

**IN VITRO ANTICANCER ACTIVITY OF 6-THIOGUANINE
AND 6-THIOGUANINE LOADED CHITOSAN
NANOPARTICLES WITH OR WITHOUT CURCUMIN AND
PHARMACOKINETIC STUDIES IN RATS**

RASHMI, R

ID. No.: GVD/15-012

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In the faculty of Veterinary Science**



**DEPARTMENT OF VETERINARY PHARMACOLOGY & TOXICOLOGY
NTR COLLEGE OF VETERINARY SCIENCE
GANNAVARAM – 521102 (A.P.), INDIA**

**SRI VENKATESWARA VETERINARY UNIVERSITY
TIRUPATI – 517502 (A.P.), INDIA**

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CERTIFICATE

RASHMI, R., I.D. No. GVD/15-012 has satisfactorily prosecuted the course of research and that the thesis entitled “***IN VITRO* ANTICANCER ACTIVITY OF 6-THIOGUANINE AND 6-THIOGUANINE LOADED CHITOSAN NANOPARTICLES WITH OR WITHOUT CURCUMIN AND PHARMACOKINETIC STUDIES IN RATS**” submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any university.

Date:

(Dr. P. Ravi Kumar)

Major Advisor

Professor & University Head

Dept. of Vet. Pharmacology and Toxicology
& Principal

Animal Husbandry Polytechnic

Venkataramannagudem-534101

CERTIFICATE

This is to certify that the thesis entitled “**IN VITRO ANTICANCER ACTIVITY OF 6-THIOGUANINE AND 6-THIOGUANINE LOADED CHITOSAN NANOPARTICLES WITH OR WITHOUT CURCUMIN AND PHARMACOKINETIC STUDIES IN RATS**” submitted in partial fulfilment of the requirements for the degree of “**DOCTOR OF PHILOSOPHY**” of **SRI VENKATESWARA VETERINARY UNIVERSITY, TIRUPATI**, is a record of the bonafide research work carried out by **RASHMI, R., I.D. NO.: GVD/2015-012** under my guidance and supervision. The subject of the thesis has been approved by the student’s advisory committee.

No part of the thesis has been submitted by the student for any other degree or diploma. All assistance and help received during the course of the investigations have been duly acknowledged by the author of the thesis.

External Examiner

(Dr. P. Ravi Kumar)

Chairman of the Advisory Committee

Thesis approved by the Student’s Advisory Committee

CHAIRMAN	: DR. P. RAVI KUMAR Professor & University Head Dept. of Veterinary Pharmacology & Toxicology & Principal Animal Husbandry Polytechnic Venkataramannagudem-534101	_____
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Affectionately Dedicated

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Gannavaram

DECLARATION

I, Rashmi, R hereby declare that the thesis entitled, “***IN VITRO*** ANTICANCER ACTIVITY OF 6-THIOGUANINE AND 6-THIOGUANINE LOADED CHITOSAN NANOPARTICLES WITH OR WITHOUT CURCUMIN AND PHARMACOKINETIC STUDIES IN RATS” submitted to Sri Venkateswara Veterinary University for the degree of **Doctor of Philosophy** is a result of original research work done by me. It is further declared that one research article from the thesis has been submitted for publication.

Date:

(Rashmi, R)

Place: Gannavaram

LIST OF ABBREVIATIONS

AP-1	Activator protein 1
AuNPs	Gold Nanoparticles
ALL	Acute Lymphoblastic leukemia
ANOVA	Analysis of variance
ATR	Ataxia telangiectasia and Rad3-related protein
AUC	Area under curve
AUC _{0-t}	Area under the curve from '0' to 't'
AUC _{0-inf}	Area under the curve from '0' to 'inf'
AUMC _{0-inf}	Area under movement curve from '0' to 'inf'
BAD	Bcl2 associated death promoter
BC	before Christ
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
CAGR	Compound Annual growth rate
CC	Calibration curve
Chk 1	Checkpoint kinase 1
CIVI	continuous intravenous infusion
Cl/F _{obs}	Clearance
CLGL-90	Chronic Large Granular Lymphocytic
C _{max}	Peak plasma concentration
COX2	Cyclooxygenase 2
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CpG	5'-Cytosine-phosphate-Guanine-3'
CS	Chitosan
CSF	Cerebrospinal fluid
C _{ss}	Plasma concentration at steady-state
Cur	Curcumin
°C	Degree Celsius
DLS	Dynamic Light Scattering
DMF	Dimethylformamide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMTIs	DNA methyltransferase inhibitors
DPBS	Dulbecco's Phosphate-Buffered Saline
DSA	Dialdehyde sodium alginate
EE	Entrapment efficiency
EGFR	Epidermal growth factor receptor
Egr-1	Early growth response protein 1
EPR	Enhanced permeability and retention
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FL1	Fluorescence channel-1
FL2	Fluorescence channel-2
FTIR	Fourier Transform Infrared Spectroscopy
g	Gram
h/H	Hour
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HDACIs	Histone deacetylase inhibitors
HER2	Human epidermal growth factor receptor 2
HL-60	Human leukaemia cell lines
HQC	Higher quality control
HMW	High molecular weight
HpaII	<i>Haemophilus parainfluenzae</i>
IAEC	Institutional Animal Ethics Committee
IC ₅₀	Inhibitory concentration
ID ₅₀	Infectious dose
IFN- α	Interferon alpha
IR	Infrared
ISTD	Internal standard
IU	International unit
i.p.	Intraperitoneal
i.v. or IV	Intravenous
KBr	Potassium bromide

kDa	kilodalton
kg	Kilogram
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LD ₅₀	Lethal dose 50
LDL	Low density lipoprotein
LLOQ	Lower limit of quantification
LMW	Low molecular weight
LOX	Lipoxygenase
LSD 1	Lysine-specific demethylase 1
LQC	Lower quality control
M	molar
MCF-7	Michigan cancer foundation-7
mg	Milligram
mg/ml or mg/mL	Milligram per millilitre
mg/m ²	Milligram per square metre
min	Minute
mL / ml	Millilitre
mm	Millimetre
mm ³	Cubic millimeter
MMP-9	Matrix mettalopeptidase 9
MMR	Mismatch repair
MQC	Medium quality control
MRI	Magnetic resonance imaging
MRT	Mean residence time
MTT	3-(4,5-dimethylthiazolyl -2)-2, 5-diphenyltetrazolium bromide
mV	millivolts
m/z	mass to charge ratio
N	Normality
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
ng/ml or ng/mL	nanogram per millilitre
nm	nanometre
NK	Natural killer
NOS	Nitric oxide synthase

NPs	Nanoparticles
PAMPA	Parallel artificial membrane permeability assay
PARP	Poly ADP- ribose polymerase
PBS	Phosphate buffer saline
PCS	Photon correlation spectroscopy
PFA	Paraformaldehyde
P-gp	P-glycoprotein
pH	Potential of Hydrogen
PI	Propidium iodide
PIR	PARP inhibitor resistant
PK	Pharmacokinetics
pK_a	Acid-base dissociation constant
pmol	picomoles
p.o.	Per oral or per os
PS	phosphatidylserine
QC	Quality control
QELS	Quasi-elastic light scattering
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
RT	Room temperature
SAM	S-adenosyl-L methionine
SEM	Scanning Electron Microscope
STD	Standard
t_{max}	time to reach maximum concentration
TNF	Tumour necrosis factor
TEM	Transmission Electron Microscope
TPP	Sodium tripolyphosphate
TSGs	Tumour suppressor genes
$t_{1/2\lambda z}$	Elimination half-life
uPA	Urokinase-type plasminogen activator
USP2	Ubiquitin- specific protease 2
V_z/F_{obs}	Volume of distribution

V_{dss}	Volume of distribution steady-state
Zeb	Zebularine
μg	Microgram
μL	Microlitre
μM	Micromole
%	Per cent
β	Beta
λ_z	Terminal rate constant
βCD	β Cyclodextrin
5meC	5-methylcytosine
6- MP/ MP	6-mercaptopurine
6- TG/ TG/ 6-tG	6-thioguanine
6-TGN	6-thioguanine nucleotides
6-TG-CNPs	6- thioguanine loaded chitosan nanoparticles
USFDA	United States Food and Drug Administration
v/v	Volume by volume
w/v	Weight by volume

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ABSTRACT

Cancer is the second leading cause of mortality in the world. Cancer nanotherapeutics are rapidly progressing and being implemented to overcome several limitations of conventional drug delivery systems. 6-thioguanine encapsulated chitosan nanoparticles (6-TG-CNPs) were formulated by ionic- gelation method. Morphologically, the 6-TG-CNPs were spherical in shape and showed mean size, PDI, zeta potential and entrapment efficiency of $261.63 \pm 6.01 \text{ nm}$, 0.34 ± 0.10 , $+15.97 \pm 0.46 \text{ mV}$ and 44.27 per cent, respectively. IR spectra confirmed 6-TG complex with chitosan. *In vitro* drug release profile of 6-TG-CNPs revealed slow but increased (91.40 ± 1.08 per cent at 48h) release at pH 4.8 compared to less and sustained (73.96 ± 1.12 per cent at 48h) release at pH 7.4. MTT assay was conducted on MCF-7 and PA-1 cell lines at 48h incubation to determine per cent cell viability. IC_{50} values of 6-TG, 6-TG-CNPs and curcumin for MCF-7 were 23.09, 17.82 and $15.73 \mu\text{M}$, respectively. Likewise, IC_{50} values of 6-TG, 6-TG-CNPs and curcumin for PA-1 were 5.81, 3.92 and $12.89 \mu\text{M}$,

respectively. Combination of 6-TG-CNPs (IC_{25}) with curcumin (IC_{25}) on PA-1 and MCF-7 showed percent cell viability of 43.67 ± 0.02 and 49.77 ± 0.05 , respectively. The *in vitro* cytotoxicity potential in terms of per cent cell viability, early apoptosis, G2/M phase arrest and global DNA demethylating activity of 6-TG-CNPs alone and in combination with curcumin proved to be more effective than that of 6-TG on PA-1 cells. The C_{max} of 6-TG in 6-TG-CNPs alone and 6-TG-CNPs in curcumin pre-treated groups was found to increased by 1.6-fold and 2-fold, respectively when compared to those observed with 6-TG treated group. The rate and extent of 6-TG absorption was increased by 2.5-fold following 6-TG-CNPs alone and 3.2-fold in curcumin followed by 6-TG-CNPs treated group compared to those observed with 6-TG treated group. The enhanced C_{max} and AUC for 6-TG were in the order of curcumin pre-treatment + 6-TG-CNPs > 6-TG-CNPs > 6-TG groups. The apparent volume of distribution was substantially lower for 6-TG in 6-TG-CNPs alone and 6-TG-CNPs in curcumin pre-treated groups when compared to those observed with 6-TG treated group. Apart from this, volume of distribution at steady-state was also found lower for 6-TG in both the 6-TG-CNPs treated groups. These PK parameters suggests the better tumour targeting efficiency of 6-TG nanoformulations with less toxicity in off-target tissues. Thus, the 6-TG encapsulated chitosan nanoparticles prepared in the present study used either alone or in combination with curcumin present a wide scope for efficient delivery of 6-TG at the target sites.

Introduction

CHAPTER: I

INTRODUCTION

Cancer continues to be one among the most difficult global healthcare problems with an estimate of 18.1 million new cancer cases and 9.6 million cancer deaths in 2018 (Bray *et al.*, 2018). Cancer represents a multistage and heterogeneous disease that is driven by progressive genetic and epigenetic abnormalities (Shinjo and Kondo, 2015). Genetic changes are irreversible, but epigenetic changes are potentially reversible with pharmacological interventions (Yen *et al.*, 2016). Aberrations in the normal DNA methylation patterns and histone modifications have been recognized as targets for epigenetic therapy. Unlike conventional chemotherapy, the epigenetic therapy reverts the abnormal state of malignant cells to a more normal condition (Yoo and Jones, 2006; Cai *et al.*, 2011).

Current treatment for various cancers includes surgery, radiation, hormone therapy and chemotherapy, but the effects of these procedures may damage not only the tumor tissue but also normal tissue (Nguyen, 2011). Consequently, these often cause severe side effects in other tissues (e.g. bone marrow suppression, cardiomyopathy and neurotoxicity) (Kim and Nie, 2005). Although there is a large library of drugs that can be used in cancer treatment, the current problems in treating cancer include low specificity, rapid drug clearance, biodegradation and limited targeting (Sinha *et al.*, 2006; Tran *et al.*, 2017).

Nanomedicine is an emerging method for treating cancer. It has ushered in a new era for drug delivery by improving the therapeutic indices of the active pharmaceutical ingredients engineered within nanoparticles. Currently, a wide variety of platforms are being investigated as nanocarriers for cancer treatment, including lipids, polymers, inorganic substances, viruses and drug conjugated nanoparticles (Tran *et al.*, 2017). The properties of nanocarriers viz., their nanoscale sizes, high surface-to-volume ratios,

sustained drug release profiles and targeting modifications can allow them to better reach target tumor tissue and release drugs in a stable and controlled manner (Wicki *et al.*, 2015). First generation nanomedicines have received widespread clinical approval over the past two decades, from Doxil[®] (liposomal doxorubicin) in 1995 to Onivyde[®] (liposomal irinotecan) in 2015 (Tran *et al.*, 2017).

Chitosan is a linear polysaccharide composed of randomly distributed β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. The advantageous biological characteristics of chitosan like relative non-toxicity, biocompatibility, biodegradability, cationic properties, bio-adhesive nature and permeability-enhancing properties have led to the extensive study of chitosan based nanoparticles for delivery of anti-cancer agents, therapeutic proteins, genes, antigens and many others (Amidi *et al.*, 2010; Kumari *et al.*, 2010; Fan *et al.*, 2012). Chitosan has been approved by the U.S. FDA for tissue engineering and drug delivery (Mohammed *et al.*, 2017). Chitosan nanoparticles can be prepared by a novel method based on ionic gelation using sodium tripolyphosphate (TPP) as cross-linking agent. Among variety of methods developed to prepare chitosan nanoparticles, ionic gelation technique has attracted considerable attention as this process is non-toxic, organic solvent free, convenient and controllable (Agnihotri *et al.*, 2004; Fan *et al.*, 2012).

Modern pharmaceutical research is concerned with all aspects of identifying new chemical substances with new modes of action. In particular, economics of treatment linked to drug dosage has led to new drug development technologies (Atal and Bedi, 2010). In advance drug design technologies, a large number of drug molecules were being introduced every year but many of these molecules had problems like their solubility, stability, bioavailability and their long-lasting side effects. Low bioavailability, a serious problem of drug molecules is due to low lipophilicity and zwitterionic character at physiological pH, poor water solubility or efflux by P-glycoprotein (P-gp) etc. (Jain and

Patil, 2015). Maximizing oral bioavailability is therapeutically important as the extent of bioavailability directly influences plasma concentrations and therapeutic efficacy after oral drug administration. The use of bioenhancers is an innovative concept and their judicious use can result in reduced drug cost and toxicity (Javed *et al.*, 2016).

Curcumin exerts anticancer effects by inhibiting cancer cell proliferation, metastasis and by inducing cell cycle arrest and apoptosis (Liu and Ho, 2018). Additionally, curcumin can epigenetically regulate the expression of important genes by reversing DNA methylation and altering histone modifications and by targeting several miRNAs that play a key role in cancer (Boyanapalli and Kong, 2015). Apart from this, curcumin also improves the bioavailability of co-administered drugs (Dudhatra *et al.*, 2012).

The thiopurines viz., azathioprine, 6-thioguanine (6-TG) and 6-mercaptopurine (6-MP) are effective anti-inflammatory, anticancer and immunosuppressive drugs (Karran and Attard, 2008). 6-TG and 6-MP were co-developed by Gertrude Elion and George Hitchings, who received the Nobel Prize for development of these and other anticancer agents (Hitchings and Elion, 1954; Munshi *et al.*, 2014). 6-TG and 6-MP were produced by substitution of oxygen by sulfur at carbon 6 of guanine and hypoxanthine, respectively. One limitation of orally administered thiopurines is their low and highly variable bioavailability due, in part, to their extensive first pass metabolism in the intestinal mucosa and liver (Jacob *et al.*, 2007). Familial mutations in the breast cancer susceptibility genes BRCA1 or BRCA2 are associated with an increased risk of several cancers, particularly breast, ovarian and prostate cancer. Recent studies revealed that, 6-TG can be efficiently used to selectively kill BRCA2 defective tumors and also for the treatment of BRCA1 or BRCA2 mutant tumors that are resistant to Poly ADP-ribose polymerase (PARP) inhibitors or platinum-based chemotherapy (Issaeva *et al.*, 2010). 6-TG is a noncompetitive and slow-binding inhibitor of ubiquitin-specific protease 2

(USP2) belonging to the family of deubiquitinases that can rescue protein targets from proteasomal degradation by reversing their ubiquitination. Thus, USP2 plays a critical role in tumor cells survival and therefore represents a therapeutic target in USP2-upregulated cancers *viz.*, prostate and ovarian cancers (Chuang *et al.*, 2018). 6-TG has been shown to be epigenetically active as a demethylating agent (Flesner *et al.*, 2014) and the effect is mediated by down-regulation of histone lysine-specific demethylase 1 expression which stimulates lysine methylation of DNA methyltransferase 1 (DNMT1) and triggers its degradation *via* the ubiquitin proteasomal pathway (Yuan *et al.*, 2011).

Keeping the above points in view, the present study was undertaken with the following objectives:

- a) To synthesize and characterize chitosan nanoparticles loaded with 6-thioguanine (6-TG).
- b) To compare the *in vitro* anti-cancerous efficacy of 6-TG, 6-TG loaded chitosan nanoparticles alone and in combination with curcumin.
- c) To determine the demethylating action of 6-TG, 6-TG loaded chitosan nanoparticles alone and in combination with curcumin.
- d) To compare the pharmacokinetic properties of 6-TG, 6-TG loaded chitosan nanoparticles alone and in combination with curcumin in rats.

Review of Literature

CHAPTER: II

REVIEW OF LITERATURE

Cancer is the second leading cause of death globally, despite good advances in tumor biology research and chemotherapy. Major obstacles in cancer treatment arise from tumor heterogeneity, drug resistance and systemic toxicities. Nanoscale delivery systems are gaining importance as vehicles for antineoplastic agents because of their potential for targeting and multifunctionality (Ediriwickrema and Saltzman, 2015).

2.1. HALLMARKS OF CANCER

The first documented case of cancer was recorded in Egypt around 3000 BC and surgical resections were historically the predominant mode of treatment. The birth of scientific oncology ensued when Rudolf Virchow examined blood samples from leukemia patients under the microscope in 1847. Emile Grubbe began using X-rays to treat recurrent breast carcinoma in 1896, while surgery and radiotherapy continued to be the mainstay of treatment. It was not until 1948, when Sidney Farber evaluated the use of antifolate compounds for treating leukemia in children, that chemotherapy became a viable treatment option (Cravo and Mersny, 2013). The ensuing decades have generated numerous advances in cancer biology and chemotherapy. Significant increase in cancer mortality were first noticeable in the 1990s and the field of targeted cancer therapies blossomed with new discoveries in cancer signaling pathways involved in tumor development, proliferation and metastasis (Ediriwickrema and Saltzman, 2015). Currently, cancer pathogenesis is considered a complex multistep process where cells attain certain hallmark properties (Fig.1) as a result of both genetic and epigenetic alterations (Luo *et al.*, 2009; Hanahan and Weinberg, 2011). The hallmarks of cancer comprises of six biological capabilities acquired during its multistep development which constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell

death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. Underlying these hallmarks are genome instability, which generates the genetic diversity that expedites their acquisition and inflammation, which fosters multiple hallmark functions. Conceptual progress in the last decade has added two emerging hallmarks of potential generality to this list - reprogramming of energy metabolism and evading immune destruction. In addition to cancer cells, tumors also exhibit another dimension of complexity: they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the “tumor microenvironment.” Recognition of the widespread applicability of these concepts will increasingly help the development of new means to treat cancer (Hanahan and Weinberg, 2011; Nogales-Cadenas *et al.*, 2016).

2.2. EPIGENETICS IN CANCER

Nowadays, the field of epigenetics is considered as one of the most rapidly expanding fields of modern biology that has enormous influence on the understanding of biological phenomena and diseases, including cancer. The term “epigenetics” was coined by Conrad H Waddington in 1942 (Wang, 2014). The word ‘epi’ is derived from the Greek meaning “above or over genetics” (Gal-Yam *et al.*, 2008). Epigenetics is the study of heritable changes in gene function that cannot be attributed to DNA sequence variations (Bennett and Licht, 2018). Epigenetic changes are reversible (Hatzimichael and Crook, 2013), heritable and influenced by the environment (Holliday, 1987). The fact that epigenetic marks are reversible and offers increased opportunities to ameliorate the disease phenotype (Agrawal *et al.*, 2018). Epigenetic activity is influenced by several exogenous and endogenous factors including, nutrition, environment, disease, ethnicity, life style, medication, toxins, physical activity, age, gender and family genetics (Gal-Yam *et al.*, 2008). Cancer epigenetic modifications include DNA methylation, histone modification and non-coding RNA *esp.* miRNA modifications (Yen *et al.*, 2016). Since

four decades of discovery, DNA methylation (Fig.2) is the most widely studied lesion of the malignant cell and remains to be a major hallmark in most cancer types. DNA methylation-mediated epigenetic silencing of cancer-related genes has greatly emphasized on the development of anti-cancer therapies that work by inhibiting DNA methylation and restore normal epigenetic landscape by reprogramming of genes involved in disease mechanisms (Agrawal *et al.*, 2018). DNA methylation is the addition of a methyl group from the universal methyl donor, S-adenosyl-L methionine (SAM) to the fifth carbon atom in the cytosine ring within 5'-Cytosine-phosphate-Guanine-3' (CpG) dinucleotides, resulting in the formation of 5-methylcytosine (5meC). Thus, methylation of DNA occurs exclusively in 5-cytosine. This replication-dependent reaction is catalyzed by DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, DNMT3B and DNMT3L, which are present at the replication fork during the S-phase (Klose and Bird, 2006; Wang, 2014). DNMT1 is the proposed maintenance methyltransferase responsible for copying DNA methylation patterns to newly biosynthesized DNA during replication. DNMT3 consists of two related proteins, DNMT3A and DNMT3B which function as *de novo* methyltransferase and set up DNA methylation patterns during early development. DNMT3L is homologous to DNMT3s but does not possess catalytic activity (Subramaniam *et al.*, 2014).

DNMT inhibitors (DNMTIs) are a promising class of anticancer therapeutics which modulate the epigenome by reversing the DNA hypermethylation patterns, leading to renewed transcription of previously silenced tumor suppressor genes (TSGs). These DNMT targeting drugs are classified as nucleoside analog inhibitors which incorporate into DNA during replication and sequester DNMTs by mediating their proteasomal degradation and non-nucleoside analog classes which directly bind to the catalytic region of DNMTs and render the enzyme inactive, without covalent enzyme trapping (Agrawal *et al.*, 2018). Currently, there are two main classes of epigenetic drugs, namely DNA

methyltransferase inhibitors (DNMTIs), histone deacetylase inhibitors (HDACIs). DNMT inhibitors such as azacytidine and decitabine and HDAC inhibitors viz., vorinostat, romidepsine and belinostat are approved by FDA (Shinjo and Kondo, 2015).

2.3. CANCER NANOMEDICINE

Nanomedicine involves the development and application of nanometer-scaled materials in the diagnosis, treatment and prevention of diseases. Nanotechnology is a known field of research since last century. Since “nanotechnology” was presented by Nobel laureate Richard P. Feynman during his well famous 1959 lecture “There’s Plenty of Room at the Bottom” (Feynman, 1960), these have made various revolutionary developments in the field of nanotechnology (Khan *et al.*, 2017). Nanotechnology provide the potential to improve drug solubility and stability, prolong drug half-lives in plasma, minimize off target effects and concentrate drugs at the target site (Ediriwickrema and Saltzman, 2015). Nanoparticles are the key components of nanomedicine and have received extensive interest as promising drug-delivery systems for cancer diagnosis and treatment (Davis *et al.*, 2008; Xin *et al.*, 2017). Nanoparticles applied as drug delivery systems are submicron-sized (100 -1000 nm) particles, devices, or systems synthesized from various materials, including polymers (e.g., polymeric nanoparticles, micelles, vesicles, or dendrimers) (Kumari *et al.*, 2010), lipids (liposomes) (Rivera, 2003), viruses (viral nanoparticles) (Manchester and Singh, 2006) and even inorganics (Ghosh *et al.*, 2008; Mornet *et al.*, 2004). By using either passive or active targeting strategies, nanoparticles can increase the intracellular concentration of drugs in cancer cells while preventing toxicity to normal cells. Nanoparticles are usually enveloped by endosomes *via* receptor-mediated endocytosis after entering tumor cells, thereby can escape P-glycoprotein recognition, which is one of the main mechanisms of drug resistance (Xin *et al.*, 2017).

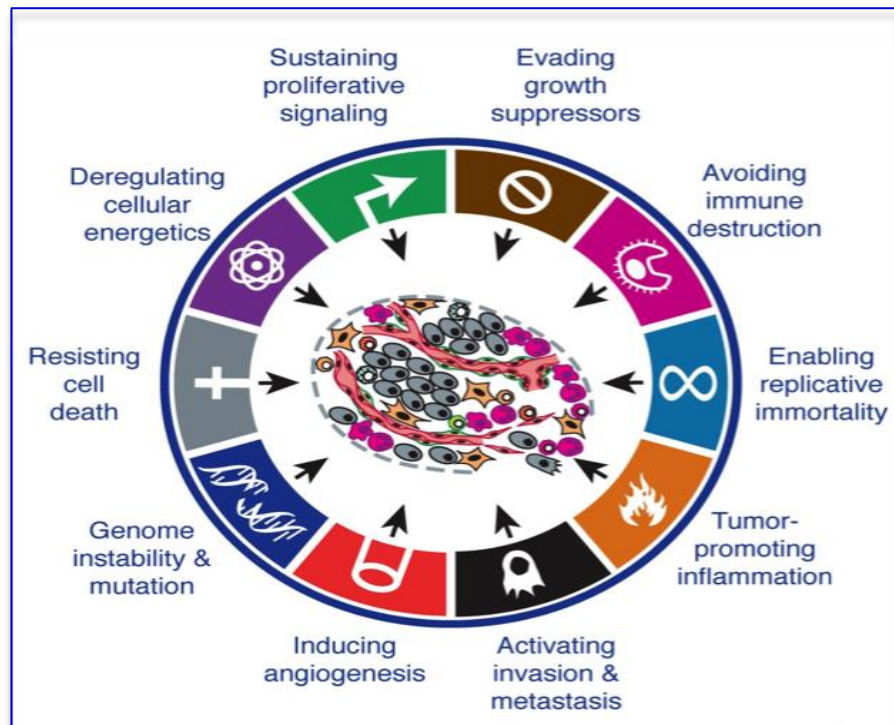


Fig.1. Hallmarks of cancer (Hanahan and Weinberg, 2011; Nogales-Cadenas *et al.* 2016)

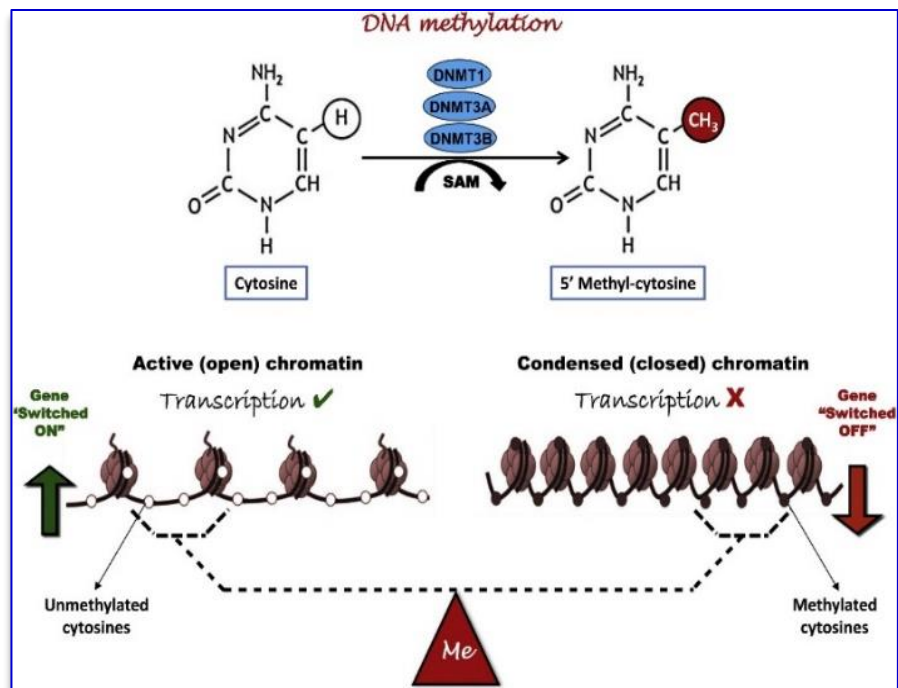


Fig.2. Interplay between DNA methylation, gene transcription and chromatin structure (Agrawal *et al.*, 2018).

Nanoparticles (NPs) are a subcategory of nanomaterials that are currently at the forefront of cutting-edge research in nearly every field imaginable due to its unique properties and tremendous applicability (Elsabahy and Wooley, 2012). In a technology market research report entitled “Global NP Market Outlook 2020” by RNCOS, it was reported that the market for NPs will grow at a compound annual growth rate (CAGR) of 16% during 2015–2020. NP technology has found a unique niche in the field of biomedicine and biotechnology with its rapidly burgeoning repertoire of applications (Shi *et al.*, 2017). For instance, nanoparticles have been applied for drug and gene delivery, bio-detection of pathogens, detection of proteins, tissue engineering, tumor imaging and targeting, tumor destruction *via* hyperthermia and magnetic resonance imaging (MRI) contrast enhancement. Owing to their small size, NPs can easily enter the cells as well as translocate across the cells, tissues and organs. NPs are widely used in biomedical applications because they are able to pass through the biological barrier and enter the cell to exert their function (Foroozandeh and Aziz, 2018).

Polymeric nanoparticles are extensively used for the nanoencapsulation of various useful bioactive molecules and medicinal drugs to develop nanomedicine. Biodegradable polymeric nanoparticles are highly preferred because they show promise in drug delivery system. Such nanoparticles provide controlled/sustained release property, subcellular size and biocompatibility with tissue and cells (Panyam and Labhasetwar, 2003; Kumari *et al.*, 2010). Apart from this, these nanomedicines are stable in blood, non-toxic, non-thrombogenic, non-immunogenic, non-inflammatory, do not activate neutrophils, biodegradable, avoid reticuloendothelial system and suitable for various molecules such as drugs, proteins, peptides, or nucleic acids (Des Rieux *et al.*, 2006; Kumari *et al.*, 2010).

2.3.1. Chitosan Nanoparticles

Chitosan is a modified natural carbohydrate polymer prepared by the partial N-deacetylation of crustacean derived natural biopolymer chitin. Chitosan is a linear co

polymer polysaccharide consisting of β (1–4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units. The degree of deacetylation of typical commercial chitosan is usually between 70 per cent and 95 per cent, and the molecular weight is between 10 and 1000 kDa (George and Abraham, 2006). The pK_a of the primary amine of chitosan is ~ 6.5 , depending on the degree of *N*-deacetylation. The systemic absorption and distribution of chitosan following oral exposure are likely influenced by the molecular weight of the polymer. The effect of molecular weight on chitosan absorption has been evaluated in male Sprague Dawley rats. Oral gavage administration of chitosan with molecular weights of 3.8, 7.5, 13, 22, or 230 kDa resulted in maximum plasma chitosan concentrations (C_{max}) of 20.23, 9.30, 5.86, 4.32 and less than 0.5 $\mu\text{g/mL}$, respectively (Chae *et al.*, 2005). The results of this study suggested that the absorption of chitosan from the gastrointestinal tract following oral exposure is inversely related to chitosan molecular weight, as there is likely low bioavailability associated with the higher molecular weight chitosan polymers. The biodegradation of chitosan *in vivo* is dependent on the degree of deacetylation (Yang *et al.*, 2007). Enzymatic degradation of chitosan depends on the ability to hydrolyze glucosamine-glucosamine, glucosamine-N-acetyl-glucosamine and N-acetyl-glucosamine-N-acetyl-glucosamine linkages. Degradation of chitosan in vertebrates occur predominantly by lysozymes and bacterial enzymes in the colon (Kean and Thanou, 2010). Hirano (1996) reported the oral LD_{50} for chitosan as 16 g/kg body weight in mice.

There are at least four methods reported for the preparation of chitosan nanoparticles *viz.*, ionotropic gelation, microemulsion, emulsification solvent diffusion and polyelectrolyte complex (Tiyaboonchai, 2003; Nagpal *et al.*, 2010). Ionotropic gelation is based on electrostatic interaction between amine group of chitosan and negatively charged groups of polyanion such as sodium tripolyphosphate (TPP). Chitosan is dissolved in acetic acid in the absence or presence of stabilizing agent. Polyanion was

then added and nanoparticles were spontaneously formed under mechanical stirring (Calvo *et al.*, 1998; Nagpal *et al.*, 2010; Kumari *et al.*, 2010). Sodium TPP is the most extensively used ion cross-linking agent due to its non-toxic and multivalent properties (Shu and Zhu, 2002). This physical cross-linking process not only avoids the use of chemical cross-linking agents and emulsifying agents which are often toxic to organisms, but also prevents the possibility of damage to drugs, particularly biological agents (Fan *et al.*, 2012).

In recent years, low molecular weight (LMW) chitosan nanoparticles have shown great potential in the applications of drug delivery and non-viral vector for gene delivery (Lavertu *et al.*, 2006; Csaba *et al.*, 2009). This is because, compared with high molecular weight (HMW) chitosan, LMW chitosan shows better solubility, biocompatibility, bioactivity, biodegradability and even less toxicity (Chae *et al.*, 2005). Positive charge of chitosan neutralizes the negative charge on the tumor cell surface, resulting in selective absorption thereby increasing the drug concentration at the tumor site which improves therapeutic effects (Wang *et al.*, 2011).

Chitosan is an excellent carrier as it is mucoadhesive polymer, permeation enhancer and capable of forming a protective barrier for the drug. Chitosan interacts with mucus (negatively charged) to form a complex by ionic or hydrogen bonding as well as through hydrophobic interactions and release the drug over time (Fig. 3) (Mohammed *et al.*, 2017). Drug get released from chitosan nanoparticles by different mechanisms such as swelling of the polymer (Liu *et al.*, 2017), diffusion of the adsorbed drug, drug diffusion through the polymeric matrix, polymer erosion or degradation and a combination of both erosion and degradation (Singh and Lillard, 2009). Chitosan acts as a penetration enhancer by transient and reversible opening of the tight junctions of the epithelium. It facilitates both paracellular and transcellular transport of drugs across the epithelium (Fig.4).

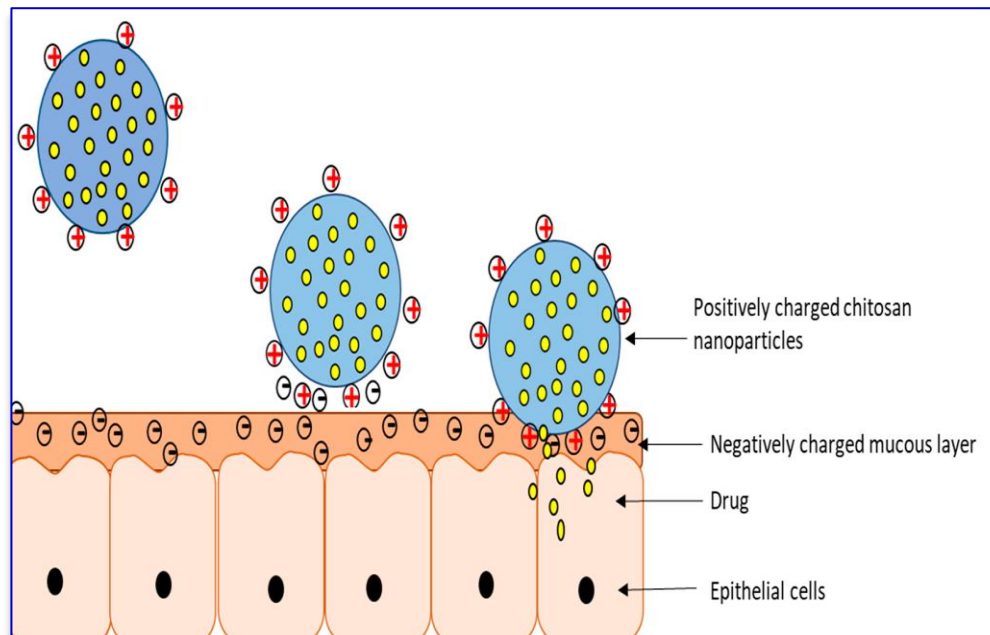


Fig.3. Electrostatic interaction occurs between drug loaded chitosan nanoparticles and mucus layer and release the drug over time (Mohammed *et al.*, 2017).

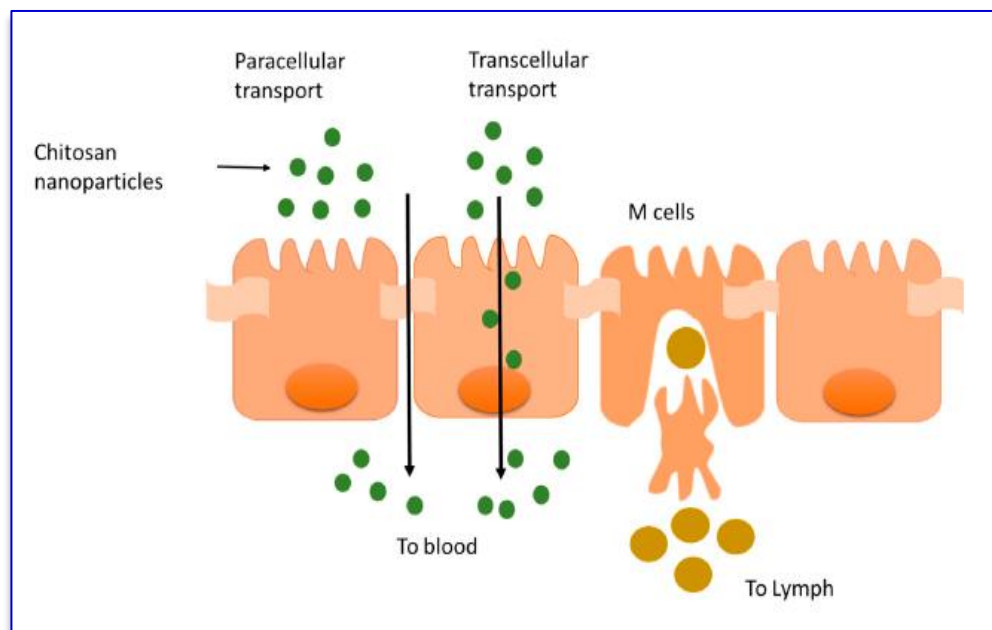


Fig.4. Schematic illustration of the presumed mechanism of transcellular and paracellular transport of chitosan nanoparticles across the epithelium (Mohammed *et al.*, 2017).

2.4. 6-THIOGUANINE

6-Thioguanine also known as thioguanine; tioguanine or 2-amino-1,7-dihydro-6H-purine-6-thione (6-tG), is a synthetic guanosine analog antimetabolite with remarkable anti-neoplastic and immuno-suppressive activity, used in maintenance therapy of childhood acute lymphoblastic leukemia (ALL) and lymphoblastic non-Hodgkin's lymphoma (Munshi *et al.*, 2014). Thiopurines are metabolized by three competing pathways (Fig. 5). In the first pathway, 6-TG and 6-MP are anabolized by hypoxanthine-guanine phosphoribosyl transferase to form the nucleotides 6-thioguanylate and 6-thioinosinate, respectively. The latter nucleotide can be further metabolized to 6-thioguanylate. 6-thioinosinate and 6-thioguanylate can inhibit *de novo* biosynthesis and interconversion of normal purines. These biochemical effects have been implicated in cytotoxicity of the thiopurines. However, a key step in the cytotoxicity of the thiopurines is the further metabolism of 6-thioguanylate resulting in the incorporation of 6-TG nucleotides into nucleic acids.

The second pathway of thiopurine metabolism constitutes the methylation of the sulfhydryl moiety of the thiopurines and their nucleosides and nucleotides by thiopurine methyltransferase. Methylated thiopurines may be demethylated to form the parent thiopurine. Methylated 6-TG and 6-MP are pharmacologically inactive and are not substrates for hypoxanthine-guanine phosphoribosyltransferase. In contrast, methylated 6-TG nucleotides and methylated 6-thioinosinate monophosphate can inhibit *de novo* purine biosynthesis, with methylated 6-thioinosinate monophosphate being more active. In the last thiopurine metabolic pathway, 6-TG is deaminated to form thioxanthine, which is then further oxidized to thiouracil, whereas 6-MP is oxidized to thioxanthine and subsequently to thiouracil. Both thioxanthine and thiouracil are inactive metabolites that are readily excreted into urine and do not contribute to the biological effects of the thiopurines. The active metabolite is able to incorporate itself into the DNA. The

cytotoxic potency of 6-TG *in vitro* and *in vivo* is greater than that of 6-MP (Elion, 1989; Adamson *et al.*, 1994; Gunnarsdottir *et al.*, 2002). During the first cell cycle the sulphur group of the active metabolite of 6-TG is methylated to S⁶-methyl thioguanine (Glaab *et al.*, 1998; Sciandrello *et al.*, 2003). During the next cell cycle the mismatch repair mechanism (MMR) recognizes the 6-TG/cytidine and 6-TG/thymidine mismatches on the daughter strand and repairs the mismatch. However, the MMR is unable to excise the faulty nucleic acid on the parent strand, inducing DNA strand breaks and the activation of various pro-apoptotic signaling pathways *i.e.* the Ataxia telangiectasia and Rad3-related protein (ATR) / Checkpoint kinase 1 (Chk 1) pathway, hyperphosphorylation of Bcl2 associated death promoter (BAD), release of cytochrome c and activation of the caspases (Stojic *et al.*, 2004; Bignami *et al.*, 2003).

2.4.1. 6-Thioguanine Anticancer Potential and Nanoformulations

The *in vitro* effect of 6-TG and α -interferon (IFN- α) was evaluated by Tan *et al.* (1989) to determine their effectiveness in combination on the therapeutically relevant events of: Human leukaemia (HL-60) cell cytotoxicity, HL-60 cell differentiation and natural killer (NK)-cell mediated cytotoxicity. 6-TG was found to be toxic to HL-60 cells (ID₅₀ = 0.6 μ M; 24h exposure) while IFN- α (up to 1000 IU/ml) had minimal cytotoxic activity. The combination of 0.5 μ M 6-TG and 100 IU/ml IFN- α produced the same level of HL-60 cell differentiation as each agent alone, suggesting no benefit from the combination on this process. The effect of 6-TG and IFN- α on NK cell-mediated cytotoxicity was found to be sequence dependent. NK cell activity was markedly stimulated by IFN- α , whereas 6-TG alone seemed to have no direct effect. However, when the NK cells were pretreated with 100 IU/ml IFN- α followed by 10 μ M 6-TG, the IFN- α -enhanced activity of NK cells was ablated. These results suggested that the immunosuppressive activity of 6-TG may be related to the acute inhibition of cytokine activation. It was concluded that 6-TG and IFN- α have considerable interactions, which

are sequence dependent and the optimal sequence for potential therapeutic application of these anticancer agents appears to be 6-TG pretreatment followed by IFN- α .

Samuels, (2008) carried out comparative *in vitro* anticancer potential evaluation of novel compounds viz., Au metal 6-thioguanine and Au-phosphine 6-thioguanine with that of 6-thioguanine. Cytotoxicity assay was carried out on cancer cell lines viz MCF 7 (mammary, adenocarcinoma, human), MCF 12A (mammary gland, human), HeLa (cervix, adenocarcinoma, human), COLO 320 (Colon, adenocarcinoma, human), A2780 (ovary, carcinoma, human), A2780cis (ovarian, carcinoma, human resistant to cisplatin), HCT 116 (colorectal, carcinoma, human) (MMR deficient cell line), Jurkat (peripheral blood, human T cell leukaemia,) and Du 145 (prostate cancer). The cells were exposed to the experimental compounds at various concentrations ranging from 100 μ M to 0.0125 μ M for a period of 7 days and the percentage of viable cells was determined by adding MTT reagent. The novel derivatives of 6-TG were highly selective towards the hormone dependent cell lines and resulted in lower IC₅₀ values compared to the non-hormonal cell lines. Apoptosis and necrosis cell death pathways were studied on HeLa cell suspension using annexin V-FITC/PI staining. Cells were treated at concentration equal to IC₅₀ and twice the IC₅₀ concentration and incubated for 24h. The results indicated that the novel compounds and 6-TG were able to induce early apoptosis significantly compared to the untreated control group. Further, HeLa cells treated with the experimental compounds showed markedly increased activation of caspase-3 compared to the untreated control group. Presence of the effector's caspases such as caspase 3, 6 and 7 are the markers of the induction of early apoptosis. Cell cycle analysis data revealed that cells treated at both the lower and higher concentration of 6-TG showed an increase in the S-phase block (43 per cent) after 24h incubation. However, at 48h the percentage of cells in the S-phase was found decreased though not significant from (43 per cent to 24 per cent) with a subsequent increase in cells in the G2/M phase (24 per cent to 44 per cent).

6-TG is as efficient as a PARP inhibitor in selectively killing BRCA2-defective tumors in a xenograft model. This was demonstrated by Issaeva *et al.* (2010) in mice bearing xenografts derived from BRCA2-deficient V-C8 and wt BRCA2-complemented V-C8+B2 cells. Mice were administered with PARP inhibitor AG014699 10 mg/kg p.o and 6-TG at the dose of 1.5 mg/kg i.p for 10 days. They found that all mice with BRCA2-defective tumors responded to both 6-TG and the PARP inhibitor equally, with significant growth delay and 3/5 complete tumor regressions in both groups. BRCA1 mammary tumors derived from K14cre, BRCA1, p53 mice were grown out and treated with PARP inhibitor olaparib. Small tumor fragments of an olaparib resistant tumor with 80-fold increased expression of the *Abcb1b* gene were transplanted orthotopically into syngeneic wild-type female mice. The animals were then treated with 6-TG at the dose of 1.5 mg/kg i.p and olaparib 50mg per kg i.p. daily for 10 days when the tumor volume reached ~200 mm³. The spontaneous BRCA1, p53-defective mammary tumors and PARP inhibitor resistant tumors in which resistance is caused by increased P-glycoprotein-mediated drug efflux showed sensitivity to 6-TG. They also showed that 6-TG can kill cells and tumors that have gained resistance to PARP inhibitors or cisplatin through genetic reversion of the BRCA2 gene.

The anti-proliferative activity of 6-thioguanine against four BRCA1-mutant (HCC1937, MDA-MB-436, SUM1315MO2 and SUM149PT) and four BRCA1- wild-type (MDA-MB-231, SUM229PE, MCF-10A, and MCF-7) breast cancer cell lines was studied by Gu *et al.* (2016). Cells were treated at a final concentration from 1nM to 10 μ M. After 72h incubation at 37°C and 5 per cent CO₂, cell viability was measured using CellTiter-Blue fluorescent assay. BRCA1-mutant cell lines viz., HCC1937, MDA-MB-436 and MDA-MB-231 were hypersensitive to 6-TG with IC₅₀ less than 1 μ M. Non-malignant mammary epithelial cell line MCF-10A demonstrated an intermediate sensitivity to 6-TG with IC₅₀ about 10 μ M. Thus, the study concluded that 6-TG might be

a promising therapeutic agent against BRCA1-deficient cancers provided that cancer cells are homologous recombination-deficient and have a functional mismatch repair machinery.

Kannaiyan *et al.* (2011) synthesized 6-thioguanine containing poly lactic acid-polyethylene glycol (PLA-co-PEG) nanocapsules in the presence and absence of gold nanoparticles *via* solvent-evaporation method. SEM and TEM measurements revealed the average size of the polymer nanocapsules and gold nanoparticles in the range of 230-260 nm and 18-20nm, respectively. The drug release was quicker in phosphate buffer saline (pH 7.4) compared to 0.1M hydrochloric acid and this may be due to higher solubility, higher swelling and penetration properties of PLA-co-PEG in PBS compared to HCl. Polymer nanocapsules with gold showed a prolonged controlled release with higher encapsulation efficiency (75 per cent) compared to that of polymer nanocapsules (45 per cent) in the absence of gold nanoparticles. It may be due to the more entrapping efficiency of gold and less diffusivity of drugs from the nanocapsules.

Thioguanine encapsulated in chitosan nanocapsules with gold, iron oxide and gold- iron oxide nanoparticles by solvent evaporation method was prepared by Vijayalakshmi *et al.* (2012). The morphology of the nanocapsules from SEM analysis revealed spherical shape and the average size of the nanocapsules were found to be around 100-200nm. Drug release profile in 0.1M HCl and PBS (pH 7.0) media showed that both chitosan nanocapsules with gold nanoparticles and chitosan nanocapsules with gold- iron oxide released thioguanine in a slow and sustained manner. The percentage of drug release was found to be more in HCl than in PBS for all the nanocapsules. Drug loading and encapsulation efficiency were observed to be high in thioguanine encapsulated chitosan nanocapsules with gold nanoparticles which were 25 per cent and 83 percent, respectively.

Sierpe *et al.* (2017) used β cyclodextrin (β CD) as a carrier to form β CD-TG and β CD-MP complexes followed by subsequent interaction with gold nanoparticles (AuNPs), generating the ternary systems: β CD-TG-AuNPs and β CD-MP-AuNPs. This modification increased their solubility, improved their stability and site-specific transport due to their nanometric dimensions. TEM image revealed that AuNPs covered with β CD-TG and β CD-MP had an average diameter of 23nm and 21nm, respectively. Parallel artificial membrane permeability assay (PAMPA) revealed an increased effective permeability using β CD while the systems containing AuNPs did not diffuse through the membrane *via* passive diffusion, which favored their controlled release.

6-TG was conjugated to dialdehyde sodium alginate (DSA) *via* acid-labile Schiff base linkage followed by the ultrasound-assisted oxidation of thiol groups to disulfide cross-links by Wan *et al.* (2018). TEM analysis revealed spherical configuration of nanoparticles with mean size of 98.4 ± 4.87 nm. Zeta potential values were between -28.7 mV and -36.5 mV indicating good stability of nanoparticles. In comparison with free 6-TG, nanoformulation exhibited slightly higher inhibition ratio on HL-60 cancer cells with an obviously reduced cytotoxicity on healthy liver cell line L-O2. *In vitro* cell line studies further confirmed an efficient cellular uptake of the nanoformulations and enhanced intracellular drug release in the HL-60 cells.

2.4.2. 6-Thioguanine Demethylating Activity – Epigenetic Regulation

The impact of 6-thioguanine on cytosine methylation mediated by two DNA methyltransferases, human DNMT1 and bacterial *Haemophilus parainfluenzae* (HpaII) by using a restriction enzyme digestion coupled with LC-MS/MS method was studied by Wang and Wang, (2009) studied. They revealed that the incorporation of 6-thioguanine into CpG site could affect the methylation of cytosine residue by both methyltransferases and the effect on cytosine methylation is dependent on the position of 6-thioguanine with respect to the cytosine to be methylated. The presence of 6-thioguanine at methylated

CpG site enhanced the DNMT1- mediated methylation of the opposing cytosine in the complementary strand, whereas the presence of 6-thioguanine at unmethylated CpG site almost completely abolished the methylation of its 5' adjacent cytosine by both DNMT1 and HpaII. Thus, they opined that the incorporation of 6-TG into DNA may perturb the epigenetic pathway of gene regulation by altering the DNA methyltransferase-mediated cytosine methylation.

Yuan *et al.* (2011) reported that 6-thioguanine treatment results in reactivation of epigenetically silenced genes in T- leukemia cells. Bisulfite genomic sequencing revealed that 6-thioguanine treatment universally elicited demethylation in the promoters and/or first exons of the genes that were reactivated. The treatment with 6-thioguanine also attenuated the expression of histone lysine-specific demethylase 1 (LSD1), thereby stimulating lysine methylation of the DNA methylase DNMT1 and triggering its degradation *via* the ubiquitin-proteasomal pathway. Further, Jurkat-T cells were treated with 3 μ M 6-TG for 6 to 24 h and the level of global cytosine methylation was assessed by HPLC method. The percentage of cytosine methylation dropped from $3.74 \pm 0.02\%$ in untreated cells to $3.57 \pm 0.12\%$, $3.52 \pm 0.08\%$ and $3.34 \pm 0.03\%$ in cells treated with 3 μ M of 6-TG for 6, 12 and 24h, respectively. Increase in mRNA levels of DCC, KCNK2, LRP1B, NKX6-1, NOPE, PCDHGA12 and RPIB9 genes was more than 4-fold after treatment with 6-TG for 48 h by using quantitative real-time RTPCR in Jurkat-T cells. Upon treatment with 3 μ M of 6-TG, the DNMT1 transcript level was similar to that of the control cells suggesting that 6-TG induced decrease in DNMT1 protein level was not due to a decline in its mRNA level. Pre- treatment of cells with proteasome inhibitor MG132 at the dose of 25 μ M for 2 h and then with 6-TG at the dose of 3 μ M indeed restored the percentage of global cytosine methylation and abolished the 6-TG mediated decrease in DNMT1 protein level, supporting that the 6-TG induced degradation of DNMT1 involved the proteasomal pathway.

The effect of 6-thioguanine and zebularine (zeb) on downregulation of DNMT1 and global demethylation of the genomic DNA of canine lymphoma cells was studied by Flesner *et al.* (2014). Canine CLGL-90 malignant T cells and CLL 17-7 cells were incubated in modified RPMI media. Cells were treated with 6-TG, Zeb or control media at biologically relevant concentrations viz., 1.5, 3.0 and 6.0 μM and 50, 100 and 200 μM respectively and cell pellets were collected at 24h and 48h incubation. DNMT1 protein and global DNA methylation were significantly decreased. A dose-dependent decrease in cell survival was also observed, with apoptosis being the primary mode of cell death in the CLGL-90 cell line. These results confirmed the demethylating action of 6-TG and Zeb in canine cells which was similar to that shown in human cell lines.

2.4.3. 6-Thioguanine Pharmacokinetics

The plasma thioguanine levels in acute myelogenous leukemia patients during remission induction (daunomycin 60 mg/m^2 on day 1, arabinosylcytosine 200 mg/m^2 for 7 days by infusion, thioguanine 100 mg/m^2 PO every 12 h for 7 days) and remission maintenance (arabinosylcytosine 200 mg/m^2 for 4 days by infusion, thioguanine 100 mg/m^2 PO every 12 h for 4 days) was studied by Brox *et al.* (1981). Hourly blood samples were taken after thioguanine administration and plasma thioguanine levels were measured by high-performance liquid chromatography with an anion-exchange column. Peak plasma levels of thioguanine were observed 2-4h after administration and varied from 0.03 - 0.94 μM . As opined by the authors the 30-fold range in thioguanine plasma levels observed in this study suggests that intermittent IV administration may provide a better means of standardizing the dosage of thioguanine.

A dose-seeking and pharmacokinetic study of 6-TG was carried out by Kovach *et al.* (1986) where 6-TG was given i.v. daily for 5 days in patients with various refractory advanced solid tumors. The starting dose was 15 mg/m^2 daily and was increased to a maximum of 65 mg/m^2 daily. Dose-limiting myelosuppression without other significant

toxicity occurred at 55-65 mg/m² daily for 5 days. After i.v. administration at 65 mg/m², the mean peak plasma concentration of 6-TG ranged from 6-10 µM. These concentrations were 8-300 times greater than peak plasma concentrations of 6-TG in plasma reported to occur after p.o. administration at 100 mg/m².

Lennard *et al.* (1993) studied the plasma 6-TG concentrations following an oral dose of 40 mg/m² and the accumulation and fluctuation of intracellular (erythrocyte, RBC) 6-TG nucleotides (6-TGNs) at regular intervals during daily oral therapy. Plasma 6-TG concentrations were low and cleared within 6h of oral dosing. At 7 days, 6-TGNs concentrations ranged from 959 to 2361 pmol 8 X 10⁻⁸ RBCs which was significantly higher than those produced by the same patients on 6-MP. The maximum 6-TG plasma concentrations ranged from 45 to 317 pmol/ml and the time to reach maximum plasma concentration was 0.75 to 2.5h. The area under the plasma concentration time curve for 0 to 6h ranged from 149 to 488 pmol/ml and the half-life of plasma 6-TG ranged from 0.8 to 6.2h. The results from the study suggested that 6-TG was well tolerated and produced higher concentrations of intracellular cytotoxic metabolites than 6-MP.

A pediatric Phase I trial of TG administered as a continuous i.v. infusion (CIVI) was carried out by Kitchen *et al.* (1997). Eighteen patients with refractory malignancies (16 solid tumors and 2 ALL) were enrolled in the study. The starting dose rate of 10 mg/m²/h administered for 24 h achieved an average C_{ss} of 0.9 µM (range, 0.7-1.2 µM). Therefore, the dose rate was escalated to 20 mg/m²/h, which achieved an average C_{ss} of 4.1 µM (range, 1.0-8.3 µM). The 6-TG clearances at the 10 and 20 mg/m²/h dose rates were 987 and 608 ml/min/m², respectively. Thus, it was recommended that the CIV dose rate and duration for CIVI of TG could be 20 mg/m²/h for 24h.

Kitchen *et al.* (1999) studied the novel metabolite of 6-TG in eighteen pediatric patients with refractory cancer. 6-TG was administered at a dose rate of 10 or 20 mg/m²/h for 24 to 36h through continuous intravenous infusion. Blood samples drawn *via* a

percutaneously placed i.v. catheter at a site distant from the infusion site, were collected at 18, 23 and 24h following initiation of the infusion. The retention time of the peak that immediately follows TG was identical with that of 8-hydroxy-Thioguanine, and co-injection of 8-OH-TG standard with patient plasma resulted in a single peak with an increase in peak height. The UV spectrum of this metabolite was identical with the UV spectrum of 8-OH-TG standard at 350 nm. Identity was confirmed by mass spectroscopy. Thus, they concluded that 8-OH-TG is the predominant circulating metabolite found in patients receiving CIVI of TG and is likely generated by the action of aldehyde oxidase.

Lancaster *et al.* (2001) studied the pharmacokinetics of 6-thioguanine in children receiving 6-thioguanine for maintenance therapy of acute lymphoblastic leukaemia (ALL) and investigated the effect of food on parent drug pharmacokinetics and the accumulation of the active metabolites 6-thioguanine nucleotides in red cells. Children with ALL demonstrated significant interindividual variation in 6-TG pharmacokinetics in $C_{\max}$ (median 313 pmol ml⁻¹, range 51±737) and AUC (median 586 pmol ml⁻¹ h, range 156 ±1306) in the fasted state. Concomitant food administration resulted in a significant reduction in $C_{\max}$ (median 71 vs 313 pmol ml⁻¹, $P=0.006$, CI from 36 to 426), AUC (median 200 vs 586 pmol ml⁻¹ h, $P=0.006$, 95% CI from 109 to 692), and time to reach $C_{\max}$ (median 1.5 vs 3 h, $P=0.013$, 95% CI from 0.74 to 2.73). The study concluded that there was no difference in the steady state concentration of red cell 6-TGNs observed after a 4-week period of 6-TG administered fasting or after food.

Plasma pharmacokinetics of oral TG and steady-state plasma and CSF TG concentrations were studied by Lowe *et al.* (2001) during a continuous intravenous infusion (CIVI) in children with newly diagnosed standard risk ALL. Blood sampling was done at 0, 0.5, 1, 1.5, 2, 3, 4, 6 and 8h after 60 mg/m² PO dosing of TG during maintenance chemotherapy. TG of 20 mg/m²/h over 24h was administered as CIVI during the consolidation phase of therapy. The peak plasma concentration was

$0.46 \pm 0.68 \mu\text{M}$ and AUC ranged from 0.18 to $9.5 \mu\text{M}$. Mean steady state plasma and CSF TG concentrations during CIVI were 2.7 and $0.5 \mu\text{M}$, respectively. The mean TG clearance was $935 \pm 463 \text{ ml/min per m}^2$. Thus, they concluded that TG concentrations that are cytotoxic to human leukaemia cell lines can be achieved in plasma after a 60 mg/m^2 oral dose of TG and in plasma and CSF during CIVI of TG.

Deibert *et al.* (2003) studied the disposition of three different 40 mg tablet preparations of thioguanine in patients with Crohn's disease. The three tablet preparations differed in one adjuvant: tablet I contained Gelita-Sol-P, tablet II contained gum arabic and tablet III contained Lutrol F 127. Blood samples for the determination of thioguanine in plasma were drawn 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 6 h after drug administration. No significant differences were found in the AUC and C_{max} values between the three tablets. The results suggested that the absorption of thioguanine is highly variable in patients with Crohn's disease. They also opined that thioguanine nucleotide levels of $>235 \text{ pmol}/8 \times 10^8$ red blood cells may serve as a biochemical marker for clinical response to thioguanine.

Jharap *et al.* (2011) studied 6-thioguanine nucleotides (6-TGNs) level and activities of thiopurine S-methyltransferase, xanthine oxidase and hypoxanthine guanine-phosphoribosyl-transferase in a two-stage (i.v. and p.o. administration of 0.3 mg/kg 6-TG) in inflammatory bowel disease patients. The median AUC per mg 6-TG was 1068 (p.o.) and 7184 (i.v.) $\text{pmol} \cdot \text{h}$. Median exposure of 6-TGN in RBC was 15%. Hypoxanthine guanine-phosphoribosyl-transferase activity correlated with peak 6-TGN and with AUC per mg. Thiopurine S-methyltransferase activity was inversely related to AUC per mg, whereas that of xanthine oxidase was correlated with a lower peak 6-TGN. The great variability of the AUC per mg for 6-TG observed after p.o. and i.v. administration of 6-TG was attributed to the variability in activities of metabolizing enzymes.

2.5. CURCUMIN

Curcumin (diferuloylmethane; 1,7-bis [4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3, 5-dione) is a polyphenol derived from the plant *Curcuma longa*, L. (*Zingiberaceae* family) commonly called turmeric. The medicinal use of this plant has been documented in Ayurveda (an Indian system of medicine) for over 6000 years. Curcumin constitutes 80 per cent of the curcuminoid complex, with the remainder constituted by demethoxycurcumin (17 per cent) and bisdemethoxycurcumin (3 per cent) (Teiten *et al.*, 2013; Boyanapalli and Kong, 2015). Extensive research over decades has indicated that curcumin reduces blood cholesterol, LDL oxidation, platelet aggregation, suppress thrombosis and myocardial infarction, symptoms associated with type II diabetes, rheumatoid arthritis, multiple sclerosis, Alzheimer and inhibits HIV replication and enhances wound healing, bile secretion (Aggarwal *et al.*, 2003).

2.5.1. Anticancer Potential of Curcumin

There is an extensive literature that suggests that curcumin has potential in the prevention and treatment of cancer. Curcumin can suppress tumor initiation, promotion and metastasis. The anticancer potential of curcumin stems from its ability to suppress proliferation of a wide variety of tumor cells, down-regulate transcription factors NF- κ B, AP-1 and Egr-1; down-regulate the expression of COX2, LOX, NOS, MMP-9, uPA, TNF, chemokines, cell surface adhesion molecules and cyclin D1; down-regulate growth factor receptors (such as EGFR and HER2) and inhibit the activity of c-Jun N-terminal kinase, protein tyrosine kinases and protein serine/threonine kinases (Aggarwal *et al.*, 2003).

2.5.2. Curcumin Demethylating Activity – Epigenetic Regulation

The epigenetic regulatory roles of curcumin include (1) inhibition of DNA methyltransferases (DNMTs), which has been well defined from the recent studies on its function as a DNA hypomethylating agent; (2) regulation of histone modifications *via*

regulation of histone acetyltransferases (HATs) and histone deacetylases (HDACs) and (3) regulation of micro RNAs (miRNA) (Boyanapalli and Kong, 2015).

Curcumin inhibits DNMT1 activity by covalently blocking the catalytic thiol group of Cys1226 binding site (Liu *et al.*, 2009). Curcumin at a concentration of 5 μ M reverses CpG methylation at the promoter region of Neurog1, a cancer methylation marker in LNCaP human prostate cancer cells and at 2.5 μ M reverses the hypermethylation of Nrf2 in transgenic adenocarcinoma of mice prostate cells (TRAMP C1 cells) (Shu *et al.*, 2011; Khor *et al.*, 2011). Curcumin was reported to induce global hypomethylation in MV4–11 leukemia cell line at IC₅₀ concentration of 30 nM after 72 h of incubation (Liu *et al.*, 2009). Downregulation of DNMT1 at mRNA and protein level was observed in melanoma cells with an IC₅₀ of 10 μ M (Abusnina *et al.*, 2011) and in MCF-7 breast cancer cells (Du *et al.*, 2012) in which curcumin induced a global decrease of DNA methylation activity. Teiten *et al.* (2013) suggested that the epigenetic event may be associated with curcumin induced disruption of an NF- κ B/Sp1 complex bound to the promoter region of DNMT1.

2.5.3. Curcumin as a Bioenhancer

There is an increasing interest and medical need for the improvement of bioavailability of a large number of drugs. Of the promising approaches, the co-administration of therapeutic agents with natural compounds possessing absorption improving activities has gained great interest in oral drug delivery. The term bioavailability enhancer was first coined by Indian scientists at the Regional Research laboratory, Jammu (RRL, now known as Indian Institute of Integrative Medicine), who discovered and scientifically validated piperine as the world's first bioavailability enhancer in 1979 (Atal and Bedi, 2010). A bioenhancer is an agent capable of enhancing bioavailability and bio-efficacy of a co-administered drug. Many natural compounds from medicinal plants have demonstrated capacity to enhance the bioavailability of co-

administered drugs by inhibiting efflux pumps and oxidative metabolism (Kang *et al.*, 2009). Natural bioenhancers include piperine, curcumin, gingerol, niaziridin, glycyrrhizin, allicin, lysergol, Aloin, sinomenine, genistein, capsaicin, quercetin, naringin and cow urine distillate. They help to reduce the dose, toxicity and development of resistance besides shortening the treatment duration. Due to dose economy, treatment becomes cost-effective (Dudhatra *et al.* 2012).

Sharma *et al.* (2017) studied the effect of pre-treatment with curcumin, a naturally occurring CYP3A and P-gp inhibitor on augmentation of paclitaxel plasma level. Paclitaxel was administered at dose of 35 mg/kg orally to swiss mice either alone or after pre-treatment with curcumin at a dose of 100 mg/kg for four days. Increase in C_{max} and AUC by 1.4 to 1.5-fold in curcumin pretreated rats when compared with oral paclitaxel alone suggested improved oral bioavailability of paclitaxel. They concluded that curcumin can be an ideal candidate for regular use to improve the bioavailability of paclitaxel followed by decrease in dose related side effects. Furthermore, anticancer effect of curcumin itself can add to that of paclitaxel for better clinical management of cancer.

The enhancement of oral bioavailability of docetaxel in curcumin pre-treated rats was studied by Yan *et al.* (2010). To improve the oral bioavailability of docetaxel, a component of turmeric, curcumin, which can down-regulate the intestinal P-glycoprotein and CYP3A protein levels was used for the pre-treatment of rats before the oral administration of docetaxel. Curcumin (100 mg/kg) did not significantly modify the pharmacokinetics of docetaxel when given orally 30 min before the administration of docetaxel. However, the C_{max} of docetaxel in rats pre-treated with curcumin for four consecutive days was significantly increased ($p < 0.01$) by about 10 times compared to that of the docetaxel control and the area under the plasma concentration–time curve (AUC) was about eight times higher than that of the control. Consequently, the absolute

bioavailability of docetaxel in the pretreatment group (four days of curcumin at 100 mg/kg) was about 40 per cent, which was a significant increase of about eightfold in comparison to the control value.

Cho *et al.* (2012) investigated the effects of curcumin on the bioavailability and pharmacokinetics of tamoxifen and its metabolite, 4-hydroxytamoxifen in rats. Tamoxifen and curcumin interact with cytochrome P450 (CYP) enzymes and P-glycoprotein. A single dose of tamoxifen was administered orally ($9 \text{ mg} \times \text{kg}^{-1}$) with or without curcumin (0.5 , 2.5 and $10 \text{ mg} \times \text{kg}^{-1}$) and intravenously ($2 \text{ mg} \times \text{kg}^{-1}$) with or without curcumin (2.5 and $10 \text{ mg} \times \text{kg}^{-1}$) to rats. Curcumin inhibited CYP3A4 activity with an IC_{50} value of $2.7 \mu\text{M}$. In addition, curcumin significantly ($p < 0.01$ at $10 \mu\text{M}$) enhanced the cellular accumulation of rhodamine-123 in MCF-7/ADR cells overexpressing P-gp in a concentration-dependent manner. This result suggested that curcumin significantly inhibited P-gp activity. Compared to the oral control group (given tamoxifen alone), the area under the plasma concentration-time curve ($\text{AUC}_{0-\text{inf}}$) and the peak plasma concentration (C_{max}) of tamoxifen were significantly ($p < 0.05$ for $2.5 \text{ mg} \times \text{kg}^{-1}$; $p < 0.01$ for $10 \text{ mg} \times \text{kg}^{-1}$) increased by 33.1-64.0 per cent and 38.9-70.6 per cent, respectively by curcumin. Consequently, the absolute bioavailability of tamoxifen in the presence of curcumin (2.5 and $10 \text{ mg} \times \text{kg}^{-1}$) was 27.2-33.5 per cent, which was significantly enhanced ($p < 0.05$ for $2.5 \text{ mg} \times \text{kg}^{-1}$; $p < 0.01$ for $10 \text{ mg} \times \text{kg}^{-1}$) compared to that in the oral control group (20.4 per cent). Moreover, the relative bioavailability of tamoxifen was 1.12 to 1.64-fold greater than that in the control group. Furthermore, concurrent use of curcumin significantly decreased ($p < 0.05$ for $10 \text{ mg} \times \text{kg}^{-1}$) the metabolite-parent AUC ratio, implying that curcumin may inhibit the CYP-mediated metabolism of tamoxifen to its active metabolite, 4-hydroxytamoxifen. The author opined that the enhanced bioavailability of tamoxifen by curcumin may be mainly due to inhibition of the CYP3A4-mediated metabolism of tamoxifen in the small intestine and/or

in the liver and inhibition of the P-gp efflux transporter in the small intestine rather than reduction of renal elimination of tamoxifen.

The effects of curcumin on the pharmacokinetics of loratadine in rats was studied by Li *et al.* (2011). Pharmacokinetic parameters of loratadine were determined after oral and intravenous administration in the presence or absence of curcumin. Curcumin inhibited CYP3A4 activity with an IC_{50} value of 2.71 μ M and the relative cellular uptake of rhodamine-123 was comparable. Compared to the oral control group, curcumin significantly increased the area under the plasma concentration-time curve and the peak plasma concentration of loratadine by 39.4-66.7 per cent and 34.2 - 61.5 per cent. Curcumin also significantly increased the absolute bioavailability of loratadine by 40.0 - 66.1 per cent compared to the oral control group. Consequently, the relative bioavailability of loratadine was increased by 1.39 to 1.67-fold. In contrast, curcumin had no effect on any pharmacokinetic parameters of loratadine given intravenously, implying that the enhanced oral bioavailability may be mainly due to increased intestinal absorption caused *via* P-gp and CYP3A4 inhibition by curcumin rather than to reduced renal and hepatic elimination of loratadine. The enhanced bioavailability of loratadine might be mainly attributed to enhanced absorption in the gastrointestinal tract *via* inhibition of P-gp and reduced first-pass metabolism of loratadine *via* the inhibition of the CYP3A subfamily in the small intestine and/or in the liver by curcumin.

Influence of curcumin pre-treatment on bioavailability of marbofloxacin following single oral administration in broiler chickens was investigated by Abo-EL-Sooud *et al.* (2017). Group-I and II birds were administered with marbofloxacin (5 mg/kg body weight) intravenously and orally, while animals in group-III and IV received similar dose of marbofloxacin (5 mg/kg body weight) intravenously and orally, after oral pre-treatment with curcumin (100 mg/kg body weight/day, 10 days). Blood samples were collected from the right-wing vein at 0, 0.166, 0.25, 0.33, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12,

18, 24 and 48h from all the groups. The pharmacokinetic data revealed that curcumin treated chickens had significantly higher area under curve (AUC), volume of distribution (V_d) and mean residential time (MRT). After oral dosing the absorption rate constant (k_{ab}) was significantly higher than the elimination rate constant (k_{el}). This could result in the presence of *in vivo* flip-flop pharmacokinetics. The delayed absorption was evident following oral administration, which limited the elimination and demonstrated sustained release from entero-hepatic circulation. Thus, they concluded that curcumin improved the systemic and relative bioavailability of marbofloxacin after oral administrations in broiler chickens.

Materials & Methods

CHAPTER: III

MATERIALS & METHODS

The present work was undertaken to synthesize and characterize 6-TG-CNPs and evaluation of *in vitro* anticancer efficacy with or without curcumin and *in vivo* pharmacokinetics of 6-thioguanine loaded chitosan nanoparticles alone and 6-TG-CNPs in curcumin pre-treated rats. The study protocols and the use of experimental animals were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. VCH/IAEC/2017/143, dated 18-11-2017, Veterinary College, Hebbal, Bengaluru, India).

3.1. CHEMICALS AND REAGENTS

6-thioguanine ($\geq 98.0\%$, #A4882), chitosan low molecular weight (deacetylation $\geq 75.0\%$, #448869), sodium tripolyphosphate (TPP) ($\geq 98\%$, #72061), glacial acetic acid ($\geq 99\%$, #A6283), Tween[®] 80 (#P1754), dimethylformamide (DMF) ($\geq 99\%$, #D4551) and curcumin ($\geq 65\%$, #C1386) were purchased from Sigma-Aldrich Chemical Co. Ltd. All other reagents were of analytical grade.

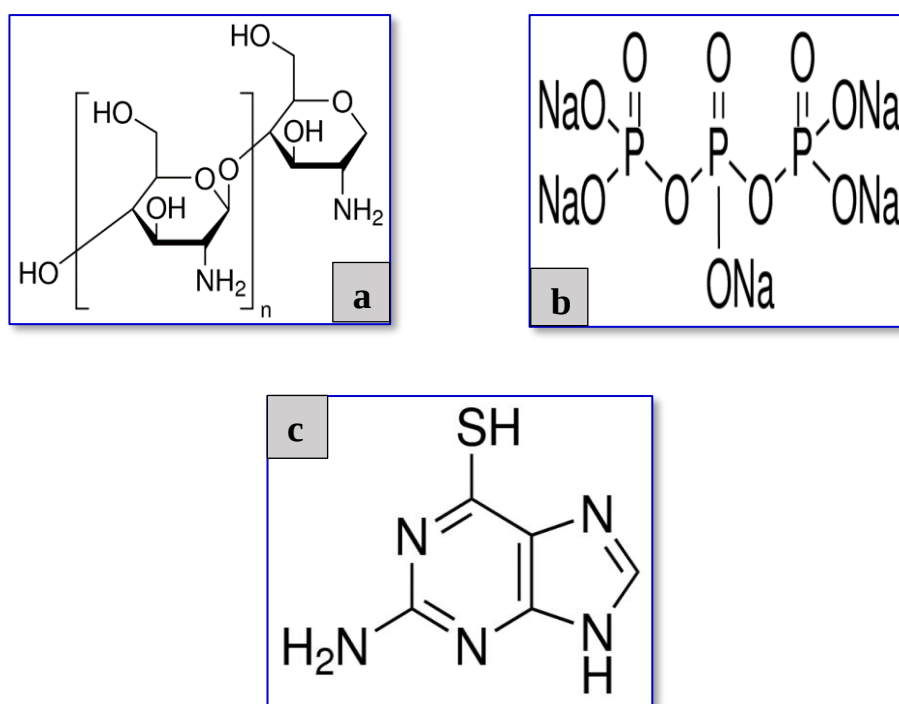


Fig.6. Structure of a) Chitosan b) Sodium tripolyphosphate and c) 6-thioguanine

3.2. SYNTHESIS OF 6-THIOGUANINE ENCAPSULATED CHITOSAN NANOPARTICLES

Chitosan was dissolved in 1% (w/v) acetic acid solution to maintain chitosan concentration at 0.5mg/mL. Prepared chitosan solution was mixed with 6-TG solution (6-TG dissolved in Dimethylformamide) and 1mg/ml 6-TG containing chitosan solution was maintained. Tween 80 (0.5% (v/v)) was added to chitosan solution and pH was adjusted to 4.6-4.8 with 1N NaOH. Thus, prepared 6-TG containing chitosan solution was mixed with 0.5mg/ml TPP solution at a volume ratio of (2:1) (v/v) (chitosan: TPP). The nanoparticle suspension was gently stirred for 30 min at room temperature to allow excess 6-TG adsorption on the nanoparticles to reach isothermal equilibrium. 6-TG encapsulated chitosan nanoparticles were centrifuged at 12000g for 30min, resuspended in water and freeze-dried for further analyses.

3.3. CHARACTERIZATION OF 6-THIOGUANINE ENCAPSULATED CHITOSAN NANOPARTICLES

3.3.1. Zetasizer Analysis

Particle size, polydispersity index and zeta potential were measured by Dynamic Light Scattering (DLS) technique employing Zetasizer (Nano ZS-90, Malvern, UK) equipped with 50mW HeNe laser beam at a wavelength of 633nm. DLS is also called as photon correlation spectroscopy (PCS) and quasi-elastic light scattering (QELS). Surface charges of the nanoparticles was measured by an electrophoretic light scattering technique, using a fold capillary cuvette (Folded Capillary Cell - DTS1070, Malvern, UK). Freshly prepared solution of 6-TG-CNPs were used for the measurement of particle size, polydispersity index and zeta potential.

3.3.2. Scanning Electron Microscopy (SEM) Analysis

The surface morphology of the prepared 6-TG-CNPs was observed using Field Emission Scanning Electron Microscope (Zeiss, Ultra 55, Germany). A pinch of sample

was placed on the stub and fixed nanoparticles were coated with gold using a sputter coater (Quorum, Q150R ES, UK). All the images were recorded at typical working distance of 8-10mm.

3.3.3. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Sample preparation and spectral recordings were carried out as described by Stuart (2004). IR spectra of 6-TG, chitosan and 6-TG-CNPs were recorded using FTIR (Perkin Elmer, Frontier, USA) operated by Spectrum 10. Potassium bromide (KBr) disc method was adopted for recording the spectra. Briefly, few milligrams of aforesaid compounds were mixed with KBr and pellets were prepared. The pellets were placed in FTIR and IR spectra from 4000 to 400 cm^{-1} in transmittance mode was recorded.

3.3.4. Determination of Entrapment Efficiency (EE) of 6-Thioguanine Encapsulated Chitosan Nanoparticles

The entrapment efficiency of 6-TG-CNPs was quantified by double beam UV-Vis Spectrophotometer (Evolution 300, Thermo Fisher Scientific, USA). The lyophilized 6-TG-CNPs were treated with 0.1N NaOH to completely redissolve the entrapped 6-TG, followed by vortexing, sonication and centrifugation. The supernatant was collected and the absorbance value was measured at 257 nm. The corresponding drug concentration was calculated from the calibration graph of 6-TG. The EE of the 6-TG-CNPs was calculated using the following equation (Papadimitriou and Bikiaris, 2009):

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Amount of drug entrapped in nanoparticles}}{\text{Initial amount of drug}} \times 100$$

3.3.5. *In Vitro* Release Profile of 6-Thioguanine Encapsulated Chitosan Nanoparticles

In vitro drug release study was carried out by dialysis membrane method (Shaikha *et al.*, 2009). Ten milligrams of 6-TG-CNPs at a concentration of 1 mg/mL in buffer solution was separately placed in dialysis tubing cellulose membrane and were incubated in 500 mL of phosphate buffered saline (pH 4.8 and 7.4) at 37°C with 50 rpm agitation.

At predetermined time intervals (0, 0.083, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 24.0 and 48.0h) an aliquot of 5 mL of the release media was withdrawn and the concentration of the 6-TG in release media was estimated by using a double beam UV-Vis spectrophotometer (Evolution 300; Thermo Fisher Scientific, USA) at 257nm. The intensity of absorption was plotted against time which gave the desorption profile of 6-TG.

3.4. *IN VITRO* CELL CULTURE ASSAYS

3.4.1. Chemicals and Reagents

The following chemicals and reagents were used for *in vitro* cell culture assays: Dimethyl sulfoxide (Sigma, #D8418), Fluorescein isothiocyanate (FITC) -annexin V (BD Biosciences, # 556547), 10X annexin V binding buffer (BD Biosciences, #556547), propidium iodide (BD Biosciences, #556547), 5-azacytidine (Sigma, #A2385), anti-5-methylcytosine (5-mc) FITC conjugated antibody (Abcam, #ab179898), Dulbecco's Modified Eagle's Medium (GE Healthcare Life Sciences, #SH30243.01), Dulbecco's phosphate-buffered saline (GE Healthcare Life Sciences, #SH30028.02). Trypan blue, MTT [3-(4, 5-dimethylthiazolyl -2)-2, 5-diphenyltetrazolium bromide], trypsin, streptomycin, penicillin, amphotericin, fetal bovine serum, absolute ethanol, paraformaldehyde (PFA), Triton-X 100, RNase all from HiMedia Labs, Mumbai, India.

3.4.2. Cell Lines and Culture Conditions

In vitro cytotoxicity study was carried out on breast adenocarcinoma (MCF-7) and ovarian teratocarcinoma (PA-1) cell lines (National Centre for Cell Science, Pune, India). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (4.5g/L Glucose), supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (4mM), penicillin 10,000 units/mL, streptomycin 1000µg/mL and Amphotericin-B solution 25µg/mL. Cells were incubated at 37°C in a humidified atmosphere of 95 per cent air and 5 per cent CO₂. Following 24h of incubation, the

adherent cells were detached using trypsin-EDTA solution 1X/0.25%. Cell count was carried out using the Luna automated cell counter (Logos Biosystems, USA) based on trypan blue dye exclusion method.

3.4.3. Cell Viability and Cytotoxicity Assay

Cytotoxicity of 6-TG, 6-TG-CNPs, curcumin and combination of 6-TG-CNPs with curcumin on MCF-7 and PA-1 cell lines was determined using MTT (3-(4,5-dimethylthiazolyl -2)-2, 5-diphenyltetrazolium bromide) assay. Cell suspension of 200 μ L was seeded in 96- well microplates (Corning[®], USA) at a density of 20,000 cells/well and incubated in 5 per cent CO₂ incubator at 37°C for 24h, after which the cells were exposed to a graded concentration (MCF-7: 6.25, 12.5, 25, 50 and 100 μ M ; PA-1: 3.125, 6.25, 12.5, 25 and 50 μ M) of 6-TG, 6-TG-CNPs and curcumin and incubated for 48h. Later media was removed from all treated and vehicle control (1% DMSO) cells and replaced with fresh media containing MTT (0.5 mg/ml) and were re-incubated for 3 hours at 37°C. This was followed by the removal of MTT solution and then addition of 100 μ L of DMSO to dissolve insoluble formazan crystals formed within mitochondria of viable cells. The plate was incubated with DMSO for 5min with gentle shaking. Cell viability was determined by measuring the absorbance on a microplate reader (ELx800, BioTek, USA) at λ_{max} 570 nm. The concentration of 6-TG, 6-TG-CNPs and curcumin that resulted in 50 per cent inhibition of cell growth was calculated as the half maximal inhibitory concentration (IC₅₀) by constructing a dose-response curve. The IC₅₀ for each cell line was determined by a linear curve fit generated after obtaining dose-response to five different concentrations of 6-TG, 6-TG-CNPs and curcumin. Cell viability percentages (y-axis) were plotted against increasing concentrations of 6-TG, 6-TG-CNPs on the x-axis. IC₅₀ values were estimated by using the linear regression equation $y=mx+c$; where 50 is substituted for y, yielding x as the IC₅₀ value.

$$\text{Cell Viability (\%)} = \frac{\text{Number of cells treated with the test item}}{\text{Number of untreated cells}} \times 100$$

Later, the cell viability per cent for the combination of 6-TG-CNPs (IC₂₅) and curcumin (IC₂₅) on MCF-7 and PA-1 cell lines was carried out at 48h incubation.

3.4.4. Analysis of Apoptosis and Necrosis using Annexin V-FITC/ PI Staining

Cell death analysis in PA-1 was performed using annexin V-FITC (Fluorescein isothiocyanate) / PI (propidium iodide) assay with flow cytometer (BD Biosciences FACSCalibur, USA). Cells were cultured in 6-well plates (Corning®, USA) for 24h at 37°C in 5% CO₂ humidified atmosphere and were later exposed to 6-TG, 6-TG-CNPs, curcumin at their respective IC₅₀ and combination of 6-TG- CNPs (IC₂₅) and curcumin (IC₂₅) for 48h.

After incubation, cells were harvested and washed once with 1X DPBS and once with 1X annexin V binding buffer (0.1M Hepes/ NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl₂). Cells were then suspended in binding buffer at 0.5X10⁶ cells/mL concentration and stained with 5µL annexin V-FITC. This was followed by an incubation period of 10-15 min at room temperature (RT) in dark. After incubation, the cells were washed with binding buffer and fixed with 4% PFA, 0.1% Triton-X 100 for 10-15 min at RT. The cells were washed with binding buffer and subjected to 0.25-0.5 mg/mL RNase treatment for 20 min at 37°C. Cells were then washed and suspended in binding buffer and stained with 5µL PI. Following incubation with PI for 10-15 min at RT, cells were suspended in 400µL of 1X annexin-V binding buffer and the apoptotic/necrotic populations were analyzed by flow cytometry using Fluorescence channel-1 (FL1) and Fluorescence channel-2 (FL2).

3.4.5. Analysis of Cell Cycle by Flow Cytometry

PA-1 cells were cultured in 6-well plates for 24h at 37°C in 5 per cent CO₂ humidified atmosphere and were exposed to 6-TG, 6-TG-CNPs, curcumin at their respective IC₅₀ and combination of 6-TG-CNPs (IC₂₅) and curcumin (IC₂₅) for 48h. Cells

were harvested and washed once with 1X DPBS. Cells were then suspended in 100 μ L binding buffer at 1X10⁶ cells/mL concentration. Fixation was carried out by adding 0.7mL chilled 70 per cent absolute ethanol drop wise with gentle vortexing. Later, cells were fixed for 30 min at 4°C, centrifuged at 2500 rpm and washed twice with 1X DPBS. The cells were then treated with 0.2 mg/mL RNase for 20 min at 37°C, followed by staining with 50 μ g/mL PI for 5-10 min in dark. Cell cycle was then analyzed by flow cytometry using Fluorescence channel-2 (FL2).

3.4.6. *In Vitro* Global DNA Demethylating Activity – The Epigenetic Regulation

Genome-wide changes in methylation have been associated with lineage-specific changes in gene expression profiles during development and in some cell-based pathologies, including oncogenesis (Celik-Uzuner *et al.*, 2016). Flow cytometry method was used for specific detection of global DNA methylation (Schneider and d'adda di Fagagna, 2012; Celik-Uzuner *et al.*, 2016) by using anti-5mec FITC conjugated antibody. PA-1 cells were cultured in 6-well plates for 24h at 37°C in 5% CO₂ humidified atmosphere and were exposed to 6-TG, 6-TG-CNPs, curcumin at their respective IC₅₀, standard nucleoside DNMT inhibitor 5-azacytidine at 5 μ M and combination of 6-TG-CNPs (IC₂₅) and curcumin (IC₂₅) for 48h. After incubation cells were harvested and washed with Ca²⁺ and Mg²⁺ free Dulbecco's phosphate buffered saline. Cells were fixed with 70% ethanol for 30 min at -20 °C and followed by 0.5 % (v/v) Triton-X 100 in DPBS for 10 min at RT. Chromatin was denatured by 2N HCl for 10 min at RT followed by 0.25% trypsin treatment for 1 min. Trypsin activity was then stopped by the addition of 10% serum followed by extensive washing. Later, the cells were labelled with FITC conjugated anti-5mec at RT in dark and then analyzed by flow cytometry (BD Biosciences FACSCalibur, USA). Fluorescence channel-1 (FL1) was used for DNA methylation detection. At least 10,000 cells were counted per sample. Staining levels

were measured as mean fluorescence intensities (MFI) using BD CellQuest™ Pro software, Version 6.

3.5. PHARMACOKINETIC EVALUATION OF 6-TG IN 6-TG, 6-TG-CNPS ALONE AND 6-TG-CNPS IN CURCUMIN PRE-TREATED WISTAR RATS BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

3.5.1. Chemicals and Reagents

2-thiouracil ($\geq 99\%$, Sigma, #T7750) was employed as internal standard. Acetic acid, ammonia and HPLC grade methanol and acetonitrile were obtained from Merck (Germany).

3.5.2. Animal Dosing and Experimentation

The study was conducted on eight to ten-week-old healthy male Wistar rats weighing around 200–250g. All animals were maintained as per the protocol outlined in publication of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2003). The animals were kept on conventional laboratory diet and *ad libitum* water and were acclimatized at temperature of $22 \pm 2^\circ\text{C}$ and relative humidity of 30-70% under 12h/12h light/dark conditions for one week before experiment. The animals (n=9) were randomly distributed into three groups (Group I: 6-TG, Group II: 6-TG-CNPs alone and Group III: 6-TG CNPs in curcumin pretreated animals). All formulations were administered orally using an oral gavage needle attached to a hypodermic syringe. Group III animals were administered with curcumin (100 mg/kg body weight with 0.1% sodium carboxymethylcellulose) for four consecutive days. On fifth day, Group I animals were administered with 6-TG (14.8 mg/kg body weight) while Group II and III animals were administered with 6-TG-CNPs (14.8 mg/kg body weight) by oral gavage. All animals were fasted overnight prior to the treatment and water was made available *ad libitum*. The blood samples (~ 0.5 mL) were collected *via* jugular vein cannulation at pre-determined intervals of 0, 0.5, 1.0, 1.5, 2.0, 4.0, 8.0, 12.0 and 24.0h

into the micro-centrifuge tubes containing K₂EDTA as an anticoagulant and was centrifuged at 4000 rpm for 10 min at 4°C to separate plasma from erythrocytes and stored at -20°C until analysis.

3.5.3. Stock Solutions

Main stock solutions of 6-Thioguanine and 2-Thiouracil were prepared by dissolving the appropriate amount of each compound in 5 per cent ammonia in water. Spiking stock solutions of 6-Thioguanine and working stock solution of 2-Thiouracil were prepared by using diluent 1 per cent ammonia in acetonitrile. Stock solutions were stored at 2-8°C until required.

3.5.4. Preparation of Calibration Standard Solutions and Quality Control Stocks

Primary stock solution of 6-thioguanine for calibration standard and quality control (QC) samples were prepared in 5% ammonia in water. From the primary stock solution, appropriate dilutions were made using 1% ammonia in acetonitrile as a diluent to produce working standard solutions of 19.54, 39.06, 78.12, 156.26, 625.00, 2500.00, 5000.00 and 10000.00 ng/mL. These solutions were used to prepare relevant calibration curve (CC) standards. Another set of working solutions of 6-TG was prepared in the diluent (from primary stock) at concentrations of 62.50, 4000.00 and 8000.00 ng/mL respectively for QC samples (LQC, MQC and HQC).

The calibration standards and quality control samples were prepared by spiking 5µl of the spiking stock solution (6-thioguanine) into 95 µl of screened blank rat plasma. K₂-EDTA anticoagulated whole blood was centrifuged at 4000 rpm for 10 min at 4°C to separate plasma from erythrocytes. The plasma fraction was stored at -20±5°C until analysis. Calibration samples were made at concentrations of 0.98, 1.95, 3.91, 7.81, 31.25, 125.00, 250.00 and 500.00 ng/mL. Quality control samples were prepared at concentrations of 3.12, 200.00 and 400.00 ng/mL (LQC, MQC and HQC).

3.5.5. Instrumentation and Chromatographic Conditions

Column used: Agilent®, Cyano RP, 7.5 cm, 3.5u, 4.6.

Mobile phase used: 70% of Acetonitrile: 30% of 0.2% Acetic acid in water (70:30% v/v).

Flow rate: 0.5 mL/min

Column oven temperature: 37 °C

Auto sampler temperature: 10 °C

Injection volume: 5 µL

Retention time of 6-TG: 1.60 min

Retention time of 2-thiouracil (Internal standard-ISTD): 1.59 min

Total chromatographic run time: 3.5 min

Working solution concentration of ISTD: 2.5 µg/mL

Quantification was achieved with MS-MS detection in positive ion mode using an SCIEX API 4000™ Mass spectrometer (CA, USA) and Agilent HPLC system (CA, USA).

3.5.6. Sample Preparation

Protein precipitation extraction method was employed for extraction of 6-thioguanine and internal standard from rat plasma. A 100µl volume of plasma sample was transferred to a 1.5mL micro centrifuge tube and into it 100µl of ISTD working solution of 2-thiouracil (2.5µg/mL) was spiked. The above mixture was vortexed to mix well. To the above mixture 400µl of 1% v/v ammonia in acetonitrile was added and vortexed for 5 min using multipulse vortexer at 2000rpm. Sample was then centrifuged for 5 min at 4000rpm at 4°C. The supernatant was separated and 5µl was injected in to LC-MS/MS.

3.5.7. Method Validation

The method was validated for specificity, selectivity, recovery, matrix effect, precision and accuracy as per USFDA guidelines.

Specificity was assessed by analyzing six blank rat plasma matrix samples.

Selectivity was determined by analyzing six replicates of plasma samples spiked with the lowest level of the calibration curve concentration.

Inter-day assay precision and accuracy was determined by analyzing six replicates at four different QC levels on two different day batches. The acceptance limit of accuracy was $\pm 30\%$.

Recovery of the analyte from the extraction procedure was determined by comparing the peak area of the analyte in spiked plasma samples (extracted samples) (six each of LQC and HQC). The area was compared with the aqueous equivalent of LQC and HQC samples.

Matrix effect was checked with six different lots of K₂EDTA plasma. Six replicate samples each of LQC and HQC were prepared from different lots of plasma. Matrix effect was assessed by injecting the LQC and HQC samples. Post spiking was performed and the area was compared with that of aqueous equivalent LQC and HQC samples.

3.5.8. Determination of Pharmacokinetic Parameters

The pharmacokinetic parameters were calculated from the time vs mean plasma concentration profile by using a menu-driven add-in program for Microsoft excel written in visual basic (PK Solver[®]; Version 2.0) validated previously (Zhang *et al.*, 2010). The PK parameters viz.,

- λ_z = terminal rate constant
- $t_{1/2\lambda_z}$ = elimination half-life
- t_{max} = time to reach maximum concentration
- C_{max} = peak plasma concentration
- AUC_{0-t} = Area under the curve from '0' to 't'
- AUC_{0-inf} = Area under the curve from '0' to 'inf'
- $AUMC_{0-inf}$ = Area under movement curve from '0' to 'inf'

- MRT = Mean residence time
- V_z/F_{obs} = Volume of distribution
- Cl/F_{obs} = Clearance
- $V_{d_{ss}}$ = Volume of distribution steady state

$V_{d_{ss}}$ was calculated using the formula: $V_{d_{ss}} = AUMC / (AUC)^2 \times \text{Dose}$

3.6. STATISTICAL ANALYSIS

The data of *in vitro* cytotoxicity studies were subjected to linear regression analysis to obtain dose-response curves and IC_{50} values. All the values were expressed as Mean $\pm$ SD (GraphPad Prism, Version 5). Apoptosis/necrosis stages, cell cycle stages and mean fluorescence intensities of FITC-anti-5mec were analyzed by using BD CellQuest™ Pro software, Version 6 and difference in percentage of population between vehicle control and treatment groups were calculated based on the statistical data generated by the system (BD Biosciences FACSCalibur, USA). The *in vitro* cell culture assays (MTT, apoptosis-necrosis, cell cycle and DNA demethylating activity) and *in vivo* pharmacokinetic data were analyzed by One-way ANOVA followed by post hoc Tukey's multiple comparison test. All the values were expressed as Mean $\pm$ SD (Graph Pad Prism, version 5).

Results

CHAPTER: IV

RESULTS

Synthesis and characterization of 6-TG loaded chitosan nanoparticles, *in vitro* cell culture studies viz., MTT, apoptosis-necrosis, cell cycle and demethylating activity and pharmacokinetic study of 6-TG in 6-TG, 6-TG-CNPs treated alone and 6-TG-CNPs in curcumin pre-treatment group were carried out in the present investigation and the results are presented hereunder.

4.1. PREPARATION OF 6-TG ENCAPSULATED CHITOSAN NANOPARTICLES

6-TG encapsulated chitosan nanoparticles were prepared by ionic gelation crosslinking method between chitosan (0.5 mg/mL) solution containing 1.0 mg/mL concentration of 6-TG and TPP (0.5 mg/mL) solution.

4.2. CHARACTERIZATION OF 6-TG ENCAPSULATED CHITOSAN NANOPARTICLES

4.2.1. Zetasizer Analysis

Particle size, polydispersity index and zeta potential were measured by dynamic light scattering technique by using Zetasizer. Colloidal stability was determined by measuring particle size and zeta potential. The mean particle size, polydispersity index and zeta potential of 6-TG loaded chitosan nanoparticles were 261.63 ± 6.01 nm, 0.34 ± 0.10 and $+15.97 \pm 0.46$ mV, respectively. The particle size and polydispersity index of 6-TG-CNPs are depicted in Fig.7 and zeta potential of 6-TG-CNPs are depicted in Fig.8.

4.2.2. Scanning Electron Microscope Analysis

Morphologically, 6-thioguanine is irregular in shape and 6-thioguanine encapsulated chitosan nanoparticles are spherical in shape by scanning electron

microscope analysis. SEM images of 6-thioguanine and 6-thioguanine encapsulated chitosan nanoparticles are depicted in Fig.9a and 9b respectively.

4.2.3. Fourier Transform Infrared Spectroscopy (FTIR) analysis

The fundamental vibrations (FTIR) of primary functional groups and their frequency (cm^{-1}) of chitosan, 6-TG and 6-TG-CNPs are shown in Fig.10a, 10b and 10c, respectively.

In chitosan (Fig.10a), the main characteristic absorption bands appeared at 1550.48 cm^{-1} and 1380.01 cm^{-1} were assigned to N-H bending vibration and C-O stretching of the alcohol group respectively. The bands at 1032.89 cm^{-1} , 3436.73 cm^{-1} and 2927.11 cm^{-1} were assigned to C-N, N-H and C-H stretching vibrations, respectively.

In 6-TG (Fig.10b), the main characteristic absorption bands appeared at 1202.01 cm^{-1} , 1373.28 cm^{-1} and 3492.18 cm^{-1} were assigned to C=S, C-N and $-\text{NH}_2$ vibrations, respectively.

The peak observed in Fig.10c for chitosan at 3436.73 cm^{-1} was assigned to the N-H stretching and the shift in the same spectral peak to interior (3418.33 cm^{-1}) in the 6-TG-CNPs confirms the formation of the 6-TG complex with chitosan. The resultant complex also evidenced missing of spectral peaks related to C=S (1202.01 cm^{-1}) and C-N (1373.28 cm^{-1}) of 6-TG in the 6-TG-CNPs. All these changes demonstrated that 6-TG was encapsulated to chitosan.

4.2.4. Determination of Entrapment Efficiency (EE) of 6-Thioguanine Encapsulated Chitosan Nanoparticles

The entrapment efficiency (EE) of 6-thioguanine encapsulated chitosan nanoparticles was determined by Double beam UV-Vis Spectrophotometer. The entrapment efficiency of 6-TG-CNPs was found to be 44.27% (Fig.11).

4.2.5. *In Vitro* Release of 6-Thioguanine from Chitosan Nanoparticles

6-TG release profiles up to 48h of incubation period at pH 4.8 and 7.4 are presented in Table 1 and Fig.12.

Desorption profile of 6-TG-CNPs in PBS of pH 4.8 showed 57.14 ± 0.91 per cent 6-TG release from the nanoparticles in the first 4h, 82.48 ± 0.92 per cent in 12h which increased up to 87.32 ± 1.16 per cent in 24h and 91.40 ± 1.08 per cent in 48h (Table 1 and Fig.12).

Desorption profile of 6-TG-CNPs in PBS of pH 7.4 showed 19.71 ± 1.29 per cent 6-TG release from the nanoparticles in the first 4h, 48.17 ± 0.95 per cent in 12h, 63.53 ± 6.44 per cent in 24h and 73.96 ± 1.12 per cent in 48h (Table 1 and Fig.12).

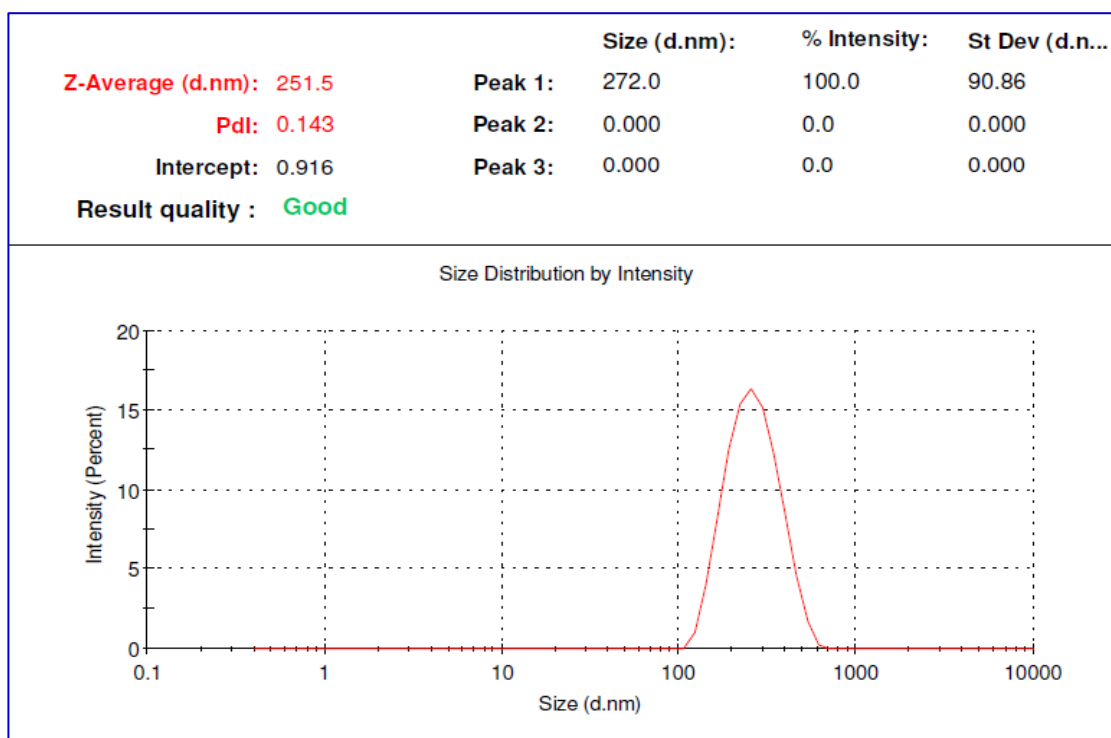


Fig.7. Representative image of particle size and polydispersity index of 6-TG-CNPs by zetasizer analysis

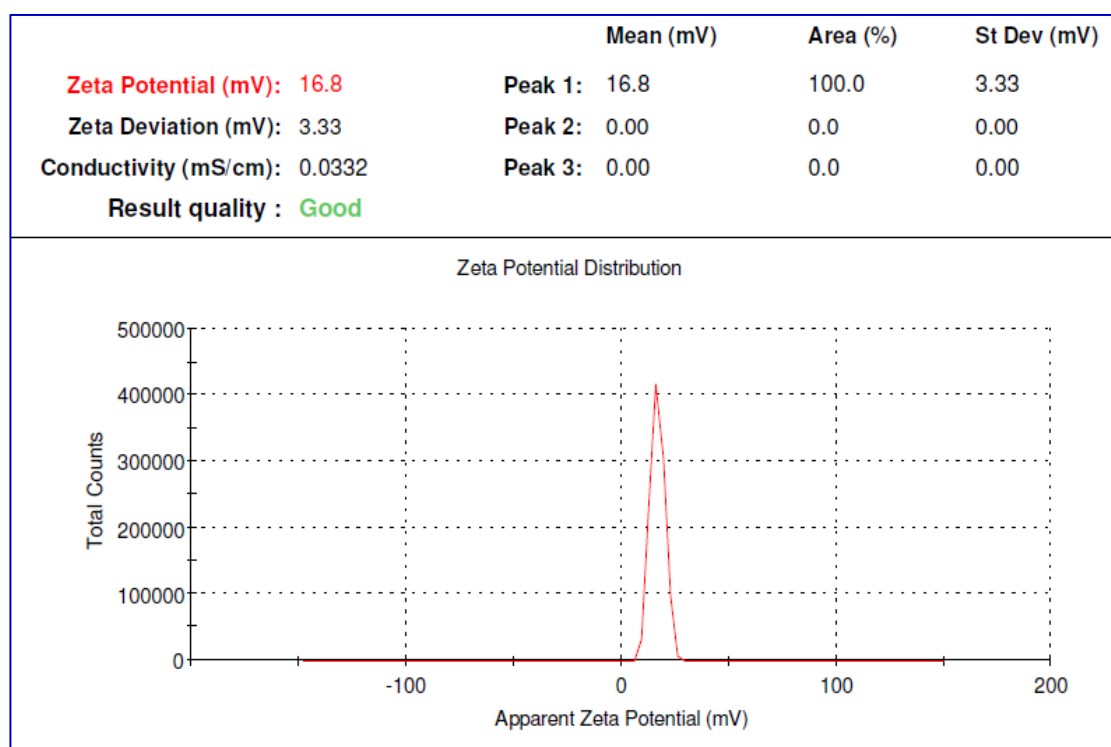


Fig.8. Representative image of zeta potential of 6-TG-CNPs by zetasizer analysis

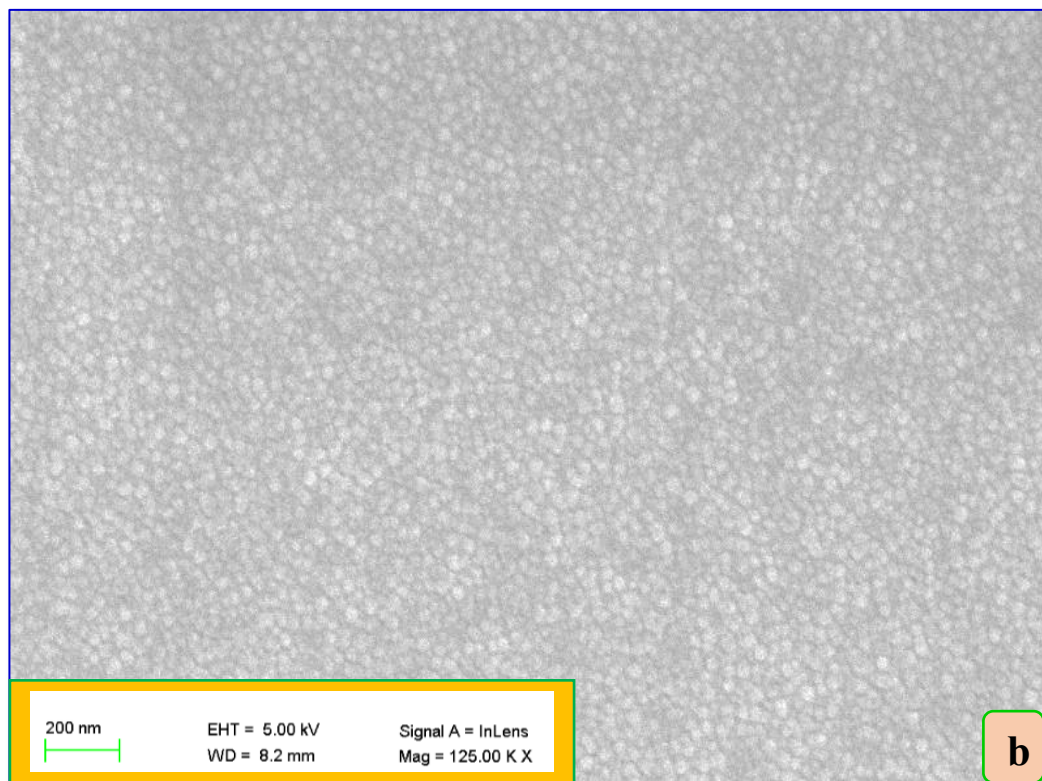
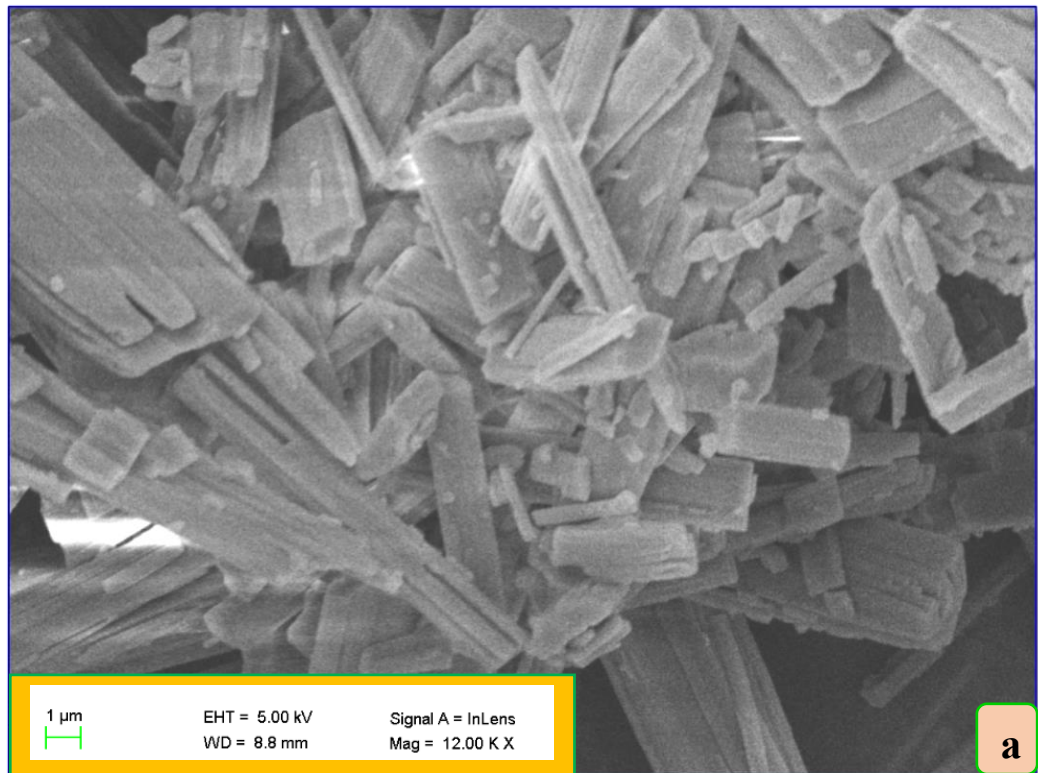


Fig.9. Representative Scanning electron microscope image of a) 6-TG and b) 6-TG-CNPs

Fig.10. Representative image of IR Spectra of a) Chitosan b) 6-TG and c) 6-TG-CNPs

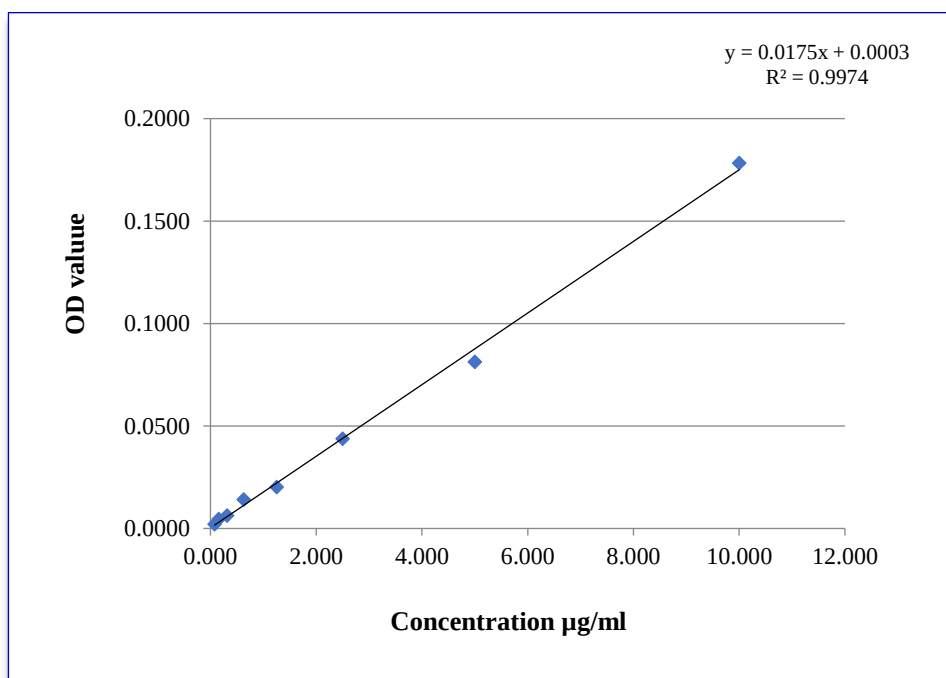


Fig.11. Standard calibration curve of 6-thioguanine (0.078 to 10 µg/ml) used to determine entrapment efficiency

Table 1: *In vitro* drug release of 6-TG from 6-TG-CNPs at pH 4.8 and 7.4. Values are Mean SD, n=3

Time (h)	% Drug release at pH: 4.8	% Drug release at pH: 7.4
0	0	0
0.083	2.84 ± 1.26	0.81 ± 0.10
0.25	10.03 ± 0.78	1.64 ± 0.60
0.5	18.35 ± 1.03	3.09 ± 0.49
1	26.30 ± 0.69	6.08 ± 0.80
2	41.48 ± 0.69	7.70 ± 1.20
4	57.14 ± 0.91	19.71 ± 1.29
8	76.47 ± 0.94	31.60 ± 1.66
12	82.48 ± 0.92	48.17 ± 0.95
24	87.32 ± 1.16	63.53 ± 6.44
48	91.40 ± 1.08	73.96 ± 1.12

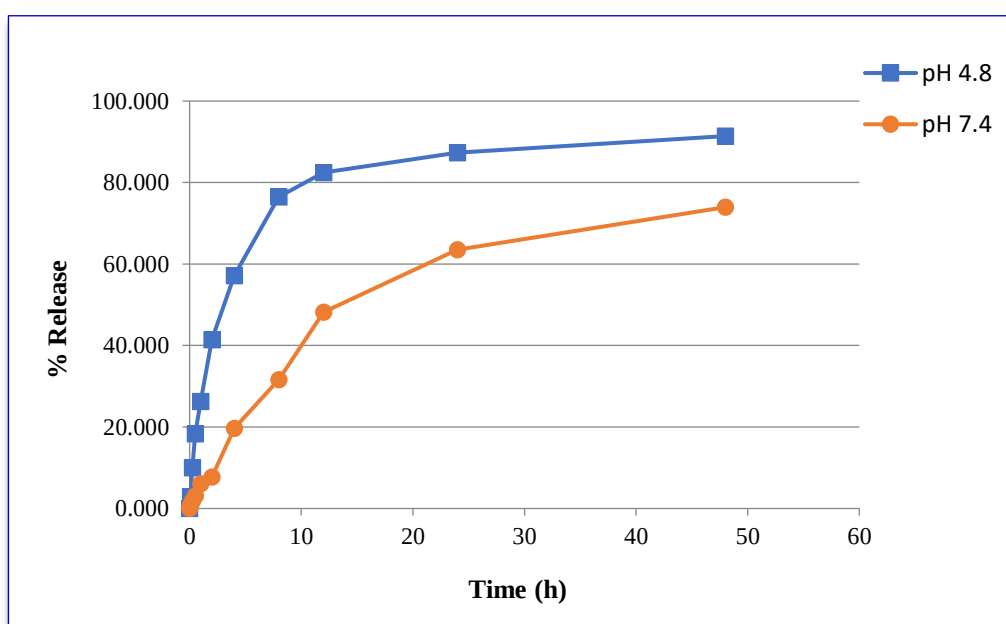


Fig.12. *In vitro* drug release profile of 6-TG from 6-TG-CNPs at pH 4.8 and 7.4

4.3. *IN VITRO* CELL LINE STUDIES

4.3.1. *In Vitro* Cytotoxicity Assay

MTT [3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] assay was conducted on MCF-7 and PA-1 cell lines at 48 h incubation for cytotoxicity evaluation. IC_{50} values of 6-TG, 6-TG loaded CNPs and curcumin for MCF-7 were 23.09, 17.82 and 15.73 μ M, respectively (Fig.13a, 13b and 13c; Fig.15b, 15c and 15d, respectively). Likewise, IC_{50} values of 6- TG, 6-TG loaded CNPs and curcumin for PA-1 were 5.81, 3.92 and 12.89 μ M, respectively (Fig.14a, 14b and 14c; Fig.16b, 16c and 16d, respectively). The effect of vehicle control on MCF-7 and PA-1 cell lines is depicted in Fig.15a and Fig.16a, respectively. The cytotoxicity of 6-TG, 6-TG-CNPs and curcumin on both MCF-7 and PA-1 cell lines was dose- dependent (Fig.17a and 17b, respectively). Combination of 6-TG-CNPs (IC_{25}) with curcumin (IC_{25}) on MCF-7 and PA-1 showed a percent cell viability of 49.77 ± 0.05 and 43.67 ± 0.02 , respectively (Fig.15e and 16e; Fig.18a and Fig.18b, respectively).

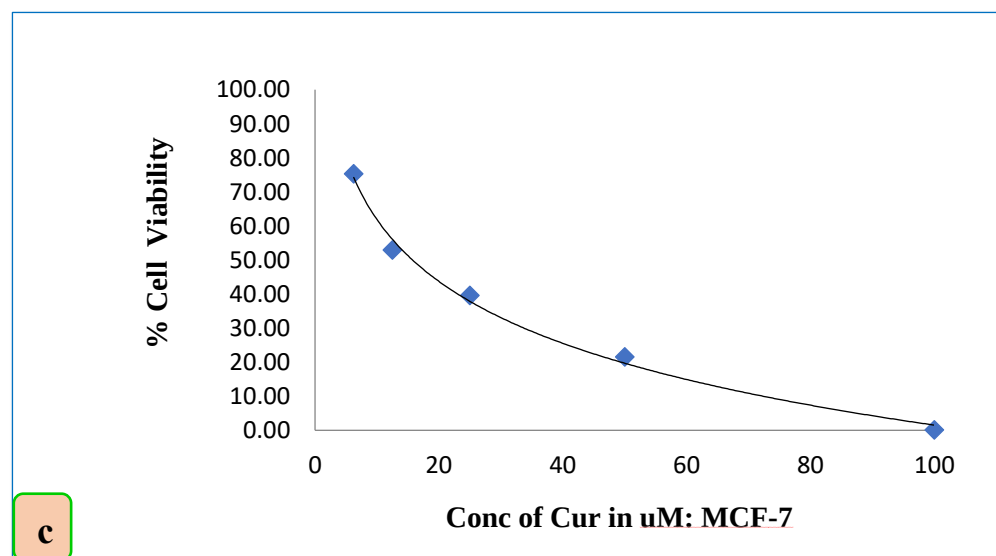
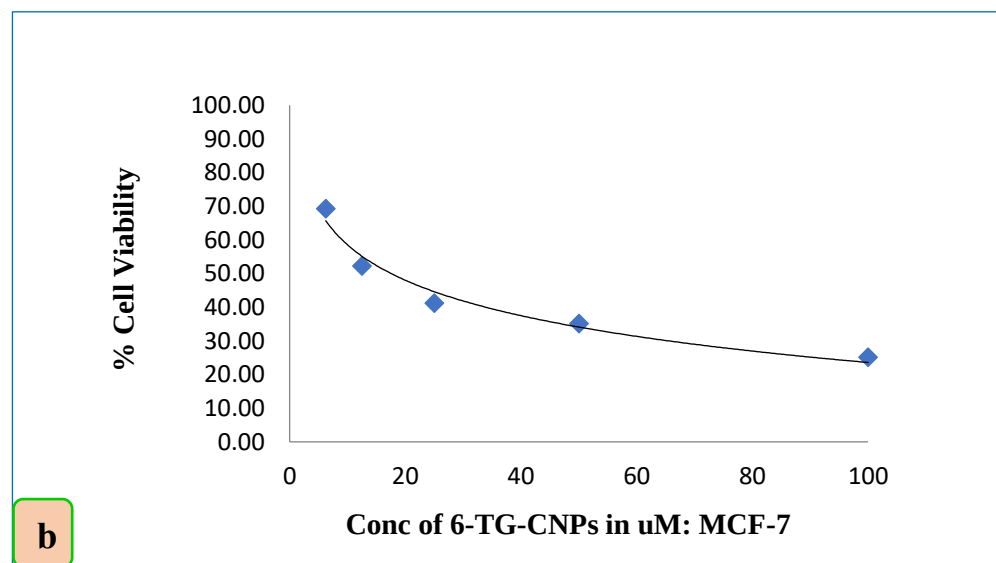
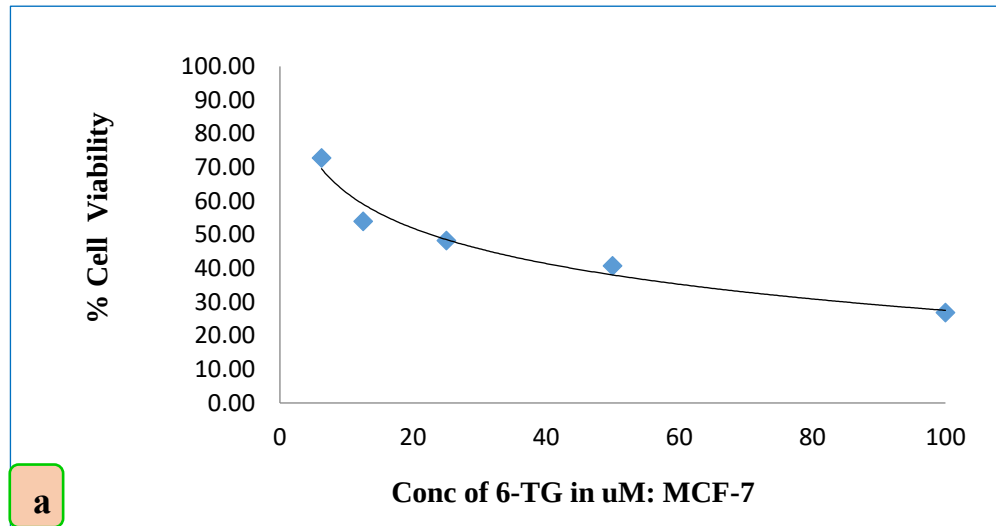


Fig.13. Effect of 6-TG, 6-TG-CNPs and curcumin on MCF-7 cancer cell line

a) 6- TG: $IC_{50} = 23.09\mu M$ (b) 6-TG-CNPs: $IC_{50} = 17.82\mu M$

(c) curcumin: $IC_{50} = 15.73\mu M$

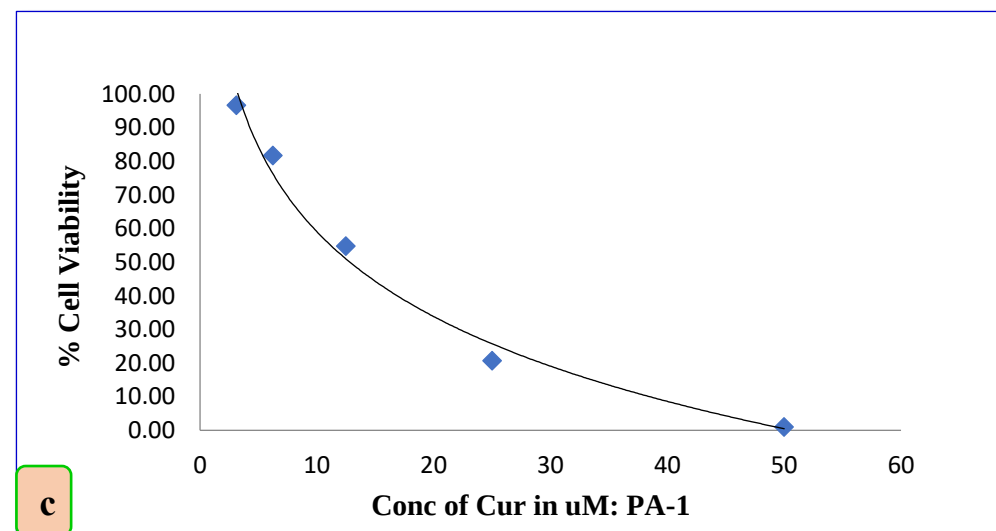
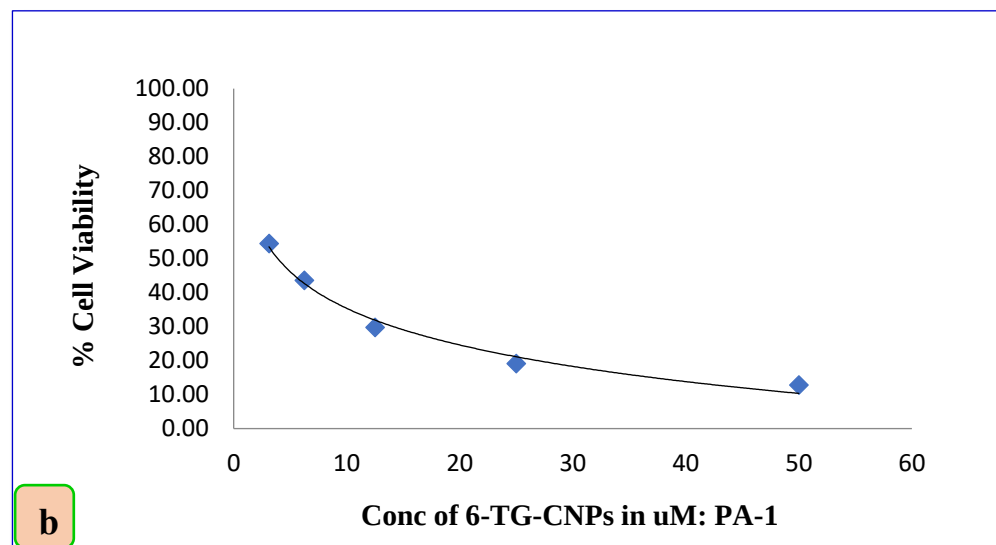
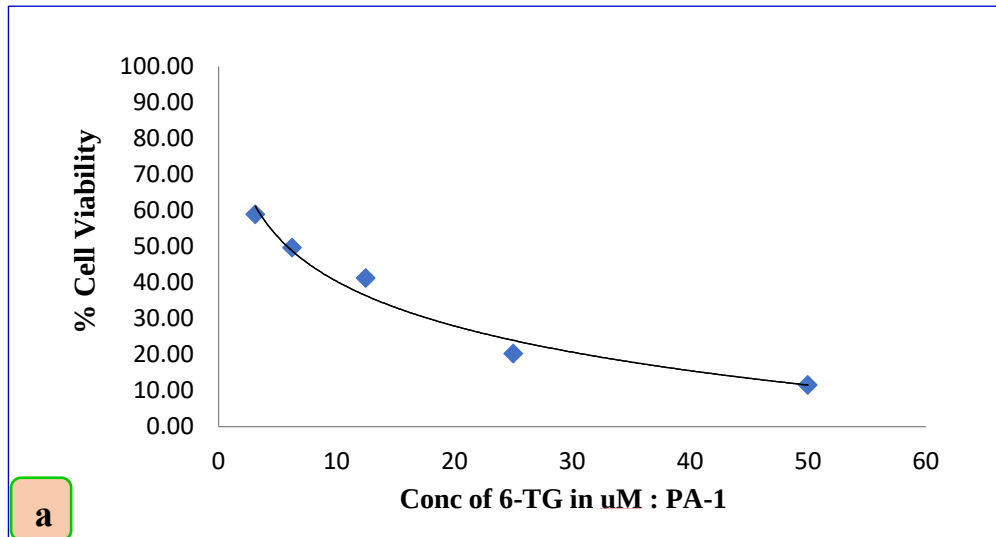


Fig.14. Effect of 6-TG, 6-TG-CNPs and curcumin on PA-1 cancer cell line

(a) 6-TG: $IC_{50} = 5.81\mu M$ (b) 6-TG-CNPs: $IC_{50} = 3.92\mu M$

(c) curcumin $IC_{50} = 12.89\mu M$

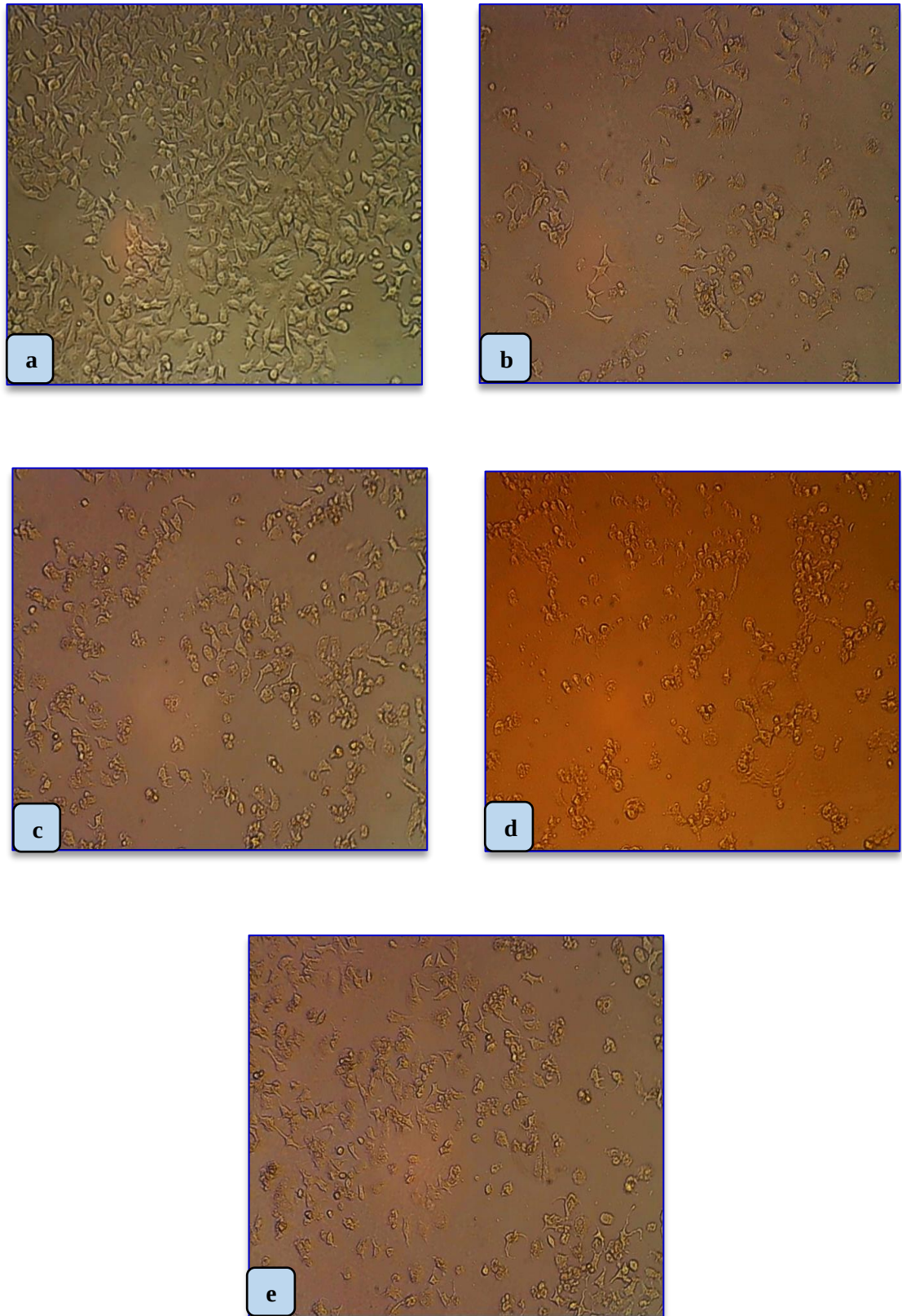


Fig.15. Representative images of cytotoxic effect of a) vehicle control b) 6-TG c) 6-TG-CNPs d) curcumin and e) combination [6-TG-CNPs (IC₂₅) + curcumin (IC₂₅)] on MCF-7 cell lines at 48h incubation (10X)

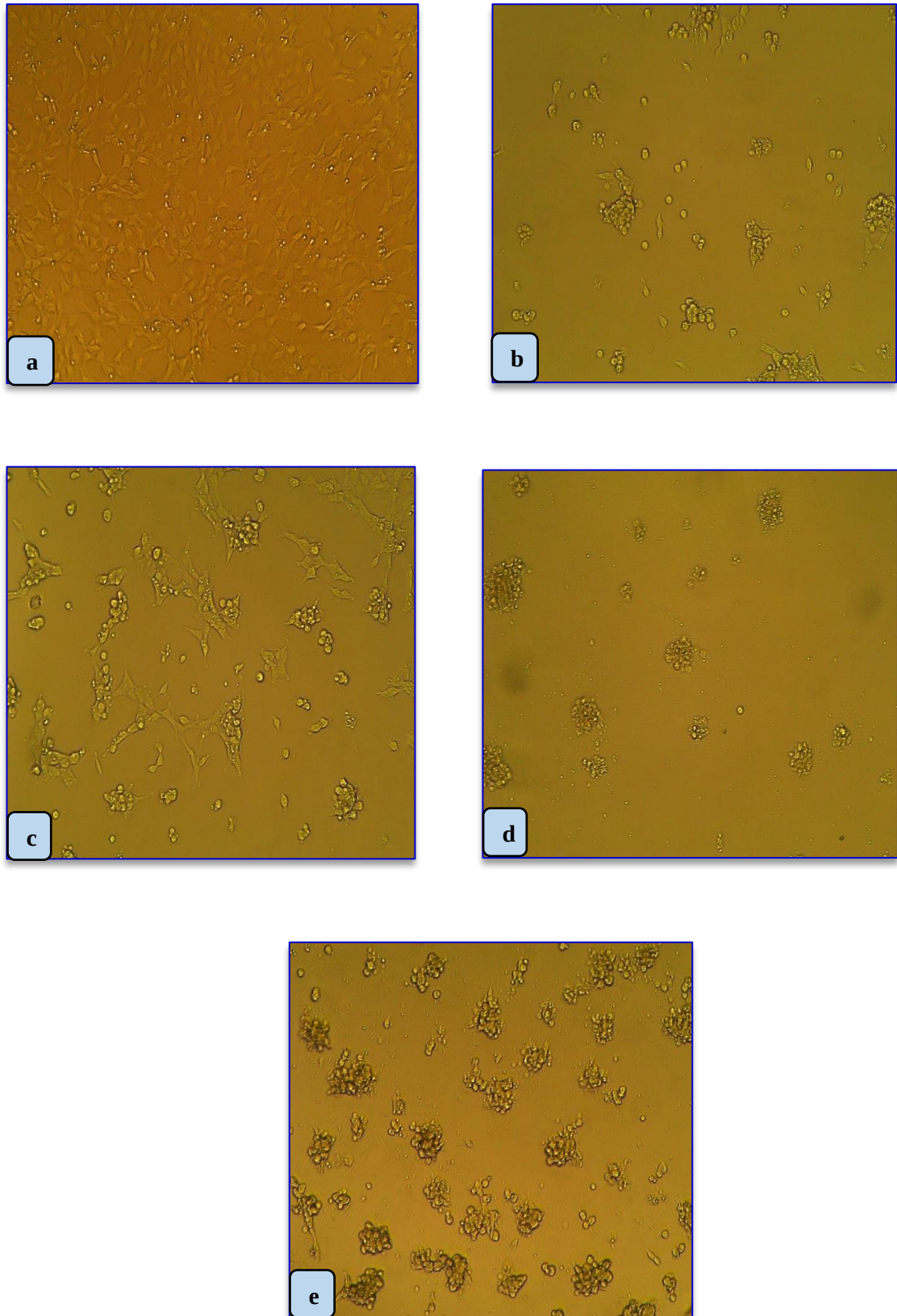


Fig.16. Representative images of cytotoxic effect of a) vehicle control b) 6-TG c) 6-TG-CNPs d) curcumin and e) combination [6-TG-CNPs (IC₂₅) + curcumin (IC₂₅)] on PA-1 cell lines at 48 h incubation (10X)

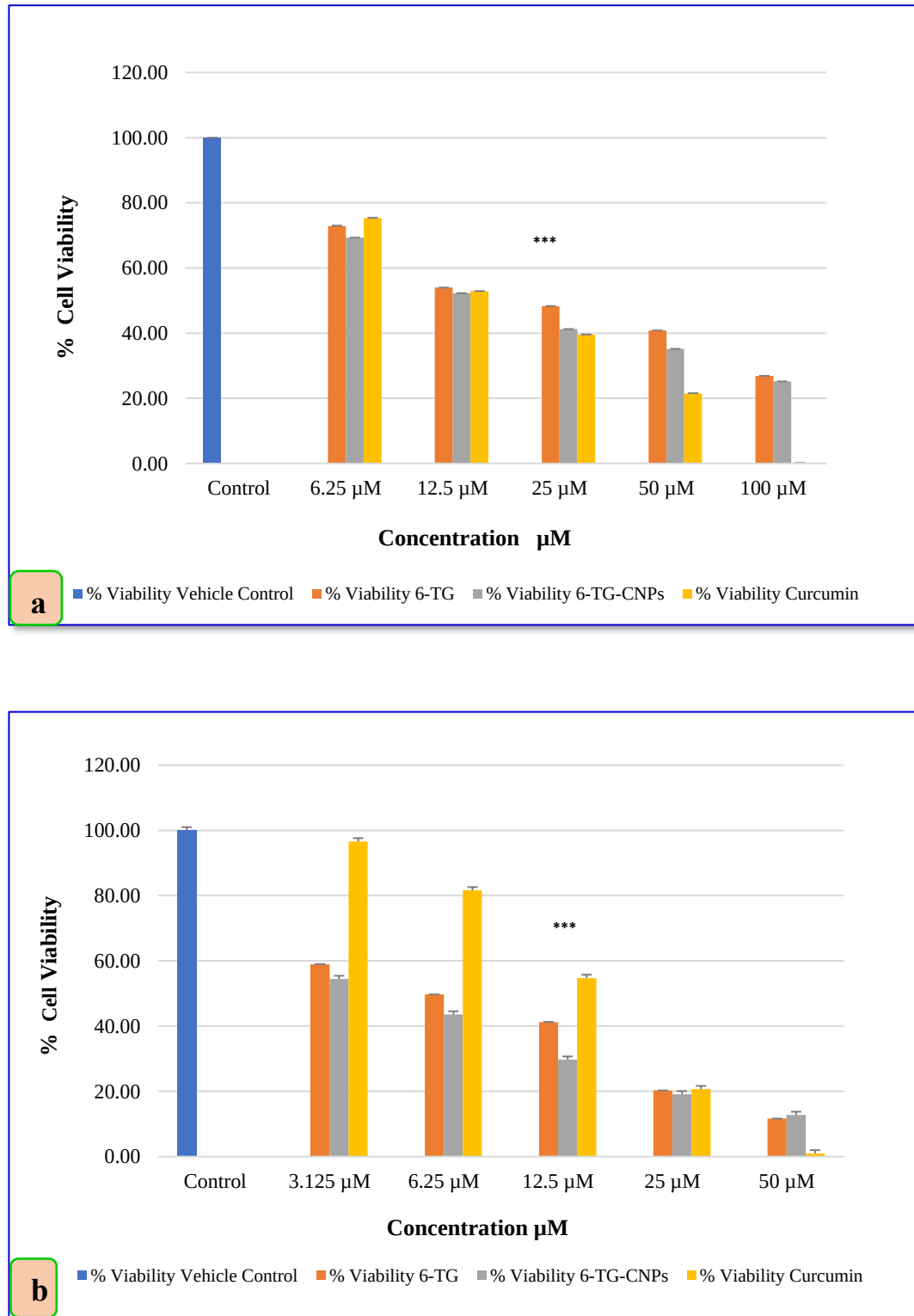


Fig.17. Histogram depiction of dose dependent cytotoxicity data of 6-TG, 6-TG-CNPs and curcumin as determined by MTT assay on (a) MCF- 7 and (b) PA- 1 cell lines. Values are Mean $\pm$ SD, n=3, (*) $p < 0.0001$)**

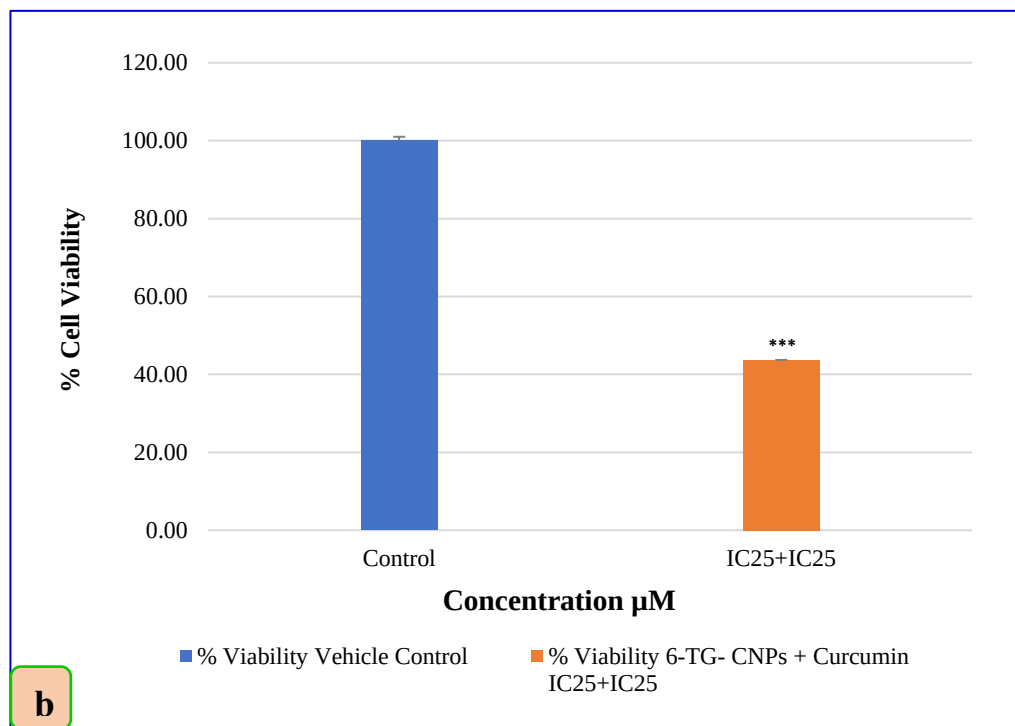
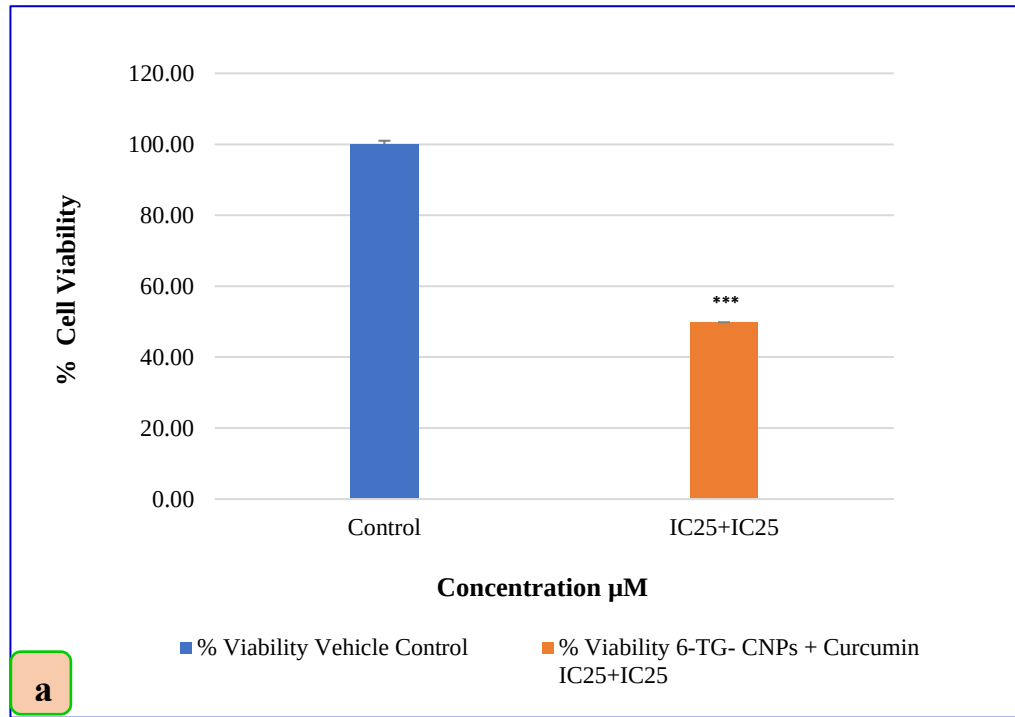


Fig.18. Bar graph depiction of cytotoxicity data of combination studies (6-TG-CNPs IC₂₅ and curcumin IC₂₅) as determined by MTT assay on (a) MCF- 7 and (b) PA-1 cell lines. Values are Mean $\pm$ SD, n=3, (*)p<0.0001)**

4.3.2. Detection of Apoptosis by Flow Cytometry Analysis

Annexin V-FITC assay was carried out to determine percentage of cells in early apoptosis and late apoptosis phase. Annexin V-FITC is a Ca^{2+} dependent phospholipid-binding protein has a high affinity for the phospholipid, phosphatidylserine (PS). The PS is translocated from the inner to the outer plasma membrane during apoptosis. Binding of annexin V-FITC to PS measure the percentage of cells undergoing apoptosis. Propidium iodide is used to detect non-viable cells which enters through damaged cell membranes and intercalates with the DNA of necrotic cells.

Cells that stain positive for the annexin V-FITC and negative for the propidium iodide are undergoing apoptosis and those cells that stain positive for both the Annexin V-FITC and propidium iodide are either at the end of apoptosis, undergoing necrosis or non-viable. Cells that do not stain with either of the two stains are viable and not undergoing measurable apoptosis (Tuschl and Schwab, 2004). Flow cytometry images (Fig.19a and 19b; Fig.20a and 20b; Fig.21) of apoptosis assay indicates the distribution of cells into four quadrants. Lower left (LL) quadrant (Annexin V-/PI-) is regarded as the population of live cells, lower right quadrant (LR) (Annexin V+/PI-) is considered as the cell population at early apoptotic stage, upper right (UR) quadrant (Annexin V+/PI+) represents the cell population at late apoptotic stage and extreme upper right (UR) and upper left (UL) quadrant (Annexin V-/PI+) is considered as necrotic cell population.

Flow cytometric data analysis revealed that PA-1 cells with vehicle control showed 96.94 ± 0.32 per cent cell viability and 3.01 ± 0.34 per cent of cells in early apoptosis phase (Fig.19a and Table 2). 6-thioguanine (IC_{50}) treated group showed 45.79 ± 2.33 per cent of cells in early apoptosis and 3.47 ± 0.87 per cent of cells in late apoptosis stage (Fig.19b and Table 2). 6-TG-CNPs (IC_{50}) treated group showed 61.92 ± 2.20 per cent of cells in early apoptosis and 2.98 ± 0.68 per cent of cells in late apoptosis stage. (Fig.20a and Table 2). Curcumin (IC_{50}) treated group showed 77.33 ± 3.97

per cent of cells in early apoptosis and 22.58 ± 3.99 per cent of cells in late apoptosis stage (Fig.20b and Table 2). The combination of 6-TG-CNPs (IC_{25}) and curcumin (IC_{25}) treated group showed 90.67 ± 1.79 per cent of cells in early apoptosis and 8.28 ± 1.71 per cent in late apoptosis stage (Fig.21 and Table 2).

The treatment groups viz., 6-TG, 6-TG-CNPs, curcumin and combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher (45.79 ± 2.33 , 61.92 ± 2.20 , 77.33 ± 3.97 and 90.67 ± 1.79 , respectively) per cent of cells in early apoptosis when compared to those observed with vehicle control (3.01 ± 0.34 per cent) group (Table 2 and Fig.28).

The treatment groups viz., 6-TG-CNPs and combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher (61.92 ± 2.20 and 90.67 ± 1.79 , respectively) per cent of cells in early apoptosis when compared to those observed with 6-TG (45.79 ± 2.33 per cent) treated group (Table 2 and Fig.28).

The combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher (90.67 ± 1.79) per cent of cells in early apoptosis when compared to those observed with 6-TG-CNPs alone (61.92 ± 2.20 per cent) treated group (Table 2 and Fig.28).

The combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.01$) higher (8.28 ± 1.71) per cent of cells in late apoptosis when compared to those observed with 6-TG (0.05 ± 0.02 per cent) treated group (Table 2 and Fig.28).

4.3.3. Study of Cell Cycle Arrest by Flow Cytometry Analysis

PA-1 cells upon treatment with 6-TG, 6-TG-CNPs and curcumin at their respective IC_{50} concentration and combination of 6-TG-CNPs with curcumin at their respective IC_{25} concentration for 48h incubation showed G2/M phase arrest. The vehicle control and 6-TG treated group arrested 33.26 ± 0.47 and 40.86 ± 0.34 per cent of cells, respectively in G2/M phase (Fig.22a and 22b; Table 3) of the cell cycle. The 6-TG-CNPs and curcumin treated group arrested 47.49 ± 0.29 and 48.59 ± 0.52 per cent of cells,

respectively in G2/M phase (Fig.23a and 23b; Table 3) of the cell cycle. The combination of 6-TG-CNPs and curcumin treated group arrested 50.42 ± 0.74 per cent of cells in G2/M phase of the cell cycle (Fig.24 and Table 3).

The treatment groups viz., 6-TG, 6-TG-CNPs, curcumin and combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher (40.86 ± 0.34 , 47.49 ± 0.29 , 48.59 ± 0.52 and 50.42 ± 0.74 , respectively) percent of cells arrest in G2/M phase of the cell cycle when compared to those observed with vehicle control (33.26 ± 0.47 per cent) group (Table 3 and Fig.29).

The treatment groups viz., 6-TG-CNPs and combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher (47.49 ± 0.29 and 50.42 ± 0.74 , respectively) percent of cells arrest in G2/M phase of the cell cycle when compared to those observed with 6-TG (40.86 ± 0.34 per cent) treated group (Table 3 and Fig.29).

The combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher (50.42 ± 0.74) percent of cells arrest in G2/M phase of the cell cycle when compared to those observed with 6-TG-CNPs alone (47.49 ± 0.29 per cent) treated group (Table 3 and Fig.29).

4.3.4. Detection of Global DNA Demethylating Activity by Flow Cytometry Analysis

In the present study, global DNA demethylating activity was measured as relative mean fluorescence intensity of anti-5mc-FITC conjugated antibody by flow cytometry.

The global DNA demethylating activity produced by vehicle control and 5-Azacytidine ($5 \mu\text{M}$) treated group were 10.32 ± 0.05 and 6.95 ± 0.09 , respectively (Fig.25a and 25b; Table 4). The global DNA demethylating activity produced by 6-TG (IC_{50}) and 6-TG-CNPs (IC_{50}) treated group were 6.86 ± 0.07 and 6.18 ± 0.06 , respectively (Fig.26a and 26b; Table 4). The global DNA demethylating activity produced by curcumin (IC_{50}) and combination of 6-TG-CNPs (IC_{25}) with curcumin (IC_{25}) treated group were 7.29 ± 0.12 and 4.95 ± 0.05 , respectively (Fig.27a and 27b; Table 4).

The treatment groups viz., 5-azacytidine, 6-TG, 6-TG-CNPs, curcumin and combination of 6-TG-CNPs with curcumin showed significant ($p < 0.0001$) increase (6.95 ± 0.09 , 6.86 ± 0.07 , 6.18 ± 0.06 , 7.29 ± 0.12 and 4.95 ± 0.05 , respectively) in global DNA demethylating activity when compared to those observed with vehicle control (10.30 ± 0.05) group (Table 4 and Fig.30).

The treatment groups viz., 6-TG-CNPs and combination of 6-TG-CNPs with curcumin showed significant ($p < 0.0001$) increase (6.18 ± 0.06 and 4.95 ± 0.05 , respectively) in global DNA demethylating activity when compared to those observed with 6-TG (6.86 ± 0.07) treated group (Table 4 and Fig.30).

The combination of 6-TG-CNPs with curcumin showed significant ($p < 0.0001$) increase (4.95 ± 0.05) in global DNA demethylating activity when compared to those observed with 6-TG-CNPs (6.18 ± 0.06) treated group (Table 4 and Fig.30).

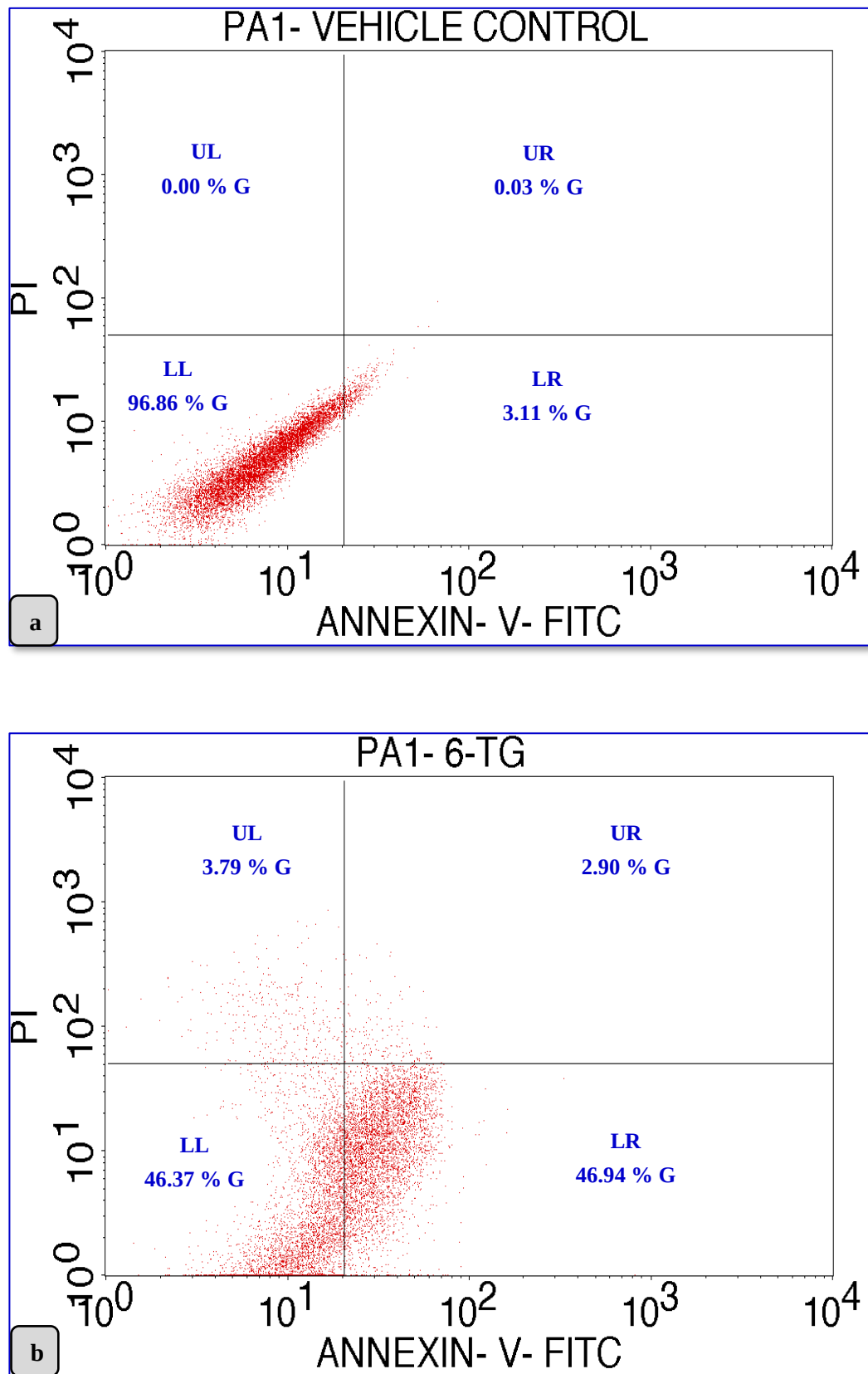


Fig.19. Representative image of flow cytometry analysis of apoptosis in PA-1 cells after staining with annexin V-FITC/PI: a) vehicle control b) 6-TG

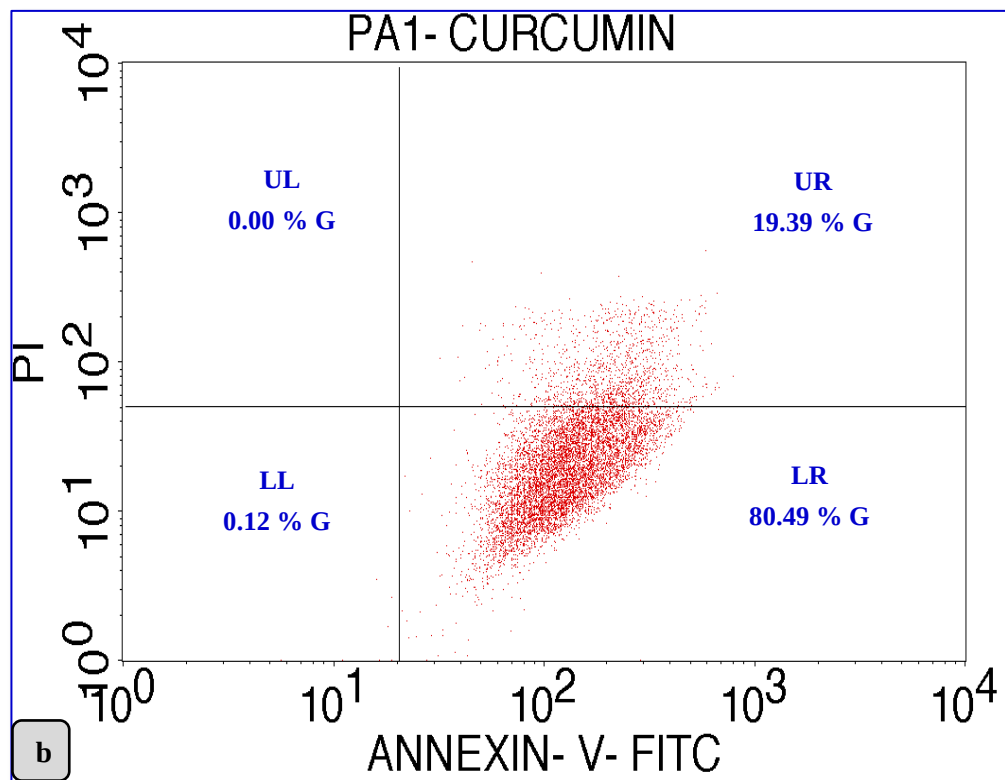
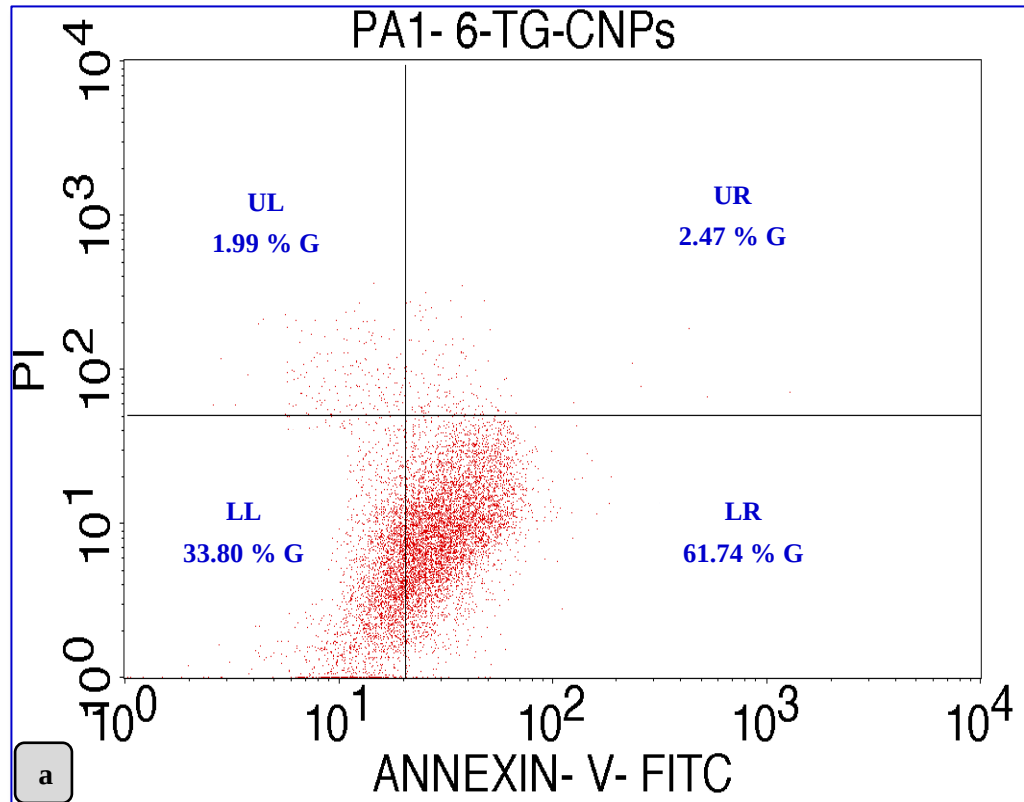


Fig.20. Representative image of flow cytometry analysis of apoptosis in PA-1 cells after staining with annexin V-FITC/PI: a) 6-TG-CNPs b) curcumin

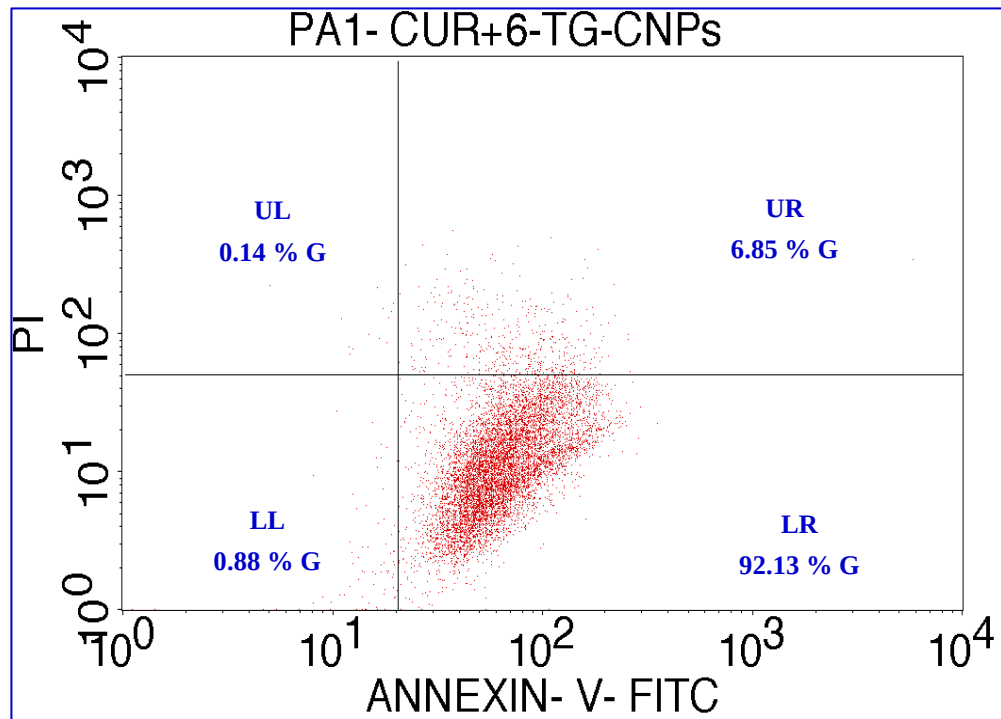


Fig.21. Representative image of flow cytometry analysis of apoptosis in PA-1 cells after staining with annexin V-FITC/PI: 6-TG-CNPs (IC₂₅) + curcumin (IC₂₅)

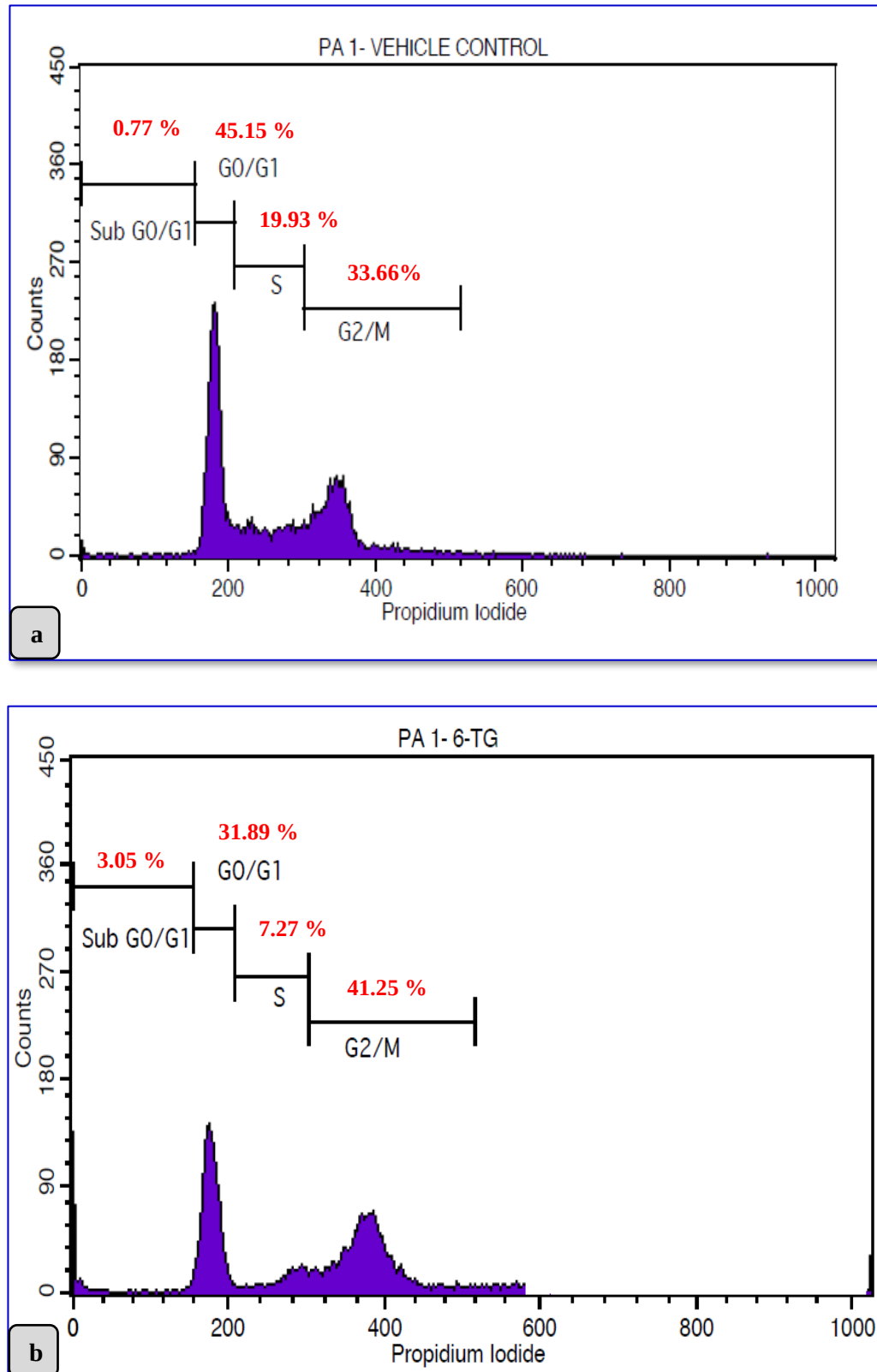


Fig.22. Representative image of cell cycle analysis in PA-1 cells at 48h incubation by flow cytometry a) vehicle control b) 6-TG

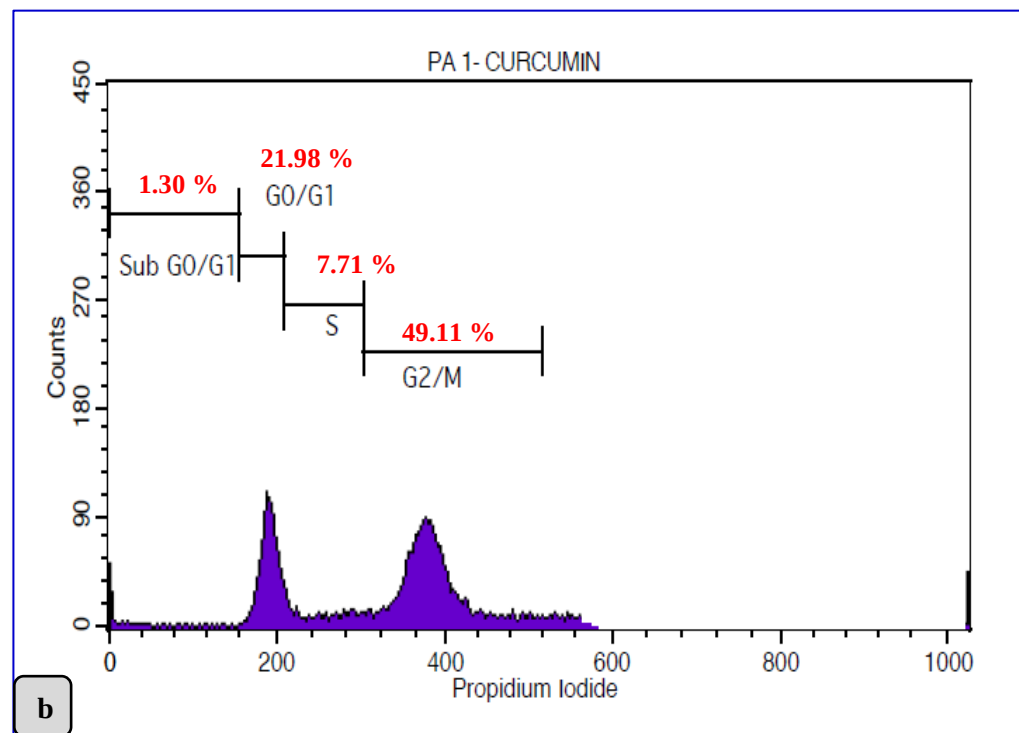
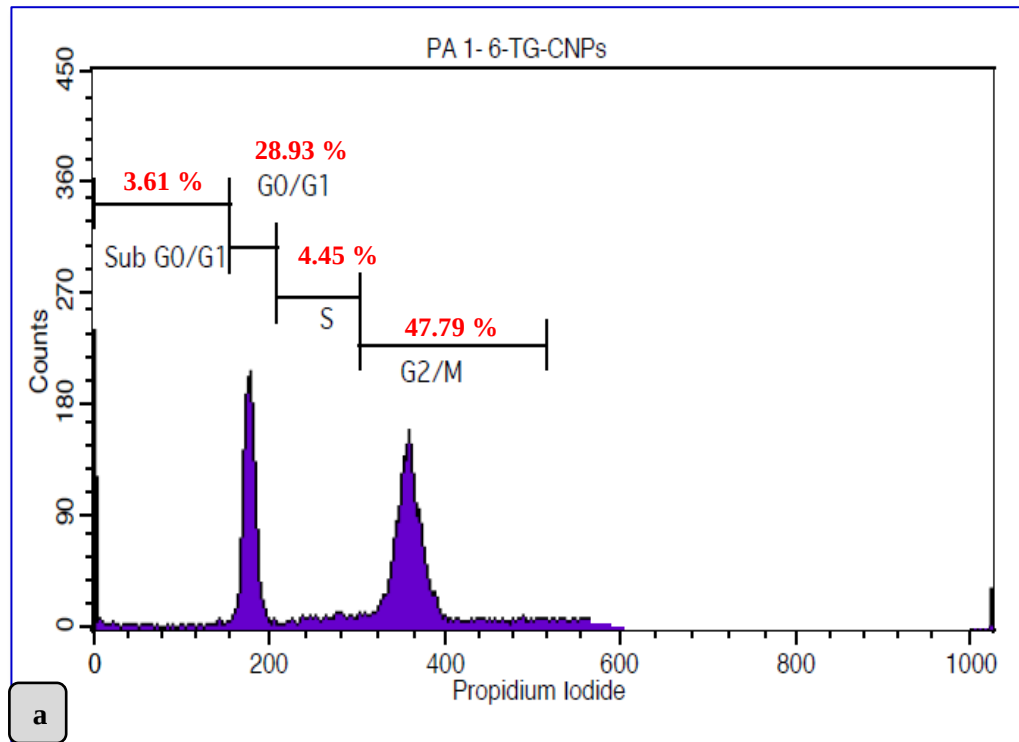


Fig.23. Representative image of cell cycle analysis in PA-1 cells at 48h incubation by flow cytometry a) 6-TG-CNPs b) curcumin

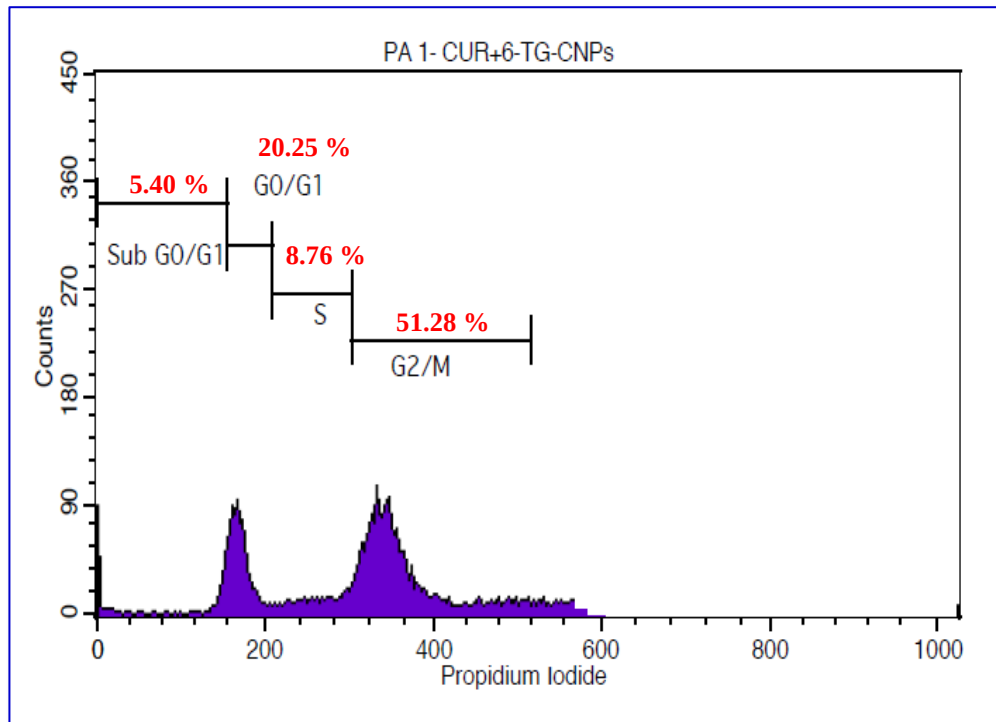


Fig.24. Representative image of cell cycle analysis in PA-1 cells at 48h incubation by flow cytometry: 6-TG-CNPs (IC₂₅) + curcumin (IC₂₅)

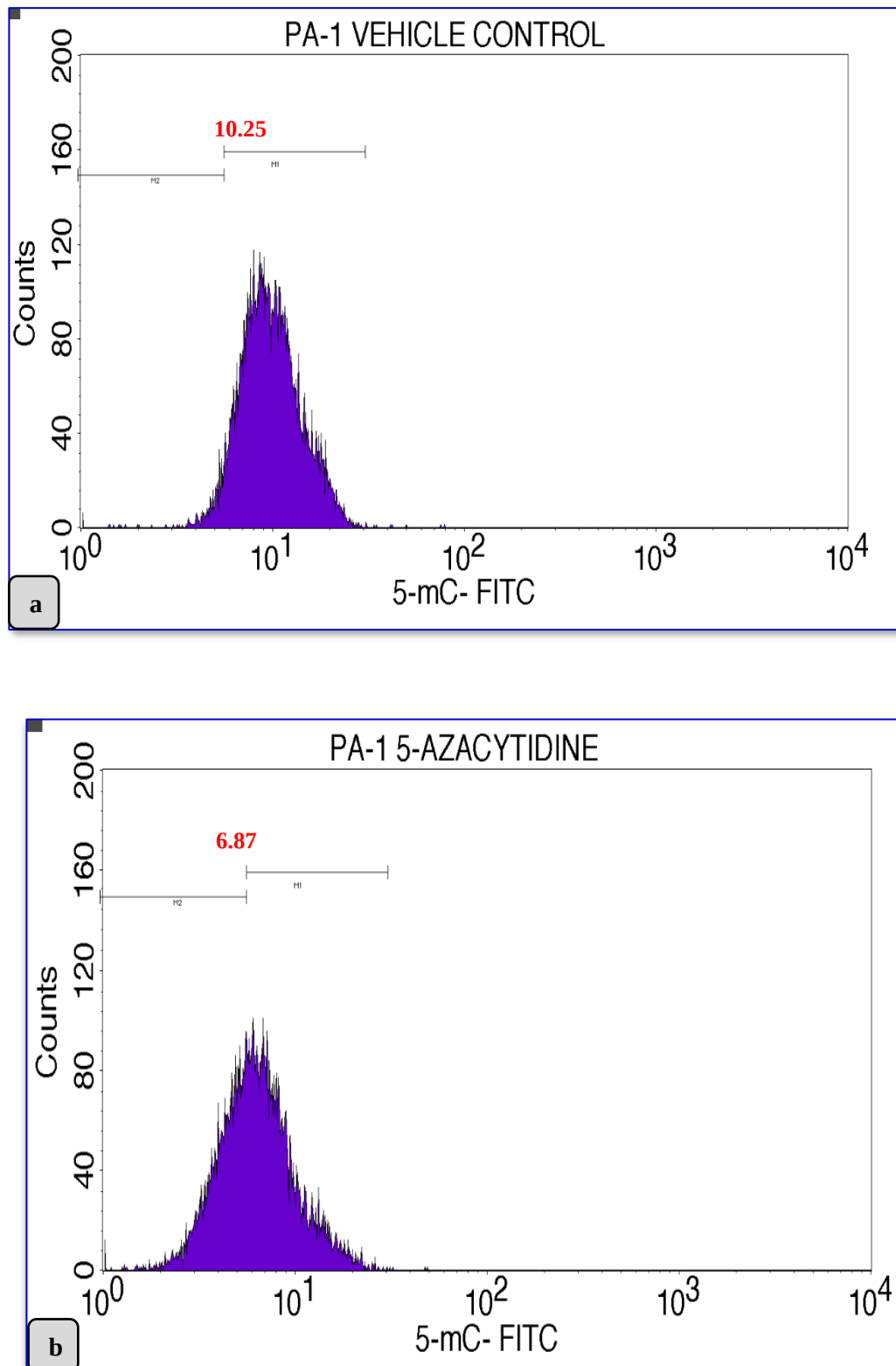


Fig.25. Representative image of flow cytometry analysis of global DNA demethylating activity in PA-1 cells at 48h incubation: a) vehicle control b) 5-azacytidine

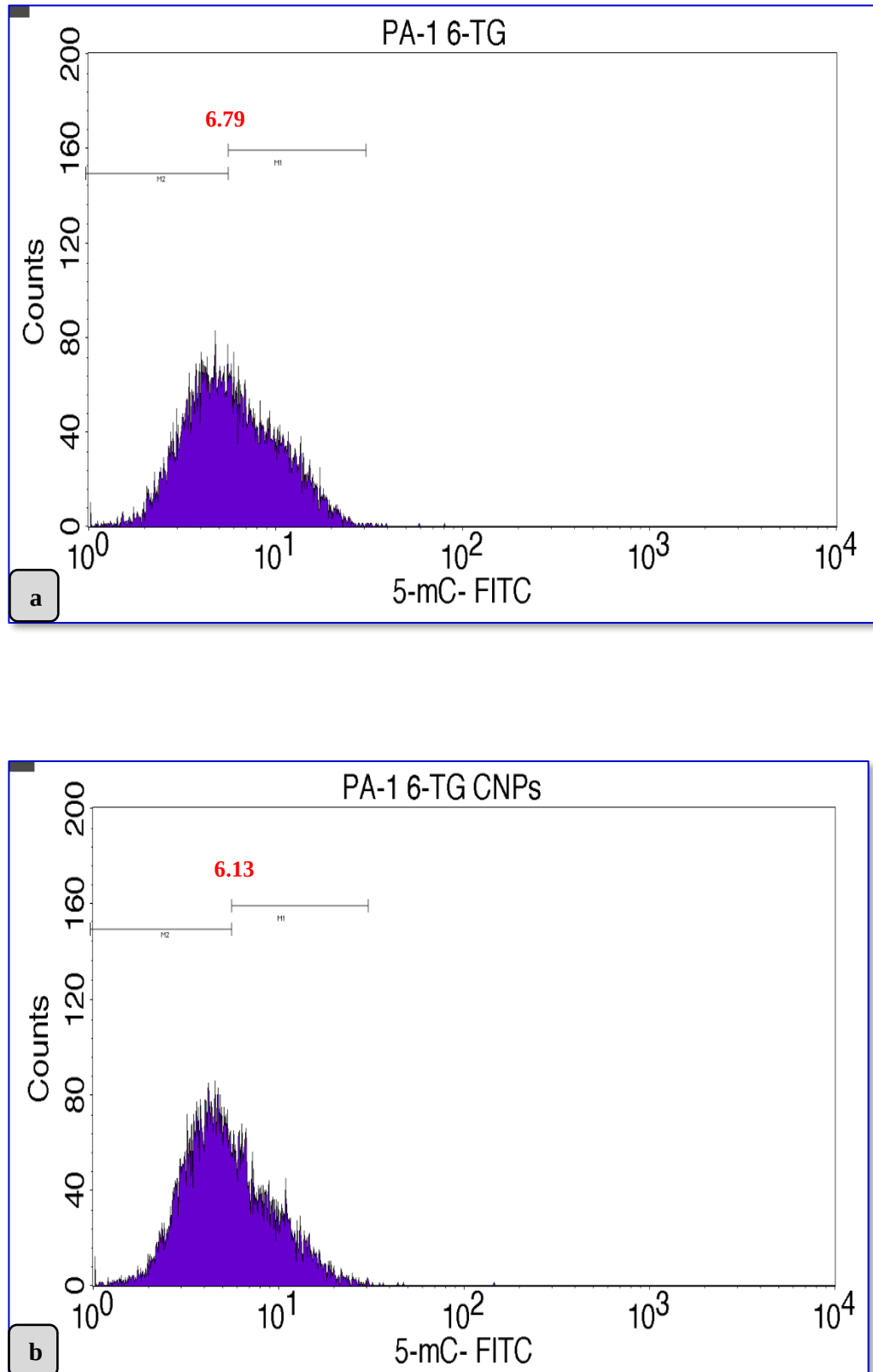


Fig.26. Representative image of flow cytometry analysis of global DNA demethylating activity in PA-1 cells at 48h incubation: a) 6-TG b) 6-TG-CNPs

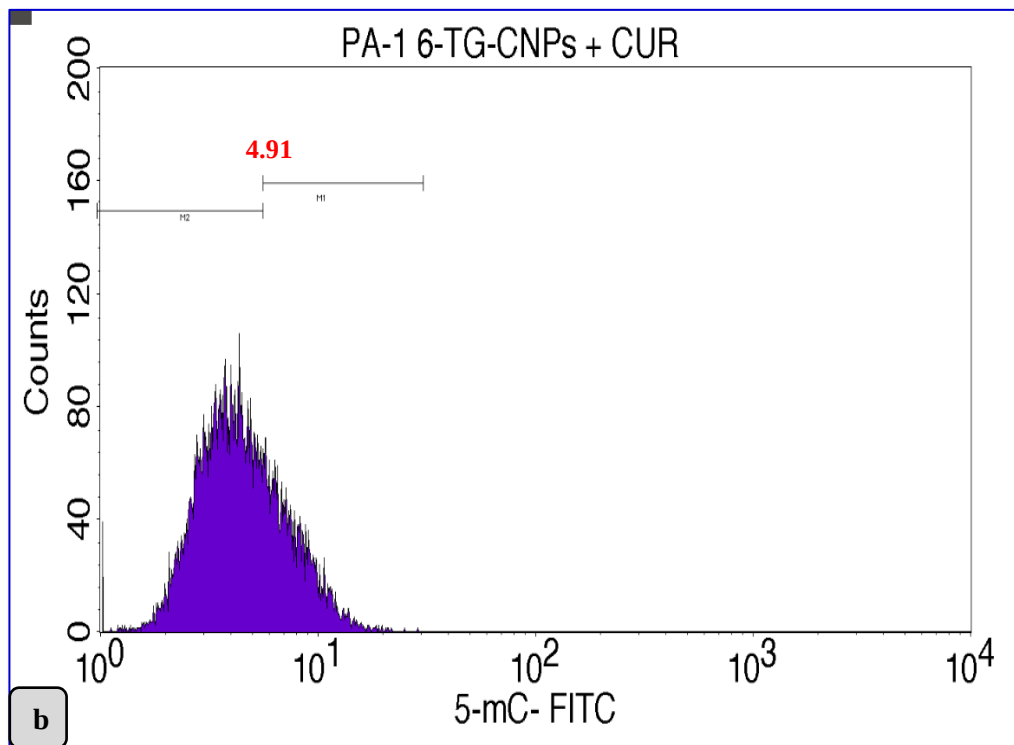
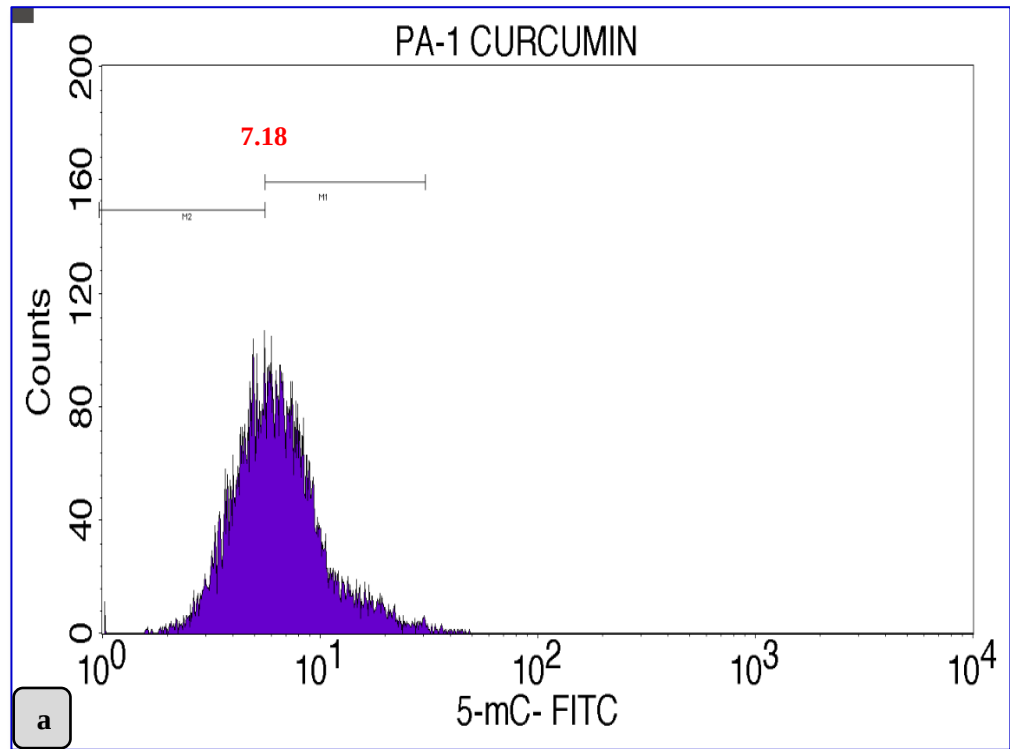


Fig.27. Representative image of flow cytometry analysis of global DNA demethylating activity in PA-1 cells at 48h incubation: a) curcumin b) 6-TG-CNPs (IC₂₅) + curcumin (IC₂₅)

Table 2. Effect of 6-TG, 6-TG-CNPs, curcumin and 6-TG-CNPs+curcumin on apoptosis and necrosis at 48h incubation from PA-1 cells. One-way ANOVA, followed by post hoc Tukey's multiple comparison test. Values are Mean $\pm$ SD, n=3, (p<0.01, ***p<0.0001)**

Groups	UL	UR	LL	LR
Vehicle Control	0.00 $\pm$ 0.00	0.05 $\pm$ 0.02	96.94 $\pm$ 0.32	3.01 $\pm$ 0.34
6-TG	4.15 $\pm$ 0.39	3.47 $\pm$ 0.87	46.58 $\pm$ 1.17	45.79 $\pm$ 2.33***
6-TG-CNPs	2.35 $\pm$ 0.38	2.98 $\pm$ 0.68	32.74 $\pm$ 1.74	61.92 $\pm$ 2.20***
Curcumin	0.00 $\pm$ 0.00	22.58 $\pm$ 3.99***	0.10 $\pm$ 0.02	77.33 $\pm$ 3.97***
6-TG-CNPs + Curcumin	0.16 $\pm$ 0.03	8.28 $\pm$ 1.71**	0.88 $\pm$ 0.06	90.67 $\pm$ 1.79***

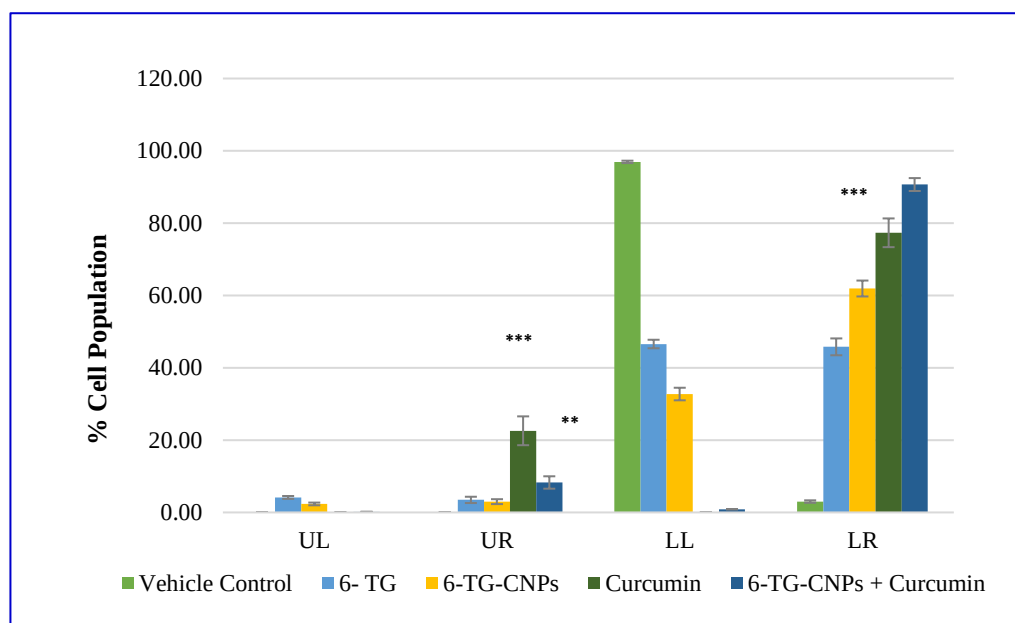
UL: Necrosis **UR:** End stage of Apoptosis **LL:** Normal cells **LR:** Early stage of Apoptosis

Table 3. Effect of 6-TG, 6-TG-CNPs, curcumin and 6-TG-CNPs+curcumin on cell cycle at 48h incubation from PA-1 cells. One-way ANOVA, followed by post hoc Tukey's multiple comparison test. Values are Mean $\pm$ SD, n=3, (p<0.01, ***p<0.0001)**

Groups	Sub G0/G1	G0/G1	S	G2/M
Vehicle Control	0.70 $\pm$ 0.07	45.78 $\pm$ 1.01	19.65 $\pm$ 1.53	33.26 $\pm$ 0.47
6-TG	2.83 $\pm$ 0.19	32.13 $\pm$ 0.25	7.39 $\pm$ 0.47	40.86 $\pm$ 0.34***
6-TG-CNPs	3.53 $\pm$ 0.07	29.05 $\pm$ 0.14	4.52 $\pm$ 0.25	47.49 $\pm$ 0.29***
Curcumin	1.29 $\pm$ 0.01	22.38 $\pm$ 0.70	7.30 $\pm$ 1.16	48.59 $\pm$ 0.52***
6-TG-CNPs + Curcumin	3.60 $\pm$ 1.59	21.92 $\pm$ 1.52	9.06 $\pm$ 0.99	50.42 $\pm$ 0.74***

Table 4. Effect of 6-TG, 6-TG-CNPs, Curcumin and 6-TG-CNPs+curcumin at 48h incubation on DNA demethylation from PA-1 cells. One-way ANOVA, followed by post hoc Tukey's multiple comparison test. Values are Mean $\pm$ SD, n=3, (p<0.0001)**

Groups	Relative Mean Fluorescence Intensity (MFI)
Vehicle Control	10.32 $\pm$ 0.05
5-Azacytidine	6.95 $\pm$ 0.09***
6-TG	6.86 $\pm$ 0.07***
6-TG-CNPs	6.18 $\pm$ 0.06***
Curcumin	7.29 $\pm$ 0.12***
6-TG-CNPs + Curcumin	4.95 $\pm$ 0.05***



UL: Necrosis UR: End stage of Apoptosis LL: Normal cells LR: Early stage of Apoptosis

Fig.28. Histogram representation of flow cytometry data for population of cells in early stage, late stage of apoptosis and necrosis from PA-1 cells treated with 6-TG, 6-TG-CNPs, curcumin and 6-TG-CNPs + curcumin at 48h incubation. Values are Mean $\pm$ SD, n=3, (**p<0.01, ***p<0.0001)

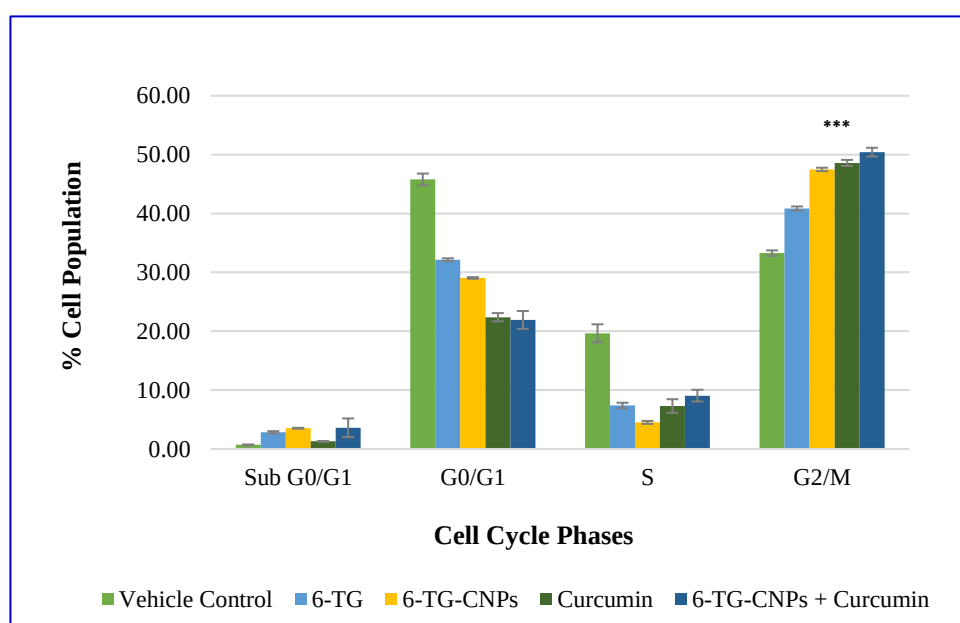


Fig.29. Histogram representation of flow cytometry data for population of cells in Sub G0/G1, G0/G1, S and G2/M phases of cell cycle from PA-1 cells treated with 6-TG, 6-TG-CNPs, curcumin and 6-TG-CNPs + curcumin at 48h incubation. Values are Mean $\pm$ SD, n=3, (**p<0.01, ***p<0.0001)

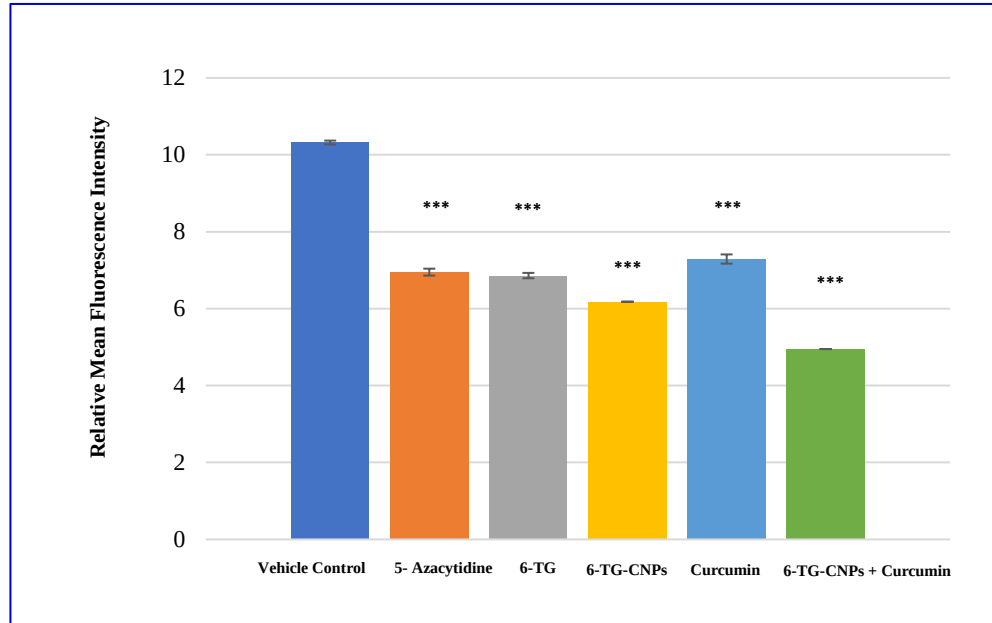


Fig.30. Bar graph representation of flow cytometry data for Mean florescence intensity of cellular 5mec staining labelled with FITC conjugated anti-5mec from PA-1 cells treated with 6-TG, 6-TG-CNPs, curcumin and 6-TG-CNPs + curcumin at 48h incubation. Values are Mean $\pm$ SD, n=3, (*) $p < 0.0001$).**

4.4. PHARMACOKINETIC STUDIES OF 6-TG IN 6-TG, 6-TG-CNPS ALONE AND 6-TG-CNPS IN CURCUMIN PRE-TREATED RATS BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

4.4.1. METHOD VALIDATION

4.4.1.1. Mass Spectrometry

Mass parameters were tuned in both positive and negative ionization modes for the analyte and internal standard. Good response was found in positive ionization mode. The most sensitive mass transition monitored was 168.1m/z to 151.0m/z for 6-thioguanine and 129.1m/z to 112.1m/z for 2-thiouracil.

4.4.1.2. Chromatographic Conditions

Chromatographic conditions and mobile phase were optimized to obtain good resolution. The presence of small amount of acetic acid in the mobile phase improved the detection of the analyte. It was found that 70% acetonitrile: 30% of 0.2% acetic acid in water (70:30% v/v) could achieve this purpose and hence was adopted as the final mobile phase. Agilent, Cyano RP, 7.5cm, 3.5 μ m, 4.6mm column resulted in providing good peak shapes and response at lowest concentration level. The mobile phase was operated at a flow rate 0.5 mL/min. The retention times of 6-thioguanine and 2-thiouracil were 1.60 and 1.59 min, respectively.

4.4.1.3. Selectivity and chromatography

The degree of interference by endogenous plasma constituents with the analyte and internal standard was assessed by the inspection of chromatograms derived from the processed blank plasma sample. No significant interference was observed in the blank plasma at the retention time of the analyte and internal standard.

4.4.1.4. Sensitivity

The lowest limit of reliable quantification for analyte was set at the concentration of LLOQQC. The precision and accuracy at LLOQQC concentration were 10.65 and 109.81 per cent, respectively (Table 7).

4.4.1.5. Precision and accuracy

The eight-point calibration curve was found to be linear in P&A-1 and P&A-2. A regression equation with a weighing factor of $1/x^2$ of drug to internal standard concentration was found to produce the best fit concentration response relationship for the analyte in rat plasma. The calibration curve standards and quality controls (LLOQQC, LQC, MQC and HQC) were found to be within $\pm 30\%$ of precision and accuracy (Table 5 to 8).

4.4.1.6. Recovery:

The recovery was observed in all six lots of rat plasma for the analyte at concentration of LQC and HQC. The percentage recovery for 6-thioguanine and 2-thiouracil was found to be within $\pm 30\%$ (Table 9).

4.4.1.7. Matrix Effect:

No significant matrix effect was observed in all six lots of rat plasma for the analyte at concentration of LQC and HQC. The percentage matrix effect for 6-thioguanine and 2-thiouracil was found to be within $\pm 30\%$ (Table 10).

4.4.1.8. PK Sample Analysis

The eight-point calibration curve standard and Quality control (LQC, MQC and HQC) were found to be within $\pm 30\%$ of the acceptance criteria (Fig. 31 to 38)

Table 5. Summary of the experimental parameters and results of the method validation for the quantification of 6-TG in rat plasma by LC-MS/MS method

Batch	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8	Slope	Intercept	r2
Spiked Concentrations (ng/mL)	1.95	3.91	7.81	15.63	31.25	125.00	250.00	500.00	0.00242	0.00193	0.9939
P&A-1-Calculated Concentrations (ng/mL)	1.88	4.22	8.38	13.25	29.95	136.31	271.78	450.75	0.00233	0.00213	0.9926
P&A-2-Calculated Concentrations (ng/mL)	1.94	4.00	8.10	13.67	31.58	132.00	292.34	418.13			
Mean	1.91	4.11	8.24	13.46	30.77	134.16	282.06	434.44			
SD	0.04	0.16	0.19	0.29	1.15	3.05	14.54	23.06			
Accuracy	97.70	105.18	105.48	86.14	98.45	107.32	112.82	86.89			
Precision	2.22	3.80	2.35	2.16	3.75	2.28	5.15	5.31			

Table 6. Precision and accuracy of 6-thioguanine in PA-1 for LLOQQC, LQC, MQC and HQC

Batch	LLOQQC	LQC	MQC	HQC
Spiked Concentrations (ng/mL)	1.95	6.25	200.00	400.00
1	2.08	5.89	227.74	349.52
2	2.36	7.38	252.42	357.08
3	2.22	7.26	253.23	379.17
4	3.15*	6.70	224.69	384.07
5	2.57	6.65	227.19	377.90
6	2.45	6.69	223.21	340.49
Mean	2.34	6.76	234.74	364.70
SD	0.19	0.53	14.10	18.07
Accuracy	119.62	108.19	117.37	91.18
Precision	8.34	7.84	6.01	4.96

LLOQQC: Lower limit of quantification quality control, **LQC:** Lower quality control, **MQC:** Medium quality control, **HQC:** High quality control

*: Doesn't meet acceptance criteria

Table 7. Precision and accuracy of 6-thioguanine in PA-2 for LLOQQC, LQC, MQC and HQC

Batch	LLOQQC	LQC	MQC	HQC
Spiked Concentrations (ng/mL)	1.95	6.25	200.00	400.00
1	2.10	5.83	232.79	371.21
2	1.90	7.17	242.67	362.21
3	1.99	7.08	236.82	402.49
4	2.55	6.30	232.64	379.69
5	2.09	7.69	237.07	371.47
6	2.23	6.72	215.03	340.91
Mean	2.14	6.80	232.84	371.33
SD	0.23	0.67	9.46	20.25
Accuracy	109.81	108.77	116.42	92.83
Precision	10.65	9.79	4.06	5.45

LLOQQC: Lower limit of quantification quality control, **LQC:** Lower quality control, **MQC:** Medium quality control, **HQC:** High quality control

Table 8. Inter-day precision and accuracy (LLOQQC, LQC, MQC and HQC) of 6-thioguanine in Precision and Accuracy-01 & Precision and Accuracy-02

Batch	LLOQQC	LQC	MQC	HQC
Spiked Concentrations (ng/mL)	1.95	6.25	200.00	400.00
1	2.08	5.89	227.74	349.52
2	2.36	7.38	252.42	357.08
3	2.22	7.26	253.23	379.17
4	3.15*	6.70	224.69	384.07
5	2.57	6.65	227.19	377.90
6	2.45	6.69	223.21	340.49
7	2.10	5.83	232.79	371.21
8	1.90	7.17	242.67	362.21
9	1.99	7.08	236.82	402.49
10	2.55	6.30	232.64	379.69
11	2.09	7.69	237.07	371.47
12	2.23	6.72	215.03	340.91
Mean	2.23	6.78	233.79	368.02
SD	0.23	0.57	11.49	18.62
Accuracy	114.27	108.48	116.89	92.00
Precision	10.15	8.47	4.92	5.06

LLOQQC: Lower limit of quantification quality control, **LQC:** Lower quality control, **MQC:** Medium quality control, **HQC:** High quality control

*: Doesn't met acceptance criteria

Table 9. Recovery of 6-thioguanine in quality control after spiking in rat plasma

Spiked Concentrations (ng/mL)	Area Counts			
	LQC		HQC	
1	55302	51349	2309701	2275507
2	49633	43598	2399340	2351470
3	50449	35934	2592661	2298377
4	48057	30188	2456115	2153568
5	49742	34148	2674873	2241741
6	46057	32078	2605360	2606562
Mean	47900	33113	2640117	2424152
% Recovery	69		92	

LQC: Lower quality control **HQC:** High quality control

Table 10. Matrix effect of 6-thioguanine in quality control after spiking in rat plasma

Spiked Concentrations (ng/mL)	Area Counts			
	LQC		HQC	
1	55302	45067	2309701	2170497
2	49633	38466	2399340	2531086
3	50449	44110	2592661	2432248
4	48057	42571	2456115	2271216
5	49742	47891	2674873	2272020
6	46057	30521	2605360	2278746
Mean	47900	39206	2640117	2275383
% Matrix Effect	82		86	

LQC: Lower quality control **HQC:** High quality control

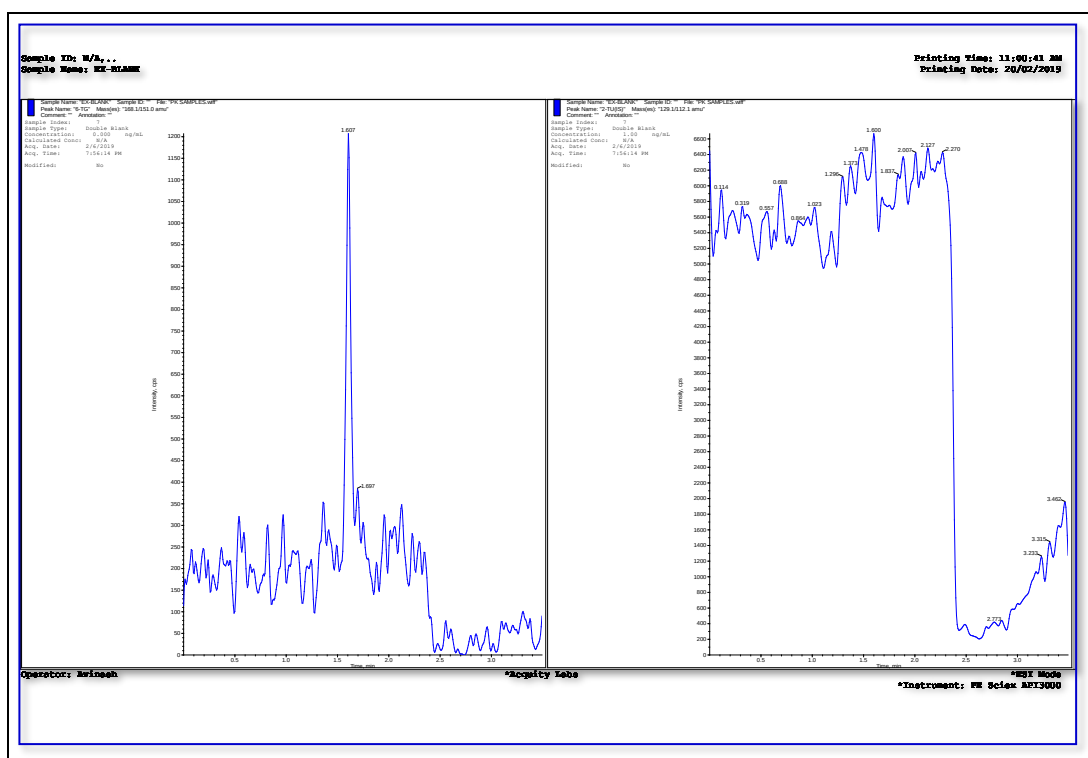


Fig.31. Representative chromatogram of the extracted blank plasma.

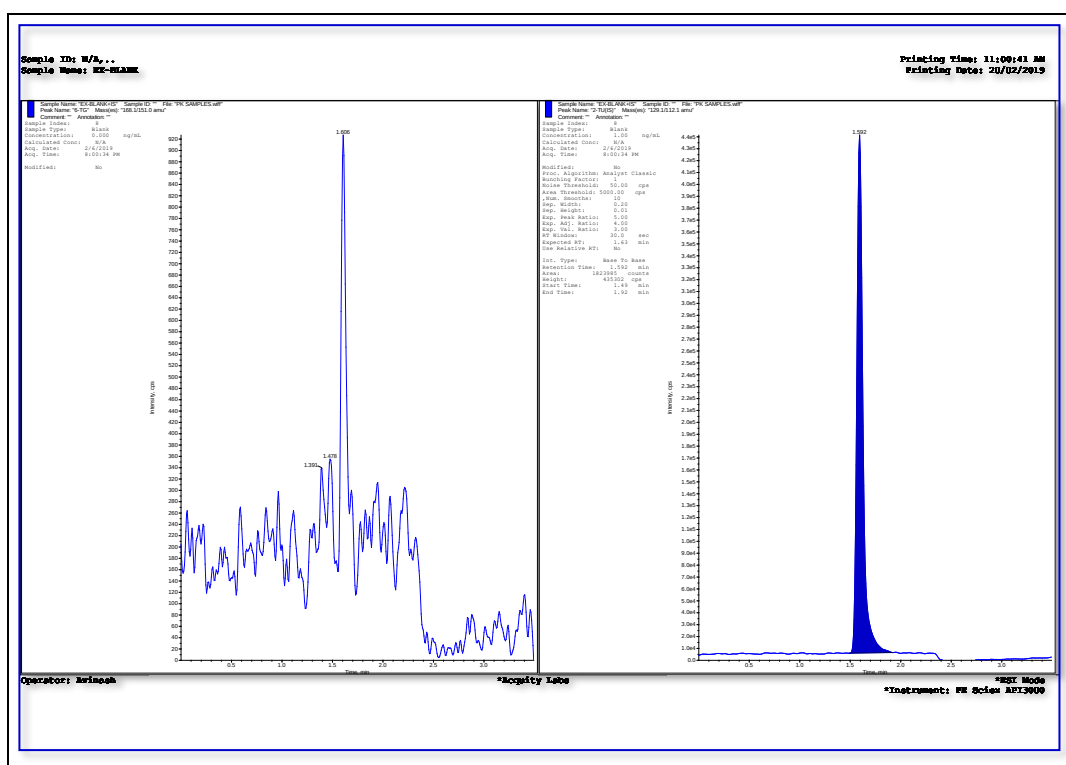


Fig.32. Representative chromatogram of the extracted blank plasma with internal standard

[illegible]

Fig.34. Representative chromatogram of standard 8.

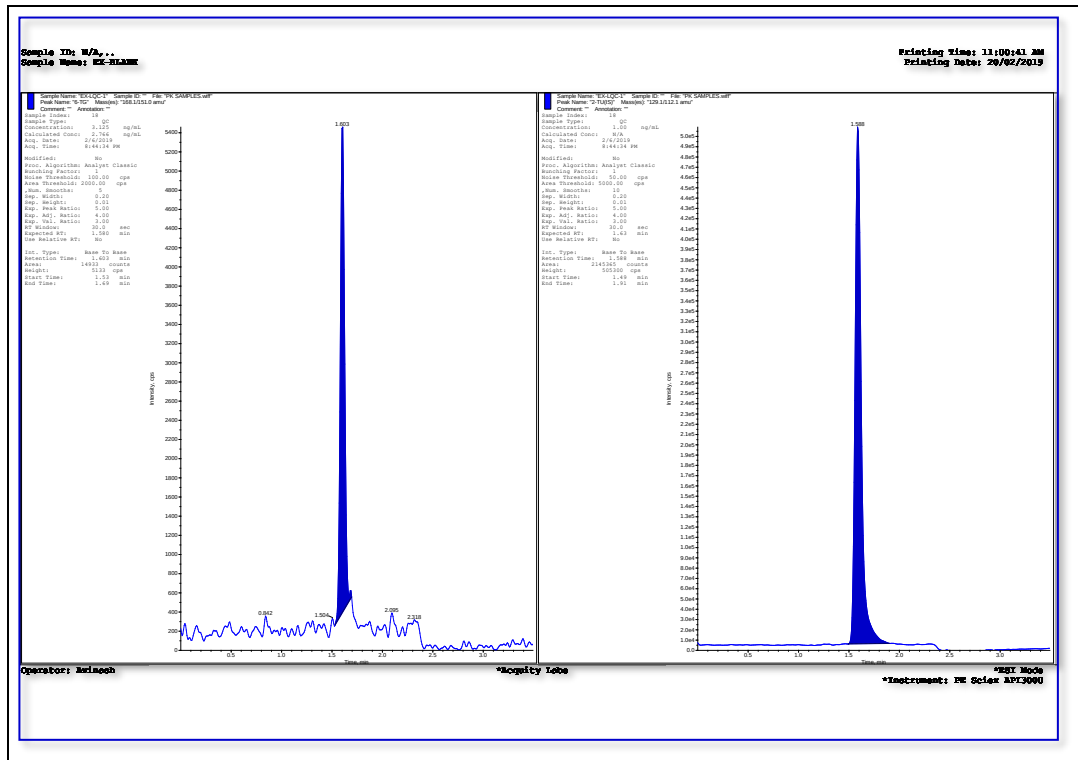


Fig.35. Representative chromatogram of lower quality control (LQC)

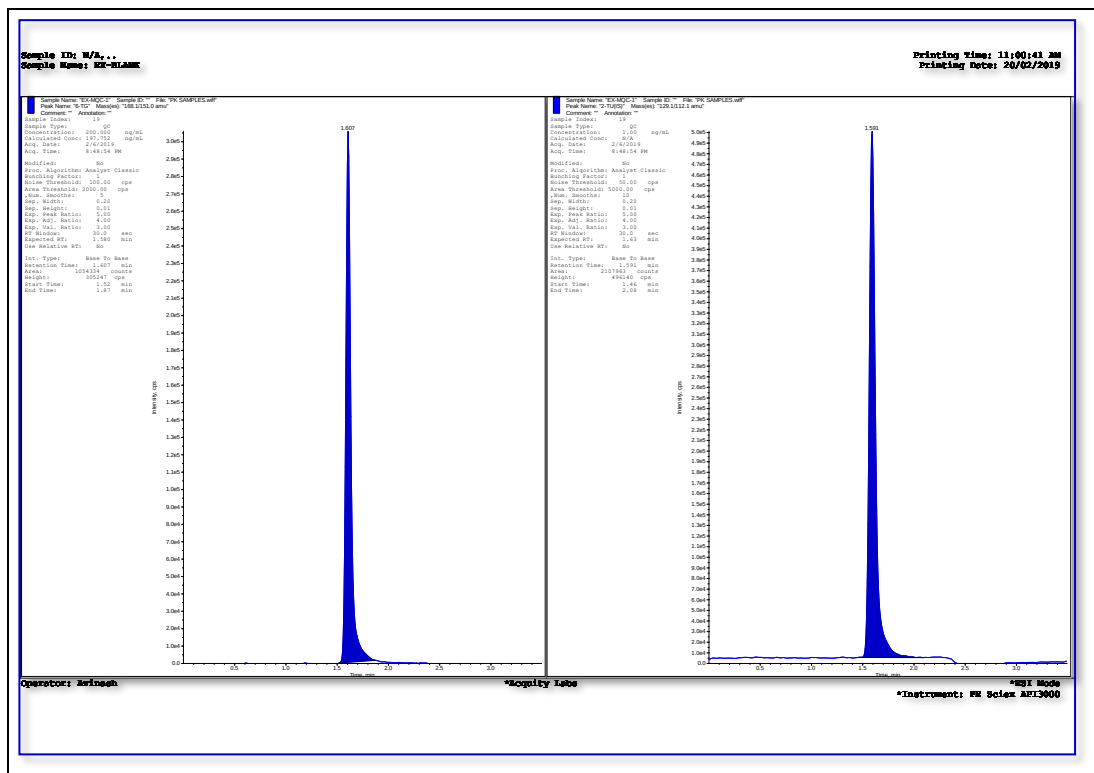
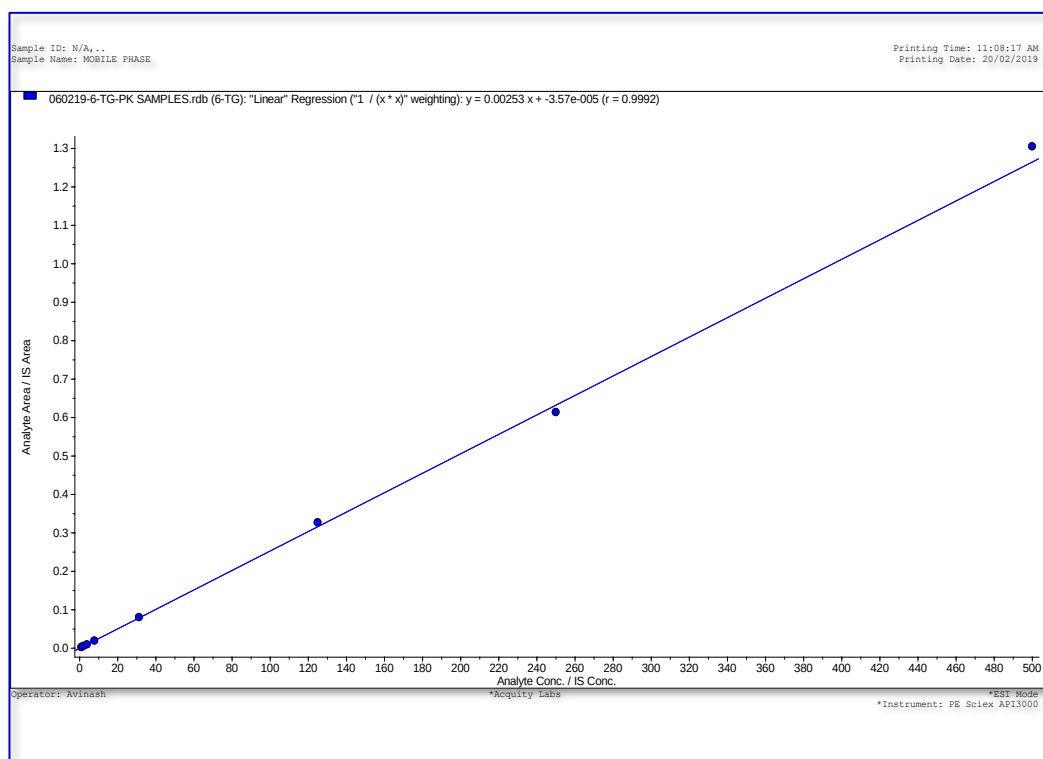


Fig.36. Representative chromatogram of medium quality control (MQC)



4.4.2. PK PARAMETERS OF 6-TG

4.4.2.1. Pharmacokinetics of 6-TG following Single Oral Dose Administration in Wistar Rats

The mean plasma concentration-time profile following single oral dose of 6-TG (14.8 mg/kg) administration in male wistar rats is depicted in Fig.39 and Table 11 and 12. A non-compartment model adequately described the plasma concentration-time profile.

The mean elimination rate constant (λ_z) and elimination half-life ($t_{1/2\lambda_z}$) for 6-TG were $0.13 \pm 0.01 \text{ h}^{-1}$ and $5.27 \pm 0.36 \text{ h}$, respectively (Table 15).

The mean values of $C_{\max}$ for 6-TG was $79.12 \pm 11.63 \text{ ng.ml}^{-1}$ and $T_{\max}$ was 2h, respectively (Table 15).

The mean area under the curve from time zero to time t (AUC_{0-24}) and mean area under the curve from time zero to time infinity ($AUC_{0-\infty}$) were 216.66 ± 25.84 and $230.59 \pm 29.94 \text{ ng.ml} \cdot \text{h}^{-1}$, respectively (Table 15). The mean area under first moment curve ($AUMC_{0-\infty}$) and mean residence time were $1481.48 \pm 281.30 \text{ ng.ml} \cdot \text{h}^2$ and $6.40 \pm 0.38 \text{ h}$, respectively (Table 15).

The mean volume of distribution (V_z/F_{obs}) and oral clearance (Cl/F_{obs}) were $0.49 \pm 0.04 \text{ (mg/kg)/(ng/ml)}$ and $0.07 \pm 0.01 \text{ (mg/kg)/(ng/ml)/h}$, respectively (Table 15).

The mean volume of distribution at steady-state ($V_{d_{ss}}$) for 6-TG was $0.41 \pm 0.03 \text{ ml}$ (Table 15).

4.4.2.2. Pharmacokinetics of 6-TG following Single Oral Dose Administration of 6-TG-CNPs in Wistar Rats

The mean plasma concentration-time profile of 6-TG following single oral dose of 6-TG-CNPs (14.8 mg/kg) in male wistar rats is depicted in Fig.40 and Table 11 and 13. A non-compartment model adequately described the plasma concentration-time profile.

The mean elimination rate constant (λ_z) and elimination half-life ($t_{1/2\lambda_z}$) were $0.35 \pm 0.13 \text{ h}^{-1}$ and $2.23 \pm 1.06 \text{ h}$, respectively. The elimination rate constant (λ_z) for 6-TG

in 6-TG-CNPs alone treated group was significantly ($p<0.05$) higher ($0.35 \pm 0.13 \text{ h}^{-1}$) when compared to those observed with 6-TG ($0.13 \pm 0.01 \text{ h}^{-1}$) treated group. The elimination half-life ($t_{1/2\lambda_z}$) for 6-TG in 6-TG-CNPs alone treated group was significantly ($p<0.01$) lower ($2.23 \pm 1.06 \text{ h}$) when compared to those observed with 6-TG ($5.27 \pm 0.36 \text{ h}$) treated group (Table 15).

The mean values of $C_{\max}$ for 6-TG was $124.07 \pm 2.73 \text{ ng.ml}^{-1}$ and $T_{\max}$ was 4h, respectively. The $C_{\max}$ for 6-TG in 6-TG-CNPs alone treated group ($124.07 \pm 2.73 \text{ ng.ml}^{-1}$) was increased by 1.6-fold when compared to those observed with 6-TG ($79.12 \pm 11.63 \text{ ng.ml}^{-1}$) treated group (Table 15).

The mean area under the curve from time zero to time t (AUC_{0-24}) and mean area under the curve from time zero to time infinity ($AUC_{0-\infty}$) were 536.52 ± 98.70 and $545.18 \pm 99.39 \text{ ng.ml} \cdot \text{h}^{-1}$, respectively. The mean area under first moment curve ($AUMC_{0-\infty}$) and mean residence time were $2613.30 \pm 561.17 \text{ ng.ml} \cdot \text{h}^2$ and $4.78 \pm 0.46 \text{ h}$, respectively. The mean Area under the curve (AUC_{0-24}) for 6-TG in 6-TG-CNPs alone treated group was significantly ($p<0.05$) higher ($536.52 \pm 98.70 \text{ ng.ml} \cdot \text{h}^{-1}$) when compared to those observed with 6-TG ($216.66 \pm 25.84 \text{ ng.ml} \cdot \text{h}^{-1}$) treated group. The mean Area under the curve ($AUC_{0-\infty}$) for 6-TG in 6-TG-CNPs alone treated group was increased by 2.4-fold ($545.18 \pm 99.39 \text{ ng.ml} \cdot \text{h}^{-1}$) when compared to those observed with 6-TG ($230.59 \pm 29.94 \text{ ng.ml} \cdot \text{h}^{-1}$) treated group. The mean area under first moment curve ($AUMC_{0-\infty}$) for 6-TG in 6-TG-CNPs alone treated group was increased by 1.8-fold ($2613.30 \pm 561.17 \text{ ng.ml} \cdot \text{h}^2$) when compared to those observed with 6-TG ($1481.48 \pm 281.30 \text{ ng.ml} \cdot \text{h}^2$) treated group. The mean residence time for 6-TG in 6-TG-CNPs alone treated group was significantly ($p<0.01$) lower ($4.78 \pm 0.46 \text{ h}$) when compared to those observed with 6-TG ($6.40 \pm 0.38 \text{ h}$) treated group (Table 15).

The mean volume of distribution (V_z/F_{obs}) for 6-TG was 0.09 ± 0.04 ($\text{mg/kg}/(\text{ng/ml})$). The volume of distribution (V_z/F_{obs}) for 6-TG in 6-TG-CNPs alone

treated group was significantly ($p < 0.0001$) lower [0.09 ± 0.04 (mg/kg)/(ng/ml)] when compared to those observed with 6-TG [0.49 ± 0.04 (mg/kg)/(ng/ml)] treated group (Table 15).

The mean oral clearance (Cl/F_{obs}) for 6-TG was 0.03 ± 0.01 (mg/kg)/(ng/ml)/h. The oral clearance (Cl/F_{obs}) for 6-TG in 6-TG-CNPs alone treated group was significantly ($p < 0.0001$) lower [0.03 ± 0.01 (mg/kg)/(ng/ml)/h] when compared to those observed with 6-TG [0.07 ± 0.01 (mg/kg)/(ng/ml)/h] treated group (Table 15).

The volume of distribution at steady-state (V_{dss}) for 6-TG was 0.13 ± 0.02 ml. The volume of distribution at steady-state (V_{dss}) for 6-TG in 6-TG-CNPs alone treated group was significantly ($p < 0.0001$) lower (0.13 ± 0.02 ml) when compared to those observed with 6-TG (0.41 ± 0.03 ml) treated group (Table 15).

4.4.2.3. Pharmacokinetics of 6-TG following Single Oral Dose Administration 6-TG-CNPs in Wistar Rats Pretreated with Curcumin for Four Consecutive Days

The mean plasma concentration-time profile of 6-TG following single oral dose of 6-TG-CNPs (14.8 mg/kg) in male wistar rats pre-treated with curcumin for four consecutive days is depicted in Fig.41 and Table 11 and 14. A non-compartment model adequately described the plasma concentration-time profile.

The mean elimination rate constant (λ_z) and elimination half-life ($t_{1/2\lambda_z}$) were $0.15 \pm 0.01 \text{ h}^{-1}$ and 4.75 ± 0.54 h respectively (Table 15).

The mean values of C_{max} and T_{max} for 6-TG were $158.70 \pm 52.06 \text{ ng.ml}^{-1}$ and 4h, respectively. The C_{max} of 6-TG ($158.70 \pm 52.06 \text{ ng.ml}^{-1}$) in curcumin pre-treated followed by 6-TG-CNPs administered group was increased by 2-fold when compared to those observed with 6-TG ($79.12 \pm 11.63 \text{ ng.ml}^{-1}$) treated group (Table 15).

The mean area under the curve from time zero to time t (AUC_{0-24}) and mean area under the curve from time zero to time infinity (AUC_{0-inf}) were $691.96 \pm 194.42 \text{ ng.ml} \cdot \text{h}^{-1}$ and $701.12 \pm 195.62 \text{ ng.ml} \cdot \text{h}^{-1}$, respectively. The mean area under first moment curve

(AUMC_{0-inf}) and mean residence time were $3538.00 \pm 882.98 \text{ ng.ml} \cdot \text{h}^2$ and $5.08 \pm 0.20 \text{ h}$, respectively. The mean Area under the curve (AUC₀₋₂₄ and AUC_{0-inf}) for 6-TG in curcumin pre-treated followed by 6-TG-CNPs administered group was significantly ($p < 0.01$) higher (691.96 ± 194.42 and $701.12 \pm 195.62 \text{ ng.ml} \cdot \text{h}^{-1}$) when compared to those observed with 6-TG (216.66 ± 25.84 and $230.59 \pm 29.94 \text{ ng.ml} \cdot \text{h}^{-1}$) treated group. The mean area under first moment curve (AUMC_{0-inf}) for 6-TG in curcumin pre-treated followed by 6-TG-CNPs administered group was significantly ($p < 0.05$) higher ($3538.00 \pm 882.98 \text{ ng.ml} \cdot \text{h}^2$) when compared to those observed with 6-TG ($1481.48 \pm 281.30 \text{ ng.ml} \cdot \text{h}^2$) treated group. The mean residence time for 6-TG in curcumin pre-treated followed by 6-TG-CNPs administered group was significantly ($p < 0.05$) lower ($5.08 \pm 0.20 \text{ h}$) when compared to those observed with 6-TG ($6.40 \pm 0.38 \text{ h}$) treated group (Table 15).

The volume of distribution (V_z/F_{obs}) for 6-TG was $0.15 \pm 0.05 \text{ (mg/kg)/(ng/ml)}$. The volume of distribution (V_z/F_{obs}) for 6-TG in curcumin pre-treated followed by 6-TG-CNPs administered group was significantly ($p < 0.0001$) lower [$0.15 \pm 0.05 \text{ (mg/kg)/(ng/ml)}$] when compared to those observed with 6-TG [$0.49 \pm 0.04 \text{ (mg/kg)/(ng/ml)}$] treated group (Table 15).

The oral clearance (Cl/F_{obs}) for 6-TG was $0.02 \pm 0.01 \text{ (mg/kg)/(ng/ml)/h}$. The oral clearance (Cl/F_{obs}) for 6-TG in curcumin pre-treated followed by 6-TG-CNPs administered group was significantly ($p < 0.0001$) lower [$0.02 \pm 0.01 \text{ (mg/kg)/(ng/ml)/h}$] when compared to those observed with 6-TG [$0.07 \pm 0.01 \text{ (mg/kg)/(ng/ml)/h}$] treated group (Table 15).

The volume of distribution at steady-state ($V_{d_{ss}}$) for 6-TG was $0.12 \pm 0.04 \text{ ml}$. The volume of distribution at steady-state ($V_{d_{ss}}$) for 6-TG in curcumin pre-treated followed by 6-TG-CNPs administered group was significantly ($p < 0.0001$) lower ($0.12 \pm 0.04 \text{ ml}$) when compared to those observed with 6-TG ($0.41 \pm 0.03 \text{ ml}$) treated group (Table 15).

Table 11. Plasma concentration of 6-TG (ng/mL) in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pre-treated group

Plasma Concentrations of 6-TG (ng/mL) LLOQ/STD-1: 0.977ng/mL and ULOQ/STD-8: 500ng/mL									
Time (h)	Group I: 6-TG			Group II: 6-TG-CNPs			Group III: Curcumin pre-treatment followed by 6-TG-CNPs		
	Animal Number			Animal Number			Animal Number		
	#A1	#A2	#A3	#A4	#A5	#A6	#A7	#A8	#A9
0	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
0.5	8.1	11.1	8.4	4.1	4.9	4.5	9.4	10.1	6.4
1	18.7	25.8	17.8	9.2	8.1	11.2	19.3	19.5	13.2
1.5	43.3	51.1	43.2	21.9	16.3	23.6	29.0	29.6	24.6
2	75.8	92.0	69.5	45.6	43.7	49.3	64.3	71.8	47.1
4	9.6	11.0	12.1	124.5	98.1	149.6	186.2	191.3	98.7
8	1.8	2.1	2.2	17.3	14.5	20.8	17.4	21.4	13.9
12	3.0	4.1	3.5	4.1	3.5	4.4	4.1	5.1	3.8
24	1.7	2.3	1.4	1.5	BLQ	BLQ	1.6	1.2	1.1

BLQ: Below limit of quantification

LLOQ: Lower limit of quantification

ULOQ: Upper limit of quantification

Table 12. Pharmacokinetic parameters derived from time vs plasma concentration profile following administration of 6-TG (14.8 mg.kg⁻¹ p.o.)

PK parameter	Unit	Animal Number			Mean $\pm$ SD
		#A1	#A2	#A3	
λ_z	h ⁻¹	0.13	0.12	0.14	0.13 $\pm$ 0.01
$t_{1/2\lambda_z}$	h	5.30	5.62	4.90	5.27 $\pm$ 0.36
T_{max}	h	2.0	2.0	2.0	2.00 $\pm$ 0.00
C_{max}	ng.ml ⁻¹	75.85	92.04	69.47	79.12 $\pm$ 11.63
$C_{last \text{ (obs)}}/C_{max}$	ratio	0.02	0.03	0.02	0.02 $\pm$ 0.005
$AUC_{(0-24)}$	ng.ml*h ⁻¹	200.08	246.43	203.47	216.66 $\pm$ 25.84
$AUC_{(0-inf.)}$	ng.ml*h ⁻¹	213.02	265.16	213.60	230.59 $\pm$ 29.94
$AUMC_{(0-inf.)}$	ng.ml*h ²	1348.17	1804.65	1291.61	1481.48 $\pm$ 281.30
MRT	h	6.33	6.81	6.05	6.40 $\pm$ 0.38
V_z/F_{obs}	(mg/kg)/(n g/ml)	0.53	0.45	0.50	0.49 $\pm$ 0.04
Cl/F_{obs}	(mg/kg)/(n g/ml)/h	0.07	0.06	0.07	0.07 $\pm$ 0.01
V_{dss}	ml	0.440	0.380	0.419	0.41 $\pm$ 0.03

Table 13. Pharmacokinetic parameters derived from time vs plasma concentration profile following administration of 6-TG-CNPs (14.8 mg.kg⁻¹ p.o.)

PK parameter	Unit	Animal Number			Mean $\pm$ SD
		#A4	#A5	#A6	
λ_z	h ⁻¹	0.20	0.42	0.44	0.35 $\pm$ 0.13
$t_{1/2\lambda_z}$	h	3.45	1.66	1.58	2.23 $\pm$ 1.06
T_{max}	h	4.0	4.0	4.0	4.00 $\pm$ 0.00
C_{max}	ng.ml ⁻¹	124.52	98.11	149.58	124.07 $\pm$ 2.73
$C_{last \text{ (obs)}}/C_{max}$	ratio	0.01	0.04	0.03	0.03 $\pm$ 0.02
$AUC_{(0-24)}$	ng.ml*h ⁻¹	558.86	428.56	622.13	536.52 $\pm$ 98.70
$AUC_{(0-inf.)}$	ng.ml*h ⁻¹	566.39	436.90	632.25	545.18 $\pm$ 99.39
$AUMC_{(0-inf.)}$	ng.ml*h ²	3009.52	1971.15	2859.23	2613.30 $\pm$ 561.17
MRT	h	5.31	4.51	4.52	4.78 $\pm$ 0.46
V_z/F_{obs}	(mg/kg)/(n g/ml)	0.13	0.08	0.05	0.09 $\pm$ 0.04
Cl/F_{obs}	(mg/kg)/(n g/ml)/h	0.03	0.03	0.02	0.03 $\pm$ 0.01
V_{dss}	ml	0.139	0.153	0.106	0.13 $\pm$ 0.02

Table 14. Pharmacokinetic parameters derived from time vs plasma concentration profile following administration (*per os*) of 6-TG-CNPs (14.8mg.kg⁻¹) in male wistar rats pre-treated with curcumin (100 mg.kg⁻¹x 4days; p.o.)

PK parameters	Unit	Animal Number			Mean ± SD
		#A7	#A8	#A9	
λ_z	h ⁻¹	0.13	0.16	0.15	0.15 ±0.01
$t_{1/2\lambda_z}$	h	5.29	4.22	4.74	4.75±0.54
T _{max} .	h	4.0	4.0	4.0	4.00 ±0.00
C _{max}	ng.ml ⁻¹	186.15	191.29	98.66	158.70±52.06
C _{last (obs)} /C _{max}	ratio	0.01	0.01	0.01	0.01 ±0.00
AUC ₍₀₋₂₄₎	ng.ml*h ⁻¹	779.86	826.91	469.12	691.96±194.42
AUC _(0-inf.)	ng.ml*h ⁻¹	792.39	834.42	476.54	701.12 ±195.62
AUMC _(0-inf.)	ng.ml*h ²	4009.96	4085.37	2519.70	3538.00±882.98
MRT	h	5.06	4.90	5.29	5.08±0.20
V _z /F _{obs}	(mg/kg)/(n g/ml)	0.14	0.11	0.21	0.15±0.05
Cl/F _{obs}	(mg/kg)/(n g/ml)/h	0.02	0.02	0.03	0.02±0.01
V _{d_{ss}}	ml	0.095	0.087	0.164	0.12±0.04

Table 15. Pharmacokinetic parameters Mean $\pm$ SD of 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pre-treated group.
One-way ANOVA, followed by post hoc Tukey's multiple comparison test. Values are Mean $\pm$ SD, n=3, (*p<0.05, **p<0.01, *p<0.0001)**

PK parameter	Unit	Group I	Group II	Group III
		6-TG	6-TG-CNPs	6-TG-CNPs pretreated with curcumin
λ_z	h^{-1}	0.13 $\pm$ 0.01	0.35 $\pm$ 0.13*	0.15 $\pm$ 0.01
$t_{1/2\lambda_z}$	h	5.27 $\pm$ 0.36	2.23 $\pm$ 1.06**	4.75 $\pm$ 0.54
T_{max}	h	2.00 $\pm$ 0.00	4.00 $\pm$ 0.00	4.00 $\pm$ 0.00
C_{max}	ng.ml ⁻¹	79.12 $\pm$ 11.63	124.07 $\pm$ 2.73	158.70 $\pm$ 52.06
$C_{last (obs)}/C_{max}$	ratio	0.02 $\pm$ 0.005	0.03 $\pm$ 0.02	0.01 $\pm$ 0.00
$AUC_{(0-24)}$	ng.ml*h ⁻¹	216.66 $\pm$ 25.84	536.52 $\pm$ 98.70*	691.96 $\pm$ 194.42**
$AUC_{(0-inf.)}$	ng.ml*h ⁻¹	230.59 $\pm$ 29.94	545.18 $\pm$ 99.39	701.12 $\pm$ 195.62**
$AUMC_{(0-inf.)}$	ng.ml*h ²	1481.48 $\pm$ 281.30	2613.30 $\pm$ 561.17	3538.00 $\pm$ 882.98*
MRT	h	6.40 $\pm$ 0.38	4.78 $\pm$ 0.46**	5.08 $\pm$ 0.20*
V_z/F_{obs}	(mg/kg)/(ng/ml)	0.49 $\pm$ 0.04	0.09 $\pm$ 0.04***	0.15 $\pm$ 0.05***
Cl/F_{obs}	(mg/kg)/(ng/ml)/h	0.07 $\pm$ 0.01	0.03 $\pm$ 0.01***	0.02 $\pm$ 0.01***
Vd_{ss}	ml	0.41 $\pm$ 0.03	0.13 $\pm$ 0.02***	0.12 $\pm$ 0.04***

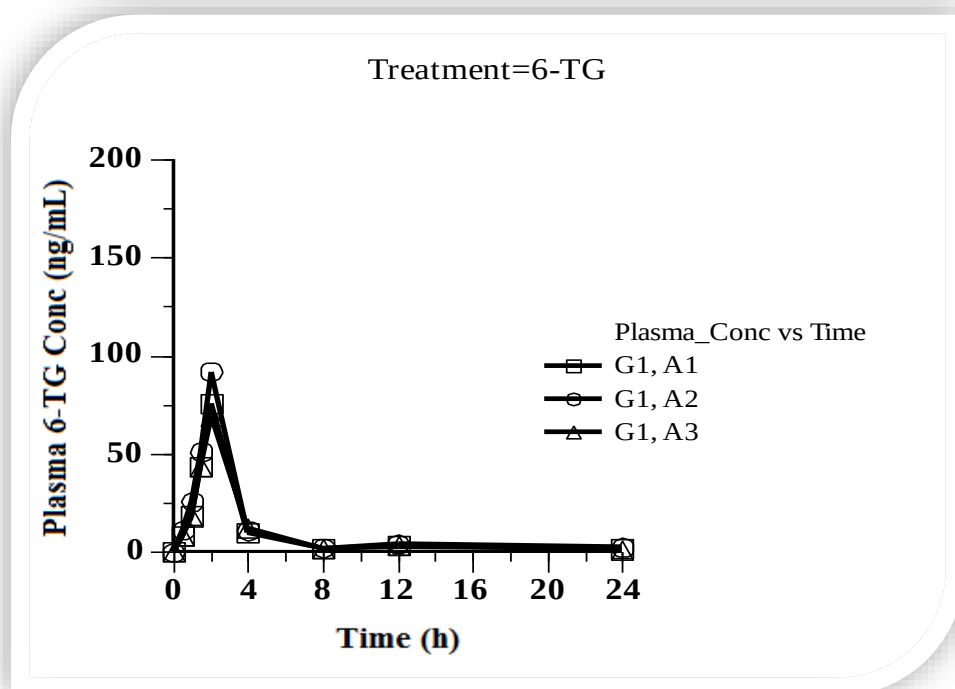


Fig.39. Mean plasma concentration time profile of 6-TG in rats administered with 6-TG

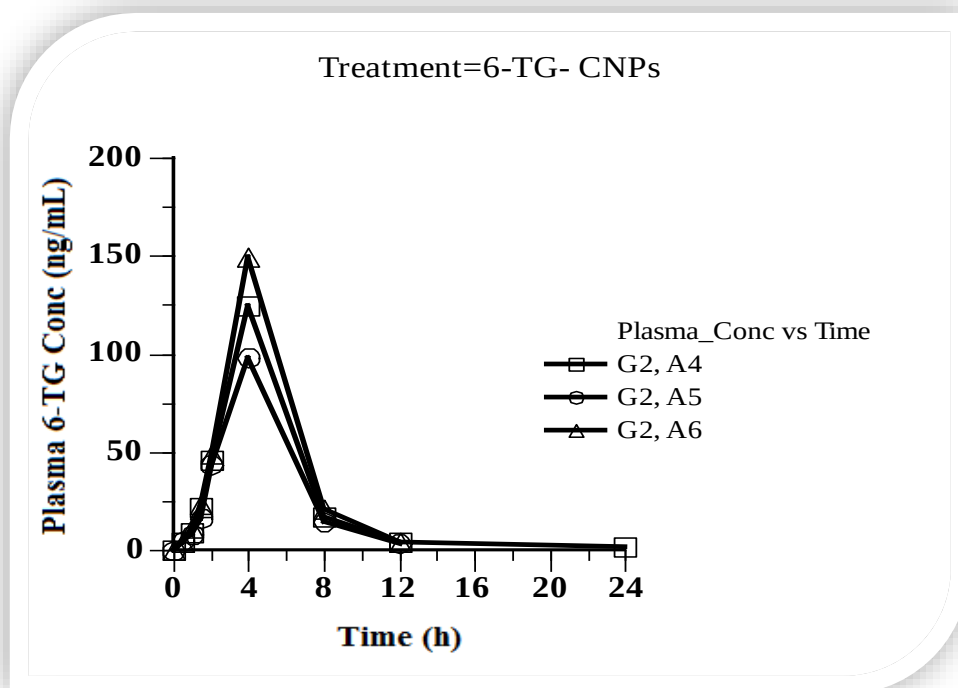


Fig.40. Mean plasma concentration time profile of 6-TG in rats administered with 6-TG-CNPs

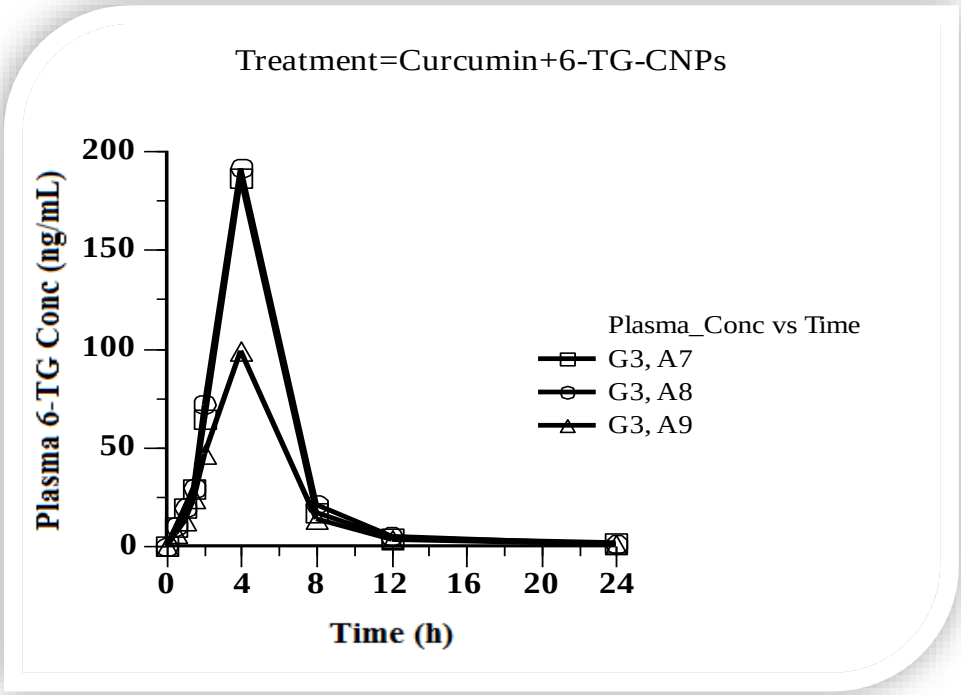


Fig.41. Mean plasma concentration time profile of 6-TG in rats pre-treated with curcumin for four consecutive days followed by 6-TG-CNPs administration

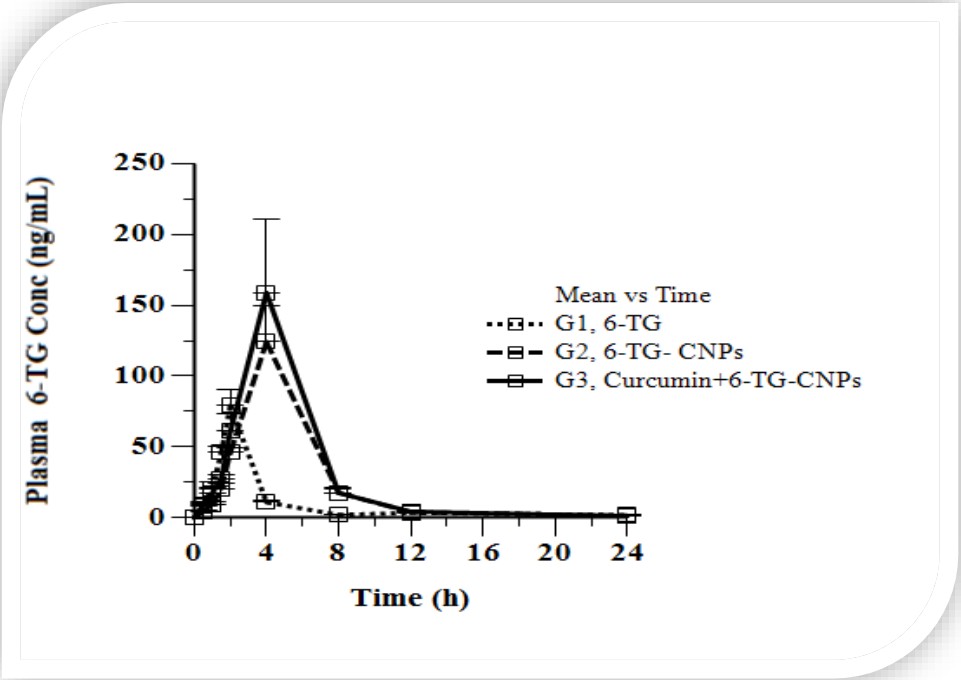


Fig.42. Mean plasma concentration time profile of 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pre-treated group

Discussion

CHAPTER: IV

DISCUSSION

The development of cancer nanotherapeutics has been attracting great interest since last decade. Cancer nanotherapeutics has the potential to overcome several limitations of conventional therapies, such as nonspecific biodistribution, poor water solubility and limited bioavailability. Nanoparticles with tuned size and surface characteristics are the key components of nanotherapeutics and are designed to passively or actively deliver anti-cancer drugs to tumor cells. A recent survey found that more than 247 nanomaterial-based medical products have been approved by the United States Food and Drug Administration (USFDA) and are currently in various stages of clinical study (Etheridge *et al.*, 2013). A distinct advantage of nanotechnology is the ability to design and optimize the unique physiochemical properties of nanoscale materials and structures. Altering the size, shape and/ or surface chemistry of nanoparticles allows to tailor their functionalities to meet different requirements (Elias *et al.*, 2013; Kulkarni and Feng 2013; Hoshyar, 2016).

Nanoparticle fabrication methods are readily scalable and applicable to a broad range of drugs. Of all the nanoparticle drug delivery approaches, polymeric nanoparticles have gained significant importance as they are biodegradable, biocompatible and their formulation methods are more widely available. Their range of applications has been expanded to include a variety of chemical drug classes and dosage forms. Chitosan has been used as a carrier in polymeric nanoparticles for drug delivery through various routes of administration. Chitosan has chemical functional groups which can be modified to achieve specific goals, making it a polymer with a tremendous range of potential applications (Mohammed *et al.*, 2017).

Curcumin exerts anticancer potential by suppressing proliferation, down-regulation of transcription factors NF- κ B, AP-1 and Egr-1, down-regulation of the

expression of COX2, LOX, NOS, MMP-9, uPA, TNF, chemokines, cell surface adhesion molecules and cyclin D1, down-regulation of growth factor receptors (such as EGFR and HER2) and inhibiting the activity of c-Jun N-terminal kinase, protein tyrosine kinases and protein serine/threonine kinases. Additionally, curcumin also combat cancer by its epigenetic regulatory role *i.e* by inhibiting DNA methyltransferases and histone deacetylases and regulating miRNA expression (Du *et al.*, 2012; Aggarwal *et al.*, 2003).

Further, curcumin is also a natural bioenhancer. It improves the bioavailability of co-administered drugs by inhibiting CPY3A mediated metabolism and P-glycoprotein efflux pump (Sharma *et al.*, 2017).

6-TG is one of the major cancer chemotherapeutic agent. However, it is known for its low and highly variable oral bioavailability. Hence, with a view to overcome this limitation and to enhance its therapeutic effect, a nanoformulation of 6-TG was synthesized using chitosan. Keeping in view of the actions of curcumin as described above it was further planned to study the combined effect of curcumin and nanoformulation of 6-TG. The study consisted of 1) synthesis and characterization of 6-TG encapsulated chitosan nanoformulation; 2) evaluation of *in vitro* anticancer activity of 6-TG-CNPs alone and in combination of curcumin and 3) study of *in vivo* pharmacokinetic profile of 6-TG and its nanoformulations given in normal and in curcumin pre-treated rats.

5.1. SYNTHESIS OF 6-TG ENCAPSULATED CHITOSAN NANOPARTICLES

6-TG encapsulated chitosan nanoparticles were prepared by ionic gelation method using chitosan (0.5 mg/mL), 6-TG (1.0 mg/mL) and TPP (0.5 mg/mL) solutions. The chitosan and TPP concentrations were selected based on the study of Fan *et al.* (2012) who stated that the concentration of chitosan and TPP should be below 1.5 mg/mL and 1.0 mg/mL, respectively in order to prevent the formation of microparticles. Chitosan is a polycation and TPP is a polyanion. The amine group of chitosan undergoes protonation

at acidic pH. The pKa of 6-TG is 8.52 and it is negatively charged (Chrzanowska *et al.*, 2009). Formation of nanoparticles is based on ionic gelation interaction between positively charged chitosan and negatively charged tripolyphosphate at room temperature. Hence, 6-TG-CNPs were formed instantaneously upon adding of TPP to readily mixed chitosan containing 6-TG solution. There may exist an electrostatic interaction between chitosan and the negatively charged drug during ionic gelation interaction between chitosan and TPP (Zhao and Wu, 2006; Csaba *et al.*, 2009; Yang and Hon, 2009) resulting in the formation of 6-TG-CNPs.

5.2. CHARACTERIZATION OF 6-TG ENCAPSULATED CHITOSAN NANOPARTICLES

5.2.1. Particle Size

In the present study, the mean particle size of 6-TG-CNPs was 261.63 ± 6.01 nm. This present size was in accordance with the findings of Vijayalakshmi *et al.* (2012) who prepared chitosan loaded 6-thioguanine nanoparticles based on solvent evaporation method with an average particle size of 100-200 nm. Kannaiyan *et al.* (2011) also reported an average particle size of 230-260 nm of 6-TG containing poly lactic acid-polyethylene glycol (PLA-co-PEG) nanocapsules which were prepared in the presence and absence of gold nanoparticles *via* the emulsification solvent-evaporation method. An average particle size of 118 ± 0.12 nm for 6-TG conjugated to dialdehyde sodium alginate *via* acid-labile schiff base linkage followed by the ultrasound-assisted oxidation of thiol groups to disulfide cross-links was reported by Wan *et al.* (2018)

5.2.2. Polydispersity Index

The polydispersity index of 6-TG-CNPs obtained in the present study was 0.34 ± 0.10 . Polydispersity index indicates particle size distribution that ranges from 0 to 1. PDI values close to 1 indicate heterogeneity whereas less than 0.5 indicates homogeneity of particle size dispersion (Kouchak *et al.*, 2012). PDI of 0.34 ± 0.10

observed in this study is less than 0.5 and it indicates that the prepared nanoparticles exhibit relatively narrow size distribution (Fan *et al.*, 2012).

5.2.3. Zeta Potential

A zeta potential of $+15.97 \pm 0.46$ mV was observed for prepared 6-TG-CNPs in this study. Zeta potential is an indicator of electrostatic or charge repulsion / attraction between the nanoparticles. Hence, it's an important fundamental parameter which affect the stability of the nanoparticles to a great extent. Nanoparticles with high zeta potential exhibit high degree of repulsion thereby resist agglomeration or flocculation. In case of nanoparticles with low zeta potential, attraction exceeds repulsion in the colloidal dispersion system leading to flocculation or agglomeration. The zeta potential of stable nanoparticles ranges from -40 mV to +40 mV (Mathew *et al.*, 2010). Zeta potential value obtained in this study was within the acceptable range thereby indicating good stability. The positive zeta potential value of 6-TG-CNPs indicates that the nanoparticle surface is positively charged due to the availability of free amine groups on the polymer (Li *et al.*, 2011a; Fan *et al.*, 2012).

5.2.4. Surface Morphology

The surface morphology of 6-TG drug was irregular in shape whereas the 6-TG-CNPs was spherical in shape from scanning electron microscope analysis. This was in agreement with the findings of Vijayalakshmi *et al.* (2012), Kannaiyan *et al.* (2011) and Wan *et al.* (2018) who also observed spherical configuration of 6-TG nanoformulations.

5.2.5. Drug Polymer Interactions

FTIR analysis was performed to determine the drug-polymer interactions, if any. The peak observed for chitosan at 3436.73 cm^{-1} was assigned to the N-H stretching and the shift in the same spectral peak to interior (3418.33 cm^{-1}) in the 6-TG-CNPs confirms the formation of the 6-TG complex with chitosan. The resultant complex also evidenced

missing of spectral peaks related to C=S (1202.01 cm^{-1}) and C-N (1373.28 cm^{-1}) of 6-TG in the 6-TG-CNPs, probably due to interaction of 6-TG with chitosan. The reduction in stretching frequency and disappearance of spectral peak could be due to strong involvement of that functional group in the bond formation (Li *et al.*, 2011a). Thus, these findings suggested that 6-TG was encapsulated to chitosan without any structural alterations. This was in concurrence with the findings of Vijayalakshmi *et al.* (2012) who observed characteristic peaks of thioguanine by FTIR at 1210 cm^{-1} , 1372 cm^{-1} and 3390 cm^{-1} pertaining to C=S, C-N and -NH_2 groups, respectively that confirmed the interaction of 6-thioguanine with chitosan. Kannaiyan *et al.* (2011) also observed characteristics bands for thioguanine by FTIR at 1210 cm^{-1} , 1372 cm^{-1} and 3436 cm^{-1} pertaining to C=S, C-N and -NH_2 groups, respectively which confirmed the presence of thioguanine in the PLA-co-PEG/TG-Au nanocapsules.

5.2.6. Entrapment Efficiency

In the present study, entrapment efficiency of 6-TG-CNPs was found to be 44.27 per cent. This was in accordance with the findings of Kannaiyan *et al.* (2011) who reported 45 per cent encapsulation efficiency for 6-TG loaded to Poly lactic acid-polyethylene glycol nanocapsules. However, Vijayalakshmi *et al.* (2012) reported 83 per cent encapsulation efficiency for 6-TG loaded chitosan nanoparticles. Apart from this, very low drug content of 17 per cent for 6-TG loaded to dialdehyde sodium alginate nanoformulations was reported by Wan *et al.* (2018). The variation in the entrapment efficiency between the studies is attributed to the different nanocarriers used for the preparation of 6-TG nanoformulations.

Thus, it appears that ionic gelation method followed in the present study for preparation of 6-TG-CNPs resulted in the good electrostatic interaction between 6-TG and chitosan.

5.2.7. *In Vitro* Drug Release Studies

In the present study, the drug release profile of 6-TG-CNPs showed a slow but increased release of 6-TG up to 87.32 ± 1.16 per cent in 24h and 91.40 ± 1.08 per cent in 48h at pH 4.8. The release of 6-TG at pH 7.4 was sustained but low which was 63.53 ± 6.44 per cent in 24h and 73.96 ± 1.12 per cent in 48h. Findings of present study are in accordance with the observations of Vijayalakshmi *et al.* (2012) who reported that thioguanine release from chitosan nanoparticles was relatively quicker in 0.1M HCl than in PBS (pH 7.0) and was about 89.3 per cent at the end of 72h. In contrast to our findings, Kannaiyan *et al.* (2011) observed quicker release of 6-TG from poly lactic acid-polyethylene glycol (PLA-co-PEG) nanocapsules in phosphate buffer saline (pH 7.4) and it was about 92 per cent. Drug release was sustained in 0.1M hydrochloric acid and was about 45 per cent for 50h.

A good drug release at pH 4.8 when compared with pH 7.4 was observed in the present study. This might be due to loosely arranged chitosan chains in acidic environment which allows drug to diffuse freely from the nanoparticles with the assistance of polymer degradation process (Mohammed *et al.*, 2017; Miladi *et al.*, 2015). At pH 7.4, chitosan gels or solidifies rapidly. This solidification process or mechanism might be responsible for sustained release of drug at pH 7.4 (Bernkop-Schnurch and Dunnhaupt 2012; Liu *et al.*, 2017). Thus, altered 6-TG release process from the nanoformulations is attributed to the pH dependent chitosan physical status/solubility process.

5.3. *IN VITRO* ANTICANCER STUDIES

5.3.1 *In Vitro* Cytotoxicity Assay

Cytotoxicity assays were performed to evaluate the sensitivity and selectivity of the 6-TG-CNPs alone and in combination with curcumin on breast adenocarcinoma (MCF-7) and ovarian teratocarcinoma (PA-1) cancer cell lines.

In the present *in vitro* cytotoxicity study on MCF-7 cell lines, the IC₅₀ values of 6-TG, 6-TG-CNPs and curcumin at 48h incubation were 23.09, 17.82 and 15.73 μ M, respectively. On PA-1 cells, the IC₅₀ values of 6-TG, 6-TG-CNPs and curcumin at 48h incubation were 5.81, 3.92 and 12.89 μ M, respectively. Combination of 6-TG-CNPs (IC₂₅) with curcumin (IC₂₅) on MCF-7 and PA-1 at 48h incubation showed percent cell viability of 49.77 $\pm$ 0.05 and 43.67 $\pm$ 0.02, respectively. Cytotoxicity of 6-TG, 6-TG loaded CNPs and curcumin was dose-dependent and 6-TG-CNPs found to be 1.5-fold more effective than that of 6-TG on PA-1 cells. Karimi-Maleh *et al.* (2018) reported more cytotoxicity for 6-TG loaded gold nanoparticles on MCF-7 cell lines with an IC₅₀ of 3.5 μ g/ml when compared to those observed with 6-TG with an IC₅₀ of >15 μ g/ml.

Based on IC₅₀ values and per cent cell viability observed in MTT assay it was concluded that PA-1 cells were more sensitive than MCF-7 cell lines to the test compounds. Hence, for further studies PA-1 cells were selected for evaluation of apoptosis and necrosis, cell cycle arrest and DNA demethylating activity.

5.3.2. Detection of Apoptosis by Flow Cytometry Analysis

Most chemotherapeutic agents have the ability to induce apoptosis by either causing intracellular stress, irreversible damage to DNA or the inhibition of vital intracellular proteins (Lowe and Lin, 2000). Apoptosis forms a part of complex cascade of events within the eukaryotic cell such as the activation of various genes, transcription of pro-apoptotic protease, kinases, phosphates and nucleases (Goldar *et al.*, 2015).

In the present study, treatment groups *viz.*, 6-TG, 6-TG-CNPs, curcumin at their respective IC₅₀ and combination of 6-TG-CNPs (IC₂₅) with curcumin (IC₂₅) showed early apoptotic effect which was significantly ($p < 0.0001$) higher when compared to those observed with the vehicle control group. The treatment groups *viz.*, 6-TG-CNPs and combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher early apoptotic effect when compared to those observed with 6-TG treated group. The

combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher early apoptotic effect when compared to those observed with 6-TG-CNPs treated group. These findings are in accordance with Samuels, (2008) who reported that 6-TG induces early apoptosis by activation of effector caspases on Hela cell lines. Aggarwal *et al.* (2003) reported induction of apoptosis *via* cytochrome C release, caspase-3 and caspase-9 activation on myeloid (HL-60) cells by curcumin.

6-thioguanine damages DNA which leads to the activation of p53 dependent intrinsic pathway of apoptosis. This further leads to mitochondrial dependent activation of executioner cytochrome-c which in turn allows activation of the effector caspases (caspase-9 and caspase-3), which leads to the characteristic apoptotic mode of death (Shi *et al.*, 2001). Thus, 6-TG-CNPs showed better efficiency by inducing early apoptosis when compared to those observed with 6-TG treated group. Further, combination of 6-TG-CNPs with curcumin showed better cytotoxicity by inducing early apoptosis than 6-TG-CNPs treated group.

5.3.3. Detection of Cell Cycle Arrest by Flow Cytometry Analysis

Diseases including cancer, Alzheimer's, Parkinson's and many more, are all caused at some level by cell cycle dysregulation. Cancer cells attain proliferative abilities through various mutations within the genome resulting in the inactivation or up regulation of various cell regulatory proteins. The cell cycle can be divided into four distinct phases: G1 (growth), S (DNA synthesis), G2 (gap) and M (mitosis), with each phase being unique according to its function (McLaughlin *et al.*, 2003). In normal proliferative cells various mechanisms tightly regulate the progression of cells through the phases by the formation, activation and deactivation of phase specific cyclins and cyclin dependent kinases (Adams *et al.*, 1995). At each phase various checkpoints within the cell cycle, allows the cell to delay transit into the next phase to repair any damage. Defects in these checkpoints and the proteins expressed during these phases are contributing factors in cancer. If the

cell is severely damaged and un-repairable it will be subjected to apoptosis (Schafer, 1998).

In the present study, PA-1 cells upon treatment with 6-TG, 6-TG-CNPs and curcumin at their respective IC_{50} concentration and combination of 6-TG-CNPs with curcumin at their respective IC_{25} concentration for 48h incubation showed G2/M phase arrest which was significantly ($p < 0.0001$) higher when compared to those observed with vehicle control group. The treatment groups viz., 6-TG-CNPs and combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher G2/M phase arrest when compared to those observed with 6-TG group treated group. The combination of 6-TG-CNPs with curcumin showed significantly ($p < 0.0001$) higher G2/M phase arrest when compared to those observed with 6-TG-CNPs treated group. These findings are in accordance with Samuels, (2008) who reported G2/M phase arrest by 6-TG on Hela cell lines. Abusnina *et al.* (2011) reported cyclin-dependent kinases mediated G2/M phase arrest by curcumin.

G2/M phase arrest by 6-TG might be due to the initial damage to the DNA by the cytotoxic metabolite of 6-TG during S phase of the cell cycle. Although damaged cells are able to continue their transit through S phase, they became arrested in G2 due to recognition of strand breaks at G2 checkpoint which was enacted by Chk1 (Azuma *et al.*, 2001). This G2 arrest is associated with an accumulation of Tyr-15-phosphorylated form of Cdk1, which in turn depends upon the activation of Chk1 (Chk1-dependent cell cycle arrest) (Liu *et al.*, 2000) and this can be therapeutically exploited to maximize cytotoxicity (Wang *et al.*, 2002). Thus, 6-TG-CNPs showed better cytotoxicity by inducing G2/M phase arrest leading to early apoptosis when compared to those observed with 6-TG treated group. Further, nanoformulation of 6-TG along with curcumin showed better cytotoxicity by inducing G2/M phase arrest leading to early apoptosis than 6-TG-CNPs treated group.

5.3.4. Detection of Demethylating Activity by Flow Cytometry Analysis

DNA methylation-mediated epigenetic silencing of cancer-related genes has greatly emphasized on the development of anti-cancer therapies that work by inhibiting DNA methylation and restore normal epigenetic landscape by reprogramming of genes involved in disease mechanisms. DNA methyltransferase inhibitors (DNMTIs) are a promising class of anticancer therapeutics which modulate the epigenome by reversing the DNA hypermethylation patterns, leading to renewed transcription of previously silenced tumor suppressor genes. These DNMT targeting drugs are classified as nucleoside analog inhibitors and non-nucleoside analog inhibitors. Nucleoside analog inhibitors incorporate into DNA during replication and sequester DNMTs by mediating their proteasomal degradation. 6-TG and curcumin are known DNMT inhibitors.

In the present study, the treatment groups *viz.*, 6-TG, 6-TG-CNPs, curcumin at their respective IC_{50} concentration, 5-azacytidine ($5\mu M$) and combination of 6-TG-CNPs (IC_{25}) with curcumin (IC_{25}) showed significant ($p < 0.0001$) increase in global DNA demethylating activity when compared to those observed with vehicle control group. The treatment groups *viz.*, 6-TG-CNPs and combination of 6-TG-CNPs with curcumin showed significant ($p < 0.0001$) increase in global DNA demethylating activity when compared to those observed with 6-TG treated group. The combination of 6-TG-CNPs with curcumin showed significant ($p < 0.0001$) increase in global DNA demethylating activity when compared to those observed with 6-TG-CNPs treated group. These findings are in concurrence with Hogarth *et al.* (2008) who reported that the cytotoxic effects of 6-TG on human embryonic kidney cell line was due to inhibition of DNA methylation. Wang and Wang, (2009) and Yuan *et al.* (2011) have also reported decrease in the level of global cytosine methylation following treatment with 6-TG on human embryonic kidney cell line and leukemia cell lines. Another study conducted in canine malignant lymphoid cells further confirmed the demethylation activity of 6-TG, evidenced by a

decrease in the level of DNMT1 protein and global DNA methylation (Flesner *et al.*, 2014). Curcumin cause global demethylating activity by inducing downregulation of DNMT1 at mRNA and protein level (Abusnina *et al.*, 2011; Du *et al.*, 2012). Liu *et al.* (2009) also reported on the global hypomethylation activity of curcumin.

The increase in global demethylating activity of 6-TG-CNPs may be due to the down-regulation of histone lysine-specific demethylase 1 expression which stimulated the lysine methylation of DNMT1 and triggered its degradation *via* the ubiquitin proteasomal pathway (Yuan *et al.*, 2011). Thus, 6-TG-CNPs were able to restore the epigenetic dysregulation and found to be more effective when compared to those observed with 6-TG treated group. Further, combination of 6-TG-CNPs with curcumin showed better effect on the restoration of epigenetic dysregulation when compared to those observed with 6-TG-CNPs treated group.

In conclusion, the enhanced anticancer effects of 6-TG-CNPs in terms of per cent cell viability, apoptosis and necrosis, cell cycle arrest and DNA demethylating activity when compared with 6-TG might be due to the efficient cellular uptake of nanoformulations by the cancer cells which in turn might be attributed to their unique properties such as small size, shape, high surface area-to-volume ratio, solubility and stability (Liu *et al.*, 2012). The increased internalization could also be due to the neutralization of negative charge on tumor cell by the positive charge of chitosan. Polymer nanoparticles with size ranging from 5 to 500nm are readily internalized by the cancer cells (Shang *et al.* 2014). Apart from this, the favorable intracellular milieu in cancer cells (pH 3.0-5.5, slightly acidic) compared to normal cells (pH 7.4) (Wan *et al.*, 2018) might have resulted in increased intracellular drug release from the nanoformulations.

Further, combination of 6-TG-CNPs with curcumin produced better anticancer effect in terms of per cent cell viability, apoptosis and necrosis, cell cycle arrest and DNA

demethylating activity when compared to those observed with 6-TG-CNPs treated group. The anticancer effect of curcumin by various cellular pathways and also its role in reversing epigenetic dysregulation by DNA demethylating activity might have additionally contributed to the increased anticancer effect of the combination.

5.4. PHARMACOKINETIC STUDIES OF 6-TG IN 6-TG, 6-TG-CNPS ALONE AND 6-TG-CNPS IN CURCUMIN PRE-TREATED RATS BY LC- MS/MS

Pharmacokinetic profile of 6-thioguanine was studied to know the superiority of its nanoformulation and the influence of curcumin pre-treatment for four consecutive days in Wistar rats.

The mean peak plasma concentration ($C_{\max}$) of 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was 79.12 ± 11.63 , 124.07 ± 2.73 and 158.70 ± 52.06 ng/mL, respectively. The $C_{\max}$ of 6-TG in 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was found to increase by 1.6-fold and 2-fold, respectively when compared to those observed with 6-TG treated group.

The $T_{\max}$ achieved for 6-TG in 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was at 4h, whereas in 6-TG treated group it was achieved at 2h post treatment.

The mean area under the curve (AUC_{0-24}) for 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was 216.66 ± 25.84 , 536.52 ± 98.70 and 691.96 ± 194.42 ng.ml*h⁻¹, respectively. The mean area under the curve (AUC_{0-24}) for 6-TG in 6-TG-CNPs alone treated group was significantly ($p < 0.05$) higher when compared to those observed with 6-TG treated group. The mean area under the curve (AUC_{0-24}) for 6-TG in curcumin followed by 6-TG-CNPs treated group was significantly ($p < 0.01$) higher when compared to those observed with 6-TG treated group.

The mean area under the curve ($AUC_{0-\infty}$) for 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was 230.59 ± 29.94 , 545.18 ± 99.39 and

701.12 $\pm$ 195.62 ng.ml*h⁻¹, respectively. The mean area under the curve (AUC_{0-inf}) for 6-TG in 6-TG-CNPs alone treated group was found to increase 2.4-fold when compared to those observed with 6-TG treated group. The mean area under the curve (AUC_{0-inf}) for 6-TG in curcumin followed by 6-TG-CNPs treated group was significantly ($p < 0.01$) higher when compared to those observed with 6-TG treated group.

Area under the curve is considered as an indicator of the extent of absorption. Thus, the rate and extent of 6-TG absorption was found increased by 2.5-fold following 6-TG-CNPs alone and 3.1-fold in curcumin followed by 6-TG-CNPs treated group when compared to those observed with 6-TG treated group. The enhanced C_{max} and AUC observed in the present study was in the order of curcumin pretreatment + 6-TG-CNPs > 6-TG-CNPs > 6-TG treated groups. Sharma *et al.* (2017) reported 1.4 to 1.5-fold increase in C_{max} and AUC for paclitaxel in curcumin pretreated rats when compared with only paclitaxel treated group. Yan *et al.* (2010) also reported increased C_{max} of docetaxel in curcumin co-administered rats when compared to only docetaxel treated group.

Chitosan is an excellent carrier as it is mucoadhesive, permeation enhancer and forms a protective barrier for the drug. It acts as a penetration enhancer by transient and reversible opening of the tight junctions of the epithelium and facilitates both paracellular and transcellular transport of drugs across the epithelium (Mohammed *et al.*, 2017). Curcumin exert its bioavailability enhancing properties by enhancing the absorption of orally administered drugs by inhibiting cytochrome P450 mediated metabolism and also efflux pump inhibition (Sharma *et al.*, 2017). The CYP3A4 isozyme found in intestinal enterocytes and hepatic cells acts as an important catalyst of first-pass drug metabolism (Guengerich, 2003). One of the potential oxidative pathways for 6-TG is mediated by cytochrome P-450 (Kitchen *et al.*, 1999). Thus, increase in C_{max}, AUC and variation in T_{max} observed in present study can be attributed to the mucoadhesive property of chitosan which increased the residence time of the nanoformulation at the site of absorption. Apart

from this, permeation enhancing property of chitosan also facilitated both paracellular and transcellular transport of drug across the epithelium. Additionally, inhibition of CYP3A4 activity by curcumin might have also contributed to the increase in the PK parameters discussed above.

The volume of distribution (V_z/F_{obs}) for 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was 0.49 ± 0.04 , 0.09 ± 0.04 and 0.15 ± 0.05 (mg/kg)/(ng/ml), respectively. The volume of distribution (V_z/F_{obs}) for 6-TG in 6-TG-CNPs alone and 6-TG-CNPs in curcumin pre-treated rats were significantly ($p < 0.0001$) lower when compared to those observed with 6-TG treated group. This indicates that apparent volume of distribution was substantially higher for 6-TG in 6-TG treated group when compared to those observed with nanoformulations alone and nanoformulation with curcumin pre-treated group. This suggest better tumor targeting efficiency for nanoformulations with less toxicity in off-target tissues. This can be extrapolated/explored in clinical or preclinical perspective because the enhanced permeability and retention (EPR) effect of the tumor microenvironment facilitates the selective internalization of nanoparticles of size 100-1000nm by the cancer cells (Xin *et al.*, 2017). Additionally, positive charge on chitosan surface neutralizes the negative charge on the tumor cell surface which in turn increases the drug concentration in the tumor site (Wang *et al.*, 2011) thereby suggesting better tumor targeting efficiency of nanoformulations over conventional 6-TG.

The volume of distribution at steady-state ($V_{d_{ss}}$) for 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was 0.41 ± 0.03 , 0.13 ± 0.02 and 0.12 ± 0.04 ml, respectively. The volume of distribution at steady-state ($V_{d_{ss}}$) for 6-TG in 6-TG-CNPs alone and 6-TG-CNPs in curcumin pre-treated rats was significantly ($p < 0.0001$) lower when compared to those observed with 6-TG treated group. $V_{d_{ss}}$ is a measure of steady-state concentration and indicate that at equilibrium the tissue

concentration is equal to that of plasma and its depletion profile in tissue is similar to that of plasma kinetics. Any drug that bound to tissue/ tissue protein is pharmacologically inactive (Baggot, 2001). Thus, decreased V_{dss} indicate rapid dissociation of nanoformulation from tissues resulting in availability of free drug molecules for pharmacological actions (Yates and Arundel, 2006).

CONCLUSIONS

The following conclusions could be drawn from the present study:

- 6-TG was successfully loaded / encapsulated to chitosan nanoparticles without any structural modification with acceptable particle size of $261.63 \pm 6.01 \text{ nm}$, PDI of 0.34 ± 0.10 , zeta potential of $+15.97 \pm 0.46 \text{ mV}$ and entrapment efficiency of 44.27 per cent.
- The 6-TG release from the chitosan nanoformulation was found to be pH dependent, with a maximum release at pH 4.8.
- When compared to 6-TG, there was 1.2-fold increase in cytotoxicity in terms of apoptosis and necrosis, cell cycle arrest and DNA demethylating activity with 6-TG-CNPs in the PA-1 cell lines. This can be attributable to the efficient cellular uptake of nanoformulation by the cancer cells. The increased internalization might be due to the neutralization of negative charge on tumor cells by positively charged chitosan. Additionally, the favorable intracellular milieu in cancer cells (pH 3.0-5.5, slightly acidic) compared to normal cells (pH 7.4) might have also favored increased intracellular drug concentration from the nanoformulations.

Further, there was 1.3-fold increase in cytotoxic effect when PA-1 cell lines were exposed to combination of 6-TG-CNPs with curcumin.

The increased cytotoxic effect observed for the combination might be due to involvement of multiple cellular cytotoxicity pathways or role of curcumin in reversing the epigenetic dysregulation by DNA demethylating activity.

- PK- studies in rats following single *per os* administration indicated significant variation with respect to C_{max} , T_{max} and AUC when compared to 6-TG alone. Similarly, curcumin pre-treatment further augmented the aforesaid PK-parameters. There was 1.6 and 2-fold increase in C_{max} values with 6-TG-CNPs and its combination with curcumin pre-treatment, respectively. This might be due to the permeation enhancing property of chitosan. Further, the delay in T_{max} observed in the current study with nanoformulations may be due the mucoadhesive properties of chitosan at the level of absorption from gastrointestinal tract.
- The $AUC_{(0-24)}$ was significantly ($p<0.05$) increased in 6-TG-CNPs treated rats when compared to 6-TG. Further, curcumin pre-treatment resulted in significant ($p<0.01$) increase in $AUC_{(0-24)}$. This increase in AUC observed in the current study could be due to bio-enhancing *vis-a-vis* CYP₄₅₀ inhibition by curcumin pre-treatment.
- The volume of distribution (V_d) of 6-TG in 6-TG-CNPs or 6-TG-CNPs following curcumin pre-treated groups were significantly ($p<0.0001$) lower when compared to 6-TG treated rats. This observation might be significant from clinical perspectives, as the permeability and retention of nanoformulation in the tumor microenvironment facilitates their internalization which depends upon size of the nanoparticles.
- The volume of distribution at steady state (V_{dss}) of 6-TG in 6-TG-CNPs with or without curcumin pre-treatment observed in the present study was significantly ($p<0.0001$) lower when compared to 6-TG treated group. This implies that chitosan-based nanoparticle dissociates from tissues thus rendering drug molecules available for their action at the target sites.

Thus, the developed 6-TG loaded chitosan nanoformulation used alone or in combination with curcumin appears to have wide scope for application in cancer therapeutics.

Summary

CHAPTER: VI

SUMMARY

Present study comprises of synthesis and characterization of 6-thioguanine encapsulated chitosan nanoparticles and study of its *in vitro* anticancer efficacy and *in vivo* pharmacokinetic profile.

6-thioguanine encapsulated chitosan nanoparticles were formulated based on the ionic gelation interaction between positively charged chitosan and negatively charged tripolyphosphate (TPP).

Morphologically, 6-TG-CNPs were spherical in shape and showed acceptable mean size, PDI, stable zeta potential and entrapment efficiency of 261.63 ± 6.01 nm, 0.34 ± 0.10 , $+15.97 \pm 0.46$ mV and 44.27 per cent, respectively. FTIR analysis revealed the formation of 6-TG complex with chitosan as evidenced by shift in the spectral peak related to N-H stretching in chitosan to interior in the 6-TG-CNPs and also missing of spectral peaks related to C=S and C-N of 6-TG in the 6-TG-CNPs. The drug release profile of 6-TG-CNPs showed a slow but increased release which was 87.32 ± 1.16 per cent in 24h and 91.40 ± 1.08 per cent in 48h at pH 4.8. However, at pH 7.4 the release was less and sustained which was 63.53 ± 6.44 per cent in 24h and 73.96 ± 1.12 per cent in 48h. Thus, 6-TG release from chitosan nanoparticles was more pH dependent.

MTT assay was conducted on MCF-7 and PA-1 cell lines. IC_{50} values of 6-TG, 6-TG-CNPs and curcumin for MCF-7 cell lines at 48h incubation were 23.09, 17.82 and $15.73 \mu\text{M}$, respectively. On PA-1 cells, the IC_{50} values of 6-TG, 6-TG-CNPs and curcumin were 5.81, 3.92 and $12.89 \mu\text{M}$, respectively. Combination of 6-TG-CNPs (IC_{25}) with curcumin (IC_{25}) on MCF-7 and PA-1 at 48h incubation showed percent cell viability of 49.77 ± 0.05 and 43.67 ± 0.02 , respectively.

Flow cytometric apoptosis assay of PA-1 cells with vehicle control showed 96.94 ± 0.32 per cent viability and 3.01 ± 0.34 per cent of cells in early apoptosis. 6-TG,

6-TG-CNPs, curcumin at their respective IC_{50} concentration showed 45.79 ± 2.33 , 61.92 ± 2.20 and 77.33 ± 3.97 per cent of cells in early apoptosis. The combination of 6-TG-CNPs (IC_{25}) and curcumin (IC_{25}) treated group showed 90.67 ± 1.79 per cent of cells in early apoptosis stage.

The percent of cells arrested at G2/M phase of the cell cycle by 6-TG, 6-TG-CNPs, curcumin at their respective IC_{50} concentration were 40.86 ± 0.34 , 47.49 ± 0.29 , 48.59 ± 0.52 , respectively. The combination of 6-TG-CNPs (IC_{25}) and curcumin (IC_{25}) treated group arrested 50.42 ± 0.74 per cent of cells in G2/M phase of the cell cycle.

The increase in global DNA demethylating activity in terms of relative mean fluorescence intensity of anti-5mc-FITC by vehicle control, 5-Azacytidine($5\mu M$), 6-TG, 6-TG-CNPs, curcumin at their respective IC_{50} concentration were 10.32 ± 0.05 , 6.95 ± 0.09 , 6.86 ± 0.07 , 6.18 ± 0.06 , 7.29 ± 0.12 , respectively. The combination of 6-TG-CNPs (IC_{25}) with curcumin (IC_{25}) showed 4.95 ± 0.05 global DNA demethylating activity.

Thus, the treatment groups viz., 6-TG, 6-TG-CNPs, curcumin and combination of 6-TG-CNPs with curcumin showed significant ($p < 0.0001$) increase in cytotoxicity potential in terms of per cent cell viability, early apoptotic effect, G2/M phase arrest and increase in global DNA demethylating activity when compared to those observed with vehicle control. The treatment groups viz., 6-TG-CNPs and combination of 6-TG-CNPs with curcumin showed significant ($p < 0.0001$) increase in cytotoxicity potential when compared to those observed with 6-TG treated group. The combination of 6-TG-CNPs with curcumin showed significant ($p < 0.0001$) increase in cytotoxicity potential when compared to those observed with 6-TG-CNPs treated group. This might be due to efficient cellular uptake of nanoformulations by the cancer cells. Apart from this, well known anticancer effect of curcumin and also its role in reversing epigenetic dysregulation by

DNA demethylating activity might have additionally contributed to the increased anticancer effect of the combination.

The mean peak plasma concentration (C_{max}) of 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was 79.12 ± 11.63 , 124.07 ± 2.73 and 158.70 ± 52.06 ng/mL, respectively. The T_{max} for 6-TG in 6-TG treated group was 2h and in both nanoformulation treated group was 4h. The mean area under the curve (AUC_{0-24}) for 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was 216.66 ± 25.84 , 536.52 ± 98.70 and 691.96 ± 194.42 ng.ml*h⁻¹, respectively. The mean area under the curve (AUC_{0-inf}) for 6-TG in 6-TG, 6-TG-CNPs alone and 6-TG-CNPs in curcumin pretreated group was 230.59 ± 29.94 , 545.18 ± 99.39 and 701.12 ± 195.62 ng.ml*h⁻¹, respectively.

Thus, the PK parameters viz., C_{max} , AUC and variation in T_{max} for 6-TG in 6-TG-CNPs alone treated group was found to increase by 1.6-fold, 2.5-fold and 4h, respectively when compared to those observed with 6-TG treated group. The PK parameters viz., C_{max} , AUC and variation in T_{max} for 6-TG in curcumin followed by 6-TG-CNPs treated group was found increased by 2-fold, 3.2-fold and 4h respectively, when compared to those observed with 6-TG treated group. This could be attributed to the mucoadhesive and permeation enhancing property of chitosan. Additionally, bio-enhancing property of curcumin might have also contributed to the increased PK parameters in combination study. The enhanced C_{max} and AUC was in the order of curcumin pre-treatment + 6-TG-CNPs > 6-TG-CNPs > 6-TG groups.

The apparent volume of distribution (V_z/F_{obs}) and volume of distribution at steady-state (V_{dss}) of 6-TG in 6-TG-CNPs with or without curcumin pre-treatment observed in the present study was significantly ($p < 0.0001$) lower when compared to those observed with 6-TG treated group. These PK parameters suggests the better tumor targeting efficiency of 6-TG nanoformulations with less toxicity in off-target tissues.

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