ISOLATION AND CHARACTERIZATION OF ANTIOXIDANT AND ANTIMICROBIAL COMPOUNDS FROM *GARUGA PINNATA*

A DISSERTATION IN PARTIAL FULFILLMENT FOR THE REQUIREMENTS OF THE DEGREE OF MASTER OF PHILOSOPHY IN ORGANIC CHEMISTRY

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DEDICATED
TO
MY BELOVED PARENTS
AND
SUPERVISORS

ACKNOWLEDGEMENT

IN THE NAME OF ALMIGHTY ALLAH! THE MOST MERCIFUL, MOST GRACIOUS.

I feel proud and honored to express my profound gratitude, indebtedness, deep appreciation, and respect to my supervisor, Dr. Tofail Ahmad Chowdhury, Professor, Department of Chemistry. The University of Dhaka. My indebtedness to him for his wise guidance and active encouragement, constructive criticism, valuable suggestions, and advice throughout the present work. His affectionate dealings highly inspired me to accomplish this work.

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DECLARATION CERTIFICATE

We declare that the thesis entitled "Isolation and characterization of antioxidant and antimicrobial compounds from *Garuga pinnata*" submitted by Abdullah Nasir Pulak, in partial fulfillment of the requirement for the degree of Master of Philosophy is the candidate's accomplishment and is not conjoint work with anyone else. This is an authentic study of the author, and no part of this thesis has been submitted to any university or institution for any degree. The author carried out his research under our supervision and guidance in the Organic Research Laboratory, Department of Chemistry, University of Dhaka and, in the Organic Research Laboratory, Institute of National Analytical Research and Services (INARS), BCSIR, Dhaka, Bangladesh.

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Declaration

This thesis work was undertaken in the Organic Research Laboratory, Department of Chemistry, University of Dhaka, and the Organic Research Laboratory, Institute of National Analytical Research and Services (INARS), BCSIR, Dhaka, Bangladesh. I believe, to the best of my knowledge, that in this thesis, all work contained is original and my own, except as acknowledged by appropriate references. I declare that I have not submitted this dissertation for a degree at this or any other university, in whole or in part.

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ABSTRACT

Phytochemical analysis was carried out in this study to isolate and characterize antibacterial and antioxidant chemicals found in the stem and bark of *Garuga pinnata* Roxburgh. *Garuga pinnata* belongs to the Burseraceae family has been investigated for evaluation of their biological activities. The powder of dried stem and bark of *Garuga pinnata* was extracted with Hexane, dichloromethane, ethyl acetate, and methanol. Different chromatographic techniques have attempted to isolate secondary metabolites from dichloromethane and ethylacetate extract of the stem and bark. It has been possible to isolate four compounds from the dichloromethane extracts of the stem and bark of *Garuga pinnata*. Different spectroscopic techniques have established the structures of the compounds.

The isolated compounds were Stigmasterol, ß-sitosterol, Isofouequrone, and Lupeol. The isolated compounds were identified by extensive analyses of their high-resolution ^{1}H-NMR (400 MHz) and ^{13}C-NMR(100MHz).

The extracts of stem and bark of *Garuga pinnata*, i.e., Methanol Soluble Fraction (MSF), Hexane Soluble Fraction (HSF), Dichloromethane Soluble Fraction (DCMSF), were subjected to assay for various biological screening such as Antioxidant activity screening and Antimicrobial screening.

The crude plant extract and its different soluble fractions were investigated to evaluate biological activities *in vitro*.

In the analysis of free radical scavenging, the EASF of bark and stem of *Garuga pinnata* with an IC_{50} value of 21.588 µg/mL had excellent free radical scavenging activity.

Test antimicrobial activity against a number of Gram-positive and Gram-negative bacteria and fungi were done for ethyl acetate extract, methanol extract, Dichloromethane extract, and a mixture of compound-1 and Compound-2 (mixture of Stigmasterol and β-sitosterol). The ethyl acetate and dichloromethane extracts exhibited antimicrobial activity against all of the test micro-organisms and fungi. The methanol extract does not show good antimicrobial activity against the Gram-positive micro-organisms. The mixture of compound-1 and compound-2(GPE-1 & 2, as a mixture of Stigmasterol and β-sitosterol) showed almost similar activity as ethyl acetate crude extract (9-12 mm inhibition zone) against nearly all the micro-organisms and fungi.

In Brine shrimp lethality bioassay, among all extractives of leaves of *Garuga pinnata*, the highest brine shrimp lethality was given by HSF (110.15 µg/mL) followed by EASF (55.48 µg/mL), and MSF (80.99 µg/mL).

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Introduction

INTRODUCTION

1.1 General

Medicinal plants have been used from the civilization of humankind in diseases, physical discomfort, injuries, and fear of dying. Herbs play a vital role in for existence of humanity on earth.

Babylonians (about 3000 years B.C.) knew about several medicinal plants and their effects. The exact purpose, like henbane (*Hyoscyamus* spp.), Opium (*papaver somniferum*), Castor oil (*Ricinus communis*), and Aloe vera (*Aloe spp.*), etc., (Ghani, A).

The Chinese have an effective and unique system of medicine. The earliest known Chinese pharmacopeia, The Pen Tsao, reported over 300 herbal plants (Shealy, N). According to the Greek physician, Hippocrates (460-370 B.C.) consists of some 300 to 400 medicinal plants. Opium, mint rosemary, sage, and Verena are of them (Wikipedia).

Greek pharmacist-physician Galen (131-200 A.D.) used many herbal plants to prepare his recipes (Ghani, A).

The Arabian physicians, Al-Razi and Ibn Sina (800 to 1400 A.D.) revolutionized medicine using herbal plants. In the Indian subcontinent, according to the Rig Veda (4500 – 1600 BC), Soma fungi (*Amanita muscaria)* are used by Indo-Aryans as medicinal agents. The Vedas made many references to healing plants, including sarpagondha (*Rauvolfia serpentine*), a comprehensive Indian Herbal, the Charaka Samhita, and more than 500 medicinal plants (Ghani, A).

Only 5-15% of the approximately 2,50,000-5,00,000 species of higher plants, of which more than 80,000 are medicinal, have been investigated pharmacologically. Thus there are many chances of finding new natural compounds with pharmacological activities helpful in developing new drugs. Chemists and pharmacists are working to make a plant product into a commercial drug (State of the World's Plants Report - 2016).

1.2 Medicinal uses of plant materials

Plants provide man with food, shelter, medicine, and oxygen. From early to the present, humans have successfully used plants' therapeutic tools against diseases. Although synthetic drugs are used in the modern age, many medicinal drugs are from herbal plants. According to research, almost 80% of medicines are from herbal plants (Zhang, G. L *et al*.).

Using medicinal plants continued with the development of human knowledge. With time, their synthetic analogs have also been prepared. In this way, the discovery of vincristine was made from *Catharanthus roseus*, which is used to treat cancer (Ward, J. L *et al*., 2003).

Vincristine

$C_{46}H_{56}N_4O_{10}$

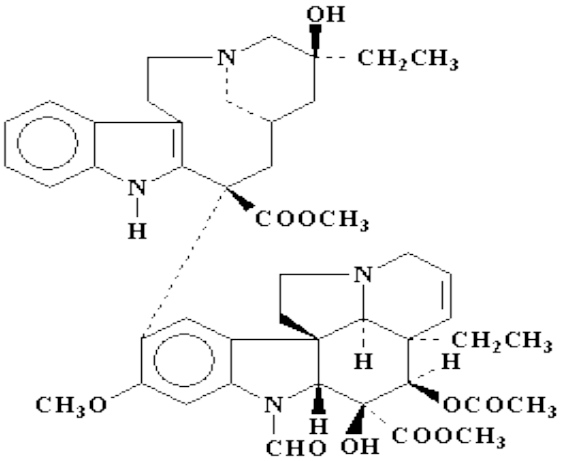


Fig: 1.1 Vincristine

Calanolide-A is a reverse-transcriptase inhibitor isolated from *Calophyllum Langerum*. It has anti-HIV activity combined with nucleoside reverse-transcriptase inhibitors, including AZT, ddI, and ddC (Schmitt, A. C *et al*.,). Medichem pharmaceuticals, Inc., and the state of Sarawak, Malaysia, have begun clinical development of Calanolide A as a potential treatment for AIDS and HIV infections.

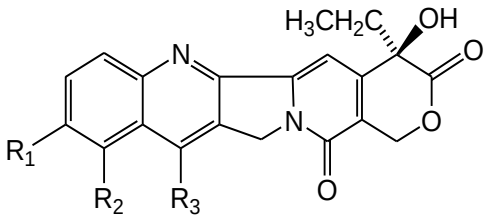


Fig: 1.2 Calanolide A

Chinese medicinal practices use *Artemisia annua* as an antimalarial medicine. From the 1960s, researchers evaluated various extracts of this herb. Isolation yielded artemisinin, a new antimalarial compound. Artemisinin effectively treats chloroquine-resistant cases and other severe cases.

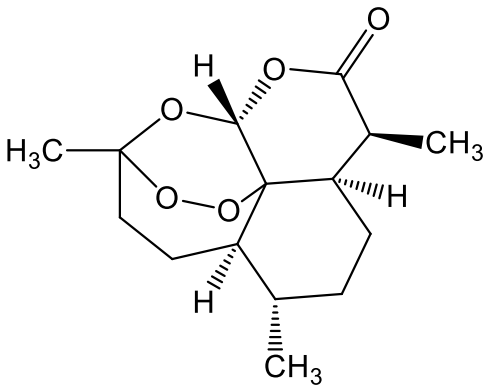


Fig:1.3 Artemisinin

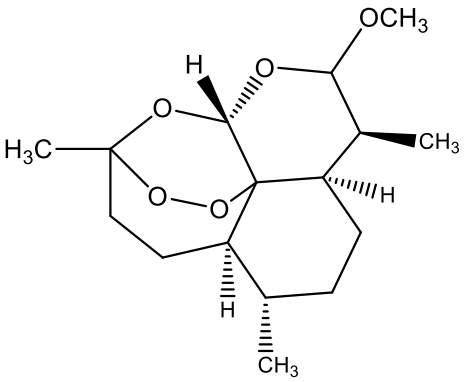


Fig:1.4 Artem ether

For several decades, older adults in some parts of Mainland China have brewed tea from the leaves of the club moss (*Huperzia serrata*) for improving memory. Huperzine, a potent, reversible, and specific acetylcholinesterase inhibitor, was discovered by Chinese scientists in the plant Club moss. Scientists developed synthesis due to deficient levels in nature. The product is suitable for treating cholinergic-related neurodegenerative disorders such as Alzheimer's disease. Trial with 103 patients, Huperzine-A was safe and superior to placebo and induced improvement in memory cognition and behavior in about 58% of patients with Alzheimer's disease.

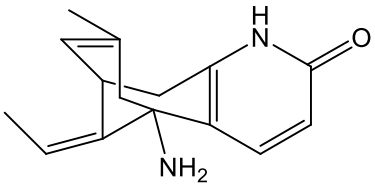


Fig: 1.5 Huperzine

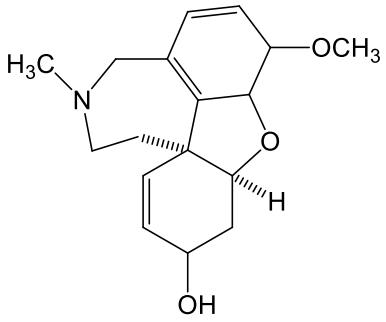


Fig: 1.6 Galathmine

Galanthamine an active competitive cholinesterase inhibitor, a natural product isolated from *Galanthus nivalis* in the 1950s. Under the name of Nivalein, Galanthamine is marketed in Austria for A.D. and Germany for other indications such as facial neuralgia (Review, Plants as Serves of Drugs, April 2000).

The past decade has witnessed the market introduction of many α-glucosidase inhibitors derived from natural merchandise within the antidiabetes space. Advanced sugar acarbose was isolated from Actinoplanes sp. acetylsalicylic acid from an inquiry for a-glycosidase protein inhibitors. By inhibiting α -glucosidase, acarbose decreases the discharge of aldohexose from eaten carbohydrates. It slows the rise of food-induced blood sugar levels. Acarbose is approved in Deutschland, Japan, the U.S. used as adjuvant medical care in polygenic disease.

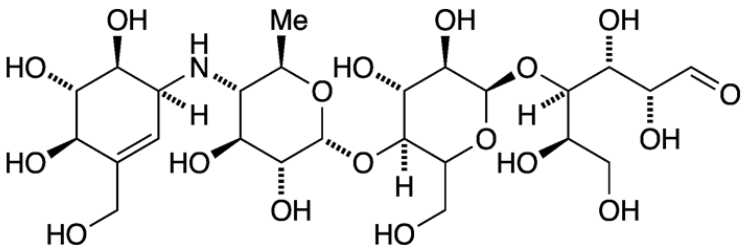


Fig: 1.7 Acarbose

Forskolin (Colforsin) has been isolated from *Coleus forskohlii* at Hoechsts research lab India. It has cardioactive properties. Forskolin was also found as an adenylate cyclase activator. Colforsin daproate (NHK-477) is a semisynthetic product of forskolin derivative. It brought clinical trials in Japan to treat cardiac insufficiency and to treat asthma.

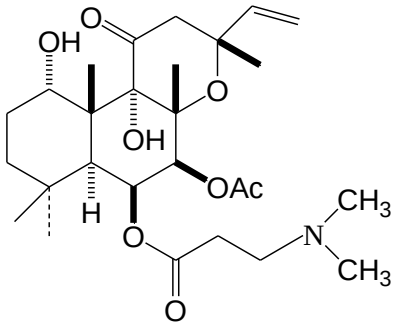


Fig: 1.8 Colforsin daproate

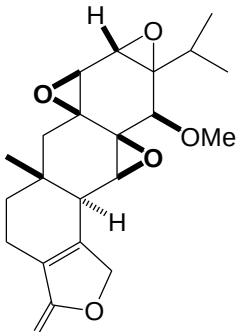
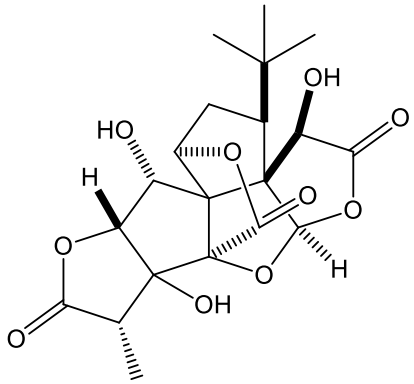


Fig: 1.9 Triptolide

Triptolide (1.**9**) is an active compound isolated from *Tripterygium wilfordii*, used to treat rheumatoid arthritis. A variety of formulations was developed, effectively in treating inflammatory and autoimmune diseases.

The Chinese medicinal tree *Ginkgo biloba* has been used for decades. More recently, extracts of the leaves have become available in many European countries as over-the-counter products to treat cerebral vascular insufficiency and tinnitus. Ginkgolides, a class of unique diterpene cage-like molecules, were isolated from the leaves of *Ginkgo biloba* and represented a group of highly selective platelet activity factor (PAF) receptor antagonists. Among them, Ginkgolide-B has been advanced to clinical trials to treat septic shock in patients. With severe sepsis caused by Gram-positive bacterial infections and good results in inflammatory and autoimmune disorders (Newman, D. J., 2003, *Journal of Natural Products*, **66,** 1022-1037.).



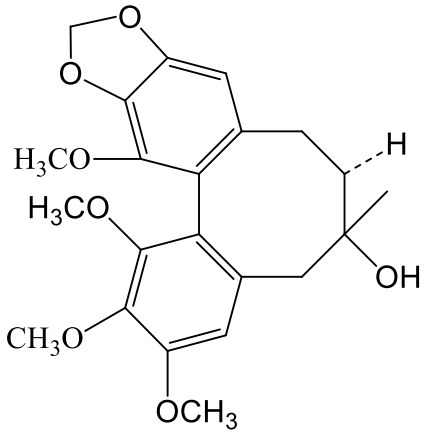


Fig:1.10 Ginkgolide B

Fig: 1.11 Gomisin A

Gomisin A (**1.11**) isolated from the dry fruit of *Schisondra chinensis*, used for the treatment of liver intoxication. Gomisin-A was found to be hepatoprotective and protect liver damage.

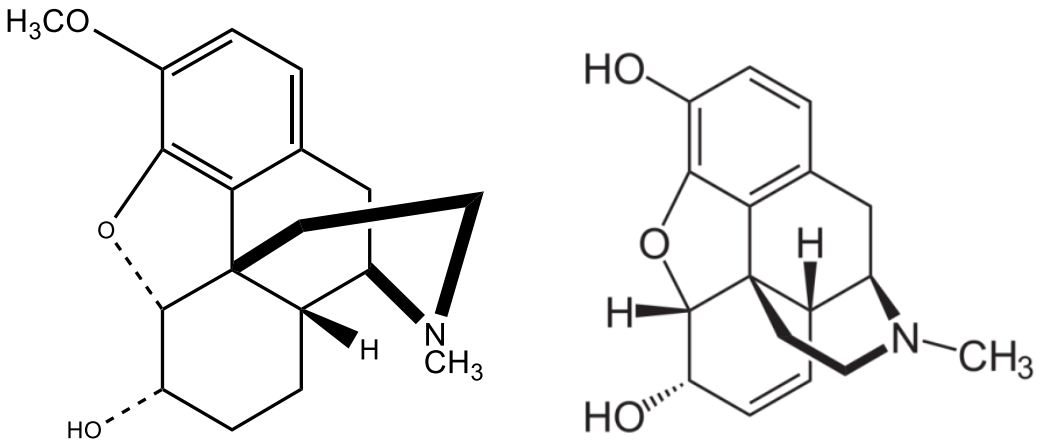
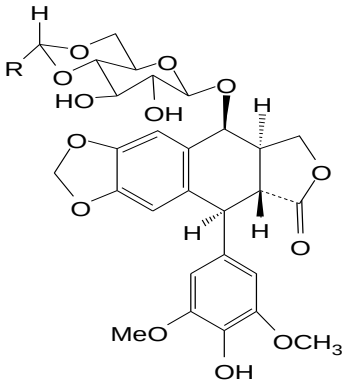


Fig: 1.12 Dextromethorphan and Morphine

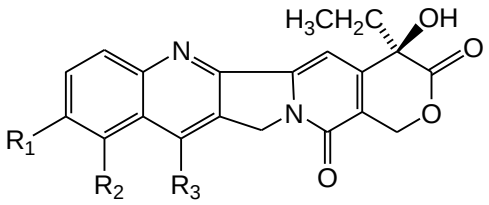
Serturner first isolated Morphine in 1806, then by Codeine (1832) by Robiquet, and then the non-morphine alkaloid papaverine by Merck in 1848 from the seeds of *Poppy*. Dextromethorphan is a semisynthetic product of Morphine used in most cough syrup today.

Etoposide and teniposide, two potent anticancer drugs, were isolated from the roots of several *Podophyllum* species. Early American and Asian societies used these plants for therapeutic purposes, including treating skin cancer and warts.



R = Me; Etoposide

R =  ; Teniposide



Camptothecin $R_1 = R_2 = R_3 = H$;

Toptican R_1 = OH, $R_2 = CH_2N(CH_3)_2$, $R_3 = H$;

Fig: 1.13 Camptothecin and Toptican

Camptothecin was isolated from the Chinese ornamental plant *Camptotheca acuminata* by Wani and Wall. Topotecan is modified camptothecin was approved for use in the USA in 1996. The discovery of quinine was made from *Cinchona* bark, which is used to treat malaria by French scientists Caventon and Pelletier (Tapsell, L. C . *et al*.,)

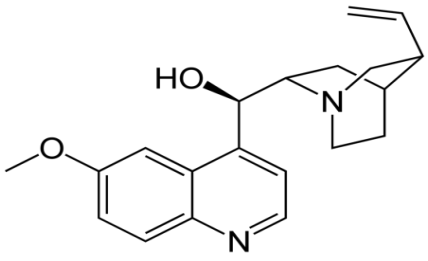


Fig: 1.14 Quinine

Uses of folks or ancient medication represent the means of cutoff discovery of contemporary medicine. A list of healthy plants compiled by the U.N. agency

supported literature from ninety-one countries. The classical text on Ayurvedic and Unani medication listed 21000 species of "medicinal plants." per the U.N. agency, around 5.76 billion populations within the developing world accept flavourer remedies for their primary care.

Plants can still be vital as a supply of the latest medicine, as proved by recent approvals. New plant-derived medicine supported the secondary metabolites of plants. Sex gland cancer treatment, a replacement drug, has recently been approved. A comparatively new antineoplastic agent that helped podophyllotoxin is etoposide. A constituent of the Mayapple magnoliid Ddicot genus petatum is beneficial in treating refractory male reproductive gland carcinomas, small cell respiratory organ carcinomas, non-Hodgkin's malignant, neoplastic disease, and nonlymphocytic leukemia.

The list of modern medicine derived from medicinal plants is very long now. Some of them are as follows:

Table 1.1: Important chemicals from a plant source and their actions. (Zhang, X . 2004)

Drug/Chemical	Action/Clinical use	Plant source
Betulinic acid	Anticancerous	*Betula alba*
Camptothecin	Anticancerous	*Camptotheca acuminata*
Chymopapain	Proteolytic, mucolytic	*Carica papaya*
Cissampeline	Skeletal muscle relaxant	*Cissampelos pareira*
Colchiceine amide	Antitumor agent	*Colchicum autumnale*

Colchicine	Antitumor agent, anti-gout	*Colchicum autumnale*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*
Danthron	Laxative	*Cassia species*
L-Dopa	Anti-parkinsonism	*Mucuna sp*
Etoposide	Antitumor agent	*Podophyllum peltatum*
Glaucarubin	Amoebicide	*Simarouba glauca*
Glycyrrhizin	Sweetener, Addison's disease	*Glycyrrhiza glabra*
Hesperidin	Capillary fragility	*Citrus species*
Irinotecan	Anticancer, antitumor agent	*Camptotheca acuminata*
Lapachol	Anticancer, antitumor	*Tabebuia species*
Menthol	Rubefacient	*Mentha species*
Papain	Proteolytic, mucolytic	*Carica papaya*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Podophyllotoxin	Antitumor anticancer agent	*Podophyllum peltatum*
Quinidine	Antiarrhythmic	*Cinchona ledgeriana*
Quinine	Antimalarial, antipyretic	*Cinchona ledgeriana*

Rutin	Capillary fragility	*Citrus species*
Sennosides A, B	Laxative	*Cassia species*
Stevioside	Sweetner	*Stevia rebaudiana*
Taxol	Antitumor agent	*Taxus brevifolia*
Teniposide	Antitumor agent	*Podophyllum peltatum*
Theobromine	Diuretic, vasodilator	*Theobroma cacao*
Theophylline	Diuretic, brochodilator	*Theobroma cacao*
Topotecan	Antitumor, anticancer agent	*Camptotheca acuminata*
Trichosanthin	Abortifacient	*Trichosanthes kirilowii*
Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum*
Vasicine	Cerebral stimulant	*Vinca minor*
Vinblastine	Antitumor, Antileukemic agent	*Catharanthus roseus*
Vincristine	Antitumor, Antileukemic agent	*Catharanthus roseus*

1.3 Status of medicinal plants in Bangladesh

Approximately 2000 therapeutic herbs are included in the ***Materia medica*** of traditional medicine in this subcontinent. In Bangladesh, between 450 and 500 medicinal plants have been identified as growing or being available. The most rural population uses medicinal plants for their primary health care. Bangladesh's

traditional healthcare systems, such as Ayurvedic, Unani, Hekimi, and other folk remedies, make extensive herbal medicine (Begum F).

1.4 Description of the Burseraceae family

The Burseraceae family is mostly herbs or shrubs comprising about 17-19 genera and 540 species, including twining forms. The plant *Garuga pinnata* is an herbaceous plant. It grows mainly in the hilly and semi-evergreen areas of Bangladesh, India, Malaysia, and the Philippines. The *Garuga* species examined by us is called *Garuga pinnata*. Its stem, roots and bark find numerous applications in medicine and are particularly useful in asthma, the opacity of the cornea, menstrual disorders, cold, stimulant, and pulmonary infections. Garuganin is the characteristic compound of the species (B. Lavanya *et al*.,).

1.5 Description of *Garuga pinnata* Roxb.

Garuga pinnata deciduous trees, to twenty-five meter high, bark gray or brown, shallowly lengthways corrugated, exfoliating in giant irregular flakes; blaze orange-red. Leaf galls copious. Fruit are greenish-yellow, rectangular or periodically global, horned; pyrenes a pair of or three; seed one, with a membranous wing (Wikipedia).

Fig 1.15: *Garuga Pinnata* leaves

Fig 1.16: Whole plant of *Garuga pinnata*

Fig 1.17: Bark of *Garuga pinnata* Roxb

1.5.1 Scientific classification

Kingdom : Plantae

Phylum : Tracheophyta

Class : Magnoliopsida

Order : Sapindales

Family : Burseraceae

Genus : *Garuga* Roxb

Species : *Garua pinnata* Roxb

Binomial name : *Garuga pinnata* Roxb.

1.5.2 Other Names: bhadi (bangla); dabudabi (bangla); dabdubi (bangla); katrang bhadi (bangla); bon kapila (bangla); jeol bhadi (bangla); nil bhadi (bangla); paharijiga (chittagong); silbhadi (chittagong); kharpata (chittagong); moroung-shishu (mogh)

Bengali: জুম jum, কপিল kapila

Sanskrit: karnikarha, kinikirath

English: Garuga, grey downy balsam

Hindi: Kharpat

Telugu: Garuga, Konda vepa

Marathi: kakad

Konkani: Kudak

Oriya: kekadogatcho

Gujarathi: Kaked,Khusimb

Tamil: Arunelli, Karuvempu

Malayalam: Annakaara, Kaattunelli

Kannada: Aranelli, Biligadde,Kaashthanelli

Assamese: Pama

Chinese: 羽叶白头树 yu ye bai tou shu

Nepal: Dabadabe, Ramasin

1.6 literature survey of *Garuga pinnata* Roxb

As a herbal plant, *Garuga pinnata* is studied by researchers. The literature survey is described below.

1.6.1 Constituents: Biflavone, macrocyclic biphenyl ether, euphane triterpene alcohol, diarylheptanoids, tetracyclic triterpenoid, and biphenyl-derived macrocyclic diarylheptanoids (Kulsum ARA *et al*.; 2012)

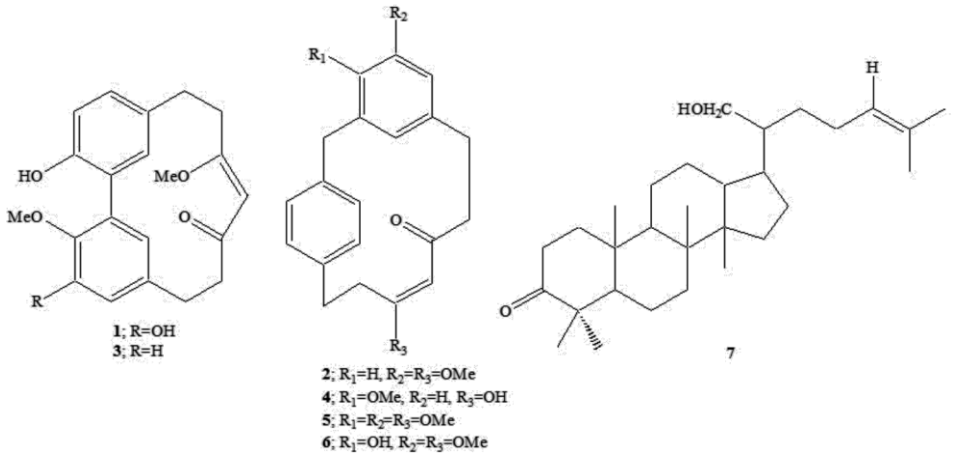


Figure 1.18: Structures of 6'-hydroxygaruganin V (**1**), garuganin IV (**2**), garuganin V (**3**), 9'-desmethylgarugamblin-I (**4**), garuganin III (**5**), 1-desmethylgaruganin III (**6**), and 21-hydroxydammar-24-en-3-one (**7**).

Actions: Adaptogen, Antibiotic; Analgesic, inhibitor, Anti-diabetic; Anticancer, Anti-carcinogenic, Antithrombotic, Antiviral, Antimicrobial, Antipyretic, Bitter tonic, Blood purifier; Cardio-protective, Choleretic, biological process.

1.6.2 Medicinal importance of *Garuga pinnata* Roxb

Garuga pinnata exhibits many healthful properties and is available to treat diabetes, abdomen issues, asthma attack, and healing of bone fractures, etc. Kathad *et al*.; (2010) reportable inhibitor activities of *Garuga pinnata* leaves wherever ethyl alcohol extract showed a significant inhibition proportion of DPPH (2,2-diphenyl-1-picrylhydrazyl), a hydroxyl group, gas, and anion. Annie Shirwaikar *et al*.; (2007) conjointly reportable effective anti-diabetic potentials of stem bark liquid extract of *Garuga pinnata* in streptozotocin-nicotinamide-induced diabetic

rats. Prapai Wongsikongman *et al*., (2002) reportable that the methanolic crude extract of *Garuga pinnata* Roxb possessed promising cytotoxic activity against human growth drug-resistant sublines.

1.6.3 Pharmacological Properties

1.6.3 .1 Bone Fracture

Suneetha *et al*.; (2011), within their work on native herbal therapy for bone fracture from the Western Ghats, declared the employment of *Garuga pinnata* in the healing of bone fracture by daubing crude stem bark paste on the realm of bone fracture.

1.6.3.2 Antioxidant Activity

Leaves, fruit, and stem bark of *Garuga pinnata* were collected and evaluated for its inhibitor activity by determinative DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, hydroxyl group activity, gas scavenging activity, superoxide radical scavenging activity, and scavenging of hydroxyl radical peroxide with completely different concentrations of methanolic extract (50,100 and 250 μg/ mL) Shahidi *et al*.; (1997). There is a high presence of polyphenols in stem bark instead of leaves and fruits.

1.6.3.3 Anti-diabetic Activity

The anti-diabetic effectualness of *Garuga pinnata* Roxburgh was evaluated by Thupurani *et al*.; (2013) wherever they used fuel and liquid extract of *Garuga pinnata* stem bark streptozotocin (oral administration) induced diabetic rats. Initially, glucose levels of animals were hyperbolic until the seventh day when streptozotocin administration. Thenceforth, there was a forceful decrease in glucose levels; those animals were treated with fuel extract at 2000 mg/kg weight. Their findings directly indicate that fuel extract of stem bark possesses anti-diabetic compounds that resulted in the reduction of glucose levels. Reduction within the aldohexose levels when seven days forward, perhaps due to the

regeneration of β-cells of the exocrine gland once treated with *Garuga pinnata* stem bark methanolic extract destroyed by streptozotocin.

1.6.3.4 Antiulcer Activity

The antiulcer activity of *Garuga pinnata* Roxb was studied by Chitra *et al*.; (2013). They used associate degree alcoholic extract of *Garuga pinnata* leaves against indomethacin-induced unusual person Wistar rat. A trial was created by Kapil *et al*.; in 2014 to check the antiulcer activity of hydro-alcoholic stem bark extract of *Garuga pinnata*. It had been utilized in 2 doses 200mg/kg and 400mg/kg. They used the orifice ligature model for their studies wherever Tagamet was used because of the customary. Orifice ligation-induced ulcers area unit thought to be caused because of the accumulated presence of acid and enzyme within the abdomen. The hydro-alcoholic stem bark extract of *Garuga pinnata* Roxburgh at a 400mg/kg dose was cared for decreased the acid and enzyme secretion within the abdomen, indicating this plant's notable antiulcer activity.

1.6.3.5 Anticancer Activity

Methanolic extract of leaf, stem, fruit, and stem bark of *Garuga pinnata* was studied to analyze malignant tumor activity. The methanol extract of *Garuga pinnata* stems bark has been noticed with important malignant tumor activity on MCF-7 human carcinoma cell lines.

1.6.3.6 Antibacterial activity

Shahidi *et al*.; (1997) assayed the bactericide activity of *Garuga pinnata* against coccus aureus ATCC 96, Bacillus globigii MTCC 441, Bacillus Cereus, enterics respiratory illness MTCC 109, E. coli ATCC 8739, Salmonella typhosa ATCC 4420, and Bacillus Cereus ATCC 9372 were methanol extracts of leaf, stem bark, fruit and stem were used. Among plant extracts tested, stem bark extract shows the best bactericide activity against various gram-positive and gram-negative microorganism strains. This could ensure that Garuganin-I and II, a famous diarylheptanoid isolated from the stem bark of this plant that exhibits structural

similarity with rifamycin S.V., a typical ansamycin antibiotic, recommend a similar mechanism for bactericide action.

1.6.3.7 Anthelmintic Activity

In 2013, a periodic survey of medicinal plants used as natural remedies by the native individuals of Manikganj district of People's Republic of Bangladesh to treat enteric worms was created by Eneh *et al*.; They came to understand the usage of juice from tip and tea made of leaves of *Garuga pinnata* for treating helminths.

1.6.3.8 Wound Healing

The wound healing potential of *Garuga pinnata* was 1st tried by Janhavi and Ashok in 2013. They used a dried alcoholic extract of *Garuga pinnata* bark at the concentration of 50mg/mL saline. It had been assessed on excision and dead area wound models victimization Swiss unusual person mice. The experimental animals, treated with the extract, showed seventy-two healing on the twelfth day of application compared to Martinmas healing of the management. The amino alkanoic acid content of the recovered space of the treated and the management mice was calculable to assess the strength of the healed wound.

1.7 Aim of the project

Bangladesh could be a sensible repository of medicinal plants happiness to numerous families and the Burseraceae family. The Bursera plants contain many chemical and distinctive pharmacologically active compounds and antiseptic, astringent, stomachic, medication, anti-rheumatic, anti-diarrhea, and medicament activities. Though an outsized range of torchwood family species is investigated domestically, a touch of attention was given to the present specific species. Therefore, an endeavor has been taken to check the chemical constituents and biological activities of *Garuga pinnata* Roxb. These investigations could offer some fascinating medicinal compounds. These are used as remedies for the treatment of some diseases. Since this plant is accessible in Asian nations and plenty of herbal health centers and herbal industries are mistreatment such as

connected seasoned plants for treatments, thus if the biological activity of this plant is studied thoroughly, this might be a cheap treatment. So, the target of the study is isolation and structural elucidation of the bioactive compounds by chemical and spectroscopic methods (U.V., FTIR, 1H-NMR, 13C-NMR, etc.) and to explore the chance of developing new drug candidates from this plant for the treatment of varied diseases.

1.8 Present study protocol

The present study is about isolating pure compounds and observing the biological activities of the isolated pure compounds with crude extract and their different fractions. The study protocol as the following steps:

- Cold extraction of the powdered stem of the plant with n-hexane, ethyl acetate, and methanol, respectively.
- Fractionation of each partitioned extract by column chromatography (CC).
- Isolation of pure compounds from different column fractions using various chromatographic methods.
- Determine the structure of compounds with the help of chemical and spectroscopic methods (U.V., FTIR, 1H-NMR, ^{13}C-NMR, etc.).
- Observation of antioxidant property of crude extracts, column fractions.
- Observation of *in vitro* antimicrobial activity of crude extracts, column fractions, and pure compounds.

Chapter-2

Experimental

EXPERIMENTAL

This chapter briefly describes the various methods followed in extraction, fractionation, & purification of the compounds in experimental works.

2.1 Solvents and chemicals

This experiment used analytical and laboratory-grade solvents and chemicals from Merck (Germany), BDH (England). The commercial-grade solvents (ethyl acetate, methanol, n-hexane, and dichloromethane) were distilled before use.

2.2 Distillation of the solvents

The analytical grade solvents (ethyl acetate, methanol, n-hexane, and dichloromethane) were distilled. Distilled solvents were used throughout the investigation.

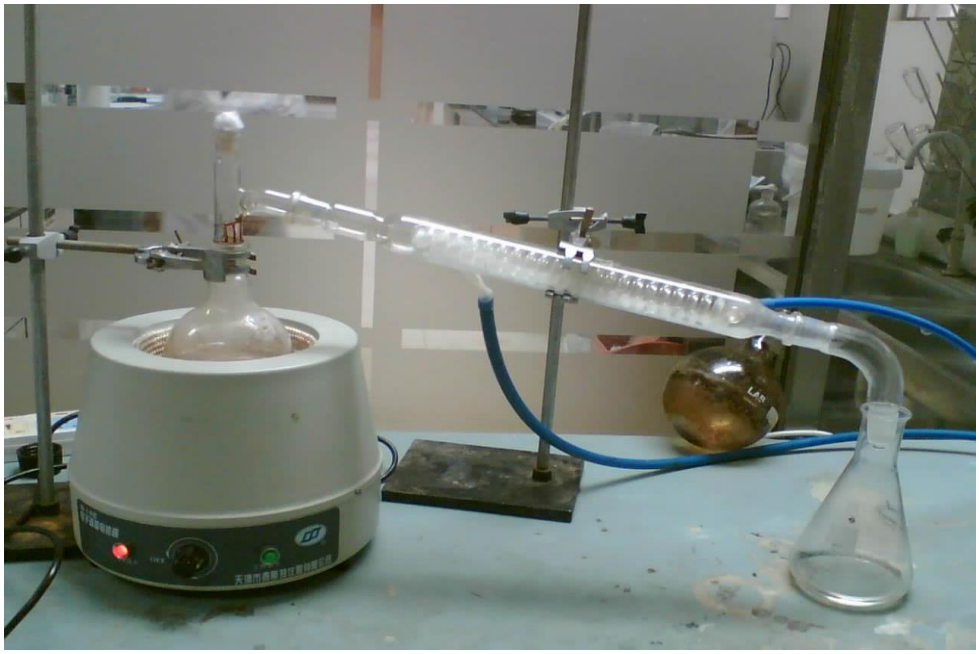


Fig 2.1: Distillation process.

2.3 Evaporation

All evaporations were performed using a rotary evaporator under 40^0C. The solvent in the extract and compounds were removed under a high vacuum.

Fig 2.2: Rotary vacuum evaporator

2.4 Preparation of the spray reagents

Fig 2.3: Spray reagent

2.4.1 Spray reagent (Developing reagent)

1mL anisaldehyde

20mL acetic acid (glacial)

10mL sulfuric acid

170 mL methanol

Anisaldehyde-sulphuric acid reagent (AS): 1 mL Anisaldehyde mixed with 20 mL glacial acetic acid, then 170 mL methanol and 10 mL concentrated sulphuric acid, in that order. The reagent is unstable and is no longer useable when the color has turned red-violet.

2.5 Chromatographic techniques

In these experiments, thin-layer chromatography (TLC), column chromatography (CC), and Vacuum liquid chromatography (VLC) were used.

2.5.1 Thin-layer chromatography (TLC)

0.2 mm thin silica gel coated aluminum TLC plates were used for the experiment.

2.5.2 Sample application

The TLC plates, by using a capillary glass tube, the capillary tube was cleaned with acetone before each sample was applied.

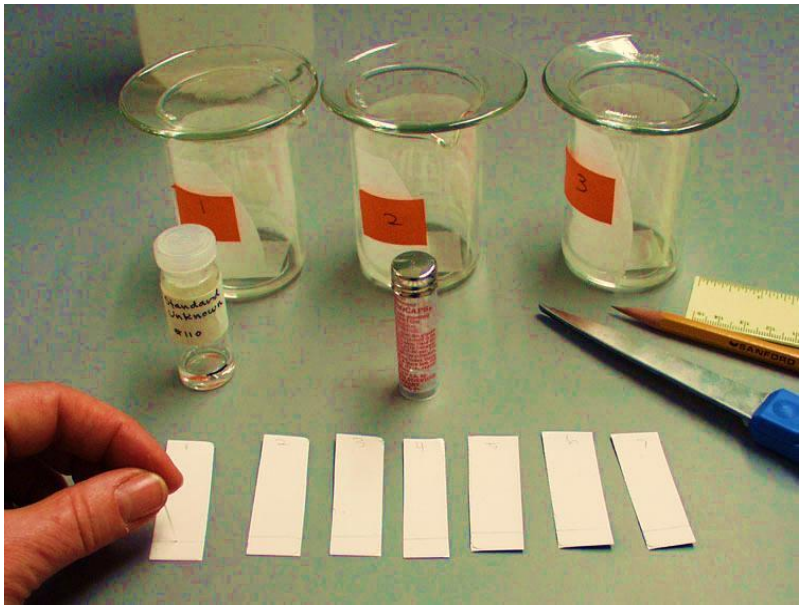


Fig 2.4: Process of spotting

2.5.3 Solvent system

Different polar and nonpolar solvents were used for TLC in different ratios are given below:

n-hexane: Ethyl acetate; Ethyl acetate: Methanol; n-hexane: dichloromethane;

Ethyl acetate: Dichloromethane; Dichloromethane: Methanol.

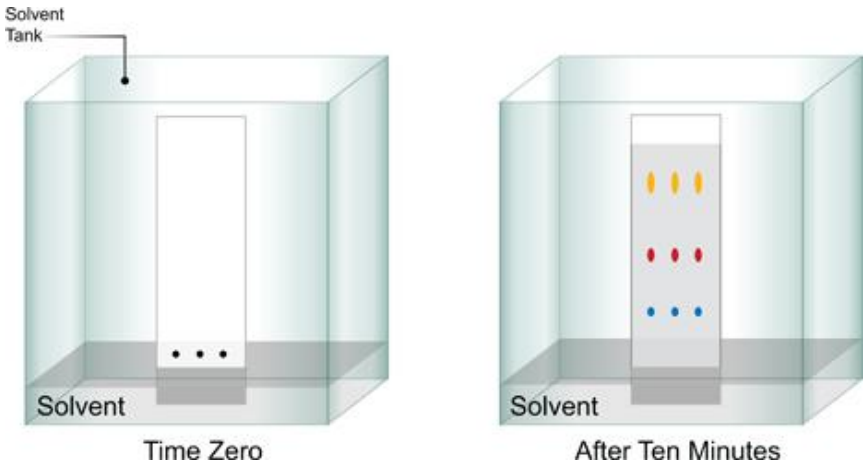


Fig 2.5: Developing of TLC plate

2.5.4 Preparation of TLC tanks

TLC plates were developed in glass jars and glass tanks. In a glass jar or tank, an appropriate solvent system was poured. Then the tank was covered and kept for allowing it to achieve saturation. The solvent level was kept underline of the spot. As the solvent rises, the plate becomes wet. When the solvent front forwards the end of the plate, the plate was then taken out and dried.

2.5.5 Detection of spots

To identify the separated compound at the plate was placed under UV light of 254 and 361 nm wavelength. The spray reagent was then used to develop the plates, which were then heated at 100°C (in an oven) for a few minutes.

Fig 2.6: TLC spot detection under UV lamp

2.5.6 The R_f value

The retardation factor (Rf) is the ratio of the distance traveled by the compound to the distance traveled by the solvent front.

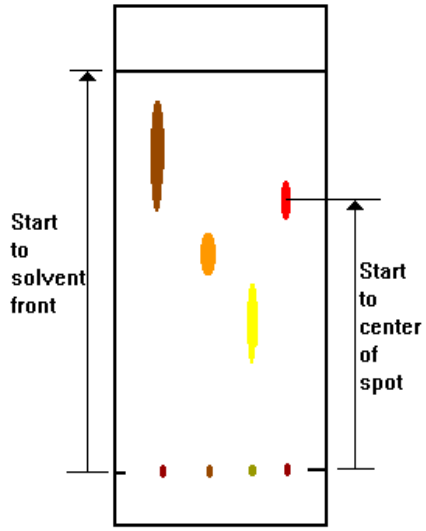


Fig 2.7: Calculation of R_f value

$$R_f = \frac{\text{Distance traveled by a substance}}{\text{Distance traveled by a solvent}}$$

R_f value is the physical property of a compound and constant for a specific compound.

$$R_f = \frac{B}{A}$$

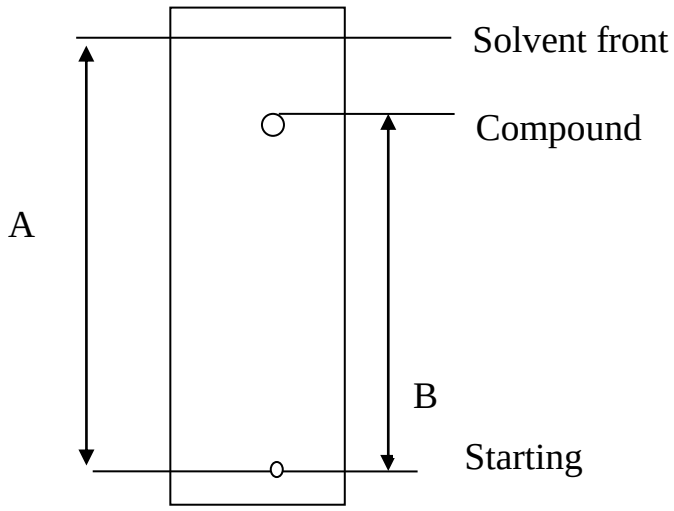


Figure 2.8: The R_f value calculation.

2.5.7 Stationary phases of column chromatography

For normal phase column chromatography, silica gel (230-400 mesh)(Merck) was used, and separation by gravitational flow with solvents of increasing polarity. The sample was applied into the column either as a solution or in a powdered form by adsorbing samples with the silica gel. The elute were stored in several test tubes and made different fractions based on R_f values.

2.5.8 Procedure for micro-scale flash column chromatography

In microscale flash chromatography, the solvent flows very slowly through the column by gravity.

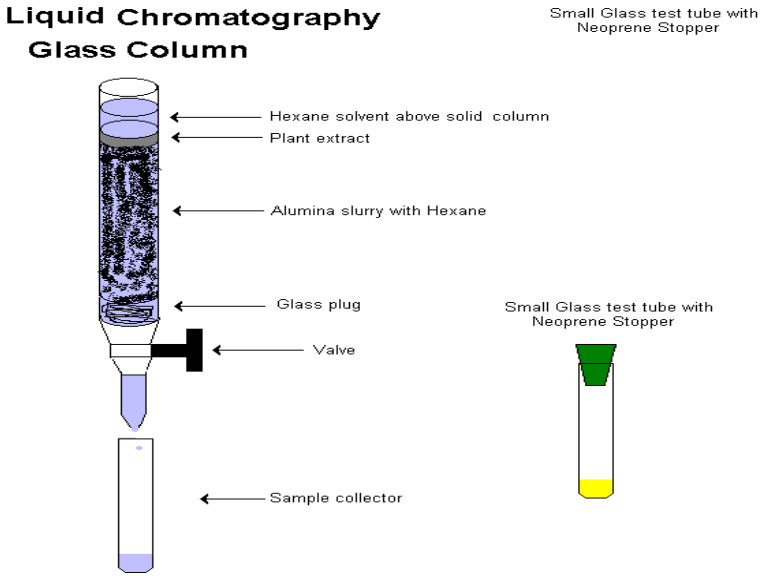


Fig 2.9: Various parts of a column

2.5.9 Preparation of column (For micro-scale operation)

A small amount of cotton was plugged at the bottom of the Pasteur pipette to prevent the adsorbent from leaking. The Pasteur pipette was filled with the slurry of column-grade silica gel with a stream of solvent using a dropper. It was ensured that the "sub-column" was free from air bubbles by several recycling solvents. The samples were applied at the top of the column. Elution was started with petroleum ether or n-hexane, followed by increasing polarity.

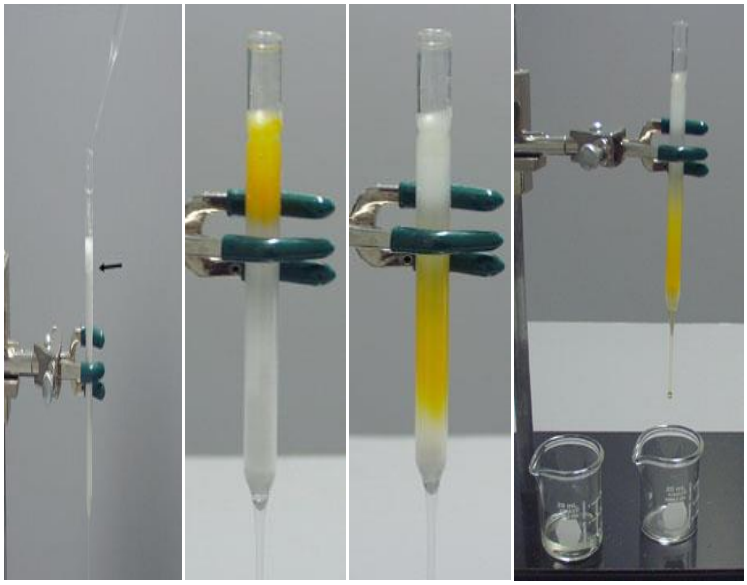


Fig 2.10: Various stages in microscale column.

2.6 Recrystallization

Recrystallization was the final procedure of purification. A minimum volume compound solution of the suitable solvent was prepared. It was then left for crystallization.

2.7 Spectroscopic Techniques

2.7.1 Nuclear Magnetic Resonance spectroscopy(NMR)

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a BRUKER NMR DPX-400 MHz instrument. Chemical shift data(ppm) reported relative to the solvent were used. The spectra were taken by using $CDCl_3$ and CD_3OD.

2.8 Investigation of *Garuga pinnata* Roxb.

2.8.1 Collection of the plant

The plant *Garuga pinnata* Roxb (Locally known as Kapil) was collected from Madhupur of Tangail district.

2.8.2 Identification of species

The Bangladesh National Herbarium's Botanist confirmed the taxonomical identification of the plant. A voucher specimen was deposited of this plant at Bangladesh National Herbarium.

2.8.3 Test of steroids

The stems powder (15 g) was extracted with a MeOH and $CHCl_3$ (1:1) 100 mL mixture. This extract was concentrated and divided into two parts. One part was treated with concentrated H_2SO_4. The reddish color indicates the presence of a steroidal compound. The other part was mixed with a few drops of concentrated H_2SO_4 with 4-6 drops of acetic anhydride. The development of a greenish color designates the presence of a steroidal compound.

2.8.4 Test of terpenoids

A few mg of sample was dissolved in a mixture of $CHCl_3$-CH_3OH and then a few drops of concentrated. H_2SO_4 and 4-6 drops of Ac_2O. The formation of red-violet color confirms the presence of terpenoid-type compounds.

2.8.5 Test of alkaloids

It extracted 2 g of powder by boiling 20 mL 1% H_2SO_4 in a 50 mL conical flask on a water bath for two minutes with occasional shaking, centrifuge, and pipette the supernatant into a tiny conical flask. To test for alkaloids, apply one drop of Meyer's reagent of 0.1 mL in a semi-micro tube. It produces an alkali-rich cream precipitate.

2.8.6 Test of carbohydrates

0.5 mL of aqueous extract was added to 5 mL of benedicts solution and boiled for 5 min. The formation of colored precipitate was due to the presence of carbohydrates.

2.8.7 Test of flavanoids

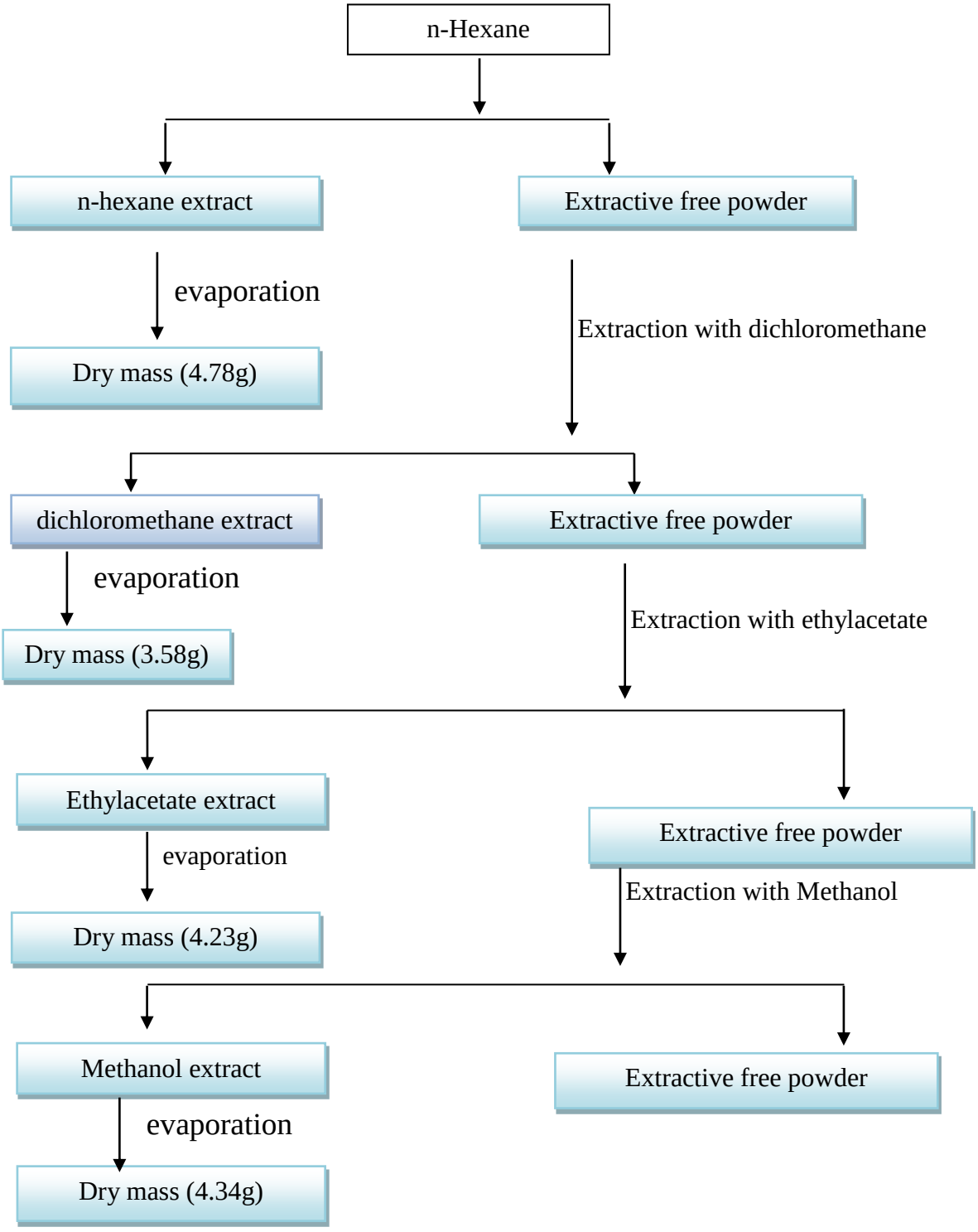
A few milligrams of alcoholic extract were mixed with 5-10 drops of dilute HCl, then a little bit of zinc was added. The presence of flavonoids was revealed by the production of pink or radish-colored precipitation.

2.8.8 Test of resins

To 0.05 mL sulphuric acid, a little quantity of alcoholic extract in 5 mL acetic anhydride was added. The presence of resins was revealed by the creation of a brilliant purplish-red tint.

2.8.9 Extraction, partition, and isolation of the compounds from *Garuga pinnata.*

Stems and barks of this plant were separated and dried under in open air and then dried in an oven at 37^{o}C. Afterward, it (~560 g) was powdered (~200 mesh) by a grinding machine. This powder (~302 g) was used throughout this investigation. The powder of *Garuga pinnata* was extracted with n-hexane, dichloromethane, ethyl acetate, and methanol. All the crude extracts were also subjected to antimicrobial tests.



Scheme 2.1: Extraction scheme of *Garuga pinnata*

2.9.1 Investigation of the ethyl acetate extract of *Garuga pinnata*

2.9.1.1 Thin-layer chromatography (TLC)

TLC analysis of the ethyl acetate extract showed several spots under UV lamp followed by the development by spray reagent on TLC plate.

2.9.2 Fractionation of the ethyl acetate extract by vacuum liquid column (VLC) chromatography

A rotary evaporator was used to condense the ethyl acetate extract to dry mass (4.23 g). The column-grade silica gel absorbed the dry mass of ethyl acetate extract. This sample was put on top of the TLC-grade silica gel-packed bed of the VLC column. The column was initially eluted with 100 percent n-hexane, then with n-hexane and ethyl acetate mixes to increase the polarity of the solvents, and lastly, with ethyl acetate methanol combinations. The eluents were collected in a succession of conical flasks in a total volume of 150 mL. Table 2.1 lists the solvent systems utilized as mobile phases in the ethyl acetate component analysis.

Table-2.1: Fractions collected from vacuum liquid chromatography (VLC) of ethyl acetate extract (4.23 g) using different solvent systems

Fraction no.	Solvent system	Volume
1	n-hexane (100%)	150 mL
2	n- hexane : ethyl acetate (90:10)	135 mL
3	n- hexane : ethyl acetate (80:20)	120 mL
4	n- hexane : ethyl acetate (70:30)	100 mL
5	n- hexane : ethyl acetate (60:40)	100 mL
6	n- hexane : ethyl acetate (50:50)	100 mL
7	n- hexane : ethyl acetate (40:60)	100 mL
8	n- hexane : ethyl acetate (30:70)	100 mL
9	n- hexane : ethyl acetate (20:80)	100 mL
10	n- hexane : ethyl acetate (10:90)	135 mL
11	ethyl acetate (100%)	200 mL
12	ethyl acetate : methanol (95:05)	200 mL
13	ethyl acetate : methanol (90:10)	200 mL
14	ethyl acetate : methanol (85:15)	200 mL
15	ethyl acetate : methanol (80:20)	200 mL
16	ethyl acetate : methanol (75:25)	200 mL
17	ethyl acetate : methanol (70:30)	200 mL
18	ethyl acetate : methanol (65:35)	200 mL
19	ethyl acetate : methanol (60:40)	200 mL
20	ethyl acetate : methanol (55:45)	200 mL
21	ethyl acetate : methanol (50:50)	200 mL
22	methanol (100%)	200 mL

2.9.3 Analysis of the collected Ethyl acetate fractions

Fraction F-1 to 15 shows no prominent spot on TLC analysis. Maybe it was oily and fatty substances. So, they were discarded. Fraction F-16 to F-22 shows spots in TLC analysis; they were mixed. Among the fractions, the TLC analysis of F16 to F22 was tailing. So they were mixed and fractionated by using a column. The VLC fractions were run in a sub-column and got column fractions P-1 to P-71. Column fractions P-51to P-53 were the same and fractionated by another column using the mobile phase MeOH: EA ≡ 20:80. They were again fractionated pipette column using EA: MeOH ≡ 50:50 solvent system. Since they contained some contamination, a pipette column was purified by PTLC and got GPE-1, 5.9mg (R_f =0.11 in EA: MeOH ≡ 50:50) .

2.10. Investigation of Dichloromethane (DCM) extract

2.10.1 Thin-layer chromatography (TLC)

TLC analysis of the DCM extract showed several spots under a UV lamp, which was further confirmed by developing a spray reagent on a TLC plate for detecting the spots.

2.10.2 Fractionation of the DCM extract by column chromatography

Using a rotary evaporator, concentrate the DCM extract to dry mass (3.58g). The column-grade silica gel absorbed the dry mass of DCM extract. This sample was placed on top of the column's silica gel bed (column-grade silica gel). The column was initially eluted with 100 percent n-hexane, followed by combinations of n-hexane, DCM, and ethyl acetate to increase the polarity of the solvents, and lastly, with ethyl acetate & methanol. **Table 2.2** lists the solvent systems that were employed as mobile phases in the DCM component analysis.

Table-2.2: Fractions collected from Solid-liquid column chromatography (CC) of DCM extract using different solvent systems

Fraction no.	Solvent system	Volume collection
1 - 7	n-hexane (100%)	140 mL
8 - 19	n- hexane : DCM (95:5)	50mL
20 – 25	n- hexane : DCM (90:10)	20 mL
26 - 36	n- hexane : DCM (85:15)	20 mL
37 - 42	n- hexane : DCM (80:20)	20 mL
43 - 47	n- hexane : DCM (75:25)	20 mL

48 - 53	n- hexane : DCM (70:30)	30 mL
54 - 59	n- hexane : DCM (60:40)	20 mL
60 - 64	n- hexane : DCM (50:50)	20 mL
65 - 69	n- hexane : DCM (60:40)	20 mL
70 - 76	DCM (100%)	20 mL
77 - 82	DCM: ethyl acetate (80: 20)	20 mL
83 - 87	DCM: ethyl acetate (60:40)	20 mL
88 - 92	DCM: ethyl acetate (50: 50)	30 mL
93 - 96	DCM: ethyl acetate (90:10)	20 mL
96 - 100	ethyl acetate 100%	20 mL
101 - 105	ethyl acetate: methanol (75:25)	20 mL
106 - 114	ethyl acetate: methanol (50:50)	20 mL
115-120	methanol (100%)	50 mL

2.11 Analysis of the properties of GPE-2 (compound-1)

Fraction F-1 to F-14 shows no prominent spot on TLC analysis. Maybe it was oily and fatty substances. So, they were discarded. Fraction F-26 to 38 were similar. Then they were treated with charcoal to get the chlorophyll absorbed by the charcoal. Further pipette column chromatography was done. GPE-2 **(compound-1 as stigmasterol)** (R_f =0.16, n-Hexane:DCM=50:50) was obtained.

Table: 2.3 Isolation and purification of GPE-2(8mg)(compound-1) by pipette column from DCM extract fraction (F-26-F-38)

Fraction No.	Solvent system	TLC Examination	Observation	Fraction. of compound
1-7	Hex : DCM (9:1)	No spot	None	Discarded
8-13	Hex: DCM (7:3)	spot with tailing	No good resolution	Discarded
* 14-20	Hex : DCM (1:1)	One spot R_f 0.16	One pure compound	GPE-2 **(comound-1)**
21-28	DCM: EA (1:1)	One spot with tailing	No good resolution	Discarded
29-35	DCM: EA (1:2)	Tailing near the baseline	No good resolution	Discarded

DCM fraction F26 to F-38 shows prominent TLC spots and ran for pipette column chromatography with the different solvent systems. Pipette fractions P1 to P7 indicate no spot. P8-P13 shows a spot with tailing, so that was discarded. P14-P20 shows a single spot and purified by preparative TLC

2.11.1 Physical properties of GPE-2 (compound-1)

The compound-1 is a white crystalline compound. The R_f value of the compound is 0.16 in Hexane: DCM ≡ 50:50. It is soluble in DCM. The white crystalline and treatment with concentrated H_2SO_4 and acetic anhydride indicate the steroidal compound.

2.11.2 Characterization of GPE-2 (compound-1) by spectroscopic method

2.11.2.1 ^{1}H-NMR spectroscopy of GPE-2 (compound-1)

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound-1 **(Fig: 3.1)** has signals at δ_H (ppm) 5.347 (1H, d), 5.148 (1H, m), 5.010 (1H, m), 3.513 (1H, m; oxymethineprotone), 2.262 (2H, m), 1.987 (2H, t), 1.835 (2H, m), 1.488(6H, s) 1.001 (6H, s), 0.913 (3H, d), 0.807 (9H, m), 0.687 (3H, d).

2.11.2.2 ^{13}C-NMR spectroscopy of GPE-2 (compound-1)

The ^{13}C-NMR spectrum (100 MHz) in $CDCl_3$ of the compound-1 has signals at δ_C (ppm) 37.30, 31.72, 71.85, 42.36, 140.81**,**121.74, 31.96, 31.96,50.2, 36.56, 21.13, 39.83, 40.50, 56.82, 24.34, 28.28, 56.12, 12.08, 19.43, 36.19, 18.82, 138.33, 129.31, 45.9, 29.23, 19.84, 19.08, 23.13, 12.26..

2.12 Analysis of the properties of GPE-3 (compound-2)

DCM Fraction F-40 to 46 were similar. Then they were treated with charcoal to get the chlorophyll absorbed by the charcoal. Further pipette column chromatography was done. Thus the compound GPE-3 **(compound-2 as β-sitosterol)** (R_f =0.12 in n-Hexane:DCM=50:50) was obtained.

Table: 2.4 Isolation and purification of GPE-3(~4.2 mg)(compound-2) by pipette column from DCM extract fraction (F-40-F-46).

Fraction No.	Solvent system	TLC Examination	Observation	Fraction. of compound
1-5	Hex : DCM (9:1)	No spot	None	Discarded
6-12	Hex: DCM (7:3)	spot with tailing	No good resolution	Discarded
* 13-19	Hex : DCM (3:7)	One spot R_f 0.12	One pure compound	GPE-3 **(comound-2)**
20-26	DCM: EA (1:1)	One spot with tailing	No good resolution	Discarded
27-33	DCM: EA (1:2)	Tailing near the baseline	No good resolution	Discarded

DCM fraction F40 to F-46 shows prominent TLC spots and ran for pipette column chromatography with the different solvent systems. Pipette fractions P1 to P5 indicate no spot. P6-P12 shows a spot with tailing, so that was discarded. P13-P19 shows a single spot and purified by PTLC. The compound was designated as **GPE-3.**

2.12.1 Physical properties of GPE-3 (compound-2)

The compound-1 is a white crystalline compound. The R_f value of the compound is 0.12 in Hexane: DCM ≡ 50:50. It is soluble in DCM. The white crystalline and treatment with concentrated H_2SO_4 and acetic anhydride indicate the steroidal compound.

2.12.2 Characterization of GPE-3 (compound-2) by spectroscopic method

2.12.2.1 ^{1}H-NMR spectroscopy of GPE-3 (compound-2)

The ^{1}H-NMR spectrum(400MHz, $CDCl_3$) of the compound-1 revealed the peaks at δ 0.667, 0.997, 0.814, 0.832, 0.850, 1.240 ppm was observed

2.12.2.2 ^{13}C-NMR spectroscopy of GPE-2 (compound-1)

The ^{13}C-NMR spectrum (100MHz, $CDCl_3$) of the compound -1 showed the main peaks at , 37.28, 31.68, 71.87, 42.35, 140.77, 31.95, 31.68, 50.17, 36.17, 21.24, 39.71, 42.88, 56.90, 23.1, 29.72, 56.09, 12.27, 19.42, 39.81, 21.11, 51.27, 31.95, 19.01, 21.24, 25.43 and 12.27 ppm.

2.13 Analysis of the properties of GPE-4 (compound-3)

The fractions of the F-57 to 69 were mixed for their similar R_f value. Then they were run by a column with the solvent system n-hexane: EA ≡ 70:30. Finally, preparative TLC was done with a solvent system of 20% ethyl acetate in n-hexane and isolated the compound GPE-4 **(compound-3 as Isofouquerone)** was obtained.

Table 2.5: Examination and purification of GPE-4 (15mg)(compound-3) by pipette column from DCM Extract fraction 57-69.

Fraction No.	Solvent system	TLC Examination	Observation	Fraction of compound
1-6	Hex: DCM(90:10)	No spot	None	Discarded
7-12	Hex: DCM(70:30)	spot with tailing	No good resolution	Discarded
13-19	Hex: DCM(50:50)	One spot with tailing	No good resolution	Discarded
* 20-27	Hex: EA(80:20) (PTLC purified)	One spot R_f 0.12 at Hex:EA(95:5)	One pure compound	GPE-4 **(compound-3)**
28-34	Hex: EA(60:40)	Tailing near baseline	No good resolution	Discarded
35-40	Hex: DCM(40:60)	No clear spot	None	Discarded

In the fractions F-57 to 69, some colorless crystals formed at the bottom of the test tube and showed similar spots in TLC analysis and mixed. But the TLC

analysis provided the information of the little contamination of another compound separated by performing a pipette column using the different solvent system as the mobile phase to obtain various fractions. According to the TLC analysis, the pipette fraction P-1 to 6, P-7 to 12, P-13 to 19, and P-28 to 40 at the various solvent systems showed no spot and no good resolution and spot with tailing near the baseline for their oily and fatty substances. So, they were discarded. On the other hand, the fraction P-20 to 27 (15 mg) showed almost a single spot at R_f 0.12 in the solvent system Hexane: ethyl acetate (95:5). The single spot indicates it may be a pure compound. Then PTLC was done. The compound was designated as **GPE-4.**

2.13.1 Physical properties of GPE-4 (compound-3)

The compound-3 is a white crystalline compound. The R_f value of compound-3 is 0.12 in 5% ethyl acetate in hexane. It is soluble in dichloromethane, ethyl acetate, methanol, and ethanol. The white crystalline and treatment with concentrated H_2SO_4 and acetic anhydride confirm that compound-3 is a steroid-type compound.

2.13.2 Characterization of GPE-4 (compound-3) by spectroscopic method

2.13.2.1 ^{1}H-NMR spectroscopy of GPE-4 (compound-3)

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound-3 (GPE-4) has main signals at δ_H (ppm) 0.741, 0.761, 1.121, 2.330, 5.269, 1.240, 0.973, 0.832, 0.850 and 1.240.

2.13.2.2 ^{13}C-NMR spectroscopy of GPE-4 (compound-3)

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of the compound-3 has signals at δ_C (ppm) 17.09, 18.32, 25.95, 30.69, 29.38, 76.71, 143.60, 122.67, 41.05, 23.59, 79.07, 14.13, 13.34, 45.90, 27.70, 32.66, 47.65, 39.30, 27.20, 22.71, 37.10, 46.54, 38.78, 33.82, 18.32, 55.24, 41.65, 182.87, 33.08, 38.43.

2.14 Analysis of the properties of compound-4 (GPE-5)

In the DCM fractions F-83 to 87 (500mg), some colorless crystals were formed at the bottom of the test tube and showed similar spots in TLC analysis, and they were mixed. But the TLC analysis provided the information of the little contamination of another compound separated by performing a pipette column using the different solvent system **(Table: 2.6)** as the mobile phase to obtain various fractions.

Table 2.6: Examination and purification of compound-4 by pipette column from fractions 83-87.

Fraction No.	Solvent system	TLC Examination	Observation	Fraction of compound
1-10	DCM: Hex (50:50)	No spot	None	Discarded
11-18	DCM: Hex (80:20)	spot with tailing	No good resolution	Discarded
*19-27	DCM (100%)	One spot R_f 0.58	One pure compound	GPE-5 **(com-4)**
28-36	DCM: EA (90:10)	One spot with tailing	No good resolution	Discarded

According to the TLC analysis of pipette fraction P-1 to 10, P-11 to 18, and P-28 to 36, the various solvent systems showed no spot and no good resolution and spot with tailing or tailing near the baseline for their oily and fatty substances. So, they were discarded. On the other hand, the fraction F-19 to 27 showed a single spot at R_f 0.0.58 at 100% DCM solvent system. The single spot indicates it may be a pure

compound. The compound was washed with 50% DCM in n-hexane. The compound was designated as **GPE-5.**

2.14.1 Physical properties

The compound-**4 (GPE-5)** (~3.5 mg) was a white powdered solid having an R_f value of 0.58 (100% DCM). It was soluble in chloroform.

2.14.2 Characterization of compound-4 (GPE-5) by spectroscopic method

2.14.2.1 ^{1}H-NMR spectroscopy of compound-4

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of the compound-3 has signals at δ_H (ppm) 0.66 (d), 0.73(s), 0.76(s), 0.80(s), 0.92(s), 0.94(s), 1.00(s), 1.65(s), 1.82-1.96(m), 2.35(dt), 3.16(dd), 4.55(br s), 4.65(br s).

CHAPTER 3

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 Primary investigation of the plant material

3.1.1 Plant material

A species of the Burseraceae family, *Garuga pinnata* Roxb, has been investigated in this work. The stems and bark were used for extraction.

3.1.2 Extraction of the plant materials

For cold extraction, air-dried and powdered plant stem material (0.302 kg) was suspended in n-Hexane, Dichloromethane, ethyl acetate, and Methanol for five days and shook randomly. Extracts were filtered through clean white fabrics every day. After that, I used Whatman No. 1 filter paper. Used a rotary evaporator at a low temperature (under 40°C) and reduced pressure to concentrate.

3.1.3 Compound isolation and characterization

Pure chemicals were obtained from raw samples using various chromatographic methods. The pure compounds were characterized using a variety of spectroscopic methods.

3.2 Characterization of isolated compounds from *Garuga pinnata*

3.2.1 Characterization of compound-1 (GPE-2) as stigmasterol

The compound-1 (~8mg) is a white crystalline compound. The R_f value of the compound is 0.16 in n-hexane: DCM ≡ 50:50. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol, and ethanol. It has been tested by the Salkawoski method, which developed a reddish color indicating that the compound may be a steroid (Melting point 138-140^{0}C).

3.2.1.1 Spectral analysis of Compound-1 (GPE-2)

The structure of the compound GPE-2 **(Fig: 3.6)** has been established by ^{1}H-NMR, ^{13}C-NMR spectral evidence.

3.2.1.2 ^{1}H-NMR spectroscopy of compound-1 (GPE-2) as stigmasterol

The ^{1}H-NMR spectrum of compound-1 revealed the peaks at δ 0.681, 1.001, 1.545 ppm were observed due to methyl groups of a steroid. Several multiplates between δ 1.042 - 2.296 ppm and a multiplate at δ 0.856 ppm are due to methylene and methyl protons present in the compound. The broad multiplate at δ 3.513 ppm indicates the presence of oxymethine proton flanked with two different methylene groups (-CH_2-CHOH-CH_2-). Two multiplates at δ 5.148 and 5.010 ppm in the spectrum indicated the presence of two olefinic protons attached with two methylene groups (>CH-CH=CH-CH<) in the side chain of the compound.

A broad singlet at δ 5.347 ppm indicated the presence of a double bond in between a quaternary carbon and a methylene carbon, *i.e.,* presence of olefinic proton.

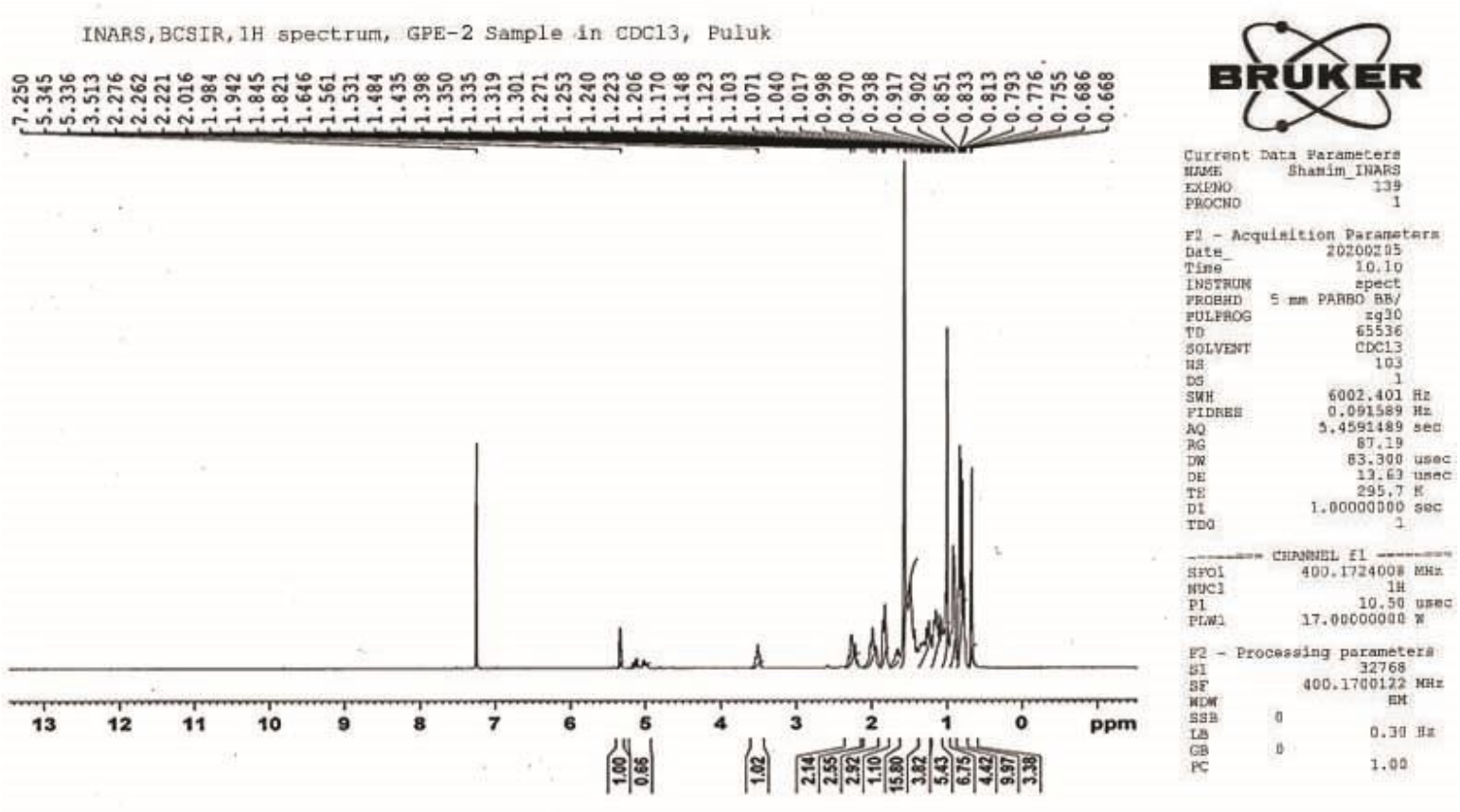


Figure 3.1: ^{1}H NMR spectrum compound-1

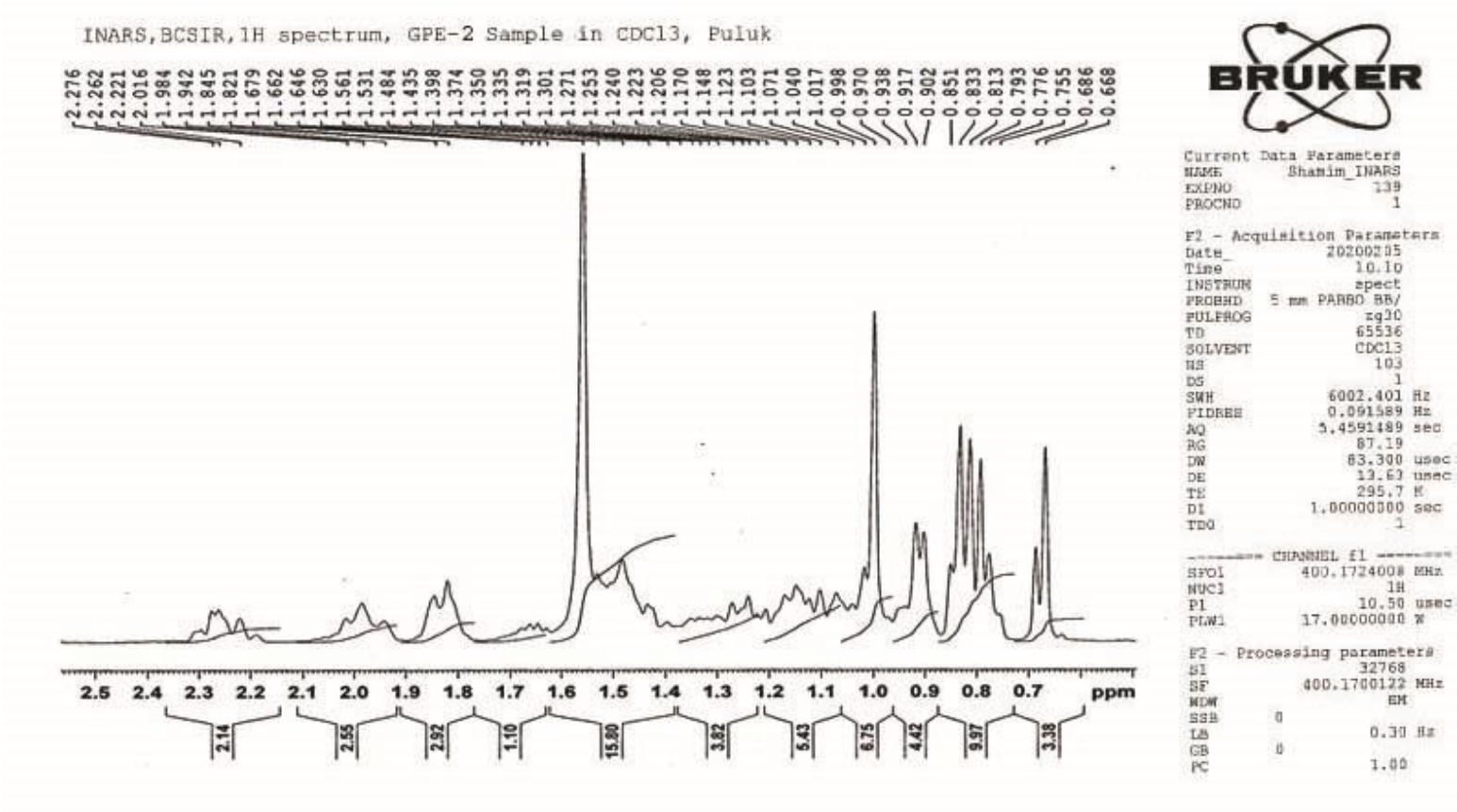


Figure 3.2: Expanded ^{1}H NMR spectrum of compound-1

3.2.1.3 ^{13}C-NMR spectroscopy of compound-1 (GPE-2) as stigmasterol

The 13C-NMR spectrum in $CDCl_3$ of the compound -1 showed various chemical shifts for various carbon. The peak at δ 71.85 (C-3) ppm confirmed the presence of the oxymethine group in the compound. The peaks at δ 138.33 (C-22) and 129.31 (C-23) ppm spectrum indicated the presence of two olefinic protons attached with two methylene groups (>CH-CH=CH-CH<) in the side chain of the compound. Peaks at δ 140.81 (C-5) and 121.74 (C-6) ppm in the spectrum indicated the presence of a double bond in a quaternary carbon and a methylene carbon.

The ^{13}C-NMR exhibited exactly 29 carbon signals which suggested the compound may be a steroid. The other peaks are 37.3 (C-1), 31.72 (C-2), 71.85 (C-3), 42.36 (C-4), 31.96 (C-7), 31.96 (C-8), 50.2 (C-9), 36.56 (C-10), 21.13 (C-11), 39.83 (C-12), 40.50 (C-13), 56.82 (C-14), 24.34 (C-15), 28.28 (C-16), 56.12 (C-17), 12.08 (C-18), 19.43 (C-19), 36.19 (C-20), 18.82 (C-21), 45.9 (C-24), 29.23 (C-25), 19.84 (C-26), 19.08 (C-27), 23.13 (C-28) and 12.26 (C-29) ppm.

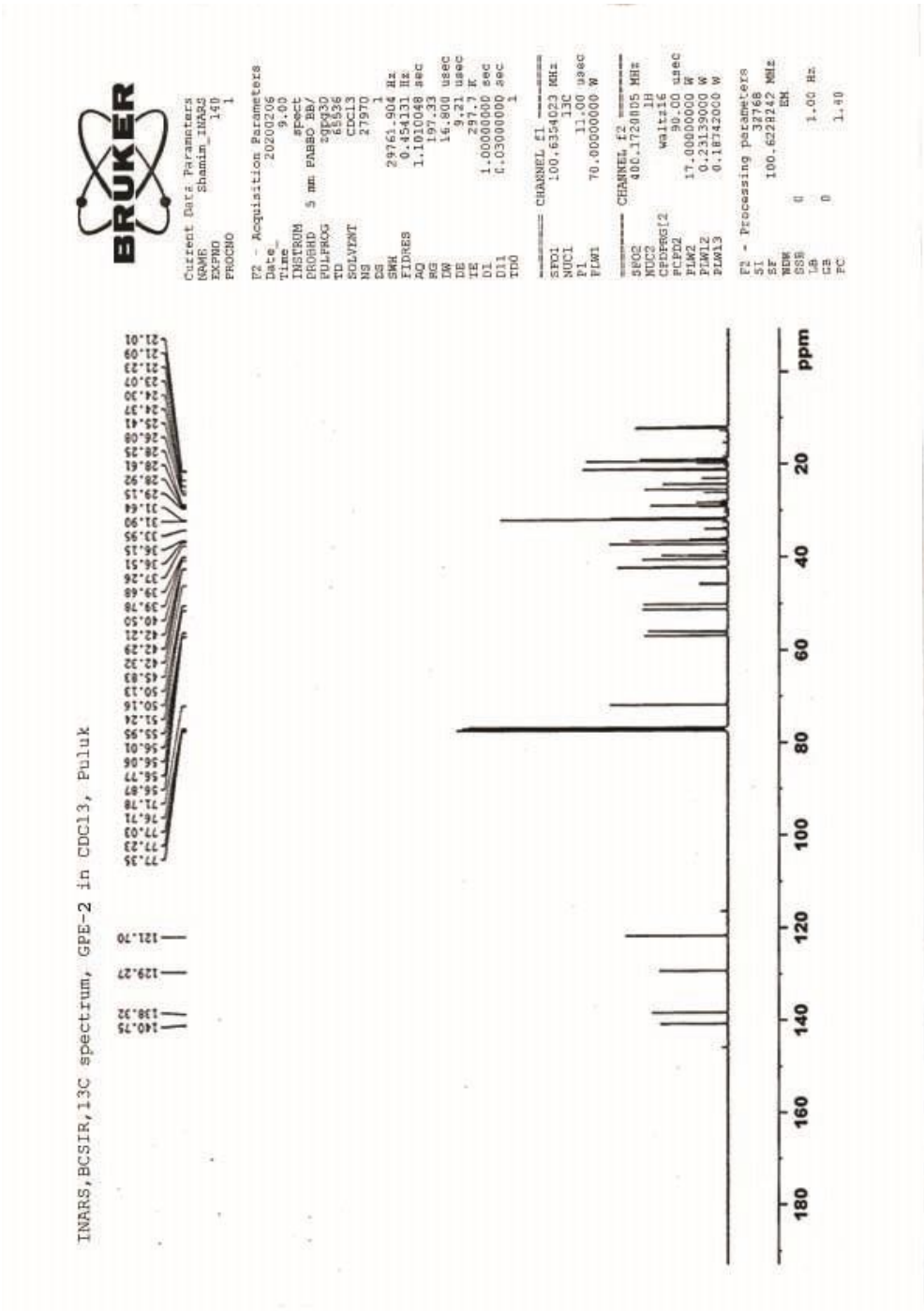


Figure 3.3: ^{13}C NMR spectrum of compound-1

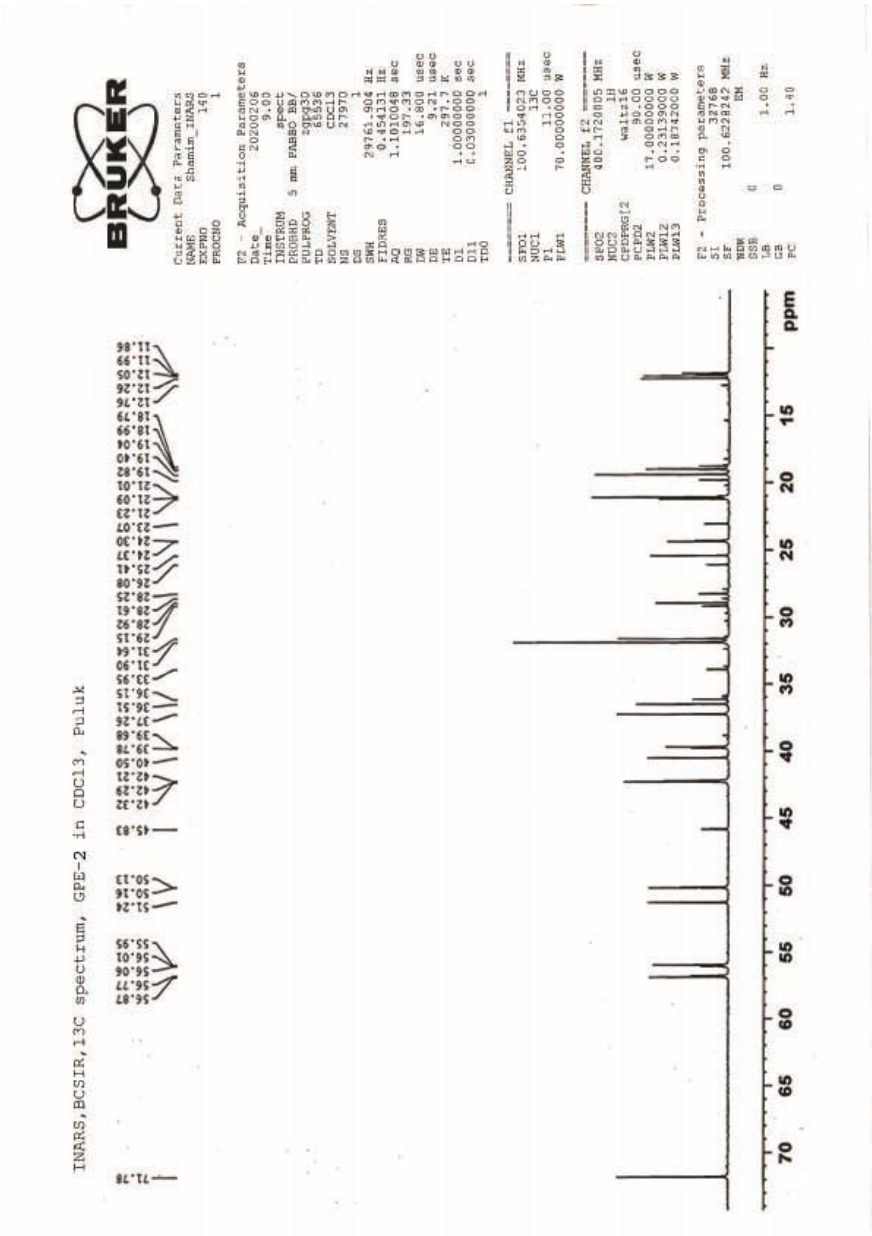


Figure 3.4: Expanded ^{13}C NMR spectrum of compound-1

The comparison of all these values with the reported value (Ali, *et al.*, 2003) has been shown in the following table 3.1:

Table 3.1: **^{13}C-NMR and ^{1}H NMR data of compound-1 (GPE-2) compared with published data of stigmasterol**

Carbon no.	Type of carbon	(Chemical shift in ppm)			
		stigmasterol	compound-1	stigmasterol	compound-1
		^{13}CNMR	^{13}CNMR	^{1}HNMR	^{1}HNMR
1	$-CH_2-$	37.31	37.30		
2	$-CH_2-$	31.69	31.72		
3	=CH-	71.81	71.85	3.517	3.513
4	$-CH_2-$	42.55	42.36		
5	=C=	140.5	140.81		
6	=CH-	121.69	121.74	5.34(1H, br,s)	5.345
7	$-CH_2-$	31.94	31.96		
8	=CH-	31.94	31.96		
9	=CH-	50.20	50.2		
10	=C=	36.56	36.56		
11	$-CH_2-$	21.11	21.13		
12	$-CH_2-$	39.77	39.83	1.40-2.00	1.017-2.276
13	=C=	42.35	40.5		
14	=CH-	56.91	56.82		
15	$-CH_2-$	24.39	24.34		
16	$-CH_2-$	28.96	28.28		
17	=CH-	56.02	56.12		
18	$-CH_3$	12.07	12.08	0.690(3H, s)	0.686(3H, s)
19	$-CH_3$	19.42	19.43	1.002(3H, s)	1.017
20	=CH-	40.54	36.19	0.85(3H, d)	0.851(3H, d)
21	$-CH_3$	21.11	18.82		
22	=CH-	138.37	138.33	5.04(1H, dd)	
23	=CH-	129.69	129.31	5.147 (1H, m)	5.336
24	=CH-	51.29	45.9		
25	=CH-	31.49	29.23		
26	$-CH_3$	21.26	19.84	0.837(3H, d)	0.833
27	$-CH_3$	19.02	19.08	0.798(3H, d)	0.793
28	$=CH_2$	25.44	23.13		
29	$-CH_3$	12.29	12.26	1.25(3H, br, s)	1.253

These values give us the confirmation the compound-1 is **stigmasterol** having the structure-

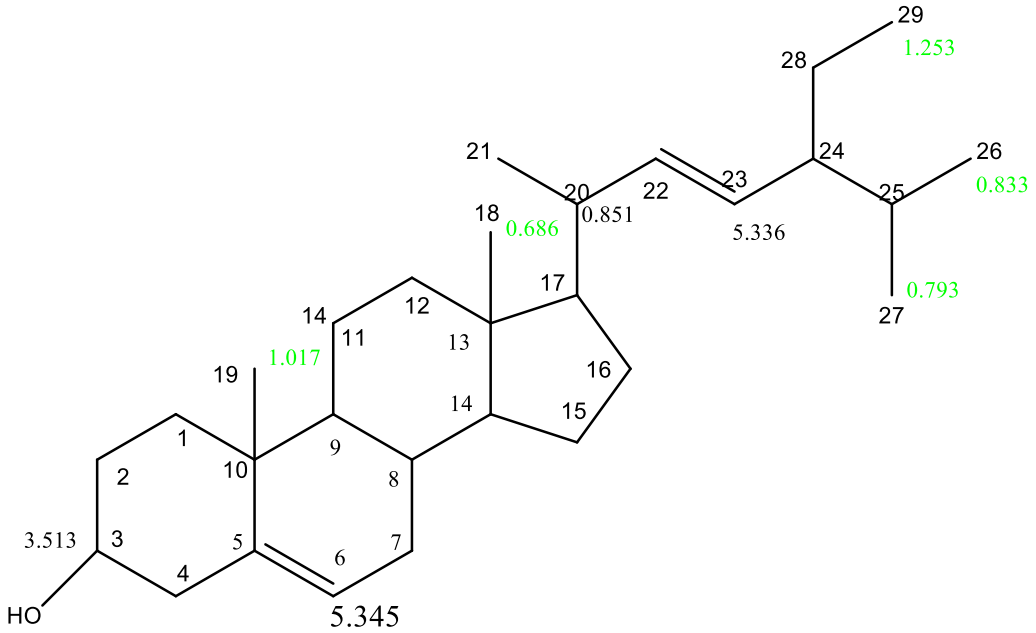


Figure: 3.6: Structure of **stigmasterol** showing ^{1}H signals

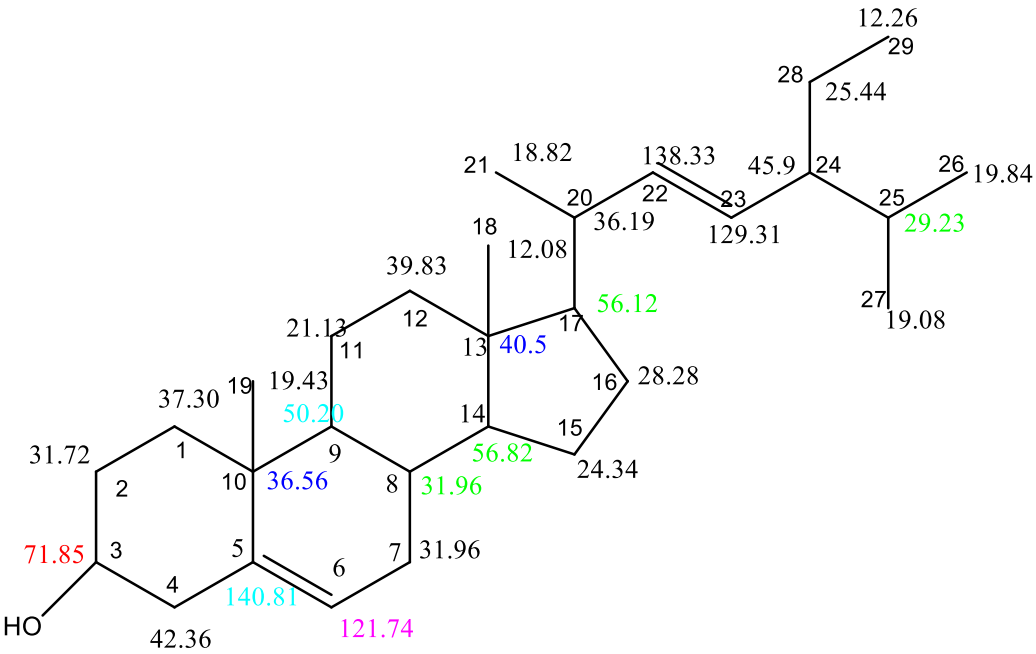


Figure: 3.7: Structure of **stigmasterol** showing ^{13}C signals

3.2.2 Characterization of compound-2 (GPE-3) as β-sitosterol

The compound-**2** (~4.2 mg) was a white crystalline solid having R_f value of 0.12 (Hexane: DCM= 50:50). It was soluble in chloroform. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 110^0C for several minutes purple colour appeared.

3.2.2.1 Spectral analysis of Compound-2 (GPE-3)

The structure of the compound GPE-2 **(Fig: 3.12)** has been established by ^{1}H-NMR, ^{13}C-NMR spectral evidence.

3.2.2.2 ^{1}H-NMR spectroscopy of compound-2 (GPE-3) as β-sitosterol

The ^{1}H-NMR spectrum of the compound-1 revealed the peaks at δ 0.667(H-18), 0.997(H-19), 0.900(H-21), 0.832(H-26), 0.809(H-27), and 0.850(H-29) ppm was observed due to methyl groups of the steroid at C-13, C-10, C-25, C-25, C-20, and C-28. Some multi plates between δ 1.240-2.354 ppm are due to methylene and methine protons present in the compound.

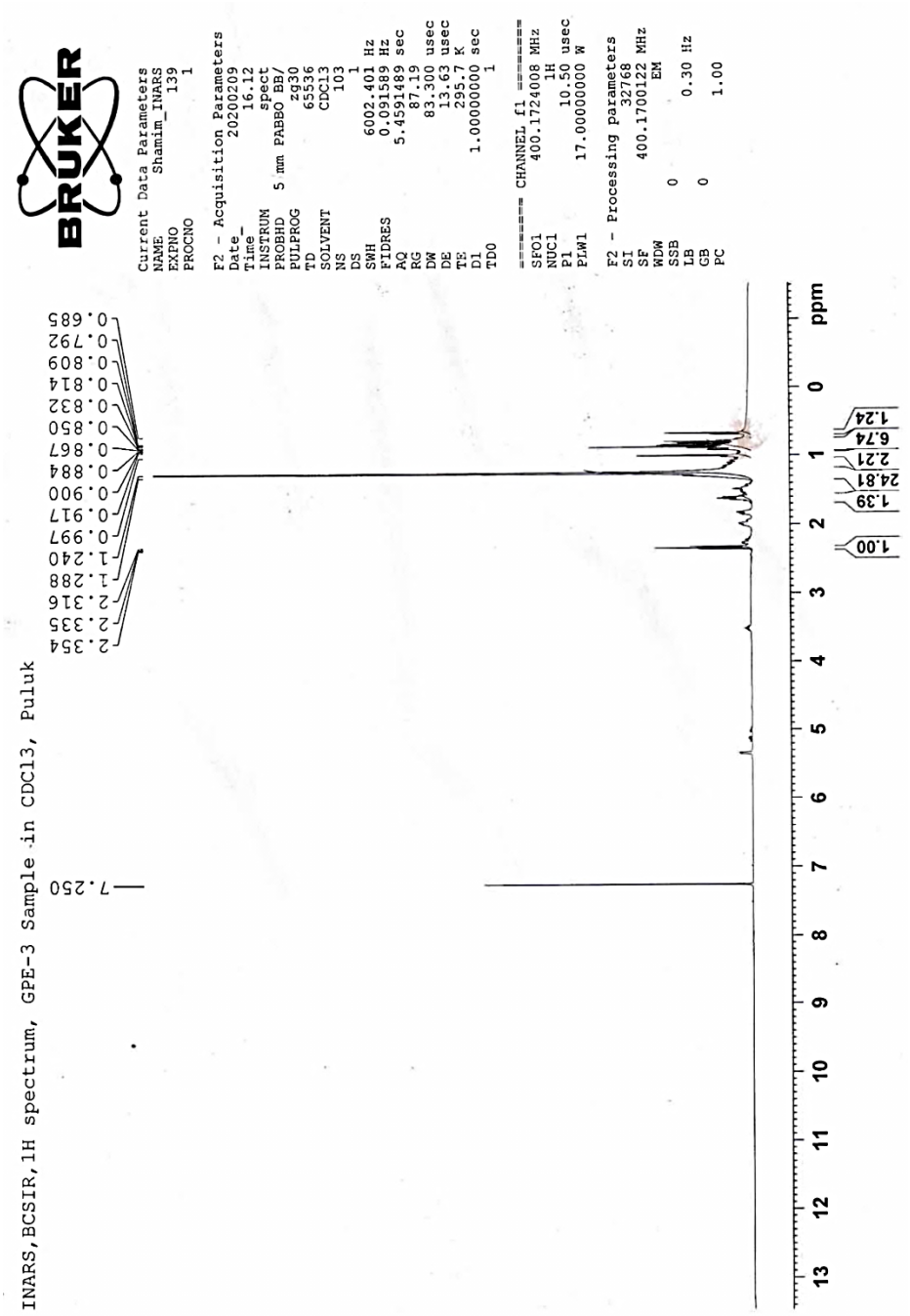


Figure 3.7: ^{1}H NMR spectrum compound-2

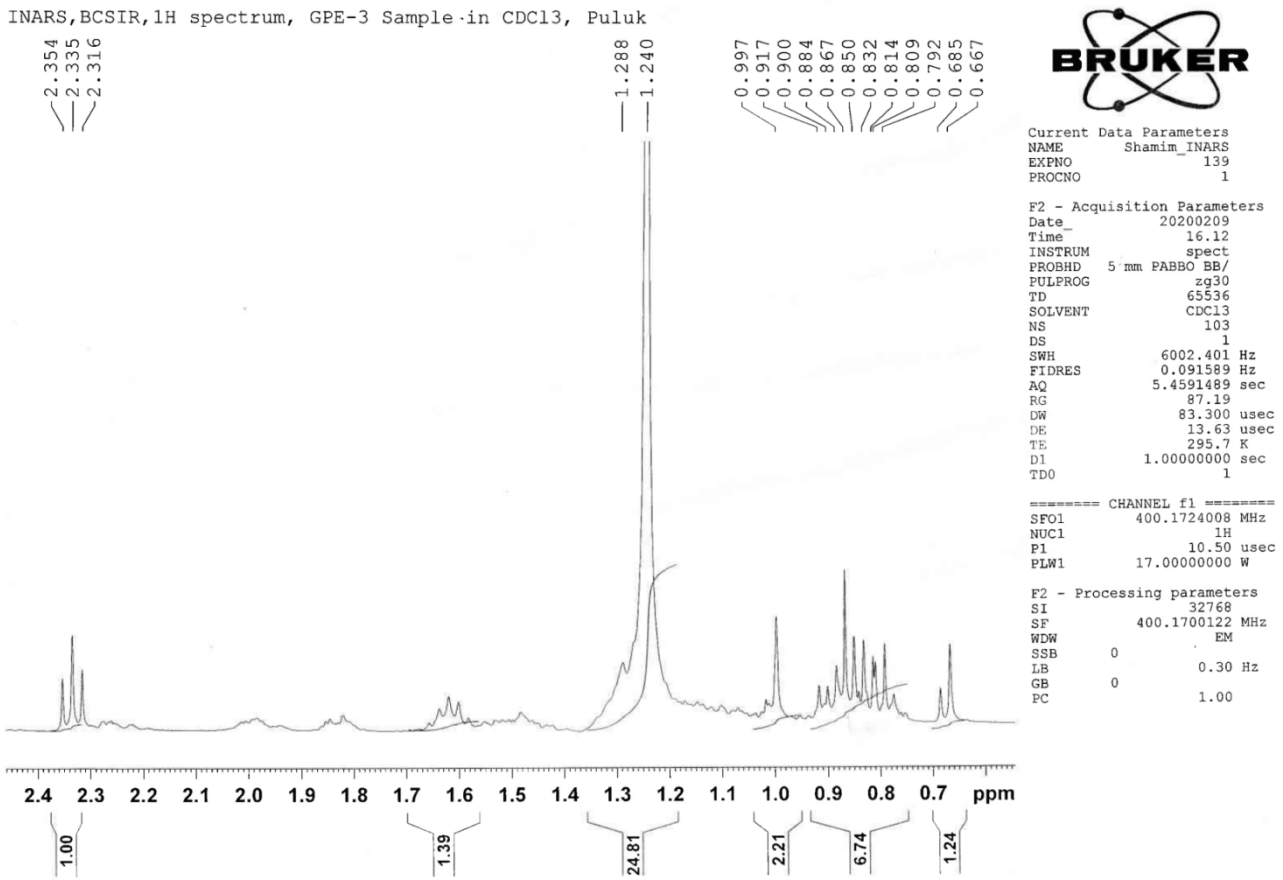


Figure 3.8: Expanded ^{1}H NMR spectrum of compound-2

3.2.2.2 ^{13}C-NMR spectroscopy of compound-2 (GPE-3) as β-sitosterol

The ^{13}C-NMR spectrum in $CDCl_3$ of the compound -2 showed various chemical shifts for various carbons, the peak at δ 71.87 (C-3) ppm confirmed the presence of oxymethine group in the compound. The peaks at δ 33.98 (C-22) and 26.12 (C-23) ppm spectrum indicated the presence of $–CH_2$- protons. The ^{13}C-NMR exhibited exactly 29 carbon signals which suggested the compound may be a steroid. The other peaks are 37.28 (C-1), 31.68 (C-2), 71.87 (C-3), 42.31 (C-4), 140.77 (C-5), 121.76 (C-6), 31.95 (C-7), 31.68 (C-8), 50.17 (C-9), 36.54 (C-10), 21.24 (C-11), 39.81 (C-12), 42.35 (C-13), 56.80 (C-14), 24.33 (C-15), 28.27 (C-16), 56.09 (C-17), 12.27 (C-18), 19.42 (C-19), 36.17 (C-20), 18.80 (C-21), 33.98 (C-22), 26.12 (C-23) 45.88 (C-24), 29.19 (C-25), 19.42 (C-26), 19.06 (C-27), 23.10 (C-28) and 12.27 (C-29) ppm.

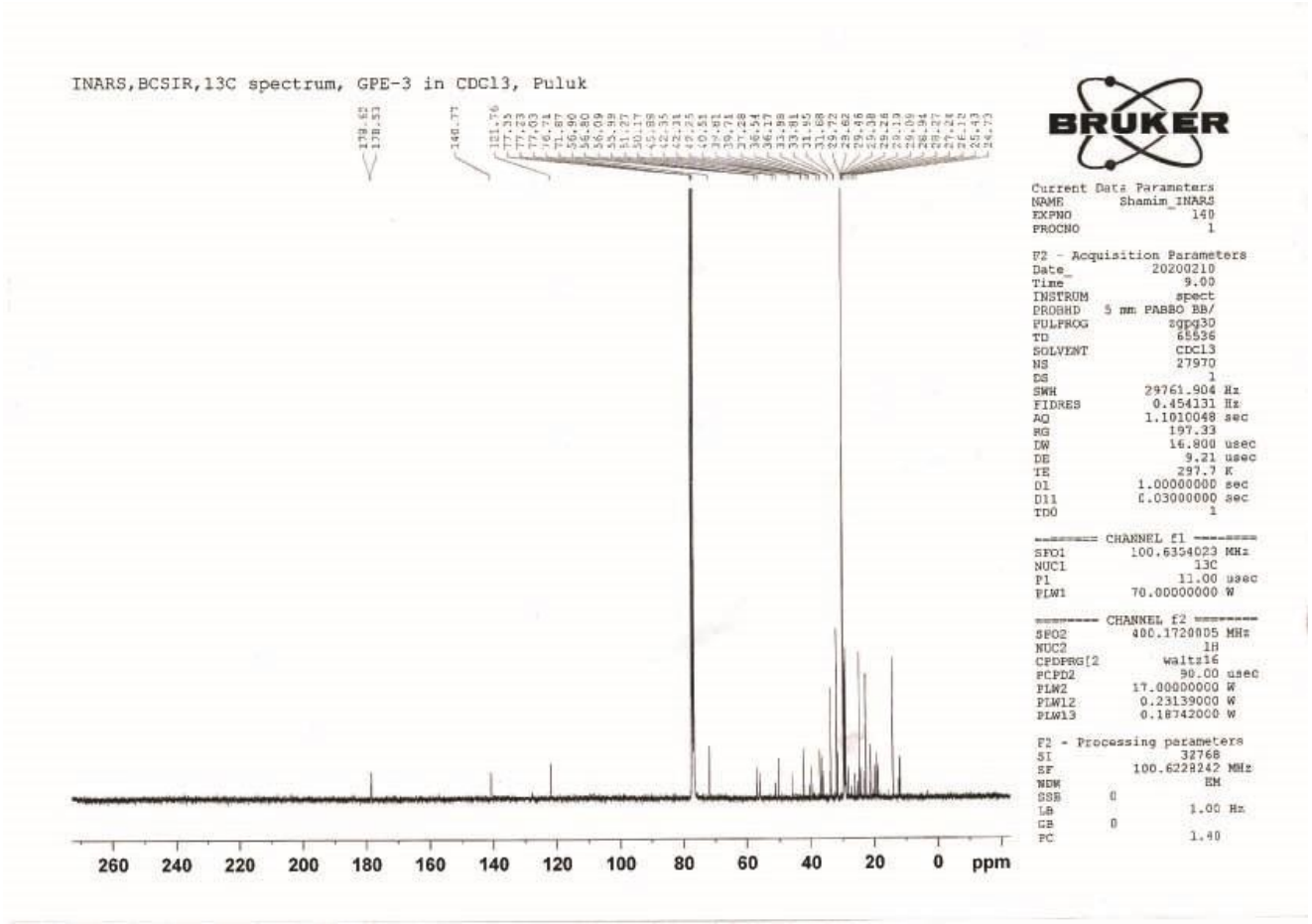


Figure 3.9: ^{13}C NMR spectrum of compound-2

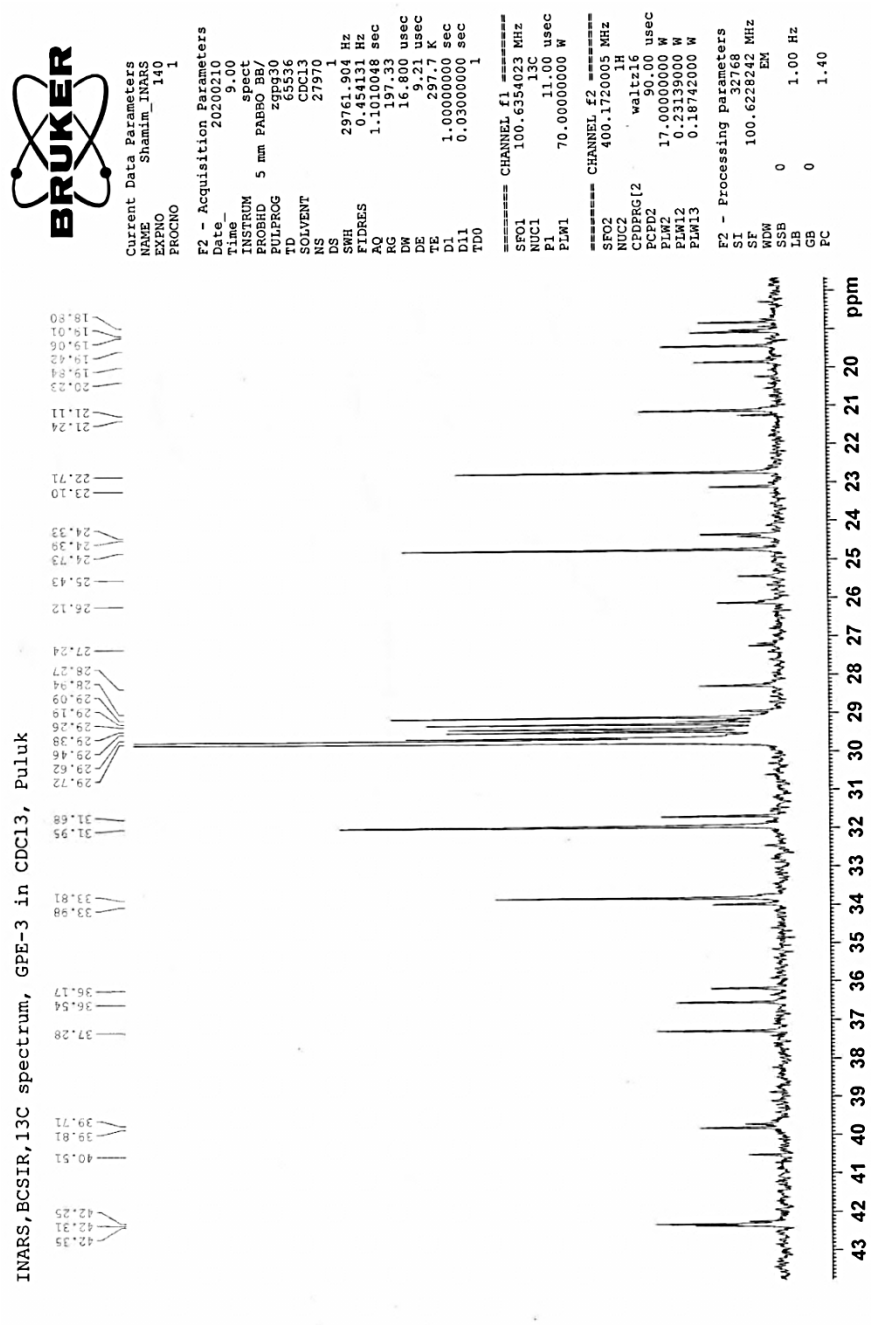


Figure 3.10: Expanded ^{13}C NMR spectrum of compound-2

The comparison of all these values with the reported value (Ali, *et al.*, 2003) has been shown in the following table 3.2:

Table 3.2: **^{13}C-NMR and ^{1}H NMR data of compound-2 (GPE-3) compared with published data of β-sitosterol**

Carbon no.	Type of carbon	(Chemical shift in ppm)			
		β-sitosterol	compound-2	β-sitosterol	compound-2
		^{13}CNMR	^{13}CNMR	^{1}HNMR	^{1}HNMR
1	$-CH_2-$	37.30	37.28		
2	$-CH_2-$	31.60	31.68		
3	=CH-	71.70	71.87	3.55(1H, br, s) 2.00(1H, br, s)	
4	$-CH_2-$	42.30	42.35		
5	=C=	140.80	140.77		
6	=CH-	121.60	121.76	5.36(1H, br, s)	
7	$-CH_2-$	32.00	31.95		
8	=CH-	31.90	31.68		
9	=CH-	50.20	50.17		
10	=C=	36.50	36.54		
11	$-CH_2-$	21.10	21.11		
12	$-CH_2-$	39.80	39.81	1.40-2.00	1.24-2.354
13	=C=	42.80	42.88		
14	=CH-	56.80	56.90		
15	$-CH_2-$	24.30	24.33		
16	$-CH_2-$	28.30	28.94		
17	=CH-	56.10	56.09		
18	$-CH_3$	11.90	12.27	0.670(3H, s)	0.667(3H, s)
19	$-CH_3$	19.40	19.42	1.01(3H, s)	0.997(3H, s)
20	=CH-	36.20	36.17		
21	$-CH_3$	18.20	18.80	0.91(3H, d)	0.900(3H, d)
22	$-CH_2-$	33.90	33.98		
23	$-CH_2-$	26.10	26.12		
24	=CH-	45.90	45.88		
25	=CH-	29.20	29.29		
26	$-CH_3$	19.60	19.42	0.83(3H, d)	0.832(3H, d)
27	$-CH_3$	19.80	19.84	0.81(3H, d)	0.809(3H, d)
28	$=CH_2$	23.10	23.10		
29	$-CH_3$	12.30	14.14	0.84(3H, br, s)	0.850(3H, br, s)

These values give us the confirmation that compound-2 is **β-sitosterol** having the structure-

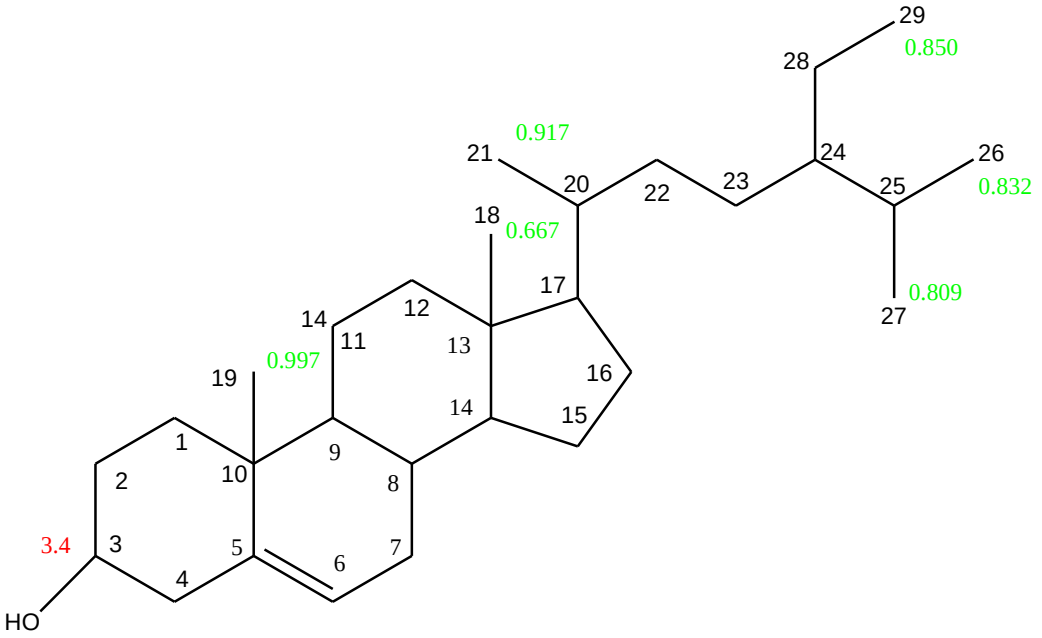


Figure: 3.11: Structure of β-sitosterol showing ^{1}H signals

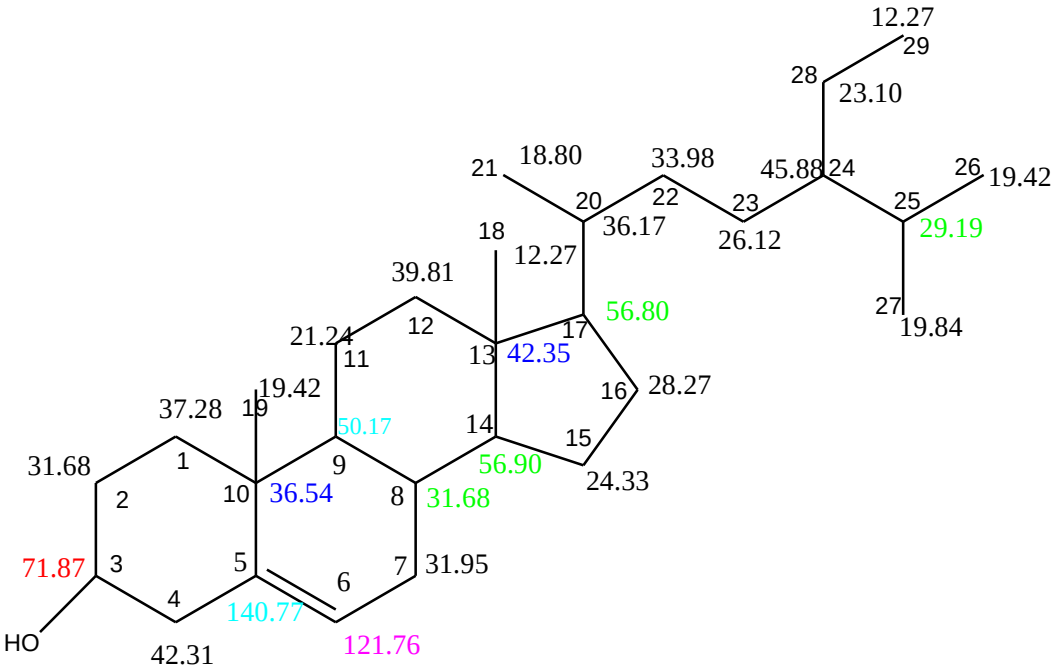


Figure: 3.12: Structure of β-sitosterol showing ^{13}C signals

3.2.3 Characterization of compound -3 (GPE-4) as Isofouquerone

The compound-3 is a white crystalline compound. The R_f value of the compound is 0.12 in 5% ethyl acetate in hexane. It is soluble in dichloromethane, ethyl acetate, methanol & ethanol. It has been tested for steroids, and a white color confirms that compound-3 is steroid type compound.

3.2.3.1 Spectral analysis of Compound-3 (GPE-4)

3.2.3.2 ^{1}H-NMR spectroscopy of Compound-3 (GPE-4)

^{1}H-NMR spectrum(400MHz, $CDCl_3$) of compound-2 revealed peaks at δ 0.741(H-18), 0.761(H-19), 0.973 (H-28), 0.832(H-26), 0.850(H-21) and 1.240(H-29) ppm was observed due to methyl groups of the steroid at C-13, C-10, C-25, C-25, C-20, and C-28. Some multi plates between δ 0.917-2.354 ppm are due to methylene and methine protons present in the compound.

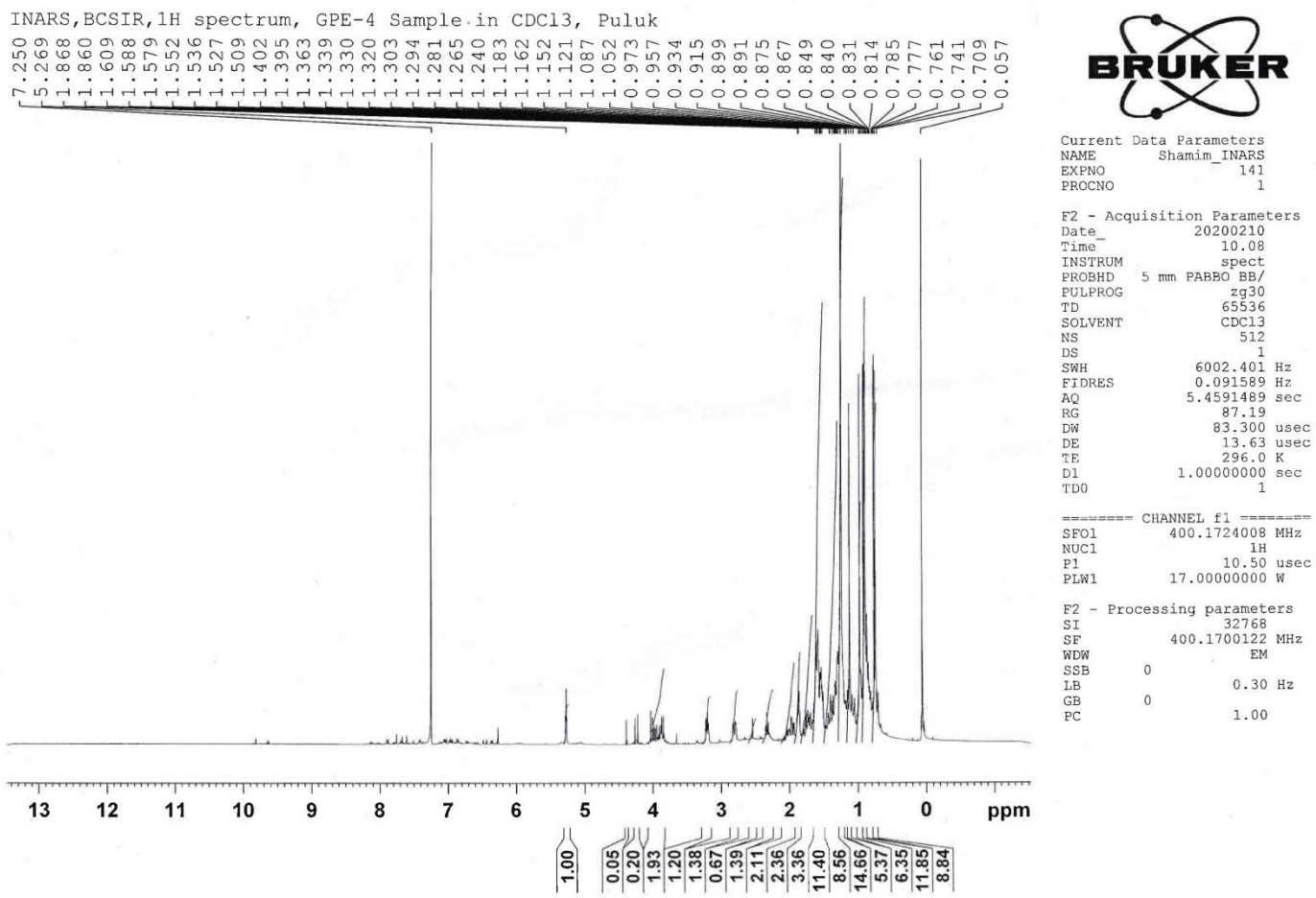


Figure 3.13: ^{1}HNMR spectrum of compound-3

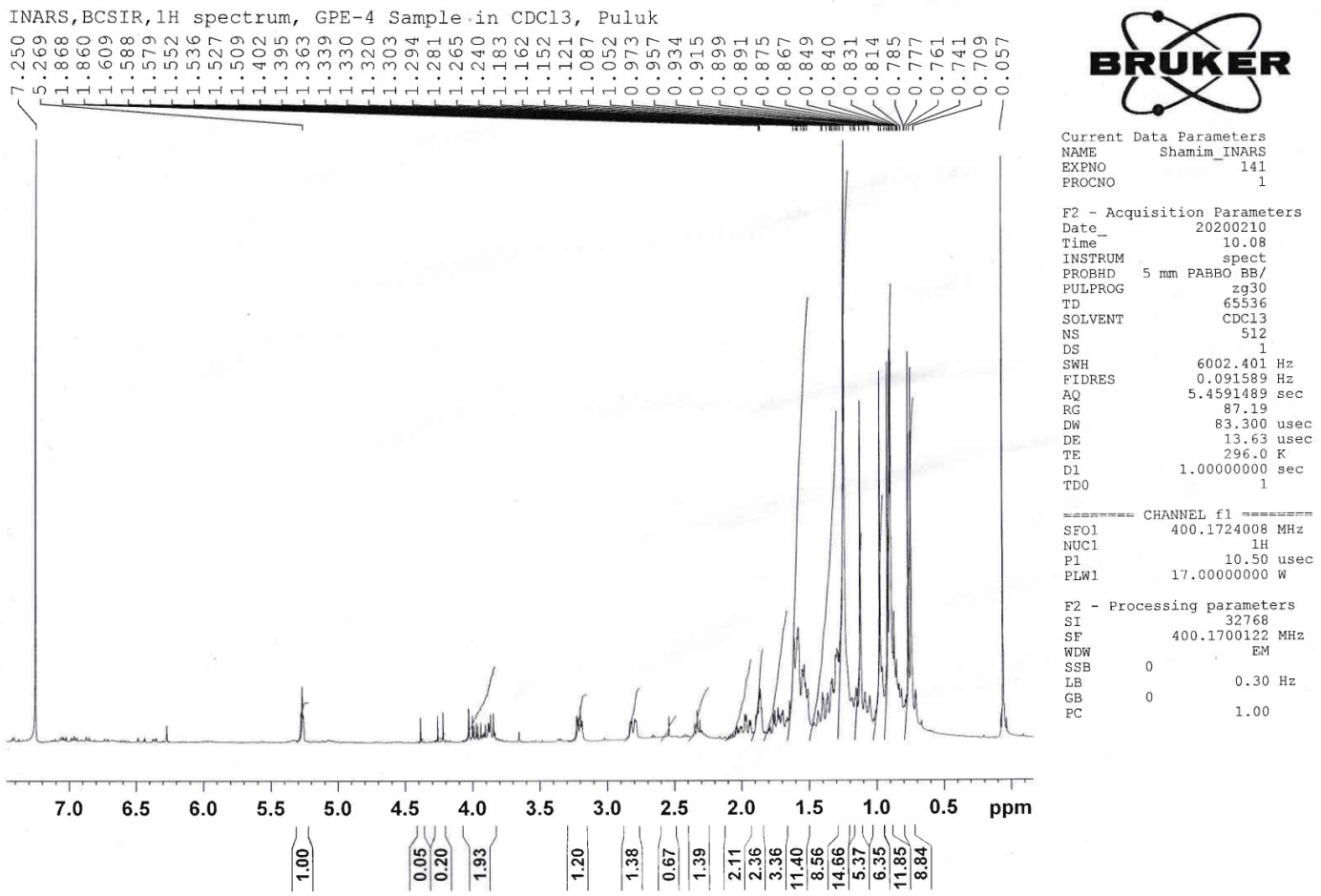


Figure 3.14: Extended ^{1}HNMR spectrum of compound-3

3.2.3.3 ^{13}C-NMR spectroscopy of compound-3 (GPE-4)

The ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) of the compound-2 has signals at δ_C (ppm) 17.09, 18.32, 25.95, 30.69, 29.38, 76.71, 143.60, 122.67, 41.05, 23.59, 79.07, 14.13, 13.34, 45.90, 27.70, 32.66, 47.65, 39.30, 27.20, 22.71, 37.10, 46.54, 38.78, 33.82, 18.32, 55.24, 41.65, 182.87, 33.08, 38.43. These signals indicate that it has 30 carbons. Among them, 3 signals were assignable to tertiary carbons, 8 signals to methyl carbons, 10 signals to methylene carbons, 6 signals for quaternary carbons, 2 signals for sp^2 carbons and 1 signal for ketonic carbon.
The signals at δ 182.87 ppm is due to ketonic carbon. Signals at δ55.24, 46.54, 39.30, and 45.90 due to 4 tertiary carbons, signals at δ 15.34, 14.13, 23.59, 29.38, 30.69, 25.95, 18.32, 17.09ppm were due to 8 methyl carbons. Signals at δ 38.43, 33.08, 18.32, 33.82, 22.71, 27.20, 32.66, 27.70, 41.05ppm were due to 9 methylene carbons. Signals at δ41.65, 38.78, 37.10, 47.65, ppm due to 4 quaternary carbons, δ79.07, 76.71ppm due to 2 hydoxymethyne carbons, and signals at δ122.67, and 143.60ppm due to 2 sp^2 olefinic carbons

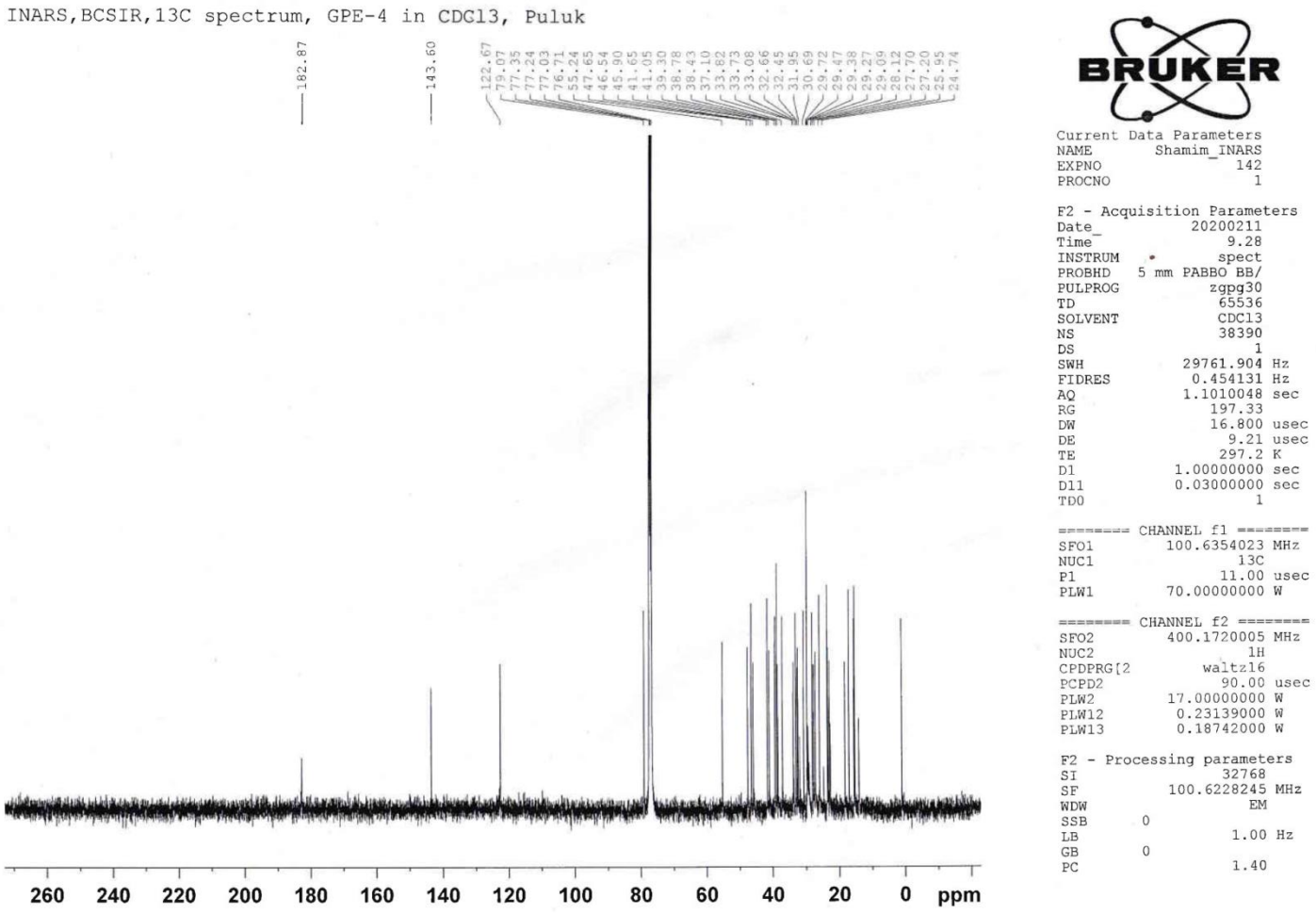


Figure 3.15: ^{13}C NMR spectrum of compound-3

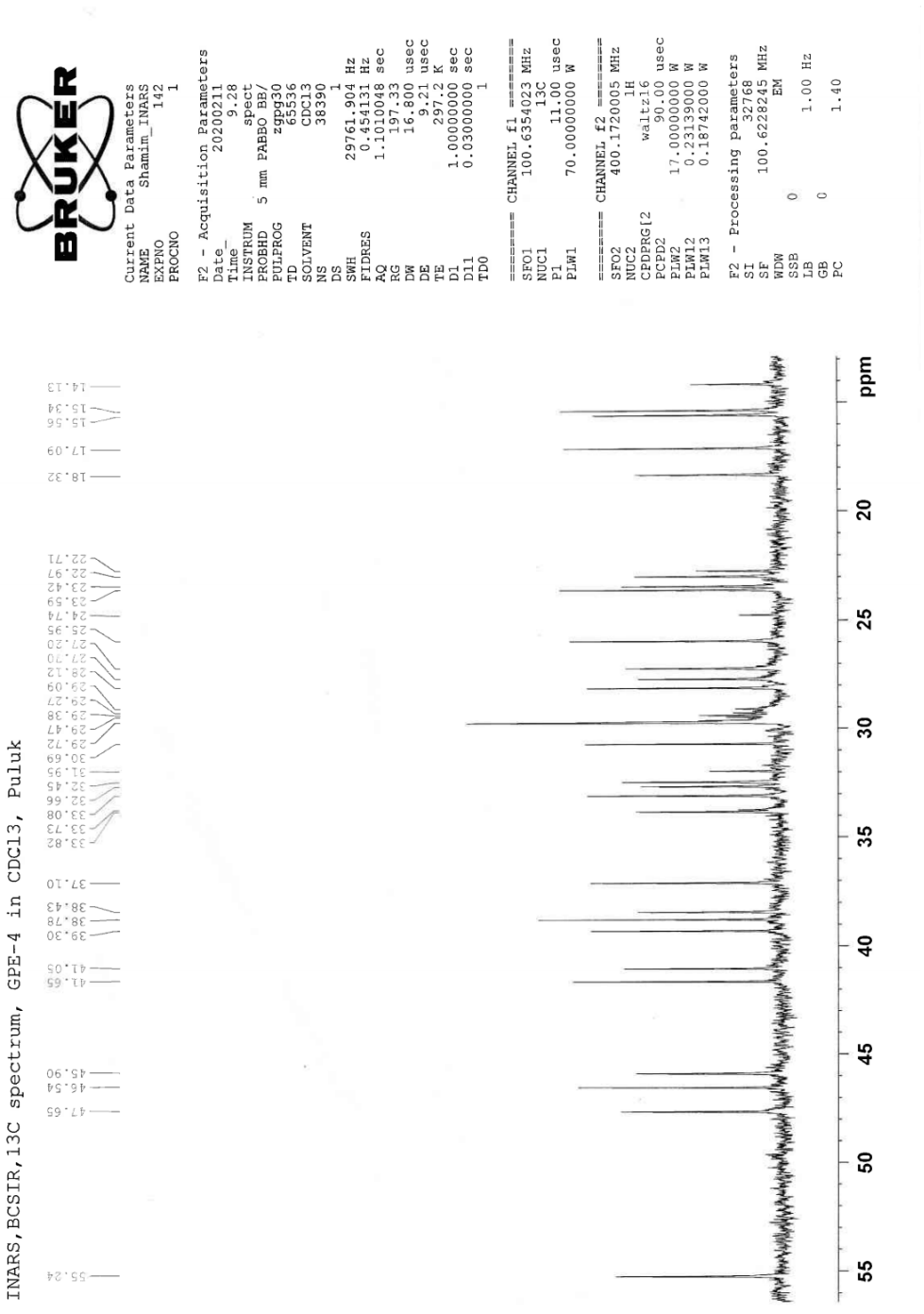


Figure 3.16: ^{13}C NMR spectrum of compound-3

The comparison of all these values with the literature survey has been shown in the following table: 3.7

Table 3.3: **^{13}C-NMR and ^{1}HNMR data of compound-3 (GPE-4) compared with published data of Isofouquierone (P G. Waterman et el., 1985)**

Carbon no.	Type of carbon	(Chemical shift in ppm)			
		Isofouquierone	compound-3	Isofouquierone	compound-3
		^{13}CNMR (90.56Mhz)	^{13}CNMR (100MHz)	^{1}HNMR(250MHz)	^{1}HNMR(400MHz)
1	-CH_2-	39.81	38.43		
2	-CH_2-	34.00	33.08	2.45	-
3	=CO	217.73	182.87		
4	=CH=	47.31	41.65		
5	=CH-	55.31	55.24		
6	-CH_2-	19.59	18.32		
7	-CH_2-	34.49	33.82		
8	=C=	40.24	38.78		
9	=CH-	49.93	46.54		
10	=C=	36.77	37.10		
11	-CH_2-	21.94	22.71		
12	-CH_2-	27.44	27.20		
13	=CH-	43.32	39.30		
14	=C=	50.18	47.65		
15	-CH_2-	31.02	32.66		
16	-CH_2-	27.44	27.70		
17	=CH-	49.83	45.90		
18	-CH_3	15.90	15.34(15.56)	0.89	0.741
19	-CH_3	15.14	14.13	0.94	0.761
20	≡C(OH)	74.86	79.07		
21	-CH_3	25.76	23.59	1.12	
22	-CH_2-	43.32	41.05	2.20	2.330
23	=CH-	122.22	122.67	5.69	5.269
24	=CH-	141.92	143.60	5.69	5.269
25	≡C(OH)	70.62	76.71		
26	-CH_3	29.83	29.38	1.31	1.240
27	-CH_3	29.88	30.69	1.31	1.240
28	-CH_3	26.65	25.95	1.00	0.973
29	-CH_3	20.92	18.32	1.03	
30	-CH_3	16.25	17.09	1.07	

This data evaluates the compound-3 (GPE-4) is Isofouquerone, having the structure-

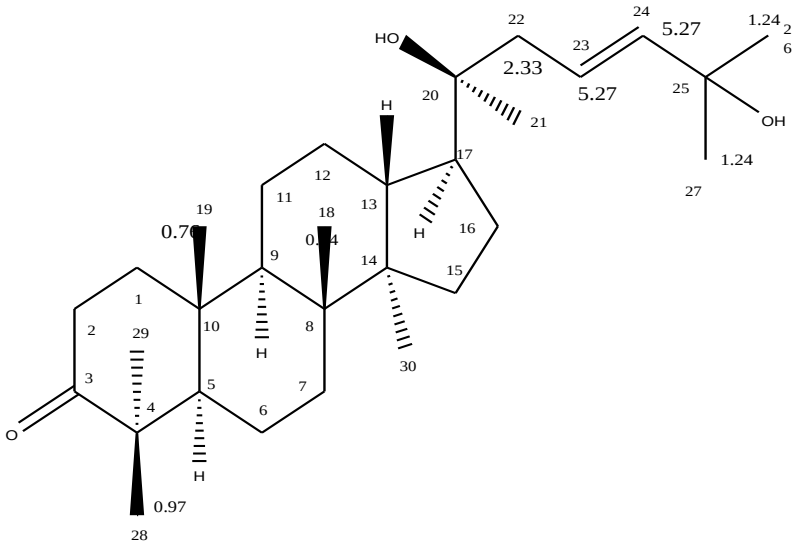


Figure 3.17: Structure of **Isofouquierone** showing ^{1}H signals

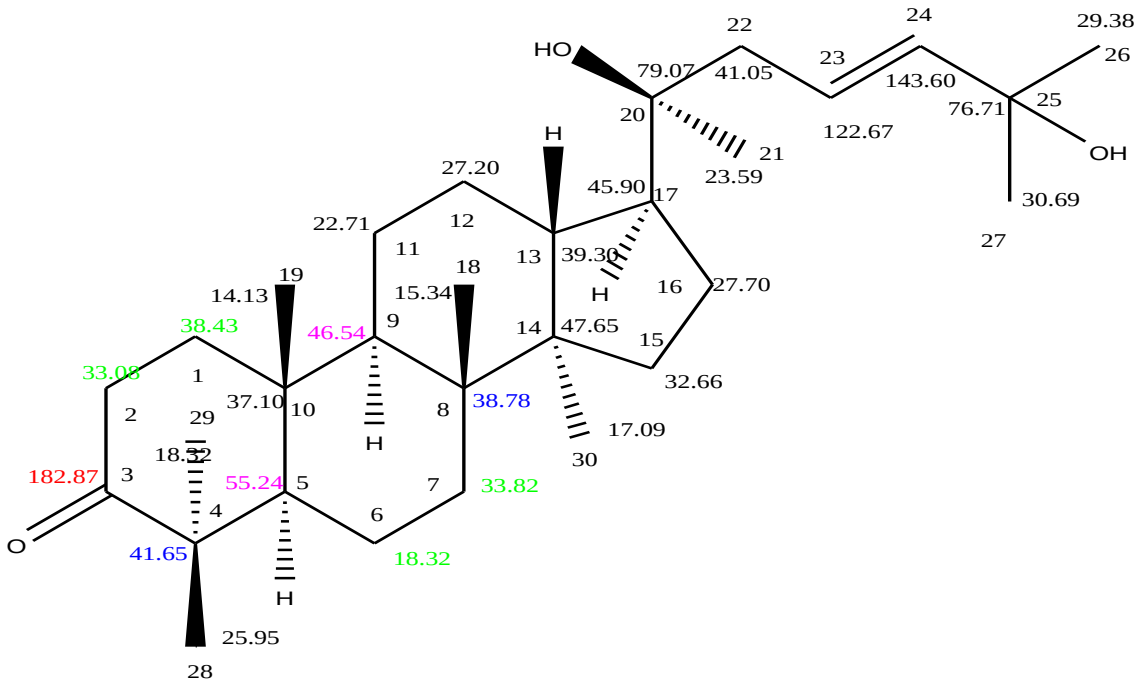


Figure 3.18: Structure of **Isofouquierone** showing ^{13}CNMR signals

3.2.4 Characterization of Compound 4(GPE-5)

The compound-4**(GPE-5)** (~3.5 mg) was a white powdered solid having an R_f value of 0.58 (100% DCM). It was soluble in chloroform. On spraying with anisaldehyde-sulfuric acid spray reagent, followed by heating at 110^0C for several minutes, it appeared purple.

3.2.4.1 ^{1}H-NMR spectral analysis:

The **^{1}H-NMR** spectrum **(Figure 3.19)** of GPE-5 (compound-4**)** was recorded, and its absorption frequencies were identified comparing the reported value of a known compound (Prachayasittikul *et al.,* 2010), which are given below **(Table 3.8)**

3.4: Comparative ^{1}H-NMR spectral data of isolated compound-4(GPE-5) with the published data (Prachayasittikul et al., 2010) of lupeol.

No. of protons	Chemical shift value(δ)	
	Experiment value (δ)	Reported value(δ)
1H (H-5)	0.67(d)	0.66 (d)
3H (H-24)	0.74(s)	0.73(s)
3H (H-28)	0.77(s)	0.76(s)
3H (H-25)	0.81(s)	0.80(s)
3H (H-27)	0.93(s)	0.92(s)
3H (H-23)	0.95(s)	0.94(s)
3H (H-26)	1.01(s)	1.00(s)
3H (H-30)	1.66(s)	1.65(s)
1H (H-21)	-	1.82-1.96(m)
1H (H-19)	2.36(dt)	2.35(dt)
1H (H-3)	3.16(dd)	3.16(dd)
1H (Ha-29)	4.55(br s)	4.55(br s)
1H (Hb-29)	4.67(br s)	4.65(br s)

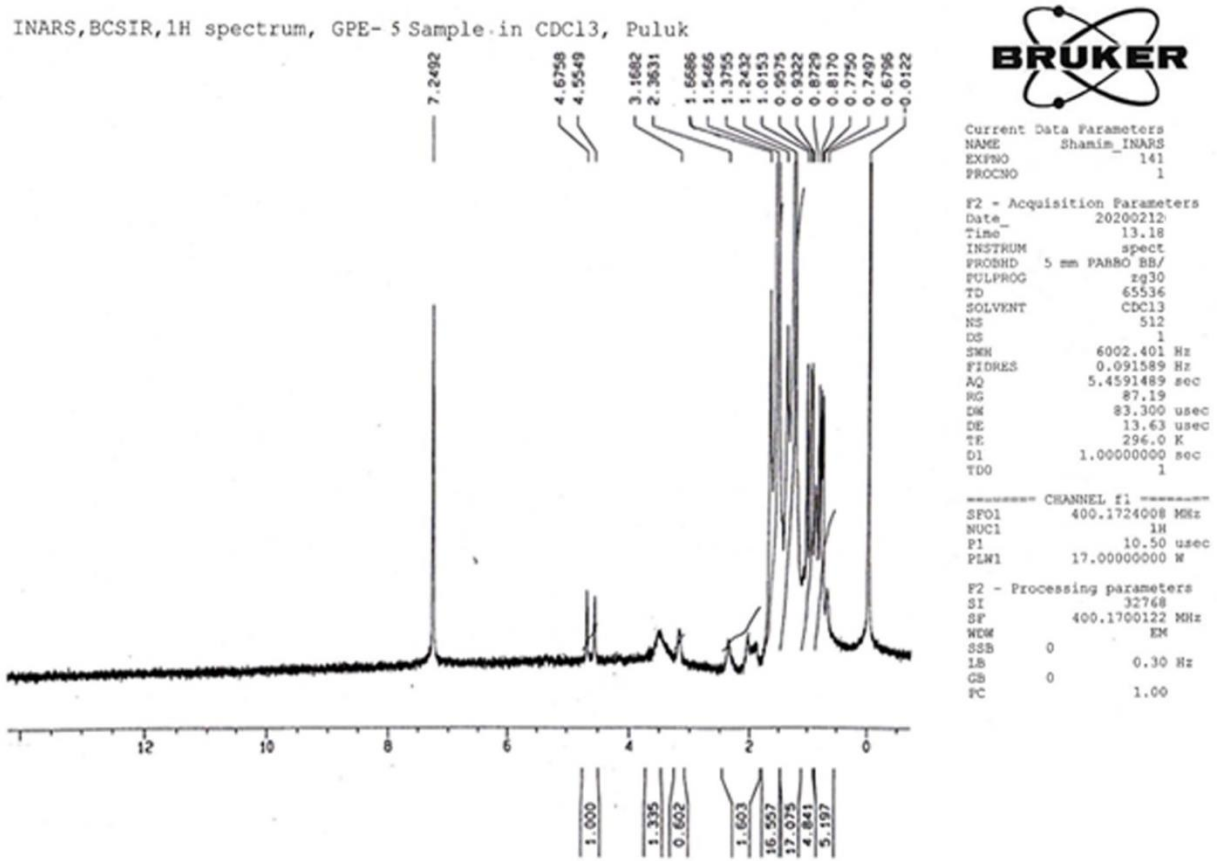
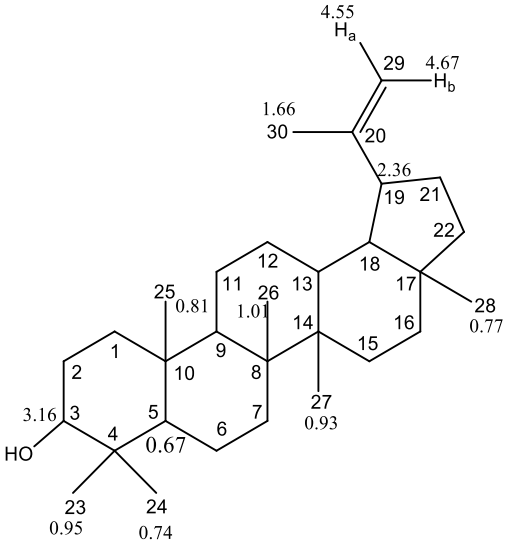


Figure 3.19: ^{1}H-NMR spectrum of compound 4

In the isolated GPE-5 (compound-4) ^{1}H-NMR (400 MHz, in $CDCl_3$) spectrum (Figure-3.19), the H-3 proton appeared as a multiplet at 3.16 ppm, while the H-29 olefinic proton appeared as two wide singlets at 4.55 and 4.67 ppm, respectively. Seven tertiary methyl singlets and one secondary hydroxyl group were detected in the ^{1}H NMR spectra. At 1.66, 1.01, 0.95, 0.93, 0.81, 0.77, and 0.74 ppm, seven methyl protons were also found. Table-3.8 shows the ^{1}H-NMR signals of GPE-5 (compound-4) which were compared to published data on lupeol [(3-)-lup-20(29)-en-3-ol], a pentacyclic triterpene (Prachayasittikul et al., 2010). The structure of GPE-5 (Compound-3) has been tentatively ascribed as



Molecular Weight: 424.70

Figure: 3.20: Structure of **Lupeol** showing ^{1}H signals

Comparing the **^{1}H-NMR** data of the compound-**4** with that of reported values (Prachayasittikul et al., 2010), **compound-4** was found to be a pentacyclic triterpene **lupeol** [(3β)-lup-20(29)-en-3-ol], having the structure-

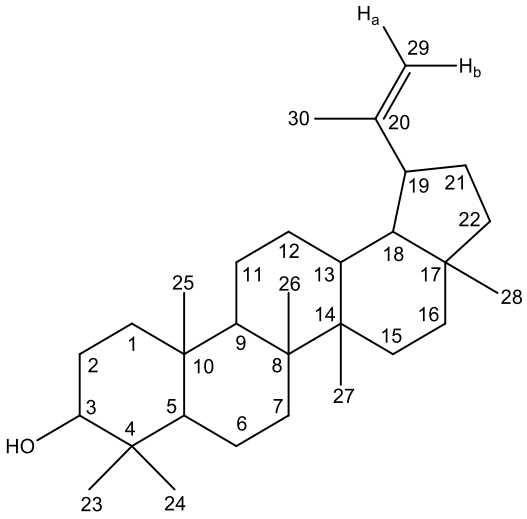


Figure 3.21: Structure of Lupeol (compound-3)

Due to time constrain, further spectral analysis ^{13}C-NMR was not performed.

Chapter-4

ANTIOXIDANT ACTIVITY

ANTIOXIDANT ACTIVITY

4.1 Introduction

For thousands of years, nature has provided medical substances. Many contemporary drugs have been extracted from natural sources. Plants generate a wide range of bioactive chemicals that are very useful in the treatment of life-threatening illnesses. Excess free radicals have been found to induce tumor development, DNA mRNA, protein, and enzyme damage, as well as cancer, cardiovascular diseases, neurological disorders, accelerated aging, Parkinson's and Alzheimer's diseases, and rheumatic and pulmonary problems. As a result, the importance of screening medicinal plants for antioxidant activity cannot be overstated. Atoms or groups of atoms having at least one unpaired electron are known as free radicals, and they are highly reactive. Reactive oxygen species (ROS) are potentially reactive oxygen derivatives (e.g., superoxide anions, hydrogen peroxide and hydroxyl, nitric oxide radicals) that play a role in oxidative damage to various biomolecules such as proteins, lipids, lipoproteins, and DNA and are linked to the pathogenesis of diseases such as diabetes, cancer, atherosclerosis, arthritis, and neurodegenerative diseases, as well as the aging process. Different synthetic antioxidants such as *tert*-butyl-1-hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), and tert-butyl hydroquinone (TBHQ) are used as food additives. These are known to have not only toxic and carcinogenic effects in humans (Ito *et al.*, 1986; Wichi,1988) but abnormal effects on enzyme systems (Inatani *et al.*, 1983). Therefore, the interest in natural antioxidants, especially those of plant origin, has dramatically increased in recent years (Jayaprakashan&JaganmohanRao, 2000). Plant polyphenols have a protective effect afforded by fruit and vegetable intake against cancer and other chronic diseases (Elen*a et al., 2*006). The antioxidant properties of plant extracts must be investigated by combining two or more separate in vitro tests due to the

complex nature of phytochemicals. A number of reports on the isolation and testing of plant-derived antioxidants have been described during the past decade.

Figure 4.1: How the antioxidant works in our immune system.

Antioxidants work in two ways:

- **Chain-breaking:** Another free radical is formed when free radical releases or gain an electron. This molecule then repeats the process with a third molecule. The process is continued until a chain-breaking antioxidant stabilizes the radical.
- **Preventive:** Antioxidant enzymes stop oxidization by reducing the speed of chain initiation.

Antioxidants in food play An significant role in health. Scientific proof suggests that antioxidants cut back the chance for chronic diseases and cancer, and heart diseases. The whole grain, fruits, vegetables, and plant-sourced food antioxidants like vitamin-C, vitamin-E, and carotene. Most of the antioxidants compound in a very typical diet. Some compounds like gallates have inhibitor activity. The characteristic of an inhibitor is its ability to entice free radicals. These free radicals could oxidize DNA, RNA, proteins, and lipids. Inhibitor compounds like synthetic resin acids, polyphenols, and flavonoids, free radicals like peroxides, hydroperoxides inhibit the aerobic mechanism. The radical scavenging activity of antioxidants in food is considerably investigated and reported within the literature.

4.2 Study of Antioxidant property by DPPH method

The consumption of fruits and vegetables containing many antioxidant compounds protects against cancer and cardiovascular diseases. This protection is explained by these antioxidants' ability to scavenge free radicals, which cause oxidative damage. In recent years, the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been used to evaluate antioxidants' free radical scavenging capacity. The determination of scavenging stable DPPH was a rapid method to assess the antioxidant activity of the extracts. The DPPH method was introduced nearly 50 years ago by Marsden Blois. The free radical-scavenging activity of the synthesized compounds was assayed according to the Blois method with some modification. DPPH, a stable free radical because of the free delocalized electron. The delocalization phenomenon also gives purple, characterized by an absorption band in ethanol solution at 520 nm. By donating a hydrogen atom, the reduced form of DPPH is formed with the loss of the purple color (although there would be expected to be a residual pale yellow color from the picryl group still present).

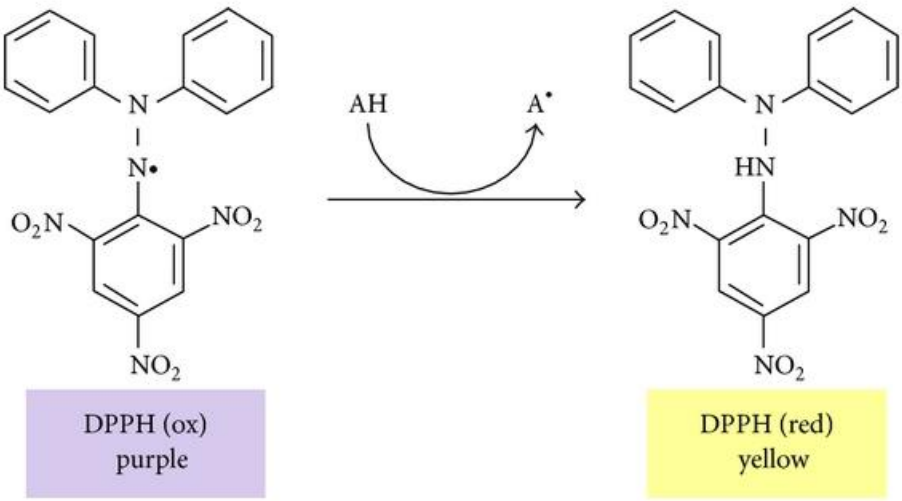


Figure 4.2: Reaction involved in DPPH method.

4.3 DPPH assay principle and mechanism

4.3.1 Standard sample preparation

Ascorbic acid (ASA) was used as a positive control. A calculated amount (about 2 mg) of ascorbic acid dissolved in methanol to get a 2000 µg/mL mother solution.

The mother solution has been serially diluted to obtain concentrations ranging from 500.0 to 3.90625µg/mL.

4.3.2 Test sample preparation

The calculated amount of different extractives (about 2 mg) were measured and dissolved in methanol to get the mother solution (concentration 2000 µg/mL). The mother solution was serially diluted to generate concentration levels varying from 500.0 to 3.90625 µg/mL, which were saved in the marked glass stopper vial.

4.3.3 DPPH solution preparation

4mg DPPH powder dissolved in 100 mL methanol to have a 40 µg/mL DPPH solution. In the amber reagent bottle, the solutions were prepared and kept in the lightproof box with ice.

4.4 Assay Procedure

The antioxidant activities of different extractives on the stable radical DDPH were determined by the Brand-Williams method (Brand-Williams). A 1000L DPPH methanol solution was combined with 200 µL of a sample solution (extractives or control) at various concentrations (500 to 3.90625 µg/mL) and 800 µL methanol (total 1000 µL). A UV spectrophotometer was used to assess the absorbance at 517 nm against methanol as a blank after 25 minutes of reaction at room temperature (25^{o}C) in the dark.

4.5 Calculation

Inhibition of free radical DPPH in percent was calculated as follows:

$$\%I = \left(1 - \frac{A_{sample}}{A_{blanck}}\right) \times 100\%$$

Where A_{blanck} = absorbance of the control reaction (all reagent except test materials), A_{sample}= absorbance of the sample reaction

Then plotted percent inhibitions VS concentration. The IC_{50} values were determined using Excel 2010 office program. The concentration of each sample is

necessary to provide 50% DPPH radical scavenging activity from the graph (linear regression curve) is **IC_{50}**.

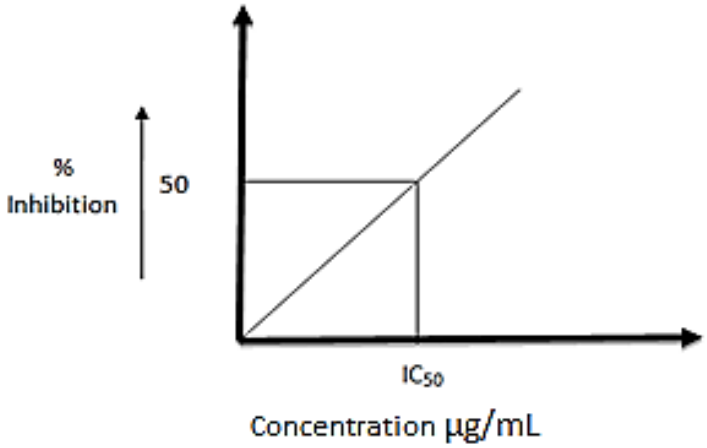


Figure 4.3: Plotting Concentration vs. % Inhibition graph to evaluate IC_{50}.

Table 4.1: For DPPH radical scavenging activity of ethyl acetate extract.

Sample	Concentration (µg/ml)	Log of concentration	Absorbance	% of inhibition	IC_{50} (µg/mL)
Ethyl acetate extract	500	2.699	0.381	71.2018	**21.588**
	250	2.3979	0.604	54.3461	
	125	2.0969	0.726	45.2272	
	62.5	1.7959	0.805	39.1534	
	31.25	1.4949	0.978	26.0771	
	15.625	1.1938	1.063	19.6523	
	7.8125	0.8928	1.133	14.3613	
	3.90625	0.5918	1.196	9.5993	
			1.323(control)		

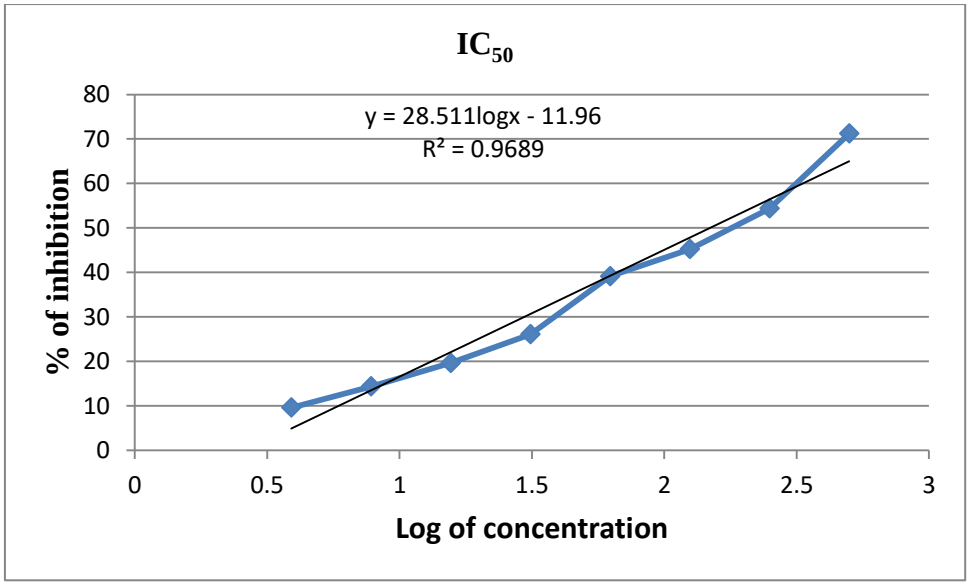


Figure 4.4: Plotting Concentration vs. % Inhibition graph to evaluate IC_{50}.

Table 4.2: For DPPH radical scavenging activity of Ascorbic acid (standard).

Sample	Concentration (µg/ml)	Log of concentration	Absorbance	%of inhibition	IC_{50} (µg/mL)
Ascorbic acid	500	2.6990	0.032	98.46	**7.629**
	250	2.3979	0.034	98.15	
	125	2.0969	0.046	92.59	
	62.5	1.7959	0.553	79.01	
	31.25	1.4949	0.738	69.75	
	15.625	1.1938	0.871	57.1	
	7.8125	0.8928	0.945	42.59	
	3.90625	0.5918	0.962	45.99	
			1.323(control)		

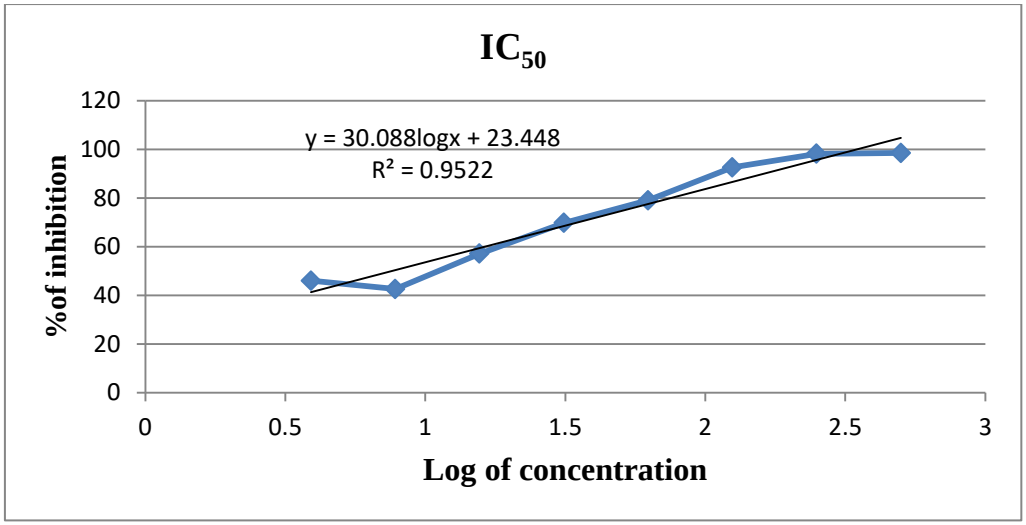


Figure 4.5: Plotting Concentration vs. % Inhibition graph to evaluate IC_{50}.

4.6 Results and Discussion

The IC_{50} is defined as the concentration at which 50% of the total DPPH free radical is scavenged and neutralized. The highest antioxidant activity is indicated by the lowest IC_{50} value, and vice versa. The ethyl acetate extract shows a good IC_{50} value (**21.588**µg/mL) for standard Ascorbic acid (**7.629** µg/mL). This value indicates that ethyl acetate extract shows good antioxidant activity.

Chapter-5

ANTIMICROBIAL SCREENING

ANTIMICROBIAL SCREENING

5.1 Introduction

Bacteria and fungus cause many infectious illnesses. The growing clinical consequences of drug-resistant fungal and bacterial infections have heightened the importance of antimicrobial drug development. The initial stage of antimicrobial drug research is antimicrobial screening, which determines the sensitivity of different fungi and bacteria to any agent. The capacity of each test material to suppress in vitro fungal and bacterial growth is measured in this test. Any of the three methods below can be used to estimate this ability.

i) Disc diffusion method

ii) Serial dilution method

iii) Bio-autographic method

But there is no standardized method to express the results of the antimicrobial screening. To limit the development of microorganisms, researchers use the diameter of the zone of inhibition and the minimal weight of the extract. However, many factors, viz., the extraction methods, inoculums volume, culture medium composition**,** pH, and incubation temperature, can influence the results.

Disc diffusion is a widely recognized in vitro research for preliminary screening of test substances that may have antibacterial action, among the techniques mentioned above. It's simply a quantitative or qualitative test that determines the microorganisms' sensitivity or resistance to the test ingredients. This method, however, cannot differentiate between bacteriostatic and bactericidal activity.

5.2 Principle of Disc Diffusion Method

Known concentration (µg/mL) solution of the test samples was made. Using a micropipette, known quantities of the test materials are impregnated onto dried and sterilized discs (6 mm). Discs holding the test material are planted equally with the test microorganisms on nutrient agar plates. Positive and negative controls are standard antibiotic discs and blank discs (impregnated with solvents). To optimize diffusion, these plates were kept at a low temperature (4^0C) for 24 hours. For optimum growth of the organisms, the plates are inverted and incubated at 37^0C for 24 hours. According to the Antibacterial, physical screening of all the substances was done using the disc diffusion technique at a concentration of 400µg/disc. Tetracycline was used as a standard drug at a concentration level of 100µg/disc. The activity of the compounds was recorded by measuring the zone of inhibition in mm and compared with the common zone of inhibition produced by Tetracycline. This determination indicates whether the organism is sensitive or resistant to the synthesized compounds.

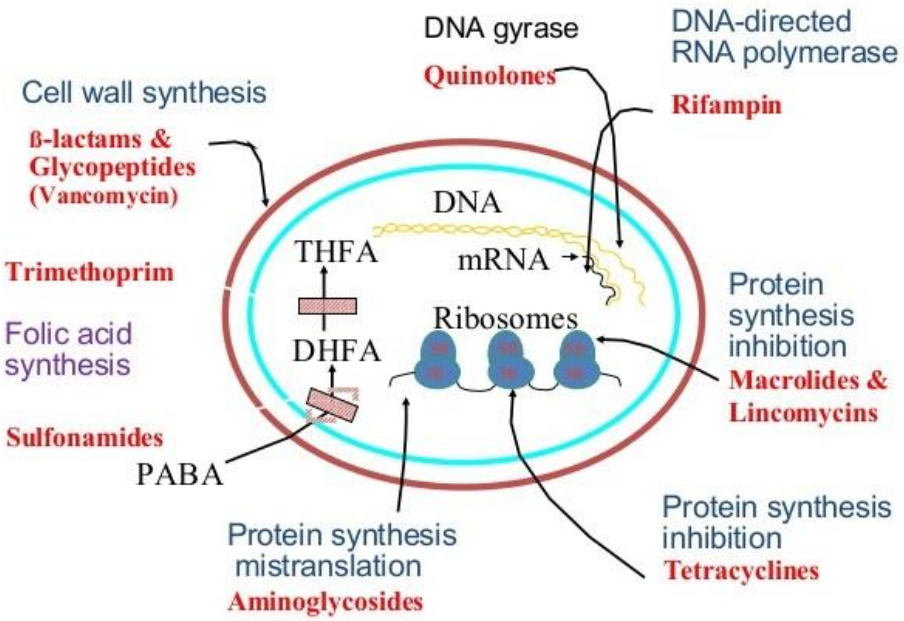


Figure 5.1: How antibiotics work in our immune system

5.3 Experimental

5.3.1 Apparatus and Reagents

Filter paper discs	Petri dishes	inoculating loop
Sterile cotton	sterile forceps	Spirit burner
Micropipette	Screwcap test tubes	Nose mask and Hand gloves
Laminar airflow hood	Autoclave	Incubator
Refrigerator	Nutrient Agar Medium	Ethanol, Chloroform

5.3.2 Test materials

5.3.2.1 Test materials of *Garuga pinnata*

1. Three partitioned crude extracts.
2. Compound-1 (mixture of Stigmasterol and β-sitosterol)

5.3.3 Test Organisms

Pure cultures of the bacterial and fungal strains were collected from the BCSIR's Institute of Food Science and Technology (IFST). Both Gram-positive and Gram-negative organisms for the test are listed in Table 5.1

Table 5.1: List of Test Bacteria and Fungi

Gram-positive Bacteria	**Gram-negative Bacteria**	**Fungi**
Bacillus cereus	*Escherichia coli*	*Candida albicans*
Bacillus megaterium	*Salmonella paratyphi*	*Sacharomyces cerevacae*
Bacillus subtilis	*Salmonella typhi*	
Staphylococcus aureus	*Shigella boydii*	
Sarcina lutea	*Shigella dysenteriae*	
	Vibrio mimicus	
	Pseudomonas aeruginosa	

5.3.4 Culture medium and their composition

The following media is usually used for antimicrobial screening.

a. Nutrient agar medium

Ingredients	Amounts
Bacto peptone	0.5 g
Sodium chloride	0.5 g
Bacto yeast extract	1.0 g
Bacto agar	2.0 g
Distilled water	100mL
pH	7.2 ± 0.1 at 25^0C

b. Nutrient broth medium

Ingredients	Amounts
Bacto beef extract	0.3 g
Bacto peptone	0.5 g
Distilled water	100mL
pH	7.2 ± 0.1 at 25^0C

c. Muller – Hunton medium

Ingredients	Amounts
Beef infusion	30 g
Casamino acid	1.75 g
Starch	0.15 g
Bacto agar	1.70 g
Distilled water	100mL
pH	7.3 ± 0.2 at 25^0 C

d. Tryptic soy broth medium (TSB)

Ingredients	Amounts
Bacto tryptone	1.7 g
Bacto soytone	0.3 g
Bacto dextrose	0.25 g
Sodium chloride	0.5 g
Dipotassium hydrogen Phosphate	0.25 g
Distilled water	100mL
pH	7.3 ± 0.2 at 25^0C

The most often used medium for determining organism resistance to test chemicals and generating new cultures is nutrient agar medium (DIFCO). A measured quantity of each element was placed in a conical flask to create the needed volume of this substance. Then it was mixed with distilled water. The components were heated in a water bath to form a transparent solution. The pH was adjusted to 7.2–7.6 at 25°C using NaOH or HCl. 10mL and 5mL of the medium were placed into screw cap test tubes to make plates and slants, respectively. After that, the test tubes were sealed and autoclaved at 121^0C for 20 minutes at 15 pounds per square inch. The tips were used to make fresh cultures of bacteria and fungi that were used for sensitivity study.

5.3.5 Sterilization procedures

The antimicrobial screening was done in a Laminar Hood to minimize contamination and cross-contamination by organisms, and all safety protocols were followed correctly. The UV light was switched on an hour before working in the Laminar Hood. Sterilization was also done on micropipette tips, cotton, forceps, blank discs, and other items.

5.3.6 Preparation of subculture

The test organisms were transferred from pure cultures to agar slants using a transfer loop in an aseptic environment under a laminar air cabinet to guarantee fresh, pure cultures. The injected strains were then incubated for 24 hours at 37°C to achieve optimum development. Fresh cultures were used in the sensitivity test.

5.3.7 Preparation of the test plates

The bacterial isolates were shifted from the subculture to the test tube. Test tube containing 10mL of melted and sterilized agar medium. The test tubes were spun to ensure that the organisms were suspended uniformly. Immediately, the bacterial and fungal suspensions were put into sterile Petri plates. The Petri dishes were rotated clockwise and anticlockwise to achieve homogenous dispersion of the test organisms in the fluid.

5.3.8 Preparation of discs

The antimicrobial screening was done using three different types of discs.

a) Standard disc

b) Blank disc

c) Sample discs

5.3.8.1 Standard discs

These positive control discs were used to ensure the activity of standard antibiotics. This test was compared the response of test organisms and by the known antimicrobial agent—kanamycin (30μg/disc) disc was used as the reference in this investigation.

5.3.8.2 Blank discs

To confirm that the filter paper and residual solvent (which remained on the discs after air-drying) were not active. Blank discs were used as negative controls.

5.3.8.3 Preparation of sample discs with test samples

In an aseptic setting, a determined quantity of each test sample was dissolved in a particular solvent volume to acquire the appropriate concentrations. Under the laminar hood, sterile metrical (BBL, Crooksville, USA) filter paper discs were placed in a blank petri dish. The discs were then submerged in test sample solutions and dried.

5.3.8.3.1 Preparation of sample discs with test samples of *Garuga pinnata* Roxb.

a) Test sample for crude ethyl acetate, methanol, and dichloromethane extract

Crude ethyl acetate, methanol, and DCM extract were tested for antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. The amount of sample per disc was 300 μg.

b) Test samples for pure compounds

A mixture of pure compound-1 and Compound-2 (**GPE-2** and **GPE-3**) was tested for antimicrobial activity using a sample concentration of 300μg per disc.

5.3.9 Preparation and application of the test samples

The test samples were weighed correctly, and determining solvents were added to the dried samples using a micropipette to achieve the necessary concentrations. Under aseptic circumstances, the test samples were pipetted onto previously sterilized discs using an adjustable micropipette.

5.3.9.1 Diffusion and incubation

The sample, standard, and control discs were carefully put on the agar plates pre-inoculated with test bacteria and fungus in the previously specified zones. Allow the discs' components to permeate the surrounding agar medium by placing the dish upside down in a refrigerator at 4°C for 24 hours. After that, the plates were inverted and stored in a 37°C incubator for 24 hours.

5.3.10 Determination of antimicrobial activity by the zone of inhibition

The capacity of the test compounds to suppress the formation of bacteria surrounding the discs, resulting in a unique zone of inhibition, is used to determine their antibacterial activity. The antibacterial activity of the test materials was

measured after incubation by measuring the width of the inhibitory zones in millimeters on a precision scale.

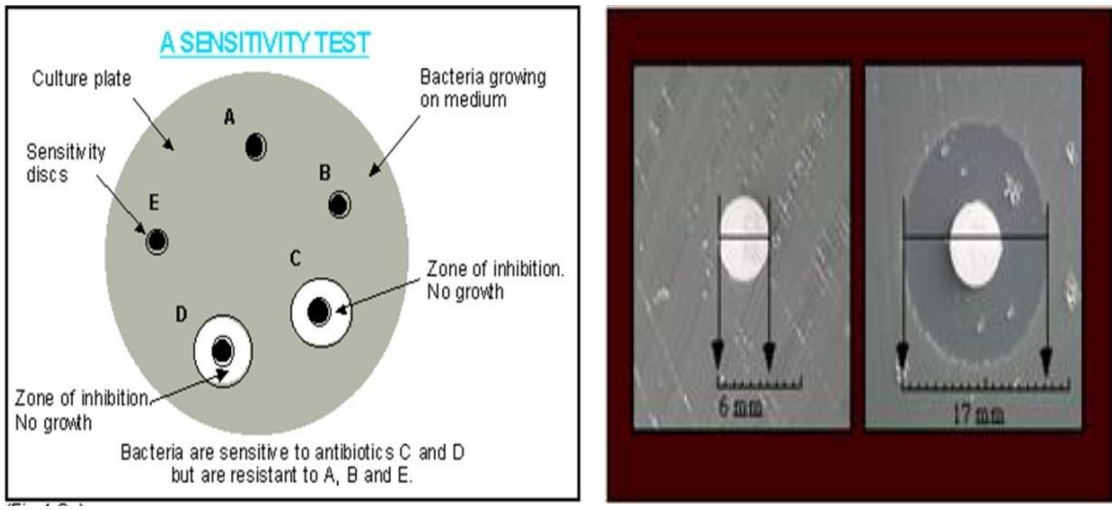


Figure 5.2: Inhibition zone measurement

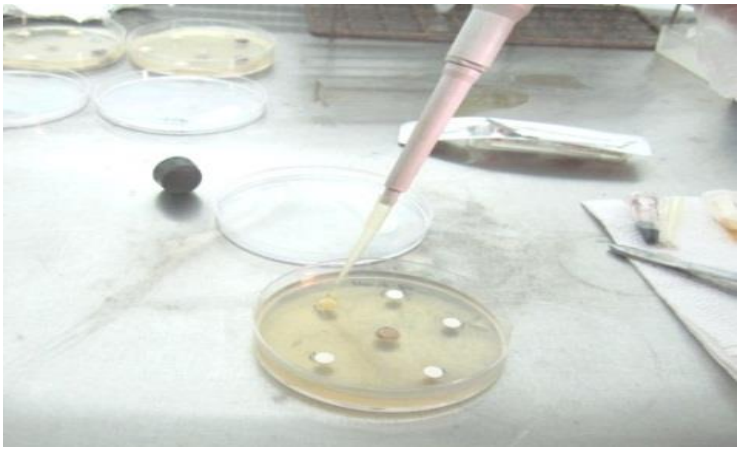


Figure 5.3: Application of samples on the discs

Figure 5.4: Antibacterial activity of various extracts against different bacteria

Table 5.2:Antimicrobial activity of crude ethyl acetate extract, methanol extract, and dichloromethane extract of *Garuga pinnata.*

Test bacteria and fungi	Ethyl acetate extract	Methanol extract	Dichloromethane extract	kanamycin
	300 (µg/disc)			30 (µg/disc)
Gram-positive bacteria				
Bacillus cereus	33	---	31	32
Bacillus megaterium	27	---	---	32
Bacillus subtilis	32	---	30	33
Staphylococcus aureus	34	---	28	33
Sarcina lutea	31	---	29	33
Gram-negative bacteria				
Escherichia coli	28	---	33	33
Salmonella paratyphi	28	28	---	33
Salmonella typhi	35	28	38	33
Shigella boydii	31	---	25	33
Shigella dysenteriae	28	---	27	33
Vibrio mimicus	30	---	27	32
Pseudomonas aeruginosa	32	21	29	33
Fungi				
Candida albicans	34	---	22	32
Sacharomyces cerevacae	27	---	---	32

"--- "Indicates 'No activity.

Table 5.3: Antimicrobial activities of compound-1 and **Compound-2 (GPE-2** and **GPE-3) of *Garuga pinnata.***

Test bacteria and fungi	Compound-1 &2 (mixture of Stigmasterol and β-sitosterol)	Kanamycin
	300 (µg / disc)	30 (µg / disc)
Gram-positive bacteria		
Bacillus cereus	---	32
Bacillus megaterium	---	32
Bacillus subtilis	9	32
Staphylococcus aureus	12	33
Sarcina lutea	....	33
Gram-negative bacteria		
Escherichia coli	11	33
Salmonella paratyphi	10	33
Salmonella typhi	11	33
Shigella boydii	9	33
Shigella dysenteriae	11	33
Vibrio mimicus	9	32
Vibrio parahemolyticus	10	33
Fungi		
Candida albicans	10	32
Sacharomyces cerevacae	9	32

"---" Indicates 'No activity.'

5.4 Results and discussion of *in vitro* Antimicrobial screening of *Garuga pinnata* Roxb**.**

Partitioned crude methanol, ethyl acetate, and dichloromethane extract, and the mixture of compounds -1 and 2 (mixture of Stigmasterol and β-sitosterol) were tested for antimicrobial activity against a number of both Gram-positive and Gram-negative bacteria and fungi. Kanamycin (30μg/disc) standard disc was used for comparison purposes.

The ethyl acetate and dichloromethane extracts (300μg/disc)were antimicrobial against all test microorganisms and fungi **(Table: 5.2)**. The methanol extract has little antimicrobial activity against the Gram-positive microorganisms *Bacillus cereus, Staphylococcus aureus*.

The pure compound-1 and 2 mixture(300μg/disc) showed similar activity as ethyl acetate crude extract (9-12 mm inhibition zone) against almost all the microorganisms and fungi. **(Table: 5.3)**.

Chapter-6

BRINE SHRIMP LETHALITY BIOASSAY

BRINE SHRIMP LETHALITY BIOASSAY

6.1 Introduction

Interest in plant-based medications has gradually increased during the last decade. The absence of a good, simple, and quick screening approach sometimes restricted the research of bioactive chemicals from plant sources and extracts in the chemical laboratory. Of fact, there are several bioassay protocols. Still, unless collaboration programs with biologists or pharmacologists are in place, the ordinary chemical laboratory is ill-equipped to execute standard bioassays on entire animals or isolated tissues and organs.

The brine shrimp lethality bioassay technique outperforms alternative testing methods because it is quick and affordable. It requires a small number of samples against a large number of organisms for statistical validation. In addition, unlike other procedures, it does not necessitate the use of animal serum.

6.2 Principle (Meyer *et al., 1*982)

By adding the estimated DMSO, brine shrimp eggs are hatched in simulated saltwater to produce nauplii. The nauplii are counted visually and then put in test tubes with 5 mL of seawater. Various concentrations of samples are added to the premarked test tubes using a micropipette. The test tubes are then left for 24 hours. The surviving are counted after 24 hours.

6.3 Materials

Artemia salina leach (brine shrimp eggs)	Micropipette.
Sea salt (NaCl)	Glass vials.
Small tank with a perforated dividing dam to hatch the shrimp	Magnifying glass.
Lamp to attract shrimps	Test tubes.
Pipettes	Test Sample of experimental plants

Table 6.1 Test Samples of experimental plants

Sample Code	Test Sample	Calculated amount (mg)
ME	Methanol Soluble Fraction	4.0
HSF	Hexane Soluable Fraction	4.0
DCMSF	Dichloromethane Soluble Fraction	4.0

6.4: Experimental Procedure

6.4.1 Preparation of seawater

A transparent solution was made of 38gram/L of sea salt (pure NaCl) and filtering it.

6.4.2 Hatching of brine shrimps

Artemia salina leach (brine shrimp eggs) obtained from pet stores. The little tank was filled with briner, and shrimp eggs were placed on one side of the tank, then covered. The shrimp hatched until mature as nauplii for one day. Throughout the hatching period, a continuous oxygen supply was maintained. The perforated dam attracted the hatched shrimps to the lamp, and they were removed for testing.

Ten live shrimps were introduced in each test tube of 5 ml of saltwater using a Pasteur pipette.

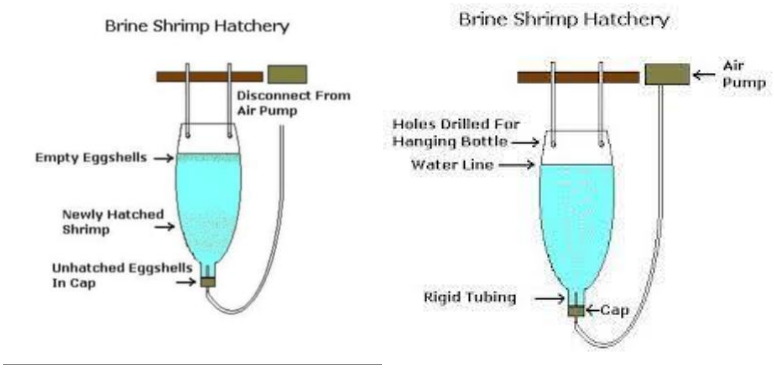


Figure 6.1: Brine shrimp Hatchery

6.4.3 Preparation of test samples of the experimental plant

All test samples were placed in vials and dissolved in 200µL of pure DMSO to make stock solutions. The solution was then transferred to the first test tube, which contained 5 mL of saltwater and ten shrimp nauplii. As a result, the prepared solution in the first test tube had a final concentration of 400µg/mL. Then, using the serial dilution procedure, a series of solutions with different concentrations were made. In each observation, the test tube was filled with 100 µl of sample solution, and the vial was filled with fresh 100µL DMSO. As a result, various concentrations were discovered in different test tubes (Table 6.2)

Table 6.2: Test sample with concentration values after serial dilution

Test tube no.	Concentration (µg/mL)
01	400.0
02	200.0
03	100.0
04	50.00
05	25.00
06	12.50
07	6.250
08	3.125
09	1.563
10	0.781

6.4.4 Preparation of the control group

In cytotoxicity studies, control groups are used to confirm the test procedure and ensure that the results obtained are solely attributable to the test agent's activity, with the effect of any other conceivable factors being neutralized. There are two sorts of control groups that are commonly utilized.

i) Positive control

ii) Negative control

6.4.4.1 Preparation of the positive control group

The outcome of the test compared of sample VS positive control. Vincristine sulfate was employed as a positive control in this investigation. The vincristine sulfate was dissolved in DMSO to provide an initial concentration of 20 µg/mL, from which serial dilutions in DMSO to obtain 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.0 µg/mL, respectively. Creating a positive control group, the premarked test tubes containing ten alive brine shrimp nauplii in 5 mL seawater.

6.4.4.2 Preparation of the negative control group

100 µL DMSO was added to three premarked glass test tubes containing 5 ml of simulated seawater and ten shrimp nauplii. But, while the brine shrimps in these vials show a high death rate, the test is invalid because the nauplii perished for reasons other than the compounds' cytotoxicity.

6.4.5 Counting of nauplii

With a magnifying glass, vials of nauplii were checked after 24 hours. For each dilution, the percent (percent) mortality was computed.

6.5 Results and discussion of the test samples of *Garuga pinnata*

At larger doses, bioactive chemicals are potentially poisonous. *In vivo* lethality in an elemental zoological creature might thus be utilized as a valuable informant for screening and fractionation when looking for novel bioactive natural compounds.

All crude extracts yielded promising findings in the current bioactivity investigation, showing that the test items are biologically active. At varied concentrations, each of the test samples revealed different fatality rates. For all test samples, there was an essentially linear connection between mortality and test results. The plots were used to compute the samples' median lethal concentration (LC_{50}, the 50% of brine shrimp nauplii die).

The extracts of *Garuga pinnata* stem and bark such as Methanol Soluble Fraction, Hexane Soluble Fraction, and Ethylacetate Soluble Fraction were examined for brine shrimp lethality using Meyer *et al.,* technique. (1982). The extractives' lethality in brine shrimp was determined, and the findings are shown in Table 6.3. A plot of the proportion of shrimps that died versus the logarithm of the sample concentration (toxicant concentration) yielded the fatal concentration (LC_{50}) of the test samples after 24 hours, and regression analysis was used to get the best-fit line from the curve data. As a positive control, vincristine sulfate (VS) was utilized, and the LC_{50} was reported to be 9.02 µg/mL. Ethylacetate Soluble Fraction 55.48 µg/mL had the most excellent brine shrimp lethality among all *Garuga pinnata* stem and bark extractions, followed by Methanol Soluble Fraction 80.99 µg/mL and Hexane Soluble Fraction 110.15 µg/mL in a Brine shrimp lethality bioassay. When compared to Vincristine sulfate, the Ethylacetate Soluble Fraction demonstrated high lethality activity.

Table 6.3: LC_{50} values of the test sample of *Garuga pinnata*

Test Samples	Regression Line	R^2	LC_{50}
VS	y = 30.803x + 20.575	R^2 = 0.9731	9.02
HSF	y = 25.169x - 1.3985	R^2 = 0.9565	110.15
EASF	y = 28.188x + 0.8343	R^2 = 0.9833	55.48
MSF	y = 26.478x - 0.5318	R^2 = 0.9421	80.99

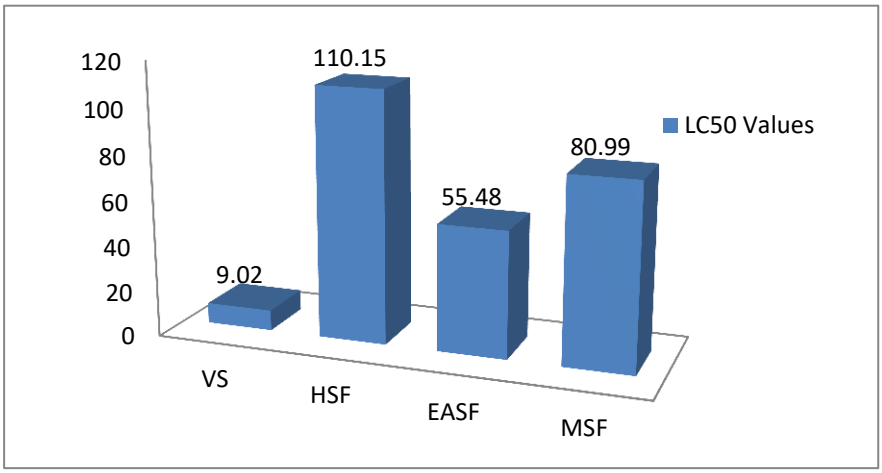


Figure 6.2 LC_{50} values of different extractives of *Garuga pinnata*

Table 6.4: Effect of Vincristine sulfate (positive control) on shrimp nauplii

Conc. (µg/mL)	Log_{10} concentration	% of Mortality	LC_{50}
400	2.60206	100	**9.02**
200	2.30103	90	
100	2	80	
50	1.69897	80	
25	1.39794	70	
12.5	1.09691	50	
6.25	0.79588	40	
3.125	0.49485	30	
1.5625	0.19382	30	
0.78125	-0.10721	20	

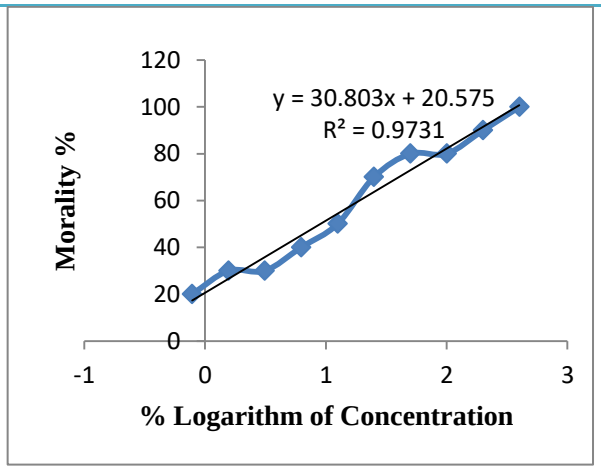


Figure6.3 Plot of % of mortality and predicted regression line of VS

Table 6.5: Effect of HSF of *G. pinnata* (positive control) on shrimp nauplii

Conc. (µg/mL)	Log_{10} concentration	% of Mortality	LC_{50}
400	2.60206	70	**110.15**
200	2.30103	60	
100	2	50	
50	1.69897	35	
25	1.39794	30	
12.5	1.09691	20	
6.25	0.79588	15	
3.125	0.49588	10	
1.563	0.19382	10	
0.781	-0.10721	0	

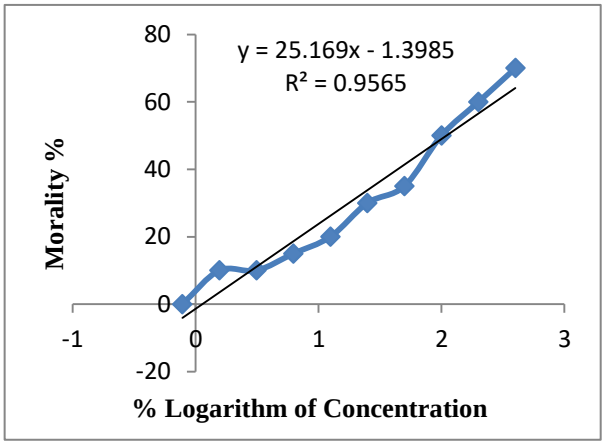


Figure6.4 Plot of % of mortality and predicted regression line of HSF

Table 6.6: Effect of EASF Extract of *G. pinnata* (positive control) on shrimp nauplii

Conc.(µg/mL)	Log_{10} concentration	% of Mortality	LC_{50}
400	2.60206	70	**55.48**
200	2.30103	70	
100	2	60	
50	1.69897	50	
25	1.39794	40	
12.5	1.09691	30	
6.25	0.79588	20	
3.125	0.49588	10	
1.563	0.19382	10	
0.781	-0.10721	0	

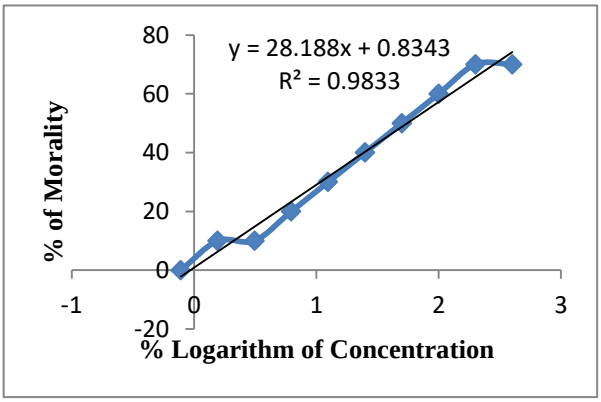


Figure6.5 Plot of % of mortality and predicted regression line of EASF

Table .7: Effect of MSF Extract of *G. pinnata* (positive control) on shrimp nauplii

Conc.(µg/mL)	Log_{10} concentration	% of Mortality	LC_{50}
400	2.60206	70	**80.99**
200	2.30103	70	
100	2	60	
50	1.69897	50	
25	1.39794	40	
12.5	1.09691	30	
6.25	0.79588	20	
3.125	0.49588	10	
1.563	0.19382	10	
0.781	-0.10721	0	

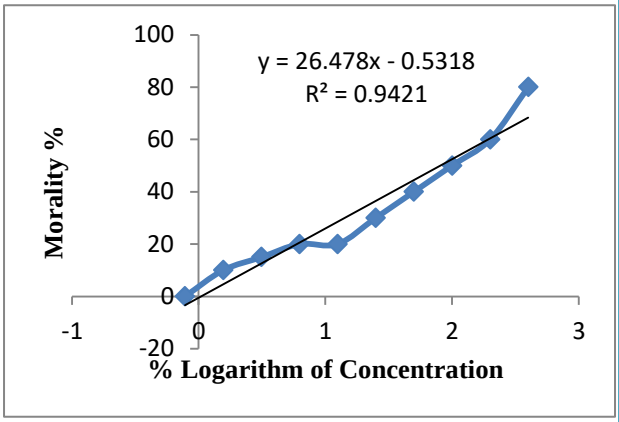


Figure6.6 Plot of % of mortality and predicted regression line of MSF

Chapter- 7

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