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Anticancer Activity and Phytochemicals Analysis of Eucalyptus species in Bangladesh

Islam, Md. Farhadul

University of Rajshahi

Anticancer Activity and Phytochemicals Study of *Eucalyptus species* in Bangladesh

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SUBMITTED BY

Md. Farhadul Islam

Examination Roll No: 10210

Registration No: 0449

Session: 2010-2011

Cell Culture Laboratory
Department of Biochemistry & Molecular Biology
University of Rajshahi
Rajshahi, Bangladesh

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Dedicated
To
My Beloved Parents
And Supervisor

DECLARATION

I do hereby declare that the materials embodied in this thesis entitled "**Anticancer Activity and Phytochemicals Analysis of *Eucalyptus species* in Bangladesh**" prepared for submission to the Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh for the degree of Master of Philosophy in Science, are the original research works of mine and have not been previously submitted for the award of any degree or diploma anywhere.

(Md. Farhadul Islam)
M. Phil. Fellow

CERTIFICATE

This is to certify that, the thesis entitled "**Anticancer Activity and Phytochemicals Analysis of *Eucalyptus species* in Bangladesh"** has been prepared by Md. Farhadul Islam under our guidance and supervision for submission to the Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh for the degree of Master of Philosophy in Science. It is also certified that, the materials included in this thesis are original work of the researcher and have not been previously submitted for the award of any degree or diploma anywhere.

(Dr. Shaikh Md. Mohsin Ali)
Professor
Department of Applied Chemistry and Chemical Engineering
Co-Supervisor

(Dr. Jahan Ara Khanam)
Professor
Department of Biochemistry and Molecular Biology
Principal Supervisor

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ABSTRACT

An attempt has been made in this dissertation to find out potential chemo-preventive drugs for effective therapeutic management of cancers. For this purpose, anticancer activity of methanol extract (ME), petroleum ether extract (PEE) and ethyl acetate extract (EAE) of *Eucalyptus camaldulensis* has been studied out against Ehrlich ascites carcinoma (EAC) cells in mice with three different doses (25.0, 50.0 and 100.0 mg/kg/day; i.p.). The study has been done by monitoring the parameters like tumor weight measurement, survival time of tumor bearing mice, growth inhibition of cancer cells, hematological profile, transplantability of tumor cells etc. All the extracts showed remarkable anticancer properties. Among them, EAE exhibited the highest activity at the dose of 100 mg/kg/day (i.p.). It showed 96 % ($P < 0.001$) cell growth inhibition and reduced tumor burden significantly (81.4 %; $P < 0.01$) when compared with the control. It also increased the life span of EAC bearing mice pronouncely (71.36 %; $P < 0.01$). It restored the altered hematological parameters more or less towards normal levels. On the other hand, ME and PEE showed 71.72 % ($P < 0.01$) and 85 % ($P < 0.001$) cell growth inhibition respectively at the same dose. They also showed potential resistance in tumor burden and increased the life span of tumor bearing mice remarkably.

EAE showed its resistance to EAC cells by inducing apoptosis as investigated by observing morphological changes of EAC cells treated with EAE under fluorescence microscope. EAC cells treated with EAE showed membrane blebbing, chromatin condensation, nuclear fragmentation (apoptotic feature) in Hoechst 33342 staining. DNA fragmentation assay in agarose gel (1.5%) electrophoresis also reflected the apoptotic death of EAC cells following EAE

treatment. Apoptosis of EAC cells treated with EAE is induced by caspase-3 mediated signaling pathway, which was observed from cellular growth in presence of caspase-3 and caspase-8 inhibitors. Caspase-8 inhibitor (caspase-8 blocked and caspase-3 active) pretreated EAC cells showed 67% ($p<0.01$) cell growth inhibition with EAE in comparison with that of control whereas caspase-3 inhibitor (caspase-3 blocked and caspase-8 active) did not show any such effect.

Biochemical and hematological parameters as well as histopathological observations of major organs of mice receiving the extracts did not show any major or minor toxicity at the dose used in this studies. Experimental results obtained from EAE extract of Eucalyptus exhibited the potential anticancer activity through apoptosis. Thus it can be considered as one of the promising resource of chemotherapeutic drugs.

The EAE was fractionated by column chromatography from which only one compound (p-menth-1-ene-4,7-diol) was isolated. Probably this compound is responsible for the anticancer activity of EAE but this assumption has not been verified here.

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LIST OF ABBREVIATIONS

^{0}C	: Degree centigrade
Fig	: Figure
ME	: Methanol extract
PEE	: Pet. Ether extract
EAE	: Ethyl acetate extract
EC-1	: Eucalyptus camaldulensis-1
ICDDR'B	: International Centre for Diarrhoeal Disease Research, Bangladesh
EAC	: Ehrlich Ascites Carcinoma
Hb	: Haemoglobin
HIV	: Human Immune Syndrome
WHO	: World Health Organization
NCI	: National Cancer institute
Kg	: Kilogram
mL	: Milliliter
RBC	: Red blood cell
WBC	: White blood cell
TC	: Total count
TLC	: Thin layer chromatography

PTLC	: Preparative thin layer chromatography
µmol	: Micromole
%	: Percentage
µg	: Micro gram
gm	: Gram
GOT	: Glutamate-oxalo-acetate transaminase
GPT	: Glutamate-pyruvate transaminase
ALP	: Alkaline phosphatase
dl	: Deciliter
DMSO	: Dimethyl sulfoxide
TPA	: 12-O-tetradecanoylphorbol-13-acetate
SEM	: Standard error of mean
i.p.	: Intraperitoneal
χ^2	: Chi squared value
df	: Degree of freedom

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INTRODUCTION

Cancer is one of the most dreaded diseases of the 20th century and spreading further continuously with increasing incidence in 21st century. It poses serious health problems both in developed and developing countries. Cancer kills more people globally than tuberculosis, HIV and malaria combined. In the 1960s, almost 25 % of global cancer burden was diagnosed in low-income and lower-middle-income countries. In 2010, nearly 55 % of the global cancer was found in these countries. It is a group of more than 100 different diseases, characterized by uncontrolled cellular growth, local tissue invasion and distant metastases[1]. Though it is a genetic disease and promoted by external (tobacco, chemicals, radiations and infectious organisms) and internal factors (mutation, hormones and immune conditions),[2-3] it can be treated with surgery, radiation, chemotherapy, hormonal and biological therapy. This health problem in Bangladesh is particularly acute because of poverty, illiteracy and other diseases associated with poor nutrition and lack of basic knowledge of health matters[4]. The prevention and control of cancer in developing countries deserve urgent attention since the disease is expected to be double in the next 20 to 25 years. Multidisciplinary scientific investigations are making best efforts to combat this disease, but the sure-shot, perfect cure is yet to be brought into world medicine. Many treatment modalities have also been developed so far and of them chemotherapy is a major option in cancer prevention. The chemopreventive drugs have provided active principles which are in clinical use for controlling advanced stages of malignancies[5]**.** However, most of these chemotherapeutic agents exhibit severe normal toxicity, resulting in undesirable side effects. Moreover, many of the active molecules available for the treatment of cancer are highly expensive, mutagenic, carcinogenic and teratogenic. Hence,

there is a need to find alternative drugs, which are highly effective at non-toxic doses, inexpensive and accessible to common man. A need is therefore felt to search newer remedies, which are cheaper economically and do not have severe side effects to the host. Therefore this work has been designed to find out noble drugs in cancer chemotherapy.

1.1 Cancer scenarios in the world

In spite of good advancements for diagnosis and treatment, cancer is still a big threat to our society[6]. This is the second most common disease after cardiovascular disorders for maximum deaths in the world[7]. The world's population is expected to be 7.5 billion by 2020 and approximations predict that about 15.0 million new cancer cases will be diagnosed which implicates

50% increment of cancer incidence rate; with deaths of about 12.0 million cancer patients[8]. In 2010, total 8.4 million people worldwide died from cancer. Approximately 55% of cancer deaths occur in low- and middle-income countries and 30 % of this cancer is preventable.

1.2 Cancer scenarios in Bangladesh

Bangladesh is still lacking a national cancer registry. Acording to GLOBOCAN 2008, the 5-year cancer survival prevalence is 291.2 thausand. The number of new cases per year is 141.1 thausand and the number of cancer deaths is 103.3 thausand. New and old cases constitute 124.8 thausand. On the other hand, according to Bangladesh Bureau of Statistics cancer is the sixth leading cause of death in Bangladesh (BBS, 2008). A few decades ago, a hospital based registry was initiated in 1967 at Radiotherapy Department of Dhaka Medical College

Hospital and continued till 1971. A few years back, hospital based cancer registry was intitited at National Institute of Cancer Research Hospital and Oncology Department of Bangabandhu Sheikh Mujib Medical University. International Agency for Research on Cancer (IARC) has been estimated death from cancer in Bangladesh is 7.5 % in 2005 and projected that it would be increased upto 13 % in 2030.With the increase of the incidence of cancer in the country; it has become a matter of serious concern for Bangladesh. Although definite figures about the incidence of cancer is not available in here, it is estimated that there are around 800,000 cancer patients in the country of the 140 million people and about 200,000 new cases are being added every year. Around 150,000 cancer patients die annually. The number of people developing cancer is expected to increase in huge number mainly because of ageing population and lifestyle factors. Cancer load is more than 1,200,000 in Bangladesh.

1.3 Common cancers in Bangladesh

Based on the data available from the Radiotherapy Department of the Dhaka Medical College and Hospital, common cancers in males and females can be figured out as shown in table-1.1.

However, according to the draft annual report 2005 of the National Institute of Cancer Research and Hospital, Dhaka, top five cancers in males are - lungs 24.7 percent, unknown primary site 8.1 percent, larynx 7.3 percent, lymphatic and lymph node 7.3 percent and esophagus 5.1 percent and in females are cervix 24.6 percent, breast 24.3 percent, lungs 5.5 percent, oral cavity 4.1 percent and ovary 3.8 percent. The common risk factors of cancer in our country are summarized in table-1.2. Statistics say some 5,000-6,000 children are affected with cancer every

year. Cancer is a serious health problem for children also. Treatment for child cancer is till now not practicable in Bangladesh. According to a recent study, two-thirds of the affected children are suffering from blood cancer due to quantitative or structural abnormality of chromosome. It is possible to detect all of cancers in 60-70 percent cases through blood test or ultra-sonogram or by other tests. Proper treatment with drugs can mitigate or rather cure cancers.

1.4 Cancer treatment

Choice of treatment is influenced by several factors; including the specific characteristics of individual's cancer, overall condition and whether the goal of treatment is to cure patient's cancer. Depending on these factors, one may receive one or more of the following; chemotherapy, surgery, radiation therapy, hormonal therapy, angiogenesis inhibitors, complementary etc. It is common to use several treatment modalities together (concurrently) or in sequence with the goal of preventing recurrence. This is referred to as multi-modality treatment of cancer[9-13].

1.4.1 Chemotherapy

Chemotherapy is any treatment involving the use of drugs (from nature or synthetic) to kill cancer cells. Cancer chemotherapy may consist of single drug or combination of drugs and can be administered through a vein, injected into a body cavity or delivered orally in the form of a pill. Chemotherapy is different from surgery or radiation therapy in that the cancer-fighting drugs circulate in the blood to parts of the body where the cancer may have spread and can kill or eliminate cancers cells even at sites far away from the original cancer. As a result, chemotherapy is considered a systemic treatment. More than half of all people diagnosed with cancer receive chemotherapy. For millions of people who have

cancers that respond well to chemotherapy. This approach helps treat their cancer effectively, enabling them to enjoy full, productive lives. Furthermore, many side effects once associated with chemotherapy are now easily prevented or controlled, allowing many people to work, travel and participate in many of their other normal activities while receiving chemotherapy.

1.4.2 Chemo-agents from nature-an ayurvedic perspective

The control of cancer, the second leading cause of death worldwide, may benefit from the potential that resides in alternative therapies. The primary carcinogens stem from a variety of agricultural, industrial, and dietary factors. Conventional therapies cause serious side effects and, at best, merely extend the patient's lifespan by a few years. There is thus the need to utilise alternative concepts or approaches to the prevention of cancer. This approach focuses on many natural products that have been implicated in cancer prevention and that promote human health without recognisable side effects. These molecules originate from vegetables, fruits, plant extracts and herbs. Several studies have been conducted on herbs under a multitude of ethno-botanical grounds. Hartwell has collected data on about 3000 plants, those of which possess anticancer properties and subsequently been used as potent anticancer drugs[14-15]. Ayurveda, a traditional Asian medicine of plant drugs has been successfully used from very early times in preventing or suppressing various tumors.

1.5 Necessity of herbal drug research in Bangladesh

Nature has endowed Bangladesh with enormous plant resources. Many of these plants grow in jungles, forests, gardens and many of them are found lying every where in the fertile region of the country. In Bangladesh, the prospect of using of plant constituents as remedy for diseases is therefore, very promising.

Vast natural resources of medicinal plants are being used for thousands of years for the cure of many diseases in Bangladesh. Till now in the age of modern science, herbal medicines are used almost parallelly with allopathic medicines for the cure of many diseases[16]. If we could use medicinal plants properly we could get medicines at low cost and then it might be possible to fulfil the demand of our medication. This will supply low cost medicine to our poor people and we could establish a better health care system. In order to achieve this goal, research and development on the traditional medicines should be given the proper privilege. Because, following this trend, the countries like China, Japan, Srilanka, India, Thailand and even UK had already developed a remarkable medication system. Thus their dependence on the allopathic drugs has been reduced to a great extent[17]. So to make the effective WHO slogan "Health for all by 21st century" for our country, we should make proper use of our traditional plant medicines.

The works cited above, can by no means be said to be exhaustive considering the vast number of medicinal plants available in the country. A thorough compositional analysis, isolation of the active principles and search for hitherto undetected therapeutic properties of these plants is therefore, of prime importance. Research works leading to extraction, isolation and biological study of plant constituents have now formed the major field of study, which needs cooperations

from all nocks and corners. This present work is just a coordinated approach along this direction.

1.6 Medicinal value of plants as anticancer agents

Natural products and related drugs are used to treat 87% of all catagorized human diseases including bacterial infection, cancer and immunological disorders[18] and about 25% of prescribed drugs in the world originate from plants[19]. WHO (2005), pointed out that more than 80% of worlds population rely on plants based products to meet their primary health care needs[20]. However, the majority of these plants have not yet undergone chemical, pharmacological and toxicological studies to investigate their bioactive compounds[21]. Traditional records and ecological diversity indicate that Bangladeshi plants represent an exiting resource for possible lead structure in drug design.

Medicinal plants are part and parcel of humans since the down of civilization. In India, they form the backbone of several indigeneous traditional systems of medicine. Pharmacological studies have acknowledged the value of medicinal plants as potential source of bioactive compounds[22]. Phytochemicals from medicinal plants serve as lead compounds in drug discovery and design[23].

Medicinal plants are rich source of novel drugs that forms the ingredients in traditional systems of medicine, modern medicines, neutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs[24]. Over exploitation of selected medicinal plant species has lead to significant reduction in number of plants in the wild[25]. In recent years, multiple drug resistance in both human and plant pathogens has been developed due to indiscriminate use of synthetic drugs. This drives the need to

screen medicinal plants for novel bioactive compounds as plant based drugs are biodegradable, safe and have fewer side effects[26]. US National Cancer Institute (NCI) had initiated intensive search for plant products, which are believed to have anti-tumor activity in 1957.

Today there are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world. Several of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances of them most important drugs that are used in cancer treatment[27-31] are given in table-1.3.

A good number of plants have been identified and utilized for pharmacological and medicinal purposes, but it is true that quite a large number of plants have still been remained untouched or less touched from which significant results can be obtained. Eucalyptus (*Eucalyptus camaldulensis*) is one of such plants under the family Myrtaceae that has not been studied in details till now. It is well known to possess therapeutic values in the Ayurvedic and Unani pharmacopoeia. Accordingly, in this present research work active components have been isolated from stem bark of *Eucalyptus camaldulensis* and studied their anticancer activities.

1.7 The plant under investigation

Eucalyptus camaldulensis

Biological name **:** *Eucalyptus camaldulensis.*

Traditional name **:** Eucalyptus

Bengali name **:** Eucalyptus

English name **:** Blue Gum tree, Eucalipto, Eucalypt.

Familly **:** Myrtaceae

Genus **:** Eucalyptus

Species : *camadulensis*

Habit **:** Eucalyptus is a diverse genus of flowering trees

Height : A mature Eucalyptus may take the form of a low shrub or a very large tree growing 10-60 m tall.

Propagation **:** Seed

Stem **:** The appearance of Eucalyptus bark varies with the age of the plant, the manner of bark shed, the length of the bark fibres.

Leaf **:** Nearly all Eucalyptus are evergreen but some tropical species lose their leaves at the end of the dry season.

Inflorescence **:** Polychasial cyme.

Flower and Fruit **:** The most readily recognisable characteristics of Eucalyptus species are the distinctive flowers and fruit.

Distribution **:** There are more than 700 species of Eucalyptus, mostly native to Australia, and a very small number are found in adjacent areas. Only 15 species occur outside Australia, and only 9 do not occur in Australia. Species of Eucalyptus are cultivated throughout the tropics and subtropics including the Americas, Europe, Africa, the Middle East, China, India, Bangladesh and subcontinent[32-34].

Fig-1. *Eucalyptus camaldulensis* plant and leaves

1.7.1 Uses of Eucalyptus

Eucalyptus have many uses which have made them economically important trees[35]. They provide many desirable characteristics for use as ornament, timber, firewood and pulpwood. Eucalyptus is considered the world's top quality pulping species due to its high fibre yields. It is also used in a number of industries, from fence posts and charcoal to cellulose extraction for biofuels[36-37].

Eucalyptus oil is readily steam distilled from the leaves and can be used for cleaning, deodorising and in very small quantities in food supplements, especially sweets, cough drops and decongestants. It also has insect repellent properties[38] and is an active ingredient in some commercial mosquito repellents[39].

1.8 Literature review

1.8.1 Chemical literature review of Eucalyptus

There are many compounds in *Eucalyptus sp*. While the same compounds exist in many of the species, some compounds can be found in only one or a few species. Within species the quantity of essential oil and the specific compounds in the essential oil and extracts of dry and fresh leaves, buds, mature fruit and bark vary with the origin of the tree and the age of the leaves. The essential oil in the leaves is commonly used for medicinal purposes. The quantity of essential oil ranges from less than 1.5 to over 3.5%. The essential oil from the fruit, buds and branches contain from 15-57%, 1,-8-cineole. Other major components in the oil are: aromadendrene, camphene, cryptone, p-cymene, d-limonene, alpha-phellandrene, alpha-pinene, beta-pinene, spathulenol, y-terpinene, alpha-thujene.

Other important compounds found in the leaves, buds, branches and bark include: antioxidants (from bark)-eriodictyol, naringenin quercetin, rhamnazin, rhamnetin, taxifolin, citriodorol, cuminaldehyde epiglobulol euglobals, d-linalool, rutin, tannins, gamma-terpinene[40-44].

1.8.2 Biological literature review of Eucalyptus

Ito *et al*., (2000) reported that three new phenol glycosides were isolated from the dried leaves of *Eucalyptus cypellocarpa*. These new compounds and a known related glucoside showed potent *in vitro* antitumor-promoting activity in a short-term bioassay evaluating the inhibitory effect on Epstein-Barr virus early antigen activation induced by 12-O-tetradecanoyl phorbol 13-acetate (TPA). These compounds also suppressed an *in vivo* two-stage carcinogenesis induced with nitric oxide and TPA on mouse skin[45].

Vigo *et al.*, (2004) reported that *Eucalyptus globulus* Labill, and *Thymus vulgaris L*. have been used in traditional medicine in the treatment of bronchitis, asthma and other respiratory diseases. Pre-treatment with *E. globulus* and *T. vulgaris* extracts significantly inhibits inducible nitric oxide synthase (iNOS) mRNA expression. This study, thus suggests that the inhibition of net NO production by these two extracts may be due to their NO scavenging activity and/or their inhibitory effects on iNOS gene expression[46].

Saraswat *et al*., (1977) reported that ursolic acid is the active material isolated from the leaves of the Eucalyptus hybrid *E. tereticornis*, it has shown a significant preventive effect *in vitro* against ethanol-induced toxicity in isolated rat hepatocytes. Compared with the incubation of isolated hepatocytes with ethanol only, the simultaneous presence of ursolic acid in the cell suspension preserved the viability of hepatocytes and reversed the ethanol-induced loss in the level of all the marker enzymes (AST, ALT and AP) studied. Ursolic acid isolated from *Eucalyptus tereticornis* protects against ethanol toxicity in rat hepatocytes[47].

Takasaki *et al.*, (2000) reported that the phlorogrucinol-monoterpene derivative, euglobal-G1 (EG-1), was obtained from the leaves of *Eucalyptus grandis* as an active constituent. EG-1 exhibited the remarkable inhibitory effect on two-stage carcinogenesis test of mouse skin tumors induced by 7, 12-dimethylbenz[a]anthracene (DMBA) as an initiator and fumonisin-B1, which has been known as one of mycotoxins produced by *Fusarium monifliforme*, as a promoter. Further, EG-1 exhibited potent anti-tumor-promoting activity on two-stage carcinogenesis test of mouse pulmonary tumor using 4-nitroquinoline-N-oxide (4-NQO) as an initiator and glycerol as a promoter[48].

Konoshima *et al.*, (1995) reported that 21 euglobals (acylphloroglucinol-monoterpene or -sesquiterpene structures) isolated from the juvenile leaves of five species of Eucalyptus plants of these compounds, euglobal-G1--G5 (1-5), -Am-2 (15) and -III (16) exhibited significant inhibitory effects on Epstein-Barr virus (EBV) activation induced by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). Furthermore, the effects of compounds 1 and 16 on the cell cycle of Raji cells were also examined by a flow cytometer and both compounds 1 and 16 exhibited strong inhibition on the effect of the cell cycle induced by TPA. These two euglobals (1 and 16) exhibited remarkable anti-tumor-promoting effects on mouse skin tumor promotion in an *in vivo* two-stage carcinogenesis test[49].

Brantner *et al.*, reported that antitumor-promoting activity (100% inhibition; 100 microg/mL) was observed in the 80% MeOH and acetone extracts of crown gall, a plant tumor, obtained from *Eucalyptus globulus* tree. In the antimicrobial screening all extracts displayed predominantly antifungal activity against *Candida sp*. The extracts also showed various levels of antibacterial activity against *E. faecalis, P. aeruginosa, B. subtilis* and *S. Epidermidis*[50].

1. 9 Research plan

The present work has been designed to find out potent antineoplastic agents from nature. For this purpose the whole research work has been divided into the following headings:

1. To extract the ingredients from Eucalyptus (stem bark) with methanol, petroleum ether and ethyl acetate.

2. To study the antitumor activity of plant extracts against EAC cells *in vivo* using the following parameters-

a) Tumor weight measurement

b) Survival time of tumor bearing mice

c) Tumor cell growth inhibition

d) Percentage increase of life span

3. To study the mechanism of anticancer activity of the plant extracts by

a) DNA fragmentation assay in gel electrophoresis

b) Morphologic observation of cancer cells under fluorescence microscope

c) Observation of apoptotic pathway using caspase inhibitors

3. To study the cytotoxic effect of the plant extracts by brine shrimp lethality test.

4. To study the host toxic effect of the plant extracts by evaluating the following parameters.

a) Hematological parameters

b) Liver functions regarding serum glucose, billirubin, ALP, GOT, GPT

c) Histopathology of liver, kidney, heart, lung, spleen and brain.

5. Phytochemical analysis of the extract.

Table-1.1 Relative percentage of common cancers in Bangladesh

Male		**Female**	
Lung	21	Cervix	24
Larynx	13	Breast	17
Oral cavity	12	Oral cavity	13
Leukaemia/Lymphoma	8	Ovary	6
Pharynx	6	Leukaemia/Lymphoma	5
Oesophagus	5	Oesophagus	4
Others	35	Others	31

Source Based on 21, 238 cancer (Male 14,222 and female 7,076) treated at the Radiotherapy Department of Dhaka Medical College Hospital during the period of 1985-92.

Table-1.2 Prevalent cancers in Bangladesh and their risk factors

Sites	Risk factors
Lung, Larynx	Tobacco smoking
Oral cavity	Tobacco chewing
Pharynx	Air pollution and chemicals: Asbestos (Lung)
Oesophagus	Poor dental care and oral hygiene (oral cavity)
Pancreas	Excessive intake of red chilli (oesophagus)
Kidney, Bladder	Early sex, early marriage, multiple sex partners, multiple pregnancies, low socio-economic status, poor personal hygiene, venereal diseases
Cervix	HSV-2, HPV, Uncircumcised male partner, nulliparous
Breast	Daughters of breast cancer patients, less breast feeding, high fatty food, alcohol drinking (+tobacco smoking)
Liver	HBV
Stomach	High fatty food
Colo-rectum	Low fibrous food, low Vitamin A,C,E, and Selenium, Zinc in food
Penis	Uncircumcised male organ

Table-1.3 Some drugs with their clinical use and plant source

Drug/chemical	Action/Clinical use	Plant source
Betulinic acid	Anticancerous	*Betula alba*
Camptothecin	Anticancerous	*Camptothecea acuminata*
Colchiceneamide	Antitumor agent	*Colchicum autumna*
Colchicine	Antitumor and antigout agent	*Colchicum autumna*
Demecocine	Antitumor agent	*Cochicum autumnale*
Etoposide	Antitumor agent	*Podophyllum peltatum*
Irinotecan	Antitumor and anticancer agent	*Camtotheca acuminata*
Lapachol	Antitumor and anticancer agent	*Tabebuia sp.*
Monocrotaline	Antitumor agent(topical)	*Crtalaria sessli flora*
Podophyllotoxin	Antitumor and anticancer agent	*Podophyllum peltatum*
Taxol	Antitumor agent	*Taxas brevifolia*
Teniposide	Antitumor agent	*Podophyllum peltatum*
Topotecan	Antitumor and anticancer agent	*Camptotheca acuminata*
Vinblastine	Antitumor, Antileukemic agent	*Catharanthus roseu*
Vincristine	Antitumor,antileukemic agent	*Catharanthus roseu*

EXPERIMENTAL

Materials and Methods

2.1 Instrument and apparatus

2.1.1 Rotary evaporator

The extracts were concentrated and the solvents were evaporated with a rotary evaporator under reduced pressure.

2.2 Chemical work

The phytochemical investigation of a plant was performed by the following five major steps:

a) Collection and proper identification of the plant sample

b) Drying and grinding

c) Extraction

d) Isolation and purification of compounds and

e) Characterization and structure determination of the isolated compounds.

a) Collection and proper identification of the plant sample

The plant part was collected from Rajshahi University area and identified by a taxonomist. A voucher specimen was submitted to a herbarium for future reference.

b) Drying and grinding

The plant materials were air-dried followed by grinding into powder using a grinder (Hammer mill).

c) Cold extraction

In cold extraction, the coarse powdered plant materials were submerged in a suitable solvent in a flat bottom flask at room temperature and allowed to stand for several days with occasional shaking and stirring. When the concentration of the extract was maximum, the content was then filtered. After evaporation of the solvent in *vacuo* in a rotary evaporator the crude extract[51] was obtained.

d) Isolation and purification of compounds

For isolation and purification of components the following three techniques were used-

i) Thin layer chromatography (TLC)

ii) Column chromatography and

iii) Preparative thin layer chromatography (PTLC)

i) Thin layer chromatography (TLC)

For the preparation of plates, a number of glass plates (20cm×5cm) were thoroughly washed and dried in a hot oven. The plates were then placed over an aligning tray specially made for TLC. The slurry (2 gm silica gel/plate) was then distributed uniformly over the carrier plates with help of a TLC spreader by adjusting the thickness of the layer. After air drying, the coated plates were activated by heating in a hot oven at 110°C for 70 minutes.

Cylindrical shaped glass chamber (TLC tank) with air tight glass lid was used for the development of a chromatoplate. The selected solvent system was poured into the tank and a smooth sheet of filter paper was laid and soaked in the solvent to saturate the internal atmosphere with the solvent vapor[52-53].

A small amount of dried extract was dissolved in suitable solvent to get (1%) solution. A small spot with the solution was applied on the activated silica gel plate with a capillary tube just about 2cm above the lower edge of the plate. The spot was then dried with an air blower. The spotted plate was then placed in a saturated tank in such a way so as to keep the applied spot above the surface of the solvent and the lid was placed. The plate was left allowing the solvent system to creep up. When the solvent front reached up to 1cm below the upper edge, the plate was taken out and dried with air blower and the sports on TLC plate was detected by spraying the plate with spray reagent and under UV light[54-55].

ii) Column chromatography

For packing the column, silica gel (60-120 mesh) was taken in a beaker and the solvent was poured. The content was covered with glass lid and kept at 20°C for at least 2 hrs, to make slurry. A glass column of required size was first plugged with a piece of clean cotton at the bottom and fitted with a stand. The stopcock of the column was opened and solvent was passed through the column. In the mean time the slurry was allowed to pour gently into the column. After settling, some solvent was allowed to drain out. Care was taken so that the solvent sufficiently covered the adsorption bed. The column was packed at 20°C to avoid cracking. After packing, the sample was applied to column. The extract was mixed with a small portion of the stationary phase (silica gel) using mortar and pestle in order to

obtain a non-sticky free flowing mass. The amount thus obtained was placed on the packed column carefully in such a way that the upper layer of the bed was not disturbed.

The column was then run with solvent mixtures of increasing polarities. The flow was maintained at a constant rate (2 mL/min). Elutes were collected in different test tubes. All organic extracts elutes were dried over anhydrous $MgSO_4$[56].

iii) Preparative thin layer chromatography (PTLC)

Preparative thin layer chromatography technique was routinely used in separating and for final purification of the compounds. For this experiment, larger plates (20cm×20cm) were used. Table 2.1 shows the amount of silica gel required for preparing plates of varying thickness.

Table 2.1: Amount of silica gel required for preparing PTLC plates of various thickness

Size (cm×cm)	Thickness (mm)	Amount of silica gel/plate (gm)
20×20	0.3	3.6
20×20	0.4	4.8
20×20	0.5	6.0

The sample was dissolved in a suitable solvent and as a narrow uniform band rather than a spot. The plates were then developed in an appropriate solvent system previously determined by TLC. In some cases, multiple development technique was adopted for improved separation. After development, the plates

were allowed to dry and the bands of compounds were visualized under UV light (254nm and 366nm) or with appropriate spray reagents on both edges of the plates. The required bands were scraped from the plates and the compounds were eluted from the silica gel treating with suitable solvents mixtures.

e) Characterization of isolated compounds

To characterize each of the isolated compounds, physical and chemical properties as well as spectral data are fully studied.

2.3 Detection of the compounds

The following techniques were generally used to detect the compound in TLC and PTLC plates[57].

- **Visual detection**

The developed chromatogram was viewed visually to detect the presence of colored compounds.

- **Fluorescence and UV light**

The developed and dried plates were observed under UV light of both long and short wavelengths (254 mm and 366 nm) to detect the spot/band of any compound. Some of the compounds appear as fluorecent spots while the others as dark spots under UV light.

- **Iodine chamber**

The developed chromatogram was placed in a closed chamber containing crystals of Iodine and kept for minutes. The compounds that appear as brown spots, were marked. Unsaturated compounds absorb iodine.

- **Spray reagents**

Different types of spray reagents were used depending upon the nature of compounds expected to be present in the fractions or the crude extract. The reagents are descried in the below:

(a) Vanillin in sulfuric acid

This reagent was prepared by dissolving vanillin (1gm) in conc. H_2SO_4 (100mL) and used as general spray reagent for non-alkaloidal compounds. The plates sprayed with this reagent were heated at 110°C for 10-15 min to develop a color or ranges of colors developed with time, which, in trun, indicated the presence of higher alcohols, steroids, essential oils and terpens[58].

(b) Modified dragendorff's reagent

This reagent was prepared by mixing equal parts (v/v) of 1.7% bismuth subnitrate dissolved in 20% acetic acid in water and 40% aqueous solution of potassium iodide. Usually orange-red color indicated the presence of alkaloids by this reagent. Some coumarins also gave a positive test with modified Dragendorff's reagent[59].

(c) Ferric chloride/EtOH [Dyeing reagents for TLC and PTLC]

Some of the phenolic compounds were detected by spraying the plates with ferric chloride (5% ferric chloride in absolute ethanol) reagent[59].

(d) Perchloric acid reagent

2% aqueous per-chloric acid produced brown sports with steroids after heating at 150°C for 10 minutes[59].

(e) Potassium permanganate reagent

Only the oxidizable compounds were detected by this reagent. After spraying with the reagent, the compound appeared as yellow or pale yellow spot on the colored (color of permanganate) plate[59].

2.4 Phytochemical study on the stem bark of *Eucalyptus camaldulensis* (Dehnh.)

2.4.1 Collection and proper identification of the plant material

The stem bark of *Eucalyptus (Eucalyptus camaldulensis*) was selected for the chemical investigation. The stem barks were collected during the month of May-June, 2010 from the relevant area of Rajshahi University campus. The plant was taxonomically identified by Professor A.T.M Naderuzzaman and Dr. Goura Pado Ghosh, Department of Botany, University of Rajshahi.

2.4.2 Drying and pulverization

The collected plant materials were washed with water in such a way that adhering dirty materials were completely removed. After washing, the upper thin and rough surfaces were removed. Then the stem barks were cut into small spices with the help of a knife. These small spices were sun dried for 7-10 days and finally kept in an electric oven for 72 hours at 40^{o}C. After complete drying; the dried pices were then pulverized into a coarse powder with the help of a grinding machine (FFC-15, China) and were stored in an airtight container for further use.

2.4.3 Extraction of powdered plant materials

After pulverization, the weight of total powdered plant material was 1.5 Kg. For extraction, the powdered material was kept immersed in 2.5 liters methanol

contained in an aspirator bottle at room temperature for 15 days with occasional shaking and stirring. Then the contents were pressed through the Tincture Press (Karlkolb, Scientific-Technical Suppliers, Frankfurt / M-Germany) in Pharmaceutical Laboratory of the Department of Applied Chemistry and Chemical Engineering, Rajshahi University, to get maximum amount of extract. The extract was then filtered through a filter paper (Whatman No.1) and concentrated with a rotary evaporator under reduced pressure at 40^{o}C to obtain a crude methanol extract (ME).

2.4.4 Phytochemical screening of plant material

In testing for the presence of bioactive agents in a plant, an extract of the plant was prepared by macerating a known weight of the fresh plant with redistilled methylated spirit in a blender. Each extract was then suction-filtered and the process was repeated until all soluble compounds had been extracted, as judged by loss of colour of the filtrate. The total extract from plant part was evaporated to dryness in vacuo at about 45°C and further dried to constant weight at the same temperature in a hot-air oven. The yield of residue was noted and a potion of it was used to test for the constituents of the medicinal plant. Following were the methods of testing used for bioactive properties of medicinal plants

A] **Tests for alkaloids**

In testing for alkaloids, Fafowora[60] described that about 0.5g of extract stirred with 5mL of 1 per cent aqueous hydrochloric acid on a water bath; 1mL of the filtrate was treated with a few drops of mayer's reagent and a second 1mL portion was treated in the same way with Dragendorff's reagent. (Green/whitish color) Turbidity of precipitation with either of those reagents was taken as preliminary

evidence for the presence of alkaloids in the extract being evaluated[61]. A confirmatory test designed to remove non-alkaliodal compounds capable of eliciting "false-positive" reactions was carried out as false with the extract which give preliminary positive test for alkaloids.

A modified form of the thin-layer chromatography (TLC) method was used. In this method, one gram of the extract was treated with 40 per cent calcium hydroxide until the extract was distinctly alkaline to litmus paper, and then extract was added twice with 10mL portions of chloroform. The extracts were combined and concentrated in vacuo to about 5mL. The methanol extract was then spotted on thin-layer plates. Four different solvent systems (of widely varying polarity) were used to develop plant extract. The presence of alkaloids in the developed chromotograms were detected by spraying the chromatograms with freshly prepared spray reagent.

A positive reaction on the chromatograms (indicated by an orange or darker-coloured spot against a pale yellow background) was confirmatory evidence for the presence of alkaloids.

B] **Tests for saponins**

The ability of saponins to produce frothing in agueous solution and to hemolyse red blood cells was used as screening test for these compounds. For the test, the method described by Wall et al. (1952 and 1954) was used[62]. About 0.5g of plant extract was shaken with water in a test tube. Frothing which persists on warming was taken as preliminary evidence for the presence of saponins. In order to remove "False-positive" results, the blood hemolysis test was needed to be performed on the extract that frothed in water. About 0.5g of extract

was to be boiled briefly with 50 mL phosphate butter at pH 7.4, and then allowed to cool and filtered. 5mL of the filtrate was passed for 3 hrs through an asbestos disc (1.5mm thick about 7mm in diameter), which was previously soaked with two drops of 1 percent cholesterol in ether and dried. After filtration, the disc was washed with 0.5mL of distilled water, dried and boiled in 20mL of oxylol for 2 hrs to decompose the complex formed between cholesterol and any saponins in the extract. The disc was then washed in ether, dried and placed on 7 percent blood nutrient ager. Complete heamolysis of red blood-cells around the disc after 6 hrs. was taken as further evidence of the presence of saponins.

C] **Test for tannins**

About 5g of plant extract was stirred with 10mL distilled water, filtered. Ferric chloride reagent was added to the filtrate. A blue-black precipitate was taken as evidence for the presence of tannins[61].

D] Test for phlobatannins

Deposition of a red precipitate when an aqueous extract of the plant was boiled with 1 per cent aqueous hydrochloric acid was taken as evidence for the presence of phlobotannins[61] (Evans, 2002).

E] **Test for anthraquinones**

Borntrager's test was used for the detection of anthraquinones. Plant extract (5g) was shaken with 10mL benzene, filtered and 5mL of 10 per cent ammonia solution was then added to the filtrate. The mixture was shaken and the presence of a pink color in the ammoniacal (lower) phase indicated the presence of free hydroxyl-anthraquinones.

For bound anthraquionones, 5g of each plant extract was boiled with 10mL aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 5mL of benzene. The benzene layer was separated and half of its own volume of 10 percent ammonia solution was added. A pink coloration in the ammonia phase (lower layer) indicated the presence of anthroquinones derivatives in the extract[61].

G] Test for cardiac glycosides

Legal test

The extract was dissolved in pryridine and a few drops of 2 per cent sodium nitorprusside together with a few drops of 20 per cent NaOH were added. A deep red colour which faded to a brownish yellow indicated the presence of cardenoloides.

Kedde test

For this test, 1mL of an 8 percent solution of the extract in methanol was mixed with 1mL of a 2 per cent solution 3, 5-dinitrobenzoic acid in methanol and 1mL of a 5.7 percent aqueous sodium hydroxide. An immediate violet colour indicated the presence of cardenolides in the extract. The colour faded gradually through reddish-brown to brownish-yellow with the precipitation of a white crystalline solid. The test indicated the presence of a lactone ring in the cardenolide.

Lieberman's test

In this experiment, 0.5g of the extract was dissolved in 2mL of acetic anhydride and cooled well in ice sulphuric acid was then carefully added. A colour change from violet to blue and then green will indicated the presence of a steroidal nucleus[63] (i.e. aglycone partion of the cardiac glycoside) (Shoppee, 1964).

Salkowski test

The extract (0.5g) was dissolved in 2mL of chloroform. Sulpuric acid was then carefully added to form a lower layer. A reddish-brown colour at the interface indicated the presence of a steroidal ring (i.e. aglycone portion of the cardiac glycoside).

Keller kiliani test

For this test, 0.5 g of extract was dissolved in 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This has then been underlayed with 1mL of concentrated sulphuric acid.

A brown ring was obtained at the interface indicated the presence of a deoxy-sugar characteristic of cardenolides. A violet ring appeared below the brown ring while, in the acetic acid layer a greenish ring formed just above the brown ring and gradually spread throughout this layer[61] (Evans 2002).

2.4.5 Fractionation with petroleum ether

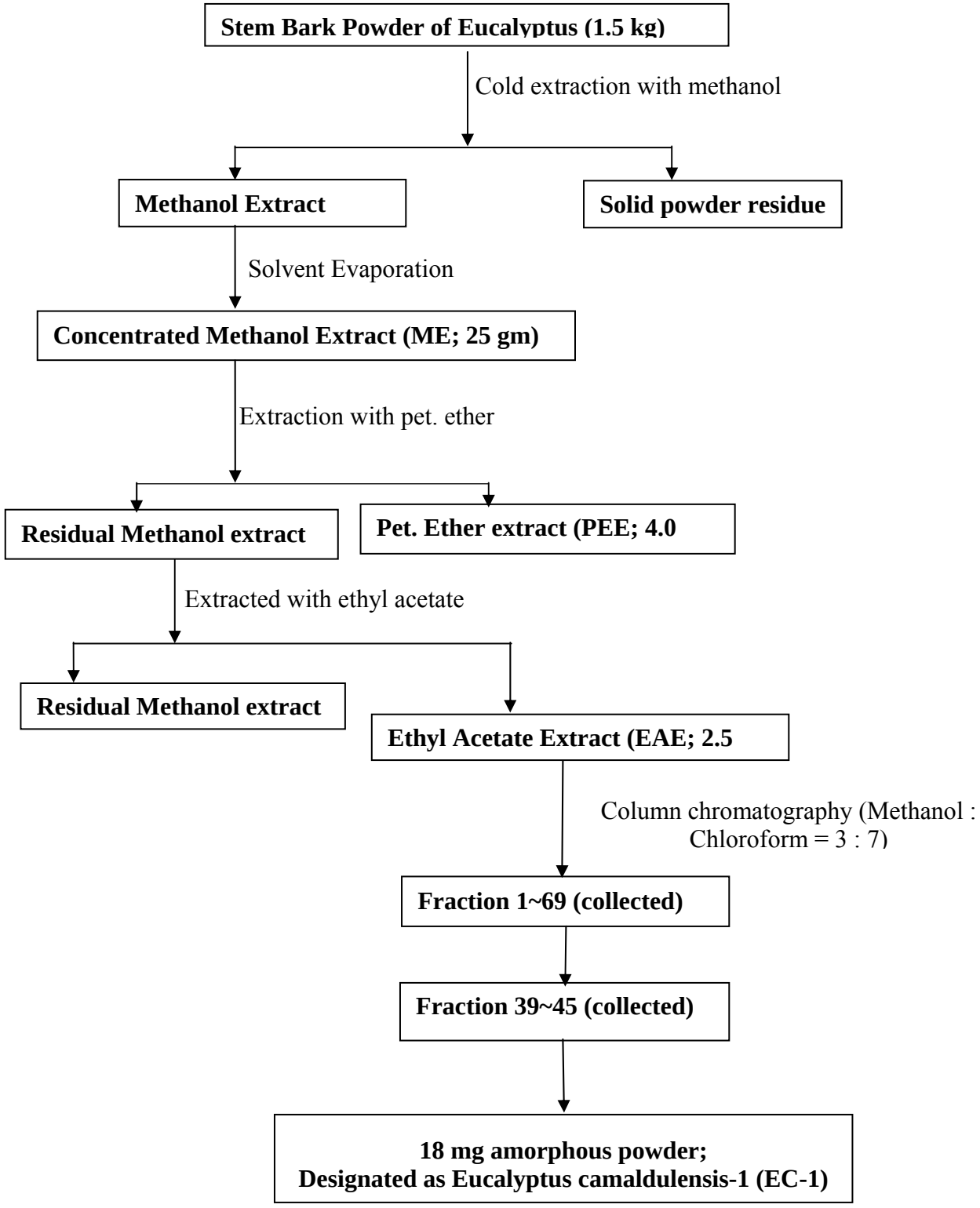
The concentrated crude methanol extract, ME (25 gm) was made slurry with 100 mL of water. The slurry was taken in a separating funnel and 40 mL petroleum ether (40^oC-60^oC) was added. The funnel was shaken vigorously and allowed to stand for few minutes. The petroleum ether layer (upper layer) was then collected.

The process was repeated three times with fresh petroleum ether. The three petroleum ether fractions were combined together and dried and concentrated in vacuo to obtain a semisolid mass (4 gm), which was orange in color and designated as PEE.

2.4.6 Fractionation with ethyl acetate

After fractionation with petroleum ether, the aqueous portion was vigorously shaken with ethyl acetate three times in the same way, described above, to obtain an ethyl acetate fraction. The fraction was dried and concentrated in vacuo, where a greenish mass (2.5 g) was obtained (EAE) extract.

2.4.7 Schematic Flow Diagram for Extraction



2.4.8 TLC screening for PEE and EAE fraction of the extract

For the preparation of plates, a number of glass plates (20 cm x 5 cm) were thoroughly washed to remove any dirt present and dried in a hot oven. The plates were then placed over an aligning tray specially made for TLC. The slurry (2gm silica gel/plate) was then distributed uniformly over the carrier plates with help of a TLC spreader by adjusting the thickness of the layer. After air-drying, the coated plates were activated by heating in a hot oven at 110^{o}C for 70 minutes.

Cylindrical shaped glass chamber (TLC tank) with air tight glass lid was used for the development of a chromatoplate. The selected solvent system was poured into the tank and a smooth sheet of filter paper was laid and soaked in the solvent to saturate the internal atmosphere with the solvent vapor[53]. A small amount of dried extract was dissolved in suitable solvent to get (1%) solution. A small spot with the solution was applied on the activated silica gel plate with a capillary tube just about 2 cm above the lower edge of the plate. The spot was then activated in a drier. The spotted plate was then placed in saturated tank in such a way as to keep the applied spot above the surface of the solvent and the lid was placed. The plate was left allowing the solvent system to creep up. When the solvent front reached up to 1 cm below the upper edge, the plate was taken out and dried with air blower and the spots on TLC plate was detected by spray the plate with spray reagent and under UV light and also with the vanillin-H_2SO_4 reagent. Through several pilot experiments, it was found that the compounds of petroleum ether fraction were separated by the solvent system of n-hexane and ethyl acetate in the proportion of 9:1 and ethyl acetate fraction in the solvent system of methanol and chloroform in the ration of 3:7 (Figure 2.1).

R_f value can be calculated by the following formula[64].

$$R_f \text{ value} = \frac{\text{Distance traveled by the sample}}{\text{Distance traveled by the solvent (solvent front)}}$$

Table 2.2 R_f values of possible compounds present in PEE and EAE extract

Fraction	Solvent system	Sample code name	R_f value	Color with vanillin-H_2SO_4	Possible compound
Petroleum ether	n-Hexane Ethyl acetate = 9 : 1	PEE-1	0.74	Bluish-violet	Saponins
		PEE-2	0.63	Pink	Steroids
Ethyl acetate	Methanol: Chloroform =3:7	EAE-1	0.51	Violet	Terpenoides
		EAE-2	0.37	Yellow	Flavonoids

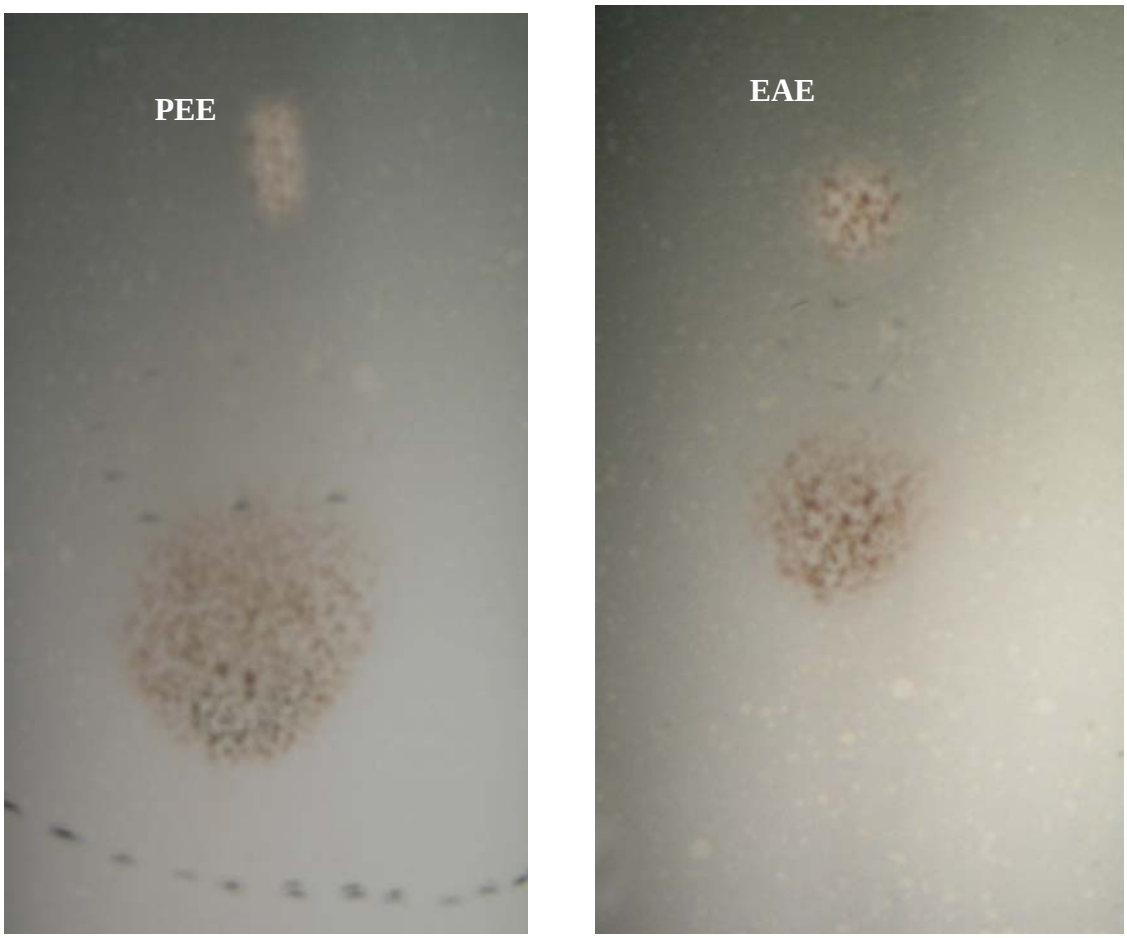


Figure 2.1 TLC profile of PEE and EAE

2.5 Isolation and purification of compounds from EAE by column chromatography

EAE showed two prominent spots with other minor spots on TLC plate and were checked under UV, Iodine vapor and vanillin-sulfuric acid reagent. EAE (1 g) was mixed well with 10 g of silica gel in a mortar and pestle. The sample was then applied carefully on the top of the prepared column without disturbing the top of the column (column height 25 cm and diameters 1.4 cm). The column was successively eluted with chloroform, with increasing portions of methanol and finally with 100% methanol as shown in the table 2.3.

Table 2.3 Solvent systems used in the column analysis of EAE

Fraction no	Solvent system	Proportion	Volume eluted (mL)
1-8	Chloroform	100%	42
9-18	Chloroform: Methanol	95 : 05	45
19-28	Chloroform: Methanol	90 : 10	60
29-34	Chloroform: Methanol	85 : 15	47
35-40	Chloroform: Methanol	80 : 20	45
41-47	Chloroform: Methanol	75 : 25	55
48-55	Chloroform: Methanol	80 : 20	90
56-61	Chloroform: Methanol	70 : 30	120
62-64	Chloroform: Methanol	60 : 40	60
65-67	Chloroform: Methanol	50 : 50	50
68	Methanol	100%	75
69	Ethyl acetate	100%	50

All the column fractions were screened by TLC under UV light and by spraying with vanillin-sulfuric acid reagent. Among these fractions, fraction 39-45 afforded **amorphous powder** (**18 mg**), which was designated as **Eucalyptus camaldulensis-1 (EC-1).** These fractions showed single spot on TLC screening after spraying with vanillin-sulfuric acid (Figure 2.2)**.**

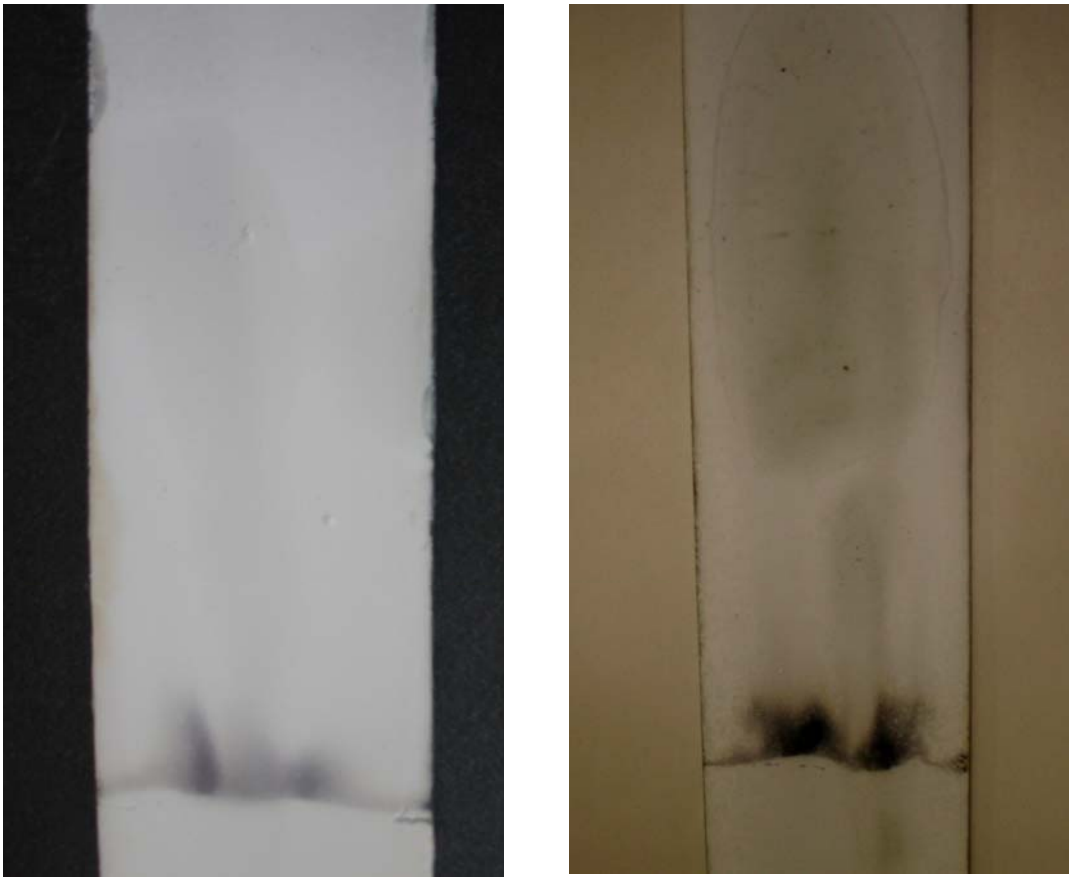


Figure 2.2 TLC profiles of 39-45 fractions (EC-1)

2.6 Results

Phytochemical screening of crude extracts of *E. camaldulensis* indicated that the plant contained terpenoides, saponins, flavonoids, tannins and also volatile oils. The components; anthraquinones, hydrolysable tannin, alkaloid and glycosides were not detected in the crude methanol extract of the plant (Table 2.4).

Table 2.4. Phytochemical components of crude methanol extract of *E. camadulensis*

Phytochemical components	*E. camadulensis*
Alkaloids	-
Saponins	+
Saponin glycosides	-
Tannins	+
Hydrolysable tannins	-
Phlobatannins	-
Anthraquinones	-
Glycosides	-
Cardiac Glycosides	-
Flavonoids	+
Steroid	-
Volatile oils	+
Phenols	-

+: Present; -: Absent

2.6.1 Physical characteristics of EC-1

Physical form : Amorphous powder

R_f value : 0.51 (Methanol : Chloroform = 3 : 7)

Melting point : 106^{o}C

Solubility data : The compound was found to be soluble in ethanol, methanol, petroleum ether and chloroform.

2.6.2 Spectral characteristics of EC-1

All the spectra (^{1}H-NMR & ^{13}C-NMR) were taken in Analytical Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR) Laboratories, Dhaka-1205, Bangladesh.

2.6.2.1 ^{1}H-NMR and ^{13}C-NMR spectrum

NMR spectra were recorded on Bruker 400 MHz FT spectrometer (DPX-400, Switzarland).

Table 2.5 ^{1}H- and ^{13}C-NMR spectral data of EC-1 in $CDCl_3$

Carbon Number	EC-1	
	δ_C	δ_H
1	112.9	-
2	148.9	5.98 (1H, s, H-2)
3	33.3	2.14 ~2.46 (2H, m, H-3)
4	76.3	-
5	47.7	1.80 (2H, ddd, J = 6, 10.5 Hz, H-5)
6	37.2	2.67 (2H, ddd, J = 6, 10.5 Hz, H-6)
7	67.2	4.37 (2H, s, H-7)
8	49.5	1.89 (1H, m, H-8)
9	17.3	1.07 (3H, d, *J* =7.0, H-9)
10	17.2	1.00 (3H, d, *J* =7.0, H-10)

Proton resonance integral, multiplicity and coupling constant (*J* = Hz) are in parentheses

Table 2.6 ^{1}H- and ^{13}C-NMR spectral data of authentic *p*-Menth-1-ene-4,7-diol in $CDCl_3$[64]

Carbon Number	*p*-Menth-1-ene-4,7-diol	
	δ_C	δ_H
1	114.9	-
2	147.9	5.98 (1H, s, H-2)
3	34.3	2.18 ~2.38 (2H, m, H-3)
4	73.8	-
5	47.7	1.80 (2H, ddd, *J* = 6, 10.5 Hz, H-5)
6	37.2	2.67 (2H, ddd, *J* = 6, 10.5 Hz, H-6)
7	68.3	4.34 (2H, s, H-7)
8	49.5	1.89 (1H, m, H-8)
9	17.3	1.10 (1H, d, *J* =7.0, H-9)
10	17.2	1.05 (1H, d, *J* = 7.0,H-10)

The ^{1}H-NMR spectrum of EC-1 showed an olefinic proton resonating at δ 5.98 as singlet which corresponds to H-2. ^{1}H-NMR spectrum of EC-1 also showed a singlet of one hydroxylated methylene with the integration of two protons at δ 4.34 (H-7). In addition, ^{1}H-NMR spectrum of EC-1 contained two methyl protons at δ 1.07 (d, *J* =7.0, H-9) and δ 1.00 (d, *J* =7.0, H-10) (Table 2.4). The ^{13}C-NMR spectrum of EC-1 showed ten (10) carbon signals (Table 2.5). Its ^{13}C-NMR spectrum showed two olefinic carbons at δ 112.9 (C-1) and δ 148.9 (C-2). In ^{13}C-NMR spectrum of EC-1, two hydroxylated carbon signals were also observed at δ 76.3 and δ 67.2. Based on the foregoing observations and a comparison of the data (Table 2.5) with the literature[65], the structure of EC-1 was confirmed as *p*-Menth-1-ene-4,7-diol. This is the first time report of this compound from this source *(Eucalyptus camaldulensis*) species in Bangladesh. The chemical structure of *p*-Menth-1-ene-4,7-diol is given in figure 2.3.

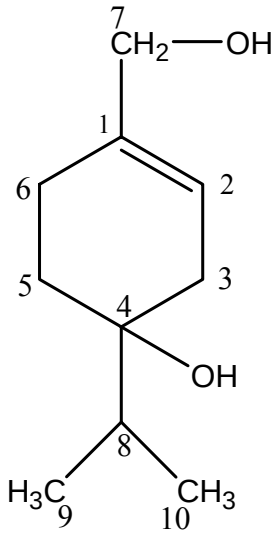


Figure 2.3 Structure of EC-1 (i.e., *p*-Menth-1-ene-4,7-diol)

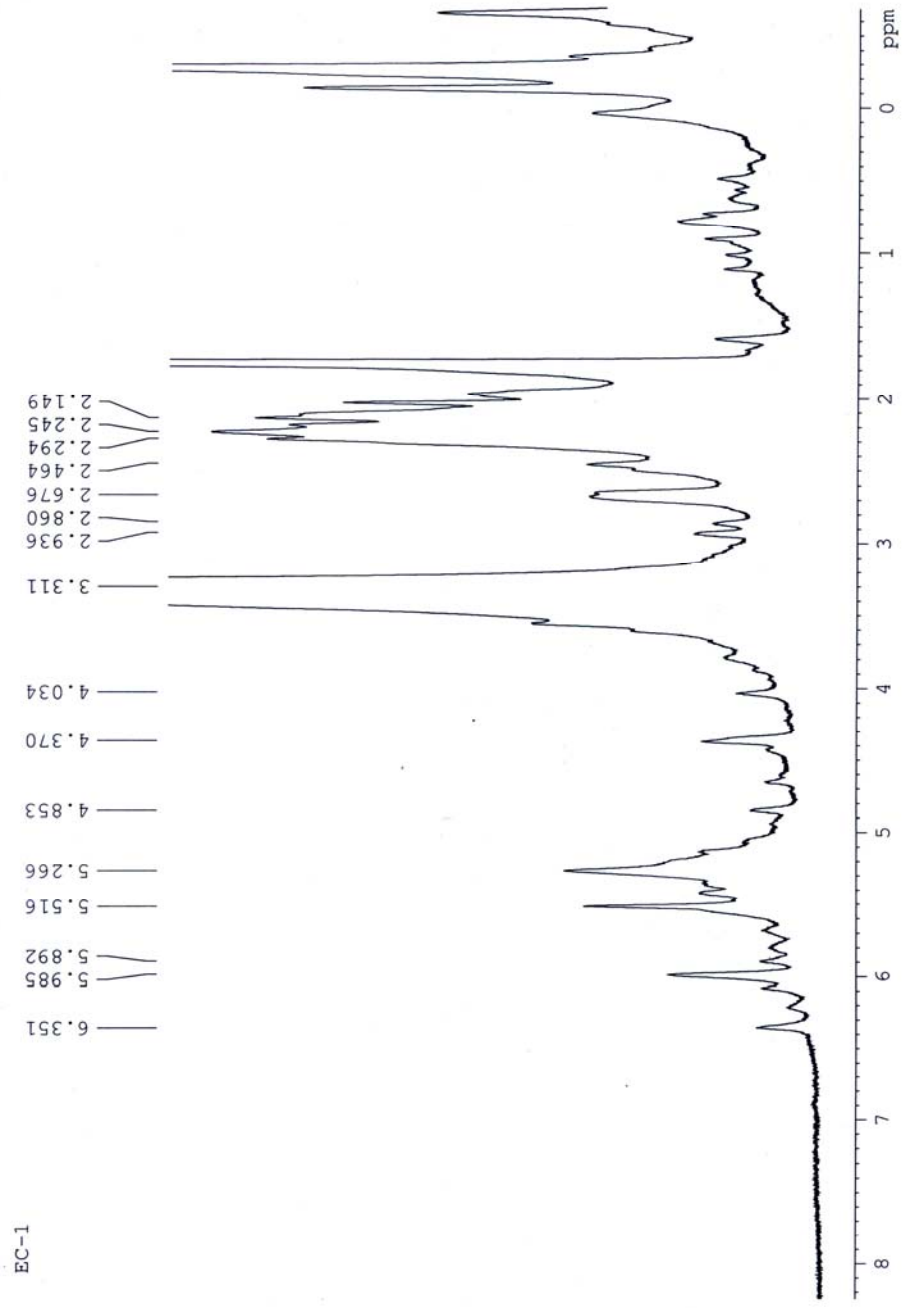


Figure 2.4 ^{1}H-NMR spectrum of EC-1

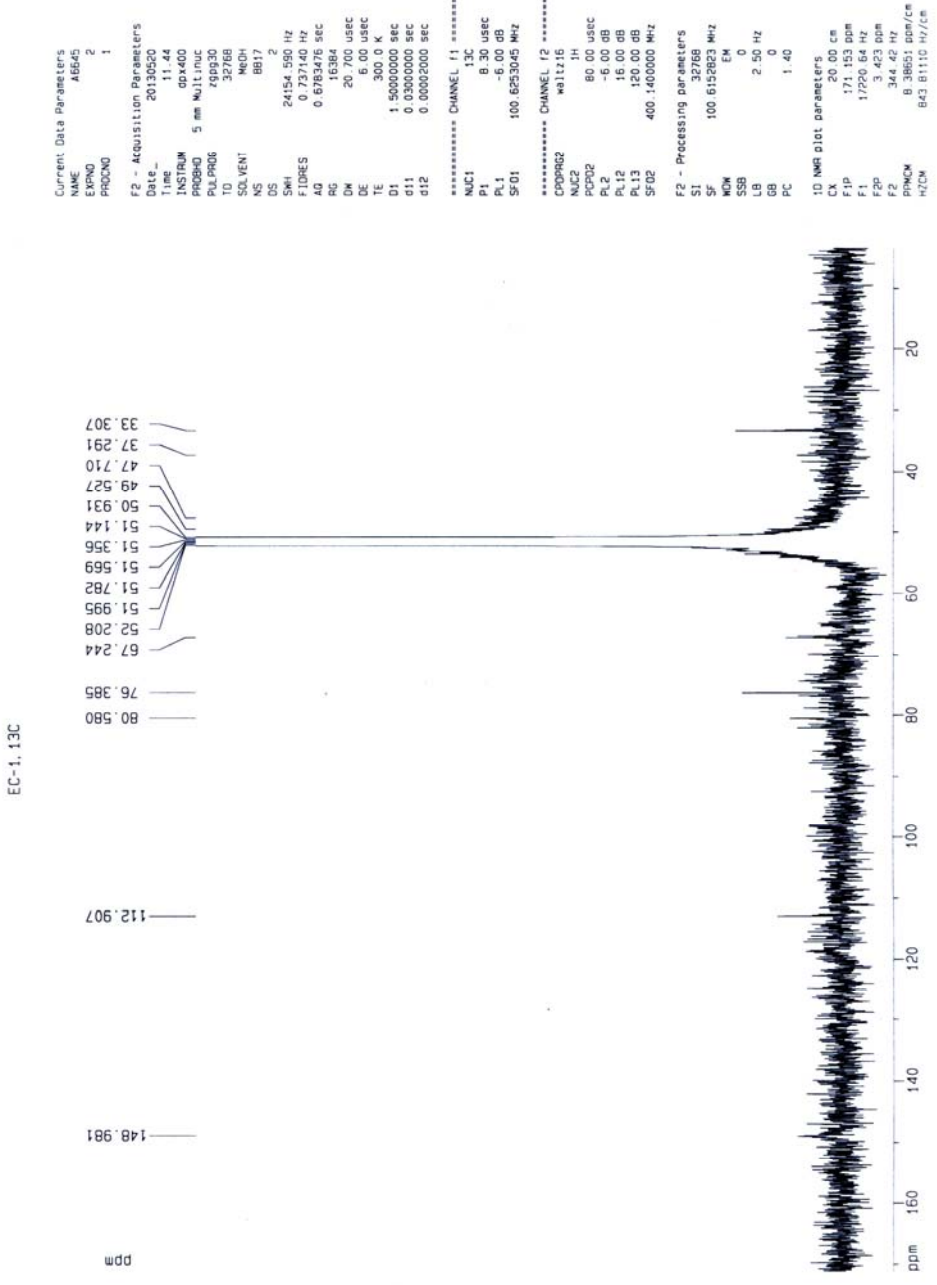


Figure 2.5 ^{13}C-NMR spectrum of EC-1

ANTICANCER ACTIVITY OF EUCALYPTUS

3.1 Introduction

The study of antineoplastic activity (*in vivo*) of the medicinal plant extract against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice has been assigned as the principal part of the work. For this purpose, the parameters monitored were tumor weight measurement, survival time of tumor bearing mice, tumor cell growth inhibition and mechanism of anticancer activity. Toxic effect of the test samples on hematological parameters both in normal and tumor bearing mice have also been studied and all such data have been compared with those of standard anticancer drug '*bleomycin*' in parallel experiments.

3.2 Materials and Methods

3.2.1 Instrument and apparatus

3.2.1.1 Hemometer

The hemoglobin concentration was measured with Hellige Sahli's Hemometer No. 304-B Hellige, USA.

3.2.1.2 Microscope

Samples on the hemocytometer were visualized with the help of a binocular microscope with magnification of 10x, 40x and 100x. Morphological characterization of the cancer cells have been studied using a fluorescence microscope (Olympus iX71, Korea).

3.2.1.3 Hemocytometer

Tumor cells, WBC, RBC, lymphocytes, neutrophils and monocytes were counted with a Neubauer hemocytometer. The subsequent cell concentration per mL was determined by using the following procedure.

$$\text{No. of cells per mL} = \frac{\text{The average count per square} \times \text{Dilution factor}}{\text{Depth of fluid under cover slip} \times \text{Area counted}}$$

$$= \frac{\text{The average count per square} \times \text{Dilution factor}}{(0.1\text{mm}) \times (1\text{mm})^2}$$

$$= \frac{\text{The average count per square} \times \text{Dilution factor}}{(0.1) \times (1\text{mm})^3}$$

$$= \frac{\text{The average count per square} \times \text{Dilution factor}}{(0.1) \times 10^{-3}\ \text{ml}}$$

$$= \text{The average count per square} \times \text{Dilution factor} \times 10^4.$$

3.3 Screening test

Screening is a test by which; it can be determined within short time and with comparably little effort whether a medicinal plant extract has any toxic effect on cancer cells. An essential part of developing a new anticancer drug is the testing of the potentiality of the new samples against animal tumors *in vivo*. *In vivo* tests, determination is done not only to study of the effects of the drug on animals (bearing transplanted tumor) but also on the host, including its toxicity and therapeutic index.

3.4 Experimental animal

Swiss albino mice of 5-7 weeks old, weighing 20±5 grams, were collected from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR'B), Mohakhali, Dhaka.

3.4.1 Mice vital statistics

i) Scientific name: *Mus Musculus*

ii) Life Span: 2-3 years

iii) Potential life span:4 years

iv) Desirable environmental temperature range: 18-27°C

v) Desirable relative humidity range: 30-70%

vi) Age at onset of puberty: 28-40 days

vii) Estrus (heat) cycle length: 4-5 days

viii) Estrus length (period during which female is receptive to male for copulation): 12 hours

ix) Gestation (pregnancy) period: 19-21 days

x) Weaving age: 21-28 days[66]

3.4.2 Animal care

a) Cage: Mice were kept in iron cages with saw dust and straw bedding which was changed once a week regularly. Standard mouse diet (recommended and prepared by ICDDR'B) and water were given in adequate.

b) Temperature, light and humidity: The room temperature was maintained around 25-32˚C and a controlled 12 hours day light and 12 hours dark were maintained in the laboratory (animal house).

c) Food: Pellet diet was collected from ICDDR'B, Dhaka.

Composition	**% (By Weight)**
Starch	66
Casein	20
Fat	8
Standard vitamins	2
Salt	4

3.5 Experimental tumor model

Transplantable tumor (Ehrlich Ascites Carcinoma) was used in this experiment. The initial inoculums of EAC cells was kindly provided by the Indian Institute of Chemical Biology (IICB), located at Kolkata, India. The EAC cells were thereafter propagated in our laboratory biweekly intra-peritoneal (i.p.) injections of $3x10^6$ cells, freshly drawn from a donor swiss albino mouse bearing 6-7 days old ascites tumor suspended in 0.1 mL sterile saline solution.

3.6 Ehrlich ascites carcinoma

Ehrlich ascites carcinoma (EAC) cells are being used in cancer research, worldwide.

In 1907, Ehrlich located this tumor in the mammary gland of a white mouse and thus the tumor was named after him. Loewenthal and John from one of the several lines of mammary gland origin[67] have developed the present form of EAC cells. External surface of EAC cells is covered with a thin section. The membrane matrix of Ehrlich Ascites tumor cells are specially strong[68] Extensive studies on the morphology of normal and cancer cells have shown that both the surface and intracellular membranes have 'unit membrane' structure a bimolecular lipid leaflet lined on both sides of protein or polysaccharide material[69-70]. EAC cell being cancer cells also possess the same type of structure. Tumor can be grown subcutaneously as solid form, but the present ascitic form is produced by infecting tumor cell suspension into the mouse peritoneal cavity. The ascitic tumor develops as a milky white fluid containing large rounded tumor cells. One million of tumor cells multiply to yield about 25-100 million tumor cells/mL. Host carrying such a tumor survives for about 14-30 days.

3.6.1 Transplantation of ascitic tumor

Ascitic fluid was drawn out from different tumor bearing Swiss albino mice at the respective log-phases of tumor cells. A 3 mL syringe fitted with 20 gauge needle was used for this tumor cell aspiration. The freshly drawn fluid was diluted with normal saline (0.98% NaCl solution) and the tumor cells number was adjusted to approximately 2×10^6 cells/mL by counting the cell number with the help of a haemocytometer. The viability of tumor cells were observed by trypen blue dye (0.4%) exclusion assay. Cell sample showing above 90% viability were used for transplantation.

Tumor suspension of 0.1 mL was injected intra-peritoneally (i. p.) to each Swiss albino mouse. Strict aseptic condition was maintained throughout the transplantation process.

3.7 Preparation of stock solution of the test samples: [Eucalyptus and *bleomycin*]

For therapeutic treatment, stock solution of *bleomycin* was made by using distilled water at the concentration of 0.075 mg/mL. Methanol extract (ME), petroleum ether extract (PEE) and ethyl acetate extract (EAE) of Eucalyptus (*Eucalyptus camaldulensis*) were dissolved in DMSO at the concentrations of 6.25mg/mL, 12.5mg/mL, 25mg/mL respectively.

3.8 *In vivo* assessment of Eucalyptus extract as an antineoplastic agent

In vivo antineoplastic activity of Eucalyptus extracts was determined by measuring the effect of the plant extracts against the parameters (tumor cell growth inhibition, survival time of tumor bearing mice, increase of body weight) of tumor induced mice. Hematological parameters of tumor bearing mice were also studied.

3.8.1 Determination of cell growth inhibition with ME, PEE and EAE (*in vivo*)

To determine the cell growth inhibition[71] of each extract, five groups of Swiss albino mice (6 in each group) weighing 20±5 gm were used. For therapeutic evaluation 2×10^6 EAC cells in every mouse were inoculated on day "0". Treatments were started after 24 hours of tumor inoculation and continued for five days. For every case group one to three received ME, PEE and EAE at the doses of 25 mg/kg (i. p.), 50 mg/kg (i. p.), and 100 mg/kg (i. p.) respectively per day. Group four received *bleomycin* at the dose of 0.3 mg/kg (i. p.) and group five was

used as control which received solvent only. Mice in each group were sacrificed on day six and the total intraperitoneal tumor cells were harvested by normal saline (0.98%). Viable cells were first identified by using trypen blue and then counted by a hemocytometer. Total numbers of viable cells in every animal of the treated groups were compared with those of control (EAC treated only) group.

3.8.2 Bioassay of EAC cells (Transplantation ability of EAC cells)

The effect of the extracts on transplantability of EAC cells was carried out by the method as described in the literature[72]. In this experiment six groups of mice (n=4) were inoculated with 115×10^5 EAC cells. Group 1, 2 and 3 was treated with ME, PEE and EAE respectively at the dose of 100.0 mg/kg (i.p.) per day for five consecutive days and group 4, 5 and 6 served as control. On day 7, tumor cells from the mice were harvested in cold (0.9 %) saline, pooled, centrifuged and re-inoculated into six fresh groups of mice (n=4) as before. No further treatment was done on these mice. On day 5, mice were sacrificed and viable tumor cells count/mouse were estimated.

3.8.3 Determination of average tumor weight and survival time with the Eucalyptus extracts

For this determination, a brief description of the method used by Sur et al[71] is given bellow, five groups of swiss albino mice (6 in each group) were used for each fraction. For therapeutic evaluation 136×10^4 EAC cells per mouse were inoculated in to each group of mice on day 0. Treatment was started after 24 hours of tumor cell inoculation and continued for 10 days. Tumor growth were monitored by recording daily weight change and host survival was recorded and

expressed as mean survival time in days and percent increase of life span was calculated by using the following formulae:

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{Total number of mice}}$$

$$\text{Percent increase of life span (ILS) \%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \right) \times 100$$

3.8.4 Effect of ME, PEE and EAE on hematological parameters in normal and tumor bearing mice

Hematological parameters were studied in both normal and tumor bearing mice by the method as described in the literature[73]. In case of tumor bearing mice, treatment was started after 24 hours of EAC cell transplantation and continued for 10 days. Treatment schedule has already been described in experiment no. 3.8.3. Blood was drawn from each group of mice on day 12 by tail puncture. The following hematological parameters were studied.

Estimation of hemoglobin %

Instrument: Sahli's haemometer

Principal Reagent: N/10 HCl

Procedure

The amount of haemoglobin was measured by using Sahli's haemometer. Blood (20 µL) was drawn into the pipette up to the mark and transferred to the cuvette (tube) in haemometer containing a little amount of N/10 HCl. Distilled water was added and stirred until a good color match was obtained. The final reading of the solution in the cuvette was noted. From the cuvette reading gram % (gm/dl) of haemoglobin was calculated.

Total WBC count

Reagents: WBC counting fluid containing

(a) 2% v/v aqueous acetic acid solution

(b) Aqueous methylene blue (0.3% w/v)

(c) Distilled water

Procedure

Exactly 10µL non coagulating blood was drawn with the tip of a micropipette and diluted with 1 mL WBC counting fluid and mixed properly; the resultant mixture was checked in Neubauer haemocytometer and the number of cells was counted with a microscope. The dilution factor was 100. Total WBC cells per mL were calculated.

Total RBC count

Reagents: RBC counting fluid containing:

(a) Tri sodium citrate (8%)

(b) Formaldehyde (37% formalin)

(c) Distilled water

Procedure

Exactly 10µl non-coagulating blood was drawn with the tip of a micropipette and diluted to 1000 times with red cell counting fluid. RBC was counted with haemocytometer like WBC counting technique.

3.8.5 Observation of morphological changes and nuclear damage of EAC cells

Cellular apoptosis induced by the EAE was studied by the method described in the literature[74]. Morphological observation of cells in absence and presence of extract (100 µg/mL) for 24 hrs were studied using a fluorescence microscope (Olympus iX71, Korea). At first EAC cells were collected from culture disc (RPMI-1064 media) treated with the extract and saline (none treated control disc) and stained with 0.1 µg/mL of Hoechst 33342 at 37^{o}C for 20 min. Then the cells were washed with phosphate buffer saline (PBS) and re-suspended in PBS for observation of morphological changes under fluorescence microscopy. In addition, to determine the necrotic or late apoptotic cell death, EAC cells further washed by 0.01% sodiun azaid containing 0.9% NaCl and then stained with propidium iodide (PI).

3.8.6 Effect of caspase inhibitors on EAC cells

In order to find out the involvement[75] of caspases in the extract-induced cell death, the cells were collected from control mice on day six of tumor inoculation and washed with PBS. Then these cells were incubated in CO_2 incubator (RPMI-1064 media) with Z-DEVD-FMK (caspase-3 inhibitor, 2µmol/mL) and Z-IETD-FMK (caspase-8 inhibitor, 2µmol/mL) for 1 h. After that, cells were treated with EAE and kept for another 24 hrs. Finally, the cells were counted using hemocytometer and determined the effects of EAE on cell growth inhibition in presence or absence of specific inhibitor.

3.8.7 DNA fragmentation assay

EAC cells obtained from mice treated with EAE (100 mg/kg; i.p.) for five consecutive days and without extract (1X 10^6/mL). The cells were washed with PBS and re-suspended again in PBS. The total DNA was isolated by using a DNA

extraction kit (Promega, USA) and analyzed by electrophoresis on 1.5% agarose gel containing 0.1 µg/mL ethidium bromide and visualized under UV illuminator[76].

3.8.9 Determination of the effect of extract on peritoneal cells

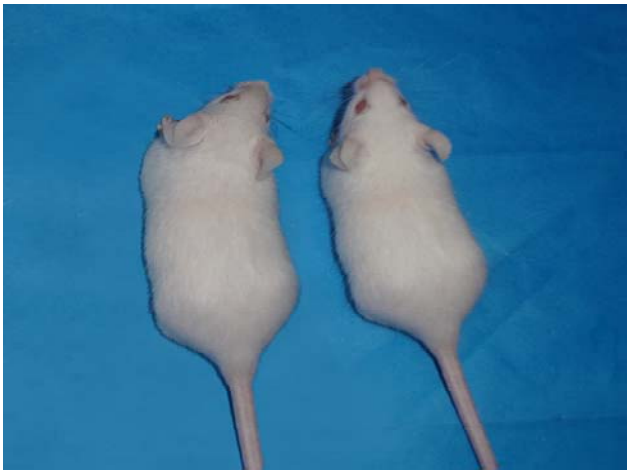
Effects of extracts on normal peritoneal cells were determined[77] by counting total peritoneal cells and number of macrophages. Three groups of mice (n=4 in each) were treated with ME, PEE and EAE at dose 100 mg/kg (i.p) per day for three consecutive days, the fourth (untreated) group (n=4) served as control. After 24 hours of last treatment, each animal was injected with 5 mL of normal saline (0.98%) into the peritoneal cavity and then sacrificed. Intraperitoneal exuded cells and number of macrophages were counted after staining with 1% neutral red by haemocytometer.

3.9 Determination of median lethal dose (LD_{50})

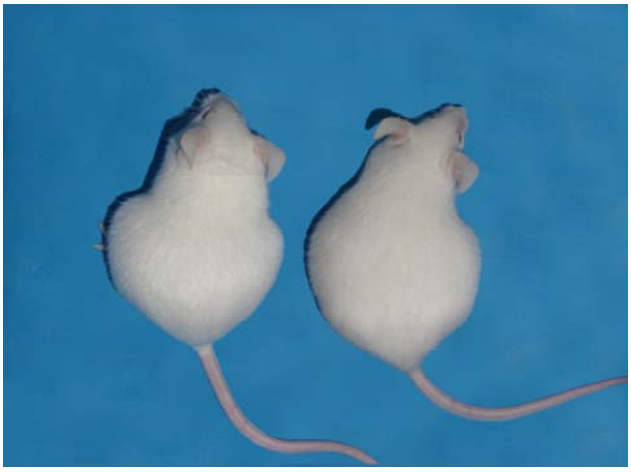
Methanol, petroleum ether and ethyl acetate extract of Eucalyptus (*Eucalyptus camaldelunsis*) were dissolved in DMSO and were injected intraperitoneally to seven groups of mice (each group containing four animals) at different doses of [900mg/kg (i.p.), 1000 mg/kg (i.p.), 1050 mg/kg (i.p.), 1100 mg/kg (i.p.), 1150 mg/kg (i.p.), 1200 mg/kg (i.p.), and 1250 mg/kg (i.p.)]. The LD_{50} value was then estimated by the procedure as described in the literature[78].

3.10 Statistical analysis

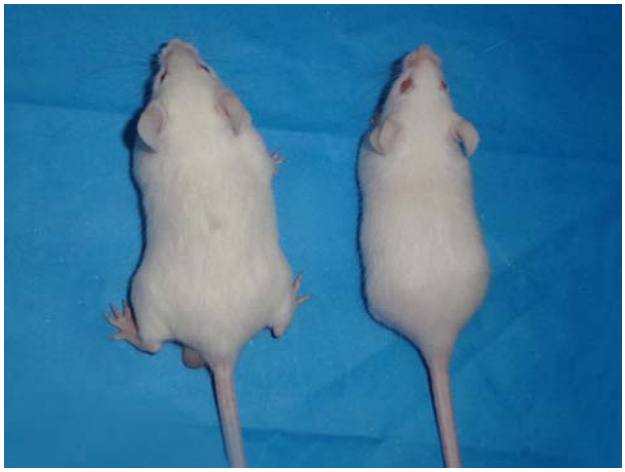
The experimental results are expressed as the mean ± SEM (Standard Error of Mean). Data have been calculated by one way ANOVA followed by Dunnett ‘t’ test using statistical package for social science (SPSS) software of 15 version.



Normal swiss albino mice



EAC cell bearing swiss albino mice on day 12



EAC cell bearing swiss albino mice treated *Eucalyptus camaldulensis*

3.11 Results

3.11.1 Effects of ME, PEE and EAE on EAC cell growth inhibition

Effects of the extracts and *bleomycin* on EAC cell growth on day six after tumor transplantation are shown in table 3.1.

Treatment with EAE resulted 96 and 85% cell growth inhibition at the doses 100 mg/kg (i. p.) and 50 mg/kg (i.p.) respectively .Treatment at the dose of 100mg/kg with the PEE and 0.3 mg/kg with *bleomycin* showed cell growth inhibition by 84.97 and 88.00 % respectively. Mice receiving ME showed 71.72 % cell growth inhibition at the dose of 100 mg/kg/per day.

3.11.2 Bioassay of EAC cells

Transplantability of EAC cell treated with ME, PEE and EAE was found to be decreased remarkably. It was found that 72.4, 65.7 and 58.3% EAC cell growth reduction were observed respectively when 5 days treated (at the dose 100 mg/kg i. p.) EAC cells were re-inoculated into fresh mice when compared to control mice. Results were shown in figure-3.1.

3.11.3 Effect of extracts on survival time of EAC cell bearing mice

All anti-cancerous drugs show a significant effect on survival time of EAC cell bearing mice. The effect of methanol, petroleum ether and ethyl acetate extracts of Eucalyptus at different doses has been summarized in table-3.2. It has been observed that tumor induced mice treated with the PEE and EAE at doses 25mg/kg, 50mg/kg and 100mg/kg resulted in, increase of life span significantly, which were 47.41, 67.13, 79.34 and 44.74, 58.08, 71.36% respectively, when compared to that of control mice. On the other hand, ME at dose 100 mg/kg

showed 65.87 % life span increment of tumor bearing mice. Thus the survival time was found to be increased with higher doses of the plant extracts was increased. *Bleomycin* increased life span by 90.14 % when compared to control. It was found that % increase of life span of 100mg/kg of Eucalyptus is quite comparable to that of standard anti cancer agent *bleomycin* (0.3 mg/kg), (Table 3.2).

3.11.4 Effects of extracts of on average tumor weight in mice

Effect of ME, PEE and EAE and *bleomycin* on average tumor weight is shown in the table-3.3, 3.4 and 3.5 as well as in figure-3.2a, 3.2b and 3.2c. Treatment of petroleum ether and ethyl acetate extracts of Eucalyptus on mice previously inoculated with EAC cells, resulted in the inhibition of tumor growth. In the case of control (EAC bearing) group, the body weight was increased by 75.34 % on 20 day when compared to the normal. Mice treated with ME, PEE and EAE at doses 25mg/kg (i.p.), 50mg/kg (i.p.), 100mg/kg (i.p.) the body weight increased by 52.8, 35.48, 22.5 and 53.7, 44 32.15 and 24.5, 22.7, 19.6% respectively on 20 day. In contrast the use of *bleomycin* as standard at the doses of 0.3 mg/kg (i. p.) the body weight was increased by 22% on day 20.

3.11.5 Effect of extracts on hematological parameters in normal and tumor bearing mice on day 12 of tumor inoculation

Hematological parameters were found to be altered from normal values along with the growth of tumor. Hemoglobin, RBC, WBC and Hb % count were found to be decreased after inoculation of EAC cells in swiss albino mice. After treatment with ME, PEE and EAE, it was found that the parameters restored moderately only at high doses. Normal mice when treated with the extracts, the

parameters were found to be changed to some extent. Results are shown in table- 3.6, 3.7 and 3.8.

3.11.6 Morphological changes of EAC cells by fluorescence microscopy

Morphological changes of EAC cells were examined by Hoechst 33342 staining after culturing the cells with EAE and without extract (100 µg/mL) for 24 hrs. EAC nuclei were round, regular and homogeneously stained with Hoechst 33342 in control group as shown in Figure 3.3A. Whereas EAE treated EAC cells showed manifest fragmented DNA in nuclei as shown in Figure 3.3B. Apoptotic morphologic alterations such as membrane blebbing and nuclear condensation were also observed clearly by fluorescence microscopy. These results indicate that EAE could induce apoptosis of EAC cells. Necrotic or late apoptotic cell death caused by extract was also observed by staining with PI as shown in Figure 3.3C. Here the numbers of necrotic cells were found to be very low.

3.11.7 Effect of caspase inhibitors on EAC cells

The involvement of specific caspase in the apoptotic cell death induced by the treatment of EAC cells with the extract (EAE) was investigated using caspase inhibitors Z-DEVD-FMK (caspase-3 inhibitor) and Z-IETD-FMK (caspase-8 inhibitor). The cytotoxicity of extract towards Z-IETD-FMK-pretreated EAC cells was significantly reduced to 67%, whereas Z-DEVD-FMK-pretreated cells did not exhibit any reduction of cytotoxicity in comparison with the control (Figure 3.4).

3.11.8 DNA fragmentation assay

The activation of the endogenous $Ca^{2+/}Mg^{2+}$-dependent endonuclease is the most distinctive biochemical hallmark of apoptosis. This activated endonuclease-

mediated the cleavage of internucleosomes and generate oligonucleotide fragments of about 180-200 bp length or their polymers. A DNA ladder bands was obtained in agarose gel electrophoresis of DNA preparation extracted from EAE treated EAC cells which is characteristic feature of apoptosis induction. On the other hand, control group shown smear-like DNA degradation. Result was shown in Figure-3.5.

3.11.9 Effect of extracts on peritoneal cells

The average number of peritoneal exudates cells of normal mice treated with the extracts (ME, PEE and EAE) were found to be $(14 \pm 1.7) \times 10^6$, $(17 \pm 3.4) \times 10^6$ and $(11 \pm 1.2) \times 10^6$ respectively whereas of which the macrophage counts were $(5.06 \pm 0.8) \times 10^6$ $(4.8 \pm 1.1) \times 10^6$ and $(3.6 \pm 0.5) \times 10^6$ respectively. Treatment with extracts at dose 100 mg/kg/day for three consecutive days significantly enhanced the number of macrophages. Results are shown in figure 3.6.

3.11.10 Lethal dose (LD_{50}) value

Lethal dose of methanol, ethyl acetate and petroleum ether extracts of Eucalyptus were found to be 1120 mg/kg, 1050mg/kg (i. p.) and 1250mg/kg (i. p.) for intraperitoneal treatment in male swiss albino mice respectively.

Table 3.1 Effect of extracts on EAC cell growth inhibition (*in vivo*)

Name of Exp.	Nature of the drugs	Dose in mg/kg/day (i.p)	No of EAC cells	% of cell growth inhibition	LD_{50} Values mg/kg
Control (EAC cell bearing mice)	-	-	$(6.79\pm0.53)\times10^{7}$	-----	…..
Bleomycin	Standard	0.3 mg/kg	$(0.81\pm0.36)\times 10^{7***}$	88.00	……
Eucalyptus camaldulensis	ME	25 mg/kg	$(3.95\pm0.29)\times 10^{7}$	41.80	1120
		50 mg/kg	$(2.48\pm0.38)\times 10^{7**}$	63.47	
		100 mg/kg	$(1.92\pm0.56)\times 10^{7**}$	71.72	
	PEE	25 mg/kg	$(3.25\pm0.85)\times 10^{7*}$	52.13	1040
		50 mg/kg	$(1.85\pm0.24)\times 10^{7**}$	72.75	
		100 mg/kg	$(1.02\pm0.21)\times 10^{7***}$	84.97	
	EAE	25 mg/kg	$(2.38\pm0.72)\times 10^{7*}$	63.15	1012
		50 mg/kg	$(0.83\pm0.15)\times 10^{7**}$	85	
		100 mg/kg	$(0.23\pm0.10)\times 10^{7***}$	96	

Results are shown as mean ± SEM, where significant values are * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ when compared with control.

Treatment was started 24 hours of EAC cell transplantation (2×10^{6} cells/mouse) and continued for 5 consecutive days at dose 25.0, 50.0 and 100.0 mg/kg (i.p.) (number of mice in each day = 6). On day six mice were sacrifice and cells were counted using hemocytometer and compared with control.

Table 3.2 Effect of extracts on survival time of EAC cell bearing mice

Name of Exp.	Drugs	Dose mg/kg/ day	Mean survival time (Days) Mean ± SEM	% Increase of life span % ILS
Control (EAC bearing mice)			21.3±1.20	…….
EAC+ *Bleomycin*	Standard	0.3	40.5±0.32	90.14***
Eucalyptus *camaldulensis*	ME	25	25.83±0.79	30.25
		50	29.0±0.61	49.00*
		100	33.0±0.52	65.23**
	PEE	25	31.4±0.74	47.41*
		50	35.6±0.41.	67.13**
		100	38.2±0.31	79.34**
	EAE	25	30.83±0.79	44.74*
		50	33.67±0.47	58.08**
		100	36.5±0.60	71.36**

Numbers of mice in each experiment were 6. The results are shown in mean ± SEM. Significant values are,*$p<0.05$,**$p<0.01$ and***$p<0.001$.

Each mouse was inoculated with 140×10^4 EAC cells and treatment was started after 24 hours of tumor cell inoculation and continued for 10 days. Host survival was recorded and expressed as mean survival time (MST) in days.

Table 3.3 Tumor weight of EAC bearing mice treated with ME at the doses of 25mg/kg (i.p.) 50mg/kg (i.p.), 100mg/kg (i.p.) and *bleomycin* at 0.3 mg/kg (i.p.)

Days	Tumour weight of untreated EAC cell bearing mice in gm	Tumour wt. of EAC cell bearing mice treated with *Bleomycin* (0.3 mg/kg)	Tumour wt. of ME treated mice in gm		
			25 mg/kg (i.p)	50 mg/kg (i.p)	100 mg/kg (i.p)
0	0.00	0.00	0.00	0.00	0.00
2	0.9±0.21	0.25±0.1	0.62±0.02	0.57 ±0.41	.50±0.17
4	1.9±0.14	0.78±0.32	1.37±.07	0.99 ±0.28	0.88±0.15
6	3.95±0.25	1.30±0.21	2.10±0.18	1.81 ±0.52	1.48 ±0.24
8	4.45±0.55	1.70±0.54	3.56±0.14	2.68 ±0.5	2.00±0.47
10	6.22±1.2	1.90±0.5	4.14±0.10	3.10±0.65	2.50 ±0.33
12	8.02±0.94	2.40±0.25	4.92±0.16	3.90 ±0.24	2.90+0.25
14	10.12±1.5	2.90±0.52	5.28±0.19	4.70 ±1.0	3.55 ±0.45
16	13.22±1.2	3.40±0.61	7.12±0.04	5.90±1.1	4.23 ±0.58
18	15.46±1.1	3.90±0.50	8.43±0.18	6.30 ±0.85	5.00±0.75
20	17.02±1.35	4.30±0.34	9.80±0.20	6.90 ±0.9	5.40 ±0.68

Results are shown in mean ±SEM.

Number of EAC cell 136 $\times 10^4$ per mL was inoculated into five groups of mice (six in each group) on day 0. Four groups were treated with ME at the doses of 25 mg/kg, 50 m/kg and 100 mg/kg and *bleomycin* (0.3 mg/kg) respectively after 24 hours of EAC cell inoculation in mice and continued for 10 days. Fifth group was considered as untreated control. Tumor weight was obtained from different mouse of each day from first day of tumor inoculation.

Table 3.4 Tumor weight of EAC bearing mice treated with PEE at the doses of 25mg/kg (i.p.), 50mg/kg (i.p.), 100mg/kg (i.p.) and *bleomycin* at 0.3 mg/kg (i.p.)

Days	Control EAC bearing mice	Standard drug *bleomycin* (0.3 mg/kg,i.p)	Petroleum ether extract of Eucalyptus (PEE)		
			25 mg/kg (i.p)	50 mg/kg (i.p)	100 mg/kg (i.p)
0	0.00	0.00	0.00	0.00	0.00
2	0.8±0.2	0.15±0.04	0.62±0.2	0.48±0.27	0.38±0.15
4	1.8±0.33	0.68±0.18	1.45±0.54	0.92±0.49	0.85±0.10
6	3.85±0.18	1.20±0.16	3.25±1.10	1.65±0.55	1.30±0.28
8	4.35±0.8	1.60±0.18	4.60±1.21	2.12±0.86	1.98±0.32
10	6.12±1.02	1.80±0.26	5.12±0.73	2.94±0.74	2.30±0.24
12	7.92±0.5	2.30±0.2	6.10±0.86	3.80±0.65	2.80±0.35
14	9.50±0.64	2.80±0.30	7.20±0.31	4.70±0.57	3.40±0.48
16	13.12±0.38	3.30±0.25	7.90±1.14	5.40±0.50	4.12±0.55
18	15.36±0.46	3.80±0.32	8.6±1.12	6.13±0.82	4.90±0.86
20	17.10±0.98	4.20±0.34	8.9±1.3	6.51±1.12	5.42±0.52

The results are shown in mean ± SEM.

Number of EAC cell 136 $\times 10^4$ per mL was inoculated into five groups of mice (six in each group) on day 0. Four groups were treated with PEE at the doses of 25 mg/kg, 50 m/kg and 100 mg/kg and *bleomycin* (0.3 mg/kg) respectively after 24 hours of EAC cell inoculation in mice and continued for 10 days. Fifth group was considered as untreated control. Tumor weight was obtained from different mouse of each day from first day of tumor inoculation.

Table 3.5 Tumor weight of EAC bearing mice treated with EAE at the doses of 25mg/kg (i.p.), 50mg/kg (i.p.), 100mg/kg (i.p.) and *bleomycin* at 0.3 mg/kg (i.p.)

Days	Control EAC bearing mice	Standard drug *bleomycin* (0.3 mg/kg,i.p)	Ethyl acetate extract of Eucalyptus (EAE)		
			25 mg/kg (i.p)	50 mg/kg (i.p)	100 mg/kg (i.p)
0	0.00	0.00	0.00	0.00	0.00
2	0.8±0.2	0.15±0.04	0.70±0.25	0.56±0.24	0.52±0.12
4	1.8±0.33	0.68±0.18	1.60±0.19	0.98±0.71	0.90±0.30
6	3.85±0.18	1.20±0.16	2.9±0.23	1.80±0.63	1.50±0.25
8	4.35±0.8	1.60±0.18	4.02±0.12	2.67±0.29	2.30±0.34
10	6.12±1.02	1.80±0.26	4.98±.29	3.00±0.57	2.70±0.50
12	7.92±0.5	2.30±0.2	5.50±0.75	4.10±0.33	3.40±0.35
14	9.50±0.64	2.80±0.30	7.00±0.81	5.6±0.25	4.00±0.19
16	13.12±0.38	3.30±0.25	7.50±.93	6.25±0.75	5.00±0.36
18	15.36±0.46	3.80±0.32	8.20±1.69	6.75±1.71	5.69±0.27
20	17.10±0.98	4.20±0.34	8.95±1.75	7.5±2.14	6.19±1.18

The results are shown in mean ± SEM.

Number of EAC cell 136 $\times 10^4$ per mL was inoculated into five groups of mice (six in each group) on day 0. Four groups were treated with EAE at the doses of 25 mg/kg, 50 m/kg and 100 mg/kg and *bleomycin* (0.3 mg/kg) respectively after 24 hours of EAC cell inoculation in mice and continued for 10 days. Fifth group was considered as untreated control. Tumor weight was obtained from different mouse of each day from first day of tumor inoculation.

Table-3.6 Effect of ME on blood parameters of tumor bearing Swiss albino mice on day 12 of tumor inoculation

Name of Exp.	RBC Cells/mL	WBC Cells/mL	% of Hb gm/dl
Normal mice	$(5.55\pm0.20)\times10^9$	$(10.7\pm1.4)\times10^6$	12.20 ±0.4
Control (EAC bearing) mice	$(2.20\pm0.15)\times10^9$	$(25.9\pm1.5)\times10^6$	4.5 ±0.7
EAC+ 25 mg/kg	$(2.48\pm0.35)\times10^9$	$(17.0\pm0.65))\times10^6$	10.4±0.21
EAC+ 50 mg/kg	$(3.6\ 0\pm0.21)\times10^9$	$(16.0\pm1.3.)\times10^6$	13.80±1.2
EAC+ 100 mg/kg	$(5.20\pm0.5)\times10^9$	$(14.0\pm1.7)\times10^6$	14.50±1.4
Normal mice + 25 mg/kg	$(4.98\pm0.8)\times10^9$	$(11.0\pm0.22)\times10^6$	11.25±0.3
Normal mice + 50 mg/kg	$(3.78\pm0.4)\times10^9$	$(8.50\pm1.2)\times10^6$	8.2±0.24
Normal mice + 100 mg/kg	$(3.42\pm0.25)\times10^9$	$(6.23\pm1.1)\times10^6$	7.70±1.2

The results are shown in mean ± SEM.

Hematological parameters were studied in eight groups of mice of them four groups were EAC bearing mice treated with ME at the doses of 25 mg/kg, 50 mg/kg and 100mg/kg (i. p.) on day 12 of tumor inoculation, where 136×10^4 EAC cells were inoculated and other four groups were normal mice treated with the same doses. Number of mice per group was 6. Results were shown in MEAN± SEM and compared with normal (without EAC bearing mice) and control (EAC bearing mice).

Table-3.7 Effect of PEE on blood parameters of tumor bearing swiss albino mice on day 12 of tumor inoculation

Name of Exp.	RBC Cells/mL	WBC Cells/mL	% of Hb gm/dl
Normal mice	$(5.55\pm0.20)\times10^9$	$(10.7\pm1.4)\times10^6$	12.6±0.4
Control (EAC bearing) mice	$(2.30\pm0.3)\times10^9$	$(26.8\pm0.6)\times10^6$	4.9±0.75
EAC+ 2 5 mg/kg	$(2.8\pm0.45)\times10^9$	$(19.2\pm0.43)\times10^6$	11.5±0.18
EAC+ 50 mg/kg	$(3.50\pm0.18)\times10^9$	$(17.0\pm0.31)\times 10^6$	12.5±0.26
EAC+ 100 mg/kg	$(5.0\pm0.45)\times10^9$	$(15.0\pm0.44)\times10^6$	13.0±0.12
Normal mice + 25 mg/kg	$(3.58\pm0.29)\times10^9$	$(13.0\pm0.35)\times10^6$	13.5±0.23
Normal mice + 50 mg/kg	$(2.98\pm0.23)\times10^9$	$(8.50\pm0.47)\times10^6$	8.0±0.32
Normal mice + 100 mg/kg	$(2.85\pm0.63)\times10^9$	$(6.23\pm0.13)\times10^6$	7.2±.82

The results are shown in mean ± SEM.

Hematological parameters were studied in eight groups of mice of them four groups were EAC bearing mice treated with PEE at the doses of 25 mg/kg, 50mg/kg and 100 mg/kg (i. p.) on day 12 of tumor inoculation, where 136×10^4 EAC cells were inoculated and other four groups were normal mice treated with the same doses. Number of mice per group was 6. Results were shown in MEAN± SEM and compared with normal (without EAC bearing mice) and control (EAC bearing mice).

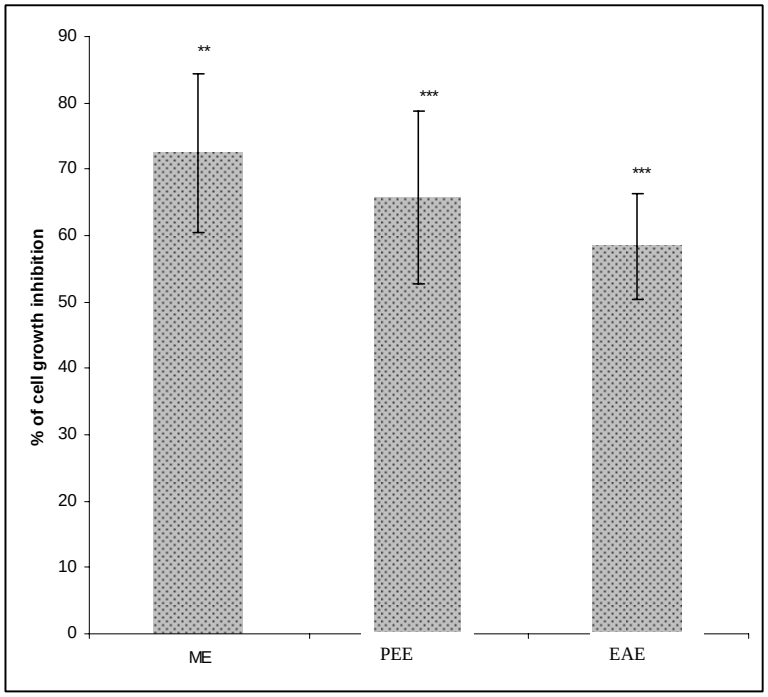
Table-3.8 Effect of EAE on blood parameters of tumor bearing swiss albino mice on day 12 of tumor inoculation

Name of Exp.	RBC Cells/mL	WBC Cells/mL	% of Hb gm/dL
Normal mice	$(5.65\pm0.20)\times10^9$	$(10.3\pm1.2)\times10^6$	12.32±0.7
Control (EAC bearing) mice	$(2.30\pm0.3)\times10^9$	$(26.8\pm0.6)\times10^6$	4.9±0.75
EAC+ 2 5 mg/kg	$(2.7\pm0.47)\times10^9$	$(18.2\pm0.44)\times10^6$	10.8±0.25
EAC+ 50 mg/kg	$(3.35\pm0.19)\times10^9$	$(16.50\pm0.33)\times 10^6$	13.7±0.37
EAC+ 100 mg/kg	$(4.98\pm0.15)\times10^9$	$(15.50\pm0.48)\times10^6$	13.1±0.13
Normal mice + 25 mg/kg	$(3.52\pm0.23)\times10^9$	$(12.5\pm0.25)\times10^6$	13.3±0.43
Normal mice + 50 mg/kg	$(2.88\pm0.63)\times10^9$	$(8.75\pm0.57)\times10^6$	7.78±0.32
Normal mice + 100 mg/kg	$(2.89\pm0.55)\times10^9$	$(6.11\pm0.12)\times10^6$	6.98±.42

The results are shown in mean ± SEM.

Hematological parameters were studied in eight groups of mice of them four groups were EAC bearing mice treated with EAE at the doses of 25 mg/kg, 50mg/kg and 100 mg/kg (i.p.) on day 12 of tumor inoculation, where 136×10^4 EAC cells were inoculated and other four groups were normal mice treated with the same doses. Number of mice per group was 6. Results were shown in MEAN± SEM and compared with normal (without EAC bearing mice) and control (EAC bearing mice).

Figure 3.1 Effect of ME, PEE and EAE on transplantabilibity of EAC cells *in vivo*



Numbers of mice in each experiment were 6. The results are shown in mean ± SEM. Where significant values are,*$p<0.05$,**$p<0.01$ and***$p<0.001$.

Mice were inoculated with EAC cells and treatment were started after 24 h of tumor inoculation and continued for five days then the mice were sacrificed. The cells were harvested with normal saline and reinoculated into fresh mice and left these mice for another five days. On day six these mice were sacrificed and the tumor cells were harvested and counted using hemacytometer. Cells growth inhibition was determined by comparing with control.

Figure 3.2a Tumor weight of EAC bearing mice treated with ME and *bleomycin*

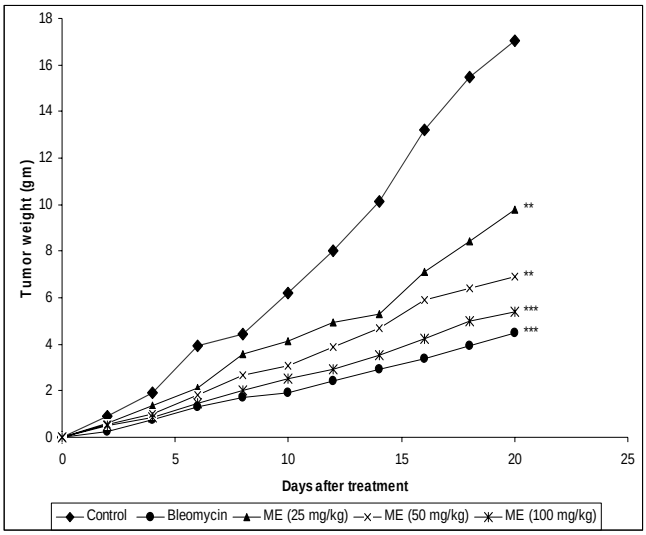
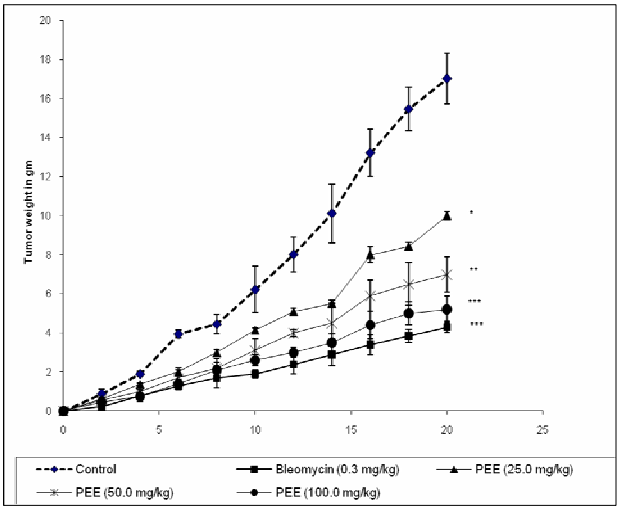


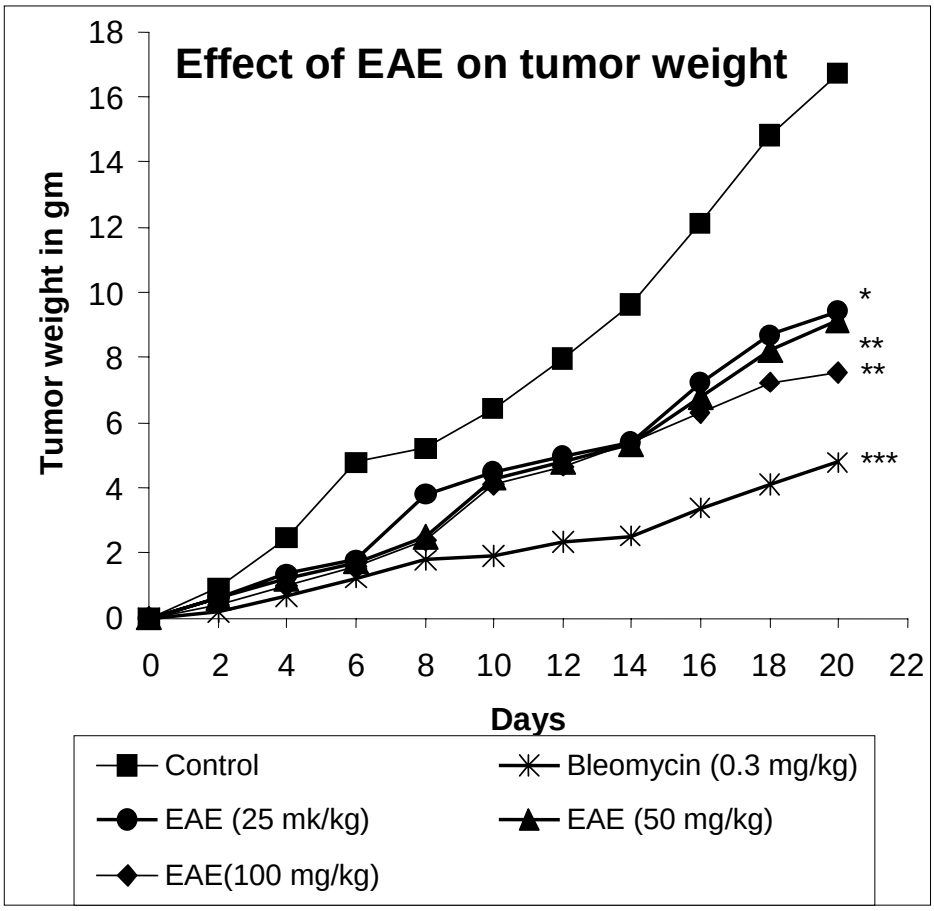
Figure 3.2b Tumor weight of EAC bearing mice treated with PEE and *bleomycin*



Numbers of mice in each experiment were 6. The results are shown in mean ± SEM. Significant values are,*$p<0.05$,**$p<0.01$ and***$p<0.001$.

Each mouse was inoculated with 140×10^4 EAC cells and treatment was started after 24 hours of tumor cell inoculation and continued for 10 days. Tumor growth was monitored by recording the weight change daily.

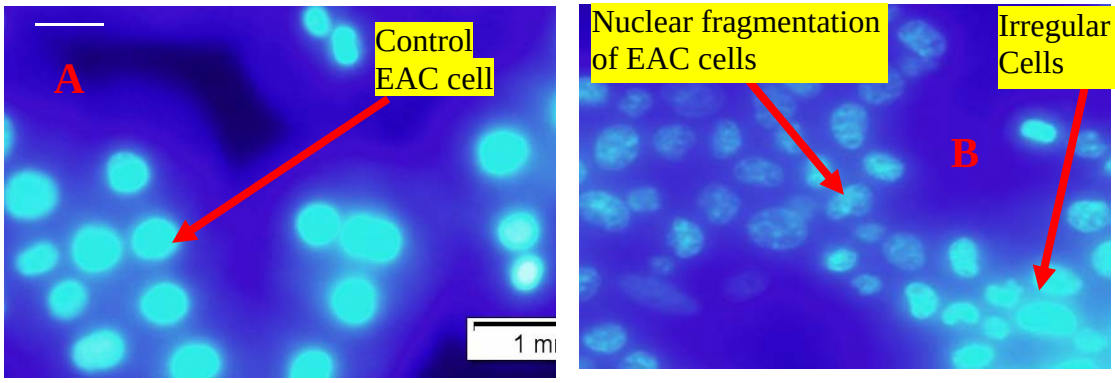
Figure 3.2c Tumor weight of EAC bearing mice treated with EAE and *bleomycin*



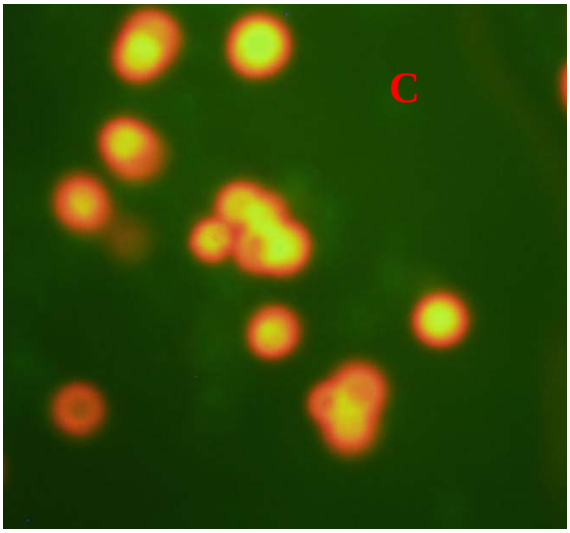
Numbers of mice in each experiment were 6. The results are shown in mean ± SEM. Significant values are,*$p<0.05$,**$p<0.01$ and***$p<0.001$.

Each mouse was inoculated with 140×10^4 EAC cells and treatment was started after 24 hours of tumor cell inoculation and continued for 10 days. Tumor growth was monitored by recording daily weight change.

Figure 3.3 Fluorescence microscopic view of control and EAE treated EAC cells



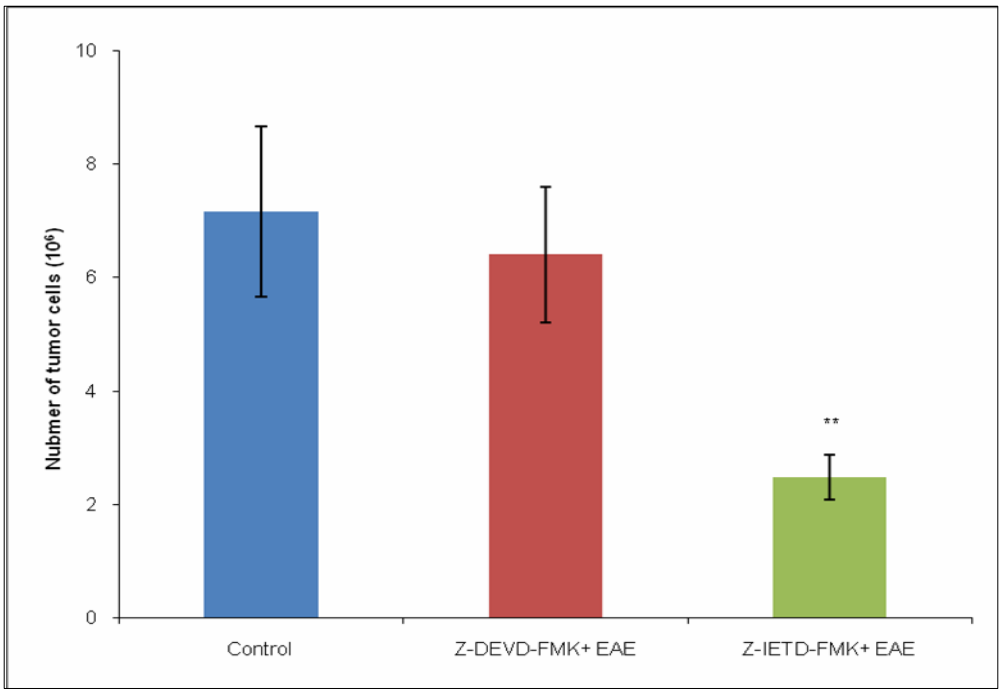
Normal EAC cells **Apoptotic EAC cells treated with the extract (EAE)**



Necrotic EAC cells treated with the extract (EAE)

EAC cells were collected from control and treated mice on day six. After washing with PBS all cells were stained with 0.1 µg/mL of Hoechst 33342 at 37^{o}C for 20 min. The cells were then washed with Phosphate buffer saline (PBS) and re-suspended in PBS for observation of morphological changes under fluorescence microscopy. Cells also stained with Propidium Iodide (PI) to observe necrotic or late apoptotic death. A) EAC of normal mice showed no apoptotic feature. B) EAC cells treated with EAE showed nuclear condensation, fragmentation, cell membrane blebbing and apoptotic bodies. C) Cells undergone late apoptosis are showed in PI staining.

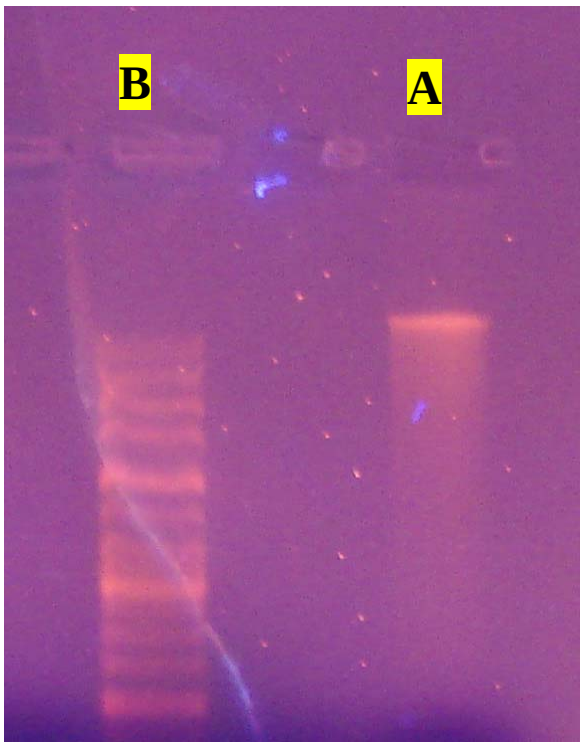
Figure 3.4 Effects of caspases inhibitors on EAC cells



Numbers of mice in each experiment were 6. The results are shown in mean ± SEM. Significant value is,**$p<0.01$ when compared to control.

Cells were collected from control mice and washed with PBS. Then these cells were incubated with Z-DEVD-FMK (caspase-3 inhibitor, 2µmol/mL) and Z-IETD-FMK (caspase-8 inhibitor, 2 µmol/mL) for 1 h, and after that the cells were treated with the EAE and kept for another 24 h. Finally, the cells were counted using hemocytometer and determined the effects of EAE on cell growth inhibition in presence or absence of the specific inhibitor.

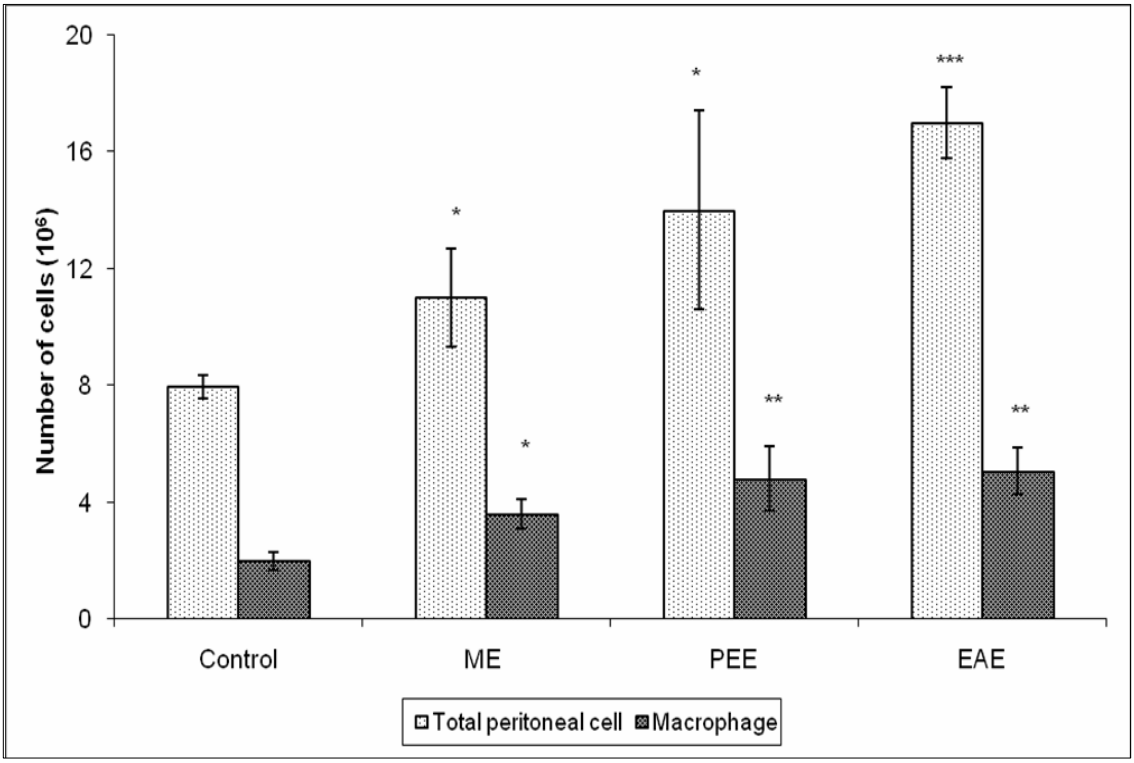
Figure 3.5 *In vivo* effects of EAE on DNA fragmentation of EAC cells



DNA on 1.5% agarose gel electrophoresis. A) DNA from control EAC cells, B) DNA from EAE treated EAC cells (DNA fragmentation detected from treated EAC cells).

EAC cells from control and treated (EAE) mice were obtained and washed with PBS. The total DNA was extracted by using a DNA extraction kit (Promega, USA) and analyzed by electrophoresis on 1.5% agarose gel containing 0.1 µg/mL ethidium bromide and visualized under UV illuminator.

Figure 3.6 Effect of extracts on macrophages and peritoneal cells



Numbers of mice in each experiment were 6. The results are shown in mean ± SEM. Significant values are,*$p<0.05$,**$p<0.01$ and***$p<0.001$.

Three group of normal mice (n=6) were treated with the extracts at dose 100.0 mg/kg (i.p) per day for three consecutive days, the fourth (untreated) group (n=4) served as control. After 24 hours of last treatment, each animal was injected with 5 mL of normal saline (0.98%) into the peritoneal cavity and then sacrificed. Intraperitoneal exuded cells and number of macrophages were counted after staining with 1% neutral red using hemocytometer.

HOST TOXICITY STUDY OF EUCALYPTUS

4.1 Introduction

As a part of the research focused bioactive compounds to find out a novel host friendly anticancer agent, the host toxic effect of extracts was reported here with the aim of determining whether the extracts while functioning as antitumour agent can exert any unacceptable toxic side effect to the host. For this purpose, the different hematological, biochemical profiles and histological investigation of mice organs have been studied.

4.2 Experimental design of toxicity studies

The sub-acute toxicity studies of the tested samples, methanol extract (ME), petroleum ether extract (PEE) and ethyl acetate extract (EAE) of Eucalyptus were performed on normal adult healthy mice by giving a daily dose of 100 mg/kg (i.p.) body weight for 14 consecutive days. The mice were kept under keen observation throughout the treatment period. The following parameters were studied during this course of time.

1. Gross general observation
2. Hematological profiles
3. Biochemical parameters of blood
4. Histopathology of liver, kidney, heart, lung, spleen and brain.

4.2.1 Preparation of the sample solutions

The methanol, petroleum ether and ethyl acetate extract of Eucalyptus were dissolved in DMSO in such a way that the concentrations of 25 mg/mL for each were obtained.

4.2.2 Gross general observation after the drug administration

The mice were injected intraperitoneally (i.p.) with the Eucalyptus extract at 100 mg/kg body weight. Following features were observed daily

Behavior

CNS excitation

CNS depression

Food intake

Salivation

Diarrhea

Muscular weakness

Reflexes

4.2.3 Checking the changes of body weight

The body weights of each mouse were measured before administration of ME, PEE and EAE of Eucalyptus and at the completion of the treatment prior to sacrifice the animals. The weights of individual mice were compared.

4.2.4 Assay of hematological profiles

The hematological profiles of the experimental mice were done to check the hematological abnormalities after administration of the extract intraperitoneally. For this purpose, the following parameters were observed.

Total RBC count

Total WBC count

Hemoglobin content

4.2.4.1 Materials and methods

To assay hematological (TC, % Hb etc) and biochemical (serum glucose, bilirubin, ALP, GOT and GPT etc) parameters four groups of mice (n=24) were taken. Group-I to III were treated with the ME, PEE and EAE at dose of 100 mg/kg (i.p.) for 14 consecutive days and group IV served as control. Blood was collected from each group on day 5, 10 and 25 for study of different parameters described above.

4.2.4.2 Treatment schedule

Hematological parameters were studied in normal mice by the method as described earlier[73]. For this treatment four groups of mice were taken. Treatment was given at dose 100 mg/kg (i.p.) and continued for 14 days. Blood was drawn from each group of mice by conventional way on days 5, 10 and 25.

4.2.4.3 Assay of the biochemical parameters of blood

The biochemical parameters which are related to liver functions such as serum GOT, GPT, ALP, bilirubin, and the parameter which are related to kidney

functions such as serum level of glucose are determined. Serum levels of these parameters change with the pathological changes of these organs. In case of hepatic necrosis, cirrhosis and obstructive jaundice, the serum level of GOT and GPT may increase up to 200 IU/L. If a drug possesses any effect on liver and kidney, several pathological changes may occur and ultimately serum level of these parameters will be altered.

The following parameters were assayed for liver function test.

I. **Assay of enzyme**:

serum glutamate-oxalo-acetate transaminase (GOT)

Serum glutamate-pyruvate transaminase (GPT)

Serum alkaline phosphatase (ALP)

II. Assay of other biomolecules

Serum glucose

Serum bilirubin

Procedure

Collection of serum

On the targeted day of treatment with the extracts, the mice of all groups were sacrificed with the help of a surgical blade No. 22 and the blood was collected in plastic centrifuge tubes. These were then allowed to clot at 40°C for 4 hours. After clotting, the blood samples were centrifuged at 4000 rpm for 15 minutes using a WIFUNG centrifuge LABO-50M. The clear straw color serum was then collected in vials with Pasteur pipette and stored at -20°C.

Biomolecules and enzymes activities were estimated[79-90] using auto-Bioanalyzer, Hitachi Ltd. Tokyo, Japan.

4.3 Histopathology of mice organs

Histopathology of liver, kidney, heart, lung, spleen and brain were performed to observe any changes in the cellular structures (degradation and regeneration) of the mice receiving the extracts at a dose of 100 mg/kg /day for 14 consecutive days with respect to control group (normal mice).

Reagents:

Formalin (10%)

Absolute alcohol (95%)

Paraffin

Xylene

D.P.X. mounting fluid

Harris hematoxylin and eozin stain.

4.3.1 Collection and processing of the tissues

The liver, kidney, heart, lungs, spleen and brain were collected from different groups of treated and controlled mice. After sacrificing them on 15 day of observation, the tissues were sliced into pieces, each measuring a few millimeters of thickness. The sliced tissues were then immersed in 10% formalin for 3 days. The tissues were then dehydrated in ascending order of ethanol and embedded in paraffin. The blocks were sectioned with the help of rotating microtome at 6 micron in thickness.

A. Staining

The sections were deparaffinized at two changes of xylene (5 min. / each) and hydrated in descending order of alcohol (2-3 min. / each) and then cleaned in two sets of xylene (5 min./each).

B. Mounting

Glass slides contained the tissue area were wiped, dried and then a drop of Canada balsam was put on the section and cover slip was gently placed on it. On the sections, thin film between the cover slip and the slide with the mounting medium (Canada balsam) was formed to attach them. Then the slides of liver, heart, lung, kidney, spleen and brain were observed under microscope and recorded by photographs.

4.4 Results

4.4.1 Effect of ME, PEE and EAE on body weight of normal mice

During the whole experimental period, general behavior, CNS excitation, CNS depression, reflexes, muscular weakness, salivation, diarrhea and food intake of all the mice were observed. The treated mice did not show any abnormalities and their food intake was also observed to be normal. No muscular numbness of the hind and forelegs, salivation or diarrhea was observed. Table 4.1, 4.2 and 4.3 shows the average body weights of all the mice before and after the treatment and the data were compared with control. No significant changes in body weight were observed.

4.4.2 Effect of ME, PEE and EAE on blood parameters of normal mice

Effect of methanol, petroleum ether and ethyl acetate extract of Eucalyptus on hematological parameters has been observed in Swiss albino mice. Hematological parameters in swiss albino mice treated at dose of 100 mg/kg for 14 consecutive days are shown in table 4.4, 4.5 and 4.6 respectively on days 5, 10 and 25. It has been observed that RBC, WBC, % hemoglobin decreased moderately during treatment but regained almost towards normal values after 25 days of the initial treatment. This indicates that, the extracts have practically no long term toxic effect.

4.4.3 Effect of extracts on biochemical parameters of normal mice

Effect of extracts on biochemical parameters has been observed in swiss albino mice. Biochemical parameters in swiss albino mice treated at dose of 100 mg/kg/day (i. p.) for 14 consecutive days are shown in table-4.7, 4.8 and 4.9 on

days 5, 10 and 25. It has been observed that the blood glucose, bilirubin ALP, GOT and GPT slightly increased during the treatment period. Liver damage via reactive oxygen species (ROS) pathway cause a remarkable increase of glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) levels in serum. As assumed from its high LD_{50} value extracts of Eucalyptus produces little toxicity in animals. The results presented here have again confirmed that the test plant extracts has no serious adverse effect to the host.

4.4.4 Histopathology of mice organ

In mice of the treated group, no major abnormalities in the histopathology of liver, heart, lung, kidney, spleen and brain were detected in comparisons with control group under microscope. This indicates that, the plant extract has no toxic effect at this dose (100 mg/kg body weight/day) on cellular structure. Results are shown in figure 4.1-4.6.

Table 4.1 Effect of ME on body weight of normal Swiss albino mice after intraperitoneal administration (dose of 100mg/kg/day)

Group	Dose (i.p.) mg/ kg /day	Day	Experimental group		Control group	
			Body weight n = 6, Mean± SEM	% change	Body weight n = 6, Mean± SEM	% change
ME	100	1	24.76 ± 3.13		23.92 ± 1.97	
		2	24.93 ± 1.5	0.69	24.50 ± 1.55	2.42
		3	25.5 ± 2.81	2.98	26.32 ± 1.60	9.85
		4	26.2 ± 2.31	5.73	26.87 ± 1.66	11.94
		5	27.16 ± 2.52	9.39	27.50 ± 1.80	14.28
		6	27.57 ± 1.65	10.90	27.78 ± 1.33	15.30
		7	29.05 ± 2.54	16.27	28.12 ± 1.29	16.52
		8	30.13 ± 3.0	18.41	28.50± 0.68	17.87
		9	30.43 ± 2.25	19.41	30.22 ± 1.15	23.91
		10	31.44± 2.56	22.73	30.54 ±1.321	24.97
		11	32.06 ± 2.05	24.70	31.85± .412	29.26
		12	32.96 ± 3.1	27.51	32.00 ± .361	29.73
		13	33.83 ± 3.54	30.15	32.31 ± 1.50	30.70
		14	34.78 ± 2.50	32.96	32.5 ± 2.51	31.29

Number of mice in each case (n=6); the results are shown as mean ± SEM.

To evaluate host toxic effects of ME, normal mice were treated with ME for 14 consecutive days at dose 100.0 mg/kg/day and body weight of individual mouse were recorded in gm.

Table 4.2 Effect of PEE on body weight of Swiss albino mice after intraperitoneal administration (dose of 100mg/kg/day)

Group	Dose (i.p.) mg/ kg /day	Day	Experimental group		Control group	
			Body weight n = 6, Mean± SEM	% change	Body weight n = 6, Mean± SEM	% change
PEE	100	1	24.83 ± 3.27		24.92 ± 1.98	
		2	25.5 ± 2.68	2.627	24.98 ± 1.582	0.24
		3	26.7 ± 2.92	7	26.2 ± 1.631	4.88
		4	27.50 ± 3.10	9.70	26.32 ± 1.75	5.32
		5	27.75 ± 2.13	10.52	26.55 ± 1.81	6.139
		6	28.2 ± 2.56	11.95	27.23 ± 1.21	8.48
		7	28.6 ± 3.2	13.18	27.45 ± 1.25	9.22
		8	29.73 ± 2.93	16.48	28.32 ± 0.78	12.00
		9	30.55 ± 2.39	18.72	29.54 ± 1.22	15.63
		10	31.13 ± 3.12	20.23	29.78 ± 1.74	16.31
		11	32.00 ± 3.31	22.40	29.98 ± 1.41	16.88
		12	32.31 ± 2.502	23.15	30.21 ± 1.76	17.51
		13	32.72 ± 4.153	24.11	30.74 ± 1.34	18.93
		14	33.25 ± 3.25	25.32	31.55 ± 2.25	21.14

Number of mice in each case (n=6); the results are shown as mean ± SEM.

To evaluate host toxic effects of PEE, normal mice were treated with PEE for 14 consecutive days at dose 100.0 mg/kg/day and body weight of individual mouse were recorded in gm.

Table 4.3 Effect of EAE on body weight of Swiss albino mice after intraperitoneal administration (dose of 100mg/kg/day)

Group	Dose (i.p.) mg/ kg /day	Day	Experimental group		Control group	
			Body weight n = 6, Mean± SEM	% change	Body weight n = 6, Mean± SEM	% change
EAE	100	1	24.76 ± 3.13		23.92 ± 1.97	
		2	25.13 ± 2.70	1.47	24.50 ± 1.55	2.36
		3	26.4 ± 2.88	6.21	26.32 ± 1.60	9.11
		4	27.44 ± 3.32	9.77	26.87 ± 1.66	10.98
		5	27.65 ± 2.17	10.45	27.50 ± 1.80	13
		6	28.28 ± 2.54	12.45	27.78 ± 1.33	13.89
		7	28.50 ± 3.35	13.12	28.12 ± 1.29	14.94
		8	28.73 ± 2.87	13.82	28.50± 0.68	16.07
		9	30.05 ± 2.46	17.60	30.22 ± 1.15	20.85
		10	31.16± 3.14	20.53	30.54 ± 1.321	21.67
		11	31.70 ± 3.37	21.89	31.85 ± 1.412	24.9
		12	32.31 ± 2.80	23.367	32.00 ± 1.361	25.25
		13	32.77 ± 4.41	24.44	32.31 ± 1.50	25.96
		14	34.45 ± 3.75	28.128	32.5 ± 2.51	26.4

Number of mice in each case (n=6); the results are shown as mean ± SEM.

To evaluate host toxic effects of EAE, normal mice were treated with EAE for 14 consecutive days at dose 100.0 mg/kg/day and body weight of individual mouse were recorded in gm.

Table 4.4 Effects of ME on blood parameters in normal mice on days, 0, 5, 10 and 25

Name of Exp.	Days	RBC Cells/mL	WBC Cells/mL	% of Hb
Normal mice	0	$(6.32\pm0.38)\times10^{9}$	$(11.1\pm1.5)\times10^{6}$	12.1±1.4
ME	5	$(4.69\pm0.27)\times10^{9*}$	$(6.97\pm0.85)\times10^{6***}$	$5.8\pm0.32^{***}$
	10	$(5.14\pm0.75)\times10^{9**}$	$(7.12\pm1.0)\times10^{6**}$	$6.88\pm0.91^{***}$
	25	$(5.85\pm0.41)\times10^{9}$	$(9.75\pm1.2)\times10^{6**}$	12.00±1.1

Number of mice in each group were six (n=6). Result are shown as Mean +SEM and compared with control, where significant values are *$p<0.05$, ** $p<0.01$ and *** $p<0.001$.

To evaluate host toxic effects of ME, normal mice were treated with ME for 14 consecutive days at dose 100.0 mg/kg/day and blood was collected from heart on 5, 10 and 25th days.

Table 4.5 Effects of PEE on blood parameters in normal mice on days, 0, 5, 10 and 25

Name of Exp.	Days	RBC Cells/mL	WBC Cells/mL	% of Hb
Normal mice	0	$(6.32\pm0.38)\times10^{9}$	$(11.1\pm1.5)\times10^{6}$	12.1±1.4
PEE	5	$(4.68\pm0.62)\times10^{9*}$	$(6.96\pm0.71)\times10^{6***}$	$5.8\pm0.21^{***}$
	10	$(5.24\pm0.9)\times10^{9**}$	$(7.2\pm0.32)\times10^{6**}$	$6.91\pm0.19^{***}$
	25	$(5.95\pm0.61)\times10^{9}$	$(9.75\pm1.5)\times10^{6**}$	12.10±1.5

Number of mice in each group were six (n=6). Result are shown as Mean +SEM and compared with control, where significant values are *$p<0.05$, ** $p<0.01$ and *** $p<0.001$.

To evaluate host toxic effects of PEE, normal mice were treated with PEE for 14 consecutive days at dose 100.0 mg/kg/day and blood was collected from heart on 5, 10 and 25th days.

Table 4.6 Effects of EAE on blood parameters in normal mice on days, 0, 5, 10 and 25

Name of Exp.	Days	RBC Cells/mL	WBC Cells/mL	% of Hb
Normal mice	0	$(5.99\pm0.24)\times10^{9}$	$(10.7\pm2.1)\times10^{6}$	10.89±1.7
EAE	5	$(4.2\pm0.52)\times10^{9**}$	$(5.91\pm0.82)\times10^{6***}$	$6.2\pm0.20^{***}$
	10	$(5.3\pm0.45)\times10^{9**}$	$(6.5\pm0.32)\times10^{6**}$	$6.98\pm0.23^{***}$
	25	$(5.80\pm0.71)\times10^{9}$	$(8.50\pm3.2)\times10^{6**}$	11.1±1.5

Number of mice in each group were six (n=6). Result are shown as Mean +SEM and compared with control, where significant values are *$p<0.05$, ** $p<0.01$ and *** $p<0.001$.

To evaluate host toxic effects of EAE, normal mice were treated with EAE for 14 consecutive days at dose 100.0 mg/kg/day and blood was collected from heart on 5, 10 and 25th days.

Table 4.7 Effect of ME (100mg/kg/day) on biochemical parameters in normal mice

Name of Experiment	Days	Serum glucose mg/dl blood	Serum bilirubin mg/dl	ALP U/L	GOT U/L	GPT U/L
Normal mice	0	145.6±5.4	1.32 ±0.11	105.7±10.2	68.3±4.5	77±.8.6
ME	5	102±10.5**	1. 07±0.2	233.2±15.1***	109±5.3**	61±6.5*
	10	138.7±8.3***	1.59 ±0.1	245±13**	144±7.4**	54±4.1
	25	156±14**	1.45±0.25	206.4±11**	95±6.5	76±11**

The number of mice in each group were 6. Results are shown in MEAN ± SEM and compared with control, where significant values are *$p<0.05$, ** $p<0.01$ and *** $p<0.001$.

To evaluate host toxic effects of ME, normal mice were treated with ME for 14 consecutive days at dose 100.0 mg/kg/day and blood was collected from heart on 5, 10 and 25th days.

Table 4.8 Effect of PEE (100mg/kg/day) on biochemical parameters in normal mice

Name of Experiment	Days	Serum glucose mg/dl blood	Serum bilirubin mg/dl	ALP U/L	GOT U/L	GPT U/L
Normal mice	0	145.6±1.4	1. 32±0.3	105.7±2.5	68.3±2	77±.5
PEE	5	146.5±3**	1. 56±0.4	133.2 ±1.6***	73±4**	83±3*
	10	163.7±4***	1.42± .37	145±1.5**	89±3**	98±3
	25	130±2.1**	1.39±0.70	106.4±1.3**	72±1.3	72±1.3

Number of mice in each group were six (n=6). Result are shown as Mean +SEM and compared with control, where significant values are *$p<0.05$, ** $p<0.01$ and *** $p<0.001$.

To evaluate host toxic effects of PEE, normal mice were treated with PEE for 14 consecutive days at dose 100.0 mg/kg/day and blood was collected from heart on 5, 10 and 25th days.

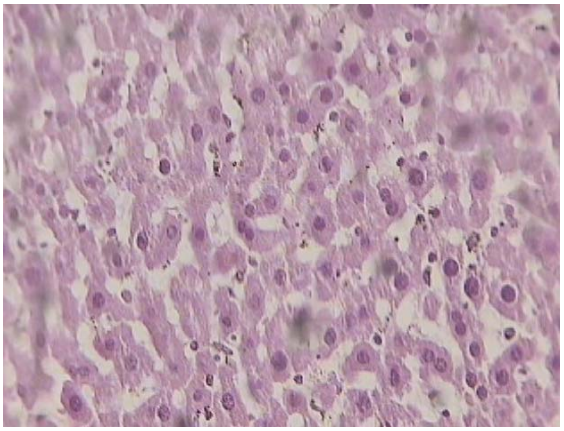
Table 4.9 Effect of EAE (100mg/kg/day) on biochemical parameters in normal mice

Name of Experiment	Days	Serum glucose mg/dl blood	Serum bilirubin mg/dl	ALP U/L	GOT U/L	GPT U/L
Normal mice	0	145.6±1.4	1. 32±0.3	105.7±2.5	68.3±2	77±.5
EAE	5	141.2±2**	1. 50±0.2	130.3±1.2***	71±3**	80±2*
	10	158.4±.97***	1.39±. 32	143±1.4**	87±4**	100±1
	25	132±1..9**	1.35±0.52	102.1±.6**	73±1.1	79±.4

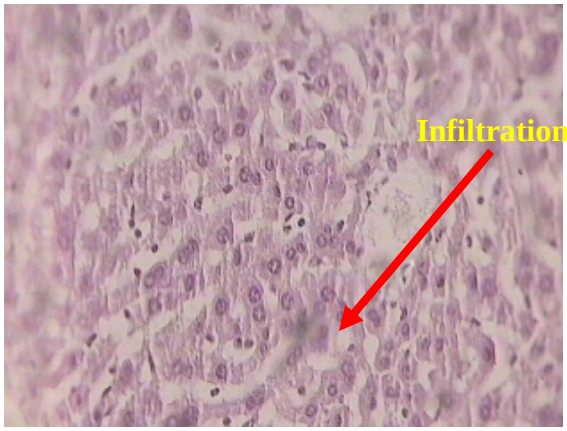
Number of mice in each group were six (n=6). Result are shown as Mean +SEM and compared with control, where significant values are *$p<0.05$, ** $p<0.01$ and *** $p<0.001$.

To evaluate host toxic effects of EAE, normal mice were treated with EAE for 14 consecutive days at dose 100.0 mg/kg/day and blood was collected from heart on 5, 10 and 25th days.

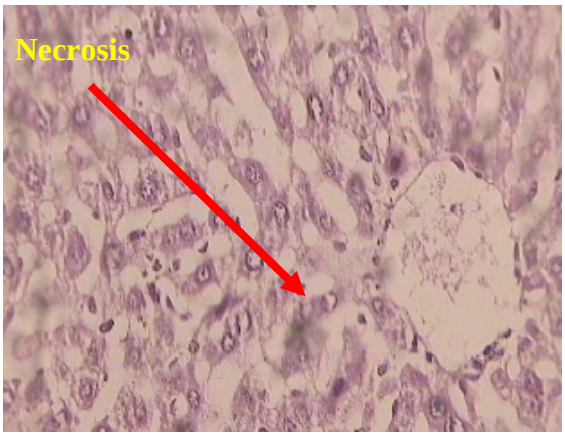
Figure 4.1 Histopathological examinations of experimental mice. a) Liver tissues from control mice with no abnormality b) Liver tissue from ME treated normal mice shown very little infiltration with no central vein dilation, fatty generation or nodule formation c) Liver tissues from PEE treated normal mice with very little necrosis, central vein dilation and d) Liver tissues from EAE treated normal mice with no necrosis and no central vein dilation.



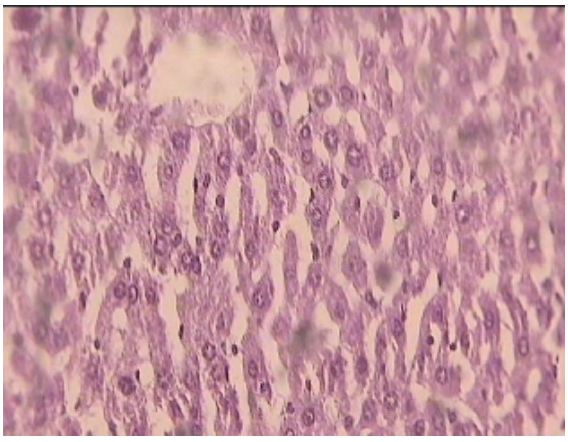
a) Liver of Control mice (normal mice)



b) Liver from ME treated mice



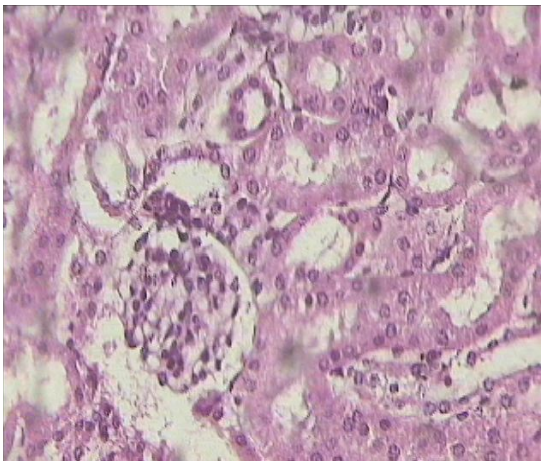
c) Liver from PEE treated mice



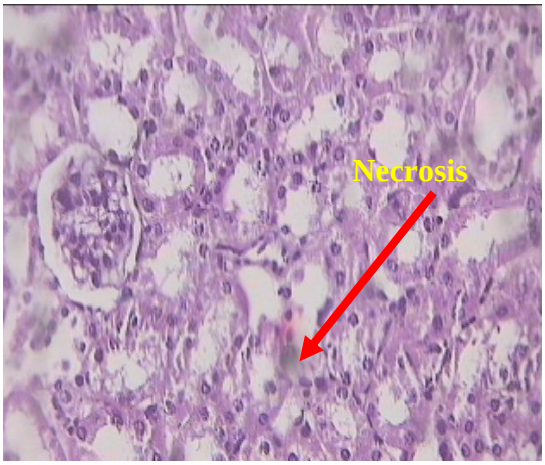
d) Liver from EAE treated mice

Treatment was continued for 14 consecutive days at dose 100.0 mg/kg (i.p). Treatment was discontinued after 14 days from the start.

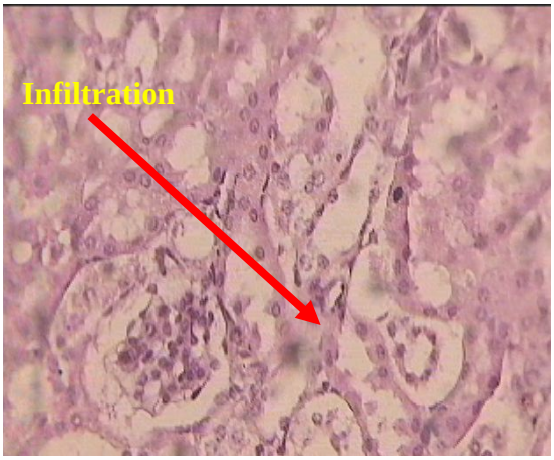
Figure 4.2 Histopathological examinations of experimental mice. a) Kidney tissues from control mice with no abnormality b) Kidney tissue from ME treated normal mice shown very little epithelial cell necrosis c) Kidney tissues from PEE treated normal mice with very little mononuclear cell infiltration and d) Kidney tissues from EAE treated normal mice with no necrosis and no central vein dilation.



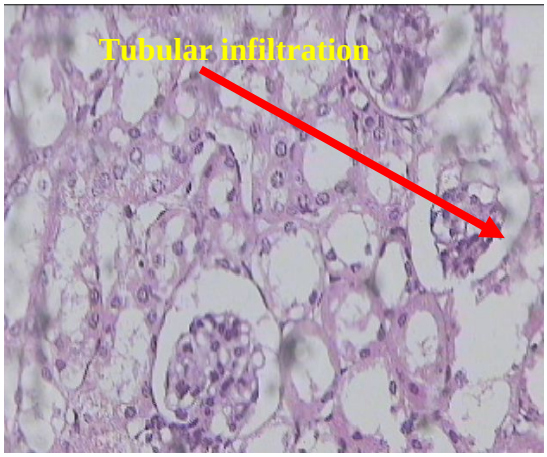
a) Kidney of Control mice (normal mice)



b) Kidney from ME treated mice



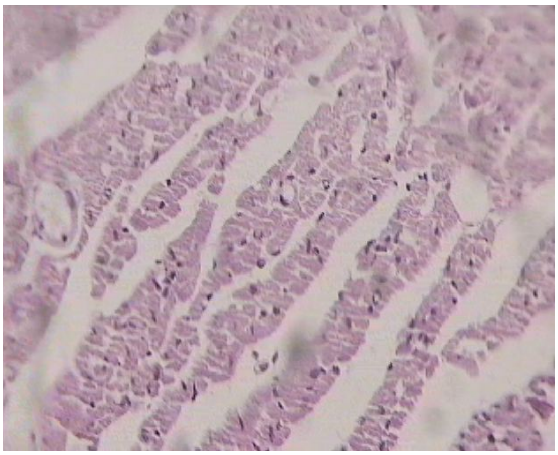
c) Kidney from PEE treated mice



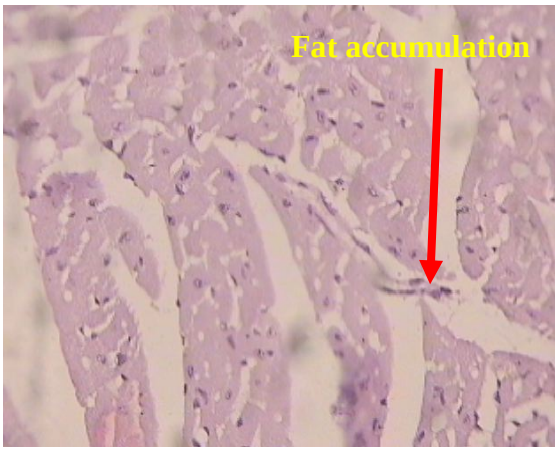
d) Kidney from EAE treated mice

Treatment was continued for 14 consecutive days at dose 100.0 mg/kg (i.p). Treatment was discontinued after 14 days from the start.

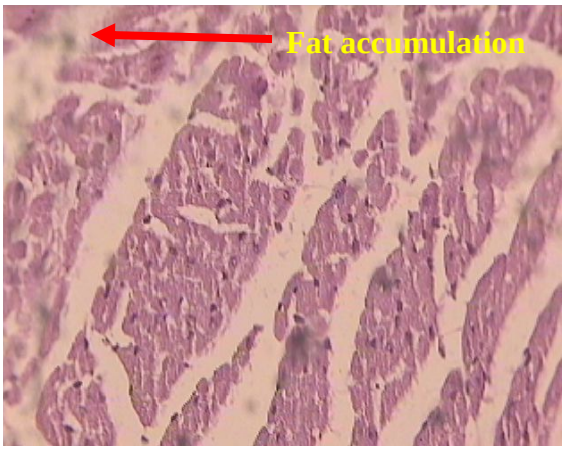
Figure 4.3 Histopathological examinations of experimental mice. a) Heart tissues from control mice with no abnormality b) Heart tissue from ME treated normal mice shown very little fatty generation c) Heart tissues from PEE treated normal mice with little fatty generation and d) Heart tissues from EAE treated normal mice with no abnormalities.



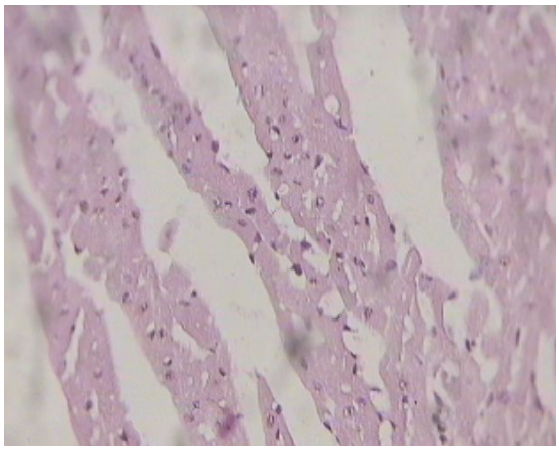
a) Heart of Control mice (normal mice)



b) Heart from ME treated mice



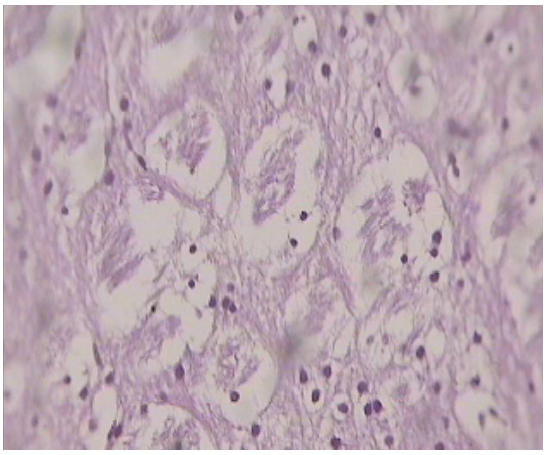
c) Heart from PEE treated mice



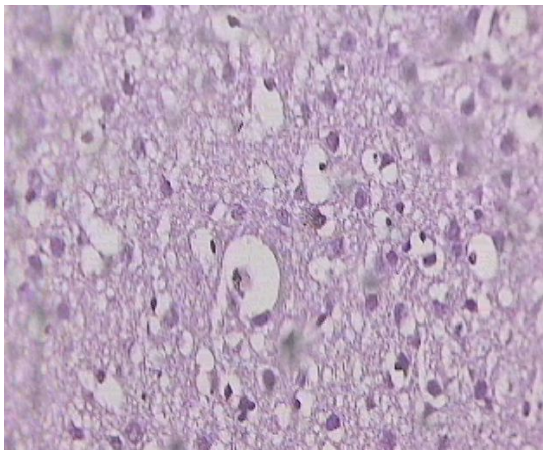
d) Heart from EAE treated mice

Treatment was continued for 14 consecutive days at dose 100.0 mg/kg (i.p). Treatment was discontinued after 14 days from the start.

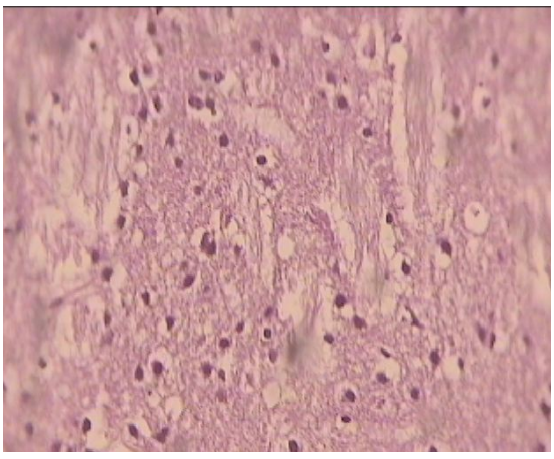
Figure 4.4 Histopathological examinations of experimental mice. a) Brain tissues from control mice with no abnormality b) Brain tissue from ME treated normal mice shown no abnormalities c) Brain tissues from PEE treated normal mice with none major or minor abnormalities and d) Brain tissues from EAE treated normal mice with no abnormalities.



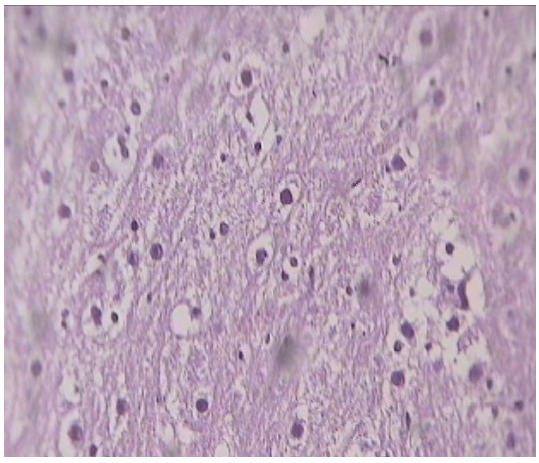
a) Brain of Control mice (normal mice)



b) Brain from ME treated mice



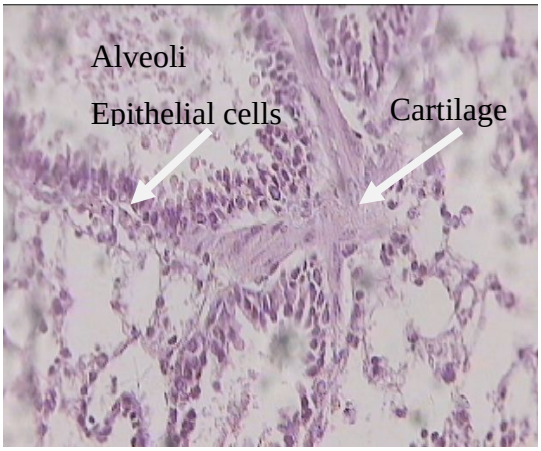
c) Brain from PEE treated mice



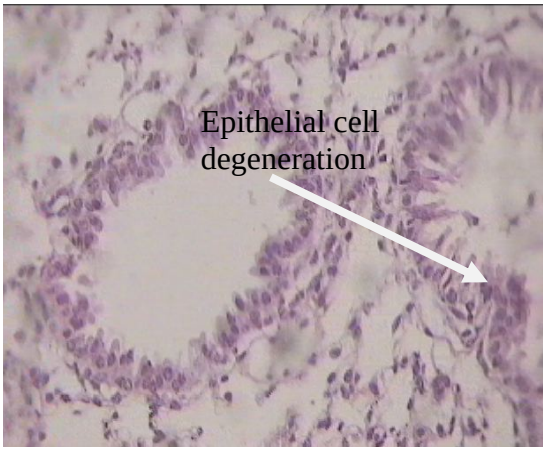
d) Brain from EAE treated mice

Treatment was continued for 14 consecutive days at dose 100.0 mg/kg (i.p). Treatment was discontinued after 14 days from the start.

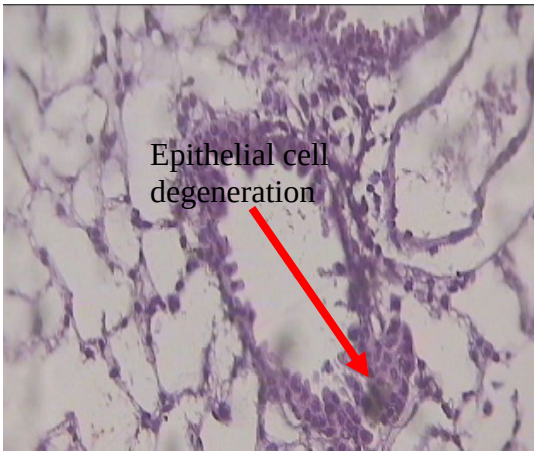
Figure 4.5 Histopathological examinations of experimental mice. a) Lung tissues from control mice with no abnormality b) Lung tissue from ME treated normal mice with minor epithelial cells degeneration c) Lung tissues from PEE treated normal mice with epithelial cells degeneration and d) Lung tissues from EAE treated normal mice with almost no abnormalities.



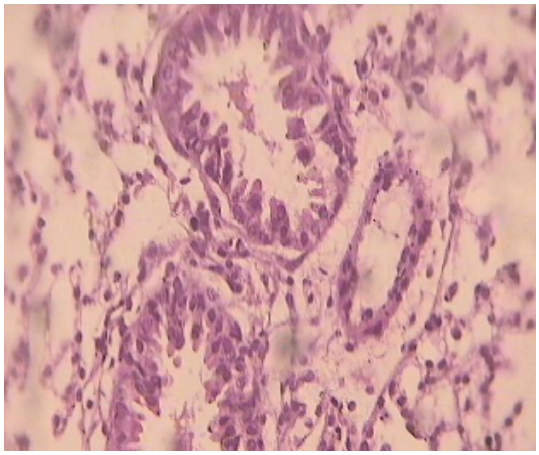
a) Lung of Control mice (normal mice)



b) Lung from ME treated mice



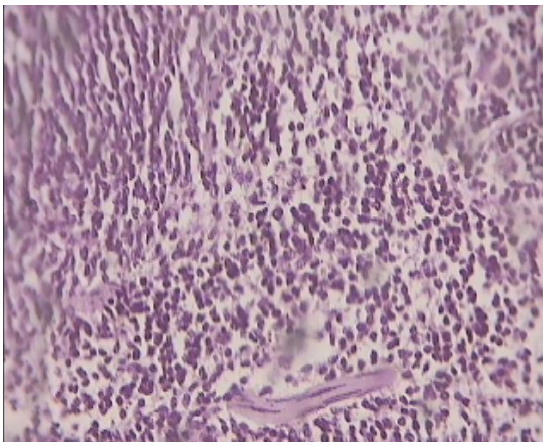
c) Lung from PEE treated mice



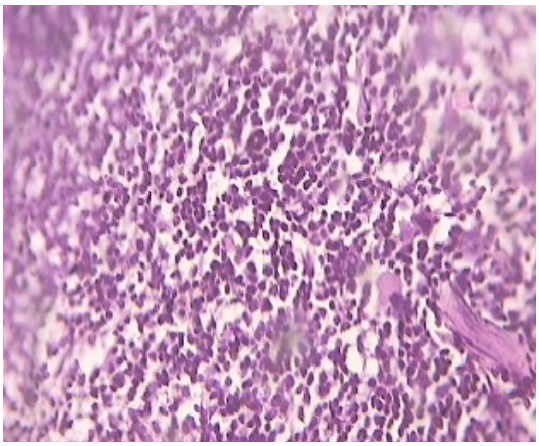
d) Lung from EAE treated mice

Treatment was continued for 14 consecutive days at dose 100.0 mg/kg (i.p). Treatment was discontinued after 14 days from the start.

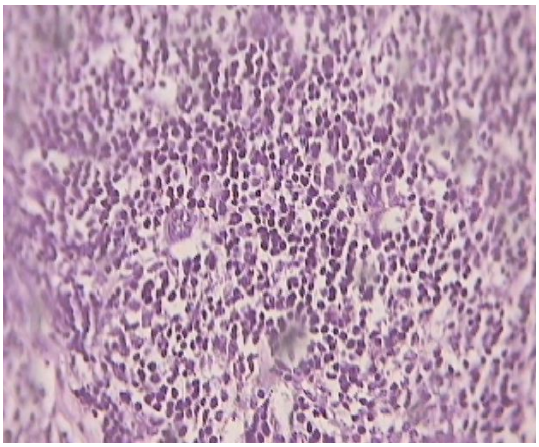
Figure 4.6 Histopathological examinations of experimental mice. a) Spleen tissues from control mice with no abnormality b) Spleen tissue from ME treated normal mice shown no abnormalities c) Spleen tissues from PEE treated normal mice with any major or minor abnormalities and d) Spleen tissues from EAE treated normal mice with no abnormalities.



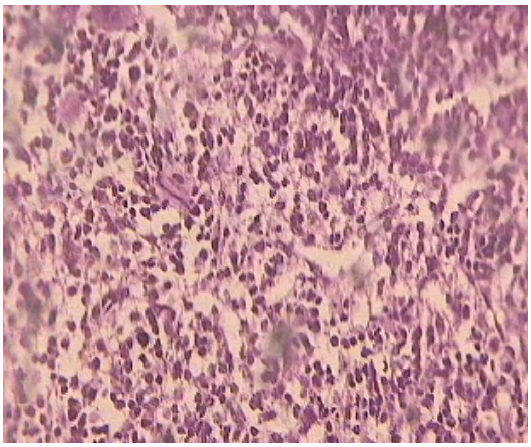
a) Spleen of Control mice (normal mice)



b) Spleen from ME treated mice



c) Spleen from PEE treated mice



d) Spleen from EAE treated mice

Treatment was continued for 14 consecutive days at dose 100.0 mg/kg (i.p). Treatment was discontinued after 14 days from the start.

BRINE SHRIMP LETHALITY BIOASSAY

5.1 Introduction

Bioactive compounds can be bioassayed in a number of methods. Brine shrimp lethality bioassay is recently developed method in this field. Extracts and isolated compounds from plant origin can be tested for their bioactivity by this method and the simple zoological organism, brine shrimp nauplii (*Artemia salina***,** Leach) can be used as a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive natural products. This bioassay indicates toxicity as well as a wide range of pharmacological activities of the compounds[91-93]. The eggs of brine shrimp are readily available in pet shops at low cost and remain viable for years in the dry state. Upon being placed in sea water, the eggs hatch within 48 hours to provide large numbers of larvae (nauplii) for experimental use.

5.2 Principle

Brine shrimp eggs are hatched in simulated sea-water to get nauplii. Test samples are prepared by the addition of calculated amount of DMSO for obtaining desired concentration of the test sample. The nauplii are counted by visual inspection and are taken in vials containing 5 mL of sea-water. Then samples of different concentrations are added to the pre-marked vials through micropipette. The vials are then left for 24 hours and then the nauplii are counted again to find out the cytotoxicity of the plant extract and compared to the results with positive control.

5.3 Experimental design

5.3.1 Materials

a) *Artemia salina* leach (brine shrimp eggs)

b) Sea salt (NaCl)

c) Small tank with perforated dividing dam to grow shrimp, cover and lamp to attract shrimp.

d) Pipettes (5 mL and 1 mL)

e) Micropipette (10 µL- 100µL)

f) Vials, (5 mL).

g) Magnifying glass. (3X magnifying glass)

Procedure

a) Preparation of simulated seawater

Sodium chloride (38 g) was weighed accurately and dissolved in one liter of sterilized distilled water and then filtered off to get a clear solution. The pH of the seawater was maintained between 8 and 9 by using $NaHCO_3$ solution.

b) Hatching of brine shrimp

Artemia salina leach (brine shrimp eggs) collected from the pet shop was used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5 gm/L) were added to one side of the tank and this side was covered with paper. The shrimps were allowed to hatch for two days to hatch and mature as nauplii (larvae). Constant oxygen supply was carried out and constant temperature (around 37˚C) was maintained during the hatching time. The hatched shrimps

were attracted to the lamp from the other side of the divided tank through dam. These nauplii were taken for this bioassay.

c) Preparation of sample

The methanol, petroleum ether and ethyl acetate extract of Eucalyptus (3 mg) were accurately weighed in a vial and dissolved in 0.6 mL (600 µL) DMSO to get a concentration of 5µg/µl for each of the sample. These samples were used as stock solution.

d) Application of the test sample and brine shrimp nauplii to the vials

Six clean vials were taken for each sample in five concentrations (one vial for each concentration) and another vial was also taken for control. Five doses (10, 20, 40, 80 and 100 µg) of each sample were used for the lethality test of brine shrimp nauplii. With the help of a micropipette 10, 20, 40, 80 and 100 µL of each sample were transferred from the stock solution in 5 different vials. Sea water (brine water) was added to each vial making the volume up to 5 mL. 10 living nauplii were transferred to each of the vials. A magnifying glass was used for convenient counting of the nauplii. For each concentration control experiment was done. The experiment was repeated three times.

e) Counting of nauplii

The above mentioned settings were kept for 24 hours. The vials were then observed and the numbers of survived nauplii in each vial were counted and the results were noted. From this, the percentage of mortality of the nauplii was calculated for each concentration and the LD_{50} values were determined using Probit analysis[94].

5.4 Results

5.4.1 Effects of extracts on brine shrimp cytotoxicity

The extracts showed positive results in brine shrimp lethality bioassay. The median lethal concentration (LC_{50}) of brine shrimp lethality was determined from Probit statistical analysis. The data and results of the sample and LC_{50} of standard *bleomycin* are given in the table 5.1, 5.2 and 5.3. The test sample, extracts of Eucalyptus found to show significant activity against brine shrimp nauplii. In this bioassay, the mortality rate of brine shrimp was found to be increased with the increase in concentration of the test sample. So it was observed that there is a positive correlation between brine shrimp toxicity and cytotoxicity. The high values of LC_{50} 20.59, 17.5 and 8.27 μg/mL indicate the low cytotoxic effect of petroleum ether and ethyl acetate extracts of Eucalyptus respectively. Standard cytotoxic agent *bleomycin* was found to exhibit higher cytotoxicity giving lower LC_{50} values of 0.41μg/mL (table 5.4). Regression line of log dose of ME, PEE and EAE against brine-shrimp nauplii after 24h of exposure are shown in figure 5.1, 5.2 and 5.3 respectively.

Table 5.1 Probit mortality data of ME on brine shrimp nauplii

Dose µg/mL	Log dose	No of nauplii taken	No of nauplii killed	% of nauplii killed	Corr%	Emp probit	Expt probit	Wrk probit	Weight	Final probit
10	0.999	10	02	20	20	4.16	1.196	4.15	5.03	8.975
20	1.301	10	05	50	38	4.69	3.047	9.61	1.31	7.616
40	1.602	10	07	70	63	5.33	4.897	5.358	6.27	6.258
80	1.903	10	08	80	75	5.67	5.980	5.68	4.71	5.463
100	1.999	10	09	90	88	6.18	6.748	5.868	2.08	4.899

Table 5.2 Probit mortality data of PEE on brine shrimp nauplii

Dose µg/mL	Log dose	No of nauplii taken	No of nauplii killed	% of nauplii killed	Corr%	Emp probit	Expt probit	Wrk probit	Weight	Final probit
10	0.999	10	05	50	10	3.52	3.752	3.72	3.36	3.793
20	1.301	10	04	60	30	4.28	4.448	4.48	5.58	4.455
40	1.602	10	07	70	60	5.25	5.144	5.24	6.34	5.118
80	1.903	10	08	80	70	5.62	5.840	5.46	5.03	5.780
100	1.999	10	09	90	90	6.41	6.064	6.21	4.39	5.993

Table 5.3 Probit mortality data of EAE on brine shrimp nauplii

Dose µg/mL	Log dose	No of nauplii taken	No of nauplii killed	% of nauplii killed	Corr%	Emp probit	Expt probit	Wrk probit	Weight	Final probit
10	0.999	10	06	40	10	3.12	3.752	3.72	3.36	3.793
20	1.301	10	04	60	30	4.18	4.448	4.48	5.58	4.455
40	1.602	10	07	70	60	5.24	5.144	5.24	6.34	5.118
80	1.903	10	07	70	70	5.12	5.840	5.46	5.03	5.780
100	1.999	10	09	90	90	6.52	6.064	6.21	4.39	5.993

Table 5.4 LC_{50} values after probit transformations of the mortality data of the extracts and *bleomycin*

Compounds	LC_{50} (µg/mL)	95%Confidense Limit(µg/mL) Lower-Upper		Regression equation	λ^2	df
ME	17.5	14.391	33.56	Y=2.173+2.0761X	0.173	3
PEE	20.59	14.39	29.46	Y=2.985+1.602X	1.548	3
EAE	8.278	3.250	20.100	Y=4.015+1.0690X	1.341	3
Bleomycin	0.41	0.27	0.62	Y=3.16+2.99X	0.62	2

Figure 5.1 Regression line of log dose of ME against brine-shrimp nauplii after 24h of exposure

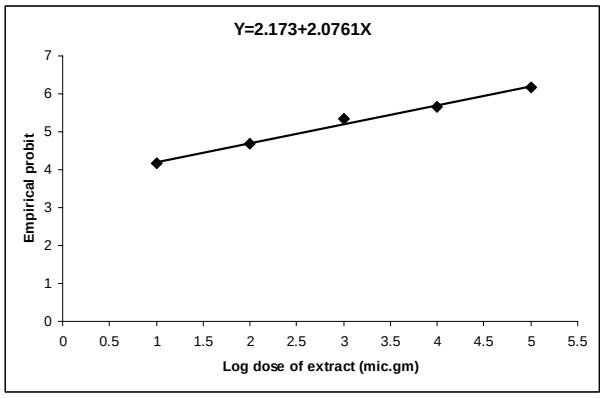


Figure 5.2 Regression line of log dose of PEE against brine-shrimp nauplii after 24h of exposure

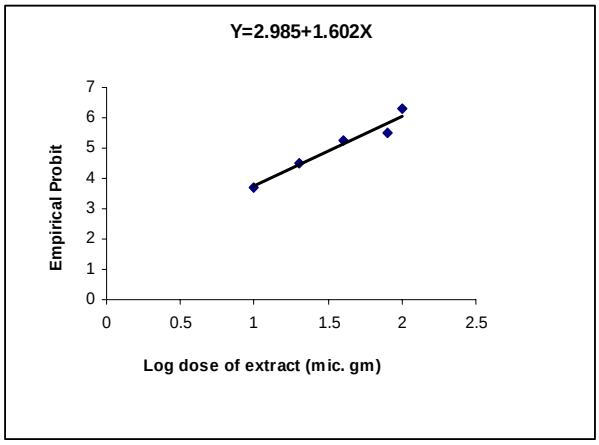
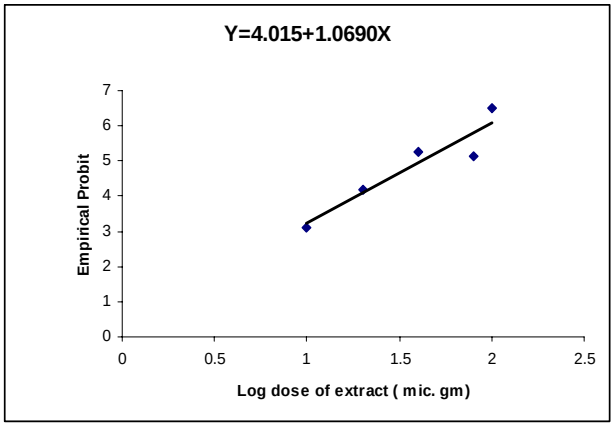


Figure 5.3 Regression line of log dose of EAE against brine-shrimp nauplii after 24h of exposure



DISCUSSION & CONCLUSION

For phytochemical investigation, cold extraction of stem bark of *Eucalyptus camadulensis* with methanol gave crude methanol extract (ME). This crude methanol extract was successively fractionated with petroleum ether (40^{o}-60^{o}C), and ethyl acetate and designated as PEE and EAE respectively. On TLC screening, it was found that the compounds of petroleum ether fraction (PEE) of ME were finely separated by the solvent system of n-hexane and ethyl acetate in the proportion of 9:1. On the other hand, the compounds of ethyl acetate fraction (EAE) of ME were finely separated by the solvent system of methanol and chloroform in the proportion of 3:7. Results from TLC profile and chemical test indicated the presence of two compounds PEE-1 and PEE-2 in the PEE fraction which were saponins and steroids respectively (Table 2.2) Two other compounds (EAE-1 and EAE-2) in EAE fraction were found to be terpenoides and flavonoids (Table 2.2) respectively. After column chromatography of EAE (methanol : chloroform = 3:7) of *Eucalyptus camaldulensis*, an amorphous powder from 39-45 fractions EC-1; was isolated (m.p., 104-106°C) giving a single spot on TLC screening. NMR spectral data analysis and literature[65] conformed that compound EC-1 is a simple mono-terpenoides namely *p*-menth-1-ene-4,7-diol, which has been reported for the first time from *Eucalyptus camaldulensis.*

ME, PEE and EAE showed dose dependent cytotoxic activity against brine shrimp nauplii. Among the extracts EAE showed lowest LC_{50} value (8.278) and PEE showed somewhat higher LC_{50} value (20.59) after 24 hours of exposure which indicated that EAE has the highest toxicity (Table 5.4).

The anticancer activity was studied against Ehrlich ascites carcinoma (EAC) cells (*in vivo*) by using Swiss albino mice as experimental animals. A comparative study was made with a highly effective clinically used antitumor agent "*bleomycin*". The parameters studied were inhibition of cell growth, increase of life span of EAC cell bearing mice and reduction of tumor weight. *In vivo* tumor cell growth inhibition was observed with *Eucalyptus camaldulensis* at various doses (25 mg/kg i.p., 50 mg/kg i.p., and 100 mg/kg i.p.). Maximum cell growth inhibition (about 96 %) was found after treatment at dose 100 mg/kg (i.p.) with EAE. *Bleomycin* at dose 0.3 mg/kg (i.p.) on the other hand inhibited the cell growth by 88 % (Table 3.1).

In vivo the mean survival time (MST) of the untreated tumor bearing mice was 21.3 days. With the treatment of PEE and EAE of Eucalyptus, this value increased significantly. About 79.34 ($p<0.01$) and 71.36% ($p<0.01$) enhancement of MST were found at the dose of 100 mg/kg (i.p.) for PEE and EAE of Eucalyptus respectively. On the contrary, *bleomycin* at the dose of 0.3 mg/kg (i.p.) increased this value to 90.14%. Methanol extract enhanced life span to about 65.23% (Table 3.2). The tumor growth rate was found to be retarded dramatically to 32.15, 22.5 and 19.6% at the dose of 100 mg/kg (i.p.) with ME, PEE and EAE respectively after 20 days as against 75.34% without any treatment. With *bleomycin* (0.3 mg/kg, i.p.) the growth rate also retarded (22 %) significantly (Figure 3.2a-3.2c).

As the extracts enhanced macrophages and other peritoneal cells remarkably, so it is speculated that they kill or destroy tumor cells by boosting cell mediated tumor immunity of the host.

The hematological parameters of both treated and normal mice were examined. In EAC cell bearing mice all the parameters such as hemoglobin, WBC, RBC were found to be significantly changed as compared to those of the normal mice. These parameters were found to be returned back towards normal values when treated with ME, PEE and EAE at the dose of 100mg/kg (i.p.). Mild host toxicity was also found with these extracts but it recovered back within a few days (25 days). This indicates that, the plant extracts have practically no long term toxic effect to the host. The high LD_{50} values 1120 mg/kg (i.p.), 1050mg/kg (i.p.), 1250mg/kg (i.p.) of the ME, PEE and EAE of Eucalyptus were also in accordance of the fact.

Biochemical parameters like glucose, cholesterol, TG, urea, bilirubin, GOT, GPT ALP etc. of the normal mice when treated with these extracts showed some extent of increase due to mild hepatotoxicity during the treatment period but they also became normal after completion of the treatment schedule. So the slight host toxic effects observed in mice during treatment time are mostly reversible. This means that, the treatments of the extracts do not cause any acute or permanent damage to the liver or other organs.

Histopathology of mice organs (liver, kidney, heart, lung, spleen and brain) after treatment with the extracts of Eucalyptus at dose 100mg/kg (i.p.) was also examined. Since no major abnormality was found, it may be assumed that the plant extracts have no adverse effect on cellular structures.

EAC cells treated with EAE showed nuclear condensation, fragmentation, cell membrane blebbing, apoptotic bodies etc. under the fluorescence microscope which implies that the extract induced EAC cells apoptosis[95-96]. Propidium iodide (PI) staining of EAC cells also indicated the late phase apoptosis induction after treatment with the extract[97-98] (Figure 3.3).

The integrity of the DNA was also assessed by agarose gel electrophoresis. DNA isolated from cells showed a ‘‘ladder’’ pattern in apoptosis[76]. Genomic DNA isolated from treated and untreated cells showed apoptotic pattern in agarose (1.5 %) electrophoresis (Figure 3.5). These characteristics of ladder like DNA band in the gel, further confirmed the induction of apoptosis in EAC cells by EAE treatment.

To understand the involvement of specific signaling pathway triggered by EAE in EAC cells, the specific inhibitors of caspase-8 and caspase-3 were used. Results obtained from the experiment showed that, EAC cells pretreated with caspase-8 inhibitor (caspase-8 blocked/inactivated and caspase-3 remain active) exhibited 67 % ($p<0.01$) growth inhibition in comparison to control. Cells pretreated with caspase-3(caspase-3 inactive and caspase-8 active) showed very little cell growth inhibition (Figure 3.4). This result leads one to conclude that caspase-3 mediated signaling pathway is involved in EAC cells apoptosis induced by the EAE of *Eucalyptus camaldulensis*[99] treatment. The anticancer activity and cytotoxic effect of EAE of *Eucalyptus camaldulensis* may be due to the presence of the isolated compound (*p*-menth-1-ene-4,7-diol) but this assumption has not been verified here.

CONCLUSION

In the light of above observations, it can be concluded that the extracts of Eucalyptus show potential anticancer activity through apoptosis and boosting host antitumor immunity and may be considered as one of the promising resource in cancer chemotherapy. However before assuming so, it is necessary to carry out extensive research works in advanced level with other cell lines and also with higher animal models in order to get novel drug for cancer treatment.

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