2018

Studies on Phytochemicals of Wedelia chinensis (Asteraceae) and Their Anticholinesterase & Antioxidant Activities

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Studies on Phytochemicals of *Wedelia chinensis* (Asteraceae) and Their Anticholinesterase & Antioxidant Activities.

A DISSERTATION
SUBMITTED TO THE RAJSHAHI UNIVERSITY,
BANGLADESH, IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

BY

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RAJSHAHI-6205, BANGLADESH.
2018

Dedicated
To
My Family

ACKNOWLEDGEMENTS

At first I do express my whole-hearted gratitude to the Almighty Allah, the merciful, the beneficent for his providence to carry out and complete this research work.

I would like to convey my best regards and profound gratefulness, honorable and sincere appreciation to my supervisor **Prof. Dr. M. Shahed Zaman,** Department of Chemistry, University of Rajshahi, Rajshahi, Bangladesh, for his constant supervision, expert guidance and inspiration throughout the entire period of my research work. I am really obliged to him forever for giving me such an opportunity to work in close association with him.

My sincerest and deepest gratitude to my co-supervisor **Prof. Dr. Md. Golam Shadik,** Department of Pharmacy, University of Rajshahi, Rajshahi, Bangladesh, for whose guidance and mentorship has provided me enthusiasm and knowledge.

I am greatly indebted to my honorable teacher Prof. Md. Belayet Hossain Howlader, Chairman, Department of Chemistry, University of Rajshahi for his advice and encouragement and also for providing me the necessary laboratory facilities in my research work.

I am grateful to my honorable teachers and former Chairman, Prof. Dr. M. Samsul Islam, Prof. Dr. M. Entazul Huque, Prof. Dr. M. Abdur Rahim Khan and Prof. Dr. Md. Nazrul Islam. Department of Chemistry, University of Rajshahi for their advice and encouragement.

I am sincerely grateful to my honorable teacher Prof. Dr. Md. Azizul Islam, Prof. Dr. M. Azhar Ali, Prof. Dr. M. Saidul Islam, Prof. Dr. Md. Hassan Ahmed, Prof. Dr. M. Akhter Farooque , Prof. Dr. Choudhury M. Zakaria, Department of Chemistry, University of Rajshahi for their useful discussion and suggestion.

I would like to express my sincere gratitude to the entire faculty members, especially Prof. Dr. Harendra Nath Roy, Prof. Dr. S.M. Monjurul Alam, Prof. Dr. A.B.M Hamidul Haque, Prof. Dr. M. Tariqul Hassan and Prof. Dr. Md. Nazrul Islam, Department of Chemistry, University of Rajshahi for their support and valuable suggestions.

I am grateful to University Grants Commission (UGC), Agargaon, Dhaka, Ministry of Education, Dhaka, Bangladesh and Director of Secondary and Higher Education (DSHE), Dhaka, Bangladesh, for providing me fellowship and sanction my deputation.

I would also like to express my thanks Mst. Naznin Akhter (Ph.D Fellow, R.U) and Md. Mominul Islam, Assistant Professor (BCS Education & Ph.D Fellow, R.U) for their advice and inspiration during my research work.

My thanks are due to the officers and staffs, Department of Chemistry, University of Rajshahi.

I also extend my sincere thanks to all officers and staffs of the Central Science Laboratory, University of Rajshahi, BCSIR, Rajshahi and BCSIR, Dhaka, Bangladesh.

Lastly, I owe a lot to my wife Bilkis Banu, beloved sons Yeamin Reza Borno and Iftefar Reza Ayon for their moral support. Finally, I thank my relatives and friends who took part in this enterprise in some form or the other.

MD. AMINUL ISLAM

DECLARATION

I do hereby declare that the materials embodied in this thesis entitled **"Studies on Phytochemicals of *Wedelia chinensis* (Asteraceae) and Their Anticholinesterase & Antioxidant Activities"** prepared for the Degree of Doctor of Philosophy, are the original research works of my own under the supervision of **Professor Dr. M. Shahed Zaman,** Department of Chemistry and **Professor Dr. Md. Golam Shadik,** Department of Pharmacy, University of Rajshahi, Rajshahi, Bangladesh. The thesis has not been concurrently submitted for the award of any Degree or Diploma anywhere.

(MD. AMINUL ISLAM)

CERTIFICATE

This is to certify that the contents in the thesis entitled **"Studies on Phytochemicals of *Wedelia chinensis* (Asteraceae) and Their Anticholinesterase & Antioxidant Activities"** are the original research work done by **MD. AMINUL ISLAM** under our guidance and supervision for the Degree of Doctor of Philosophy. It contains no material previously published or submitted for any other degree.

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PART-B

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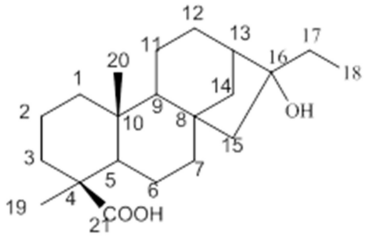
SUMMARY

This dissertation describes the results of phytochemical and Anticholinesterase & Antioxidant activities on the medicinal plant of the *Wedelia chinensis.* The whole research work has been divided into two parts. Part-A comprises with the chemical investigation of the *Wedelia chinensis.* Part-B deals with the study on biological activities on the fractions and isolated pure compounds.

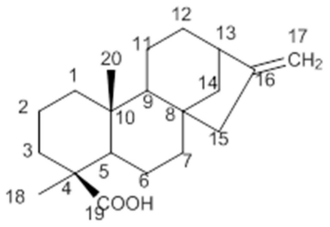
Extraction of the dried powder of whole plant of *Wedelia chnensis* was done by cold extraction method with methanol as solvent. Crude methanolic extract (CME) of the plant was further fractionated into four fractions; petroleum ether fraction (PEF), Chloroform fraction (ClF), Ethyl acetate fraction (EAF) and Dia-ion resin fraction (DRF). Chromatographic separation techniques were applied to isolate compounds from these fractions. The structures of these compounds were elucidated with the help of extensive spectroscopic techniques. The work has resulted in the isolation of seven compounds, out of which two were found to be new and one is isolated for the first time from this plant.

From chemical investigation of the Dia-ion resin (DRF) and Ethyl acetate fraction (EAF), seven compounds were isolated and characterized; DFC-1, DFC-2, DFC-3, DFC-4, DFC-5, EFC-1, EFC-2. Among the isolated compounds, compounds DFC-4 and DFC-5 are new one. Compound DFC-1 was first time isolated from this plant, previously reported from Achyranthes *Aspera.*

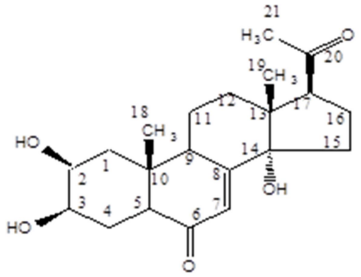
The chemical structures of the isolated compounds are presented below.



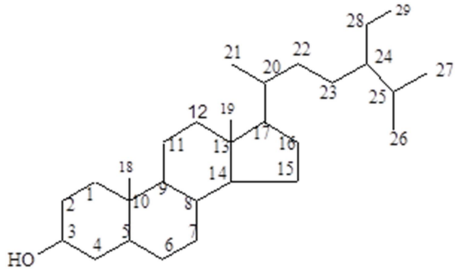
(-) Kaur-16 hydroxy-21 oic acid (EFC-1)
(Known compound)



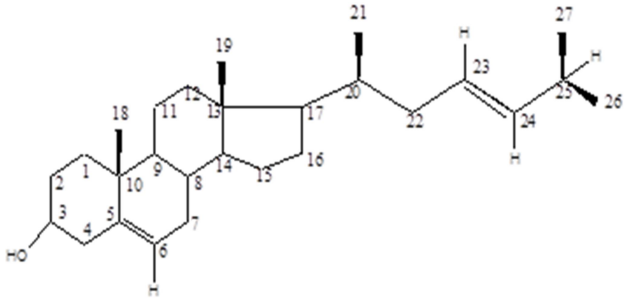
(-) Kaur-16-en-19 oic acid (EFC-2)
(Known compound)



Structure of compound DFC-1
(New source) Another source:
Achyranthes Aspera.

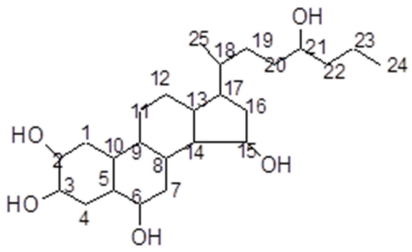


β -Sitosterol (DFC-2)
(Known compound)

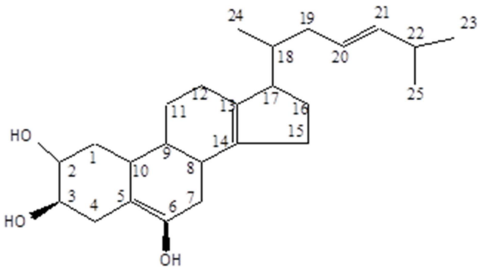


Cholesta-5, 23-dien-3-ol (DFC-3)

(Known compound)



Structure of compound DFC-4
(New compound)



Structure of compound DFC-5
(New compound)

In Part-B, Anticholinesterase & Antioxidant activities of the fractions as wel as pure compounds have been investigated. Results found are shown in decreasing order as follows:

Reducing power capacity:

Catechin > DRF > EAF > CME > CLF > PEF.

Catechin > EFC-1 > EFC-2.

DFC-3 > DFC-4 > Catechin > DFC-2 > DFC-5 > DFC-1

Total antioxidant activity

DRF > EAF > Catechin > CME > CLF > PEF.

EFC-1 > Catechin > EFC-2.

DFC-3 > DFC-4 > Catechin > DFC-1 > DFC-5 > DFC-2

DPPH radical scavenging activity:

Catechin > EAF > DRF > CME > CLF > PEF.

EFC-1 > catechin > EFC-2 .

DFC-3 > Catechin ≈ DFC-4 > DFC-5 > DFC-2 > DFC-1.

Hydroxyl radical scavenging activity

DRF > Catechin > EAF > CME.

EFC-1 > Catechin > EFC-2 .

DFC-3 > Catechin ≈ DFC-2 > DFC-4 > DFC-1 > DFC-5.

Lipid peroxidation inhibitory activity

DRF > Catechin > EAF > CME.

EFC-2 > Catechin > EFC-1.

DFC-3 > DFC-4 > Catechin > DFC-2 > DFC-1 > DFC-5.

Butyrylcholinesterase inhibitory activity

- The results demonstrated that DRF and EAF exhibited strong enzyme inhibition activity among the other fractions evaluated.

- The compound EFC-2 has strong enzyme inhibition activity than EFC-1.
- Butyrylcholinesterase inhibitory activity of isolated compounds (DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5) from dia-ion resin fraction has been investigated and IC_{50} (µg/mL) values were found at 78, 48, 20, 45 and 22.5 µg/mL respectively.
- From the IC_{50} (µg/mL) values, it was evident that compound DFC-3 & DFC-5 are Butyrylcholinesterase inhibitory active.

Acetylcholinesterase inhibitory activity

- The results demonstrated that dia-ion resin fraction and ethyl acetate fraction exhibited strong enzyme inhibition activity among the other fractions.
- From the result, it was evident that the compounds are acetylcholinesterase inhibition active and their activity decreases in the following order:

  Donepezol > EFC-2 > EFC-1

  Dopenezol > DFC-3 > DFC-4 > DFC-5 > DFC-2 > DFC-1.

PART -A
INTRODUCTION

1.1 General Discussion

Living organism produces secondary metabolites that are classes of chemical compounds. The natural product chemistry deals with the isolation of these compounds and investigation of their structure, formation, use, and purpose in the organism. Plants use these secondary metabolites for defense against predators and pathogens. Throughout evolution, plants have developed defenses against herbivore and various microbes and produced other natural products to promote competitiveness. The better defended, more competitive plants have generated more descendants, and so the capacity to produce and safely store useful metabolites has become widely established in the plant kingdom [1].

The study of natural products has led to the discovery of a variety of useful drugs for the treatment of diverse ailments. It also contributed to the development of analytical organic chemistry in the area of separation science and technology, spectroscopic methods of structure elucidation and synthetic methodologies.

One of the most important areas of application of the plant secondary metabolites is in the treatment of human and veterinary ailments. Currently, more than 100 chemical substances derived from about 90 plant species can be considered important drugs that are in use in one or more countries [2].

Since prehistoric times, humans have used natural products, such as plants, animals, microorganisms, and marine organisms, in medicines to alleviate and treat diseases. According to fossil records, the human use of plants as medicines may be traced back at least 60,000 years [3]. The use of natural products as medicines must, of course, have presented a tremendous challenge to early humans. It is highly probable that when seeking food, early humans often consumed poisonous plants, which led to vomiting, diarrhea, coma, or other toxic reactions perhaps even death. However, in this way, early humans were able to develop knowledge about edible materials and natural medicines [4]. Subsequently, humans invented fire, learned how to make alcohol, developed religions, and made technological breakthroughs, and they learned how to develop new drugs.

Thousands years ago the healing power of plants has been acknowledged by many cultures and aromatherapy can be used to stem from the various systems of traditional medicine developed by ancient civilization. Primitive peoples used plants in traditional treatment as well as in their religious rituals. All indigenous cultures also share a common acceptance of the belief that the growth and continued existence of humanity is dependent on a healthy relationship between the body and the mind and between the gods and human kind. In many cultures, fragrance odor were thought to please the gods and healing herbs were even thought to have magical qualities[1].

Since of the twenty-first century, the age of massive development the modern medicine, mankind still depends on the nature for its survival. Interestingly people use natural products and synthetic products to cure diseases. The people relieve physical sufferings because herbal medicines are once again providing a safe and natural alternative treatment for many everyday complaints. Therapeutic agents may be of plant, animals or mineral origin. Among these plants are a potential source of the primitive peoples. The plants are popularized for thousands of years and continue to rely on them for health care due to their effectiveness, easy availability, low cost and comparatively being devoid of serious toxic effects.

The earliest mention of the medicinal plants in the Indian subcontinent, founded in the *Rig Veda* (4500-1600 BC), which is the oldest book in the library of man. This great treatise supplies a lot of information about the medicinal use of plants in Indian subcontinent. The *Susuta Samhita,* probably written before 1000 BC, deals with details of surgery and therapeutics, while the comprehensive Indian herbal, *Charaka Samhita*, written about the period, deals mainly with medicinal agent and cites more then 500 medicinal plants with their medicinal properties and uses. These works are still esteemed as treasures of literature on Indian indigenous medicine, particularly *Ayurvedic* medicine. *Ayurveda* has the meaning *'the science of life'*. The earliest description of the plant medicines used in the *Ayurvedic* system were described about 1200 BC with a list of 127 plants.

The medicinal Plants are contain a large number of chemically diverse and biologically interesting compounds. The use of plants as medicine is older than recorded history. As mutewitness to this fact, marshmaliow root, hyacinth and yarrow

have been found carefully tucked around the bones of a Stone Age man in Iraq. These three medicinal herbs continue to be used today. Marshmaliow root is a demulcent herb, soothing to inflamed or irritated mucous membranes, suck as a sore throat or irritated digest tract. Hyacinth is a diuretic that encourages tissues to give up excess water. Yarrow is a time-honored cold and fever remedy that may once have been used much as aspirin today[5]. The past century has been the increasing importance of plants in the development of drugs for the treatment of diseases ranging from bacterial infection to cardiovascular problems and cancer. An impressive number of drugs have been isolated from plants.

Some of the natural products are of vital importance to the functioning of the species producing them while others appear to have no function at all. On the basis of the importance of the plants and the characteristics of the natural products, the plants are classified into following groups.

(i) Plants producing food for man and animals.
(ii) Plants used for domestic articles.
(iii) Poisonous (harmful) and medicinal (useful) plants.

Some plants found in nature are poisonous but the plants which serve us as source of energy and effective drugs[6]. Even the poisonous plants may be useful drug for recovery of human aliment if administered in small doses. The natural products such as vitamins, hormones, antibiotics, analgesics, extracted from plants and animal tissues are very important for their medicinal uses.

We are not sure that when and how man learned to use plant appropriately but it is thought that animal was the first guide to show them the use of plants. Because animals naturally know or feel how to get relief from the unusual internal conditions and during the long span of time. Men gradually developed a knowledge of naturally occurring drugs which was transmitted at one time orally, later in the written form as papyri, baked clay tablets, parchments, manuscript herbals, printed herbals, pharmacopoeias and other works and most recently by computerized information retrieval system[7]. But today these drugs are prepared synthetically in the laboratory to meet greater necessity or to confirm purity and specificity. For example, modern drugs like quinine, narcotic analgesic like Digi toxin whose source was plant and are

now prepared synthetically. Thousands of plant metabolites that are used for the treatment of various diseases were based on the use of herbal and traditional medicines. So, it is clear that primitive use of plants or herbs played an important role for the development of modern medicine.

According to WHO estimate, around 80% of the world's 5.86 billion inhabitants depend on traditional medicines, majority of which use plants or their active principles[8]. The WHO has emphasized the utilization of indigenous systems of medicines based on the locally available medicinal plant. Approximately one-third of all drugs used by common people are plant based and if bacteria and fungi are included then the picture is like that 60% of Pharmaceuticals are of plant origin [9,10]. It is quite surprising that 25% of all prescriptions dispensed from pharmacies in all over U.S.A. contained plant extracts or active principles from plants. WHO gave official recognition to these medicines in a resolution adopted by the 30th World Health Assembly in 1977. In 1993, the British Medical Association (B.M.A) published a report titled "contemporary medicine-new approach to good practice" which acknowledged that some non-conventional therapies like acupuncture, osteopathy and herbals may be good if used in consultation with general practitioners.

1.2 Prospective of Natural Product Chemistry

Actually the study of natural products chemistry began at nineteenth century with the isolation of pure morphine, the hypnotic and anesthetic principle of opium from the latex of *Papaver somniferum* capsules (1816) by scintist Serturner. This was followed by some other isolation like ‘strychnine’ from *Strychnos nuxvomica* (1817), ‘emetine’ from *Cephaelis lpecacuanha* (1819), ‘quinine’ from *Cinchona spp.* Bark (1820), ‘colchicines’ from *Colchicum autunnale* (1820), ‘nicotine’ from *tobacco* leaves and ‘cocaine’ from *Erythroxylon coca* leaves, the active chemical responsible for corresponding treatment. Subsequently a whole series of plants were investigated to detect and isolate the active principles. These led to the discovery of a group of compounds having similar structures and physiological activities.

Most of the above active principles are nitrogenous bases and are called alkaloids. The alkaloids from *Rauwolfia* group such as ‘Reserpine’ is used in medicine in conditions, including tension and anxiety as well as in the treatment of hypertension.

The alkaloid *'Vinblastin'* from Visco rosa has been subject of medicinal studies particularly in the field of cancer. Other some alkaloids like *'Atropin'* and '*Nicotine'* isolated from Solanaceae family, '*Cocaine'* from Erythroxylaceae family, *'Morphine', 'Codine', 'Thebaine'* from Papaverine family, *'Noscapine'* from Papaveraceae family and '*Quinine'* isolated from the family of Rubiaceae. Similarly steroids '*Digitoxin'* used in congestive heart failure and treatment of cardiac arrhythmias, particularly aerial fibrillation, is isolated from the plant of Scrophulariaceae family. Diterpene type compound '*Taxole'* is isolated from Taxaceae family and Trierpene type compound '*Ginseng'* and Triterpene glycoside type compound '*Ginsenoside'* isolated from genus in Araliaceae family.

Secondary metabolites which are produced by living organisms (microbes and plants) for the purpose of their own survival have a major importance in drug discovery for their therapeutic like Cyclosporine (immunosuppression), mevinolin (hypercholesterolaemia), Avernectin (parasitic disease), Artemisinin (malaria), Vinblastine, Vincristine and Taxol (cancer). Now days, natural product-based drug discovery competes with the combinatorial chemistry, which has given a significant complementary way for drug development. It produced thousands of compounds of almost similar structure in a single experiment, which are immediately available for bioassay screening. In contrast, plants and microorganisms have the potential to synthesize novel chemicals in unpredictable ways. The genes and metabolic pathways by which plants and microbes produce novel structures are now understood in many cases and this knowledge has been used in drug discovery. Plants and microbes use same technique as in combinatorial chemistry to biosynthesize secondary metabolites. Natural product biosynthesized is mostly catalyzed by enzymes, which results from an enormous gene pool that has evolved over billions of years. Recent improvements in instrumentation, robotics and bioassay technology have increased the speed of bioassay guided isolation and structure elucidation of natural products significantly. These improvements have allowed natural product to be more competitive and cost effective, as compared to synthetic medicinal chemistry.

Since 2005, thirteen natural product (NP) derived drugs have been lunched in the market (Table-1.1), in addition to the 37 late stage clinical development NP-based candidates (6 in registration and 31 in phase III). The traditional strengths of NPs in

oncologic and transmittable diseases is still explored with 19 (51%) compounds being evaluated for the treatment of cancer and 10 (27%) for the treatment of bacterial infections. The remaining compounds are for the treatment of metabolic diseases[11], pain and multiple sclerosis[12]. A large number of NP derived compounds are in various stages of clinical development which indicates that natural products are still the major feasible source of new drug candidates. In absence of NP-derived compounds, it is predicted that there would be a considerable therapeutic shortage in a number of important areas, such as neurodegenerative and cardiovascular diseases, most solid tumots and immune inflammatory diseases [Butler, 2008].

Table-1.1 : NP-derived drugs launched since 2005 [Butler, 2008].

Year	Tread Name	Lead Compound	Classification	Disease Area
2005	Dronabinol /Cannabbiol (Sativex®)	Dronabinol / Cannabbiol	NP	Pain
2005	Fumagilin (Flisint®)	Fumagilin	NP	Antiparasitic
2005	Doripenem (Finibax® / Doribax™)	Thienamycin	NP-derived	Antibacterial
2005	Tigecycline (Tygacil®)	Tetracycline	Semi-synthetic NP	Antibacterial
2005	Ziconotide (Prialt®)	Ziconotide	NP	Pain
2005	Zotarolimus (Endeavor™ Stent)	Sirolimus	Semi-synthetic NP	Cardiovascular Surgery
2006	Anidulafungin (Eraxis™ / Ecalta™)	Echinocandin B	Semi-synthetic NP	Antifungal
2006	Exenatide (Byetta™)	Exenatide-4	NP	Diabetes
2007	Lisdexamfetamine (Vyuanse™)	Amphetamine	NP-derived	ADHD
2007	Retapamulin (Altabax™ / Altargo™)	Pleuromutilin	Semi-synthetic NP	Antibacterial
2007	Temsirolimus (Torisel™)	Sirolimus	Semi-synthetic NP	Oncology
2007	Trabectedin (Yondelis™)	Trabectedin	NP	Oncology
2007	Ixabepilone (Ixempra™)	Epothilone B	Semi-synthetic NP	Oncology

1.3 Traditional Medicines (TM)

TM is the oldest form of health care in the world and is used in the prevention, and treatment of physical and mental illnesses. Different societies historically developed various useful healing methods to combat a variety of health- and life-threatening diseases. TM is also variously known as complementary and alternative, or ethnic medicine, and it still plays a key role in many countries today [13,14].

The medicaments used in TM are mostly derived from natural products. In TM, "clinical trials" have been conducted since ancient times. In the case of Traditional China Medicines (TCM), considerable experience and advances have been accumulated and developed over the past thousands of years with respect to methods of preparation, selection of herbs, identification of medicinal materials, and the best time for obtaining various different plants. Appropriate processing and dose regulation are urgently needed in TCM to improve drug efficacy and reduce drug toxicity. Considerable amounts of data have been acquired through clinical experiments, and in this way TM has assisted in the development of modern drugs. Through its use of natural products, TM offers merits over other forms of medicine in such areas as the following: discovery of lead compounds and drug candidates; examining drug-like activity; and exploring physicochemical, biochemical, pharmacokinetic, and toxicological characteristics. If any form of TM is applied successfully, it may surprisingly assist in the development of new drugs, thereby resulting in many benefits, such as significant cost reductions.

TCM is now an inseparable part of the Chinese public health system. In recent years, TCM has gradually gained considerable approval as a complementary or alternative medicine in Western countries. Chinese herbal medicine, which is the most important component of TCM, is currently used in the health care of an estimated 1.5 billion people worldwide [15,16]. It should be noted that in TCM, several herbs and ingredients are combined according to strict rules to form prescriptions, which are referred to as formulas (fang ji in Chinese). Commonly, a classic formula is composed of four elements "monarch", "minister", "assistant", and "servant" according to their different roles in the formula, each of which consists of one to several drugs. Ideally, these drugs constitute an organic group to produce the desired therapeutic effect and reduce adverse reactions [17].

Kampo is the TM of Japan. Between the fifth and sixth centuries, TCM was introduced to Japan from China; since then, TCM has been significantly altered and adapted by Japanese practitioners to meet their particular circumstances and gradually evolved into Kampo [18]. A recent study has found that some physicians in Japan use Kampo medicines in their daily practice sometimes as the preferred medication [19–21]. Together with radiotherapy or chemotherapy, some Japanese physicians frequently utilize Kampo medicines in treating cancer patients. This indicates how modern Western medicine can be well integrated with TM [20,22]. As the use of Kampo continues to rise in conjunction with Western medicine, there is growing realization of the urgent need to study the interactions between these two types of medicines [17].

Unani is an ancient Greek holistic medical system with a history that can be traced back 2500 years [23]. Since the mid-1970s, when the WHO began to place a greater focus on TM, Unani has attracted considerable attention all over the world, especially in India, where it has been integrated into the national health care system [24].

It was reckoned by WHO that a large quantity of people in the world still depend on TMs for health care [25]. The current status of TM differs in different countries. In 2012, the total value of the TCM industry was equivalent to around one-third of the total for China's pharmaceutical industry [26]. It has been determined that 80% of the population in Africa makes use of TM either alone or in conjunction with conventional medicine [27]. By contrast, traditional Aboriginal medicine in Australia is in danger of vanishing owing to the prevalence of conventional medicine [28]. In the case of Israel with its ethnic diversity, modern medicine is prevailing, and TM is declining [29]. Many practitioners of Western medical science think such TM systems as being short of reliability; however, they are adopted by the majority of people in the world [25]. It is possible to produce remarkable synergy and yield great benefits in developing reformed medicines and new drugs by connecting powerful modern scientific techniques and methods with the reasonable ethnobotanical and ethnomedical experiences of TM. Characteristics of several TM systems are summarized in Table-1.2.

1.4 Drugs Developed from Traditional Medicines

TM is too valuable to be ignored in the research and development of modern drugs. Though it has an enigmatic character, there are also wide contexts for its use in terms of non-Western medical technology or activities. In TM, a single herb or formula may contain many phytochemical constituents, such as alkaloids, terpenoids, flavonoids, etc. Generally speaking, these chemicals function alone or in conjunction with one another to produce the desired pharmacological effect [25]. It is notable that a lot of plant-originated drugs in clinical medicine today were derived from TM [30]. In addition, it has been demonstrated that the many valuable drugs derived from plants were discovered through their application in TM [3].

Almost 20 years ago, a thorough investigation of the pharmacopoeias of developed and developing nations and the associated world scientific literature was conducted as part of the WHO's TM Program. The aim of that study was to determine whether TM really had inspired modern drug discoveries and whether there was any correlation between the current use of various compounds and their application in TM. The study focused on various compounds used in drugs derived from plants in different countries, and it established that TM had indeed played a significant role in developing effective new drugs. That study focused on 122 compounds, 80% of which were found to be related to pharmaceutical effects in folk medicine, and it was determined that these compounds originated from 94 plant species.

Table 1.2 Characteristics of several important traditional medicine systems

Name	Origin and Developing Nation	Characteristics of Theory or Application	Current Role or Status	Modern Research
Traditional Chinese medicine (TCM) [15,17,31–34]	• China • Thousands of years ago.	• TCM is based on Yinyang and Wuxing concepts. • A TCM formula includes a group of various drugs that function together congenially to achieve a synergistic effect. • A classic formula is composed of four elements: monarch, minister, assistant, and servant according to their roles in the formula.	• Both TCM and conventional medicine exist at every gradation of the health-care system, and both are covered under public and private insurance. • There is a TCM division in most ordinary hospitals and TCM services are supplied for both inpatients and outpatients. • TCM is attracting increasing attention, interest, and acceptance around the world	• The pharmacology of TCM has made great advancements. • In recent decades, many TCM active compounds and compound-based therapeutics have been discovered. • Great efforts have been made to reveal the underlying molecular mechanisms of TCM.
Ayurveda [25,35]	• India • Ayurveda can be dated back to the pre-Vedic epochs (4000 BC–1500 BC)	• Ayurveda uses natural elements to eradicate the main cause of the disease by reinstating balance. • The Ayurvedic philosophy is to live a healthy life to avoid the appearance of imbalance and unnecessary pain. • In many Ayurvedic treatments, multiple herbs are united in a special quotient to create an ideal therapeutic effect and lessen the toxicity.	• More than 400,000 Ayurveda practitioners are registered. • The Indian government has an official body to ensure Ayurveda's educational efforts, quality, and practice	• Pharmacologically-active compounds of Ayurvedic medicine and their effectiveness in treatment has been increasingly recognized.

Name	Origin and Developing Nation	Characteristics of Theory or Application	Current Role or Status	Modern Research
Unani medicine [23,24,36,37]	• India • Unani medicine derived from Greco-Arabic medicine dating back 2500 years and developed during the Arab civilization.	• It treats a person's body, mind, and soul as a whole. • Unani looks upon the human body as a single unit, which consists of four basic elements which have four disparate temperaments respectively. • A person's temperament reflects their physical characteristics and natural disposition. • Disproportion in temperament makes the human body susceptible to many illnesses.	• Unani is accepted by India as meeting the health-care needs of people and has gained formal status. • Unani has been acknowledged by the WHO as an alternative health-care system. • Unani is one of the most important traditional medicine systems.	• Many bioactive ingredients have been separated from mangrove plants which are used in Unani medicine.
Kampo (traditional Japanese medicine) [20,38]	• Japan • Kampo was introduced from China via the Korean peninsula in the 5th or 6th century.	• Kampo was developed over the past 1400 years and has been organically unified with Japanese original therapies. • Kampo treats every human being as a complete and self-controlled whole in which body and mind impact mutually. • Diseases are thought to originate from the disorders of psyche and soma and herbals are trusted to affect the soul and the body equally. • Kampo therapy places emphasis on the sufferer as a whole instead of on the illness.	• Kampo is incorporated into the health-care system in Japan. • All citizens can use of Kampo herbal formulas approved by the government.	• Kampo formulas are produced by certificated drug firms under strict quality management standards. • Both the government and drug firms are deeply involved in surveillance of all processes to ensure the quality and safety of Kampo formulas. • There has also been a focus on examining the efficacy of Kampo formulas and exploring related mechanisms. Kampo is regarded as very safe.

Name	Origin and Developing Nation	Characteristics of Theory or Application	Current Role or Status	Modern Research
Traditional Korean medicine (TKM), Sasang constitutional medicine (SCM) [33,39–41]	• SCM is a division of Korean traditional medicine. • It was first introduced in the mid-19th century.	• SCM classifies persons into four Sasang types: Tae-Yang, So-Yang, Tae-Eum, and So-Eum according to his/her inborn features. • SCM is holistic. • SCM is theoretically similar to personalized medicine. • SCM supplies individualized and constitution-specific treatments for various problems.	• Although the conventional health-care organization is quite good in Korea, 86% of people still employ SCM. • Traditional medicine doctors can supply Korean SCM both in private and public hospitals. • Both national medical insurance and private insurance cover Korean SCM services.	• The Lee Jema project to supply scientific proof of SCM began in 2006 and is supported by the Korean government. • It has gained many significant achievements involving constitution-diagnostic means, constitution-specific disease vulnerabilities, and genetic research
Traditional Aboriginal medicine [28,41]	• Australia	• Indigenous peoples of Australia believe that health problems have three types of causes: natural bodily causes, harmful spirits, or witchcraft.	• Currently, there is only one national folk organization in operation. • During 2010–2011, 32.1% of the chief, indigenous health-care organizations in Australia provided some kind of traditional medicine services. • Because of colonization, traditional Aboriginal medicine is in danger of becoming extinct	

Name	Origin and Developing Nation	Characteristics of Theory or Application	Current Role or Status	Modern Research
Traditional medicine in Africa [14,27,28,42]	• Africa	• Traditional medicine doctors treat patients holistically. • They generally seek to recombine the mental and social equipoise of sufferers according to social relationships and rules. • The accessibility of traditional medicine is one of the most important reasons for its popularity across Africa. • Traditional medicine exemplifies respect for the cultural heritage	• Eighty percent of African people use traditional medicine either by itself or with conventional medicine. • Up to 80% of Ghanaians and Ethiopians depend on traditional medicine for their main health-care demands. • Ghana's traditional medical system has been integrated into the national health-care system and, therefore, it is comparatively well organized.	• Research on Hydnora africana, which is used as ethnomedicine in Africa, has demonstrated the antioxidant and antibacterial activities of natural products.
Russian herbal medicine [43]	• Russia • 10th century	• Due to the special geographical environment of Russia, Russian herbal therapy has collected and adopted traditional medicine methods that were introduced from Europe and Asia. • The Russian Federation follows the State Pharmacopoeia of the USSR; 32 of 83 individual plant monographs are found only in this Pharmacopoeia.	• Herbal therapy is a formal and independent department of medicine in Russia; thus, herbal medicinal products are regarded as official remedies. • A recent survey shows that 14% of the Russian people frequently use herbal remedies and 44% use them occasionally.	• Soviet/Russian researchers have focused mainly on the development of adaptogens derived from plants. • The collection of plants with expectorant effects shows huge potential.

The acceptability, convenience, and accessibility of TMs have been, and will be, helpful for new drug research [44]. As noted above, artemisinin and other antimalarial drugs are examples of modern drugs based on TMs. Early in China's J in Dynasty, Doctor Hong Ge (AD 284–384) recorded the efficacy and related details of Artemisia annua L. in treating malaria in his book Zhou Hou Bei Ji Fang. That is the earliest record anywhere of treating malaria with Artemisia annua L., and it shows that Chinese physicians 1700 years ago had reached a sophisticated level of medical treatment [45,46].

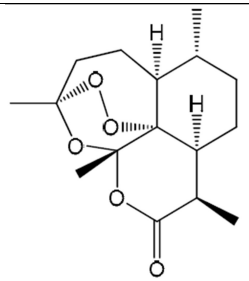
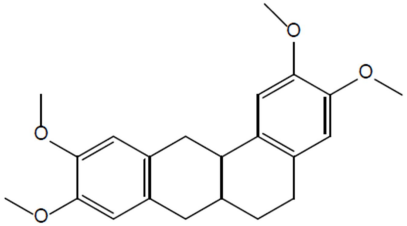
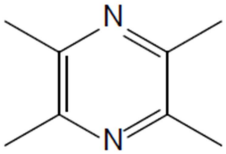
Artemisinin is known as qinghaosu in Chinese, and its study has made significant progress, including the synthesis of new artemisinin analogs and derivatives, and research efforts into the biological activities and related mechanisms. As a result, artemisinin, as well as its effective derivatives, are extensively applied throughout the world as new-type anti-malarial drugs [47].

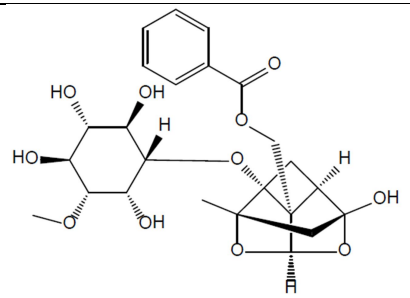
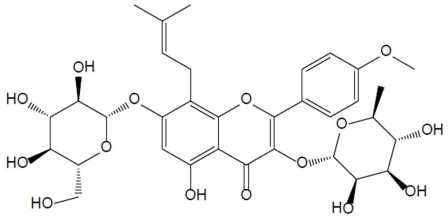
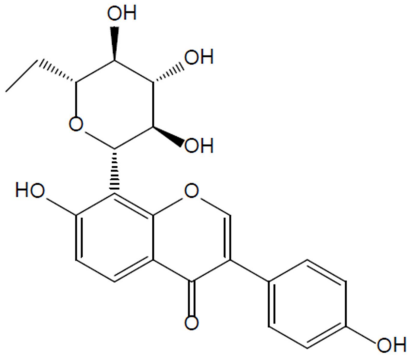
The discovery of artemisinin can be traced back to the 1960s, when tropical malaria was a serious problem during the Vietnam War. North Vietnam requested China to help tackle the malaria problem. The Chinese government approved a project for malaria control and drug research in 1967. The research group made its investigations and carried out a large-scale search of the literature on the subject. As part of the phytochemical and pharmacological research effort, a lot of Chinese herbal medicines were screened and investigated with respect to their toxicity or efficacy. Eventually artemisinin was derived from Artemisia annua L. in 1972 [45,47,48]. Artemisinin is quite different from previously-used antimalarial drugs, such as chloroquine, in that it has a novel structure, with a sesquiterpene lactone bearing a peroxy group, and it does not contain nitrogen heterocycles. Compared with previous antimalarial drugs, artemisinin has the merit of high efficiency, quick effect, and low toxicity. Artemisinin is effective in treating various forms of malaria, such as falciparum and cerebral malaria, which are resistant to chloroquine, and its mechanism of action is different from traditional antimalarial drugs. The discovery of artemisinin was a great success for TCM at a special period in China's history, and it was achieved through a well-organized team of hundreds of researchers [48]. Since that breakthrough, scientists have conducted comprehensive research in such areas as pharmaceutical chemistry, organic synthetic chemistry, and chemical biology. Through etherification

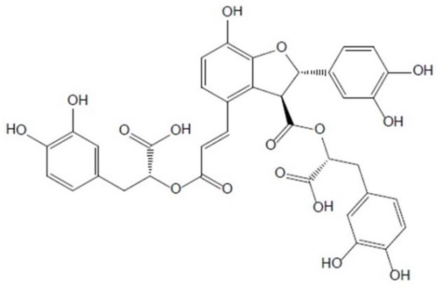
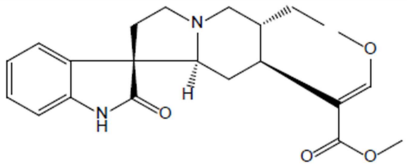
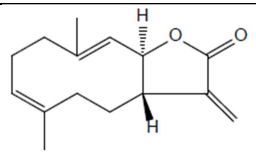
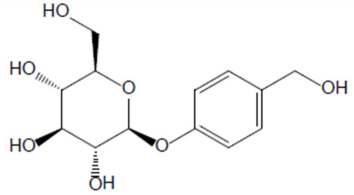
and esterification, they have produced a series of well-known new drugs, such as artemether and artesunate. Those drugs have improved efficacy and solubility, which are of benefit for patients receiving oral or intravenous administration and have overcome the high parasite recrudescence rate and low solubility of artemisinin [47–49]. Most importantly, one of these scientists, Youyou Tu, was just awarded the 2015 Nobel Medicine Prize for her significant devotion in discovering artemisinin.

The discovery of artemisinin illustrates how TCM constitutes a great store of knowledge about natural products, such as Chinese herbs, and holds much future promise. The discovery of successful new drugs can proceed by profiting from this knowledge [48]. Some drugs or compounds isolated from Chinese herbal medicines which follow the ethnomedical uses are summarized in Table 1.3.

Table 1.3. Some drugs or compounds isolated from Chinese herbal medicines which follow the traditional uses

Plant Origin	Drugs or Compounds	Chemical Structures	Effects or Indications	Ancient Chinese Literature Recording Chinese Herbal Medicines with Same Effects and the Published Time
Artemisia annua L. [45,47]	Artemisinin		Anti-malaria	Zhou Hou Bei Ji Fang (Jin Danasty, AD 266-420)
Corydalis yanhusuo W.T. Wang [50,51]	Tetrahydropalmatine		Analgesic	Lei Gong Pao Zhi Lun (Nanchao Song Dynasty, AD 420-479)
Ligusticum chuanxiong Hort. [52]	Tetrahydropalmatine		Mmyocardial ischemia-reperfusion injury	Shen Nong Ben Cao Jing (Donghan Dynasty, AD 25–220)

Plant Origin	Drugs or Compounds	Chemical Structures	Effects or Indications	Ancient Chinese Literature Recording Chinese Herbal Medicines with Same Effects and the Published Time
Paeonia lactiflora Pall. [53,54]	Paeoniflorin		Analgesic	Shen Nong Ben Cao Jing (Donghan Dynasty, AD 25–220)
Epimedium breuicornum Maxim. [55,56]	Icariin		Osteoporosis	Shen Nong Ben Cao Jing (Donghan Dynasty, AD 25–220)
Pueraria lobata (Willd.) Ohwi [57]	Puerarin		Diabetes	Shen Nong Ben Cao Jing (Donghan Dynasty, AD 25–220)

Plant Origin	Drugs or Compounds	Chemical Structures	Effects or Indications	Ancient Chinese Literature Recording Chinese Herbal Medicines with Same Effects and the Published Time
Salvia miltiorrhiza Bunge [58,59]	Salvianolic acid B		Cardiovascular and cerebrovascular diseases	Shen Nong Ben Cao Jing (Donghan Dynasty, AD 25–220)
Uncaria rhynchophylla (Miq.) Jacks. [60]	Rhynchophy-lline		Antihypertensive	Shen Nong Ben Cao Jing (Donghan Dynasty, AD 25–220)
Saussurea lappa (Decne.) C.B. Clarke [61]	Costunolide		Anti-gastric ulcer, antispasmodic	Shen Nong Ben Cao Jing (Donghan Dynasty, AD 25–220)
Cast rodia dlata Bl. [62,63]	Gastrodin		Anti-convulsion, analgesic	Shen Nong Ben Cao Jing (Donghan Dynasty, AD 25–220)

1.5 Drugs Developed from Natural Products

In clinical practice in China in the 1960s, it was found that Schisandra chinensis (Turcz.) Baill, a traditional Chinese herb had obvious enzyme-reducing and hepatoprotective effects. Chinese scientists then began isolating the chemical constituents of S. chinensis. In the subsequent total chemical synthesis and pharmacodynamic study of schisandrin C (which is one of the compounds of S. chinensis), researchers found that the intermediate compound bifendate had a stronger pharmacological activity and that the cost of preparation was low. They discovered that it may be used to lower the enzyme content in the treatment of hepatitis B virus [49].

Since the end of the 1980s, chemists and pharmacologists at the Chinese Academy of Medical Sciences have been closely cooperating in studying the structure and activity relationships of bifendate and its analogs. As part of their research, a series of novel derivatives were synthesized. After screening using a number of chemical and pharmaceutical liver injury models, it was found that the hepatoprotective activities of the derivatives were closely related to the locations of dimethylenedioxy in two benzene rings, the length of the side-chain carboxylic acid, and the heterocycle between the two benzene rings. Finally, a new compound, bicyclol formulated as 4,4”-dimethoxy-5,6,51,61-bis(methylene-dioxy)-2-hydroxy-methyl-21-methoxycarbonyl biphenyl was designed and synthesized. Bicyclol had greater in vivo absorption, and better bioavailability and biological activity, than bifendate owing to the introduction of the 6-hydroxymethyl group and 61-carbomethoxy in the side chain [64]. Pharmacological results of bicyclol showed antifibrotic and hepatoprotective effects against liver injury and liver fibrosis induced by CCl_4 or other hepatotoxins in mice and rats; it also exhibited the antihepatitis virus effect in the 2.2.15 cell line and duck model with viral hepatitis [65,66].

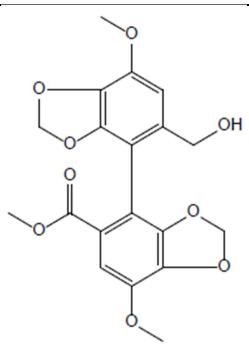
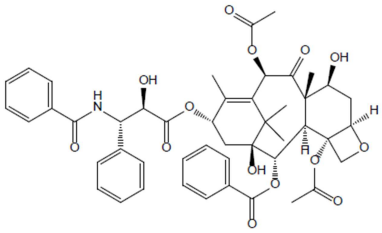
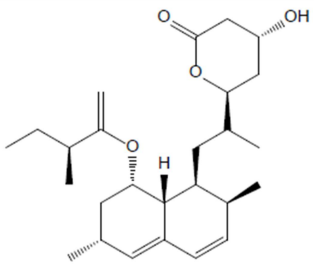
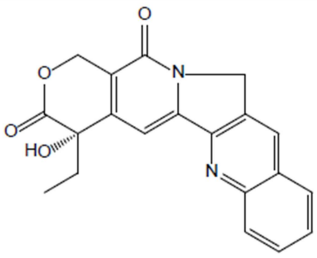
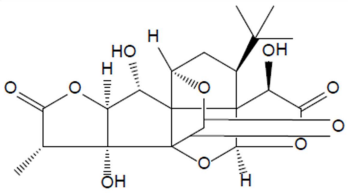
In clinical trials, it was found that the increased levels of serum alanine aminotransferase and aspartate aminotransferase were dramatically decreased by bicyclol. It was also found that bicyclol prohibited hepatitis B virus replication in chronic hepatitis B patients [67]. Compared with previous anti-hepatitis drugs, bicyclol exhibited a more consolidated effect after the drug was discontinued; the rebound rate was low, with fewer adverse reactions and higher oral bioavailability [68].

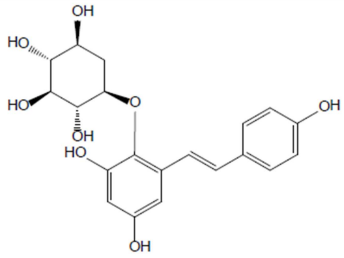
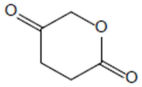
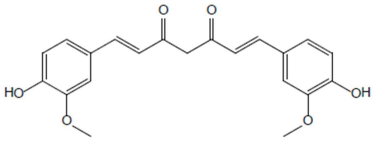
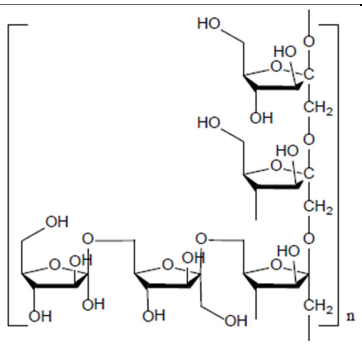
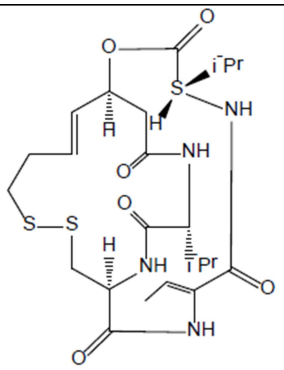
Based on previous studies in such areas as synthesis, pharmacology, toxicology, pharmacokinetics, preparation, and quality control, researchers determined that the new antihepatitis drug bicyclol offered significant hepatoprotective effects, antihepatitis virus activity, and fewer adverse reactions [49]. Bicyclol has been approved for the treatment of chronic viral hepatitis in China since 2004 [65]. Bicyclol has independent intellectual property rights and belongs to Class 1 of China's New Chemical Drug. The drug is one of the anti-inflammatory and hepatoprotective drugs recommended by the "Guidelines on Liver Disease Clinical Diagnosis and Treatment" in China, and it has been exported to many countries [49,68].

In the same decade in which Chinese scientists found that S. chinensis (Turcz.) Baill. had obvious enzyme-reducing and hepatoprotective effects, a program screening for cancer drugs from plants began in 1960 at the National Cancer Institute in the United States. Neither China nor the United States knew what the other was doing in this area. In that US project, 650 plant samples were gathered in three states. After the initial cytotoxicity tests were carried out using crude extracts, Taxus brevifolia was chosen for further research.

Taxol was isolated as a new compound from T. Brevifolia. Taxol has an unusual chemical structure and radically distinctive mechanism of action and was developed as a novel anticancer drug in subsequent decades. Nevertheless, the drug attracted little attention during the early stage of its development because of its poor solubility in water, low yield from natural products, and other disadvantages, particularly by the medical society. The story of Taxol involved many events that nearly resulted in discontinuation of the research. Fortunately, it underwent extraction, isolation, and structural determination; its activity against solid tumors and its mechanism of action were established, and it became developed for clinical practice. Finally, Taxol was approved by the US Food and Drug Administration for treating ovarian cancer in 1992—21 years after the initial breakthrough paper recording its isolation and structural identification. Taxol has remained a basic drug for treating various forms of cancer, and is still being used to develop new synergistic groups of anticancer drugs [69–71]. Some drugs or compounds isolated or developed from natural products are summarized in Table 1.4.

Table 1.4. Some drugs or compounds isolated or developed from natural products.

Origin (Plant, etc.)	Drugs or Compounds	Chemical Structures	Effects or Indication
Schisandra chinensis (Turcz.) Baill. [42,64 -68]	Schisandrin C, bicyclol, bifendate		Hepatoprotective, anti-hepatitis B virus bicyclol
Taxus breuifolia [69-72]	Taxol, docetaxel		Antitumor taxol
Aspergillus terreus [73]	Lovastatin		Hyperlipoidemia
Camptotheca acuminata Decne [2]	Camptothecin, irinotecan and topotecan		Antitumor camptothecin
Gimkgo biloba L. [74]	Ginkgolide B		Cerebral infarction

Origin (Plant, etc.)	Drugs or Compounds	Chemical Structures	Effects or Indication
Polygonum multiflorum Thunb. [75]	Stilbene glycoside		Vascular dementia
Ranunculus ternatus hunb. [76,77]	Ternatolide		Anti-tuberculosis
Curcuma longa L. [78]	Curcumin		Hypolipidemic
Ophiopogon japonicus (L.f.) Ker-Gawl. [79]	Polysaccharide MDG-1		Anti-myocardial cell injury
Chromobacterium violaceum [80]	Romidepsin		Antitumor

1.6 About the Works on *Wedelia chinensis* (Asteraceae)

1.6.1 The Plant Family-Compositae[81,82]

The Compositae or Asteraceae family is a very large plant family consisting of herbs and shrubs (e.g. Eupatorium) or rarely trees. The family comprises of about 11,000 genera and probably 8,000 species which are cosmopolitan. The members of the family can be found all over the world and in most habitats and climates. Some genera e.g. Wedelia are scabrid, pubescent or hirsute herbs or under shrubs. Leaves are alternate, rarely opposite or whorled, simple or compound; stipuls. Flowers all tubular (head discoid), or the outer, or all ligulate (head rayed), all bisexual female or neuter, some timesdiuecious. Corolla of 2 forms; 1st, tubular or companulate and 2nd, ligulale. Disk epigynous; stamens may be 4-5 and free or united.

Ovary 1-celled; style slender, 2-fid; arms sometimes linear, externally pubescent. Fruit (an achene) dry, indehiscant. Seed erect testa membranous, albumen O; embryostraight, cotyledous, plano-convex, radicle short-affinities.

Some of the Compositae species are reputed traditionally for different medicinal uses and these species have already proved to be potent anticancer, infective hepatitis, liver cirrhosis, jaundice, antimicrobial, anthelmintic, antipyretic, analgesic, antiinflammatory and diuretic agents[83,84,85]. In general, the essential oils of the plants in this family are anti-allergic, anti-fungal, anti-infectious, anti-septic and anti-inflammatory to the skin and the digestive system. They are calming to both mind and body so they are useful for cases of stress, insomnia, irritability and PMS[86].

1.6.2 Available Compositae Species in Bangladesh[87,88]

Compositae plants are found through out the Bangladesh. But wide varieties of species are found only in Dhaka, Mymensing, Tangail, Chittagong, Patuakhali and Barisal. The following species are identified in Bangladesh, listed in **Table 1.5.**

Table 1.5: Available Compositae species in Bangladesh

Sl. No	Genus	Species	Beng. Name
1.	Lamprechaenium	*Lamprechaenium microcephalum*	-
2	Elephantopus	*Elephantopus scaber*	Gojialata Shamdalan
3	Eupatorium	*Eupatorium odoratum* *Eupatorium triplonerve*	Assamlata Ayapan, Ayapana
4	Vernonia	*Vernonia cinerea* *Vernonia saligna* *Vernonia teres*	Kukshima, Kukursunnga
5	Centratherum	*Centratherum anthelminticum*	Somraj
6	Ageratum	*Ageratum conyzoides*	Fulkuri, Uchanti
7	Solidago	*Solidago virga-anrea*	-
8	Grangea	*Grangea maderespatana*	Namuti
9	Aster	*Grangea trineruus*	-
10	Eigeron	*Eigeron asteroids*	-
11	Blumea	*Blumea lacera* *Blumea eriantha* *Blumea densiflora* *Blumea balsamiflora*	Shialmutra Kukursunga
12	Pluchea	*Pluchea indica* *Pluchea lanceolata* *Pluchea pinnatifida*	Kukronda
13	Sphaeranthus	*Sphaeranthus indicus*	Murmuria Chagalnudi
14	Anaphalis	*Anaphalis neelgeriana*	-
15	Gnaphalium	*Gnaphalium Inteoalbum*	Borokamra
16	Inula	*Inula racemosa* *Inula graveolens* *Inula royeana* *Inula grantiodes*	-
17	Pulicaria	*Pulicaria crispa* *Pulicaria dysenterica*	-
18	Xanthium	*Xanthium strumarium*	Ghagra, Bichapal
19	Siegesbeckia	*Siegesbeckia orientalis*	-

Sl. No	Genus	Species	Beng. Name
20	Enhydra	*Enhydrafluctuans*	Helencha, Hincha shak
21	Eclipta	*Eclipta alba* *Eclipta erecta*	Keshuti, Kalokeshi, Bhimraj
22	Wedelia	*Wedelia chinensis* (Asteraceae) *Wedelia asperrium* *Wedelia glanca* *Wedelia trilobata* *Wedelia forsteriana* *Wedelia wallichii* *Wedelia hispida* *Wedelia prostrata* *Wedelia keatingii* *Wedelia calycina* *Wedelia scaberrima* *Wedelia hookeriana* *Wedelia buphthalmiflora* *Wedelia helianthoides* *Wedelia pinetorum* *Wedelia regis* *Wedelia rugosa* *Wedelia pludosa* *Wedelia biflora*	Keshraj, Bhimra, Kesuria
23	Spilanthes	*Spilanthes acmella* *Spilanthes acmella varoleuracea*	Marhatitga
24	Guizotia	*Guizotia abyssinica*	-
25	Helianthus	*Helianthus annuus*	Surjamukhi
26	Glossocardia	*Glossocardia bosvallia*	-
27	Bidens	*Bidens tripartita* *Bidens pilosa*	-
28	Glossogyne	*Glossogyne pinnatifida*	-
29	Achillea	*Achillea millefolium* *Achillea santolina*	-
30	Anthemis	*Anthemis gayana*	-
31	Chrysanthemum	*Chrysanthemum indicum* *Chrysanthemum coronarium*	-

Sl. No	Genus	Species	Beng. Name
32	Matricaria	*Matricaria chamomilla* *Matricaria lasiocarpa*	-
33	Tagetes	*Tagetes erecta*	Genda phool
34	Centipeda	*Centipeda orbicularis*	Mechitta
35	Cotula	*Cotula aurea* *Cotula anthemoides*	-
36	Tanacetum	*Tanacetum gracile* *Tanacetum fruticulosum*	-
37	Artemisia	*Artemisia scoparia* *Artemisia maritima* *Artemisia vulgaris* *Artemisia persica* *Artemisia absinthium* *Artemisia annua*	-
38	Tussilago	*Tussilago farfara*	-
39	Doronicum	*Doronicum hookeri*	-
40	Emillia	*Emillia sonchifolia*	Sadi modi
41	Notonia	*Notonia grandiflora*	-
42	Calendula	*Calendula officinalis*	-
43	Echinops	*Echinops echinatas*	-
44	Silybum	*Silybum mariamon*	-
45	Saussurea	*Saussurea obvallata* *Saussurea candicans* *Saussurea hypolenca* *Saussurea lappa* *Saussurea affins*	-
46	Jurinea	*Jurinea macrocephala*	-
47	Tricholepsis	*Tricholepsis glaberrima*	-
48	Centaurea	*Centaurea picrics*	-
49	Carthamus	*Carthamus tinctorius*	Kushum, Kajirah
50	Cichorium	*Cichorium intybus* *Cichorium endivia* *Cichorium noennum*	Kasni
51	Taraxacum	*Taraxacum officinale*	-

Sl. No	Genus	Species	Beng. Name
52	Lactuca	*Lactuca remotiflora* *Lactuca scariola*	-
53	Sonchus	*Sonchus oleraceus* *Sonchus arvensis* *Sonchus asper*	Bon palong
54	Carduus	*Carduus lanatus*	Silkanta
55	Volutarella	*Volutarella divaricate*	-
56	Senecio	*Senecio tenuifolias* *Senecio densiflolias*	-
57	Mikania	*Mikania cordata*	Assamlata
58	Tridax	*Tridax procumbens*	Tridhara
59	Adenostemma	*Adenostemma viscosum*	Buro-keshuti

1.6.3 Medicinal Significance of the Compositae Family[83,87-91]

The medicinal uses of some Compositae plants are listed in the Table 1.6.

Table 1.6: The medicinal uses of some Compositae plants

Sl. No	Species	Uses
1	*Ageratum conyzoides(L)*	Leaves and roots are antilithics and applied to cuts, sores and externally in ague. Whole plants are used in prolapsus
2	*Blumea lacera (Burm. f)*	Plants are aromatic, astringent, stomachic, antispasmodic and diuretic. Roots are antidysenteric and used in cholera. Leaves juice is anthelmintic, astringent, febrifuge, stimulant and diuretic.
3	*Carthamus tinctorius(L)*	Seeds are regarded as purgative and the oil is useful in rheumatism and paralysis. Roots are used in diuretic. Hot infusion of dried flowers is used in jaundice, nasal catarrh and muscular rheumatism. Cold infusion of flowers is laxative and tonic.
4	*Eclipta alba (L)*	Plants are a tonic, antipyretic, stomachic, pectoral, antiasthmatic and expectorant. Leaves juice is given in jaundice and fevers. Leaves are reputed to cure sores; used for checking hemorrhages and fluxes.

Sl. No	Species	Uses
5	*Elephantopus scaber(L)*	The herbs are diuretic, laxative, analgesic, febrifuge, cardiac tonic. Decoction of roots and leaves are used in diarrhoes, dysentery. Roots are used in fever and to arrest vomiting.
6	*Emilia sonchifolia(DC)*	Juice of leaves is used in eye inflammation, night blindness and sore throat. Roots are used for diarrhea.
7	*Enhydra fluctuans*	Leaves are laxative, antibilious, demulcent cure, inflamma-tion, bronchitis, useful in skin and nervous affectings.
8	*Eupatorium odoratum(L)*	Leaf infusions and decoctions are used in colds, influenza & fever. Decoction of flowers is used in cough & diabetes.
9	*Eupatorium triplinerve*	Decoction of plants and juice of leaves are used to clean foul ulcers. Decoctions of leaves are haemostatic and antiseptic.
10	*Gnaphalium luteo*	The leaves are used as an astringent and vulnerary. The tomentum is applied as couter irritant for gout.
11	*Grangea maderespana(L)*	Herbs are antipyretic. Leaves are stomachic, antispasmodic and used in preparing antiseptic. Roots are diuretic, antihelmintic and stimulant.
12	*Helianthus annus(L)*	Seeds are diuretic and expectorant; used in bronchial, laryngeal and pulmonary affection coughs and colds. Seeds are used in dysentery. Leaves are useful in malarial fever.
13	*Launaea sarmentosa*	The plants are given as a lactagogue. The juice of the plants is tonic, diuretic, aperient and used in rheumatic affections and as a soporific for children.
14	*Mikania cordata*	The leaves are used for itches and poulticing wounds. Leaves are useful in dyspepsia, gastric, ulcers and also efficacious in dysentery.
15	*Sonchus wightlanms*	Roots are given in jaundice, cough, bronchitis, asthma and pertussis. Leaves are used as application on swellings.

Sl. No	Species	Uses
16	*Sussaurea affinis*	The juice of the roots is given for female diseases.
17	*Spharanthus indicus(L)*	Roots are stomachic, barks are antihelmintic. Powdered seeds and roots are given as an antihelmintic. Flowers are credited with alterative, depurative and tonic properties . Rinds of the fruits are used as fish poison.
18	*Tagates erecta(L)*	Root extracts are credited with laxative properties. Leaves are used in kidney troubles, muscular pains. Leaves and flowers are emmenagogue.
19	*Tridax procumbens(L)*	The leaves are used to arrest bleeding in bruises, bronchial catarrh, dysentery and diarrhea. Leafs juice possess antiseptic, insecticides and parasiticidal properties.
20	*Vemonia patula (Merr)*	Seeds are alterative, leaves and plants are diaphoretic. Seeds are employed as antihelmintic and alexipharmic. Roots are used as on antihelmintic. The flowers are used in conjunctivitis, fever and rheumatism.
21	*Wedelia chinensis* (Asteraceae)	The leaves are used for dyeing grey hair, promoting hair growth and tonic & useful in cough, cephalalgia, diseases of skin, especially alopecia. It is also used irr enlarged liver and spleed. Decotions of the herbs are used in hepatitis-B, cancer, uterine haemorrhage and menorrhagia. The other minor uses are the juice is administered in combination with aromatics for catarrhal jaundice. The fresh plants are rubbed on the gums in toothache & applied with a little oil for relieving headache.
22	*Xanthium indicum*	The whole plants are diaphoretic, sedative, sudorific, diuretic and sialagogue; useful in malaria. Roots are useful in cancer and strumia. Fruit is cooling and demulcent. Xanthium is useful as a central nervous system depressant. Seeds are used for resolving inflammatory swellings.

1.6.4 Brief Chemistry of the Compositae Family [81,83,89]

Most of the plants of this family are regarded as medicinal plants, especially the genus are Wedelia, Eclipta, Elephantopus, Grangea and Xanthium. So obviously this family is prominent medicinal family. Chemical investigation has been done previously on various genera, notably Wedelia, Eclipta, Grangea, Aster, Pterocaulon, Artemisia, Conyza, Ixeris of this family. Research carried out on Compositae plants till present lime revealed the plants of this family possess many interesting, structurally varied secondary metabolites including alkaloids, terpenoides (novel type), steroids, flavonoides, coumarins, volatile oils, lactones (wedelolactones), acetylenic compounds, saponin, carotene, inorganic salts, and various type siliceous materials. Terpenoids are most probably the major and most wide spread group of compounds isolated from the Compositae and glycosides are the second.

In this context, a very short information about the chemistry of Compositae is given in the Table 1.7.

Table 1.7 : Brief information of constituents of Compositae family

Constituents		**Genera/Species**
A. Alkaloids	a. Simple isoquinolines b. Simple aporphines c. 7-Substiluted aporphines d. Oxo aporphines e. Phenanthrene ethylamine f. Indole type	a. Wedelia
B. Coumarins	a. Iso-coumarins b. p-Coumaric acid c. 5-Methylcoumarin deriv-atives d. p-Coumaric esters e. 6,7-Dioxygenated coumariiis	a. *Arfimi.sia dracwicnlus* b, Baccharis *a.* Chromolaena *h.* Conyza *c.* Helichrysum *d.* Mutisia *e.* Pterocaulan *f.* Gerbern

Constituents	Genera/Species	Constituents
C. Flavonoids	a. Centaureidin	*a. ICclipla erecta*
	b. Eupatolitin	*b. Eclipla erecta*
	c. Casticin	*c. Wedelia calendulacea*
	d, Eupatin	*d.* Baccharis
	e. Pututetin	*e.* Brickella
		f. Wedelia asperrima
D. Tcrpenoids	a. Monoterpenes	*a.* Artemisia
		b. Chrysan ihenmtn
	b. Sesquiterpenes	*a.* Artemisia
		b. Ambrosia
		c. Hymenollea
		d Ixeris
	C. Dilerpencs	*a. Granged maderaxpanata*
		b. Conyza
		c. Helianthus
		d. Wedelia
		e. Ichthyothere
	d. Triterpenes	*a.* Helianthus
		b. Helichrysum
	c. Tetraterpenoids	c. species of the family
E. Miscellaneous compounds	a. Acetylene compounds	*a. Dahlia pinala*
	b. Glycosides compounds	*b.* Aster

1.6.5 The Taxonomy of the Genus Wedelia[83,87]

The plants of genus Wedelia are scabrid pubescent or hirsute herbs or under shrubs. Leaves opposite, usually toothed. Heads axillary or terminal, heterogamous, radiate, yellow; ray-flowers female, fertile; disk-flowers hermaphrodite, fertile or the inner sterile. Involucre ovoid, campanulate, or subhemispheric; bracts sub-2-seriate, the exterior 3-5, usually herbraceous, or foliaceous, the inner dry or rigidly membranous. Receptacle flat or convex, furnished with folded or concave paleae embracing the hermaphrodite flowers. Corollas of female flowers ligulate, the ligules spreading, entre or 2-3 toothed; corollas of hermaphrodite flowers regular, tubular with an elongate 5-toothed limb. Another bases entire or sagittate with small blumt articles. Style-arms of hermaphrodite flowers short or elongate, with subacute hairy tips.

Achenes cuneate-oblong or obovoid, thick, laterally compressed or the outer triquetrous, tip rounded, margins obtuse or thickened. Pappus O, or a toothed cup or ring, or of short scales, with sometimes a few bristles - Distrib. Species about 65 tropical and warm temperate regions.

1.6.6 Botanical Features of *Wedelia chinensis* (Asteraceae)

Botanical name : *Wcdelia chinensis*

Kingdom : Plantac

Order : Asterales

Family : Asteraceae

Subfamily : Asteroideae

Tribe : Heliantheae

Subtribe : Ecliptinac

Genus : ***Wedelia***

Begali name : Kesharaj, Bhimraj, Bhimra, Bhringaraja, Pitabhringi, Bangra, Kesraj, Kesuria.

Identification : The plant meets all the criteria of a compositae species.

Diagnostic features : It possesses almost all characteristic features of compositae or Asteraceae.

Nature : *Wedelia chinensis* is a perennial herb, 0.3-0.9 m high; stem (6-18 inch) procumbent at the base and rooting at the lower nodes, glabrous or scabrid, terete, more or less appressedly hairy.

Habit and Habitat: An annual, erect, weak herb with elliptic leaves and terminal heads, grows in Dhaka, Mymensingh, Tangail, Patuakhali, Nijhum Dip, Barisal and sporadically in other areas.

Leaves : Leaves opposite, subsessile, 2.5-7.5 by 1-2.8 cm, Hnear-obling or oblanceolate, acute or obtuse, entire or irregularly sub-crenate, scabrous with short white hairs or at length more or less glabrate, base tapering. Heads 2-3.2 cm. diameter solitary; peduncles 2.5-15cm long, erect slender, slightly thickened beneath the heads.

Flowers : Flowers all tubular (head discoid), or the outer, or all ligulate (head rayed), all 2-sexual, or the inner 2-sexual or male, the outer female or neuter, some times diuecious. Involucral bracts herbaceous, oblong or slightly obovate, hairy, subobtuse, much longer than the disk- flowers. Ray flower ligulate, ligules yellow, 3-toothed. Style- arms of female flowers long, acute, recurved. Pappus 0 or a toothed membranous cup. Achcnes of the ray are tapering, slightly pubescent.

Fruit: Fruit (an achene) dry, indehiscent.

Seed : Seed erect, test a membranous, albumen O; embryo straight.

Distribution : In wet places, Bengal, Assam, Barisal, Patuakhali, Sylhet, Burma, Konkan, Plains districts of Madras presidency, Ceylon, Malay Islands, China, Japan etc., tropical and sub-tropical Assia and Africa.

1.7 Object of the Present Work

Medicine is an ancient art lather than modern science and for that "Back to nature" is a slogan of recent years. With centuries of tireless efforts scientists have found in plants the remedy of diverse diseases ranging from simple skin infection to complicated cancer. To achieve the target of getting new bioactive principles, research on medicinal plants is of great importance.

In the present investigation, a compositae plant was selected because the members of the family can be found all over the world and in most habitats and climates that known to produce diverse classes of pharmacologically active compounds. Some of the compositae species are reputed traditionally for different medicinal uses and these species have already proved to be potent anticancer, infective hepatitis, liver cirrhosis, jaundice, antimicrobial, anthelmintic, antipyretic, analgesics, anti-inflammatory and diuretic agents[84,85,87]. In general, the essential oils from the plants of this family are calming effect to both body and mind so they are useful for ceases of tress, insomnia and irritability[86].

Wedelia is a genus of compositae family. A lot of phytochemical works so far have been done of this genus with little biological works but the plant *Wedelia chinensis* (Asteraceae) belonging to the genus 'Wedelia' has little phytochemical work and no

antibacterial and antifungal activities tests of some compounds so far isolated from this plant has been reported. This plant has very good folk medicinal use in the treatment of liver enlargement, jaundice and other ailments of the liver and gall bladder and also in the treatment of uterine haemorrhage and menorrhagia. The leaves of the plant are used for dyeing grey hair, promoting hair growth and tonic and useful in cough, cephalalgia, diseases of skin, especially alopecia. The fresh plants are rubbed on the gums in toothache & applied with a little oil for relieving headache. It is reported to use against inflammation, cancer and hepatitis B[84,85,87,91-93].

Thus, in order to isolated bioactive principles as well as to evaluate various biological screening such as *in vitro* antibacterial and antifungal activities, minimum inhibitory concentrations (MIC), brine shrimp lethality bioassay and subacute toxicity studies of the isolated compounds, the present study was under taken on the plant *Wedelia chinensis*, which ultimately led to the proper use of this plant for better health care system of common people of Bangladesh.

1.8 Present Study Protocol

Present study was designed to isolate pure compounds as well as to determination of structure and observe biological activities of the isolated pure compounds along with crude extracts of *Wedelia chinensis* (Asteraceae). Major steps involved are:

1. Extraction of the dried powder of whole plant of *Wedelia chnensis* by cold extraction method.
2. Fractionation of the methanol extract with petroleum ether, chloroform, ethyl acetate. Another faction was made by passing the aqueous methanol extract through Dia-ion resin.
3. Determination of total phenolic and flavonoid content of the methanol extract and its different fractions (petroleum ether, chloroform, ethyl acetate and Dia-ion resin fractions) by Folin-Ciocalteu reagent and Aluminium chloride respectively.
4. Isolation and purification of the pure compounds obtained by Column Chromatography, Thin Layer Chromatography (TLC) and Preparative Thin Layer Chromatography (PTLC).
5. Observation of physical and chemical properties of the isolated compounds.

6. Determination of structures of the isolated pure compounds with the help of UV, IR, ^{1}H-NMR, ^{I3}C-NMR data.
7. Evaluation of *in vitro* anticholinesterase activity using Ellman's coupled enzyme assay.
8. Determination of the total antioxidant activity by the method of Prieto *et al*.,(1999) and reducing power capacity by the method of Oyaizu *et al.*, (1986).
9. Observation of *in vitro* radical scavenging activity using DPPH assay and hydroxyl radical scavenging assay.
10. Observation of *in vitro* lipid peroxidation inhibition activity by the method of Liu *et al*., (2000).

MATERIALS AND METHODS

2.1 Phytochemical study on *Wedelia chinensis*

Generally the following methods are used throughout the experimental work

- Collection and proper identification of the plant sample
- Preparation of the plant material
- Extraction by methanol
- Solvent-solvent partitioning of the crude methanol extract
- Determination of total phenolic content in different fractions
- Determination of total flavonoids in different fractions
- Thin layer chromatography(TLC) of ethyl acetate and dia-ion resin fractions
- Column chromatography(CC) of ethyl acetate and dia-ion resin fractions
- Isolation and purification of compounds
- Characterization of compounds
- Determination of R_f values of isolated compounds by TLC

2.2 Reagents and Solvents

All the reagents and chemicals were used for the presence work were purchased from THOMAS BAKER (MUMBAI, INDIA), BDH (ENGLAND), FLUKA (SWITZERLAND) and E. MERCK (GERMANY). Commercial alcohol (rectified spirit) and absolute alcohol were available from Carew and company, Darsana, Chuadanga. The solvents used mainly in this work were benzene, acetone, tetrahydrofuran (THF), ethyl acetate, chloroform, n-hexane, petroleum ether, methanol, absolute alcohol, toluene etc. The solvents were dried and distilled when necessary.

2.3 Purification of Solvents and Reagents

2.3.1 Solvent Purification and Drying

The purity of solvent is extremely important in chromatographic analysis as well as for crystallization purposes. The methods by which these were purified and dried are described below.

Benzene (C_6H_6):

One liter of analytical grade benzene, free from thiophene was taken in a clean dry bottle. About 150-200g of anhydrous calcium chloride was added to it. The mixture was allowed to stand for at least 24 hours with occasional shaking. Benzene was then filtered through a large fluted filter paper into another clean and dry bottle.

Fine sodium wire (7g) was then introduced directly into the benzene with the aid of a sodium press. The bottle was then closed by a rubber stopper carrying a drying tube (calcium chloride tube) and benzene was allowed to stand for about 24 hours. The bottle was then closed with a rubber stopper and preserved for use.

Ethanol (CH_3CH_2OH):

A dry 2 liter round bottom Pyrex flask fitted with a double surface reflux condenser and an anhydrous calcium chloride tube (drying tube) were taken. Clean, dry magnesium turnings (5 g) and 0.5 g of iodine were placed in the flask. About 75 mL of ethyl alcohol were added through the condenser. The mixture was warmed until iodine disappeared. Nine hundred mL of the absolute alcohol were then added and the mixture was refluxed for 30 minutes. The product was then distilled off with the exclusion of moisture. The first 25 mL of the distillate were discarded.

Acetone (CH_3COCH_3):

The analytical reagent grade acetone contains about 1% water. Commercial acetone was purified first by refluxing with successive quantities of potassium permanganate until the violet color persisted. It was then dried with anhydrous potassium carbonate (or anhydrous calcium sulphate), filtered from the desiccant and fractionated. (Precautions were taken to exclude moisture). It was preserved for crystallization purposes.

Methanol (CH_3OH):

To each 500 mL of methanol (CH_3OH), it was added CaO (50 g) and it was kept overnight. Then methanol was decanted from CaO and reflux it 5-6 h using $CaCl_2$ guard tube. After reflux, it was distilled and collected to a round bottomed flask. A white cake of Mg-turnings was formed in another round bottomed flask and the whole mass of methanol was added to it. Again, it was refluxed for 4 h, distilled out and collected it into an airtight container.

Chloroform ($CHCl_3$):

About 250 mL of $CHCl_3$ was taken in each time in a R. B. flask and CaH_2 was added slowly to it. Then the whole amount of $CHCl_3$ was stirred with an efficient magnetic stirrer at about 4-6 h. After that, chloroform was decanted from CaH_2 and distilled it out. This distilled $CHCl_3$ was collected and was kept into an airtight container.

Petroleum Ether:

To each 500 mL of petroleum ether, it was added anhydrous $CaCl_2$ (20 g) and kept overnight. Then petroleum ether was decanted from $CaCl_2$ and distilled it. This pure petroleum ether was then collected into an airtight container.

Acetic acid (CH_3COOH):

About 100 mL of acetic acid was taken in a R.B. and 50 g. of $CaCl_2$ (anhydrous) was added to it. After 6 h. then acetic acid was decanted and it was distilled and collected at reduced pressure by maintaining its b. p. 118.1°C.

Ethyl Acetate ($CH_3COOCH_2CH_3$):

About 500 mL Ethyl Acetate was taken in each time into a separating funnel and it was washed with 1N $NaHCO_3$ (3 × 100 mL) to remove trace amount of acetic acid from it. Later it was washed with water (3 × 100 mL) and dried over anhydrous $CaCl_2$. Finally, this ethyl acetate was distilled out and collected into an airtight container.

Absolute Diethyl Ether (CH_3CH_2-O-CH_2CH_3):

To 50 mL of diethyl ether, added KOH pellets (50 g) and it was kept overnight. After that, diethyl ether was decanted to another R. B. and it was refluxed for 5-6 h under N_2 prior. After reflux, it was distilled and collected into an airtight container. Addition of Na-metal with little benzophenone tested the presence of water molecule. Deep-blue coloration indicated the absence of water in diethyl ether.

Toluene (C_6H_5-CH_3):

About 500 mL toluene was taken in a Dean-stark apparatus and was refluxed for 5-6 hours. After reflux, it was distilled and collected to an airtight container. To detect the trace amount of water, Na-metal with little benzophenone was added to it. Formation of deep blue colour indicated the absence of water in it.

2.3.2 Diazotized Sulphanilic acid:

Sulphanilic acid (12.5 g) was dissolved in 10% aqueous potassium hydroxide (KOH) solution (60 mL) and was cooled in ice bath and 10% $NaNO_2$ solution was added to it. In another vessel, 20 mL of hydrochloric acid (density 1.18) was added to 10 mL of distilled water, which was kept in ice water bath. The previously cooled solution was added drop wise in the cold hydrochloric solution with constant stirring keeping the temperature below 8°C. The solid diazonium salt formed which was filtered, washed successively in ice-water, ethanol and ether and then dried at room temperature. The salt was impregnated with a few drop of acetic acid and kept in a refrigerator. It was stable for more than two months.

At the time of use, about 100 mg. of the diazotized sulphanilic acid was dissolved in 50 mL of 10% sodium carbonate solution. The resulting solution was used as the spraying reagents.

2.3.3 Dragendorff's Reagents (E. Merk, 1978):

(i) Dragendorff's reagent for alkaloids and other nitrogen containing compounds:

Solution a: Bismuth (III) nitrate, $Bi(NO_3)_2$ (1.7 g.) and tartaric acid (20 g.) were dissolved in water (8 mL) to give solution a.

Solution b: Potassium iodide, KI (16 g.) was dissolved in water (40 mL) to give solution b.

Stock solution: Equal parts of a and b were mixed together before use to give fresh Dragendorff's reagent. The solution is stable for several months, if refrigerated.

Spray solution: Tartaric acid (10 g.) was dissolved in 50 mL water and the stock solution (10 mL) was added to it.

(ii) Dragendorff's reagent for polyethylene glycols Polyethylene glycol ethers and polyethylene glycol esters:

Solution a: 1.7 g. bismuth (III) nitrate, $Bi(NO_3)_2$ was dissolved in a mixture of 20 mL glacial acetic acid and 80 mL water, A solution of 40 g. potassium iodide in 100 mL

water and 200 mL glacial acetic acid was added to it was then the resulting solution was made up to 1000 mL with required amount of water.

Solution b: 20% aqueous barium chloride ($BaCl_2$) solution.

Spray solution: 2 Parts of a and 1 part of b were mixed before use to give fresh Dragendorff's reagent.

(iii) Dragendorff's reagent for quaternary bases:

8.0 g bismuth (III) nitrate, $Bi(NO_3)_2$ was dissolved in 20-25 mL 25% nitric acid. This solution was added slowly with stirring to the slurry of 20 g potassium iodide and 1 mL 6N hydrochloric acid and 5 mL water.

Water was added to the dark precipitate until and orange-red color developed. The volume of the solution should be 95 mL. Any solid residue present was filtered off and then the solution was made up to 100 mL with water. The solution is stable for several weeks in the refrigerator when stored in an amber flask.

Spray solution: 20 mL hydrochloric acid, 2 mL stock solution and 6 mL 6N sodium hydroxide solution were mixed together. In case of bismuth hydroxide was not completely dissolved by shaking, several drops of 6N hydrochloric acid are added.

2.3.4 Vanillin-Sulphuric acid Reagent:

Sulphuric acid (400 mL) and absolute alcohol (100 mL) were mixed in a beaker (Kept in ice bath), 0.25g vanillin was added to the mixture of alcohol and sulphuric acid, cooled and used for spraying the plates, steroids, terpenoids and lignins were visualized either as a violet or a pink spot.

2.3.5 Liebermann-Burchard Reagent:

5 mL of acetic anhydride was mixed carefully with 5 mL of 98% H_2SO_4 acid and cooled. This mixture was added to 50 mL absolute ethanol with cooling. The resulting was used for testing steroids.

2.3.6 Anisaldehyde Sulfuric acid:

0.5 mL of anisaldehyde was dissolved in 50 mL of glacial acetic acid. Then 1 mL of 97% H_2SO_4 acid was added carefully. This solution was employed for testing steroids.

2.3.7 Modified anisaldehyde-Sulfuric acid:

0.5 mL of anisaldehyde was added to the solution of 9mL of ethanol and 0.1mL of acetic acid. Then 0.5 mL of 97% sulfuric acid was added cautiously. The solution was applied for testing steroids.

2.3.8 Bromocresol Green for acids (Spray solution for Paper Chromatogram):

50 mg of bromocresol green was dissolved in 50 mL of absolute ethanol. The solution was used to spray the paper chromatogram.

2.3.9 25% Pb-Acetate Solution (Spray Solution for Paper Chromatogram):

25 g of lead-acetate was dissolved in water and the total volume of the solution was increased to 100 mL by adding water. The resulting solution was employed to spray the paper chromatogram.

2.3.10 Mayer's Reagent:

A solution of mercuric chloride $HgCl_2$ (1.35 g.) in distilled water (60 mL) was poured into a solution of potassium iodide (5 g.) in distilled water (10 mL). The volume of the solution was made up to 100 mL by adding required amount of distilled water.

2.3.11 2,4-Dinitropheneyl Hydrazine Reagents:

2,4-Dinitrophenylhydrazine was suspended in 100 mL of methyl alcohol and 4.0 mL of conc. Sulphuric acid was then added cautiously and slowly. The mixture became warm and the solid was dissolved producing a clear solution.

2.4 General Methods

2.4.1 Distillation:

During the present work solvents were purified prior to use by distillation at the boiling point of the respective solvents. Impurities of solvents were removed by methods available in the literature.

2.4.2 Evaporations:

Evaporation of solvents form the extracts and other solutions were carried out on a Rotary Vacuum Evaporator under reduced pressure at bath temperature not exceeding 40^0C.

2.4.3 Crystallization:

Crystallization was employed as a final purification process where possible. The solvent was chosen in which the compound was least soluble. The compound was dissolved in a minimum volume of a solvent in hot condition and was left undisturbed for crystallization. Some times mixture of solvents was used.

2.4.4 Melting Point:

A Gallenkamp melting point apparatus was used for determination of the melting point of the substances. The heating was done carefully to ensure a steady rise of temperature.

2.4.5 Infra-Red (IR) Spectra:

Infra-red spectra were recorded on a JASCO A-302 IR SPECTROPHOTOMETER.

2.4.6 ^{1}H-NMR Spectra:

^{1}H-NMR spectra were recorded using a 300, 400 and 500 MHz BRUKER AMX-300 FT NMR, AMX-400 FT NMR, AMX-500 FT NMR Spectrometers using TMS as internal standard.

2.4.7 ^{13}C-NMR Spectra:

^{13}C-NMR spectra were recorded using a 300, 400 and 500 MHz BRUKER AMX-300 FT NMR, AMX-400 FT NMR, AMX-500 FT NMR Spectrometers using TMS as internal standard.

2.4.8 Mass Spectra:

The mass spectra were recorded on a Varian-MAT 112S and Finnigan MAT-112 and 312A double beam mass spectrometers connected to DEC PDP 11/34 and IBM-AT compatible PC based system. Electron Impact (EI), peak matching and Fast Atom Bombardment (FAB) experiments were performed on a MAT-312A or a Jeol-JMS HX-110 mass spectrometers. FAB-MS were recorded in glycerol-water (1:11) matrix in the presence of KI. High Resolution Electron Impact Mass Spectra (HR-EIMS) were recorded on a Jeol-JMS HX-110 mass spectrometer.

2.4.9 UV Spectra:

Ultraviolet spectra were recorded in methanol on a HITACHI UV-3200 spectrophotometer.

2.4.10 Optical rotation:

Optical rotation were measured on a digital Polari meter in methanol on a Jasco digital Polari meter (model DIP-3600).

2.5 Extraction and Isolation:

2.5.1 Material

The plant *Wedelia chinensis* (Asteraceae) (Family: sunflower) was selected for the Chemical and Biological investigations.

2.5.2 Collection of Plant materials

The whole plant was collected from 'Oushadye Pulle' in Nator district, Bangladesh, in March, 2012.

2.5.3 Preparation of plant material

The collected plants were first washed with water to remove adhering dirt and then shade dried for several days. The dried plant materials were ground into coarse powder by a grinding machine, (Model: FFC DISK MILLS, JIMO QINGDAO RXCO PRECISION MACHINERY CO. LTD. CHINA) in the Organic Research Laboratory department of Chemistry, University of Rajshahi, Rajshahi, Bangladesh.

2.5.4 Determination of water content:

100g of fresh plant materials were taken for the determination of water content. The fresh plant material (100g) were dried at room temperature and the dried material were weighted again and that was 39.5g. Therefore, the water content of the plant of *Wedelia chinensis* was calculated as fallows.

$$\therefore \text{ Water content} = \frac{(100 - 39.5) \times 100}{100} = 60.5\ \%$$

2.5.5 Determination of Moisture:

Dried ground of *Wedelia chinensis* plants (W_1=1.802g) were heated at 105^0C until a constant weight was reached (W_2 = 1.632g) and the moisture content was determined.

$$\text{Moisture content} = \frac{W_1 - W_2}{W_2} \times 100 = \frac{1.802 - 1.632}{1.632} \times 100 = 10.41\%$$

2.5.6 Determination of dry Matter:

Dried ground *Wedelia chinensis* plants contain 60.5% water content and 10.41% moisture content. Thus the dry matter of *Wedelia chinensis* given below:

Dry matter = 100 – (60.5+ 10.41) = 29.09 %

Table 2.1 : Some constituents hole part of *Wedelia chinensis*.

SL. No	Parameter	Quantitative (%)
1.	Water	60.5
2.	Moisture	10.41
3.	Dry Matter	29.09

2.5.7 Process of Extraction:

Dried ground plant of *Wedelia chinensis* were exhaustively extracted with methanol (MeOH, Analytical Grade, BDH Laboratory Supplies) in soxhlet apparatus. The resulting juicy extract was filtered through Whatman paper No.1 and concentrated under reduced pressure at 40°C using the Buchi Rotavapor R-200 to obtain a crude residue (23.5%). The process have done for several time to increase the crude extract. After evaporation of the methanol solvent the extracted was passed through a previously well packed dia-ion resin column which has selectivity to collect only the phenolic group containing

Fig-2.1: Soxhlet apparatus

compounds. Then the materials, which were bound in resin column, were collected by passing methanol solvent. Another portion of the crude methanol extract (CME) was triturated with petroleum ether, chloroform and ethyl acetate respectively. Finally petroleum ether, chloroform and ethyl acetate triturate were collected and evapoated.

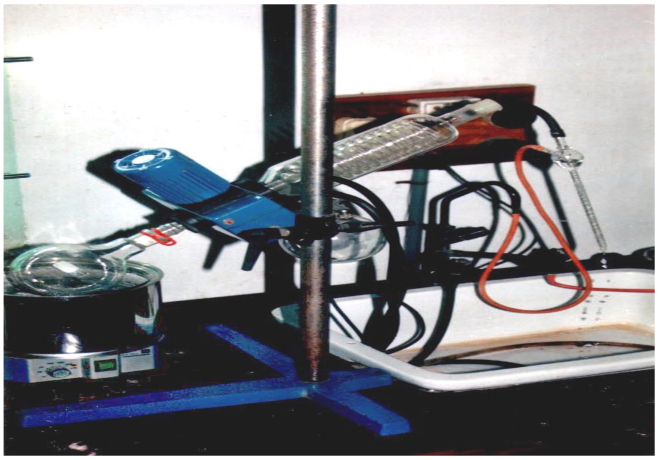
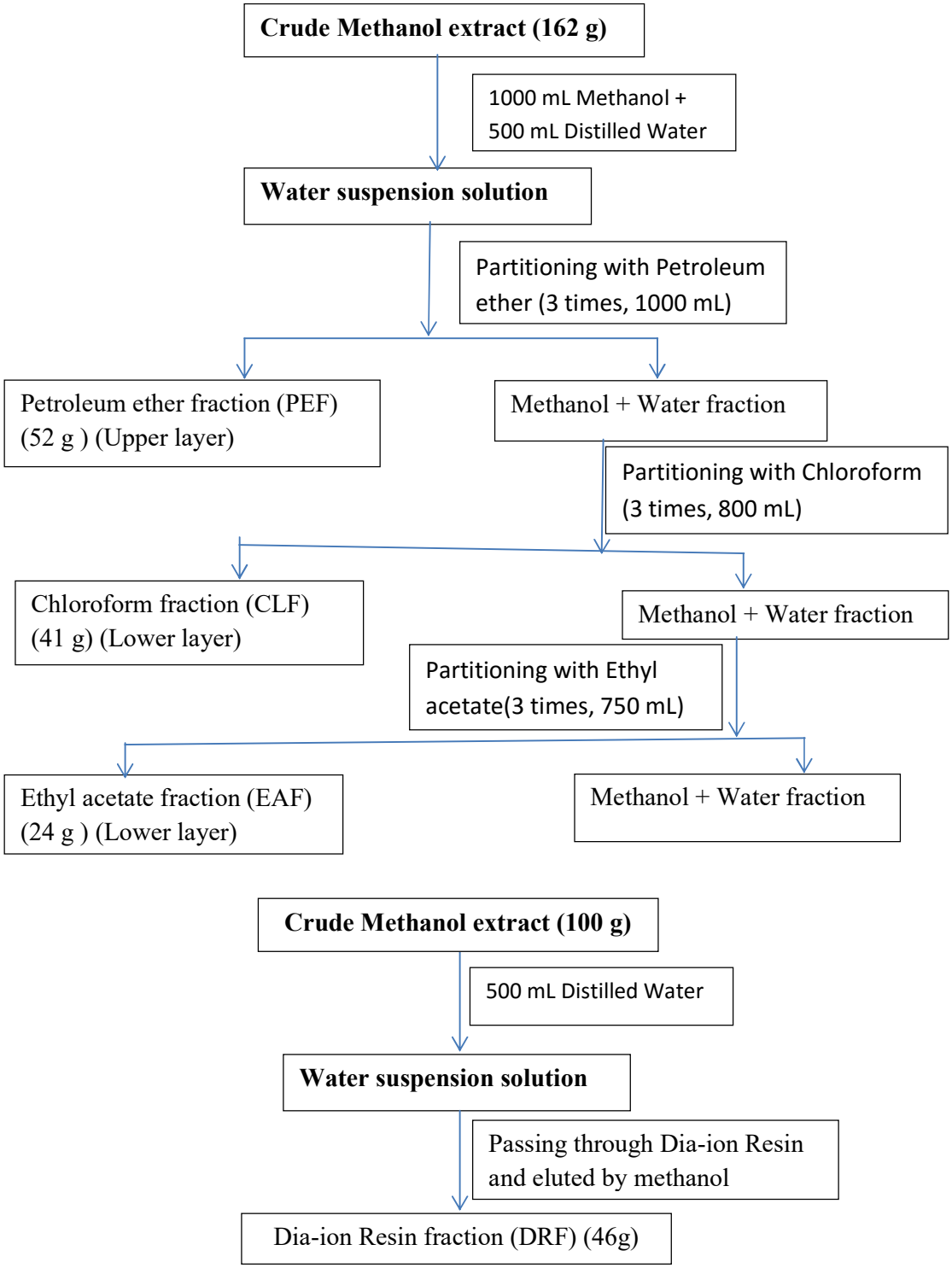


Fig 2.2: Rotatory evaporator

The obtained from the above process were used for further separation and biological activities measurements. The amount of different fractions were petrolium ether (52g), chloroform fraction(41g), ethyl acetate fraction (24g) and dia-ion resin fraction (46g) were obtained and used for the experiment purpose.

The whole extraction process is shown in the following flow chart.

FLOW CHART OF PLANT EXTRACTION



2.5.8 Phytochemical test of different fractions:

The petroleum ether, chloroform, ethyl acetate and dia-ion resin factions of *wedelia chinensis* were used for the phytochemical analysis for the identification of various classes of chemical compounds using the standard protocol.

Test for Saponins:

Frothing test: About 0.1g of different fractionates of the plant materials was taken vigorously with water. Production of a persistent frothing (which remains stable on heating) was the indication for the presence of saponins.

Test for Glycosides:

General test: A small amount of different fractionates of the plant materials was taken in a test tube and was dissolved in 1 mL of water. A few drops of aqueous sodium hydroxide solution were then added to the test tube. Development of yellow color was the indication for the presence of glycosides.

Test for Tanins:

Lead acetate test: About 5 mL of aqueous solution of different fractionates of the plant material was taken in a test tube and a few drops of a 1% solution of lead acetate were added to the test tube. A yellow or red precipitate was the indication for the presence of tannins.

Test for Steroids:

Libermann-Burchard's test: A small amount of different fractionates of the plant materials was dissolved in 1 mL of chloform, 2 mL of acetic anhydride and 1 mL of concentrated sulphuric acid were added to the solution. Formation of a greenish color which terns blue on standing was the indication for the presence of steroids.

Test for Alkaloids:

Color test: About 0.5 g of the extract was stirred with 5 mL of 1% hydrochloric acid on a steam bath and was filtered. 1 mL of the filtrate was treated with a few drops of Dragendorff's (Bismuth potassium iodide solution) reagent. Formation of orange-red precipitate was the indication for the presence of alkaloids.

2.5.9 Phytochemical Result:

Table 2.2: Phytochemical screening of crude methanol and its four fractions of *Wedelia chinensis.*

Phytochemical constituents	Crude Methanol extract	Petroleum ether fraction	Chloroform fraction	Ethyl acetate fraction	Dia-ion Resin fraction
Saponins	−	−	−	−	−
Tannins	+	−	−	−	+ + +
Glycosides	++	+	+	++	+ +
Steroids	+ +	+ +	+	+ +	++ +
Alkaloids	+ + +	−	+ +	+	+ + +

Here, + = Present in the mild amount, + + = Present in the moderate amount, + + + = Present in the large amount, − = Not present.

Results obtained for qualitative screening of phytochemical components in the different extract on different solvent extracts of *Wedelia chinensis* are presented in Table-2.2.

Crude methanol extract showed the presence of alkaloid in large amount, steroids in moderate amount, whereas glycosides and tannins in mild amount and crude methanol extracts showed the absence of saponins. Petroleum ether extracts of *wedelia chinensis* exhibited a moderate amount of steroids and glycosides in mild amount whereas saponins, tannins and alkaloids were absent. The chloroform extracts showed the presence of alkaloids in moderate amount, steroids in mild amount whereas glycosides, tannins and saponins were not found to be present in case of chloroform extracts. In case of ethyl acetate extracts, showed the presence of steroids and alkaloids were found in mild amount but saponins, tannins and glycosides were absent. Finally, dia-ion resin fraction of *Wedelia chinensis* exhibited a large amount of tannins, alkaloids and steroids whereas glycosides in moderate amount.

The presences of these phytochemical constituents are found to be comparable to the work done by us of *Wedelia chinensis* (Khyade and Vaikos, 2009; Misra *et al*., 2011).

2.6 Determination of total phenolic content of different fractions and crude extract.

Total phenolic content of the different extractives of *W. chinensis* were determined employing the method as described by Singleton *et al.,* (1965) involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard.

Principle

The content of total phenolic compounds of different fractions in the plant was determined by Folin–Ciocalteu Reagent (FCR). The method actually measures a sample's reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotungstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})_4$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI):

$$Mo\ (VI) + e^- \rightarrow Mo\ (V)$$

Materials

- Folin – ciocalteu reagent (Sigma chemical company, USA)
- Sodium carbonate (Sigma chemical company, USA)
- Methanol (Sigma chemical company, USA)
- Catechin (Wako pure chemicals Ltd., Japan)
- Micropipette (10-100 µL)
- Pipette (1-10 mL)
- UV-spectrophotometer (Shimadzu, USA)

Experimental procedure

1. 0.5 mL of plant extract or standard of different concentration solution was taken in a test tube.
2. 2.5 mL of Folin–Ciocalteu (Diluted 10 times with water) reagent solution was added into the test tube.

3. 2.5 mL of Sodium carbonate (7.5%) solution was added into the test tube.
4. The test tube was incubated for 20 minutes at 25ºC to complete the reaction.
5. Then the absorbance of the solution was measured at 760 nm using a spectrophotometer against blank.
6. A typical blank solution contained all reagents except plant extract or standard solution.
7. The total content of phenolic compounds in plant methanol extract and in different fractionates in catechin equivalents (CE) was calculate by the following formula,

$$C = \frac{c \times V}{m}$$

Where,

C = Total content of phenolic compounds, mg/g plant extract, in CE;

c = The concentration of catechin established from the calibration curve, mg/mL;

V = The volume of extract, (mL);

m = The weight of different pure plant extracts, (g).

2.7 Determination of total flavonoids content of different fractions and crude extract

Total flavonoid content of the different extractives of *W. chinensis* was determined by aluminum chloride colorimetric method. Catechin was used as standard and the flavonoid content of the extractives was expressed as mg of catechin equivalent/g of dried extract.

Principle

The content of total flavonoids in different extractives of plant extract was determined by the well known aluminum chloride colorimetric method. In this method aluminum chloride forms complex with hydroxyl groups of flavonoids present in the samples. This complex has the maximum absorbance at 510 nm

Materials

- Aluminum Chloride (Sigma chemical company, USA)
- 5% $NaNO_2$

- 1mM NaOH
- Methanol (Sigma chemical company, USA)
- Catechin (Wako pure chemicals Ltd., Japan)
- Micropipette (10-100 µL)
- Pipette (1-10 mL)
- UV-spectrophotometer (Shimadzu, USA)

Experimental procedure

1. 0.50 mL of plant extract or standard of different concentration solution was taken in a test tube.
2. 3 mL of methanol was added into the test tube.
3. 200µL of 10`% aluminum chloride solution was added into the test tube.
4. 200 µL of I M potassium acetate solution was added into the test tube. Then 5.6 mL of distilled water was added into the test tube.
5. The test tube was then incubated at room temperature for 30 minutes to complete the reaction.
6. Then the absorbance of the solution was measured at 420 nm using a spectrophotometer against blank.
7. A typical blank solution contained all reagents except plant extract or standard solution.
8. The total content of flavonoid compounds in plant extracts in Catechin equivalents was calculated by the following formula equation,

$$C = \frac{c \times V}{m}$$

Where,

C = Total content of flavonoid compounds, mg/g plant extract, in catechin equivalent (CE);

c = The concentration of catechin established from the calibration curve, mg/mL;

V = The volume of extract, mL;

m = The weight of different pure plant extracts, g.

2.8 Chromatographic Analysis

Two types of chromatographic techniques used are as follows.

A. Thin layer chromatography (TLC)

B. Column chromatography (CC)

2.8.1 A. Thin Layer Chromatography (TLC)

Thin layer chromatography is one of the most popular and widely used separation techniques for the detection of organic compounds in a mixture, which involves an adsorbent (usually silica gel, alumina) as stationary phase and a solvent or solvent mixture as a mobile phase and a support of glass plate.

Preparation of plate:

For the preparation of plates, a number of glass plates measuring 5cm × 20cm are throughly washed to remove any dirt or fatty material present and are dried at 100°C for 5-8 minutes. The plates are then placed over aligning tray specially made for TLC. A slurry made of silica gel (1.5 g/plate) and distilled water (2 mL/gm. of silica gel) is uniformly distributed over the plate with the help of a spreader. Before distribution, the plates are washed with cotton soaked in acetone to remove fatty materials. The plates are then allowed to stand for sometimes to dry and activated at a temperature of 100°C for one hour (Grafton D. *et al*.)

2.8.2 Development of chromatograph:

Cylindrical glass chamber (TLC tank) with airtight lead is used for the development of chromatoplates. The selected solvent system (20 mL) is poured into the tank and a smooth sheet of filter paper is laid and allowed to soak in the solvent. The tank is then made airtight and kept for few minutes to saturate the internal atmosphere with the solvent vapour. A small amount of dried extract is dissolved in a suitable solvent to get a solution (approximately 1% (Harbone, J.B. “Encyclopedia of Plant physiology”Vol. 8). A small spot of the solution is applied on the activated silica plate with a capillary tube just 2 cm above the lower edge of the plate. The spot is dried with a hot air blower.

The spotted plate is then placed in the tank in such a way as to keep the applied spot above the surface of the solvent system and the cap is placed again. The plate is left

for development. When the solvent front reaches up to a certain limit, the plate is taken out and air-dried (Harbone, J.B. Phytochemical Methods: "A guide to modern technique of Plant Analysis." Touchstone *et al.,* "Practice of Thin Layer Chromatography."). The properly developed plates are viewed under UV light of various wavelengths as well as treated with suitable spray reagents in order to detect the different class of compounds.

2.8.3 B. Column Chromatography (CC)

The technique of column chromatography was used to separate the individual components from a mixture having different R_f values. The chromatographic column was prepared by slurry method using silica-gel 60 (70-230 mesh, E-MERCK) as the stationary phase and freshly distilled water were used for elution. Eluent were collected and examined by TLC to monitor the separation.

Preparation of Column:

The chromatographic column was packed with 400 g silica gel (Silica gel 60-120), the most commonly used stationary phase. Silica gel was suspended in a suitable solvent, n-Hexane. It was then poured into a column of appropriate height and diameter (height 72 cm and diameter 5.5 cm). When a desired height of adsorbent bed was obtained a few hundred milliliter of solvent mixture was run for proper packing of the column. After packing the sample was applied to the top of the column.

Preparation of sample:

The 20 g semisolid mass of chloroform fraction of *W. chinensis was* mixed well with 40 g of silica gel in a mortar with pestle in order to obtain a nonstick free flowing mass. The amount thus obtained was placed on the packed column carefully in such a way that upper layer of the bed was not disturbed.

2.8.4 Thin layer chromatography (TLC):

Thin layer chromatography provides a means of separation, isolation, purification and identification of mixture of organic compounds, which involves an absorbent (usually Silica-gel) as stationary phase and a solvent or solvent mixture as the mobile phase. Due to the differential absorption into the absorbent, the components of the mixture use to migrate differentially along the TLC plate or in other words, due to the

difference in mobility at the components, the solvents separate them from each other. Generally, less polar solvents to more polar solvents were used. Two types of thin layer chromatography (TLC) plates were used.

(i) Pre-coated TLC plates: 0.20 mm thin coating on aluminum sheets.

(ii) Manually prepared glass plates.

2.8.5 Procedure for the preparation of TLC Plates

Thin layer chromatography was carried out on glass plates (20×5 cm). The glass plates were cleaned with soda water to remove greasy substances, washed with distilled water and acetone. The cleaned glass plates were dried in an electrical oven and placed on a thin layer chromatographic template. The plates were tightened with the lever and the spreader was placed in position. A suspension of silica-gel GF_{254} 60 FLUK (30 g in 65 mL distilled water for 22 plates) was transferred to the open spreader set at 0.25 mm thickness.

The spreader slotted through with the suspension was spread over the glass plates and thus a uniform layer was deposited on the plates. The plates were then allowed to stand for twenty four hours for drying and were finally activated by heating in an oven (110°C) for 20 minutes. The activated plates were kept in desiccator on a drying rack.

2.8.6 Application of sample (Spotting the Plates)

For application of sample into the TLC plates, narrow glass capillary tubes were used. The capillary was washed either acetone or ethanol before each sample was applied.

Solvent systems: The solvents of different polarity were used for TLC are given bellow:

1. n-Hexane : ethyl acetate (in different ratio)
2. Petroleum ether: ethyl acetate (in different ratio)
3. Dichloromethane: methanol (in different ratio)
4. Chloroform: methanol (in different ratio)
5. Ethyl acetate: methanol (in different ratio)
6. Toluene: Chloroform (in different ratio)

The sample solutions were applied on the TLC plates with glass capillaries at about 2 cm above from the bottom. The spotted plates were dried and then immersed vertically in the chromatographic tank containing the solvent in such a way that the spotted mark of the samples remained above the solvent surface. The tank containing developing solvent and the atmosphere inside the tank was saturated with the vapour of the same solvents. The plates were developed through ascending technique and finally removed when the solvent front reached from about 2cm apart from the top of the plates. The plates were allowed to dry and the sample spots were made visible either exposing it to UV lamp or iodine vapour or by spraying with suitable spray reagents, if it is not visible in the day light.

2.8.7 Determination of the R_f Value

R_f means retardation factor. The R_f value measures the velocity of movement of the solute zone relative to that of the solvent front. It may be defined as

$$R_f = \frac{\text{Distance (cm) travelled by solute}}{\text{Distance (cm) travelled by solvent}}$$

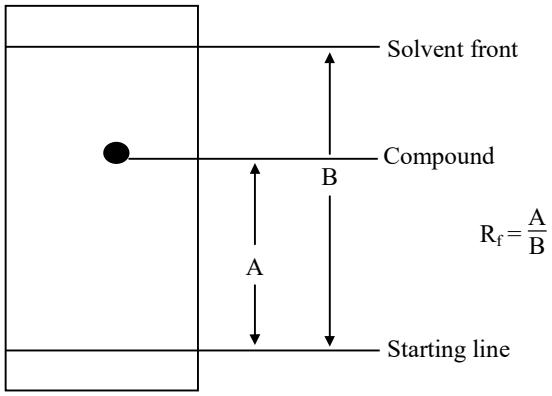


Fig. 2.3: A plate for a calculation of R_f value.

Usually, the R_f value is constant for any given compound and it correspond to a physical property of that compound (Pavia, 1976).

2.8.8 Preparative thin layer chromatography (PTLC)

Process of preparation:

Silica-gel (Merck 60 PF_{254}) was used to prepare PTLC plates. Usually glass plates (20×20 cm) were used for this purpose. At first the plates were cleaned and dried. Slurry was prepared by mixing silica-gel (60g with 120 CC distilled water) for six plates.

The slurry was then spread over the clean dry plates so that it makes a thickness at 0.5 mm. The prepared plate was then allowed to set in air at room temperature for about 24 hours. These were then activated by heating in an oven at 110°C. The activated plates were stored in a desiccator

Application of sample on PTLC

The sample was dissolved in a very small amount of a suitable solvent and applied on the plates as a fine thin band near the baseline by using glass capillary tube. The plates were then developed in an appropriate solvent system predetermined by TLC: In some cases, double or triple developments were performed in order to assure a maximum separation of the bands. The separated bands were visualized by the use of spray reagents and UV-light. Silica-gel with the adsorbed substance was scrapped off with the help of spatula and the adsorbed compound on silica-gel was dissolved in a solvent of greater polarity than that used for developing the chromatogram. The Silica-gel was filtered off and the filtrate was concentrated in a rotatory evaporator to get back the separated pure substance (Donald, 1976).

Preparative HPLC

Recycling Preparative High Performance Liquid Chromatographic separation was performed using water : methanol (50 : 50) with an equipment Model LC-908W. A JAIGEL ODS LH-80 column was used with a flow rate of 3.5 mL/min.

Detection / Visualisation of spots

For the location of the separated components, the TLC plates were examined by the following methods:

Such As:

(i) UV- Light

(ii) Iodine Vapour.

(iii) Spray Reagent.

(A) Vanillin-Sulphuric acid (Stahl, 1969)

(B) Dragendorff's reagent.

(C) Ferric chloride – Ethanol reagent

(i) UV-Light: The fluorescent compound on the developed, dried TLC and PTLC plates were observed under UV-light at 254 and 350 nm. Some of the compounds appear as fluorescing spot and the others are dark spots under the UV-Light.

(ii) Iodine Vapour: In an iodine chamber the plates were exposed to iodine vapour for several minutes for detection of spots.

(iii) TLC Spray Reagent(Stahl, Egon "Thin Layer Chromatography**:**A Laboratory Hand Book." 2nd ed.)

a) Vanillin-Sulfuric Acid: For higher alcohol's, phenols, steroids and essential oil.

Spray reagent: 1 g vanillin is dissolved in 100 ml sulfuric acid.

Treatment : The chromatogram was sprayed with 1% vanillin-sulfuric acid reagent and heating at 120^0C is carried out until the spots attain maximum intensity.

b) Dragendorff's Reagent: For alkaloids and other nitrogen-containing compounds.

Solution a: 0.85 g basic bismuth nitrate is dissolved in a mixture of 10 mL acetic acid and 40 mL water.

Solution b: A solution is made of 8 g potassium iodide in 20 mL water.

Stock solution : Equal volumes of a and b are mixed (can be stored for long time in dark glass vessels).

Spray reagent: 1 mL stock solution is mixed with 2 mL acetic acid and 10 mL water before use.

Treatment: The plates are sprayed with the reagent detect alkaloids. Alkaloid produces orange to red spot with the reagent.

c) Ferric chloride - Ethanol: For some of Phenolic compounds

Spray reagent : 5% ferric chloride in absolute ethanol

Treatment: The plates are sprayed with the reagent to detect the Phenolic compounds. Phenolic compound show blue spot with the reagent.

2.9 Different fractions were investigated for separation into individual compounds by Chromatography:

Ethyl acetate and Dia-ion resin fractions was eluted in this work as they were found biological active than other fractions. Due to the searching of petrolium ether fraction and chloroform fraction were not addvanced hear.

2.9.1 Fractionation of the ethyl acetate fraction (EAF) by column Chromatography:

A total of 300 fractions were obtained from the column chromatography of the ethyl acetate fraction (EAF) after eluting with the different ratio of solvent-solvent system as mentioned in the table 2.3. Each of the fractions was checked by TLC plates developed with the solvent system petroleum ether: ethyl acetate as mobile phase and viewed visually, under UV, I_2 chamber and with vanillin-sulphuric acid spray reagent. The fractions of the similar behaviors were combined and the fractions were designated as F-1 to F-21 respectively. Table 2.3 is a list of observation following TLC examination of the fractions obtained from column chromatography of the EAF.

Table-2.3: Solvent system used in the column analysis of ethyl acetate fraction.

Collection no.	Fraction no.	Solvent systems	Proportion	Volume eluted (ml)
1 to 10	F-1	Petroleum ether	100%	300
11 to 20	F-2	Pet .ether : ethyl acetate	95 : 5	250
21 to 38	F-3	Pet. ether : ethyl acetate	90 : 10	250
39 to 52	F-4	Pet. ether : ethyl acetate	85 : 15	250
53 to 65	F-5	Pet. ether : ethyl acetate	80 : 20	350
66 to 78	F-6	Pet. ether : ethyl acetate	75 : 25	350
79 to 89	F-7	Pet. ether : ethyl acetate	70 : 30	350
90 to 105	F-8	Pet. ether : ethyl acetate	65 : 35	400
106 to 117	F-9	Pet. ether : ethyl acetate	60 : 40	400
118 to 129	F-10	Pet. ether : ethyl acetate	55 : 45	350
130 to 143	F-11	Pet. ether : ethyl acetate	50 : 50	350
144 to 158	F-12	Pet. ether : ethyl acetate	45 : 55	350
159 to 172	F-13	Pet. ether : ethyl acetate	40 : 60	350
173 to 186	F-14	Pet. ether : ethyl acetate	35 : 65	350
187 to 198	F-15	Pet. ether : ethyl acetate	25 : 75	350
209 to 221	F-16	Pet. ether : ethyl acetate	15 : 85	350
222 to 236	F-17	Pet. ether : ethyl acetate	5 : 95	350
237 to 251	F-18	ethyl acetate	100%	350
252 to 268	F-19	methanol: ethyl acetate	25 : 75	350
269 to 281	F-20	methanol: ethyl acetate	50 : 50	350
282 to 300	F-21	methanol	100%	250

Analysis of fraction-2 (F-2):

Analysis of F-2 showed a single spot and tail on TLC using solvent system n-hexane : acetone (3:2), with R_f value of 0.82. But three spots appeared with R_f values 0.803, 0.79 and 0.76 on TLC using solvent system petroleum ether : ethyl acetate (5:3). The spots were viewed under UV and iodine chamber. These spots showed positive test (dark pink color) with vanillin-sulfuric acid spray reagent. Due to the poor amount of this sample (9.42 mg), this fraction was not further analyzed.

Analysis of fraction-3 (F-3):

Analysis of F-3 showed a single spot having R_f value 0.87 and tail on TLC using solvent system n-hexane : acetone (3:2). But in petroleum ether : ethyl acetate (5:3), it showed three sports with R_f values 0.76, 0.75, 0.74. The spots were viewed under UV and iodine chamber. All the spots showed positive test (dark pink color) with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (11.12 mg), this fraction was not further investigation.

Analysis of fraction-4 (F-4):

Fraction-4 (F-4) showed a single spot having R_f value of 0.78 on TLC using solvent system n-hexane : acetone (3:2). R_f value 0.78. The distinct spot purified of sample represented a single compound, viewed under UV and with I_2 on TLC using solvent system petroleum ether : ethyl acetate (5:4) with R_f value 0.705. This spot showed positive test with vanillin-sulfuric acid spray reagent. After a few days a gummy mass was obtained with slight impurities. The compound washed with ethyl acetate and filtered through cotton plugs and was collected in a beaker. The solvent was evaporated off under reduced pressure to afford the pure compound EFC-1 (21.47 mg) as gummy mass. The isolated compound chemical and biological analysis had been done.

Analysis of fraction-5 (F-5):

Analysis of F-5 showed two spots on TLC using solvent system n-hexane : acetone (4:3), R_f value 0.72 and 0.67. Another solvent system two spots combined on TLC using petroleum ether : ethyl acetate (5:4) with R_f values 0.67, 0.65. The spots were viewed under UV and iodine chamber. They showed positive test (dark pink color) with vanillin-sulfuric acid spray reagent. But due to small amount of this sample (8.32 mg), this fraction was not further analyzed.

Analysis of fraction-6 (F-6):

Analysis of F-6 showed two spots on TLC using solvent system n-hexane : acetone (4:3) having R_f values 0.67 and 0.62. On the other hand one band with mixture of compounds on TLC using solvent system petroleum ether : ethyl acetate (5:4) with R_f value 0.63. The spot was viewed under UV and iodine chamber. They spot showed positive test (dark pink color) with vanillin-sulfuric acid spray reagent. But due to poor amount of this sample (7.67 mg), it was not possible for further chemical investigation.

Analysis of fraction-7 (F-7):

Analysis of F-7 showed one band on TLC using solvent system n-hexane : acetone (1:1), R_f value 0.72 and three spots on TLC using solvent system petroleum ether : ethyl acetate (1:1), it showed three sports having R_f values 0.57, 0.56, 0.55. The spots were viewed under UV and I_2 chamber. All the spots showed positive test with vanillin-sulfuric acid spray reagent. But due to the poor amount of this sample (9.37 mg), this fraction was not further analyzed for chemical investigation.

Analysis of fraction-8 (F-8):

Analysis of F-8 showed mixture of several spots on TLC using solvent system n-hexane : acetone (1:1), R_f value 0.68 and one band with mixture of compounds on TLC using solvent system petroleum ether : ethyl acetate (1:1) with R_f values 0.52. This spot was viewed under UV and iodine chamber. This spot showed positive test with vanillin-sulfuric acid spray reagent. This fraction was small amount(8.42 mg) of this sample, it was not possible for further chemical investigation and purification.

Analysis of fraction-9 (F-9):

Analysis of F-9 showed two spots on TLC using solvent system n-hexane : acetone (3:4), with R_f values 0.76 and 0.62. On the other hand one band with mixture of compounds with R_f values 0.48 on TLC using solvent system petroleum ether : ethyl acetate (2:3). The spot was viewed under UV and iodine chamber. This spot showed positive test (dark pink color) with vanillin-sulfuric acid spray reagent. This fraction was small amount (5.42 mg) relative to the number of compounds observed by TLC examination. So, this fraction was not further purification and analyzed.

Analysis of fraction-10 (F-10):

Fraction-10 (F-10) showed two spots on TLC using solvent system n-hexane : acetone (3:4) with R_f values 0.69 and 0.52. Another solvent system petroleum ether : ethyl acetate (5:6) with R_f values 0.45 and 0.40. The spots were viewed under UV and I_2 chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. The amount of this sample (27.25 mg). This fraction further purification by PTLC using solvent system n-hexane : acetone (3:4). The compound washed with ethyl acetate and filtered through cotton plugs and was collected in a beaker. The solvent was evaporated off under reduced pressure to afford the pure compound EFC-2 (17.76 mg) as semi-solid. The isolated compound chemical and biological analysis had been done.

Analysis of fraction-11 (F-11):

Analysis of F-11 showed one band on TLC using solvent system n-hexane : acetone (3:5), R_f value 0.62 and two spots combined on TLC using solvent system petroleum ether : ethyl acetate (2:3) with R_f values 0.41,0.40. The spots were viewed under UV and I_2 chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But these spots were very closed to ease other and the poor amount of this sample (6.37 mg). So this fraction was not further purification by PTLC and chemical analysis.

Analysis of fraction-12 (F-12):

Analysis of F-12 showed two spots on TLC using solvent system n-hexane : acetone (3:5), R_f value 0.64, 0.61 and one band on TLC using solvent system petroleum ether : ethyl acetate (1:2) with R_f values 0.35. The spots were viewed under UV and I_2 chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But these spots were very closed to ease other and the poor amount of this sample (7.76 mg). So this fraction was not further chemical analysis and purification by PTLC.

Analysis of fraction-13 (F-13):

Analysis of F-13 showed two spots on TLC using solvent system n-hexane : acetone (2:3), R_f value 0.64, 0.61 and one band with mixture of compounds on TLC using

solvent system petroleum ether : ethyl acetate (1:2) with R_f value 0.35. The spot was viewed under UV and iodine chamber. This spot showed positive test with vanillin-sulfuric acid spray reagent. For the small quantity of the sample (8.52 mg), it was not possible further purification by PTLC.

Analysis of fraction-14 (F-14):

Analysis of F-14 showed two spots on TLC using solvent system n-hexane : acetone (2:3), R_f value 0.54, 0.51 and two spots combined on TLC using solvent system petroleum ether : ethyl acetate (1:2) with R_f values 0.32,0.31. The spots were viewed under UV and I_2 chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But these spots were very closed to ease other and the poor amount of this sample (6.37 mg). So this fraction was not possible further purification by PTLC and chemical analysis.

Analysis of fraction-15 (F-15):

Analysis of F-15 showed one broad band on TLC using solvent system n-hexane : acetone (2:3), R_f value 0.45 and three spots combined on TLC using solvent system Petroleum Ether : Ethyl acetate (2:5) with R_f values 0.28,0.27, 0.26. The spots were viewed under UV and I_2 chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But these spots were very closed to ease other and the poor amount of this sample (8.54 mg). So this fraction was not further chemical analysis and purification by PTLC.

Analysis of fraction-16 (F-16):

Analysis of F-16 showed two spots on TLC using solvent system n-hexane : acetone (2:3), R_f value 0.32, 0.31 and two spots combined on TLC using solvent system petroleum ether : ethyl acetate (2:5) with R_f values 0.24,0.23. The spots were viewed under UV and I_2 chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But these spots were very closed to ease other and the poor amount of this sample (5.18 mg). So this fraction was not possible further purification by PTLC and chemical analysis.

Analysis of fraction-17 (F-17):

Analysis of F-17 showed two spots on TLC using solvent system n-hexane : acetone (1:2), R_f value 0.45, 0.42 and one band with mixture of compounds on TLC using solvent system petroleum ether : ethyl acetate (2:5) with R_f value 0.21. The spot was viewed under UV and iodine chamber. This spot showed positive test with vanillin-sulfuric acid spray reagent. For the small quantity of the sample (5.76 mg), it was not possible further purification by PTLC.

2.9.2 Investigation of ethyl acetate fraction (EAF) by TLC

TLC analysis of a portion of the ethyl acetate soluble fraction (EAF) of *Wedelia chinensis* showed several prominent sport with small tailing at the base when developed with petroleum ether : ethyl acetate (5 : 6) and either sprayed with vanillin-sulphuric acid spray reagent or exposed in the iodine chamber.

Table 2.4: TLC analysis of fractions obtained from column chromatography of ethyl acetate fraction (EAF) obtained from *wedelia chinensis*.

Fraction no.	Solvent system	R_f Values	Appearance of sports/bands
F-1	Pet. ether : ethyl acetate (5:3)	-	-
F-2	n-hexane : acetone (3: 2) Pet .ether : ethyl acetate (5:3)	0.82 0.803, 0.79,0.76	Single spot and tail. Mixture of several spots.
F-3	n-hexane : acetone (3: 2) Pet. ether : ethyl acetate (5:3)	0.87 0.76, 0.74,0.75	One band and tail Mixture of several spots.
F-4	n-hexane : acetone (3: 2) Pet. ether : ethyl acetate (5:4)	0.78 0.705	A single spot. A single spot.
F-5	n-hexane : acetone (4: 3) Pet. ether : ethyl acetate (5:4)	0.72, 0.67 0.67, 0.65	Mixture of two spots. Mixture of two spots.
F-6	n-hexane : acetone (4: 3) Pet. ether : ethyl acetate (5:4)	0.67, 0.62 0.63	Mixture of two spots. One band and tail.
F-7	n-hexane : acetone (1:1) Pet. ether : ethyl acetate(1:1)	0.72 0.57, 0.56, 0.55	A band and tail. Mixture of several spots.
F-8	n-hexane : acetone (1: 1) Pet. ether : ethyl acetate (1:1)	0.68 0.52	One band and tail. One band and tail.
F-9	n-hexane : acetone (3: 4) Pet. ether : ethyl acetate (2:3)	0.76, 0.62 0.48	Mixed two spots. One band and tail.

Fraction no.	Solvent system	R_f Values	Appearance of sports/bands
F-10	n-hexane : acetone (3: 4) Pet. ether : ethyl acetate (2:3)	0.69, 0.57 0.45, 0.40	Mixed two spots. Mixture of two spots.
F-11	n-hexane : acetone (3: 5) Pet. ether : ethyl acetate (2:3)	0.62 0.41, 0.40	One band Mixture of two spots.
F-12	n-hexane : acetone (3: 5) Pet. ether : ethyl acetate (1:2)	0.54 0.39, 0.38, 0.37	One band and tail. Mixture of several spots.
F-13	n-hexane : acetone (2: 3) Pet. ether : ethyl acetate (1:2)	0.64, 0.61 0.35	Mixed two spots. One band and tail.
F-14	n-hexane : acetone (2: 3) Pet. ether : ethyl acetate (1:2)	0.54, 0.51 0.32, 0.31	Mixed two spots. Mixture of two spots.
F-15	n-hexane : acetone (2: 3) Pet. ether : ethyl acetate (2:5)	0.45 0.28, 0.27, 0.25	One band and tail. Mixture of several spots.
F-16	n-hexane : acetone (2: 3) Pet. ether : ethyl acetate (2:5)	0.32, 0.31 0.24, 0.23	Mixed two spots. Mixture of two spots.
F-17	n-hexane : acetone (1: 2) Pet. ether : ethyl acetate (2:5)	0.45, 0.42 0.21	Mixture of two spots. One band and tail.

2.9.3 Fractionation of the Dia-ion Resin fraction (DRF) by column chromatography:

A total of 425 fractions were obtained from the column chromatography of the dia-ion resin fraction (DRF) after eluting with the different ratio of solvent-solvent system as mentioned in the table 2.6. Each of the fractions was checked by TLC plates developed with the solvent system petroleum ether : acetone as mobile phase and viewed visually, under UV, I_2 chamber and with vanillin-sulphuric acid spray reagent.

The fractions of the similar behaviors were combined and the fractions were designated as F-1 to F-21 respectively. Table-2.6 is a list of observation following TLC examination of the fractions obtained from column chromatography of the DRF.

Table-2.5: Solvent system used in the column analysis of dia-ion resin fraction.

Collection no.	Fraction no.	Solvent systems	Proportion	Volume eluted (ml)
1 to 12	F/-1	Petroleum Ether	100%	500
13 to 29	F/-2	Pet .ether : Acetone	95 : 5	500
30 to 52	F/-3	Pet. ether : Acetone	90 : 10	450
53 to 71	F/-4	Pet. ether : Acetone	85 : 15	450
72 to 94	F/-5	Pet. ether : Acetone	80 : 20	450
95 to 118	F/-6	Pet. ether : Acetone	75 : 25	550
119 to 132	F/-7	Pet. ether : Acetone	70 : 30	550
133 to 164	F/-8	Pet. ether : Acetone	65 : 35	600
165 to 187	F/-9	Pet. ether : Acetone	60 : 40	600
188 to 212	F/-10	Pet. ether : Acetone	55 : 45	550
213 to 234	F/-11	Pet. ether : Acetone	50 : 50	550
235 to 257	F/-12	Pet. ether : Acetone	45 : 55	550
258 to 271	F/-13	Pet. ether : Acetone	40 : 60	550
272 to 297	F/-14	Pet. ether : Acetone	35 : 65	550
298 to 305	F/-15	Pet. ether : Acetone	25 : 75	500
306 to 325	F/-16	Pet. ether : Acetone	15 : 85	500
326 to 342	F/-17	Pet. ether : Acetone	5 : 95	500
343 to 367	F/-18	Acetone	100%	500
368 to 383	F/-19	Methanol: Acetone	25 : 75	500
384 to 406	F/-20	Methanol: Acetone	50 : 50	500

Analysis of fraction-2 (F/-2):

Analysis of F/-2 showed double spots and tail on TLC using solvent system petroleum ether : acetone(5:3), R_f values 0.85, 0.82 and another two spots combined on TLC using solvent system n-hexane : ethyl acetate : methanol (5:2:0.5) with R_f values 0.65,0.61. The spots were viewed under UV and iodine chamber. These spots showed positive test (dark pink color) with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (8.76 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-3 ($F^{/}$-3):

Analysis of $F^{/}$-3 showed two spots on TLC using solvent system petroleum ether : acetone(5:3), R_f values 0.76, 0.72 and another single spot on TLC using solvent system n-Hexane : Ethyl acetate : Methanol (5:2:0.5) with R_f values 0.58. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. The amount of this sample (38.76 mg), this fraction was further analyzed using PTLC for the purification. Using solvent system Pet. ether : acetone (5:3). A single compound was collect by PTLC. After a few days a solid mass was obtained with slight impurities. The compound washed with Acetone and filtered through cotton plugs and was collected in a beaker. The solvent was evaporated off under reduced pressure to afford the pure compound DFC-1 (27.32 mg) as solid mass. The isolated compound chemical and biological analysis had been done.

Analysis of fraction-4 ($F^{/}$-4):

Analysis of $F^{/}$-4 showed a band and tail on TLC using solvent system petroleum ether : acetone(5:4), R_f value 0.79 and another two spots combined on TLC using solvent system n-Hexane : Ethyl acetate : Methanol (5:2:0.5) with R_f values 0.56, 0.54. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (11.53 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-5 ($F^{/}$-5):

Analysis of $F^{/}$-5 showed double spots on TLC using solvent system petroleum ether : acetone(5:4), R_f values 0.77, 0.75 and another two spots combined on TLC using solvent system n-hexane : ethyl acetate : methanol (5:3:0.5) with R_f values 0.62,0.60. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (8.76 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-6 ($F^{/}$-6):

Analysis of $F^{/}$-6 showed double spots on TLC using solvent system petroleum ether : acetone(1:1), R_f values 0.67, 0.61 and another single spot on TLC using solvent

system n-hexane : ethyl acetate : methanol (5:3:0.5) with R_f values 0.58. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. The amount of this sample (45.56 mg), this fraction was further analyzed using PTLC for the purification. Using solvent system Pet. ether : acetone (1:1). A single compound was collect by PTLC. After a few days a solid mass was obtained with slight impurities. The compound washed with Acetone and filtered through cotton plugs and was collected in a beaker. The solvent was evaporated off under reduced pressure to afford the pure compound DFC-2 (25.76 mg) as solid mass. The isolated compound chemical and biological analysis had been done.

Analysis of fraction-7 ($F^{/}$-7):

Analysis of $F^{/}$-7 showed double spots and tail on TLC using solvent system petroleum ether : acetone(1:1), R_f values 0.64, 0.62 and another three spots combined on TLC using solvent system n-Hexane : Ethyl acetate : Methanol (5:3:0.5) with R_f values 0.55,0.52, 0.51. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (12.32 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-8 ($F^{/}$-8):

Analysis of $F^{/}$-8 showed double spots on TLC using solvent system petroleum ether : acetone(3:4), R_f values 0.68, 0.63 and another single spot on TLC using solvent system n-hexane : ethyl acetate : methanol (5:3:0.5) with R_f values 0.52. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. The amount of this sample (45.49 mg).This fraction was further analyzed using PTLC for the purification. Using solvent system Pet. ether : acetone (3:4). A single compound was collect by PTLC. After a few days a solid mass was obtained with slight impurities. The compound washed with Acetone and filtered through cotton plugs and was collected in a beaker. The solvent was evaporated off under reduced pressure to afford the pure compound DFC-3 (32.38 mg) as solid mass. The isolated compound chemical and biological analysis had been done.

Analysis of fraction-9 ($F^{/}$-9):

Analysis of $F^{/}$-9 showed double spots combined and tail on TLC using solvent system petroleum ether : acetone(3:4), R_f values 0.76, 0.62 and another single spot on TLC using solvent system n-hexane : ethyl acetate : methanol (5:3:0.5) with R_f value 0.48. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (10.73 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-10 ($F^{/}$-10):

Analysis of $F^{/}$-10 showed double spots combined on TLC using solvent system petroleum ether : acetone(3:5), R_f values 0.69, 0.57 and another two spots on TLC using solvent system n-hexane : ethyl acetate : methanol (5:4:0.5) with R_f values 0.65, 0.62. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (11.48 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-11 ($F^{/}$-11):

Analysis of $F^{/}$-11 showed A single spot on TLC using solvent system petroleum ether : acetone(3:5), R_f value 0.62 and another two spots on TLC using solvent system n-hexane : ethyl acetate : methanol (5:4:0.5) with R_f values 0.59, 0.54. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. The amount of this sample (38.78 mg).This fraction was further analyzed using PTLC for the purification. Using solvent system n-hexane : ethyl acetate : methanol (5:4:0.5). A single compound was collect by PTLC. After a few days a solid mass was obtained with slight impurities. The compound washed with Acetone and filtered through cotton plugs and was collected in a beaker. The solvent was evaporated off under reduced pressure to afford the pure compound DFC-4 (22.73 mg) as solid mass. The isolated compound chemical and biological analysis had been done.

Analysis of fraction-12 ($F^{/}$-12):

Analysis of $F^{/}$-12 showed one band on using solvent system petroleum ether : acetone(3:5), R_f values 0.54 and another three spots combined on TLC using solvent

system n-hexane : ethyl acetate : methanol (5:4:0.5) with R_f values 0.54, 0.52, 0.51. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (13.27 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-13 ($F^{/}$-13):

Analysis of $F^{/}$-13 showed double spots on using solvent system petroleum ether : acetone(2:3), R_f values 0.64, 0.61 and another two spots combined and tail on TLC using solvent system n-hexane : ethyl acetate : methanol (5:4:0.5) with R_f values 0.47, 0.43. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (11.16 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-14 ($F^{/}$-14):

Analysis of $F^{/}$-14 showed two spots on using solvent system petroleum ether : acetone(2:3), R_f values 0.54, 0.51 and another three spots combined on TLC using solvent system n-hexane : ethyl acetate : methanol (1:1:0.5) with R_f values 0.57, 0.54, 0.51. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (11.63 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-15 ($F^{/}$-15):

Analysis of $F^{/}$-15 showed one band on using solvent system petroleum ether : acetone(1:2), R_f values 0.45 and another three spots combined on TLC using solvent system n-hexane : ethyl acetate : methanol (1:1:0.5) with R_f values 0.48, 0.42, 0.38. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (14.06 mg), this fraction was not further analyzed using PTLC for the purification.

Analysis of fraction-16 ($F^{/}$-16):

Analysis of $F^{/}$-16 showed A single spot on TLC using solvent system petroleum ether: acetone(1:2), R_f value 0.32 and another two spots on TLC using solvent system

n-hexane : ethyl acetate : methanol (1:1:0.5) with R_f values 0.38, 0.29. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. The amount of this sample (41.35 mg).This fraction was further analyzed using PTLC for the purification. Using solvent system n-hexane : ethyl acetate : methanol (1:1:0.5). A single compound was collect by PTLC. After a few days a solid mass was obtained with slight impurities. The compound washed with Acetone and filtered through cotton plugs and was collected in a beaker. The solvent was evaporated off under reduced pressure to afford the pure compound DFC-5 (25.78 mg) as solid mass. The isolated compound chemical and biological analysis had been done.

Analysis of fraction-17 ($F^{/}$-17):

Analysis of $F^{/}$-17 showed two spots combined on using solvent system petroleum ether : acetone(1:2), R_f values 0.45, 0.42 and another one band on TLC using solvent system n-hexane : ethyl acetate : methanol (2:3:0.5) with R_f values 0.43. The spots were viewed under UV and iodine chamber. These spots showed positive test with vanillin-sulfuric acid spray reagent. But for the poor amount of this sample (9.65 mg), this fraction was not further analyzed using PTLC for the purification.

Table-2.6: TLC analysis of fractions obtained from column chromatography of dia-ion resin fraction (DRF).

Fraction no.	Solvent system	R_f Values	Appearance of sports/bands
$F^{/}$-1	Pet. ether : acetone (5:3)	-	-
$F^{/}$-2	Pet. ether : acetone (5:3) n-hexane: ethyl acetate : methanol (5:2:0.5)	0.85, 0.82 0.65, 0.61	Mixed of two spot and tail. Mixture of two spots.
$F^{/}$-3	Pet. ether : acetone (5:3) n-hexane: ethyl acetate: methanol (5:2:0.5)	0.76, 0.71 0.58	Mixed of two spots. A single spot.
$F^{/}$-4	Pet. ether : acetone (5:4) n-hexane: ethyl acetate: methanol (5:2:0.5)	0.79 0.56, 0.54	One band and tail. Mixed of two spots.

Fraction no.	Solvent system	R_f Values	Appearance of sports/bands
F/-5	Pet. ether : acetone (5: 4) n-hexane: ethyl acetate: methanol (5:3:0.5)	0.77, 0.75 0.62, 0.60	Mixture of two spots. Mixture of two spots.
F/-6	Pet. ether : acetone (1:1) n-hexane: ethyl acetate: methanol (5:3:0.5)	0.67, 0.61 0.58	Mixture of two spots. A single spot.
F/-7	Pet. ether : acetone (1:1) n-hexane: ethyl acetate: methanol (5:3:0.5)	0.64, 0.62 0.55, 0.52, 0.51	Mixture of two spots and tail. Mixture of several spots.
F/-8	Pet. ether : acetone (3:4) n-hexane: ethyl acetate: methanol (5:3:0.5)	0.68, 0.63 0.52	Mixture of two spots. A single spot.
F/-9	Pet. ether : acetone (3: 4) n-hexane: ethyl acetate: methanol(5:3:0.5)	0.76, 0.62 0.48	Mixed of two spots. One band and tail.
F/-10	Pet. ether : acetone (3: 5) n-hexane: ethyl acetate: methanol (5:4:0.5)	0.69, 0.57 0.65, 0.62	Mixed of two spots. Mixture of two spots.
F/-11	Pet. ether : acetone (3: 5)n-hexane: ethyl acetate: methanol (5:4:0.5)	0.62 0.59, 0.54	A single spot. Mixture two of spots.
F/-12	Pet. ether : acetone (3: 5)n-hexane: ethyl acetate: methanol (5:4:0.5)	0.54 0.54, 0.52, 0.51	One band and tail. Mixture of several spots.
F/-13	Pet. ether : acetone (2: 3) n-hexane: ethyl acetate: methanol (5:4:0.5)	0.64, 0.61 0.47, 0.43	Mixed of two spots. Mixture of two spots and tail.
F/-14	Pet. ether : acetone (2: 3) n-hexane: ethyl acetate: methanol (1:1:0.5)	0.54, 0.51 0.57, 0.54, 0.51	Mixed two spots. Mixture of several spots.
F/-15	Pet. ether : acetone (1:2) n-hexane: ethyl acetate: methanol (1:1:0.5)	0.45 0.48, 0.42, 0.38	One band and tail. Mixture of several spots.
F/-16	Pet. ether : acetone (1:2)n-hexane: ethyl acetate: methanol (1:1:0.5)	0.32 0.38, 0.29	A single spots. Mixture of two spots.
F/-17	Pet. ether : acetone (1: 2)n-hexane: ethyl acetate: methanol (2:3:0.5)	0.45, 0.42 0.43	Mixture of two spots. One band and tail.

2.10 Spectroscopic data

Spectroscopic data of isolated compounds EFC-1

UV λ_{max} (MeOH) = 201, 194 and 188 nm

IR ν_{max} (KBr) = 3412, 3017 and 1695 cm^{-1}

^{1}H-NMR (300 MHz) δ_{TMS} (CD_3OD) : 0.92, 0.96, 1.02, 1.26, 1.36, 1.78, 1.87, 2.00, 2.06, 2.08, 2.13, 2.15, 2.17, 2.71 and 3.56 ppm.

^{13}C-NMR (300 MHz) δ_{TMS} (CD_3OD) : 14.38, 15.83, 18.88, 19.28, 20.62, 22.15, 26.27, 29.17, 37.64, 38.01, 39.87, 40.69, 42.08, 43.87, 46.18, 47.86, 54.61, 56.01, 56.89, 79.45, and 183.11ppm.

Spectroscopic data of isolated compounds EFC-2

UV λ_{max} (MeOH) = 201, 194 and 188 nm

IR ν_{max} (KBr) = 3015, 3013, 3000 ~ 2850, 1634 and 1605 cm^{-1}.

^{1}H-NMR (300 MHz) δ_{TMS} (CD_3OD) : 0.83, 0.98, 1.03, 1.23, 1.43, 1.57, 1.64, 1.82, 1.87, 1.98, 2.03, 2.12, 2.62 and 4.74 ppm.

^{13}C-NMR (300 MHz) δ_{TMS} (CD_3OD) : 16.41, 18.95, 19.52, 22.27, 29.35, 33.47, 38.21, 40.13, 40.27, 41.12, 41.83, 44.17, 44.31, 44.61, 49.31, 55.47, 57.34, 103.42, 156.24 and 184.42 ppm.

Spectroscopic data of isolated compounds DFC-1

UV λ_{max} (MeOH) = 240 nm

IR ν_{max} (KBr) = 3412, 2925, 1700, 1672 and 1527 cm^{-1}

^{1}H-NMR (300 MHz) δ_{TMS} (CD_3OD) : 0.63, 0.96, 1.43, 1.45, 1.73, 1.74, 2.12, 2.17, 2.34, 2.42, 2.78, 3.34, 3.95, 4.56 and 5.83 ppm.

^{13}C-NMR (300 MHz) δ_{TMS} (CD_3OD) : 17.61, 21.73, 22.21, 24.57, 30.85, 31.54, 32.12, 32.13, 35.27, 37.37, 39.29, 48.38, 51.85, 60.13, 68.42, 68.72, 85.25, 122.83, 166.61, 212.46 and 205.47 ppm.

Spectroscopic data of isolated compounds DFC-2

UV λ_{max} (MeOH) = 210 and 182 nm

IR ν_{max} (KBr) = 3423 and 1634 cm^{-1}.

^{1}H-NMR (250 MHz) δ_{TMS} (CD_3OD) : 0.83, 0.89, 0.93, 0.98, 1.06~1.09, 1.12, 1.23, 1.45~1.51, 1.58, 1.65, 1.84 ~ 1.87, 1.87, 1.89, 1.92, 1.97, 2.02, 2.04, 2.05, 2.07, 2.08, 2.62, 3.25 and 4.87~5.22 ppm.

^{13}C-NMR (300 MHz) δ_{TMS} (CD_3OD) : 12.2, 12.4, 18.8, 19.3, 19.8, 21.1, 23.5, 24.3, 26.6, 28.7, 29.5, 31.5, 32.0, 34.3, 36.5, 36.6, 37.3, 40.5, 42.2, 42.4, 46.3, 50.20, 56.5, 57.2, 72.2, 121.5 and 140.6 ppm.

Spectroscopic data of isolated compounds DFC-3

UV λ_{max} (MeOH) = 215 and 185 nm

IR ν_{max} (KBr) = 3362 and 1634 cm^{-1}.

^{1}H-NMR (250 MHz) δ_{TMS} (CD_3OD) : 0.72, 0.83, 0.84, 0.86, 1.04, 1.06 ~ 1.09, 1.45 ~ 1.51, 1.58, 1.65, 1.84 ~ 1.87, 1.87, 1.89, 1.97, 2.02, 2.05, 2.08, 2.62, 3.25, 5.00, 5.07 and 5.27 ppm.

^{13}C-NMR (300 MHz) δ_{TMS} (CD_3OD) : 14.13, 18.85, 19.25, 21.15, 21.18, 21.25, 24.53, 24.85, 28.78, 31.75, 32.15, 32.25, 36.55, 37.35, 40.15, 41.16, 42.28, 42.42, 50.25, 52.15, 56.55, 57.12, 72.21, 121.53, 126.65, 136.35 and 140.65 ppm

Spectroscopic data of isolated compounds DFC-4

UV λ_{max} (MeOH) = 185 nm

IR ν_{max} (KBr) = 3400 – 3100 cm^{-1}.

^{1}H-NMR (250 MHz) δ_{TMS} (CD_3OD) : 0.85, 1.05 ~ 1.07, 1.06 ~ 1.08, 1.08 ~ 1.09, 1.35, 1.42, 1.43 ~ 1.54, 1.56, 1.67, 1.73, 1.79, 1.87 ~ 1.92, 1.91 ~ 1.97,1.92, 1.97, 2.03,2.03, 2.04, 2.05, 2.07, 2.08, 2.11, 2.12, 2.21 ~ 2.27, 3.23, 11.33, 11.54, 12.73, 12.78 and 13.17 ppm.

^{13}C-NMR (300 δ_{TMS} (CD_3OD) : 12.5, 19.1, 19.7, 20.8, 25.2, 25.9, 27.3, 29.3, 31.5, 31.8, 32.1, 35.7, 36.7, 37.1, 37.2, 41.3, 42.7, 43.5, 45.7, 51.2, 56.8, 57.1, 73.3, 122.3, 141.2, 161.3, 162.5, 163.7, 165.4, 165.8 ppm.

Spectroscopic data of isolated compounds DFC-5

UV λ_{max} (MeOH) = 194 and 188 nm

IR ν_{max} (KBr) = 3426 and 1635 cm^{-1}.

^{1}H-NMR (250 MHz) δ_{TMS} (CD_3OD) : 0.83, 1.06 ~ 1.09, 1.12, 1.23, 1.45 ~ 1.51, 1.58, 1.65, 1.84 ~ 1.87, 1.87, 1.89, 1.92, 1.97, 2.02, 2.04, 2.05, 2.07, 2.08, 3.25, 11.53, 12.75 and 12.78 ppm.

^{13}C-NMR (300 MHz) δ_{TMS} (CD_3OD) : 12.2, 18.8, 19.3, 21.1, 24.3, 26.6, 28.7, 29.5, 31.5, 32.05, 34.3, 36.5, 36.6, 37.3, 40.5, 42.2, 42.4, 46.3, 50.20, 56.5, 57.2, 72.2, 121.5, 140.6, 161.22, 165.28 and 165.73 ppm.

RESULTS AND DISCUSSION

3.1 Phytochemical Investigation on *Wedelia chinensis*

3.1.1 Determination of total phenolic content of crude methanol extract (CME) of *Wedelia chinensis* and its different fractions.

Phenolic content of the crude methanolic extract (CME) of *Wedelia chinensis* and its petroleum ether fraction (PEF), chloroform fraction(CLF), ethyl acetate fraction(EAF) and dia-ion resin fraction (DRF) was determined using Folin-Ciocalteu reagent. Phenolic content of the samples were calculated on the basis of the standard curve for gallic acid as shown in Table: 3.1 and in figure 3.1. The results were expressed as mg of gallic acid equivalent (GAE)/g of dried extractives.

Table-3.1: Absorbance of gallic acid at different concentrations after treatment with Folin-Ciocalteu reagent.

Concentration (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
	a	b	c	
1	0.065	0.066	0.065	0.0653 ± 0.0005
2	0.142	0.143	0.143	0.1426 ± 0.0005
4	0.274	0.274	0.275	0.2743 ± 0.0004
8	0.582	0.581	0.582	0.5816 ± 0.0008
16	1.172	1.173	1.173	1.1726 ± 0.0006
32	2.264	2.265	2.263	2.2640 ± 0.0316

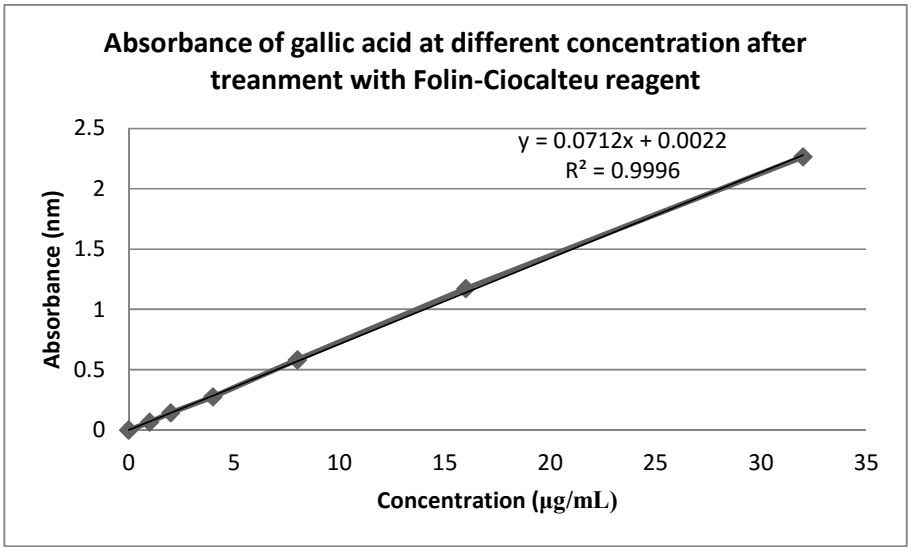


Fig 3.1 : Absorbance of gallic acid at different concentration after treatment with Folin-Ciocalteu reagent.

Table 3.2: Determination of total phenolic content of difference fractions of *wedelia chinensis.*

Name of the Sample	No. of the samples	Concentration (µg/mL)	Absorbance (nm)	GAE / g of dried sample	GAE / g of dried sample Mean ± STD
Crude methanol extract (CME)	1	50	0.573	80.89	80.00±2.589
	2	50	0.538	75.98	
	3	50	0.589	83.14	
Petroleum ether fraction (PEF)	1	50	0.051	7.15	7.34±0.155
	2	50	0.053	7.43	
	3	50	0.054	7.57	
Chloroform fraction (CLF)	1	50	0.240	33.66	33.52±0.198
	2	50	0.239	33.52	
	3	50	0.238	33.38	
Ethyl acetate fraction (EAF)	1	50	0.696	97.61	97.28±0.488
	2	50	0.697	97.75	
	3	50	0.688	96.49	
Dia-ion resin fraction (DRF)	1	50	0.433	60.73	61.05±0.206
	2	50	0.437	61.29	
	3	50	0.436	61.15	

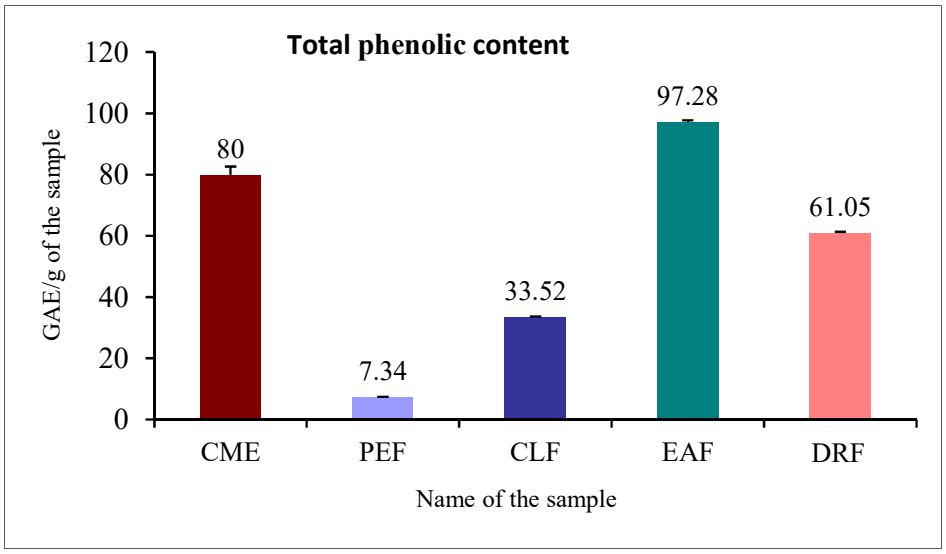


Fig 3.2: Total phenolic content of the crude methanol extract(CME) of *Wedelia chinensis* and it's different fractions (PEF, CLF, EAF and DRF).

The results of phenolic content of crude methanol extract (CME) and its different fractions were shown in table: 3.2 and in Fig. 3.2.

From the result, the phenolic content of crude methanolic extract (CME) was found to be 80 mg of GAE/g of dried extract. Among the fractions of CME, highest phenolic content was found in the ethyl acetate fraction (EAF) (97.28 mg of GAE/ g of dried extract) followed by dia-ion resin fraction (DRF) 61.05 mg of GAE/ g of dried extract, petroleum ether fraction (PEF) and chloroform fraction (CLF) were 7.38 mg and 33.52mg of GAE/ g of dried extract respectively. Comparison of the phenolic content of crude methanol extract with its different fractions, revealed that the ethyl acetate fraction (EAF) contained the highest amount of phenolics.

3.1.2 Determination of the total flavonoids of crude methanol extract (CME) of *Wedelia chinensis* and its different fractions.

Table 3.3: Absorbance of catechin at difference concentrations for quantity determination of total flavonoids.

Concentration (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
	a	b	c	
15.625	0.003	0.003	0.004	0.0033 ± 0.0259
31.25	0.007	0.006	0.008	0.0070± 0.0012
62.50	0.016	0.016	0.017	0.0163 ± 0.0005
125	0.034	0.036	0.035	0.0350 ± 0.0015
250	0.073	0.074	0.074	0.0736 ± 0.0058
500	0.165	0.166	0.167	0.1564 ± 0.0118

Total flavonoids content of the crude methanol extract (CME) and its four fractions (PEF, CLF, EAF and DRF) was determined using much known aluminum chloride colorimetric method. Flavonoid content of the samples was calculated on the basis of the standard curve for catechin as shown in Table-3.3 and in Fig. 3.3. The results were expressed as mg of catechin equivalent (CE)/g of dried sample.

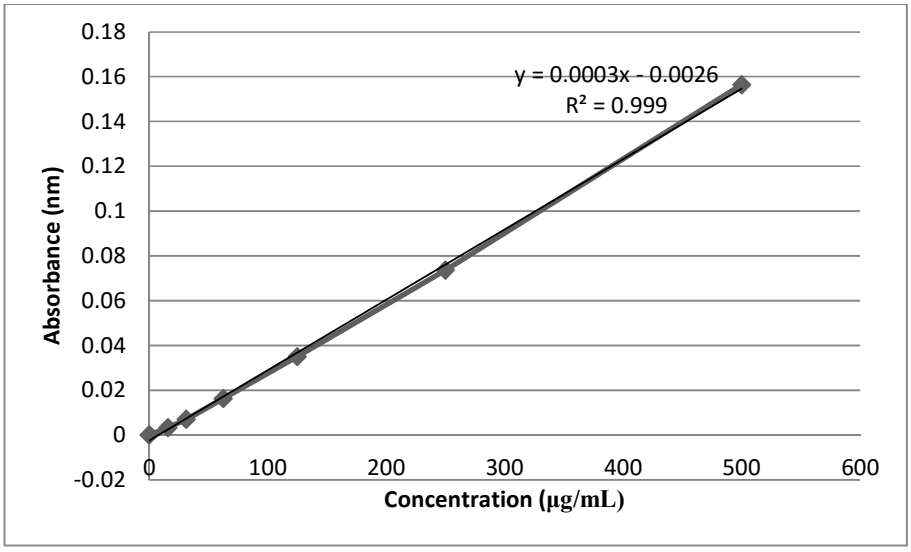


Fig. 3.3: Standard curve of catechin for the determination of total flavonoids.

Table-3.4: Determination of the total flavonoid content of different fractions of *wedeliea chinensis.*

Name of the Sample	No. of the samples	Concentration (µg/mL)	Absorbance (nm)	CE / g of dried sample	CE / g of dried sample Mean ± STD
Crude methanol extract (CME)	1	50	0.493	165.46	174.02 ±5.796
	2	50	0.521	174.80	
	3	50	0.542	181.80	
Petroleum ether fraction (PEF)	1	50	0.005	2.80	2.68±0.2267
	2	50	0.004	2.46	
	3	50	0.005	2.88	
Chloroform fraction (CLF)	1	50	0.098	33.80	33.46±0.3350
	2	50	0.096	33.13	
	3	50	0.097	33.46	
Ethyl acetate fraction (EAF)	1	50	0.430	144.46	144.35±0.5084
	2	50	0.428	143.80	
	3	50	0.431	144.80	
Dia-ion resin fraction (DRF)	1	50	0.523	175.46	175.68±0.6925
	2	50	0.526	176.46	
	3	50	0.522	175.13	

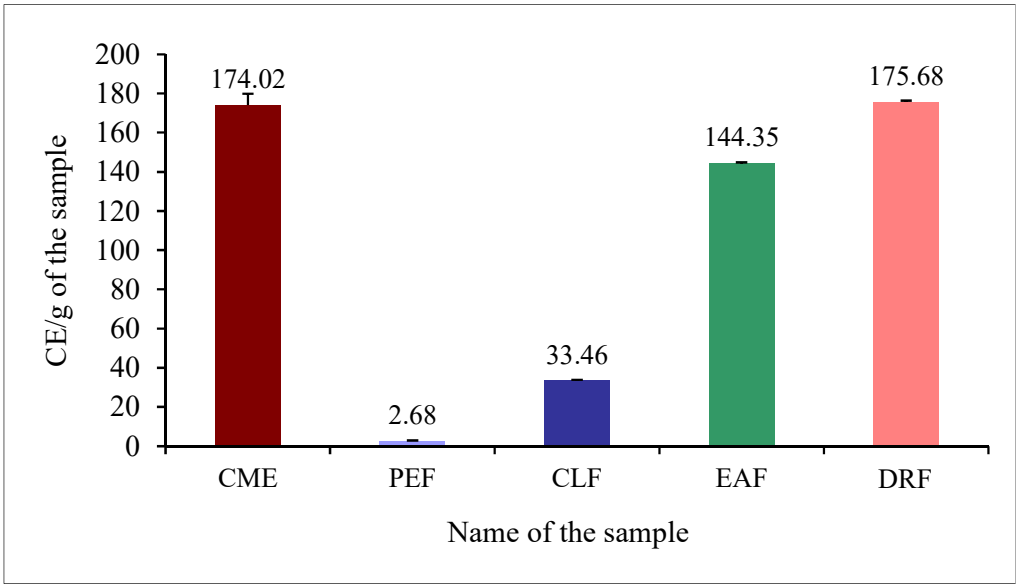


Fig 3.4: Total flavonoid content of the crude methanol extract(CME) of *Wedelia chinensis* and it's different fractions (PEF, CLF, EAF and DRF).

The flavonoid content of the crude methanol extract (CME) and its different fractions were shown in Table -3.4 and Fig. 3.4.

The flavonoid content of CME was found to be 174.02 mg of CE/g of dried extract, whereas the flavonoid content of PEF, CHF, EAF and DRF was 2.68 mg, 33.46 mg, 144.35 mg and 175.68 mg of CE/g of dried extract respectively. These results demonstrated that both dia-ion resin fraction (DRF) and ethyl acetate fraction (EAF) contained a large amount of flavonoids.

3.2 Test of purity of the isolated compounds (EFC-1 and EFC-2)

Compounds EFC-1 and EFC-2 were isolated from the column fraction F-4 and F-10 respectively of the ethyl acetate fraction. The purity of the isolated compounds were checked by using commercially available pre-coated silica gel plate (kiesel gel 60 GF 254) or plastic sheet were used for this purpose. The chromatographic analysis are presented in the Table-3.5.

Table-3.5: Observed chromatographic responses of compounds EFC-1 and EFC-2 on TLC chromatograms.

Compound code	Solvent system	R_f Value	Appearance of spot			
			Day light	UV at 365 nm	Vanillin - H_2SO_4 spray	I_2 chamber
EFC-1	petroleum ether : ethyl acetate (5:4)	0.705	Not visible	Active	Black spot	Yellow spot
	n-hexane : acetone (3:2)	0.82	Not visible	Active	Black spot	Yellow spot
EFC-2	n-hexane : acetone (3:4)	0.69	Not visible	Active	Pink spot	Black spot
	petroleum ether : ethyl acetate (3:4)	0.52	Not visible	Active	Pink spot	Black spot

3.3 Test of purity of the isolated compounds (DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5)

Compounds DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 were isolated from the column fraction F/-3, F/-6, F/-8, F/-11 and F/-16 respectively of the dia-ion resin fraction. The purity of the isolated compounds were checked by using commercially available pre-coated silica gel plate (kieselgel 60 GF 254) or plastic sheet were used for this purpose. The chromatographic analysis are presented in the Table-3.6.

Table-3.6: Observed chromatographic responses of compounds DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 on TLC chromatograms.

Compound code	Solvent system	R_f Value	Appearance of spot			
			Day light	UV at 365 nm	Vanillin - H_2SO_4 spray	I_2 chamber
DFC-1	petroleum ether : acetone (5:3)	0.76	Not visible	Active	Pink spot	Blue spot
	n-hexane : ethyl acetate: methanol (5:2:0.5)	0.58	Not visible	Active	Pink spot	Blue spot
DFC-2	petroleum ether : acetone (1:1)	0.67	Slightly visible	Active	Pink spot	Black spot
	n-hexane : ethyl acetate : methanol (5:3: 0.5)	0.58	Slightly visible	Active	Pink spot	Black spot
DFC-3	petroleum ether : acetone (3:4)	0.69	Slightly visible	Active	Blue spot	Yellow spot
	n-hexane : methanol (5:2)	0.54	Slightly visible	Active	Blue spot	Yellow spot
DFC-4	petroleum ether : acetone (3:5)	0.62	Not visible	Active	Yellow spot	Black spot
	n-hexane : methanol (5:3)	0.58	Not visible	Active	Yellow spot	Black spot
DFC-5	petroleum ether : acetone (1:2)	0.32	Not visible	Active	Pink spot	Blue spot
	n-hexane : methanol (5:4)	0.47	Not visible	Active	Pink spot	Blue spot

Table: 3.7. Physical properties of the isolated compounds (EFC-1, EFC-2, DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5) of *Wedelia chinensis*:

Code of the Comp.	Physical state	Color	Melting point	Solubility
EFC-1	Solid	Yellow	76-79^{0}c	Ethyl acetate, Acetone, DCM, MeOH.
EFC-2	Solid	White	94-97^{0}c	Ethyl acetate, Acetone, MeOH
DFC-1	Amorphous solid	Pink-White	183-186^{0}c	Acetone, MeOH, EtOH.
DFC-2	Amorphous solid	Brown White	114 -117^{0}c	Acetone, DCM, EtOH.
DFC-3	Solid	White	143 -145^{0}c	Acetone, DMSO, MeOH, EtOH
DFC-4	Solid	White	213 -216^{0}c	DMSO, MeOH, EtOH.
DFC-5	Solid	White	252 -255^{0}c	DMSO, MeOH, EtOH.

3.4 Spectral Analyses of isolated compounds

3.4.1 Isolated compound EFC-1

The compound EFC-1 was obtained from the sub-fraction F-3 of the ethyl acetate soluble fraction. Subsequent treatments of F-3 fraction with column chromatography (silica gel; Sephadex LH 20) and recycling HPLC (MeOH : water 3:2) yielded a compound EFC-1 as a white amorphous solid.

UV spectrum showed absorption at 201, 194 and 188 nm indicating the presence of any chromophore group in the compound.

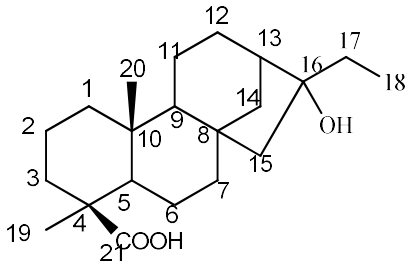
IR spectrum of the compound EFC-1 showed absorption bands at 3412 cm^{-1} for OH function indicates and 3017 cm^{-1} and 1695 cm^{-1} for -COOH group indicate the presence of hydroxyl and carbonyl functional groups in the molecule.

^{1}H-NMR spectrum (300 MHz, CD_3OD) two tertiary methyl group protons appeared at δ 0.92 and δ 1.26. The -CH_3 group protons, appeared as a singlet at δ 1.26, can be assigned as the C-19 methyl group germinal to a -COOH group, which suffers a down field shift due to the presence of -COOH. The other -CH_3 group protons, appeared as a singlet at δ 0.92, can be assigned as C-20 methyl group. Terminal methyl group protons appeared as a triplet at δ 0.96 with a coupling constant (*J*) 5.0 Hz and is

assigned as C-21 methyl group. Only one isolated CH_2 protons peaks appeared at δ 3.56 as a AB quartet with coupling (*J*) 7.60 Hz and is assigned as C-17 methylene proton. One methyl proton at C-13 appeared as a broad triplet like signal due to the presence of the protons at C-12 and at C-14. Another two methane protons at C-5 and C-9 appeared as a doublet with a coupling constant of 5.1 Hz. Methylene protons at C-15 appeared as a singlet at 2.00. Others high field peaks are not suitable for first order analysis.

^{13}C-NMR spectrum (CD_3OD, 300 MHz) of compound EFC-1 showed twenty one carbons which included three methyl, ten methylene, three methane, four quaternary carbons and one carboxyl group. Methyl groups are C-18, C-19 and C-20. Methylene groups are C-1, C-2, C-3, C-6, C-11, C-12, C-14, C-15 and C-17. Methane groups are C-5, C-9 and C-13. Quaternary carbons are in C-4, C-8, C-10 and C-16. In the compound EFC-1, there is no exocyclic double bond, instead there may be a $–CH_2$-CH_3 (ethyl group) attached at C-16 position. Another two tertiary methyl groups are attached at C-4 and at C-10 positions. The COOH group is also attached at C-4 position.

From the above spectral evidence the structure of the compound EFC-1 was established as (-) Kaur-16α hydroxyl-21 oic acid.



(-) Kaur-16 hydroxy-21 oic acid (EFC-1)

Table-3.8: ^{1}H-NMR spectral data (300 MHz, CD_3OD) of compound EFC-1.

No. of proton	δ value in ppm (*J* in Hz)
H-1	1.87 (2H, bd, J = 13.0 Hz)
H-2	1.36 (2H, bd, J = 13.0 Hz)
H-3	2.13 (2H, bd, *J*= 10.0 Hz)
-	
H-5	1.02 (each 1H, bd, *J*= 5.1 Hz)
H-6	2.08 (2H, bd, *J*= 10.0 Hz)
H-7	2.06 (2H, bd, *J*= 9.2 Hz)
-	
H-9	1.02 (each 1H, bd, *J*= 5.1 Hz)
-	
H-11	2.17 (2H, bd, *J*= 8.0 Hz)

No. of proton	δ value in ppm (*J* in Hz)
H-12	2.15 (2H, bd, *J*= 8.2 Hz)
H-13	2.71(1H, bt like)
H-14	1.78 (2H, bd, *J*= 10.0 Hz)
H-15	2.00 (2H, s)
-	
H-17	3.56 (2H, ABq, *J*= 7.60 Hz)
CH_3-18	0.96 (3H, t, *J*= 6.10 Hz)
CH_3-19	0.92 (3H, s)
CH_3-20	1.26 (3H, s)
16-OH	2.5 (1H, s)
21-COOH	11.2 (1H, s)

Table-3.9: ^{13}C-NMR spectral data (300 MHz, CD_3OD) of compound EFC-1.

^{13}C-NMR spectral data and Multiplicity			**Authentic (-) Kaur-16-en-19 oic acid**	
Number of carbon	**Multiplicity**	**δ value in ppm**	**Multiplicity**	**δ value in ppm**
C-1	CH_2	40.67	CH_2	40.90
C-2	CH_2	19.28	CH_2	19.20
C-3	CH_2	38.01	CH_2	38.00
C-4	C	43.87	C	44.00
C-5	CH	56.89	CH	57.30
C-6	CH_2	22.15	CH_2	22.00
C-7	CH_2	42.08	CH_2	41.50
C-8	C	46.18	C	44.80
C-9	CH	56.01	CH	55.40
C-10	C	39.87	C	39.80
C-11	CH_2	18.88	CH_2	18.50
C-12	CH_2	26.27	CH_2	33.20
C-13	CH	47.86	CH	44.00
C-14	CH_2	37.64	CH_2	39.80
C-15	CH_2	54.61	CH_2	49.00
C-16	C	79.45	C	156.00
C-17	CH_2	20.62	$=CH_2$	103.30
C-18	CH_3	29.17	CH_3	29.00
C-19	CH_3	14.38	COOH	185.40
C-20	CH_3	15.83	CH_3	15.70
C-21	COOH	183.11		

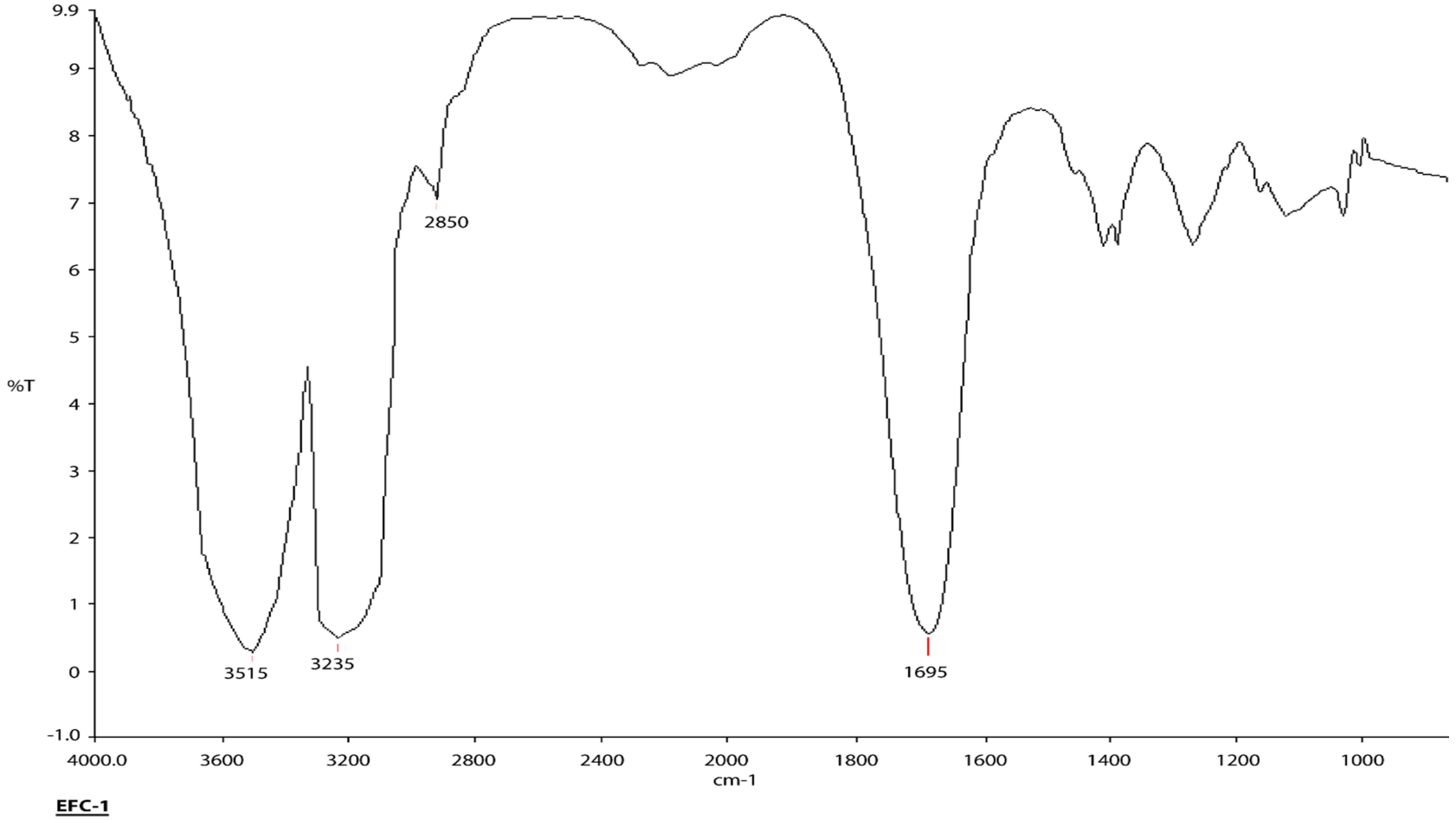


Fig 3.5: IR Spectrum of compound EFC-1

^{1}H-NMR Spectrum (300 MHz, CD_3OD) of compound EFC-1

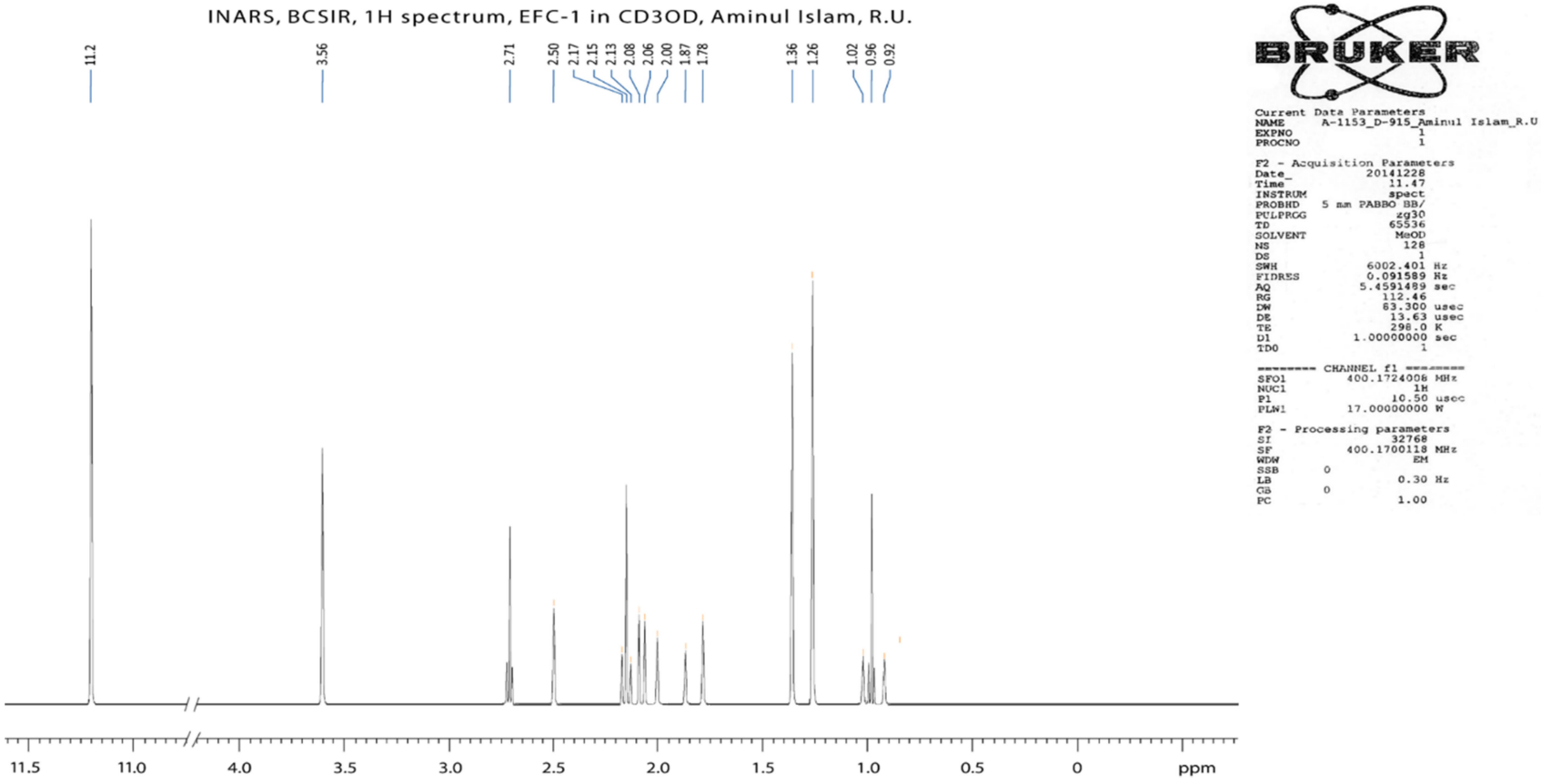


Fig 3.6 : ^{1}H-NMR spectrum of compound EFC-1

^{13}C-NMR Spectrum (300 MHz, CD_3OD) of compound EFC-1

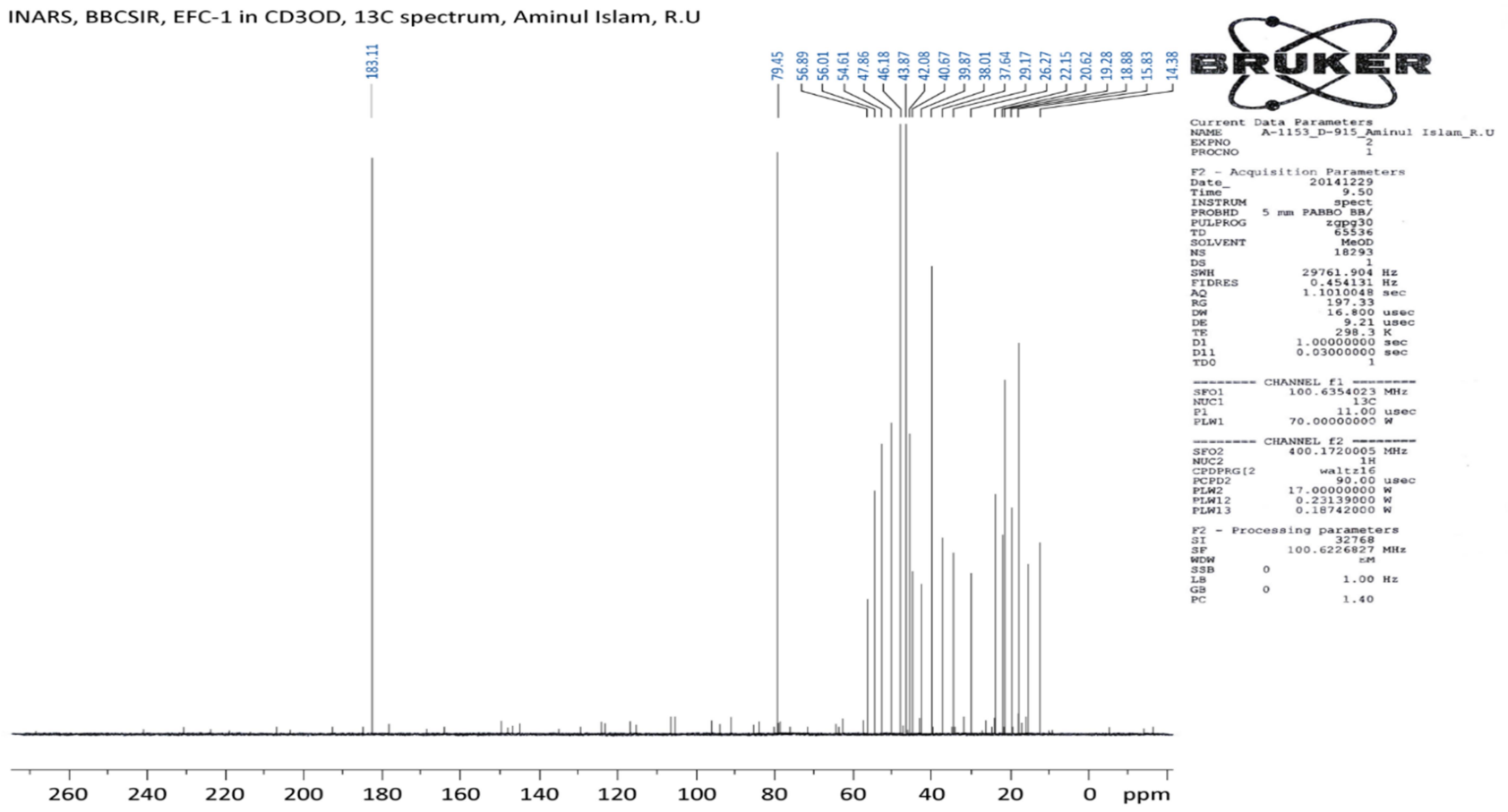


Fig 3.7 : ^{13}C-NMR spectrum of compound EFC-1

3.4.2 Isolated compound EFC-2

The compound EFC-2 was obtained from F- 10 as white crystal from ethyl acetate fraction of the plant. It showed a single spot on TLC examination under UV light at 254 nm, bound with iodine and spraying the developed plate with vanillin-sulfuric acid followed by heating at 110^0C for about 10 minutes gave pinkish violet color. The R_f value of the compound was found 0.67 in petroleum : acetone (1:1) and 0.55 in dichloromethane : ethyl acetate (2:9). It is also positively responded to Liebermann-Burchard and Salkowski tests. These chemical test and TLC behavior revealed that EFC-2 might be a steroidal or terpenoidal compound. The compound was identified as (-) kaur-16-en-19-oic acid by comparing its ^{13}C-NMR data with the literature data .

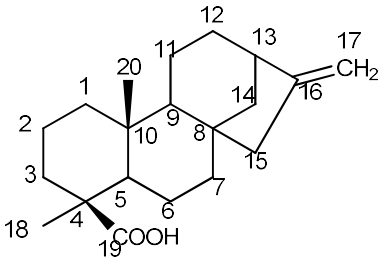
UV spectrum showed absorption at 201, 194 and 188 nm indicating the presence of any chromophore group in the compound.

From its IR spectrum the compound EFC-2 showed an absorption band at 3000 ~ 2850 cm^{-1} for $–CH_3$ group and 1710 cm^{-1} for a - COOH group and for unsaturation ($=CH_2$) stretching at 3015 cm^{-1} and (C = C) stretching at 1634 cm^{-1}. Absorption spectrum 3013 cm^{-1} indicate =C – H in aromatic ring and 1605 cm^{-1} indicate C=C in aromatic ring.

From its ^{1}H-NMR spectrum, two tertiary methyl proton signals appeared at δ 0.94 and δ 1.23. The $-CH_3$ protons, appeared as a singlet at δ 1.23, can be assigned as the C-18 methyl group germinal to a -COOH group, which suffers a down field shift due to the presence of a COOH group. The other $-CH_3$ protons, appeared as a singlet at δ 0.94, can be assigned as C-20 methyl group. The vinyl protons ($=CH_2$) at C-17 in an exocyclic double bond appeared as a doublet with a *J* value of 29.1 Hz at δ 4.74. C-13 Proton appeared as a broad triplet like signal due to the presence of the protons at C-12 and at C-14.

^{13}C-NMR spectrum (CD_3OD, 100 MHz) of compound EFC-2 showed twenty carbons which included two methyl, ten methylene, three tertiary carbons, four quaternary carbons and one carboxyl group. Methyl groups are C-4 and C-10. Methylene groups are C-1, C-2, C-3, C-6, C-7, C-11, C-12, C-14, C-15 and C-17. Tertiary carbons are C-5, C-9 and C-13. Quaternary carbons are in C-4, C-8, C-10 and C-16. In the

compound EFC-2, instead there may be a vinyl protons (=CH_2) attached at C-16 position. Another four quaternary carbons are at C-4, C-8, C-10 and C-16 positions. The COOH group is also attached at C-4 position.



Structure of compound EFC-2

Table- 3.10 : ^{1}H-NMR spectrum data (400 MHz, CD_3OD) of compound EFC-2.

Position of proton	**^{1}H-NMR spectrum data, δ values in ppm (*J* in Hz)**
H-1	1.87 (2H, bd, *J* = 14.0 Hz)
H-2	1. 43 ~ 1.48 (2H, m)
H-3	2.12 (2H, bd, *J* = 14.0 Hz)
H-5 and H-9	1.03 ~ 1.09 (1H, m like)
H-6	1.82 ~ 1.85 (2H, m)
H-7	0.83 (2H, dt, *J* = 6.5 Hz)
H-11	1.57 (2H, bd, *J* = 5.8 Hz)
H-12	1.64 (2H, bd, *J* = 5.8 Hz)
H-13	2.62 (1H, bt like)
H-14	1.98 (2H, d, *J* = 10.8 Hz)
H-15	2.03 (2H,s)
H-17	4.74 (2H, d, *J* = 29.1 Hz)
CH_3-18	1.23 (3H, s)
CH_3-20	0.94 (3H, s)
COOH-19	11.2 (1H, s)

Table -3.11: The ^{13}C-NMR spectral data of the compound EFC-2.

Position of Carbon	^{13}C-NMR spectra		Authentic (-) Kaur-16-en-19 oic acid	
	δ value in ppm	Multiplicity	δ value in ppm	Multiplicity
C-1	41.12	CH_2	40.90	CH_2
C-2	19.52	CH_2	19.20	CH_2
C-3	38.21	CH_2	38.00	CH_2
C-4	44.17	C	44.00	-C-
C-5	57.34	CH	57.30	CH
C-6	22.27	CH_2	22.00	CH_2
C-7	41.83	CH_2	41.50	CH_2
C-8	44.61	C	44.80	-C-
C-9	55.47	CH	55.40	CH
C-10	40.13	C	39.80	-C-
C-11	18.95	CH_2	18.50	CH_2
C-12	33.47	CH_2	33.20	CH_2
C-13	44.31	CH	44.00	CH
C-14	40.27	CH_2	39.80	CH_2
C-15	49.31	CH_2	49.00	CH_2
C-16	156.24	C	156.00	-C-
C-17	103.42	CH_2	103.30	CH_2
C-18	29.35	CH_3	29.00	CH_3
C-19	184.42	COOH	185.40	COOH
C-20	16.41	CH_3	15.70	CH_3

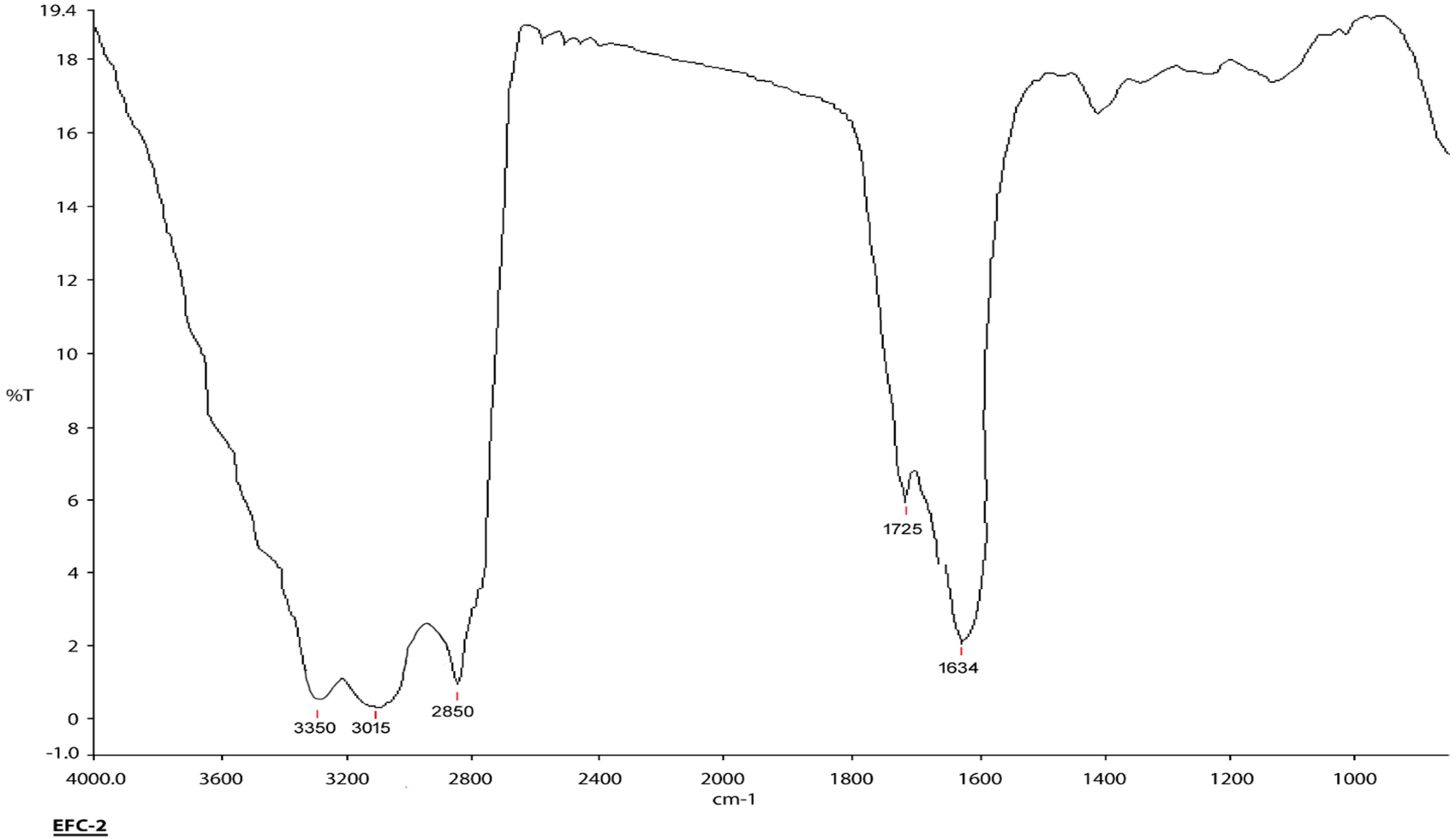


Fig 3.8: IR Spectrum of compound EFC-2

^{1}H-NMR Spectrum (300 MHz, CD_3OD) of compound EFC-2

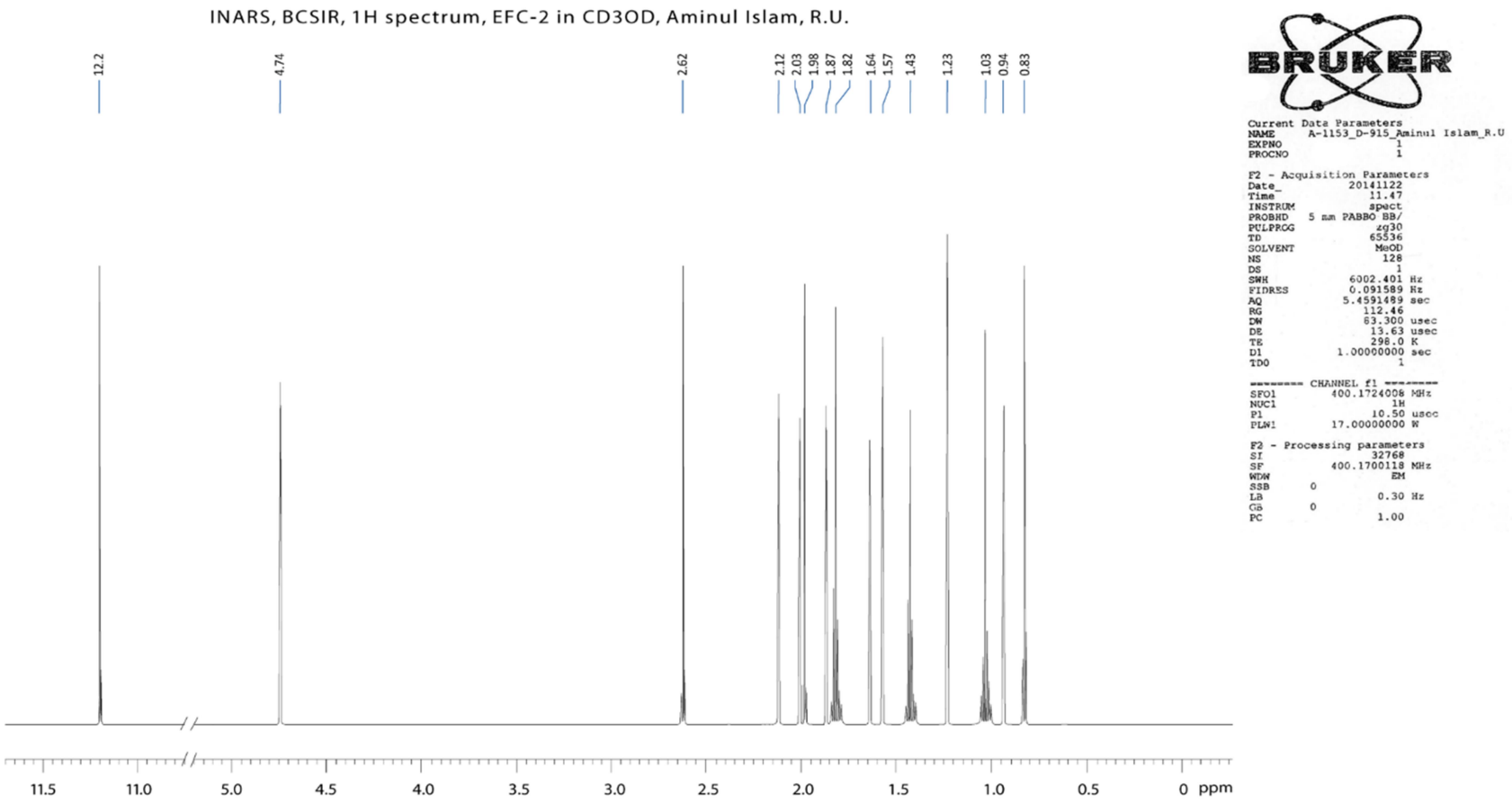
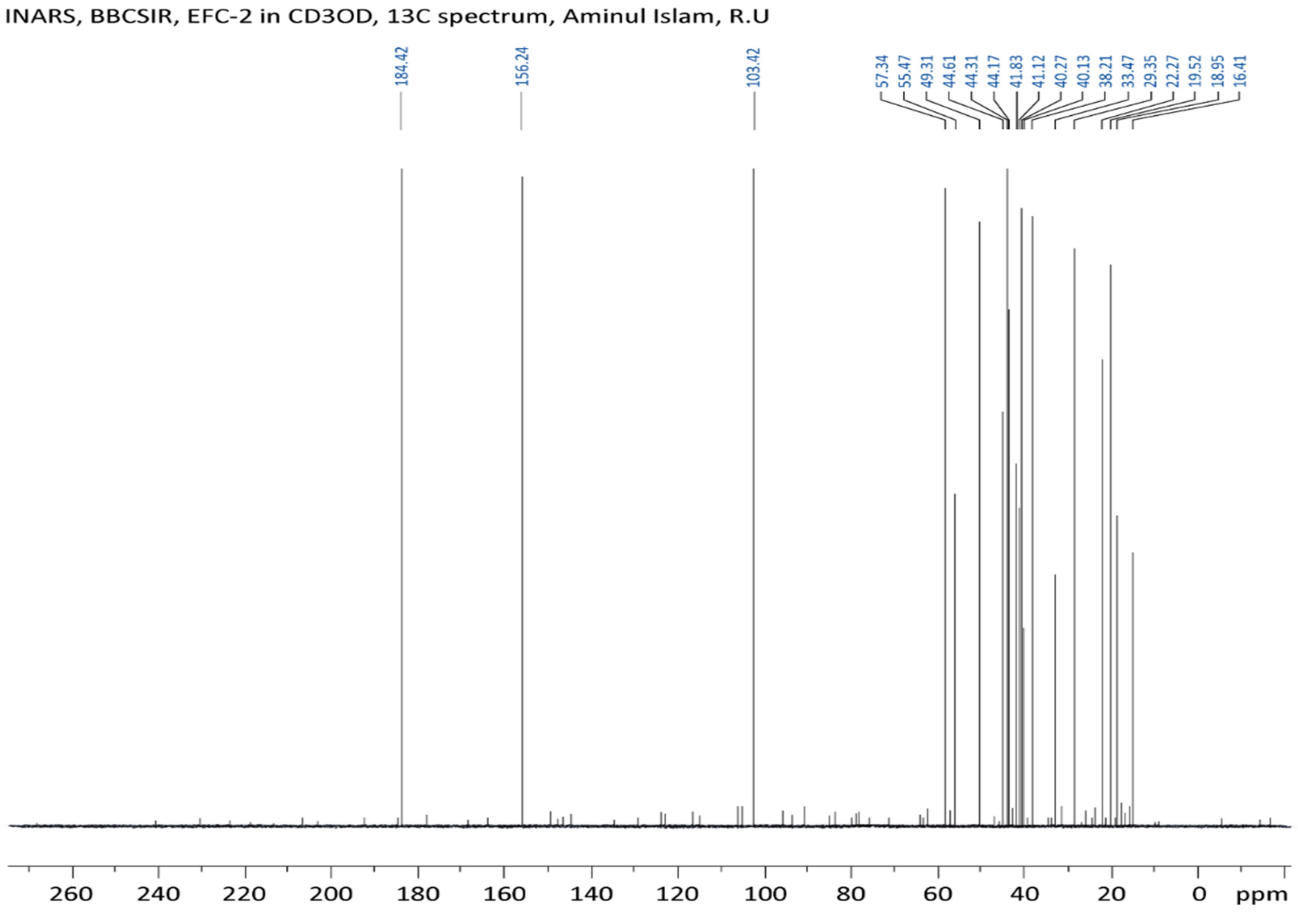


Fig 3.9 : ^{1}H-NMR Spectrum of compound EFC-2

^{13}C-NMR Spectrum (300 MHz, CD_3OD) of compound EFC-2



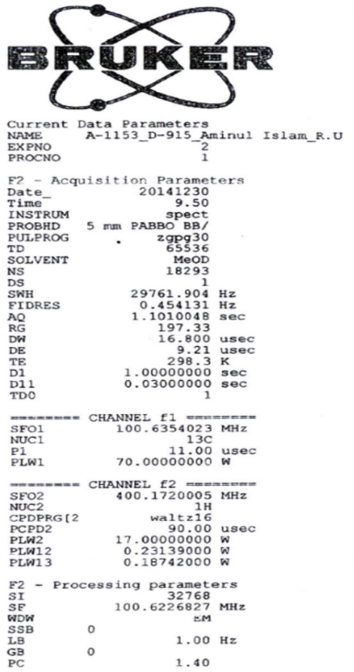


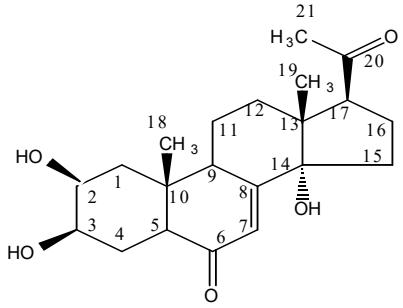
Fig 3.10 : ^{13}C-NMR Spectrum of compound EFC-2

3.4.3 Isolated compound DFC-1

The compound DFC-1 was isolated from the dia-ion resin fraction. Column chromatography (petroleum ether : acetone = 5:3) and HPLC over silica gel with n-hexane : ethyl acetate : methanol (5:2:0.5) of F/-3 fraction. This compound is pale brown amorphous solid.

The UV spectrum of compound DFC-1 displayed absorption at 240 nm.

The IR spectrum of compound DFC-1 displayed broad absorption band at 3412 cm^{-1} due to hydroxyl functions. The IR spectrum also showed absorption at 2925 and 1527 cm^{-1} due to C-H and C=C functions. The absorption at 1700 and 1672 cm^{-1} in the IR spectrum of DFC-1 were indicative of the presence of a saturated carbonyl group in addition to an α,β-unsaturated keto group.



Structure of compound DFC-1

The structure of compound DFC-1 was evident from the ^{1}H-NMR spectra (table-3.12). The downfield signals at δ 0.63, 0.97 and 2.15 were assigned to tertiary methyl protons of C-18, C-19 and C-21 respectively. Another two multiplet proton signals at δ 3.95 and 4.52 indicated the presence of hydroxyl group at C-2 and C-3 respectively. A doublet appeared at δ 5.83 with a coupling constant of 2.42 Hz for C-7 proton.

The broad-band (BB) decoupled ^{13}C-NMR spectra of the compound DFC-1 showed resonances for all 21 carbons in the molecule. The spectrum revealed the presence of three methyl, six methylene and six quaternary carbons. The downfield signals at δ 68.72, 68.42 and 85.25 were due to three hydroxyl groups at carbon C-2, C-3 and C-14 respectively. The signal at δ 205.47 indicated the presence of a carbonyl group at C-6. The downfield signals at δ 122.83 and 166.61 were probable due to tertiary

olefinic carbons of C-7 and C-8. The signal at δ 85.25 indicated the C-14 hydroxyl group with α-orientation and the signal at δ 121.46 was probably due to carbonyl group at C-20. The analysis of the spectral data indicated a cholest-7-en-6-one skeleton in the molecule.

On the basis of the IR, ^{1}H-NMR, ^{13}C-NMR spectroscopic evidences it became apparent that the compound DFC-1 belonged to the steroidal series and was characterized as 2,3,14-tri hydroxypregn-7-ene-6,20-dione.

Table- 3.12: ^{1}H-NMR spectral data (300 MHz, CD_3OD) of compounds DFC-1.

Position of proton	δ value in ppm (*J* in Hz)	Position of proton	δ value in ppm (*J* in Hz)
H-1	1.43 (d) , (9.0 Hz)	-	
H-2	3.95 (q), (2.87 Hz)	-	
H-3	4.56 (q), (2.4 Hz)	H-15	2.34 (t), (3.45 Hz)
H-4	1.74 (d), (3.5 Hz)	H-16	2.12 (q), (2.78 Hz)
H-5	2.42 (t), (2.4 Hz)	H-17	2.78 (t), (2.55 Hz)
-		H-18	0.63 (s)
H-7	5.83 (s)	H-19	0.96 (s)
-		-	-
H-9	3.34 (t), (1.95 Hz)	H-21	2.17 (s)
-		2-OH	4.2 (1H, s)
H-11	1.45 (q), (1.67 Hz)	3-OH	4.5(1H, s)
H-12	1.73 (t), (2.75 Hz)	14-OH	3.5(1H, s)

Table-3.13: ^{13}C-NMR spectral data (300 MHz, CD_3OD) of compounds DFC-1.

^{13}C-NMR spectral data and Multiplicity			**Authentic Cholest-7-en-6-one**	
Position of carbon	**Multiplicity**	**δ value in ppm**	**Multiplicity**	**δ value in ppm**
C-1	CH_2	37.37	CH_2	37.37
C-2	CH	68.72	CH	68.68
C-3	CH	68.42	CH	68.47
C-4	CH_2	32.13	CH_2	32.10
C-5	CH	51.85	CH	51.81
C-6	C	205.47	C	205.23
C-7	CH	71.25	CH	122.50
C-8	C	75.35	C	166.50
C-9	CH	35.27	CH	35.15
C-10	C	39.29	C	39.24
C-11	CH_2	21.73	CH_2	21.61
C-12	CH_2	32.12	CH_2	32.10
C-13	C	48.38	C	48.40
C-14	C	85.25	C	84.99
C-15	CH_2	30.85	CH_2	30.79
C-16	CH_2	22.21	CH_2	22.18
C-17	CH	60.13	CH	60.16
C-18	CH_3	17.61	CH_3	17.51
C-19	CH_3	24.57	CH_3	24.42
C-20	C	212.46	C	212.44
C-21	CH_3	31.54	CH_3	31.52

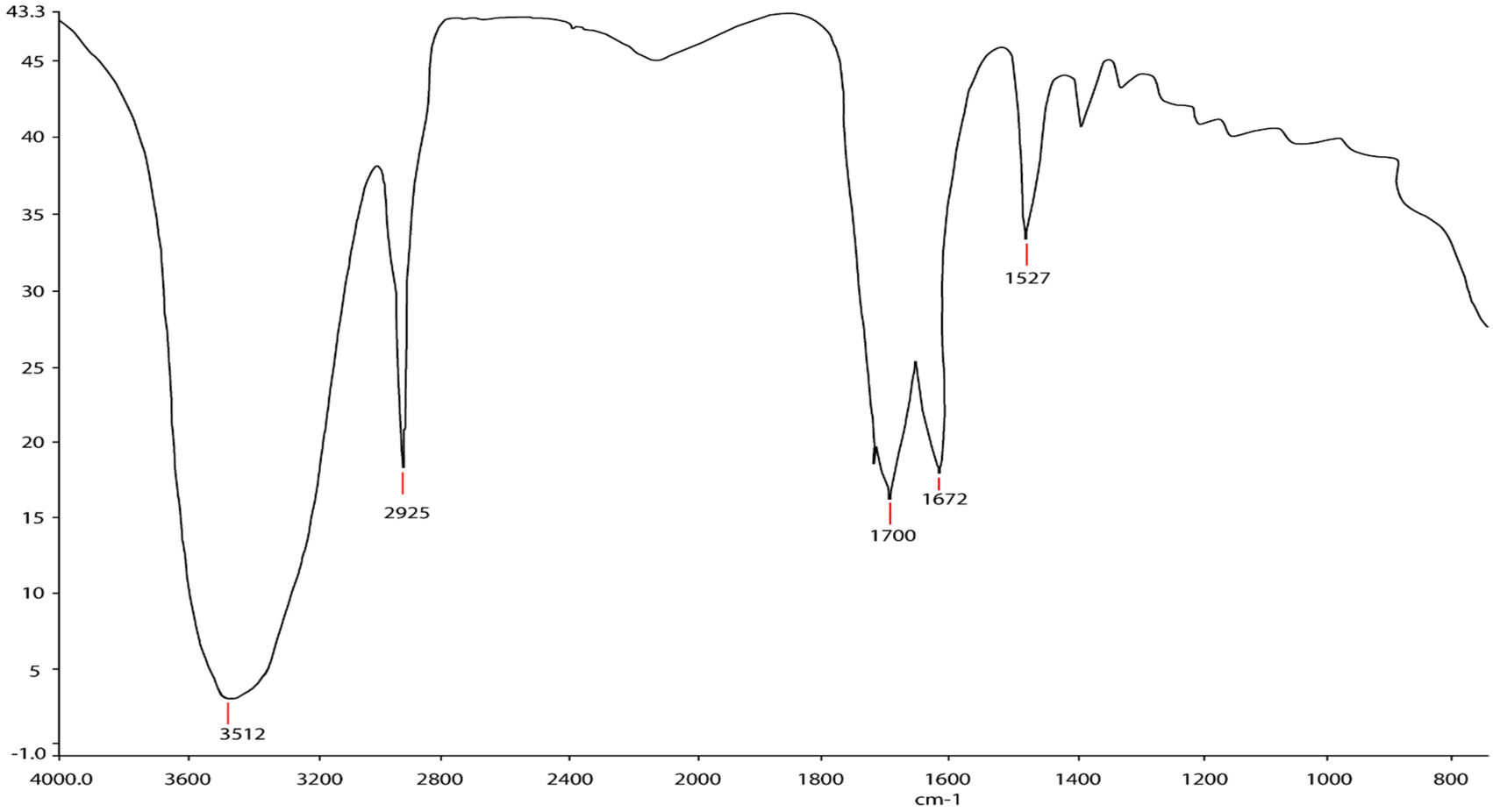


Fig 3.11 : IR Spectrum of compound DFC-1

^{1}H-NMR Spectrum (300 MHz, CD_3OD) of compound DFC-1

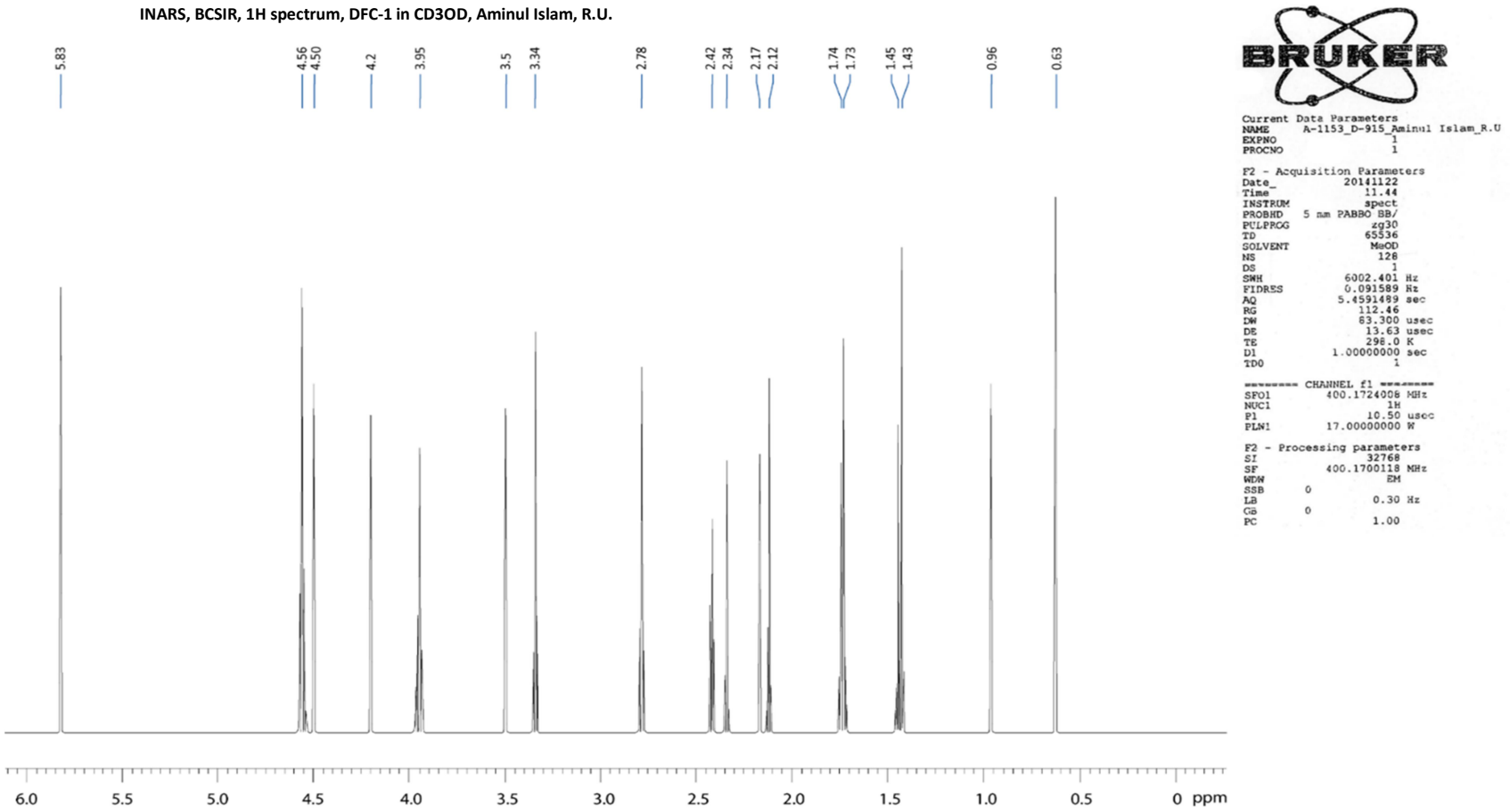


Fig 3.12: ^{1}H-NMR spectrum of compound DFC-1

^{13}C-NMR Spectrum (300 MHz, CD_3OD) of compound DFC-1

INARS, BCSIR, 13C spectrum, DFC-1 in CD3OD, Aminul Islam, R.U.

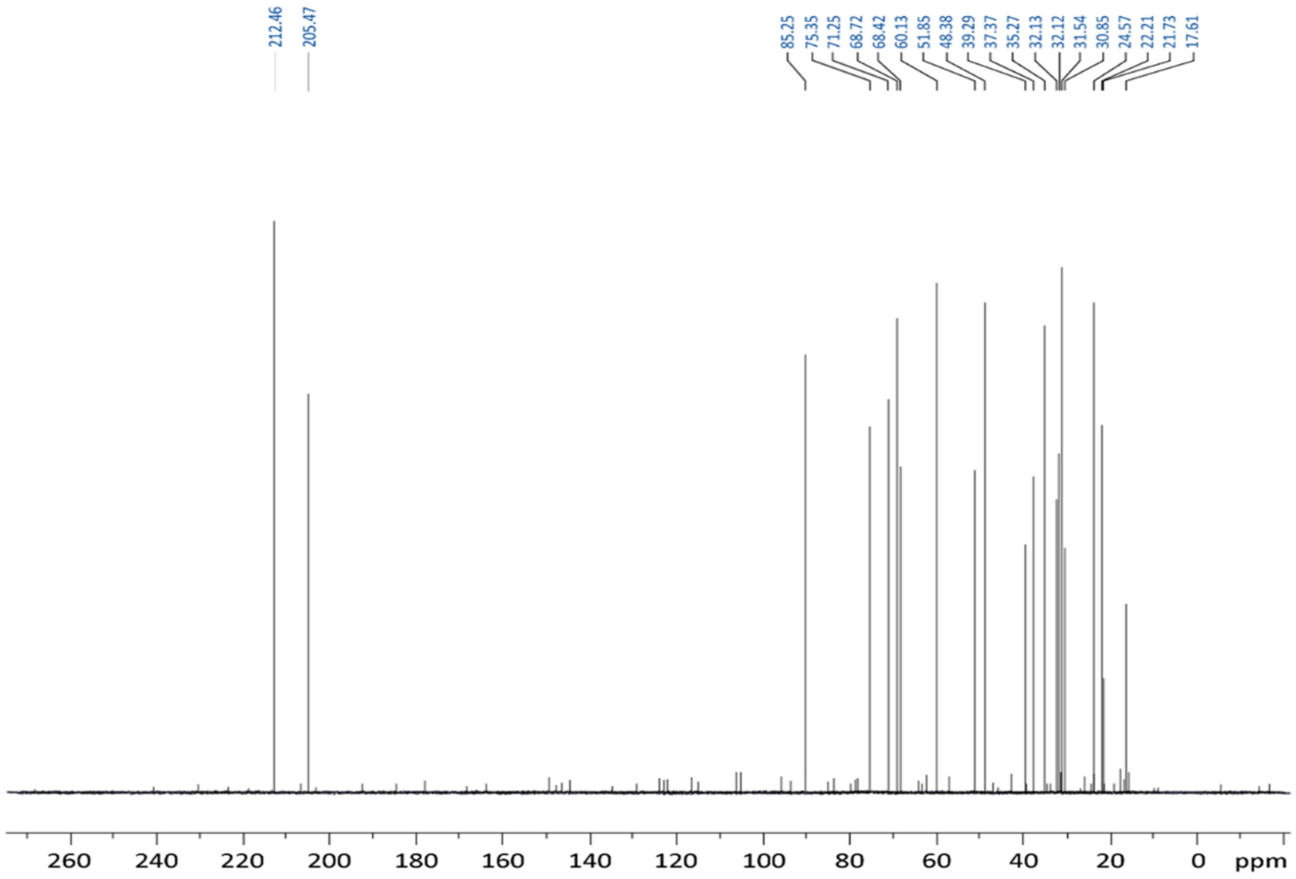


Fig 3.13: ^{13}C-NMR spectrum of compound DFC-1

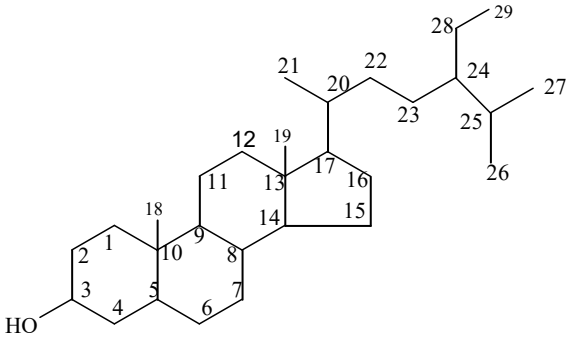
3.4.4 Isolated compound DFC-2

The compound DFC-2 was obtained as white crystal from dia-ion resin fraction of the plant. It showed a single spot on TLC examination under UV light at 254 nm, bound with iodine and spraying the developed plate with vanillin-sulfuric acid followed by heating at 110^0C for about 10 minutes gave pinkish violet color. The R_f value of the compound was found 0.69 in n-hexane : acetone (3:4) and 0.55 in dichloromethane : ethyl acetate (2:9). It is also positively responded to Liebermann-Burchard and Salkowski tests. These tests suggested that the compound was steroidal in nature.

From its IR spectrum, the compound DFC-2 showed absorption bands at 3423 cm^{-1} for -OH function indicates the presence of hydroxyl function and stretching for C = C at 1634 cm^{-1} in the molecule.

From its ^{1}H-NMR spectrum (300 MHz, CD_3OD), H-3 proton appeared as triplets of a double doublet (tdd) at δ 3.25 with coupling constants of 11.4 Hz and δ 4.2 Hz and the H-6 olefinic proton showed a multiplet between δ 4.87 ~ 5.22. Six methyl protons appeared at δ 1.23, δ 1.12, δ 0.83, δ 0.89, δ 0.89 and δ 0.98. These assignment are in good assignment for the structure of β-sitosterol.

^{13}C-NMR spectrum (CD_3OD, 300 MHz) of compound DFC-2 showed twenty nine carbons which included six methyl, twelve methylene, nine tertiary carbon, two quaternary carbons and one hydroxyl group. Methyl groups are C-18, C-19, C-21, C-26, C-27 and C-29. Methylene groups are C-1, C-2, C-4, C-6, C-7, C-11, C-12, C-15, C-16, C-22, C-23 and C-18. Tertiary carbons are in C-3, C-5, C-8, C-9, C-14, C-17, C-20, C-24 and C-25. Quaternary carbons are in C-10 and C-13. In the compound EFC-2, there is no exocyclic double bond, instead there may be a -CH_2-CH_3 (ethyl group) attached at C-24 position. Nine tertiary methyl groups are attached with C-4, C-7, C-8, C-13, C-16, C-17, C-23 and C-24 positions. The OH group is also attached at C-3 position.



β -Sitosterol (DFC-2)

Table-3.14: ^{1}H-NMR spectral data (300 MHz, CD_3OD) of compound DFC-2.

Position of proton	δ value in ppm (*J* in Hz)	Position of proton	δ value in ppm (*J* in Hz)
H-1	1.89 (2H, bd, J = 14.0 Hz)	H-16	2.05 (2H, q, J = 10.2 Hz)
H-2	1.45 ~ 1.51 (2H, m)	H-17	2.02 (1H, d, J = 9.5 Hz)
H-3	3.25 (1H, tdd, *J* = 11.4 Hz and 4.2 Hz)	H-20	1.87 (1H, m like)
H-4	1.84 ~ 1.87 (2H, m)	H-22	2.08 (2H, q, J= 8.0 Hz)
H-5	1.06 ~ 1.09 (1H, m like)	H-23	2.07 (2H, q, J = 8.0 Hz)
H-6	2.15 ~ 2.22 (1H, m)	H-24	1.92 (1H, m like)
H-7	0.83 (2H, dt, J = 6.5 Hz)	H-25	1.97 (1H, m like)
H-8	1.06 ~ 1.09 (1H, m like)	H-28	2.04 (2H, q, J= 7.5 Hz)
H-9	1.06 ~ 1.09 (1H, m like)	3-OH	3.85(1H, s)
-		CH_3-18	1.23 (3H, s)
H-11	1.58 (2H, bd, J = 6.0 Hz)	CH_3-19	1.12 (3H, s)
H-12	1.65 (2H, bd, J = 6.2 Hz)	CH_3-21	0.83 (3H, d, *J* = 16.2 Hz)
-		CH_3-26	0.89 (3H, s)
H-14	2.62 (1H, bt, like)	CH_3-27	0.93 (3H,s)
H-15	2.02 (2H, q, J = 11.0 Hz)	CH_3-29	0.98 (3H, t, *J* = 23.5 Hz)

Table-3.15: ^{13}C-NMR spectral data (300 MHz, CD_3OD) of compound DFC-2.

^{13}C-NMR spectral data and Multiplicity			Authentic β-sitosterol	
Position of carbon	**Multiplicity**	**δ value in ppm**	**Multiplicity**	**δ value in ppm**
C-1	CH_2	37.35	CH_2	37.2
C-2	CH_2	31.51	CH_2	31.5
C-3	CH	72.20	CH	71.7
C-4	CH_2	42.26	CH_2	42.3
C-5	CH	40.60	CH	14.7
C-6	CH	21.17	CH	121.7
C-7	CH_2	32.05	CH_2	31.9
C-8	CH	33.05	CH	31.9
C-9	CH	50.25	CH	50.1
C-10	C	36.52	C	36.5
C-11	CH_2	21.17	CH_2	21.1
C-12	CH_2	40.51	CH_2	39.6
C-13	C	42.40	C	42.2
C-14	CH	57.21	CH	56.8
C-15	CH_2	24.32	CH_2	24.3
C-16	CH_2	28.71	CH_2	28.2
C-17	CH	56.52	CH	56.1
C-18	CH_3	19.34	CH_3	19.4
C-19	CH_3	12.22	CH_3	11.9
C-20	CH	36.63	CH	36.1
C-21	CH_3	18.81	CH_3	19.3
C-22	CH_2	34.37	CH_2	34.9
C-23	CH_2	26.60	CH_2	29.1
C-24	CH	46.33	CH	50.1
C-25	CH	29.53	CH	26.1
C-26	CH_3	19.80	CH_3	18.7
C-27	CH_3	19.55	CH_3	29.8
C-28	CH_2	23.53	CH_2	23.1
C-29	CH_3	12.43	CH_3	11.8

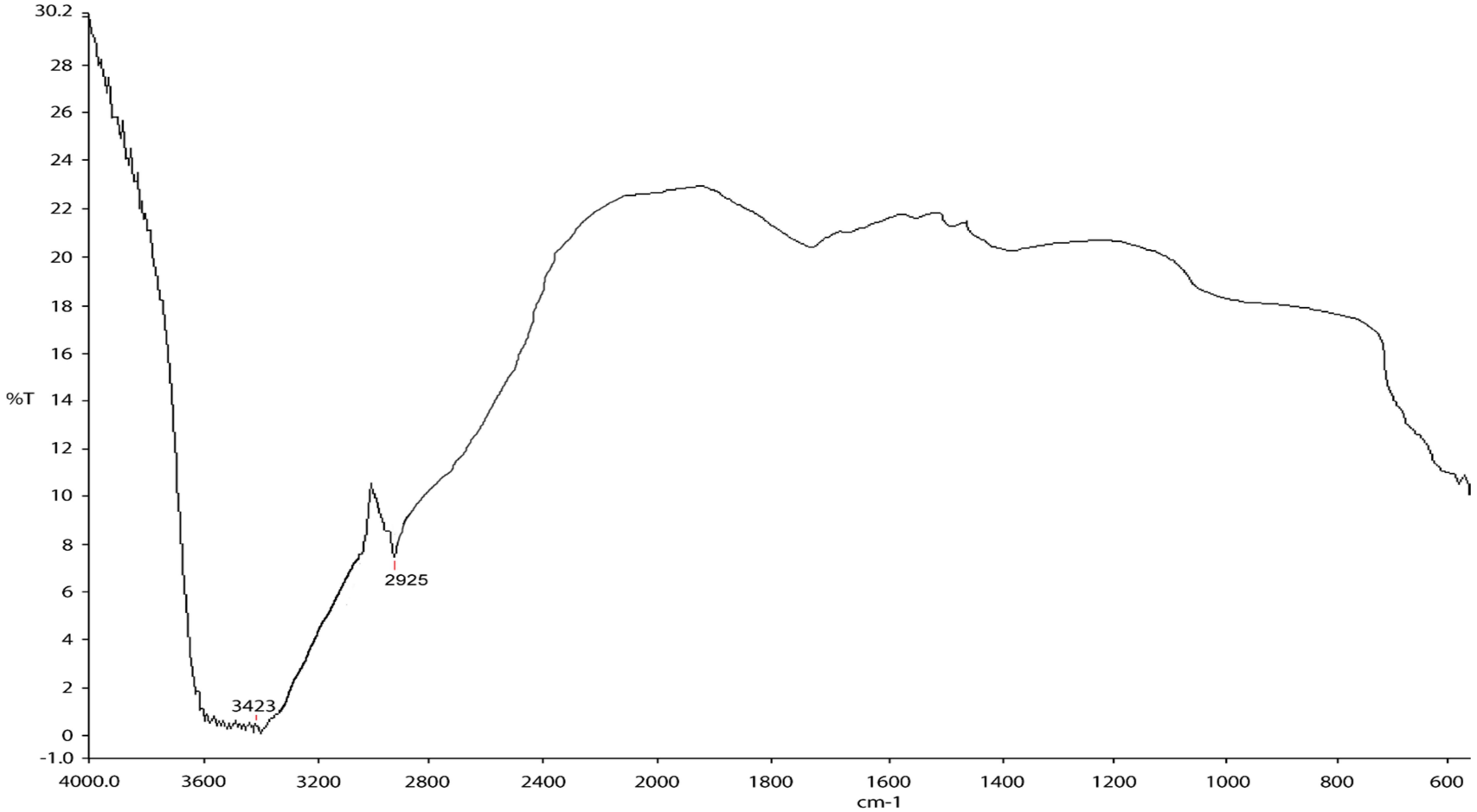
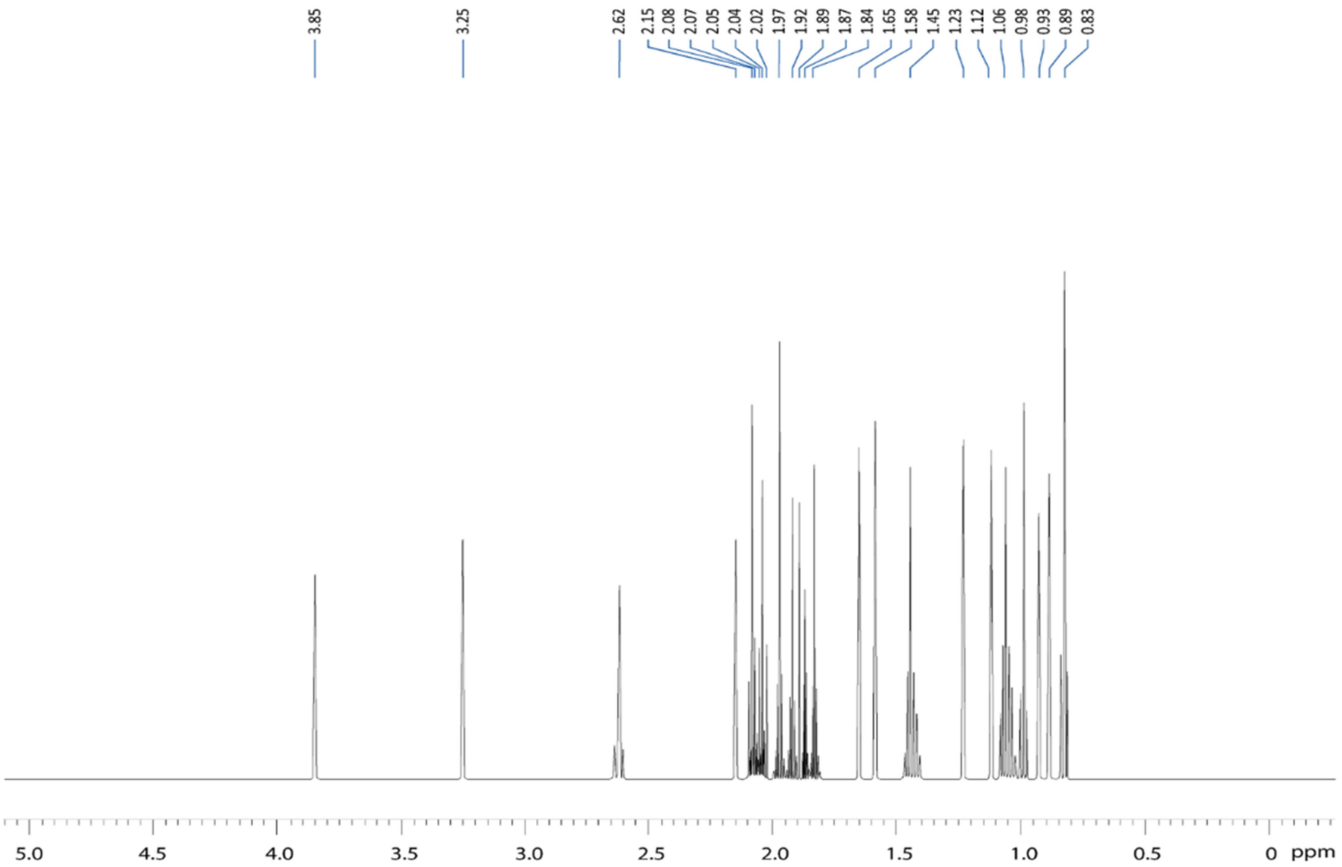


Fig 3.14 : IR Spectrum of compound DFC-2

^{1}H-NMR Spectrum (300 MHz, CD_3OD) of compound DFC-2

INARS, BCSIR, 1H spectrum, DFC-2 in CD3OD, Aminul Islam, R.U.



BRUKER

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Fig 3.15 : ^{1}H-NMR spectrum of compound DFC-2

^{13}C-NMR Spectrum (300 MHz, CD_3OD) of compound DFC-2

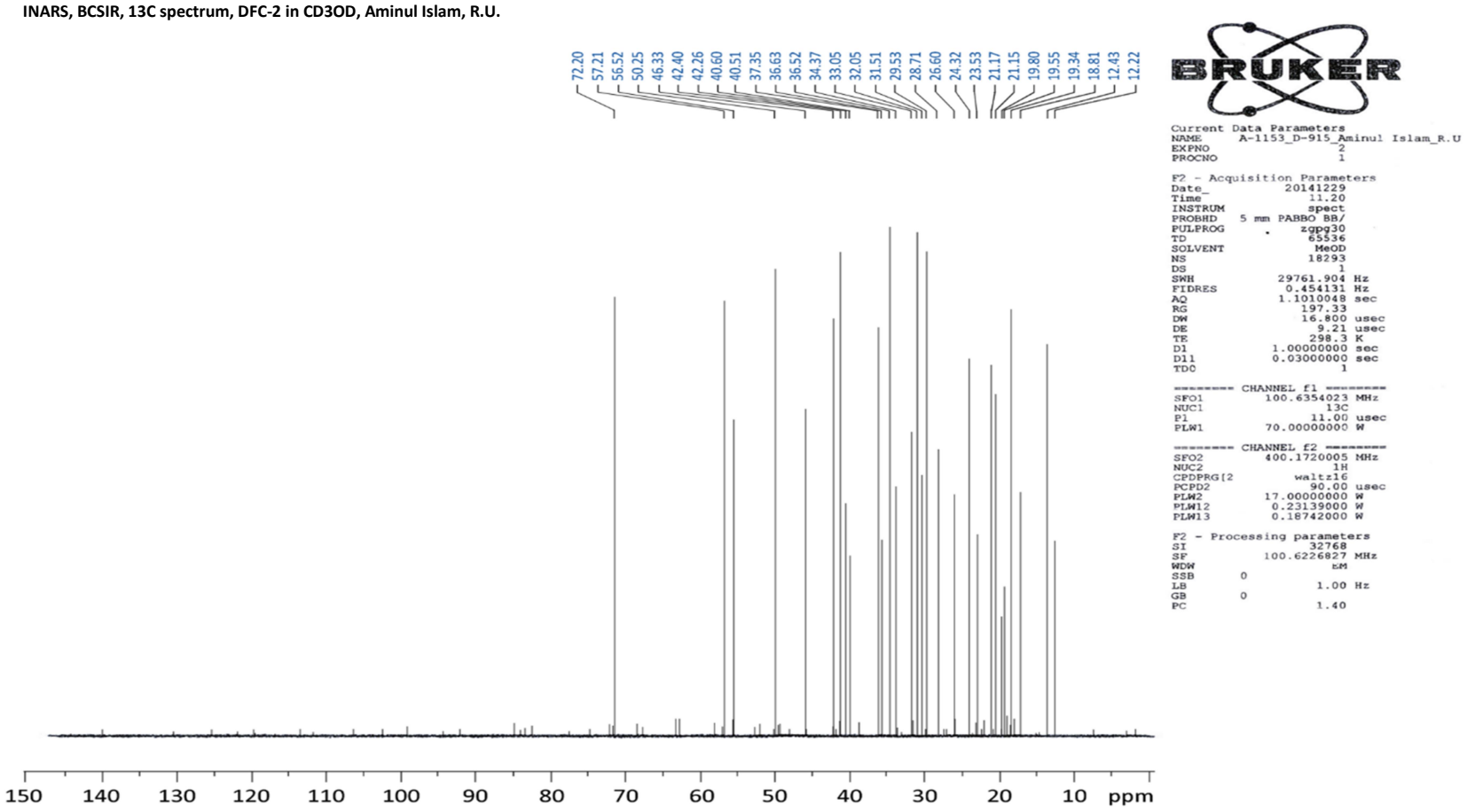


Fig 3.16 : ^{13}C-NMR spectrum of compound DFC-2

3.4.5 Isolated compound DFC-3

The compound DFC-3 was isolated from F/-8 of the dia-ion resin fraction as white needle shaped crystal. The compound was found to be highly soluble in acetone, methanol and ethanol, sparingly soluble in ethyl acetate but insoluble in petroleum ether and chloroform.

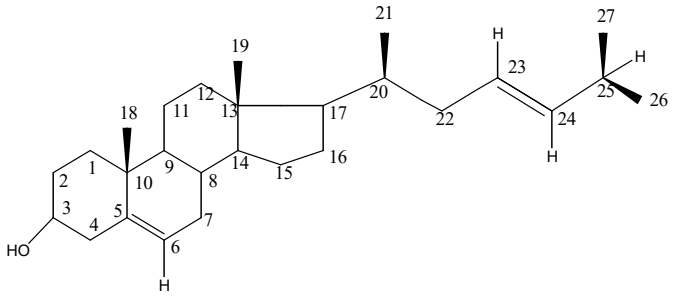
The compound showed a single spot on TLC examination under UV light at 254 nm, bound with iodine and spraying the developed plate with vanillin-sulfuric acid followed by heating at 110^0C for about 10 minutes gave pinkish violet color. The R_f value of the compound was found 0.69 in petroleum ether : acetone (3:4) and 0.54 in n-hexane : methanol (5:2). It is also positively responded to Liebermann-Burchard and Salkowski tests. These chemical test and TLC behavior revealed that DFC-3 might be a steroidal or terpenoidal compound. The compound was gave positive test for steroid and unsaturation.

From its IR spectrum the compound DFC-3 showed an absorption band at 3362 cm^{-1} for –OH group, stretching for C = C at 1634 cm^{-1}.

From its ^{1}H-NMR spectrum (500 MHz, CD_3OD), the compound showed the presence of five methyl protons at δ 0.72, δ 0.84, δ 1.04, δ 0.83, δ 0.86. Moreover, four low field signals were appeared as a sextet at δ 3.25 and is assigned for H-3 proton a signal of steroidal nucleus. Two signal at δ 5.07 and δ 5.00 both appeared as doublets of a double doublet with coupling constants of (15.0 Hz and 8.5 Hz) and (15.0 Hz and 8.5 Hz) and were assigned as H-23 and H-24 respectively. Another proton at H-6 appeared as a broad doublet at δ 5.27 with a J value of 5.12 Hz. Other peaks for saturated methyl groups appeared at high filed and are not assignable.

^{13}C-NMR spectrum (CD_3OD, 500 MHz) of compound DFC-3 showed twenty seven carbons which included five methyl, nine methylene, ten tertiary carbons, three quaternary carbons and one hydroxyl group. Methyl groups are attached with C-10, C-13, C-20 and C-25. Methylene groups are C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-

16 and C-22. Tertiary carbons are C-3, C-6, C-8, C-9, C-14, C-17, C-20, C-23, C-24 and C-25. Quaternary carbons are in C-5, C-10 and C-13. In the compound AFC-3, the OH group is also attached at C-3 position. The ^{13}C-NMR spectral data are given in Table -3.17.



Cholesta-5, 23-dien-3-ol (DFC-3)

Table-3.16: ^{1}H-NMR spectral data (300 MHz, CD_3OD) of compound DFC-3.

Position of proton	δ value in ppm (*J* in Hz)	Position of proton	δ value in ppm (*J* in Hz)
H-1	1.89 (2H, bd, J = 14.0 Hz)	H-15	2.02 (2H, q, J = 11.0 Hz)
H-2	1.45 ~ 1.51 (2H, m)	H-16	2.05 (2H, q, J = 10.2 Hz)
H-3	3.25 (1H, sextet/m)	H-17	2.02 (1H, d, J = 9.5 Hz)
H-4	1.84 ~ 1.87 (2H, m)	H-20	1.87 (1H, m like)
H-5	1.06 ~ 1.09 (1H, m like)	H-22	2.08 (2H, q, J= 8.0 Hz)
H-6	5.27 (1H, bd, J= 5.12 Hz)	H-23	5.07(1H, dd,J=15.0 Hz and 8.5 Hz)
H-7	0.83 (2H, dt, J = 6.5 Hz)	H-24	5.00 (1H,dd, J=15.0 Hz and 8.5 Hz)
H-8	1.06 ~ 1.09 (1H, m like)	H-25	1.97 (1H, m like)
H-9	1.06 ~ 1.09 (1H, m like)	CH_3-18	0.72 (3H, s)
-		CH_3-19	0.84 (3H, s)
H-11	1.58 (2H, bd, J = 6.0 Hz)	CH_3-21	1.04 (3H, d, *J* = 16.2 Hz)
H-12	1.65 (2H, bd, J = 6.2 Hz)	CH_3-26	0.83 (3H, d, J = 7.0 Hz)
-		CH_3-27	0.86 (3H, d, J = 6.5 Hz)
H-14	2.62 (1H, bt, like)	3-OH	4.25 (1H, s)

Table-3.17: ^{13}C-NMR spectral data (300 MHz, CD_3OD) of compound DFC-3.

^{13}C-NMR spectral data and Multiplicity			Authentic Cholesterol	
Position of carbon	**Multiplicity**	**δ value in ppm**	**Multiplicity**	**δ value in ppm**
C-1	CH_2	37.35	CH_2	37.3
C-2	CH_2	31.75	CH_2	32.1
C-3	CHOH	72.21	CHOH	71.9
C-4	CH_2	42.28	CH_2	42.7
C-5	C	140.65	C	141.1
C-6	CH	121.53	CH	121.6
C-7	CH_2	32.25	CH_2	32.3
C-8	CH	32.15	CH	32.1
C-9	CH	50.25	CH	50.5
C-10	C	36.55	C	36.8
C-11	CH_2	21.25	CH_2	21.4
C-12	CH_2	40.15	CH_2	40.2
C-13	C	42.42	C	42.7
C-14	CH	57.12	CH	57.2
C-15	CH_2	24.85	CH_2	24.5
C-16	CH_2	28.78	CH_2	28.3
C-17	CH	56.55	CH	56.7
C-18	CH_3	14.13	CH_3	12.0
C-19	CH_3	19.25	CH_3	19.5
C-20	CH	41.16	CH	35.9
C-21	CH_3	18.85	CH_3	18.9
C-22	CH_2	24.53	CH_2	36.5
C-23	CH	126.65	CH_2	24.1
C-24	CH	136.35	CH_2	39.8
C-25	CH	52.15	CH	28.1
C-26	CH_3	21.18	CH_3	22.6
C-27	CH_3	21.15	CH_3	22.8

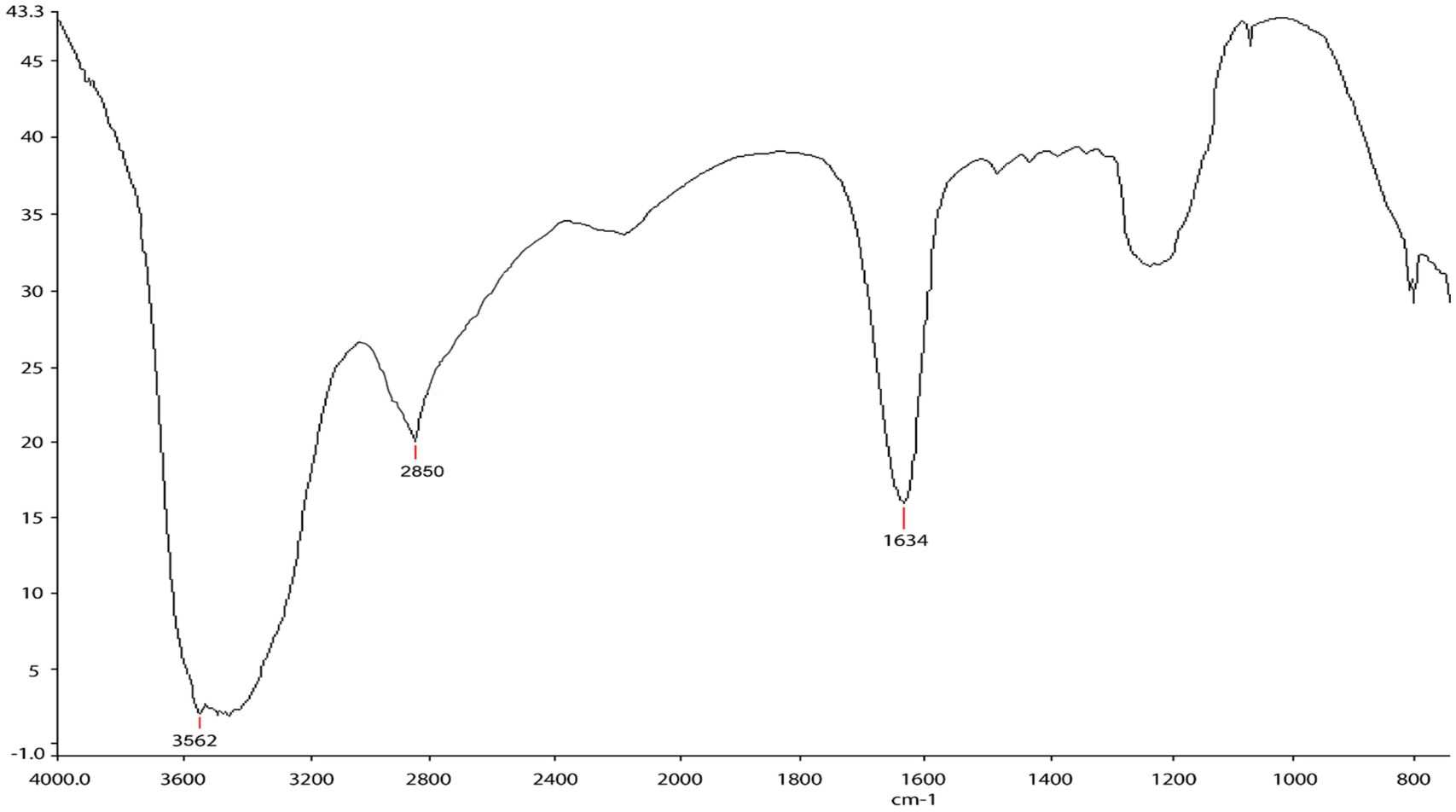


Fig 3.17 : IR Spectrum of compound DFC-3

^{1}H-NMR Spectrum (300 MHz, CD_3OD) of compound DFC-3

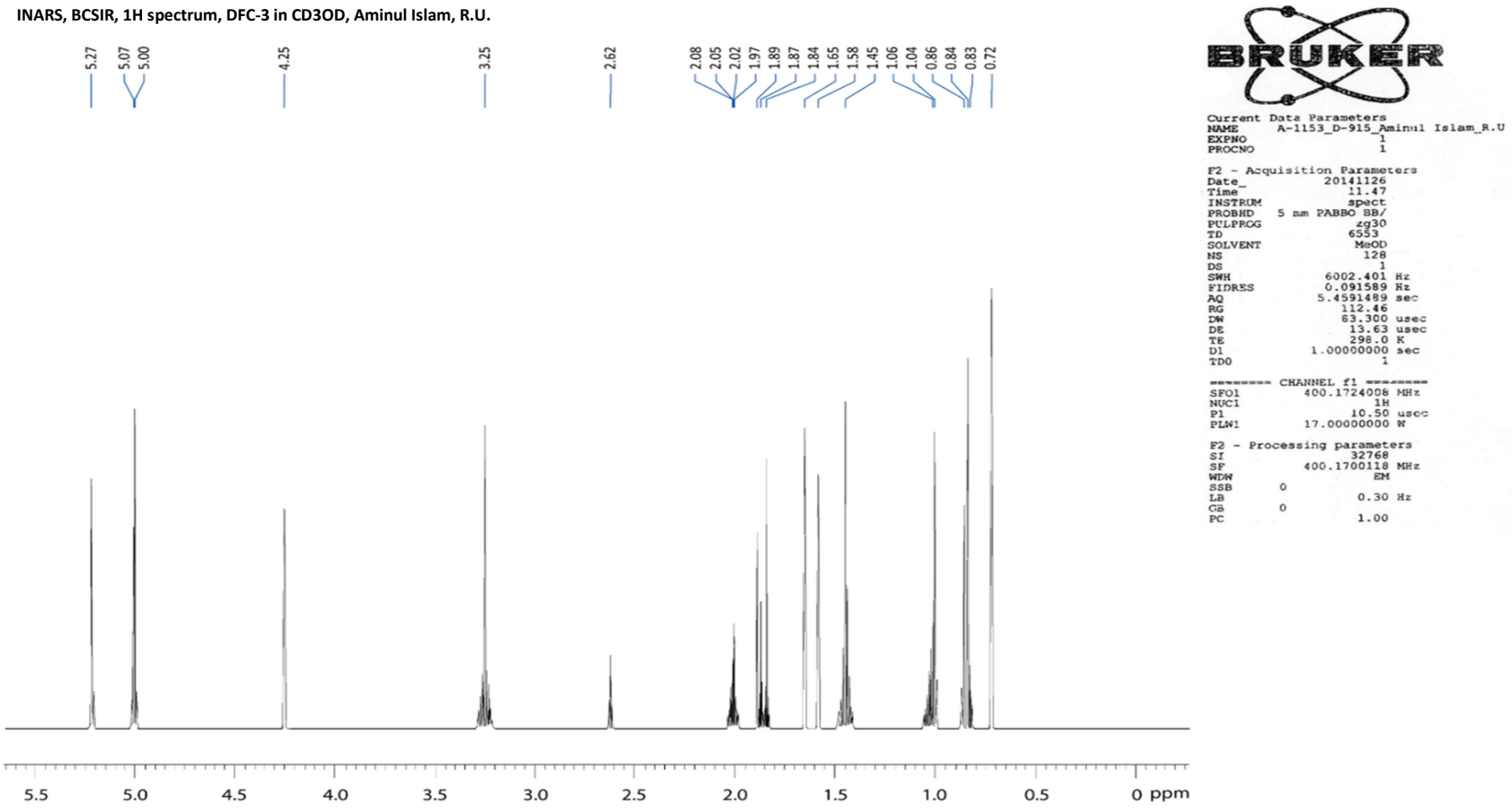


Fig 3.18 : ^{1}H-NMR spectrum of compound DFC-3

^{13}C-NMR Spectrum (300 MHz, CD_3OD) of compound DFC-3

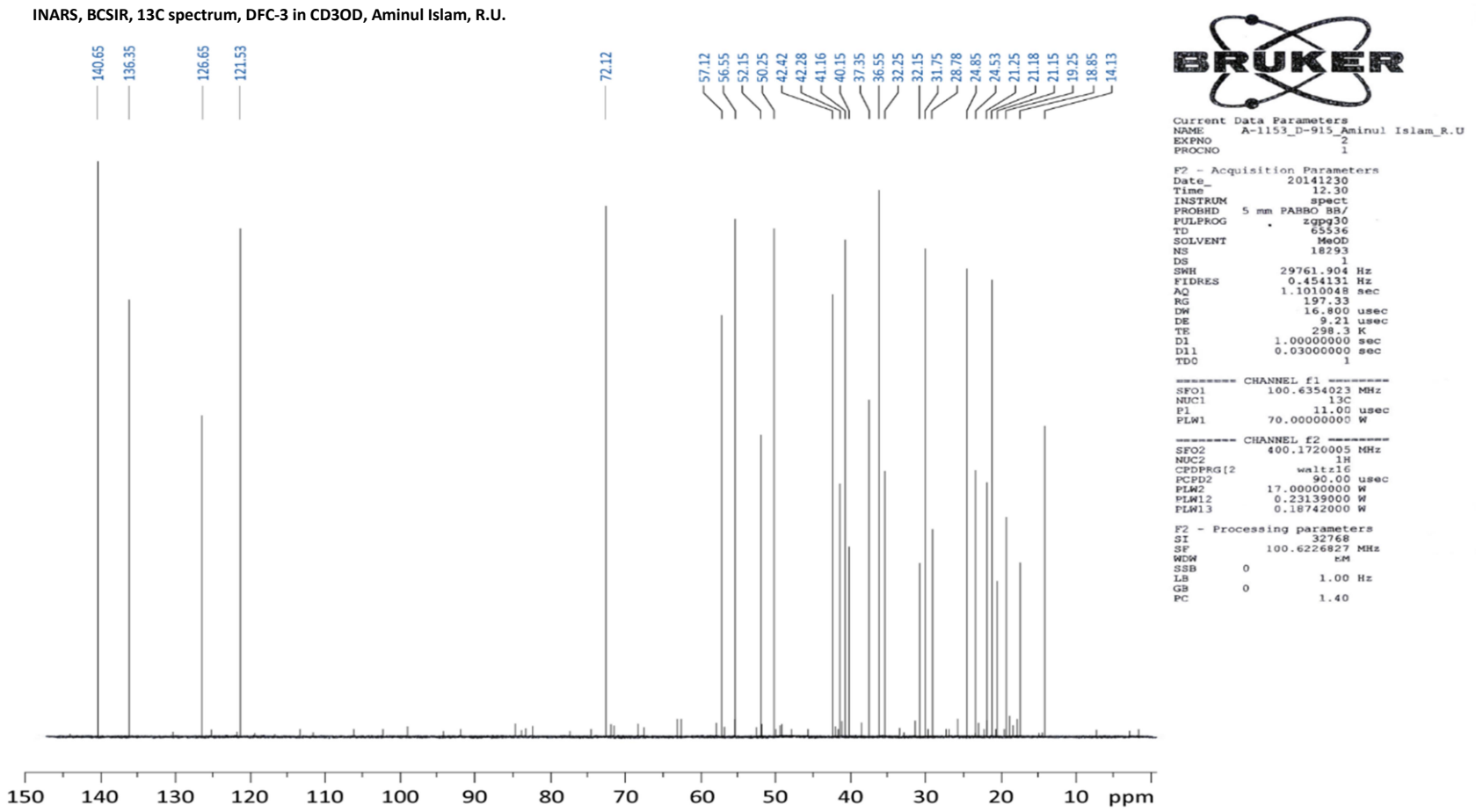


Fig 3.19 : ^{13}C-NMR spectrum of compound DFC-3

3.4.6 The compound DFC-4

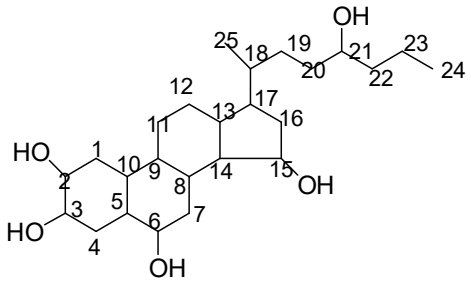
The compound DFC-4 isolated from F/-11 of the dia-ion resin fraction having R_f value 0.62 in petroleum ether : acetone (3: 5) and 0.58 in n-hexane : methanol (5 : 3) obtained as solid white crystal decomposed at 123 – 126^{0}c. The compound was found to be sparingly soluble in cold methanol and highly soluble in warmed methanol, DMSO and ethanol but the compound was insoluble in n-hexane, pet. ether, chloroform and ethyl acetate.

Preliminary the compound showed a positive test for carbonyl function, unsaturation and phenolic hydroxyl groups. Moreover, the presence of flavonoid structure was confirmed by treatment of this compound with $AlCl_3$ spray reagent when yellowish green coloured spot was developed on TLC plate.

In it's IR spectrum showed the presence of –HO groups and a broad absorption band appeared between 3400 – 3100 cm^{-1}.

From its ^{1}H-NMR spectrum (500 MHz, CD_3OD), H-3 proton appeared as triplets of a double doublet (tdd) at δ 3.23 with coupling constants of 11.6 Hz and δ 4.1 Hz. Two methyl protons appeared at δ 1.35 and δ 1.42. The hydroxyl group protons showed at δ 12.73, δ 12.78, δ 11.33, δ 13.17 and δ 11.54. These assignment are in good assignment for the structure of new compound.

^{13}C-NMR spectrum (CD_3OD, 500 MHz) of compound DFC-4 showed twenty five carbons which included two methyl, ten methylene, twelve tertiary carbon and five hydroxyl group. Two methyl groups are attached at C-18 and C-23. Methylene groups are C-1, C-4, C-7, C-11, C-12, C-16, C-19, C-20, C-22 and C-23. Tertiary carbons are C-2, C-3, C-5, C-6, C-8, C-9, C-13, C-14, C-15, C-17, C-18 and C-21. In the compound DFC-4, there is no exocyclic double bond. Five hydroxyl groups are attached with C-2, C-3, C-6, C-15 and C-21 positions.



Compound of DFC-4

Table-3.18: ^{1}H-NMR spectral data (300 MHz, CD_3OD) of compound DFC-4.

Position of proton	δ value in ppm (*J* in Hz)	Position of proton	δ value in ppm (*J* in Hz)
H-1	1.92 (2H, bd, J = 13.5 Hz)	H-16	2.07 (2H, q, J = 10.2 Hz)
H-2	3.12 ~ 3.54 (1H, m)	H-17	2.05 (1H, d, J = 9.5 Hz)
H-3	3.23 (1H, tdd, *J* = 11.6 Hz and 4.1 Hz)	H-18	1.79 (1H, m like)
H-4	1.91 ~ 1.97 (2H, m)	H-19	2.11 (2H, q, J= 8.0 Hz)
H-5	2.21 ~ 2.27 (1H, m)	H-20	2.12 (H, q, J = 8.0 Hz)
H-6	2.87 ~ 2.92 (1H, m)	H-21	3.97 (1H, m like)
H-7	0.85 (2H, dt, J = 6.5 Hz)	H-22	2.08 (1H, m like)
H-8	1.08 ~ 1.09 (1H, m like)	H-23	2.04 (2H, q, J= 7.5 Hz)
H-9	1.05 ~ 1.07 (1H, m like)	CH_3-24	1.35 (3H, s)
H-10	1.06 ~ 1.08 (1H, m like)	CH_3-25	1.42 (3H, s)
H-11	1.56 (2H, bd, J = 6.0 Hz)	OH-2	3.15 (1H, s)
H-12	1.67 (2H, bd, J = 6.2 Hz)	OH-3	3.55 (1H, s)
H-13	1.69 (1H, bd, J = 6.2 Hz)	OH-6	3.82 (1H, s)
H-14	1.73 (1H, bd, J = 6.2 Hz)	OH-15	3.25 (1H, s)
H-15	3.03 (1H, q, J = 11.0 Hz)	OH-21	3.62 (1H, s)

Table-3.19: ^{13}C-NMR spectral data (500 MHz, CD_3OD) of compound DFC-4.

^{13}C-NMR spectral data and Multiplicity			**Authentic β-sitosterol**	
Position of carbon	**Multiplicity**	**δ value in ppm**	**Multiplicity**	**δ value in ppm**
C-1	CH_2	36.72	CH_2	37.3
C-2	CH	75.15	CH	32.1
C-3	CH	73.31	CH	71.9
C-4	CH_2	42.76	CH_2	42.7
C-5	CH	45.12	C	141.1
C-6	CH	74.15	CH	121.6
C-7	CH_2	31.83	CH_2	32.3
C-8	CH	31.52	CH	32.1
C-9	CH	51.28	CH	50.5
C-10	C	37.12	C	36.8
C-11	CH_2	20.83	CH_2	21.4
C-12	CH_2	41.36	CH_2	40.2
C-13	C	43.52	C	42.7
C-14	CH	56.83	CH	57.2
C-15	CH_2	82.14	CH_2	24.5
C-16	CH_2	27.37	CH_2	28.3
C-17	CH	57.12	CH	56.7
C-18	CH	19.76	CH_3	12.0
C-19	CH_2	12.55	CH_3	19.5
C-20	CH_2	37.27	CH	35.9
C-21	CH	81.35	CH_3	18.9
C-22	CH_2	35.73	CH_2	36.5
C-23	CH_2	25.92	CH_2	24.1
C-24	CH_3	45.72	CH	39.8
C-25	CH_3	29.37	CH	28.1
C-26			CH_3	18.7
C-27			CH_3	29.8
C-28			CH_2	23.1
C-29			CH_3	11.8

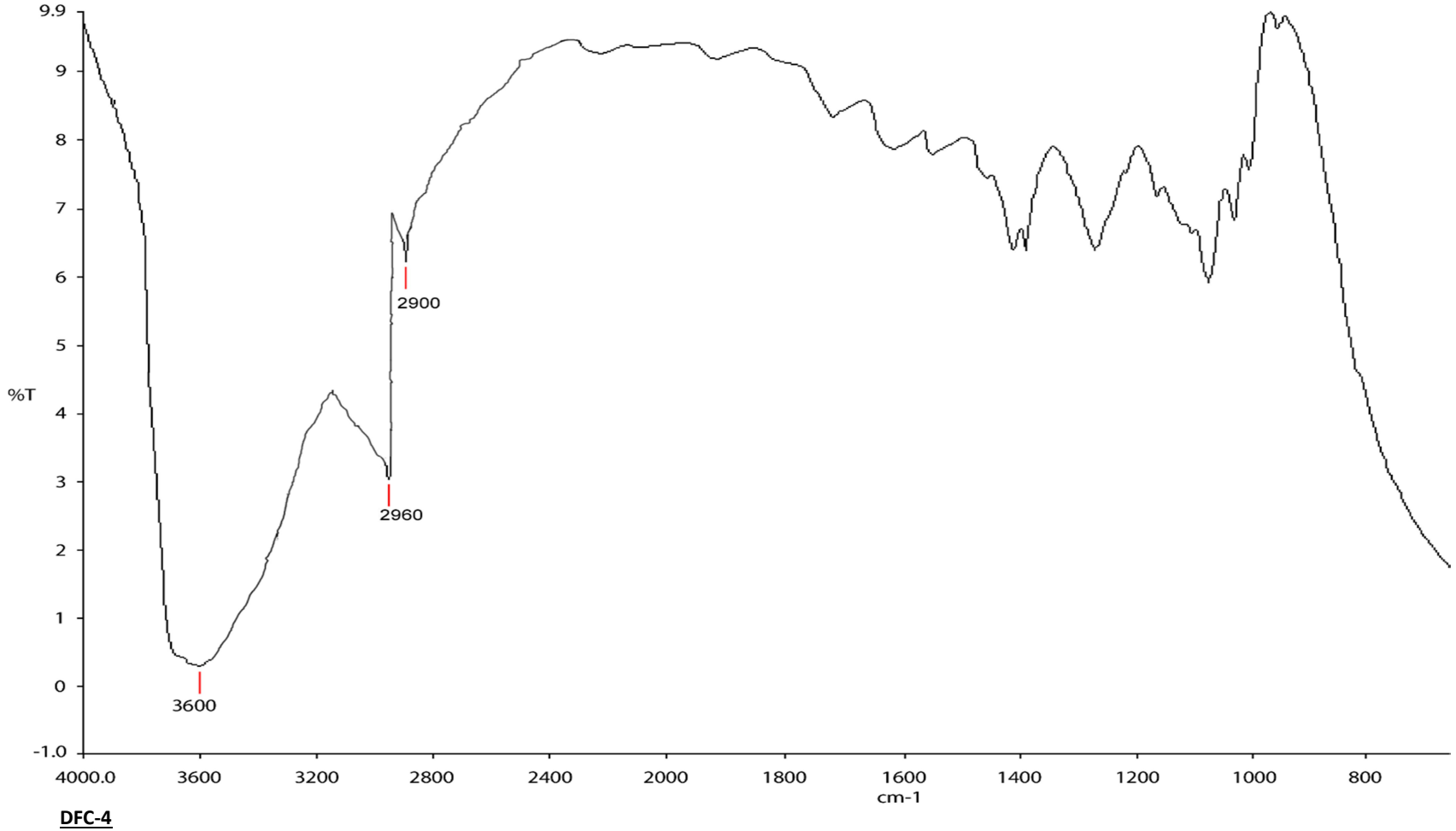


Fig 3.20: IR Spectrum of compound DFC-4

^{1}H-NMR Spectrum (300 MHz, CD_3OD) of compound DFC-4

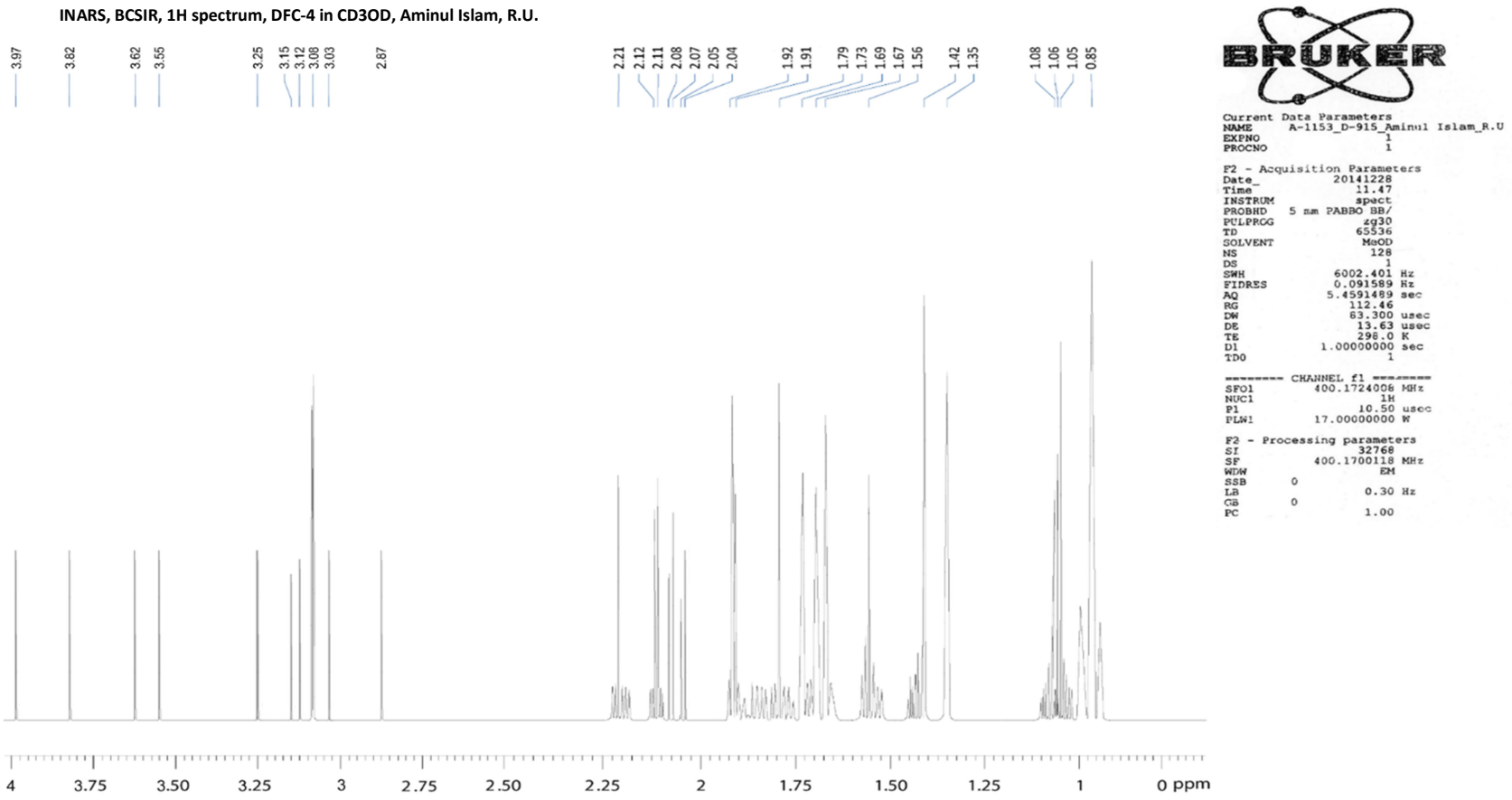


Fig 3.21 : ^{1}H-NMR spectrum of compound DFC-4

^{13}C-NMR Spectrum (500 MHz, CD_3OD) of compound DFC-4

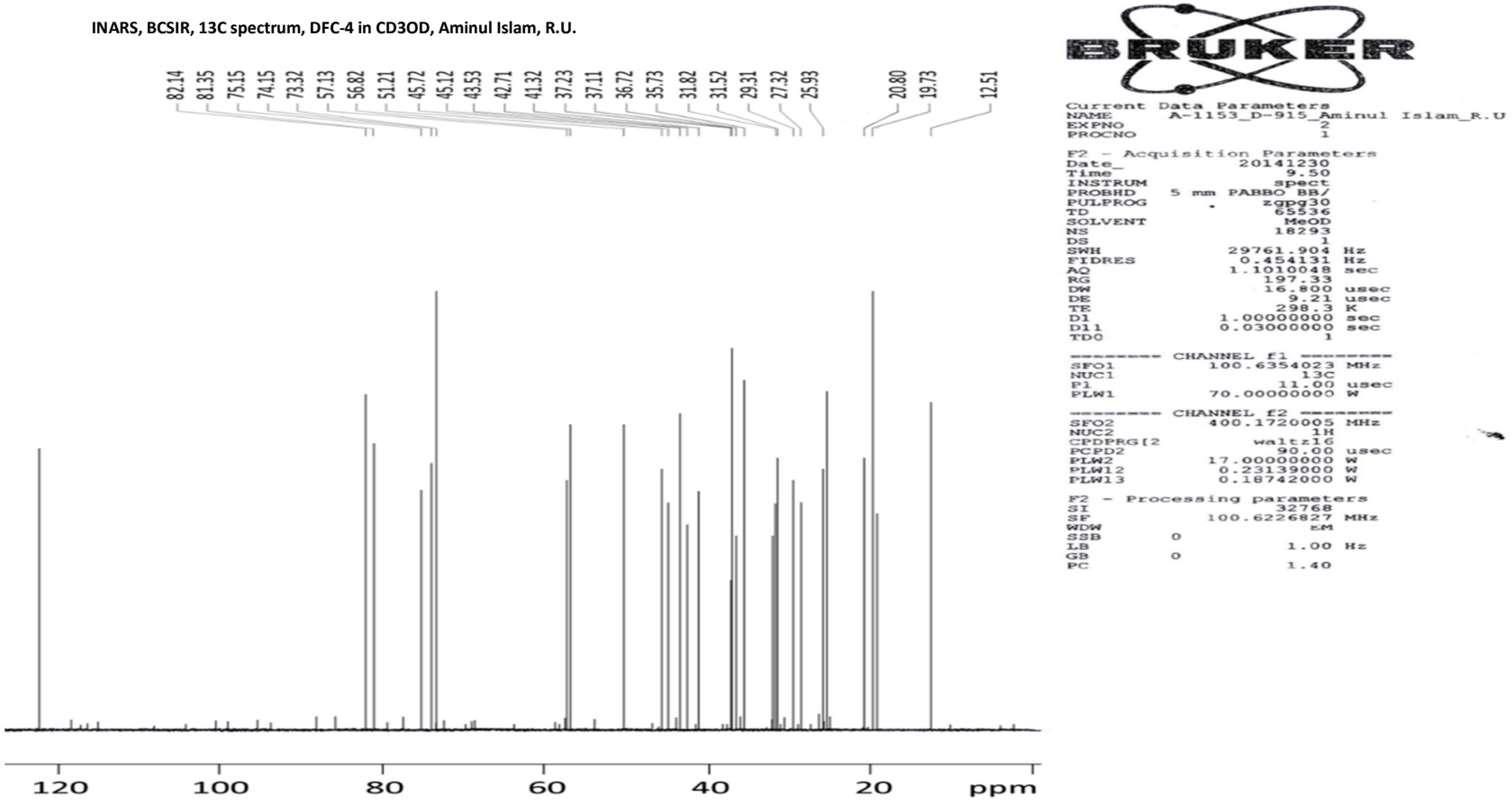


Fig 3.22: ^{13}C-NMR spectrum of compound DFC-4

3.4.7 The compound DFC-5

The compound DFC-5 was isolated from $F^{/}$-16 of the dia-ion resin fraction as white crystal. The compound was found to be highly soluble in DMSO, methanol and ethanol, sparingly soluble in acetone but insoluble in petroleum ether and chloroform.

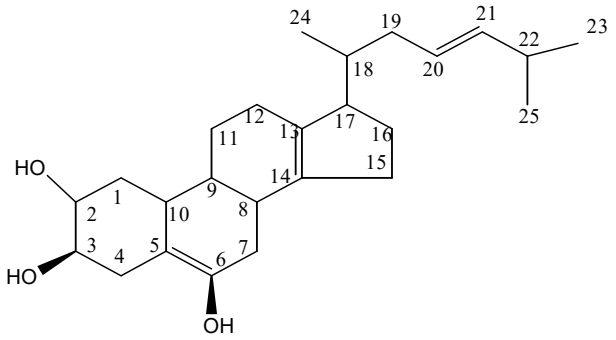
The compound showed a single spot on TLC examination under UV light at 254 nm, bound with iodine and spraying the developed plate with vanillin-sulfuric acid followed by heating at 110^0C for about 10 minutes gave pinkish violet color. The R_f value of the compound was found 0.32 in petroleum ether : acetone (1:2) and 0.47 in n-hexane : methanol (5:4). It is also positively responded to Liebermann-Burchard and Salkowski tests. These chemical test and TLC behavior revealed that DFC-5 might be a steroidal or terpenoidal compound. The compound was gave positive test for steroid and unsaturation.

UV spectrum showed absorption at 194 and 188 nm indicate the presence of any unsaturation in the compound.

From its IR spectrum, the compound DFC-5 showed absorption bands at 3426 cm^{-1} for OH function indicates the presence of hydroxyl function and stretching for C = C at 1635 in the molecule.

From its ^{1}H-NMR spectrum (500 MHz, CD_3OD), H-3 proton appeared as triplets of a double doublet (tdd) at δ 3.25 with coupling constants of 11.4 Hz and δ 4.2 Hz. Three methyl protons appeared at δ 2.04, δ 1.23, and δ 1.12. The hydroxyl group protons showed at δ 12.78, δ 12.75 and δ 11.53. These assignment are in good assignment for the structure of new compound.

^{13}C-NMR spectrum (CD_3OD, 100 MHz) of compound DFC-5 showed twenty five carbons which included three methyl, eight methylene, ten tertiary carbon, four quaternary carbons and three hydroxyl group. Methyl groups are C-18 and twice in C-20. Methylene groups are C-1, C-4, C-7, C-11, C-12, C-15, C-16 and C-19. Tertiary carbons are C-2, C-3, C-8, C-9, C-10, C-17, C-18, C-20, C-21 and C-22. Quaternary carbons are in C-5, C-6, C-13 and C-14. In the compound DFC-5, there is exocyclic double bond, instead between C-5 to C-6 and C-13 to C-14. Hydroxyl groups are attested with C-2, C-3 and C-6 position.



Structure of compound DFC-5

Table-3.20: ^{1}H-NMR spectral data (300 MHz, CD_3OD) of compound DFC-5.

Position of proton	δ value in ppm (*J* in Hz)	Position of proton	δ value in ppm (*J* in Hz)
H-1	1.89 (2H, bd, J = 14.0 Hz)	H-15	2.02 (2H, q, J = 11.0 Hz)
H-2	3.12 ~ 3.51 (2H, m)	H-16	2.05 (2H, q, J = 10.2 Hz)
H-3	2.35 (1H, tdd, *J* = 11.4 Hz and 4.2 Hz)	H-17	2.02 (1H, d, J = 9.5 Hz)
H-4	1.84 ~ 1.87 (2H, m)	H-18	1.87 (1H, m like)
-	-	H-19	2.08 (2H, q, J= 8.0 Hz)
-	-	H-20	2.07 (H, q, J = 8.0 Hz)
H-7	0.83 (2H, dt, J = 6.5 Hz)	H-21	1.92 (1H, m like)
H-8	1.06 ~ 1.09 (1H, m like)	H-22	1.97 (1H, m like)
H-9	1.06 ~ 1.09 (1H, m like)	CH_3-23	2.04 (3H, q, J= 7.5 Hz)
H-10	1.06 ~ 1.09 (1H, m like)	CH_3-24	1.23 (3H, s)
H-11	1.58 (2H, bd, J = 6.0 Hz)	CH_3-25	1.12 (3H, s)
H-12	1.65 (2H, bd, J = 6.2 Hz)	OH-2	3.25 (1H, s)
-	-	OH-3	3.18 (1H, s)
-	-	OH-6	3.27 (1H, s)

Table-3.21: ^{13}C-NMR spectral data (500 MHz, CD_3OD) of compound DFC-5.

^{13}C-NMR spectral data and Multiplicity			Authentic β-sitosterol	
Position of carbon	**Multiplicity**	**δ value in ppm**	**Multiplicity**	**δ value in ppm**
C-1	CH_2	37.3	CH_2	37.3
C-2	CH	75.12	CH_2	31.5
C-3	CH	72.2	CH	72.2
C-4	CH_2	42.7	CH_2	42.2
C-5	C	141.1	CH	140.6
C-6	C	121.6	CH_2	121.5
C-7	CH_2	32.3	CH_2	32.05
C-8	CH	32.1	CH	32.05
C-9	CH	50.5	CH	50.20
C-10	CH	36.8	C	36.5
C-11	CH_2	21.4	CH_2	21.1
C-12	CH_2	40.2	CH_2	40.5
C-13	C	132.66	C	42.4
C-14	C	145.34	CH	57.2
C-15	CH_2	24.5	CH_2	24.3
C-16	CH_2	28.3	CH_2	28.7
C-17	CH	56.7	CH	56.5
C-18	CH	12.0	CH_3	19.3
C-19	CH_2	19.5	CH_3	12.2
C-20	CH	136.6	CH	36.6
C-21	CH	118.8	CH_3	18.8
C-22	CH	36.5	CH_2	34.3
C-23	CH_3	24.1	CH_2	26.6
C-24	CH_3	39.8	CH	46.3
C-25	CH_3	28.1	CH	29.5
C-26			CH_3	18.7
C-27			CH_3	29.8
C-28			CH_2	23.1
C-29			CH_3	11.8

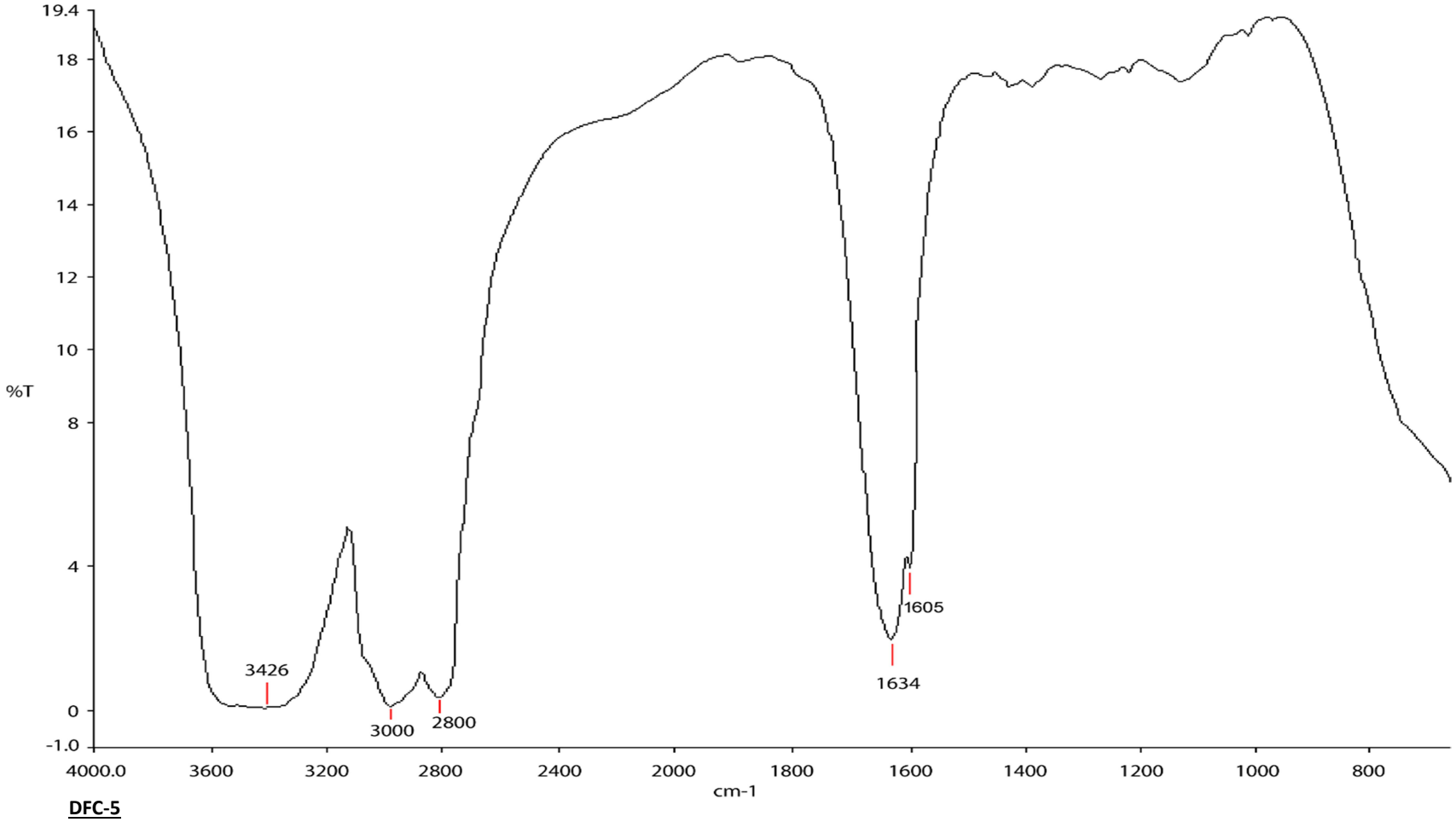


Fig 3.23: IR Spectrum of compound DFC-5

^{1}H-NMR Spectrum (300 MHz, CD_3OD) of compound DFC-5

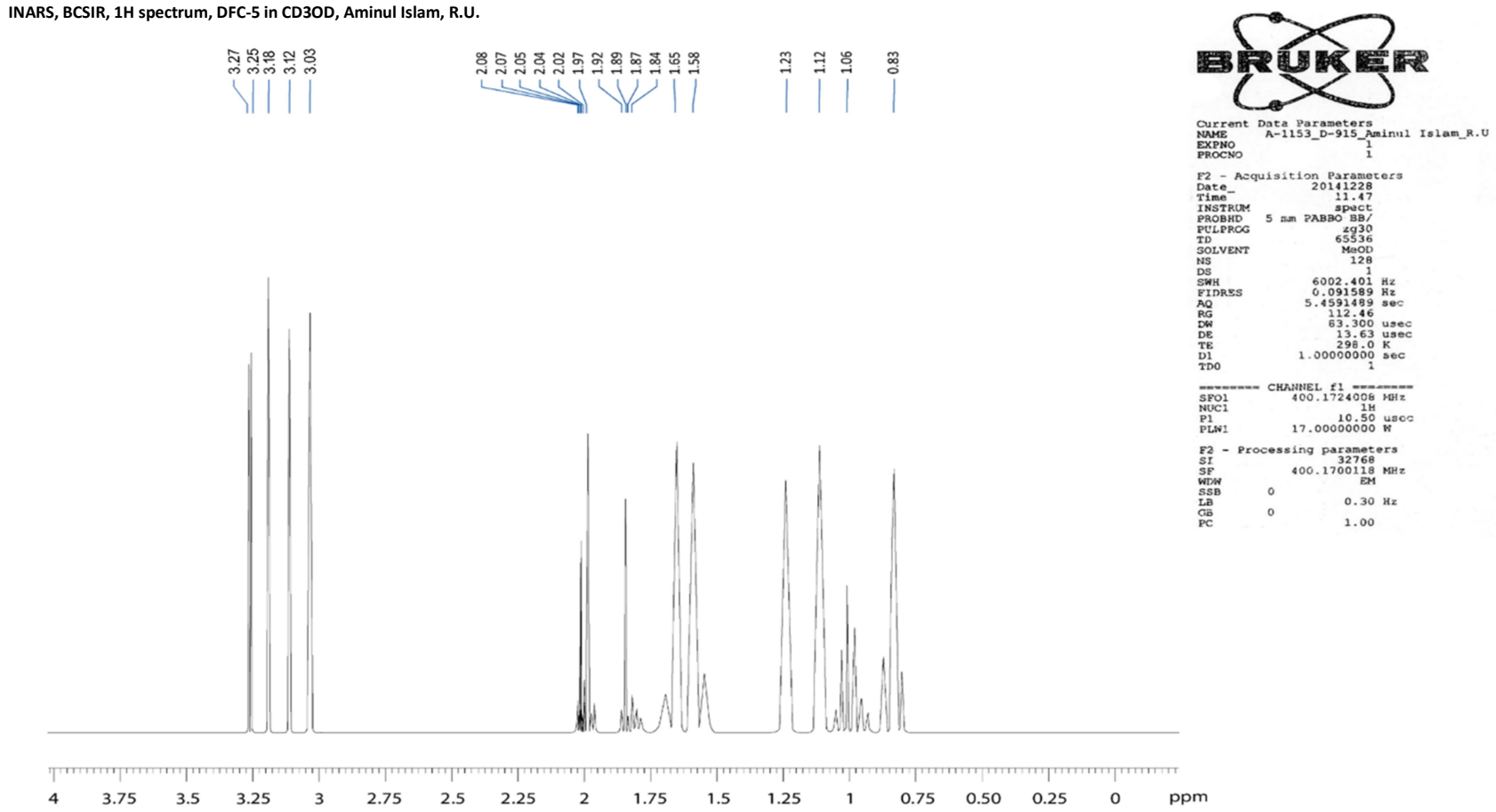


Fig 3.24: ^{1}H-NMR spectrum of compound DFC-5

^{13}C-NMR Spectrum (500 MHz, CD_3OD) of compound DFC-5

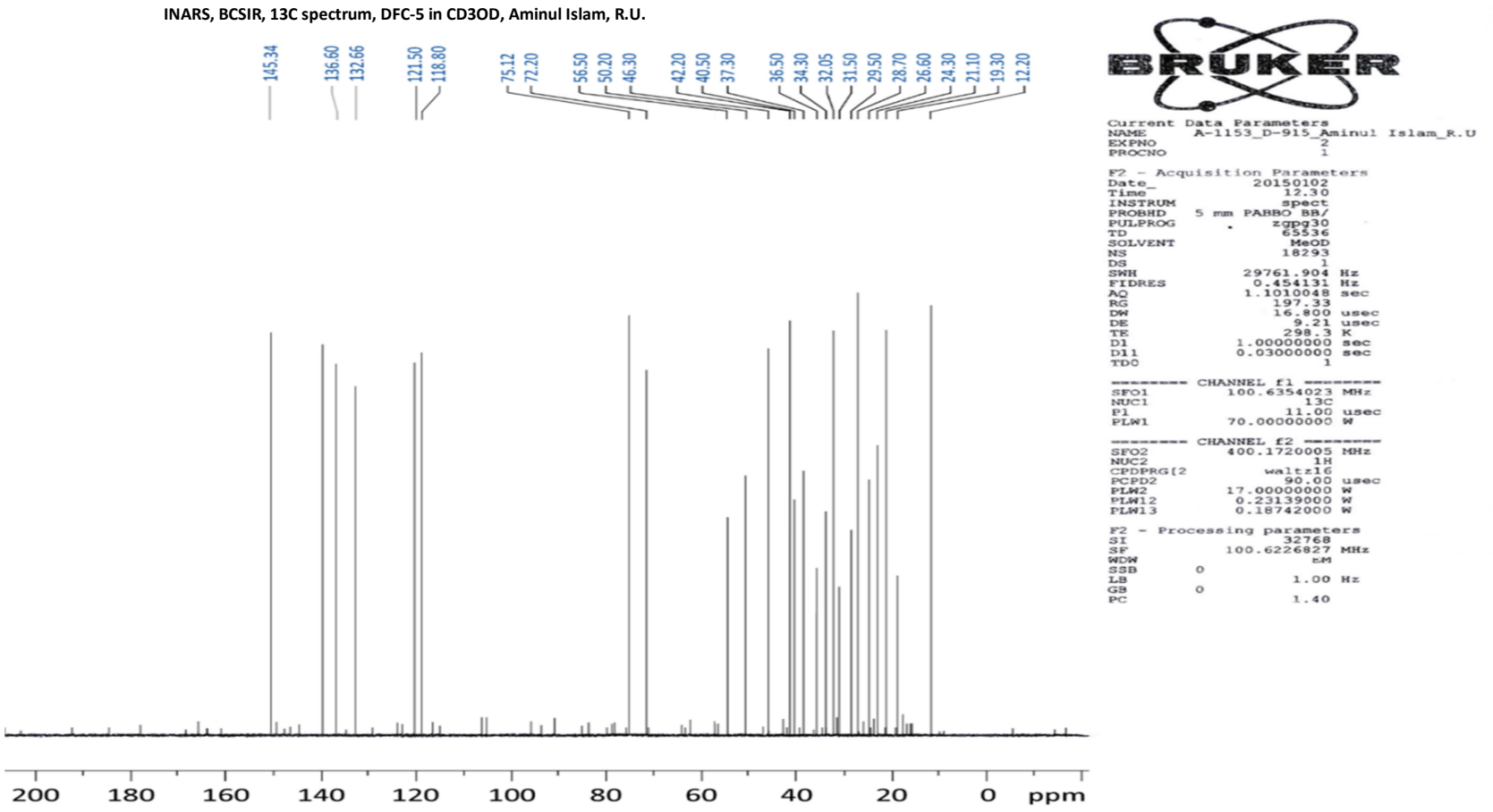


Fig 3.25: ^{13}C-NMR spectrum of compound DFC-5

PART-B
INTRODUCTION

4.1 Antioxidant

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols[94].

Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Insufficient levels of antioxidants or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. As oxidative stress appears to be an important part of many human diseases, the use of antioxidants is intensively studied, particularly as treatments for stroke and neurodegenerative diseases[95]. Moreover, oxidative stress is both the cause and the consequence of disease.

Low levels of antioxidant or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells.

4.1.1 Antioxidant defense system

To protect cells and organs from the oxidative stress induced by reactive oxygen species (ROS), living organisms have evolved with an extremely efficient and highly sophisticated protective system, the so-called “antioxidant defensive system”. It involves a variety of components, both endogenous and exogenous in origin. These components function interactively and synergistically to neutralize free radicals. Antioxidant defense system against oxidative stress is composed of several lines and the antioxidants are classified into four categories based on function [96].

- First line of defense is the preventive antioxidants, which suppress formation of free radical (enzymes; glutathione peroxidase, catalase; selenoprotein, transferrin, ferritin, lactoferrin, carotenoids etc.)
- Second line of defense is the radical scavenging antioxidants suppressing chain inhibition and / or breaking chain propagation reactions radical scavenging antioxidants.
- Third category: repair and de novo antioxidant (some proteolitic enzymes, repear enzymes of DNA etc)
- A fourth line is an adaptation where the signal for the production and reactions of free radicals induced formation and transport of the appropriate antioxidant to the right site.

4.1.2 Significance of antioxidants in relation to disease

Antioxidants may prevent and / or improved different diseased states [97]. Zinc is an essential trace element, being a co-factor for about 200 human enzyme, including the cytoplasmic antioxidant Cu-Zn superoxide dismutase (SOD), isoenzyme of SOD mainly present in cytosol. Selenium is also an essential trace element and a co-factor for glutathione peroxidase. Vitamin E and tocotrienols (such as those from palm oil) are efficient lipid soluble antioxidants that function as a 'chain breaker' during lipid peroxidation in cell membranes various lipid particles including LDL [98-99]. Vitamin E is considered as the 'standard antioxidant' to which other compounds with antioxidant activities are compound, especially in terms of its biological activity and clinical. The daily dietary allowance varies between 400 IU to 800 IU. Vitamin C (ascorbic acid) is a water soluble free radical scavenger. The daily recommended dietary allowance is 60 mg. Apart from these carotenoids such as beta-carotene, lycopene, lutein and other carotenoids function as important antioxidants and they quench IO_2 and $ROO^{\bullet}$. Flavonoids, mainly present as colouring pigments in plants also function as potent antioxidants at various levels [99-100]. Table -4.1 gives a relation of antioxidant with neurodegenerative disorders.

Table-4.1: Antioxidant with their class, chemical composition, mechanism of action and their relation with neurodegenerative disorders.

Class	Chemical composition	Mechanism of action	Neuronal disorder studied
Direct Antioxidant [101-102)	Aryl amines and indolescarotene, lycopene Polyenes-carotene, lycopene, retinol. Selenium containing compounds ebselen. Polyphenols-Flavonoids, stibenes and hydroquinone, Monophenols; tocopherols (vitamin E), 17-estradiol (estrogen), 5-hydroxytryptamine (serotonin)	Direct chemical (nonenzymatic) scavenging of ROS generated free radicals and get some of them are recycled with endogenous oxidoreductases or via intracellular reducing shuttles.	Neuroprotection in Alzheimer's disease
Indirect antioxidant [103-104]	Amino oxidase inhibitors, calcium antagonists, dopamine receptor agonists, glutamate receptor antagonists, ion chelators, nitric oxide synthase inhibitors.	Prevent excitotoxicity, ROS and free radical generation targeting inhibitors and receptors dysregulating metal homeostasis.	Parkinson's disease and dementia
Metabolic antioxidant [105-106]	N-acetyl-cysteine, glutathione, 2-oxo-thiazolidine-4-carboxylate and other thiol-delivering compounds N-butyl-phenylnitrone. Carnitine, creatine, lipoic acid (thioctic acid), ubiquinone and idebenone.	Nullify cellular damage caused by free radicals by reducing secondary metabolic burden over cellular organelle Mt, vesicles and cell membbrane	Neuroprotection in general
Metal containing antioxidant [107]	Manganese-containing mimetics of catalase/superoxide dismutase	Enzymatic mimicking prevents protein-metal interaction	Mt protection in AD and PD

4.1.3 Need for antioxidants in the treatment of neurodegenerative disorders

Antioxidants are now being looked upon as persuasive therapeutic against solemn neuronal loss, as they have capability to combat by neutralizing free radicals [108]. Diet is major source of antioxidants, as well as medicinal herbs [109] are catching attention to be commercial source of antioxidants at present. Recognition of upstream and downstream antioxidant therapy to oxidative stress has been proved an effective tool in alteration of any neuronal damage as well as free radical scavenging. Antioxidants have a wide scope to sequester metal ions involved in neuronal plaque formation to prevent oxidative stress. In addition, antioxidant therapy is vital in scavenging free radical and ROS preventing neuronal degeneration in post-oxidative stress scenario. Since our endogenous antioxidant[110] defenses are not always completely effective and since exposure to damaging factors is increasing, it seems reasonable to propose that exogenous antioxidant could be very effective in diminishing the cumulative effects of oxidative damage.

However, the therapeutic use of most of these compounds is limited since they do not cross the blood brain barrier (BBB). Although a few of them have shown limited efficiency in animal models or in small clinical studies, none of the currently available antioxidants have proven efficacious in a large-scale controlled.

Therefore, any novel antioxidant molecules designed as potential neuroprotective treatment in acute or chronic neurological disorders should have the mandatory prerequisite that they can cross the BBB after systemic administration. Due to the involvement of oxidative stress-mediated toxicity in neurodegenerative events and neuronal cell death, various experimental approaches for effective protection by antioxidants have emerged. Considering the importance of developing new antioxidant compounds and the relevance of their application in the treatment of neurodegenerative diseases, we focused our attention on.

4.1.4 Endogenous antioxidant

Biological systems have evolved with endogenous defense mechanisms to help protect against free radical induced cell damage. Glutathione peroxidase, catalase and

superoxide dismutases are antioxidant enzymes, which metabolize toxic oxidative intermediates. They require micronutrient as cofactors such as selenium, iron, copper, zinc and manganese for optimum catalytic activity and glutathione peroxidase are three primary enzymes, involved in direct elimination of active oxygen species (hydroxyl radical, superoxide radical, hydrogen peroxide) whereas glutathione reductase, glucose-6-phosphate dehydrogenase and cytosolic GST are secondary enzymes, which help in the detoxification of ROS by decreasing peroxide levels or maintaining a steady supply of metabolic intermediates like glutathione and NADPH necessary for optimum functioning of the primary antioxidant enzymes [111-112].

Glutathione, ascorbic acid, alpha-tocopherol, beta-carotene, bilirubin, selenium, NADPH, butyhydroxyanisole (BHA), mannitol, benzoate, histidine peptide, the iron bonding transferrin, dihydrolipoic acid, reduced Co Q10, melatonin, uric acid and plasma protein thiol etc, as a whole play a homoeostatic or protective role against ROS produced during normal cellular metabolism and after active oxidation insult.

Glutathione is the most significant component which directly quenches ROS such as lipid peroxides and plays major role in xenobiotic metabolism. When an individual is exposed to high levels of xenobiotics, more glutathione is utilized for conjugation making it less available to serve as an antioxidant. It also maintains (vitamin C) and alpha-tocopherol (vitamin E), in their reduced form, which also exert an antioxidant effect by quenching free radicals.

Table-4.2: Reactive oxygen species and their corresponding neutralizing antioxidants and also additional antioxidants.[113-115]

ROS	Antioxidants (endogenous)		Antioxidants (exogenous)
	Directrole	Indirectrole	
Hydroxyl radical	Glutathione peroxidase (cofactor selenium)	-	Vitamin C Lipoic acid
Lipid peroxide	Glutathione peroxidase (cofactor selenium)	-	Vitamin E beta-carotene

ROS	Antioxidants (endogenous)		Antioxidants (exogenous)
	Directrole	**Indirectrole**	
Superoxide radical	Superoxide dismutase (cofactor Cu/Zn/Mn)	Ceruloplasmin (Cu) Metallothionin (Cu) Albumin (Cu)	Vitamin C
Hydrogen peroxide	Catalase (cofactor iron)	Transferin (iron) Ferritin (iron) Myoglobin (iron)	Vitamin C beta-carotene Lipoic acid
Pro-oxidant/ antioxidant equilibrium	Thiols(GSH, Lipoic acid, N-acetyl cysteine) NADPH and NADH Ubiquinone	Bilirubin Uric acid	Flavonoids

4.1.5 Exogenous antioxidants: Contribution from the diet

The most widely studied dietary antioxidants are vitamin C, vitamin E and beta-carotene. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids, as it is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Vitamin E is a major lipid-soluble antioxidant and is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. Beta-carotene and other carotenoids also provide antioxidant protection to lipid rich tissues, Fruits and vegetables are major source of vitamin C and carotenoids, while whole grains, i.e, cereals and high quality vegetable oils are major sources of vitamin E [116-118].

A number of other dietary antioxidants exist beyond the traditional vitamins collectively known as phytonutrients or phytochemicals which are being increasingly appreciated for their antioxidant activity, one example is flavonoids which are a group of polyphenolic compounds. Many of the biological activities of flavonoids are attributed to their antioxidant properties and free radical scavenging capabilities. A number of flavonoids efficiently chelate trace metals, which play an important role in oxygen metabolism. These are widely found in plants as glucosylated derivatives.

They are responsible for the different brilliant shades such as blue, scarlet and orange. They are found in leaves, flowers, fruits, nuts, grains, spices, different medicinal plants and beverages such as wine, tea and beer [119-122].

Free iron is a potential enhancer of ROS formation as it leads to reduction of H_2O_2 and generation of the highly aggressive hydroxyl radical. Free copper mediates LDL oxidation and contributes to oxidative damage due to lipid peroxidation [123].

Due to the inefficiency of our endogenous defense systems as well as the existence of some physiopathological situations, such as, cigarette smoke, air pollutants, UV radiation, inflammation, ischaemia / reperfuion etc. ROS can be produced in excess and increasing amounts of dietary antioxidants will be needed for diminishing the cumulative of oxidative damage over an individual's life span [124]. Table 4.3 shows food stuffs containing antioxidant constituents.

Table -4.3: Food stuffs containing antioxidant constituents.

Food stuffs	Contituents act as antioxidant	Food stuffs	Constituents act as antioxidant
Citrus fruits/black tea	Quercetin, rutin, hesperetin, naringin	Cloves	Eugenol, cryophyllene
Tomato juice/green tea	Kaempferol	Fenugreek seeds	Diosgenin, sapogenin
Tomato juice/vegetables	Fiselin	Rosemay	Camosol
Tomato juice/vegetables	Myricetin	Mint (pudina)	Menthofuran, menthol
Popolis/fruits	Galangin	Garlic	Allyl sulfide
Soyabean/Soy foods	Daidzein, daidzin	Bel	Umbeliferone, mamalosin
Red clover	Biochanin A, formononetin	Cereal products	Apigenin, luteolin
Fruits/vegetables	Cyanidin, cyanin	Milk products	Casein, vitamin D
Fruits/vegetables	Chrysin	Fish	Cord oil (vitamin A)

Food stuffs	Contituents act as antioxidant	Food stuffs	Constituents act as antioxidant
Soyabeans	Genistein, genistin	Eggs	Vitamin A
Tomato	Lycopene	Green & Chili peppers	Capaisin
Cruciferous vegetables	Isothiocyanate, erucic acid	Black pepper	Piperine,piperidine, piperatine
Green and black tea	(+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin	Cinnamone	Eugenol, phellandrene
Ginkgo biloba	Bilobalide, ginkgolides	Saffron	Crocetin
Virgin olive oils	3,4-dihydroxyphenyl-ethanol (DOPET)	Ginger	Gingerol
Tulsi (basil)	Eugenol, nerol, camphene	Karela	Vicine, momoridicine
Ginseng	Ginsenosides	Amha	Corilagin,ellagic acid, gallotanins
Walnuts, almonds	Oleic acid, alpha-linolenic acid, vitamin E, minerals	Saunf	Anethole
Tumeric	Curcumin	Shahtoot	Betulinic acid

4.2 Oxidative stress in disease

Oxidative stress is thought to contribute to the development of a wide range of diseases including Alzheimer's disease[125,126], Parkinson's disease[127], the pathologies caused by diabetes[128,129], rheumatoid arthritis[130], and neurodegeneration in motor neuron diseases[131]. In many of these cases, it is unclear if oxidants trigger the disease, or if they are produced as a secondary consequence of the disease and from general tissue damage[132];One case in which this link is particularly well-understood is the role of oxidative stress in cardiovascular disease.

Here, low density lipoprotein (LDL) oxidation appears to trigger the process of atherogenesis, which results in atherosclerosis, and finally cardiovascular disease[133,134].

Oxidative damage in DNA can cause cancer. Several antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase etc. protect DNA from oxidative stress. It has been proposed that polymorphisms in these enzymes are associated with DNA damage and subsequently the individual's risk of cancer susceptibility[135].

A low calorie diet extends median and maximum lifespan in many animals. This effect may involve a reduction in oxidative stress[136].While there is some evidence to support the role of oxidative stress in aging in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*[137,138], the evidence in mammals is less clear[139-141]. Indeed, a 2009 review of experiments in mice concluded that almost all manipulations of antioxidant systems had no effect on aging[142].

Diets high in fruit and vegetables, and so possibly being rich in antioxidant vitamins, have no established effect on status of health or aging[143,144],yet may have more subtle physiological effects, such as modifying cell-to-cell communication[145,146].

4.3 Oxidative stress Behavior

The central nervous system (CNS) is especially vulnerable to free radical damage as a result of the brain’s high oxygen consumption rate, its abundant lipid content and the relative paucity of antioxidant enzymes compared with other tissues (Coyle and Puttfarcken 1993). AD brain is under extensive oxidative stress as manifested by lipid peroxidation, protein oxidation, and DNA oxidation (Butterfield and Lauderback, 2002). Aβ might be the central to the pathogenesis of AD. Aβ has been shown to induce protein oxidation and lipid peroxidation in vitro and in vivo (Butterfield and Lauderback 2002). Many studies have indicated that Aβ-induced oxidative stress is involved in the pathogenesis of AD (Butterfield *et al.,* 2001).

Lipid peroxidation is an important mechanism of neurodegeneration in AD brain. Many studies have shown increased lipid peroxidation in several regions of AD brain, where the histopathologic alterations are very noticeable (Lovell *et al.,* 1995; Marcus *et al.,* 1998). It has been shown that there is a strong regional correlation between the thiobarbituric acid reactive substances (TBARS), one indicator of lipid peroxidation, antioxidant enzymes, the presence of NPs and NFT in AD brain (Lovell *et al.* 1995). Aβ is widely reported to cause lipid peroxidation in brain cell membranes in a manner that is inhibited by free radical antioxidants (Daniels *et al.,* 1998; Mark *et al.,* 1999).

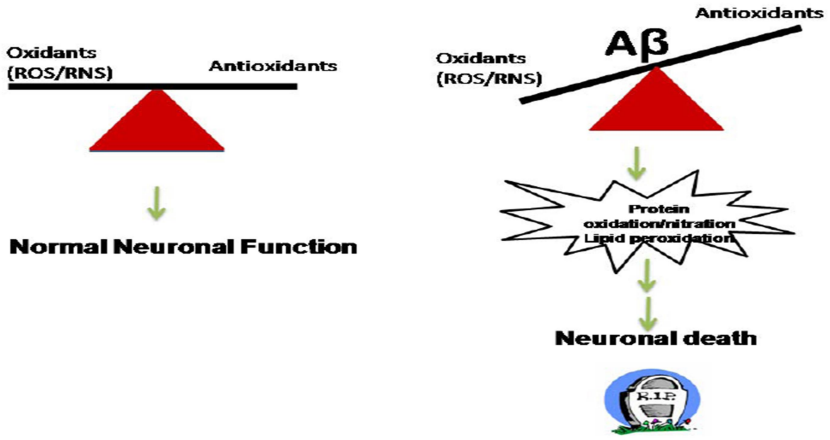


Fig-4.1: Amyloid beta (Aβ) peptide induced oxidative stress and neuronal loss.

Aβ leads to an increased level of 4-hydroxy-2-nonenal (4-FINE), one of the major products of lipid peroxidation, in hippocampal and cortical neuronal cells (Mark *et al.,* 1997). Increased 4-FINE was found in AD brain and it was proven to be toxic to hippocampal neuronal cells (Mark *et al.,* 1997). 4-HNE can also increase the vulnerability of cultured hippocampal neurons to excitotoxicity, as well as an alteration in multiple cellular functions including glucose or glutamate transport (Keller *et al.,* 1997).

4.4 Oxidative Stress Hypothesis

There is increasing evidence that free radical damage to the brain lipids, carbohydrates, proteins and DNA is involved in neuron death in neurodegenerative disorders. The largest number of studies has been performed in Alzheimer's disease (AD), where there is considerable support for the oxidative stress hypothesis in the pathogenesis of neuron degeneration.

The term oxidative stress is used when free radicals and their products are in excess of the antioxidant defense mechanism. This may occur as a result of increased radical production or a decrease in antioxidant defenses. If the increased demand on the cell's capacity to detoxify free radicals is not met, alterations occur in cells. Accumulation of oxidized products such as aldehydes or isoprostanes from lipid per oxidation, protein carbonyls from protein oxidation and oxidized base adducts from DNA oxidation, serve as markers of excess oxidative stress [147].

Oxidative damage is highly relevant in the brain for several reasons:

- It is a post mitotic tissue with a high energy demand
- It is exposed to high oxygen concentrations, utilizing about one-fifth of the oxygen consumed by the body
- It contains relatively poor concentrations of antioxidants and related enzymes
- It is rich in polyunsaturated fatty acids that are prone to oxidation
- It is enriched in iron, which accumulates in the brain as a function of age and can be a potent catalyst for oxidative species formation.
- In addition those brain regions that are rich in the catecholamine, adrenalin, noradrenalin and dopamine are exceptionally vulnerable to free radical generation. Catecholamine can induce free radicals through either spontaneous breakdown (auto oxidation) or by being metabolized by endogenous enzymes such as monoamine oxidase.

It is widely considered that the major portion (about 95-98%) of the total reactive oxygen species (ROS) produced during aerobic metabolism comes from by products of the electron transport chain of mitochondria. Under normal conditions damage by oxygen radical is controlled by an efficient array of antioxidant systems. These include antioxidant enzymes and free radical scavengers such as ascorbates, vitamin E and protein sulfhydryls.

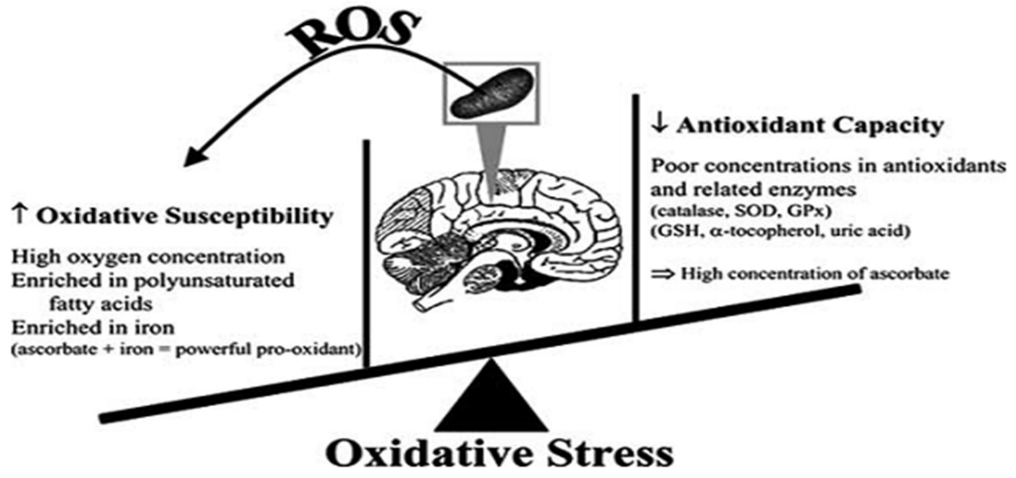


Fig 4.2: Schematic representation illustrating how brain is at risk for oxidative damage in accordance with the points illustrated in the figure and as discussed in the text. Abbreviations: glutathione peroxidase (GPx); reduced glutathione (GSH); superoxide dismutase (SOD).

However in neurodegenerative conditions, this control is altered favoring the accumulation of free radicals, mitochondrial dysfunction and neuronal injury. Oxidative damage has been found in all classes of organic molecules (proteins, lipids, nucleic acids, sugars) that are critical for neuronal, structural and functional integrity [148]. Excessive lipids per oxidation, protein oxidation, DNA and RNA oxidation and glycooxidation have all been documented in AD brains. In autopsied brain there is an increase in lipid per oxidations, a decline in polyunsaturated fatty acids (PUFA) and an increase in 4 hydroxynonenal (HNE), a neurotoxic aldehyde product of PUFA oxidation. Increased protein oxidation and a marked decline in oxidative sensitive enzymes, glutamine synthetase and creatinine kinase are found in the brain in AD. Increased DNA oxidation, especially 8-hydroxy-2-deoxyguanosine is present in the brain of AD. Immunohisto chemical studies show the presence of oxidative stress products in neurofibrillary tangles and amyloid plaques in AD [149].

If free radicals injury is a key player in the initiation and propagation of AD then antioxidant therapies envisaging the reduction of oxidative damage and the increase of endogenous antioxidant defenses should prevent, delay or ameliorate the disease symptoms.

4.3 Cholinesterase inhibitors:

Cholinesterase inhibitors are prescribed to treat symptoms related to memory, thinking, language, judgment and other thought processes. Three different cholinesterase inhibitors are commonly prescribed:

- Donepezil (marketed under the brand name Aricept), which is approved to treat all stages of Alzheimer's disease
- Galantamine (marketed under the brand name Razadyne), approved for mild to moderate stages.
- Rivastigmine (marketed under the brand name Exelon), approved for mild to moderate Alzheimer's.
- Tacrine (Cognex), the first cholinesterase inhibitor, was approved in 1993 but is rarely prescribed today because of associated side effects, including possible liver damage.

It is often regarded as providing only symptomatic relief without providing neuroprotective effects. However, invitro studies shows that donepezil offers neuroprotection by reducing glutamate excitotoxicity , diminishing βA toxicity, and consequently increasing cell longevity.(Akasofu *et al*., 2008 ; Takada *et al*., 2003). It slowed atrophy of the hippocampus in humans, which suggest a neurotective effect (Hashimoto *et al*., 2005). It is very well absorbed after oral administration and reaches peak plasmatic concentration (Cmax) in 3-4 hours. Elimination half-life of donepezil is approximately 70 hours allowing once daily administration. It binds to plasma proteins in a proportion of 96%, and is metabolised by isoenzyme 2D6 and 3A4 of cytochrome P450. Starting and minimal effective dose is 5 mg once daily. Maximal recommended dose is 10 mg daily. Overall, both the doses of 5 mg and 10 mg were beneficial, with the higher dose being marginally more effective. More side effects were reported with donepezil than with placebo. Most common side-effects were nausea, vomiting, diarrhoea, muscle cramps, dizziness, fatigue, and anorexia, and they were dose-dependent.

4.5.1 Galantamine

Galantamine is a tertiary alkaloid agent that reversibly inhibits AChE (Roberson *et al*., 2007). Galantamine, a natural AChEI (originally derived from the common snowdrop and other plants, but now synthesized), protects neurons and reduced cell death by modulating nicotinic receptors, which are significantly reduced in AD brains (Wang D *et al.,* 2007 ; Maelicke *et al*., 2000).In an animal model, galantamine also increased dopaminergic neurotransmission in the hippocampus, (Wang D *et al*., 2007) a brain area particularly important in memory.

Galantamine is rapidly absorbed after oral administration and reaches Cmax in approximately 1 hour. Elimination half-life is between 7 to 8 hours. It binds to plasma proteins in a proportion of 18% and is metabolized by isoenzyme 2D6 and 3A4 of cytochrome P450. Galantamine is commercialized as an extended-release formulation allowing once-daily dosing. Starting dose of galantamine ER is 8 mg once daily. Minimal effective dose is 16 mg daily, and maximal dose is 24 mg daily. Galantamine's side effects are comparable to other ChEI's and consist mainly of cholinergic gastrointestinal symptoms.

4.5.2 Rivastigmine

Rivastigmine is a carbamate derivative that reversibly inhibits both acetyl- (AChE) and butyryl- (BuChE) cholinesterase (Onor *et al.,* 2007). It is the only ChEI with significant inhibition of BuChE[150]. Butyrylcholinesterase is widely distributed in the central nervous system and may play a role in cholinergic function and neurodegeneration [151] (Darvesh *et al.,* 2003). It is unclear how specific BuChE inhibition relates to rivastigmine's clinical effect.

Rivastigmine is well absorbed after oral administration and reaches Cmax in one hour. Its elimination half-life is approximately 1 to 2 hours. It binds to proteins in a proportion of 40%, is hydrolysed by esterases (including cholinesterases), and is excreted in the urine. Cytochrome P450 isoenzymes are not involved in the metabolism of rivastigmine hence minimizing drug-drug interactions. Starting dose of rivastigmine is 1.5 mg twice a day and can be gradually titrated to the maximal dose of 6 mg twice a day. The minimal effective dose is 3mg twice a day. A transdermal form of rivastigmine has been developed and is available on most markets since 2008

(Blesa *et al.,*2007;Winblad *et al*., 2007). The main objective of transdermal rivastigmine is to allow titration to the highest (and most therapeutic) doses of the medication while minimizing side effects. This is achieved by slow release of the medication into the circulation as demonstrated by a Cmax of 8 hours by transdermal route. Starting dose of transdermal rivastigmine is 5 cm^2, and the effective and maximal dose is 10 cm^2. More side-effects were reported with rivastigmine than with placebo and they were dose-dependent. Most common side effects were nausea, vomiting, diarrhoea, anorexia, headache, syncope, abdominal pain and dizziness.

4.5.3 Tacrine (Cognex)

Tacrine was the first cholinesterase inhibitor approved. Doctors rarely prescribe it today because it's associated with more serious side effects than the other three drugs in this class.

4.6 Medication for moderate to severe stages

A second type of medication, memantine (Namenda) is approved by the FDA for treatment of moderate to severe Alzheimer's.

Memantine is prescribed to improve memory, attention, reason, language and the ability to perform simple tasks. It can be used alone or with other Alzheimer's disease treatments. There is some evidence that individuals with moderate to severe Alzheimer's who are taking a cholinesterase inhibitor might benefit by also taking memantine. Donepezil (Aricept) is the only cholinesterase inhibitor approved to treat all stages of Alzheimer's disease, including moderate to severe.

Memantine:

Studies have shown that enhancement of the excitatory effects of the neurotransmitter glutamate may play a role in the pathogenesis of AD (theory of "excitotoxicity") [152]. Memantine is a N - methyl - D - aspartate (NMDA) non - competitive glutatmate receptor antagonist [153]. It is well absorbed after oral administration and reaches Cmax in 3 to 8 hours. Elimination half - life is 60 - 80 hours. It binds to proteins in a proportion of 45%, and is almost completely excreted unchanged in the urine. Starting dose is 5 mg daily (in one or two doses). Minimal therapeutic dose is 10 mg daily, and maximal dose is 20 mg daily.

Vitamin E

Doctors sometimes prescribe vitamin E to treat cognitive Alzheimer's symptoms. No one should take vitamin E to treat Alzheimer's disease except under the supervision of a physician.

Vitamin E is an antioxidant, a substance that may protect brain cells and other body tissues from certain kinds of chemical wear and tear. Its use in Alzheimer's disease is based chiefly on a 1997 study showing that high doses delayed loss of ability to carry out daily activities and placement in residential care for several months. That study was conducted by the Alzheimer's disease Cooperative Study (ADCS), the clinical research consortium of the National Institute on Aging (NIA). Since the ADCS study was carried out, scientists have found evidence in other studies that high-dose vitamin E may slightly increase the risk of death, especially for those with coronary artery disease.

Table -4.4 : Treatments-at-a-glance

Generic	Brand	Approved For
Donepezil	Aricept	All stages
Galantamine	Razadyne	Mild to moderate
Memantine	Namenda	Moderate to severe
Rivastigmine	Exelon	Mild to moderate
Tacrine	Cognex	Mild to moderate
Vitamin E	Not applicable	Not approved

4.7 Side Effects of the AD Drugs

Although, acetylcholinesterase inhibitors are widely used for the treatment of AD, the benefit of cholinesterase inhibitors do not come without unpleasant side effects. The side effects of currently used drugs for AD treatment are given in the table 1.3.

Table- 4.5: Side Effects of the AD Drugs

Generic	Side Effects
Donepezil	Nausea, vomiting, dizziness, loss of appetite, weight loss, muscle cramps, tiredness, trouble sleeping and increased frequency of bowel movements.
Galantamine	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
Memantine	Headache, constipation, confusion and dizziness, drowsiness, headache, insomnia, agitation and hallucination.
Rivastigmine	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
Tacrine	Possible liver damage, nausea, emesis, diarrhea, vomiting, dyspepsia, rhinitis, myalgia, tremor and excessive urination.
Vitamin E	Can interact with medications prescribed to lower cholesterol or prevent blood clots; may slightly increase risk of death.

4.8 Anticholinesterase

Anticholinesterase, any of several drugs that prevent destruction of the neurotransmitter acetylcholine by the enzyme acetylcholinesterase within the nervous system. Acetylcholine acts to transmit nerve impulses within the parasympathetic nervous system i.e., that part of the autonomic nervous system that tends to induce secretion, to contract smooth muscles, and to dilate blood vessels. In preventing the destruction of acetylcholine, anticholinesterase permits high levels of this neurotransmitter to build up at the sites of its action, thus stimulating the parasympathetic nervous system and in turn slowing the heart action, lowering blood pressure, increasing secretion, and inducing contraction of the smooth muscles.

Anticholinesterase a drug that inhibits or inactivates the action of acetylcholinesterase. Drugs of this class cause acetylcholine to accumulate at the junctions of various cholinergic nerve fibers and their effector sites or organs, allowing potentially continuous stimulation of cholinergic fibers throughout the central and peripheral nervous systems. Anticholinesterases include physostigmine salicylate, neostigmine, edrophonium, and pyridostigmine. Neostigmine and

pyridostigmine are prescribed in the treatment of myasthenia gravis; edrophonium in the diagnosis of myasthenia gravis and the treatment of overdose of curariform drugs. Many agricultural insecticides have been developed from anticholinesterases; these are the highly toxic chemicals called organophosphates. Nerve gases developed as potential chemical warfare agents contain potent, irreversible forms of anticholinesterase.

4.9 Cholinergic hypothesis

A variety of studies in humans indicate that basal forebrain and rostral forebrain cholinergic pathways including converging projections to the thalamus serve important functional roles in conscious awareness, attention, working memory, and a number of additional mnemonic processes [154]. For more than 20 years, studies of the brains of those with advanced age and Alzheimer's disease (AD) have consistently found damage or abnormalities in these pathways (particularly basal forebrain projections) that appeared to correlate well with the level of cognitive decline. As a result, the so-called "cholinergic hypothesis" was developed, which essentially states that a loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and AD [155].

One of the most prominent features observed in AD patients is a deficiency of acetylcholine (ACh), a neurotransmitter found in the synapses of the cerebral cortex. The cholinesterase enzyme exists in two different forms in humans, AChE and butyrylcholinesterase (BuChE). AChE is the main cholinesterase in the CNS, while BuChE, originated from glial cells, is more common in serum. Decreased AChE activity and stable or increased BuChE activities were detected in the brains of AD. Most of the neocortical AChE activity in AD brain was found associated with NPs, in which it colocalized with Aβ deposits including both the diffuse amyloid deposits and the mature NPs [156]. Further studies showed that AChE promoted the aggregation of Aβ peptides and accelerated the formation of amyloid plaque, suggesting that AChE may play a pathogenic role in AD by influencing Aβ processing [157]. It has also been shown that Aβ which aggregates with AChE is more toxic to cells compared to aggregates of Aβ alone.

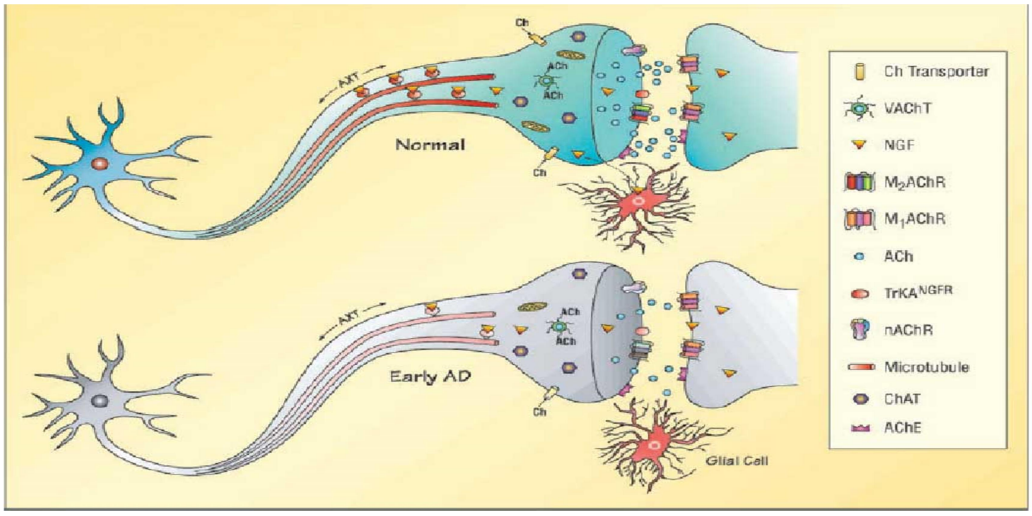


Fig 4.3 : Schematic representation of the known and proposed changes in cholinergic neurons that occur in the aged and early AD brain compared with healthy young neurons. Alterations in high-affinity choline uptake, impaired acetylcholine release, deficits in the expression of nicotinic and muscarinic receptors, dysfunctional neurotrophin support (i.e., NGF receptors), and deficits in axonal transport are represented in the early AD neuron either by a decrease in the number of symbols presented or by reduced color intensity.

4.10 Tau hypothesis

Neurofibrillary tangles, which are filamentous inclusions in pyramidal neurons, occur in Alzheimer's disease and other neurodegenerative disorders termed tauopathies. The number of neurofibrillary tangles is a pathologic marker of the severity of Alzheimer's disease. The major component of the tangles is an abnormally hyperphosphorylated and aggregated form of tau. Normally an abundant soluble protein in axons, tau promotes assembly and stability of microtubules and vesicle transport. Hyperphosphorylated tau is insoluble, lacks affinity for microtubules, and self-associates into paired helical filament structures [158]. More than 30 mutations of *tau* on chromosome 17 have been detected in front temporal dementia with Parkinsonism. By contrast, *tau* mutations do not occur in Alzheimer's disease, and the extent of neuron loss is out of proportion to the number of neurofibrillary tangles. Nevertheless, increased levels of phosphorylated and total tau in the cerebrospinal fluid correlate with reductions in scores on cognitive examinations. Elevated levels of phosphotau

amino acids T181, T231, and total tau in the cerebrospinal fluid together constitute a biomarker test with good accuracy for predicting incipient Alzheimer's disease in patients with mild cognitive impairment. Experimental evidence indicates that Aβ accumulation precedes and drives tau aggregation. Moreover, Aβ-induced degeneration of cultured neurons and cognitive deficits in mice with an Alzheimer's disease–like illness require the presence of endogenous tau.

1.11 Objectives

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting the brain. It is the most common cause of dementia, leading to deterioration in vital cognitive process such as memory, understanding and speech. About 25 million people are estimated to suffer from this disease all over the world and 66% of them live in the third world countries. These data demand for an effective treatment of AD.

AD is a multifactorial disease. Although several factors have been identified in the etiology and pathogenesis of AD, cholinergic dysfunction and oxidative stress have been implicated to be the major contributing factors in the pathogenesis of AD. According to the cholinergic hypothesis, there is extensive loss of cholinergic neurons, particularly in the basal forebrain observed in AD patients which is accompanied by deficiency of acetylcholine (ACh), a neurotransmitter found in the synapses of the cerebral cortex[159-161]. The loss of cholinergic neurons and the associated decrease in levels of acetycholine has been found to correlate well with the cognitive impairment seen in AD patients[162]. Therefore, elevation of acetylcholine by inhibiting acetylcholinesterase (AChE), which is involved in the breakdown of acetylcholine, appears to improve the symptoms of cognitive deficits in AD and serves as an important strategy in the development of drug. Based on this strategy, to date only three cholinesterase inhibitors (ChEI) such as donepezil, galantamine and rivastigmine has been approved by the Food and Drug Administration (FDA) to treat AD[163,164]; however, there are several limitations with usage, due to low bioavailability by oral administration, permeability through blood brain membrane, pharmacokinetic parameters, and hepatotoxicity (Scarpini *et al*., 2003; Sugimoto, 2008). Recently, there are also growing evidences that BuChE contributes to ACh hydrolysis and function in cholinergic transmission(Giacobini *et al*., 2003, Mesulam *et al*., 2002). In AD brain AChE activities decreased while BuChE activities increased (Perry *et al*., 1978 Darvesh *et al*., 2003). BuChE is also present in the

plaques and tangles of AD (Guillozet *et al*.,1997). AChE and BuChE amplified the toxicity of β-amyloid (Aβ) peptide in tissue culture (Yang *et al*., 2002). Greig et al. reported that high-doses of BuChE-selective cymserine analogues did not cause classical cholinergic toxicity Greig *et al*., 2005). It was suggested that BuChE-specific inhibition is unlikely to be associated with adverse events and may show clinical efficacy without remarkable side effects. Therefore, BuChE may be one of the important targets for novel drug development to treat AD patients(Chen *et al*., 2011, Nawaz *et al*., 2011, Wiêckowska *et al*., 2010, Huang *et al*.,2010). In addition, oxidative stress is extensive in AD. Excessive lipid peroxidation, protein oxidation, DNA and RNA oxidation, glycoxidation have all been documented in AD brains. Therefore, antioxidant therapies envisaging the reduction of oxidative damage and the increase of endogenous antioxidant defenses have been suggested to prevent, delay, or ameliorate the disease symptoms. At present there is special interest on natural antioxidants from the plant kingdom. The potential beneficial role of natural antioxidant has been emphasized in various diseases including AD. Therefore, researchers have paid their attention to the plant kingdom for the development of new AD drug.

Vanda roxburghii, belonging to the family Orchidaceae is an epiphytic and is widely distributed throughout the Bangladesh. The plant is traditionally used by the local people as tonic to brain in order to enhance memory and to treat the disease of nervous system including AD. According to ethno medical uses, the root of plant is reputed to have activity against inflammations, bronchitis, rheumatic pain, disease of abdomen and tremor. The plant is also used in the treatment of infectious disease including bacterial infections and tuberculosis. A comprehensive literature review revealed that the plants of this family has some promising biological activities including antiinflammation, antihepatotoxic, antidiabetic, antimicrobial, anticancer, lipoprotein oxidation, immunomodulatory effect .

A preliminary study from our laboratory reported earlier that the extract of *Vanda roxburghii,* has strong antioxidant activity and weak acetylcholinesterase inhibitory properties. But no study has yet been performed on the butyrylcholinesterase inhibitory activities of this plant. Therefore, the present work was designed to investigate the effect of this plant in the inhibition of butyrylcholinesterase *in vitro* and to isolate the compounds responsible for the activity using conventional chromatographic techniques. In addition, the correlation between the strength of these effects and total polyphenolic and flavonoid content was also analyzed.

MATERIALS AND METHODS

5.1 Biological study on *Wedelia chinensis*

5.1.1 *In-vitro* studies on *Wedelia chinensis.*

- *In- vitro* antioxidant studies
- *In-vitro* anticholinesterase studies

5.1.2 *In- vitro* antioxidant studiesof different extractive fractions and isolated compounds

5.1.2.1 Determination of Reducing Power capacity

The reducing power of different extractives of *Alstoniascholaris* was evaluated by the method of Oyaizu (1986).

Principle

In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductant's such as antioxidant substances in the samples causes the reduction of the Fe^{3+}- ferricyanide complex to the ferrous form by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

Fe^{3+} + ferricyanide + e^- $\longrightarrow$ Fe^{2+} +ferrocyanide

Materials

- Potassium ferricyanide [$K_3Fe(CN)_6$]
- Trichloro Acetic acid (Cl_3C-COOH)
- Ferric Chloride ($FeC1_3$)
- Phosphate buffer [K_2HPO_4 + KH_2PO_4]
- Ascorbic acid (Analytical or Reagent grade)
- Water bath
- Centrifuge machine
- Pipette (1-10 mL)
- UV spectrophotometer

Experimental Procedure

1. 1.0 mL of plant extract or standard of different concentration Solution was taken in a test tribe.
2. 2.5 mL of potassium buffer (0.2 M) and 2.5 mL of Potassium ferricyanide [$K_3Fe(CN)_6$], (1%) solution were added into [lie test tube.
3. The reaction mixture was incubated for 20 minutes at 50°C to complete the reaction.
4. 2.5 mL of trichloro acetic acid, (10%) solution was added into the test tube.
5. The total mixture was centrifuged at 3000 rpm for 10 mins.
6. 2.5 mL supernatant solution was withdrawn from the mixture and mix with 2.5 mL of distilled water.
7. 0.5 mL of ferric chloride ($FeCI_3$ (0.1%) solution was added to the reaction mixture.
8. Then the absorbance of the solution was measured at 700 nm using a spectrophotometer against blank.
9. A typical blank solution contained the same solution mixture without plant extract or standard and it was incubated under the same conditions as the rest of the samples solution.
10. Also the absorbance of the blank solution was measured at 700 nm against the solvent used in solution preparation. Increased absorbance of the reaction mixture indicated increase reducing power.

5.1.2.2 Determination of antioxidant capacity

Total antioxidant capacity of different extractives , different column fractions and the isolated compounds of *W. chinensis* was determined by the method of Prieto*et al.,* (1999) with some modifications.

Principle

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

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

Materials

- Sulphuric acid (Merck, Germany)
- Sodium Phosphate (Sigma chemical company, USA)
- Ammonium Molybdate (Sigma chemical company, USA)
- Ascorbic acid (Analytical or Reagent grade)
- Methanol (Sigma chemical company, USA)
- Water bath
- Micropipette (100-1000 µl)
- Pipette (1-10 ml)
- UV-spectrophotometer (Shimadzu, USA)

Experimental procedure

1. 0.5 mL of plant extract or standard of different concentration solution was taken in a test tube.
2. 3 mL of reaction mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate was added into the test tube.
3. The test tube was incubated at 95^{o}C for 10 minutes to complete the reaction.
4. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature.

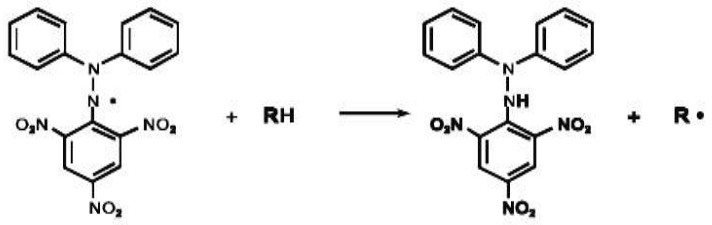
5. A typical blank solution contained 3 mL or reaction mixture and the appropriate volume (300μL) of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples solution.

5.1.2.3 Determination of DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

DPPH was used to evaluate the free radical scavenging activity of various compounds and medicinal plants (Braca et al., 2001; J. Nat. Prod., 64, 892-895).

Principle

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution. With this method it was possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH at 517 nm. Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. In the radical form this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.



DPPH
517 nm

Materials

- DPPH (Sigma chemical company, USA)
- Methanol (Sigma chemical company, USA)
- Catechin
- Pipette (1-10 ml)
- UV spectrophotometer (Shimadzu, USA)

Experimental procedure

1. 2 mL of methanol solution of plant extract or standard at different concentration was taken ina test tube.
2. 3 mL of methanol solution of DPPH was added into the test tube.
3. The test tube was incubated at room temperature for 30 minutes in dark place to complete the reaction.
4. Then the absorbance of the solution was measured at 517 nm using a spectrophotometer against blank.
5. A typical blank solution contained all reagents except plant extract or standard solution.
6. The percentage (%) of scavenging was calculated from the following equation.

$I\% = [(A_0 – A_1)/A_0] \times 100,$

Where,

I% = percentage of scavenging activity

A_0 = absorbance of the control, and

A_1 = absorbance of the extract/standard.

Then % inhibitions were plotted against concentration and from the graph IC_{50} was calculated.

5.1.2.4 Hydroxyl radical scavenging assay of sample

Hydroxyl radical scavenging activity of different extractives, column fractions and isolated compounds of *W. chinensis* was determined by the method as described by Chung *et al.,* (1997) with a slight modification.

Principle

Hydroxyl radical was generated by the Fenton reaction. When and H_2O_2 was incubated with Fe^{2+}-EDTA system at pH 7.4, hydroxyl radicals were generated, and reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA)-like product. This compound forms a pink chromogen upon heating with thiobarbituric acid (TBA) at low P^H. Samples with antiradical property can remove the hydroxyl radicals from 2-deoxy-D-ribose and prevent the formation of pink chromogen. In the radical form the pink chromogen had an absorbance at 532 nm. As the hydroxyl radicals are scavenged by antioxidant compound, the pink chromogen becomes decolorized and the intensity of color can be quantitatively measured at 532 nm.

Materials

- 2-deoxy-D-ribose (Sigma-Aldrich, Japan)
- Thiobarbituric acid (Sigma-Aldrich, Japan)
- Phosphate buffer (Sigma-Aldrich, USA)
- EDTA (Sigma chemical company, USA)
- Hydrogen peroxide (Merck, Germany)
- $FeSO_4 .7H_2O$ (Sigma chemical company, USA)
- Trichloro Acetic acid (Sigma-Aldrich, USA)
- (+)-Catechin (Sigma-Aldrich, Japan)
- Water bath
- UV spectrophotometer (Shimadzu, USA)

Experimental procedure

The Fenton reaction mixture containing 200 µL of 10mM FeSO4·7H2O, 200 µL of 10mM EDTA and 200 µL of 10mM 2-deoxyribose was mixed with 1.2mL of 0.1M phosphate buffer (pH 7.4) containing 200 µL of *samples*. Thereafter, 200 µL of 10

mM H_2O_2 was added to the mixture before incubation for 4h at 37°C. Later, 1mL of 2.8% TCA and 1mL of 1% TBA were added and placed in a boiling water bath for 10min. Then, the resultant mixture was allowed to cool up to room temperature and centrifuged at 395#*g* for 5 min. Absorbance was recorded at 532 nm in a UV-VIS spectrophotometer. The percentage (%) inhibition activity was calculated from the following equation.

$$\% I = [(A_o - A_1)/A_o] X 100$$

Where,

I% = percentage of scavenging activity

A_0 = absorbance of the control, and

A_1 = absorbance of the extract/standard.

Then % inhibitions were plotted against concentration and from the graph IC_{50} was calculated.

5.1.2.5 The inhibition of Lipid peroxidation assay

The inhibition of lipid peroxidation assay was determined according to the method as described by Liu *et al.,* (2000) with a slight modification.

Principle

Initiation of lipid peroxidation takes place either through ferryl-perferryl complex or through $OH^{\cdot}$ radical by Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction). During lipid peroxidation, low molecular weight end products, generally malondialdehyde (MDA), are formed by oxidation of polyunsaturated fatty acids that may react with two molecules of thiobarbituric acid (TBA) to give a pinkish red chromogen (Kumarasamy*et al.,* 2007). The absorbance of pinkish-red color of MDA- TBA complex was measured at 532 nm. The inhibition of lipid peroxidation could be caused by the absence of ferryl-perferryl complex or by scavenging the $OH^{\cdot}$ radical or the superoxide radical or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. The degree of lipid peroxidation was assessed by estimation of the thiobarbituric acid-reactive substances (TBARS).

Materials

- Phosphate buffer (Sigma-Aldrich, USA)
- $FeSO_4$ (Sigma-Aldrich, India)
- Ascorbic acid (Sigma chemical company, USA)
- Trichloroacetic acid (Sigma-Aldrich, USA)
- Thiobarbituric acid (Sigma-Aldrich, Japan)
- (+)-Catechin(Sigma-Aldrich, Japan)
- Centrifuge machine
- Water bath
- Micropipette (10-100 µl)
- Pipette (1-10ml)
- UV spectrophotometer (Shimadzu, USA)

Experimental procedure.

The bovine brain was homogenized with a homogenizer in ice-cold Phosphate buffer (50 mM, pH 7.4) to produce a 1/10 homogenate. The homogenate was centrifuged at 10000g for 15 min at 4^oC.The supernatant was used as liposome for *in vitro* lipid peroxidation assay.Theability of plant extract to inhibit lipid peroxidation was studied by incubating bovine brain homogenates treated with hydrogen peroxide (10 µM) and different concentrations of plant extract. Hydrogen peroxide induces lipid peroxidation in bovine brain homogenates. Lipid peroxides react with thiobarbituric acid to form a pink product, thiobarbituric acid reacting substances (TBARS), measurable colorimetrically at 532 nm. The difference between the control and plant extract treated sample is the measurement of decrease in TBARS formation, reflecting the reduction of hydroxyl radical induced lipid peroxidation.

In-vitro

5.2 butyrylcholinesterase inhibitory studies of different extractive fractions and isolated compounds

Principle

The butyrylcholinesterase inhibitory activity of different extractives and isolated compounds of *Wedelia chinensis* was determined by modifiedEllman'smethod (Ellman*et al.,* 1961). This method estimates BuchE using butyrylylthiocholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion.

Butyryllcholine → Thiocholine + Butyrate

Thiocholine + dithiobisnitro → Benzoate yellow color

The color intensity can be measured on a spectrophotometer and the enzyme activity expressed as the rate of reaction per minute.

Materials

- 5, 5′-dithio-bis-(2-nitro) benzoic acid (DTNB) (Sigma-Aldrich, Japan)
- S-butyryllthiocholine iodide (Sigma-Aldrich, Japan)
- Human blood serum (Crude enzyme)
- Tris-Hcl buffer (Merck, Germany)
- Triton X-100 (Sigma chemical company, USA)
- BCA kit (bicinchoninic acid; Sigma Co., USA)
- Bovine serum albumin (Merck, India)
- Galanthamine (Sigma-Aldrich, Japan)
- Micropipette (100-1000 µl)
- UV spectrophotometer (Shimadzu, USA)

Experimental procedure

The butyryltylcholinesterase (BChE) inhibitory assay was performed according to the colorimetric method of Ellman*et al.* (Doctor *et al.*, 1987; Ellman*et al*., 1961; Mohamed-Bassem *et al*., 1987) using butyrylthiocholine iodide as a substrate. For the enzyme source, the bovine brain was homogenized in a homogenizer with 5 volumes of a homogenation buffer [10 mMTris-HCl (pH 7.2), which contained 1 M NaCl, 50 mM $MgCl_2$ and 1% Triton X-100] (Rieger *et al*., 1980), and centrifuged at 10,000' *g* for 30 min. The resulting supernatant was used as an enzyme source. All of the extraction steps were carried out at 4°C. Protein concentration was determined using the BCA kit (bicinchoninic acid; Sigma Co., USA) with bovine serum albumin (BSA) as a protein standard. The rates of hydrolysis by acetylcholinesterase were monitored spectrophotometrically. Each sample or standard (500 µl) was mixed with an enzyme solution (500 µl) and incubated at 37°C for 15 min. Absorbance at 405 nm was read immediately after adding an Ellman's reaction mixture [3.5 ml; 0.5 mMbutyrylthiocholine, 1 mM 5, 5′-dithio-bis (2-nitro benzoic acid)] in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. Reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. The blank reaction was measured by substituting saline for the enzyme.

In- vitro

5.3 Acetylcholinesterase inhibitory studies of different extractive fractions and isolated compounds

Principle

The acetylcholinesterase inhibitory activity of the different extractives and isolated compounds of *W.chinensis* was determined by modified Ellman's method (Ellman*et al.,* 1961). This method estimates AchE using acetylthiocholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion.

$$\text{Acetylcholine} \xrightarrow[\text{enzyme}]{} \text{Thiocholine} + \text{Acetate}$$

$$\text{Thiocholine} + \text{dithiobisnitro} \rightarrow \text{Benzoate yellow color}$$

The color intensity can be measured on a spectrophotometer and the enzyme activity expressed as the rate of reaction per minute.

Materials

- 5, 5′-dithio-bis-(2-nitro) benzoic acid (DTNB) (Sigma-Aldrich, Japan)
- Acetylthiocholine iodide (Sigma-Aldrich, Japan)
- Bovine brain homogenate (Crude enzyme)
- Tris-Hcl buffer (Merck, Germany)
- Triton X-100 (Sigma chemical company, USA)
- BCA kit (bicinchoninic acid; Sigma Co., USA)
- Bovine serum albumin (Merck, India)
- Donepezil (Sigma-Aldrich, Japan)
- Micropipette (100-1000 µl)
- UV spectrophotometer (Shimadzu, USA)

Experimental procedure

The acetylcholinesterase (AChE) inhibitory assay was performed according to the colorimetric method of Ellman *et al.* (Doctor *et al*., 1987; Ellman *et al*., 1961; Mohamed-Bassem *et al*., 1987) using acetylthiocholine iodide as a substrate. For the enzyme source, the bovine brainwas homogenized in a homogenizer with 5 volumes of a homogenation buffer [10 mMTris-HCl (pH 7.2), which contained 1 M NaCl, 50 mM $MgCl_2$ and 1% Triton X-100] (Rieger *et al*., 1980), and centrifuged at 10,000 ´ *g* for 30 min. The resulting supernatant was used as an enzyme source. All of the extraction steps were carried out at 4°C. Protein concentration was determined using the BCA kit (bicinchoninic acid; Sigma Co., USA) with bovine serum albumin (BSA) as a protein standard. The rates of hydrolysis by acetylcholinesterase were monitored spectrophotometrically. Each sample or standard (500 µl) was mixed with an enzyme solution (500 µl) and incubated at 37°C for 15 min. Absorbance at 405 nm was read immediately after adding an Ellman's reaction mixture [3.5 ml; 0.5 mM acetylthiocholine, 1 mM 5, 5´-dithio-bis (2-nitro benzoic acid)] in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. Reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. The blank reaction was measured by substituting saline for the enzyme.

RESULTS AND DISCUSSIONS

In-*vitro* Study

6.1 Antioxidant activity

6.1.1 Determination of reducing power capacity

6.1.2 Determination of reducing power capacity of the crude methanol extract (CME) of *Wedelia chinensis* and its different fractions.

The Fe^{3+} reducing power of the crude methanol extract (CME) of *wedelia chinensis* and its different fractions was determined by the method of Oyaizu (1986) with sight modification. The reductive capabilities of crude methanol extract (CME) and its different fractions and the reference standard catechin are shown in Table: 6.1 and Fig:6.1& 6.2.

Table-6.1: Data for Reducing power capacity of the crude methanol extract (CME) of *Wedelia chinensis* and its different fractions.

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
(+)-Catechin (standard)	2.5	0.018	0.020	0.021	0.019±0.0015
	5	0.114	0.116	0.115	0.115±0.0010
	10	0.543	0.517	0.521	0.527±0.0140
	20	1.221	1.210	1.209	1.213±0.0066
	40	1.937	1.925	1.817	1.893±0.0661
	80	2.534	2.412	2.410	2.452±0.0710
Crude methanol extract (CME)	2.5	0.025	0.029	0.027	0.027±0.0020
	5	0.073	0.071	0.081	0.075±0.0052
	10	0.282	0.274	0.262	0.273±0.0100
	20	0.701	0.708	0.685	0.698±0.0117
	40	1.321	1.319	1.345	1.328±0.1446
	80	1.754	1.807	1.788	1.783±0.0268
Petroleum ether fraction (PEF)	2.5	0.029	0.031	0.021	0.027±0.0052
	5	0.065	0.077	0.081	0.075±0.0083
	10	0.128	0.119	0.117	0.121±0.0058
	20	0.462	0.457	0.455	0.458±0.0036
	40	0.865	0.846	0.848	0.853±0.0104
	80	0.936	0.938	0.952	0.942±0.0087

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
Chloroform fraction (CLF)	2.5	0.013	0.014	0.009	0.012±0.0026
	5	0.103	0.089	0.092	0.095±0.0073
	10	0.221	0.211	0.219	0.217±0.0052
	20	0.533	0.557	0.552	0.547±0.0126
	40	1.092	0.981	0.879	0.984±0.1065
	80	1.368	1.372	1.409	1.383±0.0226
Ethyl acetate fraction (EAF)	2.5	0.009	0.021	0.012	0.014±0.0062
	5	0.176	0.195	0.192	0.187±0.0102
	10	0.506	0.498	0.529	0.511±0.0161
	20	1.003	0.982	0.976	0.987±0.0141
	40	1.612	1.609	1.573	1.598±0.0217
	80	2.202	2.216	2.318	2.245±0.0633
Dia-ion resin fraction (DRF)	2.5	0.035	0.034	0.038	0.036±0.0020
	5	0.211	0.207	0.198	0.205±0.0066
	10	0.593	0.603	0.565	0.587±0.0196
	20	1.327	1.407	1.311	1.348±0.0514
	40	2.087	2.176	2.076	2.113±0.0548
	80	2.498	2.596	2.532	2.542±0.0497

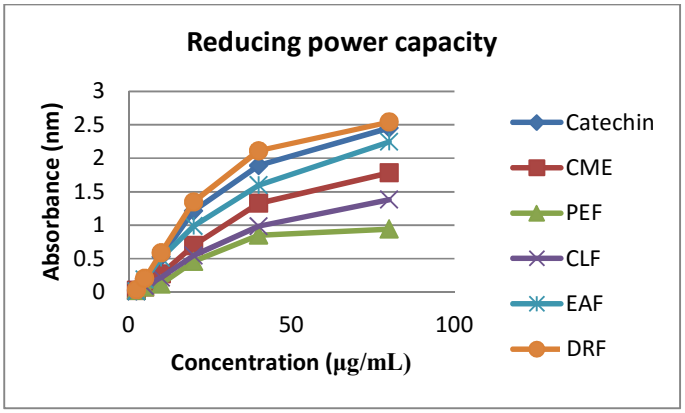


Fig 6.1: Reducing power capacity of crude methanolic extract (CME) of *wedelia chinensis* and its different fractions (PEF, CLF, EAF & DRF) and (+)-catechin (standard)

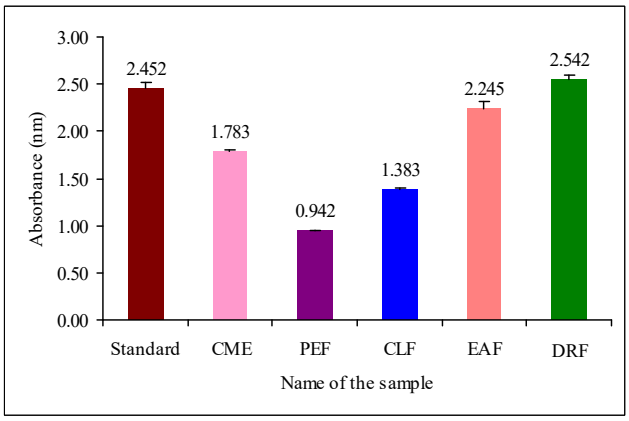


Fig 6.2: At 80 µg/mL the absorbance of crude methanolic extract (CME) of *wedelia chinensis,* its different fractions PEF, CLF, EAF & DRF and (+) -catechin (standard).

From the results, it was found that crude methanolic extract and it's different fractions tested exhibit reducing activity. Of them ethyl acetate fraction (EAF) and dia-ion resin fraction (DRF) showed the higher reducing power capacity with absorbance of 2.245 and 2.542, respectively at the concentration of 80 µg/mL, where as the reference standard catechin showed absorbance of 2.452. Petroleum ether fraction (PEF) and chloroform fraction (CLF) showed mild reducing activity.

The activity of the fractions and the reference standard catechin decreases in the following order.

Catechin > DRF > EAF > CME > CLF > PEF.

6.1.3 Determination of reducing power capacity of isolated compounds from ethyl acetate fraction of *wedelia chinensis*.

Table-6.2 : The reducing power capacity of isolated compounds EFC-1 and EFC-2.

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
(+)-Catechin (standard)	2.5	0.018	0.020	0.021	0.019±0.0015
	5	0.114	0.116	0.115	0.115±0.0010
	10	0.543	0.517	0.521	0.527±0.0140
	20	1.221	1.210	1.209	1.213±0.0066
	40	1.937	1.925	1.817	1.893±0.0661
	80	2.534	2.412	2.410	2.452±0.0710

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
EFC-1	2.5	0.025	0.021	0.027	0.024±0.0030
	5	0.119	0.122	0.131	0.124±0.0062
	10	0.455	0.454	0.462	0.458±0.0043
	20	0.973	0.972	0.979	0.975±0.0037
	40	1.543	1.553	1.542	1.546±0.0060
	80	1.893	1.863	1.873	1.876±0.0152
EFC-2	2.5	0.016	0.022	0.026	0.021±0.0050
	5	0.078	0.085	0.086	0.083±0.0043
	10	0.389	0.398	0.402	0.396±0.0066
	20	0.835	0.845	0.847	0.842±0.0064
	40	1.254	1.267	1.271	1.264±0.0088
	80	1.643	1.658	1.656	1.652±0.0081

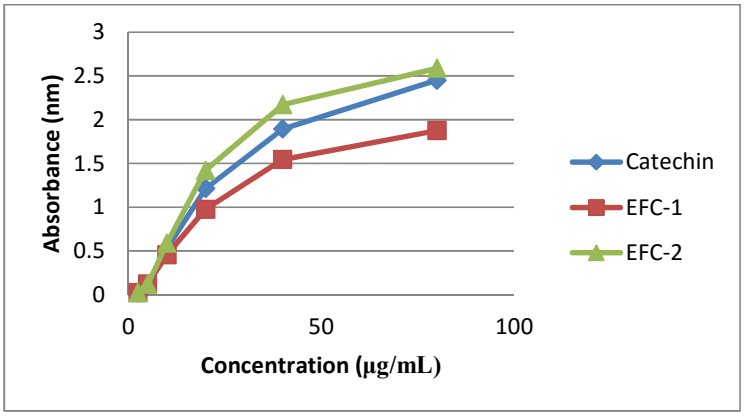


Fig 6.3: Reducing power capacity of compounds EFC-1& EFC-2 (from ethyl acetate fraction) and (+)-catechin (standard).

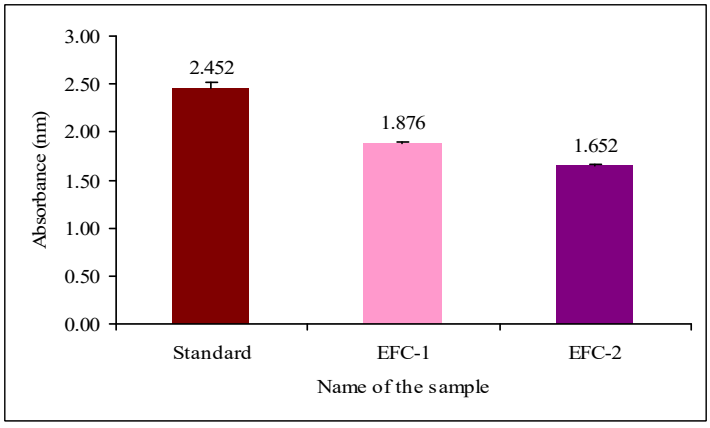


Fig 6.4: At 80 µg/mL the absorbance of compounds EFC-1 and EFC-2 from ethyl acetate fraction and (+)-catechin (standard).

From the results, it was found that the isolated compound EFC-1 and EFC-2 from the ethyl acetate fraction (EAF) and reference standard catechin tested exhibit reducing activity. Of them compound EFC-1 and EFC-2 showed the highest reducing power capacity with absorbance of 1.876 and 1.652 respectively at the concentration of 80 µg/mL which appeared to be very similar to the activity of the reference standard catechin.

It was found that the compound EFC-1 showed strong reducing activity which was very close to the activity of the reference standard catechin. The absorbance of EFC-1 and catechin at concentrates of 80 µg/mL were 1.876 and 2.452 respectively. Compound EFC-2 showed activity with absorbs of 1.652 at the same concentration.

The activity of the compounds and the reference standard catechin decreases in the following order.

Catechin > EFC-1 > EFC-2 >EAF.

6.1.4 Determination of reducing power capacity of isolated compounds from dia-ion resin fraction of *wedelia chinensis*.

Table-6.3: The reducing power capacity of isolated compounds DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 of *wedelia chinensis.*

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
(+)-Catechin (standard)	2.5	0.018	0.020	0.021	0.019±0.0015
	5	0.114	0.116	0.115	0.115±0.0010
	10	0.543	0.517	0.521	0.527±0.0140
	20	1.221	1.210	1.209	1.213±0.0066
	40	1.937	1.925	1.817	1.893±0.0661
	80	2.534	2.412	2.410	2.452±0.0710
DFC-1	2.5	0.015	0.020	0.018	0.017±0.0025
	5	0.086	0.108	0.097	0.097±0.0115
	10	0.196	0.202	0.207	0.202±0.0055
	20	0.415	0.428	0.431	0.424±0.0085
	40	0.856	0.861	0.872	0.863±0.0081
	80	1.408	1.418	1.423	1.416±0.0076

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
DFC-2	2.5	0.018	0.027	0.035	0.026±0.0085
	5	0.119	0.125	0.131	0.125±0.0063
	10	0.408	0.405	0.423	0.412±0.0096
	20	0.864	0.866	0.883	0.871±0.0104
	40	1.558	1.589	1.581	1.576±0.0161
	80	2.121	2.179	2.186	2.162±0.0356
DFC-3	2.5	0.032	0.043	0.031	0.035±0.0066
	5	0.215	0.228	0.231	0.224±0.0085
	10	0.842	0.798	0.856	0.832±0.0302
	20	1.512	1.560	1.509	1.527±0.0286
	40	2.176	2.135	2.119	2.143±0.0294
	80	2.732	2.775	2.777	2.761±0.0254
DFC-4	2.5	0.030	0.041	0.037	0.036±0.0055
	5	0.203	0.219	0.234	0.218±0.0155
	10	0.589	0.593	0.609	0.597±0.0105
	20	1.346	1.407	1.408	1.387±0.0355
	40	1.876	1.886	2.187	1.981±0.1767
	80	2.589	2.671	2.576	2.612±0.0515
DFC-5	2.5	0.049	0.048	0.056	0.051±0.0043
	5	0.275	0.288	0.283	0.282±0.0065
	10	0.397	0.389	0.394	0.394±0.0040
	20	0.579	0.581	0.595	0.585±0.0087
	40	1.256	1.248	1.281	1.261±0.0172
	80	1.871	1.878	1.882	1.877±0.0055

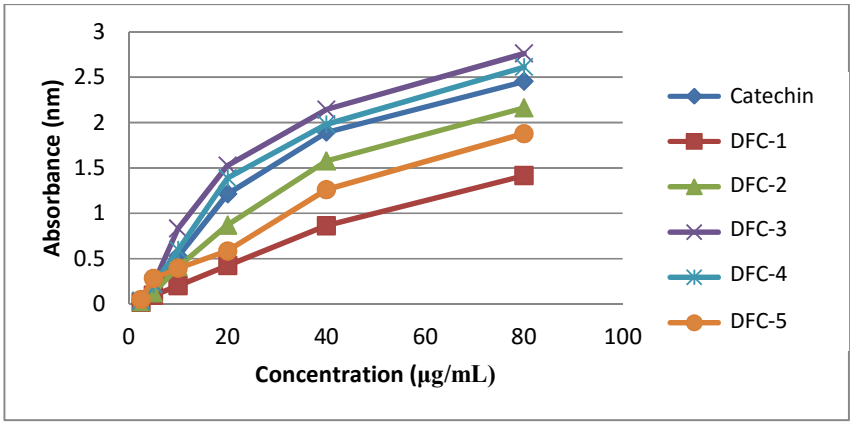


Fig 6.5: Reducing power capacity of isolated compounds DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 and (+)-catechin (standard).

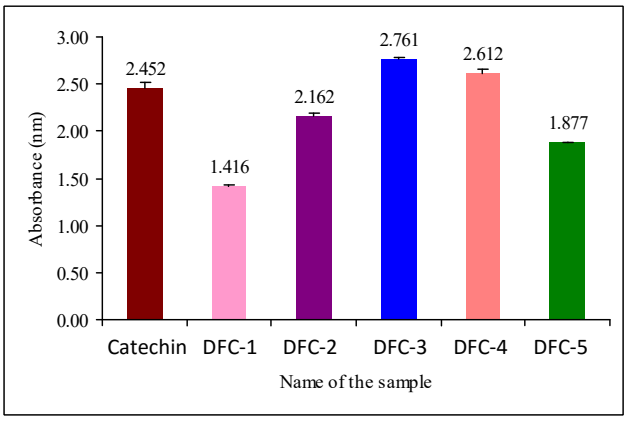


Fig 6.6 : At 80 µg/mL the absorbance of compounds DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 from dia-ion resin fraction and (+)-catechin (standard).

From the results, it was found that the isolated compounds (DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5), dia-ion resin fraction (DRF) and reference standard catechin tested exhibit reducing activity. Of them compound DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 showed the higher reducing power capacity which appeared to be very similar to the activity of the reference standard catechin.

The activity of the compounds and the reference standard catechin decreases in the following order.

Catechin > DFC-3 > DFC-5 > DFC-4 >DFC-2 > DRF > DFC-1.

6.2 Determination of total antioxidant activity

6.2.1 Determination of total antioxidant activity of the crude methanol extract (CME) of *Wedelia chinensis* and its different fractions.

Total antioxidant activity of crude methanol extract (CME) of *wedelia chinensis,* it's different fractions, isolated compounds and the reference standard catechin. The phosphomolybdenum method was based on the reduction of Mo (V1) to Mo (V) by the antioxidant compound and the formation of green phosphate / Mo (v) complex with a maximum absorption at 695 nm. The results of total antioxidant activity of the extracts, fractions and compounds are presented in Table -6.4,6.5 & 6.6 and in Fig. 6.7 & 6.8.

Table-6.4: Total antioxidant activity of difference fractions of *wedelia chinensis.*

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
+(-)Catechin (standard)	2.5	0.074	0.081	0.079	0.078 ±0.0036
	5	0.206	0.206	0.208	0.207±0.0011
	10	0.249	0.251	0.252	0.251±0.0015
	20	0.303	0.304	0.305	0.304±0.0010
	40	0.404	0.403	0.407	0.405±0.0021
	80	0.485	0.493	0.486	0.488±0.0043
Crude methanol extract (CME)	2.5	0.043	0.046	0.053	0.047±0.0051
	5	0.147	0.141	0.145	0.144±0.0030
	10	0.266	0.164	0.173	0.221±0.0564
	20	0.212	0.209	0.205	0.298±0.0035
	40	0.271	0.275	0.263	0.376±0.0061
	80	0.352	0.345	0.347	0.456±0.0036
Petroleum ether fraction (PEF)	2.5	0.023	0.027	0.035	0.028±0.0061
	5	0.130	0.131	0.135	0.133±0.0026
	10	0.184	0.197	0.201	0.194±0.0088
	20	0.231	0.243	0.253	0.242±0.0110
	40	0.291	0.303	0.301	0.298±0.0064
	80	0.343	0.345	0.353	0.347±0.0052
Chloroform fraction (CLF)	2.5	0.057	0.046	0.055	0.053±0.0058
	5	0.125	0.129	0.124	0.126±0.0026
	10	0.225	0.198	0.209	0.211±0.0135
	20	0.272	0.281	0.275	0.276±0.0045
	40	0.317	0.322	0.324	0.321±0.0036
	80	0.371	0.362	0.363	0.365±0.0049
Ethyl acetate fraction (EAF)	2.5	0.091	0.083	0.078	0.084±0.0065
	5	0.178	0.179	0.180	0.179±0.0010
	10	0.253	0.260	0.262	0.258±0.0047
	20	0.349	0.356	0.351	0.352±0.0036
	40	0.462	0.469	0.473	0.468±0.0055
	80	0.521	0.532	0.535	0.529±0.0073
Dia-ion resin fraction (DRF)	2.5	0.068	0.078	0.079	0.075±0.0060
	5	0.145	0.151	0.152	0.149±0.0037
	10	0.292	0.284	0.286	0.287±0.0041
	20	0.375	0.362	0.359	0.365±0.0085
	40	0.478	0.481	0.468	0.476±0.0068
	80	0.553	0.565	0.551	0.556±0.0075

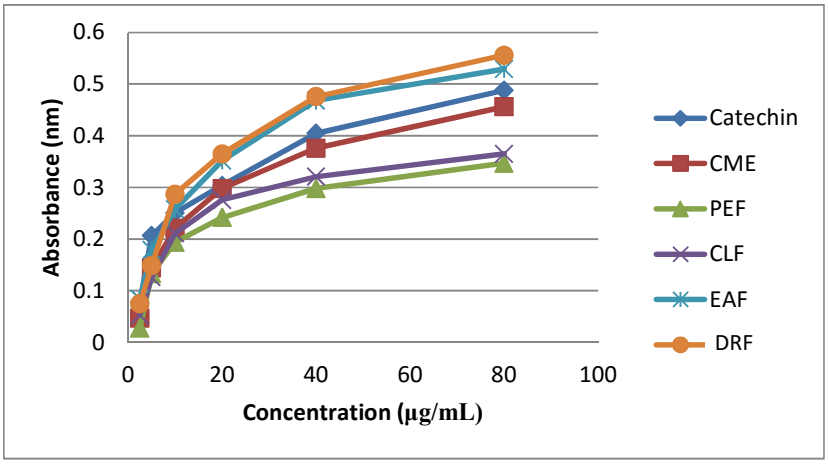


Fig 6.7: Total antioxidant activity of crude methanolic extract (CME) of *wedelia chinensis,* its different fractions and (+)-catechin (standard).

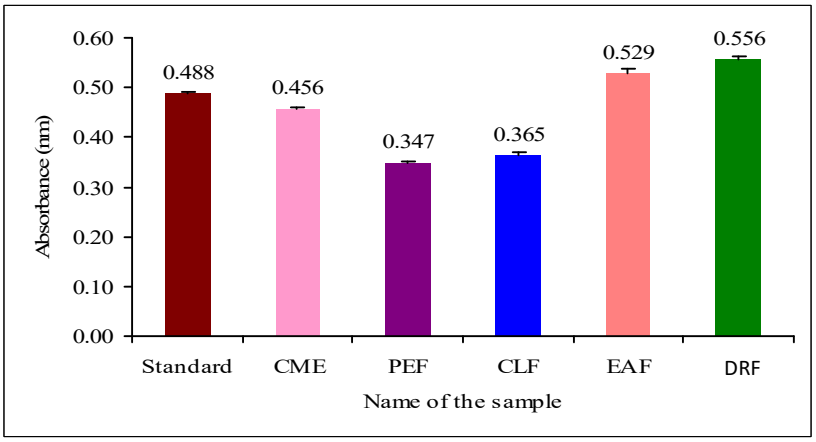


Fig 6.8: At 80 µg/mL the absorbance of crude methanolic extract (CME) of *wedelia chinensis,* its different fractions PEF, CLF, EAF & DRF and (+)-catechin (standard).

The crude methanol extract (CME) and the different fractions antioxidant activity increasing with in concentration increasing. The results demonstrated that the total antioxidant activity of ethyl acetate fraction and dia-ion resin fraction higher than crude methanolic extract. However, the activity was less than reference standard catechin. All the sample was found to increase the total antioxidant activity with the increase of the concentration. At 80 µg/mL the absorbance of methanolic extract, ethyl acetate fraction, dia-ion resin fraction and reference standard catechine were 0.456, 0.529, 0.556 and 0.488 respectively.

The total antioxidant activity of the fractions and the reference standard catechin the following order: DRF > EAF > Catechin > CME > CLF > PEF.

6.2.2 Determination of total antioxidant activity of isolated compounds from ethyl acetate fraction of *W. chinensis.*

Table-6.5 : Data for Antioxidant activity of isolated compounds from ethyl acetate fraction of *wedelia chinensis.*

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
+(-)Catechin (standard)	2.5	0.074	0.081	0.079	0.078 ±0.0036
	5	0.206	0.206	0.208	0.207±0.0011
	10	0.249	0.251	0.252	0.251±0.0015
	20	0.303	0.304	0.305	0.304±0.0010
	40	0.404	0.403	0.407	0.405±0.0021
	80	0.485	0.493	0.486	0.488±0.0043
EFC-1	2.5	0.103	0.094	0.096	0.098±0.0047
	5	0.181	0.189	0.191	0.187±0.0052
	10	0.318	0.329	0.317	0.321±0.0066
	20	0.392	0.407	0.394	0.398±0.0081
	40	0.482	0.495	0.483	0.486±0.0072
	80	0.527	0.538	0.534	0.533±0.0055
EFC-2	2.5	0.093	0.087	0.078	0.086±0.0075
	5	0.161	0.175	0.171	0.169±0.0072
	10	0.276	0.289	0.287	0.284±0.0070
	20	0.324	0.334	0.338	0.332±0.0072
	40	0.416	0.429	0.436	0.427±0.0101
	80	0.472	0.481	0.482	0.478±0.0055

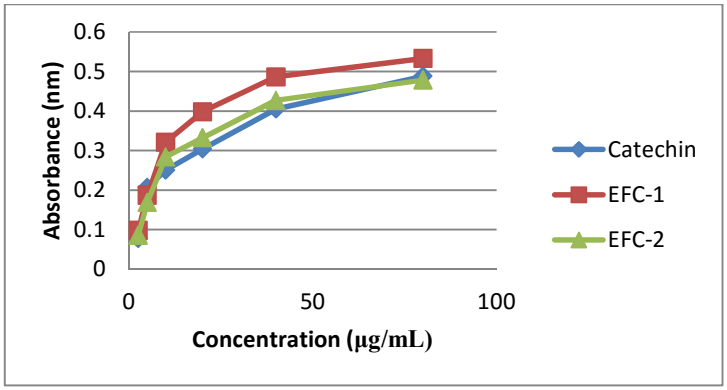


Fig 6.9: Antioxidant activity of isolated compounds EFC-1, EFC-2 and (+)-catechin (standard).

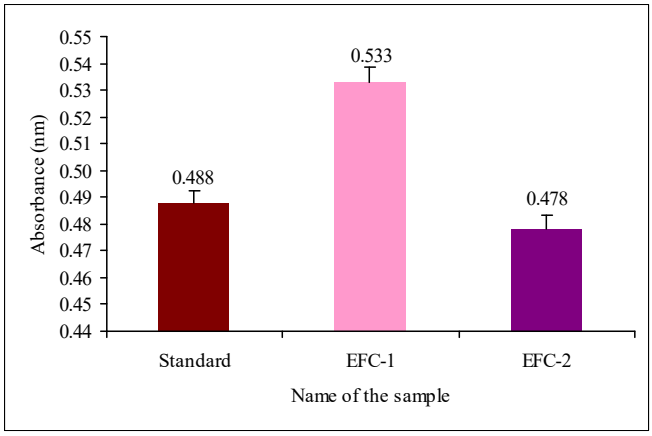


Fig 6.10 : At 80 µg/mL the absorbance of compounds EFC-1 and EFC-2 from ethyl acetate fraction and (+)-catechin (standard).

Further, among the compounds EFC-1 & EFC-2 obtained from ethyl acetate fraction. Highest antioxidant activity was found in compound EFC-1 with absorbance of 0.533 nm at 80 µg/mL, which was more than reference standard catechin and compound EFC-1 similar with catechin.

The total antioxidant activity of the isolated compounds and the reference standard owed the following order: EFC-1 > Catechin > EFC-2.

6.2.3 Determination of total antioxidant activity of isolated compounds from dia-ion resin fraction of *W. chinensis.*

Table-6.6: Data for Antioxidant activity of isolated compounds from dia-ion resin fraction of *wedelia chinensis.*

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
+(-) Catechin (standard)	2.5	0.074	0.081	0.079	0.078 ±0.0036
	5	0.206	0.206	0.208	0.207±0.0011
	10	0.249	0.251	0.252	0.251±0.0015
	20	0.303	0.304	0.305	0.304±0.0010
	40	0.404	0.403	0.407	0.405±0.0021
	80	0.485	0.493	0.486	0.488±0.0043
DFC-1	2.5	0.054	0.067	0.068	0.063±0.0043
	5	0.162	0.149	0.148	0.153±0.0078
	10	0.237	0.245	0.242	0.241±0.0040
	20	0.321	0.322	0.309	0.317±0.0072
	40	0.386	0.392	0.388	0.389±0.0030
	80	0.463	0.447	0.458	0.456±0.0081

Name of the sample	Conc. (µg/mL)	Absorbance (nm)			Absorbance (nm) Mean ± STD
		a	b	c	
DFC-2	2.5	0.048	0.056	0.055	0.053±0.0043
	5	0.147	0.138	0.141	0.142±0.0045
	10	0.192	0.178	0.180	0.183±0.0075
	20	0.249	0.247	0.239	0.245±0.0052
	40	0.317	0.325	0.322	0.321±0.0040
	80	0.384	0.379	0.372	0.378±0.0060
DFC-3	2.5	0.083	0.078	0.095	0.085±0.0087
	5	0.147	0.162	0.151	0.153±0.0077
	10	0.309	0.319	0.308	0.312±0.0061
	20	0.393	0.421	0.411	0.408±0.0141
	40	0.541	0.532	0.539	0.537±0.0047
	80	0.621	0.609	0.607	0.612±0.0075
DFC-4	2.5	0.062	0.073	0.065	0.067±0.0056
	5	0.119	0.118	0.108	0.115±0.0061
	10	0.261	0.265	0.275	0.267±0.0072
	20	0.361	0.355	0.357	0.358±0.0030
	40	0.462	0.458	0.448	0.456±0.0072
	80	0.578	0.585	0.584	0.582±0.0037
DFC-5	2.5	0.078	0.065	0.075	0.073±0.0068
	5	0.132	0.142	0.138	0.137±0.0050
	10	0.219	0.211	0.218	0.216±0.0043
	20	0.302	0.291	0.283	0.292±0.0095
	40	0.358	0.363	0.364	0.361±0.0032
	80	0.421	0.409	0.406	0.412±0.0079

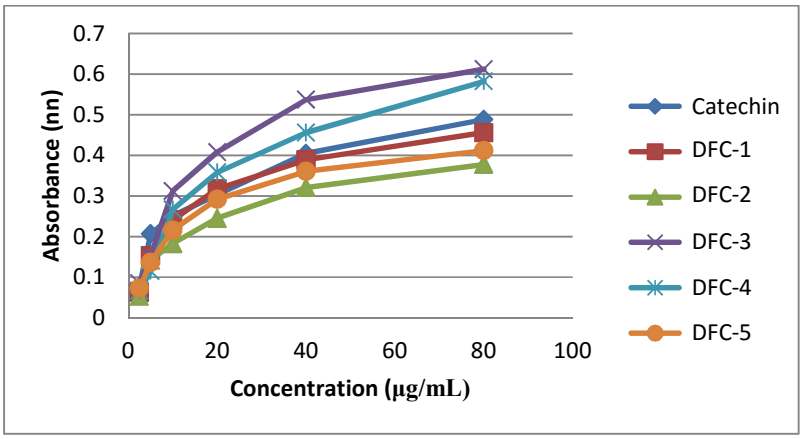


Fig 6.11: Total antioxidant activity of isolated compounds DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 from *wedelia chinensis* and (+)-catechin (standard).

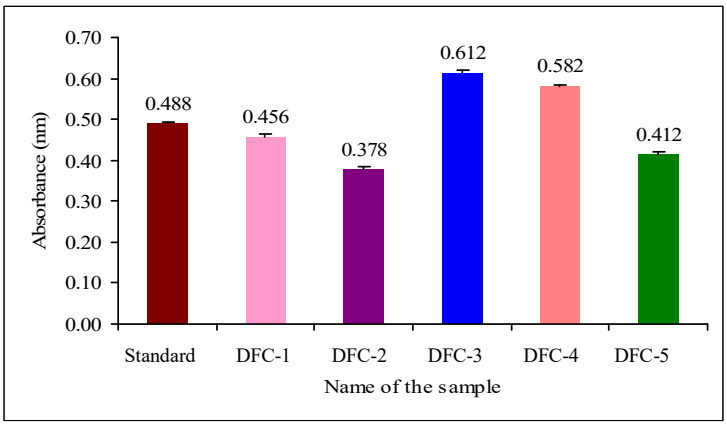


Fig 6.12 : At 80 µg/mL the absorbance of compounds DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 from dia-ion resin fraction and (+)-catechin (standard).

The compounds obtained from dia-ion resin fraction, highest antioxidant activity was found in compound DFC-3 and DFC-4 with absorbance of 0.612 nm and 0.582 nm at 80 µg/mL concentration, followed by DFC-1, DFC-2 and DFC-5 with absorbance of 0.456 nm, 0.378 nm and 0.412 nm respectively. The remaining compounds DFC-3 and DFC-4 showed high activity, compound DFC-1 & DFC-5 showed mailed activity and compound DFC-2 showed lower activity. The total antioxidant activity of the isolated compounds and the reference standard owed the following order:

DFC-3 > DFC-4 > Catechin > DFC-1 > DFC-5 > DFC-2

6.3 Determination of DPPH radical scavenging activity

6.3.1 Determination of DPPH radical scavenging activity of the crude methanol extract (CME) of *Wedelia chinensis* and its different fractions.

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples including plant extracts. DPPH antioxidant assay is based on the ability of 1, 1 diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the change in absorbance and % of scavenging activity is calculated. The antioxidant activity of the petroleum ether fraction (PEF), chloroform fraction (CLF), ethyl acetate fraction

(EAF) and dia-ion resin fraction (DRF) of *wedelia chinensis* was further evaluated by DPPH radical scavenging assay. The results of DPPH free radical scavenging assay are given in Table -6.7,6.8 & 6.9 and in Fig. 6.13, 6.14**,** 6.15, 6.16, 6.17 & 6.18.

Table-6.7: Data for DPPH radical scavenging activity of the different fractions of *wedelia chinensis.*

Name of the sample	Conc. (µg/mL)	% of scavenging			% of scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
(+)-Catechin (standard)	0.78	4.84	4.86	4.91	4.87 ± 0.0360	4.5
	1.56	25.25	25.37	25.34	25.32±0.0624	
	3.125	48.13	47.67	47.75	47.85±0.2457	
	6.25	56.21	55.37	55.32	55.63±0.5000	
	12.5	64.17	63.68	63.59	63.81±0.3121	
	25	71.54	72.06	71.45	71.68±0.3292	
	50	83.23	82.51	82.28	82.67±0.4956	
	100	93.12	92.27	92.23	92.54±0.5026	
Crude methanol extract (CME)	0.78	3.56	4.12	4.08	3.92 ± 0.3124	13.5
	1.56	13.85	14.17	14.72	14.25 ± 0.4400	
	3.125	32.59	33.32	33.59	33.16 ± 0.5173	
	6.25	41.27	43.13	42.48	42.29 ± 0.9439	
	12.5	53.43	52.85	54.47	53.58 ± 0.8208	
	25	62.73	65.12	64.38	64.07 ± 1.2235	
	50	76.34	77.26	79.11	77.57 ± 1.4107	
	100	83.76	83.72	82.87	83.45 ± 0.5026	
Petroleum ether fraction (PEF)	0.78	0.035	0.046	0.037	0.04±0.0058	35
	1.56	1.16	1.20	1.21	1.19±0.0264	
	3.125	5.65	6.01	5.92	5.86±0.1873	
	6.25	26.02	25.43	25.56	25.67±0.3119	
	12.5	38.12	37.57	37.59	37.76±0.3041	
	25	49.88	49.33	49.38	49.53±0.3041	
	50	61.51	61.64	60.65	61.26±0.5379	
	100	68.34	69.15	67.99	68.49±0.5950	
Chloroform fraction (CLF)	0.78	0.064	0.065	0.086	0.07 ± 0.0124	25
	1.56	4.59	4.64	4.67	4.63 ± 0.0404	
	3.125	17.08	16.32	16.35	16.58±0.4303	
	6.25	29.37	30.17	29.47	29.67±0.4358	
	12.5	41.51	41.67	41.12	41.43±0.2829	
	25	52.91	53.15	51.95	52.67±0.6349	
	50	65.62	66.07	65.45	65.71±0.3203	
	100	72.09	71.56	71.70	71.78±0.2746	

Name of the sample	Conc. (µg/mL)	% of scavenging			% of scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
Ethyl acetate fraction (EAF)	0.78	9.47	9.46	9.51	9.48 ± 0.0264	6.5
	1.56	27.83	27.81	27.87	27.84 ± 0.0305	
	3.125	42.37	42.31	42.40	42.36 ± 0.0458	
	6.25	57.85	57.83	57.89	57.86 ± 0.0305	
	12.5	68.11	68.12	68.19	68.14 ± 0.0435	
	25	78.77	78.80	78.85	78.81 ± 0.0404	
	50	90.07	90.10	90.17	90.11 ± 0.0513	
	100	96.25	96.26	96.32	96.28 ± 0.0378	
Dia-ion resin fraction (DRF)	0.78	4.51	4.52	4.58	4.54 ± 0.0378	10.5
	1.56	15.75	15.77	15.82	15.78 ± 0.0360	
	3.125	34.51	34.52	34.59	34.54 ± 0.0435	
	6.25	45.21	45.23	45.28	45.24 ± 0.0360	
	12.5	56.40	56.45	56.47	56.44 ± 0.0360	
	25	68.73	68.72	68.78	68.74 ± 0.0321	
	50	79.34	79.36	79.41	79.37 ± 0.0360	
	100	90.05	89.24	88.98	89.42± 0.5580	

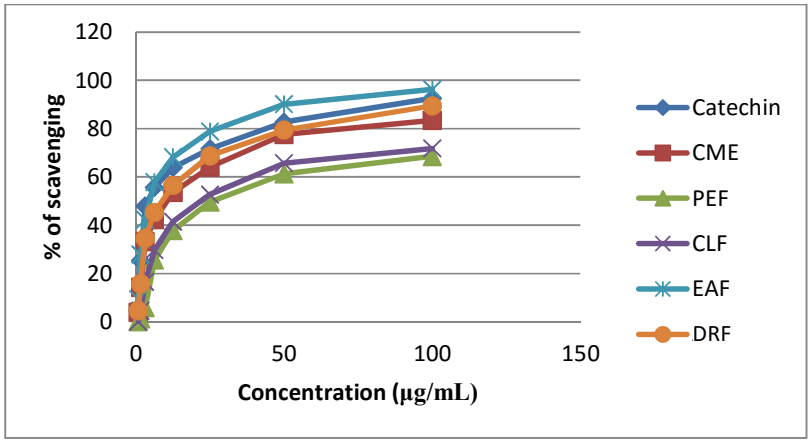


Fig 6.13 : DPPH radical scavenging activity of CME and different fractions (PEF, CLF, EAF & DRF) of *wedelia chinensis* and the reference standard (+)-catechin.

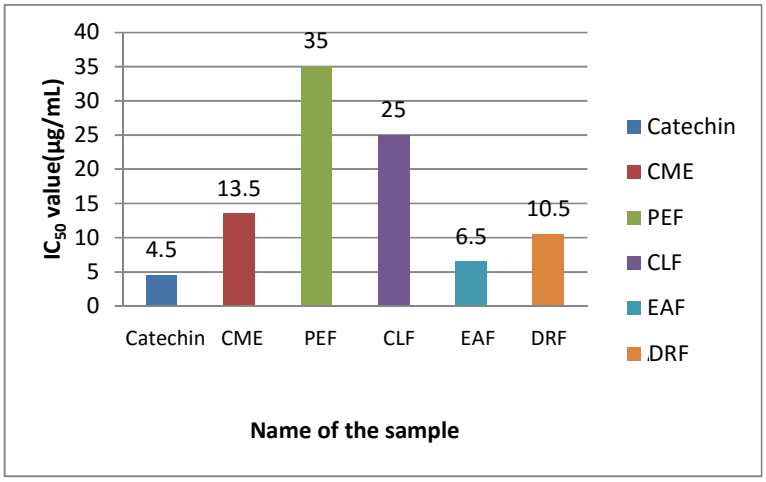


Fig 6.14 : IC_{50} values of DPPH radical scavenging activity for CME, PEF, CLF, EAF and DRF of *wedelia chinensis* and(+)-catechin (standard).

Among the fractions of *W. chinensis* EAF showed the highest scavenging activity with IC_{50} value 6.5 µg/mL. The crude methanol extract (CME) and dia-ion resin fraction (DRF) showed DPPH free radical scavenging with IC_{50} values of 13.5 and 10.5 µg/mL respectively, while the IC_{50} of (+)- catechin was 4.5 µg/mL. The results demonstrated that EAF exhibited strong scavenging activity followed by DRF and CME.

DPPH activity order: Catachin > EAF > DRF > CME > CLF > PEF.

6.3.2 Determination of DPPH radical scavenging activity of the isolated compounds from ethyl acetate fraction of *wedelia chinensis.*

Table-6.8: Data for DPPH radical scavenging activity of the isolated compounds from ethyl acetate fraction of *wedelia chinensis.*

Name of the sample	Conc. (µg/mL)	% of scavenging			% of scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
(+)-Catechin (standard)	0.78	4.84	4.86	4.91	4.87 ± 0.0360	4.5
	1.56	25.25	25.37	25.34	25.32±0.0624	
	3.125	48.13	47.67	47.75	47.85±0.2457	
	6.25	56.21	55.37	55.32	55.63±0.5000	
	12.5	64.17	63.68	63.59	63.81±0.3121	
	25	71.54	72.06	71.45	71.68±0.3292	
	50	83.23	82.51	82.28	82.67±0.4956	
	100	93.12	92.27	92.23	92.54±0.5026	

Name of the sample	Conc. (µg/mL)	% of scavenging			% of scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
EFC-1	0.78	5.67	4.98	4.96	5.20±0.4042	4.25
	1.56	39.74	36.64	38.67	36.54±1.5745	
	3.125	69.78	66.78	64.89	67.64±2.4659	
	6.25	87.45	84.86	87.38	85.73±1.4755	
	12.5	95.85	89.98	97.74	92.24±4.0465	
	25	97.54	95.95	98.54	98.83±1.3061	
	50	99.87	101.14	98.96	101.45±1.0949	
	100	105.23	104.10	102.13	103.67±1.5688	
EFC-2	0.78	4.12	4.48	5.08	4.56±0.4849	4.75
	1.56	28.84	29.19	30.11	29.38±0.6559	
	3.125	46.85	47.32	48.57	47.58±0.8889	
	6.25	63.43	62.23	62.35	62.67±0.6609	
	12.5	73.37	73.29	71.92	72.86±0.8150	
	25	80.75	81.37	82.29	81.47±0.7748	
	50	88.78	89.65	90.28	89.57±0.7531	
	100	94.05	93.36	93.07	93.49±0.5034	

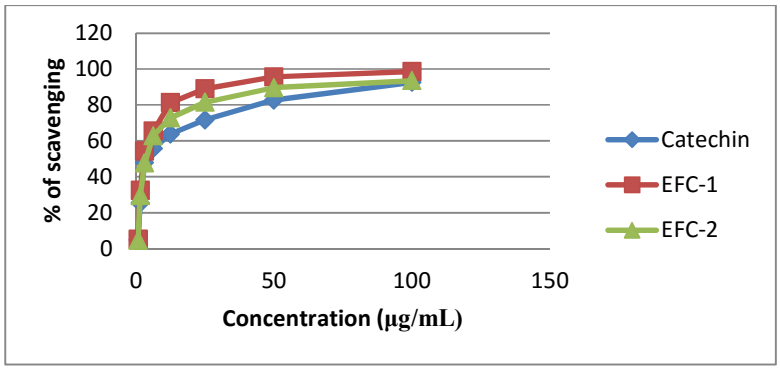


Fig 6.15: DPPH radical scavenging activity of isolated compounds EFC-1& EFC-2 and (+)-catechin (standard).

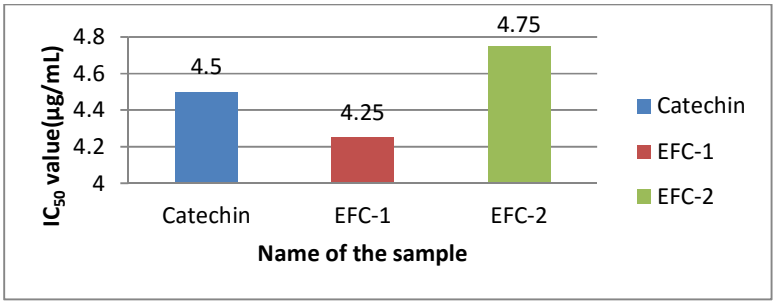


Fig 6.16 : IC_{50} values of DPPH radical scavenging activity for EFC-1 & EFC-2 of and (+)-catechin (standard).

The DPPH radical scavenging activity of the compounds EFC-1 and EFC-2 isolated from the ethyl acetate fraction of *W. chinensis* was determined in a similar manner as mentioned above and the results have been showed in Fig.6.15 & 6.16. Both the compounds EFC-1 and EFC-2 exhibited strong radical activity. The IC_{50} values of EFC-1 and EFC-2 are 4.25 and 4.75 µg/mL respectively, which was very close to the activity of the reference standard catechin, whose IC_{50} value was 4.5µg/mL. The results demonstrated that the compounds EFC-1and EFC-2 are strong radical scavenger.

DPPH activity order: EFC-1 > catachin > EFC-2 .

6.3.3 Determination of DPPH radical scavenging activity of the isolated compounds from dia-ion resin fraction of *wedelia chinensis.*

Table-6.9: Data for DPPH radical scavenging activity of isolated compounds from dia-ion resin fraction of *wedelia chinensis.*

Name of the sample	Conc. (µg/mL)	% of scavenging			% of scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
(+)-Catechin (standard)	0.78	4.84	4.86	4.91	4.87 ± 0.0360	4.5
	1.56	25.25	25.37	25.34	25.32±0.0624	
	3.125	48.13	47.67	47.75	47.85±0.2457	
	6.25	56.21	55.37	55.32	55.63±0.5000	
	12.5	64.17	63.68	63.59	63.81±0.3121	
	25	71.54	72.06	71.45	71.68±0.3292	
	50	83.23	82.51	82.28	82.67±0.4956	
	100	93.12	92.27	92.23	92.54±0.5026	
DFC-1	0.78	0.53	0.53	0.55	0.54±0.0115	21.25
	1.56	2.19	1.87	1.85	1.97±0.1907	
	3.125	5.68	5.77	5.79	5.73±0.0585	
	6.25	14.63	14.78	14.64	14.68±0.0838	
	12.5	32.51	32.59	32.38	32.49±0.1059	
	25	54.72	54.89	54.73	54.78±0.0953	
	50	67.67	68.08	67.73	67.83±0.2214	
	100	72.43	72.49	72.61	72.51±0.0916	
DFC-2	0.78	0.49	0.56	0.57	0.54±0.0435	16
	1.56	4.61	4.54	4.75	4.63±0.1069	
	3.125	9.35	9.74	10.12	9.74±0.3850	
	6.25	20.21	20.23	20.36	20.27±0.0814	
	12.5	42.56	42.72	42.73	42.67±0.0953	
	25	58.57	58.72	58.62	58.64±0.0763	
	50	70.23	69.60	69.69	69.84±0.3407	
	100	74.49	74.58	74.61	74.56±0.0624	

Name of the sample	Conc. (µg/mL)	% of scavenging			% of scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
DFC-3	0.78	9.43	9.44	9.54	9.47±0.0608	4.25
	1.56	27.78	27.86	27.89	27.84±0.0568	
	3.125	42.31	42.43	42.33	42.36±0.0642	
	6.25	57.82	57.84	57.92	57.86±0.0529	
	12.5	68.11	68.12	68.19	68.14±0.0435	
	25	78.76	78.88	78.80	78.81±0.0611	
	50	90.16	90.07	90.10	90.11±0.0458	
	100	96.26	96.28	97.29	96.61±0.5889	
DFC-4	0.78	6.65	6.83	6.44	6.64±0.1951	4.75
	1.56	25.78	25.48	25.82	25.69±0.1858	
	3.125	38.45	37.98	38.83	38.42±0.4257	
	6.25	53.53	53.82	52.96	53.44±0.4375	
	12.5	64.89	65.14	64.76	64.93±0.1931	
	25	75.36	75.72	75.86	75.65±0.2579	
	50	87.56	87.91	87.58	87.68±0.1965	
	100	93.54	93.47	92.95	93.32±0.3223	
DFC-5	0.78	4.53	4.51	4.58	4.54±0.0360	12.5
	1.56	15.77	15.69	15.85	15.77±0.0800	
	3.125	34.53	34.54	34.64	34.57±0.0608	
	6.25	45.22	45.24	44.95	45.14±0.1619	
	12.5	56.42	56.44	54.45	55.77±1.1431	
	25	68.73	68.75	68.82	68.77±0.0472	
	50	79.35	79.31	79.43	79.37±0.0611	
	100	87.76	87.61	87.59	87.65±0.0929	

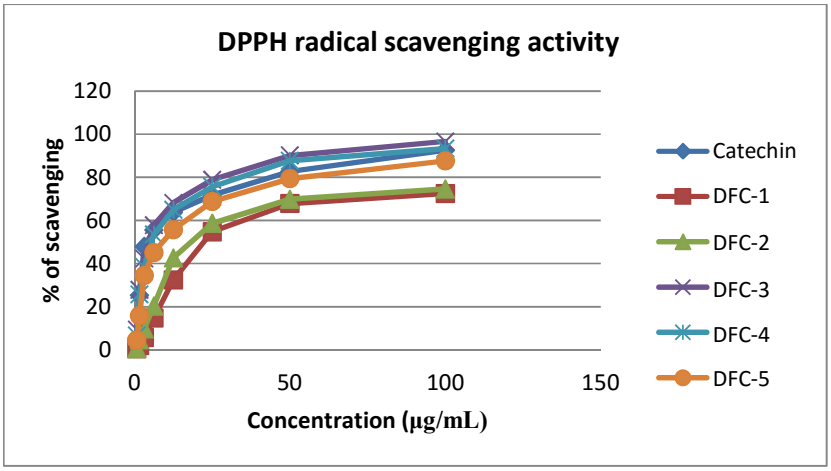


Fig 6.17 : DPPH radical scavenging activity of isolated compounds DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 of *wedelia chinensis* and (+)-catechin (standard).

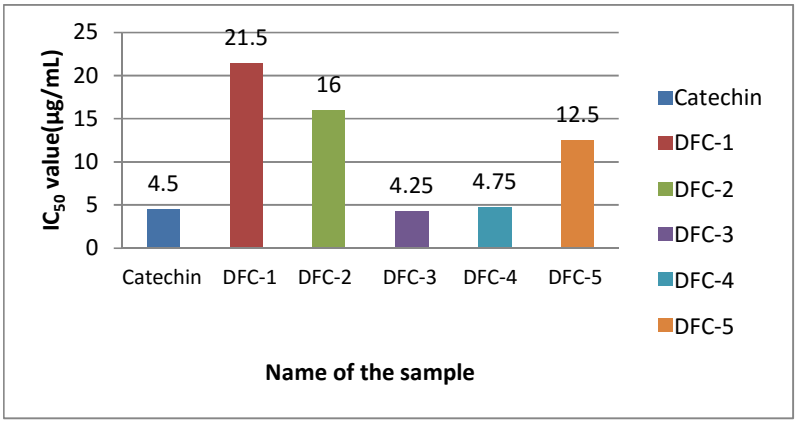


Fig 6.18 : IC_{50} values of DPPH radical scavenging activity for isolated compounds DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 and (+)-catechin (standard).

Among the isolated compounds from the dia-ion resin fraction DFC-3 and DFC-4 showed stronger DPPH radical scavenging activity with IC_{50} values 4.25 and 4.75 µg/mL respectively. While the compounds DFC-1, DFC-2 and DFC-5 showed moderate activity and the IC_{50} values were 21.5, 16 and 12.5 µg/mL, respectively.

From our result, it is evident that the compounds test edpossess DPPH radical scavenging activity and their activity decreases in the following order:

DFC-3 > Catechin ≈ DFC-4 > DFC-5 > DFC-2 > DFC-1.

6.4 Determination of hydroxyl radical scavenging activity

6.4.1 Determination of hydroxyl radical scavenging activity of the crude methanol extract (CME) of *Wedelia chinensis* and its different fractions.

Hydroxyl radicals are the major reactive oxygen species (ROS) causing lipid oxidation and enormous biological damage (Aurand *et al.,* 1977). By incubating Fe^{3+}-EDTA with H_2O_2 and ascorbic acid at pH 7.4, hydroxyl radicals can be generated in free solution and detected by their ability to degrade 2-deoxy-2-ribose into fragments that on heating with TBA at low pH forms a pink chromogen (Chung *et al.*1997) which can be quantitatively measured from the change in absorbance at 532 nm as mentioned in details in materials and method.

In the hydroxyl radical scavenging assay the ability of the crude methanolic extract of *W. chinesis* and its two fractions ethyl acetate fraction (EAF) and dia-ion resin fraction

(DRF) and different isolated compounds to remove the formed hydroxyl radical in solution was evaluated quantitatively and compared with the reference standard catechin. The percent (%) of scavenging activity of each sample was calculated. The results have been presented in table-6.10, 6.11 & 6.12 and in fig. 6.19, 6.20, 6.21, 6.22, 6.23 & 6.24. EAF, DRF showed OH free radical scavenging activity with an IC_{50} value of 24 µg/mL, 10.5 µg/mL. The activity was found to be more potent than that of the reference standard catechin having IC_{50} value of 12.5 µg/mL.

Table-6.10: Hydroxyl radical scavenging activity of catechin (standard) and different extracted of *wedelia chinensis* at different concentrations.

Name of the Sample	Conc. (µg/mL)	% of Scavenging			% of Scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
(+)-Catechin (standard)	3.125	16.16	17.08	15.61	16.28±0.7427	12.5
	6.25	38.12	36.64	37.11	37.29±0.7562	
	12.5	47.78	49.05	48.74	48.52±0.6621	
	25	56.94	56.85	58.08	57.29±0.6856	
	50	67.27	67.59	68.09	67.65±0.4132	
	100	71.87	72.32	73.17	72.45±0.6601	
Crude Methanol Extract (CME)	3.125	10.35	11.06	10.85	10.75±0.3647	35
	6.25	21.49	20.96	21.65	21.37±0.3611	
	12.5	37.72	37.84	37.78	37.78±0.0600	
	25	47.83	47.53	48.14	47.83±0.3050	
	50	52.94	52.87	53.12	52.97±0.1289	
	100	56.84	57.16	57.23	57.08±0.2079	
Ethyl acetate fraction (EAF)	3.125	10.35	11.06	10.85	10.75±0.3647	24
	6.25	24.87	25.34	26.05	25.42±0.5940	
	12.5	41.72	42.13	40.88	41.58±0.6372	
	25	52.23	51.53	51.85	51.87±0.3504	
	50	58.64	59.17	58.38	58.73±0.4026	
	100	63.81	64.09	63.56	63.82±0.2651	
Dia-ion resin fraction (DRF)	3.125	19.56	18.88	19.76	19.40±0.4613	10.5
	6.25	41.24	41.64	40.67	41.18±0.4874	
	12.5	56.83	57.23	56.74	56.93±0.2608	
	25	63.54	64.23	63.68	63.82±0.3647	
	50	70.67	71.34	70.82	70.94±0.3516	
	100	75.32	76.34	75.62	75.76±0.5142	

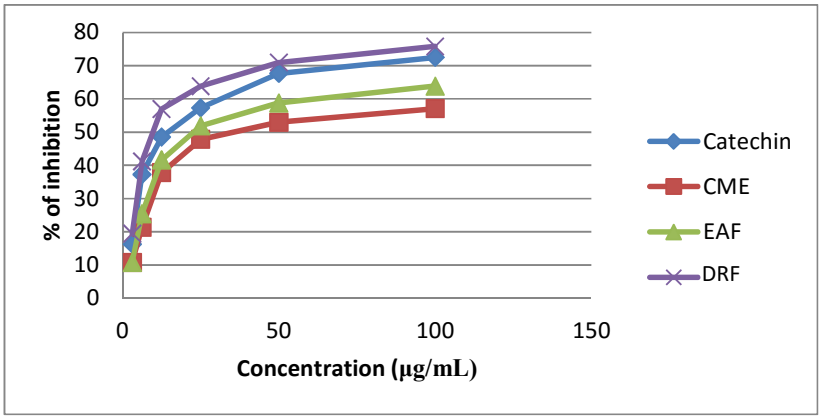


Fig 6.19 : Hydroxyl radical scavenging activity of CME, EAF, DRF of *W. chinensis* and (+)-catechin (standard) at different concentrations.

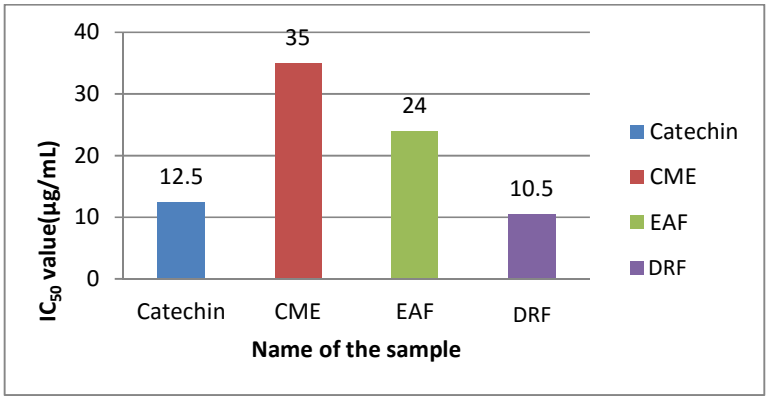


Fig 6.20 : IC_{50} values of CME, EAF, DRF of *W. chinensis* and (+)-catechin (standard) for hydroxyl radical scavenging activity.

The result demonstrated that CME and its two fractions exhibited considerable hydroxyl radical scavenging activity. Among the different extractives, DRF showed the strongly activity with IC_{50} values of 10.5 µg/mL followed by CME and EAF with IC_{50} values are 35 and 24 µg/mL.

The activity of the crude methanolic extract, fractions and the reference standard catechin decreases in the following order: DRF > Catechin > EAF > CME.

6.4.2 Determination of hydroxyl radical scavenging activity of the isolated compounds from ethyl acetate fraction of *Wedelia chinensis*.

Table-6.11: Hydroxyl radical scavenging activity of catechin (standard) and the isolated compounds of ethyl acetate fraction of *W. chinensis* at different concentrations.

Name of the Sample	Conc. (µg/mL)	% of Scavenging			% of Scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
(+)-Catechin (Standard)	3.125	16.16	17.08	15.61	16.28±0.7427	12.5
	6.25	38.12	36.64	37.11	37.29±0.7562	
	12.5	47.78	49.05	48.74	48.52±0.6621	
	25	56.94	56.85	58.08	57.29±0.6856	
	50	67.27	67.59	68.09	67.65±0.4132	
	100	71.87	72.32	73.17	72.45±0.6601	
EFC-1	3.125	09.27	10.03	09.05	09.45±0.5141	10.5
	6.25	34.98	35.47	36.56	35.67±0.8087	
	12.5	53.34	52.30	52.55	52.73±0.5428	
	25	63.66	64.38	63.12	63.72±0.6321	
	50	67.52	67.28	68.15	67.65±0.4493	
	100	76.02	75.23	76.09	75.78±0.4775	
EFC-2	3.125	12.21	11.95	13.52	12.56±0.8414	22
	6.25	25.75	26.85	26.81	26.47±0.6238	
	12.5	40.16	39.60	38.98	39.68±0.5902	
	25	53.17	52.54	52.29	52.67±0.4534	
	50	60.15	59.48	59.38	59.67±0.4186	
	100	63.27	62.14	61.97	62.46±0.7066	

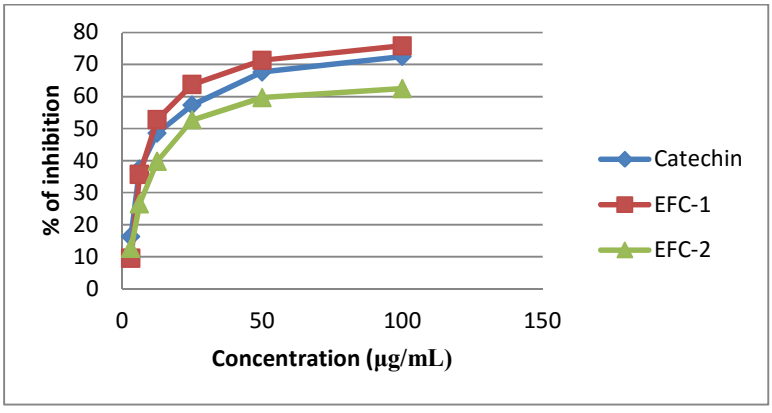


Fig 6.21 : Hydroxyl radical scavenging activity of isolated compounds EFC-1& EFC-2 of *W. chinensis* and (+)-catechin (standard) at different concentrations.

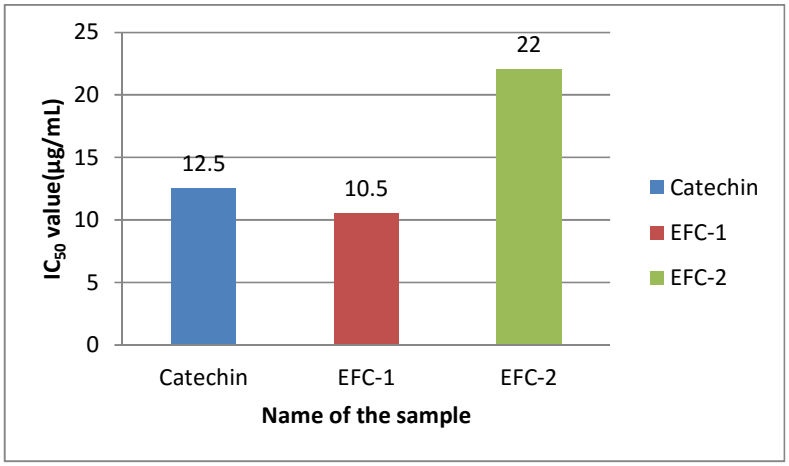


Fig 6.22 : IC_{50} values of Hydroxyl radical scavenging activity of isolated compounds EFC-1 & EFC-2 and (+)-catechin (standard).

Among the isolated compounds EFC-1 & EFC-2 obtained from the ethyl acetate fraction. Compound EFC-1 showed the highest scavenging activity against hydroxyl free radical with IC_{50} value of 10.5 µg/mL, which is more than standard reference catechin. The hydroxyl radical scavenging activity showed the following decreasing order: EFC-1 > Catechin > EFC-2.

6.4.3 Determination of hydroxyl radical scavenging activity of the isolated compounds from dia-ion resin fraction of *Wedelia chinensis*.

Table-6.12: Hydroxyl radical scavenging activity of catechin (standard) and isolated compounds of dia-ion resin fraction of *W. chinensis* at different concentrations.

Name of the Sample	Conc. (µg/mL)	% of Scavenging			% of Scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
(+)-Catechin (standard)	3.125	16.16	17.08	15.61	16.28±0.7427	12.5
	6.25	38.12	36.64	37.11	37.29±0.7562	
	12.5	47.78	49.05	48.74	48.52±0.6621	
	25	56.94	56.85	58.08	57.29±0.6856	
	50	67.27	67.59	68.09	67.65±0.4132	
	100	71.87	72.32	73.17	72.45±0.6601	
DFC-1	3.125	4.82	5.15	4.75	4.91 ± 0.2136	35
	6.25	13.95	14.28	13.38	13.87 ± 0.4553	
	12.5	32.63	32.17	33.28	32.69 ± 0.5577	
	25	45.34	46.17	45.89	45.83±0.4222	
	50	57.51	58.15	57.17	57.61±0.4975	
	100	60.87	60.93	61.44	61.08±0.3132	

Name of the Sample	Conc. (µg/mL)	% of Scavenging			% of Scavenging Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
DFC-2	3.125	6.65	7.12	6.38	6.71±0.3744	13
	6.25	28.09	26.80	26.98	27.29±0.6986	
	12.5	48.05	46.88	47.25	47.39±0.5980	
	25	59.89	60.56	59.91	60.12±0.3811	
	50	70.76	71.56	71.59	71.29±0.4707	
	100	73.12	73.08	72.03	72.74±0.6180	
DFC-3	3.125	8.85	9.26	8.21	8.77±0.5291	12.5
	6.25	21.56	21.19	22.63	32.78±0.7478	
	12.5	43.78	44.26	43.89	51.29±0.2514	
	25	54.21	55.24	54.38	65.89±0.5521	
	50	73.21	72.27	71.76	72.41±0.7355	
	100	73.74	74.38	73.93	74.02±0.3286	
DFC-4	3.125	10.73	11.17	10.46	10.76±0.3583	25
	6.25	18.84	19.24	18.51	18.86±0.3655	
	12.5	32.67	33.14	32.38	32.73±0.3835	
	25	53.82	54.16	53.63	53.87±0.2685	
	50	64.83	65.14	64.45	64.81±0.3455	
	100	68.73	69.23	68.26	68.74±0.4850	
DFC-5	3.125	4.67	5.12	4.45	4.75±0.3415	42
	6.25	11.29	10.86	12.27	11.47±0.7226	
	12.5	21.83	22.16	21.67	21.89±0.2498	
	25	42.71	43.15	42.56	42.81±0.3066	
	50	53.73	54.21	53.52	53.82±0.3536	
	100	58.62	57.89	58.94	58.48±0.5381	

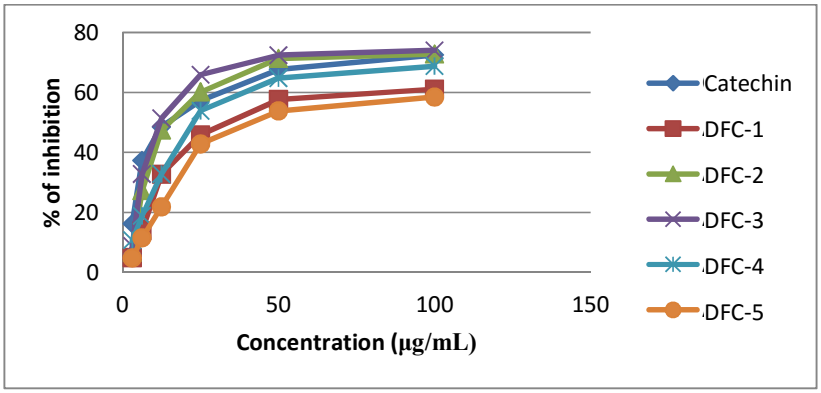


Fig 6.23 : Hydroxyl radical scavenging activity of isolated compounds DFC-1, DFC-2, DFC-3, DFC-4, DFC-5 and (+)-catechin (standard) at different concentrations.

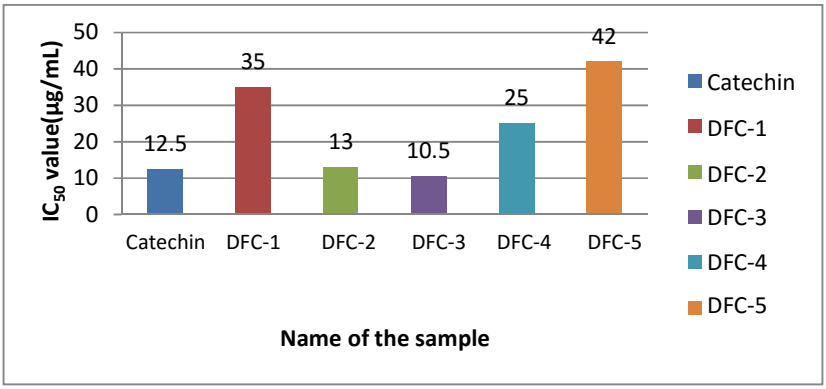


Fig 6.24 : IC_{50} values (µg/mL) of Hydroxyl radical scavenging activity of isolated compounds DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 and (+)-catechin (standard).

Among the isolated compounds (DFC-1 to DFC-5) obtained from the dia-ion resin fraction showed the highest scavenging activity against hydroxyl free radical with IC_{50} value of 35 µg/mL, 13 µg/mL, 10.5µg/mL, 25µg/mL & 42µg/mL. Compound DFC-3 were height hydroxyl radical scavenging activity, compound DFC-2 similar to catechin (standard). The hydroxyl radical scavenging activity of the different fractions showed the following decreasing order:

DFC-3 > Catechin ≈ DFC-2 > DFC-4 > DFC-1 > DFC-5.

6.5 Determination of Lipid peroxidation inhibition activity

6.5.1 Determination of Lipid peroxidation inhibition activity of the crude methanol extract (CME) of *Wedelia chinensis* and its different fractions.

Reactive oxygen species (ROS) produced by ultraviolet light, ionizing radiation, chemical reactions, and metabolic processes have numerous pathological effects, such as causing lipid peroxidation, protein peroxidation, DNA damage, and cellular degeneration related to a variety of diseases including Alzheimer's disease (Bauerova *et al.,* 1999; Finkel *et al.,* 2000; Haliwell *et al.,* 1992; Knight *et al.,* 1995; Petrone *et al.,* 1980; Visioli *et al.,* 2000). Lipid peroxidation has been reported to be elevated in the brain of AD. During lipid peroxidation, low molecular weight end products, generally malonaldehyde, are formed by oxidation of polyunsaturated fatty acids that may react with two molecules of thiobarbituric acid to give a pinkish red chromogen (Kumarasamy *et al.,* 2007). In the lipid peroxidation assay, the activity of ethyl acetate fraction (EAF), dia-ion resin fraction (DRF) and isolated compounds (EFC-1,

EFC-2, DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5) obtained from *Wedelia chinensis* was evaluated and compared with and the reference standard catechin. Addition of Fe^{2+}- ascorbate to the brain homogenate caused an increase in lipid peroxidation.

The result of lipid peroxidation inhibitory activity has been presented in Table -6.13, 6.14 & 6.15 and in Fig 6.25, 6.26, 6.27, 6.28, 6.29 & 6.30. EAF, MEF and isolated compounds inhibited lipid peroxidation in a concentration dependent manner and its IC_{50} value was calculated. Lipid peroxidation inhibition activity of catechin (standard) and different fractions of *Wedelia chinensis* at different concentrations.

Table-6.13: Lipid peroxidation inhibitory activity of different fractions of *wedelia chinensis* at different concentrations.

Name of sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
(+)-Catechin (standard)	6.25	10.09	10.23	10.25	10.19±0.0871	22
	12.5	28.38	28.63	29.17	28.72±0.4037	
	25	54.68	54.76	53.81	54.41±0.5269	
	50	63.71	64.12	63.77	63.86±0.2214	
	100	67.39	66.92	66.87	67.06±0.2868	
Crude Methanol Extract (CME)	6.25	6.82	7.24	6.57	6.88±0.3385	41
	12.5	23.73	22.98	23.52	23.41±0.3869	
	25	42.87	43.07	43.63	43.19±0.3939	
	50	55.91	55.23	55.39	55.51±0.3555	
	100	59.83	58.17	59.27	59.09±0.8445	
Ethyl acetate fraction (EAF)	6.25	7.87	8.05	7.72	7.88±0.1652	40
	12.5	29.09	28.28	27.86	28.41±0.6252	
	25	46.17	45.51	45.48	45.72±0.3900	
	50	54.32	52.94	54.21	53.82±0.7669	
	100	60.03	59.62	59.60	59.75±0.2426	
Dia-ion resin fraction (DRF)	6.25	08.18	08.31	08.19	8.23±0.0723	20
	12.5	34.83	36.03	35.70	35.52±0.6199	
	25	59.15	58.28	57.80	58.41±0.6843	
	50	66.59	66.94	67.08	66.87±0.2523	
	100	72.85	71.90	72.81	72.52±0.5373	

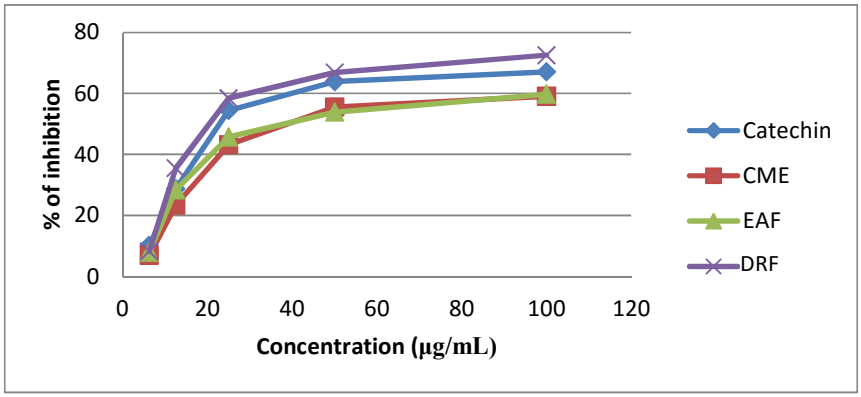


Fig 6.25 : Lipid peroxidation inhibition activity of crude methanol extract (CME) ethyl acetate fraction (EAF), dia-ion resin fraction (DRF) and (+)-catechin (standard) at different concentrations.

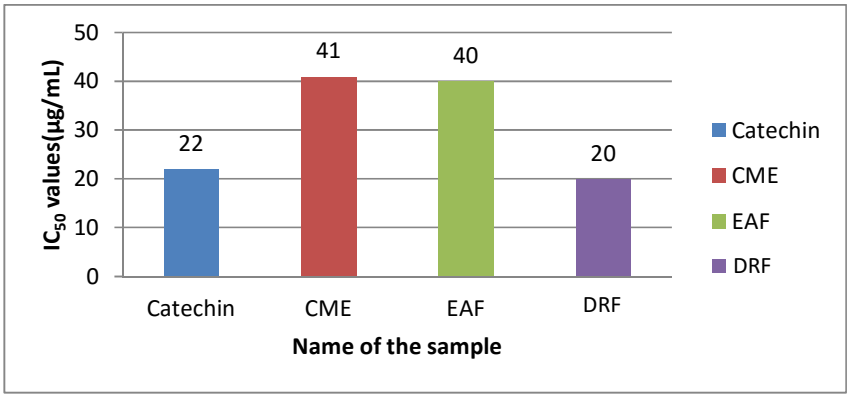


Fig 6.26 : IC_{50} values of crude methanol fraction (CMF), ethyl acetate fraction (EAF), dia-ion resin fraction (DRF) of *W. chinensis* and (+)-catechin (standard) for Lipid peroxidation inhibition activity.

The result demonstrated that CME of *W. chinensis* and its fractions exhibited considerable lipid peroxidation inhibitory activity when compared with the reference standard carechin. Comparison of the activity of CME with its fractions showed that DRF possesses the highest inhibitory activity against brain lipid peroxidation than which appeared to be very similar to that of the reference standard catechin. The IC_{50} values of CME, EAF, DRF and catechin (standard) for inhibition of lipid peroxidation were 41, 40, 20 and 22 µg/mL respectively.

The following order: DRF > Catechin > EAF > CME.

6.5.2 Determination of Lipid peroxidation inhibition activity of isolated compounds of ethyl acetate fraction of *Wedelia chinensis*.

Table-6.14 : Lipid peroxidation inhibitory activity of isolated compounds of ethyl acetate fraction of *W. chinensis* at different concentrations.

Name of the sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
(+)-Catechin (standard)	6.25	10.09	10.23	10.25	10.19±0.0871	22
	12.5	28.38	28.63	29.17	28.72±0.4037	
	25	54.68	54.76	53.81	54.41±0.5269	
	50	63.71	64.12	63.77	63.86±0.2214	
	100	67.39	66.92	66.87	67.06±0.2868	
EFC-1	6.25	5.57	6.11	5.48	5.72±0.3407	42
	12.5	28.17	27.66	27.60	27.81±0.3132	
	25	44.09	43.31	43.37	43.59±0.4340	
	50	52.84	53.38	53.26	53.16±0.2835	
	100	57.51	57.35	58.11	57.66±0.4006	
EFC-2	6.25	7.92	8.51	8.71	8.38±0.4107	21
	12.5	31.88	32.41	32.28	32.19±0.2762	
	25	58.19	57.61	57.72	57.84±0.3080	
	50	66.17	65.56	65.40	65.71±0.4063	
	100	70.03	69.72	69.75	69.83±0.1709	

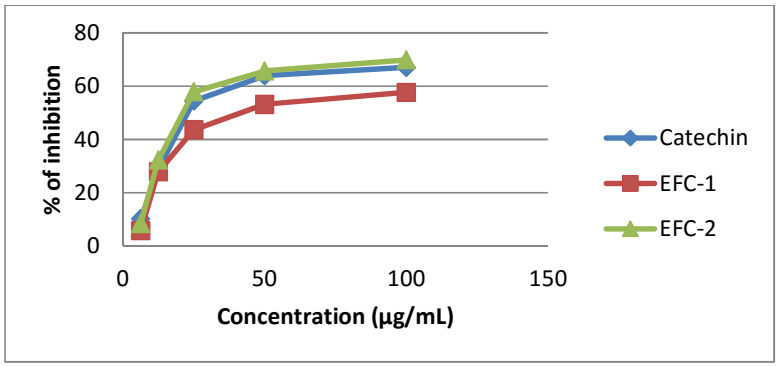


Fig 6.27 : Lipid peroxidation inhibition activity isolated compound (EFC-1, EFC-2) and (+)-catechin (standard) at different concentrations.

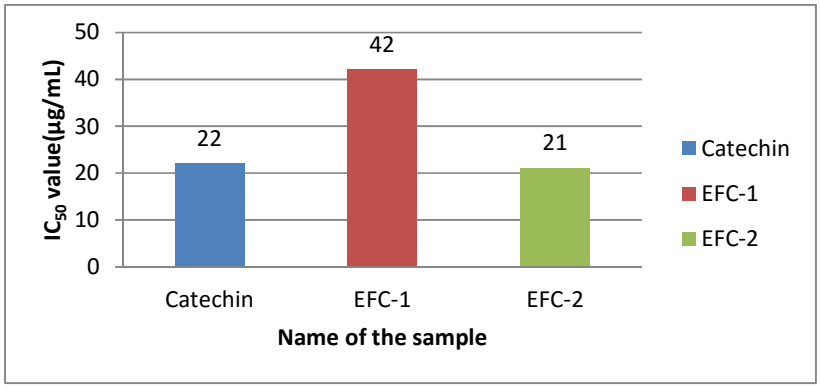


Fig 6.28 : IC_{50}values of isolated compound EFC-1 & EFC-2 and (+)-catechin (standard) for Lipid peroxidation inhibition activity.

The results of the lipid peroxidation inhibitory activity of the compound EFC-2, was found strong inhibition against lipid peroxidation of the reference standard catechin. The IC_{50} value of EFC-2 was 21µg/mL. The compound EFC-1 less then catechin was found. From the result, it was evident that the compounds test deposes anti–acetylcholinesterase inhibition activity and their activity decreases in the following order: EFC-2 > Catechin > EFC-1.

6.5.3 Determination of Lipid peroxidation inhibition activity of isolated compounds of dia-ion resin fraction of *Wedelia chinensis*.

Table-6.15 : Lipid peroxidation inhibitory activity of isolated compounds of dia-ion resin fraction of *W. chinensis* at different concentrations.

Name of sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
(+)-Catechin (standard)	6.25	10.09	10.23	10.25	10.19±0.0871	22
	12.5	28.38	28.63	29.17	28.72±0.4037	
	25	54.68	54.76	53.81	54.41±0.5269	
	50	63.71	64.12	63.77	63.86±0.2214	
	100	67.39	66.92	66.87	67.06±0.2868	
DFC-1	6.25	5.87	5.83	5.86	5.85±0.0208	58
	12.5	17.94	17.89	17.92	17.92±0.0251	
	25	36.83	36.87	36.88	36.86±0.0264	
	50	48.81	48.82	48.86	48.83±0.0264	
	100	57.34	57.42	57.41	57.39±0.0435	

Name of sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
DFC-2	6.25	3.63	3.28	3.85	3.58±0.2874	37
	12.5	22.27	23.06	22.14	22.49±0.4978	
	25	44.17	43.67	43.63	43.82±0.3008	
	50	58.38	58.14	59.08	58.53±0.4883	
	100	62.28	62.38	63.17	62.61±0.4875	
DFC-3	6.25	10.23	10.18	10.24	10.22±0.0321	19
	12.5	29.40	29.53	30.17	29.70±0.4121	
	25	61.05	60.58	60.87	60.83±0.2371	
	50	69.19	68.54	68.73	68.82±0.3342	
	100	74.13	73.41	73.29	73.61±0.4543	
DFC-4	6.25	11.87	13.21	11.95	12.34±0.7516	21
	12.5	27.06	25.87	26.52	26.48±0.5958	
	25	56.84	55.89	56.71	56.48±0.5150	
	50	66.17	65.32	65.41	65.63±0.4669	
	100	71.47	72.31	71.25	71.68±0.5594	
DFC-5	6.25	4.86	4.87	4.82	4.85±0.0264	85
	12.5	12.52	12.57	12.51	12.53±0.0321	
	25	29.48	29.83	30.08	29.79±0.3013	
	50	43.29	43.42	43.46	43.39±0.0888	
	100	52.72	53.21	52.75	52.89±0.2746	

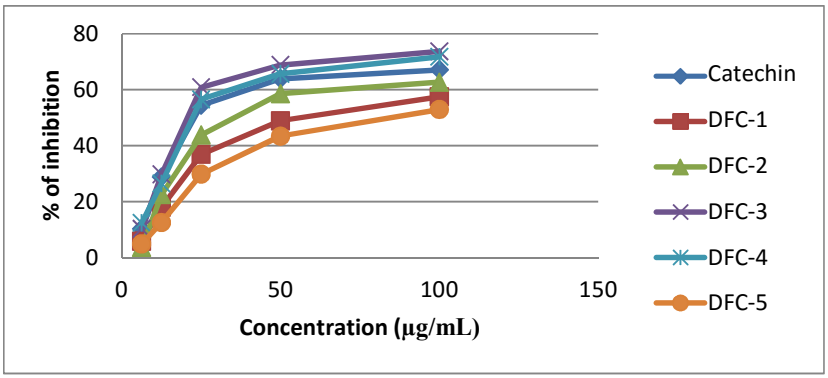


Fig 6.29 : Lipid peroxidation inhibition activity of isolated compounds DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 of *W. chinensis* and (+)-catechin (standard) at different concentrations.

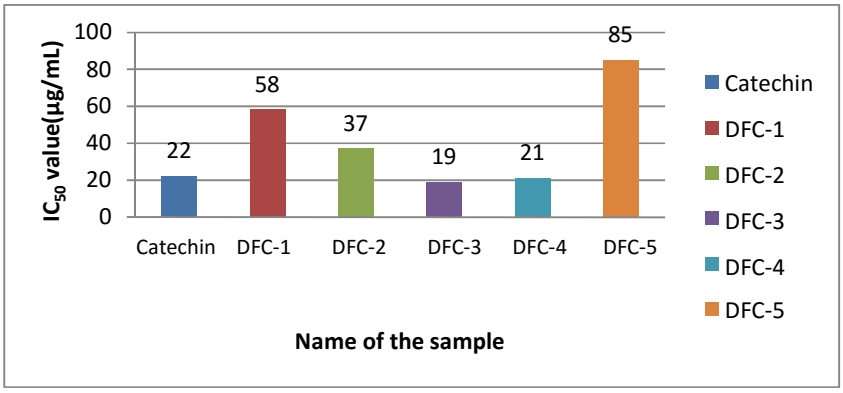


Fig 6.30: IC_{50} values of isolated compounds DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 of *W. chinensis* and (+)-catechin (standard) for Lipid peroxidation inhibition activity.

The results of the lipid peroxidation inhibitory activity of the compounds DFC-3 & DFC-4 was found strong inhibition against lipid peroxidation, compare with the reference standard catechin. Compounds DFC-1, DFC-2 & DFC-5 less inhibition lipid peroxidation than catechin. The IC_{50} of DFC-1, DFC-2, DFC-3, DFC-4, DFC-5 and catechin were found to be 58, 37, 19, 21, 85 and 22 µg/mL respectively. From the result, it is evident that the compounds test deposes anti–acetylcholinesterase inhibition activity and their activity decreases in the following order:

DFC-3 > DFC-4 > Catechin > DFC-2 > DFC-1 > DFC-5.

***In-vitro* Study**
Butyrylcholinesterase and Acetylcholinesterase inhibitory studies

6.6 Anti-butyrylcholinesterase activity assay

6.6.1 Determination of Anti-butyrylcholinesterase activity assay of the crude methanol extract (CME) of *Wedelia chinensis* and its different fractions.

Inhibition of butyrylcholinesterase, which alleviate cholinergic deficit and improve neurotransmission by reducing the enzymatic degradation of butyrylcholine, has recently emerged as a promising therapeutic target for novel drug development to treat AD patients. In this study, the butyrylcholiesterase inhibitory activity of the crude methanol extract (CME) of *wedelia chinensis* and its different fractions and isolated compounds (EFC-1 & EFC-2) of ethyl acetate fraction and (DFC-1, DFC-2,

DFC-3, DFC-4 & DFC-5) from dia-ion resin fraction was assessed by modified Ellman method and compared with the reference standard galanthamine. This method estimates butyrylylcholinesterase (BuchE) using butyrylthiocholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion.

The results of butyrylcholinesterase inhibitory activity of the different samples are given in Table -6.16, 6.17, 6.18 & 6.19 and Fig.6.31, 6.32, 6.33, 6.34, 6.35, 6.36, 6.37, 6.38 & 6.39.

Table -6.16: Butyrylcholinesterase inhibitory activity of the different fractions of *W. chinensis* at different concentrations.

Name of the sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
Crude methanol extract (CME)	12.5	7.48	7.31	8.02	7.60±0.3707	70
	25	21.27	21.59	20.89	21.25±0.3504	
	50	43.47	44.18	43.29	43.64±0.4705	
	100	62.61	63.18	62.38	62.72±0.4118	
	150	71.37	72.17	71.29	71.61±0.4866	
	200	74.92	74.27	75.19	74.79±0.4728	
Petroleum ether fraction (PEF)	12.5	1.31	1.43	1.36	1.36 ± 0.0602	135
	25	4.78	5.18	4.61	4.85 ±0.2926	
	50	17.53	17.75	17.93	17.73 ±0.2003	
	100	37.93	38.27	38.31	38.17 ±0.2088	
	150	52.62	53.26	52.48	52.78±0.4158	
	200	58.85	58.87	58.93	58.88±0.0416	
Chloroform fraction (CLF)	12.5	1.51	1.58	1.62	1.57± 0.0556	105
	25	3.93	3.87	4.08	3.96±0.1081	
	50	21.48	21.47	21.43	21.46±0.0264	
	100	48.56	48.68	48.72	48.65±0.0832	
	150	58.33	58.42	58.31	58.35±0.0585	
	200	62.47	62.63	62.48	62.52±0.0896	
Ethyl acetate fraction (EAF)	12.5	9.65	9.88	9.76	9.76±0.1150	45
	25	25.36	25.42	25.32	25.37±0.0503	
	50	55.69	55.61	55.64	55.65±0.0404	
	100	72.41	72.34	72.28	72.34 ±0.0650	
	150	81.53	82.16	81.34	81.67 ± 0.4292	
	200	88.72	88.65	88.59	88.65 ±0.0650	

Name of the sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
Dia-ion resin fraction (DRF)	12.5	16.48	16.59	16.53	16.53±0.0550	32
	25	43.42	43.47	43.53	43.47±0.0550	
	50	67.62	67.26	68.12	67.66 ±0.4318	
	100	82.51	82.38	83.19	82.69 ±0.4350	
	150	91.42	92.12	91.87	91.80 ±0.3547	
	200	94.59	94.54	93.92	94.35 ±0.3732	

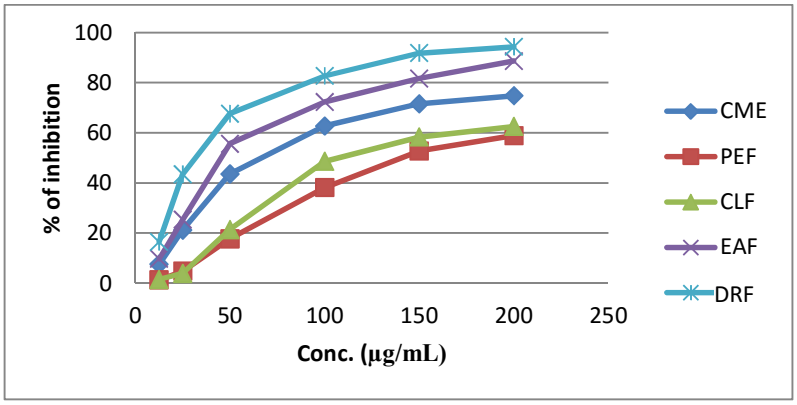


Fig 6.31: Butyrylcholinesterase inhibitory activity of crude methanol extract(CME) and the different fractions(PEF, CLF, EAF & DRF) of *Wedelia chinensis* at different concentrations.

Table-6.17: Butyrylcholinesterase inhibitory activity of galanthamine (standard) at different concentrations

Name of sample	Conc. (µg/ml)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/ml)
		a	b	c		
Galanthmine	5	34.31	33.86	35.47	34.54±0.8306	8.3
	10	60.77	60.73	60.81	60.77±0.0400	
	15	82.89	82.9	82.45	82.74±0.2569	
	20	92.56	91.32	93.87	92.58±1.2751	
	25	94.51	94.56	94.53	94.53±0.0251	
	30	94.89	95.77	96.56	95.74±0.8354	

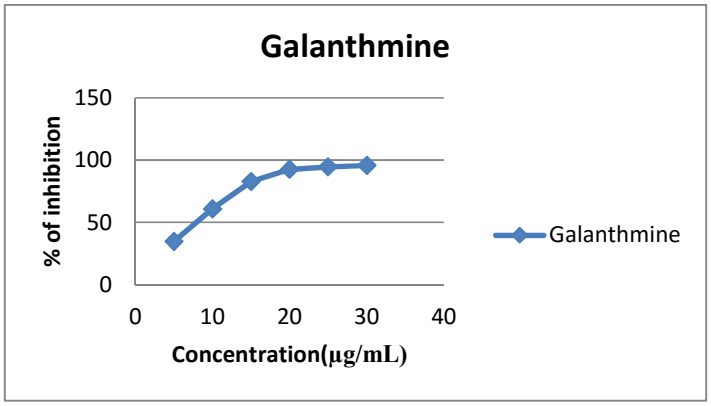


Fig 6.32 : Butyrylcholinesterase inhibitory activity of galanthamine (standard)

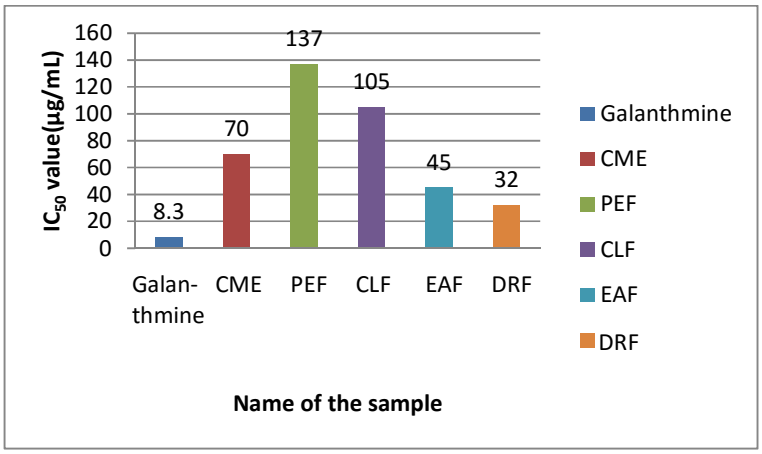


Fig 6.33 : IC_{50} values of crude dia-ion resin extract (CME) and it's different fraction (PEF, CLF, EAF & DRF) and galanthamine (standard).

All the extractives showed the butyrylcholinesterase inhibition activity. The IC_{50} values of crude methanolic extract (CME), petroleum ether fraction (PEF), chloroform fraction (CLF), ethyl acetate fraction (EAF) and dia-ion resin fraction (DRF) were 70, 135, 105, 45 and 32 µg/mL respectively. The results demonstrated that DRF and EAF exhibited strong enzyme inhibition activity among the other fractions evaluated.

6.6.2 Determination of Anti-butyrylcholinesterase activity assay of isolated compounds of the ethyl acetate fraction of *Wedelia chinensis*.

Table-6.18: Butyrylcholinesterase inhibitory activity of compounds (EFC-1 & EFC-2) obtained from the ethyl acetate fraction of *W. chinensis* and galanthamine (standard) at 100 µg/mL concentration

Name of the sample	Conc. (µg/ml)	% of inhibition			% of inhibition Mean ± STD
		a	b	c	
Galanthamine	100	96.31	96.39	96.27	96.32±0.0611
EFC-1	100	67.25	66.67	67.34	67.08±0.3636
EFC-2	100	93.15	92.33	92.11	92.53±0.5480

The galanthamine showed maximum inhibition 96.32% and EFC-1& EFC-2 showed maximum inhibition 67.08% and 92.53% respectively. From our result, it is evident that all the compounds test edpossess anti-butyrylcholinesterase activity and their activity decreases in the following order: galanthamine > EFC-2 > EFC-1

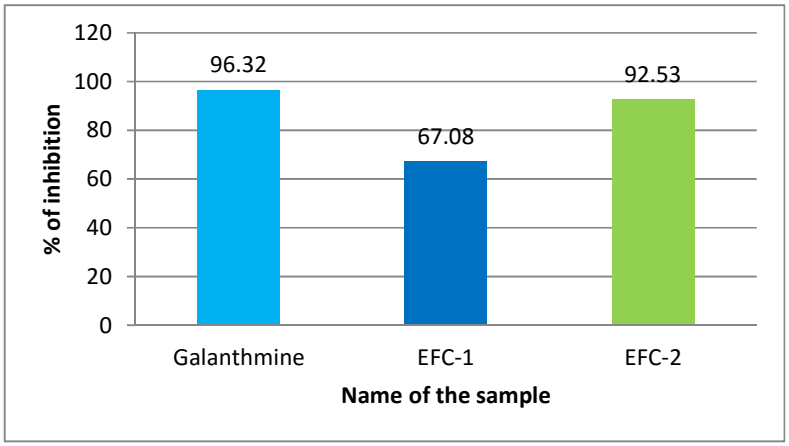


Fig 6.34 : % of Butyrylcholinesterase inhibitory activity of compounds (EFC-1 & EFC-2) and galanthamine (standard) at 100 µg/mL concentration.

Among the compounds (EFC-1 & EFC-2) tested the further exhibited inhibition activity. So it was further analyzed for it anti-butyrylcholinesterse activity having IC_{50} values.

6.6.3 Determination of Anti-butyrylcholinesterase activity assay of isolated compounds of the ethyl acetate fraction of *Wedelia chinensis* at different concentrations.

Table-6.19: Butyrylcholinesterase inhibitory activity of isolated compounds of ethyl acetate fraction of *Wedelia chinensis* at different concentrations.

Name of the sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
EFC-1	12.5	10.14	09.83	10.36	10.11±0.2662	38.5
	25	28.58	29.17	28.38	28.71±0.4107	
	50	58.68	58.15	59.13	58.65±0.4905	
	100	67.25	66.67	67.34	67.08±0.3636	
	150	73.86	74.29	73.92	74.02±0.2328	
	200	75.16	74.76	74.89	74.94±0.2040	
EFC-2	12.5	9.76	10.28	11.07	10.37±0.6596	35
	25	31.13	29.85	30.29	30.42±0.6503	
	50	64.86	65.38	67.01	65.75±1.1217	
	100	93.15	92.33	92.11	92.53±0.5480	
	150	97.47	97.56	98.63	97.89±0.6453	
	200	99.87	100.39	101.15	100.47±0.64437	

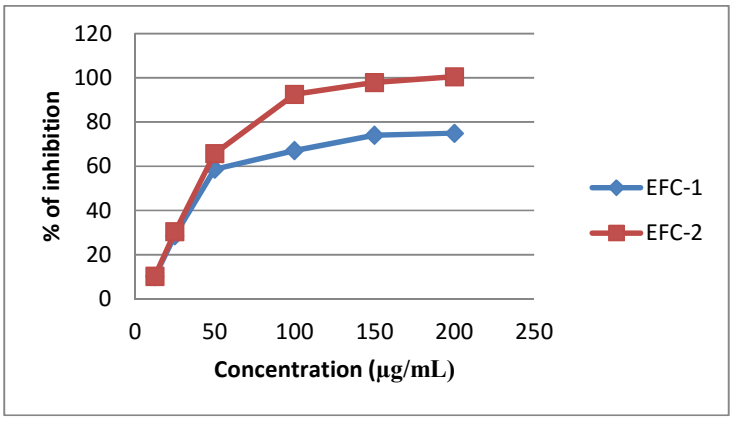


Fig 6.35 : Butyrylcholinesterase inhibitory activity of isolated compounds EFC-1 & EFC-2.

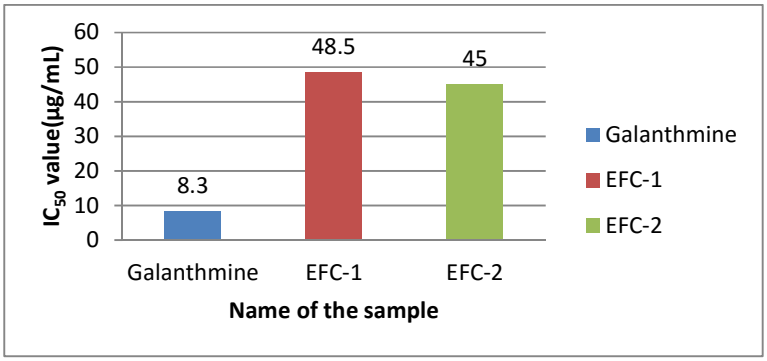


Fig 6.36 : IC_{50} (µg/mL) values of isolated compounds EAC-1, EFC-2 and galanthmine (standard).

The compounds EFC-1and EFC-2 showed the butyrylcholinesterase inhibition activity. The IC_{50} values of EFC-1, EFC-2 and galanthmine (standard) were 48.5 µg/mL, 45 µg/mL and 8.3 µg/mL respectively. The results demonstrated that EFC-2 is strong enzyme inhibition activity among the EFC-1 evaluated but less enzyme inhibition activity than galanthmine (standard).

6.6.4 Determination of Anti-butyrylcholinesterase activity assay of isolated compounds of the dia-ion resin fraction of *Wedelia chinensis*.

Table-6.20 : Butyrylcholinesterase inhibitory activity of different compounds (DFC-1 to DFC-5) obtained from the dia-ion resin fraction of *W. chinensis* and galanthamine (standard) at 100 µg/mL concentration

Name of the sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD
		a	b	c	
Galanthamine	100	96.31	96.39	96.27	96.32±0.0611
DFC-1	100	54.78	54.91	55.32	55.00±0.2818
DFC-2	100	82.75	83.32	83.51	83.19±0.3955
DFC-3	100	125.76	126.37	126.74	126.29±0.4948
DFC-4	100	102.38	103.54	104.31	103.41±0.9715
DFC-5	100	103.75	105.14	104.67	104.52±0.7070

The galanthamine showed maximum inhibition 96.32% and DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 showed 55%, 83.19%, 126.29%, 103.41% and 104.52% respectively. From our result, it is evident that all the compounds test edpossess anti-butyrylcholinesterase activity and their activity decreases in the following order:

DFC-3> DFC-5 > DFC-4 > galanthamine > DFC-2 > DFC-1

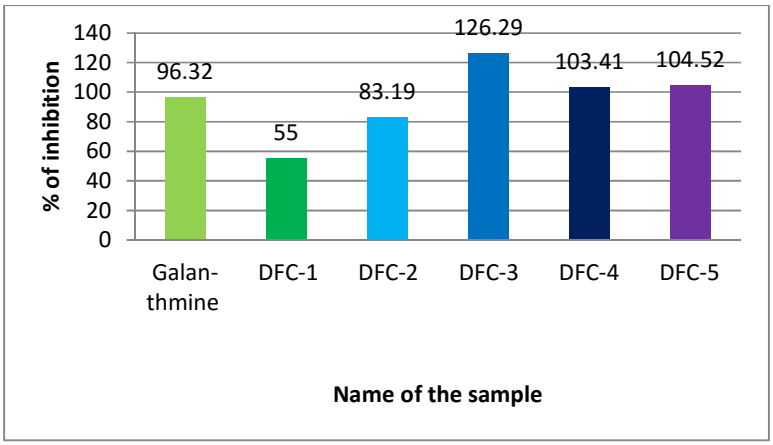


Fig 6.37 : At 100 µg/mL concentration % of Butyrylcholinesterase inhibitory activity of isolated compounds (DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5) and galanthamine (standard)

Among the compounds (DFC-1 to DFC-5) tested the further exhibited inhibition activity. So it was further analysed for it anti-butyrylcholinesterse activity having IC_{50} values.

6.6.5 Determination of Anti-butyrylcholinesterase activity assay of isolated compounds of the dia-ion resin fraction of *Wedelia chinensis* at different concentrations.

Table-6.21: Butyrylcholinesterase inhibitory activity of isolated compounds of dia-ion resin fraction of *Wedelia chinensis* at different concentrations.

Name of the sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
DFC-1	12.5	5.76	6.27	5.54	5.85±0.3744	78
	25	16.56	15.78	16.15	16.16±0.3901	
	50	38.58	37.89	38.76	38.41±0.4592	
	100	54.78	54.91	55.32	55.00±0.2818	
	150	68.33	67.76	67.13	67.74±0.6002	
	200	72.68	73.23	73.16	73.02±0.2993	
DFC-2	12.5	9.25	9.73	10.17	9.72±0.4601	48
	25	25.78	26.36	25.56	25.90±0.4132	
	50	53.89	54.24	54.13	54.08±0.1789	
	100	82.75	83.32	83.51	83.19±0.3955	
	150	91.83	92.24	92.63	92.23±0.4000	
	200	98.59	98.31	99.25	98.72±0.4826	
DFC-3	12.5	21.08	20.87	21.26	21.07±0.1951	20
	25	49.52	50.17	50.34	50.01±0.4327	
	50	81.82	82.15	82.62	82.19±0.4020	
	100	125.76	126.37	126.74	126.29±0.4948	
	150	138.57	139.27	139.09	138.97±0.3635	
	200	145.68	144.94	145.34	145.32±0.3704	
DFC-4	12.5	12.27	11.87	12.34	12.16±0.2535	45
	25	42.27	43.12	42.78	42.72±0.4278	
	50	75.42	74.76	75.62	75.26±0.4500	
	100	102.38	103.54	104.31	103.41±0.9715	
	150	112.86	111.89	113.24	112.66±0.6961	
	200	129.45	130.23	129.76	129.81±0.3927	
DFC-5	12.5	15.76	16.23	16.21	16.06±0.2657	22.5
	25	45.12	44.89	45.56	45.19±0.3404	
	50	78.17	77.86	79.12	78.38±0.6565	
	100	103.75	105.14	104.67	104.52±0.7070	
	150	122.87	123.64	123.21	123.24±0.3858	
	200	127.45	128.21	128.64	128.10±0.6025	

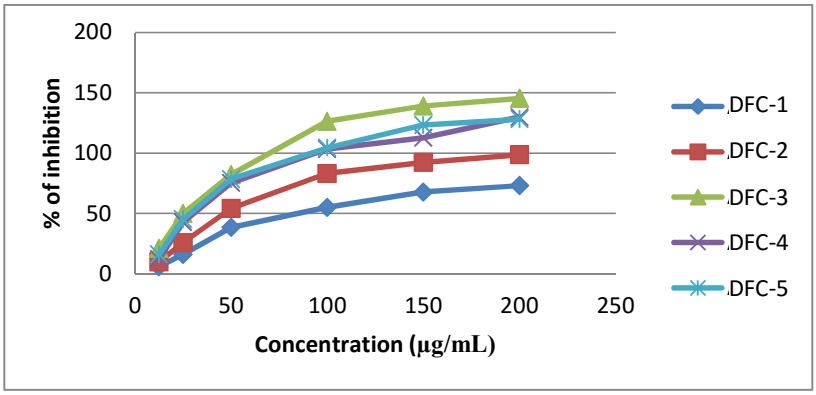


Fig: 6.38 : Butyrylcholinesterase inhibitory activity of MEF and isolated compounds DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 of *W. chinensis* at different concentrations.

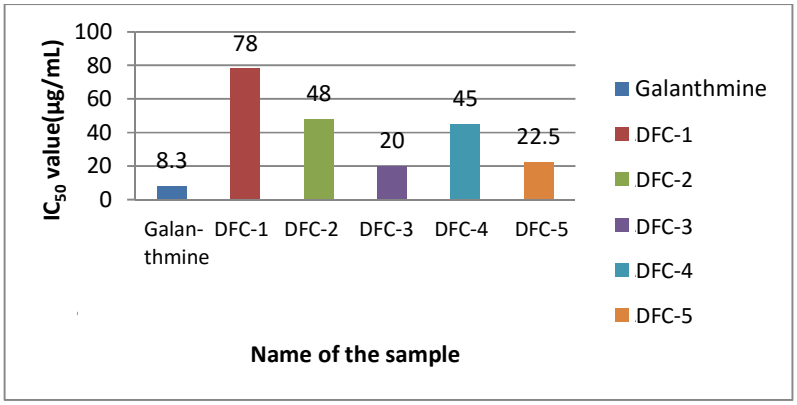


Fig 6.39 : IC_{50} (µg/mL) values of isolated compounds DFC-1, DFC-2, DFC-3, DFC-4, DFC-5 and galanthmine (standard).

Under the experimental condition maintained, the butyryllcholiesterase inhibitory activity of galanthamine (standard) was found to increase with concentration having IC_{50} value of 8.3 µg/mL (Fig. 6.32).

Further the butyrylcholinesterase inhibitory activity of isolated compounds (DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5) from dia-ion resin fraction have been investigated at 78, 48, 20, 45 and 22.5µg/mL compared with galanthamine (standard).

6.7. Anti-acetylcholinesterase activity assay

6.7.1. Anti-acetylcholinesterase activity assay of different fractions of *Wedelia chinensis.*

Inhibition of acetylcholinesterase, which enhances cholinergic transmission by reducing the enzymatic degradation of acetylcholine, is a widely accepted strategy for drug development to treat AD patients. In this study, the acetylcholiesterase inhibitory activity of the crude methanol extract(CME) of *Wedelia chinensis* and its different fractions and Colum subfractions isolated from chloroform fraction was assessed by modified Ellman method and compared with the reference standard donepezil. This method estimates acetylcholinesterase (AchE) using acetylthiocholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion.

The results of acetylcholinesterase inhibitory activity of the different samples are given in Table -6.22, 6.23, 6.24 & 6.25 and Fig. 6.40, 6.41, 6.42, 6.43, 6.44, 6.45, 6.46, 6.47 & 6.48. All the extracts showed the acetylcholinesterase inhibitory activity. Instant inhibition was found in methanol fraction followed by ethyl acetate, chloroform and petroleum ether fraction. The IC_{50} for crude methanol extract, petroleum ether, chloroform, ethyl acetate and methanol fractions were 82, 150, 100, 40 and 30 µg/mL respectively.

Table-6.22: Acetylcholinesterase inhibitory activity of the different fractions of *Wedelia chinensis* at different concentrations.

Name of the sample	Conc. (µg/ml)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/ml)
		a	b	c		
Crude methanol extract (CME)	12.5	3.51	4.11	3.37	3.66±0.3931	82
	25	15.69	16.19	15.47	15.78±0.3689	
	50	42.58	43.08	42.32	42.66±0.3862	
	100	53.82	53.31	54.13	53.75±0.4140	
	150	57.74	57.53	58.23	57.83±0.3592	
	200	63.52	63.38	63.71	63.53±0.1656	
Petroleum ether fraction (PEF)	12.5	2.53	2.36	2.73	2.54±0.1852	150
	25	8.76	9.17	8.47	8.89±0.3517	
	50	24.79	25.15	24.59	24.84±0.2837	
	100	43.68	43.37	44.09	43.71±0.3611	
	150	49.85	50.18	49.73	49.92±0.2330	
	200	53.13	53.16	53.45	53.13±0.1767	

Name of the sample	Conc. (µg/ml)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/ml)
		a	b	c		
Chloroform fraction (CLF)	12.5	2.87	3.11	2.68	2.88±0.2154	100
	25	13.59	14.27	13.45	13.77±0.4386	
	50	29.57	29.24	30.16	29.65±0.4660	
	100	46.85	47.32	46.75	46.97±0.3043	
	150	53.15	53.43	53.21	53.15±0.1474	
	200	60.21	60.54	60.53	60.21±0.1877	
Ethyl acetate fraction (EAF)	12.5	6.79	7.23	6.98	7.00±0.2206	40
	25	31.56	32.21	31.78	31.85±0.3306	
	50	54.72	54.34	55.08	54.71±0.3700	
	100	62.81	63.11	62.86	62.92±0.1607	
	150	69.83	69.27	70.35	69.81±0.5401	
	200	72.63	73.23	72.51	72.79±0.3857	
Dia-ion resin fraction (DRF)	12.5	14.78	13.97	13.64	14.13±0.5866	30
	25	38.73	39.23	38.75	38.90±0.2830	
	50	62.32	62.23	63.12	62.55±0.4899	
	100	71.53	72.35	71.56	71.81±0.4650	
	150	78.68	79.11	78.64	78.81±0.2605	
	200	82.82	83.24	82.86	82.97±0.2318	

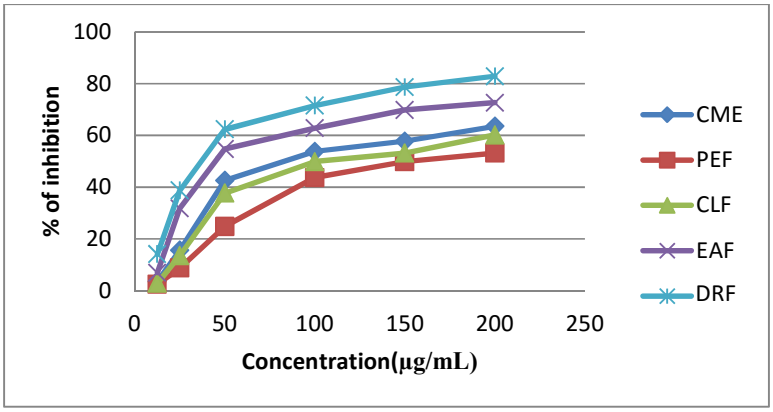


Fig 6.40: Acetylcholinesterase inhibitory activity of crude dia-ion resin extract and different fractions (PEF, CLF, EAF & DRF) of *W. chinensis* and dopenezol (standard) at different concentrations.

Table-6.23: Acetylcholinesterase inhibitory activity of donepezil (standard) at different concentrations

Name of the sample	Concentration (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
Donepezil	5	33.03	33.09	33.07	33.06±0.0305	10
	10	60.65	62.84	59.54	61.01±1.6791	
	20	86.23	86.43	86.78	86.48±0.2783	
	40	92.11	92.09	92.13	92.11±0.0200	
	80	94.57	95.29	92.89	94.25±1.2315	

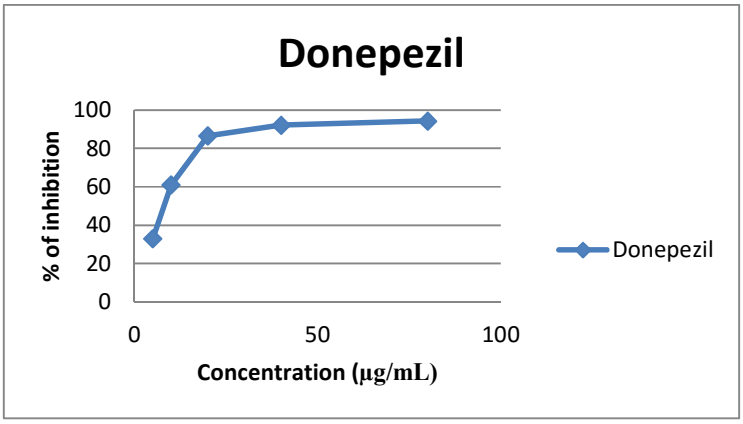


Fig 6.41: Acetylcholinesterase inhibitory activity of donepezil (standard) at different concentration.

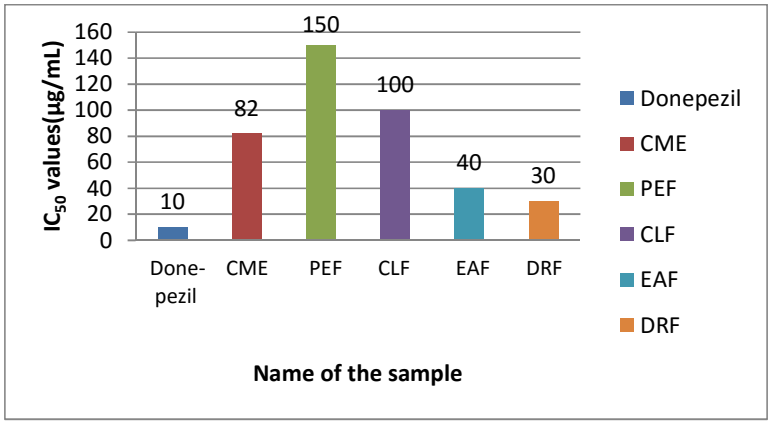


Fig 6.42 : IC_{50} values (µg/mL) of acetylcholinesterase inhibitory activity of CME and different fractions (PEF, CLF, EAF & DRF) of *W. chinensis* and donepezil (standard) at different concentrations.

The IC_{50} values of petroleum ether fraction (PEF), chloroform fraction(CLF), ethyl acetate fraction(EAF) and dia-ion resin fraction(DRF) were 150 µg/mL, 100 µg/mL, 40 µg/mL and 30 µg/mL respectively. The results demonstrated that dia-ion resin fraction and ethyl acetate fraction exhibited strong enzyme inhibition activity among the other fractions.

Under the experimental condition maintained, the aceylcholiesterase inhibitory activity donepezil exhibited with IC_{50} value of 10 µg/mL.

6.7.2. Anti-acetylcholinesterase activity assay of ethyl acetate fractions of *Wedelia chinensis.*

Table-6.24: Acetylcholinesterase inhibitory activity of compounds (EFC-1 & EFC-2) obtained from the ethyl acetate fraction of *Wedelia chinensis* and Donepezil (standard) at 100 µg/mL concentration

Name of the sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD
		a	b	c	
Donepezil	100	97.5	97.54	97.47	97.50±0.0351
EFC-1	100	75.94	75.48	75.47	75.63±0.2685
EFC-2	100	83.23	82.56	82.49	82.76±0.4085

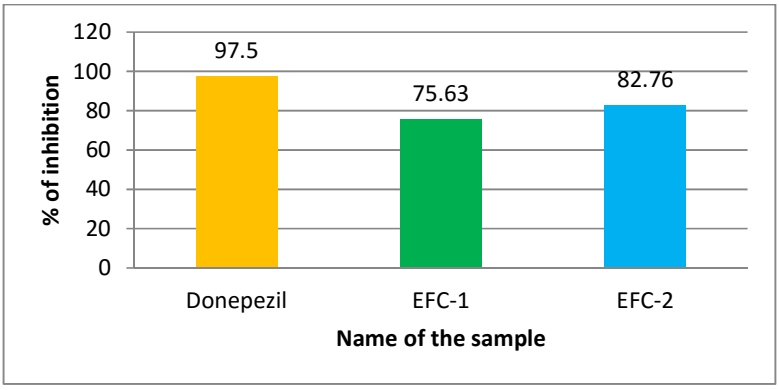


Fig 6.43 : At 100 µg/mL concentration % of Acetylcholinesterase inhibitory activity of compounds EFC-1 & EFC-2 and Donepezil (standard).

The acetylcholinesterase inhibitory activity of the compounds (EFC-1 & EFC-2) isolated from ethyl acetate fraction have been investigated at 100 µg/mL and compared with donepezil (standard). Both the compounds showed strong activity. The donepezil showed maximum inhibition 97.50 % and EFC-1 & EFC-2 showed 75.63% and 82.76% respectively. From the result, it was evident that the compounds test edpossessanti–acetylcholinesterase inhibition activity and their activity decreases in the following order: Donepezil > EFC-2 > EFC-1

Among the compounds (EFC-1 &EFC-2) were exhibited strong activity. So the compounds (EFC-1 & EFC-2) were further analyzed for it's anti-acetylcholinesterase activity having IC_{50} value of 50 µg/mL & 42.5 µg/mL.

6.7.3. Anti-acetylcholinesterase activity assay of ethyl acetate fractions of *Wedelia chinensis* at different concentrations.

Table-6.25: Acetylcholinesterase inhibitory activity of isolated compounds of ethyl acetate fraction of *Wedelia chinensis* at different concentrations.

Name of the sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
EFC-1	12.5	7.25	7.57	7.62	7.48±0.2007	50
	25	21.39	21.64	21.68	21.57±0.1571	
	50	52.27	53.79	52.34	52.50±0.8580	
	100	75.94	75.48	75.47	75.63±0.2685	
	150	85.77	86.37	86.12	86.09±0.3013	
	200	92.23	90.43	91.81	91.49±0.9417	
EFC-2	12.5	7.27	8.31	7.37	7.65±0.5737	42.5
	25	31.87	32.34	33.03	32.41±0.5834	
	50	62.78	63.29	63.71	63.25±0.4657	
	100	83.23	82.56	82.49	82.76±0.4085	
	150	97.21	96.68	96.31	96.73±0.4523	
	200	98.14	99.13	98.32	98.51±0.5273	

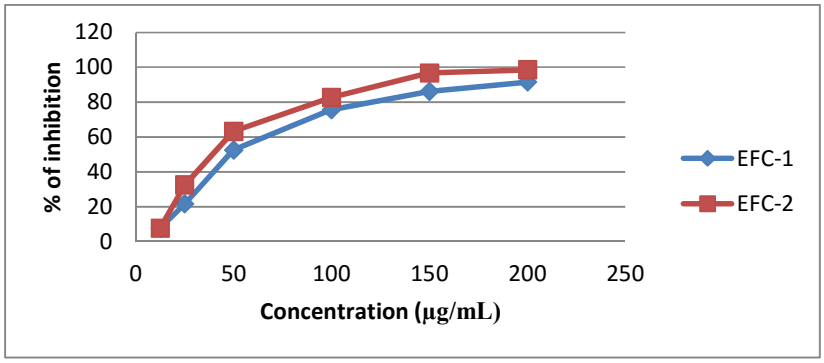


Fig 6.44 : Acetylcholinesterase inhibitory activity of isolated compounds EFC-1, EFC-2 at different concentrations.

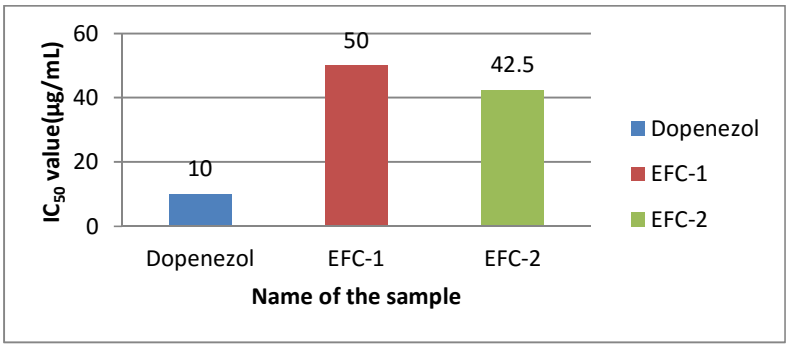


Fig 6.45 : IC_{50} values (µg/mL) acetylcholinesterase inhibitory activity of isolated compounds EFC-1 & EFC-2 of *W. chinensis* and donepezil (standard) at different concentrations.

6.7.4. Anti-acetylcholinesterase activity assay of dia-ion resin fractions of *Wedelia chinensis* at different concentrations.

Table-6.26 : Acetylcholinesterase inhibitory activity of compounds (DFC-1 to DFC-5) obtained from the dia-ion resin fraction of *wedelia chinensis* and donepezil (standard) at 100 µg/mL concentration.

Name of the sample	Conc. (µg/ml)	% of inhibition			% of inhibition Mean ± STD
		a	b	c	
Donepezil	100	97.5	97.54	97.47	97.50±0.0351
DFC-1	100	67.32	67.54	67.37	67.41±0.1153
DFC-2	100	84.54	84.76	84.73	84.68±0.1193
DFC-3	100	118.27	117.89	117.93	118.03±0.2088
DFC-4	100	106.76	106.78	106.87	106.80±0.0585
DFC-5	100	93.59	93.61	93.75	93.65±0.0871

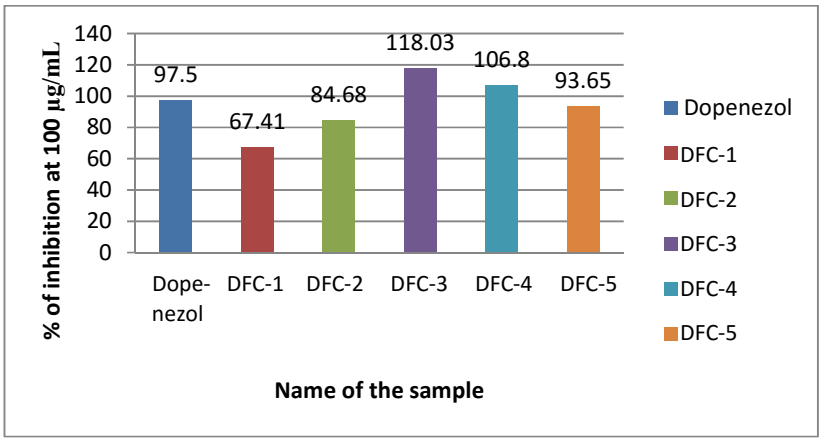


Fig 6.46 : % of Acetylcholinesterase inhibitory activity of compounds (DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5) and donepezil (standard) at 100 µg/mL concentration.

The acetylcholinesterase inhibitory activity of compounds (DFC-1 to DFC-5) isolated from dia-ion resin fraction have been investigated and compared with donepezil (standard). The donepezil showed maximum inhibition 97.50 % and DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 showed 67.41%, 84.68%, 118.03%, 106.80% and 93.65% respectively. From our result, it is evident that the compounds teste dpossess anti–acetylcholinesterase inhibition activity and their activity decreases in the following order: DFC-3 > DFC-4 > Donepezil > DFC-5 > DFC-2 > DFC-1

Since the compounds DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 exhibited high activity, so they were further analyzed for its anti-acetylcholinesterase activity. The IC_{50} values for acetylcholinesterase inhibitory activity were 75µg/mL, 48 µg/mL, 20 µg/mL, 35 µg/mL and 42 µg/mL.

6.7.5. Anti-acetylcholinesterase activity assay of dia-ion resin fractions of *Wedelia chinensis*.

Table-6.27: Acetylcholinesterase inhibitory activity of isolated compounds of dia-ion resin fraction of *W. chinensis* at different concentrations.

Name of the sample	Conc. (µg/mL)	% of inhibition			% of inhibition Mean ± STD	IC_{50} (µg/mL)
		a	b	c		
DFC-1	12.5	3.53	3.38	3.35	3.42±0.0964	75
	25	15.67	16.18	15.75	15.87±0.2742	
	50	37.76	38.12	37.79	37.89±0.1997	
	100	67.32	67.54	67.37	67.41±0.1153	
	150	75.78	76.21	75.77	75.92±0.2511	
	200	84.43	84.37	84.54	84.45±0.0862	
DFC-2	12.5	5.72	5.98	5.64	5.78±0.1777	48
	25	24.47	24.32	24.31	24.37±0.0896	
	50	51.65	51.83	51.71	51.73±0.0916	
	100	84.54	84.76	84.73	84.68±0.1193	
	150	102.26	102.57	102.37	102.40±0.1571	
	200	112.66	112.43	112.41	112.50±0.1389	
DFC-3	12.5	18.66	18.63	18.87	18.72±0.1307	20
	25	57.27	57.51	57.48	57.42±0.1307	
	50	83.55	83.59	83.78	83.64±0.1228	
	100	118.27	117.89	117.93	118.03±0.2088	
	150	132.68	132.85	132.87	132.80±0.1044	
	200	142.73	142.81	142.87	142.80±0.0702	
DFC-4	12.5	12.78	12.82	12.94	12.85±0.0832	35
	25	43.97	43.77	43.78	43.84±0.1126	
	50	72.72	72.65	72.75	72.71±0.0513	
	100	106.76	106.78	106.87	106.80±0.0585	
	150	121.87	121.76	121.82	121.82±0.0550	
	200	131.65	131.74	131.73	131.71±0.0493	
DFC-5	12.5	11.69	12.08	11.71	11.83±0.2196	42
	25	37.87	37.55	37.62	37.68±0.1682	
	50	65.68	65.62	65.86	65.72±0.1249	
	100	93.59	93.61	93.75	93.65±0.0871	
	150	111.76	111.67	111.68	111.70±0.0493	
	200	119.87	120.04	119.82	119.91±0.1153	

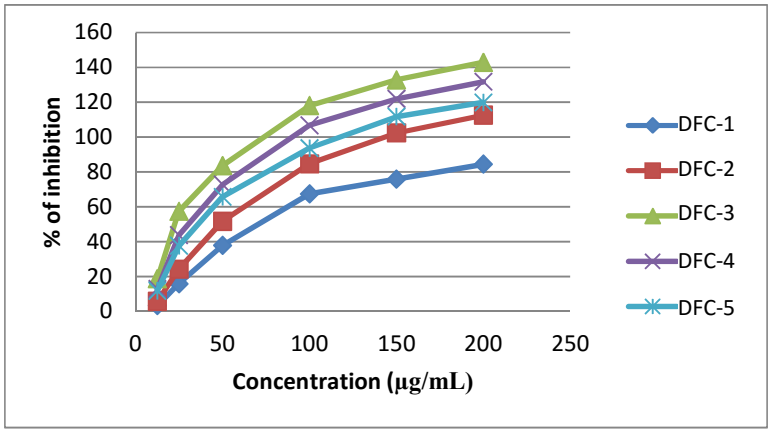


Fig 6.47: Acetylcholinesterase inhibitory activity of isolated compounds (DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5) of *W. chinensis* at different concentrations.

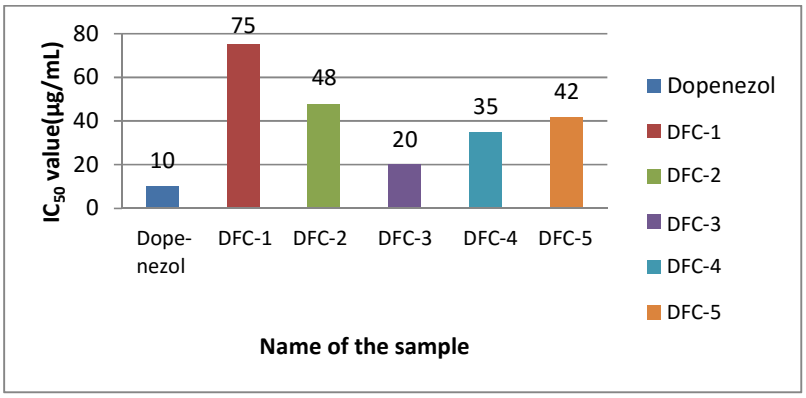


Fig 6.48 : IC_{50} Values (µg/mL) of Acetylcholinesterase inhibitory activity of isolated compounds (DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5) of *W. chinensis* and donepezil (standard) at different concentrations.

Among the compounds DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 were exhibited highest activity. So the compounds DFC-1, DFC-2, DFC-3, DFC-4 & DFC-5 were further analyzed for its anti-acetylcholinesterase activity having IC_{50} values were 75 µg/mL, 48 µg/mL, 20 µg/mL, 35 µg/mL & 42 µg/mL.

CONCLUSION

With centuries of tireless efforts scientists have found in plants the remedy of diverse diseases ranging from simple skin infection to complicated cancer. To achieve the target of getting new bioactive principles, research on medicinal plants is of great importance.

In the present investigation, a compositae plant was selected because the members of the family can be found all over the world and in most habitats and climates that known to produce diverse classes of pharmacologically active compounds. *Wedelia chinensis* is a perennial herb. This plant has very good folk medicinal use in the treatment of liver enlargement, jaundice and other ailments of the liver and gall bladder and also in the treatment of uterine haemorrhage and menorrhagia. The plant *Wedelia chinensis* (Asteraceae) belonging to the genus 'Wedelia' has little phytochemical work and no antibacterial and antifungal activities tests of some compounds so far isolated from this plant has been reported.

Thus, in order to isolate bioactive principles as well as to evaluate various biological screening such as *in vitro* antibacterial and antifungal activities, brine shrimp lethality bioassay and sub acute toxicity studies of the isolated compounds, the present study was under taken on the plant *Wedelia chinensis*, which ultimately led to the proper use of this plant for better health care system of common people of Bangladesh.

Extraction of the dried powder of whole plant of *Wedelia chnensis* was done by cold extraction method with methanol as solvent. Crude methanolic extract of the plant was further fractionated into four fractions; petroleum ether fraction (PEF), Chloroform fraction (ClF), Ethyl acetate fraction (EAF) and Dia-ion resin fraction (DRF). From chemical investigation of the Dia-ion resin (DRF) and Ethyl acetate fraction (EAF), seven compounds were isolated and characterized; DFC-1, DFC-2, DFC-3, DFC-4, DFC-5, EFC-1, EFC-2. The structures of these compounds were elucidated with the help of extensive spectroscopic techniques. Compound DFC-1, DFC-2, DFC-3, DFC-4 and DFC-5 were isolated from Dia-ion resin fraction (DRF). Compound EFC-1 and EFC-2 were isolated from Ethyl acetate fraction (EAF). Among the isolated compounds, compounds DFC-4 and DFC-5 are new one. Compound DFC-1 was first time isolated from this plant, previously reported from Achyranthes *Aspera.*

Anticholinesterase & Antioxidant activities of the fractions as well as pure compounds have been evaluated; such as total antioxidant activity, DPPH radical scavenging activity, hydroxyl radical scavenging activity, lipid peroxidation inhibitory activity, butyrylcholinesterase inhibitory activity and acetylcholinesterase inhibitory activity.

From the biological screening, it was found that among the four fractions (PEF, ClF, EAF and DRF), fractions EAF & DRF were more biologically active than others. So, first attempt was taken to investigate these two fractions. Phytochemical and biological, e.g., anticholinesterase & antioxidant activities of these fractions were investigated.

Because of time scarcity, works on the other two fractions (PEF, ClF) have not been performed. Investigation of these fractions might provide some biologically important compounds. Some other biological activities, such as, antibacterial, antifungal, insecticidal activities, sub acute toxicity could not be done which might give good result.

Further research work is needed to achieve the target of getting new bioactive principles from this plant as drug materials which would justify its use in folk medicine.

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