"CHEMOPREVENTIVE EVALUATION OF AN INDIAN MEDICINAL PLANT ON 1.2, DIMETHYL HYDRAZINE (DMH) INDUCED ABERRANT CRYPT FOCI RATS (Colon Cancer) AND IT'S ANTI OXIDANT POTENTIAL."

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CONTENTS		
S.No.	Title	Page No.
	LIST OF TABLES	iii - v
	LIST OF FIGURES	vi - xi
	LIST OF ABBREVIATIONS	xii - xiii
CHAPTER -1	**INTRODUCTION**	
	1.1 Introduction to cancer	1-5
	1.2 Introduction to colon cancer	5 - 25
	1.3 Antimicrobial activity	26
CHAPTER-2	**AIM AND OBJECTIVE**	27
CHAPTER-3	**REVIEW OF LITERATURE**	
	3.1 Plant profile	28 - 30
	3.2 Phytochemical and pharmacological reviews	30 - 32
CHAPTER-4	**SCOPE AND PLAN OF WORK**	33
CHAPTER-5	**MATERIAL AND METHODS**	
	5.1. Plant collection	34
	5.2 Physicochemical Characters of Crude Drug *Portulaca quadrifida*	34 - 36
	5.3 Phytochemical Investigation of *Portulaca quadrifida*	36 - 44
	5.4 *In vitro* antioxidant study	44 - 50
	5.5 *In vitro* cytotoxicity studies	50 - 52
	5.6 Antimicrobial Activity	52 - 53
	5.7 Screening of Acute Oral Toxicity Study of drug samples in mice (OECD 423)	53 - 54
	5.8 *In vivo* anticancer activity	54 - 78
	5.9. Isolation of aqueous extract of *Portulaca quadrifida*	78 - 82
	5.10 *In vitro* cytotoxicity assay of isolated compound of *Portulaca quadrifida* on colon cancer cell lines HT-29 and HCT-116 by determination of mitochondrial synthesis by MTT assay.	82
	5.11 GC MS Procedure	82 - 83

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

AEPQ	-	Aqueous extract of *Portulaca quadrifida*
CEPQ	-	Chloroform extract of *Portulaca quadrifida*
MEPQ	-	Methanolic extract of *Portulaca quadrifida*
DMH	-	1,2 Dimethylhydrazine
AFP	-	Alpha-feto-protein
CEA	-	Carcinoembryonic antigen
Ml	-	Millimiter
Mg	-	Milligram
Kg	-	Kilogram
Min	-	Minutes
mM	-	Millimoles
NSAIDs	-	Non Steroidal Anti-inflammatory Drugs
OECD	-	Organisation for Economic Co-operation Development.
P.O	-	Per oral
PLA_2	-	Phospholipase A_2
PPAR	-	Proliferator activated receptor
TNF	-	Tumour Necrosis Factor
IL-I	-	Interleukines
NSAIDS	-	Non steroidal Anti-inflammatory drugs
OTC	-	Over the counter
FDA	-	Food development association
GMP	-	Good manufacturing practices
SGOT	-	Serum transaminase
ALT	-	Alanine transaminase
ASP	-	Serum acid phosphate
DNA	-	Deoxyribose nucleic acid

RNA	-	Reoxyribose nucleic acid
HCL	-	Hydrochloric acid
NAOH	-	Sodium hydroxide
HNO_3	-	Nitric acid
H_2SO_4	-	Sulphuric acid
SOD	-	Superoxide dismutase
CAT	-	Catalase
GP_X	-	Glutathione Peroxidise
GSH	-	Reduced Glutathione.
GC	-	Gas Shromatography
MS	-	Mass Spectroscopy
% w/w	-	Percentage Weight by Weight

INTRODUCTION

1.1 CANCER:

The human body is complex as it is made up of few hundreds to thousands of millions of living cells.

It controls the functioning as a single body but is made up of various operational parts that work interdependently. Whole body parts are associated with a specific function essential for the normal growth of the individual.

The cells start growing in the early stage of the life of the person in a systematic manner where they grow, multiply and die. But when the person reached the stage of older in that condition most of the cells replace dying cells or worn out cells or to repair any wound.

When growing of cells becomes ungovernable in any part of the body then it leads to cancer. This occurs due to alteration in tumour suppressor genes. Cancer cells are able to disregard signals that inform to stop dividing or apoptosis, which the body uses to get rid of unwanted cells.[1]

There are more than 200 types of cancers that are reported[2] They all start because of irregular rise in number of cells. Growth of cells in cancer condition is unlike from normal cell growth as these cells form new abnormal cells instead of dying. Cancer cells occupy other tissues where as normal cells cannot. Cancer cells grow aggressively and form a tumour such as brain tumor, etc., and in advance stages it starts multiplying into other parts of the body and forms new tumours. Such condition in a person is known as metastasis. Tumors stay alike and grow with the help of immune system. In the early stage tumours appears as malignant in size where as in nature they don't and they do not spread to other parts of the body. If in the early stage tumours are removed then there is rarely chance to re grow. Benign brain tumours are usually life threatening as compared to benign tumours of other parts.

Cancer nomenclature/terminology

Cancer=”crab” in Latin

Two biggest divisions of cancer types:

i. Carcinoma – cancer of epithelial origin

- e.g. adeno-carcinomas
- note: prefix is usually the tissue type (adeno = secretory cell type)

ii. Sarcoma – cancer from mesenchymal origin (muscle, fat, cartilage)

The suffix – in most cases:

- Oma = benign tumor
- Sarcoma = malignant

(Some exceptions: melanoma, leukemia, lymphoma)

Benign	-	Tumor at a stage proceeding malignant
Malignant	-	Full blown cancer tumor
Hyperplasia	-	Growing group of cells (proliferating)
Neoplasia/neoplasm	-	Malignant group of cells
Anaplasia	-	De-differentiated group of cell (no longer looks like the original cell type)[3]

Different cancers have a different combination of genetic changes such as mutation in DNA.

The three main types of genes are affected by the genetic changes which contribute to cancer. The effected genes are as follows primitive genes–genes related to tumours, preventive genes of tumors and genes altered by DNA. These genetic changes generally called as "Drivers of cancer".

When the proto oncogenes are altered or if they become hyper active it leads to cancer causing genes which allows the cells to show in abnormal way.

DNA repair genes name itself indicates that it fixes damaged DNA. Cell mutations leads to development of additional mutations which in term makes the cells cancerous[4].

8,50,000 new types of cancer cases being diagnosed every year, Among which India ensure resulting about 5,80,000 cancer death every year because of living style. India is top listed in case of oral and throat cancer case.

In world the every 3rd oral cancer patient is from India. In males oral cancer, stomach cancer and lungs cancer cases/cancer death are common whereas in the case of females the cancer incidence/death are Cervical cancer, Breast cancer and Oral cancer. If diagnose starts in the early stage of cervical cancer then it can be prevented/diagnosed. Comparing with established countries, there were few cases of cancer in India but which can be due to in the state of diagnosis and may be due to not being reported. The factors like diet, social,

economic, or educational status might also contribute a difference in the cancer responsiveness and the prevalence. In the primitive days, cancer was mainly called as disease of olden era. Diagnosis was 60 percent at median age worldwide. Comparing with developed countries the average life span in India is 58 years where as in developed it is around 75 years[5].

Table 1: The approximate numbers of new cases of cancers/per annum.

S.No.	Type of Cancer	No. of Patients affected/Year
01	Lung	1.2 million
02	Breast	Over 1 million
03	Colorectal	9,40,000
04	Stomach	8,70,000
05	Liver	5,60,000
06	Cervical	4,70,000
07	Esophageal	4,10,000
08	Head and Neck	3,90,000
09	Urinary Bladder	3,30,000
10	Malignant Non-Hodgkin lymphomas	2,90,000
11	Leukemia	2,50,000
12	Prostate and Testicular	2,50,000
13	Pancreatic	2,16,000
14	Ovarian	1,90,000
15	Kidney	1,90,000
16	Endomaterial	1,88,000
17	Nervous system	1,75,000
18	Melanoma	1,33,000
19	Thyroid	1,23,000
20	Pharynx	65,000
21	Hodgkin disease	62,000

Cells of plants exhibit metabolites which are of two types. One of the metabolite is directly involved in both metabolism and as well as in growth usually (carbohydrates, lipids and protein are primary metabolites). These metabolites are involved in the synthesis of cell

component and also they involved in fabrication of photosynthesis. Most of the compounds which are natural in nature are obtained from primary metabolites like amino acids. Secondary metabolite are not involved in activities of metabolism viz alkaloids, phenolics, steroids, flavonoids etc., secondary metabolites plays a vital role in pharmaceutical and food industries and also in pesticides and fragrance[6].

Higher plants produce primary metabolites for commercial purpose and it is considered as low intense bulk chemicals, where as plants of medicinal use are affluent in secondary metabolites which shows intense corporal effect on system of mammalian. Thus such metabolites are considered as active constituents of plants. In comparison with secondary metabolites, several primary metabolites also shows vigorous physiological effects. which include antibiotics vaccines and hormones[7,8].

The basis of treatment of many aliments of humans from the past saga till modern era is still dependent of natural products such as minerals, plants and animal sources[9].

Drug discovery of natural products is more personalized which involves wise use of therapeutics skills in a appreciative manner so that the patients and community are assured with more benefits. Herbal drug development includes various steps, starting from raw material data, correct identification, pharmacognostics and chemical quality safety and pre clinical pharmacology. Newly commercial viable drug leads and chemical novelty are usually obtained from natural sources and they are considered as high source when compared to other sources. Plant products are rich source of lead molecules to drug discovery. 87% of all identified aliments are treated by the drugs of natural origin[10]. The secondary metabolites from natural products are exhibiting maximum adulteration of drugs and biologically affability comparing with synthetic drugs. The chemical, pharmacological and clinical studies of the traditional medicines which are derived from plants are most early medicines such as aspirin, digitalis, morphine, quinine and pilocarpine.

Herbal medicine

From the primitive saga of civilization, Man has been using plants for fighting with diseases. India is boosted by a diversity of herbage both aromatic and medicinal plants. This is due to the extensive variations of climatic conditions of India. Various category of herbs have been expertly identified and alphabetize by ecologist from Himalaya to Kanyakumari[11].

Under developing countries are actively encouraged by WHO to use medicine of herbal nature which have been used traditionally from ancient period. Already 3000 plants have been identified in the forests of India which can be used as medicines. The active ingredients from these plants are with rupees 2000 crores in the US market.

During the last several years a continuous search by researchers has given a long list of plants having medicinal benefits which has shown profound effect in the treatment of various diseases and also for promoting health of human beings as well as animals. Nowadays medicines are used to cure various ailments which are present in the form of natural or synthetic origin[12]. Studies of more than 6000 active constituents of natural origin have been isolated by the researchers.

Plants have been one of the important source of medicines since the start of human civilization. Before independence of India, the production of plant based drugs in India was confined mainly to cinchona, opium, alkaloids, galanicals and tinctures[13]. In the last three, decades bulk production of plant drugs has become an important aspect of the Indian pharmaceutical industry. Some of the drugs which are manufactured today include papaverine, thebaine, emetine, quinine, quinidine, atropine, sennosides. In India there are many well established drug manufacturers of herbal medicines in which approximately 140 manufacturers are of medium scale and about 1200 small scale pharma industries. Annually herbal drug production is approximately estimated around 100 crores and its demand is going on increasing as the years passing. In Indian market, around 1650 herbal medicine from 540 major plants are evolved in their formulations. During the last two decades, over 3000 plants have been screened in India for their biological activities. As a result, a number of new drugs have been introduced in clinical practice and some are in final phases of clinical development. In the year 2001, researchers recognised more than 100 compounds obtained from ethno medical plant sources which has been already used as traditional ethno medical treatment. Medicinal herbs played a key role in preserving the body of otztal alps for more than 5300 years[14].

1.2 COLON CANCER:

Abnormal cells when originate in the tissues of the colon and rectum are called as colorectal cancer. Both the parts are found in a long fibrous tube called large intestine that is in lower part of G.I.T. The colon is responsible for waste output of the body. The cancer is

named where it grown If the cancer began four to five feet of the large intestine then it is called as colon cancer and if it began in last part of large intestine proceeding towards anus then it is known as rectal cancer.

It is naturally begins as a growth of tissue which is called polyp. A polyp is defined as mass protruding from the mucous membrane into the lumen. They are common in large intestine when compared to small intestine. They are broadly classified in two categories non-neoplastic and neoplastic polyps.[15]

I. Colorectal polyps**:**

A. Benign polyps
   1. Hyperplastic (metaplastic) polyps.
   2. Hamartomatous polyps
      (i) Peutz-Jeghers polyps and polyposis.
      (ii) Juvenile (Retention) polyps and polyposis.
   3. Inflammatory polyps (Pseudopolyps).
   4. Lymphoid polyps.

B. Neoplastic polyps
   1. Adenoma
      (i) Tubular adenoma (Adenomatous polyp).
      (ii) Villous adenoma (villous papilloma).
      (iii) Tubulovillous adenoma (Papillary adenoma, villoglandular adenoma).
   2. Polypoid carcinoma

C. Familial polyposis syndromes
   1. Familial polyposis coli (Adenomatosis)
   2. Gardner's syndrome
   3. Turcot's syndrome
   4. Juvenile polyposis syndrome

II. Other benign colorectal tumours
(Leiomyomas, leiomyoblastoma, neurilemmoma, lipoma and vascular tumours)

III. Malignant colorectal tumours

A. Carcinoma
   1. Adenocarcinoma

2. Other carcinomas
   (Mucinous adenocarcinoma, signet-ring cell carcinoma, adenosquamous Carcinoma, undifferentiated carcinoma)

B. Other malignant tumours
   (Leiomyosarcoma, malignant lymphoma, carcinoid tumours)

IV. Tumours of the anal canal

A. Benign (viral warts or condyloma acuminate)

B. Malignant (squamous cell carcinoma, basaloid carcinoma, muscoepidermoid carcinoma, adenocarcinoma, undifferentiated carcinoma, malignant melanoma)

NON-NEOPLASTIC POLYPS:

(1) Hyperplastic Polyps are nothing but epithelial polyps found particularly in the rectosigmoid. Epithelial hyperplasia is present at the base of the crypts that is why it is known as hyperplastic. They are mostly without symptom and have no malignant potential.

(2) Hamartomatous Polyps:

These Polyps are composed of abnormal mixture of tissues. They are further subdivided into two types.

(i) PEUTZ- JEGHERS Polyps and Polyposis:

Peutz-Jeghers syndrome is autosomal dominant defect. These polyps are mostly found in the jejunum and ileum and usually found in the stomach, small intestine or colon. These polyps undergo malignant transformation if coexistent adenoma is present and if it is not present malignant transformation does not occur.

(ii) Juvenile Polyps:

This type of polyps occur commonly in children below 5 years of age. These polyps are removed in the form of rectal bleeding and they are precancerous.

(3) Inflammatory Polyps:

Inflammatory Polyps occur due to re-epitheliasiation of the Ulcers and overhanging margin in inflammatory bowel disease. These polyps have no malignant potential. They turn into cancerous on long standing cases of ulcerative colitis.

(4) Lymphoid Polyps:

Lymphoid tissue contains reactive hyperplasia which is more prominent in the rectum and terminal ileum which leads to lymphoid polyps in the form of localised or diffused polyps. Localised form is often in the rectum of geriatrics and diffused form in the rectum of paediatrics. They are benign lesions and have to be distinguished from malignant lymphoma.

B. NEOPLASTIC POLYPS:

1. Adenomas:

i). Tubular Adenoma:

These polyps are the most common neoplastic polyps and they have slight male preponderance which usually occur in the distal colon and rectum. They usually appear as benign tumour Tubular adenomas may show variable degree of cytologic atypia, Malignant transformation is seen in only 5% of tubular adenoma whereas higher incidence is usually in large adenomas. They appear in 3rd decade of life.

ii) Villous Adenoma:

Villous Adenomas are much less common when compared to tubular adenomas and they usually appear in 6th decade of life. They are invariably symptomatic and the presence of severe atypia, carcinoma in site and invasive carcinoma are most common. Invasive carcinoma has been reported in 30% of villous adenomas.

iii) Tubulovillous Adenoma:

It is an alternate form of pattern between villous adenoma and tubular adenoma. It is also called as papillary adenoma. It shows mixed pattern, characteristic vertical villi and deeper part showing tubular pattern.

C. FAMILIAL POLYPOSIS SYNDROMES:

1. Familial Polyposis Coli:-

As the name itself indicates it is a hereditary disease in which more than 100 neoplastic polyps are present on the mucosal surface of the colon. Adenomatosis can be distinguished from multiple adenomas in which the adenomas number is few, not exceeding 100. They appear in the 2nd & 3rd decades of life and malignant potential is

very high in such cases. If colectomy is not performed after few years it develops into colorectal cancer.

2. Gardner's Syndrome:-

It is a mixture of familial Polyposis Coli and certain extra colonic lesions such as connective tissue tumours.

3. Turcot's Syndrome:-

It is a mixture of malignant neoplasm and familial polyposis coli.

4. Juvenile Polyposis Syndrome:-

This types of polyps appear in the colon and stomach but when compared to familial polyposis coli, they are few in number.

In colorectal cancer, adenocaricinoma is commonly occurring cancer, because it illustrate about 95 percent of colon and rectal cancers cases/deaths and they spreads deeper to other layers. It is further subdivided into two types.

Mucinous Adenocarcinoma:

It usually represents 60% mucus. Cancer cells can spread faster and become more hostile due to mucus than typical adenocarcinoma.

Signet Ring Cell adenocarcinoma:

They usually accounts for less than 1% of adenocarcinoma. This type of cancer is more difficult to treat.

Colorectal Cancers which are rarely identified are as follows:

Gastro Intestinal Carcinoid Tumours:

This type of Cancer develops in the linings of the G.I.T and it mainly affects neuro endocrine cell. It is a rare type of colorectal cancer.

Primary Colorectal Lymphomas:

Cells of many parts of the body develop lymphoma which is known as cancer. Lymphocytes helps the body to fight against infections. This type of Cancer occurs more in males when compared to females.

Gastro Intestinal Stromal Tumors:

This type of cancer develops in the interstitial cell of cajal (ICCS) and is usually found in stomach. It is further classified as Sarcomas which begins in the connective tissue.

Leiomyosarcoma:

It is another type of Sarcoma Leiomyosarcoma represents cancer of smooth muscle of Colon and rectum which all act to escort waste through digestive tract.

Melanomas:

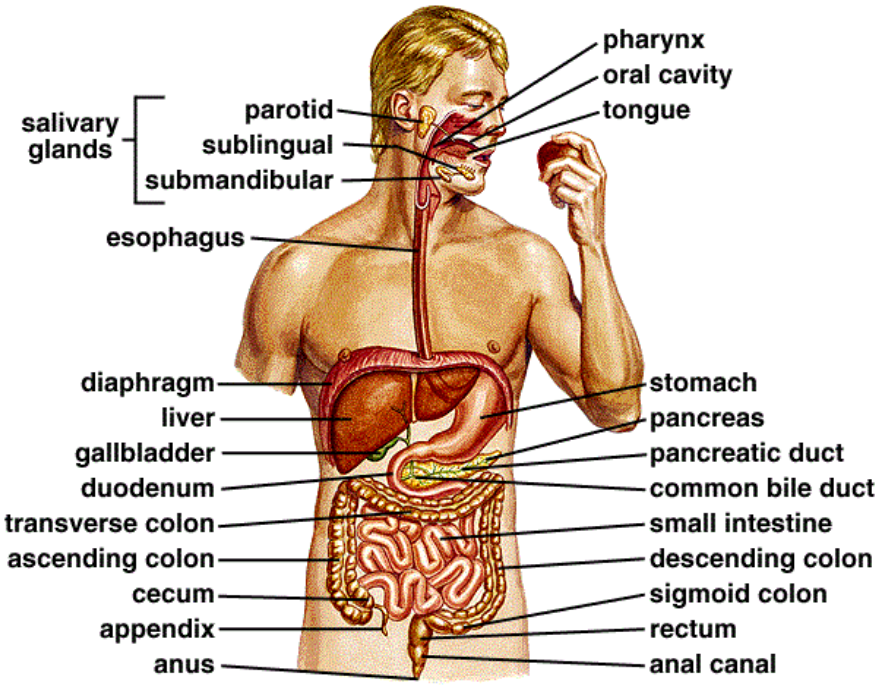
Melanomas is most commonly observed in derma cancers still it can occur anywhere which includes Colon or rectum.

Squamous Cell Carcinoma:

In the digestive system, esophagus and the lower part of anus are lined with squamous cell. Cancer which develops in such cells are known as squamous cell Carcinoma[16].

ANATOMY OF COLON

Figure 1: The normal digestive system



The large intestine, colon, rectum and anus are the parts of digestive system. The food we take is refined by stomach and small intestine for energy while the fluids are absorbed by the colon and rectum to form solid waste which is known as stool.

Large Intestine:-

Large intestine measures approximately 1.5 meters ileum of the small intestine merges in the large intestine. Ileocaecal valve which is present at the junction guards the entry of food in the colon. Large intestine resembles like small intestine but varies in diameter. The goblet cells secrete mucus and the lymph nodules are under the mucous membrane all along its course.

1) Caecum with its appendix
2) Ascending colon
3) Transverse colon
4) Descending colon
5) Sigmoid colon

Caecum:-

It is the first part of colon and is situated in the right iliac fossa. Ileocaecal function is guarded by the valve which allows the food to travel in one direction and prevents from back flow to the small intestine.

Ascending colon:-

It is located vertically on the right side of the abdomen. It merges with the transverse colon under the inner layers of the liver.

Transverse colon:-

It is located transversely below the stomach. It is suspended by its own mesentery from the posterior abdominal wall. Below the spleen it merges with the descending colon.

Descending colon:-

It is located vertically in the left side of the abdomen. It continues from the transverse colon and merges with the sigmoid colon.

Sigmoid colon:-

It is also known as pelvic colon because it lies in the pelvis. It also forms loop and has mesentery of its own.

Rectum:-

Rectum is a moderately distend section of the colon which measures approximately 13cm in length. It starts form sigmoid colon and conclude in the rectal canal.

Anal Canal:-

It is a short passage which guides the rectum to the exterior. Anus is controlled by sphincter muscles which is of two types. Internal sphincter is under the control of autonomic nervous system whereas external sphincter is under voluntary control[17].

RISK FACTORS OF COLON CANCER:

Family History and Age:-

If the colorectal cancer was diagnosed in the close blood relatives then the risk of colon cancer increases for the future generations. The risk for developing colon cancer increases with increasing age it most commonly occurs in adults over the age of 50 years but it may happen in younger age also.

Polyps:

A polyps of 1cm or more in the colon or rectum increases the risk of colon cancer as it usually starts in the inner linings of the colon. Tubular polyps are the most common with a less chance of becoming cancerous whereas villous polyps are less common with a high chance of turning into Cancerous growth.

Obese:

Colorectal Cancer transpire more often in people who are obese. Both men and women with higher body mass index (BM) increase the risk in men when compared to women.

Sex:-

Colorectal cancer incidence rate is high in men when compared to women. During the years 2000 to 2004, 69.2 per 1,00,000 population among men and 45.8 per 1,00,000 population among women.

Diet:-

It is considered as one of the most crucial step in occurrence of colon cancer. Researchers have found that high consumption of red meat and low consumption of fruits and vegetables which contains low fibre results in development cancer.

Smoking and Alcohol Consumption:-

Persons are at a high risk of developing various cancer who consume tobacco on daily basis. Colon cancer is among them. A meta analysis regulated by Botteri et al., based on survey studies it was reported that smokers are at a elevated risk of forming polyps. Consumption of alcohol is also considered to elevate the risk of developing colorectal cancer when compared to non consumption individuals[18].

STAGES OF COLON CANCER:

As cancer enhances from stage 0 to stage 4, the cells which are cancerous in nature originate in the walls of colon and further proliferate to other body parts such as lymph nodes, organs etc.,.

Stage 0:

In the innermost linings of the colon the abnormal cells are formed which in turn leads to cancerous cells and spread to nearby tissues. Such stage is known as carcinoma in situ.

Stage I:

Colon cancer which proliferate beyond the innermost layer of tissue to middle layers of colon. Such cancer is also well known as Dukes A colon cancer and it is recognised as stage-I.

Stage II:

It is also well known as Dukes B colon cancer, which is categorized into IIA and IIB.

Stage IIA:

In stage IIA the tissues affected by cancer nearby colon or rectum is recognised.

Stage IIB:

In this stage colon cancer has spread to nearby organs through the peritoneum and it is recognised as stage IIB.

Stage III:

Stage III is referred as Dukes C colon cancer which is split into stage IIIA, IIIB and IIIC.

Stage IIIA:

Colon cancer which started spreading from the underlying tissue layer to the middle layer and finally it has spread to the nearby lymph nodes. Such condition is recognised as stage IIIA.

Stage IIIB:

Colon cancer which has proliferated to nearby lymph nodes and also to the tissues of rectum or colon or beyond it into nearby organs. Such condition is considered as stage IIIB.

Stage IIIC:

In this stage cancer has escalate to most of the nearby lymph nodes and to nearby organs. Such condition is recognised as stage IIIC.

Stage IV:

It is also known as Dukes D colon cancer. Colon cancer which has spread to different parts of the body. Such as lungs etc., such condition is recognised as stage IV[19].

SURVIVAL RATE FOR COLON CANCER:

The number of relative survival rate has been estimated in table 2 from National Cancer Institute's SEER database for the years 2004 to 2010 .

Table 2. SURVIVAL RATE FOR COLON CANCER

Stage	Colon Cancer 5 years Relative Survival Rate	Rectal Cancer 5 years Survival Rate
I	92%	87%
II A	87%	80%
II B	63%	49%
III A	89%	84%
III B	69%	71%
IV	11%	12%

When compared to the above statistics patients with III A & III B, which also include III C has greater survival rate when compared to II A & II B[20].

DIAGNOSTICS:

Endoscopy:-

Endoscopy is a procedure in which Equipment with a inbuilt light is used to find abnormal changes in the body by inserting it into the body.

There are few types of endoscopic procedures to detect colorectal Cancers.

(i) Colonoscopy

(ii) Polypectomy

(iii) Flexible Sigmoidoscopy

(iv) Endoscopic ultrasound.

(v) Barium Enema.

Computed Tomography:

It helps to locate the tumour and its size and also during the treatment to assess the effectiveness.

MRI (Magnetic Resonance Imaging) it is used to distinguish between normal and diseased tissue and also to pinpoint cancerous cells. It gives more clarity and it is more safer when compared to CT Scan and X-Rays as it does not use radiation.

PET-CET Scan:

It is a most advanced nuclear imaging technology which provides a more detailed picture of cancerous tissue than any other test. The images of PET-CT provides a high level of accuracy.

Advanced Medical Treatment:

Colorectal Cancer Surgery:

It is the most Common treatment and the aim of this procedure is to remove tumors of colon as well as the surrounding normal tissue as precaution. After the surgery the patients are subjected to Chemotherapy or radiation or both depend as upon the stage of tumor. This is further divided into following types.

(i) Colectomy:

A Resection of Colon is known as colectomy. In this procedure, the cancerous portion and as well as a small portion of normal colon is removed as a precautionary method. The advance method of colectomy is laparoscopic method which provides faster recovery with less pain and with less incision around the abdomen when compared to surgical colectomy.

(ii) Colostomy:

Colostomy is performed when surgeon is unable to reconnect healthy portion of the colon and rectum. In this surgery, a healthy portion of colon is attached to the abdomen skin and an artificial opening called stoma is made outside the body. A colostomy bag is worn around the stomach to collect waste.

(iii) Hyperthermic Intrapertioneal Chemotherapy (HIPEC):

During the surgery, hyperthermic intrapertioneal chemotherapy is directly delivered to the abdomen as it improves the absorption of Chemotherapy drug by tumor and it destroy microscopic cancer cells that remain in the abdomen after surgery. It is mostly used for the patient who have advanced spread of Cancer within the abdomen. The main advantage of this procedure is that it minimizes the rest of the body exposure to chemotherapy and reduces some side effects of it.

Chemotherapy:

Chemotherapy is treatment of Cancer, using drugs which supress the cancer cell growth either by killing the cancerous cells or cease them from dividing. Chemotherapy drugs are administered by oral, subcutaneous or by intramuscular route depending upon the nature of the drug after which the drug invade the blood stream and can outreach cancerous cells throughout the body. In some cases, the chemotherapy drugs is deposited directly into the cerebrospinal fluid or cavity of body or organ so as to target the cancer cells in those

particular areas. Most of the chemotherapy drugs are derived from plants due to their less toxic effects. Chemotherapy is advised to the cancerous patient depending upon the severity, chemotherapy alone or in combination to radiation or chemotherapy, radiation and surgery is recommended. The treatment i.e., dosage and its schedule depends upon the type and stage of cancer diagnosed. and also upon the condition of the patient. It has different targets such as it may be used to cure the cancer, suppress its growth and spread or to provide comfort to the patient[21].

Common side effects of Chemotherapy:

Side effects depend upon the nature of the drug used to treat, though, certain types often have specific side effects but still it varies from patient to patient.

Fatigue:

Most of the cancer treated patient suffer with fatigue and is considered as the most common side effect[22].

Pain:

Chemotherapy drugs usually induce pain such as headache, muscle pain, stomach pain and nerve damage pain. However, this gets lesser with time[23].

Diarrhoea:

Chemotherapy drugs usually change the normal physiological pattern of the body as they are stronger in nature and lead to diarrhoea such as watery bowel movement[24].

Nausea & Vomiting:

Almost all the Chemotherapy drugs possess nausea and vomiting as their side effects. In order to avoid, the patient is given antiemetic drug along with chemotherapy drugs[25].

Constipation:

Chemotherapy patients are advised to have balanced diet with high amount of fiber content and also to have enough fluids along with enough exercise. As constipation is one of the common type of side effect[26].

Blood disorders:

In cancer treated patients, bone marrow is the first to get affected. It stops producing new blood cells. In order to find out the blood disorders, routine blood tests are done such as CBP, as-PI-RA-SHUN. However, in such cases the patients are put on alternative medicines to improve the results.

Sexual and Reproductive Issues:

Chemotherapy is not advised to pregnant patients as it may harm the fetus usually in the earlier trimester of pregnancy where the organs are still developing / growing. These drugs may also effect the fertility[27].

Appetite Loss:

Loss of appetite is the most common side effect of chemotherapy drugs. It leads to weight loss and loose muscle mass and strength which in turn leads to difficulty in recovering from the treatment[28].

Hair Loss:

Hair loss is usually noticed after few weeks of chemotherapy and it tends to increase as the duration of treatment increases[29].

Radiation Therapy:

Radiation is one of the routine treatment for cancer. To destroy cancer cells electro magnetic radiations such as X-Ray, Gamma Rays, Electron Beams are used. In most of the cases, radiation is given along with chemotherapy so as to enhance the treatment effect and in some cases if the cancer cells have spread to the surrounding organs or if the condition of the patient is not stable for surgery the radiation is the only left treatment. Radiation not only kills cancer cells but it also affects normal cells. It is given as a precautionary method and in spite of treating with radiation the tumors may reoccur. Radiation therapy schedule is mainly based on the type of cancer and its location. In colorectal cancer radiation therapy, high intensity radiations are passed through rectum for few mins. The plan of treatment in divided in to weeks. The main advantage is that it cause less damage to normal tissues as the radiation reaches directly to rectum. Such type of treatment is called as endocavitary therapy.

Radioactive Material are put into a tube and placed near or directly into the cancerous. To avoid its harmful effects on the surrounding healthy tissue such type of treatment is advised to the patients who are not fit for surgery due to various health conditions.

Skin and Rectal irritation, vomiting and nausea, fatigue are some of the common side effects observed by the patients during the treatment.

Targeted Therapy:

This type of therapy uses drugs or substances which helps in identifying and attacking specific Cancer cells without causing damage to normal cells. Monoclonal antibodies are

generally used as a targeted therapy for colorectal cancers. They are usually made from a single type of immune system cell. They attack the cancer cells by killing their growth[30].

Colorectal carcinogenesis:

The development of colon cancer occurs in two distinct pathogenetically pathways. Both pathways involve accumulation of multiple mutations stepwise. Anyhow, the mechanisms and the genes involve mutations is different.

1. APC/β: catenin is known as the first pathway which is depicted by its instability of chromosomes that lead to step wise gathering of mutations in a series of tumour onco and suppressor genes colon cancer is molecularly estimated with a chain of morphologically identified stages. In the beginning localised epithelial proliferation of colon along with the small adenomas that enlarges progressively and it will become more dysplastic and finally develops into cancer this sequence is known as adenoma carcinoma.

2. Loss of the APC tumour suppressor gene:

It is trusted to be the initial phenomenon in the formation of adenomas. In the FAP and Gardner syndromes, mutations of germ in APC gene ascend to large number of small adenomas which turns in the formation of carcinomas. APC genes duplications must be lost for adenomas to develop. The function of APC proteionre initially associated to β-catenin. APC encourages atrophy of β-catenin; with decrease in APC function, the collected β-catenin translocated to the nucleus and various genes transcriptions are activated, such as MYC and cyclic D1, as it promotes the growth of cell. Mutations of APC are available in 80% such as sporadic colon cancers.

3. Mutation of K-RAS:

The K-Ras gene conceal a signal transduction molecule that swing between an activated guanosine triphosphate-bound state and idle guanosine diphosphate-bound state. Mutated RAS is catched in an activated state that bring mitotic signals and stops apoptosis. The K-RAS mutation commonly follows the loss of APC. It is mutated in fewer that the adenomas which are above 1 cm are carcinogenic and the adenomas which are lesser than 1cm are not carcinogenic in nature.

4. Loss of 18q21 deletion:

Loss of putative cancer suppressor gene on 18q21 has been constituted in 60%-70% of colon cancers. Three genes have been portrayed to this chromosome location: DDC (deletion in colon carcinoma), DPC4/SMAD4 (deleted in pancreatic carcinoma) and SMAD2 conceal components of the transforming growth factor β (TGF-β) signalling pathway. Because TGF-β signalling inhibited the cycle of cell, the loss of these genes may permit unrestrained growth of cell which may be allowed by loss of these genes.

5. Loss of TP53:

Loss of tumor suppressor gene is notable in 70%-80% cancers of colon yet similar loss of rare adenomas, indicating that mutations in TP53 happen late in colorectal carcinogenesis.

6.Microsatellite instability pathway:

The subsequent pathway is distinguished by the genetic lesions in DNA mismatch repair genes. It is involved in 10-15% of sporadic cases. As in the APC/β- catenin schema, involved genes are unlike in nature and mutations are accumulated the, and, unlike in the most probably starting off event in colorectal cancer of this kind. Inherited mutations where DNA imbalance repair genes (MSH2, MSH6, MLH1, PMS1 and PMS2) give raise to hereditary nonpolypolips carcinoma. Loss of DNA imbalance repair genes guides to a hypermutable state in which simple reactive DNA sequence is unsteady during DNA replication, gives rise to widespread modifications in these repeats. The resulting microsatellite uncertainty is the evidence of molecular defective DNA imbalance repair and therefore this pathway is also known as MSI pathway. Most microsatellite succession are in non coding portions of the genes, hence mutation in these genes are mostly harmless. However, some microsatellite succession are discovered in the coding or genes of promoter region are participated in cell growth regulation. Such genes incorporate type II TGF-β receptor and BAX. TGF-β signalling hinders colonic epithelial cell growth, and the BAX gene causes apoptosis. Loss of imbalance repair leads to collection of mutations in these and other growth regulator genes, crowning in the disclosure of colorectal cancer[31].

Oxidative Stress:

The variation of cellular redox is induced by oxidative stress is usually noticed in cancer cells in comparison with regular cells and it may be due to stimulation of oncogene permanent genetic modification due to oxidative damage as it exhibits the first step involved in mutagenesis, carcinogenesis and ageing. DNA mutation is a censorious step in cancer and in increased oxidative levels, where rupture of DNA is found in tumours resulting in destruction of the cancer etiology. ROS-induced DNA damage includes, DNA breakdown, adenine-guanine, cytosine-thymine and DNA cross-links. Carcinogenic injury of DNA results in initiation of signal and m-RNA, fallacy and hereditary imbalance. The generation of 8-hydroxyguanine is most extensive studied DNA lesion. Easy formation of this lesion makes it potentially cancerous biomarker. Damage of DNA, mutations and restyle gene expression are the important steps of carcinogenesis whereas the oxidants involved appears to be common for all.

Reactive nitrogen species, like peroxynitrates and oxides of nitrogen have also caused in DNA destruction. Peroxynitrate has been shown to form 8-nitroguanine upon reaction with guanine and its structure is likely to induced G: C-> T:A transversion while the lesions solidity is low in DNA where as in RNA it is stable due to nitrogen adduct. The future connection between 8-nitrogenguanine and carcinogenesis procedure is unknown. Oxidative damage of DNA caused due to nucleus is thoroughly studied and there are also proofs of damage caused due to mitochondria in neoplasia [32].

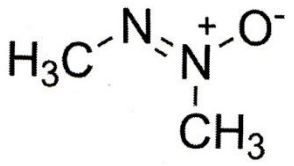
Human cancers show changed expression in gene encoding for complexes (I to V) and in hyper variable regions of mitochondrial DNA. Activation of nuclear genes which are implicated in generation of mitochondria and its genome involves the reaction between H_2O_2 and oxygen species which are reactive. Mutation of mitochondrial DNA and its alterations in cancer process does not provide satisfactory information. This link testify the fact that DNA fragment of mitochondria have been attached with DNA of nucleus, this facilitates the process of oncogenesis. Once formed, lipoperoxyl radicals by using cyclization process at can be rearranged to endoproxides with the last product of peroxidation process it is malondialdehyde. 4-hydroxynonenal is the utmost product of peroxidation of lipid other than malondialdehyde and it has strong effects on signal transduction pathways whereas malondialdehyde reacts with DNA basis Guanine, Adenine and Cytosine to construct adducts such as M1G, M1A, and M1C respectively.

The role of oxidative stress in colorectal cancer development was studied by several workers. The gastrointestinal tract inflammation leads to increase the risk of cancer development which is an incompletely understood pathway, which may involve microsatellite instability and its low frequency is referred as MSI-L" occurs frequently in chronically inflamed non neoplastic tissue. It has been reported that the cells obtained from the neoplastic tissue showed greater oxidative DNA damage when compared to the cells of normal tissue. The cell isolated from the mucosal tissue of the colon proved greater DNA breakage strand when compared to the cells isolated from normal tissue.

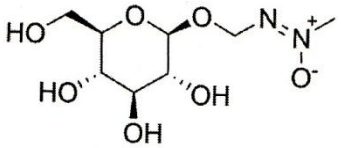
Chemical Carcinogenesis:

The synthetic compounds 1, 2-dimethyl hydrazine and azoxymethane, have proved to be of great value in their reliable and specific ability to produce colon tumors in several rodent species. Both of these chemicals are chemically associated to the naturally occurring carcinogen CYCASIN. With animal models such as these, it becomes possible to examine changes in the beginning and later on development of colon cancer under strictly controlled laboratory conditions, and thus by drawing appropriate analogies, to approach the understanding of etiology and also to attain goals of prevention, diagnosis and better management of colon cancer in man[33].

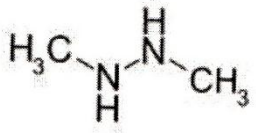
Figure 2: Chemical carcinogens.



Azoxymethane



Cycasin



1, 2-dimethyl Hydrazine

Biochemical mechanisms of chemical carcinogenesis:

Biotransformation is of primary concern to toxicology because the biological activity of chemicals are enhanced or decreased by this process. The chemical biotransformation generally leads to the formation of more polar metabolites that are more readily excreted. Biotransformation pathways is of two types, called phase I and phase II reactions. Phase I reactions incorporate reduction, oxidation and hydrolysis chiefly catalysed by cytochrome P 450. Phase II reaction (synthetic) involves the conjugation of chemicals with hydrophilic

moieties such as glutathione, glucuronides, sulphates or aminoacids by enzymes of wide range including glutathione S-transferases, glucuronyl, transferases and a number of sulphotransferases. [34]

1,2-Dimethyl hydrazine- Chemical and physical data:

Nomenclature:

Chem. Abstr. Serv. Reg. No.	**:**	540-73-8
Chem. Abstr. Name	**:**	1, 2-Dimethylhydrazine
IUPAC Systematic Name	**:**	1, 2-Dimethylhydrazine
Synonyms	**:**	DHM; hydrazomethane
Structure	**:**	C2 H8 N2
Relative molecular mass	**:**	60.10

Description**:** Flammable, hygroscopic liquid. Fumes in air and gradually turn Yellow. Characteristic ammonia-like odor of aliphatic hydrazine's. [35]

(a) Boiling-point: 810C. [36]

(b) Melting-point: 90ºC

(c) Solubility: Miscible with water with much evolution of heat. and also with ethanol, diethyl ether, and other hydrocarbons.

(d) Vapour pressure: 9 kPa at 24.50C

The term "hydrazines" is a generic name of three structurally related chemicals: hydrazine, 1, 1-dimethylhydrazine and 1, 2-dimethlhydrazine. Hydrazines are colourless and volatile liquid chemicals which contain two nitrogen atoms joined by a single covalent bond. Chemicals are used to manufacture hydrazines such as ammonia, dymethylamine, hydrogen peroxide or sodium hypochloride. They can easily evaporate in air and can be inhaled[37]. Chemically induced colon cancer in animals have provided more detailed pathogenesis, development and modulation of this disease. Dimethylhydrazine (DMH) and its metabolites, azoxymethane (AOM) and methylazoxymethanol (MAM) have been used to study colon

cancer in susceptible animals. [38] The primary target for DMH however, appears to be the colon, as this compound primarily induces colonic tumors [37].

Mechanism of action:

1,2-Dimethylhydrazine is metabolized by a series of steps of oxidation, first it leads to dehydrogenation to azomethane and then N-oxidation of this to azoxymethane and finally a C-oxidation to methylazoxymethanol.[39,40] This last metabolite decay to give methyldiazonium ion (highly reactive) to which the compound toxicity has been assessed. The continuous nature of these steps of oxidation has been shown in the isolated perfused rat liver manifest that the C-oxidation of azoxymethane to methylazoxymethanol is assembled by hepatic microsomes[41], while Schoental (1973)[42] found that methylazoxymethanol was transformed to the corresponding aldehyde by an NAD-dependent dehydrogenase.

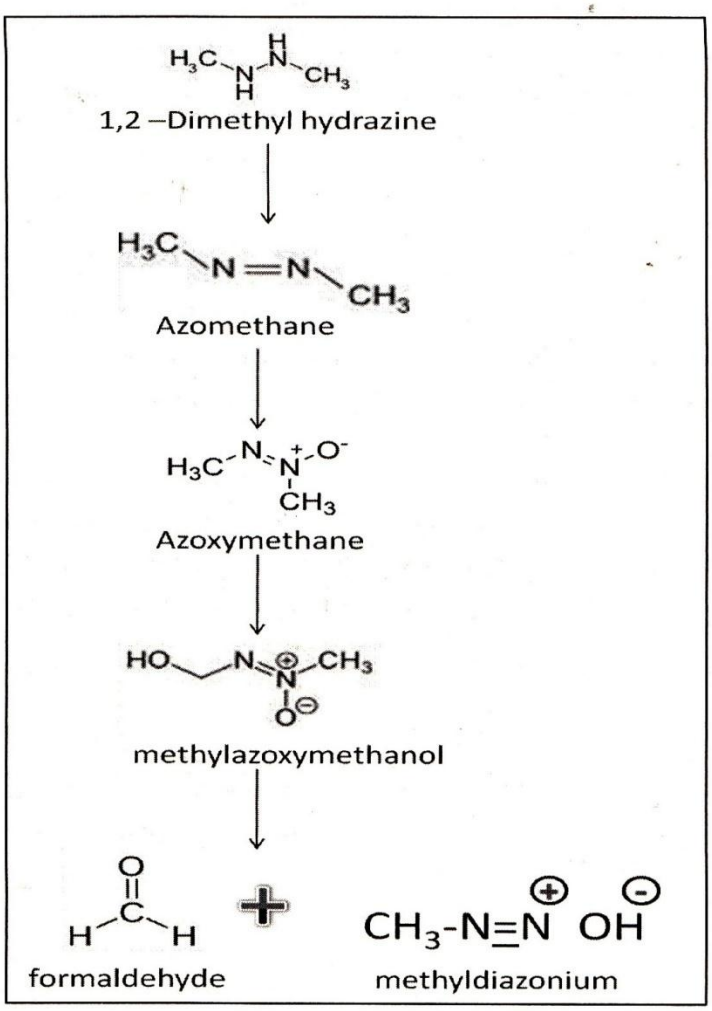


Figure 3: Mechanism of action of 1,2-Dimethylhydrazine

In inclusion to this pathway of metabolism and activation, methyl radical intermediates may also be intricate in the toxicity and metabolism of 1,2- dimethylhydrazine catalysed by haemoglobin, peroxidises and cytochrome P450.[43] A further pathway of 1, 2-dimethylhydrazine metabolism is N-demethylation, yielding monomethylhydrazine and formaldehyde. This can be catalysed by the mitochondrial enzyme monoamine oxidase[44] and, most probably, by microsomal cytochrome P 450 [45]

Table 3: Some plant derived chemo preventive agents[46]

Herb	Active Component	Molecular Mechanism of action
Turmeric	Curcumin	Apoptosis
Chasterberry/vitex	Extract polysaccharides	Apoptosis
Echinacea	Flavonoids, glycoproteins	Apoptosis
Evening primerose	Extract	Apoptosis
Fever few	Parthenolide	Growth inhibitor
Garlic	Organic sulphurs	Cell cycle, p 450 inhibitor Cox-2 inhibitor, anti-oxdant
Ginkgo biloba	Gingkolides	Anti-inflammatory, p 450 inhibitor cox-2 inhibitor, anti-oxidant
Ginseng	Ginsenosides	Inhibits fewer precancerous lesions
Hawthorn fruit	Proanthrocyanides, Flavonoids	Anti-oxidants
Liquorice	Unknown	Apoptosis, cox-2 inhibitor, topoisomerase II - inhibitor anti-oxidant
Milk thistle	Unknown	Cox-2 inhibitor
Saw palmetto	Unknown	Apoptosis, growth inhibitor
Senna	Anthraquinone	Fewer precancerous lesions
Soy	Flavonoids, genistein	Topoisomerase II inhibitor, cell cycle arrest
St john's wort	Hypericin, Rubin, Quercetin	Anti-oxidant, apoptosis, anti-proliferative
Yohimbine	Indole alkaloids	Growth inhibitor

1.3 ANTIMICROBIAL ACTIVITY:

INTRODUCTION TO MICROBES:

All living organisms, large and small, have one thing in common; the cell. Microbes are Lilliputian which is abundantly found on earth. They survive everywhere and in every climate – In rock, in soil, in air and also in water. Some microorganisms require oxygen and others does not require. The organisms are even present in plants, in animals and in human beings. They eat and grow and also they reproduce and die.

The relationship between microorganisms and human is unique and complex, as some microorganisms cause diseases in human while others are crucial for a healthy life, and Indeed the fact of life is that we could not exist without them for example, anaerobic bacteria, such as Lactobacilli acidophilus, at they exist in our intestines without harming, as it also help in digestion of food, supply of vitamins, ruin disease-causing microbes and also help in fighting against cancer cells. It is further to 4 groups such as bacteria, fungi, viruses and protozoa[47,48].

2. AIM OF THE STUDY:

This aim of this work is to deliberate for suggesting a drug to human inhabitants of the earth that can be useful with less side effects using a natural plant predominately available in developing countries where leading cause of death are cancer, unintentional injuries and cardiovascular diseases.

There are several types of cancer reported among which colon cancer is under top three [49].

Our current understanding evince that colon cancer is a disease of disrupted growth control. Screening of colon cancer and awareness can reduce mortality of colorectal cancer by uncovering the cancer at an earlier stage, where therapy of treatment has higher success rate.

The aim of the study is to evaluate *Portulaca quadrifida* for Anti oxidant and Anti colorectal cancer activity of the aqueous extract on 1, 2-Dimethyl Hydrazine induced colon cancer using rat model.

OBJECTIVE:

Lots of chemotherapeutic, cytotoxic and immune modulatory anticancer drugs are accessible in the market but the main issue associated is their serious side effects and morbidity. Because of chemotherapy treatment side effects, the patient needs secondary palliative care treatment. Still the hunt continues for an ideal treatment with less side effects and also cost effectiveness.

Portulaca quadrifida (*Portulacaceae*) is a compact, spread, annual and upright herb. The plant is acidic, tart, aromatic in taste, purgative, causes crankiness and "Kapha"; cures high temperature, respiratory problem, urinary disorders, swelling, ophthalmic disease, dermal ailments and gastric lesions. In India and China the leaf decoction is used to treat abscess and as a lotion; a soup is given in diarrhoea. In Nigeria it is used to swellings.

Plant medicines are well known for their non-toxic side effects, so the objective of the study to develop anticancer drug with antioxidant activity from the medicinal plant *Portulaca quadrifida* against DMH induced colon cancer was planned.

3. REVIEW OF LITERATURE

3.1 PLANT PROFILE

Figure: 4 ***Portulaca quadrifida*** **LINN**

3.1.1 DESCRIPTION:

Portulaca quadrifida: A small herbivore plant available in all climate of the year which is especially found in states like Andhra Pradesh, Tamil Nadu and Karnataka.

Classification for Portulaca quadrifida (Linn.)

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Caryophyllidae
Order	:	Caryophyllales
Family	:	Portulacaceae – Purslane family

Genus : Portulaca L. - purslane

Species : Portulaca quadrifida L. – chickhenweed.[50]

3.1.2 VERNACULAR NAMES

Telugu : Goddupavili

Kannada : Halibachcheli,

Hindi : Chaunlayi,

Marathi : Kathechanval

Sanskrit : Laghughonika,

Tamil : Sinnaparpukkirai

3.1.3 OCCURENCE AND DISTRIBUTION

Portulaca quadrifida: It is widely distributed in India and grown commonly in wet lands, irrigated lands and in lands beside river bank. It is cultivated in some parts of India for utilization as food.

3.1.4 MORPHOLOGY:

The leaves of *Portulaca quadrifida* are opposite and freshly

Shape : Ovate

Height : 3 to 6mm

Flowers : Terminal, solitary, surrounded by silver hair

Seeds : Minutely tubercled

3.1.5 DISTRIBUTION:

Throughout the warmer parts of India and Ceylon- tropical Asia and Africa.

3.1.6 CHEMICAL CONSTITUENTS:

Hexacosanyl acetate, eicosanoic acid, cerotin, palmitic, β-sitosterol, β-amyrin, acyclic long chain aliphatic ester heptacosanyl valerate and sugars.

3.1.7 USES:

It is used in ayurvedic medicines (Kapha), cures low fever, asthma, cough, urinary dischargers, inflammation, good for eye disease, skin disease and ulcers.[51]

3.2 PHYTOCHEMICAL AND PHARMACOLOGICAL REVIEWS:

3.2.1 PRELIMINARY PHARMACOGNOSTIC AND PHYTOCHEMICAL ASSESMENT OF *PORTULACA QUADRIFIDA* LINN.[52]

.Mulla *et al.,* (2010) has undertaken preliminary pharmacognostic and phytochemical assessment of whole plant of *Portulaca quadrifida* Linn. In this work the author subjected *Portulaca quadrifida* Linn. In successive soxhlet extraction using solvents like hexane, chloroform, methanol and finally macerated with water. Methanol and aqueous extracts showed positive result for secondary metabolites as compared to hexane and chloroform. The authors concluded these metabolites may be useful in many aliments.

3.2.2 NEUROPHARMACOLOGICAL RESULTS OF *PORTULACA QUADRIFIDA* LINN[53]

Mulla *et al*., has investigated the study of ethanolic extract in which some neuro pharmacological results of *Portulaca quadrifida* Linn were studied into various models of mice. The phytochemical study showed the visible of organic compounds. The ethanol extract showed reduction in time to recover from electrically induced convulsion as well as extracts reduced spontaneous motor activity but effects were not significant on gripping strength. According to authors the ethanol extracts on central nervous system was significant but it has not showed significant effects on peripheral nervous system.

3.2.3 ANTIMICROBIAL ACTIVITY AND IN VITRO ANTICARCINOGENIC PROPERTIES OF '*PORTULACA QUADRIFIDA* LINN'ON COLON CANCER USING DIFFERENT EXTRACTS.[54]

In my (Khasim *et al*., 2015) present study chloroform extract (CHCL3), methanol extract (CH3OH) and aqueous extract of *Portulaca quadrifida* on cell lines HT-29 of human colon was investigated by MTT assay and extracts were also investigated for antimicrobial activity following agar well diffusion method. In conclusion, my study indicates that among

all the extracts, the aqueous extract of *Portulaca quadrifida* had antiproliferative effects in vitro inducing HT-29 cells and it also showed remarkable antibacterial activity towards *Escherichia coli* and *Staphylococcus aureus* and antifungal organisms like *Aspergillus flavus* and *Aspergillus niger* when compared to standard.

3.2.4 A PROTECTIVE STUDY OF ETHANOLIC EXTRACT OF PORTULACA QUADRIFIDA LINN. ON PARACETAMOL INDUCED HEPATOTOXICITY IN RATS[55]

Mrinmay Das *et al.*, (2014) investigated ethanolic extract of *Portulaca quadrifida* Linn. (Portuleacea) aerial parts for its *In vitro* and *In vivo* antioxidant and hepatoprotective effects. According to author, the ethanolic extract of *Portulaca quadrifida* is reported to be rich in saponins. Presence of saponins in the ethanolic extract was confirmed by preliminary analysis and according to them the antioxidant property of extract is possessed by saponins. Oxidative stress plays major role for initiation and progression towards liver disease. As *Portulaca quadrifida* contains large amounts of saponins, it may be due to presence of saponins which may be responsible for the hepatoprotective activity of the extract. The protective activity of plant against oxidative damage in paracetamol induced hepatotoxicity may be due to action on lipid peroxidation and enhancing effects on cellular antioxidant.

3.2.5 COMPARISION OF PHARMACOGNOSTIC DATA ON 3 SPECIES OF *PORTULACA QUADRIFIDA*.[56]

Netala *et al.*, have compared the properties of 3species of Portulaca. According to author the plant is used as vegetable and contains a bulk amount of calcium oxalate crystals almost in all parts of plant. Quantitative and Qualitative screening showed the presence of secondary compounds like saponins, alkaloids, carbohydrates, steroids and triterpenoids. The authors have concluded the results of the research will be beneficial in setting quality parameters for the finding of detailed written study.

3.2.6 APPLICATION OF *PORTULACA QUADRIFIDA* AS ANTICANCER ON HUMAN COLON CANCER CELL LINES USING TWO EXTRACT LIKE ETHANOL AND POLYPHENOL.[57]

Mulla *et al.*, studied the effects of ethanol and polyphenol extracts of *Portulaca quadrifida* on Human colon cancer using HT-29 and a normal L-6 cell lines using MTT assay and Trypan blue dye exclusion assay behind DNA fragmentation assay. Both extracts

decreases the proliferation of HT-29 cell lines and are found less effective against L-6 cell lines indicating the cancer effect of *Portulaca quadrifida*.

3.2.7 IN VIVO ANTICANCER SCREENING OF '*PORTULACA QUADRIFIDA* LINN'ON COLON CANCER[58]

The characteristics view of my (Khasim S.M, *et al*., 2015) present study for *In vivo* study, colon cancer was induced with the chemical carcinogen (DMH) and after treatment with the aqueous extract of *Portulaca quadrifida* for 16 weeks, the various *In vivo* parameters were evaluated. The *In vivo* antioxidant levels, carbohydrate metabolizing enzymes were increased in compound plant extract treated groups compared to control animals in a dose dependent fashion. *In vivo* data thus obtained suggested that aqueous extract of *Portulaca quadrifida* is having good activity against the various biochemical parameters as compared with negative control group. The activity may be due to saponins content. Histopathalogical studies with aqueous extract of *Portulaca quadrifida* at 400mg/kg body weight showed no obvious abnormality in structure of colonic mucosa, which was very well comparable with biochemical, haematological, antioxidants and tumor markers estimation. With the above said findings we concluded that the plant *Portulaca quadrifida* posse's anti colorectal cancer activity, before its clinical usage, through toxicological profile has to be determined to confirm the safety of the drug.

SCOPE AND PLAN OF WORK

SCOPES OF THE STUDY:

Although, plants are existing from the history of the evolution of land. Plants in this modern world are respected and appreciated because of extensive role in almost all aliments. Many plants are claimed to have medicinal values but still many plants are unexplored and many researches are going on many plants.

Plant medicines are well known for their non-toxic side effects, so the main scope of the study is to develop anticancer drug with minimal side effects/no side effects.

The literature survey unveil that no technical study was carried out on *Portulaca quadrifida* to exhibit its anticancer activity. Therefore it is an attempt to explore anticancer activity of the selected plant.

PLAN OF WORK:

The present study was carried out as following:

1. Collection and authentication of plant.
2. Successive solvent extraction of plant.
3. Preliminary phytochemical screening.
4. *In vitro* Antioxidant study.
5. *In vitro* cytotoxicity study.
6. Antimicrobial activity.
7. Acute toxicity study.
8. *In vivo* anti- colorectal cancer screening using rat model.

a) Serum biochemical parameters.
b) Estimation of enzymic and non enzymic antioxidants.
c) Extraction and determination of faecal bile acids.
d) Extraction and determination of faecal neutral sterols.
e) Estimation of Tumor markers.
f) Evaluation of colon cancer by aberrant crypt foci.
g) Histopathological study of colon.

9. Isolation of active compound.
10. *In vitro* cytotoxicity study by MTT assay using different cell lines like HT-29 and HCT-116.
11. Structural elucidation by GCMS.

MATERIALS AND METHODS

5.1 Plant collection:

Freshly collected whole plant of *Portulaca quadrifida* Linn from the local fields of Hyderabad washed with tap water to remove the dust and soil. The whole plant was dried under shade, powdered and made to pass through sieve No. 40 meshes and stored in closed vessel. The plant specimen was identified and authenticated by Prof. Dr. (Mrs.) Pratibha Devi Department of Botany, Osmania University, Hyderabad. A voucher specimen is stored in the herbarium (Voucher No.024), Department of Botany, Osmania University Hyderabad.

5.2 PHYSICOCHEMICAL CHARACTERS OF CRUDE DRUG PORTULACA *QUADRIFIDA LINN.*:

1) DETERMINATION OF ASH VALUES[59]

Naturally obtained drugs such as vegetables, plants etc., are subjected to ashing so as to eliminate the organic content or else it may hinders in determination of analytical values. Normally naturally occurring drugs from plant exhibit ash content such as potassium, sodium etc.,.Crude drugs total ash values represents the quality of preparation and the care taken during the procedure. If silica or high content of calcium oxalate is present high range of acid insoluble ash is imposed

a) Determination of Total Ash:

- 30gm of the powdered drug was weighed accurately.
- It was heated at 450 ^{0}C for 4 hrs until it was devoid of carbon, cooled and weighed.
- With reference to air-dried drug the percentage of ash was evaluated .

b) Determination of Acid-insoluble Ash:

- Ash was boiled with 25 ml of 2M HCl for 5 min.
- Indissoluble matter in an ashless filter paper was collected, washed with hot water, ignited for 3-4 hrs, cooled in a desiccator and weighed.
- With reference to the air-dried drug the acid-insoluble ash percentage was estimated.

c) Determination of Water-soluble Ash:

- 25 ml of water was boiled with ash for 5 min.
- Collect the indissoluble matter on an ashless filter paper or in Gooch crucible, then wash with indissoluble matter from the total weight of ash, hot water, and for 15 min at a temperature ignite it not exceeding 450 ^{0}C.
- The difference in weight represents the water-soluble ash by subtracting the weight .
- With reference to the air-dried drug the percentage of water-soluble ash was calculated.

2) DETERMINATION OF LOSS ON DRYING:

- 2gm of powdered drug is accurately weighed and taken in Petri dish, which is uniformly distributed.
- The sample is kept for drying in oven for 4 hrs at a temperature less than 110 ^{0} C constant weighed was achieved.
- Then it was stored in desiccators and was cooled at room temperature, then weighed, the weighed was recorded.

Using the formula LOD was calculated:

Loss on drying = Loss in Weight of the Sample/ Weight of the Sample x 100

3) DETERMINATION OF EXTRACTIVE VALUES:

i) Alcohol-soluble Extractive:

- 5 gm coarse powdered was macerated in 100ml of C_2H_5OH (90 %) for 24 hours in a flask with rubber stopper, frequent shaking was required during first six hours.
- It was filtered using filter paper rapidly, taking preventive measure to bar the alcohol loss.
- 25ml of alcoholic extract was vaporized, dry at 105 ^{0} C and weigh.
- Percentage w/w ethanol soluble extract (90%) was calculated with reference to the air-dried drug.

ii) Water-soluble Extractive:

The above procedure was followed using I.P. instead of alcohol water is used.

iii) Hexane soluble Extractive:

The above procedure was followed using hexane as the solvent.

iv) Chloroform soluble Extractive:

The above procedure was followed using solvent as $CHCl_3$.

v) Alcohol soluble Extractive:

The above procedure was followed using alcohol as the solvent.

Results were tabulated (Table 4)

5.3 PHYTOCHEMICAL INVESTIGATION OF THE LEAVES OF *PORTULACA QUADRIFIDA* LINN.

5.3.1EXTRACTION PROCEDURE[60]

Successive Solvent Extraction:

The drug was subjected to systematic phytochemical screening by successively extracting with various organic solvents of increasing polarity and subjected to phytochemical investigation by qualitative chemical analysis thin layer chromatography techniques, Spectral measurement, etc.

Preparation of Plant Extracts:

Portulaca quadrifida was collected and shade dried, powdered in a mixer and sieved, powder of required particle size was obtained (sieve no 40).

The powder material was extracted in soxhlet using successive solvents of increasing polarity such as chloroform, methanol and aqueous for 48 hours using soxhlet apparatus. The filtrates were collected and evaporated to dryness under reduced pressure using a evaporator (Rotary flash). The extracts obtained was weighed from each solvent; calculated percentage

of plant air-dried weight. The dried extracts were preserved at 4˚C in small sterilized containers, for further use.

Preparation of Chloroform extract:

The air dried powder from the above process was extracted successively with Chloroform to get Chloroform extract. The marc was collected, dried and used for preparing further extraction with methanol. The percentage yield was calculated and tabulated (Table 5).

Preparation of methanol extract:

The dried marc from the above process was extracted successively with methanol to get methanolic extract. The marc was collected, dried and used for further investigation. The percentage yield was calculated and tabulated (Table 5)

Preparation of Aqueous extract:

2000 gm of powder were taken in a beaker (5000ml) and macerated with 3000ml of distilled water and 100 ml of Chloroform (preservative) for 7 days shaking daily in a closed vessel. After this extraction, the marc was separated and subjected to concentrate at 50^0C on a water bath and a semi solid mass was obtained. The percentage yield was calculated and tabulated (Table 5)

These three extracts were stored in air tight container and refrigerator. All the extracts were examined for their colour and consistency.

5.3.2 QUALITATIVES TEST[61]

The preliminary test analysis was carried out on chloroform, alcohol and water extracts of *Portulaca quadrifida.* For identification of various active ingredients.

1. Chemical tests for Carbohydrates:

Molisch's (General test): To 2-3 ml of extract add few drops of alpha-naphthol, it is shaken well then add concentrated H2SO4 from sides of the test tube. Violet ring was noticed

at junction of two liquids

Tests tor Reducing Sugars:

a. Fehling's reagent test: For one minute boil 1ml of Fehling's A and B then add some amount of test solution and again boil for 5-10 min in water bath then notice for yellow colour, then precipitate of brick red.

b. Benedict's reagent test: Mix equal volume of test solution and benedict reagent was taken in test tube and heated for 5 min on water bath. Depending on amount of reducing sugar in extract the solution may appear green, yellow or red.

Chemical tests for Monosaccharide:

Barfoed reagent test:

Heat equal amount of test solution and barfoed reagent on water bath for 1-2 min, then cooled it and then notice for any red precipitate.

Test for Hexose Sugars:

Test for Cobalt-chloride: Cool 3 ml of test solution with 2ml cobalt chloride then on NaOH solution added FeCl3 drops. Presence of Glucose is observed as greenish blue and Fructose is observed for purplish colour and mixture of glucose and fructose is observed as greenish blue on upper layer and lower layer purplish for fructose.

Non-Reducing Sugars test:

a. For presence of non-reducing sugars test like fehling's and Benedict's were conducted.

b. Test for Starch using tannic acid: Extract solution was observed for precipitation against 20% tannic acid.

2. Protein tests:

a) Biuret (General test): To 3 ml of test solution add 4% NaOH and add few drops of 1% CuSO4 and then notice for violet or pink colour.

b) Millon's reagent test: White precipitate appears when 3ml test solution was mixed with

5ml Millon's reagent. In observation precipitate converts to brick red colour.

c) Xanthoprotein test (For tyrosine or tryptophan containing protein): A white precipitate is observed when 3ml extract solution was added with 1ml concentrated sulphuric acid.

d) Protein containing sulphur test: 5ml of extract solution was mixed with 2 ml 40% NaOH and 10% lead acetate solution of 2 drops is added. , Solution turns black or brownish after boiling due to PbS formation.

e) Chemical test for precipitation: Test solution was added to the following reagents and any presence / absence of precipitate was observed:

i. Absolute alcohol

ii. 5% **mercuric chloride** solution

iii. 5% **Copper sulphate pentahydrate** solution

iv. 5% lead acetate solution

v. 5% ammonium sulphate solution

3. Chemical Tests for Steroids:

a) Salkowski Reaction: To 2ml extract solution, about 2ml chloroform and 2 ml of concentrated H2SO4 was added and Red colour was observed for chloroform layer and acid layer for fluorescence.

b) Liebermann-Burchard Reaction: Chloroform was mixed with 2ml of test solution and then add 1-2 ml acetic anhydride and 2 drops of concentrated H2SO4 from one side of test tube and notice for red, blue and green colour one after another.

c) Libermann reaction test: 3 ml acetic anhydride was mixed with 3ml of extract solution and heated then cooled and then add few drops of concentrated sulphuric acid which gives blue colour.

4. Amino Acids test:

a) Ninhydrin solution test (General test): To 3ml of test solution add 3drops of 5% Ninhydrin solution and heat on H2O bath for 10 min. It gives purple or blue colour.

b) Tyrosine test: 3 drops of Million's reagent indicating presence of Tyrosine was added to 3ml of test solution and heated. Solution turns dark red colour in observation.

c) Test for tryptophan: A few drops of glyoxalic acid was added to test solution (3ml) and then add concentrated H2SO4 was added. A ring of reddish violet colour was noticed at junction of two layers.

5. Glycosides test:

1) General test:

Part A:

Take 200 mg of extract with 5ml dilute (10%) H2SO4 in a test tube on a water bath at temperature of 1000 C for 2 min then it was centrifuged or filtered it then pipette off supernatant (or filtrate). The acid extract was neutralized with 5% sodium hydroxide and volume of NaOH was added and then going on add 0.1 ml of Fehling A solution and then Fehling B solution until it become alkaline (test with pH paper) and heated on a water bath for 2min. Red precipitate formed was noted and compared with that was formed in part B.

Part B:

To extract of 200mg used 5 ml of water was added in substitute of sulfuric acid. It was boiled and water was added equal volume of NaOH used in the above test. To this 0.1ml of A and B fehling's solution were added till it convert to alkaline in nature (tested with pH paper) then heat on a water bath for 2min. The red precipitate was noticed.

The presence of absence of glycobide will be noted it after comparing precipitate formed in Part B which was lesser then that of formed in part A. Since part-B represents free reducing sugar, whereas part-A represents free reducing sugar plus which resulted on acid hydrolysis in the drug.

2) Test for Free Sugar:

The extract was hydrolyzed with mineral acid after complete removal of free sugar and then moieties V12 (glycone and aglycone) were tested.

a) Raymond's test: Violet colour appeared when extract solution was treated with dinitro benzene in hotted methanolic alkali.

b) Legal's test: Blood red colour appeared when extract was treated with pyridine along with alkaline solution of sodium nitropruside.

c) Bromine water test: When bromine water was treated with test extract it gives precipitate which is yellow in colour.

3) Chemical Tests for Specific Glycosides:

Cardiac Glycoside tests:

a) Baljet's test: Yellow to orange colour appears when test extract was treated with sodium picrate.

b) Legal's test (For cardenolides): 1 ml of pyridine and 1ml of sodium nitroprusside were added to aqueous and alcholic solution. Pink colour changes to red colour.

c) Keller Killiani test: Glacial acetic acid was added to 2ml of test extract and one drop of $FeCl_3$ 5% and H2SO4 concentrated. At the junction of the two liquid reddish brown colour appears and upper layers viewed as bluish green.

d) Libermann's test: To 3 ml extract solution add 3ml of acetic anhydride and heat and cool and add some drops of concentrated H2SO4, a blue color appears as observation.

Tests for Saponin Glycosides:

a) Foam test: Persistent foam was observed when the test extract was vibrated forcibly with water.

b) Haemolytic test: To 1 drop of blood was added test solution settled on glass slide. Haemolytic zone presence was noticed.

Tests for Coumarin Glycosides:

A blue or green fluorescence was observed when test solution was made alkaline.

6. Tests for Flavonoids:

a) Shinoda test: To 5 ml of 95% ethanol was added to dried powder and then included some drops of concentrated HCl and magnesium turnings(0.5 g), a pink colour was noticed finally.

b) To little quantity of residue solution lead acetate was included and a precipitate of yellow colour was noticed.

c) Sodium hydroxide when added in increasing amount to the remains it showed yellow colour, which was lightened by inclusion of acid.

d) Ferric chloride test: Some drops of ferric chloride solution was added to test solution. An intense green colour was observed.

7. Tests for Alkaloids:

a) Dragendroff's test: Orange brown precipitate was observed when dragendroffs reagent was added to 2-3 ml of test solution.

b) Mayer's test: To test solution of 2-3 ml few drops of Mayer's reagent was included and noticed for precipitate.

c) Hager's test: To test solution of 2-3 ml few drops of Hagers reagent was included, yellow precipitate is noticed.

d) Wagner's test: To filtrate of 2-3 ml few drops of wagner's reagent was included, reddish brown precipitate was noticed.

8. Tests for Tannins and Phenolic Compounds: The following colour reactions were observed when test solution of 2-3 ml was treated with the following reagents:

a) 5% solution of $FeCl_3$: Dark blue-black colour appeared.

b) Lead acetate solution: White precipitate.

c) Solution of gelatin: White precipitate.

d) Bromine water: Bromine water decolourises.

e) Acetic acid solution: Red colour solution.

f) Potassium dichromate: Red precipitate.

g) iodine solution: Transient red colour.

h) Dilute HNO_3: Reddish to yellow colour.

9. Test for Vitamins:

1) Test for Vitamin-A: About 10-15 Equivalent units was dissolved to 1ml of chloroform to which antimony trichloride solution of 5ml was added, a volatile blue colour was created immediately.

2) Tests for B complex:

a) Test for Thiamine HCl (B1): In 10ml of water dissolve 20 mg was dissolved and add 1ml of 2M acetic acid and 1M NaOH of 1.6 ml was added and then heated for 30 min on water bath and it was allowed to cool shaken well for 2 min after adding 5ml of 2M NaOH, 10ml of potassium ferricyanide solution and 10ml of butanol. Light Intense blue fluorescence UV visible spectrophotometer at 365 nm was observed in upper layer using.

b) Test for Riboflavine (B2): In 100ml of water dissolve 2mg was dissolved. The solution had a light greenish yellow colour is observed by transmitting light and deep yellowish green fluorescence by reflecting light, which vanishes on inclusion of mineral acids or alkalies.

c) Tests for Nicotinic Acid:

i. Pyridine is found to be present when a small amount of quantity was heated with twice its weight of soda lime,

ii. In 20 ml water 50mg was dissolved and neutralized with 0.1M NaOH, then 3ml solution of copper sulphate was added. A blue colour precipitate is noticed.

3) Tests for Vitamin C:

i. 1ml of 2% solution (w/v) was diluted with water of 5ml and include1drop of afresh prepared 5% sodium nitroprusside solution (w/v) and 2 ml of diluted sodium hydroxide solution were added concentrated hydrochloric acid (0.6ml) dropwise was added and stirred the yellow colour turns blue.

ii. To 2 ml of a 2% test solution (w/v) add 2 ml of water, 0.1 gm of sodium bicarbonate and about 20 mg of ferrous sulphate and vibrate well and allowed to stand. The violet colour was created. On adding 1M sulphuric acid of 5ml, the colour vanishes.

4) Test for Vitamin D: A amount equal to about 1000 units of vitamin D activity was dissolved in chloroform and antimony trichloride solution of 10ml of was included. At once a pinkish-red colour appears.

Results were tabulated (Table 6)

5.4 *In-vitro* antioxidant study:

1. Scavenging of Superoxide radical[62]

Chemical used:

1. Nitro blue terazoluim: 10mg of NBT in 10ml of DMSO

2. Alkaline DMSO : Sodium hydroxide pellet of 20mg is dissolved in 1ml of DMSO and then final volume is made up with 99ml of DMSO.

Preparation of standard and test solutions:

The plant extract and abscorbic acid containing 14mg were weighed accurately and it was dissolved in DMSO of about 3ml and it was used to dilute the solutions randomly so as to obtain lower concentrations ranging between 50-250µg/ml.

Methodology

The plant extract (0.3ml) was diluted in DMSO at different concentrations to which 0.1ml of nitro blue tetrazolium was mixed to get ultimate volume of 1.4ml. Absorbance was measured at 560nm.

The activity of scavenging in terms of % was estimated using the formula

% scavenging = (Abs $_{control}$- Abs $_{sample}$- /Abs $_{control}$) X 100

Results were tabulated (Table 7,8 and 9)

Figures are shown in 5,6 and 7

2. Scavenging of hydrogen peroxide radicals[62].

Chemicals used:

Hydrogen peroxide of 0.2267ml was diluted in 100ml phosphate buffer saline (PBS).

Preparation of test and standard solutions.

The plant extract and the standard (abscorbic acid) containing 30mg was weighed accurately and it was liquefied in 10ml of methanol.

These solutions were serially mixed with methanol to acquire the lower concentrations ranging from 50-250 µg/ml.

Methodology

1. Hydrogen peroxide solution of about 20mM was prepared at a pH of 7.4 in phosphate buffer saline.
2. Various concentration of 1 ml of the plant extracts or standards in methanol was mixed to 2ml of hydrogen peroxide solutions in PBS.
3. At 230nm the absorbance was estimated against blank solution with a time interval of 10 minutes and the extracts without hydrogen peroxide was found in phosphate buffer saline.

The percentage of activity of scavenging was determined using following technique.

% scavenging = (Abs $_{control}$- Abs $_{sample}$- /Abs $_{control}$) X 100

Results were tabulated (Table 10,11 and 12)

Figures are shown in 8,9 and 10

3. Nitric Oxide Scavenging Activity[62]

Nitric Oxide Scavenging Activity

Chemicals used

1. Phosphate buffer (pH 7.4).

2. Sodium nitroprusside (5 mmol/L)

3. Griess reagent: Sulphanilamide 1%, phosphoric acid 2% and N-1-naphthylenediamine dihydrochloride 0.1%, ascorbic acid used as standard.

4. Methanol.

Preparation of test & standard solutions.

Different concentration of plant extracts ranging from 500-2500µg/ml was prepared along with the standard in methanol.

Methodology

1. Different concentration of plant extracts ranging from 500-2500µg/ml in methanol was mixed with 2.5ml of phosphate buffer and Sodium nitro prusside.

2. At 25°C the mixture was incubated for a duration of 30 minutes and 1.5ml of Griess reagent was added to reaction mixture of 1.5ml.

3. At 546nm absorbance was measured which indicates the reaction mixture has greater reducing power.

The percentage of scavenging activity was determined using following formula.

Results were tabulated (Table 13,14 and 15)

Figures are shown in 11, 12 and 13

4. β-Carotene Linoleate Model[63]

β-Carotene Linoleate Model

Chemicals used

Beta – carotene, linoleic acid, tween 40, chloroform.

Preparation of test & standard solutions

The plant extracts of different concentrations ranging from 10-200µg/ml was prepared along with the standard in methanol.

Methodology

β-carotene-linoleate model system (Miller, 1971) was used to determine the antioxidant activity.

1. 2mg of β-carotene of was dissolved in 10ml of chloroform to prepare the solution.
2. 2ml of β-carotene solution was pipette into a round bottom flask of 100ml.
3. After removal of chloroform beneath vacuum, 40mg of purified linoleic acid, 400mg of Tween 40 emulsifier and distilled water of 100 ml were mixed to the flask with aggressive shaking.
4. 4.8ml of aliquot was shifted into test tubes which hold plant extracts of various concentrations.
5. When the emulsion was add on to each test tube, absorbance at zero time was recorded at 470nm by using a spectrophotometer.
6. The tubes were put in water bath at a temperature of 500C and after 2 hours absorbance was recorded.
7. For background subtraction β-carotene blank devoid was prepared.
8. The ascorbic acid was used to separate the above procedure, as a positive control.
9. Antioxidant activity was deliberate using the below formula.

$$\text{Antioxidant activity} = \frac{\text{After 2hr of assay β-carotene content x 100}}{\text{Initial β carotene content}}$$

Results were tabulated (Table 16, 17 and 18)

Figures are shown in 14, 15 and 16

5. DPPH Radical Scavenging Assay[64]

1,1-diphenyl-2-picryl-hydrazyl a molecule (a, a-diphenyl-β-pycrylhydrazyl) was characterized as a free radical by morality of the delocalization as it give rise to deep violet colour of the spare electron above the molecule so that dimerization should no more occur as it was usually found with most of the other free radicals, which is characterized by ethanol/methanol solution absorption band at 520nm. When DPPH solution was assorted with that offer substance that can contribute a hydrogen item, which gives rise to the diminish form which change in violet colour (due to picryl group presence residual pale yellow colour is expected). DPPH radical is represented by Z● and AH represents donor molecule, the reaction is Z●+ AH=ZH+A●.

Plant extracts radical scavenging activity against stable DPPH (2, 2-diphenyl-2 picryl hydrazy1 hydrate), was spectrophotometrically estimated. Deep violet colour changes to light yellow and was recorded at 517nm by UV spectrophotometer.

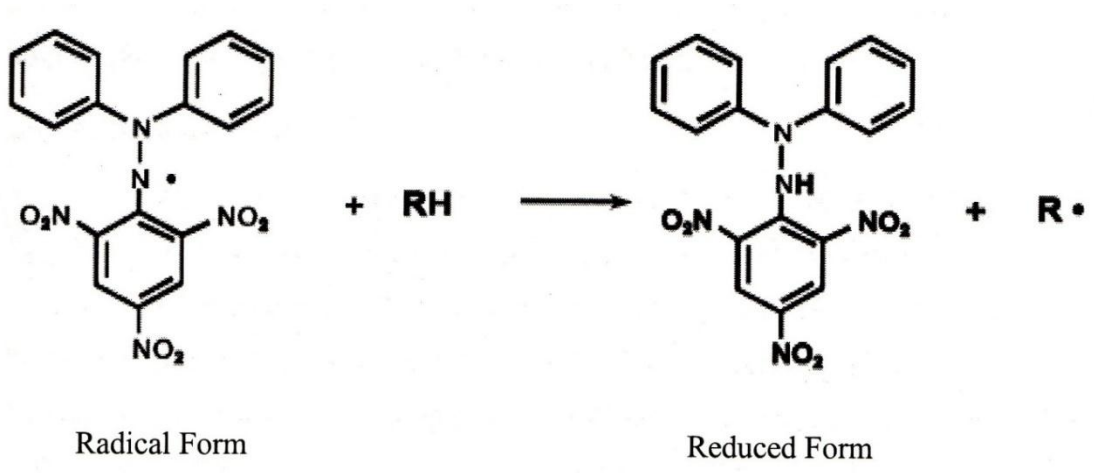


Figure 17: Reduced and Radical form of DPPH

Procedure

0.3mM DPPH solution in methanol was produced and 1ml of this solution was mixed to various concentrations of 1ml sample at 1ml and after vigorous shaking of reference compound it was sinistral in the black at room temperature for a duration of 30 minutes and then absorbance was measured at 517nm and without the test sample the control reaction was carried out. All the tests were performed in triplicate manner so as to get the mean value. The % inhibition percentage was determined comparing the test and control absorbance values. Antiradical activity was exhibited as inhibition percentage and was deliberate by the

following. In the previous equation the term Abs sample was substituted with (Abs sample-Abs blank). Calibration curves and EC50 values were obtained using different sample concentrations.

Results were tabulated (Table 19, 20 and 21)

Figures are shown in 18, 19 and 20

6. ABTS Radical Cation Decolorization Assay[65]

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate)) was liquefied in water. ABTS radical cation ($ABTS^{\bullet+}$) was processed by ABTS stock solution reaction with 4.9mM potassium/ammoniumpersulfate which is the last concentration and permitting the mixture to stand in the black at room temperature for 12-16 h before use. Because ABTS and potassium/ammoniumpersulfate act stoichiometrically at a ratio of 120.5, this leads to consequences of insufficient oxidation of the ABTS. ABTS oxidation starts immediately, but the absorbance was not ultimate and stable until more than 6 h had lapsed. The radical was in solid form for two days and more when stored in the black at room temperature. For phenolic compounds and food extracts studies, the $ABTS^{\bullet+}$ solution was mixed with ethanol and for plasma antioxidants with PBS at a pH of 7.4, to an absorbance of 0.70 (±0.02) at 734 nm.

Prior to assay, $ABTS^{\bullet+}$ solution was diluted in aqueous and absorbance was adjusted to 0.70 (±0.02) at 734 nm. Each sample of aliquot (50 µl) was assorted with 950 µl of liquefied ABTS cation radical solution. At 734 nm absorbance was measured using an UV spectrophotometer after the solution had been permitted to stand for 6 min at room temperature. A control reaction was carried out without test sample. The inhibition percentage of free radical by the sample was expressed as radical scavenging activity and was evaluated by using following calculation and IC_{50} are also estimated.

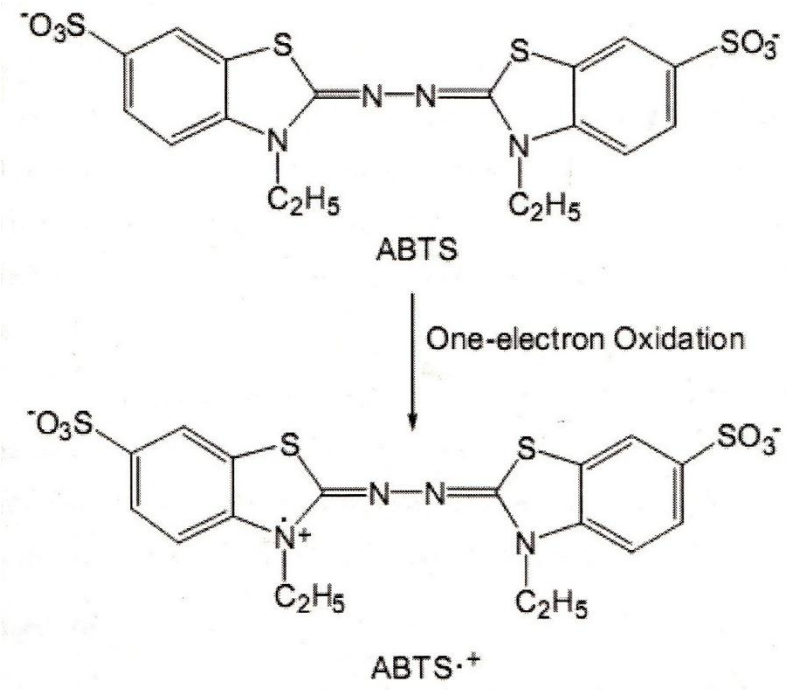


Figure 21:ABTS Radical Cation Decolorization

Inhibition percentage (I %) = Abs $_{control}$– Abs $_{sample}$ /Abs $_{control}$) X 100

Results were tabulated (Table 22, 23 and 24)

Figures are shown in 22, 23 and 24

5.5 In Vitro Cytotoxicity Studies[66]

Material and methods:

Cell Cultures used-

HT- 29 (Colon Carcinoma)

1. **Cell lines:**

Human colon cancer cell line (HT-29 and HCT-116) were acquired from National Centre for Cell Sciences, Pune. It is a adenocarcinoma human colorectal cell line with epithelial morphology. These are sensitive to oxaliplatin and 5-fluorouracil and they are used as standard chemotherapy treatment options for colorectal cancers. Additionally, for colorectal cancers an in vitro and xenograft tumor model were taken using HT-29 cell lines under standard cultured conditions in which cells were uncharged and unidentified. The

results are obtained in a distinguished and polarized morphology in spite of altered culture conditions or various inducers used to treat cells which is represented by the rearrangement of membrane antigens and growth of an apical brush-border membrane.

2. ***In vitro* cytotoxicity assay:**

Determination of mitochondrial synthesis by MTT assay[67]

Principle:

The cells ability to survive a toxic offend has been the premise of most cytotoxicity assays. Assay of MTT is mainly based on the dead cells or their products assumptions that they do not minimize tetrazolium. The number of cells present and their mitochondrial activity also depends. The division of MTT to a blue formazan fragment by living cells.

The principle involved is the cleavage of tetrazolium salt MTT (3-(4,5 dimethyl thiazole-2 yl)- 2,5-diphenyl tetrazolium bromide) into a product of blue colour (formazan) by mitochondrial enzyme succinate dehydrogenase(ref-1) . The numbers of cells were found to be proportional to formazan extent production by the cells used.

Methodology:

i. The culture of cell monolayer was trypsinized and the count of cell was altered to $1.0x10^5$ cells/ml using DMEM medium accommodating 10% FBS.

ii. To each well consisting of microtitre plate of 96 well, 100µl of the mixed suspension of cell to which about 10,000 cells/well were added.

iii. A fragmentary monolayer was formed after 24 hours, the supernatant was scanned off and then with help of medium once monolayer was washed and different extract concentrations of 100 µl prepared in maintenance media which was added per well to the microtitre plates containing partial monolayer. These plates were incubated at 37^oC for 3 days under the presence of 5% CO_2 atmosphere, and after which it was examined microscopically and after every 24 hours observations were recorded.

iv The sample solutions in the wells were discarded after 72 hours and 20 µl of MTT (2mg/ml) in MEM-PR (MEM without phenol red) was added to each well.

v. The plates after shaken gently were subjected to incubation at a temperature of 37^0C under the presence of 5% CO_2 atmosphere for about 3 hrs.

vi. 50 µl of iso-propanol was added after the removal of supernatant and the plates were shaken gently to dissolve the formed formazan.

vii. At a wavelength of 540nm absorbance was recorded using microplate reader. The inhibition of growth percentage was evaluated using the below equation and test sample concentrations or drug required to cell growth inhibition by 50% values from the curves of dose response were generated for every cell line.

$$\text{Percentage of Growth Inhibition} = \left(100 - \frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}}\right) \text{ X } 100$$

CT C_{50} was determined by plotting the cone Vs % growth inhibition.

Results were tabulated (Table 25)

Figures are shown in 25, 26 and 27.

5.6 ANTI MICROBIAL ACTIVTY:

Media preparation and its decontamination:

Agar well diffusion method and susceptibility of antimicrobial was tested on solid (Agar-agar) media placed in shallow plates. Nutrient agar (NA) (40 gm/L) for bacterial assay and for fungus Potato Dextrose Agar (39 gm/L) was used to grow surface colony. All the media developed by autoclaving for a duration of 20 mins at 121°C was sterilized.

Agar well diffusion method:

Agar well-diffusion method was escorted to discover the antimicrobial activity. Potato Dextrose Agar (PDA) and Nutrient agar (NA) plates were scrubbed (sterile cotton swabs) with culture of broth (8hrs) of respective fungi and bacteria. Wells (10mm diameter and about 2 cm a part) were made in each of these plates using cork borer. Solution of stock of extract (plant) was produced at a concentration of 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml in Ethanol and Water. About 100 µl of dissimilar concentrations of plant solvent extracts were mixed into the wells by sterile syringe and let to disperse at room temperature for at least 2hrs. Control experiments consisting substances without extract of plant were set up. At 37°C plates were incubated for a duration of 18-24 h for bacterial pathogens and at 28°C for a

duration of 48 hours for fungal pathogens. inhibition zone (mm) diameter was measured. Triplicates were continued and thrice the experiment was repeated, the recordings were taken for each replicates in three unlike fixed directions and the mean values were recorded[68].

Results were tabulated (Table 26, 27 and 28)

Figures are shown in 28 and 29

5.7 Screening of Acute Oral Toxicity Study of drug samples in mice (OECD 423) [69]

Animals

Swiss albino mice weighing 20-25gm were selected for the study. Animals were fed with standard rat pellet diet and water *ad libitum* and kept at 24-28^{0}C with 60-70% relative humidity and 12hr day and night cycle. Animals were fasted for overnight, but had access to water freely.

Procedure

The acute toxicity study was conducted according to the Organisation for Economic Co-operation and Development (OECD) Test specifications 423 using female mice using the limit test procedure . Animals were grouped into four at the dose levels of 5, 50, 300, 2000 mg/kg each group consisting of 03 animals were used; a single dose was received by animals through intragastric intubation starting with aqueous plant extract (AEPQ) of 5 mg/kg dissolved in distilled water. After the dosing it was observed for any mortality, behavioural, autonomic and toxic profile changes for one to four hours and upto 14 days, at least once daily for the immediate and delayed acute toxicity as the parameters specified below during the monitoring period as per the method outlined by OECD Test specifications

1. Motor activity
2. Grooming
3. Touch response
4. Tone response
5. Pain response
6. Tremors
7. Convulsion
8. Righting reflex

9. Gripping strength
10. Pinna reflex
11. Corneal reflex
12. Writhing
13. Pupils
14. Urination
15. Salivation
16. Skin colour
17. Lacrimation
18. Defaecation

Results are shown in table 29

5.8 *In-vivo* anti cancer activity

1. Selection and acclimatization of animals

Male Sprague-dawley rats 300-400 grams body weight were procured from The National Institute of Nutrition, Hyderabad. All rats were kept at room temperature of 22$\pm$2°C with a relative humidity of 60-70% under 12 hr light and dark cycle respectively in the animal house. Rats were fed with modified diet and water *ad libitum* throughout the study freely (including 1 week for acclimatization) shown in table . All animal procedures were carried out in accordance with CPCSEA guidelines for the proper supervision and use of laboratory animals and the protocol of present study was approved by IAEC Ref. No. IAE1012/C/10/CPCSEA-Corres–2011-12 of RVS College of Pharmaceutical Sciences, Coimbatore.

Table 30: Modified pellet diet.

Ingredients	Commercial pellet diet	Peanut oil	Total (%)
Protein	17.7	_	17.7
Fat	4.2	15.8	20.0
Carbohydrates	50.5	_	50.5
Fiber	3.4	_	3.4
Minerals	6.7	_	6.7
Vitamins	1.7	_	17.7

2.Chemicals:

The 1,2-dimethyl hydrazine (DMH) was procured from Sigma Chemical Company, Mumbai, India. After receiving, it was stored in a cool and dry place to prevent from contamination and decomposition as it rarely occurs in some cases.

3. **Preparation of DMH :**

The 1,2-dimethyl hydrazine was liquefied in 1mm EDTA just prior to use and the pH of 6.5 was modified with 1mM sodium bicarbonate to safeguard chemical stability.

4. **Induction of colon cancer**:

Sprague-dawley rats were administered on a weekly interval, subcutaneous (s.c.) injection of 1,2-dimethyl hydrazine in the groin at a dose of 20 mg/kg body weight for 15 weeks[70].

5**. Clean up the following injection:**

After the DMH injection, the excess amount of DMH and prepared area was cleaned and chemically inactivated fusing a dilute solution of sodium carbonate and was used in general and other materials used during carcinogen administration .It was disposed by incineration in compliance with Institutions bio safety guidelines.

6**. Preparation of 5 Fluorouracil solution (standard drug)** :

The 5 Fluorouracil is a standard chemotherapy which was procured and dissolved in normal saline.

7. **Preparation of drug sample(extract) :**

Plant extract was weighed accurately for 200mg/kg and 400 mg/kg doses and it was dissolved in distilled water to provide a clear solution, which was administered to the animals through oral route.

8. Treatment schedule[71] :

The animals were grouped into V groups and each group consisted of 6 animals.

Group I animals were treated as control and received only normal saline, Group II animals received DMH only for 15 weeks, Group III animals received DMH and 5-Fluororacil which is used as standard drug, Group IV animals received DMH and extract of plant at a dose of 200mg/kg (b.w) and Group V animals received DMH and extract of plant at a dose of 400mg/kg (b.w) Table 5.

Table 31: Treatment schedule of animals used.

Group	**Treatment**
Group I	Control +1 ml/kg of normal saline. p.o everyday for the entire period of the study. (Figure 30)
Group II	DMH (20mg/kg body weight for 15 weeks (s.c), once in a week) (Figure 31)
Group III	DMH (20mg/kg body weight for 15 weeks (s.c), once in a week) + 5 fluorouracil (20mg/kg) i.p (Figure 32)
Group IV	DMH (20mg/kg body weight for 15 weeks (s.c), once in a week) + plant extract (AEPQ 200mg/kg), p.o daily (Figure 33)
Group V	DMH (20mg/kg body weight for 15 weeks (s.c), once in a week) + plant extract (AEPQ 400mg/kg), p.o daily.(Figure 34)

One week before the induction of tumor, the animals were treated with extract of plant. At the end of the 7th day by administrating DMH at a dose of 20 mg/kg which was dissolved in liquefied in 1 MM EDTA with modified pH. Tumor was induced by administration of DMH and then the treatment was continued up to a period of 15 weeks.

a. Blood Collection:[72]

At the end of the 16th week, with the administration of ketamine (2mg/kg) i.p animals were anaesthetized and blood was accumulated through Retro orbital sinus with EDTA and without EDTA for the enumeration of blood cells (i.e. RBC, WBC,), estimation of Haemoglobin. and for estimation of various biochemical parameters respectively.

b. Separation of serum:[72]

Retro orbital sinus method was used to collect blood and it was centrifuged at 10,000 rpm for about 10 mins and the separated serum was collected and it was used to evaluate biochemical parameters.

c. Separation of plasma:[72]

Animals were then scarified through cervical decapitation and the body was cut open, entire liver and colon was perfused immediately with cold ice 0.9% saline and thereafter correctly removed, cropped free of extraneous tissue. During and on termination of the study, the following parameters were studied.

(a) Tumor incidence

(b) Tumor burden (average number of tumor per animal).

(c) Tumor volume.

(d) Colon weight.

(e) Body weight were recorded alternate week till the end of treatment.

Results were tabulated (Table 32 and 33)

Figures are shown in 35, 36, 37 and 38

d. Separation of plasma for tumor markers:

The estimation of tumour markers such as Alpha-feto-protein (AFP), Carcinoembryonic antigen (CEA), the blood was accumulated with EDTA, and at 10,000 rpm it was centrifuged for 5 min. the separated plasma was taken for the parameter estimation.

9. ESTIMATION OF HAEMATOLOGICAL PARAMETERS:

ENUMERATION OF RBC: [73]

Requirements:

RBC diluting fluid(Hayem'S fluid)

Counting chamber(Neubauer's chamber)

RBC Pipette

Microscope with 45X Objective lens

Procedure:

The RBC pipette was filled with blood up to the mark 0.5, "immediately RBC diluting fluid (Hayem's fluid) was filled up to the mark. 101. Pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept aside for a while. The counting chamber was placed and the RBC squares were focused under low. power first, when markings were identified then turned to high power. The first 3-4 drops of blood mixture was discarded and it was mixed once again, the counting chamber was charged with the mixed blood. After charging mount the slide was mounted and the fluid was allowed to settle. Then using a 45X lens, the RBC were counted uniformly in 7. corner and middle squares. The number of cells were expressed as 10^{12}/ Cmm.

Results are shown in Table 34 and Figure 39

ENUMERATION OF WBC: [73]

Requirements:

- WBC diluting fluid (Turk's Fluid)
- Counting chamber (Neubauer's chamber)

- WBC Pipette
- Microscope with 1oX objective lens

Procedure:

The WBC pipette was filled with blood up to the mark 0.5, immediately WBC diluting fluid (Turk's fluid) was filled up to the mark 11.Pipette was rolled between the palms to ensure thorough mixing of blood with diluting fluid and kept aside for a while. The counting chamber was placed and the WBC squares were focused under low power first, when markings were identified then turned to high power .The first 3-4 drops of blood mixture was discarded and it was mixed once again, the counting chamber was charged with the mixed blood. After charging the slide was mounted, the fluid was allowed to settle then using a 10X lens. The WBC were counted uniformly in corner squares. The number of cells was expressed as 10^9 Cmm

Results are shown in Table 34 and Figure 40

ESTIMATION OF HAEMOGLOBIN[73]

Principle:

This method is based on the conversion of Haemoglobin to acid haematin by treatment with N/10 hydrochloric acid. The brown color formed due to the acid haematin was matched against a glass, standard on a comparator.

Requirements:

- Sahli's haemoglobinometer
- Hb pipette
- N/ 10 HCl

Procedure:

The hemoglobinometer tube was filled with N/ 1 0 HCl up to the marking 10. To this 20µl of blood was added with the help of pipette. The superficial acid was sucked and it rinsed repeatedly till all the blood in the pipette washed out in mud. The contents in the tube were mixed by stirring, and allowed to stand for 10 minutes. A clear brown colour solution was formed due to the formation of acid hematin. Then distilled water was added drop by drop to dilute. The colour of diluted fluid was compared with the standard; dilution was

continued until the colour of the flu1d exactly matches the standard. The lower meniscus of the fluid was noted and reading was noted directly 31 ~ from the graduated tube as g/ 100ml or as percentage of haemoglobin

Results are shown in Table 34 and Figure 41

10. ESTMATION OF SERUM BIOCHEMICAL PARAMETERS

a. Estimation of Serum glutamate oxaloacetate transaminase (SGOT) [74]

Method:

Optimized UV- test was done according to International Federation of Clinical Chemistry and Laboratory Medicine.

Principle:

1. SGOT (ASAT) acts as by catalyzing the transfer of amino group between L-Aspartate and Ketoglutarate which leads to the formation of Oxaloacetate and Glutamate.

2. The formed Oxaloacetate further reacts With NADH in the presence of Malate Dehydrogenase, by oxidizing NADH to NAD.

3. The rate at which NADH is oxidized to NAD is measured as decreased absorbance which is proportional to the activity of SGOT (ASAT) in the sample.

Reaction:

SGOT

L-Aspartate + Ketoglutarate ⟶ Oxaloacetate + L-Glutamate

MDH

Oxaloacetate + NADH + H ⟶ Malate + NAD+

Reagents:

Reagent 1: Enzyme reagent

- TRIS Ph 7.8 80 mmol/l
- L-Aspartate 240 mmol/l

- MDH (Malate dehydrogenase) ≥600 U/1
- LDH (lactate dehydrogenase) ≥600 U/l

Reagent II: Starter reagent

- 2-Oxalog1utarate 12 mmol/l
- NADH 0.18 mmol
- Pyridoxal-5-Phosphate 0.09 mmol/l

Assay procedure:

a) To 800 µ1 of reagent-1, 200 µ1 of reagent-2 was added and mixed in a test tube.

b) To the above mixed reagents, 100 µ1 of serum was added.

It was mixed and the readings were taken immediately in a» semi auto analyser ‘ x‘ V

(Model-Photometer5010).

The level of SGOT was expressed as Units/liter

Results are shown in Table 35 and Figure 42

b. ESTIMATION OF SGPT

Serum glutamate pyruvate transaminase (SGPT) [74]

Principle:

L-alamine +2-oxoglutarate $\xleftrightarrow{ALAT}$ Glutamate + Pyruvate

Pyruvate + NADH + H^+ $\longleftrightarrow$ D Lactate + NAD^+

Inclusion of pyridoxal-5-phosphate (P-5-P) stabilizes the transaminases and keep away from wrongly low values in samples containing inadequate endogenous P-5-P, eg. Samples collected from diseased patients suffering from MI, cirrhosis etc.,

Method:

Kinetic UV test, was carried out in accordance to the international Federation of clinical chemistry and laboratory medicine (IFCC)

Reagent-1

TRIS (PH 7.5)-100 mmol/l

L-Alanine - 500 mmol/l

LDH (lactate dehydrogenase)- $\geq$ 1200 U/l

Reagent-2

2-Oxoglutarate - 15mmo1/l

NADH - 0.18 .mmol/l

Good's buffer PH 9.6 - 0.7mmol/l

Pyridoxal -5-Phosphate - 0.09 mmol/l

Assay procedure:

MIX 800 µ1 of reagent-l With 200 µ1 of reagent-2 was added in a 5 m1 test tube.

a) To this was added 100 µl of serum.
b) Mixed well and the reading was taken immediately.

Normal range : <41 µ1

The level of SGPT was expressed as Units/Liter

Results are shown in Table 35 and Figure 43

c. Total Cholesterol[75]

Total cholesterol (TC)

Principle

Estimation of cholesterol was done after hydrolysis and oxidation of enzyme. The colorimetric indicator, which is produced from 4-aminoantipyrine and phenol by hydrogen peroxide beneath the catalytic action of peroxidise is quinonemine (trinder' reaction).

Cholesterol ester $+H_2O \xrightarrow{CHE}$ Cholesterol + Fatty acid

Cholesterol $+O2 \xrightarrow{CHO}$ Cholesterol -3-one $+H_2O_2$

$2H_2O_2$ + 4- Amino antipyrine = Phenol $\xrightarrow{POD}$ quinonemine + 4 H_2O_2

Method

CHOD-PAP: enzymatic photometric test

Reagents

Goods buffer (pH 6.7)-50 mmol/1

Phenol – 5 mmol/1

4-aminoantipyrine -0.3 mmol/1

Cholesterol esterase -> 200 U/1

Cholesterol oxidase - > 100 U/1

Peroxidase – 3 KU/1

Standard – (5.2 mmol/1)

Assay procedure:

a. 1 ml (1000µl) of reagent-1 was collected in a 5 ml test tube.

b. 0.01 ml (10 µl) serum was included.

c. Mixed well and incubated at 37^0C for 5 min.

d. Reading of the test sample was taken.

Normal Range: <200 mg/dl of serum.

The level of Total Cholesterol was expressed as mg/dl.

Results are shown in Table 35 and Figure 44

d. Triglycerides (TG) [75]

Principle

Triglycerides determination (TG) alters lipoprotein lipase with enzymatic splitting. Indicator which is produced from 4-aminoantpyrine and 4-chlorophenol by peroxidise of hydrogen beneath the catalytic action of peroxidise is quinoneimine.

$$\text{Triglycerides} \xrightarrow{\text{LPL}} \text{Glycerol + fatty acid}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol -3-phosphorate+ADP}$$

$$\text{Glycerol-3-phosphate+}O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetaon phosphate+}H_2O_2$$

$$2H_2O_2\text{+4-Amino antipyrine+4-chlorophenol} \xrightarrow{\text{POD}} \text{Quinonelimine + HCL + }4H_2O$$

Method

Colorimetric enzymatic test is done utilizing glycerol-3-phosphate-oxidase (GPO).

Reagents

Content and concentrations in the test Goods buffer pH 7.2, 50mmol/1

4-chlorophenol-4 mmol/1

ATP-2 mmol/1

Mg^{2+} -15 mmol/1

Glycerokinase - >Kμ/1

Peroxidase ->2 Kμ/1

Lipoprotein lipase -> 4 Kμ/18

4-aminoantipyrine-0.5 mmol/1

Glcerol-3-phosphate-oxidase -> 1.5 Kμ/1

Standard-(2.3 mmol/1)

Assay procedure:

a. 1 ml (1000μl) of reagent-1 was collected in a 5ml test tube.
b. Added 0.01 ml (10μl) of serum.
c. It was mixed well and incubated at 37 C for 15 min.

d. Reading of the test sample was recorded.

NORMAL RANGE: <200 mg/dl in serum

The level of triglycerides was expected as mg/dl.

Results are shown in Table 35 and Figure 45

11. ESTIMATION OF TUMOR MARKERS

a. Carcinoembryonic antigen (CEA)

CEA: it is a glycoprotein of cell surface 200-kd. It was reported in 1969, that out of 36 patients, CEA was raised in most of the patients with adenocarcinoma of the colon and after surgery CEA titers decreased successfully[76].

CEA ELISA TEST: [77]

It is established on concept of solid phase enzyme associated with immunosorbent assay. The monoclonal antibody was utilized in assay system which was directed against distinct antigenic determinant on intact carcinoembryonic antigen which was used for solid phase immobilized. A goat anti-carcinoembryonic antigen antibody was conjugated to horseradish peroxide is in the antibody enzyme conjugate solution.

Simultaneously, the test sample was permitted to react with 2 antibodies which results in a sandwich of CEA between solid phase and enzyme linked antibodies. Incubation was done for one hour at room temperature and to remove antibodies of unbound labelled wells were washed with water.

Solution of TMB reagent was attached and for 20 minutes it was incubated which resulted in evolution of a blue colour. CEA concentration is directly proportional to intensity of colour intensity of sample. The development of colour was stopped with the addition of stop solution (1 NHCL) which resulted in change of colour to yellow.

Absorbance was measured spectrophotometrically at 450nm

Materials provided with the kit:-

• Microtiter plate (coated with antibody) with standards of 96 wells.

• Carcinoembryonic antigen containing ; 0,3,12,30,60 and 120 mg/ml. Ready to use (each 1ml).

• (13ml) Enzyme conjugate.

• (11ml) TMB reagent (one-step).

• (11ml) stop solution (1NHCL)

Other Materials required:-

•Pipette of precision:-1 ml, 50ml, and 100ml.

•Disposable pipette tips

•Distilled water

•Vortex mixer

•Absorbent paper or paper towel

•Microtiter plate reader

•Paper (Graph)

Reagent preparation:-

1)Before use all reagents were brought to room temperature of about 18-25^{0}C.

2)Hook effect:- expected samples with concentrations of CEA over 9000 mg/ml may be significant by dilution in order to avoid hook effects.

ASSAY PROCEDURE

1. In holder desired number of coated wells was secured.

2. Appropriate wells were taken in which standard, specimens (about 50 µl) were dispensed.

3. Enzyme Conjugate Reagent of 100 µl was dispensed to each well.

4. It was assorted thoroughly (30 seconds). In this set up it is very essential to have a complete mixing.

5. At room temperature it was incubated (18-25^{o}C) for 60 minutes.

6. The mixture of incubation was removed by vacating plate content in a drain container.

7. Wash and empty the microtiter wells were rinsed and emptied using distilled or deionized water for 5 times. (tap water should be avoided)

8. On absorbent paper bang the wells smartly (or with paper towels) to separate all residual droplets of water.

9. In each well 100 µl of TMB Reagent was dispensed and mixed gently for 10 seconds.

10. At room temperature it was incubated for about 20 minutes.

11. 100 µl of Stop Solution to each well is added to stop the reaction.

12. For 30 seconds blend gently and assure that colour changes from blue to yellow completely.

13. Within 15 minutes, read the optical density at 450 nm using a microtiter plate reader.

Results are shown in table 36 and Figure 47

b. Alpha-fetoprotein (AFP) [77]

Alpha-fetoprotein (AFP): It is a protein known as glycoprotein with a molecular weight of 70,000 daltons approximately.

AFP is normally generated during the pregnancy and neonatal. It is also produced in small concentrations by gastrointestinal tract.

After birth, there is a rapid decrease in serum AFP, and by the next year of life only little amounts are normally traced in serum. Occurrence of serum AFP is irregularly elevated with high rise values in various malignant diseases, most particularly non seminomatous testicular cancer and primary hepatocellular carcinoma (primary)

Therefore, for detection of cancer in the general population, AFP measurements are not recommended for screening.

PRINCIPLE:

AFP ELISA TEST: It is based on the concept of a solid phase enzyme-link of immunosorbent assay. The assay system utilizes a goat anti-AFP antibody directed against

intact AF P for solid phase immobilization (on the microtiter wells). In antibody-enzyme conjugate solution a monoclonal antibody is united to horseradish peroxidase (HRP). The test sample was permitted with the disabled rabbit antibody for about30 minutes. The wells were cleaned so as to remove any unbound antigen. HRP monoclonal is conjugated and for 30 minutes (at room temperature) it is reacted with immobilized antigen resulting in the AF P molecules which will be sandwiched between enzyme-linked antibodies and solid phase. To remove unbound labelled antibodies wells were washed with water. TMB reagent is added and incubated for 20 mins which results in development of blue colour. The development of colour is stopped with the addition of 0.16M sulfuric acid solution(stop solution) and colour changes to yellow. The absorption of AFP is directly proportional to the colour intensity of test sample. Measurement of absorbance was done at 450nm by spectrophotometrically.

Materials provided with the kit:

- Goat anti-APP (96 wells with microtiter plate which was coated).
- 13 ml, Zero Buffer.
- 0, 5, 20, 50, 150 and 300 µg/ml (WHO,72/225) AFP, lyophilization of reference standard is set.
- 18 ml, Enzyme Conjugate Reagent.
- 11 ml, TMB Reagent.

Stop Solution (1N HCl), 11 ml.

Materials required:

- Precision pipettes and tips: 20 pl, 100 pl, 200 pl, and 1 ml.
- Deionized water.
- Replaceable pipette tips.
- Mixer (Vortex).
- Paper towel.
- At 450 nm wavelength a microtiter plate reader with optical density and 10 nm band width and range of 0-2 OD of optical density or greater.

Assay Procedure:

1. Before use reagents should be at room temperature (18-25^{o} C).
2. Each lyophilized standard should be reconstituted with 1.0 ml of deionized water and for at least 20 minutes allow reconstituted material to stand and mix gently. Reconstituted standards will be stable for 30 days and seal it and then store at 2-8^{O}C
3. Secure the required number of wells coated in the holder.
4. 20 µl of standard, specimens, and controls were dispensed into suitable wells.
5. 100 µl of Zero buffer was dispensed in all wells.
6. It is very essential to have a whole mixing in this setup (Mixed for 30 seconds thoroughly).
7. At room temperature (18-25^{0}C) it was incubated for 30 minutes.
8. The mixture of incubation was removed by flicking plate content into a drain container.
9. Clean and flick the wells of microtiter five times with deionized water.
10. Bang wells clearly on paper towels for removal of whole residual droplets water.
11. 150 µl of conjugate enzyme reagent was dispensed in every well.
12. At room temperature it was incubated for 30 minutes.
13. Remove the mixture of incubation was removed by flicking plate contents into a drain container.
14. Clean and flick the wells of microtiter for 5 times using deionized water.
15. On absorbent paper strike the wells clearly to remove residual droplets of water.
16. TMB Reagent of 100 µl was dispensed into each well and mixed gently for about 10 seconds.
17. At room temperature it was incubated for 20 minutes.

18. The reaction is stopped by adding 100 µl of 0.16M sulphuric acid (stop solution) to every well.

19. It was mildly mixed for 30 seconds. It is necessary to make sure that yellow colour completely appears from blue colour.

20. Within 15 minutes optical density was read at 450 nm with a microtiter reader.

Results are tabulated in table 37 and fig 48

12. Collection of tissues:

After the blood collection the animals were sacrificed and the body was cut opened, and gross pathological changes were observed and the organs like liver, kidney, and colon were excised immediately and washed with normal saline and wet organ weight was determined.

Portion of that was preserved in 10% buffered neutral formalin solution for histopathologically studies and from the remaining portion of organ, was subjected to tissue homogenate preparation.

a. Preparation of tissue homogenate:

About 500mg of Liver/ colon was taken and minced with 5ml of phosphate buffer solution and it was homogenated to 10% using homogeniger which was used for enzymic and non enzymic antioxidant estimation.

b. Histopathological study:

The organ collected was washed with normal saline to remove the cell debris and preserved in 10% buffered formalin solution. The tissues were trimmed to 2-3 mm thickness & subjected to preparation of paraffin blocks and cut in to 5p thickness and H&E staining was followed any alterations in the normal tissue was examined.

13. ENZYMIC ANTIOXIDANTS ASSAYS

a. Estimation of Proteins: [78]

It was estimated as per the standard method

REQUIREMENTS:

- ✓ Alkaline copper reagent

A-Solution : sodium carbonate (2%) in 0.1 N NaOH.

B-Solution : Copper sulphate (0.5%)in 1% sodium potassium tartarate 50 ml of A solution was mixed with 1 ml of B solution just before use.

- ✓ Folin's phenol reagent (commercial reagent, 1:2 dilution)
- ✓ Bovine serum albumin (BSA).

PRINCIPLE:

This method is a combination of both Folin-ciocalteau and Biuret reaction which involves two steps.

Step: l-Protein binds with copper in alkaline medium and reduces it to Cu^{2+}.

Step:2-The cu2+ formed catalyses the oxidation reaction of aromatic amino acid by reducing Phosphomolybdotungstate to heteropolymolybdenum, which leads to the formation of blue colour and absorbance is measured at 640nm.

Results are shown in table 38 and figure 49

b. Superoxide dismutase [SOD, EC 1.15.1.1] [79]

It was evaluated as per the standard method.

Principle:

The test is established on the inhibition of NADH-phenazine-methosulfate nitroblue tetrazolium dyes formation. It was commenced by the inclusion of NADH. After 90 sec incubation, the reaction is stopped by adding glacial acetic acid. The colour obtained at the termination of the reaction is extracted into layer of n-butanol and recorded at 520 nm.

Reagents:

1. Sodium pyrophosphate buffer, 0.052 M, pH 8.3.

2. Phenazine methosulfate 186 µM.

3. Nitroblue tetrazolium 300 µM.

4. Reduced nicotinamide adenine dinucleotide 780 µM.

Procedure:

0.5 ml of the tissue homogenate (homogenized in 0.052 M sodium pyrophosphate buffer at pH 8.3) or 0.5 ml erythrocyte lysate was liquefied to 1.0 ml with ice-cold water accompanied by the inclusion of 2.5 ml ethanol and 1.5 ml chloroform (chilled reagents) and vibrate for 90 sec at a temperature of 4°C and then centrifuged. The supernatant`s activity of enzyme was decided as follows: The mixture of assay accommodated 1.2 ml sodium pyrophosphate buffer, 0.1 ml PMS, 0.3 ml NET and liquefied enzyme combination in a final volume of 3.0 ml. By the addition of 0.2ml NADH the reaction was started. At 30°C it was incubated for about 90 sec, then by the inclusion of 1.0 ml glacial acetic acid the reaction was stopped . The mixture of reaction was mixed vigorously, vibrate with 4.0 ml n-butanol and was permitted to stand for 10 min. After centrifugation the intensity of colour of chromogen in butanol layer was studied in a colorimeter at 520 nm. Control was treated as system devoid of enzyme.

The enzyme concentration needed to create 50% chromogen inhibition formation in 1 min, under quality condition was taken as one unit. The enzyme specific activity was indicated as enzyme required for 50% inhibition of NET reduction/min/mg Hb for erythrocyte lysate and enzyme essential for 50% inhibition of NET depletion/min/mg protein for tissues

The level of SOD was exhibited as units/min/mg protein.

Results are shown in table 38 and figure 50

c. Catalase [CAT, EC 1.11.1.6] [80]

The activity of CAT was determined in erythrocyte lysate and tissue homogenate by the standard method

Principle

Acetic acid containing dichromate was diminished to chromic acetate, perchromic acid was formed when heated in the presence of H_2O_2 which is an unstable intermediate. The CAT composition was permitted to split H_2O_2 for various interval of time. The preparation is terminated at different time intervals by the inclusion of dichromate-acetic acid mixture in hot conditions. H_2O_2 forms H_2O_2-chromic acetate which is recorded colorimetrically at 590 nm.

Reagents

1. Phosphate buffer, 0.01 M, pH 7.0

2. Hydrogen peroxide (H_2O_2), 0.2 M

3. 5% Potassium dichromate

4. Dichromate-acetic acid reagent - Glacial acetic acid and potassium dichromate were diversed in the ratio of 3:1. From this, 1.0 ml was liquefied again with 4.0 ml acetic acid.

5. Standard hydrogen peroxide: 0.2 mM.

Procedure

Tissue homogenate was produced in phosphate buffer. To phosphate buffer of 0.9 ml , 0.1 ml erythrocyte lysate and 0.4~ ml H_20_2 was included. The reaction was seized after 15, 30, 45 and 60 sec by including 2.0 ml mixture of dichromate-acetic acid. For about 10 min the tubes were kept in a water bath and then cooled as the developed colour was recorded at 590 nm. Standard concentration ranging from 20-100 ump were processed for test.

The specific activity of the enzyme was indicated as µmoles of H_2O_2 utilized/min/mg Hb for erythrocyte lysate and µmoles of H_2O_2 utilized/min/mg protein for tissues.

Results are shown in table 38 and figure 51

d. **Glutathione peroxidase** [GPx, EC 1.11.1.9] [81]

It was evaluated in erythrocyte lysate and tissue homogenate by the standard procedure.

Principle

A familiar quantity of enzyme preparation is allowed to react with H_20_2 and GSH for a specified time duration. GSH remaining content is calculated after the reaction by Ellman's reaction (Section 3.16.1)

$$2\ GSH + H_2O_2 \xrightarrow{\textbf{GPx}} GSSG + 2\ H_2O$$

Reagents

1. Tris-HCl buffer, 0.4 M, pH 7.0

2. Sodium azide solution, 10 mM

3. 10% TCA

4. DTA, 0.4 mM

5. H_2O_2, 1.0 111M

6. Reduced glutathione (GSH), 2.0 mM

Procedure

0.2ml of Tris-HCl buffer, 0.2ml of EDTA, sodium azide of 0.1 ml and enzyme preparation (erythrocyte lysate) of 0.2 ml were included and assorted well. To this, 0.2 m1 GSH along with 0.1 ml H_2O_2 were included. The contents were incubated at a temperature of 37°C for about 10 min. By the addition of 0.5 ml TCA, the reaction was stopped. The tubes were spin and the remaining GSH was determined colorimetrically at 340 nm.

The activities are expressed as µmoles of GSH utilized/min/mg Hb for erythrocyte lysate and umoles of GSH utilized/min/mg protein for tissues.

Results are shown in table 38 and figure 52

e. Reduced glutathione (GSH) [82]

GSH in erythrocyte lysate and tissues were estimated as per the standard method.

Principle

This procedure is based on the evolution of yellow colour when DTNB is included to compounds accommodating sulphydryl groups.

Reagents

1. Phosphate buffer, 0.2 M, pH 8.0

2. 10% TCA

3. Ellman's reagent: 40 mg DTNB in 10 ml 0.1 M phosphate buffer.

4. Stock standard: 100 mg GSH in 100 ml water.

5. Working standard: Stock was liquefied with water to get a concentration of 100 µg/ml.

Procedure

1.0 ml of erythrocyte lysate was precipitated with TCA of 2.0 ml and centrifuged. To supernatant of 1.0 ml , phosphate buffer of 3.0 ml and Ellman's reagent of 0.5 ml were included. It developed yellow colour and was read in a colorimeter at 412 nm. A sequence of standards (20-100 g) were managed in a similar manner along with buffer of 1.0 ml containing blank .

The quantity of GSH is exhibited as µ/d1 erythrocyte lysate and mmoles/mg tissue

Results are shown in table 38 and figure 53 and 54

f. ESTIMATION OF GLUCONEOGENIC ENZYMES[83]

Isolation of mitochondria and microsomes

The organelle of liver and kidney were isolated by the procedure of Johnson and Lardy (1967) and microsomes by Hanioka *et al*., (1997).

Reagents

1. 0.05 M Tris - HCl buffer, pH 7.4 containing 0.25 M sucrose.

2. 0.05 M Tris - HCl buffer, pH 7.4 containing 0.15 M potassium chloride.

A 10% (w/v) homogenate was prepared in 0.05 M Tris-HCl buffer, at a pH of 7.4 containing 0.25 M sucrose and centrifuged at 600 x g for 10 min. The supernatant fraction was gradually poured and centrifuged at 15,000 x g for 5 min. The resultant mitochondrial pellet was washed and resuspended in the buffer.

The post mitochondrial fraction was centrifuged further at 1,05,000 x g for 60 min. The microsomal pellet was suspended in 0.05 M Tris-HCl (0.05) buffer, at a pH of 7.4 containing 0.15 M KCl.

The purity of mitochondrial and microsomal fractions was evaluated by measuring the activities of succinate dehydrogenase and glucose-6-phosphate dehydrogenase respectively.

f. (i) Fructose-l, 6-diphosphatase (Fructose-1, 6-diphosphatase phospohydrolase) [84]

It was determined by an adaption of the procedure described by Gancedo and Gancedo (1971).

This enzyme catalyses the reaction

Fructose-1, 6-diphospate+H2o ⟶ Fructose-6-phosphate +pi.

Reagents

1. 0.1M Tris-HCL buffer, pH 7.0
2. Substrate : 0.05 M frustose-l,6-diphosphate solution
3. 0.1 M $Mgcl_2$
4. 0.1 M KCL
5. 0.001 M EDTA
6. 10% TCA
7. 2.5% ammonium molybdate solution
8. ANSA

Method

The test medium in a 2ml of ultimate volume contained buffer 0.1MI, substrate solution of 0.1 ml, MgCL2 of 0.23ml, KCL solution of 0.23ml, EDTA solution of 0.25 ml and enzyme of 0.1 ml . At 37^0 C it was incubated for 15 min. By addition of 1 ml of 10% TCA solution the reaction was terminated, after which it was centrifuged and supernatant contents of phosphorous was evaluated by the standard method.[85] The standard method was used to determine the protein. [78]

The enzyme activity was exhibited as n moles of Pi liberated/min/mg protein

Results are shown in table 39 and figure 55

f (ii). Glucose-6-phosphatase (Glucose-6-phosphate phospohydrolase) [86]

It was carried out according to the method of King (1965). This enzyme catalyses the reaction Glucose-6-phosphate + H2O ⟶ Glucose +Pi

Reagents

1. 0.1 M citrate buffer ,pH 6.5
2. Substrate: Glucose-6-phosphate, 0.01M in distilled water
3. 2.5% ammonium molybdate solution
4. AN SA
5. 10% TCA

Method

The incubated mixture in a entire volume of 1 ml contained buffer of 0.3 ml, substrate of 0.5 ml, and enzyme solution of 0.2 ml. At 37^0 C it was incubated for 60 min, The reaction was concluded by the inclusion of 10% TCA solution of 1 ml. It was centrifuged and the phosphorus content in the supernatant was exhibited by the procedure of Fiske and subbarow (1925).

The action of enzyme is exhibited as nmoles of Pi liberated/min/mg protein.

. Results are shown in table 40 and figure 56

g. Faecal bile acids:

Extraction and determination

During the period of study, 24 hrs stool samples were collected every week and also before sacrifice of the rats in all groups. The stool samples were weighed, homogenized with equal amounts of water (w/v) and dried at 110^0 C for 24 hrs. After drying and reweighing, the faeces was lyophilized to a fine powder and 1.0 ml KOH in ethylene glycol was added. The mixture was heated at 220^0 C for 15mins with occasional mixing. After cooling, 1.0 ml Nacl, 0.2 ml conc. HCL and 6.0 ml diethyl ether was include to the acidified solution. The tube was shaken for about 1 min and centrifuged at 2000 g for 3 mins, the upper layer was collected, evaporated at 40^0 C and the rest was dissolved in methanol of 1.0 ml and used for the determination of bile acids. [87]

Results are shown in table 41 and figure 57

h. Faecal neutral sterols:

Extraction and determination

From the collected faecal matter, 0.5 g was suspended in deionised water of 4.0 ml and hydrolyzed at 80^0 C in 4.0 ml of 2 N methanolic NaOH for 2 hrs. To the residue, 4.0 ml of 2 N methanol was added and the neutral sterols was extracted by adding 5.0 ml of petroleum ether($60\text{-}80^0$). [87]

Results are shown in table 41 and figure 58 and 59.

14. HISTOPATHOLOGY

The colon and liver were collected and washed under saline and preserved in 10% buffered neutral formalin (DNF). The tissues were trimmed and its sections were subjected to prepare paraffin blocks. Thickness of 5 micron were cut and discolour with Haematoxylin and Eosin and was observed under compound microscope. [87]

Figures are shown in 60 to 69.

15. Statistical analysis:

Data collected from the above specified studies were subjected to One way ANOVA accompanied by Dunnet's assessment comparison using Graph pad prison 5. Version 5.01.

The significance was expressed as ***P<0.001, **P<0.01, *P<0.05, #-Non Significant.

5.9. Determination of active compounds from aqueous extract (AEPQ) of *Portulaca quadrifida*

5.9.1 Isolation and determination of Total Phenolic content present in crude aqueous Extract by Folin Ciocalteu method: [88]

a. Isolation of Total Phenolic content

Sample Preparation:

A dried ground sample of 0.5 g aqueous extract was weighed and Phenolic compounds were extracted with 50 ml of 80% methanol aqueous on an accelerated bath for

about 20 min. 2ml of aliquot of the above methanol extract was ultracentrifuged for about 5 min at 1400 rpm.

b. Estimation / Calculation of the total phenol assay:

The whole phenolic content of the dry aqueous (AEPQ) crude drug was estimated with Folin- Ciocaletau assay.1ml of aliquot sample or gallic acid as standard solution (10, 20, 40, 60, 80 and 100 mg/l) was included to 25ml of volumetric flask, containing deionised water of 9 ml. Deionised water was used to prepare reagent blank. 1 ml of the Folin – Ciocalteus phenol reagent was add on to the mixture and vibrate. After 5 min.7% sodium carbonate solution of 10ml was included to mixture. The solution was liquefied to 25 ml with double deionised water and mixed. At room temperature it was incubated for 90 min, the absorbance against the produced reagent blank was recorded at 750 nm with an UV-VIS Spectrophotometer Lambda 5. The data for the whole content of phenolic of the given crude drug sample were exhibited as milligrams of gallic acid equivalents(mg GAE/100g dry weight). All samples were analysed in duplicates.

Results are shown in table 42 and figure 70.

5.9.2 Isolation and determination of Total Tannin content present in crude aqueous Extract by modified method of Broadhurst et al.,1978 [89]

The total condensed tannin content was determined by using catechin as a reference compound. A volume of 400µL of extract was added to vanillin solution of 3ml (4% in methanol) and concentrated hydrochloric acid of 1.5ml. After incubation 15 min the absorbance was recorded at 500 nm. The condensed tannin was expressed as g E.Catechin.100g-1DM.

Results are shown in table 43 and figure 71.

5.9.3 Separation and estimation of total saponin fraction from crude aqueous Extract using gravimetric technique[90,91]

The total saponin content was isolated from the 100g of crude aqueous extract using the gravimetric separation technique. Take 100g aqueous (AEPQ) crude drug powder with 500ml of 90% methanol for half an hour by refluxing. Extract the remains two more times by taking 500 ml of methanol. Combine the extract of methanol and distill off the solvent. Treat soft extract left after distillation of alcohol, with petroleum ether 60-800c, 500 ml for half an

hour by refluxing. Cool and remove the solvent by decantation. Now treat the same soft extract successively with chloroform 500 ml and ethyl acetate 500 ml and pour the solvents after cooling, keeping the soft extract in the same flask. Dissolve the soft extract (after three extractions cited above) in 500 ml of butanol. Filter and concentrate to 100 ml. Add the above drop by drop with constant stirring to 500 ml with Acetone in order to lead to the saponins. The precipitates are sieved, composed and dehumidified to a sustained weight at 105^0c.

The 100 ml of acetone filtrate obtained in the above process was concentrated to 5 ml under vacuum. To the concentrated 5 ml, 0.1% of sodium chloride of 1ml (w/v) was added and a precipitate was obtained and air dried and a 70 mg of fine brownish green powder (Figure 73).

Results are shown in table 44 and Figure 72

Figure 73: Fine brownish green powder

5.9.3.1 Isolation of Saponins from Total Saponin Fraction Using Flash Chromatography

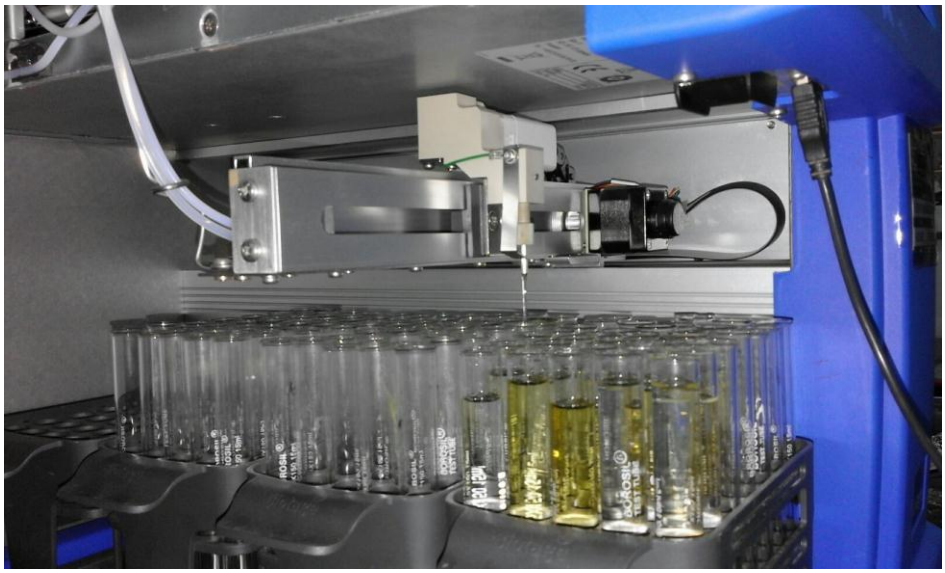


Figure 74 Isolera flash chromatography system

Flash chromatogram report of saponin isolation from total saponin fraction Isolera flash chromatography system having a touch screen display, which is a solvent-resistant, colour LCD screen with a resolution of 800 x 600 pixels which serves both as a display and as the system's input device via on-screen touch controls. A fraction collector, collects fractions into a wide variety of collection racks and vessels. A pump module, which directs the liquid flow through the system. A default flow rate is specified for each cartridge but, if required, the rate of flow can be changed. If the flow rate is increased, the system will start the run at the default flow rate and then regulates the flow rate as defined in the method. Note that the system regulates on both flow rate and pressure. If maximum(90%) allowed pressure is reached before the defined flow rate, the flow rate at 90% pressure was used. An internal detector provides the system with information on the light absorbance of the solvents and samples passing through the detector flow cell. The different fractions can be collected through an automated collector of fractions based on the Rf the interest of compound can be identified and pooled together after performing thin layer chromatographic analysis.

Based on the TLC analysis, A novel method was developed to separate the individual saponins from the total saponin fraction using Isolera flash chromatography system. 5 gms of the total saponin fraction was dissolved in 10 ml of 70% v/v Hydro ethanol. The obtained solution was passed on 5 gms of charcoal column. The obtained clear filtrate was evaporated under vacuum to get 1 gm of dried powder which was directly applied on 10 gm samplet and was dried by using rotary evaporator (Buchi R 210). The dried samplet was packed in 50 g KPSil Biotage SNAP Cartidge. A gradient flash chromatography method was developed based on TLC. A constant flow rate 50 ml / min of mobile phase chloroform : methanol is used. A total no of 51 fractions, each 18 ml was collected in different test tubes at the collection wave length of 254 nm. Each individual fractions was subjected to TLC Analysis using chloroform : methanol (9:1) and based on tlc similarity, similar fractions were pooled together, total 5 different fractions was identified (15-17, 6 – 10, 11-13, 18 – 51) These different fractions were distilled leaving 5 different brown color powders. The obtained / isolated compounds were subjected to TLC analysis using the same mobile phase chloroform : methanol (9:1) and detected for its purity using iodine chamber.

Results are shown in Figure 75.

5.9.3.2 TLC Finger Printing Of Total Saponin Fraction

Sample Preparation:

10 mg of the sample was dispersed in 10 ml of 70% v/v aqueous methanol and was sieved through filter paper. The obtained filtrate was used as sample solution.

Stationary Phase: Silica gel 60 GF_{254} precoated aluminum Tlc Plates.

Mobile Phase: Chloroform: Methanol: Water (65: 35: 10) or Chloroform: Methanol (65:35)

Saturation time: 20 minutes

Wave length of detection: 254 nm.

Results are shown in Figure 76.

5.10 *In vitro* cytotoxicity Assay of isolated compound of *Portulaca quadrifida* on colorectal different cancer cell lines HT-29 and HCT-116 by determination of mitochondrial synthesis by MTT Assay.

The procedure was well explained in the earlier chapter with reference

Results of HT-29 are shown in table 45.

Figures of HT-29 are shown in 77 to 83.

Results of HCT-116 are shown in table 46.

Figures of HCT-116 are shown in 84 to 89.

5.11 GC MS Procedure: [92]

After extraction and fractionation of the product, Just before the analysis, different fractions are to be dissolved in 70% hydro alcohol which is solvent, in order of 1000 µl, in separate vials. From each vial, 1 µl of solution is to be inj GC-MS machine, Bruker GC which comprises a Bruker gas chromatograph. The GC is usually coupled to a Mass Spectrometer (detector), a Mass Selective Detector (MSD), it is also used in noting down the mass spectrum of the chemical compounds as they emerge of the GC and after fragmentation processes by a stream of electrons in the mass spectrum. Nitrogen gas is used a carrier gas in this particular study, although other gases like helium it can also be used. It is suggested that

the GC oven should be initially held at 80°C for 1 minute, and then warmed at 4 – 6 o C/min to about 300°C, where it is to be held for 20 minutes, and the total length of time for running the analysis is 40 mins, where the injection temperature is 250o C and the detector temperature is maintained at 300o C The column used in the study is of 30 x 0.25mm,0.25µ. Peaks in the chromatograms produced by these analyses were recognised by a combination of references to their mass spectra and the NIST14 mass spectral library used for mass spectral database, and by comparsion and elution orders with those of known standards.

GC Parameters:

Instrument ID : GC-01

Comment : Column ID:ZB-5,30 x 0.25mm,0.25µ

Injection Temp : 250°C , Det Temp :300°C

Flow rate : 1.0 ml/min, Split ratio : 1:100, Carrier Gas : N2

Inj.Vol(µl):1.00.

Results are shown in table 47, 48 and 49.

Figures are shown in 90 to 98

RESULTS

The present research work was carried out in Five Phases. In the **Phase-I** of our study we have collected the plant (*Portulaca quadrifida*), authenticated, cleaned, milled and the powdered material was subjected to hot continous soxhlet extraction and got chloroform extract (CEPQ), methanolic extract (MEPQ) and aqueous extract (AEPQ) and further they were subjected to Preliminary Phytochemical studies. The results of the findings are given in Table 6,7 and 8.

6.1.1. PHYSICOCHEMICAL CHARACTERS OF CRUDE DRUG OF *PORTULACA QUADRIFIDA*LINN

Table 4: Ash value results of successive extract of *Portulaca quadrifida*

Sl.No	**Physicochemical Properties**	**Result (% W/W)**
1	Total Ash	8.14
2	Acid-insoluble ash	0.70
3	Water-soluble ash	4.5
4	Loss on drying	6.31
5	Alcohol-soluble Extractive	0.16
6	Water-soluble Extractive	0.36
7	Chloroform-soluble extractive	0.12

The ash values such as total ash, acid insoluble, water soluble, loss on drying, and extractives like alcohol, water and chloroform of *Portulaca quadrifida* was determined and the results were 8.14, 0.70, 4.5, 6.31, 0.16, 0.36 and 0.12 respectively tabulated in Table no 4.

6.1 .2 Preliminary Phytochemical Studies

Table 5: Percentage yield of successive extract of *Portulaca quadrifida*

S.No	**Extracts**	**Nature of Extract**	**Colour**	**Weight (GM)**	**Percentage Yield (%)**
1	Chloroform	SEMI-SOLID	Dark green	95.2	9.5
2	Methanol		Dark green	110.79	11.7
3	Aqueous		Dark brown	280.19	28.1

The percentage yield of *Portulaca quadrifida* was reflected in the above table with the percentage yield of 9.5gm , 11.7gm and 28.1gm for chloroform, methanol and aqueous respectively in semisolid form with dark green colour for chloroform and Methanolic extract and whereas dark brown colour for aqueous extract.

Table 6: Preliminary Phytochemical analysis of successive extract of *Portulaca quadrifida*.

S.No.	**Phytochemical Constituents**	**Extract of Chloroform**	**Extract of Methanol**	**Extract of Aqueous**
01	Alkaloids	_	+	_
02	Flavanoids	_	+	_
03	Saponins	_	+	+ +
04	Tannins	+	+	+ +
05	Phenolics	_	_	+ +
06	Glycosides	_	+	_
07	Carbohydrates	_	_	+ +
08	Amino Acids	_	+	_

\+ = Indicates presence of phytochemical and

\- = Indicates absence of phytochemicals.

++ = Shows high concentration.

Preliminary phytochemical analysis of successive extract of *Portulaca quadrifida* was conducted in which the results were as follows

- Alkaloids were showing absent in chloroform and aqueous extract where as it was present in methanolic extract.
- Flavanoids were showing absent in chloroform and aqueous extract where as it was present in Methanolic extract.
- Saponins were absent in chloroform and present in methanolic and aqueous extract.
- Tannins were present in chloroform, methanolic and aqueous extract.
- Phenolics were showing absent in chloroform and Methanolic extract where it was present in aqueous extract.
- Glycosides were showing absent in chloroform and aqueous extract where it was present in Methanolic extract.

- Carbohydrates were showing absent in chloroform and Methanolic extract where it was present in aqueous extract.
- Amino acids were showing absent in chloroform and aqueous extract where it was present in Methanolic extract.

In **Phase –II** of our study all the extracts (AEPQ, CEPQ and MEPQ) were screened for their *In vitro* antioxidant properties. The results of the findings are given in Table 7 to 24; Figure 5 to 24.

6.2 *In Vitro* Antioxidant Activity

Chloroform extract (CEPQ), methanolic extract (MEPQ) and aqueous extract (AEPQ) were subjected to *in vitro* antioxidant studies of superoxide, hydrogen peroxide, nitric oxide< DPPH, β-Carotene Linoleate, ABTS. And the results of this study are given in Table 9 to 26; Figure 9 to 26.

Table 7: Activity of Antioxidant on various concentrations of aqueous extract of *Portulaca quadrifida* and ascorbic acid in superoxide method of scavenging. Each value represents mean ± sem.

S.No	Concentration(µg/ml)	Absorbance	% Scavenging activity
	Control (blank)	**2.119**	-
Ascorbic acid (STD)			
1.	50	1.839	13.21
2.	100	1.697	19.91
3.	150	0.488	76.91
4.	200	0.408	80.74
5.	250	0.259	87.77
Aqueous Extract of *Portulaca quadrifida* (AEPQ)			
1.	50	1.654	21.94
2.	100	0.648	69.41
3.	150	0.467	77.96
4.	200	0.077	96.36
5.	250	0.041	98.06

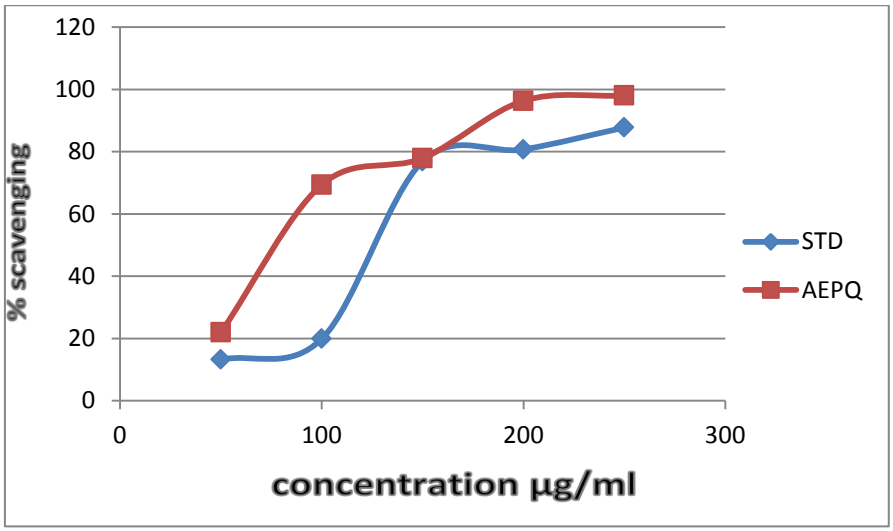


Figure 5: Scavenging of superoxide radical of ascorbic and aqueous extract of *Portulaca quadrifida* by alkaline DMSO.

The aqueous extract of *Portulaca quadrifida* and known antioxidant ascorbic acid at various concentrations produced dose dependent inhibition of superoxide radicals, the % scavenging activity values for superoxide radical against aqueous extracts of *Portulaca quadrifida* were found to be 98.06 with 250mg/ml whereas for ascorbic acid it was found to be 87.77 at 250mg/ml. The results were remarkable when compared with standard. (Table: 7; Figure: 5)

Table 8: Activity of Antioxidant on various concentrations of chloroform extract and ascorbic acid in superoxide method of scavenging.

S.No	Concentration(µg/ml)	Absorbance	% scavenging activity
	Control (blank)	**2.119**	**-**
Ascrobic acid (STD)			
1.	50	1.839	13.21
2.	100	1.697	19.91
3.	150	0.488	76.91
4.	200	0.408	80.74
5.	250	0.259	87.77
Chloroform Extract of *Portulaca quadrifida* (CEPQ)			
1.	50	1.454	17.69
2.	100	0.620	55.40
3.	150	0.421	70.10
4.	200	0.060	81.40
5.	250	0.032	83.10

Each value represents mean ± sem**.**

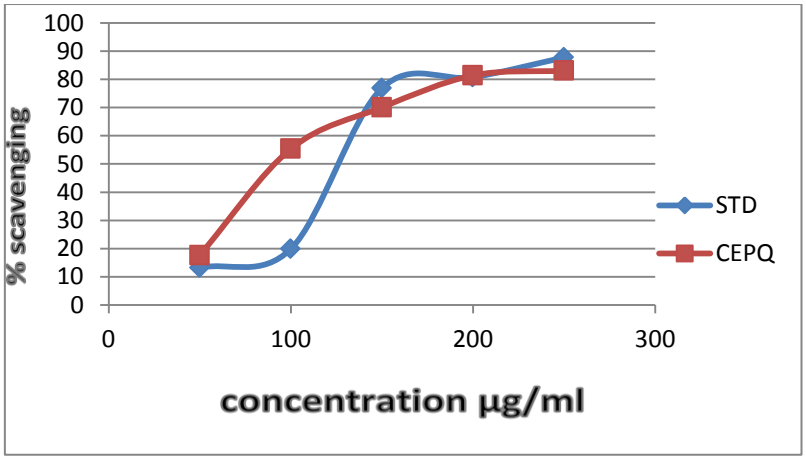


Figure 6: Scavenging of superoxide radical of ascorbic and chloroform extract of *Portulaca quadrifida* by alkaline DMSO.

The Chloroform extract of *Portulaca quadrifida* and known antioxidant ascorbic acid at various concentrations produced dose dependent inhibition of superoxide radicals, the % scavenging activity values for superoxide radical against Chloroform extracts of *Portulaca quadrifida* were found to be 83.10with 250mg/ml whereas for ascorbic acid it was found to be 87.77 at 250mg/ml. . The results were quite similar when compared with standard. (Table. 8; Figure.6)

Table 9: Activity of Antioxidant on various concentrations of methanolic extract and ascorbic acid in superoxide method of scavenging.

S.No	Concentration(µg/ml)	Absorbance	% scavenging activity
	Control (blank)	**2.119**	**-**
Ascrobic acid (STD)			
1.	50	1.839	13.21
2.	100	1.697	19.91
3.	150	0.488	76.91
4.	200	0.408	80.74
5.	250	0.259	87.77
Methanolic Extract of *Portulaca quadrifida* (MEPQ)			
1.	50	1.54	20.86
2.	100	0.682	59.62
3.	150	0.315	66.21
4.	200	0.050	78.16
5.	250	0.021	81.24

Each value represents mean ± sem.

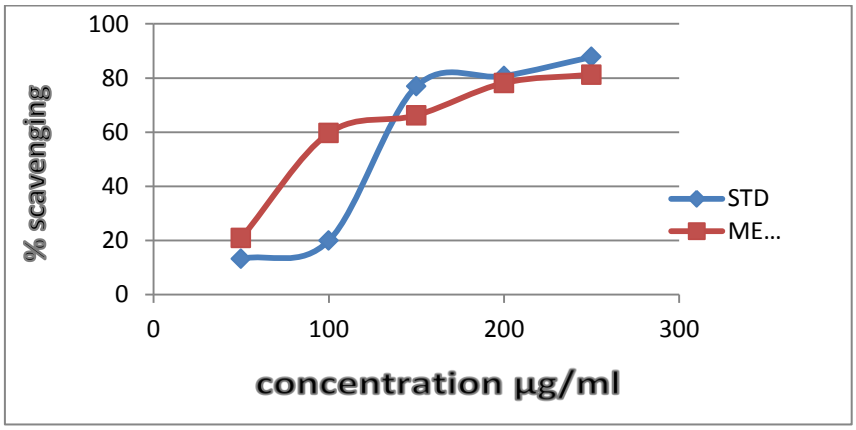


Figure 7: Scavenging of superoxide radical of ascorbic and methanolic extract of *Portulaca quadrifida* by alkaline DMSO.

The methanolic extract of *Portulaca quadrifida* and known antioxidant ascorbic acid at various concentrations produced dose dependent inhibition of superoxide radicals, the % scavenging activity values for superoxide radical against methanolic extracts of *Portulaca quadrifida* were found to be 81.24 with 250mg/ml whereas for ascorbic acid it was found to be 87.77 at 250mg/ml. The results were quite similar when compared with chloroform extract. The results were similar when compared with standard. (Table.9; Figure.7)

In DMSO model, scavenging of Superoxide radical by the AEPQ, CEPQ and MEPQ was compared with the Ascorbic acid. Among all the three extracts AEPQ, Showed the significant antioxidant potential. % scavenging activity of AEPQ was found to be 98.06 at 250 µg/ml when compared with Ascorbic acid by 87.77 at 250 µg/ml where as CEPQ and MEPQ found to be 83.10 and 81.24 at 250 µg/ml respectively. (Table 7, 8 and 9; Figure 5, 6 and 7).

Table 10: Activity of Antioxidant on various concentrations of aqueous extract of *Portulaca quadrifida* and ascorbic acid in hydrogen peroxide method of scavenging.

S.No	Concentration(µg/ml)	Absorbance	% scavenging activity
Control (blank)		**2.032**	**-**
Ascorbic acid (STD)			
1.	50	1.738	14.46
2.	100	0.805	60.38
3.	150	0.599	70.52
4.	200	0.465	77.11
5.	250	0.528	74.01
Aqueous Extract *of Portulaca quadrifida* (AEPQ)			
1.	50	0.807	60.28
2.	100	0.158	92.22
3.	150	0.177	91.28
4.	200	0.140	93.11
5.	250	0.167	91.78

Each value represents mean ± SEM.

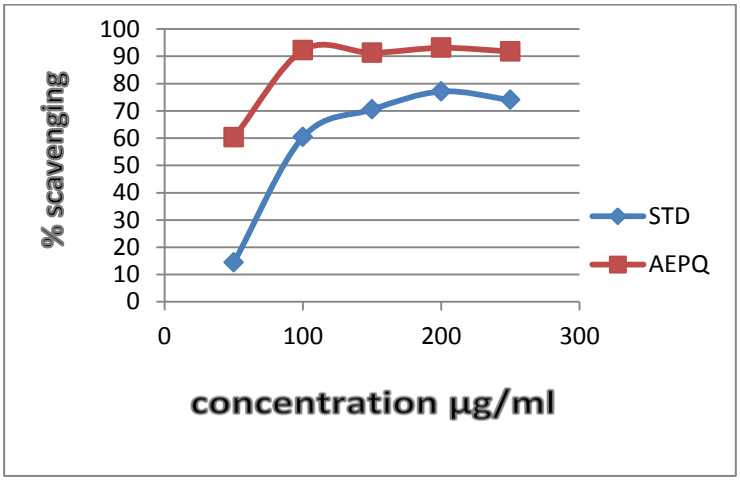


Figure 8: Scavenging activity of hydrogen peroxide radical of aqueous extract of *Portulaca quadrifida*.

The hydrogen peroxide radical was significantly scavenged by AEPQ when compare to the ascorbic acid which showed antioxidant potential of AEPQ. The % scavenging activity of AEPQ was found to be 91.78 at 250 µg/ml when compared to ascorbic acid showed 74.01 only at 250 µg/ml (Table.10; Figure.8)

Table 11: Activity of Antioxidant on various concentrations of Chloroform extract and ascorbic acid in hydrogen peroxide method of scavenging. Each value represents mean ± SEM

S.No	Concentration(µg/ml)	Absorbance	% scavenging activity
Control (blank)		**2.032**	-
Ascorbic acid (STD)			
1.	50	1.738	14.46
2.	100	0.805	60.38
3.	150	0.599	70.52
4.	200	0.465	77.11
5.	250	0.528	74.01
Chloroform Extract of *Portulaca quadrifida* (CEPQ)			
1.	50	0.759	51.62
2.	100	0.168	83.26
3.	150	0.188	80.16
4.	200	0.120	86.40
5.	250	0.178	82.30

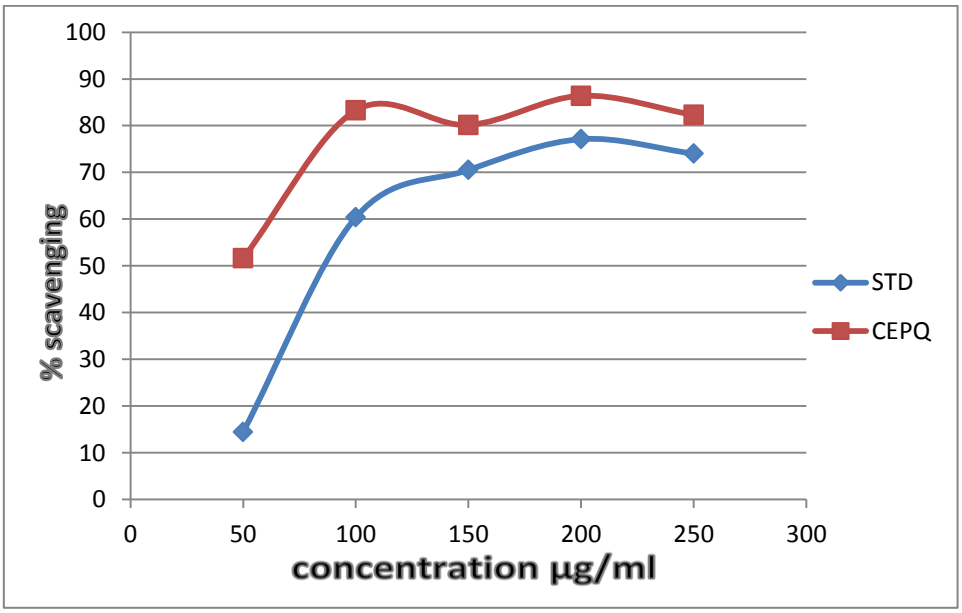


Figure 9: Scavenging hydrogen peroxide radical activity of chloroform extract of *Portulaca quadrifida*.

The hydrogen peroxide radical was significantly scavenged by CEPQ when compare to the ascorbic acid which showed antioxidant potential of CEPQ. The % scavenging activity of CEPQ was found to be 82.30 at 250 µg/ml when compared to ascorbic acid showed 74.01only at 250 µg/ml (Table.11; Figure.9)

Table 12: Activity of Antioxidant on various concentrations of Methanolic extract and ascorbic acid in hydrogen peroxide method of scavenging. Each value represents mean ± SEM

S.No	Concentration(µg/ml)	Absorbance	% scavenging activity
Control (blank)		**2.032**	**-**
Ascorbic acid (STD)			
1.	50	1.738	14.46
2.	100	0.805	60.38
3.	150	0.599	70.52
4.	200	0.465	77.11
5.	250	0.528	74.01
Methanolic Extract of *Portulaca quadrifida* (MEPQ)			
1.	50	0.790	59.14
2.	100	0.177	85.18
3.	150	0.186	75.62
4.	200	0.130	88.28
5.	250	0.158	90.19

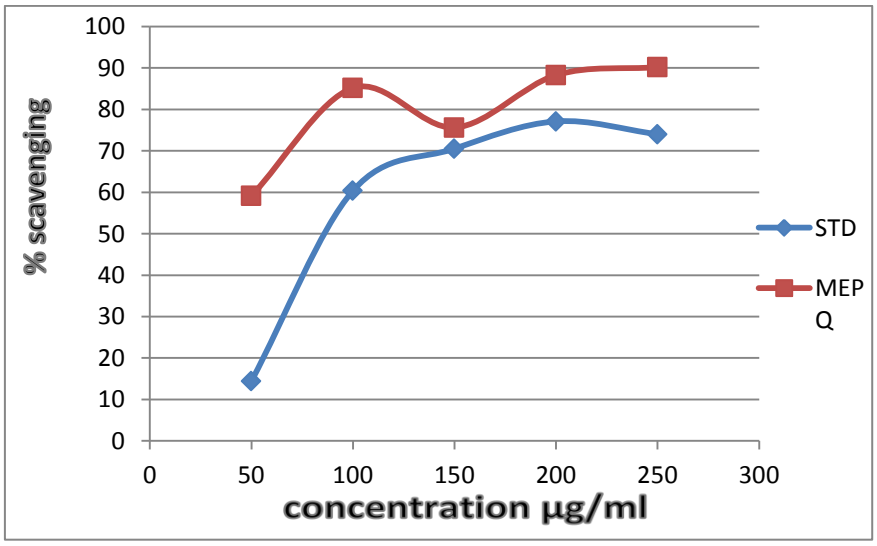


Figure 10: Scavenging hydrogen peroxide radical activity of methanol extract of *Portulaca quadrifida*.

The hydrogen peroxide radical was significantly scavenged by MEPQ when compare to the ascorbic acid which showed antioxidant potential of MEPQ. The % scavenging activity of MEPQ was found to be 90.19 at 250 µg/ml when compared to ascorbic acid showed 74.01only at 250 µg/ml (Table.12; Figure.10).

Among all the three extracts, the hydrogen peroxide radical was significantly scavenged by AEPQ when compare to the ascorbic acid which showed antioxidant potential of AEPQ. The % scavenging activity of AEPQ was found to be 91.78 at 250 µg/ml when compared to ascorbic acid showed 74.01 only at 250 µg/ml where as CEPQ and MEPQ found to be 82.30 and 91.91 at 250 µg/ml respectively (Table.10,11 and 12; Figure.8, 9 and 10)

Table 13 : Activity of Antioxidant on various concentrations of aqueous extract and ascorbic acid in nitric oxide method of scavenging. Each value represents mean ± SEM.

S.No	Concentration(µg/ml)	Absorbance	% scavenging activity
Control (blank)		**0.014**	**-**
Ascrobic acid (STD)			
1.	500	0.007	50.00
2.	1000	0.005	64.28
3.	1500	0.005	64.28
4.	2000	0.005	64.28
5.	2500	0.003	78.57
Aqueous Extract of *Portulaca quadrifida* (AEPQ)			
1.	500	0.007	50.00
2.	1000	0.007	50.00
3.	1500	0.009	62.36
4.	2000	0.019	66.82
5.	2500	0.019	79.68

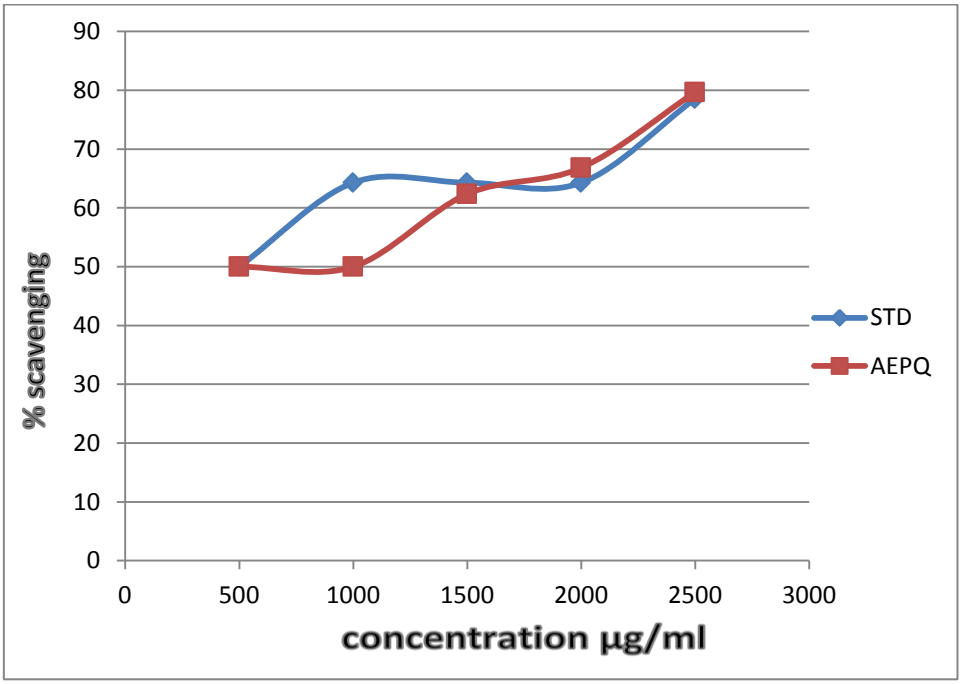


Figure 11: Scavenging nitric oxide activity of aqueous extract of *Portulaca quadrifida*.

In scavenging of Nitric oxide radical, AEPQ similarly scavenged the nitric oxide radical when compared with the Ascorbic acid scavenging effect scavenging activity of AEPQ was found to be 79.68 at 2500 µg/ml. when compared with ascorbic acid by 78.57at 2500 µg/ml (Table. 13; figure. 11).

Table 14: Activity of Antioxidant on various concentrations of Chloroform extract and ascorbic acid in nitric oxide method of scavenging. Each value represents mean ± SEM.

S.No	Concentration(µg/ml)	Absorbance	% scavenging activity
Control (blank)		**0.014**	-
Ascrobic acid (STD)			
1.	500	0.007	50.00
2.	1000	0.005	64.28
3.	1500	0.005	64.28
4.	2000	0.005	64.28
5.	2500	0.003	78.57
Chloroform Extract of *Portulaca quadrifida* (CEPQ)			
1.	500	0.006	48.00
2.	1000	0.006	55.10
3.	1500	0.007	60.40
4.	2000	0.016	63.62
5.	2500	0.017	69.10

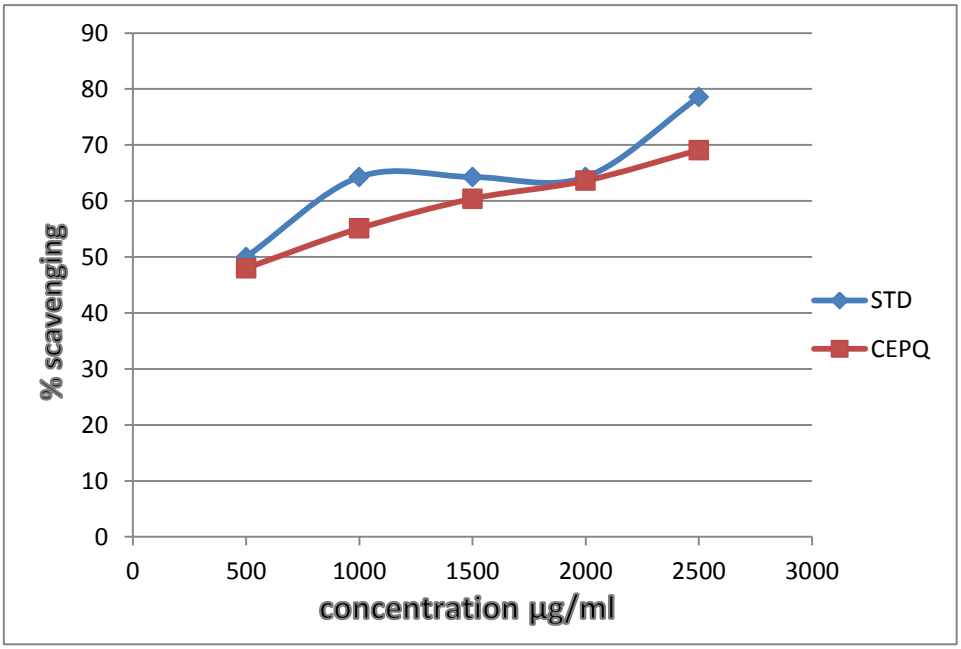


Figure 12: Scavenging nitric oxide activity of chloroform extract of *Portulaca quadrifida*.

In scavenging of Nitric oxide radical, CEPQ non significantly scavenged the nitric oxide radical when compared with the Ascorbic acid scavenging effect scavenging activity of CEPQ was found to be 69.10 at 2500 µg/ml. when compared with ascorbic acid by 78.57at 2500 µg/ml (Table.14; figure. 12)

Table 15 : Activity of Antioxidant on various concentrations of Methanolic extract and ascorbic acid in nitric oxide method of scavenging. Each value represents mean ± SEM.

S.No	Concentration(µg/ml)	Absorbance	% scavenging activity
Control (blank)		**0.014**	-
Ascrobic acid (STD)			
1.	500	0.007	50.00
2.	1000	0.005	64.28
3.	1500	0.005	64.28
4.	2000	0.005	64.28
5.	2500	0.003	78.57
Methanolic Extract of *Portulaca quadrifida* (MEPQ)			
1.	500	0.006	50.00
2.	1000	0.005	59.15
3.	1500	0.005	62.10
4.	2000	0.017	64.10
5.	2500	0.015	73.45

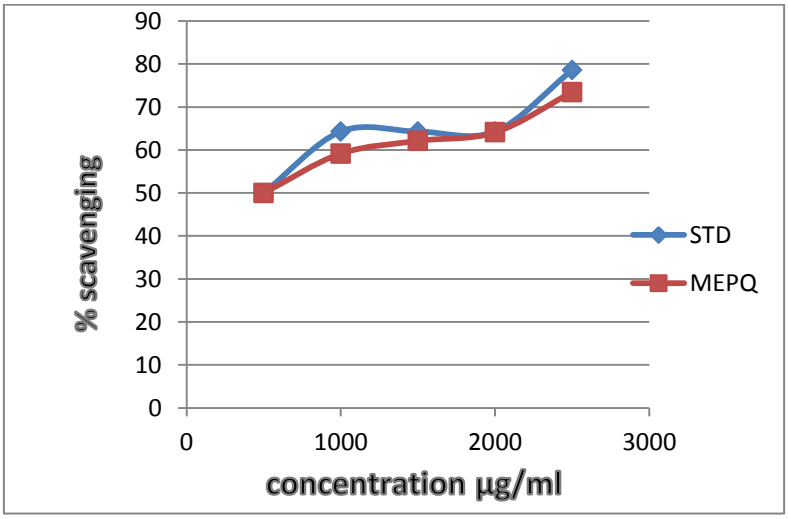


Figure 13: Scavenging nitric oxide activity of methanol extract of *Portulaca quadrifida*.

In scavenging of Nitric oxide radical, AEPQ non significantly scavenged the nitric oxide radical when compared with the Ascorbic acid scavenging effect scavenging activity of AEPQ was found to be 73.45 at 2500 µg/ml. when compared with ascorbic acid by 78.57at 2500 µg/ml (Table.15; figure. 13).

In scavenging of Nitric oxide radical, AEPQ non significantly scavenged the nitric oxide radical when compared with the Ascorbic acid scavenging effect scavenging activity of AEPQ was found to be 35.71 at 2500 µg/ml where as CEPQ and MEPQ found to be 64.28 and 46.81 µg/ml at 2500 respectively. when compared with ascorbic acid by 78.57at 2500 µg/ml (Table: 13,14 and 15; figure: 11,12 and 13)

Table 16: Scavenging of linoleic acid radical by β-Carotene Linoleate Model using Aqueous Extract of *Portulaca quadrifida*.

S.No	Concentration(µg/ml) Ascorbic acid (STD)	Initial Absorbance	Absorbance after 2 hrs	% scavenging activity
1.	500	0.063	0.038	63.33
2.	1000	0.070	0.045	64.28
3.	1500	0.074	0.042	56.75
4.	2000	0.071	0.042	59.15
5.	2500	0.071	0.049	69.01
Aqueous Extract of *Portulaca quadrifida* (AEPQ)				
1.	500	0.029	0.026	89.65
2.	1000	0.028	0.026	92.85
3.	1500	0.028	0.027	96.42
4.	2000	0.027	0.026	96.29
5.	2500	0.043	0.024	55.81

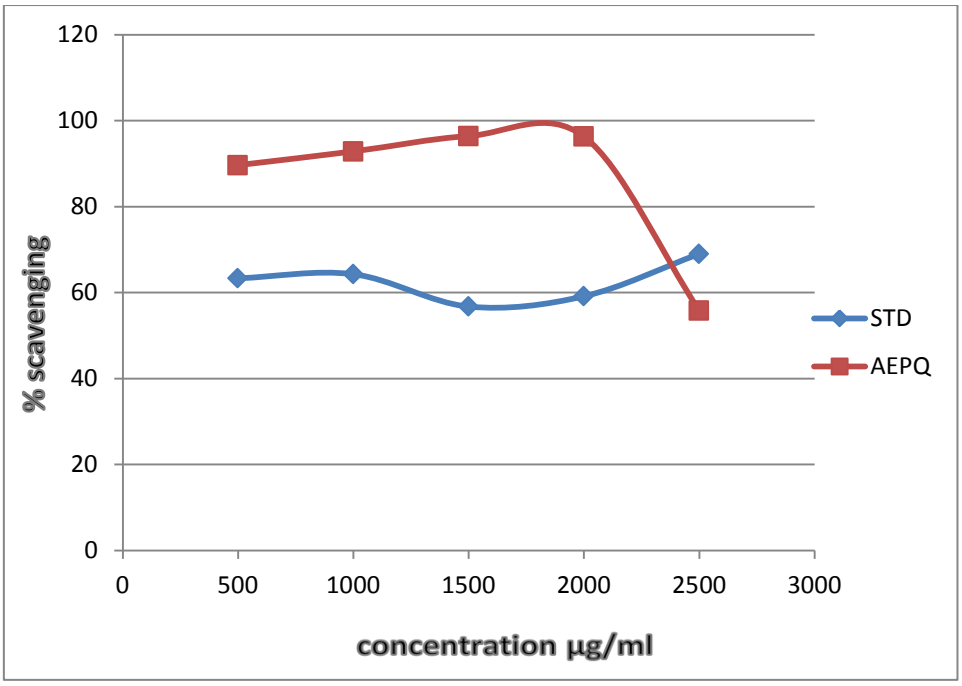


Figure 14: Scavenging of Linoleic acid radical β-Carotene Linoleate Model using aqueous extract of *Portulaca quadrifida*

The AEPQ was significantly scavenged the β-Carotene Linoleate radical when compared to the Ascorbic acid showed the antioxidant potential. AEPQ showed the scavenging activity by 96.29 at 2000µg/ml when compared to ascorbic acid showed 59.15 only at 2000µg/ml.

Table 17: Scavenging of linoleic acid radical by β-Carotene Linoleate Model using Chloroform Extract of *Portulaca quadrifida*

S.No	Concentration(µg/ml) Ascorbic acid (STD)	Initial Absorbance	Absorbance after 2 hrs	% scavenging activity
1.	500	0.063	0.038	63.33
2.	1000	0.070	0.045	64.28
3.	1500	0.074	0.042	56.75
4.	2000	0.071	0.042	59.15
5.	2500	0.071	0.049	69.01
Chloroform Extract of *Portulaca quadrifida* (CEPQ)				
1.	500	0.033	0.031	60.65
2.	1000	0.032	0.030	66.75
3.	1500	0.028	0.027	85.81
4.	2000	0.027	0.026	86.10
5.	2500	0.029	0.026	70.18

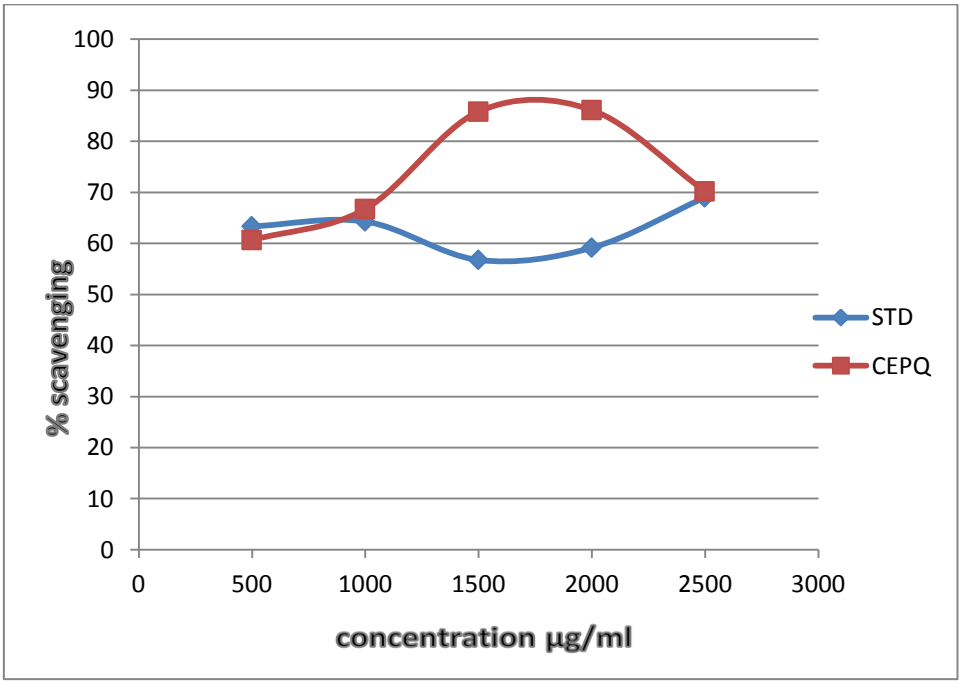


Figure 15: Scavenging of Linoleic acid radical β-Carotene Linoleate Model using chloroform extract of *Portulaca quadrifida*

The CEPQ was significantly scavenged the β-Carotene Linoleate radical when compared to the Ascorbic acid showed the antioxidant potential. CEPQ showed the scavenging activity by 86.10 at 2000µg/ml when compared to ascorbic acid showed 59.15 only at 2000µg/ml.

6.2.12 Table 18: Scavenging of linoleic acid radical by β-Carotene Linoleate Model using Methanol Extract of *Portulaca quadrifida*

S.No	Concentration(µg/ml) Ascorbic acid (STD)	Initial Absorbance	Absorbance after 2 hrs	% scavenging activity
1.	500	0.063	0.038	63.33
2.	1000	0.070	0.045	64.28
3.	1500	0.074	0.042	56.75
4.	2000	0.071	0.042	59.15
5.	2500	0.071	0.049	69.01
Methanolic Extract of *Portulaca quadrifida* (MEPQ)				
1.	500	0.029	0.028	86.10
2.	1000	0.027	0.028	88.24
3.	1500	0.027	0.026	90.12
4.	2000	0.028	0.027	93.44
5.	2500	0.034	0.022	60.16

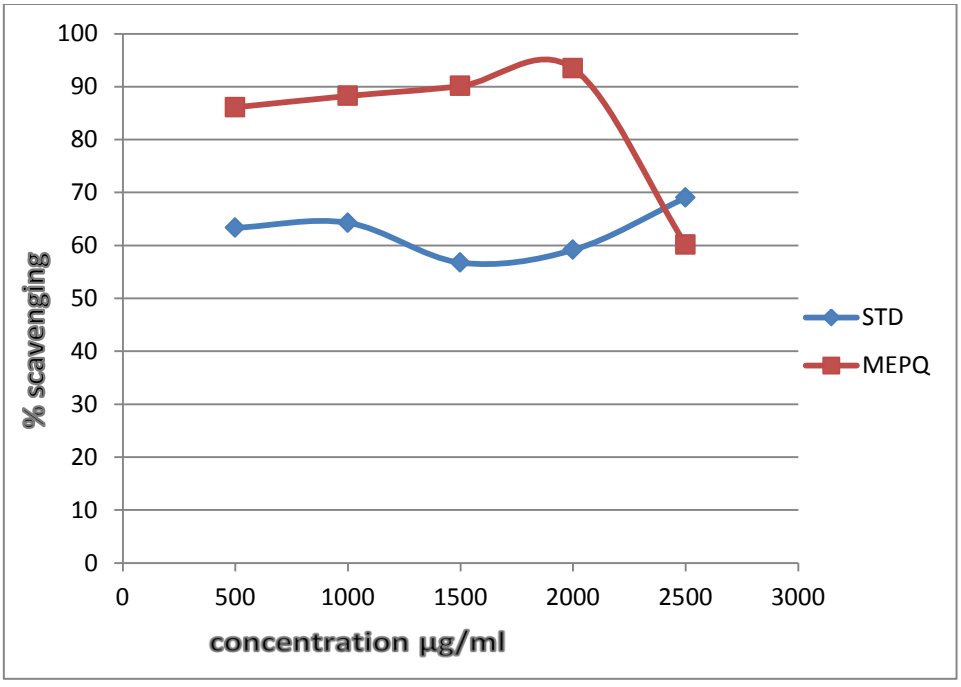


Figure 16: Scavenging of Linoleic acid radical β-Carotene Linoleate Model using methanol extract of *Portulaca quadrifida*.

The MEPQ was significantly scavenged the β-Carotene Linoleate radical when compared to the Ascorbic acid showed the antioxidant potential. MEPQ showed the scavenging activity by 93.44 at 2000µg/ml when compared to ascorbic acid showed 59.15 only at 2000µg/ml.

Table 19 : Scavenging activity of DPPH radical using Aqueous Extract of *Portulaca Quadrifida*

S.No	Concentration(µg/ml)	% scavenging activity
	STD (quercetin)	
1.	50	73.26 ± 0.015
2.	100	76.25 ± 0.022
3.	200	80.46 ± 0.01
4.	300	83.64 ± 0.02
5.	400	88.44 ± 0.024
Aqueous Extract of *Portulaca quadrifida* (AEPQ)		
1.	50	45.68 ± 0.035
2.	100	54.99 ± 0.760
3.	200	62.30 ± 0.315
4.	300	66.53 ± 0.051
5.	400	72.43 ± 0.380

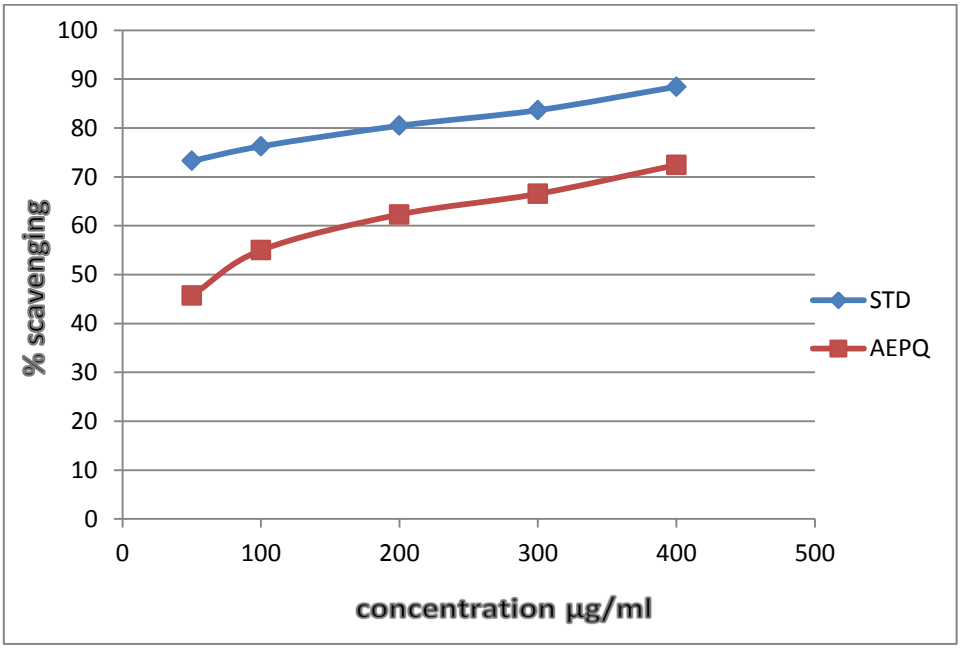


Figure 18: Scavenging activity of DPPH radical using aqueous extract of *Portulaca quadrifida*

The scavenging activity of the AEPQ was increased with increasing extract concentrations and that of the standard. AEPQ showed the % scavenging activity by 72.43 at 400 µg/ml when compared to quercetin showed 88.44 at 400 µg/ml.

Table 20: Scavenging activity of DPPH radical using Chloroform Extract of *Portulaca Quadrifida*

S.No	Concentration(µg/ml)	% scavenging activity
	STD (quercetin)	
1.	50	73.26 ± 0.015
2.	100	76.25 ± 0.022
3.	200	80.46 ± 0.01
4.	300	83.64 ± 0.02
5.	400	88.44 ± 0.024
Chloroform Extract of *Portulaca quadrifida* (CEPQ)		
1.	50	40.12±0.028
2.	100	40.99±0.697
3.	200	58.11±0.860
4.	300	62.43±0.351
5.	400	68.30±0.059

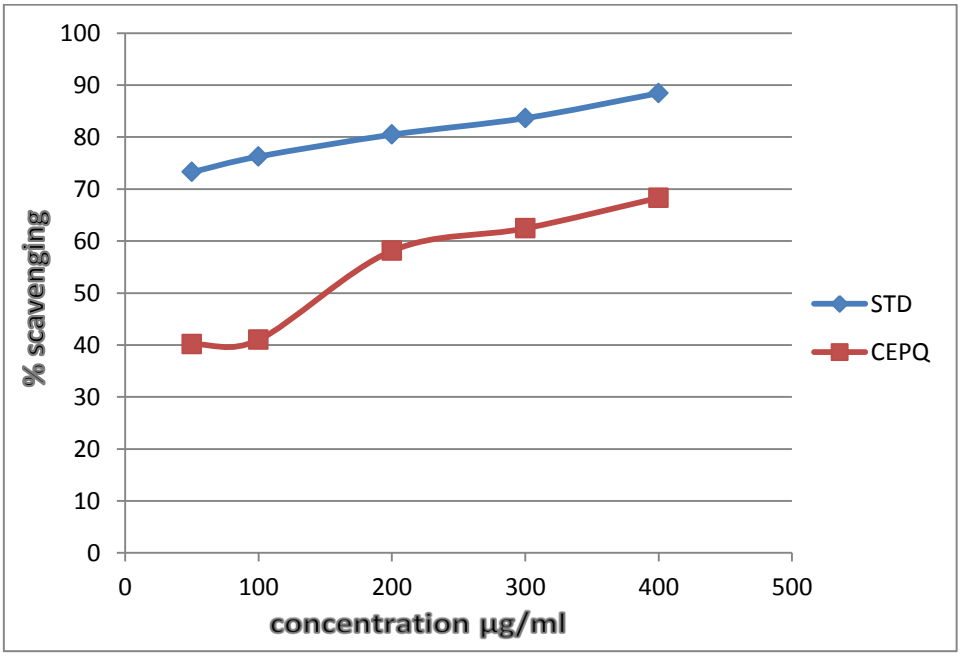


Figure 19: Scavenging activity of DPPH radical using chloroform extract of *Portulaca quadrifida*

The scavenging activity of the CEPQ was increased with increasing extract concentrations and that of the standard. CEPQ showed the % scavenging activity by 68.30 at 400 µg/ml when compared to quercetin showed 88.44 at 400 µg/ml.

Table 21 : Scavenging activity of DPPH radical using Methanol Extract of *Portulaca quadrifida.*

S.No	Concentration(µg/ml)	% scavenging activity
	STD (quercetin)	
1.	50	73.26 ± 0.015
2.	100	76.25 ± 0.022
3.	200	80.46 ± 0.01
4.	300	83.64 ± 0.02
5.	400	88.44 ± 0.024
Methanolic Extract of *Portulaca quadrifida* (MEPQ)		
1.	50	42.68±0.036
2.	100	51.30±0.729
3.	200	60.43±0449
4.	300	66.12±00.49
5.	400	69.99±0.051

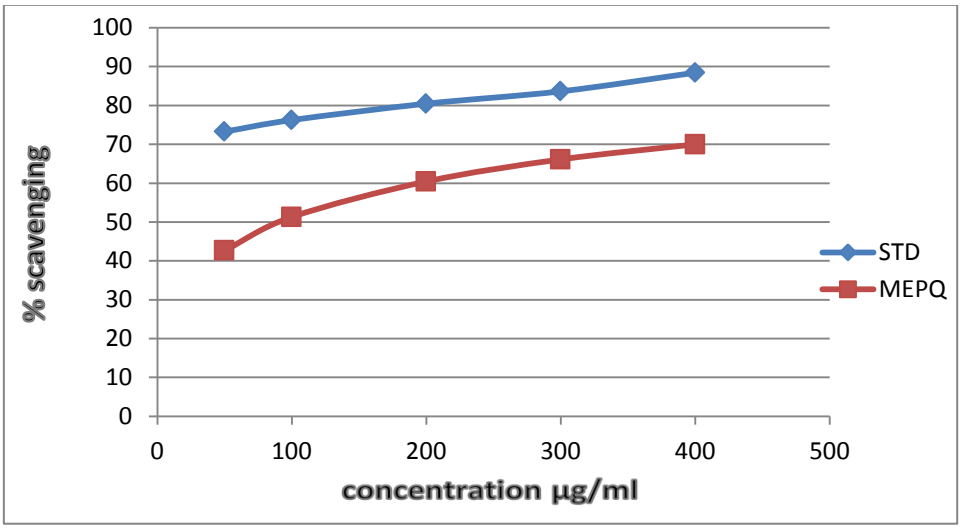


Figure 20: **Scavenging activity of DPPH radical using methanol extract of *Portulaca quadrifida***

The scavenging activity of the MEPQ was increased with increasing extract concentrations and that of the standard. MEPQ showed the % scavenging activity by 69.99 at 400 µg/ml when compared to quercetin showed 88.44 at 400 µg/ml.

By comparing all the extracts radical scavenging activity of AEPQ was evident at all the concentrations but only at low level not as significant as that of quercetin used as standard. The scavenging activity of the AEPQ was increased with increasing extract concentrations and that of the standard. AEPQ showed the % scavenging activity by 72.43 at 400 µg/ml when compared to quercetin showed 88.44 at 400 µg/ml. Whereas CEPQ and MEPQ showed 68.30 µg/ml and 69.99 µg/ml respectively.

Table 22: Scavenging activity of ABTS radical Cation assay using Aqueous Extract of *Portulaca quadrifida*

S.No	Concentration(µg/ml)	% scavenging activity
	STD	
1.	20	31.23
2.	40	42.6
3.	60	64.1
4.	80	75.1
5.	100	83.44
Aqueous Extract of *Portulaca quadrifida* (AEPQ)		
1.	20	18.76
2.	40	27.5
3.	60	43.5
4.	80	63.91
5.	100	71.2

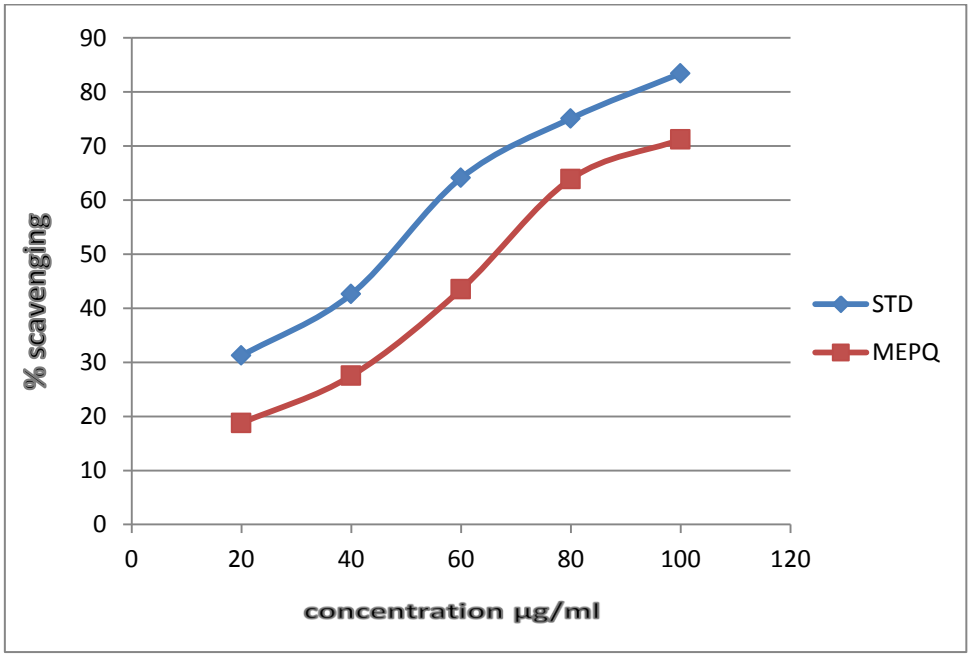


Figure 22: ABTS radical Cation assay of aqueous extract of *Portulaca quadrifida*

The antioxidant potential of AEPQ was significantly scavenged the ABTS radical by 71.2 at 100 µg/ml, when compared to the standard which was 83.44 at 100µg /ml. The antioxidant properties were expressed as concentration dependent.

Table 23: Scavenging activity of ABTS radical Cation assay using Chloroform Extract of *Portulaca quadrifida*.

S.No	Concentration(µg/ml)	% scavenging activity
	STD	
1.	20	31.23
2.	40	42.6
3.	60	64.1
4.	80	75.1
5.	100	83.44
Chloroform Extract of *Portulaca quadrifida* (CEPQ)		
1.	20	20.14
2.	40	26.45
3.	60	41.5
4.	80	56.10
5.	100	64.40

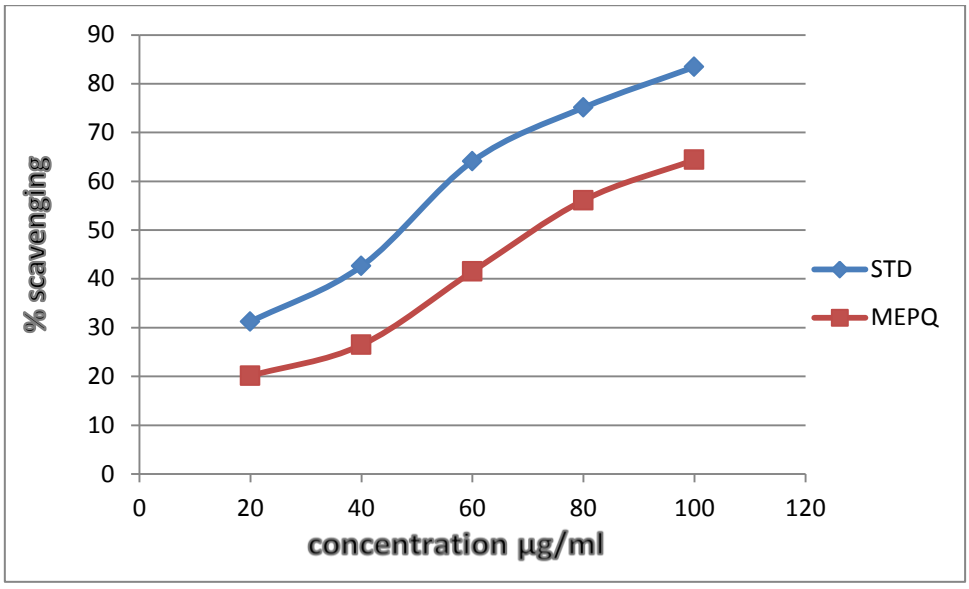


Figure 23: ABTS radical Cation assay of chloroform extract of *Portulaca quadrifida*.

The antioxidant potential of CEPQ was significantly scavenged the ABTS radical by 64.40 at 100 µg/ml, when compared to the standard which was 83.44 at 100µg /ml. The antioxidant properties were expressed as concentration dependent.

Table 24: Scavenging activity of ABTS radical Cation assay using Methanol Extract of *Portulaca quadrifida.*

S.No	Concentration(µg/ml)	% Scavenging activity
	STD	
1.	20	31.23
2.	40	42.6
3.	60	64.1
4.	80	75.1
5.	100	83.44
Methanol Extract of *Portulaca quadrifida* (MEPQ)		
1.	20	26.45
2.	40	40.10
3.	60	52.40
4.	80	60.10
5.	100	68.40

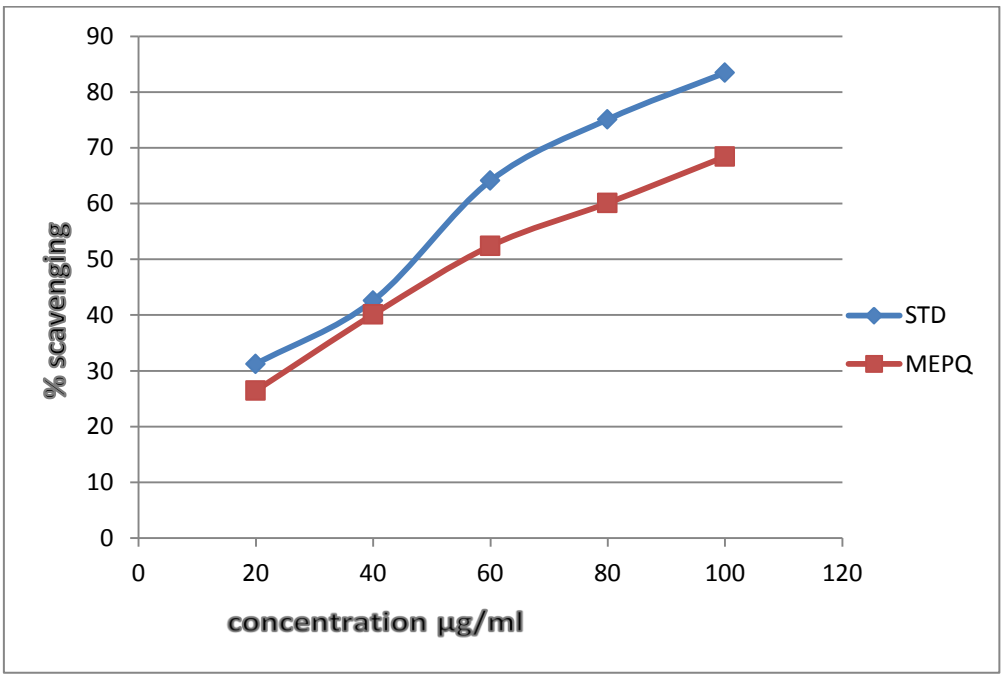


Figure 24: ABTS radical Cation assay of methanol extract of *Portulaca quadrifida*.

The antioxidant potential of MEPQ was significantly scavenged the ABTS radical by 68.40 at 100 µg/ml, when compared to the standard which was 83.44 at 100µg /ml. The antioxidant properties were expressed as concentration dependent.

The antioxidant potential of AEPQ was significantly scavenged the ABTS radical by 71.2 at 100 µg/ml, when compared to the standard, CEPQ and MEPQ which has showed 64.40 and 68.40 at 100µg /ml respectively. The antioxidant properties were expressed as concentration dependent.

From the findings of the above *In vitro* antioxidant study aqueous extract (AEPQ) was found to be potent when compared with chloroform (CEPQ) and methanol(MEPQ)

In **Phase –III** of our study all the extracts were screened for their *In vitro* cytotoxicity studies using colon cell line HT-29 ; antimicrobial screening.

. The results of the findings are given in Table 25; Figure 25 to 27, and The results of the findings are given in Table 26 to 28; Figure 30 and 31.

6.3. *In Vitro* Cytotoxicity

In Vitro Cytotoxicity study of chloroform, methanol and aqueous extract of *Portulaca quadrifida* were evaluated for the cytotoxic activity by using MTT assay on HT-29 (colon cancer cell lines) where aqueous extracts showed highest inhibition with lowest concentration of 15.87 µg /ml when compared to other extracts. (Table: 25; Figure: 25, 26 and 27).

Table 25: Cytotoxicity Studies of successive extracts of *Portulaca quadrifida* using colon cancer cell line.

Sl. No.	Sample	CTC50 µg/ml (HT- 29)
1	CHCL3	**126.28**
2	CH3OH	**398.59**
3	AQ	**15.87**

Sample - I

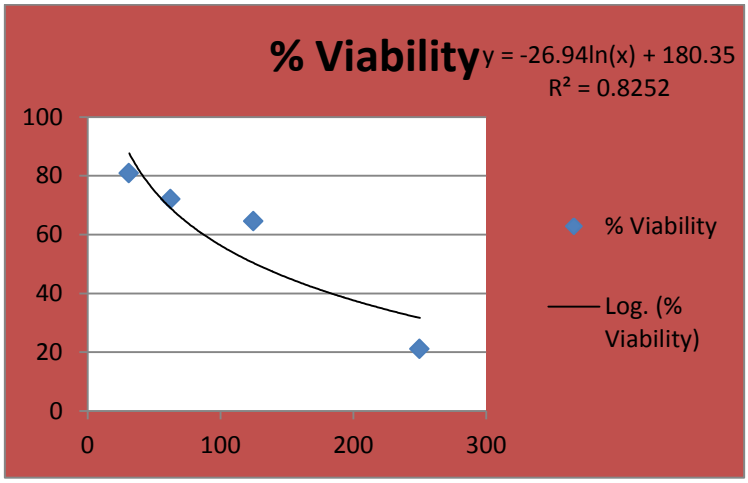


Figure 25: Inhibition of Growth of human colon cancer cell line HT-29 by chloroform extract of *Portulaca quadrifida* in MTT assay

Sample - II

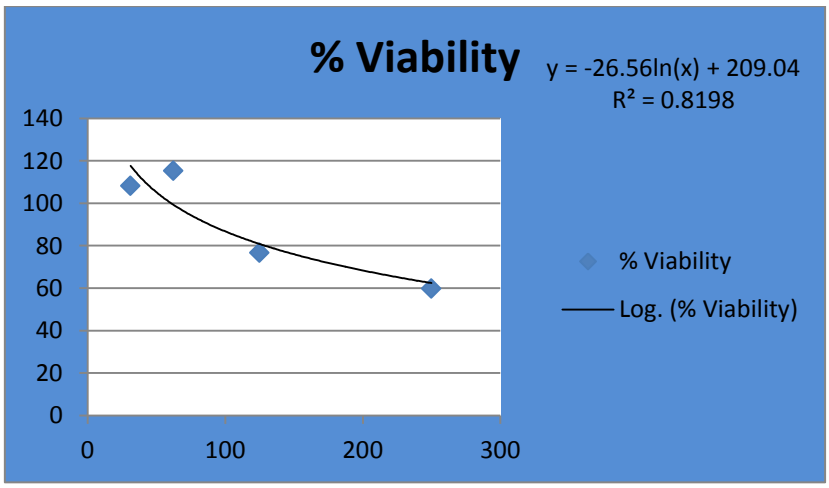


Figure 26: Inhibition of growth of human colon cancer cell line HT-29 by methanol extract of *Portulaca quadrifida* in MTT assay

Sample - III

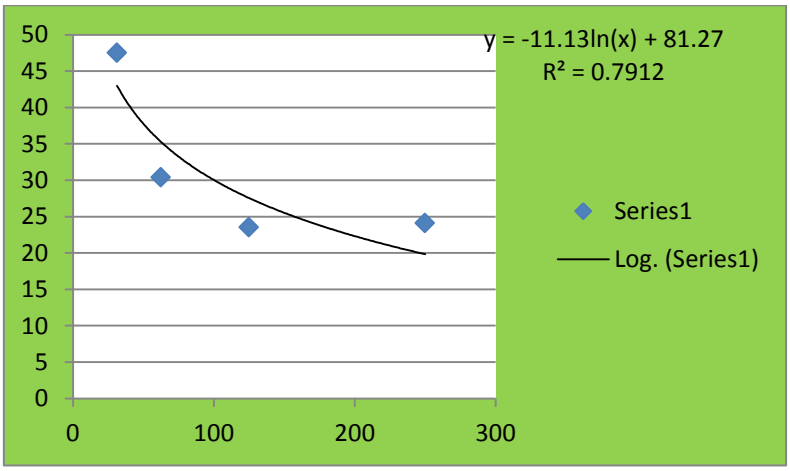


Figure 27: Inhibition of growth of human colon cancer cell line HT-29 by aqueous extract of *Portulaca quadrifida* in MTT assay

6.4 Antimicrobial activity

All the three extracts were screened for antimicrobial activity in which nutrient agar method was followed for antibacterial and potato dextrose agar method was followed for screening antifungal action of the extracts. Among chloroform extract, methanol extract and aqueous extract, aqueous extract showed significant activity by inhibiting *Escherichia coli* and *Staphylococcus aureus*, and fungal organism like *Aspergillus flavus* and *Aspergillus niger* at concentration of minimum inhibitory of 4.3, 4.2, 4.4 and 3.7 at 25mcg/ml, respectively. (Table: 26,27 and 28 ; Figure: 28 and 29).

Table 26: Antimicrobial activity of chloroform and methanol extract of *Portulaca quadrifida.*

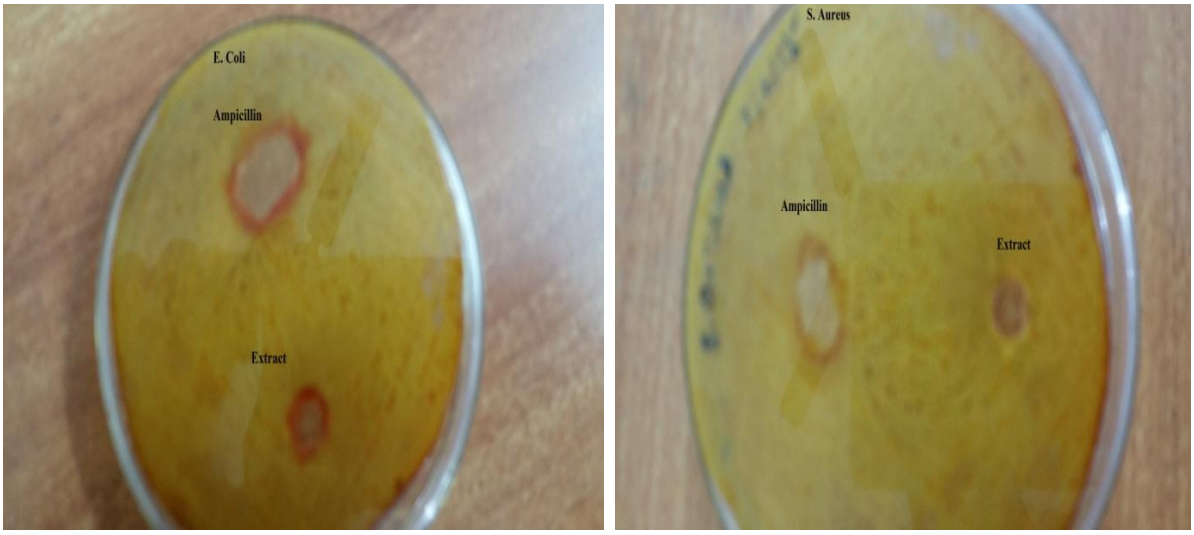
Extract	**Inhibition of Zone measured in 'mm' for bacteria and fungi differentiated with Antimicrobial agents**										
Microorganism	**Chloroform (TRIPLICATE)**						**Methanol (TRIPLICATE)**				
Bacteria		1(mm)	2(mm)	3(mm)	AVG.	STD.	1(mm)	2(mm)	3(mm)	AVG.	STD. Dv
E. Coli	Concentration mcg/ml										
	25	4.1	3.6	3.7	3.8	0.2	3.2	3	3.4	3.20	0.16
	50	9.0	10.0	9.6	9.5	0.4	7	8	8.2	7.73	0.52
	75	11.7	12.3	12.5	12.2	0.3	9	11	12.5	10.83	1.43
	100	17.4	16.9	17.1	17.1	0.2	13.4	13.9	15.1	14.13	0.71
S. Aureus	Concentration mcg/ml										
	25	3.4	3.7	4.0	3.7	0.3	3	3.2	3.3	3.17	0.12
	50	9.9	9.6	10.2	9.9	0.3	6.8	7.2	8.1	7.37	0.54
	75	12.6	12.4	12.9	12.6	0.2	8.2	9	9.7	8.97	0.61
	100	16.9	16.9	16.8	16.9	0.0	10.2	10.5	10.8	10.50	0.24
Fungi											
A. niger	Concentration mcg/ml										
	25	3.4	3.2	3.4	3.3	0.1	3.4	3.2	3.4	3.33	0.09
	50	7.9	8.0	8.2	8.0	0.1	7.9	8	8.2	8.03	0.12
	75	11.7	12.3	12.5	12.2	0.3	11.7	12.3	12.5	12.17	0.34
	100	16.8	16.0	16.6	16.5	0.3	13.8	13	13.6	13.47	0.34
A.flavus	Concentration mcg/ml										
	25	4.3	4.0	3.6	3.9	0.3	3.2	3	3.5	3.23	0.21
	50	8.1	8.5	8.8	8.5	0.3	8.1	8.5	8.8	8.47	0.29
	75	13.4	13.7	13.5	13.6	0.1	10.4	10.7	10.5	10.53	0.12
	100	17.1	17.3	17.4	17.2	0.1	12.1	12.3	12.4	12.27	0.12

Table 27: Antimicrobial activity of aqueous extract of *Portulaca quadrifida*.

Extract	**Inhibition of Zone measured in 'mm' for bacteria and fungi differentiated with Antimicrobial agents**					
Microorganism			**Aqueous(TRIPLICATE)**			
Bacteria		1(mm)	2(mm)	3(mm)	AVG.	STD.Dv
E. coli	Concentration mcg/ml					
	25	4.6	4.1	4.2	4.3	0.2
	50	10.1	11.2	10.8	10.7	0.5
	75	13.2	13.8	14	13.7	0.3
	100	19.5	19	19.2	19.2	0.2
S. aureus	Concentration mcg/ml					
	25	3.8	4.2	4.5	4.2	0.3
	50	11.1	10.8	11.5	11.1	0.3
	75	14.2	13.9	14.5	14.2	0.2
	100	19	19	18.9	19.0	0.0
Fungi						
A. niger	Concentration mcg/ml					
	25	3.8	3.6	3.8	3.7	0.1
	50	8.9	9	9.2	9.0	0.1
	75	13.2	13.8	14	13.7	0.3
	100	18.9	18	18.6	18.5	0.4
A.flavus	Concentration mcg/ml					
	25	4.8	4.5	4	4.4	0.3
	50	9.1	9.6	9.9	9.5	0.3
	75	15.1	15.4	15.2	15.2	0.1
	100	19.2	19.4	19.5	19.4	0.1

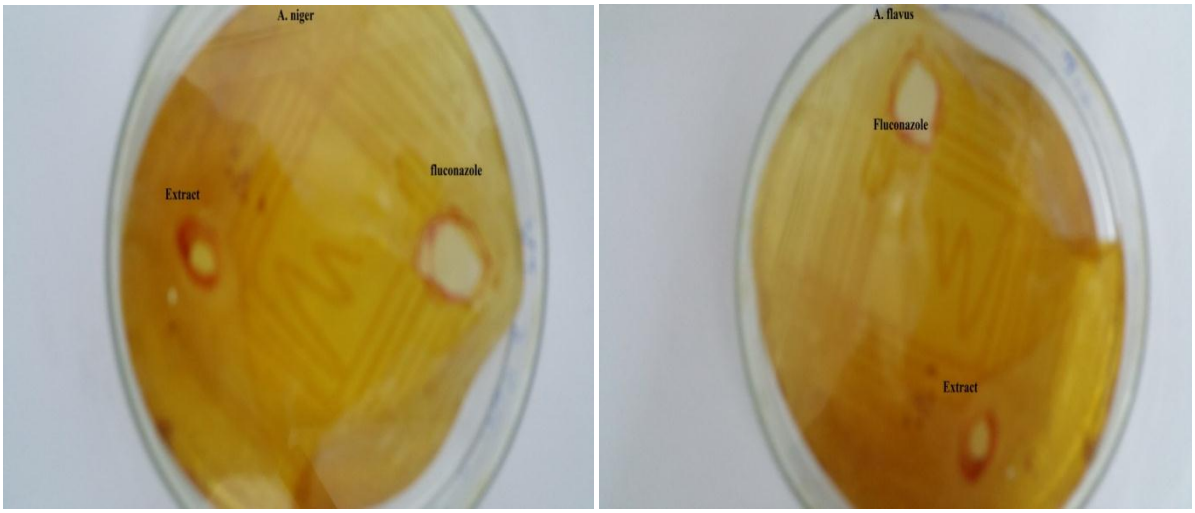
Table 28: Antimicrobial activity of standard drug.

STANDARD						
	Chloroform, Methanol and Aqueous (TRIPLICATE)					
AMPICILLIN		1(mm)	2(mm)	3(mm)	AVG.	STD. Dv.
E. Coli	Concentration mcg/ml					
	25	12.5	12.6	12.9	12.7	0.2
	50	21	21.9	20.5	21.1	0.6
	75	32	30.2	33.1	31.8	1.2
	100	40.5	40.9	41.2	40.9	0.3
S. aureus	Concentration mcg/ml					
	25	19	19.5	18.3	18.9	0.5
	50	31.5	30.6	31.2	31.1	0.4
	75	40.2	40.9	39.8	40.3	0.5
	100	46.9	46.8	47.1	46.9	0.1
FLUCONAZOLE						
A. niger	Concentration mcg/ml					
	25	15.3	15.7	14.7	15.2	0.4
	50	25.4	24.6	25.1	25.0	0.3
	75	32.4	32.9	32.0	32.4	0.4
	100	37.8	37.7	37.9	37.8	0.1
A.flavus	Concentration mcg/ml					
	25	11.6	11.7	11.9	11.7	0.2
	50	19.4	20.3	19.0	19.5	0.5
	75	29.6	27.9	30.6	29.4	1.1
	100	37.5	37.8	38.1	37.8	0.3



A B

Figure 28 : Inoculum of 24hrs E. Coli and S. Aureus growth culture A and B respectively



C **D**

Figure 29: Inoculum of 48hrs A. niger and A. flavus growth culture C and D respectively.

From the findings of the above *In vitro* cytotoxicity study and antimicrobial study aqueous extract (AEPQ) was found to be potent when compared with chloroform (CEPQ) and methanol(MEPQ). So it was comparable with *In vitro* antioxidant findings i.e aqueous extract was found to be potent so based on this findings the aqueous extract alone was used in the Acute oral toxicity study and *In vivo* anti colon cancer screening using DMH model, in the isolation of active constituents, *In vitro* cytotoxicity studies of the isolated compounds and in structure elucidation study by GC-MS.

6.5 **Acute Oral Toxicity Study**

A limit test was conducted on female mice with four dose level (5,50,300,2000 mg/kg b.wt) to find out mortality, morbidity produced by the aqueous extract of *Portulaca quadrifida* administration on acute oral toxicity study conducted as per OECD test guideline 423 and also to fix dose to be used in I*n vivo* anti colon cancer study. From the study it was found that no mortality, morbidity were produced by the extract even at 2000 mg/kg b.wt dose level tested. This confirmed the non toxic nature of the extract tested

Table 29 Acute Oral Toxicity Study Results

S.No	Response	Concentration of AEPQ			
		5 mg/kg	50 mg/kg	300 mg/kg	2000 mg/kg
01	Motor Activity	Normal	Normal	Normal	Normal
02	Grooming	Absent	Absent	Absent	Absent
03	Touch response	Absent	Absent	Absent	Absent
04	Touch response	Normal	Normal	Normal	Normal
05	Pain response	Normal	Normal	Normal	Normal
06	Tremors	Absent	Absent	Absent	Absent
07	Convulsion	Absent	Absent	Absent	Absent
08	Righting reflux	Normal	Normal	Normal	Normal
09	Gripping strength	Normal	Normal	Normal	Normal
10	Pinna reflux	Present	Present	Present	Present
11	Corneal reflux	Present	Present	Present	Present
12	Writhing	Absent	Absent	Absent	Absent
13	Pupils	Normal	Normal	Normal	Normal
14	Urination	Normal	Normal	Normal	Normal
15	Salivation	Normal	Normal	Normal	Normal
16	Skin colour	Normal	Normal	Normal	Normal
17	Lacrimation	Normal	Normal	Normal	Normal

In **Phase –IV** of our study aqueous extracts were screened for their *In vivo* anticolon cancer by using DMH model. The results of the findings are given in Table 32 to 41; Figure 30 to 69.

6.6 *IN VIVO* ANTI CANCER SCREENING ON DMH INDUCED COLON CANCER

On the termination of our study animals were fasted overnight, and anaesthesized and the abdomen was cut open to remove the colon to investigate the morphology, pathology (gross necropsy study) and anticolon cancer potential of *Portulaca quadrifida.* The results of the finding were depicted in Figure 32 to 36.

6.6.1 Gross Necropsy Study

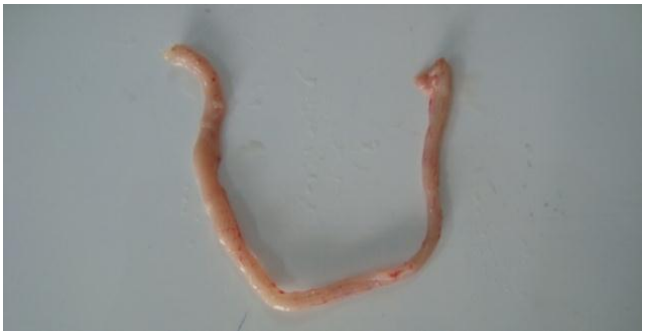


Figure 30: **GROUP – I:** CONTROL - Normal saline. (p.o)

Animals in group-I which were treated with normal saline showed no changes in colon.

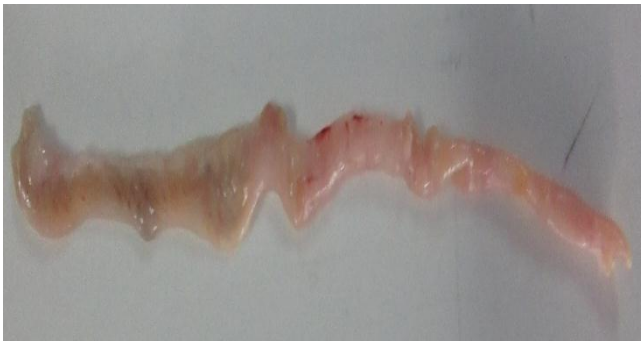


Figure 31: **GROUP – II** Only DMH (20 mg/kg)

Animals in group-II which were treated with DMH induced colon cancer major changes like polyps formation were observed when compared to control group.

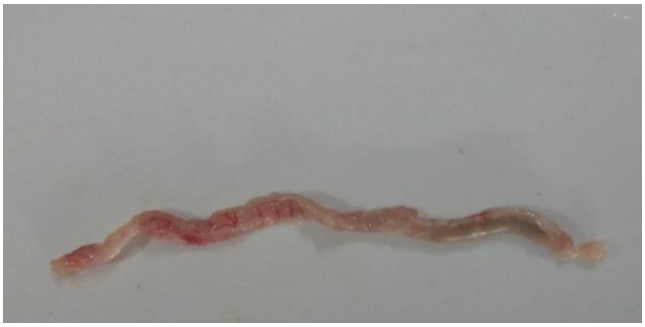


Figure 32: **GROUP – III**: DMH + 5 Fluorouracil 20mg/kg ip

Animals in group-III which were treated with DMH + 5 Fluorouracil (standard) showed delayed formation of polyps

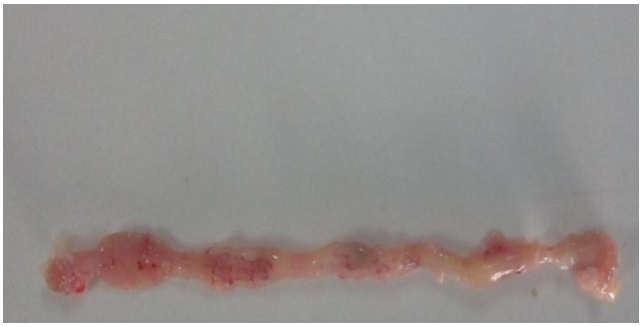


Figure 33: **GROUP – IV:** DMH (20 mg/kg) + Plant extract 200mg /kg

Animals in group-IV which were treated with DMH + Plant extract 200mg/kg showed delayed formation of polyps

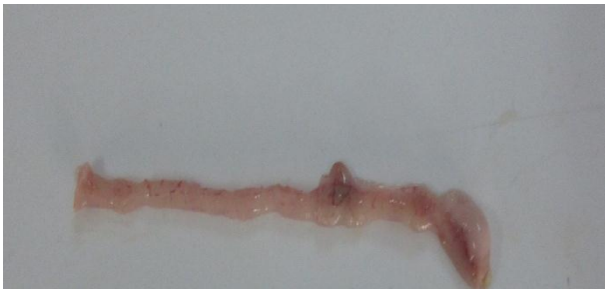


Figure 34: **GROUP-** V: DMH (20 mg/kg) + Plant extract 400mg/kg

Animals in group-V which were treated with DMH + Plant extract 400mg/kg showed dose dependant reduction and delayed formation of polyps which were very well comparable with group III animals treated with standard.

6.6.2 Analysis of Tumor volume, Burden,Tumor weight and Tumor incidence

The isolated colon was subjected to analyse tumor volume, burden ,weight and incidence and the results are depicted in Table 37; Figure 53 to 56.

Table 32: Effect of aqueous extract of *Portulaca quadrifida* (AEPQ) on tumor volume, burden, weight and tumor incidence analysis (physical parameter) on DMH induced colorectal cancer in rats.

Group	Tumor Volume	Tumor Burden	Tumor Incidence	Colon Weight gms
Normal	0.00±0.00	0.00±0.00	0.00±0.00	0.76±0.02
Control	4.55±0.67***	13.17±2.04***	22.83±3.81***	1.27±0.18***
Standard	1.96±0.41***	4.50±1.87***	9.83±1.72***	0.86±0.12***
Test I	2.99±0.59***	6.16±1.16***	15.50±1.87***	1.11±0.06ns
Test II	2.20±0.29***	5.83±1.47***	13.33±1.63***	0.92±0.22**

Values are exhibited as mean ± S.D. Statistical significance (p) was evaluated by one way ANOVA accompanied by Tukeys multiple comparison test. Control group was compared with normal Standard and test groups were compared with control. $^{***}P< 0.001$ were considered highly significant. $^{**}P< 0.001$ significant, $^{*}P< 0.001$ less significant , ns- not significant.

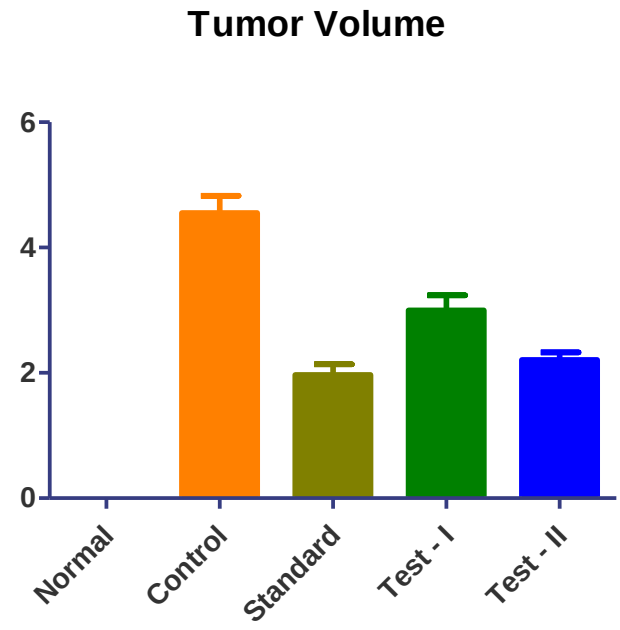


Figure 35: Effect of aqueous extract of *Portulaca quadrifida* (AEPQ) on tumor volume

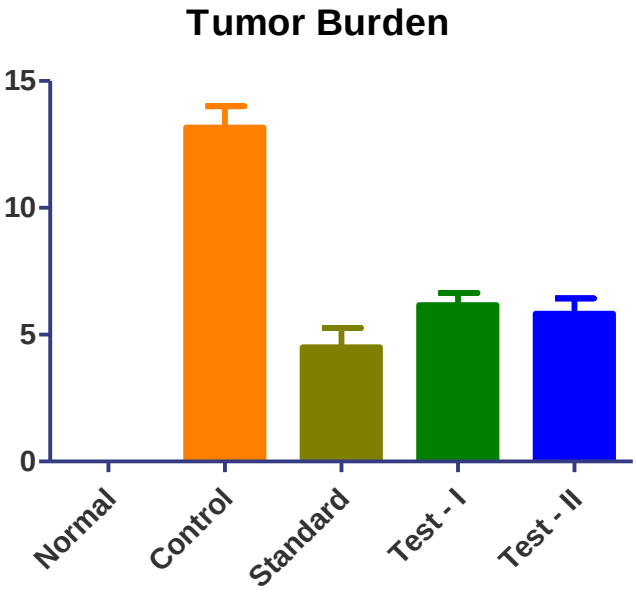


Figure 36: Effect of aqueous extract of *Portulaca quadrifida* (AEPQ) on tumor burden

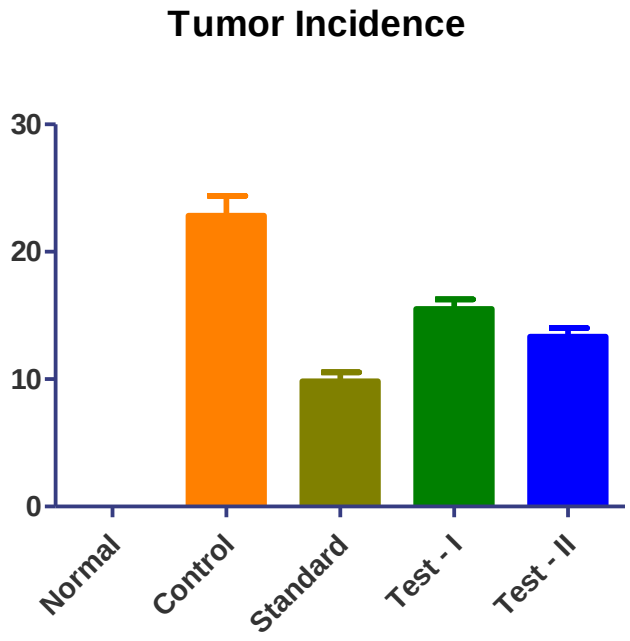


Figure 37: Effect of aqueous extract of *Portulaca quadrifida* (AEPQ) on tumor incidence

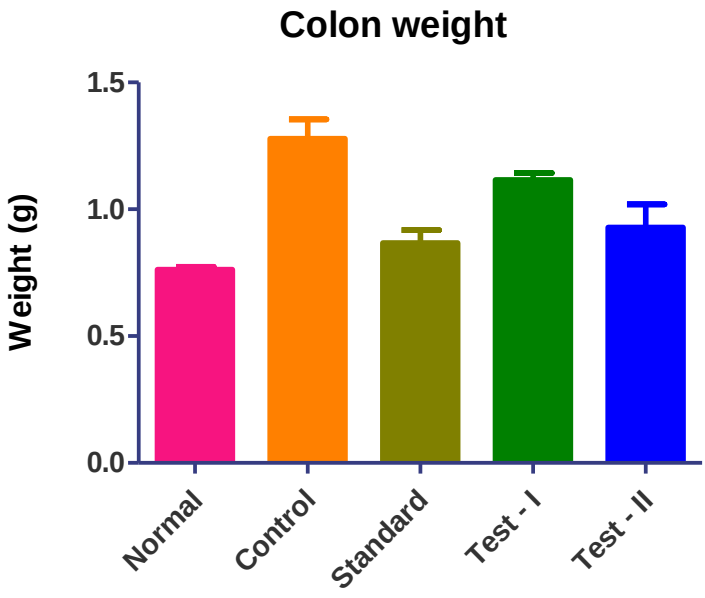


Figure 38: Effect of aqueous extract of *Portulaca quadrifida* (AEPQ) on colon weight

6.6.3 Gross Necropsy Study

Weekly body weight analysis of all the treatment group animals upto 15 weeks were analysed and the result of the study is depicted in Table 33

Table 33: Effect of aqueous extract of *Portulaca quardifida* (AEPQ) on body weight analysis (physical parameter) on DMH induced colorectal cancer in rats:

Weeks	Control Group - I	Only DMH Group - II	DMH + 5 Fluorouracil Group - III	DMH+ AEPQ (200mg/kg) Group – IV	DMH+ AEPQ (400mg/kg) Group – V
1 Week	337±1.8	330±2.4	345.3±1.4	340.08±1.8	342.12±1.7
3 Week	395±2.0	379.8±1.6	388.4±1.3	364.85±1.1	376.18±1.4
5 Week	460±1.2	420.6±2.1	434.9±2.4	393.05±2.5	412.11±2.2
7 Week	518±1.6	456.2±1.8	476.1±2.0	419.65±2.5	450.96±1.1
9 Week	574±2.2	483.1±1.1	517.3±1.1	448.16±2.0	488.84±1.9
11 Week	634±2.0	500.7±2.1	563.5±1.8	476.79±1.1	526.86±2.5
13 Week	690±1.8	509.7±2.2	609.5±2.2	504.49±1.9	565.11±2.0
15 Week	760±1.9	511.8±1.4	656.2±1.0	541.12±1.7	608.84±1.8

Group I control animals express normal body weight at the initial and final study period. Group II cancer bearing animals show decrease final body weight differentiated to group I animals. Group III standard managed animals express gradual rise in the final body weight compare to group II animals. Group IV 200mg/kg extract treated animals express increase in final body weight but Group V 400mg/kg extract treated animals showed well comparable body weight as compared to group III.

6.6.4 ESTIMATION OF HEMATOLOGICAL PARAMETERS

At the end of 16 weeks treatment, blood was collected from all the Groups of animals by rectro orbital method. In the colon cancer condition there was alteration in the normal blood cell counts. A remarkable rise in the level of WBC, and remarkable decline in the level of RBC and haemoglobin when compared to control animals were observed. The extract approximately reversed these changes towards normal values in a dose dependent and appropriate manner (Table 34; Figure 39, 40 and 41).

Table 34 : ESTIMATION OF HEMATOLOGICAL PARAMETERS

GROUPS (n=6)	Control Group - I	Only DMH Group - II	DMH + 5 Fluorouracil Group - III	DMH+ AEPQ (200mg/kg) Group – IV	DMH+ AEPQ (400mg/kg) Group – V
RBC (1 x 10^{12}/L)	5.1866±0.0882	4.5366±0.0962**	5.92333±0.1337***	4.220±0.1149***	5.400±0.1299ns
WBC (1 x 10^{19}/L)	12.43±0.3106	14.73±0.2765***	11.27±0.1725ns	15.17±0.3691***	11.87±0.5869ns
Hb (g/dL)	12.533±0.2564	10.766±0.4005*	15.066±0.532***	9.666±0.3392***	13.3000±0.4427ns
Lymphocytes %	80.00±1.932	82.666±1.1737 ns	83.666±2.8362ns	87.333±1.282ns	85.666±2.740ns
Monocytes %	3.333±0.5678	3.333±0.4216ns	4.000±0.3651ns	3.667±0.7601ns	3.33±0.5578ns
Eosinophis %	4.333±0.760	5.000±0.3651ns	4.333±0.7601ns	5.666±0.4216ns	3.666±2108ns

***P<0.001, **P<0.01, *P<0.05, #-Non significant

Data is exhibited as Mean ±SEM. (n=6, animals in each group

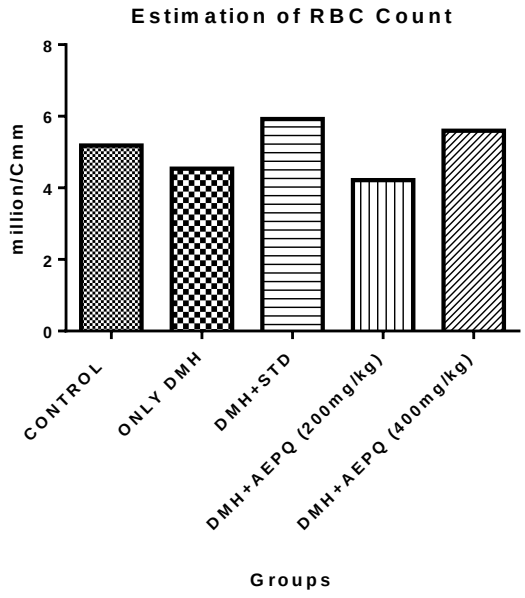


Figure: 39 Estimation of RBC Count

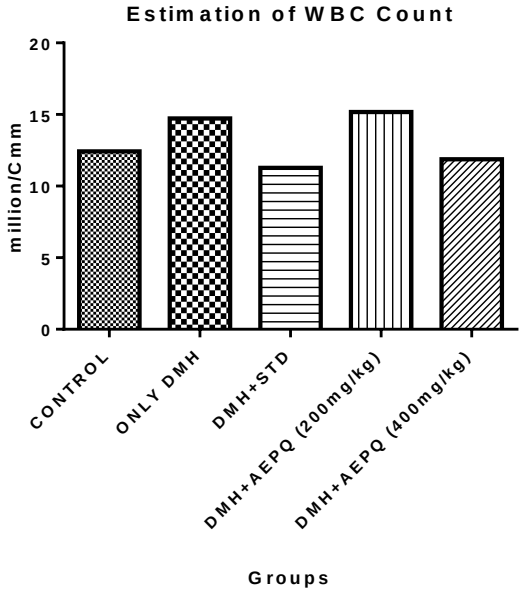


Figure 40: Estimation of WBC Count

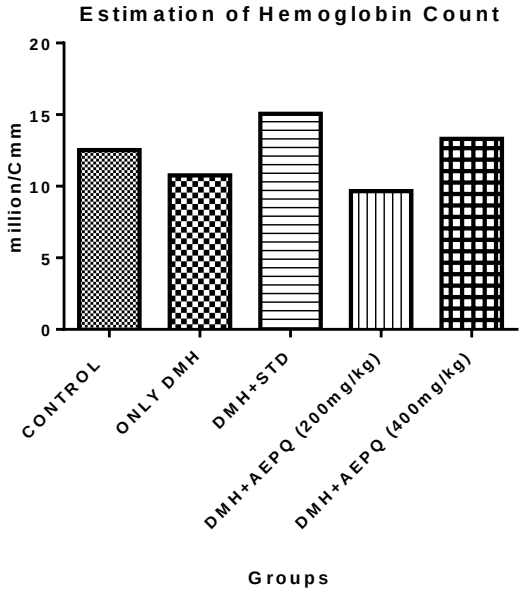


Figure: 41 Estimation of Haemoglobin Count

6.6.5 Estimation of Serum Biochemical Parameters

The separated serum was used to estimate the below biochemical parameters. It exhibited that in cancer condition there was a significant change in serum biochemical parameters. There was a remarkable decrease in triglycerides, and there is remarkable increase in SGOT, SGPT, Bilirubin levels.

Table 35: Estimation of Serum Biochemical Parameters

GROUPS **(n=6)**	Control Group – I	Only DMH Group –I I	DMH+5 Fluorouracil Group – III	DMH+AEPQ (200mg/kg) Group – IV	DMH+AEPQ (400mg/kg) Group – V
SGOT (U/L)	125.0±4.359	181.7±1.453 **	130.0±5.168*	140.0±8.021 **	142.0±0.5774 **
SGPT (U/L)	66.33±2.404	86.67±2.333 ***	68.34±3.510*	62.67±2.963 ns	64.67±4.256 ns
Total cholesterol (mg/dl)	64.00±4.041	115.0±3.215 ***	84.00±5.041**	99.33±2.186***	92.67±2.333**
Triglycerides (mg/dl)	94.17±3.491	56.13±8.781ns	98.2±4.356*	120.1±4.477**	103.6±14.47 ***
Total Bilirubin (mg/dl)	0.6667±0.03333	0.6667±0.1202	0.1568±0.0ns	0.2000±0.05774ns	0.1667±0.03333 ns

***P<0.001, **P<0.01, *P<0.05, #-Non significant

Data are exhibited as Mean ±SEM. (n=6, animals in each group)

Statistical comparison: One way ANOVA, followed by Dunnet`s comparison was performed. DMH only (Group 2) was compared with Normal control (Group 1), DMH+5 Fluorouracil (20mg/kg) Group 3, DMH+APEQ (200mg/kg) Group 4, DMH+AEPQ (400mg/kg) Group 5.

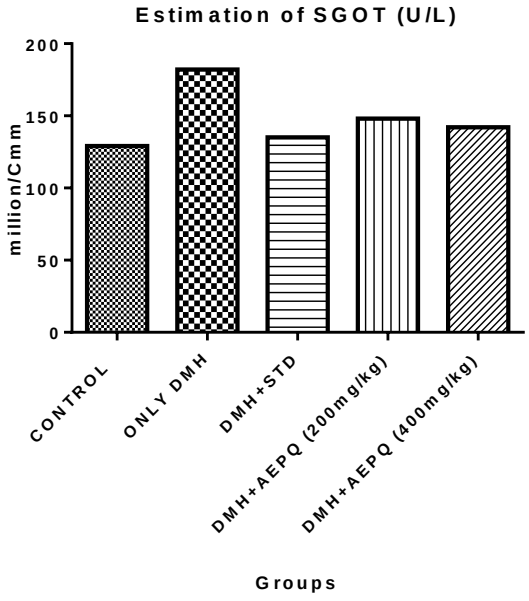


Figure: 42 Estimation of SGOT (U/L)

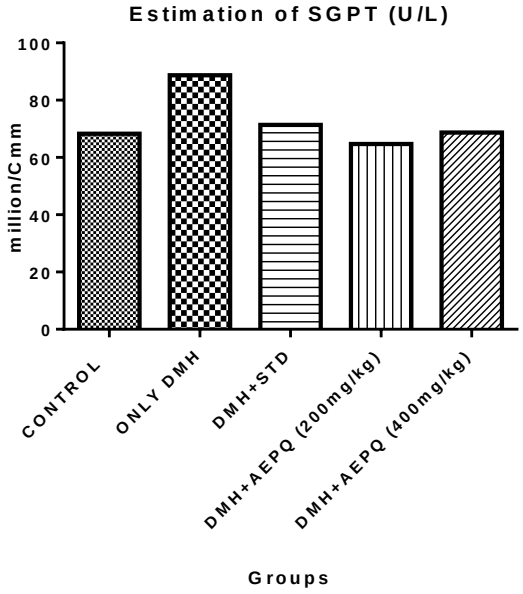


Figure: 43 Estimation of SGPT (U/L)

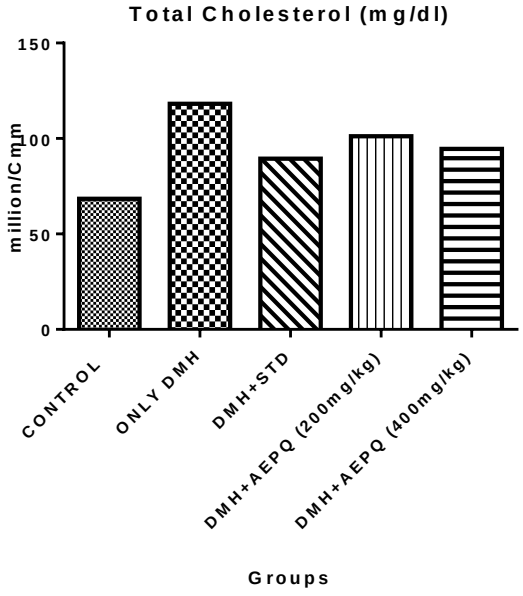


Figure: 44 Estimation of Total Cholesterol

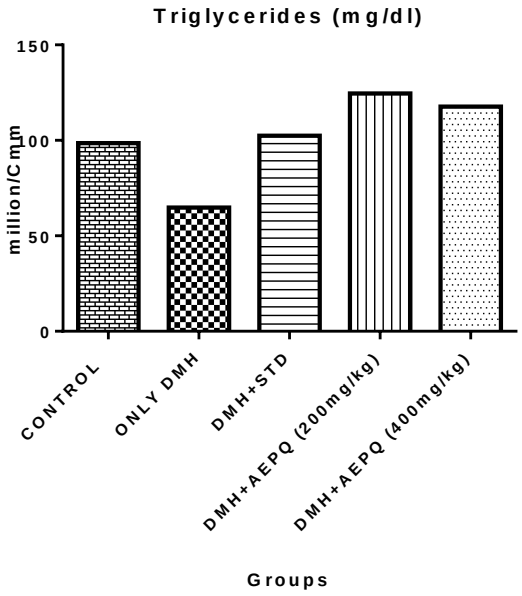


Figure: 45 Estimation of Triglycerides (mg/dl)

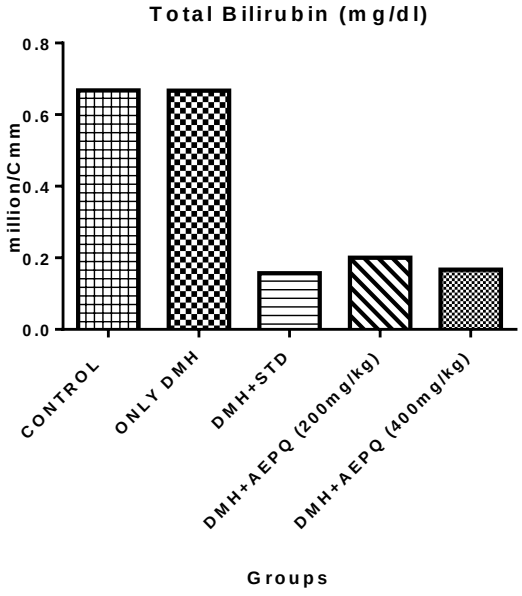


Figure: 46 Estimation of Total Bilirubin (mg/dl)

6.6.6 ESTIMATION OF TUMOR MARKERS:

The levels of CEA and AFP was increased in Group II (DMH treated group) when compared with Group I (control) which indicates the occurrence of cancer. The levels of CEA and AFP were declined in Group III (DMH+ Standard) and Group V (DMH+AEPQ 400mg/kg) as the decrease in levels prevents the neoplastic growth and reduces the level of carcinoma. (Table 36 and 37: Figure 47 and 48)

Table 36: ESTIMATION OF CARCINOABROYONIC ANTIGEN (CEA)

GROUPS (n=6)	Control Group – I	Only DMH Group –I I	DMH+5 Fluorouracil Group – III	DMH+AEPQ (200mg/kg) Group – IV	DMH+AEPQ (400mg/kg) Group - V
Carcinoembryonic antigen (ng/dL)	0.1953±0.00206	0.4733±0.007126*	0.2111±0.003000***	0.2660±0.006083***	0.2377±0.002404***

***P<0.001, **P<0.01, *P<0.05, #-Non significant

Data is exhibited as Mean ±SEM. (n=6, animals in each group)

Statistical comparison: One way ANOVA, followed by Dunnet`s comparison was performed. DMH only (Group 2) was compared with Normal control (Group 1), DMH+5 Fluorouracil (20mg/kg) Group 3, DMH+APEQ (200mg/kg) Group 4, DMH+AEPQ (400mg/kg) Group 5, the values of Carcinoembryonic Antigen was exhibited as ng/dl.

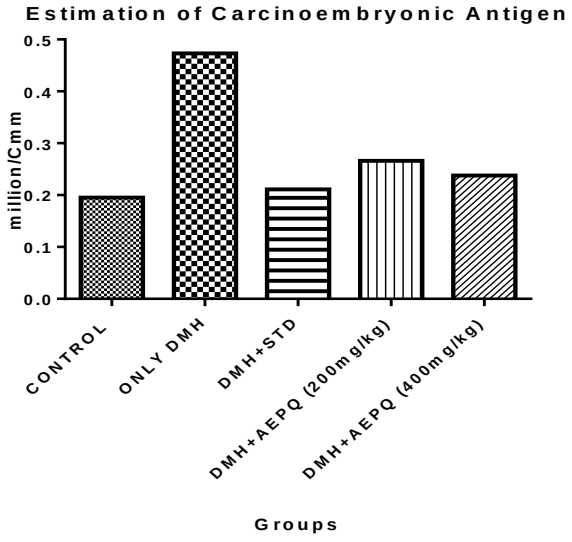


Figure 47: Estimation of Carcinoembryonic Antigen

Table 37: ESTIMATION OF ALPHA-FETO-PROTEIN (AFP)

GROUPS (n=6)	CONTROL Group – I	Only DMH Group –I I	DMH+5 Fluorouracil Group – III	DMH+AEPQ (200mg/kg) Group – IV	DMH+AEPQ (400mg/kg) Group - V
AFP (ng/dL)	0.4800±0.01528	0.6780±0.007234*	0.5000±04947***	0.6000± 0.005773***	0.5433±0.01856***

***P<0.001, **P<0.01, *P<0.05, #-Non significant

Data is exhibited as Mean ±SEM. (n=6, animals in each group)

Statistical comparison: One way ANOVA, followed by Dunnet`s comparison was performed. DMH only (Group 2) was compared with Normal control (Group 1), DMH+5 Fluorouracil (20mg/kg) Group 3, DMH+APEQ (200mg/kg) Group 4, DMH+AEPQ (400mg/kg) Group 5, the values of Alpha-Feto-Protein was exhibited as ng/dl.

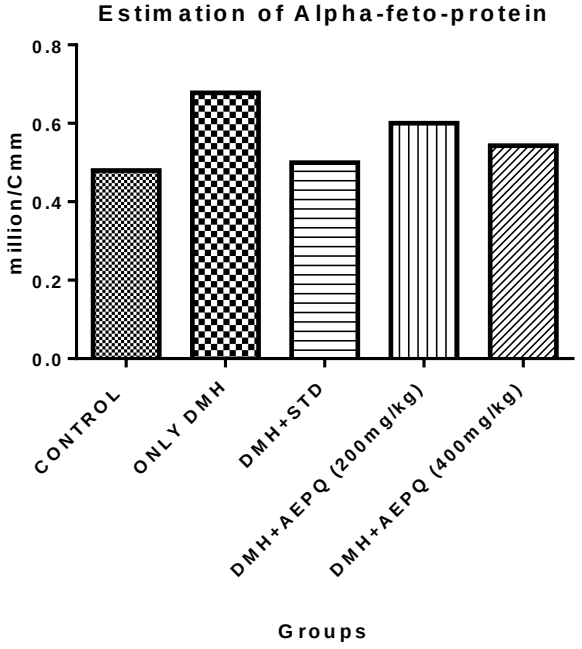


Figure 48: Estimation of Alpha-feto-protein

6.6.7 *IN VIVO* ANTIOXIDANT ACTIVITY

The various *In vivo* parameters were evaluated. The *In vivo* antioxidant levels (Protein, SOD, Catalase, GPx, GSH, LPx), Carbohydrates metabolizing enzymes were decreased in DMH induced group where as it increases in the group with standard and AEPQ (200 and 400 mg/kg, p.o.) treated groups compared to control animals in a dose dependent manner.

Table 38: *INVIVO* ANTIOXIDANT ACTIVITY IN COLON TISSUE**:**

Groups **(n=6)**	Control Group – I	Only DMH Group - II	DMH+5 Fluorouracil Group – III	DMH+AEPQ (200mg/kg) Group – IV	DMH+AEPQ (400mg/kg) Group – V
PROTEIN Mg/G Tissue	0.4900±0.02309	0.2390±0.02610ns	0.4319+0.0611 ns	0.4040±0.007024ns	0.4117±0.01922ns
SOD (Units/min/mg/protein)	5.460±0.2272	2.827±0.08090ns	4.009+0.01298 ns	3.380±0.02646 ns	3.930±0.09849ns
CATALASE µ/moles of H_2O_2 consumed / min / mg protein	41.60±0.8630	28.46±0.7681 ns	29.82+0.1412 ns	24.40±0.2797 ns	28.41±0.7664 ns
GPx µ/moles/mg protein	70.2±1.10	51.4±0.60ns	64.2+0.80ns	59.6±0.90*	68.43±1.01ns
GSH Gulatathione µ/moles /mds	87.6±3.105	59.97±3.73ns	91.20+229**	77.11±8.23ns	86.6±1159ns
LPx MDA formed/Mg protein	70.1±2.115	103.2±3.822	86.11±2.985***	78.60±1.148***	65.15±1.875***

***P<0.001, **P<0.01, *P<0.05, ns-Non significant

Data is exhibited as Mean ±SEM. (n=6, animals in each group)

Statistical comparison: One way ANOVA, followed by Dunnet`s comparison was performed. DMH only (Group 2) was compared with Normal control (Group 1), DMH+5 Fluorouracil (20mg/kg) Group 3, DMH+APEQ (200mg/kg) Group 4, DMH+AEPQ (400mg/kg) Group 5.

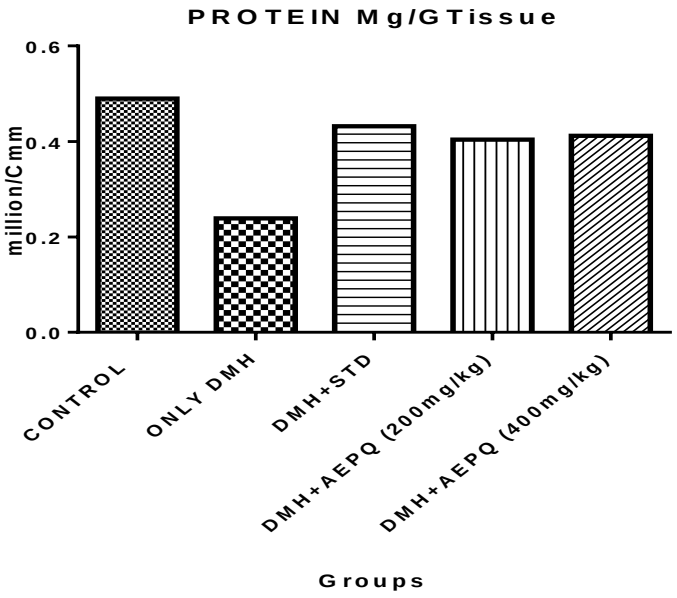


Figure: 49 Estimation of Protein (mg/g Tissue)

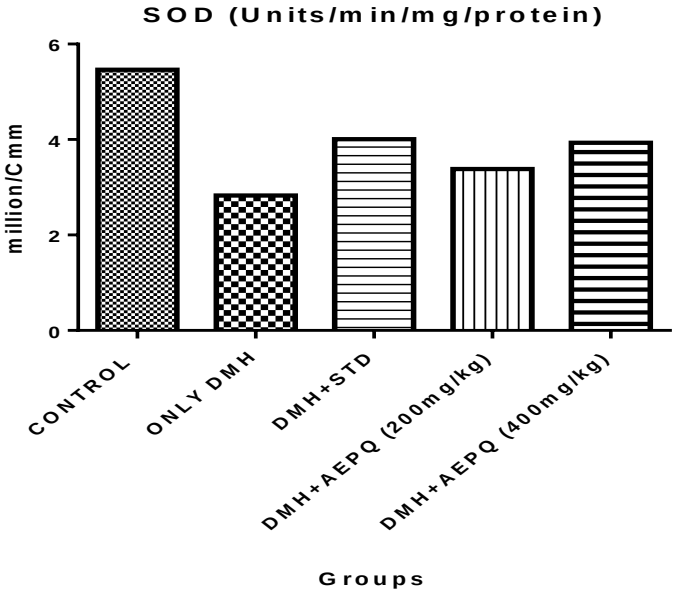


Figure: 50 Estimation of SOD (units/min/mg/protein)

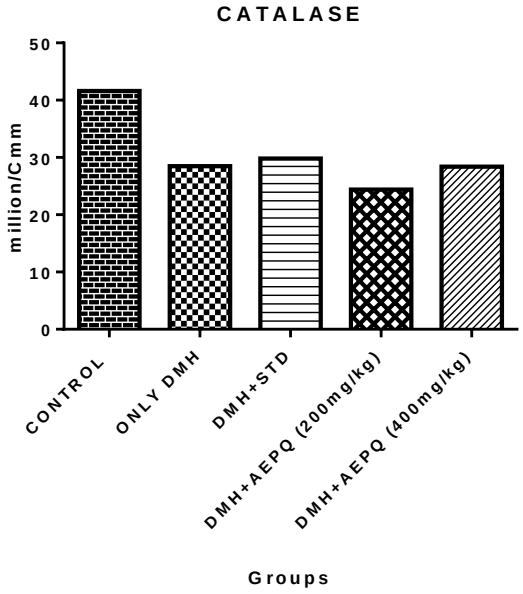


Figure: 51 Estimation of catalase

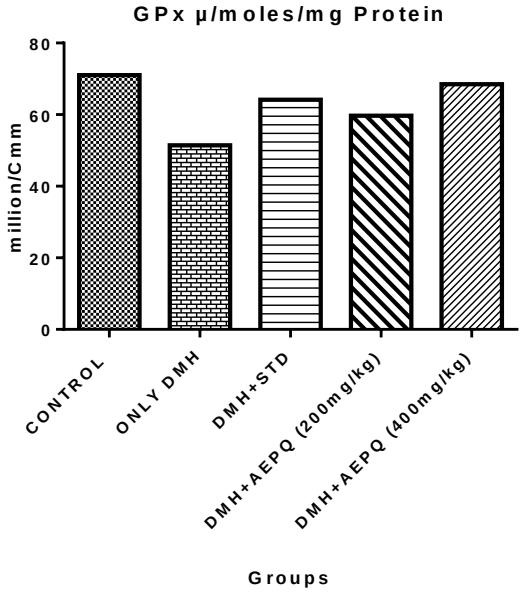


Figure: 52 Estimation of GPx µ/moles/mg Protein

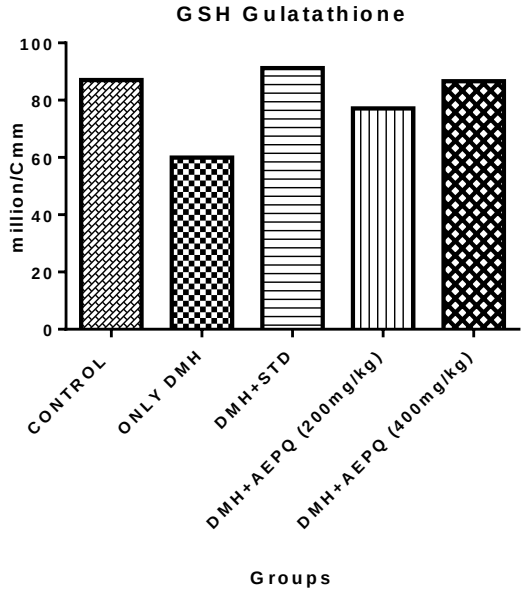


Figure: 53 Estimation of GSH Gulatathione

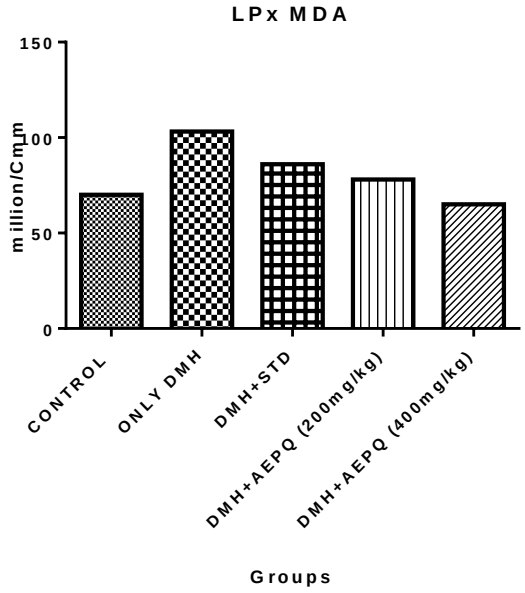


Figure 54: Estimation of LPx MDA

6.6.9 Estimation of Glycolytic Enzymes

The isolated colons were subjected to analyse the glycolytic enzyme in colon homogenate. The results were depicted in Table 39 and 40; Figure 55 and 56.

Table 39: ESTIMATION OF FRUCTOSE-1-6-DIPHOSPHATASE

Group	CONTROL Group – I	Only DMH Group – II	DMH + 5 Fluorouracil Group – III	DMH+AEPQ (200 mg/kg) Group – IV	DMH+AEPQ (400 mg/kg) Group – V
Fructose-1-6-Diphosphatase	86.83±2.850	46.67±2.657	69.89± 0.71***	54.90±2.139#	66.57±1.866**

***P<0.001, **P<0.01, *P<0.05, #-Non significant

Data is exhibited as Mean+SEM. (n=6, animals in each group).

Statistical comparison: One way ANOVA, accompanied by Dunnet's comparison was executed. DMH only (Group 2) was compared with Normal control (Group 1), DMH+AEPQ (200mg/kg) Group 3, DMH+AEPQ (400 mg/kg) Group 4, The level of F-1-6-DP was expressed as n moles of Pi liberate/min/mg protein. Group 3 and 5 showed good activity when compared to DMH group.

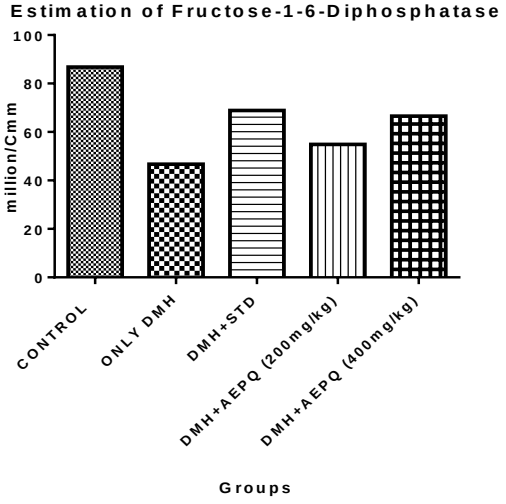


Figure 55: Estimation of fructose 1,6-diphospatase

Table 40: ESTIMATION OF GLUCOSE-6-DIPHOSPHATASE

Group	CONTROL Group – I	Only DMH Group – II	DMH + 5 Fluorouracil Group – III	DMH+AEPQ (200 mg/kg) Group – IV	DMH+AEPQ (400 mg/kg) Group – V
GLUCOSE-6-PHOSPHATE	81.54±0.8634	51.70±1.289	70.96± 0.37***	52.59±0.4795 #	66.25±1.056**

***P<0.001, **P<0.01, *P<0.05, #-Non significant.

Data is exhibited as Mean + SEM. (n=6, animals in each group).

Statistical comparison: One way ANOVA, accompanied by Dunnet's comparison was executed. DMH only (Group 2) was compared with Normal control (Group 1), DMH+AEPD (200mg/kg) Group 3, DMH+AEPD (400 mg/kg) Group 4, The level of G-6p was exhibited as nmoles of Pi liberate/min/mg protein. Group 3 and 5 showed good activity when compared to DMH group.

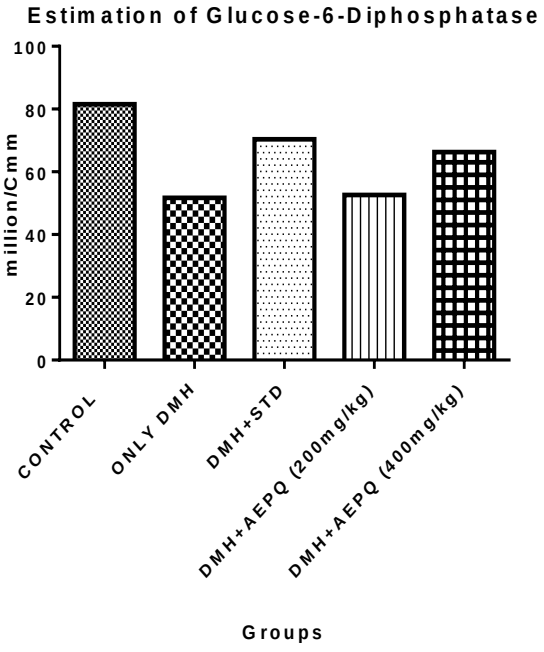


Figure 56: Estimation of glucose 6-phosphate

6.6.10 Determination of Faecal Neutral Sterols, faecal Bile Acids and faecal weight

On the termination of our study faecal matters were collected and subjected to determination of Faecal Neutral Sterols, faecal Bile Acids and faecal weight to analyse the physiological function of colon. The results are depicted in Table 41; Figure 57 to 59.

Table 41: Determination of Faecal Neutral Sterols, faecal Bile Acids and faecal weight in DMH induced colon cancer in Spraque dawley rats**.**

GROUP	CONTROL Group – I	ONLY DMH Group – II	DMH+5 FLUOROURACIL Group – III	DMH+AEPQ (200mg/kg) Group – IV	DMH+AEPQ (400mg/kg) Group – V
Dried faecal weight (g)	0.1040±0.046	0.0710±0.0317	0.0945±0.0423	0.1135±0.0507	0.097±0.0434
Faecal bile acid excretion (mg)	0.0110±0.0050	0.0223±0.0104	0.0137±0.0066	0.0127±0.0057	0.0113±0.0050
Faecal neutral sterol excretion (mg)	0.5642±0.2523	0.8547±0.4042	0.5613±0.2510	0.6132±0.2749	0.5623±0.2515

a . Data are exhibited as mean ± SEM.

b. Gain in weight.

c . Significantly different from control, $p<0.05$.

During the period of study, stool samples were collected and compared and also before sacrifice of the rats in all groups. The Faecal Weight, Determination of Faecal Neutral Sterols and Faecal Bile Acids was less in DMH induced group when compared to other groups.

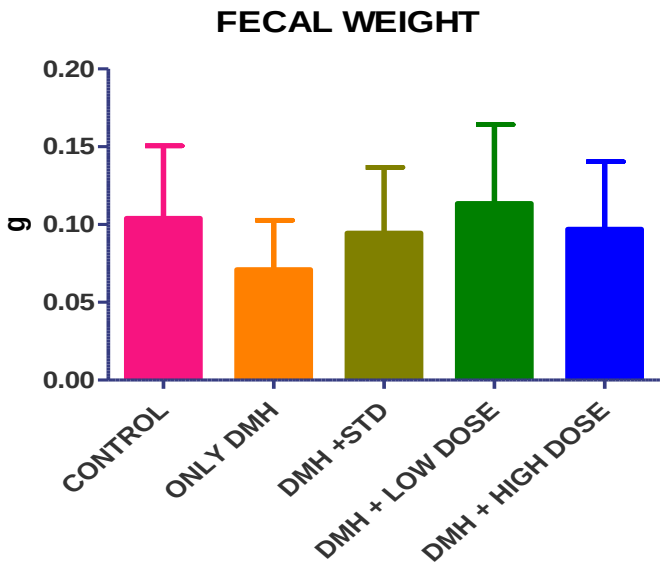


Figure 57: Determination of Fecal Weight

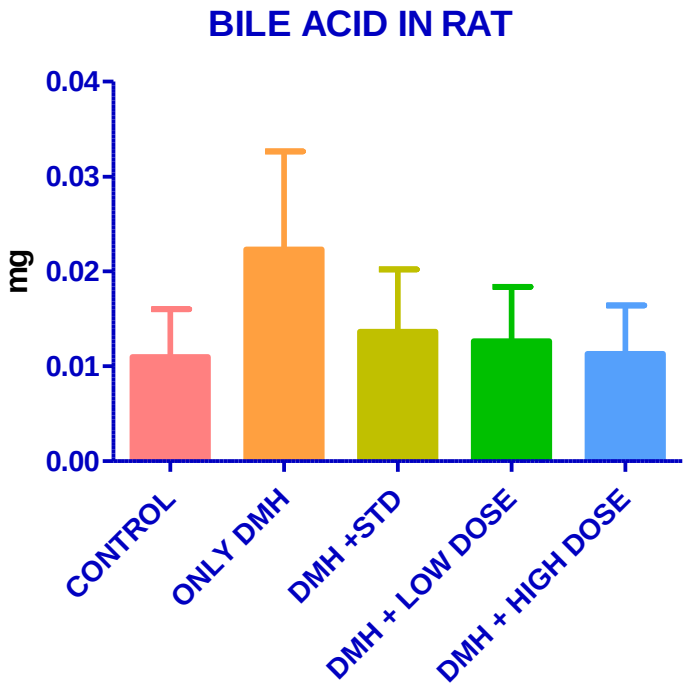


Figure 58: Determination of Bile Acid in Rat

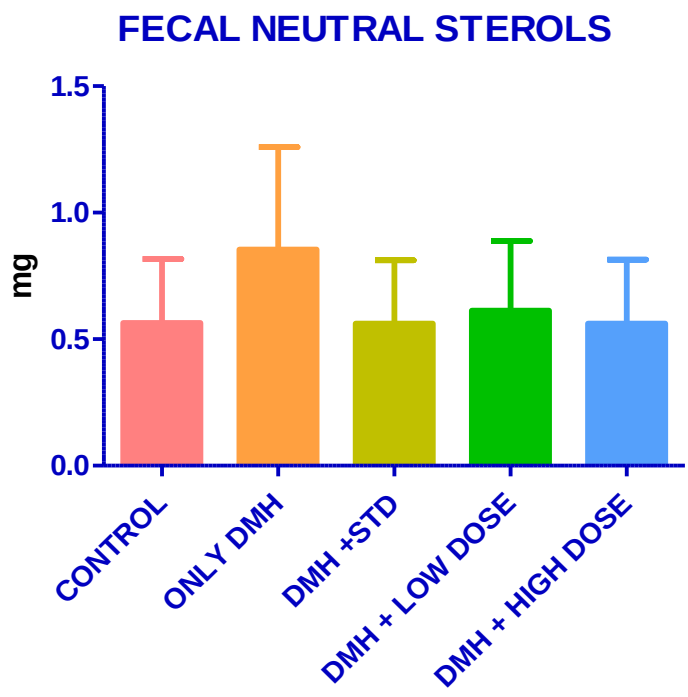


Figure 59: Determination of Fecal Neutral Sterols

6.6.10 Histopathological Studies

The isolated colon tissues from all the treated groups were preserved in buffer neutral formalin and subjected to preparation of tissue blocks trimming, cutting and staining (H&E). to analyse the histological changes occurred during the treatment period. The results of the Histopathological findings were displayed in Figure 60 to 69.

Group-I CONTROL (COLON)

40x shows mild inflammation

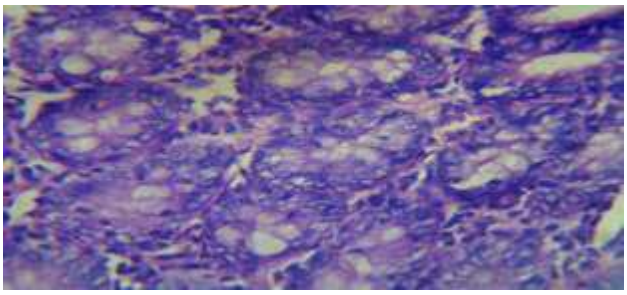


Figure 60: Histopathology of Colon

MICROSCOPIC APPEARANCY

Section studied from the colon shows normal epithelium. The lamina propria shows scattered lymphocytic infiltration. Muscular layer and serosa shows no significant pathology. There is no evidence of dysplasia/malignancy in the section studied.

Group-I CONTROL (LIVER)

40x shows normal portal tract

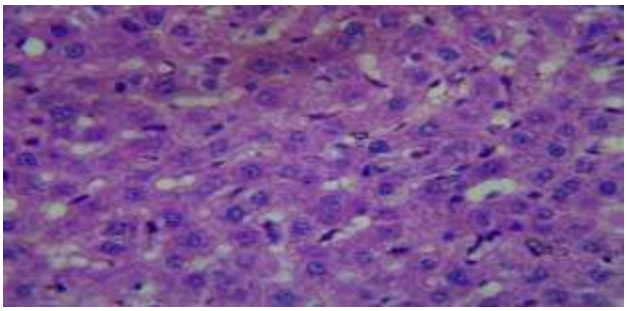


Figure 61: Histopathology of Liver

MICROSCOPIC APPEARANCY

Section studied from the liver shows maintain lobular architecture. Individual hepatocytes show no significant pathology. The central vein shows dilatation and congestion. The portal triad shows bile duct hyperplasia. The sinusoids are dilated.

Group-II ONLY DMH (COLON)

40x shows individual cells increase in number of stratification

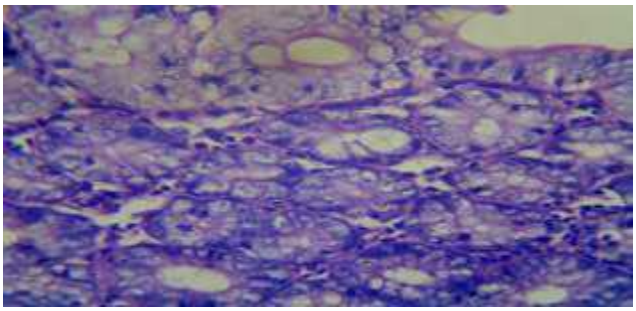


Figure 62: Histopathology of Colon

MICROSCOPIC APPEARANCY

Section studied from the colon shows increased number of glands with stratification. Individual cells are round to oval with moderate eosinophilc cytoplasm and vesicular nuclei showing mild dysplasia with prominent nucleoli. Occasional mitosis are also seen.

Group-II ONLY DMH (LIVER)

40x shows cytoplasmic vacuolation

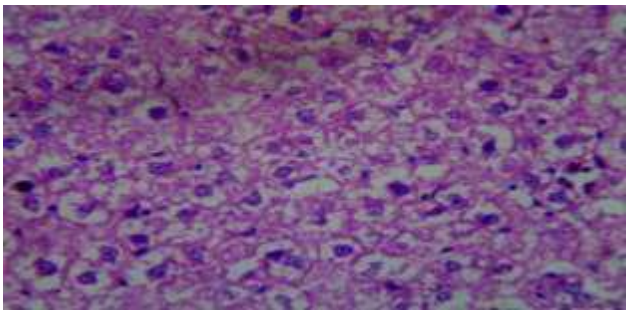


Figure 63: Histopathology of Liver

MICROSCOPIC APPEARANCY

Section studied from the liver shows distorted architecture with mild parenchymal inflammation. Individual hepatocytes show cytoplasmic vacuolation.The central vein shows normal. The portal triad shows lymphocytic infiltration. The sinusoids show no significant pathology.

Group-III DMH + STD (COLON)

40x shows normal crypts

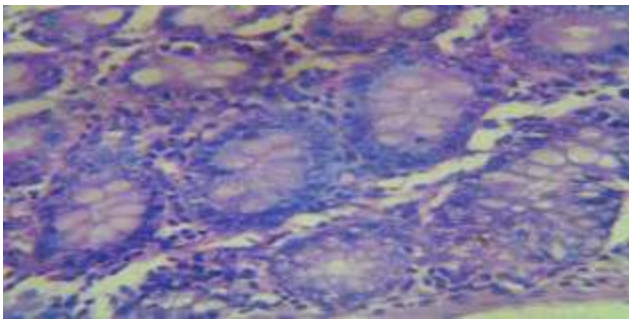


Figure 64: Histopathology of Colon

MICROSCOPIC APPEARANCY

Section studied from the colon shows normal mucosal epithelium. The lamina propria shows lymphocytic infiltration. Muscular layer and serosa shows no significant pathology. There is no evidence of dysplasia/malignancy in the section studied.

Group- III DMH + STD (LIVER)

40x shows central vein normal sinusoidal dilatation

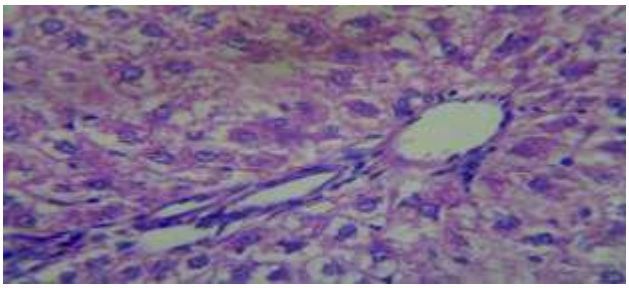


Figure 65: Histopathology of Liver

MICROSCOPIC APPEARANCY

Section studied from the liver shows mild distorted architecture. Individual hepatocytes show cytoplasmic vacuolation. The central vein shows normal. Sinusoids show mild dilatation. The portal triad shows diffuse scattered lymphocytic infiltration.

Group-IV DMH + LOW DOSE (COLON)

40x shows inflammation

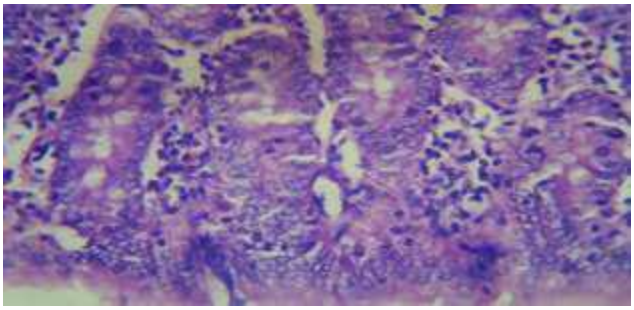


Figure 66: Histopathology of Colon

MICROSCOPIC APPEARANCY

Section studied from the colon shows normal epithelium increased number of glands with stratification are also seen. Individual cells are round to oval with moderate eosinophilc cytoplasm and vesicular nuclei showing mild dysplasia with prominent nucleoli. Occasional mitosis are also seen. The lamina propria shows scattered lymphocytic infiltration.

Group- IV DMH + LOW DOSE (LIVER)

40x shows central vein normal and sinusoidal dilatation

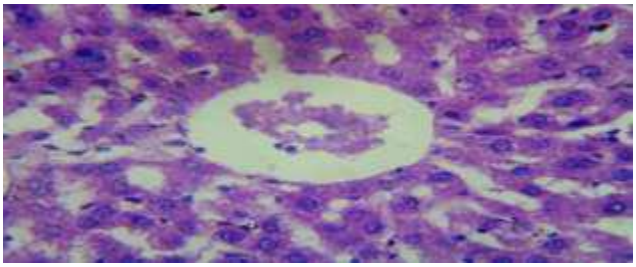


Figure 67: Histopathology of Liver

MICROSCOPIC APPEARANCY

Section studied from the liver shows distorted architecture. Individual hepatocytes show focal hepatocytic necrosis. The central vein shows normal. The portal triad shows dense lymphocytic infiltration.

Group-V DMH + HIGH DOSE. (COLON)

40x shows normal colon with mild inflammation

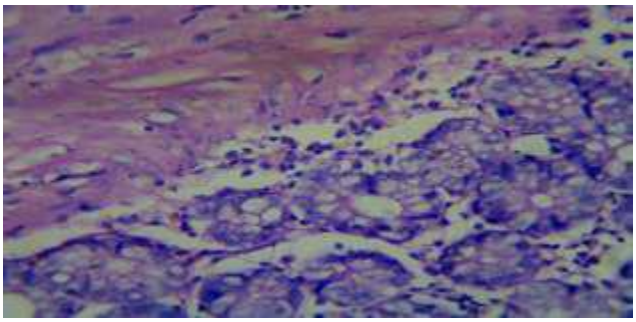


Figure 68: Histopathology of Colon

MICROSCOPIC APPEARANCY

Section studied from the colon shows normal epithelium. The lamina propria shows mild lymphocytic infiltration. Muscular layer and serosa shows well balanced. There is no evidence of dysplasia/malignancy in the section studied.

Group- V DMH + HIGH DOSE (LIVER)

40x shows portal tract inflammation with cytoplasmic vacuolation

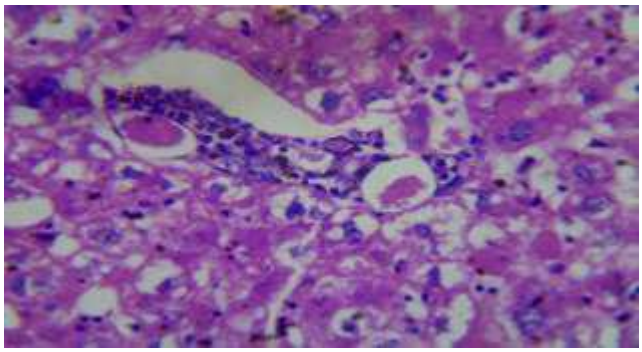


Figure 69. Histopathology of Liver

MICROSCOPIC APPEARANCY

Section studied from the liver shows lobular architecture. Individual hepatocytes show cytoplasmic vacuolation. The central vein shows dilatation. Sinusoids show mild dilatation. The portal triad shows lymphocytic infiltration.

In **Phase –V** of our study aqueous extract (AEPQ) was subjected to isolation of active compounds and followed by screening of *In vitro* cytotoxic study of the isolated compounds using different colon cell lines. Based on the In vitro cytotoxicity studies the isolated compound showing good anti cancer activity were selected and subjected for structure elucidation by Gas chromatography-Mass spectroscopy(GC-MS) The results of the findings are given in Table 42 to 49; Figure 70 to 98.

6.7. Determination of active compounds from aqueous extract (AEPQ)

The aqueous extract of *Portulaca quadrifida* was subjected for determination of total phenolic, total tannin and total saponin content by using various methods which was further subjected to TLC finger printing and isolation by flash chromatography. The results were depicted in Table 42 to 44; Figure 70 to 76.

6.7.1 Estimation of Total Phenolic Content from aqueous extract (AEPQ)

The aqueous extract of *Portulaca quadrifida* was used to isolate of total phenolic and subjected for determination. The results of determination of total phenolic content is given in Table no 41; figure No 72

Table 42: Total Phenolic Content of aqueous extract of *Portulaca quadrifida*

S.No	Concentration of standard and sample	Absorbance(nm)		
		First	Second	Average
1	10 mcg/ml	0.066	0.065	0.0655
2	20 mcg/ml	0.133	0.133	0.1330
3	40 mcg/ml	0.263	0.265	0.2640
4	60 mcg/ml	0.394	0.392	0.3930
5	80 mcg/ml	0.536	0.535	0.5355
6	100 mcg/ml	0.672	0.670	0.6710
7	Sample extract (10 mcg/ml)	0.4823	0.4821	0.4822

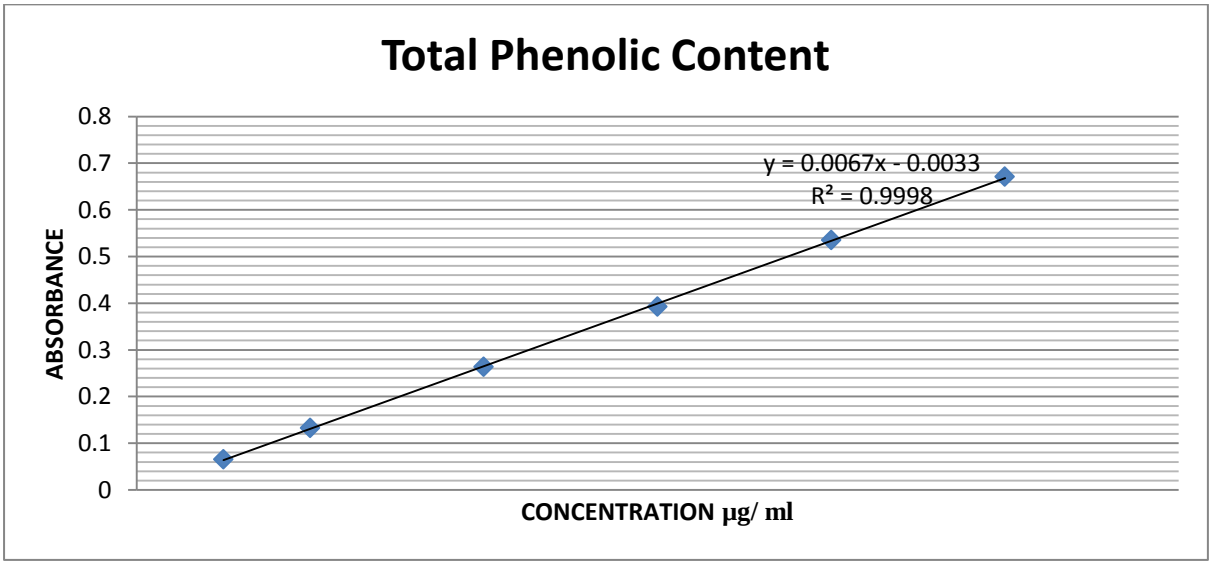


Figure 70: Total phenolic content in isolated aqueous extract of *Portulaca quadrifida*

Standard curve of standard gallic acid

Report: The whole phenolic content present in the given sample extract was found to be 71.86 mg Gallic acid equivalents /100g DW).

6.7.2 Estimation of Total Condensed tannin Content from aqueous extract (AEPQ)

The aqueous extract of *Portulaca quadrifida* was used to isolate of Total Condensed tannin Content and subjected for determination. The results of determination of Total Condensed tannin Content is given in Table No 42; Figure No 73

Table 43: Total Condensed tannin Content of isolated aqueous extract of *Portulaca quadrifida*:

S.No	Concentration of standard and sample	Absorbance(nm)		
		First	Second	Average
1	1 mcg/ml	0.094	0.098	0.143
2	2 mcg/ml	0.167	0.170	0.252
3	3 mcg/ml	0.250	0.253	0.376
4	4mcg/ml	0.336	0.334	0.503
5	5 mcg/ml	0.415	0.417	0.623
6	6 mcg/ml	0.498	0.495	0.745
7	Sample extract (10 mcg/ml) of crude drug powder	0.2014	0.2018	0.302

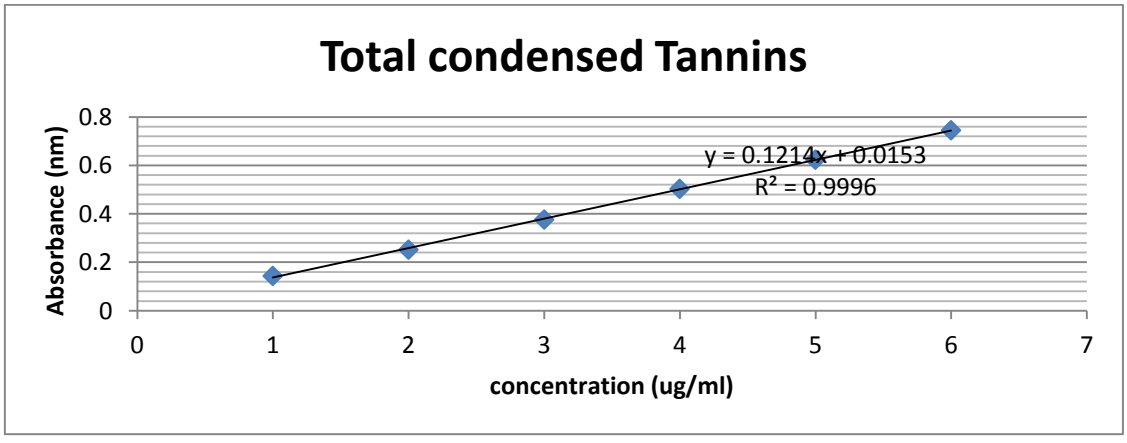


Figure 71: **Total Condensed tannin Content of aqueous extract of *Portulaca quadrifida***

Estimation of total saponin fraction of isolated aqueous extract of *Portulaca quadrifida*

The total saponin content was separated using gravimetric technique and was found to be 66.98 % w/w.

•The percentage yield of the isolated compound was found to be 0.16 % w/w.

• The Rf of the isolated compound (S3) was found to be 0.57 which is nearby R_f of standard gallic acid which was found to be 0.62. Hence the isolated compound (S3) was found to be phenolic and a gallic acid derivative which further have to confirmed by GC-MS analysis.

6.7.3 Estimation of Total Saponin fraction from aqueous extract (AEPQ)

The aqueous extract of *Portulaca quadrifida* was used to isolate Total Saponin fraction from saponins by flash chromatography and determined by Gravimetry technique and confirmation by TLC Finger printing . The results of determination of Total Saponin fraction is given in Table No 43; Figure No 74 & 75

Table 44: Estimation of total saponin fraction of aqueous extract of *Portulaca quadrifida*

Compound name & Physical state	Phytochemical Test	Melting Range	R_F (cm)
Brownish green fine powder (0.16%w/w)	Ferric chloride reagent test	250^0c to 252^0c	ying with ferric chloride (1%v/

Total four spots or solutes had been identified with R_F of 0.2, 0.54, 0.59 and 0.66.

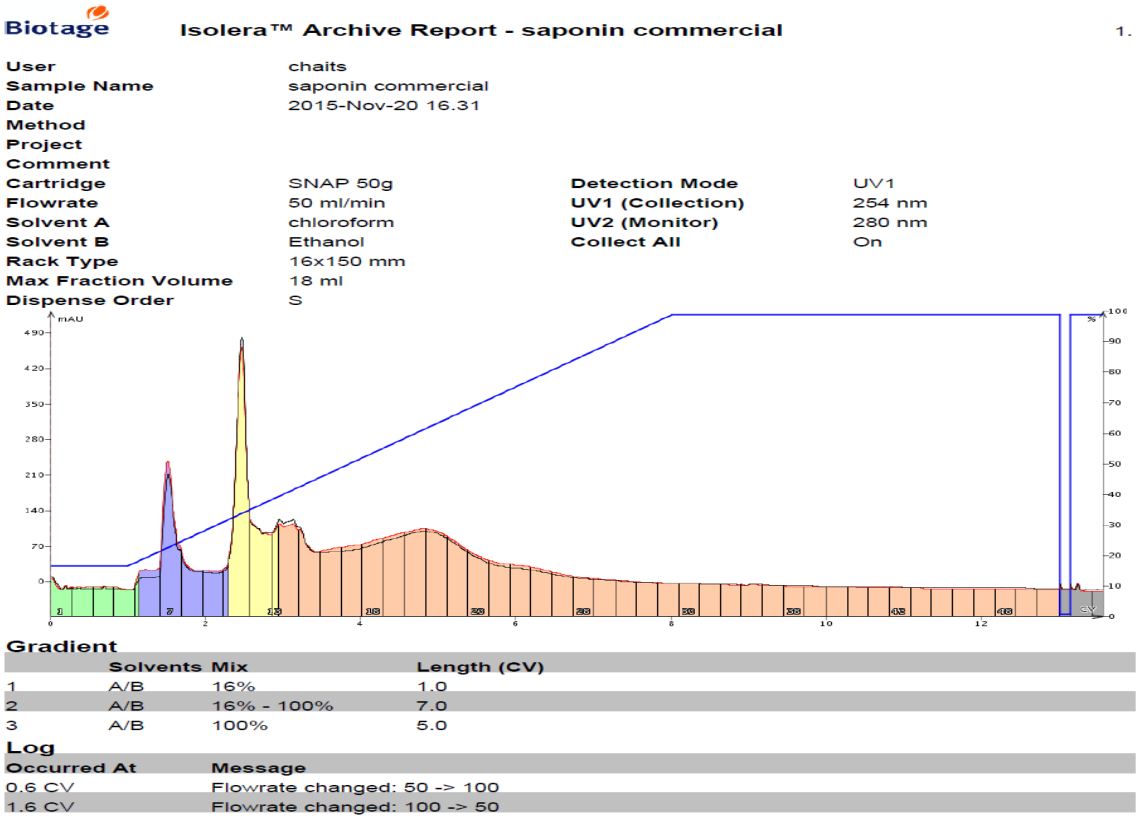


Figure 75: Isolation of saponins fractions from total saponin by flash chromatography.

1. The R_F of the fractions(15 – 17) was found to be 0.66 and the percentage yield of the compound was found to be 8.5 %w/w. S_1
2. The R_F of the fractions (6 – 10) was found to be 0.58 and the percentage yield of the compound was found to be 4.04 %w/w. S_2
3. The R_F of the fractions (11 – 13) was found to be 0.65 and the percentage yield of the compound was found to be 0.264 %w/w. S3
4. The R_F of the fractions (18 - 51) was found to be 0.73 and the percentage yield of the compound was found to be 60.0 %w/w. S4

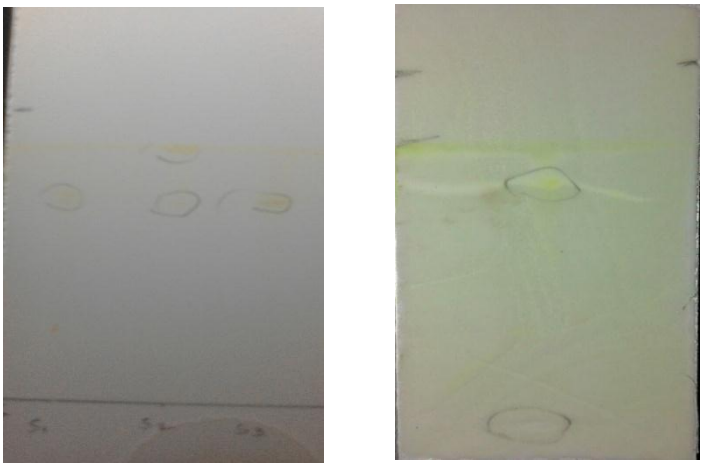


Figure 76: TLC Finger printing of isolated fractions (S1, S2, S3 & S4) from total saponin fractions

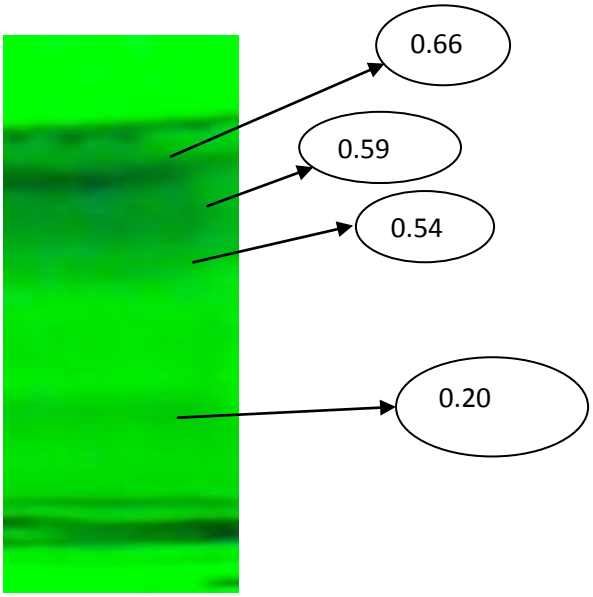


Figure 72: TLC Finger printing of total saponin fraction

6.8 *In Vitro* Cytotoxicity Studies of Isolated compound

The isolated fractions of *Portulaca quadrifida* were screened for cytotoxicity studies by using different colon cell lines such as HT-29 and HCT-116. Among all the fractions saponins fraction (S4 and S6) and total phenol (S0) Showed highest inhibition at lowest concentration. (Table 45 and 46, Figure 77 – 89).

Table 45: Cytotoxicity studies of isolated compound of *Portulaca quadrifida* by HT-29

Sl. No.	Sample	cc50
01	Total saponins	99.5915
02	Saponin fraction (6-10) (S_1)	60.38404
03	Saponin fraction (11-13) (S_2)	119.3472
04	fraction (S_3)	89.31083
05	fraction (S_4)	30.32821
06	Total phenol (S_0)	20.56636
07	fraction (S_6)	29.65764

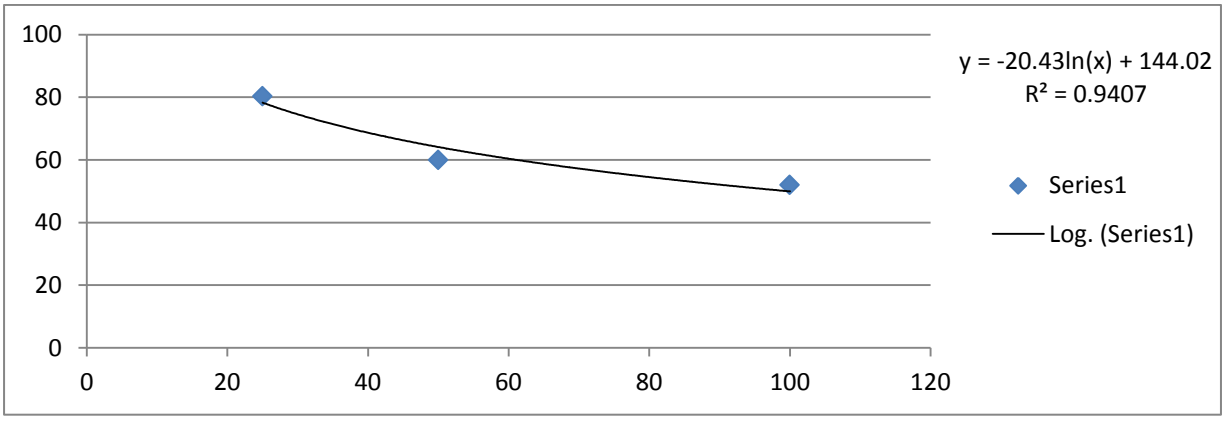


Figure 77: Inhibition of growth of human colon cancer cell line HT-29 by isolated total saponin of *Portulaca quadrifida* in MTT assay

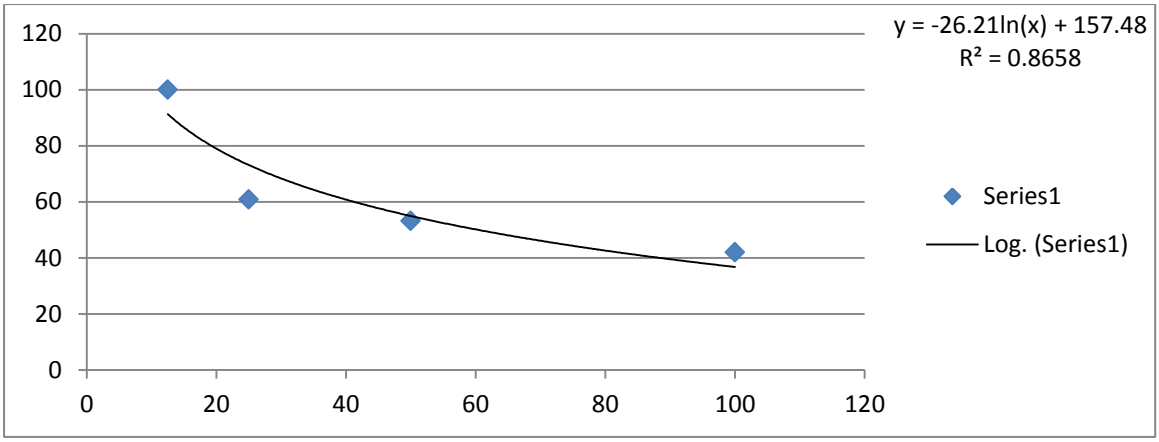


Figure 78: Inhibition of growth of human colon cancer cell line HT-29 by isolated saponin fraction (S_1) of *Portulaca quadrifida* in MTT assay

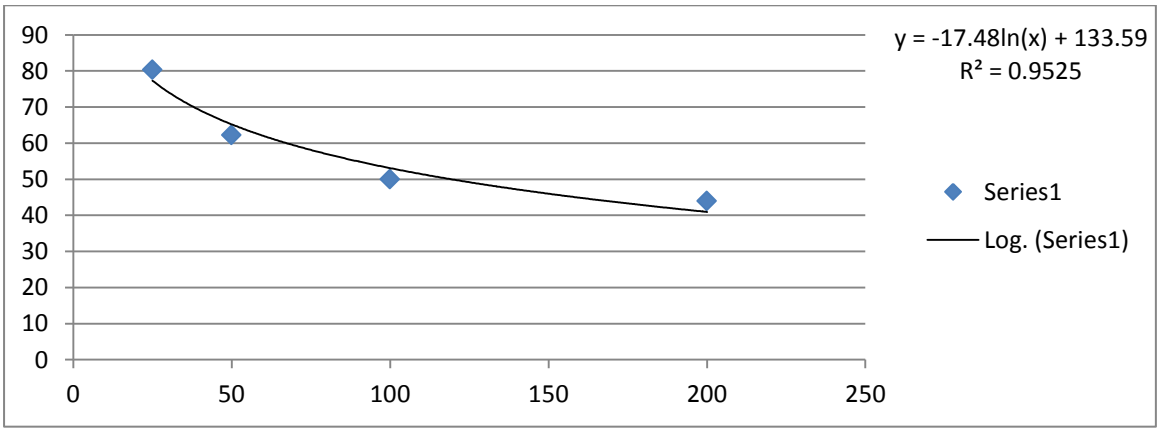


Figure 79: Inhibition of growth of human colon cancer cell line HT-29 by isolated saponin fraction (S_2) of *Portulaca quadrifida* in MTT assay

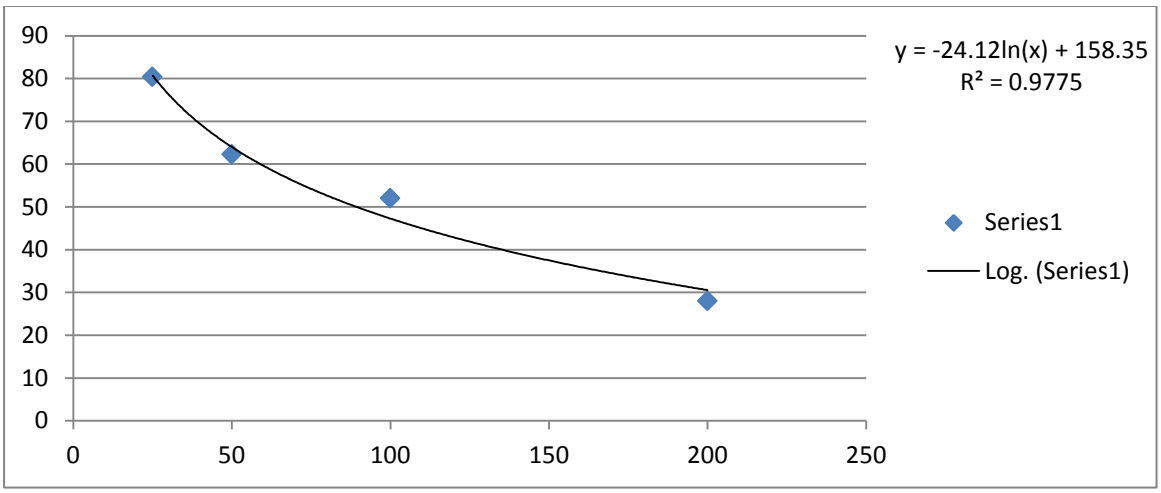


Figure 80: Inhibition of growth of human colon cancer cell line HT-29 by isolated fraction (S_3) of *Portulaca quadrifida* in MTT assay

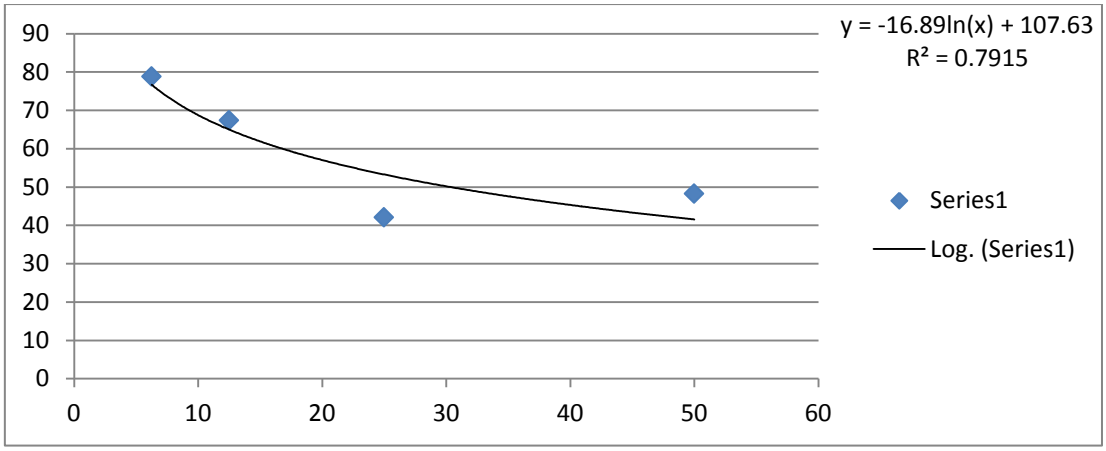


Figure 81: Inhibition of growth of human colon cancer cell line HT-29 by isolated fraction (S_4) of *Portulaca quadrifida* in MTT assay

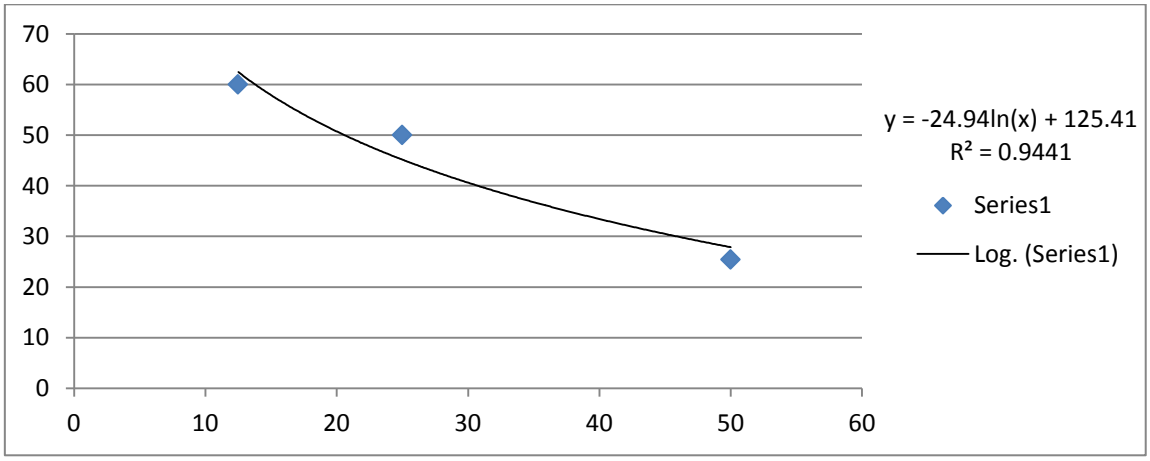


Figure 82: Inhibition of growth of human colon cancer cell line HT-29 by isolated total phenol (S_0) of *Portulaca quadrifida* in MTT assay

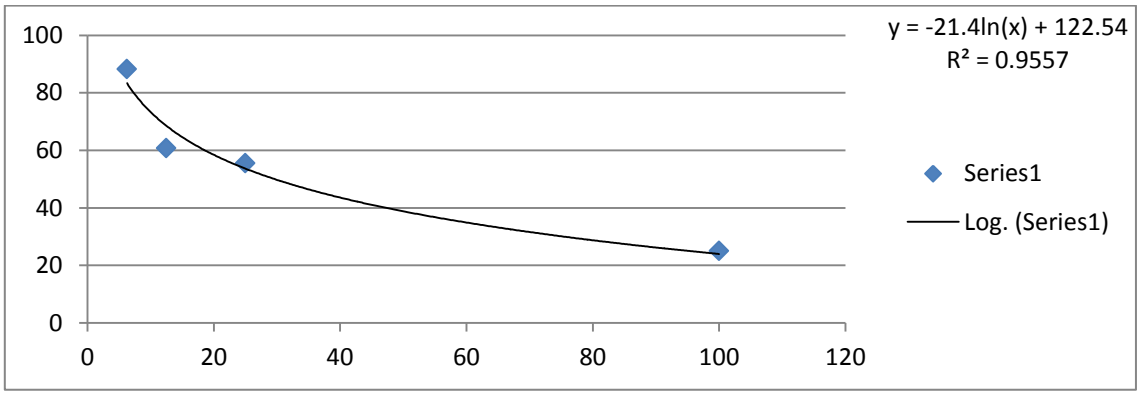


Figure 83: Inhibition of growth of human colon cancer cell line HT-29 by isolated fraction (S_6) of *Portulaca quadrifida* in MTT assay.

Table 46: I*n vitro* Cytotoxicity studies of isolated compound of portulaca quadrifida by HCT-116

Sl. No.	Sample	cc50
01	Total saponins	77.92267
02	Saponin fraction (S_1)	50.26921
03	Saponin fraction (S_2)	103.0257
04	fraction (S_3)	100.4562
05	fraction (S_4)	19.95982
06	Total phenol (S_0)	21.15221
07	fraction (S_6)	46.2586

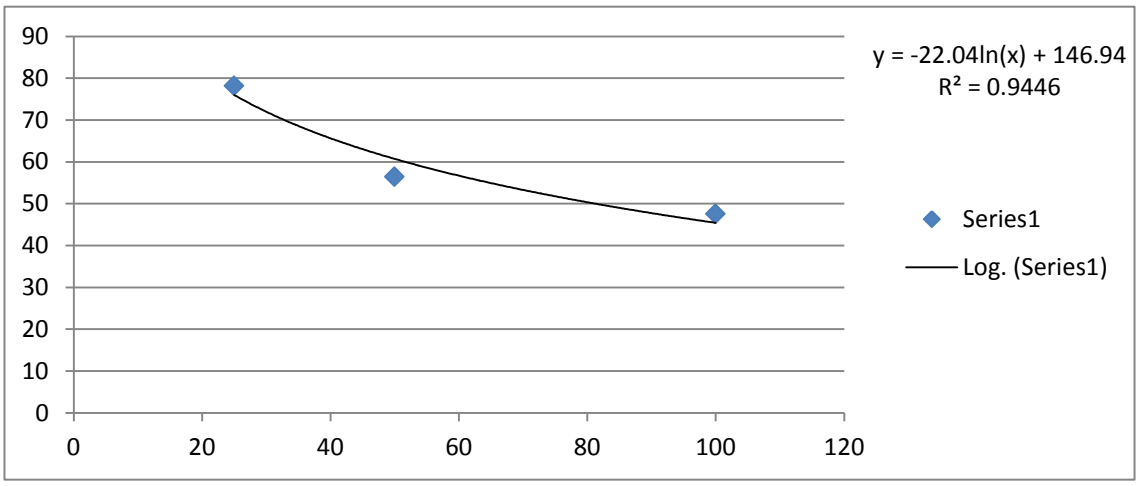


Figure 84: Inhibition of growth of human colon cancer cell line HCT-116 by isolated total saponin of *Portulaca quadrifida* in MTT assay

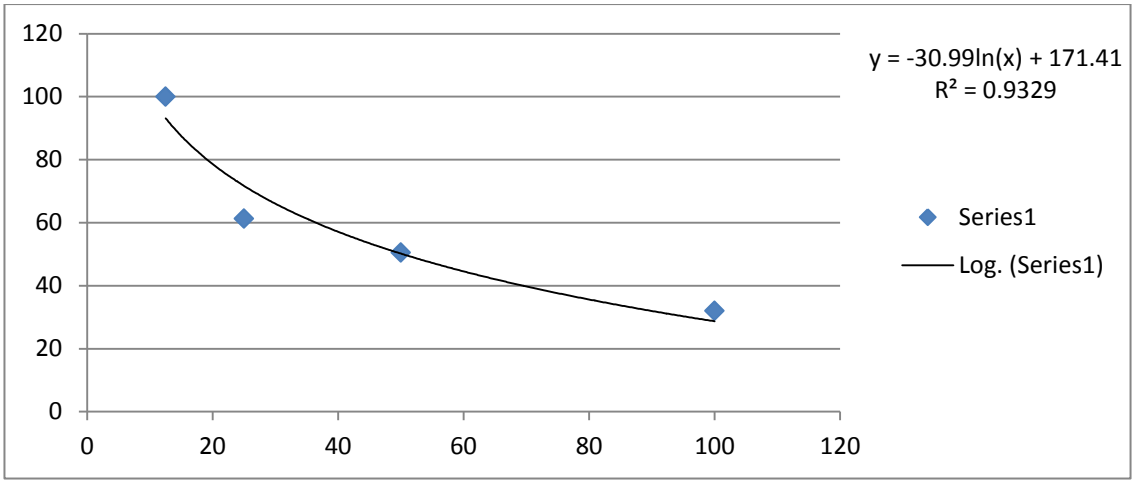
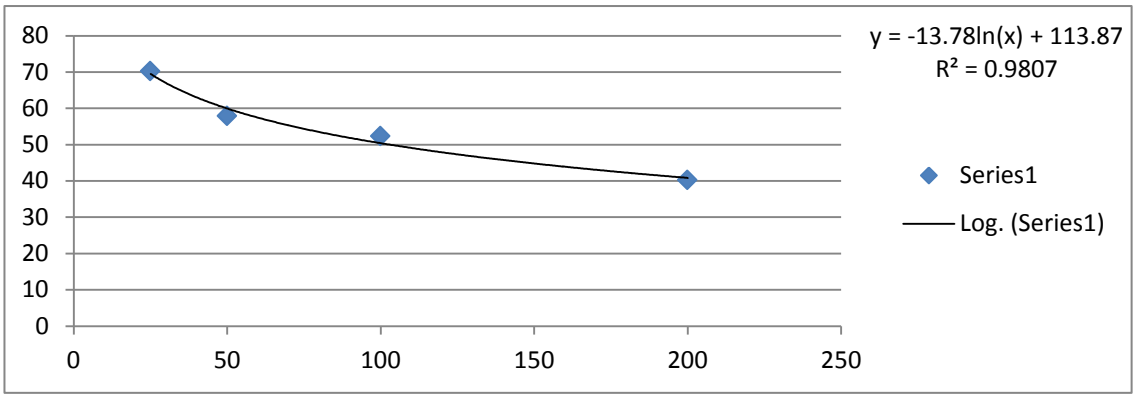


Figure 85: Inhibition of growth of human colon cancer cell line HCT-116 by isolated saponin fraction (S_1) of *Portulaca quadrifida* in MTT assay



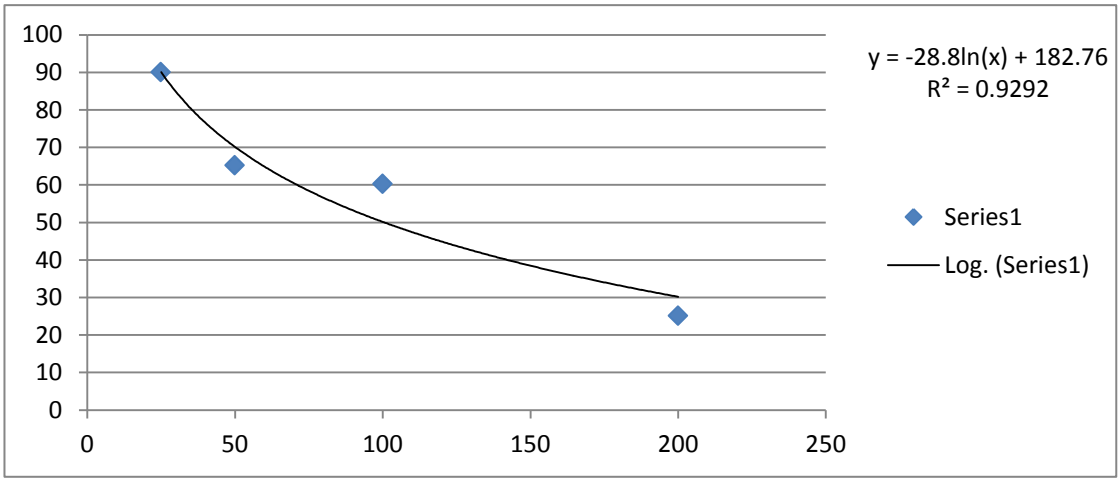


Figure 86: Inhibition of growth of human colon cancer cell line HCT-116 by isolated fraction (S_3) of *Portulaca quadrifida* in MTT assay

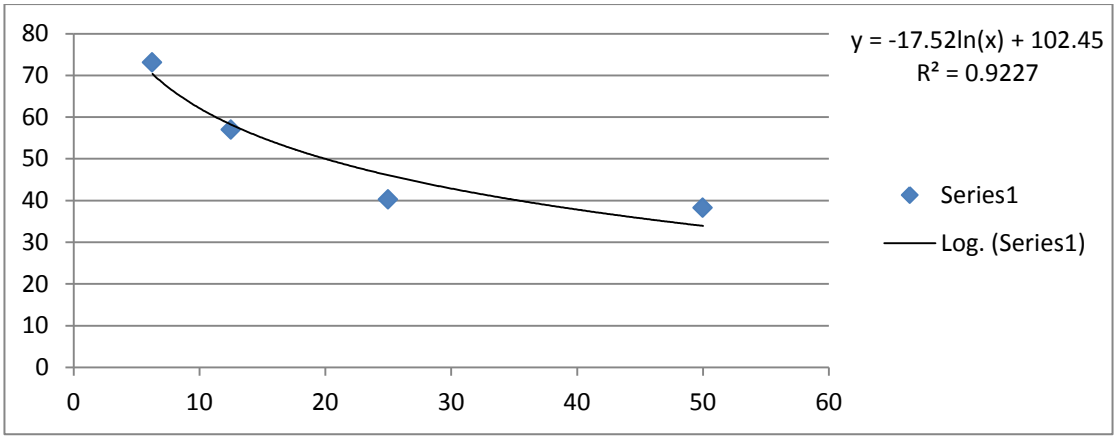


Figure 87: Inhibition of growth of human colon cancer cell line HCT-116 by isolated fraction (S_4) of *Portulaca quadrifida* in MTT assay

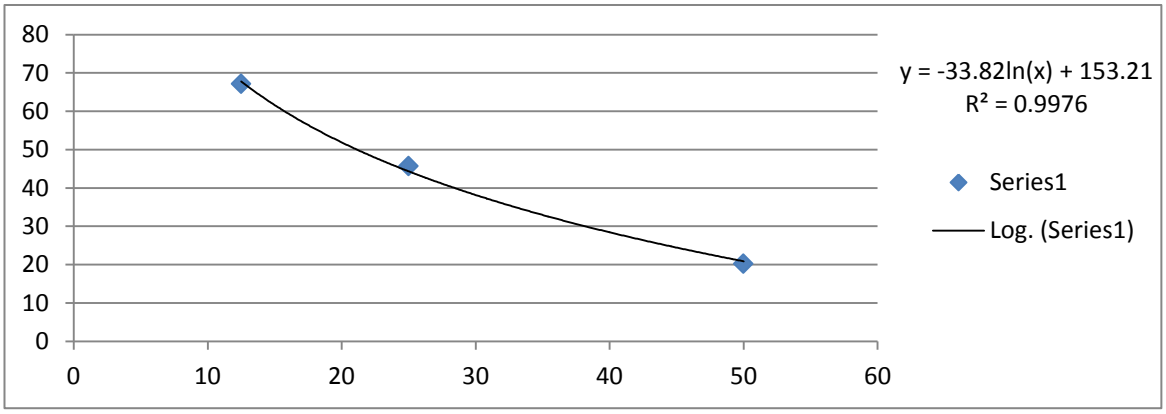


Figure 88: Inhibition of growth of human colon cancer cell line HCT-116 by isolated total phenol (S_0) of *Portulaca quadrifida* in MTT assay

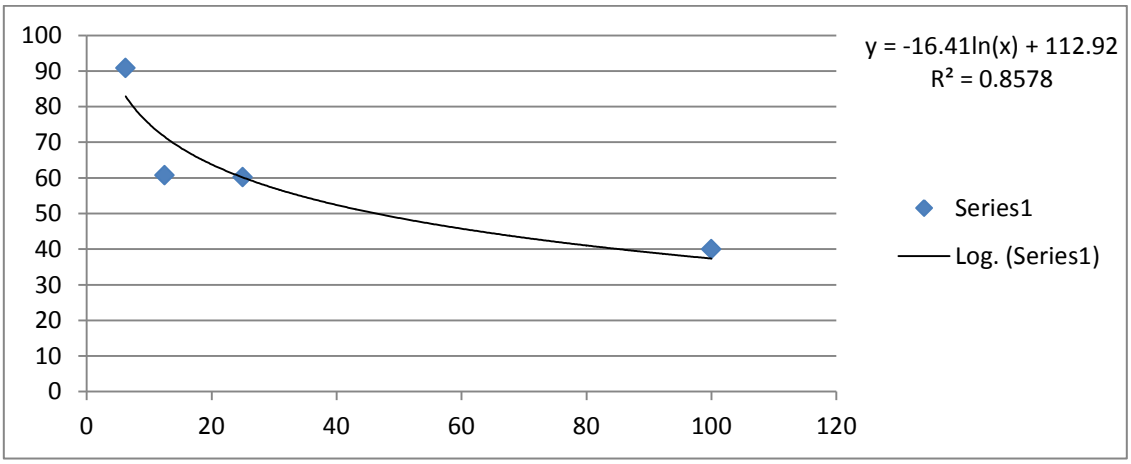


Figure 89: Inhibition of growth of human colon cancer cell line HCT-116 by isolated fraction (S_6) of *Portulaca quadrifida* in MTT assay.

6.9 Spectral Analysis and Structure elucidation by Gas Chromatography-MS

Figure 90: Gas Chromatography – Mass Spectroscopy of saponin fraction (S4)

Sample Name : AEPQ FRACTION S4

Sample ID : ---------

Analysis Date & Time: 12/23/15 15:44:54

User Name : TBC

Instrument ID : GC-01

Method Name : D:\GC Methods\Others\Genral.gcm

Comment : Column ID:ZB-5,30 x 0.25mm,0.25µ

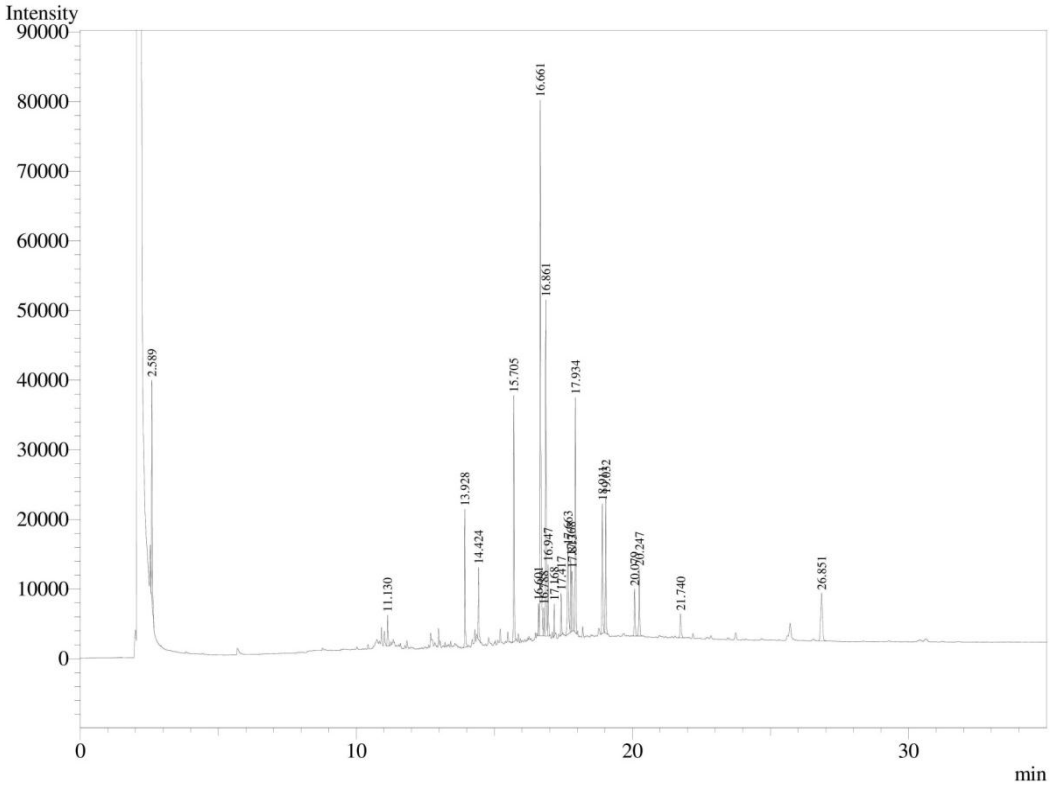
Temp Program :50-5-20-300-17.5;

Inj Temp : 250°C , Det Temp :300°C

Flow rate : 1.0 ml/min, Split ratio : 1:100, Carrier Gas : N2

Diluent: Ethanol

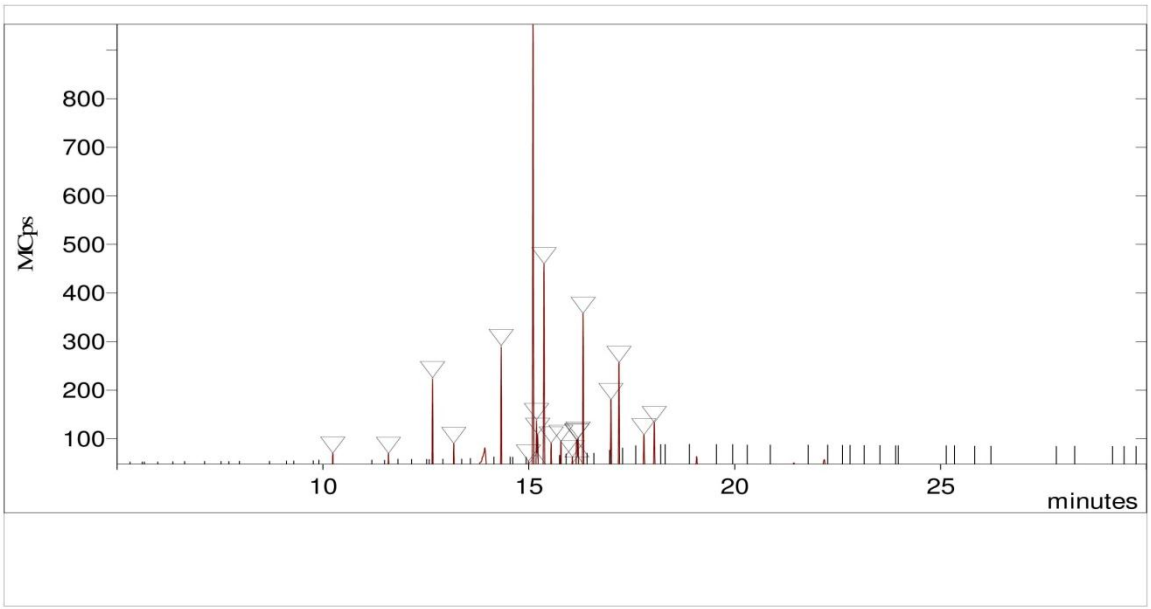
Inj.Vol(µl):2.00



ID#	Peak#	Ret.Time	Area	Theoretical Plates	Resolution	Area%
	1	2.589	63471	36530.197	0.000	7.781
	2	11.130	9644	791316.653	164.326	1.182
	3	13.928	35974	1503873.115	58.770	4.410
	4	14.424	18045	1722746.337	11.124	2.212
	5	15.705	63068	1935965.823	28.815	7.731
	6	16.601	8237	1989834.616	19.488	1.010
	7	16.661	185699	1147963.290	1.094	22.765
	8	16.788	7504	1880284.861	2.300	0.920
	9	16.861	84095	2271284.917	1.563	10.309
	10	16.947	19533	1805830.231	1.812	2.394
	11	17.168	7610	2342942.106	4.655	0.933
	12	17.417	10301	2244024.657	5.463	1.263
	13	17.663	37262	906427.104	4.080	4.568
	14	17.768	20699	0.000	0.000	2.537
	15	17.813	23511	0.000	0.000	2.882
	16	17.934	61578	2256596.329	0.000	7.549
	17	18.911	40275	1889223.788	19.055	4.937
	18	19.032	39169	2052602.278	2.244	4.802
	19	20.079	15629	1860393.226	18.754	1.916
	20	20.247	22345	1841584.665	2.830	2.739
	21	21.740	10696	1314357.565	22.096	1.311
	22	26.851	31395	795682.237	52.219	3.849
Total			815740			100.000

Table 47: Determination of peak, retention time, area, theoretical plates and resolution of saponin fraction (S4) by GC-MS

Sample ID	S4	**Operator**	BKR
Instrument ID	Bruker GC/MS #1	**Last Calib Update**	Oct-30-15 06:23
Acq Date	Dec-23-15 19:28	**Calculation Date**	Dec-23-15 19:58
Inj Sample Notes	S4		
Data File	E:\MS DATA-2012\EXTERNAL\STUDENTS\S4.XMS		
Method	E:\Methods\General.mth		



No	RT	Peak Area	Peak Height	Area%	Peak Response
1	9.651	7962513	5948129	0.189	7962513
2	10.016	27122812	25771652	0.642	27122812
3	10.240	74843032	60833152	1.773	74843032
4	11.591	65364304	56663672	1.548	65364304
5	12.661	238103440	216608976	5.640	238103440
6	12.976	15698224	16737347	0.372	15698224
7	13.178	90383712	80411616	2.141	90383712
8	14.329	283165632	273440160	6.707	283165632
9	14.993	49326412	36872288	1.168	49326412
10	15.103	1066366272	937470336	25.259	1066366272
11	15.187	207866448	117886656	4.924	207866448
12	15.214	44608036	50060896	1.057	44608036
13	15.369	550666240	433698208	13.044	550666240
14	15.542	83137864	72916336	1.969	83137864
15	15.779	84080464	76789048	1.992	84080464
16	16.032	51318300	19847910	1.216	51318300
17	16.058	35507080	31143812	0.841	35507080
18	16.160	84973512	75962728	2.013	84973512
19	16.171	68133528	76801664	1.614	68133528
20	16.198	100859112	79804624	2.389	100859112
21	16.318	372232832	335021984	8.817	372232832
22	16.995	165500080	153844592	3.920	165500080
23	17.190	245082288	226257968	5.805	245082288
24	17.795	88873008	70180792	2.105	88873008
25	18.047	120544832	94873536	2.855	120544832

Figure 91: Chromatogram plot of saponin fraction

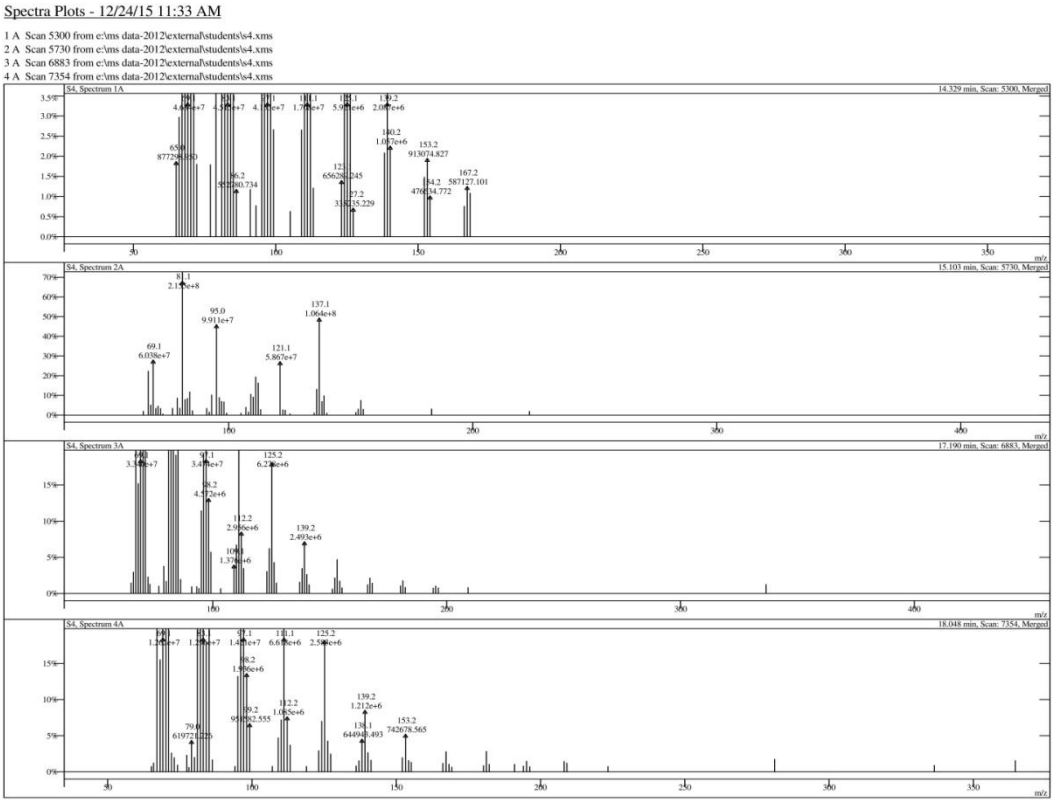


Figure 92: Characterisation of structure of isolated fraction

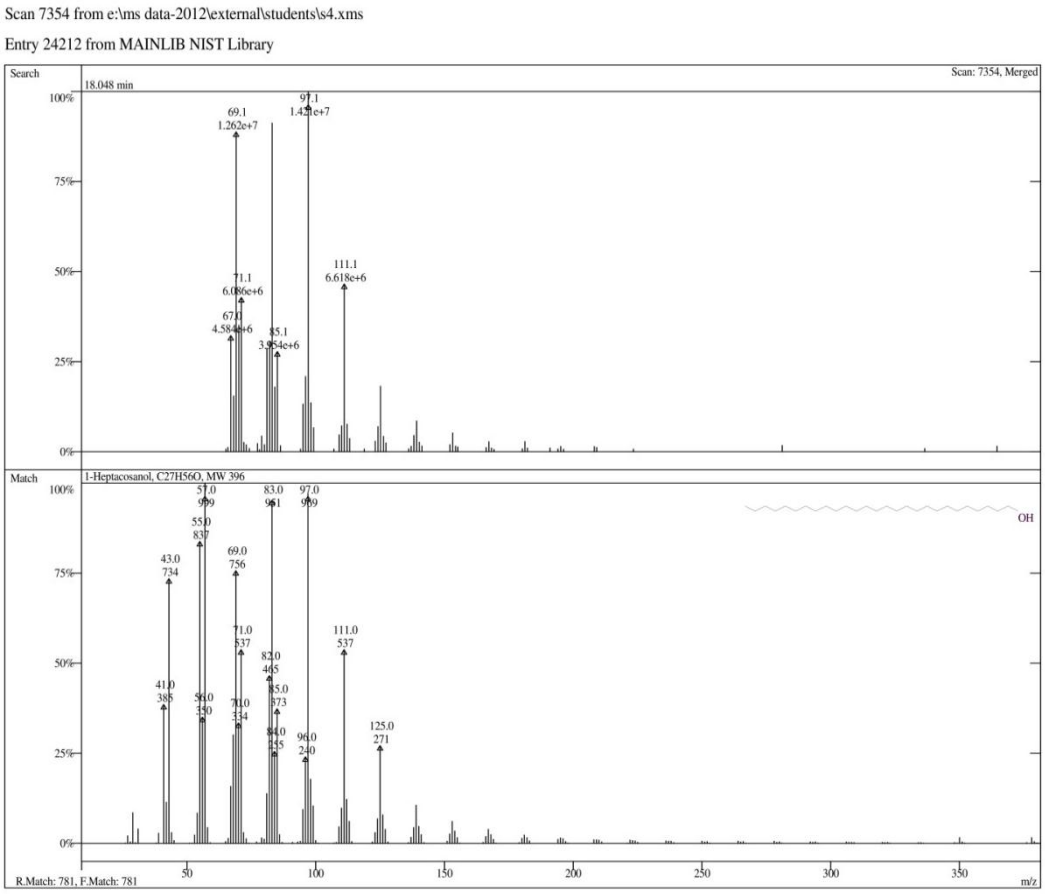


Figure 93: Gas Chromatography – Mass Spectroscopy of total phenol

Sample Name : AEPQ ISOLATED TOTAL PHENOLIC (S0)

Sample ID : ---------

Analysis Date & Time: 12/23/15 15:00:15

User Name : TBC

Instrument ID : GC-01

Method Name : D:\GC Methods\Others\Genral.gcm

Comment : Column ID:ZB-5,30 x 0.25mm,0.25µ

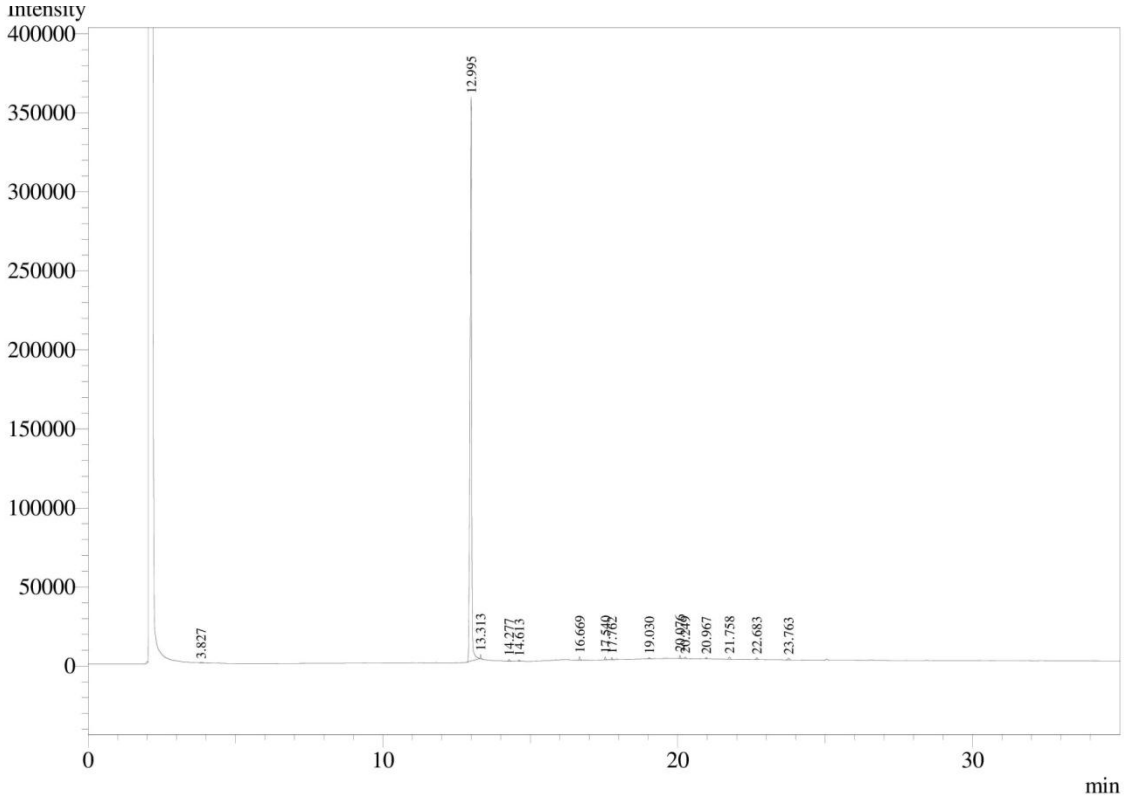
Temp Program :50-5-20-300-17.5;

Inj Temp : 250°C , Det Temp :300°C

Flow rate : 1.0 ml/min, Split ratio : 1:100, Carrier Gas : N2

Diluent:NEAT

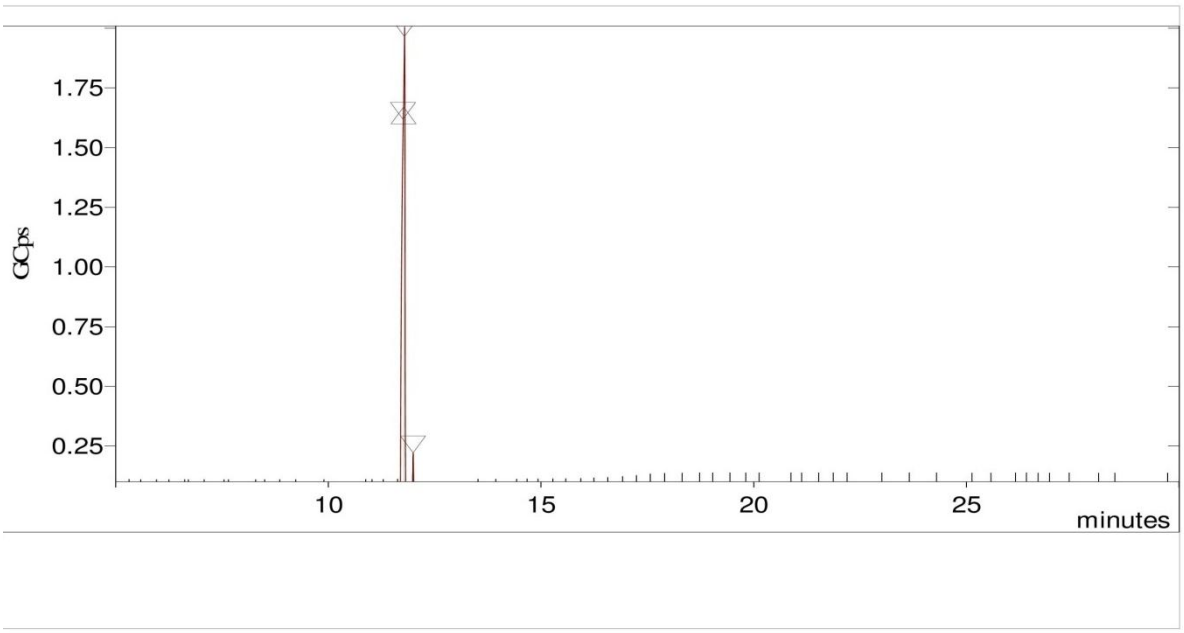
Inj.Vol(µl):2.00



ID#	Peak#	Ret.Time	Area	Theoretical Plates	Resolution	Area%
	1	3.827	1413	21291.865	0.000	0.102
	2	12.995	1332959	301557.244	92.124	96.599
	3	13.313	5182	1154209.833	4.417	0.376
	4	14.277	2566	1084662.795	18.523	0.186
	5	14.613	2171	1006510.913	5.951	0.157
	6	16.669	4825	1748978.240	37.936	0.350
	7	17.540	3861	1749071.018	16.888	0.280
	8	17.762	2905	1737552.681	4.166	0.211
	9	19.030	1913	1768339.235	22.874	0.139
	10	20.076	4443	1884978.222	18.113	0.322
	11	20.249	3102	930206.403	2.445	0.225
	12	20.967	2260	1685181.618	9.691	0.164
	13	21.758	4606	870157.160	10.040	0.334
	14	22.683	3622	1279492.082	10.695	0.262
	15	23.763	4062	873324.135	11.899	0.294
Total			1379890			100.000

Table 48: Determination of peak, retention time, area, theoretical plates and resolution of total phenol (S0) by GC-MS

Sample ID	S0	**Operator**	BKR
Instrument ID	Bruker GC/MS #1	**Last Calib Update**	Oct-30-15 06:23
Acq Date	Dec-23-15 18:48	**Calculation Date**	Dec-23-15 19:18
Inj Sample Notes	S0		
Data File	E:\MS DATA-2012\EXTERNAL\STUDENTS\S0.XMS		
Method	E:\Methods\General.mth		



No	RT	Peak Area	Peak Height	Area%	Peak Response
1	11.382	40788100	3924438	0.424	40788100
2	11.755	-2147483648	1611745280	52.528	
3	11.789	-2147483648	1957457152	43.566	
4	11.950	13535301	5638601	0.141	13535301
5	11.990	300228672	213785312	3.117	300228672
6	12.054	2556712	2111149	0.027	2556712
7	12.101	11834091	6882655	0.123	11834091
8	15.174	4615062	3433647	0.048	4615062
9	16.150	2687234	2322636	0.028	2687234

Figure 94: Chromatogram plot of total phenol

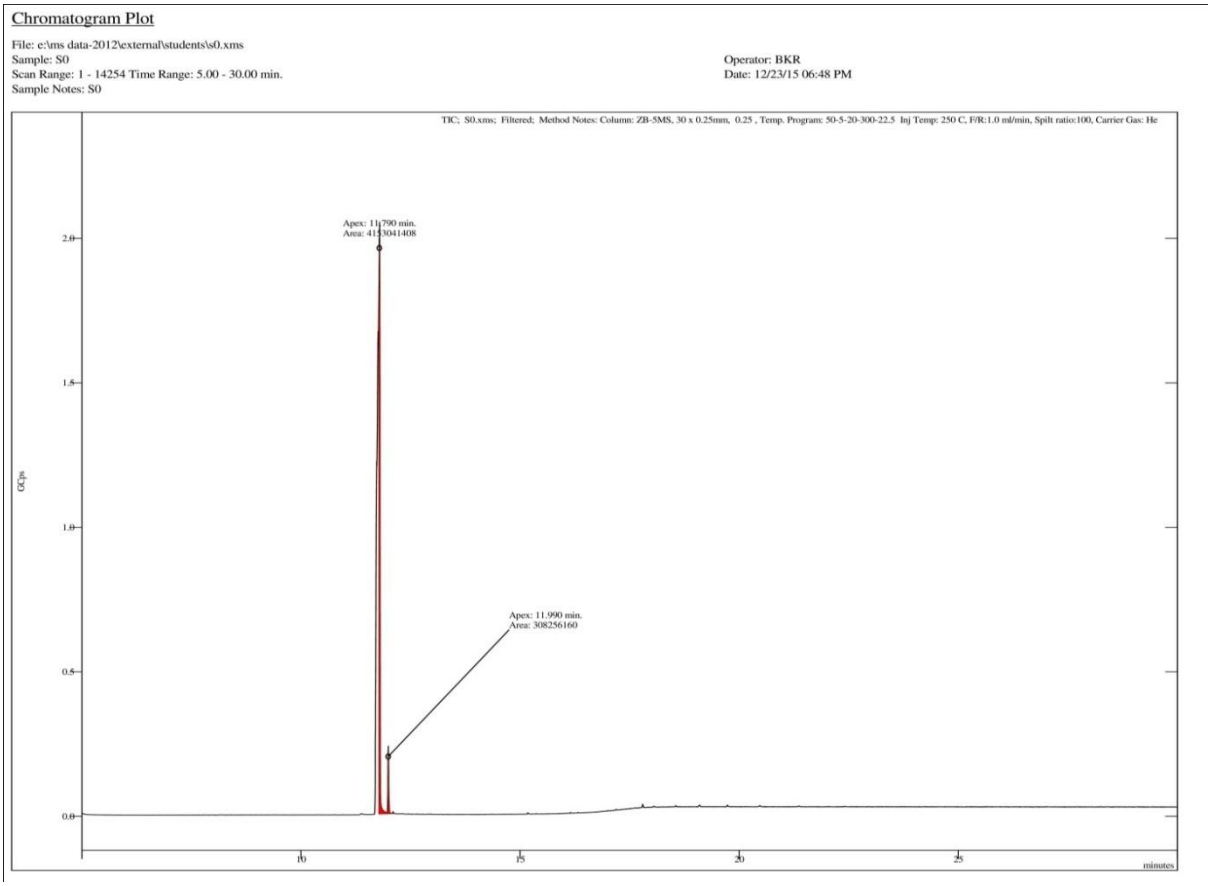


Figure 95: Characterisation of structure of total phenol

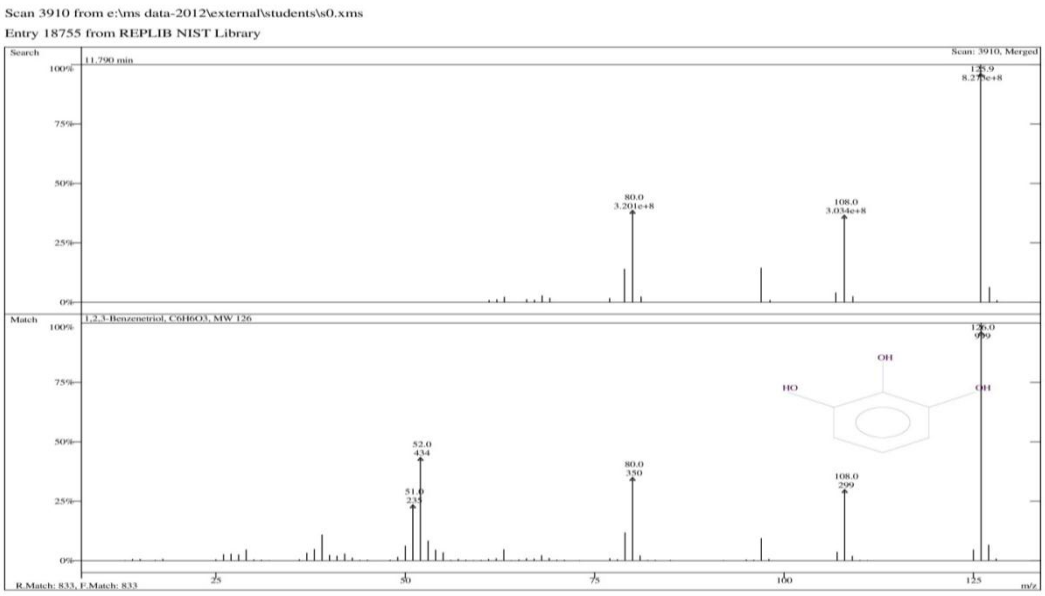
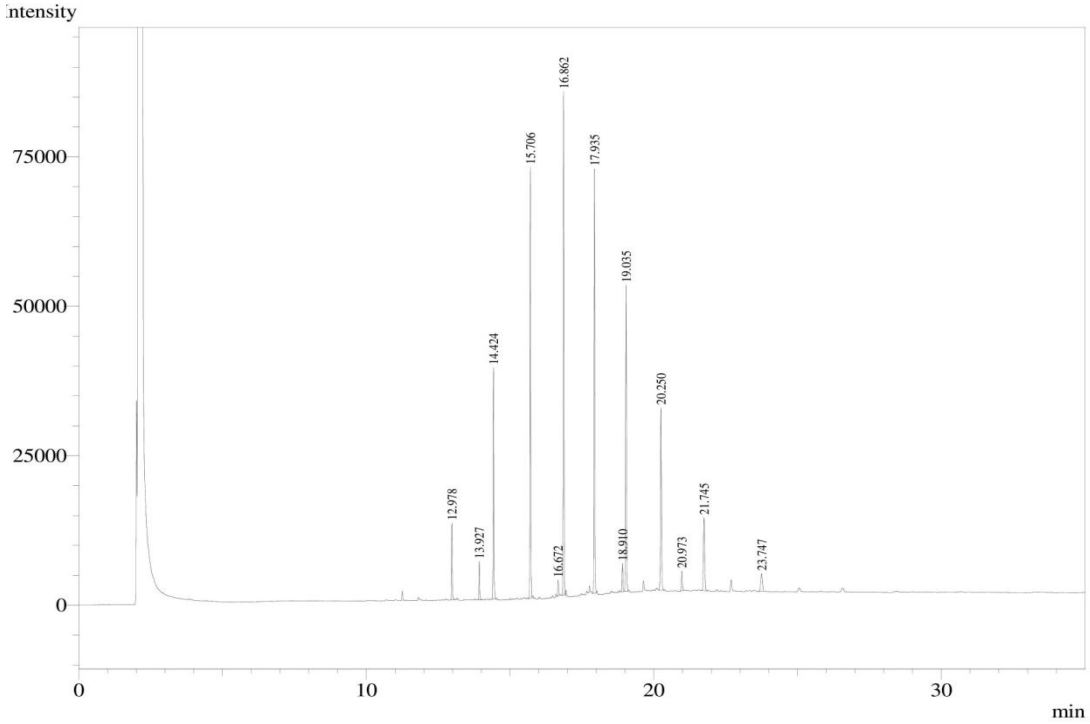


Figure 96: Gas Chromatography – Mass Spectroscopy

APEQ fraction (S_6)
Sample Name ID

Analysis Date & Time : 12/23/15 17:13:55
User Name : TBC
Instrument ID : GC-01
Method Name : D:\GC Methods\Others\Genral.gcm
Comment : Column ID:ZB-5,30 x 0.25mm,0.25µ
Temp Program :50-5-20-300-17.5;
Inj Temp : 250°C , Det Temp :300°C
Flow rate : 1.0 ml/min, Split ratio : 1:100, Carrier Gas : N2
Diluent:Ethanol
Inj.Vol(µl):2.00

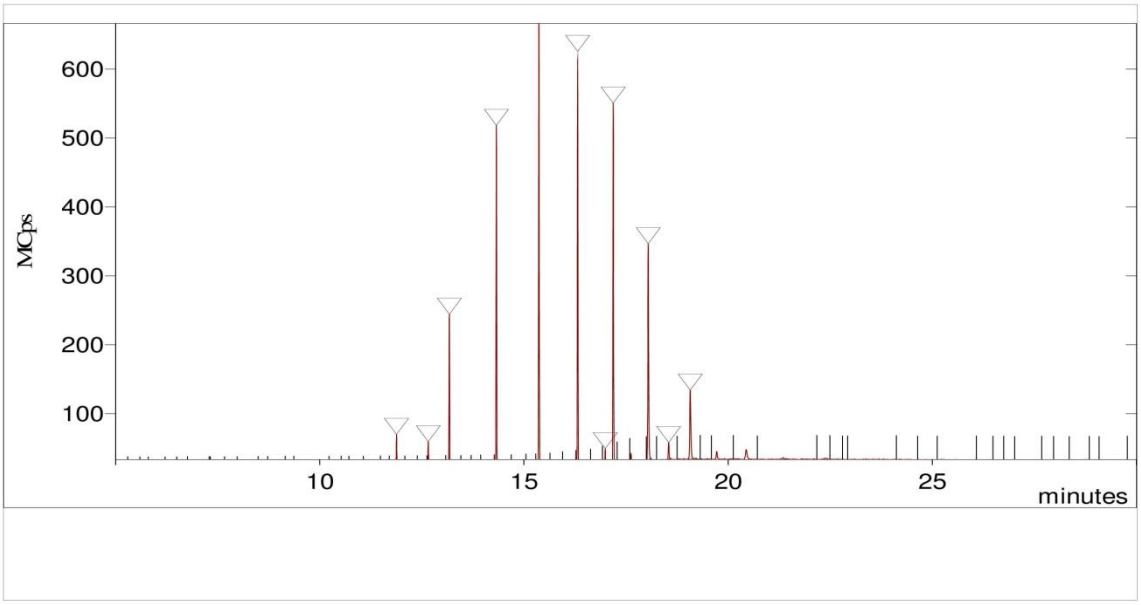


ID#	Peak#	Ret.Time	Area	Theoretical Plates	Resolution	Area%
	1	12.978	22249	1359312.703	0.000	2.971
	2	13.927	11360	1528253.408	21.246	1.517
	3	14.424	66281	1711683.757	11.181	8.850
	4	15.706	124421	2013791.183	29.081	16.614
	5	16.672	5052	1806061.312	20.641	0.675
	6	16.862	146053	2209496.879	4.001	19.502
	7	17.935	129416	2262161.406	23.121	17.281
	8	18.910	9887	1873613.551	18.993	1.320
	9	19.035	101519	2203233.838	2.344	13.556
	10	20.250	73164	1727204.750	21.583	9.770
	11	20.973	8347	1635576.131	11.401	1.115
	12	21.745	38546	1169249.138	10.600	5.147
	13	23.747	12610	714320.557	20.818	1.684

ID#	Peak#	Ret.Time	Area	Theoretical Plates	Resolution	Area%
Total			748905			100.000

Table 49: Determination of peak, retention time, area, theoretical plates and resolution of isolated fraction (S6) by GC-MS

Sample ID	S6	**Operator**	BKR
Instrument ID	Bruker GC/MS #1	**Last Calib Update**	Oct-30-15 06:23
Acq Date	Dec-23-15 20:47	**Calculation Date**	Dec-23-15 21:17
Inj Sample Notes	S6		
Data File	E:\MS DATA-2012\EXTERNAL\STUDENTS\S6.XMS		
Method	E:\Methods\General.mth		



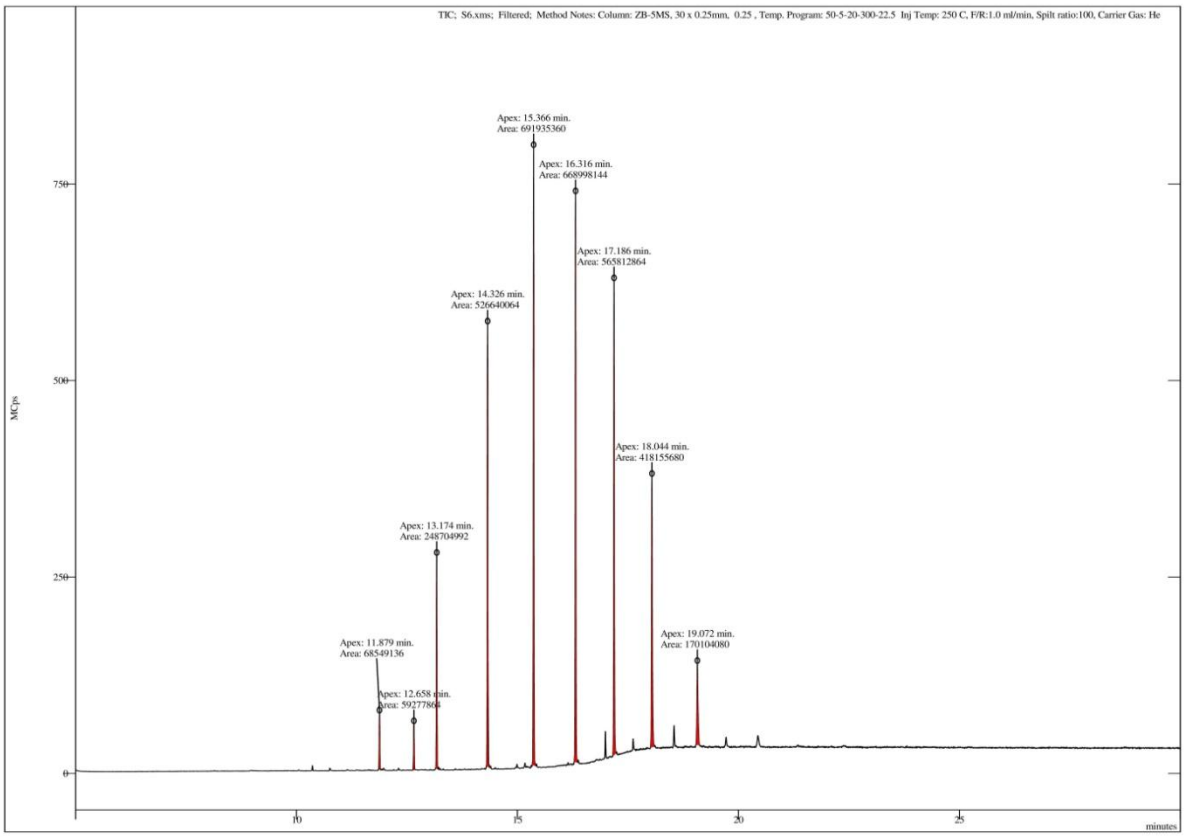
No	RT	Peak Area	Peak Height	Area%	Peak Response
1	10.357	6052901	5327227	0.172	6052901
2	11.879	70184144	65375764	1.995	70184144
3	12.658	59556136	55076744	1.693	59556136
4	13.173	253953552	239135424	7.218	253953552
5	14.325	524489920	509663200	14.908	524489920
6	15.367	696356480	657084352	19.793	696356480
7	16.315	674382208	612979392	19.168	674382208
8	16.991	32887738	30026180	0.935	32887738
9	17.186	566721344	527350304	16.108	566721344
10	18.044	421158624	313848640	11.971	421158624
11	18.544	33474772	24199424	0.951	33474772
12	19.072	179016496	99480720	5.088	179016496

Figure 97: Chromatogram plot of isolated compound

Chromatogram Plot

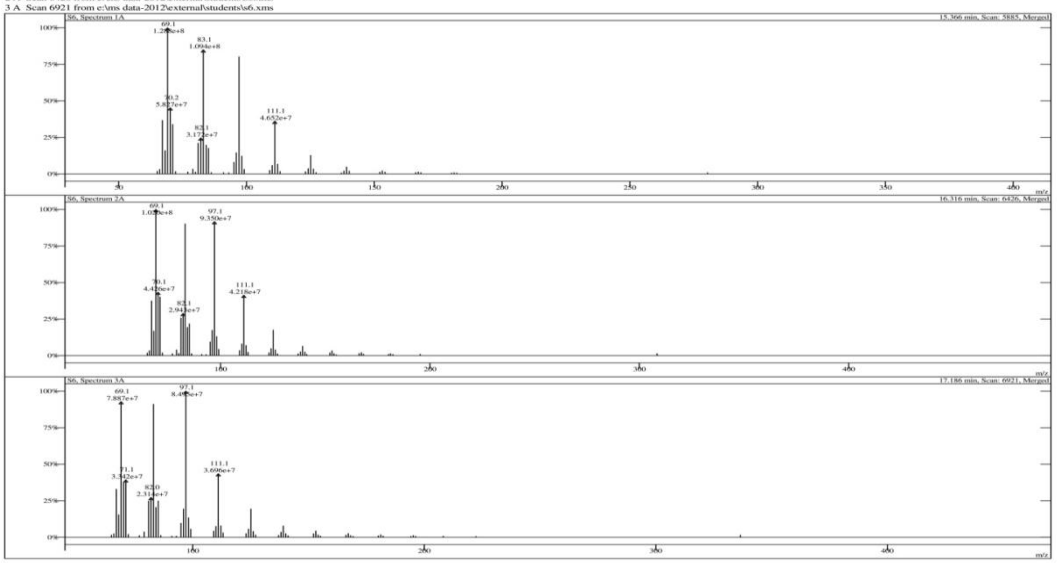
File: e:\ms data-2012\external\students\s6.xms
Sample: S6
Scan Range: 1 - 14173 Time Range: 5.00 - 30.00 min.
Sample Notes: S6

Operator: BKR
Date: 12/23/15 08:47 PM



Spectra Plots - 12/24/15 11:47 AM

1 A Scan 5885 from e:\ms data-2012\external\students\s6.xms
2 A Scan 6426 from e:\ms data-2012\external\students\s6.xms
3 A Scan 6921 from e:\ms data-2012\external\students\s6.xms



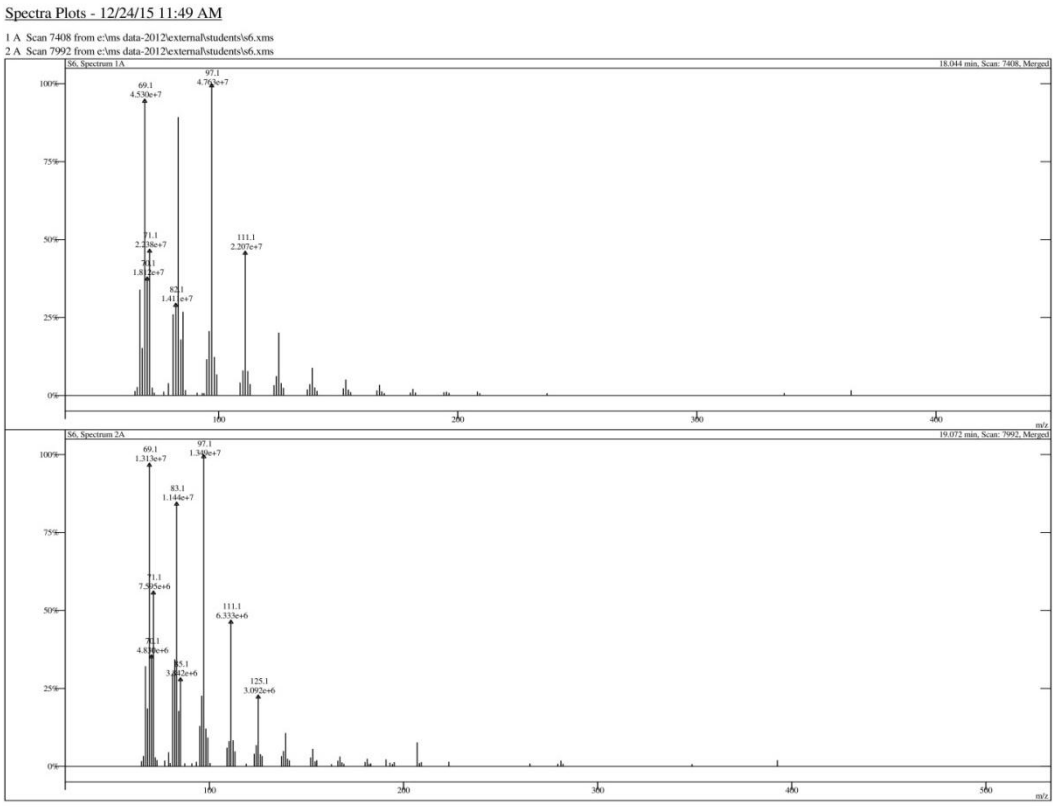
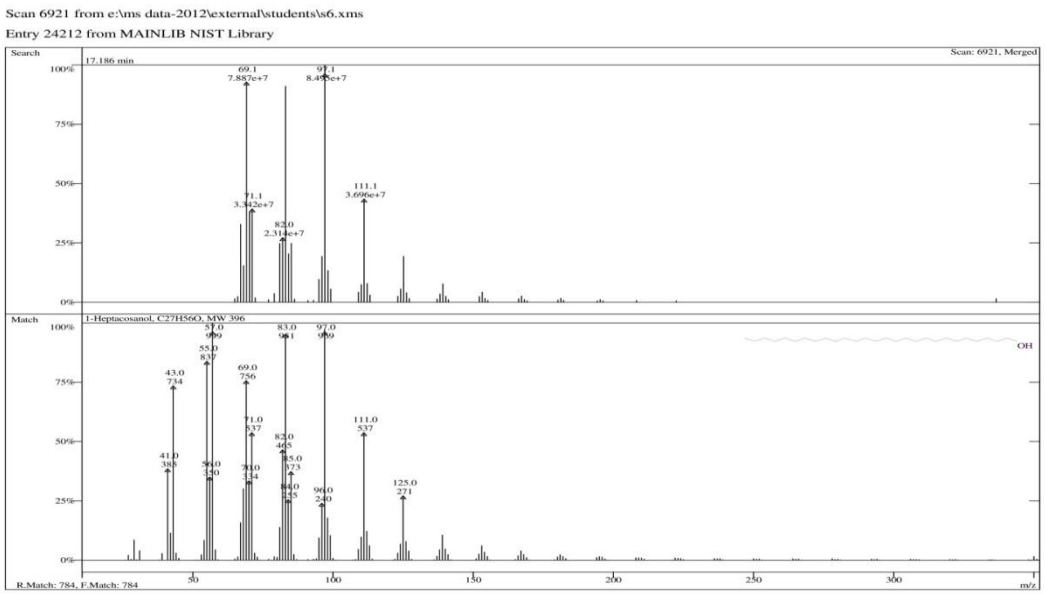
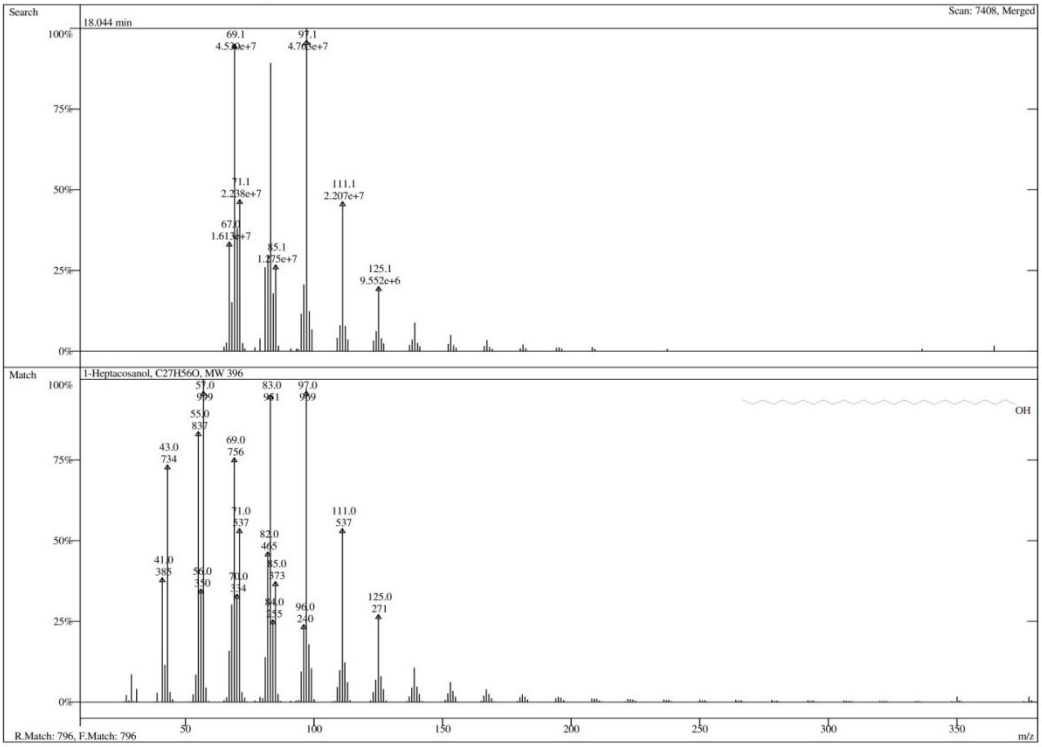


Figure 98: Characterisation of structure of isolated fraction



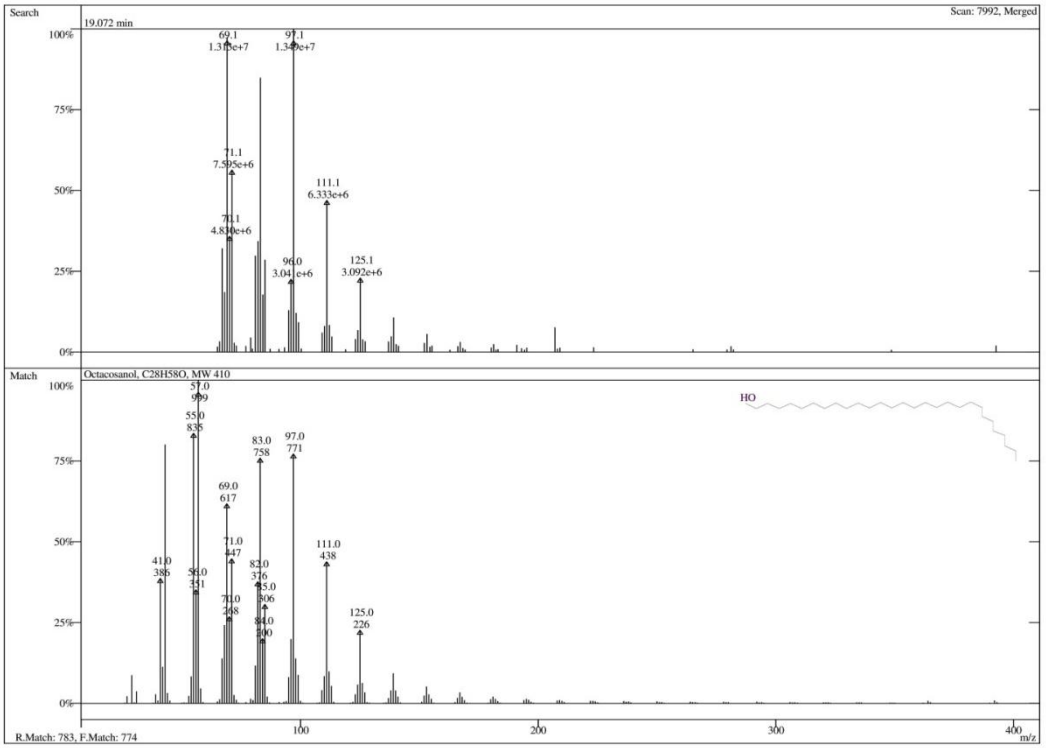
Scan 7408 from e:\ms data-2012\external\students\s6.xms

Entry 24212 from MAINLIB NIST Library



Scan 7992 from e:\ms data-2012\external\students\s6.xms

Entry 5927 from REPLIB NIST Library



GCMS ANALYSIS

In the isolated fractions of aqueous extract of *Portulaca quadrifida*, the presence of compounds such as :

Fraction S4: In S4 fraction of aqueous extract of *Portulaca quadrifida*, the presence of Compound 1-Heptacosanol (Molecular Formula $C_{27}H_{56}O$, Molecular Weight 396) was elucidated.

(The compound 1-Heptacosanol is one of the major compound contributing to the antioxidant and anticancer properties of extracts when comparing to other above compounds present in fraction S4).

Fraction S0: In S0 fraction of aqueous extract of *Portulaca quadrifida*, the presence of compound 1-2-3 Benzenetriol (Molecular Formula $C_6H_6O_3$, Molecular Weight 126) was elucidated.

(The compound Benzenetriol is one of the major compound contributing to the anticancer properties of extracts when comparing to other above compounds present in fraction S0).

Fraction S6: In S6 fraction of aqueous extract of *Portulaca quadrifida*, the presence of compound Octacosanol (Molecular Formula $C_{28}H_{58}O$, Molecular Weight 410) was elucidated.

(The compound Octacosanol is one of the major compound contributing to the antioxidant and anticancer properties of extracts when comparing to other above compounds present in fraction S6).

DISCUSSION

The world has changed radically since the turning of millennium. In day to day life new economic powers are emerging and new mechanization is renovating our societies and injustice is rising in underdeveloped countries. But still in low and middle-income countries some people believe that they are being punished for something they did wrong in past and if they had just knew what was right and what was wrong, they wouldn't have got cancer. Therefore researchers should give more importance for cancer studies especially in developing countries where mentality of peoples is backward.

Cancer is named based on location where it develops. All types of cancer start when body starts to grow cells in out of control manner or abnormal form. Cancer cells continue on growing instead of dying and forms new cancer cells. Generally in most of the cases of cancer, the cells form a tumor. Cancerous cells can also protrude into other tissues, while normal cells can't do.

There are various types of cancer(Lung, Colorectal, Stomach, Liver, Breast etc.,) which behaves differently and grow at different response that is why people need treatment with cancer.

C O L O N C A N C E R

Colorectal cancer is the most common malignancies in many regions of the world and which is one of the major source of cancer death in men and as well as in women. It is most often found in people 50 or older. The risk of colon cancer increases with the increase of age.

Usually colon cancer begins as polyps which don't show any signs of cancer, if these polyps are screened and removed then colon cancer can be prevent , The symptoms show blood in stool, Stomach pain or aches, Losing weight and other symptoms include long lasting constipation and frequent nausea.

The global scenario is focusing their face on use of herbal medicine due to lesser side effects. Folk medicinal system has deep rooted history in India of rural population. This special system of knowledge has evolved from harmonious living of aboriginal people with nature. The most majority of people on earth still depend on their traditional Material Medicine for daily health care. Medicinal plants properties lies in the active constituents

which they contain. Therefore, in many cases, the principle aim of phytochemical analysis is to detect, isolate and identify the active substances.

In the current research, an effort was made to study the anticancer activity of *Portulaca quadrifida* (aqueous extract) by *In vitro* antioxidant and cytotoxic study and *In vivo* anticolon cancer model using DMH induced colon cancer. It was executed in five phases to find out the anticancer potential of the plant selected for this study and also to find out the plant constituents responsible for the anticancer effect produced

Phase-I

The successive extracts of *Portulaca quadrifida* were named as CEPQ (Chloroform extract of *Portulaca quadrifida*), MEPQ (Methanolic extract of *Portulaca quadrifida*) and AEPQ (Aqueous extract of *Portulaca quadrifida*) and the % yield was calculated as 9.5, 11.7 and 28.1 respectively. The colour of the extract was dark green, dark green and dark brown respectively and stored in a desiccator.

In order to establish the relation between chemical content and chemo modulatory activity of successive extracts of *Portulaca quadrifida* was determined in which analysis of successive extracts of *Portulaca quadrifida* was determined showed the presence of majority of the compounds including saponins, tannins, phenols and carbohydrates.

The results obtained showed that among all the extract aqueous extracts possess higher concentration of saponins, phenols and tannins, the studies have shown that there is a clear relationship between saponins and tannins content and anticancer activity.[93]

Phase-II

***In vitro* antioxidant study**

Oxidative stress is described in common as surplus formation or inadequate destruction of highly reactive molecules of such as oxygen specie (ROS) and reactive nitrogen species (RNS). ROS comprise of free radicals such as superoxide(*O2), hydroxy1(*OH), peroxyl (*RO2), hydroxyperoxyl (*HRO) as well as non-radical species such as hydrogen peroxide (H202) and hydrochlorous acid (HOCL). RNS comprise of free

radicals like nitric oxide and nitrogen dioxide as well as non-radical such as peroxynitrite (ONOO) nitrous oxide(HNO2) and alkyl peroxynitrates (RONOO.)

Among all the three extracts tested for their antioxidant potential to scavenge Superoxide radical, hydrogen peroxide radical, Nitric oxide radical, β-Carotene Linoleate radical, DPPH radical and ABTS by *in vitro* antioxidant models, aqueous extract significantly scavenged all the free radicals tested when compare with other extracts

Hydrogen peroxide (H_20_2), which in turn produce hydroxyl radicals (*OH), resulting in propagation of lipid peroxidation.

Physiological processes such as relaxation of muscle, signalling of neuron, platelet aggregation inhibition and regulation of toxicity of cell media in biological systems by Nitric oxide (NO) since it is a strong pleiotropic inhibitor.

One of the important plant constituent Phenolic compounds possessing scavenging ability and powerful chain breaking antioxidant property due to presence of hydroxyl groups. In our study we have observed that Total phenolic content very significantly scavenged the Superoxide radical (98.06 % scavenging activity at 250 µg/ml when compared with Ascorbic acid by 87.77% at 250 µg/ml), hydrogen peroxide radical (91.78 % scavenging activity at 250 µg/ml when compared to ascorbic acid showed 74.01 %only at 250 µg/ml) , Nitric oxide radical(35.71 % at 2500 µg/ml when compared with ascorbic acid by 78.57at 250 µg/ml). This findings are very well comparable with findings of Shahidi and Wansundeara, 1992; Hatano et al., 1989[94,95]

The free radical of linoleic acid attacks the unsaturated b-carotene models and inhibit the scope of b-carotene-bleaching by equalising the free radical of linoleate[96]

By comparing all the extracts AEPQ was significantly scavenged the β-Carotene Linoleate radical when compared to the Ascorbic acid showed the antioxidant potential. AEPQ showed the scavenging activity by 96.29 at 200 µg/ml when compared to ascorbic acid showed 59.15 only at 200 µ/ml where as CEPQ and MEPQ was showing 86.10 and 93.44 respectively. (Table: 16,17 and 18; Figure: 14,15 and 16)

Electrons become matched off and it loses the colour of solution stoichiometrically based on the number of electrons utilised. Illustrates a remarkable decline in the mass of DPPH radical due to the capability of scavenging of the extract and the standard quercetin as

a reference compound. By comparing all the extracts radical scavenging activity of AEPQ was evident at all the concentrations but only at low level not as significant as that of quercetin used as standard. The scavenging activity of the AEPQ was increased with increasing extract concentrations and that of the standard. AEPQ showed the % scavenging activity by 72.43 at 400 µg/ml when compared to quercetin showed 88.44 at 400 µg/ml. Whereas CEPQ and MEPQ showed 68.30 µg/ml and 69.99 µg/ml respectively.

AEPQ significantly scavenged the DPPH radical. The l, 1-diphenyl -2-picryl hydroxyl (DPPH)radical was extensively used as the model system to examine the activities of scavenging of several pure compounds such as extract of plants in a relatively short time. Scavenging of DPPH radical by antioxidants through the contribution of proton forming the minimized DPPH)" After reduction the purple colour changes to yellow (diphenyl picryl hydrazine), which was expressed by its reduction of absorbance at 517 nm wavelength. In the present study AEPQ showed significant scavenging of DPPH radical when compared with other extracts and standard compound . (Table: 19; figure 18)

The ABTS radical cation procedure gives a count of carotenoids and phenolics antioxidant activity, and also some antioxidants of plasma, estimated by the decolorization of the ABTS-l, through determining the radical cation reduction as the inhibition of percentage of absorbance at 734 nm. ABTS-l assay of decolorization in contrast to myoglobin assay recorded at 6 min. The assay of latter involves constant development of the ABTS radical cation by ferryl myoglobin which is derived from hydrogen peroxide and met-myoglobin in the existence of the reductants.

The antioxidant potential of AEPQ was significantly scavenged the ABTS radical by 71.2 at 100 µg/ml, when compared to the standard, CEPQ and MEPQ which has showed 64.40 and 68.40 at 100µg /ml respectively. The antioxidant properties were expressed as concentration dependent.

Vitamin C is a chief antioxidants, it remarkably lowers the harmful consequences of reactive species such as ROS and RNS which can be the source for oxidative damage to macromolecules such as lipids, DNA and proteins which are involved in the development of chronic diseases by scavenging/ neutralizing them. Ascorbic acid the antioxidant vitamin, significantly lowers the harmful consequences of reactive species such as ROS and RNS which can be the source for oxidative damage to macromolecules. Vitamin C reduces the harmful consequences of reactive species such as reactive nitrogen species as it is a prime

antioxidant that can cause damage to oxidative macromolecules which are involved in chronic diseases by scavenging/ neutralizing such as lipids and DNA.[97]

Phase-III

***In vitro* cytotoxicity studies**

In this phase of study chloroform, methanol and aqueous extract of *Portulaca quadrifida* were evaluated for the cytotoxic activity by using MTT assay on HT-29 (colon cancer cell lines) where aqueous extracts showed highest inhibition with lowest concentration of 15.87 µg /ml when compared to other extracts. (Table: 25; Figure: 25, 26 and 27).

There is a clear relationship between saponins content plants and anticancer activity[98]. In preliminary phytochemical screening of all extracts, aqueous extract of *Portulaca quadrifida* possessed higher concentration of saponins and tannins when compared to other extracts.

Phase-IV

The successive extracts of *Portulaca quadrifida* were used for *in vitro* antioxidant and *in vitro* cytotoxicity studies where aqueous extract results were found to be excellent in contrast to other extracts. Based on this findings aqueous extract was selected and subjected to carry out acute oral toxicity in mice and *In vivo* anticancer in DMH induced colon cancer model in rats.

Acute oral toxicity study:

The guidelines set by the Organisation for Economic Co-operation and Development (OECD, 423) to perform acute oral toxicity study was found to be extremely safe and not produced any toxic effects on animal, Mortality was not noticed to a dose as high as 2000 mg/Kg, of AEPQ and the result (Table 29) showed that the compound can be used safely in the animals up to the dose of 2000 mg/Kg. A dose level for the present study was chosen as $1/10^{th}$ and $1/20^{th}$ of safe dose (2000 mg/kg) of plant extract.

The non-toxic nature of AEPQ is due to presence of active plant constituents like Phenolic compounds; saponins; tannins.

***In vivo* anticancer activity**

Anticancer effect of AEPQ was assessed in DMH induced colorectal cancer model, by administering DMH for 15 weeks in a dose of 20mg/kg. And the efficacy of the extract was evaluated by treating the animals with two dose levels one week prior to DMH treatment and simultaneous treatment with DMH for 15 weeks by daily dosing.

At the end of 16 weeks treatment, haematological, biochemical parameters and plasma tumor markers were estimated.

There was a alteration in the normal blood cell counts were noticed in our anticolon cancer treatment with AEPQ during the study period for 16 weeks . A remarkable rise in the level of WBC, and remarkable decline in the level of RBC and haemoglobin when compared to control animals were observed. The extract approximately reversed these changes towards normal values in a dose dependent and significant manner (Table 34; Figure 39,40 and 41).

In the cancer condition there was a significant change in serum biochemical parameters. There was a remarkable decrease in triglycerides and total cholesterol, and there is remarkable increase in SGOT, SGPT, bilirubin levels.

Many Physicians and Public Workers show curiosity towards modification of the diet, which may result in a less incidence and mortality from cancer. Recent reports mainly focused light on the possibility of cause of cancer by dietary lipids. Cholesterol, which is major cause in cardiovascular disorders and cancer

Epidemiologic data that relates serum cholesterol levels and cancer. In a recent study, a positive connection was estimated linking serum cholesterol levels and colorectal cancer risk in male.

A number of epidemiological studies have been reported in past few years showing an high risk of death from cancer subjects with low plasma cholesterol levels. [99, 100,101.]

However, some researchers believe that the cause of cancer is not hypocholesteremia, but infact it is the result. [102]

The present study examined the lipid profile of animals with colon cancer in comparison with treated cancer groups there was a remarkable rise in the total cholesterol level due to modification in the diet. The effects of AEPQ on different serum biological

parameters (Table 35; Figure 42-46) are showing significant reverse in altered serum biological parameters compared to other extracts.

The carcinoembryonic antigen (CEA) test measures the content of this protein that may exist in the blood of people who have various kinds of cancers, especially large intestine. CEA is nominally produced during the development of a fetus. Before birth the production of CEA stops and it usually is absent in the blood of healthy adults. Alpha-feto- protein is a serum protein that is identified in raised concentration in conditions of carcinoma; it is a serum protein alike in size, shape to serum albumin. The levels of AFP will be in minute quantities in adults, where there will be an elevated level in cancer condition. [103]

In the current study, a decline in the level of CEA and AFP was observed followed by AEPQ treatment (Table 36 and 37; Figure 47 and 48) indicates a positive prognosis the decrease levels on AEPQ treatment prevents the neoplastic growth and reduces the level of carcinoma, which indicates that it possesses anticancer properties.

In the present study there was significant change in the protein levels due to glyconeolytic enzymes impairment, groups treated with AEPQ shows a remarkable ($p<0.001$) reverse in the altered protein levels (Table 36)

The antioxidant and oxidant profile alterations are familiar to arise in the tissues of cancer. The outcome of the current study intelligibly indicates in the presence of AEPQ the administration of the DMH (procarcinogen) brings about intense modifications in the peroxidation of tissue of lipid and antioxidant status. When cellular macromolecules get damaged by free radical attack oxidative stress occurs such as lipids and DNA. Metabolic activation in liver is experienced by DMH so as to generate an dynamic electrophilic carboniumion, which in turn via various processes, is familiar to obtain oxidative stress. Current confirmation has showed that the production of ROS may be included in different carcinogenic activities. [104]

Superoxide dismutase can drive dismutation of 02 - into H2O2, which is then inactivated to H2O by Catalase or glutathione peroxidase. Normally, the work of superoxide dismutase with selenium dependent glutathione peroxidise is parallel, which plays a key role in the depletion of hydrogen peroxide presence of reduced glutathione (GSH) tends to form oxidized glutathione (GSSG), and thus, it shields proteins cell and membranes against stress of oxidation.

Superoxide dismutase and Catalase are two main antioxidants of enzyme that react against toxic free radicals of oxygen such as hydroxyl and superoxide ions in biological processes. By catalyzing the formation of H20 and 02 from H202 oxidative hazards are prevented by CAT. A prior study has exhibited that administration of carcinogen usually declines SOD and CAT activites[105]. Earlier results of laboratory have also exhibited related results of Vennila et.al, 2009, Aranganathan et.al, 2008.[93,106] In current study we noticed a related trend in the activities of the liver and colonic tissues on DMH administration such as SOD and CAT (Table 36; Figure 47,48 and 49). AEPQ administration to rats treated with DMH remarkably standardize the activities of SOD and CAT which may be due to scavenging of free radical and antioxidant property of AEPQ which is expected to be achieved by 02 trapping to form steady radicals of it.

The levels of lipid peroxidation were steady in the liver; DMH treated colonic tissues of rats were remarkably elevated in the liver, whereas the elevation of peroxidation of lipid products were remarkably diminished in the DMH alone (Table 36; Figure 47,48,49,50,51 and 52) prior studies have shown decreased peroxidation rates of lipid in the tissue of tumors of different cancers. Cancerous cells obtain particular features that advantage their proliferation and they multiply rapidly when the peroxidation of lipid levels are low[107]. Thus, the declined colonic peroxidation of lipid noticed in rats treated with DMH it could be due to increase in proliferation. Therefore, invasive tissues are less likely and more impervious to attack of free radical. In addition, low levels of peroxidation of lipid in targeted organs of rats treated with DMH may also be due to raised resistance and declined susceptibility for attack of free radical. Prior research have exhibited similar results. In the current study we noticed that AEPQ administration to rats treated with DMH restored the lipid peroxidation levels to nearly the values exhibited by control group rats[108]. Which may be due to the powerful properties of antioxidants of AEPQ. Phenolic hydroxyl groups are familiar to be powerful in free radicals scavenging . Therefore antioxidant properties of AEPQ may be due to the existence of the phenolic hydroxyl groups. The cells which loses their oxidative capacity by the induction of DMH are being shielded bh AEPQ and it also accredit to the anti proliferative activity of AEPQ.

GSH a major non-protein thiol, GPx in conjugation plays a important part by scavenging reactive oxygen species which protect cells against cytotoxic and carcinogenic chemicals. This tripeptide helps in the removal of many toxic carcinogens and free radicals from the environment. The breakdown of H_20_2 and organic hydroperoxides is catalyzed by

GPX an enzyme containing four selenium cofactors. Glutathione regenerating enzyme that allows transformation of oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADH to NAD+. In the current study the levels of GSH, GPx in the liver, colonic tissues were declined in rats treated with DMH alone when compared to animals of control group, which was constant with earlier results[93]. The decrease in GSH level on DMH administration may be due to the participation of these enzymes in the detoxification and feasible restore procedure in the intestinal and colonic mucosa. The decline of enzyme activity of GPx exhibits elevated capacity of detoxification and it may be a flexible mechanism by which cells of tumor obtain a selective growth benefit over the normal cells. AEPQ treatment to the rats treated with DMH rise GSH levels and the activities of G-Px (Table 38; Figure 49 to 54). lnductions of these enzymes have been estimated as a means for discovering the potency of many anti carcinogenic matters. AEPQ is familiar to inhibit reactive oxygen species and increase the levels of GSH. Therefore, increased levels of GSH, GPx in rats treated with DMH on AEPQ supplementation which may be due to the consolidated effects of the phenols which can further help in free radicals scavenging.

Aberrant crypt foci (ACF) are early morphologic changes seen in rodents after administration of specific colon carcinogen such as DMH or azoxymethane. At a high frequency similar lesions were also observed in the colons of the patients with sporadic and inherited forms of colon cancer. ACF are considered as putative pre-neoplastic lesions and are currently used as a surrogate biomarker to rapidly estimate the chemopreventive potential of several agents, including both naturally occurring and synthetic, using AOM in the Fisher 344 rat model, which accurately replicates many of the clinical, genetic, cellular, and morphologic features of human colorectal cancer. ADM-induced ACF are characterized by an increase in the size of the crypts, the lining of epithelial, and the pericryptal zone which share many morphologic and biochemical characteristics with tumors, including a comparable rise in cell proliferation

The effects of extracts on colon cancer induced by DMH was evaluated by the formation of aberrant crypt foci (ACF). After termination of the study the number of ACF in the colon was enumerated to determine the effect of extracts on DMH induced colon cancer. From the present study the extract treated groups shown significant reduce in the formation of ACF. (Table 32; Figure 37)

Aldolase, a different essential enzyme in the glycolytic pathway, was elevated in conditions of tumor. Aldolase was established to be raised in animals bearing tumor and in breast cancer. In carcinogenesis, the cells are adminster to carcinogen-induced damage and very frequently display glycolysis after increase in oxygen uptake. The high glycolytic rate of most tumors can be adopted as a major source of energy deranged cell. Increase in the activity of glycolysis results from the rise of the tumor growth rate and is accompanied by a decline in the activity of the pentose phosphate pathway and respiratory chain. The gluconeogenic enzymes activity such as fructose 1, 6-diphosphatase and glucose-6-phosphatase was inhibited significantly tumor-bearing animals. Lactate production from glucose ascend and complementary production of glucose from pyruvate declines during the development of tumor growth, Srinivasa Shanna etal 2011. The notice depletion in the activity of these enzymes in animals bearing tumors may be due to increase production of lactate of neoplastic tissue, and it confirms that tumor uses a substantial proportion of lactate for protein and glycolysis synthesis. (Table 39 and 40; Figure 55 and 56).

Histopathology reports showed a DMH treated colon tissue shows the presence of tiny pednucleated polyp probably a benign tubular adenoma, and the AEPQ treated groups does not showed any abnormality And so it suggested that AEPQ have shown a good response when compared with the first group. (Figure:61to 69).

The repair of normal haematological levels, *In vivo* antioxidant model, glycolytic enzymes, and the results obtained from the in vitro antioxidant studies as well as the effects on colon cancer and body weight and also the histopathology reports are the suggestive of strong chemopreventive action of AEPQ against the colon cancer induced by DMH and need a further studies for the potential development of more active compounds as an effective treatment against the colorectal cancer

Presences of active plant constituents like Phenolic compounds; saponins; tannins in AEPQ are responsible for *in vivo* anticolon cancer potential and in vivo antioxidant property to improve the free radical scavenging property by elevating their levels

Phase V:

Based on the above results aqueous extract of *Portulaca quadrifida* was subjected to isolation of saponins and phenols. Further, the isolated compound was screened for *In vitro* cytotoxicity discourse by different cell lines such as HT-29 and HCT-116. Among all the six

isolated fractions of *Portulaca quadrifida*, saponin fraction , S(6) and total phenol showed significant effect at lowest concentration with highest inhibition of 30.328, 29.657 and 20.566 respectively by using HT-29 cell lines whereas, the same fractions with HCT-116 cell lines also showed significant effect at lowest concentration with highest inhibition of 19.959, 46.258 and 21.152 respectively and it has been proved that isolated saponin and phenol of *Portulaca quadrifida* are aggressive towards colon cancer cell lines (Table: 45 and 46; Figure: 77-83 and 84-89)

Therefore, for identification of biochemical components, fractions are subjected to Gas Chromatography - Mass Spectroscopy.

The GCMS analysis of isolated compounds using aqueous extract of *Portulaca quadrifida* of each sample were given in (figure: 90,92,95 and 98) respectively revealed that the major compounds present are 1-Heptacosanol, 1,2,3-Benzenetriol and Octacosanol respectively which possesses anticancer activity[109,110,111] Also the comparison of the mass spectrums with the data base gave more than 95% match as well as confirmatory compound structure match. Since the plant contains the major compounds 1-Heptacosanol,1,2,3-Benzenetriol and Octacosanol with anticancer activity , it can be recommended to as a important phytopharmaceutical in field of cancer treatment.

ANTIMICROBIAL ACTIVITY: All the three extracts were screened for antimicrobial activity in which nutrient agar method was followed for antibacterial and potato dextrose agar method was followed for screening antifungal action of the extracts. Among chloroform extract, methanol extract and aqueous extract, aqueous extract showed significant activity by inhibiting *Escherichia coli* and *Staphylococcus aureus*, and fungal organism like *Aspergillus flavus* and *Aspergillus niger* at concentration of minimum inhibitory of 4.3, 4.2, 4.4 and 3.7 at 25mcg/ml, respectively. (Table: 28,29 and 30 ; Figure: 30 and 31)

SUMMARY AND CONCLUSION

In India, nature has provided tremendous output of many herbal plants which possesses many pharmacological activities.

In the present study we have taken an Indian medicinal plant (*Portulaca quadrifida*) which is used as vegetable in some places. As per epidemiological studies vegetables and fruits were used to cure many aliments as well as cancer. Therefore we have taken up the current study on one of the most common cancer that is colorectal cancer which has became more common in countries of low income both in men and women.

Colon cancer which often found in people at the age of 50 or older. Usually colon cancer begins as polyps, if these polyps are screened and removed then colon cancer can be prevented.

It is often a pathological outcome of continuous oxidative stress, governing to rupture of DNA, alterations in genes pertaining to cancer, further silencing epigenetically tumor suppressor genes leading to genomic instability in cellular excess production of reactive species of oxygen and nitrogen (ROS & RNS). ROS- induced carcinogenesis may be associated with oxidative DNA damage. Peroxidation of lipid is an ambiguity of aerobic life damaging wellness or wellbeing of man's present day life. Natural life systems which are lipid rich matrices are often open to auto oxidation if not saved from non enzymatic or endogenous enzymatic systems.

In the present study *Portulaca quadrifida* was investigated for *In vitro* antioxidant studies in which aqueous extract has shown significant scavenging activity comparing with other extracts used and then plant was concealed for *In vitro* cytotoxicity discourse on colorectal cancer using cell lines like HT-29 and HCT-116 using MTT assay, By comparing all the successive extracts screened, aqueous extract showed highest inhibition with lowest concentration of 15.87 mg/ml.

The in vitro data proved that among all the extracts AEPQ is having significant antioxidant and cytotoxicity effects hence; further in vivo studies were preceded with AEPQ.

The toxicity of AEPQ was estimated by the acute oral toxicity study in which it showed no toxicity and mortality up to the dose of 2000mg/kg. Hence the present study was carried out with two dose levels of plant extracts (200 mg/kg & 400 mg/kg).

For the *In vivo* study, colon cancer was induced with the chemical carcinogen (DMH) and after treatment with the AEPQ for 16 weeks, the various *In vivo* parameters were evaluated. The *In vivo* antioxidant levels (SOD, CAT, GPx, GSH, LPO), carbohydrate metabolizing enzymes were increased in compound treated groups (extract) compared to control animals in a dose dependent manner.

The *In vivo* data thus obtained suggested that AEPQ is having good activity against the various biochemical parameters as balanced with the negative group treated as control. The activity may be due to its hydrogen donating property.

Histopathalogical studies with aqueous extract of *Portulaca quadrifida* at 400mg/kg b.wt showed no obvious abnormality in structure of colonic mucosa, which was very well comparable with biochemical, haematological & antioxidants.

With the above said findings we conclude that the aqueous extract of plant *Portulaca quadrifida* possess anti colorectal cancer property.

Based on the results of *In vitro* antioxidant, cytotoxicity and *In vivo* studies, the aqueous extract was subjected for isolation and characterization, after which isolated compounds were screened for *In vitro* cytoxicity discourse using cancer cell lines like HT-29 and HCT116 in which S4, S0 and S6 fractions showed remarkable effect at lowest concentration with maximum inhibition of 30.328, 29.657 and 20.566 respectively with HT-29 whereas with HCT-116 it showed 19.959, 46.258 and 21.152 respectively.
GC-MS study proved the aqueous extract of *Portulaca quadrifida* possess anticancer and antimicrobial activity due to presence of 1-Heptacosanol, 1,2,3-Benzenetriol and Octacosanol

In our extended work microbial activity of aqueous extract of *Portulaca quadrifida* have undertaken because the bacterial density in large intestine is much greater than that in small intestine, which is paralleled approximately 12 folds increase in cancer of large intestine comparing with small intestine. These two observation combined point towards the theory that colon cancer may be induced by bacteria.

In which antimicrobial activity was estimated by using nutrient agar method and potato dextrose agar method used for antifungal activity by using all the three extracts. Among all the extracts aqueous extract of *Portulaca quadrifida* showed significant activity by inhibiting *Escherichia coli* and *Staphylococcus aureus* and fungal organism like *Aspergillus flavus* and *Aspergillus niger* at minimum inhibitory concentration of 4.3, 4.2, 4.4 and 3.7 at 25mcg/ml, respectively.

With the above said findings we conclude that the plant *Portulaca quadrifida* possess anti colorectal cancer, antioxidant and antimicrobial activity.

Good Luck!

RECOMMENDATIONS

1. Colon cancer, also known as colorectal cancer, has become a major problem for men's as well as in women's in the society especially in developing areas. The worrying fact is that, when treated with synthetic drugs a lot of serious side effects are produced because of various chemicals used while preparing it.
2. Besides synthetic drugs, natural medicine provides a wide range of plant resources with anticolorectal cancer effects especially vegetable and fruits are recommended for people with colon cancer. *Portulaca quadrifida* is one of the vegetative plants grown in tropical parts of India.
3. On the basis of analysis and determination of pharmacognostical research of *Portulaca quadrifida*, the major active principles identified were saponins, phenols and tannins which have remarkable therapeutic potential against anticolon cancer, antioxidant and antimicrobial properties.
4. Further processing with pharmacological screening using *In vitro* cytotoxicity study using successive extracts like chloroform, methanol and aqueous in which aqueous extract exhibited excellent effect.
5. On the basis of *In vitro* studies, further *In vivo* anticolon cancer screening was studied using aqueous extract. *Portulaca quadrifida* extract shown potent activity against the various biochemical parameters as compared with negative group. Histopathological studies with aqueous extract of *Portulaca quadrifida* at 400mg/kg body weight showed no obvious abnormality in structure of colonic mucosa, which was very well comparable with biochemical, haematological, antioxidants and tumor markers estimation. The anticolon cancer activity of aqueous extract of *Portulaca quadrifida* may be due to the presence of saponins and phenols.

   Therefore, the plant was subjected to isolation of active constituents by flash chromatography. The isolated fractions were screened for anticolon cancer activity by using different colon cell lines. After the remarkable growth inhibition at lowest concentration by isolated fractions (S0, S4 and S6), then they were subjected to GC-MS for elucidation and characterization of structure. (1-Heptacosanol, 1,2,3-Benzenetriol and Octacosanol) which confirms the anticancer activity. The plant is further recommended for *In vivo* anticancer screening by using various models.

MY FINAL CONCLUSION WORDS:

In the rest of your life and if there are possibility go for colorectal examination which can prevent cancer treatment by surgery in the future, a day of diarrhoea is a small price to pay. But ask someone who has a history of colorectal cancer or who had watched their loved ones dying from this disease which is preventable.

Good Luck!

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