EVALUATION OF SOME FRUITS FROM JAMMU SUBTROPICS: POSSIBLE ANTICANCER ROLE USING *IN VITRO* CYTOTOXICITY ASSAYS

By

Navneet Kour

(J-15-D-07-BS)

Thesis submitted to Faculty of Basic Sciences
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IN

BIOCHEMISTRY

Division of Biochemistry, Faculty of Basic Sciences
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Main Campus Chatha, Jammu-180009

2020

CERTIFICATE-I

This is to certify that the thesis entitled **"Evaluation of some fruits from Jammu subtropics: Possible anticancer role using *in vitro* cytotoxicity assays"** submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biochemistry** to the Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu is a record of bonafide research work, carried out by **Ms. Navneet Kour,** Registration No. **J-15-D-07-BS** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that such help and assistance received during the course of investigation have been duly acknowledged.

Dr. Vikas Sharma 31/01/20
(Major Advisor)

Place: Jammu
Date: 31/01/2020

Endorsed:

Head
Division of Biochemistry, FBSc.
SKUAST-Jammu, Main Campus Chatha

Date: 31.1.20

CERTIFICATE-II

We, the members of the Advisory Committee of **Ms. Navneet Kour,** Registration No. **J-15-D-07-BS,** a candidate for the degree of **Doctor of Philosophy in Biochemistry,** have gone through the manuscript of the thesis entitled **"Evaluation of some fruits from Jammu subtropics: Possible anticancer role using *in vitro* cytotoxicity assays"** and recommend that it may be submitted by the student in partial fulfillment of the requirements for the degree.

Dr. Vikas Sharma 31/01/2020

Assistant Professor, Biochemistry
Major Advisor & Chairman
Advisory Committee

Place: Jammu
Date: 31/01/2020

Advisory Committee Members:

Dr. Moni Gupta
Associate Professor, Biochemistry
Division of Biochemistry
31/01/2020

Dr. Sachin Gupta
Associate Professor, Plant Pathology
Division of Plant Pathology
31/01/2020

Dr. G.K. Rai
Assistant Professor, Biotechnology
School of Biotechnology
31/01/2020

Dr. Manish K Sharma
Professor & Head
Division of Statistics & Computer Sciences
(Dean's Nominee)
31.01.2020

CERTIFICATE – III

This is to certify that the thesis entitled "**Evaluation of some fruits from Jammu subtropics: Possible anticancer role using *in vitro* cytotoxicity assays**" submitted by **Ms. Navneet Kour,** Registration Number **J-15-D-07-BS** to the Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biochemistry** was examined and approved by the Advisory Committee and External Examiner (s) on ....13.07.2020....

Dwijendra Gupta 13/07/2020

(Dr. Dwijendra K. Gupta)
Professor & Head
Department of Biochemistry
Central University of Allahabad,
Allahabad-211002, UP
External Examiner

Dr. Vikas Sharma
Assistant Professor (Biochemistry)
Major Advisor

Vikas Sharma 19/10/20

Dr. Sanjay Guleria
Professor & Head
Division of Biochemistry

19/10/20

Dean, FBSc.
SKUAST-Jammu

19/10/2020

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Navneet Kour

Ms. Navneet Kour

Place: JAMMU
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Name of the student	**:**	**Navneet Kour**
Registration No.J-15-D-07-BS		
Major Subject	**:**	**Biochemistry**
Name and Designation of Major Advisor	**:**	**Dr. Vikas Sharma** Assistant Professor (Biochemistry)
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ABSTRACT

Cancer is one of the most life threatening diseases, which represents a substantial burden in the community and appears to be a prime cause of concern. Multidisciplinary scientific investigations are making best efforts to combat the disease, but the sure-shot, perfect cure is yet to be brought into the world of medicine. In the search of potential anticancer agents from fruits, the present research work was carried out to examine the *in vitro* anticancer potential of four fruits namely *Artocarpus heterophyllus* (katahal), *Carissa carandas* (karonda), *Grewia asiatica* (phalsa), *Syzygium cumini* (jamun). Systematic bioassays were performed on eight human cancer cell lines from five different origins such as MCF-7 and MDAMB-231 (breast), HCT-116, HT-29 and SW-620 (colon), A-549 (lung), MIA PaCa-2 (pancreatic), PC-3 (prostate). Methanolic extracts of all the above mentioned fruits were prepared and used as test material. The fractions were prepared from extracts and compounds were isolated from particular active fractions. The anticancer activity was determined by the cytotoxic potential of the test material. Cells were allowed to grow for 24 h on 96 – well flat bottom tissue culture plates. Cells were further allowed to grow in the presence of test material for 48 h. Cell growth was terminated by addition of 50% (w/v) TCA. Cells were stained with SRB dye. Excess dye was removed by washing with 1% (v/v) acetic acid and bound dye was dissolved in Tris buffer and OD was taken at 540 nm. In the primary phase of investigation, *S. cumini* was found to be active against three human cancer cell lines (A-549, HCT-116, PC-3) and the growth inhibition range was 70-74% whereas *C. carandas* suppressed the cell proliferation of MCF-7, HT-29, A-549 cancer cells by 70%, 72%, 75% respectively. The other two fruits namely phalsa and katahal exhibited <70% growth inhibition against all the human cancer cell lines. Further, fractions (n-hexane, chloroform, butanol) of *S. cumini* and *C. carandas* were tested at the conc. of 100, 50, 30, 10 and 1 µg/mL against human cancer cell lines. In *S. cumini*, IC_{50} values of n-hexane fraction calculated were 26.81±0.77 µg/mL (SW-620), 29.26±0.97 µg/mL (MIA PaCa-2), 35.16±1.46 µg/mL (A-549), 36.25±0.77 µg/mL (HCT-116) and >50 µg/mL (MDAMB-231, HT-29, PC-3). IC_{50} values obtained for chloroform fraction were 18.02±1.01 µg/mL (MDAMB-231), 40.02±1.94 µg/mL (A-549), 43.10±0.26 µg/mL

(MDAMB-231, HT-29, PC-3). IC_{50} values obtained for chloroform fraction were 18.02±1.01 µg/mL (MDAMB-231), 40.02±1.94 µg/mL (A-549), 43.10±0.26 µg/mL (MIA PaCa-2), 48.30±3.42 µg/mL (HCT-116) and >50±0.28 µg/mL (SW-620). In *C. carandas*, chloroform fraction exhibited maximum percentage of growth inhibition with less IC_{50} values of 1.28±0.02 µg/mL (HT-29), 1.48±0.002 µg/mL (A-549) and 3.98±0.24 µg/mL (MCF-7). In the secondary phase, isolation of bioactive compounds was carried out from n-hexane/chloroform fractions of *S. cumini* and chloroform fraction of *C. carandas*. The characterization was done by NMR (^{1}H and ^{13}C) and mass spectrometry. A compound namely β-sitosterol was isolated from n-hexane fraction of *S. cumini*, displayed activity against SW-620 (colon) human cancer cell line with IC_{50} value of 42.46±2.30 µM. Further, gallic acid, oleanolic acid, quercetin were isolated from chloroform fraction of *S. cumini* and maximum growth inhibition against more number of human cancer cell lines was observed in case of quercetin with IC_{50} values of 17.37±2.99 µM (HCT-116), 25.36±1.98 µM (A-549), 35.06±1.37 µM (MIA PaCa-2) and 36.18±1.85 µM (SW-620) followed by oleanolic acid and gallic acid. In case of *C. carandas,* a compound namely carissic acid was isolated from the chloroform fraction and well characterised using detailed NMR studies for the first time from any plant source, that showed significant activity with IC_{50} values of 2.65±0.35 µM (HT-29), 3.47±0.26 µM (A-549), 10.47±0.69 µM (MIA PaCa-2) and 13.58±0.59 µM (MCF-7). In the final phase of research work, a couple of mechanistic assays of carissic acid were performed on lung (A-549) cancer cell line where apoptosis was detected using DAPI staining, mitochondrial membrane potential, reactive oxygen species, scanning electron microscopy, scratch and colonogenic assay. Moreover, western blotting was performed to confirm the basis of apoptosis. The results suggest that carissic acid exhibits significant anti tumor effects by suppressing cell proliferation and promoting apoptosis. The activation of mitochondrial mediated apoptosis pathway is one of the possible mechanisms responsible for the above mentioned effects. To conclude, carissic acid may act as potent anticancer agent for treating lung cancer.

Keywords: Jamun, Katahal, Karonda, Phalsa, Anticancer, Apoptosis, Carissic acid

Signature of the Major Advisor

Signature of the Student

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LIST OF ABBREVATIONS

%	Per cent
°C	Degree celsius
µg	Microgram
µ	Micron
w/v	Weight/volume
v/v	Volume/volume
µl	Microlitre
ACHR	Advanced Centre for Horticulture Research
ACN	Acetonitrile
APS	Ammonium persulfate
BSA	Bovine serum albumin
CA	Carissic acid
CB	Control blank
CC	Column chromatography
CD_3OD	Deutriated methanol
$CDCl_3$	Deutriated chloroform
CGM	Complete growth medium
Conc.	Concentration
CT	Test control
DAPI	4,6- diamidino-2-phenylindole
DCFDA	2,7-dichlorofluorescein diacetate
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
DW	Distilled water
ECL	Enhanced chemiluminiscence
EDTA	Ethylene diamine tetra acetic acid
et. al	et alia = and other
EtoAc/hex.	Ethyl acetate/hexane
FBS	Fetal bovine serum
g	Gram
GC	Control growth
GI	Growth Inhibition
GT	Test growth
^1H-^1H COSY	Correlated spectroscopy
h	Hour
HMBC	Heteronuclear multiple bond correlation
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography

HR-ESIMS	High resolution electrospray ionisation mass spectrometry
HRP	Horseradish peroxidase
HSQC	Heteronuclear single quantum coherence
KCl	Potassium chloride
L	Litre
M	Molar
MeOH/$CHCl_3$	Methanol/chloroform
mg	Milligram
MHz	Mega hetrz
min	Minute
ml	Millilitre
mM	Millimolar
MMP	Mitochondrial membrane permeability
NaCl	Sodium chloride
Na_3VO_4	Sodium orthovandate
NaF	Sodium fluoride
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
OD	Optical density
PARP	Poly ADP-ribose polymerase
PIC	Protease inhibitor cocktail
PMSF	Phenyl methyl sulfonyl fluoride
ppm	Parts-per-million
PVDF	Polyvinylidene fluoride
Rh-123	Rhodamine 123
RIPA	Radioimmuno precipitation assay
ROS	Reactive oxygen species
rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute Medium
RRSSF	Regional Research Sub-Station for Subtropical Fruits
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SRB	Sulphorhodamine blue
TBST	Tris-buffered saline and tween-20
TCA	Trichloro acetic acid
TLC	Thin layer chromatography
TMS	Tetramethylsilane

Introduction

CHAPTER-1

INTRODUCTION

1.1 Cancer

Cancer is a group of diseases characterized by uncontrolled growth / spread of abnormal cells and is the common cause of death in humans worldwide (Wand *et al*., 2014; Goyal, 2012; Ghali *et al*., 2014). It is a multi-gene and multistep disease caused by incorrect diet, genetic predisposition and *via* the environment, but 95% of all cancers are due to life style and take 20-30 years to develop. Despite of recent developments in the tools of disease diagnosis, cancer is still one of the leading cause of death (He *et al*., 2016; Qin *et al*., 2017). The major types of cancer include carcinoma, sarcoma, melanoma, lymphoma and leukemia. Carcinomas are malignant type of neoplasm that originates in epithelial tissues (skin, lung, breast, pancreas), most commonly diagnosed and account for 80-90% of all cancer cases. Carcinomas are divided into adenocarcinoma (organ or gland) and squamous cell carcinoma (squamous epithelium). Sarcomas arise in supportive and connective tissue (bone, muscle, fat, blood vessels, cartilage) and include osteosarcoma (bone), chondrosarcoma (cartilage), leiomyosarcoma (smooth muscle), rhabdomyosarcoma (skeletal muscle), fibrosarcoma (fibrous tissue) and liposarcoma (adipose tissue). Lymphomas develop in the nodes of lymphatic system, network of vessels and organs (spleen, tonsils, thymus) that purify body fluids and produce WBC's for immune activity. Two types of lymphoma are Hodgkin (with reed-sternberg cells) and Nonhodgkin (without reed-sternberg cells). Leukemia related to blood, is basically the cancer of bone marrow where blood cells production occurs. In leukemia, the patient is more prone to infection because of overproduction of immature white blood cells. It is also known to affect the RBC's and cause poor blood clotting. It includes myeloid leukemia, acute lymphatic leukemia, chronic lymphocytic leukemia and chronic myeloid leukemia.

1.2 Aetiology of cancer

The root cause of cancerous cell is mainly genetic or epigenetic alterations (inherited or arise spontaneously) which comprises mutations in DNA repair genes

(p21, p22, p27, p51, p53 and tool box for DNA), tumor suppressor genes (p53, NF1, NF2, RB, biological breaks), oncogenes (MYC, RAF, Bcl-2, RAS) and genes involved in cell growth metabolism. In epigentic modification DNA methylation, histone modification and nucleosome positioning plays a major role in cancer formation (Portela and Esteller, 2010). Two important families of genes related to cancer are protooncogenes and oncogenes. Protooncogenes code for proteins that stimulate cell division under normal condition whereas several factors responsible for transformation of protooncogenes into oncogenes include mutations, chromosomal rearrangement, gene amplification *etc.* Prostate, lung, bronchus, colon, rectum and urinary bladder are the most commonly occurring cancers in men whereas in women most prevalent cancers are breast, lung, bronchus, colon, rectum, uterine corpus and thyroid. This data indicates that prostate and breast cancer constitutes a major portion in men and women respectively. Blood cancer and cancers related to the brain / lymph nodes, commonly occur in children (Siegel *et al*., 2016).

Both external factors (radiations, smoking, tobacco, pollutants in drinking water, food, air, chemicals, certain metals, infectious agents) and internal factors (genetic mutations, body immune system, hormonal disorders) can cause cancer. Cancer occurs by a series of successive mutations in genes so that these mutations change cell functions. Chemical compounds have an obvious role of forming gene mutations and cancer cells. In addition, smoking involves several carcinogenic chemical compounds that lead to lung cancer (Aizawa *et al*., 2016). Interestingly, environmental chemical substances with carcinogenic properties influence directly or indirectly the cytoplasm and nucleus of cells and lead to genetic disorders and gene mutations (Poon *et al*., 2014; Trafialek and Kolanowski, 2014; Cumberbatch *et al*., 2015; Antwi *et al*., 2015). Viruses, bacteria and radiation rays are other carcinogenic factors comprising about 7% of all cancers. In general, cancer disrupts cellular relations and results in the dysfunction of vital genes. This disturbance is affective in the cell cycle and leads to abnormal proliferation (Seto *et al*., 2010).

According to WHO, tobacco has been identified as the major cause of death which ultimately leads to a wide range of cardiovascular / respiratory diseases. *Nicotiana tabacum* is the main (known) tobacco plant whereas other varieties like *Nicotiana rustica* are also cultivated / used. Tobacco is responsible for about 25% of

all cancers in men, 4% in women and 16% of all cancers in both sexes in most developed and 10% in less developed countries. Recent investigations showed that tobacco causes a lot of cases of lung cancer and cancers of throat, vocal cords, oesophagus, stomach, kidneys, pancreas, liver, bladder, cervix, colon and leukemia (Yadav and Lowenfels, 2013; Gandomani *et al*., 2017). Each puff of cigarette contains thousands of compounds, 60 of them are well established carcinogens. The carcinogens belong to multiple chemical classes, including polycyclic aromatic hydrocarbon (PAHs), N-nitrosamines, aromatic amines, aldehydes, volatile organic hydrocarbons and metals. Others that are less investigated are alkylated PAHs, oxidants, free radicals and ethylating agents. Among all the carcinogens, PAHs, N-nitrosamines, aromatic amines and certain volatile organic agents play a vital role.

The carcinogens present in cigarette smoke require metabolic activation process that convert the carcinogens to forms, that can covalently bind to DNA and form DNA adducts *via* cytochrome P-450 enzymes (P-450). However, there are some carcinogens that does not require the process of metabolic activation. Various studies suggested that by the use of some non specific methods like ^{32}P-post labeling and immunoassays for measuring the adduct level in lung and other tissues are higher in smokers as compared to non smokers.

1.3 Programmed cell death (apoptosis)

It is a process where cells are born, live for a given period of time and then die. It is termed as physiological cell death, cell deletion, programmed cell death in which useless or unwanted cells are eliminated during development and other biological processes. The term apoptosis has been coined to describe the morphological processes leading to controlled cellular self destruction. Once triggered by a variety of physiological signals, series of steps occur *viz* disruption of cellular membranes, cytoplasmic and nuclear skeletons are broken down, cytosol is extruded, chromosomes get degraded, nucleus get fragmented. These all events occur within a span of 30-120 minutes. The morphological changes are consequence of both molecular and biochemical events, activation of proteolytic enzymes responsible for cleavage of DNA into fragments as well as multitude of protein substrates that help to determine the integrity and shape of cytoplasm or organelles. In early steps of apoptosis one of the component of inner layer of cell membrane *i.e.,*

phosphatidylserine (PS) get flipped out to outer layers. This allows recognition of dead cells by macrophages and leads to phagocytosis further followed by breakdown of DNA into large 50-300 kb pieces, then nucleosomal cleavage of DNA into oligonucleosomes by endonucleases, this is the characterized feature of apoptosis. Activation of group of enzymes called caspases belongs to cysteine protease family is another specific feature of apoptosis.

Two main pathways involved in this process comprises mitochondrial perturbation and death receptor activation which leads to the activation of different caspases. Activation of apoptosis signaling by treatment with cytotoxic drugs leads to the activation of intrinsic mitochondrial pathway whereas signaling through death receptor *i.e.,* extrinsic pathway contributes to senstivity of tumor cells towards cytotoxic treatment. Many proapoptotoic proteins like apoptosis inducing factor (AIF), Smac / DIABLO and cytochrome C are released from the mitochondria following the formation of pore in mitochondrial membrane. The mitochondrial pathway is dependent on the process of mitochondrial outer membrane permeabilization that causes the release of proteins from the mitochondrial intermembrane space into the cytosol. In case of any cellular stress or apoptotic signals the proapoptotic members of Bcl 2 family relocate on mitochondrial surfaceand disrupt the function of antiapoptotic Bcl2 family, causes the formation of permeability transition pore on mitochondrial surface. Cytochrome C and other proapoptotic molecules are released from intermembrane space to cytosol. The released cytochrome C in cytosol interact with apoptotic protein activating factor-1 causes the recruitment of pro caspase 9 into a multiprotein complex containing cytochrome c, apaf-1, procaspase 9 and ATP called apoptosome. This apoptosome causes the activation of caspase 9 which is followed by activation of caspase-3 and induces apoptosis. Mitochondrial outer membrane permeabilization is mediated by Bax and Bak, proapoptotic bcl-2 family members and inhibited by antiapoptotic bcl-2 family proteins. Mitochondrial outer membrane permeabilization allows the proteins of mitochondrial intermembrane space to enter cytosol and allows the formation of apoptosome. Caspase 9 in turn cleaves and activates the executioner caspase 3 & 7 to induce apoptosis.

The characteristic feature of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The caspase play crucial role in this process *via* activating Dnases, inhibiting DNA repair enzymes and breaking down structural proteins in the nucleus. PARP (poly ADP-ribose polymerase) one of the first proteins indentified as substrate for caspase. Its function is to repair DNA damge and act by synthesizing poly (ADP-ribose), then gets bind to DNA strand breaks and modify nuclear proteins. This ability to repair DNA damge is prevented following cleavage of PARP by caspase-3. Apoptosis is a complicated process with energy dependent flow of molecular events, accomplished by intrinsic and extrinsic pathways that includes the activation of caspases. It plays a significant role in multicellular organisms for survival by maintaining homeostasis as well in management of many diseases like cancer, neurodegenerative and autoimmune disorders.

1.4 Status of cancer

Cancer, a severe metabolic syndrome, is the leading cause of mortality / morbidity worldwide and the number of cases are continuously rising (Sharma *et al*., 2014). This disease ranks second in death cases after cardiovascular disorders in the developed nations (Mbaveng *et al*., 2011; Siegel *et al*., 2016).Current estimates from the American Cancer Society and from the International Union Against Cancer indicate that 12 million cases of cancer were diagnosed last year with 7 million deaths worldwide and these numbers are expected to double by 2030 (27 million cases with 17 million deaths) (Aggarwal *et al*., 2006). The mortality rate among men and women in India is almost the same as 3.56 lakh men died of cancer in 2012, the corresponding number for women was 3.26 lakh and against 4.77 lakh men, 5.37 lakh women were diagnosed with cancer in India in 2012. The estimation of 1.1 million new cancer cases, indicates India as a single country (of 184 total) contributing to 7.8% of the global cancer burden. Mortality figures were 682830, contributing to 8.33% of global cancer deaths. According to Indian Council of Medical Research (ICMR), deadly cancer disease has constantly been spreading its tentacles in Jammu and Kashmir during the past several years. In 2011, there were a total of 10688 cancer cases in Jammu and Kashmir and this number increased to 11052 in 2012 and 11428 in 2013. During the year 2013-2014, a total of 11815 cancer cases have come to the fore in

Jammu and Kashmir so far. As far as mortality cancer cases are concerned, the number was 4703 in 2011 and the same increased to 4863 in 2012 and 5028 in 2013.

1.5 Medicinal plants as indispensable source for anticancer phytochemicals

In developed countries the interest in natural products and the use of complementary and alternative medicine is increasing day by day by replacing the use of conventional pharmaceutical approaches. More and more pharmaceutical industries are interested in examining their potential as source of novel medicinal compounds (Zhang *et al*., 2013). In the field of anticancer research, natural products has made tremendous achievements *i.e.*, over 60% of clinical use of anti cancer drugs originates from natural sources like plants, marine and microbes. Several active ingredients from natural sources such as alkaloids, flavonoids, terpenoids and saponins are known to possess wide variety of antitumor properties including induction of apoptosis, autophagy and inhibition of cell proliferation (Abubakar *et al*., 2012).

Alkaloids are organic compounds with nitrogen having some biological activity and are mostly present in plants. Vinca alkaloids, isolated from *Catharanthus roseus,* are the diverse group of phytochemicals employed in several types of cancers like breast, liver, leukemia, testes, lung and the main vinca alkaloids in use are vinorelbine, vindesine, vincristine and vinblastine. To obtain vincristine, vinblastine is used as a parent drug. Both vincristine and vinblastine bind to a specific site termed as tubulin heterodimers, disrupting the function of microtubules or by arresting cell cycle at metaphase (Maryam *et al*., 2013). Taxanes are the promising agents that act by binding to microtubules and play an important role in cell division. Paclitaxel and docetaxel are strong anticancer agents with their efficacy on different molecular targets. Paclitaxel was first isolated from the bark / leaf of *Taxus baccata*, *Taxus canadensis* and *Corylus avellana* and is used for different types of cancers like ovarian, breast, lung. Binding site of paclitaxel is β-tubulin in the lumen of microtubules decreases microtubules dynamics and thus halt cell cycle at M phase whereas docetaxel from *Taxusbaccata* is used in pancreas, breast, prostate and lung cancer therapies (Xie and Zhou, 2017; Schutz *et al*., 2011).

Camptothecin is clinically active chemotherapeutic agent that possesses strong anticancer potential by inhibiting topoisomerase I. It belongs to a pentacyclic group of

quinoline alkaloids, isolated from *Camptotheca acuminata* and is mainly present in cortex, wood and fruit part. Production of carboxylic acid derivatives occurs due to hydrolysis of lactone rings present in camptothecin which leads to loss of antitumour activity. It has poor solubility in water, so water soluble semisynthetic derivatives like irinotecan and topotecan were found with clinical application. To overcome the sensitivity of camptothecin to hydrolysis, a methylene group was introduced into their hydroxylactone ring forming a stable seven membered ring which is found to be insensitive to hydrolysis. Second approach used to stabilize hydroxylactone ring is to replace the lactone ring with five membered keto analogue of the ring thereby retaining high activity against topoisomerase I. Combretastatins is an antiangiogenic agent isolated from *Combretum caffrum* (African willow) known to suppress tumour angiogenesis. It is a stilbene derivative that exist in both trans (CA-1) and cis (CA-4) forms. Like taxanes and vinca alkaloids, combretastatins are microtubule targetting drugs, belong to a class of vascular disrupting agents, that inhibit the tumour blood vessels and act by binding to β-tubulin, causing the destabilisation of microtubules.

Podophyllotoxin was obtained from *Podophyllum peltatum*, found in North America,is a toxin lignan. The constituents present in plant are α-peltatin, β-peltatin and their corresponding glycosides, along with podophyllotoxin α-D-glucoside. Podophyllotoxin and its derivatives are aryl tetralin lactone compounds with lactone ring in trans confirmation. They exhibit significant biological activity as antiviral agents and as antineoplastic drugs. Colchicine**,** a natural bioactive compound isolated from *Colchium autumnale* is an alkaloid used to treat various diseases like crystal arthritis, cirrhosis, gout *etc.* It act by binding to tubulin, stabilizes microtubule formation, arrest cell cycle at different phases and induces apoptosis,but surprisingly, its action is not very specific as it target rapidly dividing normal cells and arrest their cell cycle. So, semisynthetic deriatives like colchicinamide, deacetylcochicine have been developed with lower toxicity and used for treatment of variety of cancers.

Therefore, medicinal plants are known to help humans for better health, a source of active ingredients with major role in infectious diseases and tumour therapy. Several medicinal plant species and their phytochemicals inhibit the progression and further development of cancer (Aung *et al*., 2017). Consumption of plant derived medicines is wide spread and increasing significantly in both traditional and modern

medicine. According to the World Health Organization, more than 80% of the world population in developing countries depends primarily on plant based medicines for basic healthcare needs.

In view of the above, it is concluded that natural products have been used for centuries for the treatment of several ailments and many basic ancient medicinal systems are derived from dietary sources. Different parts of a plant may possess different level of pharmacological activity. Additive or synergistic effects of bioactive phytoconstituents might be responsible for the concerned pharmacological function rather than the purified one. In the current work, we have focussed on some of the fruits from Jammu subtropics that might play a beneficial role in discovering drugs with anticancer potential.

1.6 Objectives of the present research work

1. Determination of *in vitro* cytotoxic effect of some fruit extracts against various human cancer cell lines

2. Bioassay guided fractionation of active extracts and further evaluation

3. Characterization of most active fractions / ingredients

Review of Literature

CHAPTER-2

REVIEW OF LITERATURE

2.1Natural products / phytochemicals

Plants constitute one of the major raw materials of drugs for treating various human diseases. The modern society has been interested in drugs of natural origin due to their harmonious nature with our biological system (Paritala *et al*., 2015). Natural products contain many compounds which help in the treatment of various diseases and pharmacists are interested in these compounds because of their therapeutic performance and low toxicity. Medicinal plants have been used to treat human diseases for thousands of years because they have vast and diverse assortment of organic compounds that can produce a definite physiological action on the human body. Most important of such compounds are alkaloids, tannins, flavonoids, terpenoids, saponins and phenolic compounds (Akhtar *et al*., 2018). Polyphenols have received a great deal of attention in recent years due to their powerful antioxidant properties and are present at high concentration in a variety of fruits and vegetables. Many studies suggested that consumption of fruits and vegetables, rich in polyphenols, is related to a reduced risk of coronary heart diseases, neurodegenerative diseases and certain forms of cancer (Salerno *et al*., 2014).

A growing interest is arising around phytochemicals role in cancer prevention and treatment. Phytochemicals are plant-based non-nutrient secondary metabolites, which provide many positive health effects. These secondary metabolites are recognized as efficacious components of plants which can be used in the prevention and management of chronic disease such as cancer, diabetes and cardiovascular diseases (Shan *et al*., 2016).Recent research suggests that the investigation of "new" phytochemicals and related molecular targets can be exploited to identify novel anti-cancer drugs, following sequential steps. This approach includes preliminary selection of phytochemical candidates for cancer prevention or therapy, based on the pre-clinical results related to cell-transformation and antitumorigenic activity assays. Phytochemicals need to be further validated by means of *in vivo* models, determining pharmacokinetics and pharmacodynamics molecular interactions and targets. Clinical trials should assess anti-cancer efficacy, further investigating specific

pharmacokinetics and pharmacodynamics in humans. Phytochemicals can also contribute to cancer prevention by influencing different stages of the tumor development, from tumor initiation through all the phases of cancer (Hanahan and Weinberg, 2011) such as cell proliferation, apoptosis, invasion, metastasis, angiogenesis and immortality. Among 252 drugs considered as basic and essential by the World Health Organization, 11% are exclusively originated from plant sources (Emerenciano *et al*., 2015).

2.2Fruits as prospective reserves of active ingredients

In fruits, certain types of bioactive compounds are present and their effect on health is being investigated. Epidemiologic studies strongly suggest that high intake of fruits considerably leads to reduced risk of various chronic diseases. In addition, some wild fruit species are not taken by people, but still they possess many pharmaceutical and medicinal properties (Calixto *et al*., 2016). Citrus fruits and limonoids are known to prevent heart diseases, inflammation and arterosclerosis along with hepatoprotective, antimicrobial, neuroprotective, antioxidants, antidiabetic, anticancer properties (Duarte *et al*., 2016; Gualdani *et al*., 2016; Barreca *et al*., 2017). Several bioactive compounds are reported in berry fruits with antioxidant, antiinflammatory, cardioprotective and neuroprotective effects (Ferlemi and Lamari, 2016). The fruit extract of pomegranate is also been reported to have medicinal properties against several types of cancer (Panth *et al*., 2017; Wu *et al*., 2016). Fruit species are largely appreciated and highly consumed all over the world. There are several biological reasons regarding consumption of fruits to reduce/prevent chronic diseases. Fruits provide a rich source of nutrients, energy, vitamins, minerals, fiber and numerous other classes of biological active compounds, that are discussed below:

2.2.1 ***Phenolic acid:***Phenols are secondary plant metabolites with nutritive quality in terms of aroma, taste, flavor and colour. They include a wide variety of molecules having one or more hydroxyl groups on aromatic ring structure. Phenolic compounds classification is based on the number of phenol rings and the structural elements that bind those rings to one another. The main classes are flavonoids, tannins, stilbenes and lignans. Several berries, apples, pears and grapes contain upto 200-300 mg polyphenols per 100g fresh weight. Among berries, blueberries, raspberries and blackberries are rich in hydroxybenzoic and hydroxycinnamic acids. Some of the

derivatives of hydroxybenzoic acids are used as additives to reduce the oxidation of nutrients and to enhance the nutritional value in foods. Seeds of grape pomace are rich in phenolic compounds – gallic acid, catechins and epicatechin and epicatechin with variety of procyanidins. In particular, peels are an abundant source of natural flavonoids. The phenolic content in peel of lemons, oranges and grapefruit is about 15% more than the edible portion. One of the previous studies related to structure activity of phenolic acids and their derivatives stated that hydroxycinnamic acid derivatives has higher antioxidant activity as compared to their benzoic acid counterpoints. This ability was attributed to the presence of propenoic side chain, instead of carboxylic group of benzoic acid derivatives thereby enhancing antioxidant capacity (Ignat *et al*., 2011; Haminiuk *et al*., 2012).

Flavonoids are one of the largest groups of phenolic compounds that play a significant role in various biological activities including antioxidant, antimicrobial, antimutagenic and cytotoxic activities (Orhan *et al*., 2010). In fruits and vegetables, they are found in the form of glycosides or acylglycosides whereas acylated, methylated and sulphate molecules are less frequent and are in lower concentration. It consists of two benzene rings linked by a three carbon chain that forms a closed heterocyclic pyran ring with benzenic ring. These are divided into flavonols, flavones, catechin and anthocyanins depending on the carbon of C ring with which B ring is attached. Flavonols contain 3-hydroxyflavone backbone and an unsaturated benzo-c-pyrone displaced to a phenyl and as many as 7 hydroxyl groups surrounding their skeleton. It is reported that number and position of hydroxyl groups plays a key role in the chemical structure thereby affect the biological activity (Xiao, 2017).

Kaempferol is the most studied flavonol and recent study demonstrated that on treatment with it, myocardial ischemia-reperdusion injury in diabetic rats was greatly attenuated whereas pretreatment significantly reduced hyperglycemia, maintained hemodynamic function and also normalize oxidative stress (Suchal *et al*., 2017). Another study reported that it also induced cellular apoptosis which act as a therapeutic agent in treatment of cervical cancer. It has important role in cell signaling pathways by acting on both intracellular and extracellular targets thereby regulate the cancer progression like apoptosis, cell cycle, invasion or metastasis, angiogenesis and inflammation. The therapeutic effects of kaempferol will greatly help the scientific

community to design novel strategy for treating the dreadful diseases(Kashafi *et al*., 2017).

Quercetin is the most abundantand found to be active in several cancers, cardiovascular and neurogenerative diseases. Recent study regarding quercetin derivatives declared monochloropivaloyquercetin and chloranapthoquinon quercetin as potent therapeutic against chronic diseases like diabetes and neurodegenerative disorders (Zizkova *et al*., 2017). It is also reported that quercetin therapy might improve heat stroke outcomes in rats by attenuating excessive hyperthermia as well as myocardial injury (Lin *et al*., 2017). Myricetin is another plant flavonol that is abundant in fruits like berries and grapes andisan effective therapeutic specifically against diabetes mellitus (Li and Ding, 2012). An interesting study declared it as an effective drug target for Alzheimer's disease. Myceritin induced apoptosis and enhanced cytotoxicity in ovarian cancer cell lines.Naringenin (a citrus flavanone), is prooved as a potent candidate for dengue treatment by impairing dengue virus replication in human monocytes (Zheng *et al*., 2017).

Anthocyanins are abundant in berries and grapes with multifaceted medicinal and biological values. Various molecular mechanisms for biological activities of anthocyanins were reviewed by signaling pathways responsible for the health benefits (Li *et al*., 2017). The study also revealed that lack of synergestic or antagonistic effects of anthocyanins in carcinogenesis would generate new anthocyanin based anticancer drugs. In clinical trial experiments, therapeutic role of anthocyanin compounds was observed in both *in vitro* and *in vivo* studies and their possible benefits against chronic diseases (Putta *et al*., 2017). It is revealed from a study conducted on blueberries that anthocynanins are readily metabolized to various phenolic derivatives thus contributing to human health (Sandhu *et al*., 2017). There is a great correlation between anthocyanins intake and cardiovascular health reiterating the significance of these flavonoids (Cassidy,2017).

2.2.2 ***Carotenoids:*** Carotenoids are C_{40}tetraterpenoids formed from eight C_5isoprenoid units joined head to tail, resulting in a symmetrical structure. They are comprised of naturally occuring yellow, orange or red compounds with their structural diversity, wide distribution and multiple functions. In fruits and vegetables, around 800 carotenoids have been identified. Among carotenoids, the carotenes (β-carotene,

lycopene) are the unsaturated hydrocarbon whereas the xanthophylls (β-cryptoxanthin, canthaxanthin, violaxanthin) are oxygenative derivatives containing hydroxyl, keto, epoxy and aldehyde substituents. In fruits, xanthophylls are present in abundance.Recent studies assessed the three carotenoids namelycryptocapsin, cryptocapsin-5,6-epoxide and zeaxanthin from which only cryptocapsin represented the highest bioactivity, whereas cryptocapsin-5,6-epoxide and zeaxanthin exhibited similar activity on antiaggregation assays. This findings suggest that cryptocapsin, cryptocapsin-5,6-epoxide and zeaxanthin possess anti-amyloidogenic potential and could be used for prevention and treatment of alzheimer's disease. In fruits like mango and papaya, carotenoids are present in oil droplet in an esterified form with fatty acids. Lutein is the main carotenoid and decrease in the level of carotenes and lutein at the onset of fruit coloration with an increased level of xanthophylls was noticed (Capella *et al*., 2014; Beitia *et al*., 2017).

2.2.3 ***Tocopherols:***These are lipid soluble bioactive compounds present in fruits and vegetables, formed by a 6-chromanol ring structure methylated to varying degrees at C_5, C_7 and C_8 positions and a saturated C_{16} side chain at C_2 position. Tocopherol is the term used for vitamin-E and tocotrienols, consist of four tocopherolslike α, β, γ and δ. They act as source of antioxidants and also provide beneficial effect in the inhibition of tumor development, provide protection to membrane fatty acids from attack of free radical. Previous study demonstrated that grape seed oil provide a good source of linolenic acid with vitamin A and rich in α, β and γ tocopherols, α and γ tocotrienols, with γ tocotrienol being most important quantitatively (Beveridge *et al.*, 2005)

2.2.4 ***Stilbenes:*** Grape berries are abundant in stilbenes *i.e.*, resveratol present in fruits, leaves, stems, roots, canes and possess various biological activities like antioxidant, antibacterial, antifungal, cardioprotective, neuroprotective, antiaging and anticancer. About 13 grapevine stilbenes are quantified with e- viniferin and resveratol being more abundant. Two potential inhibitors ampelopsin A and piceatannol were identified that can be used in drugs against neurodegenerative diseases (Biais *et al*., 2017).

2.3Bioactive compounds

2.3.1 ***Extraction:*** Extraction is the separation of medicinally active ingredients *via* selective and standard procedures. It is the most important step in analysis of medicinal plant for the extraction of desired components, further their separation and characterization. Conventional techniques make use of organic solvents or water,are carried out generally at atmospheric pressure whereas modern technique use pressure and elevated temperature. According to the principle, extraction method includes solvent extraction, distillation method and sublimation. Among all these, the most widely used method is solvent extraction in which the solvents used are water, ethanol, chloroform, dichloromethane, hexane, ethyl acetate, methanol *etc*. In conventional extraction method, large volume of organic solvents and long extraction time is required. On the other hand, modern extraction method provides lower organic solvent consumption, shorter extraction time with improved extraction yield. The preliminary steps of extraction include pre-washing, drying of plant material or freeze drying, proper grinding to obtain a homogenous sample often improves the kinetics of analytic extraction and also increases the contact of sample surface with the solvent system. During the preparation of extract from plant sample, it must be assured that the active constituents should not be lost, distorted or destroyed. Different solvent systems are available for the extraction, but the selection of solvent system mainly depends on the nature of bioactive compound to be extracted. In case of hydrophilic compounds, polar solvents like methanol, ethanol or ethyl-acetate and for lipophilic compounds dichloromethane or a mixture of dichloromethane / methanol in the ratio of 1:1 are used (Njila *et al*., 2017).

2.3.2 ***Isolation/purification*****:** Different separation techniques like TLC, column chromatography, flash chromatography, sephadex chromatography and HPLC are used to obtain a pure bioactive compound. The main method used to obtain pure compound from a complex mixture is column chromatography where separation is based on the adsorption properties. It is widely used due to its simplicity, high capacity and low cost of adsorbent such as silica gel and macroporous resins. To achieve good separation selection of both stationary phase (adsorbent) and mobile phase is the crucial step and to maximize the recovery of target compound and to avoid the irreversible adsorption of target on the adsorbent. 90% of phytochemicals

separation is achieved by silica gel, a polar adsorbent with silanol groups in which molecules get retained *via* hydrogen bond and dipole-dipole interactions. In silica gel columns, polar natural products are retained for longer time than non polar ones. Sometimes, certain natural products undergo irreversible chemisorption and the deactivation process is achieved by adding water before use or using water containing mobile phase weaken the adsorption. In some cases, where alkaloids are separated on silica gel tailing might occur and to reduce the tailing small amount of ammonia or organic amines such as triethylamine are used. In order to separate alkaloids, a strong polar adsorbent *i.e.,* alumina (aluminium oxide) is used. Unlike silica gel, strong positive field of Al^{3+} and the basic sites in alumina affect easily polarized compounds to get adsorbed on alumina. For polyamide column chromatography, hydrophobic or hydrogen bond interaction occur depending on the composition of mobile phase. When aqueous solvents (polar) are used as mobile phase, polyamides act as non-polar stationary phase and is similar to reversed phase chromatography. In contrast, polyamides act as polar stationary phase and this behaviour is similar to normal phase chromatography. For the separation of natural polyphenols like anthraquinones, phenolic acids and flavonoids, polyamide chromatography is used, hydrogen bond formation occurs between polyamide adsorbents, mobile phase and target compounds. It has been investigated that polyamide act as hydrogen bond acceptor and the strength of adsorption depends on number of phenolic hydroxyls and their position in the molecule (Gao *et al*., 2011).

TLC, a commonly used planar chromatography method, is easiest / cheapest among others. It is applied in the analysis, isolation and setting the parameters for column chromatography (Bele and Khale, 2011). Silica or alumina (stationary phase) are more polar and organic solvents (mobile phase) are less polar, used in normal phase chromatography. Gas chromatography is an analytical technique based on volatilities of compounds. It provides both qualitative and quantitative information regarding compounds present in sample. Here the gas used is mobile and liquid act as stationary phase. The distribution of components in gas phase helps to determine their rate of migration. The distribution of components that occur partly in both the phases will get migrate at an intermediate rate. In this technique, the sample is vaporized, injected onto the head of column and transported by the flow of inert, gaseous mobile phase (Ingle *et al*., 2017)

HPLC, is an another technique widely used for the purpose of isolation of natural products. It provides better separation and determination of organic and inorganic solutes in any sample regarding biological, environmental, pharmaceutical *etc.* The degree of separation is mainly determined by the choice of both stationary phase and mobile phase. HPLC provides a good complement to gas chromatography as it is used for compounds that cannot be vapourized or decomposed under high temperature (Tonthubthimthong *et al*., 2011).

2.3.3 *Structure elucidation*: A wide range of spectroscopic techniques like UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy are used for the determination of structure of compounds isolated from natural sources. In spectroscopy electromagnetic radiations are passed through an organic compound which absorb some of the radiation, but not all. By measuring the amount of absorption of electromagnetic radiation, a spectrum can be produced. In UV-visible spectroscopy, qualitative analysis of certain classes of compounds and their identification occurs in both pure and biological mixtures. It can also be used for quantitative analysis because of aromatic molecules which are powerful chromophores in UV range. It is not time consuming and demands less cost as compared to other techniques. Fourier-transform infrared spectroscopy (FTIR) is another technique, used for the identification of functional groups in the plant sample and further structure elucidation of the molecule. It is a high resolution analytical tool to identify the chemical constituents and offers a rapid, non destructive investigation for herbal extracts. Nuclear magnetic resonance (NMR) spectroscopy provides physical, chemical and biological properties of matter. Two dimensional NMR is used for the complicated structure of molecules and one dimensional technique is routinely used. ^{1}H NMR helps to find out the type of hydrogen present and the connection between the H-atoms. Radiolabelled ^{13}C NMR is used for the identification of types of carbon present in the compound. In addition to this, mass spectroscopy is analytical tool for identification of unknown compounds, quantification of known compounds. It helps in the elucidation of the structure, chemical properties of the molecule and the molecular weight of the sample can be determined. From mass spectroscopy, structural elucidation of organic compounds, peptides or oligonucleotide sequencing can be achieved (Ingle *et al*., 2017).

2.4A comprehensive review of selected fruits

Natural products research is based on screening of extracts, bioassay guided isolation, structure elucidation and subsequent scale up production. Until now, most of the evidence concerning the protective effect of fruits against risk of cancer has come from case-control studies thatshowed that intake offruits is associated with a reduced risk of multiple cancers including cancer of the digestive tract, respiratory tract, pancreatic cancer, prostate cancer, breast cancer, nasopharyngeal cancer and cutaneous melanoma. Therefore, a comprehensive review was conducted to assess the safety and efficacy of some fruits in an attempt to prevent various diseases including cancer. A seminal description of the fruits that have been selected for screening *in vitro* cytotoxic potential against human cancer cell lines and further isolation of bioactive compounds, is given in the following pages. The information within braces in order is: Family, English name, Hindi name. This system has been followed throughout while describing the fruits.

2.4.1 *Artocarpus heterophyllus* [Moracea, Jackfruit, Katahal]

Artocarpus heterophyllus Lam, popularly known as jackfruit or Ceylon Jack tree, is one of the important and commonly found trees in the home gardens of India and Bangladesh (Prakash *et al*., 2009). It is known as the national fruit of Bangladesh and referred to as poor man's food as it is cheap. It is considered to be an important tree by the natives and found in plenty in case of food scarcity during summer season. The tree morphology varies with 10-30 m tall; with long tap root and dense crown (Wangchu *et al.,* 2013) producing the largest tree born fruit in the world (Baliga *et al.,* 2011; Prakash *et al.,* 2009). It is widely accepted mainly by consumers, researchers and food industries because of the bioactive compounds present and diversity products made out of it (Swami *et al.,* 2012; Saxena *et al.,* 2009; Dutta *et al.,* 2011; Lin *et al.,* 2009; Devalaraja *et al.,* 2011). Previous studies demonstrated the presence of major sugars (fructose, glucose, sucrose) and the major fatty acids (capric, myristic, lauric, palmitic, oleic, stearic, lisnoleic, arachidic acids) in jack fruit (Jagtap and Bapat, 2010; Ong *et al.,* 2006). Diversity of compounds is reported especially phenols, flavonoids, stilbenoids, arylbenzofurons, carotenids, volatile acid sterols and tannins in various *Artocarpus species* (Jagtap and Bapat, 2010; Baliga *et al.,* 2011). The seeds contain β-carotene, α-carotene, β-zeacarotene, α-zeacarotene and crocetin

which are mostly present in trans form. Major carotenoids reported are all-trans-lutein, all trans β-carotene, all trans–β- neoxanthin and 9-cis-violaxanthin, their contribution is 24-44%, 24-30%, 4-19%, 4-9% and 4-10% respectively in jackfruit(Baliga *et al.,* 2011; Jagtap and Bapat, 2010; De Faria *et al.,* 2009).

Other classes of compounds reported in jackfruit are artocarpine, artocarpetin, artocarpetinA, cycloheterophyllin, artonins A, artonins B, morin, dihydromorin, oxydihydroartocarpesin, cynomacurin, artocarpin, isoartocarpin (Prakash *et al*., 2009). Previous studies demonstrated the presence of brosimone, cudraflavone B, prenylapigenin, norartocarpin, kuwanon C, artocarpin, cudraflavone C, apigenin, albanin A, morin, 2',4'-Dihydroxyflavone, 3-Methylbut-2-en-1-ol, 2-Methylpent-2-ene, artocarpesin, norartocarpetin and oxyresveratrol in heatwood of jack fruit (Arung *et al*., 2007). Triterpenic compounds (cycloartenyl acetate, cycloartenone, heterophylol, tannin), flavone pigment (cycloheterophyllin) and betullic acid are found mainly in the bark (Prakash *et al*., 2009).

In various traditional system of medicine jackfruit tree is of great importance where all its parts are used for preparing many Ayurvedic and Yunani medicines (Saxena *et al*., 2009).The seed part contain a major protein *i.e.*, jacalin (lectin) with molecular mass 65 KDa is a tetrameric two chain lectinthat can bind to human IgA and T-antigen. Moreover a polyspecificlectin, artocarpin is also reported injackfruit seeds that can react with variety of monosaccharides. It is 159 amino acid polypeptide chain in nonglycosylated form with 52% identity in sequences (Jagtap and Bapat, 2010). These phyto-nutrients have a wide range of health benefits especially antimicrobial, anticancer, antihypertensive, antiulcer, antioxidant and anti-ageing properties (Loizzo *et al.,* 2010; Siritapetawee *et al.,* 2012). The anti-cancer studies of the diethylether extract of *Artocarpus altilis* wood was performed in human T-47D breast cancer cells and examined for its effect on cell viability, nuclear morphology and sub-G1 formation. The results demonstrated that *A. altilis* wood extract induced apoptosis and sub-G1 phase formation in breast cancer (T-47D) cells and therefore, has a potential as an anti-cancer agent (Patel and patel, 2011; Arung *et al.,* 2009).

2.4.2 *Carissa carandas* [Apocynaceae, Cranberry, Karonda]

*Carissa carandas*Linn.,is an important, exotic, minor fruit commonly known as 'Christ's thorn' which grows wild in bushes. Karonda is a large dichotomously

branched evergreen shrub with short stem and strong thorns in pairs (Wiart, 2006; Karale, 2002). In India, it is cultivated in a limited way in the tropical and subtropical Mediterranean region. Various parts (fruits, leaves, bark, roots) of *C. carandas* are popular for their medicinal use in diarrhea, constipation, malaria, epilepsy, neurological disorder, pain, myopathic spams, leprosy anorexia, cough, pharangitis, diabetes, seizures, scabies and fever(Rahmatullah*et al*., 2009). The plant possesseshepato-protective (Hegde and Joshi, 2009), anticonvulsant (Hedge *et al*., 2009), cardiotonic, antibacterial (Israr *et al*., 2012), antihypertensive (Shamim and Ahmad, 2012), antihelminthic (John *et al*., 2007), antidiabetic (Itankar *et al*., 2011) and antihyperlipidemic (Sumbul and Ahmed, 2012) properties. The fruits have been used as a dietary supplement or medicinal food for centuries and are of increasing importance to consumers (Sharma *et al*., 2007). The methanolic extract of the fruit showed the presence of reducing sugar, flavonoids, protein, cardinolides, terpenoids, steroids, phenolic compounds, saponins and acids (Arif and Fareed, 2011).

C. carandas is widely used throughout India in the treatment of scabies, intestinal worms, pruritus and its fruit have been studied for its analgesic, anti-inflammatory (Sharma *et al.,* 2007) and lipase (Mala and Dahot, 1995) activity. The karonda fruit is an astringent, antiscorbutic and as a remedy for biliousness and useful for cure of anaemia. The root extracts of *Carissa carandas* are known to have antipyretic properties (Bhaskar and Balakrishnan, 2009).*C. carandas* is known to possess wide range of phytochemicals in its parts that imparts immense medicinal value to the plant. These active constituents give medicinal value to the plant and the chemical investigations has led to the isolation of several substances including β-sitosterol, lupeol, glucosides of odoroside-H, ursolic acid and a new cardioactive substance. The leaves were reported to have triterpenoid constituents as well as tannins and carissic acid. Fruits contain a mixture of volatile principles like 2-phenyl ethanol, linalool, isoamyl alcohol, β-caryophyllene, benzyl acetate, carissol and lanost-5-en-3β-ol-21-oic acid (Arif *et al*., 2013).

Extracts of karonda were investigated on human ovarian carcinoma, Caov-3 and lung cancer cells. Chloroform extract from leaves showed good anticancer activity against Caov-3 while the n-hexane extract of the unripe fruit showed remarkable activity against the lung cancer cell line (Pino *et al.*, 2004; Pal *et al.*,1975;

Singh and Rastogi, 1975; Reisch *et al.*, 1990). Further, anti-cancer and antioxidant potential of the extracts was analyzed by unusual antioxidant enzymes such as catalase, dismutase, superoxide, glutathione-s-transferase and glutathione on MCF-7 cancer lines. This study exhibited significant antioxidant activity and fortification of cell death in MCF-7cell line pretreated with *C. carandas* extracts. The potential anti-cancer value of this medicinal plant fruit was suggested for future development of therapeutic drugs (Duaand Srivastav, 2013). Furthermore, *in-vitro* anticancer studies showed that aqueous ethanolic fruit extract (AEE) induces cytotoxicity at 800µg/mL on HeLa cancer cells maintained in Dulbecco's Modified Eagle's Medium (DMEM) (David and Karekalammanavar, 2015).

2.4.3 *Grewia asiatica*[Tilaceae, Phasla fruit, Phalsa]

Grewia asiatica, is well-known for its nutritional and therapeutic attributes. The fruits are claimed to be beneficial for heart, blood and liver disorders, anorexia, indigestion, thirst, toxemia, stomatitis, hiccough, asthma, spermatorrhoea, fever, diarrhea and are used for treating throat, tuberculosis and sexual debility troubles (Sharma and Sisodia, 2009; Pallavi *et al*., 2011; Mishra *et al*., 2012). The root bark is used for the treatment of rheumatism and urinary tract problems (Sisodia and Singh, 2009; Muhammad *et al*., 2006), while the stem bark is used in sugar refining (Haq *et al*., 2012).

Fruits contain pelargonidin 3,5-diglucoside, naringenin-7-*O*-β-D-glucoside, quercetin, quercetin 3-*O*-β-D-glucoside, tannins, catechinsand cyanidin-3-glucoside. The flower part constitute β-sitosterol, naringenin-7-O-β-D-glucoside, δ-lactone, 3, 21, 24-trimethyl-5,7-dihydroxyhentriacontanoic acid and quercetin 3-O-β-Dglucoside. Moreover, grewinol and its derivatives are present in dried flowers. Major flavonones like quercetin, kaempferol and the mixtures of their glycosides are mainly concentrated in the leaves whereas betulin, lupenone, friedelin, β-amyrin, lupeol, taraxerol and erythrodiol are present in stem and bark of phalsa (Haq *et al*., 2013). Nutritionally essential amino acids such as threonine and methionine are present in pulp and seeds respectively whereas phosphoserine, serine and taurine are the dominant amino acids in juice. The pulp contains higher concentration of phosphoserine as compared to other free amino acids, while the hydrolysed product contained aspartic acid, glycine and tyrosine in large amount. Phytochemical

screening of fruits indicated the presence of carbohydrate, tannins, phenolic compounds, flavonoids and vitamin-C in methanolic extract; flavonoids and fixed oil in petroleum ether extract; steroids in benzene extract; carbohydrate, tannins, flavonoids and phenolic compounds in ethyl acetate extract and carbohydrate, tannins, phenolic compounds and proteins in the aqueous extract (Gupta *et al*., 2006).

Amino acids such as proline, glutaric acid, lysine and phenylalanine and carbohydrates, like glucose, xylose and arabinose were identified by paper chromatography in ethanol extract of fruit. *G. asiatica* contains different phytochemicals – alkaloids, glycosides, saponins, flavonoids, functional acids and steroids which get extracted either in aqueous or in different organic solvents like petroleum ether, ethyl acetate, methanol, acetone and dichloromethane (Asghar *et al*., 2008). Aqueous extracts of leaves and fruits showed significant anticancer activity against liver cancer and breast cancer.

The *in vitro* cytotoxic activity was determined by methylthiazolyltetrazolium (MTT) assay using epidermal kidney (HEK-293), breast (MCF-7), cervical (HELA), lung (NCI-H522) and laryngeal (Hep-2) cancer cell lines. The fruit extract was found to be active on lung (IC_{50} = 59.03 μg/mL) and breast (IC_{50} = 58.65 μg/mL) cancer cell lines, while the leaf extract was active against breast (IC_{50} =50.37 μg/mL) and Hep-2 (IC_{50} = 61.23 μg/mL) cancer cell lines (Marya*et al*., 2011). Fruits of *G. asiatica* are reported for their antitumor and cytotoxic activity (Kakoti *et al*., 2011). Some *Grewia* species have free radical scavenging activities which may be responsible for therapeutic action against tissue damage (Kshirsagarand Upadhyay, 2009). *Grewia*'s extracts are also supposed to be helpful in curing hepatitis and used as herbal antidiabetic (Tripathi *et al*., 2011).

2.4.4 *Syzygium cumini* [Myrtaceae, Java Plum / Black Plum, Jamun]

Syzygium cumini (L.) is a widely distributed forest tree in India and other tropical and subtropical regions of the world. The tree has a great economic importance since most of the parts like the bark, leaves, seed and fruits are used as an alternative medicine to treat various diseases. The fruit, seeds and even juice of the fruit play an important role in the treatment of diabetes. Jamun fruit is a very rich source of anthocyanin and has anti-cancer and anti-viral properties (Jain and Seshadri, 1975; Venkateswarlu, 1962; Helmstadter, 2008; Kumar *et al*., 2008; Ayyanar and

Bubu, 2012; Tupe *et al*., 2015). Jamun possess antineoplastic (Barh and Viswanathan, 2008), radioprotective (Jagetia and Baliga, 2002 & 2003; Jagetia *et al.,* 2005 & 2008) and chemopreventive effects (Parmar *et al.,* 2010), all of which are useful in the prevention and treatment of cancer. The reasons for the myriad pharmacological effects are due to the presence of diverse phytochemicals like flavonoids, anthocyanins and terpenes (Sagrawat *et al.,* 2006).

Fruit extracts possess anti-oxidative activity (Benherial and Arumughan, 2007; Hassimotto *et al*., 2005; Faria *et al*., 2011; Aqil *et al*., 2016); anti-inflammatory properties (Kumar *et al*., 2010), antibacterial properties (Kaneria *et al*., 2009), anti-proliferative activities against human lung (Aqil *et al*., 2012) and breast cancer cells (Li *et al*., 2009; Aqil *et al*., 2016) and pro-apoptotic effects against human breast cancer cells. The biological activities of *S. cumini* extracts have been attributed to the abundant and diverse array of phenolic compounds.

Different parts of jamun - leaves, fruit, seed and bark provide a rich source of phytochemicals. In jamun leaves extract, previous studies indicated the presence of phenols, flavonoids, alkaloids, glycosides, steroids, cardiac glycosides, saponins, terpenoids and tannins (Shyamala and Vasantha, 2010; Reddy and Jose, 2013). The abundant constituents of oil reported in jamun seed is lauric (2-8%), myristic (31.7%), palmitic (4-7%), stearic (6.5%), oleic (32.2%), linoleic (16.1%), malvalic (1.2%), sterculic (1.8%) and vernolic (3.0%) acids. Gallic acid induces apoptosis in human prostate (LNCaP) and prostate cancer cell line (DU145) and human melanoma cells. In addition it inhibits proliferation of papilloma and carcinoma, radiation induced damage and peroxidation of DNA (Agarwal *et al*., 2006; Reddivari *et al*., 2010). The composition of bioactive compounds is based on both maturity level of fruit and the age of plant whereas fruit's colour intensity changes from green yellow to dark purple and black. Majority of anthocyanins increase during the period of maturation while ellagitannins, flavonols, gallic acid and ellagic acid decrease on ripening phase (Lestario *et al*., 2017).

Corilagin, 3,6-hexahydroxydi-phenoylglucose, 1-galloylglucose glucoside, 3-galloylglucose and 4,6- hexahydroxy-diphenoylglucose are mainly present in seed part. Moreover, stem and bark constitute 3,3,4-tri-O-methyl ellagic acid, 3,3-di-O-methyl ellagic acid, ellagic acid and gallic acid. The fruit is rich in anthocyanins,

primarily the diglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin (Brito *et al*., 2007; Veigas *et al*., 2007; Faria *et al*., 2011; Tavares *et al*., 2016), flavonols, mostly myricetin derivatives and flavan-3-ols, proanthocyanidins, ellagitannins and gallotannins. Anthocyanins and hydrolysable tannins are reported to be the most abundant phenolics in the fruit followed by flavanonols, flavonols and flavan-3-ols (Tavares *et al*., 2016). Among the flavonoids, anthocyanin is the most important one and the colour of jamun fruit is mainly due to this. The bioactives from berries are reported to have many roles in cancer prevention including inhibition of the formation of carcinogen-induced DNA damage, protection against oxidative DNA damage, inhibition of carcinogen-induced tumorigenesis and modulation of signaling pathways involved with cellular proliferation, inflammation and cell cycle arrest (Kausar *et al*., 2012).

Administration of jamun extract (25mg/kg b.wt/day) was effective in preventing benzo-a-pyrene-induced forestomach carcinogenesis (Goyal *et al*., 2010). Jamun possess cancer chemopreventive properties in the DMBA-induced croton oil promoted two stage skin carcinogenesis in Swiss albino mice (Parmar *et al*., 2010). The extract has been shown to exhibit cytotoxic effects on cultured human cervical cancer cells, the HeLa (HPV-18 positive) and SiHa (HPV-16 positive) and caused a concentration-dependent cell death with the effect being more pronounced in the HeLa than SiHa cells (Barh and Viswanathan, 2008). Feeding of 125 mg/kg b. wt. of the extract during pre or postinitiation phases reduced the cumulative numbers of papillomas, tumor incidence and increased latency period when compared to the control group (Parmar *et al*., 2010).

Ellagitannin, a constituent of jamun and its colonic metabolite, urolithin A, inhibit Wnt signaling crucial in the process of colon carcinogenesis (Sharma *et al*., 2010). Urolithin A reduces proliferation of colon cancer cells, induces cell cycle arrest and modulates MAPK signaling *in vitro* (Sarrias *et al*., 2009; Sarrias *et al*., 2010), while animal studies have shown it to reduce the inflammatory markers (iNOS, COX-2, PGEsynthase and PGE2) in the colonic mucosa of rat with colitis mechanisms vital in preventing / retarding the process of carcinogenesis.

Materials & Methods

CHAPTER-3

MATERIALS AND METHODS

3.1 Special requirements

1) Well equipped cell culture laboratory
2) Human cancer cell lines
3) Plant extracts / fractions / compounds (test material)
4) Standard drugs for cancer (positive controls)

3.2 Major instruments used

1) CO_2 incubator (Hera Cell)
2) Centrifuge (Thermo Scientific)
3) Cryocontainers (Thermo Electron Corporation)
4) Deep freezer (Scien Temp.)
5) Microplate reader (Thermo Scientific)
6) Inverted microscope (JVC)
7) Laminar flow (ESCO)
8) Robotic system processor (TECAN)
9) Rotary vacuum evaporator (Biomate India)
10) Ultralow deep freezer (Scien Temp.)

3.3 Preparation of reagents

3.3.1 Growth medium (RPMI-1640)

Prepared RPMI–1640 with 2 mM L-glutamine (10.4 g), 2 g of sodium bicarbonate (2 g/L) and 100 g of streptomycin (100 µg/ml) were dissolved in 1L of double distilled water andmixed thoroughly. The pH of the medium was adjusted 7.2 and the medium was sterilizedby filtration (0.22 µ filter) and stored in a refrigerator (2-8 ^{0}C).

3.3.2 Complete growth medium (CGM)

Itcontained FBS (10%) and penicillin (1%) in growth medium.

3.3.3 Medium for cryopreservation (freezing medium)

It contained FBS (20%, v/v) and DMSO (10%, v/v) in growth medium.

3.3.4 Minimum essential medium (MEM)

Prepared MEM (14.2 g), 2 g of sodium bicarbonate and 100 g of streptomycin were dissolved in 1L of distilled water. The pH was adjusted and the medium was filtered and stored as in case of RPMI–1640.

3.3.5 Gentamycin medium

It contained gentamycin (1%, w/v) in complete growth medium.

3.3.6Phosphate buffer saline (PBS)

9.6 g of PBS was dissolved in 1L of DW (pH 7.4), mixed gently, autoclaved and stored in a refrigerator (2-8 ^{0}C).

3.3.7 Trypsin-EDTA

0.05% trypsin and 0.02% EDTA were dissolved in PBS and filtered.

3.3.8 Penicillin

6.25 mg/ml in PBS and filtered.

3.3.9 Gentamycin

5 mg/ml in PBS and filtered.

3.3.10SRB dye

0.4% (w/v) SRB was dissolved in 1% (v/v) acetic acid.

3.3.11 Tris buffer

10 mM (pH 10.5), 1.210 g tris base in 1L double distilled water.

3.3.12 Trichloro acetic acid (50%, w/v)

500 g of TCA was dissolved in 1L of double distilled water.

3.3.13Separating gel buffer

1.5 M tris base in distilled water, pH 8.8.

3.3.14Stacking gel buffer

1.0 M tris base in distilled water, pH 6.8.

3.3.15Acrylamide

It contained 30% (w/v) bisacrylamide and acrylamide in 50 mL double distilled water.

3.3.16SDS

It contained 10% SDS in double distilled water.

3.3.17 APS

50 mg of APS in 500µl of distilled water.

3.3.18 Running buffer

It contained tris base, glycine, SDS in distilled water, pH 8.3.

3.3.19 Transfer buffer

It contained glycine (192 mM), tris base (25 mM), methanol (20%) in distilled water.

3.3.20 TBST

It contained tris base, NaCl, KCl, tween-20 in distilled water, pH 7.6.

3.3.21 Lysis buffer

It contained RIPA, NaF, Na_3VO_4, PMSF and PIC.

Table # 1. List of human cancer cell lines used in the present research work

S.No.	Tissue	Cell line	Doubling time (h)	Cell density /100 µL	Medium	Positive control
1.	Breast	MCF-7	25.4	8000	RPMI	Doxorubicin
2.	Breast	MDAMB-231	41.9	8000	RPMI	Doxorubicin
3.	Colon	HCT-116	17.4	7000	RPMI	5-Fluorouracil
4.	Colon	HT-29	19.5	5000	RPMI	5-Fluorouracil
5.	Colon	SW-620	20.4	13000	RPMI	5-Fluorouracil
6.	Lung	A-549	22.9	7500	RPMI	Paclitaxel
7.	Pancreas	MIA PaCa-2	40	10000	DMEM	Paclitaxel
8.	Prostate	PC-3	27.1	7500	RPMI	Mitomycin-C

3.4Sources of human cancer cell lines

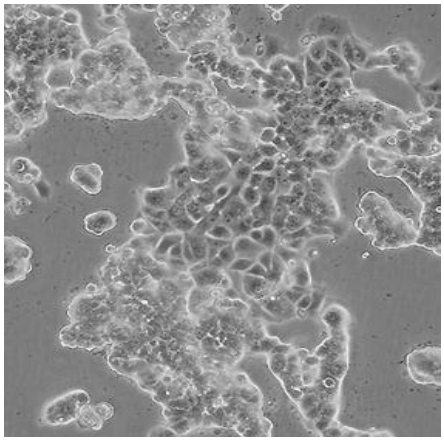
National Centre for CellScience,Ganeshkhind, Pune – 411007 (India) and National Cancer Institute, DTCD, Frederick Cancer Research and Development Center, Fairview Center, Suite 205, 1003 West – 7th Street, Frederick, MD 21701 – 8527 (U.S.A).

3.4.1Selection of human cancer cell lines

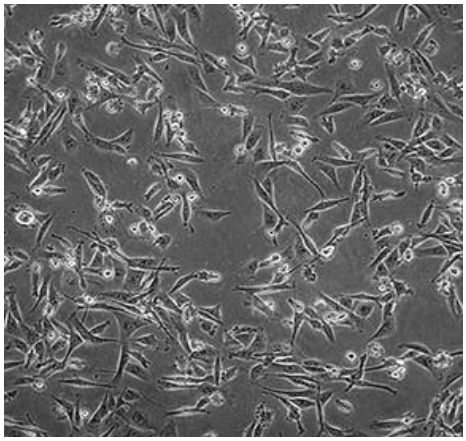
Human cancer cell lines representing different tissues were selected and the mass doubling time ofcell lines was such that enough cells were obtained for conducting assay.

3.5Selection of fruit material

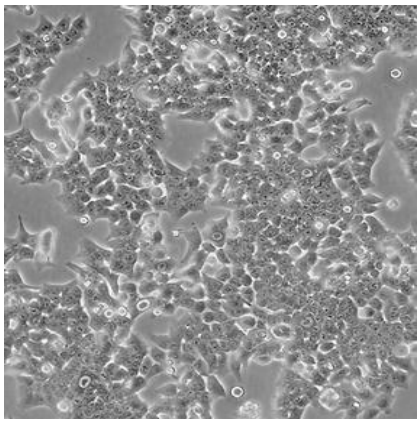
Natural dietary products have tremendous role in cancer chemoprevention and various epidemiologic studies strongly suggest that high intake of fruits considerably leads to reduced risk of chronic diseases with lower toxicity.To develop novel anticancer drugs, intense search is going on various biological sources.In addition, several phytochemicals that are present in fruits have been scientifically established to have properties that can prevent and treat this malevolent disease. Thus, in view of the above mentioned facts and the literature given in the previous chapter, the following fruits were selected for the study, with the objective to identify active fruit extracts that may have*in vitro* cytotoxic potential against human cancer cells and to further isolate the active ingredients with potent anticancer efficiency.



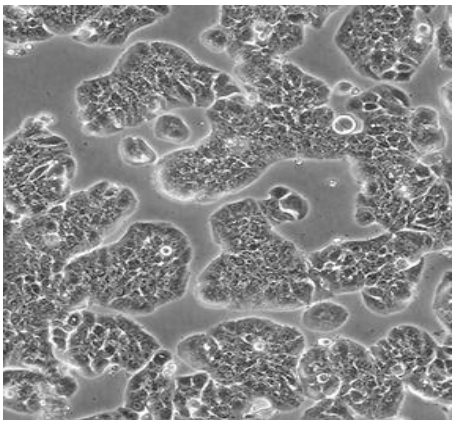
Breast (MCF-7)



Breast (MDAMB-231)

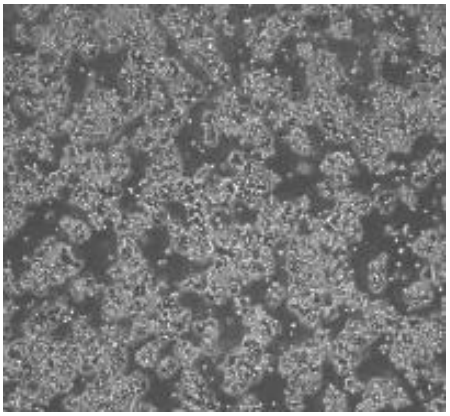


Colon (HCT-116)

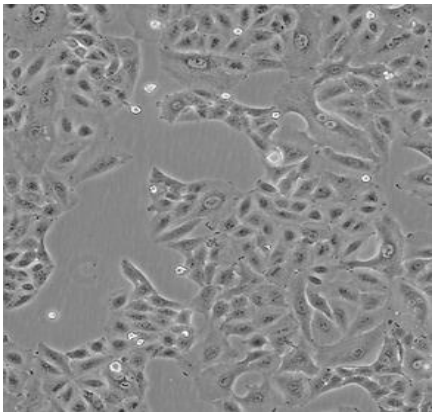


Colon (HT-29)

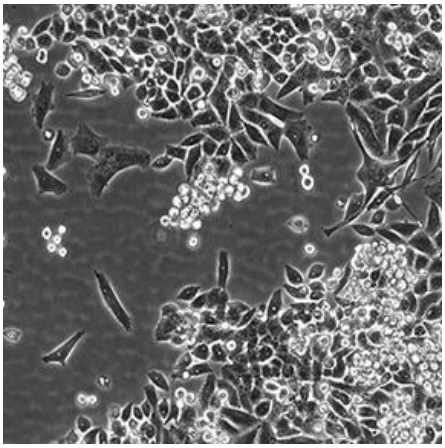
Plate #1. Human cancer cell lines from breast and colon origin



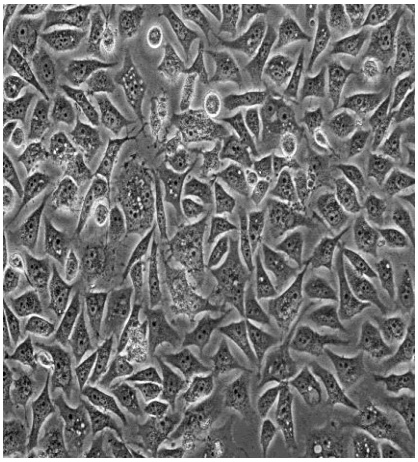
Colon (SW-620)



Lung (A-549)



Pancreatic (MIA PaCa-2)



Prostate (PC-3)

Plate #2.Human cancer cell lines from colon, lung, pancreatic and prostate origin

Table # 2.List of fruits used in the present investigation

S.No.	Name of the fruit	Collection site	Collection period
1.	*Artocarpus heterophyllus*	Village Nagri, Kathua, Jammu	June, 2018
2.	*Carrissa carandas*	RRSSF, Raya, SKUAST-J	September, 2016
3.	*Grewiaasiatica*	RRSSF, Raya, SKUAST-J	June, 2017
4.	*Syzygiumcumini*	ACHR,Udheywala, SKUAST-J	July, 2017

***Artocarpus heterophyllus* (katahal)**

***Carissa carandas* (karonda)**

***Grewia asiatica* (phalsa)**

***Syzygium cumini* (jamun)**

Plate # 3. Fruits selected for evaluation of *in vitro* cytotoxic effect against human cancer cells

3.5.1Authentication and collection of fruit material

The above mentioned fruits were authenticated at siteby Dr.Vijay Bahadhur Singh, Sr. Scientist, RRSSF, Raya SKUAST-Jammu and enough quantity of fresh fruits were collected.

3.5.2 Chopping and drying of fruit material

Freshly collected fruits were chopped, shade dried, crushed and the coarse fruit material was then extracted with methanol at room temperature (35 ^{0}C) for bioevaluation.

3.6 Extraction and fractionation of fruit material

3.6.1 Extraction with methanol

Standard protocol was followed for the extraction of different powdered fruit material (Kandil *et al*., 1994). Dried fruit material (100 g) was placed in a conical glass percolator of appropriate size.Sufficient quantity of methanol (99%, v/v) was added so as to submerge the fruit material.After standing for about 16 h (overnight), the percolate was collected and filtered if required. The process was repeated four times, which was generally sufficient for exhaustive extraction of the fruit material.The methanolic extract (collected in four attempts) was evaporated to dryness under reduced pressure at 60^{0}C using rotary vacuum evaporator and round bottom flask (RBF).The final drying was done in a vacuum desiccator.The dried extract was scrapped off from the RBF and transferred to a tared wide mouth glass container of appropriate size.The container was weighed to calculate the quantity of the extract obtained.This formed the "stock extract" of the fruit. Generally, 8 to 10 g crude extract was obtained from 100 g of the dried fruit material.The fruit extracts obtained, were stored at –20^{0}C under desiccation in deep freezer for further testing.

3.6.2Fractionation

Crude (methanolic) extract was fractionated with n-hexane, chloroform and butanol solvents. Water was added to dissolve the extract and the same volume of

hexane was used for solvent- solvent partitioning, and the upper phase (n-hexane) was removed. The residue was further partitioned using chloroform and butanol (Figure # 1-6). Different fractions obtained were evaporated using rotary vacuum evaporator. Yield of the different fractions was noted and stored at 4 ^{0}C until use.

3.7 Isolation and characterization of active ingredients

In *Grewia asiatica*, chloroform fraction (3.38 g) was purified by column chromatography (silica gel, 100-120 mesh), eluted with a gradient of hexane-ethyl acetate (250 mL collected volumes of each fraction) and concentrated, giving sixty fractions based on TLC profile. Fractions (Fr. 35-39) showed same pattern in TLC were pooled and concentrated. Obtained compound was subjected to TLC using 50% ethylacetate: hexane as mobile phase. A single spot was detected after spraying the plate with anisaldehyde reagent and heating it at 100 ^{0}C for 5 min. Spot on TLC obtained was dark blackish green in colour and the nature of compound was oil type (yellow colour).

In *Syzygium cumini*,hexane fraction (7.30 g) was purified by column chromatography (silica gel, 100-120 mesh), eluted with a gradient of hexane-ethylacetate (250 mL collected volumes of each fraction) and concentrated, giving 65 different fractions. Fr. 55-65 was again purified in silica gel mesh of 60-100 giving eight fractions (Fr. 46-53) based on TLC profile. These fractions with same pattern were pooled and concentrated. The compound 1 was subjected to TLC using 12% EtoAc/hex. as mobile phase and a single spot was detected after spraying with anisaldehyde reagent, heating the plate at 100 ^{0}C for 5 min and then about 8.41 g of chloroform fraction was purified (silica gel, 100-120 mesh) eluted with a gradient of hexane-ethyl acetate and then with methanol-chloroform. Fifty different fractions (250 mL collected volumes of each fraction) were collected, spotted on TLC and compound 2 was visualized in 25% EtoAc/hex. (mobile phase). Compound 3 was purified from 10% MeOH/$CHCl_3$ and visualized in 70% EtoAc/hex. (mobile phase) on TLC. A single spot appeared on TLC after exposing to iodine vapours in yellow colour and compound was light green in colour. Compound 4 was purified in 15% MeOH/$CHCl_3$ fromcolumn and spotted on TLC with solvent system of 10% MeOH/$CHCl_3$, visualized after spraying with anisaldehyde reagent.

In *Carissa carandas,* 13 g of chloroform fraction was purified *via* column chromatography, eluted with a gradient of EtoAc/hex. (250 mL collected volumes of each fraction) and concentrated, giving thirty fivedifferent fractions based on TLC profile. Fractions (Fr. 10-11) eluted in 25% EtoAc/hex. (mobile phase) from column showed same pattern in TLC were pooled and concentrated. Obtained compound (carissic acid) was subjected to TLC using 30% ethylacetate:hexane as mobile phase where a single spot was detected after spraying it with anisaldehyde reagent and heating at 100 ^{0}C for 5 min. Spot on TLC obtained was purple in colour and compound obtained was white (powder) in colour.

3.8 Analysis of isolated compounds

3.8.1 HPLC

HPLC purifications were performed on an Agilent 1260 Infinity II HPLC system with UV detector. HPLC solvents like MeOH and ACN were procured from Merck chemicals and water for extractions and HPLC analysis was obtained from high-purity Milli-Q Advantage A10 water system (Millipore, Molsheim, France). HPLC of crude extract, fractions and bioactive compounds were done with column ACE EXCEL, C-18 of length 25 cm with internal diameter-4.6 mm. Column temperature was adjusted to 25 ℃.

Thin Layer Chromatographty was carried out on precoated silica gel plates 60 F_{254} plates (Merck, India) with 1 mm film thickness. Anisaldehyde–H_2SO_4 reagents and iodine vapours were used to visualize spots on TLC plates.

3.8.2 General experimental procedure for NMR

All NMR spectral data were recorded on a Bruker 400 MHz spectrometer. Chemical shifts (δ) were referenced internally to the residual solvent peak (CD_3OD: ^{1}H δ 3.30, ^{13}C δ 49.0 ppm; $CDCl_3$: ^{1}H 7.26, ^{13}C 77.0 ppm) and the reference point was TMS (δ_H and δ_C: 0.00 ppm).

3.8.3 Mass analysis

HR-ESIMS spectra were recorded on an Agilent 1100 LC-Q-TOF mass spectrometer and HRMS-6540-UHD machines.

3.9 *In vitro* cytotoxic assay

3.9.1 Handling of cell lines on arrival

Immediate on receipt of the original stock of cell line in tissue culture flasks, from authentic source, the cell line was checked for apparent contamination and proper growth under microscope. In case of any contamination, the cell line was discarded and a fresh one was obtained, but if cell showed proper growth and monolayer was present, excess growth medium was collected aseptically in sterilized bottles for further use and cells were incubated at 37 ^{0}C in an atmosphere of 5% CO_2 and 90% relative humidity in CO_2 incubator. The flask was checked daily for proper growth and complete growth medium was changed whenever necessary. When the growth was 70 to 90% (sub-confluent), the passage was given.

3.9.2Subculturing of cell lines

Cells lose their viability, if they are left too long before subculturing. Thus, new cultures were established by inoculating the cells into fresh medium, called subculturing or passaging, which involved detachment of the cells from the growth surface of the one culture flask and re-inoculation of the cells into fresh medium contained in a new culture flask. The cells were detached from their anchor by the process of trypsinisation. The proteolytic enzyme trypsin was used to breakdown the proteins that binded the cells to the culture flask.

3.9.3 Steps involved in subculturing

- The complete growth medium was changed one day before giving the passage.On the day of passage, the complete growth medium was again taken out from the flask (which was at sub-confluent stage) and discarded.
- Enough volume (0.5–1 ml) of Trypsin–EDTA in PBS was added in the tissue culture flask and immediately taken out. It was done for the removal of the remaining complete growth medium present in the flask.
- In the second attempt, trypsin of enough volume (1 to 3 ml) was again added in the tissue culture flask. It was done to make a thin film on the monolayer of cells in the flask. Volume of trypsin addition was according to the size of the culture flask, as trypsin in excess could damage the cells.

- After that the flask was incubated for approximately 5 minutes or shorter at 37^0C so that cells were detached.

- The complete growth medium in enough volume (5 to 6 ml) was added in the culture flask to inactivate trypsin, as the excess of protein present in the medium reduce the trypsin activity.

- Cells were separated into single cell suspension by gentle pipetting action.

- Viable cell density for a particular human cancer cell line was adjusted and cell suspension (1 ml) was taken out from the flask and by the addition of complete growth medium, cells were inoculated into fresh tissue culture flask and incubated at 37^0C in an atmosphere of 5% CO_2 and 90% relative humidity in CO_2 incubator.

- Fresh flasks were maintained and surplus cells were cryopreserved at this stage from time to time.

3.9.4 Cryopreservation of cell lines

- The complete growth medium was changed one day before giving the passage for cryopreservation. Passage was given by the treatment of Trypsin-EDTA and complete growth medium was added to stop the action of the trypsin.

- Cells with complete growth medium were transformed into sterile centrifuge tube.

- Cells were centrifuged (05 min at 1200 rpm) as gently as possible.

- After centrifugation, supernatant was discarded and cells (pellet) were re-suspended in medium for cryogenic preservation (prewarmed at 37^0C). Pellet was the whole content of the cells in the centrifuge tube, so freezing medium was added according to the quantity of the pellet. Generally 3-4 ml of freezing medium was added in tube and mixed thoroughly.

- Viable cells were counted,appropriate cell density was adjusted with freezing medium.

- Uniform cell suspension of 1ml was then transferred into each sterile cryovial. Care was taken, as the aliquot was representative of the whole batch.

- The process of cooling was started after 20 min from addition of freezing medium as this much time was required by the cryoprotectant to enter the cells.

- The most common cooling rate used was 1^0C/min in the range of room temperature and down to -70^0C. After temperature of -70^0C was attained, the vials were transferred to liquid nitrogen (-196^0C) container.

- Cryovials were properly labelled and preferably colour coded, indicating type of cell line, passage number, date of passage and medium used. Proper record of the cell lines under cryopreservation was maintained which helped to locate the specific cell line as per requirement.

3.10 Preparation of test material

3.10.1 Stock solution

A stock solution of 20 mg/mL(extracts/fractions) was prepared in DMSO. For 99% (v/v) methanolic extracts / fractions, DMSO was used. In case of compounds, 20 mM of stock solutions were prepared atleast one day in advance. Stock solutions were not filtered / sterilized, but microbial contamination was controlled by the addition of gentamycin in complete growth medium used for dilution of stock solutions to prepare working test solutions.

3.10.2Working test solution

On the day of assay, an aliquot of frozen stock solution was thawed at room temperature. Working test solution (200 μg/mL) of extract / fraction was prepared by dilution of stock solution with gentamycin medium.(10 μl of stock solution +990 μl of gentamycin medium = 1000 μl). For compounds, working solution of 50, 30, 10 and 1μM were prepared.

3.10.3 Positive controls (standard drugs for cancer)

Positive controls were initially prepared with DW (Doxorubicin, 5–flurouracil, mitomycin-C) and DMSO (paclitaxel). They were further prepared in gentamycin medium to obtain working test solutions.

3.11 Determination of *in vitro*cytotoxicity

Cytotoxicity of a number of test samples was performed against eight human cancer cell lines from five different tissues. Number of 96-well flat bottom tissue culture plates was dependent upon the number of test samples along with appropriate positive controls.There were four types of wells in the tissue culture plates, control blank (CB, without cells, complete growth medium only) and control growth (GC, with cells alone in the absence of test material) to determine 100% growth. The growth in the presence of test material was determined from the difference of test growth (GT, cells with test material) and test control (CT, test material without cells). The method described below is for single cell line which can be easily adopted for multiple cell lines.

3.11.1 Preparation of cell suspension for assay

- The desired human cancer cell lines were grown in multiple tissue culture flasks at 37^0C in an atmosphere of 5% in CO_2 and 90% relative humidity in complete growth medium to obtain enough number of cells as per requirement depending upon number of test samples.

- The flask with cells at sub-confluent stage was selected. The cells were harvested by the treatment of Trypsin – EDTA and the complete growth medium was added to stop the action of trypsin. Cells were separated to single cell suspension by gently pipetting action.

- Viable cells were counted in haemocytometer and viable cell density was adjusted to 5,000 – 40,000 cells/100 µl depending upon the cell line(Monks *et al.*, 1991). At this stage, cell suspension was ready for addition to tissue culture plates.

3.11.2 Addition of cell suspension in tissue culture plates

- Cell suspension 100 μl/well was added into GC and GT wells.
- Complete growth medium 100 μl/well was added into CB and CT wells.
- The plates were incubated at 37^0C for 24 h in an atmosphere of 5% CO_2 and 90%relative humidity in a CO_2 incubator.
- After 24 h, test material and positive controls were added.

3.11.3 Addition of test material

- Working solutions of the test material or positive controls (100 μl) were added to respective CT and GT wells and equivalent complete growth medium was added to CB and GC wells into tissue culture plates prepared 24 h in advance, containing either cells or complete growth medium (100 μl).
- The plates were incubated at 37^0C for 48 h in an atmosphere of 5% CO_2 and 90% relative humidity in a CO_2 incubator.
- The cell growth was determined after 48 h by SRB assay.

3.12 SRB assay

- SRB assay was carried out as described by Skehan*et al.,* 1990,using SRB dye.
- After 48 h incubation of cells with test material, the plates were taken out and 50 μl of chilled 50% TCA was gently layered on top of the medium in all the wells to produce a final concentration of 10%.
- After that tissue culture plates were incubated at 4^0C in a refrigerator to fix the cells attached to the bottom of the wells.
- After 1 h, the plates were taken out from refrigerator and the contents of all the wells were pipetted out and supernatent was discarded.
- The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins *etc.*For

washing, the wells of tissue culture plates were filled with distilled water and the liquid in the wells was discarded by sharply flicking plate over a sink.

- Plates were air dried and can be stored until use.
- SRB solution (100 μl) was added to each well of the plates and the plates were incubated for 30 min at room temperature.
- The unbound SRB was removed quickly (to avoid desorption of protein bound dye) by washing the wells of the plates five times with 1% (v/v) acetic acid.
- Plates were then air dried.
- After that Tris buffer (100 μl/well) was added in the plates.
- The plates were gently stirred for 5 minutes on a mechanical shaker and the optical density was recorded on microplate reader (Thermo Scientific) at 540 nm.

3.13 Calculations

The cell growth was determined by subtracting average absorbance value of respective blank from the average absorbance value of experimental set. Percent growth in presence of test material was calculated as under:

OD Change in Presence of Control = Mean OD of Control – Mean OD of Blank

OD Change in Presence of Test Sample = Mean OD of Test sample – Mean OD of Blank

% Growth in Presence of Control = 100/OD change in presence of control

% Growth in Presence of Test Sample = (% growth in presence of control) × OD change in presence of test sample

% Growth inhibition = 100 - % growth in the presence of test sample

or

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells} - \text{Absorbance of blank cells}}{\text{Absorbance of control cells} - \text{Absorbance of blank cells}} \times 100$$

% Growth inhibition = 100 - % cell viability

3.14 Criteria for activity

The growth inhibition of 70% or above was considered active while screening extracts/fractions, but in testing of active ingredient/compounds at different molar concentrations, the growth inhibition of 50% or above was the criteria of activity.

3.15 Biological assays

3.15.1 Assessment of nuclear morphology

Human lung cancer cell line (A-549) was seeded at the density of 2×10^5 cells/mL in six well plate and incubated for 24 h. The compound CA was added at the concentration of 14, 24 and 48 µM for further 24 h, positive control used was paclitaxel (100 nM). After the incubation period cells were trypsinized, washed and resuspended in PBS. On a glass slide, a smear of cell suspension was made and stained with 1µg/mL of DAPI (4,6- diamidino-2-phenylindole) Sigma- Aldrich. Slides were fixed with chilled methanol and incubated at 37 ^{0}C for 30 min at dark. After PBS washing, the cells were mounted with glycerol: PBS (9:1). The slides were then observed under fluorescence microscopy (Olympus- 1X53 magnification) for nuclear morphological alterations (Chashoo*et al*., 2011).

3.15.2 Mitochondrial Membrane Permeability (MMP) assay

A-549 (2×10^5) cells were seeded in six well plate and treated with CA (14, 24, 48 µM) incubated for 24 h. At the end of incubation cells were trypsinized, washed with PBS and Rh-123 (2.5µg/ml) was added, incubated for 30 min at 37^{0}C in dark. The plate was again washed with PBS and resuspended in complete medium. Fluorescence intensity was determined at an excitation wavelength of 488 nm under

fluorescence microscope (Olympus-1X53 magnification). Rhodamine 123 is a cationic fluorescent dye which is non cytotoxic used to study the electrochemical gradient in mitochondria by emitting green fluorescent (Dai *et al*., 2008).

3.15.3 Detection of reactive oxygen species (ROS) accumulation

Lung cancer cell line (A-549) was seeded in six well plate (2×10^5) and treated with CA (14, 24, 48 µM) incubated for 24 h. Then H_2O_2 was added to each well containing medium 2 h before termination. The cells were further incubated with 10 µM DCFDA for 30 min at 37 ^{0}C in dark. Cells were washed with PBS and analyzed with fluorescence microscope (Olympus-1X53 magnification). The fluorescent intensity was measured at the emission and excitation wavelength of 480/530 nm (Banskota *et al*., 2015).

3.15.4 Method for colony formation assay

A-549 cells were seeded at the density of 2×10^5 cells/mL in six well plate and incubated for 24 h. After incubation period, cells were treated with compound CA at different conc. of 14, 24 and 48 µM and further incubated for next 24 h. The treated cells were then trypsinized, counted and reseeded at 1000 cells/well in fresh six well plate. The plate was kept in an incubator for at least 6 cell divisions to occur (colonies >50 cells). The medium was aspirated and cells were rinsed with PBS. Then the cells were fixed with 1% formaldehyde for 20-25 min. Fixed cells were stained with 0.5% crystal violet and plate was kept at room temperature for 30 min. At last, crystal violet was removed and the plate was rinsed with water carefully. Colonogenic survival was expressed as the number of colonies forming units in treated cells to untreated cells (Ye *et al*., 2004; Franken *et al*., 2006).

3.15.5 Scanning electron microscopy (SEM)

A-549 (1.5×10^5) cells were seeded and incubated for 24 h. After incubation cells were treated with different concentrations of CA *i.e.*, 14, 24 and 48 µM for 24 h. Then the medium was aspirated, rinsed with PBS and cells were fixed with 2.5% glutaraldehyde in PBS at 4 ^{0}C for 24 h. Cells were washed thrice with PBS for 5 min each at 4 ^{0}C. The cells were fixed in 1% Osmium tetraoxide for 4 h at 4 ^{0}C, washed with PBS and then with distilled water. Then it was dehydrated in ethanol (30% followed by 50%, 70%, 90%) and then with absolute ethanol for 15 min each for

single cell. Again cells were fixed in 1:1 ratio of HMDS and absolute ethanol and kept for 30 min at room temperature. Cells were visualized under scanning electron microscopy (JEOL JSMIT 300) (Rai *et al*., 2014).

3.15.6 Wound healing assay

A-549 (3×10^5) cells/well were seeded in 6 well plate for 24 h. Then 200 µl tips kept at the top of well, vertical scratch was made on the cell monolayer and treated with CA (14, 24, 48 µM) for next 24 h. Cells were seen at 0 h time period under the microscope. After 24 h medium was aspirated, washed with PBS and seen under phase contrast microscope (Francisco *et al*., 2012).

3.15.7 Western Blotting

A-549 cells (8×10^5) were seeded in 90 mm dish and incubated with different conc. (14, 24, 48, 55 µM) of CA for 24 h. Positive control used was paclitaxel. After incubation cells were trypsinized, washed with PBS. The cell lysates were prepared by using RIPA buffer, protease and PIC. Total protein content was determined using Quantipro BCA assay kit (SIGMA Aldrich, India) using BSA as standard. Protein lysates were mixed with 5X loading buffer and boiled at 100 ^{0}C for 5 min. An equal amount of protein sample per lane was resolved by 10 % SDS PAGE and transferred to PVDF membrane (Millipore). Blocking was done by 5% BSA in TBST solution for 1h at room temperature to avoid non specific binding. After blocking, the membrane was incubated with primary β-actin (1:500), Caspase-3 (1:1000) and cleaved PARP (1:1000) at 4^0C overnight. The blots were then incubated with HRP congujated secondary antibodies (CST, cat no.-7074). Immunoreactive proteins were detected with ECL detection blotting detection system and exposed to film (Singh *et al*., 2013).

3.16 Statistical Analysis-

The experiments were done in triplicates and each data represents the average of at least three independent experiments. The data was expressed as Mean ± S.D. One way analysis of variance technique was used for the significant difference between the groups. IC_{50} value was calculated by Graph PAD Prism software version 5.0.

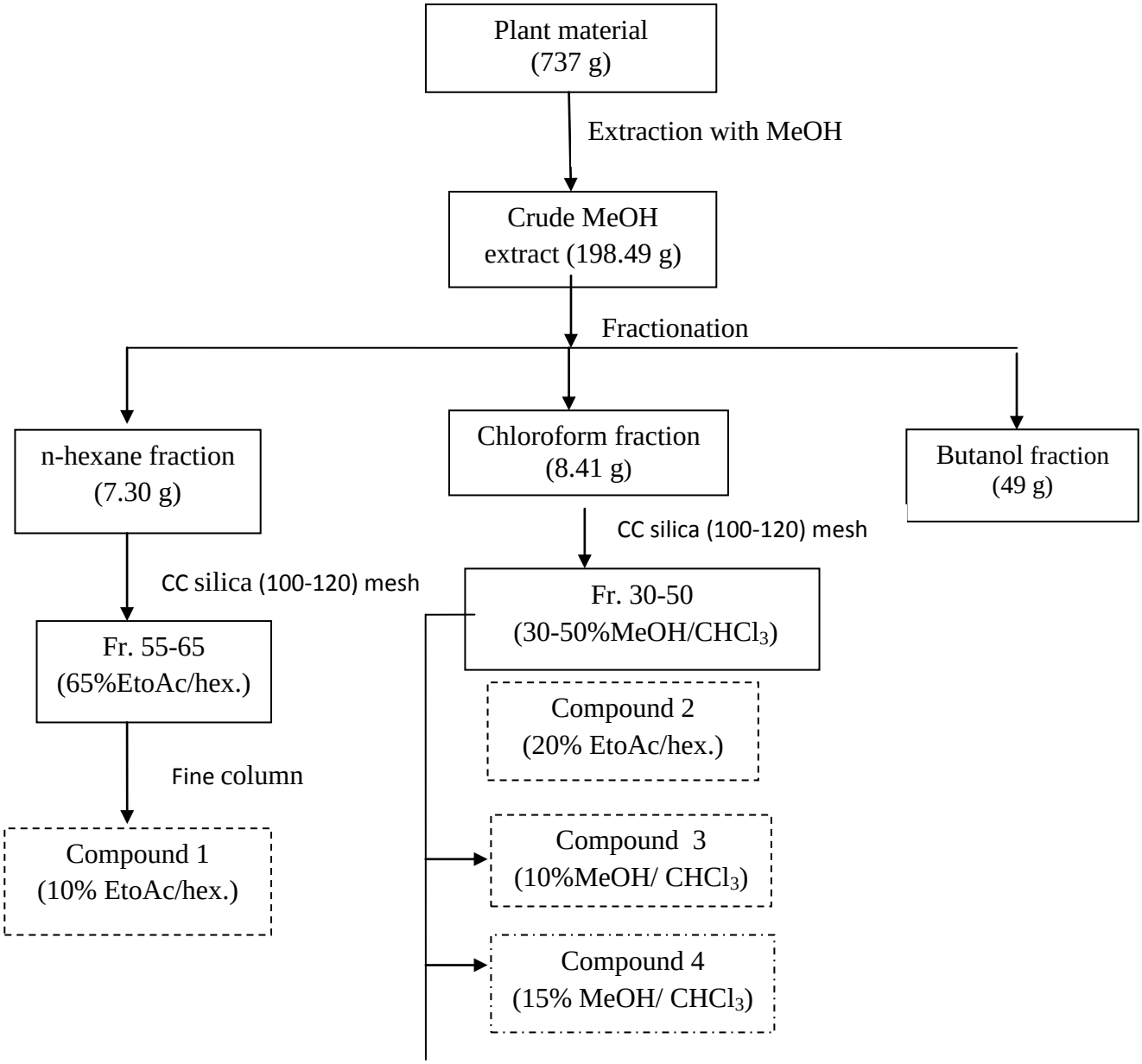


Figure # 1.Scheme for extraction, fractionation and isolation of compounds from *Syzygium cumini*: Compound-1 (beta-sitosterol), 2 (oleanolic acid), 3 (gallic acid) and 4 (quercetin)

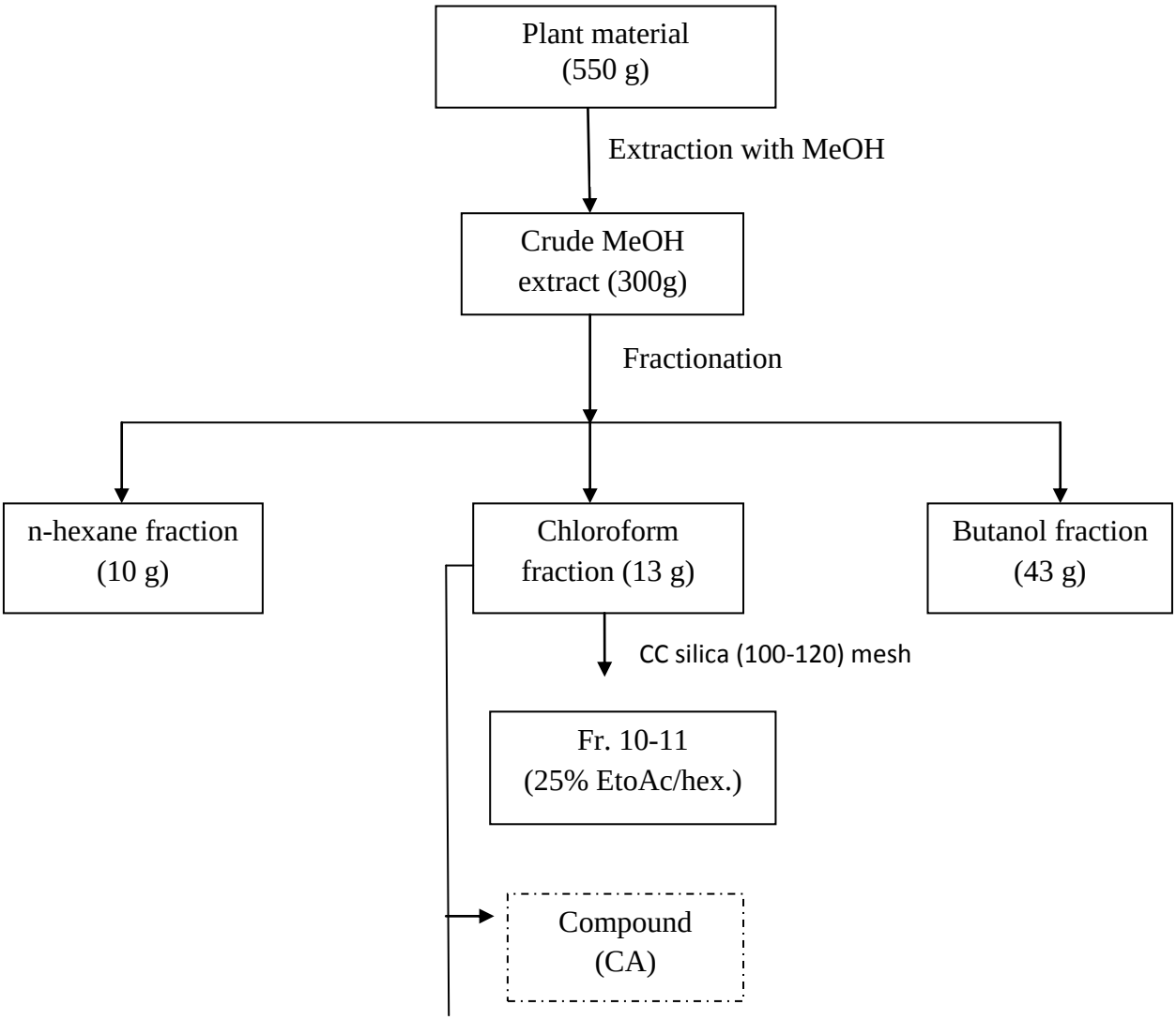


Figure # 2. Scheme for extraction, fractionation and isolation of compound from *Carissa carandas*:CA (carissic acid)

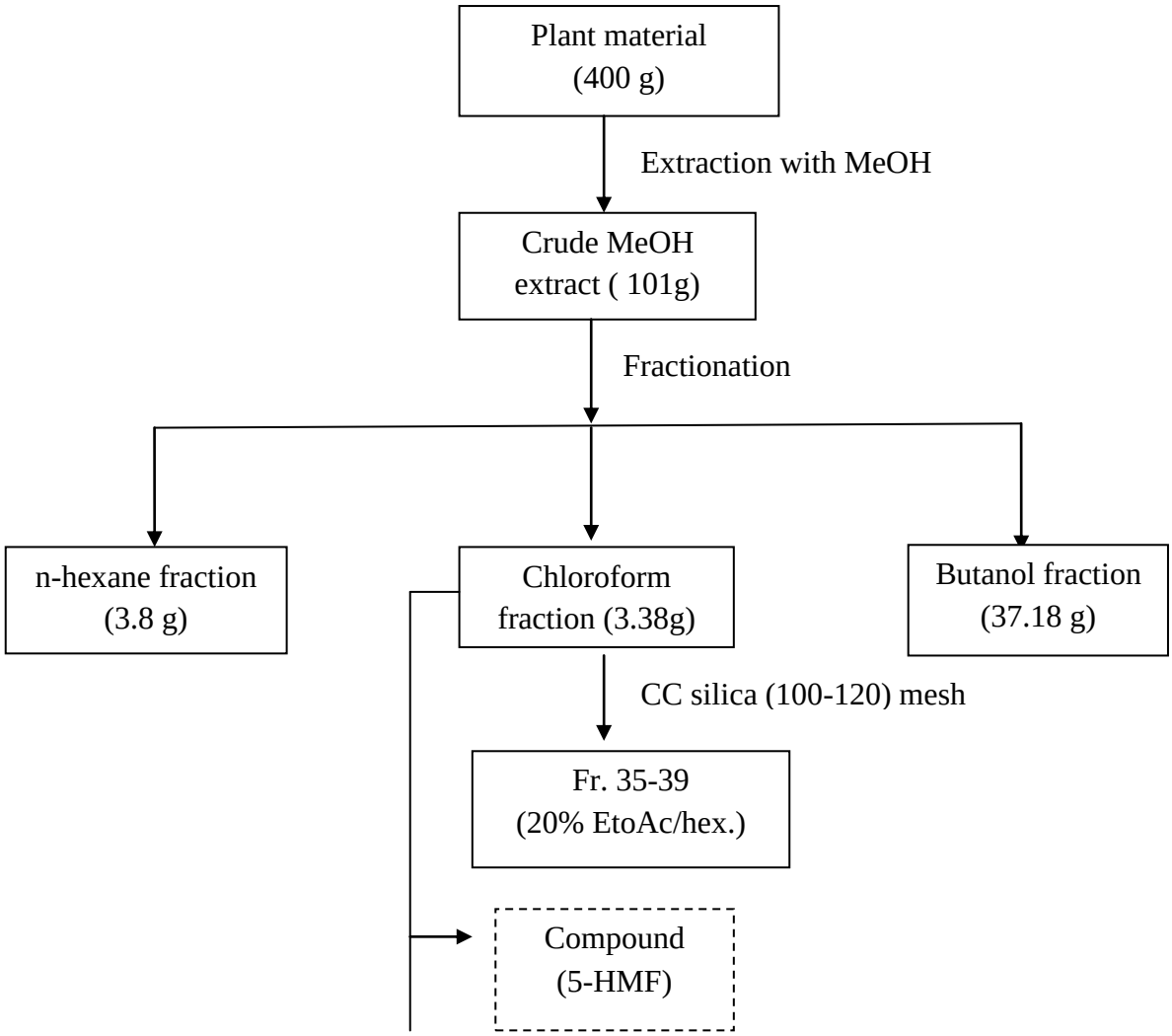


Figure # 3. Scheme for extraction, fractionation and isolation of compound from *Grewia asiatica*:5-HMF (5-hydroxy methyl furfural)

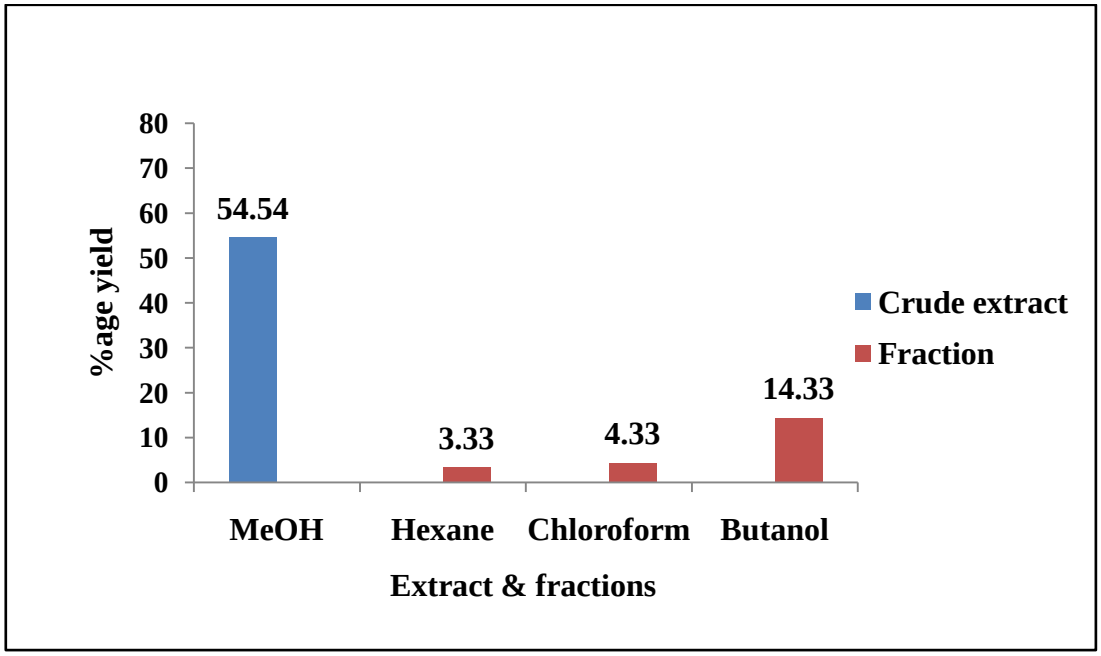


Figure # 4. Percent yield of extract and fractions of *Carissa carandas*

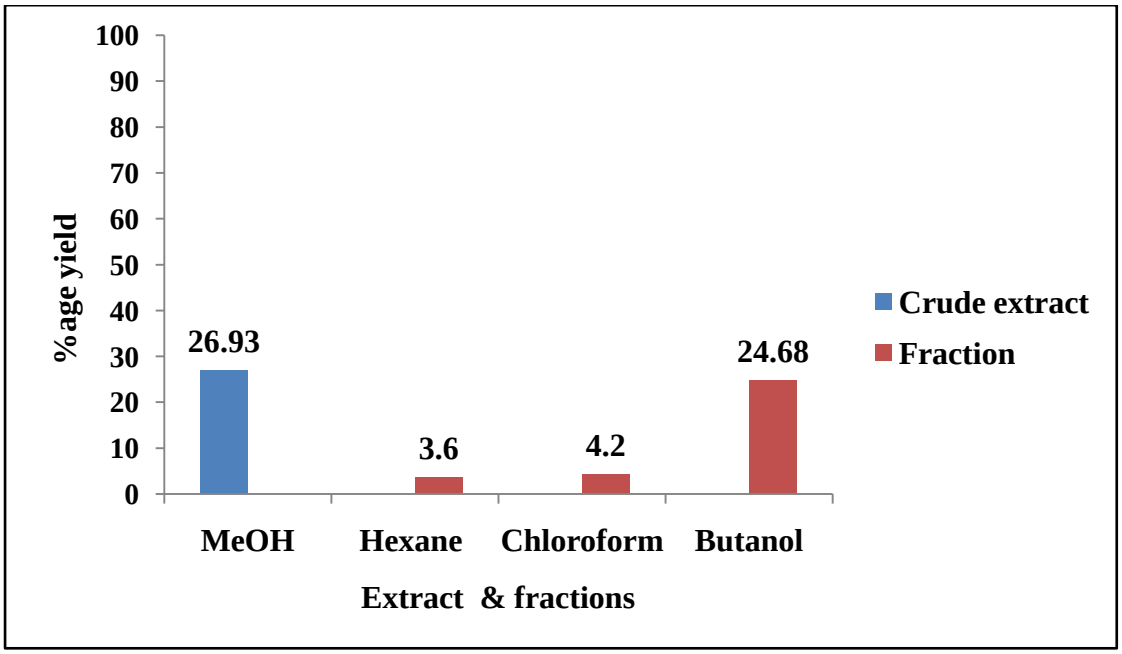


Figure # 5. Percent yield of extract and fractions of *Syzygium cumini*

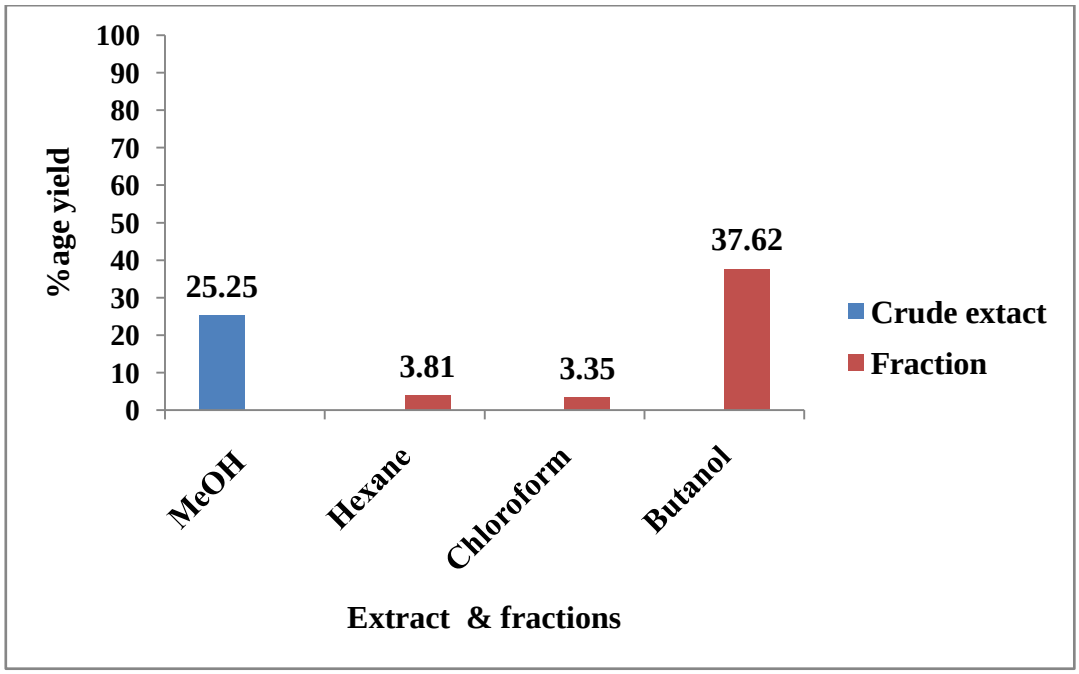


Figure # 6. Percent yield of extract and fractions of *Grewia asiatica*

Results

CHAPTER-4

RESULTS

For evaluating *in vitro* cytotoxic effect against human cancer cell lines, a total of four fruits (mentioned below) were selected from Jammu region. These fruits possess numerous medicinal properties and used in various ways against many diseases.

1. *Artocarpus heterophyllus* (katahal)
2. *Carrissa carandas* (karonda)
3. *Grewia asiatica* (phalsa)
4. *Syzygium cumini* (jamun)

Systematic bioassays were performed against eight (08) human cancer cell lines originated from five (05) different tissues, which were obtained from National Cancer Institute, Frederick, U.S.A and National Centre for Cell Science, Pune, India. These human cancer cell lines along with particular tissues are as under:

1. Breast: MCF-7
2. Breast: MDAMB-231
3. Colon: HCT-116
4. Colon: HT-29
5. Colon: SW-620
6. Lung: A-549
7. Pancreatic: MIA PaCa-2
8. Prostate: PC-3

Positive controls (standard drugs for cancer) used in the present investigation included the following:

1. Doxorubicin
2. 5-Flurouracil

3. Mitomycin-C
4. Paclitaxel

Standard protocols as given in Chapter-III of *"Materials and Methods"* were employed for the extraction and fractionation of powdered dried plant material and the following extracts and fractions were prepared / employed for bioassay:

1. Methanolic extract, 99% (v/v)
2. Fractions: n-hexane, chloroform and butanol soluble

Furthermore, isolation & characterisation of active ingredients was carried out as the procedure detailed in Chapter-III of *"Materials and Methods"*.The results thus obtained have been shown in Tables (Table # 3-11) along with important observations summarized in Figures (Figure # 7-35).

Table # 3. Growth inhibitory effect of *Artocarpus heterophyllus*(katahal) against human cancer cell lines

Extract	Conc. (µg/mL)	Human cancer cell lines from five different tissues							
		Breast	Breast	Colon	Colon	Colon	Lung	Pancreatic	Prostate
		MCF-7	MDAMB-231	HCT-116	HT-29	SW-620	A-549	MIA PaCa-2	PC-3
Methanolic	100	Growth Inhibition (%)							
		14	2	43	0	6	31	0	0
Positive controls (standard drugs)	Conc. (µM)								
Doxorubicin	1	65	65	-	-	-	-	-	-
5-Fluorouracil	20	-	-	52	52	65	-	-	-
Mitomycin-C	1	-	-	-	-	-	-	-	66
Paclitaxel	1	-	-	-	-	-	78	-	-
Paclitaxel	50	-	-	-	-	-	-	87	-

Mark (-) indicates that particular human cancer cell line was not treated with that particular positive control

Table # 4. Growth inhibitory effect of *Carissa carandas*(karonda) against human cancer cell lines

Extract & Fractions	Conc. (µg/mL)	Human cancer cell lines from five different tissues							
		Breast	Breast	Colon	Colon	Colon	Lung	Pancreatic	Prostate
		MCF-7	MDAMB-231	HCT-116	HT-29	SW-620	A-549	MIA PaCa-2	PC-3
		Growth Inhibition (%)							
Extract (Methanolic)	**100**	**70**	52	65	**72**	54	**75**	69	43
Fractions (n-hexane)	**100**	**87**	*	*	68	*	**74**	**74**	*
Chloroform	**100**	**100**	*	*	**100**	*	**100**	**100**	*
Butanol	**100**	24	*	*	0	*	61	**89**	*
Positive controls (standard drugs)	**Conc. (µM)**								
Doxorubicin	**1**	65	65	-	-	-	-	-	-
5-Fluorouracil	**20**	-	-	52	52	65	-	-	-
Mitomycin-C	**1**	-	-	-	-	-	-	-	-
Paclitaxel	**1**	-	-	-	-	-	78	-	66
Paclitaxel	**50**	-	-	-	-	-	-	87	-

Growth inhibition of 70% or more in case of extracts/fractions has been indicated in bold numbers
Mark (-) indicates that particular human cancer cell line was not treated with that particular positive control
Symbol (*) means not further evaluated

Table # 5. Growth inhibitory effect of *Carissa carandas*(karonda) against human cancer cell lines

Fraction	**Conc. (µg/mL)**	**Human cancer cell lines from three different tissues**		
Chloroform		**Breast**	**Colon**	**Lung**
		MCF-7	**HT-29**	**A-549**
		Growth Inhibition (%)		
	100	**100**	**100**	**100**
	50	**98**	**100**	**100**
	30	**89**	**99**	**100**
	10	**77**	**94**	**99**
	1	0	37	24
IC_{50} (µg/mL)		3.98±0.24	1.28±0.02	1.48±0.002
Positive controls (standard drugs)	**Conc.(µM)**			
Doxorubicin	**1**	65	-	-
Paclitaxel	**1**	-	-	78
5-Fluorouracil	**20**	-	52	-

Growth inhibition of 70% or more in case of fraction has been indicated in bold numbers
Mark (-) indicates that particular human cancer cell line was not treated with that particular positive control

Table # 6. Growth inhibitory effect of *Grewia asiatica*(phalsa) against human cancer cell lines

Extract & Fractions	Conc. (µg\mL)	Human cancer cell lines from five different tissues							
		Breast	Breast	Colon	Colon	Colon	Lung	Pancreatic	Prostate
		MCF-7	MDAMB-231	HCT-116	HT-29	SW-620	A-549	MIA PaCa-2	PC-3
		Growth Inhibition (%)							
Extract (Methanolic)	**100**	31	34	0	0	54	0	19	0
Fractions (n-hexane)	**100**	23	44	0	0	**76**	0	17	0
Chloroform	**100**	19	29	0	0	**70**	0	0	0
Butanol	**100**	29	35	0	0	32	0	1	0
Positive controls (standard drugs)	**Conc. (µM)**								
Doxorubicin	**1**	65	65	-	-	-	-	-	-
5-Fluorouracil	**20**	-	-	52	52	65	-	-	-
Mitomycin-C	**1**	-	-	-	-	-	-	-	66
Paclitaxel	**1**	-	-	-	-	-	78	-	-
Paclitaxel	**50**	-	-	-	-	-	-	87	-

Growth inhibition of 70% or more in case of extracts/fractions has been indicated in bold numbers
Mark (-) indicates that particular human cancer cell line was not treated with that particular positive control

Table # 7. Growth inhibitory effect of *Syzygium cumini* (jamun) against human cancer cell lines

Extract & Fractions	Conc. (µg/mL)	Human cancer cell lines from five different tissues							
		Breast	Breast	Colon	Colon	Colon	Lung	Pancreatic	Prostate
		MCF-7	MDAMB-231	HCT-116	HT-29	SW-620	A-549	MIA PaCa-2	PC-3
		Growth Inhibition (%)							
Extract (Methanolic)	**100**	21	44	**74**	32	65	**70**	46	**72**
Fractions (n-hexane)	**100**	28	**91**	**99**	**77**	**95**	**88**	**99**	**77**
Chloroform	**100**	21	**76**	**81**	13	**87**	**76**	**99**	12
Butanol	**100**	48	64	49	53	61	37	32	7
Positive controls (standard drugs)	**Conc. (µM)**								
Doxorubicin	**1**	65	65	-	-	-	-	-	-
5-Fluorouracil	**20**	-	-	52	52	65	-	-	-
Mitomycin-C	**1**	-	-	-	-	-	-	-	66
Paclitaxel	**1**	-	-	-	-	-	78	-	-
Paclitaxel	**50**	-	-	-	-	-	-	87	-

Growth inhibition of 70% or more in case of extracts/fractions has been indicated in boldnumbers
Mark (-) indicates that particular human cancer cell line was not treated with that particular positive control

Table # 8. Growth inhibitory effect of of *Syzygium cumini*(jamun) againsthuman cancer cell lines

Fraction	Conc. (µg/mL)	Human cancer cell lines from five different tissues						
		Breast	Colon	Colon	Colon	Lung	Pancreatic	Prostate
		MDAMB-231	HCT-116	HT-29	SW-620	A-549	MIA PaCa-2	PC-3
		Growth Inhibition (%)						
n-hexane	**100**	**91**	**99**	**77**	**95**	**88**	**99**	**77**
	50	27	65	0	59	56	58	35
	30	25	48	0	58	51	50	0
	10	22	0	0	29	16	37	0
	1	20	0	0	1	0	0	0
IC_{50} (µg/mL)		>50±2.39	36.25±0.77	>50±1.46	26.81±0.77	35.16±1.46	29.26±0.97	>50±2.17
Positive controls (standard drugs)	**Conc. (µM)**							
Doxorubicin	**1**	65	-	-	-	-	-	-
5-Fluorouracil	**20**	-	52	52	65	-	-	-
Mitomycin-C	**1**	-	-	-	-	-	-	66
Paclitaxel	**1**	-	-	-	-	78	-	-
Paclitaxel	**50**	-	-	-	-	-	87	-

Growth inhibition of 70% or more in case of fraction has been indicated in bold numbersMark (-) indicates that particular human cancer cell line was not treated with that particular positive control

Table # 9. Growth inhibitory effect of *Syzygium cumini*(jamun) against human cancer cell lines

Fraction	Conc. (µg/mL)	Human cancer cell lines from four different tissues				
		Breast	**Colon**	**Colon**	**Lung**	**Pancreatic**
		MDAMB-231	**HCT-116**	**SW-620**	**A-549**	**MIA PaCa-2**
		Growth Inhibition (%)				
Chloroform	**100**	**76**	**81**	**87**	**76**	**99**
	50	53	55	25	60	54
	30	50	30	14	45	37
	10	46	22	6	5	20
	1	5.5	8.5	0	0	1
IC_{50}(µg/mL)		18.02±1.01	48.30±3.42	>50±0.28	>50±0.28	43.10±0.26
Positive controls (standard drugs)	**Conc. (µM)**					
5-Fluorouracil	**20**	-	52	65	-	-
Doxorubicin	**1**	65	-	-	-	-
Paclitaxel	**1**	-	-	-	78	-
Paclitaxel	**50**	-	-	-	-	87

Growth inhibition of 70% or more in case of fraction has been indicated in bold numbers
Mark (-) indicates that particular human cancer cell line was not treated with that particular positive control

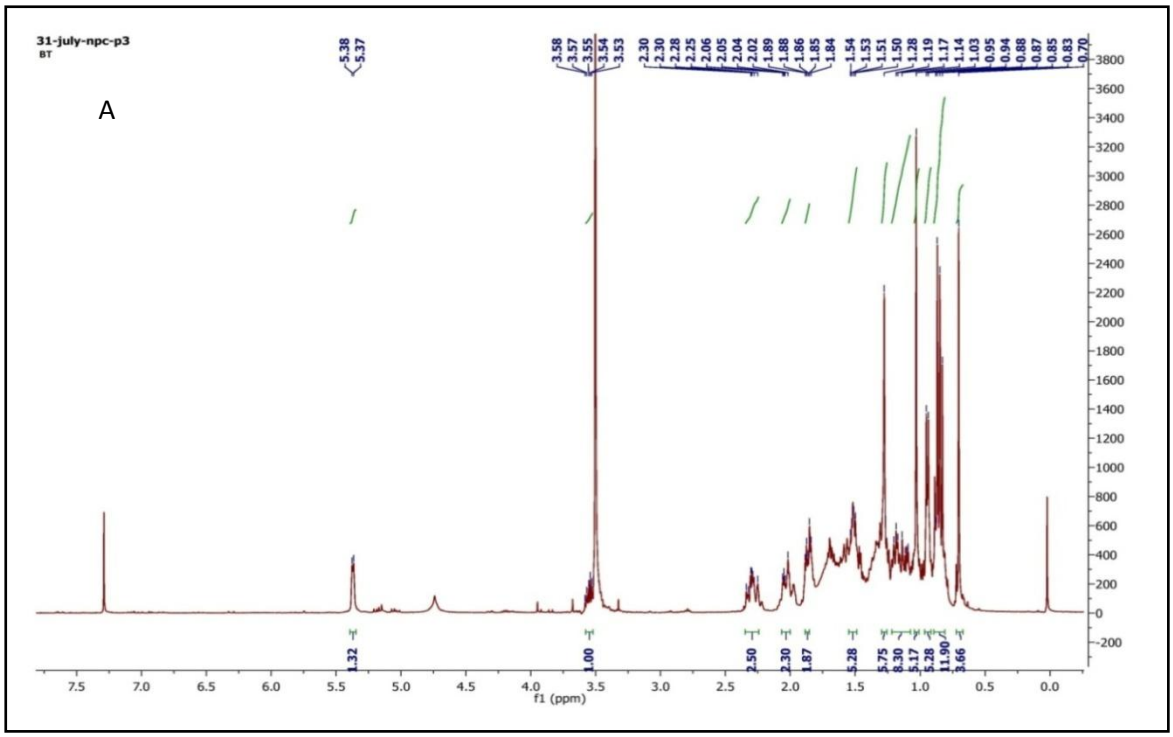
Table # 10. Growth inhibitory effect of Beta-sitosterol isolated from *Syzygium cumini* against human cancer cell lines

Compound	Conc. (µM)	Human cancer cell lines from five different tissues							
		Breast	Breast	Colon	Colon	Colon	Lung	Pancreatic	Prostate
		MCF-7	MDAMB-231	HCT-116	HT-29	SW-620	A-549	MIA PaCa-2	PC-3
		Growth Inhibition (%)							
Beta sitosterol	**50**	10	0	8	0	**58**	6	0	43
	30	*	*	*	*	36	*	*	*
	10	*	*	*	*	12.5	*	*	*
	1	*	*	*	*	0	*	*	*
IC_{50} (µM)						42.46±2.30			
Positive controls (standard drugs)	Conc. (µM)								
Doxorubicin	**1**	65	65	-	-	-	-	-	-
5-Fluorouracil	**20**	-	-	52	52	65	-	-	-
Mitomycin-C	**1**	-	-	-	-	-	-	-	-
Paclitaxel	**1**	-	-	-	-	-	78	-	66
Paclitaxel	**50**	-	-	-	-	-	-	87	-

Growth inhibition of 50% or more in case of compound has been indicated in bold numbers
Mark (-) indicates that particular human cancer cell line was not treated with that particular positive control
Symbol (*) means not further evaluated

Table # 11. Growth inhibitory effect of 5-HMF (5-hydroxy methyl furfural)isolated from *Grewia asiatica* against colon cancer cell line

Compound	Conc. (µM)	Colon cancer cell line
		SW-620
		Growth Inhibition (%)
5-HMF	50	40
	30	0
	10	0
	1	0
IC_{50} (µM)		>50±0.77
Positive control (standard drug)		
5- Fluorouracil	20	65



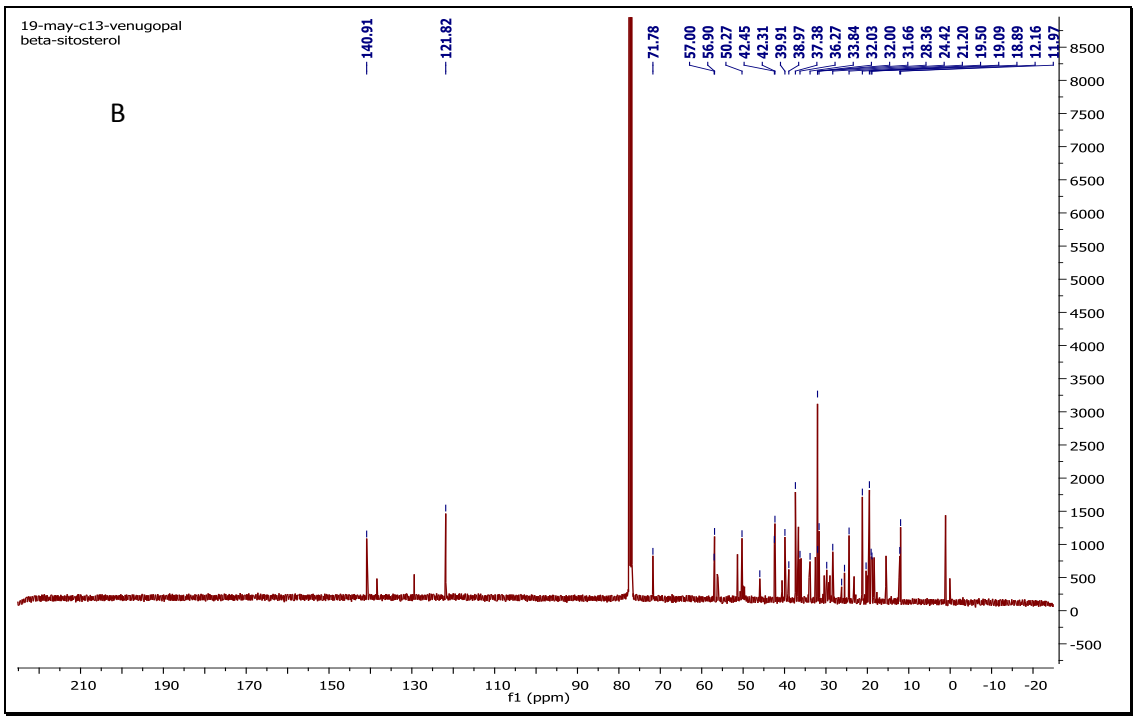
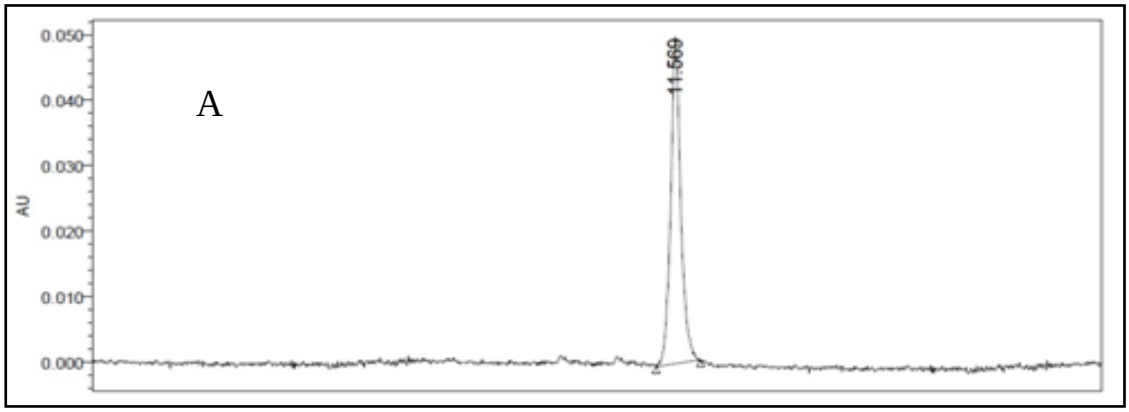


Figure # 7. NMR Spectra (A) ^{1}HNMR ($CDCl_3$& CD_3OD; 400MHz) & (B)^{13}CNMR ($CDCl_3$&CD_3OD; 100 MHz) of beta-sitosterol isolated from *Syzygium cumini*



	RT	Area	% Area	Height
1	11.569	700181	100.00	49901

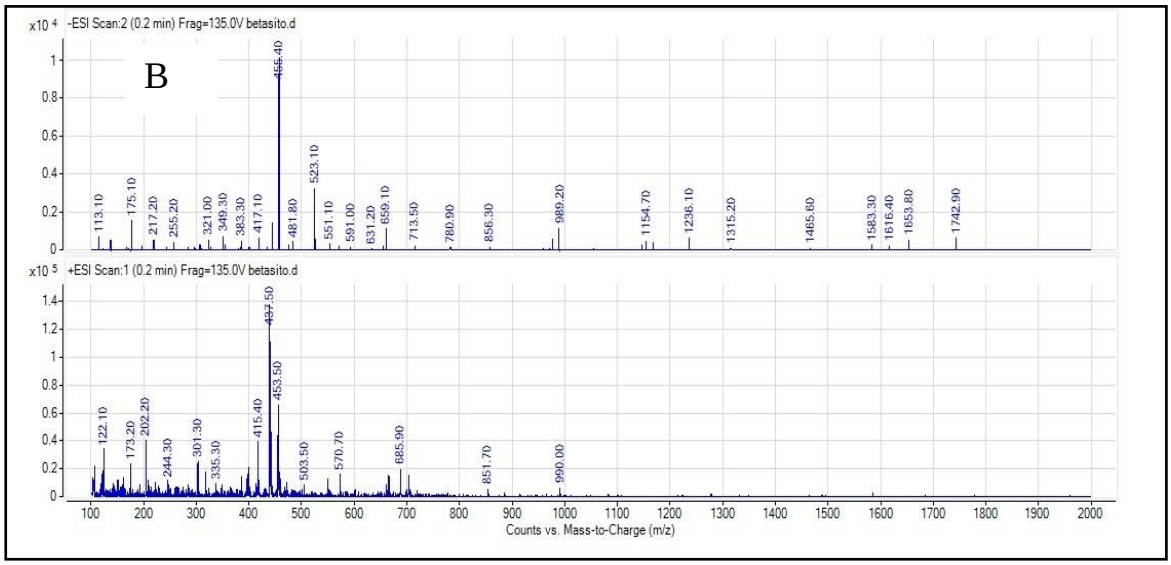
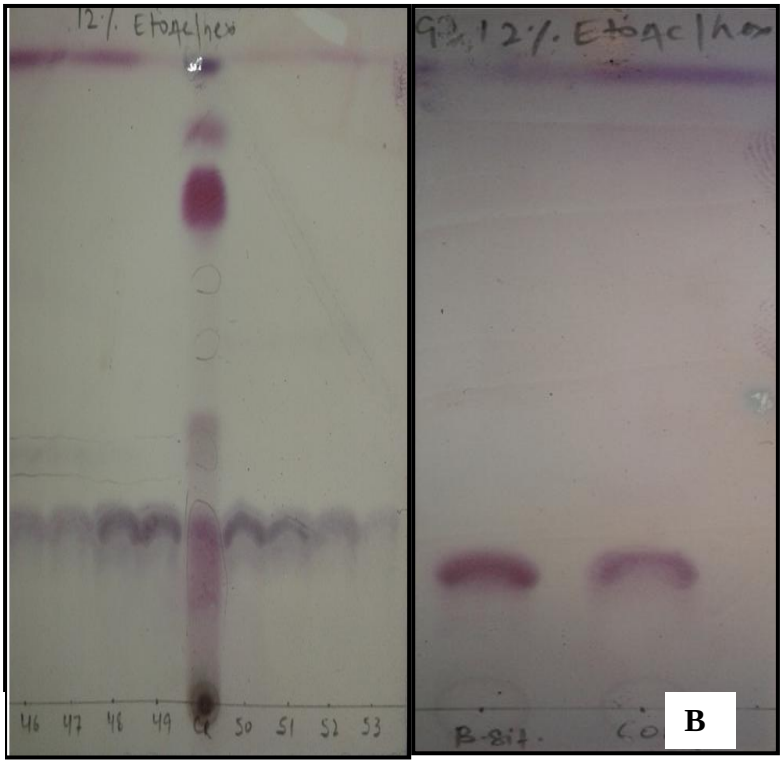
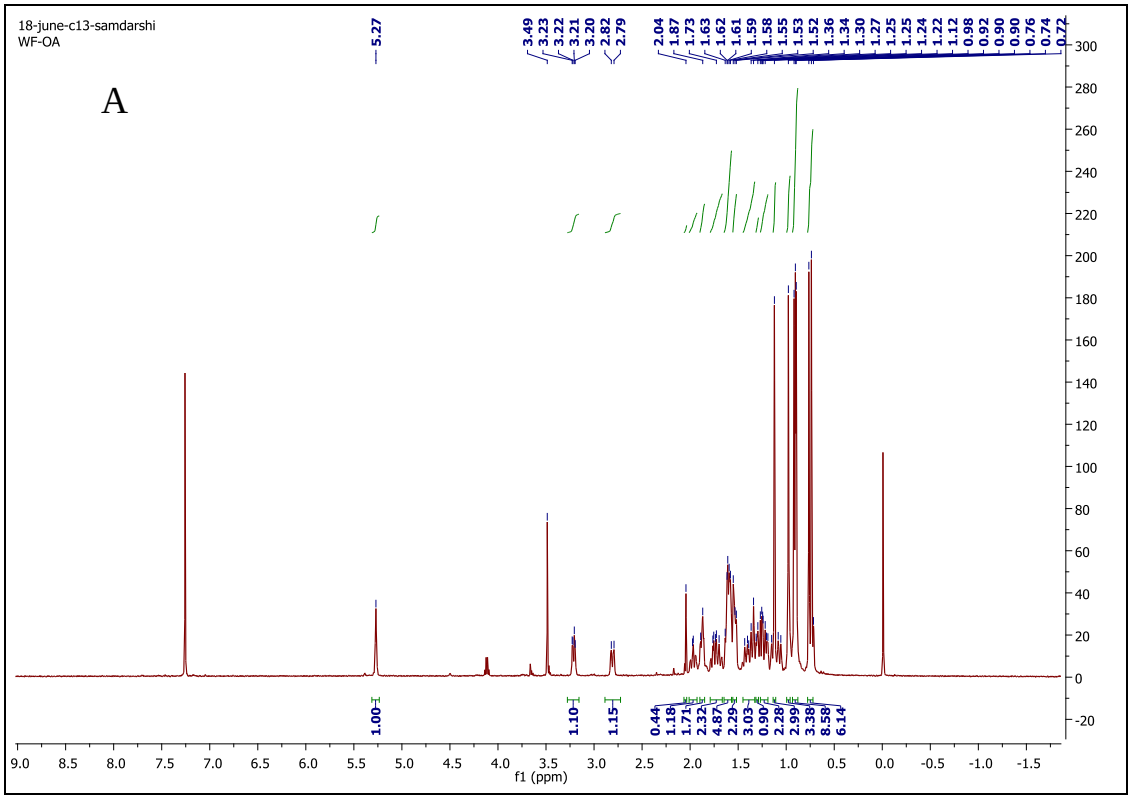


Figure # 8. (A) HPLC and (B) ESIMS chromatogram of beta-sitosterol isolated from
Syzygium cumini



A

Figure # 9. (A) TLC showing fractions (46-53)*i.e.*, beta-sitosterol from column and crude extract (CR) of *Syzygium cumini* (B) TLC of compound isolated and standard with solvent system of 12% EtoAc/hex.



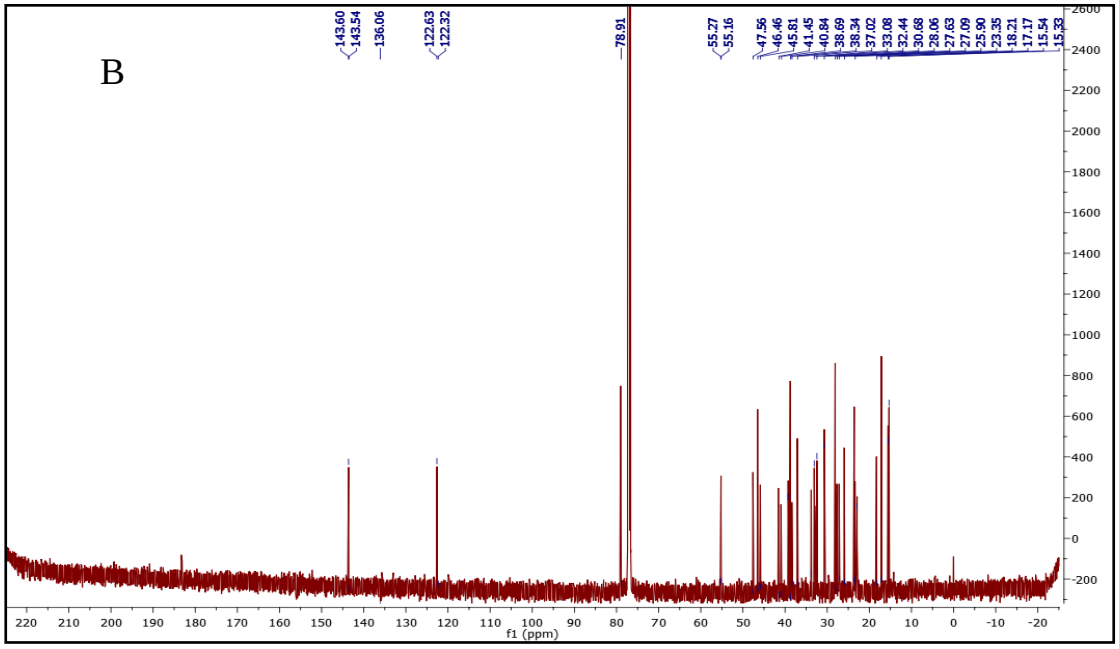


Figure # 10. NMR spectra (A) ^{1}HNMR ($CDCl_3$; 400MHz);(B) ^{13}CNMR ($CDCl_3$; 100MHz) of oleanolic acid isolated from *Syzygium cumini*

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 9: C30 H48 O3	0.419	456.3563	C30 H48 O3	C30 H48 O3	8.95	C30 H48 O3

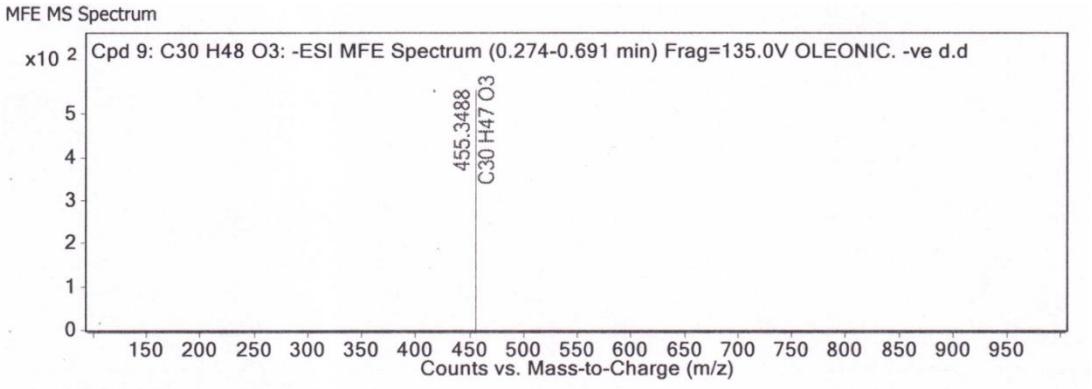


Figure # 11. HRMS chromatogram of oleanolic acid isolated from *Syzygium cumini*

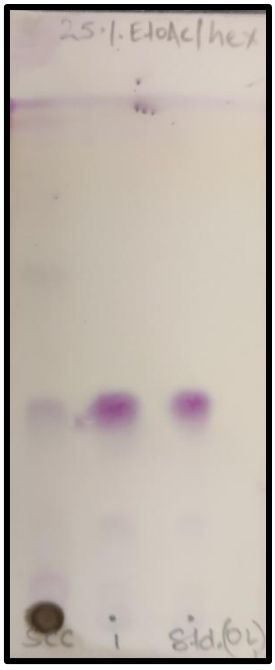
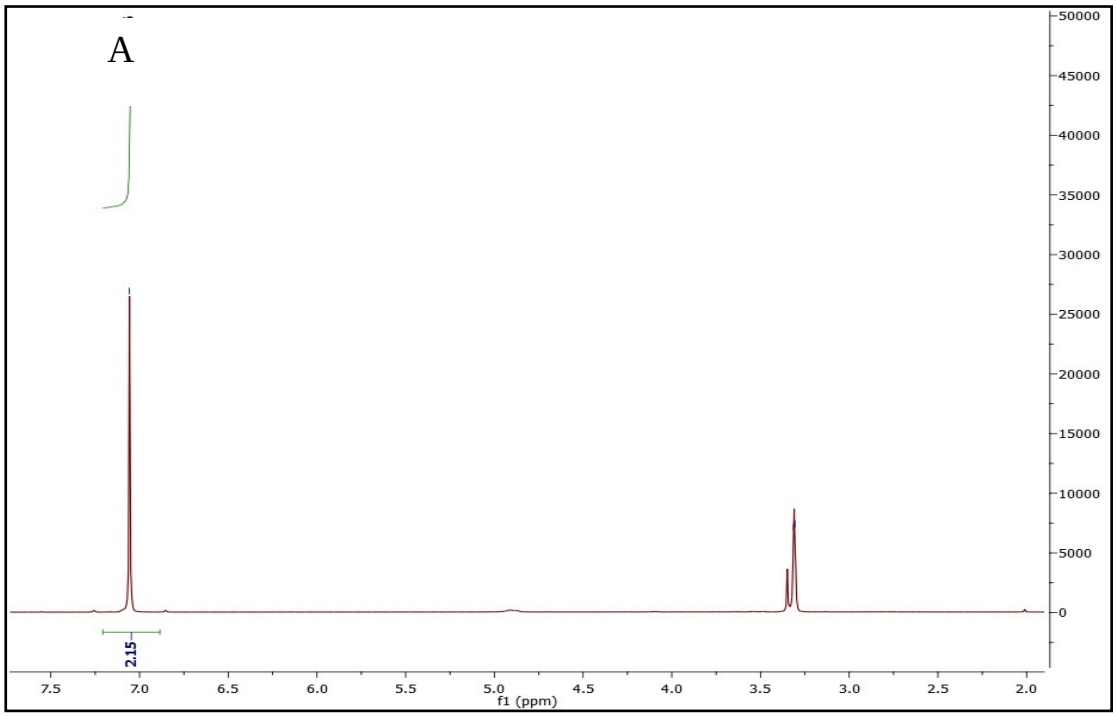


Figure # 12. TLC profile of *Syzygium cumini*crude extract (SCC), compound isolated, oleanolic acid(1) and standard (OL) with solvent system of 25% EtoAc/hex.



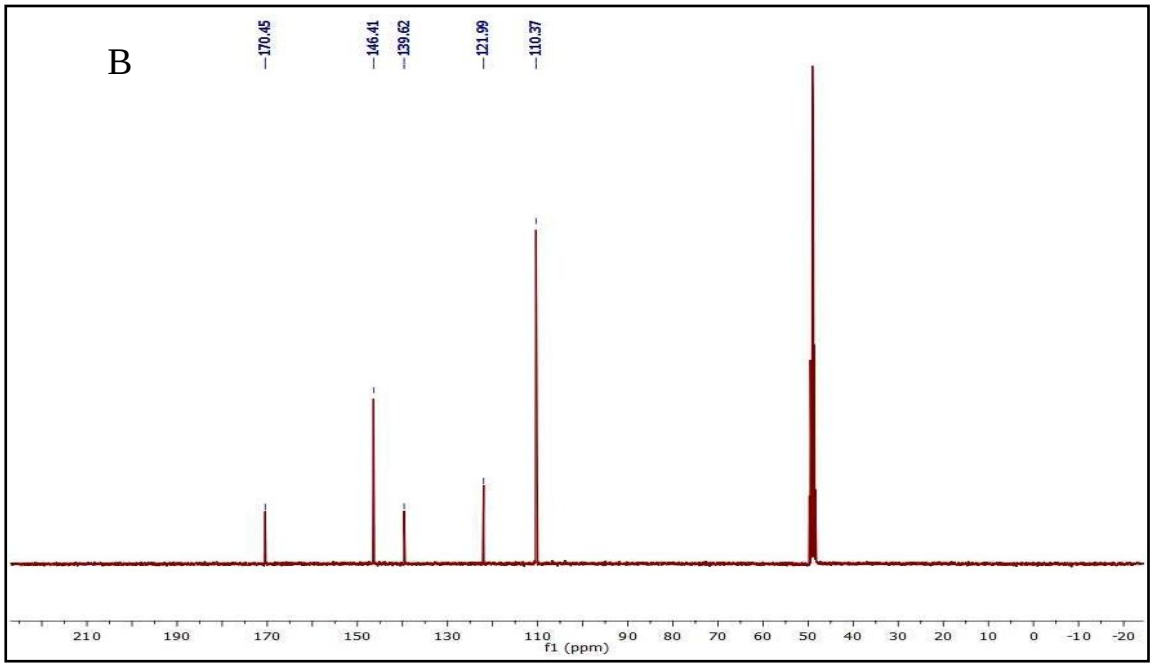


Figure # 13. NMR spectra (A) ^{1}HNMR (400 MHz; CD_3OD) & (B) ^{13}CNMR (100MHz;CD_3OD) of gallic acid isolated from *Syzygium cumini*

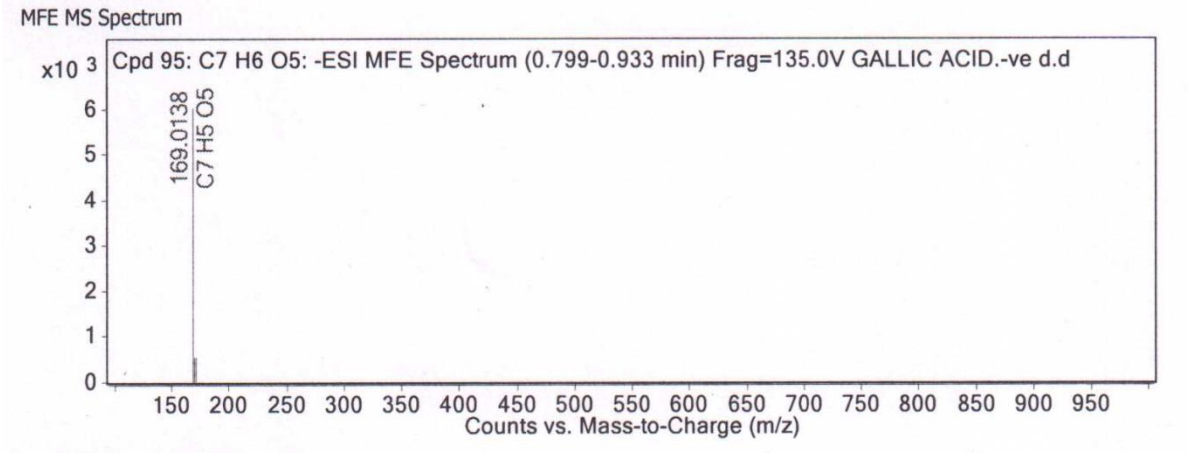


Figure # 14. HRMS chromatogram of gallic acid isolated from *Syzygium cumini*

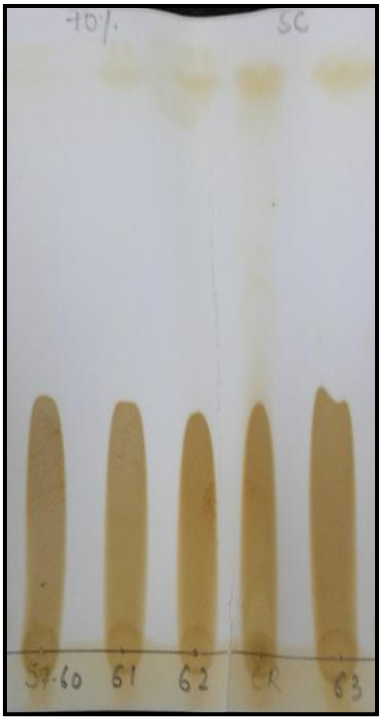
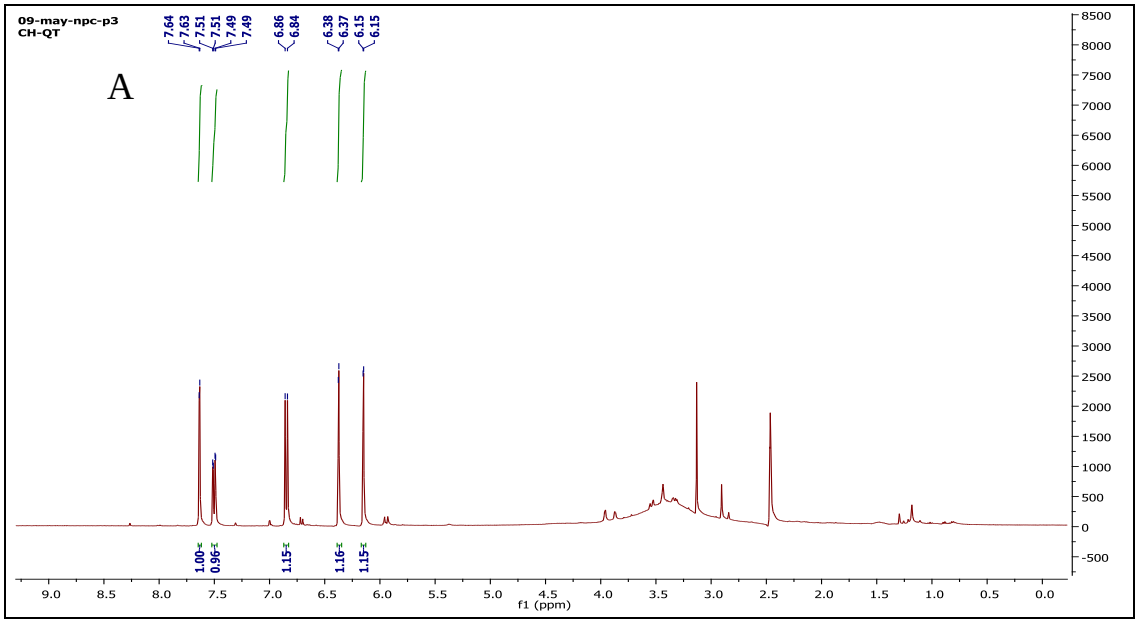


Figure # 15. TLC profile of fractions (59-63)*i.e.*, gallic acid from column and crude
extract (CR) of *Syzygium cumini* withsolvent system of 70% EtoAc/hex.



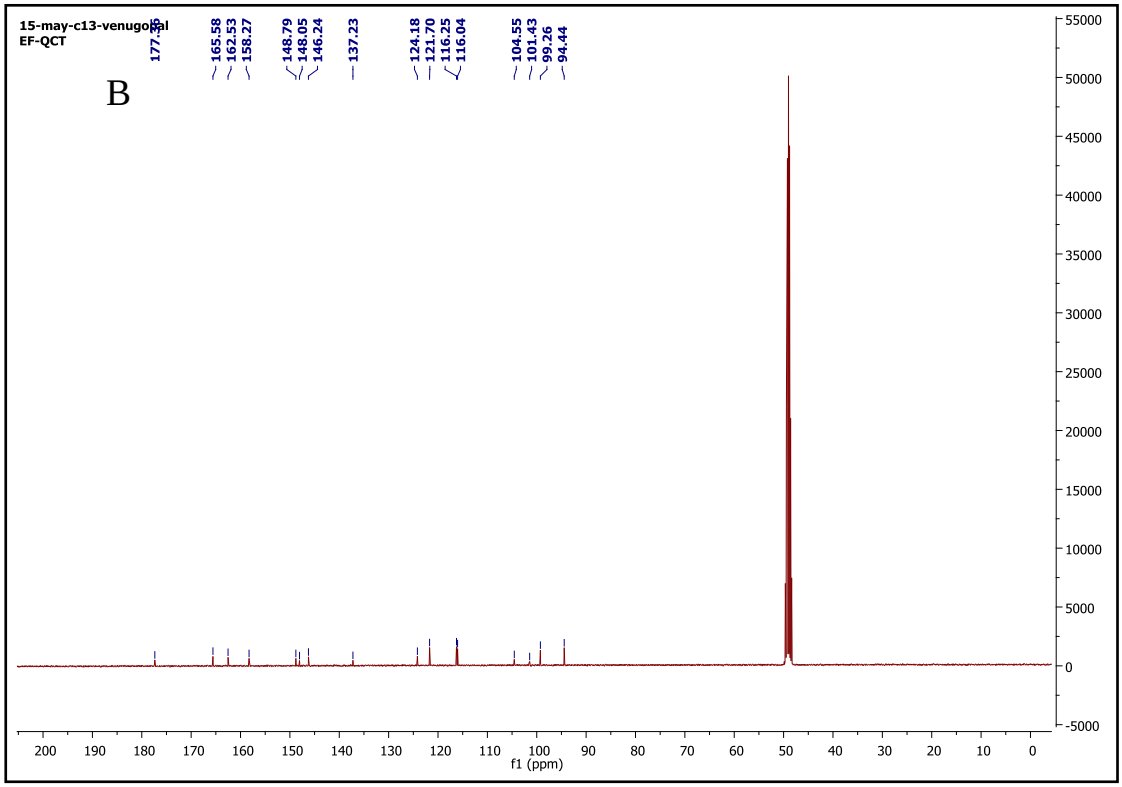
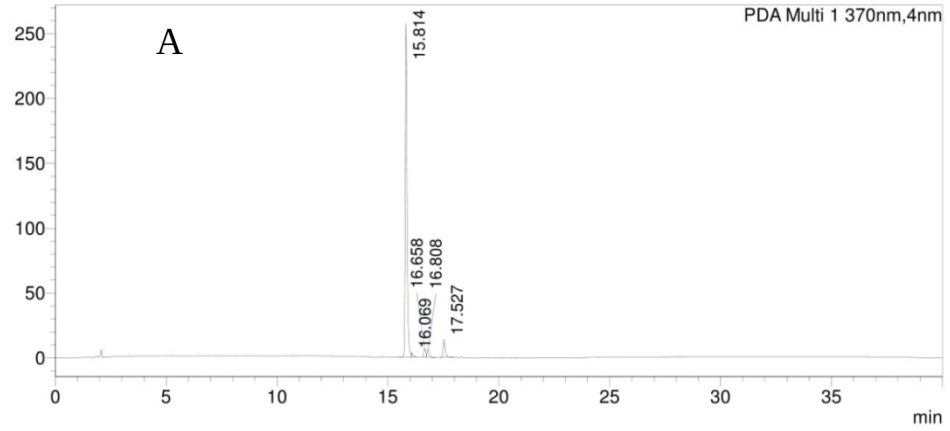


Figure # 16. NMR spectra (A) ^{1}HNMR (400 MHz; CD_3OD) & (B) ^{13}CNMR (100MHz;CD_3OD) of quercetin isolated from *Syzygium cumini*

A



Ret. Time	Area	Height	Area%
15.814	1538040	257095	89.086
16.069	15739	3236	0.912
16.658	39747	6887	2.302
16.808	42675	6385	2.472
17.527	90269	13751	5.229
	1726470	287354	100.000

B

Compound Table

Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 14: C15 H10 O7	0.341	302.0449	C15 H10 O7	C15 H10 O7	-7.57	C15 H10 O7

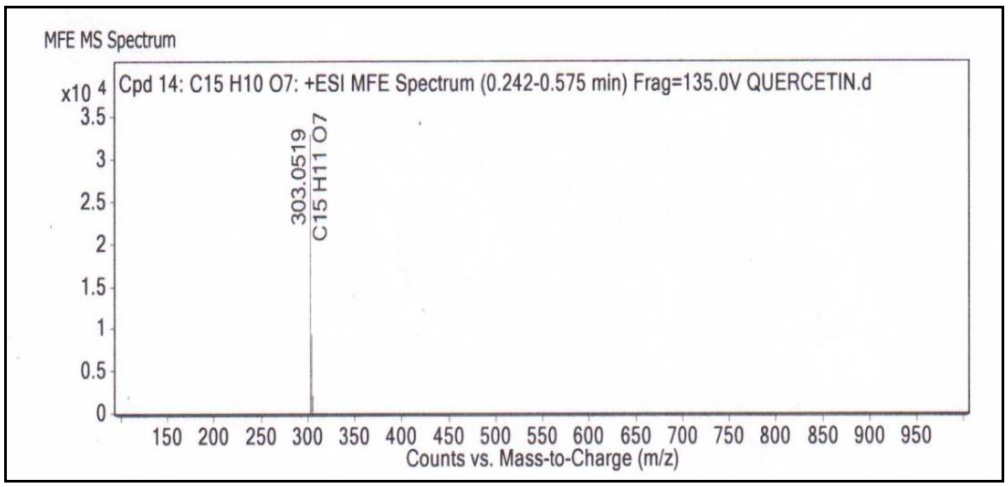


Figure # 17. (A) HPLC and (B) HRMS chromatogram of quercetin isolated from *Syzygium cumini*

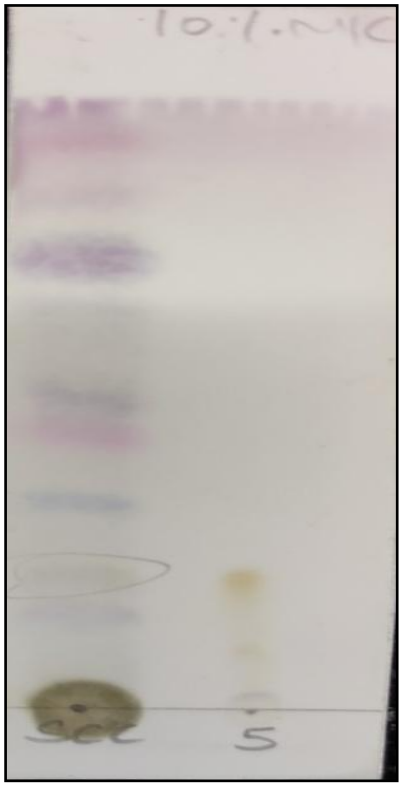


Figure # 18. TLC profile of *Syzygium cumini* crude extract (SCC) and quercetinisolated(5) with solvent system 10% MeOH/$CHCl_3$

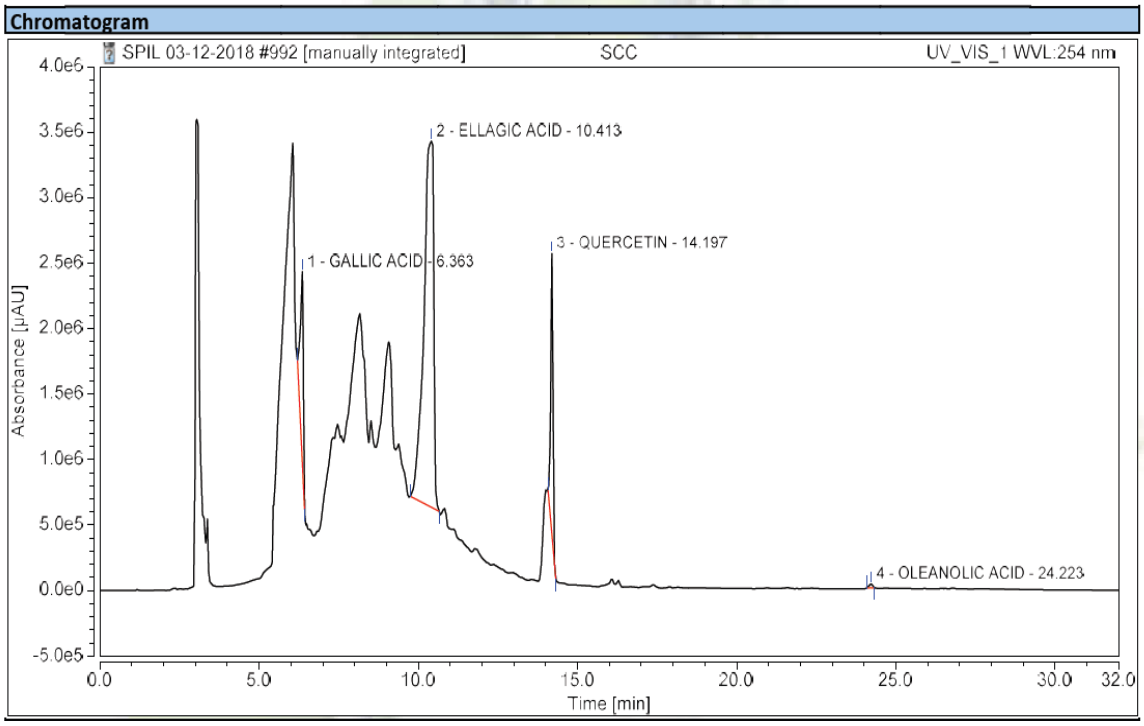


Figure # 19. HPLC chromatogram of *Syzygium cumini* crudeextract

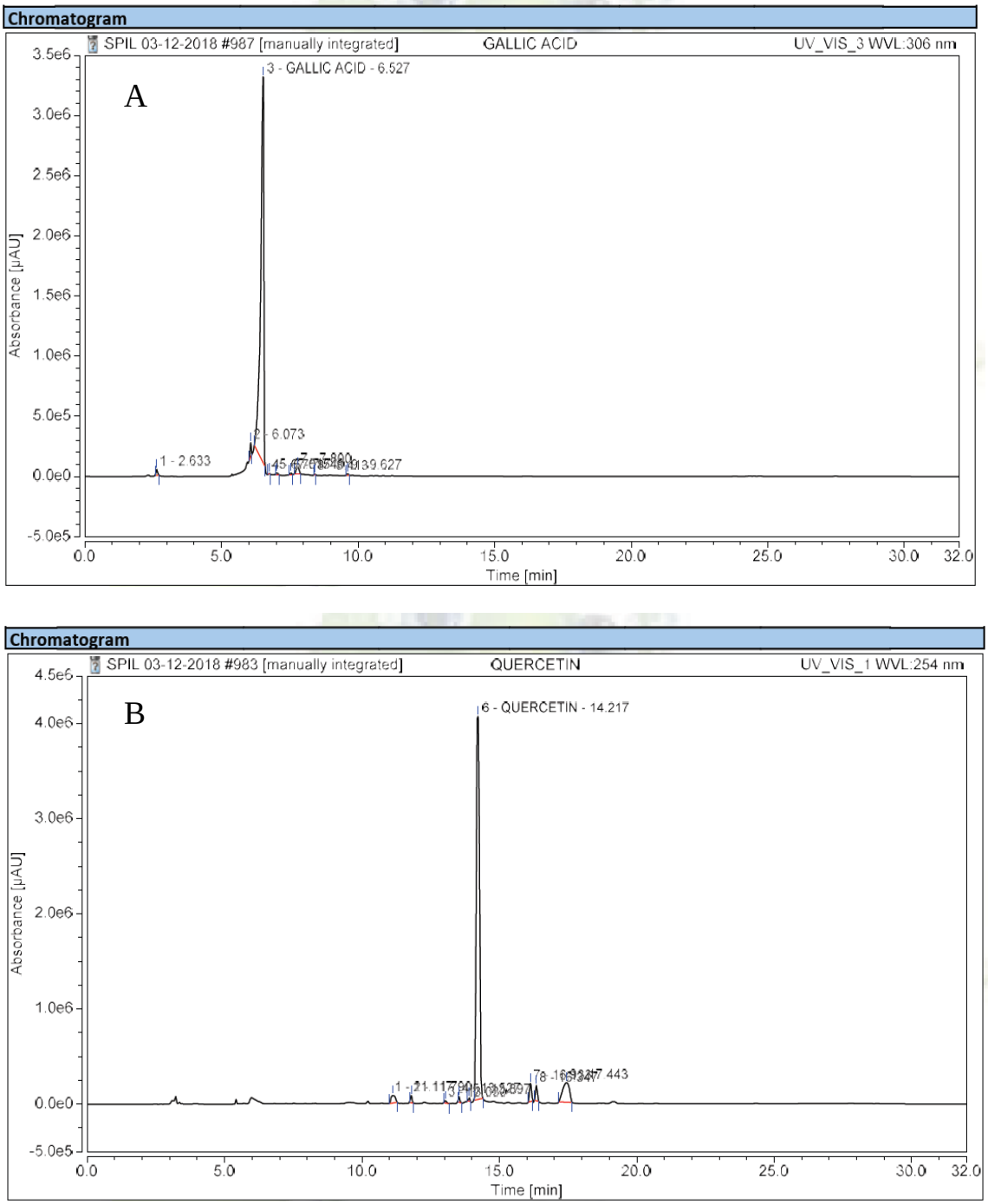
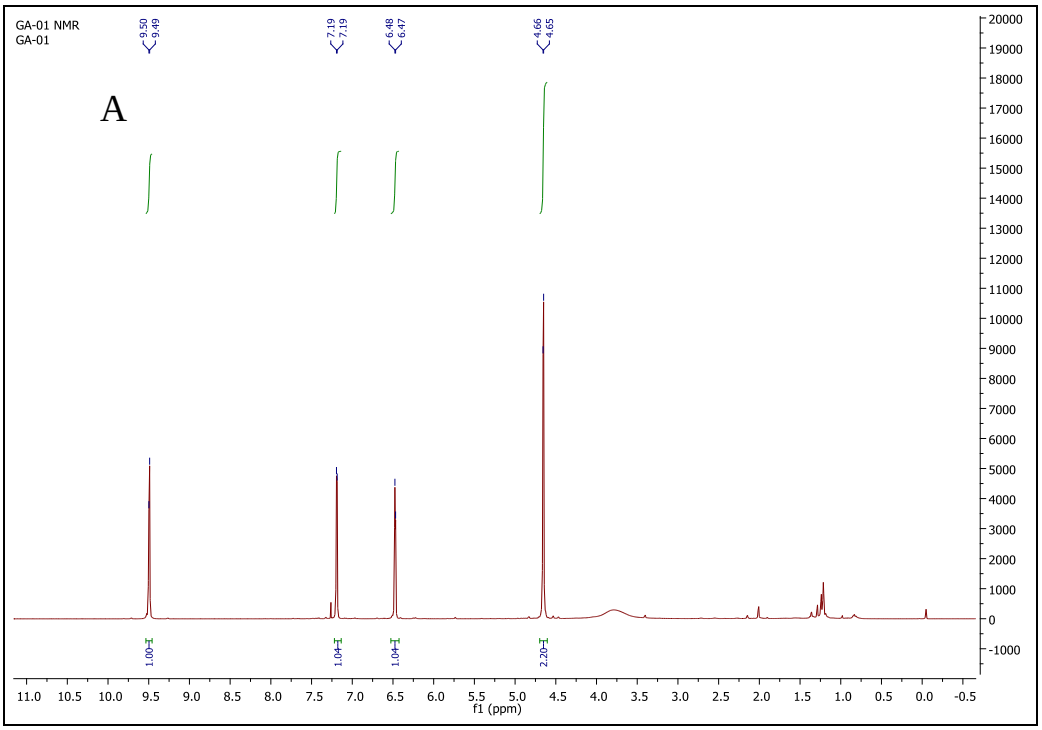


Figure # 20. HPLC chromatogram of (A) Gallic acid and (B) Quercetin isolated from *Syzygium cumini*



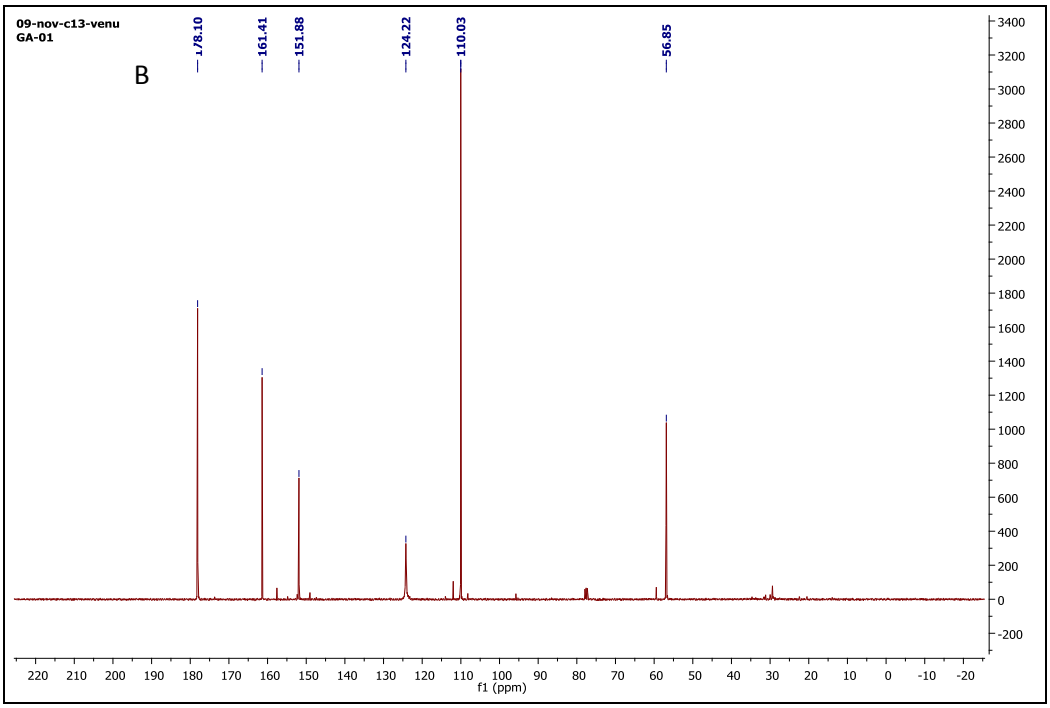
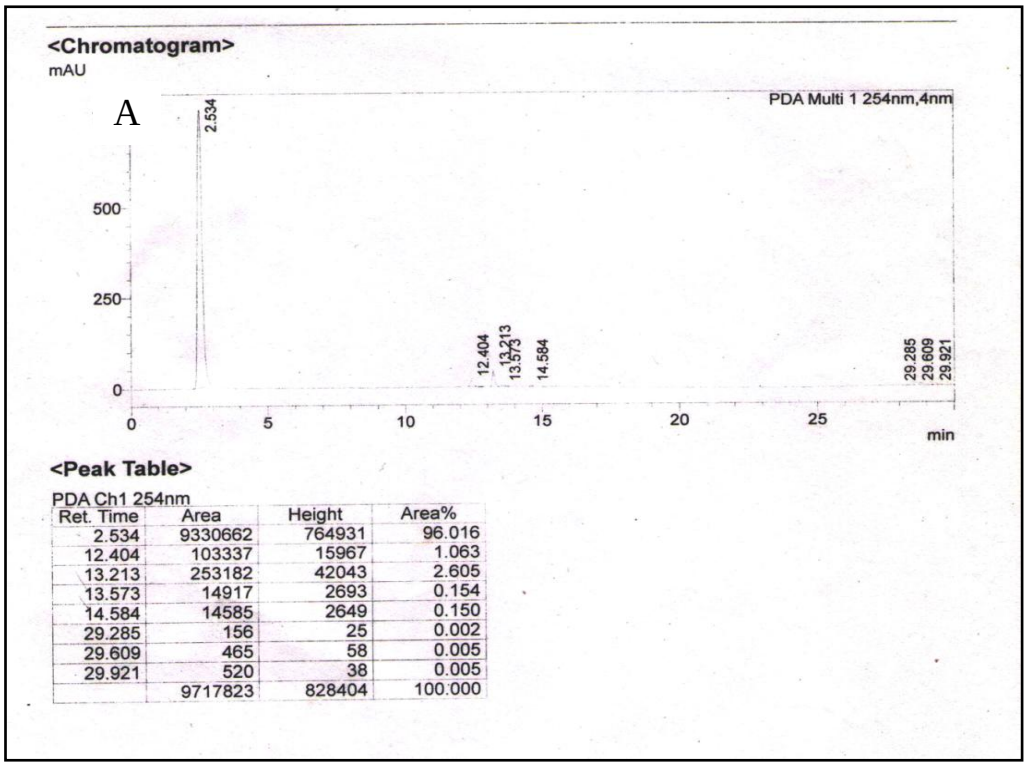


Figure # 21. NMR spectra (A) ^{1}HNMR (400 MHz; $CDCl_3$) & (B)^{13}CNMR (100MHz; $CDCl_3$) of 5-Hydroxy methyl furfural isolated from *Grewia asiatica*

A



<Peak Table>

PDA Ch1 254nm

Ret. Time	Area	Height	Area%
2.534	9330662	764931	96.016
12.404	103337	15967	1.063
13.213	253182	42043	2.605
13.573	14917	2693	0.154
14.584	14585	2649	0.150
29.285	156	25	0.002
29.609	465	58	0.005
29.921	520	38	0.005
	9717823	828404	100.000

B

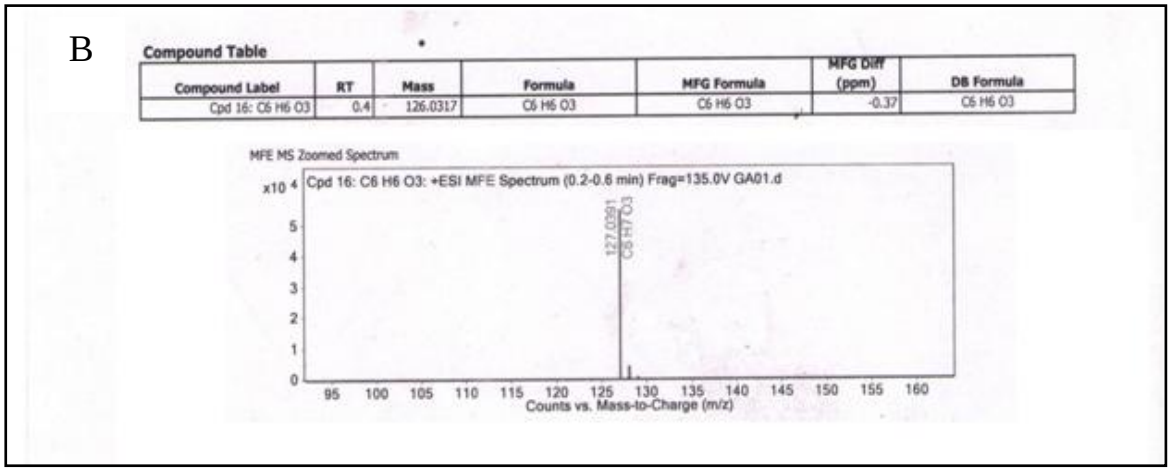


Figure # 22. (A) HPLC and (B) HRMS chromatogram of 5-Hydroxy methyl furfural isolated from *Grewia asiatica*

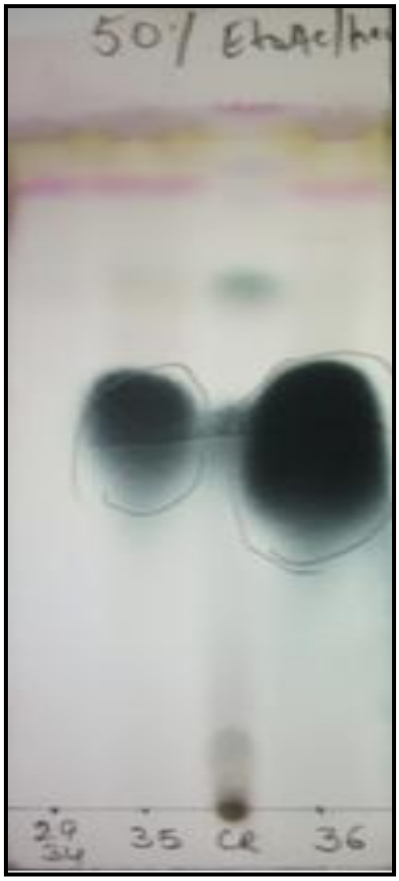


Figure # 23. TLC profile of 5-Hydroxy methyl furfural isolated (35 & 36) and crude extract (CR) of *Grewia asiatica* with solvent system of 50% EtoAc/hex.

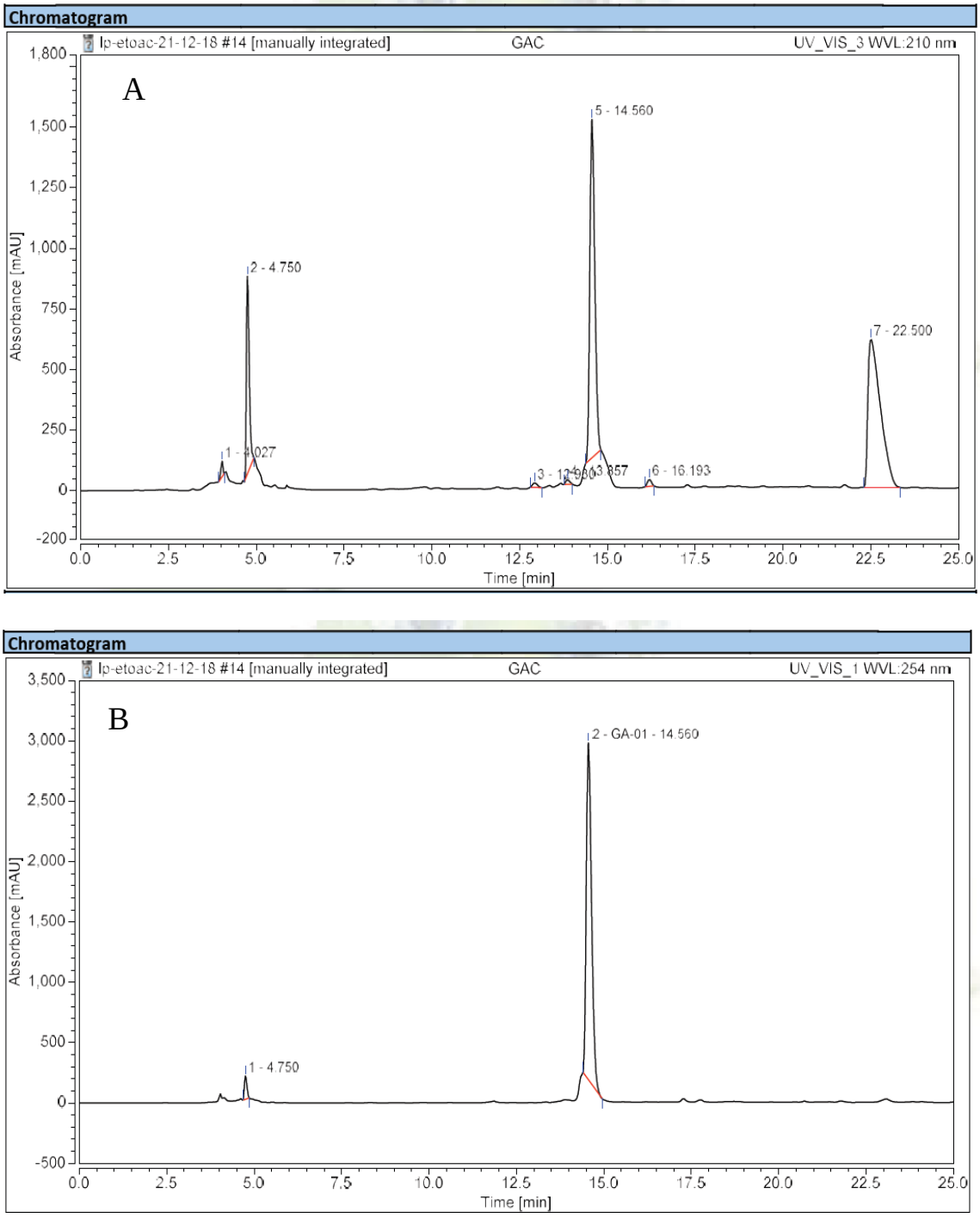
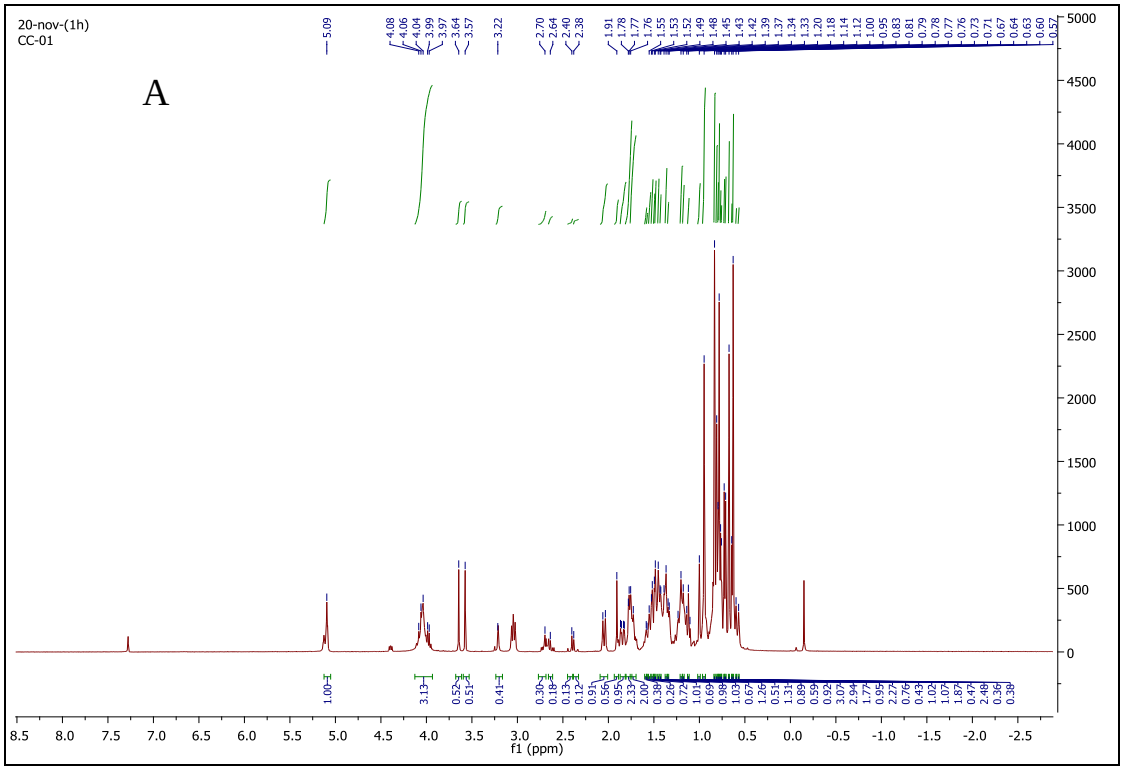


Figure # 24. HPLC chromatogram of *Grewia asiatica*(A) Chloroform fraction and (B) 5- Hydroxy methyl furfural



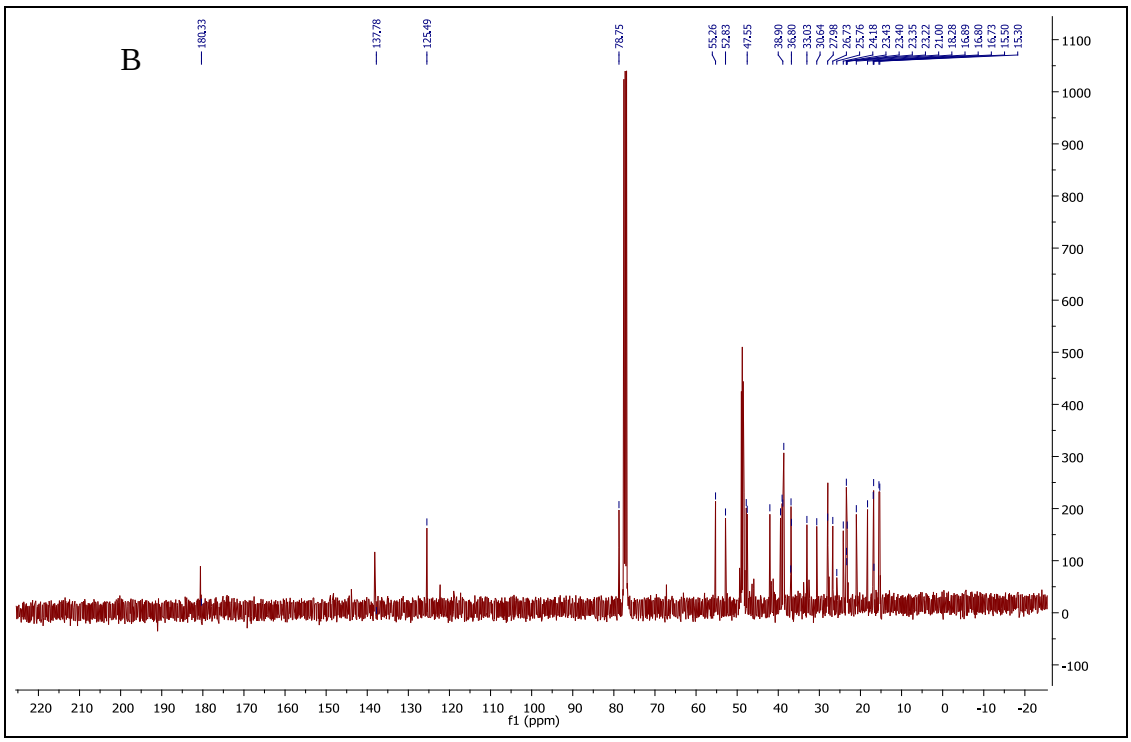


Figure # 25. NMR Spectra (A) ^{1}HNMR ($CDCl_3$& CD_3OD; 400MHz) &^{13}CNMR ($CDCl_3$&CD_3OD; 100 MHz) of carissic acid isolated from *Carissa carandas*

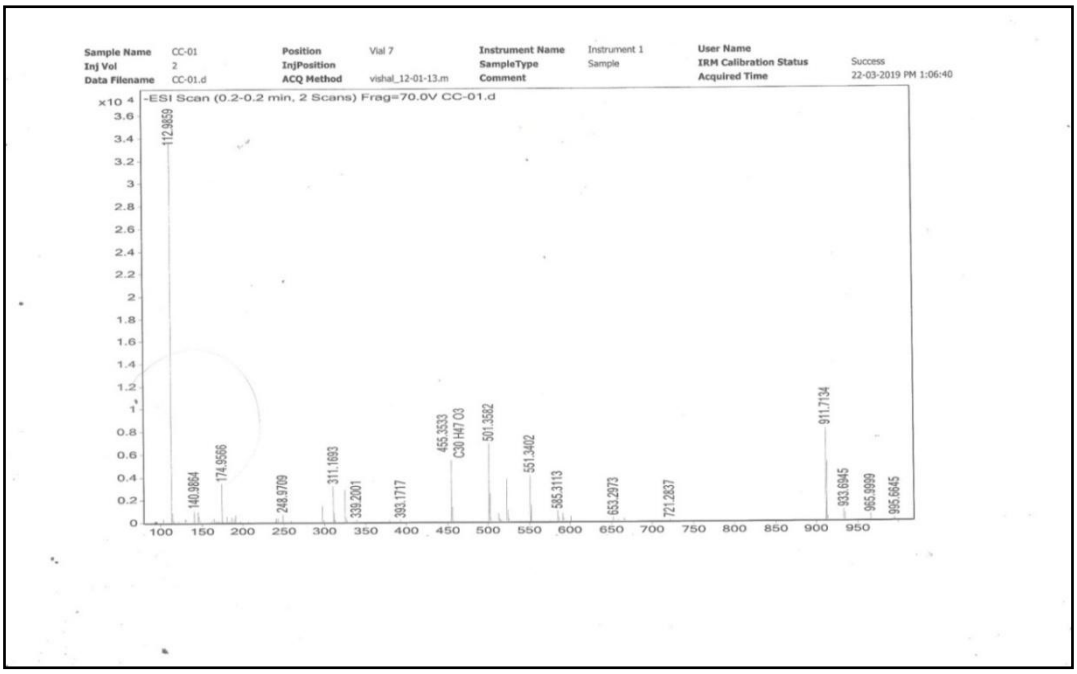


Figure # 26. HRMS chromatogram of carissic acid isolated from *Carissa carandas*

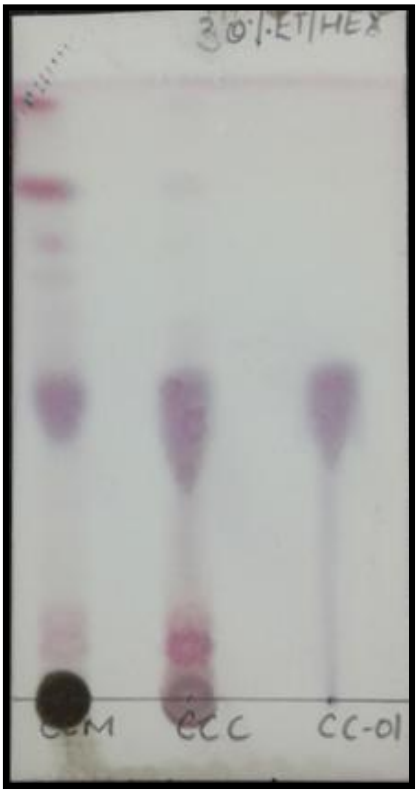
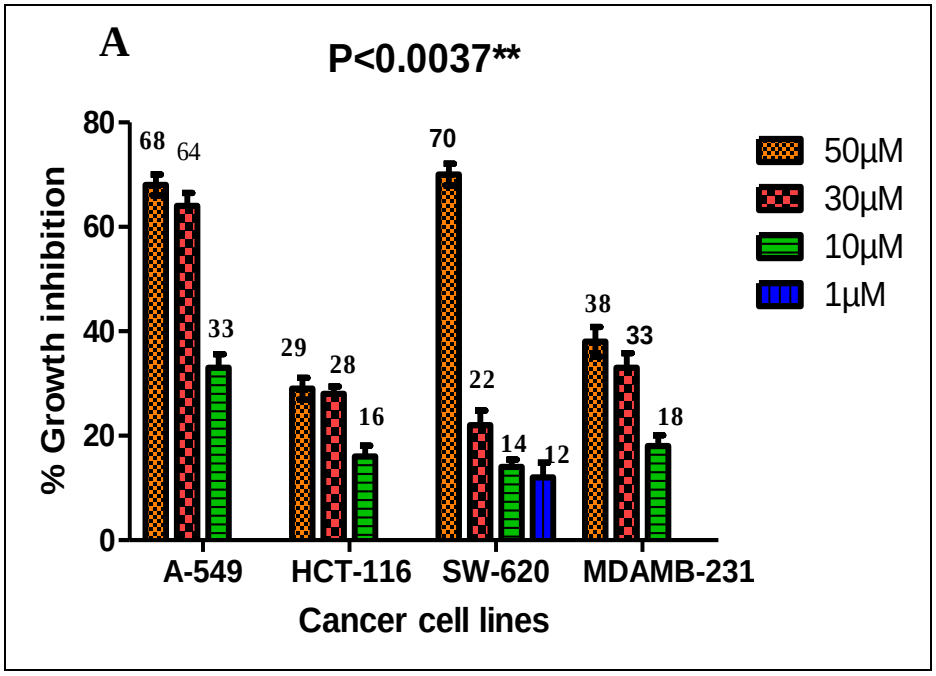


Figure # 27. TLC profile of *Carissa carandas* methanolic extract (CCM), choloroform fraction (CCC) and carissic acid isolated(CC-01) with solvent system of 30% EtoAc/hex.



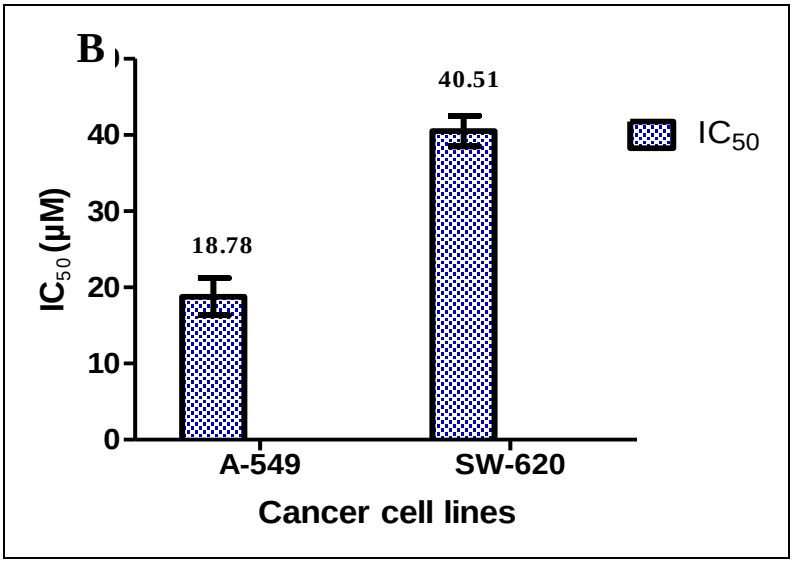
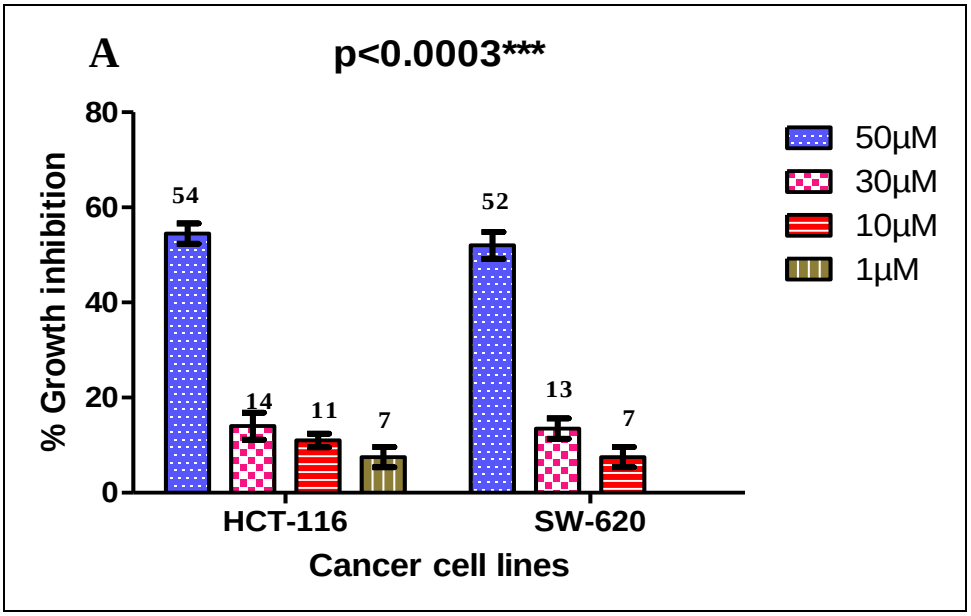


Figure # 28. (A) *In vitro* cytotoxic potential of oleanolic acid isolated from *Syzygium cumini* against human cancer cell lines and (B) IC_{50} values of oleanolic acid. Data is presented as Mean ± S.D. and P value <0.0037**



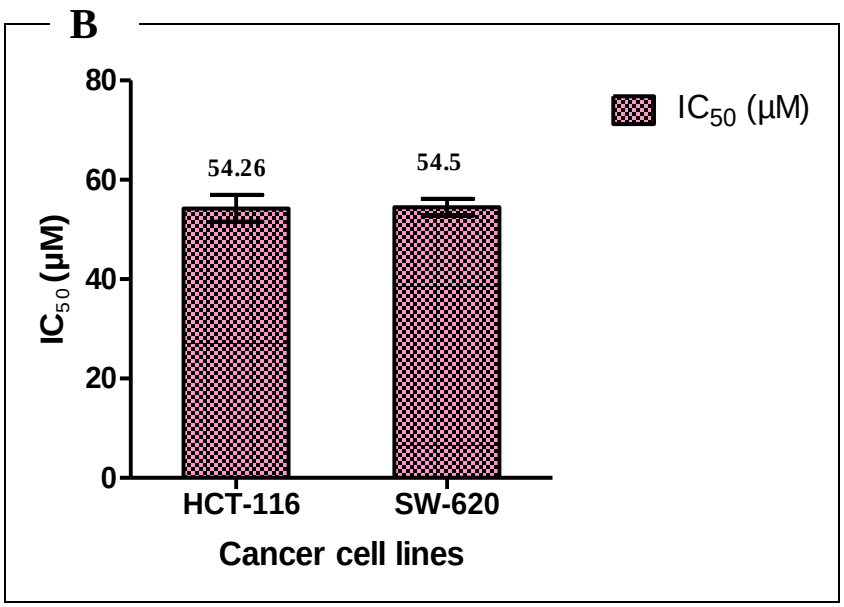
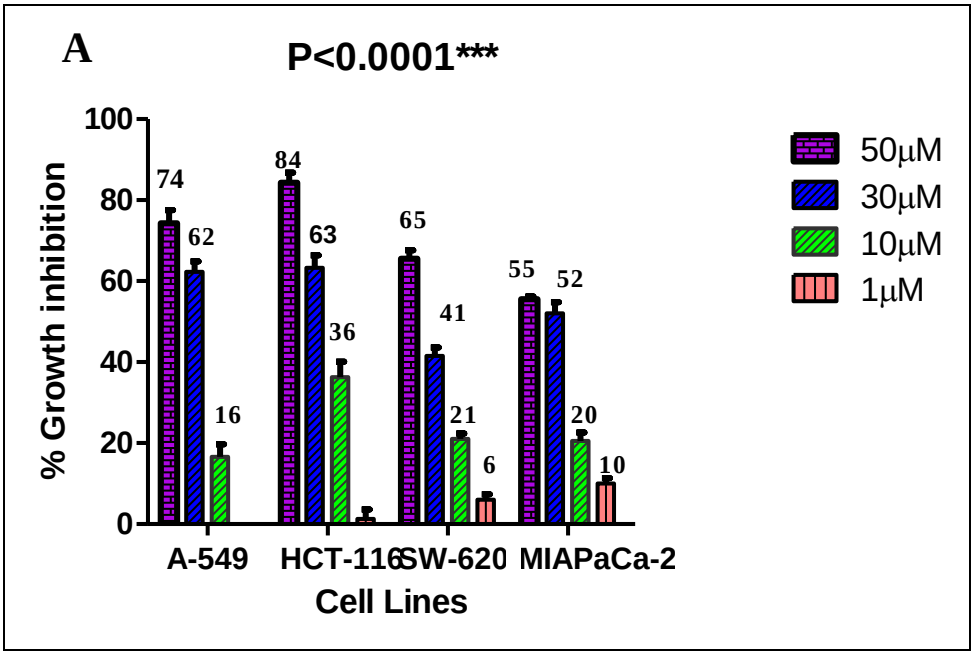


Figure # 29. (A) *In vitro* cytotoxic potential of gallic acid isolated from *Syzygium cumini* against colon cancer cell lines and (B) IC_{50} values of gallic acid. Data is presented as Mean ± S.D. and P value <0.0003***



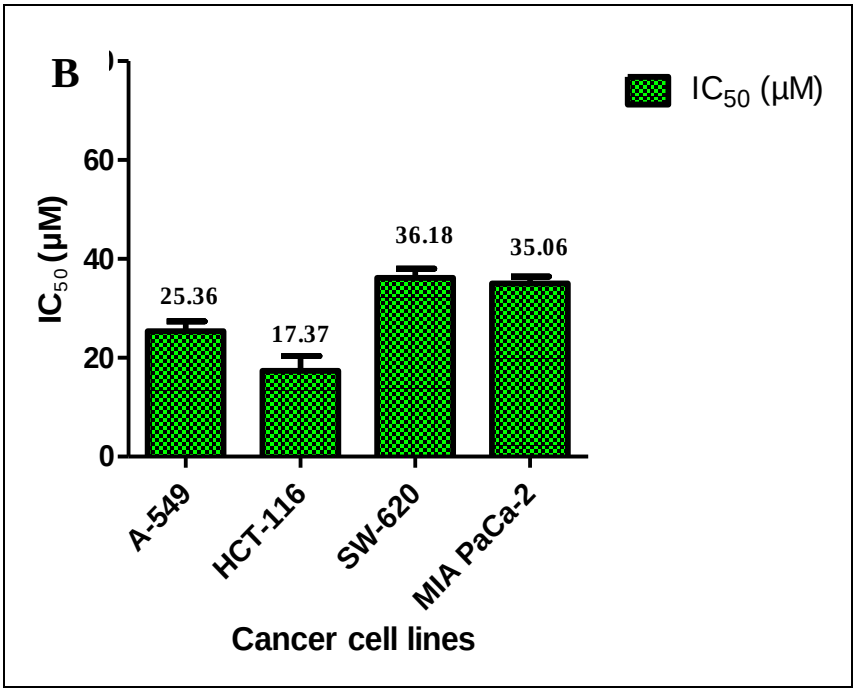
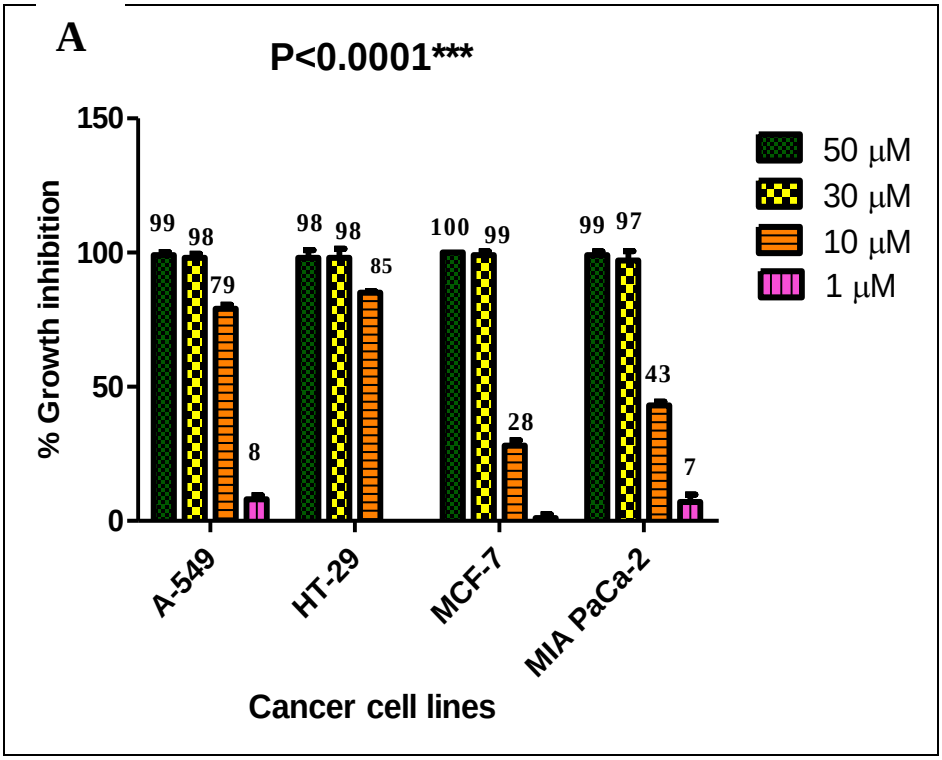


Figure # 30. (A) *In vitro* cytotoxic potential of quercetin isolated from *Syzygium cumini* against human cancer cell lines and (B) IC_{50} values of quercetin. Data is presented as Mean ± S.D. and P value <0.0001***



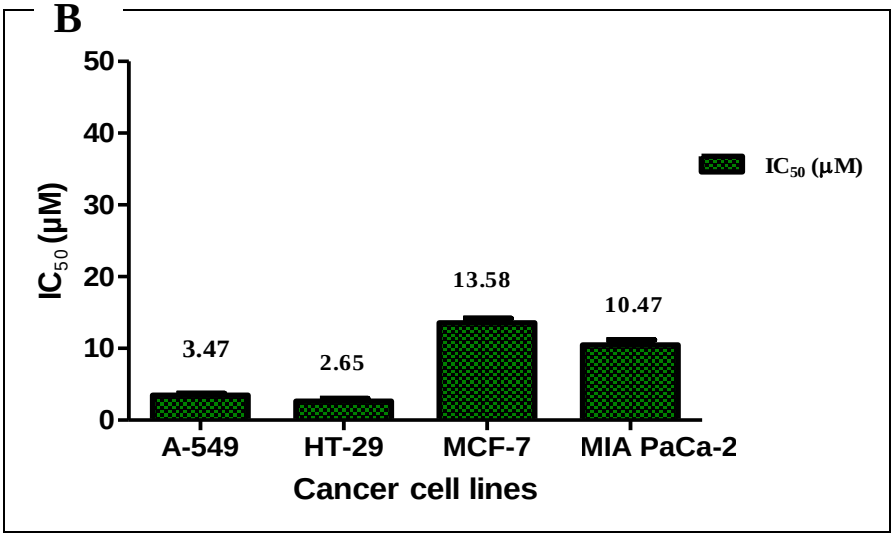


Figure # 31. (A) *In vitro* cytotoxic potential of carissic acid isolated from *Carissa carandas* against human cancer cell lines and (B) IC_{50} values of carissic acid. Data is presented as Mean±S.D. and P value <0.0001***

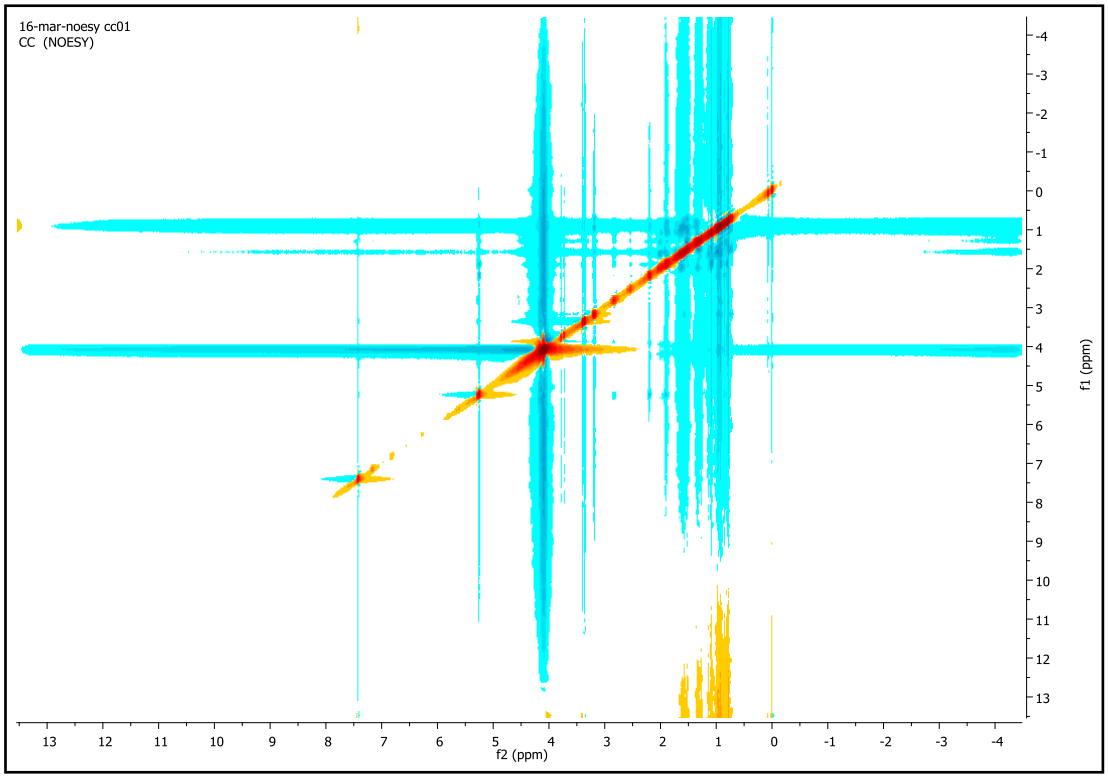


Figure # 32.NOESY spectrum of carissic acid isolated from *C. carandas*

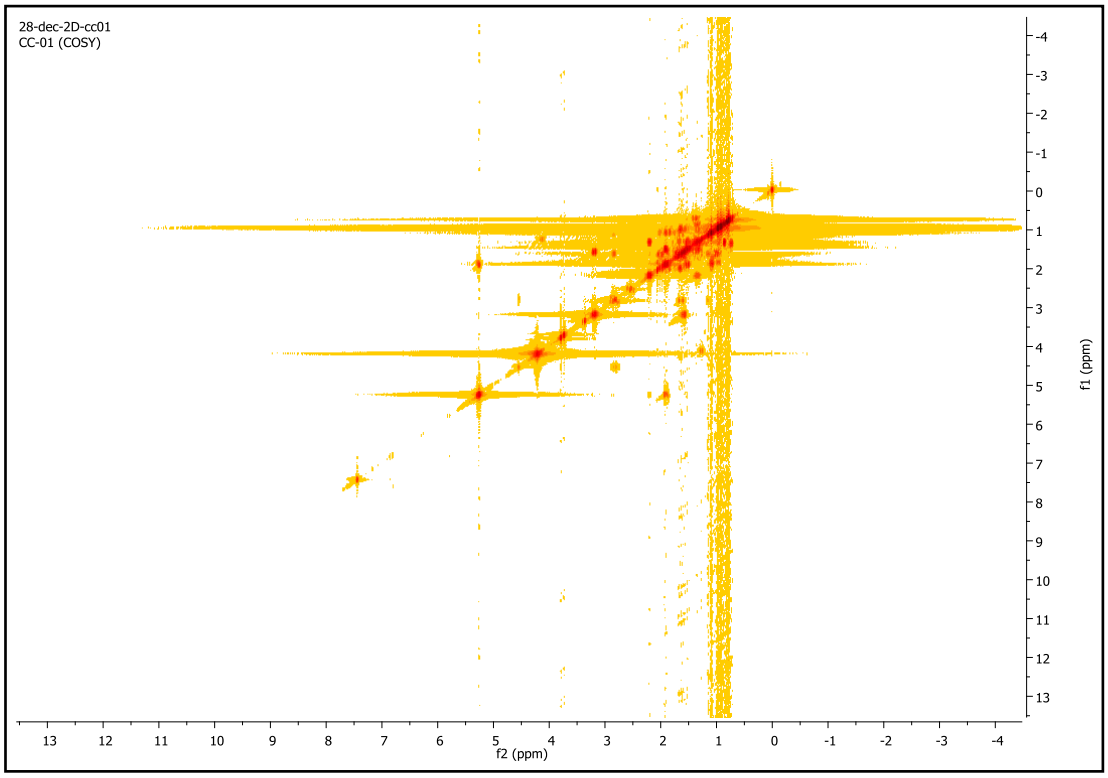


Figure # 33.^{1}H-^{1}H COSY NMR spectrum of carissic acid isolated from *C. carandas*

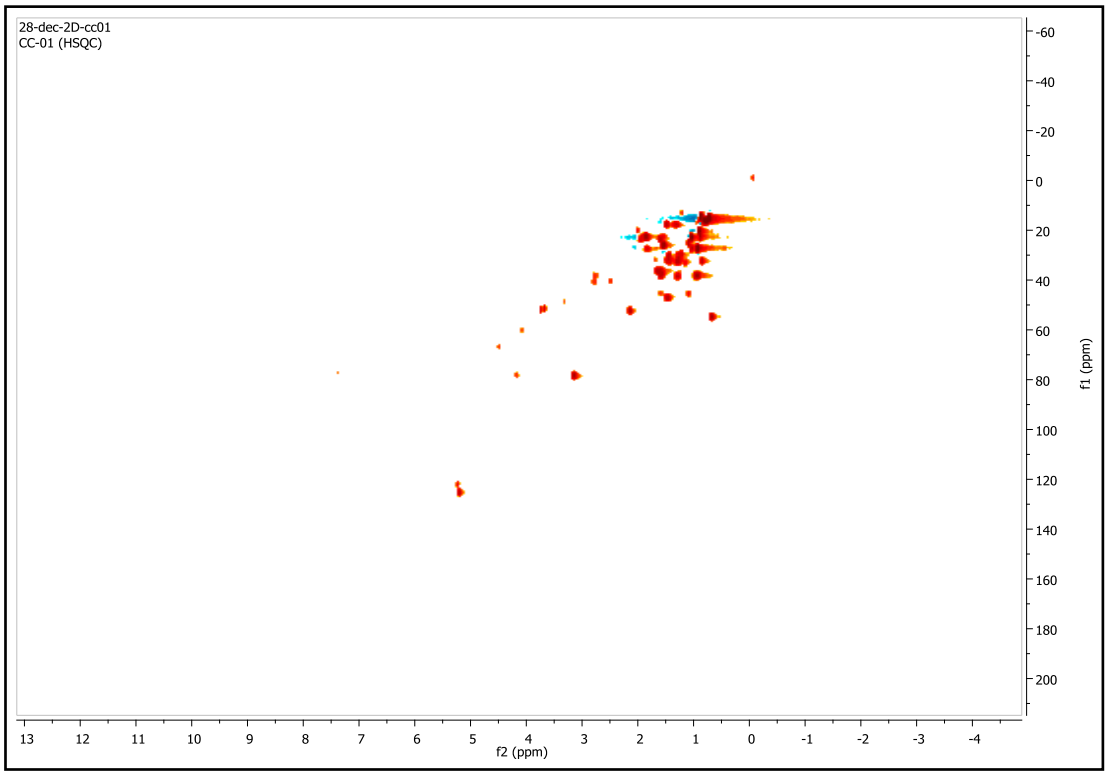


Figure # 34. HSQC NMR spectrum of carissic acid isolated from *C. carandas*

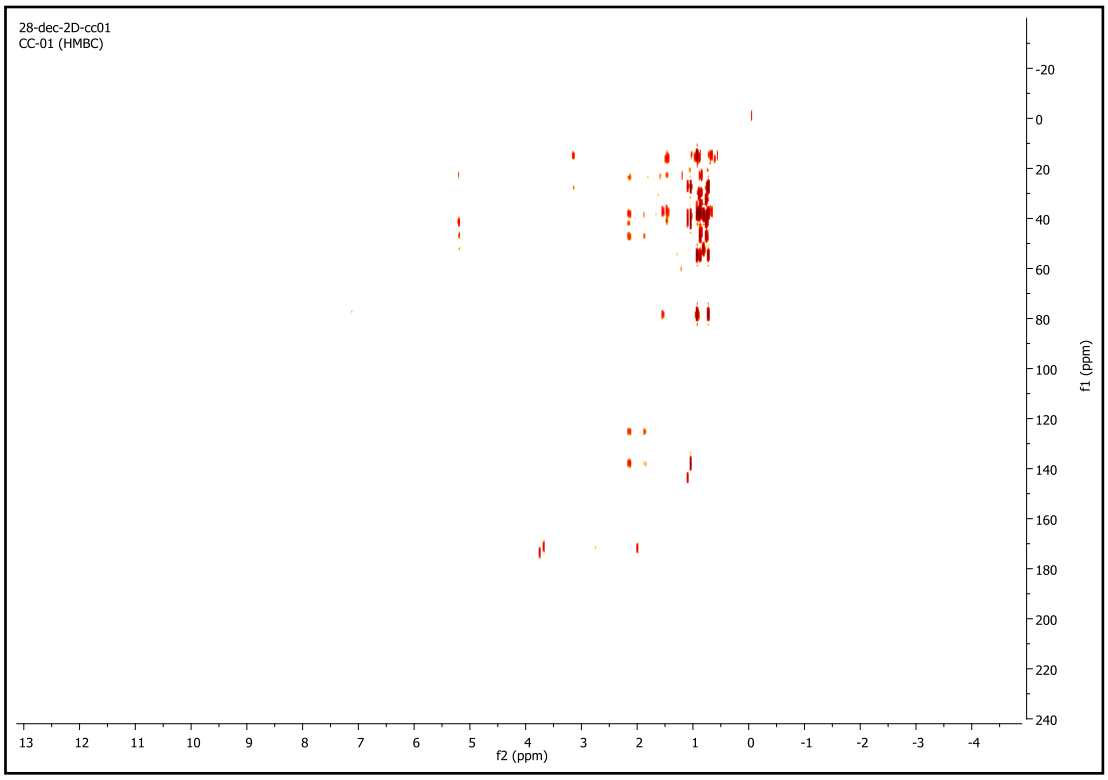


Figure # 35. HMBC NMR spectrum of carissic acid isolated from *C. carandas*

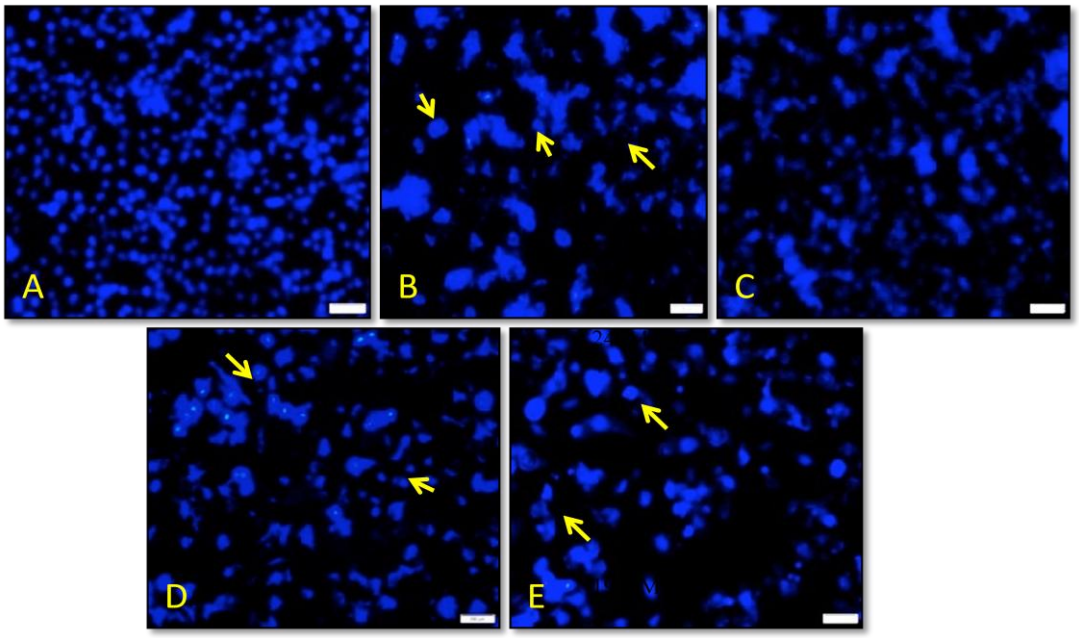


Plate # 4. Nuclear morphological changes using DAPI staining with CA treatment (A) Control (B) Paclitaxel (C) CA 14µM (D) 24µM and (E) 48µM

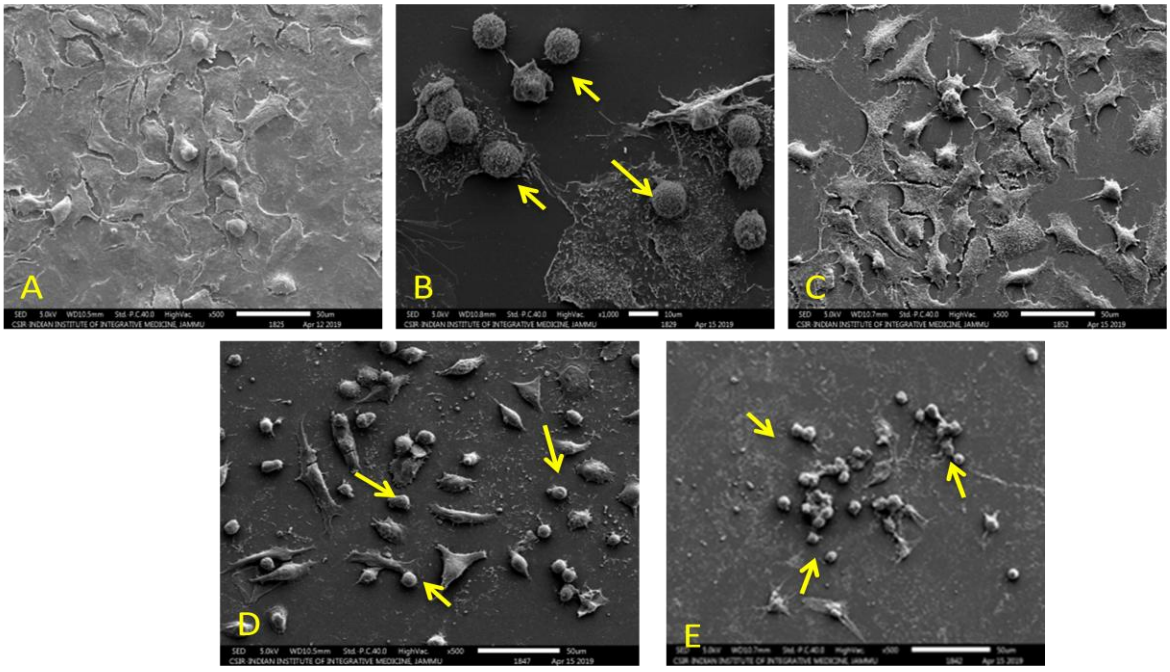


Plate # 5. Overview of A-549 cells by SEM(A)untreated cells (B) cells treated with paclitaxel (100nM) showing apoptotic bodies, (C) cells treated with CA 14 µM, (D) 24 µM and (E) 48 µM

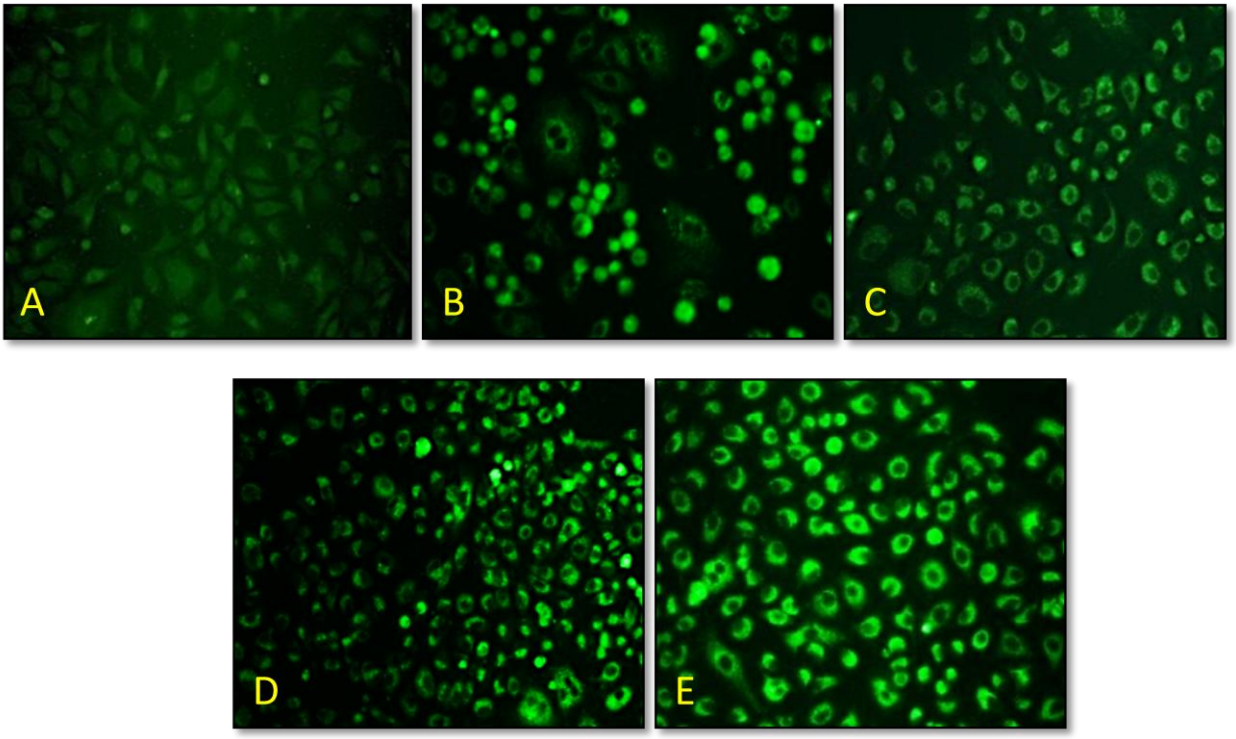


Plate # 6. Effect of CA on A-549 in ROS production assessed from the oxidation of DCFDA by hydrogen peroxide through fluorescence microscopy at 24 h posttreatment, (A) Control (B) H_2O_2 0.5% (C) 14 µM (D) 24µM (E) 48µM

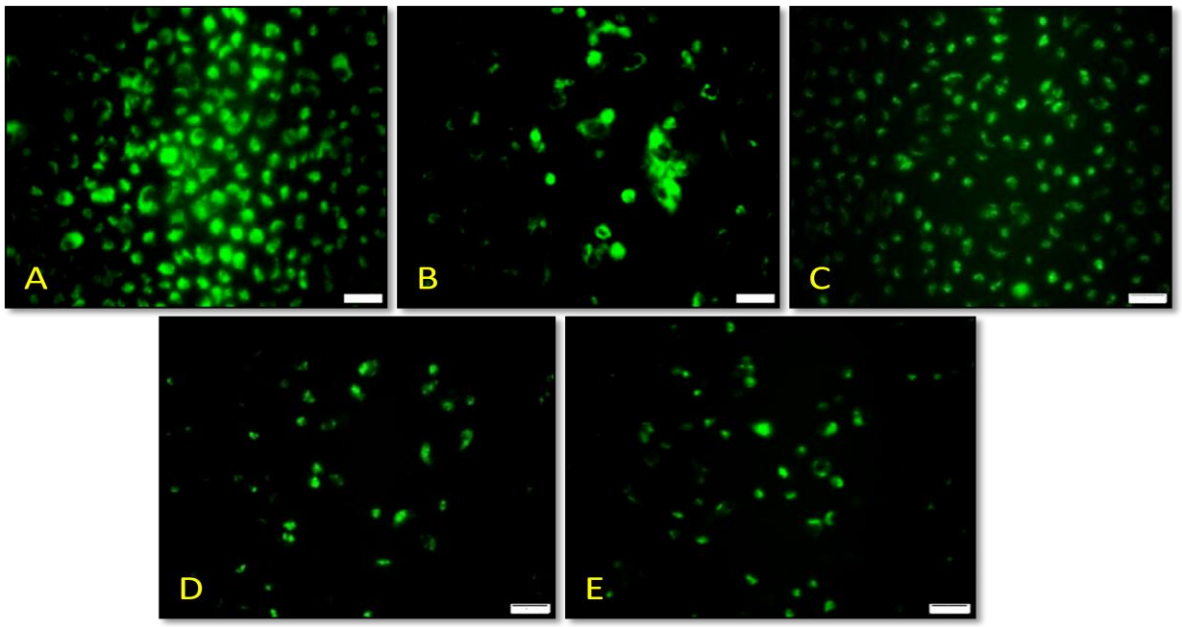
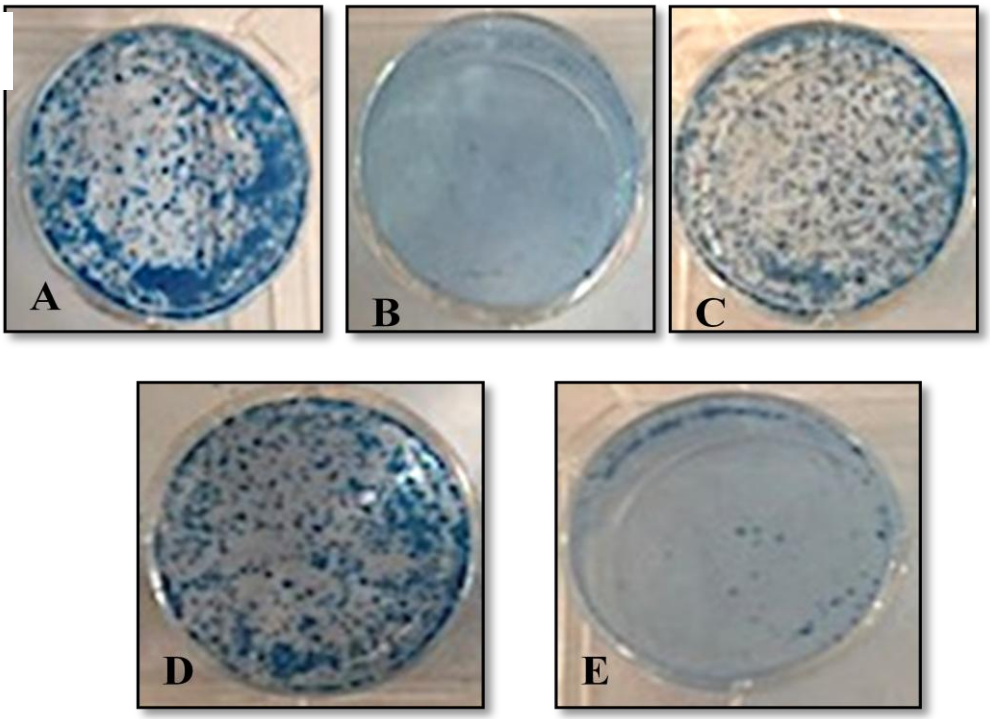


Plate # 7. Mitochondrial staining of A-549 cells using Rhodamine 123 with (A) control, (B) Paclitaxel 100nM and different conc. of CA (C) 14µM(D) 24µM (E) 48 µM

(I)



(II)

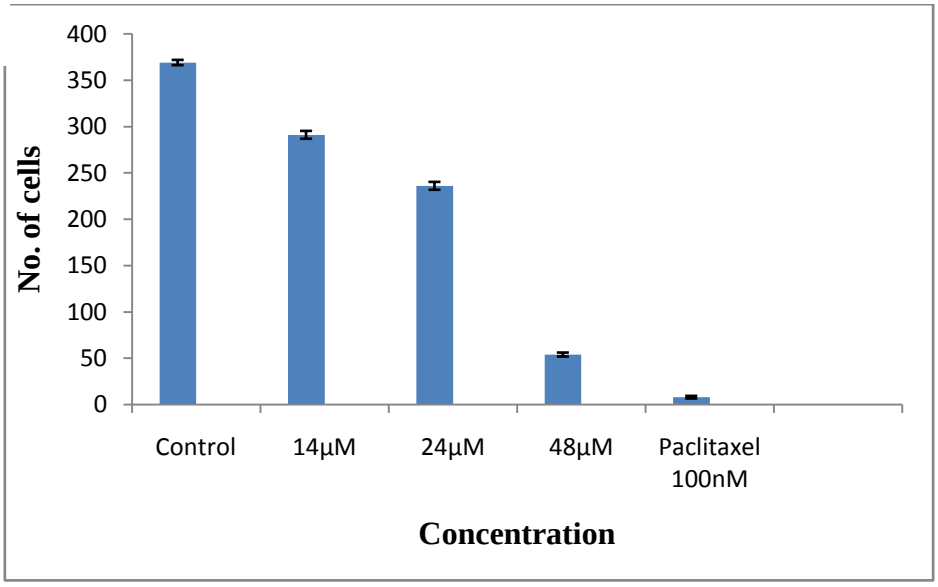


Plate # 8. (I) A-549 cells treated with (A) Control (B) Paclitaxel 100 nM (C) various conc. of CA 14 µM (D) 24 µM (E) 48 µM for 24 h and (II) quantified which were represented graphically

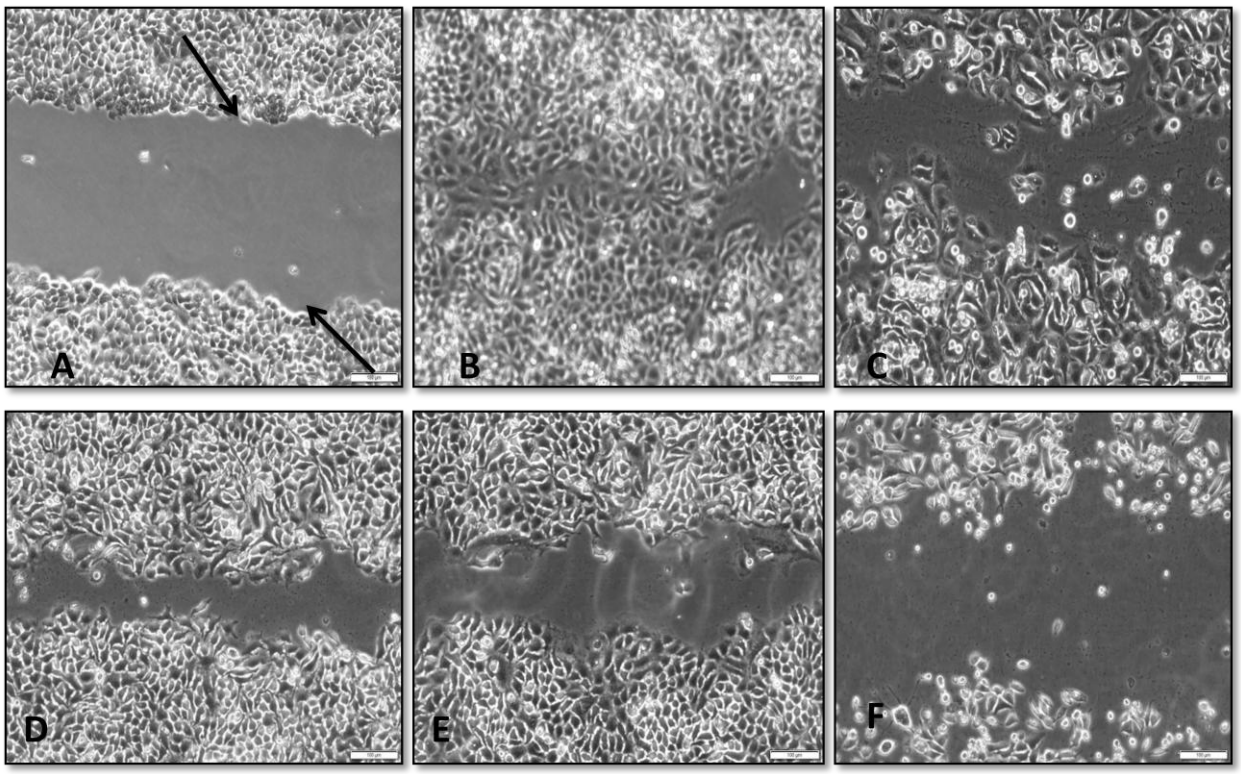


Plate # 9.*Invitro* wound healing assay on A-549 cells (A) 0h, (B) 24 h, (C) paclitaxel (100nM), (D) 14 µM, (E) 24 µM and(F) 48 µM

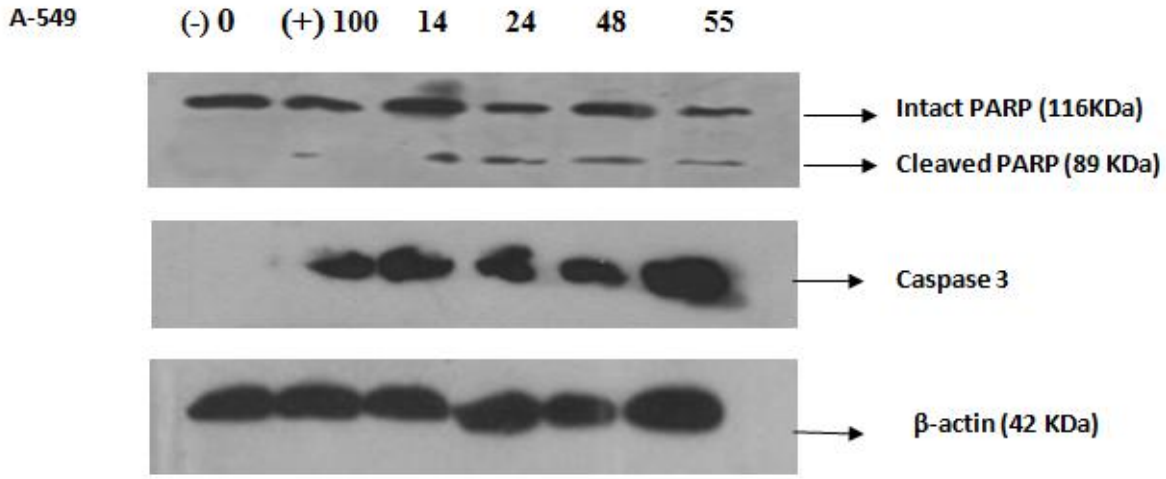


Plate # 10. Western Blot Analysis – A-549 cells were treated with CA at different conc. (14, 24, 48, 55 µM)for 48 h, revealed the expression level of intact andcleaved PARP with increased expression level of Caspase-3. Paclitaxel (100 nM) was used as positive controland β-actin as loading control which is a housekeeping gene

First phase: In this phase, screening of extracts and fractions was carried out against various human cancer cell lines and the data obtained has been discussed below:

4.1*Artocarpus heterophyllus*

The jack fruit extract (methanolic) was initially evaluated at the concentration of 100 µg/mL against eight human cancer cell lines (A-549, HCT-116, HT-29, SW-620, PC-3, MCF-7, MDAMB-231, MIA PaCa-2) from five different tissues (lung, colon, prostate, breast, pancreatic) respectively. The extract did not exhibit any significant cytotoxic effect (growth inhibition of 70% or more) against any of the human cancer cell lines mentioned above. As the growth inhibition was in the range of 0-43%, so the extract was considered as inactive and not evaluated at lower concentrations for determining the IC_{50} value and therefore not further fractionated (Table # 3).

4.2*Carissa carandas*

Karonda fruit extract (100 µg/mL) supressed the proliferation of three human cancer cell lines namely breast (MCF-7), colon (HT-29) and lung (A-549) with growth inhibition of 70%, 72% and 75% respectively. On the other hand, *C. carandas* extract seems to be inactive against five human cancer cell lines, as it suppressed growth of PC-3 by 43%, MDAMB-231 by 52%, SW-620 by 54%, HCT-116 by 65% and MIA PaCa-2 by 69%. Based on the cytotoxic effect of karonda extract, it was then fractionated with n-hexane, chloroform and butanol tofurther evaluate its *in vitro* cytotoxicity against the same human cancer cell lines that were found active as mentioned above (Table # 4).

The results revealed that chloroform fraction of *C. carandas* showed remarkable results against all the three human cancer cell lines with 100% growth inhibition in A-549, HT-29 and MCF-7 at the conc. of100 µg/mL. Surprisingly, the growth inhibition was seen 100% *i.e.,* more than methanolic extract at the same conc. (100 µg/mL). Further, at lower conc. (50, 30, 10 µg/mL), it showed 100% growth inhibition, but at 1 µg/mL it was not seemed to be active in A-549 cells. In HT-29 cell line, 100% inhibition at 50 µg/mL, 99% inhibition at 30 µg/mL, 94% at 10

µg/mLwhereas 37% inhibition (inactive) at 1 µg/mL was observed. In MCF-7 cell line, chloroform fraction was active upto 10 µg/mL,but it showed 0% growth inhibition at 1 µg/mL. These fractions were further taken up for determining the IC_{50} values which were calculated as 3.98±0.24 µg/mL (MCF-7), 1.48±0.002 µg/mL (A-549) and 1.28±0.02 µg/mL (HT-29) (Table # 5). The n-hexane fraction (karonda) was found to suppress the proliferation of three human cancer cell lines with 74% GI each in A-549 and MIA PaCa-2, 87% GI in MCF-7 at 100 µg/mL. Whereas, the butanol fraction exhibited cytotoxic effect against pancreatic (MIA PaCa-2) cancer cell line with 89% GI (Table # 4). The results demonstrated that chloroform fraction of karonda was found to be most cytotoxic against three human cancer cell lines (A-549, HT-29, MCF-7) with IC_{50} value <10 µg/mL.

4.3*Grewia asiatica*

The phalsa fruit extract did not exhibit any significant cytotoxicity (growth inhibition of 70% or more) against any of the human cancer cell lines mentioned above and the overall growth inhibition was seen in the range of 0-54%. For colon (HCT-116 & HT-29) and lung (A-549) cancer cell line, the extract was completely considered as inactive as growth inhibition was 0% whereas the results produced in fractions were quite interesting as the n-hexane fraction and chloroform fraction were found to be active specifically on colon cancer cell line *i.e.*, SW-620 with 76% and 70% growth inhibition. The butanol fraction was not found to be active on any of the human cancer cell lines as the growth inhibition was in the range of 0-35% (Table # 6).

4.4*Syzygium cumini*

Jamun fruit extract suppressed the proliferation of three human cancer cell lines - 70% in case of lung (A-549), 72% against prostate (PC-3) and 74% against colon (HCT-116). For the rest five human cancer cell lines like breast (MCF-7 & MDAMB-231), colon (HT-29 & SW-620) and pancreatic (MIA PaCa-2), the extract was considered to be inactive with the growth inhibition of 21% (MCF-7), 32% (HT-29), 44% (MDAMB-231), 46% MIA PaCa-2 and 65% (SW-620) (Table # 7).

On the basis of the preliminary investigation of fruit part of jamun, fractions obtained from particular active extract were tested with same human cancer cell lines

as mentioned above. In case of n-hexane fraction, the growth inhibition was observed in the range of 28-99% with maximum inhibition of 99% in MIA PaCa-2 and HCT-116 cancer cell lines and 95% in SW-620 cancer cell line at the conc. of 100 µg/mL. At lower conc. (50, 30, 10, 1µg/mL), it inhibited the growth of HCT-116 in the range of 0-65%, A-549 (0-56%), PC-3 (0-35%), MIA PaCa-2 (0-58%), SW-620 (1-59%), HT-29 (0%) and MDAMB-231 (20-27%) with IC_{50} values of 36.25±0.77 µg/mL, 35.16±1.46 µg/mL, >50±2.17 µg/mL, 29.26±0.97 µg/mL, 26.81±0.77 µg/mL, >50±1.46 µg/mL and >50±2.39 µg/mL respectively (Table # 8). The chloroform fraction (100 µg/mL) of jamun showed growth inhibition in the range of 76-99%. Maximum growth inhibition of 99% was observed in MIA PaCa-2 cancer cell line. It was again screened at lower concentrations of 50, 30, 10 and 1 µg/mL and IC_{50} values calculated from the results obtained were >50±0.28 µg/mL (SW-620), 48.30±3.42 µg/mL (HCT-116), 43.10±0.26 µg/mL (MIA PaCa-2), 40.02±1.94 µg/mL (A-549), 18.02±1.01 µg/mL (MDAMB-231) (Table # 9). In case of butanol fraction, no activity was visualized in any of the human cancer cell lines and the said fraction was considered completely inactive at 100 µg/mL, thereby not evaluated further at lower concentrations for determining IC_{50} value.

Second phase: The screening phase was followed by isolation and characterization of pure compounds from active fractions of *C. carandas, G. asiatica* and *S. cumini via* column chromatography. The compounds isolated were characterized *via* NMR (^{1}H and ^{13}C), mass spectroscopy (ESIMS & HRMS) and are enlisted below with their spectral description:

4.5 **<u>Beta-sitosterol</u> (1)**, White powder; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.34 (1H, m, H-11), 3.52 (1H, m, H-18), 2.28 (2H, m, H-17), 1.99 (2H, m, H-10, H-6), 1.85 (2H, m, H-14, H-15), 1.52 (3H, s, Me-19), 1.53 (2H, m, H-28, H-24), (1H, m, H-24), 1.00 (3H, d, Me-21), 1.25 (1H, d, H-9), 0.91 (3H, t, Me-29), 0.84 (3H, d, Me-26), 0.80 (3H, d, Me-27), 0.68 (3H, s, Me-18), ^{13}CNMR (100 MHz, $CDCl_3$); δ_C 140.9 (C-5), 121.8 (C-6), 71.8 (C-3), 57.0 (C-14), 56.9 (C-17), 50.3 (C-9), 45.9 (C-22), 42.5 (C-4), 42.3 (C-13), 39.9 (C-12), 37.3 (C-1), 36.3 (C-10), 36.1 (C-18), 33.8 (C-20), 32.0 (C-7), 32.00 (C-8), 31.7 (C-2), 29.8 (C-25), 28.4 (C-16), 26.2 (C-15), 25.5 (C-21), 24.4 (C-23), 21.2 (C-11), 20.3 (C-26),

19.5 (C-27), 19.1 (C-19), 18.9 (C-28), 12.1 (C-29), 11.9 (C-24). ESIMS m/z 415.71$[M+H]^+$ (cal for $C_{29}H_{50}O$, m/z 414.71) (Figure # 7 & 8).

The isolation of compound (1) was done from n-hexane fraction of *S. cumini* in 10% EtoAc/hex. from column (solvent system 12% EtoAc/hex. in TLC,Figure # 9). The testing of this crystal type compound was done on eight human cancer cell lines *namely* A-549, HCT-116, HT-29, SW-620, PC-3, MDAMB-231, MCF-7 and MIA PaCa-2. The results revealed that compound suppressed the proliferation of SW-620 by 58% at 50 µM, 36% at 30 µM, 12% at 10 µM and 0% at 1 µM and IC_{50} value calculated was 42.46±2.30 µM. In case of PC-3 and MCF-7, 43% and 10% growth inhibition was observed at 50 µM respectively. As the growth inhibition was <50%, so the compound was not further evaluated at lower concentrations for IC_{50} value. The growth inhibition lies in the range of 0-8% for MIA PaCa-2, MDAMB-231, HCT-116 and HT-29 cancer cell lines which is considered to be inactive (Table # 10).

4.6 **<u>Oleanolic acid</u> (2)** White solid; ^{1}HNMR (400 MHz, $CDCl_3$): δ_H 5.27 (1H, t, *J*=3.5 Hz, H-12), 3.23 (1H, dd, *J*=11.2, 4.4 Hz, H-3), 2.82 (1H, dd, *J*=3.6, 13.2 Hz, H-18), 1.13 (3H, s, H-27), 0.75, 0.77, 0.90, 0.91, 0.93, 0.98 (each 3H, s, CH_3 ×6); ^{13}CNMR (100 MHz, $CDCl_3$); δ_C 183.3 (C-28), 143.6 (C-13), 122.6 (C-12), 79.0 (C-3), 55.2 (C-5), 47.6 (C-9), 46.5 (C-17), 45.8 (C-19), 41.6 (C-14), 40.9 (C-18), 39.3 (C-8), 38.8 (C-4), 38.4 (C-1), 37.1 (C-10), 33.8 (C-21), 33.1 (C-29), 32.6 (C-7), 32.4 (C-22), 30.7 (C-20), 28.1(C-23), 27.7 (C-15), 27.2 (C-2), 25.9 (C-27), 23.6 (C-11), 23.4 (C-16), 22.9 (C-30), 18.3 (C-6), 17.1 (C-26), 15.5 (C-24), 15.3 (C-25); HR-ESIMS m/z 455.3488 $[M-H]^-$ (cal for $C_{30}H_{48}O_3$, m/z 456.35) (Figure # 10 & 11).

The compound (2) was isolated in 20% EtoAc/hex. solvent system from column (solvent system 25% EtoAc/hex. in TLC,Figure # 12) and was seen to be active on two cancer cell lines from lung (A-549) and colon (SW-620) origin with 68% and 70% growth inhibition at 50 µM respectively. At the conc. of 30 µM, it did not showed *in vitro* cytotoxic effect against SW-620 (22% GI) whereas in case of A-549 the activity was observed with 64% growth inhibition. However, the compound suppressed the proliferation of HCT-116 and MDAMB-231 in the range of 0-38% at four different concentrations (50, 30, 10, 1µM). For both the cell lines - A-549 and SW-620, IC_{50}values were calculated as 18.78±2.43 µM and 40.51±1.99 µM respectively (Figure # 28).

4.7 **Gallic acid (3)**, Green powder; ^{1}H NMR (CD_3OD, 400MHz); 7.06 (1H, s, H-2/6), ^{13}C NMR (CD_3OD, 100MHz); 170.0 (C-1´), 146.4 (C-3/5), 139.6 (C-4), 121.9 (C-1), 110.3 (C-2/6), HRMS *m/z* 169.0138 $[M-H]^-$ (calcd for $C_7H_6O_5$, 170.0212) (Figure # 13 & 14).

The compound (3) was isolated from 10% MeOH/$CHCl_3$ solvent system from column(solvent system 70% EtoAc/hex. in TLC,Figure # 15) and tested against four human cancer cell lines *viz.,* lung (A-549), colon (HCT-116 & SW-620) and breast (MDAMB-231). The results depicted that it has suppressed the proliferation of two colon cancer cell lines specifically HCT-116 and SW-620. Among the four concentrations (50, 30, 10, 1 µM), it was found active at 50 µM with the growth

inhibition of 54% (HCT-116) and 52% (SW-620). The growth inhibition lies in the range of 7-14% (HCT-116) and 0-13% (SW-620) at 30, 10 and 1 µM. When tested against other two human cancer cell lines (A-549 & MDAMB-231), compound showed <50% growth inhibition and was considered to be inactive. As per the results obtained, IC_{50} values calculated were 54.26±2.71 µM and 54.5±1.64 µM for HCT-116 and SW-620 respectively (Figure # 29). The overall findings suggest that the compound caused *in vitro* cytotoxic effect on specifically colon tissue with nearly same IC_{50} values.

4.8 **<u>Quercetin</u> (4)** Yellow powder; ^{1}H NMR (400 MHz, CD_3OD): δ_H 7.64 (1H, d, *J*= 2.0 Hz, H-2′), 7.50 (1H, dd, *J*=2.0, 6.4 Hz, H-6′), 6.85 (1H, d, *J*=8.8 Hz, H-5′), 6.38 (1H, d, *J*=2.0 Hz, H-8), 6.15 (1H, d, *J*=2.0 Hz, H-6); ^{13}C NMR (100MHz, CD_3OD): δ_C 177.3 (C-4), 165.6 (C-7), 162.5 (C-5), 158.3 (C-9), 148.8 (C-4′), 148.0 (C-2), 146.2 (C-3′), 137.2 (C-3), 124.1 (C-1′), 121.7 (C-6′), 116.2 (C-5′), 116.0 (C-2′), 104.5 (C-10), 99.3 (C-6), 94.4 (C-8); HRMS *m/z* 303.0499 $[M+H]^+$ (cal. for $C_{15}H_{10}O_7$, *m/z* 302.0426) (Figure # 16 & 17).

The isolation of compound 4 was done in 15% MeOH/$CHCl_3$(solvent system 10% MeOH/$CHCl_3$in TLC,Figure # 18) andthe results are quite interesting as compared to other compounds isolated from *S. cumini*. The said compound exhibited *in vitro* cytotoxic effect against all the four human cancer cell lines (A-549, HCT-116, SW-620, MIA PaCa-2). Initially at 50 µM, it suppressed the proliferation by 74% in A-549, 84% in HCT-116, 65% in SW-620 and 55% in MIA PaCa-2 cancer cells. At 30 µM, the growth inhibition observed was more than 50% for three cancer cell lines with 62% GI (A-549), 63% GI (HCT-116) and 52% GI (MIA PaCa-2) whereas in SW-620 it was 41% GI. For the last two lower concentrations (10 & 1 µM), the growth inhibition was again less than 50%. Based on *in vitro* cytotoxicity, IC_{50} values of quercetin calculated was 17.37±2.99 µM (HCT-116), 25.36±1.98 µM (A-549),

35.06±1.37 µM (MIA PaCa-2) and 36.18±1.85 µM (SW-620) (Figure # 30). This particular compound produced more *in vitro* cytotoxic effect against more number of human cancer cell lines, with less IC_{50} value in case of HCT-116.HPLC chromatogram of *S. cumini* crude extract and gallic acid, quercetin is also shown inFigure # 19 and 20 respectively).

4.9 **5-Hydroxy methyl furfural**(5-HMF), Yellowish oil; ^{1}H NMR ($CDCl_3$, 400MHz); 9.50 (1H, s, H-7), 7.19 (1H, d, *J*=3.6 Hz, H-3), 6.47 (1H, d, *J*=3.2 Hz, H-4), 4.65 (2H, d, *J*=3.2 Hz, H-6); ^{13}C NMR ($CDCl_3$, 100MHz); 178.1 (C-7), 161.4 (C-5), 151.8 (C-2), 124.2 (C-3), 110.0 (C-4), 56.8 (C-6), ESIMS *m/z*: 127.1000 $[M+H]^+$ (calcd for $C_6H_6O_3$, 126.10) (Figure # 21 & 22).

The compound 5-hydroxy methyl furfural was isolated from chloroform fraction of *G. asiatica*(solvent system 50% EtoAc/hex. in TLC,Figure # 23). The fraction was found to be active on SW-620 (colon cancer cell line).Therefore, the compound was tested against the same human cancer cell line at the conc. of 50, 30, 10 and 1 µM. Results revealed that 5-HMF exhibits 40% growth inhibition at 50 µM against SW-620, indicating it inactive, as in case of compounds the growth inhibition of 50% or more is considered to be active. At lower conc., the compound displayed negligible *in vitro* cytotoxic effect (0% GI) and the IC_{50} value calculated was >50 µM±0.77 µM (Table # 11).HPLC chromatogram of *G. asiatica*chloroform fraction and 5-HMF is shown in Figure # 24.

4.10 **Carissic acid** (CA), White powder; ^{1}H NMR (CD_3OD & $CDCl_3$, 400MHz); δ_H 5.24 (1H, d, *J*=3.6 Hz, H-12), 3.19 (1H, m, H-3), 2.20 (1H, m, H-18), 1.91(1H, m, Ha-22), 1.66 (1H, dd, m, Hb-22), 1.26 (3H, s, Me-23), 1.10 (3H,s, Me-24), 0.98 (3H, s, Me-25), 0.96 (3H, s, Me-26), 0.93 (3H, s, Me-27), 0.82 (3H, d, *J*=6.5 Hz, Me-29),0.78 (3H, d, *J*=5.9 Hz, Me-30); ^{13}CNMR (CD_3OD & $CDCl_3$,100 MHz); δ_C 180.4, 138.1, 125.5, 78.5, 55.3, 52.9, 48.9, 48.9, 41.9, 39.4,

39.0, 38.9, 38.7, 38.5, 36.8, 36.7, 32.9, 30.5, 27.9, 27.7, 26.5, 24.0, 23.1, 23.1, 20.6, 20.6, 18.2, 16.6, 15.3, 15.0; HR-ESIMS m/z 455.3495 $[M-H]^-$ (cal for $C_{30}H_{48}O_3$, m/z 456.35) (Figure # 25 & 26).

This compound was isolated from chloroform fraction prepared from the fruit part of *Carissa carandas*(solvent system 30% EtoAc/hex. in TLC,Figure # 27)and produced significant results from medicinal point of view as remarkable *in vitro* cytotoxic effect against four human cancer cell lines namely A-549, HT-29, MCF-7 and MIA PaCa-2 was observed in this case. Starting from the conc. of 50 µM, the compound suppressed the overall proliferation of A-549 cells by 99%, HT-29 by 98%, MCF-7 by 100% and MIAPACA by 99%. Further, at 30 µM, the compound again showed excellent results as the growth inhibition of 98%, 99% and 97% was observed against A-549 & HT-29, MCF-7 and MIA PaCa-2 respectively. At 10 µM, the compound showed activity against A-549 (79%) and HT-29 (85%) whereas 28% and 43% growth inhibition was observed in case of MCF-7 and MIA PaCa-2 cells. At the lowest conc. (1 µM), it did not exhibit any cytotoxic effect as the percent growth inhibition lies between 0-8.The IC_{50} values were calculated as 3.47 µM±0.26 µM for A-549, 2.65±0.35 µM for HT-29, 10.47±0.69 µM for MIA PaCa-2 and 13.58±0.59 µM for MCF-7 cells (Figure # 31). This compound displayed the potent *in vitro* cytotoxic effect against all the four human cancer cell lines with maximum growth inhibition and less IC_{50} values.

The molecular formula of the compound was determined to be $C_{30}H_{48}O_3$ based on the HR-MS revealing a molecular ion peak at *m/z* 455.35271 [M-H] (calculated for $C_{30}H_{47}O_3$, 455.3533). Based on the molecular formula, the degrees of unsaturation were found to be seven indicating that the isolated compound is probably a pentacyclic triterpenoid. Further, confirmation was provided by ^{13}C NMR and DEPT experiment revealing the presence of 30 carbon resonances distinguished into seven

methyl resonances, nine methylene moieties, seven methine carbons in addition to two carbon resonances at δ 78.5 and 125.5 ppm representing one oxygenated aliphatic (C-3) and one olefinic (C-12) carbons, respectively, together with seven quaternary carbons. The appearance of δ 125.5 and 138.1 ppm indicate the presence of a double bond. The most downfield signal at δ 180.4 indicates the carboxylic function.

The ^{1}H NMR spectrum, showed seven methyl resonances, in addition to two resonances at δ 5.24 and 3.19 ppm which displayed in HMQC spectrum C–H correlations to δ 125.5 (C-12) and 78.5 (C-3), respectively. HMBC spectrum exhibited long range correlations from a singlet methyl resonance (Me-27) at δ 0.93 ppm to four carbon resonances at δ 39.4 (C-8), 41.9 (C-14), 27.7 (C-15) and 138.1 (C-13) ppm.The relative configuration of this compound was determined by NOE correlations observed in a NOESY experiment (Figure # 32-35). NOE correlations from H-3 to H3-24, H3-25, H-5 and H-11 together with correlations from H3-23 to H3-26, established the cis-fused nature of the ring 1 and 2 in triterpene system which is totally different from ursolic acid where trans-fused system exist. An additional NOE correlation from H3-26 to H3-30 and H-18 together with correlations observed from H3-27 to H-11 and H3-29 established the trans existence of H3-29 and H3-20 methyl in ring 5 of terpene. Thus, the structure of compound is therefore defined as 3S, 5R, 9R, 25S, 26R, 27S, 18S, 28S, 29R and 30S ursan type new triterpene named carissic acid.

Third phase: Among all the phytochemicals isolated from *G. asiatica* (phalsa), *S. cumini* (jamun) and *C. carandas* (karonda), carissic acid (CA) isolated from chloroform fraction of fruit part of karonda possesses maximum *in vitro* cytotoxic efficiency against tested human cancer cell lines with less IC_{50} values. Moreover, in view of the results obtained, chloroform solvent (0.5% v/v) was also tested against human lung cancer cell line (A-549) and it was observed that growth inhibition was only 08% that confirms that the chloroform fraction possesses *in vitro* cytotoxic effect, not the solvent. Accordingly, a couple of mechanistic assays were performed on A-549 (lung) cancer cells to confirm the basis of apoptosis and the results are elaborated below:

4.11 CA induced chromatin condensation

DAPI (4,6- diamidino-2-phenylindole) is a fluorescent dye that binds to minor groove of A-T region of DNA. Induction of apoptosis results in characteristic morphological alterations like chromatin condensation (pyknosis), cell shrinkage, nuclear deformation, formation of apoptotic bodies and their degradation by adjacent cells. A-549 cells treated with different conc. of CA *i.e.,* 14, 24 and 48 µM were stained with DAPI dye and observed under fluorescence microscope. The cells in untreated condition represent the uniform appearance of blue coloured nuclei indicating healthy condition of cells. Cells treated with standard drug *i.e.,* paclitaxel showed condensed nuclei with increased intensity. On the other hand, CA treated cells also exhibited chromatin condensation of cell nuclei in dose dependent manner (Plate # 4). In treated A-549 cells, the fluorescence intensity was more as compared to untreated cells after incubation period of 24 h. Moreover, size and shape of cell nuclei was different in apparently both control and drug treated cells. So, the results indicated that CA induced fragmentation of DNA as well as decreased number of cell nuclei in concentration dependent manner.

4.12 SEM confirmed cell death induced by CA

The surface morphological changes of A-549 cells was observed by SEM and the figure clearly depicts that CA triggered apoptosis in A-549 cells in concentration dependent manner and also the apoptotic cells increased with an increase in concentration of CA (Plate # 5).

4.13 CA confirmed cell death through ROS generation

Detection of ROS was done using DCFDA (2,7-dichlorofluorescein diacetate), a non fluorescent dye, which in the presence of ROS give green fluorescent. It is a hydrogen peroxide detecting probe for measuring hydrogen peroxide in intact cells. It gets freely permeates into plasma membrane and oxidize in presence of free radicals to form a green fluoroscent byproduct *i.e.,* DCF (dichlorofluorescein) which is excited at 495 nm and emits at 520 nm. The positive control (0.5% H_2O_2) when treated with DCFDA, dye emitted green fluorescence, which was then compared with drug treated cells. The results indicate a remarkable dose dependent increase in the fluorescence of drug (CA) treated cells and thereby confirmed the ROS generation is crucial for cell death (Plate # 6).

4.14 CA induced loss of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) plays a key role in assessing the mitochondrial function and creates an electrochemical gradient through a series of chemical reactions. This electrochemical gradient drives the synthesis of ATP and generates the mitochondrial membrane potential. Rhodamine 123 staining was used to detect the integrity of mitochondrial membrane. There was loss of mitochondrial integrity which results in leakage of Rh-123 and thereby consequent decrease in fluoroscence. Results revealed loss of mitochondrial membrane potential in CA treated cells with increase in concentration as compared to untreated cells with intact mitochondria. The compound CA was found to cause concentration dependent depolarisation of MMP and these findings support the fact that alteration of mitochondria function plays a vital role in apoptotic cell death (Plate # 7).

4.15 Effect of CA on colony formation

It is an *in vitro* cell survival assay based on the ability of single cell to grow into a colony. This assay tests ability of every cell in the population to undergo unlimited division and used to determine the effectiveness of cytotoxic agent. It helps to moniter that after various treatments of drug, cells have either retained the capacity for producing large number of progenies or cause cell death. A-549 cells treated with CA at different conc. of 14, 24, 48 µM and after the treatment, the cells were seeded in appropriate dilution. The results suggest that compound effectively inhibited colony formation of A-549 cells in a dose dependent manner. Mean size of the control colonies was larger than the colonies of drug treated cells (Plate # 8).

4.16 CA leads to inhibition of cell migration

The effect of CA on the migration of A-549 cells was evaluated here by an *in vitro* wound healing assay. Following the drug treatment, the images of cell migrated into scratched area was captured. The results revealed that in untreated cells after 24 h, due to cell migration and cell proliferation, wound closure was observed clearly whereas in CA treated cells with increased concentration of 48 µM, the inhibition of cell migration occurred and the same type of activity was seen in cells treated with paclitaxel, where the scratch made was clearly visible. So, in view of above results,

the antiproliferative effect of CA treatment leads to the impaired cell migration (Plate # 9).

4.17 CA induced the activation of caspase and PARP

To assess the molecular mechanism underlying the apoptotic effect of compound CA on A-549 cancer cell line, the expression of two different apoptosis related proteins (Caspase-3 and PARP) was analyzed by western blotting. Caspases are the crucial mediators of programmed cell death and caspase-3 is a frequently activated protease for specific cleavage of many cellular proteins required for cellular function and survival. PARP (poly ADP-ribose polymerase-1) a nuclear enzyme, is one of cellular substrates of caspases which is involved in DNA repair, DNA stability and transcriptional regulation. Cleavage of PARP by caspases is considered as sign of apoptosis. The picture revealed that CA treated cells caused the activation of caspase-3 in a concentration dependent manner and the typical 89 kDa cleaved fragment of PARP was observed. Therefore, western blot analysis showed that CA treatment increased caspase-3 expression indicating caspase -3 mediated cell death *via* caspase dependent pathway (Plate # 10).

Discussion

CHAPTER-5

DISCUSSION

Cancer, a leading cause of death worldwide affecting millions of people per year, is characterized by deregulation of signaling pathways with initial loss of controlled cell growth, cell invasion and finally results in metastasis (Millan and Huerta, 2009). It is a heterogeneous group of disorders driven by genetic and epigenetic alterations that allow cells to over proliferate. Mutations that disable cell death signaling, play an important role in cancer. Chromosomal rearrangement in B-lymphocytes leads to the overexpression of Bcl-2, an antiapoptotic protein and this contribute to follicular lymphoma thus preventing cells from apoptosis. P53 also regulates apoptosis either by inducing transcription of proapoptotic regulators or by binding to proapoptotic protein *i.e.,* Bax (Green and Llambi, 2014). Loss of tumor suppressor through mutation leads to cancer. There is coordination between cell growth and metabolic processes required for the synthesis of macromolecules. Growth factor pathways are thus vital for the regulation of normal as well as tumor cells impinge/associated with metabolic pathways to meet the requirement of macromolecules to produce daughter cells (Ward and Thompson, 2012). Increased glucose uptake and glycolysis are the most common metabolic alterations in cancerous cells.

Cancer has remained a major cause of death and the number of individuals living with cancer is continuing to expand. It has emerged as an important health problem in the developed / developing countries and recognized as the important cause of morbidity, mortality, disability in India also. As per World Health Organization data, globally in the year 2012 about 14.1 million new cancer cases and 8.2 million deaths are reported. Moreover 70% of new cancer cases have been estimated in next twenty years (Ferlay *et al*., 2015; Faria *et al*., 2017). Most of the drugs used in cancer chemotherapy exhibit cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non tumor cells. Despite the recent advances in surgery, endocrine therapy, radiotherapy and chemotherapy, it is considered that the management of cancer is still not up to the mark and we are in emergent need of drugs for the treatment of cancer having no side effects. Therefore, the research for

alternative drugs of natural origin, which are less toxic, endowed with fewer side effects and more potent in their mechanism of action, is an important research line. Natural dietary products have tremendous role in cancer chemoprevention as consumption of fruits and vegetables lead to the reduced risk of cancers. To develop novel anticancer drugs, intense search is going on various biological sources to combat this dreaded disease. So far, 30 plant derived compounds have been isolated, used for clinical trials and are found to be active on various cancer cells (Nirmala *et al*., 2011). Almost 80% of world population is dependent on traditional medicine and more than 60% of clinically approved anticancer drugs are derived from these medicinal plants (Cragg *et al*., 2006; Khan, 2014). Since ancient times, plants and their bioactive constituents are known to inhibit the progression and development of cancer. Several medicinal plant species and their phytochemicals inhibit the progression and development of cancer (Aung *et al*., 2017). Various other methods for treating cancer - surgery, radiotherapy, chemotherapy, cancer vaccinations or several combinations exist with many side effects like limited bioavailability, toxicity, non specificity *etc* (Patra *et al*., 2014; Mukherjee and Patra, 2016). To minimize the side effects of drugs, efforts are being made by increasing drug efficacy in the lesion and developing novel/targetted drug delivery (Vinogradov and Wei, 2012).

In the present investigation, we have evaluated *in vitro* cytotoxic potential of four fruit extracts (methanolic), their bioactive fractions (n-hexane, chloroform and butanol) and further isolated active ingredients from specific fractions that displayed significant *in vitro* cytotoxic effect against various human cancer cell lines originated from different tissues. In the first/initial phase, screening of fruit extracts *viz*., *Artocarpus heterophyllus* (katahal), *Carissa carandas* (karonda), *Grewia asiatica* (phalsa), *Syzygium cumini* (jamun) was performed against eight human cancer cell lines. Results showed that the extracts of karonda and jamun displayed significant *in vitro* cytotoxic effect against various human cancer cell lines. Karonda fruit extract (100 µg/mL) showed 70%, 72% and 75% growth inhibition of MCF-7, HT-29 and A-549 cancer cells respectively. In case of jamun, the extract was found to be active against A-549, PC-3 and HCT-116 human cancer cell lines with growth inhibition of 70%, 72% and 74% at the conc. of 100 µg/mL. However, the fruit extracts prepared from *G. asiatica* and *A. heterophyllus* were found to be completely inactive against all the tested eight human cancer cell lines. In view of the data obtained regarding

screening of extracts, initially the bioassay guided fractionation of karonda and jamun was carried out with n-hexane, chloroform and butanol solvents, the said fractions were tested against the same human cancer cell lines, as used in case of extracts. In jamun, IC_{50} values of hexane fraction calculated were 26.81±0.77 µg/mL (SW-620), 29.26±0.97 µg/mL (MIA PaCa-2), 35.16±1.46 µg/mL (A-549), 36.25±0.77 µg/mL (HCT-116) and >50 µg/mL (MDAMB-231, HT-29, PC-3). IC_{50} values obtained in chloroform fraction were 18.02±1.01 µg/mL (MDAMB-231), 40.02±1.94 µg/mL (A-549), 43.10±0.26 µg/mL (MIA PaCa-2), 48.30±3.42 µg/mL (HCT-116) and >50±0.28 µg/mL (SW-620). The striking observations were produced by chloroform fraction of karonda as it showed remarkable results by inhibiting the growth of A-549 cells by 100% at 100, 50, 30 µg/mL and 99% at 10 µg/mL. For HT-29 cell line, growth inhibition of 100% at 100 &50 µg/mL, 99%, 94% GI at 30 and 10µg/mL was observed. In case of MCF-7 cancer cell line, chloroform fraction showed significant cytotoxic potential with growth inhibition of 100%, 98%, 89%, 77% at 100, 50, 30, 10µg/mL. On the basis of *in vitro* cytotoxic potential of chloroform fraction, the IC_{50} values obtained were 1.48±0.002 µg/mL (A-549), 1.28±0.02 µg/mL (HT-29) and 3.98±0.24 µg/mL (MCF-7). As the results indicated, that all the IC_{50} values calculated are <10 µg/mL thereby suggesting strong *in vitro* cytotoxic efficiency of chloroform fraction.

In the second phase of study, bioactive compounds were isolated from the said active fractions and characterized *via* NMR (^{1}H &^{13}C), mass spectroscopy (HRMS) and thin layer chromatography (TLC). In jamun, n-hexane and chloroform fraction were taken up for isolation of active ingredients and a total of four compounds (one from n-hexane and three from chloroform fraction) were isolated. From n-hexane fraction, a compound named beta-sitosterol (1) was isolated, tested against human cancer cell lines and found to be active against specifically SW-620 (colon) cell line with IC_{50} value of 42.46±2.30 µM. The remaining three compounds- oleanolic acid (2), gallic acid (3) and quercetin (4) were isolated from chloroform fraction of jamun. The compound 2 represented the *in vitro* cytotoxic potential against A-549 and SW-620 with IC_{50} values of 18.78±2.43 µM and 40.51±1.99 µM respectively. The compound 3 specifically targeted two cancer cell lines from colon origin with IC_{50} values of 54.26±2.71 µM (HCT-116) and 54.5±1.64 (SW-620). Interesting observation was seen in compound 4 with IC_{50} values of 17.37±2.99 µM (HCT-116),

25.36±1.98 µM (A-549), 35.06±1.37 µM (MIA PaCa-2) and 36.18±1.85 µM (SW-620). Therefore, maximum growth inhibition against more number of human cancer cell lines was observed in case of compound 4 *i.e.,* quercetin followed by oleanolic acid (2) and gallic acid (3). However, the fractionation of *G. asiatica* extract was also carried out and surprisingly, the n- hexane and chloroform fraction of the fruit exhibit *in vitro* cytotoxic effect against colon (SW-620) cancer cell line (although its extract did not possess any cytotoxic effect). Accordingly, chloroform fraction was taken up for further isolation of bioactive compound and a molecule namely 5-hydroxy methyl furfural (5-HMF) was isolated, that was tested against SW-620 and produced IC_{50} value >50±0.77 µM, therefore not considered a potent cytotoxic agent.

In karonda, a compound was isolated from chloroform fraction and HMBC correlations showed long range correlations between methyl singlet (Me-26) at δ 0.96 ppm with four carbon resonances at δ 32.9 (C-7), 39.4 (C-8), 41.9 (C-14) and 48.9 (C-9) ppm. Two methyl groups at δ 0.93 and 0.98 ppm showed long range correlations to carbon resonances at δ 38.5 (C-4), 55.3 (C-5) and 78.5 (C-3) ppm which supports that those two methyls are attached to the same carbon neighboring the oxygenated carbon at C-3. Based on these key HMBC correlations, compound can be characterized as ursan type triterpene and could be ursolic acid, however compound did not show identical TLC pattern and observed optical rotation (Papanov*et al*., 1992) which indicate that compound is different as compared to ursolic acid and may differ in relative configuration.The structure of compound is therefore defined as 3S, 5R, 9R, 25S, 26R, 27S, 18S, 28S, 29R and 30S ursan type new triterpene named carissic acid (CA).

This particular compound *i.e.,* carissic acid (CA),showed potent *in vitro* cytotoxic effect with IC_{50} values of 3.47±0.26 µM for A-549, 2.65±0.35 µM for HT-29, 10.47±0.69 µM for MIA PaCa-2 and 13.58±0.59 µM for MCF-7 cells. Results demonstrated that among all the isolated compounds, CA was found to be more cytotoxic (IC_{50}<10 µM) with maximum *in vitro* cytotoxic impact and less IC_{50} values. To gain further insight regarding anticancer potential of CA, it was investigated against lung (A-549) cancer cell line *via* different assays to confirm the basis of apoptotic cell death. It induced chromatin condensation as observed by DAPI staining, ROS generation as well as loss of mitochondrial membrane permeability.

Furthermore, it leads to impaired cell migration in dose dependent manner, thereby caused the increased activation of caspase 3 and significantly induced PARP cleavage as compared to control cells. All these observations, confirmed the pivotal role of CA in mediating apoptosis in A-549 cells.

The data was found in good agreement with the literature values. *G. asiatica* leaves and fruit extracts showed significant anticancer activity against liver and breast cancer. *In vitro* cytotoxic activity was determined by methylthiazolyltetrazolium (MTT) assay using epidermal kidney (HEK-293), breast (MCF-7), cervical (HELA), lung (NCI-H522) and laryngeal (Hep-2) cancer cell lines. The fruit extract was found to be active on lung (IC_{50} = 59.03 µg/mL) and breast (IC_{50} = 58.65 µg/mL) cancer cell lines, while the leaf extract was active against breast (IC_{50} =50.37 µg/mL) and Hep-2 (IC_{50} = 61.23 µg/mL) cancer cell lines (Marya *et al*., 2011). The activity of 5-hydroxymethyl furfural (isolated from *G. asiatica*) is already mentioned in the literature with respect to its cytotoxic potential. 5-HMF showed maximum *in vitro* cytotoxic potential on A-375 cells (human melanoma) other than common human cells *via* MTT assay. Moreover, it has been investigated to reveal its mechanism of cell death where 5-HMF induced apoptosis in A-375 cells and G0/G1 cell cycle arrest. Further, cell death was confirmed by TUNEL and DAPI co-staining assay. Many reports are showing that 5-HMF has some pharmacological effects like antioxidant, antiischemic and antityrosine enzyme effects, improve blood rheology and thereby affect the role of glycyrrhizin metabolism (Wang *et al*., 2010). 5-HMF protects L02 cells from damage induced by H_2O_2 by inhibiting the effect of cell apoptosis caused by promoting the S phase to G2/M phase with decreased caspase-3 activity and nitric oxide level (Ding *et al*., 2010).

Leaves of C. *carandas,* the unripe and ripe fruit extracts were screened for their anti-cancer activity using n-hexane, chloroform and methanol as the solvent systems against human ovarian carcinoma cells and lung cancer cells. The extracts exhibited good anti-cancerous activity (Sulaiman *et al*., 2008). The previous investigations support the fact that mitochondria, an important organelle plays major role in mediating apoptosis (Kug *et al*., 2007) and reduced MMP, is considered initial step in mitochondrial-mediated apoptosis (Muhammad *et al*., 2012). Many studies also reported the role of phytochemicals in ROS generation that target the cancer cells

above a toxic threshold to initiate the cell death (Nunes *et al*., 2011; Nianyu *et al*., 2003; Circu and Aw, 2010). *S. cumini* fruit extract possesses anti-oxidative activity (Benherial and Arumughan, 2007; Hassimotto *et al*., 2005; Faria *et al*., 2011; Aqil *et al*., 2016); anti-proliferative activities against human lung (Aqil *et al*., 2012) and breast cancer cells (Li *et al*., 2009; Aqil *et al*., 2016) and pro-apoptotic effects against human breast cancer cells (Li *et al*., 2009). The bioactives from berries are reported to have many roles in cancer prevention including inhibition of the formation of carcinogen-induced DNA damage, protection against oxidative DNA damage, inhibition of carcinogen-induced tumorigenesis and modulation of signaling pathways involved with cellular proliferation, inflammation and cell cycle arrest (Kausar *et al*., 2012). It has been previously reported that β-sitosterol (an active ingredient from *S. cumini*) showed cancer cell specific cytotoxic effects by inhibiting proliferation of COLO 320 DM cells, while showing little toxicity in VERO cells. It also possesses *in vitro* cytotoxic effects in breast cancer and Bowes cell lines (Awad *et al*., 2007; Nguyen *et al*., 2005). The anticancer potential of oleanolic acid has been evaluated in many of the cancers, including liver cancer (Liese *et al*., 2015, Shi *et al*., 2016), lung cancer (Zhao *et al*., 2015), breast cancer (Wu *et al*., 2016), colon cancer (Li *et al*., 2015) and bladder cancer (Mu *et al*., 2015), In another study, chronic oral administration of oleanolic acid (five times a week for four weeks) decreased colon carcinogenesis in rats, showing that oleanolic acid can inhibit tumor initiation (Furtado *et al*., 2016). The phenolic compound gallic acid induced apoptosis in human prostate cancer cell lines *i.e.*, LNCaP and DU145 and human melanoma cells. Moreover, it also inhibits the cell proliferation of papilloma and carcinoma, radiation induced damage and peroxidation of DNA (Agarwal *et al*., 2006; Reddivari *et al*., 2010). Many studies confirmed the antiproliferative role of gallic acid on the HCT-15 colon cancer cell line in a dose dependent manner which was similar to its effect on the human hepatoma SMMC-7721 cell proliferation in *in vitro* condition (Li *et al*., 2014). In many studies, cytotoxic effect of quercetin has been confirmed wherein *in vitro* and *in vivo* studies have suggested that it possesses anticancer activity against different tumors namely colon, lung, breast and prostate (Catanzaro *et al.*, 2015). Quercetin exert its anticancer effects through different mechanisms, including the inhibition of DNA topoisomerase I/II, the release of cytochrome *c*, the activation of caspase 3 and HSP27 and 72 elevation (Badziul *et al*., 2014).

Jackfruit is widely accepted by consumers, researchers and food industries due to the presence of bioactive compounds and diversity products made out of it (Swami *et al.,* 2012; Saxena *et al.,* 2009; Dutta *et al.,* 2011; Lin *et al.,* 2009; Devalaraja *et al.,* 2011).Fructose, glucose and sucrose are the major sugars while capric, myristic, lauric, palmitic, oleic, stearic, lisnoleic and arachidic acids are the major fatty acids present in jack fruit (Jagtap and Bapat, 2010; Ong *et al.,* 2006). The seeds contain β-carotene, α-carotene, β-zeacarotene, α-zeacarotene and crocetin which are mostly present in trans form (Baliga *et al.,* 2011; Jagtap and Bapat, 2010; De Faria *et al.,* 2009). The anti-cancer studies of the diethylether extract of *Artocarpus altilis* wood was performed in human T-47D breast cancer cells and examined for its effect on cell viability, nuclear morphology and sub-G1 formation. The results demonstrated that *Artocarpus altilis* wood extract induced apoptosis and sub-G1 phase formation in breast cancer (T-47D) cells and therefore, has a potential as an anti-cancer agent (Patel *et al.,* 2011).

The results from the present investigation forms a good basis for the selection of the anticancer compounds (particularly carissic acid,isolated and well characterised using detailed NMR studies first time from any plant source) that will serve as lead molecules for the development of anticancer drugs and will provide a great service and promise to patients especially with lung carcinoma.

Summary & Conclusion

CHAPTER-6

SUMMARY AND CONCLUSION

Cancer cells provide resistance and decreased sensitivity to the chemotherapeutic agents available, so there is an emerging need for natural drugs. Any natural compound that kills the cancer cell with either negligible or least effect on normal cells, is used for cancer therapeutic strategies. More than five decades ago, chemotherapy was introduced and in most of the cancer cases, its application is still very limited. The target of a chemotherapeutic drug should be a specific receptor, protein or DNA of neoplastic cells, thereby reduce the tumor by inducing cytoxicity with least damage to adjacent normal cells. In addition, combination effects of naturally occuring compounds and standard chemotherapeutic drugs provide a promising additive or synergistic effect. Among various phytochemicals, polyphenols/flavonoids are known to be present in many edible fruits like berries, walnut, grapes, apples *etc*.Most primitive and effective method from pre-historic time to heal injuries, cure diseases and relieve suffering, is treatment from plant extracts. Every part of plant from root to tip, participates in the production of drugs against many diseases. According to WorldHealth Organization, most lethal and leading cause of deathworldwide accounting for 7.6 million deaths (13% of all deaths) is cancer. Researchers use natural components, syntheticcompounds and even micro-organisms for making an active drugwith the capability to distinguish between healthy cells and tumorcells. Though many natural compounds are under clinical trials, yetcompounds with anti-cancerous activities still not under clinicaltrial cannot be neglected. In the search of potential anticancer agents from natural products, the present research work was carried out to evaluate the *in vitro* cytotoxic effect of fourfruits of Jammu region against eight human cancer cell lines originated from different tissues.

A. heterophyllus (katahal), *C. carandas* (karonda), *G. asiatica* (phalsa) and *S. cumini* (jamun) were collected and screened againsteight human cancer cell lines from five different origins such as MCF-7 and MDAMB-231 (breast), HCT-116, HT-29 and SW-620 (colon), A-549 (lung), MIA PaCa-2 (pancreatic) andPC-3 (prostate) *via* SRB assay.For bioevaluation, extraction procedures with powdered dried fruit

material were employed and methanolic extracts of all the above mentioned fruits were prepared and initially used as test material for bioassay. Further, fractions were prepared from active extracts and compounds were isolated from particular active/specific fractions.

Cytotoxicity of a number of test materials (extracts, fractions, compounds) was performed in 96-well flat bottom tissue culture plates in complete growth medium containing gentamycin (5mg/ml).Thetest materials specified above were evaluated further. The anticancer activity was determined by the cytotoxic potential of the test material. Cells were allowed to grow for 24 h on 96 – well flat bottom tissue culture plates. Cells were further allowed to grow in the presence of test material for 48 h. Cell growth was terminated by addition of 50% (w/v) TCA. Cells were stained with SRB dye. Excess dye was removed by washing with 1% (v/v) acetic acid and bound dye was dissolved in Tris buffer and OD was taken at 540 nm.Positive controls, *e.g.*,Doxorubicin, 5-Flurouracil, Mitomycin-C, Paclitaxel were included and growth inhibition of 70% or above was considered active for our bioassay purpose in case of extracts/fractions, but when purified compound was used at different molar concentrations, the growth inhibition of 50% or above was considered active.

Maximum yield of extract *i.e.,* 54.54% was obtained in case of karonda followed by jamun, very less in case of phalsa and jack fruit. The primary results revealed that extract of jamun was found to be active against three human cancer cell lines namely A-549, HCT-116 and PC-3whereas karonda extract suppressed growth inhibition of MCF-7, HT-29 and A-549 cancer cells. The n-hexane, chloroform and butanol fractions of jamun and karonda were prepared in the order of increasing polarity. One compound (beta-sitosterol) from n-hexane fraction and three compounds (oleanolic acid, gallic acid, quercetin) from chloroform fraction of jamun were isolated. Among all the compounds, quercetin displayed more *in vitro* cytotoxicity against more number of human cancer cell lines with less IC_{50} values. The most interesting compound was carissic acid (CA) that is isolated from chloroform fraction of karonda and exhibited potent *in vitro* cytotoxicity against A-549, MCF-7 and HT-29 cancer cells with maximum growth inhibition and IC_{50} value <10 µM. It is a pentacyclic triterpene carboxylic acid containing six isoprene units that showed cytotoxic effect on all the above mentioned human cancer cell lines leading to cell

death. So, the effect of CA has been investigated on lung cancer cell line (A-549) *via* DAPI staining, mitochondrial membrane potential (MMP), reactive oxygen species (ROS), scanning electron microscopy (SEM), scratch and colonogenic assay. It is concluded that CA induced dose dependent decrease in cell proliferation with reduced mitochondrial membrane potential and DNA fragmentation. The antiproliferative effect of compound confirmed the cell death through ROS generation and also led to the impaired cell migration. For further confirmation, changes in apoptosis associated proteins in A-549 cells were also examined through western blotting. PARP cleavage was increased accordingly suggesting the role of caspase dependent pathway *via* caspase-3.

Conclusively, carissic acid has been isolated and well characterised using detailed NMR studies for the first time from any plant source and the results confirmed the anticancer efficacy of CA on A-549 cancer cells that mediated the induction of apoptosis following the activation of caspases. The findings of present research work suggest that CA can be a potential candidate for lung cancer therapy and further studies are required to confirm its potency *in vivo.*

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Vita

VITA

Name of the student	:	Ms. Navneet Kour
Father's name	:	S. Pervinder Singh
Mother's name	:	Smt. Tajvinder Kour
Nationality	:	Indian
Date of birth	:	13th September,1991
Mobile	:	+91-9697247709
E-mail	:	kour.navneet13@gmail.com
Permanent Address	:	H. No. 204, Narwal Pain Satwari, Jammu Cantt.District-Jammu, Pin-180003, J&K, India

Educational Qualification

Bachelor degree	:	B.Sc. (Medical)
University and year of award	:	University of Jammu, 2012
Percentage of marks (OGPA)	:	69.87%
Master's Degree	:	M.Sc. Biochemistry
University and year of Award	:	SKUAST-Jammu, 2015
OGPA	:	8.49/10.0
Title of Master's thesis	:	**"Exploring *in vitro*cytotoxic effect of some minor fruitsfrom Jammu region."**
Ph.D Degree	:	Ph.DBiochemistry
University and year of Award	:	SKUAST-Jammu, 2020
OGPA	:	8.16/10.0
Title of Ph.D thesis	:	"**Evaluation of some fruits from Jammu subtropics: Possible anticancer role using *in vitro* cytotoxicity assays**"
Experience	:	06 months training on anticancer assays and 15 months training on basic cell culture technique at CSIR-IIIM, Jammu
Award / Fellowship	:	Maulana Azad National Fellowship during Ph.D.

CERTIFICATE-IV

Certified that all the necessary corrections as suggested by the External Examiner and the Advisory Committee have been duly incorporated in the thesis entitled **"Evaluation of some fruits from Jammu subtropics: Possible anticancer role using *in vitro* cytotoxicity assays"** submitted by **Ms. Navneet Kour**, Regd. No. **J-15-D-07-BS.**

19/10/2020

(Dr. Vikas Sharma)
Major Advisor and Chairman
Advisory Committee

Place: Jammu

Date: 19/10/2020

Head
19/10/20
Division of Biochemistry

भारतीय समवेत औषध संस्थान

(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद्)

केनाल रोड, जम्मू – 180001

Indian Institute of Integrative Medicine

(Council of Scientific & Industrial Research)

Canal Road, Jammu - Tawi - 180 001 (INDIA)

July 8, 2019

Abdul Rahim
Sr. Principal Scientist,
Head PME & IT Division,
CSIR-IIIM, Jammu.

TO WHOM IT MAY CONCERN

This is to certify that **Ms. Navneet Kour,** Ph.D. scholar Div. of Biochemistry, FBSc, **Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu, India** has successfully completed her training on ***"Basic Cell Culture Technique and in vitro Cytotoxicity of some Medicinal plants against various human cancer cells"*** under the guidance of Dr. Shashank K. Singh in Cancer Pharmacology Division of this institute from 05-10-2017 to 15-02-2019.

She is honest, sincere and a hard worker. I wish her all the success in her life.

(Dr. Shashank K. SIngh)
Principal Scientist
Cancer Pharmacology Division
CSIR-IIIM, Jammu

(Abdul Rahim)

Phones : EPABX (0191) 2569000-06; (Director) 2569111, 2569222, 2569333 (Fax) : PME 2569019 (P/F)
(CoA) 2569016-17 (F), PUR 2569025 (P/F), ACCTS 2569026, Website : (www.rrljammu.org)