STUDY ON TOXICITY AND ANTI CARCINOGENIC ACTIVITY SOME MEDICINAL PLANT

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In partial fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOPHOSY

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by

Dr. YASSA YONIENE PIERRE

at

Under the Guidance of

PROF. DS. KUMAR, PHD., PDF

INDIAN COUNCIL OF MEDICAL RESEARCH, NEW DELHI, INDIAN

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CERTIFICATE

This is to certify that the thesis entitled **"STUDY ON TOXICITY AND ANTI CARCINOGENIC ACTIVITY OF SOME MEDICINAL PLANT''** submitted to Indian Board of Alternative medicine, Kolkata, Indian for the award of the degree of Philosophy (Altnernative Medecine – Clinical Pathology), is a record of research work by DR. Yassa Yoniene Pierre, during the period 2015 to 2018 at the Department of Clinical Pharmacology, Indian Council of Medical Research, Chennai, Indian under my guidance.

The thesis has not previously formed the basis for the award of any other degree diploma, associateship, fellowship or other similar to the candidate and the thesis represents idependent work on the part of the candidate.

Prof DS. Kumar, Ph. D., PDF.,

(Supervisor)

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help and constant support throughout my research work.

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ABSTRACT

The objective of this study is to perform a thorough literature review of the polycarpaea plant to verity whether any scientific data is available on this plant for its anticancer activity and to evaluate the anticancer activity, its in vitro cytotoxicity, in vivo anticancer activity, in vivo antioxidant and lipid peroxidative effect.

Anti cancer activity

Materials and methods

It was an experimental study

Sollection and identification of plan

Preparation of various extracts of the whole

Plant of the polycarpaea corymbosa. Lamk

Preliminary phytochemical screening of the crude Extracts

In vitro antixodant activity

Acute toxicity study

Anti cancer activity

RESULTS

The whole plant of polycarpaea corymbosa was powdered and extracted with solvents of increasing polarity and were concentrated and the percentage yields calculated. The calculated percentage yield obtained were 9.87%w/w, 7.8%w/w and 16.56%w/w (Table 1) respectively for petroleum ether, ethyl acetate and ethanol extract. An increase in the percentage yield in a particular solvent indicates that more amount of active constituents or phyticonstituents are being present in the particular extract. In the present study was carried out to evaluate potential of various extracts of whole plant polycarpaea corymbosa on EAC induced tumor in mices. The EAC induced experimental carcinogenesis light therefore be used as an ideal method to evaluate the chemo preventive potential of medicinal plant and its active constituent Oxidative damage to cellular biomolecules such as lips, proteins and DNA is thought to play a crucial role in incidence of several diseases. Flavonoids are a group of polyphonic compounds found abundantly in the plant kingdom

CONCLUSION

Plant-based medicine plays a vital role in cancer management, and 600/0 Of anticancer drugs are derived from plant resources. in the present investigation, entitled "Study on anticarcinogcnic activity of medicinal plant-Polycarpaea Lamk", the whole plant of Polycarpaea corymbosa was screened for anticancer activity

CHAPTER I:
1 INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result death. The commono threat in all known cancers is the acquisition of abnormalities in the genetic material of the cancer cell in progeny.

A tumor may be benign (not cancerous) or malignant (cancerous).

A benign tumor may grown larger, but it does not spread to other parts of the body. It may cause serious problems, a blockage or appear unusual. But is usually can be removed and does not grow back. A malignant tumor is cancerous. The rumor invades nearby parts of the body. It can spread to others parts of the body forming new tumor (metastasis) in malignant tumors, the cells growing completely out of control.

Cancer may affect people at all ages. Even fetuses are at the risk, but the more common varieties of cancer tends to increase with age[1]. In the United States and the other developed countries, cancer is presently responsible for about 25% of all deaths[2]. On a yearly basis, 0.5% of the population is diagnosed with cancer.

CAUSES

Cancer is caused by both external factors (tobacco, chelicals, radiation and infections organisms) and internal factors (inherited mutations, hormones, immune conditions and mutations that occur from metabolism)[3]. The contribution of genetic factors and environmental factors towards cancer is 5-10% and 90-95% respectively. These causal factors may act together or bin sequence to initiate or promote carcinogenesis. The causes of cancer are discussed below in detail[4].

A. EXTERNAL FACTORS

- ✓ Chemical carcinogens
- ✓ Lionizing radiation
- ✓ Infectious diseases

B. INTERNAL FACTORS

- ✓ Heredity
- ✓ Hormonal imbalances
- ✓ Immune system dysfunction

II TYPES OF CANCER

✪ **Adult cancers**

Prostate cancer, lung cancer, colorectal cancer, bladder cancer, cutaneous melanoma prostate cancer, leukemia, breast cancer, ovarian cancer and non-hodgkins lymphoma.

✪ **Childhood cancer**

Wilm's tumor, lymphomas, rhabdomysosarcoma, teratoma, retinoblastoma, osteosarcoma and Ewing's sarcoma.

III GENERAL SYMPTOMS

Cancer symptoms can be divided into 3 groups.

- **Local symptoms**
  Unusal lymph's or swelling, hemorrhages, pain, ulcer and jaundice.
- **Symptoms of metastasis**
  Enlarged lymph nodes, cough and hemotysis, hepathomegalyu, severe in bones.
- **Systemic symptoms**
  Loss of weight, poor appetite, excessive sweating, thrombosis and hormonal changes.

EHRLICH ASCITES CARCINOMA

Ehrlich ascites carcinoma (EAC) is one of the experimental breast rumor derived from spontaneous mouse adenocarcinoma.

Intraperitoneal injection of the rumor emulsion produces ascites5. Similar to other tumors developing in body cavities. EAC cells fill the accumulation pf a fluid named ascetic fluid is also observed. On the 14th day of intraperitoneal injection of EAC cells, almost all animals showed a moderate to **marked** abdominal distention. At this time there large and small solid tumors in the abdominal wall, peritoneal surfaces of the liver, spleen, diaphragm, intestine, retroperitoneal space, pelvic cavity and around the pancreas, and cancer in the mesentery, omentum, and lymphnodes and metastases in lungs.

With the growth of the tumors the amount of the fluid increases and as a consequence, due to the pressure induced by tumor cells and the ascetic fluid and cause damage to the organism, animal dies following 17-18 days of EAC transplantation[6.7].

IV TREATMENT

In general surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods were used for the management of cancer. The option of therapy depends upon the location and grade of the tumor and stage of[8] the disease, as well as the general state of the patient. Complete removal of the cancer without damage to the rest of the body is the goal of treatment[9].

1. Surgery

The advanced stage cancers were cured by surgery, but recurrent is possible. Staging is a major determinant of prognosis and of the need for adjuvant therapy. Infrequently surgery is essential to control symptoms, such as spinal cord compression or bowel obstruction.

2. Radiation therapy

In radiation therapy the cancer cells were destroyed by ionizing radiation. The radiation therapy can be executed by external beam radiotherapy (EBRT) or insertion of radioactive implants directly into the tissue. The effects of radiation therapy are localized and confined to the region being treated. Radiation therapy injures or destroys cells in the area being treated (the ‘’target tissue’’) by damaging their genetic material, making it impossible for these cells to continue to grow and divide[10]. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. Hence, it is given in many fractions, allowing healthy tissue to recover between fractions. Radiation therapy may be used to treat almost every type of solid tumor, including cancers

of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin stomach, uterus, or soft tissue sarcomas.

Radiation is also used to treat leukemia and lymphoma[11].

3. Targeted therapy

In targeted therapy the proteins of cancers cells were deregulated. Small molecule targeted therapy drugs are generally inhibitors of enzymatic domains on mutated, over expressed or otherwise critical proteins within the cancer cells. Examples include the anti HER2/new antibody trastuzumab Herceptin used in breast cancer, and the antiCD20 antibody rituwimab, used in a variety of B-cell malignancies.

Photodynamic therapy (PDT) is a ternary treatment for cancer involving a photosensitizer, tissue oxygen, and light (often using lasers). PDT can also be useful in removing traces of malignant tissue after surgical removal of large rumors[12].

4. Immunotherapy

Cancer immunotherapy refers to a diverse set of therapeutic strategies designed to induce the patient's own immune system to fight the tumor. Contemporary methods for generating an immune response against tumors include intravesical BCG immunotherapy for superficial bladder cancer, and use of interferon and other cytokines to induce an immune response in renal cell carcinoma and melanoma patients[12].

5. Hormonal therapy

The hormones were inhibits the growth of certain cancers. Common examples of hormone-sensitive tumors certain types of breast and prostate cancer. Removing or blocking estrogen or testosterones often an important additional treatment. In certain cancers, administration of hormone agonists such as progesterone may be therapeutically
beneficial.

IV.1 CHEMOTHERAPY

Chemotherapy is the management of cancer with anticancer drugs. These agents were directly interfering with cell division pathways. Most forms of chemotherapy target13 all rapidly diving cells and are not specific for cancer cells (example: Cyclophosphamide, cisplatin). Although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while normal cells generally can. Hence, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g., intestinal lining). These cells usually repair themselves after chemotherapy. The majority of chemotherapeutic drugs can be divided in to: alkylating agents, antimetalibolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other

anti tumour agents. All of these drugs affect the cell division or DNA synthesis and have unique functions.

o **Alkylating agents**

Alkylating agents are destroying the cancerous cells DNA directly. As a class drugs, these agents are not phase-specific, in other words, they work in all phases of cell cycle. Alkylating agents are used to treat various cancers. These agents are damage the bone marrow (5 to 10 years) and cause acute leukemia with dose dependent manner. There are many different alkylating agent, including, nitrogen mustards, nitrosoureas, alkyl sulfonates, triazines, ethylenimines. The platinum drugs are sometimes grouped with alkylating agents because they kill cells in a similar way. These drugs are less likely than the alkylating agents to cause leukemia.

- **Antimetabolites**

These agents damage cells during the S phase and interfere DNA and RNA growth. They Antimetabolites were used for the management of various cancer, but it causes severe toxicity to normal cells too. Examples of antimetabolites include 5-fluoroucaril (5-FU). Capecitabine, 6-meracaptopurine (6-MP), methotrexate, gemcitabine, cytarabine, and fludarabine.

- **Anti-tumor antibiotics**
  - **anthracyclines**

These agents are interfering DNA replication process. These agents work all phases of the cell cycle. Thus, they are widely used for a variety of cancers. In higher dose it produced permanent heart damage. Examples of anthracyclines include doxorubicin, aclacinomycin A and ditrisarucin B. Mitoxantrone is an anti-tumor antibiotic that is similar to doxorubicin in many ways, including the potential for damaging the heart. This drug also acts as a topoisomerase II inhibitor and can lead to treatment related leukemia. Mitoxanthine is used to treat prostate cancer, breast cancer, lymphoma, and leukemia.

- **Topoisomerase inhibitors**

Topoisomerase II inhibitors are blocking the topoisomerase enzyme, which helos to separate the strands of DNA so they can be copied. The topoisomerase are used for management of leukemia, lung, ovarian, gastrointestinal and other cancers. Examples of topoisomerase I inhibitors include topotecan and irinotecan (CPT-11). Example of topoisomerase II inhibitprs includes etposide (VP-16), teniposide and mitoxantrone. Treatment with topoisomerase II inhibitors incancers the risk of a second cancer acute myelogenous leukemia.

- **Mitotic inhibitors**

The mitotic inhibitors were obtained from medicinal plants. An example of mitotic inhibitors from making proteins needed for cell reproduction. These drugs work during the M phase of the cell

cycle, but can damage cells in all phases. They are used to treat many different types of cancer including beast, lung, myelomas, lymphomas, and leukemia. These drugs are known for their potential to cause peripheral nerve damage, which can be a dose-limiting side effect.

- **Corticosteroids**

Steroids and steroidal analogues are useful for the management of lymphoma, leukemia and multiple myeloma. When these drugs are used to kill cancer cells or slow their growth, they are considered as chemotherapy drugs. The chemotherapy nausea and vomiting prevented by corticosteroids. Examples include methylprednisolone and dexamethasone.

IV.2 SIDE EFFECTS OF CHEMOTHERAPY

Most of the chemotherapeutic agents have been reported to exhibit cytotoxic in normal cells, accompanied by undesirable side effects. Important common side effects includes nausea and vomiting, diarrhea or constipation, anemia, malnutrition, memory loss, depression of the immune system, hence (potentially lethal) infection and sepsis, hemorrhage, secondary neoplasms, cardiotoxicity, hepatotoxicity, mutagenic and carcinogenic. Therefore, the substitute of the conventional chemotherapeutic agents to control the high mortality rate are needed which will be highly

effective at non-toxic doses and inexpensive and accessible to general people[14]. This can be achieved by screening of new molecules or natural agents with antitumor activity. Reports revealed the plant-derived extracts containing antioxidant principals showed cytotoxicity towards tumor cells[15]. Majority of the disease conditions including cancer can be effectively treated by using medical plants having antioxidant property[16].

Despite advances in understanding the molecular basis, diagnosis and treatment of this fatal disease over the past decades, this malignancy remains elusive. Therefore, the identification of new and efficient anticancer drugs has always been a focal point in cancer research[17.18].

IV.3 ANTIOXIDANTS

A free radical is a highly reactive molecule or molecular fragment that contains one or more unpaired electrons in its outer orbit and is capable of independent existence. Free radical are generally of two types: “reactive oxygen species”, (ROS) and “reactive nitrogen species” (RNS). Reactive oxygen species (ROS) are involved in a variety of important pathophysiological conditions including mutagenesis and carcinogenesis. Free radicals play an important role in tumor promotion by direct chemical reaction or alteration of cellular metabolic processes, and

their scavengers (SOD, CAT, etc.) represent inhibitors at different stage of carcigenesis. The enzymes are found in cytosolic and mitochondrial functions mainly involved in the biotransformation and detoxification of carcinogens. The continuing severity and magnitude of the cancer problems make it imperative to develop chemopreventive strategies utilizing natural antioxidants to block the initiation or arrest, or reverse the progression of pre-malignants cells. Antioxidants may protect against the toxicity of reactive oxygen species (ROS) by the prevention of ROS formation and neutralization of oxygen-free
radicals[19].

Antioxidants have the potential to prevent these oxidative damages and thereby minimize the homeostatic disturbances, by interfering with the oxidation process by reacting with free radicals, chelating catalytic metals or by acting as oxygen scavengers[25]. Butylated hydroxy Tolouse (BHT), Butylated hydroxyl anisole (BHA) and propyl gallate are used as antioxidants. However, some of these agents are, now-a-days, being for liver damage and carcinogenesis[26.27]. Therefore there is a growing interest in naturally occurring potential antioxidants, especially from plant origin. studies to shown that various common fruits and vegetables contain different promising antioxidants compounds such as Vitamin E. vitamin C, Carotenoids as well as

flavonoids trans and other polyphenolic constituents[28.29].

Despite the latest advances in medical sciences, and progress in strategies of cancer treatment, cancer currently remains a tragic disease and is one of the major causes of death worldwide. The principal methods of cancer treatment include chemotherapy radiotherapy and surgery. Chemotherapy is a systemic treatment, to which the whole body is exposed. Among the most successful chemotherapeutic agent are Cisplatin. Mitomycin and Docetaxel. All of these agents enhance serious side effects or long term complication[30.32]. These side effects include kidney damage, hearing loose, lower blood count, liver damage derve damage, and blood vessel damage[33.34].

External beams of Radiotherapy are associated with unacceptably high levels of local-regional toxicity[34]. Particularly, it affects the rapidly dividing cells of mucosa, causing initiative urinary and blood loss. Later toxic effects result from damage to the more slowly proliferating cells such as fibroblasts, endothelial, or parenchymal stem cells causing chromic fibrosis and vascular damage. Other undesired side effects such as immune suppression, bon necrosis, lung fibrosis and skin

devascularization are seen with all types of conventional therapies[35].

Plants are the chief source of natural products that are used in medicine. Even Aspirin, the world best known and most universally used medication, has its natural origins from the glycoside salicin which is found in many species of the plant genera Salix and Populus[36].

The scientific literature is rich in epidemiological studies that support significant differences in the occurrence of cancers between oriental and occidental populations[37]. Generally, populations that consume a high level of natural herbal products have a reduced incidence of cancer. An example is the low incidence of colon cancer in Asian countries with high consumption of soyabean products. Soyabeans are the major dietary source of saponins, which have been suggested as possible anticancer agents[38].

There is lately a great interest in screening of plants cancers prevention and treatment. The review of literature revealed that the whole plant of Polycarpeaa corymbosa. Lamk was used in Indian traditional medical system in inflammatory swellings and in the treatment of jaudice[39.40], lever diseases41, antimicrobial activity42, identification of stigmastanol[43] and flavonoids[44]. The literature

review has shown that anticancer activity has boot been carried on the whole plant Polycarpaea dorymbosa. Lamk and hence it was selected for the present investigation.

CHAPTER 2:
AIM AND OBJECTIVE OF THE STUDY

Natural products from medicinal plants are found to be safe and effective. Many plant species have been used in folkloric medicine to treat various ailments. The objective of this study is to identify suitable plant with anticancer activity and screen the chosen plant for anticancer activity.

There are several plants rich in phytoconstituent having potential antioxidant activity. Out of these polycarpaea corylbosa belonging to Caryphyllaceae family is a plant which is easily available in south Indian. The preliminary review of literature indicates that plant is rich in antioxidant phytochemicals. The whole plant of polycarpaea corymbosa also has a traditional and folklore claim of anticancer activity.

Hence, the aim and objective of this study is to perform a thorough literature review of this plant to verity whether any scientific data is available on this plant for its anticancer activity and then to evaluate the plant for anticancer activity.

The plant was evaluated in vitro antioxidant activity, in vitro cytotoxicity, in vivo anticancer activity, in vivo antioxidant and lipid peroxidative effect. It is also aimed to isolate the

phytoconstituents from the active extract and screen them for in vitro cytotoxicity antioxidant and anticancer activity.

CHAPTER 3:
REVIEW OF LITERATURE

The literature review comprises some of medicinal plants which have been reported to possess anticancer activity and also covers various phytochemical and pharmacological studies on the whole plant of polycarpaea corymbosa.

3.1 MEDICINAL PLANTS WITH ANTICANCER POTENTIAL

The existing natural anticancer agents such as Vinblastine and vincristine were isolated from Catharanthus roseus G are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemia lymphomas, advanced testicular cancer, breats and lung cancers and Kaosi's sarcoma45.

The discovery of paclitaxel (Taxol) from the bark of the Pacific Yew, Taxus brevifolia Nutt, is another evidence of the success in natural product drug discovery, Paslitaxel is significantly active against ovarian cancer, advanced breast cancer, small and non-small cell ling cancer 46.47.

Camptothecin, isolated from the Chinese ornamental tree Camptotheca acuminate deene, was

used in clinical trials by NCI in the 1970s, but was dropped because of severe bladder toxicity[48]. Topotecan and irinotecan are semi-synthetic derivatives of camptothecin and are used for the treatment of ovarian and small cell lung cancer and colorectal cancer respectively[49].

Etoposide and teniposide derivatives of epipodophyllotoxin were other natural anticancer agents used for the management of various cancers such as, bronchial and testicular cancers[50].

A racemic mixture of harringtonine and homoharringtonine isolated from the Chinese tree Cephalotaxux harringtonia var. drupacea has been used successfully in China for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia[51].

Elliptinium, a derivative of ellipticine, isolated from a Fijian medical plant Bleekeria A.C. Sm., is marketed in France for the treatment of breast cancer[52].

The antitumor of RC-18, isolated from Rubia cordifolia was repeadly tested as spectrum of experuimental murine tumors, viz P388. L1210, L52178Y, B16 melanoma lewi lung carcionoma and sarcoma 180, RC 18 exhibited significant increase in life span of …leukemia P388, L1210, L51178Y and solid tumor B16 melanoma. However, it failed to

show any inhibitory effect on solid tumor, lung carcinoma and sarcoma180. Promising results against a spectrum of experimental tumors suggest that RC-18 may lead to the development of a potential anticancer agent[53.54].

The antitumor activity of methanol extract of Centella asiatica and its purifier fractions tra column chropatography was investigated by both in vitro and models. Methanolic extract of Centella asiatica (100 ug/ml) showed 100% cytotoxicity to tumor cell tines (Dalton's ascites tumor cells and Ehrich ascites tumors cells) after 3h incubation at 37°C. Acetone fraction (3.5 & 8ug/ml) inhibited the proliferation of mouse lung fibroblast cells after exposure to 6-7 days at 37°C was reported[55].

The 80% hydro alcoholic extract of Andrographis paniculata has been investigated for chemo preventive potential at the dose of 50 and 180mg/kg/day for 14 days. It was found to possessed anticarcinogenic activity[56].

The ethanol extract of neem leaves on oral administration inhibited the DMBA induced hamster buccal pouch carcinogenesis as revealed by the absence of neoplasm. These result suggest that

chemo preventive effect of ethanolic extract of neem leaves may be mediated by induction of apoptosis[57].

The plant extract of curcumin can interfere the cell growth cycle of A549 cell and suppress cell growth. Suppression effect is concentration dependent and the effect depends not only from the nonspectific cytotocity but also from induced cell apoptosis. The cell proliferation was obviously suppressed after treated with different concentrations of curcumin for 72hrs. The IC50value was found to be 18μmol/l using linear regression[58].

The methanol extract of leaves and its fraction of Aesculus indica were investigated by cytotoxic activity. N-hexane, chloroform, ethyl acetate, methanol and aqueous extract was investigated againt breats adenocarcinoma cell lines (MCF-7). The cell viability was inhibited by Aesculus indica I a dose dependent manner. Methanol and aqueous extract were found to possess good cytotoxic potential[59].

One of the most popular beverages Camellia sinensis (Tea) consumption has been associated with decreased risk of developing cancer like ovary[60], oral cavity[61], stomach[62], colon[63] and prostrate[64] was studied. This benefical effect has been attributed of the catechins flavonoids) in tea[65]. Their biological benefits are due to their strong antioxidant and

angiogenic as well as inhibit the cell proliferation and modulate carcinogen metabolism[66.67].

The fruit critrus (Nimbu) contains flavonoid, flavone, limonene, nobiletin and magretin. The nobiletin, flavonoid and tangeretin are potent inhibitors of tumor cell growth and activate the detoxifying P450 enzyme system. Limonoids inhibit the tumor formation by simulating the GST enzyme and limonenes also possess anticancer activity. inhibition of treast cancer cell proliferation and delaying of mammary tumorogenesis was reported from nambu fruit. it is alsoused in leukemia and metastasis.

Bark of prunus spp[69], leaves of martynia annusa[70] and stem of Rhaphido phorapertusa have been studied by anticancer activity. These plants have used against lung, neck and abdominal cancers respectively.

3.2 REVIEW IN POLYCARPAEA CORYMBOSA LAMK

The free radical scavenging activity of leaves of Polycarpaea corymbosa (Family Caryophyllaceae) was studied. The aqueous extract of Polycarpaea corymbosa has showed highest antioxidant activity[71].

The antioxidant and antiradical activity of hydro-alcoholic extyract of polycarpaea corymbosa

was investigated by different in-vitro methods. IC_{50} values of Polycarpaea corymbosa were found to15.10±0.10, 2017.96±32.77, 134.47±21.39, 18.03±3.22, 48.73±3.39 µg/ml in DPPH radical scavenging and oxide scavenging, β-carotene linoleate model system, hydroxyl radical scavenging and anti-lipid peroxidation activity respectively[44].

The various extract like petroleum ether, chloroform, acetone and methanol extracts of polycarpaea corymbosa whole plant was ingestigated for antimicrobial activity against bacteria and six fungus. The pathogens like Streptococococus faecalis, S.pyogenees facalis. Bacillus subtillis, B.thuringiensis, Staphylococococus aureus, Serratia , klebsiella pneumonia, Proteus vulgaris, Salmonella parathypic, S.paratyphi A, S.paratyphi B, Pseudonomas aeuroginosa and Escherichia coli and fungus cultures of pascilomyceslilacinus, Mucur spp, Azospirillum lipofereum, Verticillum lecanii, Candida and Penicillium spp. The acetone and methanolic extracts showed considerably good antibacterial and antifungal activity[72].

The anti-inflammatpry activity of whole plant of ethanolic extract of Polycarpaea corymbosa was evaluated in rats using a carrageenan induced paw edema. Ethanol extract inhibits potent anti-inflammatory activity at 500mg/kg[73].

The phytoconstitutent of ethanolic extract of Polycarpaea corymbosa was studied on using GC-MS. A total of 15 compounds were isolated by GC-MS method. Furazano [3,4-b] pyrazine -5(4H)-one, 6-(1pyrrolidinyl)-, 1, (2-Acetoxyethym)-3, 6-diazahomoadamantam-9- one oxime, cycloarbital, etc was identitifed[74].

The antioxidant properties of Polycarpaea corymbosa were evaluated. The various extracts possessed good 1.1-diphenyl-2pircylhydrazyl (DPPH), hydroxyl, superoxide and ABTS radical scavenging activity with IC5028.04.26.93.31.85 and 29.83µg/ml75.

The anti-nociceptive activity of methanolic arial extract of Polycarpaea corymbosa was studied. The methanolic aerial extract of Polycarpaea corymbosa (200mg/kg b.wt) has shown significant analgesic activity than root. The results indicated that the analgesis effect of polycarpaea corymbosa methanolic extract is both peripherally and centrally significant[76].

The quantitative analysis of secondary metabolites from Polycarpaea corymbosa studied on HPLC. A total of types of flavonoids and thre types of steroids were found in the methanolic extract of ethanol of Polycarpaea corymbosa[77].

Hepatotective activity of ethanol extracts of Polycarpaea corymbosa was studied in albuno rats which were given carbon tetra chloride to induce hepatotoxicity. Significant incrasse in serum total protein, superoxide dismutase, catalase, redueced glutathione and plutathione peroxidase was observed in iextract treated groups when compared to control group[78].

The anti-diarrheal and anticancer properties of polycarpaea corymbosa were evaluated. The various extracts possessed significant aint-diarrheal and anticancer activity against experimental animals[79.80].

Fertility enhancement activity of ethanol extract of whole plant of Polycarpaea corymbosa was studied in male albino rats[81].

Morecover, the whole parts of Polycarpaea corymbosa were used for the management of inflammatory swellings, uncler and jaundice in Indian traditional medicinal system[82].

From the review of literature it was found that the whole plant of Polycarpaea corymbosa have not so far been subjected to any scientific studies for their anticancer potential. Hence this plant will be subjected to a series of tests to evaluate them for anticancer activity.

Figure 1: Polycarpaea corymbosa Lamk. Plant

3.3 PLANT PROFILE

Polycarpaea cirymbosa Lamk.

Botanical source : Polycarpaea corymbosa Lamk
Family Name : Caryophyllaceae
Synonyms : Achyrathus corymbosa
: Polycarpaea nebulosa[83]
Common Name : Old man's cap

Ternacular Names[83]:
Eng : Old man's cap
Sankrit : Bhisatta, Okharadi, Parpata
Hindi : Bygyale
Malayalam : Katu – mailosina
Tamil : Nikasedachi, Nilachadachi, Pallippuntu
Telugu : Rajuma, Bommasari
Chinese : Bai Gu Ding

Taxonomic classification[83]
Kingdom : Plantae
Phylum : Tracheophyta
Class : Magnoliopsida
Order : Caryphyllales
Family : Caryophyllaceae
Genus : Polycarpaea

Species : Polycarpaea corymbosa

Coryphyllacea is one of the major dicot family of agniosperms and is globally represented by 85 genera and 2,680 species. This family is popular known as the pink family (or) carnation family[84].

Description[85]:

It is an annual or perennial herb leaves are opposite or appear in whorls ? lenear upto 3.5cm long with a brittle at the tip. Flowers are borne in compact heads at the end of stems. Sepals are silvery white, turning rich brown with age. Petals are small, pink to orange. Fruit is a minute ellipsoid capsule.

Flowering: August – September

Part Used: Whole Plant **Habitat:**

On sandy soils on pen woolland and grassland sometimes as a weed of cultivated ground.

Distribution[85]:

It is found throughout India, Ceylon, Burma, Western Peninsula and ascending the western Himalayas to 7000 feet.

Chemical Constituents[86]:

Alkaloids Coumarine, Glycocytes, Saponins, Steroids, Phenols,

Tannins and Wanthoproteins. Polycarpaea corymbosa from which a – 1 – barrigenol camellia genin A and sigma sterol have been isolated.

Exhanomedical Uses[85]:

- ✪ Herb – Administered both internally and externally as remedy for venomous bites from reptiles and of animals;
- ✪ Pouded leaves: used cold (or) warm as poultice over boils and inflammatory swellings, used for bites from animals and givenwith molasses in form of a pill in jaundice; Roots for, liver complaints.

Other species:

P. hayoides, P. spicata, P. akkesis, P. cespitosa, P. divaricate, P. latifolia, etc.

CHAPTER 4:
SCOPE AND PLAN OF WORK

From the extensive review of literature it was found that the whole plant of polycarpaea corymbosa. Lamk not been subjected to any scientific studies for their anticancer potential so far. Hence the plant will be explored for anticancer activity by subjecting them to a series of tests.

4.1 PLAN OF WORK

1. Collection, identification and authentication of plant materials
2. Successive Soxhlet extraction of the whole plant of Polycarpaea corymbosa. Lamk (Pet ether, ethyl acetate and ethanol)
3. Phytochemical screening of the extracts obtained from successive extraction of the whole plant of Polycarpaea corymbosa. Lamk.
4. Evaluation of in-vitro antioxidant activity of different extracts of
   Polycarpaea corymbosa. Lamk by
   - DPPH free radical scavenging assay
   - Hydroxyl radical scavenging activity
   - Nitric Oxide free radical scavenging activity
   - Superoxide anion scavenging activity

5. Estimation of phenom and flavonoid content in
6. Evaluation of in-vitro cytotoxicity of various extracts of Polycarpaea corylbosa. Lamk using various cell lines by MTT assay.
7. Evaluation of in-vitro anti-caricogenic activity.
    - Acute toxicity study by OECD guidelines 423

- To estimate boby weight, mean survival time, percentage in life span, tumour volume, PCV and tumour cell count in mice treated with EAC
- Ro study the haematological Parameters such as

- RBC
- Hb
- WBC
- Differential count.

- To study the Lysosome specific cancer marker enzymes such as

- Cathepsin – D
- β-D-Glucuronidase
- Acid Phosphatase

- To study the Liver marker enzymes

- 5' – Nucleotidase
- Lacate dehydrenase

- ✪ Na^+ / K^+ AT Aase
- ✪ Mg^{2+} - ATPase
- ✪ Glycogen
- ✪ Glucose
- ✪ DNA
- ✪ RNA

➢ To study the effect of lipid peroxidation activity of various extracts of Polycarpaea corymbosa. Lamk.

➢ To assess th in-vitro antioxidant potential of various extracts of Polycarpaea corymbosa. Lamk in EAC treated mice by estimating

- ✪ Superoxide dismutase (SOD)
- ✪ Catalase (CAT)
- ✪ Glutathione peroxidase (GPx)
- ✪ Vitamin C & E
- ✪ Proteins
- ✪ Glutathione (GSH)

➢ To study the histopathological changes of various extracts of Polycarpaea corymbosa. Lamk.

8. Isolation of the active ingredients from ethanolic extract of Polycarpaea corymbosa. Lamk by column chromatographic method.
9. Identification and characterization of the isolated compounds by FT-IR, ^{13}CNMR, ^{1}HNMR and mass spectroscopy

10. Assessment of in- vitro antioxidant and in-vitro cytotoxicity of isolated compounds from rhe ethanolic extract of Polycarpaea corymbosa. Lamk
11. Studies on p53 gene expression of isolated compounds by using flow Cytometry.

CHAPTER 5:
MATERIALS AND METHODS

5.1 SOLLECTION AND DENTIFICATION OF PLANT

The whole plant of Polycarpaea corymbosa Lamk was collected in the month of august and September from Palayamkottai, Tirunelveli, Tamil Nadu, India. The taxonomic identification of the material was authenticated by Dr. GVS. Murthy, Ph.D., Scientist F & Head, Botanical Survey of India. Southern Regional centre, Coimbatore, India. The voucher specimen deposited in the department of the future reference (Voucher no: BSI/SRC/5/23/2013-14/Tech/551). The plant material was dried under shade, segregated, pulverizd by a mechanical grinder and passed through à 40 mesh sieve. The powedered plant material was stored in airtight containers and used for further research.

5.2 PREPARATION OF VARIOUS EXTRACTS OF THE WHOLE PLANT OF POLYCARPAEACORYMBOSA. LAMK

About 2 kg of air-dried powedered material from the whole plant was successively extracted by hot continuous percolation method in soxhlet

apparatus[87], using solvents of mereasing polarity like petroleum ether, ethyl acetate and ethanol for Polycarpaea corymbosa Lamk. The extract so obtained were filtered were filtered through Whatman filter paper N°. 40 and the solvents were then recovered from the extracts under reduced pressure using rotary ewaporator. The concentrated filtrate was then evaporated to dryness in vacuum at 35°C to15°C. The extracts were then stored in screw capped vials at 4°C until further use. The extract trained with each solvent was weighed and the percentage was calculated in terms of dried weight of the plant material. The colour and consistency of the extract were also noted. All the solvent used for this entire work were of analytical reagent grade (Merck, Mumbai). The percentage yield was calculated for the extract with reference to the crude taken and the extractive values for dofferent solvents were calculated.

5.3. PRELIMINARY PHYTOCHEMICAL SCREENING OF THE CRUDE EXTRACTS

Qualitative analysis refers to establishing and providing the identity of substance. The phytoconstituent present in the plants are responsible for the therapeutic properties of the plant and

therefore the pharmacological actions of crude drugs are determined by the nature of their constituents. Hence the prepared extracts were subjected to preliminary phytochemical screening for the revealing of various plant constituent present in them.

The various extracts of whole plant of Polycarpaea Corymbosa Lamk were subjected to the following chemical tests separately for the identification of various active constituents[88].

I. Tests for Alkaloids[89]

1. Dragondroff's Test: To 1ml of the extract, 1ml of Dragondroff's reagent was added formation of orange red precipitate indicated the presence of alkaloids.
2. Wagner's Test: To 1ml of the extract, 2ml of Wagner's reagent was added, the formation of a reddish brown precipitaye indicated the presence of alkaloids.
3. Mayer's Test: To 1ml of the extract, 3ml of Mayer's reagent was added, the formation of full white precipitate confirmed the presence of alkaloids.

Hager's Test: To 1ml of the extract, 3ml of Hager's reagent was added, the formation of yellow precipitated confirmed the presence of alkaloids.

II. Tests Carbohydrates[90]

1. Molisch Test: To 2ml of the extract, 1ml of α-naphthol solution was added, and concentrated sulphuric acid through the sides of test tube. Purple or reddish violet colour at the junction of the two liquids revealed the presence of carbohydrates.
2. Fehling's Test: To 1ml of the extract, equal quantities of Fehling's solution A and B were added, upon heating formation of brick red precipitate which indicated the presence of carbohydrates.
3. Benedict's Test: To 5ml of benedict's reagent, 1ml of extract solution was added and boiled for 2min and cooled. Formation of a red precipitate showed the presence of carbohydrates.

III. Test for Proteins and Amino Acids[91]

1. Biuret Test: To 1ml of the extract add 1ml of 40% sodium hydroxide solution was added followed by 2 drops of 1% copper sulphate solution Formation of a violet colour showed the presence of proteins
2. Xanthoprotein Test: To 1ml of the extract 1ml of concentrated nitric acid was added. A white precipitate is formed, it is boiled and cooled, 20% of sodium hydroxide or ammonia is subsequently added; orange colour indicated the presence of aromatic amino acids.
3. Lead Acetate Test: To the extract, 1ml of lead acetate solution is added. Formation of white precipitate which indicated the presence of proteins

Ninhydrin Test: 2 drops of frechly prepared 0.2% ninhydrin reagent was added to the extract solution and heated. Development of blue colour revealed the presence of proteins, peptides or amino acids.

IV. Test for Phytosterols[93]

1. Libermann Burchard Test: The extract was dissolved in 2ml of chloroform in a dry test tube. 10 drops of acetic anhydride and 2 drops of conc, H_2SO_4, were added. The solution became red, then blue and finally formed bluish green color, indicated the presence of steroids.
2. Salkowski Test: The extract was dissolved in chloroform and equal volume of concentrated sulphuric acid was added. The formation of bluish red to chrtry red colour in chloroform layer and green fluorescence in the acid layer represented the steroid components.

V. Tests of Glycosides[88]

1. Legal Test: The extract was dissolved in pyridine and sodium nitroprusside solution was added to make it alkaline. The formation of pink red to red colour showed the presence of glycosides.
2. Baljet Test: To 1ml of the extract, 1ml of sodium pricrate solution was added and the yellow to orange colour revealed the presence of glycosides.
3. Borntrager's Test: A few ml of dit. HCI was added to 1ml of the extract solution. It was then boiled, filtered and the filtrate was extracted with chloroform. The chloroform layer showed with 1ml of ammonia. The formation of red

colour in the aqueous layer showed the presence of anthraquinome glycosides.

Keller Killiani Test: The extract was dissolved in acetic containing traces of ferric chloride and was then transferred to a test tube containing sulphuruc acid. At the junction, formation of a reddish brown colour, which gradually turns blue, confirmed the presence of glycosides.

VI. Test for Saponins[87]

1. About 1ml of ethanol extract was diluted separately with distilled water ro 20ml, and shaken in a graduated cylinder for 15min, 1cm layer of foam indicated the presence of saponins.

VII. Test for Flavonoids **1.**

Shinoda Test: To 1ml of the extract, magnesium turnings were added followed by 1-2drops of concentrated hydrochloric acid. Produced red color which showed the presence of flavonoids.

VIII. Test for tannins and Phenolic compounds[94]

1. To 1ml of the extract, ferric chloride was added, formation of a dark blue or greenish black colour product showed the presence of tannins.

2. To the extract, potassium dichromate solution was added, formation of a precipitate showed the presence of tannins and phenolic compounds.

IX. Test for Triterpenoids

1. 1ml of the extract was added into the test tube containing two or three granules of tin metal in 2ml thionyl chloride solution. The formation of a pink colour indicated the presence of triterpenoids.

X. Test for Fixed Oils

1. Spot Test: A small quantity of extract was pressed between two filter paper. Oil stains on paper indicated the presence of fixed oils.
2. Saponification Test: To 1ml of the extract, few drops of 0.5N alcoholic potassium hydroxide were added along with a drop of phenolphthalein. The mixture was heated on a water bath for 1 to 2h. The formation of soap or partial neutralization indicated the presence of fixed oils.

5.4. IN VITRO ANTIXODANT ACTIVITY

The various extracts petroleum ether, ethyl acetate and ethanol extract of Polycarpaea corymbosa Lamk whole plant were subjected to in vitro antioxidant activity by DPPH method, superoxide radical scavenging activity, nitric oxide radical scavenging activity hydroxyl radicam

scavenging activity and estimation of total phenol and flavonoids.

5.4.1. DPPH photometric assay95Principle

The free radical scavenging activities of the extracts were evaluated by assessing their ability to reduce the colour of DPPH in ethanol according to mensor et al., 2001. DPPH stable free radical methods is an easy, rapid and sensitive way to survey the antioxidant activity of specific compounds or plant extracts. DPPH is a stable free radical with a distinctive ESR signal. Its reaction with antioxidants can be followed by the loss of absorbance at 518nm. It is widely accepted that DPPH accept an electron por hydrogen radical and become a stable diamagnetic molecule. Due to its odd electron, the ethanol solution of

DPPH (purple colour solution shows a strong absorption at 517nm.

DPPH radical with stable reducing agent where the pairing of electron takes place and the solution loses colour stochiometrically with the number of electrons take up.

- DPPH + AH □DPPH – H + A

Instrument

Shimadzu UV visible spectrometer, Model 1800

Fragents

0.4mM diphenyl pircyl hydrazyl radical in methanol

Procedure

A methanolic solution of 0.5 ml of DPPH (0.4mM) was added to 1ml of the different concentration of the plant extract and allowed to react at room temperature for 30min. rutin was used as a erefernce standard. Methanol served as the blank and DPPH in methanol without the extracts served as the positive control. After 30min, the absorbance was measured at 518nm.

The percentage scavenging was calculated using the formula [(Abs $_{Control}$-Abs$_{Test}$) Abs$_{Control}$] x 100. A graph was constructed by plotting concentration versus percentage inhibition and linear regression equation calculated. The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. A triplicate reading was take and average was calculated.

5.4.2. Superoxide radical scavenging activity[96]

Principle

Superoxide radical (02) generated from the photo reduction of riboflavin and it was by nitro blue tetrazolium dye (NBT) reduction method. The measurement of superoxide anion scavenging

activity was perfomed based on the method described by winterbourne et al., 1975.

Instrument

Shimadzu UV visible spectrometer, Model 1800

Reagents

- ✪ 1.5mM nitro blue retrazolium (NBT)
- ✪ 0.1 M EDTA
- ✪ 0.12mM riboflavin
- ✪ 0.067M phosphate buffer

Procedure

The sample was mixed with a solution containing 0.1ml of nitro tetrazolium (0.5mM NBT) solution, 0.2ml of EDTA (0.1M EDTA), 0.05ml riboflavin (0.12mM) and 0.55ml of phosphate buffer (0.067M phosphate). The control tubes were also set up wheterein DMSO was added instead of sample. The reaction mixture was illuminated for 30min and the absorbance at 560nm was measured against the control samples. Quercetin was used as a reference compound.

The percentage scavenging was calculated using the formula $[(Abs_{Control}-Abs_{Test}) Abs_{Control}] \times 100$. A graph was constructed by plotting concentration versus percentage inhibition and linear regression equation calculated. The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. A

triplicate reading was take and average was calculated.

5.4.3. Nitric oxide radical scavenging activity[97]

Principle

Nitric oxides is spontaneously generated from sodium nitroprusside in aqueous solution as physiological pH, then interact with oxygen to turn out nitritre ions, which can be estimated by the use of Griess Illosvery reaction at 540nm by the method of Garrat (1964).

Instrument

Shimadzu UV visible spectrometer, Model 1800

Reagents

- ✪ 10mM sodium nitroprusside
- ✪ 1M phosphate buffered saline
- ✪ Sulphanilic acid reagent (0.33%)
- ✪ Napthylethylene diamine dihydrochloride (1%NEDA)

Procedure

The reaction mixture (3ml) containing 2ml of sodium nitroprusside (10mM), 0.5ml of phosphate buffer saline (1M) were incubated at 25°C for 150min. after incubation, 0?5ml of the reaction mixture containing nitric was pipetted and mixed with 1ml of sulphanilic acid reagent (0.33%) and allowed to stand for 5min for completing diazotization. Then 1ml of

naphthylethylene diamine dihydrochloride (1% NEDA) was added, mixed and allowed to stand for 30min.

The percentage scavenging was calculated using the formula [(Abs $_{Control}$-Abs$_{Test}$) Abs$_{Control}$] x 100. A graph was constructed by plotting concentration versus percentage inhibition and linear regression equation calculated. The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. A triplicate reading was take and average was calculated.

5.4.4 Hydroxyl radical scavenging activity98

Principle

The assay is based on quantification of degradation product of 2deoxy ribose by condensation with TBA Hydroxyl radical was generated by the Fe^{3+} - Ascorbate – EDTA – H_20_2 system (Fenton reaction). The method described by Elizabeth and Rao (1990) was adopted.

Instrument

Shimadzu UV visible spectrometer, Model 1800

Reagents

- 2.8mM deoxyribose
- 0.1mM EDTA
- 1mM hydrogen peroxide
- 20mM KH_2PO_4-KHO

- ✪ 0.1mM ascorbic acid

Procedure

The reaction mixture contained 0.1ml deoxyribose (2.8mM), 0.1ml EDTA (0.1mM), 0.1ml H_20_2 (1mM), 0.1ml Ascorbic acid (0.1mM), 0.1ml KH_2PO_4-KHO buffer, pH 7.4 (20mM) and various concentration of plant extract in a final volume of 1ml. Rutin was used as standard. The reaction mixture was incubated for 1h at 37°C. Deoxyribose degradation was measured as TBARS.

The percentage scavenging was calculated using the formula [(Abs $_{Control}$-Abs$_{Test}$) Abs$_{Control}$] x 100. A graph was constructed by plotting concentration versus percentage inhibition and linear regression equation calculated. The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. A triplicate reading was take and average was calculated.

5.4.5. Estimation of Total Phenol and Flavonoid content in various extracts of whole plant of Polycarpaea corymbosa Lamk

Total phenol[99]

Principle

The total phenotic content was determined by Folins phenol reagent based on Mallick and Singh (1980). All the phenotic compounds are oxidized by

the Folins phenol reagent and the reaction was neutralized with sodium carbonate, which is reduced during predation of the phenotic substances, into a mixture of blue molybdenum and tungsten toxides. The blue colour produced has a maximum absorption at about 650nm. The absorption at proportional to the quantity of oxidized phenolic compounds.

Instrument

Shimadzu UV Visible spectrometer, Model 1800

Reagents

- 80% ethanol
- 0.5ml Folin Phenol reagnt
- Sodium carbonate (20%)

Procedure

To 3ml of extract, 0.5ml of Folin phenol and 2ml of sodium carbonate (20%) were added. The reaction mixture was kept in boiling water bath 1min, the absorbance was measured at 650nm in a spectrophometer. A standard was run by using Gallic acid.

A calibration curve was generated by plotting concentration of gallic acid versus absorbance. A linear regression equation was determined using regression analysis. The total phenol content was calculated using the linear regression equation and expressed in terms mg of gallic acid equivalent per gm of extract (mg GAE/g).

Total flavonoids[100]

Instrument

Shimadzu UV Visible spectrometer, Model 1800

Reagents

- 1% Vanilin in 70% conc, H_2SO_4

Procedure:

0,5ml of extract added with 4ml of the vanillin reagent (1% vanillin in 70% conc, H_2SO_4) was added and the above sample solution kept in a boilin water bath for 15min. the absorbance was read at 360nm. A standard was run by using catechol (110µg/ml). the umount of flavonoids present can be determined by linear regression analysis. The total flavonoid content was expressed as mg catechol equivalents/g of extract.

5.5. In vitro CYTOTOXICITY

In vitro cytotoxicity of various extracts of whole plant of

Polycarpaea corymbosa Lamk

Introduction

Cancer continues to represent the largest cause of mortality in the world and clams aver 6 million lives every year[101]. Drug development programme involve in the preclinical screening of a vast number of chemicals for their specific and nonspecific cytotoxicity against many types of cells. Use of in vitro assay system for the screebing of potential

anticancer drug has been a common practice almost since the origin of cancer therapy in 1946. The National Cancer Institute now regularly measure the growth inhibitory properties of every compound under test against a panel of human cancer celle lines which are representative of major human tumor types. There are numeral advantages in vitro test using cell cultures which include analysis of species specificity, feasibility of using only a small amount of test substance and facility to do mechanistic studies. A novel anticancer drug should possess cytotoxicity at low concentration against cancerous cell lines and should be safe against normal cell lines even at higher concentration[102]. The direct anticancer activity of the plant extracts can be tested under in vitro conditions using cultured or fresh preparation of various tumour cell types. One the activity has been detected, the study has to be followed up vigorously to found therapeutic efficacy and safety.

5.5.1. Human cell lines

Human brest cancer (MCF-7) and human leukemia (HL-60) cell lines were provided by Deshpandey Laboratory, Bhopal, India, HepG2 (human cancerous liver cell lines), HT29 (human colon cancer cell lines), PC3 (human prostate cancer cell lines) were obtained from National Centre for Cell Sciences, Pune, India.

5.5.2. Preparation of test material

Stock solution

DMSO was used for 95% reconstitution of various extracts and stock solutions of 20mg/ml of various extracts were prepared one day in advance. Multiple aliquots of each simple were stored for initial tests and retests, if necessary. Stock solution was filtered and sterilized.

Working test solution

On the day of assay, an aliquout of frozen stock was thawed at room temperature and 100µg/ml concentration of the extract was prepared by seriam dilution of stock solution using the complete growth medium containing 50mg/ml of gentamycin.

Positive control

The positive control used was Doxorubicin (0.01, 0.1, 1, 10, 100 µg/ml).

In vitro Assay for cytotoxic activity

The cytotoxic potential of various extracts of whole plant Polycarpaea corymbosa was determined by using human cancer cell lines. The cell lines were allowed to grown in tissue culture plates in the presence of test material. The cell grown was deliberate on ELISA reader after staining with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2Htetrazolium bromide (MTT)-reagents. The yellow dye MTT converted into a blue formazan product.

Preparation of cell suspension for assay

The desired human cancer cell line was grown in multiple TCFs at 37°C in an atmosphere of 5% in CO2 and 90% relative humidity in a complete growth medium and obtains enough number of cells as per the requirement depending upon numbers of test samples. The flasks with cells at sub-confluent stage were selected for the assay. Cells were barvested by the treatment with Trypsin – EDTA and added to complete growth medium and using to an end the action of trypsin. Cells were detached to single cell suspension by gentle pipetting action and the viable cells were counted in haemocytometer using trypan blue dye. The cell viability at this stage was >97%. The viable cell thickness was adjusted to 5,000-40,000 cells/100µl depending upon the cell line[103]. 100µl of cell suspension mixed with 100µl of complete grown medium was transferred into each well and then the plates were incubate at 37°C for 24h in an atmosphere of 5% CO2 and 90% relative humidity in a Carbon di oxide incubator. After 24h the test sample, DMSO (vehicle control) and positive control was added.

Addition of test materials

Working solutions of the test materials 100µl and positives control was added to equal quantity of

growth medium into the wells in the tissue culture plate. It was prepared 24hours in advance containing either cells or complete growth medium in a final volume of 100µl. the plates were incubated at 37°C for 48hours in an atmosphere of 5% Carbon dioxide and 90% relative humidity. The cell growth was determined after 48hours by MTT assay.

MTT assay[104.105]

The MTT assay was performed according to a slight modification of the procedure reported by Mosman (1983)[104.105]. Cells were cultured in minimum essential medium (MEME) supplemented with glutamine (0.6g/L), gentamicin (25mg/mL) and 100% fetal calf serum at 37°C and in humidified 5% CO2. For experiments, cells plated in 96-well plate (10^5 cells/ well for adherent cells or $0.3X10^6$ cells/ well for suspended cell in 100µL of medium). After 24hours, the extracts (100µg/mL) dissolved in DMSO (1%) was added to each well and incubated for 96 hour. The control groups received the same amount of DMSO. Doxorubicin (100µg/mL) was used as positive control. Growth of tumoral cells was quantified by ability of living cells to reduce the yellow dye MTT to a blue formazan product. A the end of 96 h incubation, the medium in a=each well was replaced by fresh medium containing 0.5 mg/mL of MTT. Four hour later, the formazan

product of MTT reduction was dissolved in DMSO and absorbance was measured at 550 nm. Drug effect was quantitied as the percentage of control absorbance of reduced dye at 550 nm. The experiments were performed in triplicate.

Percent growth inhibition in presence of test material was calculated as follows:

= Growth in presence of test material/Growth in absence of test material X 100

Determination of Activity

More than 70% growth inhibition at the concentration of 100µg/ml is considered to the active.

5.6. ACUTE TOXICITY STUDY

5.6.1. Acute Toxicity studies of various extracts of whole plant of *Polycarpaea corymbosa*

For assessing the safety and toxicity of the drug was studied by using animals like, rats, guinea pigs, dogs and monkeys under varying conditions of drug administration. The preclinical toxicological studies are the major key for fixation of initial dose in humans and supports for clinical trial.

The acute toxicity studies of various extracts of Polycarpaea corymbisa carried as per the Organieation for Economic Co-operation and Development (OECD) guideline number 423) Guidelines106. Depending on the mortality and/or

the morbidity statuts of the animals, an average 2-4 step may be necessary to allow judgment on the acute toxicity of the substance/extracts/ this procedure is reproducible, uses very few animals and is able to Lamk substances/extracts in a similar manner to the other acute toxicity testing method. The acute toxic class method is absed on biometric evaluation with fixed doses, adequatelyt separated to enable a substance to be ranked for classification purpose and hazard assessment. The OECD-423 method uses pre-defined doses and the results allow a substance to be ranked and classified according to the Globally Harmonized System for classification of chemicals which causes acute toxicity.

5.6.2. Acclimatization of Animals

Swiss Albino nice (25-30g) were maintained under standard laboratory condition at the center for experiemental animals in Madras Medical College. The Institutionnal Animal Medical Committee's clearance were obtained vide reference 1/243/CPCSEA dated 12/11/2013 for the study of the extracts of the whole plant of Polycarpaea corymbosa. After days of acclimatization, the animals were randomly assigned for the acute toxicity groups. Each group containing 3 animaks were housed individually in labelled cages with solid plastic studies and floor with stainless steel grid tops.

The animals were allowed free access to standard pellet diet and water ad libitum. They were maintained in controlled laboratory condition of 12h dark/light cycle, 22±2°C temperature and 45-60% humidity.

5.6.3. Administration of various extracts from whole plant of Polycarpaea corymbosa

The acute toxicity of various extracts of Polycarpaea corymbosa whole plant was carried out as per OECD-423 guidelines for deciding the safe dose of administration to animals19. Three animals were used for each step of study. The animals were fasted prior to dusing (food was withdrawn overnight and water was withdrawn 3h before drug administration) following the period of fasting.

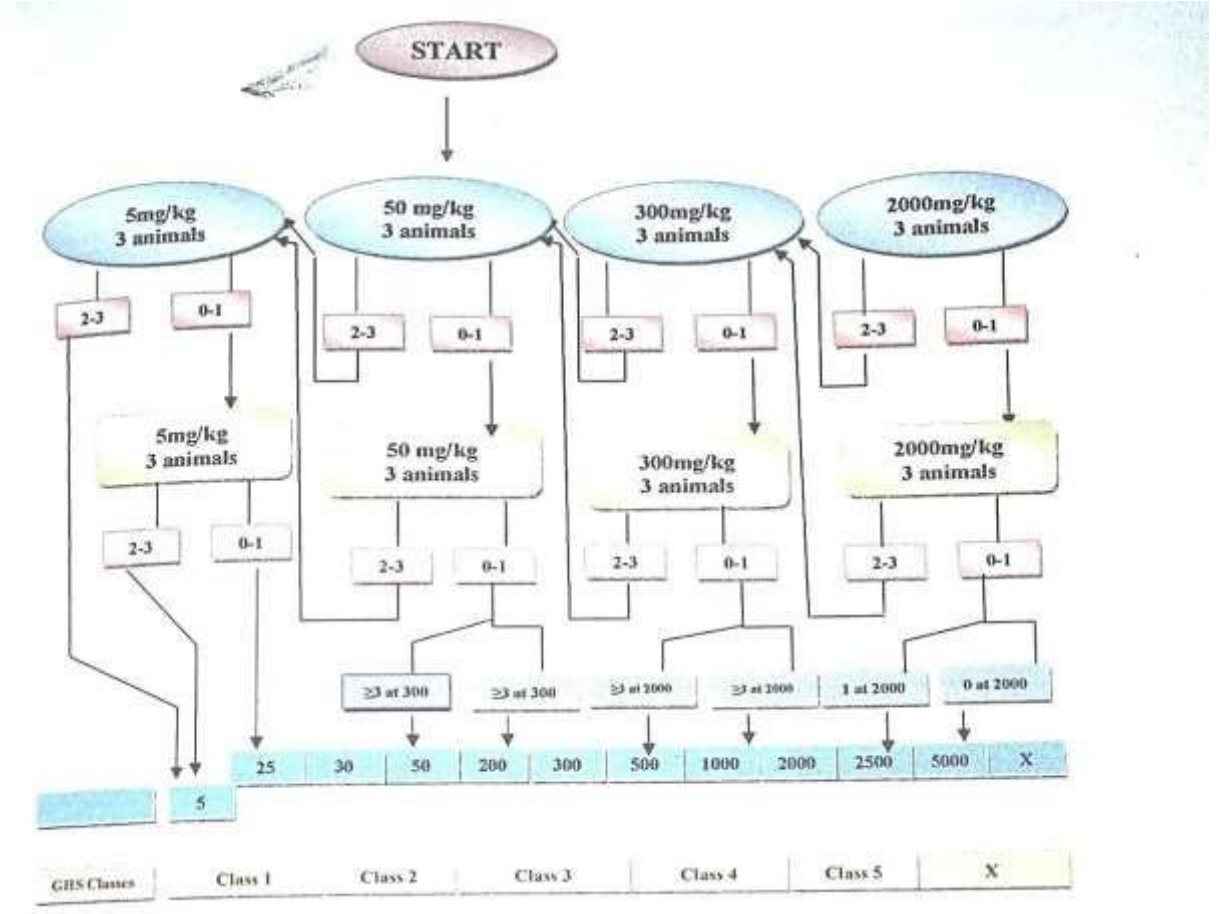
The animals were weighed and the extracts administered in a single dose, as 1% suspension in gum acacia, by oral intubation. Food was withheld for further one hour after the administration of drug. The starting dose levels selected for the study was 5mg/kg and the dose was increased step by step to 50,300 and 2000 mg/kg body weight. The mortality of the animals dosed at one step will determine the next step. The procedure flow chart described the procedure followed for each of the startings doses (Fig:2).

The time interval between treatment groups was determined by the onset, duration and severity of toxic signs. Treatment of animals at the next dose should be delayed until on is confident of survival of the previously dose animals.

Acute toxicity of various extracts from Polycarpaea corymbosa whole plant

- Group I Control (1% suspension gum acacia)
- Group II (Petroleum ether Extract)
- Group III (Ethyl acetate extract)
- Group IV (Ethanol extract)

5.6.4. Observation


START
5mg/kg
3 animals
50 mg/kg
3 animals
300mg/kg
3 animals
2000mg/kg
3 animals
2-3
0-1
5mg/kg
3 animals
50 mg/kg
3 animals
300mg/kg
3 animals
2000mg/kg
3 animals
≥3 at 300
≥3 at 300
≥3 at 2000
≥3 at 2000
1 at 2000
0 at 2000
25
30
50
200
300
500
1000
2000
2500
5000
X
5
GHS Classes
Class 1
Class 2
Class 3
Class 4
Class 5
X

The animals were observed individually every 30min after for the first 24h and frercafter daily for a total of 14 days. The time at at which signs of toxicity appear and was observed systematically and recorded for each animal. Additionals signs of toxicity such as changes in bodyweight, skin and fur, eyes and mucus membranes, respiratory system, circulatory system, autonomous system, central nervous system, somatomotor activity and behavior were also recored. Attention was given to observe the temors, convulsion, salivation, diarrhea, lethargy, sleep and coma. The absence or presences if compound-related mortality of the animals dosed at one step will determine the next step. Any mortality during the experiment for 14 days were observed and recorded.

- 0, 1, 2, 3: number of moribundn or dead animals of each step
- GHS: Globally harmonized classified system (mg/kg b.w.)
- X-Un classified

Fig 2: Flow chart for acute toxic method (OECD guidelines 423)

5.7. ANTI CANCER ACTIVITY

In vivo anticancer models animals employing transplantable tumors as well as EAC induced

carcinoma which may be used to evaluate the anticancer potential. While various medicinal plants are used for the management of cancer in Indian traditional system of medicine, most of these plants are not scientifically evaluated. If a customary and determined ethno pharmacological study is carried out, one or more plants are used in diverse ethno medical practices are sure to provide valuable anticancer drug101.

Anticancer activity investigated on various extracts from whole plant of Polycarpaea corymbosa in mice fed commercial pellet diet.

Different extracts such as petroleum ether, ethyl acetate and ethanol extracts obtained from Polycarpaea corymbosa whole plant and standard 5-flourouracil were give orally to nice, which were also fed with commercial pellet diet. The anticancer activity of the extracts was compared with standard control and cancer induced animals. The extracts were suspended in 1% gum acacia.

5.7.1. Experimental design

Male Swiss albino mice (25 to 30 gram) were procered and maintained in Central Medical House in the Madras Medical Collegge, Chennai. The Institutional Animal Ethical Committee's clearance were obtained vide reference 1/243/CPCSEA dated

22/11/2013 for the study of the extract of whole plant of Polycarpaea corymbosa.

The animals were housed in polypropylene eages with 2 per cage and placed at room temperature (27 ± 2°C) with relative humidity of 55 ± 5%, in an experimental room under a 12h light/ 12h dark cycle. They were fed on a commercial pellet diet and water provided the period of study. EAC cells were obtained through the courtesy of Amala cancer desearch center, Karala (Thrissur), Indian. The EAC cells were maintained in vivro in swiss albino mice, by intraperitoneal (i.p) transplantation of $1x10^6$ cells/ mouse after every 10 days[107].

Treatment schedule[107]

Male Swiss albino mice were divided into 6 groups (n=12) and treated as given below.

GROUPS	DESCRIPTION
Group I	Normal mice
Group II	Inoculated with $1x10^6$ EAC cells/mouse
Group III	Inoculated with $1x10^6$ EAC cells/mouse and treated with pet ether extract (200 mg/kg b.wt)
Group IV	Inoculated with $1x10^6$ EAC cells/mouse and treated with ethyl acetate extract (200 mg/kg b.wt)
Group V	Inoculated with $1x10^6$ EAC cells/mouse and treated with ethanolic extract (200 mg/kg b.wt)
Group VI	Inoculated with $1x10^6$ EAC cells/mouse and treated with 5-flourouracil (20 mg/kg b.wt i.p)

Except group I (normal), all the groups were injected with EAC cells ($1x10^6$ EAC cells/mouse) intraperitoneally. This was taken as day zero. On the first day, normal saline was administered to group I (normal) and group II (EAC-bearing). The various extracts of Polycarpaea corymbosa at the dose of 200 mg/kg b.wt. p.o was administered to groups III, IV and V for 14 days. The standard drug 5-flourouracil (20 mg/kg b.wt i.p) was administered to group VI for 14 days. After the last dose and 18-hr fasting, six mice from each group were sacrificed for various hematological and liver biochemical parameters.

Boby weights of the experimental mice were recorded both in the treated and control on day 0 and 15^{th} day. The boby weight was calculated by using the following.

% increase in boby weight = {weight of animal on day 0 – weight of animal on day 15/ weight of animal on day 0} X 100

The mean survival time and percentage increase of life span was calculated by using the following formula[107].

Mean Survival Time (MST) in days = Day of the first death + Day of the last death

Percentage increase of life span (ILS) = {(MST Test/ MST control) -

1} X 100

Timor Volume[107]

The mice were dissected and the ascetic fluid was collected from the peritoneal activity. The volume was measured by takings it in a graduated centrifuge tube and PCV determined by centrifuging at 1000g for 5 min.

Tryphan blue exclusion assay for cell viability[108]

The dye exclusion test is used to find out the number of viable cells present in a cell suspension. It is based on the principle that live cells have intact with cell membranes that excluded certain dyes; such as diazo dye Tryphan blue, acidic dye Eosin, or propidium, whereas and cells do not. A cell suspension is simply mixed with dye and then visually examined to exclude whether cells take up or exclude dye. Of the cells contain a clear cytoplasm is viable not it is non-viable.

Reagents

a) PBS (pH 7.4): 0.8 NaCI, 0.02 g KCI, 0.144 Na2 HPO4 0.024g KH2PO4. Dissolve the above reagents in 100ml of distilled water.

b) Saline (0.9% NaCI)

c) 0.4% trypan blue (Dissolve 0.4g of trypan blue in 100ml of saline). **Procedure**

Centrifuge an aliquot of cell suspension being tested for viability 5 min at 100 x g and discard the supernatant. Resuspends the cell pellet in 1ml PBS. Mix 1 part of 0.4% trypan blue (0.4% in saline and 1

part of cell suspension (dilution of cells). Allow mixture to incubate 3 min at room temperature. Apply a drop of trypan blue cell mixture to a heamocytometer. Place the heamocytometer on the stage of a microscope and focus on the cells.

- Count the unstained (viable) and stained (unviable) cells separately in the heamocytometer.
  Cell count (cells/ml) = Npo of cells counted x dilution factor/Area x thickness of liquid film.

5.7.2. Biochemical Estimation

After the examination period the animals were fasted overnight and then sacrificed by cervical dislocation method under mild anaesthesia. Blood was anti-coagulated by adding EDTA and used for determining the various hematological parameters which includes RBC, total WBC and differential count (neutrophils, lymphocytes and monocytes).

A liver tissue was examined for the determination of lysosome specific cancer markers (cathepsin-D, β-B glucuronidase and acid phosphatase), liver marker enzymes (5-inclotidase and lactate dehydrogenase), membrane bound ATPase (Na^+/K^+ ATPase and Mg^{2+} ATPase), plasma glucose, liver glycogen, DNA and RNA content.

10% Liver homogenate from different treatment groups were analyzed for in vivo

antioxidant studies. The liver tissue was cleared of, weighed accurately and portions of liver studies were blotted, homogenized with methanol (3 Volumes). The lipid extract was by method of Nichans at al[109]. It was used for the estimation of thiobarbituric acid active substances (TBARS)[109].

Liver tissue sample was used for various estmation of thiobarbituric acid reactive substances and estimation of enzymatic antioxidant like Glutathione peroxidase (GPx). Superoxide dismutase (SOD) and Catalase (CAT). Another portion of tissues was homogenized with phosphate buffet saline and it used for the estimation of non-enzymatic antioxidants like Glutathione (GSH), Vitamin E and Vitamin C.

TOTAL RED BLOOD CELL COUNT BY HEAMOCYTOMETRY[108]

Principle

The blood spectrum is diluted (usually 200 times) with red blood cell diluting fluid which not remove the white blood cells but allow the cells not to be counted under 40 x signification in a known volume of fluid. Fanilly, the number of cells in undiluted blood is calculated and reported as the number of red cells/cu mm.

Reagents

a) Red blood cell diluting fluid

Trisodium citate – 3.13g

37% formaldehyde – 1.0ml and distilled H_2O – 100ml

Procedure

Four ml of the diluting fluid was take in a test tube. Added 20µl of EDTA blodd and mixed well which resulted in 201 times dilution of blood sample. Red blood cells in the diluted sample were counted in a heamocytometer. A small quantity of diluted blood was placed on the counting chamber. Leave the counting chamber on the bench for 3 minutes as allow the cells to settle. Place the counting chamber on the stage of the microscope betweeb the clips to the hold slide so that the counting chamber cannot be moved. Switch to and power (10X) objective, adjust the light and focus on the wall of the counting chamber. Then slowly move the stage towards the middle of the slide until the rulings are visible sterpen the focus.

Locate the large square in the centre with 25 small squares. Place in the middle of the held of vision and examine the distribution of red cells on the entire area. Carefully switch to high-dry objective (40X) and move the chamber so that the smaller upper left corner which is completely in the field of vision. Count the number of red cells seen on

the small square of the upper left corner which is divided into 16 smaller squares to facilitate counting. Repeat the counting with four other squares. It is advisable to complete all counts of the corner square and then move to the center square, which is the fifth square to be counted.

Make a total of all the cells counted in 5 squares.

DETERMINATION OF HEAMOGLOBIN[108]

Cyanomethhaemoglobin method

Principle

Cyanomethhaemoglobin method is a colorimetitric procedure for determination of hemoglobin concentration. An aliquot of well mixed whole blood is take and reacted with a solution of potassium cyanide and potassium ferricyanide (called drabkins solution). The domical reaction yields a product of stable color – the cyanomethaemoglobin. The intensity of the color is proportional to the haemoglobin concentration.

TOTAL LEUKOCYTE COUNT BY HAEMOCYTOMETRY

Principle

Blood is diluted with acid solution which removes the red by haemolysis and also instituates the nuclei of white cells; thus the counting of the

counting of the white blood cells becomes easy, is done whit a microscope under low power (10X) and knowing the volume of fluid determined and the dilution of the blood, the number of white cells per cu mm (or µl) in undiluted whole blood is calculated.

Reagents

WBC diluting fluid

- ✪ Acetic acid, glacial – 2ml
- ✪ Distilled water – 100ml
- ✪ Aqueous methylene blue solution (0.3% w/v) – 10 drops

Procedure

Bito a test tube, pipette out 0.38ml of WBC diluting fluid. 20µl of EDTA anti-coagulated blooob was pipette out with the help pipette and well-mixed. Gently mixed the blood well by swirling and for 2 minutes for complete haemolysis. Dilution of the blood is (18 + 0.02)/0.02 = 20. Filled the counting chamber with 1µl of diluted sample. Placed the swirling chamber on the stage of the microscope, turned the objective to low power and the gird. Counted the cells in four squares of the haemocytometer.

DIFFERENTIAL COUNTING

Principle

Differential count is the percent distribution of various white blood cells in the peripheral blood as determined from a blood smear stained with the Leishman stain. The staining is done a polychromatic stain that includes eosin and methylene blue in its preparation eahman stain). The polychromatic stain induces multiples colors when applied to cells. In to the relative WBC count, microscopic study of a blood smear helps to get an picture of blood.

Reagents

Stain: 0.2% in acetone free methanol

Proffered water

- Disodium hydrogen phosphate – 3.76g
- Potassium dihyrogen phosphate anhydrous -2.10g
- Distilled water – 1000ml

Procedure

Differential count involved in the preparation of smear, staining of smear and microscopic observation.

Preparation of smear

Transfer a drop of well mixed EDTA-blood specimen to clean grease free slide. Place the stop approximately 1cm away from end and of about 5mm diameter. With the help of the reader a smear was made.

Staining of smear

Sherman stain contains methanol which fixes the smear in the staining process. Fixation parents distortion of cells and smears can be kept of a long time. Before staining, the smear was initially fixed with methanol for 2 to 3 minutes. Covered the smear with diluter Sherman stain for 710 minutes. Washed the stain off buffered water. Finally added wash buffered for 2-3 minutes to differentiate the film. The time taken for differentiate prend as on the stain and pH of the water used. Shaked off all water adhering to the slide was set in an upright position in a drying rack. The smear was examined under the microscope. Identify various types of white cells on the basis of the following characters as a result od staining with Leishman stain.

- **Granulocytes**
  These are cells with granulated cytoplasm which stains faint pink.
  These include neutrophils, eosinophils and basophils.
- **Neutrophils**
  Pale pinck cytoplasm with fine mauve-coloured granules, include band and segmented forms – lobes), normally 3 to 4 lobed.
- **Eosinophils**

Cytoplasm stains faint pinck and contains large red and red orange granules.

- **Basophils**
  Cytoplasmic granules large, dark, and blue which fill the cell and obscure the nucleus.
- **Lymphocytes**
  Large-sized lymphocytes have clear blue cytoplasm on the margin of the nucleus. If the smaller lymphocytes dark violet coloured nucleus almost fills the entire cell and has a rim of clear cytoplasm.
- **Monocytes**
  Largest in size of all white cells, wavy margin of cytoplasm, greyblue cytoplasm: kidney-shaped nucleus

LYSOSOME SPECIFIC CANCER MARKERS

Cathepsin athepsin-D[110]

Principle

Acid Denatured haemoglobin Cathepsin D
TCA-soluble peptides

Reagents

a) Sodium acetate buffet 0.1 M, pH3.6:92.5 ml of 0.1 M acetic acid was mixed with 7.5 ml of 0.1 M sodium acetate solution.

b) Substrate (1.5%): 1.5g of Hb was dissolved in 100 ml sodium acetate buffer.
c) TCA (5%): 5g of TCA was dissolved in 100 ml of dis H_2O.
d) Folins copper reagent (1:1 dilution).
e) Alkaline copper reagent (50:1 ratio)
   Soln A : 2% Na_2 CO_3 in NaOH
   Soln B: 0/5% $Cuso_4$, $5H_2O$ in 1% sodium potassium tartarate
f) Standard : A solution of tyrosine in the concentration of 10mg/100 ml was prepared with 0.1 NCI

Procedure

0.9 ml of buffered substrate was mixed with 0.1 ml of preparation and incubated for (2 hours at 37°C. The reaction was stopped with 1.0 ml of 5% TCA and the samples were fuged for 10 mins. To the control tubes, the enzyme preparation was added after the addition of TCA. To 1.0 ml of supernatant, 1.0ml of 5% sodium hypoxide and 4.5 ml of copper reagent were added. After 10 mins, 0.5ml of Folins phenol reagent was added and the color developed was read at 640 nm after 15 mins. The standards were treated. Enzyme activity is expressed as µmoles of tyrosine liberated/hour/mg/protein at.

Determination of β-D-Glururonidase[111]

Principle

Gluc + H_2O β-D-Glururonidase Glucuronate + Phenolphthalein

Reagents

Buffer: 0.1 M, pH 4.5

Solution A: 410.2 mg of sodium acetate was dissolved in 50 ml of dis

H_20

Solution B: 0.29 ml acetic was mixed with 50 ml dis H_20, 4.9 ml of solution A and 5.1 at of solution B were mixed before use.

Glycine buffer (pH 10.7)

This was prepared by mixing equal volume of 0.2 M glycine, 0.125 M sodium and 0.1 M sodium chloride in dis H_20

Substrate: p-nitrophenyl –β-D6glucuronidase (1mg/ml in dis H_20).

Standard: 5mg of p-nitrophenyl in 100 ml of dis H_20.

Procedure

0.5 ml of substrate, 0.05 ml of acetate buffer, 0.3 ml of homogenate was incubated at 37°C. The 1 hour. The reaction was arrested by the addition of 3.9 ml of glycine buffer. Standards were run simultaneously along with a blank. The color developed was read at 420 nm using a salorimeter. The enzyme activity is expressed as µmoles of p-nitrophenyl liberated/L.

Estimation of Acid Phosphatase112

Principle

The enzyme catalyses the hydrolysis of phosphate ester to H_3PO_4 and alcohol. Amount of H_3PO_4 produced, during hydrolysis is a measure activity. The liberated H_3PO_4 containing organic phosphate is estimated by Fiske subbarow method.

Reagents:

a) Citrate buffer, pH 5 a= 0.1 M solution of citric acid (21.01 g in 1000ml) b= 0.1 M solution of citrate (29.41 g in 1000 ml)
   20.5 ml of Citrate buffer + 29.5 ml of in diluted to a total volume of 1000 ml
b) Substrate: 0.1 M sodium β-glycerophosphate (3.15 g in 100ml of citrate buffer)
c) 10% TCA: 10g in 100 ml of distilled water.
d) Ammonium molybdate solution: 5 mgs of amùonium molybdate was dissolved in 200ml of 5N H_2SO_4.
e) ANSA: dissolve 30g sodium metasulphite, 6g sodium sulfite and 500 mg of ANSA separately in small qnatities of water. Combine all the solution and make upto 250ml with water. Allow to stand overnight and filter. Store refrigerated in an amber-coloured bottle.
f) Standard phosphate: dissolved 35.1 mg of pure KH_2PO_4 in water add 10 ml of IN H_2SO_4 and made upto 100 ml distilled water.

g) Working standard: Ten ml of the stock was diluted to hundred ml with water a standard flask with water.

Procedure

Buffered substrate (2 ml) was added to the test and control tubes and placed in a water besth at 37°C for 5 minutes. 0.1 ml of sample was added to test tubes and incubated at 37°C for 1 hour. Tubes were removed and 1.0 ml of 10% TCA was added to all the tubes. Control tubes alone 0.1 ml of sample was added and shaken well and centrifuged, 1.0 ml of supematant was take with 1.0ml of ammonium molybdate and 0.4 ml of ANSA was added the after another. Final volume was upto 10.0 ml distilled water. Standad ranging from 5-40µg concentration was added treated similarly as the above. The intensity was read at 500nm after 10 minutes.

LIVER MARKER ENZYMES

Enzymatic assay of 5' nucleotidase[113]

The activity of 5' – nucelotidase is determined as the amount of liberated phosphate from 5' –adenosine monophosphate (5'AMP) at PH 9.0 which is measured pectrophotometrically at 660 nm.

5'-AMP +H20 5'-nucleotidase

adenosine °Pi **Reagents:**

a) 200 mM glycine buffer, Ph9.0 at 37°C : (prepare in 10N sulphuric acid.
b) 66 mM adenosine 5’ monophosphate MWt: 391.91
c) 200 mM magnesium sulfate Mwt:246.48
d) Reagent A: 10% ammonium molybdate solution (prepare in 10N sulphuric acid)
e) Reagent B: Tausky-shorr reagent (TSCR): prepared by making 10ml of reagent A to 100ml with deionized water and added 5g of ferrous sulphate heptahydrate
f) Ohosphorous standard: dissolved 35.1 mg potassium dihydrogen phosphate in 1ml of 10 N H_2SO_4 and make up to 100 ml with ditilled water, 1ml of the stock contains 18 mg of phosphorous
g) Working standard: 1 in 1O dilution 10ml of the stock is diluted 100ml with distilled water, 1 ml of this solution corresponds to 8 mg of phosphorus.

Procedure

To the test and control tubes, added 1.5 ml of glycine buffer, 0.15ml of 5’-AMP and 10ml of $MgSO_4$ and the final concentration was made upto 2 ml deionized water. The final concentration in this mixture was

150mM glycine, 5.0mM adenosine 5’-monophosphate and 5mM magnesium sulfate. The mixture was equilibrated at 37°C 5 minutes. Then added and of

sample to the test. Immediately mixed and incubated at 37°C for 2 mins. Added 4ml reagent B to all the tubes. Sample (0.1 ml) was added to control tubes. Final volume was upto 4.1 ml by adding 2ml of deionized water. Read the absorbance at 660nm.

In 5 ml of working standard solutions corresponds to 8 to 4 µg of phosphorous was taken and the final volume was made up 5 ml. the after, added 4ml of reagent B to standards. Thank was read similarly as above. One unit is defined as 1.0 µmole of inorgabic phosphorus hydrolysed from adenosine 5'-monophosphate per min pH 9.0 at 37°C.

Estimation of lactate Dehydrogenase[114]

Principle

The lactate is acted upon by lactate dehydrogenase to from pyruvate in the presence of NAD. The pyruvate forms Pyruvate Phenyl Hydrazone with 2.4-dinitrophenylhydrazine. The color developed was read in a spectrophotometer at 440nm.

Reagents

a) Glycine buffer: 7.50 g of glycine and 5.85g of sodium ware dissolved in 1 litre of water.
b) Buffered substrate: 125 ml of glycine buffer and 75ml of 0.1N NaOH were added to 4g lithium lactase and mixed well.

c) Nicotinamide adecine dinucleotide: 10mg of NAD+ was dissolved in 2ml of water.
d) 2,4-dinitrophenyl hydrazine: 20mg of DNPHB was dissolved in 1OOml of IN HCI.
e) 0.4N NaOH
f) Standard: 11mg of sodium pyruvate was dissolved in 100ml of buffer (1µ mole of pyruvate/ml).

Procedure

Placed 0.5ml of buffer substrate and 5µl of sample into each of two tubes. Added 1.1ml of water to the blank. Then to the test added 0.1ml of NAD. Mixed and incubated at 37°C for 15 minutes. Exactly after 15 minutes, 0.5ml of dinitrophenylhydrazine was added to was and control tubes. Lefte for forther 15 minutes. Then added, 5.0ml of 0.4N NaOH and the color developed was read immediately at 440nm. A standard curve with sodium pyruvate solution with the concentration range 0.11.0µ mole was taken. The enzyme activity is expressed as units/mg protein in tissues.

Estimation of Na^+K^+ ATPase[115]

Principle

Na^+ K^+ ATPase transport Na, K against concentration gardient at the cost ATP molecule liberating inorganic phosphate (pi). The inorganic phosphorous liberated is estimated by Fiske and Subbarow method.

Reagents

a) 184 mM tris-HCI buffer, pH 7.5
b) 50 mM $MgSO_4$
c) 50 mM KCI
d) 600 mM Nacl
e) 1 mM EDTA
f) 40 mM ATP

Procedure

1 ml of tris buffer and 0.2ml of each of the above reagents were mixed together. Than, the assay medium in a final volume 2.0 ml, contained 92mM tris buffer, 5mM $MgSO_4$ 50mM Nacl, 1 mM EDTA and 40 mM ATP. After 10 mins, equilibration at 37°C in an hubator, reaction **was started by the addition.**

Estimation of Mg^{2+} - ATPase[116]

Principle

The activity of enzyme was estimated by the inorganic phosphorus liberated is estimated by Fiske and Subbarrow method.

Reagents

a) 357 mM Tris-HCI buffer, pH 7.6
b) 25 m,m Mgcl2
c) 10 mM ATP

The assay as estimated by the addition of 0.1 ml of homogenate to an incubation sodium containing 0.1ml of H_2O and 0.1 ml of each of the

above reagents. The final concentration of tris-buffer, $Mgcl_2$ and ATP were 75mM, 5mM and in total incubation estime of 0.5 ml. the reaction was terminated after 15 mins by the addition of 1.0 ml of 10%. The liberated phosphorous as estimated by Fiske and Subbarrow method. The onyme activity is expressed as kimoles of pi liberated/'min mg protem.

ESTIMATION OF LIVER GLYCOGEN[117]

Principle

Glycogen is treated with 45%, ethanol to remove glucose. Glucose is dehydrated sulphuric acid to furfural derivative then complexes with an anthrone green colored complex, which is read al 620nm.

Reagents

a) Extraction of Glycogen: 0.2g of the sample as homogenized with of 5%n TCA. The precipitate of was filtered and the clear filtrate was used for analysis.
b) Anthrone reagent: 0.2% anthrone in concentrated sulphuric acid
c) Stock standard: of glucose dissolved in 100ml of H_2O

d) Working standard : 10 ml of stock was made upto 100ml with H2O
e) 10N KOH = 56.1 of KOH in 100ml with
f) Glacial acetic acid

Procedure

1ml of liver sample was pippeted into a test tube. Phen 2.0 ml of 10 N KOH was added and the test tube was placed in boiling water bath I hour. After cooling. 1.0 ml of glacial acetic acid was added to neutralize the excess of alkali and the volume was made up it) ml with water. From this 1.0 ml was taken for the experiment. Glucose standards were prepared by taking 0.2 to 1.0 ml with distilled 1120. Anthrone reagent (4 ml) was added to all and heated in boiling 8 minutes. cooled and read at 620nm. The amount of glycogen present was expressed as mg g tissue.

ESTIMATION OF DNA-DIPHENYLAMINE METHOD[118,119]

Principle

Under extremely acidic conditions DNA is initially de-purinated quantitatively followed by dehydration of sugar to omega-levulinyl aldehyde. This aldehyde condenses in acidic medium with diphenylamine to produce a deep coloured condensation product with absorption maximum at 600nm

Reagents

a) DNA stock standard: 60mg of DNA was dissolved in 5mM NaOH and made up to 100ml with the same.
b) Working std: to ml of stock standard added 5.0 ml of 0.2 N perchloric acid healed at 90°C for 15 mins and cooled. 1.0 ml of this solution contains 300 µg of DNA.
c) Saline citrate: 0.15 M sodium chloride (8.78 g/L) and 0.015 M sodium citrate (4.41 g/L) was mixed.
d) Diphenylamine reagent: 1.5 g Of diphenylamine in 100 ml of glacial acetic acid and 1.5 ml of con. sulphuric acid. Warm to room temperature and swirled to remix before use.
   Stable fòr 6 months at 2°C
e) 0.6 N perchlorate
f) 0.2 N perchlorate: 18 ml of 70% perchloric acid is diluted to 250 ml with water.
g) 0.3 M KOH
h) 5% TCA
i) 10% TCA

Procedure

Sample preparation

10 % liver homogenate was prepared; 5 ml of 10 % homogenate prepared was taken in a

centrifuge tube. Then 2.5 ml of 0.6 N perchlorate was added and centrifuged for 10 minutes at 0^0 C. The supernatant was discarded and the pellet was washed twice with 2.5 ml of 0.2 N perchlorate. The supernatant was discarded and the pellet was added with 4 ml of ethanol and centrifuged for 10 minutes at 37^0

C. The supernatant was discarded again. 4 ml of 0.3 M KOH was added to the pellet and incubated for 1 hour at 37^0C. Then added 6 ml of 0.2 N perchlorate and centrifuged for 10 minutes. The supernatant was used for RNA estimation and the pellet further processed because it contained small amount of RNA. The pellet was washed twice with 5 ml of 0.2N perchlorate and the supernatant was used for RNA estimation. The pellet was added with 1.3 ml of 10 % TCA, heated for 15 minutes at 90°C. then centrifuged at 1500rpm for 10 minutes. The supernatant was collected and labeled as supernatant 1. Then the pellet was added with 2.5 ml of 5 % TCA and centrifuged for 10 minutes. The supernatant was collected and labeled as supernatant 2. The supernatant I and 2 were pooled and the pellet was discarded. The supernatant final volume was made up to 5 ml with saline citrate buffer and used for DNA estimation.

Into a series of test tubes pippeted out 0.2 to 1.0ml of the working standard DNA solution corresponding to 60-300gg values. 1.0 ml of the sample was pippeted out. Make up the volume of all the tubes to two ml with distilled 1-120. Set up a blank along with the working standard. Added 3 ml of diphenylamine reagent to each tube and after mixing heated the tubes in a water bath fòr 10 mins. Removed and cooled the tubes by immersing in tap water for 5 mins. Read the absorbance of blue solution at 600 nm against blank.

ESTIMATION OF RNA BY ORCINOL METHOD [120]

Principle

The RNA content is estimated by orcinol method based on the estimation of ribose moiety of RNA, which produce a green colour with orcinol reagent. The intensity of the colour developed is proportional to the ribose content and is measured colorimetrically at 665 nm.

Reagents

a) RNA stock standard: dissolved 100 mg of purified RNA in 10 ml of 1 N KOFI. Incubated for 16-17 hours at 37^0C and made it up to 100 ml in a standard flask with distilled water.

b) Working standard: Ten ml of the stock was diluted to hundred ml. 1.0 ml of this solution contains of RNA.
c) Orcinol reagent: dissolved 0.34 g of ferric chloride and 0.5 g of orcinol in a little amount of water and made up to 12.5 ml with water.
d) Dilute orcinol reagent: 12.5 ml of stock was added to 225 ml of conc. Hydrochloric acid and diluted to 250 ml with water.

Protocol

Sample preparation

10% liver homogenate was prepared; 5 ml of 10 % homogenate prepared was taken in a centrifuge tube. Then 2.5 ml of 0.6N perchlorate was added and centrifuged for 10 minutes at $O^{O}C$. The supernatant was discarded and the pellet was washed twice with 2.5 ml of 0.2 N perchlorate. The supernatant was discarded and the pellet was added with 4 ml of ethanol and centrifuged for 10 minutes at $37^{0}C$. The supernatant was discarded again. 4 ml of 0.3 KOH was added to the pellet and incubated for 1 hour- at $37^{0}C$. Then added 6 ml of 0.2N perchlorate and centrifuged for 10 minutes. The supernatant was used for RNA estimation and the pellet further processed because it contained small amount of RNA. The pellet was washed twice with

5 ml of 0.2N perchlorate and the supernatant was used fòr RNA estimation.

Pipetted out 0.2 to 1.0 ml of the working standard RNA solution into a series of test tubes corresponding to Pig values 20 to 100. 0.5 ml of the sample was pipetted out. The volume was made up to 2.0 ml in all the tubes with distilled water. Set up a blank along with the working standard. Added 2.0ml of diluted orcinol reagent to all the tubes. The top of the tubes were covered with marbles and kept in a boiling water bath for 20 mins. Removed and cooled the tubes at room temperature and the colour developed were read at 665 nm in a spectrophotometer against the reagent blank.

ESTIMATION OF GLUCOSE-ORTHOTOLUIDINE METHOD [121]

Principle

Aldehyde group of glucose reacts with amino group of O-toluidine to give a green colored complex which can be read at 620 nm in a colorimeter against a reagent blank.

Reagents

a) O-toluidine — 0.3 g of thiourea was made upto 155 ml with glacial acetic acid. To this, 12 ml of ortho-toluidine reagent was added.
b) Stock glucose: 2 mg/ml

c) Working standard: 1 in 10 dilution

Procedure

0.5 ml of blood and 4.5 ml of 1 0 ^{0} 0 sodium tungstate reagent vvas taken in a test tube. mixed well and the contents of the tubes were allowed to stand for 5 minutes, centrifuged and to 0.5 ml of supernatant 3 ml of O- toluidine reagent was added. Into a series of test tubes, working standard glucose solution of concentration ranging 40-200gg were pipetted out.

Determination of tissue lipid peroxidation

Lipid peroxidation is commonly observed as harmful process 22 leading to structural modification of complex lipid protein assemblies, such as biomembranes and lipoproteins is usually associated with cellular malfunction. During lipid peroxidation a polar oxygen moiety is launched in the hydrophobic tails of unsaturated fatty acids. This process is 1 15 of dual consequence: the presence of hydroperoxy groups affected the hydrophobic lipid/lipid and lipid/protein interaction which leads to structural modification of biomembranes and lipoproteins. Hydroperoxy lipids are the source for the development of free radicals when free radicals are produced they can attack poly unsaturated fatty acid in cell membrane leading to a chain of chemical reaction called lipid peroxidation. As the fatty acid

is broken down, the hydrocarbon gases and aldehyde are formed. The measurement of malonodialdehyde (MDA) formed is the thiobarbituric acid assay method[123].

The in vivo anti-oxidant and lipid peroxidation effect of various extracts petroleum ether, ethyl acetate and ethanol from Polycarpaea corymhosa whole plant was investigated in cancer induced mice with normal control mice.

1. Estimation of thiobarbituric acid reactive substances (T BARS)[109] Principle:

The level of thiobarbituric acid reactive substances was estimated by the method of Nichans et al.,(1968). In this method, malondialdehyde and other thiobarbituric acid in the acidic medium generate a pink coloured chromophore, which was read at 535nm.

Reagents

- TCA, 15% (w/v)
- HCI, 0.25N
- TBA, 0.375% in hot distilled water
- TCA-TBA-HCI Reagent
- Stock standard malondialdehyde solution
- Working standard

Stock solution was diluted to get a concentration of 50nM/ml.

Procedure:

0.5ml of homogenate was diluted to 1.5ml with double distilled and mixed well 2ml of TCA-TBA-HCI reagent was then added. The mixture was kept in boiling water bath for about 15min. After cooling, the tubes were centrifuged at 1000rpm for I()min and the supernatant was taken for colorimetric measurement. A series of standard solutions in the concentration of 2-1011M were treated in a similar manner. The absorbance of chromophore was read at 535nm against a reagent blank. The values were expressed as nM/g wet tissue.

5.7.3 DETERMINATION OF IN VIVO ANTIOXIDANT LEVEL

1. Assay of superoxide dismutase (SOD)[124] Principle:

Superoxide dismutase activity was assayed by the method of Kakkar el al (1984).The assay of SOD was based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium complex. The reaction was initiated by the addition of NADH. After incubation for 90s, the reaction was stopped by the addition of glacial acetic acid. The colour developed at the end of the reaction was extracted into butanol layer and measured at 560nm.

Reagents

- ✓ Sodium pyrophosphate buffèr, 0.025M, pH 8.3

- ✓ Phenazine methosulphate, 186þ1M
- ✓ Nitroblue tetrazolium, 300mM
- ✓ NADH-780mM
- ✓ Glacial acetic acid
- ✓ n-butanol
- ✓ Chloroform
- ✓ Ethanol

Procedure: 0.5 ml of the sample (tissue homogenate) was diluted to Iml with ice cold water. 2.4ml of ethanol and 1.5ml chloroform (in chilled condition) were added. This mixture was shaken for Imin at 4 ^{0} C and then centrifuged. The enzyme activity in the supernatant was determined. The assay mixture contained 1.2ml sodium pyrophosphate buffer, 0. Iml phenazine methosulphate, 0.3ml nitroblue tetrazolium, appropriately diluted enzyme preparation and water in a total volume of three milli litre. The reaction was initiated by the addition of 0.2 ml NADH. After incubation at 30^{0} C for 90s, the reaction was stopped by the addition of 1.0ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0ml of Il-butanol. The mixture was allowed to stand for I Omin and then centrifuged. I he colour intensity of the chromophore in butanol laser xxas measured at 56()nm against Il-butanol blank and a system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme

reaction which gave 50^0 0 inhibition of NBT reduction in one minute under the assay conditions and the activity was expressed as units/mg protein.

2. Assay of catalase (CAT)

Principle:

The activity of catalase was determined by the method of Sinha (1972). Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate when heated in the presence of hydrogen peroxide. The chromic acetate formed was measured at 620nm. Catalase was allowed to split hydrogen peroxide for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate acetic acid mixture and the remaining hydrogen peroxide as chromic acetate is determined colorimetrically.

Reagents

- Phosphate buffer, 0.01M. pH 7.0
- Hydrogen peroxide (FL(L) 0.2M
- Potassium dichromate, 5% (w/v)
- Dichromate acetic acid reagent
- Standard hydrogen peroxide, 2mM

Procedure:

To 6.0ml phosphate buffer, 0. Iml sample and 0.4 ml hydrogen peroxide were added. The reaction was stopped at 15, 30, 45 and 60s by the

addition of 2ml dichromate-acid reagent. The tubes were kept in boiling water both for I Omin and the colour developed was read at 620nm. Standards in the range of 2-1 OHM were taken and preceded similar to the test with blank containing reagent alone. The activities were expressed as HIVI of H202 consumed/ minute/ mg protein.

3. Assay of glutathione peroxidase (Gpx)[126] Principle:

Glutathione peroxidase activity was estimated by the method of Rotruck et al., 1973. A known amount of enzyme preparation was allowed to react with H202 in the presence of CJSH for a specified time period and the remaining GSH content was measured subsequently.

$$2GSH + l\text{-}H_2O_2 \xrightarrow{Gp.x} GSSG + 2H_2O$$

Reagents

- Tris buffer, 0.4 M, pH 7.0
- Sodium azide solutions 10 mM
- Tricholoro acetic acid, 10% (w/v)
- EDTA, 0.4mM
- 1-1202 solution, 20 111M

Procedure: To 0.2ml of tris buffer, O.ml EDTA, 0. Iml sodium azide. 0.5ml sample (tissue

homogenate) and 0.2ml CJSH were added followed by 0. Iml hydrogen peroxide. 1 he contents were mixed well and incubated at 37° c for 10min along with a tube containing all the reagents except the sample. After 1 Omin, the reaction was arrested by the addition of 0.5ml of 10% TCA, centrifuged and the supernatant was assayed for GSH by the method of Beutler e/ al.. 1963.

4. Estimation of glutathione (GSH)[127]

Principle:

Glutathione level was estimated by the method of Ellman et al., 1959 in which, yellow colour developed when dithio-dinitro-bis-benzoic acid (DTNB) was added to the compounds containing sulphydrvl groups.

Reagents

- Phosphate buffer, 0.2M, pH 8.0
- TCA, 5%(w/v)
- Ellman's reagent
- Disodium hydrogen phosphate (Na_2HPO_4). 0.3M, pH 8.0
- Standard glutathione solution
- Precipitating reagent

Procedure:

A known weight of tissue was homogenized in phosphate buffer. To 0.5ml of the sample, 3.0ml

of precipitating reagent was added, mixed thoroughly and allowed to stand fòr 5minu and centrifuged. A set of standards (20-100þtg) were taken and made upto 2.0ml with distilled water. 2.0ml of the supernatant along with 2.0ml of blank containing distilled water was also taken. To all the tubes 4.0ml 0.3M disodium hydrogen phosphate and Iml of DTNB reagent were added. The colour developed was read at 412nm. The reduced glutathione leve s were expressed as mg/g wet tissue.

5. Estimation of protein128

Principle:

The protein content was estimated by the methods of Lowry et al., (1951). Proteins react with Folin-Ciocalteau reagent to give a complex. The colour so formed was due to the reaction of alkaline copper with protein and the reduction of phosphomolybdate by tyrosine and trytophan present in the protein. The intensity of the colour depends on the amount o these aromatic amino acids present.

Reagents

- Alkaline copper reagent
- Folin's phenol reagent
- Standard bovine serum albumin (BSA)

Procedure:

0. Iml of homogenate was made upto Iml with saline, then Iml 10% TCA was added. The mixture was centrifuged, supernatant discarded and the precipitate was dissolved in Iml of 0. IN sodium hydroxide. l' rom this aliquots were taken for the estimation. 4.5ml of alkaline copper reagent was added and the contents were allowed to stand at 37°C for 10min. Then 0.5ml dilute Folin's phenol reagent was added and mixed. A series of standards of concentration range 20100ktg and a blank were processed as for the test. The blue colour developed was read at 620nm after 20min.

6. Estimation of Vitamin E (a-Tocopherol) [129] Principle:

Vitamin E levels were estimated by the method of Baker et al (1980). This method involves the reduction of ferric ions to ferrous ions by tocopherol and the formation of a pink coloured complex with l, 10phenanthroline-ortho phosphoric acid. The reaction mixture consisted of redistilled ethanol, petroleum ether, 4,7-dipyridyl-l-10phenanthroline, 0.01M ferric chloride and 0.001M Ortho phosphoric acid. The colour developed was

measured at 520nm. The vitamin E values were expressed as mg/100mg tissue for tissues.

Reagents

- Petroleum ether : 60- 80°C
- Double distilled ethanol.
- 2, 2' dipyridyl solution: 0.2% in double distilled ethanol.
- Ferric chloride solution: 0.5% in double distilled ethanol.
- Stock standard: 10 mg ol' (1 — tocopherol in 100 ml. of distilled ethanol.
- Working standard: Stock solution was diluted with ethanol to a concentration of 10µg/ml.

Procedure

To 0.5 ml. of sample, 1.5 mL of ethanol was added, mixed and centrifuged. The supernatant was evaporated and to the precipitate, 3.0 mL of petroleum ether, 0.2 mL of 2, 2' dipyridyl solution and 0.2 ml, of ferric chloride solution were added and kept in dark for 5 min. An intense red colour was developed. 4.0 ml. of n-butanol was added to all the tubes and mixed well. Standard tocopherol in the range of 10-100 Pig was taken and treated similarly along with a blank containing only the reagent. The colour in the n-butanol layer was read at 520 nm. The values were expressed as mg/dl for plasma or mg/l()()g of wet tissue.

7. Estimation of Vitamin C[130]

Principle:

Vitamin C was measured by the method of Roe and Kuether (1943). In this reaction mixture consist of ethanol, petroleum ether, batho phenanthroline, ferric chloride, and *O* phosphoric acid. The colour developed was measured at 530nm. Vitamin C values were expressed as mg/dl.

Reagents

- TCA: 6%
- 2, 4 DNPH reagent: 2.0 g of DNPH was dissolved in 100 mL of 9 N sulphuric acid. To this. 4.0 g of thiourea was added and mixed.
- Sulphuric acid : 85%
- Stock ascorbic acid solution: 10 mg of L-ascorbic acid in 100 ml. of 4 ^{0} 0 TCA. Working ascorbic acid solution: 1 in 10 dilution of stock ascorbic acid solution with 4% TCA to obtain a concentration of 0.1 mg/ml..

Procedure

To 0.5 ml. of sample, 1.5 ml, of 4% TCA was added and allowed to stand for 5 min and centrifuged. To the supernatant. 0.3 g of acid washed norit was added. shaken vigorously and filtered. This converts ascorbic acid to dehydroascorbic acid. 0.5 ml. of the filtrate was

taken and 0.5 mL of DNPH was added, stoppered and placed in a water bath at 37 ^{o} c for exactly 3 h. Removed, placed in ice-cold water and added 2.5 ml. of 85% sulphuric acid drop by drop. The contents of the tubes were mixed well and allowed to stand at room temperature for 30 min. A set of standards containing 20-100 pig of ascorbic acid were taken and processed similarly along with a blank containing 2.0 ml. of 4% TCA. The color developed was read at 540 nm. The values were expressed as mg/dl of plasma or mg/l oog of wet tissue.

STATISTICAL ANALYSIS

The results were expressed as mean SEM of 6 mice in each group and statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Duncan multiple comparison method was used to correlate the difference between the variables. was considered as statistically significant.

5.7.4 HISTOPATHOLOGICAL STUDIES

When the tissues are exposed to any toxicants, alterations in the tissue architecture are more prominent. This can be monitored by histochemical examination 3 1

TISSUE PROCESSING

A fraction of the tissues was fixed in 10 % formalin immediately after autopsy. The fixed tissues were placed in (10 % formalin in 0.9 % Nacl) for one hour to resolve shrinkage due to higher concentration of formalin solution. They were left overnight in running water after securing the mouths of the vessels with cotton gauze. [he tissues were dehydrated in ascending grades of isopropanol (by immersing in 80% isopropanol overnight Iòllowed by 100% isopropanol for one hour). The dehydrated tissues were cleared in two changes of xylene, one hour each. Then the tissues were drenched with histology grade paraffin wax (melting point 58-60^0 C. The wax impregnated tissues were implanted in paraffin blocks using the same grade wax. The paraffin blocks were mounted and cut with rotary microtome at three micron thickness. The sections were drift on a tissue flotation bath at 40^{0}C and taken on a glass slide smeared with equal parts of egg albumin and glycerol. The sections were then melted in an incubator at 60^{0}C and allowed to cool for 5 minutes.

TISSUE STAINING

The sections were deparaffinised by dipped in xylene for 10 minutes in a staining jar. The deparaffinised sections were washed in hundred

percentage of isopropanol and stained in Ehrlich's hematoxylin for 8 minutes. After staining in hematoxylin, the sections were washed in tap water and immersed in acid alcohol (8.3 % I-ICI in 70 % alcohol) to remove excess stain. The sections were counterstained with 1 % aqueous solution of eosin for I minute. The excess stain was washed in tap water and the section was allowed to dry. Complete dehydration of the stained section was ensured by placing the section in an incubator at 60 ^{o}c for 5 minutes. When the sections were cooled, they were mounted in DPX mountant. The cell architecture in the liver was observed under high power objective in a microscope.

5.8 ISOLATION BY COLUMN CHROMATOGRAPHY

COLUMN CHROMATOGRAPHY [132]

Column chromatography is an isolation technique in which the phytoconstituents are being eluted by adsorption. The principle involved in this separation of constituents is adsorption at the interface between solid and liquid. The component must have various degree of affinity towards adsorbent and also reversible interaction to achieve successful separation. No two compounds are alike

in the above aspect. Low affinity compounds will elute first. The columns of different sizes were used for the present studies.

Since the ethanolic extract of Polycarpaea corymbosa was found to possess significant pharmacological activity when compared to other extracts an attempt was made to fractionate the ethanol extract by column chromatography.

5.8.1 MATERIALS AND METHODS

Type of extract : Ethanol extract

Method : Wet packing method

Packing material : Silica gel G 70 -325

The ethanolic extract of Polycarpaea corymbosa whole plant was placed to column chromatographic separation using normal phase silica gel column.

5.8.2 PREPARATION OF ADMIXTURE

20g of the ethanolic extract of whole plant of Polycarpaea corymhosa was admixed with 20g of silica gel (60/120 meshes) to get uniform mixing.

Column packing

Two hundred gram of silica gel (70/325 meshes) was taken in a suitable column and packed very carefully without air bubbles using hexane as

filling solvent. The column was kept aside for one hour and allowed for close packing. Admixture was then introduced at the top of the stationary phase and started separation of compounds by the eluting with various solvent mixtures with increasing order of polarity. All the column fractions were collected separately and concentrated under reduced pressure. Finally the column was washed with ethyl acetate and methanol.

The EEPC was subjected to column chromatographic separation using normal phase silica gel column. The dark brown solid (20 gram of EEPC) was adsorbed on silica gel (20 g) and transferred to a column of silica gel (200g equilibrated with Il-hexane). Elution was performed with n-hexane (100%), Il-hexane: chloroform (90: 10), Il-hexane: chloroform (70:30), n-hexane: chloroform (50:50), n-hexane: chloroform (30: 70), chloroform (100), chloroform: ethyl acetate (70:30), chloroform: ethyl acetate (5():50), chlorofòrm: ethyl acetate, (30: 70), ethyl acetate (100), ethyl acetate: ethanol (80:20), ethyl acetate: ethanol (70:30) ethyl acetate: ethanol (50:50), ethyl acetate: ethanol (30:70) and ethanol(100).

Fractions were collected, distilled off the solvent and the homogenecity of the resulting residues was examined on TLC by using different

solvent systems and similar fractions, identified by their TLC behavior mixed together.

Fractions 27-61 (eluted with Il-hexane: Chloroform 70:30), fractions 73-95 (eluted with ethyl acetate: ethanol 50:50). Fractions 2761 (eluted with Il-hexane: Chloroform 70:30) gave a solid designated as compound 1 (125mg), fractions 73-95 (eluted with ethyl acetate: ethanol 50:50, v/v) gave a solid designated as compound 2 (168mg).

5.8.3 THIN LAYER CHROMATOGRAPHY STUDIES

TLC characterization of Polycarpaea corymbosa whole plant

Chromatography is the separation of mixture of compounds into individual compounds using a mobile phase and stationary phase. The principle of separation is either partition or adsorption. The constituent which is having more affinity for mobile phase moves with it. while the constituent which is having more affinity Ibr stationary phase gets adsorbed on it. This way various compounds appear as a band on the TLC plate, having different Rt• values. The EEPC was subjected to TLC study for the separation and identification of its components.

TLC is an important analytical tool in the separation, identification and estimation of different

classes of natural products. This technique allows the separation of different components by the differential migration of solute between two phases- a stationary phase and a mobile phase. The main principle involved in this technique is adsorption chromatography.

Preparation of plates

Hundred grams of silica eel G was v\eighed and made into a homogenous suspension with 200ml of distilled water to form slurry. "I he slurry was added into a TLC applicator. which was adjusted to 0.25mm thickness on flat glass plate of different dimensions (l OX2, 10X5, 30X5, 20X10 cm etc,). The coated plate was allowed to dry in air, followed by heating at 100 to 105 ^{0}C for one hour, cooled and protected from moisture. Before using, the plate was activated at I for ten minutes.

Separation of components

The ethanol extract of the plant was dissolved in methanol separately and spotted using a capillary tube on TLC plates 2cm above from the bottom of the plate.

Selection of a mobile phase

The selection of a mobile phase depends upon various factors as mentioned below:

- Nature of substance to be separated
- 3 Nature of stationary phase
- Chromatographic mode

The selection of solvent systems was based on increasing the order of polarity. The different spots developed in each system were detected by means of iodine staining.

5.8.4 CHARACTERISTIC ANALYSIS OF THE ISOLATED COMPOUNDS FROM ETHANOLIC EXTRACT OF Polycarpaea corymbosa

WHOLE PLANT USING VARIOUS ANALYTICAL TECHNIQUES [132]

The isolated compounds 1-2 were characterized and identified by IR, NMR and GCMS spectrophotometric methods and chemical structure of the compounds were subsequently elucidated.

Spectral analysis of the compounds using FT-IR

The FT-IR spectra of the isolated compounds from the whole plant of ***Polycarpaea corymbosa*** by using Nicolet 170SX. The spectral resolution for the Nicolet 170SX was 0.25cm-l, and the spectral data were stored in the database at intervals of 0.5cm-l at 2000-4000cm^{-l} ,

and of 0.25cm-l at 2000-400cm -l . The samples were measured by using KBr disc methods.

Spectral analysis of the compounds using ^{I}l-I NMR

The ^{1}H NMR spectra of the isolated compounds from the whole plant ***Polycarpaea corymbosa*** by using a JEOL AL-400 (399.65 MHz). The measuring conditions for the most of the spectra were as follows: flip angle of 22.5-30.0 degrees, pulse repetition time of 30s. The long pulse repetition time and small flip angle was used to ensure precise relative intensities. The ^{I} H NMR chemical shifts were referred to TMS in organic solvents and TSP in mo.

Spectral analysis of the compounds using ^{13}c NMR

The ^{13}C NMR spectra of the compounds isolated from the whole plant ***Polycarpaea corymbosa*** by using a Bruker AC-200 (50.323 MHz). The measuring conditions for the most of the spectra were as follows: a pulse flips angle of 22.45-45 degrees, a pulse repetition time of 4-7 seconds, and a resolution of 0.025-0.045 ppm. The chemical shift was referred to a TMS for all solvents.

Spectral analysis of the compounds using GC-MS

Mass spectra of the compounds isolated from the whole plant ***Polycarpaea corymbosa*** by using a

JEOL JMS-700 by the electron impact method where an electron is accelerating voltage 75eV and an ion accelerating voltage of 8-1 OnV. The reservoir inlet systems were used. The dynamic range for the peak intensities were 3 digits and the accuracy of the mass number was 0.5.

5.9 IN VITRO ANTIOXIDANT AND CYTOTOXICITY FOR ISOLATED COMPOUNDS

The isolated compound 1 namely 6-methoxyflavone and compound 2 namely 3, 5, 7-trihydroxy-2-(4-methoxyphenyl)- 41-1 - chromen — 4 — one from ethanolic extract of Polycarpaea corymbosa whole plant was evaluated for in vitro anti-oxidant (DPPH, superoxide radical scavenging and nitric oxide scavenging activity) and anti cancer [human cancerous liver cell lines (HepG2), human colon cancer cell lines (HT29)] activity by the method adopted for screening of the extracts.

5.10 P53 GENE EXPRESSION FOR ISOLATED COMPOUNDS IN HepG2 CELLS

The P53 gene expression for isolated compounds was studied by using HepG2 cells. The

total RNA was isolated from HepG2 cells for real time-PCR analysis of p53 genes [i33] HepG2 cells were cultured for 24 h then incubated for 48 h with fresh medium containing isolated compounds (test cells) or 1% DMSO (control cells). The concentrations of compound I and compound 2 used were 2.50, 7.50 and 10.45 ug/mL and triplicate cell cultures were exposed to each concentration. The quantity and quality of the extracted RNA were determined by measuring the absorbance at 230, 260 and 280 nm of the spectrophotometer and 2 characteristic bands of RNA were appeared by gel electrophoresis. The primers were obtained from NCBI site and real-time PCR was performed by using Syber Green dye. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was preserved as a reference gene.

Primer sequences for real time-PCR.

Name of Primer	forward	Primer reverse gene
p53	TGAGGTGCGTGTTTGTGCCTGT	TCGGAACATCTCGAAGCGCTCA
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTT

Determination of p53 by flow cytometry[133]

The tested and control cells were fixed V\ith 4 ^{0} 0 paraformaldehyde (10 min) and then permeabilized with 0.1% PBS-Tween for 20 min [i33] . The cells were incubated in I-fold PBS/ 10% normal goat serum/ 0.3 mol/L glycine to block non-specific protein-protein interactions followed by the Anti-p53 antibody [DO-I] (USA) for 30 min at 22^{0} C and then analyzed using FAC Star caliber (Becton Dickinson).

CHAPTER 6: RESULTS

6.1 PRELIMINARY PHYTOCHEMICAL SCREENING OF EXTRACTS OF *Polycarpaea corvmhosa*

Whole plant of Polycarpaea corymbosa was collected from Tirunelveli District, Tamil Nadu. India and plant authentication was done by the Botanical survey of India. The whole plant of Polycarpaea corymbosa was dried under shade, segregated, pulverized by a mechanical grinder and passed through a 40 mesh sieve. The dried powder of whole plant of Polycarpaea corymbosa was extracted sequentially by hot continuous percolation method using Soxhlet apparatus, using different solvents like petroleum ether, ethyl acetate and ethanol. The resultant extracts were concentrated by using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained. The extracts were stored in screw cap vials at 4^0 C until further use. The percentage yields of the whole plant of Polvcarpaea corvmhosa are shown

Table 1: Percentage yield of extracts of the whole plant of P.corymbosa

Plant name	Parts used	Method of extraction	Solvent system	Percen tage yield (%w/w)
P. corymbosa	Whole plant	Continuous Hot Percolation	Petroleum ether Ethyl acetate Ethanol	9.87 7.88 16.56

6.1.1 PRELIMINARY PHYTOCHEMICAL SCREENING

The various extracts of Polycarpaea corymbosa were subjected to screening for its phytochemical constituents. The phytochemical screening results are shown in Table 2.1 he petroleum ether extract of Polycarpaea corymbosa was contains phytosterols, fixed oils & fats. Ethyl acetate extracts containing alkaloids, carbohydrates, glycoside, phenolic compounds & tannins, protein and amino acid compounds, saponins and fixed oils & fats. The ethanolic extract containing alkaloids, carbohydrates, glycoside, phenolic compounds, saponins, tannins, protein and aminoacid & flavonoids.

Table 2: Ph tochemical anal sis of various extracts of P.corvmbosa

S.N 0.	Test	Petroleum ether	Ethyl Acetate	Ethanol
	Alkaloids	-	+	+
	Carbohydrates glycosides	-	+	+
111	Phytosterols	+	-	-

IV	Fixed oil and fats	+	+	-
V	Saponins	-	+	+
VI	Phenolic compounds and tannins	-	+	+
Vil	Protein and Amino Acid	-	+	+
Vill	Gum and Mucilage	-	-	-
IX	Test for flavanoids	-	-	+

+ positive
- Negative

6.2 In vitro ANTI-OXIDANT ACTIVITY RESULTS AND ANALYSIS

The various extracts petroleum ether, ethyl acetate and ethanol extract of the whole plant of Polycarpaea corymbosa were subjected to in vitro antioxidant activity.

6.2.1.DPPH radical scavenging activity of the extracts

The results obtained for the DPPH radical scavenging activity of the extracts of the whole plant of

Polycarpaea corymhosa are presented in Table 3 and Fig. 3.

From Table 3 and Fig. 3, it was observed that the maximum scavenging activity at

1000gg/ml and IC5() value of pet ether extract was found to be 47.53% and 1250gg/ml and for ethyl acetate extract, it was found to be 53.45% and 875gg/ml while for ethanolic extract of Polycarpaea corymbosa was found to be 72.02% and 225gg/ml respectively, while for standard rutin it was found to be 70.65% and 270gg/ml respectively.

Table 3: DPPH scavenging potential of various extracts of the whole plant of Polvcarpaea corymbosa

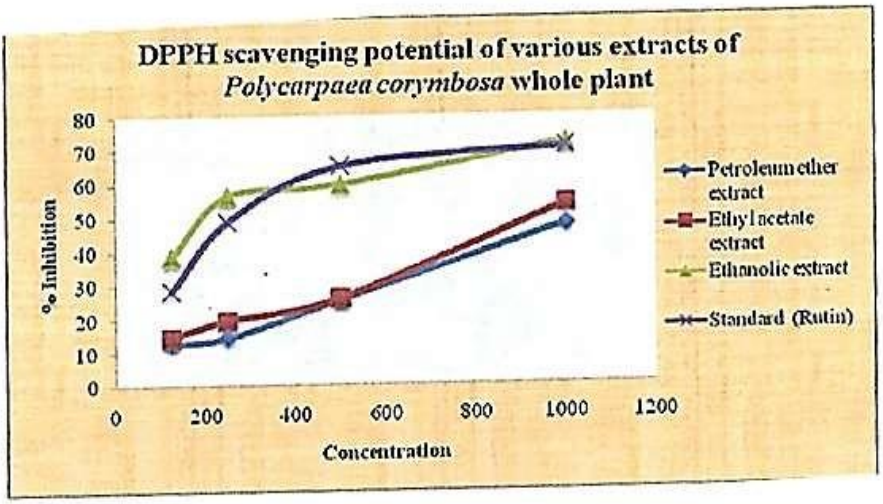
	Conc. in Itg/ml		% scavenging ± SEM*		
		Pet ether extract	Ethyl acetate extract	Ethanolic Extract	Standard (Rutin)
1	125	12.64± 0.03	14.61	38.65± 0.16	28.39± 0.25
2	250	14.43± 0.09	19.58 ± 0.06	56.51 ± 0.03	48.98± 0.46
3	500	25.45± 0.30	25.97 ± 0.27	59.76 ± 0.18	65.18 ± 0.13

4	1000	47.53 0.02	53.45 ± 0.14	72.02 ± 0.43	70.65 ± 0.20
IC50 (ptg/ml)		1250	875	225	270

All values are expressed as mean ± SEM for three determinations

• Values that are not sharing a common superscript letter in the same column differ significantly differ at P<O.05 (DMRT).

Fig 3: DPP H scavenging potential of various extracts of the whole



6.2.2. Inhibition of superoxide anion radical scavenging activity

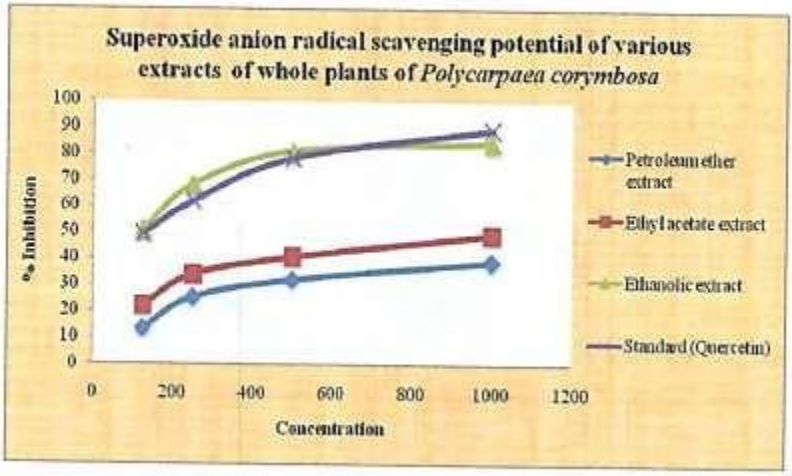
The results obtained for superoxide anion radical scavenging potential of the extracts of the whole plant of Polycarpaea corymbosa are presented in Table 4 and Fig. 4.

Table 4: Superoxide anion radical scavenging potential of various extracts of whole plant of Polycarpaea corymbosa

S. No	Conc. in pg/ml	**% inhibition ± SEM***			
		Petroleum ether extract	Ethyl acetate extract	Ethanolic extract	Standard (Quercetin)
1	125	13.26 0.48	21.87 0.33	51.11 +0.50	49.44 ± 0.48
2	250	25.17± 0.42	33.84 ± 0.58	68.22 0.11	61.88 4: 0.49
3	500	32.06 ± 0.52	40.67 ± 0.22	81.10 ± 0.31	78.39 ± 0.18
4	1000	39.14 ± 0.43	49.42± 0.15	84.41 ± 0.03	89.28 ± 0.09
IC50 in pg/ml		1365	1005	110	145

***All values are expressed as mean ± SEM for three determinations ❖ Values that are not sharing a common superscript letter in the same column differ significantly differ at (DMRT).**

Fig 4: Superoxide anion radical scavenging potential of various extracts of whole plant of Polycarpaea corymbosa



From Table 4 and Fig. 4, it was observed that the maximum superoxide radical scavenging activity at 1000gg/ml and IC50 value of petroleum ether extract was found to be 39.14% and 1365gg/ml, for ethyl acetate extract it was found to be 49.42% and 1005gg/ml while for ethanol extract of *Polycarpaea corymbosa* it was found to be 84.41% and 110 pg/ml respectively, while for standard quercetin, it was found to be 89.28% and 145gg/ml respectively.

6.2.3. Nitric oxide radical scavenging activity

The results obtained for nitric oxide radical scavenging potential of the extracts of the whole plant of Polycarpaea corymbosa are presented in Table 5 and Fig. 5.

Table 5: Nitric oxide of

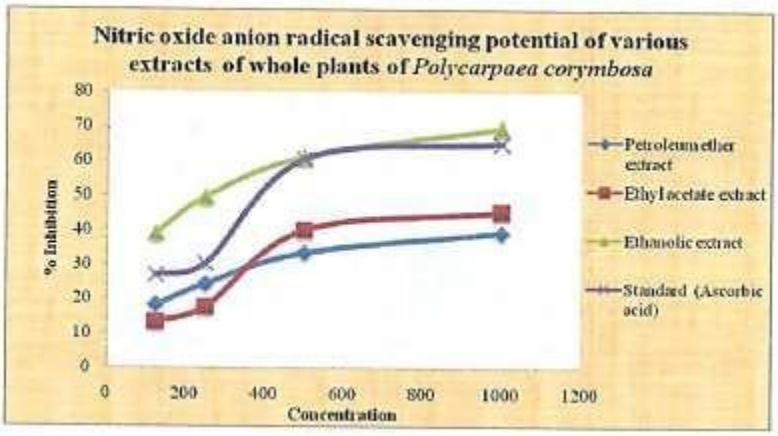
S. No	Conc. in Itg/ml		^{0}/0 inhibition ± SEM*		
		Petroleum ether extract	Ethyl acetate extract	Ethanolic extract	Standard (Ascorbic acid)
	125	18.26± 0.09	13.36± 0.16	39.20±0.02	26.87 ± 0.08
2	250	24.39± 0.19	17.71± 0.50	49.73±0.29	30.30± 0.05
3	500	33.42± 0.27	40. 12± 0.43	61 .29±0.70	60.64± 0.02
4	1 000	39.41 ± 0.14	45.49± 0.16	69.65±0.05	65.23± 0.01
IC_{50} in µg/ml		1320	1090	255	410

***All values are expressed as mean ± SEM for three determinations**

- **Values that are not sharing a common superscript letter in the same column differ significantly differ at P<0.05 (DMRT).**

From Table 5 and Fig. 5, it was observed that the maximum of nitric oxide radical scavenging activity at 1000µg/ml and IC_{50} value of petroleum ether extract of Polycarpaea corymbosa was found to be 39.41% and 1320µg/ml, for ethyl acetate extract, it was fòund to be
45.49% and 1090µg/ml and for ethanol extract, it was found to be 69.65% and 255µg/ml respectively while for standard ascorbic acid was found to be 55.23% and 410µg/ml respectively.

Fig 5: Nitric oxide anion radical scavening potential of various extracts of whole plant of Polycarpaea corymbosa



6.2.4. Hydroxyl radical scavenging activity

The results obtained for hydroxyl radical scavenging activity of the extracts of the whole plant of Polycarpaea corymbosa are presented in Table 6 and Fig. 6.

From Table 6 and Fig. 6, it was observed that the maximum of hydroxide radical scavenging activity at 1000µg/ml and IC_{50} value of petroleum ether extract of Polycarpaea corymbosa was found to be 29.48% and 1480µg/ml, for ethyl acetate extract, it was found to be
65.36% and 595µg/ml and for ethanol extract it was found to be 62.58% and 265µg/ml respectively, while for that of standard rutin was found to be 75.23% and 280µg/ml.

Table 6: Hydroxyl radical scavenging potential of various extracts of whole plant of Polycarpaea corymbosa

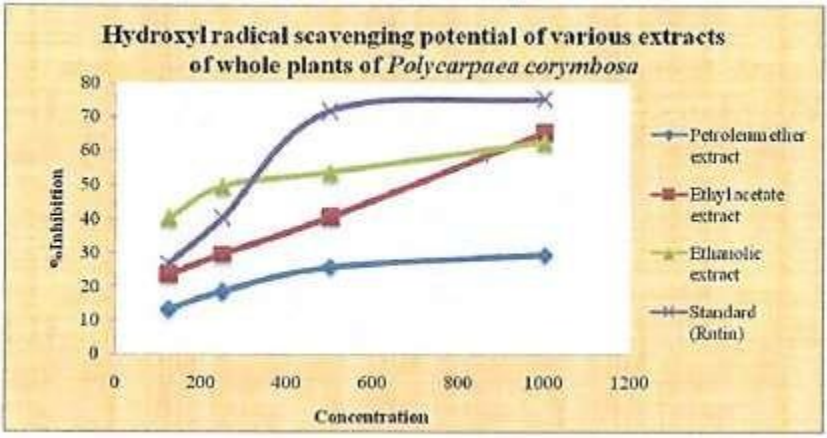
S.	Conc. in pg/ml		**% inhibition ± SEM***		
		Petroleum ether extract	Ethyl acetate extract	Ethanolic extract	Standard (Rutin)
1	125	13.26 0.47	23.66 0.04	40.36 ± 0.07	26.87 ± 0.09

2	250	18.47 ± 0.96	29.78 ± 0.93	49.63 ± 0.31	40.38 ± 0.08
3	500	25.66 ± 0.92	40.66 ± 0.22	53.74 ± 0.97	71.64 ± 0.43
4	1000	29.48 +0.07	65.36 ± 0.02	62.58 ± 0.11	75.23 0.02
IC_{50} in µg/ml		1480	595	265	280

All values are expressed as mean ± SEM for three determinations

- ***Values that are not sharing a common superscript letter in the same column differ significantly differ at P<O.05 (DMRT).***

Fig 6: Hydroxyl radical scavenging potential of various extracts of whole plant of Polycarpaea corymbosa



6.2.5. Total phenolic and flavonoid content

The results obtained for total phenolic and flavonoid content of various extracts of the whole plant of Polycarpaea corymbosa are depicted in Table 7 and Fig. 7.

The phenolic and flavonoid content in ethanolic extract of Polycarpaea corymbosa was found to 4.60 ± 0.05 mg and 3.63±0.93 mg respectively which was higher than that present in other extracts.

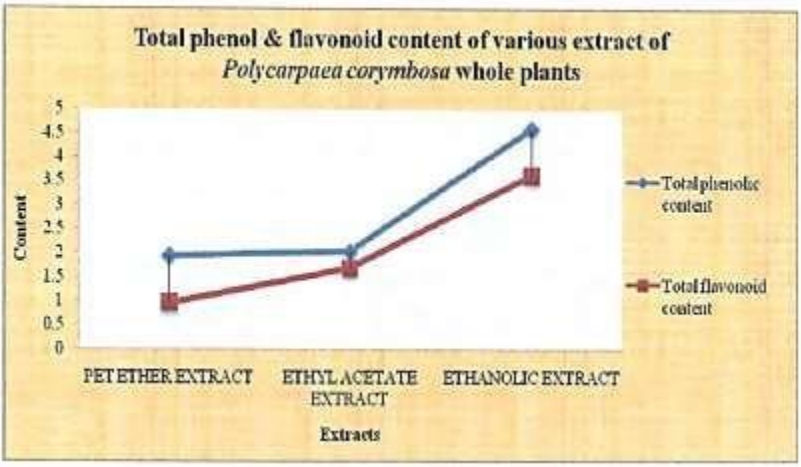
Table 7: Total phenol and flavonoid content of various extracts of whole plant Polycarpaea corymbosa

s. No	Name of the extract	Total phenol content (mg/g of Gallic acid) ±SEM	Total flavonoid content (mg/g of Catechol) ±SEM *
	Petroleum ether extract	1.94 ±0.22	0.97 ±0.06
2	Ethyl acetate extract	2.04± 0.73	1.7± 0.09
3	Ethanol extract	4.60 ± 0.05	3.63 ± 0.93

**All values are expressed as mean ± SEM for three determinations*

- *Values that are not sharing a common superscript letter in the same column differ significantly differ at (DMRT).*

Fig 7: The total phenolic and flavonoid content of various extracts of whole plant of Polycarpaea corymbosa



6.3 In-vitro CYTOTOXICITY

6.3.1 Percentage growth inhibition of different cell lines by various extracts of whole plant of Polycarpaea corymbosa

The results obtained for inhibition of the growth of various cell lines by various extracts of the

whole plant of Polycarpaea corymbosa are shown in Table 8 and Fig.8 and 9-28.

The results obtained for inhibition of the growth of control cell lines by doxorubicin are shown in Fig.9-13.

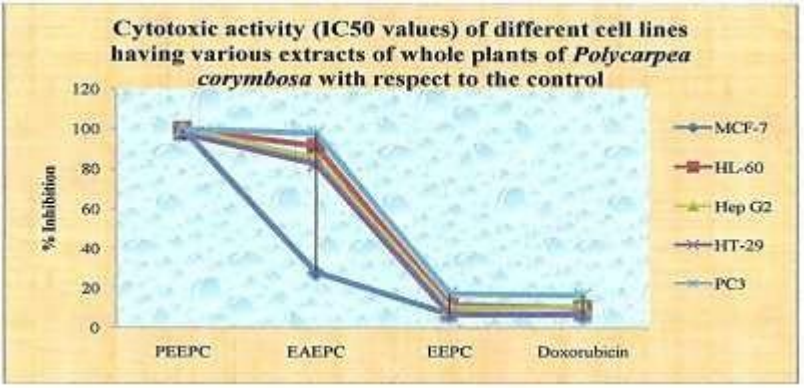
Table 8: Cytotoxic activity (IC50 values) of different cell lines by various extracts of the whole plant of Polycarpaea corymbosa

S. No.	Treatment	Conc. in pg/ml			Cell lines		
			MCF-7	I-IL-60	HepG2	HT29	PC3
	PEEPC	10	> 100	> 100	> 1 00	97.55± 0.75	> 100
2	EAEPC	10	28.05± 0.02	91.55± O.Ol	85.55± 0.71	82.69± 0.05	98.02± 0.05
3	FEPC	1 0	6.80± 0.05	11.50± 0.45	10.00± 0.02	7.05± 0.60	17.20± 0.85
4	DXN	1 0	6.50± 0.07	10.25± 0.93	10.780± 0.1	7.25± 0.1 1	16.47± 0.24

- Values are means of three independent analyses ± standard deviation
- Values that are not sharing a common superscript letter in the same column differ significantly differ at (I)MRT).
- PEEPC- Pet ether extract of Polycarpaea corymbosa; EAEPC -

Ethyl acetate extract of Polycarpaea corymbosa; EEPC - Ethanolic extract of Polycarpaea

corymbosa; DXN- Doxrubicin. **Fig: 8 Cytotoxic activity (IC50 values) of different cell lines by various extracts of the whole plant of Polycarpaea corymbosa**



The ethanolic extract of plant extract was active on all cell lines (MCF-7, HL-60, HepG2, I-IT-29 and PC3). The IC50 value of ethanolic extract P.corymbosa on various cell lines like MCF-7 (6.80±0.05), HL-60 (11.50±0.45), HepG2 (10.00±0.02), HT-29 (7.05±0.60) and PC3 (17.20±0.85) (Fig. 24-28). The ethyl acetate extract was found moderately active on MCF 7(28.05±0.02) cell line and inactive on HL-60, HepG2, HT-29 and PC3 cell lines (Fig. 19-23).

On the other hand petroleum ether extract were not found active on all cell lines (Fig. 14-18)

Fig: 9 Effect of Doxorubicin on MCF-7 cell line	Fig: 10 Effect of Doxorubicin on HL-60 cell line

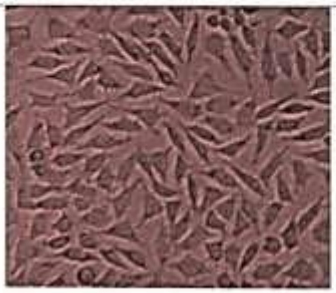
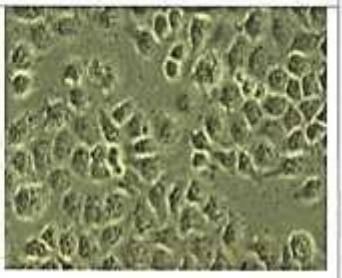

	

Fig: 11 Effect of Doxorubicin on Hep G2 cell line	Fig:12 Effect of Doxorubicin on HT-29 cell line

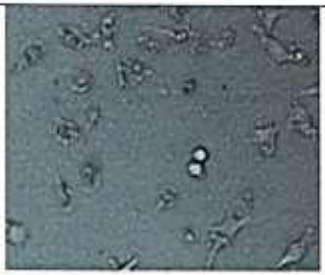
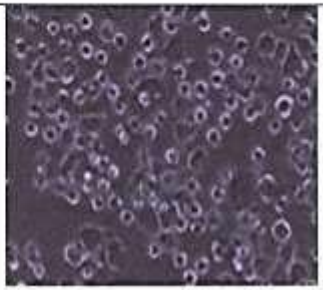

	

Fig:13 Effect of Doxorubicin on PC3 cell line	
	

Percent Growth inhibition of different cell lines by Petroleum ether Extract of *Polycarpaea corymbosa*

Fig: 14 Petroleum ether extract of *Polycarpaea corymbosa* on MCF-7 cell line

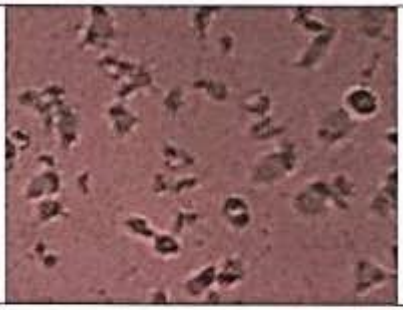


Fig: 15 Petroleum ether extract of *Polycarpaea corymbosa* on HL-60 cell line

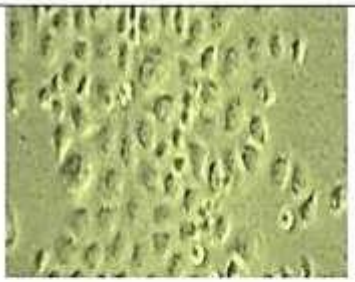


Fig: 16 Petroleumether extract of *Polycarpaea corymbosa* on Hep G2 cell line

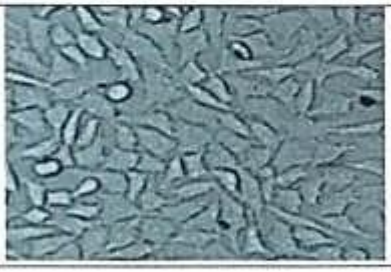


Fig: 17 Petroleum ether extract of *Polycarpaea corymbosa* on HT-29 cell line

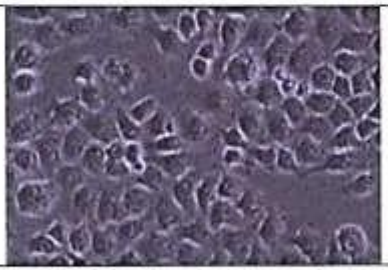


Fig: 18 Pet ether extract of *Polycarpaea corymbosa* on PC3 cell line

Percent Growth inhibition of different cell lines by ethyl acetate extract of *Polycarpaea corymbosa*

Fig: 19 Ethyl acetate Extract of *Polycarpaea corymbosa* on MCF-7 cell line

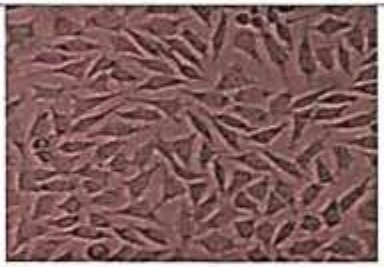


Fig: 20 Ethyl acetate extract of *Polycarpaea corymbosa* on HL-60 cell line

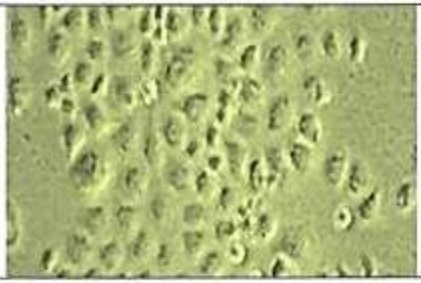


Fig: 21 Ethyl acetate extract of *Polycarpaea corymbosa* on Hep G2 cell line

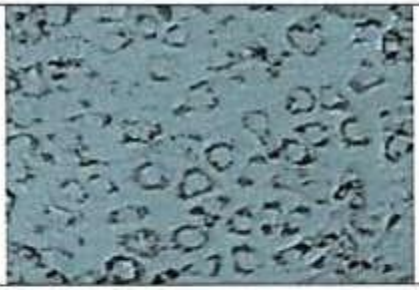


Fig: 22 Ethyl acetate extract of *Polycarpaea corymbosa* on HT-29 cell line

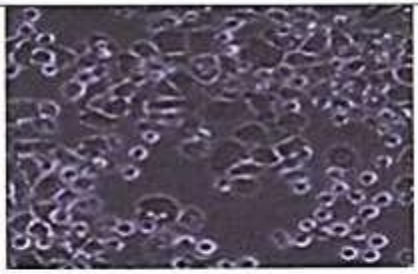


Fig: 23 Ethyl acetate extract of *Polycarpaea corymbosa* on PC3 cell line

Percent Growth inhibition of different cell lines by ethanol Extract of *Polycarpaea corymbosa*

Fig:24 Ethanol Extract of *Polycarpaea corymbosa* on MCF-7 cell line

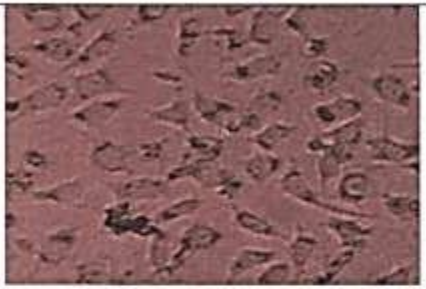


Fig: 25 Ethanol Extract of *Polycarpaea corymbosa* on HL-60 cell line

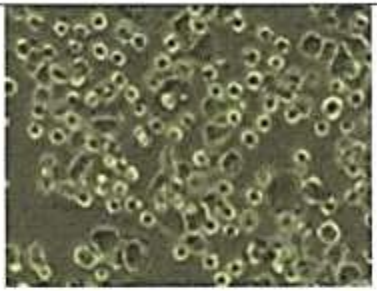


Fig: 26 Ethanol Extract of *Polycarpaea corymbosa* on HepG2 cell line

Fig: 27 Ethanol Extract of *Polycarpaea corymbosa* on HT-29 cell line

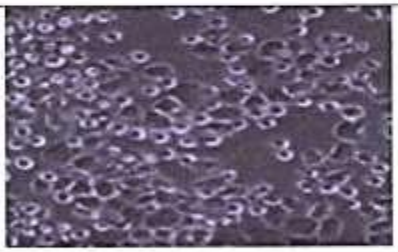


Fig: 28 Ethanol extract of *Polycarpaea corymbosa* on PC3 cell line

6.4 ACUTE TOXICITY RESULTS

6.4. I Acute toxicity studies on various extracts from whole plant of Polrcarpaea corrmbosa (Lamk)

The acute toxicity studies of various extracts of the whole plant of Polycarpaea corymbosa (Lamk) was carried out as per OECD-423 guidelines are presented in Fig. 2

The results of acute toxicity study revealed that LD50 values of various extract of whole plant of Polycarpaea corymbosa (Lamk) whole plant were high and apparently showed the safety of those extracts.

The results of general behavioural studies in mice after administration of various extracts of Polycarpaea corymbosa whole plant are presented in Table 9.

Table 9: Results of general behavioural studies in mice on administration of various extracts of Polycarpaea corymbosa (Lamk) at a dose of 2000mg/kg

s. No	Parameters	Pct ether extract	Ethyl acetate extract	Ethanolic Extract

I	Motor activit	Absent	Absent	Absent
2	Tremors	Absent	Absent	Absent
3	Convulsion	Absent	Absent	Absent
4	Straub reaction	Absent	A bsent	Absent
5	Pile erection	Absent	Absent	Absent
6	Loss of li ht reflex	Absent	Absent	Absent
7	Sedation	Absent	Absent	Absent
8	Muscle relaxation	Absent	Absent	Absent
9	H nosis	Absent	Absent	Absent
10	Anal yesia	Absent	Absent	Absent
I l	Ptosis	Absent	Absent	Absent
12	Lacrimation	Absent	Absent	Absent
13	Diarrhoea	Absent	Absent	Absent
14	Chane in colour	No change	No change	No change

	skin			

From Table 9, it can be observed that the treatment of mice with various extracts of the whole plant of Polycarpaea corymbosa (Lamk) showed no changes in the autonomic or behavioural responses in mice. The zero percent mortality was obtained for various extracts of the whole plant of Polycarpaea corymbosa (Lamk) and hence the extracts were found to be non toxic up to the dose of 2000mg/kg.

6.5 ANTI-CANCER STUDY

Anticancer activity was investigated for the various extracts obtained from the whole plant of Polycarpaea corymbosa in mice.

6.5.1. Animal Body weight, tumor incidence and tumor volume

The body weight, Mean survival time, percentage in life span. tumour volume and tumour cell count observed for various extracts of the whole plant of Po/vcarpaea corvmho.st/ are depicted in Table 10 and Fig.29-33.

It can be seen from Table 10 and Figs. 29-33, animals treated with EAC alone showed a increased

in body weight, decrease in Mean survival time and percentage in life span, increased in tumour volume, PC V and viable cell count than the plant extract treated Group.

Post treatment with ethanolic extract of Polvcarpaea *corymbosa* and standard drug (5F U) significantly reduced the weight gain of animals in group V and VI. Similar result was not found in other extracts treated animals (group Ill & IV). Tumor mice died after 18 days of inoculation whereas the life span of group V and VI mice was observed to be 28 days. In EAC bearing mice, the asciatic volume and viable cell count was noted to be increased. Supplementation of Polycarpaea corymbosa reverses the above alterations and incremented the nonviable cell count indicating its cytotoxicity towards cancer cells.

Table 10: The Body weight, Mean survival time, percentage in life span, tumor volume, PC V and tumor cell count of various experimental animals treated Polrcarpaea corrmbosa extracts

Groups	Body-weig t	Mean survival time (days)	% in life span	Tumor volume	Packed cell volume	Tumor cell count IX 10^7 cells/ml .	
						Viable	Nonviable
EAC control	26.10 ± 1.30	17.08 ± 0.68	-	2.81 ± 0.11	0.10	1.82± .92	0.34 ± 0.02
EAC + 200 mg of PEEPC	27.20 ± 1.90	1 8.610.98	28.06	2.67 ± 0.08	0.81	10.32 ± 0.87	0.38 ± 0.1 1
EAC I200 mg of EAEPC	26.88 ± 2.01	20.19 ± 0.71	37.48	2.10 ± 0.09	0.98 ± 0.17	9.11± 0.25	0.51± 0.22

EAC + 200 mg of EEPC	26.91±0.91	29.18 ± 0.91	59.67	I.90± 1.16*	0.53± O. 07 *	7.62±0.07*	0.68± 0.03*
EAC l20mg of 5FU	27.01±0.06	32.17 ± 2.01	86.93	-	-	-	-

- Values are expressed as mean ± S.D. for 6 mice in each group.
- Tumor volume was measured using the formula V = 4/371 (1)1/2) (1)2/2) (1)3/2), where DI, 1)2, and 1)3 are the three diameters (in mm) of the tumor.
- Values not sharing a common superscript letter in the same row differ significantly at $p < 0.05$ (DMRT).
- PEEPC- Pet ether extract of Polycarpaea corymbosa; EAEPC - Ethyl acetate extract of Polycarpaea corrmbosa; EEPC - Ethanolic extract of Polvcarpaea corrmbosa; 5-FC- 5 - Fluorouracil.

Fig: 29 Body weights of various experimental animals treated with *Polycarpaea corymbosa*

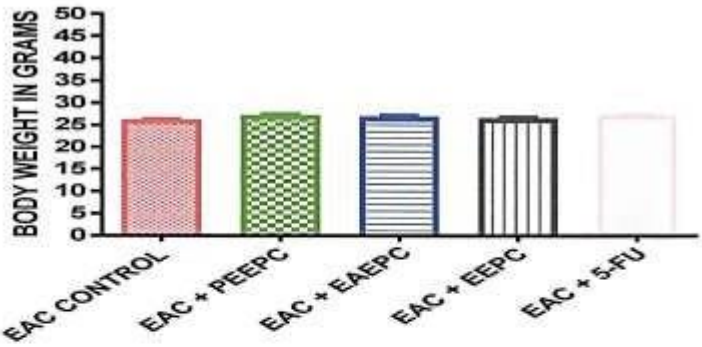


Fig: 30 Mean Survival Time of various experimental animals treated with *Polycarpaea corymbosa*

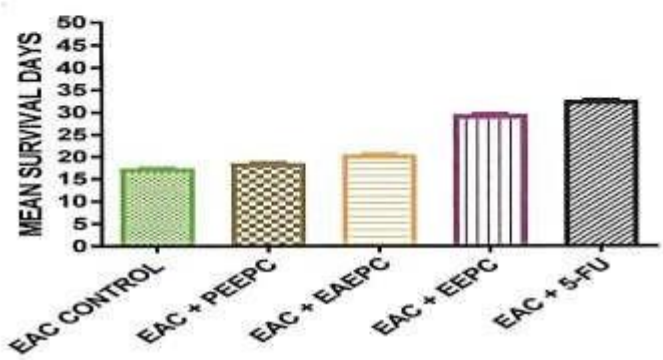


Fig: 31 Percentage in Life span of various experimental animals treated with *Polycarpaea corymbosa*

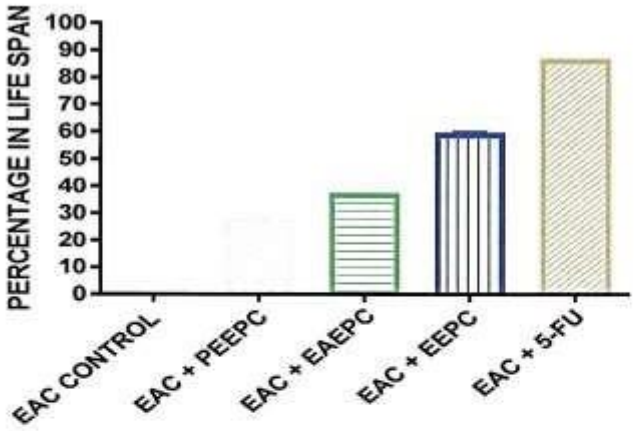


Fig: 32 Tumor volume of various experimental animals treated with *Polycarpaea corymbosa*

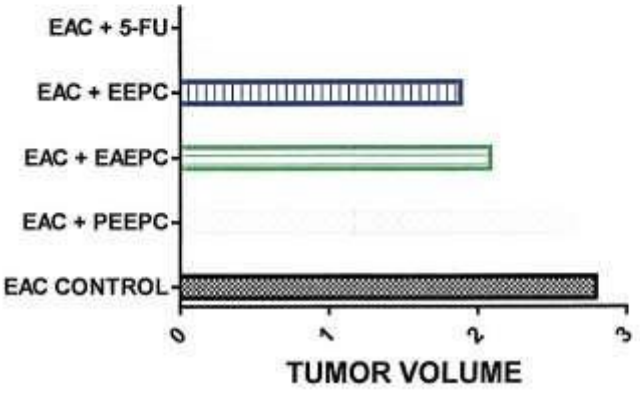
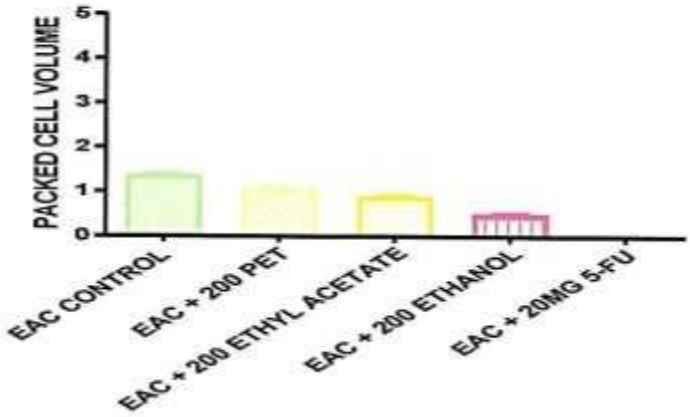


Fig: 33 Packed Cell Volume of various experimental animals treated with *Polycarpaea corymbosa*



6.5.2. Haemotological Parameters

The haemotological parameters namely RBC, HI), Total WBC count observed for various extracts of the whole plant of Polycarpaea corymbosa are depicted in Table 12 and Fig 34-37.

The EDTA-anticoagulated blood was analyzed for the abnormalities of haemotological parameters namely RBC, HI), Total WBC count and differential count. In group Il mice, RBC, HI), Lymphocytes and monocytes were found to be decremented whereas total WBC count and neutrophilis

were observed to be incremented. Ethanolic extract of Polycarpaea corymbosa and 5-Fluorouracil administration to EAC bearing animals has restored RBC, Hb, total WBC count and differential count to a significant extent. This indicates the protective nature of the extract on the haematopoietic system.

Table 11: Anti tumour effect of various extract of Polycarpaea corymbosa in normal and experimental group of mice

Groups	RBC (millions/ cu.mm)	(g/dl)	WBC (10^3/cu.mm)
Normal	4.54 0.28	1 1.92 ± 0.31	9.75 0.53
EAC control	2.1 1	6.92 0.1 1	15.73 0.36
EAC 200 mg of PEEPC	3.45 0.1 1	8.44 0.96	13.31 + 0.35
EAC + 200 mg of EAEPC	0.22	10.08 0.62	1 1.78 ± 0.42

EAC + 200 mg ofEEPC	4.21 ± 0.72	1 1.2 0.52	10.93 0.39
EAC + of	4.48 0.21	1 1.59 ± 0.17	9.67 ± 0.35

- Values are expressed as mean ± S.D. for 6 mice in each group.
- Values not sharing a common superscript letter in the same row differ significantly at $p < 0.05$ (DMRT).
- PEEPC- Pet ether extract of Polycarpaea corymbosa; EAEPC - Ethyl acetate extract of Polycarpaea corymbosa; EEPC - Ethanolic extract of Polycarpaea corymbosa; 5-FC- 5 - Fluorouracil.

Table 12: Antitumor activity of various extract of Polvcarpaea corvmbosa on haematological parameters in normal and experimental group

Goups	**Differential count (%)**		
	Neurophils	Lymphocytes	Monocytes

Normal	16.40±0.63	81.48±3.12	1.49±0.11
EAC — control (IXI 0^6 cells/ mouse)	61.17 ± 2.17	35.24 ± 1.24	0.84±0.03
EAC + 200 mg PEEPC	17.04 0.62	40.92 2.44	0.95±0.06
EAC + 200 mg of EAEPC	38.27± 2.31	53.42 ± 2.79	1.07±0.06
EAC + 200 mg of EEPC	52.27±2.31	71.42 ± 2.79	1.25±0.06
EAC + 20mg of 5-FU	54.62 ± 1.97	75.11±1.98	1.32±0.04

- Values are expressed as mean ± S.D. for 6 mice in each group.
- Values not sharing a common superscript letter in the same row differ significantly at $p < 0.05$ (DMRT).
- PEEPC- Pet ether extract of Polycarpaea corymbosa;

EAEPC - Ethyl acetate extract of Polvcarpaea corymbosa; EEPC - Ethanolic extract of Polvcarpaea corymbosa; 5-FC- 5 - Fluorouracil.

Fig 34: Hemoglobin content of various experimental animals treated with *Polycarpaea corymbosa*

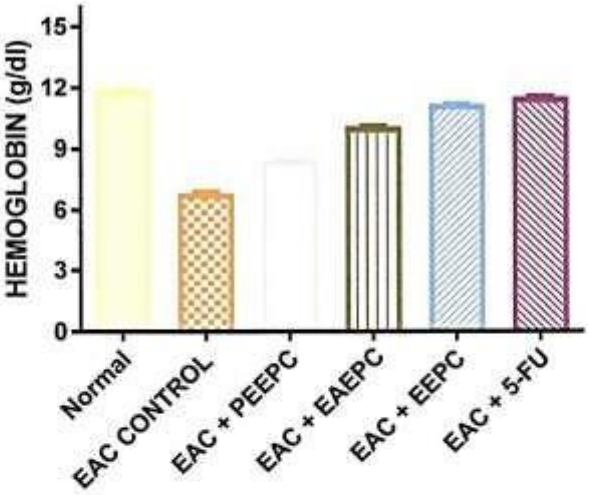


Fig. 35 RBC content of various experimental animals treated with *Polycarpaea corymbosa*

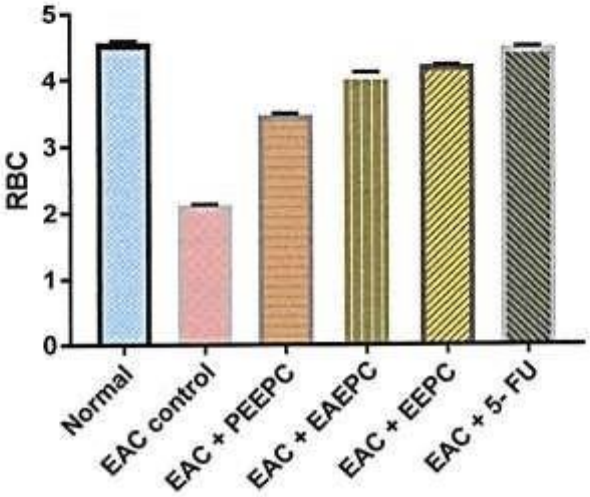


Fig: 36 White blood Cell count of various experimental animals treated with *Polycarpaea corymbosa*

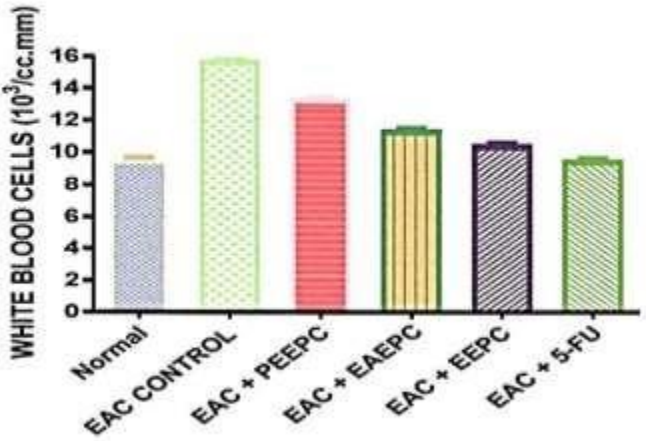
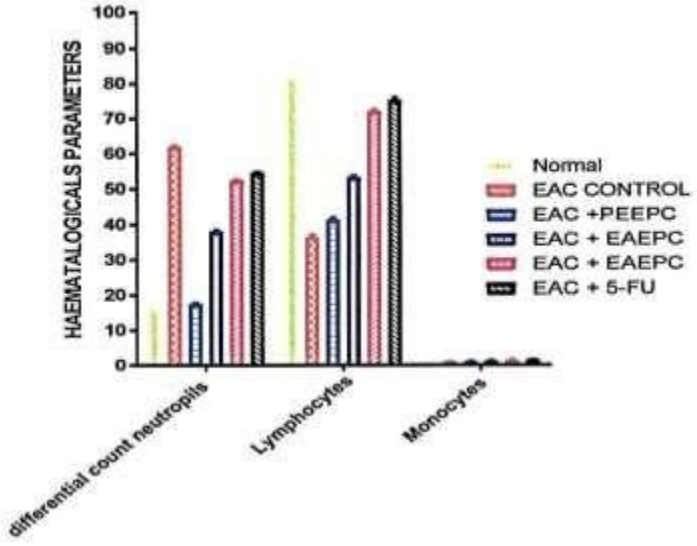


Fig: 37 White cell count of various experimental animals treated with *Polycarpaea corymbosa*



6.5.3. Lysosomal Marker enzymes, Liver Markers, Na^+/K^+ - ATPase and Mg -AT Pase, Plasma glucose, Plasma glycogen, DNA and RNA

The Lysosomal Marker enzymes, Liver Markers, Na^+/K^+- ATPase and Mg+-ATPase, Plasma glucose, Plasma glycogen, DNA and RNA levels observed for various extracts of the whole plant of Polycarpaea corymbosa are depicted in Table 13-17 and Fig. 38-42.

A liver tissue was examined for the determination of lysososme specific cancer markers (cathepsin-D, (B-D glucuronidase and acid phosphatase), liver marker enzymes (5-nuclotidase and lactate dehydrogenase), membrane bound AT Pase (Na /K AT Pase and Mg^2 ATPase), plasma glucose, liver glycogen, DNA and RNA content. In group Il mice, increased in the activities of lysosomal marker enzymes and liver marker enzyme 5-nuclotidase was observed. Administration of ethanolic extract of Polvcarpaea corvmbosa and 5-fluorouracil restored the enzvme activities to near normal. Several studies have reported that flavonoids have the abilits to stabilize the biological membranes of a cell. The restoration of lysosomal and liver marker enzymes towards normal by Polycarpaea corymbosa extract may due to the presence higher amount of flavonoid.

The levels of Plasma glucose and liver glycogen were significantly decreased in EAC mice. In group V mice (Ethanolic extract of Polycarpaea corymhosa treated) and group VI (5fluorouracil treated) ameliorated levels of plasma

glucose and liver glycogen was observed. The elevated levels of DNA and RNA in liver of EAC-bearing mice were strikingly decreased after treatment.

Table 13: Effect of various extract of Polycarpaea corvmbosa on the activities of lysosomal markers enzymes in liver of normal and experimental group of animals.

Groups	Cathepsin-D (pmoles of tyrosine liberated/hr/mg protein)	P-l)-Glucuronidase (ltmoles of p-Nitrophenol formed/min/mg protein)	Acid phosphatise (vunole of Pi liberated /min/mg protein)
Normal	21.73 ± 0.19	24.93 ± 0.79	3.79 ± 0.1 1
EAC— control (l X 10^6 cells/ mouse)	1 .75	38.78 ± 1 .25	9.14±0.24
EAC + 200 mg of PEEPC	33.27 ± 1.53	33.14± 1.17	8.75 ± 0.80

EAC + 200 mg of EAEPC	29.30 ± 1 .10	30.10± 0.70	6.27±0.20
EAC + 200 mg of EEPC	26.12 ± 1 .27	25.90 0.59	4.18 ± 0.14
EAC + 20mg of 5-	22.52± 0.77	24.32 ± 0.82	3.38 ± 0.09

- **Values are expressed as mean ± S.D. for 6 mice in each group.**
- **Values not sharing a common superscript letter in the same row differ significantly at p < 0.05 (DMRT).**
- **PEEP C- Pet ether extract of Polycarpaea corymbosa; EAEPC**
  - Ethyl acetate extract of Polycarpaea corymbosa; EEPC -
  Ethanolic extract of Polvcarpaea corvmbosa; 5-FC- 5 - Fluorouracil.

Table 14: Effect of various extract of Polycarpaea corvmhosa on the activities of liver markers enzymes in normal and experimental group of animals.

Groups	5'-Nucleotidase (units/mg protein)	Lactate dehydrogenase (units/mg protein)
Normal	2.58 ± 0.10	1.59 ± 0.10
EAC —control (IXI 0^6 cells/ mouse)	6.46 ±0.25	0.41±0.12
EAC + 200 mg of PEEPC	5.88 ± 0.17	0.43 ± 0.53
EAC + 200 mg of EAEPC	4.37± 0.16	2.22 ± 0.09
EAC + 200 mg of EEPC	3.17 ± 0.17	1 .18 ± 0.08
EAC + 20mg of 5- FU	3.38 ± 0.16	I .25 ± 0.08

- **Values are expressed as mean ± S.D. for 12 mice in each group.**
- **Values not sharing a common superscript letter in the same row differ significantly at $p < 0.05$ (DMRT).**

- **1 unit of 5'-nucleotidase = moles of pi liberated/min; 1 unit of lactate dehydrogenase = moles of pyruvate liberated/min**
- **PEEPC- Pet ether extract of Polycarpaea corymbosa; EAEPC**
  - Ethyl acetate extract of Polycarpaea corymbosa; EEPC -
  Ethanolic extract of Polycarpaea corymbosa; 5-FC- 5 - Fluorouracil.

Table 15: Effect of various extract of Polycarpaea corymbosa on the activities of Na /K ATPase and Mg - ATPase in liver of normal and experimental group of animals.

Groups	Na 1K - ATPase (units/mg protein)	Mg -ATPase (units/mg protein)
Normal	1.88 ± 0.1 1	2.71±0.12
EAC —control (l X 10^6 cells/ mouse)	0.93 ± 0.15	1.22±0.07
EAC + 200 mg ofPEEPC	1.06±0.08	1.52± 0.09

EAC + 200 mg ofEAEPC	1.37±0.08	2.27 0.08
EAC + 200 mg of IEEPC	1.61±0.08	1.25±0.08
EAC + 20mgof 5- FUJ	1.83 ± 0.08	2.58±0.08

- **Values are expressed as mean ± S.D. for 6 mice in each group.**
- **Values not sharing a common superscript letter in the same row differ significantly at p < 0.05 (DMRT).**
- **I unit of = u moles of pi liberated/min**
- **PEEPC- Pet ether extract of Polycarpaea corvmbosa; EAEPC**
  - **Ethyl acetate extract of Polycarpaea corymbosa; EEPC - Ethanolic extract of Polycarpaea corymbosa; 5-FC- 5 - Fluorouracil.**

Table 16: Effect of various extract of Polycarpaea corymbosa on the levels of plasma glucose and liver glycogen in normal and experimental group of animals.

Groups	Glucose (mg/dl)	Glycogen tissue (mg/g)
Normal	1 15.75 ± 4.93	19.81±0.65
EAC —control (IX 10^6 cells/ mouse)	49.01±1.98	8.75 ±0.51
EAC + 200 mg of PEEPC	62.17 ± 1 .73	0.17±0.76
EAC + 200 mg of EAEPC	4.73	0.63
EAC + 200 mg of EEPC	107.73 ± 4.51	18.79 ±0.59
EAC + 20mg of 5-FU	112.33 ±5.97	19.24±0.69

- **Values are expressed as mean ± S.D. for 6 mice in each group.**

- **Values not sharing a common superscript letter in the same row differ significantly at p < 0.05 (DMRT).**
- **PEEPC- Pet ether extract of Polvcarpaea corvmbosa,• EAEPC**
  - **Ethyl acetate extract of Polycarpaea corymbosa; EEPC - Ethanolic extract of Polycarpaea corymbosa; 5-FC- 5 - Fluorouracil.**

Table 17: Effect of various extract of Polycarpaea corymbosa on the levels of DNA and RNA in liver of normal and experimental group of animals.

Groups	DNA (mg/g tissue)	RNA (mg/g tissue)
Normal	3.67 ± 0.15	10.85 0.52
EAC —control ($IX10^6$ cells/ mouse)	7.49 0.34	16.84 0.67
EAC + 200 mg of pet. Ether extract	5.98 ± 0.81	14.67 0.27

EAC + 200 mg of ethyl acetate extract	4.45 ± 0.17	13.14 ± 0.50
EAC + 200 mg of Ethanol extract	3.98 ±0.61	1 1.52 ± 0.59
EAC + 20mg of 5-FU	3.72 ±4:	1 1.08 ± 0.43

- Values are expressed as mean ± S.D. for 6 mice in each group.
- Values not sharing a common superscript letter in the same row differ significantly at $p < 0.05$ (DMRT).
- PEEPC- Pet ether extract of Polycarpaea corymbosa; EAEPC -
  Ethyl acetate extract of Polycarpaea corymbosa; EEPC -
  Ethanolic extract of Polycarpaea corymbosa; 5-FC- 5 - Fluorouracil.

Fig: 38 Lysosome marker levels of various experimental animals treated with *Polycarpaea corymbosa*

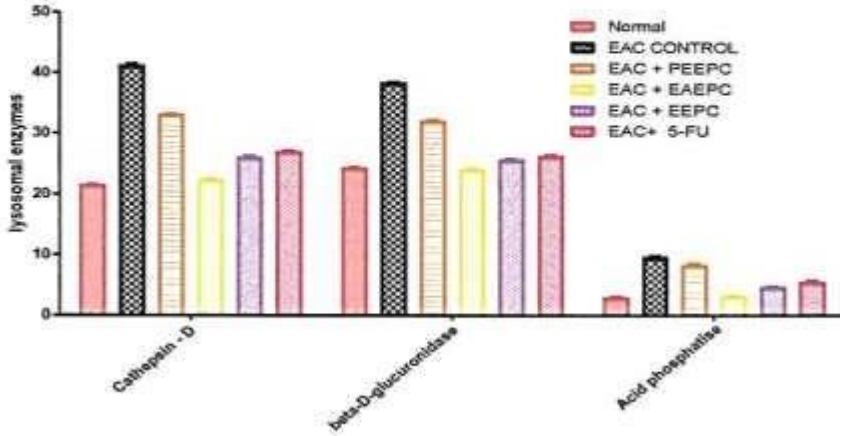


Fig: 39 Liver marker levels of various experimental animals treated with *Polycarpaea corymbosa*

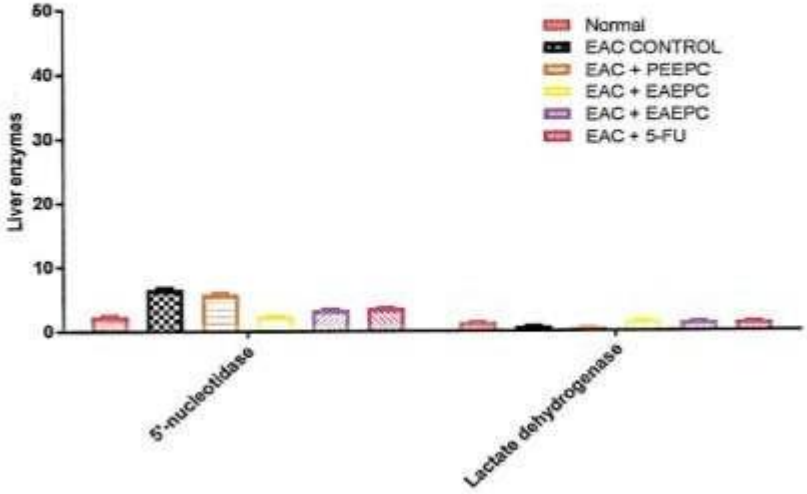


Fig: 40 Na^+/K^+- ATPase and Mg^+-ATPase levels of various experimental animals treated with *Polycarpaea corymbosa*

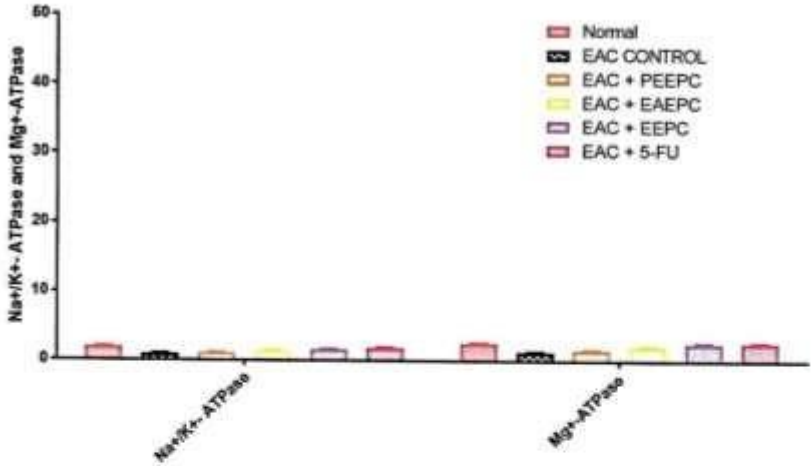


Fig: 41 Glucose and Glycogen levels of various experimental animals treated with *Polycarpaea corymbosa*

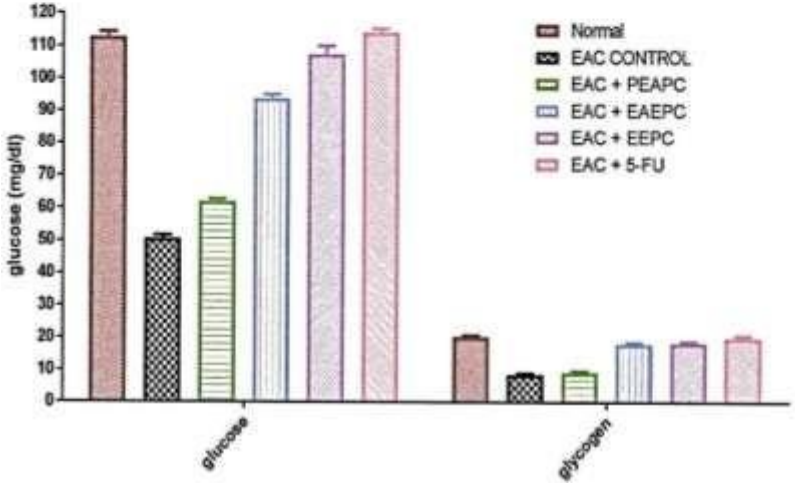
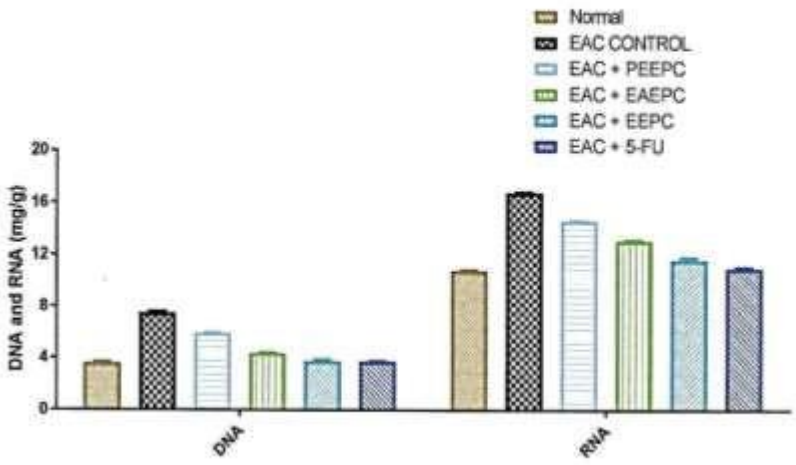


Fig: 42 DNA and RNA levels of various experimental animals treated with *Polycarpaea corymbosa*



6.5.4. ESTIMATION OF (TBARS) LEVEL

The TBARs levels in the liver tissues of various experimental groups treated with whole plant of *Polycarpaea corymbosa* extracts are illustrated in **Table 18** and **Fig. 43.**

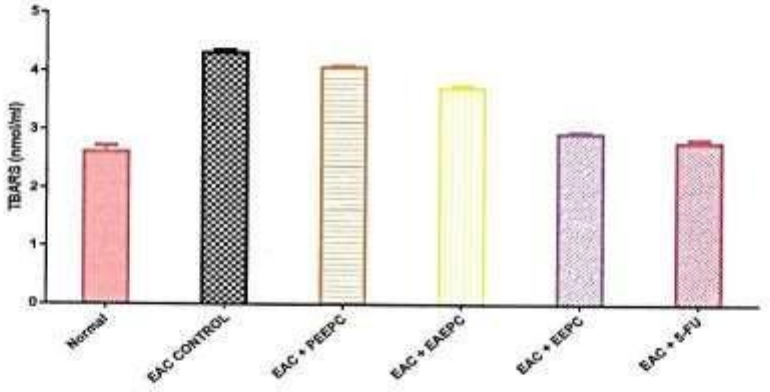
Table 18: The levels of TBARS in the liver tissues of various experimental groups treated with *Polycarpaea corymbosa* extracts

Groups	Tissues TBARS (nmol/ml)
Control	2.65±0.70
EAC	4.34±0.86
EAC + PEEPC (200 mg/kg.bt)	4.09±0.17
EAC + EAEPC (200 mg/kg.bt)	3.78± 0.69

EAC + EEPC (200 mg/kg.bt)	2.95 ±0.74
EAC + 5-FC (20mg/kg.bt)	2.89±0.49

- **Values are expressed as mean ± S.D. for 6 mice in each group.**
- **Values not sharing a common superscript letter in the same row differ significantly at $p < 0.05$ (DMRT).**
- **PEEPC- Pet ether extract of *Polycarpaea corymbosa;* EAEPC - Ethyl acetate extract of *Polycarpaea corymbosa*; EEPC - Ethanolic extract of *Polycarpaea corymbosa;* 5-FC- 5 - Fluorouracil.**

Fig: 43 TBARS levels of various experimental animals treated with *Polycarpaea corymbosa* extracts



It can be observed from the **table 18,** and **Fig: 43,** the TBARs levels were high in EAC alone treated animals (group 2) than those of untreated control (Group 1) animals. Thiobarbituric acid reactive substance (TBARS) is a product of LPO which is the oxidation of polyunsaturated fatty acids in membranes induced by free radicals, is an indicator of oxidative damage. Lipid peroxidation was enhanced in EAC bearing mice. The increased levels of lipid peroxidation were significantly reduced by the administration of ethanolic extract of *Polycarpaea corymbosa* (200 mg/kg) than that of other extracts treated groups.

6.5.5 ESTIMATION OF ENZYMATIC ANTIOXIDANTS

The results for levels of enzymatic antioxidants like superoxide dismutase, catalase and glutathione peroxidase obtained when animals are treated with extracts of the whole plant of Polycarpaea corymbosa are presented in Table 19 and Fig. 44-46.

Table 19: Levels of enzymatic antioxidants in experimental animals treated with Polycarpaea corymbosa extracts

Groups	SOD ('A/ml)	CAT (^{B}/ml)	GPx (^{C}/ml)
Normal	4.39 ±0.35	24.75 ±0.65	48.78 ± 1 .70
EAC -control (l x 1 0^{6} cells/ mouse)	2.52 ± 0.12	10.46 ±0.65	28.25± 0.28
EAC + 200 mg of PEEPC	2.78 ± 0.13	12.78 ± 0.81	30.25 ± 0.25
EAC + 200 mg of EAEPC	3.57 ± 0.22	20.07±0.64	33.47± 0.41

EAC + 200 mg of EEPC	3.94±0.1 1	22.85 ±1.21	40.25± 0.87
EAC + 20mg of 5- FU	4.15±0.13	23.34±0.81	45.18± 0.66

- Values are expressed as mean ± S.D. for 6 mice in each group.
- Values not sharing a common superscript letter in the same row differ significantly at $p < 0.05$ (DMRT).
- The amount of enzyme required to inhibit 50% nitroblue tetrazolium reduction; B: Micromoles of 11202 utilized/s; C: Micromoles of glutathione utilized/min.
- PEEPC- Pet ether extract of Polycarpaea corymbosa; EAEPC - Ethyl acetate extract of Polycarpaea corymbosa; EEPC - Ethanolic extract of Polycarpaea corvmbosa; 5-FC- 5 - Fluorouracil.

Fig: 44 Levels of enzymatic antioxidant SOD in experimental animals treated with *Polycarpaea corymbosa* extracts

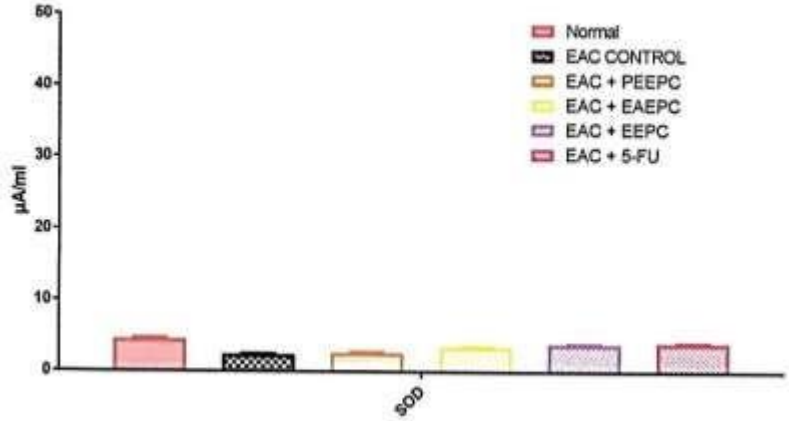


Fig: 45 Levels of enzymatic antioxidant CAT in experimental animals treated with *Polycarpaea corymbosa* extracts

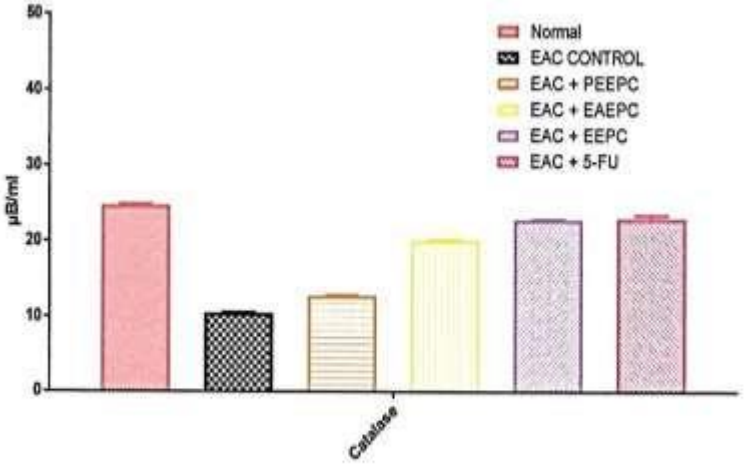
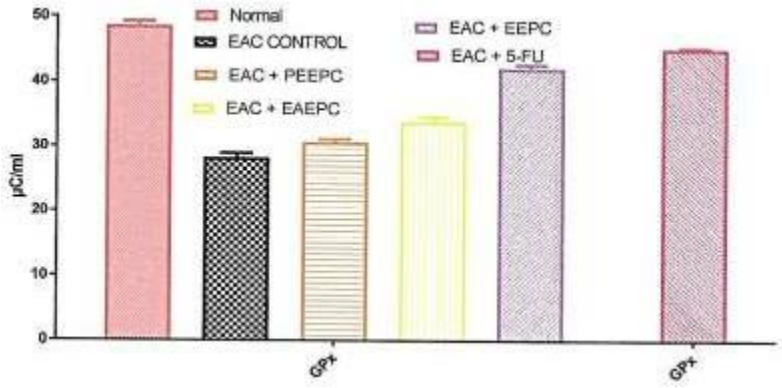


Fig: 46 Levels of enzymatic antioxidant GPx in experimental animals treated with *Polycarpaea corymbosa* extracts



It can be observed from the Table 19, Fig 44-46 that the level of enzymatic antioxidant like superoxide dismutase, catalase and glutathione peroxidase in the tissues of EAC treated (Group 2) animals decreased significantly when compared with Control (Group I) animals. After administration of various extracts of whole plant of Polycarpaea corymbosa in EAC treated mice especially those animals treated with ethanolic extract showed an increased in the amount of SOD, CAT and
GPx in liver tissues of mice

6.5.6 ESTIMATION OF NON-ENZYMATIC ANTIOXIDANTS

The results obtained for the amount of non- enzymatic antioxidants GSH, Vitamin C and Vitamin E present in the

liver tissue of animals are treated with extracts of the whole plant of Polycarpaea corymbosa are presented in Table 20 and Fig. 47.

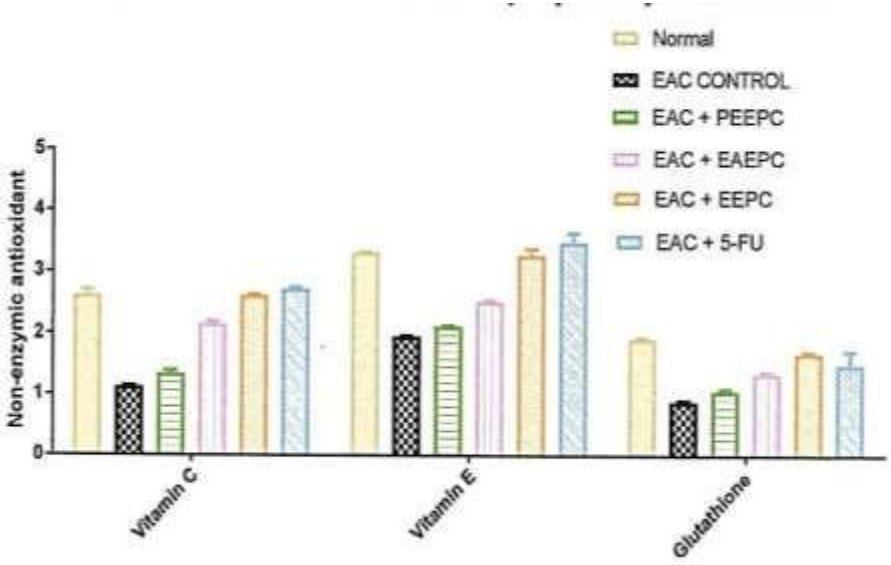
Table 20: Estimation of non-enzymatic antioxidants in liver tissue in experimental animals treated with Polycarpaea corymbosa extracts

Groups	Vitamin C m /tissue	Vitamin E m / tissue	Glutathione m / tissue
Normal	2.76 ± 0.08	3.31 ± 0.13	1.93 ± 0.07
EAC — control (IXI 0^6 cells/ mouse)	1.13 ±0.10	1.95 ± 0.07	0.89 ± 0.22
EAC-control + 200mg of PEPC	1.34 ±0.10	2.09 ± 0.12	1.05 ±0.07
EAC + 200 mg of EAEPC	2.15 ±0.10	2.52 ± 0.12	1.39 ± 0.07
EAC + 200 mg of EEPC	2.61 ±0.08	3.13 0.16	1.68 ± 0.07

EAC + 20 mg of 5FU	2.69 ± 0.07	3.25 ± 0.14	1.78 ± 0.07

- **Values are expressed as mean ± S.D. for 6 mice in each group.**
- **Values not sharing a common superscript letter in the same row differ significantly at $p < 0.05$ (DMRT).**
- **PEEPC- Pet ether extract of Polycarpaea corymbosa; EAEPC - Ethyl acetate extract of Polycarpaea corymbosa; EEPC - Ethanolic extract of Polycarpaea corymbosa; 5-FC-5 - Fluorouracil.**

Fig: 47 Estimation of non-enzymatic antioxidants GSH, Vit C and



Vit E in liver tissues in experimental animals treated with Polycarpaea corymbosa extracts

It can be observed from the Table 20, Fig: 47 the amount of GSH, Vitamin C and Vitamin E in the liver tissue was significantly lowered in EAC alone treated (group 2) animals than untreated control animals.

After administration of various extracts of whole plant of Polycarpaea corymbosa in EAC treated mice especially those animals treated with ethanolic extract showed an increase in the amount of GPII, Vitamin C and Vitamin E in

the liver tissue of mice when compared to other extracts treated group of animals.

6.5.7 HISTOPATHOLOGICAL STUDIES

6.5.7.1 Histopathological changes in liver on various extracts of whole plant of Polvcarpaea corymbosa

Histopathological observation of liver in control and experimental group of mice were studied for confirming the biochemical findings (Fig. 48-53). The liver of normal animals showed normal hepatic cells with well preserved cytoplasm, nucleus, nucleolus and central vein. The liver slice of EAC mice reveled extensive hepatocellular lesions. In addition, hepatocytes appeared unequal in shape and nuclei in the cells were diffused and enlarged upon EAC inoculation. In contrast, the cellular architecture and fine vacuolar of hepatocytes seemed to be almost like that of normal liver in group V and group VI after treatment. The size of nuclei in group V and group VI was essentialls the sam as that 01 normal cells. No significant alteration in the hepatic architecture of was observed in other extracts treated animals.

Fig. 48: Control liver Tissue

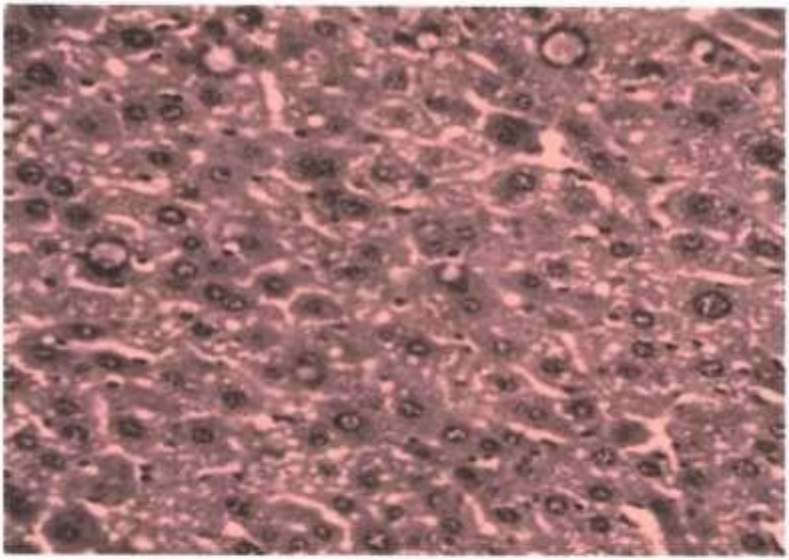


Fig 49: EAC-induced liver tissue

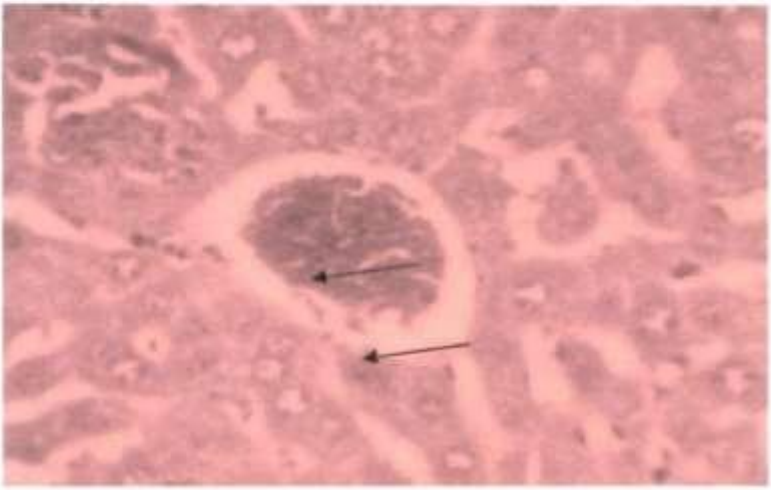


Fig 50: EAC+ Pet ether extract of *Polycarpaea corymbosa*

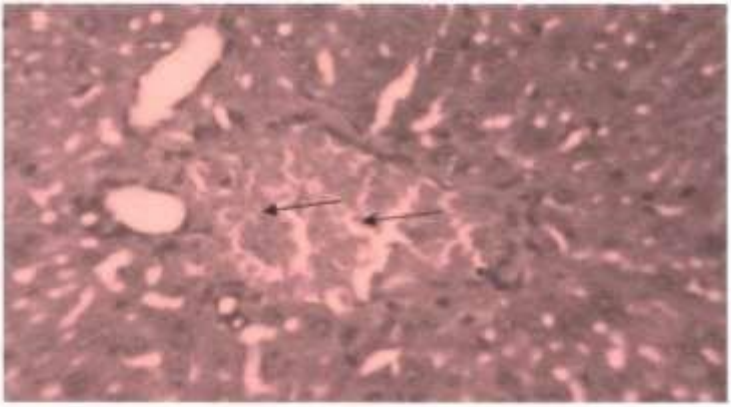


Fig 51: EAC+ Ethyl acetate extract of *Polycarpaea corymbosa*

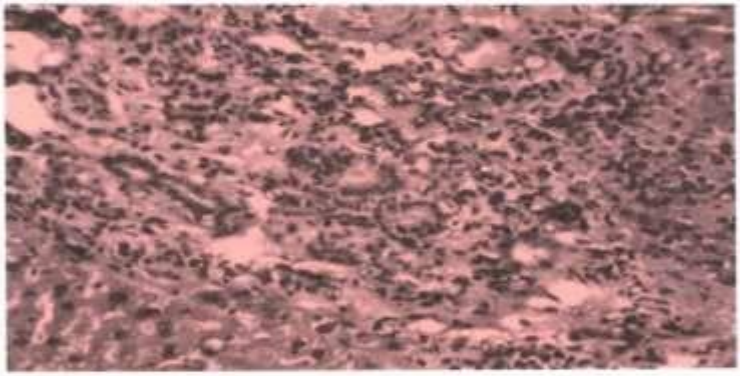


Fig 52: EAC+ Ethanol extract of *Polycarpaea corymbosa*

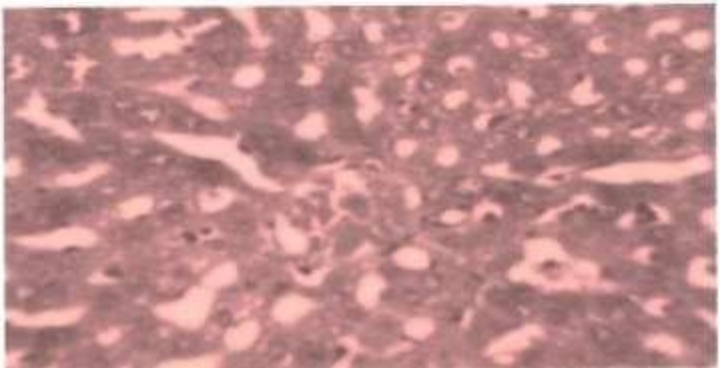
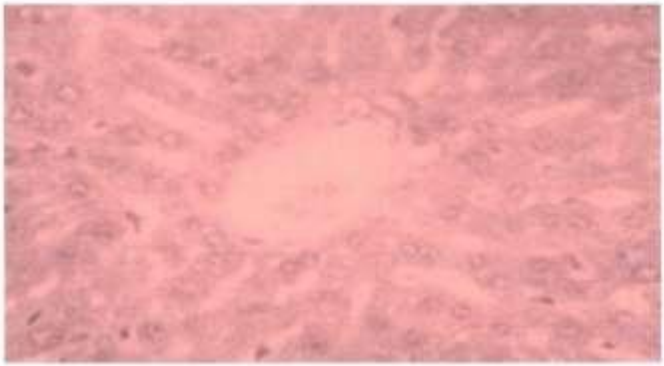


Fig. 53: EAC+ Standard Drug (5FU)



6.6 ISOLATION OF ACTIVE PRINCIPLES FROM ETHANOLIC EXTRACT OF WHOLE PLANT OF Polycarpaea corymbosa

6.6.1. Isolation and characterization of active principle from ethanolic extract of whole plant of Polycarpaea corymbosa

TLC Chromatographic Profiles

Based on the anticancer and antioxidant activities, the ethanolic extract of Polycarpaea corymbosa was found to possess significant activity than other extracts. So, the ethanolic extract of Polycarpaea corymbosa was subjected

to the TLC chromatographic profile and column chromatographic separation.

The ethanolic extract of Polycarpaea corymhosa dissolved in methanol and was taken in a capillary tube and spotted on TLC plates 2cm above its bottom. Most of the sample for application were between 0. I — 1%.

The applied spots were of equal size as far as possible and diameter ranging from 2-3mm. The solvent system for ethanolic extract was developed by trial and error method using various solvents which were differing in polarities. The isolated compounds were subjected to spectral analysis. The no of spots obtained and the Rf value are given in Table. 21

Table 21: TLC profiles of ethanolic extract of whole plant of Polycarpaea corymbosa

s.N0	Solvent system	No. of Spots	Rf Value
	Benzene : Chloroform (90: 1 0)	2	0.73, 0.36
2.	Benzene : Chloroform (80:20)	2	0.78, 0.24
3.	Benzene .• Chloroform	2	0.59, 0.36

	(70:30)		
4.	Ethyl acetate: Ethanol (70:30)	2	0.88, 0.65
5.	Ethyl acetate: Ethanol (50:50)	2	0.68,0.47

Separation using Column Chromatography

The ethanolic extract of P.corymbosa was subjected to column chromatographic separation using normal phase silica gel column. The dark brown solid (20 g ethanolic extract of P.corymbosa) was adsorbed on silica gel (20 g) and transfèrred to a column of silica gel (200g equilibrated with benzene). Elution was perlòrmed as per schedule in materials and methods. Fractions of 100ml were collected every time, the solvent was distilled off and the homogeneity of the resulting residues was examined by TLC by using different solvent systems and similar fractions identified by their TLC behavior were pooled together.

Qualitative chromatographic analysis of ethanolic extract of whole plant of P.corymbosa using thin layer chromatography was performed to separate and identify the single or mixture of constituents in ethanolic extract. The solvents ratio of benzene: chloroform (90:10) showed two spots (Rf values 0.73, 0.36), benzene: chloroform (80:20) solvent showed two spots (Rf values 0.78, 0.24), benzene: chloroform (70:30) solvent showed two spots (Rf values 0.59, 0.36), ethyl acetate:ethanol (70:30) solvent showed two spots (Rf values 0.86, 0.65) and ethyl acetate:ethanol (50:50) solvent showed two spots (Rf values 0.68, 0.47).

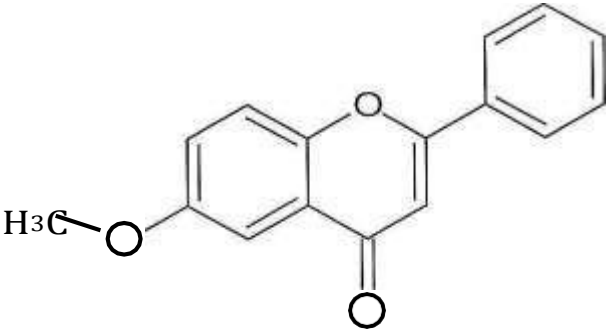
The fractions 27-61 eluted with Benzene: Chloroform 70:30 v/v gave a solid which was designated as compound 1 (125mg). Fractions 73-95 eluted with ethyl acetate: ethanol, 50:50 v/v gave another solid which was designated as compound 2 (168mg). The isolated compounds were subjected to spectral analysis. The results obtained and the spectra are given in Figs. (54- 57) & (5861).

6.7 CHARACTERISATION OF ISOLATED COMPOUNDS FROM ETHANOLIC EXTRACT OF Polycarpaea corvmbosa.

6.7.1 STRUCTURE AND IDENTIFICATION OF COMPOUND 1

The spectral data IR, ^{I} H NMR & ^{13} c NMR and Mass of the isolated compound 1 is good in agreement with the structure proposed for the compound. Based

upon the spectral characterization data, compound 1 was found to be 6methoxyflavone (molecular formula C16111203)



IR Spectrum

The IR data of the compound 1 is analyzed from the IR spectrum (Fig.54). The appearance of peaks at 3062.73 cm-I and 1589.23 cm -I confirms the presence of At' C-H and Ar C-C (in ring) stretching. respectively. Furthermore the appearance of peaks at
2923.87 cm
1288.35 cm- , 1674.09 cm and 1234.35 cm confirms the presence of C H R, ether C-(), keto
C=O and C-O-C stretching, respectively.

H NMR Spectrum

The ^{I} l-I NMR data of the compound 1 is analyzed from the ^{I} l-I NMR spectrum (Fig.55). The appearance of singlet at around 3.9-4.1 ppm shows the presence of OCHR in ^{I} H NMR spectra. The presence of singlet at around 5.3-5.6 ppm shows the presence of -C H— group. The presence of aromatic protons was observed in the aromatic region of ^{I} H NMR spectra at 7.5-
8.3, 8.5-9.3 and 9.6-10. 1 ppm, which confirms the structure.

^{13}C NMR Spectrum

The ^{13} c NMR data of the compound 1 is analyzed from the ^{3} C
NMR spectrum (Fig.56). The appearance of peak at 70.04 ppm in ^{13}c NMR spectrum shows the presence of methoxy carbon. The presence of peaks at 1 15.26 and 126.16 ppm shows the presence of ethylene carbons. The carbonyl carbon peak was observed at 195.98 ppm. The presence of peaks at δ128.28, 128.43, 128.51, 128.66, 128.97, 129.01, 132.27, 133.14, 133.82, 136.51, 136.91 and 141.24 in ^{3} C NMR spectrum corresponds to the aromatic carbons.

Mass Spectrum

The mass spectrum of the isolated compound 1 is presented in the Fig.57. The molecular ion peak of the isolated compound I was found to be 252.90 (M) which confirms the relative mass of the compound.

Spectral Data of Compound 1

IR (KBr, cm -I	1234.35 (C-O-C), 1288.35 (ether C-0), 1 589.23 (At, C-C {in ring}), 1674.09 (carbonyl C=0), 2923.87 (O-CH3), 3062.73 (Ar c-H).
^{I} H NMR (DMSO-DO	3.9-4. 1 (s, 3H, O-CH3), 5.3-5.6 (s, 1 M, =CH-), 7.5-8.3 (m. 4M, Ar-H), 8.5-9.3 (d, 3M, Ar-H), 9.6-10. 1 (s, I H. At•-H).
^{13}C NMR (DMSO-1)6) t,	70.04, 1 15.26, 126.16, 128.28, 128.43, 128.5 1 128.66, 1 28.97, 129.01 132.27, 133.14, 133.82, 136.51 136.91 195.98.
MASS SPECTROSCOPY	252.90 (M+).

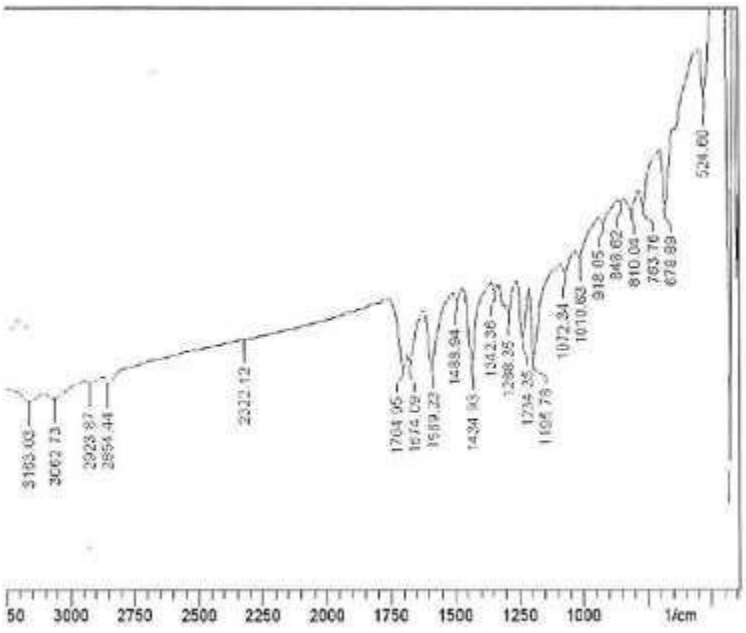


Fig 54: FT-IR Spectrum of Compound 1

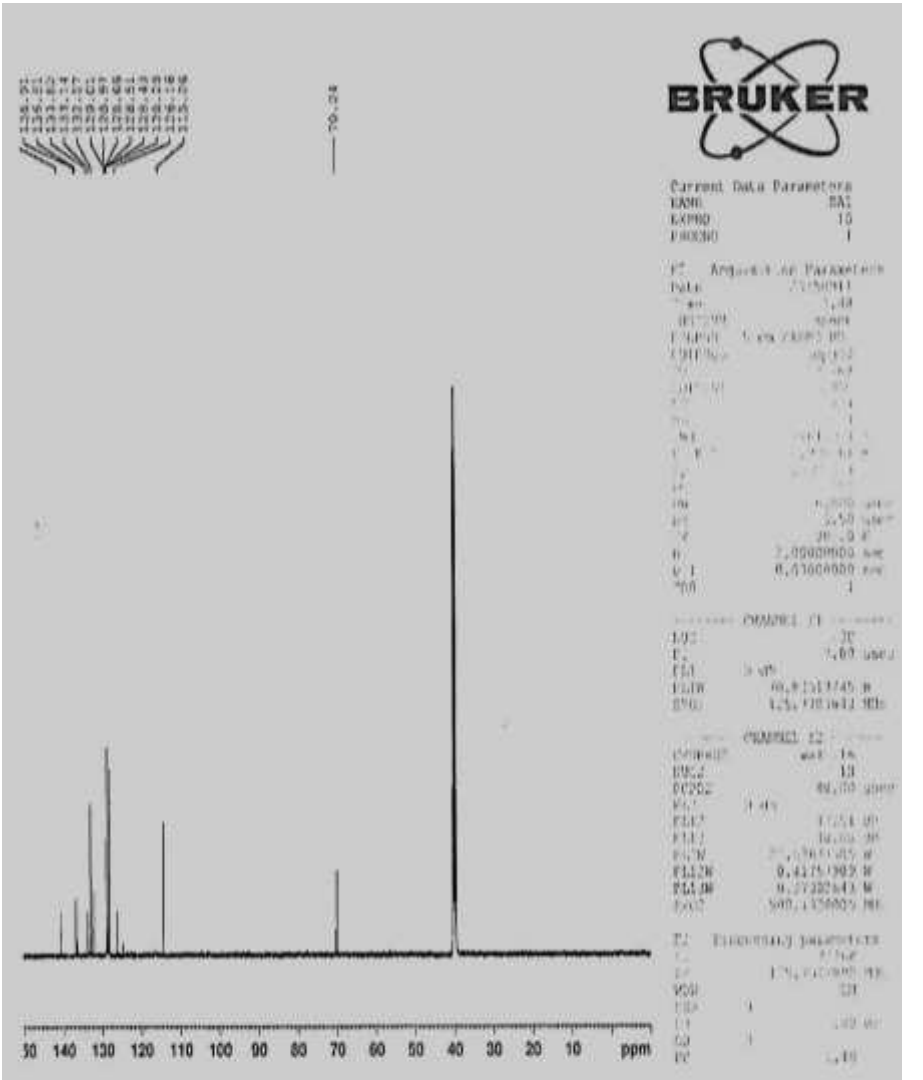


Fig 56: ^{13}C NMR Spectrum of Compound 1

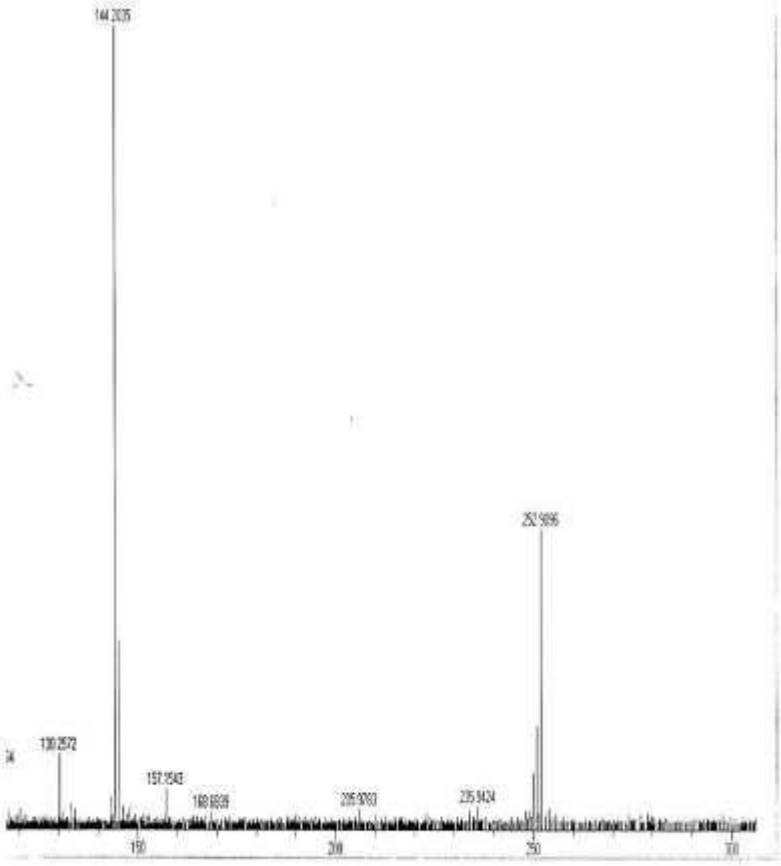
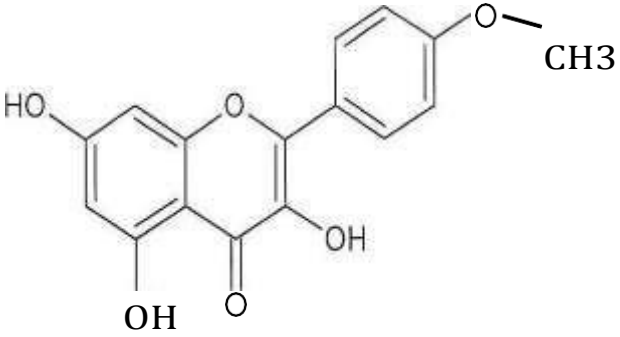


Fig 57: Mass Spectrum of compound 1

6.7.2 STRUCTURE AND IDENTIFICATION OF COMPOUND 2

The spectral data IR, ^{I}H NMR & ^{13}c NMR and Mass of the isolated compound 2 is good in agreement with the

structure proposed for the compound. Based upon the spectral characterization data, compound
2 was found to be 3, 5, 7-trihydroxy-2-(4-methoxyphenyl)-4Hchromen-4-one (Molecular Formula C 16H 1206)



IR Spectrum

The IR data of the compound 2 is analyzed from the IR spectrum (Fig.58). The appearance of peaks at 3089.57 cm -I and 1617.30 cm I confirms the presence of Ar C-H and Ar C-C (in ring) stretching, respectively. Furthermore the appearance of peaks at 3612.36 cm 2849.32 cm -I , 1291 .62 cm ^{l} , 1 655.4 1 cm ^{l} , 1220.86 cm ^{I} and 1060.74 cm ^{I} confirms the presence of alcoholic O-H, CH3, ether C-O, keto C=O, C-O-C and alcoholic C-O stretching, respectively.

^{I} H NMR Spectrum

The ^{I} H NMR data of the compound 2 is analyzed from the ^{I} H NMR spectrum (Fig.59). The appearance of singlet at around 3.1-3.6 ppm shows the presence of OCH3 in ^{I} H NMR spectra. The presence of broad humps at around 6.8-7.0 and 8.9-9.7 ppm shows the presence of alcoholic 0-1-1 group. The presence of aromatic protons was observed in the aromatic region of ^{I} H NMR spectra at 7.4-7.6, 8.1-8.2, 8.3-8.4,
8.4-8.5 and 8.7-8.9 ppm, which confirms the structure.

^{13}C NMR Spectrum

The ^{13} c NMR data of the compound 2 is analyzed from the ^{13} c
NMR spectrum (Fig.60). The appearance of peak at 56.1 1 ppm in ^{3}C NMR spectrum shows the presence of methoxy carbon. The presence of peaks at 1 13.80 and 123.97 ppm shows the presence of ethylene carbons. The carbonyl carbon peak was observed at 195.82 ppm. The presence of peaks at 125.05, 125.36, 126.33, 126.55, 128.34,
128.89, 129.56, 129.77, 129.84, 130.93, 131 .90 and
139.48 in ^{13} c NMR spectrum corresponds to the aromatic carbons.

Mass Spectrum

The mass spectrum of' the isolated compound 2 is presented in the Fig.61. The molecular ion peak of the isolated compound 2 was found to be 300.16 (M) which confirms the relative mass of the compound.

Spectral Data of Compound 2	
IR (KBr, vmax) cm-I	1060.74 (alcoholic C-0), 1220.86 (C-o-C), 1291.62 (ether C-0), 1617.30 (Ar C-C {in ring}), 1655.41 (carbonyl C=0), 2849.32 (O-CH3), 3089.57 (Ar c-H), 3612.36 (0-H).
[I] H NMR (DMSO-DO	3.1-3.6 (s, 3H, O-CH*), 6.8-7.0 (br hump, 21-1, phenolic 0-H), 7.4-7.6 (m, IH, At•-H), 8.1-8.2 (m, IH, Ar-H), 8.38.4 (s, IH, At-H), 8.4-8.5 (s, IH, Ar.H), 8.7-8.9 (d, 2H, Ar-H), 8.9-9.7 (br hump, I H, alcoholic O-H).

^{13}C NMR (DMSO-DO	70.04. 1 15.26, 126.16, 128.28, 128.43, 128.51, 128.66, 128.97, 129.01, 132.27, 133.14, 133.82, 136.51, 136.91, 141.24, 195.98.
MASS SPECTROSCOPY	300.16 (M

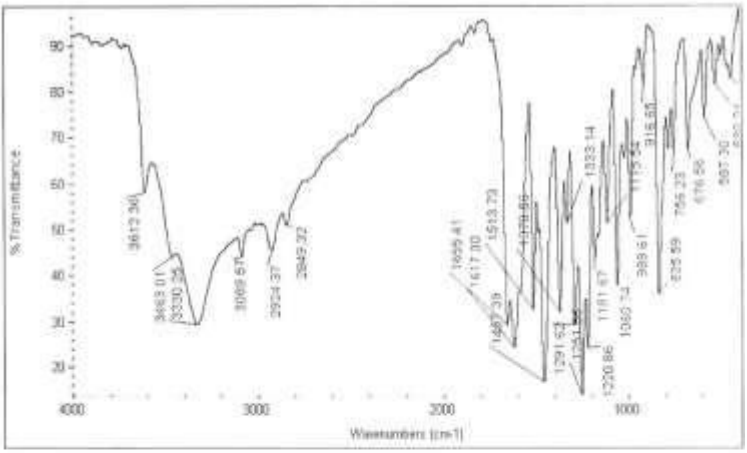


Fig 58: FT-IR Spectrum of compound 2

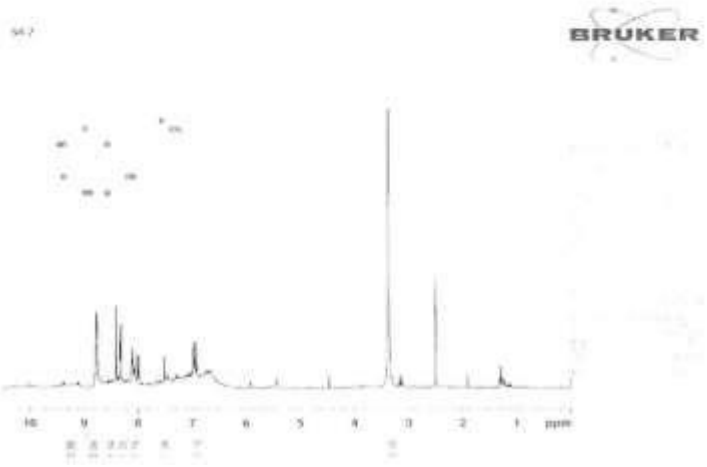


Fig 59: ^{1}H NMR Spectrum of compound 2

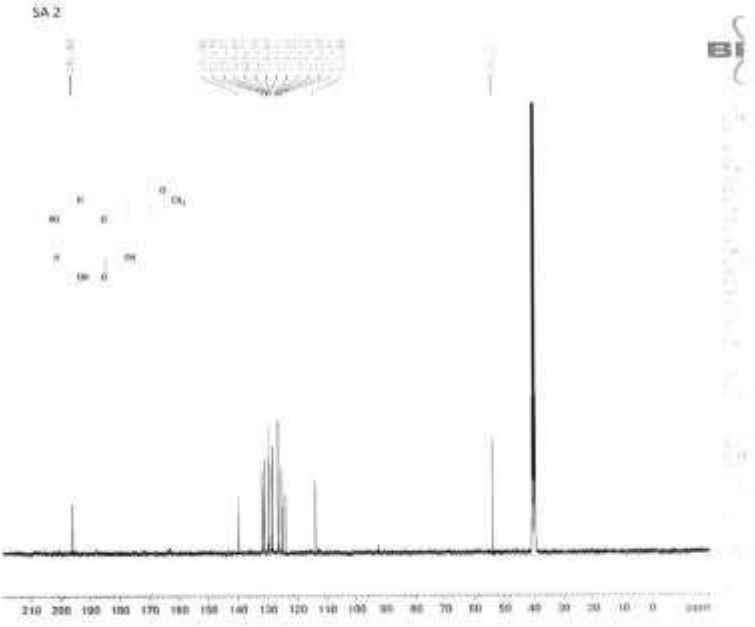


Fig 60: ^{13}C NMR Spectrum of Compound 2

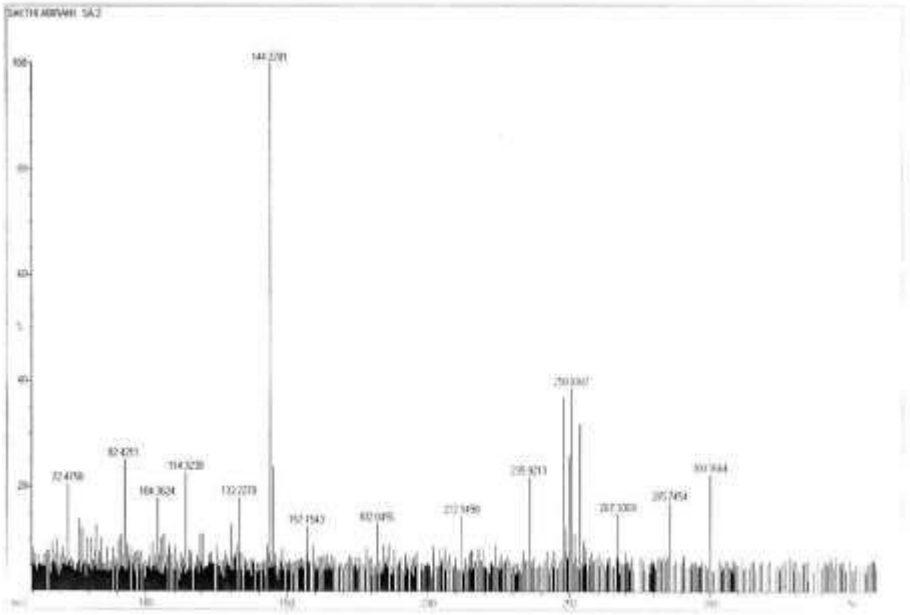


Fig 61: Mass Spectrum of compound 2

6.8 In-vitro cytotoxicity of isolated compound from ethanolic extract of whole plant of Polycarpaea corymbosa

The percentage growth inhibition of the isolated compounds namely compound 1 and compound 2 on different cell lines are presented in Table 22.

Table 22: In vitro cytotoxicity studies of the isolated compounds from the ethanolic extract of whole plant of Polycarpaea corymbosa on different cell lines.

	Treatment	Conc. pg/ml In	Percentage of Inhibition	
			HepG2	HT-29
1.	Compound 1	100	72	76
2.	Compound 2	100	91	95
3.	Mitomycin -C		88	80

 Values not sharing a common superscript letter in the same row differ significantly at $p < 0.05$ (DMRT).

From table 22, it can be seen that the isolated compound I from the ethanolic extract of whole plant of Polycarpaea corymbosa showed 72% and 76% growth inhibition on HepG2 and I-IT-29 cell lines respectively while the isolated compound 2 from the ethanolic extract of whole plant of Polycarpaea corymbosa showed 9 land 95 % growth inhibition. Mitomycin-C showed 88% and 80% growth inhibition on IIepG2 and H T-29 cell lines respectively.

The isolated compound 2 from ethanolic extract of whole plant of Polycarpaea corymbosa showed a significant

cytotoxicity against HepG2 & I-IT-29 cell lines. Similar result was not found in isolated compound 1 from ethanolic extract of whole plant of Polycarpaea corymbosa.

6.8 In-vitro antioxidant activities of isolated compound from ethanolic extract of whole plant of Polycarpaea corymbosa

The percentage free radical scavenging of the isolated compounds namely compound 1 and compound 2 on different in-vitro methods are presented in Table 23.

Table 23: DPP H scavenging potential of isolated compounds from the ethanolic extract of whole plant of Polycarpaea corymbosa

S.N0	Concentration(pg/ml)	% of activi ±SEM *		
		Compound 1	Compound 2	Standard Rutin
	125	31.07 ± O.24	53.51+0.03	17.75±0.45
2	250	40.64± 0.01	61.53+0.98	59.95±0.82

3	500	52.09± 0.04	74.73±0.21	63.73+0.20
4	1000	69.94± 0.18	89.83±0.78	79.84±0.19
		IC_{50}=430 ug/ml	ICso=110 ltg/ml	IC50=380pg/ ml

- All values are expressed as mean ± SEM for three determinations
- Values that are not sharing a common superscript letter in the same column differ significantly differ at (DMRT).

From Table 23, it was observed that the maximum scavenging activity at1000µg/ and IC5() value of compound 1 was found to be 69.94% and 430 ug/ml, and compound 2 it was found to be and 89.83% and I respectively, while for standard rutin, it was found to be 79.84% and 380gg/ml respectively.

Table 24: Superoxide anion radical scavenging potential of isolated compounds from the ethanolic extract of whole plant of Polycarpaea corymbosa

	Concen-tration Wg/ml)	% of activi ty(±SEM)*		
		Compou nd 1	Compou nd 2	Standard Quercetin
1	125	45.12± 0.04	0.39	68.85 ± 0.66
2	250	56.64± 0.60	92.32 ± o. 18	74.81 ± 1.09
3	500	76.93± 0.98	93.72 ± 0.05	92.90 ± 0.1 1
4	1000	78.98± 0.12	95.93 ± 0.04	98.13 ± 1.92
		IC5(F22 0	ICso =125	ICso =11 () pg/ml

- *All values are expressed as mean ± SEM for three determinations

- Values that are not sharing a common superscript letter in the same column differ significantly differ at (DMRT).

From Table 24, it was observed that the maximum scavenging activity at1000µg/

and IC50 value of compound 1 was found to be 78.98% and 220 gg/ml, and compound 2 it was found to be and 95.9% and 125 gg/ml respectively, while for standard quercetin, it was found to be 98.13% and respectively.

Table 25: Nitric oxide anion radical scavenging potential of isolated compounds from the ethanolic extract of whole plant of Polycarpaea corymbosa

S.N0	Concentra-tion (pg/ml)	0/0 of (±SEM *		
		Comp-ound I	Comp-ound 2	Standard Ascorbic acid
	125	31.07 ± 0.24	47.37 ± 0.03	26.87 ± 0.09
2	250	40.64 ± 0.01	58.36 0.98	51.38 ± 0.08
3	500	59.09 ± 0.04	62.14 ± 0.21	71.64 ± 0.43

4	1000	69.94 ± 0.18	78.89 ± 0.78	75.23 ± 0.02
		IC50=330 ltg/ml	IC50=180 Ptg/ml	IC50= 2301tg/ml

- All values are expressed as mean ± SEM for three determinations
- Values that are not sharing a common superscript letter in the same column differ significantly differ at (DMRT).

From Table 25, it was observed that the maximum scavenging activity at 1000µg/and IC50 value of compound 1 was found to be 69.94% and 330 ug/ml and compound 2 it was found to be and 78.89% and 180 ug/ml respectively, while for standard ascorbic acid, it vvas found to be 75.23% and 230gg/ml respectively.

The isolated compound 2 from ethanolic extract of whole plant of Polvcarpaet corymbosa showed a significant free radical scavenging activity against DPPH radical scavenging, superoxide radical scavenging and nitric oxide radical scavenging methods. Similar result was not found in isolated compound I from ethanolic extract of whole plant of Polycarpaea corymbosa.

6.9 P53 gene expression in HepG2 cells of isolated compound from ethanolic extract of whole plant of Polycarpaea corymbosa

P 53 gene expression was normalized to the house keeping GAPDH gene. The real time PCR evaluation of HepG2 cells treated with different concentrations of the compound 2 was showed in a significant dose and time dependent increase in p53 mRNA expression than that of compound l. Flow cytometer analysis of p53 protein expression in HepG2 cells (2 ties 10^5 cells/mL) treated with different concentrations of the isolated compounds for 4811 also resulted in a time and dose dependent up regulation of p53 when compared to control samples which confirmed the results from q-PCR analysis of p53 gene expression in HepG2 cells.

Fig 62: P53 gene expression HepG2 Cells - Control

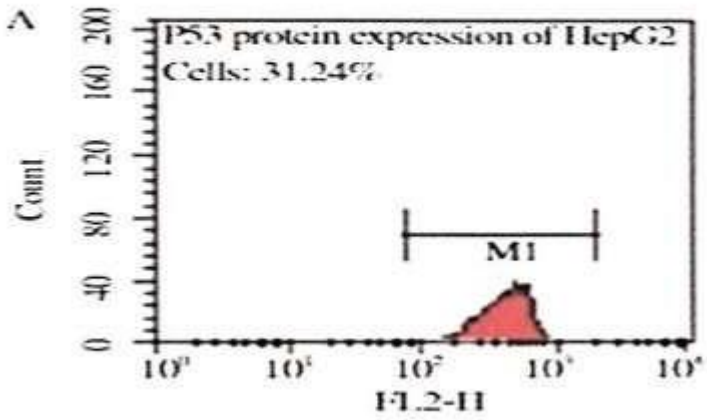


Fig 63: P53 gene expression HepG2 Cells- Compound 1

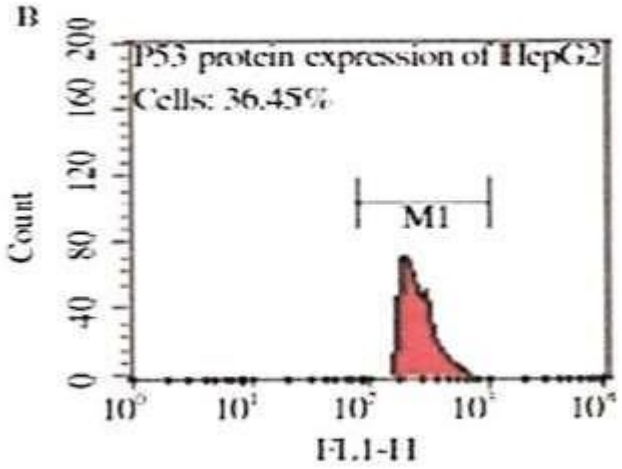
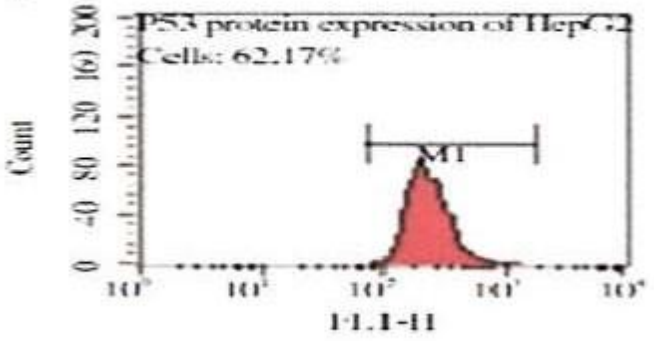


Fig 64: P53 gene expression HepG2 Cells- Compound 2

C



CHAPTER 7: DISCUSSION

For thousands of years' plants have been used as food, spices and medicines. There are least 250000 plants species existing worldwide out of which more than 1000 plants have been found to possess anti-cancer activity. The world health organization has stated that about 80% of the world's inhabitants are dependent on medicinal herbal drugs for their primary health care.

7.1. Preliminary phytochemical screening

The whole plant of polycarpaea corymbosa was powdered and extracted with solvents of increasing polarity and were concentrated and the percentage yields calculated. The calculated percentage yield obtained were 9.87%w/w, 7.8%w/w and 16.56%w/w (**Table 1**) respectively for petroleum ether, ethyl acetate and ethanol extract. An increase in the percentage yield in a particular solvent indicates that more amount of active constituents or phyticonstituents are being present in the particular extract.

The phytochemical screening of the petroleum ether extract of.corymbosa was contain phytosterols, fixed oils and fats. Ethyl acetate extract containing alkaloids, carbohydrate,

glycosides, phenolic compounds and tannins, protein and amino acid compounds, saponins and fixed oil and flats. The ethanolic extract containing alkaloids, carbohydrates, glycosides, phenolic, compound, saponins, tannins, protein, and aminoacid & flavonoids which were presented in **table 2**

7.2. In-vitro antioxidant activity of various extracts of whole plant of polycarpaea corymbosa

There is growing interest towards natural antioxidant from herbal plants and have been employed in protecting against free radical mediate damages. During the process of oxygen utilization in normal physiological and metabolic process approximately 5% of oxygen gets reduced to oxygen derived free radicals which are capable of attacking lipids, proteins and DNA leading to several diseases. An antioxidant may reduce ROS by donating hydrogen atom. Relative antioxidant content provides an indication of important of plants. In general, in vitro screening methods are preliminary methods which pave way for the in vivo evaluation.

7.2 .1. DPPH radical scavenging activity

DPPH (2,2-dipheny1-1 Picrylhydrazyl) is a stable free radical system, which is employed as an essential model by in vitro antioxidant evaluation. It accepts an electron or hydrogen radical to become a stable diamagnetic molecule namely 1-1 dipheny1-2-picryl hydrazine and the degree of

discoloration indicates the scavenging activity of drugs. The reduction capacity of DPPH radical is determined by the decrease in its absorbance at 516nm induced by antioxidant. The percentages of DPPH radical scavenging activity of various extract of whole plant of polycarpaea corymbosa are presented in **table 3** and **Fig.3.** All the extract tested processed DPPH scavenging effect and the maximum DPPH radical scavenging activity of 72.02% at a concentration of 1000µg /ml and IC_{50} value of 225µg/ml was observed for the ethanolic extract if polycarpaea corymbosa while it was 70.65% and 270µg/ml for rutin. The IC50 value for ethanolic extract was lesser than that of the standard which indicates that the extract has significant radical scavenging activity than that exhibited by the standard rutin.

7.2.2. Superoxide radical scavenging activity

Superoxide free radical formed by alkaline DMSO which reacts with nitro blue tetrazolium (NBT) to produce colored diformazan. The HATP scavenging suproxide radical and thus inhibit formazan.

Superoxide radical known to be very harmful to the cellular component. The percentage of superoxide free radical scavenging activity of various extract of polycarpaea corymbosa is presented in **table 4 Fig 4**. All the extract test

and possessed superoxide radical scavenging effect and the maximum superoxide scavenging activity of ethanolic extract was 84.41% at a concentration of 1000µg/ml and IC_{50} value of 110µg/ml while it was 89.28% and 145µg/ml for quercetin. The IC_{50} value for ethanolic extract was lesser than that of standard which indicates that the extract has significant radical scavenging activity than that exhibited by the standard quercetin.

7.2.3. Nitric oxide radical scavenging activity

The excessive and unregulated NO synthesis has been implicated and contributing to pathophysiological condition including cancer. The various cancers such as cervical, breast, central nervous system, laryngeal and head and neck cancer has been detected by expression of NOS. NO has been suggested to module different cancer-related event, however, several research have indicated that NOS may have dual effects in cancer. In many types of clinical samples, NO are measured in different concentrations. NO seems to promote tumor growth and proliferation. In contrast to this, NO is said to have tumoricidal effects, various direct and indirect mechanisms have been proposed for its antitumor properties although there is lack of data directly on cancer patients.

Nitric oxide was generated from sodium nitroprusside and measured by the greiss reagent reduction. Sodium nitroprusside in aqueous solution at physiological $_{p}H$ spontaneously generate nitric oxide which interact with oxygen to produce nitrate ions that can be estimated by using

greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide.

The percentage of nitric oxide radical scavenging activity of various extract of polycarpaea corymbosa is presented in table 5 Fig 5. All extract tested and possessed superoxide radical scavenging effect and the maximum nitric oxide scavenging activity of 69.65% at a concentration of 1000µg/ml and IC_{50} value of 255µg/ml was observed for the ethanolic extract of polycarpaea corymbosa while it was 65.23% and 410µg/ml for ascorbic acid. The IC_{50} value of ethanolic extract was lesser than that of the standard which indicate the extract has significant radical scavenging activity than that exhibited by the standard ascorbic acid.

7.2.4. Hydroxide radical scavenging activity

Hydroxide peroxide is an important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxide radical in which donate electron to H_2OH_2 by the plant extract may be attributed to their phenolic, which donate electron to H_2OH_2, thus reducing it to water. The was capable of scavenging hydrogen peroxide in a concentration dependent manner.

The percentage of hydroxyl radical scavenging activity various extract of polycarpaea corymbosa was presented in table 6 and Fig 6. All the extract tested and possessed hydroxyl radical scavenging effect and maximum hydroxyl radical scavenging activity of 62.58% at a concentration of 1000µg/ml and IC_{50} of 265µg/ml was observed for the ethanolic extract of polycarpaea corymbosa respectively while it was 73.23% and 280µg/ml for rutin. The IC_{50} value of ethanolic extract was lesser that that of the standard which indicates that the extract has significant radical scavenging activity than that exhibited by standard rutin.

7.2.5. Total phenol and flavonoid

In vitro antioxidant studies are mostly used to screen various plant containing phenolic and flavonoid constituents. The phenolic and flavonoid compound have received attention because of their physiological effect like anti-inflammatory, antitumor activities and low toxicity compared with phenolic antioxidant butylated hydroxylanisole butylated hydroxytoluene and propyl gallate. Phenols are important plant constituent because of their scavenging ability due to their hydroxyl groups. The phenolic compound may contribute directly to antioxidative activity. Flavonoids present in food of plant origin are also potential antioxidants. The total phenolic and flavonoid content of various extract of whole plant of polycarpaea corymbosa result was presented in table 7 and Fig 7. All the extract tested and possessed phenolic and flavonoid was content and maximum of

4.60±0.05 and 3.63±0.93 phenolic and flavonoid was observed for the ethanolic extract of polycarpaea corymbosa whole plant than that of other extract.

Previous finding suggests that the ethanolic extract was found to have maximum phenolic components and which may be on of it's to possess maximum antioxidant activity than other extracts. Flavonoids play some important pharmacological roles against diseases such as cardiovascular disease, cancer, inflammation and allergy and other oxidative stress related diseases. From, above discussion it was clear that the most powerful anti-oxidant effect of extract is ethanolic extract of whole plant of polycarpaea corymbosa.

7.3 In vitro cytotoxicity

The use of plant derived natural compounds as the part of herbal preparation and alternative sources of drugs continues to play major roles in the wellness of people all over the world. Cytotoxicity screening model provide important preliminary data to help selecting plan extracts with potential anti-neoplastic properties for future work.

MTT assay is a well-established in vitro method for cytotoxicity against cancer cell lines and non-cancer cell lines. The enzymes present the cancer cells are capable of reducing the tetrazolium dye MTT 3-(4,5dimethylthiazol-2-

Yl)-2,5-diphenyltertazolium bromide to its formazan, which has color.

All the extracts of whole plant of Polycarpaea corymbosa tested and possessed inhibition of growth against the following cell lines MCF7, HepG2, HT-29 and PC3 by MTT assay. From the **table 8 and Fig 8 and 9-28.** The ethanolic extract of plant extract was active on all cell lines **(MCF-7, HL-60, HepG2, HT-29 and PC3).**

The IC_{50} value of ethanolic extract polycarpaea corymbosa on various cell lines like MCF-7 (6.80±0.50), HL-60 (11.50±0.45), HepG2 (10.00±0.02), HT-29 (7.05±0.60) and PC3 (17.2±0.85). The ethylacetate extract was found moderately active on MCF-7(28.5±0.02) cell line and inactive on HL-60, HepG2, HT-29 and PC3 cell line. On the other hand, petroleum ether extract was not found active on all cell line. The cytotoxic activity might be due to the present if phenols and flavonoid active component in ethanolic extract which has been proved by earlier studies.

7.4. Acute toxicity studies

The acute toxicity of petroleum ether, ethyl acetate and ethanol extract of polycarpaea corymbosa whole plant were carried out as per OECD-423 guideline for determination of safe dose.

The treatment of mice with various extracts of polycarpaea corymbosa whole plant did not change the autonomic or behavioral responses in mice which were present in table 9. The zero percent mortality was observed for different extracts

of polycarpaea corymbosa whole plant at the dose of 2000mg/kg. hence, there is no toxicity up to a dose of 2000mg/kg body weight of the animal.

7.5. Anti-cancer activity

The general concepts put forward by many researchers in the anticancer potential of compounds which are present in plants at subpharmaceutical doses could synergize to delay or disrupt the development of aggressive disease. Plant-based medicine plays an important role in cancer treatment, and 60% of currently used anticancer agents are derived from plan resources. The ROS leading to oxidative stress involved in a variety of pathophysiological condition including mutagenesis. The conventional anticancer drugs cause nonspecific killing of cells, whereas medicinal plants offer protective and therapeutic actions to cells with low cytotoxicity and beneficial in producing nutrient repletion to compromised people. Therefore, there is a need for new plan derived potential chemotherapeutic agents for the management of cancer.

In the present study was carried out to evaluate potential of various extracts of whole plant polycarpaea corymbosa on EAC induced tumor in mices. The EAC induced experimental carcinogenesis light therefore be used as an ideal method to

evaluate the chemo preventive potential of medicinal plant and its active constituent

Oxidative damage to cellular biomolecules such as lips, proteins and DNA is thought to play a crucial role in incidence of several diseases. Flavonoids are a group of polyphonic compounds found abundantly in the plant kingdom. Preliminary phytochemical study indicated the presence of flavonoid, alkaloids and tannins in ethanolic extract of polycaepaea corymbosa whole plant. Flavonoids have been shown to possess antimutagenic and antimalignant effect. Furthermore, flavonoid have a chemo preventive role in cancer through their effect on signal transduction in cell proliferation and angiogenesis.

EAC is one of the experimental breast tumor derived from spontaneous mouse adenocarcinoma. Intraperitoneal injection of the tumor emulsion produces ascites. A regular volume was observed in EAC bearing mice. Ascetic fluid is the direct nutritional source for tumor growth because it meets the nutritional requirement of tumor cells.

From the table 10 and Fig 29-33, the body weight, mean survival timen percentage in life span tumor volume, packed cell volume and tumor cell count of various experimental animal treated with polycarpaea corymbosa extract are presented. In the present findings body weight of mice inoculated with EAC cells incremented due to ascites volume proliferating peritoneal cells. Post treatment with various extract polycarpaea corymbosa and standard drug

(5-FU) significantly reduce the weight gain of animals, similar result was not observed in animal treated with EAC alone (group II). Decrease lifespan is due to the low Hb levels observed cancerous condition. The altered levels of body weight, mean survival time, percentage in life span. tumour volume. Packed cell volume and tumour cell count were significantly improved by various extracts of Polycarpaea corymbosa treated groups. The change in the body weight and increased lifespan of animals in the treated group propose the tumor growth inhibitory property of the ethanolic extract of Polycarpae corymbosa.

The cytotoxicity and anticancer potenial of ethanolic extract of Polycarpea corymbosa whole plant is probably due to presence of flavonoids. This present study suggests that the ethanolic extract of Polycarpea corymbosa whole plant possess potent anticancer activity.

Tumor growth is normally related with noticeable changes in haematopoiesis,, immuneresponse, myelosuppression and anemia. The alterations in the levels of haematological parameters namely RBC, Hb and WBC were presented **in table 11 and Fig**
34-37. From the table 11, it was noted that RBC count, WBC and Hb content in the EAC bearing mice was markedly

($p<0.05$) declined as compared to nomarl mice. The decrease in RBC and haemoglobin percentage may be due to the deficiency of iron or due to the haemolytic or myelopathic conditions in EAC mice.

The changes in the levels of haematological parameters namely Differential count presented in **table 12 and Fig 34-37.** The WBC count was significantly enhanced. Among various white blood cells. neutrophils were found to be elevated. while the lymphocytes and monocytes were decremented. The significant increase in W BC count and neutrophils in tumour bearing mice is due to its primary defense mechanism supplementation of ethanolic extract or Polycaypea corymhosa has restored BC counts differential count, Hb and RBC count to a significant extent. No significant difference was observed between normal and plant control animals which evidenced the protective action of plant extract on the haematopoietic system. Earlier studies resealed that administration of plant extract to EAC inoculated mice resulted in the improved Hb and leukocytes recoupment of RBC Hb. total W BC count and differential count in injection of EAC mice with copper-benzohydroxamic acid complex.

Table 13 and Fig 38 demonstrate the effect of various extract of polycaepaea colymbosa on the activities of lysosomal markers enzymes in liver of nomal and experimental group of mice.

In EAC bearing animal (41.19±1.75), the activity of cathepsin-D was elevated twice the normal (21.73±0.19) β-D glucuronidase activity was increased by 56% in EAC control

when compared to normal. The increase in the activity of ACP was 3-fold (9.14±0.20) when compared to untreated animal.

Administration of various extracts of Polycarpea corymbosa lowered the leakage marker enzymes most likely via stabilizing the membrane architecture, This be attributed to the presence of flavonoids in the extract have an inhibiting property on all membranes have documented that localization of flavonoids within the lysosomal membranes may modify membrane fluidity and lipid peroxidation. Various extracts of polycarpea corymbosa was found to stabilize the lysosomal integrity and retrieve the normal functioning of lysosomes.

Lysosomes are a group of cytoplasmic organelles which are characterized by their content of acid hydrolase that are capable of digesting the macromolecules like polysaccharides, nucleic acids and lipids188. The huge production of free radicals in the cancerous leads to the irregular vulnerability of the lysosomes results in the elevated of lysosomal enzymes Lysosome specific marker enzymes include Cathepsin-D, Acid phosphates and ß-D-glucuronidase.

Cathepsin-D plays a proteolytic role in the digestion of extracellular matrix (ECM) components and is implicated in tumor invasion and metastasis. Earlier studies reported that the Cathepsin-D plays an important role in stimulating cancer cells proliferation demonstrated that transfection of rat with tumor cells resulted in over expression of CD. Several studies have reported the elevated activities of Cathepsin-D in various types of cancers.

ß-D-glucuronidase is considered to be both a microsomal and a lysosomal enzyme They are glycosidase family of enzymes that catalyses the breakdown of complex Carbohydrates. It is shown to be a sensitive marker of lysosomal integrity.

The activity of ß-D-glucuronidase depends on the rate of invasiveness of tumor Acid phosphatase is also a cytoplasmic enzyme that has been considered to be related with the lysosomes which catalyze the hydrolysis of organic phosphate.

Table 14 and Fig 39 depicts the activity of liver marker enzymes 5'-nucleotidase and lactate dehydrogenase (LDH) in normal and experimental group of mice. The activity of 5'– nucleotidase in EAC translated mice was elevated thrice (6.46±0.25) when compared to normal mice (2.58±0.10). Nucleotidase activity is increased when tumor occluding the bile ducts. LDH activity in the liver of EAC inoculate mice

(0.41±0.12) was markedly declined in comparison to normal (1.59±0.10) . The decremented activity was of LDH in liver was also reported by Rizk and Ibrahim (2008). Administration of various extracts of plant of Polycarpaea corymbosa and 5-FU, the activity was retained towards normal.

NO significant variations were noted between normal and plant control in the activities of 5-nucleotidase and LDH. The observed tumor inhibitory property of extract is impaired by the presence of flavonoids which possess antiproliferative action on cancer cells.

5 'nucleotidase is a glycoprotein having phosphatase activity. It is widely distributed through the tissues of the body and is principally localized in the cytoplasmic membrane of cells. It acts on nucleoside5'- phosphates such as adenosine-5'- phosphate (AMP) and adenylic acid to release inorganic phosphate. Lactate dehyrogenase is a tetrameric enzyme recognized as potential tumor marker in assessing the progression of the proliferating malignant cells. LDH enzyme is cytoplasmic in orgin and released into circulation after cellular damage. Veena et al., (2006), Walia et al., (1980) have reported higher activities of 5'nucleotidase activity on various types of cancers in animal models.

Table 15 and Fig 40 illustrate the effect of various extract of Polycarpaea Corymbosa whole plant on the activities of ATPases in liver of normal and experimental group of animals. When compared to normal mice, Na+/K+-ATPase activity was decreased significantly ($p<O.05$) by 2-fold in EAC inoculated mice. In the untreated mice, the marked decrement in the activity of Mg+-ATPase was found to be in comparison to normal animal. The significant decrease in the activities of Na+/K+- ATPase and Mg+-ATPase in Liver of cancer bearing mice may be due to production or their cytotoxic effects by causing peroxidation or membrane perturbation in the ion-dependent ATPases ion channels are seen in lipids peroxidation of membrane. This leads to disturbances in the ion homeostasis resulting in impaired signal ransduction. altered cellular metabolism. changes in cell membrane permeability and integrity, an elevation in membrane fluidity and disturbances of vital cellular function.

In the various extract of Polycarpaeet corymbosa whole plant treated EAC-control. Membrane bound ATPase activities were regained to a significant extent. This may be due to the stabilization of membranes imparted by the presence of flavonoids in the extract. Flavonoids influence the permeability of biomembranes by interacting with ATPase pumps in the animal cell there by regains their normal efficiency and assured normal properties.

Biological membranes encompass a group of which maintain ionic gradients between aqueous intra and extra eel

phases- They are lipid dependent membrane bound enzymes and any alterations in the lipid bilayer may the activities of ATPases and in turn in normal cellular functions- Membrane bound enzymes such as Na+/K•+- ATPase, Mg+—ATPase are responsible for the transport of sodium/potassium and magnesium across the cell membranes at the expense of ATP by hydrolysis.The activities of ATPases in liver tissues have been shown to be inhibited in cancer bearing animals. Earlier studies reported that the decreased activities of membrane bound ATPases in several types of tumours.

The levels of plasma glucose and liver glycogen clue to effect extract of polycarpaea corymbosa whole plant in normal and experimental group of mice are illustrated in **table 16 and Fig 41-** The levels of plasma glucose and liver glycogen were Significantly declined. The development of hypoglycaemia in experimental animals with carcinoma has been previously reported. Numerous studies have documented.

Hypoglycaemia and depleted liver glycogen content in other tumor bearing animals of EAC mice with various extract of Polycarpaea corymbosa whole plant ameliorated the levels of liver glycogen to a significant extent.

Glucose is an important energy fuel in all living cells of an organism. The protein of oncogens and tumor

suppressor gene P53 are involved in stimulating the production of glycolytic enzymes. Hence, glucose uptake and glycolysis proceed at faster rate in tumor cells than noncancerous tissues. The high glycolytic rate may also result in part from smaller number of mitochondria in tumor cells which results in less ATP production during mitochondrial oxidative phosphorylation. Due to hypoxia condition experienced in cancer cells. they also depend on anaerobic glycolysis for much of their ATP production.

Glycogen is the storage form of polysaccharide in animals. Tumor formation and associated increase in utilization of glucose has a way' of triggering the body into a constant state of glycogenolysis. Increased glycogenolysis could be deduced from the obtained significant decrease in liver glycogen and the increased activity of glucose-6-phosphatase which is greatly supported by several previous findings Fahim et al., (2003) have noted that the retained levels of plasma glucose and liver glycogen in solid carcinoma in after the injection of EAC mice with iodoacetate and dimethylsulphoxide.

Table 17 and Fig 42 represents the effect of various extract of Polycarpaea Corymbosa whole plant on the levels of nucleic acids in the liver of normal and experimental group of animals. In the untreated mice, the level of DNA was strikingly elevated by 4-fold when compared to normal. The increment in the levels of RNA in diseased mice was found to be when compared to group I, several studies reported that the

elevated levels of hepatic nucleic acids in cancerous condition Administration of ethanolic extract of Polycarpaea corymbosa whole plant to EAC-inoculated mice resulted in attainment acids levels to near normal. No similar result was observed from other extract treated oops.

Fahim et al., (2003) have reported that nucleic acid levels in liver cells of solid carcinoma were regained to near normal levels after the injection of EAC mice with iodoacetate and dimethylsulphoxide. Hussein et al., (2013) and Luksiene (2003) have observed the reduced levels of nucleic acid in liver cells of EAC control treated with antineoplastic drug and photodynamic therapy.

Aerobic organisms are prone to free radical attacks. Nucleic acids are the primary target for oxidative damage which causes DNA strand breaks, chromosome deletions and rearrangements which
results in the progression of tumor. Abnormal cell proliferation is associated with the increased synthesis of nucleic acids in cancerous tissues.

7.6 Level of Thiobarbituric acid reactive substance on liver tissues

Thiobarbituric acid reactive substances (TBARS) are knov\Ä1 to be the byproducts of lipid peroxidation of

poly unsaturated fatty acids present in cell membrane. TBARS is an indicator of oxidative damage. Elevation of Lipid Peroxides, as indicated by increased MDA was observed in breast cancer bearing animals the significant increase in LPO in Carcinogenic process may be due to abnormal levels of ROS. ROS production in cellular antioxidant capacity may result in damage to protein, lipid, DNA and RNA or other effects

From **table 18 and Fig 43**, an increase in the levels of LPO (Thiobarbituric acid reactive Substances in liver tissue) was found in EAC induced animals. All the extracts tested and possessed inhibition of LPO level and the significant inhibition of LPO was observed in ethanol extract of Polycarpaea corymbosa whole plant at the dose 200mg/kg body weight.

Thiobarbituric acid reactive substances, which are markers of lipid peroxidation were found to be decreased, suggesting decreased levels of oxygen free radicals which could be their increased quenching or scavenging by the increased levels of antioxidants by ethanolic extract of Polycarpaea corymbosa whole plant at the dose 200mg/kg body weight suggesting it to be an efficient antioxidant in in-vivo system.

7.7 In-vivo antioxidant studies

Elevated oxidative stress can change a number of

cellular targets and cause cell damage and the subsequent lack of repair has been responsible for carcinogenesis234. The Natural antioxidants have wide variety of biochemical activities, including inhibition of ROS generation, scavenging of free radicals by directly or indirectly and changing of anti-oxidant potentia. The antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase are present in oxygen handling cells which are the first line cellular defense against oxidative injury decomposing 0_2 and H_20_2 before they interact to form more reactive radicals. SOD detoxifies the superoxide radicals to H_20_2, which has been eliminated by Catalase. The results for enzymatic antioxidants SOD, CAT and GPx obtained are presented **in table 19, Fig 44-46.**

7.7.1 Superoxide Dismutase

McCord and Fridorich described the metallo enzyme superoxide dismutase237 Cartinogensis238 Superoxide dismutase plays an important role in catalyzing the dismutation of radicals and increase in

SOD activity accelerates the removal of the SOD act as an anti carcinogen inhibitor during the initiation and promotion stages of ROS.

superoxide dismutase is the major intracellular antioxidant enzyme, which is essential for the survival of aerobic cells. It catalytically scavenges the superoxide radical, which appears to important agent for toxicity of oxygen and thus provides a defense against oxygen. In the present study, decline in SOD level was observed in EAC induced mice which due to the increase in circulating lipid peroxides which results in accumulation of anion. A highly diffusible and potent oxidizing radical capable of traversing causing deleterious effects at sites far from the tumor. From **table 19 and Fig 44**, the extracts are tested and possessed increased in SOD level and the significant improvement in SOD was observed in the ethanolic extract of whole plant of Polycarpaea corymbosa when compared to other extracts.

Catalase Catalase is an enzyme present in the cells of animals, plants and aerobic bacteria catalase is located in a cell organelle called peroxisome. In animal's catalase is present in all major body organs. The role of catalase is to scavenge hydrogen peroxide and prevent oxidative damage in the cancerous ce. CAT and GPx detoxify significant amount of H_20_2 produced during electron transport chain and protect mitochondrial membranes from lipid damage. The enzymatic antioxidant Decreased in the activities of the antioxidant enzyme catalase level was noted.1n in thethe induced tumor group is regarded as a marker of malignant transformation Present study, decline in CAT level was observed in EAC induced mice. From the **table 19 Fig 45**, all the extracts are tested and possessed increased in CAT level and significant in

CAT was observed in ethanolic extract of Polycarpaea corymbosa whole plant when compared to other extracts. catalase is widely distributed in all tissues catalyses the breakdown of hydrogen peroxide produced by tumor cells. Glutathione PeroxidaseGlutathione Peroxidase

GPX is an important defense enzyme against oxidative damage and this in turn requires? Several studies have reported the decreased activities of GPx in lutathione as a cofactor cancerous conditions. There was a decline in the activities of GPx which may be due antioxidant defense system caused by enormous production of free radicals in the C f XC. induccd carcinogenesis. In the present study, decline in GPx level was observed in EAC induced mice. From 19 and Fig 46, all the extracts are tested and possessed increased in GPx level and the significant improvement in GPX was observed in the ethanolic extract of whole plant of corymbosa when compared to other extracts. The increased level of enzymatic antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase seen aftert. catment with Polycarpaea corymhosa could be either because of their increased expression orenhanced activity. This point needs further experimental evaluation. 14 Glutathione, Vitamin C and Vitamin E Cancer cells may sequester essential antioxidants from circulation to supply the demands of growing tumor. Apart from the enzymatic antioxidants, non-

enzymatic antioxidants such as GSH, Vitamins C and E play an excellent role in protecting the cells from oxidative stress The non-enzymatic antioxidant systems are the second line of defense against free radical damage. In the present study showed a decrease in the GSH, Vitamin C and E level was observed in EAC induced mice. From the **table 20, Fig 47,** All the extracts are tested and possessed increased in the activity of nonenzymatic antioxidants like GSH, vitamin C and E level in the liver tissue of control and experimental animals was observed and the improvement was observed in the whole plant of Polycarpaea corymbosa at the dose of 200mg/kg body weight when compared to other extracts.

Increased ascorbic acid and vitamin E content in response to ethanolic extract of Polycarpaea whole plant (200mg/kg body weight) treatment could be attributed either to them.

I. Histopathology

The antitumor activity was further confirmed by histopathological findings. The cells are exposed to chemical carcinogens or radiation; changes ate prominent at cellular tissue levels which are usually reflected in the Histopathological observation of liver the biochemical findings (Fig 48-53). The liver of normal animals showed normal hepatic cells with well-preserved

cytoplasm, nucleus, nucleolous and central vein. The liver slice of EAC mice revealed extensive hepatocellular lesions. In addition, hepatocytes appeared irregular in shape

and nuclei in the cells were diffused and enlarged upon EAC inoculation. In constrast, the cellular architecture and fine vacuolar of hepatocyte seemed to be almost like that of normal liver in group Ill and group IV after treatment, The size of nuclei. In group V and group VI (Fig 52-53) was essentially the same as that of normal cells and No significant alterations in the hepatic architecture of other extract treated groups were Observed. Chakraborty et al., (2007/49 have repofled the alterations in the liver of EAC tranSplanted mice. Bairy et al., (2003) have demonstrated that intraperitoneal injection of uncristin to EAC bearing mice resulted in the reduction of neoplastic lesions in the hepatocytes. Isolation and Characterization of active principle from ethanolic extract of Plant of Polycarpaea corymbosa.

in control and experimental histopathological architecture group of mice were studied for conforming .

7.9.1 TLC Chromatographic profiles

Based on the anti-cancer and antioxidant activities, the extract corymbosa was found to possess significant than that of other extracts. So, the ethanolic of Polycarpaea corymbosa was subjected to the TLC chromatographic profile and chromatographic separation. The no of spots Obtained and the Rf value are given in from the TLC

chromatographic profile ethanolic extract of whole plant Of corymbosa, the 27-61 eluted with Benzene: Chloroform 70:30 v/v gave a solid which was designated as compound I (125mg). Fractions 73-95 eluted with ethyl acetate: gave another solid which was designated as compound 2 (168mg). ethanol, 50:50v/v.

The results obtained and the spectra are given in Figs. The structure and identification of isolated compounds were done using FT-IR, IHNMR, & 13CNMR and Mass spectra, as per the spectral analysis, the structure of compound 1 is proposed to be 6methoxyflavone and its molecular formula was deduced as Compound 2 was proposed to be 3,5,7-trihydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one and its molecular formula was deduced as $\mathbf{C_{16}H_{12}}$ In vitro cytotoxicity of isolated compound from whole plant of Polycarpaea corymbosa The isolated compounds from whole plant of Polycarpaea corymbosa were evaluated for in-vitro cytotoxicity studies against the following cell lines HepG2 & HT- cells as these tumor cells are absorbed the anticancer drug by direct absorption in target tissue and this anticancer agent may lysis the cells by direct In-vitro antioxidant activities of isolated compound Cytotoxic Mechanism The in-vitro antioxidant studies of isolated compounds from whole plant of corymbosa were evaluated by DPI) H scavenging, radical scavenging nitric oxide scavenging methods. The isolated compound 2 extract of plant from ethanolic Polycarpaea corymbosa showed a significant radical scavenging against DPPH radical scavenging, superoxide

radical scavenging and nitric oxide radical scavenging methods. Similar result was not found in isolated compound I from ethanolic extract of whole plant of Polycarpaea corymbosa. The results obtained are presented in Table 23-25. The phenolic compounds may contribute directly to anti-oxidative actionli3

The phenolic Flavonoids present in food of plant origin are also potential antioxidants and flavonoids compounds have received attention because of their physiological effect like anti-inflammatory, antioxidant, antitumor activities and low toxicity compared such phenolic antioxidant butylated hydroxylanisole, butylated and
Gallate increase in p53 mRNA expression than that of compound 1. The compound 2 could induce Cytotoxic and genotoxic activities on human carcinoma (Hep G2) cells through induction of apoptotic pathway

CONCLUSION AND RECOMMANDATIONS

Plant-based medicine plays a vital role in cancer management, and 600/0 Of anticancer drugs are derived from plant resources; In the present investigation, entitled "Study on anticarcinogcnic activity of medicinal plant-Polycarpaea Lamk", the whole plant of Polycarpaea corymbosa was screened for anticancer activity.

The whole plant of Polycarpaea corymbosa dried powder was extracted sequentially by, hot continuous percolation method using soxhlet apparatus, using different polarities of solvents like petroleum ether, ethyl acetate and ethanol. The ethanolic extract of polycarpaea corymbosa whole plant has the highest yield (16,560/0) when compared to other extracts.

The phytochemical screening of petroleum ether extract of Pxorymhosa contains phytosterols, fixed oils & fats. Ethyl acetate extract containing alkaloids, carbohydrates, phenolic compounds & tannins, protein and amino acid compounds, Saponins and fixed oils & fats. The ethanolic extract containing alkaloids, carbohydrates, glycoside. phenolic

compounds, saponins, tannins, protein and aminoacid & flavonoid

The findings of the present studies indicate the ethanolic extract of whole plant of Polycarpaea corymbosa showed antioxidant activity by inhibiting DPPH and hydroxyl radical, super oxide anion scavenging, nitric oxide and hydrogen peroxide scavenging activities. In addition, the ethanolic extract of whole plants of Polycarpaea corymbosa was found to contain noticeabled amount of phenolics and flavonoid which play a major role in controlling oxidative stress.

The various extracts polycarpaea corymbosa were subjected to in vitro cytotoxic activity on MCF-7, HL-60, HepG2, HT-29, and Pc3 cell lines. The ethanolic extract of plant extract was **active on cell lines (MCF-7, 111.-60, Ilep (.'2, 11T-29 and PC3).** The cytotoxic activity might be due to the presence of phenols and flavonoids active components in ethanolic extract of Polycarpaea corymbosa.

The acute toxicity of various extracts from whole plant of Polycarpaea carried out as per OECD 423 guidelines for safe dose administration to animals. The of mice with various extracts from whole plant of Polycarpaea corymhosa did not change the autonomic or behavioural responses in mice. Zero percent mortality observed for different extracts of whole plant

of Polycarpaea corymbosa at the dose of 2000mg/kg. Hence there is no toxicity up to a dose of 2000mg/kg body weight of the animal and did not cause any death of the tested animals.

The various extracts of Polycarpaea corymbosa were subjected to invivo anti-cancer study. The altered levels of the body weight, mean survival time, percentage in life span, tumour volume, packed cell volume, tumour cell count, RBC, Hb. WBC, differential count, cathepsin-D, acid phosphatase, β-D-glucuronidasen 5'nucleotidase, lactate dehydrogenase, Na+/K+-ATPase, Mg+-ATPase, plasma glucose, liver glycogen and Nucleic acids (DNA &RNA) were significantly improved with administration of the extract of whole plant of polycarpaea corymbosa.

The level of thiobarbituric acid reactive substances (TBARS) is the tissues were significantly reduced after the treatment of extracts of whole plants of polycarpaea corymbosa. The anzymatic antioxidants like superoxide dismutase, catalese and glutathione peroxidase in the tissues were significantly proved and the level of non aneymatic anti-oxidants of GSH, vitamin C and E in the tissues were significantly proved

after the treatment of extract of whole plant of polycarpaea corymbosa shich were decreased in cancerous conditions which was seen in EAC alone treated animals.

The ethanolic extract of TBARs levels while enzymatic anti-oxidants like dismutase.glutathione peroxidase and the level of non-enzymatic antioxidants like C and E in the liver tissue were significantly improved when compared other extracts, The in vivo study indicates that the plant extracts were significant source of natural antioxidant, which might be helpful in preventing various oxidative stresses. The phenolic compounds and flavonoids present in the ethanolic extract may be responsible for this anticancer effect.

Histopathological studies in liver Shous that, carcinoma of the mice lÁer extensive hepatocellular lesions, unequal shape of hepatocytes and enlarged cells (Fig. 49) in EAC alone induced mice (Group Il).

The petroleum ether and ethyl acetate extract of Polycarpaea corymbosa (Fig. 50-51) treated (Group Ill - IV) animals showed mild changes in cellular architecture and shape of the hepatocytes. The ethanolic extract of Polycarpaea cownbosa treated animals (Group V) and standard drug treated animals (Group VI) showed the cellular architecture and fine vacuolar of hepatocytes similar to the normal control (Fig. 52-53) The present study has shown that the ethanolic extract of whole plant

of Polycarpaea Combosa has significant anticancer activity which confirmed by in-vitro studies and in-vivo anticancer studies.

Isolation and characterization of active compounds

The ethanolic extract containing noticeable amount phenolic compounds and which showed significant in vivo anti-cancer potential formed the basis for isolation of by column chromatography. Two compounds were isolated the ethanolic extract of Polycarpaea corymbosa namely as 6- methoxyflavone from the ethanolic extract of polycarpaea corymbosa. Namely as 6-methoxyflavone (compound 1) and
3,5,7-trihydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one (compoind2).
These compounds were characterized by IR, H NMR, C NMR and GC MS.

The isolated compounds from ethanolic extract of whole plant of Polycarpaea corymbosa were subjected to in vitro antioxidant (DPPH, superoxide radical scavenging and nitric oxide scavenging activity) and in-vitro cytotoxic activity using cell lines human cancerous liver cell lines (HepG2), human colon cancer cell lines (HT29). The isolated compound from whole plant of Polycarpaea

corymbosa (compound 2) showed a significant antioxidant and cytotoxicity properties. Similar results were not seen with isolated compound I from whole plant of Polycarpaea corymbosa. The isolated compounds from ethanolic extract of whole plant of Polycarpaea corymbosa were subjected to P53 gene expression in Hep (12 cells.

The real time PCR evaluation of HepG2 cells treated with different concentrations of the compound 2 was showed in a significant dose and time dependent increase in p53 mRNA expression than that of compound l. Flow cytometer analysis of p53 and Bcl-2 protein expression in HepG2 cells (2 x 105 cells/mL) treated with different concentrations of the isolated compounds for 4811 also resulted in a time and dose dependent up regulation of p53 when compared to control sample which confirmed the results from q-PCR analysis of p53 gene expression in HepG2 cells.

This finding provides some biochemical basis for the use of compound from polycarpaea corymbosa could have anticarcionogenic effect at least in part through modulation of apoptosis. Effectiveness along with safety is the advantage of this compound will be the future choice of drug in cancer treatment. Furthermore, in vitro studies are needed to explore the possible mechanism of action of isolated compounds derived from plant against carcinoma.

RECOMMENDATIONS

- This finding provides some biochemical basis for the use of compounds from polycarpaea corymbosa could have

  Anticarcinogenic effect, at least in part, through modulation of apoptosis.
- Effectiveness along with safety is the advantage of this compound will be the future choice of drug in cancer treatment
- Polycarpaea corymbosa may be ingested more frequently in different forms, if not to treat cancers, then as a prophylactic against them.
- Furthermore, in-vivo studies are needed to explore the possible mechanism of action of isolated compounds derived from the plant against carcinoma.

SCOPE FOR FUTURE WORK

Both plants extracts have shown promising antioxidant and anticancer potential.

Following are the future scope of the current research to develop these herbal extracts as a drug,

- Same method may be applied for extraction of plant material from same species from different geographical area and the extracts may be analyzed for phytochemical and biomedicinal properties.
- Further studies are required in animal model for the analyses of efficacy and toxicity to develop these extracts as natural antioxidant and anticancer drugs.
- If these extracts found effective and safe in animal models, clinical trials are required before licensing these extract as drug.

- Both plant extracts affecting various molecular pathways are needed to be analyzed.
- These herbal extracts may be used as a source of various neutraceutical supplements or herbal pharmaceuticals.

BIBLIOGRAPHY

1. Sinha SK, Dogra JV. A Survey of the Plants of Bhaglpur and Sanrhalpargana for saponin; flavonoids and Alkaloids. PharlaceutBio1.1985;23(2):77-86.
2. Bailabh B and Chaurasia OP. Traditional medicinal plants of cold desert Ladakh-used in treatment of cold, cough and fever. J Ethno pharmacol.2007; 112:341-349.
3. Kamboj VP. Herbal medicine. Curr sci.2000;297
4. World Cancer Report, world Health Organization. 2006 ;297.
5. Sankaran DM, Deshmukh VN. Ethno pharmacological review ofTraditional Medicinal plants for anticancer activity. Int J Pharm Tech. Res.2011; 3(1): 298-308
6. StojanRadi, ZoricaStanojevi, Bris Dindic. The pathogenesis of neoplasia. Arch Oncol. 2004 ;12(1) : 35-37
7. Franks LIM. The spread of tumours. Introduction to the Cellular and Molecular Biology of Cancer. 3rd ed. Franks LIM, Teich NM, editors, London: Oxford University Press; 2001. p. 1-17.

8. Varricchio, Claudette G. A cancer source book for nurses. Boston: Jones and Bartlett Publishers 2004; p.229.
9. Siegel R, Ma J, Zou Z, Jemal A. Cancerstatistics.CA. Cancer J Clin. 2014 ; 64 : 9-29.
10. Feriay J, Soerjomataram 1, Ervik, M, Dikshit R, et al. Cancer Incidence and mortality worldwide: IARC cancer base No.11. Int Agent for Res Cancer.2013;1.
11. Bozovic-Spasojevic1, AzambujaE,McCaskill Stevens W, BIBLIOGRAPHYDinh P, et al. Chemoprevent for breast. Cancer Treat Rev. 20012,38; 329-339.
12. Datta K, Choudhuri M, Guha S, Biswas J Al. Breast cancer scenario in a regional cancer centre in Eastern India over eight years' public health problem. Asian Pac J Cancer Prev. still a major public health problem. Asian Pac J Nat Cancer Inst. 2012; 13:809-813
13. Feuer EJ, wun LM, Boring CC, Flanders WD.et al, The lifetime
risk of developing breast cancer. J Nat Cancer Inst. 1993 ;85 :892-896.
14. Laurance, Jeremy. "Breast cancer cases rise 80% since Seventies". The Independent (London). Retrieved. 2006-10-09.

15. Culver JB, Hull J, LahadEL, Daly M, et al. Breast cancer genetics-an overview. Gene Clinics. 2000; 1-12.
16. Breast Cancer Treatment (PDQOO)". NCI. 2014; Retrieved 29 June 2014.
17. Saunders, Christobel, Jassal, Sunil, et al. (2009). Breast cancer (Led). Oxford: Oxford University Press. p. Chapter 13. ISBN 9780-19-955869-8.
18. World Cancer Report, World Health Organization. 2014. pp.
Chapter 5.2. ISBN 92-832-0429-8.
19. Halliwell B. Free radicals, antioxidants and human disease: Curiosity, cause or consequence? Lancet. 1994 ; 344 :721-724.
20. Rackova L, Oblozinsky M, Kostalova D, Kettmann V and Bezakova L, Free radical scavenging activity and lipoxygenase inhibition of Mahoniaaquifolium extract and isoquinoline alkaloids. J Inflam. 2007 ;4 : 15-22
21. Chanda S and Dave R. In vitro models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. Afri J Microbiol Res.2009; 3:981-996.

22. Wang S, Melnyk JP, Tsao R and Marcon MF, et al. How nature dietary antioxidants in fruits, vegetables and legumes promote vascular health. Food Res Int. 2011; 44: 14-22
23. Singh RP, Khanna R, Kaw JL, Khanna SK and Das M, et al. Comparative effect ofbenzanrhore and 3-bromobenzanthore on hepatic xenobiotic metabolism and antioxidative defense system in guinea pigs. Arch Toxicol. 2003; 77: 94-99
24. Jayakumar T. Thomas PA, JR and P, et al. In vitro vivo antioxidant effects of the oyster mushroom Pleurotusostreatus. Food Res Int. 2011; 44: 851-861
25. Bagchi K and Puri S. Free radicals and antioxidants in health and East. Mediterr. Health J.1998 ;4 :350-360
26. Samy RP, Gopalakrishnakone P and Ignacimuthu S, ChemjcoJBíological Interactions. 2006; 164:1-14.
27. nchez-Moreno C, Larrauri JA, Saura-Calixto F, a procedure to measure the antiradical efficiency of polyphenols. J Sci Food and the antiradical efficiency of polyphenols. Agric. 1998;7(2):270-76
28. Garlick P B, Davies M.J, Hearse DJ, Slater TF, e/ cd, Direct detection of free radicals in the perfused rat heart using electron spin resonance spectroscopy. Circ res. 1987; 61(5): 757-760
29. Turrens JF. Superoxide production by the mitochondrial respiratory chain. Biosci Rep. 1997 ;17(1) : 3-8

30. Dreher D. Junod AE. Role of oxygen radicals in cancer development. Eur J Cancer. 1996;32 (1): 30-38
31. Knight JA. Reactive oxygen species and the neurodegenerative disorders. 31. Ann clin Lab sci.1997 ;27(1) : 11-25
32. Fukuda A, Osawa T, Oda Tanaka T.et al. Oxidative stress response in iron-induced acutenephrotoxicity:enhanced expression of heat shock protein 90. BiochemBi0phys Res Common. 1996: 219(1) :76-81
33. Van AS. van Den Tromp M, Griffioen DEI. e antioxidant actiuty of flavonoids. Free Rad Biol and Med. 1996 ;20(3) : 331-342
34. licijnen CG, Haenen G, van Acker FA, BastA.et Flavonoids as pcroxynitrite scavengers: the role of the hydroxyl groups. Toxicolinvitro.2001; 15 (1):3-6
35. MarzoukMSA. Moharram FA- Mohamed MAs Gamal-Eldeen AM and Aboutabl EA. Anticancer and antioxidant tannins from Pimentadioica Icavcs, J Biosci. 2006 ; 62 : 526-536
36. Al-JabcrNA, Awaad AS and Moses Jlž. Review on some antioxidant plants grossing in Arab world J Saudi ChemSoci 20011; 15: 293-307

37. Farombi EO, Britton G, Emerole GO. Evaluation of the antioxidant activity and partial characterization of extracts from browned yam flour diet. Food Res. Int. 2000 ; 33(6) : 493-499
38. Williams GM, Iatropoulos MJ, Whysner J. Safety assessment of butylated hvdroxyl anisole and butylated hydroxyl toluene as antioxidant food additnes. Food and ChemToxicol. 1999;37 (9-10) : 1027-1038
39. Fan W, Johnson KR and Miller MC. In-vitro evaluation of combination Chemotherapy against human tumour cells. Oncol Rep.1998;5(5): 1035-1042
40. Jonsson E, Fridorg H, Asoka K, Dhar S, el 'IIS Cytotoxic activity of topotecan in human tumour cell lines and primary cultures of human tumour cells from patients. British J Cancer. 1997; 76(2):211-9.
41. Martinsson P, Liminga G, Dhar S, Lukinius A. al. Temporal effects of the novel antitumourpyridylcyanoguanidine (CHs 828) on human lymphoma cells. Eur J Cancer 2001 ;37(2):260-7.
42. Laskova A and Majtas J. Quality of life in chemotherapy of ovarian cancer. ActaFacultatisPharmaceutUnivComenianae. 2004 ;51 : 136-142.

43. Oconnor K and Fitzpatrick J. Side effects of treatments for locallv advanced prostate cancer. British J Uro. 2005; 97: 22-28
44. Indap MA, Radhika S, Motivale L, Rao KV, et al. Anti-cancer activity of phenolic antioxidants against breast cancer cells and a spontaneous mammary tumour. Ind J Phama Sciences. 2010 ;19 :470-474.
45. Elemar GM, Rafael DA, Costa H, Renato IMR, et al: Pharmacological evidences for the extracts and secondary metabolites from plants of the genus. Hibiscus. Food Chem. 2001; 118:1-10.
46. Surh Y. Molecular mechanisms of chemopreventive effects of selected dietary and medical phenolic substances. Mutation Res.
1999: 428(1-2): 305-27.
47. Figueroa -Hernandez JL, Sandoval G, Jayme AV, FigueroaEspitia JL, et al. Plant products with anti-cancer properties employed in the treatment of Bowel cancer. Proceed West Pharmacol Soci.2005 ;48 : 7-83.
48. Weed HG, McGandey R and Kennedy AR. Protection against dimethyl hydrazine- induced adenomatous tumors of the mouse colon by the dietary addition of an extract of soybeans containing the Bowman-Birk protease inhibitor. Carcinogen.

1985;6(8): 1239.pan L, Chai Il and Kinghorn AD. The continuing search for antitumor

49. agents from higher plants. Phytochem Letters. 2010; 3:1-8. Cragg GM, Grothaus PG and Newman DJ. Impact of natural products on

50. developing new anti-cancer agents. ChemReviews. 2009; 109:3012-3043•

51. Ferguson PJ, Kurowska E, Freeman DJ, Chambers AF, et al. A navonoid fraction from cranberry extract inhibits proliferation of human tumor cell lines. J Nutri 2004; 134: 1529-1535.

52. Liu X, Zhao M, wu K, Chai x,et al. Immunomodulatory and anticancer activities of phenolics from emblica fruit (Phyllanthusemblica Food Chem. 2012; 131: 685-690.

53. Suffness M and Douros J. Drugs of Plant origin. In: De Vita VT. Methods in Cancer Research, New York, New Academic Press. 1979 ; 16 :73-94.

54. Cragg GM and Newman DJs Plants as source of anticancer agents. J. Ethno. Pharmacol. 2005;100: 72-79.

55. Wani MC, Taylor I-IL, Wall M E, Coggon P et al., Plant anti-tumor agents, VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxusbrevifolia. J Am ChemSoci. 1971 ;93 :2325-2327.

56. Rowinsky EK, Onetto N,Canetta RM and Arbuck SG. Taxol-the 1st of thetaxanes, an important new

class of anti-tumor agents. Semi Oncol. 1992 ; 19 :646-62.
57. Potmeisel M and Pinedo H. Camptothecins: new anticancer agents. Boca Raton, Florida, CRC Press. 1995 ; 149-150.
58. Bertino JR. Irinotecan for colorectal cancer. Semi.Oncol. 1997 ;24 : S1 8-S23.
59. Harvey AL. Medicines from nature: are natural products still relevant to drug discovery? Trends in Pharmacol.Sci. 1999;20: 196-198.
60. Itokawa H, Wang X and Lee KH - Homoharringtonine and related compounds. In: Cragg GM, Kingston, DGI, New-man D, (Eds). Anticancer agents from natural products. Boca Raton, Florida, Brunner-Routledge psychology Press, Taylor & Francis
61. Kantarjian HM, (DC) Brien S,Anderlini P chronic myelogenousleukemia: current status and investigational options.Blood. 1996; 87;3069-3081.
62. ParagR, Bhuvan PR, HamsrajA.Karanth, e/ (d. Potent antitumor activity of Rubiacordifolia. Int J Phytomed 2010 ;244-46.
63. Adwankar MK, ChitinisMP.ln vivo anticancer activity or RC-18: a plant isolate from Rubiacardi/ô/ia, Linn against spectrum of

experimental tumor models. Chemother. 1982 ;28(4) :291-3.

64. BabuTD, Kuttan G, PadikkalaJ.Cyt0t0xic and antiutumor properties of certain taxa umbelliferae with special reference to Centellaasiatica(L). Urban J Ethno Pharmacol.1995 ; 48 :53-57.
65. Singh RP, Banerjee S, Rao AR. Modulatory influence of Andrographispanicula/on mouse hepatic and extrahepatic carcinogen metabolizing enzyme and oxidant status. PhytotherRes.2001; 15(5):382-390.
66. Subapriya R, BhuvaneswariV,NaginiS.Ethanolicneem (Azardirachtaindica) leaf extract induces apoptosis in the hamster buccal pouch carcinogenesis model by modulation of BC 12,13im,Caspase 8 and Caspase 3.Asian Pac J Cancer Prev .2005; 6(4): 515-520.
67. Zhang J Qi H, Wu C. Research cells of anti-proliferation and its mechanism. Zhong of curcumin on A549Yao human lung cancer cells and its mechanism. Zhong Yao Cai.2004;27(2):923927.
68. YaminiBibi, SobiaNisa, Muhammad Zia, Abdul waheed, el al. In vitro 68cytotoxic property of Aesculusindica against breast adenocarcinoma cell line (MCF-7) and phytochemical analysis. Pak J Pharm Sci. 2012;25(1): 183-187.
69. Zhang M,BinnsCW,Lee AH. Tea consumption and ovarian cancer risk: A case-control study in china.

CancEpidemiolBiomarkrs Rev.2002 ;1 1 :317318.

70. Hsu SD, Singh 13B, Lewis JB, Broke JL, al. Chemoprevention of oral cancer by green tea, Gen. 2000; Dent.50:140-146.
71. Su LJ, Arab L. Tea consumption and the reduced risk of colon Results from a national prospective cohort study. Pub Health Nutr 2002;5: 419-425
72. Setiawan VW, Zhang zp, Yu GP, Lu QY, et al. Protective effect of green tea in the risk of chronic gastritis and stomach cancer. Int J Cancer. 2001 ; 92 :600-604
73. Jian L, Xie LP, Lee AH, Binns CW. Protective effect of green tea against prostate cancer: A case — control study in southestchina.

Int J Cancer 2004; 108:130-135.

74. Demeule M,Levesyue-J Michaud, Annabi B, Gingras D, et al. Green tea catechins as novel antitumor and antiangiogenic compounds.Curr Med Chem Anti-CancAgents.2002;2 :441-463.
75. Kazi A, Smith DM, Daniel K, Thong S, et al. Potential molecular targets of tea polyphenols in human tumour cells: Significan. Canc. Preven. In vivo. 16 :397-403.

76. Heber D. Vegetables, fruits and phytoestrogens in the prevention of diseases.J Postgrad Med.2004;50: 145-149.
77. Hirano T, Abe K, Gotoh M, Oka K.Citrus flavone tangeretin inhibits leukemic I-IL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. Br J Cancer. 1995 ;82 :1380-1 388.
78. Prabha AL, Nandagobalan V, KannanV.Cancer and plant based anticancer drugs. In:Role of Biotechnology in Medicinal and Aromatic plants. Vol. V,1st Ed. (Khan IA and Khamum A, eds). Hyderabad, India: Ukaaz Publications; 2002.p.32-67.
79. Prajapati ND, Purohit SS, Sharma AK, Kumar T. A Hand Book of Medicinal Plants. Agrobios, India: 2003.
80. Datta PR, Diwakar J3T, Viswanatha S, Munhy KN, Naidu KA. safety evaluation study on garden cress (LepidiumsativumLinn.) seed in wister rats. Int J Applied ResNaturProducts. 2011;4 (1):37-43
81. Yogesh CY, Aviieet Jain, Sri Vastava DN, Anurekha J. Fracture healing activity of ethanolic extract of Lepidiumsa/ivum. Linn.seeds in internally fixed rats femoral osteotomy model.Int. J PharmaPharmaceut.Sci,2011;3(2).
82. Eddouks M, Maghrani M, Zeggwagh NA, Michel JB,et al. Study of thehypoglycemic activity of

Lepidiumsativum L aqueous extract in normal and diabetic rats. J EthnoPharmacol.2005;97;391-395.

83. MuhamedEddouks and MhamedMaghrani.Effect of LepidiumsativumLinn., on renal glucose reabsorption and urinary TGF-l levels in Diabetic rats.PhytotherRes. 2008; 22:1-5.
84. WafekaAbdulah AI Hamedan. Protective effect of LepidiumsativumLinn, L seeds powder and extract on hypercholestrolemic tars. J Am Sci.2010;6(11)
85. TahaouiA, EI-Hilaly J, Israili ZH, Lyovrssi B et al.? Ethno pharmacologic survey of plant used in the traditional treatment of hypertension and diabetes in south-estern Morocco (Errachidiaprovince) phytotherapy of hypertension and diabetes in oriental Morocco. J Ethno phaemacol.1997;58(1):45-54
86. Annie Shirwaikar, Bharatkumar Patel, YogeshKarmariya, VinitParmar, et al. In vitrofree radical scavenging potential of defatted ethanolic ext. of seeds of Lepidiumsativum Linn. Science Direct.Chinese J NatulMedi.2011;9(6):0435-0440.

87. AL-Yahya MA, Mossa Js, AM and S, Pharmacological on L seeds. Phytomed.1994; 1:155-159
88. AlokShukla, Chandra shekharsingh and papaya Bigoniya. Phyto chemical and CNS activity of Lepidiumsa/ivum Linn total alkaloid. scholars research library Der Pharmacia Lctter. 2011;3(2):226-237,
89. Afaf IA, Nuha Hs, Mohammed AH, Hepatoprotective effect of Lepidiumsativum against carbon tetra chloride induced damage in rats. Res. 2008; 3:20-23
90. Shama IYA, ShaymaAM, Salih and warda S, Abdulgadir. In vitro antimicrobial assessment of Lepidiumsativum L seed extract, Asian J Med Sci. 2011; 3(6): 261ISSN: 2040-8773.
91. Najeeb-urRehman, Malik HM, Khalid M, Alkharfyet al., Studies on Antidiarrheal and Antispasmodic activities of Lepidiumsativum crude extract in rats. Phytother Res. 2012 ;26: 136-141.
92. Najeeb-ur-Rehman, Malik 1-1M, Khalid M, Alkharfyel al., Prokinetic and Laxative activities of Lepidiumsalivumseed extract with species and tissue selective gut stimulatory actions. J EthnoPharmacol. 2011; 134:878-883.
93. Umang Patel, MukulKulkarni, VaishaliUndale and Ashok Bhosale. Evaluation of diuretic activity of aqueous and methanol extracts of Lepidiumsativum

Garden Cress (Cruciferae) in rats. Tropi J PharmaceutRes.2009;80 (3): 215-219,

94. Diwakar BT, Dutta PIC, Lokesh BR, Naidu KA. Bio-availability and metabolism of n-3 fatty acid rich garden cress
(Lepidiumsativum) seed oil in albino rats. Prostagland.Leukotri Essen Fatty acids. 2008; 78; 123-130.
95. MuhamedMaghrani, Naoufe, Zeggwagh NA, Michael JB, et al., Anti-Hypertensive effect of Lepidiumsativum L in spontaneously hypertensive rats. J EthnoPharmacol. 2005; 100:193-197.
96. Archana N. Paranjape, Anita A, Mehta. A et al. of Lepidiumsativum seeds in treatment of Bronchial asthnn Iran J Pharmacontherapeut. 2006; 5:55-59.
97. sawsan Hassan, Mahassni&Roaa Mahdi Al-Reemi. CMotoxic effect of an aqueOUS extract of LepidiumsativumL On human breast cancer cells-Ind J Tradit Knowled. 2013;12(4): 605-614.
98. Laith Al-Obaidi. study the Anticancer Effect of
Lepidiumsativum extract on Squamous Cell Carcinoma (CAL27) cell Lines. J Natul Sci Res. 2014; 4(17): 48-52.

99. Pramod J Kasabe, Pranit N Patil, Dayanand D Ramble, Padrna B Dandge. Nutritional, elemental analysis and antioxidant activity of garden cres (Lepidiumsativuml.) seeds. Int J PharmaPhamaceut Sci.2012;4(3).
100. FekaduKassie, Sylvia Rabot, Maria ULI, Wolfgang Huber et al. Chemoprotective effects of GC (L.S) and its Constituents towards 2-amino3 methyl imidazo (4,5F quinoline (IQ) induced genotoxic effect and colonic preneoplastic lesions.
Carcinogenesis.2002;23(7):1 155-1161.
101. Orlouskaya TV and ChelombitkoVA. Phenolic compounds from Lepidiumsa/ivumL. ChemNatul Compo.2007; 43(3).
102. JyotiAgarwal& D.L. Verma. Antioxidant Activity-Guided fractionation of aqueous extracts from Lepidiumsa/ivumand identification of active flavonolglycosides. Academia Arena.201 1 12) : 14-17.
103. Movrin M, Maysinger D, Marok E. Biologically active Mannich bases derived from nitroxoline. Pharmazie.1980;35(8):458-60.
104. Ziska P, Kind A, Franz H, Isolation and characterization of a Lectin from garden cress(LepidiumsaÍivum). ActaHistochem. 1982:71(1):29-33.
105. Radwan 1-1M, El-Mssiry MM, Al-said WM, Ismail As et al., Investigation of the Glucosinolates of

Lepidiumsativum growing in Egypt and their Biological activity. Res J Medi Medical Sci. 2007;2(2): 127732.

106. Chitra M, Nithyanandi K. Radical Trianthematriquetra in male albino rats intoxicated with CCI_4. J Environ Bioligy.2007;28(2):283-285.
107. DeshpandeDJ.Lepidiumsativum Handbook of Herbal Remedies, 102-105.
108. The Plant List, http://ipni.org/urn:lsid:ipni.org: names: 1: 138141-2
109. Chopra RN, Nayar SK, Chopra IC. New Delhi: CSIR; 1956. An Introduction of the plant taxonomy. Glossary of Indian Medicinal Plants.
110. Divanji M, Viswanatha GL, Nagesh S, Shiva Prasad FIN et al., Ethnopharmacology of Lepidiumsativum Linn Brassiaceae: A Review.
111. Lawrence George HM. United States of America: 1959. An Introduction of the plant Taxanomy.
112. Nita DR, Pandya TN. Pharmacognostic study of Lepidiumsativum Linn (Chandrasura) Ayu. 2011;32(1):1 16-119.doi: 10.4103/0974-8520.85742
113. Neueschriften Get. Naturf. Freunde Berlin,

TrianthemaTriquetraRottler Ex. Willd Flora Base: The Western Australian flora. 1803 ;4: 181.

114. ForsskVahl. Australian plant census, Published in: Black, J.M. 1948, Flora of South AustraliaEdn. 2.2 255-521.

115. Australian Plant Census. Published in: Eichler, Hj.1 965, Supplement to J.M. Black's Flora of South Australia (Second Edition, 1943-195

116. Australian Plant Census. Trianthemaglaucifolia F. Muell. Published in: Hartmann, H.E.K., Meve, U. &Liede-Schumann, S. 2011, Towards a revision of Trianthema, the Cinderella of Aizoaceae. Plant Ecology and Evolution. 144(2): 177-21.

117.Mansoorahmeed, Muhammad ashraf, F. Al-Quriany, Tahira Nawaz. Medicinal flora of the Cholistan desert: a review Pak. J Bot.2011; 43:39-50

118.Mudaliar&Rao, 191; Klebb, Bull, Coun, Sci, Indcestr, Res.

Aust., FI Delhi; 175; No.232, 1948; 14.

119. www. efloras.org. Flora of Pakistan. TrianthematriquetraRottlerex.Willd.

120. Jacobs WL, Highet J, Taxon concept: A Prescott 1984.Australian National Botanic Garden.

121. Harborne JB. Phytochemical methods 1l Edn. In Chapman &, Hall. New York. 1984; 4-5.

122. Kokate CK, 1999.Practical Pharmacognosy. 4th Edn.

Vallabhprakasan publications, New Delhi, India.

123. Evans WC, An index of medicinal plants. A Text book of Pharmacognosy.
14th ed. 1997 ; 7(5) : 12-14
124. Ramakrishnan S, Rajan (1994): Text book of Medical Biochemistry Oriantlongman, New Delhi, India.
125. Fisher DD. Protein staining of ribbonedepon section for light microscopy. Histochem. 1968; 16: 81-96.
126. Ruthman AC. (1970): Methods in Research, Cornell University Press, New york. USA.
127. Finar IL. (1986): Stereochemistry and the chemistry of natural products. v012.Longman. Singapur.
128. Mace ME. Histochemical localization of phenols in healthy and diseased tomato roots.Phytochem. 1963; 1 6:915-925.
129. Mensor LL, Meneze FS, Leitao GG, Reis AS et al., Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother Res. 2001 ; 15 :127-130.
130. Winterbourne CC, Hawkins RE, Brain, M and Carrel RW. The estimation of red cell superoxide dismutase activity. J Lab Chem Med 1975 ;85 : 337341.
131. Garrat DC. The quantitative analysis of drugs, Champman and Hall, Japan, 1964;3: 456-458.

132. Elizabeth K and Rao, MNA. Oxygen radical scavenging activity of curcumin. Int J Pharm. 1990 ;58 : 237-240.
133. Mallick CP and Singh MB. Plant enzymology and Histoenzymology (1Edn), Kalyani publishers, New Delhi, 1980;286.
134. Cameron GR, Milton RF and Allen J W. Measurement of flavonoids in plant samples. Lancet 1943 ; 179.
135. Abdullaev FI, Luna RR, Roitenburd BV, Espinosa AJ, et al Pattern of childhood cancer mortality in Mexico, Arch. Med Res.
2000;3: 1526-31.
136. Masters JRW. 2000. Animal cell culture-A practical approach. oxford university press, great clarendon str,3rd Ed, pp.82,
137. Skehan P. New colorimetric cytotoxicity assay for anticancer drug screening. J NatlCanc Inst. 1990 ;82 :1107-1112.
138. Monks. Feasibility of a high-flux anticancer drug screening using a diverse panel of cultured human tumor cell lines. J NatlCanc Inst. 1991 ; 83 :757-766.
139. OECD 423. OECD Guideline for Testing of Chemicals Acute Oral
Toxicity-Acute Toxic Class Method Adopted: 17th December 2001. 140.Barros ACSD, Muranaka ENK, Mori LJ, Pelizon CHT, et al., Induction of experimental mammary carcinogenesis

in rats with 7,12-dimethylbenz (a) anthracene, Rev lospClinFac Med S Paulo. 2004; 59:257-61

141. Nichans WH, Samulelson B. Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur JBiochem.1968 ;6 :126-30.
142. Sevanian A and Ursini F. LPO in membranes and low density lipoproteinssimilarities and differences. JBiolMed.2000 ;29 :306-311
143. Halliwell B and Chirico S. LPO- its mechanism and significance.Am.J .Clin.Nutrition. 1993; 57:715-724.
144. Kakkar P, Das B, Visvanathan PN. A modified spectrophotometric assay of SOD. Ind J Biochem Biophys.1984 ;21 :130-2.
145. Sinha AK. Colorimetric assay of catalase. Anal. Biochem.1917 ;47 :389.
146. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hatman DG and Hoekstra WG, Selenium; Biochemical roles as a component of glutathione peroxidise. Sci.1973; 179:588.
147. Ellman GL. Tissue sulfhydryl groups Arch. BiochemBiopsy.1959; 82:70-77.

148. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ,et al. Protein
measurement with the Folin phenol reagent. J Biol Chem.1951 ;193(1) :265-75.
149. Baker H, Frank O, De Angelis B et al. Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol.
NutrRes.1980 ;21 :531536.
150. Roe JH, Kuether CA. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenlyhydrazine derivative of dehydroascorbic acid. J BiolChem. 1943 ; 11 : 145164.
151. Rates S. Plants as source of drugs. Toxicon.2001 ; 39 :603-613.
152. Larson RA- The antioxidants of higher plants. Phytochem.1998 ; 27(4) :969-978.
153. Gazzani G, Papetti A, Massolini G and Daglia M. Anti and prooxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment. J Agric Food Chem.1988 ;46 :4118-4122.
154. Velioglu Y S, Mazza G, Gao L and Oomah BD. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. JAgric Food Chem.1988 ;46 :4113-4117.
155. Jayprakash GK, Singh RP, Sakariah KK. Antioxidant açtivity of grape seedextracts on

peroxidation models in vitro. J Agric Food Chem. 2011; 55:1018-1022.

156. Elayaraja A, Vijayalakshmi M, and Devalarao G. In vitro free radical scavenging activity of various root and rhizome extracts of acoruscalamuslinn. Int J Pharma Bio Sci.2010 ;4 :1.
157. Thomson LL, Lawton FG, Knowles RG, Basley JE, Riversomoreno V, Moncada S. NO synthase activity in human gynecological cancer. Canc Res.1994 ;11:1352-1354.
158. Reveneau S, Arnould L, Jolimoy G, Hilpert S, Lejeune P, Saint- Giorgio V, Belichard C, Jeannin JF. Nitric oxide synthase in human breast cancer is associated with tumor grade, proliferation rate, and expression of progesterone receptor. Lab Invest. 1999 ; 11 : 1215-1225.
159. Cabs CS, Brenman JE, Aldape KD, Bredt DS, Isrnael MA. Expression of NOS in human central nervous system tumors. Canc Res. 1995; 11: 727-730
160. Taysi S, Uslu C, Akcay F, Sutbeyaz MY. MIDA and nitric oxide in the cancer. Surg plasma of patients with advanced laryngeal Today 2003 ;11(9) :651-654.
161. Prazma J, Pertrusz P, Mims W, Ball SS, Weissler MC.

Immunohistochemical characterization of NOS activity in squamous cell carcinoma of head and neck.Otolaryn Head Neck Surg. 1995; 1:541-54.

162. Ying L, Hofseth LJ. An emerging role for endothelial nitric oxide synthase in chronic inflammation and cancer. Canc Res. 2007 ; 1 1 : 1407-1410.

163. Shang ZJ, Li JR, Li ZB. Effects of exogenous nitric oxide on oral squamous cell carcinoma: an in vitro study. J Oral Maxillofaci surg.2002 ;11(8) :905-910.

164.Harada K, Supriatno, Kawaguchi S, Tomitaro O, Yoshida H, Sato M. Overexpression of iNOS gene suppresses the tumorigenicity and metastasis of oral cancer cells. In Vivo. 2004 ;11(4) :449-455.

165. Gulcin I, Oktay M, Kirecci E, Kufrevioglu 0I: Screening of antioxidant and Antimicrobial activities of anise (*PimpinellaanisumL*) seed extracts. Food Chem 2003 ;83 :371382.

166. Kumar S, Kumar D, Prakash O. Evaluation of antioxidant potential of phenolic and flavonoid contnt of Hibiscus tiliaceus flowers. EJEAF Chem.2008; 7:2863-71.

167. Sharififar F, Dehghan-Nudeh G,MirtajaldiniM.Major flavonoids with antioxidant activity from Teucriumpolium.Food Chem. 2009; 112:885-888.

168. Duh PD, Tu YY, Yen GC. Antioxidant activity of water extract of HarngJyur (*Chrysanthemum*

morifoliumRamat). LMT- Food Sci Tech. 1999; 32: 269-277.

169. Salah N, Miller NJ, Paganga G, Tijburg L, et al. Polyphenolic flavonoids as scavengers of aqueous phase radicals and as chainbreaking antioxidants. Arch Biochem Biophys.1995 ;322(2) :339346.

170. Van Acker SABE, Van den Vijgh WJF and BastF.Structural aspects of antioxidant activity of flavonoids. FreeRadi Bio Med.
1996 ; 20(3) : 331-342.

171. Habbu PV, Mahadevan KM, Kulkarni PV, Daulatsingh C, et al. Adaptogenic and in vitro antioxidant activity of flavanoids and other fractions of *Argyreiaspeciosa* (Burm.f) Boj. in acute and chronic stress paradigms in rodents. Indian. J Exp. Biol.2010 ;48(1) :53-60.

172. Cardellina JH, Fuller Rw, Gamble WR, Westergard C, et al. Evolving strategies for the selection dereplication and prioritization of anti-tumour and HIV -inhibitory natural products extracts. In: Bohlin L, Bruhn JG. (eds.) Bioassay methods in natural product research and development.
Dordrecht: Kluwer Academic Publisher. 1999 ;25-36.

173. Baskar AA, Ignacimuthu S, Paulraj GM, Numair KSA. Chemo preventive potential of ß-sitosterol in

experimental colon cancer model an in-vitro and in-vivo study. BMC Complement Altern Med-2010 ;10 : 2-10.

174. Skehan P, Storeng R, Scudiero D, Monks A, et al. Evaluation of Colorimetric Protein and Biomass Stains for Assaying Drug Effects upon HumanTumor Cell Lines. Proceed. Am AssociCanc Res. 1989; 30: 612-621.

175. Skehan P, Storeng R, Scudiero D, McMahon J, et al. New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. J NatCanc Inst. 1990; 8203): 107-1 1 12.

176. Jainu M, Devi CSS. Antioxidant effect of methanolic extract of Solanumnigrum berries on aspirin-induced gastric mucosa injury. Ind JClinBiochem. 2004; 19 :57-66.

177. Kennedy DO, Kojima A, Hasuma T, Yano Y, et al.Growth inhibitory effect of green tea extract and (-)-epigallocatechin in Ehrlich ascites tumor cells involves a cellular thiol-dependent activation of mitogenic-activated protein kinases. Chem Biol.2001; 134: 113-133.

178. Ye F, Xui L, Yi Jria. Zhang D Y. Anticancer activity of Scutellabaicalensis and its potential mechanism. J Altern Complement Med.2002 ;25 :1801-7.

179. Lu X, Hsieh TC, wu JM. Equiguard suppress androgen dependent LNCaP prostate cancer cell

proliferation by targeting cell cycle control via downregulation off the retinoblastoma protein RB and induction of apoptosis via the release of cytochrom
c. Int J Oncol. 2004 ;25 :1801-7).

180. Aruoma 0I. Nurition and health aspects of free radicals and antioxidants.FoodChemToxicol. 1994; 32:671-685.

181. Conklin KA.Chemothrapy-associated oxidative stress:lmpact on chemotherapeutic effecivness.IntegrCancrTher.2004;3:294-300.

182.loset JR,Marston A,Gupta MP, Hostettman K. A methyl flavonoid with free radical scavenging properties from
Pancratiumlittorale. Fitoterapia.2001; 72: 3 5-39.

183. Russo J, Russo JH. Experimentally induced mammary tumors in rats. Breast canc. Res Treat. 1996 ;39 :7-20.

184. Butler MS. Natural products to drugs:natural product-derived compounds in clinical trials. Nat Prod Rep. 2008 ;25 :475-516.

185. Dias M, Cabrita E, Sousa E, Franca B, e/ al. Benign and malignant tumors induced by DMBA in female Wister rats.Eur J GynaecolOncol.1999;4:285-8.

186. SahaD,HaitM.An oncological design:two stage mouse skin carcinogenesis induced by DMBA and promoted by croton oil. Asian J Pharm Sci.2012 ;2 :1-3.
187. Flohe L. Otting Superoxide dismutase assays. Meth Enzymol. 1984; 105:93.
188. Krishnamurthy V, Palanivelu SB and Panchanatham S. Antioxidant activity of bark of brideliaretusaspring on DMBA induced mammary carcinogenesis in female Sprague dawley rats. Bio Pharm Bull. 2006;29(3):55-59.
189. Cordeiro Mc and Kaliwal BB. Antioxidant activity of bark extract of Brideliaretusaspreng on DMBA induced mammary carcinogenesis in female Sprague dawley rats. JPharmacog.2011 ; 20) : 14-20.
190. VennilaR,Thirunavukkarasu SV and Muthumarya J. Asian J PharmacutClini Res.2010;3(4):30-34.
191. Emel Z, Pinar A, Emin K, Koray GM t al. Alterations in lipid peroxidation and antioxidant status in different types intracranial tumors within their relative peritumoral tissues. ClinNurolNurosurg.2009; 111 :45-51.)
192. Amin A. Chemopreventive effect of chlorella on the antioxidant system in DMBA-induced oxidative stress in liver. Int JPharmacol. 2008;4(3):169-176.
193. Samy RP, Gopalakrishnan P and Ignacimuthu S.Antitumor promoting potential of luteolin against

7,12-dimethylbenz(a) anthracene-induced mammary tumors in rats.ChemBiol Interac.2006;164:1-14.

194. Mates JIM, Perrez-Gomez C and Decastro TN.Antioxidants, enzymes and Human Diseases. ClinBiochem. 1999 ;32 :595-603.

195. Li Y and Goodwin DC. Vital roles of an inter helical insertion in catalase peroxidase biofunctionality.BiochemBiophysi Res Communi.2004;318:970-76.

196. Kavitha K, Manokaran S. Anti-carcinognic and antilipoperoxidative effects of Tephrosiapurpurea (Linn). In 7,12dimethyl benz(a)anthracne (DMBA) induced hamster buccal pouch carcinoma.lnd J Pharmacol.2006;38: 185-9.)

197. Arivazhagan S, Balasenthil S and Nagini S. Chemopreventive potential of garlic and neem during gastric carcinogenesis induced by N-methylN '-nitro-N- Nitrosoguanidine. MediSciRes. 1998; 27: 527-529.

198. Frenandez-Checa JC, Kaplowitz N, Garcia Ruiz C, Colell A, et al. GSH transport in mitochondria: defense against TNF-induced oxidative stress and alcohol induced defect. Am J Physiol.1997 ; 273 :7-17.

199. YU BP. Cellular, defences against damage from reactive oxygen species. PhysiolRev.1994;74:139-162.
200. Hemila H, Roberts P, Wikstrom M. Activatedpolymorphonuclear leucocytes consume vitamin C. FEBSLett.1985;178:25-30.
201. Ki Won Lee, HyongJoo Lee, Young-JoonSurh and Chang Yong Lee. Vitamin C and cancer chemoprevention: reappraisal 1-3. Am J ClinNutr. 2003; 78: 1074-8.
202. ArtiShukla, Anamika M. Rasik and Bhola N. Dhawan. Asiaticosideinduced Elevation of Antioxidant Levels in Healing Wounds. Phytother Res. 1999 ; 13 : 50-54.
203. Sailesh Narayan, Abilasha Mittal. In vitro antioxidant activity and free radical scavenging potential of roots of Red sage. Asian J Pharmaceut.ClinicRes.2015 ;8(2) :417-421.
204. Fotsis T, Pepper M S, Aktas E, Breit S, et al. Flavonoid, dietaryderived inhibitors of cell proliferation and in vitro angiogenesis. Canc Res. 1997; 57 :2916-2921.
205. Wagner H. Geyer B, Yoshinobu K. and Govind SR. Coumestan as the main active principles of liver drugs EcliptaalbaandWedelicacalendulaceae. Planta Medica. 1986 ;5 : 370-372.

206. Folkmam J, D'Abore PA. Blood vessel formation: what is he molecular basis? Cell. 1996;87: 1 153-1155.
207. Watson K, Folkman J, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to Neoplasia.1989;339:58-61
208. ShahidPervez and Hassan Khan. Infiltrating ductal carcinoma breast with central necrosis closely mimicking ductal carcinoma in situ (comedo type): a series.J Med Case Reports-2007;1:83.
209. Hatano T, Edamatsu R, Mori A. Effect of interaction of tannins with coexisting substances. VI. Effects of tannins and related polyphenols on superoxide anion radical and on DPPH radical. ChemPharmaceut Bulletin.1989 ;37 :2016-2021.

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