**A DISSERTATION ON**

# Elucidating the role of Ursolic Acid on epithelial to mesenchymal transition and autophagy in lung cancer

**SUBMITTED TO THE**

**DEPARTMENT OF BIOENGINEERING**
**FACULTY OF ENGINEERING**
**INTEGRAL UNIVERSITY, LUCKNOW**

**IN PARTIAL FULFILMENT**
**FOR THE**
**DEGREE OF MASTER OF TECHNOLOGY**
**IN BIOTECHNOLOGY**

**BY**
**VIVEK SINGH**
**M. Tech Biotechnology (IV Semester)**
**Roll No: 2001361018**

**UNDER THE SUPERVISION OF**

**Dr. Snober S. Mir**
**Associate Professor and Head**
**Department of Biosciences**

**INTEGRAL UNIVERSITY, DASAULI, KURSI ROAD**
**LUCKNOW- 226026**

# DECLARATION FORM

I, **Vivek Singh**, a student of **M.Tech. Biotechnology** ( **II year/ IV Semester**), Integral University have completed my six months dissertation work entitled "**Elucidating the role of Ursolic Acid on epithelial to mesenchymal transition and autophagy in lung cancer**" successfully from **Integral university** under the able guidance of **Dr. Snober S. Mir.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

**Vivek Singh**

**Dr. Salman Akhtar**
**Course Coordinator**

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Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)


## CERTIFICATE

Certificate that **Mr. Vivek Singh (2001361018)** has carried out the research work presented in this thesis entitled **"Elucidating the role of Ursolic Acid on epithelial to mesenchymal transition and autophagy in lung cancer"** for the award of **M.Tech. Biotechnology** from **Integral University**, Lucknow under my supervision. The thesis embodies the results of original work and studies carried out by the student himself. The contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of his **M.Tech. Biotechnology**.

I wish him good luck and a bright future.

**Dr. Snober S. Mir**
**Associate Professor & Head**
**Department of Biosciences**
**Integral University, Lucknow**

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## CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Vivek Singh,** a student of **M.Tech. Biotechnology** ( II year/ IV Semester), Integral University has completed his six months dissertation work entitled **"Elucidating the role of Ursolic Acid on epithelial to mesenchymal transition and autophagy in lung cancer"** successfully. He has completed this work from **Integral University** under the guidance of **Dr. Snober S. Mir**, Associate Professor, Assistant Director IIRC and head department of Biosciences. The dissertation was a compulsory part of his **M.Tech Biotechnology.**

I wish him good luck and a bright future.

**Dr. Punit kumar Singh**
Assistant Professor
Department of Bioengineering
Faculty of Engineering

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## TO WHOM IT MAY CONCERN

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I wish him good luck and a bright future.

**Dr. Alvina Farooqui**
Head
Department of Bioengineering
Faculty of Engineering

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**Date** **Vivek Singh**

## Contents

# LIST OF SYMBOLS AND ABBREVIATIONS

- μl Micro-litre
- μg Micro- gram
- μM Micro-molar
- ml Milli-litre
- Mg Milli-gram
- mM Milli-molar
- SCLC Small Cell Lung Cancer
- NSCLC Non-Small Cell Lung Cancer
- p53 Tumor Protein 53
- PCD Programmed Cell Death
- MOMP Mitochondrial Outer Membrane Permeabilization
- BAX BCL2-associated X Protein
- BID BH3-interacting domain death agonist
- BCL2 B-cell lymphoma 2
- CDKs Cyclin Dependent Kinases

- LC3 Microtubule-associated protein light chain
- BBC3 BCL2 Binding Component 3
- TNF Tumor Necrosis Factor
- TRAIL TNF-related apoptosis-inducing ligand
- FLIP FLICE inhibitory protein
- EGFR Epidermal Growth Factor Receptor
- UA Ursolic acid
- EMT Epithelial to mesenchymal transition
- DMSO Dimethyl sulfoxide
- IAP Inhibitors of apoptosis proteins
- FBS Fetal Bovine Serum
- Caspases Cysteine-aspartic proteases

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# 1. INTRODUCTION

The term "nutraceutical" is an amalgamation of "nutrition" and "pharmaceutical." Nutraceuticals, broadly speaking, are present in food items as their components that significantly alter and sustain the normal physiological function necessary to keep healthy humans alive (Das *et al.*, 2012).

In the first instance, the phrase "nutraceutical" was used in a study from the UK, Germany, and France, where consumers believed that nutrition was more crucial to maintain health than exercise or genetic traits (Pandey *et al.*, 2010). Stephen De Felice, the founder and chairman of the Foundation for Innovation in Medicine (FIM), Cranford, New Jersey, invented the phrase "nutraceutical" in 1989 by combining "nutrition" and "pharmaceutical"(Maddi *et al.*, 2007). A chemical compound, found in diet, that offers medical or health benefits and protect us from various diseases, is referred to as a nutraceutical, in De Felice's definition. Nutraceuticals are described by Health Canada as "a product derived from foods, but offered in the form of pills, or powder (potions), or in other therapeutic forms, not normally associated with foods" (Wildman *et al.*, 2016). These dietary supplements aid in the fight against different deadly health issues of the twenty-first century, including corpulence, cardiovascular disease, weakening of bones, joint pain, hyperglycemia, hypercholesteremia, cancer, etc (Das *et al.*, 2012).

Recently, it was discovered that the pentacyclicterpenoid carboxylic acid ursolic acid (3-hydroxy-urs-12-en-28-oic acid, UA) controls numerous significant factors and proteins associated with inflammation, cell cycle and growth, and enzymatic reactions, etc. (Shanmugam *et al.*, 2013). Many plants include triterpenoids, such as the oleanane, ursane, and lupane families, which are thought to be responsible for many of these advantageous benefits (Woźniak, *et al.*, 2015). Additionally, Ursolic acid influences a variety of intra/extracellular protein molecules which may have essential effect on the processes of apoptosis, metastasis, angiogenesis, and inflammation. (Kashyap *et al.*, 2016).

It is recommended by the clinical studies that practical application of UA may possibly lead to the advancements in the therapy to treat inflammatory diseases, such as cancer or type 2 diabetes mellitus and their consequences (Silva *et al.*, 2016). These findings have raised interest and awareness regarding implications of UA as a potent treatment approach. The

anti-tumor effects of UA were reported by Zhang et al (Zang, *et al.,* 2014). Which include inhibition of angiogenesis, suppression of cancer cell growth, control of the immune surveillance system, prevention of cancer cell aggressiveness, and induction of apoptosis (Wang *et al.,* 2017).

Cancer is a disease which is characterised by uncontrollably and repeatedly dividing cells. In order to produce two genetically identical cells, cell division is carefully regulated by a number of evolutionarily conserved cell cycle regulation systems. Checkpoints in the cell cycle act as DNA monitoring systems to stop the accumulation and spread of genetic mistakes during cell division. Checkpoints can halt the course of the cell cycle or, in the case of irreparable destruction in DNA molecule, trigger cell cycle exit or death. Cancer-related mutations that affect cell cycle regulation enable continued cell division, primarily through impairing cells' capacity to exit the cell cycle. To avoid catastrophic levels of damage and sustain cell viability, however, repeated rounds of division increase reliance on other cell cycle regulatory mechanisms (Kops *et al.,* 2004).

People have been curious about "what causes cancer" for many years. The vast differences in the forms of cancer observed around the world intrigued the attendees of an international conference that the World Health Organization hosted in 1950 (Blackdar *et al.,* 2016).

It was shown that immigrants tended to get cancers that were more common in their new nations than in their original ones. This suggested that environmental exposures rather than inherited genetic elements were the main cause of most malignancies. The symposium resulted in the founding of the International Agency for Research on Cancer (IARC) in 1965, with the mandate to carry out multidisciplinary studies on the plausible reasons behind human malignancies (Shimkin at. al., 1977). Initial IARC assessments relied solely on epidemiological evidence, but later criteria were expanded to take into account experimental findings (Tomatis *et al.,* 1978).

Nowadays, chemotherapy resistance and cancer relapse have worsened the disease and are a matter of concern worldwide. As discussed above, autophagy has dual role in cancer and is responsible to support the growth of malignant cancer cells undergoing EMT and also generates resistance towards anticancer drugs however there is no clear molecular link between the autophagy and EMT. Thus, the study entitled "**Elucidating the role of Ursolic acid on epithelial to mesenchymal transition and autophagy in lung cancer**" was

designed to evaluate whether the compound is potent enough to regulate and control the expression of EMT and autophagy marker genes in lung carcinoma cell lines.

## AIM& OBJECTIVES

This study aims to evaluate the effect of Ursolic Acid on autophagy and EMT marker genes.

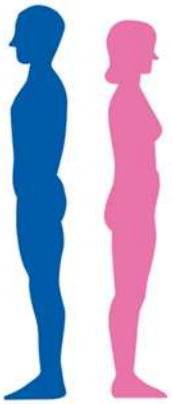
- *In silico*studies of Ursolic Acid with EMT and autophagy marker genes.
- To evaluate the cytotoxic potential of Ursolic Acid on lung cancer cell line.
- To check the effect of Ursolic Acid on EMT and autophagy marker genes.

# 2. REVIEW OF LITERATURE

The replication of genomic DNA and the ensuing segregation of daughter cells during different cell cycle phases in eukaryotic cells are the two fundamental events that are the focus of cell cycle control. It is frequently believed that cancerous cells crosses the cell cycle uncontrollably and that the majority, if not all, cell cycle checkpoints must be damaged for a cell to develop into cancer. A significant body of recent research, however, has produced compelling evidence that just particular parts of cell cycle control must be compromised so that cancerous cells continue to proliferate. Cancer cells frequently have a defective DNA damage checkpoint, allowing continued cell division despite the accumulation of genetic mistakes (Kops *et al.,* 2004).

As per the statistics report, nearly 10 million fatality worldwide may have arisen due to cancer in 2020, making it the top cause of death. Breast cancer (2.26 million cases) and lung cancer (2.21 million cases) were the most prevalent malignancies in 2020 (in terms of new cases), according to the reports of WHO (World Health Organization). Nearly half (48%) of all incidence instances of cancer in men are of the prostate, lung, and colorectal kind; prostate cancer alone accounts for 27% of diagnosed cases. Breast cancer alone affects over one-third of all new diagnoses in women, followed by CRC, lung cancer, and breast cancer, which together account for 51% of all diagnoses (Yang *et al.,* 2022).

**Estimated New Cases**

| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| Prostate | 268,490 | 27% |  | Breast | 287,850 | 31% |
| Lung & bronchus | 117,910 | 12% | | Lung & bronchus | 118,830 | 13% |
| Colon & rectum | 80,690 | 8% | | Colon & rectum | 70,340 | 8% |
| Urinary bladder | 61,700 | 6% | | Uterine corpus | 65,950 | 7% |
| Melanoma of the skin | 57,180 | 6% | | Melanoma of the skin | 42,600 | 5% |
| Kidney & renal pelvis | 50,290 | 5% | | Non-Hodgkin lymphoma | 36,350 | 4% |
| Non-Hodgkin lymphoma | 44,120 | 4% | | Thyroid | 31,940 | 3% |
| Oral cavity & pharynx | 38,700 | 4% | | Pancreas | 29,240 | 3% |
| Leukemia | 35,810 | 4% | | Kidney & renal pelvis | 28,710 | 3% |
| Pancreas | 32,970 | 3% | | Leukemia | 24,840 | 3% |
| **All Sites** | **983,160** | **100%** | | **All Sites** | **934,870** | **100%** |

**Estimated Deaths**

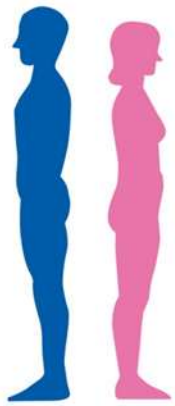
| | Males | | | Females | | |
|---|---|---|---|---|---|---|
| Lung & bronchus | 68,820 | 21% |  | Lung & bronchus | 61,360 | 21% |
| Prostate | 34,500 | 11% | | Breast | 43,250 | 15% |
| Colon & rectum | 28,400 | 9% | | Colon & rectum | 24,180 | 8% |
| Pancreas | 25,970 | 8% | | Pancreas | 23,860 | 8% |
| Liver & intrahepatic bile duct | 20,420 | 6% | | Ovary | 12,810 | 4% |
| Leukemia | 14,020 | 4% | | Uterine corpus | 12,550 | 4% |
| Esophagus | 13,250 | 4% | | Liver & intrahepatic bile duct | 10,100 | 4% |
| Urinary bladder | 12,120 | 4% | | Leukemia | 9,980 | 3% |
| Non-Hodgkin lymphoma | 11,700 | 4% | | Non-Hodgkin lymphoma | 8,550 | 3% |
| Brain & other nervous system | 10,710 | 3% | | Brain & other nervous system | 7,570 | 3% |
| **All Sites** | **322,090** | **100%** | | **All Sites** | **287,270** | **100%** |

**Figure 2.1:** Estimated new cases and deaths of cancers (*Globocan Cancer Statistics, 2021*).

## 2.1 LUNG CANCER

Among the total 1.2 million new cases in the year 2000, lung cancer was the highly prevalent cancer in the globe with 12.3% of all malignancies (Parkin *et al.*, 2001).

Lung cancer is mostly brought on by tobacco use, with 80 to 90 percent of cases occurring in smokers. There are substantial regional, racial, and gender differences in lung cancer occurrence and some literatures indicate that contact to the carcinogens in tobacco smoke may put females at an increased chances of developing lung carcinoma. However, lifetime smokers have a 20–30 fold higher risk of acquiring lung cancer unlikely to lifelong non-smokers. Since smoking is becoming less common in the United States, it is wide-ranging in China and Eastern Europe and will cause tens of millions of new cases in this century (Parkin *et al.*, 2001).

Lung cancer is therefore the most inevitable cancer among all, and after a 7-year lag, quitting smoking reduces risk. In USA, lung cancer is now increasingly a disease of people who used to smoke earlier, despite the fact that this decreased probability rate never returns to the baseline. Despite advances in treatment, 90% of patients with lung cancer will pass away from their condition. Lung cancer is thought to have killed 1.1 million individuals worldwide in 2000, with 17.8% of all cancer-related fatalities. Although only 11% of chain smokers eventually acquire lung cancer, this finding raises the possibility that there are hereditary variables that enhances the probability of this disease (Lippman *et al.,* 2001).

## 2.2 OVERVIEW OF MOLECULAR BASIS OF LUNG CANCER

Numerous molecular and genetic investigations have shown that various genetic and epigenetic modifications are present in lung malignancies, including both known and hypothetical non-dominant like tumor suppressors as well as various dominant cancer causing genes (Zochbauer *et al.,* 2000). The main kinds of lung cancer are the subject of the majority of the molecular and genetic investigations on lung malignancies (Minna *et al.,* 2002). In the lung, cancerous cells and nearby ordinary cells overexpress a variety of growth signalling or regulatory molecules and their receptors. Consequently, this neoplasm has a number of growth promoting stimuli acting in either autocrine or paracrine manner (Viallet *et al.,* 1996).

(Lu *et al.,* 2017). The size and characteristics of the cancerous cells that a histopathologist can observe under a microscope while examining a lung carcinoma are used to classify it. There are two main types differentiated for therapeutic purposes: non-small-cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC) (Kumar *et.al.,* 2013).

### 2.2.1 Non-small-cell lung carcinoma

NSCLC has three primary subtypes: squamous-cell carcinoma, large-cell carcinoma and adenocarcinoma (Wang *et al.,* 2018; Cai-Xia *et al.,* 2014). Adenocarcinomas, which typically originate from peripheral lung tissue, account for over 40 percent of the total of lung cancer incidents (Cetin *et al*., 2017).

### 2.2.2. **Small-cell lung carcinoma**

SCLC has a relationship with an endocrine or paraneoplastic illness due to the presence of numerous neurosecretory granules in its cells (vesicles carrying neuroendocrine hormones) (Rosti *et al.,* 2006). The majority of incidents develop in the primary and secondary bronchi region of bigger airways (Collins *et al.,* 2007).

**Others**

There are four recognised primary histological subtypes, while some tumours may include many of these kinds (Bagchi *et al.,* 2019).

## 2.3 CELL DEATH PATHWAYS

Apoptotic, autophagic, necrotic and mitotic catastrophe-related cell deaths can all be classified according to morphological criteria and are each given their own classification. Enzymological criteria, such as the involvement of several protease classes (calpain, cathepsin, caspases, etc.), functional sides (planned or unplanned, in normal or diseased state), or traits associated with immune system are often used to characterise cell death (Galluzzi *et al.,* 2007). According to the current paradigm, the main cell death process is caspase-dependent apoptosis, but (1) that caspase-unrelated pathways can work along with caspases (or act in their place) to carry out deadly signalling pathways, (2) that the serine/threonine kinases receptor interacting protein 1 and 3 are a significant route for necrotic cell death (RIP3), (3) that pyroptosis signalling is distinct from traditional caspase-dependent apoptosis, and (4) that "autophagic cell death" is a kind of cell demise that can occur alongside (but not always as a result of) autophagic vacuolization (Duprez *et al.,* 2009).

### 2.3.1APOPTOTIC CELL DEATH

A form of cell death linked to particular morphological characteristics was first referred to as "apoptosis" in 1972. Since then, apoptosis has undergone substantial research, and the underlying signalling processes are now well understood. Morphological featuresof apoptosis include cell contraction, bud formation on cellular membranes, and condensation of chromatin material. The regulated lysis of the cell into small apoptotic vesicles, which are afterwards recognised and ingested by neighbouring cells and macrophages, is the outcome of a cell-intrinsic programmed suicide mechanism. The Bcl-2 family of proteins, which regulates integrity of mitochondria, is one of two major evolutionary conserved protein families implicated in apoptosis (Youle *et al.,* 2008) and the caspases, which lead to the final stage of apoptosis and are specific to the amino acid cysteinyl aspartate (Fuentes *et al.,* 2004).

#### 2.3.1.1. Intrinsic pathway

Intrinsic pathway is also known as mitochondrial dependent pathway of apoptosis. The existence of many cells requires mitochondria. Without them, a cell stops breathing aerobically and degrades swiftly. Some apoptotic pathways are founded on this principle.

Different apoptotic proteins that target mitochondria have an impact on them. They might create membrane pores that enlarge the mitochondria, or they might make the mitochondrial membrane more permeable and let apoptotic effectors seep out (Cotran *et al.,* 2004 ///// Gonzalez *et al.,* 2010).

#### 2.3.1.2. Extrinsic pathway

The transmembrane death receptors are found on the dying cell in the extrinsic route, where the death ligands attach to them. Receptor clustering on the cell membrane is brought about by the docking of ligand on the target cell with the help of receptors. In order to construct a death-inducing signalling complex, this aggregation attracts adaptor proteins to the cytoplasmic location of the receptors (DISC). Procaspase molecules are brought together during DISC formation, facilitating their autocatalytic activation and release into the cytoplasm where they initiate the caspase cascade. The cleavage of proapoptotic protein BID by active caspase-8, which releases mitochondrial proapoptotic factors as a result, facilitates the connection between the two routes (Rampal *et al.,* 2012).

### 2.3.2 AUTOPHAGY

Through a lysosome-dependent controlled mechanism, autophagy is the non-specific breakdown of the cell that eliminates extraneous or defective costituents (Klionsky *et al.,* 2008). It enables the controlled deterioration and recycling of cellular parts (Mizushima *et al.,* 2011//// Kobayashi *et al.,* 2015).

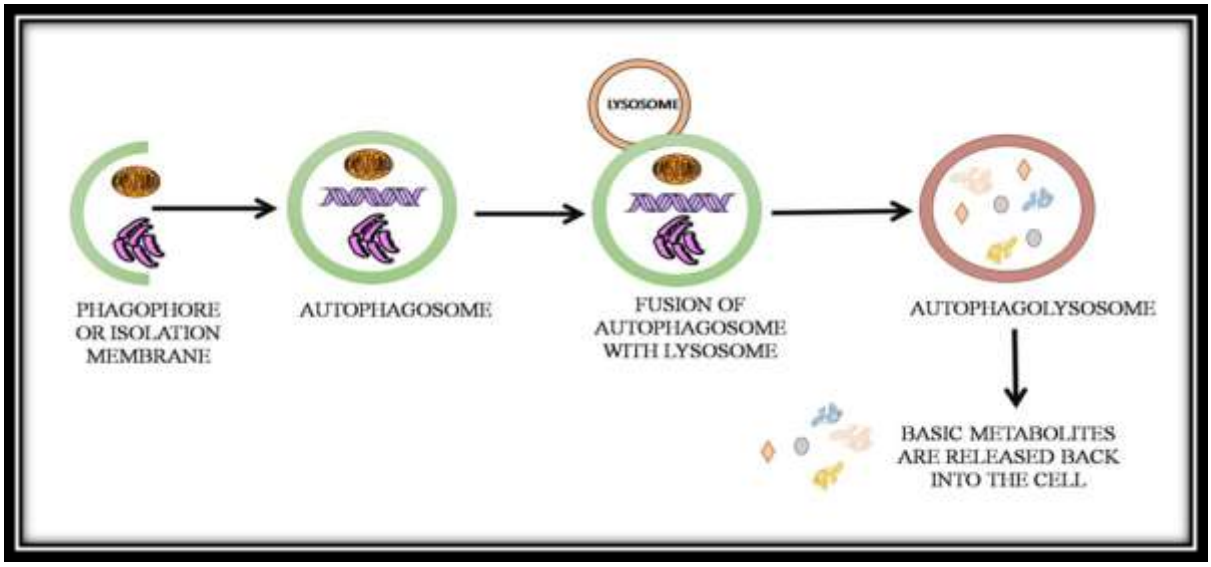



**Figure 2.2:** Mechanism of Macroautophagy

In response to environmental stressors including food deprivation and pathogen infection, autophagy occurs, which either can help cells adapt and survive or cause them to perish. In diseased situations such inherited myopathies and neurodegenerative disorders, autophagy is frequently seen (Larsen *et al.,* 2002).

To destroy and recycle proteins and organelles, internal membrane structures sequester them during the dynamic process known as autophagy. In all eukaryotic cells, from yeast to mammals, it is an evolutionarily conserved mechanism (Klionsky *et al.,* 2000).

Our knowledge of autophagy's function in cancer is still in its infancy and even the most basic question — whether it eliminates cancer cells or shields them from harmful conditions — is not yet fully resolved (Yasuko *et al.,* 2005).

The primary mechanism, macroautophagy, is employed mostly to eliminate destroyed misfunctional cell components (Levine *et al.,* 2011). Prior to forming an autophagosome around the organelle designated for eradication, the phagophore ingests the cellular molecules that needs to be destroyed (Mizushima *et al.,* 2002; Česen *et al.,* 2012). After merging with a lysosome in a mammalian cell or a vacuole in a yeast or plant cell, the autophagosome moves through the cell's cytoplasm to another organelle. The autophagosome's contents are destroyed by acidic lysosomal hydrolase inside the lysosome/vacuole (Rabie *et al.,* 2019).

As reported in literatures autophagy also has pro-survival role in cancer due to the supplication of nutrients to the starving malignant cells undergoing EMT, thus there must be some molecular link between these two pathways.

### 2.4 EMT (Epithelial to Mesenchymal Transition)

The loss of cell polarity and cell-cell adhesion in epithelial cells occurs during the epithelial-mesenchymal transition (EMT), become mesenchymal stem cells, which are multipotent stromal cells with the property of getting differentiated into numerous cell lineages, and acquire migratory and invasive capabilities. EMT is thought to be initiated by the loss of epithelial surface marker i.e. E-cadherin. Proteins which affect transcription of E-cadherin leading to its suppression are referred to as EMT-TFs (EMT inducing Transcription Factors). While factor like Twist indirectly suppress CDH1 (E-Cadherin), SNAI1/Snail 1, SNAI2/Snail 2 and ZEB1/2 can bind to the regulatory region of E-cadherin gene and restrict its transcription (Peinado *et al.,* 2007; Yang *et al.,* 2008).

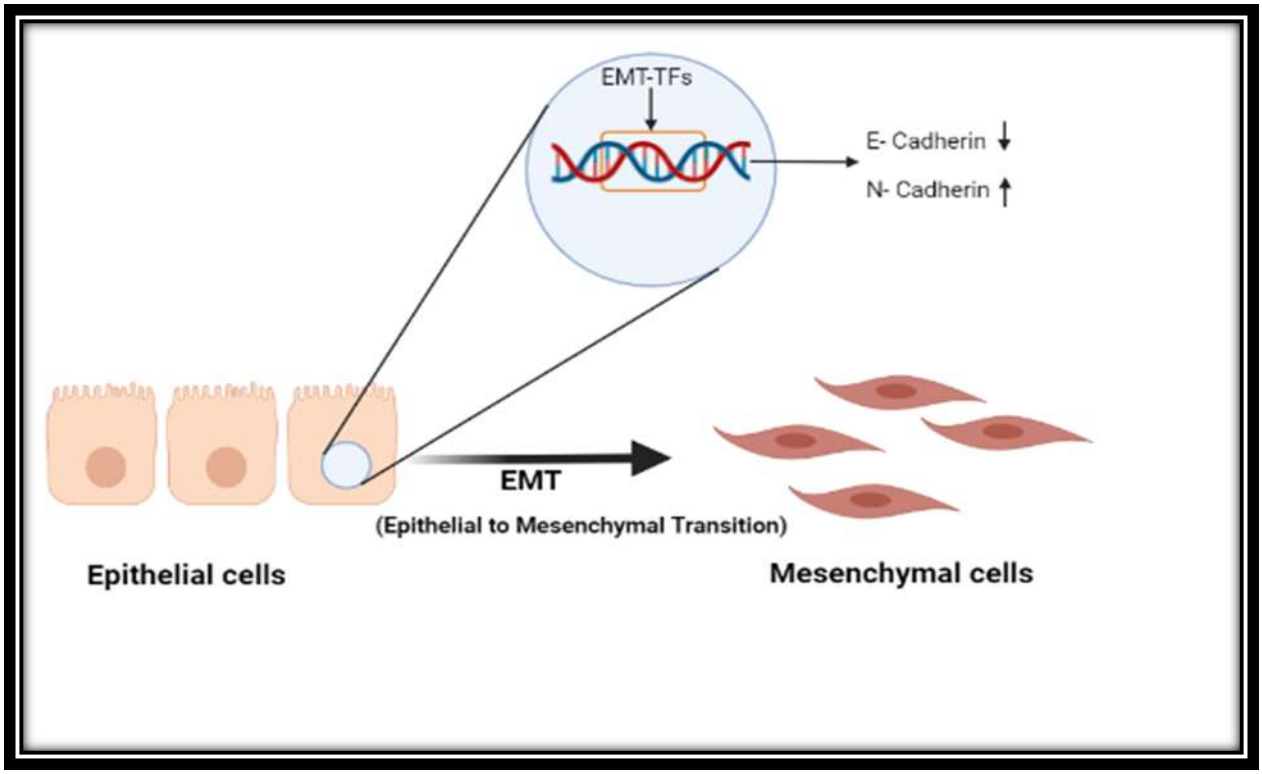



**Figure 2.3:** Epithelial to mesenchymal transition

During the process of development, wound healing, and cell culture propagation, epithelial cells have the ability to suppress their epithelial shape, elongate, and obtain the feature of motility and invasiveness. Because of the induction of some genes that are typically produced in mesenchymal cells and the repression of certain epithelial features, this reversible transition is known as the "epithelial to mesenchymal transition" (EMT). It was the prevailing belief that growth factors should only control cell growth and cannot affect cellular differentiation into different other cell types. However, after the discovery of fibroblast growth factor or transforming growth factor beta this belief initially met with scepticism that they could also cause phenotypic changes in cell culture leading to EMT (Lamouille *et al.*, 2014).

### 2.4.1. Role of EMT-Transcription Factors (EMT-TFs)

EMT has been divided into three categories based on the biological context: cancer, fibrosis, wound healing, and developmental (Kalluri *et al.*, 2009; Sciacovelli *et al.*, 2017).

### 2.4.2. EMT in Carcinoma Cells: More Than Epithelial-Mesenchymal Transition

Within a tumour, EMT processes have an impact on the stromal cells as well as the carcinoma cells directly (Shibue *et al.*, 2017). Partial EMT in cancer cells is frequently

observed, as shown by an enhanced expression of EMT associated proteins as EMT-TFs. As previously mentioned, it is presumably uncommon for epithelial cells to completely lose their prior phenotypic and transition into a mesenchymal condition. As cancer progresses, an increasing percentage of carcinoma cells exhibit incomplete EMT characteristics, resulting in cell subpopulations that differ in their invasive abilities and preferred location within the tumour as well as their patterns of intermediate EMT-associated gene expression (Aiello *et al.*, 2018).

**2.4.3. Effects of EMT on cancer malignancy and metastasis:**

Invasion is necessary for metastasis to begin, and EMT makes this possible (Weinberg *et al.*, 2000). A primary tumor's carcinoma cells lose cell-cell adhesion due to E-cadherin repression, penetrate the basement membrane with increased invasiveness, and intravasate into the bloodstream. These circulating tumour cells (CTCs) undergo MET for clonal expansion at these metastatic locations once they exit the bloodstream to generate micro-metastases. Thus, the invasion-metastasis cascade is started and ended by EMT and MET (Chaffer *et al.*, 2011).

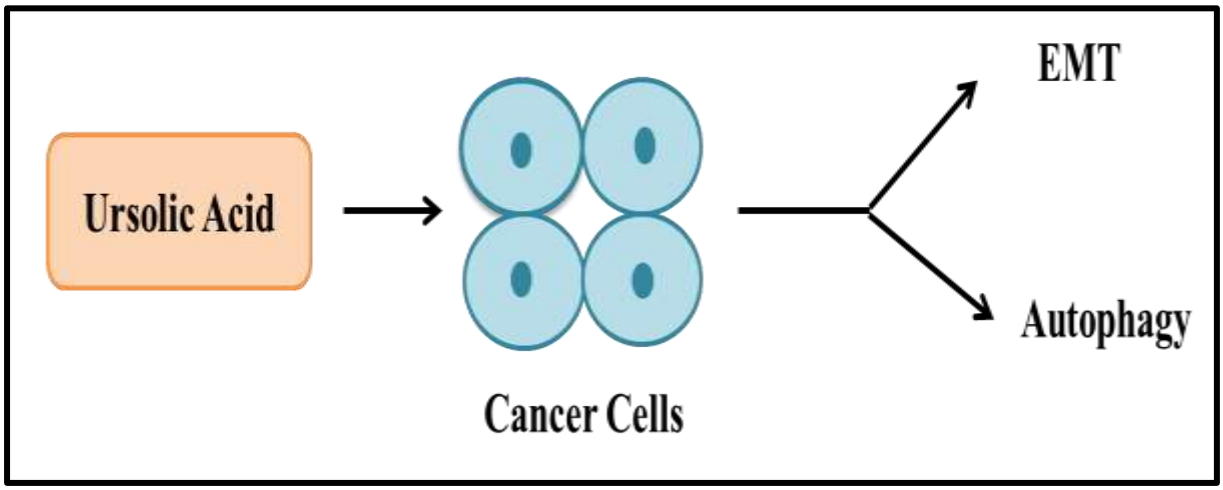



**Figure 2.4: Rationale of the study to check the role of Ursolic Acid on EMT and autophagy in lung cancer**

# 3 MATERIAL AND METHODS

## 3.1 *in-silico* analysis analysis of target proteins with Ursolic Acid and Positive control 5-FU.

### 3.1.1 Obtaining protein and ligand structure from databases

The 3-dimensional structure of *LC3* (PDB ID: 1UGM), *ATG5* (PDB ID: 4TQ1) *E-CADHERIN* (PDB ID: 6CXY) and *N-CADHERIN* (PDB ID: 1NCJ) were taken from Protein Data Bank. The structure of the ligand Ursolic Acid (CID: 64945) and the positive control 5-Fluorouracil (CID: 3385) were obtained from the PubChem database.

### 3.1.2 Ligand was docked to various proteins involved in lung cancer using 'AutoDock 1.4.5'

Swiss PDB Viewer is used for energy minimization of the ligand and receptor molecules. Gasteiger partial charges were added to the ligand atom. Non-polar hydrogen atoms were merged, and rotatable bonds were defined.

1. Docking calculation was carried out on the protein molecule. Essential hydrogen atoms, Kollman united atom type charges were added with the aid of Auto Dock tools. Prior to docking conserved water molecules were duly added to the binding pocket in ordered to mimic the *in vivo*environment.

2. Blind docking was performed as grid co-ordinates x, y and z are not known. Affinity (grid) maps of 126 x 126 x 126 Å grid points were generated with the help of Auto grid program aimed to target grid co-ordinates.

3. Auto Dock parameter set and the distance dependent dielectric functions were used in calculation of the Vander Waals and the electrostatic terms, respectively.

4. Docking simulation was performed using the "Lamarckian genetic algorithm" and the torsions of the ligand molecule was set randomly. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 2,500,000 energy evaluations. The population size was set to150.

5. The final figure was generated with the help of Discovery Studio Visualizer (Accelrys)4.0.

**3.2 Physicochemical property evaluation:**

Chemical structures and smiles notations of the synthetic compounds were obtained by PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Smiles notations of active compounds then fed in freely available online software Molinspiration chem. informatics to calculate many molecular Physiochemical properties.

**3.3 Toxicity risk prediction:**

Toxicity analysis was performed through online Data warrior software. Toxicity parameters, such as Tumorigenesis, Irritant, Reproductive Effect and Mutagenic were evaluated. Toxicity is important to evaluate in drug designing as it helps in determining the toxic dose in animal model studies and also lessens the number of animal model studies. It takes SDF file format of drugs for toxicity prediction (Sander *et al.,* 2015).

**3.4 Compounds Used:**

Ursolic Acid (RN 77-52-1-100mg) was purchased from Sigma Aldrich, and 5- fluorouracil (5-FU) from Fisher Scientific (A0305173). Ursolic Acid was dissolved in 100% DMSO and 5-FU was dissolved in nuclease-free water at 1 mM stock and stored as small aliquots at $-20^0$C. The drugs were diluted in the cell culture media immediately before use.

**3.5 Cell lines:**

The cell lines used in this study was adenocarcinomic human alveolar basal epithelial cells (A549). Cell line was procured from NCCS, Pune, India. When A549 cells are cultured *in-vitro*, they grow as monolayer cells, adherent to the flask.

**3.6 Growth conditions for A549 cell line**

The A549 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) at 37°C and 5% $CO_2$ and 95% humid atmosphere inside a $CO_2$ incubator.

**3.7 Media Preparation**

The media composition used for culturing of A549 cells was

1. DMEM/F-12 powder with 4mM L-glutamine and 4.5g/l glucose
2. Fetal Bovine Serum (FBS) (10% w/v)
3. Antibiotic-Antimycotic (1% w/v)
4. Sodium bicarbonate (2gm/l)

The final volume of the media was made 200ml using autoclaved distilled water. The media was filtered and stored at 4°C.

### 3.8 Sub-culturing

Cells should be passaged when the cells are 80%-90% confluent. The following steps were employed to passage the cells:

1. The exhausted media was discarded from the flask and the flask was washed by 1ml media.
2. The media used for washing was then discarded.
3. 1ml trypsin-EDTA was added to the flask to remove the cells from the adherent surface.
4. After the cells were detached from the flasks, 1ml media was added to neutralize the activity of trypsin. The liquid suspension obtained now contains the cells.
5. The suspension was pipetted out from the flask and transferred to 2ml centrifuge tube. The suspension was centrifuged at 1500rpm for 2 minutes and pellet was obtained.
6. The supernatant was removed from the centrifuge tube and the pellet was suspended in 1ml media.
7. Appropriate volume of cell suspension was aliquot to the freshly prepared T-25 flask containing 4ml fresh media.
8. The flasks were then kept in incubator at 37°C for the growth of the cells.

### 3.9 Cell counting using haemocytometer

The cell suspension was transferred to the edge of the haemocytometer and the suspension was allowed to spread evenly using capillary action. The counting was made for all the cells in 1mm center square and four 1mm corner squares of the haemocytometer. The circle indicates the approximate area covered at 100x microscope magnification (10x ocular and 10x objective). Top and left touching middle line cells were also counted whereas cells touching middle line at bottom and right were not counted.

Formula for the calculation of cell number is:

4n/4*10*1000*dilution=cells/ml

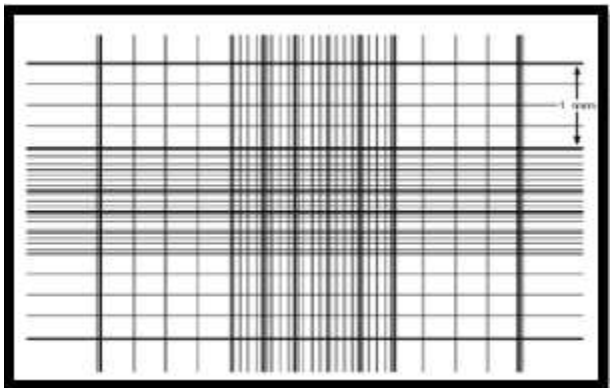


**Figure 3.1: Grids in haemocytometer**(incyto.com)

### 3.10 MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay to assess the effect of compounds on cell viability

MTT assay was used to check cell viability. It is a colorimetric assay to test the action of cellular enzymes which causes the reduction of tetrazolium salt i.e., MTT into purple colored formazan crystals. MTT was performed as described earlier by Hasan*et al.,* (2020). A vehicle control was set to check the effect of the 1% methanol on A549 cells. The viability of cells was determined as a percentage of the value in untreated control cells.

### 3.11 Mito Tracker Red staining to check the effect of compounds on mitochondrial membrane potential (MMP)

MMP was measured in treated and untreated cells after incubation with 300nM of Mito Tracker Red CMXRos fluorescent dye (Molecular Probes-M7512, Invitrogen) according to the manufacturer's protocol and as performed by Hasan*et al.,* (2020).

### 3.12 4, 6-diamidine-2-phenylindole (DAPI) staining to check the effect of compounds on DNA integrity

DAPI (Molecular Probes-D1306, Invitrogen) staining was performed in 5-FU and Ursolic Acid treated cells in a 24-well plate after staining with 300nM DAPI according to the manufacturer's protocol as performed by Hasan *et al.,* 2020.

### 3.13 Reverse transcriptase-polymerase chain reaction (RT-PCR) to check the effect of compounds on expression of selected genes

The relative expression of*LC-3, ATG-5, E-CADHERIN, N-CADHERIN, SNAIL, BCL-XL* In the presence and absence of compounds was determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Cells were grown in six-well plates and treated with the selected compounds. RNA was extracted using RNA extraction kit (Himedia, Cat-MB602) according to manufacturer's protocol. Extracted RNA was reverse transcribed using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences, cat- K1622) and was further used to analyze the expression of selected genes. Primer sequences used in the study are mentioned in Table 3.1. The expressions of genes were normalized using *GAPDH* as an internal control. PCR products were separated in a 1% agarose gel and visualized by ethidium bromide staining.

**Table 3.1: Details of primer sequence used in the study**

| **Gene** | **Primer (5'-3')** | **PCR Product Size (base pair)** | **Annealing Temp (°C)** |
|---|---|---|---|
| *LC3* | F- AAAGCTGTGGATGATCCACG<br>R- AGCAGGTGACAGGAACTCCT | 480bp | 52 |
| *ATG5* | F- AGTATCAGACACGATCATGG<br>R- TGCAAAGGCCTGACACTGGT | 500bp | 58 |
| *E-CADHERIN* | F- GGAACTGCAAAGCACCTGTG<br>R- ATTCCAGAAACGGAGGCCTG | 519bp | 57 |
| *N-CADHERIN* | F- CGTGTGAAGGTTTGCCAGTG<br>R- GTCCTGCTCACCACCACTAC | 561bp | 55.6 |
| *SNAIL* | F- GAATTCCCTCCTGAGTGCCC<br>R- GGAGACACATCGGTCAGACC | 561bp | 56.4 |
| *BCL-XL* | F-GGCTAGAGTCCTCACTCCCA<br>R-CCAAGGGTGGAGCAGAAGAG | 550bp | 53 |
| *GAPDH* | F- GATTTGGTCGTATTGGGCGC<br>R- AGTGATGGCATGGACTGTGG | 500bp | 55 |

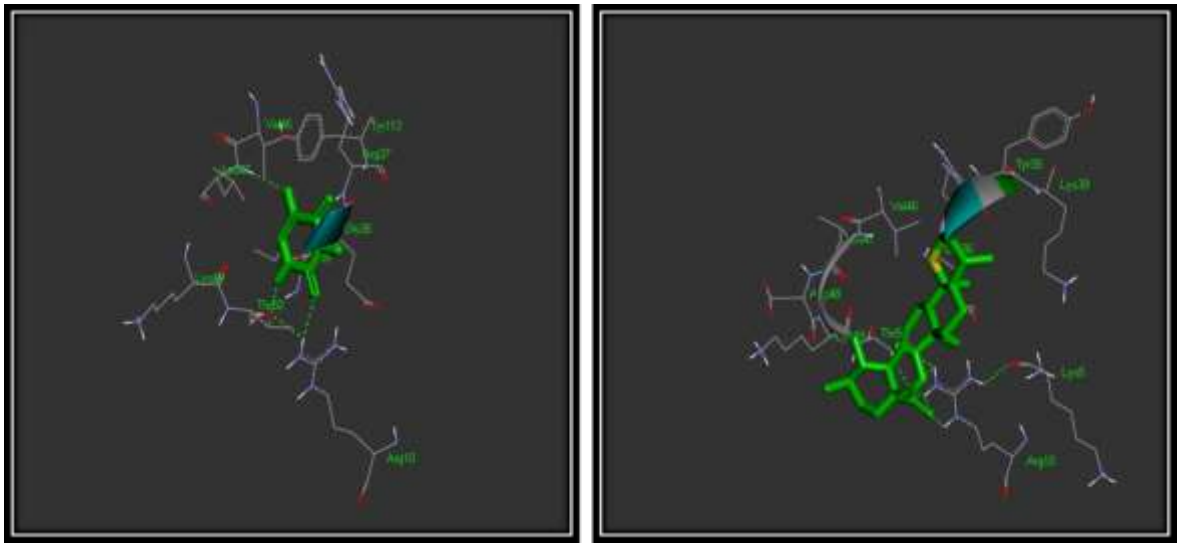
### 3.14 Statistical analysis

Statistical analysis was done by performing one-way ANOVA through Graph Pad prism 5. Data were presented as mean ± S.D of at least triplicate determinations. (Non-significant (ns), *p <0.05, ** p < 0.01, *** p< 0.001 versus untreated control).

# 4 RESULTS

## 4.1 *in-silico* analysis depicts interaction of 5-FU and Ursolic Acid with *LC3, ATG5, E-Cadherin* and *N-Cadherin*

In this study it has been observed that Ursolic Acid has good binding interaction with LC3, ATG5, E-Cadherin and N-Cadherin compared to the positive control 5-FU. The interacting amino acid residues found for LC3 and Ursolic Acid interaction are 11 namely Lys5, Arg10, Glu36, Arg37, Tyr38, Lys39, Val46, Leu47, Asp48, Lys49 , Thr50while the same for LC3 and 5-FU interaction are 9 namely Arg10, Ile35, Glu36, Arg37, Val46 , Leu47, Lys49, Thr50, Tyr113. The Gibbs free energy/binding energy (ΔG) and estimated inhibition constant (Ki) for LC3:Ursolic Acid interaction is-6.41kcal/mol and 20.17μMrespectively; however for LC3:5-FU interaction it is-3.72kcal/mol and 1880 μM respectively. (Figure 4.1 (a),Table 4.1)

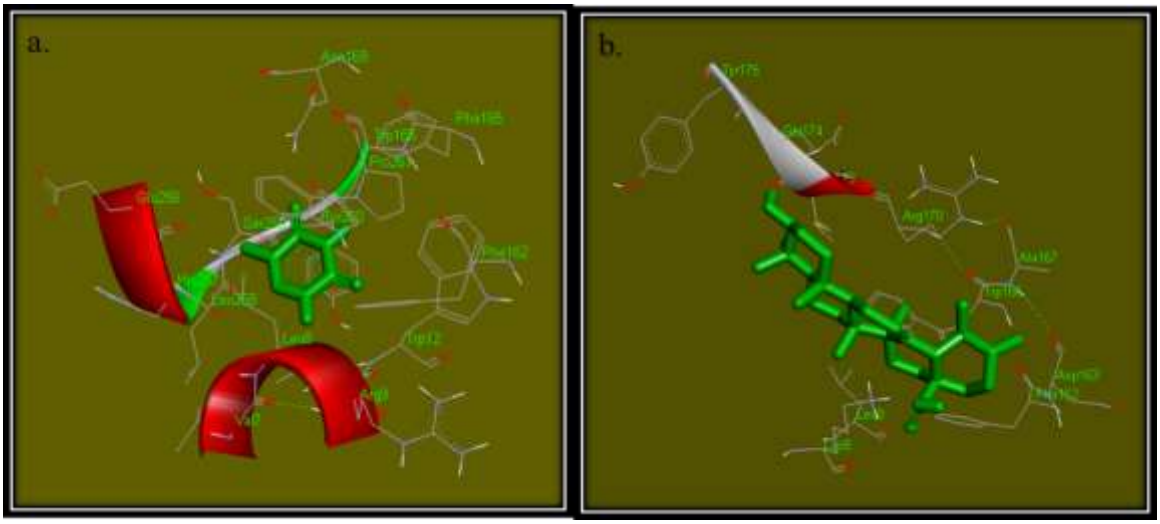


(a) (b)

**Figure 4.1(a):** Molecular docking interaction of LC3 with (a) 5-FU and (b) Ursolic Acid.

ATG5 interacts with both i.e. positive control 5-FU and Ursolic Acid where the best binding affinity is shown by Ursolic Acid. The binding energy (ΔG) and estimated inhibition constant (Ki) for ATG5:Ursolic Acid interaction is -9.44kcal/mol and0.12μMrespectively; while it is -4.70 kcal/mol and356.51μMrespectively for ATG5:5-FU interaction (Figure 4.1 (b), Table 4.1). The interacting amino acid residues found for ATG5 and Ursolic Acid interaction are10 namely Lys5, Leu8, Phe162, Asp163, Trp166, Ala167, Arg170, Met173, Glu174, Tyr175

while the same for ATG5 and 5-FU interaction are15 namely Val7, Leu8, Arg9, Val11, Trp12, Phe162, Phe165, Trp166, Asn169, Glu256, His257, Leu258, Ser259, Tyr260, Pro261.




(a) (b)

**Figure 4.1(b):** Molecular docking interaction of ATG5 with (a) 5-FU and (b) Ursolic Acid.

Epithelial surface marker gene E-Cadherin shows good binding interaction with 5-FU and Ursolic Acid. The results of E-Cadherin:Ursolic Acid interaction gave -7.01 kcal/mol binding energy and 7.29 μM inhibition constant, however for E-Cadherin:5-FU interaction values for the same are -3.26 kcal/mol and 4100μM respectively, which shows that Ursolic Acid has more affinity towards E-Cadherin than 5-FU (Figure 4.1 (c), Table 4.1). The interacting amino acid residues found for E-Cadherin and Ursolic Acid interaction are 4 namely Thr141,Tyr142,Asn143,Leu196while the same for E-Cadherin and 5-FU interaction are 8 namely Thr133, Asp134,Ala132, Ala144,Ala145, Ile146,Tyr148, Asn168.

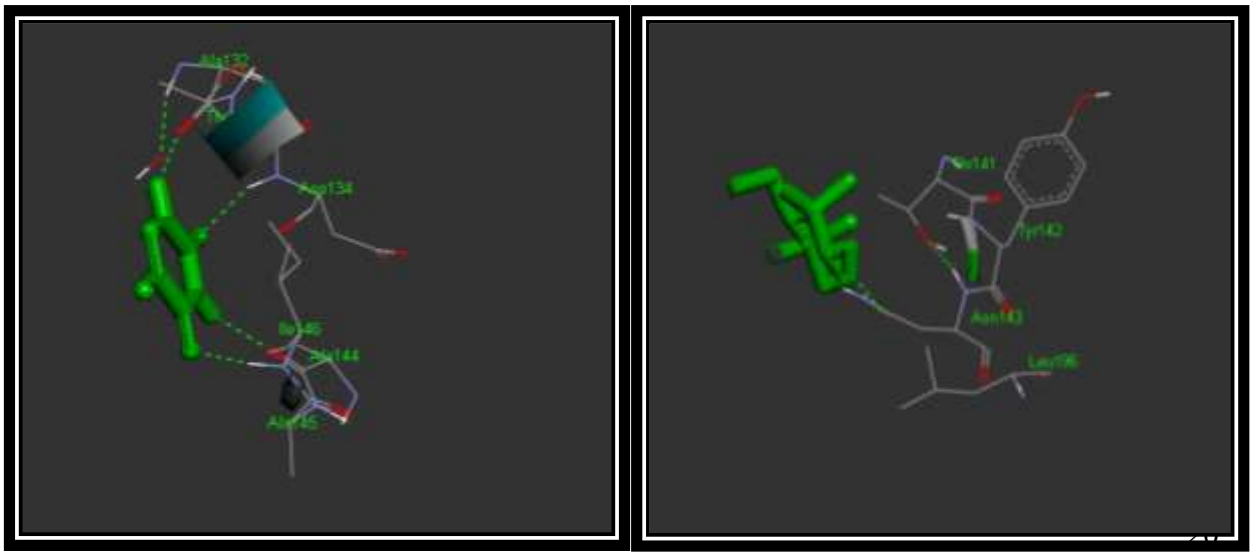
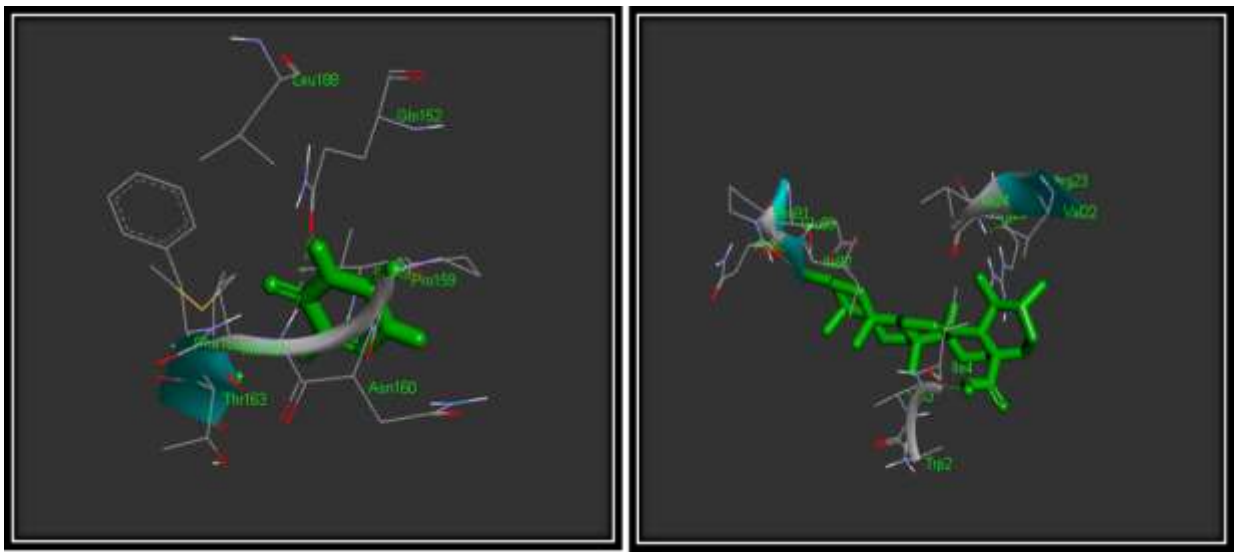


**Figure 4.1(c):** Molecular docking interaction of E-Cadherin with (a) 5-FU and (b) Ursolic Acid.

The Gibbs free energy/binding energy (ΔG) and estimated inhibition constant (Ki) for N-Cadherin:Ursolic Acid interaction is-5.88 kcal/mol and 49.12 μMrespectively; however for N-Cadherin:5-FU interaction it is-3.13 kcal/mol and 5040μM respectively. (Figure 4.1 (d),Table 4.1). The interacting amino acid residues found for N-Cadherin and Ursolic Acid interaction are 11 namely Trp2, Val3, Ile4, Val22,Arg23, Ile24,Arg25, Glu89,Asn90, Pro91,Ile92while the same for interaction with 5-FU are 9 namely Ile149, Gln152,Pro159, Asn160,Met161, Phe162,Thr163, ,Ile164,Leu188.




(a) (b)

**Figure 4.1(d):** Molecular docking interaction of N-Cadherin with (a) 5-FU and (b) Ursolic Acid.

**Table 4.1:** Binding energy along with inhibition constant and interacting amino acids of LC3,ATG5, E-Cadherin, N-Cadherinwith 5-FU and Ursolic Acid

| Target | Drug Name | Binding energy (ΔG) (Kcal/mol) | Inhibition Constant (Ki)(μM) | Interacting amino acids | No. of intermolecular H-bonds | Interacting amino acids with intermolecular H-bond |
|---|---|---|---|---|---|---|
| **LC3** | 5-FU | -3.72 | 1880 | Arg10, Ile35, Glu36, Arg37, Val46 , Leu47, Lys49, Thr50, | 8 | A:THR6:HG1 - A:GLU36:OE1 A:PHE7:HN - A:GLU36:OE1 A:ARG10:HH21 - A:THR50:OG1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | Tyr113 | | A:ARG10:HH21 - :UNK0:O3<br>A:ARG37:HN - :UNK0:O2<br>A:LEU47:HN - :UNK0:F1<br>A:TYR113:HH - A:PRO45:O<br>:UNK0:H11 - A:THR50:OG1 |
| | Ursolic Acid | -6.41 | 20.17 | Lys5, Arg10, Glu36, Arg37, Tyr38, Lys39, Val46, Leu47, Asp48, Lys49 , Thr50 | 10 | A:ARG10:HE - :UNK0:O3<br>A:ARG10:HH12 - A:LYS5:O<br>A:ARG10:HH21 - A:THR50:OG1<br>A:ARG10:HH21 - :UNK0:O2<br>A:ARG10:HH22 - :UNK0:O3<br>A:ARG10:HH22 - :UNK0:O2<br>A:ARG37:HH21 - :UNK0:O1<br>A:ARG37:HH22 - :UNK0:O1<br>A:GLU41:HN - A:TYR38:O<br>A:THR50:HG1 - :UNK0:O2 |
| **ATG5** | 5-FU | -4.70 | 356.51 | Val7, Leu8, Arg9, Val11, Trp12, Phe162, Phe165, Trp166, Asn169, Glu256, His257, Leu258, Ser259, Tyr260, Pro261 | 6 | A:VAL11:HN - A:VAL7:O,<br>A:TRP12:HN - A:LEU8:O,<br>A:TRP166:HE1 - :UNK0:O3,<br>A:LEU258:HN - A:LEU254:O,<br>A:ASN263:HN - A:TYR260:O,<br>:UNK0:H11 - A:LEU8:O |
| | Ursolic Acid | -9.44 | 0.12 | Lys5, Leu8, Phe162, Asp163, Trp166, Ala167, Arg170, Met173, Glu174, Tyr175 | 3 | A:ALA167:HN - A:ASP163:O<br>A:ARG170:HN - A:TRP166:O<br>A:ARG170:HE - A:ALA167:O |
| **E-Cadherin** | 5-FU | -3.26 | 4100 | Thr133, Asp134, Ala132, Ala144, Ala145, Ile146, Tyr148, Asn168 | 5 | C:ALA132:HN - C:TYR148:OH<br>C:ASP134:HN - :UNK0:O3<br>C:ILE146:HN - :UNK0:O2<br>:UNK0:H10 - C:ALA144:O<br>:UNK0:H11 - C:ALA132:O |
| | Ursolic Acid | -7.01 | 7.29 | Thr141, Tyr142, Asn143, Leu196 | 3 | C:ASN143:HN - C:THR141:OG1<br>C:ASN143:HD22 - C:ASP100:OD2<br>:UNK0:H77 - C:ASN143:OD1 |
| **N-Cadherin** | 5-FU | -3.13 | 5040 | Ile149, Gln152, Pro159, Asn160, Met161, Phe162, Thr163, ,Ile164, Leu188 | 4 | A:ASN160:HN - :UNK0:O3<br>A:MET161:HN - A:GLN152:OE1<br>A:THR163:HN - A:ILE172:O<br>:UNK0:H10 - A:GLN152:OE1 |
| | Ursolic Acid | -5.88 | 49.12 | Trp2, Val3, Ile4, Val22, Arg23, Ile24, Arg25, Glu89, Asn90, Pro91, Ile92 | 5 | A:ARG25:HN - A:ARG23:O<br>A:ARG28:HN - A:GLU89:OE2<br>A:ILE92:HN - A:ALA78:O<br>:UNK0:H81 - A:VAL3:O<br>:UNK0:H77 - A:ASN90:O |

**4.2 Physicochemical properties of Ursolic Acid and 5-FU suggests that Ursolic Acidshows one violationof Lipinski rule of five when compared to 5-FU**

Physicochemical properties of Ursolic Acid and 5-FU were evaluated by Molinspiration Online Calculator and are shown in table 4.2. It includes molecular descriptors of specific values, which shows whether the drug violates or not and are based on Lipinski rule.

The rules are as follows-

- H bond donor should be less than 5
- H bond acceptor should be less than 10
- No. of rotable bond should be less than 10
- Molecular weight should be less than 500KDa
- mi log P should be less than 5

**Table 4.2: The physicochemical properties of selected compounds as calculated by Molinspiration Online Calculator**

| S.N. | Compound | Mi LogP | TPSA | natoms | Mol Wt | nON | nOHNH | nviolations | nrotb | % absorption |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. | Positive Control (5-FU) | -0.59 | 65.72 | 9 | 130.08 | 4 | 2 | 0 | 0 | 86.326 |
| 2. | Ursolic Acid | 6.79 | 57.53 | 33 | 456.71 | 3 | 2 | 1 | 1 | 89.152 |

- Percentage of Absorption (% of Absorption) was calculated by: % of Absorption = 109 – (0.345 × Topological Polar Surface Area)
- Mi Log P is Logarithm of compound partition coefficient between *n*-octanol and water

### 4.3 Toxicity risk prediction of 5-FU and Ursolic Acid suggests that Ursolic Acid is not toxic when compared to 5-FU

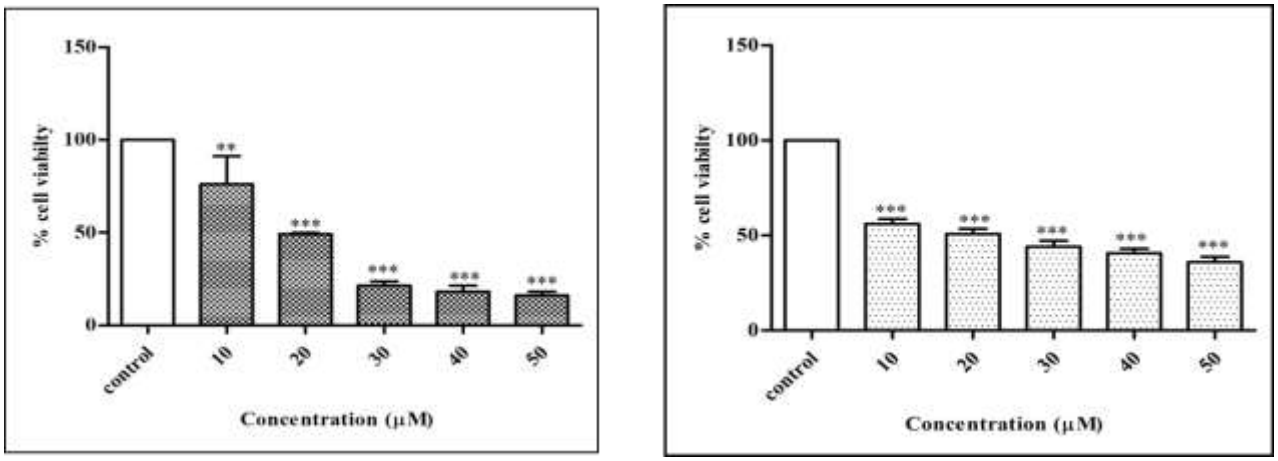
Toxicity of Ursolic Acid and 5-FU was calculated by Data Warrior Tool. It is an important data analysis tool for drug discovery to analyze any drugs mutagenecity, tumorogenicity, its irritant nature and reproductive effect and is a vital backbone for research process. Ursolic Acid shows no toxicity while 5-FU has toxic effects against all the parameters as shown in table 4.3.

**Table 4.3: Toxicity risk factors of the selected compounds**

| S.N. | Compound | Mutagenic | Tumorigenic | Reproductive effect | Irritant |
|---|---|---|---|---|---|
| 1. | Positive Control (5-FU) | high | high | high | high |
| 2. | Ursolic Acid | none | none | none | none |

### 4.4 Cytotoxic activity of Ursolic Acid and 5-FU on A549 cells

To evaluate the cytotoxic potential of Ursolic Acid and 5-FU, human lung cancer cell line (A549) were treated with varying concentrations of compound ranging from and 10μM to 50μM in case of both Ursolic Acid and 5-FU for 24h, and the cell viability was determined by MTT assay.




**(a)** **(b)**

**Figure 4.1:**MTT assay showed inhibition of A549 lung cancer cells by (a) Ursolic Acid and (b) 5-Fluorouracil (5-FU) after 24 h of treatment (***p< 0.0001, **p < 0.001, *p < 0.05, Nonsignificant (ns) versus untreated control).

As shown in Figure 4.1, Ursolic Acid treatment reduced the cell viability in A549 cell line. Ursolic Acid showed 49% decrease in cell viability at 20 µM concentration, indicating the $IC_{50}$ value of Ursolic Acid. For 5-FU, 50% cell viability was obtained at 20 µM indicating the $IC_{50}$ value of 5-FU.

### 4.5 Ursolic Acid causes more oxidative stress in A549 cells than 5-FU

Since the treatment with Ursolic Acid caused significant cell death in A549 cells, we sought to investigate whether the cytotoxicity of Ursolic Acid is result of the generation of reactive oxygen species (ROS). The level of ROS at different concentrations of Ursolic Acid and 5-FU after 24h of treatment was detected by DCFDAstaining.

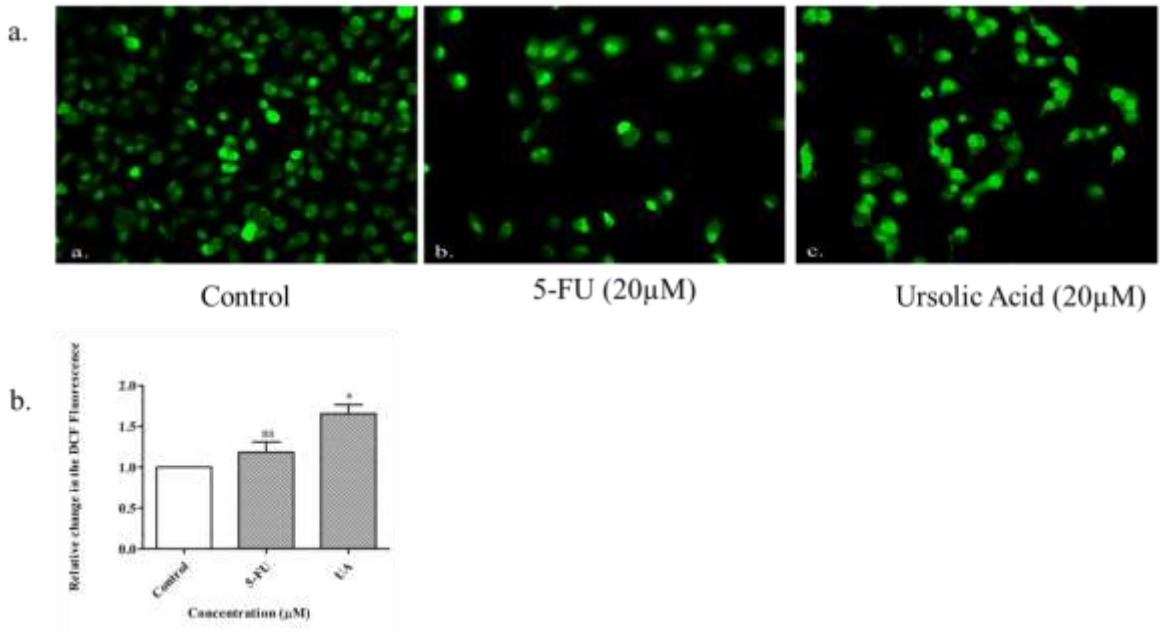



**Figure 4.2** (a) Fluorescence micrographs of ROS generation after treatment with 5-FU and Ursolic Acid for 24h obtained in A549 lung cancer cells. (b) Graphical representation of fluorescence intensity of DCF-DA staining in the A549 cells as quantified using Image J software. (***p< 0.0001, **p < 0.001, *p < 0.05, Nonsignificant (ns) versus untreated control). Key: UA- Ursolic Acid.

We quantified the images for fluorescence intensity of ROS generation using Image J software. Quantification of fluorescence intensity of DCF-DA staining also showed that Ursolic Acid treated A549 cells produced comparatively increased levels of ROS, increase being 1.65 fold at 20µM as compared to untreated control cells. In 5-FU treated cells, the increase in fluorescence intensity of ROS was found to be 1.18 fold at 20µM as compared to untreated control. This result suggested that the growth inhibition observed in the A549 cells, in response to Ursolic Acid and 5-FU is may be due to oxidative stress generated by ROS.

### 4.6 Ursolic Acid initiates apoptosis by reducing mitochondrial membrane potential (ΔΨ) in A549 cells

Mitochondria playa an important role in controlling cell survival and death so; we checked effect of Ursolic Acid and 5-FU on mitochondrial membrane potential in A549 cells by Mito Tracker Red staining. We observed that ΔΨ decreased to a greater extent in A549 cells after 24h treatment with Ursolic Acid. More MMP loss was observed in Ursolic Acid treated cells than compared to 5-FU treated cells. The fluorescence images of Mito Tracker Red staining in A549 cells were further quantified using Image J software.

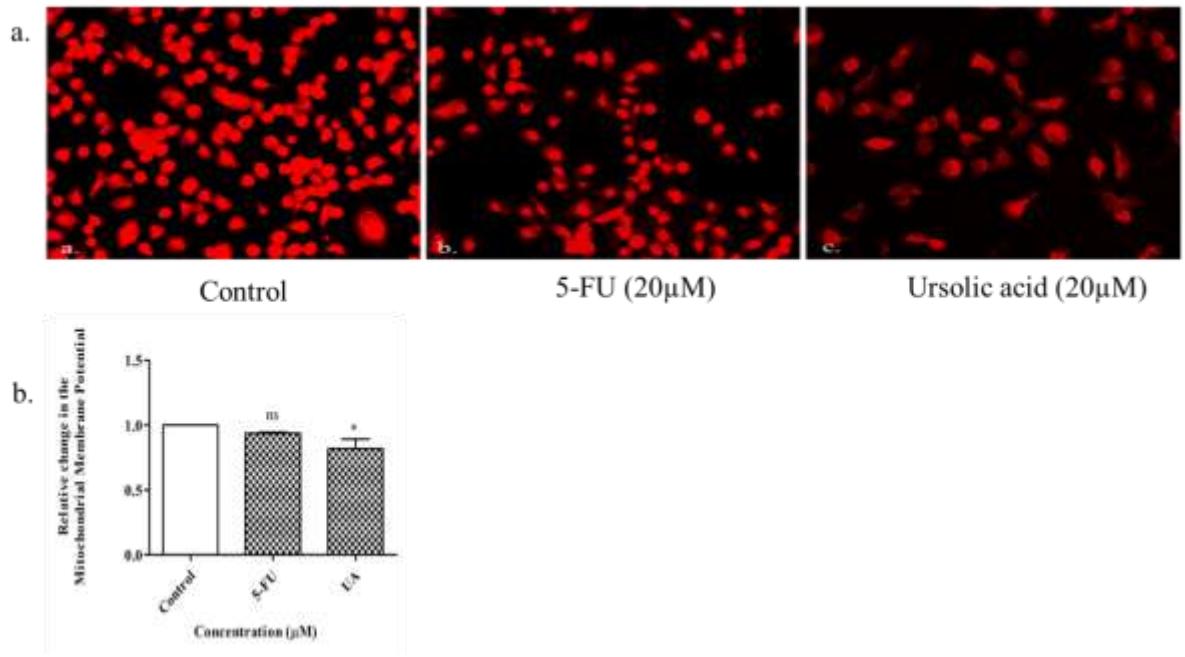



**Figure 4.3** (a) Change in level of mitochondrial membrane potential in A549 cells was measured using Mito Tracker Red staining. (b) Graphical representation of fluorescence intensity of Mito Tracker Red staining in the A549 cells as quantified using Image J software. (***p< 0.0001, **p < 0.001, *p < 0.05, Nonsignificant (ns) versus untreated control).Key: UA- Ursolic Acid.

As observed in Figure 4.3 quantification of fluorescence intensity of Mito Tracker Red staining showed that Ursolic Acid treatment at 20µMshowed 0.936 fold reduction in ΔΨ respectively whereas 5-FU treatment at 20µMshowed 0.818 fold reduction respectively as compared to untreated control. The quantified data also indicates that Ursolic Acid treated A549 cells showed more loss of mitochondrial membrane potential as compared to 5-FU.

### 4.7 Ursolic Acid induces more chromatin condensation in A549 cells compared to 5-FU

We assessed the hallmarks of apoptosis-like chromatin alteration by DAPI staining. A549 cells were treated with the compounds (Ursolic Acid and 5-FU) for 24h and analyzed to check the effect of compound on the DNA integrity. The fluorescence images of DAPI staining showed chromatin condensation in treated cells as compared to control. However, treatment with Ursolic Acid caused more chromatin condensation as compared to 5-FU. There was 1.76 fold increase in fluorescence intensity as a result of chromatin condensation in Ursolic Acid treated cells at the concentration of 20µM as compared to untreated control.

5-FU treated cells exhibited 1.19 fold increase in fluorescence intensity at the concentration of 20µM due to chromatin condensation in A549 cells as compared to untreated control.

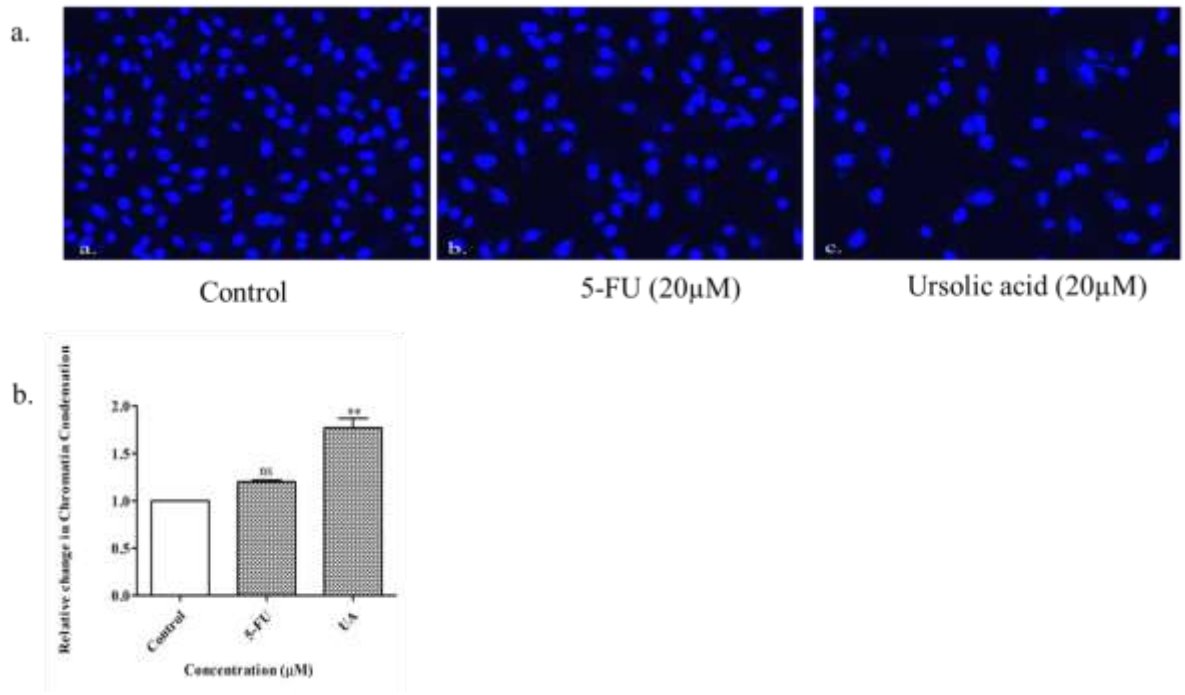



**Figure 4.4:** (a) Effect of 5-FU and Ursolic Acid on chromatin condensation in A549 cells was explored by DAPI staining. (b) Graphical representation of fluorescence intensity of DAPI staining in the A549 cells as quantified using Image J software. (∗∗∗p< 0.0001, ∗∗p < 0.001, ∗p < 0.05, Nonsignificant (ns) versus untreated control). Key: UA- Ursolic Acid.

### 4.8 Ursolic Acid modulates the expression of *LC3, ATG5, E-CADHERIN, N-CADHERIN, SNAIL* and *BCL-XL* in A549 cells

The effect of Ursolic Acid and 5-FU on the mRNA expression of selected marker genes was checked by semi-quantitative RT-PCR.

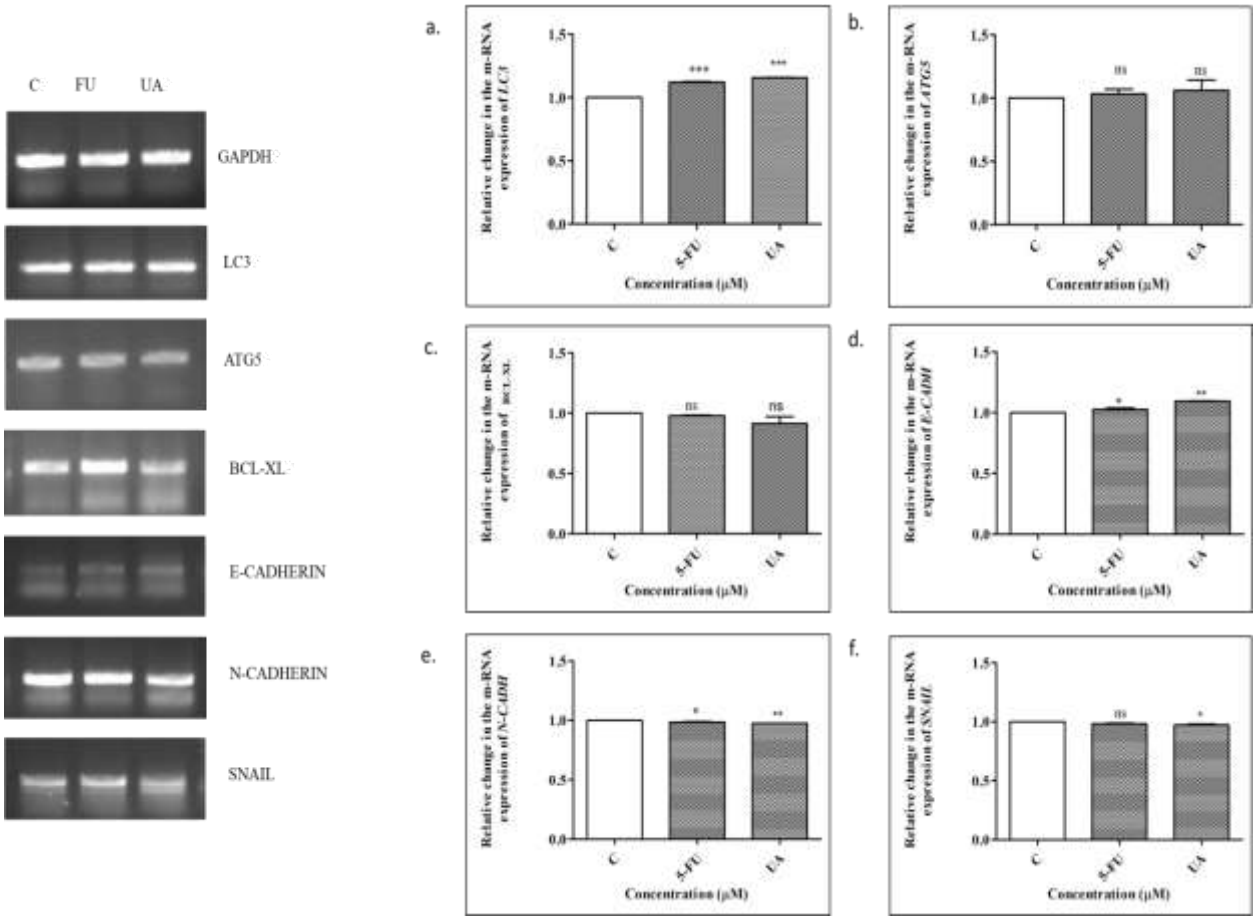



**Figure 4.6:** (a) mRNA expression of selected genes after treatment with 5-FU and Ursolic Acid compared to untreated control in A549 cells. Relative change in fold of *LC3, ATG5, E-CADHERIN, N-CADHERIN, SNAIL* and *BCL-XL*. (∗∗∗p< 0.0001, ∗∗p < 0.001, ∗p < 0.05, Nonsignificant (ns) versus untreated control.)

It is clear from the mRNA expression analysis that Ursolic Acid upregulates*LC3* and non-significantly affects *ATG5* expression in A549 cells which indicates that it may have role in the induction of autophagy. Further, the mRNA expression of *BCL-XL*, an anti-apoptotic marker gene was also checked to analyze the effect of the compound on apoptotic pathway. We observed that Ursolic Acid slightly downregulated the expression of *BCL-XL* hence sensitizing the cells towards apoptosis. Besides this, it has also influenced the expression of EMT markers i.e. *E-CADHERIN, N-CADHERIN* and *SNAIL*. It can be concluded from mRNA results that Ursolic Acid upregulates the expression of epithelial surface marker gene *E-CADHERIN* while it downregulated the expression of mesenchymal marker *SNAIL*. However, non-significant effect was observed on *N-CADHERIN* expression.

**Table 4.4:**Fold change in the expression of targeted genes as observed in 5-FU and Ursolic Acid treated A549 cells

| S.No. | Target | Fold change in the expression of targeted genes after treatment with 5-FU compared to untreated cells | Fold change in the expression of targeted genes after treatment with Ursolic Acid compared to untreated cells |
|---|---|---|---|
| 1 | *LC3* | 1.12 ↑ | 1.56 ↑ |
| 2 | *ATG5* | 1.03 ns | 1.06 ns |
| 3 | *BCL-XL* | 0.98 ns | 0.91 ns |
| 4 | *E-CADHERIN* | 1.02 ↑ | 1.09 ↕ |
| 5 | *N-CADHERIN* | 0.98 ns | 0.97 ↕ |
| 6 | *SNAIL* | 0.98 ns | 0.97 ↓ |

# 5 DISCUSSION

In this study, we tried to evaluate the effect of Ursolic Acid on the expression on autophagy and EMT marker genes in lung cancer cells.

According to the *in silico* analysis it can be said that Ursolic Acid has shown good interaction towards autophagy and EMT marker genes compared to the positive control 5-FU. Besides, Ursolic Acid is a druggable compound and less toxic than 5-FU according to different parameters.

As per the results of MTT analysis both Ursolic Acid and 5-FU exert cytotoxic effects on A549 cells at 20μM concentration.

In order to elucidate the reason behind the cytotoxicity of Ursolic Acid we further checked the amount of ROS generated in the presence of Ursolic Acid to confirm the ROS mediated toxicity. The level of ROS was detected by DCF-DA (2, 7-dichlorodihydrofluorescein diacetate) stainingat $IC_{50}$ dose of Ursolic Acidand 5-FU after 24h of treatments. ROS levels increased substantially in the presence of both the compounds. Nevertheless, level of ROS generation was higher in A549 cells treated with Ursolic Acid than 5-FU. ROS is a potent regulator of both apoptosis and autophagy. ROS plays a critical role in the cannabidiol (CBD) induced apoptosis and autophagy of breast cancer cells as restricted ROS levels with alpha-tocopherol (α-TOC) decreased the CBD induced expression of apoptosis and autophagy marker proteins (Shrivastava E et. al., 2011).

An important hallmark of apoptosis is mitochondrial membrane damage whichin turn causes the release of pro-apoptotic proteins and ultimately results in cellular apoptosis (Ghelli A *et. al*,.2003). It is noticeably clear from our study that Ursolic Acid causes greater loss of mitochondrial membrane potential in A549 cells than the standard anticancer drug 5-FU.

Moreover, any damage to the DNA integrity is also one of the common hallmarks of apoptosis (McCarthy & Evan, 1998). It has been observed that Ursolic Acid causes higher chromatin condensation in A549 cells than 5-FU.

It has been reported in literatures that autophagy has dual role in cancer and also generates resistance towards anticancer drugs (S. Chen *et al*., 2010) leading to the inhibition of apoptosis. Thus, in this study, we tried to evaluate the effect of the compounds on autophagy as well as apoptosis marker genes. As per the mRNA results, Ursolic Acid increases the expression of key marker of autophagy i.e. *LC3* indicating that it causes autophagy induction in lung cancer cells. Ursolic Acid downregulates *BCL-XL* which denotes its significance

inapoptotic pathway as well. Collectively, it can be said that Ursolic Acid exposure activates pro-death role of autophagy leading to cell death through apoptosis.

Moreover, Ursolic Acid also affects the EMT marker genes. As reported in literatures that EMT induces aggressiveness and stemness in cancer cells and generates resistance towards cancer therapies (Pérez-Pomares *et al.,* 2002), thus we ought to find out the effect of Ursolic Acid on EMT marker genes. Ursolic Acid treatment results in the upregulation of epithelial surface marker (*E-CADHERIN*) while it non-significantly affects mesenchymal surface marker gene (*SNAIL*) which indicates that it may inhibit the EMT in lung cancer cells.

# 6 CONCLUSION

Chemotherapeutic resistance and cancer relapse are the major problems faced in the treatment of cancer. Moreover, various pathways like autophagy and EMT play crucial role in the survival and aggressiveness of cancer cells. Off late, cancer therapies focus towards the prevention of disease recurrence by targeting the genes responsible for invasive potential in cancer. Thus, in our study we tried to evaluate the effect of Ursolic Acid on autophagy as well as EMT marker genes. We observed that Ursolic Acidupregulated ROS levels and inhibited mitochondrial membrane potential. Ursolic Acid also promoted DNA fragmentation. Further, at mRNA level, the expression of *LC3* and *E-CADHERIN* was upregulated and expression of *SNAIL* was downregulated. However, no effect was observed on the expression of *N-CADHERIN*and *BCL-XL*. Consequently, it indicates that Ursolic Acidaffects autophagy and EMT however further studies are required to completely understand its mechanism of action on epithelial to mesenchymal transition.

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