ANTICANCER AND ANTIVIRAL ACTIVITY OF MARINE ALGAE

Thesis Submitted To

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY, GUINDY, CHENNAI

As a partial fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

Submitted by

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CERTIFICATE

This is to certify that the thesis entitled "**ANTICANCER AND ANTIVIRAL ACTIVITY OF MARINE ALGAE**" is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the requirements for the award of degree of Doctor of Philosophy is the record of original research work done by **Mr. V.Lavakumar**, M.Pharm., for the academic year 2008 – 2012 under my guidance, and the thesis has not formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title.

Date:

Place: **Prof. Dr. V. RAVICHANDIRAN**

DECLARATION

This is to certify that the thesis, entitled "**ANTICANCER AND ANTIVIRAL ACTIVITY OF MARINE ALGAE**" is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the requirements for the award of degree of Doctor of Philosophy is the record of original research work done by me under the guidance of **Dr. V. Ravichandiran**, Principal, Vels College of Pharmacy, Chennai-600117 for the academic year 2008 – 2012 and the thesis has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title.

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ABBRIVATIONS

WHO	-	World Health Organisation
DNA	-	Deoxyribo Nucleic Acid
E_2F	-	E2 Transcription Factor
TNF	-	Tumor Necrosis Factor
FADD	-	Fas-Associated protein with Death Domain
P53	-	Protein 53
BCl_2	-	B-cell lymphoma 2
HCV	-	Hepatitis *C virus*
HBV	-	Hepatitis *B virus*
NF-kB	-	Nuclear factor kappa
GIT	-	Gastro Intestinal Tract
PAHs	-	Polycyclic aromatic hydrocarbons
PE	-	Pet Ether
Bu-OH	-	Butanol
SOD	-	Super Oxide Dismutase
NMR	-	Nuclear magnetic resonance
IR	-	Infrare*d* spectroscopy
ROS	-	Reactive oxygen species

CMC	-	Carboxy Methyl Cellulose
OECD	-	Organisation for Economic Co-operation and Development
EAC	-	Earlich Ascites Carcinoma
KCl	-	Potassium chloride
TBARS	-	Thiobarbituric acid reactive substances
GSH	-	Glutathione
GPx	-	Glutathione peroxidase
GST	-	Glutathione S-transferase
CAT	-	Catalase
PCV	-	Packed cell volume
MSD	-	Mean Survival Day
5-FU	-	5-fluorouracil
Hb	-	Haemoglobin
MDA	-	Malondialdehyde
ASE	-	*Acanthophora spicifera* Extract
BSLT	-	Brine Shrimp Lethality Test
DPPH	-	diphenyl-1-picrylhydrazyl
MTT	-	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
HPLC	-	High-performance liquid chromatography
HPTLC	-	High performance thin layer chromatography
ASW	-	Artificial seawater

DMSO	-	Dimethyl sulfoxide
DMEM	-	Dulbecco’s modified Eagle's medium
FBS	-	Fetal bovine serum
ELISA	-	Enzyme-linked immunosorbent assay
TLC	-	Thin layer chromatography
GLC	-	Gas chromatography
µg/ml	-	micro gram/milli liter
w/w	-	Weight/weight
v/v	-	Volume/volume
ng	-	nano gram
ADP	-	Adenosine diphosphate
AP-1	-	Activator protein 1
FITC	-	Secondary dye in flow cytometry (Fluorescein isothiocyanate)
PI	-	Propidium Iodide (Primary Dye)
EDTA	-	Ethylene di-amine tetra acetic acid
NaOH	-	Sodium hydroxide
PBS	-	phosphate Buffer Saline
$HepG_2$	-	Hepatocellular carcinoma
HT-29	-	Human colon cancercell lines
A549	-	Epithelial cell cancer cell line
MCF7	-	Breast cancer cell line

IC_{50}	-	Half maximal inhibitory concentration
HCC	-	Hepatocellular carcinoma
NADPH	-	Nicotinamide adenine dinucleotide phosphate
DEN	-	Diethylnitrosamine
LFT	-	Liver function test
AST	-	Aspartate transaminase
ALP	-	Alkaline phosphatase
ALT	-	Alanine transaminase
TBST	-	Tris-Buffered Saline and Tween 20
ISOQN	-	Isoquercetin
CHIKV	-	Chikungunya virus
HIV	-	Human immunodeficiency virus
CPE	-	Cytopathic effect
MNCC	-	Maximum Non Toxic Concentration

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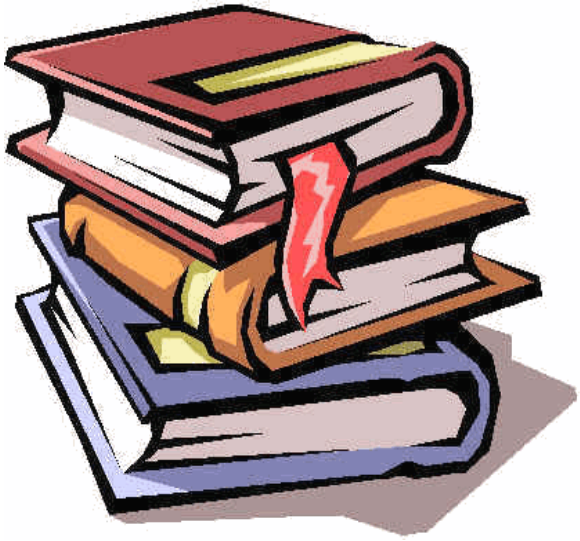


CHAPTER 1
Introduction

1.1 Introduction

Cancer continues to be a global killer, despite the enormous research has developed during the past few decade. According to recent statistics, cancer accounts 23% of total deaths in the India and is the second most common cause of death after the heart disease[1]. Cancer accounts for nearly 1 of every 4 deaths" (Source: Cancer Facts and Figs. 2010 of the American Cancer Society). Moreover, according to Lee Jong-Wook, former Director General of the WHO, "by the year 2020, cancer could kill more than 10.3 million people per year unless action is taken in both the field of prevention and treatment"[2] **.** Cancer is caused by both intrinsic factors (such as inherited mutations, hormones, and immune conditions) and extrinsic factors (such as tobacco, diet, radiation, and infectious organisms; **Fig. 1**).

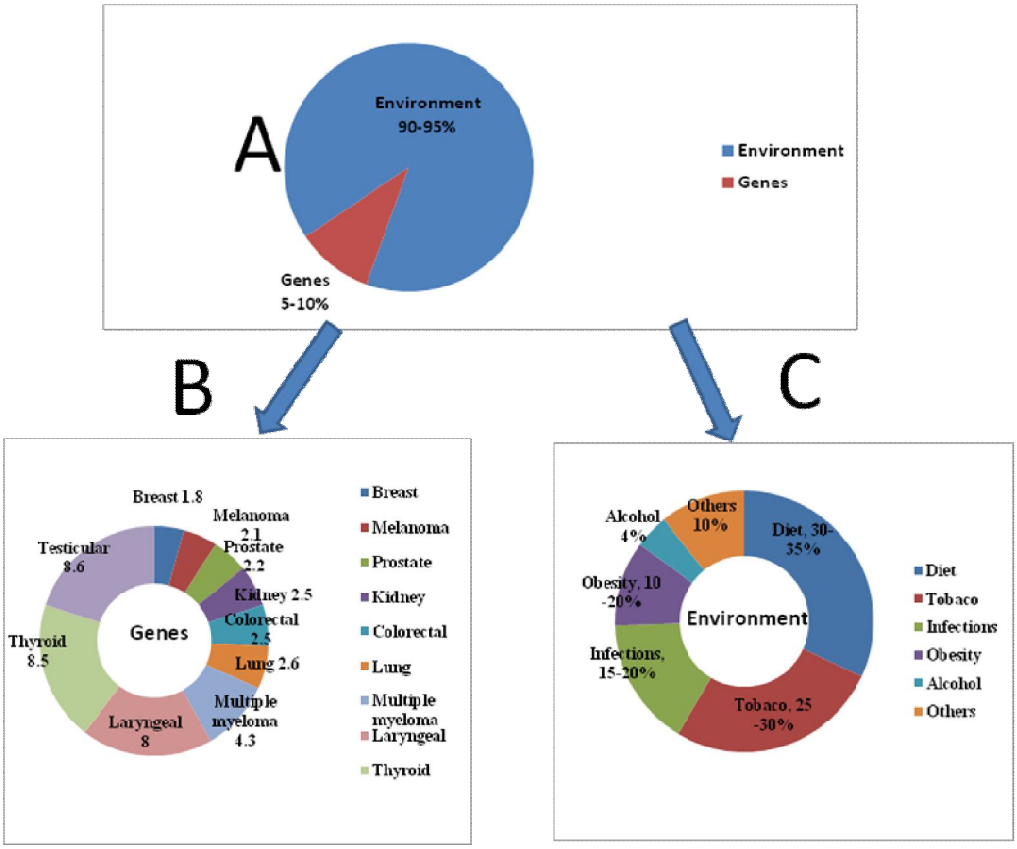


Fig:1 Represents the Cancer is caused by both Intrinsic factors and Extrinsic factors

Although all cancers are result of multiple mutations[3] and these mutations are mainly due to the interaction with the environment[4]. Infact only 5–10% of all cancers are due to an inherited gene defects which is shown in the fig 2. These observations indicate that most cancers are not of hereditary origin and that lifestyle factors, such as dietary habits, smoking, alcohol consumption, and infections, have a profound influence on cancer development[5].

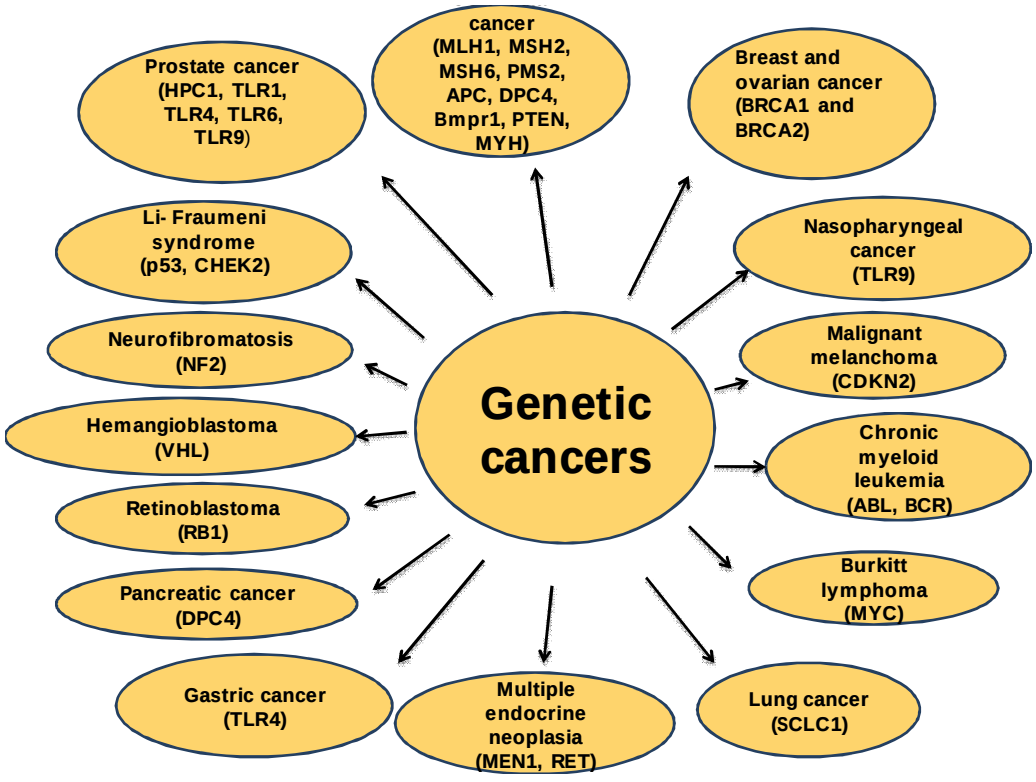


Fig:2 Represents the various cancers through genetic mutation

Modern man is suffering with an increasing incidence of cancer and cancer deaths every year. Statistics indicates that men are largely effected by lung, colon, rectum, and prostate cancer, whilst women increasingly suffers from colon, breast, rectum, and stomach cancer[6]. There is overwhelming evidence that lifestyle factors impact cancer risk and that positive, population wide changes can significantly reduce the cancer burden. Although the hereditary factors cannot be modified nor changed but the lifestyle and environmental factors are comfortably modifiable to prevent these dreadful diseases.

For millennia, these diseases processes remained the major area of detailed understanding to mankind. Current epidemiologic evidences links behavioural factors to a variety of malignancies which includes the most common cancers diagnosed in the developed world like lung, colorectal, prostate, and breast cancers. Owing to the tremendous impact of modifiable factors on risk, especially for the most prevalent cancers, it has been estimated that 50% of cancer is preventable[7]

However, to bring about dramatic reductions in cancer incidence there should be widespread of lifestyle changes are necessary. Cancer, in all forms, has been poorly understood, feared and usually fatal. This disease, thought to be a heterogeneous group of related disorders, was often diagnosed in the past on the basis of macroscopic features such as mass, relentless growth and metastatic spread. With better understanding of pathophysiology of the disease and advances in molecular biological techniques and the scenario is fast changing in the area of cancer biology.

1.1.a. An Insight in to the Carcinogenesis[8]

The transformation of a normal cell into a cancerous cell is believed to proceed through many stages over a number of years or even decades. The stages of carcinogenesis include initiation, promotion, and progression (Fig 3). The first stage involves a reaction between the cancer-producing substance (carcinogen) and the DNA of tissue cells. There may be a genetic susceptibility. This stage may remain dormant, and the subject may only be at risk for developing cancer at a later stage. The second stage occurs very slowly over a period ranging from several months to years. During this stage, a change in diet and lifestyle can have a beneficial effect so that the person may not develop cancer during his or her lifetime. The third and final stage involves progression and spread of the cancer, at which point diet may have less of an impact. Preventing initiation is an important anticancer strategy, as are the opportunities to inhibit cancer throughout the latter stages of malignancy. One of the most important mechanisms contributing to cancer is considered to be oxidative damage to the DNA. If a cell containing damaged DNA divides before the DNA can be repaired, the result is likely to be a permanent genetic alteration constituting a

first step in carcinogenesis. Body cells that divide rapidly are more susceptible to carcinogenesis because there is less opportunity for DNA repair before cell division. Mutagenic changes in the components of signalling pathways lead to cellular transformation (cancer).

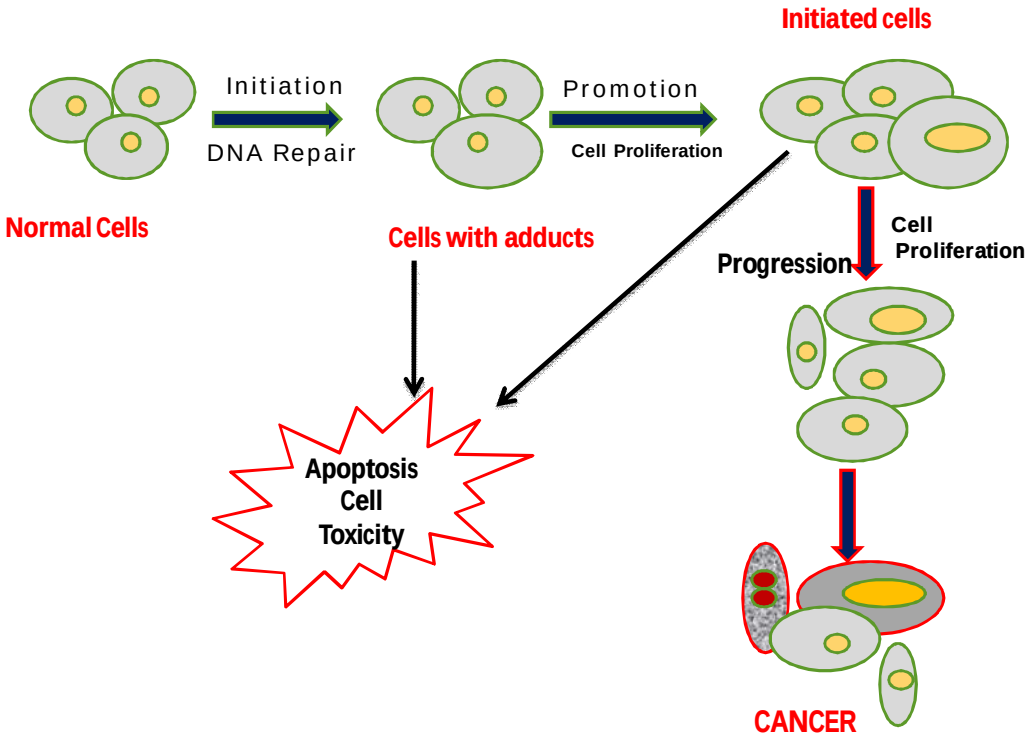


Fig 3 Represents the tumour progression towards the normal cell to cancerous cell leads to apoptosis by cell cycle arrest

Normally, human cell life cycles consist of proliferation, differentiation, and cell death. The cell reproductive life cycle has four phases G_1, S, G_2, M. G_0 is a stage of quiescence, during which cell carries out its ordinary role for the organism (Fig 4). If there is any proliferation, then purines and pyrimidines, the building blocks for DNA synthesis, must be produced. The cell then enters G1 phase in which nucleosides and enzymes are synthesised. In the S phase DNA synthesis occurs. One enzyme responsible for replication of DNA for the new cell that seems to be particularly vulnerable to exogenous plant chemicals is topoisomerase. The

next phase is G2 when the cell prepares other structures needed for mitosis. The M phase is mitosis itself , where two daughter cells are produced

There are two distinct of hallmarks of cancer. The first family describes the conditions the cell of a tissue needs to exhibit uncontrolled proliferation. These are self sufficiency in growth signals, ignoring of anti-growth signals, evading apoptosis and immortalization. Acquiring all these four properties is a necessary condition for a cell to become malignant. However, in order to cause certain death of the organism, a tumor must also have the two hallmarks belonging to the second family: angiogenesis and metastasis. Angiogenesis, the process of generating new blood vessels which infiltrate the tumor, is essential for its growth beyond a (very small) critical size[9]

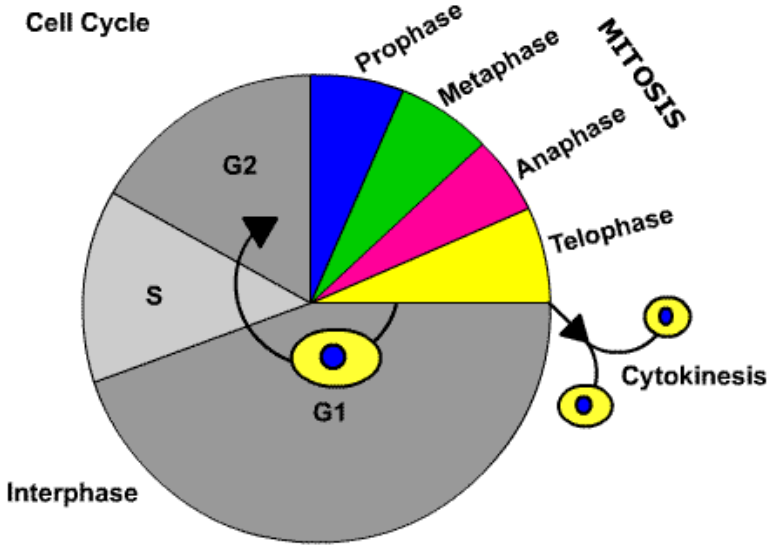


Fig 4 represents the normal cell cycle and its various phases

In a healthy organism, proliferation is a very tightly controlled process which is achieved by all kinds of regulatory networks. The breakdown of these networks results in uncontrolled proliferation. The important network is growth factors which are used as signals to transfer information between two cells (paracrine signalling). There is an unequivocal production of growth factors which lead to uncontrolled proliferation. The second is over-expression of growth factor receptors. In other

words imbalance between quantity of the signalling molecules (growth factors) and the number of receptors, i.e. the efficiency of receptions, is increased. The gene which encodes for the receptor can undergo a mutation. Over expression or mutation of a gene drive the cell towards cancer, the affected gene is called an oncogenes.

A healthy cell receives signals to proliferate as well as signals to not divide. Some of these signals are of extracellular, like e.g. anti-growth factors, other are internal. If such an anti-growth factor binds to its receptor, the complex initiates a cascade which blocks transcription. The main controller of these processes is the Rb-protein. It works as follows: When a transcription factor called E2F (elongation factor 2) is free and activated, the transcription of a large number of genes is initiated, the biological material needed for growth is synthesized and cell proliferation can proceed. When Rb is bound to E2F, it neutralizes E2F and makes it inactive[10].

However, as soon as Rb is deactivated, it releases the E2F molecule which subsequently enters the nucleus and transcription starts. The transition from the active to the inactive state of Rb is induced by its phosphorylation, i.e. adding a phosphate group to the protein. Rb belongs to a family of proteins, or genes, which are called tumor suppressors. Under normal conditions wild type Rb arrests growth; if it undergoes a mutation such that freezes it in its inactive state, it loses its function. Finally, it is worth noticing that modification of one of the two copies of the oncogenes and tumor suppressors have different effects: while a mutation into an oncogene of one of the two copies may be sufficient for a physiological effect, generally both copies of the tumor suppressor have to be removed or inactivated in order to affect the cell.

1.2 Apoptosis

Programmed cell death or apoptosis has several common pathways regardless of the endogenous biochemical pathways[11] shown in fig 5. First, a cellular sensor detects the presence of a death-inducing signal which activates a signal transduction

pathway. Signal transduction may occur by two distinct pathways: extrinsic or receptor-linked apoptosis or intrinsic or mitochondria- mediated apoptosis[12].

1.2.a. Extrinsic apoptotic pathway

This receptor-linked pathway requires the binding of a ligand to a death receptor on the cell surface. The cytokine, tumor necrosis factor (TNF), binds to the death receptor, TNF receptor type 1, which recruits two signal transducing molecules: TNFR 1-associated death domain protein (TRADD) and Fas-associated protein with death domain (FADD). This complex then binds to procaspase 8 to activate caspase 8, which initiates the protease cascade leading to apoptosis[13]

1.2.b. Intrinsic apoptotic pathway

This pathway is mediated by the mitochondrial release of cytochrome-c[14]. Cytosolic cytochrome c induces the formation of the multisubunit apoptosome composed of apoptotic protease activating factor-1 (Apaf-1), procaspase 9 and either ATP or dATP. Activated caspase 9 is not required for the apoptosome to recruit procaspase 3 and activate it by cleavage[15] to caspase 3. Active caspase 3 then mediates the apoptotic cascade. Initiation of apoptosis may occur immediately or be delayed after the DNA damage has occurred. The response may or may not be dependent on the presence of the nuclear transcription factor, p53. If p53 is upregulated, it is activated by the phosphorylation of serine 46 by homeodomain-interacting protein kinase-2, and the two proteins cooperate in the activation of p53-dependent transcription[16]. Genes induced by p53 include Bax, a bcl-2 homologous protein, which may oligomerize and form pores in the outer mitochondrial membrane and their by, releasing cytochrome C from the space between the inner and outer mitochondrial membranes. In some situations, caspase 8 may cleave Bid, a member of the BH3 only domain subgroup of the Bcl-2 family, to a truncated form, tBid, which binds with another Bax-related protein, Bak, to release cytochrome C (Wie et al., 2000 ;). All mitochondria release cytochrome C within 5 min once the process is finally initiated and other mitochondrial contents are also released

including Smac/DIABLO (caspase activator) and apoptosis-inducing factor[17]. Next, caspase 3 activation is detected following the formation of the apoptosome

1.3 Immortalization

A normal cell cannot divide an infinite number of times and there is a kind of clock which keeps count. This clock uses the fact that during cell division DNA is replicated. Replication of DNA is done by a protein called DNA polymerase. During cell division it attaches itself to a single strand of DNA of the mother cell, runs along it and synthesizes a matching strand of DNA. This way the number of copies of the entire DNA content of the dividing cell doubles, and subsequently, during mitosis, it is divided evenly between the two daughter cells. However, during each copying process the DNA polymerase cannot replicate the last stretch of the mother DNA strand to which it binds (these ends of each DNA are called telomeres). Consequently, the resulting DNA of the daughter cells is shortened compared to the original template. Each division reduces the length of DNA by about 100 nucleotides. In fully mature differentiated cells expression of telomerase is suppressed and therefore these cells cannot divide infinitely often. The division of cancer cells, on the other hand, is not restricted to a certain number of times. Cancer cells circumvent this limitation and acquire the ability to elongate their telomeres. This process is called immortalization[18]

1.4. Metastasis and angiogenesis

Metastasis is the process by which cancer cells detach from the primary tumor mass, enter the blood (or lymphatic) circulation (Fig 6) and wander around in the organism and attach and colonize some vital organ. In order to metastasize a cell has to solve a large number of problems: normal cells do not detach from their tissue of origin and do not migrate freely; and re-attachment to cells that are very different is also not an easy task

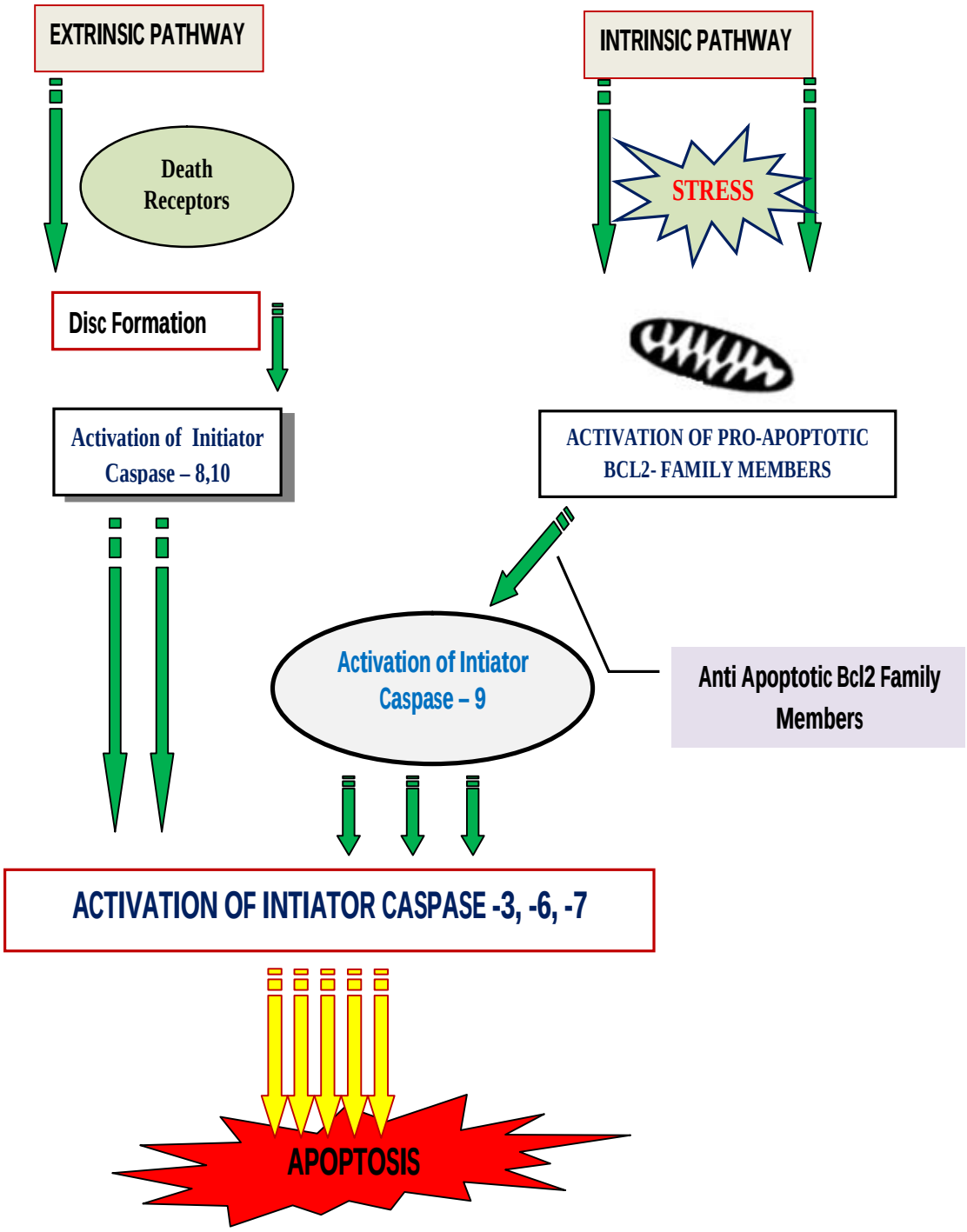


Fig: 5 represents the Apoptotic mechanism

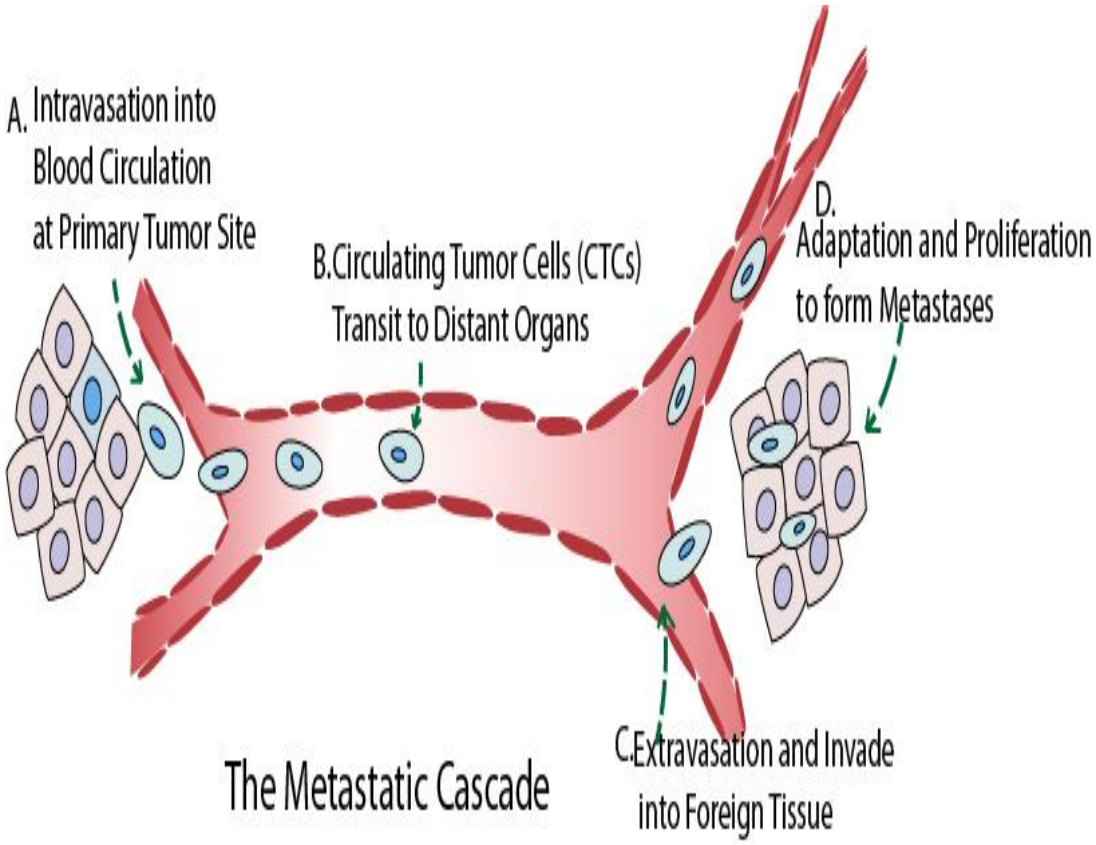


Fig 6 represents the Metastatic Cascade

But before being able to metastase, a tumor faces another problem, namely the supply of nutrients and oxygen. In normal tissues, those are delivered via the bloodstream but as a tumor mass expand (by division of its cells), only its edges can grow to the vicinity of existing blood vessels. Hence, during growth most of the tumor mass becomes increasingly distant from blood vessels and is consequently deprived of oxygen (the diffusion length of oxygen in human tissue is on the order of a few hundred microns). Thus, in order to grow beyond a critical size, a tumor induces vascularization: the in growth of blood vessels. This process is called angiogenesis[19].

1.5. Risk Factors of Cancer

Worldwide Tobacco Smoking is the primary cause of lung cancer. The tobacco smoking and chewing increases the risk of developing at least most of cancers which listed in fig 7**.** The tobacco smoke contains at least 50 carcinogens in which

active carcinogen was said to be benzopyrenediol epoxide, where as a this particular carcinogen is having the direct etiological link with lung cancer[20]. Among all developed countries the prevalence of smoking has been slowly declining due to strong governamnt policies and awareness programmes. At the similar time the similar scenario exits in developing countries like India. The prevalence of smoking is increasing due to lack of governament policies and awareness towards smoking. The only way to minimise the sigar smoking is to accelerated tobacco control programs, will be the only way to reduce the rates of tobacco-related cancer mortality. How smoking contributes to cancer is not fully understood. It has been known that smoking can alter a large number of cell signalling pathways in lung tissue and it has been established that a direct link through the activation of NF-kB[21].

1.5.a. Alcohol

The first report between the alcohol consumption and an increased risk of esophageal cancer was published in 1910[22]. Since then, a number of studies have revealed that chronic alcohol consumption is a high risk factor for cancers which primly lays on upper aero digestive tract, including cancers of the oral cavity, pharynx, hypopharynx, larynx, and esophagus[23, 24] as well as for cancers of the liver, pancreas, mouth, and breast **(Fig. 6).** There is also evidence that a synergistic effect between heavy alcohol ingestion and hepatitis C virus (HCV) or hepatitis B virus (HBV), which presumably increases the risk of hepatocellular carcinoma (HCC) by more actively promoting cirrhosis. The relationship between alcohol and inflammation has also been well established, especially in terms of alcohol-induced inflammation of the liver. How this particular alcohol contributes carcinogenesis is not fully understood but, ethanol may play a role in formation of cancer. Specifically, when ethanol is metabolized, acetaldehyde and free radicals are generated where these free radicals are believed to be predominantly responsible for alcohol-associated carcinogenesis through their binding to DNA and proteins, which destroys folate and results in secondary hyper proliferation.

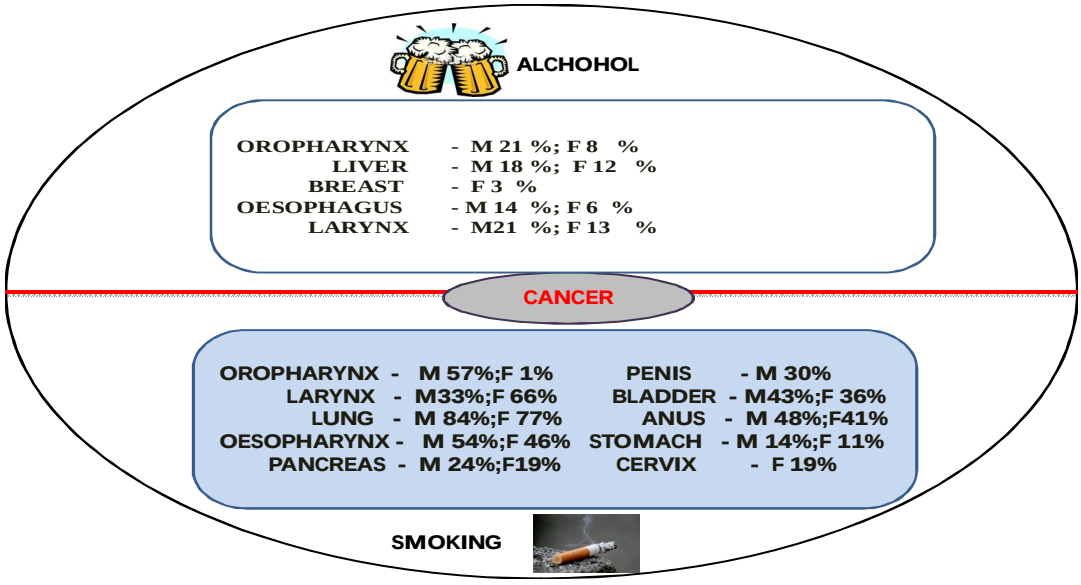


Fig 7 represents Cancers that have been linked to alcohol and smoking

1.5.b. Diet

Primly diet influences the lower GIT cancer deaths in world wide which is directly relay on diet (Fig. 7). The extent to which diet contributes to cancer deaths varies a great deal, but it purely depends on type of cancer[25]. Most carcinogens that are ingested, such as nitrates, nitrosamines, pesticides, and dioxins, come from food or food additives or from cooking. Another consecutive factor for many types of cancers is consumption of red meat. Especially, for those of the gastrointestinal tract, but also for colorectal, prostate[26], bladder, breast, gastric, pancreatic, and oral cancers[27].

Nitrites and nitrates are used in meat because they bind to myoglobin, inhibiting botulinum exotoxin production which are said to be powerful carcinogens[28]. Long-term exposure to food additives such as nitrite preservatives and Azo dyes has been associated with the induction of various types of cancer. Furthermore, the migration of bisphenol from plastic food containers may increase the risk of breast and prostate[29] cancers. Ingestion of arsenic may increase the risk of bladder, kidney, liver, and lung cancers.

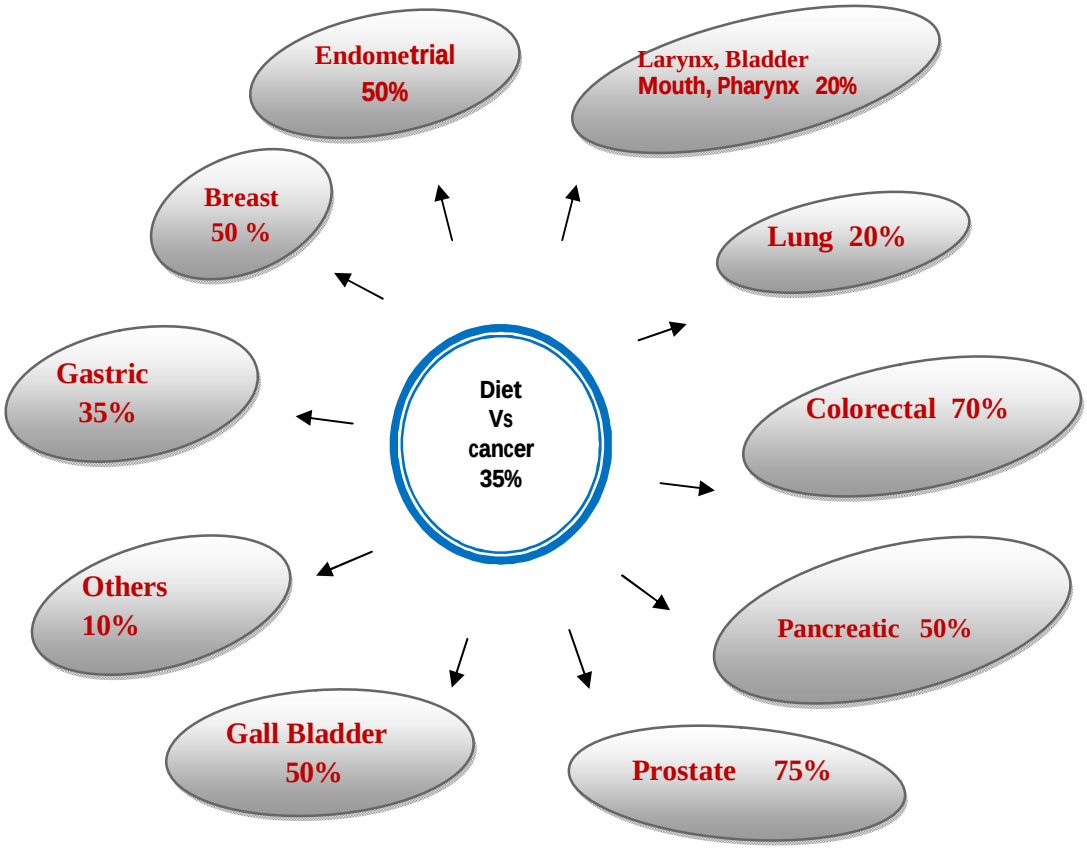


Fig 8 represents the influence of diet on carcinogenesis

1.5.c. Obesity

Obesity has shown the influence on increased mortality from cancers of the colon, breast (in postmenopausal women), endometrium, kidneys (renal cell), esophagus (adenocarcinoma), gastric cardia, pancreas, prostate, gallbladder, and liver (Fig 8). Increased modernization and a westernized diet and lifestyle have been associated with an increased prevalence of overweight people in many developing countries[30] .

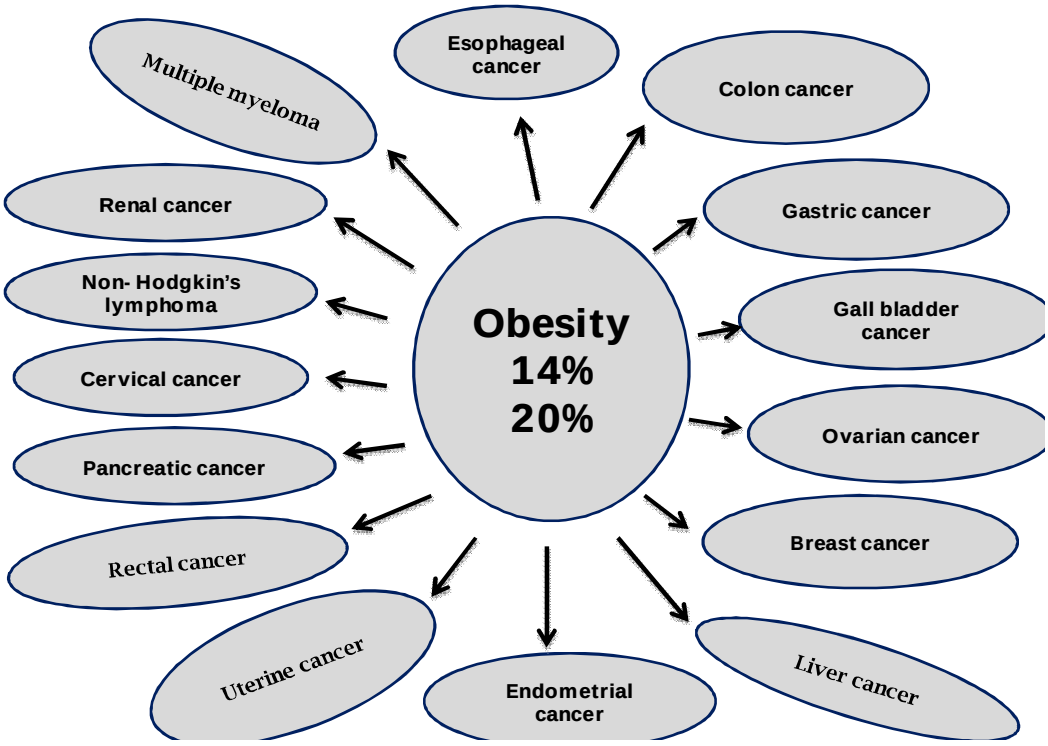


Fig 9 Inflence on obesity on cancer

1.5.d. Infectious Agents

Worldwide, an estimated 17.8% of neoplasms are associated with infections especially with viruses which accounts for most infection related cancers (Fig 9). The mechanisms by which infectious agents promote cancer are becoming increasingly evident. Infection-related inflammation is the major risk factor for cancer, and almost all viruses linked to cancer have been shown to activate the inflammatory markers[31]. Human papillomavirus, Epstein Barr virus, Kaposi's sarcoma associated herpes virus, human T-lymphotropic virus 1, HIV,HBV, and HCV are associated with risks for anogenital cancer, cervical cancer, skin cancer, Burkitt's lymphoma, nasopharyngeal cancer, Hodgkin's lymphoma, Kaposi's sarcoma, adult T-cell leukemia, B-cell lymphoma, and liver cancer. However, other microorganisms, including selected parasites such as *Opisthorchis viverrini* or *Schistosoma haematobium* and bacteria such as *Helicobacter pylori*, may also be involved, acting as cofactors and/or carcinogens.

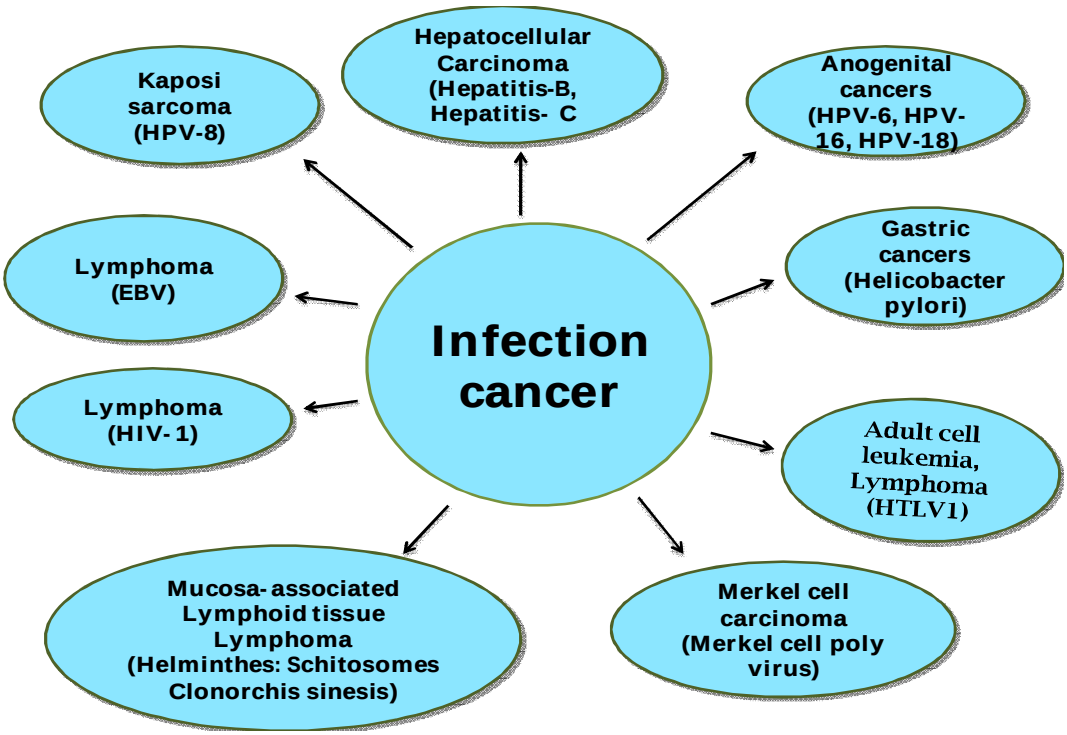


Fig 10 Represents the various neoplasm associated with infection

1.5.e. Environmental pollution

Environmental pollution has been linked to various types of cancers (Fig 10). It includes outdoor air pollution by carbon particles which is associated with polycyclic aromatic hydrocarbons (PAHs); indoor air pollution by environmental like tobacco smoke, formaldehyde, and volatile organic compounds such as benzene and 1,3-butadiene (which may particularly affect children); food pollution by food additives and by carcinogenic contaminants such as nitrates, pesticides, dioxins, and other organochlorines, carcinogenic metals and metalloids,pharmaceutical medicines and cosmetics[32]. The increased risk of childhood leukemia associated with exposure to motor vehicle exhaust was also increased now a days. Indoor air pollutants such as volatile organic compounds and pesticides increase the risk of childhood leukemia and lymphoma, and children as well as adults exposed to pesticides have increased risk of brain tumors, Wilm's tumors, Ewing's sarcoma, and germ cell tumors.

1.5.f. Radiation

Radiation like ionizing and nonionizing, typically from radioactive substances and ultraviolet (UV), pulsed electromagnetic fields increases the 10% of total cancer cases world wide. The cancers induced by radiations, includes some types of leukemia, lymphoma, thyroid cancers, skin cancers, sarcomas, lung and breast carcinomas[32].

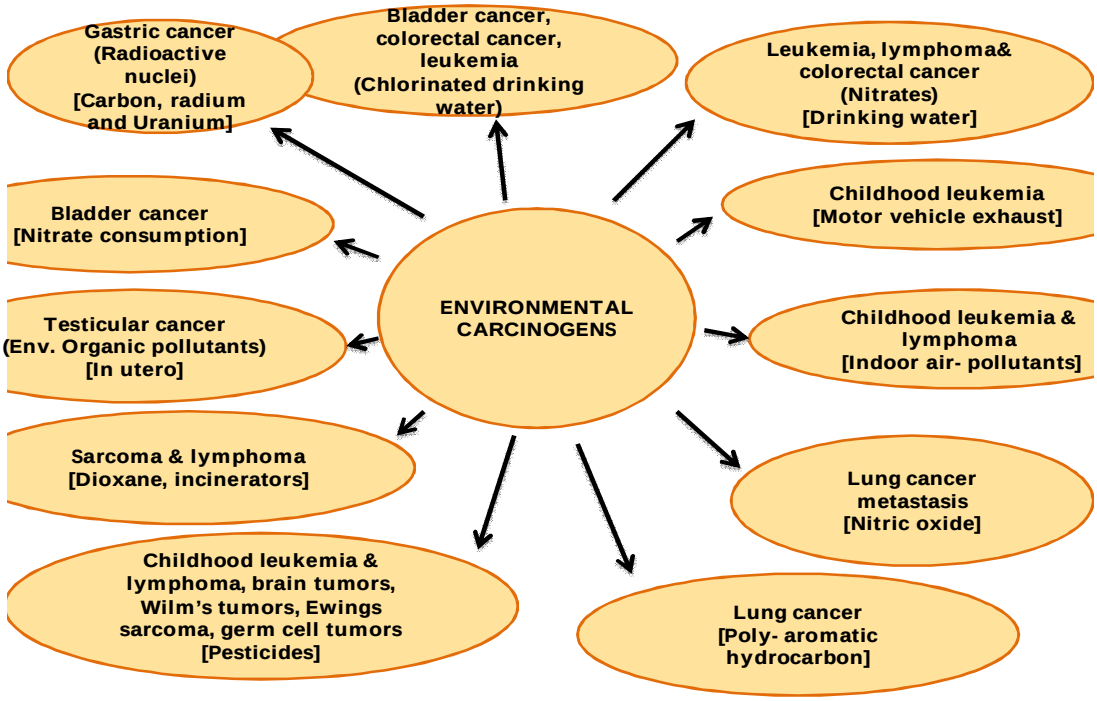


Fig 11. Represents the effect of environmental factors associated with carcinogenesis

Natural products and their derivatives represent more than 50% of all the drugs in clinical use of the world. These natural products and their derivatives have historically been a major source of new pharmaceuticals and have made enormous contributions towards human health. Their role in the drug discovery process is especially renounced in the areas of anti cancer and infectious disease agents, where the fractions of the drugs derived from natural products amount to 60 and 75%, respectively.

1.6. Role of natural products on cancer

Over the past 50 years, natural products (NP, here the term is restricted to small molecules) have been the cornerstone of anti cancer pharmacology. The discovery of these antitumor NP opened the route to the ‘‘forty glory cytotoxic’’ four decades of cytotoxic agents, among which a significant number of molecules from plants used today for the treatment of cancer. Immediately, the anti tumor pharmacology community embraced the new concept and this vision of molecular targetbased drug discovery (or reverse pharmacology) became a standard anti cancer research.

Old good drugs from plants and microbes remain essential. Since the 1950s, a large number of novel antitumor drugs have been identified and validated clinically. There is no doubt that the treatment of cancers has profoundly changed, with the advances of targeted therapies. For example, the discovery of specific tyrosine kinase inhibitors like imatinib (Gleevec1), or nilotinib (Tasigna1) have opened the new vistas in pharmacotherapeutics in cancer[33]. There is a strong need for novel anticancer drugs effective against solid tumors, especially at advanced stages of the disease. This can be achieved by the innovation of small molecules from NP should continue to be consider as one of the most important sources of innovative products.

Marine flora occupying almost 71% of globe, the ocean is rich in biodiversity, and the microflora and microalgae alone constitute more than 90% of oceanic biomass[34]. Marine floras include microflora (bacteria, actinobacteria, cyanobacteria and fungi), microalgae, macroalgae (seaweeds), and flowering plants (mangroves and other halophytes). This vast marine floral resource will offer a great scope for discovery of new drugs. It is increasingly recognized that ocean contains a huge number of natural products and novel chemical entities with unique biological activities that may be useful in finding the potential drugs with greater efficacy and specificity for the treatment of human diseases[35].

Marine floras have been used in India, China, the Near East and Europe, since ancient times for the purpose of food supplement. Ancient history reveals that maritime countries have been using seaweeds as vermifuge, anaesthetics and

ointment as well as for the treatment of cough, wounds, gout, goiter, venereal disease, and so forth. But modern scientific world proves that pleothra of the biological potential of seaweeds as antioxidant, antimutagenic, anticoagulant, and antitumor. The seaweeds also play an important role in modification of lipid metabolism in the human body. Seaweed extract is interestingly similar to human blood plasma (Langseth, 1995). Although, the use of seaweeds in medicine is not as wide spread as once it was, the use of seaweed polymer extract in pharmacy, medicine, and biochemistry is well established. Clinical trials are also in progress to make diabetic patients free from injection by introducing insulin secreting "jelly capsule"made of seaweed-derived alginic acid[36].

1.7. Anticancer Agents from Marine Floras

Marine microorganisms are a source of new genes, and exploitation his likely to the discovery of new drugs and targets. In this marine resources red algae are considered to be one of the potential organisms which can be the richest sources of known and novel bioactive compounds including toxins with potential for pharmaceutical applications[37]. Scytonemin is a protein serine/threonine kinase inhibitor, isolated from the cyanobacterium *Stigonema* sp. *Scytonemin* regulates mitotic spindle formation as well as enzyme kinases involved in cell cycle control and the compound also inhibits proliferation of human fibroblasts and endothelial cells. Thus scytonemin may provide an excellent drug as protein kinase inhibitors to have antiproliferative and anti-inflammatory activities[38].

More than 50% of the marine cyanobacteria are potentially exploitable for extracting bioactive substances which are effective in either killing the cancer cells by inducing apoptotic death, or affecting the cell signaling through activation of the members of protein kinase-c family of signaling enzymes. Macro algae (Seaweed). Seaweeds are important sources of protein, iodine, vitamins, and minerals and hence, their metabolites have shown promising activities against cancer incidences. In the past three decades, many researchers have worked on the antioxidant, antitumor, and immunomodulating activities of seaweeds[39] .

Edible seaweed like *Palmaria palmate* is shown to be effective antioxidant, capable of inhibiting cancer cell proliferation[40]. Algae have gained special interest owing to their biological properties. There are many reports on the immunomodulating and antitumor activities of algae[41]. An extract from the brown seaweed *Sargassum thunbergii* has shown antitumour activity and inhibition of tumour metastasis in the rat mammary adeno carcinoma cell.

Recent studies in the field of cancer research have revealed promising compounds, isolated from natural sources, with proven anticancer activity. Three examples are trabectedin, cytarabine and eribulin mesylate[42,43], which represent the first three described marine anticancer drugs. Indeed, almost 50% of the anti tumor agents approved in the last 50 years of the 20th century were either compounds derived from natural sources or (semi-) synthetic analogs[44]. Natural compounds remain a high out put source of promising chemotherapeutic or chemo preventive agents in current cancer research[45]. Marine organisms are potentially prolific sources of highly bioactive secondary metabolites that might represent useful leads in the development of new pharmaceutical agents. During the last four decades, numerous novel compounds have been isolated from marine organisms and many of these substances have been demonstrated to possess interesting biological activities[46].

Algae are heterogeneous group of plants with a long fossil history. Two major types of algae can be identified: the macroalgae (seaweeds) occupy the littoral zone, which included green algae, brown algae and red algae, and the micro algae are found in both benthic and littoral habitats and also throughout the ocean waters as phytoplankton. Phytoplankton comprises organisms such as diatoms (bacillariophyta), dinoflagellates (dinophyta), green and yellow–brown flagellates (chlorophyta; prasino-phyta; rymnesiophyta, cryptophyta, chrysophyta and rhaphidiophyta) and blue–green algae (cyano- phyta). As photosynthetic organisms, this group plays a key role in the productivity of oceans and constitutes the basis of the marine food chain[47].

The recent literature review over the past decades on marine seaweeds emphasize the anti tumour and cytotoxic potential established in various *in vivo* and

in vitro preclinical studies (Table 1). Numerous biological active cytotoxic molecules have been isolated marine algae and few of them are in different phases of clinical trails. So this clearly indicates the beneficial biological antitumour effect of marine derived compounds is ever increasing

Table: 1

Marine Flora	Chemical Composition	Biological Activity
Acanthophora spicifera	Crude Extracts	Antioxidants and inhibiting cancer cell proliferation[48]
Acanthophora spicifera	Crude Extracts	Tumoricidal activity on Ehrlich's ascites carcinoma developed in mice[48]
Ascophyllum nodosum	Fucoidan	Antiproliferative antitumour, anticancer, antimetastatic, and fibrinolytic[49]
Caulerpa sp.	Caulerpenyne	Cytotoxicity, anticancer, antitumour, and antiprolifer- ating activity[50]
Chondria sp.	Condriamide A	Cytotoxicity[51]
Cyanobacteria	Apratoxins	Inhibit a variety of cancer cell lines[52]
Cyanobacteria Nostoc *linckia and Nostoc* *spongiaeforme var.* *Tenue*	Borophycin	Cytotoxicity against human epidermoid carcinoma (LoVo) and human colorectal adenocarcinoma activity[53]
Cystophora sp	Meroterpenes and Usneoidone	Antitumour[54]
Eisenia bicyclis	Phloroglucinol and its polymers, namely eckol (a trimer), phlorofucofuroeckol	Antioxidant activity of the phlorotannins[55]

	A-(a pentamer), dieckol, and 8,8-bieckol (hexamers)	
Leptolyngbya sp.	coibamide A	Cytotoxicity against NCIH460 lung and mouse neuro-2a cells[56]
Nostoc linckia	Cyptophycin 1	Cytotoxicity against human tumor cell lines and human solid tumors[57]
Nostoc spongiaeforme	Cryptophycin 8	Greater therapeutic efficiency and lower toxicity than cryptophycin 14 in vivo[58]
Palmaria palmata	Phloroglucinol and its polymers, namely, Eisenia bicyclis eckol (a trimer), phlorofucofuroeckol A-(a pentamer), dieckol, and 8,8-bieckol(hexamers)	Antioxidant activity of the phlorotannins[59]
Stigonema sp.	Scytonemin	Antiproliferative and anti-inflammatory activities[60]
Stylopodium sp.	Stypoldione	Cytotoxic[61]

CAHPTER 2
Seaweed

2.1. Classification

Kingdom	:	Plantae
Phylum/division	:	Rhodophyta
Class	:	Rhodophyceae
Order	:	Ceramiales
Family	:	Rhodomelaceae
Genus	:	*Acanthophora*
Species name	:	*Acanthophora Spicifera (vahl) borgesen*
Common name	:	Spiny Seaweed

Plate 1 : Habitat of *Acanthophora spicifera*

2.2. Species description

Acanthophora spicifera is a Rhodophycean alga with wide distribution throughout the tropics and subtropics . It occurs on a wide variety of substrata, from hard bottom, as an epiphyte on other algae, or as a free living drift alga. It is often a large component of drift algae biomass.

2.3. Morphology

Shape : Large irregularly shaped holdfast for attachment to hard bottoms.

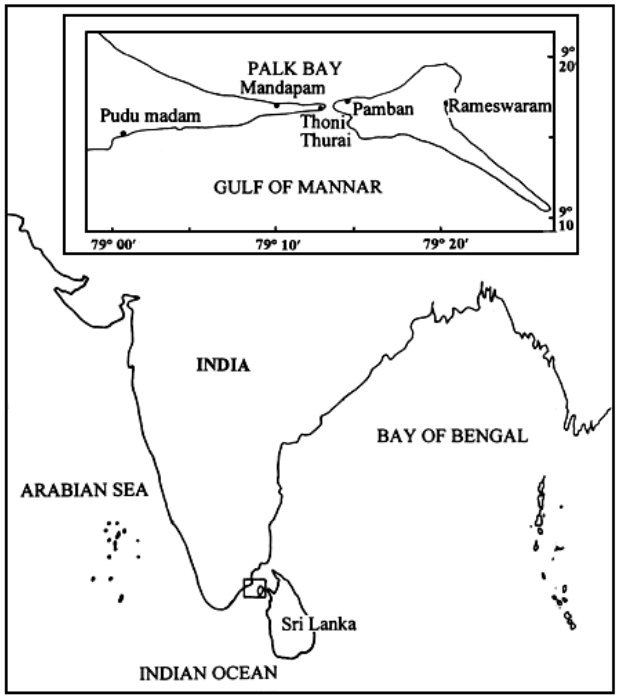
Branches : Short, Determinate branchlets that are irregularly and Spinose. Branchlets are hook-like, brittle and fragment easily under heavy wave action.

Colour : Shades of red, purple, or brown[62]

Height : Upright to approximately 25 cm.

2.4. Regional Occurrence

Acanthophora spicifera is one of the most abundant red algal species found on reef flats. It has a wide distribution in both tropical and subtropical habitats, occurring primarily in the tidal and subtidal zones. *Acanthophora spicifera* (Vahl.) Boergesen is an economically important red alga located in Mandapam, Ramaeshwaram Cost in the latitude of 78°8' E, 9° 17' N[63] .



2.5. Use

In folk medicine seaweeds are utilized for variety of residual purpose such as eczema gall stone etc. and this marine flora shows the interest in the biological activities of the compound which is derived from marine sources. This seaweed yields a maximum of 12% agaroid. It is also a good source of food for human consumption[64]. It is also used in vegetable salads, as soup flavouring and as a thickening agent in the Philippines, and is reported to contain carrageenan, used as an emulsifying agent. The liquid seaweed fertilizer obtained from seaweed extract is used as foliar spray for inducing faster growth and yield in leafy and fleshy vegetables, fruits, orchards, and horticultural plants

CHAPTER 3
Aim and Objective

Aim & Objective

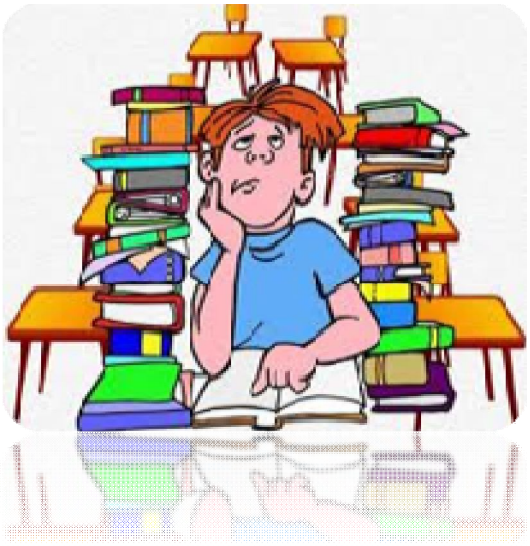
The cytotoxic and anticancer investigations of marine resources are increasing worldwide because these marine resources are economical, safe and effective, apart from this these marine resources posses enormous hidden phytoconstituents in the ocean with particular to the development of new chemical entity as anticancer drugs which has isolated from marine algae, seaweeds, tunicates etc..

The present aim of the investigation is to explore the anticancer and antiviral effect of marine algae *Acanthophora spicifera* belonging to the family: Rhodomelaceae which is widely distributed in Mandapam, Costal region of Tamilnadu, Gulf of Mannar, Bay of Bengal which is chosen its for *in vivo* and *in vitro* studies.

The objective of the present investigations is as follows.

- Collection of sufficient quantity of marine alga, *Acanthophora spicifera* for the present research investigation, identification and preservation of the marine algae
- Extraction of marine algae using conventional extraction procedure followed by filtration and purification
- Preliminary phytochemical studies of *Acanthophora Spicifera* extract followed by qualitative and quantitative phytochemical investigations.
- Preliminary anticancer effect of crude extract in Earlich Ascites Carcinoma challenged mouse model
- Bioactive guided fraction of crude extracts of *Acanthophora Spicifera* against various human cancer cell lines and Free radical scavenging assay
- Column chromatographic isolation of bioactive fraction High performance thin layer chromatography (HPTLC) finger print technique
- *In vitro* screening of isolates from *Acanthophora spicifera* against various cancer cell lines

- Cell cycle analysis of anticancer isolates against HepG 2 and HT 29 cell lines
- Anticancer efficacy of isolated compounds against Diethyl Nitrosamine (DEN) induced Hepato carcinoma in rats
- Antiviral screening of bioactive isolates against human viral cell line.



CHAPTER 4
Review of Literature

1. Mohamed et al., 2012 reported that edible seaweeds are rich in bioactive antioxidants, soluble dietary fibers, proteins, minerals, vitamins, phytochemicals, and polyunsaturated fatty acids. Although previously the seaweeds were only used as gelling and thickening agents in the food or pharmaceutical industries, recent researches have revealed their potential as complementary medicine. The red, brown and green seaweeds have been shown to have therapeutic properties for health and disease management, such as anticancer, antiobesity, antidiabetic, antihypertensive, antihyperlipidemic, antioxidant, anticoagulant, anti-inflammatory, immunomodu -latory, antiestrogenic, thyroid stimulating, neuro protective, antiviral, antifungal, antibacterial and tissue healing properties in *in vivo*. Active compounds include sulphated polysaccharides, phlorotannins, carotenoids (e.g. fucoxanthin), minerals, peptides and sulfolipids, with proven benefits against degenerative metabolic diseases.

2. Lavanya and Veerappan, 2011 has reported that *in vitro* antibacterial activity of six selected marine algae (seaweeds) which have been selected and their extracts have been tested as an alternative to commonly used antibiotics. Extracts of six seaweed samples namely *Codium decorticatum, Caulerpa scalpelliformis, Gracilaria crassa, Acanthophora spicifera, Sargassum wightii and Turbinaria conoides* were collected from Gulf of Mannar, southeast cost region. The collected algae were subjected for antibacterial activity. Extraction was done by using the following solvents viz., Acetone, methanol, chloroform, diethyl ether, ethyl acetate, hexane and aqueous and screened against selected human pathogens like *Vibrio parahaemolyticus, Salmonella sp, Shewanella sp, Escherichia coli, Klebsiella pneumoniae, Streptococcus pyogenes, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa* and *Proteus mirabilis.* All the seaweeds extracts have shown moderate antibacterial activity <10mm of zone of inhibition, out of which only methanolic extract has shown significant activity. Results showed higher antibacterial activity were found in *Acanthophora spicifera* and minimum found in *Codium decorticatum.* From these further studies on phytochemical analysis of seaweeds is necessitated to confirm them as a better source for antimicrobial properties.

3. Mayer et al., 2011 has clearly explained that preclinical pharmacology of structurally characterized marine compounds isolated from marine animals, algae, fungi and bacteria is discussed in a comprehensive manner. From 74 marine natural compounds various biological activities like antibacterial, anticoagulant, antifungal, antimalarial, antiprotozoal, antituberculosis and antiviral activities were reported. Additionally, 59 marine compounds were reported to affect the cardiovascular, immune and nervous systems as well as to possess antiinflammatory effects.

4. Schumacher et al., 2011 explained that the cancer is one of the most deadly diseases in the world. Although advances in the field of chemopreventive and therapeutic medicine have been made regularly over the last ten years, the search for novel anticancer treatments continues. In this field, the marine environment, with its rich variety of organisms, is a largely untapped source of novel compounds with potent antitumor activity.

5. Wijesekara et al., 2011 reported recently, a great deal of interest has been developed to isolate novel bioactive compounds from marine resources because of their numerous health beneficial effects. Among marine resources, marine algae are valuable sources of structurally diverse bioactive compounds. The cell walls of marine algae are rich in sulfated polysaccharides (SPs) such as fucoidans in brown algae, carrageenans in red algae and ulvans in green algae. These SPs exhibit many beneficial biological activities such as anticoagulant, antiviral, antioxidative, anticancer and immunomodulating activities. Therefore, marine algae derived SPs have great potential for further development as products in nutraceutical, pharmaceutical and cosmoceutical areas. This contribution presents an overview of biological activities and potential health benefits of SPs derived from marine algae.

6. Boopathy and Kathiresan, 2010 were shown that Marine floras, such as bacteria, actinobacteria, cyanobacteria, fungi, microalgae, seaweeds, mangroves, and other halophytes are extremely important oceanic resources, constituting over 90% of the oceanic biomass. They are taxonomically diverse, largely productive, biologically active, and chemically unique

offering a great scope for discovery of new anticancer drugs. The marine floras are rich in medicinally potent chemicals predominantly belonging to polyphenols and sulphated polysaccharides. The chemicals have displayed an array of pharmacological properties especially antioxidant, immunostimulatory, and anti tumour activities. The phytochemicals possibly activate macrophages, induce apoptosis, and prevent oxidative damage of DNA, thereby controlling carcinogenesis. In spite of vast resources enriched with chemicals, the marine floras are largely unexplored for anticancer lead compounds. Hence, this paper reviews the works so far conducted on this aspect with a view to provide a baseline information for promoting the marine flora-based anticancer research in the present context of increasing cancer incidence, deprived of the cheaper, safer, and potent medicines to challenge the dreadful human disease.

7. Nurul et al., 2010 said that the antimicrobial activity of eight crude extracts of *Acanthophora spicifera* were screened against 18 bacteria, 3 yeast and 6 fungi by disc diffusion method. The results clearly reveals that methanol and ethyl acetate extracts exhibits broad spectrum activity against all tested bacterial strains like *Bacillus Cereus*, *Pseudomonas aeruginosa*, *yersinia sp* and *Citrobacter freuidii*. The extract does not shown the any activity against tested strains of yeast and fungi.

8. Yasuhara-Bell and Lu, 2010 has reported that available treatments for many infectious diseases are limited. In particular, diseases caused by viral pathogens have demonstrated the need for new medicines, due to the increasing appearance of resistance to this available treatment, thousands of novel compounds have been isolated from various marine organisms and tested for pharmacological properties, many of which are commercially available. The screening of natural products derived from marine species for antiviral activity has yielded a considerable number of active crude aqueous and organic solvent extracts. Today, over 40 compounds are commercially available in pharmacological markets, including alternative antiviral medicines or those being tested as potential antiviral drugs. Many more are being tested as potential antiviral drugs at the preclinical and clinical stages.

The growing interest in marine-derived antiviral compounds, along with the development of new technology in marine cultures and extraction, will significantly expedite the current exploration of the marine environment for compounds with significant pharmacological applications, which will continue to be a promising strategy and new trend for modern medicine.

9. Liu et al., 2009 has stated that prevention and treatment of cancer require the continued development of novel and improved chemopreventive and chemotherapeutic agents. Throughout history, natural products have afforded a rich source of anticancer agents with diverse chemical structures and bioactivities. Recent technological and methodologic advances in structure elucidation, organic synthesis, and biological assay have resulted in the isolation and clinical evaluation of various novel anticancer agents. In this review, they present the anticancer activities, mechanism of action, structure and activity relationships of six important anticancer agents from natural products, that is, taxol, betulinic acid, camptothecin, resveratrol, podophyllotoxin and curcumin.

10. Moon et al., 2009 the invention of Laurinterol (LOEL) which was isolated from *Laurencia okamurai* is considered as invention for the prevention and inhibition of melanoma, LOEL can effectively inhibit the growth of melanoma cells by inducing apoptosis therein without adverse effect as in synthetic medicines. Thus, LOEL exhibited a dose dependent inhibitory effect on the growth of melanoma cells as it was observed that cells are treated with LOEL at 10 μg/ml and the growth of melanoma cells by was inhibited 50%. Addition of 1 μg/ml of LEOL exerted 30% inhibition on the growth of melanoma cells in the presence of fetal bovine serum (FBS)

11. Reyes-Zurita et al., 2009 has investigated the mechanisms of masilinic acid with regard to its inhibitory effects on the growth of colon-cancer cells (HT29). High concentrations of masilinic acid are present in the protective wax-like coating of olives. Our results show that treatment with maslinic acid results in a significant inhibition of cell proliferation in a dose-dependent manner and causes apoptotic death in colon-cancer cells. We found that it

inhibits considerably the expression of Bcl-2 whilst increasing that of Bax; it also stimulates the release of mitochondrial cytochrome-c and activates caspase-9 and caspase-3. All these results point clearly to the activation of the mitochondrial apoptotic pathway in response to the treatment of HT29 colon-cancer cells with masilinic acid. Our results suggest that masilinic acid has the potential to provide significant natural defence against particular colon-cancer cell lines.

12. Bacac and Stamenkovic, 2008 said that metastasis is the result of cancer cell adaptation to a tissue microenvironment at a distance from the primary tumor. Metastatic cancer cells require properties that allow them not only to adapt to a foreign microenvironment but to subvert it in a way that is conducive to their continued proliferation and survival. Recent conceptual and technological advances have contributed to our understanding of the role of the host tissue stroma in promoting tumor cell growth and dissemination and have provided new insight into the genetic makeup of cancers with high metastatic proclivity.

13. Desai et al., 2008 has reported that cancer is the second leading cause of death worldwide. Although great advancements have been made in the treatment and control of cancer progression, significant deficiencies and room for improvement remain. A number of undesired side effects sometimes occur during chemotherapy. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects. Currently, a few plant products are being used to treat cancer. However, a myriad of many plant products exist that have shown very promising anti-cancer properties *in vit*ro, but have yet to be evaluated in humans. Further study is required to determine the efficacy of these plant products in treating cancers in humans. This review will focus on the various plant-derived chemical compounds that have, in recent years, shown promise as anticancer agents which posses the potential mechanism of action.

14. Ganesan et al., 2008 has reported that *in vitro* antioxidant activities of three selected Indian red seaweeds – viz., *Euchema kappaphycus, Gracilaria edulis*

and *Acanthophora spicifera* were evaluated. Total phenolic content and reducing power of crude methanol extract was determined. The antioxidant activities of total methanol extract and five different solvent fractions (viz., petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), butanol (BuOH) and aqueous) were also evaluated. EA fraction of *A. spicifera* exhibited higher total antioxidant activity (32.01 mg ascorbic acid equivalent/g extract) among all the fractions. Higher phenolic content (16.26 mg gallic acid equivalent/g extract) was noticed in PE fraction of *G.edulis.* Reducing power of crude methanol extract increased with increasing concentration of the extract. Reducing power and hydroxyl radical scavenging activity of *E. kappaphycus* were higher compared to standard antioxidant (α-tocopherol). The total phenol content of all the seaweeds was significantly different ($P < 0.05$). *In vitro* antioxidant activities of methanol extracts of all the three seaweeds exhibited dose dependency and increased with increasing concentration of the extract.

15. Lang et al., 2007 has done the phytochemical investigation on *Acanthophora spicifera* (Vahl) Borgesen (Ceramiales: Rhodophyta) rhodophycean alga and chemical substances was identified as 5α-cholestane-3,6-dione, cholest-4-ene-3-one, 11α-hydroxy-5α-cholestane-3,6-dione, cholest-5-en-3α-diol and cholest-4-ene-3α,6β-diol from *Acanthophora spicifera* were described. Flavonoids such as quercetin, (-)- catechin and tiliroside, acanthophorin A and B, acid derivates, dipeptides and anthraxanthin were also identified from the above species.

16. Ma et al., 2006 has stated that the red alga *Rhodomela confervoides* was the source of four bromophenols and they exhibited moderate cytotoxicity against several human cancer cell lines.

17. Sun et al., 2006 said that the red alga *Gracilaria asiatica* was the source of three cyclopropyl derivatives, the cerebroside gracilarioside and the ceramides gracilamides A and B exihibits mildly cytotoxic to the human A375-S2 melanoma cell line.

18. Vasanti et al., 2006 screened *Acanthophora spicifera* from the Gulf of Mannar for their biological activity. This particular species was screened for hepatoprotective effect and antioxidant effect in CCl_4 intoxicated male albino rats. Liver damage was induced in rats by injecting CCl_4. The effect of the seaweed extract (ethanolic) at different doses was determined by comparing with the controls. The algal extract at a dose of 200 mg/kg b.w orally was found to exhibit a significant decrease in the level of SGOT, SGPT and LDH as compared to those of the CCl_4 induced liver damaged controls. The level of lipid peroxidation was also decreased in the 200 mg/kg extract treated rats. The antioxidant status of the extract treated rats showed tremendous increase in the levels of the antioxidant enzymes SOD, catalase and glutathione peroxidase. The biological activity was related to the phyto-constituents such as flavonoids, vitamin – A, E, C, present in the algae.

19. Abatis et al., 2005 has screened brown alga *Taonia atomaria, which* was a source of meroditerpenes atomarianones A and B stated as cytotoxic agents which has screened against the NSCLC-N6 and A-549 cell lines.

20. Kubanek et al., 2005 explained that the red alga *Callophyeus serratus* was the source of three antibacterial and antifungal diterpene-benzoate compounds, bromophycolides A and B, and a non-halogenated compound. Bromophycolide A was cytotoxic against several human tumour cell lines by specific induction of apoptosis.

21. Simmons et al., 2005 has explained that the chemical and biological diversity of the marine environment is immeasurable and extra ordinary resource for the discovery of new anticancer drugs. Recent technological and methodologic advances in structure elucidation, organic synthesis makes easy to the chemical and biological diversity of the marine environment, and biological assay have resulted in the isolation and clinical evaluation of various novel anticancer agents. These compounds range in structural class from simple linear peptides, such as dolastatin, to complex macrocyclic polyethers, such as halichondrin; equally as diverse are the molecular modes of action by which these molecules impart their biological activity.

22. Bhaskar et al., 2004 has screened the three species of red marine macro algae (Rhodophyta) from the Indian Ocean and subjected to analysis for the occurrence of conjugated polyenes. The composition of different lipid classes in these seaweeds along with their fatty acid composition has also been reported. Analysis of lipid classes of these seaweeds revealed that both *Acanthophora spicifera* (Ceramiales, Rhodophyta) and two species of *Gracilaria*, viz. *G.edulis* and *G.folifera* (Gracilariales, Rhodophyta) were rich in glycolipids. However, *A. spicifera* had significantly higher amounts of eicosapentaenoic acid (EPA) and arachidonic acid (AA) as compared to negligible amount of these fatty acids in both species of *Gracilaria*. The red seaweed Acanthophora *spicifera* contain conjugated eicosapentaenoic acid (CEPA) and conjugated arachidonic acid.

23. de Ines et al., 2004 has stated that furoplocamioid C, perfuroplocamioid, pirene and tetrachlorinated cyclohexane from the red alga *Plocumium carttilagineum* exhibited selective cytotoxicity against human tumour cell lines with pirene showing a specific and irreversible effect on SW480 cells.

24. Stein and Colditz , 2004 stated that over 6 million people around the world die from cancer each year. Modifiable risk factors have been linked to a wide range of malignancies, including cancers of the oropharynx, oesophagus, larynx, lung, kidney, bladder, pancreas, skin, stomach, ovary, breast, cervix, uterus, prostate, and colon. Research indicates that over half of all cancers in developed countries could be prevented if we implemented population-wide measures to promote the following behaviours: reduce tobacco use, increase physical activity, control weight, improve diet, limit alcohol, utilise safer sex practices, get routine cancer screening tests, and avoid excess sun expore.

25. Goncalves et al., 2002 has reported that the water-soluble acid agaran isolated from *Acanthophora spicifera* (Rhodophyta) was submitted to alkaline treatment for the complete cyclization of α-L-Galp 6-sulfate to 3,6-An-α-L-Gal*p* units. The modified agaran was then partially depolymerized using partial reductive hydrolysis. The resulting oligosaccharide mixture was fractionated by adsorption and ion-exchange chromatography. Fractions were

purified by gel-filtration chromatography and studied by ESIMS and NMR spectroscopy, including 1D, ^{1}H,^{13}C, DEPT and 2D ^{1}H, ^{1}H COSY, TOCSY and ^{1}H,^{13}C HMQC procedures

26. Dorta et al., 2002 showed the Stypolactone, a diterpenoid of mixed biogenesis has been isolated from the brown algae *Stypopdium zonale* and showed weak cytotoxic activity in vitro against the A-549 and H-116 cell lines.

27. Kiechle and Zhang, 2002 reported that apoptosis and necrosis represent two distinct types of cell death. Apoptosis possesses unique morphologic and biochemical features which distinguish this mechanism of programmed cell death from necrosis. Extrinsic apoptotic cell death is receptor linked and initiates apoptosis by activating caspase 8. Intrinsic apoptotic cell death is mediated by the release of cytochrome c from mitochondrial and initiates apoptosis by activating caspase 3. Cancer chemotherapy utilizes apoptosis to eliminate tumor cells. Agents which bind to the minor groove of DNA, like camptothecin and Hoechst 33342, inhibit topoisomerase I, RNA polymerase II, DNA polymerase and initiate intrinsic apoptotic cell death. Hoechst 33342-induced apoptosis is associated with disruption of TATA box binding protein/TATA box complexes, replication protein A/single-stranded DNA complexes, topoisomerase I/DNA cleavable complexes and with an increased intracellular concentration of E2F-1 transcription factor and nitric oxide concentration. Nitric oxide and transcription factor activation or respression also regulate the two apoptotic pathways. Some human diseases were associated with excess or deficient rates of apoptosis, and therapeutic strategies to regulate the rate of apoptosis include inhibition or activation of caspases, mRNA antisense to reduce antiapoptotic factors like Bcl-2 and survivin and recombinant TRAIL to activate proapoptotic receptors, DR4 and DR5.

28. Mei et al., 2001 has explained that the two new flavonoids, acanthophorin A and acanthophorin B, was isolated along with three known compounds naming tiliroside,(-)-catechin and quercetin were from the red alga

Acanthophora spicifera. The structures were determined to be kaempferol 3-O-α-L-fucopyranoside(1) and quercetin 3-O-α-L-fucopyrinoside by spectroscopic methods and these compounds shown significant antioxidant activity.

29. Guardia et al.,1999 has stated that the enantioselective synthesis is of natural anti tumour(-) – Bifurcadio involving an alkylation key-step reaction is reported. In this paper, we report the first enantioselective synthesis of (-)-Bifurcadiol 6 involving an alkylation key-step reaction between a cyanohydrin and an allyl bromide (Scheme 1). *trans trans* Farnesol 1 was converted into the corresponding ether 2 by treatment with dihydropyran and subsequently oxidized using a mixture of pyridine, selenium dioxide and PDC to afford the expected aldehyde 3 in 34% yield. 4 Compound 3 was added to a solution of trimethylsilyl cyanide in the presence of a catalytic amount of sodium cyanide and 18-crown-6 ether complex to lead, after addition to a solution of LiN(SiMe3)2 in anhydrous THF (-78°C) and condensation with 1-bromo-3-methylbut-2-ene, to the silyl cyanide derivative 45 (52% yield). Subsequent treatment with triethylamine trihydrofluoride salt afforded the expected ketone 5 in 70% yield.

30. Iwamoto et al., 1998 explains the Penostatins F–I have been isolated from a strain of *Penicillium sp*. originally separated from the marine alga *Enteromorpha intestinalis*, and their stereostructures have been established on the basis of spectral analyses. All the compounds exhibit significant cytotoxicity against culture

31. Wahidulla et al., 1998 has isolated new steroid cholest-4-ene-3α, 6β-diol together with the known cholest-4-ene-3-one, lauric acid and o-phthalic acid bis-(2-ethyl nonyl)-ester were isolated from the red alga *Acantophora spicifera*. The structures of these compounds were established on the basis of their spectral data.

32. Takahashi et al., 1995b showed new cytotoxic epipolysulfanyldioxo piperazme dimers, leptosins K, K l and K 2, have been isolated from a strain

of *Leptosphaeia sp.* originally isolated from the marine alga *Sargassum tortile*. Their stereo structures, with a different configuration from that of leptosins A-C, have been elucidated by spectral and X-ray analyses and some chemical transformations. X-Ray and NMR and NOE spectral analyses of 4 revealed that it exists in a mixture of four conformers, of which two each closely resemble, in a single crystal, and in a single conformer in solution. NOE experiments of 5 and 6 demonstrated that they exist in a mixture of two conformers slowly exchanging in CDCI3.

33. Gerwick et al., 1994 taken an account on bioassay-guided fractionation of the organic extract of *Lyngbya majuscula* led to the isolation of a new lipid, curacin A, with exceptional brine shrimp toxic and antiproliferative activities. Its unique thiazoline-containing structure has been deduced from spectroscopic information. Pure curacin A is an antimitotic agent (IC60 values in three cell lines ranging from 7 to 200 nM)that inhibits microtubule assembly and the binding of colchicine to tubulin.

34. Numata et al., 1993 has stated that the isolated Communesins A and B, exhibiting cytotoxic activity against the cultured P-388 cells, were isolated from the mycelium of a strain of *pencillium sp.* stuck on the marine alga *enteromorpha intestinalis* and their structures was elucidated by spectroscopic analyses.

35. Fuller et al., 1992, has isolated polyhalogenated monoterpene from the red alga *Portieria hornemanii* is considered as a novel *in vitro* antitumor agent by National Cancer institute (NCl). The NCI Decision Network Committee selected halmon as a pre-clinical drug for development.

36. Gupta et al., 1991 stated that the methanolic extracts in 5 of 7 algae from the Atlantic coast of Panama: *Caulerpa racemosa, Halimeda opuntia, Gelidiela acerosa, Laurencia papillosay* and *Acanthophora spicifera,* showed antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* in a concentration of 50 mg/ml by the cylinder plate method. None of the algae studies showed activity against *Escherichia coli, Pseudomonas aeruginosa,*

Aspergillus niger and *Candida albicans*. The content of agar in *Acanthophora spicifera* was found to be the highest (33.5%) of all the species studied.

37. Numata et al., 1991 shown that partition fraction of hexane, Chloroform, $CHCL_3$ for the methanolic extracts of marine algae were examined for cytotoxicity activities against cultured P-388 lymphocytic leukemia cells. Cytotoxic activities were found for partition fractions of 21 species of seaweed. Bioactivity guided fraction of the CCl_4 partition fraction from *sargassum tortile*, exhibiting the most prominent activity, afforded dihydroxysargaquinone and sargatriol previously isolated from the alga. The former was evaluated as a cytotoxic principle and the latter showing the moderate activity, suggested to be an artefact from 1 during the isolation procedure.

38. Solambi et al., 1991 has done investigation on red algae, *Acanthophora spicifera* afforded the know peptide, aurantiamide acetate and new diastereoisomer of this dipeptide(dia- aurantiamide acetate). This is the first report of aurantiamide acetate form a marine source and of the natural occurrence of the diastereoisomer.

39. Cortes et al., 1990 reported that 2-acetoxy-15-bromo-6,17-dihydroxy3-palmitoyl-neoparguera- 4(19), 9(11)-diene, a novel seco-parguerane skeleton have been isolated from the red alga *laurencia obtuse* from Okinawa and showed a cytotoxic activity against various cancer cell lines.

40. Wall et al., 1989 stated that the two new compounds, cymobarbatol and 4-isocymobarbatol, were isolated from the marine alga *Cymopolia barbata*. The complete structures and absolute stereo chemistries of these compounds were elucidated by a variety of spectroscopic techniques and X-ray crystallography. Both compounds were found to be nontoxic over a broad concentration range to *Salmonella typhimurirrm* strains T-98 and T- 100. Both compounds exhibited strong inhibition of the mutagenicity of 2-

aminoanthracene and ethyl methanesulfonate toward, respectively, the T-98 strain plus a metabolic activator and T- 100.

41. Kalaiperumal et al., 1986 has done experimental field cultivation of the red alga *Acanthophora spicifera* (Vahl.) Boergesen, by vegetative-propagation method, Vegetative fragments 5 cm in length were tied into clusters with polypropylene straw and were fastened to nylon fishing lines. The weight of seed material thus introduced was 4.85 kg. The algae grew rapidly and reached harvestable size of 15.9 cm mean length in 25 days. The weight of fresh harvested plants was 12.85 kg, having had a 2.6 fold increase over the weight of the seed material, indicating that the near shore area of Hare Island in Gulf of Mannar, where the experiment was conducted, is suitable for large-scale cultivation of this seaweed.

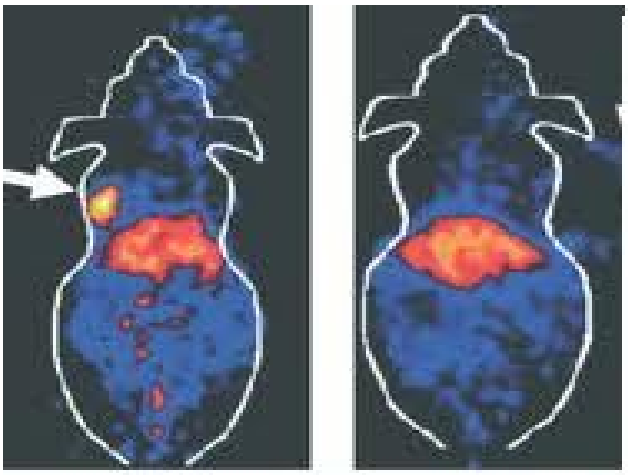
42. Higa, 1985 reported that Several cyclic monoterpenes have been isolated from the Japanese red alga *Desmia hornemanni*, and some chemical modification have been done on these compounds to get the most active moiety for cytotoxic activity and these compound exhibited relatively high activity against P-388 lymphoblastic cell line, A-549 lung carcinoma, and HCT-8 human colon adenocarcinoma.

43. Two new cyclic ethers consisting of squalene carbon skeleton, teurilene and thyrsiferyl 23-acetate, have been isolated from the red alga *Laurencia obtuse* (Suzuki et al., 1985). Thysiferyl 23-acetate (bromo ether) showed remarkably cytotoxic property (EDso of 0.3 µg/ml) against P388 in vitro cell line.

44. Carter et al., 1984 has isolated the Majuscuiamide C a cyclic compound depsipeptide from the deep water variety of *Lyngby majusculea* that inhibit the fungal plant pathogens.

45. Moore , 1982 has stated that marine prokaryotic organisms, in particular blue-green algae belonging to the *Oscillatoriaceae*, are potentially good sources of new antineoplastic agents. The dermatitis-producing substances in the blue green alga *Lyngbya majuscula*, however, which exhibit marginal anticancer activities, are powerful tumor promoters. The potencies and modes

of action of two of these tumor promoters, aplysiatoxin and lyngbyatoxin A, are essentially identical with the potency and mode of action of the well known tumor promoter from Croton oil, 12-O-tetradecanoylphorbol 13-acetate (TPA). Structure-activity relationships are presented for aplysiatoxin, lyngbyatoxin A, TPA, and related compounds. The absolute stereochemistries of the aplysiatoxin and lyngbyatoxin A are discussed.



CHAPTER 5
Anticancer, Antioxidant Effect of Alcoholic Extracts of *Acanthophora Spicifera*

5.1. Introduction

Free radical and reactive oxygen species (ROS) are by products produced in body due to various physiological and biochemical processes. Generally, most of these free radicals generated from cellular metabolism are scavenged by endogenous defence system such as superoxide dismutase, Catalase and Peroxidase–glutathione system[110]. However, in many cases, such as in unhealthy physical condition, ageing, or under stress environments, the endogenous antioxidants are either exhausted or insufficient to scavenge these free radicals.

Imbalance of these free radicals can cause oxidative damage to biomolecules like lipids, proteins and DNA[111]. These reactive oxygen species (ROS) has been implicated and having major role on human biological system which leads to diseases, like ageing, cancer, and neurodegenerative disorders etc[112]. Among this, cancer is a complex disease characterized by proliferation (uncontrolled cell division), cell transformation, and cape of apoptosis, invasion, angiogenesis and metastasis.

Cancerous cells are to produce reactive oxygen species (ROS) and their inflammatory mediators. ROS may cause DNA mutation, which may be followed by oncogenes activation and down regulation of tumour suppressor genes[113]. By the activity of ROS, scavenging system gets altered in tumour cells[114]. Over the past decades, seaweeds or their extracts have been shown to produce a variety of compounds and some of them have been reported to possess biological activity of potential medicinal values[115].

The identification and exploitation of potent anticancer molecules from the marine environment such as marine algae has generated great interest in recent years. Extensive screening of marine microalgae has led to the isolation and chemical determination of over 15,000 compounds, including fatty acids, sterols, phenolic compounds, terpenes, enzymes, polysaccharides, alkaloids, flavonoids[116]. More recent reports revealed that marine algae possess rich sources of antioxidant compounds with potential free radical scavenging activity as in *Halimeda tuna*[117]

and *Acanthophora spicifera*[118]. In this study *Acanthophora spicifera* (Vahl) Borgrsen (Ceramiales:Rhodophyta) are commonly known as spiny seaweed, is widely distributed throughout the tropic and subtropics throughout the Gulf of Mannar, Rameswaram[63].

To the date, research on biologically active substances from this species is rather limited or not been clearly established[119]. Therefore the aim of the present study was to evaluate anticancer and antioxidant activity of *Acanthophora spicifera*, a red algae in suitable experimental conditions.

5.2. Materials and Methods

5.2.a. Marine algae collection

The red algae *Acanthophora spicifera* (Family: Rhodomelaceae, Ceramiales) was collected from Mandapam, during the month of March 2008 from Ramaeshwaram coast, Tamil Nadu, India. It is identified and authenticated by Dr. Krishnamurthy, Institute of algology, Anna nagar, Chennai. The voucher specimen (VCP/09-345) was deposited in the Department of Pharmacognosy, Vels College of Pharmacy, Chennai 117.

5.2.b. Extraction of *Acanthophora spicifera*

Dried, pulverized *A. spicifera* (1 kg) was extracted with 5 liters of ethanol using soxhlet apparatus for 24 hrs. The extract was filtered, and the filtrate was evaporated by rotary vacuum evaporator and sample was freeze dried for further use. The percentage yield of ethanol extract was found to be 20.22% w/w. The ethanolic extract was subjected to qualitative chemical test and thin layer chromatography studies[121].

5.2.c. Chemicals and drugs

The chemicals used were 5-Fluorouracil (Ranbaxy Laboratories, Ltd., India), Corboxy methyl cellulose (CMC) (Ranbaxy Laboratories, Ltd., India), Adrenaline

bi-tartrate (Sisco chemicals, Mumbai), Thiobarbituric acid (Sisco chemicals, Mumbai), Elman's reagent reagent (SRL Chemicals, Mumbai) and all other chemicals and reagents used were purely of analytical grade.

5.2.d. Animals

Swiss male albino mice weighing around 20-22g were used for present investigation. Animals were obtained from the central animal house, Vels College of Pharmacy, Chennai. Mice were grouped and housed in poly acrylic cages (n=6) and maintained in standard laboratory conditions under the temperature 25 ± 2^0 C dark/light cycle. They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were acclimatized into a laboratory conditions for 7 days before the experiment. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee.

5.2.e. Acute Toxicity Study

Toxicity study - up and down procedure was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD). Oral toxicity study was done according to OECD guidelines 423. In this experiment two groups of wistar rats (n=3) were used. Animals were fasted over night with water *ad libitum* and foods were withheld for 3-4 hrs after oral administration of the extracts. One group of animals were treated with starting dose of the *Acanthophora spicifera* extract of 2000mg/kg b.wt orally. Another group of rats were treated with normal saline. Observation includes mortality and clinical signs, which includes changes in skin, fur, eyes and mucous membranes. The gross behaviors like body positions, locomotion, rearing, tremors, gait was observed. The effect of *Acanthophora spicifera* on passivity, grip strength, pain response, stereotypy, vocalization, writhing reflex, body weight and water intake was observed[122].

5.2. f. Transplantation of tumour

Ehrlich ascites carcinoma (EAC) cells were obtained through the courtesy of Amala Cancer Research Centre, Thrissur, Kerala. The EAC cells were maintained *in vivo* in swiss albino mice by intraperitoneal inoculation of 2×10^6 cells per mouse. From the peritoneal cavity of the mice, the EAC cells were aspirated, washed with saline and were given intraperitoneally to develop ascitic tumor [123].

5.2.g. Animal grouping and Drug treatment

Animals were divided into five groups (n=6)

Group-I was served as normal control treated with saline control (5 ml/kg i.p.)

Group-II was served as EAC treated control group

Group-III was served as EAC tumour control administered with 5 FU (20 mg/kg, i.p.)

Group-IV was served as EAC treated tumour control treated with alcoholic extract of *Acanthophora spicifera* (100 mg/kg/p.o)

Group-V was served as EAC treated tumour control treated with alcoholic extract of *Acanthophora spicifera* (200 mg/kg/p.o)

The standard 5-FU and the test drug *Acanthophora spicifera* extract were administered after 72 hrs of tumour inoculation. All the drugs were administered for 21 days continuously and the following parameters were evaluated periodically.

5.2. h. Tumor volume, Tumour weight and Mean survival Day

The ascetic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube. The tumor weight was measured by taking the weight of the mice before and after the collection of the ascetic fluid[124].

The Mean survival day were calculated by animals survived from the date of tumor inoculation to the date at which the animal death occurs. Mean survival day is calculated by using the formula given below.

$$\text{Mean survival day} = \frac{\text{No. of days animals survived in test group}}{\text{No. of days animals survived in control group}} \times 100$$

5.2. i. Measurement of Antioxidant / Oxidative stress markers

Animals (n=3) were sacrificed from each group. Blood samples were collected transcordially for the following haematological and biochemical estimations.

5.2.j. Biochemical parameters

After the collection of blood samples, the mice were sacrificed. Liver was excised, rinsed in ice cold normal saline followed by ice-cold 10% KCl solution, blotted, dried and weighed. A 10% w/v liver homogenate was prepared in ice-cold KCl solution and centrifuged at 1500rpm for 15 min at 4^{o}C. The supernatant thus obtained were used for the estimation of thio-barbituric acid reactive substances[125] (TBARS), glutathione[126] (GSH), superoxide dismutase [127](SOD) catalase[128] (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase[129] (GST) and total protein[130] (TP) .

5.2.k. Haematological parameters

At the end of the experimental period, next day after an overnight fasting, blood was collected from freely flowing tail vein and used for the estimation of haemoglobin (Hb) content, red blood cell (RBC) count, white blood cell (WBC) count, protein content, packed cell volume (PVC) and differential count of WBC was measured using standard procedures.

5.2.l . Statistical analysis

All the data were expressed as Mean ± SEM. All the groups were compared by one way (ANNOVA) followed by Dunnett's post hoc test. Probability of $P< 0.05$ was considered as significant.

5.3. Results and Discussion

The preliminary phytochemical analysis of *Acanthophora spicifera* showed that the presence of flavonoids, tannins, terpinoids and glycosides (Table:2)

5.3.a. Effect of *Acanthophora spicifera* on Tumour weight, Tumour volume and Mean survival day

Fig:1 represents the effect of the *Acanthophora spicifera* on tumour weight in EAC in mice. Inoculation of EAC cell lines into mice, significantly ($P <0.001$) increased the body weight of the mice as measured the indices of the tumour mice. Oral administration of ethanolic extract of *Acanthophora spicifera* (100 and 200 mg/kg bd wt) significantly ($P<0.001$) decreased the tumour weight in EAC treated carcinoma mice than the saline treated control tumour mice. In addition 5-FU also significantly ($P<0.001$) decrease the tumour weight in EAC inoculated mice as compared with saline treated EAC bearing tumour mice.

Fig: 2 represent the effect of the ethanolic extract of *Acanthophora spicifera* on tumour volume in EAC treated cancerous mice. Increase in tumour volume was noted in saline treated EAC mice during the 3 weeks of study period. The tumour volume decreases significantly ($P<0.01$) in EAC mice treated with *Acanthophora spicifera* (100 and 200 mg/kg bd wt). The effect was found to be dose dependent. In addition there was a significant ($P< 0.001$) decrease in tumour volume in 5-FU treated tumour mice than saline treated tumour mice.

Fig: 3 represents the mean survival day of the tumor bearing mice treated with 5 FU and ethanolic extract of *Acanthophora spicifera* 100 & 200 mg/kg/orally. It was observed from the fig 3 that significant increase ($P<0.001$) in mean survival day

in 5 FU treated tumor mice group as compared with saline treated tumor control. Oral administration of *Acanthophora spicifera* had significantly ($P< 0.01$) increased the MSD of the tumor bearing mice as compared to that of tumor control group. The increase in mean survival day of the tumor bearing mice treated with *Acanthophora spicifera* extract was dose dependent

Fig: 4 represents the effect of *Acanthophora spicifera* extract on various blood haematological parameters treated with mice bearing with EAC cell lines.

5.3.b. Effect of *Acanthophora spicifera* on haemoglobin

Mice treated with EAC had showed significant decrease in ($P<0.001$) haemoglobin (Hb) content as compared with saline treated control normal mice. Interestingly the tumor bearing mice administered with high dose of ethanolic extract of *Acanthophora spicifera* (200mg/kg) significantly ($p<0.01$) increases the haemoglobin content as compared to that of tumor control group animals.

5.3.c. Effect of extracts on WBC

Induction of tumor in mice had significantly ($P<0.001$) increase the WBC count as compared to that of vehicle treated control normal animal. Oral administration of *Acanthophora spicifera* extracts to EAC bearing mice showed significant reduction ($P<0.001$) in WBC levels as compared to that of vehicle treated control tumor mice. The effect was dose dependant and the high dose of *Acanthophora spicifera* (200 mg/kg/bd wt) almost reverses the WBC count towards normal.

5.3.d. Effect of *Acanthophora spicifera* extract on packed cell volume

There was a significant increase in PCV cells in EAC treated cancer group mice as compared to that of saline treated control group. It was interesting to observe that administration of *Acanthophora spicifera* ethanolic extract at the dose of 100 and 200mg /kg bd wt orally significantly ($P<0.001$) decreases PCV levels towards the normal in tumor bearing treated mice.

Fig: 5 represents the blood haematological parameters (lymphocytes, neutrophils and monocytes) of control and EAC treated tumor mice treated with *Acanthophora spicifera* extract. Significant (P<0.001) increase in lymphocytes and decreases in neutrophils was observed in EAC challenged tumor bearing animal group compared to that of saline treated control group. Administration of *Acanthophora spicifera* significantly (P<0.001) increases the lymphocytes percentage as compared to that of EAC treated tumor control mice whereas the high dose level of 200mg/kg orally of *Acanthophora spicifera* extract exhibited significant decrease (P<0.01) in the neutrophil percentage of tumor bearing mice group. However no effect was observed in monocytes percentage of vehicle treated and EAC treated cancer group.

Fig: 6 represents the effect of 5FU and *A. spicifera* extract on antioxidant and TBARS level in the liver homogenate of EAC treated cancer group. Administration of EAC significantly decreases (P<0.001) SOD and CAT level as compared to that of vehicle treated normal control group. The high dose of *Acanthophora spicifera* extract administered orally to the EAC treated mice significantly (P<0.01) increases the SOD and CAT levels of the EAC treated mice as compared to that vehicle treated tumor control group. Whereas EAC inoculation to normal mice significantly (P<0.001) increases the levels of LPO, GPx & GST as compared to that of vehicle treated control mice group and this effect was significantly decreases (P<0.01) significantly elevated LPO, GPx & GST levels as compared to that of saline treated tumor control group.

5.4. Discussion

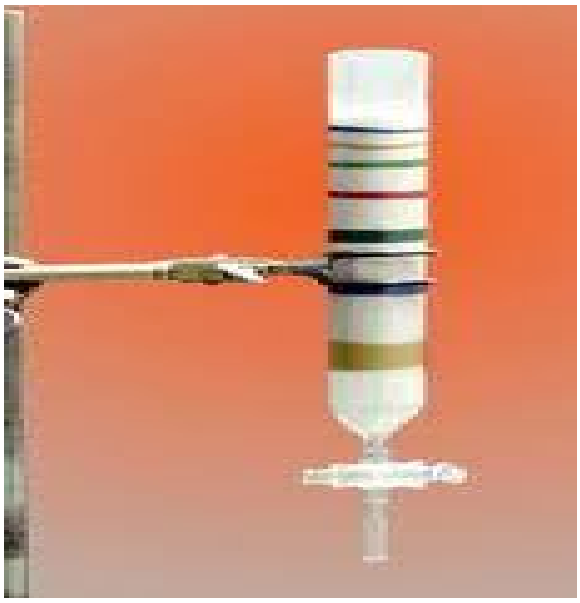
The present study high lights the *in vivo* antitumor effects of crude extract of *Acanthophora spicifera extract* in EAC treated mouse model. In this present investigation, the oral administration of *Acanthophora spicifera* extract given for 21 days has significantly decreased the tumor volume and tumor weight following the EAC inoculation in mice. The assessment of tumour volume and tumor weight is the direct parameter for assessment of tumour growth as well as anti tumour efficacy of the test drugs. The results from Mean survival day suggested that EAC treated mice shows decrease in MSD than the 5FU and *Acanthophora spicifera* treated EAC

mice. The decreased tumour volume and tumour weight, with increased MSD in EAC bearing mice treated with *Acanthophora spicifera* 100 and 200 mg /kg body weight suggested the anti tumour properties of the extracts. It has been shown earlier that the decrease in life span of the tumour control was directly proportional to the propagation to the tumor growth. It has been reported by many investigators that the life span expectancy or mean survival day get increased with the decrease in tumour size[131]. These results clearly suggests that direct antitumor efficacy of *Acanthophora spicifera* in *in-vivo* model. It was further observed that beneficial impact of EAC inoculation on haematological parameters of the EAC treated mice in which there was decrease in RBC, increase in WBC with decrease in haemoglobin levels in tumor bearing mice. The reversal and restoration of haematological parameters like RBC, WBC and haemoglobin (Hb) levels in EAC treated mice with *Acanthophora spicifera* suggested that it possess the protective effect against haematopoietic system. It was interesting to observe that restoration of lymphocytes and neutrophils towards its normal suggest the influence of *Acanthophora spicifera* on granulocytes and agranulocytes. The involvement of reactive oxygen species has been postulated in various degenerative decease and cancer as well[132]. The reactive oxygen species like Superoxide (SOD), catalase (CAT), Glutathione (GSH), GPx and melonaldyaldehyde (MDA) was considered as vulnerable species and continuous accumulation in any tissue suffers from oxidative stress. In general, the cancerous tissue can produce oxidative spurt with above said ROS which can be used as adjuvant for triggering for cancer proliferation to metastasis. Antioxidant from vegetables, fruits and herbal extracts can prevent cancer due to its powerful free radical scavenging activity and enhances the antioxidant system. Ever growing scientific incidence during last two decades has been support that antioxidant effects of various phytochemicals like flavanoids, terpenoids, tannins etc. against various types of cancers[133].

In our study decrease in endogenous antioxidant such as SOD,CAT with increase in lipid peroxidase products like TBRs, GST and GPx was noted in liver homogenate. This clearly indicates that the impaired endogenous antioxidant system with increased lipid peroxidase system suggest mice treated with EAC under oxidative stress. Preliminary phytochemical investigation suggestes that it has

flavonoids, terpenoids, tannins and glycosides. However the antitumor and antioxidant role of individual phytoconstituents yet to be explored in detail.

5.5. Figures



CHAPTER 6
Bioactive guided Fractionation, Isolation and Characterization of isolates from *Acanthophora spicifera*

6.1. Introduction

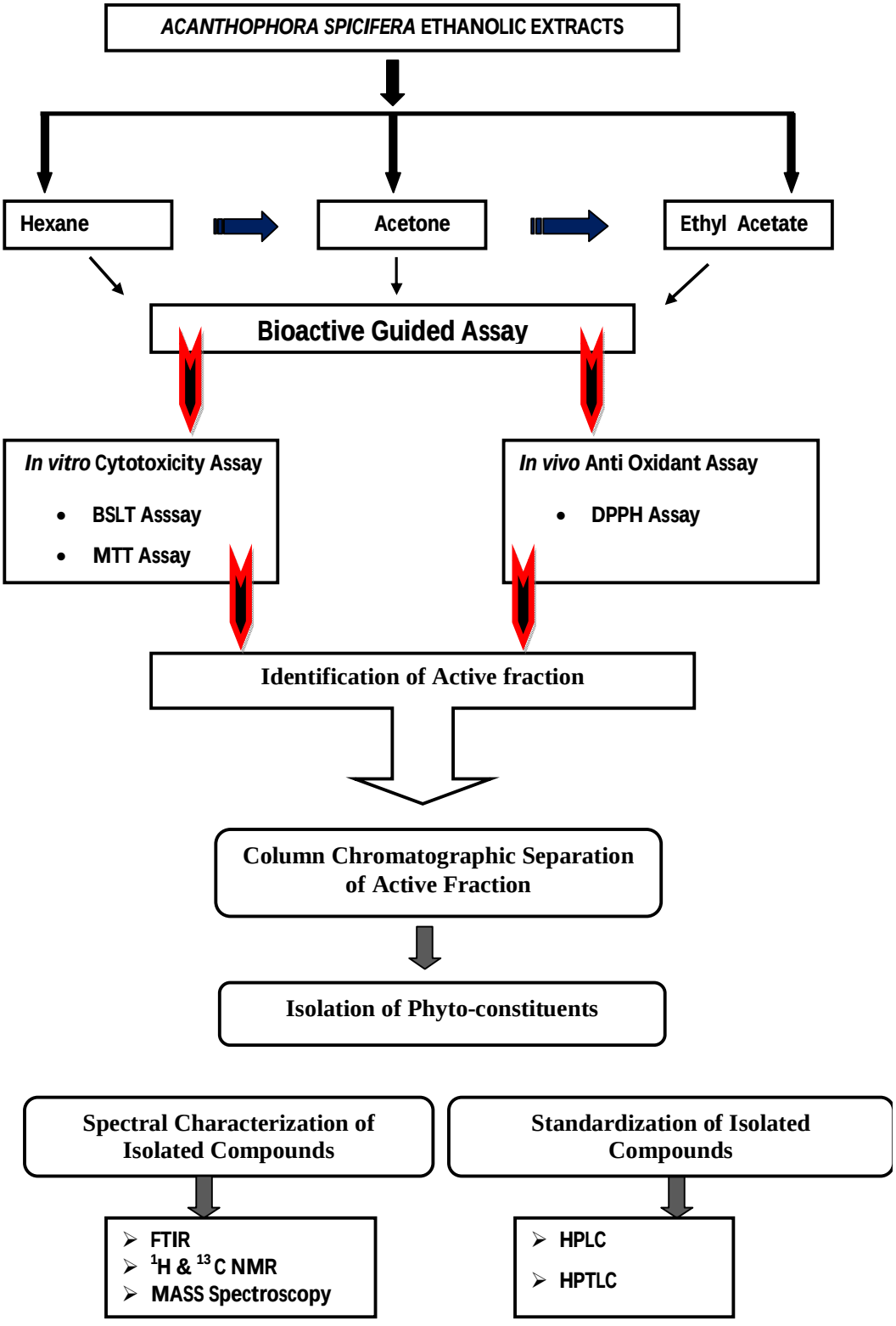
Bioactive compounds from natural products have long been and will continue to be extremely important as source of medicinal agents[134]. Many bioactive compounds have been discovered from medicinal herbs and marine resources which vary widely in chemical structure and function[135]. Enormous growing evidence suggests that many marine derived bioactive compounds have been identified, and experimental studies were conducted to evaluate their bioactive potential[136]. The advent of bioassay for the monitoring of bioactive potential is frequently included in natural product research and screening through these bioassays has simplifiedthis particular natural product research leading to drug discovery[137] .

In addition to the biologically active secondary metabolites from marine resources, which have found direct medicinal application as drug entities, many other bioactive compounds have proven useful as "leads" or model compounds for drug syntheses or semi syntheses[138]. Bioassay methods for the screening and testing of these seaweed extracts have varied over the years but eventually they evolved into pre clinical trials. A rapid, robust and inexpensive *in-vivo* Brine Shrimp Lethality (BSLT) bioassay has been used for screening for isolated biologically active natural compounds[139]. In contrast, *In-vitro* cytotoxicity evaluation in other hand will provide information about toxicity and possible application of bioactive compounds for cancer.

6.2. Materials and methods

The crude extract of *Acanthophora spicifera* was fractionated with various solvents ranging from nonpolar to polar solvents like, hexane, acetone, ethyl acetate. The alcoholic crude extract was subjected for fractionation with hexane to give a hexane-soluble fraction and hexane insoluble residue. The hexane-insoluble residue was partitioned with acetone and acetone soluble fraction was collected. The acetone insoluble fractions were further partitioned with ethyl acetate. There is no remaining insoluble residue was noted. The ethyl acetate fraction was dried in a rotary evaporator at 40◦C to yield a dry extract. The below schematic representation represents the fractionation procedure for alcoholic extract of *Acanthophora spicifera*

BIO ACTIVE GUIDED ACTIVE FRACTANATION



6.2.a. Drugs and Chemicals

RPMI 1640 medium, DME medium, fetal bovine serum, gentamycin, penicillin, streptomycin, agarose were purchased from Sigma Chemical Co. (St. Louis . MO). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], etoposide and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis . MO). Brine shrimp- *Artemia salina* cysts were obtained from laboratory of aquaculture, Belgium. In-vitro cytotoxicity study was performed with various fractions Acanthophona spicifera alcoholic extract as per standard protocol.

6.2.b. Brine shrimp lethality assay

This assay uses brine shrimps, *Artemia salina* Leach, which is used to determine the toxicity of marine seaweed extract. Brine shrimp eggs of *Artemia salina* Leach were hatched in artificial sea water (ASW) (aqueous solution of NaCl 3.8%w/v) and incubated at 25^0C. The starting pH of the ASW was 8–8.5. After 48 hours of hatching the larves (nauplii) were collected and used for BSL bioassay[140]. The brine shrimp lethality assay of the various fractions (hexane , acetone, ethyl acetate) of *Acanthophora spicifera* were carried out by the method described by Mayer[141]. All the fractions were tested at concentration levels of 10 μg/ml to 1000 μg/ml.

Each test was done in six replicates. A suspension of 10 nauplli (100 μl) was added into each well of a 24 well microplate and covered. The microplate was incubated for 24 h at room temperature. After this period the number of dead nauplii in each well was counted using binocular microscope. Potassium permanganate (100 μg/ml) was used as a standard (positive control) and a control reaction was carried out without the sample (negative control). The results were calculated statistically against their respective controls. The statistical method of probit analysis was used to calculate the concentration of the extract or fraction that would kill 50% of brine shrimps with in the 24 hrs exposure, i.e. the LC_{50} with the 95% confidence intervals. The fractions were considered bioactive when LC_{50} was 1000 μg/ml or less.

6.2.c Cell Cultures

Human liver cancer cell lines(HepG2), Human colon cancer cell line (HT29), Human colon cancer cell lines(A549) and Human Breast cancer cell lines(MCF7) was obtained from the AIIMS, New Delhi, India. The cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM). All culture media were supplemented with 10 % fetel bovine serum (FBS), 1 % antibiotic and antimycotic solution (50,00 units/ L of streptomycin) and 2 nM glutamine. Cultures were held in 75 cm^2 culture flasks at 37 ^{0} C, 5 % CO_2 and 95 % relative humidity changing media at least twice week. The cell lines were maintained at 37^{o}c with 5% CO_2 in CO_2 incubator. 1X10^5 cells were taken in each well of 96 well plates.

6.2.d. Cytotoxicity Assessment by MTT assay

Cytotoxicity was assessed by the MTT assay. Exponentially growing cells (1x10^4) were plated in 96-well plates and after 48h of growth the cells were treated with a series of concentrations of the various fractions of *Acanthophora spicifera* (10, 20, 40, 80, 100 μg/ml) dissolved in DMSO (final concentration 0.1%). Control cells were treated with DMSO alone and positive controls with various amounts of Etoposide. Incubation was carried out at 37^{o}C for 48h. MTT solution was added to each well (1.2 mg/ml) and incubated for 4h. The reaction results in the reduction of MTT by mitochondrial dehydrogenases of viable cells to form a purple coloured formazan product. The formazan product was dissolved in DMSO and the amount was estimated by measuring absorbance at 570 nm in an ELISA plate reader[142].

6.2.e. Antioxidant assay by DPPH Method

The effect of extracts on DPPH radical was estimated by Lim[143] with minor modification. In brief, 2mL of DPPH in methanol (3.6 x 10^{-5} M) were added to 50 μL of various concentrations of fractions. The mixture was vortexed for 15 sec and left to stand at 370C for 30 min. The decrease in the absorbance at 515 nm was continuously recorded in a spectrophotometer for 15 min at room temperature. All determination was performed in triplicate. The DPPH scavenging activity (decrease

of absorbance at 515 nm) of extracts and compounds were plotted against time and the (%) percentage of DPPH radical scavenging ability of the sample was calculated from the absorbance value at the end of 15 min duration as follows:

% Inhibition = [Abs Control – Abs Sample]/Abs Control X 100

Where Abs Control is absorbance of control at time = 0 and Abs Sample is absorbance of test sample at time = 15 min.

6.3. Results

6.3.a. Brine shrimp lethality assay

The result of the brine shrimp lethality assay of successive fractions from acs have been represented in the Table:1 It is noted that except ethyl acetae fraction other fraction were in active in showing lethality against brine shrimps. However ethyl acetate fraction was found to posses brine shrimp lethality at least concentration. The LC_{50} of ethyl acetate fraction was found to be in between 10µg/ml – 100 µg/ml which was compared with that of potassium permanganate (LC_{50} 40.8µg/ml) as standard.

6.3.b. DPPH assay

Fig:1 represents the free radical scavenging assay of ethyl acetate fraction using DPPH Radicals in in-vitro. There is a lenier relationship was observed with increasing in concentration of ethyl acetate fraction with increase in inhibition of DPPH radical. The IC_{50} value of EA fraction was found to be 54 µg /ml. The standard ascorbic acid was found to be 126 µg / ml.

6.3.c. Cytotoxicity

The results of cytotoxicity of ethyl acetate fraction and etoposide (as a standard) was carried out in various cancer cell lines were shown in the Fig 2 & 3 Potent selective cytotoxic activity was observed in the ethyl acetate in A549, MCF7, HT29 (Human colon cancer) and HepG2 (hepatocarcinoma) cell lines. The

MTT assay, IC_{50} value of the ethyl acetate fraction was found to be 38, 45, 16 and 17 μg/ml respectively and the IC_{50} value of standard etoposide was found to be 16, 8, 4 and 7μg/ml.

6.4. Column Chromatography Separation of Phytoconstituents From Ethy Acetate Fraction

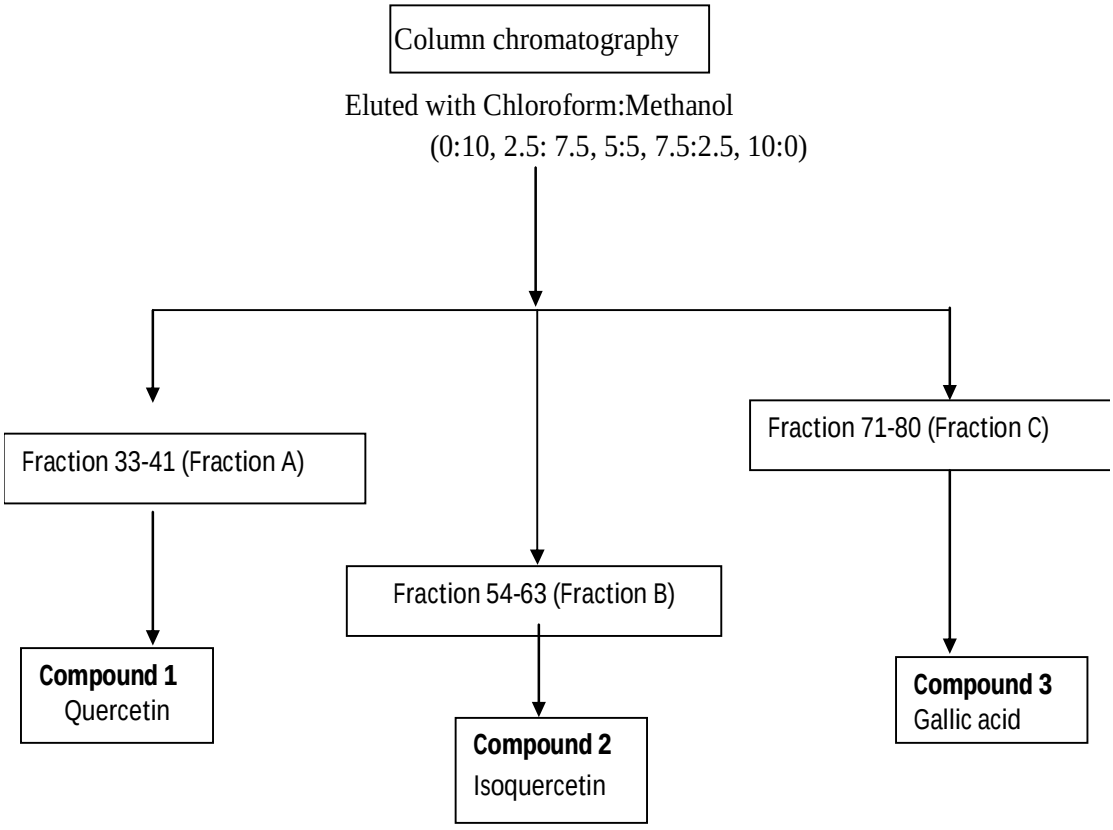
The ethyl acetate fraction was separated from red alga *Acanthophora spicifera*. The concentrated ethyl acetate fraction was subjected to column chromatography. The ethyl acetate fraction (3.5g) was chromatographed on a silica gel column, eluting with Chloroform, Chloroform:Methanol gradient system with increasing polarity (10:0, 7.5:2.5, 5.0:5.0, 2.5:7.5, 0:10) to obtain 80 fractions. Various fractions of 75 ml were collected from the column. Fraction 33 to 41 obtain chloroform:Methanol (2.5:7.5) had the same R_f value (0.47) while developed in solvent system containing toluene: ethyl acetate: formic acid (4.5:5.0:0.5). These fractions were mixed together and labeled as fraction A. The fraction A was evaporated under reduced pressure, which produced yelloe amorphous compound (Compound-1).

Another fraction 54 to 63 obtain from chloroform:Methanol (5: 5) showed the same R_f value (0.44) in toluene: ethyl acetate: formic acid (4.5:5.0:0.5). These fractions were mixed together and labeled as fraction B. Fractions B was evaporated under reduced pressure to produce yellow amorphous powder (Compound-2).

Further fraction 71-80 obtain from chloroform:Methanol (2.5: 7.5) showed compound 3 with R_f value (0.37) in toluene: ethyl acetate: formic acid (4.5:5.0:0.5). Fractions C was evaporated under reduced pressure to produce white amorphous powder (Compound-3)

Final purification of isolated compounds were achieved in Sephadex LH 20. The following figure further described the isolation of bioactive compounds by column chromatography.

ETHYL ACETATE FRACTION



6.5. Characterization of isolated compounds

6.5.a. Qualitative analysis Compounds-1 (FTIR,NMR and MASS)

Fig:4,5&6 represents the NMR,MASS and FTIR of the compound-1 respectively. A yellow, odourless, amorphous powder. It is insoluble in water, slightly soluble in alcohol, freely soluble in DMSO. The compound has a melting rang of 310 – 317^{0}C. The isolated compound-3 was tested for flavonoids chemical tests .The compound showed positive test for flavonoids. Further compound-1 was dissolved in DMSO subjected to thin layer chromatographic studies toluene: ethyl acetate: formic acid (4.5:5.0:0.5). Solvent was allowed to run up to 10 cm (solvent front) and the plates were dried. The spot were observed under UV light and then after spraying with natural products- poly ethylene glycol reagent (NP/PEG). The R_f value of the compound 1 was found to be 0.47.

High-resolution ESI mass spectrometry (HR-ESI-MS) was carried out on a JEOL GC mate spectrometer. Compound was obtained as a pale yellow powder of melting point 300°C (decomposed) and EI-MS *m/z*: [M−H]−(m/z) was found 301.0314, MS2 fragment ions (m/z)- 179.0044, 151.0042. IR absorption band at 3293.82, 1616.06, 1511.92 and 1166.72 were consistent with the presence of hydroxyl, carbonyl, aromatic ring and ether groups respectively. The ^{1}H-NMR (400MHz, CD_3OD): 4.53 (1H, s, H-6), 4.90 (1H, s, H-8), 5.90 (1H, *d*, J=8.4, H-5'), 6.79 (1H, *d*, J=8.6, H-6'), 7.02 (1H, s, H-2'). The 13C-NMR (100MHz, CD3OD): 71.26 (C-8), 72.26 (C-6), 81.51 (C-10), 83.55 (C-2'), 83.70 (C-5'), 94.90 (C-6'), 95.78 (C-1'), 95.92 (C-3), 100.44 (C-3'), 148.19 (C-4'), 148.93 (C-2), 158.41 (C-9), 162.67 (C-5), 165.72 (C-7), 177.50 (C-4). Spectral data of the compound is corresponding to the molecular formula $C_{15}H_{10}O_7$ for quercetin

6.5.b. Qualitative analysis Compounds-2 (FTIR,NMR and MASS)

Fig:7,8 & 9 represents the NMR,MASS and FTIR of the compound-2 respectively A yellow, odourless, amorphous powder. It is insoluble in water, slightly soluble in alcohol, freely soluble in Methanol. The compound has a melting rang of 226-228 ^{0}C. The isolated compound-2 was tested for flavonoids chemical tests .The compound showed positive test for flavonoids. Further compound-2 was dissolved in Methanol subjected to thin layer chromatographic studies toluene: ethyl acetate: formic acid (4.5:5.0:0.5). Solvent was allowed to run up to 10 cm (solvent front) and the plates were dried. The spot were observed under UV light and then after spraying with natural products- poly ethylene glycol reagent (NP/PEG). The R_f value of the compound 2 was found to be 0.44.

Yellow powder (methanol). (-)ESI-MS: [M−H]−(m/z) found was 463 , MS2 fragment ions (m/z) 301.0348, 193.0832, 178.9979, 151.0090, 107.0149 [M – Glu], λmax (MeOH) 258, 355 nm, ^{1}H-NMR (DMSO-*d*6) 6.20 (1H, *d*, *J* = 2.0 Hz, H-6), 6.41 (1H, *d*, *J* = 2.0 Hz, H-8), 6.82 (1H, *d*, *J* = 8.5 Hz, H-5'), 7.54 (1H, *dd*, *J* = 8.5 and 2.5 Hz, H-6'), 7.76 (1H , *d*, *J* = 2.5 Hz, H-2'), 5.345 (*d*, *J*= 7.5 Hz, H-1 glucose), 3.08- 3.88 (*m*, six sugar protons), ^{13}C-NMR(DMSOd_6)156.164 (C-2), 133.344 (C-3), 177.78 (C-4), 161.241(C-5), 98.935 (C-6), 164.606 (C-7), 3.628 (C-8), 156.40(C-9),

103.787 (C-10), 121.106 (C-1'), 115.254 (C-2'),144.864 (C-3'), 148.549 (C-4'), 116.212 (C-5'), 121.625 (C-6'), 100.991 (C-1"), 61.027 (C-6"), 69.992 (C-4"), 74.158 (C-2"), 76.561 (C-3"), 77.557 (C-5"). Spectral data of the compound is corresponding to the molecular formula $C_{21}H_{20}O_{12}$ for isoquercetin

6.5.c. Qualitative analysis Compounds-3 (FTIR,NMR and MASS)

Fig:10,11&12 represents the NMR,MASS and FTIR of the compound-3 respectively. A white odourless amorphous powder. It is insoluble in water, slightly soluble in alcohol, freely soluble in ethanol. The compound has a melting rang of 190-192 ^{0}C. The isolated compound-2 was tested for flavonoids chemical tests as discussed in previous chapter. The compound showed positive test for flavonoids. Further compound-3 was dissolved in ethanol subjected to thin layer chromatographic studies (as described in chapter 2) with toluene: ethyl acetate: formic acid (4.5:5.0:0.5). Solvent was allowed to run up to 10 cm (solvent front) and the plates were dried. The spot were observed under UV light and then after spraying with natural products- poly ethylene glycol reagent (NP/PEG). The R_f value of the compound 1 was found to be 0.37.

White amorphous powder; TLC: (toluene:ethyl acetate:formic acid, (4.5:5.0:0.5). v/v/v) R_f 0.37; IR absorption band at 3491, 3377, 1703, 1617, 1539, 1453, 1254 cm^{-1} (KBr) were consistent with the presence of hydroxyl, carbonyl, aromatic ring and ether groups respectively. off white amorphous powder, λmax (MeOH) 272 nm, ^{1}H-NMR (DMSO-*d*6) 6.91(1H, s, H-2, 6), ^{13}C-NMR (DMSO-*d*6): 121.0 (C-1), 109.0 (C-2 & C-6), 145.9 (C-3 & C-5), 138.3 (C-4), and 168.0 (C-7).

In EIMS, the single fragment ion peak of gallic acid at m/z 180.10 MS2 fragment ions (m/z)- 171 (M+H) was an indicator of trihydroxy phenol moiety. Spectral data of the compound is corresponding to the molecular formula $C_7H_6O_5$ for gallic acid (3,4,5-trihydroxybenzoic acid)

6.6. Discussion

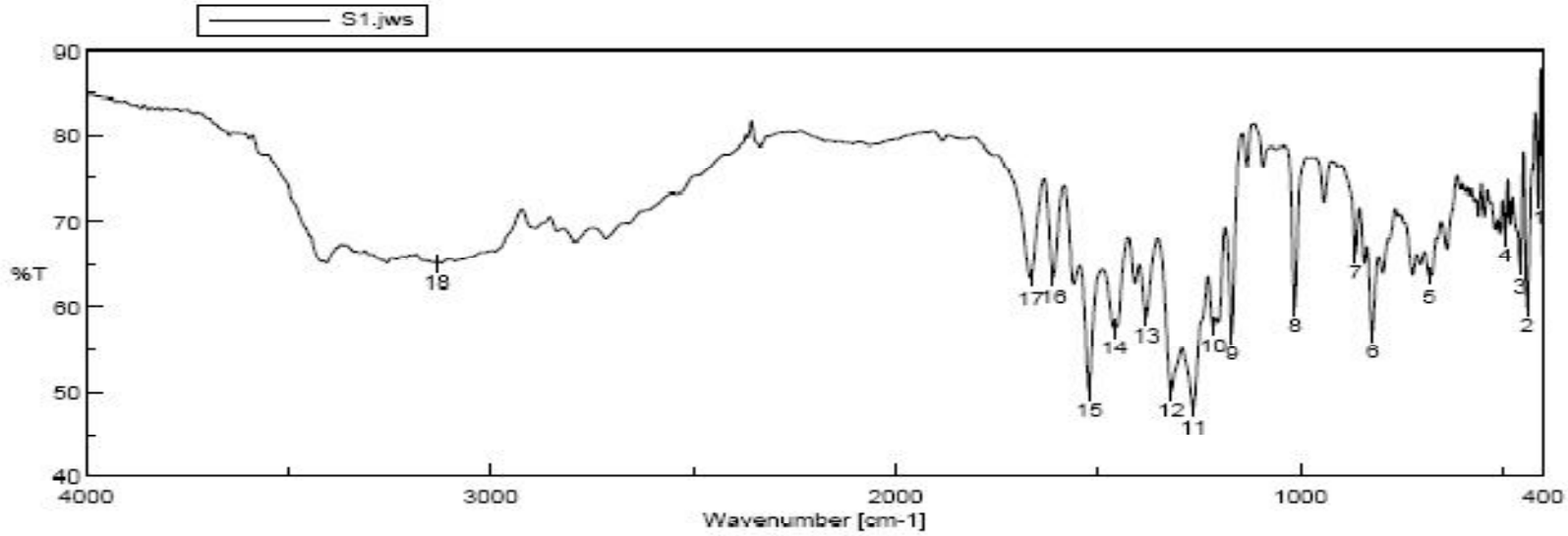
The present study is to carry out the bioactive guided *in vitro* assays like *in vitro* cytotoxicity assay and *in vitro* antioxidant assay to monitor bioactivity of various fractions separated from *acanthophora spicifera*.

The results from brine shrimp lethality assay suggest that the bidirectional effect of ethylacetate fraction against brine shrimps and cancer cell lines in *in vitro*. All the three fractions namely hexane, acetone and ethylacetate was exhibited cytotoxicity in the range of 10-100µg/ml. This prompted as to screen ethyl acetate fraction for its cytotoxic potential against various human cancer cell lines namely A549, MCF7, HT29 and HepG2 using MTT and etoposide as a standard. The data from the MTT assay suggest that ethyl acetate fraction has exhibited highly cytotoxic against HT29, HepG2 cell lines than A549 and MCF7. The *in vitro* antioxidant assay of ethyl acetate fraction was carried out by using DPPH assay. In this assay the results expressed as IC_{50} value of the ethyl acetate fraction against inhibition of DPPH free radical was found to be 54µg/ml, which is more than 5 times of IC_{50} value of *in vitro* cytotoxic assay. This clearly explains pro-oxidant and antioxidant role of ethyl acetate fraction from acanthophora *spicifera* in *in vitro* bioactive guided fractions. The other hexane and acetone fractions did not shown significant cytotoxic activity and antioxidant assay (data not included). The qualitative phytochemical investigations of ethyl acetate fraction reveals that ethylacetate has presence of only flavonoids and tannins, further the column chromatography separation of ethyl acetate fraction offers 3 compounds in which 2 compounds were identified phenolic compounds and rest of 1 is identified as tannin compounds. This spectral characterization of the isolated compounds were analyzed by FTIR, NMR and MASS.

6.7

Fig: 4

FTIR of compound -1

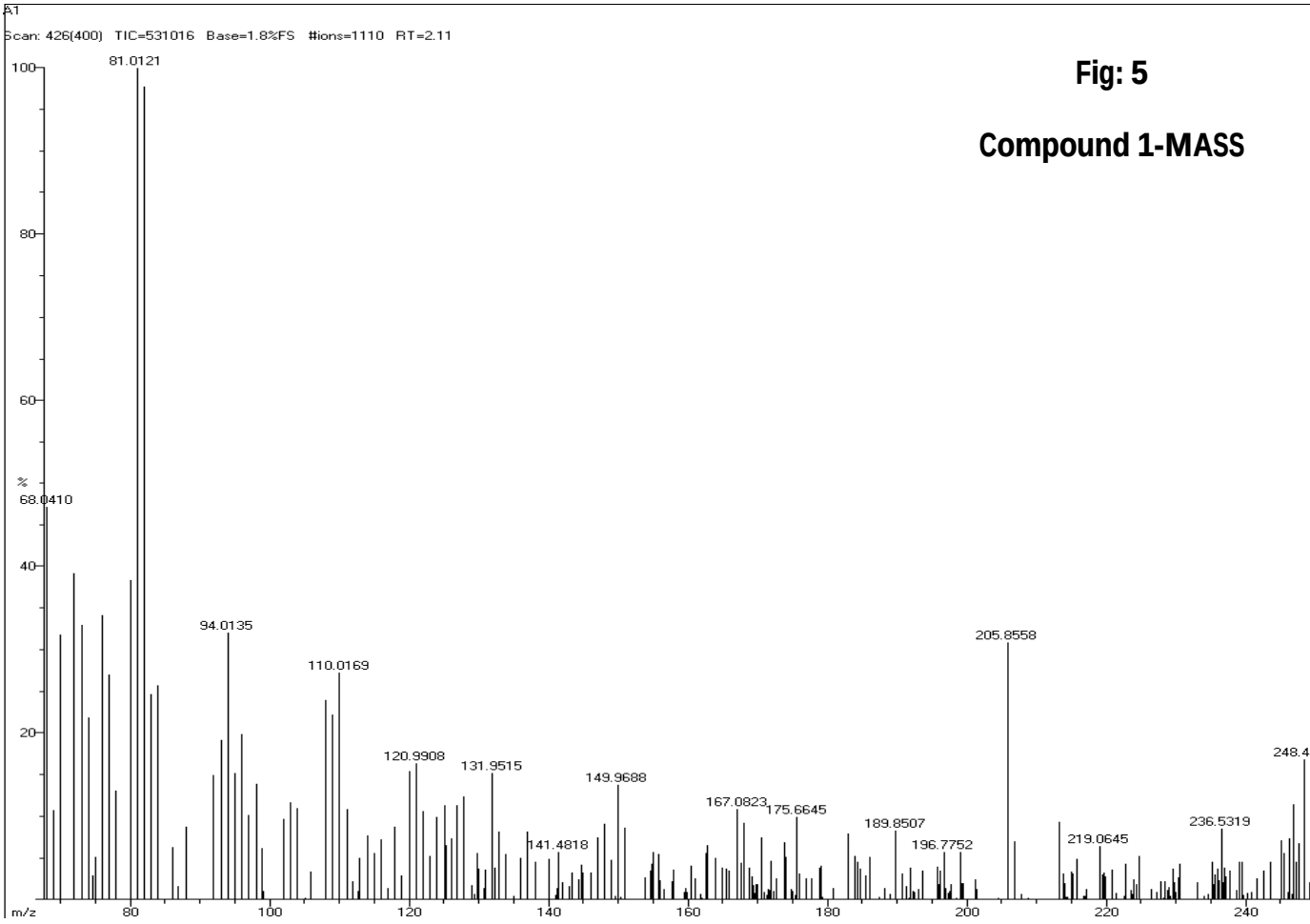


Results of Peak Find

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	412.892	72.5571	2	439.69	59.8753	3	458.011	64.6239
4	494.852	68.1887	5	681.713	63.5081	6	823.455	56.874
7	864.917	66.1963	8	1014.37	59.9439	9	1169.62	56.7211
10	1215.9	57.5456	11	1265.07	48.0522	12	1320.04	50.0289
13	1381.75	58.724	14	1460.81	57.4006	15	1522.52	50.0726
16	1611.23	63.3039	17	1667.16	63.2282	18	3133.76	65.0474

Fig: 5

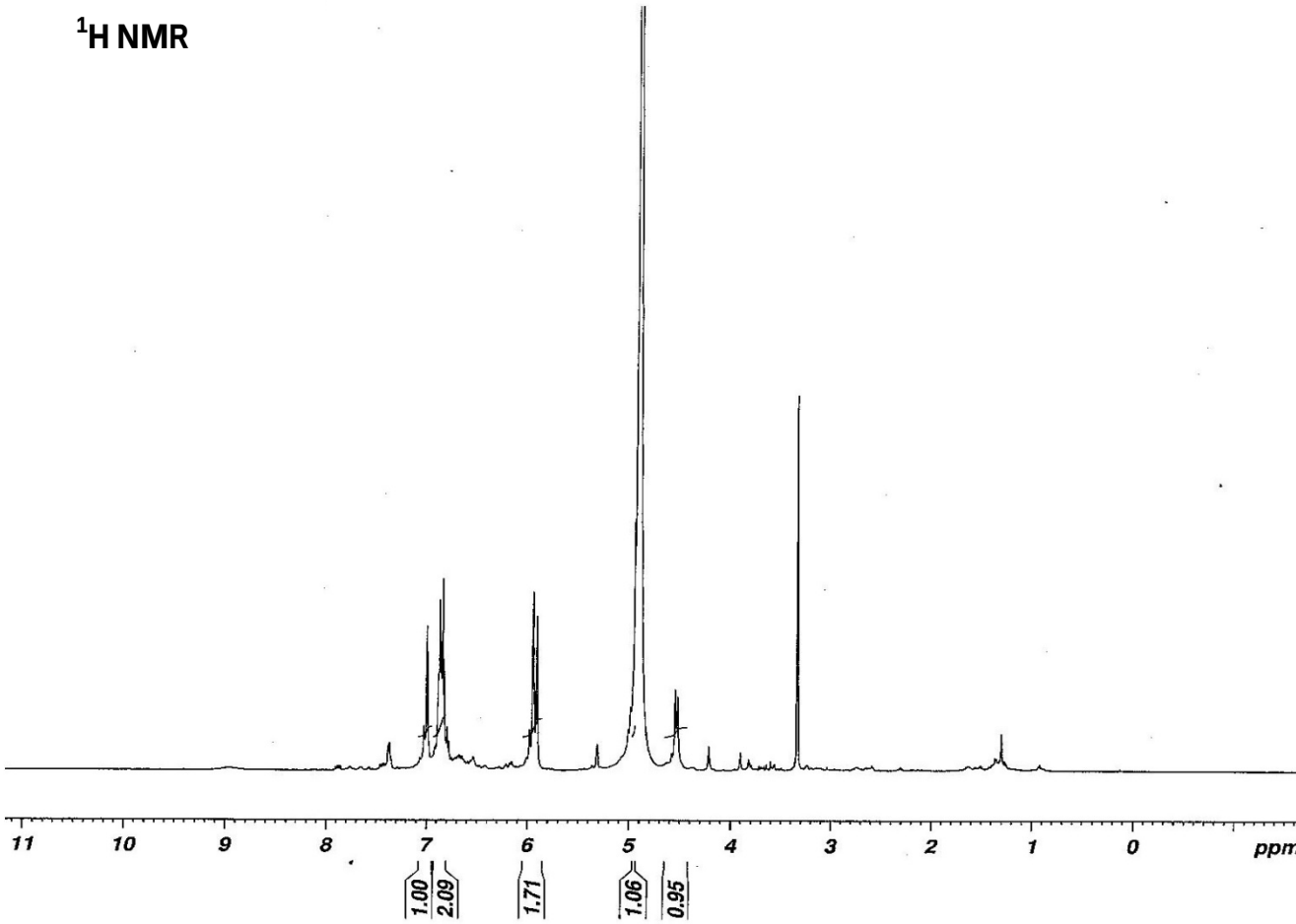
Compound 1-MASS



Mass Spectrum of Compound-1

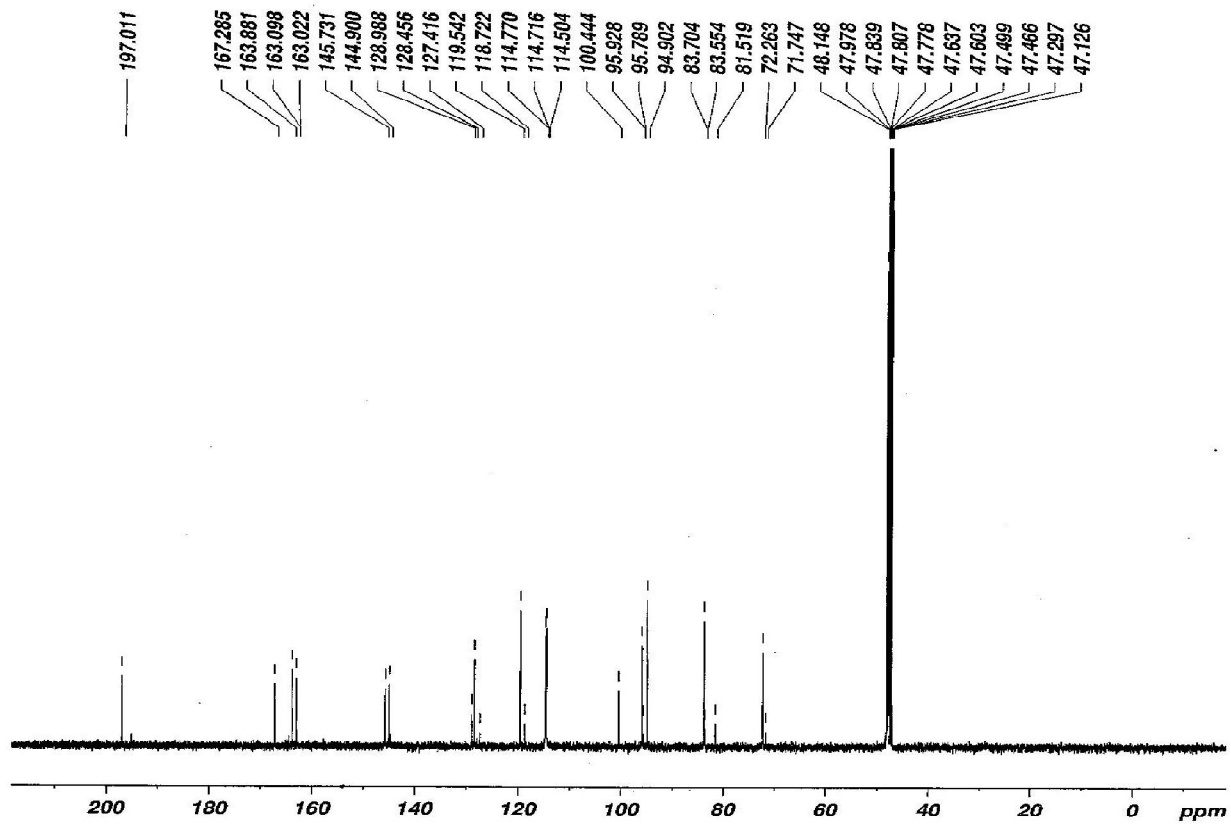
Fig:6

^{1}H NMR



^{1}H NMR Spectrum of Compound-1

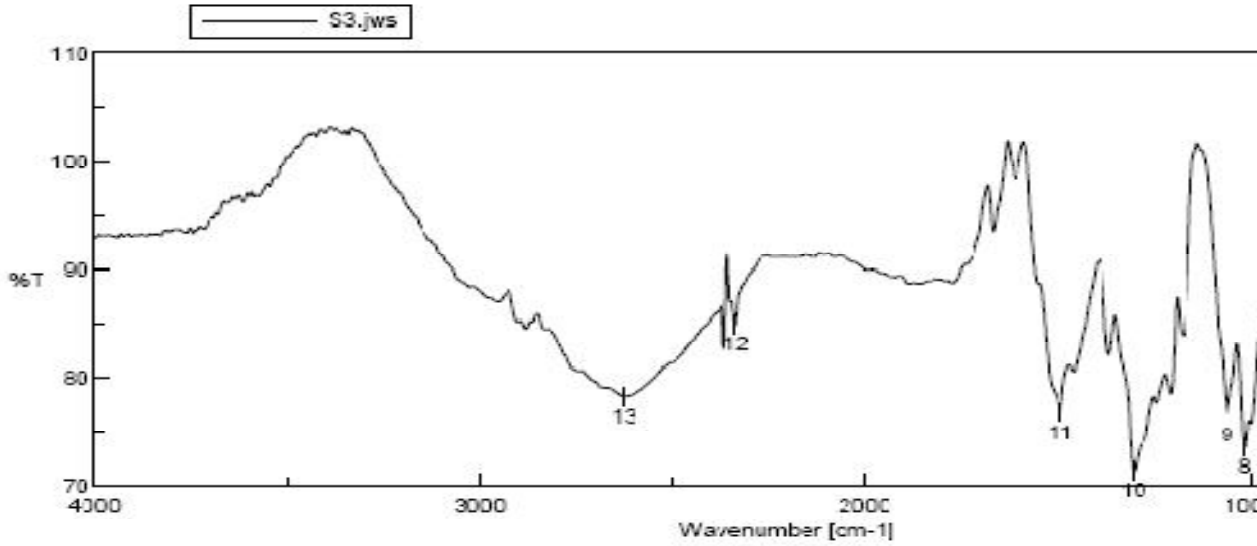
A1.....Anusha,


197.011
167.285
163.881
163.098
163.022
145.731
144.900
128.988
128.456
127.416
119.542
118.722
114.770
114.716
114.504
100.444
95.928
95.789
94.902
83.704
83.554
81.519
72.263
71.747
48.148
47.978
47.839
47.807
47.778
47.637
47.603
47.499
47.466
47.297
47.126
200
180
160
140
120
100
80
60
40
20
0
ppm

^{13}C NMR spectrum of Compound 1

Fig :7

FTIR of Compound-2



Results of Peak Find

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	415.585	71.2283	2	430.048	93.2738	3	440.655	90.7553
4	464.154	83.1582	5	481.153	83.1187	6	566.006	88.1847
7	936.271	81.4898	8	1012.45	73.5263	9	1059.69	76.582
10	1301.72	71.3285	11	1493.6	78.8313	12	2336.34	84.8154
13	2624.64	78.2355						

Fig:8

MASS spectrum of Compound-2

2..... Mass/Lavakumar.

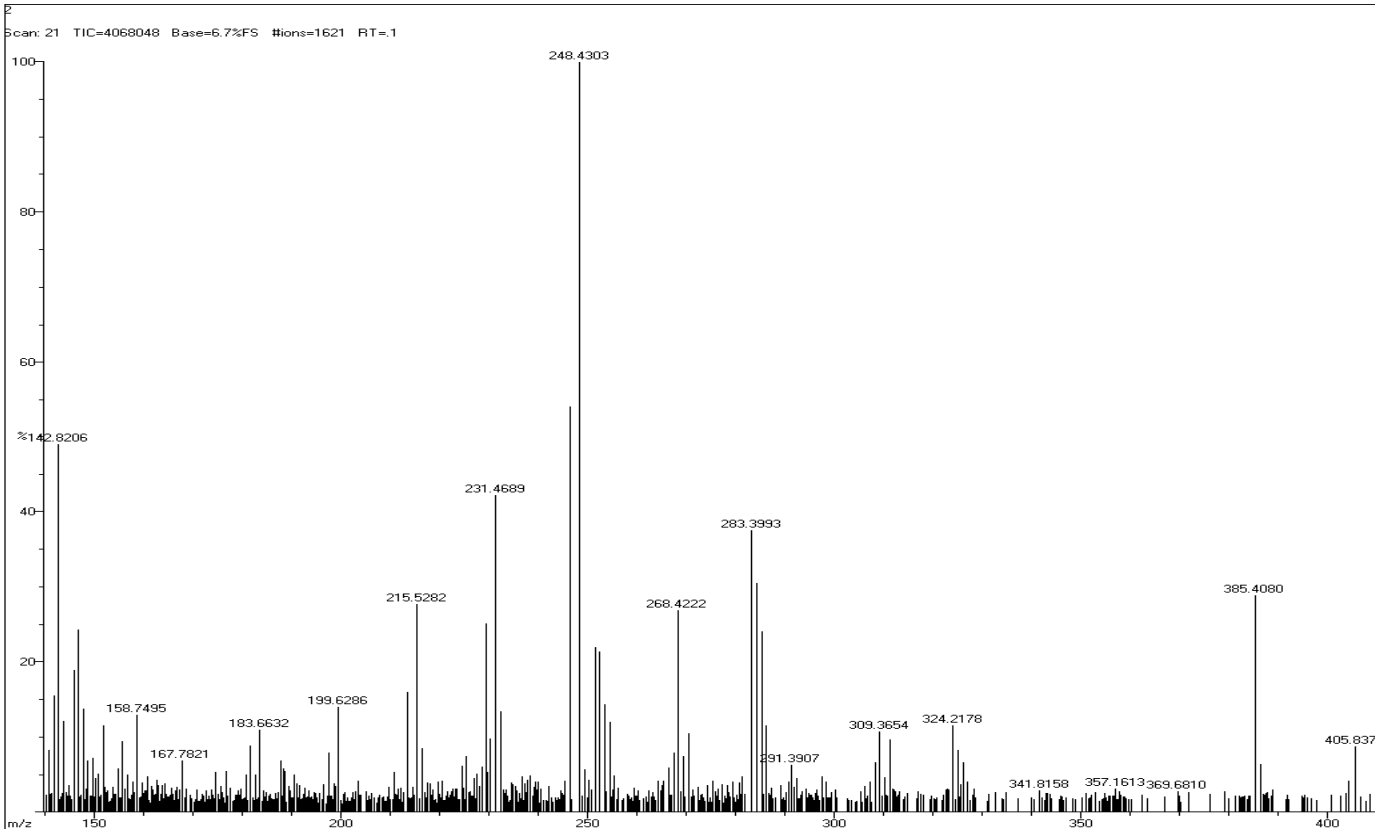
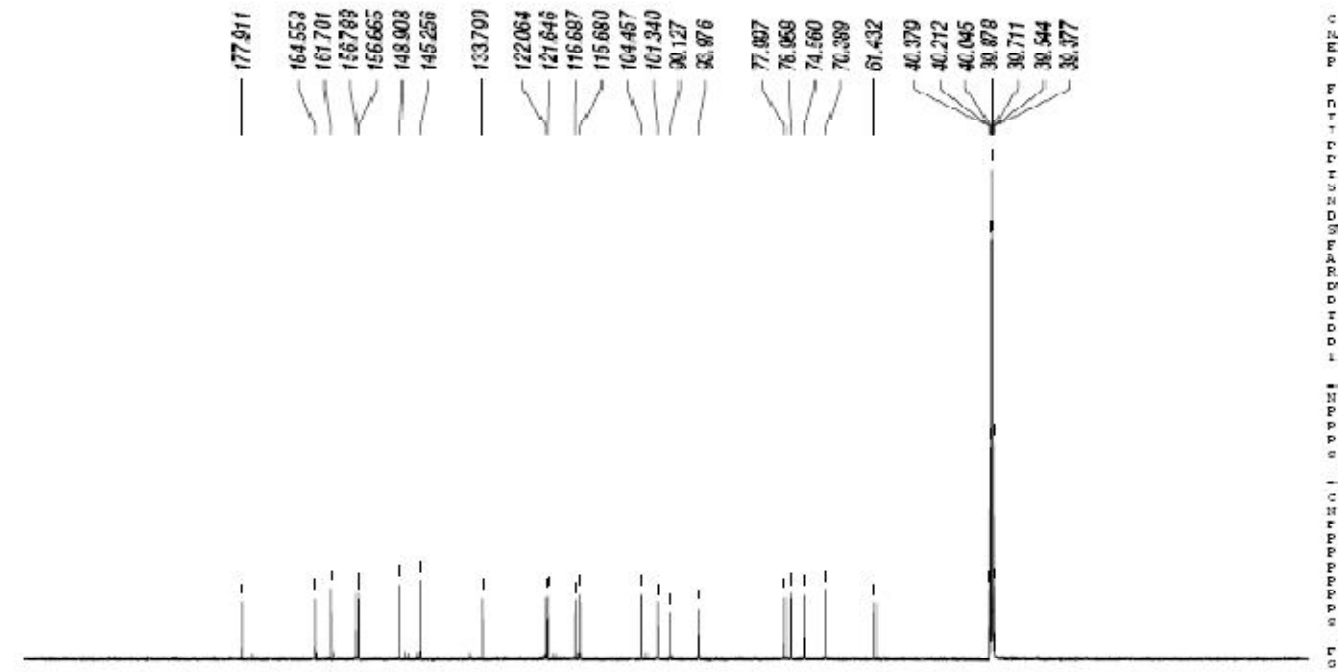


Fig: 9

^{13}C NMR of compound -2



^{1}H NMR of compound -2

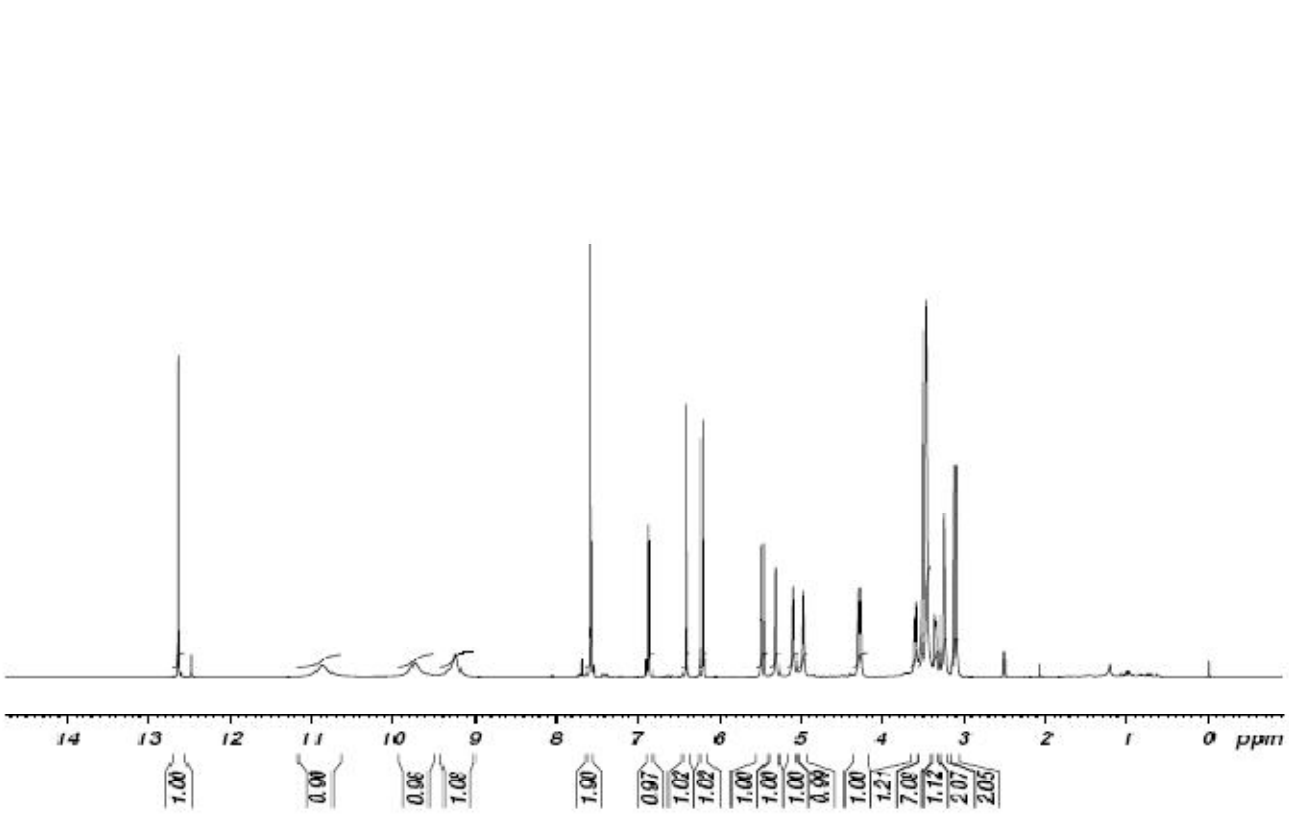
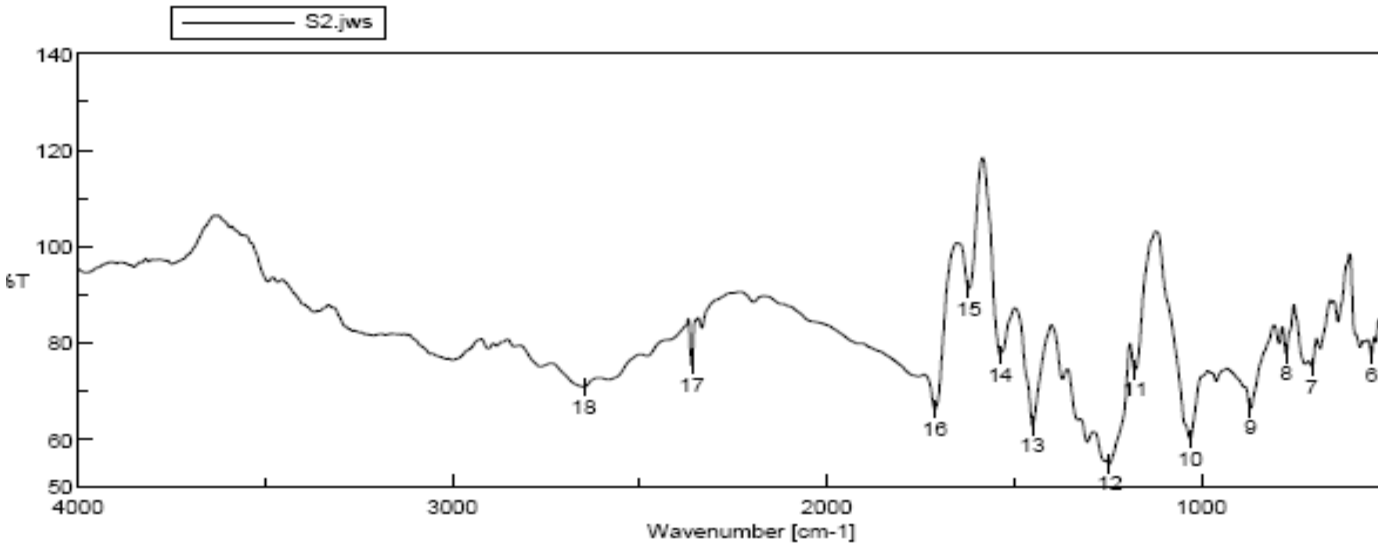

14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
ppm
1.00
0.90
0.96
1.08
1.90
0.97
1.02
1.02
1.00
1.00
1.00
0.99
1.00
1.21
7.08
1.14
2.07
2.05

Fig :10

FTIR of Compound-3



Results of Peak Find

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	415.585	74.2886	2	429.084	94.3529	3	445.476	81.8752
4	478.26	86.9595	5	494.652	84.0214	6	544.792	77.3008
7	703.89	74.931	8	772.351	77.6032	9	867.81	66.3614
10	1029.8	59.9741	11	1176.36	74.3286	12	1244.83	54.6482
13	1450.21	62.6524	14	1536.99	77.3799	15	1620.88	91.1078
16	1711.51	66.1282	17	2360.44	75.236	18	2649.71	70.7647

Fig: 11

MASS spectrum of Compound-3

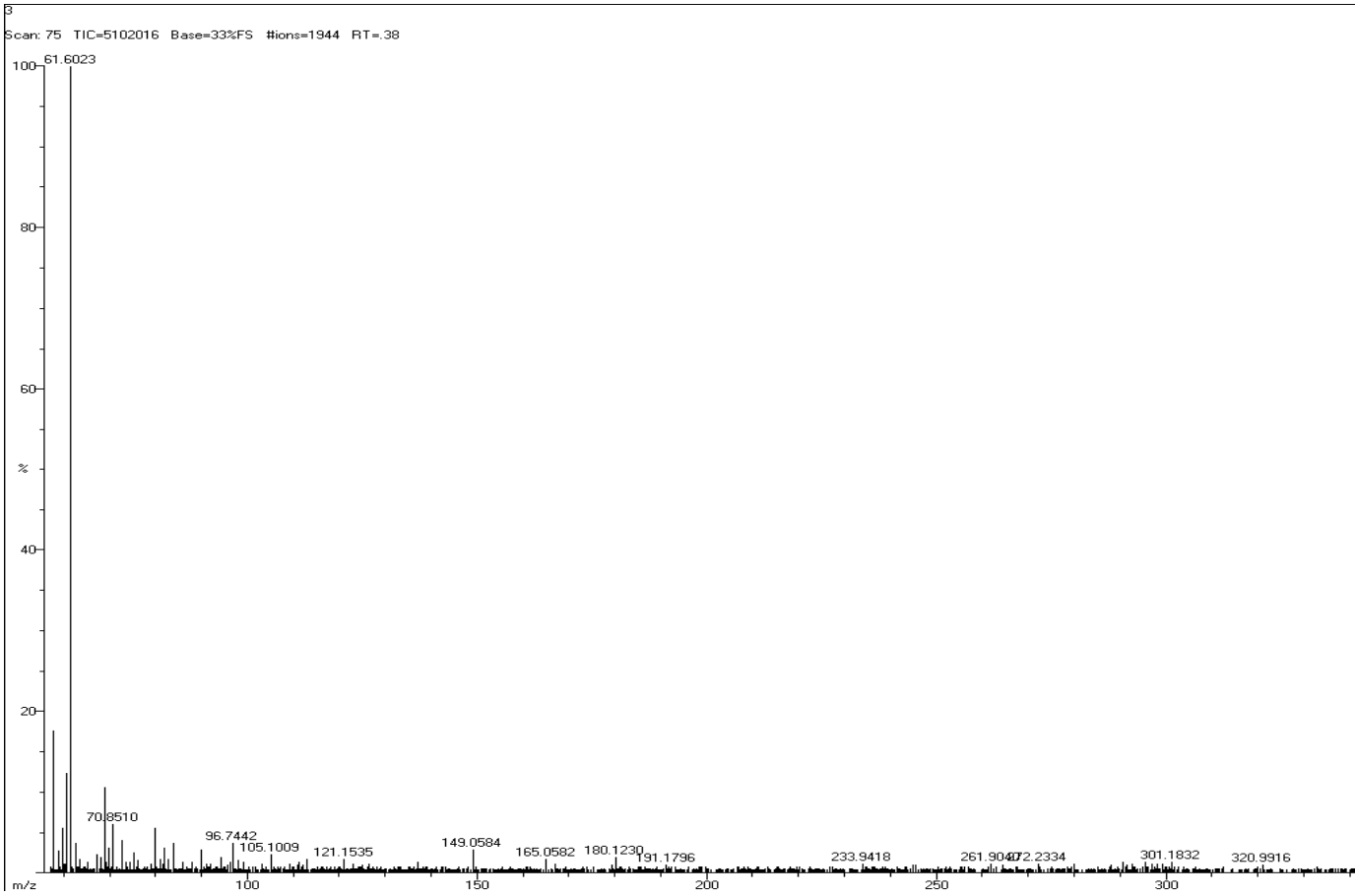
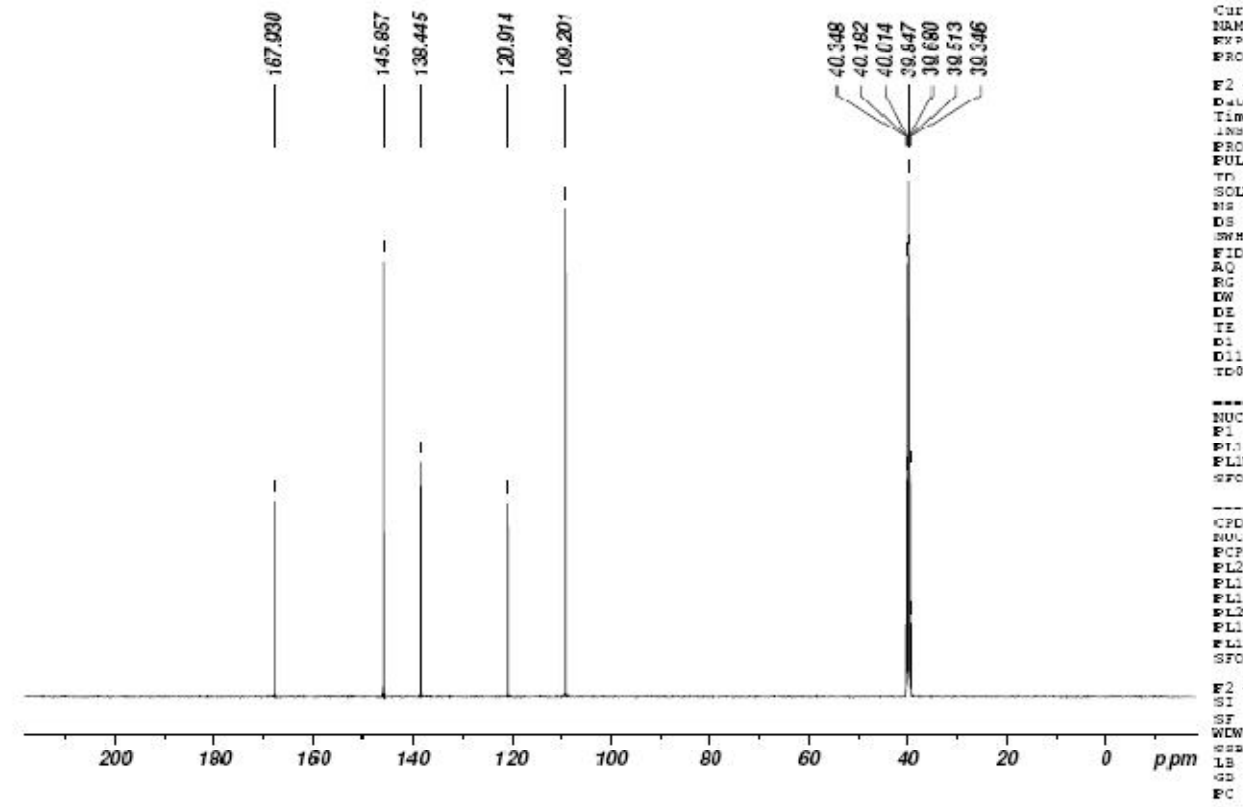


Fig: 12

^{13}C NMR of compound -3



^{1}H NMR of compound -3

3.....Lavakumar.

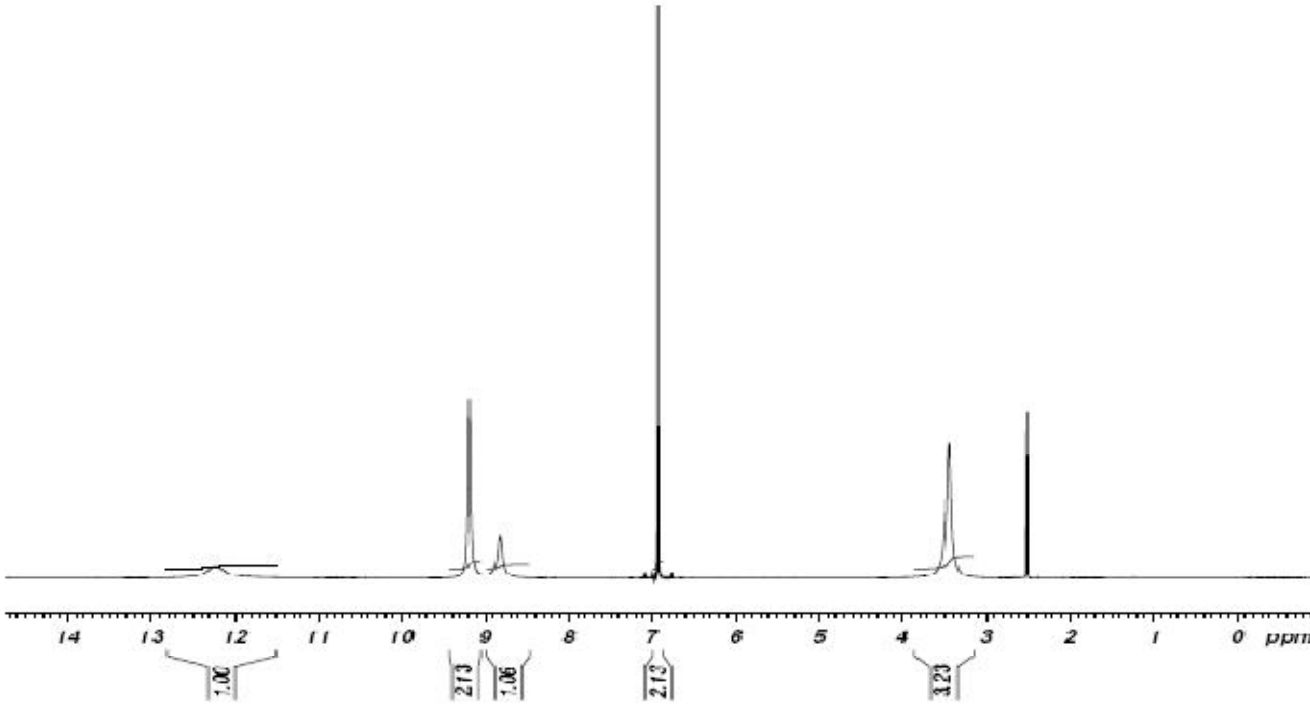


Table: 5.7 Effect of various fraction of *Acanthophora spicifera in* BSLT assay

Treatment	LC_{50} μg/ml		
	10 μg/ml	**100 μg/ml**	**1000 μg/ml**
Control	¶	¶	¶
Potassium permanganate	P <0.01	P<0.001	P<0.001
Hexane fraction	ns	Ns	ns
Acetone fraction	ns	Ns	Ns
Ethylacetate Fraction	P<0.05	P<0.001	P<0.001

Each dose was done in triplicate; LC_{50} –Lethal concentration; ¶ - no mortality of shrimp; n.s - not significant ; P < 0.05, P < 0.01, P < 0.001 sample vs controls.

CHAPTER-7
HPLC and HPTLC studies of *Acanthophora spicifera*

7.1. Introduction

Chromatography is a physical method of separation in which the components to be separated are distributed between the two phases; one of these is a stationary phase bed and the other is a mobile phase which percolates through this bed. Basically two chromatographic techniques are used for the standardization viz. HPTLC, HPLC with GLC used very rarely. These techniques give out the chromatograms, which serves as the fingerprint. The fingerprint of a particular plant, its extract or its product(s) will be same, if the conditions are maintained. Thus chromatography technique offers the best method for recording the fingerprint which can be reproduced anywhere, provided the conditions are maintained. Chemical standardization of herbal drug is defined as quantification of active components using different chemical techniques. These active components popularly known as marker compounds or reference compounds which, represents the quality and efficacy of the herbal drugs[144].

The information obtained by the chromatographic experiment is called the chromatogram, a record of the concentration or the mass profile of the sample components as a function of the movement of the mobile phase. Information that can be extracted from a chromatogram includes (a) an indication of the sample complexity or the number of components present based on the number of peaks. (b) qualitative identification of the samples based on the accurate measurement of the peak positions, (c) quantitative assessment of the relative concentration or the amount of substance present based on the peak size.

Unlike synthetic organic medicinal compounds that exhibit predictable pharmacological activity at a given dosage, the world of botanicals is quite different in the sense that it is not always known with certainty what constitutes the active ingredient(s). It is generally believed that the reported pharmacological action of a botanical is due to more than one constituent acting synergistically with other constituents present. From the pharmacopoeial perspective, a better quality control of raw material can be achieved by specifying a quantitative test procedure for the

determination of the range or a minimum content of the marker substance or the "active" ingredient.

According to the definition of chromatographic fingerprints of a herbal drugs a chromatographic fingerprint is in practice a chromatographic pattern of some common kinds of pharmacologically active and chemically characteristic components in the herbal drugs under study. It suggests that with the help of the chromatographic fingerprints obtained, the authentication and identification of a herbal drugs can be accurately conducted even if the number and/or concentration of chemically characteristic constituents are not very similar in different samples of herbal drugs or, chromatographic fingerprints could successfully demonstrate both the ''sameness'' and ''differences'' between various samples[145]. Thus, we should globally (considering multiple constituents) not locally (considering only few marker components) evaluate the quality. In the case of herbal drugs there are always hundreds of components and many of them are in too low concentrations. On the other hand, there usually exists variability within the different and even the same herbal materials. As a result, to obtain reliable chromatographic fingerprints chemically representing pharmacologically active and characteristic components is not a trivial work. The performance of a chromatographic fingerprint obtained is closely dependent on the chromatographic separation degrees and concentration distribution of all chemical components[146].

It is well known that chromatography has very powerful separation ability, suggesting the separation of complex systems into many relatively simple sub-systems. Furthermore, hyphenated chromatographic and spectrometric approaches such as high-performance liquid chromatography-diode array detection (HPLC-DAD), gas chromatography- mass spectrometry (GC-MS), capillary electrophoresis (CE)-DAD and HPLC-MS, could show greatly improved performances in terms of the elimination of instrumental interference, retention time shift correction, selectivity, chromatographic separation abilities and measurement precision

7.1.a. Liquid Chromatography (LC)

Liquid chromatography with a isocratic/gradient elution remain to be the method of choice in the pharmacopeia and for the analysis of marker compounds that are thermally labile in botanicals and herbal preparations.

HPLC is one of the latest analytical techniques, which is very essential for both quantification and standardization of the herbal materials. The technique is based on the same modes of separation as of column chromatography, i.e., adsorption, partition, ion exchange and gel permeation, but it differs from column chromatography in that the mobile phase in HPLC is pumped through the packed column under high pressure. The principle advantages of HPLC when compared to classical column chromatography are improved resolution of the separated substances, faster separation times and the increased accuracy, precision and sensitivity with which the separated substances may be quantified.

The reversed octadecyl silica (C-18) is most commonly used. In the course of our experiments, we found that columns with smaller inner diameter, such as 1.0 or 2.1mm i.d. were well suited to the analysis of components present in botanicals. For columns with smaller inner diameter, it was observed that the system precision for the retention time and peak area/height were comparable to analytical columns with 4.6mmi.d. Most important of all, methods using columns with smaller inner diameter and the right mobile phase can be readily adopted to mass spectrometry. The most common mode of detection remains to be ultraviolet detection.Methods using gradient elution HPLC with reversed phase columns had been applied for the analysis of multiple constituents present in medicinal plants and herbal preparations[147, 148].

Gradient elution HPLC with ultraviolet detection, using a C18 reversed phase column had been used to profile components present in C. rhizoma, Radix aristolochiae, ginseng, R. glycyrrhizae (liquorice), S. radix, R. codonopsis pilosula and S. miltiorrhiza. The advantages of liquid chromatography include its high reproducibility, good linear range, ease of automation and its ability to analyze the

number of constituents in botanicals and herbal preparation. However, for the analysis of marker compounds in herbal preparations with two or more medicinal plants, co-eluting peaks were often observed in the chromatograms obtained due to the complexity of the matrix[149]. The complexity of matrix may be reduced with additional sample preparation steps, such as liquid-liquid partitioning, solid phase extraction, preparative LC and TLC fractionation.

7.2. Materials and Methods

7.2.a. HPLC analysis[150]

Qualitative analysis of the flavonoid quercetin, isoquercetin and tannin gallic acid in the sample was performed using HPLC system. Analytical HPLC was performed on (**D**) a computer-controlled high-pressure-gradient LaChrom-HPLC-system (Merck-Hitachi), containing an Interface L-7000; two pumps L- 7100 (one for each eluent); diode array detector L-7450; autosampler L-7200 with 100µL sample loop; solvent degasser L-7612; high-pressure gradient mixer; Rheodyne injection valve 7725i, 20µL sample loop; administration of the device, data recording and analysis was performed with the LaChrome Software version 4.0. The column was Thermo ODS Hypersil C18 (250 x 4.6 mm, 5 µm) in isocratic mode. The separation was achieved using a mobile phase of acetonitrile - 0.1M phosphate buffer - glacial acetic acid (15: 85: 1, *v/v/v*) with pH adjusted to 4.0 using phosphoric acid at a flow-rate of 1.0 ml/min. The effluent was monitored using UV detection at a wavelength of 300 nm. The mobile phase was filtered through 0.45 µm nylon filter prior to use. The presence of quercetin, isoquercetin and gallic acid in *Acanthophora spicifera* was confirmed and the resultant graph was shown in Fig. 1.

7.2.b. HPLC Conditions

HPLC Make	:	LC 10 (Shimadzu, Japan)
Composition	:	LC 10- 80 (Dual Pump)
		SPD-M20A PDA Detector

DEW DINE Injector (20 µl)

Column : RP-C 18(Merck) with guard column of same chemistry

Size : 250nm X 4.6 (i.d) with 5µm pore size

Mobile Phase : Component A-H_2O

Component B-Methanol

Both derated in ultrasonic path and filtered through 0.45 µm nylon filter

TIME in Min	SOLVENT –A	SOLVENT-B
0	75	25
3	50	50
18	20	80
25	75	25
30	75	25

7.2.c. HPTLC analysis with known marker Quercetin and Gallic acid[151]

The ethyl acetate extract of *Acanthophora spicifera* was further subjected to HPTLC for the conformation of the active constituents. HPTLC was performed on 10 cm × 10 cm aluminum backed plates coated with silica gel 60F254 (Merck, Mumbai, India). Standard solution of quercetin and sample solution were applied to the plates as bands 8.0 mm wide, 30.0 mm apart, and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100-µL Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2°C), with Toluene: Ethyl acetate: Formic acid (5 : 4: 1) (v/v/v), as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried with a hair dryer and then scanned at 254 nm with a Camag TLC Scanner with WINCAT software,

using the deuterium lamp. The data of peak for standard and test was shown in Fig. 1 & 2.

Test solution

5 g of powdered drug was defatted with 25 ml petroleum ether (60-90°C). Solvent was removed and marc refluxed with acetone (25 ml) for 15 min consecutively 3 times on a water bath, filtered and concentrated under reduced pressure. 10 mg of extract was dissolved in 5 ml of 50% aqueous methanol and used for estimation of gallic acid and quercetin.

Standard solution for Gallic acid and Quercetin

10 mg of gallic acid was dissolved in 10 ml of methanol. From this stock solution standard solutions of 20-100 μg/ml concentrations were prepared by transferring aliquots (0.2 to 1 ml) of stock solution to 10 ml volumetric flasks and adjusting the volume to 10 ml with methanol. In the same way the standard solution for Quercetin was prepared.

Calibration curve:

10 μl of each of standard solutions (200 ng-1000 ng per spot) were applied on precoated TLC plates. The plate was then developed in the solvent system to a height of 8 cm, dried and scanned densitometrically at 272 nm, the peak area was recorded and the calibration curve was prepared by plotting the peak area against concentration of the gallic acid applied.

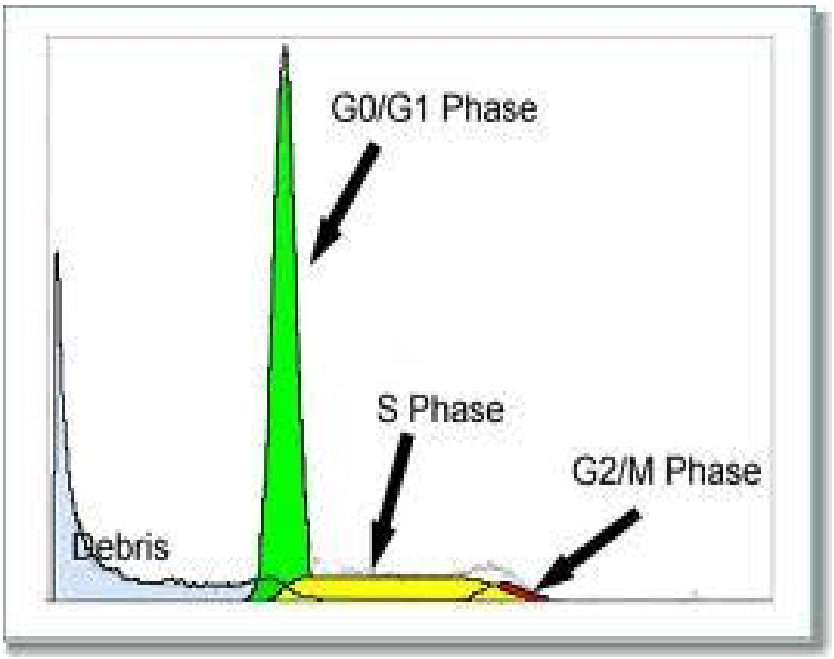
7.3. Results & Discussion

7.3.a. High performance liquid chromatography

Comparing the HPLC chromatograms from the *Acanthophora spicifera* with the standard showed 2 flavonoids quercetin, isoquercetin and gallic acid by comparing the peak eluted at 10.10 min, 10.45 min and 16.20 min respectively. (Fig. 1 & 2).

7.3.b. High performance thin layer chromatography

In the current study, the flavonoid quercetin and tannin gallic acid was detected and quantified using HPTLC silica gel 60 F254 pre-coated plates with the mobile phase made of Toluene: Ethyl Acetate: Formic acid (5:4:1 v/v/v). It was conformed to the flavonoid quercetin and gallic acid were the active constituent for the plant *Acanthophora spicifera*. The Quercetin was already reported in this particular species *Acanthophora spicifera*[92]. The same was quantified by HPTLC and the phytochemical screening of the total extract also shown the presence of Tannins. Hence, an effort was made to identify quercetin and tannin from the ethyl acetate extract using quercetin and Gallic acid as a standard for the HPTLC. Isoquercetin was also isolated in our previous protocol by column chromatography. However, HPTLC is a tool which could not separated the Quercetin and Isoquercetin. Hence Gallic acid (Fig. 3, 4 & 5) and Quercetin (Fig. 6,7 & 8) were quantified by HPTLC. The method used in this work resulted in good peak shape and enabled good resolution of gallic acid and quercetin from other constituents of the plant material. Because recovery (98.33%) was close to 100%, there was no interference with the gallic acid peak from other constituents present in the plant. The detection of gallic acid and quercetin was observed to be linear over a concentration range of 200 ng-1000 ng/ml with correlation coefficient of 0.9991. The concentration of gallic acid was found to be 0.1134 mg/gm and the concentration quercetin was found to be 0.2028 mg/gm.



CHAPTER-8
Effect of Isoquercetin on Cell Cycle Analysis of HepG2 and HT29 Cell Lines by Flowcytometry

8.1. Introduction

Apoptosis is a physiological process that plays an important role in the regulation of tissue development and homeostasis[152]. The outstanding feature of apoptosis is its morphological changes, showing condensation of nuclear heterochromatin, cell shrinkage and loss of positional organization of organelles in the cytoplasm and DNA fragmentation is regarded as the characteristic feature of apoptosis[153]. This resulting in death and degradation of cells follow the activation of a highly regulated set of cytosolic and nuclear proteases and DNAses[154]. In cells undergoing apoptosis there will be an activation of a family of proteases called caspases, and activation of caspases appears to be directly responsible formany of the molecular and structural changes in apoptosis[155,156]. These include degradation of DNA repair enzyme poly (ADP) ribose polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) and cleavage of chromatin at inter-nucleosomal sites mediated by caspase-activated[157] DNase (CAD).

Natural products are an excellent source of complex chemicals possessing a wide variety of biological activities and having great potential therapeutic value[158,159]. Crude extracts or components isolated from plants are important source to screen as apoptotic inducers. Understanding the mechanism of action of these compounds should provide useful information for their possible application in cancer therapy and also in cancer prevention[160]. Chemopreventive agents include anethol, allicin, S-allyl cysteine, capsaicin, catechins, curcumin, ellagic acid, eugenol, genistein, 6- gingerol, lycopene, resveratrol, silymarin and ursolic acid comprise a diverse group of compounds with different mechanisms of action, but their decisive ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention[161,162]. These agents have been shown to suppress cancer cell proliferation, inhibit growth factor signalling pathways, induce apoptosis, inhibit NF**k**B, AP-1 and JAK-STAT activation pathways, inhibit angiogenesis, suppress the expression of anti-apoptotic proteins, and inhibit cyclooxygenase-2[163]

8.2. Materials and Mathods

Cell lines, Medium for cell maintaience FITC-Annexin V (sigma) propidium iodide (PI) (Sigma) sodium chloride, sodium hydroxide, magnesium chloride, potassium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, acetic acid, bromophenol blue, dimethyl sulfoxide (DMSO), glycerol, Tris base, EDTA (SRL, India), hemocytometer, sterile petri dishes (35 and 55 mm) (Greiner), T25 tissue culture flasks (Greiner), 96 well microtitre plates (Corning costar), 24 well microtitre plates (Corning costar), micro pipettes, glass slides with coverslips, falcon tubes (15 ml V bottomed and 3 ml round bottomed with double sealing caps), eppendorf tubes, MilliQ water treatment plant (Waters microtitre plate reader (BioRad).

8.2.a. Cell Cultures and maintenance

Two human cancer cell lines were used for the present investigation. HT29 (Human colon cancer cell lines) and HepG2 (Liver cancer cell lines) were maintained in RPMI 1640 supplemented with 15% heat inactivated fetal bovine serum and gentamycin (40 μg/ml), penicillin (100 units/ml) and streptomycin (10 μg/ml). Cells were grown at 37^{o}C in a humidified atmosphere of 5% CO_2 / 95% air.

8.2.b. MTT Assay[164]

1) 1 x 10^4 Cells were plated in each well of a 96 well microtitre plate in 180μl of medium.

2) In the first row first six wells are kept as blank (no cells are added: negative control) and in the last six wells (cells are added without any treatment: positive control).

3) 24 Hours after seeding the plate in each row four concentrations of a single compound were added in triplicate. Stock solutions of the compounds and extracts (100mg/ml) were made in DMSO and diluted

with the medium to a final concentration of 0.5, 1, 10, 50, 100, 200, 500 μg /ml in the plate.

4) After 48 hours of incubation at 37^{o} C in a CO_2 incubator, 50 μl of MTT solution (6 mg in 5ml) was added to each well and the plates were incubated at 37^{o} C for 4 hours.

5) The plates were then centrifuged at 1000 rpm in a centrifuge with a proper rotor for 10 minutes.

6) 175 μL of the supernatant was pipetted out from each well. 175 μL of dimethylsulfoxide (DMSO) was added to each well and the pellet was dissolved well to yield a uniform solution.

7) The plates were read at 540 nm in a microtitre plate reader.

8.2.c. Trypan Blue Assay[164]

1) 2×10^5 Cells were plated in each 35mm sterile petri dishes in 1800μl of appropriate medium.

2) 24 Hours after seeding the petri dishes compounds and extracts were added in different concentrations. Stock solutions of the compounds and extracts (100 mg/ml) were made in DMSO and diluted with the medium to a final concentration of 0.5, 1, 10, 50, 100, 200, 500 μg /ml in the petri dish.

3) After 48 hours of incubation at 37^{o} C in a CO_2 incubator the cells were harvested by centrifugation at 1200rpm for 8 minutes. The pellet was washed once in PBS and resuspended in 100 μl of Phosphate Buffer Saline.

4) A 25 μl aliquot of cell suspension was treated with 25μl of 0.4% trypan blue dye and kept for 5 minutes.

The cells were then counted on a hemocytometer slide. Dead cells take up the stain while the live ones remain unstained

8.2.d. Flow cytometry Annexin V – FITC Staining[165,166]

1. $2x10^6$ Cells were seeded in 35 mm plates and treated with each compound at several doses for 12hours.

2. The treated cells were harvested after 12h of drug exposure by centrifugation at 1200 rpm for 8minutes.

3. The cells were washed in 1ml of Hepes buffer [10 mM Hepes, 140 mM NaCl, 2.5.mM $CaCl_2$ pH 7.4 (adjusted by NaOH)].

4. The cell pellet was resuspended in 1ml Hepes buffer and Annexin V-FITC was added to a final concentration of 2.5µg /ml.

5. The cells were incubated in the dark at room temperature for 30 minutes.

6. Propidium iodide was added to a final concentration of 1µg /ml, to gate out dead cells.

8.2.e. Flow cytometry procedure[167,168]

1. $2x10^5$ Cells were seeded in 35 mm plates and treated with each compound at several doses for 48 hour.

2. Treated cells were harvested after 48 hours of drug exposure by centrifugation at 1200 rpm for 8 minutes.

3. The cells were then fixed in 1 ml of chilled 80% ethanol for 2hour on ice.

4. The fixed cells were washed once in 70% ethanol and kept at -20 ^{o}C for 16hours. Fixed cells can be kept in this manner upto 1 week.

5. The cells were then centrifuged at 1500 rpm, the fixative decanted and washed once in 1 ml PBS.

6. The cell pellet was resuspended in 800 µl of PBS to which was added 20µl of RNase A stock to a final concentration of 200µg /ml.

7. After incubation for 20 minutes at room temperature PI was added to a final concentration of 20 µg /ml and kept in dark for 30 minutes.

8. The cell suspension was then analyzed by a flow cytometer (Becton Dickinson FACS Calibur) using a Nitrogen Argon laser operating at 480/500 excitation emission and the fluorescence being captured on FL2H channel with logarithmic amplification.

9. Forward scatter (FSC) was plotted against side scatter (SSC) in a dot plot.

8.2.f. Statistical analysis

All in vitro experiments were done in triplicate, and the experiments were repeated at least thrice. The statistical software SPSS version 17.0 was used for analysis. P values were determined using the t test; P value <0.001 was considered significant.

8.3. Results

8.3.a. MTT Assay (Isoquercetin) using HT 29 and HepG2 cancer cell lines

Fig 1 represents *invitro* cytotoxicity effect of Isoquercetin using HT29 and Hep G2 cellline using MTT assay.The results suggest that Dose dependent cytotoxic effect of Isoquercetin (6.25-100 µg/ml)against HT29 and HepG2 cell lines. The

cytotoxic effect was superimposed in 2 cell lines. The IC_{50} value of Isoquercetin against HT29 was found to be 20.48 μg/mland HepG2 was found to be 19.71 μg/ml.

8.3.b. Tryphan blue assay (Isoquercetin)

Fig 2 represents the cell viability assay of HT29 , HepG2 cell lines. In cell viability assay the effect of Isoquercetin against HepG2 was more Significant in terms of decrease in cell viability with increase in isoquercetin concentration (6.25-100 μg/ml). However the effect of Isoquercetin against Ht29 was found to be lesser than HepG2 cell lines The IC_{50} value of Isoquercetin against HT29 was found to be 23 μg/mland HepG2 was found to be 46.61μg/ml respectively.

8.3.c. Flowcytometry Analysis

Figure 3-5. Effect of Isoquercetin on cell cycle progression in HepG2 (Liver) cancer cells (DNA histogram)

HepG2 (Liver) cancer cells were treated with treated with vehicle (DMSO) or Isoquercetin (50μg) for 24 & 48 hours. Both floating and attached cells were collected, stained with propidium iodide and analyzed for cell cycle distribution by flow cytometry. Characteristic appearance of sub-G1 phase indicates apoptosis of cells.

8.3.d. Cell cycle analysis by flow cytometry (HepG2)

In the current investigation the effect of Isoquercetin on cell cycle progression of HepG2 cells was determined. HepG2 cells treated with Isoquercetin (50μg/ml) of for 24 & 48 hours showed a dose dependent increase in G2/M phase of cells (Figures 4 and 5). At 24h and 48h of Isoquercetin exposure the cells accumulated in G2/M phase of cell cycle whereas, the control cells (Figure 3) showed small percent of cells in G2/M phase. These results indicate that Isoquercetin at the tested concentration induces G2/M phase cell cycle arrest in HepG2 cells and characteristic appearance of sub-G1 phase indicates apoptosis of cells.

8.3.e. Figure 6-8. Effect of Isoquercetin on cell cycle progression in HT29 (Liver) cancer cells (DNA histogram)

HT29 (Colon) cancer cells were treated with treated with vehicle (DMSO) or Isoquercetin (50µg) for 24 & 48 hours. Both floating and attached cells were collected, stained with propidium iodide and analyzed for cell cycle distribution by flow cytometry. Characteristic appearance of sub-G1 phase indicates apoptosis of cells.

8.3.f. Cell cycle analysis by flow cytometry

In the current investigation the effect of Isoquercetin on cell cycle progression of HT-29 cells was determined. HT-29 cells treated with Isoquercetin (50µg/ml) of for 24 & 48 hours showed a dose dependent increase in G1/S phase of cells (Figures 7 and 8). At 24h and 48h of Isoquercetin exposure the cells accumulated in G1/S phase of cell cycle whereas, the control cells (Figure 6) showed small percent of cells in G1/S phase. These results indicate that Isoquercetin at the tested concentration induces G1/S phase cell cycle arrest in HT-29 cells and characteristic appearance of sub-G1 phase indicates apoptosis of cells.

8.4. Discussion

Cell-cycle control has been proven to be a major event in ensuring the accurate cell division. Flow cytometry has become a method of choice for the analysis of apoptosis in variety of cell systems. During apoptosis DNA becomes fragmented by endonucleases and these small DNA fragments can leak out from the cells resulting in reduced DNA content. Further, alcohol fixation does not fully preserve the degraded DNA within apoptotic cells as a result sub-G1 fluorescence peak arises from the apoptotic cells[167]. In the present study treatment with Isoquercetin resulted in significant accumulation of HepG2 cells in G2/M phase, accompanied by a decrease of S-phase cells and a moderate decrease of G-1 phase cells. The appearance of a ‘sub-G1’ population further strengthens the incidence of apoptosis among the treated cells.

8.5. Figures

Fig:1 MTT Assay (Isoquercetin) using HT 29 and HepG2 cancer cell lines

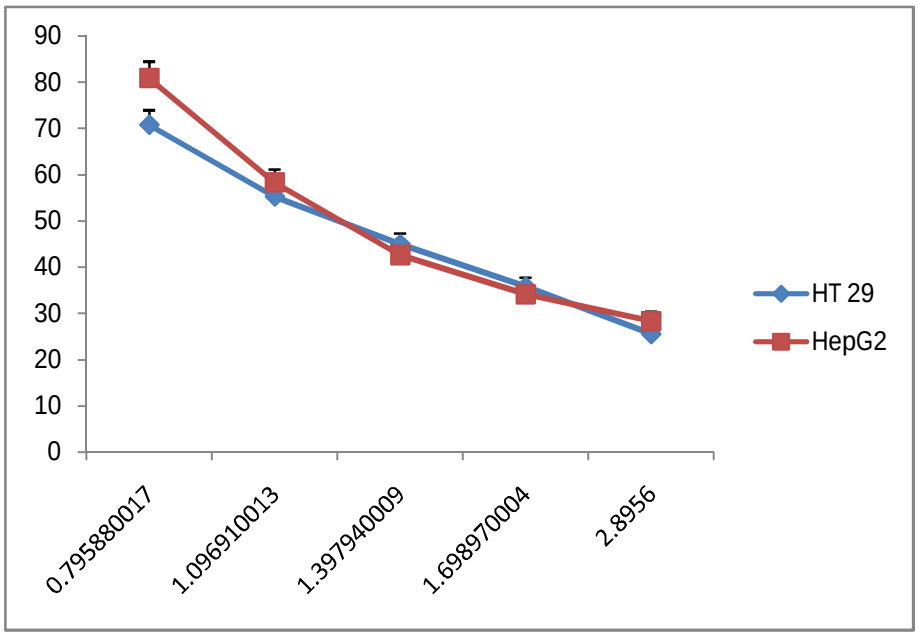
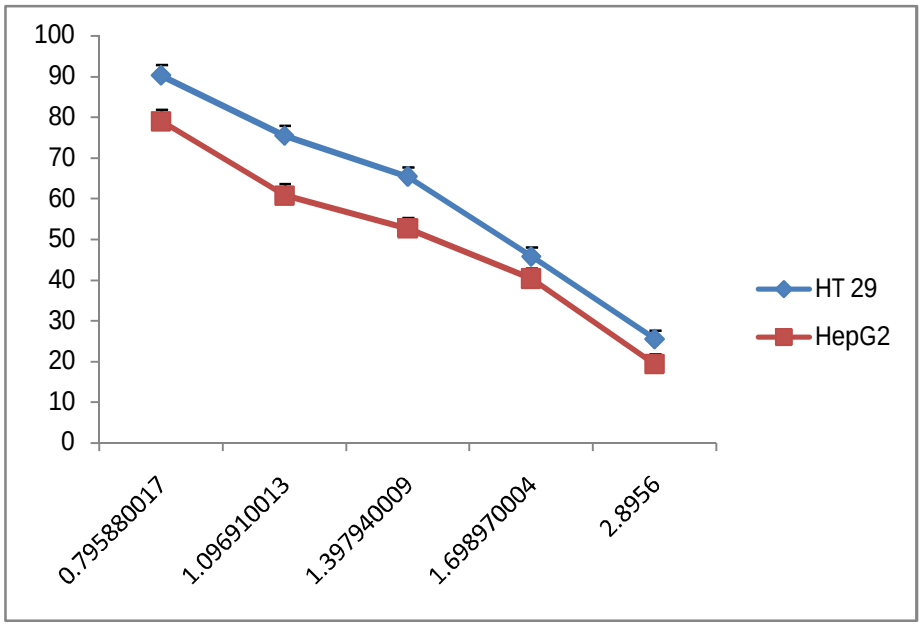
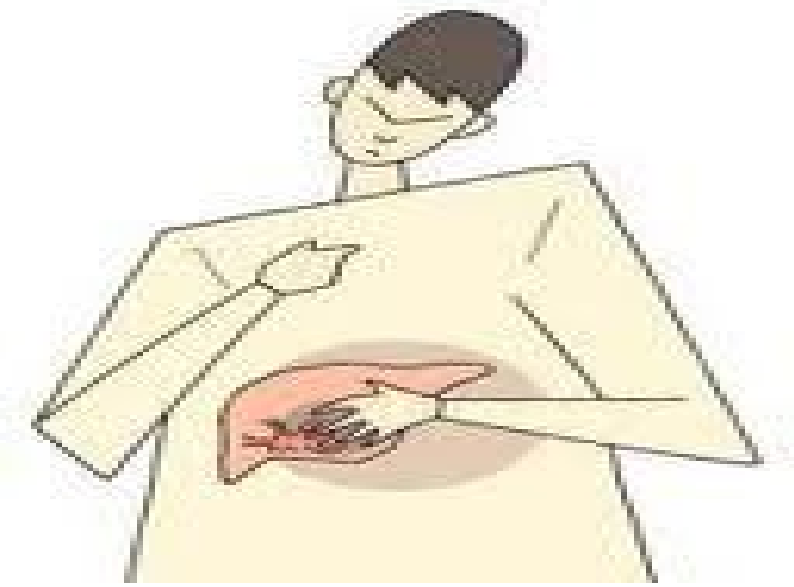


Fig 2: Tryphan blue assay (Isoquercetin)





CHAPTER:9

Effect of Isoquercetin in DEN Induced Hepatocellular Carcinoma in Rats

9.1. Introduction

Chronic liver damage is a worldwide common pathology which characterized by inflammation and fibrosis which further leads to chronic hepatitis, cirrhosis and cancer[169,170]. Hepatocellular carcinoma (HCC) is the most frequest cause of death in Indians with the age group of 35-69. The incidence rate is 2–3 times higher in developing countries like India than in developed countries.

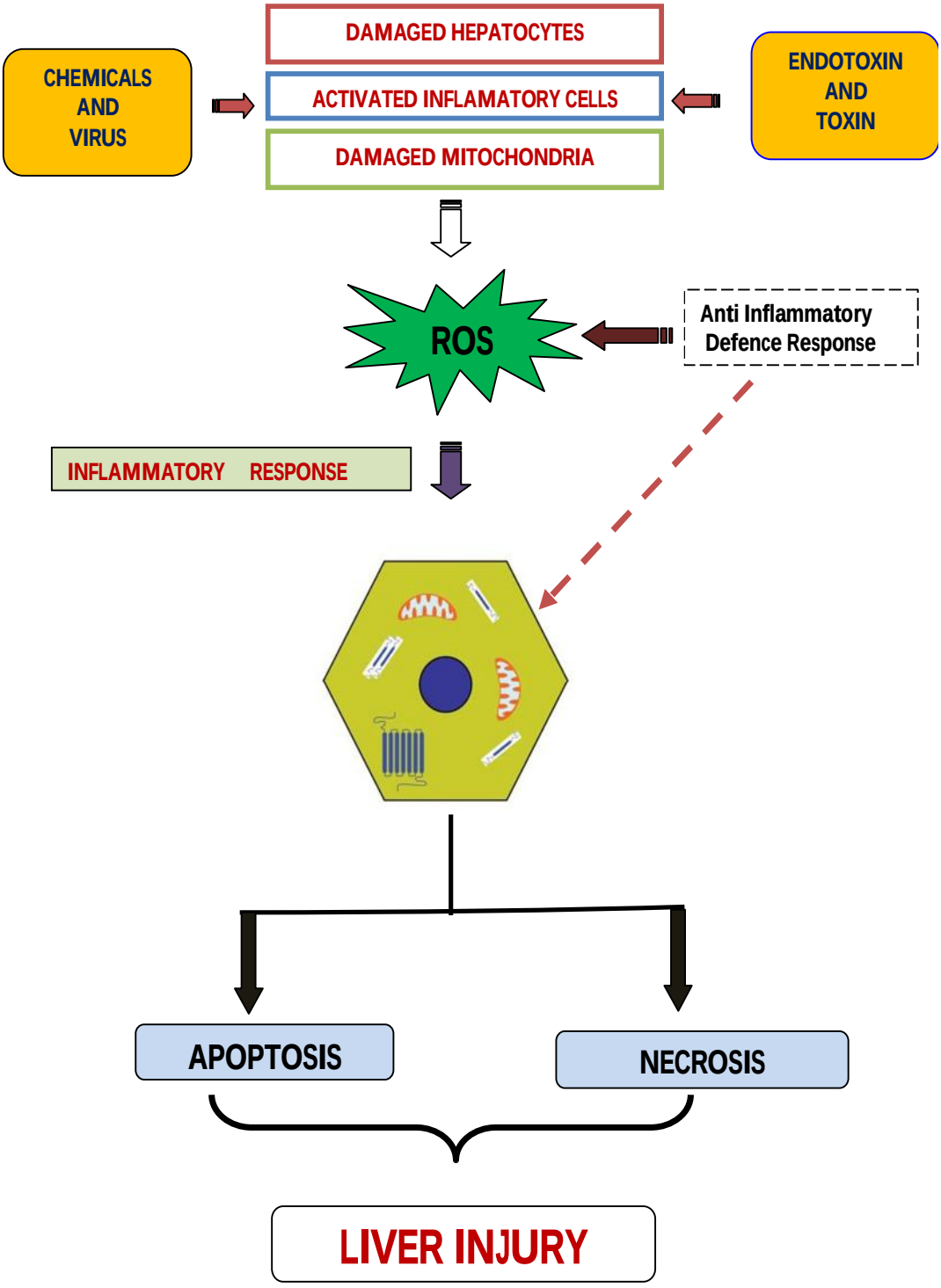
It has been well established that oxidative stress plays a causative role at the initiation, promotion and progression of hepatic diseases[171] and that the liver is the main target for several toxic agents that can provoke free radical-mediated apoptosis[172]. The knowledge about the molecular events that sustain liver damage and disease progression is continuously improving. There is increasing evidence that an alteration of the cellular redox state with production of reactive oxygen species (ROS) plays a crucial role in the various steps that initiate and regulate the progression of liver diseases independently from the type of etiologic agents. ROS are involved in the liver damages induced by alcohol, virus, alteration of lipid and carbohydrate metabolism and xenobiotics[173].

9.1.a. Hepatocarcinoma Role of antioxidant in liver cancer

The major sources of ROS in the liver are the activated inflammatory cells (neutrophils, Kupffer cells, macrophages), the mitochondria enzymes and particularly cytochrome P450 of damaged hepatocytes. The extent of ROS effects depends on a number of individual characteristics such as age, obesity, use of ethanol, blood iron concentration, as well as by available intracellular and plasma antioxidant defences[174].

The imbalance of oxidative stress affects the transcription of numerous biochemical mediators (mainly cytokines) able to modulate the tissue and cellular events that characterize different types of liver diseases such as apoptosis, necrosis, fibrosis, cholestasis and regeneration.6 In particular, pro-inflammatory cytokines as Tumor Necrosis Factor-α (TNF-α) and fibrogenic cytokines as Transforming Growth Factor (TGF-β) have been emerged as key mediators in the chronic liver

damage. In fact, the type of cytokine-mediated event addresses the occurrence and the entity of different liver damages[175].



9.1.b. Role of oxidative stress in liver carcinoma

At the molecular level, a series of studies have shown that oxidative stress is commonly induced in all forms of chronic liver injury and plays a crucial role in hepatic fibrogenesis[176] and cancer development[177]. Exogenous reactive oxygen species (ROS) released by damaged parenchymal cells directly contribute to cell degeneration process and would also activate redox-sensitive intracellular pathways in HSCs, inducing their activation and increasing collagen synthesis[178]. Furthermore, HSCs are also an important source of ROS in liver fibrosis[179]. Cytochrome P450 2E1 is the main source of ROS in hepatocytes, while phagocytic and non-phagocytic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the key source, respectively, in Kupffer cells and HSCs[180]. The phagocytic form of NADPH oxidase expressed in Kupffer cells has several important functions: besides its defensive effect against bacterial products reaching the liver through the portal system, NADPH oxidase in Kupffer cells is also activated by several stimuli (i.e. alcohol metabolites and tumor necrosis factor-α) to produce ROS. Kupffer cells derived ROS consequently drive proinflammatory effects and sensitize hepatocytes to undergo apoptosis, being involved in fibrogenesis and carcinogenesis. Conversely, recent data indicate that HSCs express the non-phagocytic form of NADPH oxidase and demonstrate that ROS participate in the activation and fibrogenic actions of HSCs *in vitro*[181]. Thus in summary, several sources of ROS in parenchymal and non-parenchymal cells actively contribute to the development and activation of pathways involved either in fibrogenic or in cancer processes.

9.1.c. Role of dietary anti oxidant against liver cancer

These compounds are considered to be effective health hazards to man. One approach to control liver cancer is chemoprevention–when disease is prevented, slowed or reversed substantially by the administration of one or more non-toxic naturally occurring or synthetic agents. In this regard, recently naturally occurring polyphenols are receiving increased attention because of their promising efficacy in several cancer models[182]. Flavonoids are naturally occurring compounds present in many plant foods such as carrot, tomato, strawberry and blueberry[183]. It has been

documented that natural compounds possess anti-oxidative activities such as scavenging free radicals and chelating metal ions[184]. A marker is synthesized by the tumor and released into the circulation, but it may be produced by normal tissues in response to invasion by cancer cells. A variety of substances, including enzymes, hormones, and proteins can be considered as tumor markers. Analysis of tumor markers can be used as an indicator of tumor response to therapy. Sensitive and specific liver cancer marker enzymes are used as indicators of liver injury. Analysis of these marker enzymes reflects mechanisms of cellular damageand subsequent release of proteins and extracellular turnover[185]. Lipid peroxidation generates a complex variety of products, many of which are reactive electrophiles; some of these react withprotein and DNA and as a result are toxic and mutagenic[186]. Taking the above into account, our present study was carried out to assess the efficacy of EA on DEN-induced HCC in experimental rats.

9.1.d. Mechanism of DEN Induced Hepatocarcinoma

Diethylnitrosamine (DEN), one of the most important environmental carcinogens, which is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication. DEN is stated as chemical carcinogen which cause causes acute liver injury and inhibits hepatic protein synthesis shortly after administration[187]. It has been suggested that the biological action of DEN requires metabolic conversion into a toxic intermediate ,and there is evidence that nucleic acids and proteins become methylated in the liver and some other organs of rats treated with dimethylnitrosamine[188].Further DEN shows the inhibition of hepatic protein synthesis in *in vivo* is accompanied by breakdown of the liver ribosomal aggregates and has suggested that methylation of messenger RNA may occur and be related to the inhibition of protein synthesis.

9.2. Materials and Methods

9.2.a. Chemicals

N-nitrosodiethylamine (DEN) was purchased from Sigma Chemical (Banglore), anti oxidant chemicals, LFT kits were purchesed from Sisco chemicals,

Mumbai, Bcl-2, Caspase - 3 and β-Actin were purchase from Sigma Chemicals Pvt ltd, Mumbai. Anti-rabbit IgGs were purchased from Cell Signaling, Inc. All other chemicals used were of the highest pure grade .

9.2.b. Experimental design for Hepatocarcinogenesis using diethyl nitrosamine (DEN)

The experimental hepatocarcinogenesis was induced by using DEN (Sigma, USA). DEN is the most important environmental carcinogen among nitrosamines and primarily induces tumors of liver[189]. The presence of nitrosamines and their precursors in human environment, together with the possibility of their endogenous formation in human body from ingested secondary amines and nitrites, have led to the suggestions of their potential involvement in HCC[190]. It is now widely used as a standard experimental model for HCC.

9.2.c. Experimental Design

Forty five male Sprague Dawley rats were obtained from animal House from Vels College of Pharmacy. Mice were grouped and housed in poly acrylic cages (n=6) and maintained in standard laboratory conditions under the temperature 25 ± 2^0 C dark/light cycle. They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were acclimatized into a laboratory conditions for 7 days before the experiment. All procedures described were reviewed and approved by the institutional Animal Ethical Committee. Animals were analysed for total experimental period of 6 weeks.

Group 1: normal control rats treated with pure drinking water

Group 2: rats were orally give DEN (200 mg/kg) at 14^{th} and 28^{th} day

Group 3: rats treated with Isoquercetin 5mg/kg/ bd wt

Group 4: rats treated with Isoquercetin 10 mg/kg/ bd wt

At the end of the 6 weeks study, Experimental Animals were scarified. The liver tissue were collected and homogenised and sample were stored at -80^{0}Cfor further assays

9.2.d. Biochemical studies

The protein content was estimated by the method of Lowry [130,] using bovine serum albumin (BSA) as standard. The macromolecular damagesuch as LPO was estimated by the method of Ohkawa[125,] .The activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase(LDH) were estimated by the method of King[191.192,193] and the activities of the antioxidant enzymes like super oxide dismutase[194] (SOD) catalase[194] (CAT) glutathione peroxidase[195] (GPx) and glutathione transferase[196] (GST) were estimated in the liver.

9.2.e. Histopathalogical studies

A portion of the liver was cut into two to three pieces ofapproximately 6 mm^3 sizes and fixed in phosphate buffered 10%formaldehyde solution. After embedding in paraffin wax, thin sectionsof 5 µm thickness of liver tissues were cut and stainedwith haematoxylin–eosin. The thin sections of liver were madeinto permanent slides and examined[197] under high-resolution microscope with photographic facility and photomicrographs were taken.

9.2.f. Preparation of cell lysates for Western blotting

Samples of frozen liver were homogenized 1:10 (w:v) in extraction buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 10 mM Na4P2O7, 10 mM NaF, 2 mM EDTA, 1% Nonidet P-40, 2 mM Na3VO4, 5 lg/mL leupeptin, 20 µg/mL aprotinin, 2 mM benzamidin and 2 mM phenylmethylsulphonyl fluoride (PMSF)] to detect Bcl2 and Caspase -3, (Gavete et al., 2005). Homogenates were centrifuged at 14,000g for 60 min and the supernatants were collected, assayed for protein concentration by using the Bradford reagent, aliquoted and stored at -80^0 C until use for Western blot analyses[198].

9.2.g. Western blot analyses[199]

Samples (80 µg each) were separated by denaturing SDS–PAGE and transferred to a PVDF membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA) by electrophoretic transfer (Bio-Rad Laboratories, Inc., USA). The membrane was preblocked with 5% non-fat milk and 0.1% Tween-20 in Tris-buffered saline (TBST), incubated overnight with the primary antibody (in TBST with 5% non-fat dried milk). Each membrane was washed three times for 15 min and incubated with the secondary horseradish peroxidase-linked antibodies (Santa Cruz Biotechnology,CA and Cell Signaling Technology, Beverly, MA, respectively). Quantitation of detected bands was performed with the Scion Image analysis software (Scion Corp., Frederick, MD, USA). To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Chemicon International Inc., Temecula, CA). Each density was normalized using each corresponding β-actin density as an internal control and averaged from three samples, and we standardized the density of vehicle control for relative comparison as 1.0 to compare other groups

9.3. Results

9.3.a. Effect of ISQN on body weight change in DEN induced Hepatacarcinoma in rats

Figure 1 represents the effect of isoquercetin (ISQN) on body weight of the DEN treated rats. The result suggests that DEN injection on 12th day significantly $P<0.001$ decrease the body weight measured on 42nd day than compared with saline treated control rats where gradual increase in body weight was noted. Administration of ISQN 10 mg/kg but not 5mg/kg had significant effect $P<0.001$ in increasing b.wt of the DEN treated rats than DEN treated group alone. In parallel with the body weight change, results from the weight gain revels that there was similar over lapping in weight gain in DEN and DEN + ISOQN treatment.

9.3.b. Effect of ISQN on liver weight in DEN induced Hepatocarcinoma in rats

Figure 2 represents the liver weight and liver to body weight ratio of the DEN and DEN+ISOQ treated rats. DEN administration significantly $p<0.001$ decreased the liver wt compared with normal control rats. Administration of ISQN six week to DEN treated rats showed significant $P<0.001$ elevated liver weight than DEN treated cancer control. Increase in liver wt was observed in ISOQ 5 mg/kg however it was not statistically significant.

9.3.c. Effect of ISQN on liver function test in rats treated with DEN

Figure 3 represents the serum ALT level of the rats treated with DEN and DEN + isoquercetin. Administration of DEN had significantly $P<0.01$ increased the ALT indicating impaired liver function. The reversal of ALT was significant $P<0.05$ with ISQN at the dose of 10 mg/kg b.wt treated DEN treated rats than compared with saline treated DEN. The effect of ISQN on the serum AST level of rats treated with DEN induced hepatic carcinoma (fig 3). Administration of DEN 100mg/kg body weight significantly ($P<0.05$) decreases the serum AST levels as compared to that of saline treated control animal group. Interestingly ISQN significantly ($p<0.05$) elevated decreased AST level induced by DEN. The effect was dose dependent. The effect of ISQN on serum alkaline phosphatase level in rats with DEN induced hepato carcinoma. It is observed from the figure 3 that there is a significant increase ($p<0.001$)in ALP with normal saline control animal group . This effect was further attenuated by ISQN at the dose of 5&10 mg/kg .The effect was dose dependent and significant ($p<0.05$) as compared with DEN treated animal group.

9.3.d. Effect of ISQN on liver SOD, CAT and TBARS

It is observed from figure 4 that ingestion with DEN had significantly $p<0.01$ decrease the SOD and CAT level in liver homogenate than saline treated normal control rat liver homogenate. Interestingly antioxidant effect of ISQN at the dose of 10 mg/kg was observed and it was significant $P<0.05$ by increasing SOD and CAT level towards normal than compared with DEN control group. DEN ingestion

leads to oxidative stress in liver as observed by significant P<0.001 increase in LPO in DEN treated control. Decrease in LPO product was observed by administration of ISQN and the decreasing effect was found to be statistically significant P<0.01.

9.3.e. Effect of ISQN on liver GPX, GR and GST

It is observed from figure 5 that ingestion with DEN had significantly p<0.01 depletes the GPX, GR and GST level from the normal control value rat liver homogenate. Interestingly, increase in Glutathione family antioxidant effect of ISQN was observed and it was significant P<0.05 by increasing GPX, GST and GSH level towards normal than compared with DEN control group. However there was increase in glutathione related enzyme was noted with ISOQ 5 mg/kg but it was not statistically significant.

9.3.f. Effect of ISQN on liver apoptotic and anti-apoptotic markers

Lane A: Normal Control. Lane B: DEN Control

Lane C: ISOQ 5 mg/kg Lane D: ISOQ 10 mg/kg

Fig 8 shows the representative caspase-3 and Bcl-2 protein expression in liver mitochondrial homogenates β-Actin was blotted as a standard and indicated no changes during the time course of the experiment.

9.3.g. Histopathology

The histopathology revels that DEN treated rats showing increase in neoplastic cells, granular cytoplasm and irregular architecture(Fig B).This deformities was less evident ie, isoquercetin treatment has shown improved architecture with few neoplastically transformed cells(Fig C). There is no such deformities was observed in saline treated normal liver cells represents in Fig A.

9.4. Discussion

The present work highlights hepatoprotective effect isoquercetin isolated from ethyl acetate fraction of *A. spicifera* against diethyl nitrosamine (DEN) induced hepatocarcinoma in rats.

DEN is a chemical carcinogen,and infusion of DEN in rodents causes inflammation, liver cirrhosis and hepato carcinogenesis and progression. It is accepted as a model to study the relations among liver necrosis, cancer initiation and replication[200, 201]. The incidence rate of liver cancer was 100%. Almost all these liver cancers developed on the basis of cirrhosis. This process might imitate the pathogenesis of human liver cancer to some extent. It has been shown that DEN toxicity is also associated to an excessive production of free radicals in the liver. As a consequence, reactive electrophilic intermediates are formed, which overwhelms the antioxidant defences and ultimately proceeds to oxidative stress paving way to liver damage[202].

In our study reduction of body weight gain was observed in DEN treated groups at 42 day and which leads to reduction in body weight gain was noted. This results in parallel with the established reports with DEN inhibitory effect on weight gain after 7^{th} week and continues for 14^{th} week, which could be associated with losses from skeletal muscle and adipose tissue as previously shown[203], and it could beconsidered as an indirect indication of the declining hepatic function following exposure to DEN[204]. Intervention with ISQN 10mg/kg administered daily for 42 days has significant effect in increasing liver wt. in DEN treated animals but nor with low dose of ISOQN 5 mg/kg. However liver to body weight ratio is similar in DEN as well as ISQN + DEN treated animal groups.

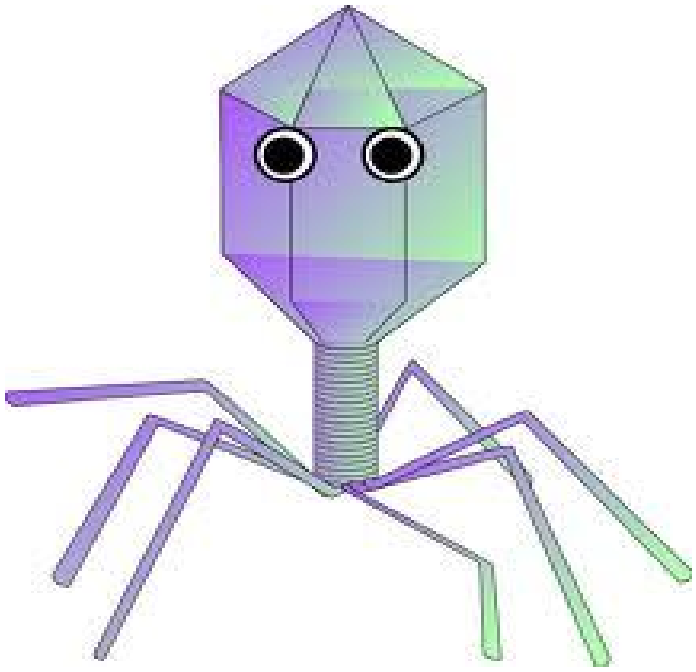
Liver damage caused by DEN generally reflects instability of liver cell metabolism which leads to distinctive changes in the serum enzyme activities. Serum transaminases and ALP, are representative of liver function; their increased levels are indicators of liver damage. The elevation of ALT activity is repeatedly credited to hepatocellular damage and is usually accompanied by a rise in AST[205]. It is apparent from this study is that ISQN at 10 mg/kg significantly revert the elevated ALT and AST level of DEN treated animals suggesting its direct enzymatic role in hepatocytes. However the low dose of ISOQN did not influence or affect the altered liver funcation enzymes induced by DEN.

GSH is an important non-enzymatic antioxidant defence requiredto maintain the normal redox state of cells and to counteractdeleterious effects of oxidative stress. GSH depletion ultimatelypromotes oxidative stress, with a cascade of effects therebyaffecting functional and structural integrity of cell and organelle membran[206]. Our results showed that thesignificant decrease in GSH levels in rat liver homogenates exposed to DEN wasrestored by ISOQN which suggest antioxidant efficacy of ISOQN to counteract the oxidative damage inducedby DEN. Emerging scientific evidence conducted during last decade reported for the hepatoprotecitve potential of natural flavonoids, flavones and isoflavones against chemical and experimentally induced oxidative stress[204].

Catalase converts H_2O_2 to H_2O and GPx catalyses the transformationof H_2O_2 to harmless byproducts. During H_2O_2 scavenging, GSHis oxidized to GSSG by GPx. The reduction of GSSG to GSH is catalyzed by GR using NADPH as reducing potential. CAT, GPx and GR activitieswere significantly get reduced inDEN treated control animals indicates the severith of oxidative stress induced during the exposure to DEN. In contrary, ISQN fed DEN treated animal group have shown increase in all above said endogenous antioxidant enzymes or ISQN counteracted the hepatic oxidative damage by preventing thereduction of these parameters provoked by DEN.

In line with CAT, Gpx and GSH we found increase in GST was observed in ISOQN treatment which catalyses the reaction of endogenous GSH with numerous electrophiles to yield less toxic conjugates that are easily eliminated[206]. Although increased and decreased liver andserum GST activities have been reported after DEN induced damage[207]. In this regard, it has been demonstratedthat different natural compounds and poly phenols, as well a cocoa bean product, induce GST as one of the principal anti-carcinogenic mechanisms[208,206]. Moreover, some studies point to the inductionof GST as a mechanism to protect against chemically induced cancer and oxidative stress by increasing the metabolism of electrophilic intermediates and ROS[206].

Activation of caspase 3, the most important enzyme responsiblefor apoptosis, has been considered as a sensitive method of detecting liver damage and has been associated with progressive liver fibrosis[209]. Moreover, high rates of apoptosishave been demonstrated in liver cancer[210,211]. This finding could be a consequence of a reduction in the number of viable hepatocytes due to enhanced cell death in liver, as these animals also showed the highest levels of caspase 3. Thus, the decreased caspase-3 activity in animals not injected with DEN and fed with ISOQN suggested a potential protective effect of ISOQN from *A. Spicifera* against cell death under oxidative conditions.



CHAPTER-10
Antiviral Effect of Isoquercetin Against Chickungunya Virus

10.1. Introduction

The prevalence and occurrence of viral infections were increasing day by day in developing countries like india. Among this recent epidemics have revealed that Chikungunya virus (CHIKV) is a one among them as a dangerous, important, emerging arbovirus. The virus is belonging to the family *Togaviridae*, genus *Alphavirus*. It is transmitted by haematophagous arthropod vectors, particularly by *Aedes aegypti or Aedes albopictus* mosquitoes which were discovered in mid 1950s from the serum of a febrile patient during a dengue fever-like epidemic in Tanzania[212]. Indeed, Chikungunya virus causes an acute fever–arthralgia syndrome which leads into chronic arthritis.

Most infected individuals show symptoms; only about 5% of cases of asymptomatic CHIKV infection have been reported[213]. The global spread of CHIKV infection in recent years have provided opportunities for greater knowledge of its clinical features[214]. CHIKV infection outbreaks were reported in Asia since 1958, when the virus was first isolated in Bangkok. Chikungunya virus infection is documented in many parts of Southern and Southeast Asia. In India, its reported first in 1963, in Calcutta, and subsequently in Madras, affecting overall more than 3 million people [215,216].

Complications of Chikungunya virus (CHIKV) infection are Vasculitis, Fulminant hepatitis, neurological complications, encephalitis (CHIKV-infected neonates), facial paralysis, Heart failure, myocarditis, Pneumonia, Mild hemorrhage, Pre-renal failure and finally leads to Death. Mode of transmission of CHIKV has two distinct transmission cycles ie. sylvatic and human–mosquito–human. Sylvatic transmission involves wild primates, such as monkeys and forest-dwelling Aedes species of mosquitoes, primarily *Aedes furcifer*, *Aedes taylori*, *Aedes luteocephalus*, *Aedes africanus* and *Aedes Neoafricanus*[217]. The human–mosquito–human transmission cycle maintains CHIKV infection in urban areas.

It involves primarily a vector, the *Aedes aegypti* mosquito, which is an extremely efficient urban vector because it preferentially feeds on humans and often bites several people during a single blood meal and is adapted to live and breed per domestically; the mosquito is therefore the most likely initiator of the large regional and global outbreaks of the disease[218].

10.1.a. Risk factors for epidemics

Massive urbanisation has facilitated the spread of contagious diseases in human populations as has increased fast travel over distances in worldwide[219.]. Furthermore, the risk of emerging or re-emerging CHIKV disease epidemics is associated with the worldwide distribution of well-adapted vectors and the pronounced viraemia in acutely infected humans[220]. The high titre viraemia in humans is sufficient to infect mosquitoes, which permits an urban transmission cycle between humans and mosquitoes. Climate change is also considered a major risk factor for spreading of CHIKV. Studies have shown that an increase of 1–2^{o} C in temperature results in augmented virus replication.[221]

However, breakthoughts for the treatment for this particular virus was the challenging task in rural and urban areas of India due to the dense population. Further the drugs which can be stated as antiviral against particular virus get resistance due to the use and misuse of antibiotics. However, the problem has escalated as the prevalence of antibiotic-resistant for the particular strains have emerged against many species that cause disease in humans. So there will be in search of newer drugs from the alternative source for the treatment of these dreadful viruses has been initiated. As a consequence of an increasing demand for biodiversity in the screening program seeking therapeutic drugs from natural product there is greater interest particularly in the oceans throughout the world[222]

Mankind has known for the several thousand of years that marine organisms containing substances capable of potent biological activity. However, the first investigation of marine organisms has started only half a century ago. Since then, all forms of life in the marine environment (e.g. bacteria, algae, sponges, fungi,

corals, ascidians, etc.) have been investigated for their active content and their biological activity. Currently, important pharmacological and therapeutic products are being obtained from the ocean[223,224]. The compounds were listed in the table 1

Among this, micro and macroalgae were one of the first marine resources of natural compounds showing *in vitro* anti-HIV activity[225]. Marine antiviral agents (MAVAs) represent a significantly unique natural marine resource whose multipotential uses include the following applications: (1) The biological control of human enteropathogenic virus contamination and disease transmission in sewage-polluted waters. This application would be particularly important to communities that utilize their coastal waters for recreational activities and for food industries (e.g. fish, shellfish), as well as to those regions of the country, such as Hawaii, where the loss of these marine resources would have a devastating effect on the lifestyle and economy of the people. (2) The chemotherapy of viral diseases of humans and lower animals. To be of practical use, it is imperative that MAVAs be isolated in pure culture, identified, characterized, and their spectrum and mechanisms of antiviral activity be clearly established. Also the active principle(s) and moieties should be identified and chemically characterized in order to facilitate application of biotechnological methods for increased yields and cost effective production. (3) The biological control of viral diseases of marine animals. Under natural conditions, there are only a few practical ways to prevent viral transmission to marine mammals. This is especially troublesome when marine mammals are kept in captivity for various uses. The seeding of MAVAs to these special environments could control viral disease transmission.

In account flavonoids are a large group of naturally occurring phenylchromones found in marine algae. They have been shown to have a wide range of biological activities, including antiallergic, antibacterial, antidiabetic, antiinflammatory, antiviral, anti- proliferative, antimutagenic, antithrombotic, anticarcinogenic, hepatoprotective, oestrogenic, insecticidal, and antioxidant activities. In this account, flavonoid namely isoquercetin isolated from ethylacetate fraction of *Acanthophora spicifera* has screened for the antiviral activity against chikungunya virus

10.2. Materials and Methods

BHK21 cell were maintained and propagated using Dulbecos Modified Eagle's Medium (DMEM) (Highmedia, Bombay, Cat No. AT007). Penicillin and Streptomycin (Sigma chemicals Pvt Ltd). All glasswares are of Borosil and Schott Duran type, For preparation of reagents, buffer and media Milli Q and double glass distilled water were used. All Chemicals were of Anala R or Excela R grade (Sigma Aldrich, Qualigens, SRL chemicals, SD fine chemicals and HIGHMEDIA).

10.2.a. Preparation of Basal Medium for BHK-21 cell line maintenance

The basal medium (1X) was prepared by dissolving 13.54g powder in 950ml of sterile MilliQ water. The pH of the medium was adjusted to 7.2 by adding sterile 8% sodium carbonate and volume made up to 1 liter with sterile MilliQ water. Thus prepared medium was sterilized by filtering through 0.22µm membrane filter. Benzyl pencillin and Streptomycin were added to the medium at a concentration of 100 IU/ml and 100 µg/ml respectively. The medium was then checked for sterility and stored at 4^0C until used.

Fetal calf serum:

Fetal calf (High media, Bombay) was used for preparation, maintenance and growth.

Growth Medium:

Prepared by adding 10% fetal calf serum to the basal medium.

Maintenance medium:

Prepared by supplementation with 2% fetal calf serum.

10.2.b. Chikungunya virus and titration

Chikungunya virus (CHIKV) *FJ225403 with the strain of CHIK-AP-KDP were grown as monolayer in BHK 21(Host cell lines) which is supplemented with (DMEM) containing 10% fetal calf serum. BHK 21 clone 13 cell lines at 46th

passage level was Procured from Department of Virology, SV Univeristy, Tirupathy. The cell were at 37^0C in a humidified chamber with 5% CO_2 and were subculture twice a week

Virus titration was performed by the limit dilution method,using a 96-well microtitre plate with 6 wells per dilution. The virus titer was estimated from cytopathogenicity of cells induced by viral infection and expressed as 50% tissue culture infectious doses/mL ($TCID_{50}$ /mL) [226].

10.2.c. Cytotoxicity assay

For growth inhibition studies, 2×10^4 cells in 0.1mL DMEM plus 10% fetal calf serum (FCS) were seeded into each well of 96-well microtitre plate, cultured for 24 h at 37^0 C, and allowed to grow for additional 72 h in the presence of serial two-fold dilutions of the sample. For the cell control, 0.1mL maintenance medium without the sample was added. The morphology of cells was inspected daily and observed for microscopically detectable alterations, e.g. loss of monolayer, rounding, shrinking of the cells, granulation, and vacuolization in the cytoplasm. The cytopathic effect (CPE) was scored under the light microscope. The maximal non-cytotoxic concentration (MNCC) was determined as the maximal concentration of the sample that did not exert toxic effect as detected by microscopic monitoring after 72 h of incubation [227].

10.2.d. Anti-viral assay

The antiviral activity of Isoquercetin (50 µg/ml) against Chikungunya virus (CHIKV-*FJ225403) was measured by the cytopathic effect reduction assay. BHK21 cell cultures were prepared in 96-well microtitre plate (Falcon Plastics, Oxnard, CA, USA). After 24 h of incubation at 37^0C in a 5% CO_2 incubator, the culture medium was removed from the monolayer cells. To confluent monolayers of BHK21 cells in 96-well microtire plate, 0.1mL of CHIKV21 suspension containing 100 times of $TCID_{50}$ and 0.1mL of maintenance medium containing an appropriate serially diluted concentrations of the test sample were added pre,post and concominent

administration. The plates were incubated at 37^0C with 5% CO_2 usually for 4–5 days until the virus in the control wells showed complete virus-induced CPE as observed under light microscope[228]

10.3. Results

The cytotoxic effect of Isoquercetin isolated from ethylacetate fraction from *Acanthophora spicifera* was studied against CHIKV human viral cell. The viral cells were seeded on the BHK21 cell lines and the infection was observed from 0 hrs to 96 hrs (Fig 2a-2d). The cytopathic effect was determined in order to asses the cytotoxicity of the isoquercetin. Treatment with isoquercetin has exhibited cytotoxic effect as observed in predominant occurrences of microscopical cytoplasmic changes with granulation, vacuolization and shell shrinkage. From the Cytopathic effect the cytotoxic concentration of Isoquercetin was was found to be 50 µg/ml.

In the present study, Antiviral effect of isoquercetin was evidenced by its viral inhibition effect detected at the fixed concentration with treatment of 3 different ways Viz., pretreatment before infection, at the time of infection (concominent) and post infection. The significant antiviral inhibition of Isoquercetin was due to complete inhibition on the development of virus and its enhancement of infection. There was remarkable reduction in term of vacuolization, granulation and shrinkage of cytoplasmic region microscopically after Isoquercetin treatment at 3 different levels likewise pre-treatment (fig 3), concominent treatment (Fig 5) and post treatment (fig 4a&4b) where as untreated group completely unveiled no changes with respect to cell cytology.

10.4. Discussion

Polyphenols have been studied for their antioxidant and anti inflammatory effects, and recently it was shown that some of flavonoids exhibit antiviral actions against some viruses[229].Quercetin has been reported to have inhibitory effect on several virus[230] Isoquercetin is a glucose-bound derivative of quercetin, and is also reported to have anti-inflammatory and antioxidant activity[231,232]. However, there

has been no previous report of antiviral activity of isoquercetin against Chikungunya virus virus. To our knowledge, this is the first report that isoquercetin has antiviral effects on chickengunya virus. In the present study we have demonstrated that the Isoquercetin isolated form Ethyl acetate fraction of *Acanthophora spicifera* effectively inhibited infection of BHK21 cell lines by CHIKV in *in vitro.* Chikenguniya is know as virulent life threatening pathogen that cause variety of illness[233]. Among the flavonoids tested the literature suggest that Quercetin Q-7 rhamnoside (Q7R) and Quercetin - 3- rhamnoside has been shown antiviral effect against human herpes virus and adenovirus[234].They found the antiviral activity among the flavonoid glycoside containing the Quercetin moiety might by correlated with the species of sugar group at 3 position. However, the Quercetin with non sugar linkage did not show less viral or viral inhibitory effect. Our results suggest the antiviral activity of Isoquercetin which is devoid of sugar linkage in 3rd or 7th position. We found that isoquercetin may not have significant direct antiviral effect but incubation of cells with Isoquercetin at 3 different phases to viral infection which significantly inhibited viral replication. In this study we have adopted 3 different protocols Viz., Pre, concominent and post treatment with single dose of isoquercetin (50 µg/ml) for 48 hrs. The mechanism of action of virus inhibition is investigated for the subset of the isoquercetin at 3 different phases and it is appeared to inhibit viral replication. The direct inactivation of the isoquercetin inhibits the attachment of virons to the cell surface. This selective virucidal effect against CHIKV was noted with protection of cell monolayer or in other way without damaging the host cell lines through the study time. This strong antiviral potential could be attributed by different mechanisms of actions which may includes inhibition of viral DNA synthesis,[235] Inhibition of early stage infection cycle (virus adsorption and penetration) in the host cell,[236] direct interaction withvirus particle or by blcage of virus axis to host cell. It is possible that these effects could be either the results of strong interaction (may be irreversible) among the virus tested and the compound Isoquercetin. The finding of the present study clearly suggest further leeds in terms of antiviral approach with respect to isoquercetin with systemic scientific documentation on the exact mechanism how the inhibition occours could

be the pave away in the explaration of isoquercetin as a potent bioactive template from an algal source in the prfilaxix, management and treatment of viral infections.

CHAPTER- 11
General Discussion and Conclusion

Increasing incidence of cancer prevalence in India is ever-growing. The management of cancer by surgical intervention and chemotherapy is not always convencing because the vulnerable virulence of the cancer cell transcends into secondary cancer sites. Ever-growing interest in cytotoxic natural compounds has been prior interest among the scientist working in the area of cancer biology. It is evidenced by literature that natural compounds from Indian system of medicine, fruits and vegetables have been shown excellent cytotoxic anticancer property with its unique phytoconstituents. The search of anticancer entities from marine algae from marine resources has another phase of dice to find out the hidden gold under deep sea. There are three important species of algae namely the red, brown and green algae in the ocean ecology. In particular red algae species *acanthophora spicifera* are viewed as special floral for its nutritive and biological properties. However, the exploitation of *acanthophora spicifera* in not expanded up to the mark. This is one of the main reason we have chosen this species which is widely distributed in the Gulf of Mannar, Mandapam, for scientific exploration. The work presented in thesis is pure quantitative data from collection of marine algae to proven anticancer role in human cancer cell line as well as antiviral role against human viral pathogens.

Earlier literature on this species revels phytoconstituents like sterols, acanthophorin A, acanthophorin B and many flavonoids. In addition antioxidant effect of *acanthophora spicifera* have been proven. We have demonstrated for the first time that crude alcoholic extracts of *Acanthophora spicifera* was injected orally shown the anticancer properties by exerting its effect by reducing tumor weight, tumor volume, mean survival day with restoration haematological abnormalities in *in vivo* cancer model. This preliminary work has prompted us to fractionate crude extract and locate active bioactive fraction. In order to achieve above statements we have used bioactive guided fractionation tool using robust *in vitro* assays namely cytotoxic assay and free radical scavenging assay. The ethyl acetate fraction from *Acanthophora spicifera* was identified as bioactive by means of its significant effects in showing selective cytotoxic activity and free radical scavenging activity. It is interesting to observe bidirectional bioactive property of ethyl acetate fraction as

pro-oxidant and antioxidant with qualitative phytochemical like phenolic flavanoids and tannins. Further our interest is isolate bioactive cytotoxic constituents from ethyl acetate fraction of *Acanthophora spicifera* using conventional column chromatography with the mobile phase of chloroform: methanol. We were identified 3 compounds in which 2 compounds are flavonoids and 1 served as tannins.The compounds are namely Quercetin, Isoquercetin and Gallic acid. The quantitative estimation of these compounds is assayed by HPLC and HPTLC. The identified compounds basically possess antioxidant properties. It is known fact that participation of oxidative stress in cancer directly links those antioxidants on cancer prevention. The flow cytometry analysis of isoquercetin HT29 and HepG2 cells at the concentration of 50 μg/ml clearly shown G1/S phase and sub-G1 phase of apoptosis respectively. In order to prove the above experiments *in vivo* set up, we have used DEN induced hepatocellular carcinoma in rats. Similar to *in vitro* the isoquercetin 5 and 10 mg/kg body weight treated DEN rats has exhibited anticancer property by following mechanism.

a) it influence the liver morphology by restoring the abnormal liver function markers induced by DEN.

b) It exerted its antioxidant role by enhancing antioxidant enzymes including non-enzymatic enzymes like glutathione and decrease in oxidative stress markers.

c) In influences the liver apoptosis markers and produces beneficial effects on liver morphology.

The reason for antiviral screening for isoquercetin form *acanthophora spicifera* is because most of the viral pathogens irreversible influence the cancer induction and also it has been very strong evidence of literature suggests the antiviral effect of red algae in *in vitro* set up.

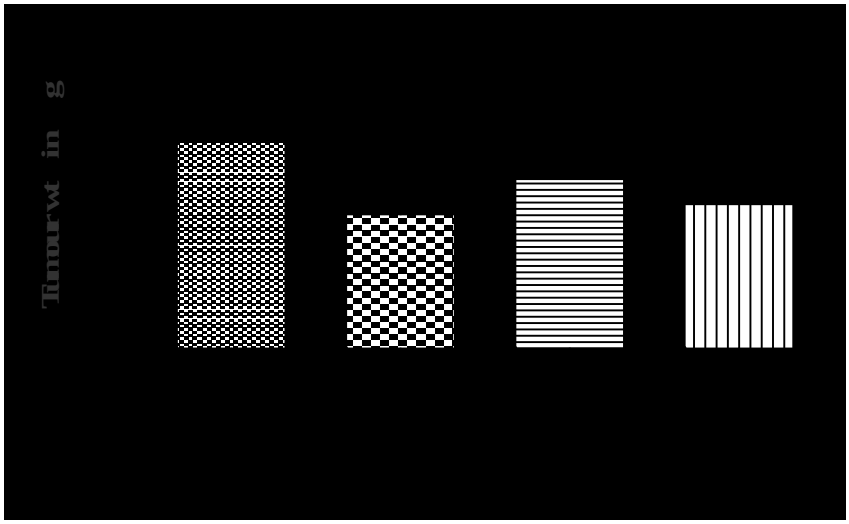
Conclusion

It is concluded that the red algae species *Acanthophora spicifera* has been explored in chemical and biological aspects. The poly-phenolic and tannins present in the *acanthophora spicifera* possesses significant anticancer, antioxidant and antiviral properties in *in vitro* and *in vivo* experimental conditions. However thear are further studies need to be established in molecular and nuclear level. Due to limitation and time line which restricts us to proceed further. It is also concluded that further exploration of this species in various biological properties beyond antiviral and anticancer is warranted.

List of figures

Chapter:5

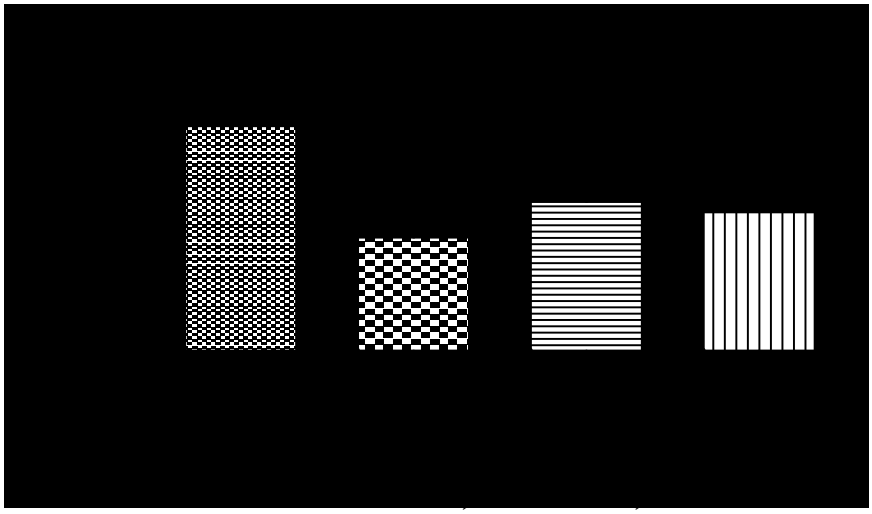
Fig: 1 Effect of *Acanthophora spicifera* on tumour weight in EAC treated mice



*** represents the $P<0.001$, 5FU Vs tumour control

** represents $P<0.01$, ASE (100 & 200 mg/kg) Vs Tumour control

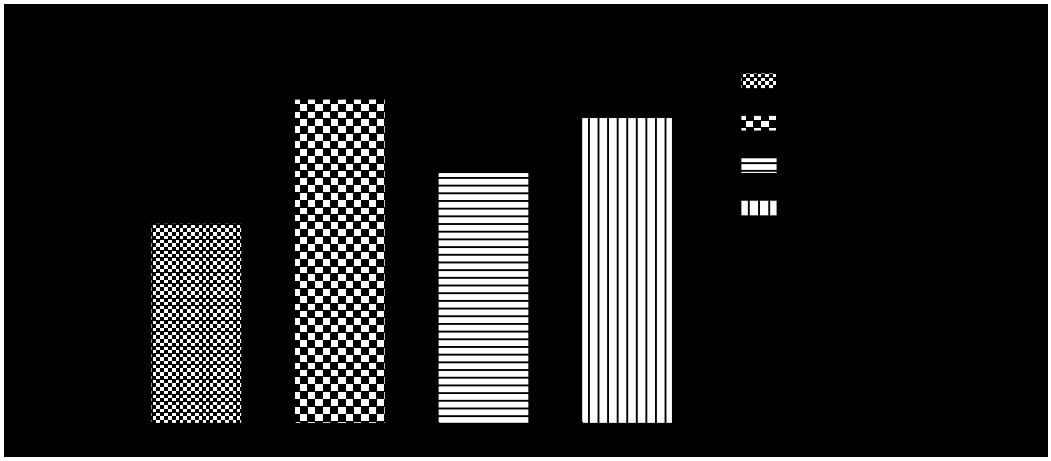
Fig: 2. The effect of the *Acanthophora spicifera* on tumour volume in EAC treated cancerous mice



*** $P<0.001$represents 5FU Vs normal control

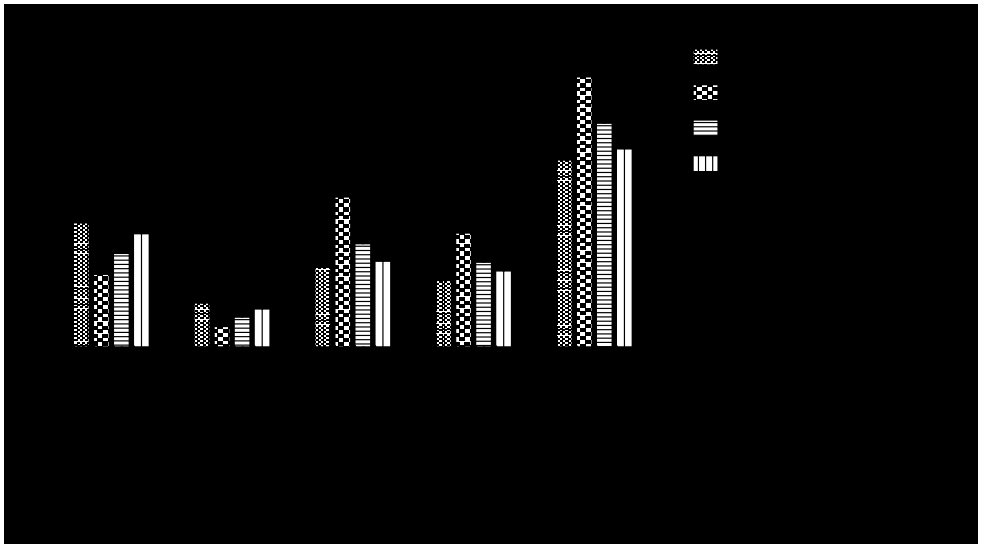
** $P<0.01$ represents ASE (100 & 200 mg/kg) Vs tumour control

Fig: 3. Mean survival day of the tumour bearing mice treated with 5 FU and ASE (100 & 200 mg/kg)



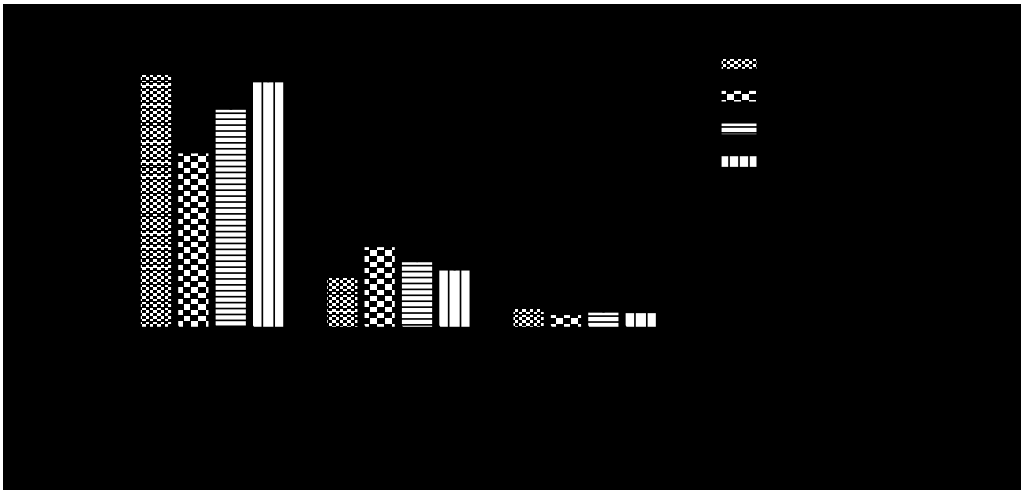
*** *P*<0.001 represents 5FU Vs Tumour control
** *P*< 0.01 represents ASE 100 & 200 mg/kg Vs Tumour control

Fig: 4. The effect of *Acanthophora spicifera* on various blood haematological parameters treated with mice bearing with EAC cell lines



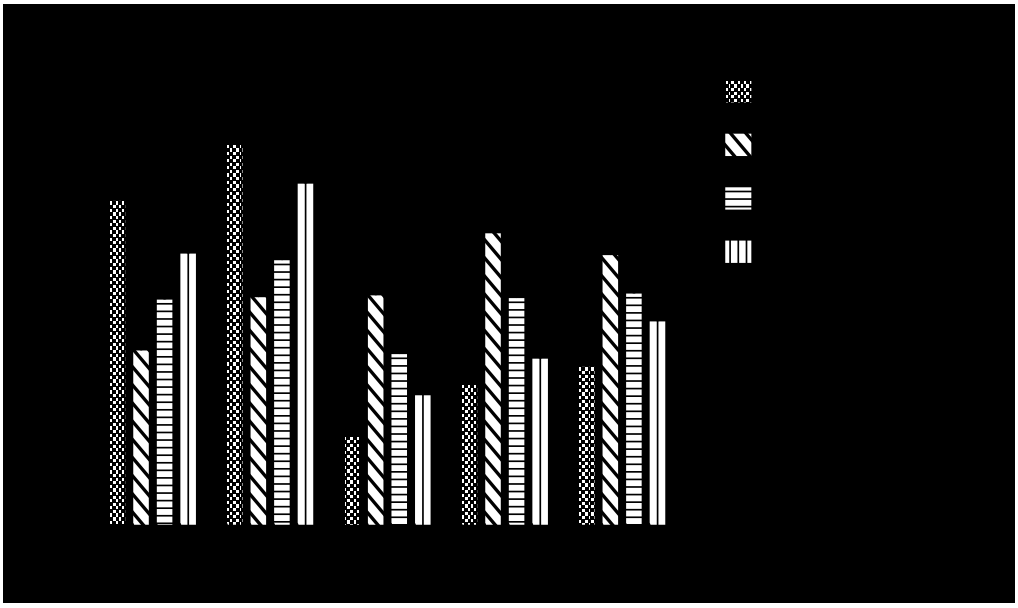
*** *P*<0.001 represents Tumour control Vs normal control
** *P*<0.01 represents ASE 200 mg/kg Vs Tumour control

Fig: 5. The effect of *Acanthophora spicifera* on blood haematological parameters like Lymphocytes, Nuetrophils and Monocytes



*** $P<0.001$ represents Tumour control Vs normal control
** $P<0.01$ represents ASE 200 mg/kg Vs Tumour control

Fig: 6. The effect of *Acanthophora spicifera* on biochemical parameters



*** $P<0.001$ represents Tumour control Vs normal control
** $P<0.01$ represents ASE 200 mg/kg Vs Tumour control
*$p<0.05$ represents ASE 100 mg/kg Vs Tumour control

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6.7 Figures

Fig: 1 DPPH assay of ascorbicoaid and EAF

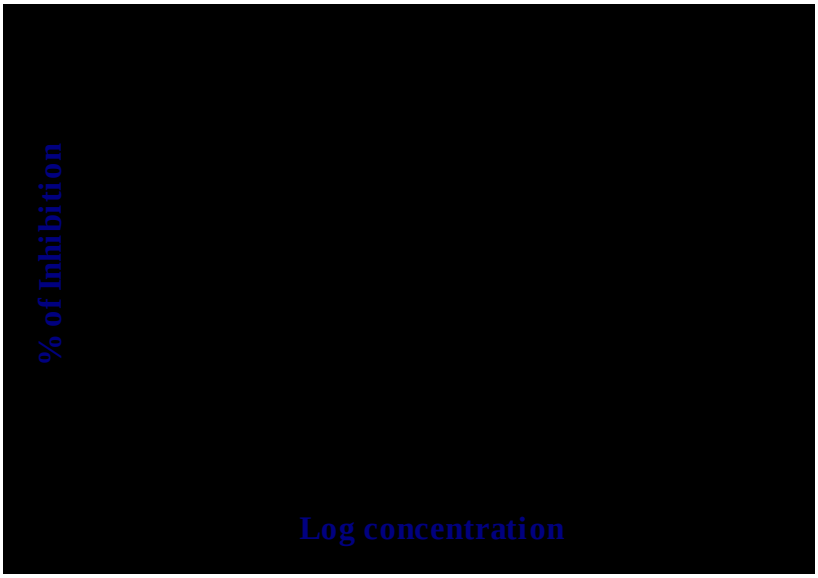


Fig 2:MTT assay of EAAS

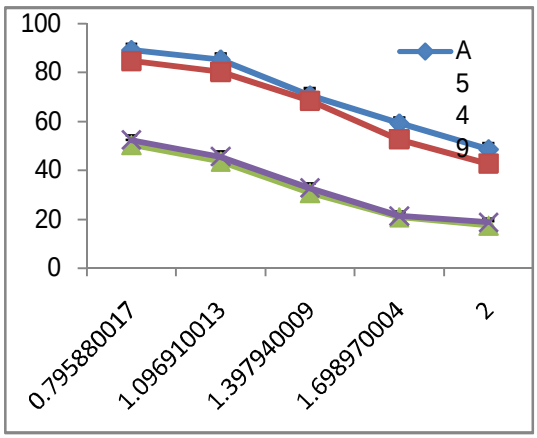
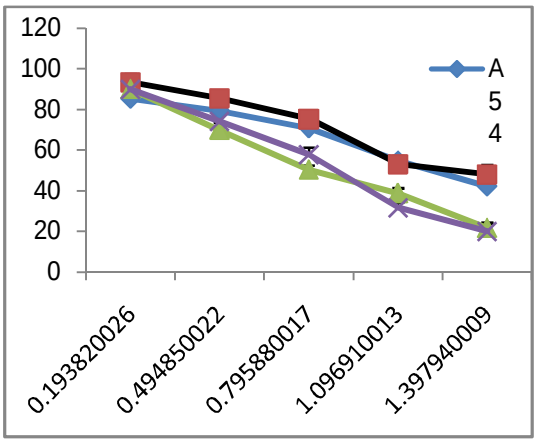


Fig 3:MTT assay of Etoposide



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7.4 Figures

Fig 1& 2(A&B) **Represents HPTLC document for Quercetin and Gallic acid STD-standard; Q-Quercetin; G-Gallic acid**

A **B**

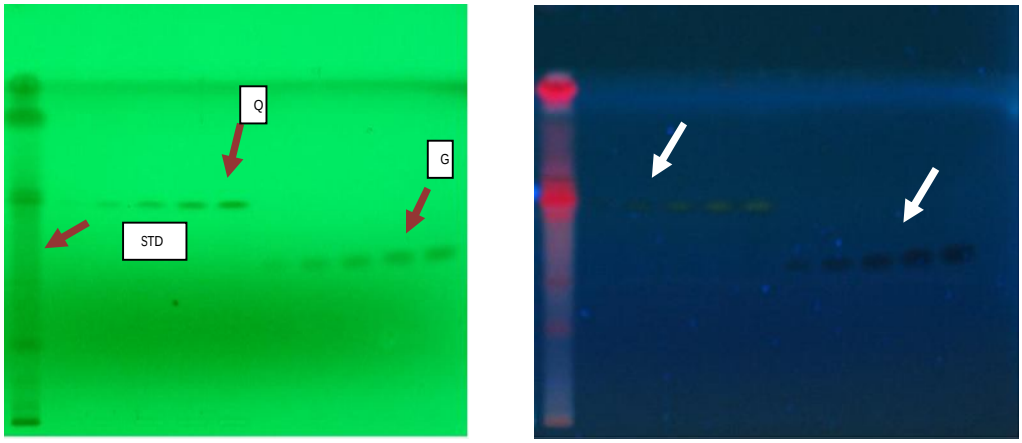

Fig 3: **An overlay of densitometric scan of *Acanthophora spicifera* and Gallic acid**

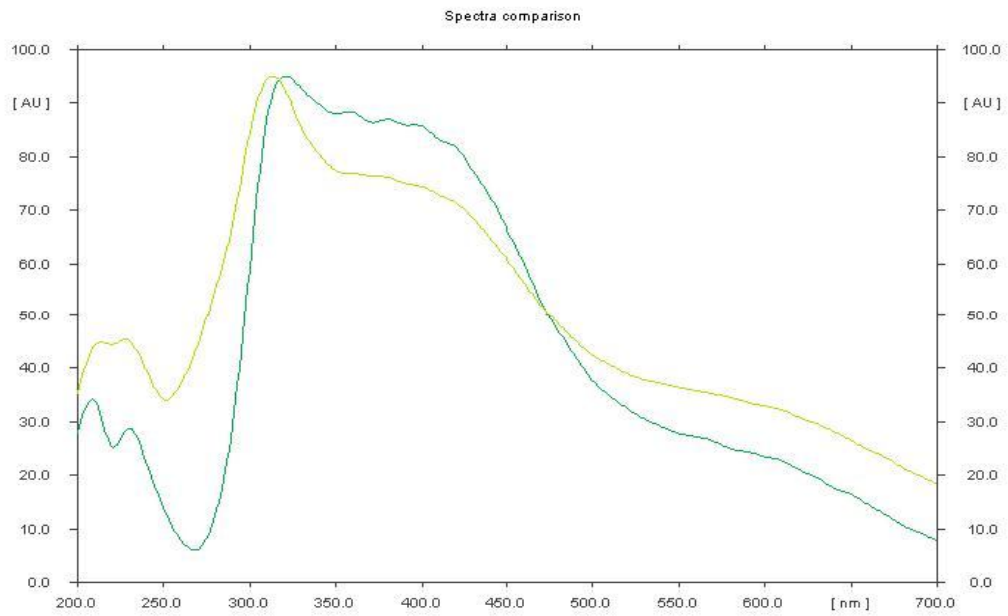

Fig 5 : HPTLC Chromatogram of Gallic acid in *Acanthophora spicifera*

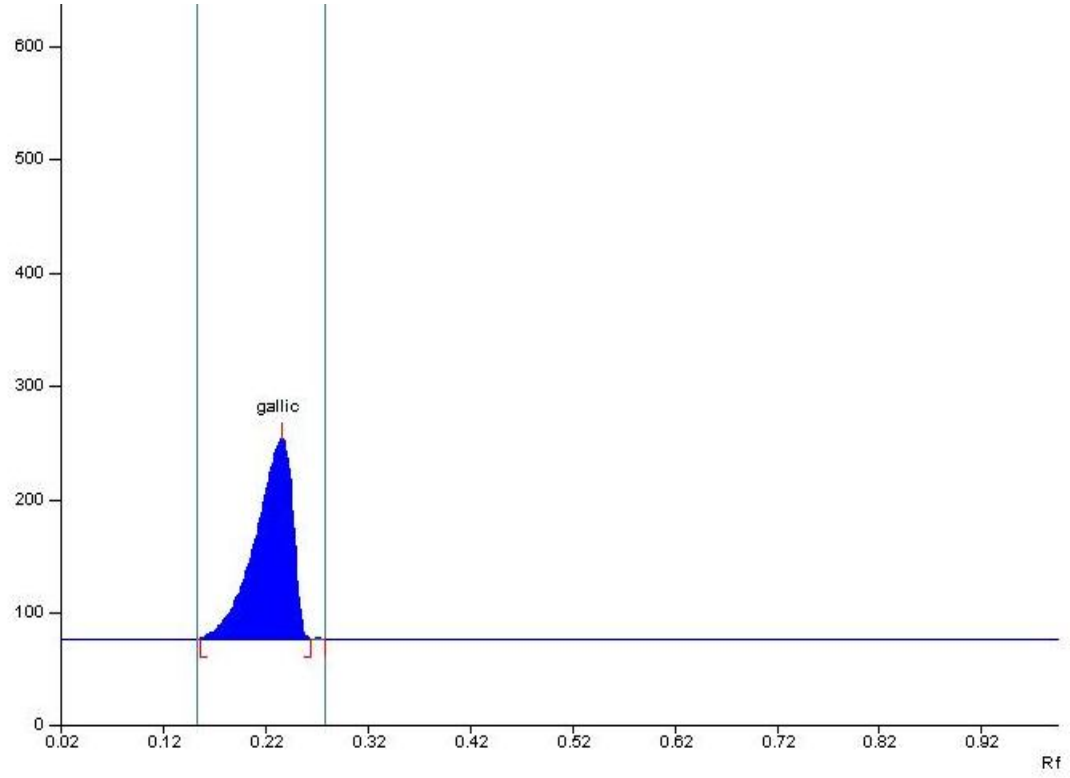

Fig 6: Represents the standard protocol for the samples Quercetin and Gallic acid

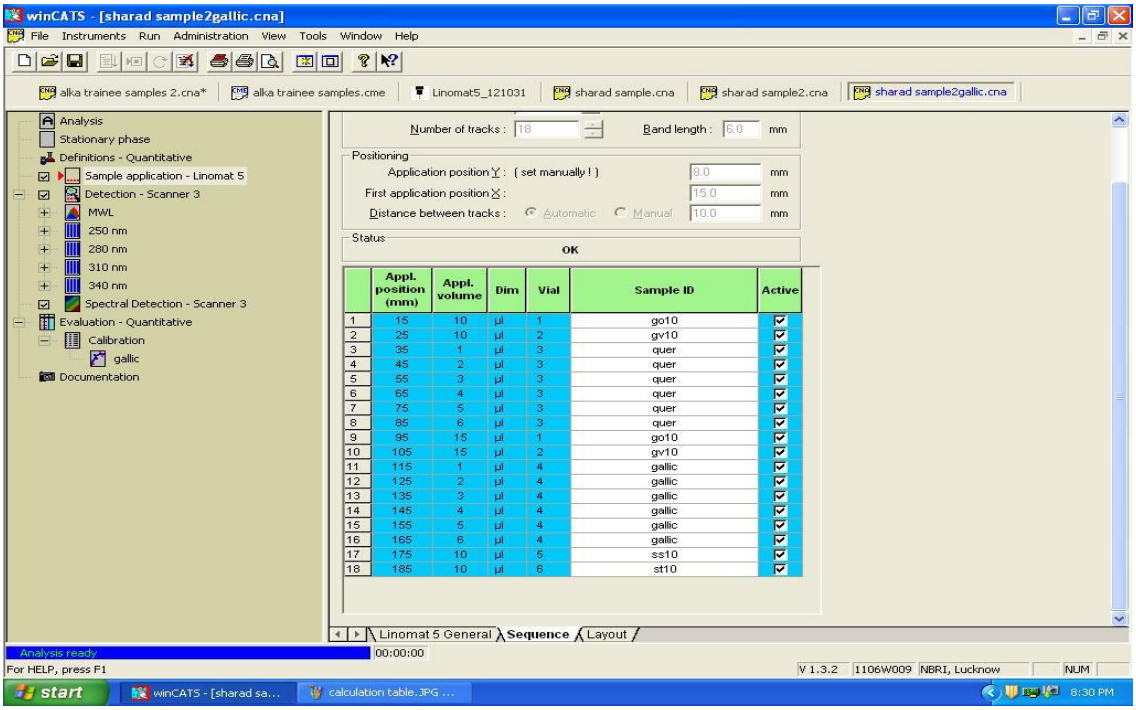

Fig 7: Profile of Gallic acid

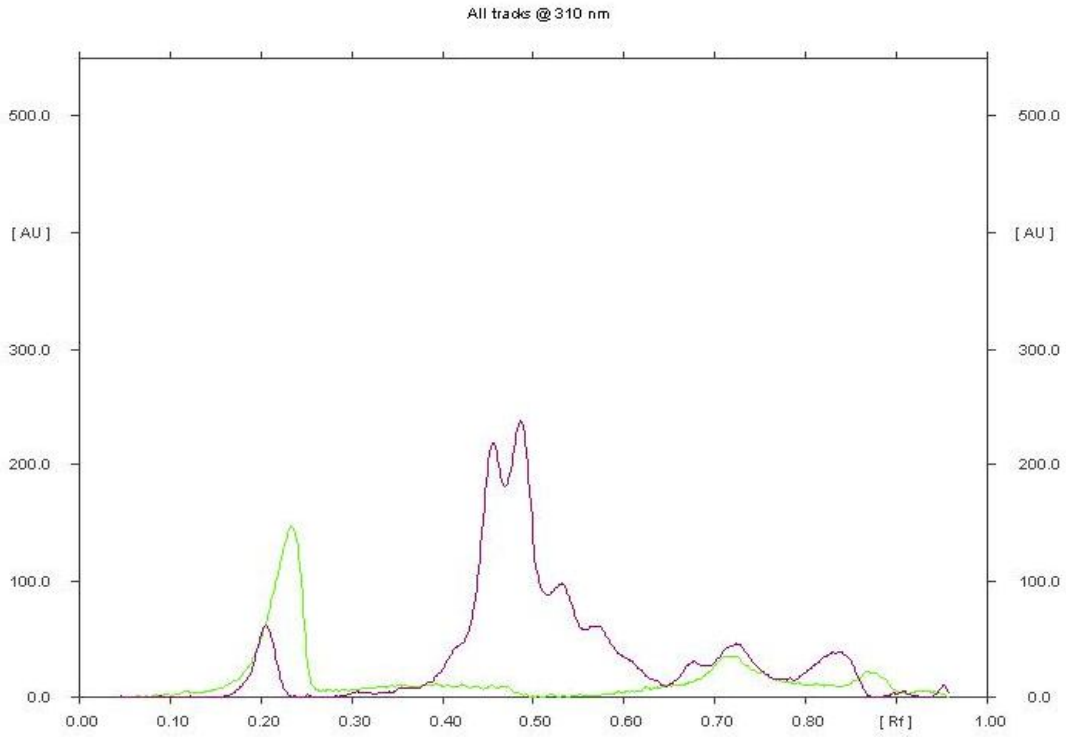

Fig 8 Represents linearity curve for gallic acid based on the area under the curve of the peak (after scanning at 310 nm)

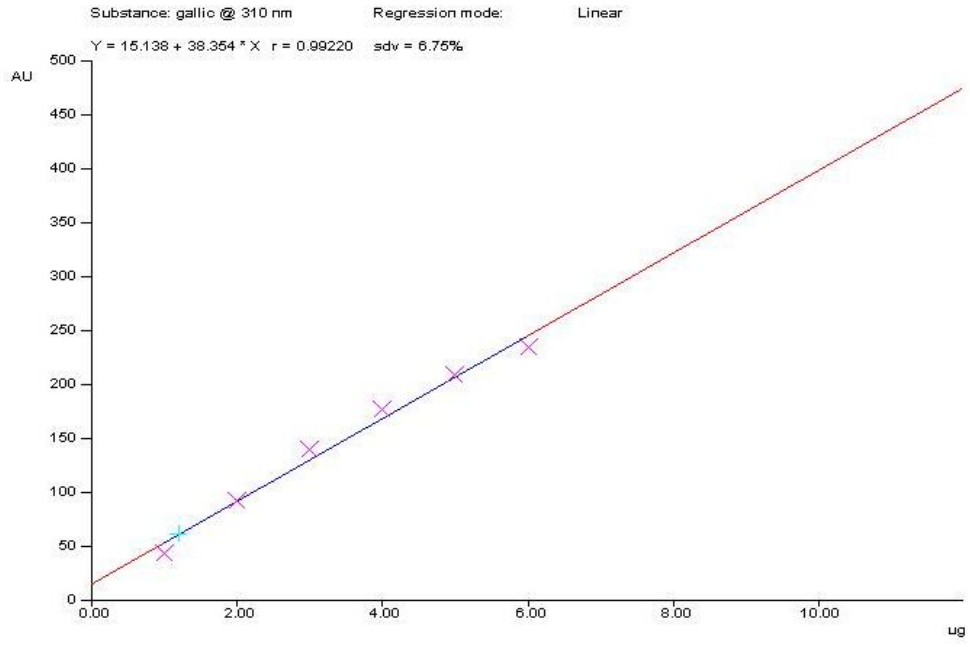

Fig:9 Linearity curve for gallic acid based on the height of the peak (after scanning at 310 nm) (both are for gallic acid, this is to show that the linearity is intact by both height and area of the peak)

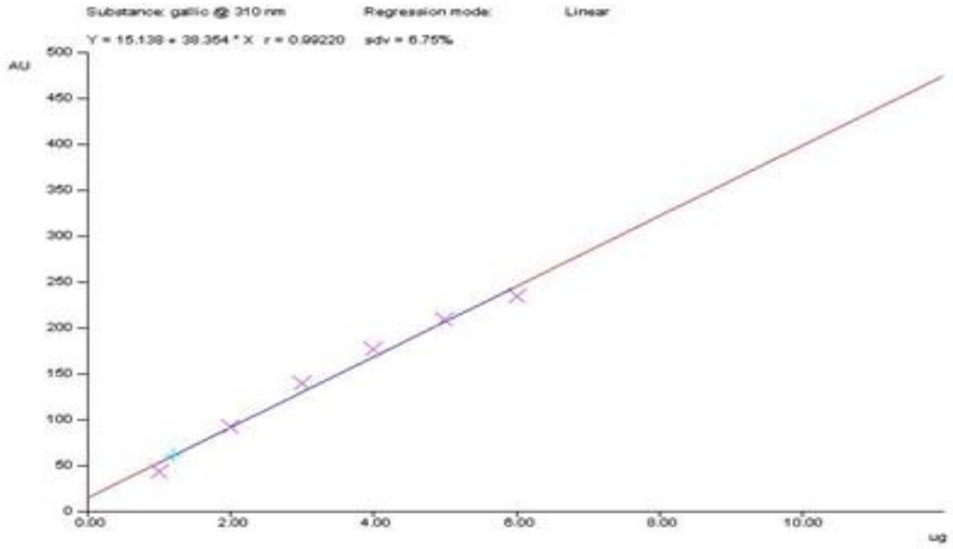

Fig 10: An overlay of densitometric scan of *Acanthophora spicifera* and Quercetin

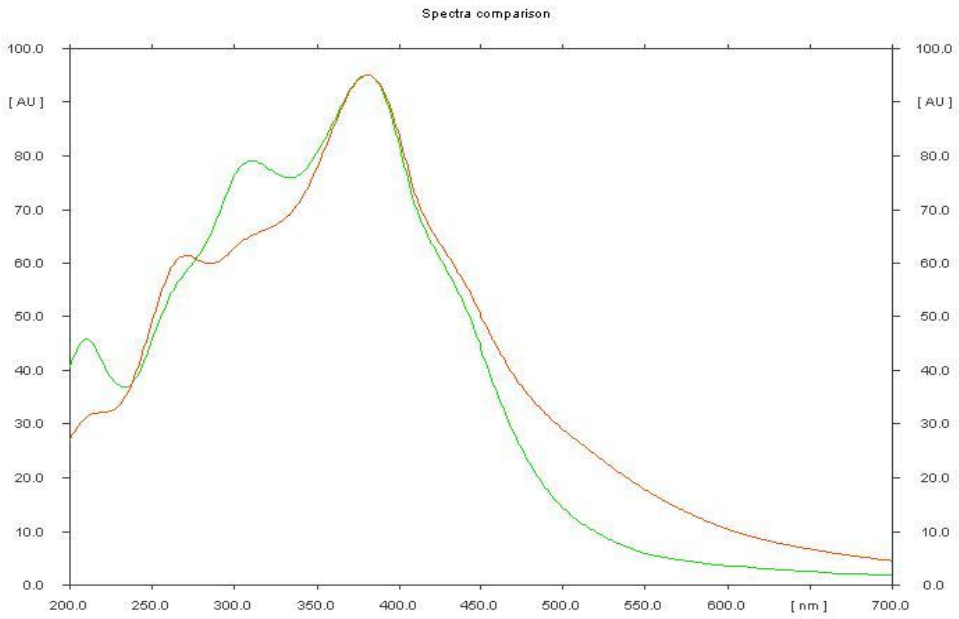

Fig.11: HPTLC Chromatogram of Ethyl acetate extract (Quercetin) of *Acanthophora spicifera*

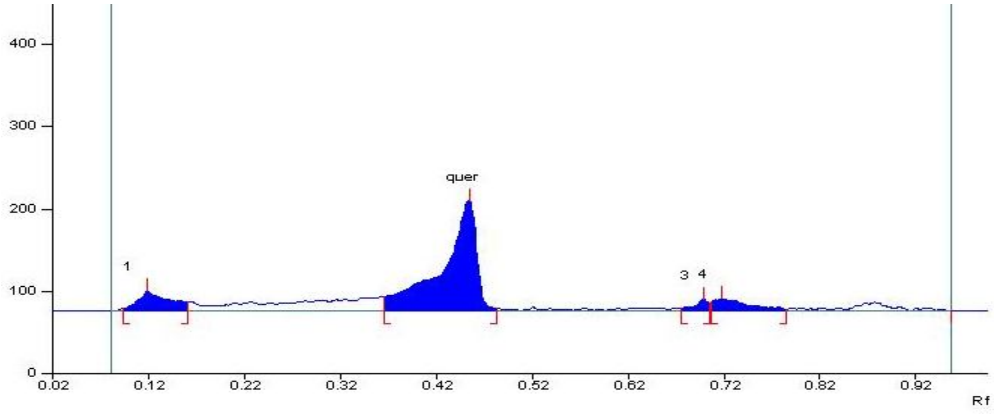

Fig 12: HPTLC Chromatogram of Quercetin in *Acanthophora spicifera*

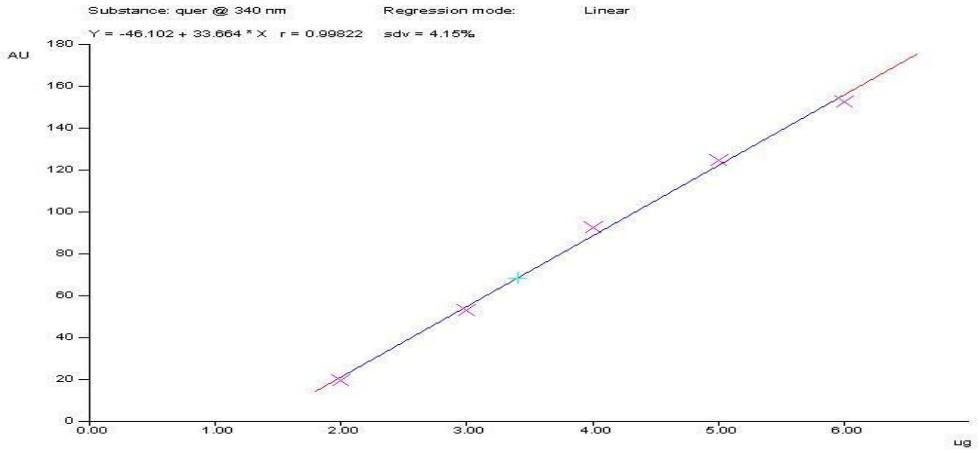

Fig 13: Linearity curve for quercetin based on the area of the peak (after scanning at 340 nm)

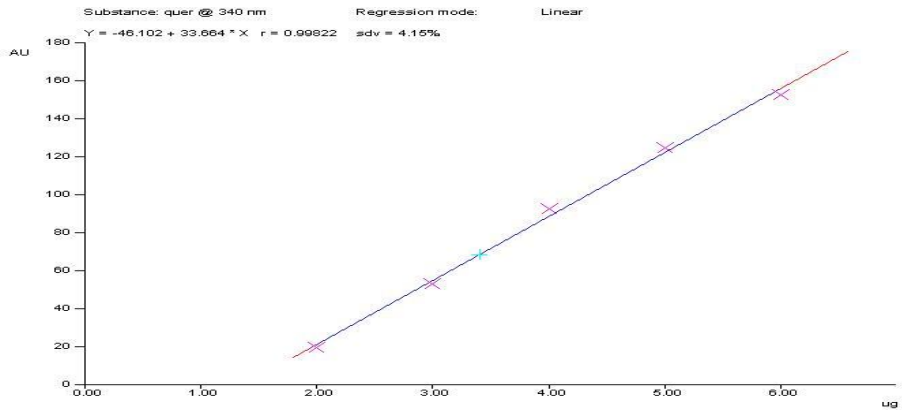

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Fig:1 MTT Assay (Isoquercetin) using HT 29 and HepG2 cancer cell lines

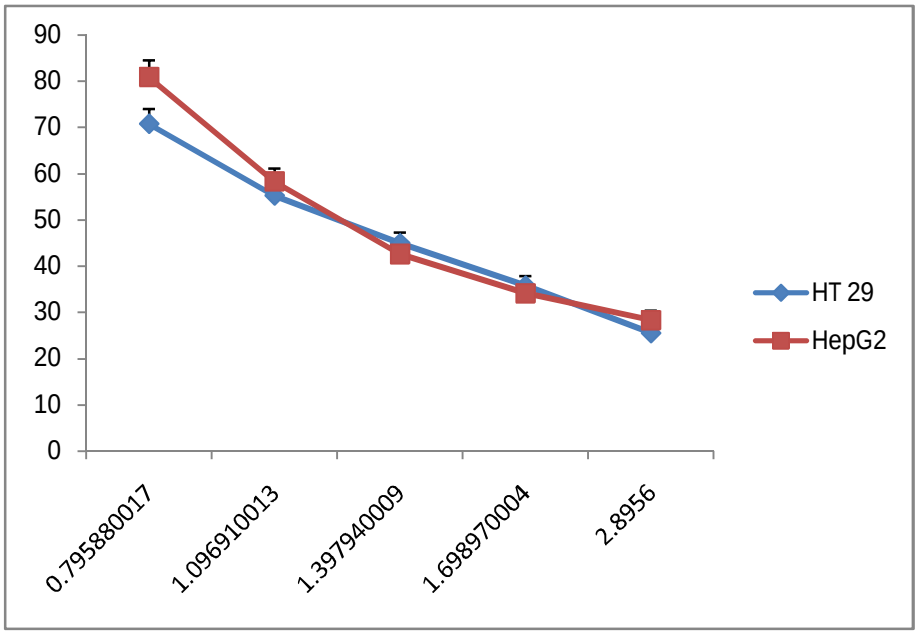
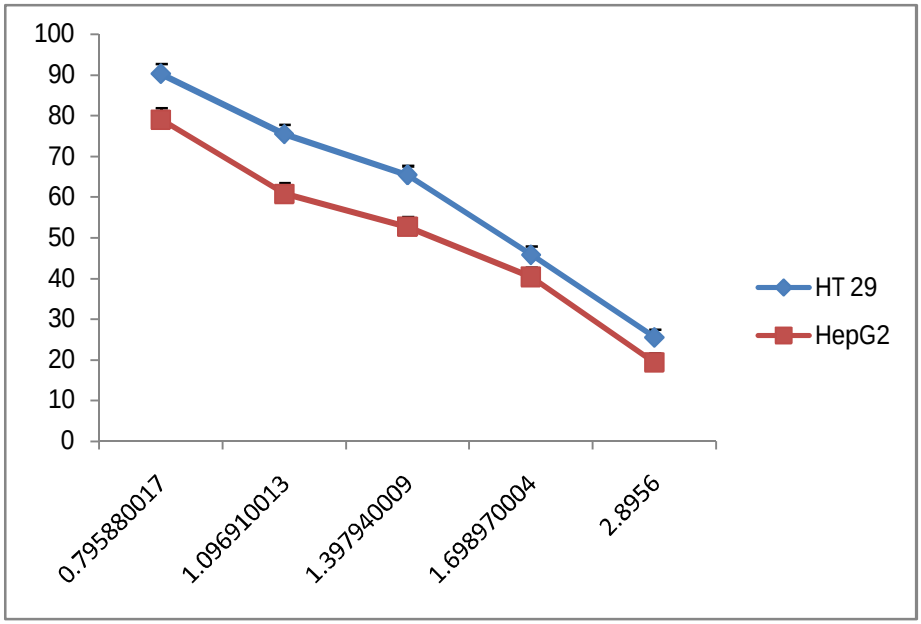


Fig 2: Tryphan blue assay (Isoquercetin)



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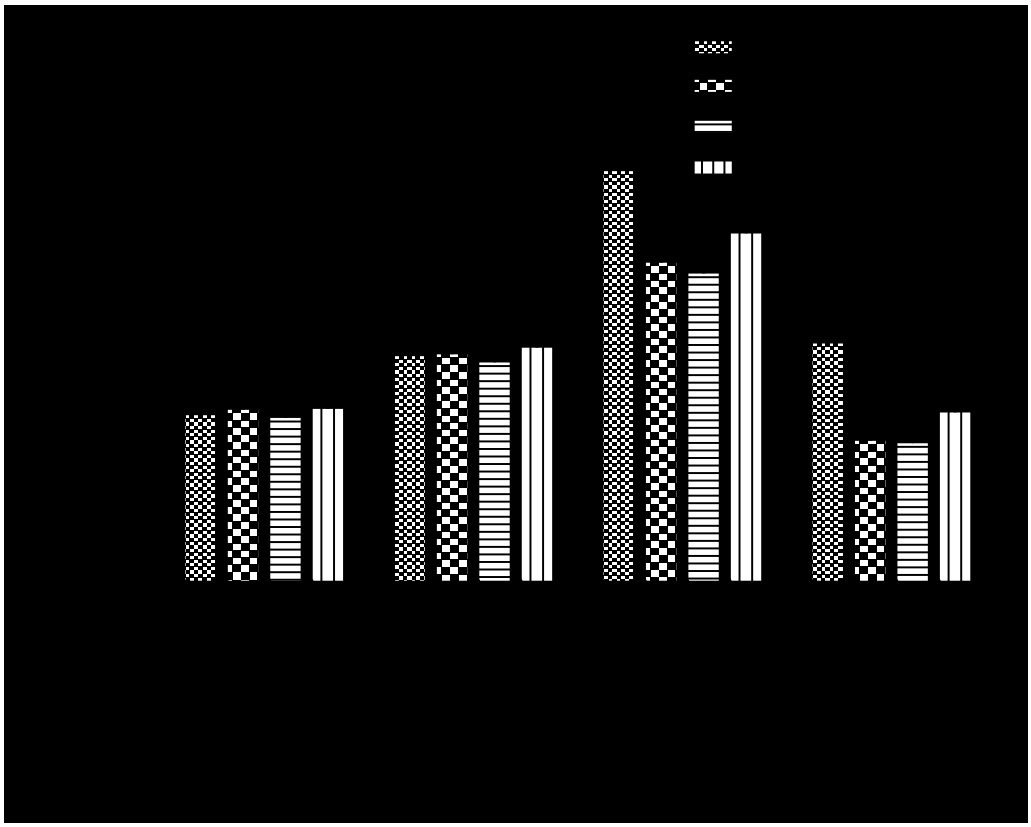
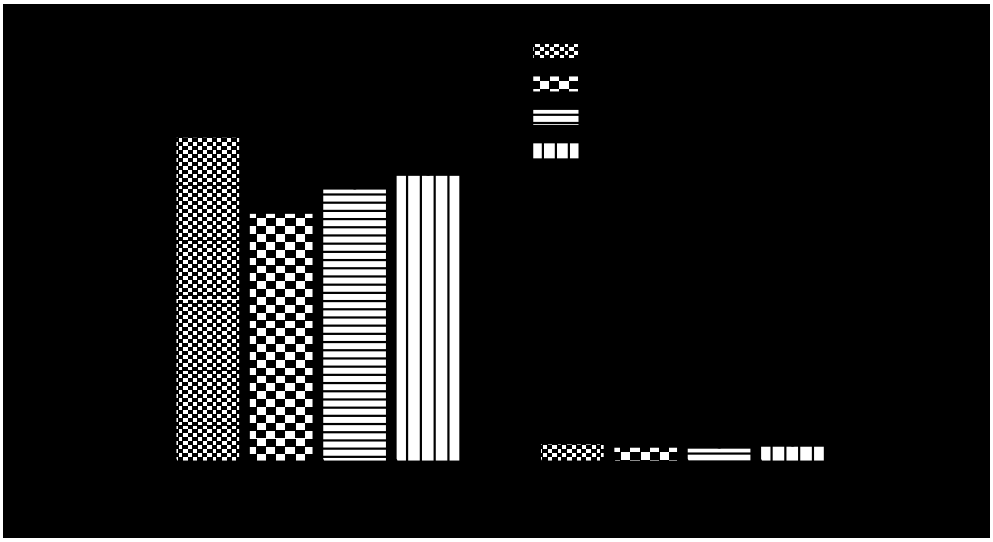
9.5 Figures

Fig 1: Effect of ISQN on body weight change in DEN induced Hepatacarcinoma in rats



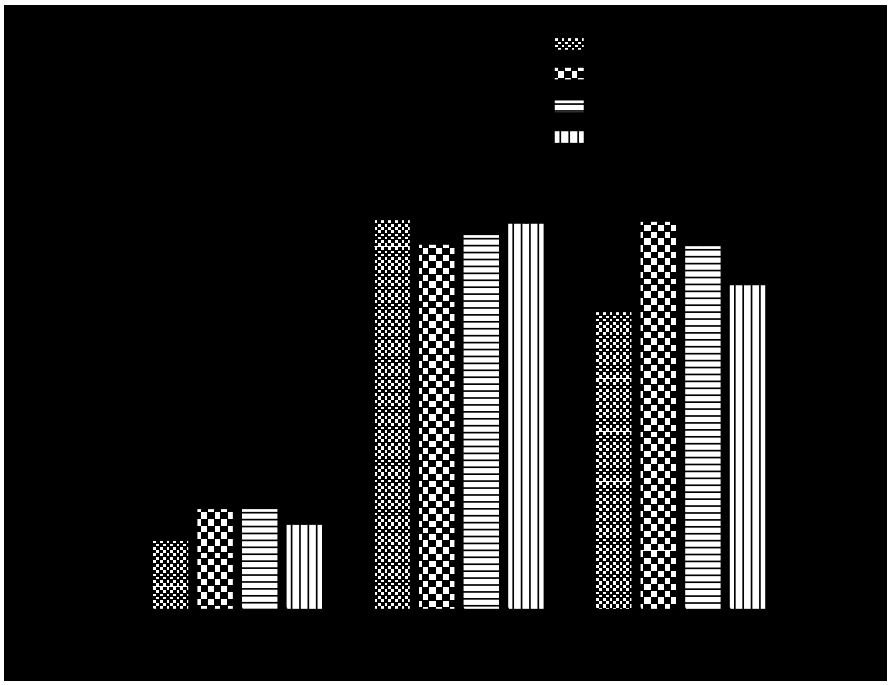
*** p<0.001 represents DEN control Vs normal control
** p<0.01 represents ISQN 10 mg/kg Vs DEN control

Fig 2 : Effect of ISQN on liver weight in DEN induced Hepatocarcinoma in rats



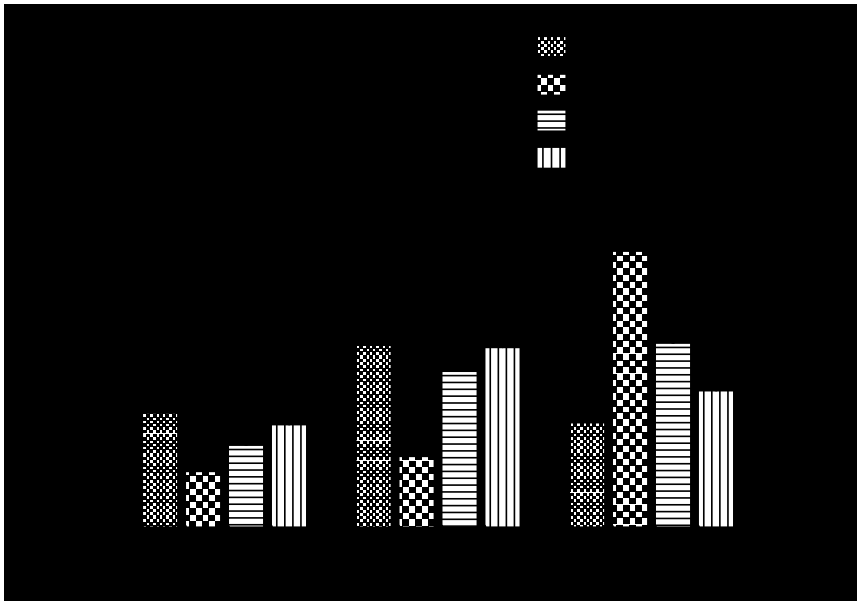
*** p<0.001 represents DEN control Vs normal control
** p<0.01 represents ISQN 10 mg/kg Vs DEN control

Fig 3: Effect of ISQN on liver function test in rats treated with DEN



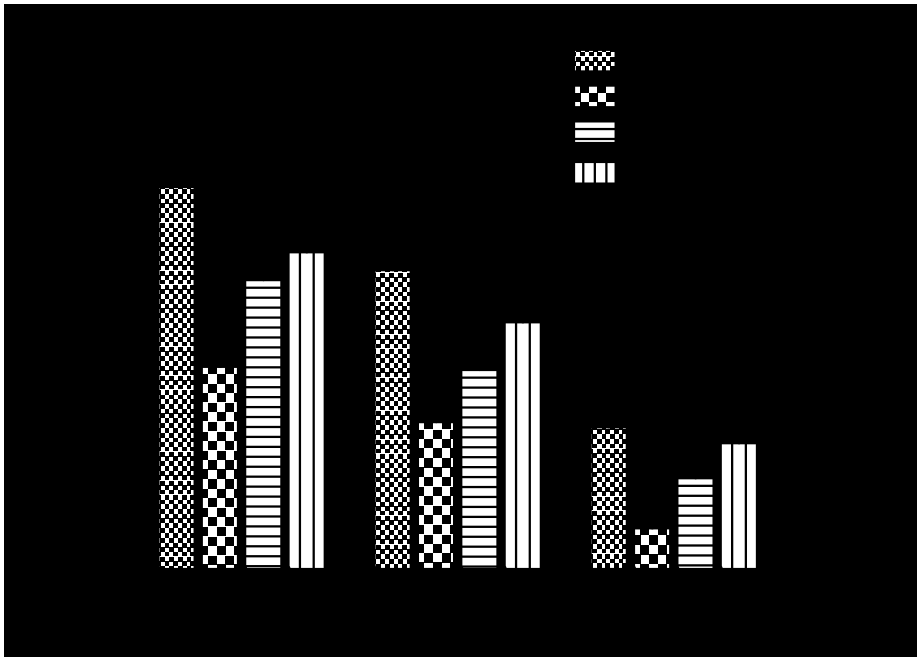
*p<0.05 represents ISQN 10 mg/kg Vs DEN control
**p<0.01 represents DEN control Vs normal control, ISQN 10 mg/kg Vs DEN control
***p<0.001 represents DEN control Vs normal control

Fig 4 : Effect of ISQN on liver SOD, CAT and TBARS



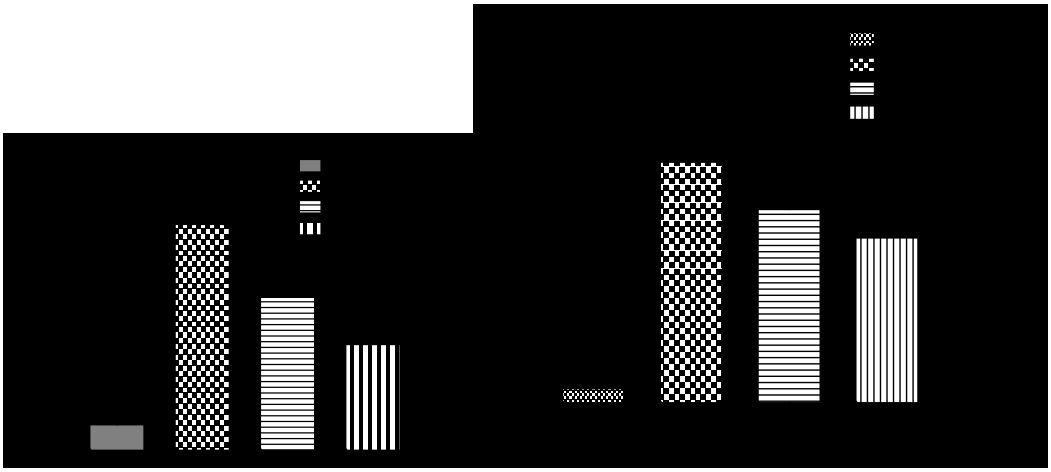
*p<0.05 represents ISQN 10 mg/kg Vs DEN control
**p<0.01 represents DEN control Vs normal control, ISQN 10 mg/kg Vs DEN control
***p<0.001 represents DEN control Vs normal control

Fig 5 :Effect of ISQN on liver GPX, GR and GST



p<0.05 represents ISQN 10 mg/kg Vs DEN control, DEN control Vs normal control
**p<0.01 represents DEN control Vs normal control
***p<0.001 represents DEN control Vs normal control

Fig 6 & 7: Effect of ISQN on liver apoptotic and anti apoptotic markers

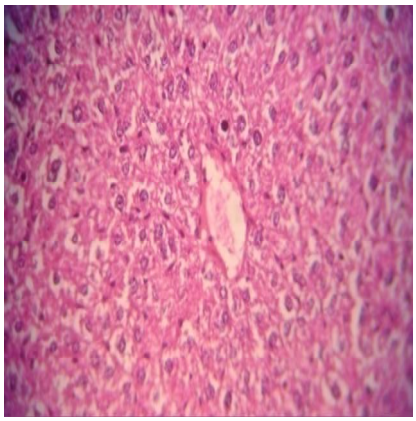


Lane A: Normal Control. Lane B: DEN Control
Lane C: ISOQ 5 mg/kg Lane D: ISOQ 10 mg/kg

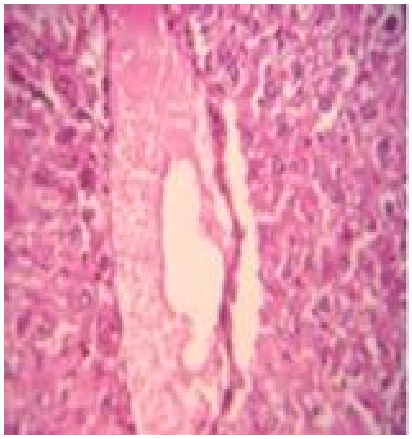
*p<0.05 represents ISQN 5 &10 mg/kg Vs DEN control
*** p<0.001 represents DEN control Vs normal control

Fig 8 Shows the representative caspase-3 and Bcl-2 protein expression in liver mitochondrial homogenates β-Actin was blotted as a standard and indicated no changes during the time course of the experiment.

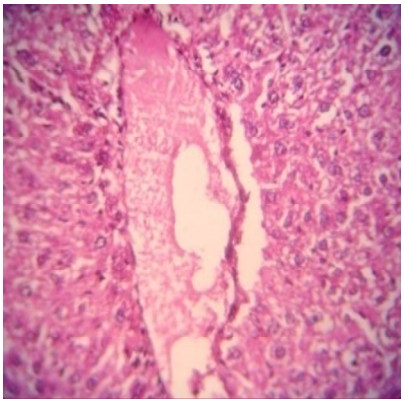
Fig 9: Histopathology report



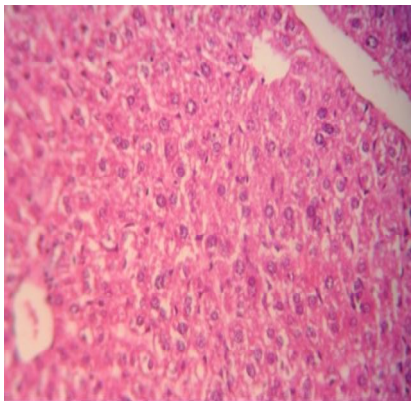
A. Normal



B.Control



C.Isoquercetin 5 mg/kg



D. Isoquercetin 10 mg/kg

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10.5 Figures

Fig 1 Represents the Host Control monolayer with uni(BHK 21)

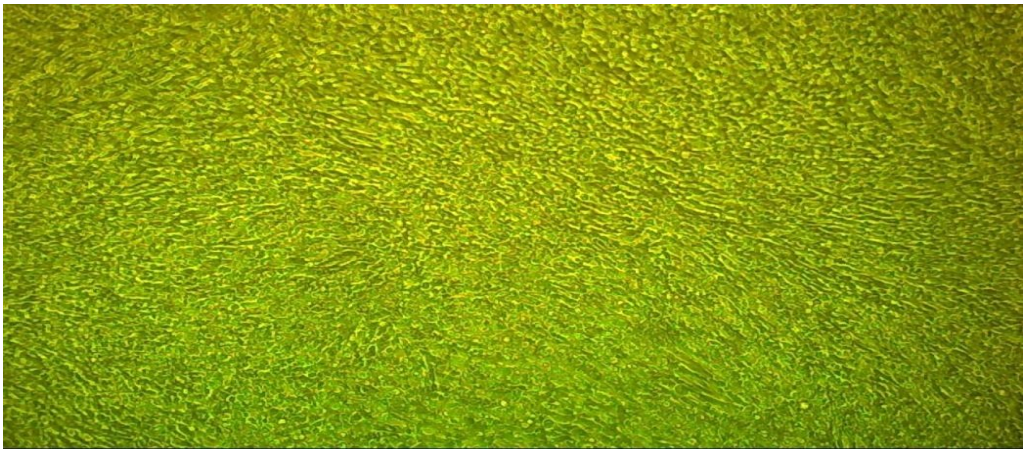

Fig 2 Represents the Virus control (CHIKV infected in to BHK21)

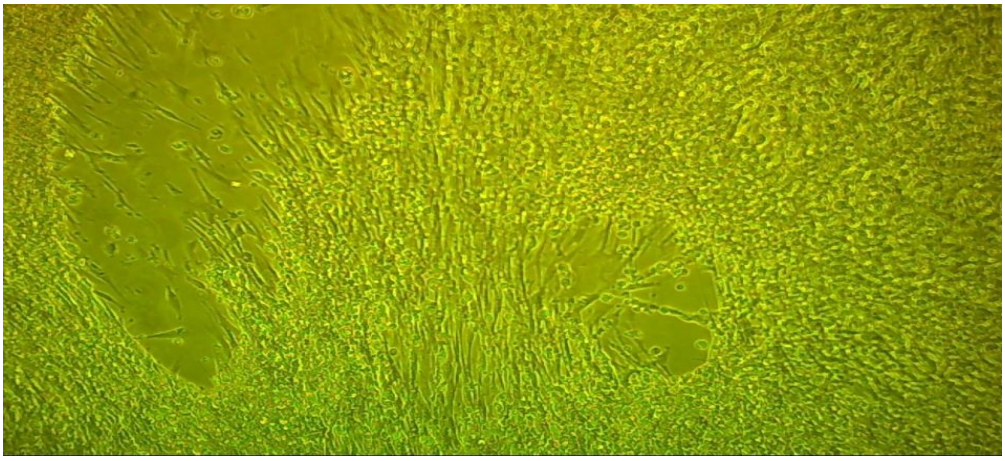

Fig 3 (Pre treatment) represents the Confluent monolayer of BHK21 cell lines without cell death after treating with compound Isoquercetin (24-48 hrs) with slight aggregation of cell and cencetia was observed

Fig 4a (Post treatment) represents the virus infection was intiated on BHK21 host cell before 24hrs of Isoquercetin treatment and observed the viral infection

Fig 4b (Post treatment) represents the regain of monolayer after 24 hrs treatment of the Isoquercetin with reference to control

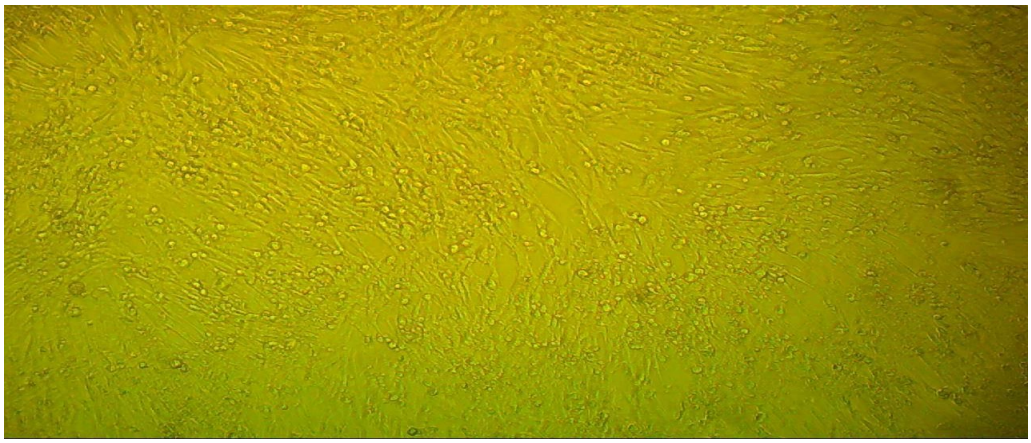

Fig 5 Represents (Same time innoculation test drug+ Virus) the monolayer without any CPE when inoculated both the Isoquercetin and CHIKGV. It was observed that no change in the monolayer except slight cencetia

10.6. Tables

Table: 1

COMPOUND	ORGANISM	ACTIVITY
A- l and A-2	Microalgae, Cochlodinium polykrikoides	[237]Influenza virus A and B; RSV A and B; HSV-1
Ac Da-1	*Dictyota menstrualis*	HIV-1 replication and RNA-dependent DNA polymerase activity [238] of the viral RT
Calcium spirulan	Cyanobacteria, *Arthrospira platensis* (previously called Spirulina platensis)	HSV-1 replication; Measles replication; Mumps replication;Influenza replication; Polio replication; Coxsackie replication;HIV-1 replication; HCMV replication; Selectively inhibition of penetration into host cells[239]
Cyanovirin-N	Cyanobacteria, *Nostoc ellipsosporum*	HIV-1 and HIV-2 and SIV fusion, replication and CPE [240]
Fucoidan	Brown seaweed, *Fucus sp*	vesiculosus HSV-1 and HSV-2; HCMV; VSV; Sinbis virus; HIV-1 RT [241]

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13.**Publications**

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