

EVALUATION OF ANTI-CANCEROUS EFFICACY AND TOXICITY OF C₆₀ FULLERENE NANOPARTICLES IN PNEUMOCYTES

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

by

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(DEEMED TO BE UNIVERSITY)**

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This is to certify that this Project Report is the bonafide work of Britlin Deva Jebasta. N (39230008) who carried out the project entitled "EVALUATION OF ANTI-CANCEROUS EFFICACY AND TOXICITY OF C₆₀ FULLERENE NANOPARTICLES IN PNEUMOCYTES" under our supervision from January 2023 to May 2023.

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ABSTRACT

In recent years, cancer is one of the leading causes of death worldwide. In particular, lung cancer is one of the most common types of cancer due to increased smoking and pollution. C₆₀ Fullerene, an allotrope of carbon, is highly beneficial in the treatment of cancer in both pristine and doped forms. It is highly antioxidant and has anticancer properties. It has shown high efficiency in targeted drug delivery, as it can pass through cell membranes and the blood-brain barrier. In addition, their spherical shape is less likely to damage healthy tissues than other drug-delivery vehicles. For further implementation of C₆₀ into cancer therapeutics, its anticancer activity and toxicity needs to be evaluated. The present study evaluates anti-cancerous efficacy, comparative toxicity on cancer and normal cells, and the differential toxicity based on the preparation method of C₆₀ Fullerene aqueous solution. The physical and chemical characteristics of C₆₀ nanoparticles was analyzed and confirmed using various characterization and spectroscopic techniques. The cytotoxic effect of C₆₀ fullerene on A549 cancer cells and Vero cells was assessed by MTT assay. The production of Reactive Oxygen Species was analyzed by DCFH-DA assay. The influence of C₆₀ on cancer cells was analyzed by dual staining using FITC and PI. The binding affinities of cancer-related proteins and C₆₀ fullerene were obtained by Molecular Docking using PyRx. The results from this study concluded that pristine C₆₀ is selectively toxic towards cancerous cells. The antioxidant assay proved the high production of ROS in cancer cells, which may be used as a new approach in the treatment of cancer. Toluene-based preparation of C₆₀FAS was determined to be the most efficient technique to solubilize C₆₀ nanoparticles for therapeutic purposes. Combined, the results suggested that C₆₀ Fullerene nanoparticles are a good candidate for lung cancer treatment as an anticancer drug.

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LIST OF ABBREVIATIONS

ARDS	Acute Respiratory Distress Syndrome
ATP	Adenosine triphosphate
A549	Human lung carcinoma epithelial cell line
BAL	Bronchoalveolar lavage
BBB	Blood Brain Barrier
CCRF-CEM	Human T lymphoblasts
C ₆₀ FAS	C ₆₀ Fullerene Aqueous Solution
Cis-Pt	Cisplatin
COPD	Chronic Obstructive Pulmonary Disease
COVID	Corona Virus Disease
DCFH-DA	Dichlorodihydrofluorescein diacetate
DEN	Diethylnitrosoamine
2 Θ	Diffraction angle
DMBA	7,12-Dimethylbenz[a]anthracene
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
FBS	Fetal Bovine Serum
FE-SEM	Field-Emission Scanning Electron Microscopy
FITC	Fluorecein isothiocyanate
FTIR	Fourier-transform infrared spectroscopy
FWHM	Full width at Half Maximum

GST	Glutathione S-Transferase
HCC	Hepatocellular carcinoma
HA	Hyaluronic Acid
HEK293	Human Embryonic Kidney cell line
HepG2	Hepatoblastoma cell line
HL-60	human leukaemia cell line
HRTEM	High-Resolution Transmission Electron Microscopy
IC ₅₀	Half-maximal inhibitory concentration
J	Joules
LC3A	LC3/Atg13 LIR complex
LB	Lower bound
LED	Light Emitting Diode
LLC	Lewis lung carcinoma
MCF-7	human breast cancer cell line
MDA-MB231	Epithelial human breast cancer cell line
MDR	Multidrug Resistance
MG-63	Human osteoblast-like cell
MHC	Major Histocompatibility Complex
MTT	3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NBO	Natural Bond Orbital
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NADPH	Nicotinamide adenine dinucleotide phosphate
NCCS	National Centre for Cell Science
Nps	Nanoparticles

nm	Nanometre
OD	Optical density
PBS	Phosphate buffer saline
PDT	Photodynamic Therapy
PEG	Polyethylene glycol
PHF	Polyhydroxy Fullerene
PI	Propidium Iodide
ROS	Reactive Oxygen Species
RMSD	Root Mean Square Deviation
RTT	Radiofrequency Thermal Therapy
SDT	Sonodynamic Therapy
UB	Upper Bound
UV-Vis	Ultraviolet-visible spectroscopy
US	Ultrasound
Vero	African Monkey kidney tissue
XRD	X-Ray Diffraction
4T1	Mammary carcinoma cell line
TNF	Tumour Necrosis Factor
W	Watts
ζ	Zeta

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CHAPTER 1

INTRODUCTION

Globally, cancer is one of the major causes of mortality in recent days. Cancer is the uncontrolled growth and proliferation of cells forming a tumour, which gradually undergoes angiogenesis and metastases to nearby organs. In particular, Lung cancer is one of the most common types of cancer due to increased smoking and pollution(Wood *et al.*, 2014). Chronic obstructive pulmonary disease (COPD) also increases the risk of lung cancer(Durham & Adcock, 2015). Despite tremendous scientific advances in understanding the mechanisms that lead to cancer, the therapy's efficacy remains limited.

Currently, available cancer treatment options include surgical treatment, chemotherapy, radiation therapy, or an amalgamation of these methods based on the stage of cancer. The main mechanism by which traditional chemotherapy kills proliferating cancer cells is by interfering with DNA synthesis and mitosis. The anticancer agents used in chemotherapy cause unwanted and unfavourable adverse effects, such as nausea and appetite loss. As anticancer drugs are non-specific, can harm healthy normal tissues as well. One of the causes of the increased death rate among cancer patients is the severe health consequences that chemotherapy medications have on healthy tissues and organs. This is a result of increased toxicity in normal cells and a rise in the occurrence of chemoresistance(Senapati *et al.*, 2018).

However, the rapid development of new technologies offers new opportunities to improve the efficiency of cancer treatments. Nanotechnology has been increasingly studied as a potential cancer treatment in recent years. Tiny bead structures called nanoshells are used in tumour targeting(Anajwala *et al.*, 2010). Nanoshell features exterior metallic layers that target cancer cells and destroy them while leaving healthy cells undisturbed(Bhakta & Barthunia, 2020). Nanobots are small devices programmed to detect, analyse and kill cancer cells(Sharma, 2012). The nanocarriers are useful in targeted drug delivery for cancer treatment. A nanocarrier's effectiveness is influenced by its biodegradability, bioavailability, circulation stability, and dispersion. An effective targeted drug-delivering agent or a

medication with anti-cancerous activity and less toxicity can be helpful in the treatment of lung diseases and malignancies.

Fullerene, an allotrope of carbon is found to be useful in cancer treatment in form of pristine, doped and as drug and gene carriers. Fullerenes or buckyballs are a family of molecules made up of carbon atoms arranged with 20 hexagons and 12 pentagons(Madannejad *et al.*, 2019), with fused rings(Chen, Mao, and Liu 2012) and conjugated double bonds(Augustine *et al.*, 2017)(Fig 1.1).

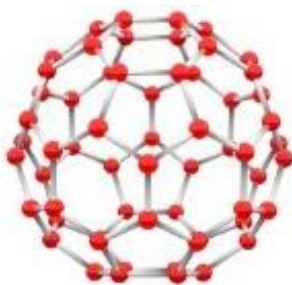


Fig 1.1: Arrangement of C- atoms in C₆₀ fullerene (created using Canva software).

They are highly symmetrical and extremely stable. C₆₀ fullerenes can pass through cell membranes and blood-brain barriers(BBB). Fullerenes are bioresorbable and readily enter cells, which makes them attractive candidates for tumour therapy. Fullerene with amphiphilic and self-assembly characteristics (Chen & Elimelech, 2006) which is highly desirable for efficient drug delivery and tumour imaging(Naz *et al.*, 2019). Fullerenes in pristine and doped forms have high antineoplastic efficiency(Skivka *et al.*, 2018). In addition, their spherical shape means that they are less likely to damage healthy tissues than other drug-delivery vehicles that are commonly used in chemotherapy. Depending on whether the molecule has a surplus or shortage of unpaired electrons, fullerenes can act as both donors and acceptors of unpaired electrons, attaching them to the double bonds(Shytikov *et al.*, 2021). Fullerenes are biocompatible with strong free radical scavenging and antioxidant potential (Shytikov *et al.*, 2021)(Svitlana Prylutska *et al.*, 2014).

Nanoconjugates of fullerene and anticancer drugs are proven to be more effective at reducing toxicity, enhancing efficacy, and controlling drug release. The

bioavailability of the drug has been significantly improved by fullerenes. In addition, their spherical shape means that they are less likely to damage healthy tissues than other drug-delivery vehicles. C₆₀ due to its cage-like structure makes it useful in drug delivery applications. Due to their adaptability, nanoparticle-based drug delivery systems have emerged as the perfect platform for the treatment of cancer. As its goal is to restrict the proliferation of tumour cells, the nano-carrier has demonstrated great potential in the treatment of lung cancer(Sharma *et al.* 2019). Fullerene as a nanocarrier of anticancer drugs including cisplatin, gemcitabine, docetaxel, paclitaxel, doxorubicin, landomycin, fluorouracil etc... has shown a tremendous decrease in toxicity *in vivo* and *in vitro*. Research on delayed drug release with fullerene nanovesicles has synthesized three amphiphilic functionalized fullerenes C₆₀R₅Cl (R = methyl ester of 4-aminobutyric/glutamic acid or phenylalanine) for effective drug release through regulation of vesicle polarity and size by amino acid methyl ester groups(Lin *et al.*, 2017). There are several possible approaches to cancer treatment with fullerenes and their nanoconjugates as anticancer agents.

With such tremendous applications, the toxicological aspect of fullerene also needs to be analyzed. When present in high quantities, fullerenes are expected to have inflammatory, cell-damaging, and carcinogenic capabilities, giving them a "two-edged sword" quality. The pristine fullerene exhibits significantly superior safety. Following pulmonary exposure, C₆₀ fullerenes seem to be capable of inducing localized reactions that are neither inflammatory nor toxic in character, but rather anti-inflammatory. During the inhalation exposure period, C₆₀ fullerene particles have shown no significant inflammation or tissue injury. Meanwhile, the immune system-related genes have been up-regulated by particles of C₆₀ fullerene(Fujita *et al.*, 2009).

Acute respiratory distress syndrome (ARDS) is characterized by the death of lung epithelial cells. ARDS is a leading cause of death in respiratory infections such as COVID-19, a disease caused by the SARS-CoV-2 virus. It has been shown that fullerene nanoformulation can be used to regenerate cells treated with apoptosis-inducing molecules, implying its potential for ARDS therapy(Nabodita Sinha and Ashwani Kumar Thakur, 2019).

CHAPTER 2

LITERATURE SURVEY

2.1 PROPERTIES OF C₆₀

The third carbon allotrope, fullerene (C₆₀), has distinct photochemical, electrochemical, and physical properties(Y. Yan *et al.*, 2020). Although C₆₀ contains 240 valence electrons, there are three sigma bonds between each carbon atom, with 180 electrons. Carbon atoms in fullerenes are strongly pyramidalized and accordingly very reactive due to their spherical form. Through the orbital rehybridization of the sp² orbitals to the sp^{2.27} orbitals with a p character gain, the conjugated carbon atoms depart from planarity(Castro *et al.*, 2017). These electrons' energy is in the subsurface of the Fermi surface. They help in structure stabilization but not in conduction. The remaining 60 electrons are distributed to carbon-carbon π orbitals around the molecule(Forro & Mihaly, 2001). This closely packed structure has large voids between the ions, atoms or molecules. The unsaturated character of the C–C bonds on the fullerene enables various electronic states to accept electrons from appropriate donors. Due to its inclination to avoid double bonds in pentagonal rings, it has poor electron delocalization. As a result, C₆₀ behaves like an electron-poor alkene and interacts easily with electron-rich molecules(Gokhale & Somani, 2006).

Alkali and alkaline earth elements combined with fullerenes provide a unique range of MnC₆₀ materials, which are primarily near-stoichiometric in nature (M can stand for Na, K, Rb, Cs Ca, Sr and Ba). Charge transfer salts with fullerenes were made using both organic and inorganic compounds. Doped fullerene refers to a small, non-stoichiometric amount of charge transfer by adding a metal ion on or into the fullerene. Conduction bands are filled when C₆₀ is doped with n-type alkali metals(Romberg *et al.*, 2003). The C₆₀ molecule's cage-like structure naturally provides two alternatives for doping: by adding a foreign atom M inside the cage (or) by swapping out one or more carbon atoms for atoms with different electrical structures (Forro & Mihaly, 2001) (Fig 2.1).

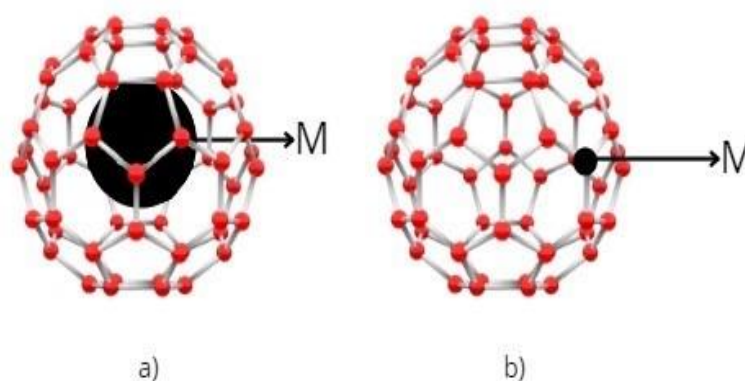


Fig 2.1: Illustration of two alternative approaches of doping; a) Addition of foreign atom M inside the cage; b) Addition of foreign atom M by swapping one C atom (created using Canva software).

2.2 ANTI-CANCEROUS ACTIVITY OF FULLERENE C₆₀

2.2.1 Pristine C₆₀

The hydrophobicity, difficulty in obtaining a suitable concentration, and aggregation in the water of pristine C₆₀ fullerene restrict the research on its biological effects (Svitlana Prylutska *et al.*, 2014). The water-soluble, pure C₆₀ fullerenes can pass through cell membranes and are highly antioxidant. At low physiological quantities, they are non-toxic. C₆₀ fullerenes have significant antioxidant activity, which can block certain cell receptors and neutralise excess ROS in the cell (S. V. Prylutska, Burlaka, Prylutsky, *et al.*, 2011). C₆₀ fullerene has been reported to improve animal survival in a rat HCC model produced by DEN + CCl₄ and to suppress fibrotic and neoplastic degeneration and metastasis. The antioxidant capabilities of C₆₀ fullerene, such as its capacity to restore liver redox status and cytostatic effects, were the reason for better results. The ability of C₆₀ fullerene to decrease *G6PD* and upregulate the expression of *p53* has been reported as the reason for decreased metastasis (Kuznietsova *et al.*, 2020). According to a study, C₆₀ fullerenes have significant antioxidant activity, neutralising excess ROS in cells and potentially inhibiting some cell receptors, such as endothelial growth factor. C₆₀FAS therapy has been demonstrated to reduce Lewis lung cancer tumour volume in male C57Bl/6J mice (S. V. Prylutska, Burlaka, Klymenko, *et al.*, 2011).

Treatment of DMBA-induced breast cancer with pristine C₆₀ Nps has shown encouraging results when tested with Wistar albino female rats. Treatment of MCF-7 cells *in vitro* with fullerene C₆₀ at varying concentrations (0.1 to 100 mg/ml) has shown decreased cell survival in a dose-dependent manner. *In vivo*, treatment with fullerene C₆₀ on rats has shown significantly elevated levels of Caspase-3 but reduced levels of pro-survival protein leading to DNA fragmentation (Beyaz *et al.*, 2022).

In BAL cells, C₆₀ fullerenes have increased sub-G1 and G1 arrest. Moreover, over the course of the experiment, lung tissue cell infiltration and the expression of genes related to tissue damage have been continuously observed confirming that C₆₀ fullerenes cause inflammatory reactions in the lung of the mouse (Park *et al.*, 2010).

2.2.2 Doped C₆₀ as a drug and gene carrier

C₆₀ has been proven to be compatible with mammalian cells for carrying drugs (Lucafò *et al.*, 2012). Nanoconjugates of fullerene and anticancer drugs are proven to be more effective at reducing cancer cell toxicity, enhancing efficacy, and controlling drug release. The simultaneous administration of the drug and fullerene has proved that the drug is protected from the reactive environment (Bulavin *et al.*, 2018). C₆₀ in both pristine and doped forms can carry a drug as conjugate. The bioavailability of the drug has been proven to be significantly improved by fullerenes (Raza *et al.*, 2015).

Few studies suggest that doped fullerene has a higher affinity in carrying drugs than pristine fullerene. The complexation of anti-cancer drugs with C₆₀ fullerene has increased their toxicity towards several human tumour cell lines, including those with distinct mechanisms of drug resistance (R. Panchuk *et al.*, 2017). The interaction of 4-Phenylpyridine (4-ppy) with B or Si-doped C₆₀ fullerene and undoped fullerene, has revealed that doped fullerene had a higher binding affinity to carry the drug (Gökpek *et al.*, 2017). The study on the interaction of penicillamine with Al-doped and undoped fullerene has shown that the binding energy of the Al-C₆₀-NH₂ complex is much stronger than that of pristine C₆₀ (Ghasemi *et al.*, 2018). The doping of C₆₀ fullerene with alkali metals has shown greater adsorption with fluorouracil molecules and easy release of the drug, near the cancer cells (Esrafil & Khan, 2022). The doping of silicon atoms to the C₆₀ fullerene has been

demonstrated to improve the adsorption mechanism. By examining the band changes in the carbonyl stretching vibrations and the natural bond orbital (NBO) features of the investigated complexes, the impact of favipiravir adsorption on the SiC₅₉ fullerene system and the nature of interaction have been evaluated (Parlak *et al.*, 2017). The C₆₀-serPF conjugate has been found in most tissues in a mouse model of liver cancer, passing undetected via the BBB and the tumour's altered vasculature while bypassing the reticuloendothelial system (Raouf *et al.*, 2012). Compilation of different drugs that use fullerene as a drug carrier is tabulated in Table 2.1.

Table 2.1: Compilation of various drugs using C₆₀ as a drug carrier

Drug	Type of analysis	Model	Effect of C ₆₀	Reference
Berberine	<i>In vitro</i>	CCRF-CEM cells and LLC cells	Greater antiproliferative potential	(Grebinyk, Prylutska, Buchelnikov, <i>et al.</i> , 2019) (Grebinyk <i>et al.</i> , 2021)
Cisplatin	<i>In vitro</i>	Hela cells	Increase in tumour growth inhibition	(Bychko <i>et al.</i> , 2016) (Svitlana Prylutska <i>et al.</i> , 2017)
	<i>In vitro</i>	HL-60/adr and HL-60/vinc tumour cells	Increase in drug cytotoxicity towards cancer cells including cisplatin-resistant sublines.	(Dzhemilev <i>et al.</i> , 2019)
	<i>In vitro</i>	LLC cells	enhanced Cis-Pt entry and intracellular accumulation,	(Svitlana Prylutska <i>et al.</i> , 2019)

			boosting the drug's cytotoxic effects	
Docetaxel	<i>In vitro</i>	MCF-7 and MDA-MB231 cell lines	Improved cytotoxicity against cancer cells and high compatibility with erythrocytes	(Raza <i>et al.</i> , 2015)
Doxorubicin	<i>In vivo</i>	Mice with LLC	Reduction in tumour volume	(Svitlana V. Prylutska <i>et al.</i> , 2015) (R. R. Panchuk <i>et al.</i> , 2015)
Fluorouracil	<i>In vivo</i>	Rats with Colon lesions	Decrease in the number and the size of lesions	(Lynchak <i>et al.</i> , 2017)
Gemcitabine	<i>In vitro</i>	Pancreatic cancer cell lines	Increase in production of ROS	(Nalepa <i>et al.</i> , 2020)
Landomycin	<i>In vitro</i>	MG-63, 4T1 and Mesenchymal stem cells	high toxicity for cancer cells, but not for the mesenchymal stem cells	(Bilobrov <i>et al.</i> , 2019)

Fullerene has shown tremendous potential in disease prevention and therapy by delivering exogenous nucleic acids such as DNA into cells, known as transfection. It has been reported that any water-soluble amino-fullerene could bind to double-stranded DNA. Tetrapiperidinofullerene made from fullerene, piperazine, and oxygen is the most effective in transfection. The fullerene derivative demonstrated significantly better efficiency for stable transfection and a stronger potential to transfect confluent cells as compared to the conventional approach (Z. Chen, Mao,

et al., 2012). The research on fullerene/DNA interactions led to the identification of tetraamino fullerene as a promising option for a fullerene method of gene transfer to mammalian cells. This cationic compound adheres to duplex DNA, delivers the fullerene/DNA complex into the cytoplasm and allows the encoded gene to be expressed either transiently or steadily with efficiency comparable to or higher than that achieved by lipofection(Djordjević *et al.*, 2006). The DNA ingested by endosomes were protected from enzymatic digestion by fullerene. Water-soluble lysine-based C₆₀-fullerene nanoconjugates (CF-LYS-TEG-MMF) have been discovered to have significant biocompatibility with erythrocytes and enhanced cytotoxicity on neuroblastoma cells. A pH-based drug release pattern was included in the developed nanoconjugate, which reduced drug leaching at plasma pH (Kumar *et al.*, 2018). Even though these C₆₀-based transfection vectors of the first generation had promising potential, some of them also showed substantial cytotoxicity.

2.3 VARIOUS APPROACHES USING FULLERENE C₆₀ IN CANCER TREATMENT

The photodynamic effect, anti-angiogenesis, efficient immunomodulation, control of oxidative stress, induction of autophagy, and sonodynamic effect are all different approaches for treating cancer with fullerene(Z. Chen, Mao, *et al.*, 2012)(Z. Chen, Ma, *et al.*, 2012). Following exposure to C₆₀ fullerene, immune cells have shown increased expression of the genes related to oxidative stress, metalloendopeptidase activity, the inflammatory response, and MHC. In rats with experimental adjuvant-induced arthritis, C₆₀ fullerene has inhibited pro-inflammatory (classically)-activated macrophages. With or without photoexcitation, C₆₀ Fullerenes can both quench ROS and generate it. C₆₀ Fullerenes also induce oxidative metabolism. A combination of direct effects on tumour cells, effects on the tumour microenvironment, and host immune system activation constitute the fundamental mechanism of the C₆₀ fullerene-based cancer treatment (Skivka *et al.*, 2018).

A study has revealed a double-strike approach against cancer cells based on Dox's antiproliferative activity and C₆₀'s photoinduced pro-oxidant activity(Grebinyk,

Prylutska, Chepurna, *et al.*, 2019). *In vivo* and *in vitro* experiments on the on-off mechanism using C₆₀-DOX-NGR NPs have shown its capacity to target tumours, remote-control drug release capability, integrated treatment effect, high antitumor efficacy and low toxicity to normal tissues (Shi *et al.*, 2016).

2.3.1 Photodynamic therapy

PDT combines laser light, molecular oxygen, and photosensitizer, to cause oxidative stress by the light of a certain wavelength to eradicate the tumour. Each of these elements is safe by itself, but when combined, they can produce ROS that can kill cancer cells. PDT is a non-invasive treatment approach (Juarranz *et al.*, 2008). The photosensitizer is a molecule which often absorbs a photon that excites an outer shell electron to a singlet state (S₁), after which it can either return to its ground state or undergo intersystem crossing to a triplet state. Fullerenes form ROS upon illumination due to the absorption of visible light and an effective intersystem crossing to a long-lived triplet state, making them photosensitizers. The triplet state reacts with oxygen to produce ROS including free radicals or singlet oxygen species (Y. Yan *et al.*, 2020) (Dos Santos *et al.*, 2019).

ROS is an aggressive chemical oxygen species that can quickly react with any adjacent biomolecules, killing any nearby cells through apoptosis or necrosis. This includes both normal and pathological cells. Free radicals produced from fullerenes can be produced by two key mechanisms, both of which have anti-cancer effects. In type 1 photochemical mechanisms, Fullerenes receives electrons from reducing agents like NADH or NADPH that are present in the cell causing them to contain an additional unpaired electron and behave like a radical anion C₆₀^{•-}. This transfers an electron to oxygen during the reaction, creating ROS like superoxide or hydroxyl radical. In a type 2 reaction, energy is directly transferred to the oxygen molecule to create exceptionally cytotoxic singlet oxygen (Benedicta *et al.*, 2022). The mechanism of the photodynamic effect is illustrated in Fig 2.2.

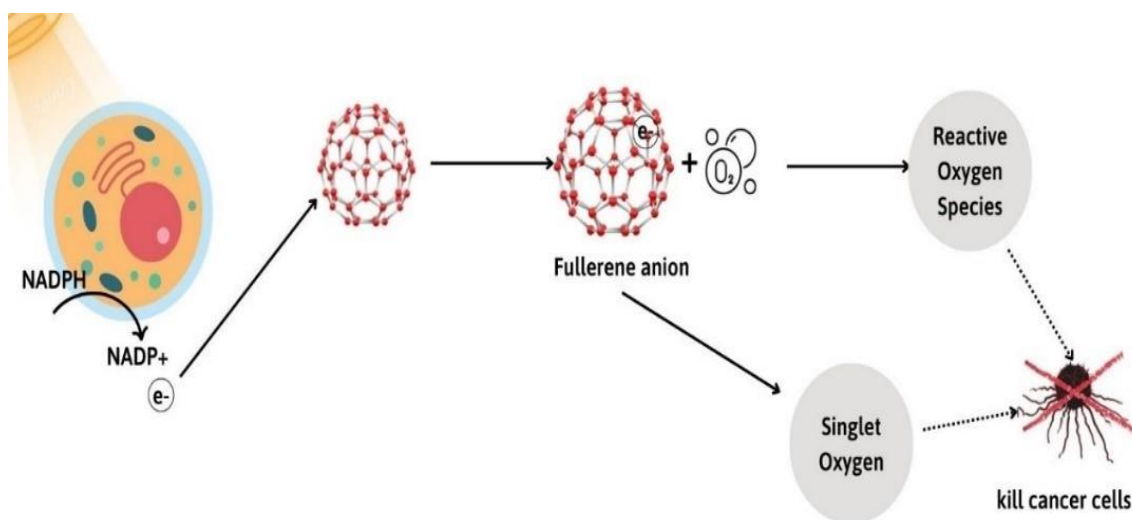


Fig 2.2: The mechanism of the Photodynamic effect by fullerenes on cancer cells (created using canva software).

Photosensitizer that is selectively localized in the target tissue, activated by a specific wavelength of light produces ROS and resulted in photodamage and subsequent cell death causing tumour ablation(Guan *et al.*, 2016). PDT can cause three pathways of cell death including apoptosis, necrosis and autophagy-mediated cell death(Agostinis *et al.*, 2011).

Photosensitizers can be classified as porphyrins (tetrapyrrole) and non-porphyrins (non-tetrapyrrole). Fullerene–porphyrin complex is highly efficient in singlet oxygen generation and cell penetration(Constantin *et al.*, 2010). C₆₀ modified with polyethene glycol (PEG) is reported to have the ability to accumulate in tumour tissue and have a photodynamic impact on tumours. The volume of the tumour has significantly reduced after intravenous injections of C₆₀-PEG conjugate into tumour-bearing mice, along with the exposure of visible light to the tumour site. The C₆₀ conjugation has also demonstrated a better anticancer impact than photofrin. It has been observed that the largest inhibition of tumour growth was seen in the C₆₀-PEG conjugates made with the highest molecular weight methyl-terminated PEG. This conjugate displayed the longest half-life period in blood circulation and the maximum tumour accumulation(Shi *et al.*, 2014).

Radiofrequency absorbing substances are used in radio frequency thermal therapy (RTT) to transform RF irradiation energy into heat, which causes the thermal

ablation of cancer cells. MCF-7 cells and tumour-bearing mouse models have been used to assess the PDT, RTT, and tumour-targeting effectiveness of C₆₀eIONPePEGeFA. Additionally, C₆₀eIONPePEGeFA is also useful for *in vivo* imaging. Using the outstanding active tumour targeting and magnetic targeting capabilities of C₆₀e IONPePEGeFA, cancer cells can be selectively targeted in highly concentrated areas. C₆₀e IONPePEGeFA has demonstrated good PDT, RTT efficacies, tumour targeting properties, and tumour diagnostic ability *in vitro* and *in vivo* (Y. Yan *et al.*, 2020). The *in vitro* and *in vivo* studies of the C₆₀-IONP-PEG/HMME complex have shown excellent PDT efficacy, magnetic targeting property and MRI ability when tested with mice-bearing tumours (Shi *et al.*, 2013).

High solubility sugar derivatives of C₆₀ are also proven to eradicate cancer cells with exposure to light. Glyco-conjugated C₆₀, when illuminated has worked as photoactive sweet C₆₀(Rondags *et al.*, 2017) by targeting pancreatic stellate cells of pancreatic adenocarcinoma(Serda *et al.*, 2018). A water-soluble polysaccharide known as pullulan with a high affinity for asialoglycoprotein receptors has been chemically combined with C₆₀ to provide a novel cytospecific photosensitizer for photodynamic anticancer therapy. In comparison to HeLa cells lacking asialoglycoprotein receptors, the C₆₀-pullulan conjugates greatly reduced the proliferation of HepG2 cells. Additionally, compared to C₆₀-PEG conjugates and saline, C₆₀-pullulan conjugates have shown a significantly higher photodynamic effect on the HepG2 neoplasm mice(Liu & Tabata, 2010).

In vitro, investigations show that the malonic acid derivatives of C₆₀ also exhibit considerable cytotoxicity and visible photosensitive action. The synthesized fullerene-maleic anhydride compound had stronger anticancer effects on HeLa cells and bone tumour cells. After being injected with the derivatives and lit with an I-W light, the tumour *in vivo* suffered significant damage (500 W)(Z. Chen, Mao, *et al.*, 2012). According to a study, C₆₀ Nps activate *p38MAPK* by promoting the formation of ROS, which causes cancer cell damage(Z. Li *et al.*, 2013). A study has concluded that HA-C₆₀ could produce a significant amount of ROS during PDT(Hou *et al.*, 2017). A study has confirmed that C₆₀ fullerene and high-power LEDs with a 405 nm wavelength could be used as the light source for PDT on cancer cells. When C₆₀ fullerene's potential phototoxicity was evaluated using LEDs of various wavelengths on human leukemic cells, the most beneficial effect had been

at 405 nm, 10 J/cm²(Grebinyk *et al.*, 2018). When combined with light irradiation, pure C₆₀ had a lethal impact on T-lymphocytes that had transformed. The longer exposure period and higher C₆₀ content increased the photodynamic impact. According to research work, C₆₀-amino acid complexes (C₆₀-phe or C₆₀-gly) are effective PDT-enhancing agents for killing cancer cells by inducing apoptosis upon illumination. The photochemical mechanism may be mediated by both superoxide and singlet oxygen(Z. Li *et al.*, 2014).

PDT has high efficiency with endoscopically accessible tumours including lung cancer, bladder cancer, gastrointestinal cancer and fallopian cancers, and also in dermatology for the treatment of non-melanoma skin cancers (Juarranz *et al.*, 2008). A recent study has revealed the ability of bacterial cellulose(BC)- C₆₀ nanocomposite to treat skin cancer with PDT (Chu *et al.*, 2018).

2.3.2 Sonodynamic therapy

SDT, inspired by PDT, has recently developed as a non-invasive cancer treatment technique that relies on the activation of particular chemical sensitizers with Ultrasound(US). US waves have an acoustic pressure, focussing energy into small volumes and have a suitable tissue attenuation coefficient for permeating biological tissues(Yumita *et al.*, 2016). Another key characteristic of US is its ability to produce sonoluminescence or light emission within an excited medium. It is commonly believed that the SDT mechanism is caused by the cavitation impact of US. When US waves travel through liquids, a peculiar physical process known as acoustic cavitation causes bubbles to form, expand, and then burst. Sonoluminescence results from the bubble explosion, which releases the stored energy.

The combination of the US of 1 MHz and pristine C₆₀ together for the dynamic treatment of cancer cells has induced sonoexcitation of pristine C₆₀ thereby killing carcinoma cells. *In vitro*, tests with HeLa cells have shown a sizable proapoptotic sonotoxic activity(Radivoievyh *et al.*, 2022).

The high correlation in the presence of Polyhydroxy fullerene shows that membrane lipid peroxidation is an important intermediary step in sonodynamically produced cellular damage. The inhibitory effects of histidine and tryptophan have shown that singlet oxygen plays a significant role in Polyhydroxy fullerene-mediated

membrane sonosensitization and sonodynamic cell lysis(Yumita *et al.*, 2014). The combination of US exposure and PHF treatment has inhibited the growth of implanted colon cancer cells(Yumita *et al.*, 2013).

2.3.3 Reversing chemoresistance

The treatment of tumours in healthcare facilities is frequently hindered by drug resistance, often reduces the accumulation and activation of the drug increases the drug-induced damage repair, changes drug targets and gene expression, destroys anatomical drug barriers, and results in the treatment of cancer not being successful(Z. Chen, Mao, *et al.*, 2012). Chemotherapy resistance has been classified into two types: noncellular and cellular. Non-cellular resistance is a term used to describe extracellular elements like the tumour micro conditions and the reachability of constrained vascular systems. The cellular resistance mechanism is further divided into conventional transport-based and non-transport-based resistance. The transport-based cellular resistance mechanism includes ATP-binding cassette transporters, like P-glycoprotein (P-gp), breast cancer resistance protein, Multidrug resistance (MDR)-associated protein, and solute carrier proteins. The non-transport-based cellular resistance mechanisms are related to changes in specific enzymatic activity and several cell death systems, particularly type I programmed cell death - apoptosis and type II programmed cell death - autophagy(C. Chen *et al.*, 2018).

Following prolonged chemotherapy, MDR frequently develops, leading to refractory cancer and tumour recurrence (Y. J. Li *et al.*, 2017). The primary mechanisms involved in the reversal of MDR based on the biological pathways involved include a reduction in drug efflux pumps, a rise in intracellular drug concentration and endosomal escape, disruption of cell membranes and intracellular organelles, and phototherapy(Curcio *et al.*, 2020).

The C₆₀-Dox Complex has demonstrated an increase in its toxicity toward numerous human tumour cell lines, including those with varied drug resistance mechanisms(R. R. Panchuk *et al.*, 2015). The photodynamic impact of C₆₀ has been observed on leukemic L1210 cells that are both sensitive and resistant to cisplatin. After C₆₀ photoexcitation, drug-resistant cell survival decreased, which was closely related to a considerable decline in mitochondrial potential. According to the study,

C₆₀-based PDT may be a viable option for regaining drug-resistant leukemic cells' sensitivity to the induction of mitochondrial death, thereby reversing chemoresistance(Franskevych *et al.*, 2017).

2.3.4 Immunotherapy

Malignant cells have discovered ways to evade the immune system, which allows them to grow into resistant tumours. The surveillance and the prevention of malignancy are the crucial roles played by our immune system. Autophagy is a balanced, destructive degradation process that transports cargo to the lysosome for breakdown. To maintain correct cell function and metabolism, autophagy is crucial for the elimination of defective or dysfunctional organelles and protein aggregates. It also enables the recycling of cellular components(C. Chen *et al.*, 2018). Nps are widely recognised for their interaction and impact on the immune system.

A high dose of nano-C₆₀ fullerene in glioma cells causes necrosis, whereas low doses usually cause autophagy(Sepand *et al.*, 2020). It has been demonstrated that the fullerene C₆₀ nanoparticle (nC₆₀) induces autophagy and makes cancer cells more susceptible to chemotherapy death. In a sequential process of autophagy, phagophores start to envelop and pack organelles to form autophagosomes. Afterwards, autophagosomes and lysosomes combine to generate autolysosomes(Wei *et al.*, 2010). Depending on the conditions, the digestion of the ingested cytoplasmic contents results in the production of small molecules for the synthesis of macromolecules as an energy source, ultimately leading to either cell survival or cell death.

Tumour growth as well as malignant transformation can be inhibited by autophagy. It is understood that spontaneous cancers are frequently caused by a decrease in autophagic capability or faulty autophagy. The importance of autophagy in cancer cells, and how nC₆₀(Nd) improved chemotherapeutic susceptibility via modulating autophagy has been demonstrated(Wei *et al.*, 2010). Compared to Nano-C₆₀, fullerene Nano-C₆₀/C₆₀(Nd) has shown more ability to induce autophagy and improve the chemotherapeutic elimination of both normal cancer cells and chemo-resistant cancer cells(Z. Chen, Mao, *et al.*, 2012). Under conditions of acute colitis and colon cancer, C₆₀ FAS has partially corrected local and systemic morphofunctional abnormalities in the colon, liver, and kidney. Also, it has

exhibited anti-inflammatory and anticancer activities. Increased serum urea and creatinine were brought back to normal levels by C₆₀FAS, indicating that renal function has been restored(Kuznietsova *et al.*, 2017).

The interaction of Nano-C₆₀ with the cellular autophagic signalling system has shown that the *Atg5* gene was the mediator of the chemosensitization effect of Nano-C₆₀. Nano-C₆₀ sensitises chemotherapeutic death of cancer cells in a photo-enhanced and ROS-dependent manner at noncytotoxic doses(Zhang *et al.*, 2009). C₆₀-doped Melanin nanoparticles (MNP) combined with hyaluronic acid have shown effective theranostic and immunotherapy of tumours when excited by near-infrared light. This study is the first to describe the characteristic of encouraging ROS production of C₆₀ by MNP, a natural biomaterial, and MC₆₀H in the application of a coactive anti-tumour approach without the dependence of conventional up-conversion material(J. H. Yan *et al.*, 2020).

2.3.5 Anti-angiogenesis

Angiogenesis is the process of the formation of new blood vessels. Angiogenesis related to tumours is the development of new blood vessels both outside and inside the tumour. Since malignant neoplasms require a sufficient blood supply to support their rapid growth and proliferation, angiogenesis is crucial for the survival of solid tumours(Meng *et al.*, 2010). By elevating angiogenic factors, oxidative stress in carcinoma cells stimulates blood flow to tumours. Hence, anti-angiogenesis is a successful cancer therapeutic approach. By preventing oxidative stress, tumour vascularization can be inhibited(Ye *et al.*, 2021). Gd@C₈₂(OH)₂₂ fullerenic Nps have shown down-regulation of anti-angiogenic properties(Meng *et al.*, 2010).

2.4 TOXICOLOGICAL ASPECTS OF C₆₀

The wide use of nanotechnology has raised concerns about the compound's safety and toxicity after human contact. Several studies have revealed that fullerenes are found to be incredibly advantageous in many ways while possessing toxicity due to various factors such as physicochemical properties, interaction with the environment, pharmacokinetics and pharmacodynamics(Holmannova *et al.*, 2022).

2.4.1 *In vivo analysis*

The capacity of both undoped and doped C₆₀ fullerene to induce inflammatory responses *in vivo* has been identified as one of the potential dangers of chronic fullerene exposure (Trpkovic *et al.*, 2012). A study with freshwater fish, *Anabas testudineus*, has demonstrated the potential of fullerene C₆₀ to cause genotoxicity even at sublethal concentrations (Sumi & Chitra, 2019). In C₆₀ exposure experiments even after 48 hours, *Daphnia magna* were still unable to eliminate C₆₀ from their digestive tracts, exhibiting rapid absorption rates but even slower disposal rates (Freixa *et al.*, 2018). C₆₀ has shown hepatotoxicity and cardiotoxicity in an analysis using rats. The inactivation of Kupffer cells (macrophages of liver cells) upon C₆₀ interaction has been observed (Elshater *et al.*, 2018) (Abd-Elraheim A. Elshater, 2017).

At higher exposure concentrations, increased pulmonary inflammation and cytokine levels have been observed in an experiment involving nose-only inhalation using rats (Etc & Das C, Lucia MS, 2019). It has been demonstrated through research on rabbits that C₆₀ fullerene Nps can alter the mechanical characteristics of the red blood cell membrane, making it "softer" than usual and more brittle (Halenova *et al.*, 2020). C₆₀-exposed earthworms have been shown to have high energy requirements. The analysis using *Eisenia fetida* has observed a decrease in free amino acids which is suspected to be caused due to the high production of enzymes to tolerate the toxicity of C₆₀ (Lankadurai *et al.*, 2015).

2.4.2 *In vitro analysis*

C₆₀Nps have significantly accelerated the growth of melanoma in experimental animals, in contrast to its powerful cytotoxic and antiproliferative effects on melanoma cells (Zogovic *et al.*, 2009). An experiment on the toxicity of the pristine C₆₀ fullerene aqueous colloid solution (C₆₀FAS) with human embryonic kidney (HEK293) cells revealed no toxic effects at concentrations of 75-150mg/kg, yet haematological effects were observed at higher levels (Svitlana V. Prylutska *et al.*, 2019).

Using kidney human embryonic (HEK293) cells, the toxicity of the C₆₀-Cis-Pt nanocomplex in comparison to the undoped Cis-Pt had been investigated. The examined results show that the nano complex with Cis-Pt equivalent concentrations

has no harmful effect. It has been reported that Cis-Pt's toxic effect against normal cells is reduced by its complexation with C₆₀ fullerene(S. V. Prylutska, 2020).

2.4.3 Microbial analysis

Analysis in bacteria using a reversion test and chromosomal aberration test *in vitro* using C₆₀ Nps has shown negative genotoxicity, even with radiation exposure(Shinohara *et al.*, 2009). In the presence of significant amounts of C₆₀-COOH, *Phanerochaete chrysosporium* liquid culture showed a complete loss of decomposition activity, thereby disrupting the carbon cycle(Ming *et al.*, 2018).

CHAPTER 3

AIM AND SCOPE

- To evaluate the physical and chemical characteristics of C₆₀ fullerene Nps.
- To understand the anti-cancerous activity of C₆₀ fullerene on A549 cancer cells *in vitro*.
- To investigate the mode of action and impact of C₆₀ fullerene on A549 cancer cells.
- To compare the cytotoxicity of C₆₀ fullerene based on the different preparation methods *in vitro*.
- To compare the cytotoxic effect of C₆₀ on cancer and normal cells.
- To determine the interaction of C₆₀ fullerene and cancer-related protein using *in silico* analysis.
- The scope of this study is to analyse the anti-cancerous activity and cytotoxicity aspects of C₆₀ fullerene to ensure its safe and efficient therapeutic use in the treatment of various lung diseases and cancer

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS USED

C₆₀ fullerene was purchased from SRL (Sisco Research Laboratories) with 99.99% purity. All chemicals were used in the present study without further purification. A549 lung cancer cell line was purchased from NCCS, Pune. Vero cell lines were used from TANUVAS, Chennai. Double distilled water was used throughout the project.

4.2 METHODS

4.2.1 Physical Characterization

4.2.1.1 UV-Vis

An analytical examination of various types of solvents and compounds is done using a technique known as UV-VIS spectroscopy. The analytical approach is based on calculating how much monochromatic light is absorbed by colourless substances throughout spectrums near the ultraviolet path. The process required to ascertain the "identification, strength, quality, and purity" of such chemicals is included in the pharmaceutical analysis (Verma & Mishra, 2018). C₆₀ fullerene NPs (powder sample) were taken for evaluation and the characteristic absorbance range from 200-500nm was measured in this measurement. Absorption spectra were recorded using a UV-Visible NIR Spectrophotometer (JASCO, V670 from Japan).

4.2.1.2 FTIR

The structural organisation of pristine C₆₀ fullerene in an aqueous solution was investigated using FTIR spectroscopy. FTIR spectroscopic analysis is used to identify significant functional groups present in the sample. (Berthomieu & Hienerwadel, 2009). C₆₀ fullerene NPs were ground with Kbr pellet using a motor pistil. The sample was subjected to Agilent Cary630 FTIR (FTIR instrumentation) at a range of wavenumber 650 to 3000 per cm.

4.2.1.3 Raman spectroscopy

In Raman spectroscopy, inelastic scattering of light is investigated. The substance under inquiry and the incident radiation field exchange energy, which leads to inelasticity. The method offers crucial knowledge regarding the vibrational state of matter. These films' Raman spectra often exhibit two unique peaks known as the D- and G- peaks. The position, width, and intensity ratio of the two peaks are utilised to characterise the thin film material. For instance, an increase in the quantity and/or size of sp^2 clusters are linked to an increase in the I_D/I_G ratio (Petrova *et al.*, 2011). By determining the ratio between these two bands ($I_D: I_G$), a quantitative measure of defect density in the C_{60} surface can be determined. Consequently, $I_D: I_G$ band analysis can be used to obtain information regarding structural changes as a result of functionalization. Prepared powder samples of C_{60} were subjected to Raman spectra and defect intensities of the nanomaterial were evaluated using a 785 nm Micro-Raman Spectrophotometer, (Renishaw, UK, Invia0719-17) in spectral frequency 100 –3,500 cm^{-1} with a spectral resolution of 0.75 cm^{-1} (FWHM).

4.2.1.4 Zeta potential and Particle size analysis

The electrical potential difference between a particle's surface and the surrounding fluid is measured by ζ potential analysis. It is a measure of the electrostatic attraction or repelling force between particles and is affected by variables including pH, ionic strength, and surface charge. Many details regarding the stability, aggregation, and interaction of particles in a solution can be obtained from the size and sign of the ζ potential (Guindani *et al.*, 2020). C_{60} fullerene in toluene was adequately diluted in water to prepare the solution and sonicated for 30 min. Aliquots of the C_{60} FAS, at a concentration of 10 $\mu g/ml$ from a stock [1 mg/ml], were prepared. The C_{60} FAS was subjected to Horiba nanopartica analyser (SZ-100V2 HORIBA, Japan) to analyse the ζ potential and particle size. Data were recorded at 25.1 $^{\circ}C$ in backscattering modus at a scattering angle of $\sim 90^{\circ}$.

4.2.1.5 XRD

X-Ray diffraction (XRD) was used to ascertain the crystallographic properties of the sample. When X-rays are focused on a sample, the resulting diffraction pattern reveals details about the atom arrangement within the substance. The unique arrangement of atoms in the crystal lattice causes X-rays to diffract in various

directions when they interact with a crystalline sample. The atom locations and the distance between them are ascertained by detecting and analysing the diffraction pattern. This data can be used to determine the material's crystal structure, phase composition, and other physical characteristics. XRD analysis was carried out on Thermofisher Model - Equinox 3000. The radiation used was Cu-K α source at 1.8 kW power in a Θ/Θ scan mode with a wavelength of 0.15406 nm to determine the crystalline structure and lattice planes in the material. The samples were scanned at diffraction angles ranging from $2\theta = 5^\circ$ to 80° (the step size is 0.025 degree/step) with an exposure time of 0.1 s/step. Different lattice planes against specific diffraction angles were calculated and used to determine the intrinsic details of the structure based on the diffractogram.

4.2.1.6 FE-SEM

The surface topography of the powdered C₆₀ was observed using FE-SEM [EVO-60, Zeiss company]. The powdered samples of C₆₀ were drop-casted onto the surface of a glass coverslip and dried overnight. The resulting samples were viewed under an FE-SEM at an operating voltage of 20 kV. The particle size of the C₆₀ was calculated using a technique for determining the layer thickness and the particle diameter based on the use of a raster scanning mode.

4.2.1.7 HRTEM

TEM uses a high-energy electron beam (up to 300 keV) to image powdered C₆₀ samples based on electron transmission. The length and dispersion state of C₆₀ can be assessed by TEM at low magnification. At high magnifications, TEM can be used to count the number of walls in C₆₀, measure inner and outer diameter, assess structural integrity, and identify structural changes caused by surface modification.

Although TEM is capable of imaging the structure of C₆₀, including the presence of sidewall damage, the electrons used for imaging can themselves also cause sidewall damage and structural modification. To avoid this problem, it has been recommended that analysis areas only be exposed to the electron beam for very short durations (<15 s) and that beam energies should be limited to 100 keV. As in many imaging techniques, verification that an observed structure remains unchanged with respect to irradiation time is the best way to ensure that the analysis technique is non-destructive.

For the analysis, the powdered samples of pristine C₆₀ were deposited by placing droplets of finely prepared ethanol-dispersed samples on carbon-coated, copper grids [300 mesh size, regular grip, spi 2030 C] and allowed to dry overnight. The resulting samples were viewed in HR-STEM [Tallos F200S G2 TEM] at a frequency of 80 Hz.

4.2.2 Preparation of C₆₀ FAS

4.2.2.1 Tol/C₆₀

The C₆₀ fullerene aqueous solutions (C₆₀FAS) used for the anticancer screening experiments were prepared by mixing C₆₀ fullerenes (purity 99.5%) in toluene and distilled water (Prylutsky et al., 2014). The sample solution was continuously ultrasonicated for 3 hrs until the complete evaporation of toluene. The Tol/ C₆₀FAS working solution was aliquoted to different concentrations of 5, 10, 20, 40, 80 and 100 µg/ml.

4.2.2.2 Aqs/C₆₀

Another method of preparation of C₆₀FAS was performed, to understand the differential toxicity of C₆₀ due to the various preparation techniques. In this method, the C₆₀ powdered sample was added to double distilled water and was ultrasonicated for several days. A soluble working solution obtained was filtered and used (Bouchard et al., 2009) (Andrievsky et al., 2002). The Aqs/ C₆₀FAS working solution was aliquoted to different concentrations of 5, 10, 20, 40, 80 and 100 µg/ml.

4.2.3 Cell culture maintenance

A549 (Human lung carcinoma epithelial cell line) were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. They were maintained at 37°C with 5% CO₂ in 95% air-humidified incubator.

4.2.4 In vitro Anticancer Screening

4.2.4.1 MTT Assay

The cytotoxic effect of the sample was tested against A549 cancer cells by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cells were seeded in 96-well microplates (1×10^6 cells/well) and incubated at 37°C for 48 h in 5% CO₂ incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the cells were treated with different concentrations of sample and incubated for 24 h. The morphological changes of untreated (control) and treated cells were observed under a digital inverted microscope (20X magnification) after 24 h and photographed. The cells were then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 µL of (MTT) solution (5 mg/mL in PBS) was added to each well. The plates were then stood at 37°C in the dark for 2 h. The formazan crystals were dissolved in 100 µL DMSO and the absorbance was read at 570 nm. Based on cytotoxicity%, the graphs were plotted and IC₅₀ value was calculated.

The percentage of cytotoxicity was calculated using the formula,
Cytotoxicity (%) = 100 - ((Sample OD / Control OD) X 100).

4.2.4.2 Dual FITC and PI staining

Dual staining assay is based on the phosphatidylserine residue exposure on the outer side of the cell plasma membrane to detect apoptosis. Exposure of phosphatidylserine on the cell surface is an “eat me” signal, which facilitates phagocytic recognition of apoptotic cells and their destruction (Svitlana Prylutska *et al.*, 2019).

A549 cancer cells were seeded in six-well plates. When cells reached 70% confluence, they were treated with C₆₀ (containing 40 µg/mL carbon nanomaterials). The cells were incubated at 37 °C for a further 24 h and then the cells were collected and suspended in 400 µL binding buffer to adjust appropriate cell density. Each well was labelled as control and test for respective probes. 5 µL of Annexin V-FITC and 5 µL of propidium iodide (PI) to the respective wells. Dual staining of Annexin V-FITC and PI was performed in the labelled well to identify the early and late apoptotic and necrotic cells. After adding Annexin V-FITC and PI, the cells were kept for 15 min at room temperature in the dark for cell apoptosis test. The morphological change, DNA damage, and cell membrane integrity were identified and

photographed using *life* EVOS-FLc fluorescence microscope. In this assay, FITC binds to phosphatidylserine and PI binds to DNA (Grebinyk *et al.*, 2018).

4.2.4.3 DCFH-DA Assay

The fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to investigate ROS production. DCFH-DA can enter cells where it is deacylated by esterases into DCFH, a non-fluorescent form. When DCFH interacts with intracellular ROS, it is transformed into DCF, which exhibits a strong green fluorescence. DCFH is mostly sensitive to H₂O₂, peroxonitrite, and the hydroxyl radical. A549 cancer cells were seeded in six-well plates. When cells reached 70% confluence, C₆₀ (containing 40 µg/mL carbon nanomaterials). The well without C₆₀ acted as the control group. Following incubation with C₆₀ for 6 h, culture medium containing these nanomaterials was removed and DCFH-DA was added to the cells. After 30 min incubation, cells were washed twice with PBS and then fluorescence images of treated cells were acquired using a fluorescence microscope (*life* EVOS FLc).

4.2.5 Differential Cytotoxicity Analysis

4.2.5.1 MTT Assay

The cytotoxic effect of the sample was tested against Vero cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The cells were seeded in 96-well microplates (1 x 10⁶ cells/well) and incubated at 37°C for 48 h in 5% CO₂ incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the cells were treated with different concentrations of Two different samples of C₆₀ FAS (Tol/C₆₀ and Aqs/C₆₀) and incubated for 24 h. The colour changes of untreated (control) and treated cells were observed after 24 h. The cells were then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 µL of (MTT) solution (5 mg/mL in PBS) was added to each well. The plates were then stood at 37°C in the dark for 2 h. The formazan crystals were dissolved in 100 µL DMSO and the absorbance was read at 570 nm.

The percentage of cytotoxicity was calculated using the formula,

Cytotoxicity (%) = 100- ((Absorbance of sample/Absorbance of control) X 100).

With cytotoxicity % and concentrations line graphs were plotted for Tol/C₆₀ and Aqs/C₆₀. The IC₅₀ values for each assay were calculated based on the graphs.

4.2.6 *In silico* analysis

In silico tools were applied to understand the protein interactions of fullerene C₆₀ against cancer. Pyrx is a virtual screening software for computational drug discovery to screen compounds against potential drug targets. The results of Pyrx consists of Binding affinity and RMSD (Root mean Square Deviation). RMSD values are calculated relative to the best mode and use only movable heavy atoms. The two variants RMSD/UB(Upper Bound) and RMSD/LB(Lower Bound) are calculated based on how the atoms are matched in the distance calculations and conformations. The anticancer activity potential of fullerene C₆₀ was evaluated by molecular docking studies with Pyrx and Autodock Vina Software. The interactions of fullerene C₆₀ with the active site of previously identified or predicted cancer macromolecules were obtained by auto dock vina wizard.

Energy minimization of ligand fullerene C₆₀ was performed using Open Babel in Pyrx software. PDB files of macromolecules were obtained from the protein data bank (<https://www.rcsb.org/>) and modified using Open Babel. 8 protein studies were performed using standard settings for all macromolecules. The proteins were selected based on their involvement in apoptosis and autophagy pathways. Docking scores were obtained using Auto Dock Vina Software(Beyaz *et al.*, 2022). The binding energy and RMSD(root mean square deviation), metric of distance of proteins and ligand(C₆₀) of each docked molecule was obtained and evaluated.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 PHYSICAL CHARACTERIZATION

5.1.1 UV-Vis

Fig 5.1. represents the UV-Vis absorption spectra of the pristine C₆₀ nanoparticle. The nanoparticle gave a sharp absorption peak at 200nm. This indicates the presence of Nps in the solution. Absorbance peaks were observed at ~223 nm and ~278 nm respectively. Similar absorbance spectrum peaks with C₆₀ FAS have been observed in previous studies(Grebinyk, Prylutska, Buchelnikov, *et al.*, 2019)(Scharff *et al.*, 2004).

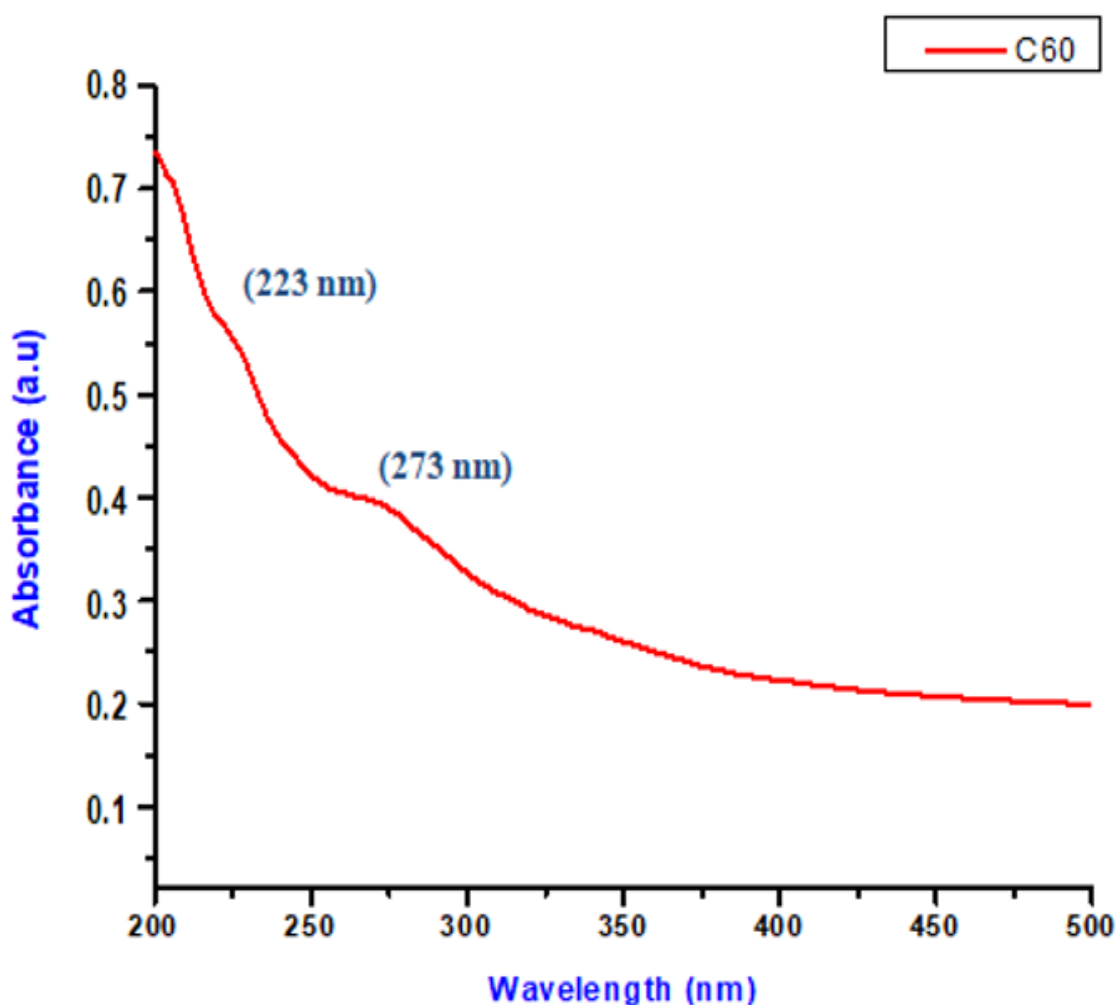


Fig 5.1: UV-Vis spectroscopy of C₆₀FAS

5.1.2 FTIR

The FTIR results indicated various chemical groups involved in the formation of Nps. The peaks at $\sim 2356\text{ cm}^{-1}$ and $\sim 2111\text{ cm}^{-1}$ indicate the presence of functional groups of C-O. The peak at $\sim 2660\text{ cm}^{-1}$ corresponds to C-H vibrations involved in the stabilization of the nanoparticle. Fig 5.2 shows the Inverse dipping of peaks ~ 981 , 2111, 2356 and 2660 corresponding to different C-O-C vibrations and C-OH vibrations in C_{60} structures along with some wagging of -OH vibrations in the solution of C_{60} .

The lowest wavenumbers bands (below 1000 cm^{-1}) signify radial displacement of carbon atoms, whereas above 1000 cm^{-1} signifies tangential modes of carbon atoms in C_{60} structures.

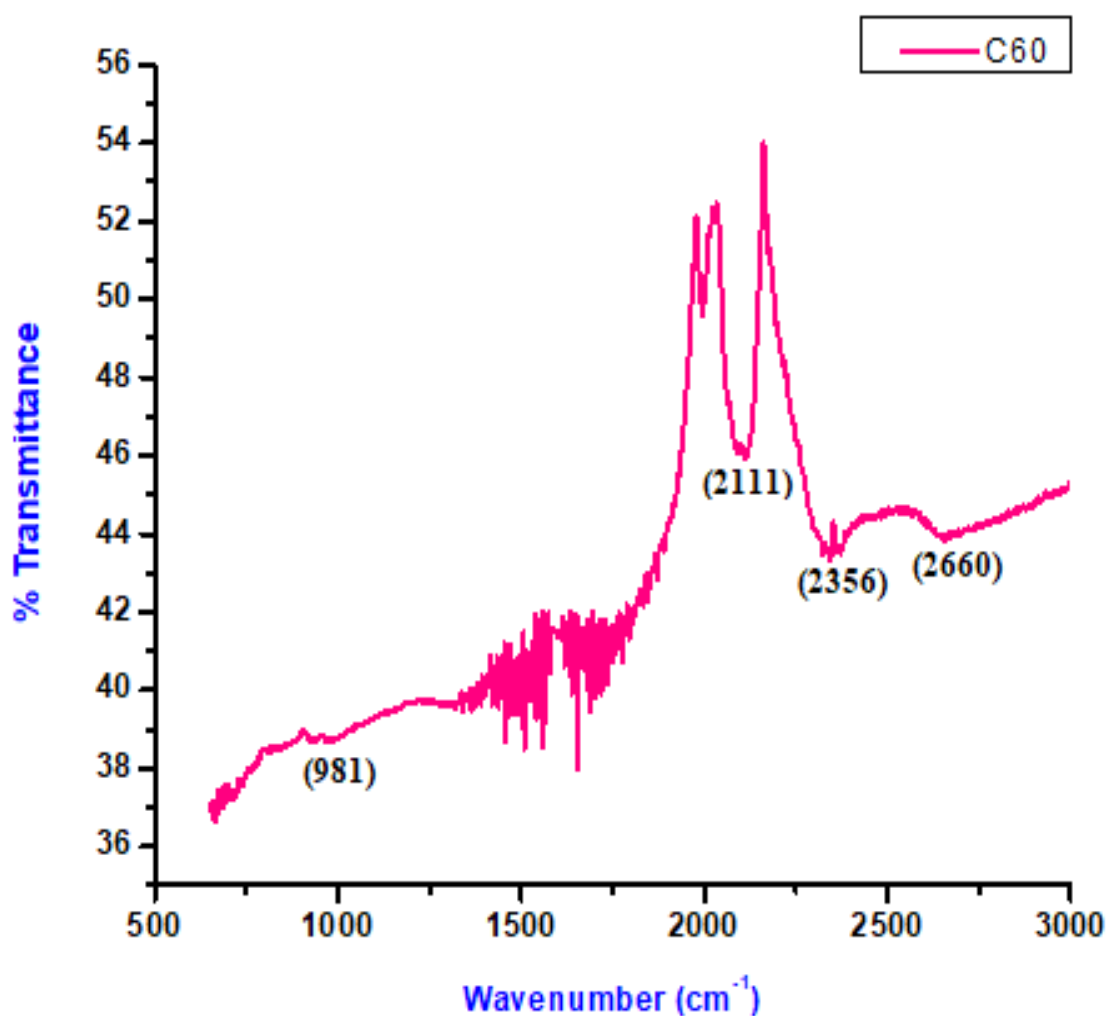


Fig 5.2: FTIR analysis of C_{60} fullerene

5.1.3 Raman spectroscopy

Raman Spectroscopy determines the I_D/I_G ratio from the spectra, Smaller is the I_D (1314 cm^{-1}) and the steeper peak is I_G (1599 cm^{-1}). The ratio of I_D/I_G was estimated as 0.82, the characteristic defect ratio determined the nature and proportion of defect concentration in the sample. The absorption peak was observed at 1314 cm^{-1} as shown in Fig 5.3. Similar absorbance spectra peaks have been observed in previous studies (Chu *et al.*, 2018).

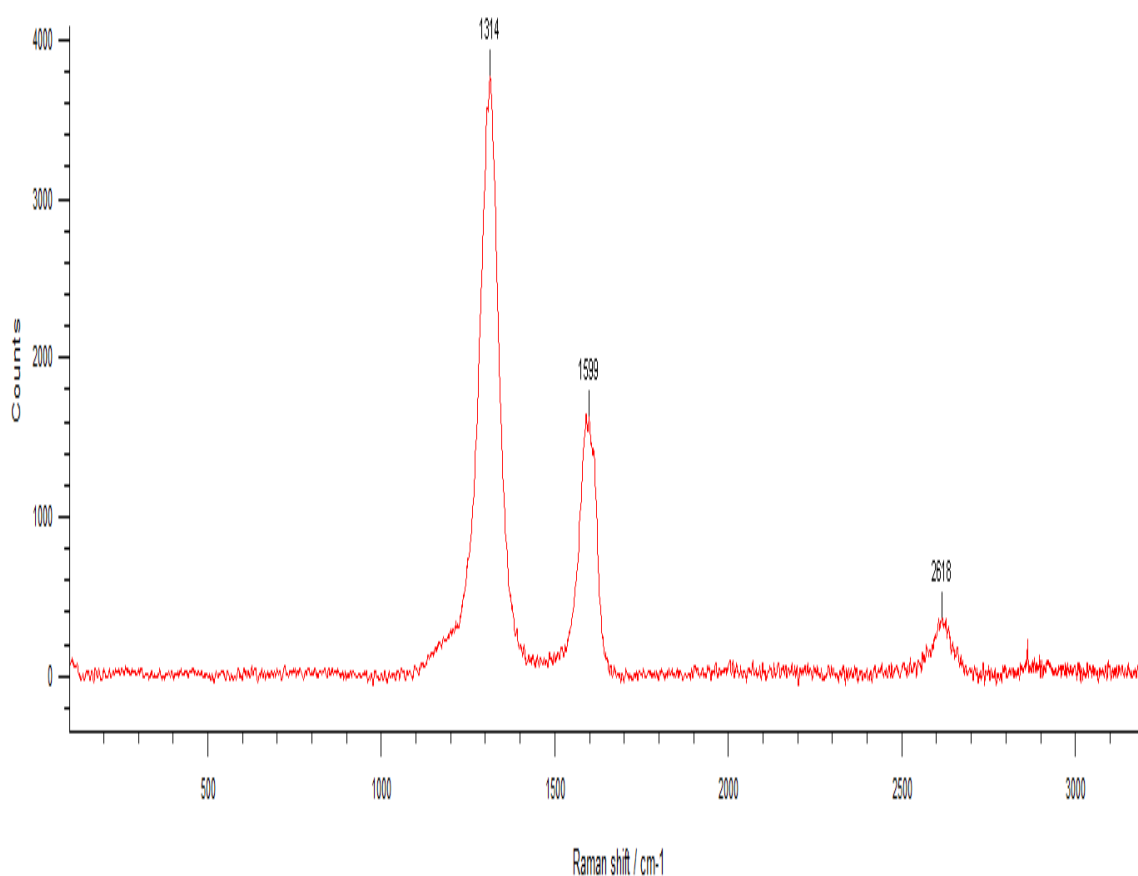


Fig 5.3: Raman spectroscopy of C₆₀ fullerene

5.1.4 Zeta potential and Particle size analysis

Zeta potential measurements were used for ascertaining the hydrodynamic size and electrokinetic potential of C₆₀ fullerene particles in an aqueous solution. ζ potential was recorded as -52.8 mv. Following dilution, the absolute value of ζ potential increased. Fig 5.4 shows the 3 different peaks observed with dilution. Bouchard *et al.* have observed a similar ζ potential of -55.7 mV.

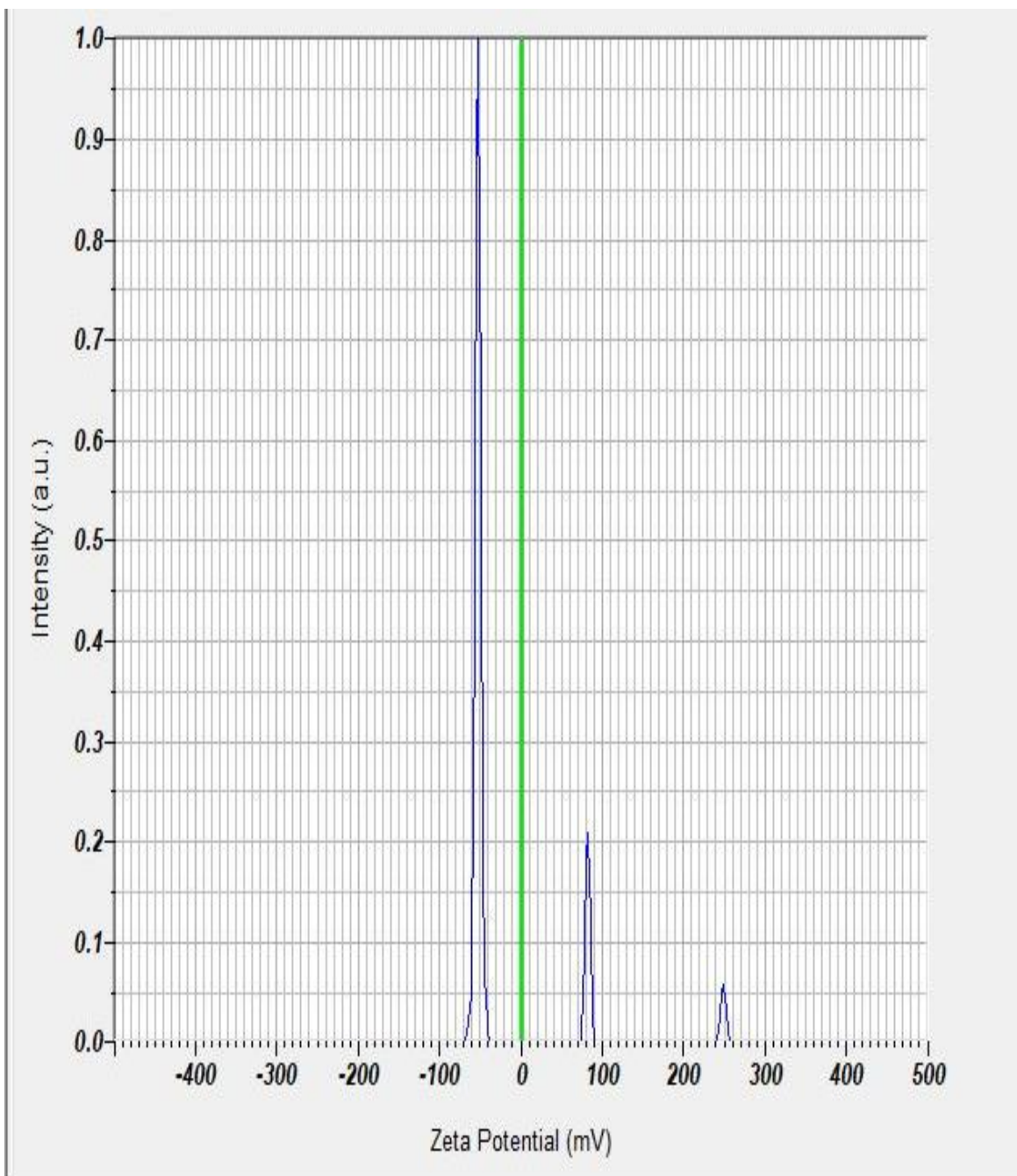


Fig 5.4 : Zetapotential of C₆₀ fullerene

In water, the stability of negatively charged clusters depends on two opposite forces such as the electrostatic repulsion of negatively charged C₆₀ molecules and attraction of the C₆₀ fullerenes due to hydrophobic and Van der Waals forces. Thus, the negative potential of C₆₀ is an important factor for the stability of the nanoparticle. Fig 5.5 represents the plot of the particle size analysis of C₆₀ Nps.

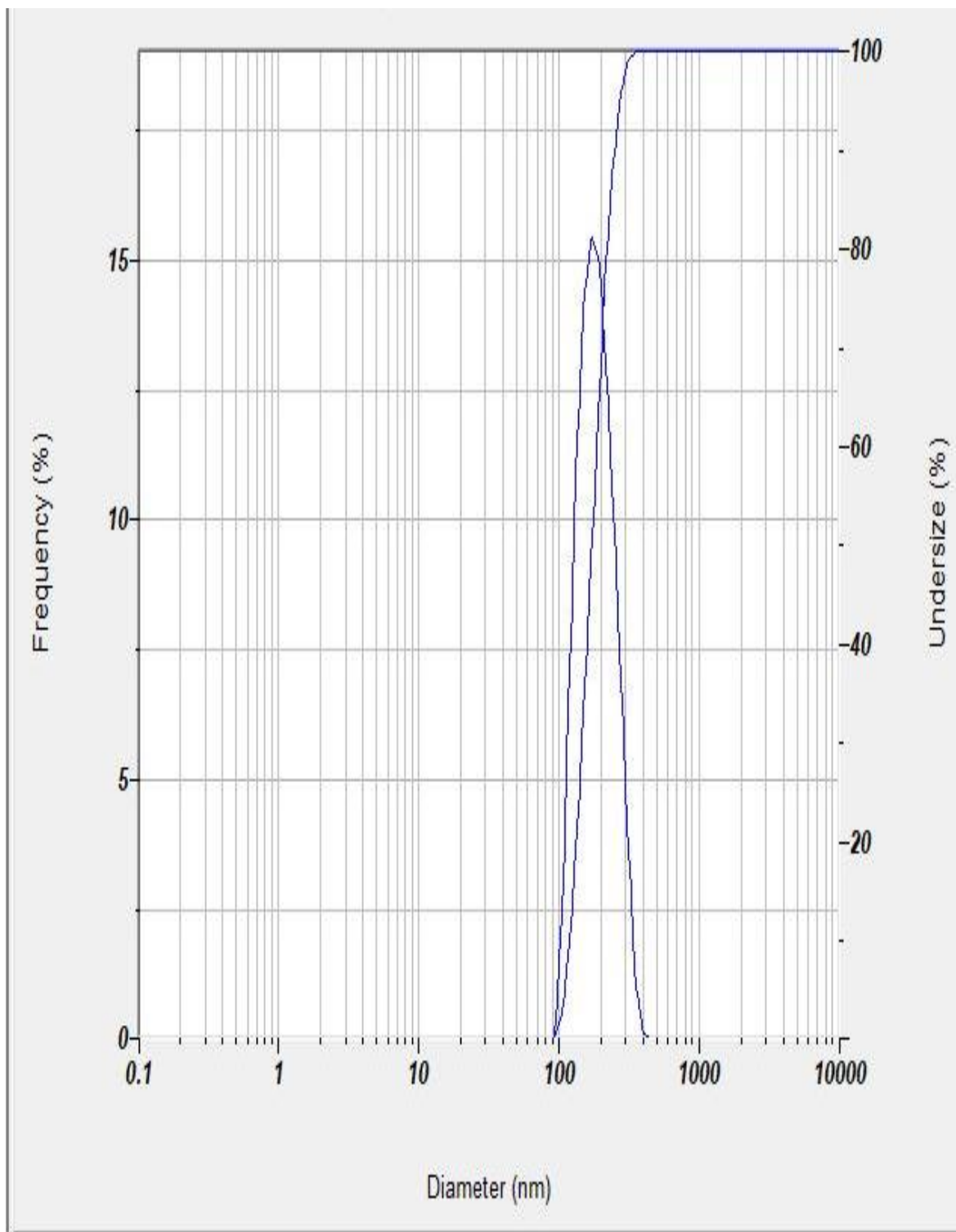


Fig 5.5: Particle size analysis of C₆₀ fullerene

From Fig 5.5 , it was observed that the C₆₀ NPs were mainly distributed in the range of 105 - 403 nm with an average particle size of 179.7 nm. Particle size analysis has a key role in determining the bulk properties of the powder. The C₆₀ NPs sample was categorized to be fine particles based on its size range.

5.1.5 XRD

The XRD analysis revealed the presence of lattice planes (111), (220), (311), (420) and (422) that are characteristic of C₆₀ fullerene. A similar XRD pattern has been observed in previous studies (Labille *et al.*, 2009).

Lattice planes observed at a characteristic diffraction angle (2θ) denotes characteristic crystallinity in pristine undoped C₆₀ molecule. The nature of crystallinity indicates and determines the behaviour of the material when introduced into complex biological systems. The 2θ was observed as 220 degrees. The sharp peak can be observed in Fig 5.6, which confirms the C₆₀ particle is crystalline.

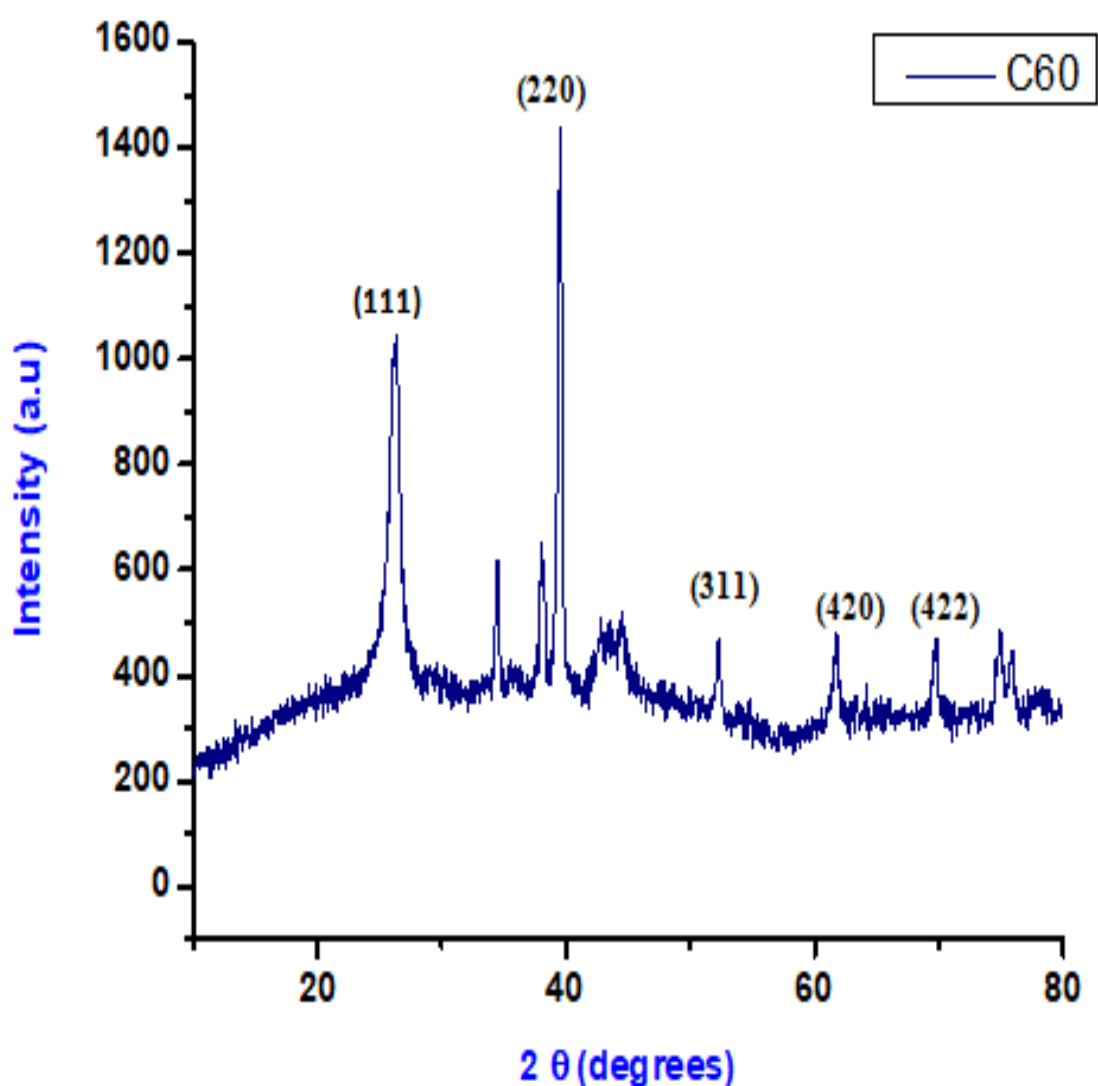


Fig 5.6: XRD pattern of C₆₀ fullerene

5.1.6 FE-SEM

The typical surface topography of C₆₀ was evident in the FESEM analysis. A scanning electron microscope is used to know the size, shape and morphology of the synthesized nanoparticle. Fig 5.7 shows the approximate size and the morphology of the nanoparticle. Data on surface morphology is highly useful regarding the potential use of nanomaterials in medical devices and for other applications. The images showed relatively symmetric surfaces of C₆₀ fullerenes. For C₆₀ fullerenes, the observed size range in SEM was from 300 nm to 800 nm.

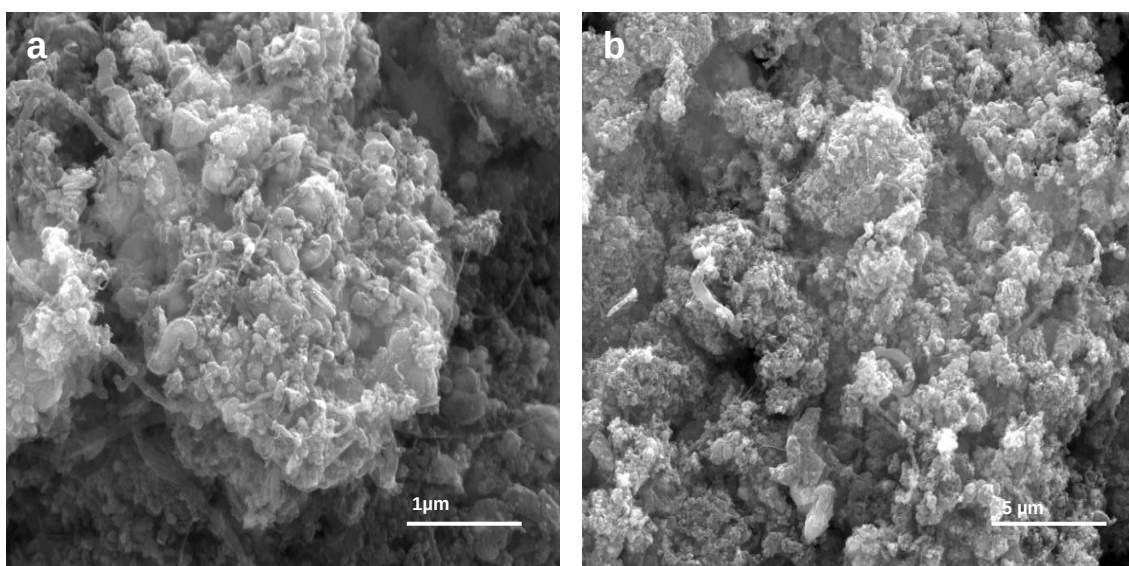


Fig 5.7: FESEM images of C₆₀ Fullerene; a) Image obtained at a magnification of 80000X; b) Image obtained at a magnification of 20000X

5.1.7 HRTEM

High-resolution Transmission Electron Microscopy (HRTEM) has been used to investigate the individual C₆₀ fullerene morphology, crystallography, and microstructure. HR-TEM observations indicated that the internal structure of the C₆₀ sample possesses a spherical morphology. HR-TEM imaging enables one to visualize the internal, structural architecture of the prepared C₆₀. The diameter of C₆₀ was ~ 10 nm. A crystalline structure for C₆₀ with a diameter of about 0.5 nm was observed. The HRTEM images have shown no aggregation of spherical particles.

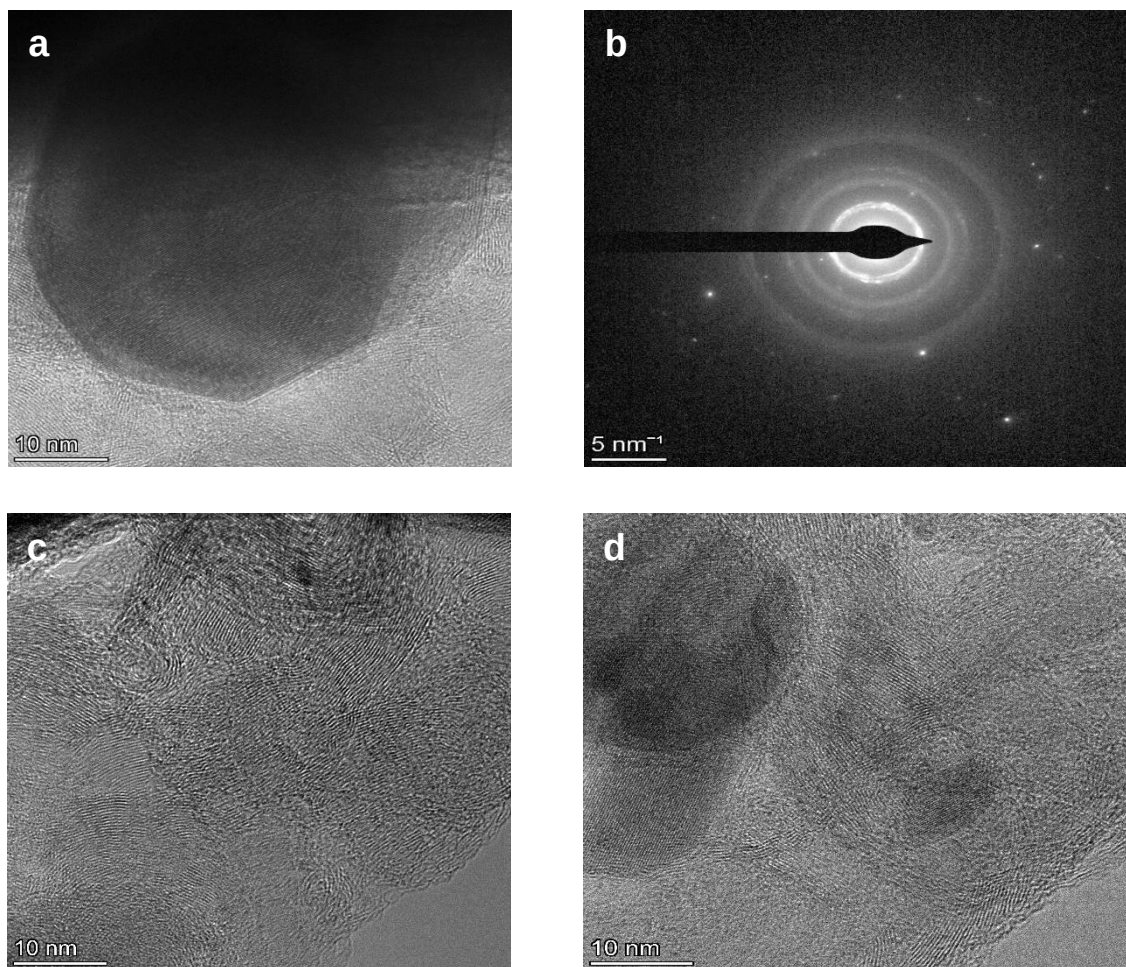


Fig 5.8: TEM images of C₆₀ fullerene; a) clear spherical structure of C₆₀ fullerene; b) corresponding SAED pattern; c),d) magnified images of C₆₀ Nps

5.2 IN VITRO ANTICANCER SCREENING

5.2.1 Cytotoxicity activity

In order to determine the effects of fullerene C₆₀ on cytotoxicity, cells were incubated following 5, 10, 20, 40, 80, and 100 µg/ml treatment. The data obtained showed that there was a statistically significant difference in cytotoxicity at 5, 10, 20, 40, 80, and 100 µg/ml doses compared to the control group. On the other hand, there was a significant decrease in cell viability of the group exposed to the highest dose of 100 µg/ml C₆₀. The decrease in cell population with increasing concentration is visible in Fig 5.8. The exponential decrease in cells was observed with the increasing concentration of C₆₀ in Fig 5.9.

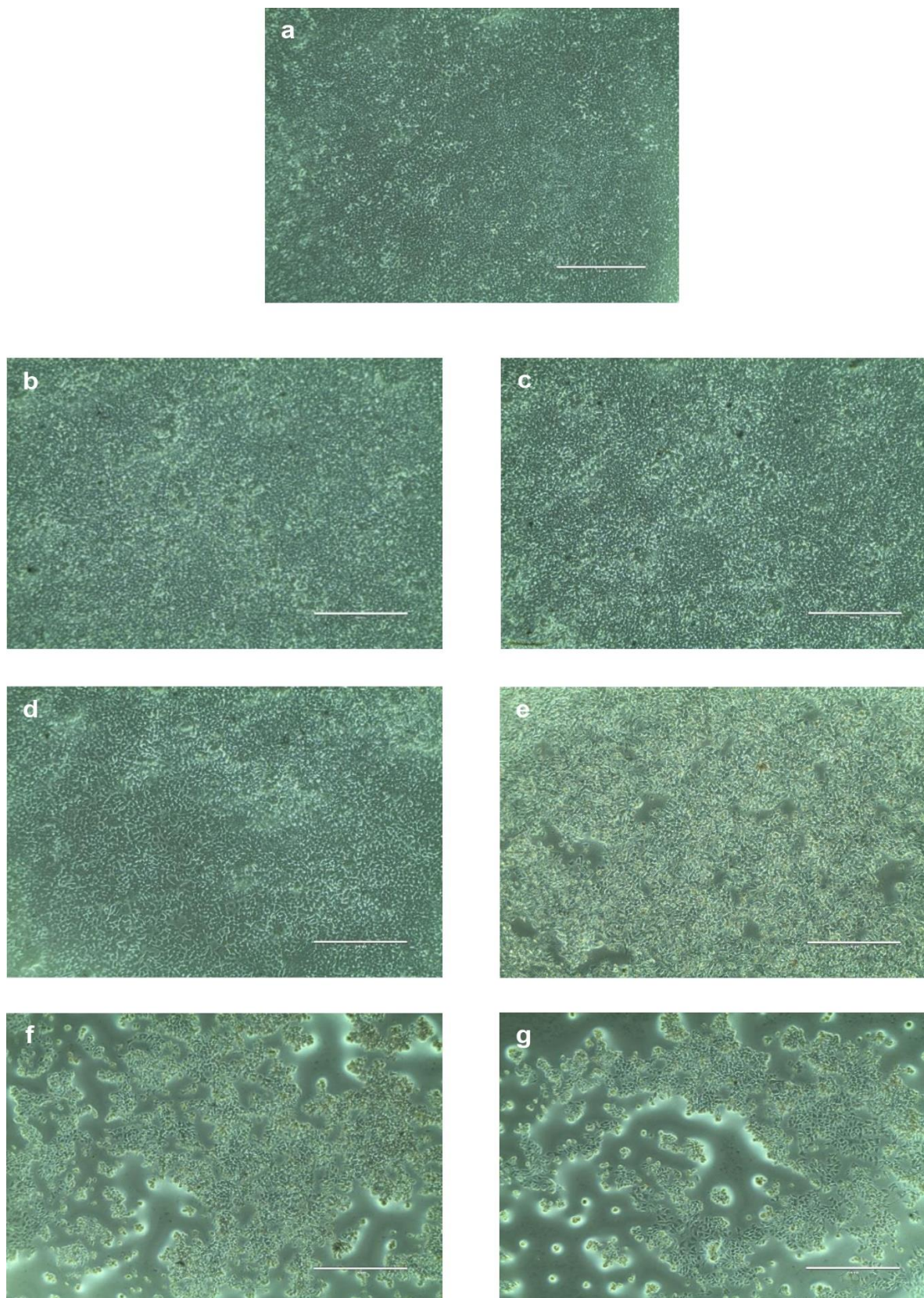


Fig 5.9: Microscopic images of A549 cancer cells treated with different concentrations of Tol/C₆₀FAS; a) Control with C₆₀ untreated A549 cells; b) 5 µg/ml; c) 10 µg/ml; d) 20 µg/ml; e) 40 µg/ml; f) 80 µg/ml; g) 100 µg/ml

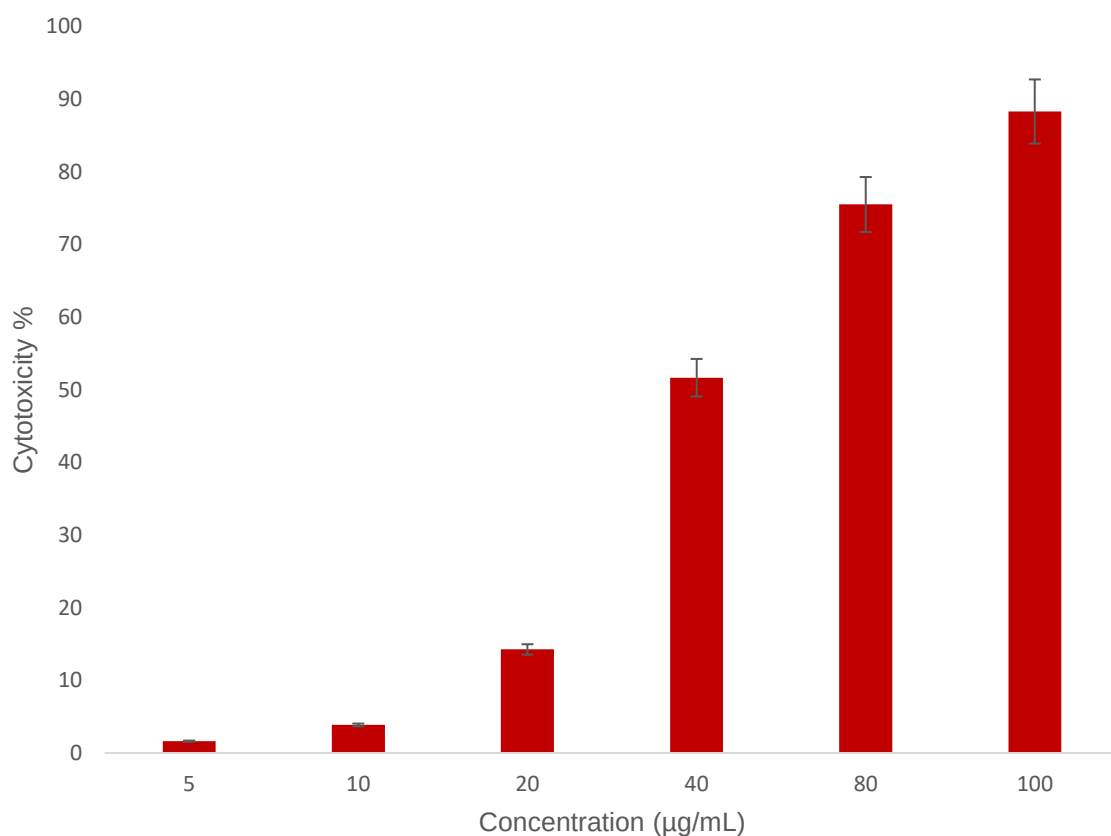


Fig 5.10: Bar graph representing the dose dependant cytotoxicity of Tol/C₆₀FAS in A549 cancer cells

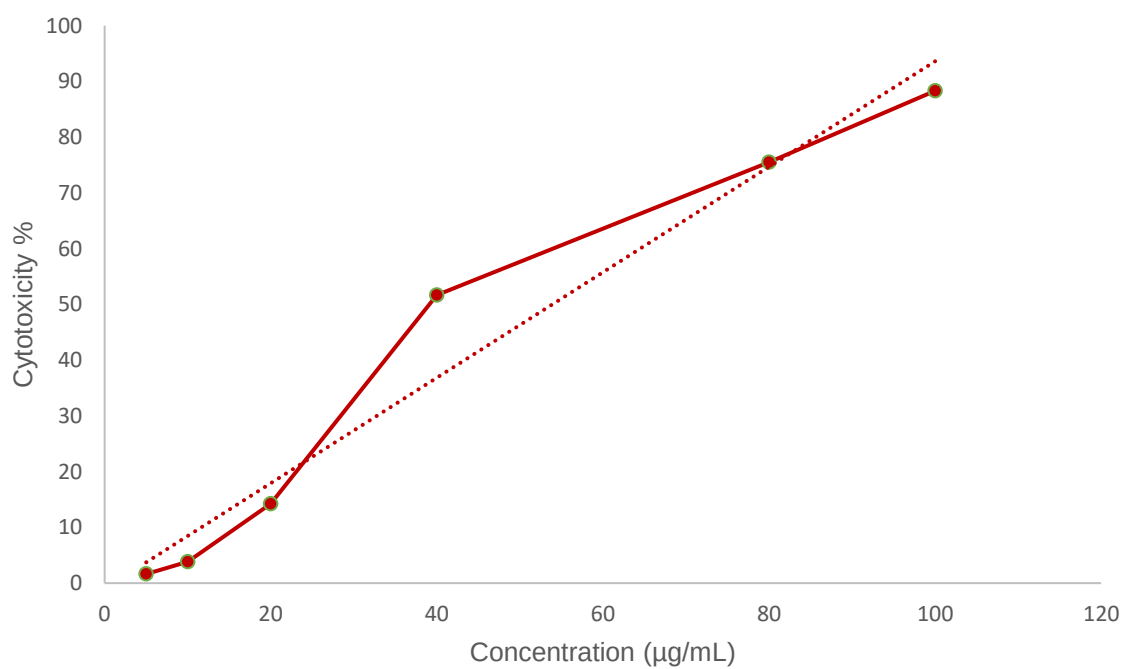


Fig 5.11: IC₅₀ inhibition line graph for cytotoxicity of Tol/C₆₀FAS in A549 cancer cells

Fig 5.10 represents the dose-dependent cytotoxicity of C₆₀ with standard error. The bar graph clearly shows the exponential increase in cytotoxicity with the increase in C₆₀ concentration. Fig 5.11 represents the cytotoxicity of C₆₀ in cancer cells in a line graph with linear intercept (R^2 almost equals 1)
 $y = mx - c$, where $m = 0.9459$ and $c = -0.9968$
 To calculate the IC₅₀ value substitute $y = 50$ in the above equation,
 IC₅₀ value was estimated to be 53.91 µg/ml.
 The observed results show that C₆₀ shows high toxicity towards cancer cells proving its anticancer activity.

5.2.2 Apoptosis activity

The apoptosis can be triggered by ROS, external factors and internal factors by activating proapoptotic genes. C₆₀ inducing apoptosis after entering the cell is depicted in Fig 5.12.

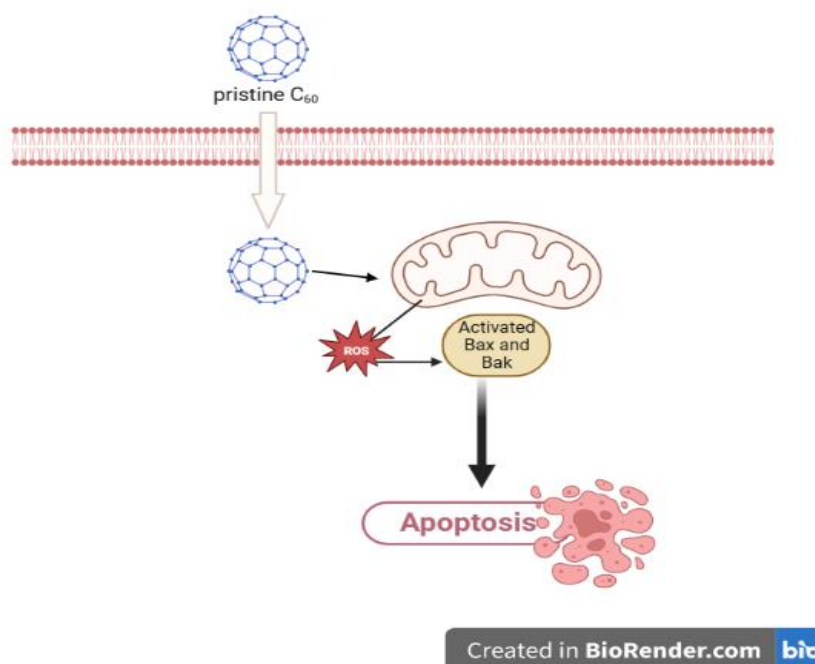


Fig 5.12: Apoptotic pathway induced by C₆₀ in cancer cell

The PI staining images in Fig 5.13(a and b) shows no fluorescence in the control group proving that the cells are intact and in the treated group with bright red cells proving that the treatment with Tol/C₆₀FAS has induced cell disruption and cell death. The bright green fluorescence in Tol/C₆₀FAS treated A549 cancer cells along with damaged cell morphology in Fig 5.13(d) shows the externalisation of

phosphatidylserine from the cell plasma membrane. This confirmed that C₆₀ had caused apoptosis.

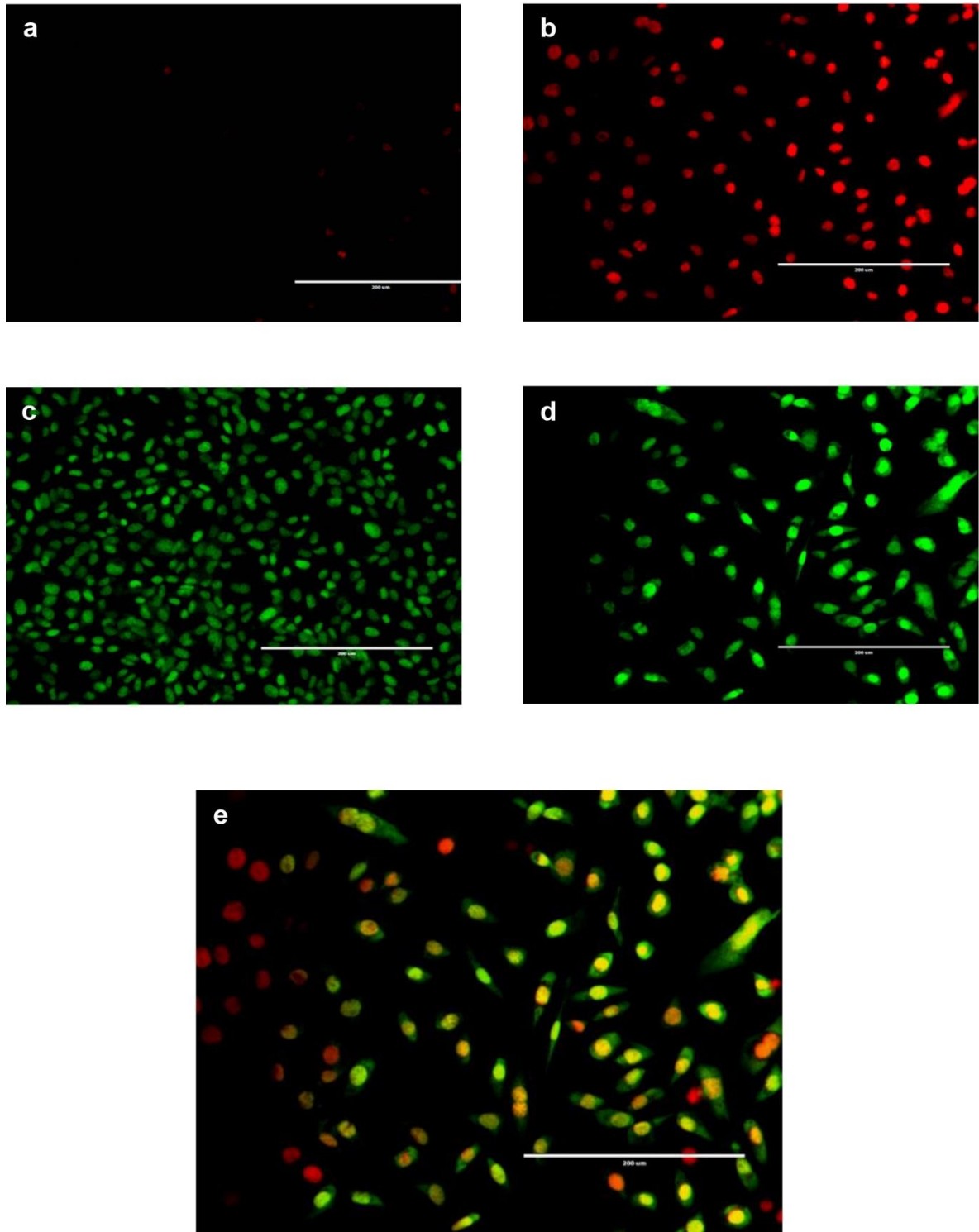


Fig 5.13: Dual staining with FITC and PI fluorescent images of C₆₀ treated A549 cancer cells for cell apoptosis; a) Control stained with PI; b) C₆₀ treated cells stained with PI; c) Control stained with FITC; d) C₆₀ treated cells stained with FITC; e) Dual staining of PI and FITC of C₆₀ treated cells

Dual staining of PI and FITC distinguished the cell populations of viable (FITC negative, PI negative), early apoptotic (FITC positive, PI negative), late apoptotic (FITC positive, PI positive), and necrotic (FITC negative, PI positive) cells (Svitlana Prylutska *et al.*, 2019) (Grebinyk *et al.*, 2018).

5.2.3 ROS Generation

ROS such as superoxide radical anion, hydrogen peroxide, singlet oxygen, and hydroxyl radical, can cause oxidative stress and are an important factor in the mechanism of carcinogenesis. The antioxidant potential of fullerene was associated with ROS, and the degree of ROS generated in the cells was examined. ROS productions were observed in A549 cells incubated with Tol/C₆₀FAS by using DCFH-DA fluorescent probe. As shown in Fig 5.14, the bright green fluorescence of DCFH was observed in A549 cells incubated with C₆₀ at the concentration: 40 µg/ml. Control groups showed negligible DCFH fluorescence while green fluorescence of DCFH was observed in cells treated with C₆₀ representing the elevated levels of ROS in the test group (Zhang *et al.*, 2009).

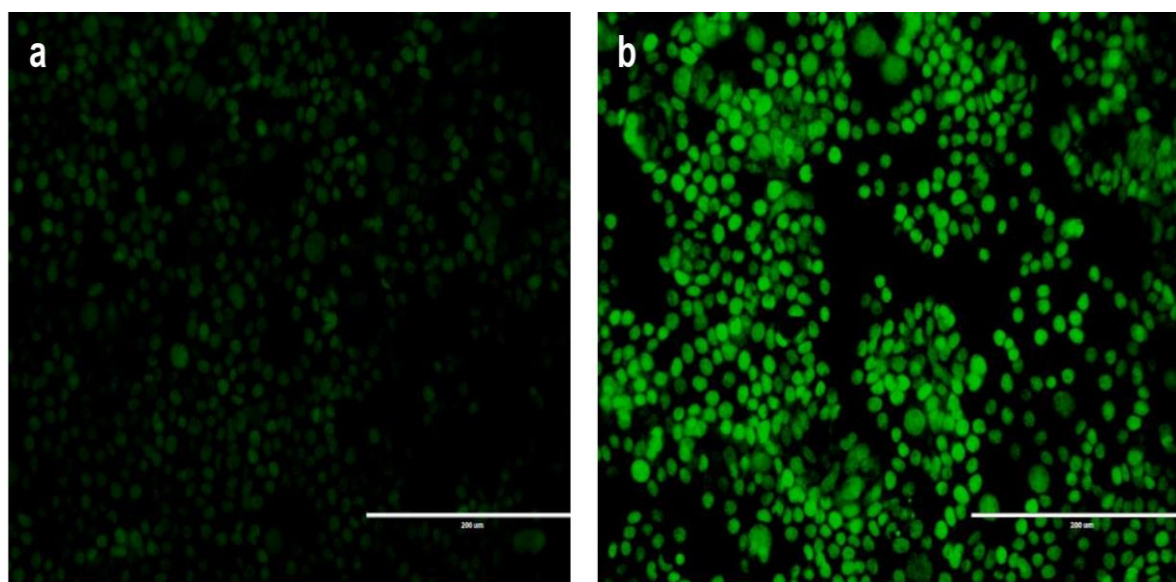


Fig 5.14: Analysis of Intracellular ROS formation using DCFH-DA staining on A549 cancer cells treated with C₆₀; a)Control; b)Test

Due to the hypermetabolism of Redox equilibrium, cancer cells produce more ROS than normal cells, but because of their strong antioxidant capacity, cancer cells can maintain the redox equilibrium. The results observed prove that C₆₀ has a high

potential ROS formation than scavenging. Thus, by accelerating the production of accumulating ROS redox balance can be disturbed and cancer cells can be damaged (Kim *et al.*, 2019).

5.3 DIFFERENTIAL CYTOTOXICITY ANALYSIS

5.3.1 Comparative Cytotoxicity of C₆₀ Based on the Method of Preparation

The cytotoxic activity of pristine C₆₀ fullerene in a concentration range of 5-100 µg/mL against Vero cells at 24 h of incubation was investigated using MTT assay. Fig 5.15 shows the colour variation in different concentrations of the two different samples.

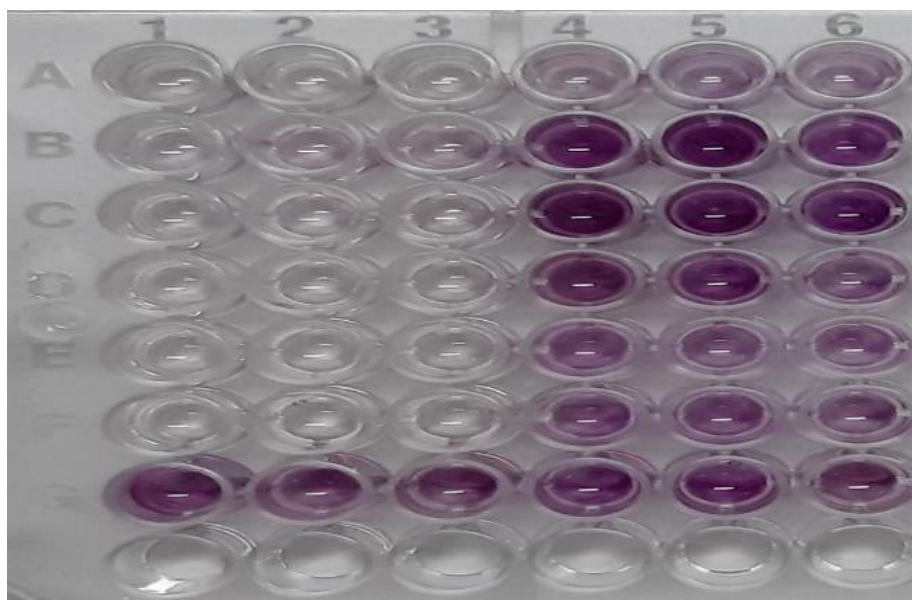


Fig 5.15: Variation in colour change observed due to differential cytotoxicity on Vero cells treated with Aqs/C₆₀ FAS and Tol/C₆₀ FAS

Fig 5.16 represents the cytotoxicity of Tol/C₆₀ FAS and Aqs/C₆₀ FAS in line graphs with straight intercept (R^2 almost equals 1)

In line graph for Tol/C₆₀ FAS,

$$y = mx + c, \text{ where } m = 0.0627 \text{ and } c = 5.1831$$

To calculate the IC₅₀ value, $y = 50$ was substituted in the above equation,

IC₅₀ was estimated to be 714.78 µg/ml.

In line graph for Aqs/C₆₀ FAS,

$y = mx + c$, where $m = 0.2234$ and $c = 45.746$

To calculate the IC_{50} value, $y = 50$ was substituted in the above equation,

IC_{50} value was estimated to be $19.042 \mu\text{g/ml}$.

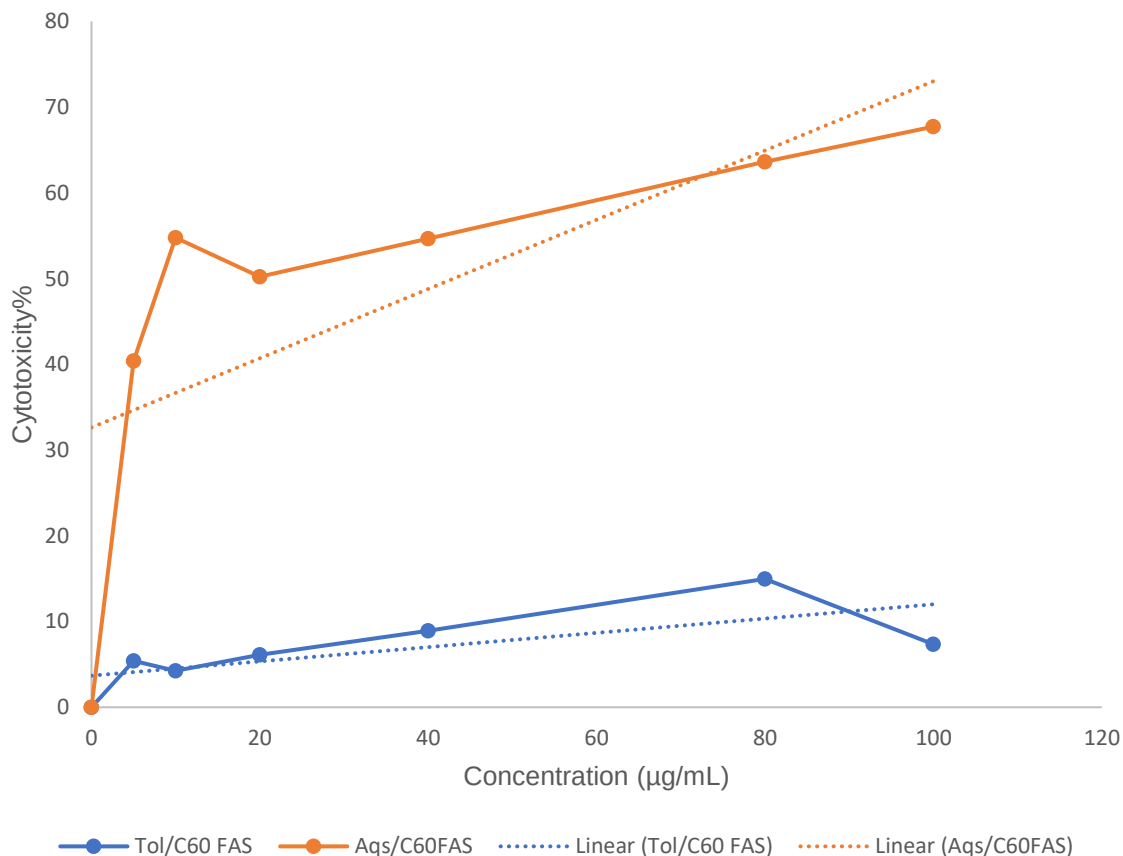


Fig 5.16: IC_{50} inhibition line graph for toxicity of Tol/ C_{60} FAS and Aqs/ C_{60} FAS

The IC_{50} value for Tol/ C_{60} FAS-treated Vero cells were calculated to be $714.78 \mu\text{g/mL}$. Fig 5.16 shows very low toxicity of C_{60} fullerene even at high concentrations against Vero cells. The IC_{50} value for Aqs/ C_{60} FAS was estimated to be $19.042 \mu\text{g/mL}$. A study on toxicity of Aqueous based nano C_{60} formulation has shown that it is highly cytotoxic based on its aggregative nature triggering oxidative stress thereby killing cells (Sayes *et al.*, 2004).

The line graphs of both Tol/ C_{60} FAS and Aqs/ C_{60} FAS show a statistically significant difference in cytotoxicity. Fig 5.17, shows the differential toxicity of C_{60} with two different preparation methods. Higher the IC_{50} value lower the toxicity, the graph depicts that the toxicity of Tol/ C_{60} FAS in vero cells are very less compared to Aqs/ C_{60} FAS.

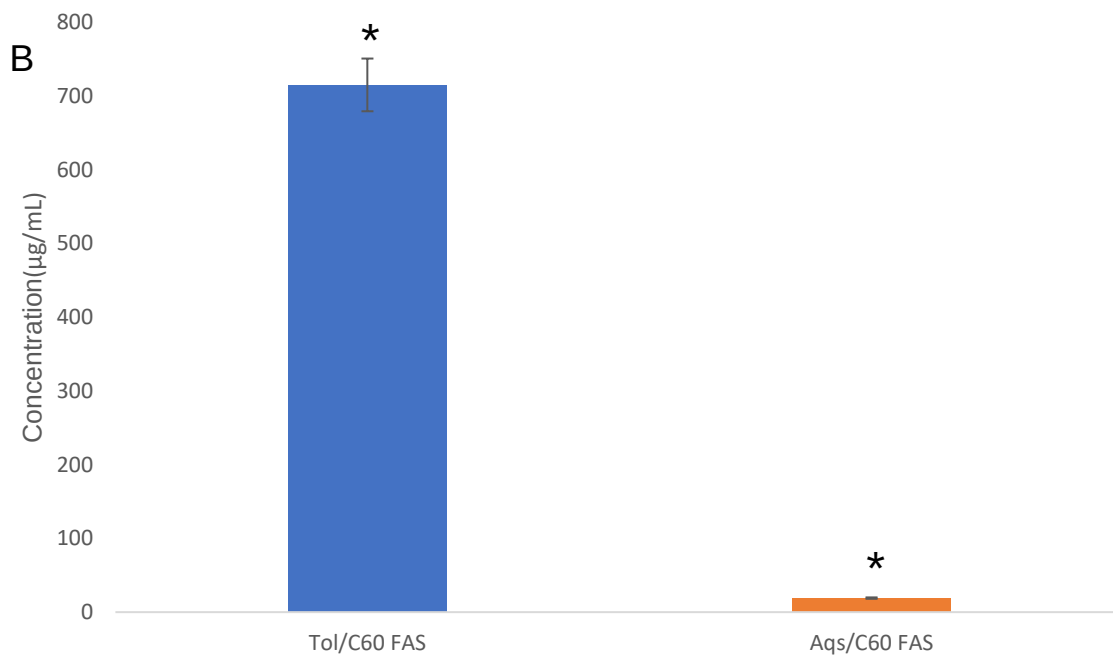
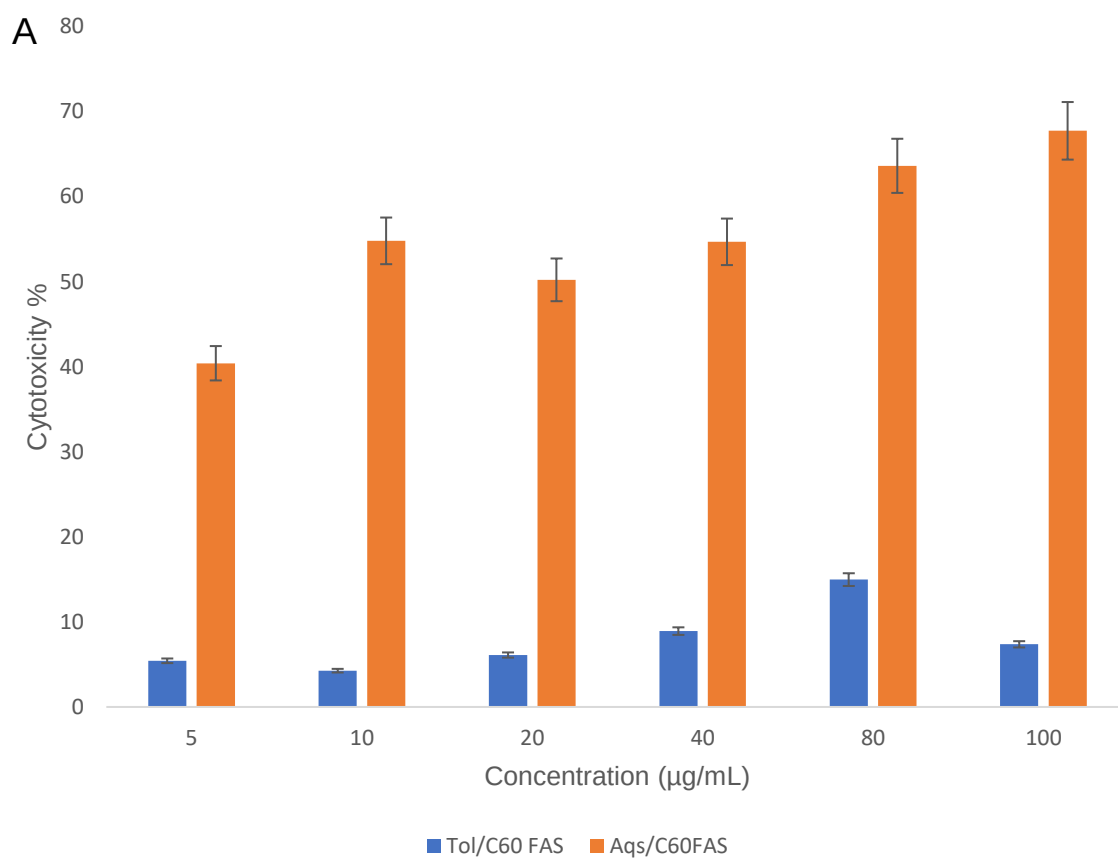


Fig 5.17: Representation of Comparative Toxicity of Tol/C₆₀ FAS and Aqs/C₆₀ FAS;A) Comparative Toxicity of Tol/C₆₀ FAS and Aqs/C₆₀ FAS: B) Comparison of IC₅₀ values of vero cells treated with Tol/C₆₀ FAS and Aqs/C₆₀ FAS,* represents $p < 0.05$

In toluene-based C₆₀ FAS preparation, all the Nps are completely solubilized in water as the toluene evaporates, whereas in Aqs/C₆₀ FAS preparation, the particles got dissolved during sonication but later formed aggregates. The observations proved that the Aqs/C₆₀ FAS preparation technique is inefficient due to the aggregate formation, thereby a higher level of cytotoxicity. The student's t-test was applied using Microsoft Excel. The estimated $p < 0.05$ for the data was considered to be a significant difference.

5.3.2 Comparative cytotoxicity in cancer and normal cells

The cytotoxicity of C₆₀ was evaluated with both cancer cells (A549 cell line) and normal cells (Vero cell line) to evaluate its use in therapeutics. The comparative bar graph in Fig 5.18 depicts the difference in cytotoxicity of C₆₀ in normal and cancer cells.

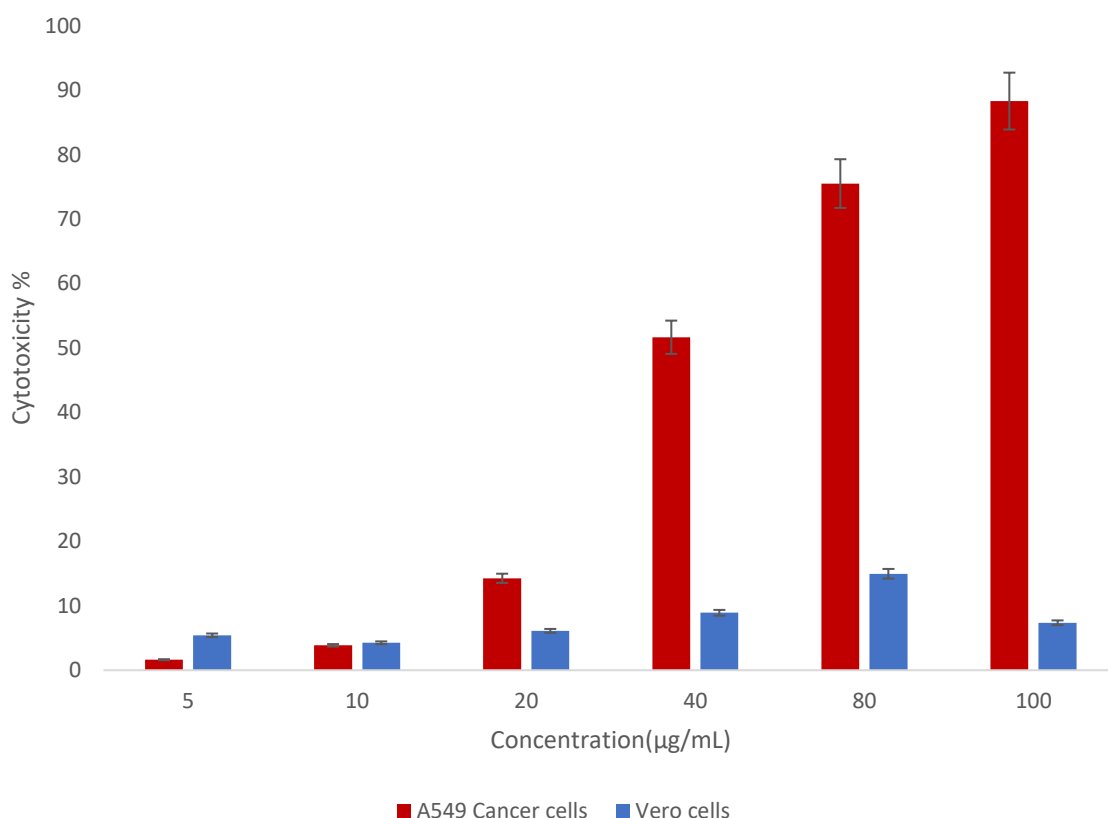


Fig 5.18: Representation of Comparative Toxicity of C₆₀ in A549 cancer cells and normal cells

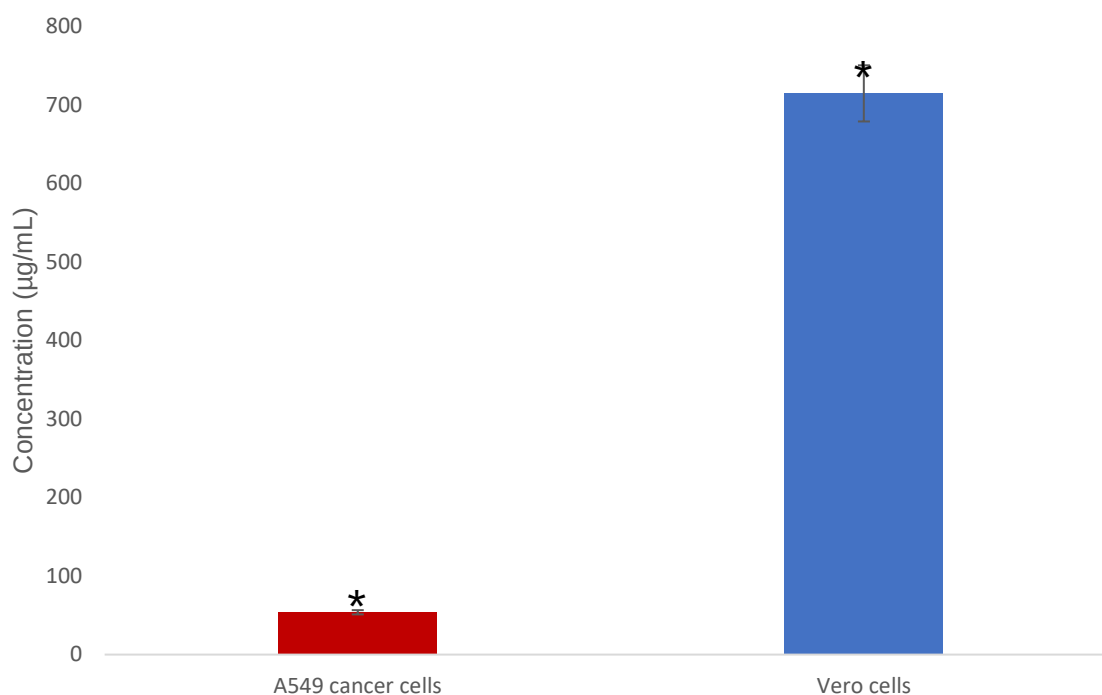


Fig 5.19: Comparison of IC_{50} values of A549 cells and Vero cells treated with C_{60} ; * represents $p < 0.05$

Fig 5.19 represents the variation in IC_{50} of Cancer cells and normal cells treated with C_{60} . Higher the IC_{50} value lower the toxicity, the graph depicts that the toxicity of C_{60} in vero cells are very less compared to A549 cancer cells.

The student's t-test was applied using Microsoft Excel, and the estimated $p < 0.05$ for the data was considered to be a significant difference. The results indicated that C_{60} can be used in cancer therapeutics as it shows selectively high toxicity towards cancer cells and less toxicity towards normal cells.

5.4 IN SILICO ANALYSIS

Fullerene C_{60} NPs showed significant activity for different drug targets in lung cancer treatment by forming good interactions with the binding sites of different protein targets. The binding energies of best docked compounds ranged between -8.0 kcal/mol and -11.71 kcal/mol. The minimized energy of C_{60} as ligand was estimated to be 10706.39 kcal. Fig 5.20 shows the docked images of various proteins and C_{60} as the drug.

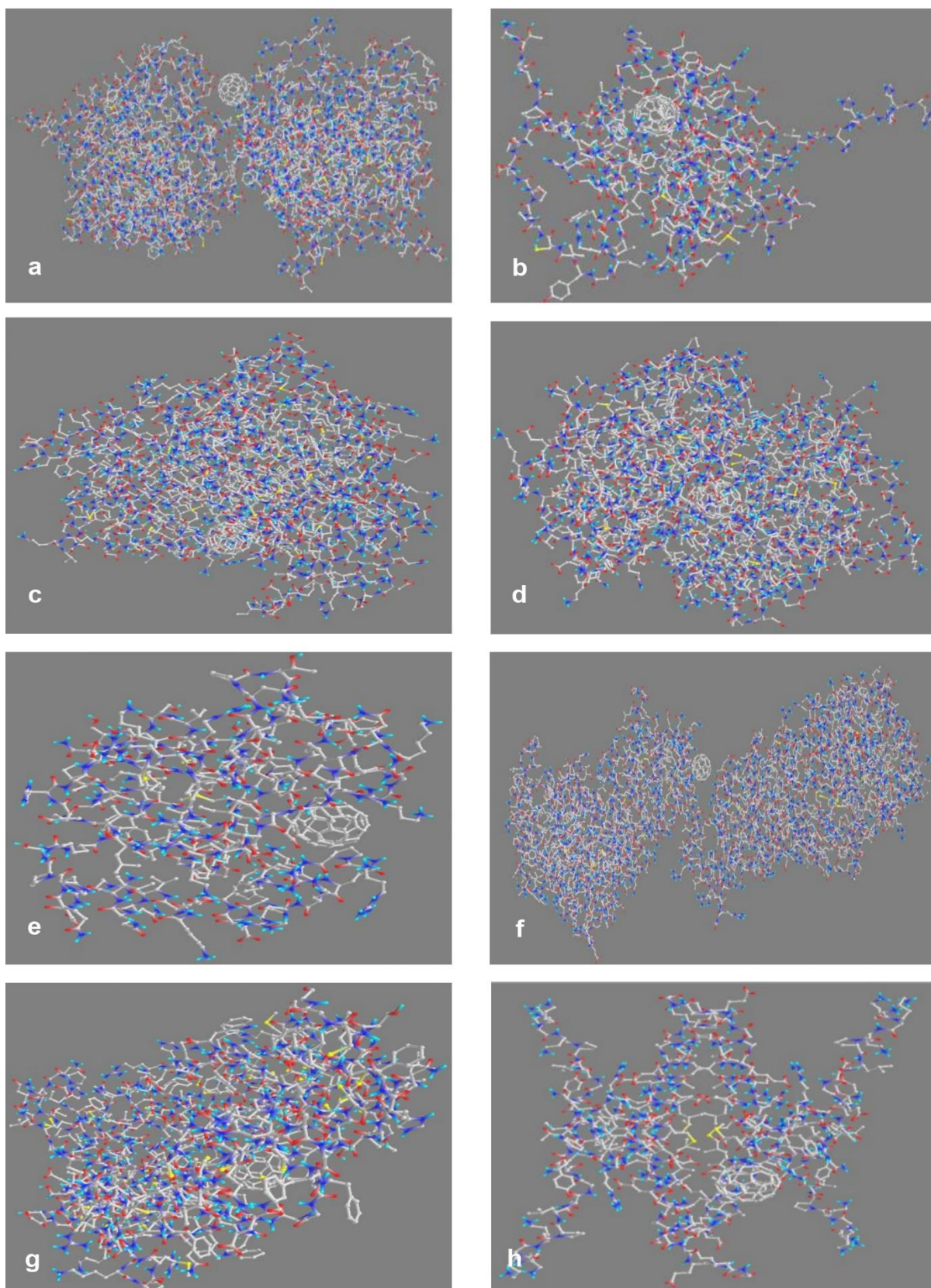


Fig 5.20: Molecular screening of docking between C_{60} and proteins related to cancer; a) Atg 4b; b) BCL-w; c) Caspase 7; d) GS-T; e) LC3A; f) Menin; g) TNF; h) P53

Apoptotic proteins such as Caspase 7, BCL-w and p53 showed higher binding energy proving it to be with the least binding affinity. Caspase7 is an important factor in the apoptotic pathway, any molecular interaction with this protein can lead to activation or inactivation of apoptosis. BCL-w is an antiapoptotic protein from the BCL-2 family, it inhibits the apoptosis pathway. P53 protein is a tumour suppressor protein which is involved in the cell cycle checkpoints and induces apoptosis in case of cell or DNA damage.

The results of molecular screening of Caspase7, BCL-w and p53 with C₆₀ inferred that C₆₀ may not affect the apoptotic pathway. Also, the results of docking of C₆₀ and Glutathione S-Transferase(GST) have shown less binding affinity. GST is an enzyme responsible for the detoxification of the cell. Since the binding affinity of GST and C₆₀ is less, the effect of C₆₀ inside the cell will not be affected by Glutathione.

Autophagic proteins such as LC3/Atg13 LIR complex (LC3A), and Atg4b were observed to be best docked with C₆₀. LC3A is a protein complex majorly associated with autophagosome formation same as that of Atg4b. Various studies have shown the autophagosome-inducing capacity of C₆₀ and the observed binding energy also proves the same.

A high binding affinity of Menin protein and C₆₀ was also observed. Menin is a tumour suppressor protein which is been recently found to be highly associated with lung cancers. Likewise, the Tumour Necrosis Factor (TNF) has also shown high binding affinity with C₆₀.

Table 5.1 compiles the binding affinities and RMSD values of UB and LB. A comparison between the binding energies of the selected proteins is represented in Fig 5.21. The graph depicts the best-docked molecules and least-docked molecules.

Table 5.1 Binding affinities of the protein docked with C₆₀

Proteins	Binding Affinity	RMSD/UB	RMSD/LB
Atg 4b	-8.6	0	0
	-8.6	4.877	0.121

	-8.6	3.366	0.038
	-8.6	3.938	0.584
	-8.6	5.68	0.116
	-8.6	5.398	0.086
	-8.6	5.507	0.598
	-8.6	5.681	0.151
	-8.6	5.682	0.136
BCL-w	82.3	0	0
	82.4	5.426	0.119
	82.4	5.68	0.104
	82.4	5.372	0.136
	82.4	3.263	0.135
	82.4	5.679	0.123
	82.4	5.681	0.197
	82.4	5.68	0.209
	82.4	5.678	0.238
Caspase 7	97.9	0	0
	97.9	3.39	0.628
	98.1	3.118	0.577
	98.7	5.199	0.697
	99.9	5.329	0.637
	99.9	5.314	0.598
	100.1	5.438	0.214
	100.6	5.663	0.934
	100.7	5.702	0.563
GS-T	0.1	0	0
	0.2	5.403	0.044
	0.2	4.935	0.05
	0.2	4.934	0.046
	0.2	4.939	0.107

	0.2	5.41	0.027
	0.2	4.888	0.082
	0.2	5.679	0.127
	0.2	3.408	0.105
LC3A	-10.1	0	0
	-10.1	5.682	0.15
	-10.1	4.91	0.152
	-10.1	5.438	0.164
	-10.1	5.401	0.153
	-10.1	4.955	0.144
	-10.1	5.414	0.153
	-10.1	3.314	0.148
	-10.1	4.928	0.152
Menin	-9.2	0	0
	-9.2	5.681	0.005
	-9.2	17.543	13.967
	-9.2	17.375	14.015
	-9.2	17.472	13.973
	-9.2	17.892	13.944
	-9.2	17.863	13.986
	-9.2	17.44	14.016
	-9.2	17.638	14.001
TNF	-9.6	0	0
	-9.6	5.679	0.136
	-9.6	3.338	0.08
	-9.6	4.934	0.084
	-9.6	5.68	0.061
	-9.6	5.679	0.061
	-9.6	4.892	0.119
	-9.6	3.364	0.115

	-9.6	5.41	0.066
P53	99.2	0	0
	100.4	5.507	0.697

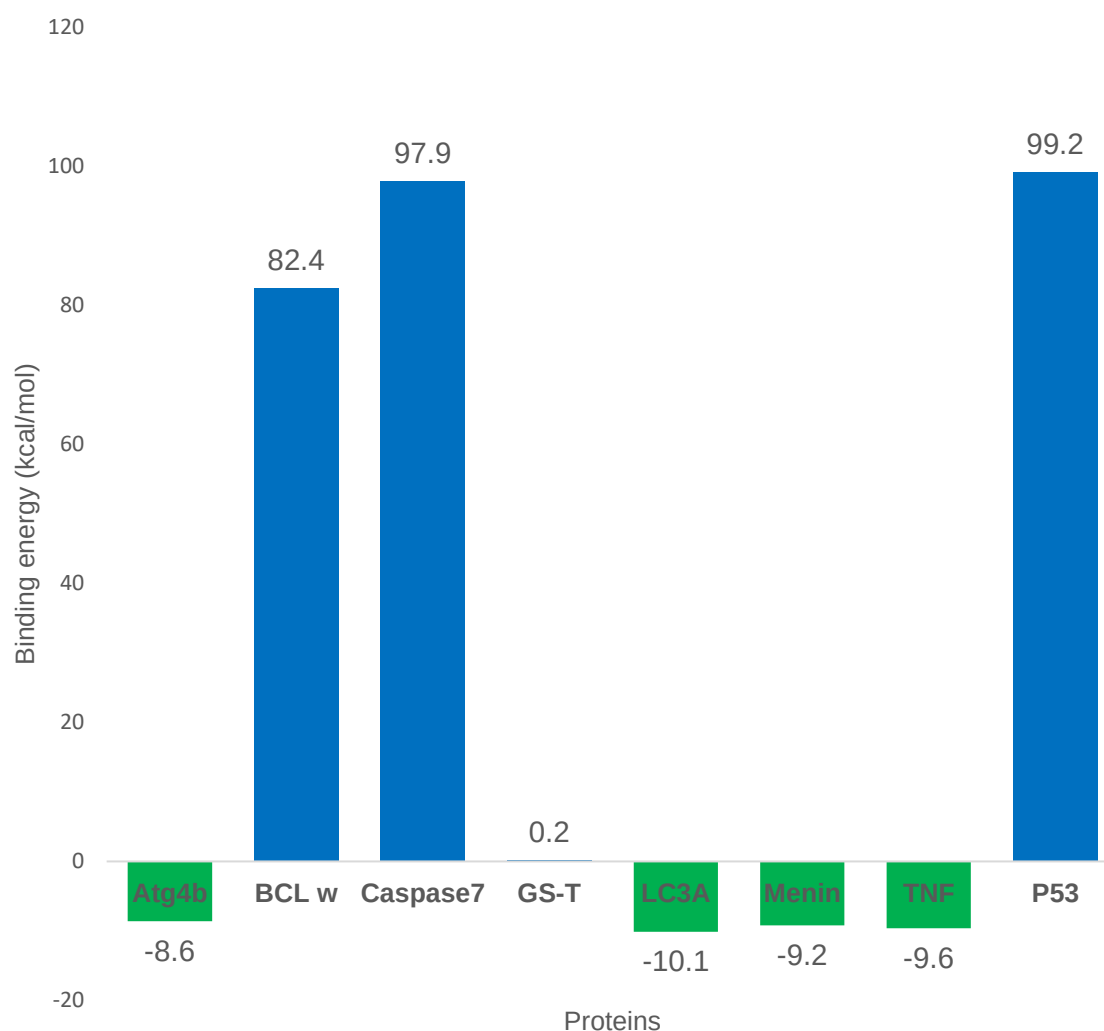


Fig 5.21: Comparative bar graph representing the binding energy of various proteins and C₆₀. The blue bars represent the least-docked compounds whereas the green bars represent the best-docked compounds

5.5 STATISTICAL ANALYSIS

Data analysis was performed using Microsoft Excel. Statistical analysis of data from studies on cultured cells was performed using Student's t-test for comparison. The difference was considered statistically significant at $p < 0.05$.

CHAPTER 6

SUMMARY AND CONCLUSION

In this study, the anti-cancerous efficacy of C₆₀ on A549 lung cancer cells and the toxicity of C₆₀ were evaluated. The C₆₀ Nps used in this study was pristine and undoped particles. Various physical characterization techniques were used to analyse the particle. UV–Vis absorbance peaks indicated the presence of Nps in the solution. FTIR analysis revealed the functional groups present in the sample due to the interaction with the environment. ζ potential and particle size analysis determined the C₆₀ Nps to be stable with a negative potential. XRD signified the crystallinity of the particle. FESEM analysis showed the symmetric surfaces of C₆₀. HRTEM images confirmed no aggregation of C₆₀ particles.

The cytotoxicity assay on A549 lung cancer cells proved that C₆₀ even at low concentration has a high anti-cancerous effect. The toxicity analysis on the Vero cells showed that the fullerene shows the least cytotoxicity even at high concentrations. The PI staining images proved that the treatment with C₆₀ induced cell disruption and cell death. The externalisation of phosphatidylserine from the cell plasma membrane was indicated by FITC staining. It was evident with the dual staining that C₆₀ causes cell death through apoptosis. DCFH-DA staining images proved that C₆₀ has a high potential in ROS formation than scavenging, this result supports the latest therapeutic approach of ROS-based therapy.

The differential toxicity analysis based on two different preparation methods was useful to conclude that Aqs/C₆₀ FAS was inefficient due to aggregation. The comparative cytotoxicity analysis with cancer and Vero cells proved that Tol/C₆₀ FAS shows selectively high toxicity towards cancer cells and less toxicity towards normal cells.

The *in silico* results have paved a way for future research on a molecular level. Apoptotic proteins such as Caspase 7, BCL-w and p53 showed higher binding energy proving it to be with least binding affinity whereas autophagic proteins such as LC3A and Atg4b was observed to be best docked with C₆₀. Various studies have

shown the autophagosome-inducing capacity of C₆₀ and the observed binding energy of the proteins also proves the same.

Collectively, the results from the study showed that pristine C₆₀ is selectively toxic towards cancerous cells. The DCFH-DA assay proved the high production of ROS in cancer cells treated with C₆₀, which may be used as a new approach in the treatment of cancer. Toluene-based preparation of C₆₀FAS was concluded to be the most efficient technique to solubilize C₆₀ Nps for therapeutic purposes. The study also suggests that when C₆₀ Nps are used for therapeutics, the influence of the C₆₀ on various gene expressions needs to be analysed on a molecular level. The results have concluded that C₆₀ Nps are a good candidate for lung cancer therapeutics. The achieved results are in full agreement with the literature.

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ANNEXURE

The book chapter on “Emerging Graphene-Based Nanomaterials As DNA Biosensors Using Lab-On-Chip, Computational Approaches And Robotics” was submitted on 12th August to Elsevier, CRC Press (Taylor & Francis) and is **ACCEPTED** for publication.



Britlin Deva Jebasta . N <britsam1977@gmail.com>

Fwd: Acceptance for the Contributed book chapter

Dr. Kunal Biswas <kunal.sapiens@gmail.com>

Wed, Feb 1, 2023 at 8:20 PM

To: sandhya selvakumar <sandy1162105@gmail.com>, mithrintha shankar <mithrinthaashankar@gmail.com>, "Britlin Deva Jebasta . N" <britsam1977@gmail.com>, "Rakshi A.D" <rakshising@gmail.com>, Bavani Latha <bavagold@gmail.com>, Jayashree Shanmugam <jayashree005@gmail.com>, sudhak7phd@gmail.com, thyagarajan@gmail.com

Dear all,

This is to inform :

Our book chapter has been accepted in CRC Press (Taylor and Francis).

Congratulations to all of you!

Regards,

Dr Kunal Biswas
Scientist C,
IRC

----- Forwarded message -----

From: Dr. Yugal Kishore Mohanta <ykmohanta@gmail.com>

Date: Wed, 1 Feb, 2023, 8:02 pm

Subject: Acceptance for the Contributed book chapter

To: Dr. Kunal Biswas <kunalbiswas.irc@sathyabama.ac.in>, Dr. Kunal Biswas <kunal.sapiens@gmail.com>

Dear Dr K Biswas

Greetings!

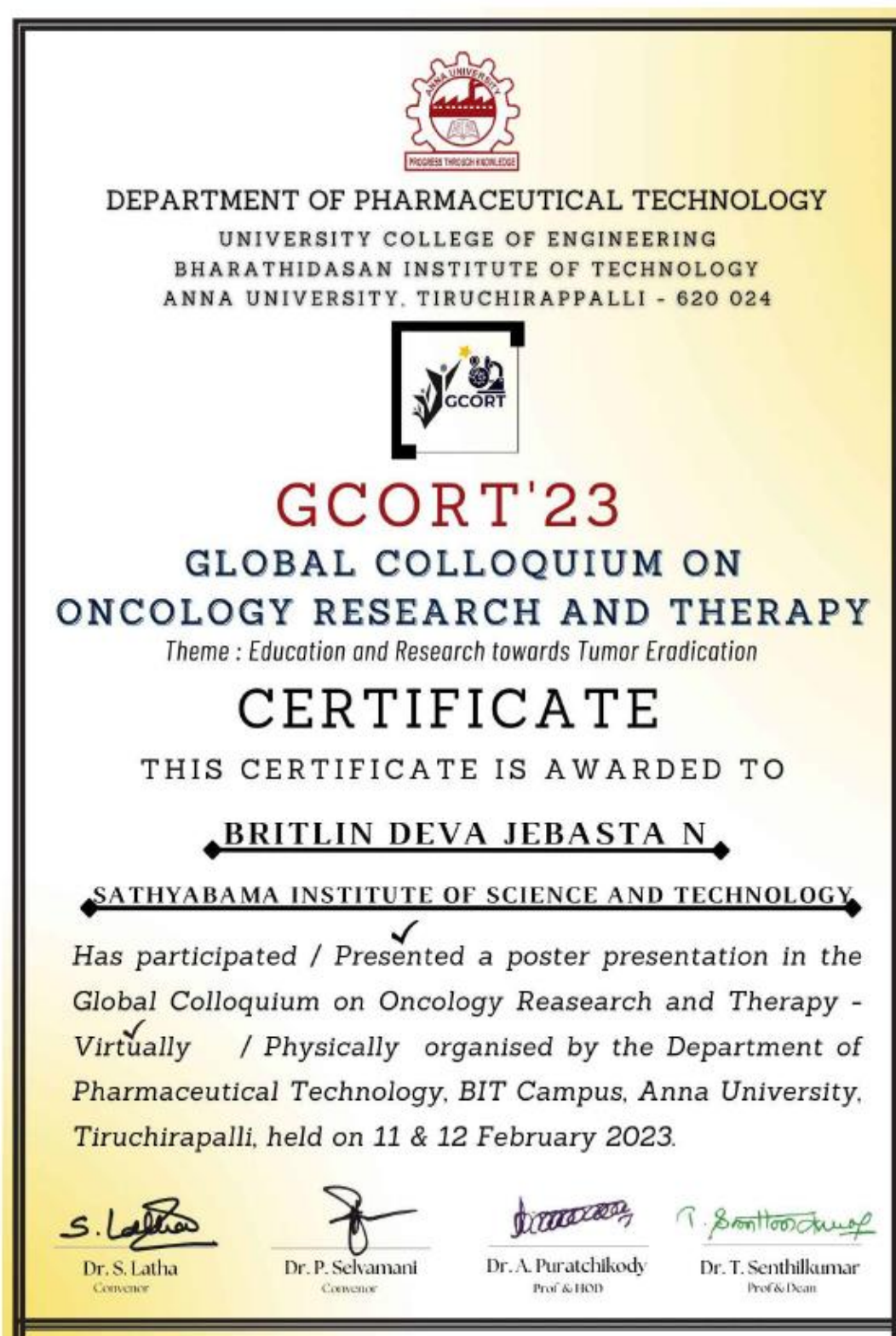
I am glad to inform you that the submitted chapter from your team EMERGING GRAPHENE-BASED NANOMATERIALS AS DNA BIOSENSOR(S) USING LAB-ON-CHIP, COMPUTATIONAL APPROACHES, AND ROBOTICS has been accepted for publication in the book Graphene Nanomaterials in Food, Health & Agriculture sectors published by CRC Press.

The final proof will be sent to you by the CRC publisher for your final corrections.

Best Wishes
Editorial Team

Dr Yugal Kishore Mohanta (he/him/his)

Presented a poster virtually on the topic **“FULLERENE AS A THERAPEUTIC APPROACH IN LUNG CANCER TREATMENT: A REVIEW”** in GCORT conducted by Anna University, Tiruchirappalli. The abstract is **published** in GCORT Proceedings by **“Association of Pharmacy Professionals”**



POSTER PRESENTATION (virtual mode)

FULLERENE AS A THERAPEUTIC APPROACH IN LUNG CANCER TREATMENT: A REVIEW

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Globally, cancer is one of the major causes of mortality in recent years. Lung cancer is one of the most common types of cancer due to increased smoking and pollution. Chronic obstructive pulmonary diseases also increase the risk of lung cancer. Currently available cancer treatment options include surgical treatment, chemotherapy, radiation therapy, or an amalgamation of these methods based on the stage of cancer. The traditional cancer treatment options caused complications and the adverse effect such as chemoresistance. However, the rapid development of new technologies offers new opportunities to improve the efficiency of cancer treatments.

Nanotechnology has been increasingly studied as a potential cancer treatment in recent years. The nanocarriers are useful in targeted drug delivery for cancer treatment. Fullerenes are allotropes of carbon made up of carbon atoms arranged with 20 hexagons and 12 pentagons, with fused rings and conjugated double bonds. They are highly symmetrical and extremely stable. C₆₀ fullerenes can pass through cell membranes and blood-brain barriers. They are bioresorbable and readily enter cells. Due to its cage-like structure and adaptability, it is useful as a drug delivery system. Nanoconjugates of fullerene and anticancer drugs are proven to be more effective at reducing toxicity, enhancing efficacy, and controlling drug release.

According to *in vivo* analysis, C₆₀ fullerenes have significant antioxidant activity, neutralising excess ROS in cells and potentially inhibiting some cell receptors. C₆₀FAS therapy has shown a reduction in tumour volume in mice models with Lewis lung cancer. Fullerene and Doxorubicin nanoformulation has shown a considerable decrease in tumour growth and improvement in the average lifespan of the animals to a greater extent. Additionally, Fullerene-based nanocomplexes have shown a significant amount of anti-cancer potential for improving alkaloid berberine's effectiveness against lung cancer. The C₆₀ fullerene being a part of the C₆₀-cis-pt nanocomplex has enhanced cis-pt entry and intracellular accumulation, boosting the cytotoxic effect of the drug on lung cancer cells. Recently, fullerene nanoformulation demonstrated the regeneration of lung epithelial cells that were treated with apoptosis-inducing molecules, implying its potential for Acute respiratory distress syndrome therapy. The present study compiles and analyses the uses of fullerene in the treatment of lung cancer.

Presented a poster on “**Toxicity of fullerene and its interaction with biosystems: A review**” for poster presentation at the 7th International Conference On Nanoscience and Nanotechnology (ICONN 2023) held at SRMIST from 27th – 29th March 2023.



Toxicity of fullerene and its interaction with biosystems: A review

Britlin Deva Jebasta Newton^{1*}, Bavanilatha Muthiah¹, Kunal Biswas²

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Abstract

Fullerene is an allotrope of carbon. It is bioresorbable and readily enters cells, making it a promising candidate in therapeutic. It also has amphiphilic and self-assembling properties, which are highly desired for effective drug delivery and tumour imaging. With such tremendous biomedical applications, toxicity is a major concern. Fullerenes can cause acute and long-term tissue damage by passing through biological barriers such as the blood-alveolus, blood-brain, and blood placenta. Fullerene has a negative impact on the liver and can alter the expression of its genes. Despite the cardiotoxic effects of some Carbon nanomaterials, fullerene has shown cardio-protective benefits against doxorubicin-induced cardiotoxicity.

In vivo analysis using freshwater fish, *Anabas testudineus*, fullerene C60 has shown genotoxicity even at sublethal concentrations. Even with radiation exposure, the interaction of fullerene and bacteria has shown negative genotoxicity. One of the potential risks of long-term fullerene exposure is the ability of both undoped and doped C60 fullerene to trigger inflammatory responses. The present study emphasizes the various aspects of the toxicity of fullerene.

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5. Trpkovic A, Todorovic-Markovic B, Trajkovic V (2012) *Arch Toxicol* 86:1809–1827.

Submitted the review article **“UNVEILING THE ANTI-CANCEROUS THERAPEUTIC APPLICATIONS OF PRISTINE AND DOPED C₆₀ FULLERENE: A REVIEW”** in **Journal of Pharmaceutical Investigation, Springer** and is currently **UNDER REVIEW**.



Britlin Deva Jebasta . N <britsam1977@gmail.com>

**JPIN-D-23-00087 - Submission Notification to co-author -
[EMID:0d0a67d1a5070967]**

1 message

Journal of Pharmaceutical Investigation <em@editorialmanager.com>

Wed, Apr 5, 2023 at 12:03 PM

Reply-To: Journal of Pharmaceutical Investigation <saranya.sekar@springer.com>

To: Britlin Deva Jebasta Newton <britsam1977@gmail.com>

Re: "UNVEILING THE ANTICANCEROUS THERAPEUTIC APPLICATIONS OF PRISTINE AND DOPED C₆₀ FULLERENE: A REVIEW"

Full author list: Britlin Deva Jebasta Newton; Bavanilatha Muthiah; Kunal Biswas

Dear Ms Newton,

We have received the submission entitled: "UNVEILING THE ANTICANCEROUS THERAPEUTIC APPLICATIONS OF PRISTINE AND DOPED C₆₀ FULLERENE: A REVIEW" for possible publication in Journal of Pharmaceutical Investigation, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dr Kunal Biswas who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

Springer Journals Editorial Office
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UNVEILING THE ANTICANCEROUS THERAPEUTIC APPLICATIONS OF PRISTINE AND DOPED C₆₀ FULLERENE: A REVIEW

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ABSTRACT

Background:

Cancer is one of the primary causes of mortality in recent days. Cancer is the uncontrolled growth and proliferation of cells forming a tumour, which gradually undergoes angiogenesis and metastases to nearby organs. Fullerene, an allotrope of carbon is found to be useful in cancer treatment in pristine and doped forms as well as drug and gene carriers. C₆₀ Fullerene, an allotrope of carbon, is highly beneficial in the treatment of cancer in both pristine and doped forms with the ability to form complexes with various anticancer drugs. C₆₀ fullerene nanoconjugates have shown high efficiency in cancer treatment with reduced toxicity of drugs on normal cells. Drug resistance is usually the limiting factor in the efficiency of cancer treatment, C₆₀ fullerene nanoconjugates have shown promising results in reversing chemoresistance.

Area Covered:

This review highlights the use of doped and pristine fullerene in cancer treatment. C₆₀ fullerene and its conjugates with different anticancer drugs have been clearly outlined. Various approaches have been extensively explained, including immunotherapy, photodynamic therapy, sonodynamic therapy, anti-angiogenesis, and reversing chemoresistance for cancer treatment. The toxicity aspect of the use of fullerenes and its nanocomplexes has been analyzed and clarified.

Expert Opinion:

The nanoconjugates of fullerene and anticancer drugs are highly efficient in cancer treatment with targeting, slow drug release, high efficiency and reduced cytotoxicity on normal cells. Photodynamic therapy and immunotherapy with doped and pristine fullerene nanoconjugates are promising approaches to eradicating cancer.

Keywords: Nanotechnology, Nanoconjugate, Autophagy, Photodynamic, Sonodynamic, Chemoresistance

1. INTRODUCTION:

Globally, cancer is one of the primary causes of mortality in recent days. Cancer is the uncontrolled growth and proliferation of cells forming a tumour, which gradually undergoes angiogenesis and metastases to nearby organs. Despite tremendous scientific advances in understanding the mechanisms that lead to cancer, the therapy's efficacy remains limited. Currently available cancer treatment options include surgical treatment, chemotherapy, radiation therapy, or an amalgamation of these methods based on the stage of cancer. The main mechanism by which traditional chemotherapy kills proliferating cancer cells is by interfering with DNA(Deoxyribonucleic acid) synthesis and mitosis. The anticancer agents used in chemotherapy cause unwanted and unfavourable adverse effects. As anticancer drugs are non-specific, they can harm healthy normal tissues as well. One of the causes of the increased death rate among cancer patients is the severe health consequences that chemotherapy medications have on healthy tissues and organs. This is a result of increased toxicity in normal cells and a rise in the occurrence of chemoresistance(Senapati et al. 2018).

However, the rapid development of new technologies offers new opportunities to improve the efficiency of cancer treatments. Nanotechnology has been increasingly studied as a potential cancer treatment in recent years. Tiny bead structures called nanoshells are used in tumour targeting (Anajwala et al. 2010). Nanoshell features exterior metallic layers that target cancer cells and destroy them while leaving healthy cells undisturbed(Bhakta and Barthunia 2020). Nanobots are small devices programmed to detect, analyse and kill cancer cells(Sharma 2012). The use of nanocarriers is useful in targeted drug delivery for cancer treatment. A nanocarrier's effectiveness is influenced by its biodegradability, bioavailability, circulation stability, and dispersion.

Fullerene, an allotrope of carbon, is useful in cancer treatment in the form of pristine, doped and as drug and gene carriers. Fullerenes or buckyballs are a family of molecules made up of carbon atoms arranged with 20 hexagons and 12 pentagons(Madannejad et al. 2019), with fused rings(Chen et al. 2012b) and conjugated double bonds(Augustine et al. 2017)(Figure 1). They are highly symmetrical and extremely stable. Fullerenes are bioresorbable and readily enter cells, which makes them attractive candidates for tumour therapy. Fullerene with amphiphilic and self-assembly characters(Chen and Elimelech 2006) is highly desirable for efficient drug delivery and tumour imaging(Naz et al. 2019).

Fullerenes in pristine and doped forms have high antineoplastic efficiency(Skivka et al. 2018). In addition, their spherical shape means that they are less likely to damage healthy tissues than other drug-delivery vehicles that are commonly used in chemotherapy. Depending on whether the molecule has a surplus or shortage of unpaired electrons, fullerenes can act as both donors and acceptors of unpaired electrons, attaching them to the double bonds(Shytikov et al. 2021). Fullerenes are biocompatible with strong free radical scavenging and antioxidant potential(Prylutska et al. 2014).

Particularly intriguing applications of fullerene include continuous and delayed drug release and tumour imaging (Naz et al. 2019). Fullerenes have been investigated in several preclinical studies as a treatment for a variety of different cancers. C₆₀ due to its cage-like structure makes it useful in drug delivery applications. Due to their adaptability, nanoparticle-based drug delivery systems have emerged as the perfect platform for the treatment of cancer. As its goal is to restrict the proliferation of tumour cells, the nano-carrier has demonstrated great potential in the treatment of lung cancer(Sharma et al. 2019). Fullerene as a nanocarrier of anticancer drugs including cisplatin, gemcitabine, docetaxel, paclitaxel, doxorubicin, landomycin, fluorouracil etc... has shown a tremendous decrease in toxicity *in vivo* and *in vitro*. Research on delayed drug release with fullerene nanovesicles has synthesized three amphiphilic functionalized fullerenes C₆₀R₅Cl (R = methyl ester of 4-aminobutyric/glutamic acid or phenylalanine) for effective drug release through regulation of vesicle polarity and size by amino acid methyl ester groups(Lin et al. 2017). There are several possible approaches to cancer treatment with fullerenes and nanoconjugates as anticancer agents. It includes Photodynamic Therapy(PDT), immunotherapy, Sonodynamic Therapy (SDT), anti-angiogenesis and reversing chemoresistance. Fullerene and its nano complexes show promising photodynamic effects for cancer therapy due to its ability to produce Reactive Oxygen Species (ROS) with efficient intersystem and illumination. This review explores the use of fullerene in cancer therapy. The toxicity aspects of fullerene are emphasized in the present study.

2. PROPERTIES OF C₆₀:

The third carbon allotrope, fullerene (C₆₀), has distinct photochemical, electrochemical, and physical properties(Yan et al. 2020b). Although C₆₀ contains 240 valence electrons, there are three sigma bonds between each carbon atom, with 180 electrons. Carbon atoms in

fullerenes are strongly pyramidalized and accordingly very reactive due to their spherical form. Through the orbital rehybridization of the sp^2 orbitals to the $sp^{2.27}$ orbitals with a p character gain, the conjugated carbon atoms depart from planarity (Castro et al. 2017). These electrons' energy is in the subsurface of the Fermi surface. They help in structure stabilization but not in conduction. The remaining 60 electrons are distributed to carbon-carbon π orbitals around the molecule (Forró and Mihály 2001). This closely packed structure has large voids between the ions, atoms or molecules. The unsaturated character of the C–C bonds on the fullerene enables various electronic states to accept electrons from appropriate donors. Due to its inclination to avoid double bonds in pentagonal rings, it has poor electron delocalization. As a result, C_{60} behaves like an electron-poor alkene and interacts easily with electron-rich molecules (Gokhale and Somani 1996).

Alkali and alkaline earth elements combined with fullerenes provide a unique range of MnC_{60} materials, which are primarily near-stoichiometric in nature (M can stand for Na, K, Rb, Cs, Ca, Sr and Ba). Charge transfer salts with fullerenes were made using both organic and inorganic compounds. Doped fullerene refers to a small, non-stoichiometric amount of charge transfer by adding a metal ion on or into the fullerene. Conduction bands are filled when C_{60} is doped with n-type alkali metals (Romberg et al. 1993). The C_{60} molecule's cage-like structure naturally provides two alternatives for doping: by adding a foreign atom M inside the cage (or) by swapping out one or more carbon atoms for atoms with different electrical structures (Forró and Mihály 2001) (Figure 2). Metals including Y, Sc, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Yb, Lu, Ca, Sr, Ba, and Ti could react with the carbon cage and enter it.

Endohedral metallofullerenes are produced using the laser furnace process, electric arc discharge, and ion implantation; however, due to their better yield, the electric arc is more frequently utilised (Chen et al. 2012b). The electric arc method involves accelerating ions and implanting them into the C_{60} cage. Just enough power should be provided for the ions to open and enter the cage. To prevent the atoms from escaping the cage, a significant portion of the initial kinetic energy should be absorbed and redistributed at the first collision. Due to their exceptional physicochemical properties, endohedral metallic fullerenes have a variety of uses. Particularly, polyhydroxylated Endohedral Metallofullerene was identified to have antineoplastic activity. The C_{60} molecule's unexpected capacity to create covalently linked polymers can be achieved through ionisation, high-pressure and high-temperature treatments, molecular collisions, and photo-excitation (Forró and Mihály 2001).

3. ANTI-CANCEROUS ACTIVITY OF FULLERENE C₆₀ :

3.1 PRISTINE C₆₀ :

The hydrophobicity, difficulty in obtaining a suitable concentration, and aggregation in the water of pristine C₆₀ fullerene restrict the research of its biological effects(Prylutska et al. 2014). The water-soluble, pure C₆₀ fullerenes can pass through cell membranes and are highly antioxidant. At low physiological quantities, they are non-toxic. C₆₀ fullerenes have significant antioxidant activity, which can block certain cell receptors and neutralise excess ROS in the cell(Prylutska et al. 2011b). C₆₀ fullerene has been reported to improve animal survival in a rat HCC model produced by DEN + CCl₄ and to suppress fibrotic and neoplastic degeneration and metastasis. The antioxidant capabilities of C₆₀ fullerene, such as its capacity to restore liver redox status and cytostatic effects, were the reason for better results. The ability of C₆₀ fullerene to decrease G6PD and upregulate the expression of p53 has been reported as the reason for decreased metastasis(Kuznietsova et al. 2020). According to a study, C₆₀ fullerenes have significant antioxidant activity, neutralising excess ROS in cells and potentially inhibiting some cell receptors, such as endothelial growth factor. C₆₀FAS therapy has been demonstrated to reduce Lewis lung cancer tumour volume in male C57Bl/6J mice(Prylutska et al. 2011a).

Treatment of DMBA-induced breast cancer with pristine C₆₀ nanoparticles has shown encouraging results when tested with Wistar albino female rats. Treatment of MCF-7 cells *in vitro* with fullerene C₆₀ at varying concentrations (0.1 to 100 mg/ml) has shown decreased cell survival in a dose-dependent manner. *In vivo* treatment with fullerene C₆₀ on rats has shown significantly elevated levels of caspase-3 but reduced levels of pro-survival protein leading to DNA fragmentation (Beyaz et al. 2022).

In BAL cells, C₆₀ fullerenes have increased sub-G1 and G1 arrest. Moreover, over the course of the experiment, lung tissue cell infiltration and the expression of genes related to tissue damage have been continuously observed confirming that C₆₀ fullerenes cause inflammatory reactions in the lung of the mouse(Park et al. 2010).

3.2 DOPED C₆₀ AS A DRUG AND GENE CARRIER:

C₆₀ has been proven to be compatible with mammalian cells for carrying drugs(Lucafò et al. 2012). Nanoconjugates of fullerene and anticancer drugs are proven to be more

effective at reducing cancer cell toxicity, enhancing efficacy, and controlling drug release. The simultaneous administration of the drug and fullerene has proved that the drug is protected from the reactive environment(Bulavin et al. 2018). C₆₀ in both pristine and doped forms can carry a drug as conjugate. The bioavailability of the drug has been proven to be significantly improved by fullerenes(Raza et al. 2015).

Few studies suggest that doped fullerene has a higher affinity in carrying drugs than pristine fullerene. The complexation of anti-cancer drugs with C₆₀ fullerene has increased their toxicity towards several human tumour cell lines, including those with distinct mechanisms of drug resistance(Panchuk et al. 2017). The interaction of 4-Phenylpyridine (4-phpy) with B or Si-doped C₆₀ fullerene and undoped fullerene, has revealed that doped fullerene had a higher binding affinity to carry the drug (Gökpek et al. 2017). The study on the interaction of penicillamine with Al-doped and undoped fullerene has shown that the binding energy of the Al-C₆₀-NH₂ complex is much stronger than that of pristine C₆₀(Ghasemi et al. 2018). The doping of C₆₀ fullerene with alkali metals has shown greater adsorption with fluorouracil molecules and easy release of the drug, near the cancer cells(Esrafil and Khan 2022). The doping of silicon atoms to the C₆₀ fullerene has been demonstrated to improve the adsorption mechanism. By examining the band changes in the carbonyl stretching vibrations and the natural bond orbital (NBO) features of the investigated complexes, the impact of favipiravir adsorption on the SiC₅₉ fullerene system and the nature of interaction have been evaluated(Parlak et al. 2017). The C₆₀-serPF conjugate has been found in most tissues in a mouse model of liver cancer, passing undetected via the blood-brain barrier and the tumour's altered vasculature while bypassing the reticuloendothelial system(Raoof et al. 2012).

3.2.1 Cisplatin

Currently, One of the most effective therapeutic agents used to treat cancer-related diseases is the water-soluble inorganic bi-valent platinum derivative known as cisplatin (cis-[Pt(II)(NH₃)₂Cl₂], Cis). This drug is especially effective against ovarian cancer, bladder cancer, oesophageal cancer, lung cancer, and head and neck cancer. Cisplatin, as an anticancer metal-containing drug has an alkylating effect and binds covalently to DNA, causing specific suppression of DNA synthesis and replication in malignant cells(Prylutska et al. 2017b). The use of Cis in anticancer chemotherapy is confined by the negative effects that are associated

with the drug's activity. There have been reports of Cis-induced nephrotoxicity, hepatotoxicity, and cardiotoxicity as well as abnormalities of the central nervous system and sensory organs.

In an aqueous solution, C₆₀ fullerene has been shown to form non-covalent nanocomplexes with Cis. The stability of the C₆₀ + Cis complex is determined by its concentration, lipid composition, and thickness of the lipid membrane's hydrophobic region. In *in vitro* tests with Hela cells, the C₆₀ + Cis complex has shown membranotropic effects at increasing drug concentrations (Bychko et al. 2016). The strength of its intercalation into lipid membrane has also increased at high concentrations without the disruption of the complex. Compared to Cis and FC₆₀ alone, the C₆₀+Cis complex has produced a two-fold increase in tumour growth inhibition (Prylutska et al. 2017a). Cisplatin's cytotoxicity toward the HL-60/adr and HL-60/vinc tumour cells, including cisplatin-resistant sublines, has been improved by fullerene C₆₀ (Dzhemilev et al. 2019). Caspase 3/7 activation and the externalisation of phosphatidylserine on the outside of LLC cells have been used to demonstrate that the C₆₀- Cis-Pt nanocomplex causes apoptosis. The C₆₀ fullerene being a part of the C₆₀-Cis-Pt nanocomplex is considered to have enhanced Cis-Pt entry and intracellular accumulation, boosting the drug's cytotoxic effects on lung cancer cells (Prylutska et al. 2019a).

3.2.2 Docetaxel

Docetaxel, also known as DTX [(N-debenzoyl-N-tert-butoxy-carbonyl)-10-deacetyl paclitaxel], is an anti-metabolite and a type of anti-neoplastic drug (Suzuki and Tokuda 2001). It is stated that DTX is more effective than paclitaxel (Raza et al. 2015). DTX is used to treat several cancers, including ovarian cancer, prostate cancer, small cell lung cancer, breast cancer, head cancer, neck cancer, and non-small cell lung cancer. In cases of hormone-refractory prostate malignancies, DTX helps to increase life expectancy and quality of life. DTX often causes cytotoxicity by stabilising microtubule arrays, which then prevents microtubule depolymerization. The scarcity of free tubulin, which is necessary for the formation of microtubules, is caused due to this stabilisation. Therefore, it limits the anaphase and metaphase stages of mitotic cell division, which stops the proliferation of cancer cells and triggers the expression of the IL-8 (interleukin) gene (Thotakura et al.

2018). Microtubule depolymerization results in apoptosis. Apoptosis can also be induced by inhibiting the apoptosis-inhibitory bcl-2 oncoprotein.

DTX has been successfully conjugated to the C₆₀-fullerenes to overcome the numerous problems with DTX distribution and take into account the many beneficial characteristics of C₆₀-fullerenes in drug delivery. This strategy achieved the expected advantages of improved cytotoxicity against cancer cells and pharmacokinetic profile. The system was largely compatible with erythrocytes(Raza et al. 2015).

3.2.3 Doxorubicin

Doxorubicin (DOX) is yet another extremely efficient cancer chemotherapy drug(Prylutsky et al. 2015). Dox is an anthracycline antibiotic(Hanušová et al. 2011) with a broad spectrum of therapeutic action against solid tumours and hemoblastoses, lung cancer, and acute leukaemia. Dox non-covalently binds to DNA, inhibiting nucleic acid synthesis, and has significant antimitotic activity and a strong mutagenesis effect, but is harmful to normal tissues and cells.C₆₀ fullerene has an antioxidant and antitumor nanostructure that may be useful in modulating cellular responses to Dox. C₆₀ fullerene protects against doxorubicin (Dox)-induced oxidative stress in Dox-induced chronic cardio- and hepatotoxicity *in vitro*(Prylutska et al. 2014).C₆₀ fullerene and Dox combined anticancer therapy is more effective than Dox alone.

A covalent compound of C₆₀ and DOX has been recently developed to make the resultant conjugation, hydrophilic. The amide linkage appears to have preserved the anti-neoplastic activities of DOX as well as the intrinsic properties of the fullerene cage. Surprisingly, conjugates frequently differ significantly from free drug molecules in cellular absorption and dispersion. C₆₀-DOX conjugate was found largely in the cytoplasm, in contrast to free DOX molecules, which were found mostly in the cell nucleus(Chen et al. 2012b). C₆₀ + Dox complex treated mice had tumour volumes that were 1.4 times less than those of untreated control animals(Prylutska et al. 2015). A male C57Bl/6J mice with Lewis lung cancer had been treated with a C₆₀+Dox compound, which considerably (by 2.5 times) suppressed tumour growth and improved the average lifespan of the animals to a greater extent by 63% than was seen with the administration of Dox or C₆₀ fullerene alone(Panchuk et al. 2015). A study has shown that the fullerene-Dox conjugate, due to its lack of

cytotoxicity, seems to have the scope for the safest delivery of toxic drugs into cancer cells(Butowska et al. 2019).

3.2.4 Fluorouracil

Fluorouracil (FU) is an antineoplastic, immunosuppressive, radio-sensitizing agent(Shakerzadeh 2021). FU is a commonly used drug in chemotherapy to treat breast cancer, head and neck cancer, liver cancer, colon cancer, stomach cancer, anal cancer and skin cancer(Hazrati and Hadipour 2016). 5-FU has undesirable side effects, including myelotoxicity, hand-foot syndrome, stomatitis, neurotoxicity, and cardiotoxicity. In order to reduce the side effects, the potential for targeted delivery using 5-fluorouracil (5-FU) loaded fullerene in cancer therapy has been investigated(Hazrati et al. 2017). Drug delivery clusters are thought to be strongly protonated in low pH circumstances in malignant cells, which would improve drug separation from fullerene and release kinetics from the cluster's surface, according to *in vitro* drug release profiles(Naz et al. 2019). The combination of pure C₆₀FAS, 5-fluorouracil (5-FU), and pyrrole derivative was reported to reduce the number of 1,2-dimethylhydrazine-induced tumours and the size of colon lesions in rats(Lynchak et al. 2017).

3.2.5 Landomycin

Streptomyces bacteria produce a class of angucycline polyketides known as landomycins (LAs) with antiproliferative abilities(Yushchuk et al. 2019). Since their linear glycosidic chains solely comprise di- and tri-deoxy sugars, all natural LAs have the same aglycon (landomycinone), and their only difference is the number of sugar residues in those chains (b-D-olivose and a-L-rhodinose). It has been demonstrated that LAs with longer saccharide chains have greater action against tumour cell lines *in vitro*, with LA-A (which has six sugar residues) being the most effective member of the family. The non-covalent complexation between C₆₀ fullerene and angucycline antibiotic Landomycin A has been reported as the most potent molecule within the family of angucycline drugs(Prylutskyy et al. 2016). The vital importance of unique extramitochondrial hydrogen peroxide generation and early caspase-7 activation upstream of mitochondria has been demonstrated by LA with three sugar residues (LA-E)(Bilobrov et al. 2019). An experiment with landomycin on

human cancer cell type KB-3-1 resulted in the appearance of classic morphological indications of apoptosis such as cell shrinkage, chromatin condensation, and the development of apoptotic bodies(Korynevskaya et al. 2007). When tested *in vitro* on two cancer cell lines, the C₆₀-LA nano complex has exhibited significant toxicity on tumour cells and much lower toxicity on mesenchymal stem cells(Bilobrov et al. 2019).

3.2.6 Paclitaxel

The C₆₀-paclitaxel conjugate is the first C₆₀-based chemotherapy to be synthesised(Mekhail and Markman 2002). It was created as a slow-release drug administration system for inhaling liposome aerosol. This conjugate demonstrated strong antitumor activity in tissue cultures and possesses therapeutically applicable hydrolytic kinetics. Animal model testing for its anticancer efficacy is still being conducted. According to the research, the conjugate's anticancer potency *in vitro* was comparable to that of free paclitaxel, and it showed potential for improving paclitaxel's therapeutic efficacy *in vivo*(Benedicta et al. 2022). The *in vitro* therapeutic effectiveness of the paclitaxel-embedded C₆₀ formulation of paclitaxel was compared to that of Abraxane, a paclitaxel formulation that is commercially available and bound to nanoparticles of albumin. The results showed that paclitaxel-embedded fullerene had similar efficacy to Abraxane in cell viability experiments; additionally, pristine fullerene was not cytotoxic(Chen et al. 2012b). This conjugate has been proven to be efficient in lung, ovarian and breast cancer(Wagner et al. 1997).

3.2.7 Berberine

Berberine (Ber) is a herbal alkaloid with a great anticancer effect. It has been discovered that intracellular uptake of Ber is promoted by complexation with C₆₀. The C₆₀-Ber nanocomplexes have shown greater antiproliferative potential toward CCRF-CEM cells as C₆₀ concentration was increased. When CCRF-CEM cells were exposed to C₆₀-Ber nanocomplexes, apoptosis has been induced by the activation of caspase 3/7 and accumulation in the sub-G1 phase(Grebinyk et al. 2019a). Additionally, it has been found that C₆₀-based nanocomplexes offer a significant amount of anti-cancer potential for improving alkaloid Ber's effectiveness against lung cancer(Grebinyk et al. 2021).

3.2.8 Crizotinib

The drug Crizotinib (CZT) fights lung cancer. The protein made by the ALK fusion gene is silenced by the oral tyrosine kinase inhibitor (TKI) crizotinib (Rothschild and Gautschi 2013). As a multikinase inhibitor, crizotinib has strong anti-MET activity. Met exon 14 alterations are the primary causes of non-small cell lung cancers (NSCLCs) (Drilon et al. 2020). Though crizotinib has significant advantages in the treatment of cancer, it also has harmful side effects. Due to certain characteristics, including great stability in energy efficiency, hydrogen bonding, good solubility in the aqueous phase, and physical absorption of the drug with fullerene, the interaction between CZT and C₆₀COOH has been identified in AIM and NBO evaluation. The study has revealed a decrease in toxicity and an increase in crizotinib's antineoplastic activity (Ghasemi et al. 2020).

3.2.9 Gemcitabine

One of the most effective anticancer drugs is gemcitabine (GEM), a pyrimidine nucleoside antimetabolite. This drug is licenced for the treatment of non-small cell lung cancer, pancreatic, bladder, liver, ovarian and breast cancer and has demonstrated antineoplastic activity in a wide range of solid tumours. Gemcitabine has a low incidence of non-haematological events and a low incidence of myelosuppression, thus with less toxicity (Toschi et al. 2005). The cytotoxic effects of nanoC₆₀GEM have been demonstrated with pancreatic cancer cell lines which expressed the production of ROS after blue LED irradiation (Nalepa et al. 2020).

Summary of different drugs that use fullerene as a drug carrier is tabulated in Table 1. Fullerene has shown tremendous potential in disease prevention and therapy by delivering exogenous nucleic acids such as DNA into cells, known as transfection. It has been reported that any water-soluble amino-fullerene could bind to double-stranded DNA. Tetrapiperidinofullerene made from fullerene, piperazine, and oxygen is the most effective in transfection. The fullerene derivative demonstrated significantly better efficiency for stable transfection and a stronger potential to transfect confluent cells as compared to the conventional approach (Chen et al. 2012b). The research on fullerene/DNA interactions led to the identification of tetraamino fullerene as a promising option for a fullerene method of gene transfer to mammalian cells. This cationic compound adheres to duplex DNA, delivers the fullerene/DNA complex into the cytoplasm and allows the

encoded gene to be expressed either transiently or steadily with efficiency comparable to or higher than that achieved by lipofection(Djordjević et al. 2006). The DNA ingested by endosomes were protected from enzymatic digestion by fullerene. Water-soluble lysine-based C₆₀-fullerene nanoconjugates (CF-LYS-TEG-MMF) have been discovered to have significant biocompatibility with erythrocytes and enhanced cytotoxicity on neuroblastoma cells. A pH-based drug release pattern was included in the developed nanoconjugate, which reduced drug leaching at plasma pH (Kumar et al. 2018). Even though these C₆₀-based transfection vectors of the first generation had promising potential, some of them also showed substantial cytotoxicity.

4. VARIOUS APPROACHES USING FULLERENE C₆₀ IN CANCER TREATMENT:

The photodynamic effect, anti-angiogenesis, efficient immunomodulation, control of oxidative stress, induction of autophagy, and sonodynamic effect are all different approaches for treating cancer with fullerene(Chen et al. 2012b)(Chen et al. 2012a). Following exposure to C₆₀ fullerene, immune cells have shown increased expression of the genes related to oxidative stress, metalloendopeptidase activity, the inflammatory response, and MHC. In rats with experimental adjuvant-induced arthritis, C₆₀ fullerene has inhibited pro-inflammatory (classically)-activated macrophages. With or without photoexcitation, C₆₀ Fullerenes can both quench ROS and generate it. C₆₀ Fullerenes also induce oxidative metabolism. A combination of direct effects on tumour cells, effects on the tumour microenvironment, and host immune system activation constitute the fundamental mechanism of the C₆₀ fullerene-based cancer treatment (Skivka et al. 2018). A study has revealed a double-strike approach against cancer cells based on Dox's antiproliferative activity and C₆₀'s photoinduced pro-oxidant activity(Grebinyk et al. 2019b). *In vivo* and *in vitro* experiments on the on-off mechanism using C₆₀-DOX-NGR NPs have shown its capacity to target tumours, remote-control drug release capability, and integrated treatment effect, high antitumor efficacy and low toxicity to normal tissues (Shi et al. 2016).

4.1 PHOTODYNAMIC THERAPY

PDT combines laser light, molecular oxygen, and photosensitizer, to cause oxidative stress by the light of a certain wavelength to eradicate the tumour. Each of these elements

is safe by itself, but when combined, they can produce ROS that can kill cancer cells. PDT is a non-invasive treatment approach (Juarranz et al. 2008). The photosensitizer is a molecule which often absorbs a photon that excites an outer shell electron to a singlet state (S₁), after which it can either return to its ground state or undergo intersystem crossing to a triplet state. Fullerenes form ROS upon illumination due to the absorption of visible light and an effective intersystem crossing to a long-lived triplet state, making them photosensitizers. The triplet state reacts with oxygen to produce ROS including free radicals or singlet oxygen species (Yan et al. 2020b)(Dos Santos et al. 2019).

ROS is an aggressive chemical oxygen species that can quickly react with any adjacent biomolecules, killing any nearby cells through apoptosis or necrosis. This includes both normal and pathological cells. Free radicals produced from fullerenes can be produced by two key mechanisms, both of which have anti-cancer effects. In type 1 photochemical mechanisms, Fullerenes receives electrons from reducing agents like NADH or NADPH that is present in the cell causing them to contain an additional unpaired electron and behave like a radical anion C₆₀^{•-}. This transfers an electron to oxygen during the reaction, creating ROS like superoxide or hydroxyl radical. In a type 2 reaction, energy is directly transferred to the oxygen molecule to create exceptionally cytotoxic singlet oxygen (Benedicta et al. 2022). The mechanism of the photodynamic effect is illustrated in Figure 3. Photosensitizer that is selectively localized in the target tissue, activated by a specific wavelength of light produces ROS and resulted in photodamage and subsequent cell death causing tumour ablation (Guan et al. 2016). PDT can cause three pathways of cell death including apoptosis, necrosis and autophagy-mediated cell death (Agostinis et al. 2011).

Photosensitizers can be classified as porphyrins (tetrapyrrole) and non-porphyrins (non-tetrapyrrole). Fullerene–porphyrin complex is highly efficient in singlet oxygen generation and cell penetration (Constantin et al. 2010). C₆₀ modified with polyethene glycol (PEG) is reported to have the ability to accumulate in tumour tissue and have a photodynamic impact on tumours. The volume of the tumour has significantly reduced after intravenous injections of C₆₀-PEG conjugate into tumour-bearing mice, along with the exposure of visible light to the tumour site. The C₆₀ conjugation has also demonstrated a better anticancer impact than Photofrin. It has been observed that the largest inhibition of tumour growth was seen in the C₆₀-PEG conjugates made with the highest molecular weight methyl-terminated PEG. This conjugate displayed the longest half-life period in blood circulation and the maximum tumour accumulation (Shi et al. 2014).

Radiofrequency absorbing substances are used in radio frequency thermal therapy (RTT) to transform RF irradiation energy into heat, which causes the thermal ablation of cancer cells. MCF-7 cells and tumour-bearing mouse models have been used to assess the PDT, RTT, and tumour-targeting effectiveness of C₆₀eIONPePEGeFA. Additionally, C₆₀eIONPePEGeFA is also useful for *in vivo* imaging. Using the outstanding active tumour targeting and magnetic targeting capabilities of C₆₀e IONPePEGeFA, cancer cells can be selectively targeted in highly concentrated areas. C₆₀e IONPePEGeFA has demonstrated good PDT, RTT efficacies, tumour targeting properties, and tumour diagnostic ability *in vitro* and *in vivo* (Yan et al. 2020b). The *in vitro* and *in vivo* studies of the C₆₀-IONP-PEG/HMME complex have shown excellent PDT efficacy, magnetic targeting property and MRI ability when tested with mice-bearing tumours (Shi et al. 2013).

High solubility sugar derivatives of C₆₀ are also proven to eradicate cancer cells with exposure to light. Glyco-conjugated C₆₀, when illuminated has worked as photoactive sweet C₆₀(Rondags et al. 2017) by targeting pancreatic stellate cells of pancreatic adenocarcinoma(Serda et al. 2018). A water-soluble polysaccharide known as pullulan with a high affinity for asialoglycoprotein receptors has been chemically combined with C₆₀ to provide a novel cytospecific photosensitizer for photodynamic anticancer therapy. In comparison to HeLa cells lacking asialoglycoprotein receptors, the C₆₀-pullulan conjugates greatly reduced the proliferation of HepG2 cells. Additionally, compared to C₆₀-PEG conjugates and saline, C₆₀-pullulan conjugates have shown a significantly higher photodynamic effect on the HepG2 neoplasm mice(Liu and Tabata 2010).

In vitro investigations show that the malonic acid derivatives of C₆₀ also exhibit considerable cytotoxicity and visible photosensitive action. The synthesized fullerene-maleic anhydride compound had stronger anticancer effects on HeLa cells and bone tumour cells. After being injected with the derivatives and lit with an I-W light, the tumour *in vivo* suffered significant damage (500 W)(Chen et al. 2012b). According to a study, C60 nanoparticles activate p38MAPK by promoting the formation of ROS, which causes cancer cell damage(Li et al. 2013). A study has concluded that HA-C₆₀ could produce a significant amount of ROS during PDT(Hou et al. 2017). A study has confirmed that C₆₀ fullerene and high-power LEDs with a 405 nm wavelength could be used as the light source for PDT on cancer cells. When C₆₀ fullerene's potential phototoxicity was evaluated using LEDs of various wavelengths on human leukemic cells, the most beneficial effect had been at 405 nm, 10 J/cm²(Grebinyk et al. 2018). When combined with light irradiation, pure C₆₀ had a

lethal impact on T-lymphocytes that had transformed. The longer exposure period and higher C₆₀ content increased the photodynamic impact. According to research work, C₆₀-amino acid complexes (C₆₀-phe or C₆₀-gly) are effective PDT-enhancing agents for killing cancer cells by inducing apoptosis upon illumination. The photochemical mechanism may be mediated by both superoxide and singlet oxygen (Li et al. 2014).

PDT has high efficiency with endoscopically accessible tumours including lung cancer, bladder cancer, gastrointestinal cancer and fallopian cancers, and also in dermatology for the treatment of non-melanoma skin cancers (Juarranz et al. 2008). A recent study has revealed the ability of bacterial cellulose(BC)- C₆₀ nanocomposite to treat skin cancer with PDT (Chu et al. 2018).

4.2 SONODYNAMIC THERAPY

SDT, inspired by PDT, has recently developed as a non-invasive cancer treatment technique that relies on the activation of particular chemical sensitizers with Ultrasound(US). US waves have an acoustic pressure, focussing energy into small volumes and have a suitable tissue attenuation coefficient for permeating biological tissues (Yumita et al. 2016). Another key characteristic of US is its ability to produce sonoluminescence or light emission within an excited medium. It is commonly believed that the SDT mechanism is caused by the cavitation impact of US. When US waves travel through liquids, a peculiar physical process known as acoustic cavitation causes bubbles to form, expand, and then burst. Sonoluminescence results from the bubble explosion, which releases the stored energy.

The combination of the US of 1 MHz and pristine C₆₀ together for the dynamic treatment of cancer cells has induced sonoexcitation of pristine C₆₀ thereby killing carcinoma cells. *In vitro* tests with HeLa cells have shown a sizable proapoptotic sonotoxic activity (Radivoievyh et al.).

The high correlation in the presence of Polyhydroxy fullerene shows that membrane lipid peroxidation is an important intermediary step in sonodynamically produced cellular damage. The inhibitory effects of histidine and tryptophan have shown that singlet oxygen plays a significant role in Polyhydroxy fullerene-mediated membrane sonosensitization

and sonodynamic cell lysis(Yumita et al. 2014). The combination of US exposure and PHF treatment has inhibited the growth of implanted colon cancer cells(Yumita et al. 2013).

4.3 REVERSING CHEMORESISTANCE

The treatment of tumours in healthcare facilities is frequently hindered by drug resistance, often reduces the accumulation and activation of the drug increases the drug-induced damage repair, changes drug targets and gene expression, destroys anatomical drug barriers, and results in the treatment of cancer not being successful(Chen et al. 2012b). Chemotherapy resistance has been classified into two types: noncellular and cellular. Non-cellular resistance is a term used to describe extracellular elements like the tumour micro conditions and the reachability of constrained vascular systems. The cellular resistance mechanism is further divided into conventional transport-based and non-transport-based resistance. The transport-based cellular resistance mechanism includes ATP-binding cassette transporters, like P-glycoprotein (P-gp), breast cancer resistance protein, Multidrug resistance (MDR)-associated protein, and solute carrier proteins. The non-transport-based cellular resistance mechanisms are related to changes in specific enzymatic activity and several cell death systems, particularly type I programmed cell death - apoptosis and type II programmed cell death - autophagy(Chen et al. 2018). Following prolonged chemotherapy, MDR frequently develops, leading to refractory cancer and tumour recurrence (Li et al. 2017). The primary mechanisms involved in the reversal of MDR based on the biological pathways involved include a reduction in drug efflux pumps, a rise in intracellular drug concentration and endosomal escape, disruption of cell membranes and intracellular organelles, and phototherapy(Curcio et al. 2020).

The C₆₀-Dox Complex has demonstrated an increase in its toxicity toward numerous human tumour cell lines, including those with varied drug resistance mechanisms(Panchuk et al. 2015). The photodynamic impact of C₆₀ has been observed on leukemic L1210 cells that are both sensitive and resistant to cisplatin. After C₆₀ photoexcitation, drug-resistant cell survival decreased, which was closely related to a considerable decline in mitochondrial potential. According to the study, C₆₀-based PDT may be a viable option for regaining drug-resistant leukemic cells' sensitivity to the induction of mitochondrial death, thereby reversing chemoresistance(Franskevych et al. 2017).

4.4 IMMUNOTHERAPY

Malignant cells have discovered ways to evade the immune system, which allows them to grow into resistant tumours. The surveillance and the prevention of malignancy are the crucial roles played by our immune system. Autophagy is a balanced, destructive degradation process that transports cargo to the lysosome for breakdown. To maintain correct cell function and metabolism, autophagy is crucial for the elimination of defective or dysfunctional organelles and protein aggregates. It also enables the recycling of cellular components (Chen et al. 2018). Nanoparticles are widely recognised for their interaction and impact on the immune system.

A high dose of nano-C₆₀ fullerene in glioma cells causes necrosis, whereas low doses usually cause autophagy (Sepand et al. 2020). It has been demonstrated that the fullerene C₆₀ nanoparticle (nC₆₀) induces autophagy and makes cancer cells more susceptible to chemotherapy death. In a sequential process of autophagy, phagophores start to envelop and pack organelles to form autophagosomes. Afterwards, autophagosomes and lysosomes combine to generate autolysosomes (Wei et al. 2010). Depending on the conditions, the digestion of the ingested cytoplasmic contents results in the production of small molecules for the synthesis of macromolecules as an energy source, ultimately leading to either cell survival or cell death. The mechanism is illustrated in Figure 4.

Tumour growth as well as malignant transformation can be inhibited by autophagy. It is understood that spontaneous cancers are frequently caused by a decrease in autophagic capability or faulty autophagy. The importance of autophagy in cancer cells, and how nC₆₀(Nd) improved chemotherapeutic susceptibility via modulating autophagy has been demonstrated (Wei et al. 2010). Compared to Nano-C₆₀, fullerene Nano-C₆₀/C₆₀(Nd) has shown more ability to induce autophagy and improve the chemotherapeutic elimination of both normal cancer cells and chemo-resistant cancer cells (Chen et al. 2012b). Under conditions of acute colitis and colon cancer, C₆₀ FAS has partially corrected local and systemic morphofunctional abnormalities in the colon, liver, and kidney. Also, it has exhibited anti-inflammatory and anticancer activities. Increased serum urea and creatinine were brought back to normal levels by C₆₀FAS, indicating that renal function has been restored (Kuznietsova et al. 2017).

The interaction of Nano-C₆₀ with the cellular autophagic signalling system has shown that the *Atg5* gene was the mediator of the chemosensitization effect of Nano-C₆₀. Nano-C₆₀

sensitises chemotherapeutic death of cancer cells in a photo-enhanced and ROS-dependent manner at noncytotoxic doses(Zhang et al. 2009). C₆₀-doped Melanin nanoparticles (MNP) combined with hyaluronic acid have shown effective theranostic and immunotherapy of tumours when excited by near-infrared light. This study is the first to describe the characteristic of encouraging ROS production of C₆₀ by MNP, a natural biomaterial, and MC₆₀H in the application of a coactive anti-tumour approach without the dependence of conventional up-conversion material(Yan et al. 2020a).

4.5 ANTI-ANGIOGENESIS

Angiogenesis is the process of the formation of new blood vessels. Angiogenesis related to tumours is the development of new blood vessels both outside and inside the tumour. Since malignant neoplasms require a sufficient blood supply to support their rapid growth and proliferation, angiogenesis is crucial for the survival of solid tumours(Meng et al. 2010). By elevating angiogenic factors, oxidative stress in carcinoma cells stimulates blood flow to tumours. Hence, anti-angiogenesis is a successful cancer therapeutic approach. By preventing oxidative stress, tumour vascularization can be inhibited(Ye et al. 2021). Gd@C₈₂(OH)₂₂ fullerene nanoparticles have shown down-regulation of anti-angiogenic properties(Meng et al. 2010).

5. TOXICOLOGICAL AND BIOCOMPATIBILITY ASPECTS OF C₆₀

The wide use of nanotechnology has raised concerns about the compound's safety and toxicity after human contact. Several studies have revealed that fullerenes are found to be incredibly advantageous in many ways while possessing toxicity due to various factors as illustrated in Figure 5(Holmannova et al. 2022).

5.1 *In vivo* analysis

The capacity of both undoped and doped C₆₀ fullerene to induce inflammatory responses *in vivo* has been identified as one of the potential dangers of chronic fullerene exposure(Trpkovic et al. 2012). A study with freshwater fish, *Anabas testudineus*, has demonstrated the potential of fullerene C₆₀ to cause genotoxicity even at sublethal concentrations(Sumi and Chitra 2019). In C₆₀ exposure experiments even after 48 hours,

Daphnia magna were still unable to eliminate C₆₀ from their digestive tracts, exhibiting rapid absorption rates but even slower disposal rates(Freixa et al. 2018). C₆₀ has shown hepatotoxicity and cardiotoxicity in an analysis using rats. The inactivation of Kupffer cells (macrophages of liver cells) upon C₆₀ interaction has been observed(Elshater et al. 2018)(Abd-Elraheim A. Elshater 2017). At higher exposure concentrations, increased pulmonary inflammation and cytokine levels have been observed in an experiment involving nose-only inhalation using rats(Etc and Das C, Lucia MS 2019). It has been demonstrated through research on rabbits that C₆₀ fullerene nanoparticles can alter the mechanical characteristics of red blood cell membrane, making it "softer" than usual and more brittle(Halenova et al. 2020). C₆₀ exposed earthworms has shown to have high energy requirement. The analysis using *Eisenia fetida* has observed a decrease in free amino acids which is suspected to be caused due to the high production of enzymes to tolerate the toxicity of C₆₀(Lankadurai et al. 2015).

5.2 *In vitro* analysis:

C₆₀ Nanoparticles have significantly accelerated the growth of melanoma in experimental animals, in contrast to its powerful cytotoxic and antiproliferative effects on melanoma cells(Zogovic et al. 2009). An experiment on the toxicity of the pristine C₆₀ fullerene aqueous colloid solution (C₆₀FAS) with human embryonic kidney (HEK293) cells revealed no toxic effects at concentrations of 75-150mg/kg, yet haematological effects were observed at higher levels(Prylutska et al. 2019b). Using kidney human embryonic (HEK293) cells, the toxicity of the C₆₀-Cis-Pt nanocomplex in comparison to the undoped Cis-Pt had been investigated. The examined results show that the nano complex with Cis-Pt equivalent concentrations has no harmful effect. It has been reported that Cis-Pt's toxic effect against normal cells is reduced by its complexation with C₆₀ fullerene(Prylutska 2020).

5.3 Microbial analysis

Analysis in bacteria using reversion test and chromosomal aberration test *in vitro* using C₆₀ Nanoparticles has shown negative genotoxicity, even with radiation exposure(Shinohara et al. 2009). In the presence of significant amounts of C₆₀-COOH, *Phanerochaete*

chrysosporium liquid culture showed a complete loss of decomposition activity, thereby disrupting the carbon cycle(Ming et al. 2018).

6. DISCUSSION

Nanotechnology has been increasingly studied as a potential cancer treatment in recent years. The nanocarriers are useful in targeted drug delivery for cancer treatment. Fullerenes in both pristine and doped forms possess high antineoplastic and antioxidant activity. Fullerene is an allotrope of carbon. It is bioresorbable and readily enters cells, making it a promising candidate in therapeutics. It also has amphiphilic and self-assembling properties, which are highly desired for effective drug delivery and tumour imaging. C₆₀ fullerenes can pass through cell membranes and are blood-brain barriers.

Nanoconjugates of fullerene and anticancer drugs are proven to be more effective at reducing the toxicity of drugs towards normal cells, enhancing efficacy, and controlling drug release. The bioavailability of the drug has been significantly improved by fullerenes. The different anticancer treatment using fullerene includes PDT, immunotherapy and SDT. The treatment of tumours in healthcare facilities is often hindered by drug resistance. The combination of PDT and immunotherapy is useful in reversing MDR. In fullerene nanoconjugates, fullerenes are known to reduce the toxicity of anticancer drugs to normal cells.

With such tremendous biomedical applications, toxicity is a major concern. The pristine fullerene exhibits significantly superior safety. Toxicology involving various biosystems shows that fullerene also has an extent of toxicity based on the concentration, aggregation and interaction with the environment. Fullerenes can cause acute and long-term tissue damage by passing through biological barriers such as the blood-alveolus, blood-brain, and blood placenta. Fullerene has a negative impact on the liver and can alter the expression of its genes. Despite the cardiotoxic effects of some Carbon nanomaterials, fullerene has shown cardio-protective benefits against doxorubicin-induced cardiotoxicity. When present in high quantities, fullerenes are expected to have inflammatory, cell-damaging, and carcinogenic capabilities, giving them a "two-edged sword" quality.

7. CONCLUSION

In this review, the anti-cancerous activity of C₆₀ fullerene and its conjugates have been compiled. The toxicological aspects of C₆₀ fullerene have been emphasized based on various research with different organisms. The latest approaches to cancer therapy based on C₆₀ fullerenes were discussed. The nanoconjugates of fullerene and anticancer drugs are highly efficient in cancer treatment with targeting, slow drug release, high efficiency and reduced cytotoxicity on normal cells. PDT and immunotherapy with doped and pristine fullerene nanoconjugates are promising approaches to eradicating cancer. With tremendous applications, future research should be based on the pharmacokinetics of fullerene and its nanoconjugates.

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9. COMPLIANCE WITH ETHICAL STANDARDS

9.1 Conflict of Interest: All authors (Britlin Deva Jebasta Newton, Bavanilatha Muthiah, Kunal Biswas) declare that they have no conflict of interest.

9.2 Statement of Human and Animal Rights’: This article does not contain any studies with human and animal subjects performed by any of the authors.

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11.TABLES:

Table 1: Compilation of various drugs using C₆₀ as a drug carrier

Drug	Type of analysis	Model	Effect of C ₆₀	Reference
Berberine	<i>In vitro</i>	CCRF-CEM ¹ cells and LLC ² cells	Greater antiproliferative potential	(Grebinyk et al. 2019a) (Grebinyk et al. 2021)
Cisplatin	<i>In vitro</i>	Hela cells	Increase in tumour growth inhibition	(Bychko et al. 2016) (Prylutska et al. 2017a)
	<i>In vitro</i>	HL-60 ³ /adr and HL-60/vinc tumour cells	Increase in drug cytotoxicity towards cancer cells including cisplatin-resistant sublines.	(Dzhemilev et al. 2019)
	<i>In vitro</i>	LLC cells	enhanced Cis-Pt entry and intracellular accumulation, boosting the drug's cytotoxic effects	(Prylutska et al. 2019a)
Docetaxel	<i>In vitro</i>	MCF-7 ⁴ and MDA-MB231 ⁵ cell lines	Improved cytotoxicity against cancer cells and high compatibility with erythrocytes	(Raza et al. 2015)
Doxorubicin	<i>In vivo</i>	Mice with LLC	Reduction in tumour volume	(Prylutska et al. 2015) (Panchuk et al. 2015)

¹ CCRF-CEM: T- Lymphoblasts

² LLC: Lewis lung carcinoma

³ HL60: Human leukemia cell line

⁴ MCF-7: Breast cancer cell line

⁵ MDA-MB231: Epithelial human breast cancer cell line

Fluorouracil	<i>In vivo</i>	Rats with Colon lesions	Decrease in the number and the size of lesions	(Lynchak et al. 2017)
Gemcitabine	<i>In vitro</i>	Pancreatic cancer cell lines	Increase in production of ROS	(Nalepa et al. 2020)
Landomycin	<i>In vitro</i>	MG-63 ⁶ , 4T1 ⁷ and Mesenchymal stem cells	high toxicity for cancer cells, but not for the mesenchymal stem cells	(Bilobrov et al. 2019)

⁶ MG-63: Human osteoblast like cells

⁷ 4T1: mammary carcinoma cell line

12.FIGURE LEGENDS:

Figure 1: Arrangement of C- atoms in C₆₀ fullerene (created using canva software).

Figure 2: Illustration of two alternative approaches of doping; a) Addition of foreign atom M inside the cage; b) Addition of foreign atom M by swapping one C atom (created using canva software).

Figure 3: The mechanism of Photodynamic effect by fullerenes on cancer cells (created using canva software).

Figure 4: The mechanism of autophagy inducement by fullerene: 1) Autophagy inducer – fullerene entry; 2) Phagopore formation; 3) Phagopore engulfing an organelle forming autophagosome; 4) Lysosome and autophagosome combine to form autolysosome; 5) Cell lysis (created using canva software).

Figure 5: Factors affecting toxicity of C₆₀. The various factors determining the toxicity of fullerene C₆₀ include particle size, surface area, solubility, biological accumulation, formation of ROS, lipid peroxidation, the technique of manufacture, their delivery method, mode of cellular uptake, cellular toxicity pathways, accumulation and metabolism(created using canva software)..

13.FIGURES:

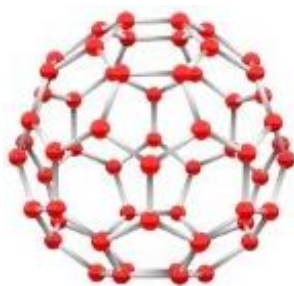


Figure 1: Arrangement of C- atoms in C₆₀ fullerene

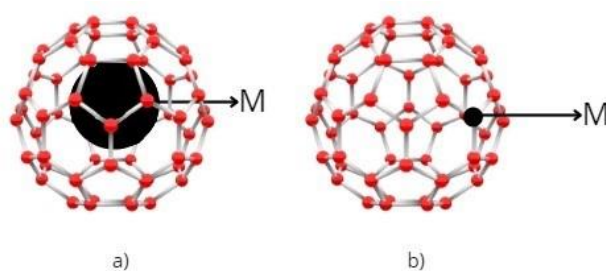


Figure 2: Illustration of two alternative approaches of doping

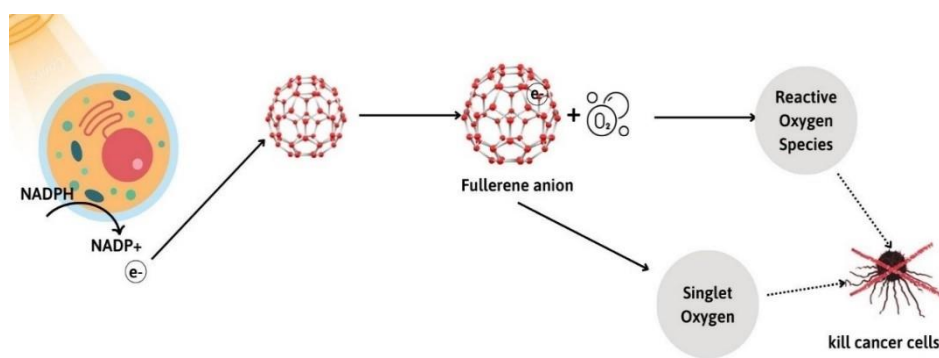


Figure 3: The mechanism of Photodynamic effect by fullerenes on cancer cells

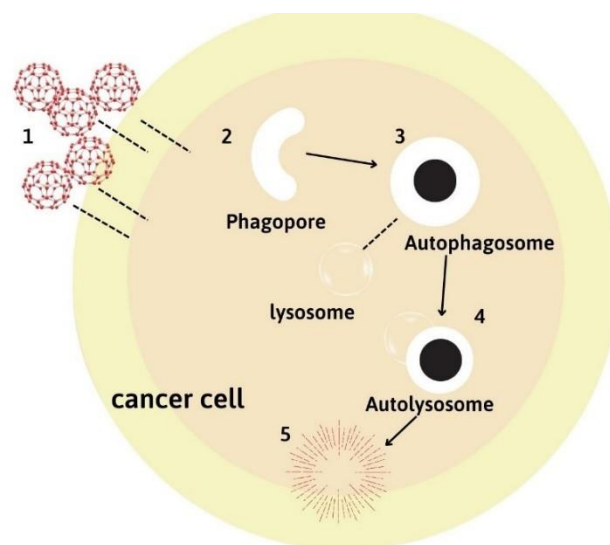


Figure 4: The mechanism of autophagy inducement by fullerene C₆₀



Figure 5: Factors affecting toxicity of Fullerene C₆₀

PLAGIARISM REPORT



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