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Phytochemical and Biological Studies on the Plants Calotropis gigantea (Linn) and Amoora rohituka Roxb

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University of Rajshahi

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THESIS SUBMITTED FOR THE DEGREE OF
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Ph.D. THESIS

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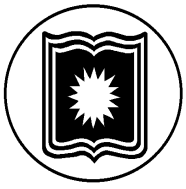

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DECLARATION

I do hereby declare that, the work presented in this thesis entitled "**Phytochemical and Biological Studies on the Plants *Calotropis gigantea* (Linn.) and *Amoora rohituka* (Roxb.)**" submitted to the University of Rajshahi, Bangladesh, for the degree of **Doctor of Philosophy** in Science are the original research work of mine and neither of this thesis nor any part of it has been submitted previously for any degree or diploma anywhere.

M. Rowshanul Habib

June, 2013

CERTIFICATE

This is to certify that the thesis entitled "**Phytochemical and Biological Studies on the Plants *Calotropis gigantea* (Linn) and *Amoora rohituka* Roxb.**" submitted by **M. Rowshanul Habib** for the award of a Ph.D. (Science) degree of Rajshahi University, is absolutely based on his own work under my supervision and neither of this thesis nor any part of it has been submitted for any degree/deploma or other academic award anywhere before.

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The author

M. Rowshanul Habib

Abstract

The universal role of medicinal plants in the treatment of diseases is established by their employment in all important systems of medicine. Many active drugs have been derived from different medicinal plants, and the process is going on. As a preliminary approach along this direction, this study was designed to carry out phytochemical and biological studies on the two important medicinal plants, *Calotropis gigantea* (Linn.) and *Amoora rohituka* (Roxb.).

The plant *Calotropis gigantea* (Linn.) (Bengali Name: Boro Akanda) belonging to the Asclepiadaceae family has wide folk medicinal uses and various parts of this plant possess different pharmacological properties. As a part of phytochemical investigation of this study, the root bark and flower powder of *Calotropis gigantea* was extracted with methanol and ethyl acetate, respectively, at room temperature to get methanol (ME) and ethyl acetate (EECF) extracts. ME was fractionated with petroleum ether and chloroform successively to yield petroleum ether (PEF) and chloroform (CF) soluble fractions, respectively. EECF was applied on silica gel column chromatography using n-hexane with a gradient of ethyl acetate. Fractions 21~30 afforded white crystals as compound-2 whereas fractions 40~48 were combined and subjected on preparative thin layer chromatography (PTLC) to find colorless oily liquid as compound-1**.** Based on TLC profile, fractions 7~13 were combined and rechromatographed on silica gel column eluting with n-hexane and ethyl acetate (19 : 1) and three compounds were purified as compound-3 (white crystals), compound-4 (white amorphous powder) and compound-5 (colourless crystals) from different fractions of this second column.

Amoora rohituka (Roxb) (Bengali Name: Pithraj) another plant of this study, belongs to the Meliaceae family and according to ayurvedic classical texts, the stem bark of *Amoora ruhituka* is being prescribed in liver and spleen diseases, oedema, anaemia, intestinal worms, urinary disorders, internal tumours and abdominal complaints. As a part of phytochemical study of this research work, the stem bark powder of *Amoora rohituka* was successively extracted with ethyl acetate and dichloromethane at room temperature to have ethyl acetate (EAEAR) and dichloromethane (DMEAR) extracts. Preparative thin layer chromatography (PTLC) was then applied on DMEAR and an UV active band afforded orange oily liquid as compound-6.

Finally based on analysis and comparison of spectroscopic evidences (Mass, IR, ^{1}H- and ^{13}C-NMR data) of each compound with the literature, compound-1, -2, -3, -4, -5 and -6 were identified as di-(2-ethylhexyl) phthalate, anhydrosophoradiol-3-acetate, taraxasteryl acetate, lup-12,20(29)-dien-3β,28-diol, β-boswellic acid and 2-methoxy-14-calamenenone, respectively. Except taraxasteryl acetate, all of the five isolated compounds are reported here for the first time from the corresponding plant.

In brine shrimp lethality bioassay, the cytotoxicity exhibited by EECF, compound-1, compound-2, compound-5 and DMEAR was promising with the LC_{50} values of 14.61, 9.19, 15.55, 15.26 and 17.67 µg/mL, respectively. But in comparison to ampicillin trihydrate (LC_{50}:

7.21 µg/mL), compound-3, compound-4 and EAEAR demonstrated moderate activity with the LC_{50} values of 45.46, 30.58 and 26.59 µg/mL, respectively.

EECF and compound-1 showed a better broad spectrum of antibacterial activity against pathogenic bacteria *In vitro* than the other isolated compounds and extracts. The intensity of antibacterial activity was found in the order of EECF > compound-1 > EAEAR > compound-5 > DMEAR > compound-2 > compound-4. The zone of inhibition produced by these extracts and compounds was found in the range 06 to 24 mm whereas the lowest minimum inhibitory concentration (MIC) values for these samples were in the range 16 to 64 µg/ml. In antifungal activity test, only EECF and compound-1 exhibited activity against the test fungi and produced zone of inhibition between 07 to 15 mm.

In vivo antineoplastic effect of EECF (50, 100 and 200 mg/kg), compound-1 (10, 20 and 40 mg/kg), compound-2 (10 and 20 mg/kg), ME (10 and 20 mg/kg), PEF (40 and 80 mg/kg), CF (20 and 40 mg/kg), EAEAR (20 and 40 mg/kg) and DMEAR (20 and 40 mg/kg) was assessed by evaluating the viable tumour cell count, survival time, body weight gain due to tumour burden, heamatological (hemoglobin content, RBC and WBC count) and biochemical (Glucose, cholesterol, triglyceride, blood urea, SALP, SGPT and SGOT) parameters of Ehrlich ascites carcinoma (EAC) bearing mice. Treatment with the all above samples dose dependently and significantly ($P < 0.05$; $P< 0.01$ and $P < 0.001$) decreased the viable EAC cells and body weight gain thereby increasing the life span of EAC bearing mice and also brought back the altered hematological (Hb, total RBC and total WBC) and biochemical parameters more or less to normal level. Among the test samples, EECF, CF and DMEAR and compound-2 showed prominent antineoplastic activity as compared with standard drug bleomycin. In addition, treatment of normal mice with EECF did not cause any extreme abnormality at the three doses used in this study.

In vitro cytotoxicity assay against A431 cell line (human vulval-derived epidermoid carcinoma), compound-1 (IC_{50}: 0.34 µg/mL) and compound-5 (IC_{50}: 0.36 µg/mL) showed strong cytotoxic effect than the others in comparison with doxorubicin (IC_{50}: 0.31 µg/mL).

In insecticidal activity against both larvae and adults of *Tribolium castaneum* (Herbst), EECF caused the highest mortality of the 1st instars larvae in comparison with other larval instars indicating high susceptibility to the newly hatched larvae with lowest LD_{50} value (0.134 mg/cm^2) and less susceptibility to the adult (after 72 hr exposure) with highest LD_{50} value (1.371 mg/cm^2).

From the overall results of this study, it is concluded that extracts and purified compounds from flower of *Calotropis gigantea* and stem bark of *Amoora rohituka* have noteworthy antibacterial, cytotoxic and antineoplastic effects that might be a source of herbal drugs in respective therapeutic area.

CONTENTS

Page No

Page No

Page No

Page No

LIST OF TABLES

LIST OF FIGURES

List of Abbreviations

BCSIR	.....	Bangladesh Council of Scientific & Industrial Research
MH_z	.....	Megahertz
NMR	.....	Nuclear Magnetic Resonance
IR	.....	Infra-red
m.p.	.....	Melting point
TLC	.....	Thin Layer Chromatography
PTLC	.....	Preparative Thin Layer Chromatography
TMS	.....	Tetramethylsilane
µg	.....	Microgram
^{1}H-NMR	.....	Proton nuclear magnetic resonance
^{13}C-NMR	.....	Carbon-13 nuclear magnetic resonance
DEPT	.....	Distortion enhancement of polarization transfer
ME	.....	Methanol extract of root bark of *Calotropis gigantea*
EECF	.....	Ethyl acetate extract of flower of *Calotropis gigantea*
PEF	.....	Petroleum ether soluble fraction of ME
CF	.....	Chloroform soluble fraction of ME
EAEAR	.....	Ethyl acetate extract of stem bark of *Amoora rohituka*
DMEAR	.....	Dichloromethane extract of stem bark of *Amoora rohituka*
δ	.....	Chemical shif in ppm
ν	.....	frequency
s	.....	singlet
d	.....	doublet
dd	.....	double boublet
m	.....	Multiplet
mL	.....	Milliliter
mg	.....	Milligram
g	.....	Gram
cm	.....	Centimeter
cm^{-1}	.....	Per-centimeter
R_f	.....	Retardation factor
ppm	.....	Parts per million

μL	---------	Micro liter
IPM	---------	Integrated pest management
GC-MS	---------	Gas chromatography-Mass spectrum
MS	---------	Mass Spectrometry
m/z	---------	mass / charge
IPM	---------	Integrated pest management
DMSO	---------	Dimethylsulfoxide
LD_{50}	---------	Dose required to kill 50% of test organism
LC_{50}	----------	Concentration required to kill 50% of test organism
χ^2	----------	chi-squared
J	----------	Coupling constant
δ_C	----------	Carbon 13 chemical shift
δ_H	----------	Proton chemical shift
Kg	----------	Kilogram
Hz	----------	Hertz
$[M^+]$	----------	molecular ion
SALP	----------	Serum alkaline phosphatase
SGPT	----------	Serum glutamate pyruvate transaminase
SGOT	----------	Serum glutamate oxaloacetate transaminase
Hb	---------	Haemoglobin
WBC	---------	White blood corpuscle
RBC	---------	Red blood corpuscle
MST	---------	Mean survival time
ILS	---------	Increase in life span
MIC	---------	Minimum inhibitory concentration
EAC	---------	Ehrlich ascites carcinoma
ICDDR,B	---------	International Centre for Diarrhoeal Diseases and Research, Bangladesh
$CDCl_3$	.....	Deuteriochloroform

List of published papers from this dissertation

1) **M. Rowshanul Habib,** M. A. Islam, M. Rezaul Karim. Cytotoxic chemicals from *Calotropis gigantea* Flower. *Chemistry of Natural Compounds*, 49(1): 165-166 (2013) [Impact Factor (2011): **1.029**]
2) **M. Rowshanul Habib,** M. Rezaul Karim. Antitumour evaluation of di-(2-ethylhexyl) phthalate (DEHP) isolated from *Calotropis gigantea* L. flower. *Acta Pharmaceutica*, 62(4): 607-615 (2012) [Impact Factor (2011): **0.912**]
3) **M. Rowshanul Habib**, M. Rezaul Karim. Evaluation of antitumour activity of *Calotropis gigantea* L. root bark against Ehrlich ascites carcinoma in Swiss albino mice. *Asian Pacific Journal of Tropical Medicine*, **4**(10): 786-790 (2011) [Impact Factor (2010): **0.172**]
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5) **M. Rowshanul Habib**, M. Abdul Aziz, M. Rezaul Karim. Inhibition of Ehrlich's ascites carcinoma by ethyl acetate extract of the flower of *Calotropis gigantea* L. in mice. *Journal of Applied Biomedicine*, **8**(1): 47-54 (2010) [Impact Factor (2010): **1.687**]
6) **M. Rowshanul Habib** and M. Rezaul Karim. Antimicrobial and Cytotoxic Activity of Di-(2-ethylhexyl) phthalate and Anhydrosophoradiol-3-acetate Isolated from *Calotropis gigantea* (Linn.) Flower. *Mycobiology*, **37**(1): 31-36 (2009).
7) **M. Rowshanul Habib**, M. Mohaimenul Islam and M. Rezaul Karim. Antimicrobial activity of *Amoora rohituka* Roxb. Stem. *Plant Archives*, **9**(1): 239-241 (2009).
8) **M. Rowshanul Habib** and M. Rezaul Karim. Toxicity Studies on the Extracts of *Amoora rohituka* Roxb. Stem. *Journal of Pharmacy Research*, **2**(2): 185-188 (2009).
9) **M. Rowshanul Habib** and M. Rezaul Karim. Effect of anhydrosophoradiol-3-Acetate of *Calotropis gigantea* (Linn.) flower as antitumouric agent against Ehrlich's ascites carcinoma in mice. *Pharmacological reports* (accepted) [Impact Factor (2011): **2.445**]

INTRODUCTION

Plants have been part of human lives since the beginning of time. We get numerous products from plants, most of them not only good and beneficial but also crucial to our existence. Over the centuries humans have relied on plants for basic needs such as food, clothing, and shelter, all produced or manufactured from plant matrices (leaves, woods, fibers) and storage parts (fruits, tubers). Plants have also been utilized for additional purposes, namely as arrow and dart poisons for hunting, poisons for murder, hallucinogens used for ritualistic purposes, stimulants for endurance, and hunger suppression, as well as inebriants and medicines. The plant chemicals used for these latter purposes are largely the secondary metabolites, which are derived biosynthetically from plant primary metabolites (e.g., carbohydrates, amino acids, and lipids) and are not directly involved in the growth, development and reproduction of plants. These secondary metabolites can be classified into several groups according to their chemical classes, such as alkaloids, terpenoids, steroids, glycosides, phenolics etc[1,2]. Secondary metabolites have no apparent function in a plant's primary metabolism, but often have an ecological role, such as pollinator attractants or as chemicals for adaptations to environmental stresses or as defensive agent against micro-organisms, insects and higher predators and even other plants (allelochemics). These secondary constituents of plant have been used directly as therapeutic agents or as starting materials for drug synthesis or as models for pharmacologically active compounds in drug manufacture by the pharmaceutical industries. In contrast to primary metabolites, secondary metabolites are synthesized in specialized cell types and at distinct developmental stages, making their extraction and purification difficult. Secondary metabolites are frequently accumulated by plants in smaller quantities than the primary metabolites[3,4] and these metabolites are purified from plant materials by steam distillation or by extraction with organic or aqueous solvents followed by different chromatographic purification techniques[5]. So the study of medicinal plants which include the proper selection of medicinal plants, preparation of crude extracts, phytochemical and biological screening have opened the door to the development of new drug for the health care products[6].

1.1. History of plant based tratiditional medicine

The use of plants as medicines has a long history. The beginning of human life on this earth was not at all smooth or comfortable. From the very start of human life on earth man had to face many obstacles, such as ferocious attacks by animals causing injuries and wounds, adverse environmental and climatic factors causing diseases, decay of health causing death etc. In order to survive and consolidate his existence on earth by

overcoming these obstacles, the primitive man had started thinking about diseases and their treatment at the very dawn of human intellect. In that process, when the primitive man realized that he could get relief from the sufferings of diseases by using plants or plant parts or their products, he had utilized them in a variety of forms[7]. In this way plants had been serving as the major source of drugs and medicine for human against diseases from the very beginning of their existence on earth.

From historical records, it is apparent that most of the early peoples like Assyrians. Babylonians, Egyptians and ancient Hebrews, were familiar with the properties of many medicinal plants. Babylonians (about 3000 BC) were aware of a large number of medicinal plants and their properties. Historical records of Assyria and Babylonia indicated that by 560 BC there were about 250 plant medicines in use in that region[8]. As evident from the Papyrus Ebers (written about 1500 BC), the ancient Egyptians possessed a good knowledge of the medicinal properties of hundreds of plants. Important plant based drugs like henbane, mandrake, opium, pomegranate, castor oil, aloe cannabis, senna and many other were in common use in Egypt about 4500 years ago[9].

The earliest mention of the medicinal use of plants in the Indian subcontinent is found in the Rig Veda (4500-1600BC), the oldest book in the library of man. This great treatise supplies various information's on the medicinal use of plants in the Indian subcontinent. The Vedas made many references to healing plants. Susruta Samhita and Charaka Samhita are another two of the oldest and most authoritative works written on Indian medical systems and medicinal plants. The Susruta Samhita, probably written before 1000 BC, deals with details of surgery and therapeutics, while the comprehensive Indian Herbal, Charaka Samhita, written about the same period, deals mainly with medicinal agents and cites more than 500 medicinal plants with their medicinal properties and uses. These works are still esteemed as treasures of literature on Indian indigenous medicine, particularly Ayurvedic medicine[10].

The practice of medicine using medicinal plants flourished mostly during the Greek civilization when historical personalities like Hpippocrates (born in 460 BC) and Theophrastus (born in 370 BC) practiced herbal medicine. The Materia medica of the great Greek physician Hippocrates (460-370BC) consists of some 300 to 400 medicinal plants. The far-ranging scientific work of Aristotle (384-322BC), included an effort to catalogue the properties of the various medicinal herbs known at that time. The encyclopedic work of Dioscorides (1st Century AD), *De* Materia Medica (published in 78 AD), was the forerunner of all modern pharmacopoeias and an authoritative text on botanical medicine. This work featured about 600 medicinal plants[11].

The Arabian Muslim physicians, like Al-Razi and Ibn Sina (9th to 12th century AD), brought about a revolution in the history of medicine by bringing new drugs of plant and mineral origin into general use. The famous medical book Al-Kanun of Ibn Sina was the prescribed book of medicine in the schools of western medicine for several centuries[12].

The use of medicinal plants in Europe in the 13th and 14th centuries was based on the Doctrine of signatures or Similar developed by Paraceisus (1490-1514 AD), a Swiss alchemist and physician. The South American countries have provided the world with many useful medicinal plants, grown naturally in their forests and planted in the medicinal plant gardens. Many centuries ago, the medicinal plants used by the Australian aborigines tremendously enriched the stock of medicinal plants of the world. The current list of the medicinal plants growing around the world includes more than a thousand items[11].

1.2. Contribution of medicinal plants to modern medicine

Many drugs commonly used today are of plant origin. The array of medicines derived from medicinal plants is impressive and includes hypotensive drugs, analgesics, anesthetic, anticancerous and antiparasitic compounds, anti-inflammatory drugs, steroids, laxatives, diuretics and many others[11]. About 25% of the prescription drugs available in markets contain at least one active ingredient derived from plant material. The World Health Organization (WHO) estimates that 4 billion people, 80% of the world population, presently use plant based medicine for some aspect of primary health care. According to WHO, 122 plant-derived pharmaceutical medicines are used in modern medicine that correlated directly with their traditional uses as plant medicines by native cultures[13]. Between 1994 and 2007, it is estimated that almost 50% of new chemical entities authorized to initiate clinical studies were natural products or natural products related[14]. Moreover, about 60% of anticancer drugs and 75% of compounds for infectious diseases are either natural products or their derivatives[15,16]. Recently, all over the world, there is an upsurge and interest among scientific institutions, biological research institutions in the use of medicinal plants, crude extracts or active ingredients to treat various ailments[17,18]. Most of the research that is done on medicinal plants continues to focus on identifying and isolating active ingredients, rather than studying the medicinal properties of whole plants.

1.3. Drugs derived from medicinal plants

During the long journey from ancient time to modern age, the human has successfully used plants and plant products as effective therapeutic tools for fighting against diseases and various other health hazards. Plants provide a multitude of medicines for all types of

ailments and diseases. Several of the drugs sold today are simple synthetic modifications or copies of the substances derived from plants. List of some important drug derived from medicinal plants[19] are presented in table 1.1.

Table 1.1 Drugs of plant origin used in modern medicine[19]

Name of Chemical used as drug	**Action/Clinical Use**	**Plant Source**
Vinblastine	Antitumor, Antileukemic agent	*Catharanthus roseus*
Vincristine	Antitumor, Antileukemic agent	*Catharanthus roseus*
Anisodine	Anticholinergic	*Anisodus tanguticus*
Taxol	Antitumor agent	*Taxus brevifolia*
Teniposide	Antitumor agent	*Podophyllum peltatum*
Adoniside	Cardiotonic	*Adonis vernalis*
Atropine	Anticholinergic	*Atropa belladonna*
Glasiovine	Antidepressant	*Octea glaziovii*
(+)-Catechin	Haemostatic	*Potentilla fragarioides*
Cocaine	Local anaesthetic	*Erythroxylum coca*
Codeine	Analgesic, antitussive	*Papaver somniferum*
Ephedrine	Sympathomimetic, antihistamine	*Ephedra sinica*
Etoposide	Antitumor agent	*Podophyllum peltatum*
Palmatine	Antipyretic, detoxicant	*Coptis japonica*
Papavarine	Smooth muscle relaxant	*Papaver somniferum*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Caffeine	CNS stimulant	*Camellia sinensis*
Rotenone	Pesticide, Insecticide	*Lonchocarpus nicou*
Tetrandrine	Antihypertensive	*Stephania tetrandra*
Theobromine	Diuretic, vasodilator	*Theobroma cacao*

In addition to these natural drugs of modern medicine, plants have also contributed and are still contributing to the development of modern synthetic drugs and medicines. Novel structures of biologically active chemical compounds, isolated from plant sources, often prompt the chemists to synthesise similar or biologically more potent semi-synthetic compounds. Synthetic drugs with similar or more potent therapeutic activity are often prepared by structural modification of the plan-derived compounds with known biological activity[20].

1.4. Importance of plant derived product as antibiotic, antineoplastic agent and pesticide

1.4.1. As antibiotic

Bacterial infection is one of the serious global health issues in 21st century[21]. The emergence of human pathogenic microorganisms that are resistant to major classes of antibiotics has been increased in recent years, due to the indiscriminate use of antimicrobial drugs like tetracycline, ampicillin and chloramphanical, nalidixic acid, cotrimoxazole, mecillinam, ciprofloxacin etc[22,23]. This has caused many clinical problems in the treatment of infectious diseases and the antibiotics commonly used are sometimes associated with adverse effects on the host, which include hypersensitivity, allergic reaction and immunosuppression[24,25]. So there is a need of an alternative source for new antibiotics in the drug development pipeline. Plants are known to produce some chemicals, which are naturally toxic to bacteria and fungi[25,26]. Therefore plant based products, either as pure compounds or as standardized extract provides unlimited opportunities for new drug development because of the unmatched availability of chemical diversity[27].

1.4.2. As antineoplastic agent

Cancer is one of the most life-threatening diseases with more than 100 different types. Due to lack of effective drugs, expensive cost of chemotherapeutic agents and side effects of anticancer drugs, cancer can be a cause of death[28]. Hence researchers are increasingly turning their attention to natural products and looking for new leads to develop better drugs against cancer. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis only in cancer cells. Recent studies on tumor inhibitory compounds of plant origin have yielded an impressive array of novel structures that are known to induce apoptosis in cancer cells but not in normal cells[29-31]. So information on the ethnopharmacologic use of plants has given important lead in the cancer drug development.

1.4.3. As pesticides

Pest control is a major issue for underdeveloped agricultural countries. More than 2000 species of field and storage pests annually destroy approximately one third of world's food production, valued US $ 100 billion among which highest losses (43% of potential production) occur in developing Asian countries[32]. Synthetic pesticides are the easy control technology for pest. Continuous or heavy usages of some pesticides has

created undesirable changes in the gene-pool for the presence of some mutagenic agents and thereby increasing pesticide resistance in insects. Heavy usages of pesticides has also created serious problem such as direct toxicity to parasites, predators, pollinators, fish and man[33,34]. Due to the problems associated with the indiscriminate use of synthetic insecticides, like insect resistance and impact on non-target organisms, many scientists have given an extra impulse to search alternative ways in pest control. Natural products derived from plants as an alternative to conventional strategies for pest control is now a day very popular among the integrated pest management (IPM) practitioners[34,35]. Plant-derived pesticides are considered as an alternative to the synthetic chemicals for being biodegradable, pest specific, non-hazardous to human health and environment and leaving no toxic residue in nature[36]. Farmers have been using plant extracts in pest control for centuries[37]. Therefore, pest control with plant derived products provides an ideal source of low cost, safe and effective pesticides.

1.5. Necessity of research on medicinal plants in Bangladesh

Bangladesh is situated at the complex interface of the Indian, Himalayan and Southeast Asian biographic regions, and historically it is well-endowed with very diverse complements of terrestrial and aquatic flora and fauna that include a considerable number of medicinal plant resources[38]. Approximately, 500 of these medicinal plants are being used in the traditional medication system for the treatment of different types of diseases in Bangladesh. Most of these medicinal plants have been grown in jungles, forests, gardens and fertile region of Dhaka, Rajshahi, Shylet and Chittagong division of Bangladesh whereas some of these plants are found lying every where of this country[39].

About 80% of the total population of Bangladesh is still live in the villages. Approximately 14% of them go to the qualified doctors (simple MBBS) and rests of the peoples are still dependent on the different types of traditional medicine practitioners[40]. Most of the practitioners use plant materials as such without knowing the side effect and toxicity and their preparation is substandard. This type of use sometimes causes serious health problem. To overcome this problem, research on our enormous medicinal plant resources is necessary to maintain a safer traditional practice by determining their chemical entities and biological activity properly. If we could use medicinal plants properly we could get medicines at low cost and then it might be possible to fulfil the demand of our medication. This will supply low cost medicine to our poor people and we could establish a better health care system. In order to achieve this goal, research and development on the traditional medicines should be given the proper privilege.

1.6. Plants investigated in this study

1.6.1. *Calotropis gigantea* (Linn)

Calotropis gigantea (Linn), locally known as 'Boro Akanda' belongs to the Asclepiadaceae family. It grows in tropical region and most abundant in Bangladesh, India, Burma, Pakistan and in the sub Himalayan tract[41]. In order to scientifically appraise some of the folkloric and ethnomedical uses of *Calotropis gigantea*, this study have taken root bark (Figure 1.1) and flowers (Figure 1.2) of this plant to conduct phytochemical and biological studies.

1.6.1.1. Scientific classification of the investigated plant: *Calotropis gigantea*

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Order	: Gentianales
Family	: Asclepiadaceae
Genus	: *Calotropis*
Species	: *Calotropis gigantea* (Linn)

1.6.2.2. Common name of the plant

Traditional name: Akand

Bangali name: Boro Akanda

English name: Calotrope, Mudar

1.6.1.3. Botanical description of the investigated plant: *Calotropis gigantea*[41]

Habit	: Shrub or a small tree up to 2.5 m (maxmum 6 m) height.
Root	: Simple, branched, woody at base and covered with a fissured; corky bark; branches somewhat succulent and densely white tomentose; early glabrescent. All parts of the plant exude white latex when cut or broken.
Leaves	: Opposite-decussate, simple, sub sessile, extipulate; blade-oblong obovate to broadly obovate, 5-30 x 2.5-15.5 cm, apex abruptly and shortly acuminate to apiculate, base cordate, margins entire, succulent, white tomentose when young, later glabrescent and glacouse.
Flowers	: Bracteate, complete, bisexual, actinomorphic, pentamerous,

hypogynous, pedicellate, pedicel 1-3 cm long.

Calyx : Sepal 5, Polysepalous, 5 lobed, shortly united at the base, glabrescent, quincuncial aestivation.

Corolla : Petals five, gamopetalous, five lobed, twisted aestivation.

Fruit : A simple, fleshy, inflated, subglobose to obliquely ovoid follicle up to 10 cm or more in diameter.

Seed : Many, small, flat, obovate, 6x5 mm, compressed with silky white pappus, 3 cm or more long.

Inflorescence : A dense, multiflowered, umbellate, peducled cymes, arising from the nodes and appearing axillary or terminal

Figure 1.1 *Calotropis gigantea* plant with flowers

Figure 1.2 Roots of *Calotropis gigantea*

1.6.1.4. About the family: Asclepiadaceae[41]

Asclepiadaceae is a family with 175-180 genera and 2200 species and these species are distributed mainly in the tropical and subtropical regions of the world. The *Asclepiadaceae* constitute a derived family showing complex floral structure, which are dedicated to the service of highly specialized pollination biology. The presence of fascinating floral structures and colours in connection with facilities fostering deceit, trapping and attachment as well as the possession of pollen-masses (pollinia) warrant the *Asclepiadaceae* to be seen as the 'orchids' among the dicotyledons.

Common features of Asclepiadaceae species are given below:

Plant : Herbs, vines and shrubs

Stem : Usually with milky juice or sap (Latex)

Leaves : Simple, usually entire, mostly paired and opposite, or in whorls of 4, rarely alternate; stipules very small or absent

Flowers	: Perfect, regular (actinomorphic), complex flower, solitary or usually clusters (umbels) that are flat or round
Root	: Pods (follicles) often paired, many seeds with tuffs of silky hairs

1.6.1.5. Some important species of Asclepiadaceae family available in Bangladesh

The genus *Calotropis* R. Br of Asclepiadaceae family comprises six species but in Indian subcontinents including Bangladesh, it is mainly represented by two popular species viz, *Calotropis procera* and *Calotropis gigantea*. Some important species (available in Bangladesh) of Asclepiadaceae family with their pharmacological and folk medicinal uses[42] are listed in table 1.2.

Table 1.2. Some medicinally important species of Asclepiadaceae family available in Bangladesh[42].

Plant's Name [Local name]	Pharmacological properties with plant parts	Folk medicinal uses with plant parts
Asclepias curassavica L. [Ban Kapas]	Purgative, emetic, febrifuge, cardio tonic, anthelmentic and astringent. (WP)	Blood dysentery, asthma, piles, gonorrhea, fever.(WP)
Calotropis acia F. Ham [Mader]	Tonic, antispasmodic, emetic	Swelling rheumatic pains and toothache
Calotropis procera R.Br. [Akand]	Tonic, antispasmodic and expectorant	Swellings, asthma, rheumatic pains
Marsdenia tinctoria R.Br [Roin]	Stimulant, stomachic, antiseptic	Stomachache and intestinal disease
Oxystelma secamone (L.) [Dudhi]	Antiseptic, depurative, galactagogue, diuretic, aphrodisiac, anthelmintic	Bronchitis, gonorrhoea, aphthous ulceration of the mouth and in sore throat
Pergularia daemia (Forssk.) [Dudhi lata]	Expectorant, diuretic, emetic, anthelmintic, laxative, antipyretic	Diarrhoea, cough, menstrual troubles, rheumatic cases
Tylophora indica (Burm.f) [Antamul]	Expectorant, stornachic, cathartic, emetic, diaphoretic, stimulant, diaphoretic	Asthma, diarrhoea, cough, bronchitis, rheumatism, gouty pains, dysentery
Wattakaka volubilis (L.f.) [Madhumalati]	Antiseptic, emetic, tonic, aphrodisiac, antipyretic, astringentexpectorant.	Piles, tumours, leucoderma, asthma, boils, abscesses, urinary dishagges

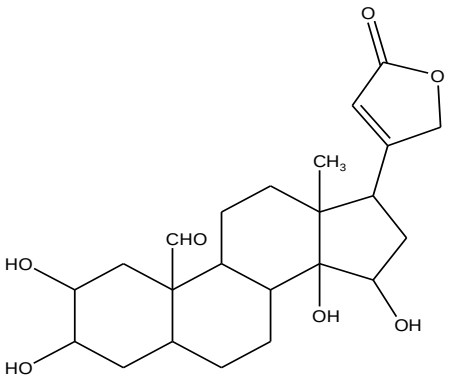
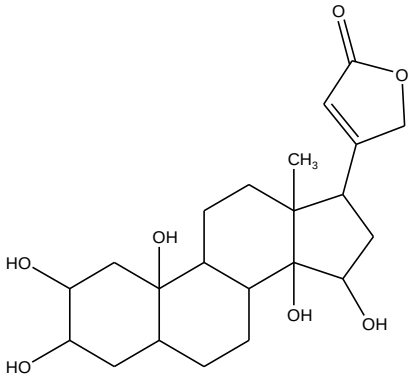
1.6.1.6. Traditional uses of *Calotropis gigantea*

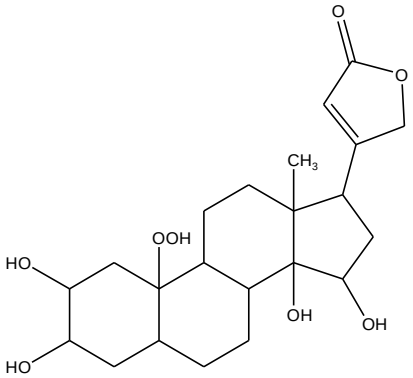
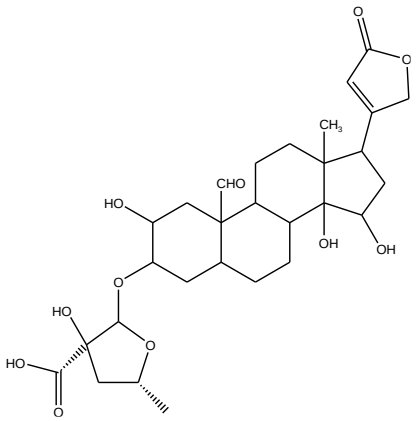
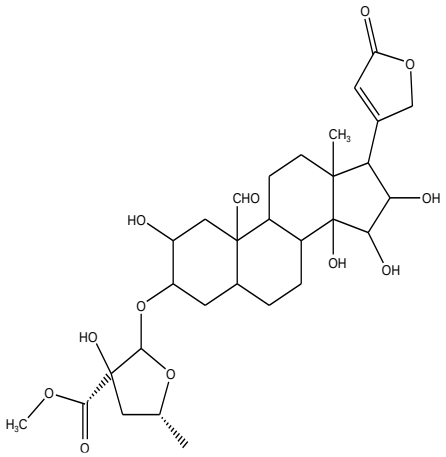
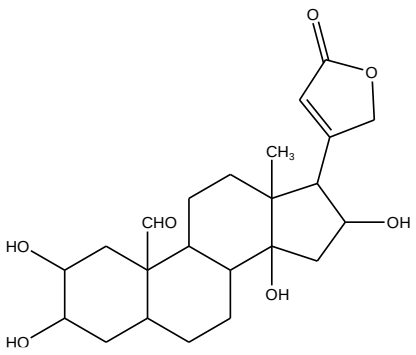
The roots and leaves of *Calotropis gigantea* are used traditionally for the treatment of abdominal tumors, boils, syphilis, leprosy, skin diseases, piles, wounds, rheumatism, insect-bites, ulceration and elephantiasis[38]. The dried bark of the root is stated to be an excellent substitute for Ipecacuanha. The latex of this plant is used in indigenous medicine as a purgative and a local irritant. In addition, people in Indian-subcontinent including Bangladesh use flowers of *Calotropis gigantea* as a traditional flock medicine in small pox, muscular pain, convulsions, scabies, and a number of ailments. Powdered flowers, in small doses, are useful in the treatment of colds, cough, asthma, catarrh, indigestion, inflammatory diseases and loss of appetite[38,43].

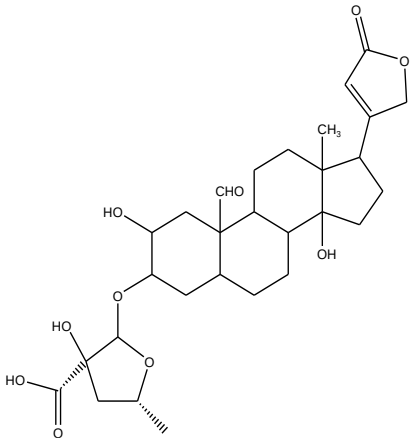
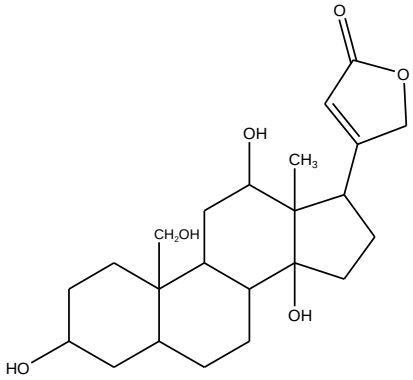
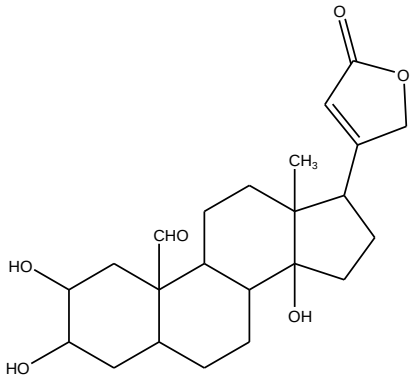
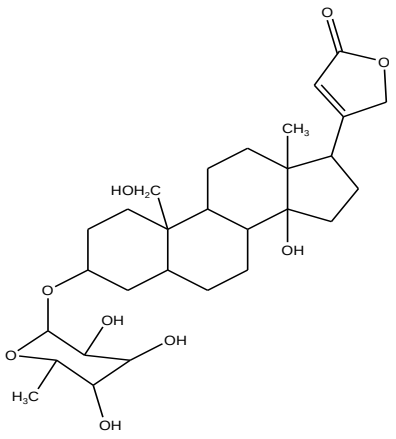
1.6.1.7. Chemical literature review on the *Calotropis gigantea*

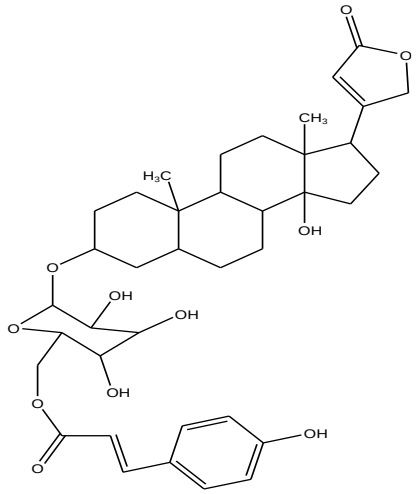
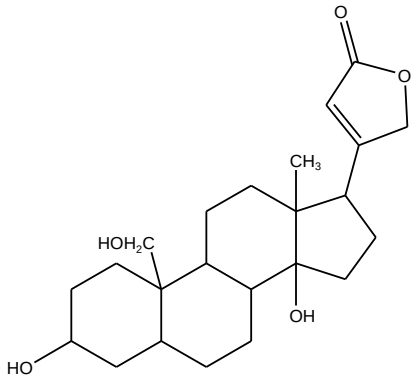
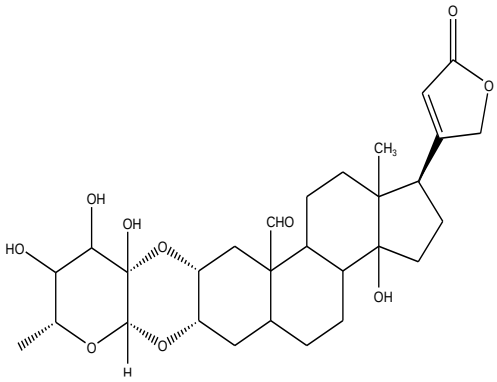
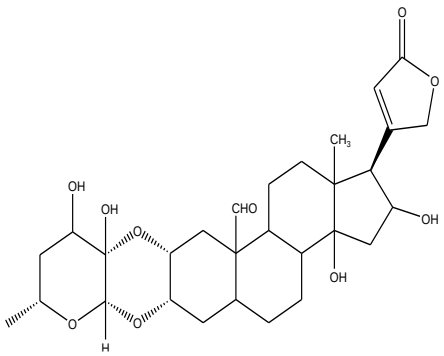
Several phytochemicals have been isolated from *Calotropis gigantea* and they include cytotoxic cardenolides[44-47], antifeedant nonprotein amino acid[48], naphthalene and terpene derivatives[49], flavonol glycosides[50], pregnanes[51,59-60], ursane-type triterpenoids[53-58,61] and steroids[52,62-63]. On the basis of the survey, compounds that were isolated from different parts of *Calotropis gigantea* are mentioned in table 1.3.

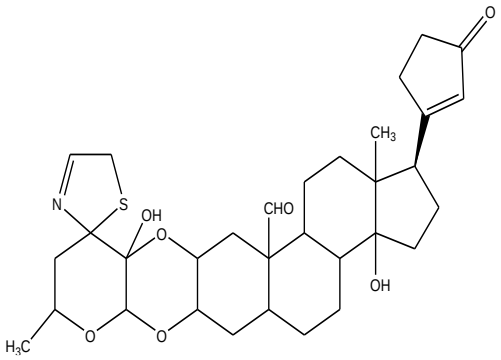
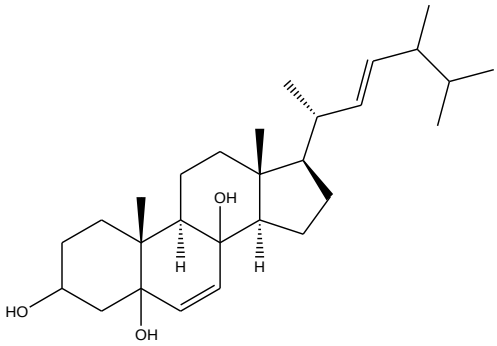
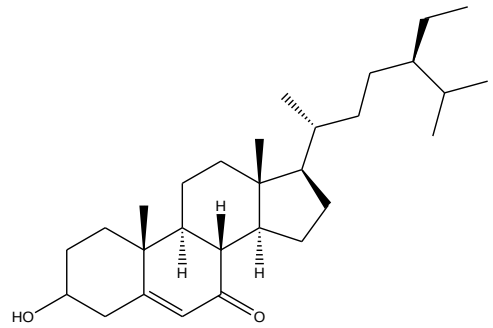
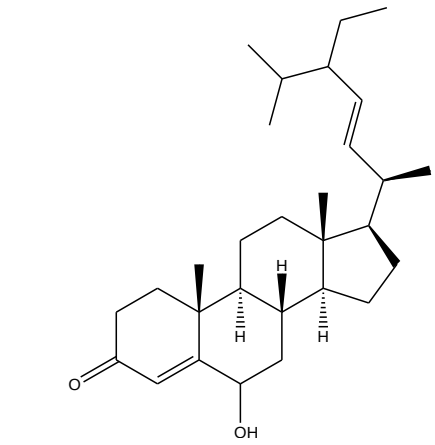
Table 1.3 List of compounds isolated from different parts of *Calotropis gigantea*

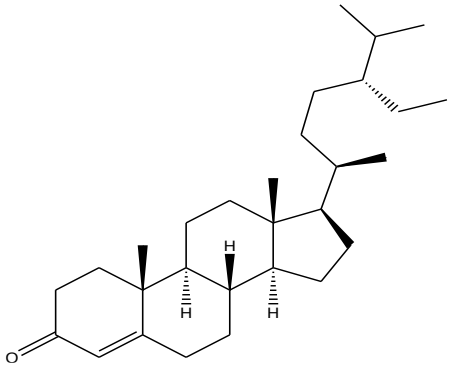
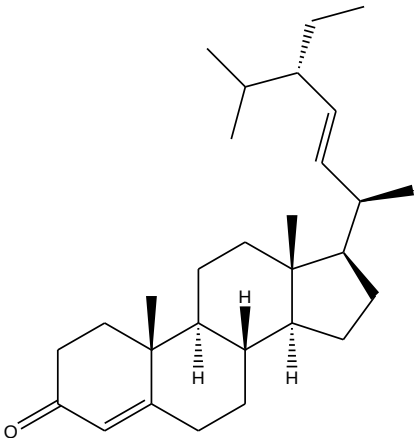
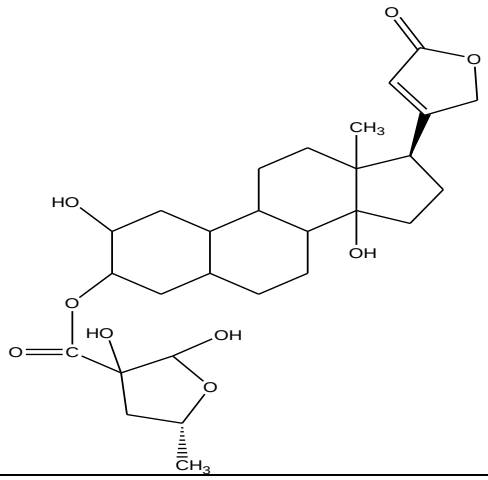
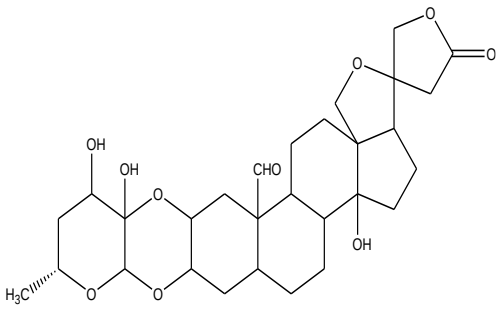
Chemical structure	Name of the compound	Source
	2α,15β-Dihydroxy-19-oxo-uzarigenin[44]	Leaves of *Calotropis gigantea* L.
	19-Nor-2α,10,15β-trihydroxyuzarigenin[44]	Leaves of *Calotropis gigantea* L.

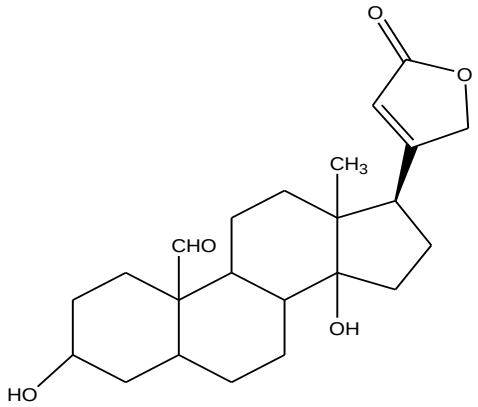
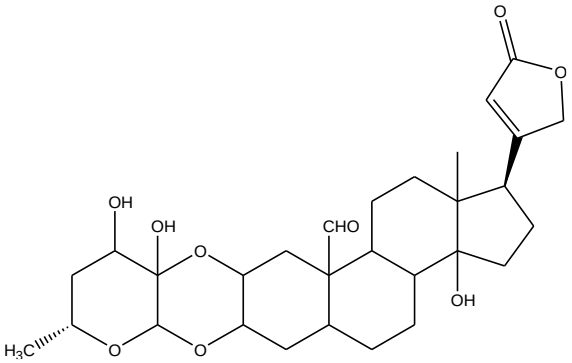
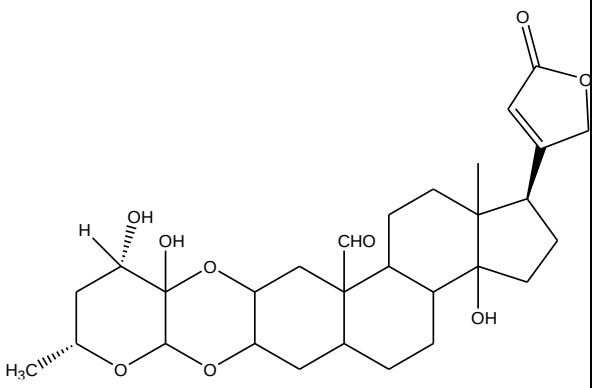
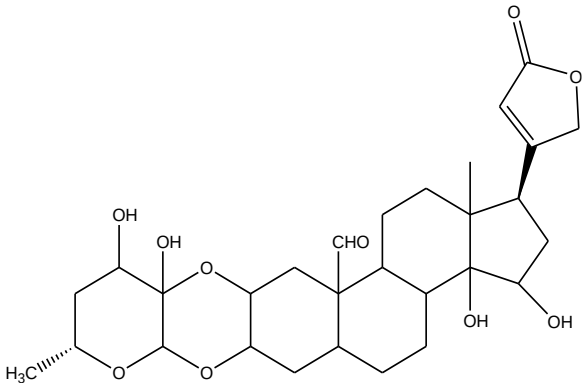
Chemical structure	Name of the compound	Source
	19-Nor-10-hydroperoxy-2α,15β-dihydroxyuzarigenin[44]	Leaves of *Calotropis gigantea* L.
	15 β-Hydroxycalactinic acid[44]	Leaves of *Calotropis gigantea* L.
	16α-Hydroxycalactinic acid methyl ester[44]	Leaves of *Calotropis gigantea* L.
	16α-Hydroxycalotropagenin[44]	Leaves of *Calotropis gigantea* L.

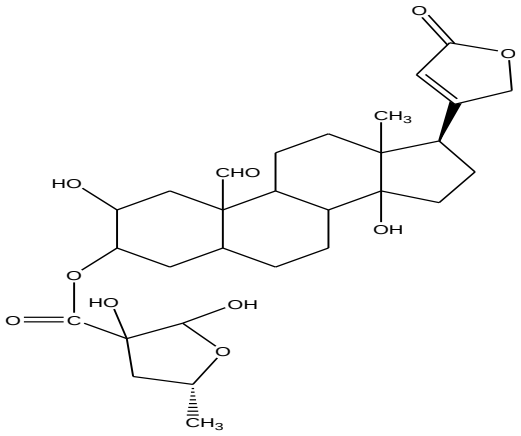
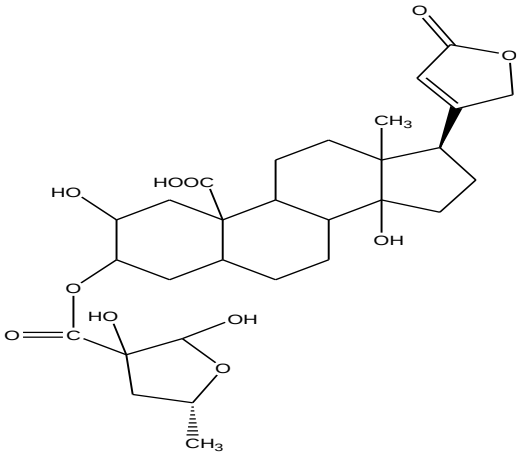
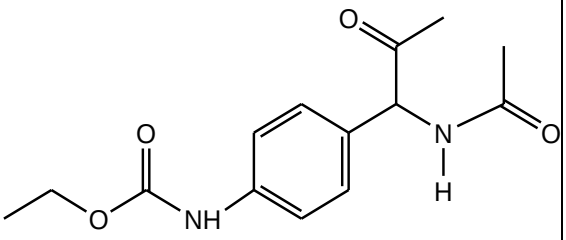
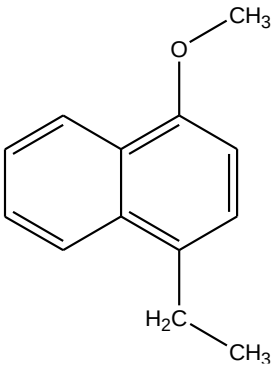
Chemical structure	Name of the compound	Source
	Calactinic acid[44]	Leaves of *Calotropis gigantea* L.
	16β-Hydroxycoroglaucigenin[44]	Leaves of *Calotropis gigantea* L.
	Calotropagenin[44]	Leaves of *Calotropis gigantea* L.
	Frugoside[44,46]	Leaves and root bark of *Calotropis gigantea* L.

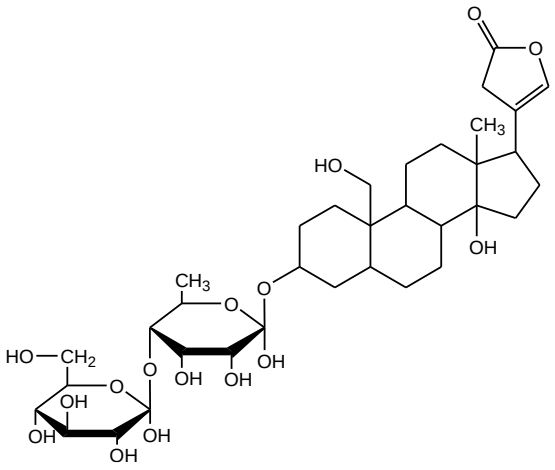
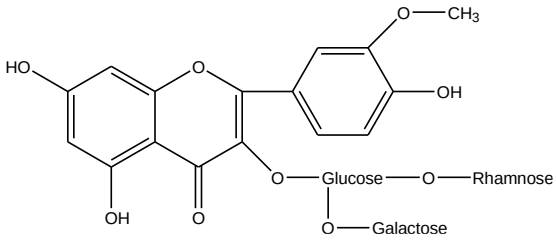
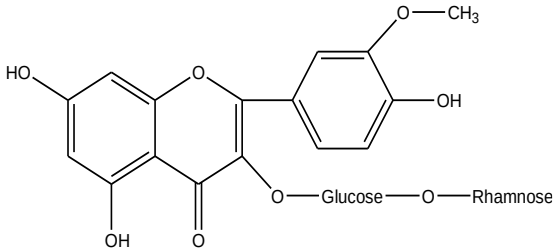
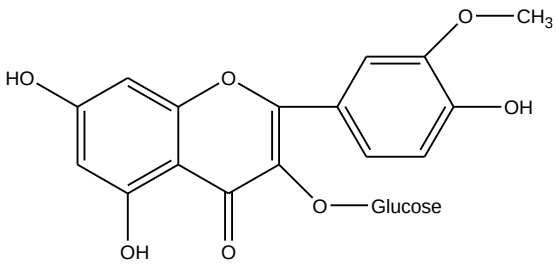
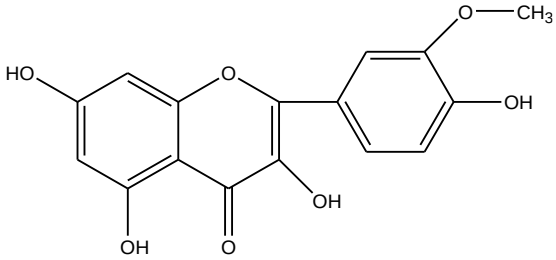
Chemical structure	Name of the compound	Source
	6′-O-(E-4-hydroxycinnamoyl) desglucouzarin[44]	Leaves of *Calotropis gigantea* L.
	Coroglaucigenin[44]	Leaves of *Calotropis gigantea* L.
	Calotoxin[44]	Leaves of *Calotropis gigantea* L.
	16α-Hydroxycalotropin[44]	Leaves of *Calotropis gigantea* L.

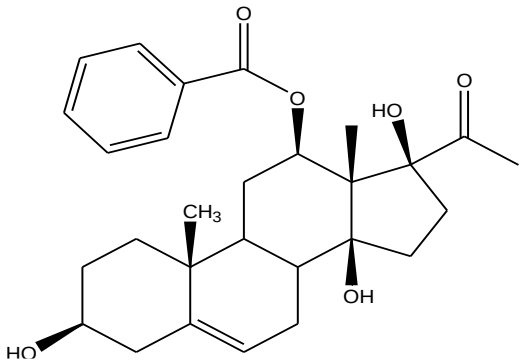
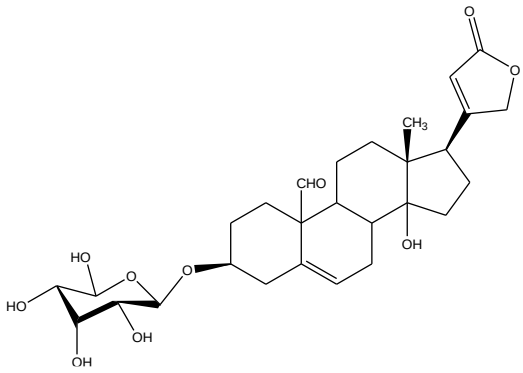
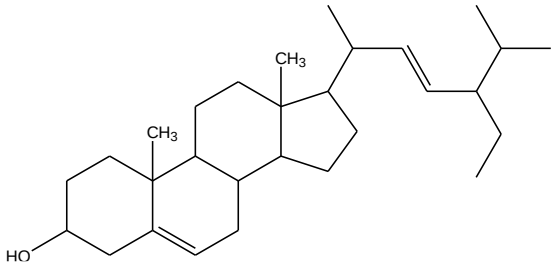
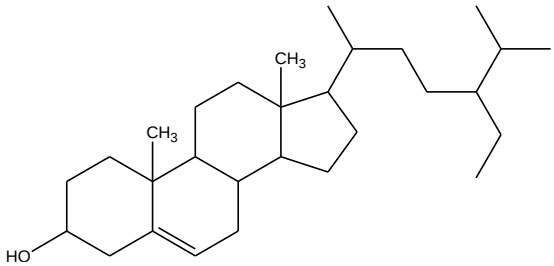
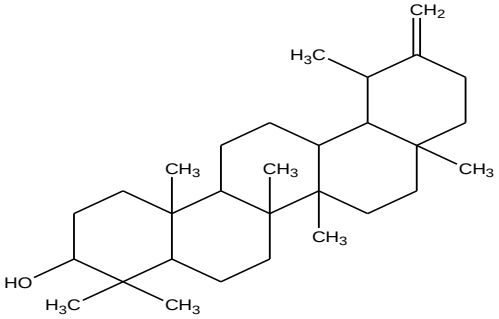
Chemical structure	Name of the compound	Source
	Uscharin[43]	Leaves of *Calotropis gigantea* L.
	3,5,8-trihydroxy-24-methylcholest-6,22-diene[47]	Leaves of *Calotropis gigantea* L.
	(24R)-3.-hydroxy-24-ethylcholest-5-en-7-one[47]	Leaves of *Calotropis gigantea* L.
	6β-hydroxy-24-ethylcholest-4,22-dien-3-one[47]	Leaves of *Calotropis gigantea* L.

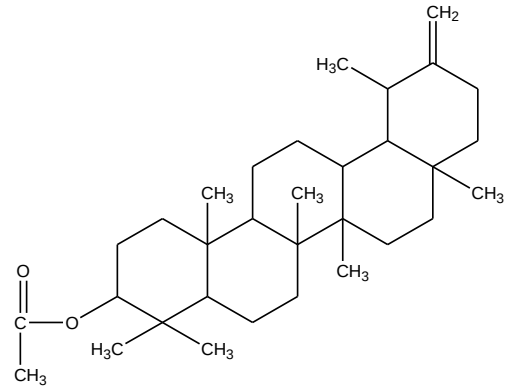
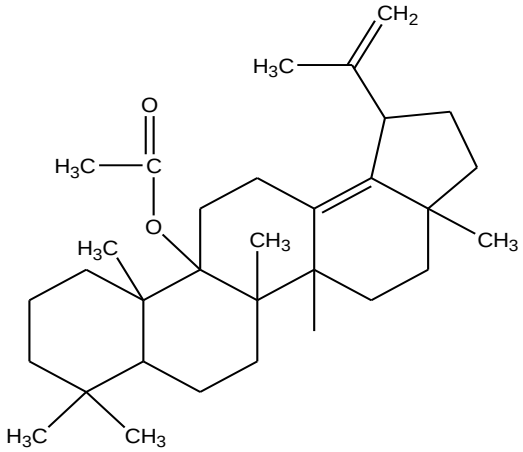
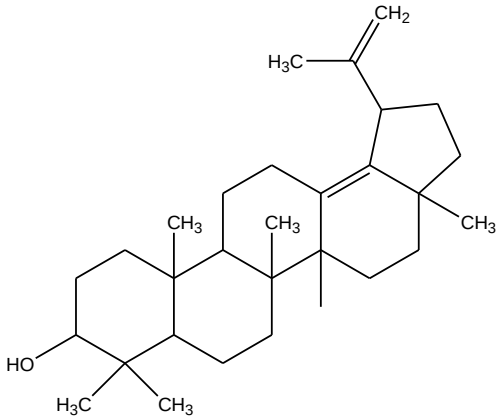
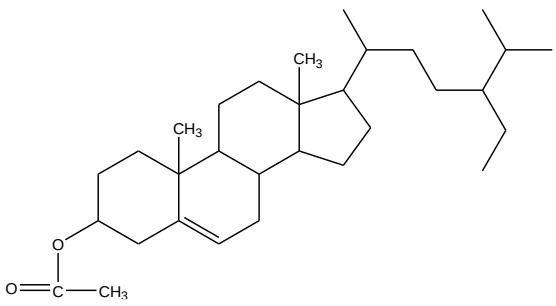
Chemical structure	Name of the compound	Source
	(24R)-24-ethylcholest-4-en-3-one[47]	Leaves of *Calotropis gigantea* L.
	(24S)-24-ethylcholest-4,22-dien-3-one[47]	Leaves of *Calotropis gigantea* L.
	19-Nor-10-hydrocalactinic acid methyl ester[45]	Leaves of *Calotropis gigantea* L.
	18,20-Epoxycalotropin[45]	Leaves of *Calotropis gigantea* L.

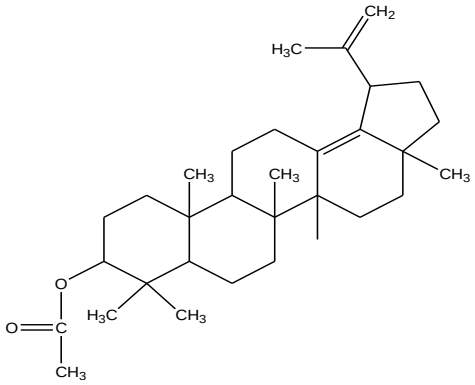
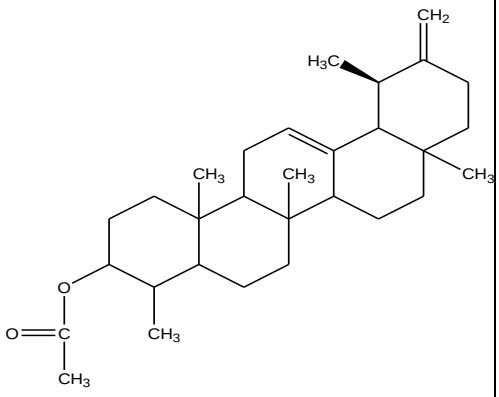
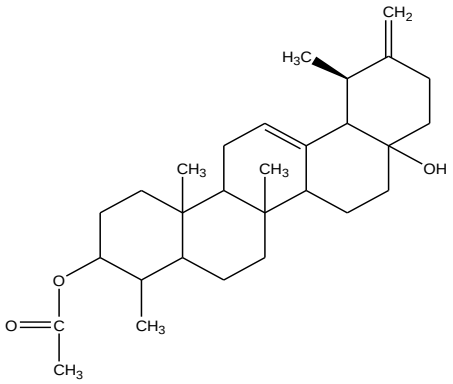
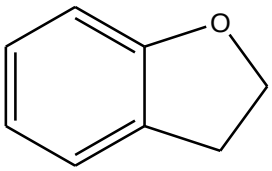
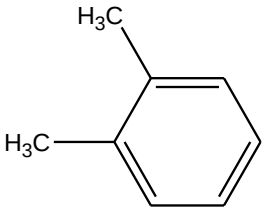
Chemical structure	Name of the compound	Source
	Uzarigenin[45]	Leaves of *Calotropis gigantea* L.
	Calactin[45]	Leaves of *Calotropis gigantea* L.
	Calotropin[46]	Leaves and root bark of *Calotropis gigantea* L.
	15β-hydroxycalotropin[45]	Leaves of *Calotropis gigantea* L.

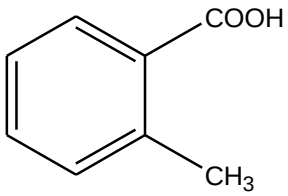
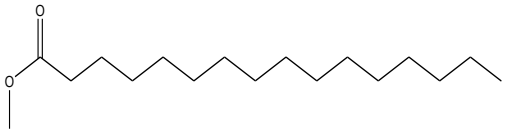
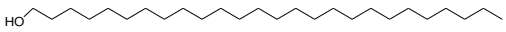
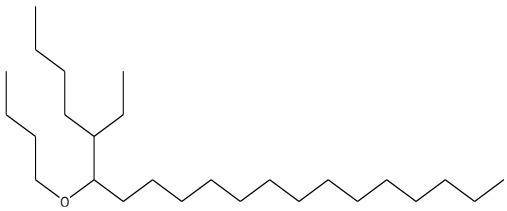
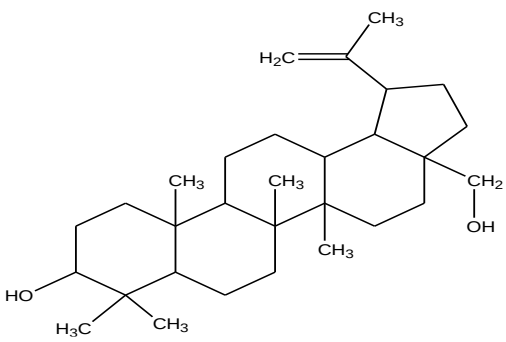
Chemical structure	Name of the compound	Source
	Calactinic acid methyl ester[45]	Leaves of *Calotropis gigantea* L.
	19-carboxylcalactinic acid methyl ester[45]	Leaves of *Calotropis gigantea* L.
	Giganticine[48]	Root bark of *Calotropis gigantea* L.
	1-Methoxy-4-ethyl naphthalene[49]	Root bark of *Calotropis gigantea* L.

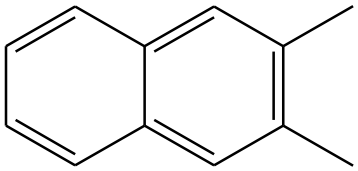
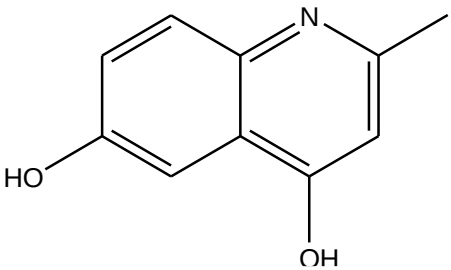
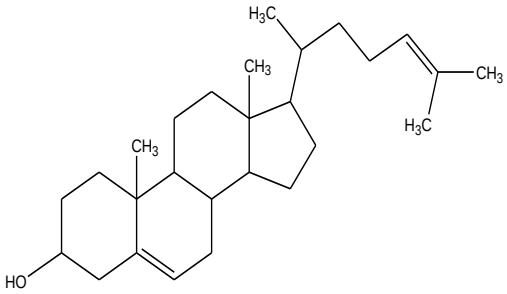
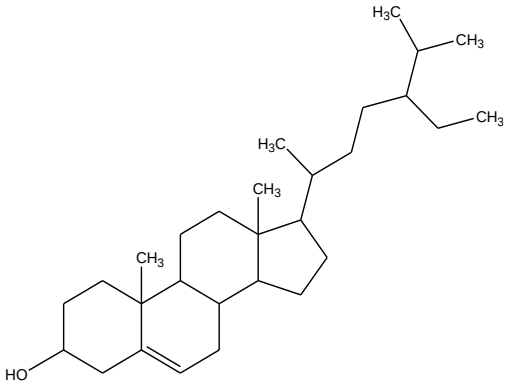
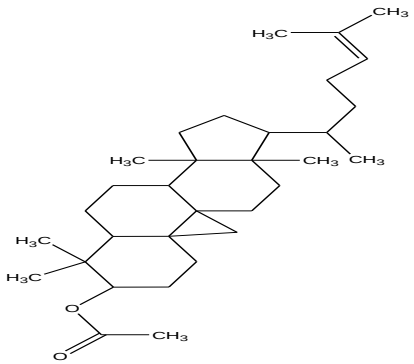
Chemical structure	Name of the compound	Source
	4′-*O*-β-D-glucopyranosylfrugoside[46]	Root bark of *Calotropis gigantea* L.
	Isorhamnetin-3-O-rutinoside[50]	Root bark of *Calotropis gigantea* L.
	Isorhamnetin rhamnoglucoside[50]	Root bark of *Calotropis gigantea* L.
	Isorhamnetin-3-O-glucoside[50]	Root bark of *Calotropis gigantea* L.
	Isorhamnetin[50]	Root bark of *Calotropis gigantea* L.

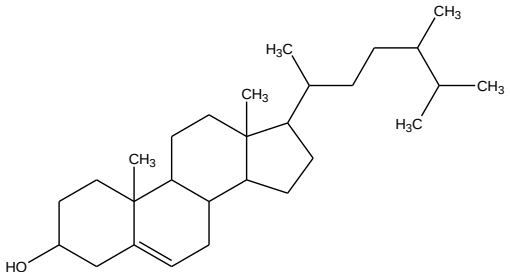
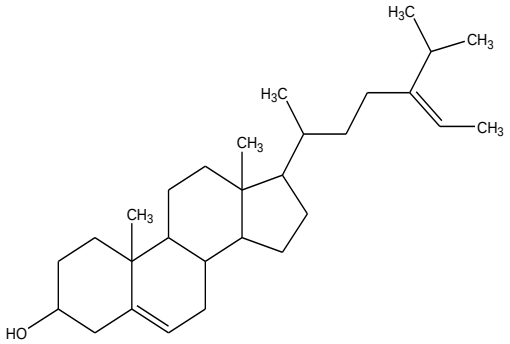
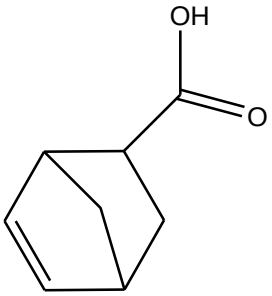
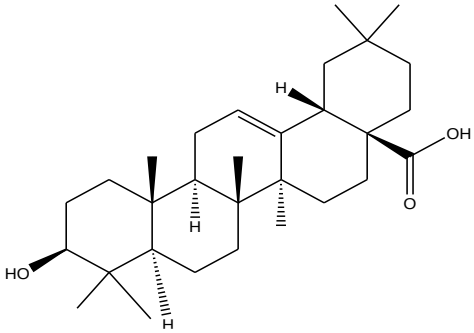
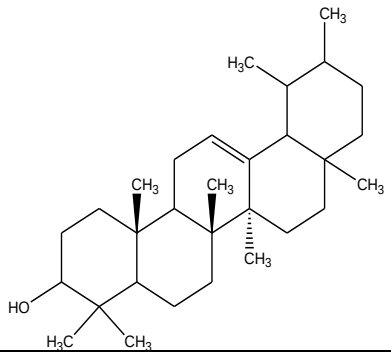
Chemical structure	Name of the compound	Source
	Calotropone[51]	Root bark of *Calotropis gigantea* L.
	Gofruside[51]	Root bark of *Calotropis gigantea* L.
	Stigmasterol[52]	Root bark of *Calotropis gigantea* L.
	β-sitosterol[52]	Root bark of *Calotropis gigantea* L.
	Taraxasterol[43]	Root bark of *Calotropis gigantea* L.

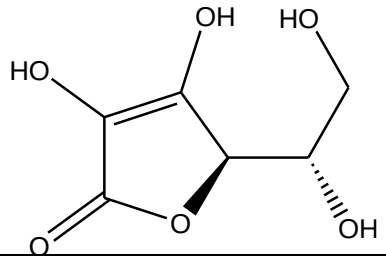
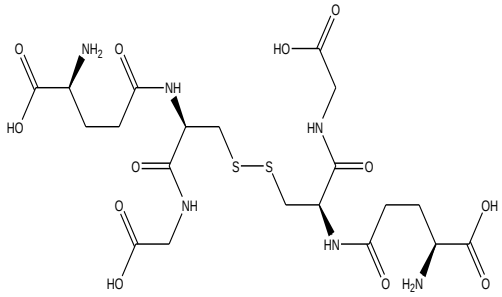
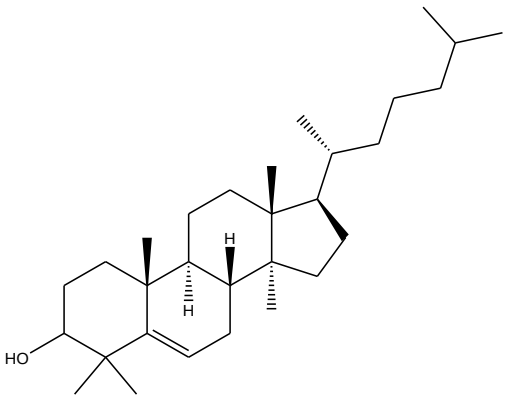
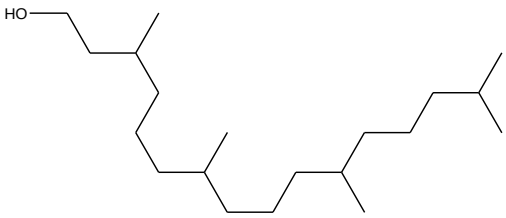
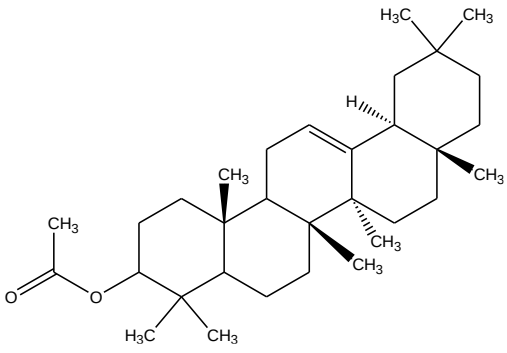
Chemical structure	Name of the compound	Source
	Taraxasterol acetate[45]	Root bark of *Calotropis gigantea* L.
	Lup-13(18),19(29)-dien-9α-yl acetate[53]	Root bark of *Calotropis gigantea* L.
	Lupeol[53,62]	Root bark and latex of *Calotropis gigantea* L.
	β-Sitosterol acetate[54]	Root bark of *Calotropis gigantea* L.

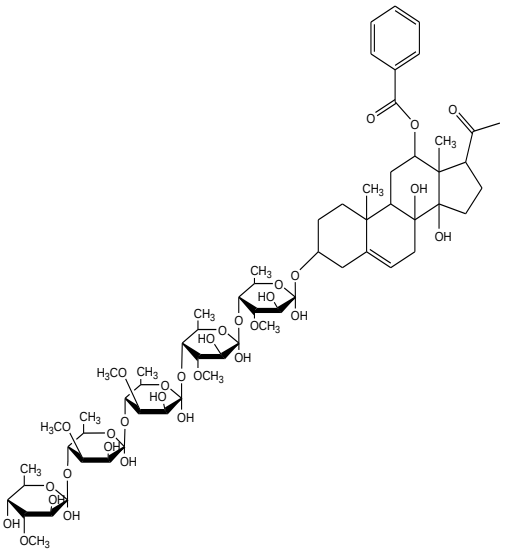
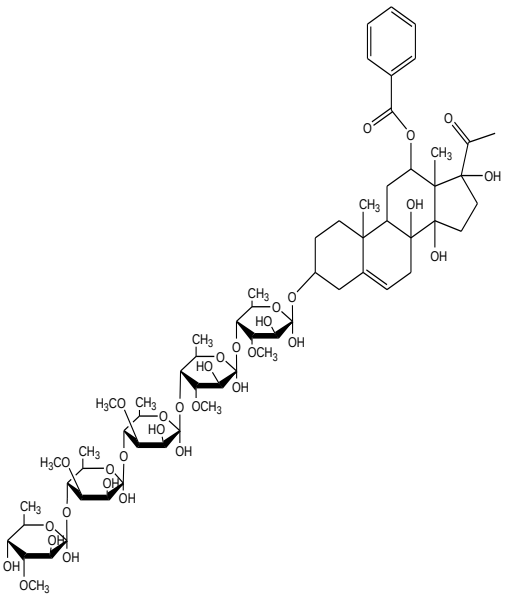
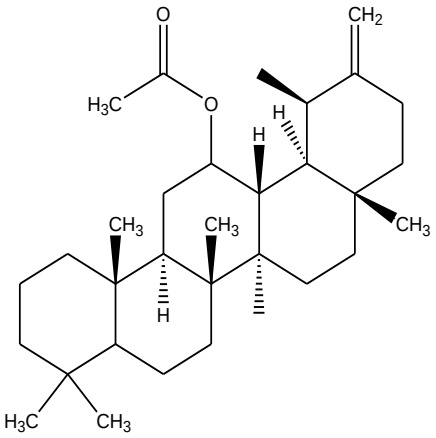
Chemical structure	Name of the compound	Source
	Lupeol acetate[53]	Root bark of *Calotropis gigantea* L.
	Urs-18β-H-12,20(30)-diene-3β-yl acetate[53]	Root bark of *Calotropis gigantea* L.
	17β-Hydroxy-28-normethylurs-18αH-12,20(30)-dien-3β-yl acetate[53]	Root bark of *Calotropis gigantea* L.
	2,3-dihydrobenzofuran[55]	Aerial part of *Calotropis gigantea* L.
	1,2-Dimethyl benzene[55]	Aerial part of *Calotropis gigantea* L.

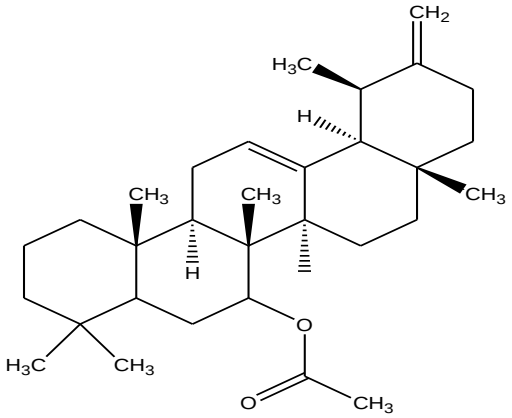
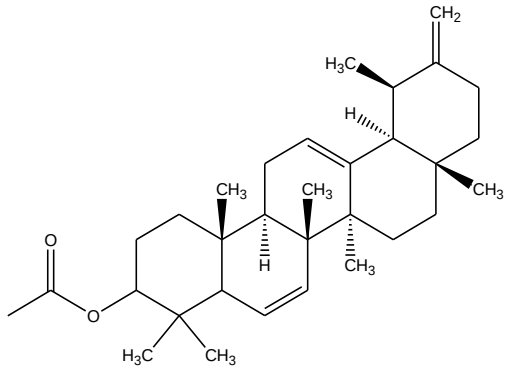
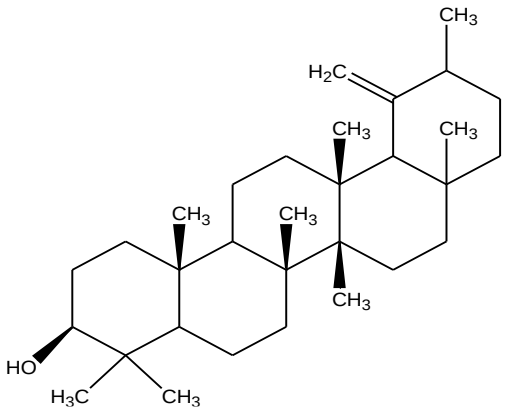
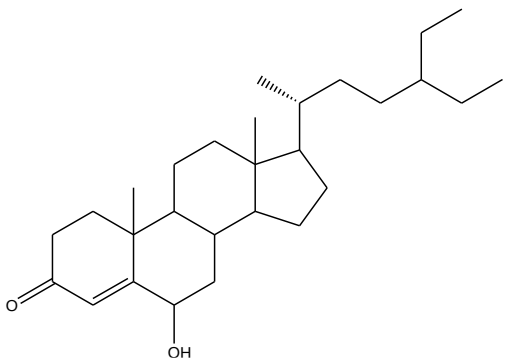
Chemical structure	Name of the compound	Source
	2-Methylbenzoic acid[55]	Aerial part of *Calotropis gigantea* L.
	Oxirane[55]	Aerial part of *Calotropis gigantea* L.
	Henicosane[55]	Aerial part of *Calotropis gigantea* L.
	Methyl hexadecanoate[55]	Aerial part of *Calotropis gigantea* L.
	Hexacosan-1-ol[55]	Aerial part of *Calotropis gigantea* L.
	6-Butoxy-5-ethylicosane[55]	Aerial part of *Calotropis gigantea* L.
	Betulin[55]	Aerial part of *Calotropis gigantea* L.

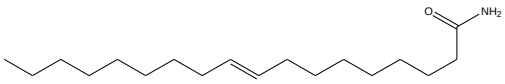
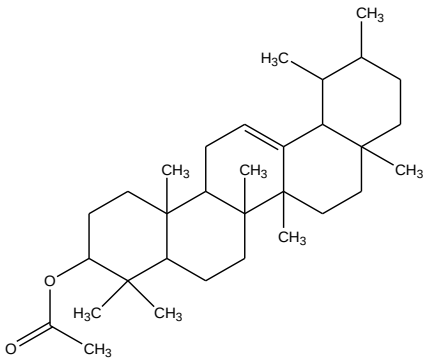
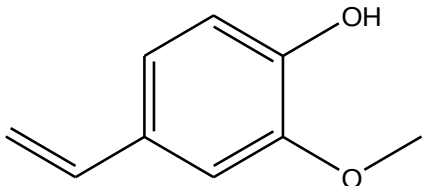
Chemical structure	Name of the compound	Source
	2,3-Dimethyl naphthalene[55]	Aerial part of *Calotropis gigantea* L.
	2-Methyl-4,6 quinolinediol[55]	Aerial part of *Calotropis gigantea* L.
	Desmosterol[56]	Aerial part of *Calotropis gigantea* L.
	Gamma-sitosterol[56]	Aerial part of *Calotropis gigantea* L.
	9,19-Cyclo-9β-lanost-24-en-3β-ol, acetate[56]	Aerial part of *Calotropis gigantea* L.

Chemical structure	Name of the compound	Source
	Campesterol[56]	Aerial part of *Calotropis gigantea* L.
	Fucosterol[56]	Aerial part of *Calotropis gigantea* L.
	5-Norbornene-2-carboxylic acid[57]	Aerial part of *Calotropis gigantea* L.
	Oleanolic acid[57]	Aerial part of *Calotropis gigantea* L.
	α/β-Amyrin[57]	Aerial part of *Calotropis gigantea* L.

Chemical structure	Name of the compound	Source
	Ascorbic acid[38]	Aerial part of *Calotropis gigantea* L.
	Glutathione[38]	Aerial part of *Calotropis gigantea* L.
	Lanosterol[58]	Aerial part of *Calotropis gigantea* L.
	Phytol[58]	Aerial part of *Calotropis gigantea* L.
	12-Oleanen-3-yl acetate[58]	Aerial part of *Calotropis gigantea* L.

Chemical structure	Name of the compound	Source
	Calotroposide-A[59]	Root bark of *Calotropis gigantea* L.
	Calotroposide-B[59]	Root bark of *Calotropis gigantea* L.
	Urs-20(30)-en-12-ol acetate[61]	Root bark of *Calotropis gigantea* L.

Chemical structure	Name of the compound	Source
	Urs-12,20(30)-dien-7-ol acetate[61]	Root bark of *Calotropis gigantea* L.
	Urs-6(7),12,20(30)-trien-3-ol acetate[61]	Root bark of *Calotropis gigantea* L.
	α/β-Calotropeol[38]	Root bark of *Calotropis gigantea* L.
	Procesterol[63]	Root bark of *Calotropis gigantea* L.

Chemical structure	Name of the compound	Source
	9-Octadecenamide[58]	Aerial part of *Calotropis gigantea* L.
	α/β-Amyrin acetate[38]	Aerial part of *Calotropis gigantea* L.
	2-Methoxy-4-vinylphenol[58]	Aerial part of *Calotropis gigantea* L.

1.6.1.8. Biological literature review on the *Calotropis gigantea*

The various pharmacological activities of whole plant or plant parts including latex of *Calotropis gigantea* (Linn.) have been documented. Reported activities include analgesic[64], hepatoprotective and antidiabetic[65-66] activities in flowers; antipyretic[67], anticonvulsant[68], anxiolytic[68], sedative[68], anti-diarrhoeal[69], pregnancy interceptive[70] insecticidal[71] and wound healing[72] activities in roots and hypoglycemic[66], antibacterial[43], mosquitocidal[73] and cytotoxic[44,45] activities in leaves. The crude latex extract of *Calotropis gigantea* is reported to exhibit strong proteolytic activity, being able to hydrolyse casein, human fibrinogen and crude fibrin clot in a dose dependent manner[43]. The latex of *Calotropis gigantea* exhibited marked stimulant action on the spontaneous activity of isolated non-gravid rat uterus[43]. In addition, extracts from leaves of *Calotropis gigantea* caused marked contraction in isolated preparation of rabbit's duodenum, rat's ileum and uterine horn from rat[43].

1.6.2. *Amoora rohituka* (Roxb.)

Amoora rohituka (Roxb.) which belongs to Meliaceae family, is an evergreen tree that grows wild and planated in forests and roadsides in Dhaka, Mymensing, Chittagong, Sylhet and Rajshahi of Bangladesh[74]. The stem bark (Figure 1.3) of *Amoora rohituka* plant (Figure 1.4) have been taken in this study for phytochemical and biological studies.

1.6.2.1. Scientific classification of the investigated plant: *Amoora rohituka*

Kingdom : Plantae
Division : Tracheophyta
Class : Magnoliopsida
Order : Rutales
Family : Meliaceae
Genus : *Amoora*
Species : *Amoora rohituka* (Roxb.)
[**Syno nym**: *Aphanamixis polystachya* (Wall.) Parker]

1.6.2.2. Common name of the plant

Traditional name: Roina, Royena

Bangali name: Pitraj

English name: Rohituka tree

1.6.2.3. Botanical description of the investigated plant: *Amoora rohituka*[41]

Habit : large handsome ever green tree with a dense spreading crown and a straight cylindrical bole upto 15 m in height and 1.5-1.8 m in girth.

Leaves : Leaves are large imparipinate, 30-75 cm long; lea flets opposite 4-8 pairs and an odd one, 7.5-23 by 3.4-10 cm, elliptic oblong or oblong lanceolate, acuminate, glabrous on both surfaces, very inequilateral obtuse or acute at the base.

Flowers : Male flowers numerous, erect 4 mm long subglobular, in solitary axillary panicles more than half as long as the leaves. Female or hermaphrodite flowers larger than the male, in axillary or supraaxillary solitary spikes much shorter than the leaves.

Stem bark : Stem bark are dark brown, rough, corky cracked, cut reddish.

Fruit : Fruit 2.5-3.8 cm diameter, globular, yellow when ripe,

pericarp coriaceous, smooth, 3-celled, opening by 3 valves.

Seed : Seeds are oblong with scarlet aril

Wood : The wood is reddish brown when fresh, a ging to deep reddish brown.

Figure 1.3. *Amoora rohituka* plant

Figure 1.4. Stem bark of *Amoora rohituka*

1.6.1.4. About the family: Meliaceae[41]

The Meliaceae family includes 50 genera and 1400 species, with a pantropical distribution one genus extends north into temperate China and south into southeast Australia, and another nearly as far north[41]. It is a family of dicotyledonous plants.

Common features of Meliaceae species are given below:

Plant : flowering plant family of mostly trees and shrubs (and a few herbaceous plants, mangroves) in the order Sapindales. Most species are evergreen, but some are deciduous, either in the dry season or in winter.

Stem : Stem bark sometimes with a milky latex; indumentum simple or stellate

Leaves : Leaves alternate, usually 1-pinnate, rarely 2–3-pinnate or rarely simple, margins mostly entire.

Flowers : Flowers actinomorphic, bisexual or unisexual. Sepals 3–5, free or fused. Petals usually 3–6, rarely up to 14, fused at base or coherent with each other and staminal tube.

Fruits : Fruit a capsule, berry or drupe or rarely a nut.

Seed : Seeds sometimes winged or arillate

1.6.2.5. Some important species of Meliaceae family available in Bangladesh

Some species of Meliaceae family that grow in different areas of Bangladesh, have been used in folk medicine for the treatment of different diseases. Some important species (available in Bangladesh) of Meliaceae family with their pharmacological and folk medicinal uses[75] are listed in table 1.4.

Table 1.4 Some medicinally important species of Meliaceae family available in Bangladesh[75].

Plant's Name [Local name]	Pharmacological properties with plant parts	Folk medicinal uses with plant parts
Azadirachta indica A. Juss [Neem]	Antiseptic, antineoplastic, antidiabetic, antihyperlipaemic, antipyretic, tonic	Gingivitis, sores, malaria, tumours, smallpox, boils, ulcer, eczema, ringworm, scabies, catarrh, diarrhoea, cholera, head scald
Melia sempervirens (Linn.) [Ghora Nim]	Deobstruent, resolvent, alexipharmic, anthelmintic, antilithic, diuretic, antipyretic	Leprosy, scrofula, rheumatism, skin diseases, diarrhea, abscesses
Toona ciliate J. Roem [Rangi, Piya Tun]	Antiperiodic, astringent, tonic, antipyretic	Dysentery, rheumatism, fevers, menstrual disorders, ulcer
Carapa moluccensis Lam. [Tutul]	Astringent, febrifuge, ointment	Diarrhoea, dysentery, abdominal problems, elephantiasis, itch

1.6.2.6. Traditional uses of *Amoora rohituka*

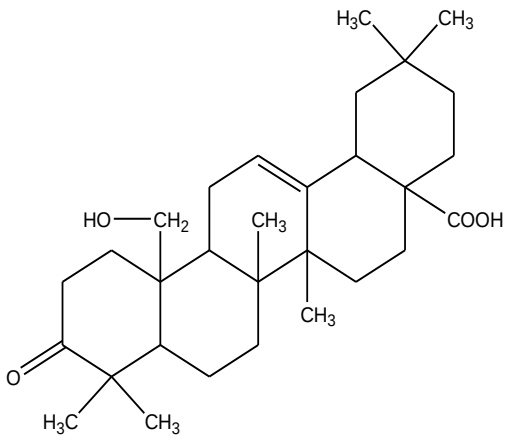
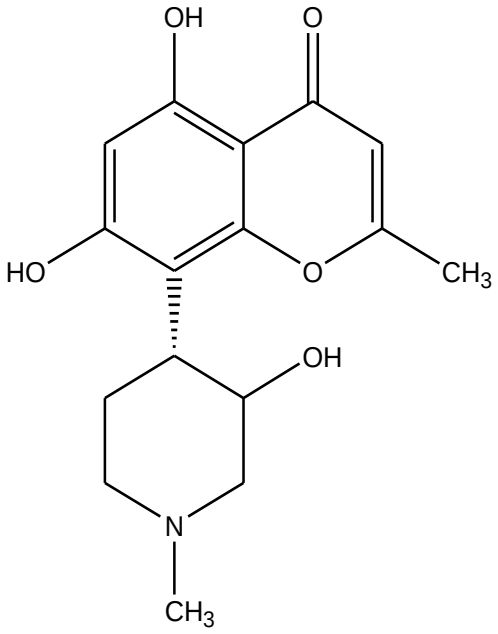
Various parts of *Amoora rohituka* are used in Bengali traditional medicine because of their anticancer, antimicrobial, antiinammatory and hepatoprotective properties. The stem bark of *Amoora rohituka* is acrid, astringent, bitter, vulnerary, digestive, anthelmintic, depurative, urinary astringent, ophthalmic and refrigerant[75]. Stem bark powder of this plant is traditionally effective in hepatomegaly and also used in spleen diseases, tumours, ulcer and abdominal complaints. The seeds of *Amoora rohituka* have a folkloric

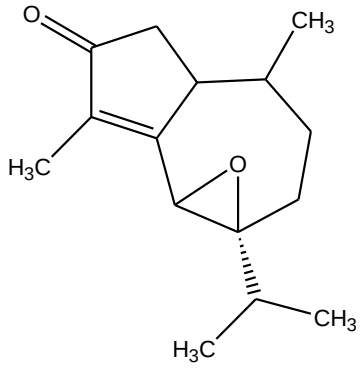
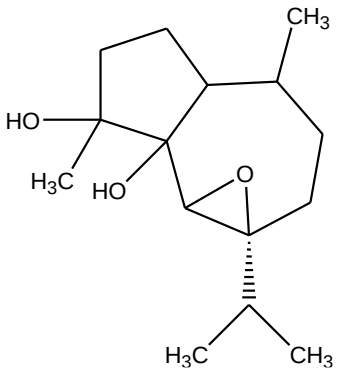
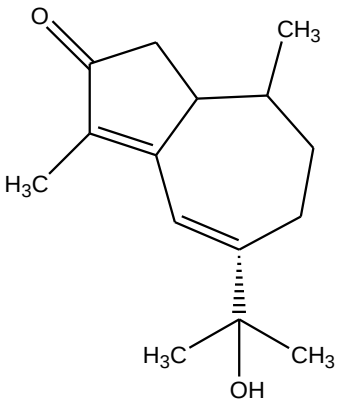
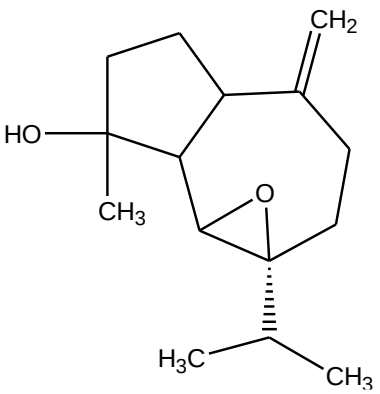
reputation to exhibit laxative, anthelmintic and antiulcer properties. They are useful in ulcers. The seed oil is used as liniment in muscular pains[76].

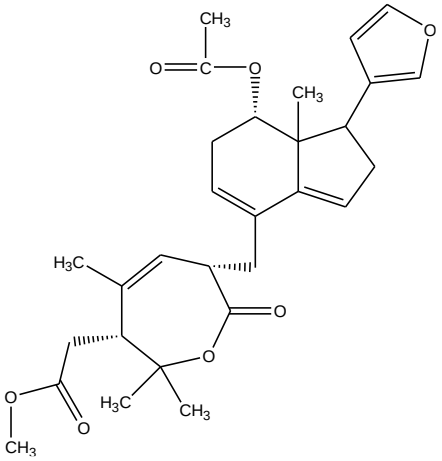
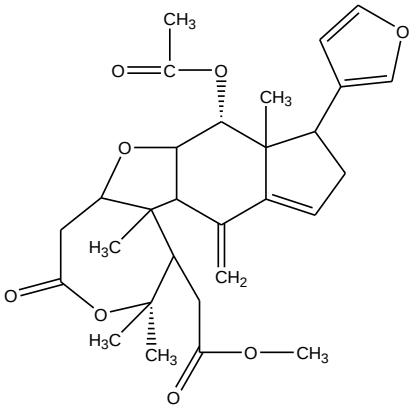
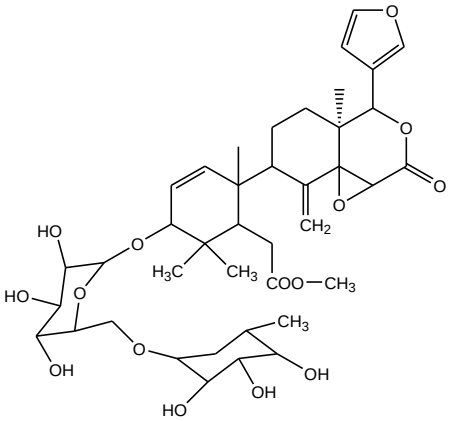
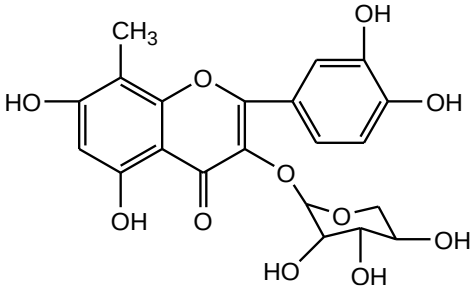
1.6.2.7. Chemical literature review on the *Amoora rohituka*

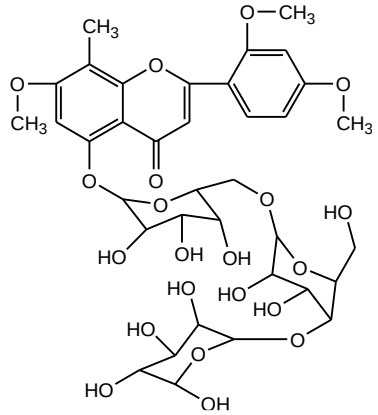
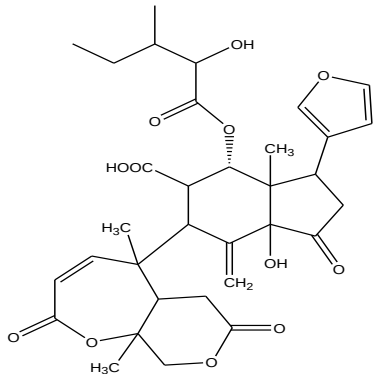
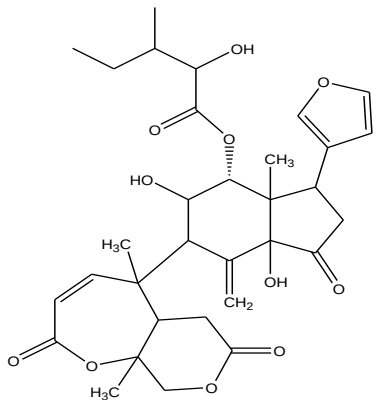
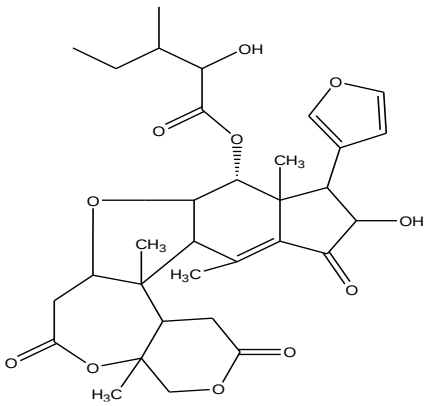
Chemical investigations of *Amoora rohituka* afforded a number of sesquiterpenes[79] limonoids[80-90], , amooramin (a triterpene acid)[77], alkaloid[78], flavonoid glycosides[81] and saponin[91-93]. The compounds isolated from different part of *Amoora rohituka* are listed in table 1.5.

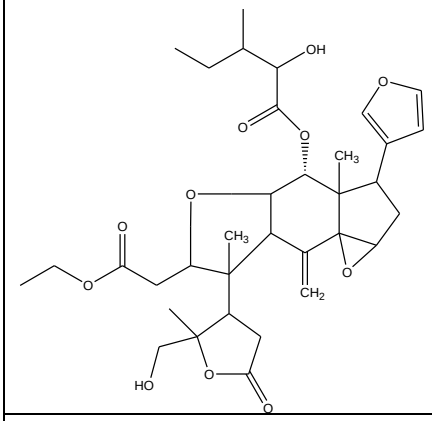
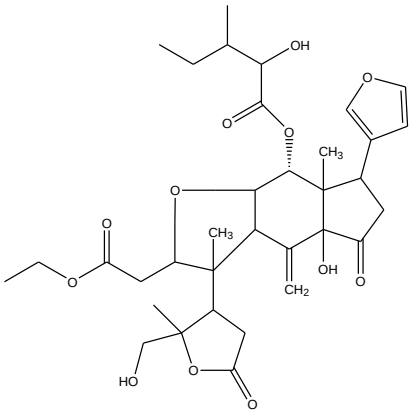
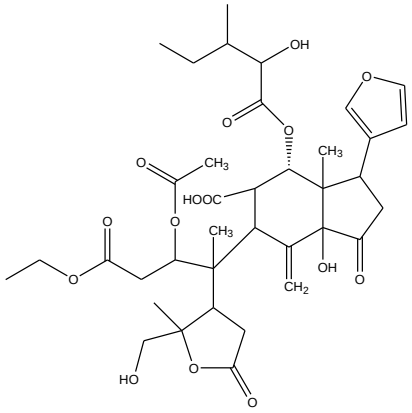
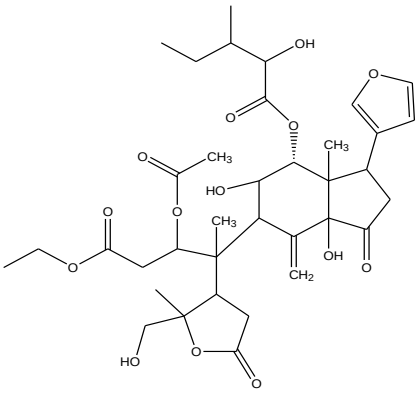
Table 1.5 List of compounds isolated from different parts of *Amoora rohituka*

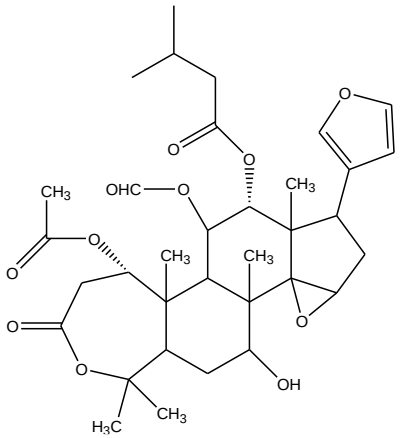
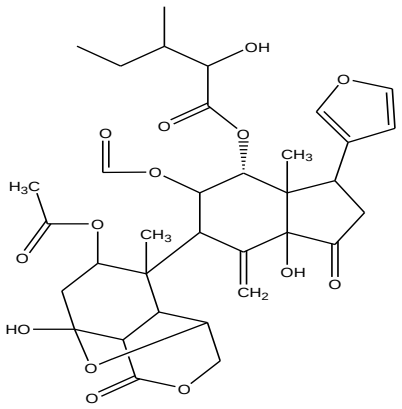
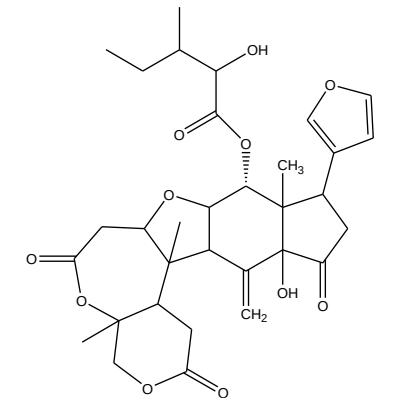
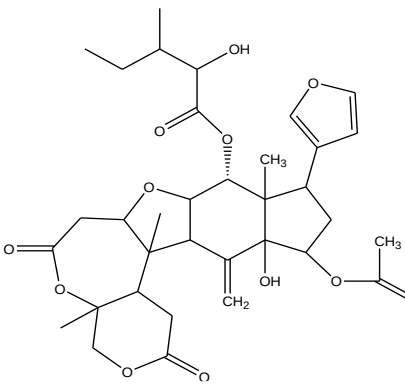
Chemical structure	Name of the compound	Source
	Amooranin[77]	Stem bark of *Amoora rohituka*
	Rohitukine[78]	Stem bark of *Amoora rohituka*

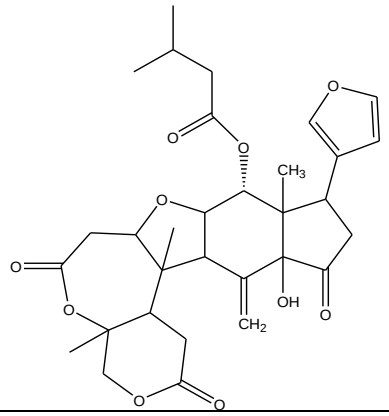
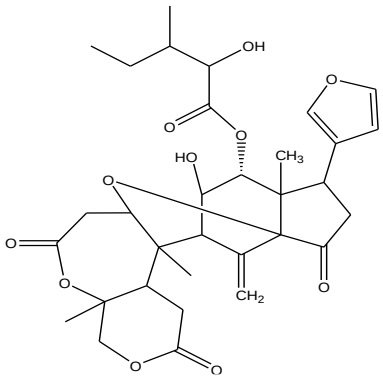
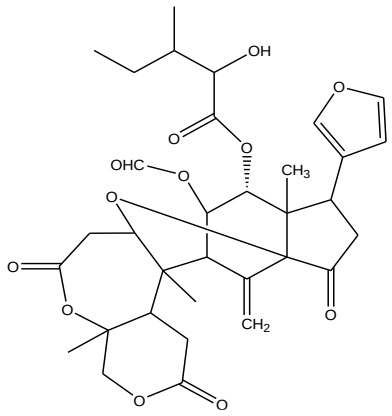
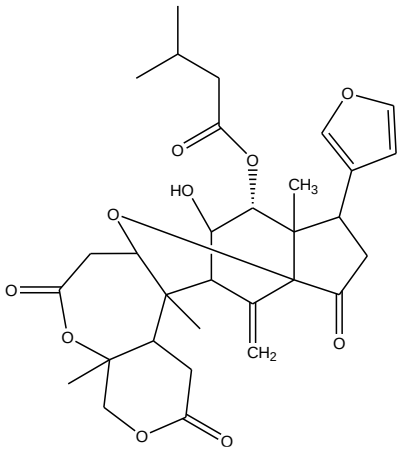
Chemical structure	Name of the compound	Source
	6β,7β-epoxyguai-4-en-3-one[79]	Stem bark of *Amoora rohituka*
	6β,7β-epoxy-4β,5-dihydroxyguaiane[79]	Stem bark of *Amoora rohituka*
	Sootepdienone[79]	Stem bark of *Amoora rohituka*
	Orientalol-C[79]	Stem bark of *Amoora rohituka*

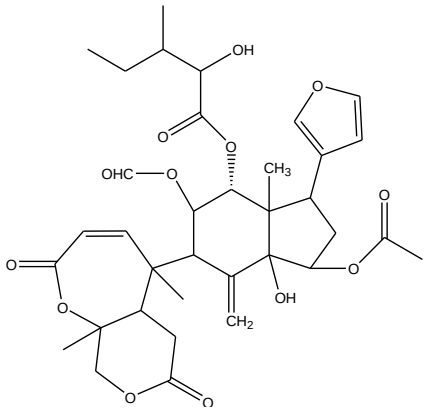
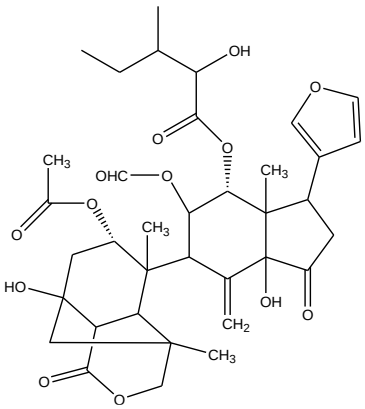
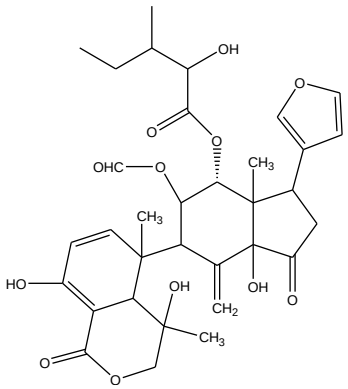
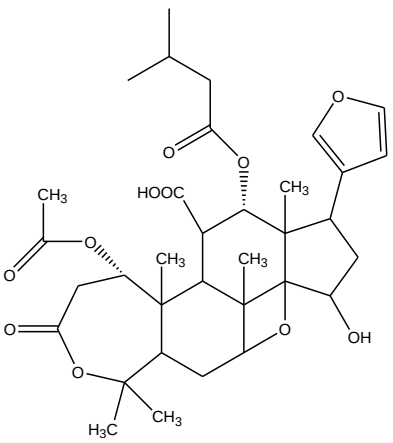
Chemical structure	Name of the compound	Source
	Aphanamixoids-A[80]	Leaves of *Amoora rohituka*
	Aphanamixoids-B[80]	Leaves of *Amoora rohituka*
	Amoorinin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside[81]	Root of *Amoora rohituka*
	8-Methyl-5,7,3′,4′-tetrahydroxyflavone-3-*O*-α-L- arabinopyranoside[81]	Root of *Amoora rohituka*

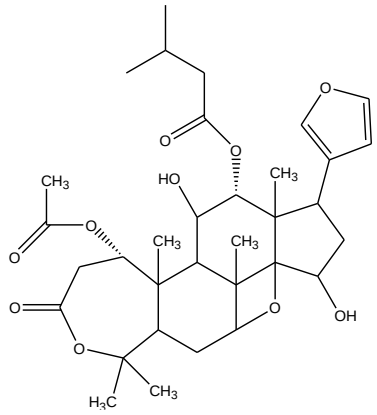
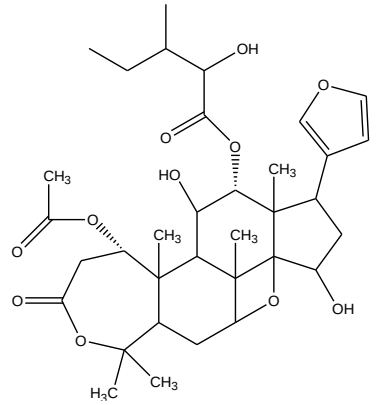
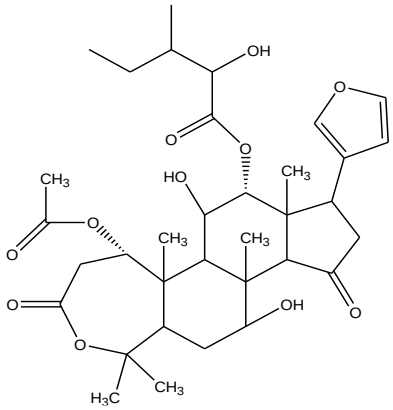
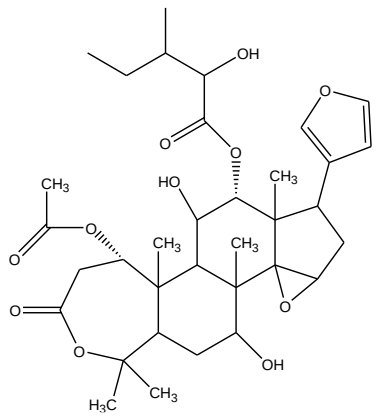
Chemical structure	Name of the compound	Source
	8-methyl-7,2′,4′-trimethyl-flavanone-5-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside[81]	Root of *Amoora rohituka*
	Aphapolynin-C[82]	Fruit of *Amoora rohituka*
	Aphapolynin-D[82]	Fruit of *Amoora rohituka*
	Aphapolynin-E[82]	Fruit of *Amoora rohituka*

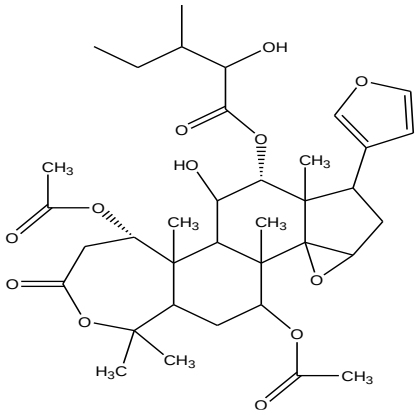
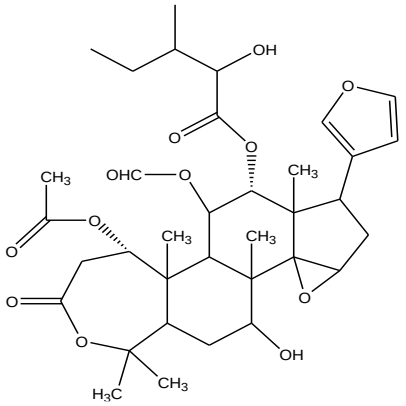
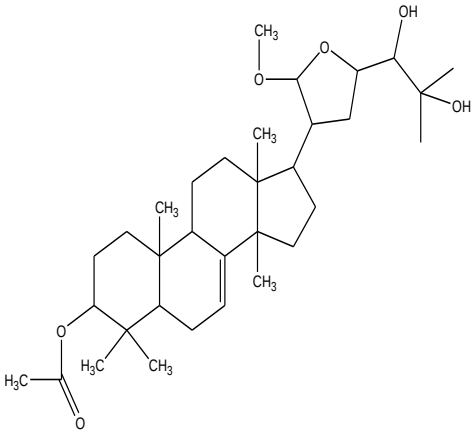
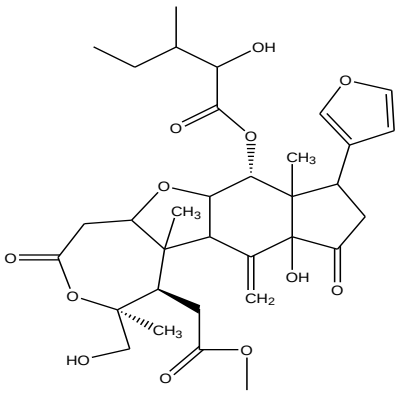
Chemical structure	Name of the compound	Source
	Aphapolynin-F[82]	Fruit of *Amoora rohituka*
	Aphapolynin-G[82]	Fruit of *Amoora rohituka*
	Aphapolynin-H[82]	Fruit of *Amoora rohituka*
	Aphapolynin-I[82]	Fruit of *Amoora rohituka*

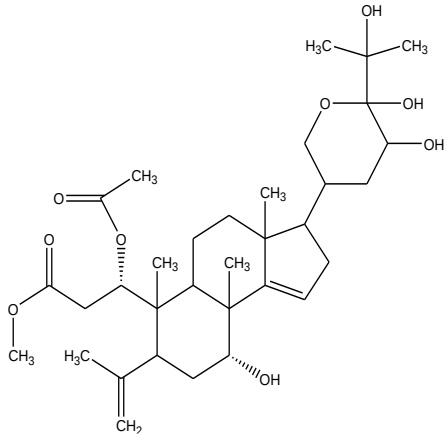
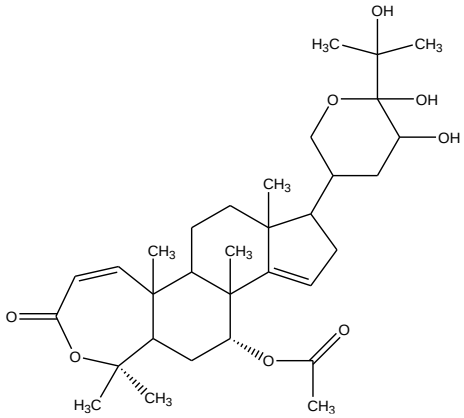
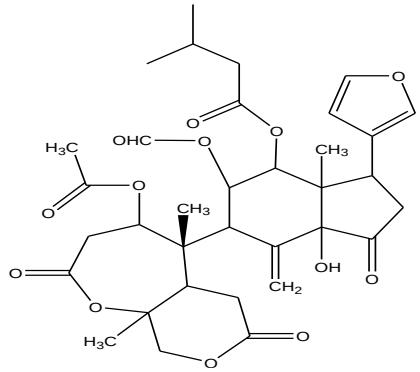
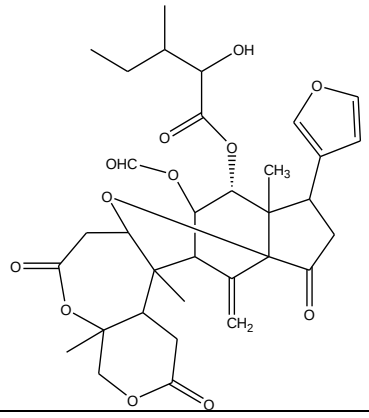
Chemical structure	Name of the compound	Source
	Aphanalides-H[82, 83]	Fruit of *Amoora rohituka*
	Aphanamolide-B[82]	Fruit and seed of *Amoora rohituka*
	Rohituka-3[82]	Fruit and seed of *Amoora rohituka*
	Rohituka-7[82]	Fruit and seed of *Amoora rohituka*

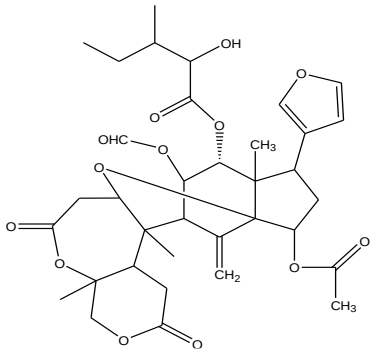
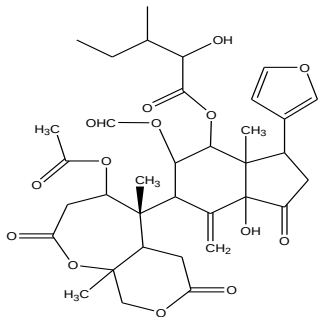
Chemical structure	Name of the compound	Source
	Rohituka-9[82]	Fruit and seed of *Amoora rohituka*
	Rohituka-12[82]	Fruit and seed of *Amoora rohituka*
	Rohituka-14[82]	Fruit and seed of *Amoora rohituka*
	Rohituka-15[82]	Fruit and seed of *Amoora rohituka*

Chemical structure	Name of the compound	Source
	Dregenana-1[82]	Fruit and seed of *Amoora rohituka*
	Aphanamolide-A[82]	Fruit and seed of *Amoora rohituka*
	Aphapolynin-A[82]	Fruit of *Amoora rohituka*
	Aphanalides-A[82, 83]	Fruit of *Amoora rohituka*

Chemical structure	Name of the compound	Source
	Aphanalides-B[82, 83]	Fruit of *Amoora rohituka*
	Aphanalides-C[82, 83]	Fruit of *Amoora rohituka*
	Aphanalides-D[82, 83]	Fruit of *Amoora rohituka*
	Aphanalides-E[82, 83]	Fruit of *Amoora rohituka*

Chemical structure	Name of the compound	Source
	Aphanalides-F[82, 83]	Fruit of *Amoora rohituka*
	Aphanalides-G[82,83]	Fruits of *Amoora rohituka*
	Polystanins-C[84]	Fruits of *Amoora rohituka*
	Aphapolynins-B[85]	Fruits of *Amoora rohituka*

Chemical structure	Name of the compound	Source
	Polystanins-A[84]	Fruit of *Amoora rohituka*
	Polystanins-B[84]	Fruit of *Amoora rohituka*
	Rohitukin[86]	Seed of *Amoora rohituka*
	Polystachin[87]	Seed of *Amoora rohituka*

Chemical structure	Name of the compound	Source
	Rohituka-13[88]	Seed of *Amoora rohituka*
	Tr-B[86]	Seed of *Amoora rohituka*

In addition to the above listed chemical, several researchers have also reported the isolarion of poriferasterol-3-rhamnoside, aphanamixinin, aphanamixol, eperu-13-en-8*β*,15-diol, 3',4',5'-trihydroxyflavonone-7-*O*-β-D-xylopyranosyl-β-D-arabinopyranoside and stigma-5,24-diene-3-β-o-β-D-glucopyranoyl-o-α-L-rhamnopyranoside from *Amoora rohituka*[81,91-93].

1.6.2.8. Biological literature review on the *Amoora rohituka*

A literature survey has shown that different pharmacological studies have been carried out on different parts of *Amoora rohituka* plant. Ethyl acetate extract of the stem bark of *Amoora ruhituka* showed anti-tumour activity against Dalton's lymphoma ascites cells (DLA) in mice[94]. Treatment of mice with stem bark extract of *Amoora rohituka* reduced radiation-induced chromosome damage[95]. The hepatoprotective activity of the plant extract[96,97], antimicrobial activity of volatile oils from seed, antiviral and antibacterial activity of the isolated limonoid rohitukin[98], cytotoxicity of amoorastatin[99], growth inhibitory effect of 12**a**-hydroxyamoorastatin against murine P388 lymphocytic leukaemia cell lines[99], feeding deterrence of some secondary metabolites against *Tribolium castaneum*[100] have been reported earlier. *In vivo* amooranin, a triterpene acid isolated from stem bark of *Amoora rohituka*, exhibited antitumor activity against N-nitrosomethylurea induced mammary adenocarcinoma in rats[101]. Amooranin (25-hydroxy-3-oxoolean-12-en-28-oic) induced the growth arrest and apoptosis[102] activity in human

breast cancer cells through caspase[103] and it overcame multidrug resistance in human leukemia and colon carcinoma cell lines[104]. In addition, *in vitro* amooranin was to be cytotoxic against human colon carcinoma cell line with an IC_{50} value of 2.9 μg/mL[105].

1.7. Merits of *Calotropis gigantea* and *Amoora rohituka* for phytochemical and biological studies

Many plants with antioxidant ingredients have been used traditionally in treatment of tumour[106]. Generally imbalance between cellular oxidant species production and antioxidant capability produces reactive oxygen species (ROS) which are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis[106]. Many natural compounds of plant-derived extracts have vital roles in balancing the intracellular redox status and in antioxidant function. So natural compounds with antioxidative effects are important therapeutic prospects for cancer chemotherapy[107-108].

Literature on *Calotropis gigantea* showed that its flowers were found to increase the antioxidative parameters and free radical scavenging activity in diabetic rats[66]. But there are no reports on isolation of phytochemicals from flower of *Calotropis gigantea* with antimicrobial, cytotoxic and antineoplastic activities *in vivo* and *in vitro* model. These evidences indicate that flower of *Calotropis gigantea* have merits for investigation in isolating the active constituents to evaluate their antimicrobial, cytotoxic and antineoplastic properties.

Moreover, root bark of *Calotropis gigantea* showed anti-inflammatory activity in various biological systems[109] and cardenolides with potent cytotoxic properties (*in vitro*) were also isolated from root bark of this plant[46,51]. But no reports regarding the antineoplastic activity of root bark of *Calotropis gigantea in vivo* model are available in literature. So the root bark of this plant is the promising candidate to evaluate its *in vivo* antineoplastic activity against Ehrlich ascites carcinoma (EAC) in mice.

On the other hand, stem bark of *Amoora rohituka* showed potent antioxidative and free-radical scavenging ability *in vitro* assays[110]. Due to its potent antioxidative and free-radical scavenging ability, the stem bark of *Amoora rohituka* could be used as a potential preventive action taken to improve free radical-mediated diseases like cancer. Majority of the recent studies on alternative medicine suggested that *Amoora rohituka* possesses considerable antitumor[75] and antibacterial properties[98,111]. An ethyl acetate extract derived from the stem bark of *Amoora rohituka* exhibited antitumor activity on mice inoculated with Dalton's lymphoma ascites cells (DLA)[94]. In addition, many researchers have reported on isolation of phytochemicals with antineoplastic[101-102], cytotoxic[77,99] and

antimicrobial[75] properties from different parts of this plant. But it is expected that more phytochemicals with potent antineoplastic, cytotoxic and antimicrobial properties may be present in the stem bark of *Amoora rohituka*. So there is a scope to isolate more active constituents from the stem bark of this plant and put them on different biological screening to assay their pharmacological properties. In this aspect, stem bark of *Amoora rohituka* is an important natural source to carry out phytochemical and biological screening.

1.8. Objective of this research work

In the present investigation two plants *Calotropis gigantea* (Linn.) and *Amoora rohituka* (Roxb.) were selected for phytochemical and biological studies.

Both *Calotropis gigantea* and *Amoora rohituka* have tremendous uses in traditional medicines. But the folk medicinal practitioners use these two plants without having knowledge about their side effects and toxicity. If it is possible to isolate and identify the secondary components present in these plants with their pharmacological properties, we can properly use these two plants for the treatment of various diseases and it will ultimately lead to the discovery of new drug. In order to do so, research on *Calotropis gigantea* and *Amoora rohituka* is of great importance to identify and characterize secondary metabolites with biological studies.

So the objective of this research work is to isolate and determine the structure of bioactive principles from *Calotropis gigantea* (Linn.) and *Amoora rohituka* (Roxb.) and study of their effects on bacteria, fungi, cancer cell and insects

The following protocols have been used for achieving the research goal:

- Collection, authentication and processing of plant materials
- Extraction of plant materials by organic solvents
- Isolation and purification of compounds from extracts by chromatographic techniques.
- Structure elucidation of isolated compounds through analysis of their IR, NMR & Mass spectrum.
- *In vitro* antimicrobial study of isolated compounds and extracts
- *In vivo* antineoplastic study of isolated compounds and extracts against Ehrlich ascites carcinoma
- *In vitro* cytotoxic activity study of isolated compounds against human vulval-derived epidermoid carcinoma cells (A431 cell line)
- Study on insecticidal activity of isolated extracts against both larvae and adults of *Tribolium castaneum*

2.1. Introduction

Medicinal plants are the most reliable foundation to discover the new chemical entities for the health care products. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body[112]. The study of medicinal plants opened the door to the development of purified and defined chemical compounds as dose-controlled medicines. As therapeutic uses of plants continued with the progress of civilization and development of human knowledge, scientists endeavored to isolate different chemical constituents from medicinal plants, put them to biological and pharmacological tests and thus have been used to identify and isolate therapeutically active compounds[113]. This overall research has ultimately developed modern medicines. This study has been designed to isolate and identify bioactive compounds from two important medicinal plants *Calotropis gigantea* (Linn.) and *Amoora rohituka* (Roxb.).

2.2. Materials and methods used in phytochemical study

The materials and methods used in this investigation are briefly described below:

2.2.1. Chemicals

The following chemicals were used in the phytochemical study:

i) Silical gel for column chromatography (60-120 mesh; E-Mark, Germany)

ii) Silica gel for thin layer chromatography (kiselgel 60 GF_{254}, E-Merck, Germany)

iii) n-Hexane (E-Merck, Germany)

iv) Ethyl acetate (E-Merck, Germany)

v) Chloroform (E-Merck, Germany)

vi) Methanol (E-Merck, Germany)

vii) Petroleum ether (40-60ºC; Sigma, India)

viii) Concentrated H_2SO_4 (Sigma, India)

ix) Vanillin (Loba, India)

x) Bismuth subnitrate (E-Merck, Germany)

xi) Glacial acetic acid (E-Merck, Germany)

xii) Potassium iodide (Loba, India)

xiii) Perchloric acid (Sigma, India)

2.2.2. Thin layer chromatographic plates:

Preparation: For the preparation of thin layer chromatographic plates, a number of glass plates (20 cm × 5 cm) were thoroughly washed to remove any dirt present and dried

in a hot oven. The plates were then placed over an aligning tray specially made for TLC (Thin layer chromatography). The slurry (2 g silica gel/plate) was then distributed uniformly over the carrier plates with the help of a TLC spreader by adjusting the thickness (0.3 mm) of the layer. After air drying, the coated plates were preserved for use[114].

Plates for preparative thin layer chromatography (PTLC) were prepared by coating glass plates (20×20 cm) to a thickness of 0.5 mm using a slurry of 40 g (for 6 plates) silica gel and 85 mL of distilled water. The plates were allowed to air dry for a time and then activated at 110°C for 2-3 hours in an oven[114].

2.2.3. TLC spreader

A TLC spreader (Richmond, USA) which was moved over the glass plates, was used to apply the adsorbent (here silica gel) of desired thickness over the entire surface of the plate.

2.2.4. TLC jar

Cylindrical shaped glass chamber (TLC tank; Sigma-Aldrich, USA) with air tight glass lid was used for the development of a chromatoplate.

2.2.5. UV light

The developed and dried plates were exposed to UV light (Wave length: 254 mm and 366 nm) to observe quenching or fluorescing compounds.

2.2.6. Iodine Vapor

The developed chromatogram was exposed to iodine vapor in a closed jar containing few crystals of iodine. Brownish spots indicate resolution of the compounds.

2.2.7. Spray Reagents

The following different types of spray reagents were used depending upon the nature of compounds expected to be present in the crude extracts or fraction.

1) Vanillin in sulfuric acid: This reagent was prepared by dissolving vanillin (1g) in concentrated H_2SO_4 (100 mL) and used as general reagent for non-alkaloidal compounds. The plates sprayed with this reagent were heated at 110°C for 10-15 minutes for the development of colors with time, which, in turn, indicated the presence of higher alcohols, steroids, heterocyclic compounds, flavonoids, glycosides and terpenes[115].

2) Modified Dragendorff's reagent: This reagent was prepared by mixing equal parts (v/v) of 1.7% bismuth subnitrate dissolved in 20% acetic acid in water and 40% aqueous

solution of potassium iodide. Usually orange-red color indicates the presence of alkaloids by this reagent[116].

3) Perchloric acid reagent: When the TLC plates were sprayed with aqueous perchloric acid (2%) and the plates were heated at 150ºC for 10 minutes, steroids (if present) produced brown spots[114].

2.2.8. Melting point

A pure solid substance will be melted sharply at a definite temperature, while an impure substance will have a lower and indefinite melting point. Melting points were determined on melting point apparatus (PIC, India).

2.2.9. Instruments used for spectroscopic data

All the spectra were taken in Analytical Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR) Laboratories, Dhaka-1205, Bangladesh, using the following instruments:.

1) Infrared (IR) spectra: IR-spectra were taken on FTIR-8900 spectrophotometer (Shimadzu Kyoto, Japan)

2) Mass spectra: High Resolution TOF Mass Spectra were obtained using a Waters LCT Premier mass spectrometer (UK) coupled with a Waters AQUITY HPLC system, with data acquisition achieved using MassLynx software, version 4.0.

GCMS-QP2010S (Shimadzu Kyoto, Japan) spectrometer was used for taking gas chromatography-mass spectrum (GC-MS).

3) NMR spectra: NMR spectra were recorded on Bruker 400 MHz FT spectrometer (DPX-400, Switzarland). In this study, deuterated chloroform ($CDCl_3$) was used as NMR solvent. In ^{1}H-NMR spectrum, the solvent peak was observed at δ 7.25 (singlet) whereas it was found at 77.34 (triplet) in ^{13}C-NMR spectrum.

2.3. Phytochemical study on *Calotropis gigantea* (Linn.)

2.3.1. Collection of plant material

The root bark and flowers of *Calotropis gigantea* (Linn.) (Family: Asclepiadaceae) were selected for the chemical investigation. The root and flowers of *Calotropis gigantea* were collected during the month of May-June, 2007 from the relevant area (Meherchandi) of Rajshahi University campus. The plant and root were taxonomically identified by Professor A.T.M Naderuzzaman and Dr. Goura Pado Ghosh, Associate Professor, Department of Botany, University of Rajshahi. Voucher specimen (No. 1A. Alam, collection date 15.08.2004) was kept in the Department of Botany, University of Rajshahi.

2.3.2. Drying and pulverization of plant materials

The collected roots and flowers were washed with water in such a way that adhering dirty materials were completely removed. After washing, the upper thin and rough surfaces of root were removed. Then the root barks were peeled out from the wooden sticks and cut into small pieces. These small pieces along with flowers were sun dried for 7-10 days and finally kept in an electric oven for 72 hours at 40°C. After complete drying, the dried pieces of root bark and flower were then separately pulverized into a coarse powder with the help of a grinding machine (FFC-15, China) and were stored in airtight containers for further use.

2.3.3. Extraction of powered root bark with methanol

After pulverization the weight of total root bark powder was 1.5 kg. For cold extraction the root bark powder was kept immersed in 2.0 liters methanol in an aspirator bottle at room temperature for 15 days with occasional shaking and stirring. Then the contents were pressed through the Tincture Press (Karlkolb, Scientific-Technical Suppliers, Germany) to get maximum amount of extract. The extract was then filtered through a filter paper (Whatman No.1) and concentrated with a rotary evaporator (Sigma-Aldrich, USA) under reduced pressure at 60°C to obtain a crude extract of 40.6 g and designated as methanol extract of root bark of *Calotropis gigantea* (ME)[114].

2.3.4. Extraction of flower powder with ethyl acetate

The flower powder (1.0 kg) was similarly extracted with ethyl acetate (1.5 L) at room temperature for 7 days by the method as described earlier for root bark powder. The solvent was completely removed by rotary vacuum evaporator from the crude extract to yield a residue of 38 g and it was designated as ethyl acetate extract of *Calotropis gigantea* flower (EECF)[114]. Both ME and EECF were stored in vacuum desiccators for further use.

2.3.5. Fractionation of methanol extract of root bark of *Calotropis gigantea* (ME)[117]

2.3.5.1. Preparation of mother solution

The crude methanol extract (25.0 g) was triturated with 90 mL of methanol containing 10 mL of distilled water. The crude extract was dissolved completely. This aqueous methanolic solution was termed as mother solution.

2.3.5.2. Partitioning with petroleum ether (40-60°C)

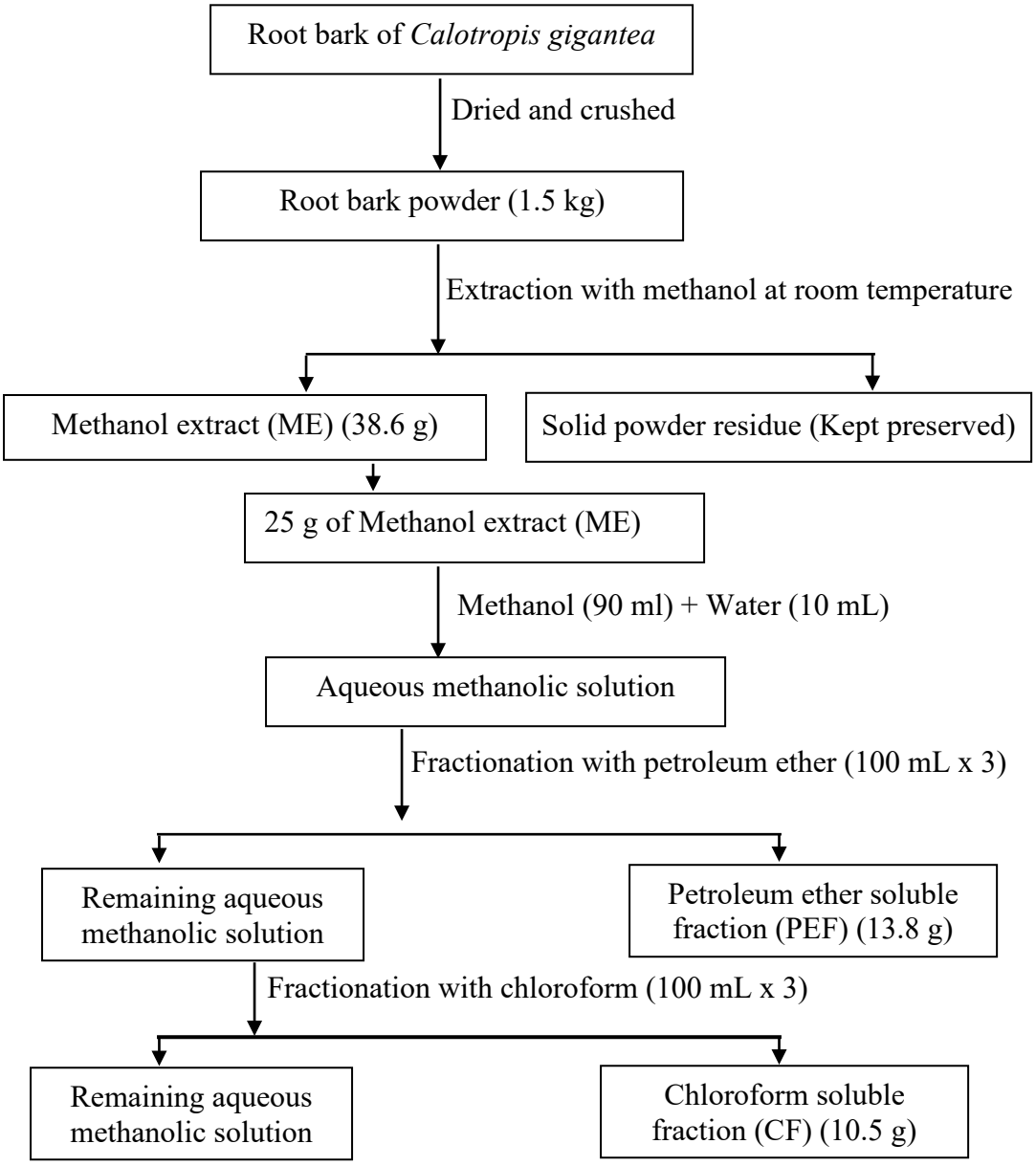
The mother solution was taken in a separatory funnel. 100 mL of the petroleum ether (40-60°C) was added to it, shaken vigorously and allowed to stand for layer separation. The organic portion (upper layer) was then collected. The process was repeated three times. Petroleum ether

soluble fractions were collected together and evaporated to obtain a semisolid mass (13.8 g), which was orange in color and designated as petroleum ether soluble fraction of methanol extract (PEF). The aqueous fraction was used for the next step.

2.3.5.3 Partitioning with chloroform

After fractionation with petroleum ether, the aqueous methanolic phase was shaken vigorously with (100 mL) chloroform and allowed to stand for the complete separation of the layer. The lower organic layer was collected. The process was repeated three times as before. The chloroform layers were then dried and concentrated under reduced pressure when a reddish brown colored mass (10.5 g) was obtained and it was designated as chloroform soluble fraction of methanol extract (CF).

2.3.6 Schematic flow diagram for extraction and fractionation of root bark of *Calotropis gigantea* (Linn.)



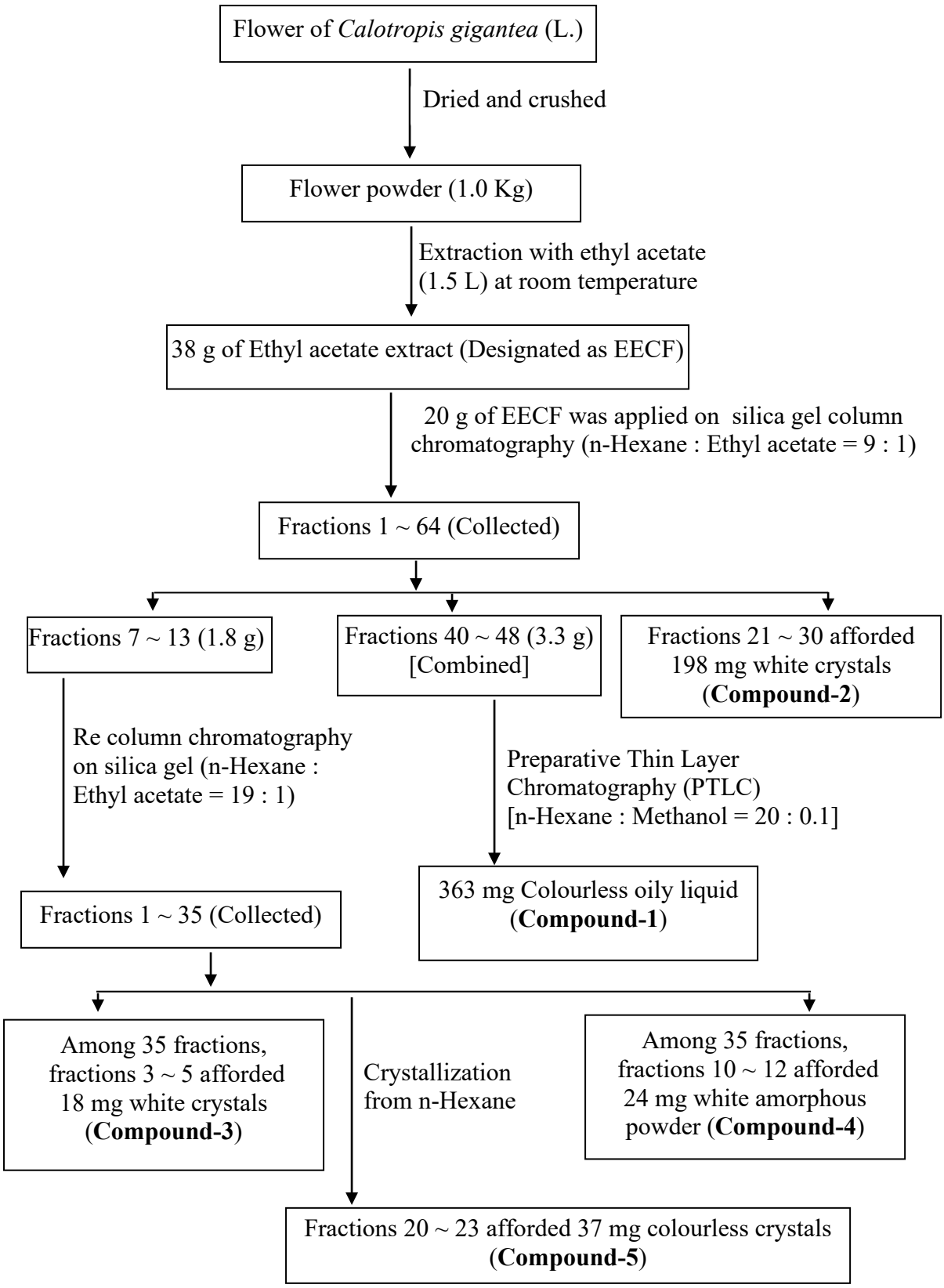
2.3.7. Preliminary TLC screening on EECF, ME, PEF and CF

A small portion of EECF, ME, PEF and CF were dissolved in respective solvent and the solutions were spotted on TLC plates. Then the TLC plates were run by different specific solvent system and were viewed individually under UV light and also with the vanillin-H_2SO_4 reagent[114]. Through several pilot experiments, it was found that each extract and fractions contained different type of compounds and the compounds of each sample were separated by different specific solvent systems. The overall findings from preliminary TLC screening are summarized in table 2.1.

Table 2.1 Types of compounds present in EECF, ME, PEF and CF

Sample	Solvent system	Code No. of each spot	R_f value	Colour with vanillin-H_2SO_4	Possible compound
EECF	n-Hexane : Ethyl acetate (9 : 1)	EECF-1	0.91	Violet	Terpene
		EECF-2	0.85	Pink	Steroid
		EECF-3	0.80	Violet	Terpene
		EECF-4	0.78	Violet	Terpene
		EECF-5	0.69	Black	Glycoside
		EECF-6	0.48	Yellow	Flavonoid
		EECF-7	0.43	Brown	Heterocyclic
ME	n-Hexane : Chloroform (7 : 3)	ME-1	0.88	Pink	Steroid
		ME-2	0.82	Violet	Terpene
		ME-3	0.74	Violet	Terpene
		ME-4	0.57	Black	Glycoside
		ME-5	0.54	Yellow	Flavonoid
PEF	n-Hexane : Ethyl acetate (9 : 1)	PEFM-1	0.72	Pink	Steroid
		PEFM-2	0.67	Brown	Heterocyclic
		PEFM-3	0.52	Violet	Terpene
		PEFM-4	0.35	Black	Glycoside
CF	n-Hexane : Chloroform : Ethyl acetate (8 : 1.5 : 0.5)	CFM-1	0.86	Black	Glycoside
		CFM-2	0.81	Violet	Terpene
		CFM-3	0.70	Black	Glycoside
		CFM-4	0.44	Pink	Steroid
		CFM-5	0.32	Brown	Heterocyclic

2.3.8. Schematic flow diagram for phytochemical study on *Calotropis gigantea* (Linn.) flower



2.3.9. Column Chromatography on EECF

For packing the column, 35 g silica gel was taken in a beaker and solvent was poured. The content was covered and kept at 20°C for at least 2 hours, to make slurry. A glass column of required size (80 cm in height and 1.4 cm in diameter) was first plugged with a piece of clean cotton at the bottom and fitted with a stand. The stopcock of the column was opened and solvent was passed through the column. In the mean time the slurry was allowed to pour gently into the column. After settling, some solvent was allowed to drain out. The column was packed at 20°C to avoid cracking. After packing, the sample, EECF (20 g) was mixed with a small portion of the stationary phase (silica gel) using mortar and pestle in order to obtain a non-sticky free flowing mass. The amount thus obtained is placed on the packed column carefully in such a way that the upper layer of the bed is not disturbed. The column was then run using n-hexane with a gradient of ethyl acetate up to 100% and followed by chloroform[114]. Sixty four (64) fractions were collected (Table 2.2). Among these fractions, fraction 21~30 afforded white crystals (198 mg). It was designated as **Compound-2**.

Based on TLC profile, fractions 40~48 were combined and subjected to preparative thin layer chromatography (PTLC). The sample (fractions 40~48) to be separated was dissolved in a small amount of ethyl acetate and applied to the plates as a uniform band 2 cm from the bottom edge. The plates were developed to upper edge of plates with a mobile phase of n-hexane-methanol (20: 0.1). After development, the plates were allowed to dry and observed under UV light (254 nm and 366 nm). UV active band was scraped off from the plates with the help of a spatula and the compounds were eluted from the silica matrix by dissolving in ethyl acetate and removing from the silica gel by filtration. Thus the fractions 40~48 afforded the pure compound (363 mg) as colorless oily liquid and it was designated as **Compound-1**.

From TLC profile, it was found that fractions 7~13 eluted with n-hexane and ethyl acetate (94 : 06) contained seven spots in TLC plate. These fractions were combined (1.8 g) and rechromatographed on silica gel column eluting with n-hexane and ethyl acetate (19:1) with increasing portions of ethyl acetate and finally with methanol. Thirty five (35) fractions were collected (Table 2.3). Among the 35 fractions, fraction 3~5 gave 18 mg white crystals and it was designated as **Compound-3** whereas fractions 10~12 afforded 24 mg white amorphous powder (designated as **Compound-4).** For crystallization, residue of fraction 20~23 was dissolved partially in n-hexane at room temperature and

completely dissolved on heating. The warm solvent was then allowed to cool undisturbed till crystallization was completed. The crystals were then separated from the mother liquor by filtration and dried[117]. Thus 20~23 fractions afforded **Compound-5** as colourless crystals (37 mg)**.** The purity of the isolated compounds was checked on TLC plates (Figure 2.1 – 2.5)

Figure 2.1 Purity of compound-1 on TLC plate using the solvent system of n-hexane and ethyl acetate (9 : 1)

Figure 2.2 Purity of compound-2 on TLC plate using the solvent system of n-hexane and ethyl acetate (9 : 1)

Figure 2.3 Purity of compound-3 on TLC plate using the solvent system of n-hexane and ethyl acetate (19 : 1)

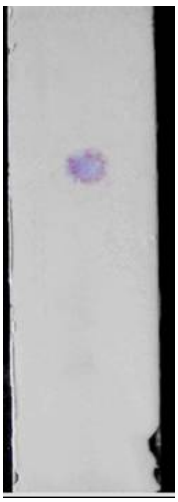


Figure 2.4 Purity of compound-4 on TLC plate using the solvent system of n-hexane and ethyl acetate (19 : 1)

Figure 2.5 Purity of compound-5 on TLC plate using the solvent system of n-hexane and ethyl acetate (19 : 1)

Table 2.2 Different solvent systems used for the column chromatographic analysis for EECF.

Fraction No.	Solvent systems	Proportion	Volume collected (mL)
1-2	n-Hexane	100%	300
3-6	n-Hexane : Ethyl acetate	98 :02	450
7-13	n-Hexane : Ethyl acetate	94 : 06	650
14-20	n-Hexane : Ethyl acetate	92 : 08	550
21-30	n-Hexane : Ethyl acetate	90 : 10	600
30-35	n-Hexane : Ethyl acetate	85 : 15	500
35-39	n-Hexane : Ethyl acetate	80 : 20	400
40-45	n-Hexane : Ethyl acetate	75 : 25	450
46-52	n-Hexane : Ethyl acetate	70: 30	350
53-57	n-Hexane : Ethyl acetate	60 : 40	200
58-59	n-Hexane : Ethyl acetate	50 : 50	250
60-61	n-Hexane : Ethyl acetate	30 : 70	300
62-63	Ethyl acetate	100%	250
64	Chloroform	100%	200

Table 2.3 Different solvent systems used for the column chromatographic analysis for fraction 7-13.

Fraction No.	Solvent systems	Proportion	Volume collected (mL)
1-2	n-Hexane	100%	50
3-7	n-Hexane : Ethyl acetate	99 : 1	100
8-13	n-Hexane : Ethyl acetate	98 : 2	150
14-17	n-Hexane : Ethyl acetate	97 : 3	100
18-23	n-Hexane : Ethyl acetate	96 : 4	150
24-30	n-Hexane : Ethyl acetate	95 : 5	200
31-33	n-Hexane : Ethyl acetate	92 : 7	100
29	Ethyl acetate	100%	50
35	Chloroform	100%	50

2.4. Phytochemical study on *Amoora rohituka* (Roxb.)

2.4.1. Collection of plant material

Stem bark of *Amoora rohituka* (Roxb.) (Family: Meliaceae) were collected in the month of August 2007 from Rajshahi district of Bangladesh. The plant material was taxonomically identified by Professor A.T.M Naderuzzaman, Department of Botany, University of Rajshahi and a voucher specimen was deposited under the accession number DACB-28927 at the Bangladesh National Herbarium.

2.4.2. Drying and pulverization of plant materials

The collected stem bark were cut into small pieces. These small pieces were sun dried for 7-10 days and finally kept in an electric oven for 72 hours at 40°C. After drying, the dried pieces of stem bark and flower were then separately pulverized into a coarse powder with the help of a grinding machine (FFC-15, China) and were stored in airtight containers for further use.

2.4.3. Successive extraction of stem bark powder with ethyl acetate and dichloromethane

The stem bark powder of *Amoora rohituka* was successively extracted with ethyl acetate and dichloromethane at room temperature. After pulverization the weight of total stem bark powder was 650 g. For extraction the stem bark powder was kept immersed in 1.2 liters ethyl acetate in an aspirator bottle at room temperature for 15 days with occasional shaking. Then the contents were pressed through the Tincture Press to get maximum amount of extract. The extract was filtered through a filter paper and concentrated with a rotary evaporator under reduced pressure at 60°C to obtain a crude extract 7.8 g and designated as ethyl acetate extract of stem bark of *Amoora rohituka* (EAEAR).

After extraction with ethyl acetate, the remaining powder residue was further kept immersed in 1.0 liter dichloromethane and the above procedure was followed to get a crude extract 4.3 g which was designated as dichloromethane extract of stem bark of *Amoora rohituka* (DMEAR).

2.4.4. Preliminary TLC screening on EAEAR and DMEAR

A small portion of EAEAR and DMEAR were dissolved in respective solvent and the solutions were spotted on TLC plates. The TLC plates were run by different specific solvent system and viewed individually under UV light and also with the vanillin-H_2SO_4 reagent[114,118]. Through several pilot experiments, it was found that EAEAR and DMEAR

contained different type of compounds and the compounds of each sample were separated by different specific solvent systems. The findings from preliminary TLC screening on EAERA and DMEAR are summarized in table 2.4.

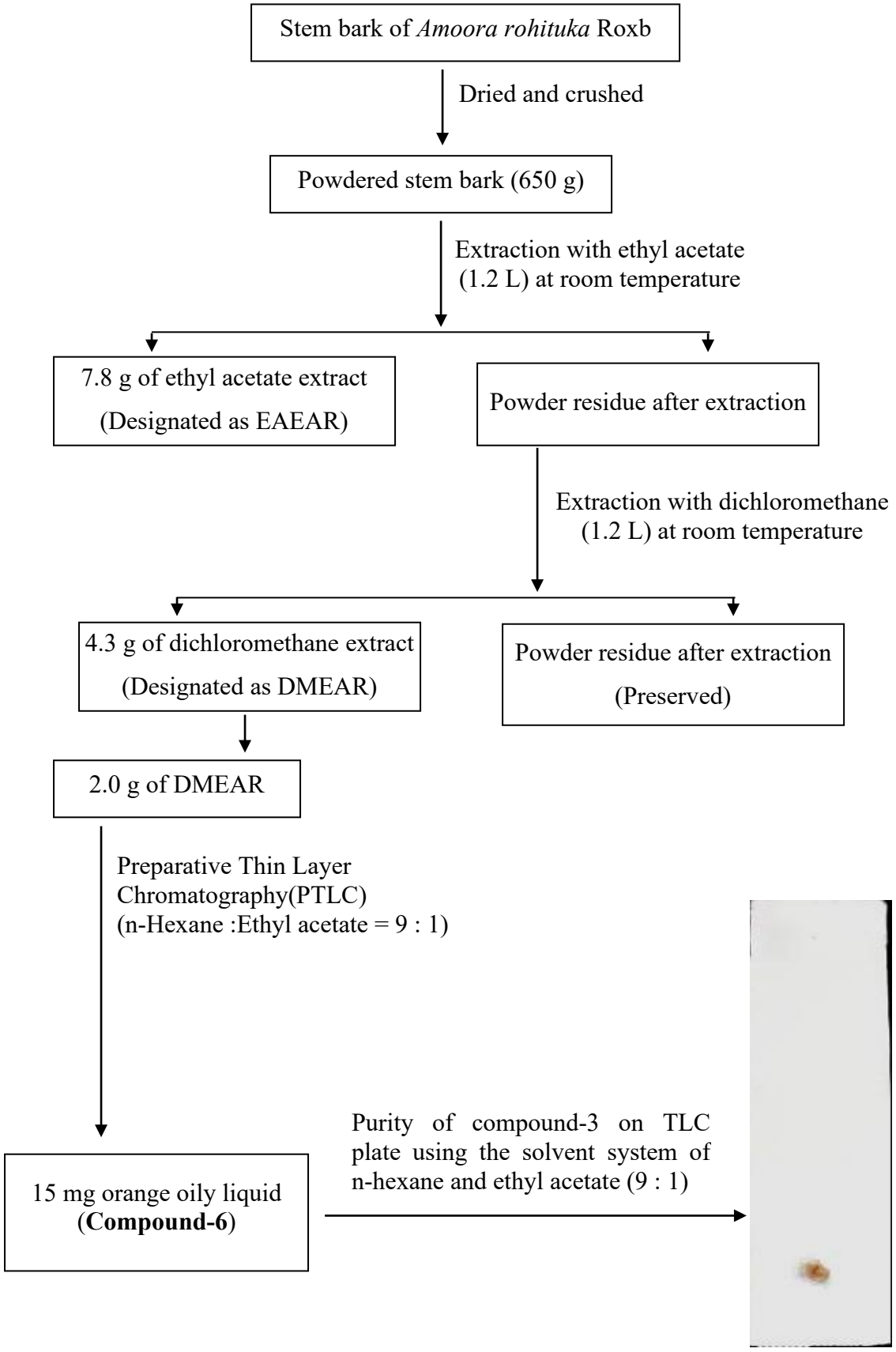
Table 2.4 Types of compounds present in EAEAR and DMEAR.

Sample	Solvent system	Code No. of each spot	R_f value	Colour with vanillin-H_2SO_4	Possible compound
EAEAR	n-Hexane : Ethyl acetate (8 : 2)	EAEAR-1	0.98	Pink	steroid
		EAEAR -2	0.87	Violet	Terpene
		EAEAR -3	0.74	Violet	Terpene
		EAEAR -4	0.58	Pink	steroid
		EAEAR -5	0.44	Black	Glycoside
		EAEAR -6	0.41	Violet	Terpene
		EAEAR -7	0.36	Yellow	Flavonoid
		EAEAR -8	0.32	Pink	steroid
DMEAR	n-Hexane : Ethyl acetate (9 : 1)	DMEAR-1	0.93	Violet	Terpene
		DMEAR -2	0.78	Violet	Terpene
		DMEAR -3	0.73	Violet	Terpene
		DMEAR -4	0.68	Black	Glycoside
		DMEAR -5	0.52	Violet	Terpene
		DMEAR -6	0.38	Brown	Heterocyclic
		DMEAR -7	0.22	Black	Glycoside

2.4.5. Preparative Thin Layer Chromatography (PTLC) on DMEAR

Preparative TLC was applied to the separation and final purification of compound from DMEAR. 2.0 g of DMEAR was dissolved in a small amount of dichloromethane and applied to the plates for PTLC as a uniform band 2 cm from the bottom edge. The plates were developed to upper edge of plates using the solvent system of n-hexane and ethyl acetate in 9:1 proportion. After development, the plates were allowed to dry and observed under UV light (254 nm and 366 nm). An UV-active band (R_f value-0.93) were scraped off from the plates with the help of a spatula. Then the silica matrix was dissolved in dichloromethane and the silica gel was removed by filtration[114,118]. Finally 15 mg orange oily liquid was obtained by evaporation of solvent in *vacuo* and purified compound was designated as **compound-6**.

2.4.6. Schematic flow diagram for phytochemical study on *Amoora rohituka* Roxb. stem bark.



2.5. Results and discussion

2.5.1. Characterization and structure elucidation of isolated compound-1

Compound-1 was isolated from the ethyl acetate extract of *Calotropis gigantea* (L.) flower (EECF) as colourless oily liquid. Compound-1 was characterized by IR, mass and NMR spectral data to elucidate its chemical structure. Molecular formula for compound-1 was deduced as $C_{24}H_{38}O_4$ through mass spectrum (Figure 2.8) which showed the molecular ion (M^+) peak at m/z 390.3617 corresponding to the molecular formula $C_{24}H_{38}O_4$. In mass spectrum the presence of a phthalate was inferred from peaks at m/z 167.1017 (48) and m/z 149.1021 (100). The IR spectrum (Figure 2.9) revealed a carbonyl band observed at 1739.7 cm^{-1} and strong C-O bands in the range 1047.3 ~ 1238.2 cm^{-1}. The aromatic signals between δ 6.96 (1H, dd, J = 6.3 ~ 2.2 Hz) and δ 7.11 (1H, dd, J = 6.3 ~ 2.2 Hz) ppm (Table 2.5) on the ^{1}H-NMR spectrum (Figure 2.10) of compound-1 had reasonable coupling constants for protons at the ortho-substituted ring. Signal at δ 4.15 (m) ppm was assigned to one methylene group (H-6) geminal to the ester alcohol group whereas the signal at δ 2.30 (dq, J = 4.3 Hz), was assigned to another methylene group (H-8). In ^{1}H-NMR spectrum of compound-1, signals at δ 0.84 (t, J = 5.3 Hz) and δ 0.93 (t, J = 4.3 Hz) were observed for protons of two tertiary methyl group and signal at δ 2.606 (m) was assigned to a methine proton (Table 2.5). The ^{13}C-NMR spectrum of compound-1 (Figure 2.11), confirming the symmetry of the molecule (Figure 2.6), exhibited the expected 12 carbon resonances (Table 2.6) assigned by DEPT experiment (Figure 2.12) to two quaternary, three methine and five methylene carbons with two methyl groups. One carbonyl carbon signal at δ 171.10 ppm and three olefinic carbon signals at δ 124.75, δ 118.95 and δ 132.65 ppm, were observed in the ^{13}C-NMR spectrum of compound-1 (Table 2.6). By comparison of ^{1}H and ^{13}C-NMR data to those published in literature[119,120], compound-1 was identified as di-(2-ethylhexyl) phthalate (DEHP) (Figure 2.7). DEHP (compound-1) is a well known synthetic plasticizer, already reported to be present in *Alchornea cordifolia*[121], *Aloe vera*[122], *Euphorbia cyparissias* and *Euphorbia seguieriana*[123]. But to the best of our knowledge, DEHP is reported from *Calotropis gigantea* and also from *Calotropis* genus for the first time. The effective presence of compound-1 in flowers, not as a contaminant from solvents, was further confirmed by GC-MS analysis of ethyl acetate. The plant flowers were not conserved in plastic bags, so these could be discounted as a source of DEHP. The present study could not determine if DEHP is synthesized by the plant, absorbed by the roots or adsorbed

from external atmosphere, but this compound, whatever its origin, appears likely to be present in preparations of *Calotropis gigantea* flower.

Properties of Compound-1 (i.e., Di-(2-ethylhexyl) phthalate). Colorless oily liquid, IR bands (neat): 3384.8, 2929.7, 2854.5, 1739.7 (s), 1635.5, 1515.9, 1436.9, 1375.2, 1238.2, 1124.4, 1047.3 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 0.84 (3H, t, *J* = 5.3, H-1/H-1′), 1.23 ~1.40 (2H, m, H-2/H-2′), 1.23 ~1.40 (2H, m, H-3/H-3′), 1.23 ~1.40 (2H, m, H-4/H-4′), 2.606 (1H, m, H-5/H-5′), 4.15 (2H, m, H-6/H-6′), 2.30 (2H, dq, *J* = 4.3, H-8/H-8′), 0.93 (3H, t, *J* = 4.3, H-9/H-9′), 6.96 (1H, dd, *J* = 6.3 ~ 2.2, H-11/H-11′), 7.11 (1H, dd, *J* = 6.3 ~ 2.2, H-12/H-12′); ^{13}C NMR (150 MHz, CDCl3, δ, ppm): 14.11 (C-1/C-1′), 24.80 (C-2/C-2′), 22.68 (C-3/C-3′), 29.50 (C-4/C-4′), 40.76 (C-5/C-5′), 65.21 (C-6/C-6′), 171.10 (C-7/C-7′), 29.67 (C-8/C-8′), 20.79 (C-9/C-9′), 124.75 (C-10/C-10′), 118.95 (C-11/C-11′), 132.65 (C-12/C-12′). EI-MS *m/z*: 390.3617 (M^+), 279.2022 (94), 223.2144 (36), 167.1017 (58), 149.1021 (100), 113.2833 (32).

Table 2.5 Comparison of ^{1}H-NMR spectral data of compound-1 and authentic di-(2-ethylhexyl) phthalate.

Carbon No.	δ_H of compound-1 (in ppm)	δ_H of authentic di-(2-ethylhexyl) phthalate[119,120] (in ppm)
1 (1′)	0.84 (3H, t, *J* = 5.3 Hz)	0.82 (3H, t, *J* = 5.3 Hz)
2 (2′)	1.23 ~1.40, m	1.15 ~1.30, m
3 (3′)	1.23 ~ 1.40, m	1.15 ~ 1.30, m
4 (4′)	1.23 ~ 1.40, m	1.15 ~ 1.30, m
5 (5′)	2.606, m	1.597, m
6 (6′)	4.15 (2H, m)	4.13 (2H, m)
7 (7′)	-	-
8 (8′)	2.30 (2H, dq, *J* = 4.3 Hz)	2.32 (2H, dq, *J* = 4.3 Hz)
9 (9′)	0.93 (3H, t, *J* = 4.3 Hz)	0.91 (3H, t, *J* = 4.3 Hz)
10 (10′)	-	-
11 (11′)	6.96 (1H, dd, *J* = 6.3 ~ 2.2 Hz)	6.98 (1H, dd, *J* = 6.3 ~ 2.2 Hz)
12 (12′)	7.11(1H, dd, *J* = 6.3 ~ 2.2 Hz)	7.14 (1H, dd, *J* = 6.3 ~ 2.2 Hz)

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

Table 2.6 Comparison of ^{13}C-NMR spectral data of compound-1 and authentic di-(2-ethylhexyl) phthalate.

Carbon No.	Carbon type (confirmed by DEPT experiment)	δ_C of compound-1 (in ppm)	δ_C of authentic di-(2-ethylhexyl) phthalate[119,120] (in ppm)
1 (1′)	- CH_3	14.11	14.08
2 (2′)	= CH_2	24.80	24.71
3 (3′)	= CH_2	22.68	22.79
4 (4′)	= CH_2	29.50	27.92
5 (5′)	= CH -	40.76	40.61
6 (6′)	= CH_2	65.21	67.02
7 (7′)	O= CO (quaternary)	171.10	166.58
8 (8′)	= CH_2	29.67	29.39
9 (9′)	- CH_3	20.79	20.53
10 (10′)	= C = (quaternary)	124.75	124.70
11 (11′)	= CH – (olefinic)	118.95	118.41
12 (12′)	= CH - (olefinic)	132.65	132.62

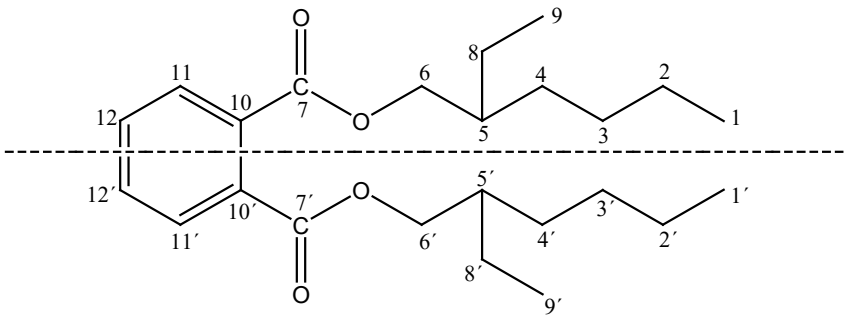


Figure 2.6 The symmetry of compound-1

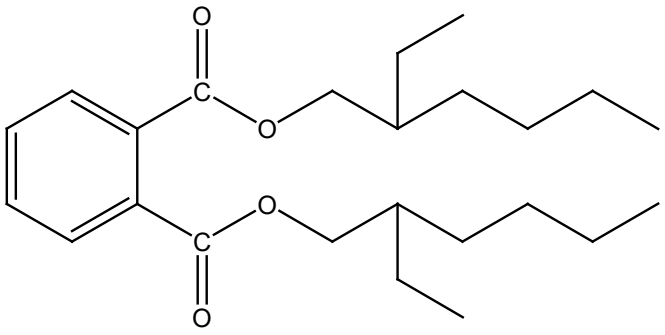


Figure 2.7 Chemical structure of compound-1: Di-(2-ethylhexyl) phthalate

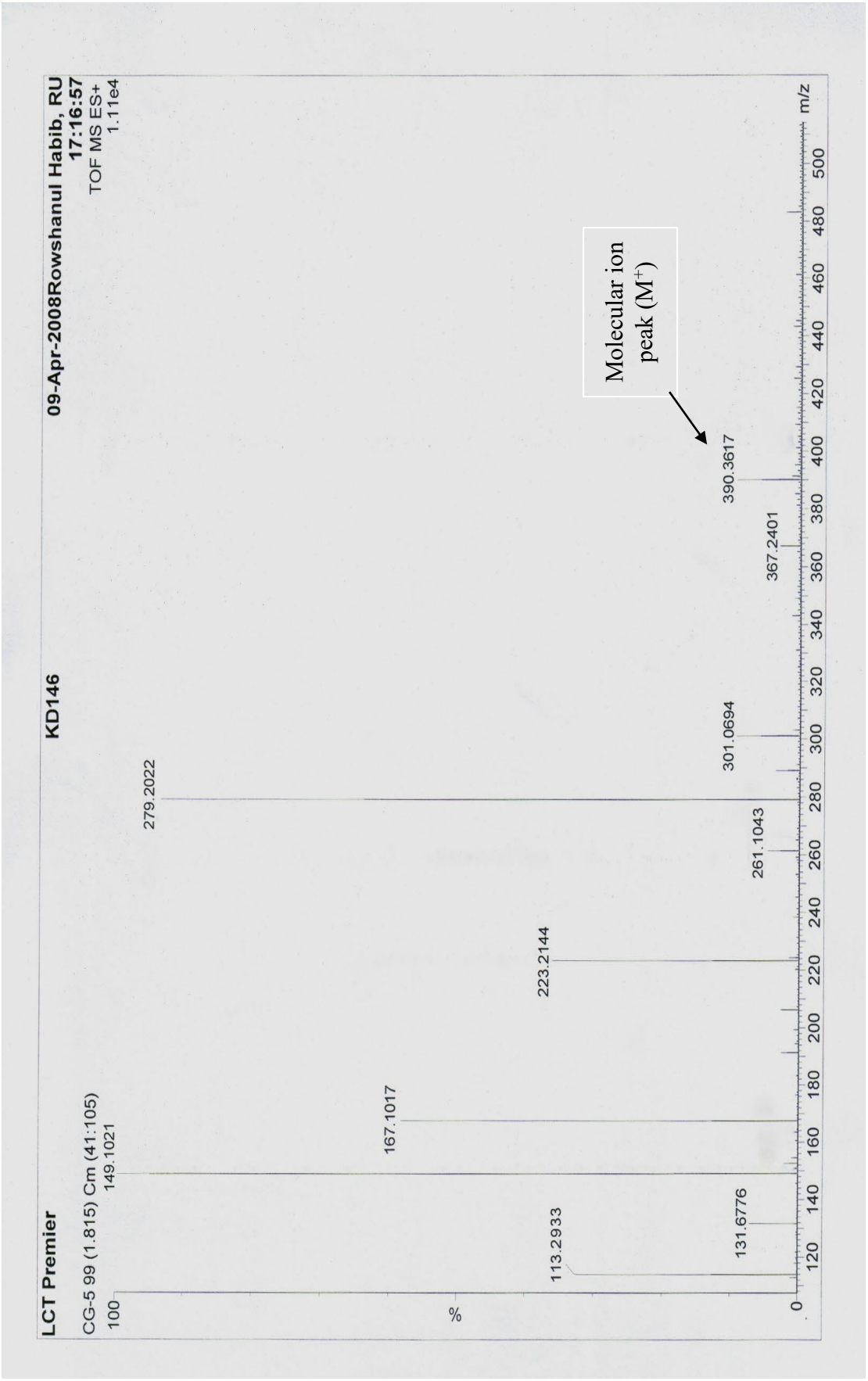


Figure 2.8 Mass spectrum of compound-1

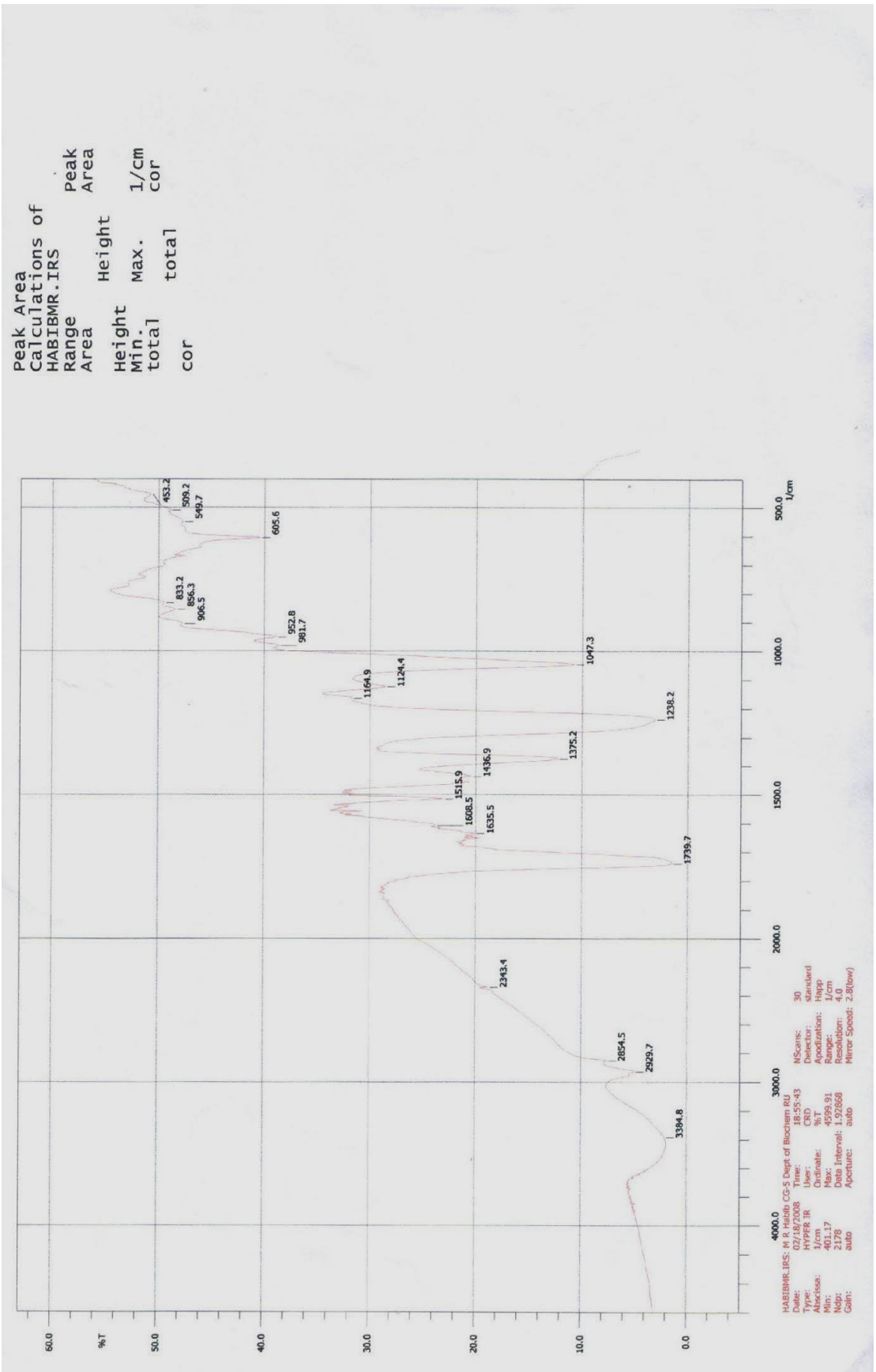


Figure 2.9 IR spectrum of compound-1

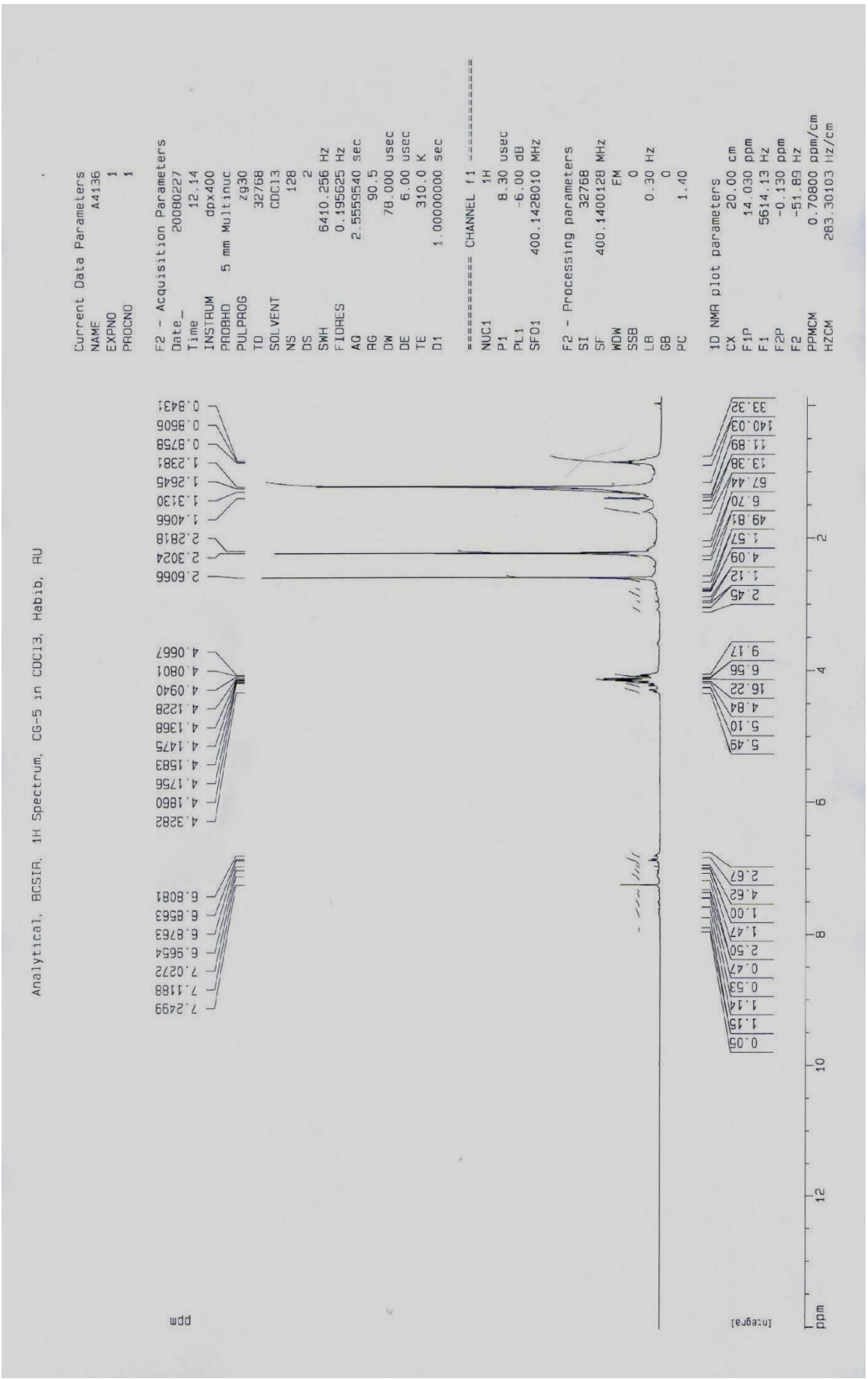


Figure 2.10 ^{1}H-NMR spectrum of compound-1

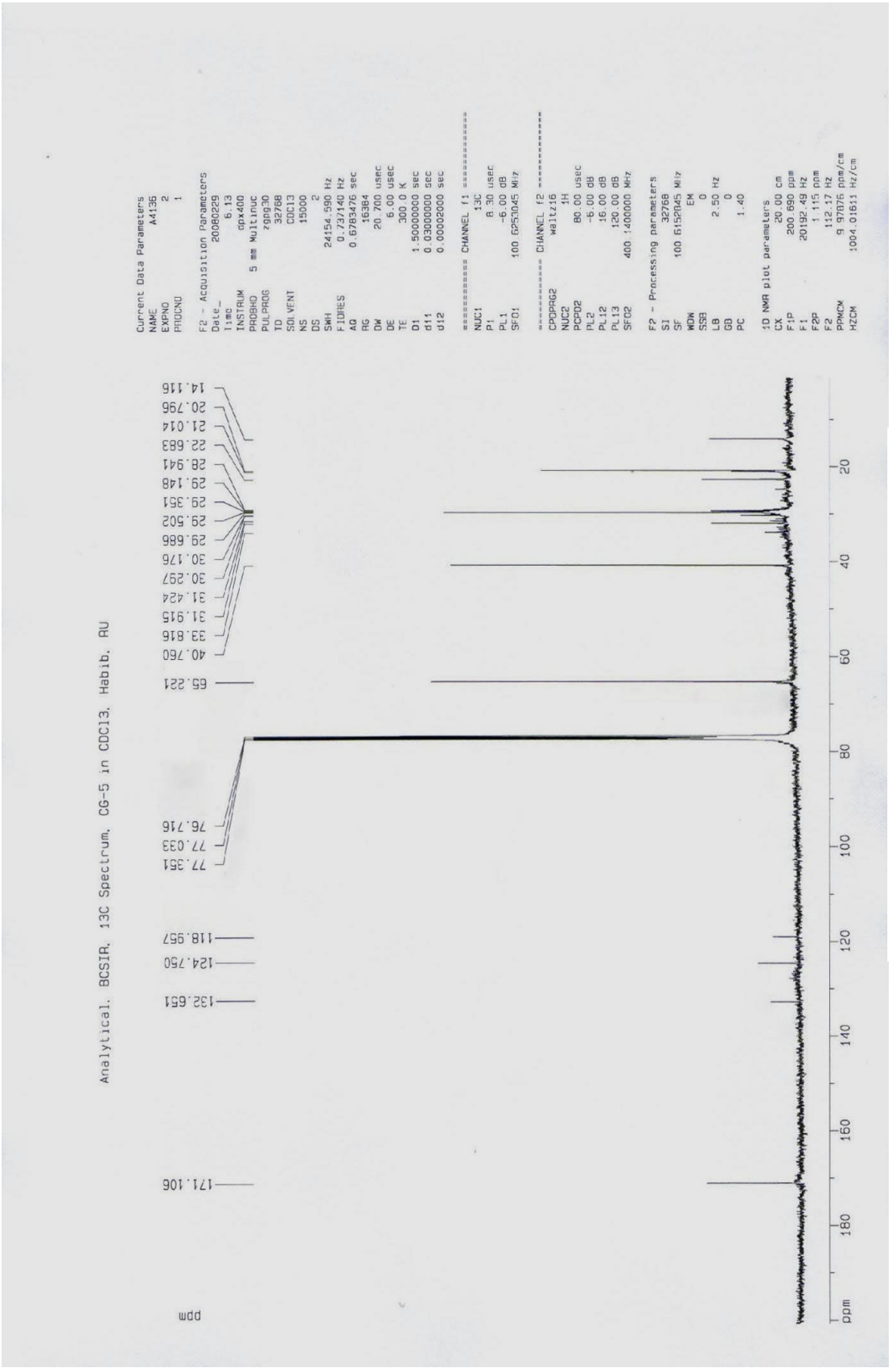


Figure 2.11 ^{13}C-NMR spectrum of compound-1

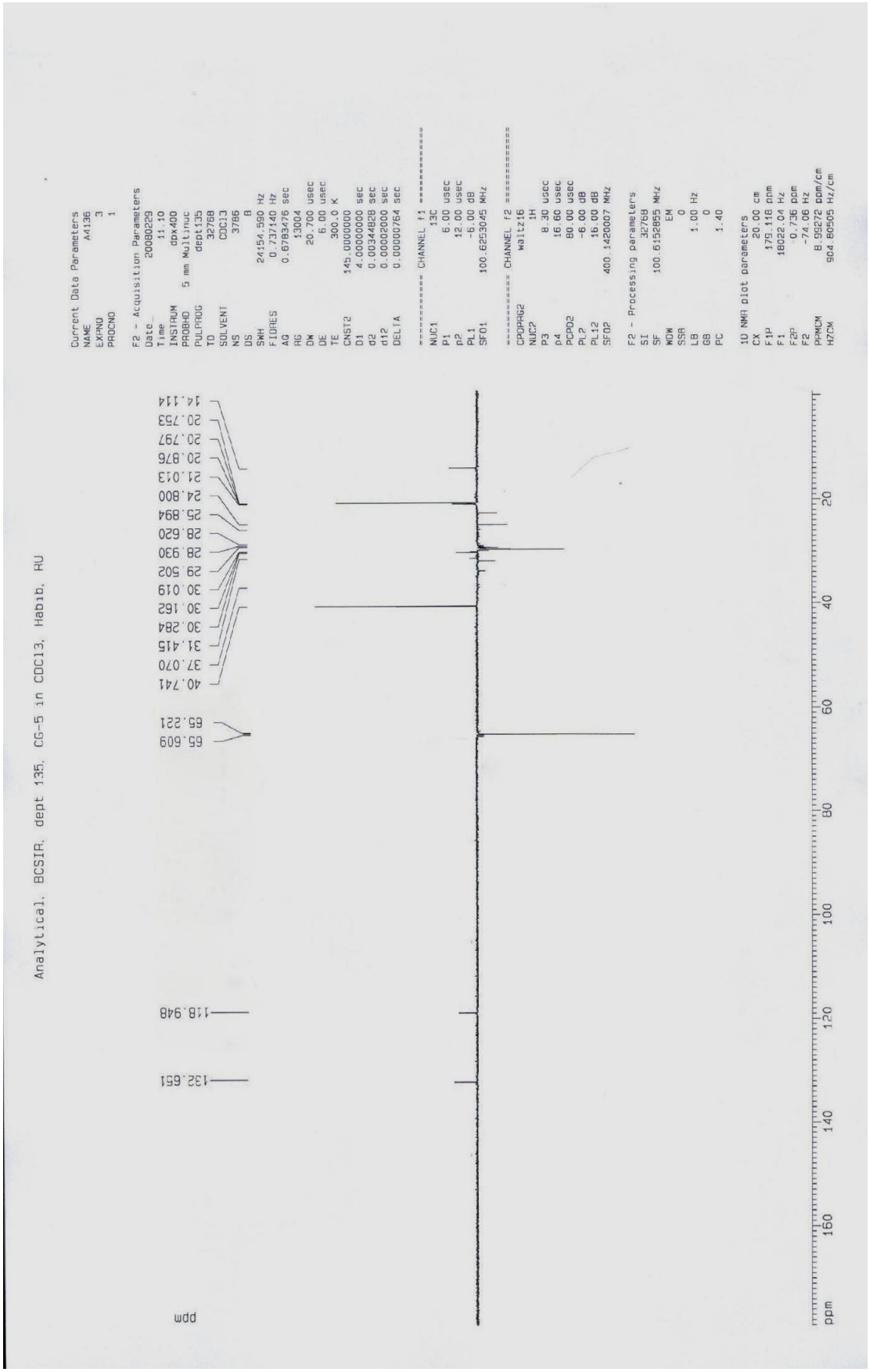


Figure 2.12 DEPT spectrum of compound-1

2.5.2. Characterization and structure elucidation of isolated compound-2

Compound-2 was obtained as white crystal and its melting point was 234°C. Its molecular formula was determined to be $C_{32}H_{50}O_2$ by mass spectrum (Figure 2.14) showing a molecular ion (M^+) peak at m/z 466.4023 (calcd for $C_{32}H_{50}O_2$). The other peaks were observed at m/z 465.3787 (20), 443.3477 (31), 409.3716 (100), 391.3050 (19). Its IR spectrum (Figure 2.15) showed a sharp absorption band for carbonyl group (1735.8 cm^{-1}). The ^{1}H-NMR spectrum of compound-2 (Figure 2.16) exhibited three olefinic proton at δ 5.34 (1H, t, *J* = 3.5 Hz), δ 5.18 (1H, t, *J* = 3.5 Hz) and δ 5.12 (1H, t, *J* = 3.5 Hz) and an acetyl methyl proton at δ 2.04 (3H, s) (shown in table 2.7). Its ^{1}H-NMR spectrum demonstrated the presence of eight methyl proton signals as singlets at δ 1.25, δ 1.13, δ 1.06, δ 0.98, δ 0.97, δ 0.91, δ 0.87 and δ 0.86 (Table 2.7). In addition, an acetylated methine proton signal was observed at δ 4.50 (1H, t, *J* = 11.6 Hz) in its ^{1}H-NMR spectrum (Table 2.7). The ^{13}C-NMR spectrum (Figure 2.17) of compound-2 showed an acetyl carbonyl carbon at δ 171.01, four olefinic carbons at δ 122.5 (C-12), δ 143.75 (C-13), δ 139.67 (C-21), δ 124.37 (C-22) and acetyl methyl carbon at 15.76 (Table 2.8). Its DEPT spectrum (Figure 2.18) displayed thirty two carbon signals comprised of eight quaternary, seven methine, eight methylene and nine methyl carbons. On the basis of all the above evidences and comparison with reported data[124], the structure of the compound-2 was deduced as anhydrosophoradiol-3-acetate (3β-acetoxyolean-12, 21-diene) (Figure 2.13). Isolation of anhydrosophoradiol-3-acetate is reported for the first time from this plant.

Properties of Compound-2 (i.e., Anhydrosophoradiol-3-acetate). White crystal; m.p. 234°C; IR bands (neat): 3448.5, 2979.8, 2956.7, 2908.5, 1735.8 (s), 1456.2, 1365.5, 1244.0, 1022.2, 1002.9 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 2.01 (2H, *d*, *J* = 11.5 Hz, H-1), 1.67 (2H, *s*, H-2), 4.50 (1H, *t*, *J* = 11.6 Hz, H-3), 1.67 (1H, *s*, H-5), 1.66 (2H, *s*, H-6), 1.65 (1H, *s*, H-7), 2.00 (1H, *s*, H-9), 1.92 (2H, *m*, H-11), 5.12 (1H, *t*, *J* = 3.5 Hz, H-12), 1.90 (2H, *m*, H-15), 1.97 (2H, *d*, J = 11.5 Hz, H-16), 1.89 (2H, *m*, H-19), 5.18 (1H, *t*, *J* = 3.5 Hz, H-21), 5.34 (1H, *t*, *J* = 3.5 Hz, H-22), 1.25 (3H, *s*, H-23), 1.13 (3H, *s*, H-24), 1.06 (3H, *s*, H-25), 0.98 (3H, *s*, H-26), 0.97 (3H, *s*, H-27), 0.91 (3H, *s*, H-28), 0.87 (3H, *s*, H-29), 0.86 (3H, *s*, H-30), 2.04 (3H, *s*, -CO-C<u>H</u>$_3$). ^{13}C-NMR (150 MHz, $CDCl_3$, δ, ppm): 28.1 (C-1), 23.4 (C-2), 81.0 (C-3), 28.1 (C-4), 55.3 (C-5), 18.2 (C-6), 29.7 (C-7), 39.6 (C-8), 47.7 (C-9), 28.7 (C-10), 23.6 (C-11), 122.5 (C-12), 143.7 (C-13), 40.0 (C-14), 32.9 (C-15), 38.5 (C-16), 36.8 (C-17), 42.1 (C-18), 41.5 (C-19), 41.5 (C-20), 139.6 (C-21), 124.3 (C-22), 23.2 (C-23), 16.9 (C-24), 16.7 (C-25), 17.5 (C-26), 17.6 (C-27), 23.6

(C-28), 21.4 (C-29), 21.3 (C-30), 15.7 (-CO-$\underline{C}H_3$), 171.0 (-$\underline{C}O$-CH_3); EI-MS (m/z): 466.4023 (M^+), 465.3787 (20), 443.3477 (31), 410.3904 (33), 409.3716 (100), 391.3050 (19).

Table 2.7 Comparison of ^{1}H-NMR spectral data of compound-2 and authentic anhydrosophoradiol-3-acetate.

δ_H of compound-2 (in ppm)	δ_H of authentic anhydrosophoradiol-3-acetate[124] (in ppm)
2.01 (2H, d, *J* = 11.5 Hz, H-1)	2.01 (2H, d, *J* = 11.5 Hz, H-1)
1.67 (2H, s, H-2)	1.67 (2H, s, H-2)
4.50 (1H, t, *J* = 11.6 Hz, H-3)	4.49 (1H, t, *J* = 11.6 Hz, H-3)
1.67 (1H, s, H-5)	1.68 (1H, s, H-5)
1.66 (2H, s, H-6)	1.66 (2H, s, H-6)
1.65 (1H, s, H-7)	1.69 (1H, s, H-7)
2.00 (1H, s, H-9)	2.00 (1H, s, H-9)
1.92 (2H, m, H-11)	1.98 (2H, m, H-11)
5.12 (1H, t, *J* = 3.5 Hz, H-12)	5.12 (1H, t, *J* = 3.5 Hz, H-12)
1.90 (2H, m, H-15)	1.95 (2H, m, H-15)
1.97 (2H, d, J = 11.5 Hz, H-16)	1.97 (2H, d, J = 11.5 Hz, H-16)
2.34 (1H, s, H-18)	2.35 (1H, s, H-18)
1.89 (2H, m, H-19)	1.89 (2H, m, H-19)
5.18 (1H, t, *J* = 3.5 Hz, H-21)	5.19 (1H, t, *J* = 3.5 Hz, H-21)
5.34 (1H, t, *J* = 3.5 Hz, H-22)	5.29 (1H, t, *J* = 3.5 Hz, H-22)
1.25 (3H, s, H-23)	1.28 (3H, s, H-23)
1.13 (3H, s, H-24)	1.13 (3H, s, H-24)
1.06 (3H, s, H-25)	1.06 (3H, s, H-25)
0.98 (3H, s, H-26)	0.98 (3H, s, H-26)
0.97 (3H, s, H-27)	0.97 (3H, s, H-27)
0.91 (3H, s, H-28)	0.92 (3H, s, H-28)
0.87 (3H, s, H-29)	0.88 (3H, s, H-29)
0.86 (3H, s, H-30)	0.87 (3H, s, H-30)
2.04 (3H, s, -CO-CH_3)	2.05 (3H, s, -CO-CH_3)

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

Table 2.8 Comparison of ^{13}C-NMR spectral data of compound-2 and authentic anhydrosophoradiol-3-acetate.

Carbon No.	Carbon type (confirmed by DEPT experiment)	δ_C of compound-2 (in ppm)	δ_C of authentic anhydrosophoradiol-3-acetate[124] (in ppm)
1	$=CH_2$	28.14	28.07
2	$=CH_2$	23.41	23.55
3	=CH-	81.0	80.9
4	=C= (quaternary)	28.10	28.14
5	=CH-	55.32	55.32
6	$=CH_2$	18.29	18.30
7	$=CH_2$	29.72	29.71
8	=C= (quaternary)	39.69	39.49
9	=CH-	47.70	47.71
10	=C= (quaternary)	28.77	28.79
11	$=CH_2$	23.64	25.89
12	=CH-	122.50	122.47
13	=C= (quaternary)	143.75	143.75
14	=C= (quaternary)	40.08	45.89
15	$=CH_2$	32.92	32.54
16	$=CH_2$	38.52	38.50
17	=C= (quaternary)	36.84	36.91
18	=CH-	42.13	42.15
19	$=CH_2$	41.57	41.59
20	=C= (quaternary)	41.58	41.60
21	=CH-	139.67	138.01
22	=CH-	124.37	125.60
23	$-CH_3$	23.26	23.24
24	$-CH_3$	16.91	16.90
25	$-CH_3$	16.76	16.78
26	$-CH_3$	17.53	18.18
27	$-CH_3$	17.60	17.03
28	$-CH_3$	23.61	23.61
29	$-CH_3$	21.41	21.41
30	$-CH_3$	21.32	21.32
CO-$\underline{C}H_3$	$-CH_3$	15.76	15.76
$\underline{C}O$-CH_3	=C= (quaternary)	171.01	171.01

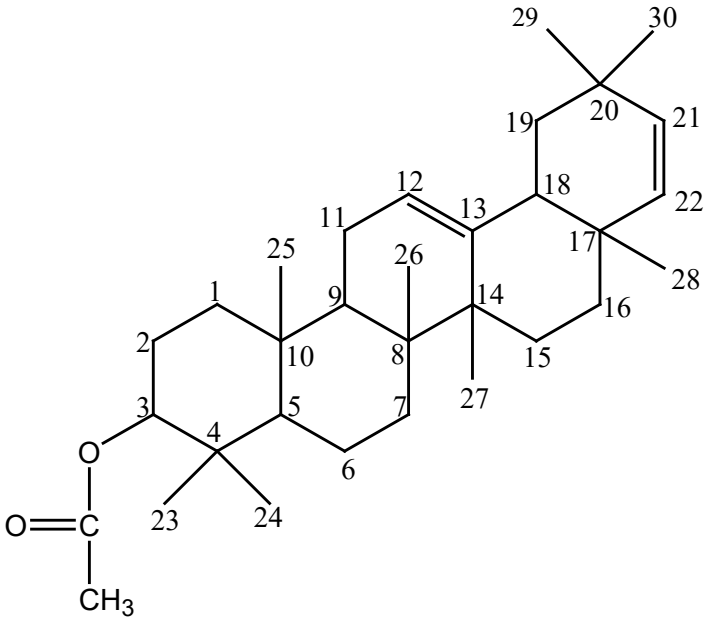


Figure 2.13 Chemical structure of compound-2: anhydrosophoradiol-3-acetate

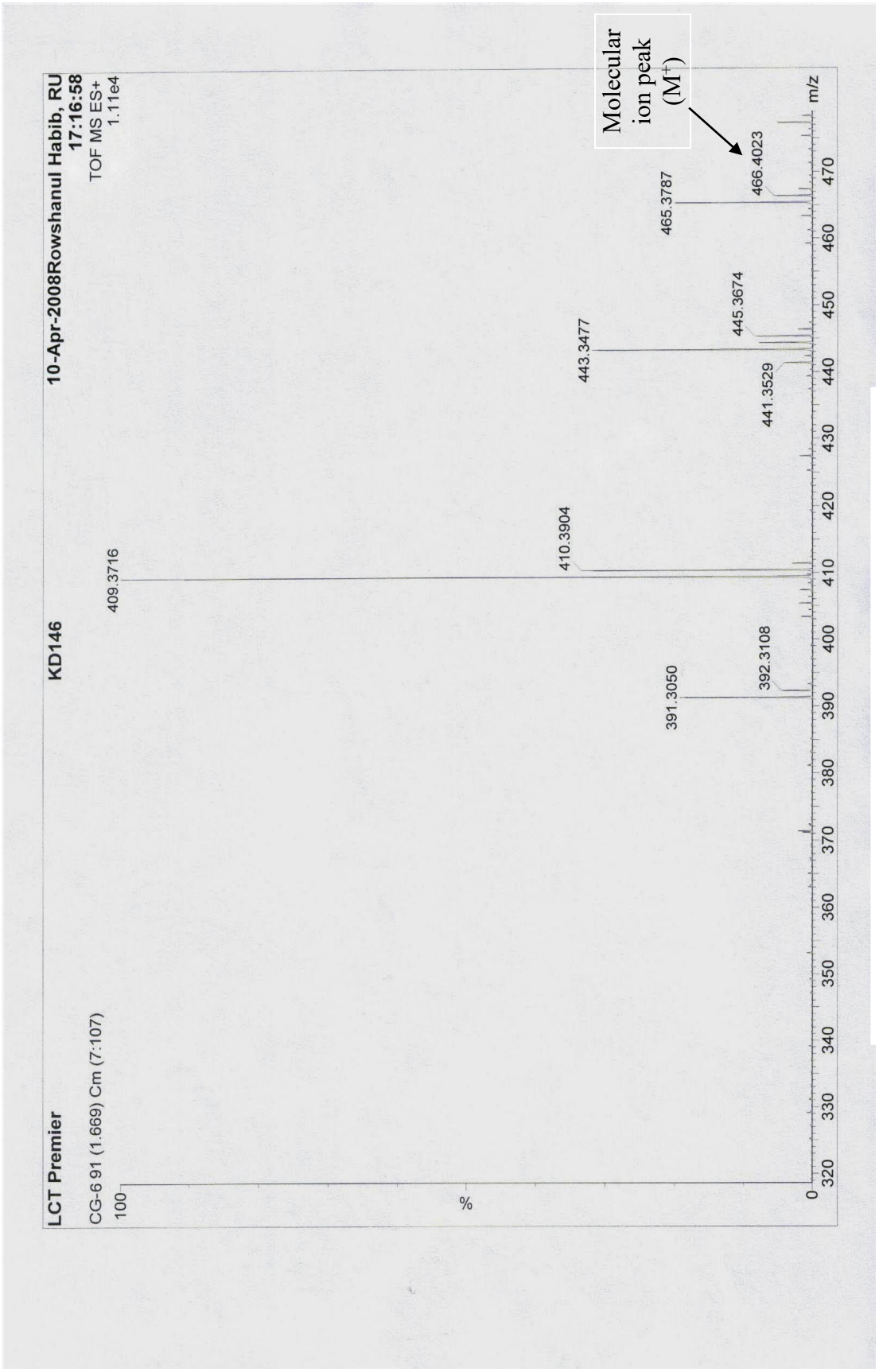


Figure 2.14 Mass spectrum of compound-2

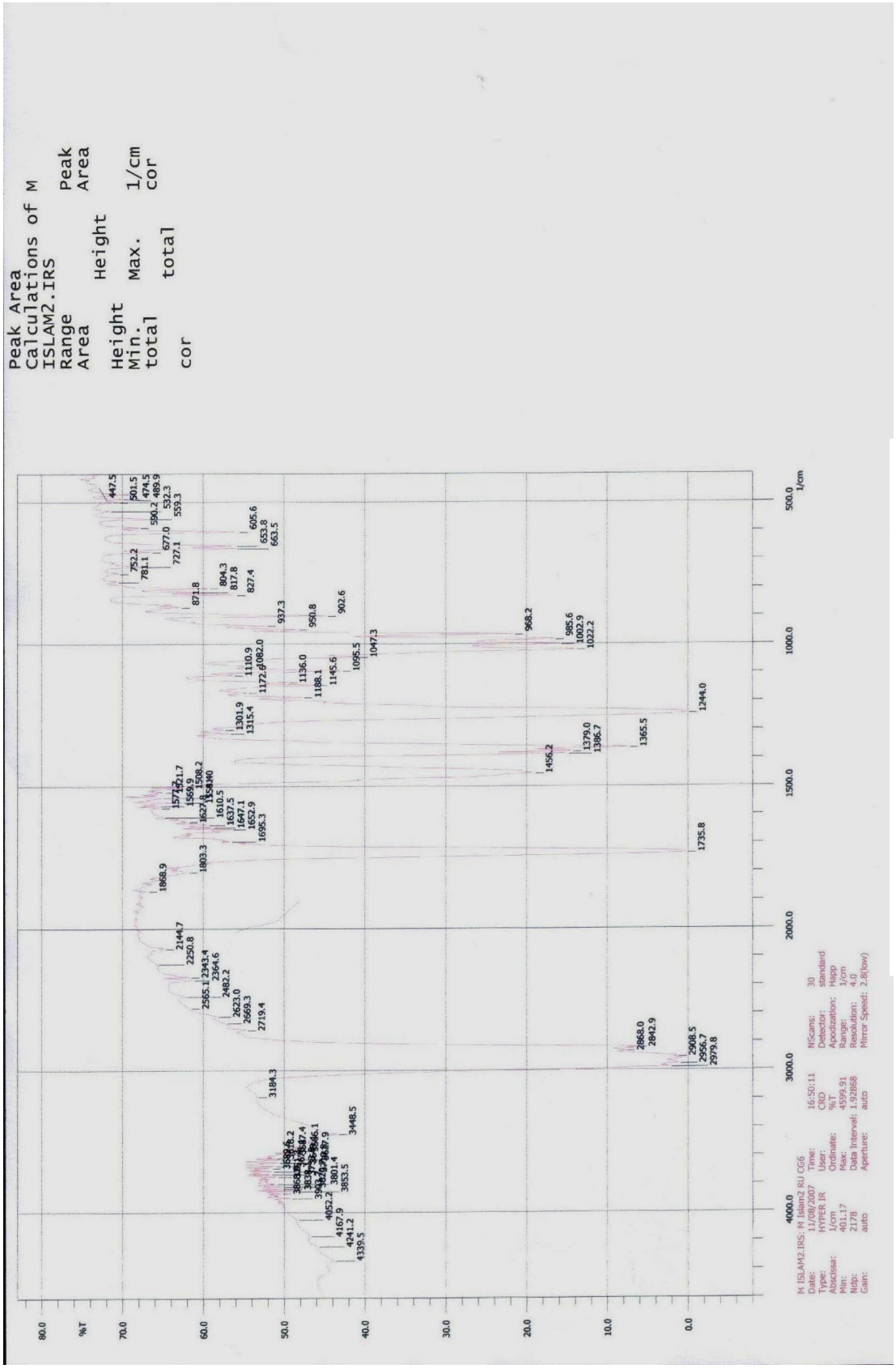


Figure 2.15 IR spectrum of compound-2

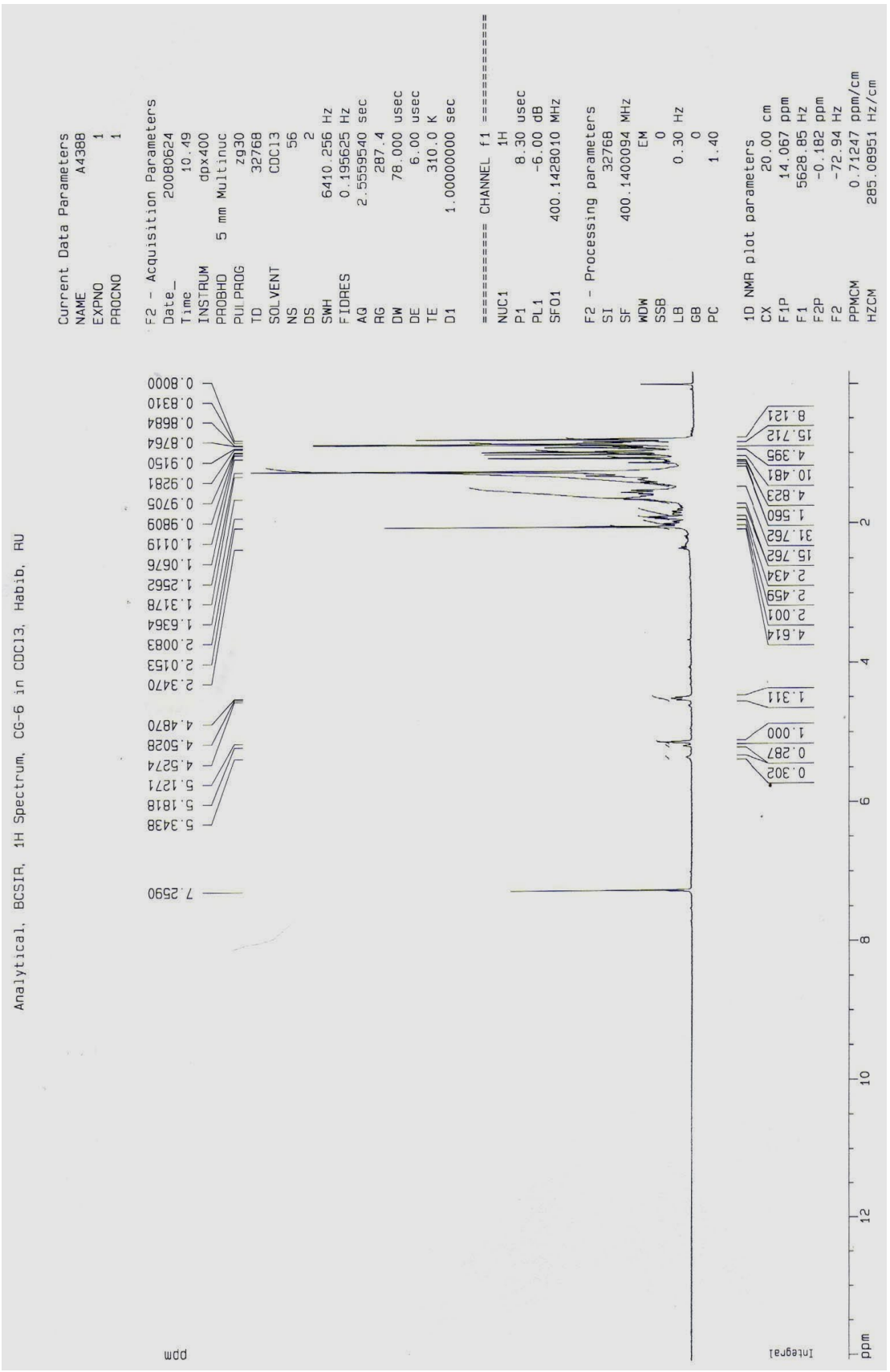


Figure 2.16 ^{1}H-NMR spectrum of compound-2

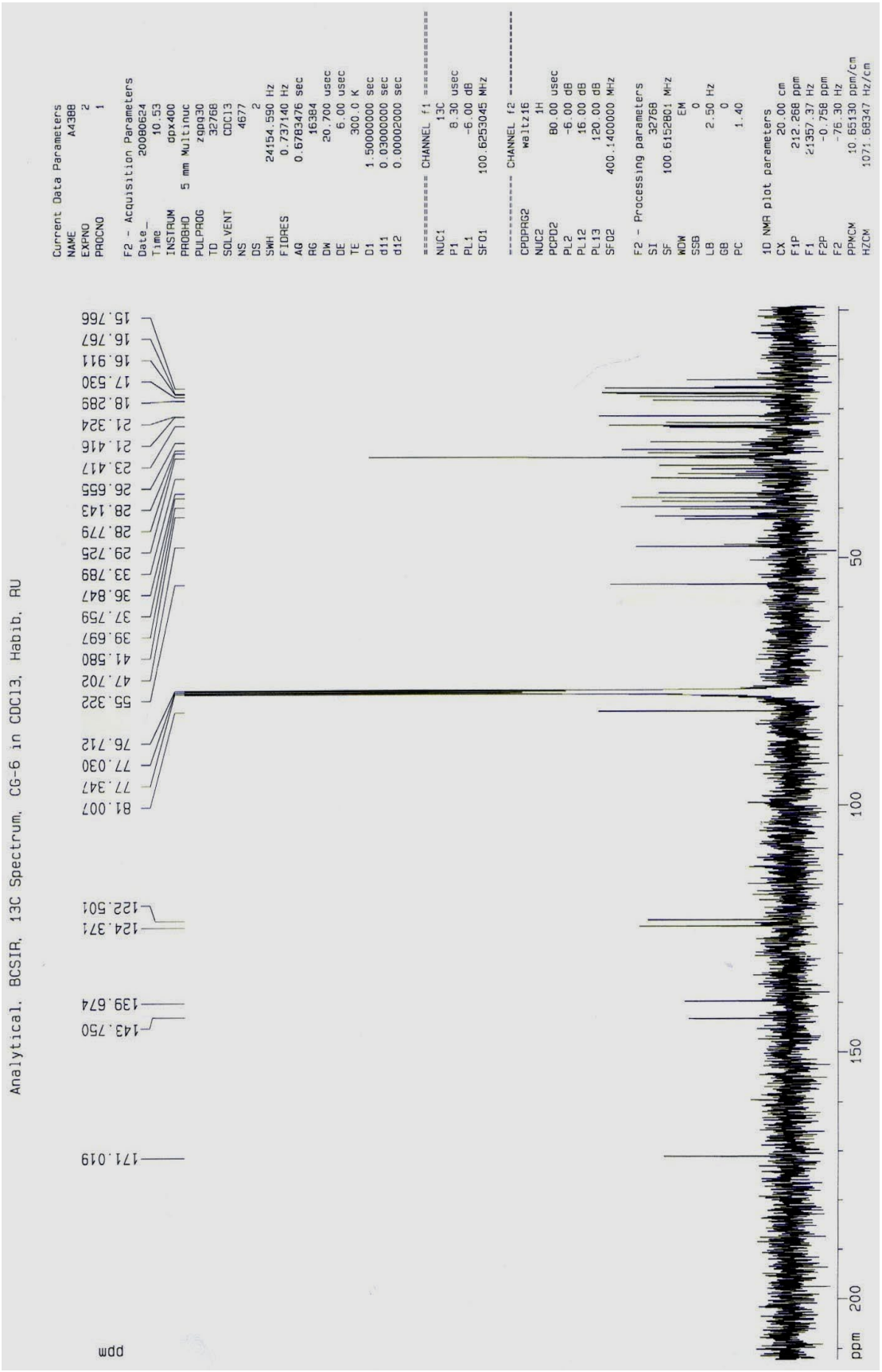


Figure 2.17 ^{13}C-NMR spectrum of compound-2

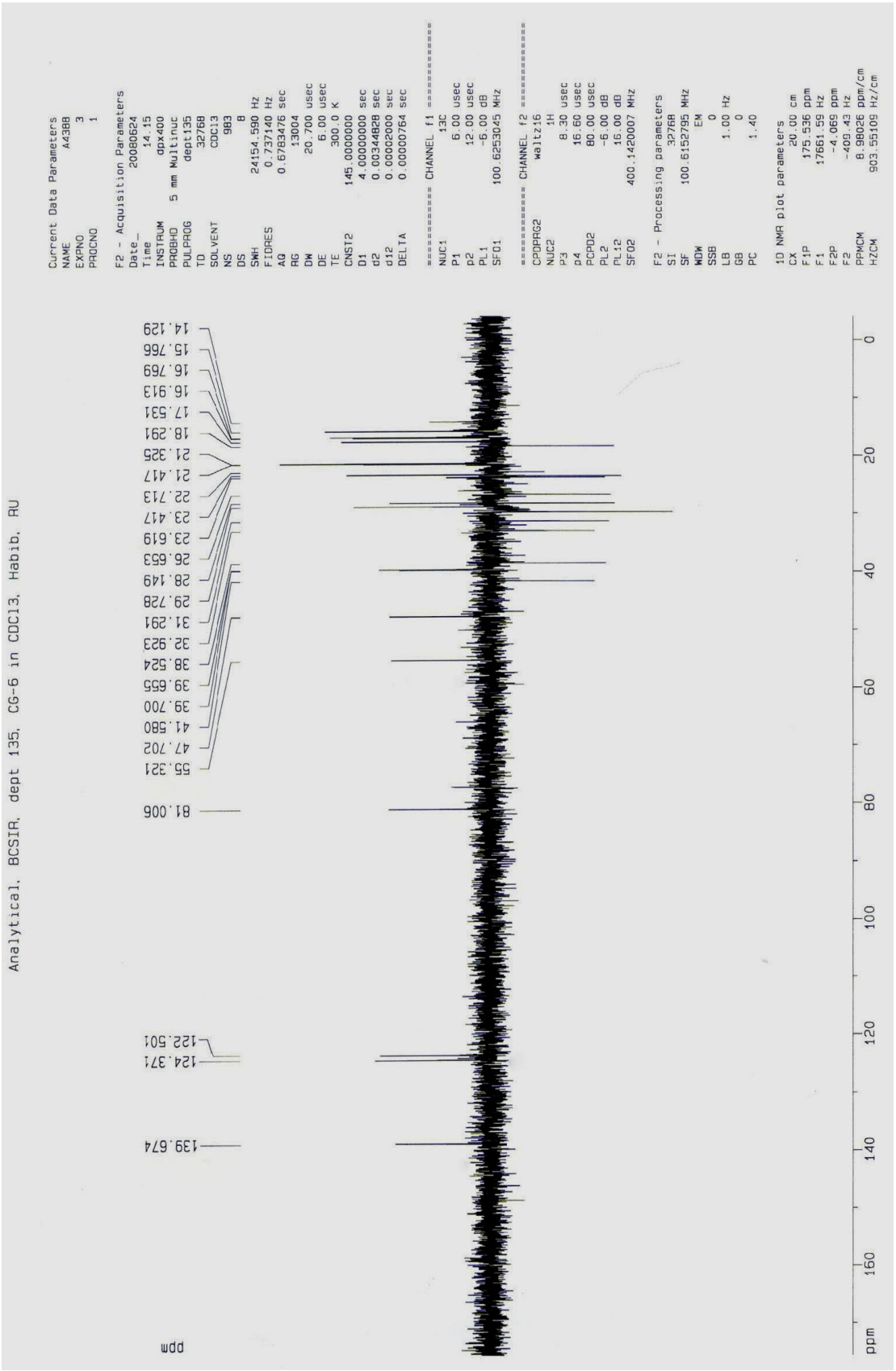


Figure 2.18 DEPT spectrum of compound-2

2.5.3. Characterization and structure elucidation of isolated compound-3

Compound-3 was obtained as white crystals and its melting point was 237°C. Compound-3 showed a molecular ion peak at m/z 468.4023 $[M]^+$ in the mass spectrum (Figure 2.20), corresponding to the molecular formula of $C_{32}H_{52}O_2$. Mass spectrum also exhibited other fragments at m/z 408.6416 (28), 393.5350 (15), 334.4026 (22), 285.2954 (13), 249.5924 (11), 218.3131 (55), 203.3934 (24), 135.3054 (43) and a base paek was found at m/z 189.3864 (100). In the ^{1}H-NMR spectrum (Figure 2.21) of compound-3, typical triterpene signals were observed, and the presence of methyl signals was shown at δ 0.84, δ 0.85, δ 0.86, δ 0.91, δ 0.94, δ 1.02 and δ 1.08 (Table 2.9). Additionally, a methyl signal at δ 2.03 (1H, s) indicated the acetyl group in the structure. Two olefinic proton signals at δ 4.60 (2H, s, H-30) and a acetylated methine proton signal at δ 4.49 (1H, dd, *J* = 10.0 and 6.4 Hz, H-3) were also observed in its ^{1}H-NMR spectrum (Table 2.9 and Figure 2.21). The ^{13}C-NMR spectrum (Figure 2.22) of compound-3 showed eight methyl carbon [δ 27.9 (C-23), δ 16.5 (C-24), δ 16.3 (C-25), δ 15.9 (C-26), δ 14.7 (C-27), δ 19.5 (C-28), δ 25.5 (C-29), δ 21.3 (C$\underline{H}_3$-CO-)] signals; a secondary acetyl group bearing carbon signal at δ 81.0 (C-3) and an carbonyl carbon signal at δ 171.03 (Table 2.10). Finally the comparison with published data[125] confirmed compound-3 as taraxasteryl acetate the chemical structure of which is given in figure 2.19. Isolation and identification of this compound have already been reported in this plant.

Properties of Compound-3 (i.e., Taraxasteryl acetate). White crystal; m.p. 237°C; ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 1.72 (2H, t, *J* = 7.5 Hz, H-1), 1.68 (2H, s, H-2), 4.49 (1H, t, *J* = 10.0 and 6.4 Hz, H-3), 1.62 (1H, s, H-5), 1.39 (1H, m, H-6a), 1.58 (1H, m, H-6b), 1.37 (1H, *s*, H-7), 1.30 (1H, m, H-9), 1.15 (1H, m, H-11a), 1.53 (1H, m, H-11b), 1.11 (1H, m, H-12a), 1.65 (1H, m, H-12b), 1.62 (1H, m, H-13), 0.96 (1H, m, H-15a), 1.26 (1H, m, H-15b), 1.20 (1H, m, H-16a), 1.24 (1H, m, H-16b), 0.98 (1H, m, H-18), 2.06 (1H, m, H-19), 2.22 (1H, m, H-21a), 2.43 (1H, m, H-21b), 1.32 (1H, m, H-22a), 1.42 (1H, m, H-22b), 0.84 (3H, *s*, H-23), 0.85 (3H, *s*, H-24), 0.91 (3H, *s*, H-25), 1.08 (3H, *s*, H-26), 0.94 (3H, *s*, H-27), 0.86 (3H, *s*, H-28), 1.02 (3H, *d*, *J* = 7.0 Hz, H-29), 4.60 (2H, d, *J* = 3.5 Hz, H-30), 2.03 (3H, *s*, -CO-C$\underline{H}_3$). ^{13}C-NMR (150 MHz, $CDCl_3$, δ, ppm): 38.4 (C-1), 23.7 (C-2), 81.0 (C-3), 37.8 (C-4), 55.5 (C-5), 18.2 (C-6), 34.0 (C-7), 40.9 (C-8), 50.4 (C-9), 37.1 (C-10), 21.5 (C-11), 26.1 (C-12), 39.2 (C-13), 42.0 (C-14), 26.6 (C-15), 38.3 (C-16), 34.5 (C-17), 48.7 (C-18), 39.4 (C-19), 154.6 (C-20), 25.6 (C-21), 38.9 (C-22), 27.9 (C-23), 16.5 (C-24), 16.3 (C-25), 15.9 (C-26), 14.7 (C-27), 19.5 (C-28), 25.5

(C-29), 107.1 (C-30), 21.3 (-CO-$\underline{C}H_3$), 171.0 (-$\underline{C}O$-CH_3); EI-MS (m/z): 408.6416 (28), 393.5350 (15), 334.4026 (22), 285.2954 (23), 249.5924 (11), 218.3131 (55), 203.3934 (24), 135.3054 (43),189.3864 (100).

Table 2.9 Comparison of ^{1}H-NMR spectral data of compound-3 and authentic taraxasteryl acetate.

δ_H of compound-3 (in ppm)	δ_H of authentic taraxasteryl acetate[125] (in ppm)
1.08 (1H, m, H-1a), 1.72 (1H, m, H-1b)	1.06 (1H, m, H-1a), 1.73 (1H, m, H-1b)
1.68 (2H, s, H-2)	1.68 (2H, s, H-2)
4.49 (1H, t, *J* = 10.0 and 6.4 Hz, H-3)	4.48 (1H, t, *J* = 10.0 and 6.4 Hz, H-3)
1.62 (1H, s, H-5)	1.62 (1H, s, H-5)
1.39 (1H, m, H-6a), 1.58 (1H, m, H-6b)	1.39 (1H, m, H-6a), 1.56 (1H, m, H-6b)
1.37 (2H, *s*, H-7)	1.36 (2H, *s*, H-7)
1.30 (1H, m, H-9)	1.30 (1H, m, H-9)
1.15 (1H, m, H-11a), 1.53 (1H, m, H-11b)	1.17 (1H, m, H-11a), 1.56 (1H, m, H-11b)
1.11 (1H, m, H-12a), 1.65 (1H, m, H-12b)	1.12 (1H, m, H-12a), 1.67 (1H, m, H-12b)
1.62 (1H, m, H-13)	1.61 (1H, m, H-13)
0.96 (1H, m, H-15a), 1.26 (1H, m, H-15b)	0.95 (1H, m, H-15a), 1.69 (1H, m, H-15b)
1.20 (1H, m, H-16a), 1.24 (1H, m, H-16b)	1.20 (1H, m, H-16a), 1.25 (1H, m, H-16b)
0.98 (1H, m, H-18),	0.97 (1H, m, H-18),
2.06 (1H, m, H-19)	2.05 (1H, m, H-19)
2.22 (1H, m, H-21a), 2.43 (1H, m, H-21b)	2.23 (1H, m, H-21a), 2.40 (1H, m, H-21b)
1.32 (1H, m, H-22a), 1.42 (1H, m, H-22b)	1.36 (1H, m, H-22a), 1.42 (1H, m, H-22b)
0.84 (3H, *s*, H-23)	0.88 (3H, *s*, H-23)
0.85 (3H, *s*, H-24)	0.84 (3H, *s*, H-24)
0.91 (3H, *s*, H-25)	0.87 (3H, *s*, H-25)
1.08 (3H, *s*, H-26),	1.09 (3H, *s*, H-26),
0.94 (3H, *s*, H-27)	0.94 (3H, *s*, H-27)
0.86 (3H, *s*, H-28)	0.85 (3H, *s*, H-28)
1.02 (3H, *d*, *J* = 7.0 Hz, H-29)	1.02 (3H, *d*, *J* = 7.0 Hz, H-29)
4.60 (2H, d, *J* = 3.5 Hz, H-30)	4.61 (2H, d, *J* = 3.5 Hz, H-30)
2.03 (3H, *s*, -CO-C$\underline{H}_3$)	2.03 (3H, *s*, -CO-C$\underline{H}_3$)

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

Table 2.10 Comparison of ^{13}C-NMR spectral data of compound-3 and authentic taraxasteryl acetate.

Carbon No.	δ_C of compound-3 (in ppm)	δ_C of authentic taraxasteryl acetate[125] (in ppm)
1	38.4	38.4
2	23.7	23.6
3	81.0	80.9
4	37.8	37.8
5	55.5	55.4
6	18.2	18.2
7	34.0	34.0
8	40.9	40.9
9	50.4	50.4
10	37.1	37.0
11	21.5	21.5
12	26.1	26.2
13	39.2	39.2
14	42.0	42.0
15	26.6	26.6
16	38.3	38.3
17	34.5	34.5
18	48.7	48.6
19	39.4	39.4
20	154.6	154.6
21	25.6	25.6
22	38.9	38.9
23	27.9	27.9
24	16.5	16.5
25	16.3	16.3
26	15.9	15.9
27	14.7	14.7
28	19.5	19.5
29	25.5	25.5
30	107.1	107.1
CO-$\underline{C}H_3$	21.3	21.3
$\underline{C}O$-CH_3	171.0	171.0

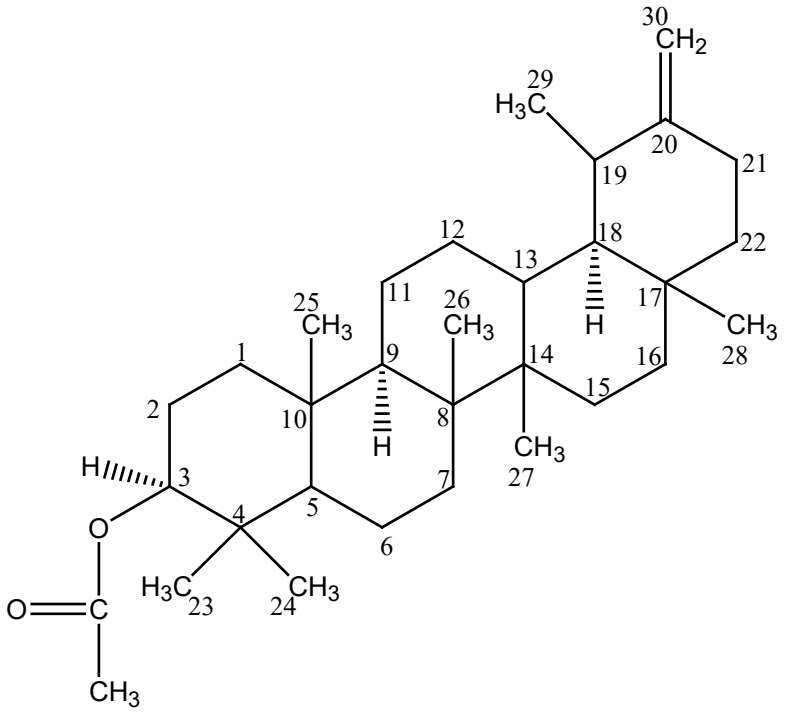


Figure 2.19 Chemical structure of compound-3: Taraxasteryl acetate

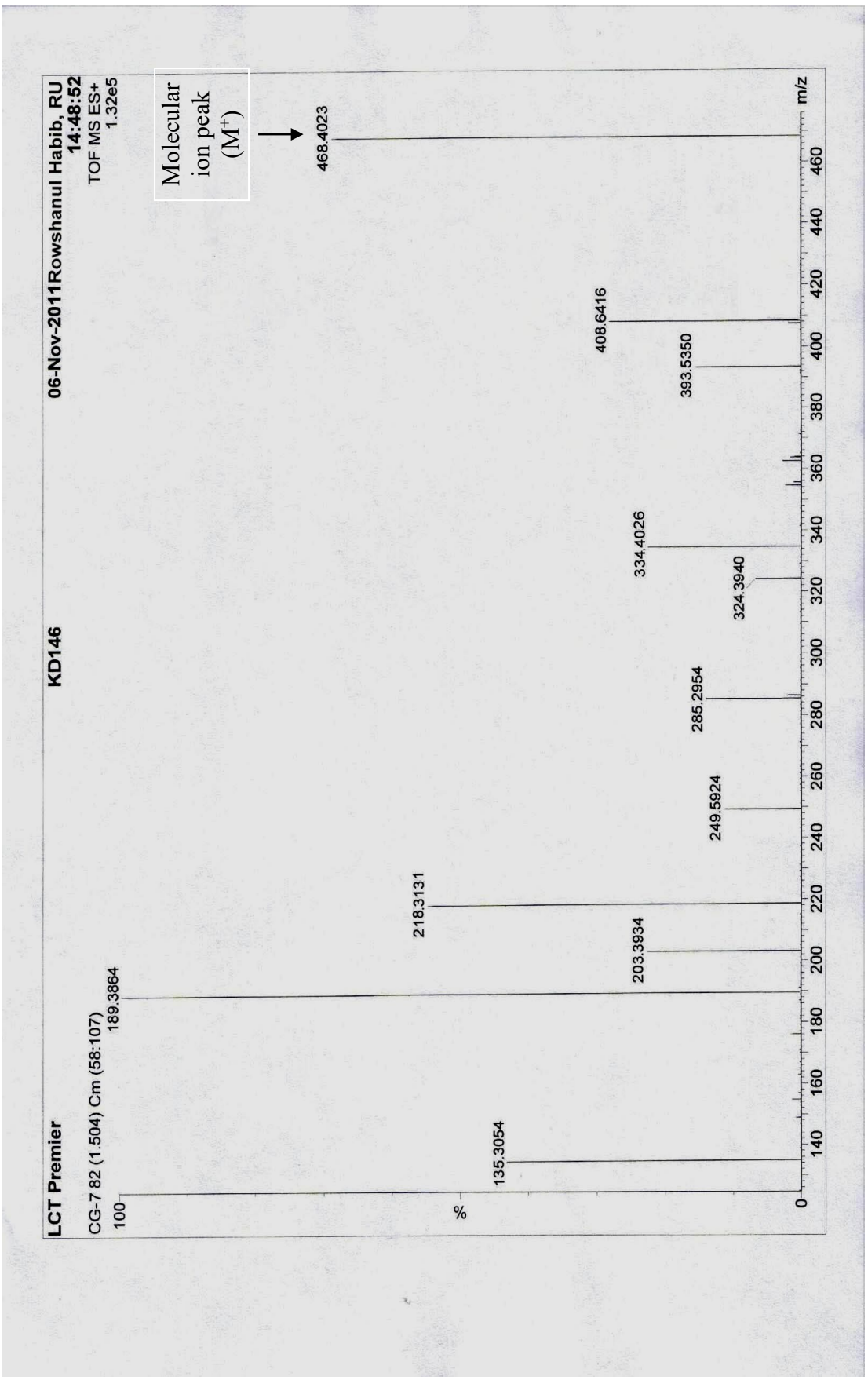


Figure 2.20 Mass spectrum of compound-3

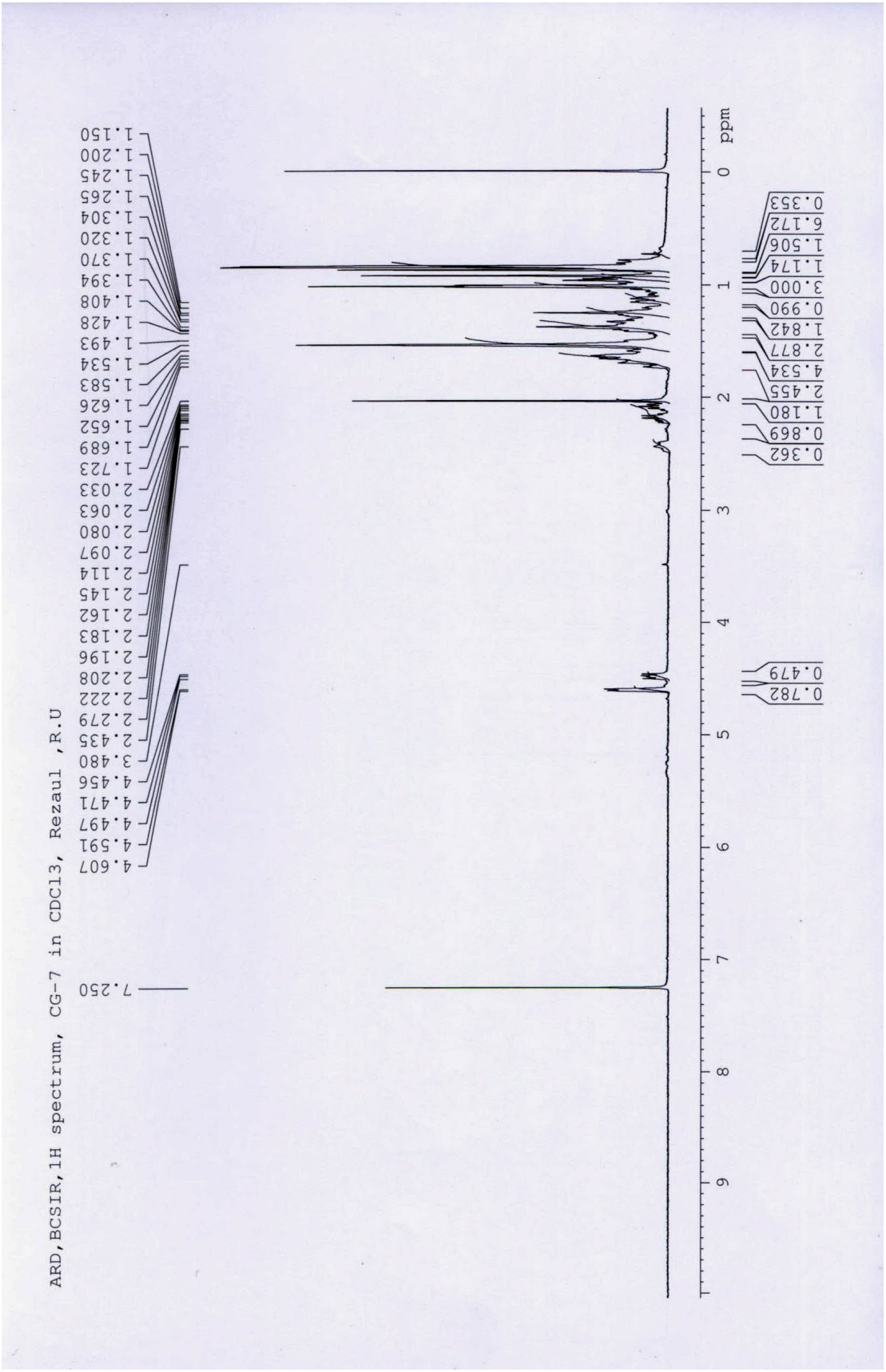


Figure 2.21 ^{1}H-NMR spectrum of compound-3

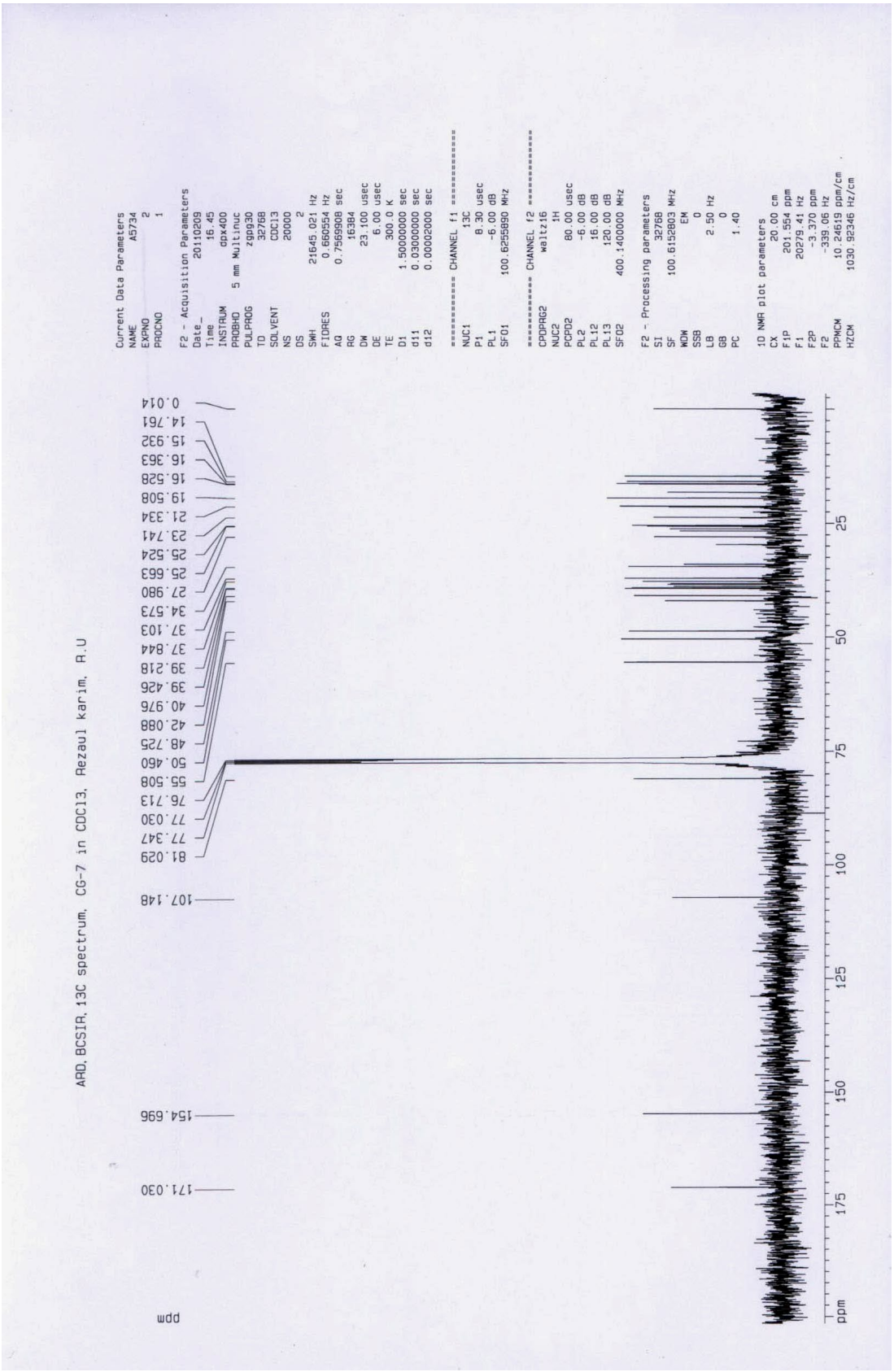


Figure 2.22 ^{13}C-NMR spectrum of compound-3

2.5.4. Characterization and structure elucidation of isolated compound-4

The compound-4 was obtained as a white amorphous powder and its melting point was 214°C. The mass spectrum of compound-4 (Figure 2.24) established the molecular weight with the molecular ion at m/z 440.0566 and the molecular formula as $C_{30}H_{48}O_2$. In its mass spectrum, the significant fragments at m/z 232.1850 and 208.3394 derived from the characteristic retro-Diels-Alder cleavages of the molecule, and the ion at m/z 191.1594 [232- isoprenyl group]$^+$, strongly indicated that the compound is of a lup-12-ene type[126]. Further, the base peak at m/z 201.0438 [232- CH_2OH]$^+$, instead of m/z 232 allowed the location of the second hydroxyl group at C-28. The ^{1}H-NMR data of compound-4 (Table 2.11 and figure-2.25) indicated that the compound was a terpenoid with six tertiary methyl groups at δ 0.84 ~ 1.68 (3H each, s), two olefinic protons at δ 5.73, (2H, s, H-29) and δ 5.40 (1H, t, *J* = 7.5 Hz, H-12), a hydroxylated methine proton at δ 3.52 (1H, t, *J* = 6.4 Hz, H-3) and a hydroxylated methylene at δ 3.34 and δ 3.84 (each 1H, d, *J* = 10.4 Hz, H-28). The ^{13}C-NMR spectrum of compound-4 (Figure 2.26) exhibited the presence of thirty carbon signals incluiding four olefinic carbon signals at δ 150.3 (C-20), δ 140.8 (C-13), δ 126.7 (C-12) and δ 108.9 (C-29) (Table 2.12). This data suggested that the compound-4 might be a lupane-type triterpene. In addition, the carbon signals observed at δ 78.5 (C-3) and δ 61.8 (C-28) indicated the presence of a secondary and a primary hydroxyl group and it was an important evidence for presence of an additional primary hydroxyl group its structure. Assignment of this additional primary hydroxyl group to C-28 was confirmed by comparison of ^{1}H-NMR and ^{13}C-NMR spectral data of compound-4 (Table 2.11 & 2.12) with betulin[127]. All these analyses and comparison of its spectral data with literature[128-130] led this compound-4 to be identified as lupa-12,20(29)-dien-3b,28-diol (Figure 2.23). Isolation of lupa-12,20(29)-dien-3β,28-diol is reported for the first time from flower of *Calotropis gigantea*.

Properties of Compound-4 (i.e., Lupa-12,20(29)-dien-3β,28-diol). white amorphous powder; m.p. 214°C; ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 0.92 (1H, m, H-1a), 1.53 (1H, m, H-1b), 1.22 (2H, m, H-2), 3.52 (1H, t, *J* = 6.4 Hz, H-3), 0.69 (1H, m, H-5), 1.49 (1H, m, H-6a), 1.40 (1H, m, H-6b), 1.27 (1H, m, H-7), 1.24 (1H, m, H-9), 1.94 (1H, m, H-11), 5.40 (1H, t, *J* = 7.5 Hz, H-12), 1.44 (2H, m, H-15), 1.15 (1H, m, H-16a), 1.10 (1H, m, H-16b), 2.27 (1H, d, J = 3.5, H-18), 2.68 (1H, d, *J* = 10.3 Hz, H-19), 1.94 (1H, m, H-21a), 1.17 (1H, m, H-21b), 1.84 (2H, d, *J* = 16.3 Hz, H-22), 0.83 (3H, s, H-23), 0.87 (3H, s, H-24), 0.90 (3H, s, H-25), 1.00 (3H, s, H-26), 1.02 (3H, s, H-26), 3.32 (1H, d, *J* = 10.4 Hz, H-28a), 3.84 (1H, d, *J* = 10.4 Hz, H-28b), 5.74 (2H, s, H-29), 1.66 (3H, s, H-30); ^{13}C-NMR (150 MHz, $CDCl_3$, δ, ppm): 38.7 (C-1), 27.5 (C-2), 78.5 (C-3), 37.3 (C-4), 55.8

(C-5), 18.8 (C-6), 36.5 (C-7), 42.3 (C-8), 51.2 (C-9), 37.6 (C-10), 23.1 (C-11), 126.7 (C-12), 140.8 (C-13), 50.2 (C-14), 27.1 (C-15), 29.2 (C-16), 42.3 (C-17), 47.1 (C-18), 39.8 (C-19), 150.3 (C-20), 24.5 (C-21), 31.7 (C-22), 21.1 (C-23), 12.2 (C-24), 18.8 (C-25), 19.0 (C-26), 19.4 (C-27), 61.8 (C-28), 109.3 (C-29), 19.9 (C-30); EI-MS (m/z): 440.0566 (M^+) 391.3050 (15), 340.2716 (52), 326.2864 (23), 289.3424 (14) 232.1850 (16), 208.3394 (18), 201.0438 (100), 191.1594 (12).

Table 2.11 Comparison of ^{1}H-NMR spectral data of compound-4 and authentic lupa-12,20(29)-dien-3β,28-diol.

δ_H of compound-4 (in ppm)	δ_H of authentic lupa-12,20(29)-dien-3β,28-diol[128-130] (in ppm)
0.92 (1H, m, H-1a), 1.53 (1H, m, H-1b)	0.93 (1H, m, H-1a), 1.54 (1H, m, H-1b)
1.22 (2H, m, H-2)	1.23 (2H, m, H-2)
3.52 (1H, t, *J* = 6.4 Hz, H-3)	3.54 (1H, t, *J* = 6.4 Hz, H-3)
0.69 (1H, m, H-5)	0.69 (1H, m, H-5)
1.49 (1H, m, H-6a), 1.40 (1H, m, H-6b)	1.48 (1H, m, H-6a), 1.42 (1H, m, H-6b)
1.27 (1H, m, H-7)	1.29 (1H, m, H-7)
1.24 (1H, m, H-9)	1.25 (1H, m, H-9)
1.94 (1H, m, H-11)	1.94 (1H, m, H-11)
5.40 (1H, t, *J* = 7.5 Hz, H-12)	5.42 (1H, t, *J* = 7.5 Hz, H-12)
1.44 (2H, m, H-15)	1.44 (2H, m, H-15)
1.15 (1H, m, H-16a), 1.10 (1H, m, H-16b)	1.16 (1H, m, H-16a), 1.12 (1H, m, H-16b)
2.27 (1H, d, *J* = 3.5, H-18)	1.94 (1H, d, *J* = 3.5, H-18)
2.68 (1H, d, *J* = 10.3 Hz, H-19)	2.68 (1H, d, *J* = 10.3 Hz, H-19)
1.94 (1H, m, H-21a), 1.17 (1H, m, H-21b)	2.28 (1H, m, H-21a), 1.17 (1H, m, H-21b)
1.84 (2H, d, *J* = 16.3 Hz, H-22)	1.84 (2H, d, *J* = 16.3 Hz, H-22)
0.83 (3H, s, H-23)	0.84 (3H, s, H-23)
0.87 (3H, s, H-24)	0.86 (3H, s, H-24)
0.90 (3H, s, H-25)	0.88 (3H, s, H-25)
1.00 (3H, s, H-26)	0.98 (3H, s, H-26)
1.02 (3H, s, H-26)	1.01 (3H, s, H-26)
3.32 & 3.84 (each 1H, d, *J* = 10.4 Hz, H-28)	3.34 & 3.85 (each 1H, d, *J* = 10.4 Hz, H-28)
5.74 (2H, s, H-29)	5.73 (2H, s, H-29)
1.66 (3H, s, H-30)	1.68 (3H, s, H-30)

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

Table 2.12 Comparison of ^{13}C-NMR spectral data of compound-4 and authentic lupa-12,20(29)-dien-3b,28-diol.

Carbon No.	δ_C of compound-4 (in ppm)	δ_C of authentic lupa-12,20(29)-dien-3β,28-diol[128-130] (in ppm)
1	38.7	38.9
2	27.5	27.6
3	78.5	78.8
4	37.3	37.4
5	55.8	55.7
6	18.8	18.2
7	36.5	36.7
8	42.3	42.3
9	51.2	51.7
10	37.3	37.6
11	23.1	23.6
12	126.7	126.6
13	140.8	141.0
14	50.2	50.1
15	27.1	27.3
16	29.2	29.5
17	42.3	42.4
18	47.1	47.2
19	39.8	40.0
20	150.3	150.1
21	24.3	24.5
22	31.7	31.8
23	21.1	21.1
24	12.2	12.3
25	18.8	18.8
26	19.0	19.0
27	19.4	19.4
28	61.8	61.6
29	108.9	109.3
30	19.9	19.9

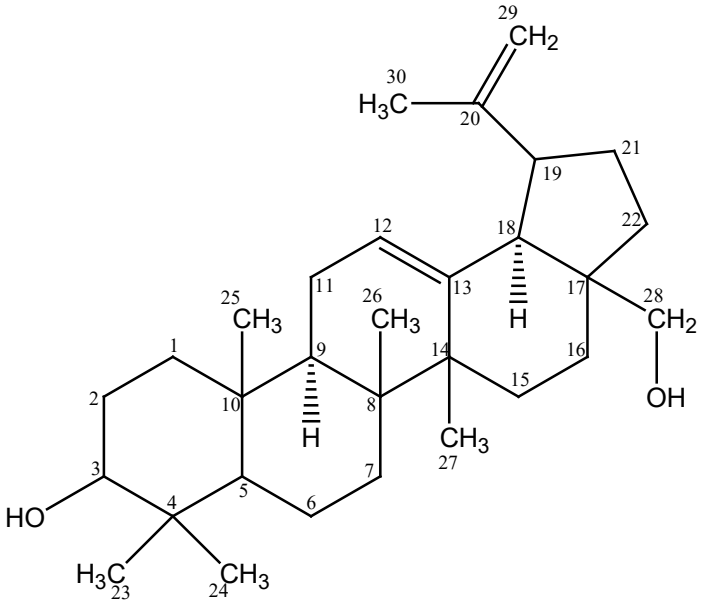


Figure 2.23 Chemical structure of compound-4: Lupa-12,20(29)-dien-3β,28-diol

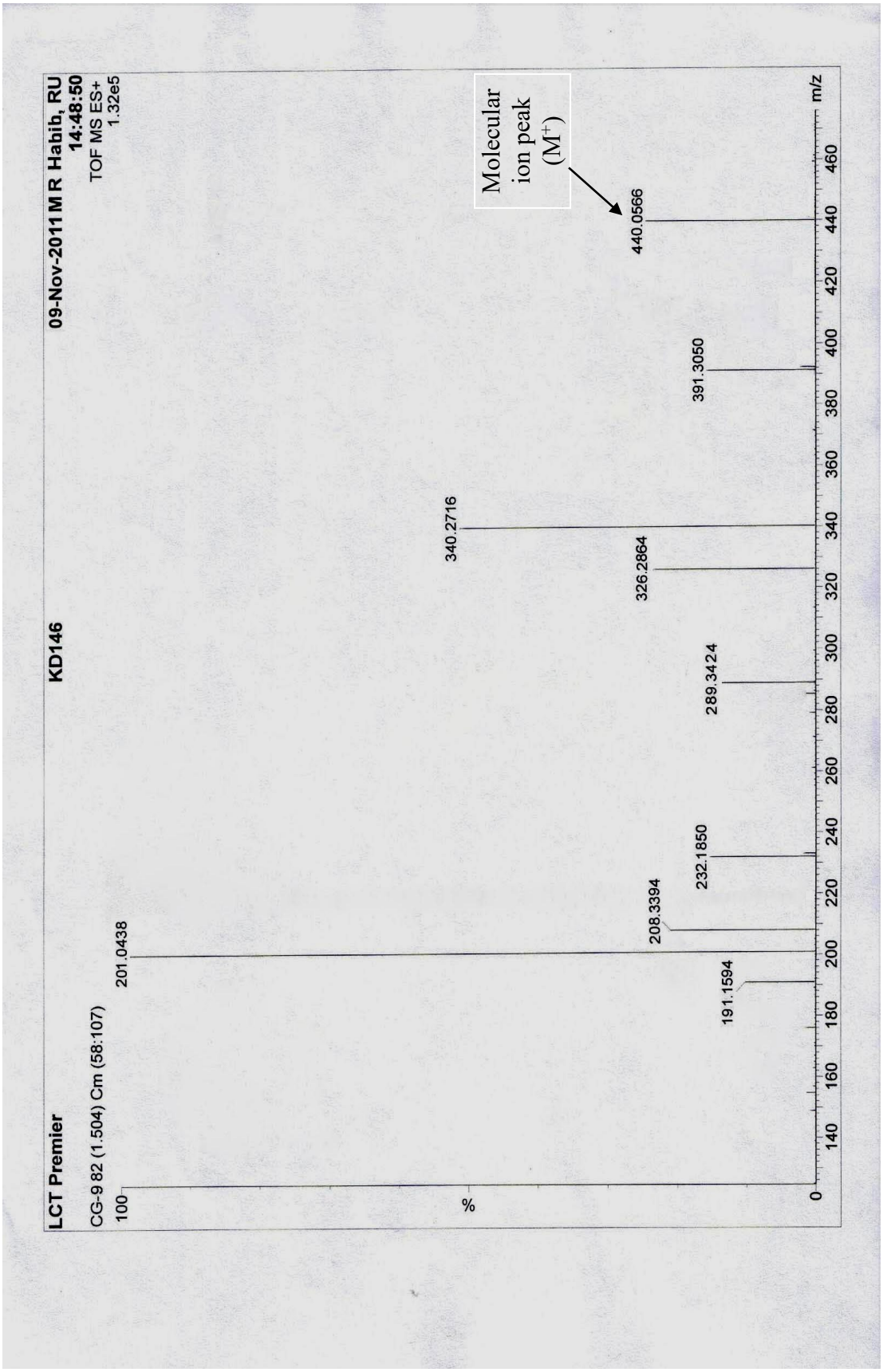


Figure 2.24 Mass spectrum of compound-4

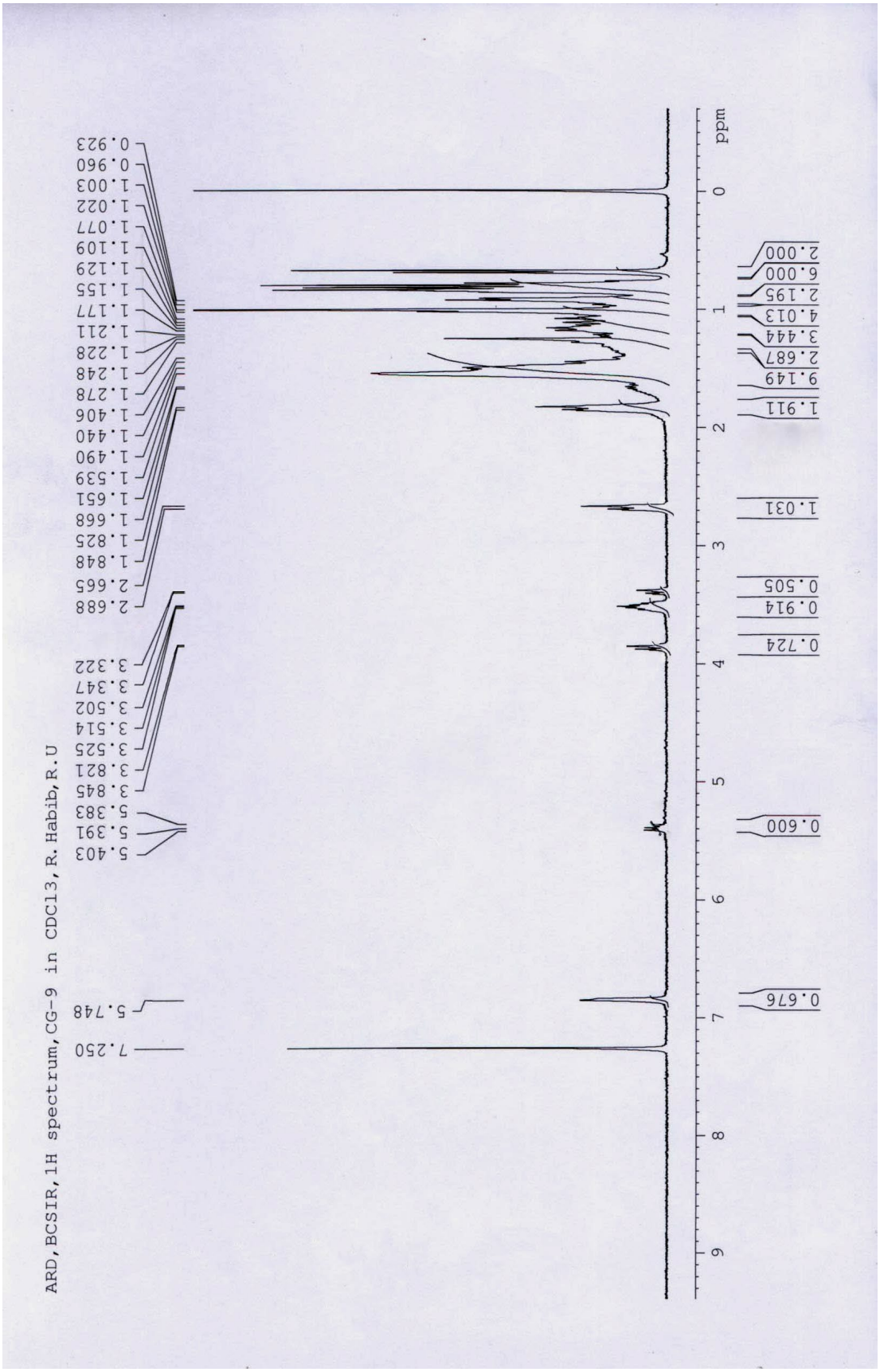


Figure 2.25 ^{1}H-NMR spectrum of compound-4

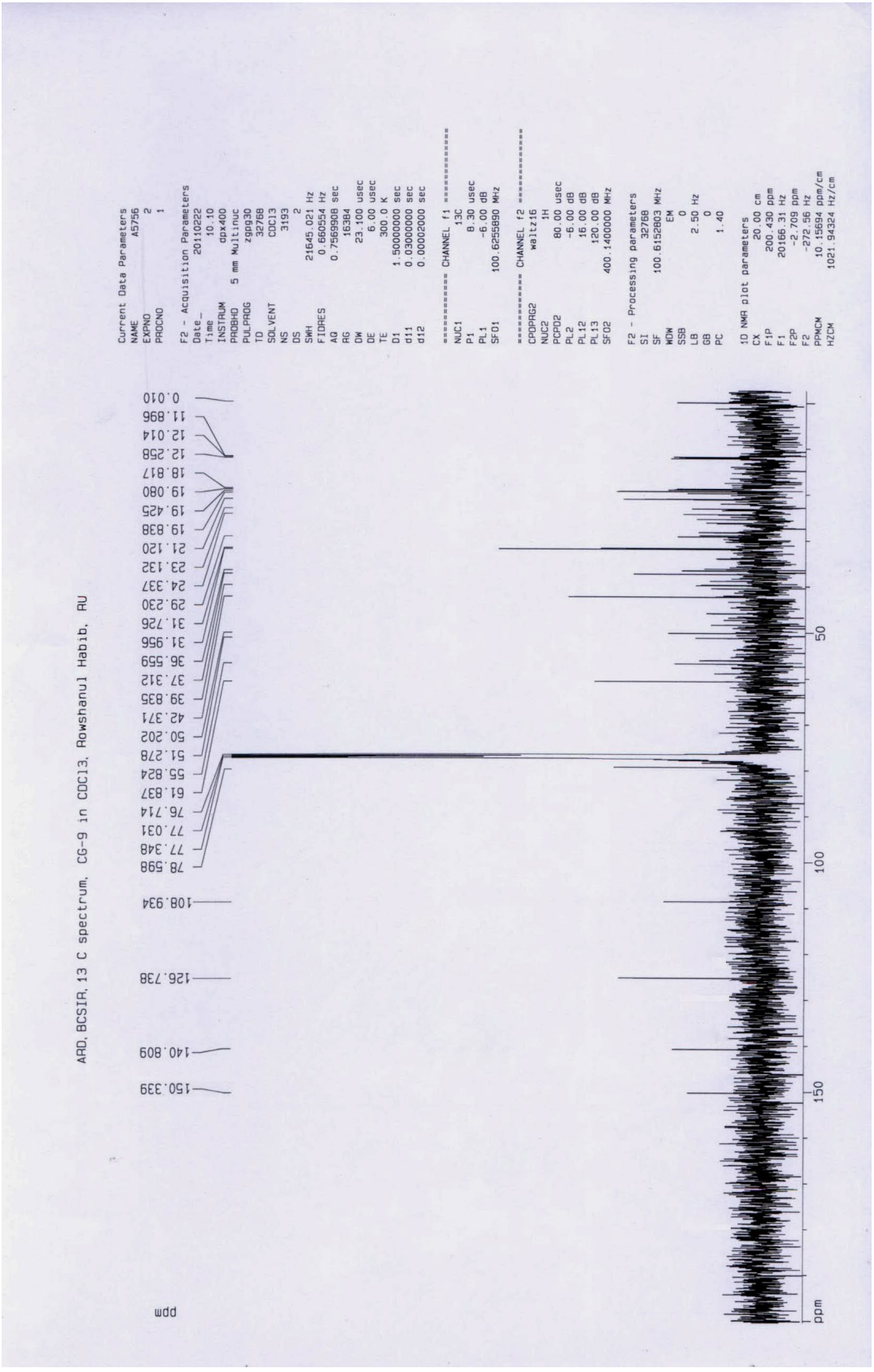


Figure 2.26 ^{13}C-NMR spectrum of compound-4

2.5.5. Characterization and structure elucidation of isolated compound-5

Compound-5 was isolated as colourless crystals and its melting point was determined as 228ºC. The IR spectrum of compound-5 (Figure 2.29) exhibited the absorptions for a hydroxyl group (3448.5 cm^{-1}), a carbonyl carbon of a carboxylic acid group (1705.8 cm^{-1}) and an olefinic structure (1627.8 cm^{-1}). The mass spectrum of compound-5 (Figure 2.28) showed a molecular ion peak at m/z 456.3603 suggesting a molecular formula of $C_{30}H_{48}O_3$. The other peaks were observed at m/z 443.3375 (54), 425.3866 (28), 407.3864 (29), 238.0080 (21), 218.2954 (100), 203.3854 (26), 159.4275 (9), 133.5075 (15). In ^{1}H-NMR spectrum of compound-5 (Figure 2.30), the appearance of a olefinic proton at δ 5.14 (1H, t, *J* = 3.5 Hz, H-12), a methine proton at δ 1.30 (1H, d, *J* = 8.9 Hz, H-18) and two methyl group signals at δ 0.78 (3H, d, *J* = 5.6 Hz, H-29) and δ 0.79 (3H, d, *J* = 3.2 Hz, H-30) unambiguously confirmed the presence of Δ^{12}-ursane skeleton. In addition, the ^{1}H-NMR spectrum of compound-5 also showed a hydroxylated methine proton signal at δ 4.08 (1H, t, *J* = 3.5, H-3) and five methyl group signals, each as singlet at δ 1.32 (C-23), δ 0.89 (C-25), δ 0.96 (C-26), δ 0.92 (C-27) and δ 0.99 (C-28) (Table 2.13). ^{13}C-NMR spectrum of compound-5 (Figure 2.31) showed the presence of 30 signals, one of which was assigned to a carbonyl carbon (δ 183.7) of a carboxylic acid group (Table 2.14). In ^{13}C-NMR spectrum of compound-5, two signals at δ 124.8 (C-12) and δ 139.6 (C-13) (Table 2.14) were assigned to a double bond that was consistent with that of Δ^{12}-ursane skeleton. The location of the carboxylic acid group was found to be at 24-position based on the downfield shift of C-4 resonating at δ 47.7 ppm relative to similar compounds cited in literature[131]. In addition, a hydroxylated methine carbon signal at δ 71.9 (C-3) was also observed in its ^{13}C-NMR spectrum (Figure 2.31). Finally the structure of compound-5 was confirmed as β-boswellic acid (3β-hydroxy-urs-12-ene-24β-oic acid) (Figure 2.27) by a comparison with the ^{1}H-, ^{13}C-NMR and mass spectral data reported in the literature[132]. This study reported for the first time β-boswellic acid in the *Calotropis gigantea*.

Properties of Compound-4 (i.e., β-boswellic acid). white amorphous powder; m.p. 228ºC; IR bands (neat): 3448.5, 2956.7, 1705.8 (s), 1627.8, 1456.2, 1244.0, 1022.2 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 1.91 (2H, dd, *J* = 10.5 & 4.0 Hz, H-1), 2.21 (2H, dd, *J* = 14.0 & 4.0 Hz, H-2), 4.08 (1H, t, *J* = 3.5 Hz, H-3), 1.46 (1H, dd, *J* = 11.8 & 3.1 Hz, H-5), 1.67 (2H, dd, *J* = 14.1 & 3.0 Hz, H-6), 1.50 (2H, dd, *J* = 14.0 & 3.2 Hz, H-

7), 1.60 (1H, t, *J* = 3.5 Hz, H-9), 1.98 (2H, t, J = 3.5, H-11), 5.14 (1H, t, *J* = 3.5 Hz, H-12), 0.84 (2H, d, *J* = 12.3 Hz, H-15), 1.03 (2H, dd, *J* = 16.0 & 2.0 Hz, H-16), 1.30 (1H, d, *J* = 8.9 Hz, H-18), 1.24 (1H, t, *J* = 3.5 Hz, H-19), 1.29 (1H, m, H-20), 1.63 (2H, m, H-21), 1.20 (2H, t, *J* = 4.0 Hz, H-22), 1.32 (3H, s, H-23), 0.89 (3H, s, H-25), 0.96 (3H, s, H-26), 0.92 (3H, s, H-27), 0.99 (3H, s, H-28), 0.78 (3H, d, *J* = 5.6 Hz, H-29), 0.79 (3H, d, *J* = 3.2 Hz, H-30); ^{13}C-NMR (150 MHz, $CDCl_3$, δ, ppm): 34.2 (C-1), 26.4 (C-2), 71.9 (C-3), 47.7 (C-4), 55.3 (C-5), 21.3 (C-6), 33.7 (C-7), 39.6 (C-8), 47.2 (C-9), 37.7 (C-10), 23.6 (C-11), 124.8 (C-12), 139.6 (C-13), 39.7 (C-14), 28.1 (C-15), 26.3 (C-16), 33.2 (C-17), 59.1 (C-18), 40.2 (C-19), 40.1 (C-20), 31.2 (C-21), 41.5 (C-22), 23.4 (C-23), 183.7 (C-24), 15.7 (C-25), 16.9 (C-26), 21.3 (C-27), 23.2 (C-28), 17.5 (C-29), 28.7 (C-30); EI-MS (m/z): 456.3603 (M^+), 443.3375 (54), 425.3866 (28), 407.3864 (29), 238.0080 (21), 218.2954 (100), 203.3854 (26), 159.4275 (9), 133.5075 (15).

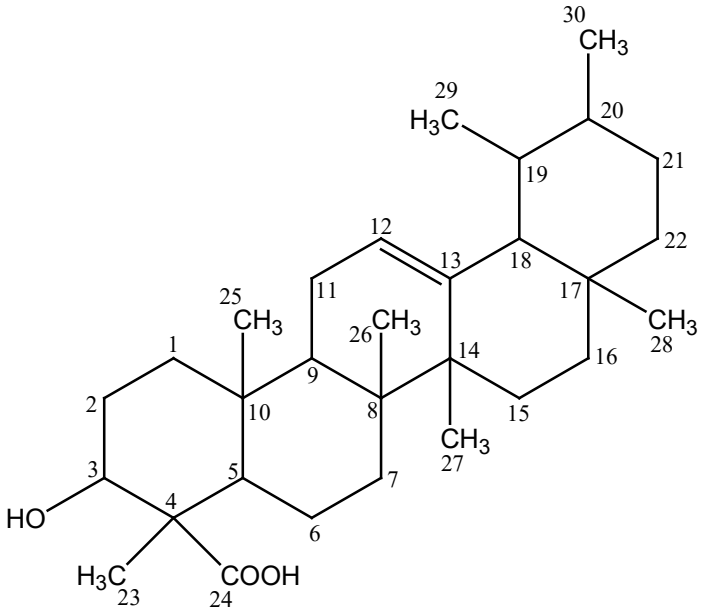


Figure 2.27. Chemical structure of compound-5: β-boswellic acid (3β-hydroxy-urs-12-ene-24β-oic acid)

Table 2.13 Comparison of ^{1}H-NMR spectral data of compound-5 and authentic β-boswellic acid.

δ_H of compound-5 (in ppm)	δ_H of authentic β-boswellic acid[132] (in ppm)
1.91 (2H, dd, *J* = 10.5 & 4.0 Hz, H-1)	1.92 (2H, dd, *J* = 10.5 & 4.0 Hz, H-1)
2.21 (2H, dd, *J* = 14.0 & 4.0 Hz, H-2)	2.22(2H, dd, *J* = 14.0 & 4.0 Hz, H-2)
4.08 (1H, t, *J* = 3.5 Hz, H-3)	4.08 (1H, t, *J* = 3.5 Hz, H-3)
1.46 (1H, dd, *J* = 11.8 & 3.1 Hz, H-5)	1.48 (1H, dd, *J* = 11.8 & 3.1 Hz, H-5)
1.67 (2H, dd, *J* = 14.1 & 3.0 Hz, H-6)	1.68 (2H, dd, *J* = 14.1 & 3.0 Hz, H-6)
1.50 (2H, dd, *J* = 14.0 & 3.2 Hz, H-7)	1.52 (2H, dd, *J* = 14.0 & 3.2 Hz, H-7)
1.60 (1H, t, *J* = 3.5 Hz, H-9)	1.60 (1H, t, *J* = 3.5 Hz, H-9)
1.98 (2H, t, J = 3.5, H-11)	1.99 (2H, t, J = 3.5, H-11)
5.14 (1H, t, *J* = 3.5 Hz, H-12)	5.14 (1H, t, *J* = 3.5 Hz, H-12)
0.84 (2H, d, *J* = 12.3 Hz, H-15)	0.82 (2H, d, *J* = 12.3 Hz, H-15)
1.03 (2H, dd, *J* = 16.0 & 2.0 Hz, H-16)	1.02 (2H, dd, *J* = 16.0 & 2.0 Hz, H-16)
1.30 (1H, d, *J* = 8.9 Hz, H-18)	1.30 (1H, d, *J* = 8.9 Hz, H-18)
1.24 (1H, t, *J* = 3.5 Hz, H-19)	1.25 (1H, t, *J* = 3.5 Hz, H-19)
1.29 (1H, m, H-20)	1.27 (1H, m, H-20)
1.63 (2H, m, H-21)	1.64 (2H, m, H-21)
1.20 (2H, t, *J* = 4.0 Hz, H-22)	1.21 (2H, t, *J* = 4.0 Hz, H-22)
1.32 (3H, s, H-23)	1.34 (3H, s, H-23)
0.89 (3H, s, H-25)	0.89 (3H, s, H-25)
0.96 (3H, s, H-26)	0.96 (3H, s, H-26)
0.92 (3H, s, H-27)	0.91 (3H, s, H-27)
0.99 (3H, s, H-28)	0.99 (3H, s, H-28)
0.78 (3H, d, *J* = 5.6 Hz, H-29)	0.78 (3H, d, *J* = 5.6 Hz, H-29)
0.79 (3H, d, *J* = 3.2 Hz, H-30)	0.79 (3H, d, *J* = 3.2 Hz, H-30)

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

Table 2.14 Comparison of ^{13}C-NMR spectral data of compound-5 and authentic β-boswellic acid.

Carbon No.	δ_C of compound-5 (in ppm)	δ_C of authentic β-boswellic acid[132] (in ppm)
1	34.2	34.3
2	26.4	26.6
3	71.9	71.8
4	47.7	47.6
5	55.3	55.2
6	21.3	20.1
7	33.7	33.5
8	39.6	39.8
9	47.2	47.3
10	37.7	37.7
11	23.6	23.8
12	124.8	124.9
13	139.6	139.8
14	39.7	39.9
15	28.1	28.5
16	26.3	26.9
17	33.2	33.5
18	59.1	59.6
19	40.2	40.1
20	40.1	40.0
21	31.2	31.7
22	41.5	41.9
23	23.4	24.6
24	183.7	183.6
25	15.7	13.7
26	16.9	17.3
27	21.3	21.8
28	23.1	23.6
29	17.5	17.8
30	28.7	29.2

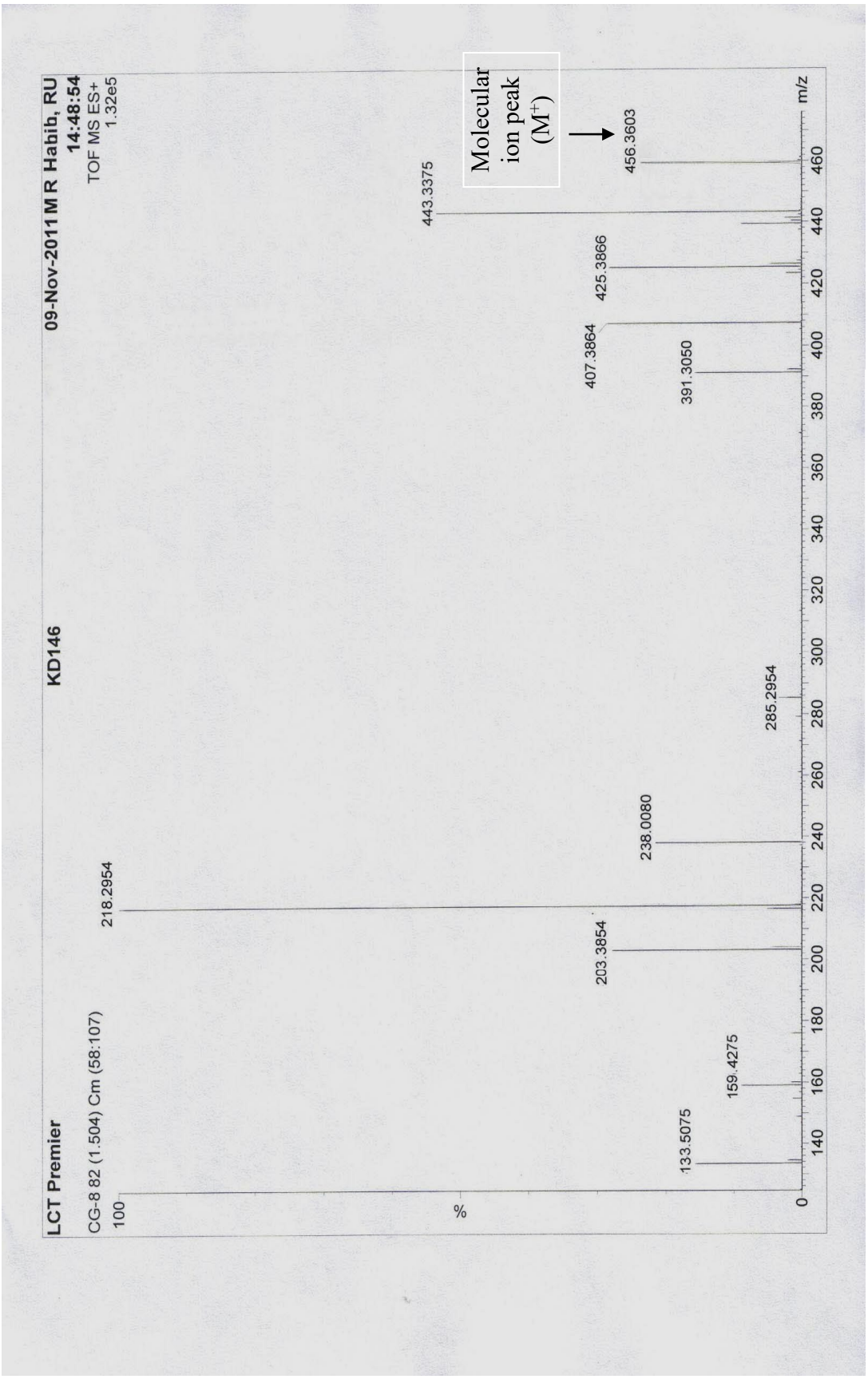


Figure 2.28 Mass spectrum of compound-5

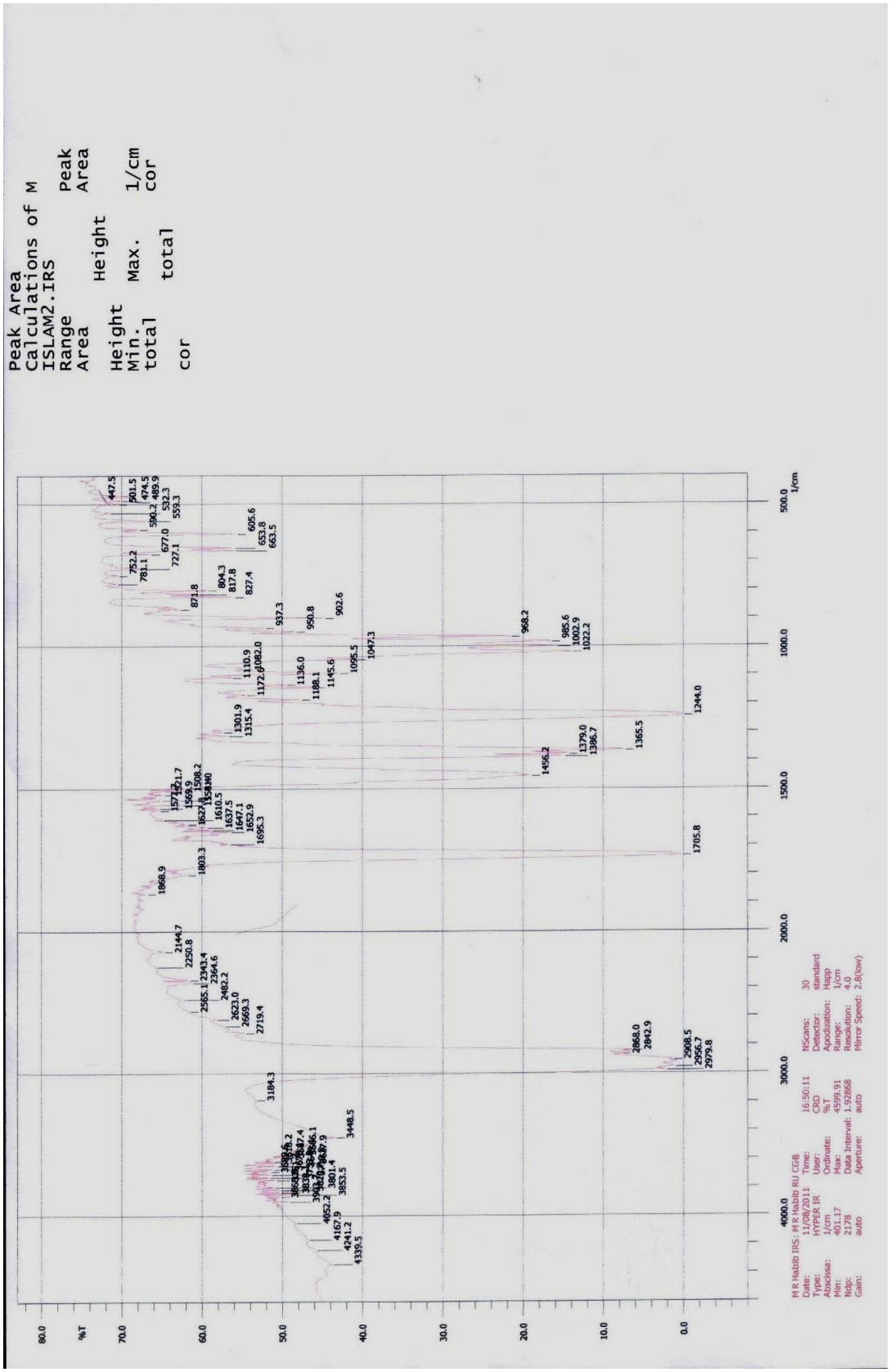


Figure 2.29 IR spectrum of compound-5

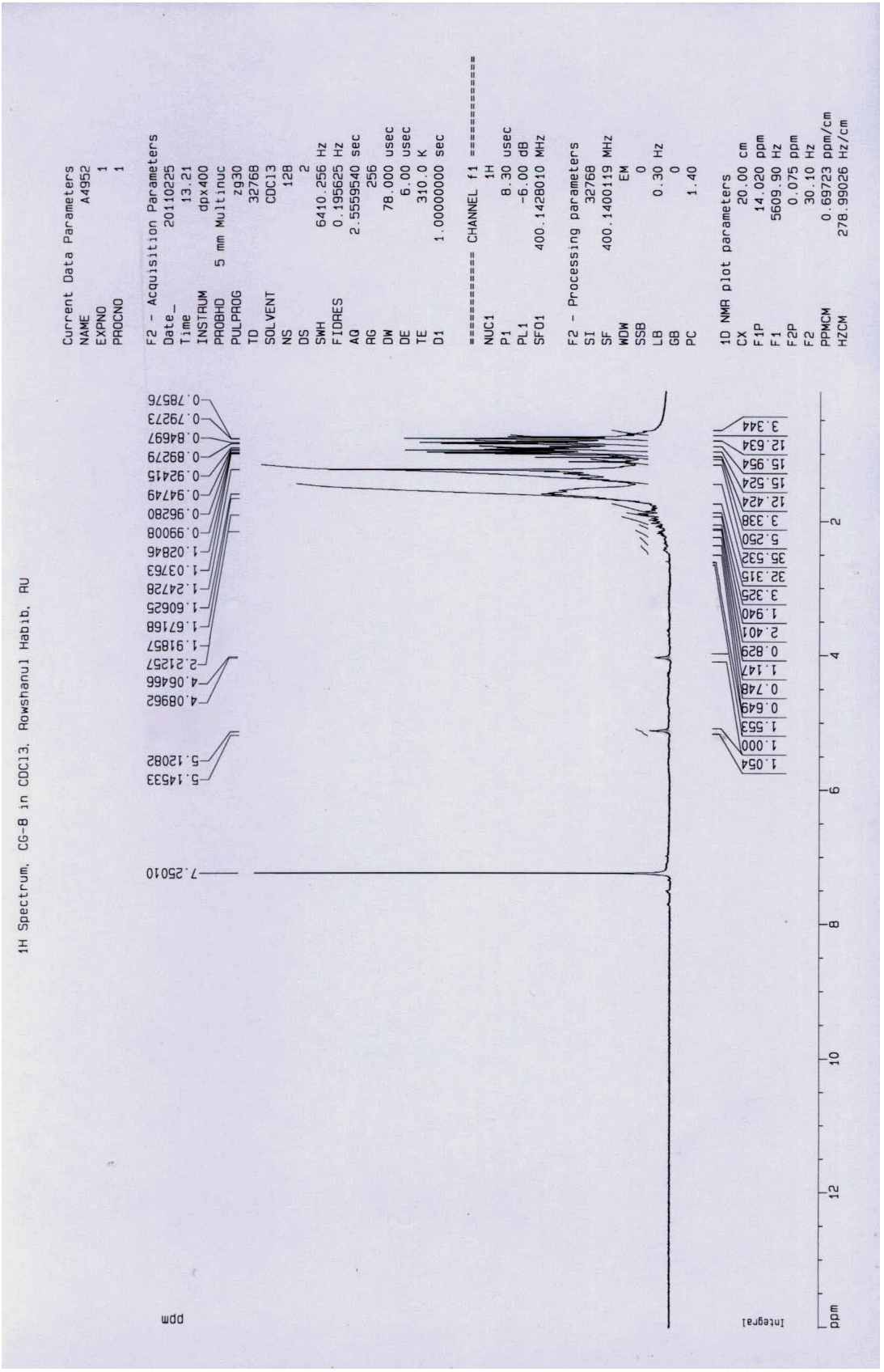


Figure 2.30 ^{1}H-NMR spectrum of compound-5

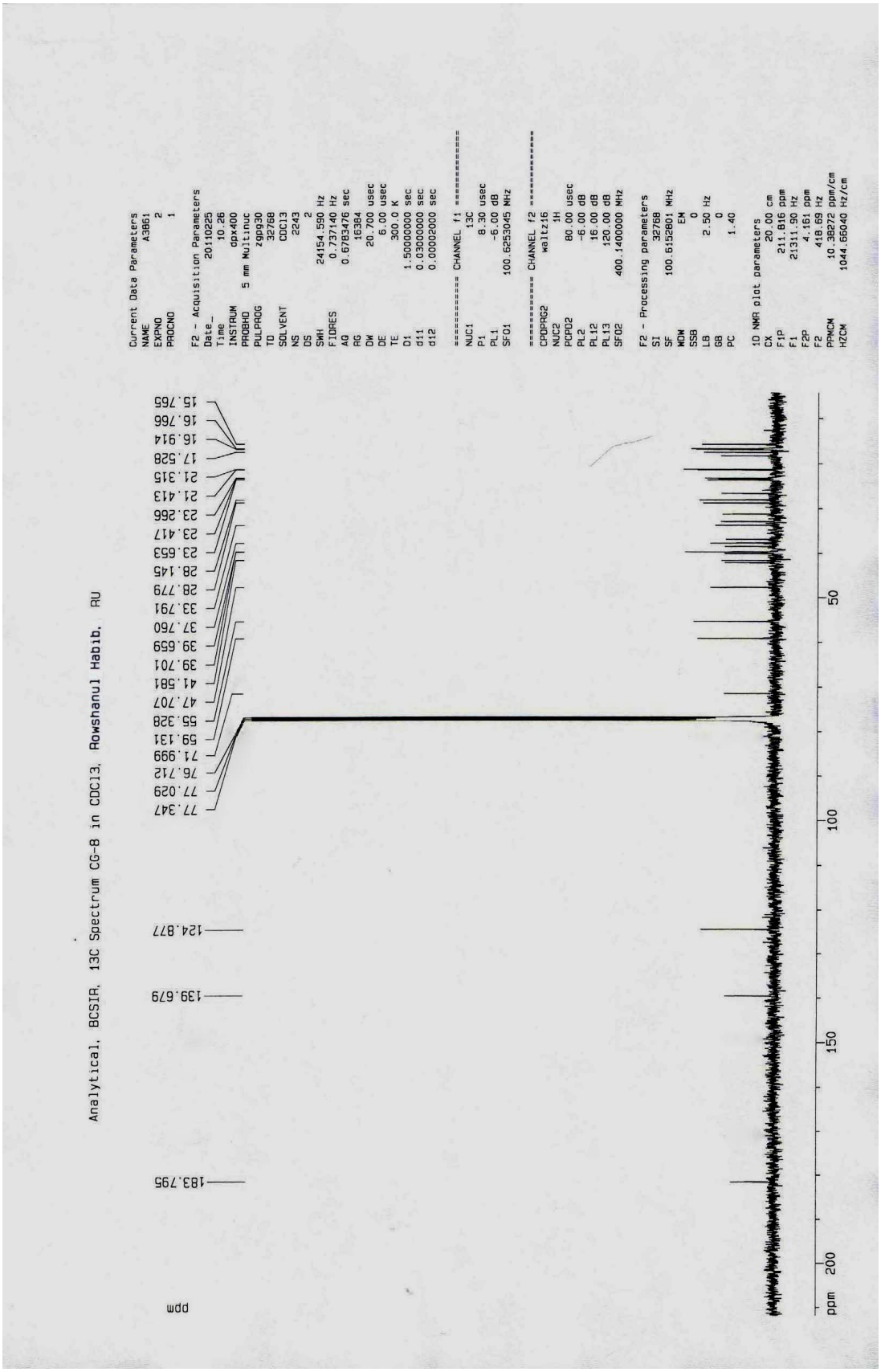


Figure 2.31 ^{13}C-NMR spectrum of compound-5

2.5.6. Characterization and structure elucidation of isolated compound-6

Compound-6 was isolated as orange oil. The ^{1}H-NMR spectrum of compound-6 (Figure 2.33) indicated two aromatic proton signals at δ 6.72 (1H, s, H-1) and δ 6.98 (1H, s, H-4) (Table 2.15). One methoxyl signal at δ 3.78 (3H, s, CH_3-O-) and two methyl signals at δ 0.99 and δ 0.76 (each 3H, d, *J* = 6.5, H-12 &13) were observed in its ^{1}H-NMR (Table 2.15). In addition, ^{1}H-NMR spectrum of compound-6 also exhibited one downfield methyl proton signal at δ 2.19 (3H, s, H-15) which is conjugated with benzene ring. The ^{13}C-NMR spectrum of compound-6 (Figure 2.34) displayed 14 signals while the DEPT experiment (Figure 2.35) indicated the presence of four methyls, two methylenes, four methines and five quarternary carbons. The ^{13}C-NMR spectrum of compound-6 included six olefinic carbon signals (δ 108.9, δ 156.7, δ 133.2, δ 131.2, δ 131.5 and δ 140.3) indicating the presence of a one benzene ring in its structure (Table 2.16). In addition, its ^{13}C-NMR spectrum also showed one carbonyl carbon signal (δ_C 198.7) with four methyl carbon signals (δ 20.7, δ 20.2, δ 14.5 & δ 61.8) (Table 2.16). Finally based on spectroscopic analysis and a comparison of the NMR data with literature[133], compound-6 has been identified as 2-Methoxy-14-calamenenone (a sesquiterpene) (Figure 2.32). In this study, we have reported 2-Methoxy-14-calamenenone from the stem bark of *Amoora rohituka* for the first time.

Properties of Compound-6 (i.e., 2-Methoxy-14-calamenenone). orange oil; ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm, *J*/Hz): 6.72 (1H, s, H-1), 6.98 (1H, s, H-4), 1.88 ~ 1.98 (2H, m, H-8), 2.55 ~ 2.59 (2H, m, H-9), 2.30 ~ 2.39 (2H, m, H-10), 2.30 ~ 2.39 (2H, m, H-11), 0.99 (3H, d, J = 6.5 Hz, H-12), 0.76 (3H, d, J = 6.5 Hz, H-13), 2.19 (3H, s, H-14), 3.78 (3H, s, CH_3-O-); ^{13}C-NMR (150 MHz, $CDCl_3$, δ, ppm): 108.9 (C-1), 156.7 (C-2), 133.2 (C-3), 131.2 (C-4), 131.5 (C-5), 140.3 (C-6), 198.7 (C-7), 38.4 (C-8), 24.5 (C-9), 44.2 (C-10), 31.7 (C-11), 20.7 (C-12), 20.2 (C-13), 14.5 (C-14), 61.8 ($\underline{C}H_3$-O-).

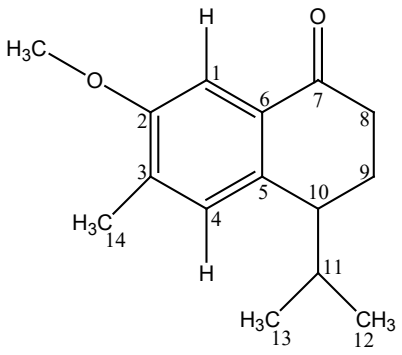


Figure 2.32. Chemical structure of compound-6: 2-Methoxy-14-calamenenone

Table 2.15. Comparison of ^{1}H-NMR spectral data of compound-6 and authentic 2-Methoxy-14-calamenenone.

δ_H of compound-6 (in ppm)	δ_H of 2-Methoxy-14-calamenenone[133] (in ppm)
6.72 (1H, s, H-1)	6.71 (1H, s, H-1)
6.98 (1H, s, H-4)	6.97 (1H, s, H-4)
1.88 ~ 1.98 (2H, m, H-8)	1.88 ~ 1.99 (2H, m, H-8)
2.55 ~ 2.59 (2H, m, H-9)	2.55 ~ 2.59 (2H, m, H-9)
2.30 ~ 2.39 (2H, m, H-10)	2.31 ~ 2.40 (2H, m, H-10)
2.30 ~ 2.39 (2H, m, H-11)	2.31 ~ 2.40 (2H, m, H-11)
0.99 (3H, d, J = 6.5 Hz, H-12)	0.99 (3H, d, J = 6.5 Hz, H-12)
0.76 (3H, d, J = 6.5 Hz, H-13)	0.75 (3H, d, J = 6.5 Hz, H-13)
2.19 (3H, s, H-14)	2.17 (3H, s, H-14)
3.78 (3H, s, CH_3-O-)	3.79 (3H, s, CH_3-O-)

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

Table 2.16. Comparison of ^{13}C-NMR spectral data of compound-6 and authentic 2-Methoxy-14-calamenenone.

Carbon No.	Carbon type (confirmed by DEPT experiment)	δ_C of compound-6 (in ppm)	δ_C of authentic 2-Methoxy-14-calamenenone[133] (in ppm)
1	= CH -	108.9	108.2
2	= C = (quaternary)	156.7	156.7
3	= C = (quaternary)	133.2	133.2
4	= CH -	131.2	131.2
5	= C = (quaternary)	131.5	131.4
6	= CH_2	140.3	140.3
7	OC= (quaternary)	198.7	198.8
8	= CH_2	38.4	38.5
9	= CH_2	24.5	24.6
10	= CH -	44.2	44.2
11	= CH -	31.7	30.5
12	-CH_3	20.7	20.8
13	-CH_3	20.2	20.0
14	-CH_3	14.5	14.9
$\underline{C}H_3$-O-	$\underline{C}H_3$-O-	61.8	61.9

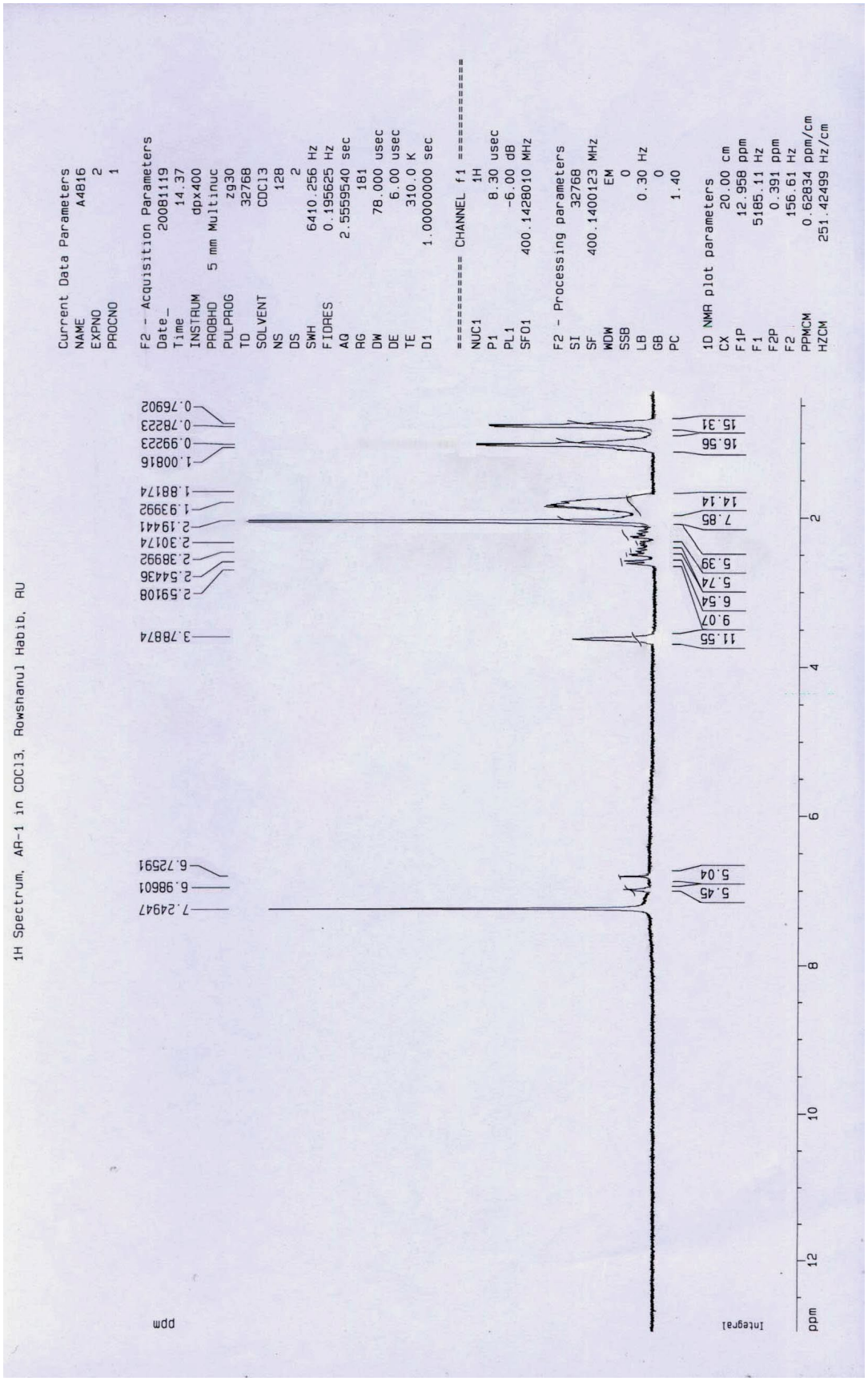


Figure 2.33 ^{1}H-NMR spectrum of compound-6

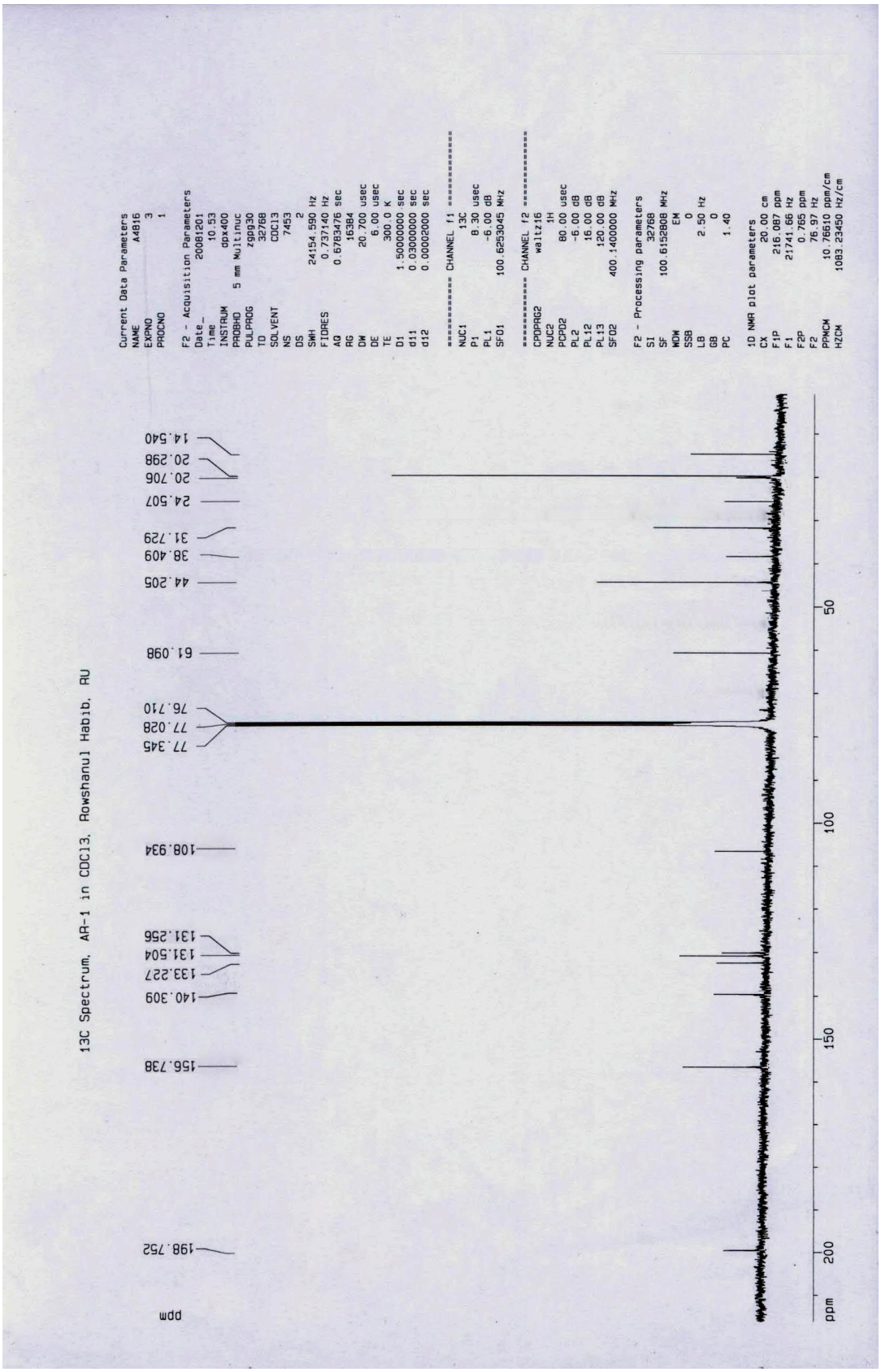


Figure 2.34 ^{13}C-NMR spectrum of compound-6

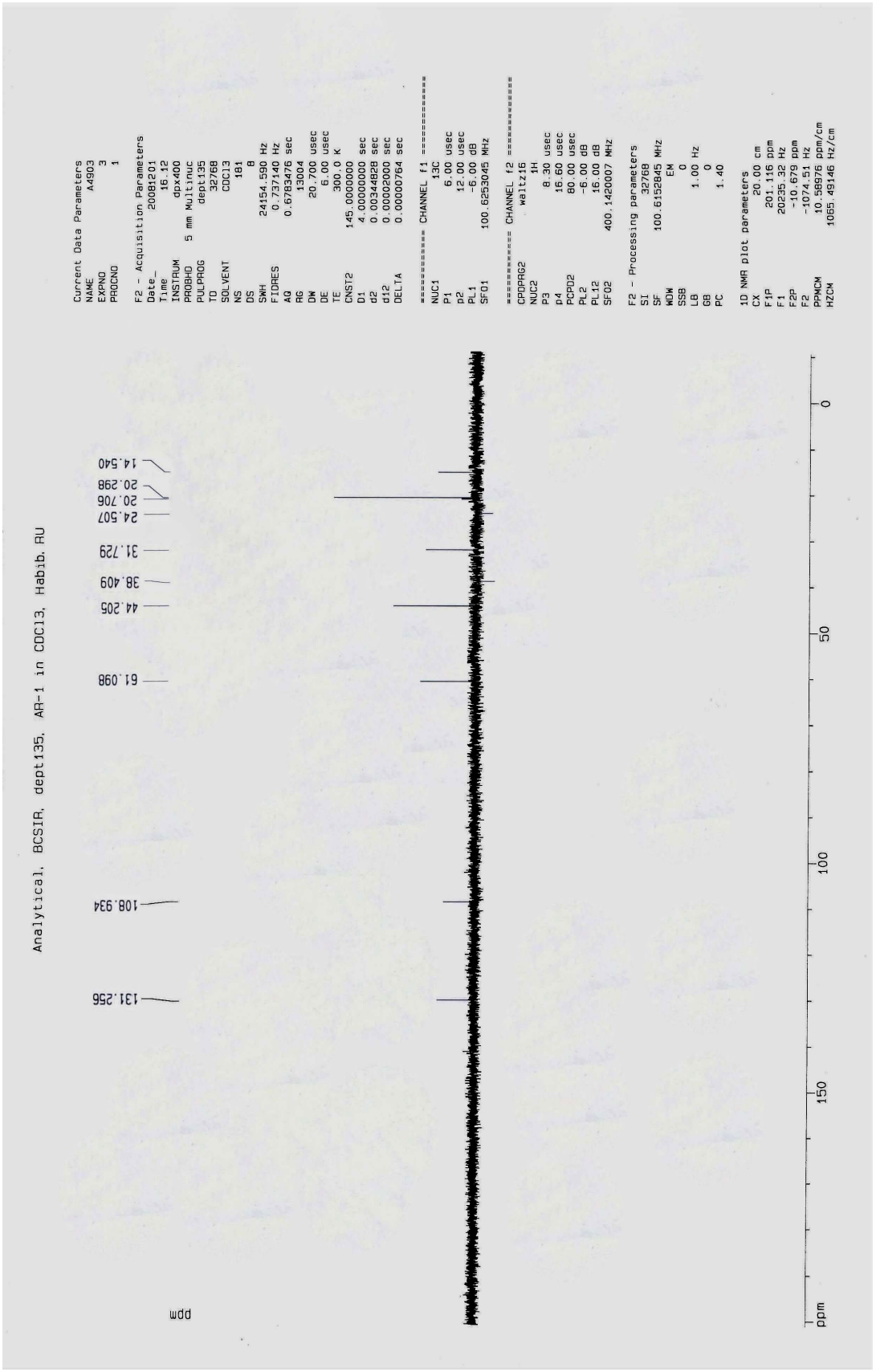


Figure 2.35 DEPT spectrum of compound-6

3.1. Brine shrimp lethality bioassay

3.1.1. Introduction

Plants used in traditional medicine are more likely to yield pharmacologically active compounds. Over the last decade, interest in drugs of plant origin has been growing steadily. The selection of the plant species for screening of bioactive plant constituents, is obviously a crucial factor for the ultimate success of the investigation. The study of bioactive compounds from plant sources and extracts in the chemical laboratory is often hampered by the lack of a suitable, simple, and rapid screening procedure[134-135]. The brine shrimp lethality bioassay in which the simple zoological organism, brine shrimp nauplii (*Artemia salina,* Leach) is used, is efficient, rapid and inexpensive tests[136-137] that correlates reasonably well with cytotoxic and other biological properties[137]. Brine shrimp have been previously utilized in various bioassay systems. There have been many reports on the use of zoological organism for environmental studies[138-139], screening for natural toxins[140] and as a general screening for bioactive substances in plant extracts[136]. Therefore, we have applied the brine shrimp bioassay to screen the cytotoxic activity of isolated compounds and extracts of *Calotropis gigantea* flower and *Amoora rohituka* stem bark.

3.1.2. Materials

The following reagents and equipments were used in brine shrimp lethality bioassay:

i. *Artemia salina*, Leach (brine shrimp eggs)
ii. Sea salt (non-ionized NaCl)
iii. Sodium bicarbonate ($NaHCO_3$) [E-Merck, Germany]
iv. Small tank (to hatch the shrimp)
v. Lamp (to attract the nauplii)
vi. Hatching pump
vii. Pipette (2 mL and 5 mL)
viii. Micropipette (1-100 μL adjustable)
ix. Glass vials (10 mL)
x. Magnifying glass
xi. Test Samples of experimental plants (as shown in table 3.1)

Table 3.1 Test samples of experimental plant

Plant	Test Samples	Measured amount (mg)
Calotropis gigantea (Linn.)	EECF (Ethyl acetate extract of *Calotropis gigantea* flower)	3.0
	Compund-1	3.0
	Compund-2	3.0
	Compund-3	3.0
	Compund-4	3.0
	Compund-5	3.0
Amoora rohituka Roxb.	EAEAR (Ethyl acetate extract of *Amoora rohituka* Roxb. stem bark)	3.0
	DMEAR (Dichloromethane extract of *Amoora rohituka* Roxb. stem bark)	3.0

In our laboratory the effect of methanol extract (ME) of root bark of *Calotropis gigantea* and its petroleum ether (PEF) and chloroform (CF) soluble fractions against brine shrimp nauplii has already been examined and the results of this experiment have been published[141]. In addition, moderate activity of compound-6 against brine shrimp nauplii has also been cited[142]. So ME, PEF, CF and compound-6 were not included in this assay.

3.1.3. Preparation of brine water

38 g of sea-salt (non-ionized sodium chloride; NaCl) was dissolved in one liter of sterilized distilled water and then filtered off to get clear solution. The P^H of the seawater was maintained between 8 and 9 using $NaHCO_3$ solution.

3.1.4. Hatching of brine shrimp eggs

Brine shrimp eggs were collected from a shop of Kawran Bazar, Dhaka. Seawater was taken in the small tank and the shrimp eggs (1.5 g/L) were added to one side of the tank. The eggs were allowed to hatch and mature as nauplii (Larvae) for two days. Constant oxygen supply was carried out during the hatching time. The hatched shrimps were attracted to the lamp on the one side of the tank. These mature nauplli were taken for this bioassay.

3.1.5. Preparation of stock solution for test samples

3.0 mg of each samples (i.e., EECF, EAEAR, DMEAR, compound-1, 2, 3, 4 & 5) was measured and dissolved in 0.6 mL (600 μL) DMSO to get a concentration of 5 mg/mL for each of the sample. These solutions were used as stock solutions.

3.1.6. Preparation of stock solution for positive control

In the present study, ampicillin trihydrate was used as the positive control. 3 mg of ampicillin trihydrate was dissolved in 0.6 mL (600 μL) of DMSO to get a concentration of 5 mg/mL and used as stock solution of ampicillin trihydrate.

3.1.7. Procedure

Five doses (10, 20, 40, 80 and 100 μg/mL) of each sample were used for the lethality test against brine shrimp nauplii. 10, 20, 40, 80 and 100 μL of each sample were transferred from their corresponding stock solution in 5 different glass vials using a micropipette. Brine water was added to each vial making the volume up to 5 mL. During this addition, 10 living nauplii were transferred to each of the vials with the help of a Pasteur pipette. A magnifying glass was used for convenient counting of the nauplii. If the counting of 10 nauplii was not being possible accurately, then a variation in counting from 09-15 might be allowed. After making the volume up to 5 mL, the final concentration of each sample in these vials becomes 10, 20, 40, 80 and 100 μg/mL respectively. For each concentration, solvent control was done. The experiment was repeated three times.

3.1.8. Counting of Nauplii

After 24-hours of incubation, the vials were observed using a magnifying glass and the numbers of survivors in each vial were counted and noted. The percentage of mortality of the nauplii was calculated for each concentration and the LC_{50} values were determined using probit analysis[143].

3.1.9. Results and discussion

In brine shrimp lethality bioassay, all the test samples from the experimental plants (*Calotropis gigantea* and *Amoora rohituka*) showed positive results indicating that these were biologically active. The LC_{50} results of the test samples evaluated in this screening are listed in table 3.2. In comparison to ampicillin trihydrate (LC_{50}: 7.21 μg/mL) used as positive control, the cytotoxicity exhibited by EECF, compound-1, compound-2, compound-5 and DMEAR was promising with the LC_{50} values of 14.61, 9.19, 15.55,

15.26 and 17.67 µg/mL, respectively. On the other hand, compound-3, compound-4 and EAEAR demonstrated moderate activity with the LC_{50} values of 45.46, 30.58 and 26.59 µg/mL, respectively (as shown in table 3.2). Regression equation for each test sample was obtained by plotting empirical probit against the logarithm of the sample concentration (figure 3.1 to 3.9) and after 24 hrs the LC_{50} of each test sample was calculated from the corresponding regression equation[143]. In this bioassay, the mortality rate of brine shrimp nauplii by each test sample was found to increased with the increase in concentration of each sample (figure 3.10 to 3.18).

Table 3.2 Toxicity of crude extracts and isolated compounds of *Calotropis gigantea* and *Amoora rohituka* against brine shrimp nauplii.

Test sample	LC_{50} (µg/mL)	95% Confidence limits (µg/mL)	Regression equation	χ^2 value (Degrees of freedom)
Amphicillin trihydrate	7.21 ± 0.47	4.00 to 13.03	y = 2.213x + 3.117	0.049 (1)
EECF	14.61 ± 0.71	9.64 to 22.16	y = 1.650x + 3.081	0.016 (2)
Compound-1	9.19 ± 0.42	5.01 to 16.85	y = 1.551x + 3.509	0.026 (2)
Compound-2	15.55 ± 0.37	11.65 to 20.76	y = 2.343x + 2.213	0.982 (2)
Compound-3	45.46 ± 0.28	33.09 to 62.44	y = 1.571x + 2.397	2.202 (3)
Compound-4	30.58 ± 0.64	13.03 to 24.42	y = 2.147x + 1.817	3.151 (3)
Compound-5	15.26 ± 0.57	10.87 to 21.42	y = 1.980x + 2.663	1.362 (2)
EAEAR	26.59 ± 0.40	18.44 to 38.33	y = 1.417x + 2.982	1.458 (3)
DMEAR	17.67 ± 0.75	12.44 to 25.10	y = 1.785x + 2.776	1.936 (3)

LC_{50} values are expressed as mean ± S.E.M (Standard error of mean)

From the above results it is evident that EECF, compound-1, compound-2 and compound-5 have potent toxic effects against brine shrimp nauplii and their toxicity is as nearer as ampicillin trihydrate. This result indicates that compound-1 (LC_{50}: 9.19 µg/mL),

compound-2 (LC_{50}: 15.55 µg/mL) and compound-5 (LC_{50}: 15.26 µg/mL) play an important role in the cytotoxicity of EECF (14.61 µg/mL**)**. On the other hand, DMEAR was more active than EAEAR and it can be regarded as a promising candidate for phytochemical and biological analysis.

Several studies have shown that brine shrimp bioassay has been an excellent method to screen the cytotoxic property of medicinal plants and isolated biologically active compounds[144]. More over 300 novel antitumor and pesticidal natural products have now been isolated using this bioassay[145]. In this study, extracts and isolated compounds of flower of *Calotropis gigantea* and stem bark of *Amoora rohituka* was found to be effective against brine shrimp nauplli. So this finding may be a support for using these as larvicide, insecticide and anticancer agent, if further research parameters could be furnished out.

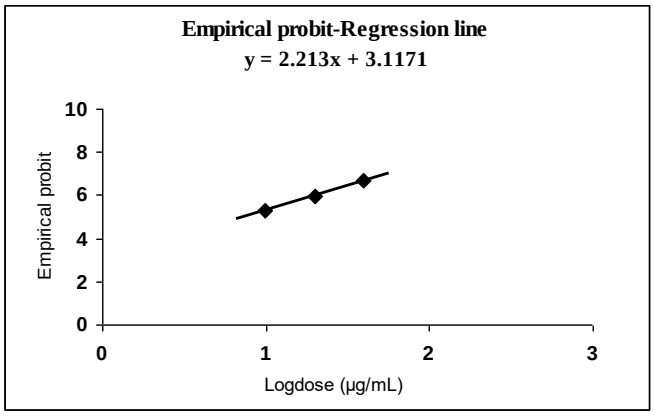


Figure 3.1 Probit mortality line of amphicillin trihydrate against brine shrimp nauplii after 24 hours of exposure.

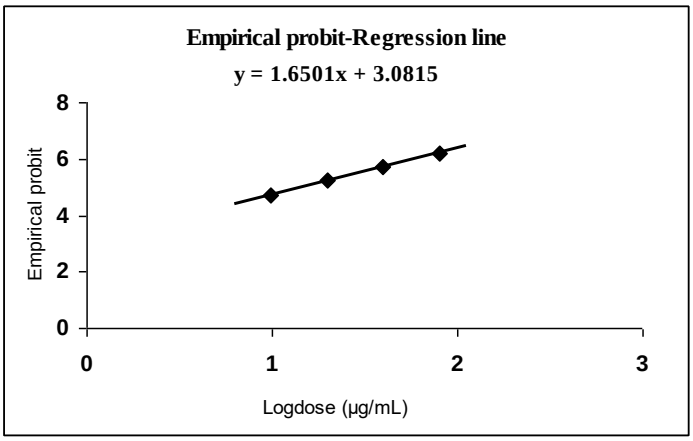


Figure 3.2 Probit mortality line of EECF against brine shrimp nauplii after 24 hours of exposure.

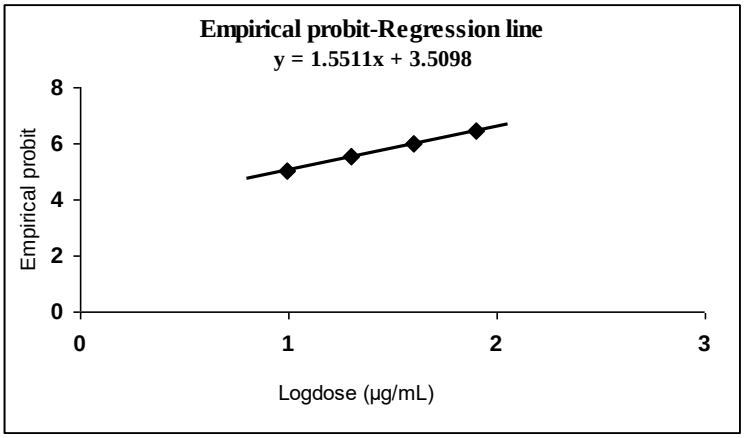


Figure 3.3 Probit mortality line of compound-1 against brine shrimp nauplii after 24 hours of exposure.

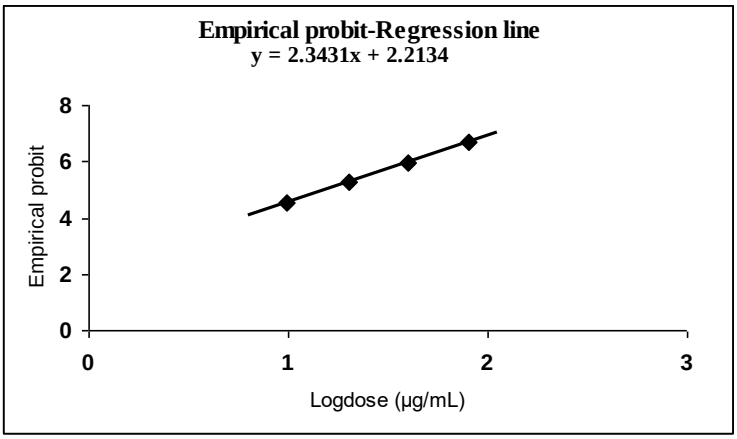


Figure 3.4 Probit mortality line of compound-2 against brine shrimp nauplii after 24 hours of exposure.

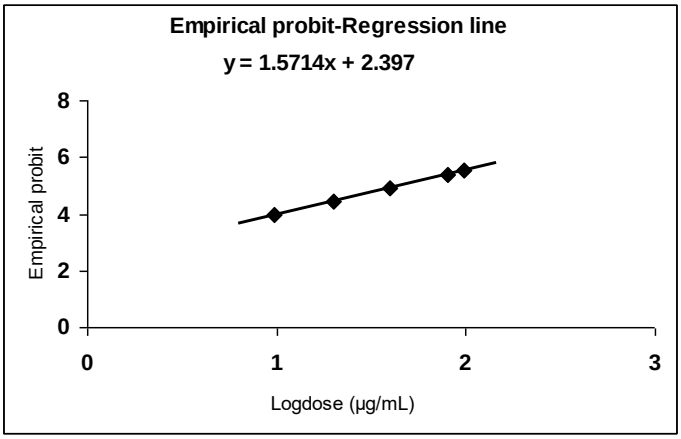


Figure 3.5 Probit mortality line of compound-3 against brine shrimp nauplii after 24 hours of exposure.

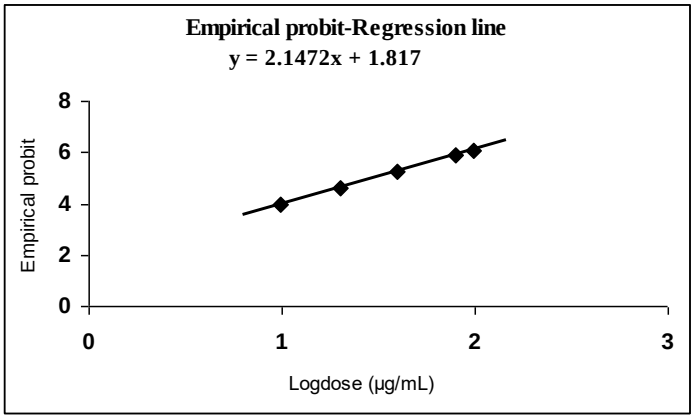


Figure 3.6 Probit mortality line of compound-4 against brine shrimp nauplii after 24 hours of exposure.

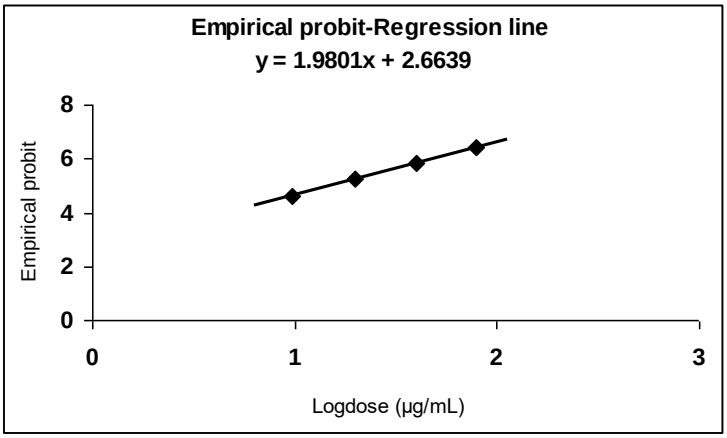


Figure 3.7 Probit mortality line of compound-5 against brine shrimp nauplii after 24 hours of exposure.

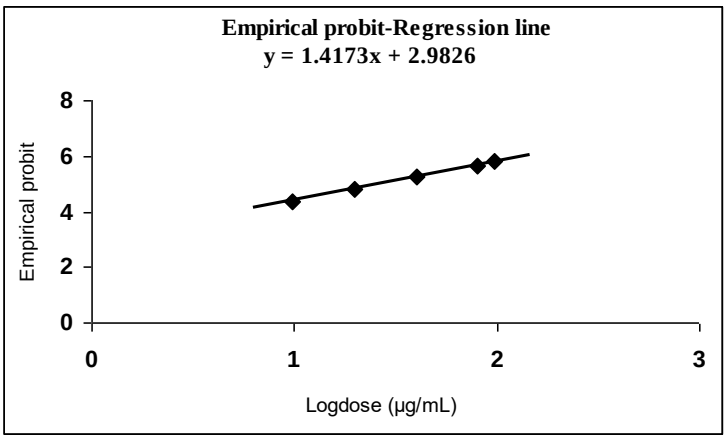


Figure 3.8 Probit mortality line of EAEAR against brine shrimp nauplii after 24 hours of exposure.

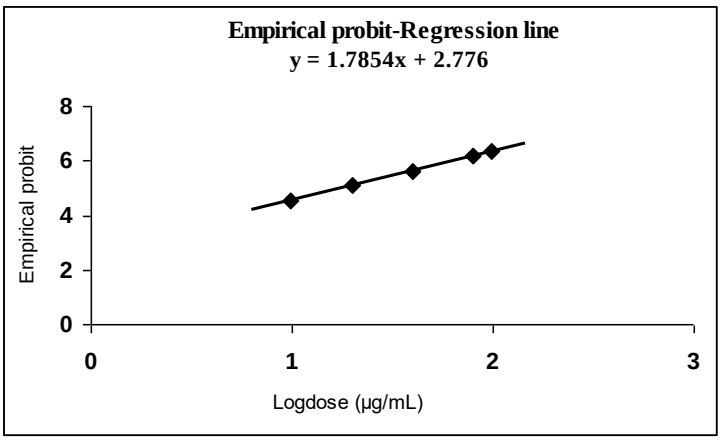


Figure 3.9 Probit mortality line of DMEAR against brine shrimp nauplii after 24 hours of exposure.

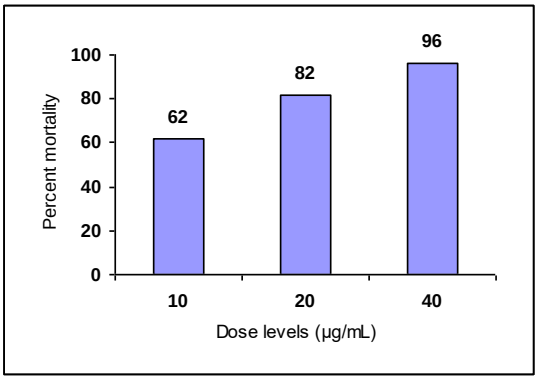


Figure 3.10 Percent mortality of brine shrimp by amphicillin trihydrate after 24 hours of exposure.

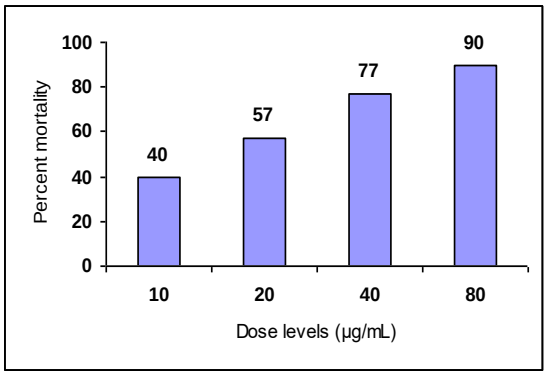


Figure 3.11 Percent mortality of brine shrimp by EECF after 24 hours of exposure.

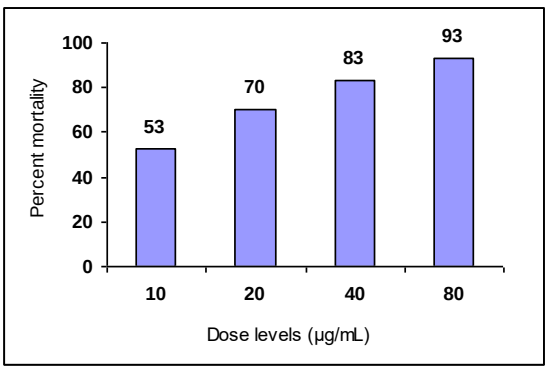


Figure 3.12 Percent mortality of brine shrimp by compound-1 after 24 hours of exposure.

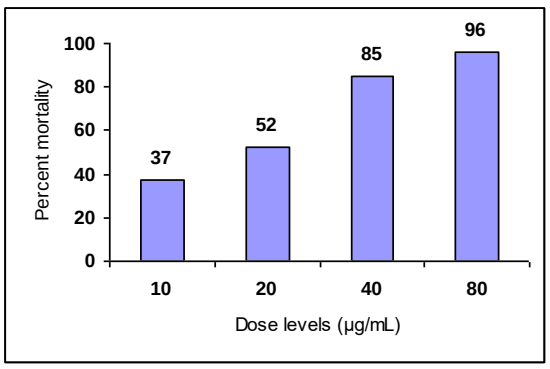


Figure 3.13 Percent mortality of brine shrimp by compound-2 after 24 hours of exposure.

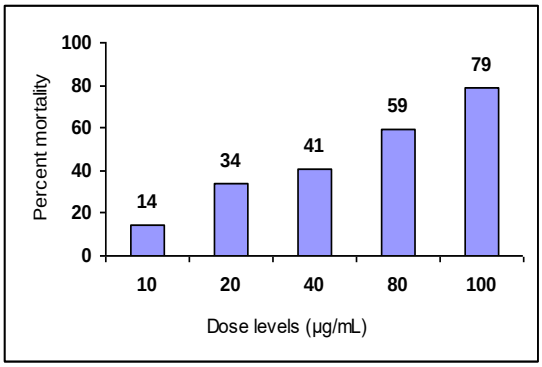


Figure 3.14 Percent mortality of brine shrimp by compound-3 after 24 hours of exposure.

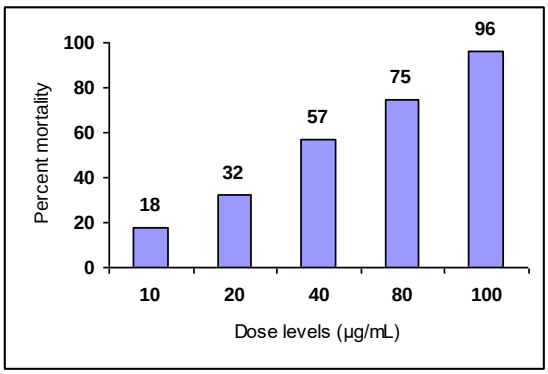


Figure 3.15 Percent mortality of brine shrimp by compound-4 after 24 hours of exposure.

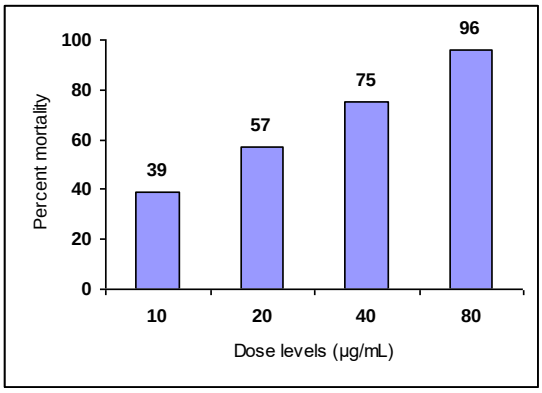


Figure 3.16 Percent mortality of brine shrimp by compound-5 after 24 hours of exposure.

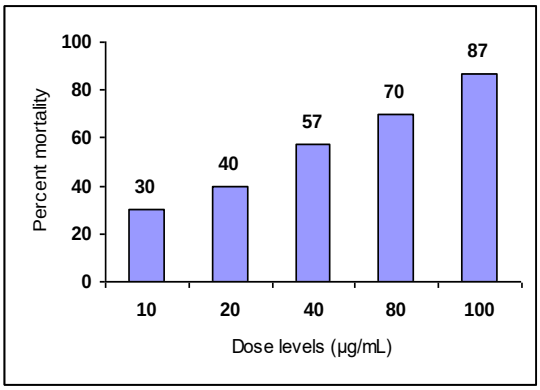


Figure 3.17 Percent mortality of brine shrimp by EAEAR after 24 hours of exposure.

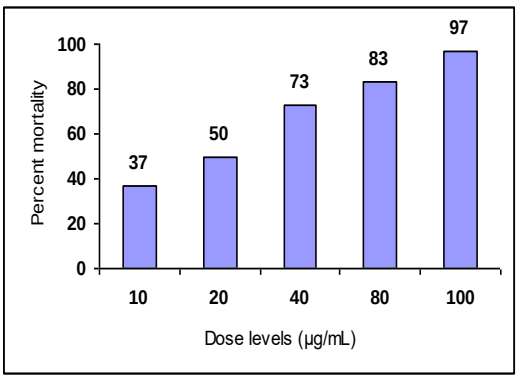
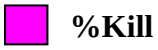


Figure 3.18 Percent mortality of brine shrimp by DMEAR after 24 hours of exposure.



3.2. *In vitro* antimicrobial activity study

3.2.1. Antibacterial and antifungal screening

3.2.1.1 Introduction

During the last decade, development of antibiotic resistance as well as undesirable side effects of some drugs has led to the search for new antimicrobial agents. Many researchers have shown that the plants and their essential oils have antimicrobial activity and other biological effects[146-147]. Therefore, research for development of new antimicrobial agents from plants is an urgent need. Here we have investigated *in vitro* antimicrobial activity of some isolated compounds and extracts from *Calotropis gigantea* flower and *Amoora rohituka* stem bark against some pathogenic bacteria and fungi.

Bayer et al.,[148] published a detailed description of a standardized single-disk method for performing the anti-microbial susceptibility test. It is a widely accepted *in vitro* investigation for preliminary screening of test agents, which may possess antibacterial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method.

In this classical method, samples diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Solutions of known concentrations (µg/ml) of the test samples are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (5 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion. During this time, dried discs absorb water from the surrounding media & then the test materials are dissolved & diffused out of the sample disc. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel[149]. As a result there is a gradual change of test materials concentration in the media surrounding the discs.

The plates are then incubated at 37°C for 24 hours for optimum growth of the micoorganisms. The test materials having antimicrobial property will inhibit microbial growth in the media surrounding the discs to yield a clear distinct area, defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter[148,150].

3.2.1.2. Apparatus and reagents

i) Filter paper discs (sterilized)
ii) Petridishes
iii) Inoculating loop
iv) Sterile cotton
v) Sterile forceps
vi) Spirit burner
vii) Standard disc (Kanamycin-30 µg/disc)
viii) Nutrient Agar Media (Difco)
ix) Nutrient Broth Medium (Difco)
x) Ethanol (95%; E-Merck, Germany)
xi) Bacto peptone (Sigma, India)
xii) Bacto yeast extract (E-Merck, Germany)
xiii) Bacto agar (E-Merck, Germany)
xiv) Shaker (Sigma-Aldrich, USA)
xv) Micropipette (10-100 µL)
xvi) Incubator (OSK-9639A, Japan)
xvii) Nose mask and Hand gloves
xviii) Laminar airflow unit (Bio-Craft & Scientific Industries, India)
xix) Autoclave (ALP Ltd. KT-30L, Tokyo)
xx) Refrigerator (Aritstion, Italy)

3.2.1.3. Test microorganism

Nine pathogenic bacterial strains (four gram positive and five gram negative), and five pathogenic fungal strains were collected from the Institute of Biological Science (IBS), University of Rajshahi, Bangladesh. The pathogenic microorganisms used for this investigation are listed in table 3.3

Table 3.3 List of microorganisms used in antimicrobial assay

Microorganisms	**Strain No.**
Gram positive (+) bacteria	
Staphylococcus aureus	ATCC25923
Bacillus subtilis	QL40
Bacillus megaterium	QL38
Sarcina lutea	QL166
Gram negative (-) bacteria	
Escherichia coli	ATCC27853
Shigella sonnei	C182
Shigella shiga	C180
Shigella dysenteriae	ATCC26131
Pseudomonas aeruginosa	ATCC14228
Fungi	
Aspergillus flavus	ATCC10558
Aspergillus niger	ATCC235561
Aspergillus fumigatus	ATCC10231
Candida albicans	ATCC25889
Fusarium sp	ATCC56390

3.2.1.4. Test samples

The test samples for antimicrobial screening are listed in table 3.4.

Table 3.4. List of test samples of experimental plant

Plant	Test Samples
Calotropis gigantea (Linn.)	EECF (Ethyl acetate extract of *Calotropis gigantea* flower)
	Compund-1
	Compund-2
	Compund-4
	Compund-5
Amoora rohituka Roxb.	EAEAR (Ethyl acetate extract of *Amoora rohituka* Roxb. stem bark)
	DMEAR (Dichloromethane extract of *Amoora rohituka* Roxb. stem bark)

In our laboratory, the antimicrobial effect of the methanol extract (ME) of root bark of *Calotropis gigantea* and its petroleum ether (PEF) and chloroform (CF) soluble fractions have been evaluated and the results of this investigation have already been published[141]. In addition, the antimicrobial efficacy of compound-3 (identified here as taraxasterol acetate) and compound-6 (identified here as 2-methoxy-14-calamenenone) has also been reported[142,151]. So ME, PEF, CF, compound-3 and compound-6 were not included in this assay.

3.2.1.5. Composition and preparation of culture medium

For demonstrating the antibacterial activity and subculture of the test organisms, nutrient agar media and nutrient broth media were used. The composition of these two media is given in table 3.5

Specified amount of each component for a particular required volume of nutrient agar or nutrient broth was measured and taken in a conical flask. Then distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The pH (at 25 °C) was adjusted at 7.2-7.6 using NaOH or HCl[152].

For antifungal activity test and subculture of the test fungi, potato dextrose agar (PDA) medium was used. To prepare potato dextrose agar medium, 200 g of peeled and sliced potatoes were boiled in 1 liter of water until the potatoes were soft. Then it was

strained through cheesecloth and adjusted the filtrate to 1 liter with adding distilled water. Finally 20 g dextrose and 20 g agar powder were added to it and the medium was sterilized by autoclaving to use it in antifungal activity test[153].

Table 3.5 Composition of nutrient agar & nutrient broth medium per 100 mL

Ingredients	Amounts
Nutrient agar medium	
Bacto peptone	0.5 g
Sodium chloride	0.5 g
Bacto yeast extract	1.0 g
Bacto agar	2.0 g
Distilled water	100 mL
Nutrient broth medium	
Bacto peptone	0.5 g
Sodium chloride	0.5 g
Bacto yeast extract	1.0 mg
Distilled water	100 mL

3.2.1.6. Sterilization procedure

To avoid any type of contamination the antimicrobial screening was carried out in laminar airflow unit maintaining all types of precautions. UV light was switched on one hour earlier of the start of the experiment to avoid contamination in the laminar airflow unit. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121°C and a pressure of 15 lb/sq inch for 30 minutes. Blank discs, micropipette tips, cotton, forceps were also sterilized and kept in laminar hood under UV light for 30 minutes before starting the experiment.

3.2.1.7. Preparation of fresh culture

The nutrient agar medium was prepared and dispersed in a number of clean test tubes to prepare slants (5 mL in each test tube). The test tubes were plugged with cotton and sterilized for 30 minutes. After sterilization, the test tubes were kept in an inclined position (45^{o}) for solidification. These were then incubated at 37.5^{o}C to ensure sterilization. The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in an aseptic condition. The loop was red heated carefully after each transfer of microorganism to avoid contamination. The

inoculated slants were then incubated at 37.5ºC for 24 hours to assure the growth of test organisms. These fresh cultures were used for preparation of culture in nutrient broth medium.

3.2.1.8. Preparation of culture in nutrient broth medium for antibacterial screening

500 mL nutrient broth medium was prepared and then uniformly distributed in nine 100 mL conical flasks. The conical flasks were plugged with cotton and sterilized for 30 minutes. After sterilization, these flasks were placed in laminar airflow unit. The test microorganisms were transferred to these liquid medium from the fresh cultures with the help of an inoculating loop in an aseptic condition. The loop was red heated carefully after each transfer of microorganism to avoid contamination. The inoculated cultures were then incubated on a shaker at 37.5ºC for 24 hours to assure the growth of test organisms. These liquid cultures were used for the sensitivity test.

3.2.1.9. Preparation of the test plates for antibacterial screening

The test plates for antibacterial screening were prepared according to the following procedure:

(i) A number of petridishes and nutrient agar media were sterilized in an autoclave for 30 minutes and were transferred into laminar airflow unit.

(ii) Nutrient agar media was then transferred to the sterile petridishes in aseptic condition. The media were poured into petridishes in such a way as to give a uniform depth of approximately 4 mm. The petridishes were rotated clockwise and anticlockwise for several times.

(iii) 200 µL of cultured test organism in nutrient broth media was spread on the surface of solid nutrient agar media and kept preserved for applying of sample and standard discs.

3.2.1.10. Preparation of the test plates for antifungal screening

For antifungal screening, the test plates were prepared according to the following procedure:

(i) About 10 mL in quantity of distilled water was poured in several clean test tubes and plugged with cotton.

(ii) The test tubes, a number of petridishes, glass rods, a piece of cotton and the potato dextrose agar medium were sterilized by autoclave and then transferred to the laminar air flow cabinet.

(iii) About 6 ml of the medium was poured carefully in each petridishes. The petridishes were rotated clockwise and anticlockwise for several times to assure homogenous thickness of the medium and allowed to cool and solidify at about 30°C.

(iv) The test tubes containing distilled water were inoculated with fresh culture of the test fungi and were shaken gently to form a uniform suspension of the microorganism because of their high prevalence of sporulation process.

(v) A piece of cotton was immersed in the test tubes with the help of individual glass rod and then gently rubbed the medium and the cotton was discarded.

(vi) Finally, the plates were kept preserved for applying of sample and standard discs.

3.2.1.11. Preparation of disc

Three types of discs were used for antimicrobial screening.

A) Sample discs:

The antibacterial activity of EECF, compound-1, compound-2, compound-4 and compound-5 was tested against each bacterium at the concentrations of 30 μg/disc, 60 μg/disc and 90 μg/disc whereas EAEAR and DMEAR were tested against each bacterium at the concentrations of 30 μg/disc, 100 μg/disc and 200 μg/disc.

To prepare sample disc for antimicrobial screening, sterilized filter paper (BBL, U.S.A) were folded into three layers. Then it was cut rounded (5 mm in diameter) with the help of a punch machine. Then discs were taken in a blank petridish and sterilized before use.

In antibacterial activity test, 1.5, 3 and 4.5 mg of each EECF, compound-1, compound-2, compound-4 and compound-5 were measured and dissolved in 0.5 mL chloroform in separate glass vials to get the concentrations of 3 μg/ μL, 6 μg/μL and 9 μg/ μL for each sample. For EAEAR and DMEAR, 1.5, 5 and 10 mg of each extract were dissolved in 0.5 mL chloroform in separate glass vial to get the concentrations of 3 μg/

μL, 10 μg/ μL and 20 μg/ μL for each sample. Then 10 μl of desired concentration was applied on the discs with the help of a micropipette in an aseptic condition.

For antifungal activity screening, EECF, compound-1 and compound-2 were tested at the concentrations of 100 μg/disc, 200 μg/disc and 400 μg/disc whereas EAEAR and DMEAR were tested at the concentration of 100 μg/disc, 200 μg/disc and 300 μg/disc. In antifungal assay 2, 4 and 8 mg of each EECF, compound-1 and compound-2 were dissolved in 0.2 mL chloroform to concentrations of 10 μg/μL, 20 μg/μL and 40 μg/μL for each sample. For EAEAR and DMEAR, 2, 4 and 6 mg of each extract were dissolved in 0.2 mL to get the concentrations of 10 μg/μL, 20 μg/μL and 30 μg/μL. Then 10 μl of desired concentration was applied on the discs with the help of a micropipette in an aseptic condition.

B) Standard discs:

Kanamycin disc (30 μg/disc) and *Nystatin* disc (100 μg/disc) were used as positive antibacterial and antifungal control, respectively, to ensure the activity of standard antibiotic against the test microorganisms as well as for the comparison with the response produced by the test samples.

C) Solvent control discs:

These were prepared using similar filter paper (5 mm in diameter) and same volume of residual solvent without sample following the same process and condition. These were used as negative control to ensure that the residual solvent and the filter paper themselves was not active.

3.2.1.12. Placement of the discs and incubation

The following procedure was adopted for the placement of the discs:

(i) The sample discs, standard discs and solvent control discs were placed gently on the solidified agar plates seeded with the test organisms with the help of a pair of sterile forceps to ensure contact with the medium.

(ii) Then the plates were kept in a refrigerator at 4ºC for 24 hours in order to provide sufficient time to diffuse the materials into the surrounding medium.

(iii) Finally, the plates were incubated at 37.5ºC for 24 hours in an incubator.

3.2.1.13. Precaution

The discs were placed in such way that they were separated from each other about 25 mm and 15 mm far from the edge of the plate to prevent the overlapping of zones of inhibition.

3.2.1.14. Measurement of the zones of inhibition

The antimicrobial potency of the test samples was determined by measuring the diameter of the zones of inhibition in millimeter resulted by the microbial growth inhibitory activity of test samples surrounding the discs.

3.2.1.15. Results and discussion

In vitro antibacterial activity study, both EECF and compound-1 showed a better broad spectrum of antibacterial activity against both Gram positive (*Staphylococcus aureus*, *Bacillus subtilis*, and *Sarcina lutea*) and Gram negative (*Escherchia coli*, *Shigella sonnei*, *Shigella shiga* and *Shigella dysenteriae*) bacteria, with inhibition zones in the range of 07-20 mm (Table 3.6 and figure 3.19 − 3.33). Although EECF showed activity, compound-1 was inactive against *Bacillus megaterium* (Table 3.6). Compound-2 showed moderate activity against *Staphylococcus aureus*, *Sarcina lutea* and *Escherchia coli* (Figure 3.34 − 3.36). It produced inhibition zone ranging from 08 to 15 mm (Table 3.6).

In antibacterial study, the efficacy of compound-4 and compound-5 to inhibit the growth of gram positive bacteria and gram negative bacteria is shown in table 3.7 and figure 3.37 − 3.42. Compound-5 moderately inhibited the growth of *Escherchia coli*, *Shigella dysenteriae, Shigella shiga* and *Shigella sonnei* with the zone of inhibition in the range 06 to 14 mm. Compound-4 showed mild activity against *Staphylococcus aureus* and *Escherichia coli* (Figure 3.37 & 3.38) and produced zone of inhibition between 06 to 10 mm.

The pattern of above results indicated that compound-1 had a potential contribution on the antibacterial activity of EECF and the intensity of activity was found in the order of EECF > compound-1 > compound-5 > compound-2 > compound-4. The similar type of results has been reported by Hayet *et al.*[154] where they evaluated antimicrobial activity of *Salvia sclarea*. In addtition, Sastry and Rao[155] showed the activity of Di-(2-ethylhexyl) phthalate against *Proteus vulgaris*, *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella typhimurium* and *Pseudomonas aurioginosa.* The present study revealed the antishigellosis activity of compound-1 [Di-(2-ethylhexyl) phthalate] because compound-1 had better activity against *Shigella shiga*, *Shigella sonnei* and *Shigella dysenteriae* (Table 3.6).

As shown in table 3.8, EAEAR moderately inhibited the growth of *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus* and *Escherchia coli* with the zone of inhibition in the range 07 to 24 mm (Figure 3.43 − 3.46). DMEAR showed activity

against *Shigella sonnei*, *Shigella shiga* and *Pseudomonas aeruginosa* (Figure 3.47 − 3.49) and produced zone of inhibition between 08 to 18 mm (Table 3.8). The presence of terpenoids, flavonoids and glycosides like chemicals in crude extract plays an important role for producing antimicrobial activity[156-158]. *Amoora rohituka* stem bark contained limonoids with antimicrobial activity[99] and here TLC screening also showed the presence of steroid, flavonoid, triterpene and glycoside type compounds on EAEAR and DMEAR. In addition, this study identified a sesquiterpene as 2-methoxy-14-calamenenone (compound-6) in DMEAR that have previously reported antimicrobial activity[142]. So these chemicals may be responsible for producing antimicrobial activity of EAEAR and DMEAR.

In antifungal activity test, EECF produced zone of inhibition between 07 to 15 mm against *Aspergillus flavus* and *Aspergillus fumigatus* (Table 3.9 and figure 3.50 −3.51) whereas compound-1 exhibited activity against *Aspergillus flavus* (Table 3.9 and figure 3.50). Compound-2 had no antifungal activity. In antifungal screening both EAEAR and DMEAR were active against *Aspergillus niger* and *Candida albican* (Table 3.10 and figure 3.52 −3.53).

In this study it was found that the activity of each extract and compound against bacteria and fungi was increased with increasing concentration of the sample and this finding was consistence with the results found in case of antimicrobial activity of *Dendranthema zawadskii*[159].

Table 3.6 *In vitro* antibacterial activity of EECF, compound-1 and compound-2.

Microorganisms	Zone of Inhibition									
	EECF			Compound-1			Compound-2			*Kanamycin*
	Dose (µg/disc)									
	30	60	90	30	60	90	30	60	90	30
Staphylococcus aureus	08 ± 1.0	11 ± 0.5	15 ± 0.5	09 ± 0.3	12 ± 0.6	14 ± 0.5	10 ± 0.6	12 ± 0.3	15 ± 0.6	31 ± 1.0
Bacillus subtilis	10 ± 1.7	16 ± 0.8	18 ± 1.0	13 ± 0.6	16 ± 0.5	18 ± 0.6	R	R	R	27 ± 1.0
Bacillus megaterium	07 ± 0.6	09 ± 0.7	12 ± 1.1	R	R	R	R	R	R	26 ± 1.1
Sarcina lutea	12 ± 0.6	16 ± 0.6	19 ± 0.6	12 ± 0.6	16 ± 0.3	20 ± 0.5	08 ± 0.6	11 ± 0.5	13 ± 1.0	27 ± 0.6
Escherchia coli	13 ± 1.1	17 ± 0.6	20 ± 0.8	10 ± 0.3	13 ± 0.5	15 ± 0.6	09 ± 0.6	11 ± 0.7	14 ± 0.6	32 ± 1.1
Shigella sonnei	10 ± 0.6	14 ± 0.5	16 ± 0.6	11 ± 0.6	13 ± 1.0	17 ± 0.6	R	R	R	29 ± 0.6
Shigella shiga	08 ± 1.0	10 ± 0.8	20 ± 0.6	12 ± 1.1	15 ± 0.6	19 ± 0.3	R	R	R	31 ± 1.7
Shigella dysenteriae	09 ± 0.6	12 ± 0.3	14 ± 0.5	07 ± 0.6	10 ± 0.6	13 ± 1.0	R	R	R	28 ± 1.5

Data are expressed as mean ± S.E.M (Standard error of mean); R = Resistance

Table 3.7 *In vitro* antibacterial activity of compound-4 and compound-5.

Microorganisms	Zone of Inhibition						
	Compound-4			Compound-5			*Kanamycin*
	Dose (µg/disc)						
	30	60	90	30	60	90	30
Staphylococcus aureus	06 ± 0.3	08 ± 0.6	10 ± 0.9	R	R	R	29 ± 0.3
Bacillus subtilis	R	R	R	R	R	R	31 ± 0.6
Sarcina lutea	R	R	R	R	R	R	28 ± 0.5
Escherchia coli	R	07 ± 0.5	08 ± 0.3	09 ± 0.6	11 ± 0.3	14 ± 1.1	26 ± 1.1
Shigella dysenteriae	R	R	R	07 ± 0.6	09 ± 1.0	11 ± 0.6	27 ± 0.6
Shigella shiga	R	R	R	06 ± 0.3	08 ± 0.5	10 ± 0.6	29 ± 0.3
Shigella sonnei	R	R	R	R	08 ± 1.1	12 ± 0.6	30 ± 0.5

Data are expressed as mean ± S.E.M (Standard error of mean); R = Resistance

Table 3.8 *In vitro* antibacterial activity of EAEAR and DMEAR.

Microorganisms	Zone of Inhibition						
	EAEAR			DMEAR			*Kanamycin*
	Dose (µg/disc)						
	30	100	200	30	100	200	30
Bacillus subtilis	11 ± 0.5	17 ± 0.6	24 ± 0.9	R	R	R	32 ± 0.8
Bacillus megaterium	08 ± 1.0	14 ± 0.5	19 ± 0.3	R	R	R	31 ± 0.6
Staphylococcus aureus	07 ± 0.6	10 ± 0.6	14 ± 0.6	R	R	R	30 ± 0.5
Escherchia coli	R	09 ± 0.5	13 ± 0.6	R	R	R	29 ± 1.1
Shigella shiga	R	R	R	08 ± 0.3	10 ± 0.6	15 ± 0.5	30 ± 1.0
Shigella sonnei	R	R	R	10 ± 1.7	13 ± 0.5	16 ± 0.5	28 ± 1.5
Pseudomonas aeruginosa	R	R	R	12 ± 0.6	15 ± 0.5	18 ± 0.9	31 ± 1.0

Data are expressed as mean ± S.E.M (Standard error of mean); R = Resistance

Table 3.9 *In vitro* antifungal activity of EECF, compound-1 and compound-2.

Microorganisms	Zone of inhibition									
	EECF			Compound -1			Compound-2			*Nystatin*
	Dose (µg/disc)									
	100	200	400	100	200	400	100	200	400	100
Aspergillus flavus	10 ± 0.8	12 ± 0.3	15 ± 0.7	08 ± 0.3	11 ± 0.6	13 ± 0.6	R	R	R	26 ± 1.1
Aspergillus fumigatus	07 ± 0.6	09 ± 0.3	12 ± 0.3	R	R	R	R	R	R	30 ± 1.0
Aspergillus niger	R	R	R	R	R	R	R	R	R	25 ± 0.6
Fusarium sp	R	R	R	R	R	R	R	R	R	31 ± 0.3

Data are expressed as mean ± S.E.M (Standard error of mean); R = Resistance

Table 3.10 *In vitro* antifungal activity of EAEAR and DMEAR.

Microorganisms	Zone of inhibition						
	EAEAR			DMEAR			*Nystatin*
	Dose (µg/disc)						
	100	200	300	100	200	300	100
Aspergillus flavus	R	R	R	R	R	R	32 ± 1.2
Aspergillus niger	10 ± 0.8	15 ± 0.7	18 ± 0.6	R	11 ± 0.5	16 ± 1.0	30 ± 1.0
Aspergillus fumigatus	R	R	R	R	R	R	31 ± 1.7
Candida albicans	09 ± 0.3	13 ± 1.1	16 ± 0.8	08 ± 0.5	10 ± 0.3	13 ± 0.7	31 ± 0.8
Fusarium sp	R	R	R	R	R	R	28 ± 1.2

Data are expressed as mean ± S.E.M (Standard error of mean); R = Resistance

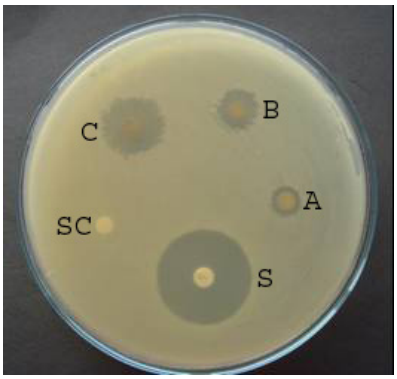


Figure 3.19 Antibacterial effect of EECF against *Staphylococcus aureus*

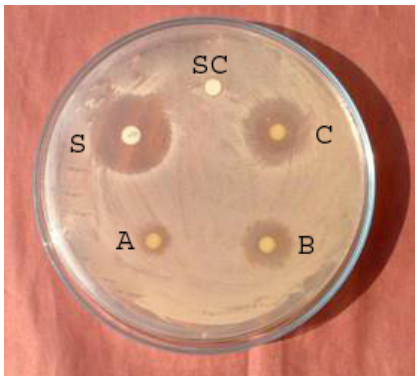


Figure 3.20 Antibacterial effect of EECF against *Bacillus subtilis*

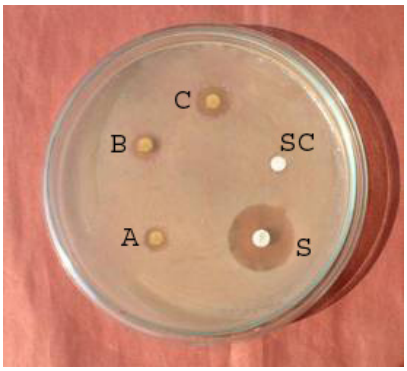


Figure 3.21 Antibacterial effect of EECF against *Bacillus megaterium*

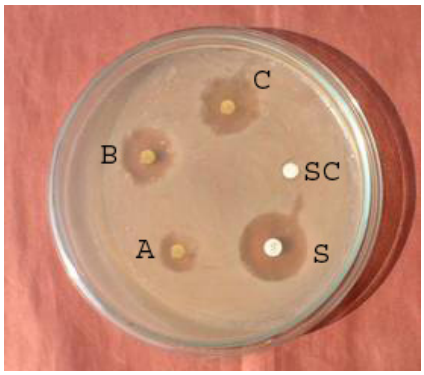


Figure 3.22 Antibacterial effect of EECF against *Sarcina lutea*

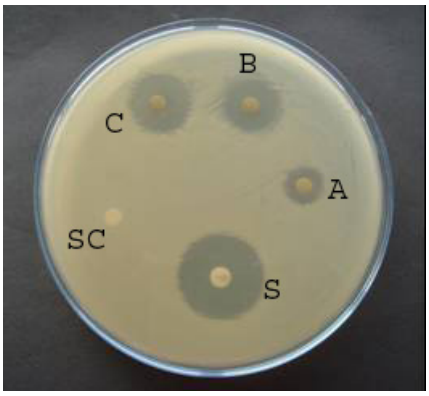


Figure 3.23 Antibacterial effect of EECF against *Escherchia coli*

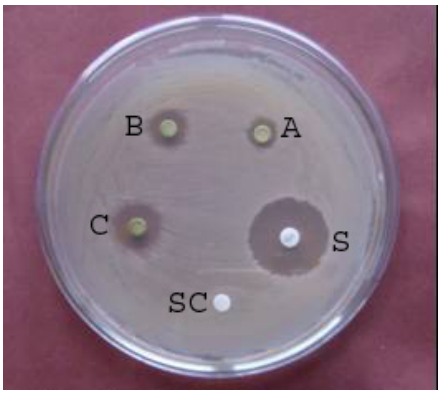


Figure 3.24 Antibacterial effect of EECF against *Shigella sonnei*

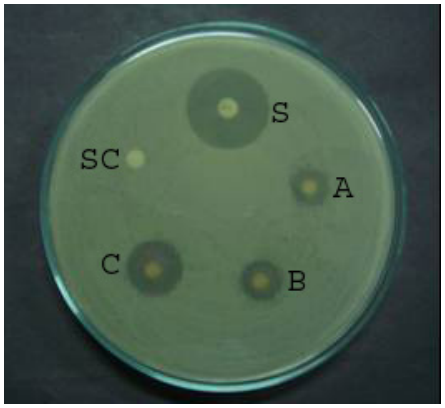


Figure 3.25 Antibacterial effect of EECF against *Shigella shiga*

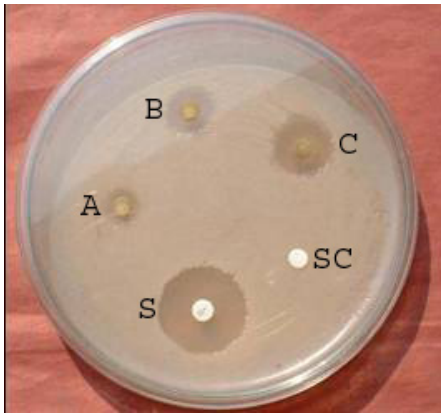


Figure 3.26 Antibacterial effect of EECF against *Shigella dysenteriae*

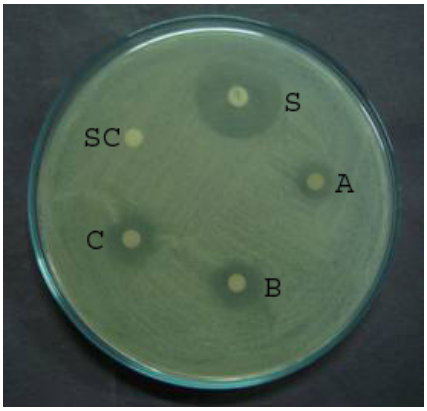


Figure 3.27 Antibacterial effect of compound-1 against *Staphylococcus aureus*

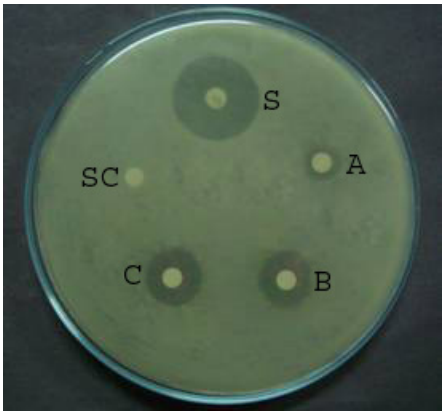


Figure 3.28 Antibacterial effect of compound-1 against *Bacillus subtilis*

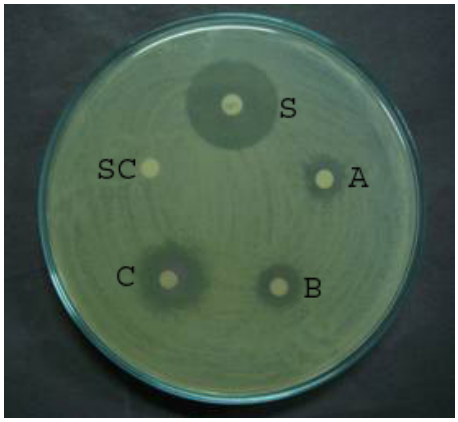


Figure 3.29 Antibacterial effect of compound-1 against *Sarcina lutea*

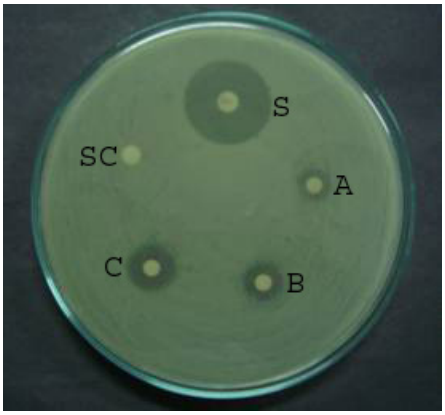


Figure 3.30 Antibacterial effect of compound-1 against *Escherchia coli*

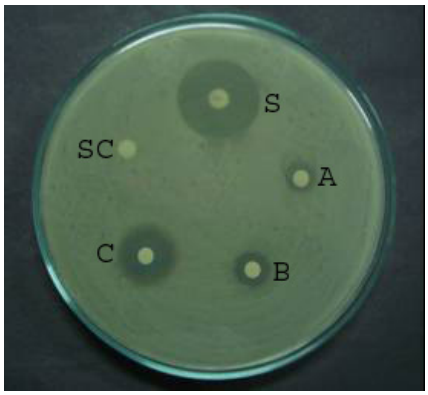


Figure 3.31 Antibacterial effect of compound-1 against *Shigella sonnei*

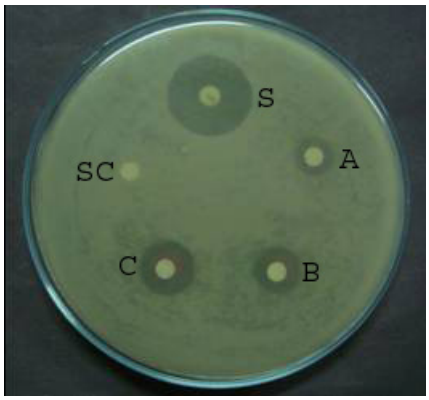


Figure 3.32 Antibacterial effect of compound-1 against *Shigella shiga*

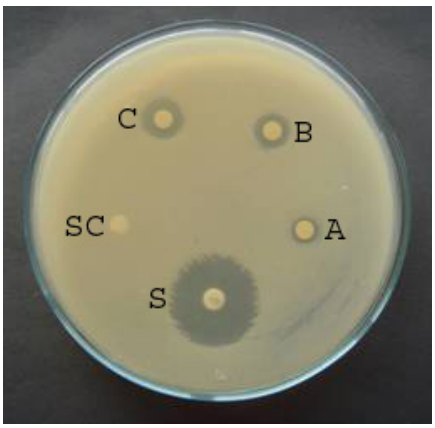


Figure 3.33 Antibacterial effect of compound-1 against *Shigella dysenteriae*

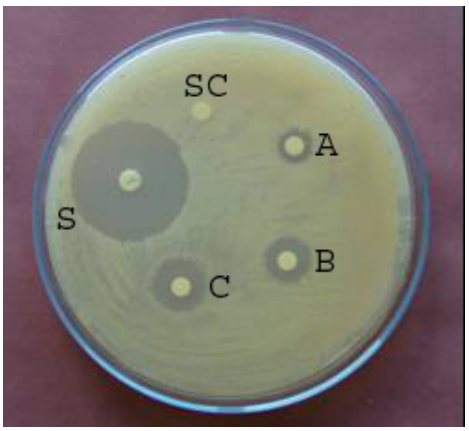


Figure 3.34 Antibacterial effect of compound-2 against *Sarcina lutea*

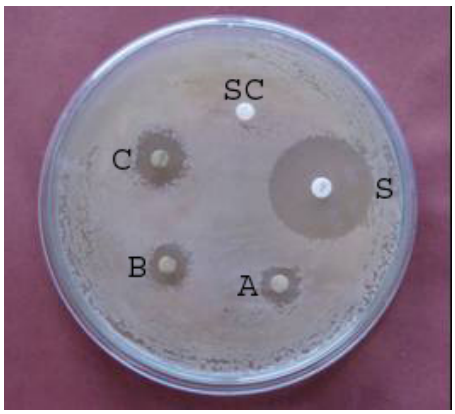


Figure 3.35 Antibacterial effect of compound-2 against *Staphylococcus aureus*

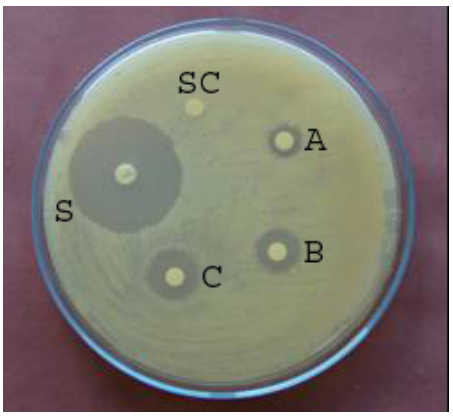


Figure 3.36 Antibacterial effect of compound-2 against *Escherchia coli*

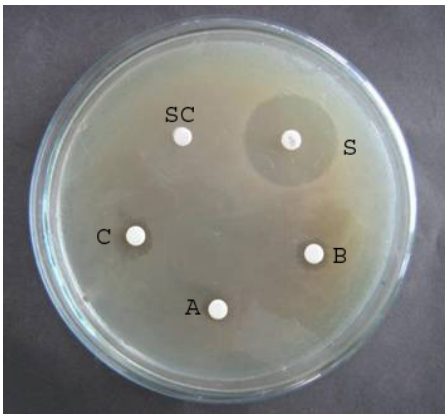


Figure 3.37 Antibacterial effect of compound-4 against *Staphylococcus aureus*

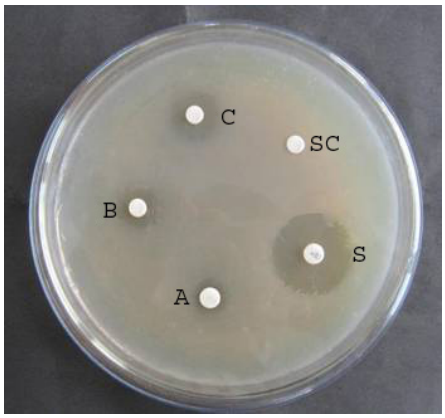


Figure 3.38 Antibacterial effect of compound-4 against *Escherchia coli*

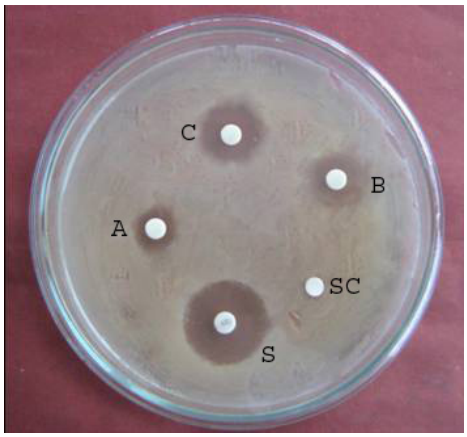


Figure 3.39 Antibacterial effect of compound-5 against *Escherchia coli*

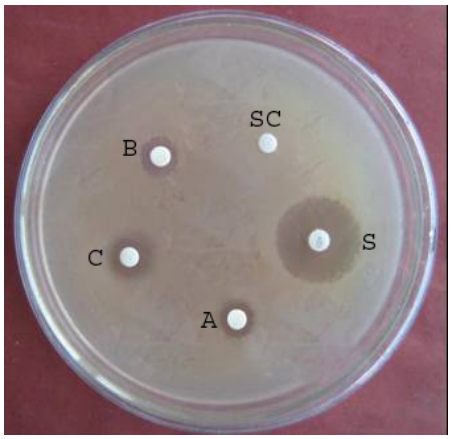


Figure 3.40 Antibacterial effect of compound-5 against *Shigella shiga*

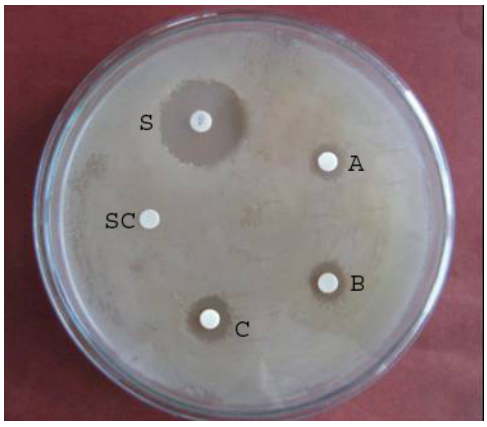


Figure 3.41 Antibacterial effect of compound-5 against *Shigella dysenteriae*

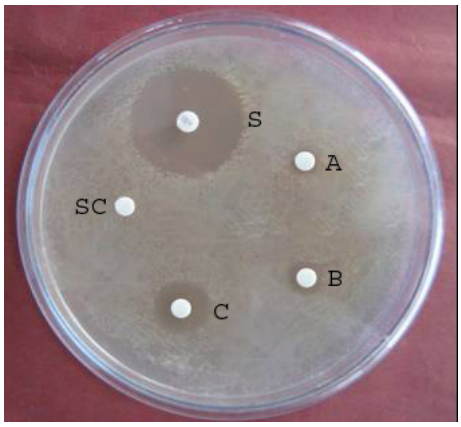


Figure 3.42 Antibacterial effect of compound-5 against *Shigella sonnei*

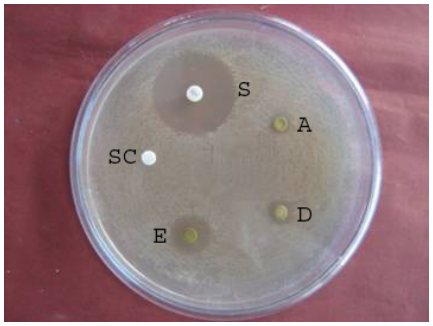


Figure 3.43 Antibacterial effect of EAEAR against *Staphylococcus aureus*

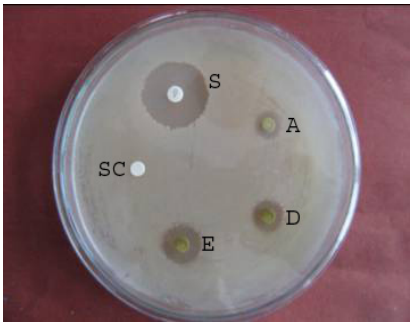


Figure 3.44 Antibacterial effect of EAEAR against *Bacillus subtilis*

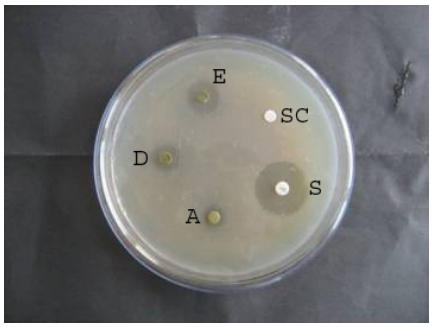


Figure 3.45 Antibacterial effect of EAEAR against *Bacillus megaterium*

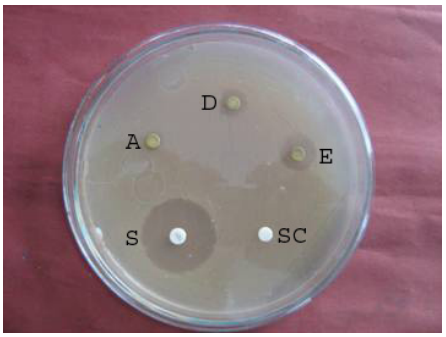


Figure 3.46 Antibacterial effect of EAEAR against *Escherchia coli*

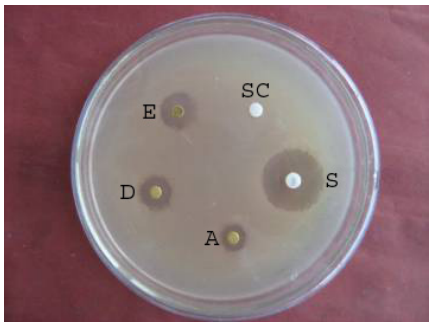


Figure 3.47 Antibacterial effect of DMEAR against *Shigella shiga*

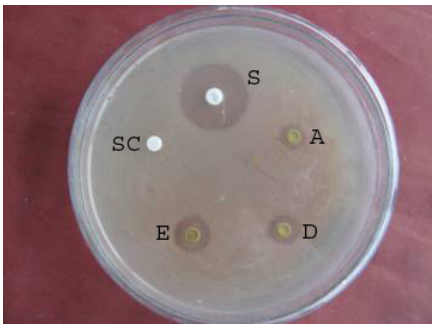


Figure 3.48 Antibacterial effect of DMEAR against *Shigella sonnei*

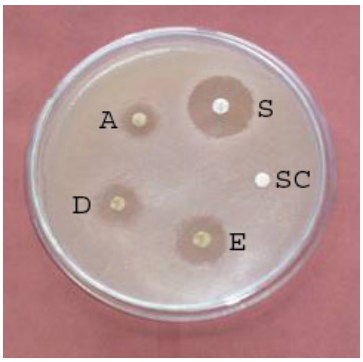


Figure 3.49 Antibacterial effect of DMEAR against *Pseudomonas aeruginosa*

A = 30 µg/disc;
B = 60 µg/disc;
C = 90 µg/disc;
D = 100 µg/disc;
E = 200 µg/disc ;
SC = Solvent Control
S = Kanamycin (30 µg/disc);

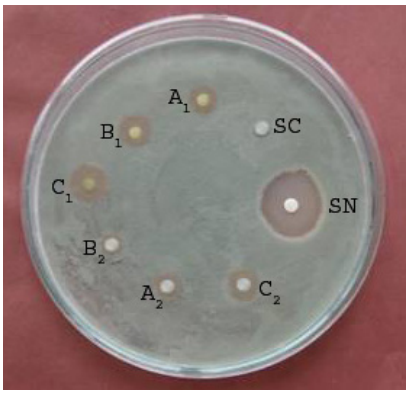


Figure 3.50 Antifungal effect of EECF and compound-1 against *Aspergillus flavus*

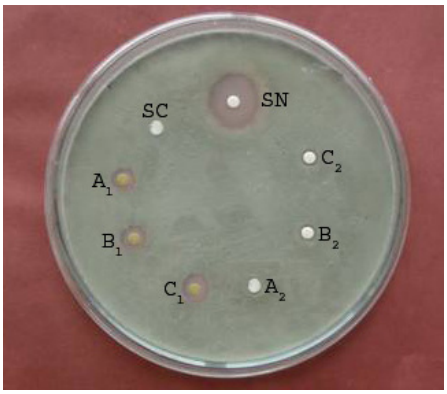


Figure 3.51 Antifungal effect of EECF and compound-1 against *Aspergillus fumigatus*

Figure 3.52 Antifungal effect of EAEAR and DMEAR against *Aspergillus niger*

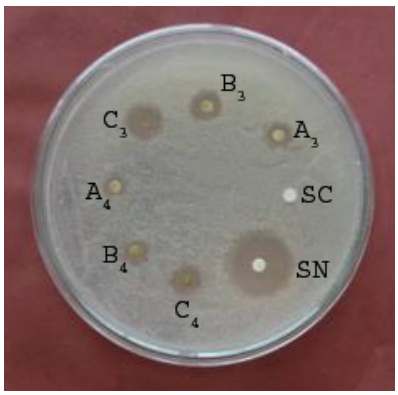


Figure 3.53 Antifungal effect of EAEAR and DMEAR against *Candida albican*

A_1 = EECF (100 µg/disc)
B_1 = EECF (200 µg/disc)
C_1 = EECF (400 µg/disc)
A_2 = Compound-1 (100 µg/disc)
B_2 = Compound-1 (200 µg/disc)
C_2 = Compound-1 (400 µg/disc)
A_3 = EAEAR (100 µg/disc)
B_3 = EAEAR (200 µg/disc)
C_3 = EAEAR (300 µg/disc)
A_4 = DMEAR (100 µg/disc)
B_4 = DMEAR (200 µg/disc)
C_4 = DMEAR (300 µg/disc)
SN = Nystatin (100 µg/disc)
SC = Solvent Control

3.2.2. Determination of minimum inhibitory concentration (MIC)

3.2.2.1. Introduction

The minimum inhibitory concentration (MIC) may be defined as the lowest concentration of the test sample or drug at which it shows the highest activity against the growth of the pathogenic microorganisms. There are two methods for determining the minimum inhibitory concentration (MIC) values. They are as follows:

(i) Serial tube dilution technique or turbidimetric assay[160]

(ii) Paper disc plate technique or agar diffusion assay[161]

Here "serial tube dilution technique"[160] was followed using nutrient broth medium to determine the MIC values of EECF, compound-1, compound-2,compound-5, EAEAR and DMEAR against those gram positive and gram negative bacteria which were sensitive to these test samples in antibacterial screening . The following pathogenic bacterial strains were used for determination of MIC (Table 3.11)

Table 3.11 List of bacteria used for determination of MIC (the Institute of Biological Science, University of Rajshahi, Bangladesh).

Microorganisms	**Strain No.**
Gram positive (+) bacteria	
Staphylococcus aureus	ATCC25923
Bacillus subtilis	QL40
Bacillus megaterium	QL38
Sarcina lutea	QL166
Gram negative (-) bacteria	
Escherichia coli	ATCC27853
Shigella sonnei	C182
Shigella shiga	C180
Shigella dysenteriae	ATCC26131
Pseudomonas aeruginosa	ATCC14228

3.2.2.2. Preparation of inoculums

Fresh cultures of the test organisms were grown at 37.5°C for over night on nutrient agar medium. Bacterial suspensions were then prepared in sterile nutrient broth

medium in such a manner so that the suspension contains 10^7 CFU/ml. These suspensions were used as inoculums[160].

3.2.2.3. Preparation of the sample solution

To determine the MICs values against one bacterium, each test sample was measured accurately (2.048 mg each) and were taken in different vials. Then 2 mL of 2% dimethyl sulfoxide (DMSO) was added to each of the vials and mixed well to make sample solution whose concentration becomes 1024 μg/ml.

3.2.2.4. Procedure of serial tube dilution technique

(i) Twelve (12) autoclaved test tubes were taken, nine of which marked as 1, 2, 3, 4, 5, 6, 7, 8, 9, and the rest three were assigned as C_M (medium), C_{MS} (medium + compound) and C_{MI} (Medium + inoculum).

(ii) 1 ml of sterile nutrient broth medium was added to each of the twelve test tubes.

(iii) 1 ml of the sample solution was added to the first test tube and mixed well.

(iv) 1 ml content from the first test tube was transferred by the sterile pipette to the second test tube and mixed uniformly. Then 1 ml of this mixture was transferred to the third test tube. This process of serial dilution was continued up to the ninth (9^{th}) test tube. 1 mL of the mixture was discarded from the 9^{th} test tube to make uniform volume (1 mL) in all tubes. Then the concentration of sample in the following nine test tubes became 512, 256, 128, 64, 32, 16, 8, 4 and 2 μg/mL.

(v) 1 drop (10 μL) of properly diluted inoculums was added to each of the nine test tubes and mixed well.

(vi) For the control test tube C_{MS}, 1 ml of the sample solution was added, mixed well and 1 ml of this mixed content was discarded. This was used to check the clarity of the medium in presence of diluted solution of the compound.

(vii) 10 μL of the inoculums was added to the control test tube $C_{MI,}$ to observe the growth of the organism in the medium used.

(viii) The control test tube $C_{M,}$ containing medium only was used to confirm the sterility of the medium.

(ix) All the test tubes were incubated at 37.5°C for 18-24 hours.

3.2.2.5. Results and discussion

Minimum inhibitory concentration (MIC) values of EECF, compound-1, compound-2, EAEAR and DMEAR were evaluated (Table 3.12). The lowest MIC values were observed for EECF (16 µg/ml) and compound-1 (32 µg/ml) against *Bacillus subtilis* and *Sarcina lutea* (Table 3.12). Compound-2 (64 µg/ml) and compound-5 (64 µg/ml) showed lowest MIC value against *Staphylococcus aureus* and *Escherchia coli*, respectively (Table 3.12).

The MIC values of EAEAR and DMEAR were also determined and the results were presented in the table 3.12. The lowest MIC values were observed for EAEAR (32 µg/ml) and DMEAR (32µg/ml) against *Bacillus subtilis* and *Pseudomonas aeruginosa*, respectively. This minimum inhibitory concentration was very effective when compared with data obtained by Edziri *et al.* for *Salvia sclarea*[162].

Table 3.12 Minimum inhibitory concentrations (MICs) of EECF, compound-1, compound-2, EAEAR and DMEAR.

Microorganisms	EECF (µg/mL)	Compound-1 (µg/mL)	Compound-2 (µg/mL)	Compound-5 (µg/mL)	EAEAR (µg/mL)	DMEAR (µg/mL)
Staphylococcus aureus	32	64	64	-	64	-
Bacillus subtilis	16	32	-	-	32	-
Bacillus megaterium	128	-	-	-	64	-
Sarcina lutea	16	32	128	-	-	-
Escherchia coli	32	64	128	64	128	-
Shigella sonnei	32	64	-	64	-	64
Shigella Shiga	64	64	-	128	-	64
Shigella dysenteriae	128	128	-	128	-	-
Pseudomonas aeruginosa	-	-	-	-	-	32

3.3. Antineoplastic study

3.3.1. Introduction

Cancer represents the largest cause of mortality in the world and claims over 6 million lives every year[163]. In modern medicine, chemotherapy, radiotherapy and surgery are the major possibilities to treat cancer[164]. Today an extremely promising strategy for cancer prevention is chemoprevention, which is defined as the use of synthetic or natural agents (alone or combination) to block the development of cancer in humans. Intervention with chemopreventive agents in the early stage of carcinogenesis is theoretically more rational than attempting to eradicate fully developed tumors with chemotherapeutic drugs[165]. These agents have a narrow margin of safety and the therapy may fail due to drug resistance and dose-limiting toxicities, which may severely affect the host normal cells[166]. Hence the use of natural products has been contemplated in the control of cancer and its eradication programme[167]. Over 60% of currently used anti-cancer agents are derived in one-way or another from natural sources, including plants, marine organisms and microorganisms[168]. Plants have a long history of use in the treatment of cancer. A survey on medicinal plants reported that over 3000 species of plants have alleged anticancer properties[169]. Plant derived natural products such as flavonoids, terpenes, alkaloids and so on have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive properties[170-171].

However, an essential part of drug development against cancer is the testing of potential new compounds against animal tumors both *in vitro* and *in vivo*. *In vitro* tests determine whether the drug has any effect against tumour cells or not and *in vivo* tests gives an indication of the effects of the drug not only on the tumour but also on the host, thereby indicating its toxicity and therapeutic index. Ehrlich ascites carcinoma (EAC) is a rapidly growing experimental tumor with very aggressive behavior and resembles human tumors[172]. Medicinal plants used as folk medicine, have strong antitumour activity against Ehrlich ascites carcinoma (EAC) cell line[173]. The potent inhibitory effects of isolated principles of different medicinal plants against EAC have also been reported in literature[174-175]. So here we have taken EAC to evaluate the *in vivo* antitumor activity of isolated extracts (i.e., EECF, EAEAR and DMEAR) and compounds (i.e., compound-1, and compound-2) from *Calotropis gigantea* flower and *Amoora rohituka* stem bark in Swiss albino mice.

3.3.2. Materials

The important chemicals and equipments used in this study are mentioned below:

List of chemicals:

i) Sodium chloride (Loba, India)

ii) Dimethyl sulfoxide; DMSO (E-Merck, Germany)

iii) Trypan blue (Sigma, India)

iv) Hydrochloric acid; HCl (E-Merck, Germany)

v) WBC counting fluid: 100 ml WBC counting fluid containing 1.5 ml glacial acetic acid solution, 1.0 ml of 1% aqueous solution of Gention violet and 100 ml distilled water.

vi) RBC counting fluid: 100 ml RBC counting fluid containing 3.0 g Sodium citrate, 1 ml formalin and 100 ml distilled water.

vii) Leishman stain: 100 ml Leishman stain contains 0.3 gm Leishman Powdered and 100 ml acetone free methanol.

viii) Ethylenediaminetetraacetic acid; EDTA (Sigma-Aldrich, Germany)

ix) Glucose assay kit (Randox, USA)

x) Cholesterol assay kit (Cypress diagnostics, China)

xi) Triglycerides assay kit (Atlas Medica, UK)

xii) Urea assay kit (Atlas Medica, UK)

xiii) Serum alkaline phosphatase (SALP) assay kit (Atlas Medica, UK)

xiv) Serum glutamate pyruvate transaminase (SGPT) assay kit (Atlas Medica, UK)

xv) Serum glutamate oxaloacetate transaminase (SGOT) assay kit (Atlas Medica, UK)

xvi) Bleomycin (Biochem Pharmaceutical, India)

List of equipments:

i) Haemocytometer (Chang Bioscience, Inc., China)

ii) Shali's Haemometer (Hospital Equipment Mfg. Co., India)

iii) Microscope with magnification of 10x, 40x and 100x (Ample Scientific CM502 Nexcope Plan Objectives Professional Binocular Microscope China)

iv) Bioanalyser (Microlab 200, Vital Scientific, Dieren, Netherlands)

v) Eppendorf tube & Disposable syringe

vi) Micropipette (10-100 µL)

vii) Centrifuge machine (Eppendorf, Model-5415 C)

viii) Digital electric balance (FA1004N, China)

ix) Refrigerator (-80°C) (Model: CVK-UB, SANYO Electric Co. Ltd., Japan)

3.3.3. Experimental animal

Male and female Swiss albino mice (20-25 g) were collected from the Animal Research Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR,B). The mice were grouped and housed in iron cages with ten animals per cage and maintained under standard laboratory conditions (temperature 25±2 ^{o}C; humidity 55±5 %) with 12 hrs dark/light cycle. The mice were allowed free access to standard dry pellet diet (Collected from ICDDR,B) and water *ad libitum*. The composition of pellet diet is given in table 3.13. The mice were acclimatized to laboratory conditions for 10 days before beginning of the experiment. The experiments were carried out after approval of the protocol by the Institutional Ethics Committee for Experimentations on animal, human, microbes and living natural sources (225/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Table 3.13 The nutrient composition of the pellet diet (Per hundred grams of diet)

Nutrient	Grams
Starch	66
Casein	20
Fat	8
Standard vitamins	2
Salt	4

3.3.4. Experimental tumour model

Transplantable tumor (Ehrlich's Ascites Carcinoma; EAC) used in this study were obtained by the courtesy of Indian Institute for Chemical Biology, Kolkata, India and were maintained by weekly intraperitoneal (i. p.) inoculation of 10^5 cells per mouse in the laboratory. After inoculation mice were separated as EAC cell bearing mouse (Figure 3.55) from normal (Figure 3.54).

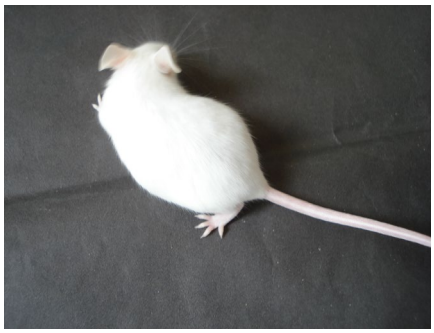


Figure 3.54 Normal Swiss albino mice

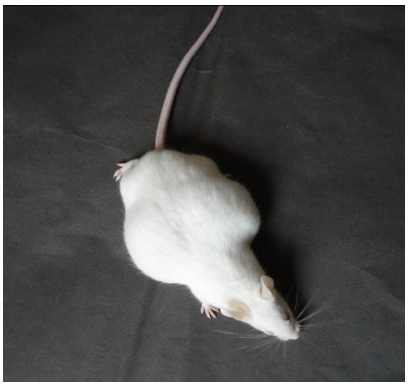


Figure 3.55 EAC cell bearing Swiss albino mice

3.3.5. Ehrlich's ascites carcinoma (EAC) cell line

Experimental tumors have great importance for the purposes of modeling and Ehrlich ascites carcinoma (EAC) is one of the commonest. It was appeared firstly as a spontaneous breast cancer in a female mouse and then Ehrlich and Apolant used it as an experimental tumor by transplanting tumor tissues subcutaneously from mouse to mouse[176]. In 1932, Loewenthal and Jahn[177] obtained the liquid form in the peritoneum of the mouse and named it as "Ehrlich ascites carcinoma (EAC)" due to the ascites liquid, together with the carcinoma cells. The ascitic tumor developes as a milky white fluid containing rounded tumor cells. One million of tumor cells multiply to yield about 25-100 million tumor cells/ml. Host survives for 14-30 days[176].

3.3.6. Counting of cells using haemocytometer

The simplest, most convenient and cheapest means of accurately determining the numbers of cells in a sample is to use a haemocytometer and a microscope. A haemocytometer is a specialized slide that has a counting chamber with a known volume of liquid.

The haemocytometer consists of a heavy glass slide with two counting chambers, each of which is divided into nine large 1 mm squares, on an etched and silvered surface separated by a trough (Figure 3.56). A cover slip sits on top of the raised supports of the 'H' shaped toughs enclosing both chambers. There is a 'V' or notch at either end where the cell suspension is loaded into the haemocytometer. When loaded with the cell suspension it contains a defined volume of liquid. The engraved grid on the surface of the counting chamber ensures that the number of particles in a defined volume of liquid is

counted. The haemocytometer is placed on the microscope stage and the cell suspension is counted.

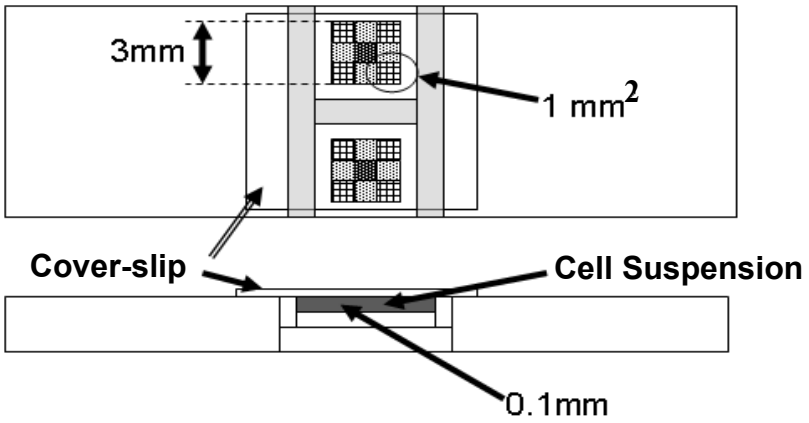


Figure 3.56 Haemocytometer arrangement and dimensions

The subsequent cell concentration per ml were determined using the following procedure

$$\text{Cells/ml} = \frac{\text{The average count per square } \mathbf{x} \text{ Dilution factor}}{\text{Depth of fluid under cover slip x Area counted}}$$

$$= \frac{\text{The average count per square } \mathbf{x} \text{ Dilution factor}}{0.1 \text{ mm x } 1 \text{ mm x } 1 \text{ mm}}$$

$$= \frac{\text{The average count per square } \mathbf{x} \text{ Dilution factor}}{0.01\text{cm x } 0.1 \text{ cm x } 0.1 \text{ cm}}$$

$$= \frac{\text{The average count per square } \mathbf{x} \text{ Dilution factor}}{0.0001 \text{ cm}^3}$$

$$= \frac{\text{The average count per square } \mathbf{x} \text{ Dilution factor}}{1 \text{ x } 10^{-4} \text{ ml}}$$

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

3.3.7. Collection of blood sample and processing

For measurement of heamatological parameters, blood was collected separately in EDTA-containing eppendorf tubes from freely flowing tail vein blood of each mouse. When mice were sacrificed, blood was collected from each individual mouse by heart puncture in the sterilized eppendorf tubes. Whole blood was then placed immediately on ice and subsequently centrifuged at 4000 rpm for 10 minutes at 4^{o}C. The supernatant was then taken as serum and stored at -80^{o}C for analysis.

3.3.8. Measurement of haematological and biochemical parameters

Haematological (hemoglobin, total RBC, total WBC and differential count of WBC) and biochemical parameters (glucose, total cholesterol, urea, triglyceride, serum alkaline phosphatase; SALP, serum glutamate pyruvate transaminase; SGPT, serum glutamate oxaloacetate trans aminase; SGOT) were measured from serum by the following ways:

3.3.8.1. Measurement of hemoglobin (Hgb)[178]

The amount of haemoglobin (Hgb) was measured by using Shali's haemometer. 20 µL non coagulating blood was transferred to the cuvette (tube) in haemometer containing a little amount of N/10 HCl. Distilled water was added and stirred until a good color match was obtained. The final reading of the solution in the cuvette was noted. From the cuvette reading gram % (g/dL) of hemoglobin was calculated.

3.3.8.2. Total count of WBC[178]

Exactly 10µL non coagulating blood was diluted with 1 ml WBC counting fluid and mixed properly. The resultant mixture was checked in haemocytometer and the number of cells was counted with a Microscope. Here the dilution factor was 100. Total WBC cells per ml were calculated.

3.3.8.3. Total count of RBC[178]

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

3.3.8.4. Differential count of WBC[178]

Blood smear was prepared from freshly drawn blood on clean glass slides. The smear was covered with undiluted Leishman stain and kept for one minute. The stain was diluted with two volumes of distilled water. The slides remained in this condition for 10-12 minutes and then flooded with tap water. The slides were air dried and observed under microscope. Different types of white blood cells were scored on their nuclear and cytoplasmic characteristics. The percent distribution of each type of cell was calculated after scoring at least 100 leukocytes per slide.

3.3.8.5. Measurement of glucose in serum

Glucose was measured by using glucose assay kit according to the manufacture's protocol (Randox, USA) through bioanalyser. The reaction principle, reagent composition, procedure and calculation are briefly described below:

Principle:

Glucose is determined after its enzymatic oxidation in the presence of glucose oxidase (GOD). GOD converts the sample glucose into gluconic acid and hydrogen peroxide. Then the hydrogen peroxide formed reacts with phenol and 4-aminoantipyrine under catalysis of peroxidase (POD), to form red quinine which is measurable at 505 nm. The increase in absorbance correlates with the glucose concentration of the sample[179-180].

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$4H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Red quinine} + 4H_2O$$

Reagent composition:

The composition of reagent for measurement of glucose is shown in table 3.14

Table 3.14 Reagents composition for glucose assay

Contents	**Concentration**
Reagent-1(R1); P^H 7.5 :	
Phosphate buffer	90 mmol/L
Phenol	0.5 mmol/L
Reagent-2 (R2); Enzymes:	
Glucose oxidase	≥ 10000U/L
Peroxidase	1000 U/L
4-Aminoantipyrine	2.5 mmol/L
Standard (Glucose)	5.56 mmol/L

Procedure:

The working reagent was prepared by mixing one vial of (R2) in appropriate amount of R1. This working reagent was stable at 20-25 °C for 14 days and at 2-8 °C for 30 days. Then test tubes were set as follows:

	Blank	Standard	Sample
Working reagent	1 mL	1 mL	1 mL
Distilled water	10 µL	-	-
Standard	-	10 µL	-
Sample (serum)	-	-	10 µL

The content of each tube was mixed and reading was taken after 10 minutes at room temperature.

Calculation:

Glucose concentration (mg/dL) = $(A_{sample}/A_{standard})$ **x** $C_{standard}$ **x** 18

Where A = Absorbance and C = Concentration

3.3.8.6. Measurement of total cholesterol in serum

Total serum cholesterol was measured by using cholesterol assay kit according to the manufacture's protocol (Cypress diagnostics) through bioanalyser. The reaction principle, reagent composition, procedure and calculation are briefly described below:

Reaction principle:

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyses the esters and H_2O_2 is formed in the subsequent enzymatic oxidation of cholesterol by cholesterol-oxidase according to the following equation[181-182].

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{4-Cholestenon} + H_2O_2$$

$$4H_2O_2 + \text{4-Aminophenazone} + \text{Phenol} \xrightarrow{\text{Cholesterol esterase}} \text{Quinonimine} + 4H_2O$$

In last reaction a red dye quinonimine is formed of which the intensity is proportional to the cholesterol concentration.

Reagents composition:

The composition of reagent for measurement of cholesterol is shown in table 3.15

Table 3.15. Reagents composition for total cholesterol assay

Contents	**Concentration**
Reagent-1(R1); P^H 6.9 :	
Pipes buffer	90 mmol/L
Phenol	26 mmol/L
Reagent-2 (R2); Enzymes:	
Peroxidase	1250 U/L
Cholesterol esterase	300 U/L
Cholesterol oxidase	300 U/L
4-Aminophenazone	0.4 mmol/L
Standard (Cholesterol aqueous)	200 mg/dL

Procedure:

The working reagent was prepared by mixing one bottle R2 reagent with one bottle R1 reagent. Then test tubes were set as follows:

	Blank	Standard	Sample
Standard	-	10 µL	-
Sample (serum)	-	-	10 µL
Working reagent	1 mL	1 mL	1 mL

The content of each tube was mixed and incubated at 37ºC for 5 minutes. Finally reading was taken.

Calculation:

Cholesterol concentration (mg/dL) = (Absorbance of sample/Absorbance of standard) x 200 (Concentration of cholesterol in standard)

Conversion factor: mg/dL x 0.0258 = mmol/L

3.3.8.7. Measurement of triglycerides in serum

Serum triglyceride was measured by using triglyceride assay kit according to the manufacture's protocol (Atlas Medica) through bioanalyser. The reaction principle, reagent composition, procedure and calculation are briefly described below:

Principle:

Sample triglycerides incubated with lipoproteinlipase (LPL), liberate glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase and ATP. Glycerol-3-phosphate (G3P) is then converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2) by glycerol-3-oxidase (GPO). In the last reaction, hydrogen peroxide (H_2O_2) reacts with 4-aminophenazone (4-AP) and p-chlorophenol in presence of peroxidase (POD) to give a red colored dye (Quinone)[183-184]:

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + \text{Free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{G3P} + \text{ADP}$$

$$\text{G3P} + \text{ATP} \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-AP} + \text{p-chlorophenol} \xrightarrow{\text{POD}} \text{Quinone} + H_2O$$

The intensity of the color formed is proportional to the triglycerides concentration in the sample·

Reagent composition:

The composition of reagent for measurement of triglyceride is shown in table 3.16

Table 3.16. Reagents composition for triglyceride assay

Contents	**Concentration**
Reagent (R); P^H 7.5 :	
Tampone Good	50 mmol/L
p-Chlorophenol	2 mmol/L
Lipoproteinlipase (LPL)	150000 U/L
Glycerol kinase	500 U/L
Glycerol-3-oxidase (GPO)	3500 U/L
Peroxidase (POD)	5000 U/L
4-Aminophenazone (4-AP)	0.1 mmol/L
ATP	0.1 mmol/L
TRIGLYCERIDES STANDARD: triglycerides primary calibrator 200 mg/dL	

Procedure:

The test tubes are set as follows:

	Blank	Standard	Sample
Standard		10 µL	
Sample (serum)		-	10 µL
R (mL)	1 mL	1 mL	1 mL

The content of each tube was mixed and incubated at 37ºC for 5 minutes. Finally reading was taken.

Calculation:

Triglycerides concentration (mg/dL) = (Absorbance of sample/Absorbance of standard) x 200 (Concentration of triglyceride in standard)

Conversion factor: mg/dL x 0.0113 = mmol/L

3.3.8.8. Measurement of urea in serum

Serum urea was measured by using urea assay kit according to the manufacture's protocol (Atlas Medica) through bioanalyser. The reaction principle, reagent composition, procedure and calculation are briefly described below:

Principle:

Urea in the sample is hydrolized enzymatically into ammonia (NH_4) and carbon dioxide (CO_2). Ammonia ions formed reacts with salicylate and hypochlorite (NaClO), in presence of the catalyst nitroprusside, to form a green indophenol[185-186].

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} (NH_4)_2 + CO_2$$

$$NH_4 + \text{Salicylate} + NaOCl \xrightarrow{\text{Nitroprusside}} \text{Indophenol}$$

The intensity of the color formed is proportional to the urea concentration in the sample.

Reagent composition:

The composition of reagent for measurement of urea is shown in table 3.17

Table 3.17. Reagents composition for urea assay

Contents	**Concentration**
Reagent-1(R1); P^H 6.7:	
Phosphate buffer	50 mmol/L
EDTA	2 mmol/L
Sodium salicylate	60 mmol/L
Sodium nitroprusside	3.2 mmol/L
Reagent-2 (R2):	
Sodium hypochlorite (NaClO)	140 mmol/L
Sodium hydroxide	150 mmol/L
Reagent-3 (R3); Enzymes:	
Urease	30000 U/L
Standard (Urea aqueous)	50 mg/dL

Procedure:

The content of R3 was dissolved in one bottle R1 reagent to prepare working reagent. It was stable in refrigerator for 4 weeks.

The test tubes are set as follows

	Blank	Standard	Sample
Standard		10 µL	
Sample (serum)		-	10 µL
Working reagent (mL)	1 mL	1 mL	1 mL

The content of each tube was mixed and incubated at 37ºC for 5 minutes.Then 1 mL of R2 reagent was added to each of the test tubes and the tubes were again incubated at 37ºC for 5 minutes. Finally reading was taken.

Calculation:

Urea concentration (mg/dL) = (Absorbance of sample/Absorbance of standard) x 50
(Concentration of triglyceride in standard)

Conversion factor: mg/dL x 0.1665 = mmol/L

3.3.8.9. Measurement of serum alkaline phosphatase (SALP) activity in serum

Serum alkaline phosphatase (SALP) activity was measured by using alkaline phosphatase assay kit according to the manufacture's protocol (Atlas Medica) through bioanalyser. The reaction principle, reagent composition, procedure and calculation are briefly described below:

Principle:

Serum Alkaline phosphatase (SALP) catalyses the hydrolysis of p-nitrophenyl phosphate at pH 10.4, liberating p-nitrophenol and phosphate, according to the following reaction:

$$\text{p-Nitrophenylphosphate} + H_2O \xrightarrow{\text{SALP}} \text{p-Nitrophenol} + \text{Phosphate}$$

The rate of p-Nitrophenol formation, measured photometrically, is proportional to the catalytic concentration of alkaline phosphatase present in the sample[187-188].

Reagent composition:

The composition of reagent for measurement of SALP acivity is shown in table 3.18

Table 3.18. Reagents composition for SALP acivity assay

Contents	**Concentration**
Reagent-1(R1): p-Nitrophenyl phosphate	 16 mmol/L
Reagent-2 (R2); P^H 10.5: AMP buffer Magnesium chloride ($MgCl_2$)	 350 mmol/L 2 mmol/L

Procedure:

One bottle of R1 was dissolved with a ration of R2 (Refer to label on R1) as working reagent. The working reagent was allowed to equilibrate to the reaction temperature prior to processing. Then test tubes are set as follows

	Blank	Sample
Standard	20 µL	-
Sample (serum)	-	20 µL
Working reagent (mL)	1 mL	1 mL

The content of each tube was mixed thoroughly and at reaction temperature for 60 seconds. Finally, SALP activity was measured at 25^0C (absorbance at 405 nm).

Calculation:

SALP (U/L) = (ΔA_{sample}/min-ΔA_{blank}/min) x F

and F = V_t/(V_s x extinction coefficient) x 1000 = 2757

Where ΔA/min = Changes of absorbance per minute at 25°C,

V_t = Total volume and V_s = Sample volume

The extinction coefficient of p-nitrophenyl at 405 nm is 18.50

3.3.8.10. Measurement of serum glutamate pyruvate transaminase (SGPT) activity in serum.

Serum glutamate pyruvate transaminase (SGPT) activity was measured by using commercial kit according to the manufacture's protocol (*Atlas Medica*) through bioanalyser. The reaction principle, reagent composition, procedure and calculation are briefly described below:

Principle:

Serum glutamate pyruvate transaminase (SGPT) catalyses the reversible transfer of an amino group from alanine to α-ketoglutarate forming glutamate and piruvate. The piruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH[189-190].

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{SGPT}} \text{Glutamate} + \text{Piruvate}$$

$$\text{Piruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of SGPT present in the sample

Reagent composition:

The composition of reagent for measurement of SGPT acivity is shown in table 3.19

Table 3.19. Reagents composition for SGPT acivity assay

Contents	**Concentration**
Reagent-1(R1):	
α-Ketoglutarate	15 mmol/L
NADH	0.18 mmol/L
Lactate dehydrogenase	5000 U/L
Reagent-2 (R2); P^H 7.3:	
Tris Buffer	100 mmol/L
L-Alanine	500 mmol/L

Procedure:

One bottle of R1 was dissolved with a ration of R2 (Refer to label on R1) as working reagent. The working reagent was allowed to equilibrate to the reaction temperature prior to processing. Then test tubes are set as follows

	Blank	Sample
Standard	70 µL	-
Sample (serum)	-	70 µL
Working reagent (mL)	1 mL	1 mL

The content of each tube was mixed thoroughly and at reaction temperature for 60 seconds. Finally, SGPT activity was measured at 25^0C (absorbance at 405 nm).

Calculation:

SGPT (U/L) = $(\Delta A_{sample}/min - \Delta A_{blank}/min) \times F$

and $F = V_t/(V_s \times \text{extinction coefficient}) \times 1000 = 1746$

Where $\Delta A/min$ = Changes of absorbance per minute at 25^0C,

V_t = Total volume and V_s = Sample volume

The extinction coefficient of NADH at 340 nm is 6.3/mmol

3.3.8.11. Measurement of serum glutamate oxaloacetate transaminase (SGOT) activity in serum.

Serum glutamate oxaloacetate transaminase (SGOT) activity was measured by using commercial kit according to the manufacture's protocol (*Atlas Medica*) through bioanalyser. The reaction principle, reagent composition, procedure and calculation are briefly described below:

Principle:

Serum glutamate oxaloacetate transaminase (SGOT) catalyses the reversible transfer of an amino group from L-aspertate to α-ketoglutarate forming glutamate and oxaloacetate. The oxaloacetate produced is reduced to L-malate by malate dehydrogenase (MDH) and NADH[189-190].

$$\text{L-aspertate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{SGOT}} \text{Oxaloacetate} + \text{L-glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-malate} + NAD^+$$

The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of SGOT present in the sample

Reagent composition:

The composition of reagent for measurement of SGOT acivity is shown in table 3.20

Table 3.20 Reagents composition for SGOT acivity assay

Contents	**Concentration**
Reagent-1(R1):	
α-Ketoglutarate	12 mmol/L
NADH	0.18 mmol/L
Malate dehydrogenase (MDH)	1000 U/L
Reagent-2 (R2); P^H 7.8:	
Tris Buffer	80 mmol/L
L- aspertate	240 mmol/L

Procedure:

One bottle of R1 was dissolved with a ration of R2 (Refer to label on R1) as working reagent. The working reagent was allowed to equilibrate to the reaction temperature prior to processing. Then test tubes are set as follows

	Blank	Sample
Standard	70 µL	-
Sample (serum)	-	70 µL
Working reagent (mL)	1 mL	1 mL

The content of each tube was mixed thoroughly and at reaction temperature for 60 seconds. Finally, SGOT activity was measured at 25^0C (absorbance at 405 nm).

Calculation:

SGOT (U/L) = (ΔA_{sample}/min-ΔA_{blank}/min) x F

and F = V_t/(V_s x extinction coefficient) x 1000 = 1746

Where ΔA/min = Changes of absorbance per minute at 25^0C,

V_t = Total volume and V_s = Sample volume

The extinction coefficient of NADH at 340 nm is 6.3/mmol

3.3.9. Transplantation of EAC cells

Ascitic fluid was drawn out from different EAC cell bearing Swiss albino mouse at the respective log-phases of tumor cells. A 5 ml syringe fitted with 20 gauge needle was used for this tumor cell aspiration. The freshly drawn fluid was diluted with normal saline and the tumor cells number was adjusted to approximate $1.5x10^5$ cells/mL by counting the number with a haemocytometer. The viability of tumor cells was checked by trypan blue dye (0.4%) exclusion assay. Cell samples showing above 90% viability were used for transplantation. Tumor suspension of 0.1 ml was injected intraperitoneally to each Swiss albino mouse during experiment. Strict aseptic condition was maintained throughout the transplantation process.

3.3.10. Evaluation of antitumour activity of EECF against EAC

3.3.10.1. Acute toxicity study (LD_{50}) for EECF

The acute toxicity study was conducted by the method of Lorke[191] to determine the LD_{50} value of EECF in mice and LD_{50} was required to select the doses for EECF in antitumour activity study. This method was carried out by a single intraperitoneal injection in thirty six animals (6 in each group) at different doses (100, 200, 400, 800, 1600 and 3200 mg/kg body weight). LD_{50} was evaluated by recording mortality after 24 hours using probit analysis[143].

3.3.10.2. Studies on EAC cell growth inhibition

In vivo cell growth inhibition was carried out as described by Sur et al. and Rajkapoor et. al.[192-193]. For this study the mice were divided into five groups (8 mice in each group) and for therapeutic evaluation the mice of all groups were inoculated with $1.5x10^5$ cells/mouse on the day zero. After 24 hours of EAC cell inoculation, treatment was started and continued for 5 days. The mice in group 1 were given 2% v/v dimethylsulfoxide (DMSO) at 5 mL/kg/mouse/day and considered as untreated EAC

control. EECF (50, 100 and 200 mg/kg/mouse/day) and standard drug, bleomycin (0.3 mg/kg/mouse/day) were administered intraperitonealy (i.p.) in groups 2, 3, 4 and 5, respectively. The mice were sacrificed on the 6th day after transplantation of tumour cells and EAC cells were collected by repeated intraperitoneal wash with normal saline (0.9 % NaCl). Viable EAC cells were counted with a haemocytometer using trypan blue and total number of viable cells per mouse of the treated group was compared with those of control.

3.3.10.3. Studies on survival time

Animals were divided into five groups, consisting of 8 mice in each and inoculated with $1.5x10^5$ cells/mouse on the day zero. The control group (group 1) was treated with only 2% DMSO solution at 5 mL/kg/mouse/day. After 24 hours of inoculation, treatment (i.p.) with EECF was started on group 2, 3 and 4 at doses of 50, 100 and 200 mg/kg/mouse/day, respectively and continued for 10 days. The antitumour efficacy of EECF was compared with that of bleomycin which was administered (i.p.) in group 5 at 0.3 mg/kg/mouse/day for 10 days. The average body weight of each group was noted on 15^{th} day after EAC cell inoculation. The survival time was recorded and expressed as mean survival time (MST) in days and percent increase of life span (%ILS) was calculated[192-193] as follows:

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

$$\text{Where, MST (mean survival time)} = \frac{\sum \text{Survival time in days of each mouse in a group}}{\text{Total number of mice}} \text{ - ------ (2)}$$

3.3.10.4. Studies on heamatological and biochemical

In order to detect the effect of EECF on hematological and biochemical parameters of EAC cell bearing mice, a comparison was made among six groups (n = 8) of mice on the 15^{th} day after inoculation[194]. All the groups were injected with EAC cells (0.1 mL of $1.5x10^5$ cells/mouse) intraperitoneally except the normal group at the day zero. After 24 hours of inoculation, normal saline (5 mL/kg/mouse/day) and 2% DMSO (5 mL/kg/mouse/day) were administered intraperitoneally to normal (group 1) and EAC control (group 2), respectively, for 10 days. EECF at 50, 100 and 200 mg/kg/mouse/day

and bleomycin at 0.3 mg/kg/mouse/day, were administered in groups 3, 4, 5 and 6, respectively. On 15th day after EAC cell inoculation, hematological parameters (Hemoglobin, RBC, WBC and Differential count of WBC) were measured from freely flowing tail vein blood of each mice of each group[178]. Then every mouse was sacrificed and serum was analyzed for glucose, total cholesterol, urea, triglyceride, SALP, SGPT and SGOT in a Bioanalyzer using commercial kits. Previously described methods (i.e., 3.3.7. Measurement of haematological and biochemical parameters) have been used to measure these haematological and biochemical parameters.

3.3.10.5. Sub-chronic toxicity studies

To determine sub-chronic toxicity, healthy Swiss albino mice were divided into four groups of 8 animals in each. Mice in group 1 were received (ip.) 2% DMSO at 5 ml/kg/mouse/day and group 2, 3 and 4 received (ip.) EECF at 50, 100 and 200 mg/kg/mouse/day, respectively for 14 days. At twenty-four hours after the last treatment and 18 hrs fasting, the mice were sacrificed. Hematological and biochemical parameters (glucose, total cholesterol, urea, triglyceride, SALP, SGPT and SGOT) were determined as described above.

3.3.11. Evaluation of antitumour activity of compound-1 against EAC

3.3.11.1. Acute toxicity study

The scientific committee emphasized that di-2-ethylhexyl phthalate (i.e., compound-1) has a low toxic potential by oral or intraperitoneal administration (LD_{50} > 25 g/kg) in mice and rats[195].

3.3.11.2. Studies on EAC cell growth inhibition

In order to determine the effect of compound-1 on EAC cell growth, 30 mice were randomly divided into five groups (6 animals in each group) and for therapeutic evaluation, the mice of all groups were inoculated with $1.5x10^5$ cells/mouse on the day zero. After 24 hrs of EAC cell inoculation, treatment was started and continued for 5 days. The mice in group 1, 2, 3, 4 and 5 received intraperitonelly 2% v/v DMSO (5 mL/kg/mouse/day), compound-1 (10, 20 and 40 mg/kg/mouse/day) and standard drug, bleomycin (0.3 mg/kg/mouse/day), respectively and treatment was continued for 5 days. On 6th day after EAC cell transplantation, animals were sacrificed. EAC cells were collected by repeated washing with 0.9% saline and viable EAC cells were counted with a haemocytometer using trypan blue. Total number of viable cells per mouse of the treated group was compared with those of EAC control (i.e., group 1)[192-193]

3.3.11.3. Studies on survival time

Mice in five groups (6 animals per group) were inoculated with $1.3x10^5$ cells/mouse on the day 0. After 24 hrs of inoculation, mice in group 1, 2, 3, 4 and 5 were treated (i.p) with 2% v/v DMSO (5 mL/kg/mouse/day), compound-1 (10, 20 and 40 mg/kg/mouse/day) and bleomycin (0.3 mg/kg/mouse/day), respectively and continued for 10 days. The average body weight gain (after 12 days) and mean survival time of each group were noted. The mean survival time (MST) and percentage increase in life span (%ILS) of the treated groups were calculated by equation-1 and equation-2 and then compared with that of the untreated EAC control group[192-193].

3.3.11.4. Studies on heamatological and biochemical parameters

Swiss Albino mice were divided into six groups (n = 6). All the animals were injected with EAC cells ($2x10^5$ cells/mouse) intraperitoneally except for the normal group. This was taken as day 0. Group 1 served as the normal control and group 2 served as the EAC control. Group 1 and 2 received normal saline (5 mL/kg/mouse/day) and 2% (v/v) DMSO (5 mL/kg/mouse/day). Group 3, 4 and 5 were treated with compound-1 at 10, 20 and 40 mg/kg/mouse/day, respectively. Group 6, which served as the positive control, was treated with bleomycin at 0.3 mg/kg/mouse/day. All these treatment were given 24 h after the EAC cell inoculation, once daily for 10 days. On 12^{th} day after inoculation, hematological parameters (Hemoglobin, RBC, WBC and Differential count of WBC) were measured from freely flowing tail vein blood of each mice of each group[178]. Then every mouse was sacrificed and serum was analyzed for glucose, total cholesterol, urea, triglyceride, SALP, SGPT and SGOT in a Bioanalyzer using commercial kits. Previously described methods (i.e., 3.3.7. Measurement of haematological and biochemical parameters) have been used to measure these haematological and biochemical parameters.

3.3.12. Evaluation of antitumour activity of compound-2 against EAC

3.3.12.1. Acute toxicity study

For dose selection of compound-2, male Swiss albino mice were divided into four groups (n = 6). After 2 hrs of a single intraperitoneal administration of compound-2 on the animals of group 1, 2 and 3 at 25, 50 and 100 mg/kg doses did not show any gross changes in behavioral, neurological and autonomic profiles when compared with untreated control (group 4) and no mortality was observed after 24 hrs. Then to optimize the dose levels, 10 and 20 mg/kg body weight were selected for the evaluation of compound-2.

3.3.12.2. Studies on EAC cell growth inhibition

In order to determine the effect of compound-2 on EAC cell growth, 24 mice were randomly divided into four groups (6 animals in each group) and for therapeutic evaluation, the mice of all groups were inoculated with $1.5x10^5$ cells/mouse on the day zero. After 24 hrs of tumour inoculation treatment was started and continued for 5 days. The mice in group 1, 2, 3 and 4 received intraperitonelly 2% (v/v) DMSO (5 mL/kg/mouse/day), compound-2 (10 and 20 mg/kg/mouse/day) and standard drug, bleomycin (0.3 mg/kg/mouse/day), respectively. On day 6 after EAC cell transplantation, animals were sacrificed. EAC cells were collected by repeated washing with 0.9% saline and viable EAC cells per mouse of the treated group were compared with EAC control (i.e., group 1)[192-193].

3.3.12.3. Studies on survival time

Mice in four groups (6 animals per group) were inoculated with $1.5x10^5$ cells/mouse on the day 0. After 24 hrs of inoculation, mice in group 1, 2, 3 and 4 were treated (i.p) with 2% (v/v) DMSO (5 mL/kg/mouse/day), compound-2 (10 and 20 mg/kg/mouse/day) and bleomycin (0.3 mg/kg/mouse/day), respectively and continued for 10 days. The mice in group 1 were considered as untreated EAC control. The average body weight changes (after 12 days) and mean survival time of each group were noted. The mean survival time (MST) and percentage increase in life span (%ILS) of the treated groups were calculated by equation-1 and equation-2 and then compared with that of the control group[193].

3.3.12.4. Studies on heamatological and biochemical parameters

Swiss Albino mice were divided into five groups (n = 6). All the animals were injected with EAC cells ($2x10^5$ cells/mouse) intraperitoneally except for the normal group. This was taken as day 0. Group 1 served as the normal control and group 2 served as the EAC control. Group 1 and 2 received normal saline (5 mL/kg/mouse/day) and 2% (v/v) DMSO (5 mL/kg/mouse/day). Group 3 and 4 were treated with compound-2 at 10 and 20 mg/kg/mouse/day, respectively. Group 5, which served as the positive control, was treated with bleomycin at 0.3 mg/kg/mouse/day. All these treatment were given 24 h after the EAC cell inoculation, once daily for 10 days. Six mice from each group were sacrificed on 12^{th} day after tumor inoculation for the study of hematological and biochemical parameters. Hematological parameters (Hemoglobin, RBC, WBC and Differential count of WBC) were measured from freely flowing tail vein blood of each mice of each group[178]. Then every mouse was sacrificed and serum was analyzed for glucose, total cholesterol, urea, triglyceride, SALP, SGPT and SGOT in a Bioanalyzer

using commercial kits. Previously described methods (i.e., 3.3.7. Measurement of haematological and biochemical parameters) have been used to measure these haematological and biochemical parameters.

3.3.13. Evaluation of antitumour activity of ME, PEF and CF against EAC

3.3.13.1. Acute toxicity study

An acute toxicity study relating to the determination of LD_{50} of each ME, PEF and CF was performed in mice by the method of Lorke[191]. For each sample, this method was carried out in twenty animals, four per treatment group and widely different dose ranges of 100, 200, 400, 800 and 1600 mg/kg body weight. Then after 24 hrs, the mortality number caused by each test sample was observed from which the median lethal dose (LD_{50}) of each sample was determined using probit analysis[143].

3.3.13.2. Studies on EAC cell growth inhibition

In vivo tumour cell growth inhibition was carried out by the method as described by Senthilkumar et al[194]. $2x10^5$ EAC cells were inoculated into 8 groups of mice (6 in each) on day 0. The groups and the design of the experiment were as follows:

Group 1: EAC + 2% DMSO (5 mL/kg/mouse/day) (EAC control)

Group 2: EAC + ME (10 mg/kg/mouse/day; i.p.)

Group 3: EAC + ME (20 mg/kg/mouse/day; i.p.)

Group 4: EAC + PEF (40 mg/kg/mouse/day; i.p.)

Group 5: EAC + PEF (80 mg/kg/mouse/day; i.p.)

Group 6: EAC + CF (20 mg/kg/mouse/day; i.p.)

Group 7: EAC + CF (40 mg/kg/mouse/day; i.p.)

Group 8: EAC + Bleomycin (0.3 mg/kg/mouse/day; i.p.)

Treatment was continued for 5 days and on day 6 after EAC cell transplantation, animals were sacrificed. EAC cells were collected by repeated washing with 0.9% saline and viable EAC cells per mouse of the treated group were compared with those of control.

3.3.13.3. Studies on survival time, heamatological and biochemical parameters

Swiss Albino mice were divided into nine groups (n = 12). All the animals were injected with EAC cells ($2x10^5$ cells/mouse) intraperitoneally except for the normal group. This was taken as day 0. Group 1 served as the normal control and group 2 served as the EAC control. Group 1 and 2 received normal saline (5 mL/kg/mouse/day) and 2% (v/v) DMSO (5 mL/kg/mouse/day). Group 3, 4, 5, 6, 7 and 8 were treated with ME, PEF

and CF at same doses as described above. Group 9, which served as the positive control, was treated with bleomycin at 0.3 mg/kg/mouse/day. All these treatment were given 24 h after the EAC cell inoculation, once daily for 10 days. Six mice from each group were sacrificed on 15th day after tumor inoculation for the study of hematological and biochemical parameters whereas the rest of the animals in each group were kept to determine the survival time and percentage increase in life span (%ILS) of EAC cell bearing mice using equation-1 and equation-2[192-193]. Hematological parameters (Hemoglobin, RBC, WBC and Differential count of WBC) were measured from freely flowing tail vein blood of each mice of each group[178]. Then every mouse was sacrificed and serum was analyzed for SALP, SGPT and SGOT in a Bioanalyzer using commercial kits. Previously described methods (i.e., 3.3.7. Measurement of haematological and biochemical parameters) have been used to measure these haematological and biochemical parameters.

3.3.14. Evaluation of antitumour activity of EAEAR and DMEAR against EAC

3.3.14.1. Acute toxicity study

The acute toxicity study was conducted by the method of Lorke[191] to determine the LD_{50} value of EAEAR and DMEAR in mice. For each sample, this method was carried out by a single intraperitoneal injection in twenty five animals (5 in each group) at different doses (100, 200, 400, 800 and 1600 mg/kg body weight). LD_{50} was evaluated by recording mortality after 24 hours using probit analysis[143].

3.3.13.2. Studies on EAC cell growth inhibition

In vivo cell growth inhibition was carried out by the method as described by Sur and Ganguly[192]. For therapeutic evaluation $2x10^5$ cells/mouse were inoculated into the mice of six groups (6 mice in each group) on the day zero. Treatment was started after 24 hours of tumour inoculation and continued for 5 days. Group 1, 2, 3, 4, 5 and 6 received the 2% (v/v) DMSO (5 mL/kg/mouse/day), EAEAR (20 and 40 mg/kg/mouse/day), DMEAR (20 and 40 mg/kg/mouse/day) and standard drug, *bleomycin* (Biochem Pharmaceutical, India; 0.3 mg/kg/mouse/day), respectively. The mice were sacrificed on the 6th day after transplantation and viable EAC cells per mouse of the treated group were compared with those of untreated control (i.e., group 1) using trypan blue.

3.3.14.3. Studies on survival time

Animals were divided into six groups (6 mice in each group) and inoculated with $1x10^5$ cells/mouse on day '0'. The control group was treated with 2% DMSO solution at 5 mL/kg/mouse/day. Treatment (i.p.) with EAEAR (20 and 40 mg/kg/mouse/day), DMEAR (20 and 40 mg/kg/mouse/day) and *bleomycin* (0.3 mg/kg/mouse/day) were started after 24 hours of inoculation and continued for 10 days. Then average body weight gain of each group was noted on the 14^{th} day after EAC cell inoculation. Mean survival time (MST) and percentage increase in life span (%ILS) of each group were calculated by equation-1 and equation-2[193].

3.3.14.4. Studies on heamatological and biochemical parameters

Forty two mice in seven groups (6 mice in each group) were injected with EAC cells (2×10^5 cells/mouse) intraperitoneally except the normal group at the day "0". From the first day normal saline and 2% DMSO were intraperitoneally administered to normal (group 1) and EAC control (group 2) at 5 mL/kg/mouse/day for 10 days. Similarly both EAEAR and DMEAR at 20 and 40 mg/kg/mouse/day doses were administered in groups 3, 4, 5 and 6, respectively. Mice in group 7 were treated with standard *bleomycin* (0.3 mg/kg/mouse/day). Heamatological parameters were measured on 14^{th} day after EAC cell inoculation from freely flowing tail vein blood of each mouse of each group[178]. Then every mouse was sacrificed and serum was analyzed for SALP, SGPT and SGOT in a Bioanalyzer using commercial kits. Previously described methods (i.e., 3.3.7. Measurement of haematological and biochemical parameters) have been used to measure these haematological and biochemical parameters.

3.3.15. Statistical analysis

Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Dunnett's 't' test using SPSS statistical software of 14 version. All values were expressed as mean ± SEM.

3.3.16. Results and discussion

3.3.16.1. Effect of EECF against EAC

In acute toxicity study, intraperitoneal administration of graded doses of EECF to Swiss albino mice, produced a LD_{50} of 2225.0 mg/kg body weight.

Effect of EECF on EAC cell growth is shown in figure 3.57. In this study, the number of viable EAC cells in peritoneum of EECF-treated mice, were significantly ($P<0.05$ and $P<0.001$) lower when compared to the EAC control group (Table 3.21 and figure 3.57). EECF at 200 mg/kg dose, showed highest inhibition (71.24%) of EAC cell growth. EAC cell growth inhibition for bleomycin was 92.37% inhibition (Table 3.21). EECF also prolonged the average life span of animals (Table 3.22 and figure 3.58). The animals of the EAC control group inoculated with EAC cells survived for a period of 21.5 days (Table 3.22 and figure 3.58). The treatment with EECF at 50, 100 and 200 mg/kg and with bleomycin at 0.3 mg/kg increased the mean survival time (MST) by 25.25 ± 0.47 ($P<0.05$), 27.7 ± 0.63 ($P<0.05$), 35.5 ± 0.86 ($P<0.01$) and 39.0 ± 0.85 ($P<0.001$) days, respectively (Table 3.22 and figure 3.58). The EECF at 200 mg/kg was found to be the most potent in inhibiting the proliferation of EAC with percentage increase in life span (ILS) of 65.1% (Table 3.22 and figure 3.58). As shown in figure 3.59**,** the EECF treatment at 100 and 200 mg/kg doses significantly (($P<0.05$) inhibited the body weight gain due to tumor burden when compared to the EAC control. Microscopic observations of EAC cell smear of control and EECF treated group have also been presented in figure 3.63 and 3.64.

Table 3.21 Effect of EECF on Ehrlich ascites carcinoma (EAC) cell growth

Group No.	Treatment	Viable EAC cells on day 6 after inoculation (x 10^7 cells/mL)	Percentage (%) cell growth inhibition
1	EAC + 2% (v/v) DMSO	3.425 ± 0.07	-
2	EAC + EECF (50 mg/kg)	2.025 ± 0.11[a]	40.87 ± 1.52
3	EAC + EECF (100 mg/kg)	1.425 ± 0.06[c]	58.39 ± 1.98
4	EAC + EECF (200 mg/kg)	0.985 ± 0.12[c]	71.24 ± 2.31
5	EAC + Bleomycin (0.3 mg/kg)	0.261 ± 0.05[c]	92.37 ± 1.20

Data are expressed as the mean ± S.E.M (n = 8);

Significantly different from group 1; [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$

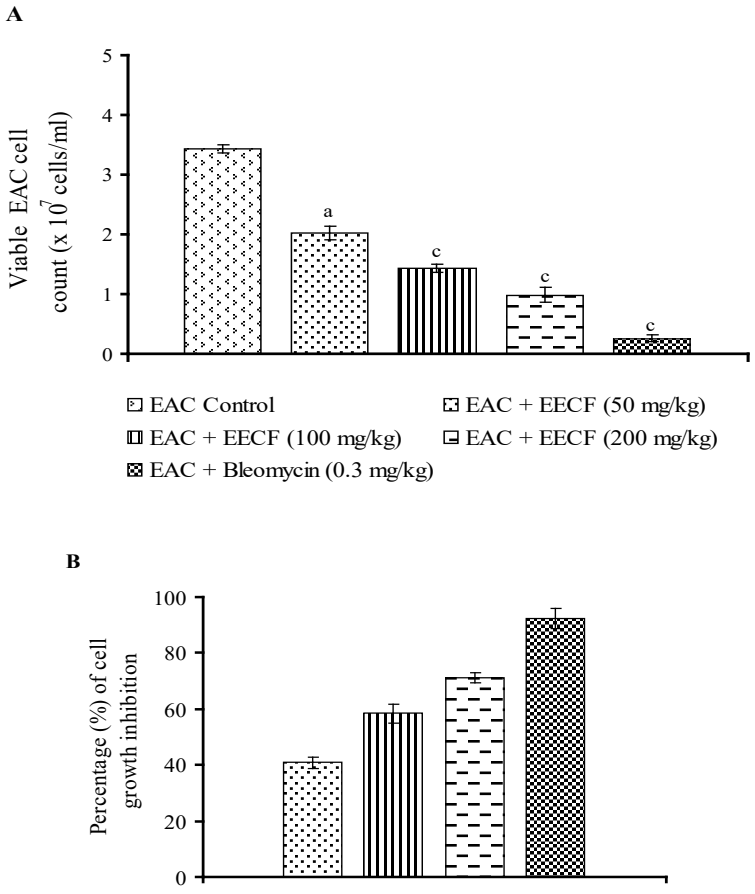


Figure 3.57. Effect of EECF on EAC cell growth. **A**: Viable EAC cells on day 6 after tumor cell inoculation, **B**: % of cell growth inhibition; Data are expressed as mean ± S.E.M (n = 8); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

Table 3.22 Effect of EECF on survival time and body weight gain of EAC cell bearing mice

Group No.	Treatment	MST (in days)	%ILS	Body weight gain (g) after 15 days
1	EAC + 2% (v/v) DMSO	21.5 ± 0.64	-	13.5 ± 0.43
2	EAC + EECF (50 mg/kg)	25.2 ± 0.47^{a}	17.2 ± 2.21	12.2 ± 0.68
3	EAC + EECF (100 mg/kg)	27.7 ± 0.62^{a}	28.8 ± 2.92	09.1 ± 0.42^{a}
4	EAC + EECF (200 mg/kg)	35.5 ± 0.86^{b}	65.1 ± 4.02	07.8 ± 0.73^{a}
5	EAC + *Bleomycin* (0.3 mg/kg)	39.0 ± 0.85^{c}	81.4 ± 3.97	05.8 ± 0.57^{c}

Data are expressed as the mean ± S.E.M (n = 8); Significantly different from group 1: $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$; MST: Mean survival time; %ILS: Percentage (%) increase in life span

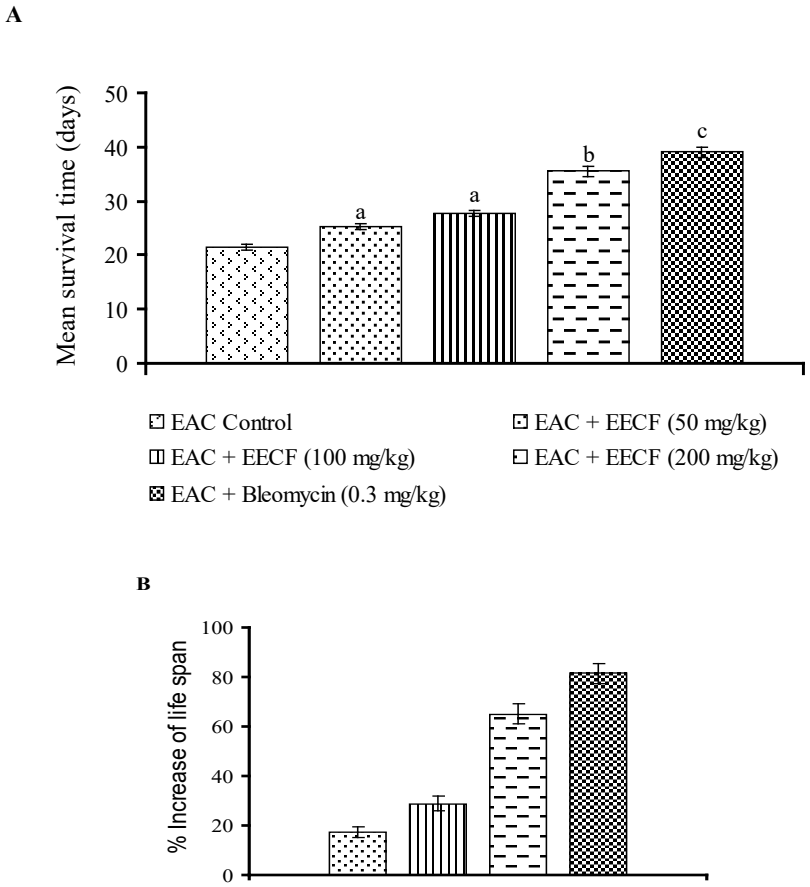


Figure 3.58. Effect of EECF on survival time of EAC cell bearing mice. **A**: Mean survival time (MST), **B**: Percentage increase in life span (%ILS); Data are expressed as mean ± S.E.M (n = 8); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

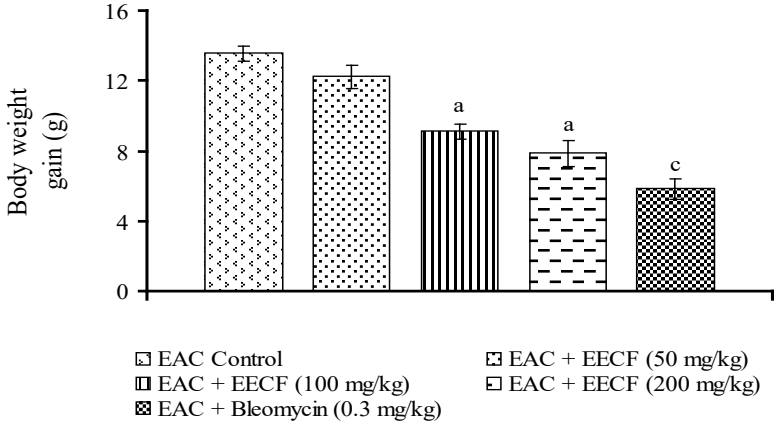


Figure 3.59 Effect of EECF on body weight gain of EAC cell bearing mice after 15 days. Data are expressed as mean ± S.E.M (n = 8); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

In this study, heamatological and biochemical parameters of only EAC cell bearing mice on the day 15 were changed significantly ($P<0.001$) when compared to normal mice. The WBC count, neutrophils, cholesterol, triglyceride, blood urea, SALP and SGOT were found to be increased with a reduction in heamoglobin, RBC count, lymphocytes, glucose and SGPT (Table 3.23). Treatment with EECF at all doses, restored altered heamoglobin level, RBC count, WBC count, percentage lymphocytes and neutrophils, glucose level, total cholesterol, triglycerides and serum urea more or less to normal levels (Table 3.23). The enzymatic activities of SALP, SGPT and SGOT were also restored to normal level in EECF-treated mice (Table 3.23). At 0.3 mg/kg, standard drug bleomycin significantly ($P<0.001$) restored all heamatological and biochemical parameters to normal.

Ehrlich tumor is able to grow in almost all strains of mice and its implantation induces *per se* a local inflammatory reaction with increasing vascular permeability, which results in an intense edema formation, cellular migration and a progressive ascitic fluid formation[196-197]. The ascitic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells[198]. The inhibition of EAC cells in this study could indicate either a direct cytotoxic effect of EECF on tumor cells or an indirect local effect, which may involve macrophage activation, vascular permeability inhibition and nutritional fluid deficiency.

In cancer chemotherapy the major problem are of myelosuppression and anemia[199]. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions[199]. Recovery of the hemoglobin content, RBC and WBC cell count in the experimental mice indicated the protective action of EECF on the heamopoietic system. The development of hypoglycemia and hyperlipidemia in experimental animals with carcinoma has been previously reported[200-201]. In this experiment, reduced glucose level and elevated cholesterol, triglycerides and serum urea were returned more or less to normal level in EECF-treated mice, thereby indicating the potent antitumour efficacy of EECF (Table 3.23).

It is well known that there are significant elevations in the levels of SGPT, SGOT and SALP in liver diseases and disorders and in hepatocellular damages caused by a number of agents[202]. Biochemical measurements of these parameters showed that some extent of hepatotoxicity was associated after 15 days inoculation with EAC. Treatment with the

EECF depicted that values were remained near the normal range in the treated groups (Table 3.23).

In short term toxicity study, administration of EECF at 50, 100 and 200 mg/kg body weight for 14 days, did not influence the body weight of the normal mice. Heamatological and biological parameters remained unaltered in EECF-treated mice at 50 and 100 mg/kg but haemoglobin, RBC, WBC, glucose, and cholesterol were increased significantly ($P<0.05$) at 200 mg/kg (Table 3.24). This indicates that after short term treatment the extract did not cause any extreme abnormality at the three doses used in this study.

Potential antitumour activity of EECF at 200 mg/kg is comparable to that of bleomycin (0.3 mg/kg), which is commonly used as an active antitumour agent in vast series of preclinical and clinical studies[203]. The preliminary phytochemical studies in our laboratory indicated the presence of flavonoid, triterpene, glycoside and steroid type compounds in the EECF. Many such compounds are known to possess potent antitumor properties[204]. In addition, both *Calotropis gigantea* and *Calotropis procera* plants possess hepatoprotective and antioxidant properties[65,205]. Many natural compounds of plant-derived extracts have vital roles in balancing the intracellular redox status and in antioxidant function. Imbalance between cellular oxidant species production and antioxidant capability produces reactive oxygen species (ROS) which are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis. So natural compounds with antioxidants effects are important therapeutic prospects for cancer[106-107]. These evidences help us to state that the antitumor properties of EECF may be due to the presence of phytoconstituents with antioxidative activity.

Table 3.23 Effect of EECF on hematological and biochemical parameters of EAC cell bearing mice.

Parameters	Treatment (mg/kg body weight)					
	Normal	EAC + 2% DMSO (EAC control)	EAC + EECF (50)	EAC + EECF (100)	EAC + EECF (200)	EAC + *Bleomycin* (0.3)
Hgb (g/dL)	15.48 ± 0.22	7.35 ± 0.20*	8.10 ± 0.14	8.37 ± 0.15^{t}	10.66 ± 0.15^{t}	14.37 ± 0.25^{t}
RBC(x10^{9} cells/mL)	5.67 ± 0.10	2.27 ± 0.06*	2.64 ± 0.05^{a}	2.76 ± 0.08^{a}	3.84 ± 0.07^{b}	4.90 ± 0.09^{c}
WBC(x10^{6} cells/mL)	8.75 ± 0.53	25.4 ± 1.19*	22.6 ± 0.84	20.2 ± 0.73^{a}	16.3 ± 0.57^{b}	9.37 ± 0.59^{c}
Lymphocytes (%)	75.5 ± 1.36	33.8 ± 1.35*	36.6 ± 1.13	41.3 ± 1.09^{a}	56.3 ± 0.91^{c}	68.2 ± 0.90^{c}
Neutrophils (%)	19.6 ± 1.38	63.7 ± 1.04*	60.0 ± 1.05	55.7 ± 1.10^{a}	41.1 ± 0.72^{a}	28.8 ± 0.93^{b}
Monocytes (%)	1.87 ± 0.40	1.50 ± 0.38	1.75 ± 0.25	1.70 ± 0.31	1.62 ± 0.26	2.00 ± 0.27
Glucose(mg/dL)	138.9 ±1.15	54.3 ± 0.99*	60.6 ± 0.64^{a}	91.9 ± 0.77^{b}	111.3 ± 0.99^{b}	134.1 ± 1.21^{c}
Cholesterol (mg/dL)	103.8 ± 0.85	153.5 ± 0.78*	149.9 ± 1.27	148.3 ± 0.32^{a}	130.2 ± 0.49^{b}	112.4 ± 0.71^{c}
Triglyceride (mg/dL)	105.5 ± 1.02	186.8 ± 0.75*	182.5 ± 1.32	179.2 ± 1.67^{a}	160.6 ± 0.82^{a}	126.8 ± 1.28^{c}
Blood Urea (mg/dL)	24.5 ± 0.44	64.1 ± 0.88*	58.9 ± 1.01^{a}	43.7 ± 0.75^{a}	36.1 ± 0.40^{b}	32.2 ± 0.52^{c}
SALP (U/L)	106.4 ± 0.74	238.9 ± 0.66*	232.6 ± 1.20^{a}	203.4 ± 0.97^{a}	165.8 ± 0.89^{b}	133.1 ± 0.72^{c}
SGPT (U/L)	71.9 ± 0.52	61.6 ± 0.49*	60.9 ± 0.46	65.2 ± 1.68	66.3 ± 0.70^{a}	71.3 ± 0.36^{a}
SGOT (U/L)	43.7 ± 0.84	242.6 ± 0.93*	236.0 ± 0.92^{a}	174.9 ± 0.72^{b}	140.5 ± 0.80^{c}	80.0 ± 0.78^{c}

Data are expressed as mean ± S.E.M. for eight animals in each group.

$^{*}P<0.001$: against normal group and $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: against EAC control group.

Table 3.24 Effect of EECF on hematological, biochemical parameters and body weight of normal mice.

Parameters	Treatment (mg/kg body weight)			
	Normal + 2% DMSO (5 mL/kg)	Normal + EECF (50)	Normal + EECF (100)	Normal + EECF (200)
Hgb (g/dL)	12.70 ± 0.20	12.07 ± 0.18	13.36 ± 0.19	13.93 ± 0.15[a]
RBC (x10^9 cells/mL)	6.11 ± 0.13	6.25 ± 0.63	6.05 ± 0.05	6.79 ± 0.03[a]
WBC (x10^6 cells/mL)	7.87 ± 0.52	7.37 ± 0.38	9.50 ± 0.46	10.12 ± 0.44[a]
Lymphocytes (%)	73.5 ± 1.21	72.0 ± 0.71	69.1 ± 0.77	70.25 ± 0.59
Neutrophils (%)	23.6 ± 1.05	25.7 ± 0.45	27.7 ± 0.90	26.3 ± 1.31
Monocytes (%)	1.87 ± 0.35	1.62 ± 0.32	2.12 ± 0.35	2.12 ± 0.23
Glucose (mg/dL)	149.2 ± 0.58	151.9 ± 0.73	150.0 ± 0.48	155.2 ± 0.90[a]
Cholesterol (mg/dL)	108.4 ± 0.70	106.3 ± 1.65	105.5 ± 0.94	101.5 ± 0.93[a]
Triglyceride (mg/dL)	98.3 ± 1.29	97.9 ± 0.73	99.7 ± 0.56	99.5 ± 1.28
Blood Urea (mg/dL)	28.8 ± 0.34	30.4 ± 0.47	30.2 ± 0.42	28.2 ± 0.76
SALP (U/L)	109.5 ± 0.70	108.2 ± 0.72	105.1 ± 1.17	106.7 ± 1.51
SGPT (U/L)	68.5 ± 0.82	67.4 ± 0.79	65.3 ± 0.89	70.4 ± 0.77
SGOT (U/L)	51.2 ± 0.77	51.6 ± 0.80	52.5 ± 0.55	56.8 ± 1.71
Body weight gain (g) after 15 day	5.52 ± 0.31	5.25 ± 0.19	5.86 ± 0.30	5.11 ± 0.29

Data are expressed as mean ± S.E.M. for eight animals in each group. [a]P<0.05, [b]P<0.01 and [c]P<0.001:between normal and EECF-treated groups.

3.3.16.2. Effect of compound-1 against EAC

It has been found that compound-1 is capable of reducing viable EAC cells (Table 3.25, figure 3.60 & figure 3.65) and weight gain of EAC cell bearing mice (Figure 3.62). For compound-1, the reduction ability increases with a dose-dependent manner. With 40 mg/kg (i.p.), compound-1 showed a maximum reduction of viable EAC cells when compared with that of control (Table 3.25 & figure 3.60).

The effect of compound-1 on the survival of EAC bearing mice is shown in table 3.26. The MST (Mean survival time) of the control group was 21.50 ± 0.50 days, whereas it was 26.02 ± 1.86, 29.25 ± 1.14 ($P<0.05$), 32.37 ± 1.08 ($P<0.05$) and 39.20 ± 0.85 ($P<0.001$) for the group treated with compound-1 (10, 20 and 40 mg/kg) and bleomycin (0.3 mg/kg), respectively. Compound-1 at the doses of 20 and 40 mg/kg body weight, significantly ($P<0.05$) elevated the MST and ILS (Increase in life span) of EAC tumor bearing mice (Table 3.26 & figure 3.61). The prolongation of life span is a reliable criterion for judging the efficacy of any anticancer drugs[206] and compound-1 was able to meet this criterion.

In this study, hematological parameters of EAC cell bearing mice on day 12 showed significant ($P<0.001$) changes when compared with the normal mice (Table 3.27). The total WBC count was found to increase with a reduction in the hemoglobin content and total RBC count. The differential count of WBC showed that the percentage of neutrophils was increased while that of lymphocytes was decreased. At the same time interval, treatment with compound-1 (20 and 40 mg/kg) could significantly ($P<0.05$) change these altered parameters more or less to normal (Table 3.27). This indicates that compound-1 possesses protective action on the heamopoietic system thereby suppressing myelosuppression and anemia found as major problems in cancer chemotherapy[199].

Table 3.25 Effect of compound-1 on Ehrlich ascites carcinoma (EAC) cell growth

Group No.	Treatment	Viable EAC cells on day 6 after inoculation (x 10^7 cells/mL)	Percentage (%) cell growth inhibition
1	EAC control	4.050 ± 0.25	-
2	EAC + Compound-1 (10 mg/kg)	2.375 ± 0.19[b]	41.35 ± 4.74
3	EAC + Compound-1 (20 mg/kg)	1.878 ± 0.17[b]	53.62 ± 4.35
4	EAC + Compound-1 (40 mg/kg)	1.275 ± 0.13[c]	68.51 ± 3.41
5	EAC + Bleomycin (0.3 mg/kg)	0.261 ± 0.05[c]	93.55 ± 1.20

Data are expressed as mean ± S.E.M (n = 6);

[a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$: Significantly different from group 1

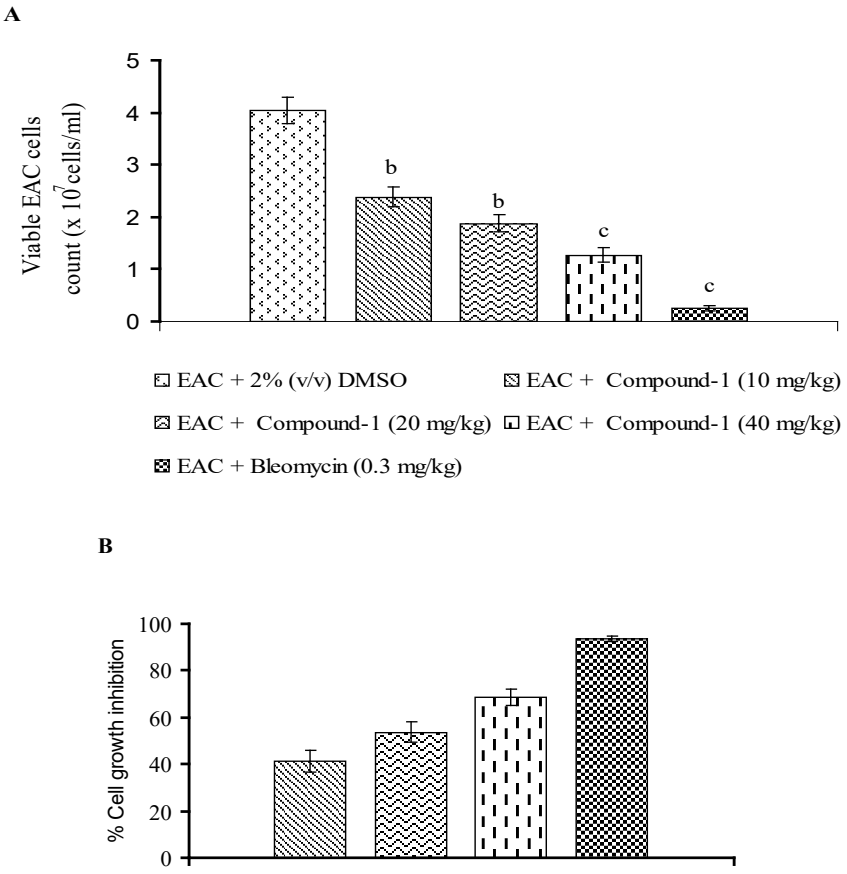


Figure 3.60 Effect of compound-1 on EAC cell growth. **A**: Viable EAC cells on day 6 after tumor cell inoculation, **B**: % of cell growth inhibition; Data are expressed as mean ± S.E.M (n = 6); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

Table 3.26 Effect of compound-1 on survival time and body weight gain of EAC cell bearing mice

Group No.	Treatment	MST (in days)	%ILS	Body weight gain (g) after 12 days
1	EAC control	21.50 ± 0.50	-	14.2 ± 0.85
2	EAC + Compound-1 (10 mg/kg)	26.02 ± 1.86	21.02 ± 4.02	11.1 ± 0.73
3	EAC + Compound-1 (20 mg/kg)	29.25 ± 1.14^{a}	36.04 ± 5.32	9.7 ± 1.05^{a}
4	EAC + Compound-1 (40 mg/kg)	32.37 ± 1.08^{a}	50.55 ± 5.04	7.2 ± 0.89^{b}
5	EAC + Bleomycin (0.3 mg/kg)	39.20 ± 0.85^{c}	82.32 ± 3.97	4.5 ± 0.36^{c}

Data are expressed as the mean ± S.E.M (n = 6); Significantly different from group 1: $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$; MST: Mean survival time; %ILS: Percentage (%) increase of life span.

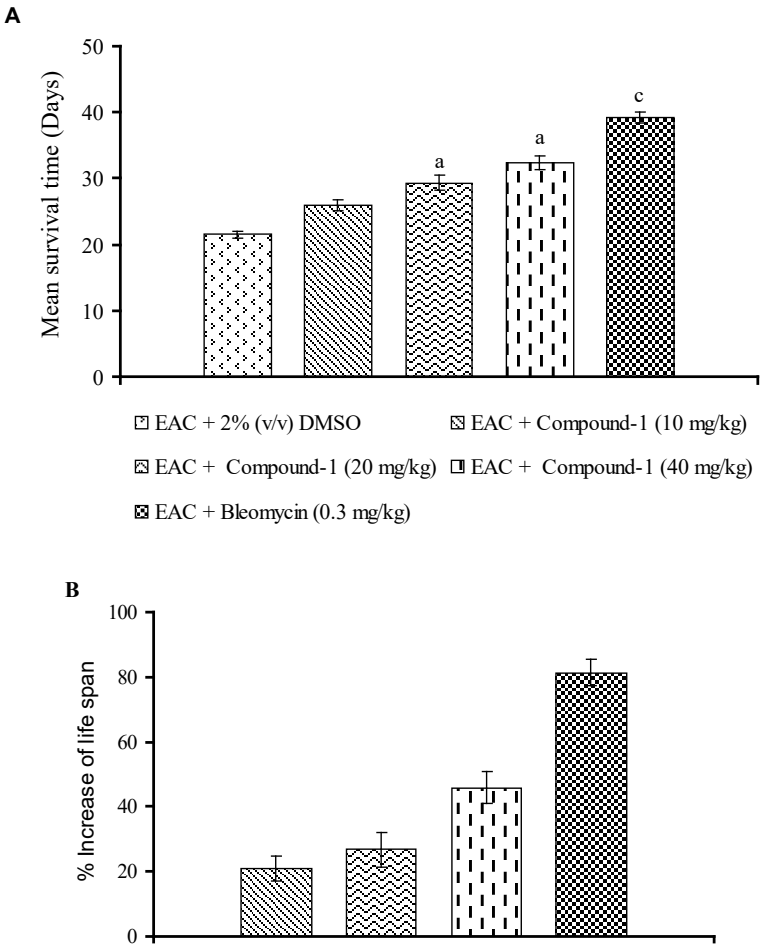


Figure 3.61 Effect of compound-1 on survival time of EAC cell bearing mice. **A**: Mean survival time (MST), **B**: Percentage increase of life span (%ILS); Data are expressed as mean ± S.E.M (n = 6); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

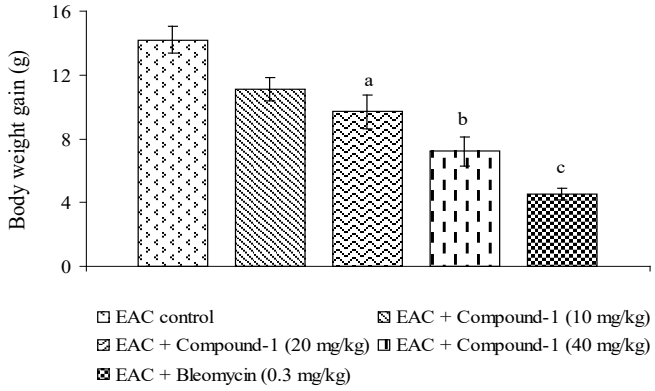


Figure 3.62 Effect of compound-1 on body weight gain of EAC cell bearing mice after 12 days. Data are expressed as mean ± S.E.M (n = 8); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

Biochemical estimation as shown in table 3.27 indicated the elevated level of cholesterol, triglycerides, urea, SALP and SGOT in serum in untreated EAC control group with respect to normal animals while glucose level was significantly ($P<0.001$) reduced from normal value. The development of hypoglycemia and hyperlipidemia in experimental animals with carcinoma has been previously reported[200-201]. In this experiment, reduced glucose level and elevated cholesterol and triglycerides were returned more or less to normal level in compound-1 treated mice, thereby again indicating its antitumour efficacy (Table 3.27). Inoculation and progression of EAC proliferation in mice have also an association with some extent of kidney damage and elevation of blood urea[207]. Compound-1 reduced the elevated level of blood urea.

Significant elevation in the levels of SGOT and SALP reflected the some extent cellular damages that were associated after 12 days of inoculation with EAC[208]. Treatment with the compound-1 restored the elevated biochemical parameters more or less to normal range (Table 3.27), indicating the protection of the tumor cell induced cellular damages by compound-1. However, no such inference could be drawn from the SGPT assay, as it was not notably affected by the tumour growth. But significant elevation in SGPT activity was observed for treatment of compound-1 at 10, 20 and 40 mg/kg (Table 3.27).

In vitro anti-leukaemic and anti-mutagenic effects of isolated di-(2-ethylhexyl) phthalate (DEHP) from *Aloe vera* Linn were also reported against human tumour cell lines[122]. In this study, we demonstrated *in vivo* the remarkable antitumour activity of compound-1 (here identified as di-(2-ethylhexyl) phthalate) from *Calotropis gigantea* flower against EAC.

Table 3.27 Effect of compound-1 on hematological and biochemical parameters of EAC cell bearing mice.

Parameters	Treatment (mg/kg body weight)					
	Normal	EAC + 2 % DMSO	EAC + Compound-1 (10)	EAC + Compound-1 (20)	EAC + Compound-1 (40)	EAC + *Bleomycin* (0.3)
Hgb (g/dL)	15.4 ± 0.22	7.35 ± 0.20*	7.76 ± 0.82	9.83 ± 0.68^{t}	9.92 ± 0.58^{a}	14.3 ± 0.25^{c}
RBC(x10^9 cells/mL)	5.67 ± 0.10	2.27 ± 0.06*	2.69 ± 0.13	3.41 ± 0.27^{a}	4.51 ± 0.42^{b}	4.90 ± 0.09^{c}
WBC(x10^6 cells/mL)	8.75 ± 0.53	25.3 ± 1.19*	16.1 ± 2.01^{a}	17.1 ± 1.75^{a}	15.7 ± 1.75^{b}	9.37 ± 0.59^{c}
Lymphocytes (%)	77.5 ± 1.36	33.8 ± 1.35*	34.5 ± 3.28	40.0 ± 2.29	49.6 ± 3.18^{a}	68.2 ± 0.90^{c}
Neutrophils (%)	19.6 ± 1.38	63.7 ± 1.04*	59.2 ± 3.47	53.0 ± 2.17^{a}	44.8 ± 2.93^{a}	28.8 ± 0.93^{b}
Monocytes (%)	1.87 ± 0.40	1.50 ± 0.37	3.87 ± 0.71	4.75 ± 0.64^{a}	3.75 ± 0.56^{a}	2.00 ± 0.27
Glucose(mg/dL)	138.9 ± 1.15	54.3 ± 0.99*	63.8 ± 4.30	82.2 ± 6.44^{a}	107.6 ± 6.77^{b}	134.1 ± 1.21^{c}
Cholesterol (mg/dL)	103.8 ± 0.85	153.5 ± 0.78*	139.3 ± 4.52	133.4 ± 4.67^{b}	130.4 ± 3.95^{b}	112.4 ± 0.71^{c}
Triglyceride (mg/dL)	105.5 ± 1.02	186.8 ± 0.75*	151.2 ± 9.45	148.0 ± 6.51^{b}	124.0 ± 8.11^{b}	126.8 ± 1.28^{c}
Blood Urea (mg/dL)	24.5 ± 0.44	64.1 ± 0.88*	52.2 ± 4.53	49.1 ± 3.99^{b}	41.7 ± 3.22^{b}	32.2 ± 0.52^{b}
SALP (U/L)	106.4 ± 0.74	238.9 ± 0.66*	213.7 ± 8.49	193.2 ± 9.34^{b}	180.1 ± 6.23^{t}	133.1 ± 0.72^{c}
SGPT (U/L)	71.9 ± 0.52	61.6 ± 0.49	82.9 ± 4.90^{t}	89.5 ± 5.87^{a}	94.6 ± 6.30^{t}	71.3 ± 0.36
SGOT (U/L)	43.7 ± 0.84	242.6 ± 0.93*	221.5 ± 3.52	197.3 ± 3.94^{a}	199.6 ± 3.75^{b}	80.0 ± 0.78^{c}

Data are expressed as mean ± S.E.M. for six animals in each group. $^{*}P<0.001$: against normal group and $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: against EAC control group.

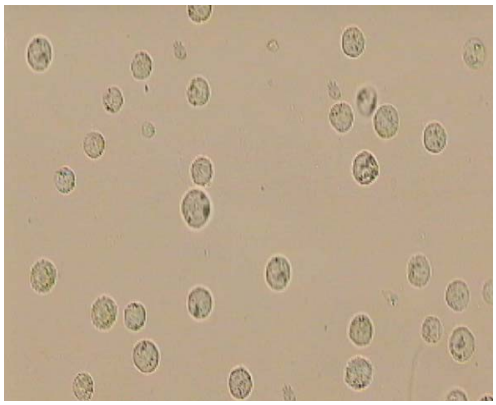


Figure 3.63 EAC cell smear of untreated EAC control

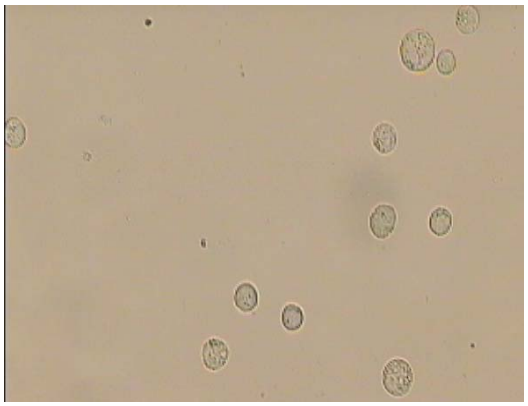


Figure 3.64 EAC cell smear of EECF treated mice

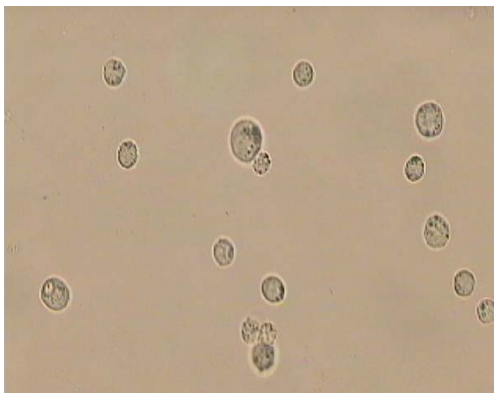


Figure 3.65 EAC cell smear of compound-1 treated mice.

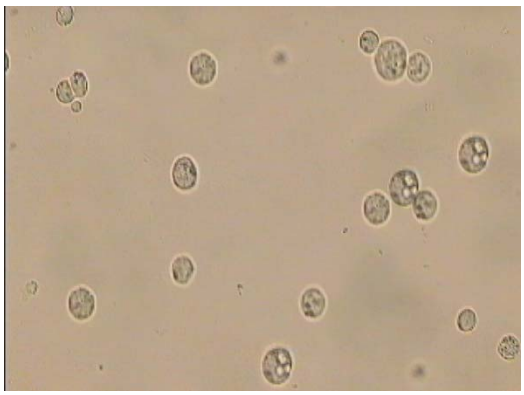


Figure 3.66 EAC cell smear of compound-2 treated mice.

3.3.16.3. Effect of compound-2 against EAC

The effects of compound-2 at the doses of 10 and 20 mg/kg on survival time, viable tumor cell count and body weight gain due to tumour burden are shown in table 3.28-3.29 and figure 3.66 to figure 3.69. The prolongation of life span is a reliable criterion for judging efficacy of anticancer drugs[206] and the compound-2 was able to meet this criterion through reducing the viable EAC cells. Treatment with compound-2 at the doses of 10 and 20 mg/kg significantly ($P<0.05$ & $P<0.01$) reduced viable tumor cell count and body weight gain in a dose-dependent manner as compared to that of the EAC control group (Table 3.28 and figure 3.67 & 3.69). In the EAC control group, the mean survival time was 19.8±1.40 days, while it increased to 25.6±1.58 (10 mg/kg), and 31.0±0.96 (20 mg/kg) days ($P<0.05$), respectively, in the compound-2 treated groups (Table 3.29). The standard drug bleomycin (0.3 mg/kg) treated group had a mean survival time of 39.0±0.85 days ($P<0.001$) (Figure 3.68). The percentage increase in the lifespan (%ILS) of EAC cell bearing mice treated with compound-2 (20 mg/kg) was found to be 56.32% as compared to the control group whereas it was 96.97% for bleomycin (0.3 mg/kg) (Figure 3.68).

Table 3.28 Effect of compound-2 on Ehrlich ascites carcinoma (EAC) cell growth.

Group No.	Treatment	Viable EAC cells on day 6 after inoculation (x 10^7 cells/mL)	Percentage (%) cell growth inhibition
1	EAC control	5.241 ± 0.30	-
2	EAC + Compound-2 (10 mg/kg)	2.404 ± 0.24[a]	54.13 ± 4.51
3	EAC + Compound-2 (20 mg/kg)	1.482 ± 0.11[b]	71.72 ± 2.18
4	EAC + Bleomycin (0.3 mg/kg)	0.261 ± 0.05[c]	95.02 ± 1.20

Data are expressed as mean ± S.E.M (n = 6);

[a]P<0.05, [b]P<0.01 and [c]P<0.001: Significantly different from group 1

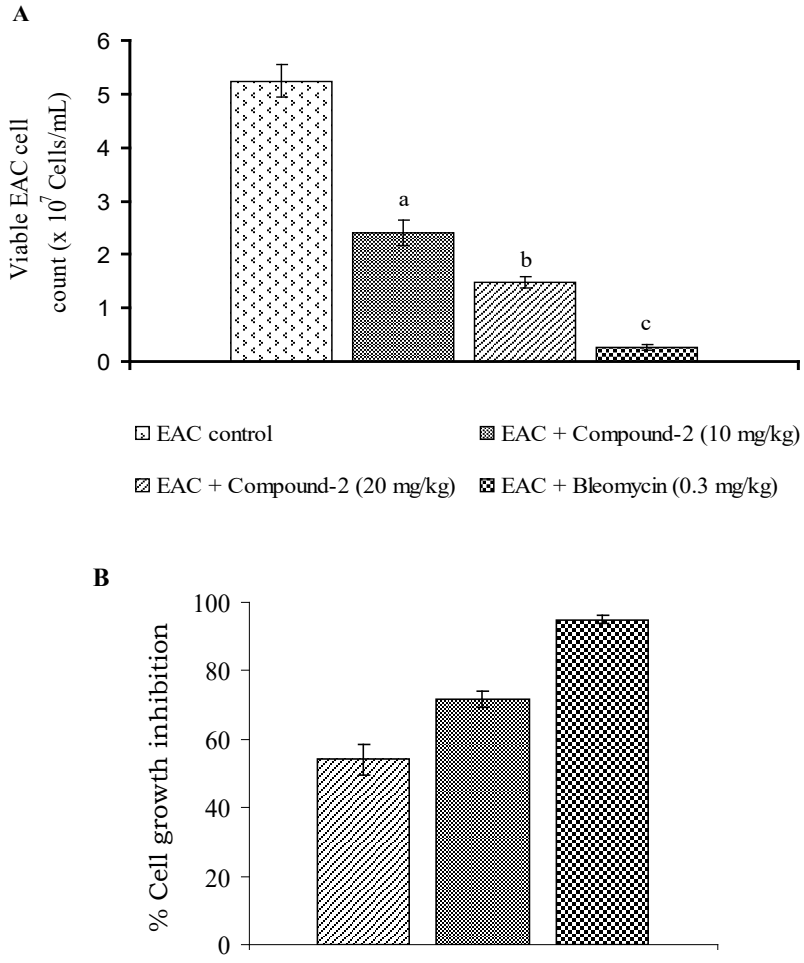


Figure 3.67 Effect of compound-2 on EAC cell growth. **A**: Viable EAC cells on day 6 after tumor cell inoculation, **B**: % of cell growth inhibition; Data are expressed as mean ± S.E.M (n = 6); [a]P<0.05, [b]P<0.01 and [c]P<0.001: Significance difference with respect to EAC control.

Table 3.29 Effect of compound-2 on survival time and body weight gain of EAC cell bearing mice

Group No.	Treatment	MST (in days)	%ILS	Body weight gain (g) after 12 days
1	EAC control	19.83 ± 1.40	-	16.2 ± 0.85
2	EAC + Compound-2 (10 mg/kg)	25.66 ± 1.08[a]	29.39 ± 5.41	10.1 ± 0.73[a]
3	EAC + Compound-2 (20 mg/kg)	31.00 ± 0.96[a]	56.32 ± 4.87	7.9 ± 0.65[b]
4	EAC + Bleomycin (0.3 mg/kg)	39.00 ± 0.85[c]	96.67 ± 3.97	4.5 ± 0.36[c]

Data are expressed as the mean ± S.E.M (n = 6); Significantly different from group 1: [a]P<0.05, [b]P<0.01 and [c]P<0.001; MST: Mean survival time; %ILS: Percentage (%) increase of life span.

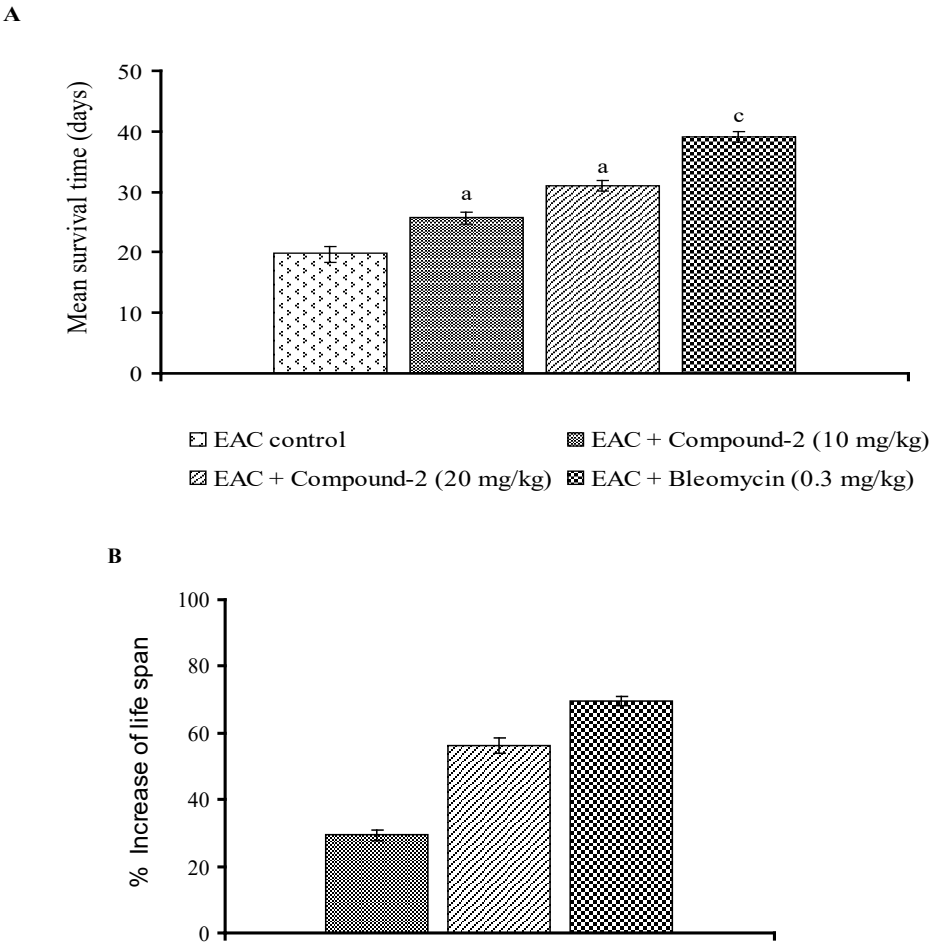


Figure 3.68 Effect of compound-2 on survival time of EAC cell bearing mice. **A**: Mean survival time (MST), **B**: Percentage increase of life span (%ILS); Data are expressed as mean ± S.E.M (n = 6); [a]P<0.05, [b]P<0.01 and [c]P<0.001: Significance difference with respect to EAC control.

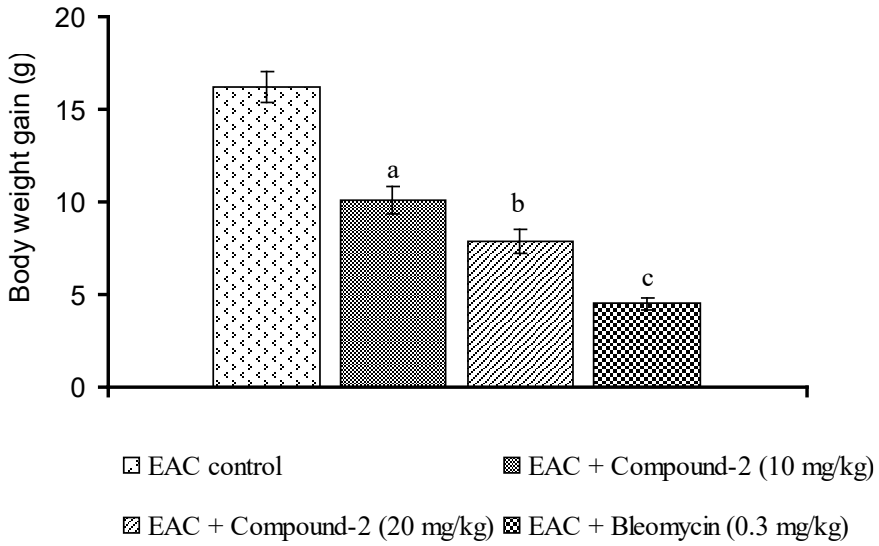


Figure 3.69 Effect of compound-2 on body weight gain of EAC cell bearing mice after 12 days. Data are expressed as mean ± S.E.M (n = 8); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

Myelosuppression and anemia (reduced haemoglobin) have been frequently observed in ascites carcinoma[199]. Anemia encountered in ascites carcinoma mainly due to iron deficiency, either by haemolytic or myelopathic conditions which finally lead to reduced RBC number[199]. In this study, elevated WBC count, reduced haemoglobin and RBC count were observed in EAC control mice (Table 3.30). Administration of compound-2 at 20 mg/kg significantly ($P<0.01$) reduced total WBC count in respect to that of EAC control group. RBC count and hemoglobin content were found to be significantly ($P<0.05$; $P<0.01$) restored to the normal levels in the animals treated with compound-2 (10 and 20 mg/kg) as well as standard drug bleomycin (0.3 mg/kg) (Table 3.30). The restoring capability of compound-2 on altered hemoglobin, total RBC and WBC count has indicated its haematopoietic protecting activity without inducing myelotoxicity, the most common side effects of cancer chemotherapy[199]. Compound-2 did not show any significant efficacy to bring the altered differential count of WBC.

Numerous studies on the enzymes of carbohydrate metabolism in cancer showed that actively dividing neoplastic tissues require more energy than normal cells[209]. The consequent display of a high rate of glycolysis in malignant conditions is clinically manifested in the increased activity of several serum enzymes[210-211]. In our study, twelve (12) days of inoculation with EAC brought the significant ($P<0.001$) elevation in the levels of SGOT and SALP (Table 3.30). Treatment with compound-2 restored the

elevated biochemical parameters more or less to normal range thereby supporting its protective effect on the tumour induced complications.

The development of hypoglycemia and hyperlipidemia in experimental animals with carcinoma has been previously reported[200-201]. In this experiment, reduced glucose level and elevated cholesterol and triglycerides were returned more or less to normal level in compound-2 treated mice (Table 3.30). Inoculation and progression of EAC proliferation in mice have also an association with some extent of kidney damage and elevation of blood urea[212]. Compound-2 reduced the elevated level of blood urea.

Compound-2 was a pentacyclic terpenoids and *in vitro* it exhibited potent cytotoxicity against A549, SK-OV-3, SK-MEL-2, MES-SA and HCT-15 tumour cell lines[124]. Generally pentacyclic triterpenoids exert their antitumor activity through apoptosis induction. They were associated with early disruption of the endoplasmic reticulum and alterations in calcium homeostasis which are early events in activation of caspases[213-214]. In this study, we demonstrated *in vivo* the remarkable antitumour activity of compound-2 against EAC but the mechanisms of action should be further addressed.

Table 3.30 Effect of compound-2 on hematological and biochemical parameters of EAC cell bearing mice.

Parameters	Normal	EAC control	EAC + Compound-2 (10 mg/kg)	EAC + Compound-2 (20 mg/kg)	EAC + *Bleomycin* (0.3 mg/kg)
Hgb (g/dL)	12.2 ± 0.55	5.8 ± 0.53*	7.9 ± 0.47	9.6 ± 0.58[b]	15.8 ± 0.25[c]
RBC(x10^9 cells/mL)	5.4 ± 0.28	2.5 ± 0.27*	3.6 ± 0.27[a]	4.2 ± 0.24[b]	5.16 ± 0.09[c]
WBC(x10^6 cells/mL)	9.1 ± 1.57	36.1 ± 3.89*	27.6 ± 3.05	21.3 ± 2.07[b]	8.62 ± 0.59[c]
Lymphocytes (%)	70.0 ± 1.82	30.3 ± 1.83*	38.5 ± 1.76	36.3 ± 2.21	69.4 ± 0.90[c]
Neutrophils (%)	25.5 ± 1.64	64.1 ± 1.64*	56.3 ± 1.54	58.5 ± 2.32	26.1 ± 0.93[c]
Monocytes (%)	2.6 ± 0.49	4.3 ± 0.80	3.83 ± 0.47	3.83 ± 0.47	2.00 ± 0.27
Glucose(mg/dL)	142.3 ± 2.74	76.9 ± 2.87*	103.9 ± 5.02[a]	108.0 ± 4.02[b]	140.5 ± 1.21[c]
Cholesterol (mg/dL)	109.3 ± 2.50	183.7 ± 3.68*	155.4 ± 5.87	141.9 ± 4.46[b]	120.4 ± 0.71[c]
Triglyceride (mg/dL)	125.4 ± 1.86	179.6 ± 4.09*	173.1 ± 2.92	150.2 ± 3.81[b]	126.7 ± 1.28[c]
Blood Urea (mg/dL)	27.3 ± 1.93	85.1 ± 3.48*	75.5 ± 2.08	42.9 ± 1.66[b]	30.5 ± 0.52[c]
SALP (U/L)	122.2 ± 4.28	234.6 ± 2.38*	196.0 ± 3.36[a]	180.3 ± 3.75[b]	129.6 ± 0.72[c]
SGPT (U/L)	67.0 ± 2.33	73.3 ± 2.70	68.5 ± 3.12	70.5 ± 2.18	73.3 ± 0.36
SGOT (U/L)	39.3 ± 0.84	229.0 ± 3.75*	216.0 ± 4.61	197.3 ± 4.17[b]	94.2 ± 0.78[c]

Data are expressed as mean ± SEM. for six animals in each group.

*P<0.001: against normal group and [a]P<0.05, [b]P<0.01 and [c]P<0.001: against EAC control group.

3.3.16.4. Effect of ME, PEF and CF against EAC

The LD_{50} value of ME, PEF and CF was evaluated in Swiss albino mice and found to be 259.2, 905.1 and 482.5 mg/kg body weight, respectively. The average number of viable EAC cells per mouse of EAC control group was found to be (5.12 ± 0.18) x 10^7 cells/mL. Treatment with ME (10 and 20 mg/kg), PEF (80 mg/kg) and CF (20 and 40 mg/kg) decreased the viable cells significantly ($P<0.05$; $P<0.01$)) (Table 3.31 and figure 3.70). In addition, microscopic observations of EAC cell smear of control, ME, PEF and CF treated group have been shown in figure 3.75 to 3.78.

The effect of ME, PEF and CF on the survival of tumor-bearing mice is shown in table 3.31 and figure 3.71. The MST for the control group was 19.8±0.47 days, whereas it was 26.8±2.14, 28.5±1.62 ($P<0.05$), 24.1±0.92, 25.1±1.80, 26.5±1.43, 31.1±1.52 ($P<0.01$) and 39.0±0.85 ($P<0.001$) days for the groups treated with ME (10 and 20 mg/kg), PEF (40 and 80 mg/kg) and CF (20 and 40 mg/kg) and bleomycin (0.3 mg/kg) respectively (Table 3.32 and figure 3.71). Among the test samples, the highest increase in the lifespan of tumor-bearing mice treated with CF (40 mg/kg) was found to be 57.07% as compared to the control group whereas it was 96.97% for bleomycin (0.3 mg/kg). PEP did not show significant efficacy to increase the MST of EAC cell bearing mice.

Table 3.31 Effect of ME, PEF and CF on Ehrlich ascites carcinoma (EAC) cell growth.

Group No.	Treatment	Viable EAC cells on day 6 after inoculation (x 10^7 cells/mL)	Percentage (%) cell growth inhibition
1	EAC control	5.12 ± 0.18	-
2	EAC + ME (10 mg/kg)	2.35 ± 0.06[a]	54.10 ± 4.51
3	EAC + ME (20 mg/kg)	1.52 ± 0.07[b]	70.31 ± 2.18
4	EAC + PEF (40 mg/kg)	3.54 ± 0.16	30.85 ± 3.89
5	EAC + PEF (80 mg/kg)	2.67 ± 0.06[a]	47.85 ± 2.50
6	EAC + CF (20 mg/kg)	2.45 ± 0.08[a]	52.14 ± 3.13
7	EAC + CF (40 mg/kg)	1.19 ± 0.15[b]	76.75 ± 2.66
8	EAC + Bleomycin (0.3 mg/kg)	0.47 ± 0.06[c]	90.82 ± 2.20

Data are expressed as mean ± S.E.M (n = 6);

[a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$: Significantly different from group 1

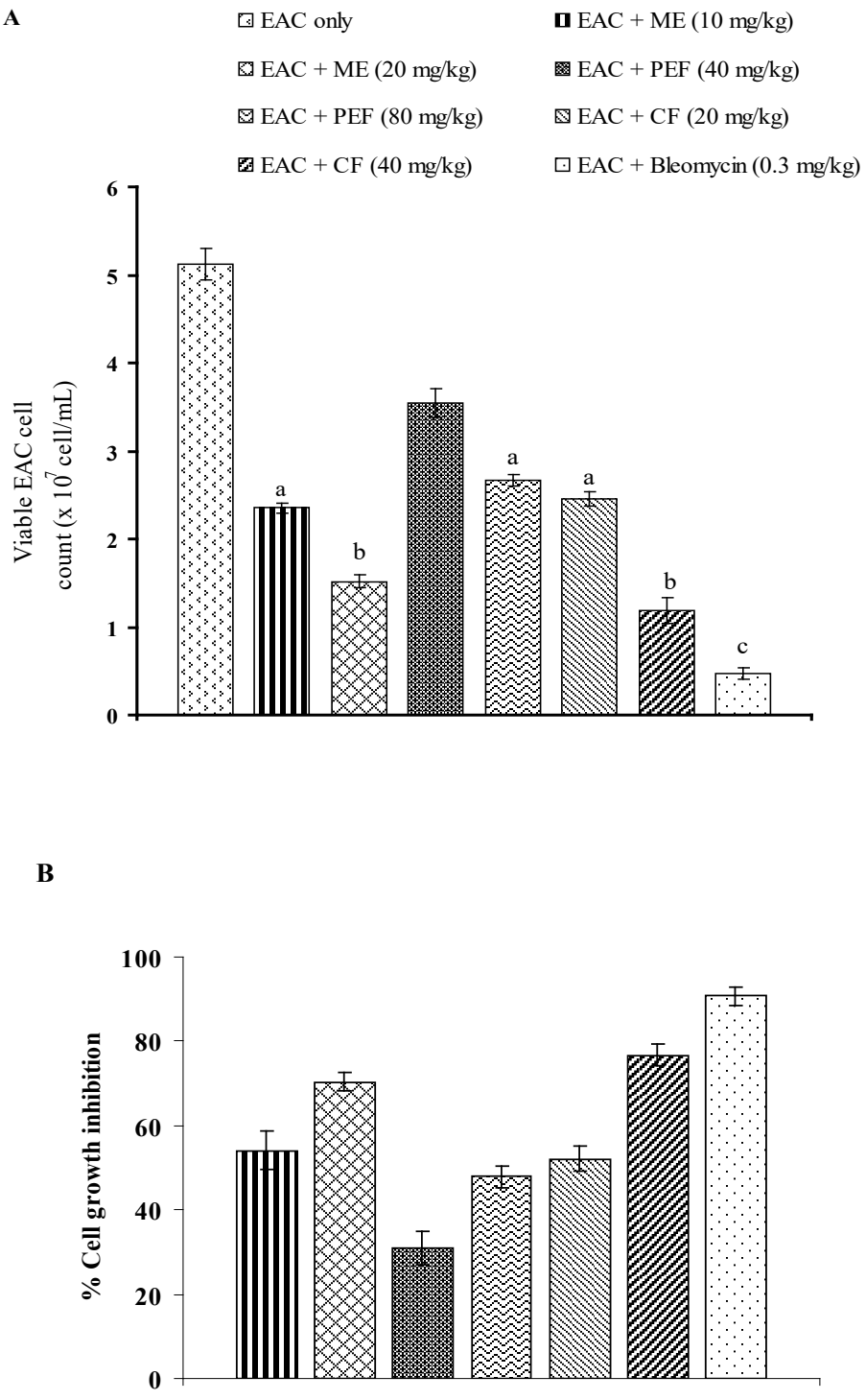
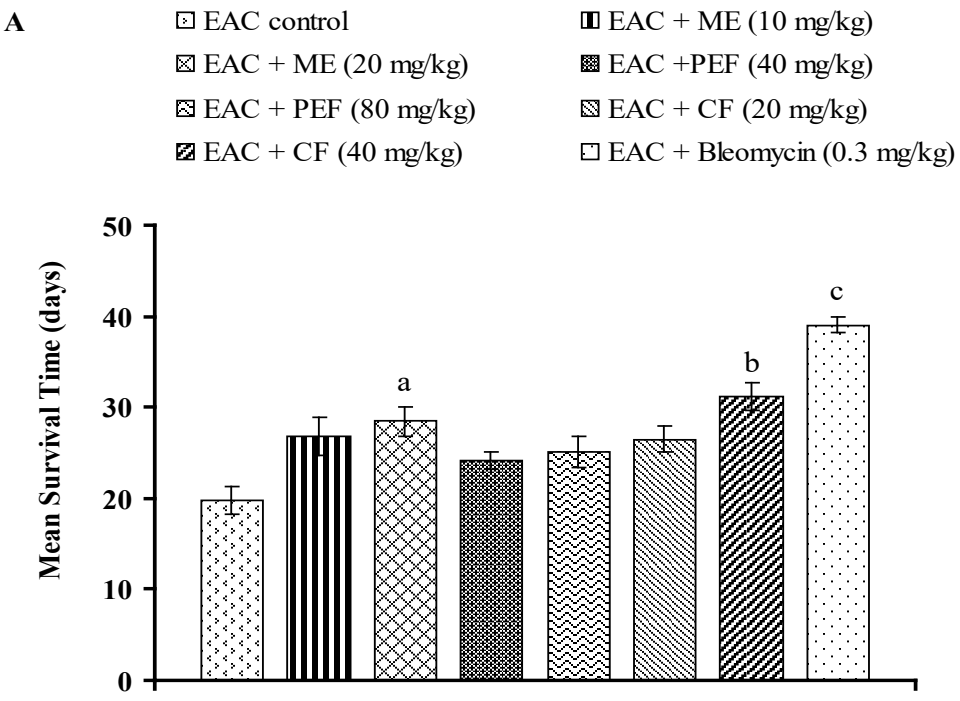


Figure 3.70 Effect of ME, PEF and CF on EAC cell growth. **A**: Viable EAC cells on day 6 after tumor cell inoculation, **B**: % of cell growth inhibition; Data are expressed as mean ± S.E.M (n = 6); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

Table 3.32 Effect of ME, PEF and CF on survival time and body weight gain of EAC cell bearing mice

Group No.	Treatment	MST (in days)	%ILS
1	EAC control	19.8 ± 1.47	-
2	EAC + ME (10 mg/kg)	26.8 ± 2.14	35.3 ± 1.38
3	EAC + ME (20 mg/kg)	28.5 ± 1.62[a]	43.9 ± 2.15
4	EAC + PEF (40 mg/kg)	24.1 ± 0.92	21.7 ± 1.05
5	EAC + PEF (80 mg/kg)	25.1 ± 1.80	26.7 ± 1.57
6	EAC + CF (20 mg/kg)	26.5 ± 1.43	33.8 ± 1.90
7	EAC + CF (40 mg/kg)	31.1 ± 1.52[b]	57.0 ± 2.36
8	EAC + Bleomycin (0.3 mg/kg)	39.0 ± 0.85[c]	96.67 ± 3.24

Data are expressed as the mean ± S.E.M (n = 6); Significantly different from group 1: [a]P<0.05, [b]P<0.01 and [c]P<0.001; MST: Mean survival time; %ILS: Percentage (%) increase of life span.



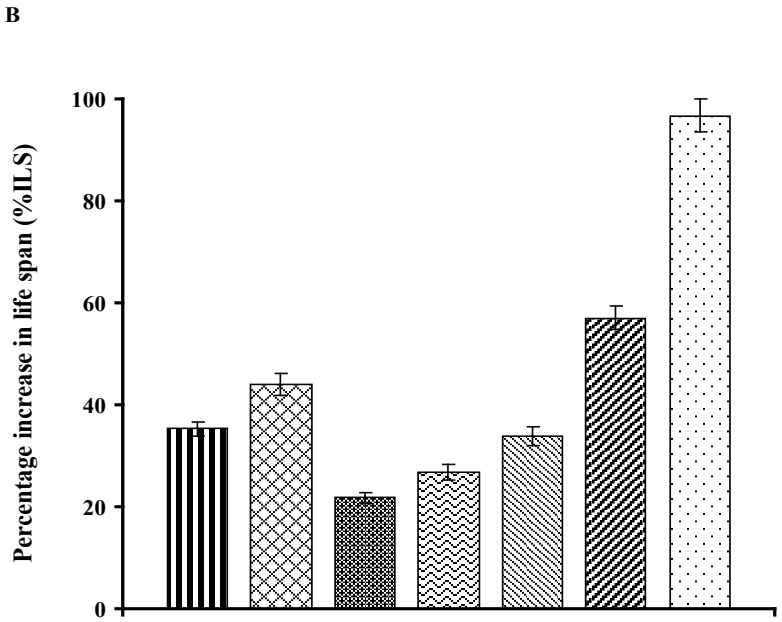


Figure 3.71 Effect of ME, PEF and CF on survival time of EAC cell bearing mice. **A**: Mean survival time (MST), **B**: Percentage increase of life span (%ILS); Data are expressed as mean ± S.E.M (n = 6); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

In EAC cell bearing mice, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells[198]. In this investigation, treatment with ME and CF decreased the viable EAC cell count and increased the life span of the tumor bearing mice, (Table 3.31 & 3.32 and figure 3.70 & 3.71). The reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals[206]. ME and CF by decreasing the nutritional fluid volume and arresting the tumor growth, increases the life span of EAC-bearing mice. Thus, ME and CF has potent antitumor activity against EAC bearing mice.
Anemia is a common complication in cancer and the situation aggravates further during chemotherapy since a majority of antineoplastic agents exert suppressive effects on erythropoiesis[199] and thereby limiting the use of these drugs. On day 14th after inoculation, hematological parameters of untreated EAC cell bearing mice showed significant ($P<0.001$) changes when compared with the normal mice (Table 3.33). At the same time interval, ME (10 and 20 mg/kg) and CF (20 and 40 mg/kg) treatment could change ($P<0.05$; $P<0.01$) these altered parameters to near normal whereas no significant change was found for PEF (Table 3.33).

Table 3.33 Effect of ME, PEF and CF on hematological and biochemical parameters of EAC cell bearing mice.

Parameters	Treatment (mg/kg body weight)								
	Normal	EAC + Vehicle	EAC + ME (10 mg/kg)	EAC + ME (20 mg/kg)	EAC + PEF (40 mg/kg)	EAC + PEF (80 mg/kg)	EAC + CF (20 mg/kg)	EAC + CF (40 mg/kg)	EAC + Bleomycin (0.3 mg/kg)
Hgb (g/dL)	12.05 ± 0.34	6.61 ± 0.11*	8.16 ± 0.21[a]	9.78 ± 0.19[b]	6.21 ± 0.29	6.73 ± 0.16	9.51 ± 0.24[a]	10.15 ± 0.25[b]	14.37 ± 0.25[c]
RBC ($x10^9$ cells/mL)	6.10 ± 0.05	2.98 ± 0.04*	3.76 ± 0.03[a]	4.24 ± 0.03[a]	2.97 ± 0.04	3.28 ± 0.09	4.01 ± 0.05[a]	4.56 ± 0.04[b]	4.90 ± 0.09[c]
WBC ($x10^6$ cells/mL)	5.83 ± 0.53	17.5 ± 0.99*	12.3 ± 0.66[a]	10.2 ± 0.98[b]	17.6 ± 1.22	16.5 ± 0.61	11.3 ± 0.71[a]	8.16 ± 0.60[b]	9.37 ± 0.59[c]
Lymphocytes (%)	69.5 ± 0.76	35.3 ± 1.17*	42.2 ± 1.30[a]	46.6 ± 0.49[a]	34.2 ± 1.24	38.6 ± 1.33	45.8 ± 1.49[a]	53.1 ± 1.55[b]	68.25 ± 0.90[c]
Neutrophils (%)	26.6 ± 1.28	60.5 ± 1.33*	53.6 ± 1.62	49.0 ± 1.06[a]	62.5 ± 1.64	55.8 ± 1.70	50.3 ± 1.40[a]	43.6 ± 1.22[a]	28.87 ± 0.93[c]
Monocytes (%)	2.5 ± 0.3	2.6 ± 0.33	2.7 ± 0.34	2.5 ± 0.42	2.0 ± 0.44	3.0 ± 0.36	2.5 ± 0.22	2.1 ± 0.42	2.00 ± 0.27
SALP (U/L)	113.1 ± 1.35	293.0 ± 1.70*	253.6 ± 1.38[a]	168.5 ± 1.24[b]	287.2 ± 1.66	266.6 ± 3.62	187.3 ± 1.57[a]	163.7 ± 1.25[b]	133.1 ± 0.72[c]
SGPT (U/L)	56.3 ± 0.52	51.3 ± 1.12	53.0 ± 1.85	79.1 ± 2.93[b]	51.5 ± 1.84	56.3 ± 0.70	62.4 ± 1.47	83.3 ± 1.16[b]	71.3 ± 0.36[b]
SGOT (U/L)	45.1 ± 1.10	290.3 ± 1.00*	205.2 ± 1.55[a]	154.3 ± 1.95[b]	287.9 ± 1.94	277.9 ± 1.58	206.1 ± 0.92[a]	184.7 ± 1.51[b]	80.0 ± 0.78[c]

Data are expressed as mean ± S.E.M. for eight animals in each group.

*P<0.001: against normal group and [a]P<0.05, [b]P<0.01 and [c]P<0.001: against EAC control group

In this study, fourteen (14) days of inoculation with EAC brought the significant ($P<0.001$) elevation in the activities of SALP and SGOT when compared with the respective normal values (Table 3.33). Significant ($P<0.05$; $P<0.01$) depletion in the activities of SALP and SGOT was found by treatment with ME (at 10 and 20 mg/kg) and CF (20 and 40 mg/kg). ME and CF restored the elevated activities of SALP and SGOT more or less to normal range thereby indicating their protective effect on the tumour induced complications. At 0.3 mg/kg dose, *bleomycin* significantly ($P<0.01$; $P<0.001$) also decreases the activities of SALP and SGOT. However some extent of hepatotoxicity was associated with the treatment of ME (20 mg/kg) and CF (40 mg/kg) as indicated by the elevation in the levels of SGPT (Table 3.33).

Preliminary thin layer chromatography (TLC) screening also showed that ME and CF contained flavonoid, glycosides, steroids and terpenoids type compounds. Many such type of compounds are known to possess potent antitumor properties[204]. In addition, *In vitro* some phytochemicals with potent cytotoxic effect have already been reported from the root bark of *Calotropis gigantea*[46,51]. So findings of this study were consistence with previous one.

3.3.16.5. Effect of EAEAR and DMEAR against EAC

Intraperitoneal administration of graded doses of EAEAR and DMEAR to Swiss albino mice, in our toxicity study produced a LD_{50} of 723±0.62 and 563±0.95 mg/kg body weight, respectively.

Effect of EAEAR and DMEAR on EAC cell growth on day 5 after EAC cell transplantation is shown in table 3.34 and figure 3.72. Treatment with DMEAR resulted in pronounced cell growth inhibition at doses 20 mg/kg and 40 mg/kg. 37.69% and 57.69% cell growth inhibition was resulted by treatment with EAEAR at 20 mg/kg and 40 mg/kg doses, respectively. On the other hand, the established antitumour drug *bleomycin* showed the highest inhibition (91.74%) at dose 0.3 mg/kg. Reduction of viable EAC cell by EAEAR and DMEAR was also evidenced by microscopic observation (figure 3.79 & figure 3.80).

The effect of EAEAR and DMEAR on the survival of tumour bearing mice is shown in table 3.35 and figure 3.73. In the EAC control group, the mean survival time (MST) was 19.0±2.16 days and it is increased dose dependently at 20 mg/kg (23±1.70) and 40 mg/kg (26±1.41) ($P<0.05$) in the EAEAR treated groups. The MST of DMEAR treated groups were 28±1.41 ($P<0.05$) and 32±1.87 $P<0.01$) days at 20 mg/kg and 40 mg/kg doses, respectively whereas the standard drug *bleomycin* (0.3 mg/kg)-treated group had a MST of 37±0.95 ($P<0.001$). The increase in the life span of EAC cell bearing mice treated with

EAEAR (20 and 40 mg/kg), DMEAR (20 and 40 mg/kg) and *bleomycin* (0.3 mg/kg) was found to be 21.05%, 36.84%, 47.36%, 68.84% and 89.47%, respectively. The effect of EAEAR and DMEAR on the inhibition of average increase in body weight is also shown in table 3.35 and figure 3.74. The average weight gain of tumour control group was 18.7±0.83 g whereas it was 16.1±0.80 ($P<0.05$), 13.6±0.71 ($P<0.05$), 10.4±0.58 ($P<0.05$), 8.6±0.98 ($P<0.01$) and 6.8±0.65 ($P<0.001$) for the groups treated with EAEAR (20 and 40 mg/kg), DMEAR (20 and 40 mg/kg) and *bleomycin* (0.3 mg/kg), respectively. In this study, treatment with EAEAR and DMEAR showed enhancement of mean survival time (MST), decrease in body weight gain and tumour cell growth inhibition thereby exhibiting the reliable criteria for judging their value as any anticancer drug[206].

As shown in table 3.37, hemoglobin content and RBC count in the EAC control group was significantly ($P<0.001$) decreased as compared to the normal group. Treatment with EAEAR (40 mg/kg) and DMEAR (40 mg/kg) significantly ($P<0.05$; $P<0.01$)) increased the hemoglobin content and RBC count more or less to normal levels. The total WBC count was found to be increased significantly ($P<0.05$) in the EAC control group when compared with the normal group. Administration of DMEAR and EARAR in EAC bearing mice significantly ($P<0.05$; $P<0.01$) reduced the WBC count at 40 mg/kg. The differential count of WBC showed that the percentage of neutrophils was increased while that of lymphocytes was decreased significantly ($P<0.05$) in the EAC control group when compared to normal mice. DMEAR at 20 and 40 mg/kg doses reverted these altered parameters more or less to the normal values. At 0.3 mg/kg, standard drug *bleomycin* restored all hematological parameters to normal level. So treatment with EAEAR and DMEAR reverted the hemoglobin content, RBC and WBC cell count near to normal values. This indicates the protective action of EAEAR and DMEAR on the heamopoietic system to overcome myelosuppression and anemia that are encountered as major problems in cancer chemotherapy[199].

Elevation in the activity of several serum enzymes were clinically manifested in malignant conditions[209-211]. Here mice of EAC control group showed significant ($P<0.001$) increase in the activities of ALP and SGOT when compared with the respective normal values (Table 3.36). Significant ($P<0.05$) depletion in the activities of ALP and SGOT was found by treatment with EAEAR (40 mg/kg) and DMEAR (20 and 40 mg/kg). At 0.3 mg/kg dose, *bleomycin* significantly ($P<0.001$) decreased the activities of ALP and SGOT. However SGPT was not significantly altered by tumour growth in only tumour bearing mice but DMEAR at 40 mg/kg dose, increased SGPT ($P<0.05$) when compared with only EAC cell bearing mice.

In our studies, DMEAR have potential antitumour activity than EAEAR and it is comparable to that of *bleomycin*, which is commonly used as an active antitumour agent in vast series of preclinical and clinical studies[203]. Preliminary phytochemical screening of EAEAR and DMEAR showed the presence of terpenes, flavonoids and glycosides. Terpenes and glycosides have antitumour effects[204,215]. Flavonoids have been shown to possess antimutagenic and antimalignant effects[216]. The antitumour properties of the extracts may due to the presence of these compounds.

Table 3.34 Effect of EAEAR and DMEAR on Ehrlich ascites carcinoma (EAC) cell growth.

Group No.	Treatment	Viable EAC cells on day 6 after inoculation (x 10^7 cells/mL)	Percentage (%) cell growth inhibition
1	EAC + 2% (v/v) DMSO	3.515±0.18	-
2	EAC + EAEAR (20 mg/kg)	2.19±0.35[a]	37.69 ± 1.77
3	EAC + EAEAR (40 mg/kg)	1.48±0.37[b]	57.89 ± 2.43
4	EAC + DMEAR (20 mg/kg)	1.53±0.43[b]	56.47 ± 2.80
5	EAC + DMEAR (40 mg/kg)	0.87±0.43[c]	75.10 ± 3.16
6	EAC + *Bleomycin* (0.3 mg/kg)	0.29±0.09[c]	91.74 ± 2.54

Data are expressed as mean ± S.E.M (n = 6); [a]P<0.05, [b]P<0.01 and [c]P<0.001: Significantly different from group 1

Table 3.35 Effect of EAEAR and DMEAR on survival time and body weight gain of EAC cell bearing mice

Group No.	Treatment	MST (in days)	% ILS	Average increase in body weight (g)
1	EAC + 2% (v/v) DMSO	19 ± 2.16	-	18.7±0.83
2	EAC + EAEAR (20 mg/kg)	23 ± 1.70	21.05 ± 1.90	16.1±0.80[a]
3	EAC + EAEAR (40 mg/kg)	26 ± 1.41[a]	36.84 ± 2.07	13.6±0.71[a]
4	EAC + DMEAR (20 mg/kg)	28 ± 1.41[a]	47.36 ± 3.26	10.4±0.58[a]
5	EAC + DMEAR (40 mg/kg)	32 ± 1.87[b]	68.84 ± 2.60	8.6±0.98[b]
6	EAC + *Bleomycin* (0.3 mg/kg)	37 ± 0.95[c]	89.47 ± 2.18	6.8±0.65[c]

Data are expressed as the mean ± S.E.M (n = 6); Significantly different from group 1: [a]P<0.05, [b]P<0.01 and [c]P<0.001; MST: Mean survival time; %ILS: Percentage (%) increase of life span.

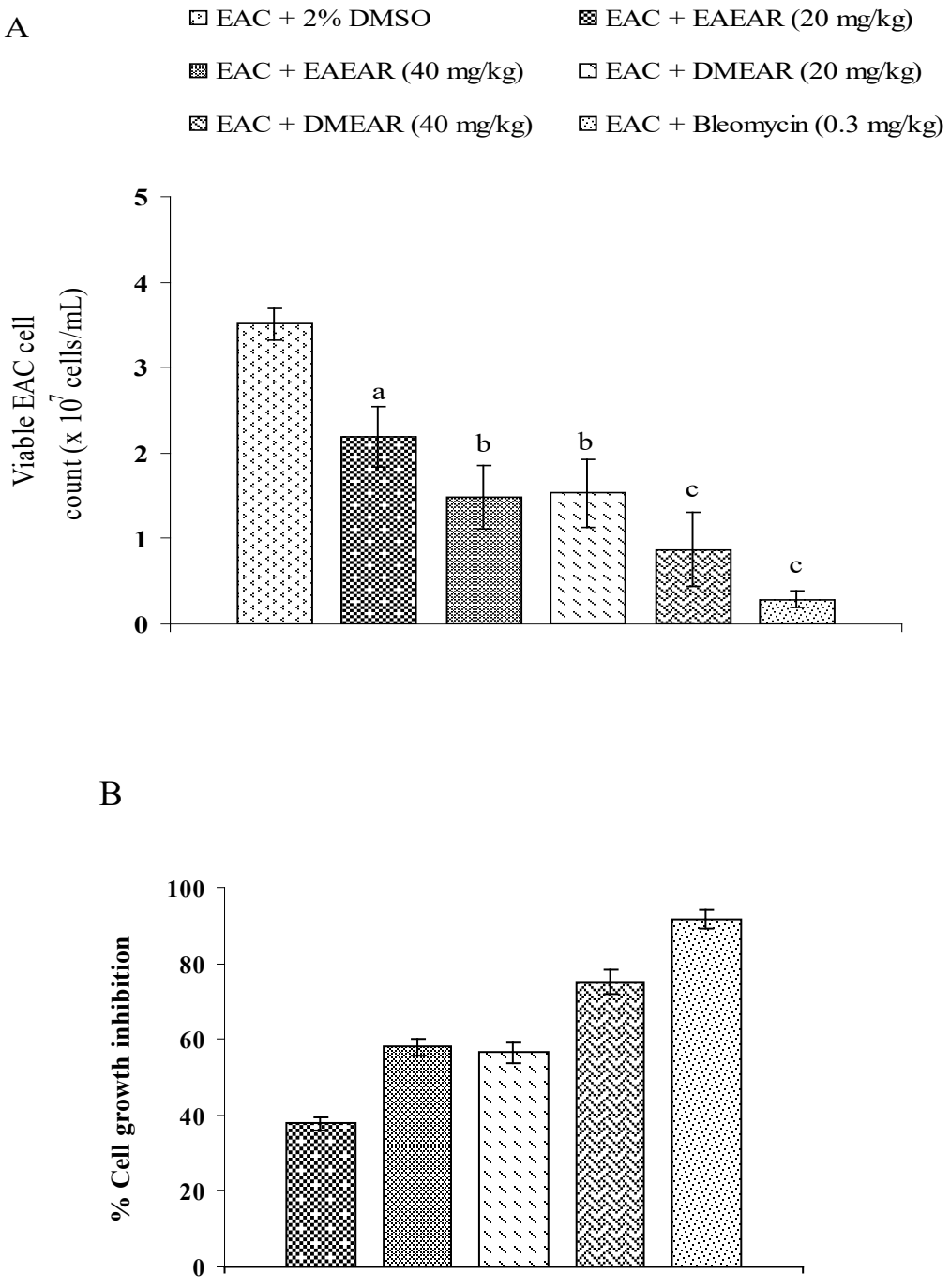


Figure 3.72 Effect of EAEAR and DMEAR on EAC cell growth. **A**: Viable EAC cells on day 6 after tumor cell inoculation, **B**: % of cell growth inhibition; Data are expressed as mean ± S.E.M (n = 6); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

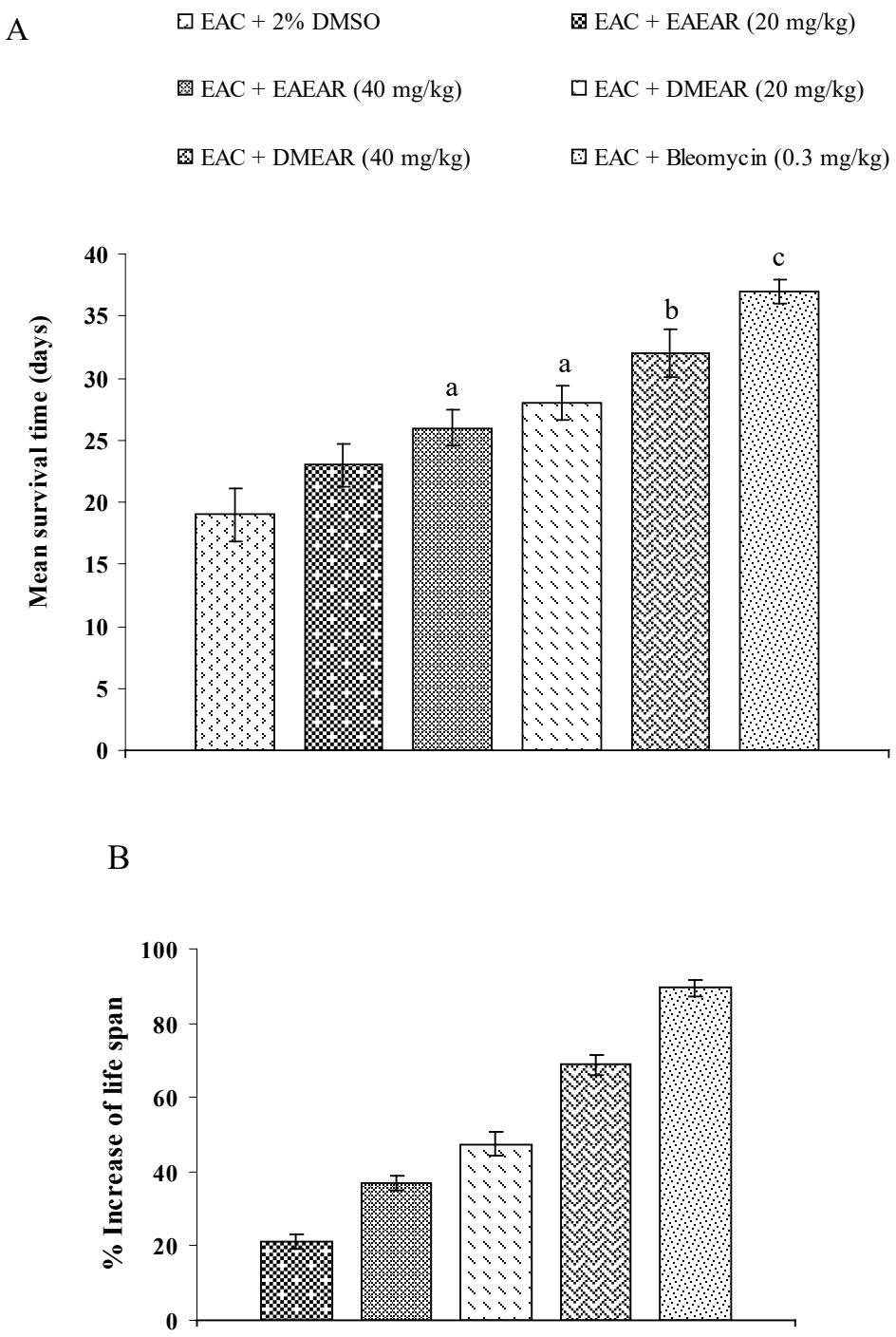


Figure 3.73 Effect of EAEAR and DMEAR on survival time of EAC cell bearing mice. **A**: Mean survival time (MST), **B**: Percentage increase of life span (%ILS); Data are expressed as mean ± S.E.M (n = 6); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

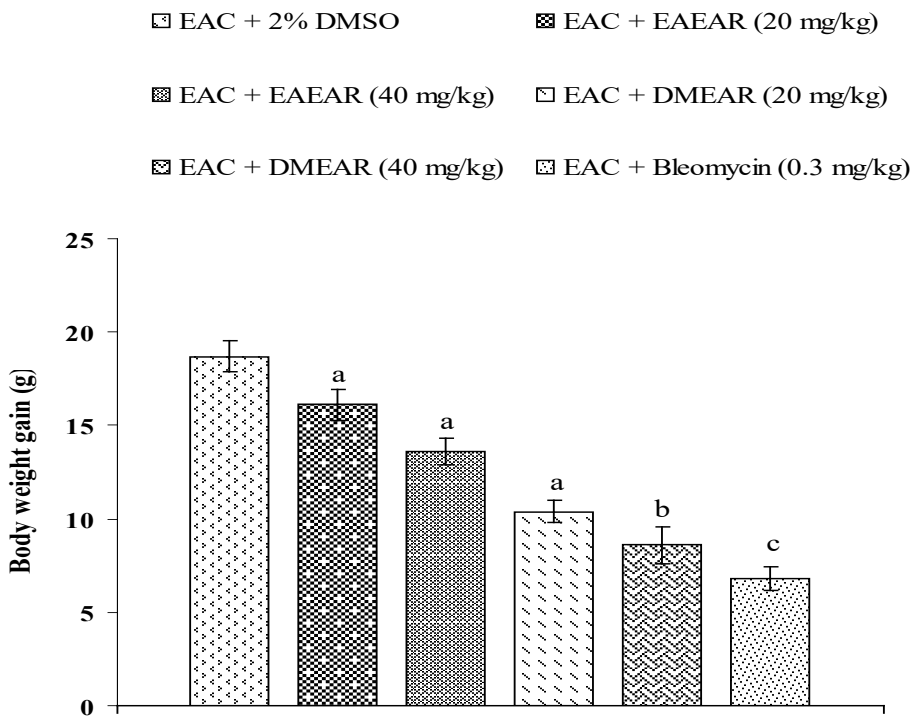


Figure 3.74 Effect of EAEAR and DMEAR on body weight gain of EAC cell bearing mice after 12 days. Data are expressed as mean ± S.E.M (n = 6); $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: Significance difference with respect to EAC control.

Table 3.36 Effect of EAEAR and DMEAR on ALP, SGPT and SGOT.

Group	Treatment	SALP (U/L)	SGPT (U/L)	SGOT (U/L)
1	Normal + 2% (v/v) DMSO	122.5 ± 3.7	68.9 ± 2.6	39.6 ± 1.4
2	EAC + 2% (v/v) DMSO	234.6 ± 5.1*	66.6 ± 1.9	84.1 ± 2.3*
3	EAC + EAEAR (20 mg/kg)	225.0 ± 7.7	68.3 ± 2.3	80.3 ± 1.2
4	EAC + EAEAR (40 mg/kg)	184.1 ± 5.0^{a}	66.9 ±1.9	75.3 ± 0.9^{a}
5	EAC + DMEAR (20 mg/kg)	144.3 ± 3.1^{a}	65.2 ± 2.0	58.6 ± 0.9^{b}
6	EAC + DMEAR (40 mg/kg)	126.5 ± 4.8^{c}	75.6 ± 2.4^{a}	51.4 ± 0.7^{b}
7	EAC + Bleomycin (0.3 mg/kg)	127.6 ± 4.1^{c}	68.0 ± 2.4	37.3 ± 1.6^{c}

Data are expressed as mean ± S.E.M. for eight animals in each group. $^{*}P<0.001$: against normal group and $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$: against EAC control group.

Table 3.37 Effect of EAEAR and DMEAR on hematological parameters

Treatment	Hb (g/dL)	RBC ($x10^9$cells/mL)	WBC ($x10^6$cells/mL)	Differential Count (%)		
				Lymphocytes	Neutrophils	Monocytes
Normal + 0.9% NaCl	12.1±1.5	5.4±0.5	7.8±2.8	73.6±4.9	24.8±4.0	1.5±1.0
EAC + 2% (v/v) DMSO	6.6±0.9*	2.1±0.4*	25.5±3.1*	34.3±5.0*	64.0±5.5*	1.6±1.0
EAC + EAEAR (20 mg/kg)	7.8±1.0	2.6±0.2	17.6±6.6	32.8±5.6	66.1±5.9	1.0±0.6
EAC + EAEAR (40 mg/kg)	8.7±1.0[a]	3.1±0.4[a]	15.1±4.0[a]	34.6±5.2	63.7±4.9	1.3±0.8
EAC + DMEAR (20 mg/kg)	8.4±0.9	2.8±0.2	14.8±3.2[a]	45.8±4.2[a]	52.6±3.9[a]	1.5±0.5
EAC + DMEAR (40 mg/kg)	9.7±0.9[a]	4.8±0.3[a]	14.8±3.2[b]	61.2±6.7[b]	35.6±5.6[c]	1.3±1.0
EAC + Bleomycin (0.3 mg/kg)	11.7±0.7[c]	5.2±0.9[c]	10.5±2.8[c]	70.3±1.2[c]	28.2±0.9[c]	1.5±0.7[c]

Data are expressed as mean ± S.E.M. for eight animals in each group.

*P<0.001: against normal group and [a]P<0.05, [b]P<0.01 and [c]P<0.001: against EAC control group.

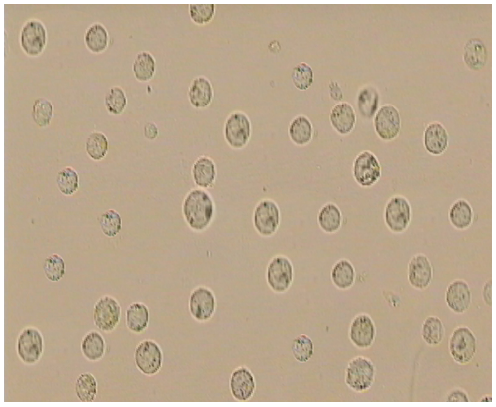


Figure 3.75. EAC cell smear of untreated EAC control.

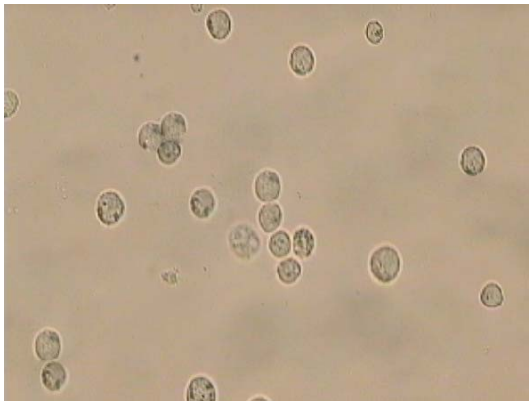


Figure 3.76. EAC cell smear of ME treated mice.

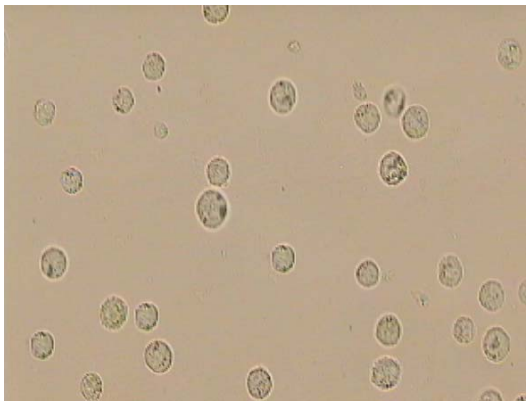


Figure 3.77. EAC cell smear of PEF treated mice.

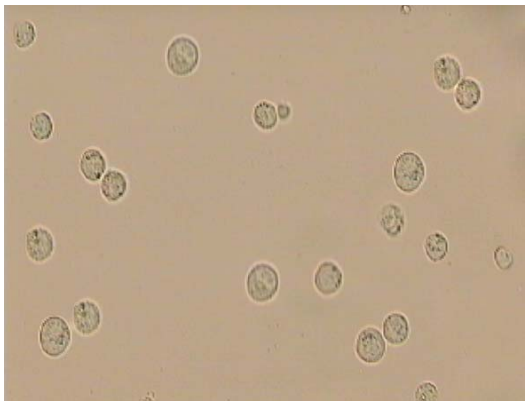


Figure 3.78. EAC cell smear of CF treated mice.

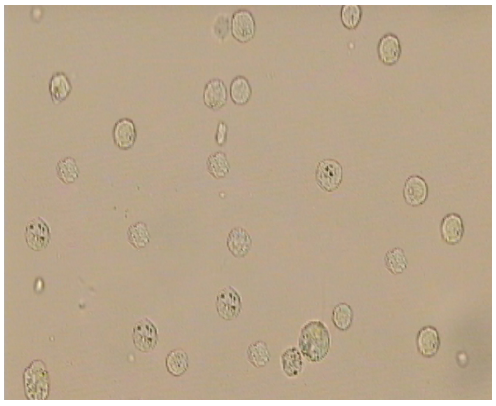


Figure 3.79. EAC cell smear of EAEAR treated mice.

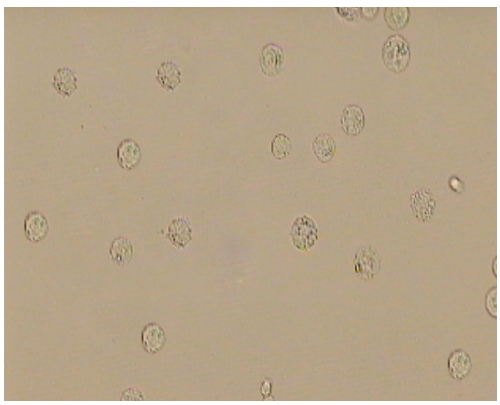


Figure 3.80. EAC cell smear of DMEAR treated mice.

3.4. *In vitro* cytotoxic assay against A431 cell line

3.4.1. Introduction

Drug screening is a long and costly process confronted with low productivity and challenges in using animals, which limit the discovery of new drugs. In addition, the number and diversity of compounds that need to be tested for activity against targets has rapidly expanded in recent years[217]. To improve drug screening efficacy and minimize animal testing, i*n vitro* cytotoxicity assays has become an integral aspect of drug discovery because it is a convenient, cost effective and predictive means of characterizing the toxic potential of new chemical entities. *In vitro* cytotoxicity test provides a crucial means of ranking compounds for consideration in drug discovery[218]. If researchers are interested in developing a therapeutic that target rapidly dividing cancer cells, they can screen a large number of compounds using defined cytotoxicity assays[219]. Eukaryotic cell culture is accepted as the model system of choice to get a first approximation of cytotoxicity. Moreover, advances in assay and signal detection technology have allowed miniaturization of cell-based assays, making it more convenient to perform dose-response experiments during primary screens of drug[220]. This study was designed to determine *in vitro* the cytotoxic activity of isolated compounds from *Calotropis gigantea* (Linn) and *Amoora rohituka* (Roxb.) using A431 (human vulval-derived epidermoid carcinoma) cells line.

3.4.2. Materials

The important chemicals and equipments used in this study are mentioned below:

i) Insulin (Sigma-Aldrich, Germany)

ii) Transferring (Sigma-Aldrich, Germany)

iii) 2-Mercaptoethanol (Sigma-Aldrich, Germany)

iv) 2-Aminoethanol (Sigma-Aldrich, Germany)

v) Selenite (Sigma-Aldrich, Germany)

vi) 24-well tissue culture plates (BD Bioscience, NJ, USA)

vii) Trypsin (Sigma-Aldrich, Germany)

viii) Ethylenediaminetetraacetic acid; EDTA (E-Mark, Germany)

ix) CO_2 incubator (RS Biotech, mini galaxy A)

x) Zf counter (Coulter Electronics Inc., Hialeah, FL, USA)

xi) Cytotoxic safety cabinets (Esco Technologies, Inc., USA)

3.4.3. Human vulval-derived epidermoid carcinoma (A431) cell line

Human vulval-derived epidermoid carcinoma (A431) cell line is a model cell line used in biomedical research and it was derived from an epidermal carcinoma of the vulva taken from an 85 years old female. They are used in studies of the cell cycle and cancer-associated cell signalling pathways since they express abnormally high levels of the epidermal growth factor receptor (EGFR)[221]. As such they are often used as a positive control for EGFR expression. Epidermal growth factor (EGF) stimulation of A431 cells induces rapid tyrosine phosphorylation of intracellular signalling proteins which control cellular processes such as growth, proliferation and apoptosis[222]. A431 cells contain no functional p53, a potent tumor suppressor gene, and so are highly sensitive to mitogenic stimuli[223].

3.4.4. Test samples

The test samples for *in vitro* cytotoxicity assay are listed in table 3.38.

Table 3.38 List of test samples of experimental plant

Plant	Test Samples
Calotropis gigantea (Linn.)	Compund-1
	Compund-2
	Compund-5
Amoora rohituka Roxb.	Compound-6

3.4.5. Procedure for cytotoxicity assay

To examine the cytotoxicity of isolated compounds, A431 human vulval-derived epidermoid carcinoma cell line[224] was used in this study. This study was conducted in Graduate School of Bioagricultural Sciences, Nagoya University, Japan. The cytotoxicity of each compound was evaluated by previously reported procedures[225-226]. The A431 cell line was cultured in a serum-free RD medium [RPMI 1640 medium (Kyokuto, Tokyo Japan)-DMEM (Kyokuto) 1:1, vol/vol] containing five factors (10 μg/ml of insulin, 5 μg/ml of transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol and 10 nM Selenite)[227-228]. In the proliferation assays, the cells were plated at 1.0 x 10^4 per well into 24-well tissue culture plates coated with type-I collagen and cultured in the same

medium. The cells were allowed to attach and spread for 12 hrs prior to their incubation with each test compound and doxorubicin (used as standard drug) at various concentrations (none, 0.02, 0.2 and 20 µg/ml). On day 4, the cells were harvested by 0.05% trypsin/0.01% EDTA and the cell numbers were counted with a Zf counter to determine IC_{50} values.

3.4.6. Result and discussion

In this cytotoxicity assay, relationships between concentration (µg/mL) of each test sample and number of viable A431 cells are presented in figure 3.81 to figure 3.85. Cell viability was reduced in a dose-dependent manner after exposure of A431 cells to test samples. The IC_{50} values of test samples on the A431 cells are summarised in table 3.39

Table 3.39 *In vitro* cytotoxicity of isolated compounds against A431 cell line

Compound	IC_{50} (µg/mL)
Compound-1	0.34
Compound-2	0.78
Compound-5	0.36
Compound-6	0.56
Doxorubicin	0.31

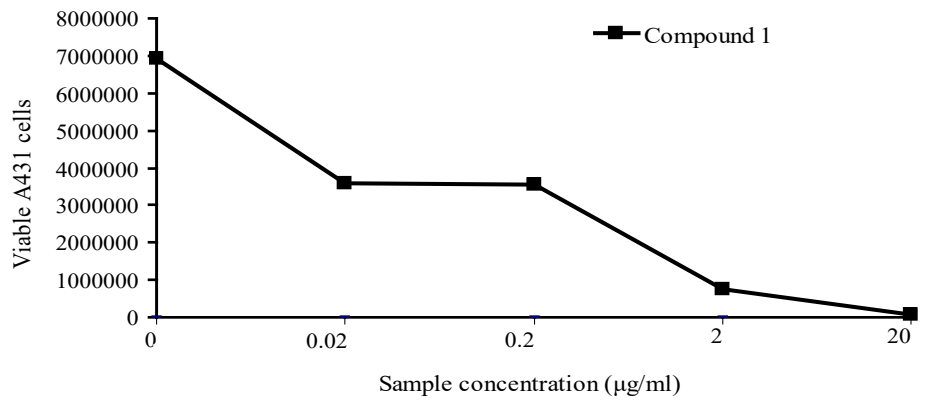


Figure 3.81 Concentration-cell viability curves of compound-1 following 12 hour exposure on A431 cell line.

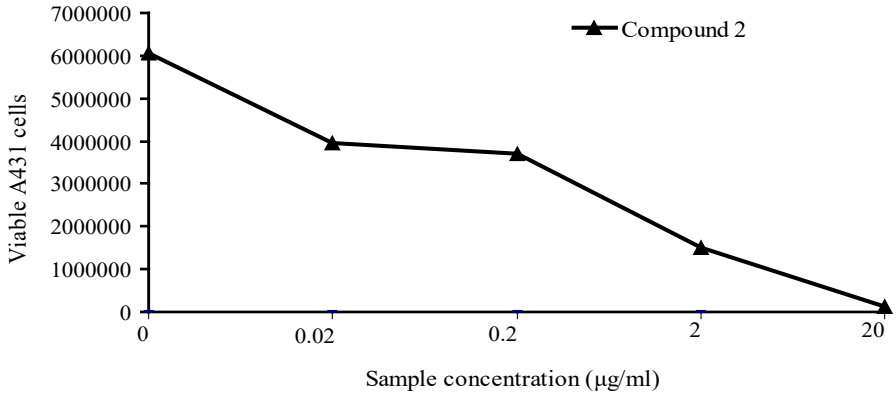


Figure 3.82 Concentration-cell viability curves of compound-2 following 12 hour exposure on A431 cell line.

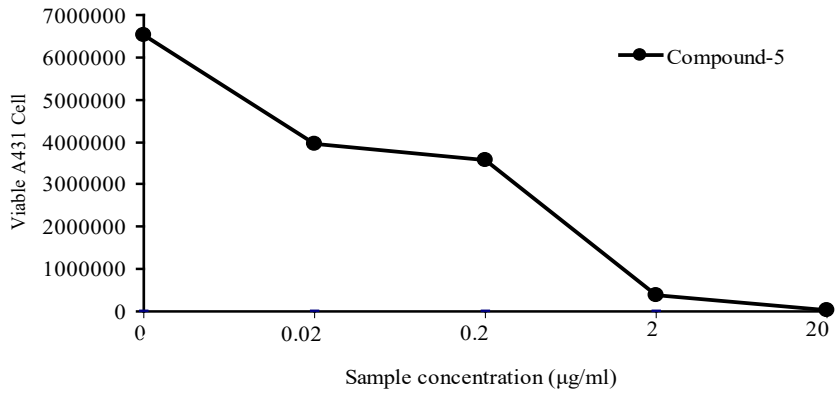


Figure 3.83 Concentration-cell viability curves of compound-5 following 12 hour exposure on A431 cell line.

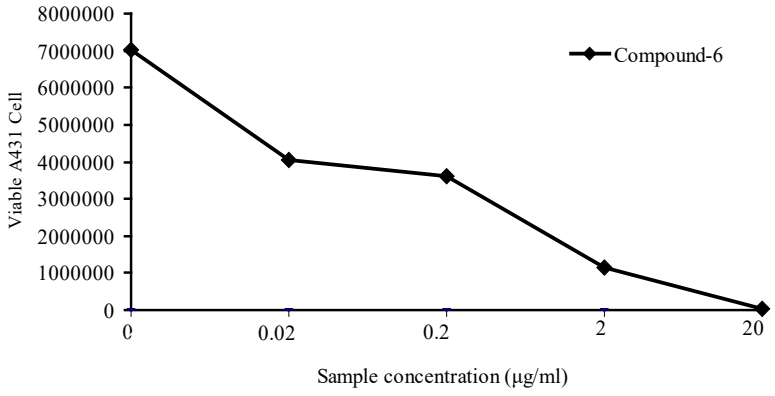


Figure 3.84 Concentration-cell viability curves of compound-6 following 12 hour exposure on A431 cell line.

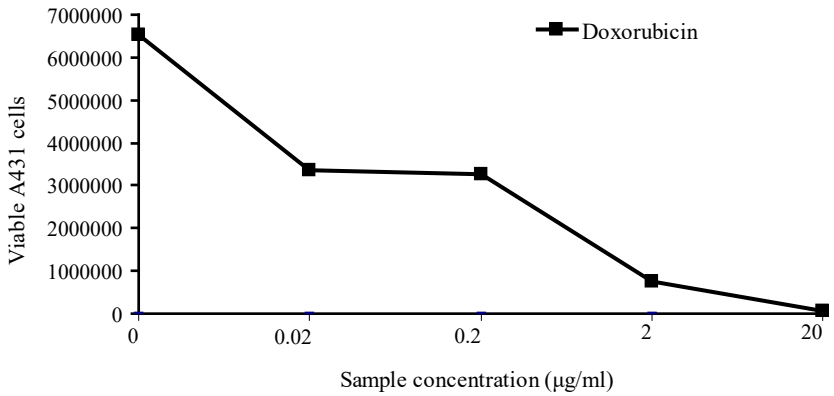


Figure 3.85 Concentration-cell viability curves of doxorubicin following 12 hour exposure on A431 cell line.

In this assay, compound-1 and compound-5 showed strong cytotoxic effect on A431 cells (table 3.39) than other two test samples and IC_{50} values were found to be 0.34 µg/mL for compound-1 and 0.36 µg/mL for compound-5. Compound-2 (IC_{50}: 0.78 µg/mL) and compound-5 (IC_{50}: 0.56 µg/mL) also exhibited moderate cytotoxic effect in comparison with doxorubicin (IC_{50}: 0.31 µg/mL). Here compound-1, compound-2, compound-5 and compound-6 was identified as di-(2-ethylhexyl) phthalate, anhydrosophoradiol-3-acetate, β-boswellic acid and 2-methoxy-14-calamenenone, respectively. Anti-leukaemic and anti-mutagenic effects of di-(2-ethylhexyl) phthalate (i.e., compound-1) have been reported[122]. In addition, previous studies also showed that *in vitro* anhydrosophoradiol-3-acetate (i.e., compound-2) exhibited cytotoxicity against five human cancer cell lines[124]. But this is the first time report on cytotoxicity of anhydrosophoradiol-3-acetate against A431 cell line.

3.5. Insecticidal activity study

3.5.1. Introduction

Chemicals largely used as pesticides in crop protection could be environmental pollutants and have undesirable effects on animals and human beings. Therefore, the development of bioinsecticides has been focused as a viable pest control strategy in recent years[229-231]. Phytochemicals have been established to be antifeedants, repellents, growth inhibitors or as insecticides. The trend to utilize botanical pest control agents led to the study of the efficacy of many plant extracts as insecticides[232-234].

Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae), is considered as a common and most destructive pest throughout the world. This species has been found associated with a wide range of commodities including grain, flour, peas, beans, cacao, nuts, dried fruits and spices[235]. This pest has been reported to attack the germ part (embryo portion) of the grain. Their presence in stored foods directly affects both the quantity and quality of the commodity[236]. Currently different kinds of preventive and curative control measures are practiced to get protection from this pest. Among those, chemical pesticides have been used for a long time, but have serious drawbacks, such as direct toxicity to beneficial insects, fishes and human due to their effects on non-target organisms[237-239]. However, few works has been done in Bangladesh to determine the efficacy of our locally available plant materials against *Tribolium castaneum*. The insecticidal activity of root bark of *Calotropis gigantea* L. and stem bark of *Amoora rohituka* (Roxb.) has already been demonstrated against *Tribolium castaneum* (Herbst)[71,240]. Therefore, this investigation deals with the effects of EECF (Ethyl acetate extract of *Calotropis gigantea* flower) against the *Tribolium castaneum* of different larval and adult stages.

3.5.2. Materials

The following reagents and equipments were used in insecticidal activity study:

i) Earthen pots
ii) Beakers (500ml)
iii) Petridishes (7 cm in diameter)
iv) 250 and 500- micrometer aperture sieve
v) Camel hairbrush and filter paper
vi) Ethyl acetate (E-Mark, Germany)
vii) Incubator
viii) Autoclave (ALP Co. Ltd. KT-30L, Tokyo)

3.5.3. Origin of beetles

The insects *Tribolium castaneum* (Herbst) (as shown in figure 3.86) used in the experiment were obtained from a culture maintained in the Entomology Laboratory,

Department of Zoology and IPM (Integrated Pest Management) Laboratory, Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Figure 3.86. *Tribolium castaneum* (Herbst) Adult

3.5.4. Culture of the beetles

Cultures were maintained in 1 L glass jar containing food medium. A filter paper was placed inside each jar for easy movement of the insects. The jar was covered with a piece of cloth and kept in an incubator at 30±0.5°C.

3.5.5. Food medium

The wheat flour was used as the food medium for *Tribolium castaneum*. A standard mixture of whole-wheat flour with powdered dry yeast in the ratio of 19:1[241-242] was used as food medium throughout the experimental period. Both the flour and the powdered dry yeast were previously passed through a 250-micrometer aperture sieve and mixed thoroughly using an electric blender. The food medium was sterilized in an oven at 120°C for 6 hours. Food was not used until at least 15 days after sterilization to allow its moisture content to equilibrate with that of environment.

3.5.6. Collection of eggs

About one hundred beetles were placed in a 500 mL beaker containing food medium. The beaker was covered with a piece of cloth and kept in an incubator at 30±5°C. On the following day the eggs were collected by sieving the food medium using 500 and 250-micrometer aperture sieves (Khan and Selman, 1981). Eggs were transferred to glass petridish (7 cm in diameter) and incubated at 30±0.5°C.

3.5.7. Collection of newly hatched larvae

Larvae emerged after 3 days in that condition. Newly hatched larvae were then collected with a fine camel hair brush and transferred to fresh food medium.

3.5.8. Determination of larval instars

Most larvae had six instars as reported by Good[243]. The second, third, fourth, fifth and sixth instars larvae were obtained form the larval culture on the 3rd, 6th, 9th, 12th and 16th day from the hatching, respectively while the newly hatched larvae was used as first instar[244-245]. Every three days the food medium was changed by fresh one to avoid conditioning by the larvae[241-242].

3.5.9. Collection of adults

A huge number of flour beetles were thus rared to get a regular supply of the newly formed adults. When sufficient adults produced in the sub-cultures they were collected from the food medium. Some pieces of filter paper were kept inside the beaker on the food. Adults crawled upon the paper and then the paper was taken out with a forceps. Adults were then collected in a small beaker (50 ml) with the help of a fine camel hair brush.

3.5.10. Precaution

All glassware's and sieves were regularly cleaned using washing liquid detergent and sterilized on an oven at 120^{o}C for six hours. The working bench and other equipments were also cleaned before used.

3.5.11. Preparation and application of doses

Residual film method[246] was used to test the mortality rate of larvae and adults of *Tribolium castaneum* (Herbst). A preliminary screening of different doses was performed on several insters of larvae and adults to obtain 0% to 100% mortalities. Then 400 mg, 200 mg, 100 mg, 50 mg and 25 mg of EECF were dissolved separately in 5 mL of ethyl acetate to get the concentrations of 80 mg/mL, 40 mg/mL, 20 mg/mL, 10 mg/mL and 5 mg/mL, respectively, which were used as stock solutions. 1 mL of various concentrations for EECF was applied on different petridishes (7 cm diameter) in such a way that it made a uniform film over the petridishes. For solvent evaporation, the petridishes were air dried leaving the extract on it. The actual extract present in 1 mL was calculated and the dose per square centimeter was determined by dividing the value present in one mL with the area (πr^2 where r = diameter/2 = 3.5 cm) of the petridish. So calculated doses were 2.078, 1.039, 0.519, 0.259 and 0.129 mg/cm^2. After drying, 10 beetles were released in each petridish with two replication. A control batch was also maintained with the same number of insects after preparing the petridish by applying and evaporating the solvent only. The treated beetles were placed in an incubator at the same temperature as reared in stock

cultures and the mortality of the beetles were counted after 24 and 48 hour post-exposure[247].

3.5.12. Statistical analysis

The mortality data were subjected to Probit analysis[143] for the determination of LD_{50} values using the computer software SPSS of 14 version. Results with $p<0.05$ were considered to be statistically significant.

3.5.13. Results and discussion

In the present investigation, the residual toxicity of EECF was tested against both larvae and adults of *Tribolium castaneum*. The mortality were recorded and statistical data regarding LD_{50}, 95% confidence limit and chi-square values were calculated and presented in table 3.40. After 24 hrs of exposure, the LD_{50} value of EECF against 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} instar larvae and adult *Tribolium castaneum* were found to be 0.206, 0.199, 0.705, 0.738, 0.754, 0.451 and 1.371 mg/cm^2, respectively (Table 3.40). The effectiveness of EECF was increased with the increase of exposure time and after 48 hrs of exposure, the maximum residual toxicity was observed with LD_{50} of 0.134, 0.174, 0.455, 0.440, 0.559, 0.390 and 0.716 mg/cm^2 for 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} instar larvae and adults *Tribolium castaneum*, respectively. No mortality was observed in control. Regression equations for EECF against larvae of different stages and adult beetles were obtained by plotting empirical probit against the logarithm of the sample doses (Figure 3.87 to 3.100). After 24 and 48 hrs of exposure, the LD_{50} of EECF against larvae of each instar and adult *Tribolium castaneum* was calculated from the corresponding regression equation[248-249]. In this bioassay, the mortality rate of *Tribolium castaneum* of different larval and adult stages was found to increased with the increase in concentration of EECF (Figure 3.101 to 3.114). The EECF caused the highest mortality of the 1^{st} instars larvae in comparison with other larval instars which indicated that the newly hatched larvae were the most susceptible with lowest LD_{50} value (0.134 mg/cm^2) whereas the adult (after 72 hr exposure) were less susceptible with highest LD_{50} values (1.371 mg/cm^2).

Results of this study demonstrated that toxicity of the plant extracts decreased with the increase of age of the larvae. This may clearly support of others that insect's age play an important role in influencing susceptibility[250]. The present result is more or less similar to the findings of Upadhyay[251] who revealed the insecticidal properties of *Piper nigrum* against *Tribolium castaneum*.

Table 3.40 Insecticidal activity of EECF against *Tribolium castaneum* (Herbst).

Sample	Life stage	Exposure time (hrs)	LD_{50} (mg/cm^2)	95% Confidence Limits		Chi-squre; χ^2 (Degree of freedom)
				Lower	Upper	
EECF	1st instar	24	0.206	0.131	0.324	1.022 (2)
		48	0.134	0.071	0.254	0.219 (1)
	2nd instar	24	0.199	0.106	0.372	0.398 (2)
		48	0.174	0.097	0.313	0.136 (2)
	3rd instar	24	0.705	0.484	1.027	0.558 (3)
		48	0.455	0.329	0.628	1.525 (3)
	4th instar	24	0.738	0.573	0.952	2.932 (3)
		48	0.440	0.324	0.598	1.338 (2)
	5th instar	24	0.754	0.574	0.990	1.368 (3)
		48	0.559	0.409	0.763	0.238 (3)
	6th instar	24	0.451	0.315	0.647	2.801 (3)
		48	0.390	0.255	0.597	0.232 (2)
	Adult	24	1.371	0.708	2.653	0.183 (3)
		48	0.716	0.493	1.052	3.624 (3)

#Values were based on four doses with 20 insects each. *Significant at $P<0.05$ level

Developing countries in Asia and Africa including Bangladesh, have a long history to protect stored grains with locally available herbal substances, where the application of plant materials is simple and aqueous extracts in several cases proved to be highly effective against stored product insects[252]. Moreover, the crude extracts, oil, leaf powder etc. are easy to prepare and handle and very much cheaper in comparison to the imported chemical pesticides[253]. Therefore, the use of plant products (crude extracts, oil, powders etc.) as insecticides in stored product protection might benefit the farmers by a reduction of protection costs, insecticide resistance development and environmental impact in term of insecticidal hazard. The result of this experiment first time indicates that like other plant oils extracts, flower of *Calotropis gigantea* may be used in the control of *Tribolium castaneum* population with integrated pest management system which seems to be economically feasible and ecologically sound.

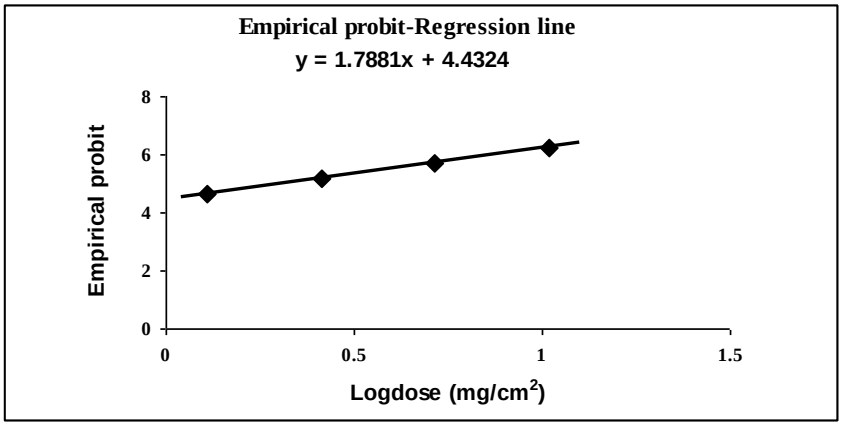


Figure 3.87 Probit mortality line of EECF against 1st instar larvae of *Tribolium castaneum* after 24 hours of exposure.

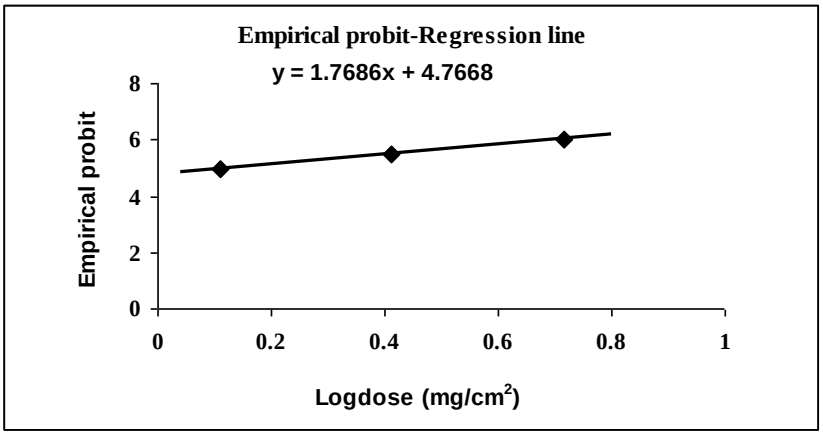


Figure 3.88 Probit mortality line of EECF against 1st instar larvae of *Tribolium castaneum* after 48 hours of exposure.

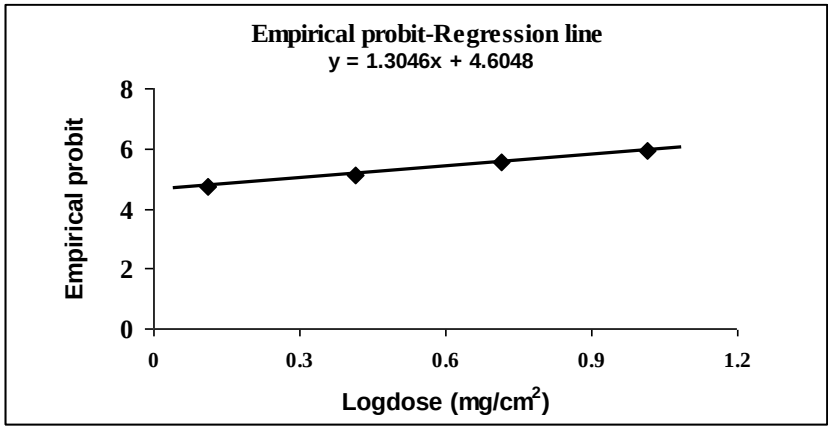


Figure 3.89 Probit mortality line of EECF against 2nd instar larvae of *Tribolium castaneum* after 24 hours of exposure.

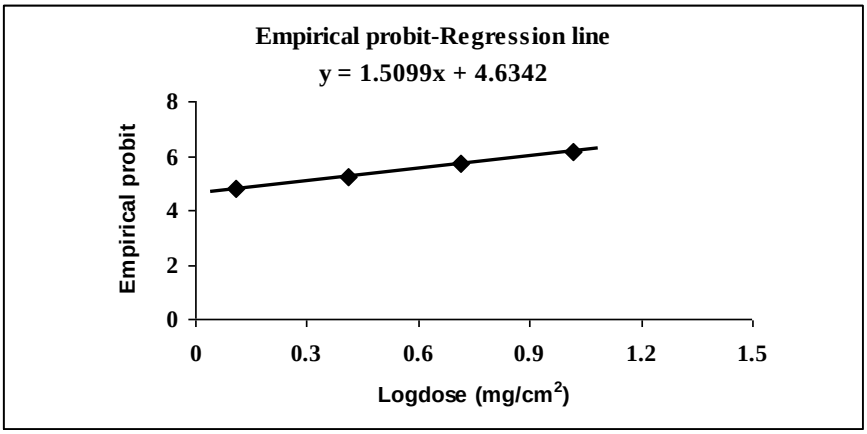


Figure 3.90 Probit mortality line of EECF against 2nd instar larvae of *Tribolium castaneum* after 48 hours of exposure.

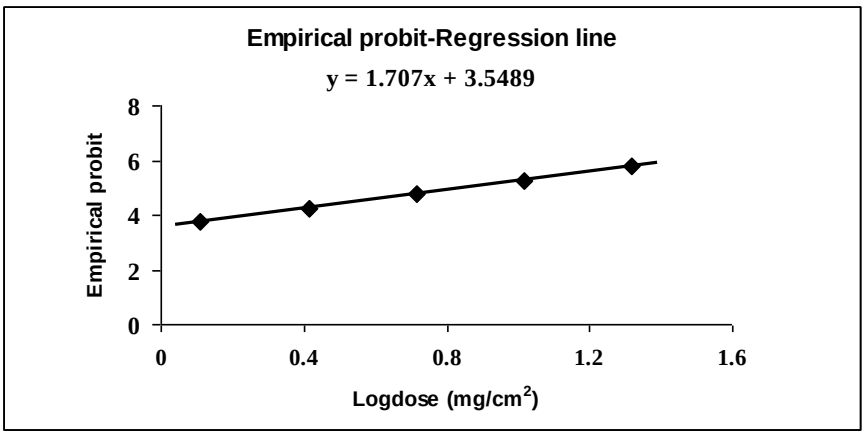


Figure 3.91 Probit mortality line of EECF against 3rd instar larvae of *Tribolium castaneum* after 24 hours of exposure.

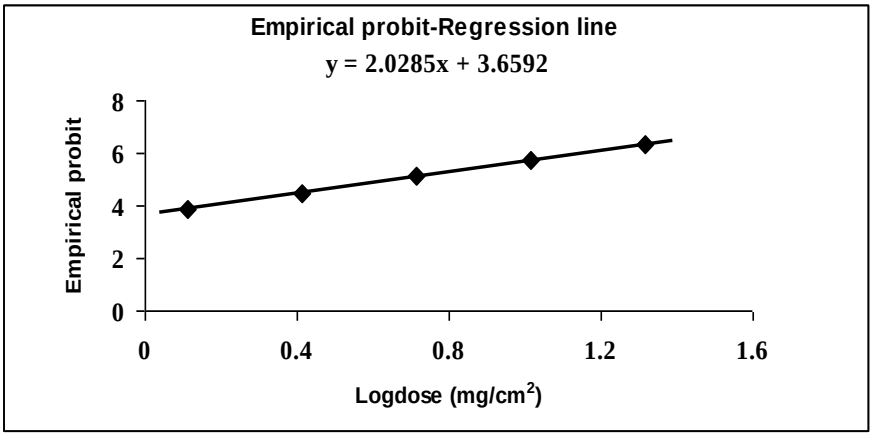


Figure 3.92 Probit mortality line of EECF against 3rd instar larvae of *Tribolium castaneum* after 48 hours of exposure.

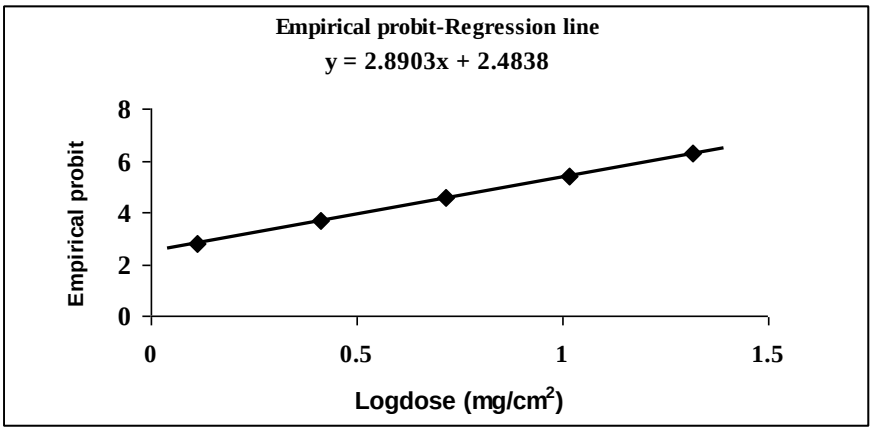


Figure 3.93 Probit mortality line of EECF against 4th instar larvae of *Tribolium castaneum* after 24 hours of exposure.

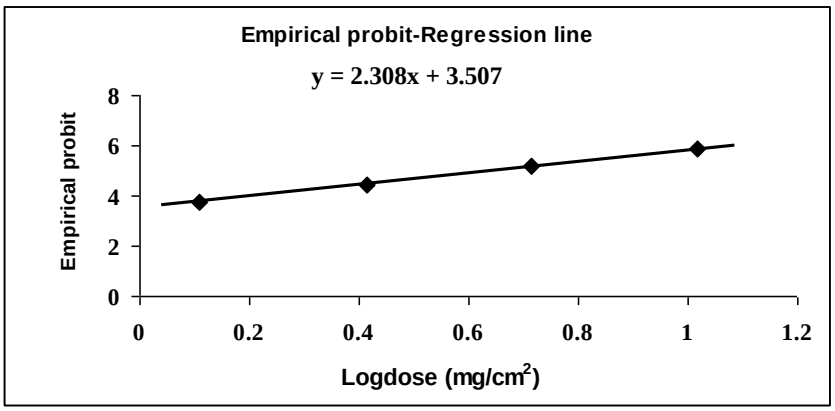


Figure 3.94 Probit mortality line of EECF against 4th instar larvae of *Tribolium castaneum* after 48 hours of exposure.

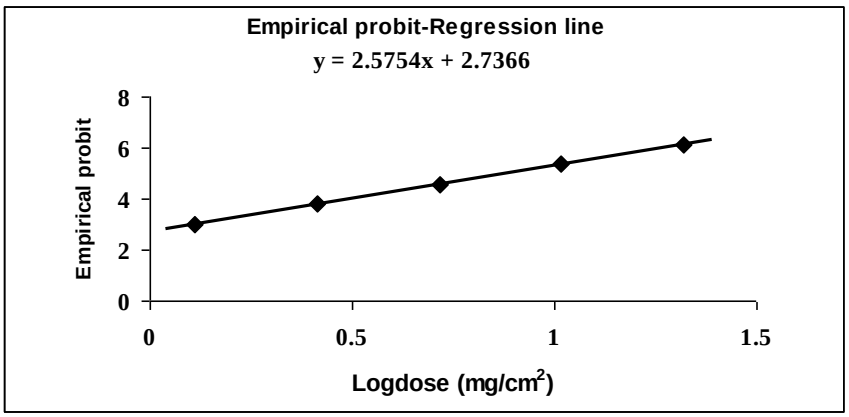


Figure 3.95 Probit mortality line of EECF against 5th instar larvae of *Tribolium castaneum* after 24 hours of exposure.

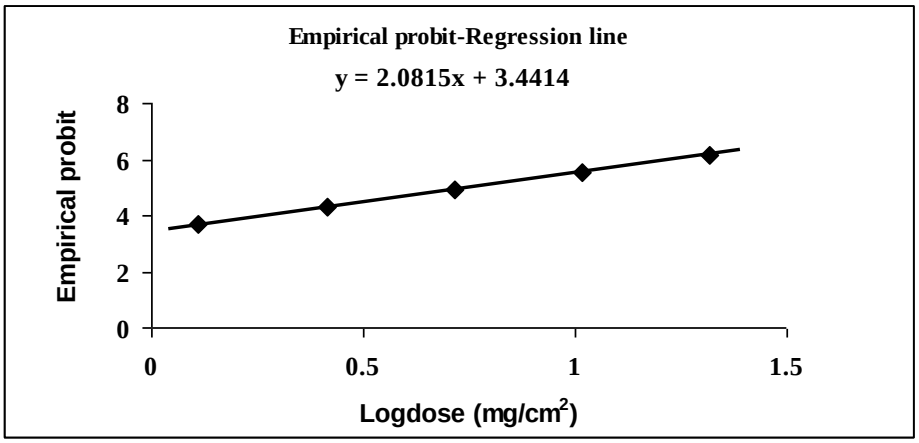


Figure 3.96 Probit mortality line of EECF against 5th instar larvae of *Tribolium castaneum* after 48 hours of exposure.

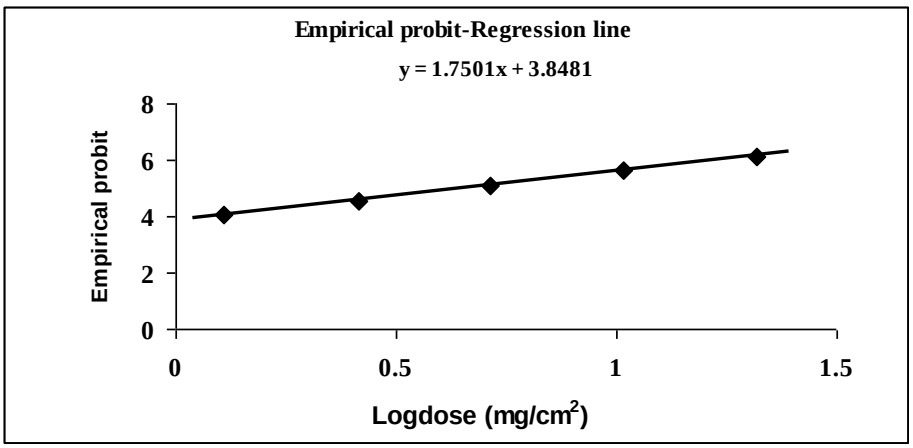


Figure 3.97 Probit mortality line of EECF against 6th instar larvae of *Tribolium castaneum* after 24 hours of exposure.

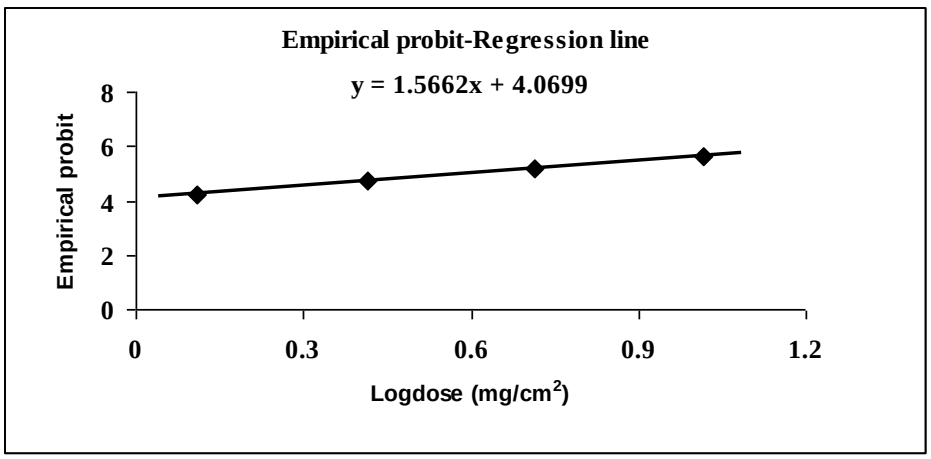


Figure 3.98 Probit mortality line of EECF against 6th instar larvae of *Tribolium castaneum* after 48 hours of exposure.

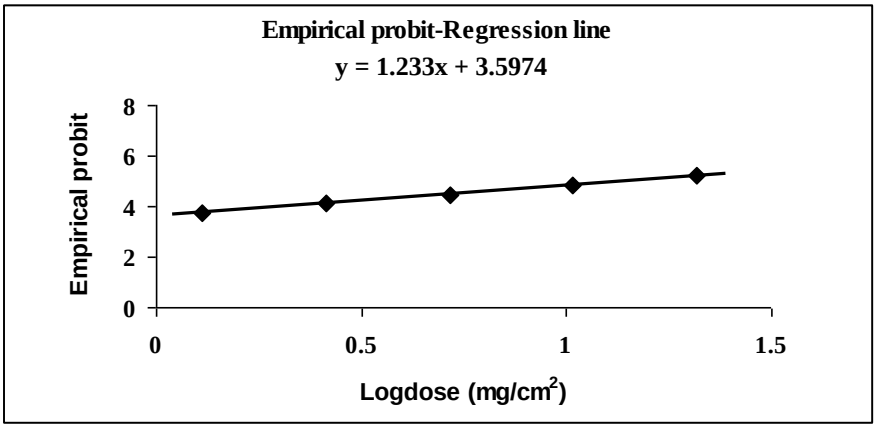


Figure 3.99 Probit mortality line of EECF against adult *Tribolium castaneum* after 24 hours of exposure.

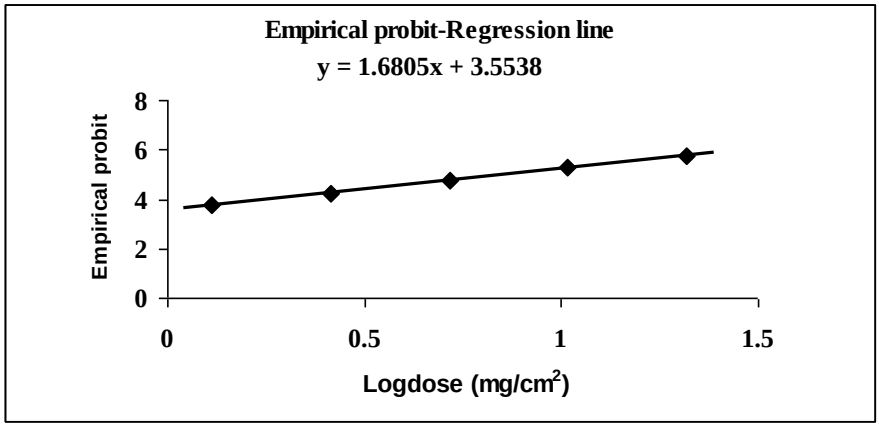


Figure 3.100 Probit mortality line of EECF against adult *Tribolium castaneum* after 48 hours of exposure.

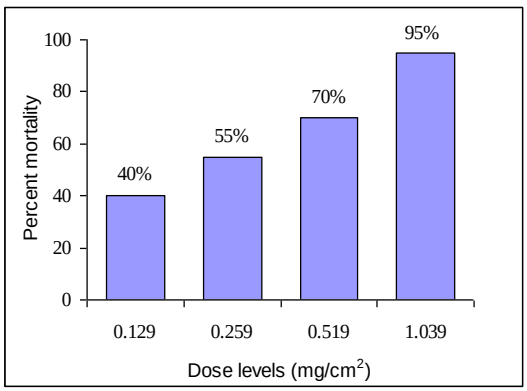


Figure 3.101 Percent mortality of 1st instar larvae of *Tribolium castaneum* after 24 hours of exposure.

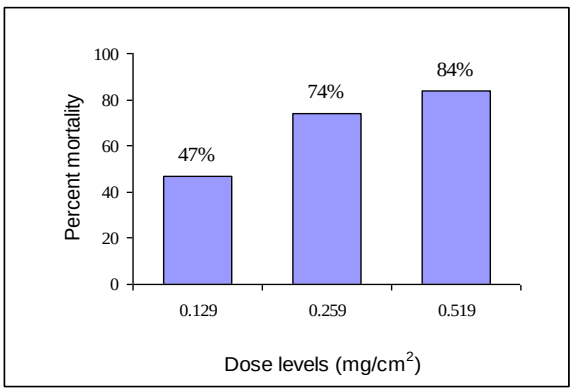


Figure 102 Percent mortality of 1st instar larvae of *Tribolium castaneum* after 48 hours of exposure.

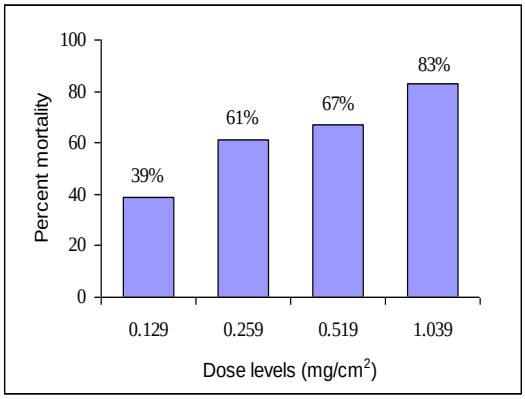


Figure 3.103 Percent mortality of 2nd instar larvae of *Tribolium castaneum* after 24 hours of exposure.

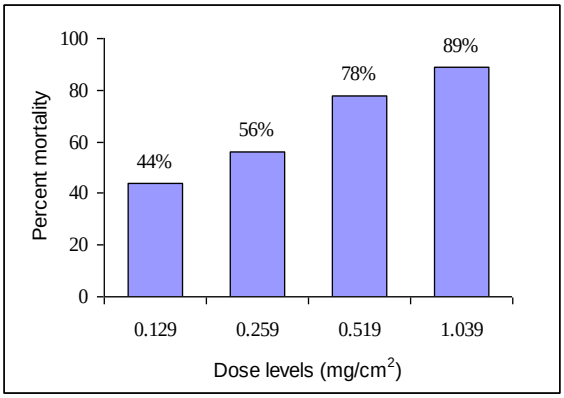


Figure 3.104 Percent mortality of 2nd instar larvae of *Tribolium castaneum* after 48 hours of exposure.

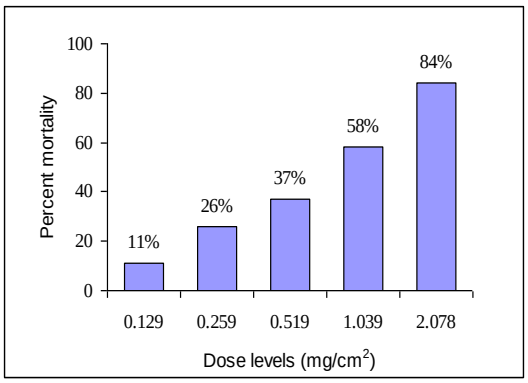


Figure 3.105 Percent mortality of 3rd instar larvae of *Tribolium castaneum* after 24 hours of exposure.

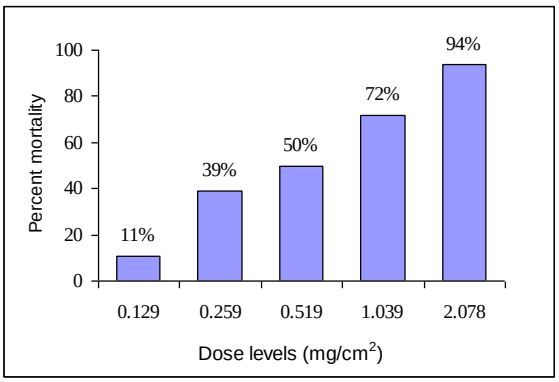


Figure 3.106 Percent mortality of 3rd instar larvae of *Tribolium castaneum* after 48 hours of exposure.

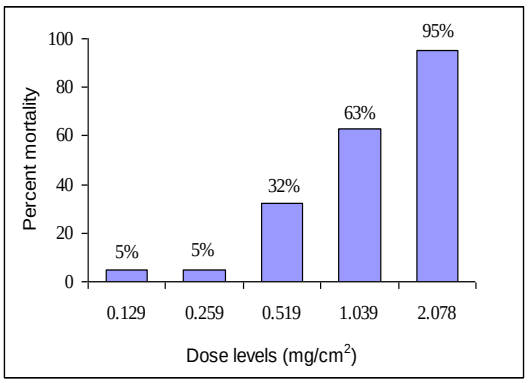


Figure 3.107 Percent mortality of 4th instar larvae of *Tribolium castaneum* after 24 hours of exposure.

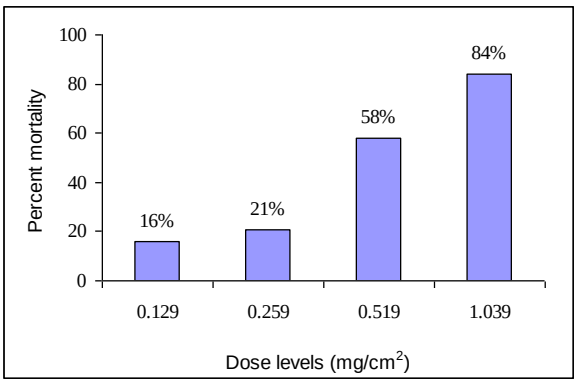


Figure 3.108 Percent mortality of 4th instar larvae of *Tribolium castaneum* after 48 hours of exposure.

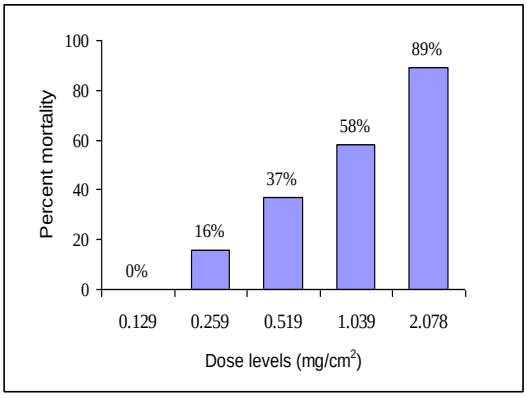


Figure 3.109 Percent mortality of 5th instar larvae of *Tribolium castaneum* after 24 hours of exposure.

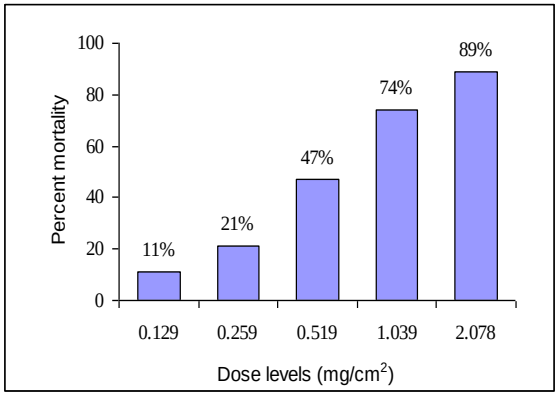


Figure 3.110 Percent mortality of 5th instar larvae of *Tribolium castaneum* after 48 hours of exposure.

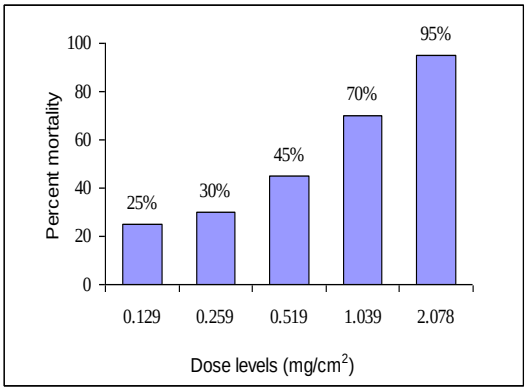


Figure 3.111 Percent mortality of 6th instar larvae of *Tribolium castaneum* after 24 hours of exposure.

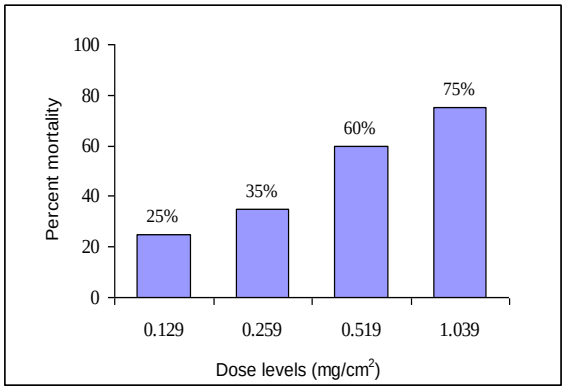


Figure 3.112 Percent mortality of 6th instar larvae of *Tribolium castaneum* after 48 hours of exposure.

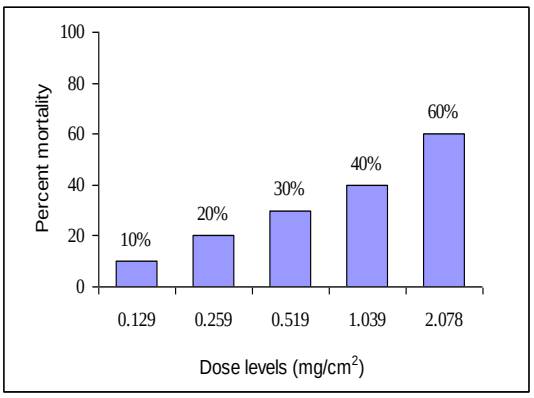


Figure 3.113. Percent mortality of adult *Tribolium castaneum* after 24 hours of exposure.

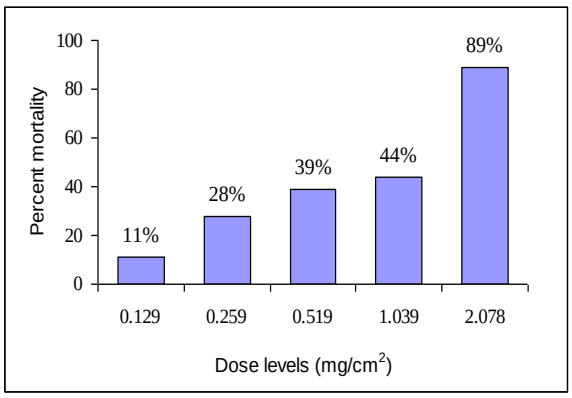


Figure 3.114. Percent mortality of adult *Tribolium castaneum* after 48 hours of exposure.

Conclusion

In this study, six compounds have been isolated from the flower of *Calotropis gigantea* and stem bark of *Amoora rohituka*. Based on spectroscopic (NMR, Mass & IR) evidences, the isolated and purified compounds have been identified as di-(2-ethylhexyl) phthalate, anhydrosophoradiol-3-acetate, taraxasteryl acetate, lup-12,20(29)-dien-3β,28-diol, β-boswellic acid and 2-methoxy-14-calamenenone. Among these, three compounds have a basic polycyclic triterpene skeleton. Except taraxasteryl acetate, the other five compounds have been isolated first time from the corresponding plant. Actually this new source report explores the chemical versatility of *Calotropis gigantea* and *Amoora rohituka* plants as well as Asclepiadaceae and Meliaceae families.

The present study demonstrates that different extracts, fractions and purified compounds from *Calotropis gigantea* and *Amoora rohituka* have noteworthy antibacterial, cytotoxic, insecticidal and antineoplastic effects that may be a source of herbal drugs in respective therapeutic area. To evaluate the mechanism by which these extracts, fractions and purified compounds showed biological activities, further studies should be carried out.

Moreover, in antimicrobial and antineoplastic studies, isolated compounds specially di-(2-ethylhexyl) phthalate, anhydrosophoradiol-3-acetate and β-boswellic acid have been found to be very effective against microorganisms and cancer cells. So these compounds can be chosen as lead compounds to conduct research on structural modification and structure–activity relationship for the development of new antimicrobial and antineoplastic agents.

The overall findings of this study are consistence with some traditional uses of *Calotropis gigantea* and *Amoora rohituka* and might be helpful in the proper use of these important medicinal plants for better health care system of common people in Bangladesh.

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