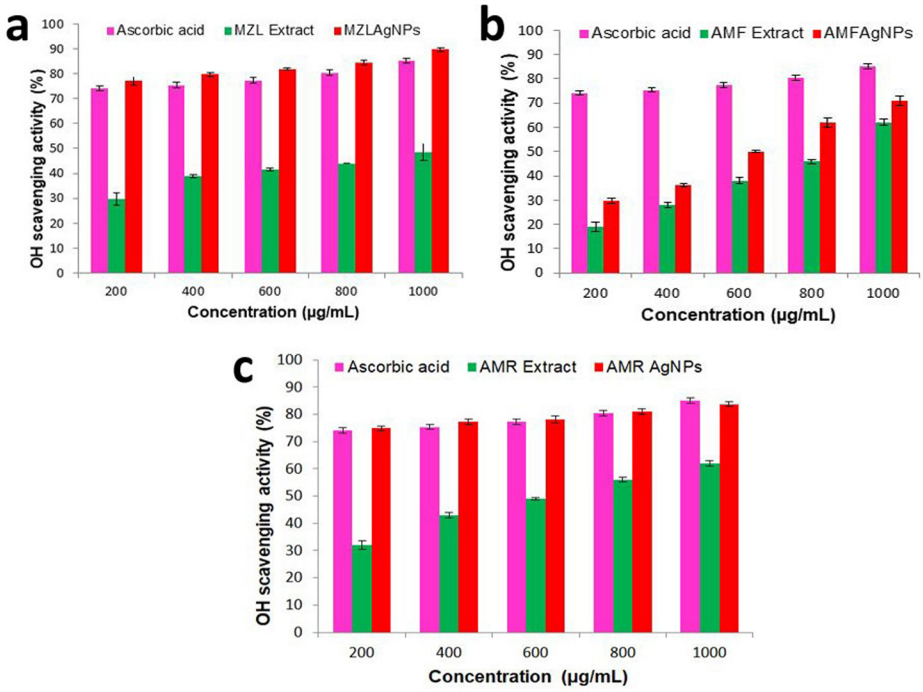

Fig. 4.13. ***Hydroxyl radical scavenging activity of (a) MZL (b) AMF and (c) AMR derived AgNPs,*** **corresponding extracts and the standard, ascorbic acid** (*Values represent mean ± S.D. of three experiments; P<0.05*).

Ferrous ion chelating activity

Iron is a metal essential for life since it is required for oxygen transport, respiration and many enzyme activities. The compound ferrozine can quantitatively form a magenta colored stable complex with Fe^{2+} ions which decreases in the presence of chelating agents (Ebrahimzadeh et al., 2008). This measurement of color reduction, therefore, allows estimation of the chelating ability. As early as 1990, scientists reported that the chelating agents are effective as secondary antioxidants since they reduce the redox potential and stabilize the oxidized form of the metal ion (Pavithra and Vadivukkarasi, 2015). An evaluation of the ferrous ion chelation capacity of biogenic AgNPs and the precursor extracts revealed that

MZLAgNPs possessed Fe ion chelation capacity equivalent to that of the standard EDTA solution prepared at a strength of 1.0 mg/mL. The activities were recorded in the decreasing order of MZLAgNPs > AMFAgNPs > AMF Extract > AMRAgNPs > MZL Extract > AMR Extract (Fig. 4.14).

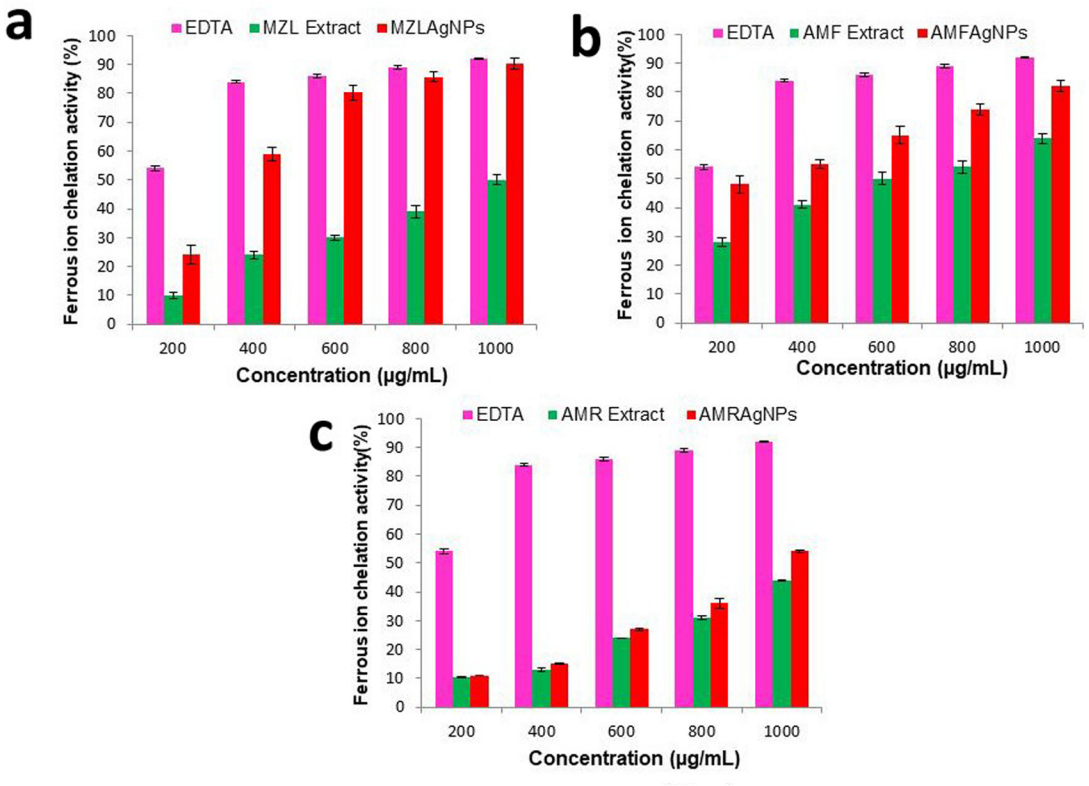

Fig. 4.14. ***Ferrous ion chelation activity (a) MZL (b) AMF and (c) AMR derived AgNPs*****, corresponding extracts and the standard, ascorbic acid** (*Values represent mean ± S.D. of three experiments; P<0.05*)

Determination of reducing power

Fe (III) reduction is often used as an index of electron donating activity, a mechanism involved in antioxidant action of phenolics (Nabavi et al., 2009). The reducing ability of the plants generally depends on the presence of reductones (antioxidants), which cause reduction of Fe^{3+} - ferricyanide complex to Fe^{2+} form which can be monitored by measuring the formation of Perls' Prussian blue at 700 nm (Fernando and Soysa, 2014). A dose-dependent increase in the reducing power of the AgNPs / extracts was observed and the activities were found to decrease in the order of MZL extract > AMFAgNPs > AMRAgNPs > AMF extract > AMR extract

> MZLAgNPs (Fig 4.15). Though the reducing power of MZL Extract was found to be higher than that of the standard, ascorbic acid, AgNPs, however, displayed far lower (< 50%) activity when compared to that of the extract showed least reducing power.

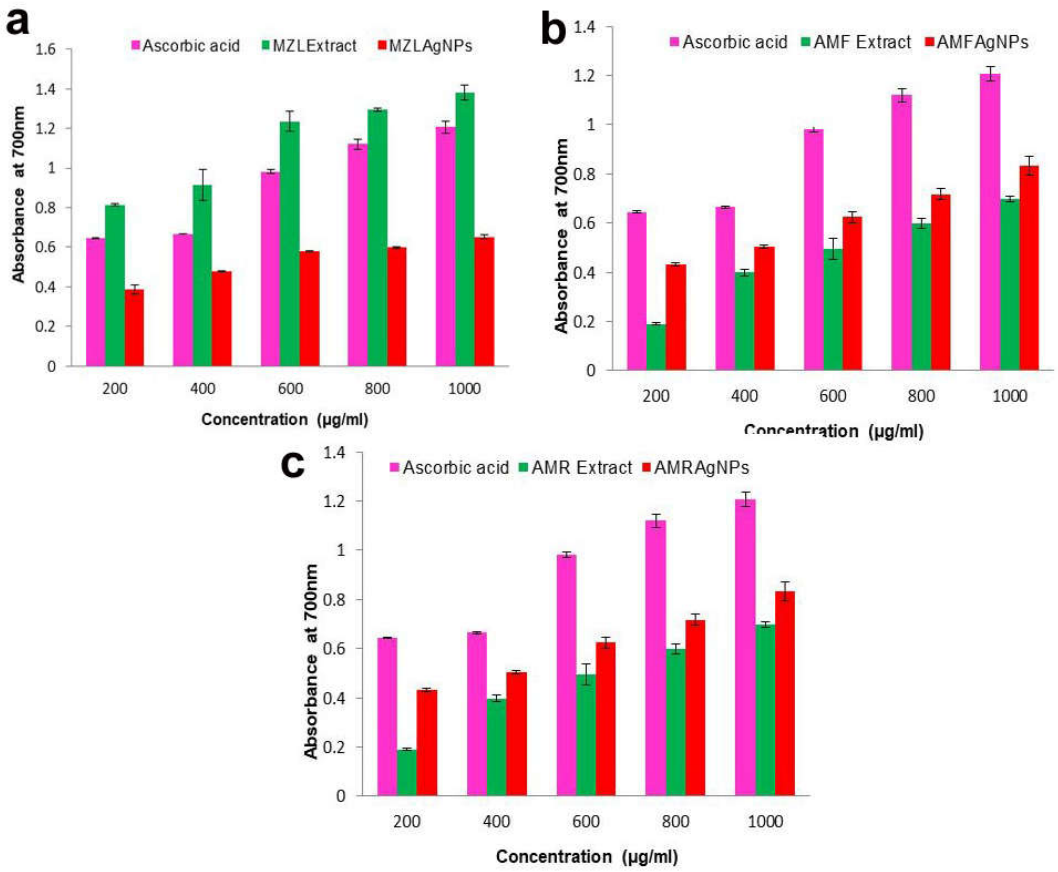

Fig. 4.15. ***Reducing power assay of (a) MZL (b) AMF and (c) AMR derived AgNPs,*** **corresponding extracts and the standard, ascorbic acid**(*Values represent mean ± S.D. of three experiments; P<0.05*)

Determination of total antioxidant capacity

Finally, an assessment of the total antioxidant capacity of the different biogenic AgNPs and the plant extracts employed in the present study was carried out using phosphomolybdenum assay. This method is based on the reduction of molybdenum (VI) by antioxidants and the formation of a green phosphate / molybdenum (V) compound with maximum absorption at 695 nm (Shah et al., 2013). The total antioxidant capacity of the samples were found to decrease in the

order of AMRAgNPs > AMFAgNPs > AMR extract > AMF extract > MZL extract > MZLAgNPs (Fig. 4. 16).

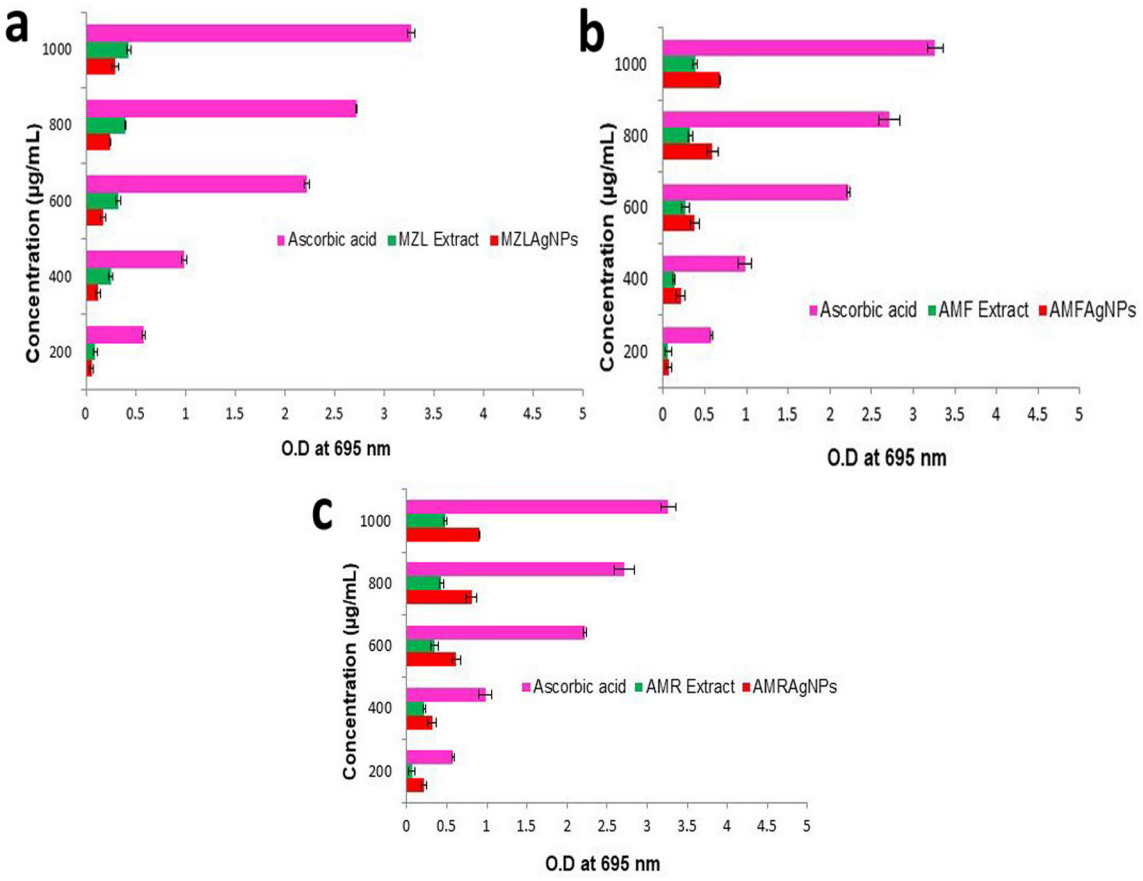

Fig. 4.16. ***Total antioxidant capacity of (a) MZL (b) AMF and (c) AMR derived AgNPs,*** **corresponding extracts and the standard, ascorbic acid**(*Values represent mean ± S.D. of three experiments; P<0.05*)

Taken together, these results suggest that of the three types of biogenic nanparticles generated and evaluated in the present study, MZLAgNPs were found to possess the highest hydroxyl / DPPH scavenging and metal ion chelation capacities. Incidentally, these particles displayed the lowest antioxidant activity and reducing power. AMFAgNPs, on the other hand, were found to possess the highest NO scavenging and reducing power. Though, among the three biogenic nanoparticles, AMRAgNPs relatively displayed the highest antioxidant activity, it

was far below (< 3 folds) than that of standard compound (ascorbic acid). The antioxidant property of the biogenic nanoparticles as evidenced by the positive results obtained with various assays might be attributed to two aspects - one associated with capping agents such as phenols or flavonoids and the other related to their large surface to volume ratios and crystallographic surface structures as noted by He et al., 2017.

4.5. *Antibacterial activity*

The well known antibacterial effect of silver nanoparticles against both Gram-negative and Gram-positive bacteria is attributable to mechanisms such as (i) attachment of silver nanoparticles to the negatively charged cell surface leading to alterations in the physical and chemical properties of the cell membranes or attachment to cell wall disrupting physiological functions of permeability, osmoregulation, electron transport and respiration (Su et al., 2009; Marambio-Jones and Hoek, 2010) (ii) formation of free radicals and (iii) interaction of AgNPs with DNA, interruption of DNA replication / translation or by dephosphorylation of tyrosine residues on peptides inhibiting signal transduction and growth in bacteria (AshaRani et al., 2009; Soo et al., 2011). Release of silver ions from the nanoparticles is thought to be the underlying cause of bactericidal activity (Sondi and Salopek-Sondi, 2004; Morones et al., 2005). The antibacterial activities of the different biogenic silver nanoparticles were evaluated in the present study using agar-well diffusion method followed by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

4.5.1. *Agar well diffusion assay*

The results of the disc diffusion studies carried out as described in sub-section 3.3.4 (Chapter 3) showed clear zones around the wells (Fig. 4.17). The mean diameters of the inhibition zones obtained are given in Table 4.3.

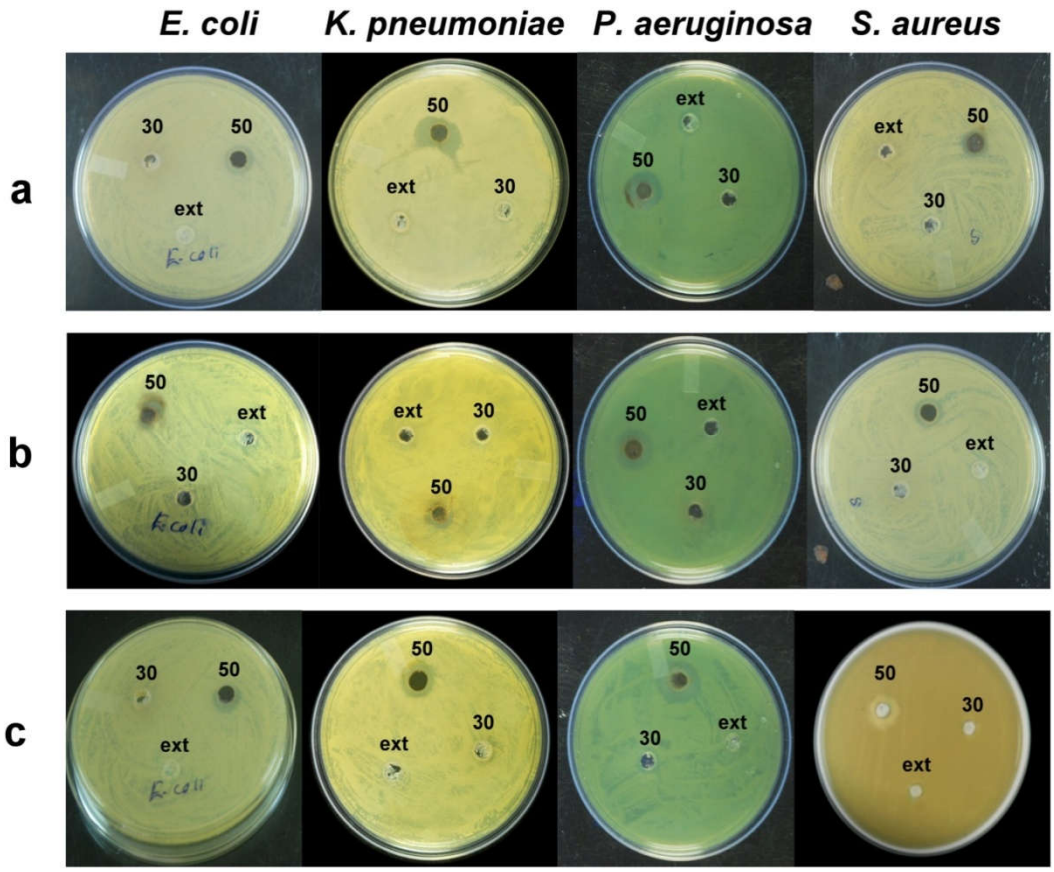

Fig. 4.17. ***Illustration of the antimicrobial activity of (a) MZL (b) AMF and (c) AMR derived AgNPs against four clinical pathogenic bacterial isolates employing agar well diffusion method.*** *The values, 30 and 50, denote concentrations of nanoparticle stock solution (30µL=150 ng; 50µL = 250ng); 'ext' denotes invidual plant extracts (50µL = 250 ng) loaded into the wells* (*Values represent mean ± S.D. of three experiments; P<0.05*)

Table 4.3. Zone of inhibition (in mm) demonstrating antibacterial activity of biogenic AgNPs against clinical strains.

Types of AgNPs	***E.coli***	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***
MZLAgNPs	17 mm ± 0.2	27 mm ± 0.32	28 mm ± 0.4	12 mm ± 0.13
AMFAgNPs	18 mm ± 0.5	16 mm ± 0.43	24 mm ± 0.3	17 mm ± 0.2
AMRAgNPs	17 mm ± 0.3	18 mm ± 0.23	24 mm ± 0.5	17 mm ± 0.4

The inhibition zones indicated that all three biogenic AgNPs suppressed growth of all clinical bacteria tested. The highest antibacterial activity was obtained against *P.aeruginosa* compared to that against *E.coli*, *K. pneumoniae* and *S.aureus*.

The results also showed that all the three type AgNPs exhibited more or less similar antibacterial activity. Similar antibacterial activity of plant-derived AgNPs have been reported earlier (Jyothi et al., 2012). Plant extracts, however, did not exhibit any antibacterial activity. Hence, the observed antibacterial activity may be attributable to the well established antibacterial activity of silver nanoparticles *per se* without any contribution from phytoconstituents of the extract. Alternatively, very low activity of the phytoconstituents or inextractability of putative antibacterial compounds in the aqueous plant extract might also might lead to the absence of antibacterial activity as observed by Ahmed et al. (2016).

4.5.2. *Determination of MIC and MBC*

The antimicrobial activities of biogenic silver nanoparticles were evaluated by determining the MIC and MBC values employing the broth dilution method. The lowest concentration of nanoaprticles that inhibited bacterial growth was defined as MIC and the lowest concentration of nanoparticles that prevented microbial growth was designated as the MBC. The MIC and MBC values determined for each of the biogenic AgNPs are given in Table 4.4.

Table. 4.4. MIC and MBC values of biogenic AgNPs

MIC (µg/mL)	***E.coli***	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***
MZLAgNPs	7.0 ± 0.32	18 ± 0.7	15 ± 0.43	18 ± 1.5
AMFAgNPs	16 ± 0.78	44 ± 1.5	25 ± 0.32	20 ± 0.8
AMRAgNPs	7.0 ± 0.5	30 ± 0.76	18 ± 1.3	10.5 ± 0.5
MBC (µg/mL) MZLAgNPs	8 ± 0.45	21 ± 0.78	18 ± 0.62	22 ± 0.82
AMFAgNPs	18 ± 0.48	50 ± 1.2	30 ± 0.46	26 ± 1.2
AMRAgNPs	7.5±0.4	35 ± 0.53	20 ± 0.8	12 ± 0.45

(*Values represent mean ± S.D. of three experiments;* $p<0.05$).

Of the three biogenic silver nanoparticles studied, AMFAgNPs showed the lowest MIC and MBC values for *E.coli* and *S.aureus* whilst MZLAgNPs showed lowest MIC and MBC values for *P. aeruginosa* and *K. pneumoniae*. The variations in the effective concentration of AgNPs could be due to the differential modes of action against individual bacterial isolates . As already mentioned, the extremely

large surface to volume ratio of the nanoparticles provide better interaction with the organisms leading to high bactericidal activity (Arokiyaraj et al., 2014).

III. Cytotoxicity evaluation of AgNPs on human cancer and normal cells

4.6. *Human cancer cells*

4.6.1. *MTT assay*

The three types of biogenic AgNPs and cisplatin, which served as a control chemotherapeutic agent for comparison, were employed against HCT 116, Hela and A549 cells for cytotoxicity evaluation. Cell samples, individually treated with varying concentrations of biogenic silver nanoparticles / cisplatin for 24, 48 and 72 h durations, were subjected to MTT assay. The results are shown in Fig. 4.18. All three AgNPs showed the highest cytotoxicities against HCT 116 cells compared to that against Hela and A549 cells. Against HCT 116 cells, MZLAgNPs displayed the highest cytotoxicity with the lowest IC_{50} value of 25±2 µg/mL for 24 h whilst that of AMFAgNPs and AMRAgNPs were found to be relatively higher at 63±1.5 and 69±2.0µg/mL respectively (Table 4.5). At the end of 72 h treatment period, the IC_{50} values with respect to all three AgNPs types were found to taper within 7.5 – 8.5 µg/mL. The IC_{50} values of the three types of AgNPs against HeLa and A549 cell ranged from 52 >100 µg/mL. The IC_{50} values for cisplatin against the three cell lines tested were found to be 16.0 and 5.0 µg/mL for 24 and 72 h treatment periods respectively. The cytotoxicity, if any, of the individual plant extracts *per se* were also tested within the concentration range of 10 -100 µg/mL against all cell lines. Interestingly, the results of the MTT assay showed that the cells were viable (Fig. 4.19.) and also appeared to be cytomorphologically normal at all tested concentrations.

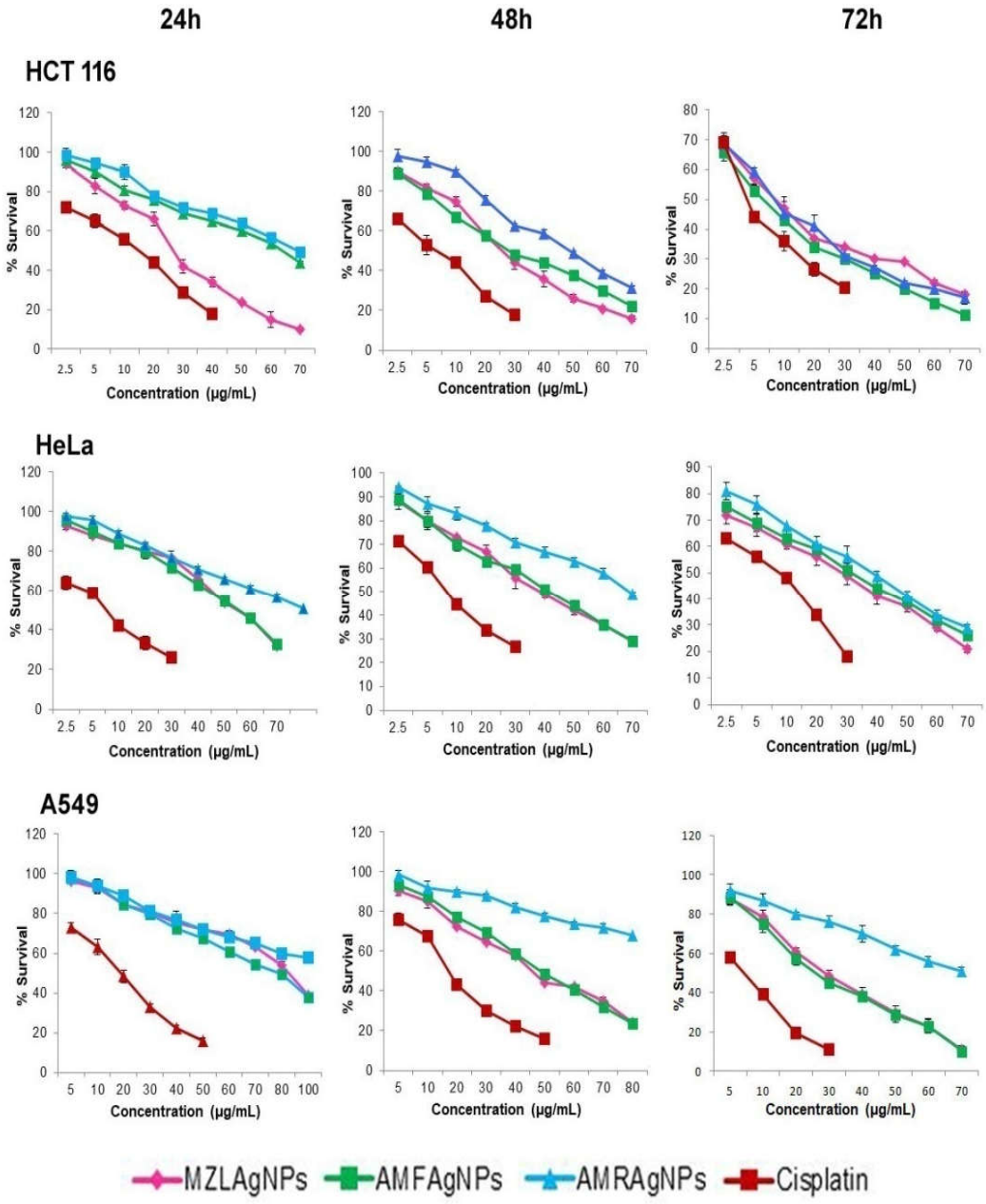

Fig. 4.18. Cytotoxicity evaluation of biogenic AgNPs / cisplatin against different cancer cell lines. *Values represent mean ± S.D. of three experiments; $p < 0.05$.*

Table 4.5. IC_{50} values of biogenic AgNPs against different human cancer cell lines

Biogenic nanoparticles	Treatment duration (h) 24	48	72
HCT 116			
MZLAgNPs	25 ± 1.8 µg/mL	23 ± 1.0 µg/mL	8.0 ± µg/mL
AMFAgNPs	44±1.0 µg/mL	25± 2.0 µg/mL	7.5 µg/mL
AMRAgNPs	69 ± 2.0 µg/mL	63 ± 1.5 µg/mL	8.5 µg/mL
Cisplatin	16 ±2.0 µg/mL	7 ±1.0µg/mL	5.0 µg/mL
HeLa			
MZLAgNPs	52 ± 2.0 µg/mL	38 ±3.0 µg/mL	24 ± 1.9 µg/mL
AMFAgNPs	53 ± 1.6 µg/mL	42 ± 2.0 µg/mL	37 ± µg/mL
AMRAgNPs	>70 µg/mL	>70 ± 2.0µg/mL	68 µg/mL
	9 ± 1.3 µg/mL	8.5 ± 2.0 µg/mL	7 ± 1.9 µg/mL
A549			
MZLAgNPs	83 ± 1.5 µg/mL	44±1.0µg/mL	25 ±3.4
AMFAgNPs	74 ± 2.0 µg/mL	48±1.0 µg/mL	23.5 ± 3.5
AMRAgNPs	> 100 µg/mL	> 80 µg/mL	> 70 µg/mL
	16.5 ± 1.7 µg/mL	13 ± 1.9 µg/mL	7.5 ± 1.4 µg/mL

(*Values represent mean ± S.D. of three experiments; $p < 0.05$*)

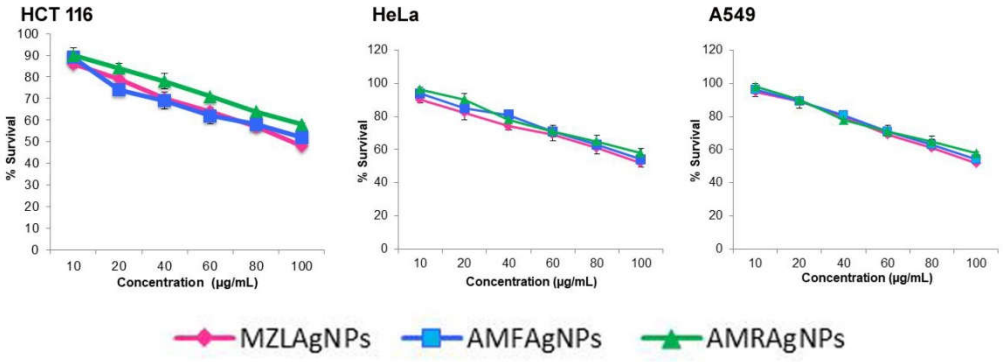

Fig. 4.19. Cytotoxicity evaluation of plant extracts *per se* on different cancer cell lines. *Values represent mean ± S.D. of three experiments; $p < 0.05$.*

The results mentioned above reveal time and dose-dependent cytotoxicity of biogenic AgNPs. This is in line with previous reports on different types of biogenic silver nanoparticles with IC_{50} values ranging within 5-50 µg/mL obtained against HeLa, Hep2 and MCF-7 cells (Jacob and Narayanan 2012; Shankar et al., 2014; Vasanth et al., 2014). It is thus apparent that nanoparticle toxicity may vary in a cell type - specific manner. It is relevant to note here that the size of the metal NPs is also

known to influence cytotoxicities against cancer cells (Dipankar and Murugan, 2012; Gurunathan et al., 2013; Vasanth et al., 2014; Zhu et al., 2016).

4.6.2. *Trypan blue dye exclusion assay*

Cell viability as evaluated by the trypan blue dye exclusion method, corroborated well with the results of MTT assay. For MZLAgNPs and AMFAgNPs, the concentrations tested ranging from 10-80 µg/mL, were found to be highly toxic to HCT 116 cells compared to the relatively lower toxicity of AMRAgNPs. Cisplatin also showed 47% cell death at the IC_{50} concentration (Fig. 4.20).

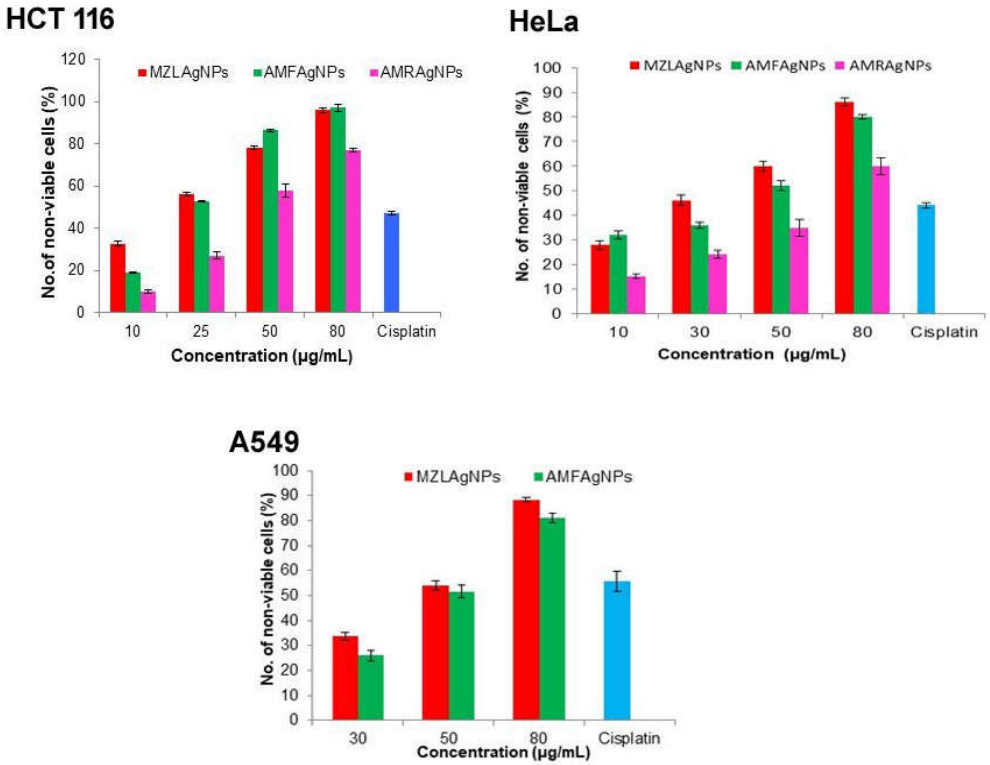

Fig. 4.20. ***Trypan blue dye exclusion assay employing biogenic AgNPs / cisplatin against (a) HCT 116 cells (b) HeLa cells and (c) A549 cells.***

In the case of HeLa cells, following treatment at 80 µg/mL, a concentrations higher than IC_{50} value, 86% non-viable cells were obtained; the non-viable cell percentage obtained in respect of A549 being 87% in MZLAgNPs treated cells. This was marginally higher than that obtained for AMFAgNPs treated cells. This was clearly indicative of a dose-dependent effect of biogenic nanoparticles was evident from the MTT assay. Again, the fact that at IC_{50} concentration of cisplatin, 56 % of

non-viable cells were observed following Trypan blue staining falls in line with the results of MTT assay data - within experimental limits.

4.7. *Human Lymphocytes and erythrocytes*

The effects of varying concentrations (10-80 µg/mL) of the three types of AgNPs /cisplatin on hPBLs was determined by MTT assay. The results clearly indicated that none of them displayed any toxicity towards the normal human lymphocytes. At IC_{50} concentration lymphocyte proliferation remain unaffected with 100 % viability (Fig. 4.21) contrary to severe toxicity observed with the chemotherapeutic drug cisplatin. Strikingly, use of concentrations which were 2-3 folds higher than the IC_{50} value, only elicited 20 % death in lymphocyte.

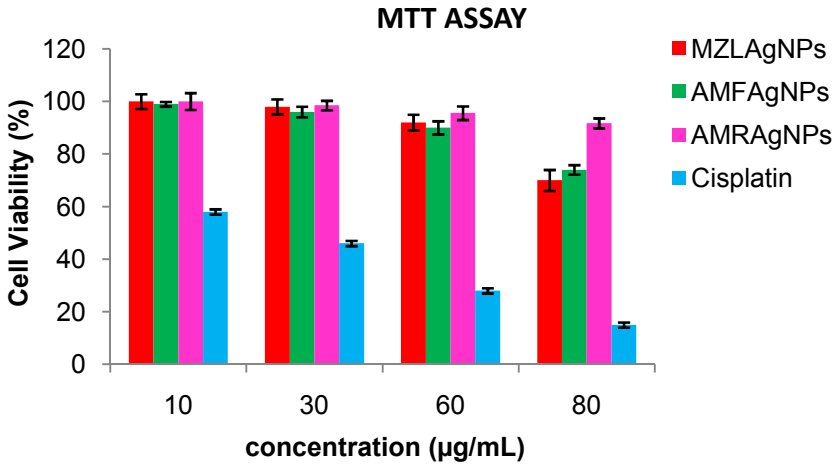

Fig. 4.21. Cytotoxicity evaluation of biogenic *AgNPs / cisplatin against hPBLs.* *Values represent mean ± S.D. of three experiments; $p < 0.05$.*

The extent of hemolysis was studied in human erythrocytes at concentrations ranging from 50-200 µg/mL. All the three types of biogenic AgNPs failed to show any toxicity towards them. In other words, the biogenic nanoparticles apparently did not cause any harmful effect on human erythrocytes (Fig. 4. 22). Given the fact that interaction of NPs with erythrocyte is biologically relevant, our results of hemolytic assay reveal the hemocompatibility of MZLAgNPs for drug delivery applications. Here, it is relevant to point out that only a few studies have been conducted on the toxicity of biogenic nanoparticles on human lymphocytes and erythrocytes (Vergallo

et al., 2014; Bhanumathi et al., 2017). On the whole, these results provide compelling evidence in support of application of biogenic silver nanoparticles for selective killing of cancer cells as well as on the biosafety of these particles on normal human cells.

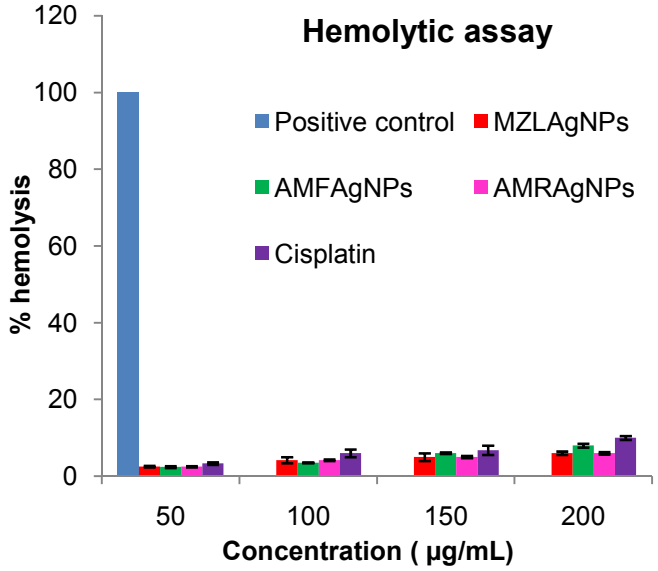

Fig. 4.22. Cytotoxicity evaluation of biogenic *AgNPs / cisplatin against human erythrocytes.* *Toxicity was measured as percentage hemolysis. Values represent mean ± S.D. of three experiments; $p < 0.05$.*

IV. Detailed cellular and molecular studies to elucidate mode of action of biogenic AgNPs

In continuation with the aforementioned results confirming cytotoxicities of biogenic AgNPs, all further experiments conducted as part of the present study were carried out only on HCT 116 and A549 cells. This was due to the fact that in comparison to the large number of reports available on the effect of biogenic silver nanoparticles on HeLa cells, a literature scan revealed that only scanty information was available in respect of the two selected cell lines.

The results of MTT and Trypan blue dye exclusion assays clearly showed that all the three biosynthesized silver nanoparticles displayed significant cytotoxicities against the colorectal cell line, HCT 116. On the other hand, non-small

lung carcinoma cell line, A549 was found to be refractory to AMRAgNPs within the concentration range tested. Hence, to provide a deeper insight into the cellular / molecular effects of the biogenic nanoparticles and their mode of action, further detailed experiments were carried; the results obtained have been enumerated below.

4.8. *Clonogenic assay*

The results of the clonogenic assay performed to determine cell's reproductive ability when exposed to the biogenic nanoparticles below, at and above the IC_{50} value are given in Fig. 4.23. At a concentration higher than the IC_{50} value, biogenic AgNPs were found to drastically inhibit proliferation of the HCT 116 cells, with a 60-80% reduction in colony numbers when compared to untreated controls. Likewise, the positive control cells treated with cisplatin also displayed a comparable - 75% inhibition – in colony count. A concentration-dependent reduction in colony formation capability was apparent which suggested that biogenic AgNPs effectively suppressed growth and proliferation of these cells. In this regard, MZLAgNPs were the most effective followed by AMF and AMR derived AgNPs. Likewise, the inhibition of colony forming ability of A549 cells by MZLAgNPs and AMFAgNPs was relatively lower close to 45 % compared to that observed with HCT 116 cells (Fig. 4. 24). In other words, HCT cells displayed higher sensitivity toward the biogenic AgNPs. Notably, this differential sensitivity was not displayed by both the cell lines against cisplatin since both displayed more or less similar reduction in colony number. It has been reported that tumours resistant to radiation and chemotherapeutic drugs possess enhanced colony forming ability (Varun and Sellappa, 2014). In this context, the therapeutic potential of biogenic AgNPs assumes much significance.

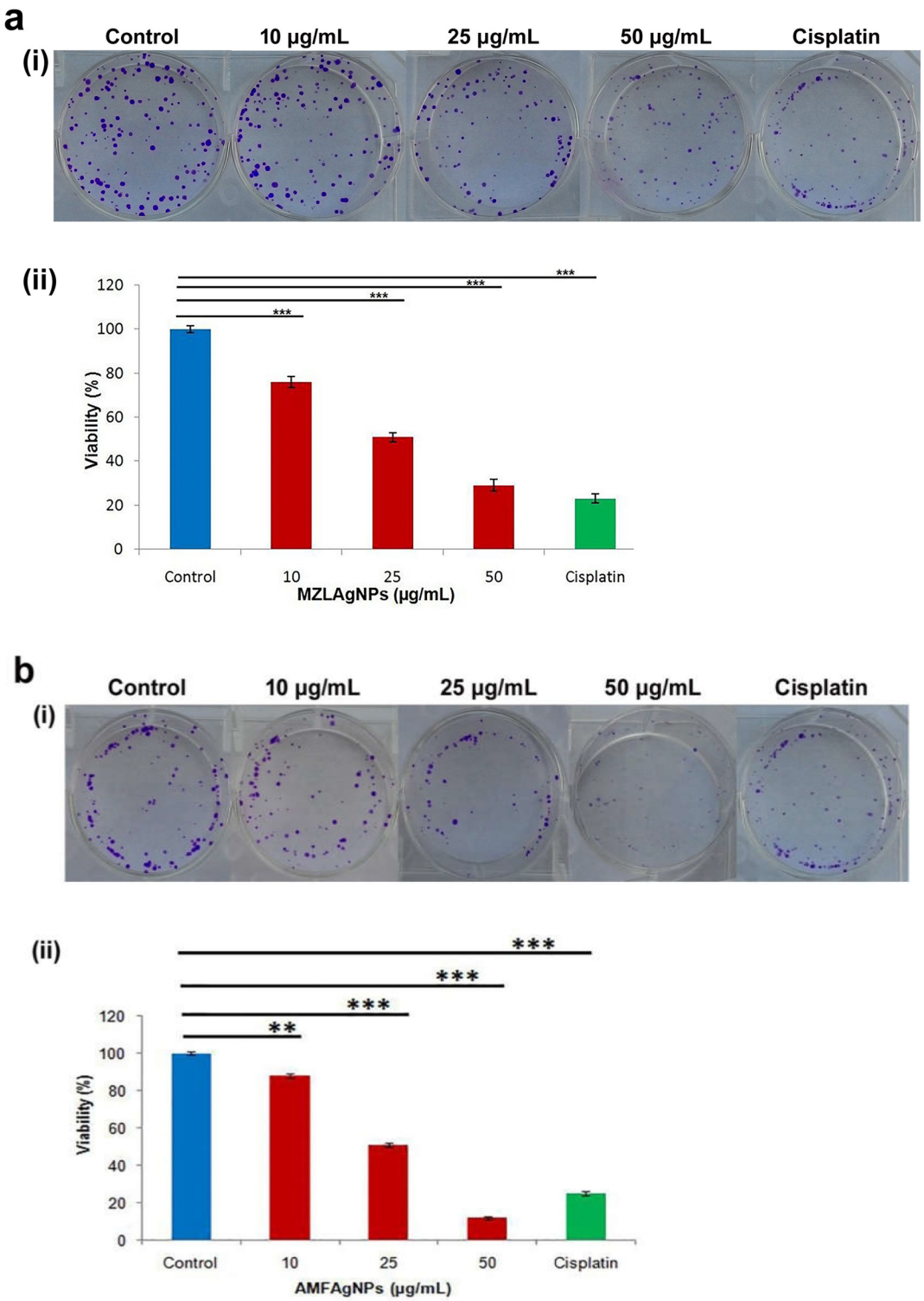

Fig. 4.23 a and b. ***Colony forming capacity of HCT 116 cells treated with MZL and AMF derived AgNPs/cisplatin****; (i) colonies stained with crystal violet (ii) histogram showing significant reduction in the number of colonies in comparison to the controls. ***P≤0.001.*

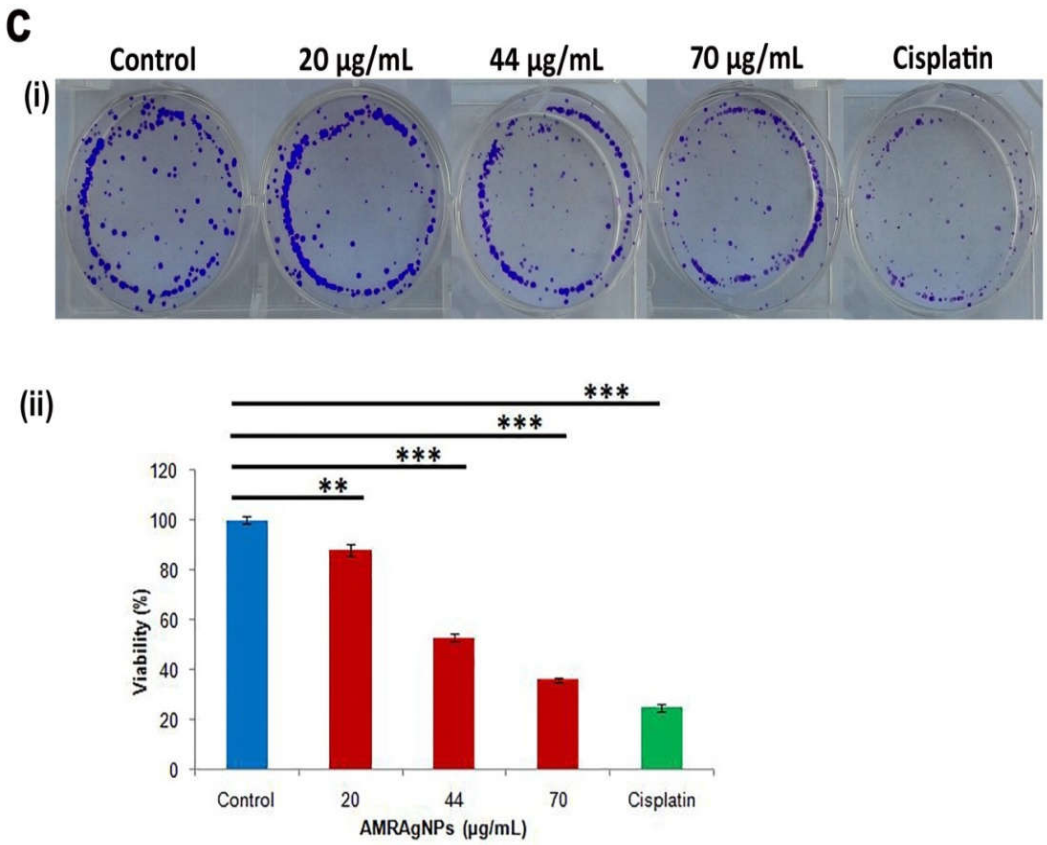

Fig. 4.23 c. ***Colony forming capacity of HCT 116 cells treated with AMRAgNPs/cisplatin****; (i) colonies stained with crystal violet (ii) histogram showing significant reduction in the number of colonies in comparison to the controls. ***P≤0.001.*

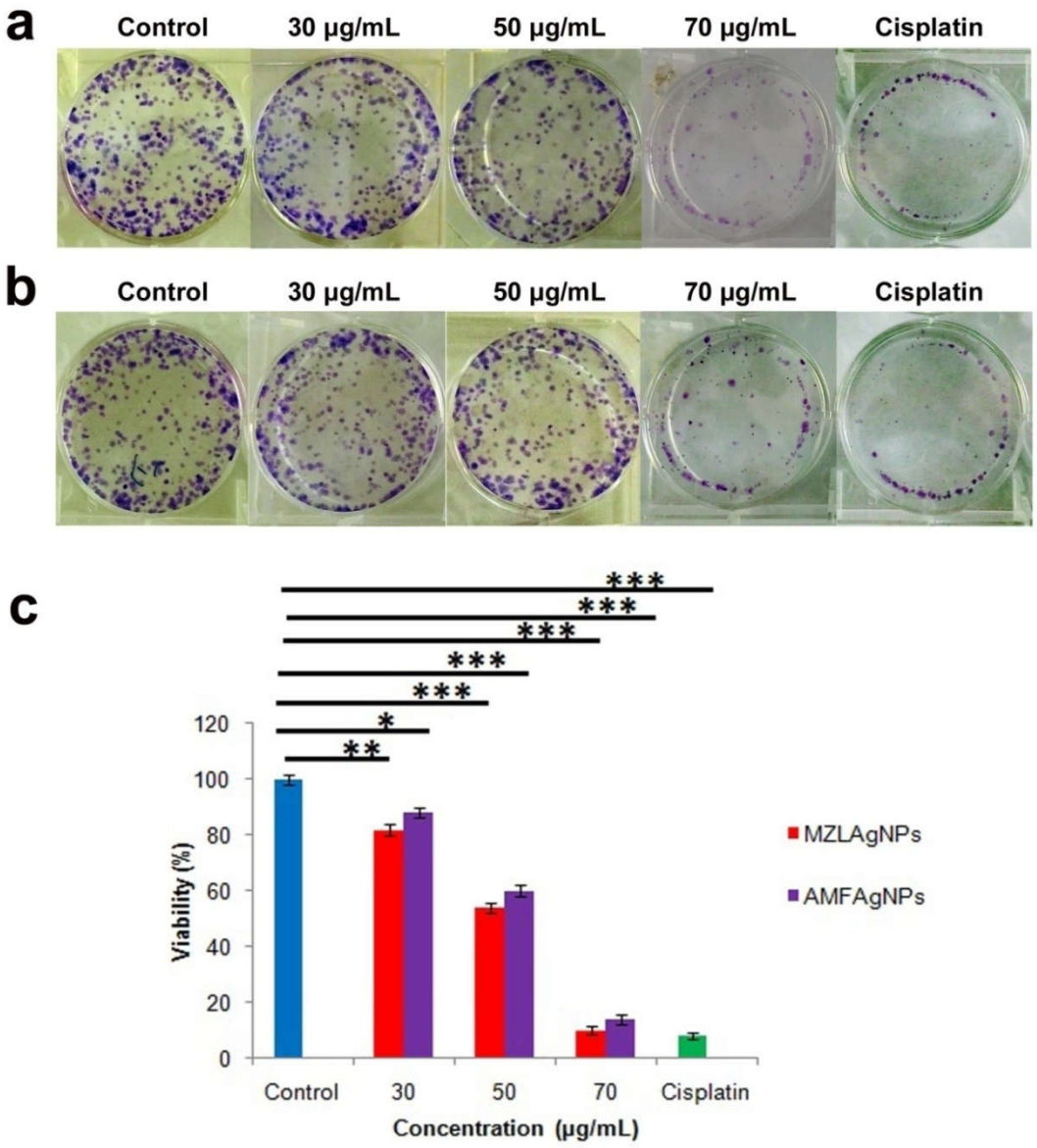

Fig. 4.24. ***Effect of biogenic AgNPs / cisplatin on colony forming capacity in A549 cells*** *(a)MZL and (b) AMF derived AgNPs treated colonies stained with crystal violet (c)) histogram showing significant reduction in the number of colonies in comparison to the controls. ***P≤0.00.*

4.9. *Cell migration assay*

Cell migration is a pivotal step in the metastatic process predominantly responsible for patient deaths from solid tumours (Hanahan et al., 2011; Paul et al., 2017). Cell migration assay, also known as wound healing or scratch assay was carried out to evaluate the effects of treatment with biogenic AgNPs / cisplatin on HCT 116 cell / A549 cell migration following a 12-24 h exposure period (Fig. 4.25

and 4.26). MZLAgNPs were found to be the most potent in inhibiting cell migration with respect to both cell types, with a two-fold higher inhibition against HCT 116 compared to that against A549 cells. Biogenic silver nanoparticles synthesized using leaf extracts of sweet-scented snout bean, *Rhynchosia suaveolens* have been reported with antimigratory effects against human ovarian carcinoma cells (Bethu et al., 2018).

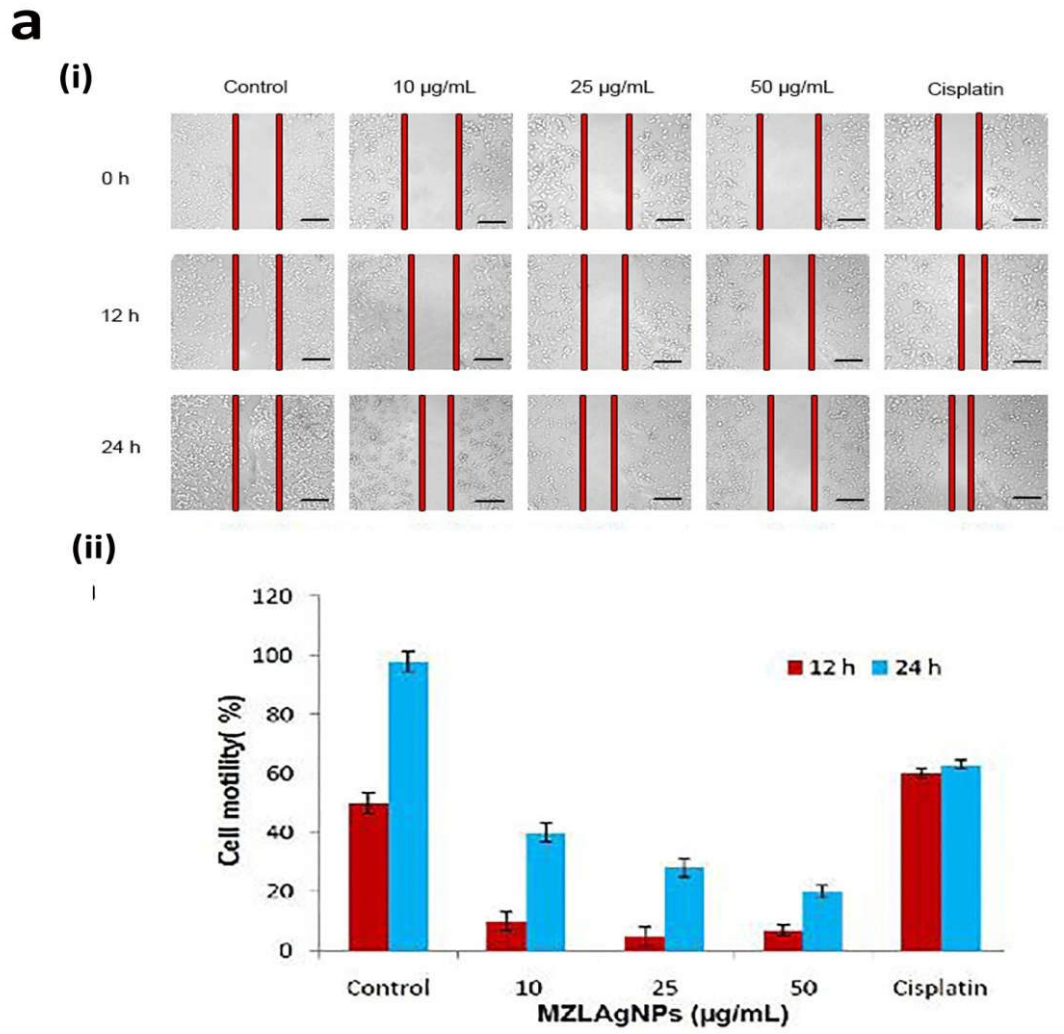

Fig. 4.25 a. ***Effect of biogenic AgNPs / cisplatin on cell migration in HCT 116 cells treated with MZLAgNPs;*** *(i) Light microscopic images of scratched area (scale bar: 20 µm)(ii) histogram showing the cell motility at 12 h and 24 h. ***P≤0.001, **P≤ 0.01, *P≤0.05.*

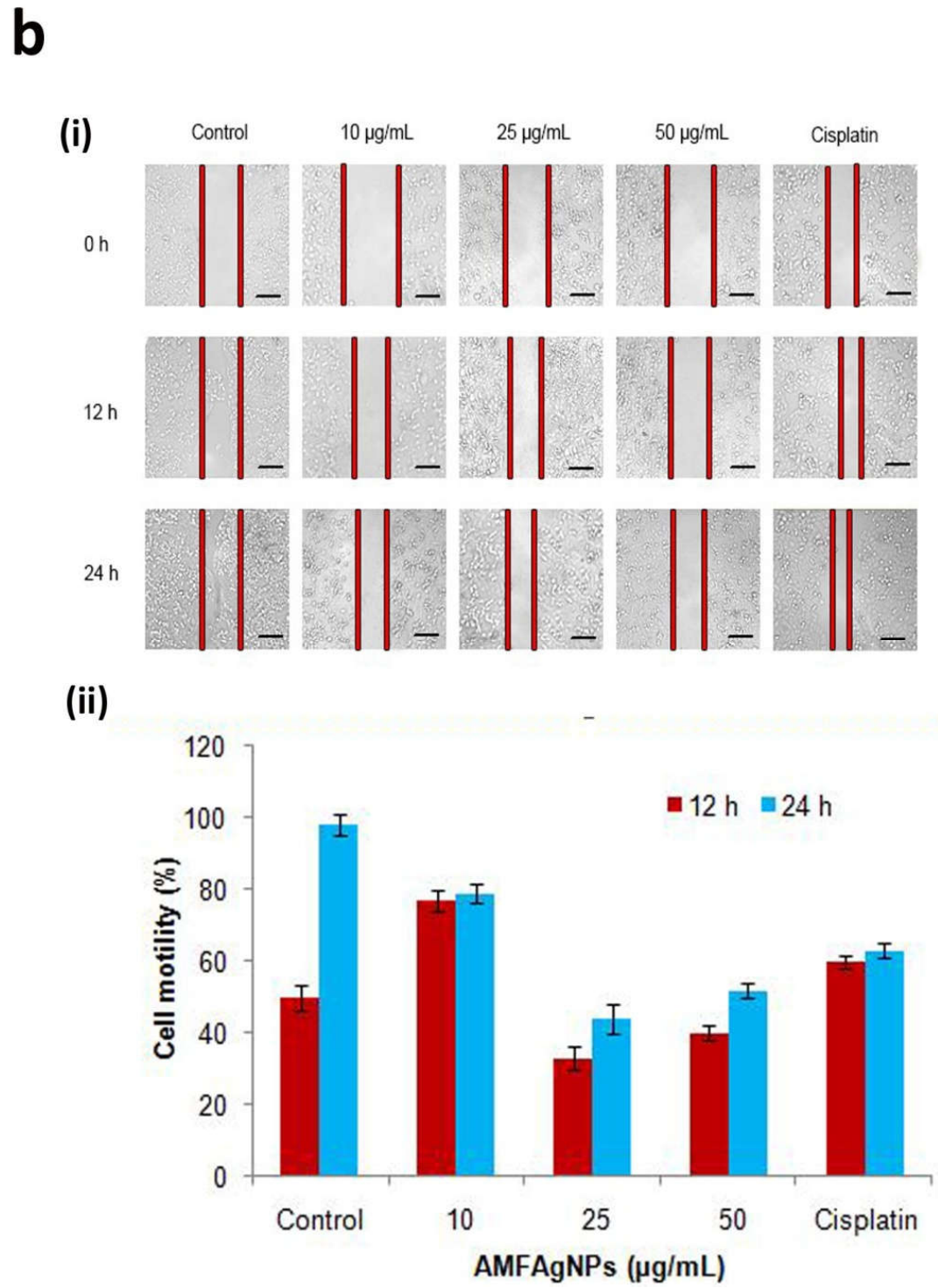

Fig. 4.25 b. ***Effect of biogenic AgNPs / cisplatin on cell migration on HCT 116 cells treated with AMFAgNPs;*** *(i) Light microscopic images of scratched area (scale bar: 20 µm) (ii) histogram showing the cell motility at 12 h and 24 h. ***P≤0.001, **P≤ 0.01, *P≤0.05.*

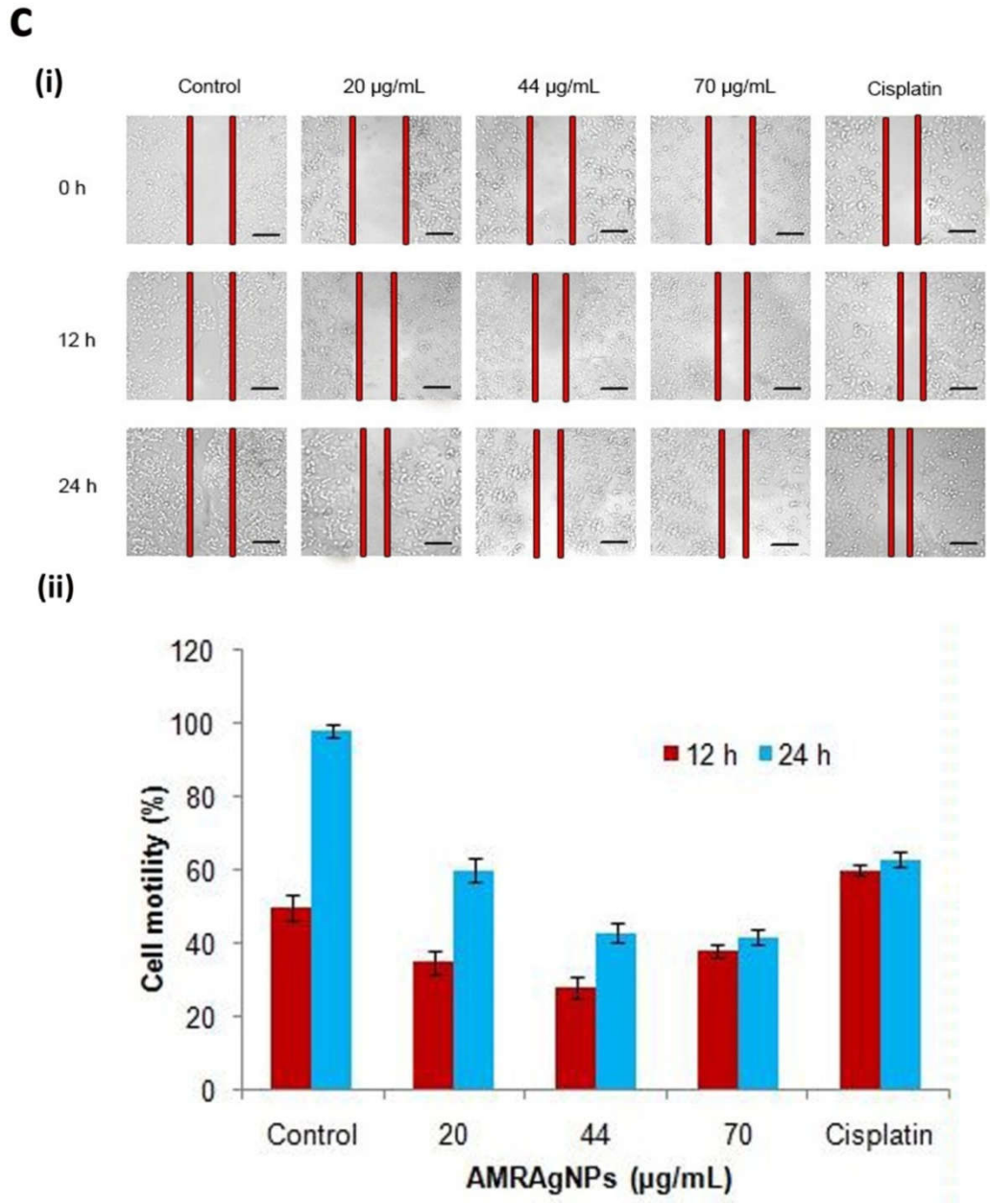

Fig. 4.25 c. ***Effect of biogenic AgNPs / cisplatin on cell migration in HCT 116 cells treated with AMRAgNPs;*** *(i) Light microscopic images of scratched area (scale bar: 20 µm) (ii) histogram showing the cell motility at 12 h and 24 h ***P≤0.001, **P≤ 0.01, *P≤0.05.*

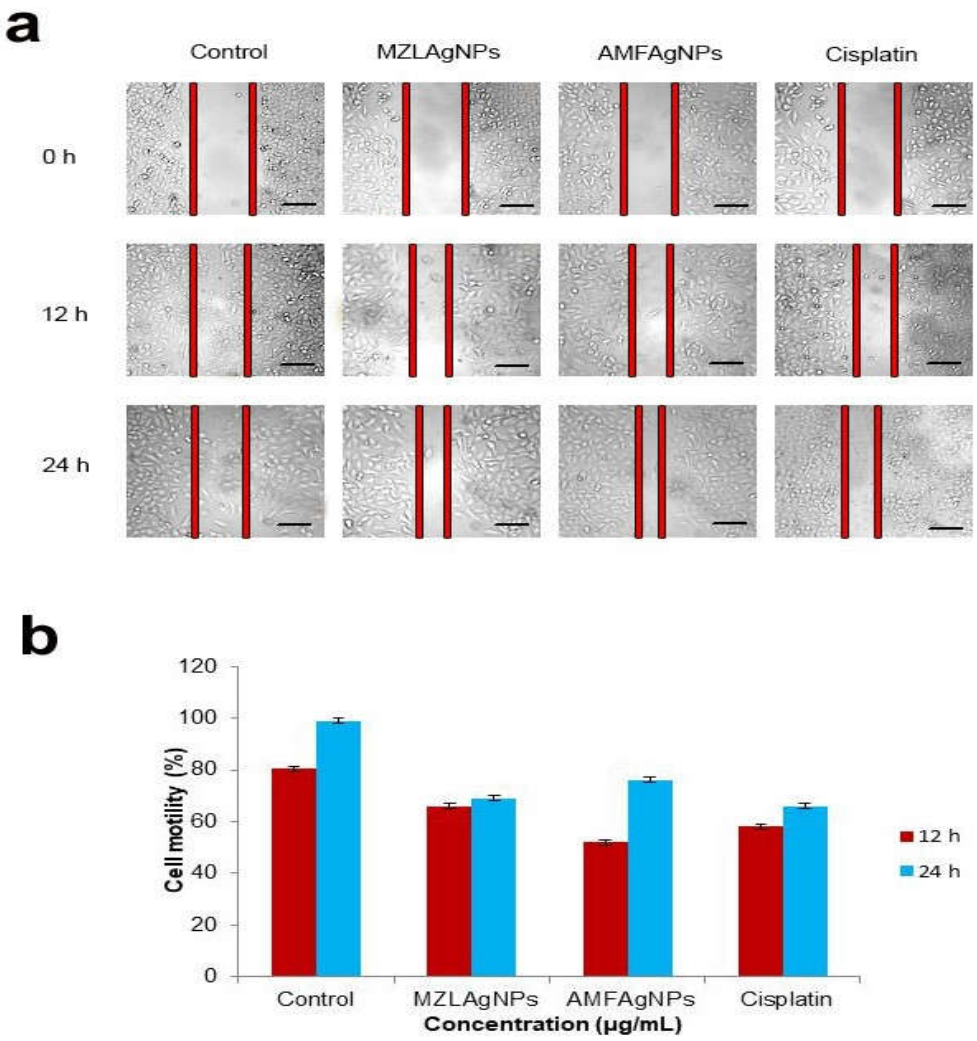

Fig. 4.26. ***Effect of MZL and AMF derived AgNPs-treatment on migration of A549 cells;*** *(a) Light microscopic images of scratched area (scale bar: 30 µm)* ***(b)*** *histogram showing the cell motility at 12 h and 24 h.***P≤0.001, **P≤ 0.01, *P≤0.05.*

4.10. *Analysis of cytomorphological alterations*

4.10.1. *Light and scanning electron microscopy*

The first and most readily noticeable effect following exposure of cells to toxic materials was the alterations observed in cell shape and morphology of the monolayer cultures. Light and scanning electron microscopy of biogenic AgNPs / cisplatin treated HCT 116 cells showed distinct cellular morphological alterations such as cell shrinkage, extensive membrane blebbing with a restricted cell-spreading pattern. The control cells appeared normal as irregular confluent aggregates of

rounded and polygonal cells. Dose-dependent cellular changes were apparent in micrographs of treated cells obtained with both of the techniques (Fig. 4.26).

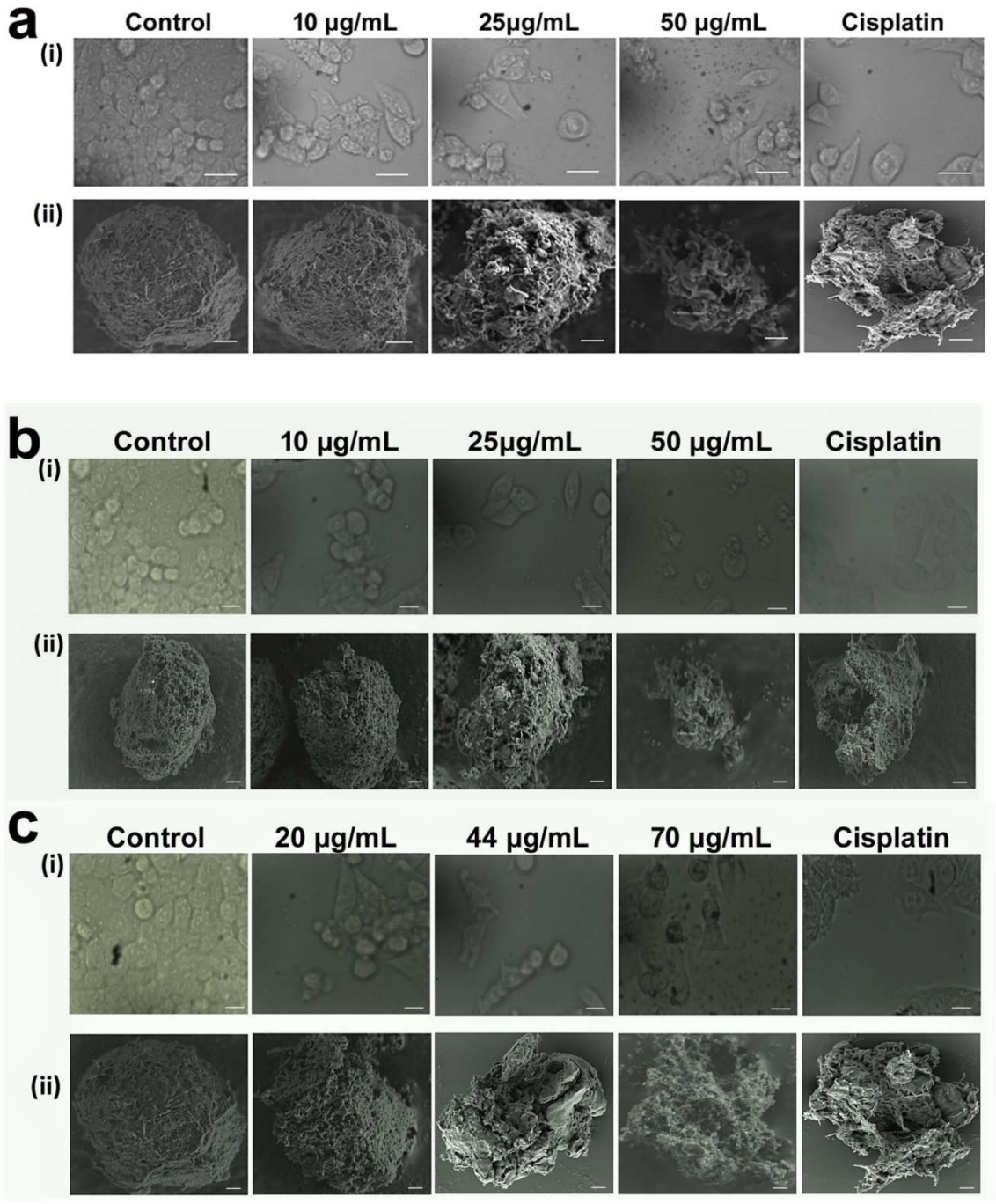

Fig. 4.27. ***Morphological changes in HCT 116 cells after 48 h treatment with (a) MZL (b) AMF and (c) AMR derived AgNPs****; (i) light microscopic images (scale bars: 20µm) (ii) Field emission scanning electron microscopic images (scale bars: 2µm).*

AgNP treated A549 cells also showed distinct cytomorphological changes such as cell shrinkage and detachment from the adjacent cells (Fig. 4. 28). These results corroborated well with a previous study where *Vitex negundo* leaf extract-derived AgNPs were reported to induce cellular shrinkage in HCT 15 colon cancer cell lines (Prabhu et al., 2013).

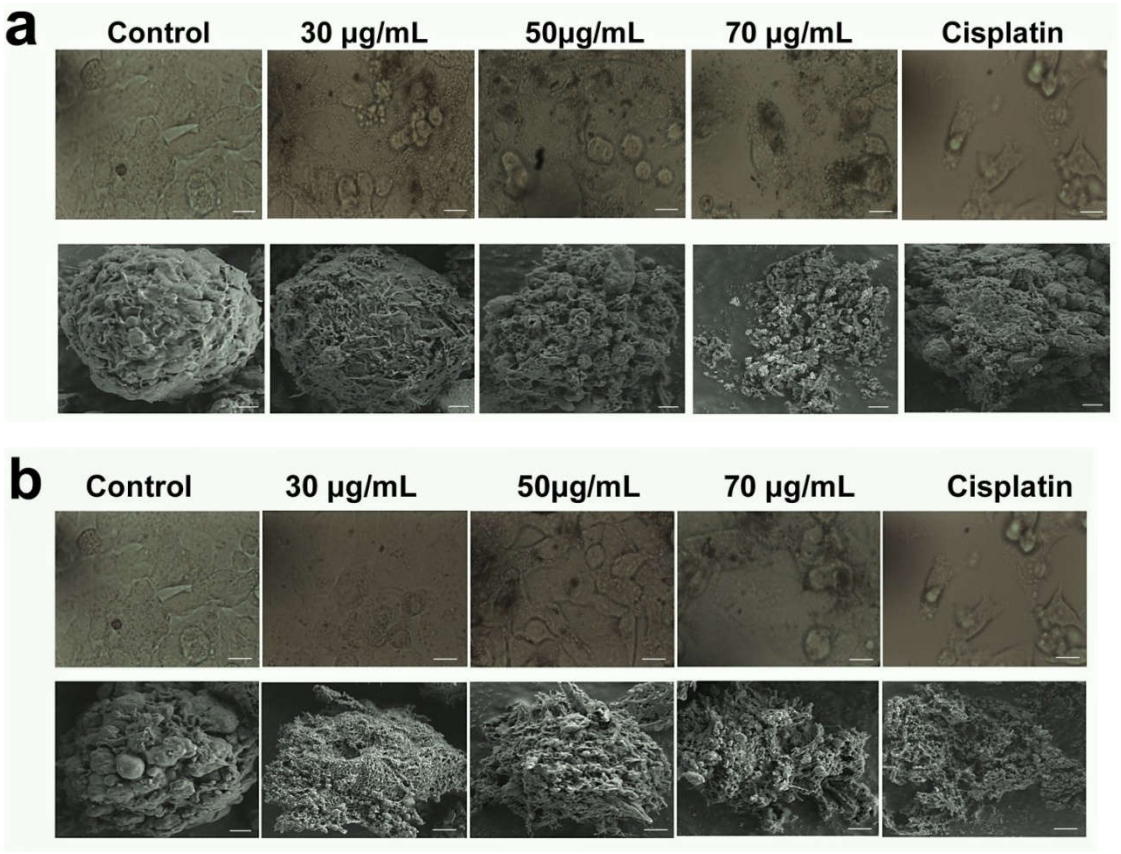

Fig. 4.28. ***Morphological changes in A549 cells after 48 h treatment.*** *Light microscopic (upper panel; scale bars: 30µm) and Field emission scanning electron microscopic images (lower panel; scale bars: 2µm) of cells treated with (a) MZL and (b) AMF derived AgNPs.*

4.10.2. *Fluorescence microscopy for detection of apoptosis induction*

Studying apoptosis is of utmost importance in cancer therapeutic intervention. Evasion of apoptosis or inhibition of apoptotic pathways accompany tumour development. The apoptotic pathways preferentially kill cells damaged beyond repair without affecting the normal healthy cells. Recent cancer research employing such a 'molecular targetting' approach has laid greater emphasis on finding agents capable of apoptosis induction in cancer cells (Baig et al., 2016).

The results of the various experiments conducted to detect apoptosis induction and cell death are enumerated in the following sub-sections.

Hoechst 33258 staining

Cells were stained with Hoechst 33258, a membrane-permeable, blue fluorescent nuclear dye to check for the presence of condensed chromatin known to be associated with the execution of the cell death programme. Following treatment with biogenic AgNPs for 48 h, cells exhibited apoptosis-related nuclear condensation and fragmentation resulting from intense uptake of stain into such regions (Baghbani-Arani et al., 2017; Fig. 4.29). Compared to AgNP - treated HCT cells, A549 showed lesser nuclear condensation and fragmentation (Fig. 4.30).

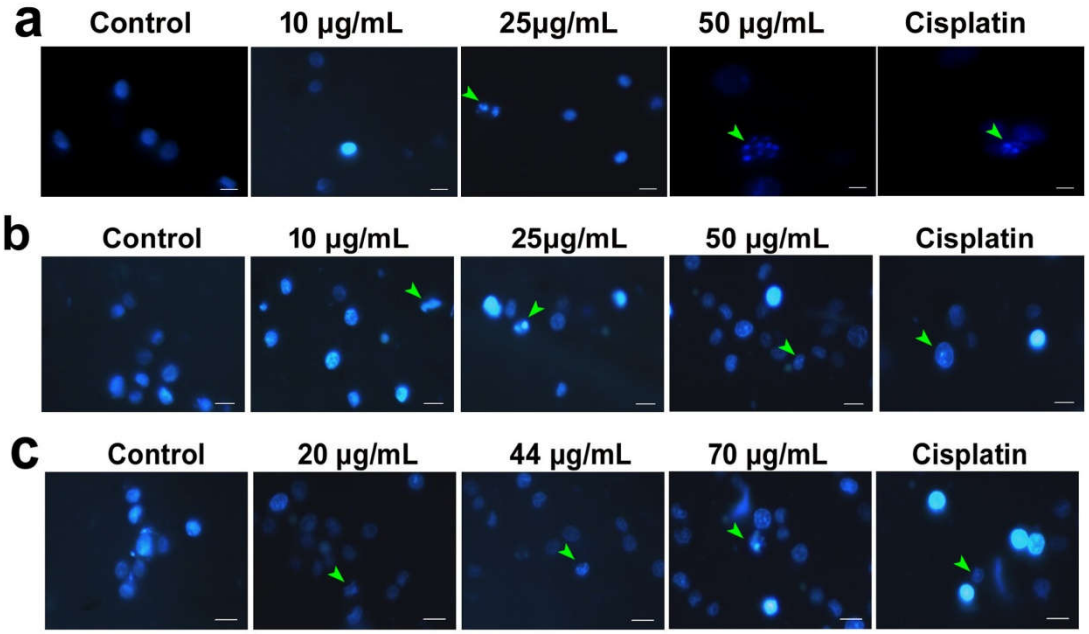

Fig. 4.29. ***Fluorescence microscopy images of HCT 116 cells treated with (a) MZL (b) AMF and (c) AMR derived AgNPs and stained with Hoechst 33258*** (*arrow heads indicate apoptotic nuclear condensation and DNA fragmentation; scale bars : 20 µm*)**.**

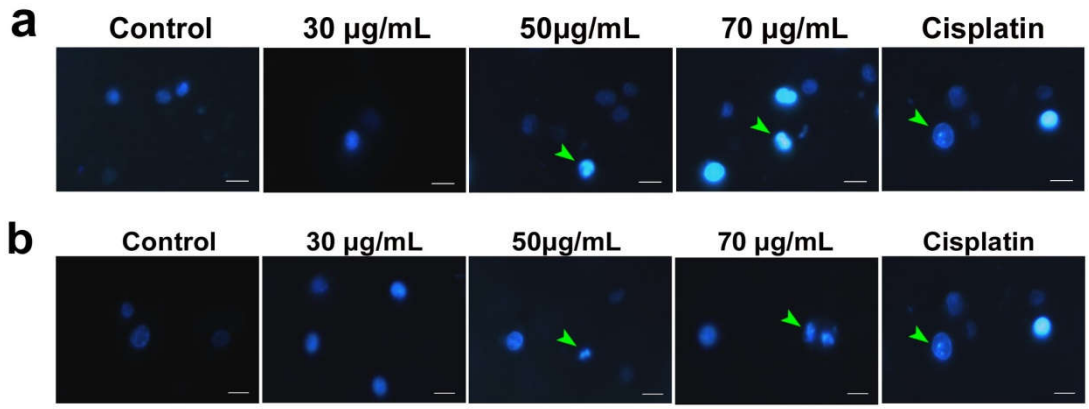

Fig. 4.30. ***Fluorescence microscopy images of A549 cells treated with (a) MZL and (b) AMF derived AgNPs and stained with Hoechst 33258*** *(arrow heads indicate apoptotic nuclear condensation and DNA fragmentation; scale bars : 30 µm).*

Acridine orange - ethidium bromide dual staining

In acridine orange - ethidium bromide dual staining technique, viable cells are characterized by possession of uniformly bright green nuclei, early to late apoptotic cells with bright green and orange fluorescent areas denoting condensed or fragmented chromatin and necrotic cells with uniformly bright orange nuclei. Based on these criteria, a quantitative analysis of HCT 116 cells treated with MZL and AMF derived AgNPs showed a dose-dependent induction of apoptosis leading to more than 80% cell death at the highest test concentration of 50 µg/mL. About 89% cell death was also observed following treatment at the highest concentration of 70µg/mL in the case of AMRAgNPs (Fig. 4. 31). MZLAgNP and AMFAgNP treated A549 cells showed 75 % cell death when treated with the highest concentration of 70 µg/mL (Fig. 4. 32).

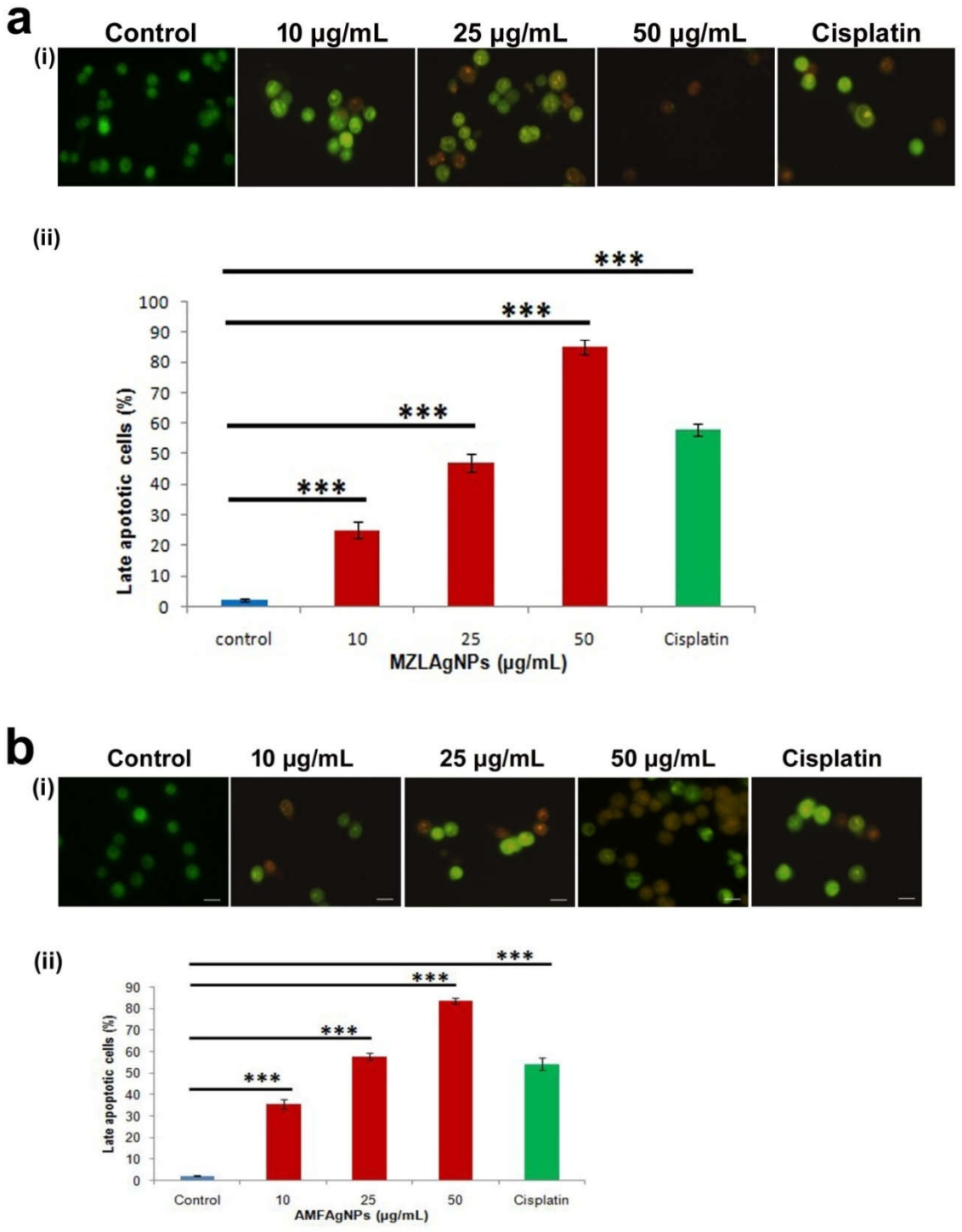

Fig. 4.31 a and b. ***Apoptosis induction in HCT 116 cells treated with MZL and AMF derived AgNPs and stained with AO / EtBr;*** *(i) fluorescence microscopy images (ii) quantitative analysis of late apoptotic cells (scale bars : 20µm); ***P≤0.001,**P≤ 0.01, *P≤0.05.*

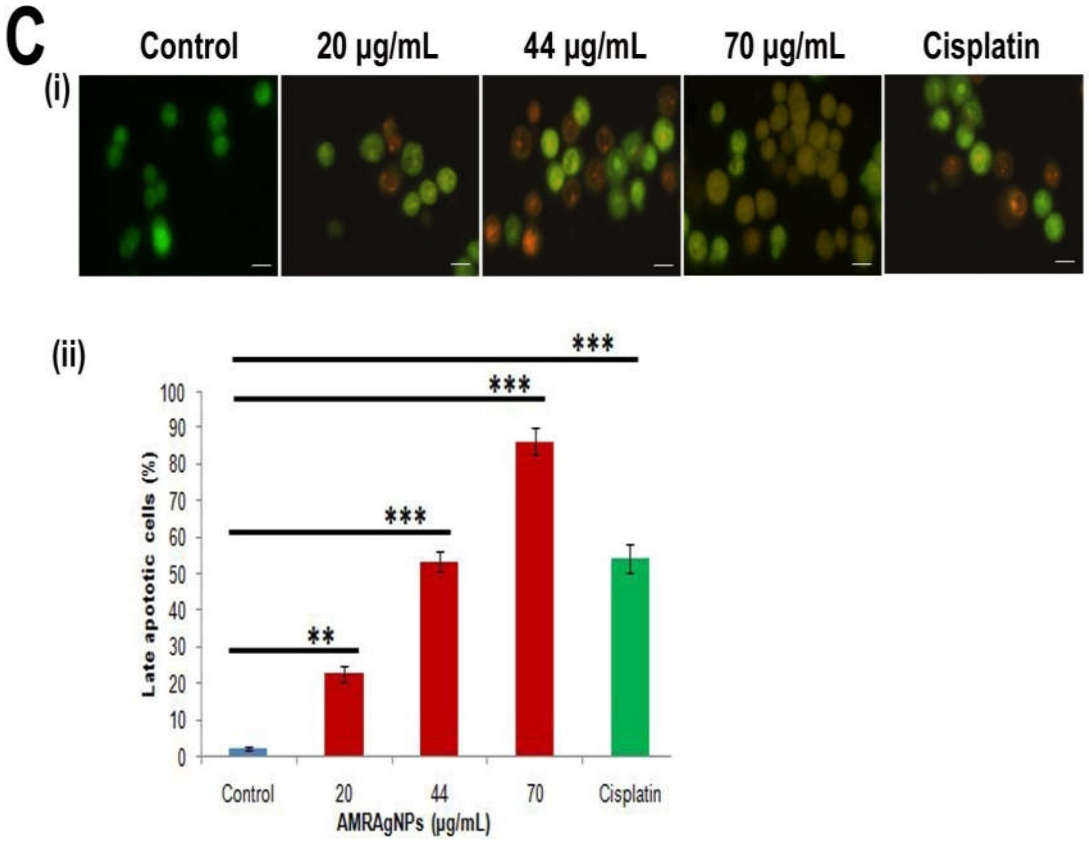

Fig. 4.31 c. ***Apoptosis induction in HCT 116 cells treated with AMRAgNPs and stained with AO / EtBr****; (i) fluorescence microscopy images (ii) quantitative analysis of late apoptotic cells (scale bars : 20µm); ***P≤0.001,**P≤ 0.01, *P≤0.05.*

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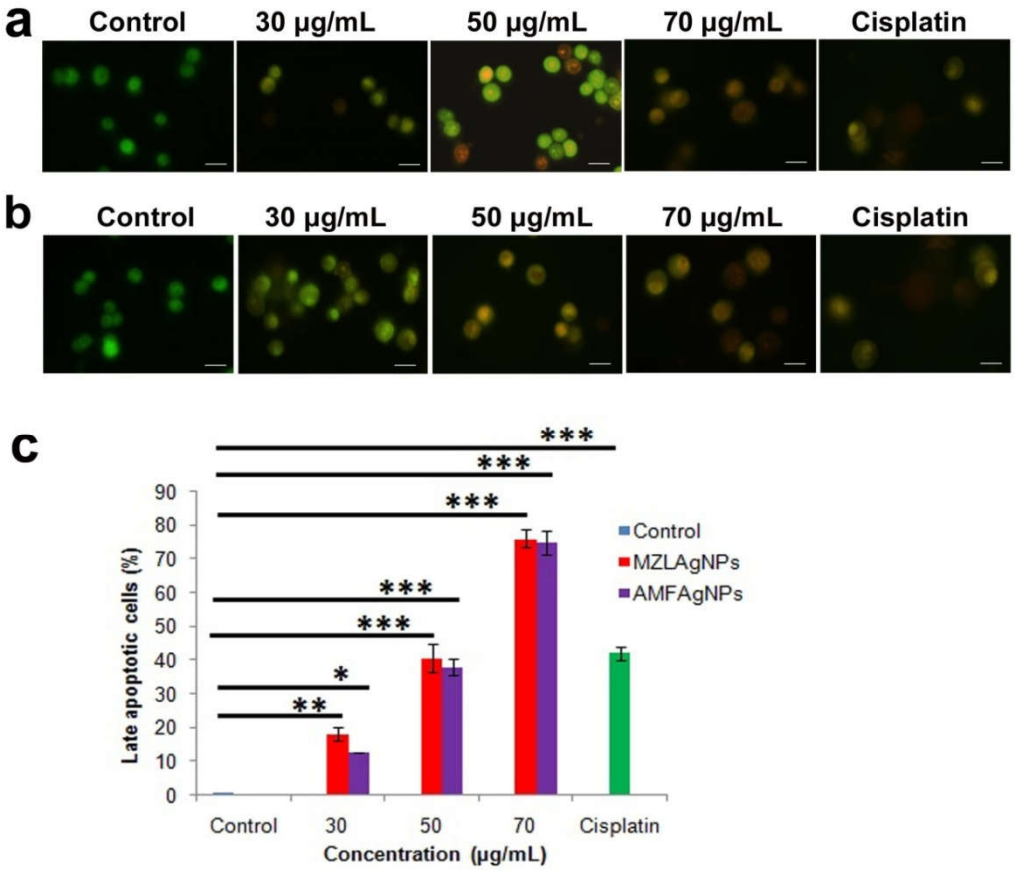

Fig. 4.32. ***Apoptosis induction in A549 cells treated with (a) MZL (b) AMF derived AgNPs and stained with AO / EtBr*** *; (i) fluorescence microscopy images (ii) quantitative analysis of late apoptotic cells (scale bars : 20µm); ***P≤0.001,**P≤ 0.01, *P≤0.05.*

4.11. *Effect of biogenic silver nanoparticles on cell cycle analysis*

Accumulations of mutations leading to alterations in cell cycle machinery and impaired DNA damage response interfere with cell cycle checkpoint pathways. This is reflected in abnormal cell cycle distribution, which in turn serves as a hallmark of human cancer (Alimbetov et al., 2018). In view of this, the cell cycle distribution of HCT116 cells, treated with biogenic nanoparticles and stained with the stoichiometric DNA binding dye, propidium iodide, was analyzed by measuring the DNA content using flow cytometry. For each experiment 10,000 events per sample were recorded. It is pertinent to note here that the presence of cells undergoing apoptosis in a population can also be assessed by flow cytometry. This is due to the fact that DNA fragmentation accompanying induction of cell death

programme leads to reduced cellular DNA content due to leaching out of nucleosomal DNA multimers during cell fixation / permeabilization with 70 % ethanol. The results obtained showing the proportion of cells in G_0/G_1, S, G_2/M phases are represented as DNA histograms (Fig. 4.33).

FACS analysis revealed discernible changes induced by treatment with MZLAgNPs in the cell cycle phase-specific distribution of the HCT 116 cells. An increase of upto 60% of the cell population was found in G_1 phase compared to 48% in untreated controls and about 32 % in cisplatin-treated cells which was indicative of a predominant MZLAgNPs-induced G_1 arrest. But the G_2 phase sub-population showed a dose-dependent decrease from about 27% in untreated control cells to 20% following MZLAgNP treatment compared to a drastic increase of 48% observed in respect of cisplatin-treated cells. The percentages of sub-G_1 fraction representing apoptotic cells was observed to increase from about 4.5% in controls to about 10 -11 % following exposure to MZLAgNPs or cisplatin. In other words, the cell killing ability of the biogenic nanoparticles and cisplatin were almost comparable. Though the cells in S phase showed a marginal decrease from 14% cells in untreated controls to about 11% and 10% following nanoparticle and cisplatin treatment respectively, a dose dependent effect was not observed (Fig. 4.33a)

AMFAgNPs treatment resulted in an increase of upto 88% of the cells in G1 phase compared with 65% in untreated controls indicative of a predominant G_1 arrest. The fact that the G1 phase constituted only 42 % in cisplatin-treated cells is in line with the previous reports on G2 arrest induction by this compound in HCT 116 (Kuppusamy et al., 2016). Interestingly, G_2/M phase sub-population showed a dose-dependent decrease from about 16% in untreated control cells to 6% on exposure to AMFAgNPs. A slight increase in the sub-G1 fraction from 1.5 to about 5-6% following AMFAgNPs / cisplatin treatment was observed indicative of comparable cell killing potential for both agents as observed with MZLAgNPs. Cells in S-phase were found to decrease from 6% in untreated controls to about 4.6 % (Fig. 4.33b).

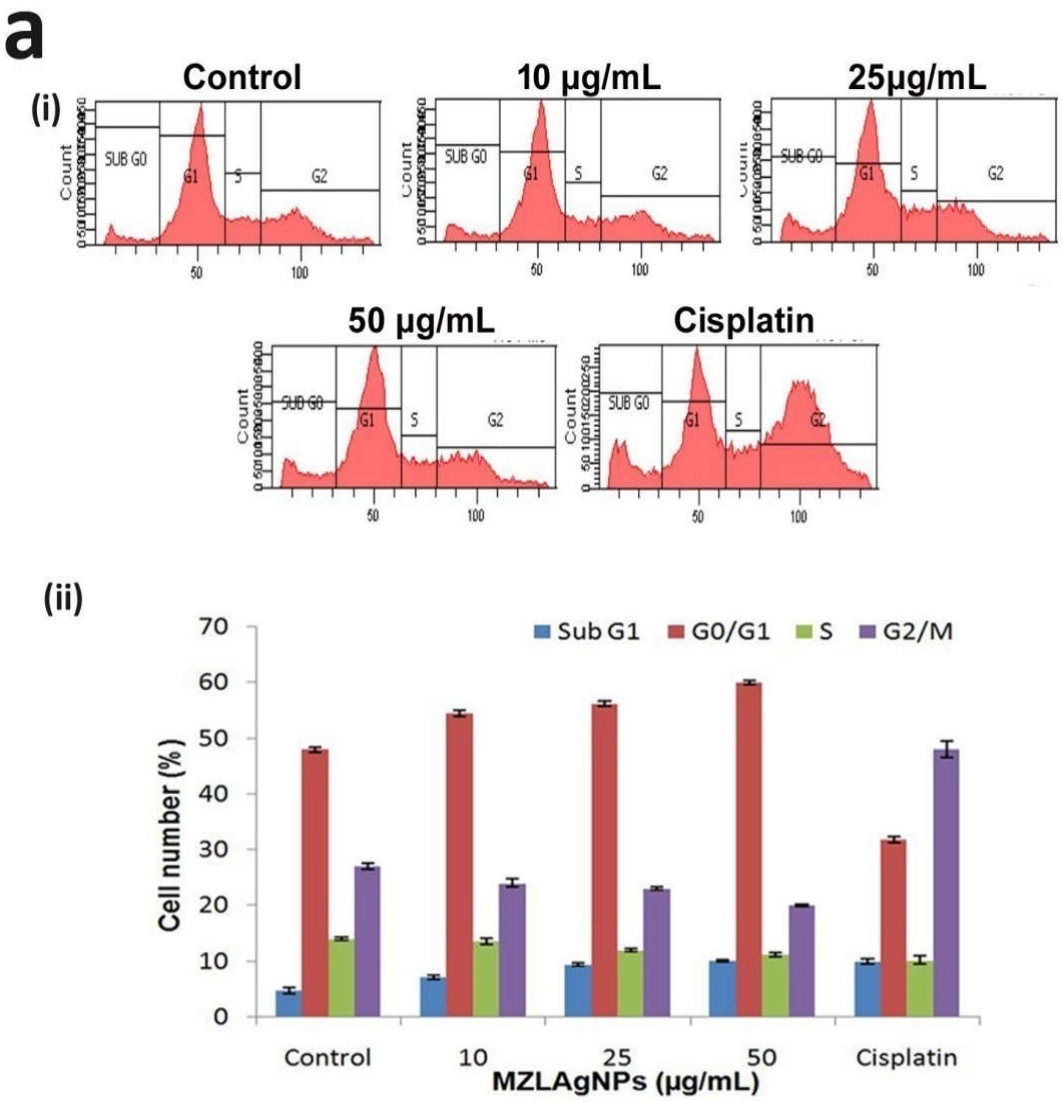

Fig. 4.33 a. ***Cell cycle distribution of HCT 116 cells treated with MZLAgNPs*** *; (i) FACS data (ii) histogram showing percentage of cells in Sub-G_1, G_0/ G_1, S, G_2/M. Data shown as mean ± SD of three independent experiments.*

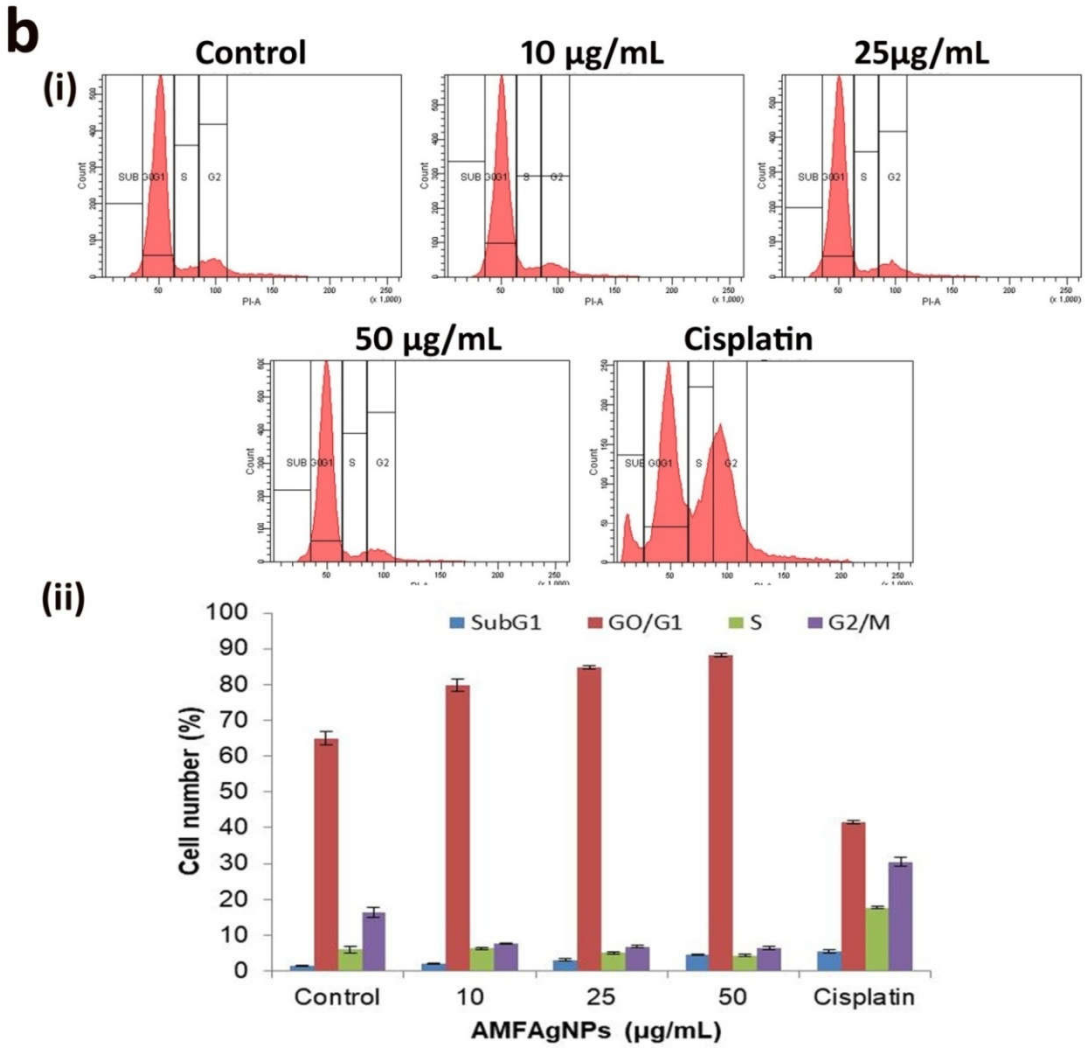

Fig. 4.33 b. ***Cell cycle distribution of HCT 116 cells treated with AMFAgNPs****; (i) FACS data (ii) histogram showing percentage of cells in Sub-G_1, G_0/ G_1, S, G_2/M. Data shown as mean ± SD of three independent experiments.*

In AMRAgNP- treated cells, an alteration in the percentage of cells in each stage of the cell cycle, namely, sub-G_1, G_0/G_1, S and G_2/M was observed in comparison to controls. AMRAgNP-treatment ranging from 20 to 70 µg/mL resulted in a concentration-dependent increased accumulation of apoptotic cells at sub-G_1 phase from 7.8 to 19.9 % respectively, in comparison to controls with only about 5.4% in cisplatin-treated cells. Both in AgNP / cisplatin treated cells, a concomitant decrease in G_0/G_1 from 71.6 in untreated controls to 58.3% and 38.5 %

respectively was observed. On the other hand, percentage increase in S phase cells from 9.0 in controls to 16% and 17.6% were obtained following AMRAgNP and cisplatin treatments respectively. A slight decrease in G_2/M phase subpopulation (7 to 5%) compared to an increase of 42% was recorded in respect of cisplatin treated cells (Fig. 4.33c).

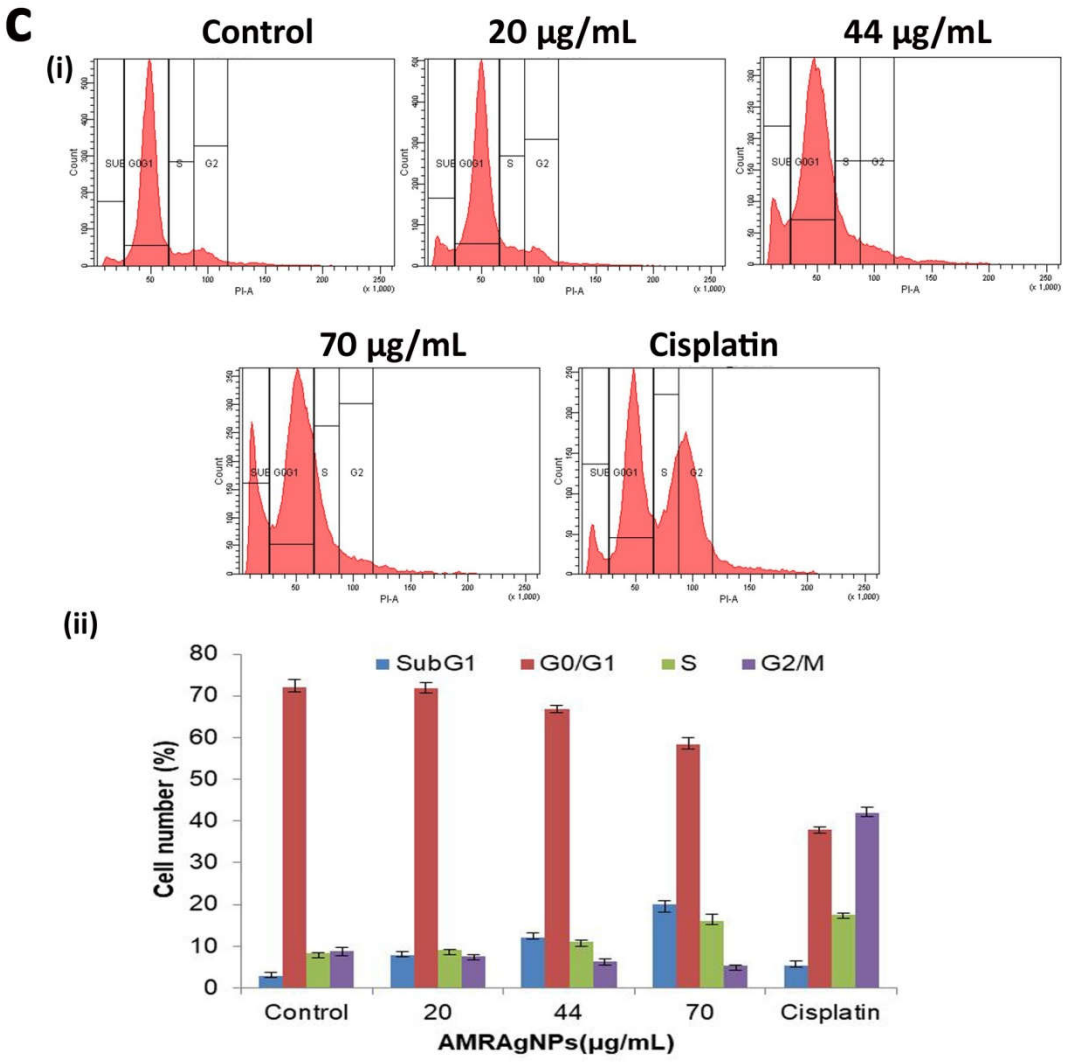

Fig. 4.33 c. ***Cell cycle distribution of HCT 116 cells treated with AMRAgNPs****; (i) FACS data (ii) histogram showing percentage of cells in Sub-G_1, G_0/ G_1, S, G_2/M. Data shown as mean ± SD of three independent experiments.*

Treatment with MZLAgNPs, AMFAgNPs and cisplatin were found to induce G_0/G_1 arrest in A549 cells. An increase of upto 84% of the cell population was found in G_1 phase compared to 74% in untreated controls which was indicative of G_0 / G_1 arrest. A Concomitant decrease in the number of cells in S and G_2 /M phases was also observed (Fig. 4.34).

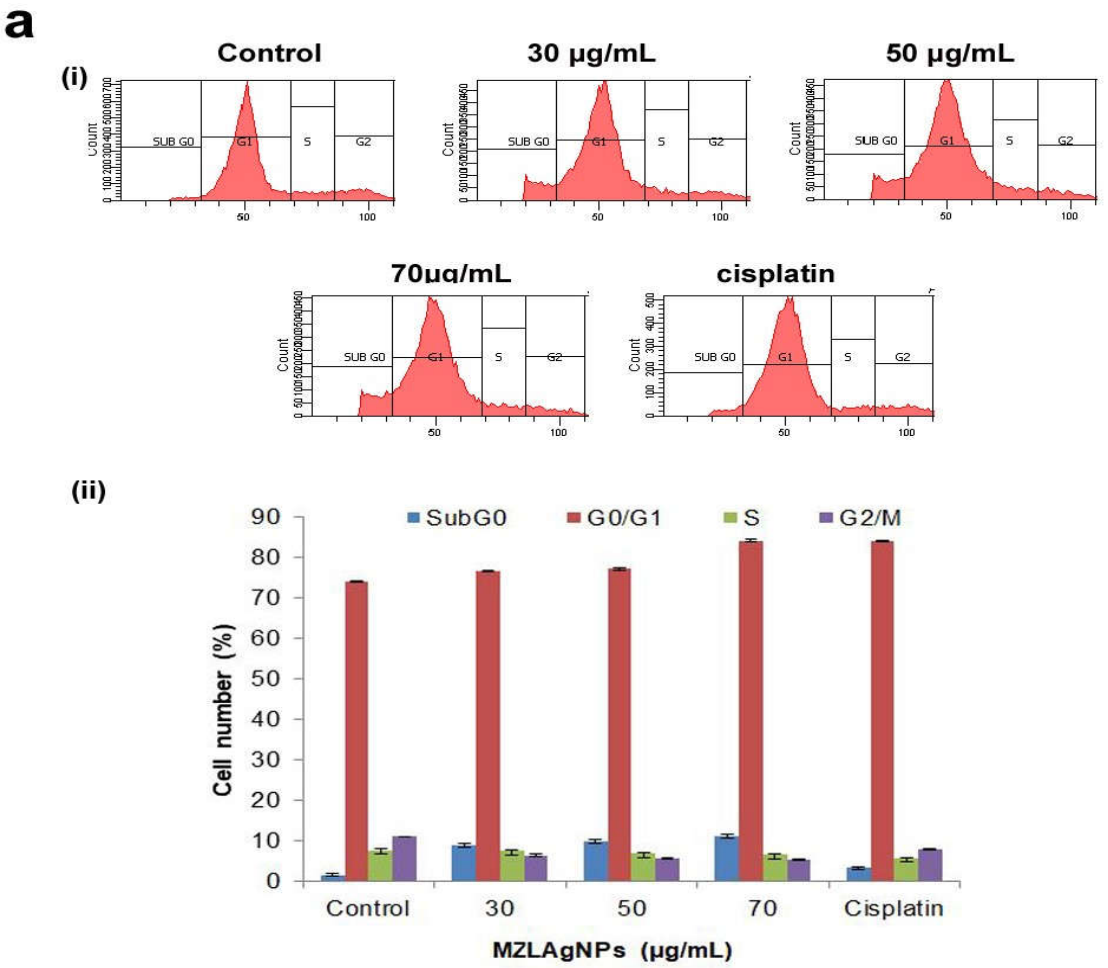

Fig. 4.34a. ***Cell cycle distribution of A549 cells treated with MZLAgNPs*** *; (i) FACS data (ii) histogram showing percentage of cells in sub-G_1, G_0/ G_1, S, G_2/M; Data shown as mean ± SD of three independent experiments.*

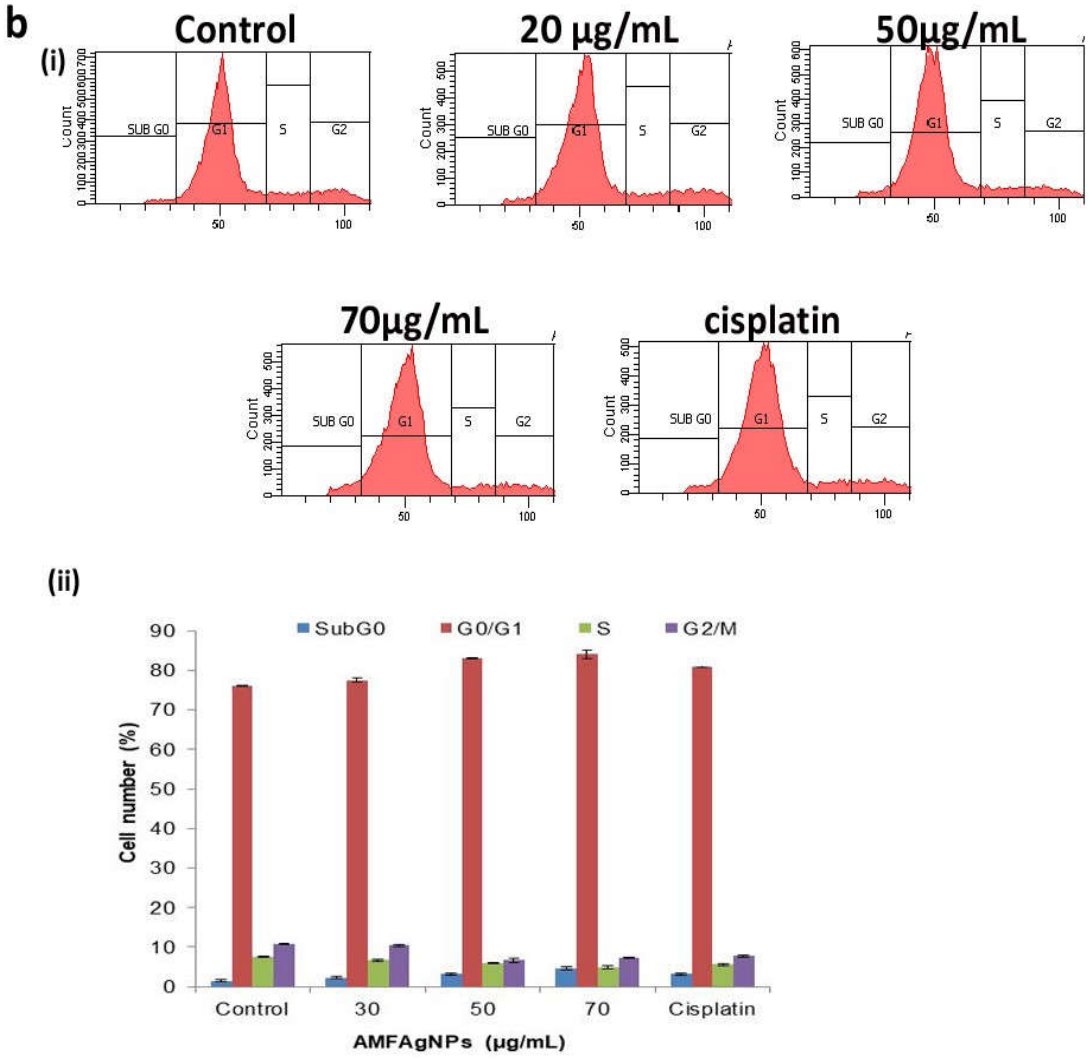

Fig. 4.34 b. ***Cell cycle distribution of A549 cells treated with AMFAgNPs*** *; (i) FACS data (ii) histogram showing percentage of cells in Sub-G_1, G_0/ G_1, S, G_2/M; Data shown as mean ± SD of three independent experiments.*

To sum up, FACS analysis showed that - of the three biogenic silver nanoparticles, MZL and AMF-derived nanoparticles arrested both HCT 116 / A549 cells at G_0/G_1 phase of cell cycle. Treatment with AMRAgNPs resulted in a 20% increase in the sub-G_1 population. Prabhu et al. (2013) reported that biosynthesized AgNPs arrested HCT-15 cells at G_0/G_1 and G_2/M phases of the cell cycle. Other studies carried out in the recent past have also shown that silver nanoparticles were

capable of inducing DNA fragmentation and cell cycle arrest at all stages (Durai et al., 2014; Thirunavukkarasu et al., 2014; Kuppusamy et al., 2016).

4.12. *Effect of AgNPs on intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and phosphatidylserine (PS) externalization*

ROS generation, in cells undergoing chemical or environmental stress, results in oxidative damage to macromolecules such as lipids, proteins and DNA (Pieme et al., 2013). The use of 6-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for indirect estimation of intracellular ROS concentration involves measuring intracellular generation of hydrogen peroxide. This probe is cell-permeable and is hydrolyzed intracellularly to DCFH carboxylate anion which is retained in the cell. Oxidation of DCFH results in the formation of dihydrofluorescein which emits fluorescence directly proportional to the amount of ROS generated. HCT 116 cells incubated with the three individual biogenic AgNPs for 48 h showed a dose-dependent increase in ROS production compared to that observed in controls (Fig. 4.35). ROS generation in response to treatment with the three types of nanoparticles ranged from about 35 - 53% cells exhibiting fluorescence compared to that in controls (<1.0 %) and with ~ 30 % cells fluorescing following cisplatin treatment.

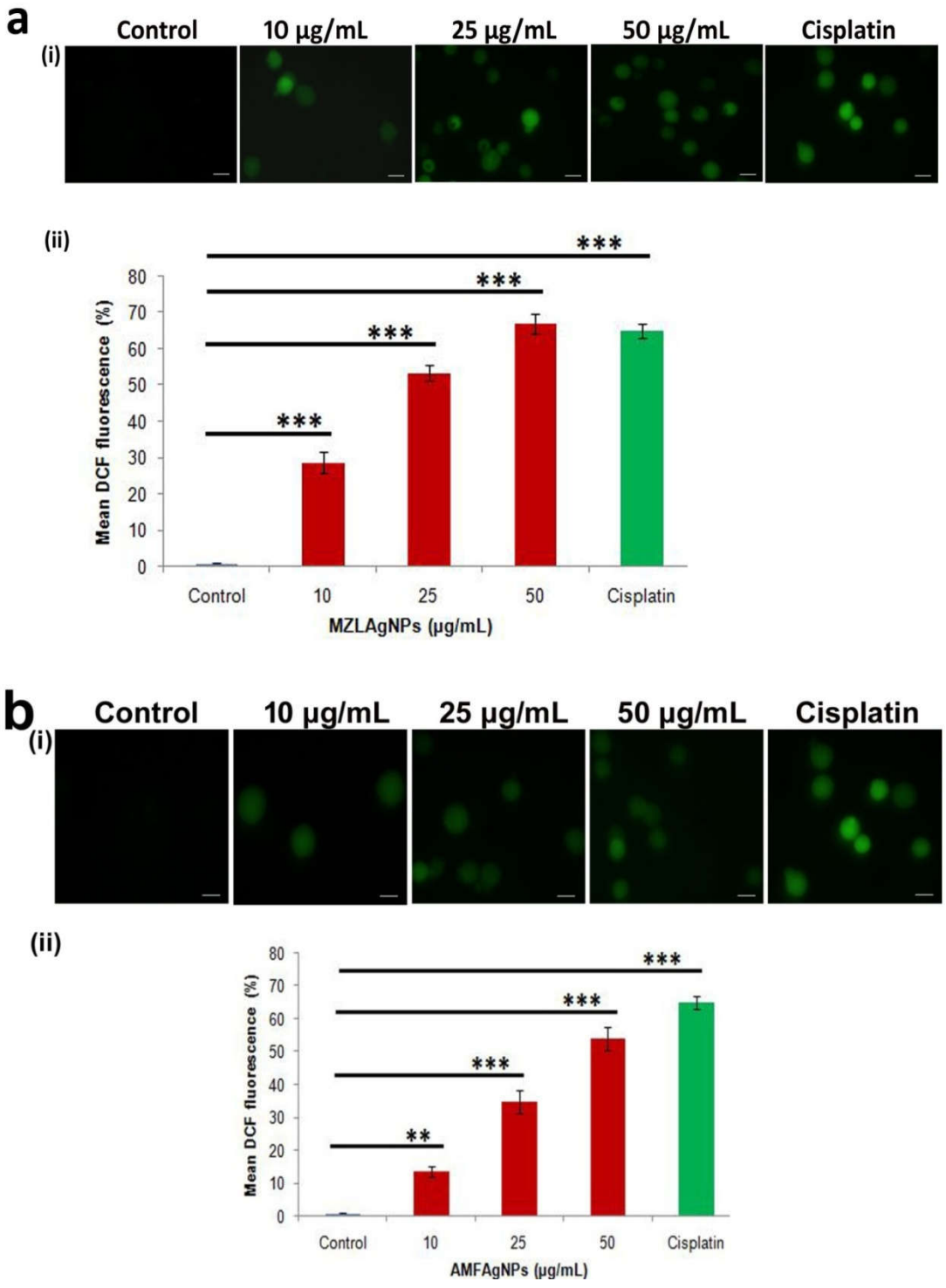

Fig. 4.35 a and b. Measurement of cellular ROS in HCT 116 cells treated with *MZL and AMF derived AgNPs*; *(i) fluorescence microscopy images* (*scale bar:20µm*) *(ii) mean fluorescent intensity of cells stained with ROS indicator DCFH-DA; ***P≤ 0.001, **P≤0.01*).

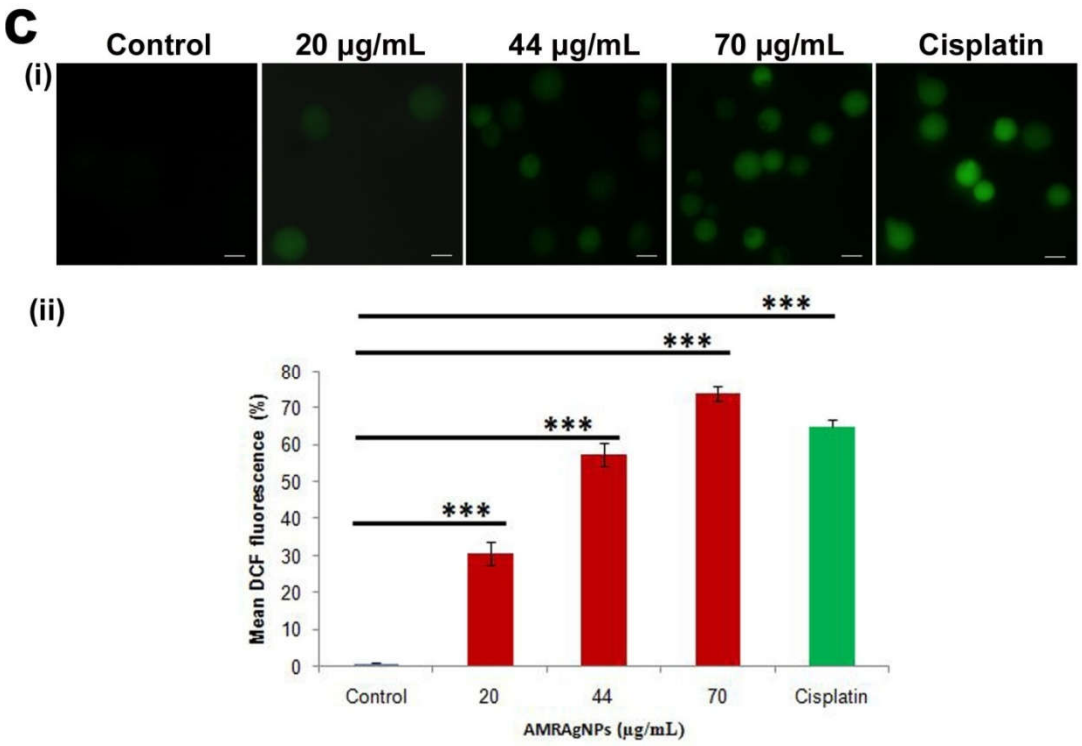

Fig. 4.35 c. Measurement of cellular ROS in HCT 116 cells treated with *AMRAgNPs;* *(i) fluorescence microscopy images* (*scale bar:20µm*) *(ii) mean fluorescent intensity of cells stained with ROS indicator DCFH-DA; ***P≤ 0.001, **P≤0.01.*

The levels of ROS production observed in A549 cells, following treatment with AMF and MZL derived AgNPs (Fig. 4.36), were comparable to those observed in HCT 116 cells. The abundance of ROS in the treated cells presumably induced oxidative stress in mitochondria resulting in impaired ATP generation, induction of DNA damage and apoptosis (Trachootham et al., 2009; El-Sonbaty, 2013; Mukherjee et al., 2015; Juarez-Moreno et al., 2017). Apoptosis induction on exposure to AgNPs mediated by oxidative stress in fibroblasts, muscle and colon cells has been previously reported (Foldbjerg et al., 2009).

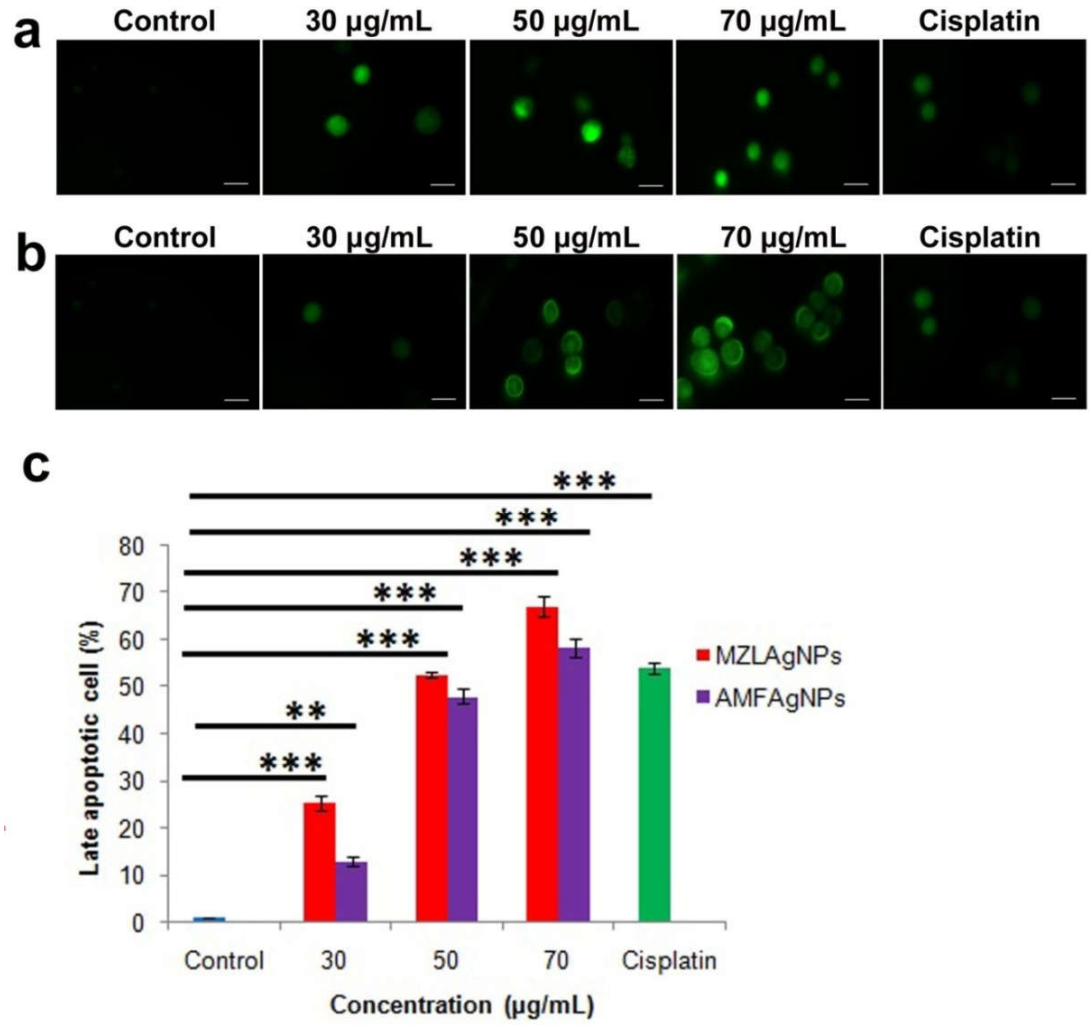

Fig. 4.36. Measurement of cellular ROS in A549 cells. *Fluorescence microscopy images* of cells treated with *(a) MZLAgNPs and (b) AMFAgNPs (scale bars : 30µm); (c) mean fluorescent intensity of cells stained with ROS indicator, DCFH-DA ; ***P≤ 0.001, **P≤0.01.*

The depletion of MMP, an early marker of apoptosis, was observed employing mitochondria-specific and voltage-dependent, fluorescent lipophilic cationic dye, Rhodamine123 in AgNPs / cisplatin treated cells. Treatment with increasing concentrations of each type of biogenic nanoparticle resulted in significant loss of mitochondrial membrane potential in HCT 116 cells which was found to be higher in magnitude than that observed in cisplatin treated cells (Fig. 4.37). A549 cells, however, displayed extremely weak fluorescence as evident from the micrographs shown in Fig. 4.38; hence, histogram of fluorescence measurement is not shown. It may be noted that proof of apoptosis induction was

clearly evident in the fluorescence micrographs obtained following dual staining with AO-EtBr as well as with DCFH-DA (see Fig.4.32 and 4.36). Thus, the weak fluorescence observed following rhodamine123 staining could be due to technical reasons which remain unclear.

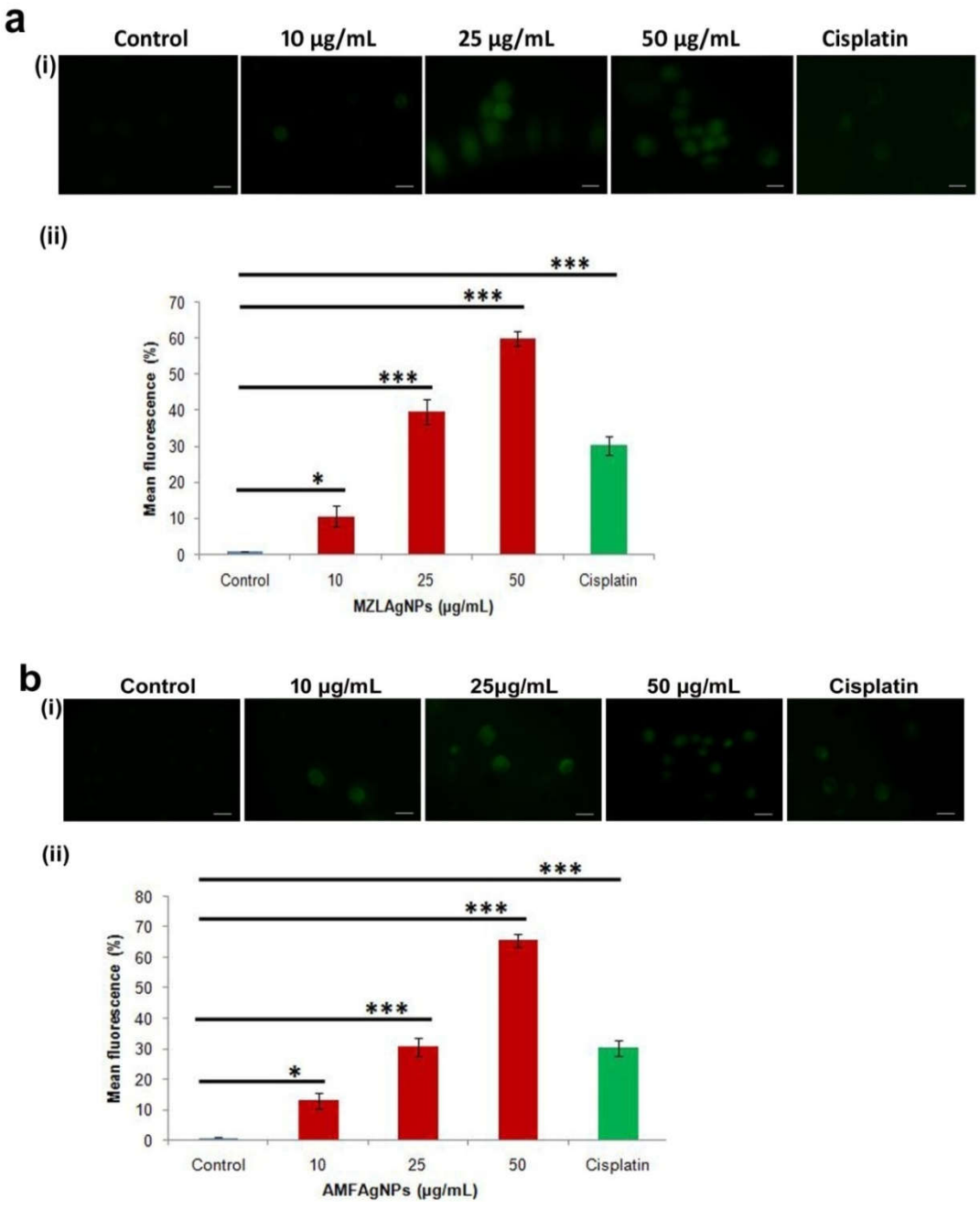

Fig.* 4.37a and b.** ***Measurement of mitochondrial membrane potential in HCT 116 cells treated with (a) MZL and (b) AMF derived AgNPs*; (i) fluorescence microscopy images (scale bar:20µm) (ii) mean fluorescent intensity of cells stained with Rhodamine 123; ***P≤ 0.001, **P≤0.01).*

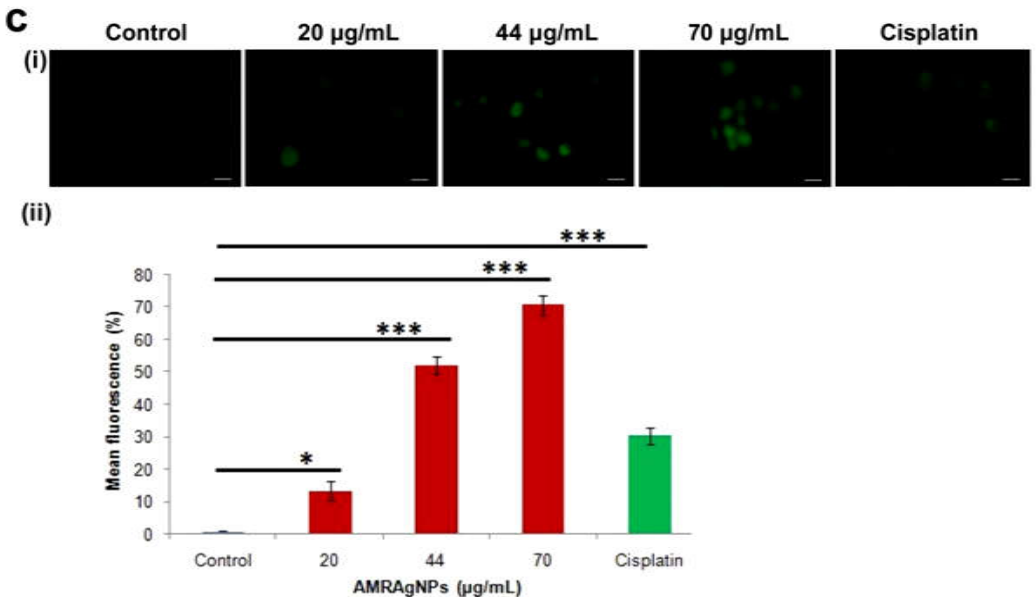

Fig.* 4.37 c.** ***Measurement of mitochondrial membrane potential in HCT 116 cells treated with AMRAgNPs *; (i) fluorescence microscopy images (scale bar:20µm) (ii) mean fluorescent intensity of cells stained with Rhodamine 123; ***P≤ 0.001, **P≤0.01.*

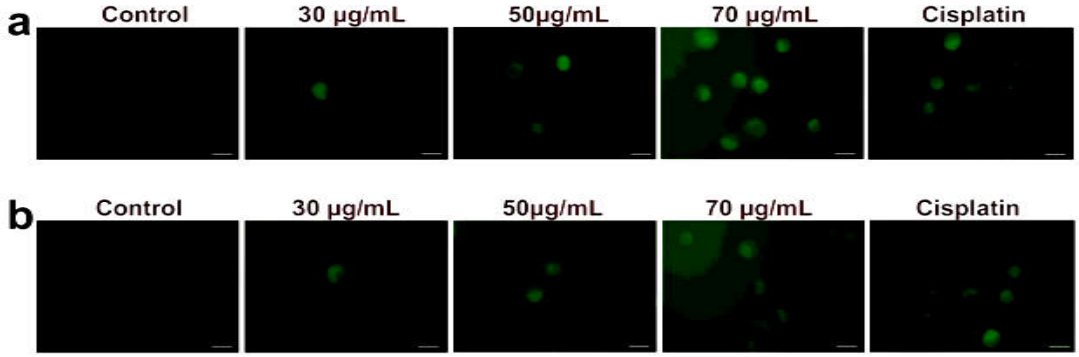

Fig. 4.38. Fluorescence microscopic images of A549 cells stained with treated Rhodamine 123 following treatment with (a) MZL and (b) AMF derived AgNPs *(scale bar : 30µm).*

Annexin V-FITC staining carried out to determine the extent and mode of cell death induced by AgNPs revealed a dose-dependent increase in the number of annexin V positive HCT 116 cells which enabled better determination of apoptosis (Fig. 4.39). MZLAgNP-treatment resulted in a dose-dependent increase in the number of apoptotic cells compared to that observed following treatment with AMF or AMR derived nanoparticles. As observed with MMP assessment, A549 cells failed to display fluorescence-associated with annexin V-FITC staining presumably due to low levels of apoptosis induction.

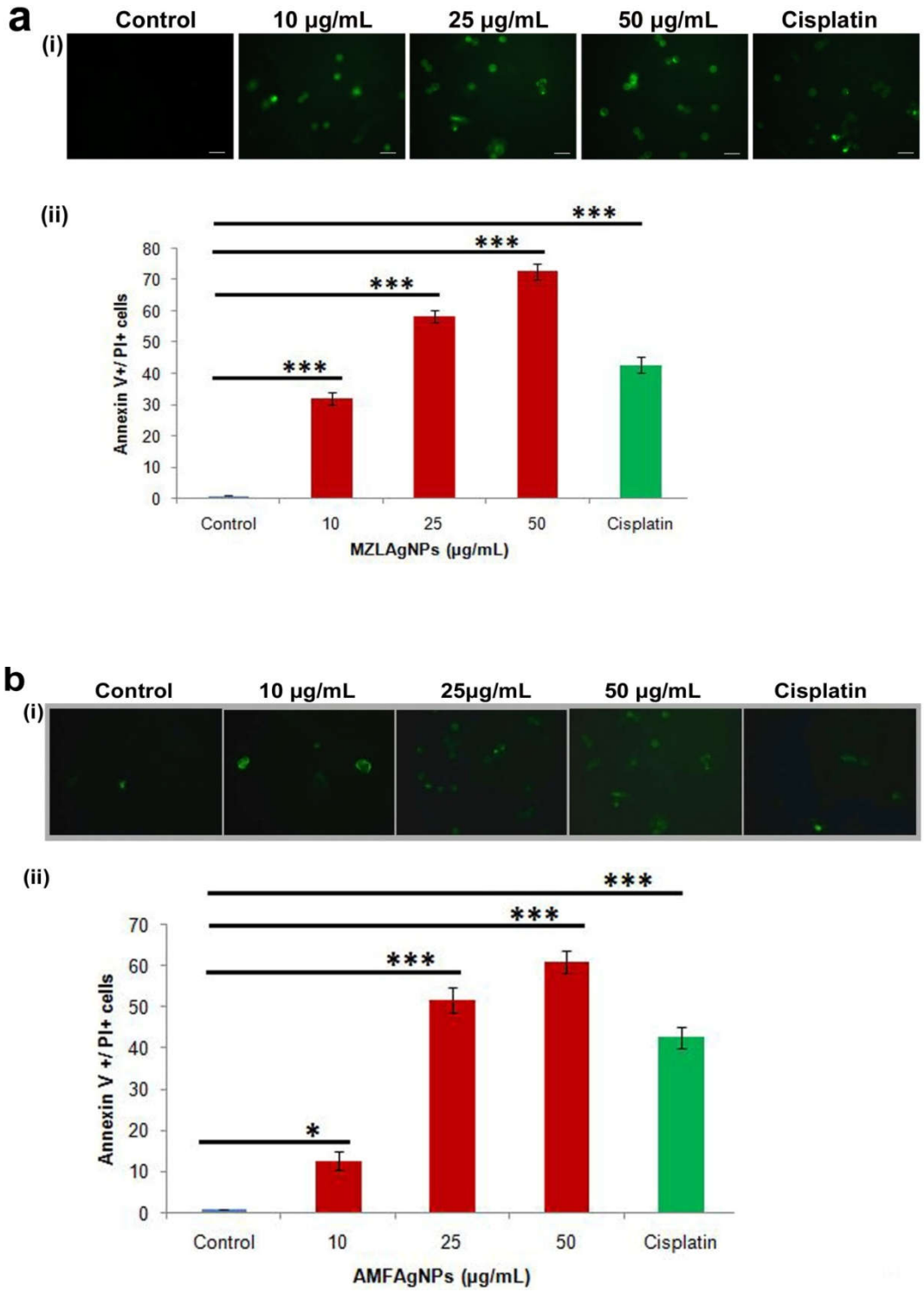

Fig. 4.39 a and b. ***Detection of apoptosis by Annexin V-FITC / PI staining in HCT 116 cells treated with MZL and AMF derived AgNPs;*** *(i) fluorescence microscopy images (scale bar : 20µm) ; (ii) quantitative analysis of annexin* V^+/PI^+ *cells.*

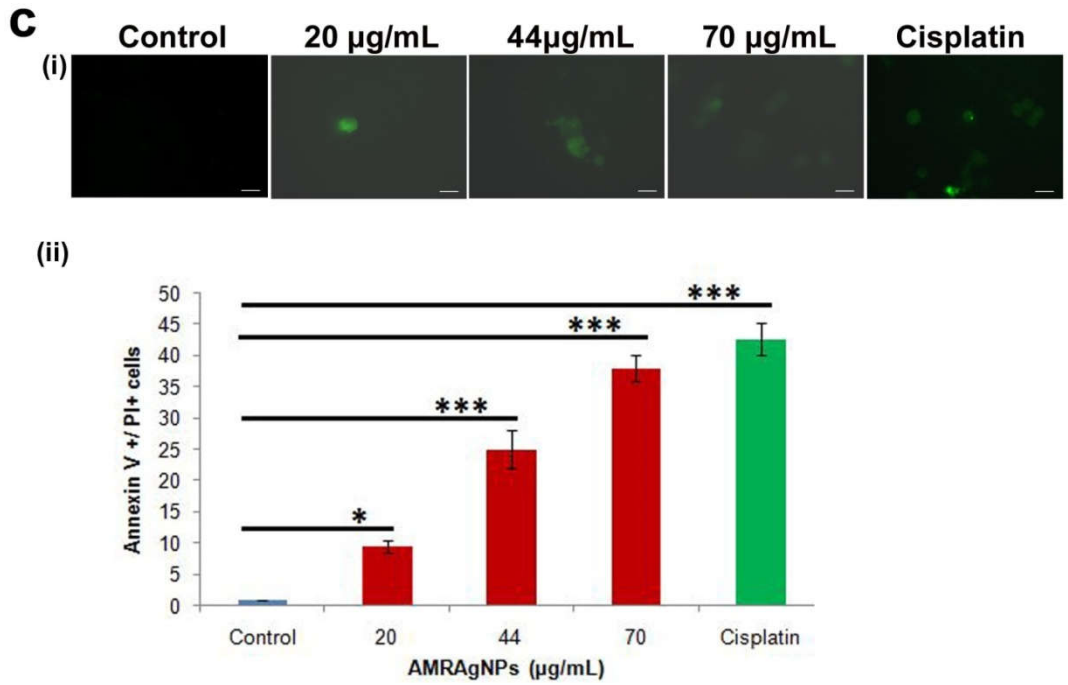

Fig. 4.39 c. ***Detection of apoptosis by Annexin V-FITC / PI staining in HCT 116 cells treated with AMRAgNPs;*** *(i) fluorescence microscopy images (scale bar : 20µm) ; (ii) quantitative analysis of annexin V^+/PI^+ cells.*

4.13. Genotoxicity of AgNPs

DNA damaging potential of AgNPs was assessed by the comet assay. A concentration dependent increase in the tail moment was observed in the treated cells which was found to be absent in the untreated controls (Fig. 4.40). A comet-like tail implied presence of damaged DNA strands trailing behind an intact nucleus during electrophoresis. Genotoxic effects of biogenic silver nanoparticles were confirmed by the observation of DNA strand breaks in the form of diffuse comet tails as reported previously (Jeyaraj et al., 2013). A549 cells treated with MZL and AMF derived AgNPs, however, failed to exhibit comet tails apparently indicating lack of nanoparticle-induced DNA strand breaks.

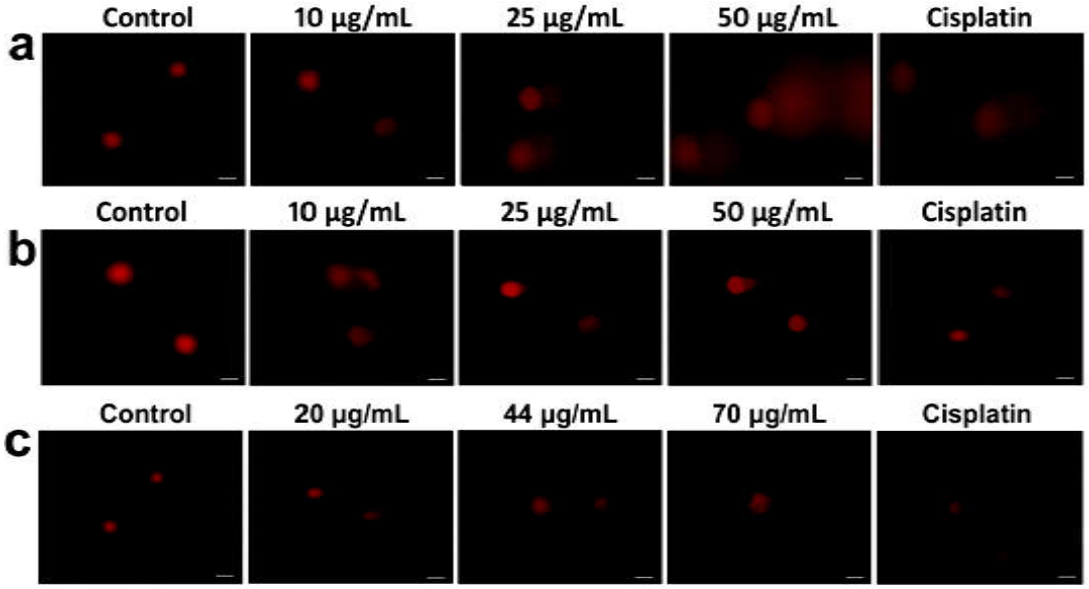

Fig. 4.40. ***Detection of DNA strand breaks by alkaline comet assay.*** *HCT 116 cells treated with (a) MZLAgNPs (b) AMFAgNPs and (c) AMRAgNPs (scale bars : 20µm).*

4.14. RTq-PCR analysis

RTq-PCR is one of the most sensitive techniques for detection and quantification of specific mRNAs and / or identification of patterns of gene expression in cells, also termed as expression profiling. Expression levels of apoptosis-related genes such as p53-upregulated modulator of apoptosis (PUMA), caspase-3, -8, -9, Bcl-2 and Bax were also determined for studying biogenic AgNP-induced changes in the transcriptional profiles of HCT 116 and A549 cells. Exposure to MZLAgNPs resulted in more than 4 fold upregulation in the expression of PUMA, caspase-3 and -9 in both cancer cell types at the IC_{50} concentration. Caspase-8 mRNA expression was significantly higher by about 4 folds in HCT 116 cells than in A549. Contrastingly, the upregulation of Bax was found to be 4 folds higher in A549 than in HCT cells. However, Bcl-2 expression was found to be marginally greater in HCT 116 than in A549 cells (Table. 4.6 and 4.7).

The activation of caspases is fundamental in triggering apoptotic cell death. Among the different subgroups, caspase-3 plays a decisive role in apoptosis-associated processes such as loss of membrane integrity and DNA damage enhancing apoptotic cell death in a caspase-3 dependant pathway. AMFAgNP-

treatment resulted in a drastic concentration-dependent increase of upto 32 fold in caspase-3 expression in A549 cells. However, the fold increase observed in HCT 116 cells was only about 5 fold higher than that in the control cells. PUMA and caspase-9 expression were found to be higher in HCT cells but caspase-8 expression was higher in A549 cells. Interplay between the pro-apototic Bax and the antiapoptotic Bcl-2 is known to play a key role in mitochondria-mediated apoptosis. Strikingly, Bax expression was found upregulated about 27-28 folds following AMFAgNP-treatment in HCT cells but only 6.5 fold in A549 cells. On the contrary, Bcl-2 expression in both HCT and A549 was found to be marginally lower than that in control cells.

AMRAgNP treatment resulted in >2 folds increase in expression of caspase-3, -8, -9 and PUMA. Notably, a 20-fold increase in Bax expression concomitant with a 0.20 fold decrease of anti-apoptotic Bcl-2 expression at IC_{50} concentration was also observed (Table. 4.7). Overall, transcript quantitation by RTq-PCR technique clearly demonstrated induction of apoptosis through upregulation of the key genes - PUMA, caspase-3, -8, -9 and Bax and down regulation of anti-apoptotic Bcl-2 expression.

Table 4.6. The relative quantitation of mRNA expression of apoptosis-related genes using RTq-PCR in HCT 116 cells treated with biogenic AgNPs /cisplatin.

Samples	Concentration (µg/mL)	Genes					
		PUMA	Caspase-3	Caspase-8	Caspase-9	Bax	Bcl-2
MZLAgNPs	10	8.5± 0.9	3.36 ± 0.5	8.1 ±0.5	2.1 ± 0.7	1.42 ±0.43	0.72± 0.8
	25	10.7 ±0.5	5.45 ± 0.8	19.7± 1.0	5.1 ± 0.5	1.6±0.5	0.6 ± 0.4
	50	0.8 ±0.4	10.48± 0.7	1.8 ± 0.9	8.0 ± 0.63	4.0 ±0.48	0.22 ±0.8
AMFAgNPs	10	5.2 ± 0.3	1.4 ±0.6	2.5 ± 0.4	22 ±0.69	1.2 ± 0.5	4.3 ± 0.5
	25	10.46 ±0.8	2.0 ±0.7	3.2± 0.43	26 ±1.3	16.4 ± 2.7	0.8 ± 0.6
	50	7.1 ±0.3	4.9 ±0.17	1.86± 0.6	32 ±1.52	26.5 ± 1.5	0.1 ± 0.9
AMRAgNPs	20	2.16 ± 2.7	1.3 ± 1.4	1.58 ±1.2	1.58 ±0 .8	1.9 ± 0.5	3.13 ±1.4
	44	12.9 ± 1.5	2 ± 0.8	3.7±0.9	7.06 ± 0.9	4.92 ± 1.5	0.2 ±0.9
	80	1.8 ± .9	4.5 ± 1.5	2.4 ± 1.0	12.2 ± 1.3	20 ± 1.6	0.099 ±0.7
Cisplatin	6.5	2.14 ± 0.8	1.55 ± 0.4	4.7 ± 1.3	11.55± 0.5	11.71±0.6	0.11 ±0.3

Table 4.6. The relative quantitation of mRNA expression of apoptosis-related genes using RTq-PCR in A549 cells treated with Biogenic AgNPs /cisplatin.

Samples	Concentration (µg/mL)	Genes					
		PUMA	Caspase-3	Caspase-8	Caspase-9	Bax	Bcl-2
MZLAgNPs	30	1.07 ± 2.0	1.1± 2.7	1.01 ± 0.24	8.51 ± 2.5	1.3 ± 0.43	1.0 ±0.8
	44	4.6 ± 3.3	5.4 ± 1.4	4.92 ±.1.8	10.5 ± 0.45	6.3 ± 1.2	0.8 ±0.5
	70	0.2 ± 1.9	6.3 ± 0.50	18 ± 1.3	22 ± 0.74	8.6 ± 0.84	0.53±0.3
AMFAgNPs	30	1.8 ± 3.0	1.48 ±1.0	1.24 ± 0.7	0.98 ± 1.6	1.1 ± 1.4	1.8 ± 0.7
	48	5.2 ± 2.5	26 ± 2.1	10.8 ± 2.2	1.5 ± 0 .4	6.5 ± 2.0	1.0 ± 1.3
	70	3.4 ± 1.93	32 ± 1.4	23± 1.7	1.8 ± 1.0	35 ± 2.6	0.6±0.65
Cisplatin	13	3.8± 2.8	2.3 ± 0.9	5.6 ± .62	4.9 ± 2.4	20 ± 1.2	0.34±0.2

Transcript profiling by RT-qPCR technique clearly demonstrated that all three types of biogenic AgNPs generated in this study are capable of significantly elevating expression of pro-apopotic genes, caspase-3 and -9, in a dose-dependent manner. Notably, at concentrations beyond IC_{50}, caspase-8 downregulation was observed as already reported earlier in connection with silver nanoparticle treatment (Abbasi et al., 2016) along with that of PUMA following treatment with all three types of AgNPs. Previous reports on biogenic AgNPs have also shown increased PUMA expression in colon cancer cells in a mitochondria-dependent manner (Mustata et al., 2013; Kuppusamy et al., 2016; Kovacs et al., 2016). Likewise, A549 NSCLC cells treated with silver nanoparticles were reported to upregulate expresssion of caspase-3 and -8 (Karthik et al., 2014). The striking highlight of the results obtained with transcription profiling is the enhanced expression of the proapoptotic genes PUMA and caspase-3 induced by all the three nanoparticles over the standard anticancer drug cisplatin. Higher expression of caspase-8 and caspase-9 following MZLAgNP and AMFAgNP treatments respectively was also observed in HCT 116 cells.

4.15 . Western blot analysis

To confirm induction of apoptosis by biogenic AgNPs, the expression of the canonical hallmark proteins, namely, cleaved PARP, cleaved caspase-3 and -9 were also examined by western blot analysis. Increased expression of these proteins is

associated with mitochondria-mediated intrinsic pathway of apoptotic cell death. Moderate levels of the tumour suppressor protein p53 were found to be present as expected in the wild type HCT 116 cells. A dose-dependent increase in the levels of CDK inhibitor, p21 - a well established DNA damage-inducible, direct transcriptional target of p53 capable of effecting cell cycle arrest in G1- was also clearly evident (Fig. 4. 41). The highest induction of p21 was observed in cisplatin-treated cells.

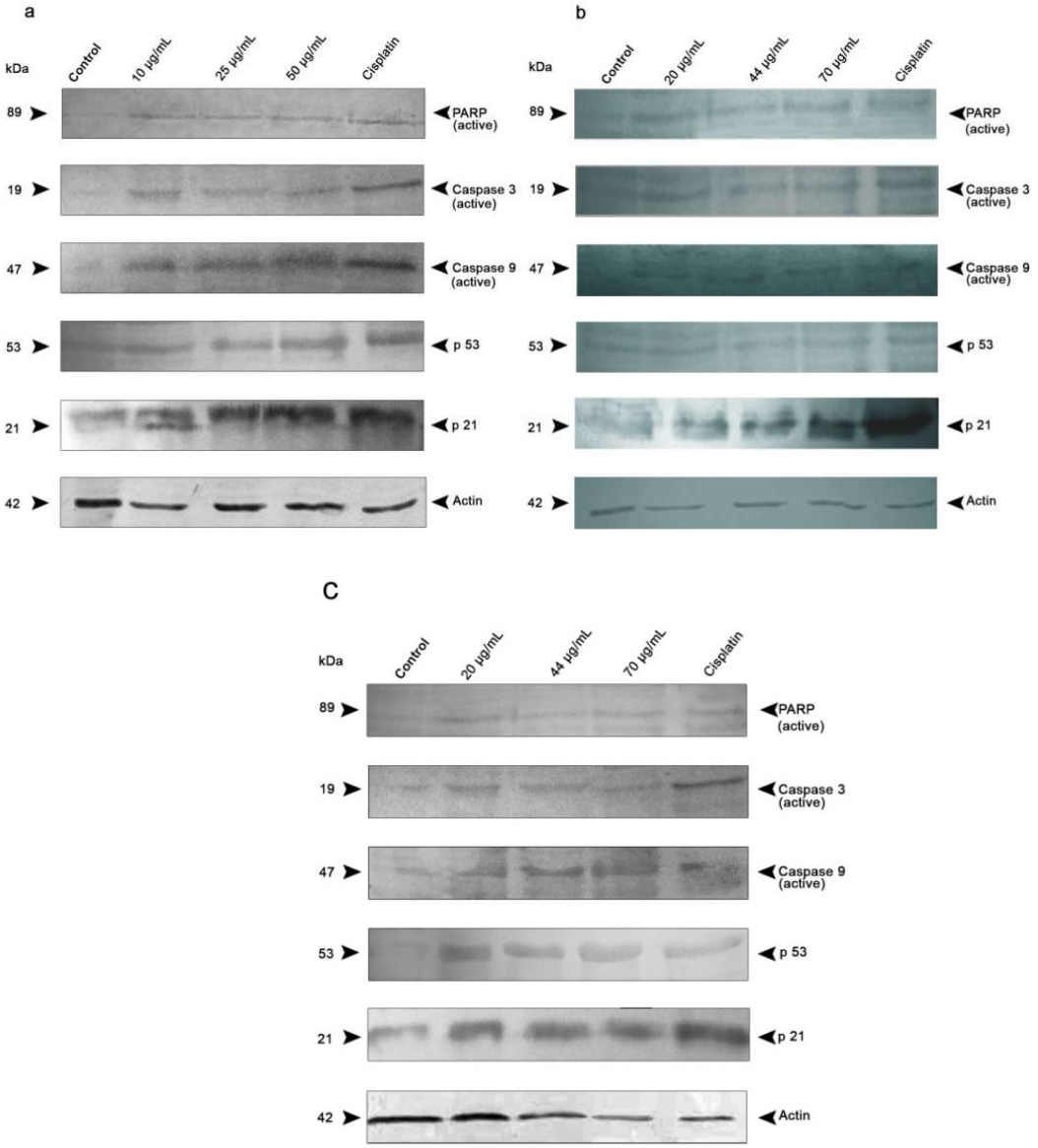

Fig. 4.41. Western blot analysis of apoptosis-related protein expression of PARP (active), caspase -3, -9 (active) and cell cycle-related protein expression of p53 and p21in HCT 116 cells treated with (a) MZL (b) AMF and (c) AMR derived AgNPs. *All proteins were normalized against beta actin taken as the loading control.*

The fold increase observed in the transcript levels of caspases-3 and -9 were not proportionate with the expression of these proteins on the western blots. Interestingly, interaction of 20nm AgNPs with human neutrophils were found to induce apoptosis along with potent inhibition of *de novo* protein synthesis (Poirier et al., 2014). Earlier, Yoon et al. (2009) have also shown the involvement of DNA damage-associated inhibition of translation linked with p53/PKR/eIF2α phosphorylation pathway in HCT 116 cells. Whether the moderate levels of p53 observed on western blots is a consequence of such an effect is unclear and remains to be established. However, in the context of AgNP-induced DNA damage and dose-dependent increase in ROS generation observed in HCT 116 cells in the present study, expression of $p21^{WAFI/Cip1}$ is meaningful since it is a direct transcriptional target of p53 and is strongly induced by DNA damage in cells. Incidentally, it has been observed that cyclin-dependent kinase inhibitor, p21, can be activated by both p53-dependent and p53-independent mechanisms and can assume pro- or -anti-apototic functions, depending on the cellular context (Ravizza et al., 2004). However, MZLAgNP and AMFAgNP-induced G1 arrest and apoptosis induction as evidenced by the flow cytometric data tilts in favour of the well established role of p21- mediated G_1 arrest (Han et al., 2002) in HCT 116 cells. On the other hand, in AMRAgNP-treated cells, commensurate with the increased sub-G_1 fraction of apoptotic cells observed during FACS analysis, apoptosis related expression was apparent on the western blots. In the case of A549 cells also, AgNP-treatment resulted elicited a similar expression profile. Further, both MZL and AMF-extract derived AgNP-treated cells displayed G_1 arrest as evidenced by the flow cytometric data. This was corroborated by the higher expression of p21, a target of p53, observed in the western blots, given the fact that moderate levels of p53 were also observed. In this context, it may be relevant to note here that in A549 cells apoptosis induction has been shown to occur in a p-53 dependent and independent manner (Shapiro et al., 1999; Oleinik and Krupenko, 2003).

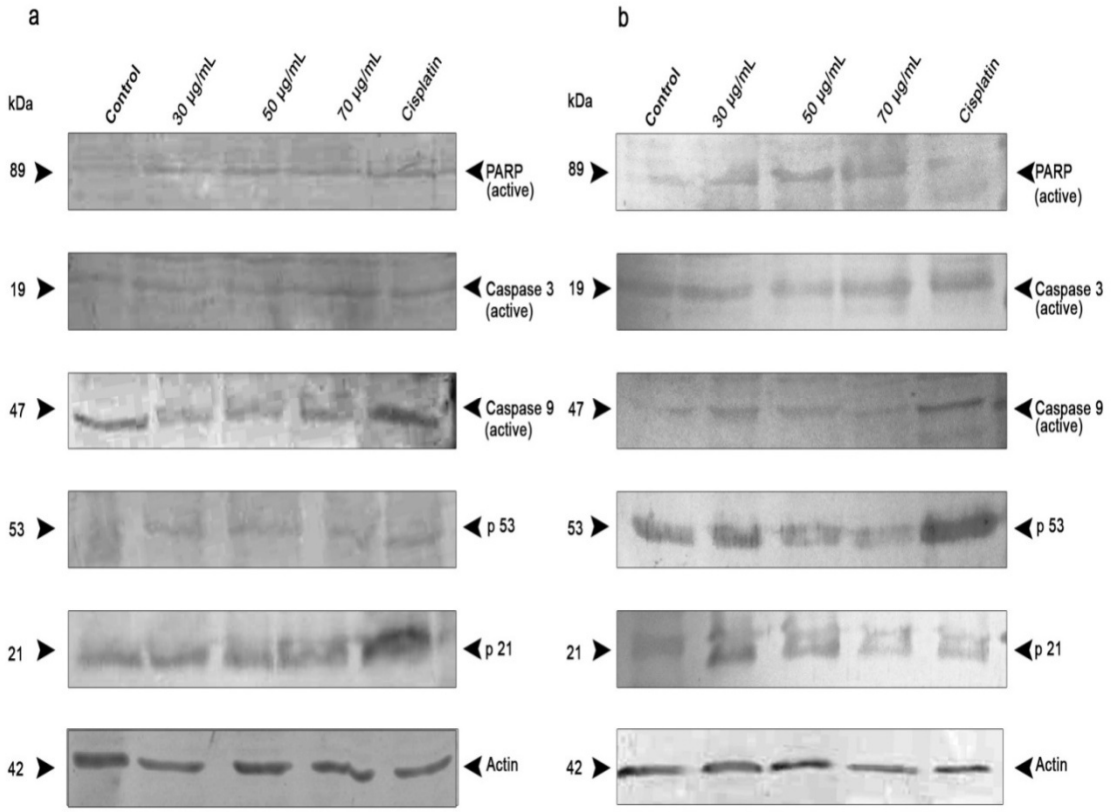

Fig. 4.42. Western blot analysis of apoptosis-related protein expression of PARP (active), caspase -3, -9 (active) and cell cycle-related protein expression of p53 and p21 in A549 cells treated with (a) MZL and (b) AMF derived AgNPs. *All proteins were normalized against beta actin taken as the loading control.*

SUMMARY AND CONCLUSIONS

Nanotechnology encompasses a recent, revolutionary, eco-friendly, cutting edge, general purpose technology driven by the desire to fabricate materials with novel and improved qualities. It continues to impact on virtually all areas of physical, chemical, biological and health sciences since it provides cleaner, safer and smarter options.

Nanoparticles are clusters of atoms below 100 nm endowed with extraordinary size and shape-dependent properties useful for applications in the fields of molecular biology, material sciences, biomedical engineering, electronics and medicine. Among the several noble metal nanoparticles, silver nanoparticles (AgNPs) stand out as the most used in all nanotechnology products. Their unique properties of catalysis, chemical stability, antimicrobial, anti-viral, antifungal and anti-inflammatory activities have led to a spurt of biomedical applications including cancer diagnosis, drug delivery and treatment

India is a tropical country blessed with a vast biodiversity of plant resources and ancient wisdom as evident in the practice of traditional phytopharma systems including Ayurveda. Medicinal plants thus offer an attractive platforms for phyto-mediated synthesis of nanoparticles. The present study exploited the bioreduction and capping potential of phytoconstituents present in medicinal plant extracts for biosynthesis of silver nanoparticles. Adopting an extracellular method, biosynthesis of silver nanoparticles was initially attempted employing several medicinal plants. Impediments encountered with the various extracts included lack of bioreducing potential, excessive time-limit for production of nanoparticles, extremely low yields and improper size range. Successful biosynthesis was achieved with the deployment of plant extracts prepared from (i) the leaves of *Manilkara zapota* and (ii) fruits and roots of *Annona muricata*.

Physico-chemical parameters such as extract concentration, precursor metal ion concentration, pH, temperature and period of incubation were optimized for

producing stable biogenic AgNPs, as evidenced by the generation of visually discernible, different shades of brown solution. These nanoparticles were designated as MZLAgNPs (***M**anilkara **z**apota **l**eaf-derived*), AMFAgNPs (***A**nnona **m**uricata **f**ruit-derived*) and AMRAgNPs (***A**nnona **m**uricata **r**oot-derived*). The characteristic absorbance profile of AgNPs, particularly in the range of 420-450 nm was used as an indicator of reduction of Ag^{+} to metallic Ag, was confirmed by Ultraviolet-vis spectroscopy. FTIR (Fourier-transform infrared) spectroscopy analysis confirmed that the three extracts - MZL, AMF and AMR possessed the biochemical components such as proteins and other functional groups capable of performing dual functions of reduction encapsulation and stabilization essential for biogenesis of silver nanoparticles. Further, XRD (X-ray diffraction) patterns confirmed the cubic crystalline nature and size range of the nanoparticles. Additional confirmation on details of the size, shape and morphologies of the particles were obtained by FESEM (Field-emission scanning electron microscopy) imaging. The presence and quantification of elemental silver and the detection of plant-derived carbon, oxygen, phosphorous and chlorine in the nanoparticles were achieved using EDX (Energy-dispersive X-ray). FESEM images revealed the presence of predominantly uniform, spherically-shaped nanoparticles averaging 28.59 ±3.0 nm for MZLAgNPs, 21.36 ± 3.4 nm for AMFAgNPs and 31 ± 3.7 nm for AMRAgNPs. Notably, these values were found to be closely similar to those determined by the XRD technique.

Qualitative and quantitative phytochemical analysis revealed that significantly large amounts of up to 80% of phenolics,were found associated with MZLAgNPs. Interestingly, despite the presence of much lower amounts of phenolics in the AMF extract (51%), upto 95% of it was found to be associated with AMFAgNPs. AMRAgNPs, on the other hand were found to contain the lowest phenolic content (75%), compared to that found in the extract. The highest flavonoid content was found in AMRAgNPs with 410±0.02 mg QE/g of the nanoparticles followed by that in AMFAgNPs (250±1.58 mg QE/g) and MZLAgNPs (190±0.5 mg QE/g). Antioxidant assays revealed that MZLAgNPs possessed the highest hydroxyl / DPPH scavenging and metal ion chelation capacities. AMFAgNPs, on the other

hand, were found to possess the highest NO scavenging and reducing power. Among the three biogenic nanoparticles, AMRAgNPs relatively displayed the highest antioxidant activity attributable to the capping agents such as phenols or flavonoids.

All the three types of AgNPs displayed antibacterial activity against the four clinical pathogenic strains – *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* - tested in the study. Incidentally, *P.aeruginosa* exhibited maximum sensitivity towards all the three types of nanoparticles. The MIC and MBC values obtained ranged from 7- 50 µg/mL. Interestingly, the plant extracts *per se* did not exhibit any antibacterial activity.

Cytotoxicity evaluation of biogenic AgNPs using three cancer cell lines, colorectal carcinoma (HCT 116), cervical carcinoma (HeLa) and non-small lung carcinoma (A549), revealed that all three AgNPs displayed the highest cytotoxicity against HCT 116 cells in comparison to the other two cell types. The toxicity was found to vary in a cell type - specific manner. Interestingly, these variations were found to be similar to those obtained with the standard chemotherapeutic compound, cisplatin, employed as a drug control for comparison. Of the three cell types, Hela and A549 cells responded in a similar fashion to the nanoparticles. The results of nanoparticle toxicity assays employing normal human peripheral blood lymphocyte cultures and erythrocytes revealed that all particle types were non-toxic within experimental limits. Cisplatin, on the other hand, was found to be cytotoxic to these normal cells.

The results of the detailed experiments carried out to elucidate the cellular and molecular effects / mode of anticancer activity of the biogenic AgNPs against HCT 116 and A549 cells are summarized as follows : clonogenic and scratch assays confirmed the reduction in colony formation capacity and inhibition of cell migration, comparable to that of cisplatin. MZLAgNPs were found to be the most potent in inhibiting cell migration with respect to both cell types, with a two-fold higher inhibition against HCT 116 compared to that against A549 cells. Interestingly, MZLAgNPs treated HCT cells showed greater inhibition of cell migration than that obtained with cisplatin. AgNP-treated cells displayed apoptosis

related cytomorphological alterations such as cell shrinkage and membrane blebbing as evidenced by light and scanning microscopy. Flow cytometric analysis revealed the accumulation of cells in G_0/G_1 phase consequent to cell cycle arrest induced by nanoparticles. Increase in the sub-G_1 population of AMRAgNPs treated HCT cells was also clearly indicative of apoptosis induction. The expression profile of the apoptosis-associated proteins such as PUMA, caspase-3, -8, -9, Bax and Bcl-2 further confirmed apoptosis induction. The results of transcription profiling revealed that all three nanoparticles induced higher expression of the proapoptotic genes PUMA and caspase-3 in HCT as well as A549 cells than obtained with cisplatin. Likewise, increased levels of ROS, mitochondrial membrane depolarization observed in cells combined with the presence of p53 and p21, cleaved PARP, caspase-3, -9, on western blots unambiguously confirmed occurrence of cell cycle arrest and mitochondrial apoptosis in both cell types.

FUTURE PROSPECTIVES

Improvement in nanoscale technologies including nanomedicines has paved the way for emergence of new vistas towards revolutionizing the fundamentals of disease diagnosis, treatment and prevention. They have the potential to transform molecular discoveries arising from genomics and proteomics analysis into widespread benefits for patients. The flare-up of emerging and re-emerging epidemic diseases continues to be a substantial burden on worldwide economies and public health. Broad spectrum bioactivities of nanoparticles make them promising agents not only in fighting infections but also in tackling serious problem of tumors and particularly, multi-drug resistant cancers.

The present study has been successful in the generation, characterization and unraveling of the cellular and molecular action as well as the therapeutic potential of phyto-mediated silver nanoparticles as evidenced by an impressive array of cytotoxic and molecular assays. This is the first study utilizing extracts from the leaves of *Manilkara zapota* as well as the fruits and roots of *Annona muricata* to generate silver nanoparticles which effectively target colorectal carcinoma HCT 116 and lung carcinoma A549 cells without having any damaging effects on normal human cells. These results assume importance since the conventional cancer treatment strategies such as surgery, radiotherapy and use of chemotherapeutic drugs have gained notoriety due to lack of cell-specificity and harm caused to normal human cells. More studies like the present one are required to explore full potential as well as the versatility of 'biogenic medicine' to overcome some of the most challenging impediments to human health.

Recent advances in the development of multifunctional nanoparticles having more than one-end application – diagnosis and therapy with single conjugate – has added a new dimension in translational applications of nanomedicine. In this context it is worthwhile to note that in a tropical country like India with a vast biodiversity of plant resources including ancient wisdom associated with medicinal plant use, tremendous potential exists for labscale generation of nanoconjugates of potent phytopharmaceuticals. Such studies can then be extended to animal experiments followed by clinical trials and commercial production of efficacious 'green', ecofriendly drugs.

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