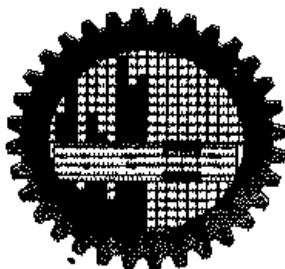# PHYTOCHEMICAL AND MICROBIAL ANALYSIS OF
Andrographis peniculata

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF PHILOSOPHY (M. PHIL.) IN CHEMISTRY

SUBMTTED

BY

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OCTOBER, 2011

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TO
MY PARENTS

Dr. Md. Abdul WAHHAB
and
RAWSHAN ARA BEGUM

CERTIFICATE

This is to certify that the research work embodying in this thesis has been carried out under my supervision. The work presented herein is original. This thesis has not been submitted elsewhere for the award of any other degree or diploma in any University or Institution.

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DECLARATION

This thesis work has been done by the candidate himself and does not contain any material extracted from elsewhere or from a work published by anybody else. The work for this thesis has not been presented elsewhere by the author for any degree or diploma.

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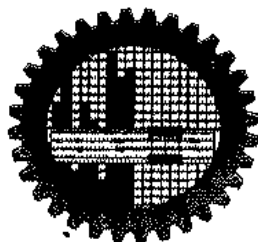
THESIS ACCEPTANCE LETTER

This thesis titled "**Phytochemical and Microbial Analysis of** *Andrographis peniculata*" submitted by R aquibur R ahman, Roll N o. 040803106 F, S ession- April, 2008 h as be en a ccepted a s satisfactory in partial fulfillment of the requirement for the degree of Masters of Philosophy (M. Phil) in Chemistry on October 30, 2011.

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ACKNOWLEDGEMENT

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Contents

PART-2
BIOLOGICAL ACTIVITIES OF *Andrographis peniculata*

ABSTRACT

Acanthaceae plants such as *Andrographis peniculata* is enriched with important biologically active compounds. F rom t he pr ofound i nterest of t hese c ompounds and their us eful biological activities, the leaves along with the stems were extracted with ethanol. Then the ethanol extract was partitioned with n-hexane, ethyl acetate and 1-butanol, respectively. The partitioned extracts were subjected to various chromatographic techniques to isolate the pure compounds f or c haracterization a nd de termination of t heir biological a ctivities. Seven compounds were characterized from the plant extract. Among the compounds stigmasterol, andrographolide, •-sitosterol, daucosterol were isolated from the n-hexane part. Three other compounds a ndrographanin, 14- deoxyandrographolide, 12-hydroxyneoandrographolide were isolated from the ethyl acetate part. All the structures of the compounds isolated from the plant are as follows:
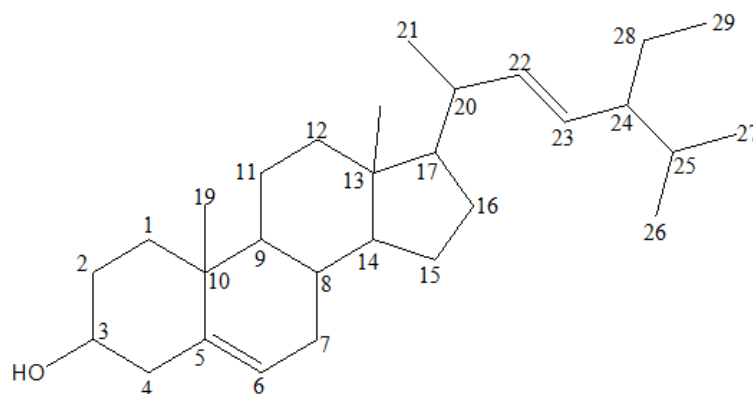
Stigmasterol (Compound: 1)
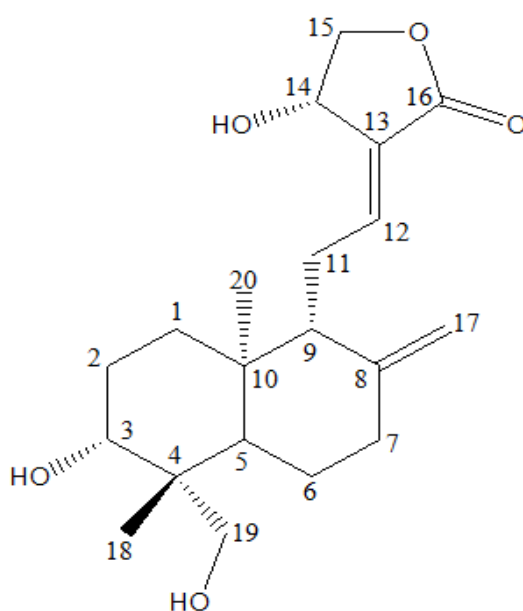
Andrographolide (Compound: 2)
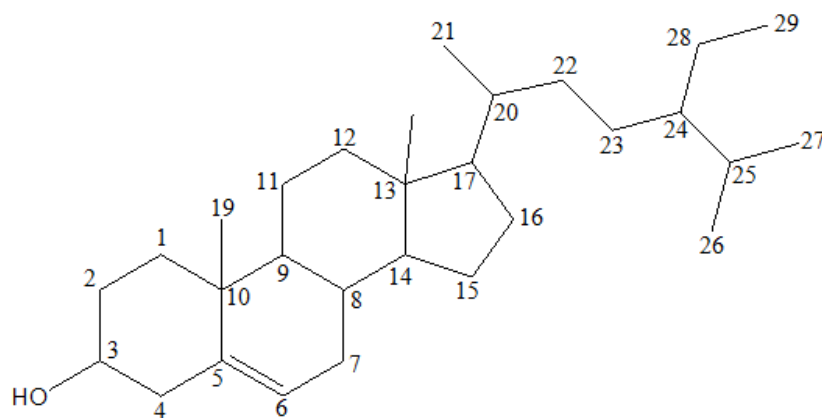
• -sitosterol (Compound: 3)
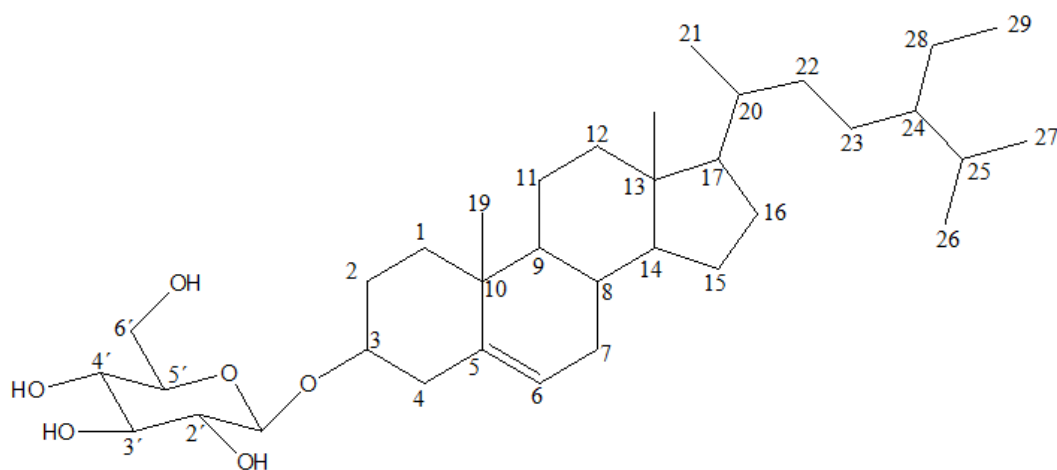
Daucosterol (Compound: 4)
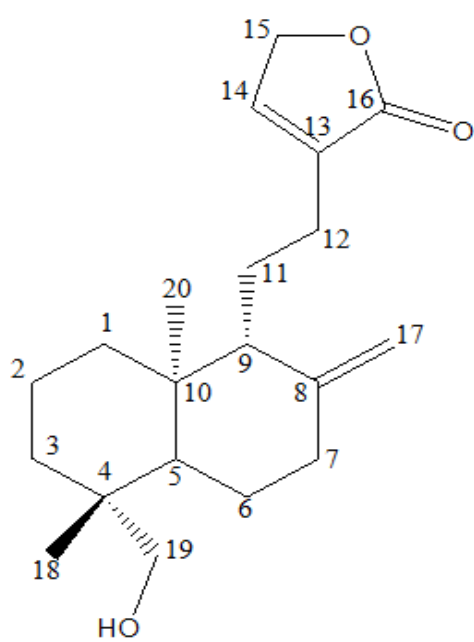
Andrographanin (Compound: 5)
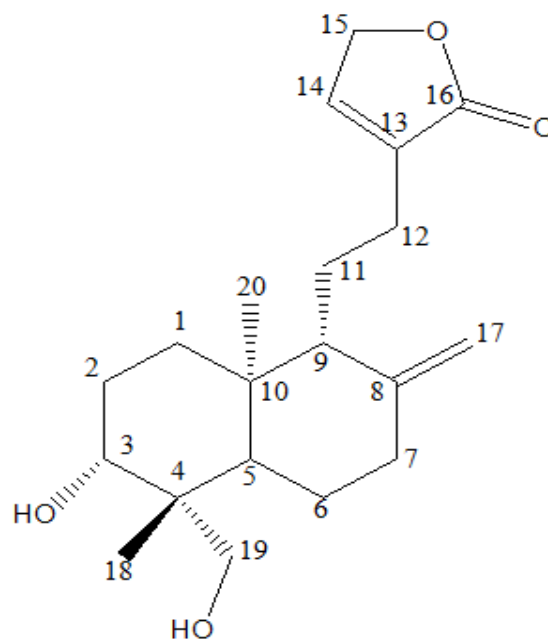
14- deoxyandrographolide (Compound: 6)
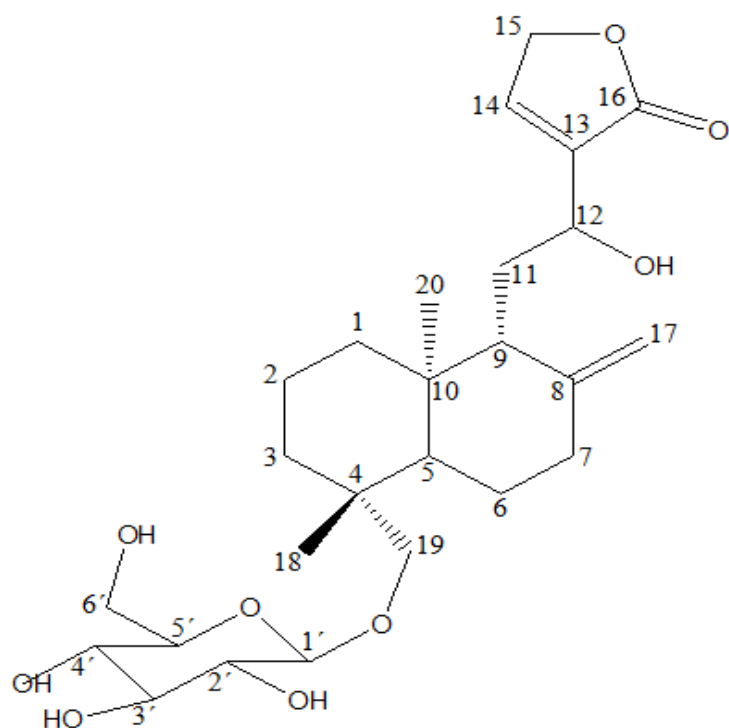
12- hydroxyneoandrographolide (Compound: 7)

The above structures of the compounds were elucidated by various spectroscopic methods (UV, FTIR, ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HSQC, HMBC etc.). Among the isolated compounds, 12-hydroxyneoandrographolide (compound-7) was found to be isolated and elucidated for the first time according to the literature survey.

The compounds andrographolide, 14-deoxyandrographolide, 12-hydroxyneoandrographolide were found to have high anti-oxidant activity. The other steroidal compounds (compound 1, 4, 5) showed very low antioxidant activity. Among the isolated compounds only andrographolide showed moderate antimicrobial activity.

Thus the isolation, characterization and investigation of potent antioxidant and antimicrobial activity of the compounds might generate much synthetic and biological interest in this class of the natural products.

Part-1

PHYTOCHEMICAL ANALYSIS OF *Andrographis peniculata*

Chapter One

INTRODUCTION

1.1 General

The use of medicinal plants for alleviating diseases had its origin in the activities of the most primitive man of the remote past. Illness, physical discomfort, injuries, wounds and fear of death had forced early man to use any natural substance for relieving the pain and suffering caused by these abnormal conditions and for preserving health against diseases and death. Plants play a vital role for the existence of life in the universe. Primitive man started to distinguish nutritional and pharmacologically active plants for their survival. By their experience, this knowledge of herbal remedies was transferred from one generation to another at first orally and later in written from a papyri, backed clay tablets, parchments, manuscripts, pharmacopoeias and other works. Therefore, medicinal plants have been in use for the eradication and human suffering since ancient time.

As far as record goes, it appears that Babylonians (about 3000 years B.C.) were aware of a large number of medicinal plants and their properties. Some of them are still in use for the same purpose like henbane (*Hyoscyamus* Spp.), Opium (*papaver somniferum*), Castor oil (*Ricinus communis*), Aloevera (*Aloe spp.*) etc[1].

The Chinese have an effective and unique system of medicine. The earliest known Chinese pharmacopoeia, The Pen Tsao, described over 300 medicinal plants and their uses. Although various of several ancient pharmacopoeia still exist today, the main surviving text book is on herbalism written in the 16th century by the physician Li Shih-Cheu describing almost 200 herbs and 10,000 herbal remedies[2]. Today Chinese herbalism is very much an orthodox form of therapy and preventive treatment in China and it is increasingly practiced in the west. Chinese herbalism can be used for a wide range of ailments, including asthma, skin diseases, menstrual problems, digestive disturbances, migraine and is effective when used on its own or in conjunction with another therapy such as acupuncture.

The material media of the great Greek physician Hippocrates (460-370 B.C.) consists of some 300 to 400 medicinal plants which included opium, mint rosemary, sage and verena. In the middle age, the great Greek pharmacist-physician Galen (131-200 A.D.) used a large number of medicinal plants in preparing his recipes.

The Arabian Muslim physicians like Al-Razi and Ibne Sina (9th to 14th century A.D.) brought about a revolution in the medicine by bringing new drugs of plant and mineral origin into

general us e. Enriching t he or iginal G reek system of medicine b y i ntroducing t hese ne w materials and knowledge they laid down the foundation stone of modern western medicine. The medicinal use of pl ants in the Indian subcontinent i s the R ig V eda (4500 – 1600 BC), which noted that Indo-Aryans used the Soma plant (*Amanita muscaria)* as a medicinal agent. The V edas m ade m any r eferences t o he aling plants i ncluding s arpagondha (*Rauvolfia serpentine*), while a co mprehensive Indian Herbal, the C haraka S amhita, cities m ore t han 500 medicinal plants[1].

Since di sease, de cay and death have al ways co -existed with life, the s tudy of di seases and their treatment must also have been contemporaneous with the dawn of the human intellect. It is appa rent that whatever progress science might have made in the field of medicine over the years, plants still remain the primary source of supply of many important drugs used in modern medicine. Indeed, the potential of obtaining new drugs from plant sources is so great that thousands of substances of plant origin are now being studied for activity against such formidable foes as h eart di seases, cancer, diabetes and AIDS. This t ype of study i s s ure t o bring f ruitful r esults, b ecause of t he f act t hat t he pl ant ki ngdom r epresents a vi rtually untapped reservoir of new chemical compounds and it has been estimated that only 5-15% of the approximately 2,50,000-5,00,000 species of higher plants of which more than 80,000 are medicinal ha s be en investigated pharmacologically. Thus there ar e cons iderable ch ances of finding new natural compounds with pharmacological activities, useful for the development of new drugs.

Scientists are now working together to find out new drug for incurable diseases. Taxonomist, Chemist, Biochemist, Pharmacologist and Pharmacist a re w orking unde r c ollaborative program for making a plant product(s) into a commercial drug.

1.2 Medicinal importance of plant materials

Plants not only provide man with food, shelter, and medicine, but also the sustaining oxygen. From anc ient t ime t o m odern a ge t he hum an ha s be en s uccessfully used pl ants a nd pl ant products a s e ffective t herapeutic t ools f or f ighting a gainst di seases a nd va rious he alth hazards.

Although with the advent of synthetic drugs the use and procurement of plant derived drugs have de clined t o a l arge e xtent, a l arge num ber of dr ugs of m odern m edicine a re obt ained

from plant sources. According to some generous estimates, almost 80% of the present day medicines are directly or indirectly obtained from plants[3].

As therapeutic use of plants continued with the progress of civilization and development of human know ledge, s cientists e ndeavored t o i solate di fferent chemical constituents f rom plants, put them to biological and pharmacological tests and thus have been able to identify and i solate t herapeutically a ctive c ompounds, which ha ve b een us ed t o pr epare m odern medicines. In course of time their synthetic analogues have also been prepared. In this way, the di scovery of vi ncristine w as don e f rom *Catharanthus roseus*, w hich i s, us ed i n t he treatment of cancer[4].
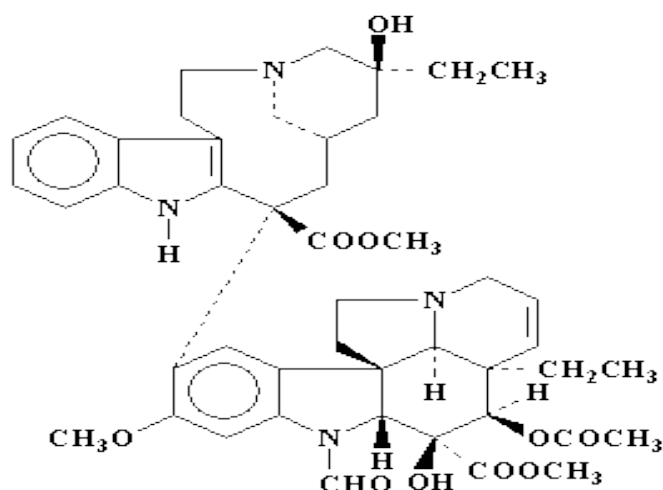
Fig: 1 Vincristine

Calanolide A is a r everse-transcriptase inhibitor isolated from the Malaysian rainforest tree, *Calophyllum Langerum* by the U S N CI. It h as exhibited synergistic a nti-HIV a ctivity in combination w ith nuc leoside r everse-transcriptase i nhibitor, i ncluding A ZT, d dI a nd ddC[5]. Medichem ph armaceuticals, Inc., and the s tate o f S arawak, Malaysia have begun c linical development of Calanolide A as a potential treatment for AIDS and HIV infections.
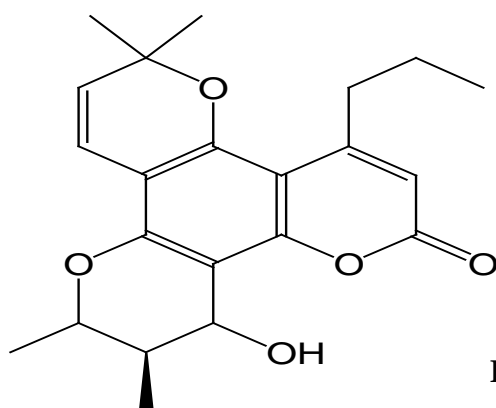
Fig: 2 Calanolide A

Ancient Chinese medical texts written 2000 years ago describe the herb *Artemisia annua* as a remedy for malaria. From this indication, in the late 1960s Chinese researchers initiated evaluation of various extracts of this herb. Bioassay guided isolation yielded the new anti-malarial compound artemisinin which is effective in treating chloroquine resistant cases and other severe cases without major toxicity.
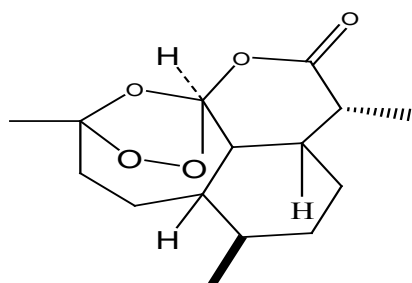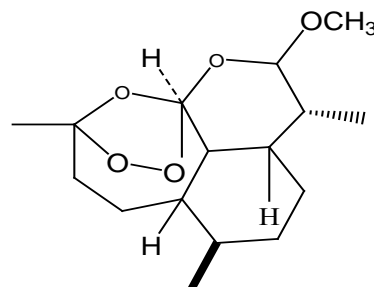
Fig: 3 Artemisinin   **Fig: 4** Artem ether

Artemether, a synthetic analogue of artemisinin has been developed in the People's Republic of China. Two recent clinical studies suggested that artemether is as effective as quinine in the treatment of severe malaria.

For several centuries, elderly people in some parts of Mainland China have brewed tea from the leaves of the club moss (*Huperzia serrata*) for improvement of their memory. In the early 1980s, Chinese scientist isolated huperzine from the plant Club moss as a potent, reversible and selective inhibitor of acetycholinesterase. A total synthesis has been developed due to very low levels in nature, and the product is found to be a promising candidate for the treatment of cholinergic related neurodegenerative disorders such as Alzheimer's disease (AD). In a prospective, multicenter, double-blind trial with 103 patients, huperzine A was found to be safe and superior to placebo and induced improvement in memory cognition and behavior in about 58% of patients with AD.
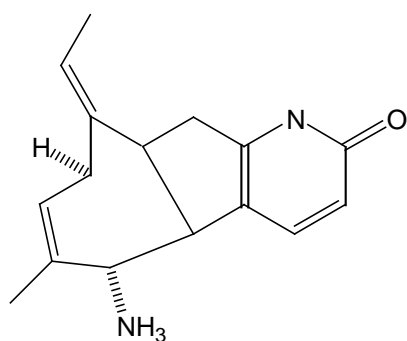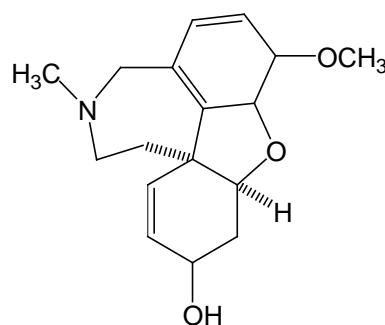
Fig: 5 Huperzine   **Fig: 6** Galathmine

Galanthamine is a long acting, centrally active competitive cholinesterase inhibitor; a natural product i solated from *Galanthus nivalis* in t he 1950s . G alanthamine under t he na me o f Nivalein is marketed in Austria for AD and in Germany for other indication such as facial neuralgia[6].

In the antidiabetes area, the past decade has witnessed the market introduction of several α-glucosidase inhibitors derived from natural products. A carbose, a c omplex ol igosaccharide one of them, was isolated from *Actinoplanes* sp. At Bayer from a s earch for α-glucosidase enzyme inhibitors. B y i nhibiting α-glucosidase, acar bose de creases t he r elease of g lucose from i ngested c arbohydrate a nd s lows t he i ncrease of f ood-induced bl ood g lucose l evels. Acarbose is now approved in Germany, Japan, the US and other countries and has been used as adjuvant therapy in diabetes.
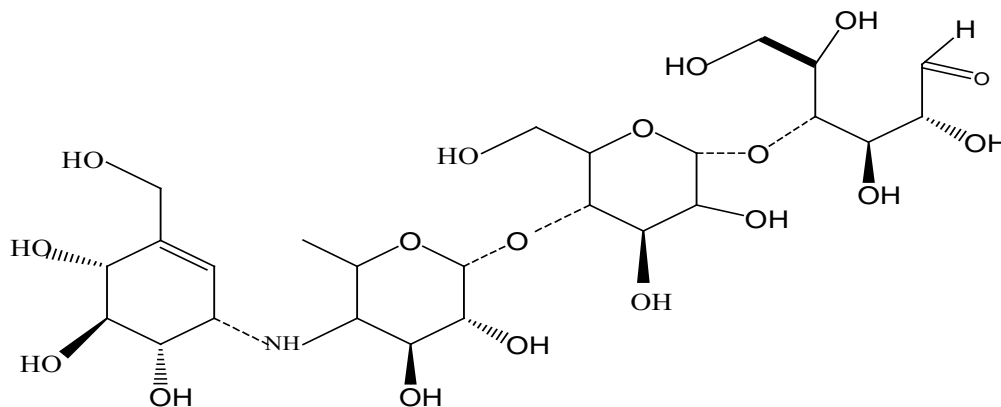
Fig: 7 Acarbose

Forskolin (Colforsin) i s a di terpene na tural pr oduct i solated f rom t he Indian pl ant *Coleus forskohlii* at H oechsts research l abs i n India a nd ha ve bl ood pr essure l owering a nd cardioactive pr operties. L ater F orskolin w as found a s a pot ent a denylate c yclase a ctivator. Colforsin da proate (NHK-477) i s a s emisynthetic pr oduct o f f orskolin derivative and w as then brought into phase III clinical trials in Japan for treatment of cardiac insufficiency and phase II trials for treatment of asthma.
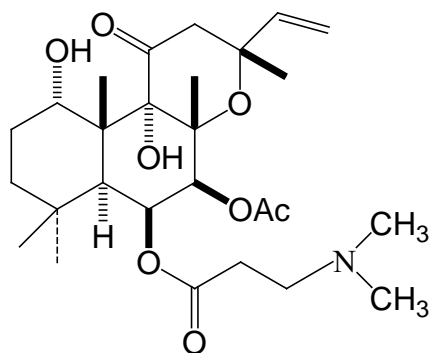
Fig: 8 Colforsin daproate
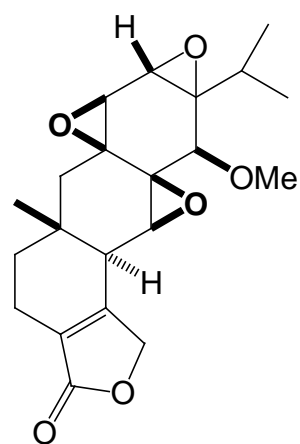
Fig: 9 Triptolide

Triptolide (**9**) is an active component isolated from the Chinese plant *Tripterygium wilfordii*, a plant traditionally used for the treatment of rheumatoid arthritis. Variety of formulations were developed in Mainland China and are shown to be effective in the treatment of inflammatory and autoimmune diseases. Triptolide was demonstrated to significantly inhibit arthritis in animal modal and have potent cytotoxicity.

The Chinese tree *Ginkgo biloba* has been used therapeutically for thousands of years. More recently, extracts of the leaves have become available in many European countries as over-the counter products for the treatment of cerebral vascular insufficiency and tinnitus. Ginkgolides, a class of unique diterpene cage like molecules were isolated from the leaves of *Ginkgo biloba* and represent a group of highly selective platelet activity factor (PAF) receptor antagonists. Among them, Ginkgolide B (BN-52021, **10**) has been advanced to phase III clinical trials for the treatment of septic shock in patients with severe sepsis caused by Gram positive bacterial infections and also good result in inflammatory and autoimmune disorders[7].
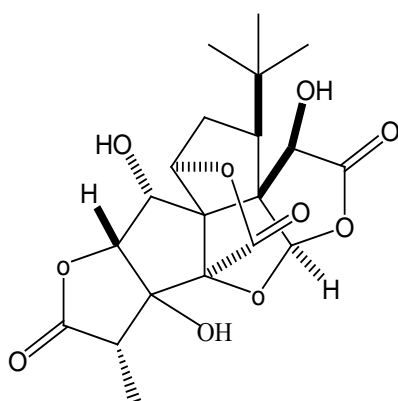
Fig:10 Ginkgolide B
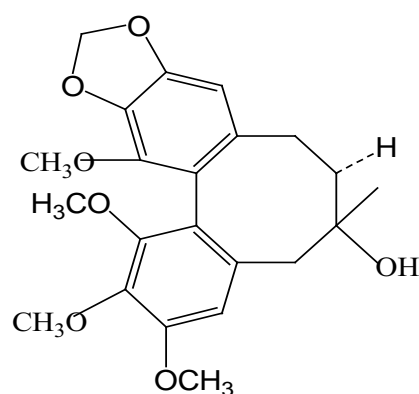
Fig: 11 Gomisin A

Gomisin A (**11**) is a lignin derivative isolated from the dry fruit of *Schisondra chinensis*, a traditional Chinese medicine used for the treatment of liver intoxication. Gomisin A was found to be hepatoprotectant and protect liver damage in various animal models.
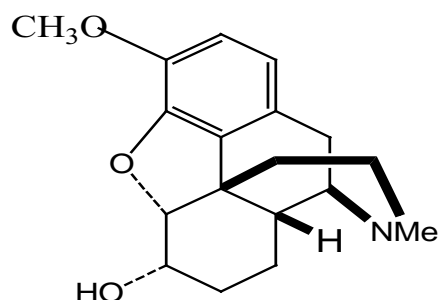
Fig: 12 Dextromethorphan

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

Two active anti-tumor agents etoposide and teniposide was isolated from the root of various species of the genus *Podophyllum*. These plants possess a long history of medicinal use by early American and Asian cultures, including the treatment of skin cancer and wart.

Camptothecin $R_1 = R_2 = R_3 = H$;

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

R = Me; Etoposide

R = ; Teniposide
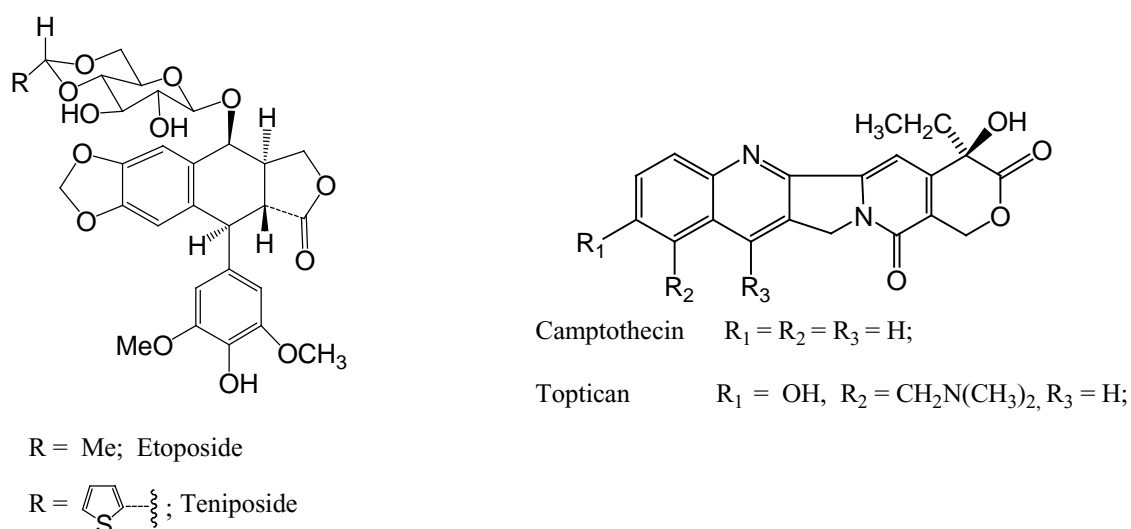
Fig: 13 Camptothecin and Toptican

Camptothectin was isolated from the Chinese ornamental tree *Camptotheca acuminata* by Wani and Wall. It was advanced to clinical trial by NCI in the 1970s but was dropped because of severe bladder toxicity. But toptican is a modified camptothecin was approved for

use in the USA in 1996. The discovery of quinine was done from *Cinchona* bark, which is, used in the treatment of malaria by French scientist Caventon and Pelletier[8].
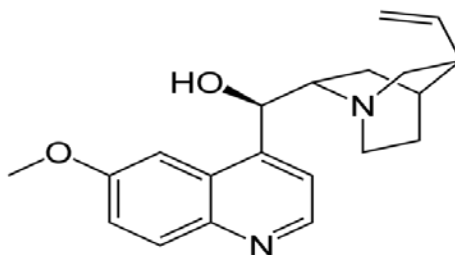
Fig: 14 Quinine

Uses of folk or traditional medicine represent the way of shortcut discovery of modern medicine. An inventory of medicinal plants compiled by WHO on the basis of literature from 91 countries including the classical text on Ayurvedic and Unani medicine list 21000 species of "medicinal plants". According to WHO, around 80% of the world's 5.76 billion populations in the developing world rely on herbal remedies for their basic health care need. The use of medicinal plants as a source for relief from illness can be traced back over five millennia from written documents of the early civilizations in China, India and near east but it is doubtless an art as old as mankind. Even today, plants are the almost exclusive source of drugs for the majority of the world's population. In industrialized countries, medicinal plant research has had its ups and downs during the last decades.

Plants will continue to be extremely important as source of new drugs as evidenced by recent approvals in the United States of several new plant derived drugs based on the secondary metabolites of plants. For example, in the treatment of refractory ovarian cancer, new drug has recently been approved in the United States from taxol, an anti cancer taxane diterpenoid derived from the relativity scarce pacific western Yew tree, *Taxus brevifolia* Nutt. A relatively new semi-synthetic antineoplastic agent based on podophylotoxin is etoposide a constituent of the Mayapple *Podophyllum petatum*, which is useful in the chemotherapeutic treatment of refractory testicular carcinomas, small cell lung carcinomas, non-Hodgkin's lymphoma and non lymphocytic leukemia.

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

Table 1.1: Important drugs/chemicals from plant source and their actions/chemical uses.

Drug/Chemical	Action/Clinical use	Plant source
Betulinic acid	Anticancerous	*Betula alba*
Camptothecin	Anticancerous	*Camptotheca acuminata*
Chymopapain	Proteolytic, mucolytic	*Carica papaya*
Cissampeline	Skeletal muscle relaxant	*Cissampelos pareira*
Colchiceine amide	Antitumor agent	*Colchicum autumnale*
Colchicine	Antitumor agent, anti-gout	*Colchicum autumnale*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*
Danthron	Laxative	*Cassia species*
L-Dopa	Anti-parkinsonism	*Mucuna sp*
Etoposide	Antitumor agent	*Podophyllum peltatum*
Glaucarubin	Amoebicide	*Simarouba glauca*
Glycyrrhizin	Sweetener, Addison's disease	*Glycyrrhiza glabra*
Hesperidin	Capillary fragility	*Citrus species*

Drug/Chemical	Action/Clinical use	Plant source
Irinotecan	Anticancer, antitumor agent	*Camptotheca acuminata*
Lapachol	Anticancer, antitumor	*Tabebuia sp.*
Menthol	Rubefacient	*Mentha species*
Papain	Proteolytic, mucolytic	*Carica papaya*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Podophyllotoxin	Antitumor anticancer agent	*Podophyllum peltatum*
Quinidine	Antiarrhythmic	*Cinchona ledgeriana*
Quinine	Antimalarial, antipyretic	*Cinchona ledgeriana*
Rutin	Capillary fragility	*Citrus species*
Sennosides A, B	Laxative	*Cassia species*
Stevioside	Sweetner	*Stevia rebaudiana*
Taxol	Antitumor agent	*Taxus brevifolia*
Teniposide	Antitumor agent	*Podophyllum peltatum*
a-Tetrahydrocannabinol (THC)	Antiemetic, decrease occular tension	*Cannabis sativa*
Theobromine	Diuretic, vasodilator	*Theobroma cacao*

Drug/Chemical	Action/Clinical use	Plant source
Theophylline	Diuretic, brochodilator	*Theobroma cacao*
Topotecan	Antitumor, anticancer agent	*Camptotheca acuminata*
Trichosanthin	Abortifacient	*Trichosanthes kirilowii*
Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum*
Vasicine	Cerebral stimulant	*Vinca minor*
Vinblastine	Antitumor, Antileukemic agent	*Catharanthus roseus*
Vincristine	Antitumor, Antileukemic agent	*Catharanthus roseus*

1.3 Status of medicinal plants in Bangladesh

The number of medicinal herbs included in the *Materia Medica* of traditional medicine in this subcontinent at present stands at about 2000. About 450 to 500 of such medicinal herbs have so far been enlisted as growing or available in Bangladesh. Almost 80 % of rural population is dependent on medicinal plants for their primary health care. Herbal medicine is widely used in Bangladesh in traditional healthcare system such as Ayurvedic, Unani, Hekimi and other form of folk treatments.

1.4   Description of the Family Acanthaceae

The Acanthaceae are mostly herbs or shrubs comprising about 250 genera and 2,500 species, including twining forms. The leaves are simple, opposite and decussate; stipules are lacking. The flowers are bisexual, zygomorphic and usually are associated with conspicuous, often brightly colored bracts. The calyx is usually deeply 4-5 lobed or sometimes is highly reduced with more numerous minute teeth. The corolla is sympetalous, usually 5-merous, mostly,

zygomorphic and commonly 2 lipped. The androecium usually consists of 4 di dynamous stamens or only 2 stamens adnate to the corolla tube or epigynous zone, alternate with the lobes. The gynoecium consists of a single compound pistil of 2 carpels, a single style and a superior ovary with 2 locules, each with usually 2-10 axial ovules in one or two collateral vertical tiers. An annular nectar disk is usually found around the base of the ovary. The fruit is commonly an elastically dehiscent loculicidal capsule. The seed stalk or funiculus of each seed is modified into a hook shaped jaculator or retinaculum that functions in flinging out the seeds during dehiscence.

1.5 Description of *Andrographis peniculata*

Fig: 15 Morphology of *Andrugraphis peniculata*

1.5.1 Scientific classification

Kingdom   : Plantae

Division   : Angiosperms

Class   : Eudicots

Sub-class   : Asterids

Order   : Lamiales

Family   : Acanthaceae

Genus   : *Andrographis*

Species: *Andrographis peniculata* (Burm.f.)

Binomial name: *Andrographis peniculata*

Other names: Kalmegh (both Bengali and in Hindi), King of bitters (English).

1.6 General Description and Chemical Investigation of *Andrographis peniculata*

King of Bitters, also called Andrographis, is a traditional Chinese, Southeast Asian and Indian herb, and used for centuries in Ayurvedic medicine. The herb has been revered for treating infectious diseases and highly regarded also as having a preventative effect from many diseases, due to its powerful immune strengthening benefits. The global flu epidemic of 1919 was one of the most devastating infectious out breaks in world history, killing millions worldwide, in many countries. However, in India, the amazing prophylactic benefits of Andrographis were credited with stopping the deadly virus. It is a potent stimulator of the immune system by two direct ways:

(1) Antigen- specific response: antibodies are made to counteract invading microbes,

(2) Non-specific immune response: macrophage cells scavenger and destroy invaders. King of B itters activates bot h r esponses, making it e ffective against a va riety of inf ections a nd oncogenic, cancer-causing agents.

Constituents: flavonoids, diterpenoid, lactones (Andrographolides), pa niculides, farnesols, polyphenols, a rabinogalactan, pr otein. Aaccording to the lite rature s urvey the al ready isolated compounds are as follows:
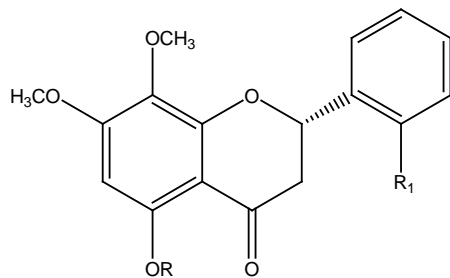
Fig: 16 5-hydroxy-7,8-dimethoxyflavone **Fig: 17** 5-hydroxy-2•,7,8-trymethoxyflavone
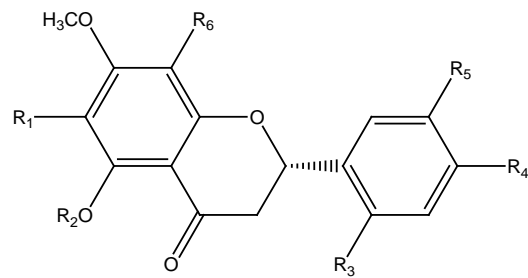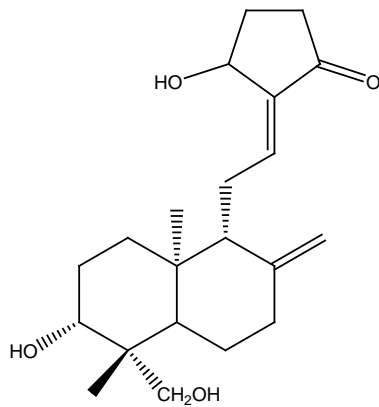
Fig: 18 Andrographolide **Fig: 19** Neoandrographolide
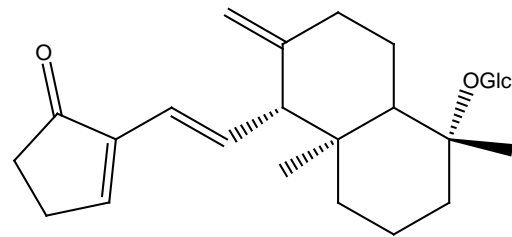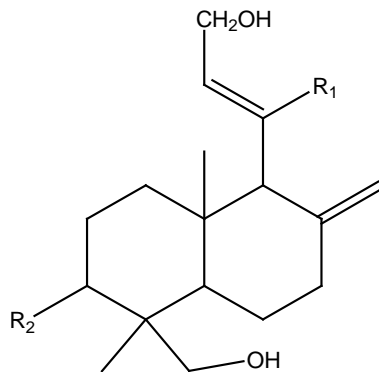
Fig: 20 8(17),13-*ent*-labdadiene-15,16,19-triol **Fig: 21** 3,12,19-trihydroxy-13,14,15,16-
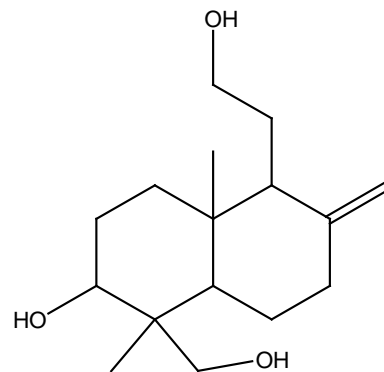
tetranor-*ent*-labd-8(17-ene)

Actions: Adaptogen (helps to normalise a physical function, depending on what the individual needs, e.g. it will lower high blood pressure, but raise low blood pressure; Antibacterial (fights bacterial activity); Antibiotic; Analgesic (pain reliever); Anti-inflammatory (reduces swelling); Antioxidant (helps in protecting the body from free radical damage); Anti-diabetic; Anti-acne (protects skin from pimples); Anti-carcinogenic (activity against different types of cancer, and leukemia; Anti-thrombotic (blood clot preventative); Anti-viral (inhibits viral activity); Anti-microbial (significant activity in fighting the common cold, flu, respiratory infections); Antiperiodic (counteracts periodic/intermittent diseases such as malaria); Antipyretic (reduces fever, usually caused by multiple infections or toxins); Bitter tonic; Blood purifier; Cardio-protective (protects heart muscles); Choleretic (alters the properties and flow of bile); Digestive (promotes digestion); Depurative (acts to clean and purify the body, particularly the blood); Expectorant (promotes mucus discharge from respiratory system); Hepato-protective (helps to protect liver and gall bladder functions); Hypoglycemic (blood sugar reducer); Immuno-stimulant; Laxative; Prophylactic (helps prevent disease); Sedative; Thrombolytic (blood clot buster); Vermicidal (used to kill intestinal worms and helps support the intestines).

1.7 Medicinal Importance of *Andrgraphis peniculata:*

Nowadays, research on medicinal plants has attracted a lot of attention globally. A number of evidence has been accumulated to demonstrate promising potential of medicinal plants used in various traditional, complementary, and alternative systems. In recent years, a medicinal plant, *Andrographis peniculata,* and its major active phytochemicals have been extensively studied for several pharmacological activities. To understand the mechanism of action, researches have to be carried out at molecular levels. The present review aims at compiling consequential compendium of pharmacological benefits of health on this plant and its major diterpenoid constituent andrographolide that have been tested in various experimental models using modern scientific methodologies.

An herb is a plant or plant part used for its scent, flavor, or therapeutic properties, and medicinal products made of them are frequently taken to improve health as dietary supplements. Herbs are usually eaten for a long time in combinations, in relatively large, unmeasured quantities under folklore remedies. Therefore, the real challenge lies not in proving whether herbs have health benefits, but in defining what these benefits are and developing the methods to expose them by scientific means.[9]

Andrographis peniculata (Burm.f.) Nees is an herbaceous plant, commonly known as "King of Bitters," in the family Acanthaceae. It is widely cultivated in southern Asia. Mostly the leaves and roots have been traditionally used over the centuries for different medicinal purposes in Asia and Europe as a folklore remedy for a wide spectrum of ailments or as an herbal supplement for health promotion (**Table 1**). The *Indian Pharmacopoeia* narrates that it is a predominant constituent of at least 26 Ayurvedic formulations.[10,11] In traditional Chinese medicine, it is an important "cold property" herb used to rid the body of heat, as in fevers, and to dispel toxins from the body.[12] In Scandinavian countries, it is commonly used to prevent and treat the common cold.[13] In Thailand, this plant was selected by the Ministry of Public Health as one of the medicinal plants to be included in "The National List of Essential Drugs A.D. 1999" (List of Herbal Medicinal Products).[14] Extensive research has revealed that *Andrographis peniculata* has a surprisingly broad range of pharmacological effects and some of them are extremely beneficial, such as antiinflaniinatory,[15] antidiarrhoeal, antiviral, anti-malarial,[16] hepatoprotective,[17-29] cardiovascular,[30,31] anticancer,[32-39] and immunostimulatory[40-44] activities. On the other hand, male reproductive toxicity[45] and cytotoxicity[46] of this plant have been reported as well.

1.8 Morphology, Chemistry and Biotransformation

Andrographis peniculata is an annual herb, extremely bitter in taste in every part of the plant body. It grows erect to a height of 30-1l0 cm in moist shady places with glabrous leaves and white flowers with rose-purple spots oil the petals. The stem is dark green, 0.3-1.0 in height, 2–6 mn in diameter, quadrangular with longitudinal furrows and wings on the angles of the younger pails, slightly enlarged at the nodes; leaves glabrous, up to 8.0 cm long and 2.5 cm broad, lanceolate, pinnate; flowers small, in lax spreading axillary and terminal racemes or panicles; capsules linear-oblong, acute at both ends, 1.9 cm x 0.3 cm; seeds numerous, sub quadrate, yellowish brown ( ). It grows abundantly in southeastern Asia. i.e., India, Sri Lanka, Pakistan, Java, Malaysia, and Indonesia, while it cultivated extensively in India, China, and Thailand[47].

The aerial parts of the plant (leaves and stems) are used to extract the active phytochemicals. Previous investigations oil the chemical composition of *Andrographis*

peniculata showed that it is a rich source of diterpenoid and $2^/$-oxygenated flavonoids including a ndrographolide **(fig:18)**, neoandrographolide **(fig:19)**, 14 -deoxy- 11, 12 - didehydroandrographolide. 14 -deoxyandrographolide, i soandrographolide, a nd 14 - deoxyandrographolide 19 •-Dglucoside, hom oandrographolide, a ndrographan, andrographosterin, a nd s tigmasterol.[48-49] The pr imary bioactive c omponent of t he medicinal p lant *Andrographis peniculata is* andrographolide.[50-51] Andrographolide [$C_{20}H_{30}O_5$; (3-2-{decahydro-6-hydroxy-5-(hydroxyinethyl)-5,8•-dimethyl-2-methylene-1-napthalenyl}ethylidene] di hydro-4-hydroxy 2(3H)-furanone); is a colorless cr ystalline b icyclic d iterpenoid l actone an d h as a v ery b itter t aste.[52] It presents in all parts of the plant, maximally in the leaves (> 2%).

When or ally c onsumed, a ndrographolide a ppears t o a ccumulate i n or gans t hroughout the viscera. Pharmacokinetic studies showed that andrographolide is quickly absorbed and ex tensively metabolized in rats and hunians.[53] Ninety percent is eliminated within 48 hr. Andrographolide metabolites are mainly identified as sulfonic acid adducts and sulfate c ompounds, a s w ell a s g lucuronide c onjugations. T en m etabolites of andrographolide a s s ulfonates, s ulfate e ster c ompounds, a nd a ndrographolide analogues were i solated f rom r at u rine, f eces, an d t he co ntents o f t he s mall i ntestine after t he d rug w as o rally a dministrated to r ats.[54-56] While th ose me tabolites is olated from h uman u rine were as s ulfates, cy steine S -conjugate, a nd g lucuronide conjugates.[57] One of t he m etabolites. 14 -deoxy-12(R)-sulfo-andrographolide (M-1 12R, was r eported to be id entical to the antiinflammatory drug. Lian-bi-zhi, which is being clinically used in China.[58]

1.9 Pharmacological Potential
1.9.1 Hepatoprotective Activity

In A yurvedic m edicine, t here ar e 2 6 d ifferent r emedies co ntaining *Andrographis peniculata* used t o t reat l iver di sorders. A dministration of *Andrographis peniculata* prevented he xachlorocyclohexane i nduced i ncrease i n the a ctivities o f glutamyl transpeptidase. g lutathione-S-transferase a nd l ipid pe roxidation i n m ouse l iver, a n indication of pot ential a ntioxidant a nd he patoprotective e ffects of *Andrographis puniculata*.[59] Leaf ex tract o f *Andrographis peniculata* and a ndrographolide w ere

found to be effective in preventing carbon tetrachloride induced liver damage in rats and mice. [60]

Andrographolide also showed significant hepatoprotective effect against various types of liver damage induced by galactosamine,[61,62] paracetamol,[63] and had a higher capability than a classical antioxidant silymarin in preventing a decrease of bile production induced by paracetamol.[64] Andrographolide played a hepatoprotective role by reducing a lipid peroxidation product malondialdehyde (MDA), in which the lowering of MDA formation conveyed the free radical seas engine property of diterpene lactones of andrographolide, as well as by maintaining high level of glutathione, glutamicpyruvate transaininased and alkaline phosphatase in carbontetrachloride or *tart*-butylhydroperoxide treated mice.[65] Antihepatotoxic action of andrographolide against *Plasmodium berghei* K173-induced hepatic damage of *Mastomys natalensis* was noted.[66] In other studies, andrographolide was suggested to play a role as a potent stimulator of gall-bladder function by producing a significant increase in bile flow, bile salts, and bile acid in conscious rats and anaesthetized guinea pigs. There was marked improvement, *i.e.,* improvement of appetite and liver function tests, gradual recovery from jaundice, subsidence of fever, in the majority of infective hepatitis patients after continuous treatment with *Andrographis peniculata.*[67]

1.9.2 Immunological Potential

Recent research has excitingly indicated that extracts of *Andrographis peniculata* may have the potential for interfering with the viability of the human immuno-deficiency virus (HIV) and advised that *Andrographis peniculata* could combine with modern medicines against acquired immuno-deficiency syndromes (AIDS).[68] *Andrographis peniculata* contains substances, one of which is andrographolide, which interrupted or modified the cellular signal transduction pathway of the virus, resulting in interfering the key enzymes and viral reproduction consequently.[69,70] It was proposed as a potent stimulator of immune system by two approaches. The first was an antigen-specific response; antibodies were made to counteract invading microbes and the second was a nonspecific immune response: macrophage cells scavenged and destroyed invaders. Since *Andrographis peniculata* activated both responses, it may be effective against a variety of infectious and oncogenic agents.

Andrographolide e xhibited pr o-hormone/ pr o-protein convertase (PC) en zyme inhibition a gainst furin, though t he a ctivity was r elatively weak, c ompared t o t hat of its succinoylated derivatives. The findings suggested a specific structural modification of the andrographolide skeleton by virtue of the protease inhibitory property, possibly acted by suppressing the proteolytic cleavage of envelope glycoprotein gp160 of HIV, which i s know n t o be P C-mediated, p articularly by f urin.[71] Furthermore. andrographolide ha s be en r eported t o pos sess a n i nhibitory effect o n HIV r eplication and i nterference w ith H IV-induced c ell f usion.[72,73] Andrographolide pr omoted interferon (IFN), tumor ne crosis f actor (TNF) inductions of pe ripheral bl ood mononuclear cells (PBMCs), enhanced phagocytosis activity of peritoneal macrophage from g uinea pi g t o pha gocytosis c ock e rythrocyte, a s w ell a s a ugmented t he cytotoxicity mediated by n atural k iller c ells from P BMCs to d amage th e K 562 cell lines. T hese obs ervations s uggested a ndrographolide a s a n i mmunostimulant a gent which c ould m odulate both a ntigen s pecific a nd nons pecific i mmune f unction b y means of i ts na tural ki ller c ells a nd m acrophage a nd c ytokines i nduction.[74] Besides anti-HIV a ctivity, a ndrographolide s howed vi ricidal a ctivity against he rpes s implex virus 1.[75]

A phase I dose-escalating clinical trial conducted in 13 HIV positive patients showed a significant r ise in the m ean C D4 (+) l ymphocyte le vel b ut w ith n o s tatistically significant ch anges i n mean p lasma H IV-1 R NA l evels o f H IV-1 i nfected s ubjects after administration of the regimen (andrographolide at a dose of 5 mg/kg for 3 weeks, escalating to th at o f 1 0 mg /kg for 3 w eeks a nd t o (hat of 2 0 m g/kg f or a f inal 3 weeks). T he f indings not ed t hat a ndrographolide m ay i nhibit H IV-induced cel l c ycle dysregulation, l eading t o a rise i n CD4 (+) l ymphocyte le vels in HIV-1 i nfected individuals. [76]

Andrographolide t reatment i nhibited t he i n vi tro pr oliferation of di fferent hum an cancer an d i mmune c ell l ines b y ex erting d irect an ticancer activity at t he G O/G I phase o f c ell c ycle a rrest t hrough i nduction of i nhibitory protein p 27 a nd de creased expression of c yclin-dependent ki nase 4 (CDK4). Immunostimulatory a ctivity of andrographolide w as e videnced b y i ncreased pr oliferation of l ymphocytes a nd production of i nterleukin-2 (IL-2).[77] Likewise, *Andrographis peniculata* extract an d andrographolide w as f ound t o s ignificantly inhibit g rowth of hum an a cute m yeloid

leukemic cells by inducing cell cycle arrest and affecting an intrinsic mitochondria-dependent pathway of apoptosis by regulating expression of some pro-apoptotic markers, namely causing chromosomal DNA fragmentations, an occurrence of apoptosis, induction of Bcl2-associated X protein (Bax) expression, and decrease of B-cell lymphoma 2 (Bel-2) proteins in the inhibited cells.[78] Moreover, induction of cell cycle arrest at G2/M phase and late apoptosis of the cells with the collapse of mitochondria) membrane potential and an intracellular increase of hydrogen peroxide but a decrease of superoxide radicals and reduction of glutathione have been reported as well. The cytotoxic effect of andrographolide on HepG2 cells was suggested to be the primary attribute to the induction of cell cycle arrest via the alteration of cellular red-ox status.[79] In addition, Andrographolide activated apoptosis in human cancer cell lines via activation of caspase-3 and caspase-8 with the participation of mitochondria has been noted.[80,81] Recently, Andrographolide was reported to possess cytotoxicity to a human oral epidermis carcinoma cell line.[82]

Andrographolide also enhanced production of TNF and CD marker expression, resulting in increase of cytotoxic activity of lymphocytes against cancer cells, which may contribute for its indirect anticancer activity. Andrographolide also showed *in vivo* anticancer activity against B16FO melanoma syngenic and HT-29 xenograft models.[83] Another study, an extract of *Andrographis peniculata* significantly inhibited the proliferation of HT-29 colon cancer cells and augmented the proliferation of human peripheral blood lymphocytes (HPBLs).[84]

An ethanolic extract of *Andrographis peniculata* and andrographolide showed promising immunostimulant activity.[85] Andrographolide reciprocally showed anticancer and immunostimulatory activities on diverse cancer cells representing different types of human cancers by enhanced proliferation and IL-2 induction in HPBLs. Furthermore, andrographolide demonstrated potent cell differentiation-inducing activity toward mouse myeloid leukemia cells[86] and significantly enhanced natural killer cell activity in normal and tumor-bearing animals; antibody-dependent cellular cytotoxicity, mitogen-induced proliferation of tumor-bearing animals; antibody-dependent cellular cytotoxicity, mitogen-induced proliferation of splenocyte, thymocyte and bone marrow cells were increased with significant elevation of

production of I L-2 a nd i nterferon in n ormal an d E hrlich as cites car cinoma-bearing animals.[87]

Besides c ausing cancer c ell m aturity o r d ifferentiation, a ndrographolide s howed selective c ytotoxicity to p rostate can cer P C-3 c ells b y i nducing c ell de ath t hrough activation of an extrinsic caspase cascade of the apoptotic pathway comparable to that of t he w idely used a nd hi ghly t oxic dr ug, cisplatin.[88] Further, a ndrographolide w as able t o ef ficiently block T cel l act ivation b y completely ab olishing a nd in terfering with t he m aturation of dendritic c ells, as w ell as by drastically d iminishing a ntibody response to a thymus-dependent an tigen and delayed t ype h ypersensitivity in mic e, a feature that could be useful for interfering with detrimental T cell responses.[89]

1.9.3 Anti-inflammatory Activity

Andrographis peniculata is also u sed as a f olk m edicinal r emedy f or f ever, p ain reduction, and disorders of the intestinal tract. The ability of *Andrographis peniculata* to l ower fever ha s be en de monstrated i ndependently in s everal r eports. It ha s s hown that a ndrographolide l owered t he f ever pr oduced b y di fferent f ever-inducing a cents, such as bacterial endotoxins, pneumococcus, hemolytic streptococcus, typhoid, paratyphoid a nd 2, 4-dinitrophenol. T he a nalgesic a ctivity of a ndrographolide w as weaker than aspirin while anti-pyretic activity was comparable to that of as pirin.[66,67]

Andrographolide a ttenuated t he T NF-induced intercellular a dhesion mo lecule-1 (ICAM-1) ex pression a nd a lso inhibited the T NF-induced e ndothelial-monocyte adhesion; t hose w ere ke y s teps i n the de velopment of i nflammation.[61] An a nti-inflammatory benefit of a ndrographolide by reduction of i nducible n itric o xide synthase (iNOS) p rotein e xpression t hrough pr evention of t he *de novo* protein synthesis and decreasing the protein stability via a post-transcriptional mechanism has been i nferred. Furthermore, a ndrographolide e xerted a nti-inflammatory e ffects by inhibiting nuc lear factor (NF)-$_K$B bi nding t o D NA, a nd t hus r educing t he expression of p ro-inflammatory p roteins, s uch a s c yclooxygenase2 (C OX-2).[69] Another s tudy showed w eak a nti-inflammatory effects of a ndrographolide c ompared t o ot her diterpenoid c onstituents i n t he pl ant, i n w hich t he a nti-inflammatory effect p robably worked b y a m echanism t hat i nvolves s ecreted m aterial f rom ad renal g lands. s ince

such effects disappeared in adrenalectoinized animals.[4] The anti-inflammatory effect of andrographolide has also been explained by its ability to inhibit neutrophil adhesion/transmigration through suppression of macrophage adhesion molecule-1 (Mac-1) up-regulation which could be mediated by down regulation of reactive oxygen species (ROS) production via a protein kinase C (PKC)-dependent niechanism.[90] The potent immunosuppressant effects in marine T-cells by significant reduction of IFN production and extracellular-signal-regulated protein kinase (ERKI/2) phosphorylation induced by concanavaline A has been mentioned. The ability of andrographolide to inhibit T cell activation was applied to interfere with the onset of Experimental Autoimmune Encephalomyelitis (EAE), an inflammatory demyelinating disease of the central nervous system that is primarily mediated by CD4(+) T cells and serves as an animal model for human multiple sclerosis. Treatment with andrographolide was able to significantly reduce EAE symptoms in mice by inhibiting T cell and antibody responses directed to myelin antigens.[91]

In addition, andrographolide significantly inhibited carrageenin-, kaolin- and nystatin-induced paw oedema. Moreover, it significantly inhibited the weight of granuloma induced by cotton pellet and decreased oedema in adjuvant-induced arthritis and also inhibited dye leakage in acetic acid-induced vascular permeability. It was devoid of L11-cerogenic effect oil the stomach of rats.[70] Presently, a novel formulation comprising of andrographolide, a diterpene lactone, and diterpene triepoxide lactones for synergistic inhibition of COX-2 has been achieved.[71]

Inflammation plays all important roles in the pathogenesis of several neurodegenerative diseases including Parkinson's disease. Andrographolide has been reported to possess all anti-inflammatory effect *in vitro* by modulating macrophage and neutrophil activity.

Treatment with andrographolide exhibited a significant protective effect against lipopolysaccharide-induced neurotoxicity in mixed neuron-glia cultures. These findings demonstrate that andrographolide reduced inflammation-mediated dopaminergic neurodegeneration in mesencephalic neuron-glia cultures by inhibiting microglial activation, thus indicating that andrographolide may have clinical utility for the treatment of inflammation-related neurodegenerative disorders such as Parkinson's disease.[72]

1.9.4 Respiratory System Benefits

Andrographis peniculata has been reviewed to be superior to placebo in alleviating the subjective symptoms of uncomplicated upper respiratory tract infection (URI) and being preliminary evidence of a prevalence effect.[73] There was reasonably strong evidence from clinical trials to suggest that *Andrographis peniculata was* effective in reducing the severity and the duration of URI when treatment was started within the first 36-48 hr symptoms.[74]

Prevention of common cold with *Andrographis peniculata* was studied in a pilot double-blind study. There was a significant decrease in prevalence and intensity of the symptoms in the uncomplicated common colds, compared to the placebo after the *Andrographis peniculata* regimen intakc.[75] The relative risk of catching a cold indicated that the preventive effect could be due to immunostimulatory effects of andrographolide. Moreover, *Andrographis peniculata* accelerated recuperation of common cold patients with symptoms including nasal discharge, nasal stuffiness, sore throat, earache, cough, fever, headache and malaisc.[75]

The mechanism of action of *Andrographis peniculata* in treatment of URI remains unclear to date. The apparent effectiveness of the plant may be based either on its anti-inflammatory properties or on its immunomodulatory properties. Further research is needed both to clarify whether *Andrographis peniculata* is as effective in treatment of URI and to clarify the mechanism through which this benefit is mediated.

1.9.5 Anti-malarial Activity

Malaria is still a prevalent disease in many tropical and subtropical countries. *Andrographis peniculata* was found to considerably inhibit the multiplication of *Plasmodium berghei.* The protective action of *Andrographis peniculata* is proposed to be due to reactivation of the key antioxidant enzyme superoxide dismutase. In dog, *Andrographis peniculata* extracts effectively killed filaria that obstructed lymph channels, consequently leading to elephantiasis. Another study also reported anti-malarial effect of *Andrographis peniculata* against *Plasmodium falciparum.*

1.9.6 Antidiarrhoeal and Intestinal Effects

Diarrhea is one of the top ten causes of death worldwide and is a leading cause of death in children in developing countries; especially under five years of age. Many modern drugs used to relieve the symptoms. i.e., kaolin-pectin, bismuth, loperamide, has undesirable side effects. It had been believed that *Andrographis peniculata* was effective against bacterial dysentery and diarrhea, but how it was accomplished has been unclear up to date. Extracts of *Andrographis peniculata* have been shown significant anti-diarrheal activity against *Escherichia coli* associated diarrhea, while andrographolide exhibited similar activity to loperamide the most common anti-diarrheal drug. In a double blind study, patients with acute diarrhea and bacillary dysentery responded favorably to *Andrographis peniculata.*[77]

1.9.7 Cardiovascular Activity

The effects of *Andrographis peniculata* in an atherosclerotic rabbit model showed increases of the nitric oxide, cyclic guanosine monophosphate, and activity of superoxide dimutase with declines of lipid peroxide and endothelin. These observations suggested the potential of *Andrographis peniculata* as an antioxidant to preserve endothelial function. resulting in maintenance of the balance of nitric oxidefendothelin.[78] In another study, Andrographis panic/data demonstrated an increase of blood clotting time, thus pre- and post-treatment with the extracts of *Andrographis peniculata* before angioplasty and after surgery significantly prevented constriction of blood vessels, resulting in decreasing risk of subsequent closing of blood vessels (restenosis) after angioplasty procedures.[78] The arterial narrowing caused by injury to the inner lining of the blood vessel and by high cholesterol in the diet was also found to be decreased by *Andrographis peniculata.*[79]

Besides the benefits on the surgery, it was reported that *Andrographis peniculata* given to dogs one hour after development of myocardial infraction decreased the damage of the heart muscle[80] and activated fibrinolysis. *Andrographis peniculata* additionally showed antihypertensive effects by relaxing the smooth muscle wall of the blood vessels, consequently resulting in lowering of blood pressure in noradrenaline-treated rats.[81] These observations hinted at the potential of *Andrographis peniculata as* a good option for cardiovascular therapy.

The car diovascular a ctivities o f a crude extract of *Andrographis peniculata* and andrographolide were elucidated in anaesthetized rats. The hypotensive effects of the *Andrographis peniculata* extract occurred in the absence of significant change in heart rate, i ndicating m ore contribution of t he hy potensive r esponse o n c ardiovascular activity than a di rect a ction on t he he art, a nd w ere f inally pr oposed t o b e m ediated through adrenoceptors, a utonomic g anglion a nd hi staminergic r eceptors. H owever, i t was c onsidered t hat a ndrographolide w as not t he h ypotensive a ctive c ompound of *Andrographis peniculata.*

1.9.8 Psycho-pharmacological Activity

Psycho-pharmacological s tudies w ere co nducted w ith an e xtract o f *Andrographis peniculata.* It was evident that the extract had a p otent central nervous system (CNS) depressant act ion as i ndicated b y its h ypnotic p otentiation e ffect; it p roduced hypothermia and exhibited an analgesic action against acetic acid-induced writhing, by the s ame m echanism as r eserpine an d ch lorpromazine.[82] Moreover, r eduction i n exploratory behaviors w ith th e e xtract w as in c onformity with s imilar a ctions produced by other CNS depressant drug[83] The extract also exhibited significant motor incoordination an d m uscle r elaxant act ivity. T hese f indings r evealed a potent C NS-depressant act ion of *Andrographis peniculata,* though i t c ould not pr edict i ts CNS category a t pr esent. T he e xtract a lso pr oduced a pr olongation of t he pe ntobarbitone-induced s leeping t ime a nd l owered t he body temperature i n di fferent e xperimental animal models.

1.9.9 Hypoglycemic Activity

A w ater ex tract o f *Andrographis peniculata* significantly prevented i nduction o f hyperglycemia induced by oral administration of glucose in rabbits, but it failed to do so i n a drenaline-induced h yperglycemia. A dditionally, l ong-term a dministration (6 weeks) o f *Andrographis peniculata was* incapable to demonstrate fasting bl ood s ugar lowering effect. Hence, might prevent glucose absorption from gut.[84]

1.9.10 Anti-fertility Activity

Andrographis peniculata possessed a nti-fertility and pregnancy-terminating e ffects and s topped s permatogenesis i n male r ats. N one o f t he f emale m ice t hat d aily consumed *Andrographis peniculata* mixed food became pregnant when mated with the male o f p otential f ertility who d id n ot r eceive th e tr eatment.[93] The observations suggested a n a nti-spermatogenic o r an ti-androgenic abilities a s w ell a s o vulation preventive e ffect of t he pl ant. H ence, us ing of t he he rb dur ing pr egnancy s hould be avoided. *Andrographis peniculata* and a ndrographolide p roved t o a ffect spermatogenesis i n rats b y preventing c ytokinesis o f th e d ividing s permatogenic c ell lines w ith ap pearances o f s ertoli cel l d amage an d a s permatotoxic e ffect. T he s tudy pointed to a male reproductive toxic effect of a therapeutic use of andrographolide and confirmed t he pos sible pr ospective us e of a ndrographolide a s a male co ntraceptive. Changes in the biochemical parameters in rats, such as significant decreases in protein content, but m arked i ncreases i n cholesterol, a cid phos phatase, a nd a lkaline phosphatase l evels w ith ap pearance o f f ructose i n the r eproductive system, s uggested anti-fertility effects of the andrographolide.[94]

1.10 Aim of the project

Bangladesh is a good repository of medicinal plants belonging to various families, including Acanthaceae. The Acanthaceous plants contain wide range of chemical and unique pharmacologically active compounds, including antiseptic, astringent, stomachic, anti-inflammatory, anti-rheumatic, anti-diarrhea and anti-emetic activities.

Though a large number of *Andrographis* species have been investigated but locally a little attention was given to this particular species. Therefore, an attempt has been taken to study the chemical constituents and biological activities of *Andrographis peniculata.*

These investigations may provide some interesting compounds, which may be pharmacologically active. If significant results are obtained, these can be used as remedies for the treatment of some diseases. Since this plant is available in Bangladesh and a lot of herbal health centers and herbal industries are using such related herbal plants for treatments, so if the biological activity of this plant can be studied thoroughly, this may be a cost-effective treatment. So, the objective of the study is isolation and structural elucidation of the bioactive compounds by chemical and spectroscopic methods (UV, FTIR, ^{1}H-NMR, ^{13}C-NMR, DEPT etc.) and to explore the possibility of developing new drug candidates from this plant for the treatment of various diseases.

1.11 Present study protocol

The present study was designed to isolate pure compounds as well as to observe biological activities of the isolated pure compounds with crude extract and their different fractions. The study protocol consisted of the following steps:

- ❖ Cold extraction of the powdered leaves of the plant with ethanol.
- ❖ Partition of the crude ethanol extract by n-hexane, ethyl acetate, 1-butanol respectively.
- ❖ Fractionation of each partitioned extract by vacuum liquid chromatography (VLC).
- ❖ Isolation and purification of the pure compounds from different column fractions by various chromatographic methods.
- ❖ Determination of the structure of the isolated compounds with the help of chemical and spectroscopic methods (UV, FTIR, 1H-NMR, 13C-NMR, DEPT etc.).
- ❖ Observation of *in vitro* antimicrobial activity of crude extracts, column fractions and pure compounds.
- ❖ Observation of antioxidant property of crude extracts, column fractions and pure compounds.

Chapter Two

EXPERIMENTAL

EXPERIMENTALS

2.0 General methods

The following sections of this chapter are a brief description of the various method followed in extraction, fractionation & purification of the compounds in the course of experimental works.

2.1 Solvents and chemicals

Analytical or laboratory grade solvents and chemicals had used in the experiments. All solvents and reagents used in the experiments were produced from E. Merck (Germany), BDH (England). The commercial grade solvents (ethyl acetate, chloroform, methanol, ethanol, n-hexane, DMSO, dichloromethane and 1-butanol) were distilled before use.

2.2 Distillation of the solvents

The analytical grade solvents (ethyl acetate, chloroform, methanol, ethanol, n-hexane, DMSO, dichloromethane and 1-butanol) were distilled. Distilled solvents were used throughout the investigation.

Fig 2.1: Distillation process.

2.3 Evaporation

All evaporations were carried out under reduced pressure using a rotary evaporator at a bath temperature not more than 40^0C. The residual solvent in the extract and compounds were removed under high vacuum.

Fig 2.2: Rotary vacuum evaporator

2.4 Preparation of the reagents

Fig 2.3: Spray reagent

2.4.1 Spray reagent (Developing reagent)

1 ml anisaldehyde

20 ml acetic acid (glacial)

10 ml sulfuric acid

170 ml methanol

2.5 Chromatographic techniques

Three types of chromatographic techniques such as Thin Layer Chromatography (TLC) and Column Chromatography (CC) & Vacuum Layer Chromatography (VLC) were used.

2.5.1 Thin layer chromatography (TLC)

Pre-coated 0.2 mm thin coatings TLC plates on aluminum sheets were used throughout the experiment.

2.5.2 Sample application

The TLC plates were spotted with a small amount of the crude extract by using a narrow glass capillary. The capillary was washed with acetone before each sample applied.
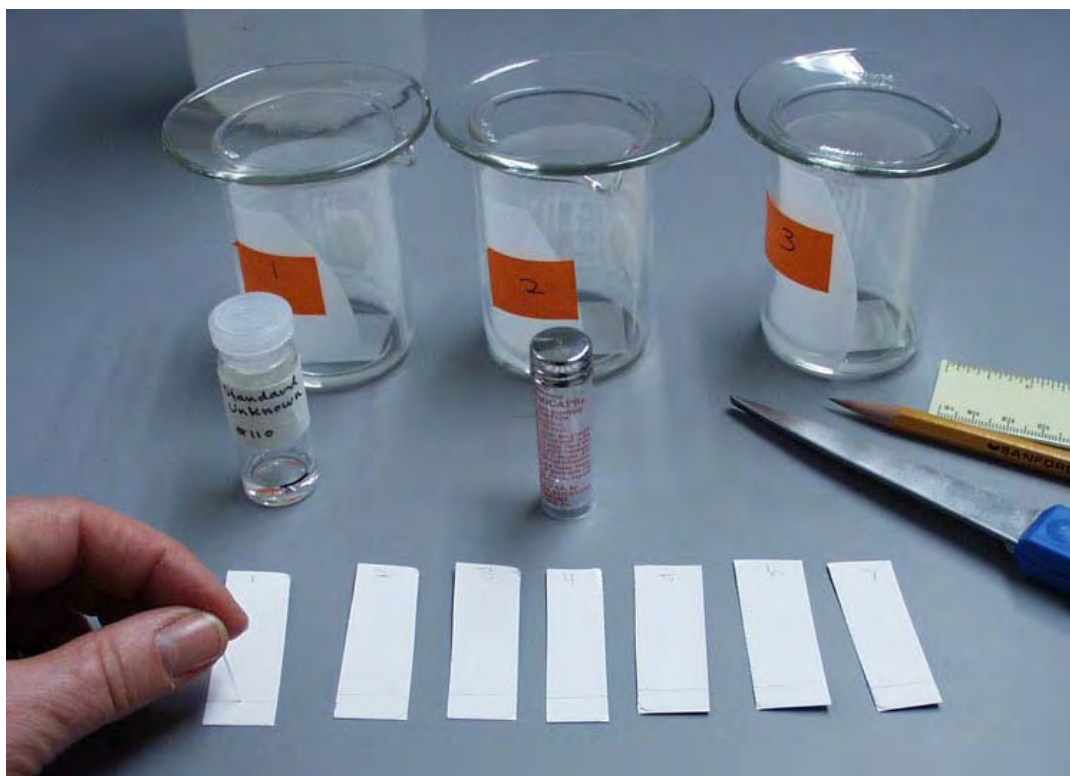
Fig 2.4: Process of spotting

2.5.3 Solvent system

The solvents of different polarity used for TLC are given below:

n-hexane : Ethyl acetate (in different ratios)

Ethyl acetate : Methanol (in different ratios)

Chloroform: Methanol (in different ratios)

n-hexane: Chloroform (in different ratios)

Ethyl acetate : Dichloromethane (in different ratios)

Dichloromethane : Methanol (in different ratios)
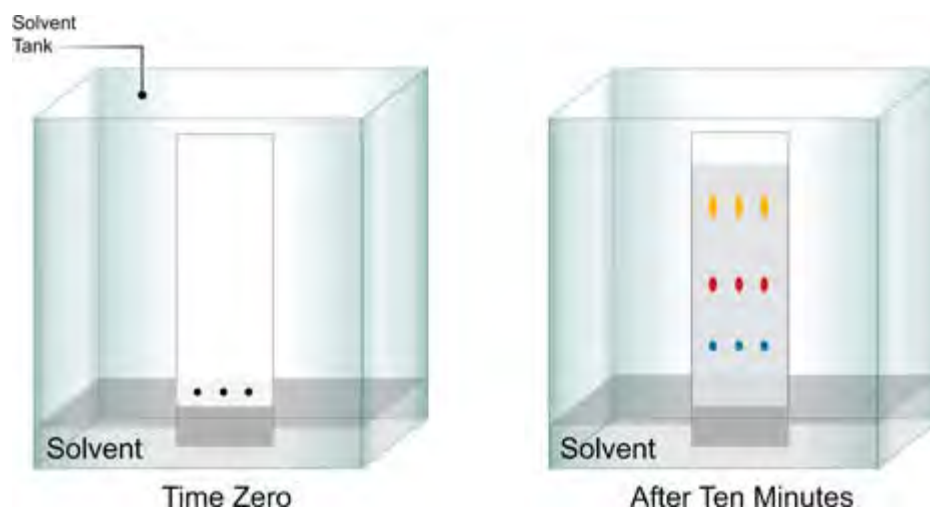
Fig 2.5: Developing of TLC plate

2.5.4 Preparation of TLC tanks

The ascending technique in glass jars & glass tanks were used to develop TLC plates. A suitable solvent system was poured into glass jar or tank in a required amount. The tank was then covered with a lid and kept for a certain period for allowing it to achieve saturation. A filter paper was usually introduced into the tank to promote the saturation process. The solvent level at the bottom of the tank must not be above the line of the spot where the sample solution was applied to the plate. As the solvent rises, the plate becomes moistened. When the solvent front moves almost near the end of the plate, the plate was then taken out and dried. The solvent front was not allowed to travel beyond the end of the silica-coated surface.

2.5.5 Detection of spots

For the location of the separated compounds, the plates were examined under UV light in different wavelengths, 254 and 361 nm.

The plates were developed by the spray reagent followed by heating in an oven at 80°C at for 5 minutes.
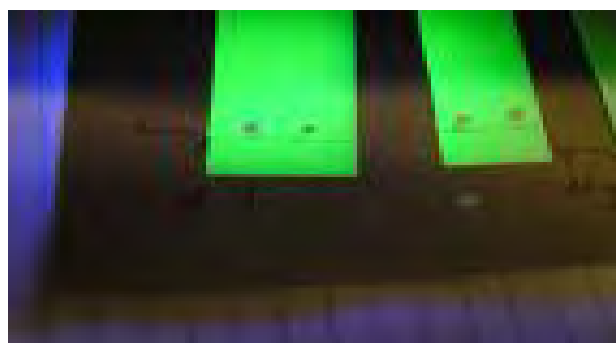
Fig 2.6: TLC plates under UV lamp

2.5.6 The R_f value

Retardation factor (R_f) is the ratio of the distance the compound travels and the distance the solvent front moves.
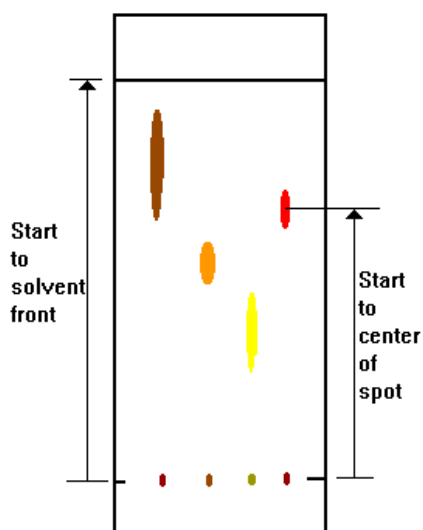
Fig 2.7: Calculation of R_f value

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

Usually, the R_f value is constant for any given compound and it corresponds to a physical property of that compound.
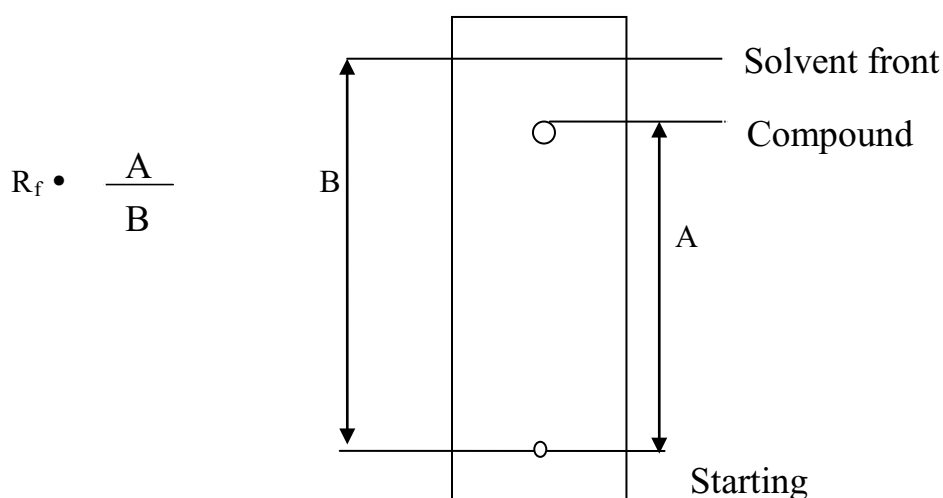
Figure-2.8: A plate for the calculation of R_f value.

2.5.7 Stationary phases of column chromatography

For normal phase column chromatography, silica gel of particle size 230-400 mesh (Merck) was used and separation was performed by gravitational flow with solvents of increasing polarity. The sample was applied into the column either as a solution or in a powdered form or by adsorbing samples by the silica gel. The eluted samples were collected in several test tubes and were monitored by TLC to make different fractions on the basis of R_f values.

For preparation of Sephadex LH-20 column, the required amount of Sephadex LH-20 gel (25-100 μm, Pharmacia, Sweden) was suspended in chloroform or DCM or methanol and the column was packed with this suspended gel.

2.5.8 Procedure for micro scale flash column chromatography

In micro scale flash chromatography, the column does not need either a pinch clamp or a stopcock at the bottom of the column to control the flow, nor does it need a ir-pressure connection at the top of the column. Instead, the solvent flows very slowly through the column by gravity until we apply air pressure at the top of the column with an ordinary Pasteur Pipette Bulb.
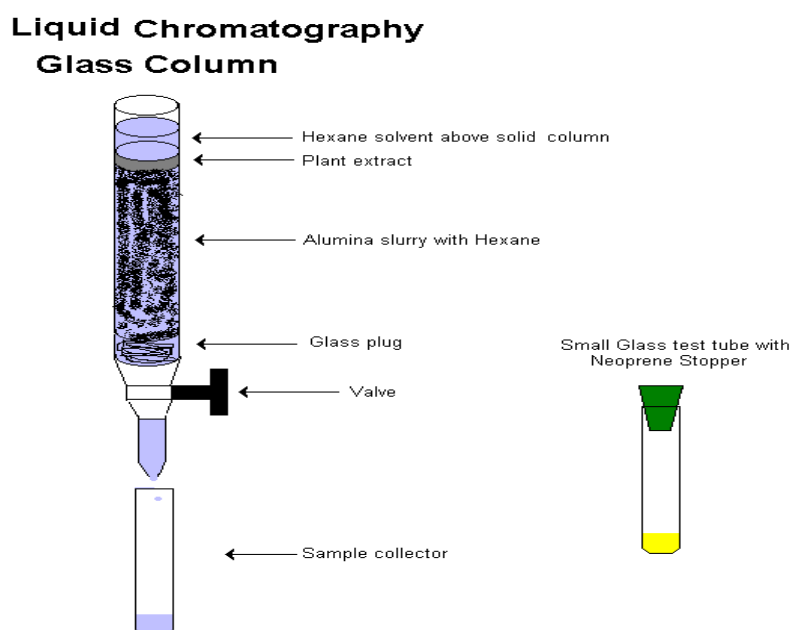
Fig 2.9: Various part of a column

2.5.9 Preparation of column (For micro scale operation)

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

2.6 Re-crystallization

Re-crystallization was employed as a final purification process. A solution of the compound in a minimum volume of the solvent in which it is soluble was prepared in hot condition. It was then left for crystallization. Sometimes, a mixture of solvents was used.
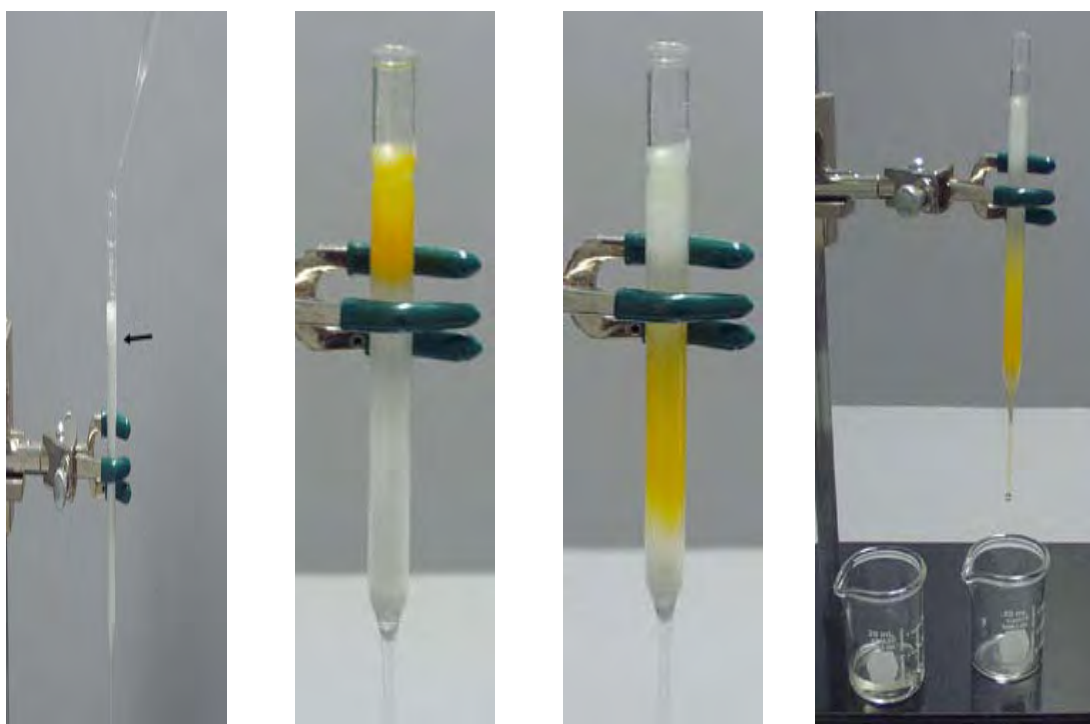
Fig 2.10: Various stages in micro scale column.

2.7 Spectroscopic Techniques

2.7.1 Infra-Red Spectroscopy (IR)

A Shimadzu IR prestige-21(FT-IR) spectrometer was used for recording infrared spectrum. Major bands (ν_{max}) were recorded in wave number (cm^{-1}) as KBr pellets.

2.7.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

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

2.7.3 Ultra-Violet Spectroscopy

UV absorbance for obtaining the •-max of the isolated compounds was taken by Shimadzu UV-1601 UV spectrophotometer.

2.8 Investigation of *Andrographis peniculata*

2.8.1 Collection of the plant

The plant *Andrographis peniculata* (Locally known as Kalmegh) was collected from Medicine Polli, Bogra.

2.8.2 Identification of species

The taxonomy of the plant was confirmed consulting with the National Herbarium's Botanist. A voucher specimen of this plant was deposited at Bangladesh National Herbarium.

2.8.3 Test of steroids

The leaves powder (15 g) was extracted with a mixture of MeOH and $CHCl_3$ (100 ml, 1:1). This extract was concentrated and divided into two parts. One part was treated with concentrated H_2SO_4. Development of a reddish color indicates the presence of steroidal compound.

The other part was treated with a few drops of concentrated H_2SO_4 and 4-6 drops of acetic anhydride. Development of a greenish color indicates the presence of steroidal compound.

2.8.4 Test of terpenoids

A few mg of sample was dissolved in a mixture of $CHCl_3$-CH_3OH, and then few drops of conc. H_2SO_4 were added to it followed by 4-6 drops of Ac_2O. Formation of red-violet color confirms the presence of terpenoid type compounds.

2.8.5 Test of alkaloids

Extracted 2 g of powder by warming for two minutes with 20 ml 1% H_2SO_4 in a 50 ml conical flask on a water bath, with intermittent shaking, centrifuge; pipette off supernatant into a small conical flask. Make an initial test for alkaloids by adding to 0.1 ml in a semi-micro tube, one drop of Meyer's reagent. It gives a cream precipitate with alkaloids.

2.8.6 Extraction, partition and isolation of the compounds from *Andrographis peniculata*

The leaves with the stems of this plant were separated and dried under in open air & then dried in oven at 37 ^oC. Afterwards it was powdered (~200 mesh) by a grinding machine. This powder (207.6 g) was used throughout this investigation. The leaf powder of *Andrographis peniculata* was extracted with ethanol. The extract was concentrated to dry mass (18.6 g) using rotary evaporator. The ethanol extract was then partitioned by separatory funnel by using n-hexane, ethyl acetate and 1-butanol successively. All the crude extracts were also subjected to antimicrobial tests.

```
                        ┌─────────────────────┐
                        │   Ethanol extract   │
                        └─────────────────────┘
              Dissolved in H₂O & shaken with n-hexane
          ┌──────────────────────────┴──────────────────┐
  ┌──────────────────┐                         ┌──────────────────┐
  │  n-hexane part   │                         │    Water part    │
  │     (4.2 g)      │                         └──────────────────┘
  └──────────────────┘         Partitioned with ethyl acetate
          ┌──────────────────────────┴──────────────────┐
  ┌──────────────────┐                         ┌──────────────────┐
  │ Ethyl acetate part │                       │    Water part    │
  │     (10.3 g)     │                         └──────────────────┘
  └──────────────────┘           Partitioned with 1-butanol
          ┌──────────────────────────┴──────────────────┐
  ┌──────────────────┐                         ┌──────────────────┐
  │  1-butanol part  │                         │    Water part    │
  │     (2.7 g)      │                         └──────────────────┘
  └──────────────────┘
```
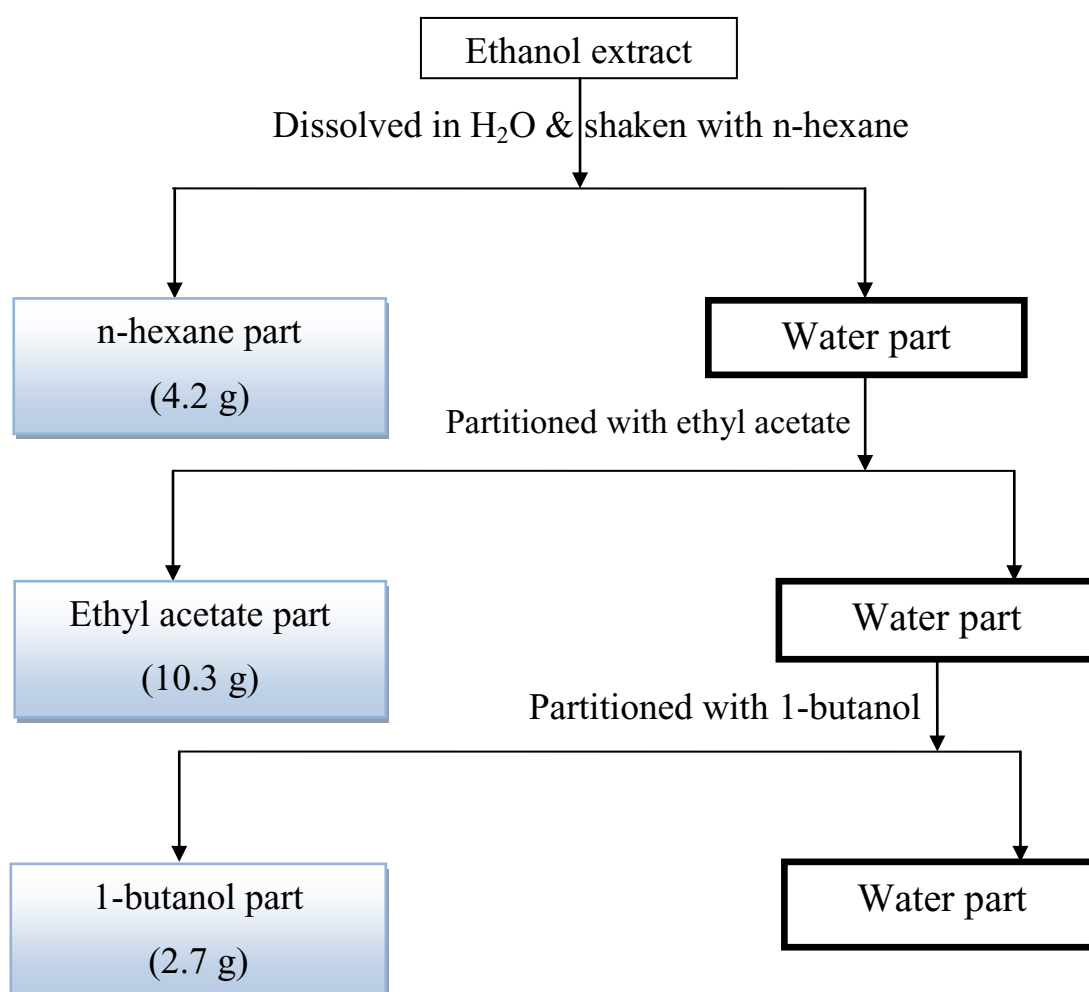
Scheme 2.1: Partition of ethanol extract

2.8.6.1 Investigation of n-hexane extract

2.8.6.1.1 Thin layer chromatography (TLC)

TLC an alysis of t he n -hexane ex tract s howed several s pots unde r U V l amp, w hich w as further confirmed by the development by spray reagent on TLC plate for detecting the spots.

2.8.6.1.2 Fractionation of the extract by vacuum liquid column chromatography (VLC)

The n-hexane extract was concentrated to dry mass (4.2 g) using rotary evaporator. The dry mass of n -hexane ex tract w as ads orbed by t he c olumn grade s ilica gel. This s ample w as placed on t he top of the bed of column packed with TLC grade silica gel. The column was first eluted with 100% n-hexane and then eluted with mixtures of n-hexane and ethyl acetate increasing t he pol arity of t he s olvents a nd finally w ith t he m ixtures of e thyl a cetate & methanol. The eluents were collected in an amount of 200 ml in a series of conical flasks. Solvent systems used as mobile phases in the analysis of n-hexane part are listed in table-2.1.

Table-2.1: Fractions collected from vacuum liquid column chromatography (VLC) of n-hexane extract using different solvent systems

Fraction no.	Solvent system
H_1-1	n-hexane (100%)
H_1-2	n- hexane : ethyl acetate (90:10)
H_1-3	n- hexane : ethyl acetate (80:20)
H_1-4	n- hexane : ethyl acetate (70:30)
H_1-5	n- hexane : ethyl acetate (60:40)
H_1-6	n- hexane : ethyl acetate (50:50)
H_1-7	n- hexane : ethyl acetate (40:60)
H_1-8	n- hexane : ethyl acetate (30:70)
H_1-9	n- hexane : ethyl acetate (20:80)
H_1-10	n- hexane : ethyl acetate (10:90)
H_1-11	ethyl acetate (100%)
H_1-12	ethyl acetate: methanol (97.5: 2.5)
H_1-13	ethyl acetate: methanol (95:5)
H_1-14	ethyl acetate: methanol (90:10)
H_1-15	ethyl acetate: methanol (87.5:12.5)
H_1-16	ethyl acetate: methanol (85:15)
H_1-17	ethyl acetate: methanol (80:20)
H_1-18	ethyl acetate: methanol (70:30)

```
                         ┌──────────────────┐
                         │   n-hexane part  │
                         └──────────────────┘
                                  │ VLC fractions
          ┌───────────────────────┼──────────────────────────┐
          │                       │                          │
          │              ┌──────────────────┐       ┌──────────────────┐
          │              │  H₁-8 & H₁-9     │       │  H₁-10 & H₁-11   │
          │              └──────────────────┘       └──────────────────┘
          │                   │ Column with 2%              │
          │                   │ MeOH in CHCl₃               │
          │              ┌──────────┐           ┌──────────────────────┐
          │              │  R_{H-4} │           │  Crystal obtained at │
          │              └──────────┘           │  the bottom of flask │
          │                                     └──────────────────────┘
   ┌──────────────┐                              Column with 100% EA │
   │ H₁-4 to H₁-7 │                                   ┌──────────┐
   └──────────────┘                                   │  R_{H-5} │
          │ Column with sephadex                      └──────────┘
          │ LH-20 with CH₂Cl₂
```

H_1-8 & H_1-9 → Column with 2% MeOH in CHCl$_3$ → $\mathbf{R_{H-4}}$

H_1-10 & H_1-11 → Crystal obtained at the bottom of the flask → Column with 100% EA → $\mathbf{R_{H-5}}$

H_1-4 to H_1-7 → Column with sephadex LH-20 with CH$_2$Cl$_2$

H_2-3 to H_2-6	H_2-7	H_2-8 to H_2-12
Column H:EA • 3:2	Column H:EA • 50:50	Column H:EA • 50:50
H_3-14 to H_3-22	H_3-1 to H_3-10	H_3-5 & H_3-6
Mix	Column H:EA • 50:50	Column H:EA • 80:20
Adsorbed by charcoal	H_4-2 to H_4-4	$\mathbf{R_{H-3}}$
$\mathbf{R_{H-1}}$	Column H:EA • 50:50	
	H_5-13 to H_5-15	
	Mix	
	$\mathbf{R_{H-2}}$	

Scheme 2.2: Scheme of the isolation of compounds from n-hexane part.

2.8.6.1.3 Analysis of the fractions by column chromatography

Fraction H_1-1 to H_1-3 & H_1-12 to H_1-18 shows no prominent spot on TLC analysis. So, they were discarded.

Fraction H_1-4 to H_1-7 shows similar spots in TLC analysis; they were got mixed. As they contain chlorophyll, so they were done column using sephadex LH-20 as stationary phase

& chloroform as mobile phase. Among the fractions, the TLC analyses of H_{2-3} to H_{2-6} were similar. So they got mixed & had done a column using n-hexane : EA• 3:2. Then they were treated with charcoal to get the chlorophyll adsorbed by the charcoal & thus the first compound **R_H-1** was obtained. On the other hand, the second fraction H_2-7 shows a single compound with a little bit contamination. So, it was done a column using n-hexane : EA • 50:50. But still containing some tailing in the TLC analysis, it was further treated with column with the same solvent system to get the purified compound **R_H-2.** Rest of the fractions of the H_1 fractions (i.e. H_1-8 to H_1-12) were mixed together for their similar R_f value. Then they were done column with the solvent system n-hexane : EA• 50:50 & finally with 20% EA in n-hexane & the isolated compound **R_H-3** was obtained.

Since the R_f value of the fractions H_1-8 & H_1-9 were same so they were mixed together & had done a column using 2% MeOH in $CHCl_3$ to get the pure compound **R_H-4.** In the fractions H_1-10 & H_1-11, a number of colorless crystals were formed at the bottom of the conical flasks. The TLC analysis provided the information of the little contamination of another compound which was excluded by performing a column by 100% EA as mobile phase to obtain pure compound **R_H-5.**

2.8.6.2 Investigation of the ethyl acetate extract

2.8.6.2.1 Thin layer chromatography (TLC)

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

2.8.6.2.2 Fractionation of the extract by vacuum liquid column chromatography

The ethyl acetate extract was concentrated to dry mass (10.3 g) using rotary evaporator. The dry mass of ethyl acetate extract was adsorbed by the column grade silica gel. This sample was placed on the top of the bed of column packed with TLC grade silica gel. The column was first eluted with 100% n-hexane and then eluted with mixtures of n-hexane and ethyl acetate increasing the polarity of the solvents and finally with the mixtures of ethyl acetate & methanol. The eluents were collected in an amount of 200 ml in a series of conical flasks. Solvent systems used as mobile phases in the analysis of ethyl acetate part were listed in table-2.2.

Table-2.2: Fractions collected from vacuum liquid column chromatography (VLC) of ethyl acetate extract using different solvent systems

Fraction no.	Solvent system
E_1-1	n-hexane (100%)
E_1-2	n- hexane : ethyl acetate (90:10)
E_1-3	n- hexane : ethyl acetate (80:20)
E_1-4	n- hexane : ethyl acetate (70:30)
E_1-5	n- hexane : ethyl acetate (60:40)
E_1-6	n- hexane : ethyl acetate (50:50)
E_1-7	n- hexane : ethyl acetate (40:60)
E_1-8	n- hexane : ethyl acetate (30:70)
E_1-9	n- hexane : ethyl acetate (20:80)
E_1-10	n- hexane : ethyl acetate (10:90)
E_1-11	ethyl acetate (100%)
E_1-12	ethyl acetate: methanol (97.5: 2.5)
E_1-13	ethyl acetate: methanol (95:5)
E_1-14	ethyl acetate: methanol (90:10)
E_1-15	ethyl acetate: methanol (87.5:12.5)
E_1-16	ethyl acetate: methanol (85:15)
E_1-17	ethyl acetate: methanol (80:20)
E_1-18	ethyl acetate: methanol (70:30)
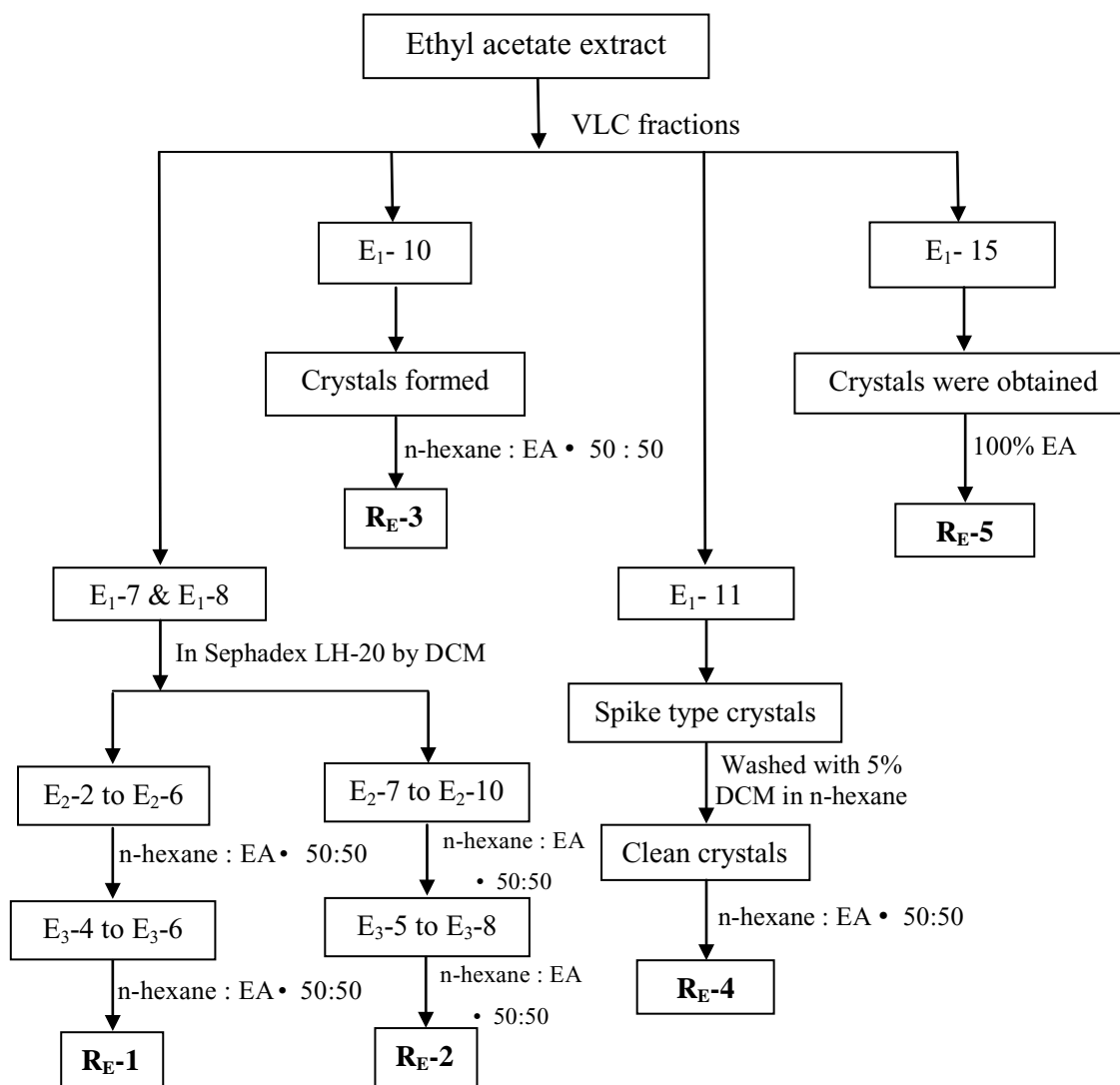
Scheme 2.3: Scheme of isolating different compounds from the ethyl acetate part.

2.8.6.2.3 Analysis of the fractions by column chromatography

Fraction E_1-1 to E_1-6, E_1-9, E_1-12, E_1-13 & E_1-16 to E_1-18 shows no prominent spot on TLC analysis. So, they were discarded.

Fraction E_1-7 & E_1-8 shows similar spots in TLC analysis; they were got mixed. As they contain chlorophyll, so they were done column using Sephadex LH-20 as stationary phase & dichloromethane as mobile phase. Among the fractions, the TLC analyses of E_2-2 to E_2-6 were similar. So they got mixed & had done a column using . Owing to find evidence of the presence of other trace compounds the fractions E_3-4 to E_3-6 were again columned with n-hexane : EA• 50:50 & thus the compound **R_E-1** was obtained. On the other hand, the second fraction E_2-7 to E_2-10 shows a single compound with a little bit contamination. So, it was done a column using n-hexane : EA • 50:50 to get the purified compound **R_E-2.**

In the fractions E_1-10, E_1-11 & E_1-12 a number of colorless crystals were formed at the bottom of the conical flasks. The TLC analysis provided the information of the little contamination of another compound which was excluded by performing a column by n-hexane : EA• 50:50 as mobile phase to obtain pure compound **R$_E$-3.** The crystals of the E_1-11 part was first washed by n-hexane : DCM• 60:40. Afterwards a column was done with mobile phase n-hexane : EA• 50:50 to obtain the single compound **R$_E$-4.** The final fraction of the ethyl acetate part E_1-15 was first done a column using 100% EA as mobile phase. Being the similar R$_f$ value of part E_2-20 to E_2-24 were mixed together to get pure compound **R$_E$-5.**

2.9 Characterization of the isolated compounds

2.9.1Properties of compound R$_H$-1

2.9.1.1 Physical properties

The compound R$_H$-1 is a white crystalline compound. The R$_f$ value of the compound is 0.86 in n-hexane : EA • 50:50. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It has tested by Salkawoski method which developed a reddish color indicating that the compound may be steroid.

2.9.1.2 Characterization of R$_H$-1 by spectroscopic method

2.9.1.2.1 Ultraviolet (UV) spectroscopy of R$_H$-1

The UV spectrum of the compound R$_H$-1**(Fig: 5.1.1)** has absorption at •$_{max}$ 280 nm & 219 nm in methanol.

2.9.1.2.2 Infrared (IR) spectroscopy of R$_H$-1

The IR spectrum (in KBr) of the compound R$_H$-1**(Fig: 5.1.2)** has important transmittance at 3446 (OH stretching), 2960 (-HC=CH- cyclic), 2937 (-C-H stretching), 1637 (C=C), 1458 (CH$_2$, n), 1062 (cyclic alkane) cm^{-1}.

2.9.1.2.3 ^{1}H-NMR spectroscopy of R$_H$-1

The ^{1}H-NMR spectrum (400 MHz, CDCl$_3$) of the compound R$_H$-1**(Fig: 5.1.3)** has signals at •$_H$ (ppm) 5.347 (1H, d), 5.148 (1H, m), 5.010 (1H, m), 3.513 (1H, m; oxymethine proton), 2.262 (2H, m), 1.987 (2H, t), 1.835 (2H, m), 1.488(6H, s) 1.001 (6H, s), 0.913 (3H, d), 0.807 (9H, m), 0.687 (3H, d).

2.9.1.2.4 ^{13}C-NMR spectroscopy of R$_H$-1

The ^{13}C-NMR spectrum (100 MHz) in CDCl$_3$ of the compound R$_H$-1**(Fig: 5.1.4)** has signals at •$_C$ (ppm) 37.30, 31.72, 71.85, 42.36, 140.81 , 121.74, 31.96 , 31.96, 50.2, 36.56 , 21.13, 39.83, 40.50 , 56.82 , 24.34, 28.28 , 56.12, 12.08, 19.43, 36.19, 18.82, 138.33 , 129.31, 45.9, 29.23, 19.84, 19.08, 23.13, 12.26.

2.9.2 Properties of compound R$_H$-3

2.9.2.1 Physical properties

The compound R$_H$-3 is a white crystalline compound. The R$_f$ value of the compound is 0.71 in 2% MeOH in CHCl$_3$. It is soluble in dichloromethane, ethyl acetate, methanol & ethanol. It has tested for terpenoid & a red-violet color confirms that the compound R$_H$-3 is terpenoid type compound.

2.9.2.2 Characterization of R$_H$-3 by spectroscopic method

2.9.2.2.1 Ultraviolet (UV) spectroscopy of R$_H$-3

The UV spectrum of the compound R$_H$-3 **(Fig: 5.2.1)** has absorption at •$_{max}$ 235 nm in methanol.

2.9.2.2.2 Infrared (IR) spectroscopy of R$_H$-3

The IR spectrum (in KBr) of the compound R$_H$-1 **(Fig: 5.2.2)** has important transmittance at 3398 (OH stretching), 2848 & 2927 (C-H stretching), 1728 (lactone ring), 1674 (C=C unsaturation), 906 (exocyclic methylene) cm^{-1}.

2.9.2.2.3 ^{1}H-NMR spectroscopy of R$_H$-3

The ^{1}H-NMR spectrum (400 MHz, CDCl$_3$ + few drops of MeOH) of the compound R$_H$-3 **(Fig: 5.2.3)** has signals at •$_H$ (ppm) 6.8 (1H, t), 4.84 (1H, d), 4.76 (1H, d), 4.5 (1H, s), 4.33 (1H, m), 4.07 (2H, m), 2.45 (2H, m), 2.3 (1H, d), 1.86 (1H, m), 1.69 (6H, m), 1.11 (3H, s), 0.58 (3H, s).

2.9.2.2.4 ^{13}C-NMR spectroscopy of R$_H$-3

The ^{13}C-NMR spectrum (100 MHz) (in CDCl$_3$ + few drops of MeOH) of the compound R$_H$-3 **(Fig: 5.2.4)** has signals at •$_C$ (ppm) 37.5, 23.5, 79.8, 42.3, 55, 27.6, 36.8, 146.5, 55.8, 38.6, 24.5, 149, 127.9, 65.3, 63.8, 170.9, 108.5, 22.4, 74.6, 14.8.

2.9.3 Properties of compound R_H-4

2.9.3.1 Physical properties

The compound R_H-4 is a white crystalline compound. The R_f value of the compound is 0.82 in n -hexane : E A • 50:50. It i s s oluble i n c hloroform, di chloromethane, e thyl a cetate, methanol & ethanol. It has tested by Salkawoski method which developed a reddish color indicating that the compound may be steroid.

2.9.3.2 Characterization of R_H-4 by spectroscopic method

2.9.3.2.1 Ultraviolet (UV) spectroscopy of R_H-4

The U V s pectrum o f t he c ompound R $_H$-4(**Fig: 5.3.1**) has absorption a t •$_{max}$ 280 nm i n methanol.

2.9.3.2.2 Infrared (IR) spectroscopy of R_H-4

The IR spectrum (in KBr) of the compound R_H-4 **(Fig: 5.3.2)** has important transmittance at 3290 (OH s tretching), 1673 (C=C s treching), 15 65 (CH be nding due to m ethylene gr oup), 1445 (CH bending due to methyl group), 1275 (C-O stretching for alcoholic group) cm^{-1}.

2.9.3.2.3 ^{1}H-NMR spectroscopy of R_H-4

The ^{1}H-NMR spectrum (400 MHz, CDCl$_3$) of the compound R_H-4 **(Fig: 5.3.3)** has signals at •$_H$ (ppm) 5.34 (1H, d), 3.51 (1H, m), 2.26 (2H, m), 1.98 (1H, m), 1.86 (3H, m), 1.56 (3H, s), 0.99 (4H, s), 0.67 (3H, s).

2.9.3.2.4 ^{13}C-NMR spectroscopy of R_H-4

The ^{13}C-NMR spectrum (100 MHz) in CDCl$_3$ of the compound R_H-4 **(Fig: 5.3.4)** has signals at •$_C$ (ppm) 37.29, 28.2 6, 71.84, 42.36, 140.80, 121.73, 31.71, 31.95 , 50.19, 36.54, 21.12 , 39.82, 42.36, 56.81, 24.33, 26.16, 56.11, 11.88, 19.41, 40.50, 19.07, 34, 26.16, 45.89, 31.95, 19.83, 21.12, 24.33, 12.01.

2.9.4 Properties of compound R_H-5

2.9.4.1 Physical properties

The compound R_H-5 is a white crystalline compound. The R_f value of the compound is 0.78 in n -hexane : E A • 50:50. It i s s oluble i n di chloromethane, e thyl a cetate, m ethanol & ethanol. It has tested by Salkawoski method which developed a reddish color indicating that the compound may be steroid.

2.9.4.2 Characterization of R_H-5 by spectroscopic method

2.9.4.2.1 Ultraviolet (UV) spectroscopy of R_H-5

The UV spectrum of the compound R_H-5 **(Fig: 5.4.1)** has absorption at λ_{max} 219 nm in methanol.

2.9.4.2.2 Infrared (IR) spectroscopy of R_H-5

The IR spectrum (in KBr) of the compound R_H-5 **(Fig: 5.4.2)** has important transmittance at 3490 (OH stretching), 1640 (C=C streching), 1560 (CH bending due to methylene group), 1515 (CH bending due to methyl group), 1360 (C-O stretching for alcoholic group) cm^{-1}.

2.9.4.2.3 ^{1}H-NMR spectroscopy of R_H-5

The ^{1}H-NMR spectrum (400 MHz, CDCl$_3$ + MeOH) of the compound R_H-4 **(Fig: 5.4.3)** has signals at δ_H (ppm) 5.37 (1H, br s), 4.40 (1H, d), 3.85 (1H, dd), 3.72 (1H, dd), 3.60 (1H, m), 3.42 (2H, t), 3.229(1H, br s), 3.22 (1H, t), 2.42 (1H, dd), 2.28 (1H, s), 1.03 (6H, s), 0.94 (5H, d), 0.85 (9H, m), 0.70 (3H, s).

2.9.4.2.4 ^{13}C-NMR spectroscopy of R_H-5

The ^{13}C-NMR spectrum (100 MHz) in CDCl$_3$ + MeOH of the compound R_H-5 **(Fig: 5.4.4)** has signals at δ_C (ppm) 36.8, 31.5, 78.7, 39.3, 139.9, 121.6, 31.5, 31.9, 49.8, 36.2, 20.6, 38.1, 41.9, 56.3, 23.9, 28.7, 55.6, 11.2, 19.1, 36.2, 18.4, 33.5, 27.7, 45.5, 28.7, 19.4, 18.7, 22.6, 11.2, 100.7, 73.1, 76.1, 69.6, 75.6, 61.7.

2.9.5 Properties of compound R_E-1

2.9.5.1 Physical properties

The compound R_E-1 is a colorless crystalline compound. The R_f value of the compound is 0.89 in 2% MeOH in Ethyl acetate. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It has tested for terpenoid & a red-violet color confirms that the compound R_E-1 is terpenoid type compound.

2.9.5.2 Characterization of R_E-1 by spectroscopic method

2.9.5.2.1 Ultraviolet (UV) spectroscopy of R_E-1

The UV spectrum of the compound R_E-1 **(Fig: 5.5.1)** has absorption at λ_{max} 237 nm in methanol.

2.9.5.2.2 Infrared (IR) spectroscopy of R_E-1

The IR spectrum (in KBr) of the compound R_E-1 **(Fig: 5.5.2)** has important transmittance at 3302 (OH stretching), 2839 & 2924 (C-H stretching), 1749 (lactone ring), 1637 (C=C un-saturation), 900 (exocyclic methylene) cm^{-1}.

2.9.5.2.3 ^{1}H-NMR spectroscopy of R_E-1

The ^{1}H-NMR spectrum (400 MHz, CDCl$_3$) of the compound R_E-1 **(Fig: 5.5.3)** has signals at • $_H$ (ppm) 7.08 (1H, s), 4.83 (1H, s), 4.74 (2H, s), 4.56 (1H, s), 3.72 (1H, d), 3.37 (1H, d), 2.43 (2H, m), 2.11 (1H, m), 1.9 (1H, m), 1.74 (5H, m), 0.96 (3H, s), 0.64 (3H, s).

2.9.5.2.4 ^{13}C-NMR spectroscopy of R_E-1

The ^{13}C-NMR spectrum (100 ppm) in CDCl$_3$ of the compound R_E-1 **(Fig: 5.5.4)** has signals at • $_C$ (ppm) 38.5, 18.9, 24.5, 38.8, 56.2, 24.4, 35.3, 147.5, 56.4, 38.5, 27, 39, 134.8, 143.8, 64.9, 174.3, 106.7, 21.7, 70, 15.2.

2.9.6 Properties of compound R_E-2

2.9.6.1 Physical properties

The compound R_E-2 is a brownish crystalline compound. The R_f value of the compound is 0.76 in 2% MeOH in Ethyl acetate. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It has tested for terpenoid & a red-violet color confirms that the compound R_E-2 is terpenoid type compound.

2.9.6.2 Characterization of R_E-2 by spectroscopic method

2.9.6.2.1 Ultraviolet (UV) spectroscopy of R_E-2

The UV spectrum of the compound R_E-2 **(Fig: 5.6.1)** has absorption at • $_{max}$ 255 nm in methanol.

2.9.6.2.2 Infrared (IR) spectroscopy of R_E-2

The IR spectrum (in KBr) of the compound R_E-2 **(Fig: 5.6.2)** has important transmittance at 3286 (OH stretching), 2870 & 2929 (C-H stretching), 1753 (lactone ring), 1635 (C=C un-saturation), 902 (exocyclic methylene) cm^{-1}.

2.9.6.2.3 ^{1}H-NMR spectroscopy of R_E-2

The ^{1}H-NMR spectrum (400 MHz, CDCl$_3$) of the compound R_E-2 **(Fig: 5.6.3)** has signals at • $_H$ (ppm) 7.09 (1H, s), 5.03 (1H, s), 4.92 (2H, s), 4.74 (1H, s), 3.32 (1H, d), 3.6 (1H, t),

3.45 (1H, d), 3.28 (2H, s), 2.57 (2H, m), 2.24 (1H, m), 2.08 (1H, m), 1.92 (6H, m), 1.38 (3H, s), 0.78 (3H, s).

2.9.6.2.4 ^{13}C-NMR spectroscopy of R_E-2

The ^{13}C-NMR spectrum (ppm) in CDCl$_3$ of the compound R_E-2 **(Fig: 5.6.4)** has signals at $\bullet_C$ 38.1, 21.8, 80.5, 42.8, 55.3, 24.4, 36.8, 146.8, 56, 39, 23.8, 28.1, 134.6, 144.1, 64.1, 174.3, 107.4, 22.7, 70.1, 15.2.

2.9.7 Properties of compound R_E-3

2.9.7.1 Physical properties

The compound R_E-3 is a colorless crystalline compound. The R_f value of the compound is 0.8 in 2% MeOH in Ethyl acetate. It is soluble in dichloromethane, ethyl acetate, methanol & ethanol. It has tested for terpenoid & a red-violet color confirms that the compound R_E-3 is terpenoid type compound.

2.9.7.2 Characterization of R_E-3 by spectroscopic method

2.9.7.2.1 Ultraviolet (UV) spectroscopy of R_E-3

The UV spectrum of the compound R_E-3 **(Fig: 5.7.1)** has absorption at $\bullet_{max}$ 270 & 427 nm in methanol.

2.9.7.2.2 Infrared (IR) spectroscopy of R_E-3

The IR spectrum (in KBr) of the compound R_E-3 **(Fig: 5.7.2)** has important transmittance at 3421 (OH stretching), 2868 & 2941 (C-H stretching), 1747 (lactone ring), 1647 (C=C un-saturation), 910 (exocyclic methylene) cm^{-1}.

2.9.7.2.3 ^{1}H-NMR spectroscopy of R_E-3

The ^{1}H-NMR spectrum (400 MHz, CDCl$_3$ + few drops of methanol) of the compound R_E-3 **(Fig: 5.7.3)** has signals at $\bullet_H$ (ppm) 7.49 (1H, s), 4.88 (1H, d), 4.63 (1H, s), 4.23 (2H, d), 4.1 (1H, d), 3.97 (3H, s), 3.26 (2H, m), 2.45 (1H, m), 2.16 (1H, m), 1.08 (3H, s), 0.72 (3H, s).

2.9.7.2.4 ^{13}C-NMR spectroscopy of R_E-3

The ^{13}C-NMR spectrum (100 MHz) (in CDCl$_3$ + few drops of methanol) of the compound R_E-3 **(Fig: 5.7.4)** has signals at $\bullet_C$ (ppm) 38.13, 18.5, 35.53, 39.21, 55. 95, 24. 09, 38.63, 147.23, 56.16, 37.89, 21.38, 69.76, 134.12, 144.23, 70.28, 175.05, 106.37, 27.12, 72.31, 14.86, 103.32, 73.4, 76.13, 61.39, 75.42, 61.27.

Chapter Three

RESULTS & DISCUSSION

3.1 Preliminary investigation of the plant material

3.1.1 Plant material

A species of the Acanthaceae family, *Andrographis peniculata* has been investigated in this work. The leaves along with the stems were used for crude extraction.

3.1.2 Extraction of the plant material

The air-dried and powdered plant material (1.4 kg) was suspended in ethanol for eight days with shaking after every 2/3 hours for the purpose of cold extraction. Every day the extract was filtered through clean white cloth and finally with Whatman No.1 filters paper. The volume of the filtrate was concentrated with a rotary evaporator at low temperature (not more than 40^0C) under reduced pressure.

3.1.3 Isolation and characterization of compounds

From the crude extracts pure compounds were isolated applying various chromatographic techniques. The isolated pure compounds were then characterized using different spectroscopic techniques.

3.2 Characterization of isolated compounds from *Andrographis peniculata*

3.2.1 Characterization of R_H-1 as stigmasterol

The compound R_H-1 is a white crystalline compound. The R_f value of the compound is 0.86 in n-hexane : EA 50:50. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It has been tested by Salkawoski method which developed a reddish color indicating that the compound may be steroid.
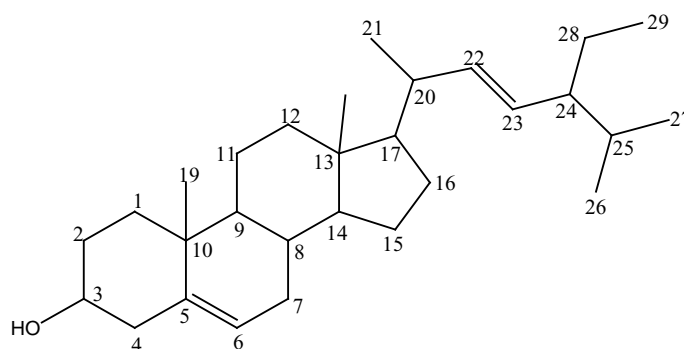
Fig: 3.1 Structure of stigmasterol

3.2.1.1 Characterization of R$_H$-1 by spectroscopic methods

The structure of the compound R$_H$-1 (Fig: 3.1) has been established by UV, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 & DEPT-135 spectral evidences.

3.2.1.2 Ultraviolet (UV) spectroscopy of R$_H$-1

The UV spectrum of the compound R$_H$-1 (Fig: 5.1.1) has absorption at • $_{max}$ 280 nm & 219 nm in methanol.

3.2.1.3 Infrared (IR) spectroscopy of R$_H$-1

The I.R Spectroscopic analysis (in KBr) of the compound R$_H$-1 (Fig: 5.1.2), the observed absorption bands are at 3446 cm^{-1} that is characteristic of O-H stretching. Absorption at 2960 cm^{-1} is due to cyclic olefinic –C=CH- stretching, 2937 cm^{-1} due to =CH stretching and 2866 cm^{-1} assigned to C-H stretching. Other absorption frequencies include 1637 cm^{-1} as a result of C=C absorption, however, this band is weak. These absorption frequencies resemble the absorption frequencies observed for stigmasterol.

3.2.2.3 ^{1}H-NMR spectroscopy of R$_H$-1

The ^{1}H-NMR spectrum of the compound R$_H$-1 (Fig: 5.1.3) revealed the peaks at • 0.681, 1.001, 1.545 ppm due to methyl groups of a steroid. A number of multiplates between • 1.042 - 2.296 ppm and a multiplate at • 0.856 ppm are due to methylene and methyl protons present in the compound. The broad multiplate at • 3.513 ppm indicates the presence of oxymethine proton flanked with two different methylene groups (-CH$_2$-CHOH-CH$_2$-). Two multiplates at • 5.148 and 5.010 ppm in the spectrum indicated the presence of two olefinic protons attached with two methylene groups (>CH-CH=CH-CH<) in side chain of the compound.

A broad singlet at • 5.347 ppm indicated the presence of a double bond in between a quarternary carbon and a methylene carbon i.e. presence of olefinic proton.

3.2.2.4 ^{13}C-NMR spectroscopy of R$_H$-1

In ^{13}C-NMR spectrum in CDCl$_3$ of the compound R$_H$-1 (Fig: 5.1.4) the peak at • 71.85 (C-3) ppm confirmed the presence of oxymethine group in the compound. The peaks at • 138.33 (C-22) & 129.31 (C-23) ppm spectrum indicated the presence of two olefinic protons attached with two methylene groups (>CH-CH=CH-CH<) in the side chain of the compound. Peaks at • 140.81 (C-5) and 121.74 (C-6) ppm in the spectrum indicated presence of double bond in a quarternary carbon & a methylene carbon.

The ^{13}C-NMR exhibited exactly 29 carbon signals which suggested the compound may be a steroid. The other peaks are 37.3 (C-1), 31.72 (C-2), 71.85 (C-3), 42.36 (C-4), 31.96 (C-7), 31.96 (C-8), 50.2 (C-9), 36.56 (C-10), 21.13 (C-11), 39.83 (C-12), 40.50 (C-13), 56.82 (C-14), 24.34 (C-15), 28.28 (C-16), 56.12 (C-17), 12.08 (C-18), 19.43 (C-19), 36.19 (C-20), 18.82 (C-21), 45.9 (C-24), 29.23 (C-25), 19.84 (C-26), 19.08 (C-27), 23.13 (C-28), 12.26 (C-29) ppm. These values give us the conformation of the compound R $_H$-1 is stigmasterol. The comparison of all these values of with the literature survey[102] has been showed in the following table 3.1:

Table 3.1: ^{13}C-NMR da ta of compound R $_H$-1 c ompared w ith pub lished da ta[102] of stigmasterol:

Carbon no.	Type of carbon	Stigmasterol (Chemical shift in ppm)	R$_H$-1 (Chemical shift in ppm)
1	CH$_2$	37.31	37.30
2	CH$_2$	31.69	31.72
3	CH	71.81	71.85
4	CH$_2$	42.55	42.36
5	C	140.5	140.81
6	CH	121.69	121.74
7	CH$_2$	31.94	31.96
8	CH	31.94	31.96
9	CH	50.20	50.2
10	C	36.56	36.56
11	CH$_2$	21.11	21.13
12	CH$_2$	39.77	39.83
13	C	42.35	40.5
14	CH	56.91	56.82
15	CH$_2$	24.39	24.34
16	CH$_2$	28.96	28.28
17	CH	56.02	56.12
18	CH$_3$	12.07	12.08
19	CH$_3$	19.42	19.43
20	CH	40.54	36.19
21	CH$_3$	21.11	18.82
22	CH	138.37	138.33
23	CH	129.69	129.31
24	CH	51.29	45.9
25	CH	31.49	29.23
26	CH$_3$	21.26	19.84
27	CH$_3$	19.02	19.08
28	CH$_2$	25.44	23.13
29	CH$_3$	12.29	12.26

3.2.2 Characterization of R_H-3 as andrographolide

The compound R_H-3 is a white crystalline compound. The R_f value of the compound is 0.71 in 2% MeOH in CHCl$_3$. It is soluble in dichloromethane, ethyl acetate, methanol & ethanol. It ha s been tested for terpenoid & a r ed-violet c olor c onfirms t hat t he c ompound R $_H$-3 is terpenoid type compound.
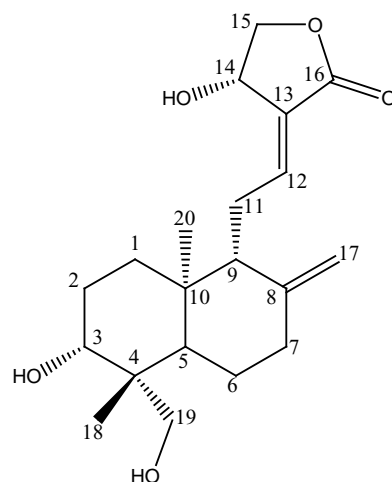
Fig: 3.2 Structure of andrographolide

3.2.2.1 Characterization of R_H-3 by spectroscopic methods

The structure of the compound R_H-3(**Fig: 3.2**) has been established by UV, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 & DEPT-135 spectral evidences.

3.2.2.2 Ultraviolet (UV) spectroscopy of R_H-3

The UV spectrum of the compound R_H-3 (**Fig: 5.2.1**) has absorption at • $_{max}$ 235 nm in methanol.

3.2.2.3 Infrared (IR) spectroscopy of R_H-3

The IR spectrum (in K Br) of the compound R_H-3 (**Fig: 5.2.2**) has i mportant absorbance at 3398 (OH s tretching), 2848 & 2927 (C-H s tretching), 1728 (lactone r ing), 1674 (C=C unsaturation), 906 (exocyclic methylene) cm^{-1}.

3.2.2.4 ^{1}H-NMR spectroscopy of R_H-3

The ^{1}H-NMR s pectrum of R $_H$-3 (**Fig: 5.2.3**) indicated t he pr esence of t wo m ethyl groups with t hree pr oton s ignals a t • 1.11 and 0.58 pp m. It also e xhibited t wo s ignals with fine splitting a t • 4.76 (1H) a nd 4.51 ppm (1H) pr escribed t he two pr otons of t he m ethylene group of H-17. The triplet at • 6.8 and a doublet at • 4.84 ppm were attributed to H-12 and H-14, respectively. Two double doublets at • 4.33 and 4.07 r epresents two protons of H-15. On the other hand, the multiplate at • 2.45 and a doublet at 2.30 ppm are responsible for the

protons at C_{19}-OH & C_3-OH respectively. The peaks of methylene groups of H-19 & peaks of H-14 might have apparently merged with the solvent signals.

3.2.2.5 ^{13}C-NMR spectroscopy of R_H-3

In ^{13}C-NMR spectrum in $CDCl_3$ + few drops of methanol of the compound R_H-3 **(Fig: 5.2.4)** the peaks at • 79.8 (C-3) and 65.3 (C-14) ppm spectrum confirmed the presence of two hydroxyl groups in the compound. The peaks at • 146.5 (C-8) & 108.5 (C-17) ppm spectrum indicated the presence of an exocyclic CH_2 group of the compound. Peaks at • 149(C-12) and 127.9(C-13) ppm in the spectrum indicated presence of double bonded carbons.

The ^{13}C-NMR exhibited exactly 20 carbon signals which suggested the compound may be a diterpene. The other singlets are • 37.5 (C-1), 23.5 (C-2), 42.3 (C-4), 55 (C-5), 27.6 (C-6), 36.8 (C-7), 55.8 (C-9), 38.6 (C-10), 24.5 (C-11), 65.3 (C-14), 63.8 (C-15), 170.9 (C-16), 22.4 (C-18), 74.6 (C-19), 14.8 (C-20) ppm. The DEPT 90 confirms the presence of five –CH- groups & the DEPT 135 shows the presence of eight –CH$_2$- groups & two –CH$_3$ groups of the identifying compound.

These values give us the conformation of the compound R_H-3 is andrographolide. The comparison of all these values of with the literature survey[102] has been showed in the following table 3.2:

Table 3.2: ^{13}C-NMR da ta of compound R $_H$-3 compared w ith pub lished da ta^{102} of androgapholide:

Carbon no.	Type of carbon	Andrographolide (Chemical shift in ppm)	R$_H$-3 (Chemical shift in ppm)
1	CH$_2$	37.5	37.5
2	CH$_2$	29.1	23.5
3	CH	80.0	79.8
4	C	43.4	42.3
5	CH	55.5	55.0
6	CH$_2$	24.5	27.6
7	CH$_2$	38.3	36.8
8	C	148.0	146.5
9	CH	56.5	55.8
10	C	39.3	38.6
11	CH$_2$	25.1	24.5
12	CH	147.0	149.0
13	C	130.2	127.9
14	CH	66.1	65.3
15	CH$_2$	75.3	63.8
16	C	170.7	170.9
17	CH$_2$	108.8	108.5
18	CH$_3$	23.8	22.4
19	CH$_2$	64.2	74.6
20	CH$_3$	15.3	14.8

3.2.3 Characterization of R_H-4 as • -sitosterol

The compound R_H-4 is a white crystalline compound. The R_f value of the compound is 0.82 in n -hexane : E A • 50:50. It i s s oluble i n c hloroform, di chloromethane, e thyl acetate, methanol & e thanol. It ha s been tested b y S alkawoski m ethod w hich de veloped a r eddish color indicating that the compound may be steroid.
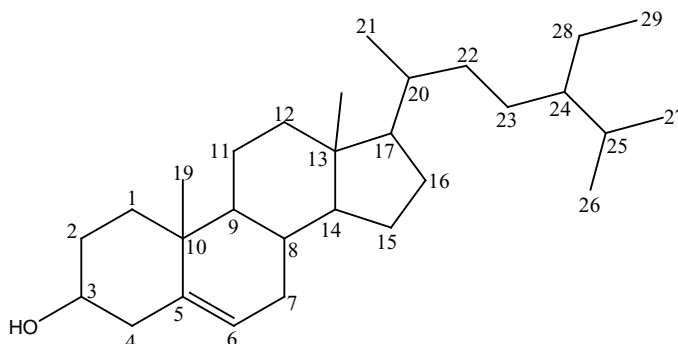
Fig: 3.3 Structure of • -sitosterol

3.2.1.1 Characterization of R_H-4 by spectroscopic methods

The structure of the compound R_H-4 **(Fig: 3.3)** has been established by UV, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 & DEPT-135 spectral evidences.

3.2.2.1.1 Ultraviolet (UV) spectroscopy of R_H-4

The UV spectrum of the compound R_H-4 **(Fig: 5.3.1)** has absorption at • $_{max}$ 280 nm in methanol.

3.2.2.1.2 Infrared (IR) spectroscopy of R_H-4

The I.R S pectroscopic a nalysis (in K Br) of t he compound R $_H$-4 **(Fig: 5.3.2)**, t he obs erved absorption bands are at 3290 cm^{-1} that is characteristic of O-H stretching. Absorption at 2910 cm^{-1} is due to cyclic olefinic –C=CH- stretching. Other absorption frequencies include 1673 cm^{-1} as a r esult o f C =C a bsorption, how ever, t his ba nd i s w eak. 1445 c m^{-1} is a be nding frequency fo r c yclic (CH$_2$)$_n$. T hese a bsorption f requencies r esemble t he absorption frequencies observed for • -sitosterol.

3.2.1.1.3 ^{1}H-NMR spectroscopy of R_H-4

The ^{1}H-NMR s pectrum of c ompound R$_H$-4 **(Fig: 5.3.3)** showed mul tiplet s ignal a t • 3· 51 due t o ox ymethine pr oton. T he ol efinic pr oton s ignal a·34• v5 as r epresents of   H-5 steroids. T he s pectrum showed s ignals a t • 0· 67 &·99(3H e ach) assignable t o t wo tertiary methyl groups at C-18 and C-19 respectively, the resonance at • 0·85 (J=7·0 Hz) & •

0·81 (J=7·0 Hz) were due to secondary methyl groups at C-26 and C-27 respectively. The doublet at • 0· 82 (d, J=1.2 H z) and the triplet of three proton intensity at • 0· 83 were demonstrated by a secondary methyl group at C-21 and a primary methyl group at C-29 respectively.

3.2.1.1.4 ^{13}C-NMR spectroscopy of R_H-1

The ^{13}C- NMR spectrum showed 29 carbons including an oxymethine carbon signal at • 71·84 ppm and two olefinic carbons at • 140·80 & • 121·74 ppm. The double bonded unsaturation at • 140·80 & • 121·74 ppm was characteristics of spirostene. The DEPT 135 & DEPT 90 experiments confirmed that this compound was having six methyl groups at • 11·88 (C-18), 19·41 (C-19), 19·07 (C-21), 19·83 (C-26), 21·12 (C-27) and 12·01 ppm (C-29), eleven methylene, nine methine and three quaternary carbons. These values give us the conformation of the compound R_H-4 is •-sitosterol. The comparison of all these values of with the literary survey[102] has showed in the following table:

Table 3.3: ^{13}C-NMR da ta of c ompound R $_H$-4 compared w ith publ ished da ta[102] of •-sitosterol:

Carbon no.	Type of carbon	R$_H$-4 (Chemical shift in ppm)	•-sitosterol (Chemical shift in ppm)
1	CH$_2$	37·29	37.7
2	CH$_2$	28·26	32.3
3	CH	71·84	72.2
4	CH$_2$	42·36	42.8
5	C	140·80	141.2
6	CH	121·73	122.1
7	CH$_2$	31·71	32.1
8	CH	31·95	32.3
9	CH	50·19	50.6
10	C	36·54	36.9
11	CH$_2$	21·12	21.5
12	CH$_2$	39·82	40.2
13	C	42·36	42.8
14	CH	56·81	57.2
15	CH$_2$	24·33	24.7
16	CH$_2$	26·16	28.7
17	CH	56·11	56.5
18	CH$_3$	11·88	12.4
19	CH$_3$	19·41	19.8
20	CH	40·50	36.6
21	CH$_3$	19·07	19.2
22	CH$_2$	34·00	34.4
23	CH$_2$	26·16	26.5
24	CH	45·89	46.2
25	CH	31·95	29.6
26	CH$_3$	19·83	20.2
27	CH$_3$	21·12	19.5
28	CH$_2$	24·33	23.5
29	CH$_3$	12·01	12.3

3.2.4 Characterization of R_H-5 as daucosterol

The compound R_H-5 is a white crystalline compound. The R_f value of the compound is 0.78 in n -hexane : E A • 50:50. It i s s oluble i n c hloroform, di chloromethane, e thyl acetate, methanol & e thanol. It has been tested b y S alkawoski m ethod w hich de veloped a r eddish color indicating that the compound may be steroid.
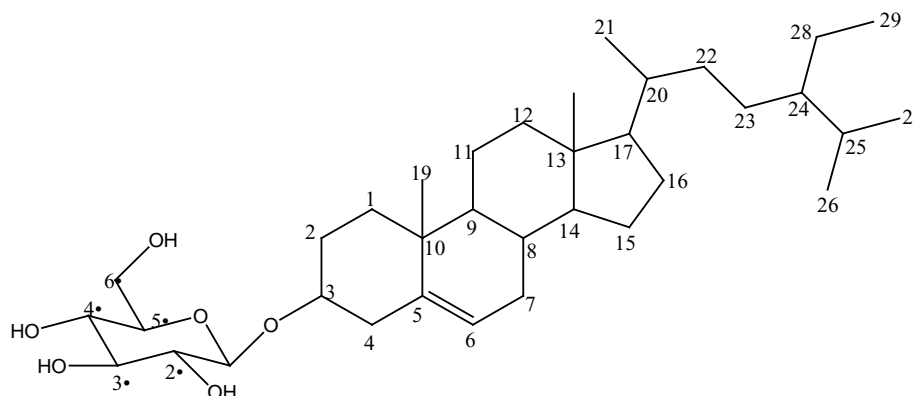
Fig: 3.4 Structure of daucosterol

3.2.4.1 Characterization of R_H-5 by spectroscopic methods

The structure of the compound R_H-5 **(Fig: 3.4)** has established by UV, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 & DEPT-135 spectral evidences.

3.2.2.1.1 Ultraviolet (UV) spectroscopy of R_H-5

The U V s pectrum o f t he c ompound R H-5 **(Fig: 5.4.1)** has a bsorption at • max 219 nm i n methanol.

3.2.2.1.2 Infrared (IR) spectroscopy of R_H-5

On s ubjection t o I .R S pectroscopic a nalysis (in K Br), t he obs erved a bsorption ba nds a re 3490 cm^{-1} that i s cha racteristic of O-H stretching. A bsorption at 2960 c m[-1] is due to cyclic olefinic –C=CH- stretching. O ther a bsorption f requencies i nclude 1640 c m[-1] as a r esult of C=C a bsorption, how ever, t his ba nd i s w eak. 14 58 c m[-1] is a be nding f requency f or cyclic (CH_2) n and 1360 cm^{-1} for $–CH_2$ $(CH_3)2•$.

3.2.1.1.3 ^{1}H-NMR spectroscopy of R_H-5

The ^{1}H-NMR spectra of the compound R_H-5 **(Fig: 5.4.3)** gave a pattern very similar to that of • -sitosterol with the e xception than an additional signal for a glucose moiety. The ^{1}H-NMR spectrum of the compound R_H-1 revealed the peaks at • 0.71, .09 3, 1.03 ppm due to methyl gr oups of a s teroid. A num ber of m ultiplates be tween • 1.09-2.05 ppm a nd a multiplate a t • 0.084 p pm w ere due t o m ethylene a nd m ethyl pr otons pr esent in the compound. The broad multiplate at • 3.60 ppm indicates the presence of oxymethine proton

flanked with two different methylene groups (-CH_2-CHOH-CH_2-). Two low intense multiplates at • 5.16 and 5.05 ppm in the spectrum indicated the presence of two olefinic protons attached with two methylene groups (>CH-CH=CH-CH<) in side chain of the compound.

A broad singlet at • 5.38 ppm indicated the presence of a double bond in between a quarternary carbon and a methylene carbon i.e. presence of olefinic proton. The peaks between • 4.40 - 3.61 ppm and • 3.30 – 3.20 ppm are confirms the glucose moiety in the compound.

3.2.1.1.4 ^{13}C-NMR spectroscopy of R$_H$-5

The ^{13}C-NMR spectrum of the compound R$_H$-5 (**Fig: 5.4.4**) showed 35 carbons including an oxymethine carbon signal at • 78.7 and two olefinic carbons at • 139.9 & • 121·6 ppm. The double bonded unsaturation at • 139.9 & • 121·6 was characteristics of spirostene. It gave signals for six methyl groups at • 11·2 (C-18), 19·1 (C-19), 18.4 (C-21), 19·4 (C-26), 18.7 (C-27) and 11.2 (C-29) ppm, twelve methylene, fourteen methine and three quaternary carbons. Distinction among methyle, methylene, methane & quaternary carbons was approved by ^{13}C-NMR spectrum recorded by DEPT 135 & DEPT 90 experiments.

Table 3.4: ^{13}C-NMR data of compound R$_H$-5 compared with published data of daucosterol:

Carbon no.	Type of carbon	R$_H$-5 (Chemical shift in ppm)	Daucosterol (Chemical shift in ppm)
1	CH$_2$	36.8	37.0
2	CH$_2$	31.5	31.6
3	CH	78.7	78.9
4	CH$_2$	39.3	39.5
5	C	139.9	141.6
6	CH	121.6	121.9
7	CH$_2$	31.5	31.6
8	CH	31.9	31.7
9	CH	49.8	50.0
10	C	36.2	36.5
11	CH$_2$	20.6	20.8
12	CH$_2$	38.1	38.5
13	C	41.9	42.1
14	CH	56.3	56.5
15	CH$_2$	23.9	24.5
16	CH$_2$	28.7	28.7
17	CH	55.6	55.8
18	CH$_3$	11.2	12.0
19	CH$_3$	19.1	19.0
20	CH	36.2	36.0
21	CH$_3$	18.4	18.5
22	CH$_2$	33.5	34.0
23	CH$_2$	27.7	28.0
24	CH	45.5	45.7
25	CH	28.7	28.9
26	CH$_3$	19.4	19.5
27	CH$_3$	18.7	18.7
28	CH$_2$	22.6	22.8
29	CH$_3$	11.2	12.0
1•	CH	100.7	100.9
2•	CH	73.1	73.3
3•	CH	76.1	76.2
4•	CH	69.6	70.0
5•	CH	75.6	75.4
6•	CH$_2$	61.7	61.7

3.2.5 Characterization of R_E-1 as andrograpanin

The compound R_E-1 is a colorless crystalline compound. The R_f value of the compound is 0.89 in 2% MeOH in Ethyl acetate. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It has been tested for terpenoid & a red-violet color confirms that the compound R_E-1 is terpenoid type compound.
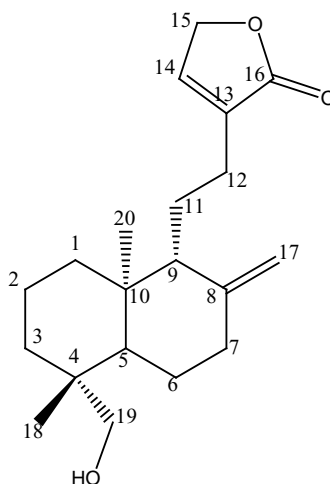
Fig: 3.5 Structure of andrographanin

3.2.5.1 Characterization of R_E-1 by spectroscopic methods

The structure of the compound R_E-1 **(Fig: 3.5)** has been established by UV, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 & DEPT-135 spectral evidences.

3.2.2.1.1 Ultraviolet (UV) spectroscopy of R_E-1
The UV spectrum of the compound R_E-1 **(Fig: 5.5.1)** has absorption at •max 237 nm in methanol.

3.2.2.1.2 Infrared (IR) spectroscopy of R_E-1
The IR spectrum (in KBr) of the compound R_E-1 **(Fig: 5.5.2)** has important absorbance at 3302 (OH stretching), 2839 & 2924 (C-H stretching), 1749 (lactone ring), 1637 (C=C unsaturation), 900 (exocyclic methylene) cm^{-1}.

3.2.1.1.3 ^{1}H-NMR spectroscopy of R_E-1
The ^{1}H-NMR spectrum of R_E-1 **(Fig: 5.5.3)** indicated the presence of two methyl groups at C-18 and C-20 with three protons signals at • 0.96 (3H) and 0.64 (3H) ppm. It also exhibited two signals with fine splitting at • 4.83 (1H) and 4.56 ppm (1H) prescribed to the exocyclic methylene protons of H-17. The singlets at • 7.08 and at • 4.74 ppm were attributed to H-14 and H-15. On the other hand, the two doublets at • 3.72 and • 3.37 ppm are responsible for the protons at H-19. The peak of hydroxyl group at H-19 is at • 2.39 ppm.

3.2.1.1.4 ^{13}C-NMR spectroscopy of R$_E$-1

In ^{13}C-NMR spectrum in CDCl$_3$ of the compound R$_E$-1 **(Fig: 5.5.3)** the peak at • 70 (C-19) ppm confirmed the presence of a hydroxyl group in the compound. The peaks at • 147.5 (C-8) & 106.7 (C-17) ppm spectrum indicated the presence of an exocyclic CH$_2$ group of the compound. Peaks at • 134.8 (C-13) and 143.8 (C-14) ppm in the spectrum indicated presence of double bonded carbons.

The ^{13}C-NMR exhibited exactly 20 carbon signals which suggested the compound may be a diterpene. The other singlets are • 38.5 (C-1), 18.9 (C-2), 24.5 (C-3), 38.8 (C-4), 56.2 (C-5), 24.4 (C-6), 35.3 (C-7), 56.4 (C-9), 38.5 (C-10), 27 (C-11), 39 (C-12), 64.9 (C-15), 174.3 (C-16), 21.7 (C-18), 70 (C-19), 15.2 (C-20) ppm. The DEPT 90 confirms the presence of three –CH- groups & the DEPT 135 shows the presence of ten –CH$_2$- groups & two –CH$_3$ groups of the identifying compound.

These values give us the conformation of the compound R$_E$-1 is andrographanin. The comparison of all these values of with the literature survey has been showed in the following table 3.5:

Table 3.5: ^{13}C-NMR da ta of c ompound R $_E$-1 compared with publ ished da ta[102] of andrographanin:

Carbon no.	Type of carbon	Andrographanin (Chemical shift in ppm)	R$_E$-1 (Chemical shift in ppm)
1	CH$_2$	39.3	38.5
2	CH$_2$	19.5	18.9
3	CH$_2$	36.6	24.5
4	C	40	38.8
5	CH	56.4	56.2
6	CH$_2$	24.9	24.4
7	CH$_2$	38.9	35.3
8	C	148.4	147.5
9	CH	56.9	56.4
10	C	38.7	38.5
11	CH$_2$	22.3	27
12	CH$_2$	25.1	39
13	C	134.3	134.8
14	CH	145.3	143.8
15	CH$_2$	70.6	64.9
16	C	174.6	174.3
17	CH$_2$	107	106.7
18	CH$_3$	28.2	21.7
19	CH$_2$	72.7	70
20	CH$_3$	15.5	15.2

3.2.6 Characterization of R_E-2 as 14-deoxyandrographolide

The compound R_E-2 is a brownish crystalline compound. The R_f value of the compound is 0.76 in 2% MeOH in Ethyl acetate. It is soluble in chloroform, dichloromethane, ethyl acetate, methanol & ethanol. It has tested for terpenoid & a red-violet color confirms that the compound R_E-2 is terpenoid type compound.
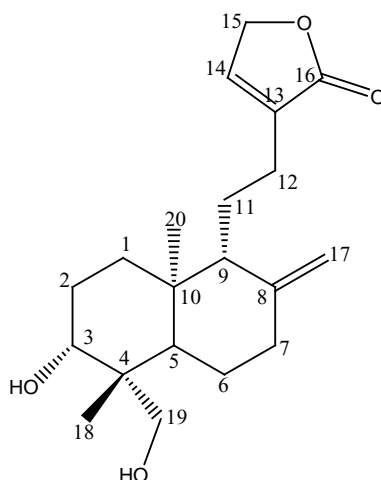
Fig: 3.6 Structure of 14- deoxyandrographolide

3.2.6.1 Characterization of R_E-2 by spectroscopic method

The structure of the compound R_E-2 **(Fig: 3.6)** has established by UV, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 & DEPT-135 spectral evidences.

3.2.2.1.1 Ultraviolet (UV) spectroscopy of R_E-2

The UV spectrum of the compound R_E-2 **(Fig: 5.6.1)** has absorption at •$_{max}$ 255 nm in methanol.

3.2.2.1.2 Infrared (IR) spectroscopy of R_E-2

The IR spectrum (in KBr) of the compound R_E-2 **(Fig: 5.6.2)** has important absorbance at 3286 (OH stretching), 2870 & 2929 (C-H stretching), 1753 (lactone ring), 1635 (C=C un-saturation), 902 (exocyclic methylene) cm^{-1}.

3.2.1.1.3 ^{1}H-NMR spectroscopy of R_E-2

The ^{1}H-NMR spectrum of R_E-2 **(Fig: 5.6.3)** indicated the presence of two methyl groups at C-18 and C-20 with three protons signals at • 1.38 (3H) and 0.78 (3H) ppm. It also exhibited two signals with fine splitting at • 5.03 (1H) and 4.74 ppm (1H) prescribed to the exocyclic methylene protons of H-17. The singlets at • 7.42 and at 4.92 ppm were attributed to H-14 and H-15. On the other hand, the one double doublet & a doublet at • 3.62 and • 3.46 ppm

are responsible for the protons at H-19. The peak of hydroxyl group at H-19 is at • 2.54 ppm.

3.2.1.1.4 ^{13}C-NMR spectroscopy of R$_E$-2

In ^{13}C-NMR spectrum in CDCl$_3$ of the compound R$_E$-2 **(Fig: 5.6.4)** the peaks at • 70 (C-19) & 80.5 (C-3) ppm confirmed the presence of two hydroxyl groups in the compound. The peaks at • 146.8 (C-8) & 107.2 (C-17) ppm spectrum indicated the presence of an exocyclic CH$_2$ group of the compound. Peaks at • 134.6 (C-13) and 144.1 (C-14) ppm in the spectrum indicated presence of double bonded carbons.

The ^{13}C-NMR exhibited exactly 20 carbon signals which suggested the compound may be a diterpene. The other singlets are 38.1 (C-1), 21.8 (C-2), 80.5 (C-3), 42.8 (C-4), 55.3 (C-5), 24.4 (C-6), 36.8 (C-7), 56 (C-9), 39 (C-10), 23.8 (C-11), 28.1 (C-12), 64.1 (C-15), 174.3 (C-16), 22.6 (C-18), 70 (C-19) and 15.1 (C-20) ppm. The DEPT 90 confirms the presence of five –CH- groups & the DEPT 135 shows the presence of nine –CH$_2$- groups & two –CH$_3$ groups of the identifying compound.

These values give us the conformation of the compound R$_E$-2 is 14-deoxyandrographolide. The comparison of all these values of with the literary survey has showed in the following table 3.6:

Table 3.6: ^{13}C-NMR da ta of c ompound R $_E$-2 compared w ith publ ished da ta[102] of 14-deoxyandrographolide:

Carbon no.	Type of carbon	14-deoxyandrographolide (Chemical shift in ppm)	R$_E$-2 (Chemical shift in ppm)
1	CH$_2$	33.2	38.1
2	CH$_2$	27.8	21.8
3	CH	74.4	80.5
4	C	45.8	42.8
5	CH	47.3	55.3
6	CH$_2$	26	24.4
7	CH$_2$	36.7	36.8
8	C	148.1	146.8
9	CH	56.4	56
10	C	40.2	39
11	CH$_2$	25.7	23.8
12	CH$_2$	31.7	28.1
13	C	155.1	134.6
14	CH	124.9	144.1
15	CH$_2$	79.5	64.1
16	C	176.7	174.3
17	CH$_2$	109.1	107.2
18	CH$_3$	21.4	22.6
19	CH$_2$	65.4	70
20	CH$_3$	13.3	15.1

3.2.7 Characterization of R$_E$-3 as 12-hydroxyneoandrographolide

The compound R$_E$-3 is a colorless crystalline compound. The R$_f$ value of the compound is 0.8 in 2% MeOH in Ethyl acetate. It is soluble in dichloromethane, ethyl acetate, methanol & ethanol. It has been tested for terpenoid & a red-violet color confirms that the compound R$_E$-3 is terpenoid type compound.
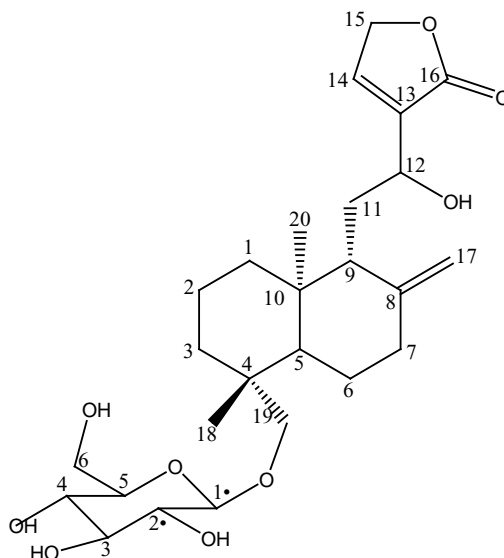
Fig: 3.7 Structure of 12- hydroxyneoandrographolide

3.2.7.1 Characterization of R$_E$-3 by spectroscopic method

The structure of the compound R$_E$-3 **(Fig: 3.7)** has established by UV, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-90 & DEPT-135 spectral evidences.

3.2.2.1.1 Ultraviolet (UV) spectroscopy of R$_E$-3

The UV spectrum of the compound R$_E$-3 **(Fig: 5.7.1)** has absorption at • $_{max}$ 270 & 427 nm in methanol.

3.2.2.1.2 Infrared (IR) spectroscopy of R$_E$-3

The IR spectrum (in KBr) of the compound R$_E$-3 **(Fig: 5.7.2)** has important absorbance at 3421 (OH stretching), 2868 & 2941 (C-H stretching), 1747 (lactone ring), 1647 (C=C un-saturation), 910 (exocyclic methylene) cm^{-1}.

3.2.1.1.3 ^{1}H-NMR spectroscopy of R$_E$-3

The ^{1}H-NMR spectrum of R$_E$-3 **(Fig: 5.7.3)** indicated the presence of two methyl groups at C-18 and C-20 with three protons signals at • 1.07 (3H) and 0.72 (3H) ppm. It also exhibited two signals with fine splitting at • 4.90 (1H) and 4.63 (1H) ppm prescribed to the exocyclic methylene protons of H-17. The singlets at • 7.49 and at 4.86 (2H) ppm were attributed to H-

14 and H-15. The peak of hydroxyl group at H-12 is at • 2.16 ppm. On the other hand, the peaks between • 3.26 & 4.23 ppm are responsible for the glucose ring.

3.2.1.1.4 ^{13}C-NMR spectroscopy of R$_E$-3

In ^{13}C-NMR spectrum in CDCl$_3$ + few drops of methanol of the compound R$_E$-3 **(Fig: 5.7.4)** the peaks at • 72.31 (C-19) & 69.76 (C-12) ppm confirmed the presence of hydroxyl methyl & hydroxyl group in the compound respectively. The peaks at • 147.23 (C-8) & 106.37 (C-17) ppm spectrum indicated the presence of an exocyclic =CH$_2$ group of the compound. Peaks at • 134.12 (C-13) and 144.23 (C-14) ppm in the spectrum indicated presence of double bonded carbons.

The ^{13}C-NMR exhibited exactly 26 carbon signals. The other peaks are • 38.12 (C-1), 18.5 (C-2), 35.53 (C-3), 39.21 (C-4), 55.95 (C-5), 24.09 (C-6), 38.63 (C-7), 56.16 (C-9), 37.89 (C-10), 21.38 (C-11), 27.12 (C-18), 72.31 (C-19), 14.86 (C-20), 103.32 (C-1•), 73.4 (C-2•), 76.13 (C-3•), 61.39 (C-4•), 75.42 (C-5•) and 61.27 (C-6•) ppm. The DEPT 90 confirms the presence of nine –CH- groups & the DEPT 135 shows the presence of ten –CH$_2$- groups & two –CH$_3$ groups of the identifying compound.

These values give us the conformation of the compound R$_E$-3 is 12-hydroxyneoandrographolide. The ^{13}C-NMR peaks have been showed in the following table 3.7:

Table 3.7: ^{13}C-NMR da ta of c ompound R $_E$-3 compared w ith publ ished da ta[102] of 12-hydrohyneoandrographolide:

Carbon no.	Type of carbon	R$_E$-3 (Chemical shift in ppm)	Neoandographolide (Chemical shift in ppm)
1	CH$_2$	38.13	39.3
2	CH$_2$	18.5	20.4
3	CH	35.53	38.5
4	C	39.21	44.6
5	CH	55.95	56.6
6	CH$_2$	24.09	26.4
7	CH$_2$	38.63	39.0
8	C	147.23	148.2
9	CH	56.16	56.0
10	C	37.89	40.9
11	CH$_2$	21.38	22.4
12	CH	69.76	25.2
13	C	134.12	134.2
14	CH	144.23	145.5
15	CH$_2$	70.28	70.7
16	C	175.05	174.4
17	CH$_2$	106.37	106.9
18	CH$_3$	27.12	28.9
19	CH$_2$	72.31	78.9
20	CH$_3$	14.86	13.5
1•	CH	103.32	102.9
2•	CH	73.4	73.3
3•	CH	76.13	76.2
4•	CH	61.39	70.0
5•	CH	75.42	75.4
6•	CH$_2$	61.27	61.7

Part-2

BIOLOGICAL ACTIVITY OF *Andrographis peniculata*

Chapter One

ANTIOXIDENT ACTIVITY

"Free Radical Scavenging Activity analysis of the crude extracts and isolated compounds of *Andrgraphis peniculata*"

4.1 INTRODUCTION

Free radical species & reactive oxygen species (ROS) may cause oxidative damage. Free radicals induced by ultraviolet light or oxidative stress contributes to skin & can adversely affect the skin health, which means antioxidants active in skin cells may support skin health.

The term "antioxidant" refers to a variety of vitamins, minerals and enzymes that help to protect the body from the formation and disposal of free radicals. The body can also manufacture its own antioxidants. Yet an increase in free radicals would place stress on the body if sufficient dietary antioxidants were not available.

Free radicals are an atom or group of atoms containing at least one unpaired electron. An electron is a particle that normally occurs in pairs and is negatively charged which is a stable arrangement.

When an electron become unpaired, it becomes unstable, and to create balance again, it will bond with another molecule, which then sets off a whole chain reaction.

Free radicals are created in normal biochemical processes and the body can normally keep them in check. But a problem is created when too many free radicals are present in the body for too long a time. It is normal to have some free radicals in a body, since they do perform certain necessary functions. Yet, modern lifestyle can lead to an excess of these free radicals being formed.

Free radicals are generated when oxygen is used to produce energy but are also produced by other external factors.

Free radicals can:

1. Affect the rate at which we age.
2. Start cancers by damaging the DNA in cells.
3. Increase heart disease by making LDL cholesterol more likely to stick to artery walls.
4. Produce cataracts and encourage degeneration of the lens of the eye that ultimately leads to blindness.

5. Contribute to inflammation of the joints, as in arthritis.

6. Damage brain cells, promoting neurological conditions such as Parkinson's or Alzheimer's disease

Foods rich in vitamin E and flavanoids are recommended components of a healthy diet. These foods include vegetables, fruits, unsaturated and monounsaturated fats (especially extra virgin olive oils), nuts, soybeans, tea and grape products. Possible benefits of supplemental antioxidants are limited to vitamin E on current evidence, although even in this case the evidence is equivocal.

Existing evidence suggests that intakes of antioxidant vitamins may need to be rather higher than current recommended dietary intakes (RDIs) – the protective effects of a diet high in fruits and vegetables occurs at dietary levels of nutrient antioxidants in the 2-3 times RDI range.

However, because of the complexity of the antioxidant system, it may be that optimal benefit from antioxidant supplementation will only be obtained from a mixture of these substances. Ideally, the best way to get them is from food – this will ensure the consumption of other unidentified but beneficial compounds.
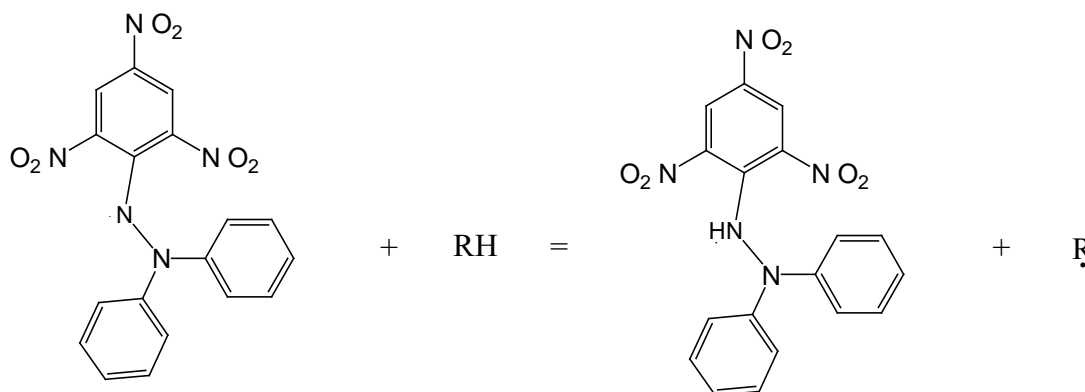
Fig: 6.1 Free radical scavenging reaction between DPPH & active compound

4.2 MATERIALS & METHODS

4.2.1 Reagents & Instrument

1. DPPH (2,2–diphenyl-1-picrylhydrazyl), (3.9 mg dissolved in 50 mL 95% EtOH).
2. 70% EtOH or dimethylsulphoxide (DMSO) was used as solvent to get the samples soluble.
3. PERKIN ELMER LAMBDA-25 UV-Visible Spectrophotometer (The absorption was measured at λ_{max} 525nm).
4. Standard: Vitamin C (Sigma Chemical Co.) vitamin E (Sigma Chemical Co.).
5. Sample (1.8-2.0 mg) preparation: The concentration of the samples was 1 mg / 1 mL (W/V) in 70% ethanol. The solutions were prepared by using vortex & sonication for 15min at 25˚C.

4.2.2 Free Radical Scavenging Activity Test (DPPH Method)

The free radical scavenging activity was assayed spectrophotometrically. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical has a deep violet color due to its unpaired electron, and radical scavenging activity can be followed spectrophotometrically absorbance at 525 nm. Sample stock solutions (1 mg/mL) were diluted to final concentrations of 200, 100, 50 & 10 μg/mL in 70% ethanol. DPPH ethanol solution (0.2 mM, 0.5 mL) was added to 1 mL of sample solutions of different concentrations, shaken well by vortex, and allowed to react at room temperature. The absorbance values were measured after 10 min at 525 nm by UV spectrophotometer. The free radical scavenging activity of samples was calculated according to the formula:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (\text{Abs}_{sample} - \text{Abs}_{blank}) / \text{Abs}_{control}] \times 100$$

Where, Abs_{sample} = absorbance of the experimental sample,

Abs_{blank} = absorbance of the blank,

$\text{Abs}_{control}$ = absorbance of the control.

As a blank, 70% EtOH (0.5 mL) and sample solution (1.0 mL) were used. DPPH solution (0.5 mL, 0.2 mM) & 70% EtOH (1.0 mL) was used as a negative control. The ascorbic acid (vitamin C) was used as a positive control. Each treatment was replicated twice.

4.3 RESULTS & DISCUSSION

Free Radical Scavenging Activity Test (DPPH Method) results were given below by the tabular and graphical form.

Table 4.1 Antioxidant property studies of R_H-1, R_H-3, R_H-4, R_H-5, R_E-1, R_E-2, R_E-3 at 200 µg/mL

Sample name	Absorbance	DPPH Inhibition			
Control 200	0.200		Control	Sample	Blank	Inhibition (%)
Vit. C	0.182	Vit. C		0.182		94
Vit. E	0.200	Vit. E		0.200		85
R_H-1	0.288	R_H-1		0.288		29
R_H-3	0.258	R_H-2		0.258		56
R_H-4	0.312	R_H-3	0.200	0.312	0.170	41
R_H-5	0.328	R_H-4		0.328		21
R_E-1	0.208	R_E-1		0.208		81
R_E-2	0.272	R_E-2		0.272		49
R_E-3	0.245	R_E-3		0.245		62.5

Table 4.2 Antioxidant property studies of R_H-1, R_H-3, R_H-4, R_H-5, R_E-1, R_E-2, R_E-3 at 100 µg/mL

Sample name	Absorbance	DPPH Inhibition				
			Control	Sample	Blank	Inhibition (%)
Control 100	0.193					
Vit. C	0.188	Vit. C		0.188		81.3
Vit. E	0.199	Vit. E		0.199		75
R_H-1	0.260	R_H-1		0.260		31.1
R_H-3	0.217	R_H-2		0.217		66.32
R_H-4	0.285	R_H-3	0.193	0.285	0.152	44.04
R_H-5	0.295	R_H-4		0.295		25.91
R_E-1	0.237	R_E-1		0.237		56
R_E-2	0.213	R_E-2		0.213		68.3
R_E-3	0.210	R_E-3		0.210		69.9

Table 4.3 Antioxidant property studies of R_H-1, R_H-3, R_H-4, R_H-5, R_E-1, R_E-2, R_E-3 at 50 µg/mL

Sample name	Absorbance	DPPH Inhibition				
			Control	Sample	Blank	Inhibition (%)
Control 50	0.175					
Vit. C	0.184	Vit. C		0.184		96
Vit. E	0.195	Vit. E		0.195		89.7
R_H-1	0.284	R_H-1		0.284		34.49
R_H-3	0.234	R_H-2		0.234		67.4
R_H-4	0.291	R_H-3	0.175	0.291	0.177	38.86
R_H-5	0.312	R_H-4		0.312		22.86
R_E-1	0.215	R_E-1		0.215		78
R_E-2	0.188	R_E-2		0.188		93.7
R_E-3	0.238	R_E-3		0.238		65.14

Table 4.4 Antioxidant property studies of R_H-1, R_H-3, R_H-4, R_H-5, R_E-1, R_E-2, R_E-3 at 10 µg/mL

Sample name	Absorbance	DPPH Inhibition				
Control 10	0.213		Control	Sample	Blank	Inhibition (%)
Vit. C	0.224	Vit. C		0.224		93.8
Vit. E	0.246	Vit. E		0.246		83.5
R_H-1	0.324	R_H-1		0.324		32.86
R_H-3	0.271	R_H-2		0.271		71.8
R_H-4	0.354	R_H-3	0.213	0.354	0.211	46.95
R_H-5	0.362	R_H-4		0.362		29.11
R_E-1	0.254	R_E-1		0.254		84.04
R_E-2	0.230	R_E-2		0.230		91
R_E-3	0.277	R_E-3		0.277		70.89

From the above four table tables it is found that compound R_H-1, R_H-4, R_H-5 shows little to moderate activity. While the other diterpene compounds (R_H-3, R_E-1, R_E-2, R_E-3) show satisfactory level of activity in DPPH method.

The above test results can be showed by line diagram for at a glance comparison:
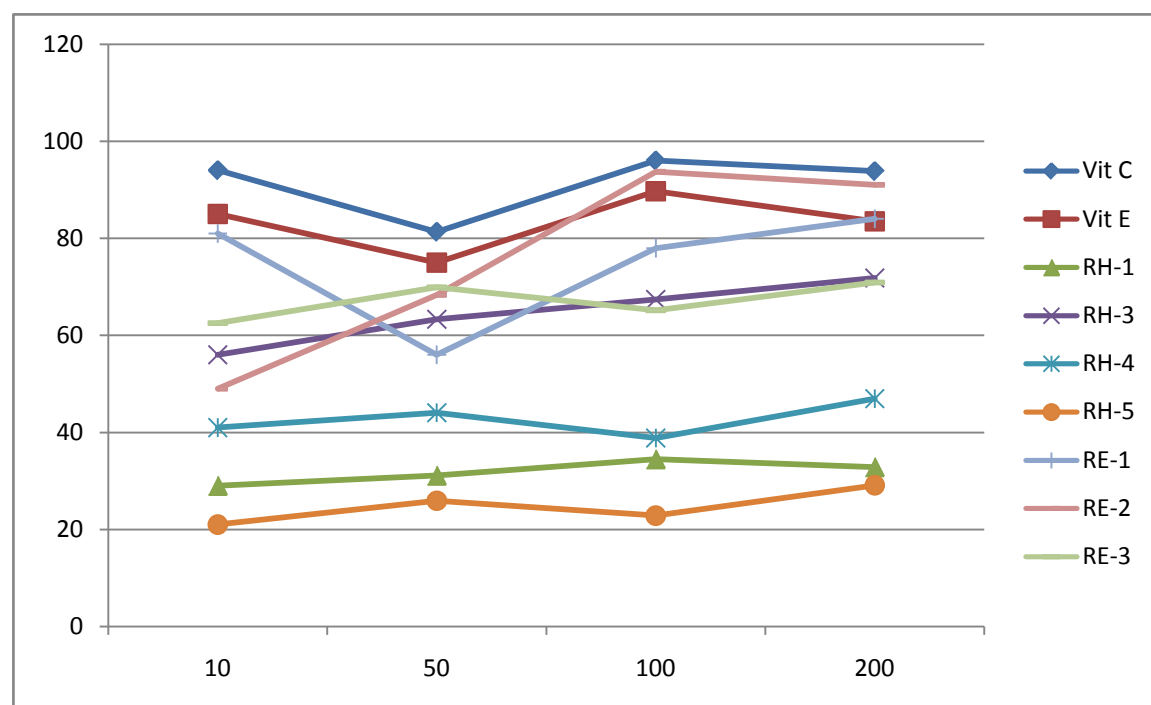
Fig:4.2 Antioxident property of the isolated compounds & standards (where the Y-axis represents percent of inhibision and the X-axis represents concentration)

Chapter Two

ANTIMICROBIAL ACTIVITY

5.1 Introduction

Bacteria and fungi are responsible for many infectious diseases. The increasing clinical implications of drug resistant fungal and bacterial pathogens have lent additional urgency to antimicrobial drug research. The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by any of the following three methods.

      i)   Disc diffusion method

      ii)   Serial dilution method

      iii)   Bio-autographic method

But there is no standardized method for expressing the results of antimicrobial screening. Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculums volume, culture medium composition, pH and incubation temperature can influence the results.

Among the above mentioned techniques the disc diffusion is a widely accepted *in vitro* investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the micro-organisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method.

5.2 Principle of Disc Diffusion Method

Solutions of known concentration (μg/mL) of the test samples are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) are then impregnated with known amounts of the test substances using micropipette. Discs containing the test material are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are then kept at low temperature ($4\,^{0}$C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the sample disc. The diffusion occurs according to the physical

law that controls the diffusion of molecules through agar gel. As a result there is a gradual change of test materials concentration in the media surrounding the discs.

The plates are then incubated at 37 ^{0}C for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment is carried out more than once and the mean of the readings is required.

In the present study the partitioned crude extracts and six purified compounds were tested for antimicrobial activity by disc diffusion method.

5.3 Experimental

5.3.1 Apparatus and Reagents

Filter paper discs	Petri dishes	Inoculating loop
Sterile cotton	Sterile forceps	Spirit burner
Micropipette	Screw cap test tubes	Nose mask and Hand gloves
Laminar air flow hood	Autoclave	Incubator
Refrigerator	Nutrient Agar Medium	Ethanol
Chloroform		

5.3.2 Test materials

5.3.2.1 Test materials of *Andrographis peniculata*

1. Three partitioned crude extracts.
2. Six pure compounds (**R$_H$-1, R$_H$-3, R$_E$-1, R$_E$-2, R$_E$-3, R$_E$-5**)

5.3.3 Test Organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both Gram positive and Gram-negative organisms were taken for the test and they are listed in the Table 5.1.

Table 5.1: List of Test Bacteria and Fungi

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

5.3.4 Culture medium and their composition

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

a. Nutrient agar medium

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

b. Nutrient broth medium

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

c. Muller – Hunton medium

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

d. Tryptic soya broth medium (TSB)

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

Nutrient agar medium (DIFCO) is used most frequently for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

5.3.4.1 Preparation of medium

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The pH (at 25^0C) was adjusted at 7.2 – 7.6 using NaOH or HCl. 10mL and 5 mL of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure/sq. inch at 121^0C for 20 minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

5.3.5 Sterilization procedures

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri dishes and other glassware were sterilized by autoclaving at a temperature of 121^0C and a pressure of 15-lbs./sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

5.3.6 Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37^0C for their optimum growth. These fresh cultures were used for the sensitivity test.

5.3.7 Preparation of the test plates

The test organisms were transferred from the subculture to the test tubes containing about 10mL of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petri dishes. The petri dishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

5.3.8 Preparation of discs

Three types of discs were used for antimicrobial screening.

   a) Standard disc
   b) Blank disc
   c) Sample discs

5.3.8.1 Standard discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, kanamycin (30μg/disc) disc was used as the reference.

5.3.8.2 Blank discs

These w ere us ed as ne gative c ontrols w hich e nsure t hat t he r esidual s olvent (left ov er t he discs even after air-drying) and the filter paper were not active themselves.

5.3.8.3 Preparation of sample discs with test samples

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the de sired concentrations in an aseptic c ondition. Sterilized metrical (BBL, Cocksville, USA) filter paper di scs were taken in a blank petri dish under the laminar hood. T hen discs were soaked with solutions of test samples and dried.

5.3.8.3.1 Preparation of sample discs with test samples of *Andrographis peniculata*

Partitioned crude 1-butanol, n-hexane and ethyl acetate extract and six pure compounds (R_H-1, R_H-3, R_H-5, R_E-1, R_E-2, R_E-3) were tested for antimicrobial activity against a number of both Gram-positive and Gram-negative bacteria and fungi.

a) Test sample for crude ethanol extract

The amount of sample per disc was 500 μg.

b) Test samples for pure compounds

Pure compounds (R_H-1, R_H-3, R_H-5, R_E-1, R_E-2, R_E-3) were tested f or a ntimicrobial activity using sample concentration of 100μg per disc.

5.3.9 Preparation and application of the test samples

The t est s amples were weighed accurately and cal culated amounts of the s olvents w ere added accordingly using micropipette to the dried samples to get desired concentrations. The test s amples w ere a pplied t o previously s terilized di scs us ing a djustable m icropipette unde r aseptic conditions.

5.3.9.1 Diffusion and Incubation

The s ample di scs, t he s tandard a ntibiotic di scs a nd t he c ontrol di scs w ere pl aced gently on the pr eviously m arked z ones i n t he a gar pl ates pr e-inoculated w ith t est b acteria a nd fungi. The plates were then kept in a refrigerator at 4^0C for about 24 hour s upside dow n to a llow sufficient di ffusion of t he m aterials f rom t he di scs t o t he s urrounding a gar m edium. T he plates were then inverted and kept in an incubator at 37^0C for 24 hours.

5.3.10 Determination of antimicrobial activity by the zone of inhibition

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the micro-organisms surrounding the discs which gives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

5.4 Results and Discussion of *in vitro* Antimicrobial screening of *Andrographis peniculata*

Partitioned crude 1-butanol, n-hexane and ethyl acetate extract and six pure compounds (**R$_H$-1, R$_H$-3, R$_H$-5, R$_E$-1, R$_E$-2, R$_E$-3**) were tested for antimicrobial activity against a number of both Gram-positive and Gram-negative bacteria and fungi. Standard disc of kanamycin (30μg/disc) was used for comparison purpose.

The crude n-hexane extract exhibited mild antimicrobial activity against most of the test organisms **(Table: 5.2)** and the ethyl acetate and 1-butanol extracts exhibited no antimicrobial activity against the test organisms **(Table: 5.2)**. The two pure compounds (**R$_H$-1, R$_H$-5**) of n-hexane extract and the three pure compounds (**R$_E$-1, R$_E$-2, R$_E$-3**) of ethyl acetate extract exhibited no antimicrobial activity against most of the test organisms **(Table: 5.3)**.

The only one pure compound **R$_H$-3** showed the similar activity as n-hexane crude extract (9-13 mm inhibition zone) against all the micro-organisms and fungi. The rest of the pure compounds showed no resistance against the micro-organisms and the fungi.

Table 5.2: Antimicrobial activity of crude ethanol extract, n-hexane part, ethyl acetate part, 1-butanol part of *Andrographis peniculata.*

Test bacteria and fungi	Ethanol extract	n-hexane part	Ethyl acetate part	1-butanol part	Kanamycin
	500 (µg/disc)				30 (µg/disc)
Gram-positive bacteria					
Bacillus cereus	---	---	---	---	32
Bacillus megaterium	---	---	--	---	32
Bacillus subtilis	14	13	---	---	33
Staphylococcus aureus	13	11	---	---	33
Sarcina lutea	12	12	---	---	33
Gram-negative bacteria					
Escherichia coli	12	11	---	---	33
Salmonella paratyphi	10	10	---	---	33
Salmonella typhi	12	11	---	---	33
Shigella boydii	11	9	---	---	33
Shigella dysenteriae	12	11	---	---	33
Vibrio mimicus	13	9	---	---	32
Vibrio parahemolyticus	10	10	--	---	33
Fungi					
Candida albicans	13	10	---	---	32
Aspergillus niger	10	10	--	---	32
Sacharomyces cerevacae	13	9	---	---	32

"---" Indicates 'No activity'.

Table 4.3: Antimicrobial activity of the six pure compounds (R_H-3, R_H-1, R_H-5, R_E-1, R_E-2, R_E-3) of n-hexane and ethyl acetate extract of *Andrgraphis peniculata.*

Test bacteria and fungi	R_H-3	R_H-1	R_H-5	R_E-1	R_E-2	R_E-3	Kanamycin
	300 (µg/disc)						30 (µg / disc)
Gram-positive bacteria							
Bacillus cereus	---	---	---	---	---	---	32
Bacillus megaterium	---	---	---	---	---	---	32
Bacillus subtilis	13	---	---	---	---	---	32
Staphylococcus aureus	11	---	---	---	---	---	33
Sarcina lutea	12	---	---	---	---	---	33
Gram-negative bacteria							
Escherichia coli	11	---	---	---	---	---	33
Salmonella paratyphi	10	---	**---**	---	---	---	33
Salmonella typhi	11	---	---	---	---	---	33
Shigella boydii	9	---	---	---	---	---	33
Shigella dysenteriae	11	---	---	---	---	---	33
Vibrio mimicus	9	---	---	---	---	---	32
Vibrio parahemolyticus	10	---	---	---	---	---	33
Fungi							
Candida albicans	10	---	---	---	---	---	32
Aspergillus niger	10	---	---	---	---	---	32
Sacharomyces cerevacae	9	---	---	---	---	---	32

"---" Indicates 'No activity'

Conclusion

A detailed phytochemical analysis has been carried out on the herb *Andrographis peniculata.* During thi s in vestigation e ight compounds w ere isolated from thi s plant e xtract. A mong them s tructural e lucidation of t he s even c ompounds w ere performed. The s tructural elucidation of the remaining compound was not carried out as its ^{1}H-NMR gives evidence for being a f atty ma terial. All t he c ompounds were identified preliminarily b y chemical methods a nd then the structural elucidations of the c ompounds w ere performed by va rious spectral methods (UV, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HMBC, HSQC e tc.) a nd were confirmed by th e c omparison with the results tha t a re a vailable i n t he published literatures. Three steroidal t ype c ompounds sitgmasterol, •-sitosterol, daucosterol and a diterpinoid c ompound, a ndrographolide, w ere isolated f rom t he n -hexane pa rt. The t hree other c ompounds, andrographanin, 14 -deoxyandrographolide and 12-hydroxyneoandrographolide w ere isolated from t he et hyl a cetate pa rt. The compound 12-hydroxyneoandrographolide, having th e f ollowing c hemical s tructure so far has b een isolated as a new compound for the first time.
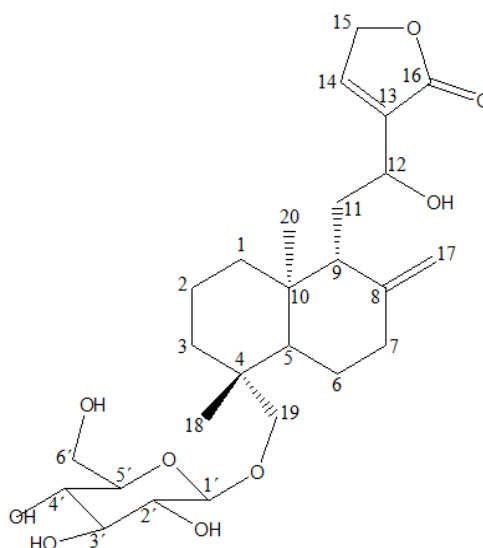
12-hydroxyneoandrographolide

The i solated di terpinoid c ompounds (andrographolide, andrographanin, 14 -deoxyandrographolide, 12-hydroxyneoandrographolide and 12-hydroxyneoandrographolide) were found to ha ve hi gh a nti-oxidant pr operty. In a ddition, andrographolide ha s a lso a nti-microbial activity.

Since the plant Andrographis peniculata contains various bioactive compounds, so it may be concluded that this plant might play a vital role as a medicinal plant.

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