EVALUATION OF THE ANTIOXIDANT AND *IN VIVO* ANTICANCER ACTIVITY OF *Excoecaria agallocha* BARK EXTRACT

By

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A thesis submitted to the Department of Mathematics and Natural science in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

Department of Mathematics and Natural Sciences
BRAC University
October 2019

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis/project titled "Evaluation of the Antioxidant and *in vivo* Anticancer activity of *Excoecaria agallocha* bark extract" submitted by Sharmin Sultana (Student ID 18176002) of fifth semester, 2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science on October 24, 2019.

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Ethics Statement

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Abstract

Mangrove ecosystems have been known to possess diverse secondary metabolites and a good number of bioactive compounds of pharmaceutical importance have been reported from the mangrove plants. Mangrove plants are biochemically unique, producing wide array of natural products with unique bioactivity. This study was undertaken to evaluate the *in vitro* antioxidant and *in vivo* anticancer activity of *Excoecaria agallocha* (bark) which is a mangrove plant. *E. agallocha* possess various therapeutically important properties and their different parts have been experimented for antioxidant and anticancer activities in various studies. The objective of this study was to evaluate the bark extract of *E. agallocha* and to find their antioxidant content and also *in vivo* anticanceractivity in mice model. For this purpose, dried powdered bark of *E. agallocha was* extracted by kupchan method of solvent-solvent partitioning and after that polarity band was observed by Thin layer of Chromatography (TLC). In the*in vitro* antioxidant activity test, the extracts were tested for CUPRAC reducing capacity, total antioxidant capacity, total phenolic content and total flavonoid content. Where, Methanolic bark extract showed highest antioxidant content than other solvent extracts. After that anticancer activity was done in mice model, where comparison of the body weight among the groups of mice and hematological parameters was measured by analyzing clinical tests. In sub-chronic toxicity study, methanolic bark extracts of *E. agallocha* did not produce any significant changes in body weight of mice model when compared to both control groups (negative and positive) of mice. Moreover, there was significant rising of serum SGPT level during the treatment with the plant extract at a drug 400 mg/kg, but no significant changes were observed with 200 mg/kg dose, indicating no acute or sub-chronic toxicity of the extract. In conclusion, *E. agallocha* bark extract can be considered as a potential anticancer compound which should be validated from other animal model trials. Further studies are required to fractionate the extract, to identify the bioactive compounds, and which may be directed to carry out *in vivo* studies of its medicinal active components.

Keywords: Kupchan method; Thin layer Chromatography; Antioxidant activity; Anticancer activity; Hemotological parameters.

Dedicated

To

My amazing parents

Acknowledgement

First and foremost, I express my deepest gratitude to almighty Allah for endowing me with health, patience, protection and faith in all aspect of my life. During the time of my study I have worked with many people for whom I have great regard, and I wish to extend my warmest thanks to all those who have helped me to complete the thesis.

I am grateful to Professor Dr. A F M Yusuf Haider, Chairperson, MNS department for allowing me to pursue my postgraduate studies in the department of MNS and for his constant guidance and help throughout my entire period of study in thedepartment.

I am overwhelmed to express my respect, sincere gratitude and heartfelt thanks to Dr. Chowdhury Rafiqul Ahsan, Professor, Department of Microbiology, University of Dhaka for his inspiration, constructive criticism, affectionate guidance, permissions to work in various laboratories in order to get my work done, positive attitude and specially for his scholastic knowledge in helping me to complete my thesis paper. Without him, my study and research would not be complete. I owe him my deepest and utmost gratitude.

I would like to convey my indebtedness to Dr. Mahboob Hossain, Professor, Microbiology Discipline, Department of Mathematics and Natural Sciences, BRAC University, Dhaka, for his inspiration, prudent advice, and immense patience in guiding me and for giving me the opportunity to work whole heartedly.

I would like to convey my heightened appreciation to all my respected teachers of the Department of Mathematics and Natural Sciences, BRAC University, for their academic counsel and encouragement.

I would particularly like to express my deepest thanks to Akash Ahmed, Lecturer, who provided me with constant knowledge, his valuable time and tolerance towards my work, sound advice and good company throughout the time of my the study. I gratefully acknowledge him for all that he has done for me.

I owe a debt of gratitude to Dr. Dr. Md. Hossain Sohrab, Principle Scientific Officer, Phytochemical laboratory, BCSIR, for allowing me to work in his laboratory, use their services with complete comfort and helping me complete my work in peace.

I owe a great debt of gratitude to Professor Masum Shariar, Department of Pharmacy, Jahangirnagar University, Savar, Dhaka, for his complete guidance, knowledge and opening his laboratory and allowing me to work with specialized equipment's without which my work would remain incomplete.

I am also grateful to the people of each of the laboratories I have worked in. I thank them from the bottom of my heart for their support, and concerns. I am grateful to my loving friends, who constantly encouraged me and pushed me to get through whenever I felt like giving up.

Finally, I have no words to thank my Mother, who made me get admission for my Master's Program before she departed, her love and blessings are still upon me. My Father, without whom I wouldn't be here where I stand today, I thank him for his love, care, encouragement, the biggest pillar of support anyone could ever give me and for always being there, my brother, who never gave up supporting me throughout my time of research and my family members for their unconditional love and support through all the hassles and unavoidable circumstances and helping me pull through with ease.

The Author

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BRAC University, October
2019.

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List of Acronyms

DMSO	Dimethyl Sulfoxide	
SGOT	Serum Glutamic Oxaloacetic Transaminase	
SGPT	Serum Glutamic Pyruvic Transaminase	
ALT	Alkaline Phosphatase	
Nc.	Neucuproine	
BHT	Butylated Hydroxytoluene	
Mo	Molybdenum	
GAE	Gallic Acid Equivalent	
HEPA	High efficiency particulate air	
PBS	Phosphate buffered saline	
NH	N-Hexane	
PEE	Petroleum ether extract	
ME	Methanol Extract	
CHE	Chloroform extract	
AA	Ascorbic Acid	
GA	Gallic Acid	
QUER	Quercetin	
WHO	World Health Organization	
UV	Ultraviolet	
ml	milliliter	
µl	microliter	
mg	milligram	
µg	microgram	
R^2	Co-efficient of Determination	
Lymph	Lymphocyte	
Mon	Monocyte	

Eosin	Eosinophil	
Baso	Basophil	
Neu	Neutrophil	
VS	Vincristine Sulfate (Criston 2)	

CHAPTER 1
Introduction

1.0 Introduction

Natural products have been recognized as a source for medicines since ancient times across the world for treating and preventing human diseases (Cragg et al., 1997). They have been derived from various sources including plants, microorganisms, marine organisms, vertebrates and invertebrates (Newman et al., 2000). Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural products still make an enormous contribution to drug discovery today. Natural sources possess chemical diversity and therefore serve as an important reservoir of bioactive leads in the development of new drugs by providing novel templates for new drugs, as well as patterns for structural modifications to produce more potent and safer drugs (Cragg et al., 2011).

Natural sources are limitless inspiration for novel drug development because there are many promising drug candidates available (Newman and Cragg, 2012). Among these sources "phytochemicals" (derived from plants) have been the single most productive source of leads for the development of drugs. Plants synthesize these chemicals for their particular needs, mainly in their defensive roles and they may become a base and natural blueprint for the development of new drugs. These biologically active compounds would provide selective ligands for disease-related targets and influence the disease-related pathways and eventually shift the biological network from disease status to the healthy status (Clardy and Walsh, 2004;Gu et al., 2013). At least 12,000 such compounds have been isolated and a number estimated to be less than 10% of the total (Lai and Roy, 2004;Tapsell et al., 2006). Chemical diversity of these chemicals may provide the core scaffolds for future drugs. The past few years, however, have experienced a renewed interest in the use of phytochemicals and more importantly their role as a basis for drug development. Approximately one-half of all licensed drugs that were registered worldwide in the last 25 years period were natural products or their synthetic derivatives (Newman et al., 2007).

Natural products and their derivatives represent over 60% of all drugs clinically used worldwide where natural products from medicinal plants alone contribute to 25% of total drugs (Gurib-Fakim, 2011). Natural products and related drugs are reportedly used for example as antibacterial, anticancer, anticoagulant, antiparasitic and immunosuppressant agents to treat 87% of all categorized human diseases (Newman et al., 2003). More than 28% of new chemical entities introduced into the market are derived from natural products (Xiong et al., 2013).

Approximately, 80% of 122 pure compounds on the market derived from 94 plant species were originally used for the same or related ethno medical purposes (Cragg and Newman, 2013). Plant studies indicate that there are about 250,000 to 350,000 plant species identified so far , and of them about 35,000 have been used for medicinal purpose across the world (Kong et al., 2003).

Plants have been utilized as medicines for centuries. Still, plants remain as the principal source of new drugs, new lead compounds and new chemical entities. Plant-based medicinal systems play a significant role in the World health Organization (WHO), about 65% of the world's population and 80% of developing countries population depend primarily on about 85% of plant derived traditional medicines (Craggand newman, 2013). From the early steps of human civilization the use of medicinal plants has been recorded in various parts of the world, especially in India, China, and other Asian countries. About 2000 plant species including their medicinal properties are listed in the *MateriaMedica* of traditional medicine in the Asian subcontinent (Ghani, 1998). Medicinal uses of plants play an important role in providing primary health care services to people in rural areas. A report from 2004 states that about 80% of the population in the third world countries rely on traditional medicines, mainly plant drugs, for their primary health care needs, whereas, the percentage of people using traditional medicines in developed countries such as Germany, USA, Australia and France, lies between 40-50% (Titz, 2004).

Antioxidants are most important agent obtained from various sources like plant parts, fruits etc. because they inhibit the initiation of lipid peroxidation, which is related to aging and the pathogenesis of diseases such as cardiovascular disorders, cancer, inflammation, and brain dysfunction (Ames, 1983; Shon *et al.*, 2004). Various epidemiological studies have suggested that consumption of fruit and vegetables is associated with reduced risk of cardiovascular diseases and cancers (Ethertone*t al.*, 2002). Bangladesh is abundant in supply of its typical fruits which possess functional characteristics such as antioxidant content, cytotoxicity content, antitumor content etc. Even though individual works of these plants' extracts of various parts exist, this document is the only one where all three plants' leaf extracts are evaluated to record their antioxidant value and cytotoxic content on cancer cells. People of Bangladesh are mainly poor and are in dire need of good but cheaper medicines for cancer. The fruit plants used in this research are all very cheap and are readily available in rural areas as well as the urban. The aim

of this study is to provide a document investigating the leaf extracts of these three fruit trees and provide suitable information regarding the experiments done.

According to the journal published in October 2018 by the department of oncology of Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh, cancer-related death rate in Bangladesh was 7.5% in 2017 and is expected to hit 13% in 2030. Cancer is a deadly disease all over the world. But unlike the first world countries, Bangladesh is far behind in treating and controlling it and is in need of alternative drugs which are cheaper and available in the country. There is 13 to 15 lakh cancer patients in Bangladesh as per the journal published by the oncology department of Bangabandhu Sheikh Mujib Medical University, with about 2 lakh are being newly diagnosed with cancer each year. In the near future, the increase in population will result in an increase in the number of cancer patients in Bangladesh.

The treatment of cancer currently includes surgical removal of tumor, radiotherapy, chemotherapy, chemo radiotherapy and targeted therapy. Anticancer drug in the form of chemotherapy are the major therapeutic strategy against cancer. The use of anticancer drugs, is often more beneficial when used in conjunction with radiation therapy or surgery. Cytotoxic drugs are the mainstay of cancer chemotherapy and it is therefore important to discover novel cytotoxic agents with diverse activity and toxicity (Guilbaud et al., 2001). The main classes of anti-cancer drugs that exist today are alkylating agents, anti-tumor antibiotics, anti-metabolites, spindle poisons and kinase inhibitors. A number of plant derived anticancer drugs are currently in use in the treatment of cancer such as vincristine, vinbalstine, etoposide, teniposide, paclitaxel (taxol), docetaxel (taxotere), campotothecin, homoharringtonnine and elliptinium(Mans et al., 2000;Cragget al., 2005;Shoeb, 2006). Cancers are capable of developing resistance to the drugs used in chemotherapy; this coupled with an increased cancer-related death rate means that there is an ever increasing demand for new anticancer agents.

The term mangrove is used to designate an intertidal wetland ecosystem which has a special association with animals and plants which proliferate luxuriantly in the coastal areas and rivers. Mangroves are also designated as halophytic (salt loving) and salt resistant marine tidal forests, which consists of trees, shrubs, palms, epiphytes, ferns, grasses and are associated together in

stands of groves (Premanathan et al., 1999;Bandaranayake, 2002). Mangrove and mangrove associates have long been widely used medicinal purposes throughout the world. Plants of mangrove origin have been a source of several bioactive compounds and have been used in folklore medicines. There are reports of extracts from plants of mangrove origin to have proven activity against human, animal and plant pathogens. The diversity in activity of these plants could be due to the peculiar environment (high moisture, large tidal difference, high salinity, an abundance of living organisms and insects etc.) in which they exist, producing stressful conditions, which might change their morphology, physiognomy and biosynthetic pathways to survive (Bandaranayake, 2002). Several novel bioactive chemical lasses have been characterized by mangroves, and therefore, they have clinical, toxicological and economic importance (Ravikumar, Muthuraja et al. 2010).

In this study mangrove species *Excoecariaagallocha*was collected from tidal forests in the coastal Sundarbans of Bangladesh. This plant has been chosen for scientifically validated the usage in traditional medicine or ethno medicine and investigate their potentiality for a number of bioactivities. The traditional uses of those plants as rheumatism, asthma, diabetes, treatment wounds, stop bleeding, fish poison and skin disease.

Therefore, the broad objective of this present study is to investigate the scientific basis of the medicinal uses and subsequently evaluating the biological potential of these plants. The following objectives were considered:

- Determination of *in vitro* antioxidant properties of *E. agallocha*bark extractsdissolving in methanol, chloroform, N-hexane and Petroleum benzene solvents.

- Investigation of*in vivo* anticancer activity of*E. agallocha* bark extracts dissolving in methanolic solvent in mice model.

CHAPTER 2
Literature Review

2.1 Review on general concept and methodology

Terrestrial plants, marine organisms, microorganisms and fungi are rich sources of novel bioactive, structurally diverse compounds. Most biologically active natural products are secondary metabolites, which are organic compounds with complex structures. Plants, marine organisms and fungi synthesize and accumulate secondary metabolites such as polysaccharides, alkaloids, terpinoids, polyketides, glycosides, tannins and volatile oils. These metabolites are not directly involved in normal growth, primary metabolism, development or reproduction of organisms but improve survival of the organisms (such as pollination) (Williams et al., 1989, Mulabagal and Tsay, 2004). These metabolites often possess biological activity and can be potential curative agents in various diseases.

Bangladesh is a developing country and is one of the most densely populated countries of the world. According to Global cancer (GLOBOCAN), in Bangladesh 291,200 patient were diagnosed with cancer in a 5 year period (2003-2007). In Bangladesh, the top five malignancies in men and women occur in lung, breast, cervical, lip and oral cavity and esophagus. Lung cancer is estimated as being the most prevalent in men, whereas breast cancer is most prevalent in women. The top five pediatric cancers identified in Bangladesh are retinoblastoma, Ewing's sarcoma (a rare bone cancer worldwide), osteosarcoma, rhabdomyosarcoma, and nephrobalstoma(Uddin, et al. 2013).

Although new strategies in cancer prevention, diagnosis and treatment, such as gene therapy and cancer vaccines are emerging, new lead structures useful for the development of selective anti-cancer drugs are still desired. A large number of agents are available which are cytotoxic, but only a small number of compounds have been developed further into a potent anti-cancer drugs are mostly non-selective, thus affecting healthy and cancerous cells. Therefore, there is great potential to search for new anti-cancer drugs, delivering more effective and selective treatments of cancer (Uddin, 2011).

2.2 Pharmaceutical lead compounds derived from plants

Plants contain a wide range of secondary metabolites that can possess varied biological activities and therefore can be useful sources of medicinal or pharmaceutical products. Plants have been used for therapeutic purposes throughout human history and many modern pharmaceuticals were

derived from plants. In 1805, morphine was the first recorded pharmaceutically active compound which had been isolated from plant *Papaversomniferum*. The discovery of morphine initiated an era wherein drugs from plants began to purified, studied, and administered in precise dosages. Recently there has been growing interest in alternative therapies and the therapeutic use of natural products, especially in plant derived drugs and near about 25% of prescribed drugs in the world originate from plants (Alongi, 2001).

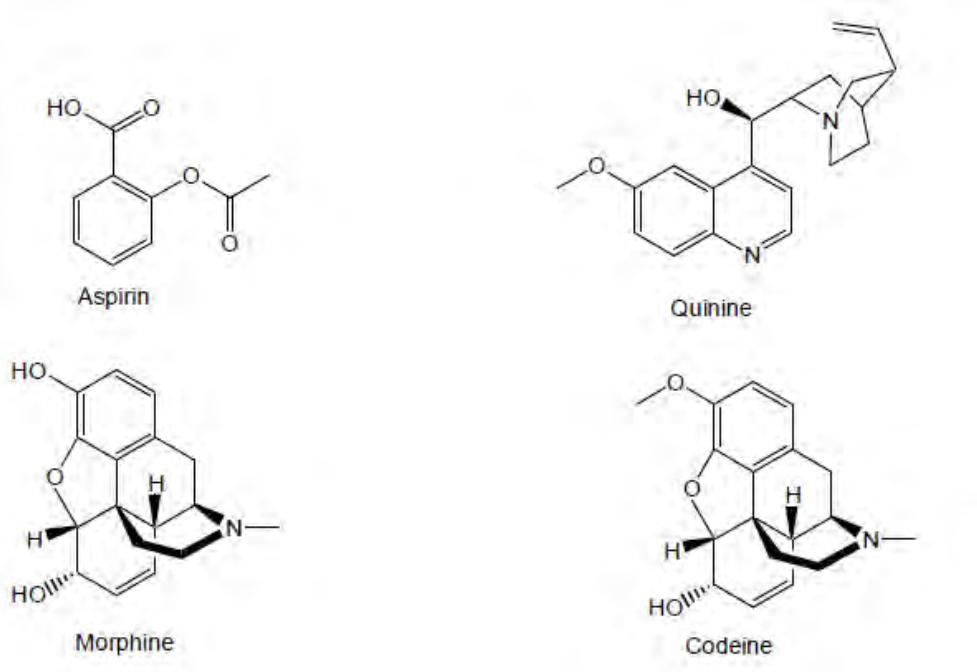


Figure 2.1: Pharmaceutical lead compounds structure.

2.3 Anticancer drugs from plants

Over the centuries, plants have been used in the treatment of cancer. Out of an estimated 250,000 plant species worldwide more than 3,000 have reported with significant anticancer potential (Mans et al., 2000;Kaur et al., 2011). Plant-based drug discovery has contributed and is contributing to the development of anti-cancer drugs as well as new anticancer lead compounds in clinical trials (Shah, Shah et al. 2013). For example, an extract of *Camptotheca acuminate* showed anticancer activity which lead to the isolation of an anticancer drug, camptothecin(Kaur et al., 2011). Over 60% of cancer patients use vitamins or herbs in their cancer therapy (Kaur et al., 2011). Plants with good immunomodulatory and antioxidant effects have potential anticancer

activity (Kaur et al., 2011). Naturally occurring anti-inflammatory or immunomodulatory plant metabolites provide anticancer activity through induction or suppression of specific cellular inflammatory activities. In addition, they also act as anticancer agents by scavenging reactive oxygen species (ROS). For instance, shikonin provides anticancer activity through inhibition of TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF). Whereas resveratrol from grapes induces anticarcinogenesis through antioxidant activity (Aravindaram and Yang, 2010).

However, there are numerous plants identified with anticancer potential that have not to date been investigated for the isolation and development of new anticancer compounds such as vincristine, etoposide, teniposide, paclitaxel, topotecan, homoharringtonine, flavopiridol, 4-ipomeanol and silvestrol coming from such studies (Mans et al., 2000;Kaur et al., 2011;Shah, Shah et al., 2013)

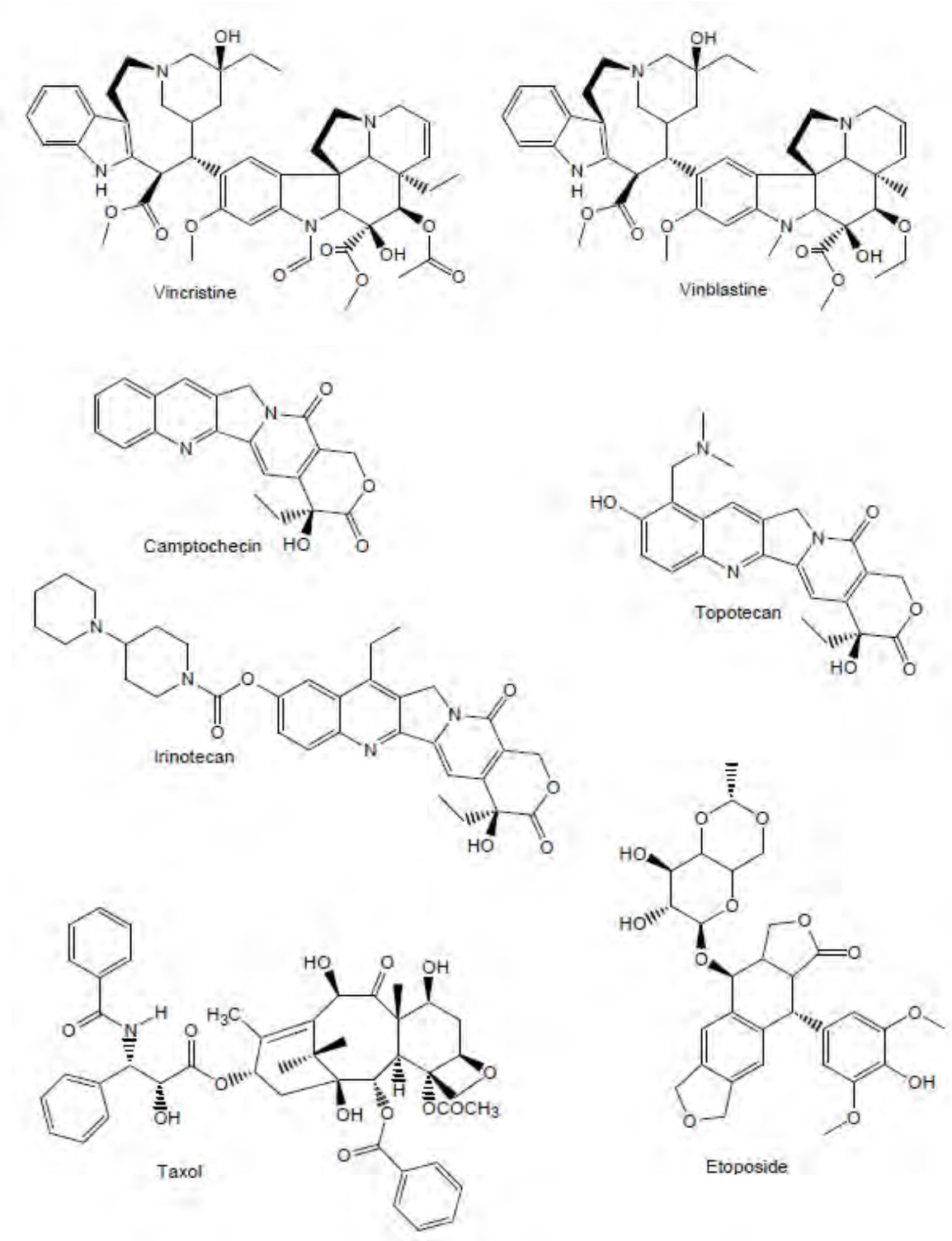


Figure 2.2: Some representative plant-derived anticancer drugs in clinical use or development.

2.4Literature Review of *Excoecariaagallocha*

General Information

E. agallocha L. (Euphorbiaceae), is a medium dioecious tree up to 15-20 m tall. Most of the names refer to its poisonous or blinding nature. The plant is well-protected by chemical defenses; these include diterpenoids, triterpenoids and flavonoids(Zou, 2006).

Scientific classification

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Eudicots

Order: Malpighiales

Family: Euphorbiaceae

Genus:*Excoecaria*

Species:*E. agallocha*

Binomial name:*Excoecariaagallocha*L.

Local name: Geoa or Gneoa or Genwa.

Common Names: Guan (Oriya), kampetti (Tamil), thilla (Telugu), buta-buta(Malay), milky mangrove, blind-your-eye mangrove and river poison tree.

Etymology

The genus name Excoecaria is derived from the Latin word ex-caecare which means 'to make blind'. Its Malay name of 'Buta-buta' also reflects this, meaning 'blind'.

Description

This is a small evergreen or deciduous tree that grows to 15 m tall with young leaves that are pink and old leaves that turn scarlet. Leaves are simple, 2.5-11 x 2-6 cm, elliptic and down-pointing with up curled sides. It is dioecious, with male and female flowers borne on different trees. Flowers are arranged in a catkin-like inflorescence. Male inflorescences are in 5-10 cm long while female inflorescences, if shorter, at 1.2-4 cm long. Among local mangrove plants, the drooping flowering shoot is a unique characteristic. Fruits are 3-shouldered capsules, about 7mm wide, resembling a miniature rubber fruit. Trees are either male or female. Male flowers form drooping tassels, while female flowers appear as shorter spikes. Pollinators such as bees commonly visit the flowers. The fruit is a small dark capsule.

Figure 2.3: Pictorial view of leaves and bark of *E. agallocha*.

Ethno medical Uses

The plant has been used in traditional medicine against rheumatism, leprosy, epilepsy, paralysis, conjunctivitis and dermatitis and for as a dart and fish poison (Patil et al., 2012). Parts of the plant possess medicinal properties and are used to treat hematuria and toothache (Simlai and Roy, 2013).

Biological investigation

Literature shows that the plant parts of *E.agallocha* have not evaluated in a great detail for the presence of a number of bioactivities.

A series of diterpenoid, triterpenoids derivatives and alkaloids were isolated from *E. agallocha* and some diterpenes extracted from the plant were also found to possess anti-tumor promoting activity. It is commonly used in folklore medicine to treat swollen hands and feet in leprosy, flatulence, epilepsy, and as an aphrodisiac agent. The milky latex exuded from the bark of *E. agallocha* has been used as a poison for fish by adding it to water and to poison arrowheads. Modern clinical trials show that the plant may have anti-HIV, anti-cancer, anti-bacterial and anti-viral properties. But the cytotoxic potential of *E. agallocha* has not been evaluated yet (Patil et al., 2012).

The brine shrimp assay is a simple and useful tool for the isolation of potentially cytotoxic compounds from plant extracts. It has been established that the cytotoxic compounds usually show good activity in the BSL assay, and this assay can be recommended as a guide for the detection of antitumor and pesticidal compounds because of its simplicity and cost-effectiveness. The extract of *E. agallocha* showed considerable brine shrimp toxicity with an LC_{50} value less than 20 μg/mL (Subhan et al., 2008).

Another study demonstrated a few biological activities such as neuropharmacological, antimicrobial and cytotoxicity effect of the ethanolic extract from the plant bark of Bangladeshi origin. Also potent antinociceptive and gastroprotective effect of the crude ethanolic extracts of bark from *E. agallocha* of Bangladeshi Sundarbans. In this study, the extract was found to possess potential effect on the CNS, exhibited significant antimicrobial activity and considerable cytotoxic effect on brine shrimps; however, the extract showed low level of toxicity in mice (Subhan et al., 2008).

The chemical characteristics of *E. agallocha* along with few other mangrove species from Bangladeshi Sundarbans demonstrating the dichloromethane, lignin, pentosane, α-cellulose etc. content in them (Mun et al., 2011).

E. agallocha is a milky mangrove widely distributed in Indian coastal regions. There are 20 different polyphenols, 15 terpenoids and more than 50 volatile derivatives were identified from leaves, stem, latex and root extract. Enormous number of compounds isolated from ethanolicextact of leaves. In conclusion, *E. agallocha* has huge amount of polyphenols and terpenoids, which was reported to have endocrine, epidemic and endemic disease control as anti-microbial, anti-cancer and anti-diabetic agent (Kaliamurthi and Selvaraj, 2016).

Phytochemical constituents

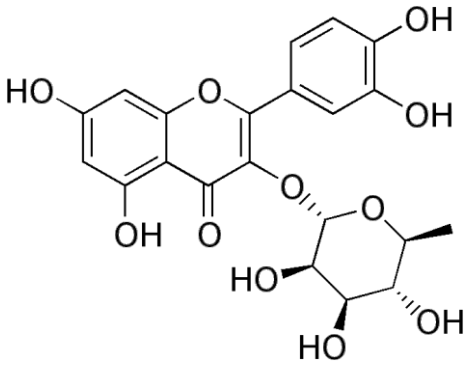
Phytochemicals are chemical compounds produced by plants, generally to help them thrive or thwart competitors, predators, or pathogens. The name comes from the Greek word *phyto*, meaning plant. Some phytochemicals have been used as poisons and others as traditional medicine.

Some phytochemicals are known phytotoxins that are toxic to humans; for example aristolochic acid is carcinogenic at low doses. Some phytochemicals are antinutrients that interfere with the absorption of nutrients. Others, such as some polyphenols and flavonoids, may be pro-oxidants in high ingested amounts. Non-digestibledietary fibers from plant foods, often considered as a phytochemical, are now generally regarded as a nutrient group having approved health claims for reducing the risk of some types of cancer and coronary heart disease.

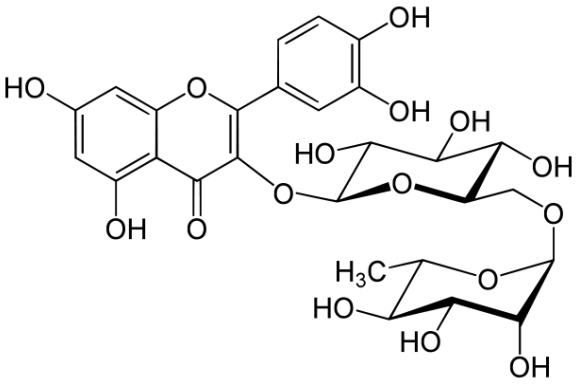
Phytochemical screening of the methanolic leaf and bark extract of *E. agallocha* indicate the presence of flavonoid, diterpenoid and triterpenoid classes of compounds, which have anti-cancer, antitumor and cytotoxic activities as shown in table 2.1.

Table 2.1: Isolated compounds of *E. agallocha* bark extracts.

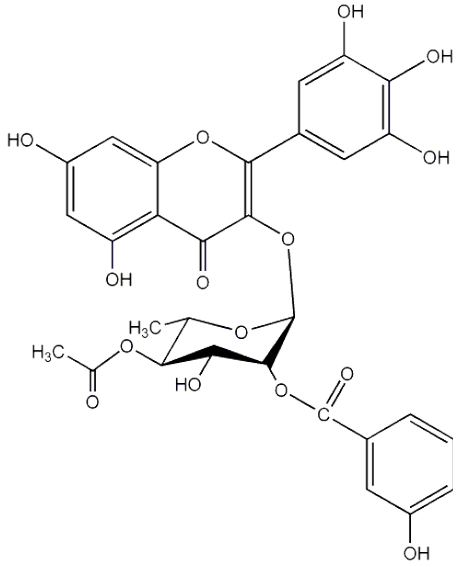
Sources	Classes	Compounds	References
Bark	Flavonoid	Quercetin-3-O-rutinoside	(Konishi et al., 1998;Tian et al., 2008;Kaliamurthi, 2016)
		Quercetin 3-O- α -L-rhamnoside	
		Kaempferol-3-O-(2-O-acetyl-α-L-rhamnopyranoside)	
		Kaempferol 3-O-α-L-rhamnopyranoside	
	Diterpenoid	Excoecarin A	
		Excoecarin G1	
		Excoecarin G2	
	Triterpenoid	Taraxerone	
		3beta-[(2E,4E)-6-oxo-decadienoyloxy]-olean-12-ene	
	Diterpenoid	2,3-secoatisane	
		Exoecarin B	
		Exoecarin C	
		Exoecarin D	
		Excoecarin E	
		Exoecarin F	
		Exoecarin H	



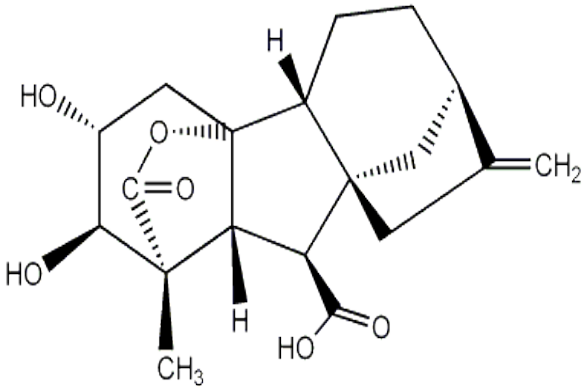
Quercetin 3-*O*- α -L-rhamnoside



Quercetin-3-O- rutinoside

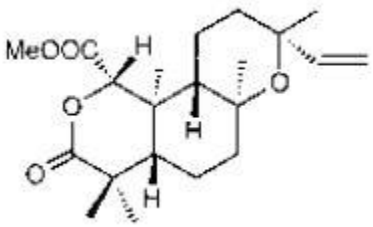


Kaempferol-3-O-(2-O-acetyl-α-L-rhamnopyranoside)

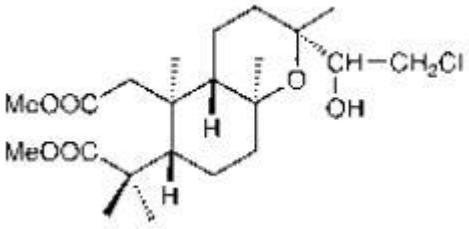


3 α, 18-dihydroxy-3 beta, 20-epoxy beyer-15-ene (Excoecarin D)

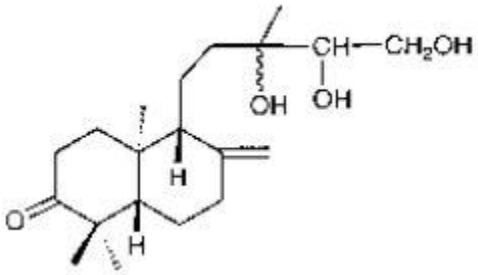
Figure 2.4: Structure of active chemical constituents of *E. agallocha* L.



Compound 1



Compound 2



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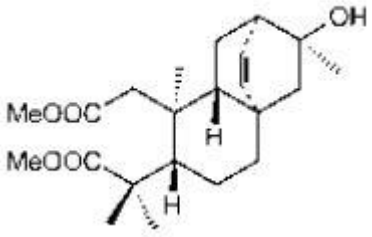

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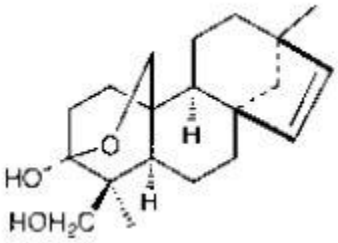

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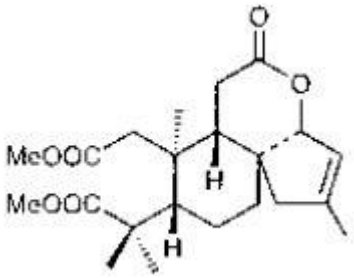
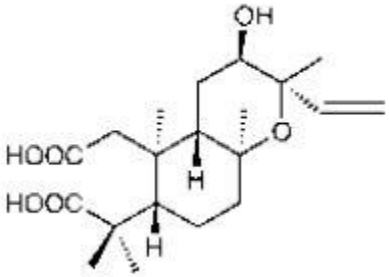
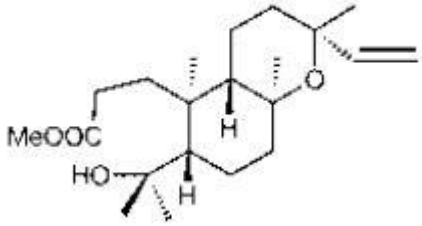


Compound 6



Compound 7



Compound 8

Figure 2.5: Diterpenoids, isolated from the Bark of *E. agallocha*.

2.5 Antioxidantactivity of *E. agallocha*

An antioxidant can be defined as: "any substance that when present in low concentrations compared to that of an oxidizable substrate significantly delays or inhibits the oxidation of that substrate". The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals (Young and Woodside, 2001). Free radical is constantly generated in all living cells and is a part of normal cellular function. However, excess free radical originating from endogenous or exogenous sources are responsible for aging and causing various human diseases. Free radicals cause oxidative damage to different molecules, such as lipids, proteins and nucleic acids and thus are involved in the initiation phase of some degenerative diseases. Research has shown that free radical mediated oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others. Antioxidants prevent free radicals from doing harm to our DNA, proteins, and cells by donating electrons tostabilizeandneutralizetheharmfuleffectsofthefreeradicals.Thisactionhelpsinprotecting the body from degenerative diseases. With that, the role of antioxidants has drawn much attention as a candidate to combat certain diseases and prevent the aging process. An antioxidant can be defined as: any substance that when present in low concentrations compared to that of an oxidizable substrate significantly delays or inhibits the oxidation of molecules, by inhibiting the initiation or propagation of oxidizing chain reactions (Khatoon*et al*., 2013).

A free radical is defined as any atom or molecule possessing unpaired electrons. Antioxidants are agents which scavenge the free radicals and prevent the damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). Antioxidants can greatly reduce the damage due to oxidants by neutralizing the free radicals before they can attack the cells prevent damage to lipids, proteins, enzymes, carbohydrates DNA. A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases (Kalita*et al*., 2013).

The antioxidant activities were determined by using the four following methods:

1. Cupric Reducing Antioxidant Capacity (CUPRAC).

2. Determination of Total AntioxidantContent.

3. Determination of Total PhenolicContent.

4. Determination of Total Flavonoid Content.

Cupric Reducing Antioxidant Capacity (CUPRAC) Principle

This assay is based on the changes in absorption characteristics of the neocuproine (Nc) copper (II) complex when it is reduced by an antioxidant. The reduction potential of the sample or standard effectively converts Cu^{+2} to Cu^{+1}. This reduced complex absorption is measured at 450nm. This is a colorimetric assay with the reagents changing colour from blue-green to yellow in the presence of an antioxidant (Apak*et al*., 2004).

Total antioxidant content Principle

The phospho-molybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, α-tocopherol, and carotenoids. The phospho-molybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and subsequent formation of green phosphate/ Mo (V) complex. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI) and the formation of a green phosphate/Mo (V) complex with maximal absorption at 695 nm (Prieto *et al.,* 1999).

$$Mo\ (VI) + e \longrightarrow Mo\ (V)$$

Total phenolic content Principle

Total phenolic content of all the extracts was evaluated with Folin-Ciocalteu method. Samples containing polyphenols are reduced by the Folin-Ciocalteu reagent by producing blue colored complex. The phenolic concentration of extracts was evaluated from a Gallic acid calibration curve. After incubation at 20°C for 60 min, the quantitative phenolic estimation was performed at 765 nm against reagent blank by UV Spectrophotometer (1650 Shimadzu, Japan). Total phenolic content was expressed as milligrams of Gallic acid equivalent (GAE) per gm of extract (Khatoon*et al*., 2013).

Total flavonoid content Principle

Determining the total flavonoids by using aluminum chloride method is based upon the formation of stable complex between aluminum chloride and keto and hydroxyl groups of flavones and flavonoids. For building the calibration curve, quercetin was used as standard. Various concentrations of standard quercetin solution were used to make a standard calibration curve (Pallab*et al*., 2013).

Literature review of anti-oxidant potential of *E. agallocha*

Studies have shown that the leaf (Patra*et al*., 2009; Poorna*et al*., 2012; Deepa*et al*., 2015; Laith*et al*., 2016) and bark (Subhan*et al*., 2008a; Hossain *et al*., 2009) extracts of *E. agallocha*possess significant antioxidant activities when assessed using well-established assays. Based on 2,2-diphenyl- 1-picrylhydrazyl (DPPH) free radical scavenging of different plant parts extracted with different solvents, the ranking was as follows: leaf > root > stem, and methanol > ethyl acetate > ethanol > chloroform (Sofia and Teresa, 2016). Similarly, studies have shown that leaf (Agoramoorthy*et al*., 2007; Vadlapudi*et al*., 2009; Raja *et al*., 2010; Laith and Najiah, 2014; Deepa*et al*., 2015; Laith*et al*., 2016) and bark (Subhan*et al*., 2008b) extracts of *E. agallocha*inhibited bacterial growth. Of interest was the inhibition of bacteria causing fish-related diseases (Laith and Najiah, 2014; Laith*et al*., 2016). One such study reported that the bark extract of *E. agallocha*(1−3 mg/ml) exhibited no antibacterial activity against bacteria from clinical isolates (Rajia*et al*., 2006). Recently, the antibacterial activity of *E. agallocha*bark

extract (500 µg/disc) (Shanmugapriya and Ramanathan, 2015) and of silver nanoparticles of *E. agallocha*fruit extract have been reported (Nagababu and Rao, 2017). Another interesting study was on the anti-quorum sensing and anti-biofilm properties of *E. agallocha*leaf extract on *Pseudomonas aeruginosa.*At 2 mg/ml, the leaf extract inhibited violacein production, biofilm formation includingthe motilitybehaviour of *P. aeruginosa* (Karuppiah, 2017).

2.6Anticancer activity of *E. agallocha*

Impact of *E.agallocha*on cancer chemotherapy

One of the major health issue all around the world is cancer. The major risk factors responsible causes of cancer are tobacco/alcohol consumption, preserved food products, family heredity, environmental pollution, sexual behavior, medicines and its treatment procedures. Then compared to other factors, alcohol consumption increases the occurrence of cancer at oral, esophagus, pharynx, stomach and liver region respectively. In India, the breast and cervical cancer are predominantly identified in women. Cancer is the second leading disease factor becauseof more death in United states of America. Siegel et al. estimated the death rate and various cancer sites (Oral cavity and pharynx, Digestive system, Respiratory system, Bones and joint, skin, breast, urinary system, eye, brain, endocrine system, lymphoma, myeloma) in both male and female subjects in United States of America.

2.6.1. In *vivo* model

Induction of Ehrlich ascites carcinoma(Devi et al., 1998)

Antitumor activity of the test compounds is determined using Ehrlich Ascites Carcinoma (EAC) tumor model in mice. The ascitic carcinoma bearing mice (donor) are used for the study, 15 days after tumor transplantation. The animals are divided into groups:

i. Normal mice
ii. Tumor-bearing mice
iii. Tumor-bearing mice treated with standard drug

iv. Tumor-bearing mice groups treated with test drug

The ascitic fluid is drawn using an 18-gauge needle with sterile syringe. A small amount is testing for microbial contamination. Tumor viability is determine by Tryphan blue exclusion test and cells are counted using haemocytometer. The ascitic fluid is suitably diluted in normal saline to get a concentration of 10^6cells/ml of tumor cell suspension. This is injected intraperitoneally to obtain ascitic tumor. The mice are weighed on the day of tumor inoculation and then once in three days thereafter. Treatment is started on the 10^{th} day of tumor inoculation. Standard (one dose) is injected on 10^{th} day intraperitoneally. The drug is administered from 10^{th} day for 5days intraperitoneally. After the administration of last dose followed by 18h fasting, six mice from each group are sacrifice for the study of antitumor activity and hematological parameters. The remaining animals in each of the groups are kept to check the Mean Survival Time (MST) of the tumor-bearing hosts. Antitumor effects of drug are assessed by observation of following parameters.

i. Percentage increase in weight as compared to day-0 weight
ii. Median survival time and increase in lifespan [% ILS]
iii. Hematological parameters

The human pancreatic cancer cell lines Miapaca-2, PANC-1, Capan-1 and BxPC-3 were used to test the anticancer activity of ethanol extract of stem of *E. agallocha*(Patil et al., 2012). The activity was determined by using MTS *in vitro* assay. The effects of this fraction of *E. agallocha* on the growth of various human pancreatic cell lines were tested under *in vitro* conditions using different concentrations (1, 3, 10, 30 and 100µg/ml) and cell survival after 48h of treatment.

Table 2.2: Cytotoxic activity of ethanol fraction of stem ethanol extracts of different Concentrations of *E. agallocha*(Patil et al., 2012).

Conc. of extract (µg/ml)	Percentage of Growth Inhibition			
	Cell Line			
	Capan-1	Miapaca-2	PANC-1	BxPC-3
1	0	8	0	0
3	0	19	0	0
10	58	59	0	0
30	73	73	0	0
100	81	79	35	36

From this table it is clear that, a high degree of inhibition over the growth of two cell lines- Capan-1 and Miapaca-2, which was significantly more than that on the other two cell lines- PANC-1, and BxPC-3. The IC_{50} values were calculated to be 4µg/ml and 7µg/ml for Capan-1 and Miapaca-2 respectively (Table 14). The inhibition was found to be dose dependent with greater inhibition at the highest concentration (100µg/ml). The cytotoxic activity was compared with the effect of flavopiridol (positive control), which showed significant cytotoxic activity on all of the four cell lines with IC_{50} values in the range of 0.03 to 0.11µg/ml.

Table 2.3: Mean IC_{50} values of ethanol fraction of stem ethanol extract of *E. agallocha* in human pancreatic cancer cell lines(Patil et al., 2012).

Cell Line	Mean IC_{50} (µg/ml)	
	***E. agallocha* ethanol extract**	**Flavopiridol**
Capan-1	4	0.08
Miapaca-2	7	0.04
PANC-1	>100	0.03
BxPC-3	>100	0.11

The IC_{50} values calculated by plotting cytotoxicity curves were 4µg/ml and 7µg/ml respectively which were <20µg/ml and hence the extract can be considered as 'active' according to National Cancer Institute's guidelines (Boyd, 1997).

So it is assume that, the stem extracts of *E. agallocha* has the potential to be developed as an anticancer drug. So, molecular studies are needed to elucidate the mechanism(s) of action of these extracts on cancer cells.

Reported anticancer compound:

Diterpenes from the wood of *E. agallocha*had been isolated and for searching potential anti-tumor-promoting effect both *in vitro* and *in vivo* tests were performed (Konoshima et al., 2001). Their inhibitory effects on the induction of Epstein - Barr virus Early Antigen (EBV-EA) in Raji cells were tested. Here, 12-O-tetradecanoylphorbol-13-acetate (TPA) was used as a promoter and 7, 12-dimethylbenz[a] anthracene (DMBA) was used as an initiator. Among these diterpenes, 4, 5, 7and 8 exhibited stronger inhibitory effects than that of Glycyrrhetic acid (an anti-tumor-promoting reagent). Compound 7 (secolabdane-type diterpinoid) exhibited strongest inhibitory effects of all these diterpenes.

In vivo Two-Stage Mouse Skin Carcinogenesis Test was performed in female ICR mice (6-weeks old) for investigating anti-tumor-promoting effect of compound 7. Here also, 12-O-tetradecanoylphorbol-13-acetate (TPA) was used as a promoter and 7, 12-dimethylbenz[a]anthracene (DMBA) was used as an initiator. Fifteen mice were divided in three groups. Each mouse was treated with DMBA for initiation. One week after initiation they were promoted by applying TPA and 1h before promotion Group II was treated with compound 7 and Group III was treated with Glycyrrhetic acid. In this experiment, compound 7 exhibited a significant inhibitory effect while comparing with positive control group I and the group treated with glycyrrhetic acid (group III). The inhibitory effect was determined by both the rate (%) of papilloma-bearing mice and the average number of papillomas per mouse.

Table 2.4: Rate (%) of papilloma-bearing mice (Konoshima et al., 2001)

Groups	Group I	Group II			Group III		
Weeks of promotion	10	10	15	20	10	15	20
Incidence of papillomas (%)	100	43	70	95	<20	60	95

Table 2.5: Average number of papillomas per mouse (Konoshima et al., 2001)

Groups	Group I			Group II			Group III		
Weeks of promotion	10	15	20	10	15	20	10	15	20
Average number of papillomas per mouse	4.8	8.1	9.1	2.6	5.0	5.8	<1.0	3.1	4.7

Results demonstrated that, compound 7 remarkably delayed the formation of papillomas as well as significantly reduced the number of papillomas. A secolabdane-type diterpenoid like compound 7 is the first example of an anti-tumor-promoter of chemical carcinogenesis, although several abietane- or rearranged lab Danetypediterpenoids were isolated from Pinaceous plants and their potential anti-tumor-promoting effects were reported(Kinouchi et al., 2000; Ohtsu et al., 2001; Ohtsu et al., 1999). Further studies are needed to find out the mechanism of the antitumor-promoting effect of this compound.

2.7 Vincristine Sulfate as positive control

Vincristine Sulfate Injection, USP (vincristine sulfate) is the salt of an alkaloid obtained from a common flowering herb, the periwinkle plant (*Catharanthusroseus*). Originally known as leurocristine, it has also been referred to as LCR and VCR. The molecular formula for Vincristine Sulfate, USP is $C_{46}H_{56}N_4O_{10}•H_2SO_4$. It has a molecular weight of 923.04.

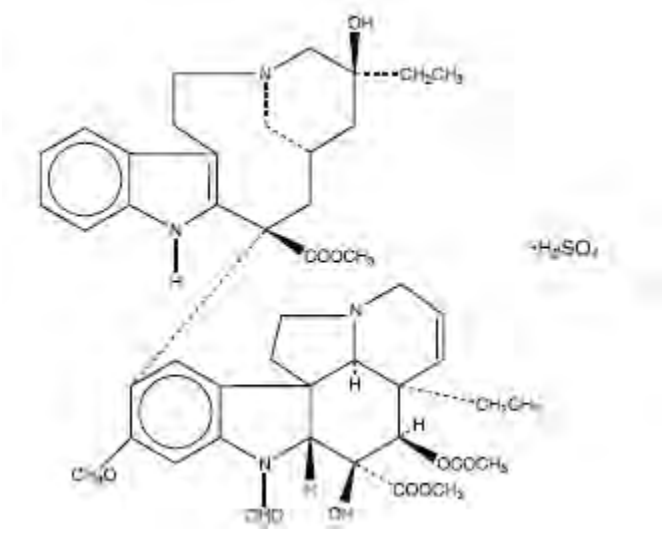


Figure 2.6: The structural formula of Vincristine Sulfate.

Vincristine Sulfate, USP is a white to off-white powder. It is soluble in methanol, freely soluble in water, but only slightly soluble in 95% ethanol. In 98% ethanol, Vincristine Sulfate, USP has an ultraviolet spectrum with maxima at 221 nm.

Vincristine Sulfate Injection, USP (vincristine sulfate) is a sterile, preservative-free, single use only solution available for intravenous use in 2 mL (1 mg and 2 mg) vials. Each mL contains 1mg Vincristine Sulfate, USP, 100 mg mannitol and Water for Injection, USP q.s. Sulfuric acid or sodium hydroxide have been added for pH control. The pH of Vincristine Sulfate Injection, USP (vincristine sulfate) ranges from 4.0 to 5.0. At the time of manufacture, the air in the containers is replaced by nitrogen.

CHAPTER 3
Materials and Methods

3.0 Materials and Methods

3.1 Plant extracts preparation

Collection and authentication of plant material

In order to investigate *in vitro* antioxidant and cytotoxic potential, the medicinal plant *E. agallocha*was chosen. As no significant amount of work had been conducted on this plant, thereby the plant was decided to be chosen for investigating the antioxidant and cytotoxic potential by the assistance of comprehensive literature and also its availability.

Chemical investigation of *E. agallocha*

Name of the plant: *E. agallocha*
Family: Euphorbiaceae
Plant part: Bark

The plant part of *E. agallocha*was collected fromHodda substation, Nolian forest, East zone of the Sundarbans, Khulna, Bangladesh on 11th October, 2018.

Extraction procedure

The following processes are involved in the extraction procedure.
A. Plant material preparation and drying (3-steps)
B. Extraction process (5-steps)

Preparation of the plant material

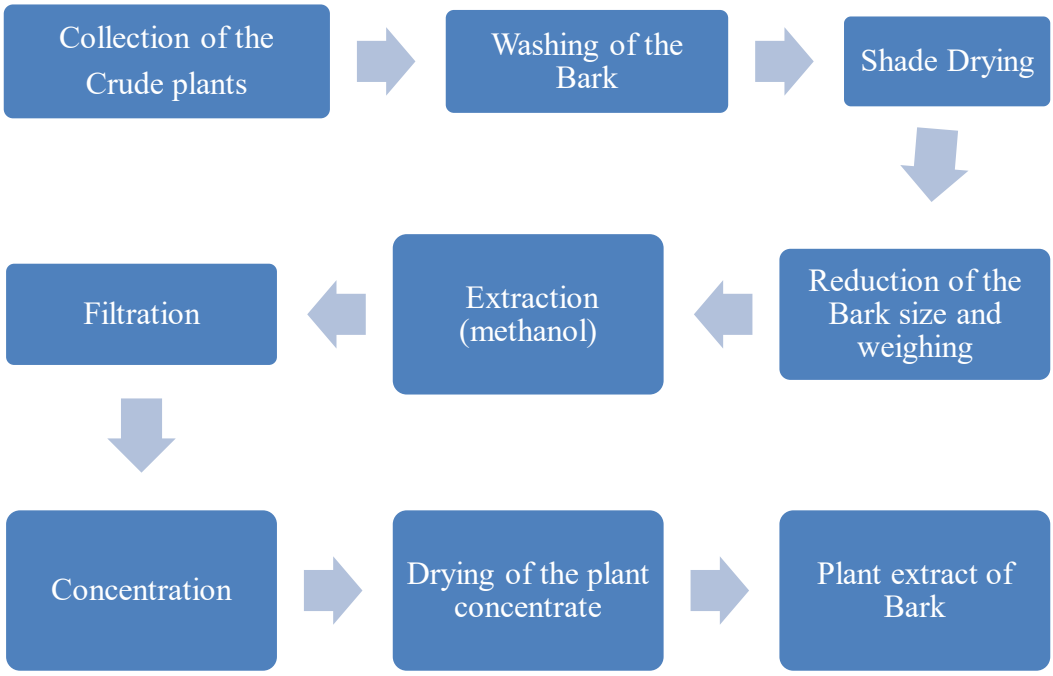


Figure 3.1: Flow chart of the extraction procedure of the crude medicinal plant.

Plant material preparation and drying

This process occurs in two steps.

a. Washing

b. Shade drying

At first, the bark was separated and peeling away from the stem of the plant. After that, it was washed carefully with clean water in order to remove any dirt. After that, the plant was shade-dried for several days until it is completely dried. Then, the dried bark was used in the extraction process.

3.2 Extraction process

It occurs in five steps.

Size reduction and weighing

1. Size reduction and weighing

2. Extraction
3. Filtration
4. Concentration
5. Drying of the plant concentrate

Size reduction and weighing

The clean dried bark was grounded by using a high capacity grinding machine in order to make a coarse powder. After that, the coarse powder poured in an air-tight plastic container in order to avoid the risk of cross-contamination. Thereafter, the plastic container was properly labeled with all the necessary information and then it was kept in dried, cooled and dark place until it is needed for further investigation.

Then, the powder of the bark was poured into a beaker and the amount was determined by weighing. Finally, the amount was recorded.

Extraction

Extraction of plant materials are performed by maceration process. Methanolicsolventwas used for the extraction of the plant material.

Here 1kg powdered bark of*E. agallocha*were soaked in methanol 3.5 L in for five days at room temperature (22-25°C) and agitation was performed occasionally to yield 156.2 gm of gummy extracts.

Filtration

At first, the contents of the beaker were decanted by using a Whatman filter paper. It was then filtered after five days of maceration process.

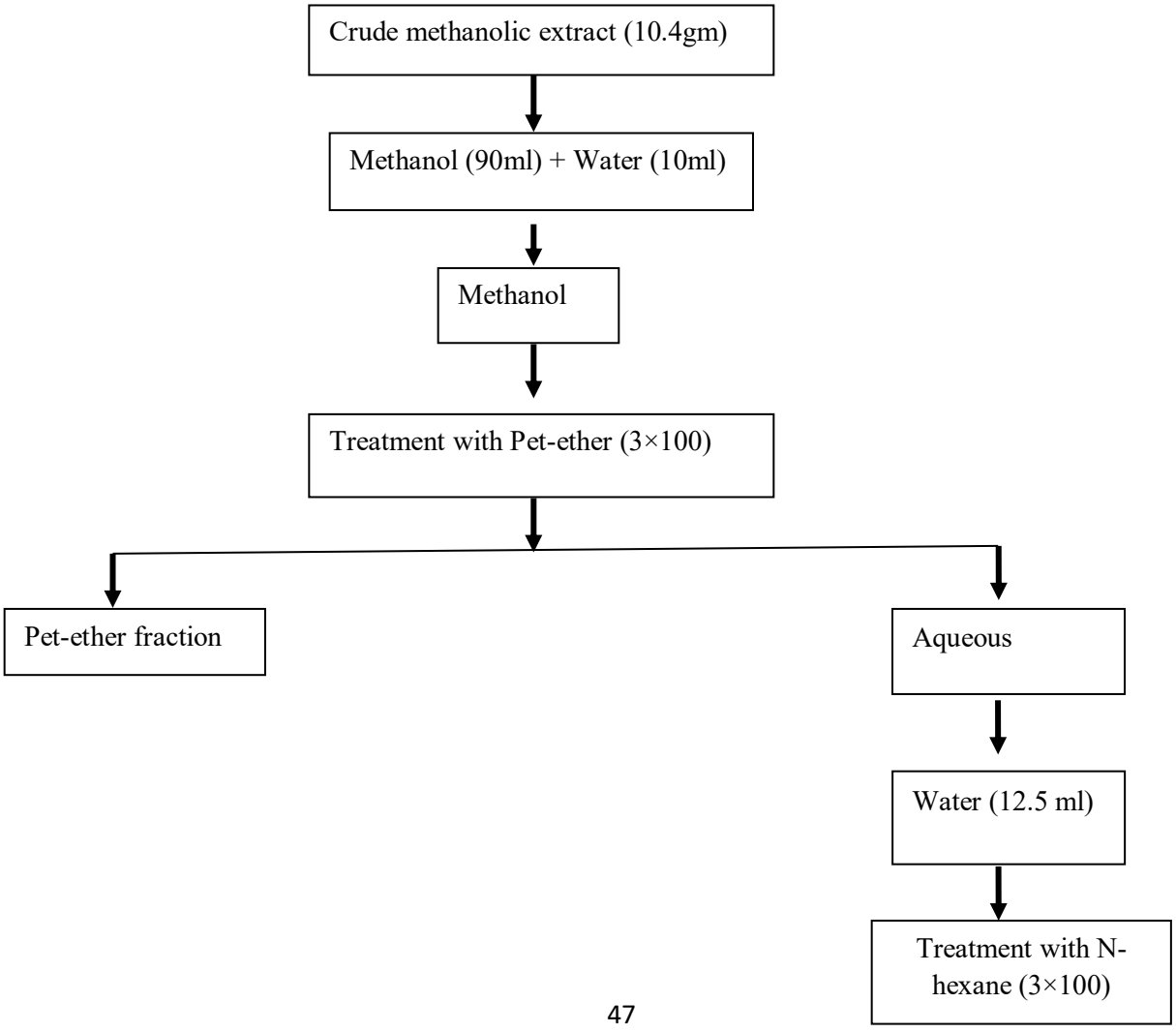
Concentration

After filtration, the filtrate was collected and then concentrated with the help of a rotary evaporator (Heidolph) at 100 rpm at 65°C, until concentrated extract was produced. After that, in order to dry under LAF (Laminar Air Flow), the mixture was transferred onto the petri-dishes.

Then the petri-dishes were kept under Laminar Air Flow (LAF).When the extract was successfully dried, the petri-dishes were safely covered with aluminum foil and it was kept in the refrigerator for further investigation.

3.3 Solvent-solvent partitioning

Solvent-solvent partitioning of the crude concentrated methanolic extract was done using the protocol designed by Kupchan and modified by wagenen et al. (1993). The extract was dissolved in 90% methanol. It was extracted with petroleum ether (60-80)ºC, then with carbon tetrachloride (CCl_4), and finally with chloroform ($CHCl_3$). The whole partition process is schematically shown in figure 3.2. All the fractions were concentrated with a rotary evaporator at low temperature (40-50)ºC and reduced pressure. These were collected for further analysis.



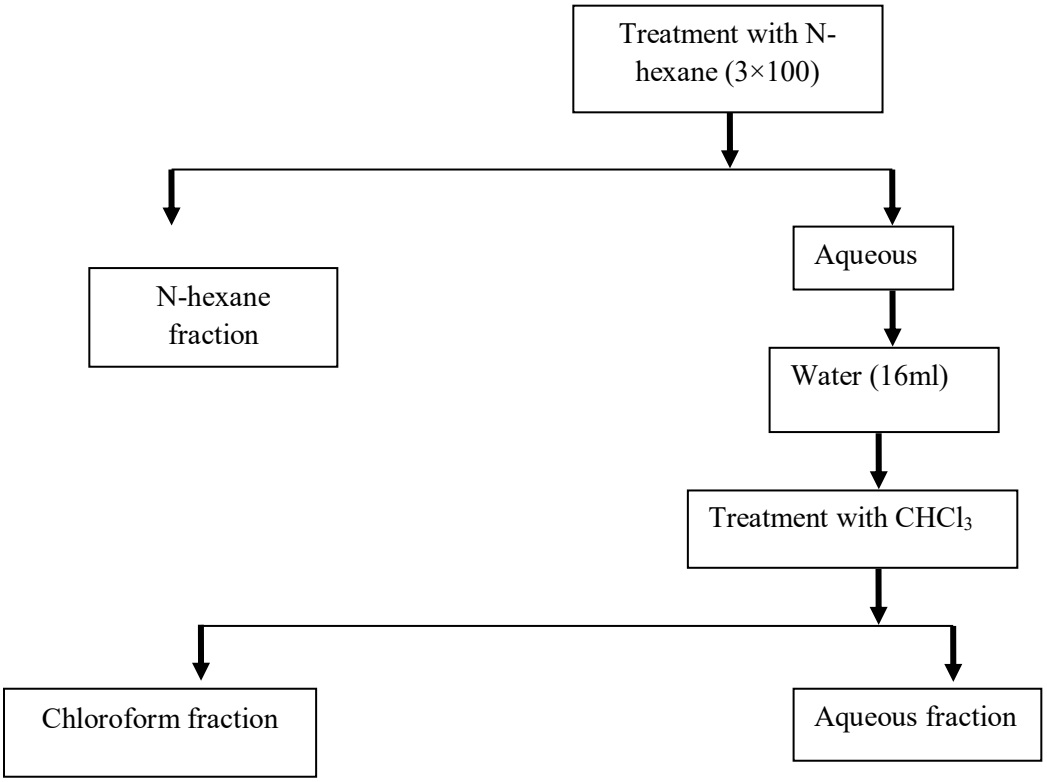


Figure 3.2: Schematic representation of modified kupchan partitioning of the crude methanolic extract of *Excoecariaagallocha*.

Drying

In order to evaporate the solvent from the extract as well as to avoid the growth of microorganisms at the time of drying the extract, the petri dishes were kept under Laminar Air Flow (LAF).When the extract was successfully dried, the petri-dishes were safely covered with aluminum foil and it was kept in the refrigerator for further investigation .

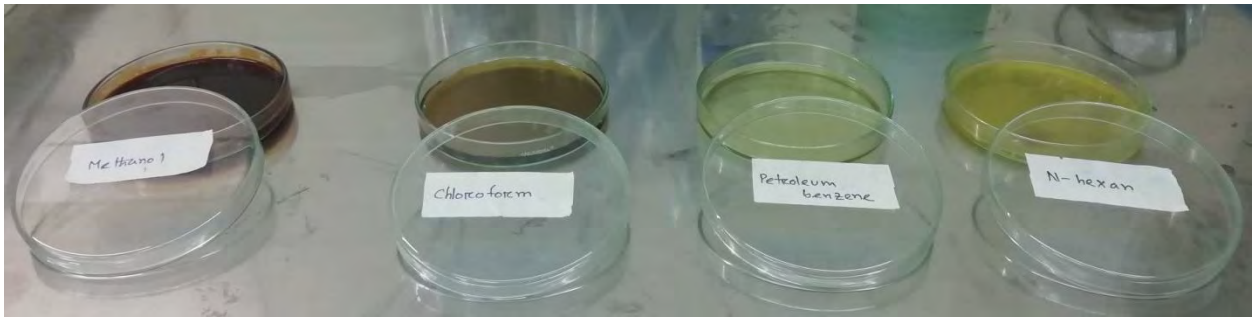


Figure 3.3: The petri-dishes that contains the bark extracts were placed under LAF for drying.

3.4 TLC (Thin Layer Chromatography)

Ascending one-dimensional thin layer chromatographic technique is used for the initial screening of the extracts and column fractions and checking the purity of isolated compounds. For the latter purpose commercially available precoated silica gel (kieselgel 60 PF_{254}).

Extract preparation

A small amount of dried extract is dissolved in a suitable solvent (Acetone & DCM) to get a solution (approximately 1%) (Harbone, 1976; Touchstone and Dobbins, 1978).

Preparing of TLC plates

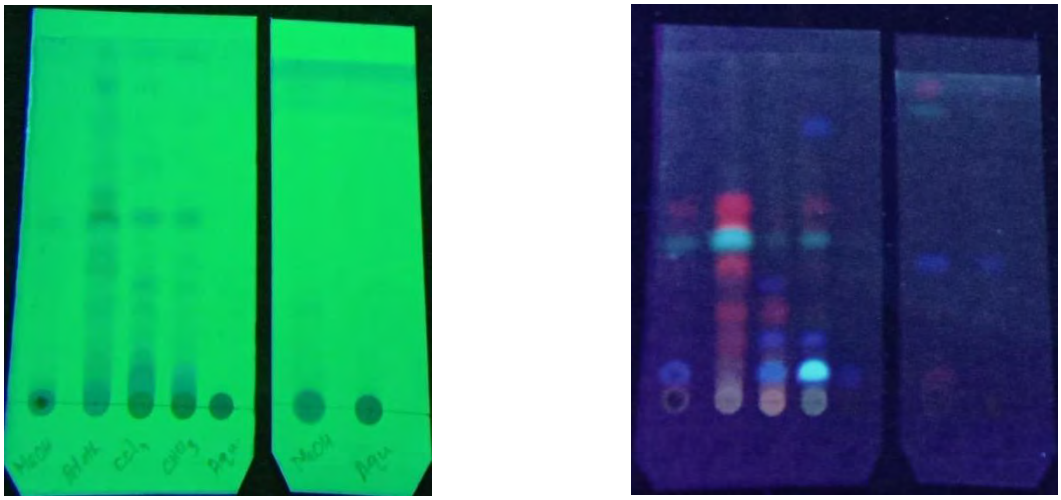
The glass plates were washed well in order to remove any fatty acid residue and dried. Then the samples were applied as spots at one end of the plates, about 1 cm above from the bottom, with capillary tube. The spots were kept as small as possible and dried in hot air.

Chamber saturation and development of TLC plates

The developing solvent mixtures were freshly prepared and transferred to respective chromatographic tank in such a way that the surface of the solvent system remained within 1cm from the lower surface tank. A smooth filter paper sheet was placed in the tank to help in chamber saturation. The tank was close with glass lid and left for development. When the solvent run being the pre-marked point, the plate are taken out and air-dried.

Detection of the compounds

The properly developed plates are viewed under UV light at 254nm and 365nm to detect the spot/band of any fluorescent or quenching compounds.



(a) **(b)**

Figure 3.4: (a) Under UV light at 254nm and (b) Under UV light at 365nm.

Extraction obtained from solvent-solvent partitioning (Kupchan method) and after that TLC (Thin Layer Chromatography) observation under UV light, there are various valuable compound present in the *E.agallocha* bark extract and extract in CCl_4 and $CHCl_3$ solvent showed higher level of compound present So that, both of them can be applied for further investigation for Anti-oxidant, Anti-microbial , Anti-cancer etc.

From the observation (Figure 3.4) given below, extract in CCl_4 and $CHCl_3$ solvent obtained from Kupchan partitioning method showed higher level of compound present then extract in other solvent.

3.5 Evaluation of Antioxidant Activity

1. Cupric Reducing Antioxidant Capacity (CUPRAC)

This assay is based on the changes in absorption characteristics of the neocuproine (Nc) copper (II) complex when it is reduced by an antioxidant. The reduction potential of the sample or standard effectively converts Cu+2 to Cu+1. This reduced complex absorption is measured at 450nm. This is a colorimetric assay with the reagents changing color from blue-green to yellow in the presence of an antioxidant (Apak*et al*., 2004).

Table 3.1: Materials needed for Cupric reducing antioxidant capacity

Reagents	Apparatus
➢ Cupric chloride *(*$CuCl_2.2H_2O$*)* ➢ Ammonium acetate buffer, pH 7.0 ➢ Neocuproine ➢ Butylatedhydroxyl toluene (BHT) (standard) ➢ Distilled water ➢ Ethanol (solvent)	➢ Screw cap test tubes ➢ Test tubes ➢ Pipettes (1ml, 5ml, 10ml) ➢ Micropipette (10-100µl) ➢ Spatula ➢ Beaker ➢ Volumetric flasks (250 ml, 100ml) ➢ Test tube racks ➢ Pipette filler ➢ Electronic Balance

Preparation of reagents

Preparation of 0.01 M solution of cupric chloride

0.4262 gm of $CuCl_2.2H_2O$, after measuring in an electric balance was taken in a 250ml of volumetric flask and distilled water was added to adjust the volume.

Preparation of 1 M ammonium acetate buffer, pH 7.0

19.27 gm of ammonium acetate was taken and placed in a 250 ml volumetric flask and the volume was adjusted with distilled water.

Preparation of 0.0075 M solution of Neocuproine

0.156 gm of neocuproine was taken and placed in a 100ml volumetric flask and the volume was adjusted with 96% ethanol.

Preparation of Standard and Extract solution

The standard and extract solutions were prepared separately by weighing them and adding ethanol to a volume to yield a concentration of 5mg/ml. The experimental concentrations were prepared by serial dilution of this stock solution.

- BHT (Butylatedhydroxyl toluene) was used as the standard

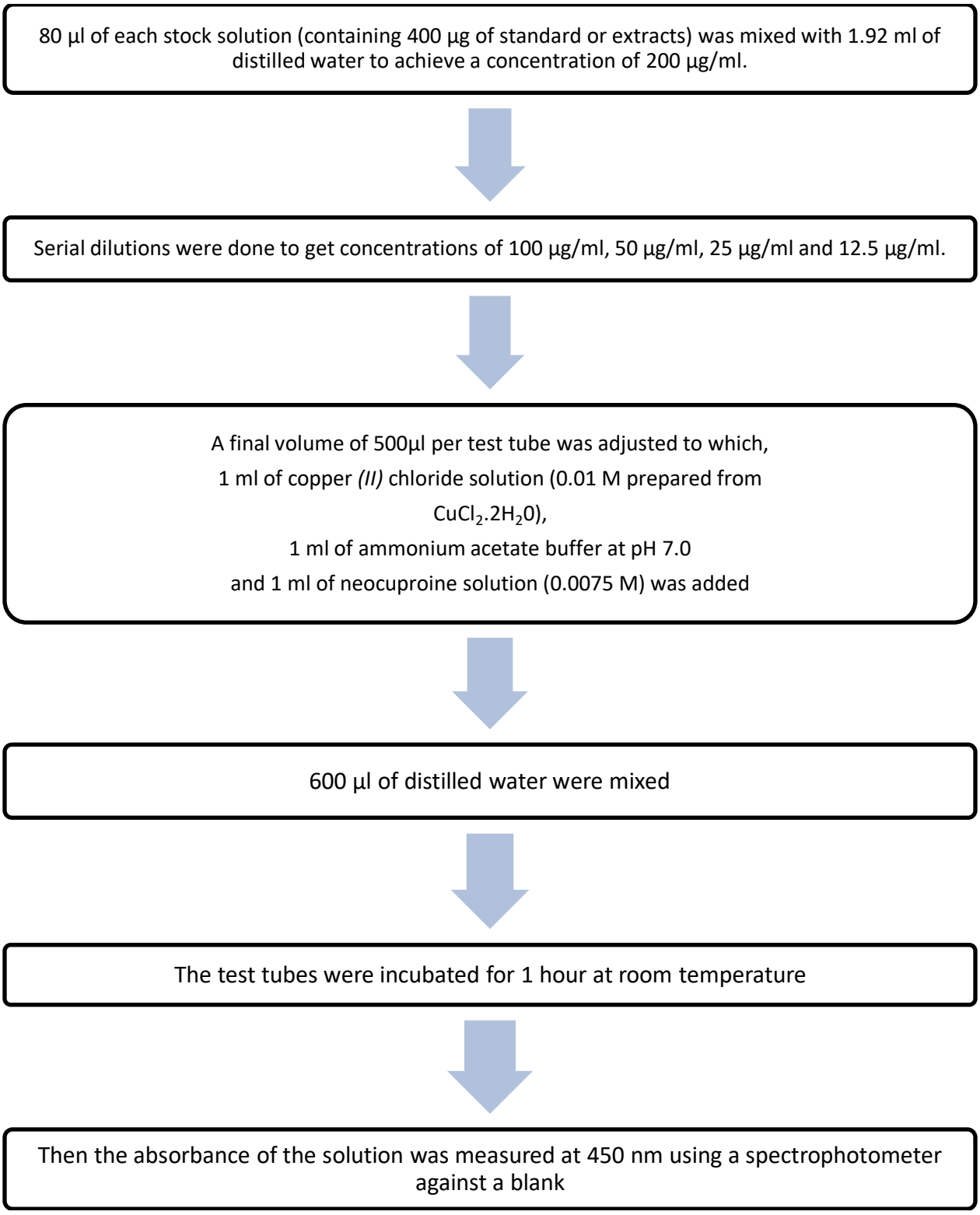


Figure 3.5: Experimental procedure of Cupric Reducing Antioxidant Capacity (CUPRAC).

2. Total antioxidant content

The phospho-molybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, α-tocopherol, and carotenoids. The phospho-molybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and subsequent formation of green phosphate/ Mo (V) complex. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI) and the formation of a green phosphate/Mo (V) complex with maximal absorption at 695 nm (Prieto *et al.,* 1999).

$$Mo\ (VI) + e \longrightarrow Mo\ (V)$$

Table 3.2: Materials needed for total antioxidant content

Reagents	Apparatus
➢ 96 % Sulphuric Acid (H_2SO_4) ➢ Sodium Phosphate (Na_3P0_4) ➢ Ammonium Molybdate ➢ Ascorbic acid (standard) ➢ Ethanol (solvent) ➢ Distilled Water	➢ Screw cap test tubes ➢ Test tubes ➢ Pipettes (1ml, 5ml, 10ml) ➢ Micropipette (10-100µl) ➢ Spatula ➢ Beaker ➢ Volumetric flasks (250 ml, 100ml) ➢ Test tube racks ➢ Pipette filler ➢ Electronic Balance

Preparation of reagents

Preparation of 96% Sulphuric Acid (H_2SO_4)

0.333ml of H_2SO_4 of 96% concentration was taken in a 100ml volumetric flask and distilled water was added to adjust the volume.

Preparation of 0.02295 M Sodium Phosphate (Na_3P0_4)

0.459 gm of Na_3P0_4 was taken in a 100ml volumetric flask and distilled water was added to adjust the volume.

Preparation of 0.004 M Ammonium Molybdate

0.4943 gm of ammonium molybdate was taken in a 100ml volumetric flask and distilled water was added to adjust the volume.

Preparation of standard and stock solution

The standard and extract solutions were prepared separately by weighing them, and adding ethanol to a volume to yield a concentration of 5mg/ml. The experimental concentrations were prepared by serial dilution of this stock solution.

- Ascorbic acid was used as the standard.

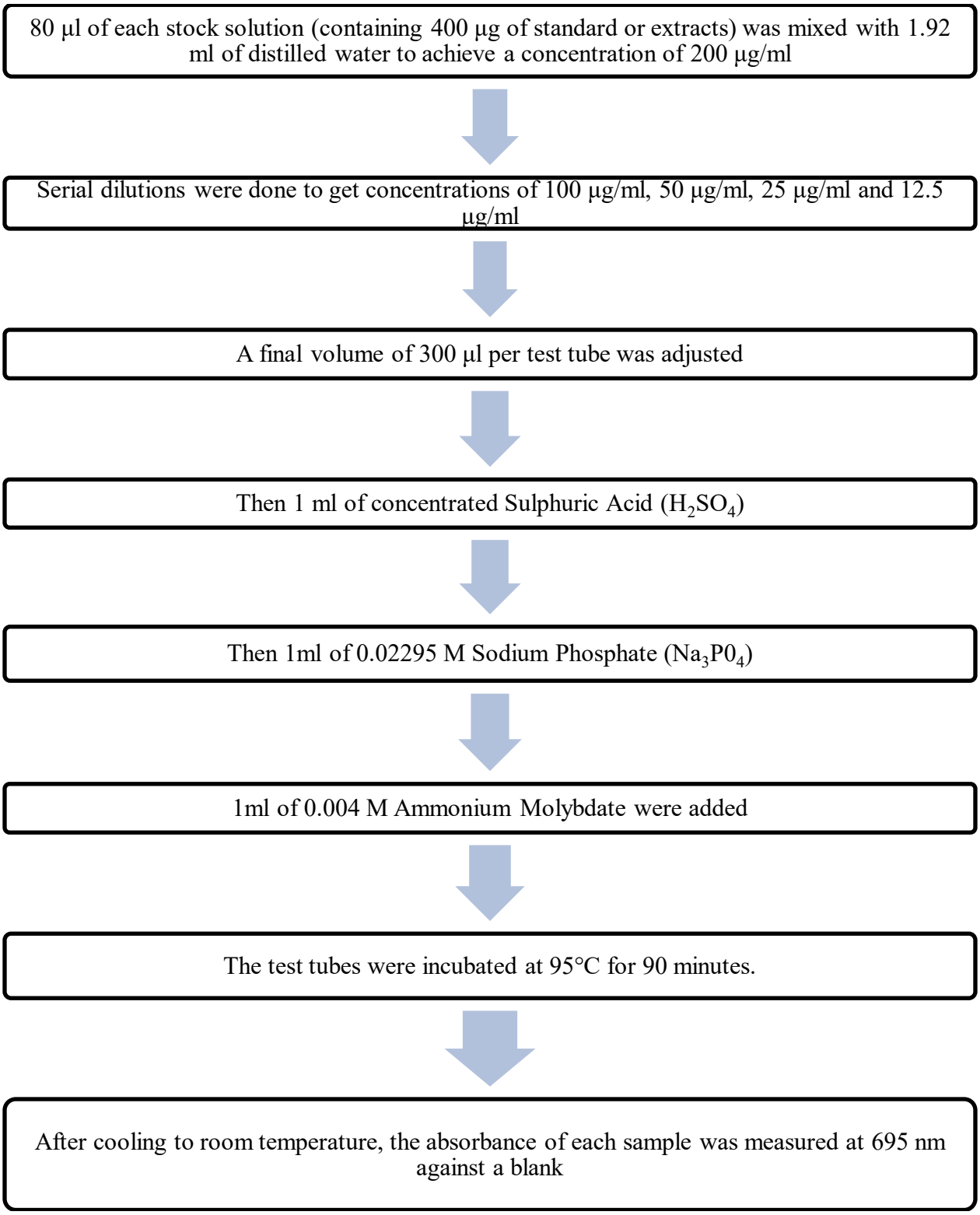


Figure 3.6:Experimental procedure of determination of total antioxidant content.

Calculation

The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was Calculated by the following equation:

$A = (c \times V)/m$

Where,
A = total content of Antioxidant compounds in mg/gm plant extract, in Ascorbic acid Equivalent.
c = the concentration of Ascorbic acid established from the calibration curve in mg/ml
v = the volume of extract in ml
m = the weight of crude plant extract in gm

3. Total phenolic content

Total phenolic content of all the extracts was evaluated with Folin-Ciocalteu method. Samples containing polyphenols are reduced by the Folin-Ciocalteu reagent by producing blue colored complex. The phenolic concentration of extracts was evaluated from a Gallic acid calibration curve. After incubation at 20°C for 60 min, the quantitative phenolic estimation was performed at 765 nm against reagent blank by UV Spectrophotometer (1650 Shimadzu, Japan). Total phenolic content was expressed as milligrams of Gallic acid equivalent (GAE) per gm of extract (Khatoon*et al*., 2013).

Table 3.3: Materials needed for total phenolic content

Reagents	Apparatus
➢ Folin -ciocalteu reagent ➢ 7.5% Sodium carbonate solution ➢ Methanol (solvent) ➢ Gallic acid (standard) ➢ Distilled water	➢ Screw cap test tubes ➢ Test tubes ➢ Pipettes (1ml, 5ml, 10ml) ➢ Micropipette (10-100µl) ➢ Spatula ➢ Beaker ➢ Volumetric flasks (250 ml, 100ml) ➢ Test tube racks ➢ Pipette filler ➢ Electronic Balance

Preparation of reagent

Preparation of 7.5% Sodium Carbonate solution

7.5 gm of Na_2C0_3 was taken into a 100 ml of a volumetric flask and the volume was adjusted by distilled water.

Preparation of 1:10 v/v Ratio Folin-ciocalteuslolution

20 ml Folin-ciocalteu phenol reagent was dissolved in 200 ml of distilled water to get a ratio of 1:10

Preparation of standard and stock solution

The standard and extract solutions were prepared separately by weighing them and adding methanol to a volume to yield a concentration of 5mg/ml. The experimental concentrations were prepared by serial dilution of this stock solution.

- Gallic acid was used as the standard.

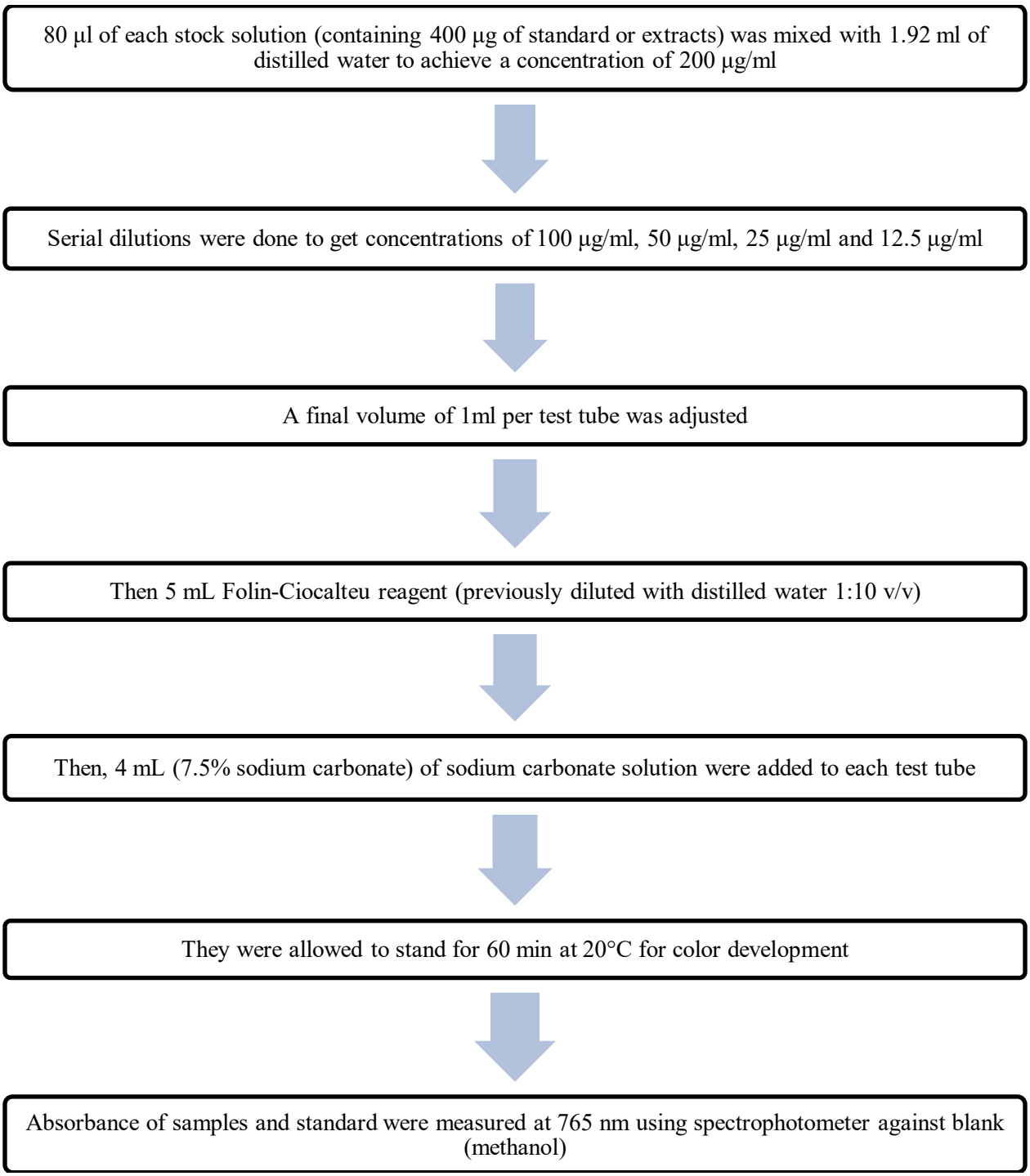


Figure 3.7: Experimental procedure of determination of total phenolic content.

Calculation

The total content of phenolic compounds plant extracts in Gallic acid equivalents (GAE) calculated using the following equation:

$C = (c \times V)/m$

Where;

C = total content of phenolic compounds in mg/gm plant extract, in GAE

c = the concentration of Gallic acid established from the calibration curve (mg/ml)

V =the volume of extract in ml

m = the weight of crude plant extract in gm

4. Total flavonoid content

Determining the total flavonoids by using aluminum chloride method is based upon the formation of stable complex between aluminum chloride and keto and hydroxyl groups of flavones and flavonoids. For building the calibration curve, quercetin was used as standard. Various concentrations of standard quercetin solution were used to make a standard calibration curve (Pallab*et al*., 2013).

Table 3.4: Materials needed for Total Flavonoid content

Reagents	Apparatus
➢ 10% Aluminium Chloride solution ➢ 1M Potassium Acetate solution ➢ Methanol (solvent) ➢ Quercertine (standard) ➢ Distilled water	➢ Screw cap test tubes ➢ Test tubes ➢ Pipettes (1ml, 5ml, 10ml) ➢ Micropipette (10-100µl) ➢ Spatula ➢ Beaker ➢ Volumetric flasks (250 ml, 100ml) ➢ Test tube racks ➢ Pipette filler ➢ Electronic Balance

Preparation of reagents

Preparation of 10% Aluminum Chloride ($AlCl_3$) solution

10 gm of $AlCl_3$ was taken into a 100 ml of a volumetric flask and the volume was made up to 100ml using distilled water.

Preparation of 1M Potassium Acetate solution

9.815 gm of potassium acetate was taken into a 100 ml of ii volumetric flask and the volume adjusted up to 100ml by distilled water.

Preparation of standard and stock solution

The standard and extract solutions were prepared separately by weighing them and adding methanol to a volume to yield a concentration of 5mg/ml. The experimental concentrations were prepared by serial dilution of this stock solution.

- ❖ Quercertin was used as the standard.

Calculation

The total content of flavonoid compounds in plant methanol extracts in quercetin equivalents was calculated by the following formula equation:

C = {c x V)/m

Where;
C = total content of flavonoid compounds in mg/gm plant extract, in quercetin equivalent
c = the concentration of quercetin established from the calibration curve (mg/ml)
V =the volume of extract in ml
m = the weight of crude plant extract in gm

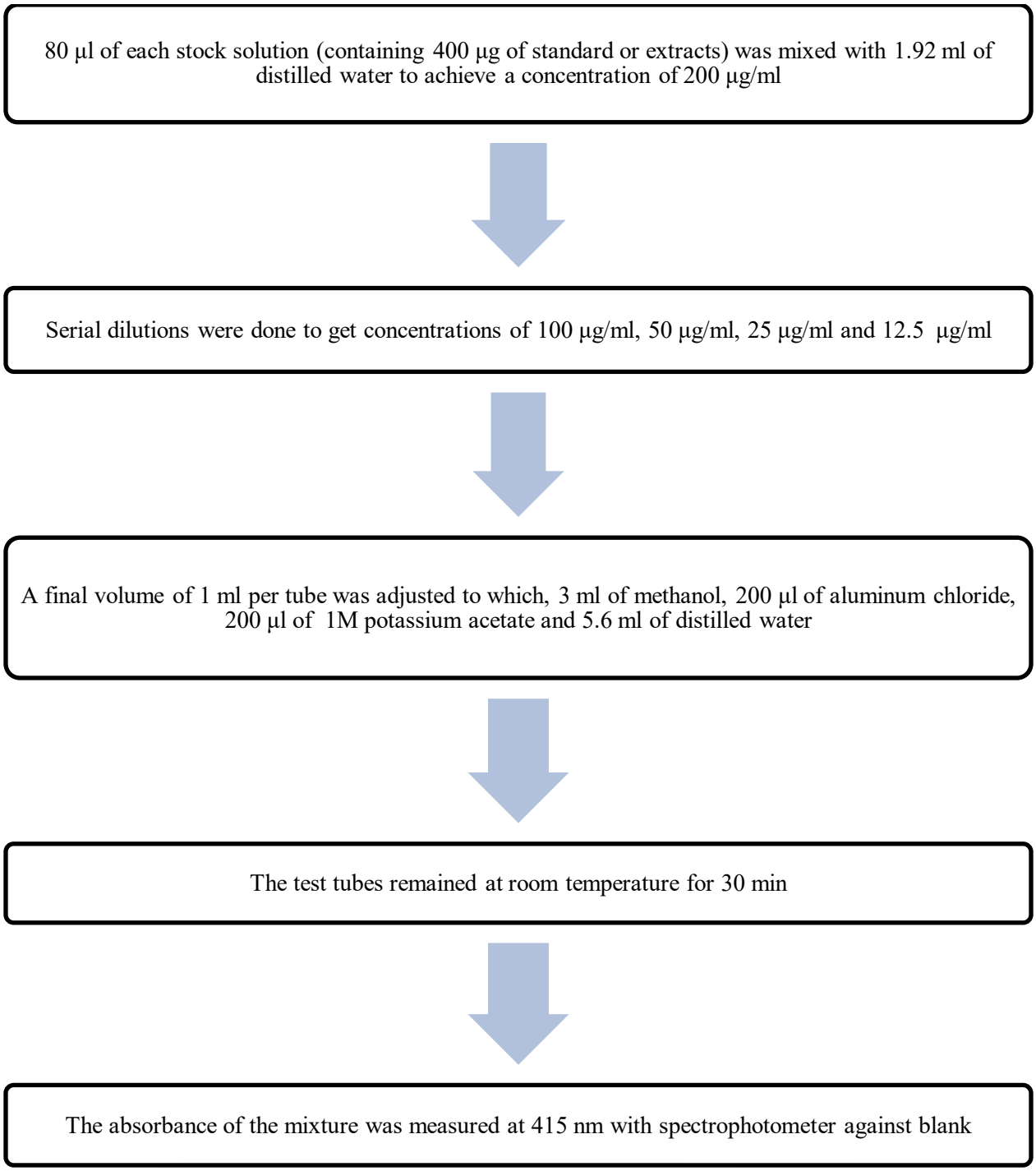


Figure 3.8: Experimental procedure of Total flavonoid content.

3.6 *In vivo* anticancer activity

Antitumor activity of the test compounds is determined using Ehrlich Ascites Carcinoma (EAC) cancer model in mice. The ascitic carcinoma bearing mice (donor) are used for the study, 15 days after tumor transplantation. The animals are divided into groups:

i. Tumor-bearing mice
ii. Tumor-bearing mice treated with standard drug
iii. Tumor-bearing mice groups treated with test drug

The ascitic fluid was drawn using an 18-gauge needle with sterile syringe. The ascitic fluid is suitably diluted in normal saline to get a concentration of 10^6cells/ml of tumor cell suspension. This is injected intraperitoneally to obtain ascitic tumor. The mice are weighed on the day of cancer cell inoculation and then once in three days thereafter. Treatment is started on the 5^{th} day of cancer inoculation. Standard (vincristine sulfate) is injected on 5^{th} day intraperitoneally. The drug is administered from 5^{th} day for 15days intraperitoneally. After the administration of last dose followed by 18h fasting, six mice from each group were sacrifice for the study of antitumor activity and hematological parameters. Antitumor effects of drug were assessed by observation of the following parameters.

1. Measurement of body weight increase in day-0,7 and 15 among the group
2. Hematological parameters

Experimental animals

For the experiment Swiss albino female mice, 6-7 weeks of age, weighing between 25-30gm, were collected from the pharmacology lab, department of pharmacy, Jahangirnagar University, savar, Dhaka. Animals were maintained under standard environmental conditions (temperature: (27.0 ± 1.0)ºC, relative humidity: (65-70) % and 12hr light/ 12hr dark cycle) and had free access to feed and water added libitum. The animals were acclimatized to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

Acute Toxicity study

Acute toxicity describes the adverse effects of a substance which result either from a single exposure or from multiple exposures in a short space of time (usually less than 24 hours). The test samples were administered orally to the test animals at different concentrations (200 and 400 mg/kg body weight). After administration of the extract solutions mortality or sign of any toxicity was observed for 1 hour. Then the test animals were observed every 1 hour for next 5-6 hours. The animals were kept under observation for 15 days (Walum, 1998).

Experimental Design

Animals were divided into four groups of six animals in each group.

Table 3.5: Group distribution and administered substance with their dose.

Group	Administered substance and Dose
Group I	Negative Control (only water)
Group II	Positive Control (Vincristine Sulfate)
Group III	Plant Extract (200 mg/kg)
Group IV	Plant Extract (400mg/kg)

Methanolic extracts of*Excoecariaagallocha* bark extract was used in the acute toxicity study. All doses are administered per orally.

Identification of Animals during Experiment

Each group consists of ten mice and hence it is difficult to identify and observe at a time ten mice receiving same treatment. Thus it was important to identify individual animal of a group during the treatment. To denote individual animal, they were marked or coded I, II, III……none (for no. ten) on their tails.

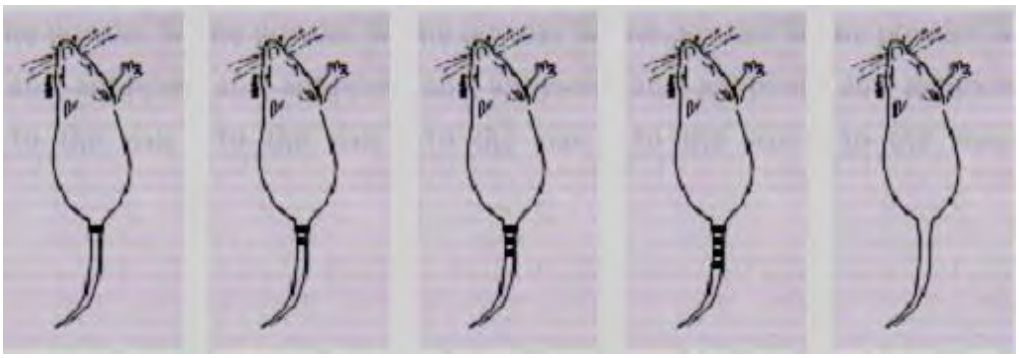


Figure 3.9: Identification of Animals.

Results

After the acute toxicity studies no mortality was observed up to dose as high as 400 mg/kg of the extracts.

Preparation of Sample and Standard

The sample was prepared by dissolving the methanolic extract of *Excoecariaagallocha*and diluted in distilled water at two doses (200 mg/kg body weight and 400mg/kg bodyweight).05 gmextractwasmeasuredandaddedwithit250mlofdistilled water andmixingwith the help of vortex apparatus. From this solution 200 mg/kg dose and 400mg/kg dose were feeded orally depending on the body weight of the mice. Vincristine Sulfate (Cristone 2) wasusedaspositivecontrol and forthepreparationofVincristine Sulfate atthedose1 ml was taken and a suspension of 20 ml was made with normal saline. From this solution per dose was injected Intra peritoneal (IP) way depending on their body weight (12.5 µg/ 250 µl).Figure 3.10: Preparation of the solution of plant extract with the help of vortex apparatus.

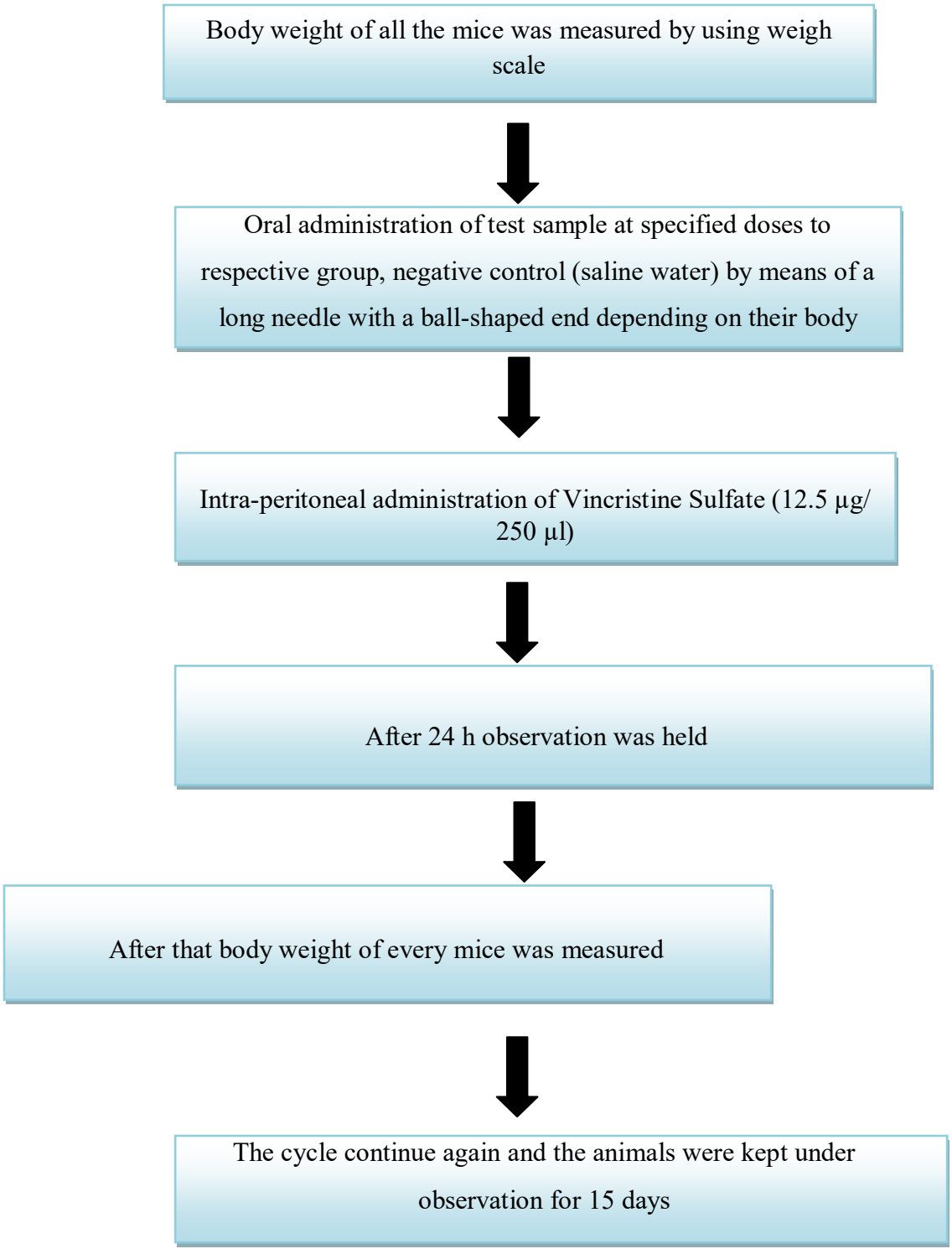


Figure 3.11: Schematic Diagram of Anticancer activity Test procedure.

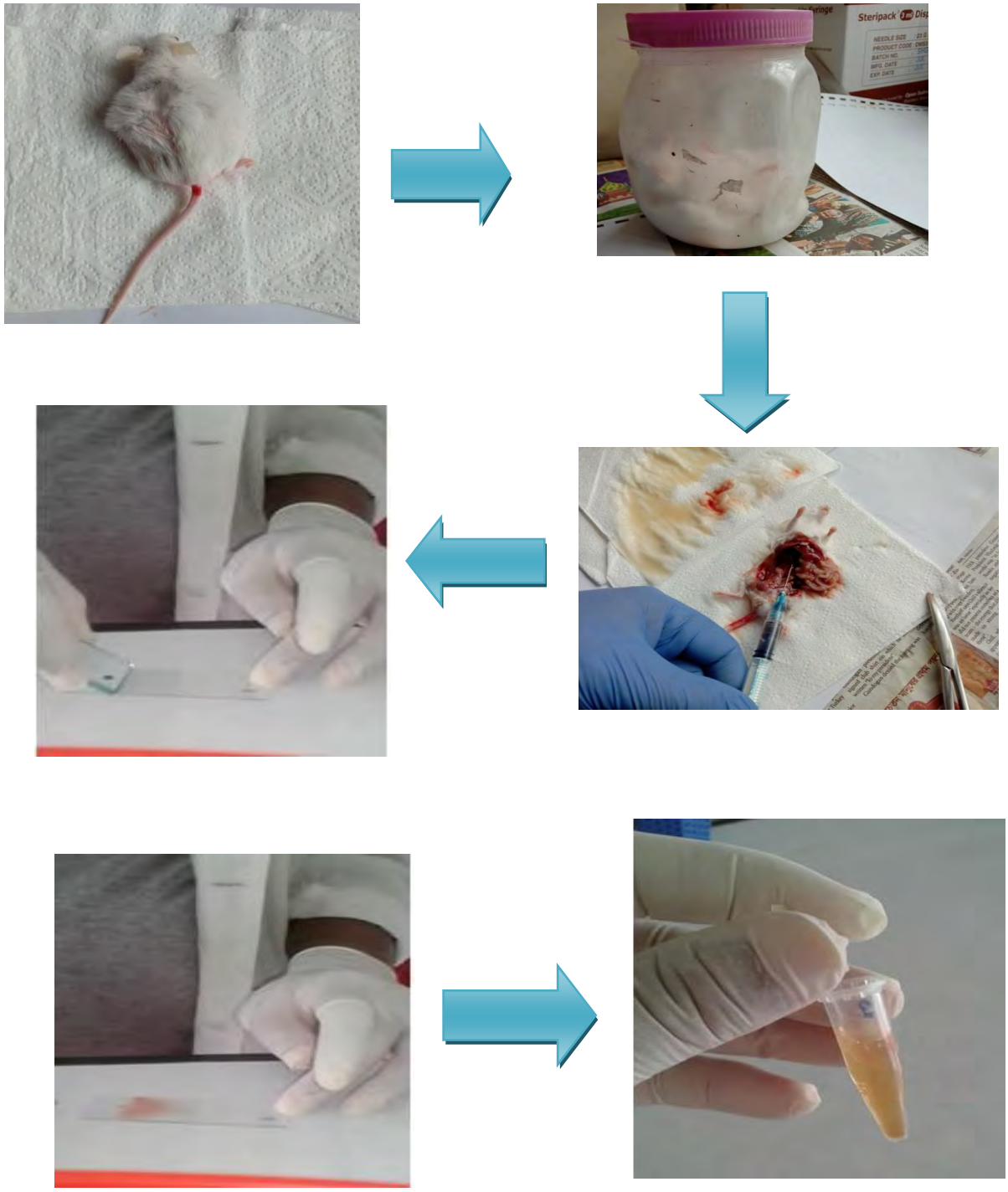


Figure 3.12: Flow chart of Dissection and Blood collection for the test.

Measurement of Hematological parameters in mice

The heparinized blood was used for hematological determination. Hematology analyses were performed on whole blood, using the automatic hematology system to evaluate the following parameters; LYMPH=Lymphocyte, MON=Monocyte, NEU=Neutrophils, EOSIN=Eosinophils, BASO= Basophils, SGPT= Serum Glutamic Pyruvic Transaminase, SGOT= SerumglutamicOxaloacetic Transaminase, ALP= Serum Alkaline Phosphatase.

Statistical analysis

Statistical analysis for animal experiments were carried out by Single factor One way ANOVA using Microsoft Excel. Data was presented as Mean= SEM. The result obtained were compared with the control group and $p<0.05$, $p<0.01$and $p<0.001$ were considered to be statistically significant, highly significant and very highly significant respectively.

CHAPTER 4

Results and Discussion

4.0 Results and Discussion

4.1 Antioxidant activity of *E. agallocha* bark extracts

4.1.1 Cupric Reducing Antioxidant Capacities

Result

Reduction of Cu^{2+} ion to Cu^{1+} was found to rise with increasing concentrations of the different extracts. The standard BHT showed the highest reducing capacity. Among the extracts the methanolic extract of *E. agallocha*showed maximum reducing capacity that is comparable to BHT (Figure 4.1).

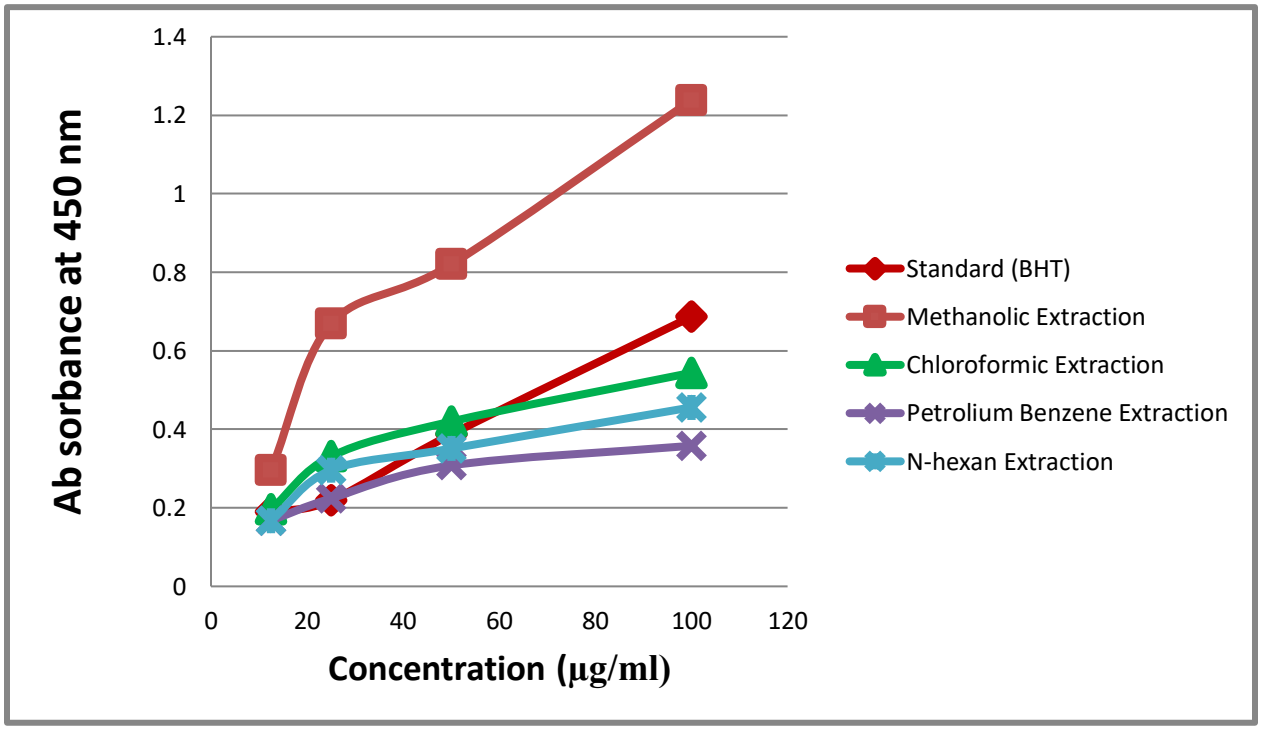


Figure 4.1: Comparative reducing powers of *E. agallocha*extracts andButylated hydroxyl toluene (BHT).

Discussion

This method was based on the principle of increase in the absorbance of the reaction mixtures. Increase in the absorbance indicates increase in the antioxidant activity. Increase in absorbance of the reaction mixture indicates the reducing power of the samples (Jayaprakasha et al., 2001).

Reducing power is associated with antioxidant activity and may serve as a significant reflection· of the antioxidant activity (Oktay*et al,* 2003). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995).

4.1.2 Total antioxidant Contents

Result

Total antioxidant capacity of the bark extracts of *E. agallocha*was evaluated by the phosphor molybdenum method and was expressed as ascorbic acid equivalents (AAE) per gram of plant extract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid (y = 0.002x + 0.064; R^2=0.957) (Figure4.2). Methanolicextraction of*E. agallocha*was found to possess the highest total antioxidant capacity (Table 4.5).Total antioxidant capacity of the extracts was found to decrease in the following order:

Methanol extract > Chloroform extract > N-hexane extract > Petroleum benzene extract (Figure 4.3).

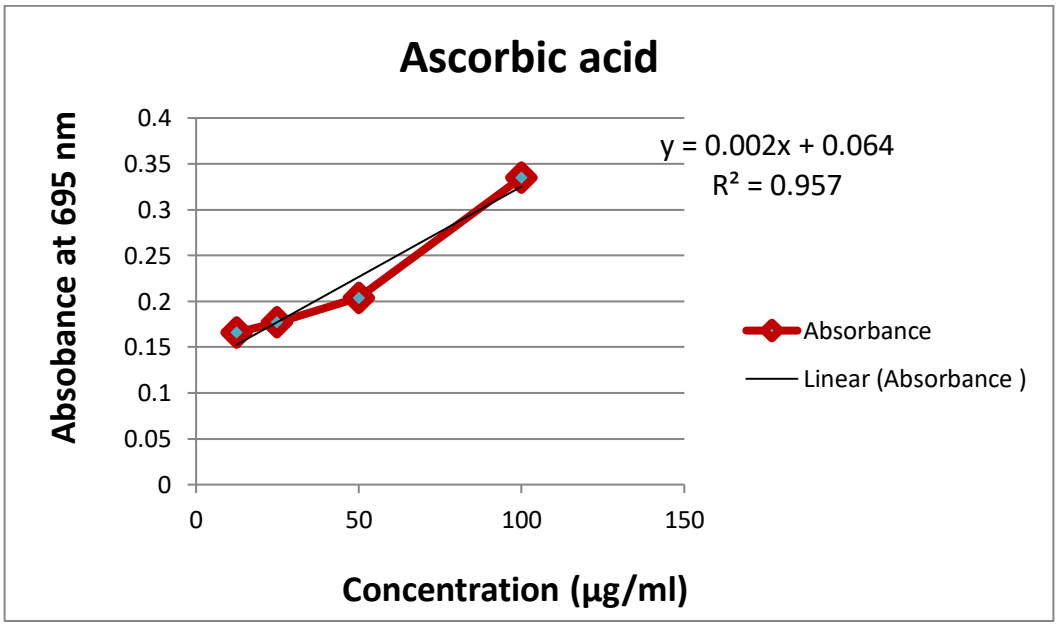


Figure 4.2: Calibration curve of ascorbic acid (Standard).

Table 4.1: Total antioxidant content of the bark extract of *E. agallocha*(Methanolic extraction).

Weight of the dry extract (gm) (m)	Absorbance of the extract (695 nm)	Ascorbic Acid concentration (µg/ml) (c)	Ascorbic Acid concentration (mg/ml) (c)	TAC as AA , A=(c×v)/m	Mean± SEM (mg/gm)
0.005	3.531	1733.5	1.7335	346.7	344.63± 1.386
	3..484	1710	1.710	342	
	3.516	1726	1.726	345.2	

Table 4.2: Total antioxidant content of the bark extract of *E. agallocha*(Chloroformic extraction).

Weight of the dry extract (gm) (m)	Absorbance of the extract (695 nm)	Ascorbic Acid concentration (µg/ml) (c)	Ascorbic Acid concentration (mg/ml) (c)	TAC as AA , A=(c×v)/m mg/gm	Mean± SEM (mg/gm)
0.005	2.405	1170.5	1.1705	234.1	237.67± 3.3268
	2.414	1175	1.175	235	
	2.506	1221	1.221	244.5	

Table4.3: Total antioxidant content of the bark extract of *E. agallocha*(N-hexane extraction).

Weight of the dry extract (gm) (m)	Absorbance of the extract (695 nm)	Ascorbic Acid concentration (µg/ml) (c)	Ascorbic Acid concentration (mg/ml) (c)	TAC as AA , A=(c×v)/m mg/gm	Mean± SEM (mg/gm)
0.005	2.104	1020	1.02	204	207.63± 0.048827
	2.08	1008	1.008	201.6	
	2.237	1086.5	1.0865	217.3	

Table 4.4: Total antioxidant content of the bark extract of *E. agallocha*(Petroleum benzene extraction).

Weight of the dry extract (gm) (m)	Absorbance of the extract (695 nm)	Ascorbic Acid concentration (µg/ml) (c)	Ascorbic Acid concentration (mg/ml) (c)	TAC as AA , A=(c×v)/m mg/gm	Mean± SEM (mg/gm)
0.005	2.069	1002.5	1.0025	200.5	202.067± 1.667
	2.067	1001.5	1.0015	200.3	
	2.118	1027	1.027	205.4	

Table 4.5: Total antioxidant content of the different extracts of *E. agallocha*extracts.

Extract	Total Antioxidant Capacity (mg/gm, Ascorbic Acid Equivalent)
Methanol	344.63± 1.386
Chloroform	237.67± 3.3268
N-hexane	207.63± 4.8827
Petroleum Benzene	202.067± 1.667

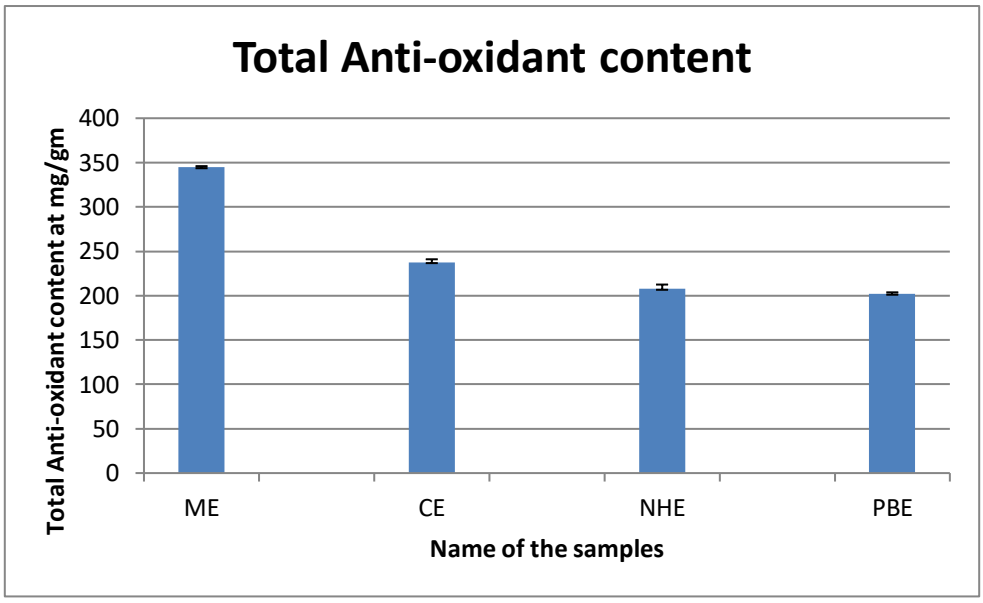


Figure 4.3: Total antioxidant content of the bark extracts of *E. agallocha* after extraction with different solvent.

Discussion

The reducing capacity of an extracts may serve as a significant indicator of its potential antioxidant activity. From the quantitative estimation of total antioxidant potential of*E. agallocha*, methanolic and chloroformic extract of *E. agallocha*exhibited 344.63±

1.386mgand237.67± 3.3268mg AA/gmequivalent respectively. Methanolic extract of *E. agallocha*showed significant antioxidant power. Investigating the results of the present study, Petroleum Benzeneextract of *E. agallocha*bark showed the lowest antioxidant capacity which was 202.067± 1.667mg AA/gm equivalent then other solvent extract. Among the entire extracts *E. agllocha*bark extract showed highest antioxidant capacity, specially its methanolic extract.

In this research work, previous assays also showed that the methanolic extract had a greater antioxidant potentiality than other extract.

4.1.3 Determination of Total Phenolic content

Result

Total phenolic content of the different extracts of *E. agallocha*were determined by usingtheFolinCiocalteu reagent and were expressed as Gallic acid equivalents (GAE) per gram ofplant extract. The total phenolic contents of the test fractions were calculated using the standardcurve of Gallic acid ($y = 0.0034x + 0.3777$; $R^2 = 0.8555$) (Figure 4.4). Methanolic extract of*E. agallocha*was found to contain the highest amount of phenols (Table 4.6). Phenol contents of the extracts were found to decrease in the following order:

Methanol extract> Chloroform extract> N-hexane extract> Petroleum benzene extract (Figure 4.5)

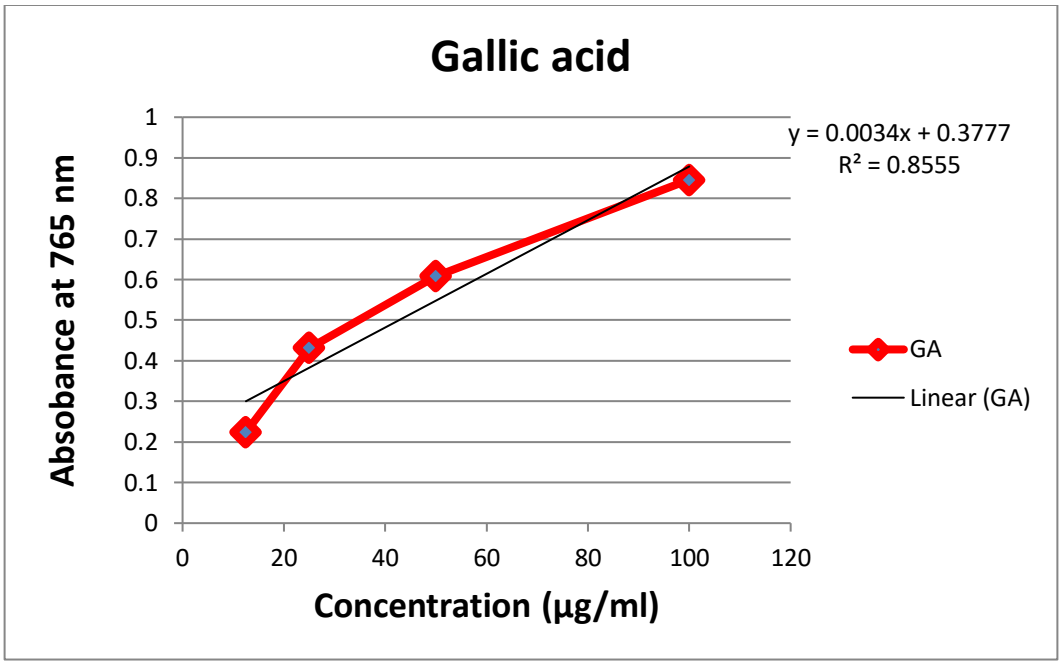


Figure 4.4: Calibration curve of Gallic Acid.

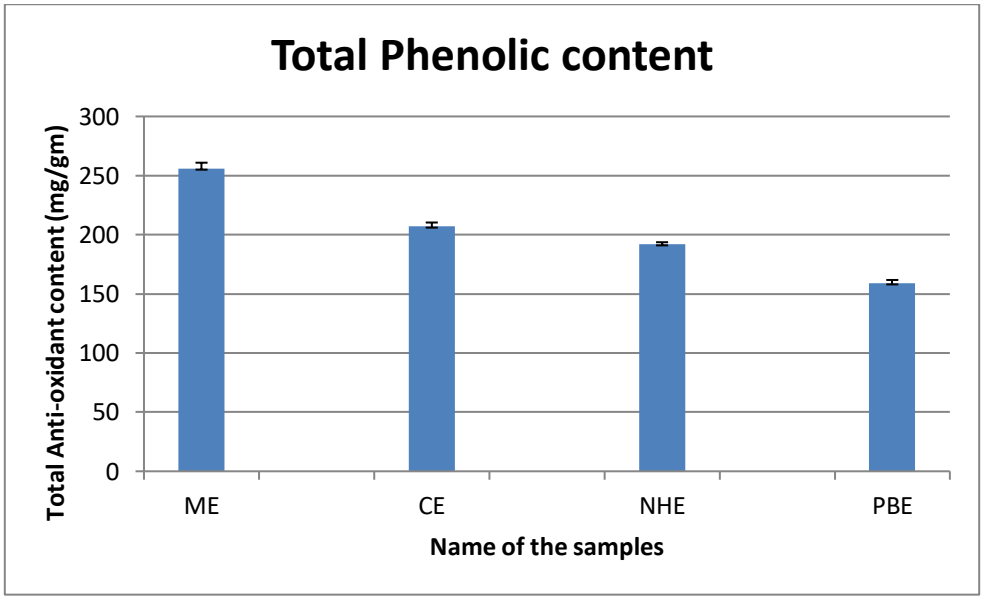


Figure 4.5: Total Phenolic content of the bark extracts of *E. agallocha* after extraction with different solvent.

Table 4.6 Total Phenolic content of the different extracts of *E. agallocha*extracts.

Extract	Total Phenolic Content (mg/gm, Gallic Acid Equivalent)
Methanol	181.37± 2.316
Chloroform	84.135± 4.3286
N-hexane	67.6647± 6.129
Petroleum Benzene	51.84± 1.767

Discussion

Phenolic or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Many of the phenolic compounds have been shown to contain high levels of antioxidant activities (Razali et al., 20080. The antioxidant properties of phenolic compounds originate from their properties of proton loss, chelate formation and dismutation of radicals. In fact, in some studies, theoretical methods have been proposed to estimate the antioxidant activities of phenolic substances. Their structure activity relationships are examined for this purpose. Phenols are compounds that have the ability to destroy radicals because they contain hydroxyl groups (Das and Pereira, 1990; Younes, 1981). These important plant components give up hydrogen atoms from their hydroxyl groups to radicals and from stable phenoxyl radicals; hence, they play an important role in antioxidant activity. Therefore, determination of the quantity of phenolic compounds is very important in order to determine the antioxidant capacity of plant extracts (De Gaulejac et al., 1999; Hatano et al., 1989).

Investigating the results of the present study, the quantitative estimation of total phenolic content of methanolicextract of *E. agallocha*exhibited 181.37± 2.316 mg GAE/g of extract. Petroleum Benzeneextract of *E. agallocha*showed the amount of total phenolic content is 51.84± 1.767 mg GAE/g of extract. The amount of total phenol in methanolic extract of *E. agallocha bark* was the highest compared to all the other extracts in this research work. According to the literature,

polyphenols are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants (Okawa et al., 2001).

4.1.4 Determination of Total flavonoid content

Result

Aluminium chloride colorimetric method was used to determine the total flavonoid contents of the different extracts of*E. Agallocha*. Total flavonoid contents were calculated using the standard curve of quercetin (y = 0.0025x + 0.0959; R^2 = 0.9858) **(Figure 4.6)** and was expressed as quercetin equivalents (QE) per gram of the plant extract. Petroleum ether of *A. marmelos*was found to contain the highest amount of flavonoid **(Table 8).** Flavonoid contents of the extracts were found to decrease in the following order:

Methanol extract > Chloroform extract > N-hexane extract > Petroleum ether extract(Figure 4.7).

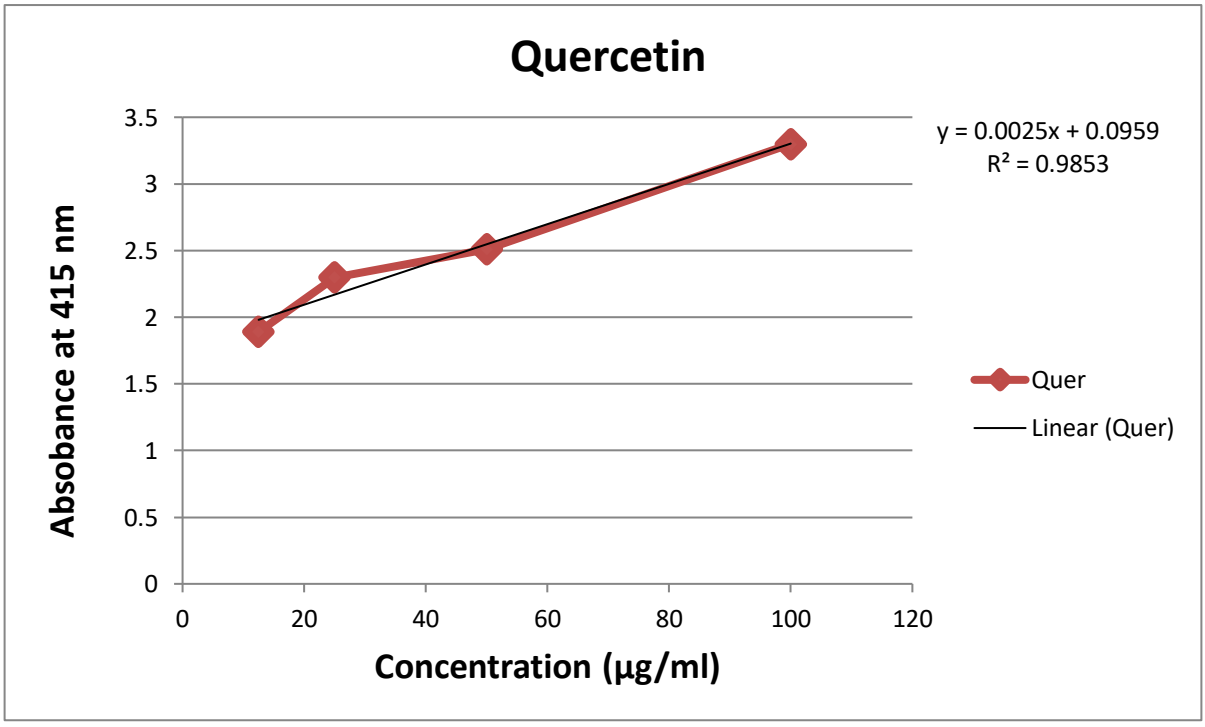


Figure 4.6: Calibration curve of Quercetin.

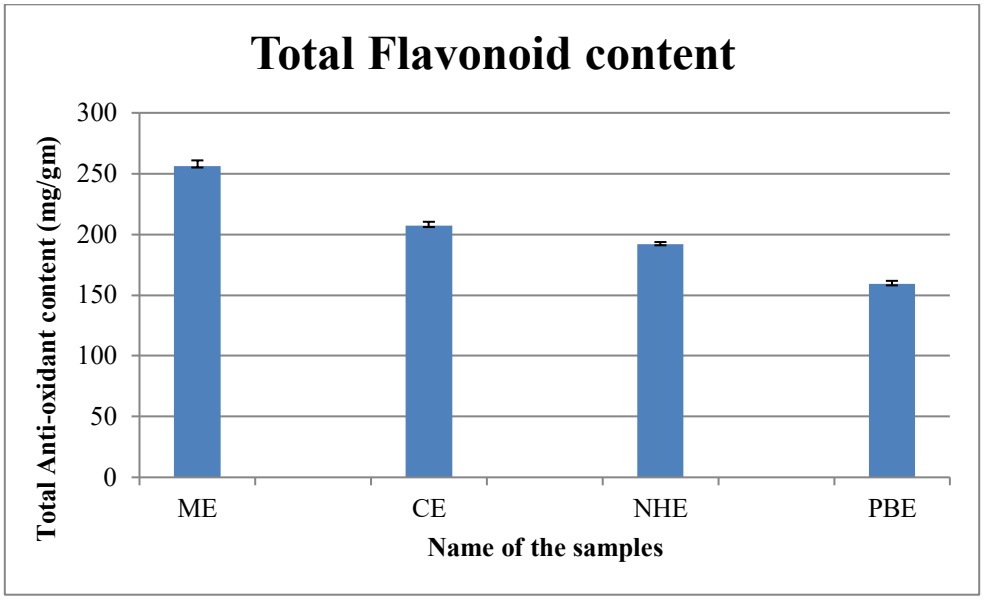


Figure 4.7: Total Flavonoid content of the different extracts of *E. agallocha*extracts.

Table 4.7: Total Flavonoid content of the different extracts of *E. agallocha*extracts.

Extract	**Total Phenolic Content (mg/gm, Gallic Acid Equivalent)**
Methanol	256± 4.8827
Chloroform	207± 3.326
N-hexane	192± 1.667
Petroleum Benzene	152± 2.667

Discussion

Flavonoids constitute a wide array of biological active compounds that are found abundantly in plant kingdom and dietary intake (Cushnie et al., 2005). They are gaining interest due to their wide variants and number of members. These are reported to be effective in pathogenesis of majority of diseases. Antioxidant activity is the foundation of many actions which lead to its beneficial effects in majority of the diseases. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers (Pal et al., 2009). This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available ‘‘H” atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure (Tripoli et al., 2007). Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical, thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain [Cook et al. 1996; Ferreira et al., 2010]. The flavonols having ortho or para hydroxyl group in the 2-phenyl ring are known to have strong antioxidant properties, while free hydroxyl at the 5, 7- positions proved to have a pro-oxidant effect (Heim et al., 2002). A positive correlation between the content of flavonoids and the antioxidant activity in plant extract has been found (Nagarajan et al., 2008). For that reason, determination of the quantity of flavonoids compounds is very important in order to determine the antioxidant activity of plant extracts.

Investigating the results of the present study, the quantitative estimation of total flavonoid content of methanolic extract of *e. agallohca*exhibited256± 4.8827 mg QE/g of extract. From the quantitative estimation of total flavonoid content of Petroleum benzene extract of *E. agallocha* bark shows that the amount of total flavonoid content is 159± 2.729mg QE/g of extract. In this research work, we found methanolic extract of *E. agallocha*showed a quite good amount of flavonoid content. But we also found in the literature, polyphenols or flavonoids are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants (Okawa et al., 2001).

Statistical analysis

Statistical analysis was done with correlation regression analysis using Microsoft Excel. All the data were statistically significant at $p<0.05$ level of significance.

4.2 Anticancer activity of *E. agallocha* bark extracts

4.2.1 Measurement of body weight

Measurement of body weight increase in day-0, 7 and 15 among the four groups of mice.

1. **Measurement of body weight at Day 0**

Table 4.8: Body weight at Day 0 of the four groups of mice.

Observation day	Negative control (gm)	Positive control(gm)	Plant extracts 200 mg/kg(gm)	Plant extracts 400 mg/kg (gm)
	27	27	25	26
	28	25	25	28
	26	28	24	25
Day 0	27	26	24	24
	24	27	27	24
	25	25	26	25

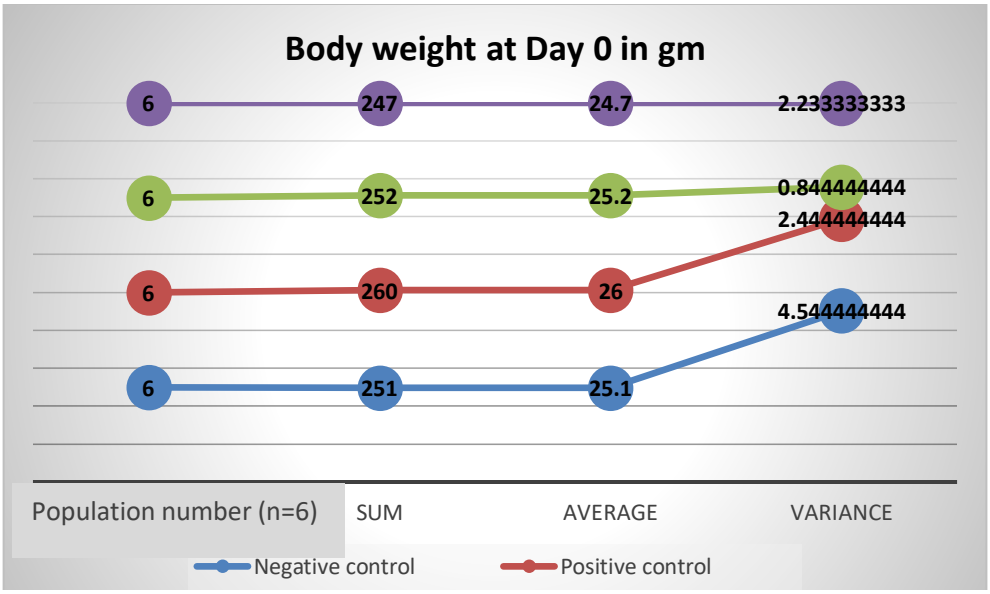


Figure 4.8: Comparison of body weight ay Day 0 among the mice model.

2. Measurement of body weight at Day 7

Table 4.9: Body weight at Day 7 of the four groups of mice.

Observation day	Negative control(gm)	Positive control(gm)	Plant extracts 200 mg/kg(gm)	Plant extracts 400 mg/kg(gm)
	36	22	31	32
	38	26	32	35
Day 7	34	29	31	33
	32	30	28	32
	37	29	32	30
	32	26	34	30

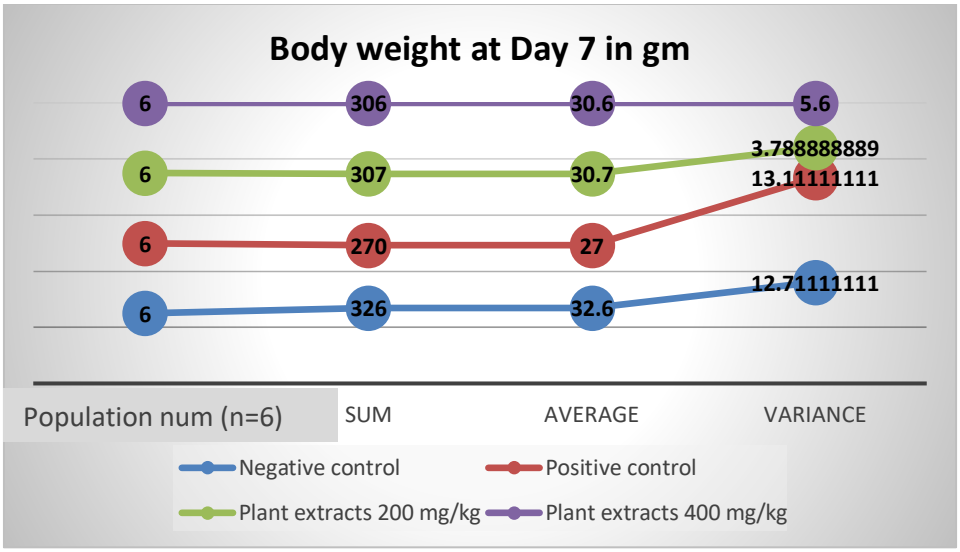


Figure 4.9: Comparison of body weight ay Day 7 among the mice model.

3. Measurement of body weight at Day 15

Table 4.10: Body weight at Day 15 of the four groups of mice.

Observation day	Negative control (gm)	Positive control (gm)	Plant extracts 200 mg/kg(gm)	Plant extracts 400 mg/kg(gm)
	43	32	40	39
	46	31	39	43
	41	37	38	39
Day 15	42	38	35	42
	39	41	43	29
	38	33	42	33

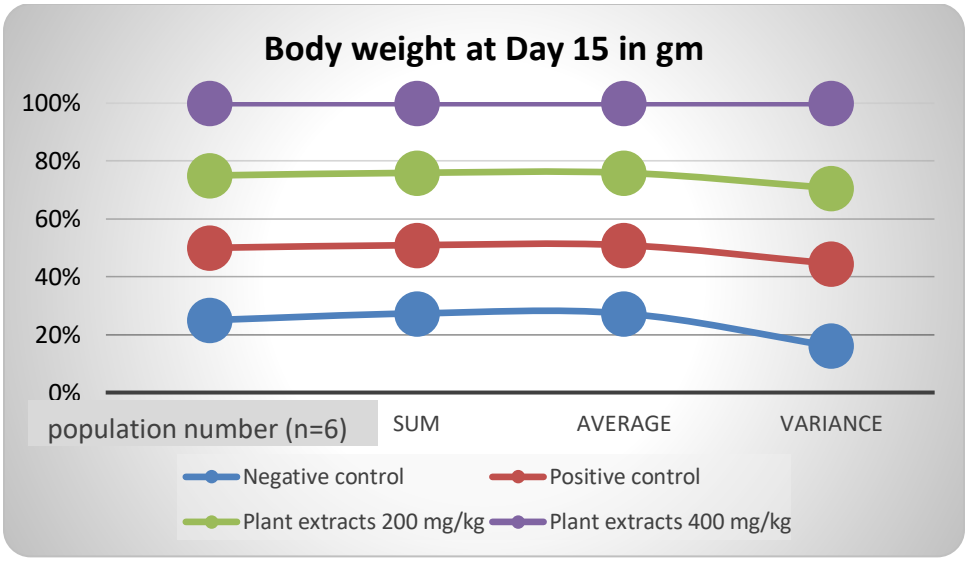


Figure 4.10: Comparison of body weight ay Day 15 among the mice model.

Table 4.11: Comparison of the body weight of day 0, 7 and 15 among the four groups of mice model.

Observation day	Analysis	Negative control (gm)	Positive control (gm)	Plant extracts 200 mg/kg (gm)	Plant extracts 400 mg/kg (gm)
Day 0	Mean ± SEM	25.1 ± 4.544444	26 ± 2.444444	25.2 ± 0.844444	24.7 ± 2.233333
	p-value			0.406099	0.254052
Day 7	Mean ± SEM	32.6 ± 12.71111	27 ± 13.11111	30.7 ± 3.788889	30.6 ± 5.6
	p-value			0.001632	0.002275
Day 15	Mean ± SEM	41 ± 13.55556	35.4 ± 24.04444	37.5 ± 22.05556	36.1 ± 24.76667
	p-value			0.029526	0.020911

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way body weight is increasing day by day among the four groups of mice.

Statistical analysis

All the statistical analysis was done using Microsoft Excel. Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

Discussion

Here, Body weight day 0, comparison of plant extract 200 mg/kg with control (negative and positive control) groups shows non-significance $P<0.05$ at significance level. So that we can accept null hypothesis that means there is difference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows non-significance $P<0.05$ at significance level. So that we can accept null hypothesis that means there is difference among the control groups.

Body weight at day 7, comparison of plant extracts 200 mg/kg with control (negative and positive control) groups shows significance $P<0.05$ at significance level. So that we can reject null hypothesis that means there is no difference among the control groups. Again, comparison of plant extracts400 mg/kg with control (negative and positive control) groups shows significance $P<0.05$ at significance level. So that we can reject null hypothesis that means there is no difference among the control groups.

Body weight at day 15, comparison of plant extracts 200 mg/kg with control (negative and positive control) groups shows significance $P<0.05$ at significance level. So that we can reject null hypothesis that means there is no difference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows significance $P<0.05$ at significance level. So that we can reject null hypothesis that means there is no difference among the control groups.

4.2.2 Hematologicalparameters

1. RBC count

Table 4.12: Effect of *E. agallocha* on Red Blood cell (RBC) count in female mice.

RBC Count (per microliter)	Negative control (per microliter)	Positive control (per microliter)	Plant extract 200 mg/kg(per microliter)	Plant extract 400 mg/kg(per microliter)
Mean ±SEM	501666.7±966666	491666±1.58E+09	473333.3±1.95E+09	486666.7±2.15E+0
p-value			0.382033	0.763416

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way RBC count was decreasing when tested with plant extract both 200 mg/kg and 400 mg/kg comparing with control groups (negative and positive).

Statistical analysis

Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

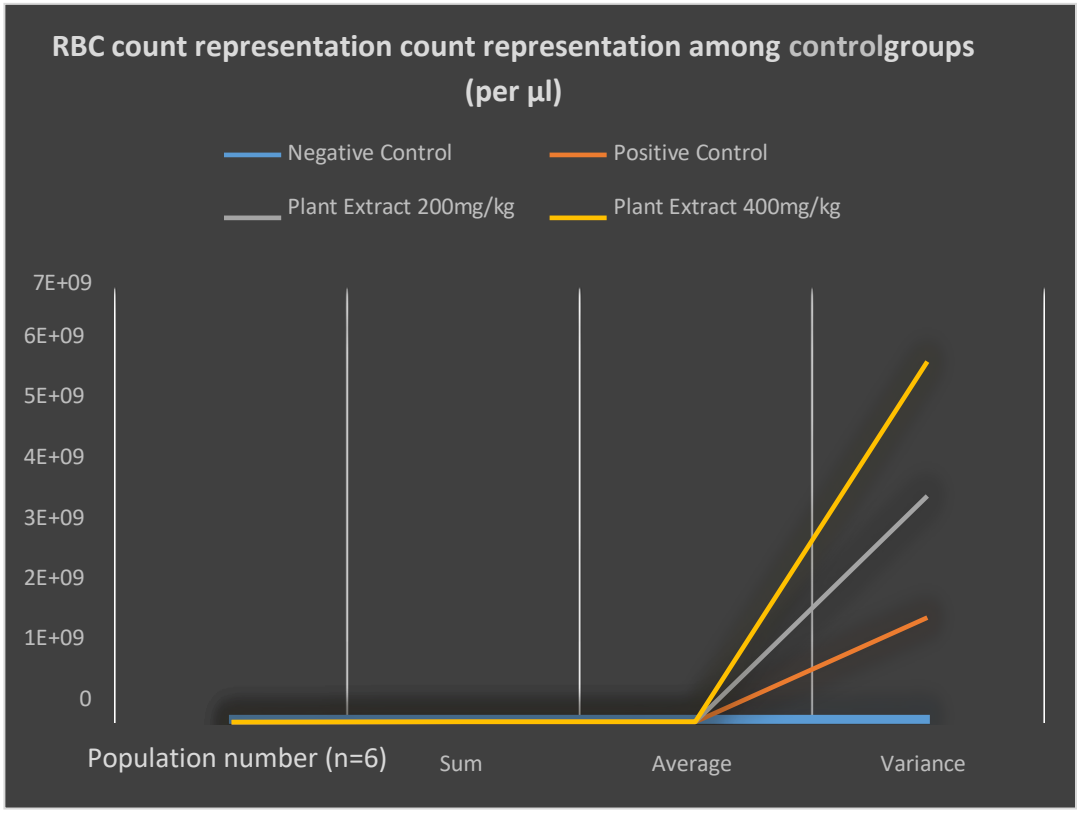


Figure 4.11:Effect of *E. agallocha* on Red Blood cell (RBC) count in female mice.

Discussion

Comparison of plant extracts 200 mg/kg with control (negative and positive control) groups shows non-significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows non-significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups.

2. **WBC count**

Table 4.13:Effect of *E. agallocha* on White Blood cell (WBC) count in female mice.

WBC count (per microliter)	Negative control (per microliter)	Positive control (per microliter)	Plant extract 200 mg/kg(per microliter)	Plant extract 400 mg/kg(per microliter)
Mean ±SEM	88333.33 ± 9.07E+08	81333.33 ± 1.09E+08	70666.67 ± 72666667	71166.67 ± 44166667
p-value			0.299122	0.310459

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way WBC count was decreasing when tested with plant extract both 200 mg/kg and 400 mg/kg comparing with control groups (negative and positive).

Statistical analysis

Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

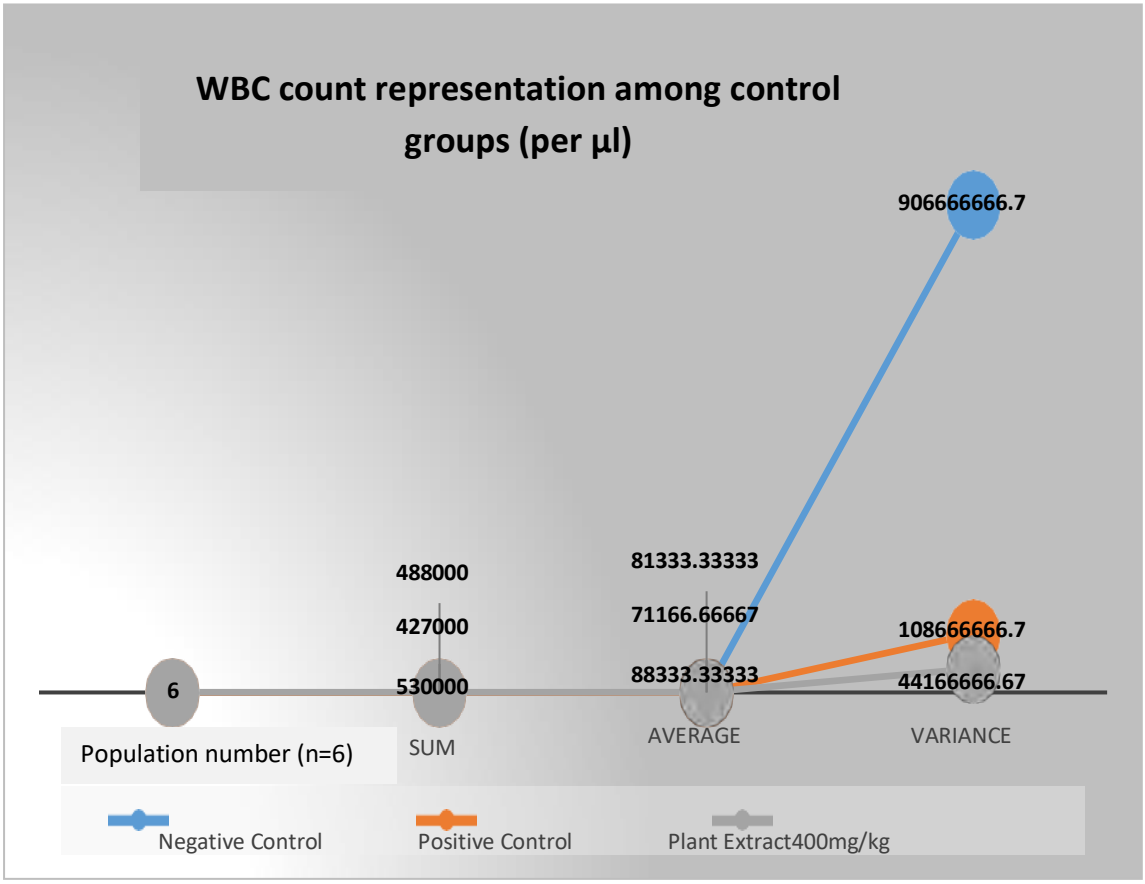


Figure 4.12: Effect of *E. agallocha* on white Blood cell (WBC) count in female mice.

Discussion

Comparison of plant extracts 200 mg/kg with control (negative and positive control) groups shows non-significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows non-significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups.

Research Findings from effect on RBC and WBC count in female mice

After 15 days of chronic administration of the *E. agallocha* bark extract the total count of red blood cell473333.3 (plantextract 200 mg/kg) and 486666.7(plantextract 400 mg/kg) and

whiteblood cell70666.67 (plant extract 200mg/kg) and 71166.67 (plant extract 400 mg/kg) were determined in the female mice group.

At a low dose, there is a decrease in the number of white blood cell count of the female mice, the decrease though not significant yet it was prominent (p=0.299).

Whereas at the higher dose, there is a statistically not significant (p= 0.31045) decreases in the number of white blood cell count of the female mice.

Significance of WBC count

WBC

White blood cells are responsible for detecting and destroying diseases that come into our body (Moffat,2013). Blood leucocytes (WBC's) consist of five cell lines (neutrophils, monocytes, eosinophils, basophils, and lymphocytes). In man, there are ~ 4-11×10^3 WBC's/microliter blood, whereas in mice ~ 2-10 ×10^3 WBC's /microliter blood. In man, Granulocyte (neutrophils, eosinophils, and basophils) comprise ~50-70% of the total number of circulating WBC's. Lymphocytes comprise ~ 20-40% and monocytes ~8%; whereas mice have ~10-25% granulocytes, ~% lymphocytes and ~ 1-5% monocytes (Travlos, 2007).

From study of Swiss albino female mice (n=6) the mean for WBC count was found to be 9.46×10^3 per microliter with a standard deviation of 3.09×10^3 per microliter(Matsuzawa et al., 1993).

From an agewise study of 6-7 weeks old Swiss albino mice (n= 6) the mean ± std.dev.for WBC count was found to be 12.9 ± 2.4 × 10^3 per microliter with a minimum value of 8.1 and a maximum value of 21.5; and with 13 weeks old mice (n=6) the mean± std.dev for WBC count was found to be 10.8±2.4 ×10^3 per microliter with a minimum value of 5.9 and a maximum value of 19.0 (Petterino et al., 2006).

The WBC in female mice range between 10× 10^3 to 15× 10^3per microliters with a mean 12.4× $10^3$of per microliter(Car et al., 2006).

Significance of WBC Count Result

WBC

White Blood Count too high: Rule out infection and leukemia, known as leukocytosis (Moffat, 2013)

-Malignant Neoplasms, especially bronchogenic carcinoma

-Infection, myeloproliferative disorders, Allergies, asthma

-Tissue death (trauma, burns, heart attack)

1. Leukocytosis:

    a. It is usually caused by an increase of only one type of leukocyte, and it is given the name of type of cell that shows the main increase:

        (1) Neutrophilic leukocytosis or neutrophilia
        (2) Lymphocytic leukocytosis or lymphocytosis
        (3) Monocyte leukocytosis or monocytosis
        (4) Basophilic leukocytosis or basophilic
        (5) Eosinophilic leukocytosis or eosinophilia

    b. An increase in circulating leukocyte is rarely caused by a proportional increase in leukocytes of all types. When this does occur, it is usually of hem concentration.
    c. In certain diseases (e.g., measles, pertussis, Sepsis), the increase of leukocytes is so great that the blood picture suggests leukemia. Leukocytosis of a temporary nature (leukemia) must be distinguished from leukemia. In leukemia, the leukocytosis is permanent and progressive.
    d. Leukocytosis occurs in acute infections, in which the degree of increase of leukocytes depends on severity of the infection, patient's resistance, patient's age, and marrow efficiency and reserve.

e. Other causes of leukocytosis include the following :
   (1) Leukemia, myeloid proliferative disorders
   (2) Trauma or tissue injury (e.g., surgery)
   (3) Toxins, uremia, coma, eclampsia, thyroid stoma
   (4) Drugs, especially ether, chloroform, quinine, epinephrine (Adrenalin), colony-stimulating factors
   (5) Acute hemolysis
   (6) Hemorrhage (acute)
   (7) After splenectomy
   (8) Polycythemia Vera
   (9) Tissue necrosis
f. Occasionally, leukocytosis is found when there is no evidence of clinical disease. Such findings suggest the presence of:
   (1) Sunlight, ultraviolet irradiation
   (2) Physiologic leukocytosis resulting from excitement, stress, exercise, pain, cold or heat, anesthesia
   (3) Nausea, vomiting, seizers
g. Steroid therapy modifies the leukocyte response
   (1) When corticotrophin (adrenocorticotropic hormone, or ACTH) is given to a healthy person, leukocytosis occurs
   (2) When ACTH is given to a patient with severe infection, the infection can spread rapidly without producing the expected leukocytosis; therefore, what would normally be an important sign is obscured.

3. Lymphocyte count

Table 4.14: Effect of *E. agallocha* on Lymphocyte cell count in female mice.

Lymphocyte count (per microliter)	Negative control (per microliter)	Positive control (per microliter)	Plant extract 200 mg/kg(per microliter)	Plant extract 400 mg/kg(per microliter)
Mean ±SEM	25 ± 348.8	37.33333 ± 20.26667	33.16667 ± 96.96667	32.66667 ± 4.266667
p-value			0.250501	0.188381

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way Lymphocyte count was decreasing when tested with plant extract both 200 mg/kg and 400 mg/kg comparing with control groups (negative and positive).

Statistical analysis

Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

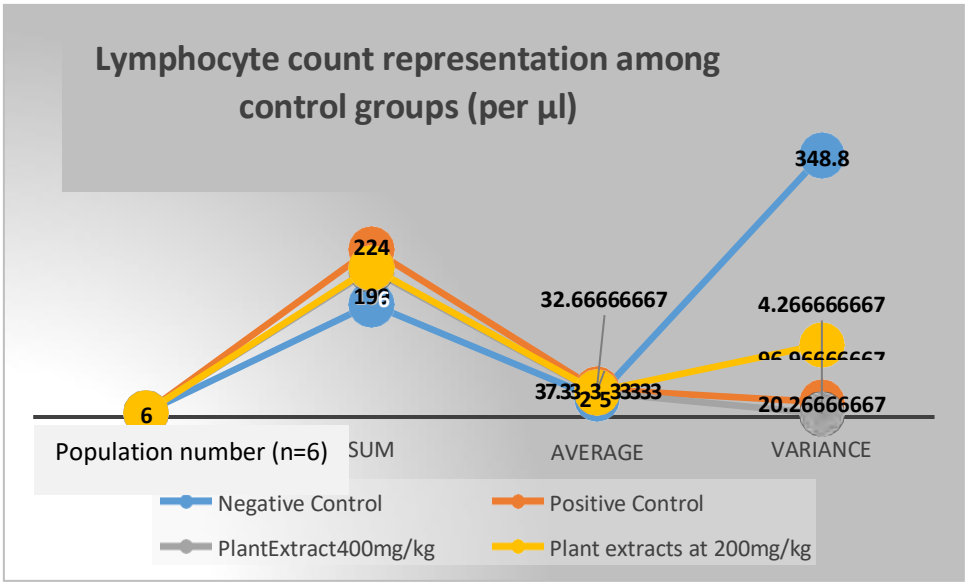


Figure 4.13:Effect of *E. agallocha* on Lymphocyte cell count in female mice.

Discussion

Comparison of plant extract 200 mg/kg with control (negative and positive control) groups shows non-significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows non-significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups.

4. Monocyte count

Table 4.15:Effect of *E. agallocha* on Monocyte cell count in female mice.

Monocyte count (per microliter)	Negative control(per microliter)	Positive control (per microliter)	Plant extract 200 mg/kg(per microliter)	Plant extract 400 mg/kg(per microliter)
Mean ±SEM	2.5±0.3	3±0	2.666667±0.266667	2.833333±0.166667
p-value			0.162153	0.1156

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way Monocyte count was decreasing when tested with plant extract both 200 mg/kg and 400 mg/kg comparing with control groups (negative and positive).

Statistical analysis

Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

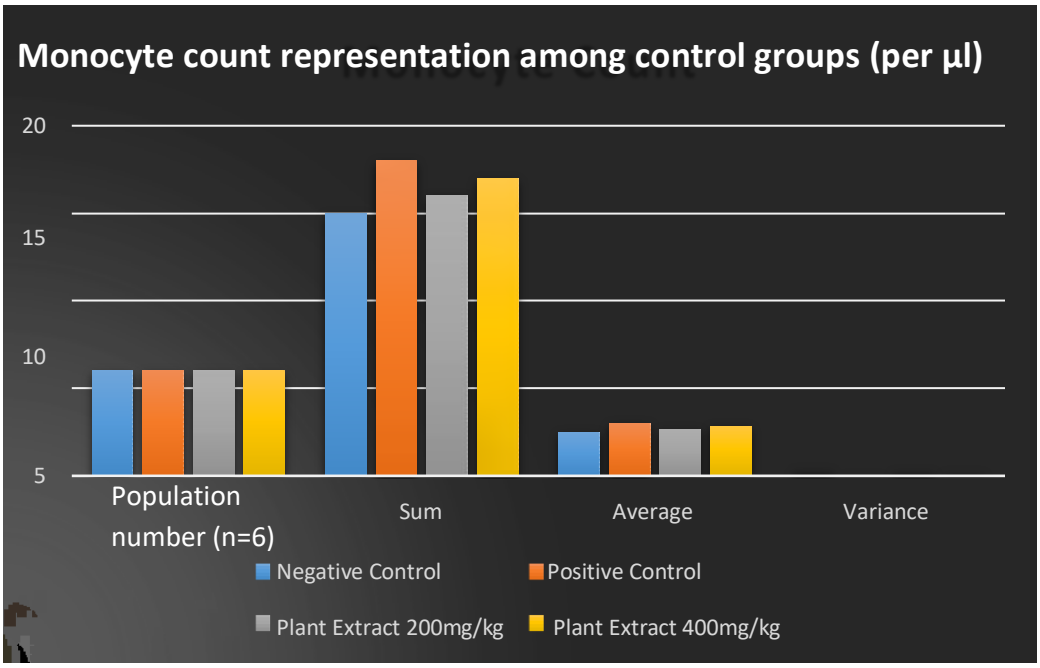


Figure 4.14:Effect of *E. agallocha* on Monocyte cell count in female mice.

Discussion

Comparison of plant extract 200 mg/kg with control (negative and positive control) groups shows non-significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows not significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups.

Lymphocytes

Lymphocytes can be the most numerous cell type in rodents. Lymphocytes, unlike the other leucocytes, are produced in lymphoid tissue rather than in marrow. Most of the lymphocytes in blood are long-lived cells that recirculate between blood and tissue. Changes in distribution rather than changes in production or loss (French et al., 2013).

Lymphocytes live in the immune rich tissue like the lymph nodes, spleen, tonsils, thymus, gastrointestinal lymphoid tissue, bronchial lymphoid tissue, bone marrow and in the blood. There are lots of lymph nodes in the breast, under the arm, behind the knees, in the neck, and in the groin areas. Lymphocytes are specifically attracted to viruses, pollens and cells that have anything wrong on them on the outside of the cell (this is called cell-mediated immunity). They live a lot longer (weeks) than neutrophils and recirculate from the tissue to the blood and back. They go where they are needed (Moffat, 2013).

The lifespan of rat lymphocytes are 60-100 days, in male Sprague-Dawley rats the lymphocytes range between 6.10-18.45 $\times 10^3$ per microliter with a mean of 10.85 $\times 10^3$ per microliter (Car et al., 2006).

Monocytes

Monocytes share a common committed stem cell with neutrophils. They are produced in marrow, circulate briefly in blood, and migrate into tissues where they differentiate further to become macrophages. There is no storage pool of monocytes in marrow at a given time are very small. Monocytes in blood are unevenly distributed between a marinated and circulating pool. Corticosteroid affect differs among species. Factors produced at sites of inflammation can increase monocyte production (French et al., 2013).

The lifespan of [mouse] monocytes are 18 hours, in male Sprague-Dawley rats the Monocytes range between 0.04-0.50$\times 10^3$ per microliter with a mean of 0.20$\times 10^3$ per microliter (Car et al., 2006).

Research findings

Lymphocyte

Lymphocytes too high (Lymphocytosis) (Moffat et al., 2013)

- Chronic inflammatory disorder (e.g., ulcerative colitis)
- Lymphocytic leukemia, lymphoma
- Stress (acute)

1. Lymphocytosis: >4000/mm^3 or 4.0× 10^9/L in adults; > 7200/mm^3 or 7.2× 10^9 in children; and > 9000/mm^3 or 9.0 × 10^9/L in infants occur in:

    a. Lymphatic leukemia (acute and chronic) lymphoma
    b. Infectious lymphocytosis
    c. Infectious mononucleosis
        (1) Caused by Epstein-Barr virus
        (2) Most common in adolescents and young adults
        (3) Characterized by atypical by a typical lymphocytes (Downey cells) that are larger, deeply indented, with deep blue (basophilic) cytoplasm
        (4) Differential diagnosis-positive heterophil test
    d. Other viral diseases:
        (1) Viral infections of the upper respiratory tract (pneumonia)
        (2) Cytomegalovirus
        (3) Measles, mumps, chickenpox
        (4) Acute HIV infection
        (5) Infectious hepatitis (acute viral hepatitis)
        (6) Toxoplasmosis
    e. Some bacterial diseases such as tuberculosis, brucellosis (undulant fever), and pertussis
    f. Crohn's disease
    g. Serum sickness, drug hypersensitivity
    h. Hypo-adrenalism, Addison's disease

i. Thyrotoxicosis
j. Neutropenia

Monocyte

In the low dose (50 mg/kg), CTRK caused monocytes too low

Repeated low counts can indicate (Moffat, 2013):

a. Bone marrow damage or failure
b. Hairy cell leukemia

Decreased monocyte count (<100 cells/mm^3 or < 0.1×10^9/L) is not usually identified with specific diseases:

a. Prednisone treatment
b. Hairy cell leukemia
c. Overwhelming infection that also causes neutropenia
d. Human immunodeficiency virus (HIV) infection
e. Aplastic anemia (bone marrow injury)

5. Neutrophil count

Table 4.16:Effect of *E. agallocha* on neutrophil count in female mice.

Neutrophil count (per microliter)	Negative control (per microliter)	Positive control (per microliter)	Plant extract 200 mg/kg(per microliter)	Plant extract 400 mg/kg(per microliter)
Mean ±SEM	63.16667±296.5667	47.33333±18.66667	51±95.2	43.66667±18.26667
p-value			0.079453	0.013643

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way neutrophil count was decreasing when tested with plant extract both 200 mg/kg and 400 mg/kg comparing with negative control group but increasing then positive control group.

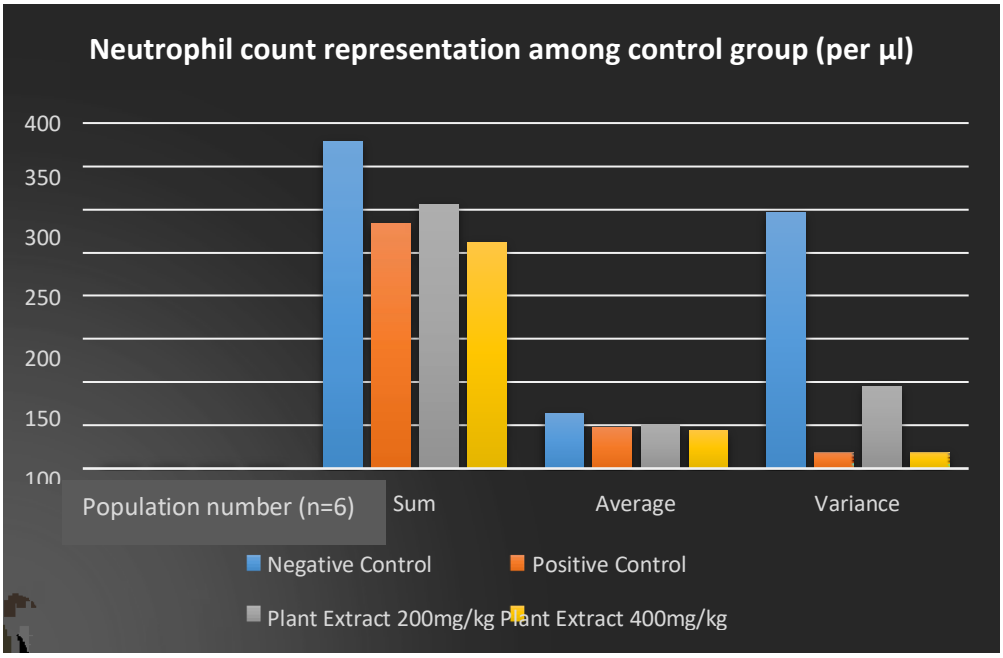


Figure 4.15: Effect of *E. agallocha* on neutrophil count in female mice.

Statistical analysis

Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

Discussion

Comparison of plant extracts 200 mg/kg with control (negative and positive control) groups shows significance P<0.05 at significance level. So that we can reject null hypothesis that means there is nodifference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows significance P<0.05 at significance level. So that we can reject null hypothesis that means there is no difference among the control groups.

6. Eosinophil count

Table 4.17:Effect of *E. agallocha* on eosinophil count in female mice.

Eosinophil count (per microliter)	Negative control (per microliter)	Positive control (per microliter)	Plant extract 200 mg/kg (per microliter)	Plant extract 400 mg/kg (per microliter)
Mean ±SEM	2.333333±0.266667	3.166667±0.566667	2.666667±0.666667	2.5±0.3
p-value			0.155653	0.075298

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way eosinophil count was increasing when tested with plant extract both 200 mg/kg and 400 mg/kg comparing with negative control group but decreasing then positive control group.

Statistical analysis

Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

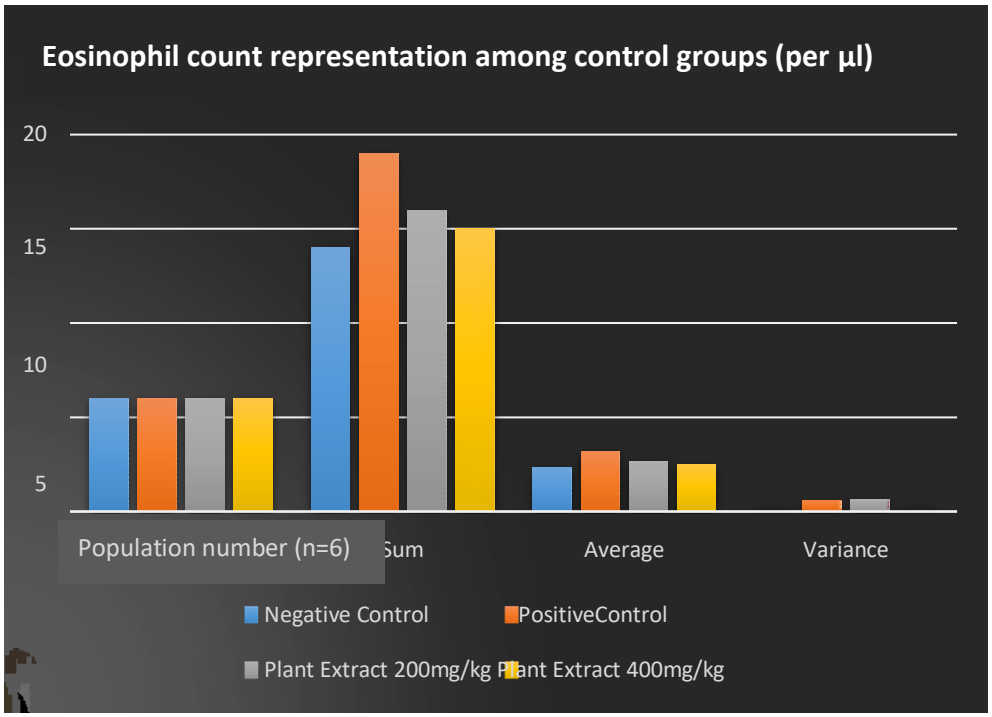


Figure 4.16:Effect of *E. agallocha* on eosinophil count in female mice.

Discussion

Comparison of plant extracts 200 mg/kg with control (negative and positive control) groups shows non-significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows non-significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups.

7. Basophil count

Table 4.18:Effect of *E. agallocha* on basophil count in female mice.

Basophil Count (per microliter)	Negative control (per microliter)	Positive control (per microliter)	Plant extract 200 mg/kg (per microliter)	Plant extract 400 mg/kg (per microliter)
Mean ±SEM	7±4.4	9.833333±10.96667	11.16667±4.166667	14.66667±21.86667
p-value			0.036247	0.006219

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way basophil count was increased when tested with plant extract both 200 mg/kg and 400 mg/kg compared with both negative and positive control group.

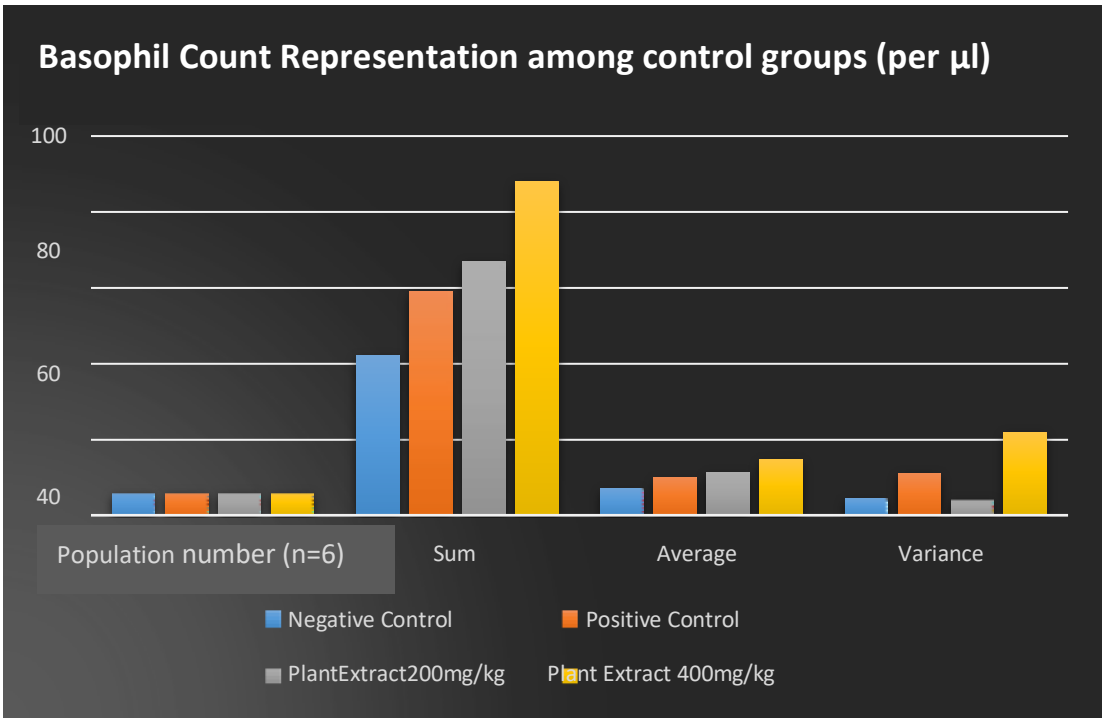


Figure 4.17: Effect of *E. agallocha* on basophil count in female mice.

Statistical analysis

Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

Discussion

Comparison of the plant extractsat 200 mg/kg with control (negative and positive control) groups shows significance $P<0.05$ at significance level. So that we can reject null hypothesis that means there is nodifference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows significance $P<0.05$ at significance level. So that we can reject null hypothesis that means there is no difference among the control groups.

Significance of Neutrophil, Eosinophil, Basophil:

Neutrophils

Neutrophil is a type of white blood cell that circulates in both tissues and blood. Once they move from the circulation into the tissues, they don't return. They are replaced two times or more each day, so that these cells can really multiply when there is a problem (Moffat, 2013).

Neutrophils are produced in bone marrow, released into blood after maturation in marrow, circulate for less than a day (10-15hours), and migrate out of the vessels into tissues or into alveoli and gut lumen. While circulating, neutrophils are distributed between large vessels (neutrophils here from the circulating neutrophil pool or CNP; these are the neutrophils in the usual blood sample) and small vessels (neutrophils here from the marinating neutrophil pool or MNP). The total body neutrophil pool or TNP comprises the CNP, MNP, and the pool of post-mitotic neutrophils in marrow (French et al., 2013).

Humoral factors produced at sites of inflammation stimulate increased production of neutrophils in marrow and increased release of neutrophils from marrow. Chemotactic factors produced at sites of inflammation direct migration of neutrophils from blood vessels into tissue at those sites (French et al., 2013).

Normally, neutrophils are released from marrow in an age-dependent manner, i.e., the most mature cells are released before less mature cells. Corticosteroids tend to cause movement of neutrophils from the MNP to the CNP and release of some cells from the marrow pool, thereby raising the neutrophil count in a blood sample. Endotoxin tends to cause sequestration of neutrophils in the spleen, liver, and lung, thereby lowering the neutrophil count in blood sample; endotoxin also stimulates release of neutrophils from marrow and increases granulocytopoiesis resulting in a rebound rise in neutrophil count with presence of immature neutrophils (bands, matamyelocytes) (French et al., 2013)

The life span of mice neutrophils are 6 hours, in male Sprague-Dawley rats the Neutrophil range between 0.37-2.63×10^3 per microliter with a mean of 0.95 ×10^3 per microliter (Car et al., 2006).

Neutrophils (lab)-Decreased

Neutropenia (decreased neutrophils)

a. Causes associated with decreased or ineffective production:

    (1) Inherited stem cell disorders and genetic disorders of cellular development
    (2) Acute overwhelming bacterial infections (poor prognosis) and septicemia
    (3) Viral infections (e.g. mononucleosis, hepatitis, influenza, measles)
    (4) Some rickettsial and parasitical (protozoan) diseases (malaria)
    (5) Drug, chemicals, ionizing, radiation, venoms
    (6) Hematopoietic disease (e.g., a plastic anemia, megalobasticanemias, iron-deficiency anemia, a leukemia, myeloprolifirative diseases)

b. Causes associated with decresed survival:
    (1) Infections mainly in persons with little or no marrow reserves, elderly people and infants
    (2) Collagen vascular diseases with antineutrophil antibodies (e.g. systemic lupus erythematosus [SLE] and Felty's syndrome)
    (3) Autoimmune and isoimmune causes

(4) Drug sensitivity (There is an extensive list of drugs that continue to grow. Women are more likely than men to have a drug sensitivity. Removal of offending drug results in return to normal).

(5) Splenic sequestration

c. Neutropenia in neonates

(1) Maternal neutropenia, maternal drug ingestion, maternal isoimmunization to fetal leukocytes (maternal immunoglobulin G[IgG] antibodies to fetal neutrophils)

(2) Inborn errors of metabolism (e.g., maple syrup urine disease)

(3) Immune deficits- acquired

(4) Deficits and disorders of myeloid stem cell (e.g., Kostomann'sagranulocytosis, begin chronic granulocytopenia of childhood)

(5) Congenital neutropenia

d. Pregnancy-progressive decrease until labor

Eosinophil

Characteristics

1. Function: Response to Allergy and Parasitis infection
    - Respond to mast cell, Basophil chemotactic factors
2. Morphology on blood Smear
    - Granulocyte stains brightly with Eosin Stain

Eosinophils are produced in marrow, circulate in blood for a few hours, and migrate into tissues where they survive for several days. Increased production of eosinophils is mediated by factors produced by some activated T lymphocytes. Corticosteroids decrease blood eosinophil numbers but increase the marrow pool of eosinophils(French et al., 2013).

Increased numbers of circulating eosinophils may be seen in hypersensitivity reactions, as certainforms of parasitism and allergic conditions. Eosinophils are elevated (eosinophilia) with

parasite infestation, allergies, myositis, eosinophilicgastroenteritis, panosteitis and mast cell neoplasia (Moffat, 2013).

Eosinophils (LAB) - Decreased

Eosinopenia (decreased circulating eosinophils) is usually caused by an increased renal steroid production that accompanies most conditions of bodily stress and is associated with:

a. Cushing's syndrome (acute adrenal failure): $<50/mm^3$
b. Use of certain drugs such as ACTH, epinephrine, thyroxine, prostaglandins
c. Acute bacterial infections with marked shift to the left (increase in immature leukocytes)

Basophils

Characteristics

1. Function: Allergic Response
   -Similar mechanism of action to mast cells
   -Related to delayed hypersensitivity response
   -Triggered by IgE binding to antigen
   -Releases inflammatory mediators
   a. performed mediators (e.g. Histamine, Bradykinin)
   b. New Mediators (e.g. prostaglandins, Leukotrienes)

2. Morphology on Blood smear

   a. Granulocyte cytoplasm stains darkly with wright stain

b. Two lobed nucleus

Basophils are normally of similar size to neutrophils or may be slightly larger. The distinctive feature of basophils their lavender to purple cytoplasmic granules (containing histamine, heparin, chondroitin sulfate, and substances) and their long "ribbon-like" nuclei. Basophils are produced in marrow. The number in blood is very small. Basophilia sometimes occurs concurrent with eosinophilia (French et al., 2013).

In female mice Basophil range between 0.01-0.02×10^3 per microliter with a mean of 0.05×10^3 per microliter (Car eta l., 2006). Basophils are the least numerous of the white blood cells. Observing even a few cells on the blood smear usually attracts attention (Moffat, 2013).

8. SGPT

Table 4.19: Effect of *E. agallocha* on SGPT in female mice.

SGPT	Negative control (U/L)	Positive control (U/L)	Plant extract 200 mg/kg (U/L)	Plant extract 400 mg/kg (U/L)
Mean ±SEM	35.33333±71.86667	29.66667±91.06667	33.66667±91.46667	53.33333±701.8667
p-value			0.561409	0.070659

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way SGPT level is increasing when tested with plant extract both 200 mg/kg and 400 mg/kg comparing with both negative and positive control group.

Statistical analysis

Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

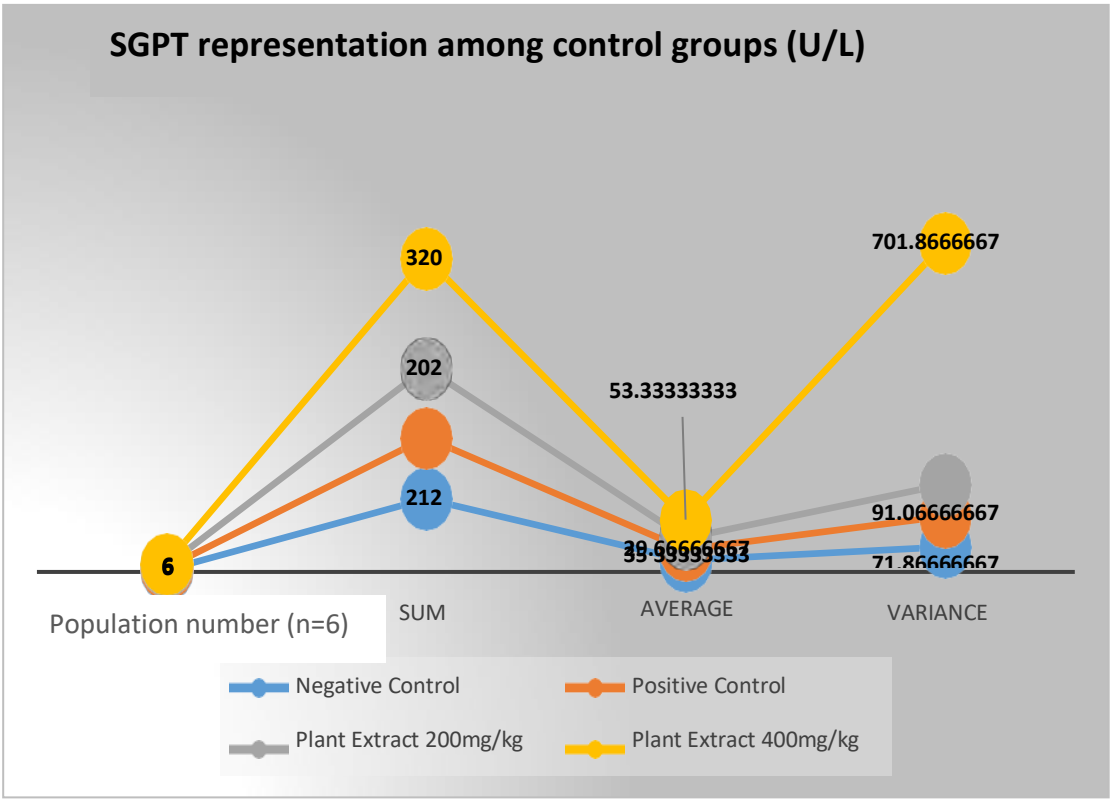


Figure 4.18:Effect of *E. agallocha* on SGPT in female mice.

Discussion

Comparison of plant extracts 200 mg/kg with control (negative and positive control) groups shows not significant P<0.05 at significance level. So that we can accept null hypothesis that means there isdifference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows not significant P<0.05 at significance level. So that we can reject null hypothesis that means there is difference among the groups.

9. SGOT

Table 4.20: Effect of *E. agallocha* on SGOT in female mice.

SGOT	Negative control (U/L)	Positive control (U/L)	Plant extract 200 mg/kg (U/L)	Plant extract 400 mg/kg (U/L)
Mean ±SEM	43.83333±153.7667	39±70	46.66667±52.66667	56±152.8
p-value			0.398614	0.05049

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way SGOT level is increasing when tested with plant extract both 200 mg/kg and 400 mg/kg comparing with both negative and positive control group.

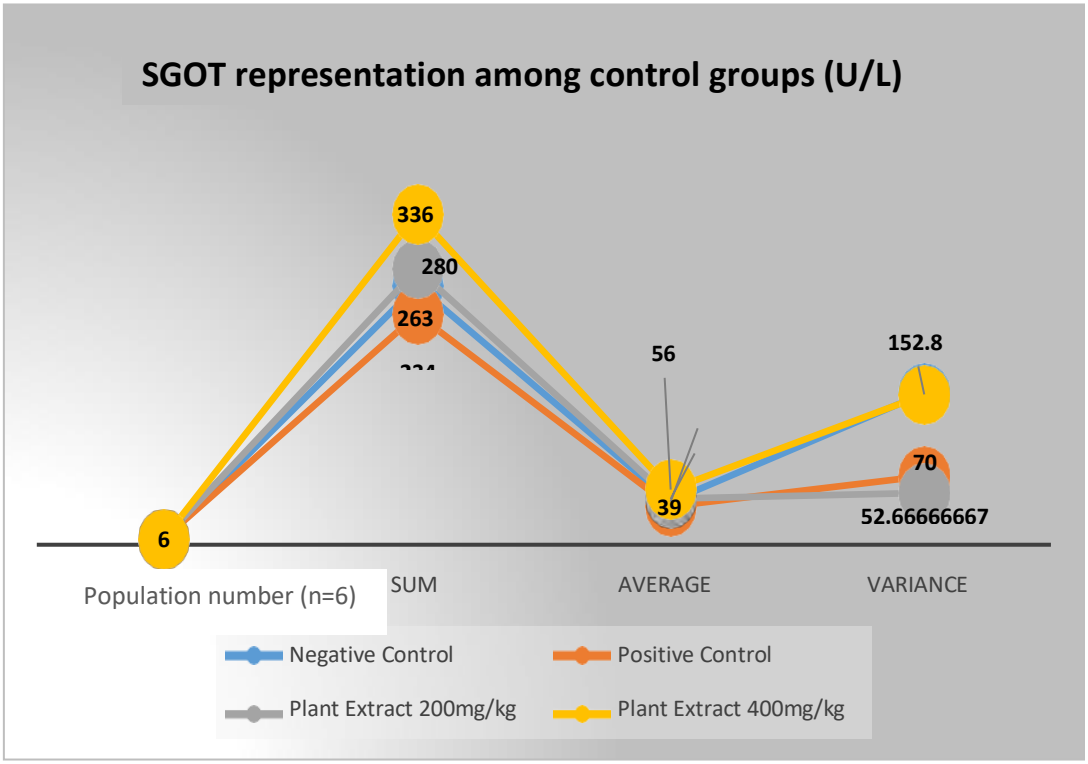


Figure 4.19:Effect of *E. agallocha* on SGOT in female mice.

Statistical analysis

Values are presented as mean ± SEM (n=6). Single factor ANOVA analysis was performed to analyze this dataset when compared against control (negative and positive control).

Discussion

Comparison of plant extracts 200 mg/kg with control (negative and positive control) groups shows not significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows significance P<0.05 at significance level. So that we can reject null hypothesis that means there is no difference among the control groups.

10. ALP

Table 4.21: Effect of *E. agallocha* on ALP in female mice.

ALP	Negative control (U/L)	Positive control (U/L)	Plant extract 200 mg/kg (U/L)	Plant extract 400 mg/kg (U/L)
Mean ±SEM	71.66667±51.46667	78.5±124.3	73±25.2	86±334.8
p-value			0.335645	0.197298

Result

After injecting EAC cell line in the mice model Intraperitoneal (IP) way basophil count is increasing when tested with plant extract both 200 mg/kg and 400 mg/kg comparing with both negative and positive control group.

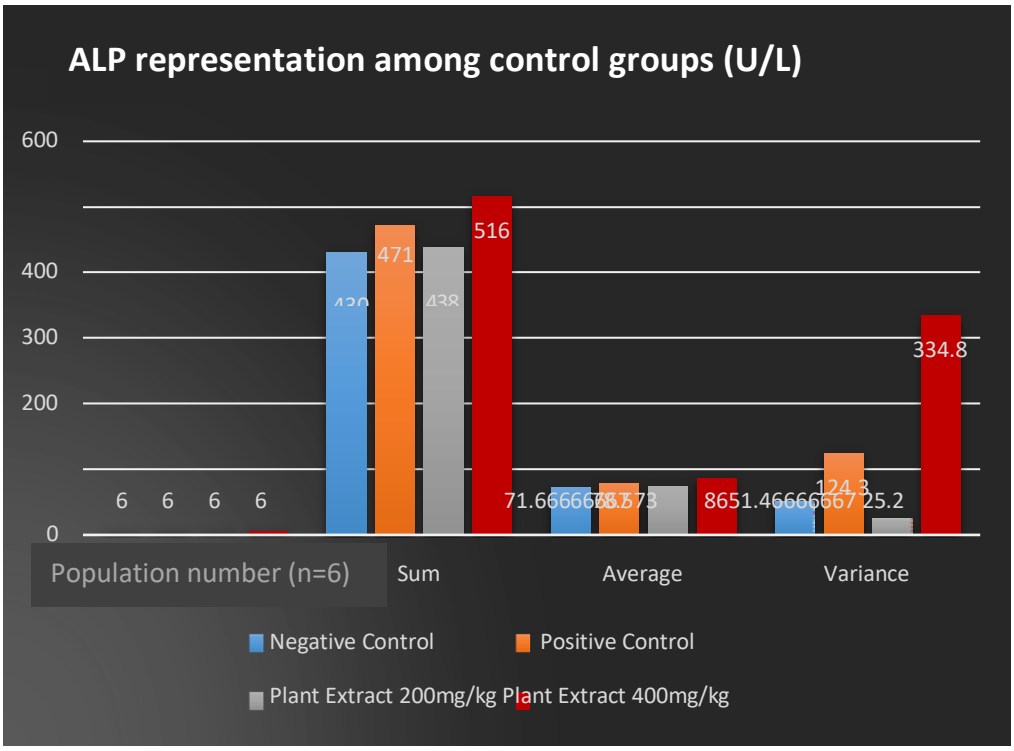


Figure 4.20:Effect of *E. agallocha* on ALP in female mice.

Discussion

Comparison of plant extracts 200 mg/kg with control (negative and positive control) groups shows not significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups. Again, comparison of plant extracts 400 mg/kg with control (negative and positive control) groups shows not significance P<0.05 at significance level. So that we can accept null hypothesis that means there is difference among the control groups.

Research findings of SGPT, SGOT and ALP

Among the most sensitive and widely used liver enzymes are the aminotransferases. They include aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT). These enzymes are normally predominantly contained within liver cells and to a lesser

degree in the muscle cells. If the liver is injured or damaged, the liver cells spill these enzymes into the blood, raising the AST and ALT enzyme blood levels and signaling liver disease.

AST (SGOT) is normally found in a variety of tissues including liver, heart, muscle, kidney, and the brain. It is released into the serum when any one of these tissues is damaged. For example, AST level in serum is elevated in heart attacks or with muscle injury. It is therefore, not a highly specific indicator of liver injury as its elevation can occur as a result of other injured tissues.

ALT (SGPT) is, by contrast, normally found largely in the liver. This is not to say that it is exclusively located in the liver, but that is where it is most concentrated. It is released into the bloodstream as the result of liver injury. Thus, it serves as a fairly specific indicator of liver status.

ALP (alkaline phosphatase): The liver synthesizes the highest amounts of this enzyme so high levels in the blood may suggest liver injury among other causes.

In human

The normal range of values for AST (SGOT) is about 5 to 40 units per liter of serum (the liquid part of the blood).

- The normal range of values for ALT (SGPT) is about 7 to 56 units per liter of serum.

- Normal levels of ALP are about 45 to 115 U/L.

High level causes

- Acute viral hepatitis A may develop very high AST and ALT levels
- Chronic hepatitis C infection
- Celiac disease (celiac sprue) is a disease of the small intestine where a person has an allergy to gluten and develops gas, bloating, diarrhea, and in advanced cases malnutrition.

Table 4.22: All the data of hematological parameters.

Hematological test	Data analysis	Negative Control	Positive Control	Plant Extract 200mg/kg	Plant Extract 400mg/kg
WBC	Mean ± SEM	88333.33 ± 9.07E+08	81333.33 ± 1.09E+08	70666.67 ± 72666667	71166.67 ± 44166667
	p-value			0.299122	0.310459
Lymph	Mean ± SEM	25 ± 348.8	37.33333 ± 20.26667	33.16667 ± 96.96667	32.66667 ± 4.266667
	p-value			0.250501	0.188381
Mono	Mean ± SEM	2.5±0.3	3±0	2.666667±0.266667	2.833333±0.166667
	p-value			0.162153	0.1156
Neu	Mean ± SEM	63.16667±296.5667	47.33333±18.66667	51±95.2	43.66667±18.26667
	p-value			0.079453	0.013643
Eosino	Mean ± SEM	2.333333±0.266667	3.166667±0.566667	2.666667±0.666667	2.5±0.3
	p-value			0.155653	0.075298
Baso	Mean ± SEM	7±4.4	9.833333±10.96667	11.16667±4.166667	14.66667±21.86667
	p-value			0.036247	0.006219
RBC	Mean ± SEM	501666.7±96666667	491666.7±1.58E+09	473333.3±1.95E+09	486666.7±2.15E+09
	p-value			0.382033	0.763416
SGPT	Mean ± SEM	35.33333±71.86667	29.66667±91.06667	33.66667±91.46667	53.33333±701.8667
	p-value			0.561409	0.070659
SGOT	Mean ± SEM	43.83333±153.7667	39±70	46.66667±52.66667	56±152.8
	p-value			0.398614	0.05049
ALP	Mean ± SEM	71.66667±51.46667	78.5±124.3	73±25.2	86±334.8
	p-value			0.335645	0.197298

Summary of the test result of homology parameters

Effect of RBC: After the effect of plant extract RBC count decreases than control groups (negative and positive).

Effect of WBC: After the effect of plant extract WBC count decreases than control groups (negative and positive).

Effect of Lymphocyte: After the effect of plant extract lymphocyte count increases than negative control group and decreases then positive control group.

Effect of Monocyte: After the effect of plant extract monocyte count increases than negative control group but decreases then positive control group.

Effect of Neutrophil: After the effect of plant extract neutrophil count decreases than control groups (negative and positive).

Effect of Eosinophil: After the effect of plant extract eosinophil count increases than negative control group but decreases then positive control group.

Effect of Basophil: After the effect of plant extract basophil count increases than control groups (negative and positive).

Effect of SGPT: After the effect of plant extract SGPT level increases than control groups (negative and positive).

Effect of SGOT: After the effect of plant extract SGOT level increases than control groups (negative and positive).

Effect of ALP: After the effect of plant extract ALP level increases than control groups (negative and positive).

Conclusion

Mangroves are potentially of great commercial value but this is often not recognized. Mangrove plants have the ability to grow where no other vascular plants can, as shown by their existence in calm, nutrient-rich environments. They thrive under stressful and extreme tropical environmental conditions such as high concentration of moisture and high temperature. They exist in muddy, shifting, saline and anaerobic conditions, soil acidity and high-low tides of brackish water. Hence, the plants that can survive in these extreme habitats have evolved special methods to survive. Production of unique chemicals may be one such strategy. It is therefore reasonable to assume that the mangrove plants produce metabolites which in turn are unique to them and are of interest to the scientist.

In this study, an exploratory extraction was performed on an adequate amount of mangrove plant*E. agallocha*(bark)to obtain an extract of varying polarity using methanol, chloroform, N-hexane and petroleum benzene to gain medium-polar and polar compounds extract by kuchan method of solvent-solvent partitioning. Furthermore, *in vitro* antioxidant activity was determined by four tests (the determination of cupric reducing antioxidant capacity, total antioxidant content, total phenolic and flavonoid content)and *in vivo* anticancer activity of *E. agallocha*bark extracthave a significant role both *in vitro* and *in vivo* model.

Our *in vitro* studies provide sound scientific footing to enhance confidence on the traditional claims of *E. agallocha bark*. The present research study deals with the evaluation of antioxidant and in vivo anticancer activity of methanolic*E. agallocha* bark extract.

In this research study, phytochemical screening of the plants revealed that methanolic*E. agallocha* bark extract exhibited significant antioxidant activity as measured by various antioxidant assays.

The administration of traditional preparation without any standard dosage coupled with scarcity of adequate scientific studies on their safety have raised concerns on their toxicity. In the*in vivo* anticancer activity, the effects of chronic administration of *E. agallocha* bark extract werestudied over the period of 15 days. Science there was Blood cell count (WBC, Lymphocyte count, Monocyte count, etc.) showed lower cell count and also high effect was observed on the levels

oftransaminases (SGOT, SGPT, and ALT), which are good indicators of liver and kidneyfunctions. It is reasonable to suggest that the *E. agallocha* bark extract did not induce anydamage to liver and kidneys. There was also no alteration of plasma protein and albumin levelsof treated animals compared to respective control group. In addition, all the hematological valuesin the treatment groups were in the range of the normal groups. Thus no acute or sub-chronictoxicity was observed. In conclusion, *E. agallocha* bark extract can be considered devoid of anytoxic risk.

Further studies are required to fractionate the extract, to identify the bioactive compounds and should be directed to carry out in vivo studies of its medicinal active components in order to determine their extract mechanism of action and to improve nutritional profile and health benefits as well as to prepare natural pharmaceutical products of high value.

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