# CHEMICAL AND BIOLOGICAL STUDIES OF LEAVES AND TWIGS OF *ANOGEISSUS RIVULARIS* AND SOME HYPOGLYCEMIC HERBAL FORMULATIONS

**A Dissertation for the Degree**
**of**
**Masters of Philosophy**

**Submitted by**

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# DECLARATION

Experimental work described in this thesis has been carried out by himself at the Laboratory of Organic Section, Department of Chemistry, University of Dhaka, Dhaka-1000, Bangladesh and Department of Chemistry, Mahidol University, Bangkok-10400, Thailand under our supervision. The work has not been and will not be presented for any other degree.

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**DEDICATED**
**TO**

**My Beloved Parents**

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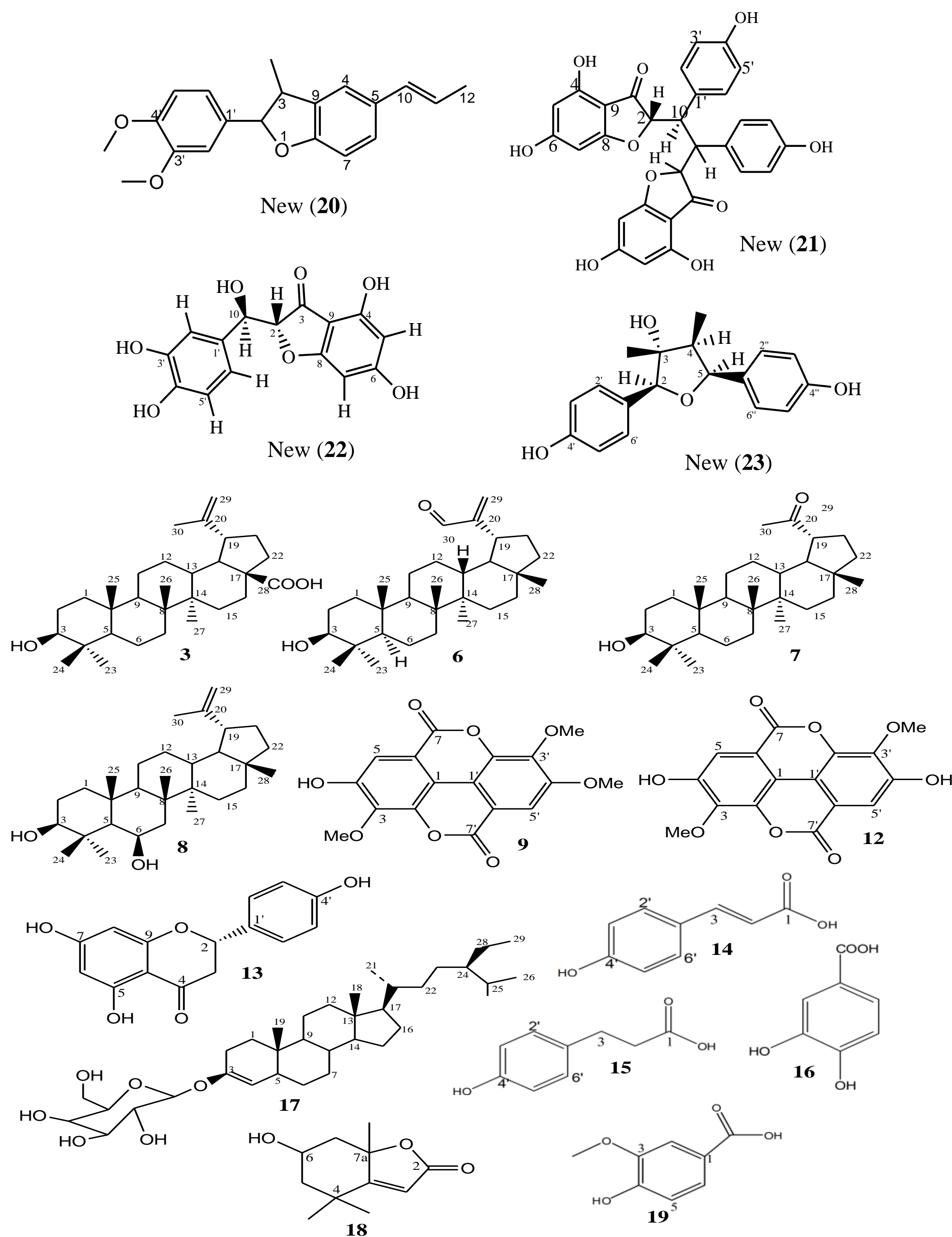
# ABSTRACT

Hexane and Ethyl acetate extracts of leaves and twigs of *Anogeissusrivularis* and their sub-fractions were tested for anticancer activities on the six common human cancer cell lines; P-388, KB, HT-29, MCF-7, A549and two normal human kidney cell lines ASK and Hek293. Sub-fractions, 1F-3, 1F-5, Et-1F-2, Et-1F-3, and Et-1F-4 were found to possess significant anticancer activities (ED50 <4, 3.40, 4.50, 4.60 and 6.37 µg/mL respectively) on P-388 cell lines. The same extracts and their sub-fractions were also tested for anti-HIV-1 RT. The two extracts showed moderate activity but the two sub-fractions, 1F-5& Et-1F-7 were found to be very active (growth inhibition 97% & 92%, respectively). From the active extracts four new compounds namely, 2-(3′,4′-Dimethoxy-phenyl)-3-methyl-5-propenyl-2,3-dihydro-benzofuran (**20**), Dimer of 4,6-Dihydroxy-2-(4′-hydroxy-benzyl)-benzofuran-3-one (**21**), 2-[(3′,4′-Dihydroxy-phenyl)-hydroxy-methyl]-4,6-dihydroxy-benzofuran-3-one (**22**), 2,5-Bis-[(4′-hydroxy-phenyl)(4″-hydroxy-phenyl)]-3,4-dimethyl-tetrahydro-furan-3-ol (**23**), together with thirteen known compounds namely, betulinic acid (3β-Hydroxy-19β-hydrogen-lup-20-(29)-en-28-oic acid)(**3**), 3β-Hydroxy-20(29)-en-lupan-30-al (**6**),29-*Nor*-20-oxolupeol (**7**), 3β,6β-Dihydroxylup-20(29)-ene(**8**),3,3′,4′-Tri-*O*-methylellagic acid (**9**),3,3′-Di-*O*-methylellagic acid(**12**), (*S*)-naringenin(4′,5,7-Trihydroxyflavanone) (**13**),*p*-Cumaric acid ((*E*)-3-(4′-Hydroxyphenyl)-2-propenoic acid)(**14**),2,3-dihydro-*p*-cumaric acid(3-(4′-Hydroxyphenyl)-propanoic acid)(**15**), protocatechuic acid (3,4-Dihydroxybenzoic acid)(**16**), Stigmast-5-en-3-*O*-β-glucoside(**17**),loliolide (6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydro-4H-benzofuran-2-one) (**18**),vanillic acid (3-Methoxy 4-hydroxybenzoic acid) (**19**) were isolated by chromatographic techniques. The structure of this compounds were elucidated by spectroscopic methods using UV, FTIR, high resolution $^{1}$H &$^{13}$C NMR and Mass spectrometry (HRMS-ESI-TOP) and Single Crystal X-ray Diffraction.




A few local herbal formulations (13) were evaluated for antimicrobial and heavy metal contaminants (Cu, Cd, Cr, Mn, Pb and Zn). All of the herbal formulations showed the presence of different bacteria & fungi and their level were also slightly higher the safe level for consumption. Lead content in almost all of the samples (except two) exceeded the permissible limit according to WHO and US FDA.

# DECLARATION

Md. Anowar Hosen made necessary correction and modifications of his thesis according to the report of one of the external examiners of examination committee. The correction and modifications were done under my supervision.

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# CHAPTER I

# INTRODUCTION

# PART-A

# Biological and Chemical Studies of *Anogeissusrivularis*Gannep O. Lecompte

## 1.1 General Introduction

Plant plays a vital role throughout our life. A major part of the four basic needs, that is food, clothing, medicine and shelter are obtain from the plant kingdom. We depend on plants directly or indirectly for all our needs. The primary compound glucose and by product oxygen, which are very essential for our life are produced in plant by the process of photosynthesis. Plants are important to human in many other ways. It is also important for oxygen balance in the atmosphere. The role of forest in controlling climate regulation is well known to us. A major of global energy is also supplied from the plants as fuel.

Plants have been used extensively as a source of medicine for the majority of the world population from the primeval age. Still now 80% of world population use plant as their primary medicine. Plants produce a lot of compounds such as glycosides, steroids, terpenoids, alkaloids, tannins, essential oils, flavonoids, pigments etc. in their cell through different metabolic pathway. These are usually called secondary metabolites and are mainly responsible for their various therapeutic properties and pharmacological actions. Isolation of the useful compounds from the plant sources started in the last century and a number of important drugs have been developed from the plants.

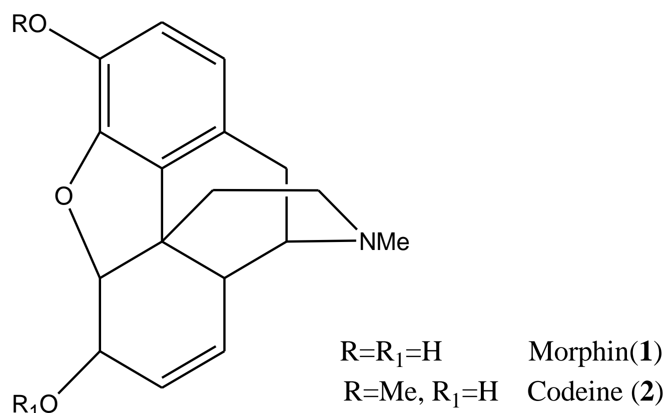
Mankind has known the important of herbs, shrubs trees for medicinal purpose from primeval age. Primitive people learned from experience that some of the plants made them sick and could cure them. Using that experience and knowledge, the natural product researchers have been trying to find out active constituents from plant source for developing new drug against various diseases including diseases like cancer, AIDS, diabetics etc. The people of different part of the world are working under collaborative research program between Chemists, Biochemists, Pharmacists, Pharmacologists, and Taxonomists to find out new active compound of plant, which have therapeutic value or making a plant products into a commercial drug.

## 1.2 Medicinal Importance of Plant Materials

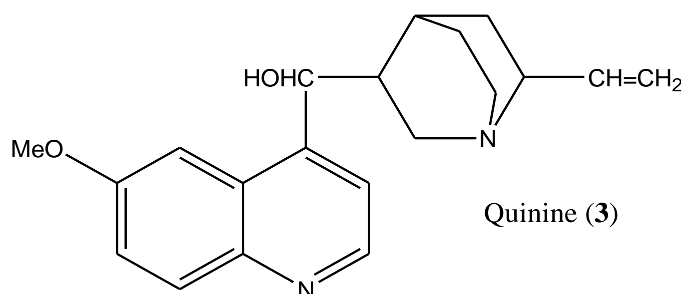
Phyto-medicines are as old as human civilization. Phyto-medicine comes to traditional medicine with or without clinical trial and phyto-medicine laid the foundation of stone of all forms of medicinal treatment that are practiced today. Illness, physical discomfort, injuries, wounds and fear of death had forced the early man to use any natural substance that he could be lay his hands on. Thus, the human started using plant as means of treatment of diseases and injuries from the early days of men's life on the earth and it is long journey from ancient time to modern age the human race successfully use plant product as a effect therapeutic tools for fighting against diseases and various health hazards.

People of the developing country countries are mostly users of traditional medicine. The World Health Organization (WHO) estimates that 80% of the people in developing countries rely on traditional medicine for their primary health care, and above 85% of the traditional medicine involve the use of plant extract. This means that the above 4 billion people in the world rely on plant as sources of drugs. In all type of diseases natural products played and continue to play a unique role in the therapeutic system. Some natural product that are used as a therapeutic agents are describe below-

The natural analgesic drug morphine (**1**) and Codeine (**2**) were isolated first in 1804 from latex of *papeversomniferium* capsules (opium). These are probably the first drug by which plant has directly contributed to the modern medicine. They are used for the depressant action on the nervous system.

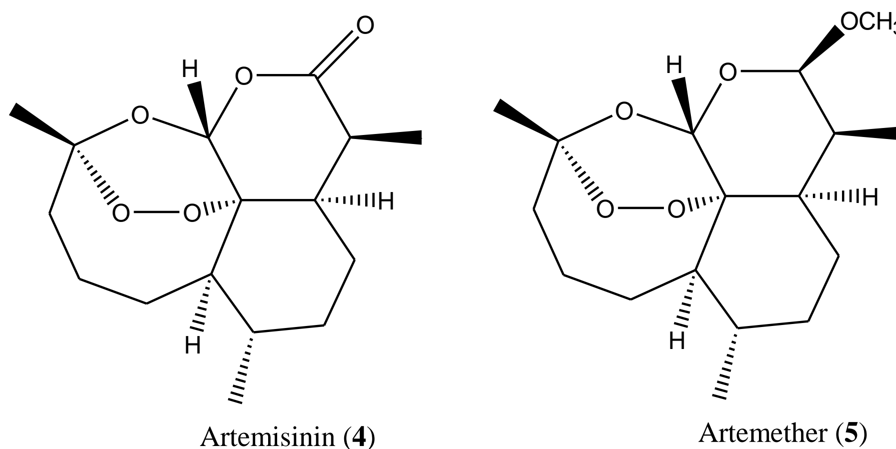


In this way quinine (**3**) was isolated cinchona bark, which still used for the treatment of malaria. Malaria is still the most important diseases and the number of clinical case is estimated to ca. 200 million annually.




Quinine (**3**)

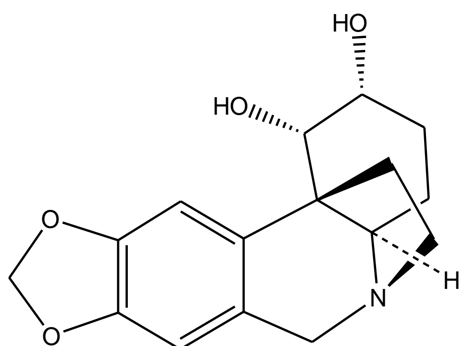
The most promising antibacterial drug artemisinin (**4**) was isolated from the herb *Artemisia annua*. The herb was used previously as remedy for malaria, which was written in ancient Chinese medical text. From this indication, in the late 1960s, Chinese researcher initiated evaluation of the various extract of this plant. Bioassay guided isolation yielded the new anti-malaria compound artemisinin(**4**) and was found to be effect in treating chloroquine resistance case and other severe case without major toxicity.



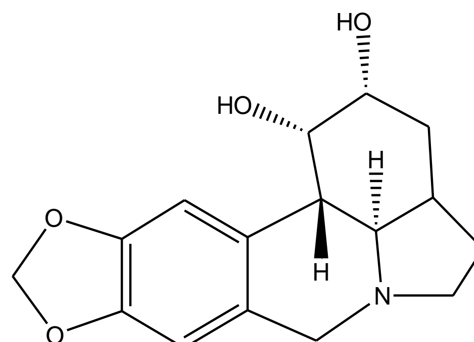

Artemisinin (**4**) Artemether (**5**)

A synthetic analogy artimisinin named artemether (**5**) was developed in the People's Republic of China [1]. Two recent clinical studies suggested that artemether (**5**) was effective as quinie (**3**) in the treatment of severe malaria [2].

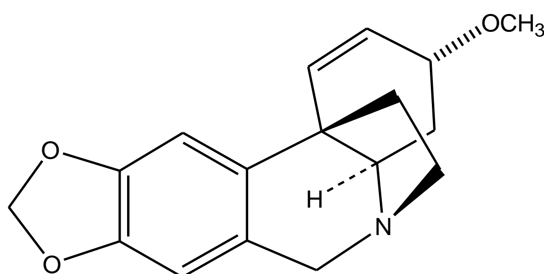
Likhitwitawuid et al. (1993) isolated some alkaloids (-) ambiline (**6**), (-) Lycorine(7), Buphanisine (**8**), (-) Augustine (**9**), (+) crinamine (**10)** from the blubs of *Crinum amabile*, which have effective antimalarial activity.



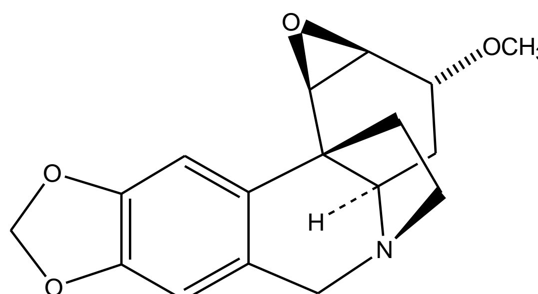

(-)- Amabiline (**6**)



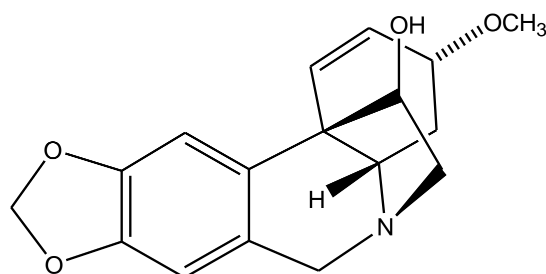

(-)- Lycorine (**7**)




(-)- Buphanisine (**8**)

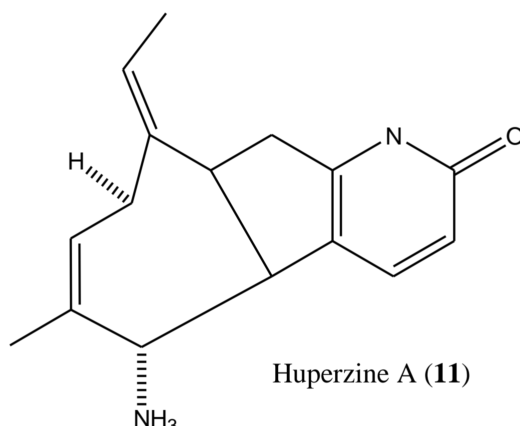



(-)-Augustine (**9**)



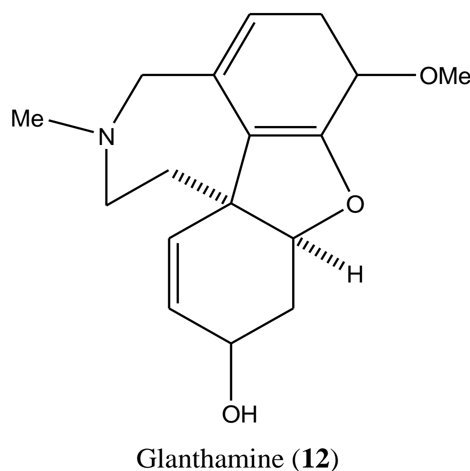

(-)- Crinamine (**10**)

In the early 1980s, a Chinese scientist isolated huperzineA (**11**) from the plant club moss as a potent, reversible and selective inhibitor of acetycholinesterase [1]. A total synthesis was developed due to very small amount present in nature and to be promising candidate for the treatment of cholinergic related nurodegenerative disorders such as Alzheimer's diseases (AD).



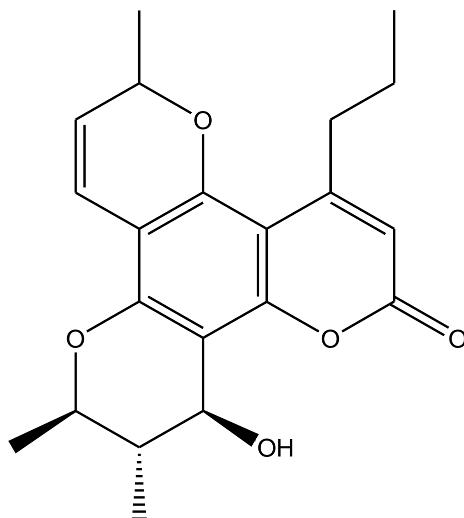
Huperzine A (**11**)

Anatural product,galanthamine(**12**), isolated from *Galanthusnivalis* in 1950s is a long acting, certainly active competitive cholinesterase inhibitor. Galanthamine under the name of nivalein is marketed in Austria for AD and in Germany for other indication such as facial neuralgia [1].



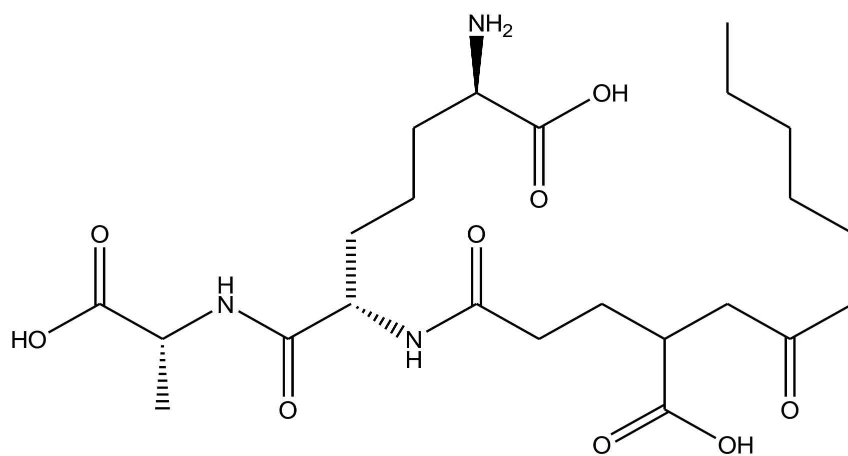
Glanthamine (**12**)

A novel coumarin derivative Calanolide A (**13**) is a reverse-transcript inhibitor isolated from Malaysian rainforest tree, *Callophylamlangerum* by NIC USA. It exhibited synergistic anti-HIV activity in combination with nucleoside reversed-transcript inhibitor, including AZT DDI and DCC [3]. In June 1997, MedichemPhermaceuticals, Inc. and the state of Sarawak, Malaysian began clinical development of Calanilide A as a potential drug for the treatment of ADIS and HIV infactions.




Calanolide (**13**)

The immune stimulating peptide FK 565(**14**), a secondary metabolites from *Streptomyces sp.* Was discovered by Fujisawa. It possesses antitumor activities against marine (p-388) leukemia. The activity appeared to be a host media effect. FK565 was brought into phase II clinical trials in the USA for the treatment of cancer and AIDS [1].




FK 565 (**14**)

## 1.3 *Anogeissus* genus

The Combretaceae family comprises 20 genera and more than 600 species. Combretum and Terminalia are the largest genera, with over 200 species each. The investigation by Smttinand tem indicated the presence of 6genera, mainly Anogeissus, Combritum, Getonia, Lumnitzera, Quisqualis and Terminalia in Thailand.[4]

Anogeissus is a genus of trees, native to South Asia, Africa and Arabian Peninsula. It belongs to the family Combretaceae.[5-6] This genus was elevated from the section of Conocurpus and distinguish from Conocurpus by the fruits.[7] Anogeissus fruits are aggregated into cone -like heads with wings or ribs and a beak at the apex, representing the persistence calyx-stalks. Conocurpus fruits have a deciduous Calyx-stalk. The genus Anogeissus comprises 19 species, namely *A. rivularis,A. acuminate, A. pierrei, A. tokinensis, A. lotifolia, A. leiocarpus, A. bentile, A.dhofarica, A. rotundifolia, A. schimperia, A. pendula, A. coronate, A. fischeri, A.sericea, A. harmandii, A. hirta, A. myrtofolai, A. nummulria*and *A. phillyreaefoli*. In Thailand onely two species are found, namely *A. acuminata*(Roxb. Ex DC) Guill&Perr. Var. *lanceolata*and *A.rivularies* (Gagnep) O. Lecopte[ST][4].

*Anogeissusrivularis* is a shrub or small tree, growing up to 15m with limb weakness, leaf stlak bract, stem and other tubes sepals short with soft hairs. It was found in Laos and Thailand. In Thailand it grows in KhongChaim district of UbonRatchathani province and Phichit province. *Anogeissusrivularis* is called by local name ‘TakhianNarm’ in UbonRatchathani province and ‘Cro-thein’ in phichit province [8]. The picture of *Anogeissusrivularis* showing bellow-

**Figure-1.1**Anogeissusrivularis Tree and Leave & Flower

Plants of *Anogeussis* are used in folkloric medicine as indicated in NAPRALERT DATA BASE. *Anogeussislatifolia* is used for the treatment of cancer on the face [9], stomach ache [10], diarrhea and urinary disease [11], treatment of snake and scorpion bite [10], colic and cough [10]. *Anogeussisleiocarpus* is used to treat syphilitic ulcers [12], malaria, diarrhea [13] typhoid fever, gonorrhea and toothache [14]. *Anogeussisshimperi* is used as chewing stick [15], as a blood purifier [16], as well as for fever and whooping cough [17]. *Anogeussissericea*is used for typhoid fever [18]. Biological activities of extracts of *Angeissus* species found in NAPRALERT DATABASE are listed in Table-1.1. Preliminary result indicate that methanol extract of stem of *Anogeissusacuminata* var. *lanceolata* exhibited HIV-1 reverse transcriptase inhibitory activity in the cell line by Raimond et al. in 1994 [19].

**Table-1.1:** Biological activities of selected *Anogeissus* species-

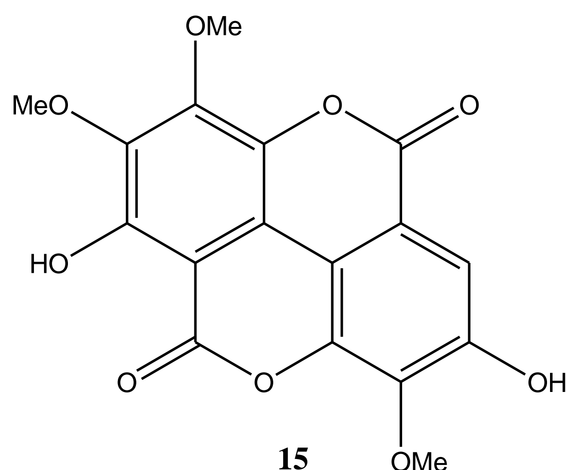
| Species | Type of Biological Activities | Ref. |
|---|---|---|
| *A. Latifolia* | Antioxident | 10,20 |
| | Antiulcer | 21 |
| | Antimicrobial | 21 |
| | Antibacterial | 22 |
| | Antihyperglycemim | 23 |
| | Antihelmintic. | 24 |
| *A. leoicarpus* | Antimicrobial | 14 |
| | Antibacterial | 25 |
| | Antifungal | 26 |
| | Anti-plasmodial | 27 |
| *A. accuminata* | Antidermatophytic | 28 |
| | Cytotoxicity | 29 |
| | HIV-1reverse transcriptase. | 19 |
| *A. pendulaEdgew* | Anti-inflamatory | 30 |
| | Antioxident. | 31 |
| *A. dhofarica* | Antioxident | 32 |

On the basis of SciFinder Scholar 2007(developed by CAS Chemical Abstracts Service) and NAPRALERT DATABASE indicated that the compounds isolated from *Anogeissus*genus were classified as follows-

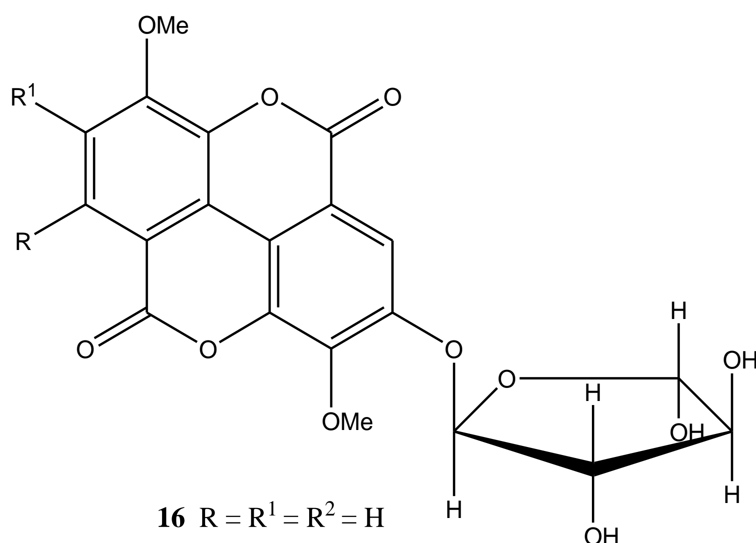
1) Coumarins
2) Lignans
3) Benzenoides
4) Triterpenoides
5) Tannins
6) Flavonoides

## 1.4Coumarins from plants in *Anogeissus* genus

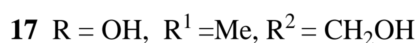
3,3′,4-tri-O-methylflavagelic acid (**15**) was repoted from the chloroform extract of the bark of *A. latifolia* by Row and Raju [33] in 1974. The extract of this plant was studied by Deshpande coworkers [34]. The investigation leading to the isolation of new glycosides of ellagic acid and flavegellic acid, i.e. 3,3′-di-O-methylellagic acid-4′-β-D-xyloside (**16**) and 3,4,3′-tri-O-methylflavellagic acid-4′-β-D- glucoside (**17**) was repoted in 1976. In 1988, Ndjui and Okwute [35] reported the identification of ellagic acid derivatives, 3,3′,4-tri-O-methylflavellagic acid (**18**) and 3,3′-di-O-methylellagic acid (**19**) from the bark of *A. schimperii* collected from Nigeria.



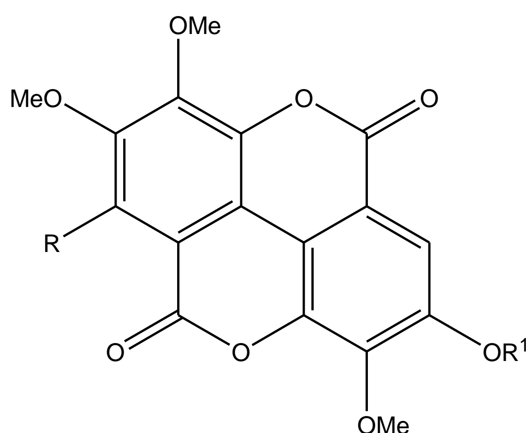

**15**




**16** R = R$^1$ = R$^2$ = H



**17** R = OH, R$^1$ =Me, R$^2$ = $CH_2OH$



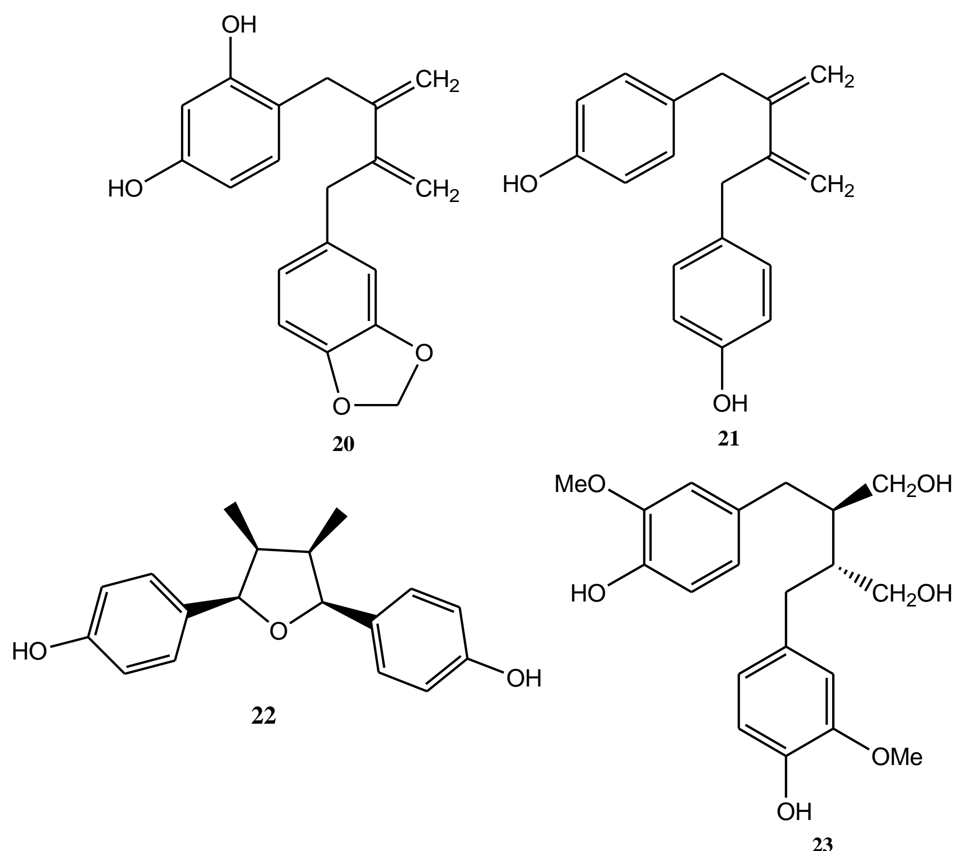
**18** R= OH, R$^1$= Me

**19** R= R$^1$= H

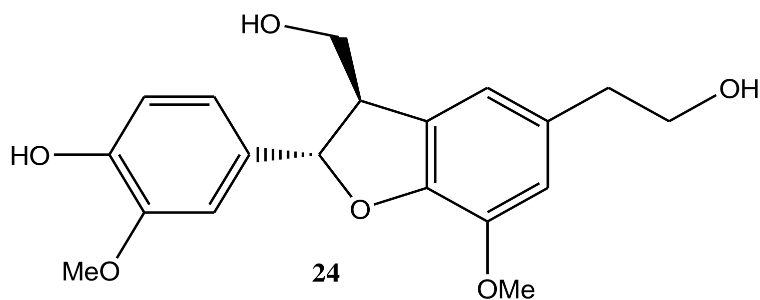
## 1.5Lignans from plant of Anogeissus genus

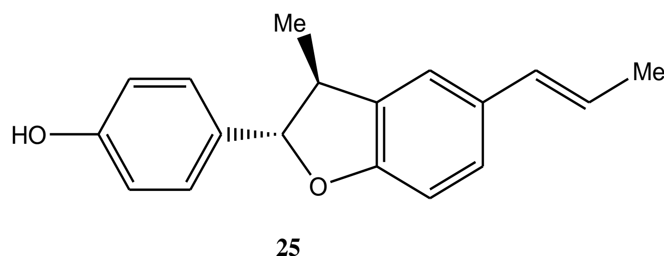
A. acuminata (Roxb.Ex DC) Guill&Perr. Var. *lanceolata* Wall ex C.B. Clarke stem, collected in Thailand was found to contain lignans. In 1994, Rimando*et al* [19] reported the isolation of four lignans, anolignan A (**20**), anolinan B (**21**), anolignan C (**22**) and (-)-secoisolaricciresinol (**23**) from methanol extract. Compound **20** and **21** were identified as the active HIV-1 reverse transcriptase (RT) inhibitory constituents of this plant. Compound **21**, which was weakly active when tested alone, showed high activity when combine with **20**. In other word, the activity of **20** was enhanced in the presence of **21**. Compounds **22** and

**23** did not have high activity against HIV-1RT. Compound **20** and **21** were weakly cytotoxic while compound **22** moderate cytotoxcity against ZR-75-1 cell time.




Another report on the isolation of compounds from the same plant in 1994 by Rimando*et al.* [29] was on the isolation of two neolignans, dihydrodehydro-diconiferyl alcohol (**24**) and conocarpan (**25**). Compound (**24**) showed moderate cytotoxic activity human melanoma cancer cell line with $Ed_{50}$ value of 11.8 µg/mL and compound (**25**) exhibited cytotoxic against several cancer cell lines, Lu-1, col-1, P-388, and ZR-75-1 with $ED_{50}$ values of 17.6, 15.7, 3.0 and 8.7 µg/mL respectively.

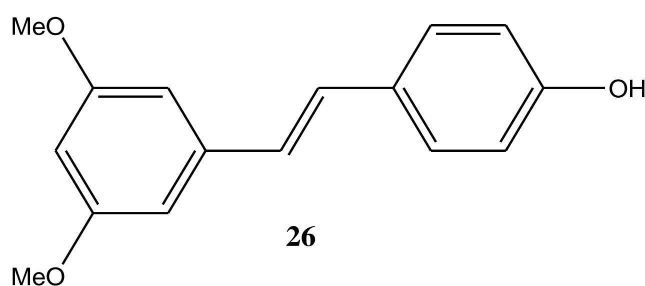





25

## 1.6Benzenoides from plants in *Anogeissus* genus

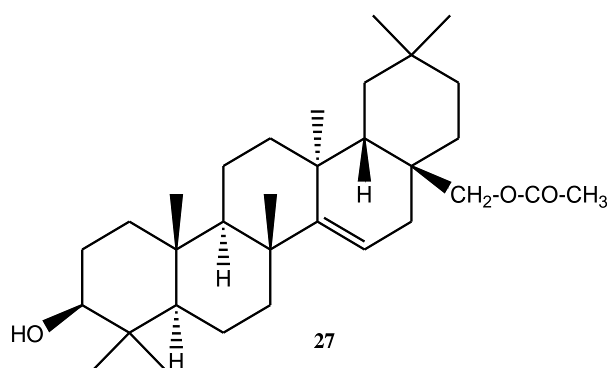
Pterostilbene (**26**) from the methanol extract of stems of *A. acuminata* (collected in Thailand) was reported by Rimando*et al* [29] in 1994. This compound was found to exhibit in vitro cytotoxicity against BC-1, HT-1080, Lu-1, Col-1, KB, KB-V1, P-388, A-341, LNCaP, ZR-75-1, and U-373, cells with $ED_{50}$ values of 3.5, 6.6, 9.2, 8.0, 6.5, 16.7, 2.3, 15.0, 10.1, 6.6 and 8.1μg/mL respectively.



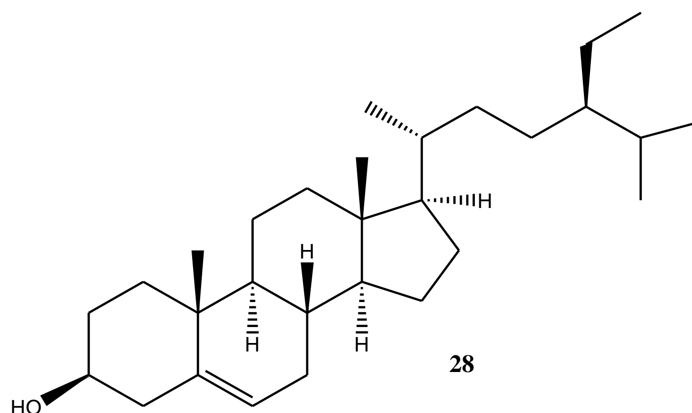

26

## 1.7Triterpenoids from plants in *Anogeissus* genus

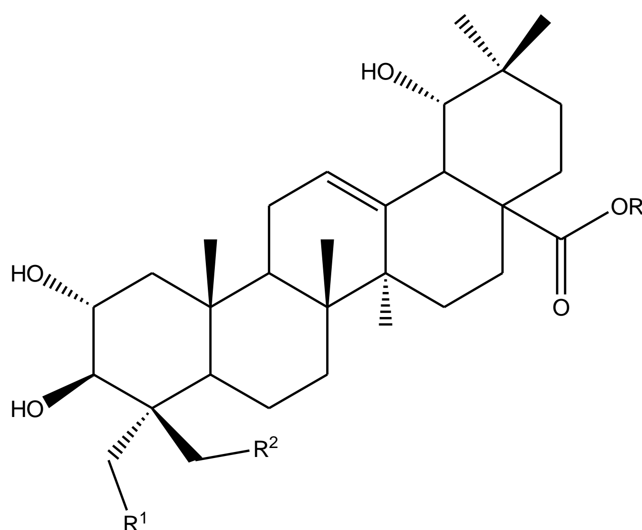
The investigation of the stem bark of *Anogeissuslatifolia* collected from Dhaka, Bangladesh, was reported by Mohammads group in 2007[37]. Two triterpenes namely 3-β-hydroxy-28- acetyltaraxaren (**27**) and β- sitosterol (**28**) were isolated from the ethyl acetate extract.




27




Furthermore the bark extract of Anogeissusleiocarpus collected from Nikola Koba National Park in southeastern Senegal, was investigated by Chaabi and Co-workers [36]. The isolation and identification of two triterpenoid glycosides, Stericosides (**29**) and tracheloperoxide E1 (**31**) as well as triterpenoides namely, Sericic acid (**30**), Trachelosperogenin E (**32**) and Arjungenin (**33**) were reported in 2008.




**29** R= H, $R^2$ = OH, R=glucose

**30** R= $R^1$= H, $R^2$ = OH,

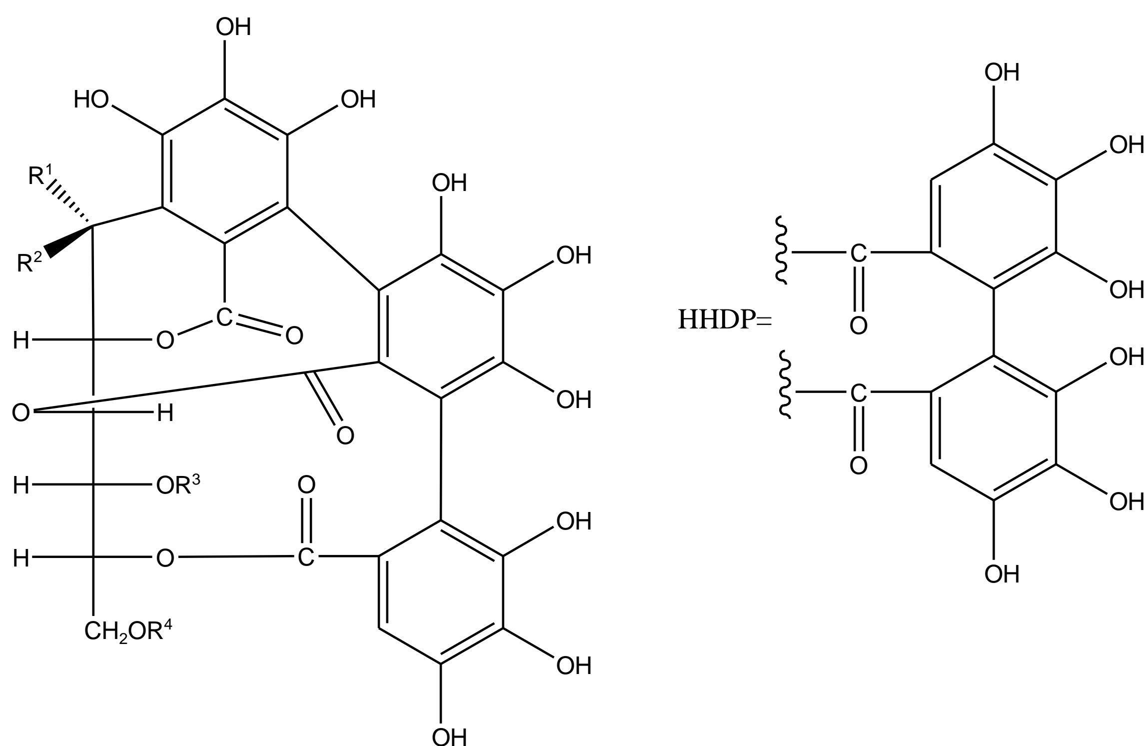
**31** R=glucose

**32** R= H, $R^1$=$R^2$ = OH,

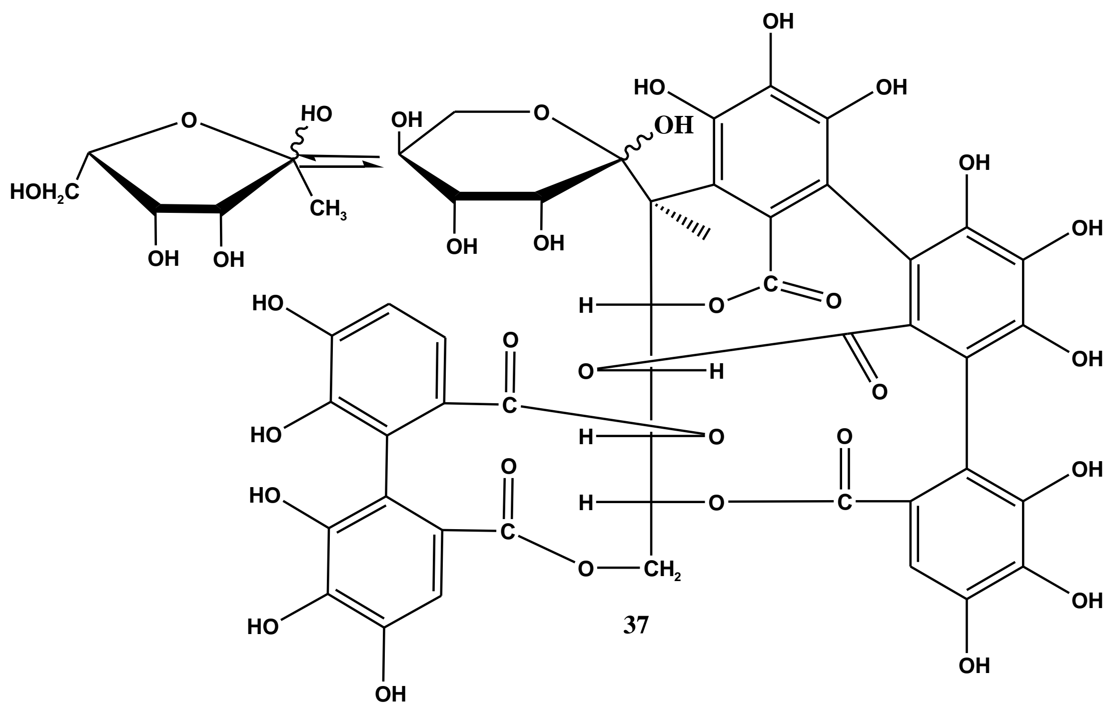
**33** $R^1$= OH, $R^2$ = R=H

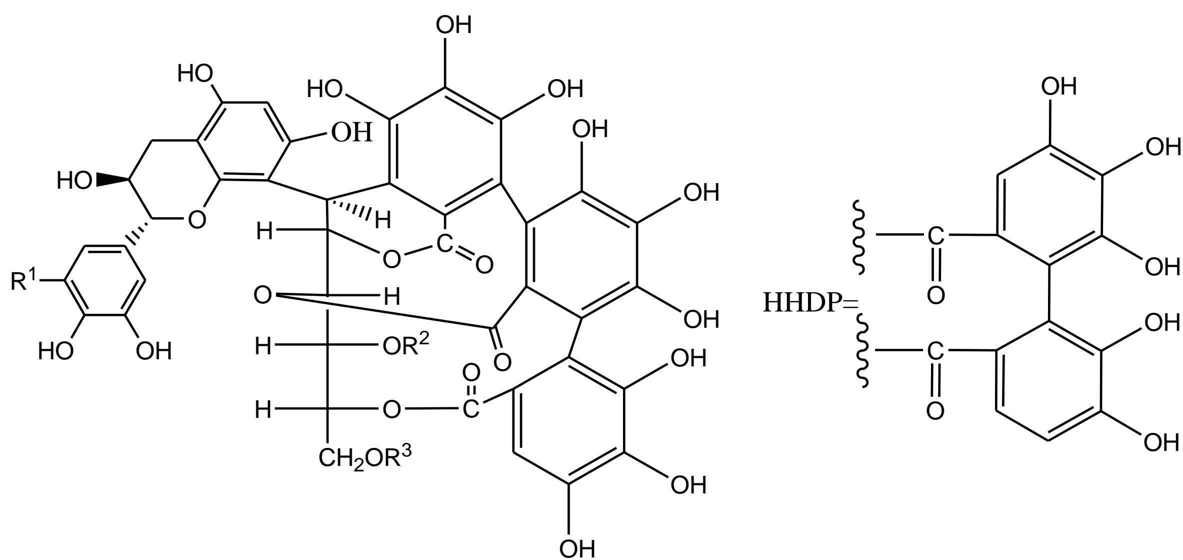
## 1.8 Tannins from plants in *Anogeissus* genus

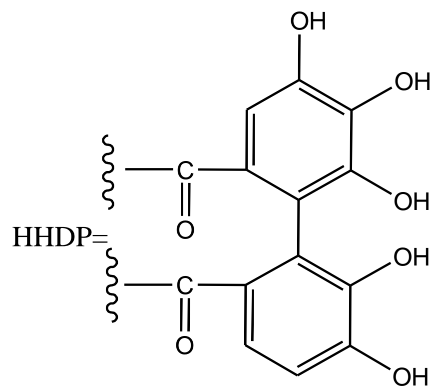
C-Glycosidichydrolyzable tannins, castalin (**34**), castalagin (**35**), vescalagin carboxylic acid(**36**), grandinin(**37**), acutissinim C(**38**), acutissinim A (**39**), eugenigrandin A (**40**) and castamollinin, together with three complex tannins (flavano- ellagitannins), anogeissinin, anoguissusins A and anogeissusin B, were isolated by Lin *et al* [38] in 1991from the bark of *A. acuminate* (Roxb. Ex DC) Guill.&Perr. Var. lanceolata wall ex C.B. Clake collected from Yunnan in Chaina.

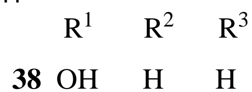



| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| **34** | OH | H | H | H |
| **35** | OH | H | HHDP | |
| **36** | H | $CO_2H$ | HHDP | |



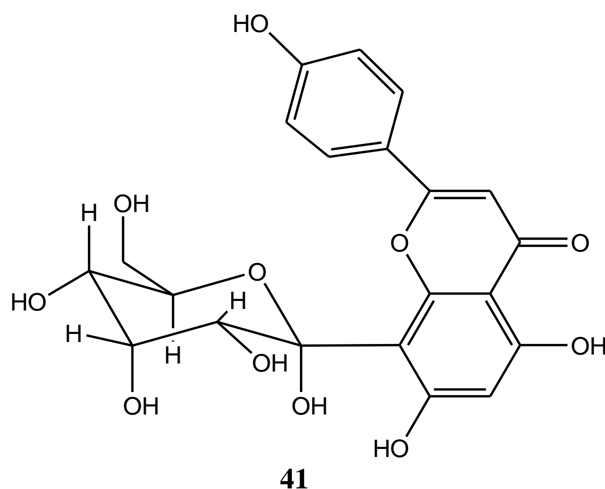


| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **38** | OH | H | H |
| **39** | OH | HHDP | |
| **40** | H | HHDP | |

### 1.9 Flavonoid from plants in *Anogeissus* genus

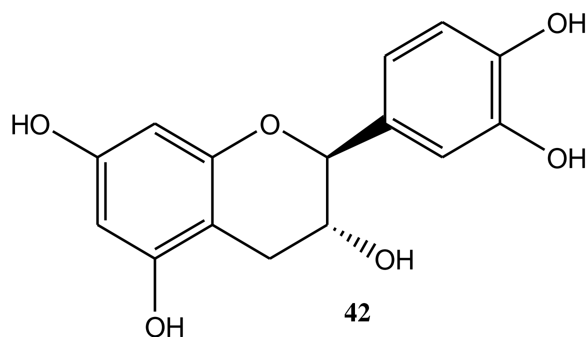
Flavonoid C- glycoside (**41**) was isolated from a methanol extract of stem of *Anogeissuslatifolia* collected in India. S.K. Chaturvedi and B.S. Chhabra [39] reported their work on this extract in 2005.



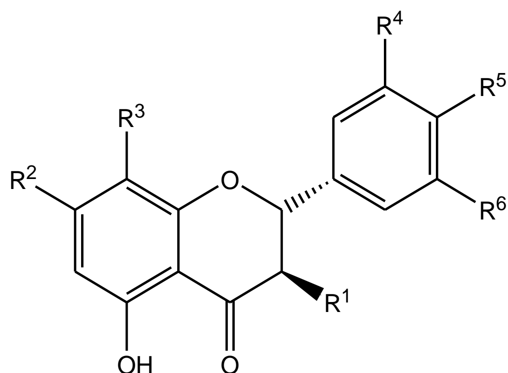

**41**

The investigation on an alcoholic extract from leaves of *Anogeissuspendula* by S. Lata and BM Bhadoria [40] in 2010 revealed the presence of a new chromone- substituted dihydrotriflavanol.

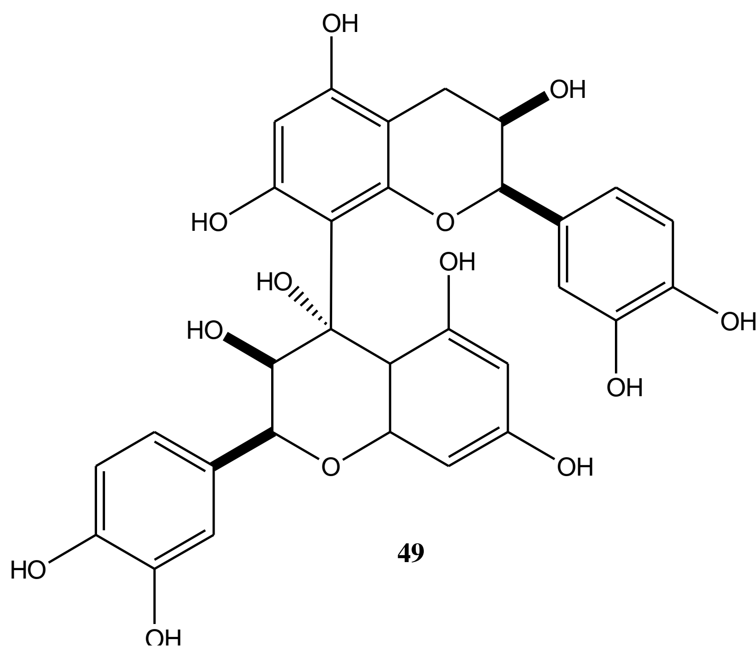
In 2011, another report by Attioua and co-worker [41] demonstrated the isolation of eight known flavonoids, cathechin (**42**), 4H-1- benzopyran-4-one-[(6-deoxy-α-L-manno-pyranosyl)oxy]-5-hydroxy-2-(4-hydroxy-3-methoxyphenul) (**43**), quercetin (**44**), isoqucercetin(**45**), rutin (**46**), vitexin (**47**), kaempferol (**48**) and procyanidin (**49**) from an ethyl acetate extract of leaves of Anogeissusleiocarpus collected near Segula (North Ivory Coast). Compound **44** and **49** showed antiplasmodial activity with $IC_{50}$ values of 6.6 and 5.3 μM respectively whereas compound (**46**) exhibited in vitro anti leishmanial activity with best $IC_{50}$ value of 1.6 μM.




**42**

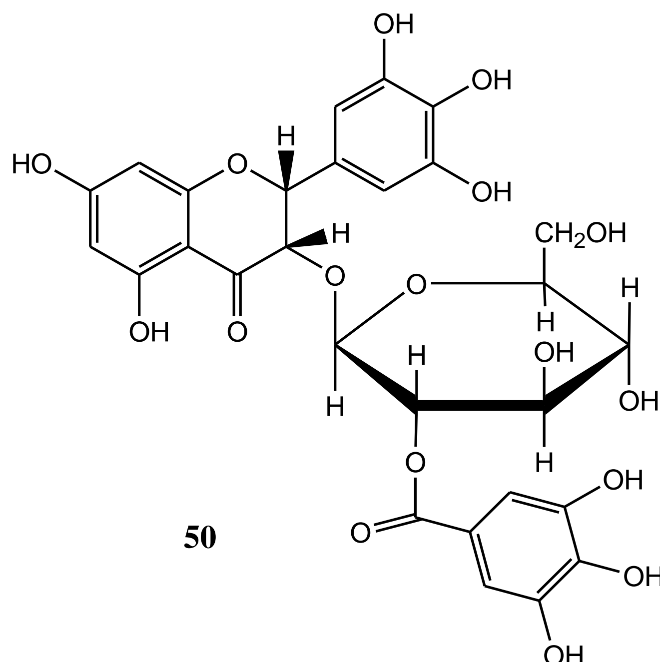



| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $R^6$ |
|---|---|---|---|---|---|---|
| **43:** | H | O-Rha | H | H | OMe | OH |
| **44:** | OH | OH | H | OH | OH | H |
| **45:** | O-Glcu | OH | H | OH | OH | H |
| **46:** | O-Gluc-Rha | OH | H | OH | OH | H |
| **47:** | H | OH | Rha | H | OH | H |
| **48:** | OH | OH | H | H | OH | H |




**49**

The isolation of 5,7,3′,4′,5′-pentahydroxydrihydroflavanol-O-(2″-O galloy)-β-D-glucopyroside (AP1) (41) was reported by Arunadevi*et al* [42] from *Anogeissuspendula*Edgew in the same year.




This type of compounds has biological activities especially Anti HIV, Antimicrobial, Cytotoxicity etc. Ongoing works to search for bioactive compound from plants, numerous biologically testing including cytotoxic, anti-inflammatory, anti-microbial and anti-oxidant activities are the development of folk-medicine. In this thesis *Anogeissusrivularis* was chosen to be investigated for its chemical constituent, as well as biological activities of the isolated compounds.

## 1.10 Objective of the present research work

Infectious diseases are the world's leading cause of premature death and killing a large number of people in every year. Since diseases, decay, and death have always coexisted with our life, so the study of diseases and their treatment must have been contemporaneous with the down of intellect. Among all other diseases the most dangerous and deadly diseases are cancer and AIDS. Still no unique therapeutic agent has been discovered which could completely cure this diseases. Plants have been the almost exclusive source of drugs for the majority of the world population since the primeval age. It is apparent that science made a lot of progress in the field of medicine, but still plant

materials continue to play a major role in primary health care and as therapeutic remedies in many developing countries. The approach to discover and developed new drugs from plant origin is therefore an alternative area along with complementary synthetic and biosynthetic approaches.

The phytochemical studies on medicinal plants serve dual purpose of bringing up the new therapeutic agent and provide useful lead for further studies directed towards the synthesis of new drugs, modeled on the basis of chemical structure of the natural product. Moreover, they promote studies in the correlation of chemical structures of the plant materials (cordell 2000). Bangladesh is a developing country where different types of in curable diseases are prevalent. But the country is blessed with large number of medicinal plants and marine organisms.

During the course work of this study, a number of plant materials were investigating on the basis of ethenomedical and folkloric reputations. On the basis of above studies **Leaves and Twigs of *Anogeissusrivularis*** were selected for isolation and characterization of bioactive compounds. Cytotoxicity of the plant extract was studied on different cell lines (P-388, KB, HT29, MCF-7, A549, ASK AND Hek293) and Anti-HIV-1RT was also studied. Isolation and characterization of compounds from plant part were also done by chromatographic and spectroscopic methods. The whole works were carried out in the Department of Chemistry, **Mahidol University (MU)**, **Thailand**, under a collaborative research program between MU and University of Dhaka.

# PART-B

## Herbal Formulations

### 1.11 General Introduction

Over the resent times, the popularity of herbal medicine is increasing to such an extent that around 20% of world population is now using herbal medicine in different forms for different purposes[43]. In developing countries, it is estimated that 70-80% of the populations somehow relay on nonconventional medicines mainly herbal origins for the primary health care [44], as they are cheap and easily accessible[45]. Herbal preparation are produced from any raw or processed part of a plant, which includes leaves, stems, flowers, roots, seeds and in most of the case it is a complex mixture of organic chemicals from natural sources[43,46,47]. As different plant parts are used in a herbal preparation, it may carry a large number of various kinds of microbes originating from soil usually adhering to different part of herbs [47]. More over in some of the herbal preparations, particularly Ayurvedic formulation, the use of heavy metals is intentional, as some of these heavy metals are believed to have beneficial effect on our body. In general, most of the common contaminants are heavy metals, pesticides, microbes and mycotoxine [48,49].

### 1.12 Use of Herbal formulation for Diabetic Mellitus

The range of the usage [50] of herbal preparations is vast as they are frequently used in the treatment of several chronic diseases including type 2 diabetics (diabetic mellitus). Diabetics is a noncommunicable heterogeneous group of disorder and affects approximately 200 million individual globally. More over it predicted that over 300 million people will be diabetic by 2015[50,51]. In general this posses challenges to the health care and social welfare but in particular, it is a huge challenge to developing country like Bangladesh because of it limited resources and weak economy. The trend of use of antidiabetic herbal preparations (mostly based on based on Ayurvedic and Unani formulary) is increasing day by day among the population of Bangladesh. In parallel, there is a rising concern regarding the safety and efficacy of these herbal preparations as most of them contain contain different contaminant including microbial contaminants and heavy metals (particularly in Ayurvedic preparation). In the most of the developed countries, herbal preparations are defined as dietary supplement. As a result, unlike pharmaceutical preparations, manufacturers are producing, selling and marketing herbal preparations without any

evidence based scientific study regarding their safety and efficacy [43]. Although in several countries herbal medicine (a part of complementary and alternative medicine) is the officially approve system, there is no guidelines and regulations for assuring the safety of this preparations.

## 1.13 Safety and Efficacy of Herbal Preparation

The safety of these herbal preparations is very important because Ayurvedic formulation contains several heavy metals as therapeutic ingredients. But the use of these heavy metal beyond the limit could be toxic. Moreover the level of microbial contamination of herbal preparation is dependent on the quality of raw materials used and manufacturing environment. Most raw materials for herbal preparations support some form of microbial growth, as medicinal plants used in herbal preparations provide nutrition to microorganisms and facilitate the multiplication of microorganism. In addition, inappropriate cleaning, unsuitable transportation, prolonged drying and storage, in adequate hygiene of producers, and congenital climatic conditions render the medicinal plants vulnerable to infestations and exposed them to many microbial contaminations. Inadvertent contamination, like fungal contamination during the production stage can also lead to deterioration in safety and quality as the risk of mycotoxin production, specially aflatoxin, may arise which has proven mutagenic, carcinogenic, teratogenic, neurotoxic, nephrotoxic, and immunosuppressive activities [52-58]. Therefore, it is important to evaluate the safety of these anti diabetic herbal preparations based on relavent scientific investigation. This research project focus on the safety of antidiabetic herbal preparation available in Bangladesh particularly related to heavy metal and microbial contamination.

## 1.14 Objective of the present research work

In Bangladesh several herbal antibiadetic herbal preparations are readily available and are being used, but studies regarding microbial contaminants and heavy metal content in locally produced herbal preparations are limited. Therefore, it is necessary to evaluate microbial contamination as well as heavy metal content in some locally produced and widely used herbal preparations. In this study, we investigated the level of microbial contamination and heavy metal content present in antidiabetic herbal preparations widely used and formulated in Bangladesh.

**Figure-1.2** Some herbal formulation of Bangladesh

# CHAPTER II

# EXPERIMENTAL

# PART-A

## Biological and Chemical Studies of *Anogeissus rivularis* Gannep O. Lecompte

## 2.1 General Methods

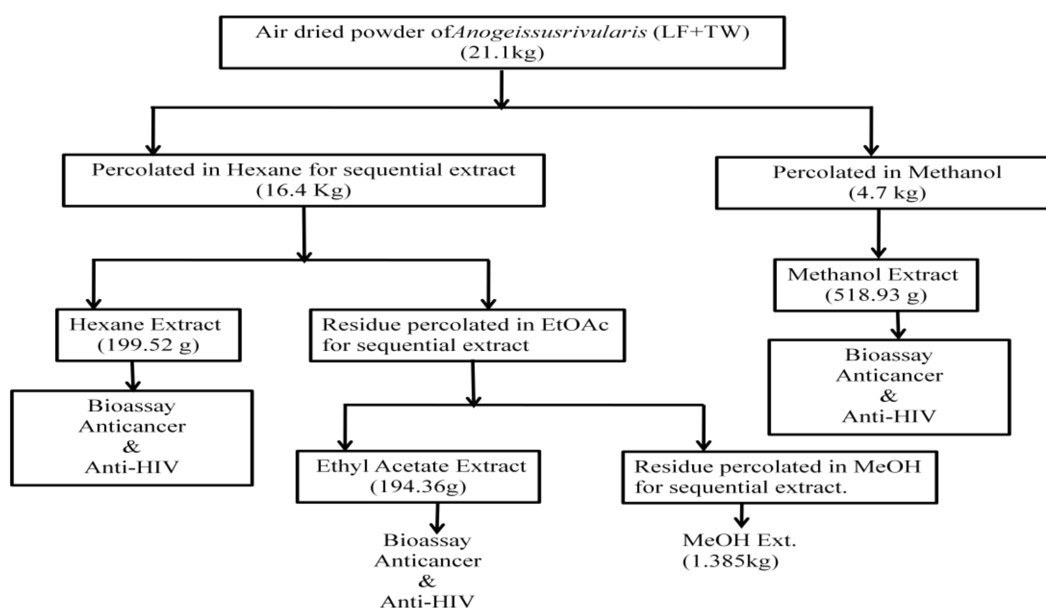
Melting points (uncorrected) were recorded in $^{o}C$ and were determined on a digital Electrothermal Melting apparatus. Infrared spectra were recorded by using Perkin Elmer System 2000 FT-IR. Major bands ($\nu_{max}$) were recorded in wave number ($cm^{-1}$). Optical rotations were measured on a JASCO DIP 370 digital polarimeter by using a 50 mm microcell (1 ml). Ultraviolet absorption spectra were measured in ethanol, methanol and dichloromethane solutions on a JASCO 530 spectrophotometer. Principle bands ($\lambda_{max}$) were reported as wavelengths (nm) and log ε. Low resolution EI mass spectra were recorded on a Thermo Finnigan Polaris Q mass spectrometer at 70 eV (probe). The high resolution mass spectra (HRMS) were recorded on a Micromass model VQ-TOF2. The high resolution nuclear magnetic resonance spectra were mainly recorded on Bruker AV-500 spectrometer. Solvents for extraction, chromatography and recrystallization were distilled at their boiling point ranges prior to use. Pre-coated TLC aluminum sheets of silica gel 60 $PF_{254}$ (20 x 20 cm, layer thickness 0.2 mm) were used for analytical purposes and the bands were visualized by ultraviolet light (at $\lambda_{max}$ 254 and$\lambda_{max}$ 366 nm) and/or spraying solution of anisaldehyde [abs. EtOH (90 mL), $H_2O$ (3 mL), anisaldehyde (2 mL), conc. $H_2SO_4$ (2 mL)]. Plates of silica gel $PF_{254}$(Art. no. 7747), 20 x 50 cm, thickness 1.25 mm, activated at 120 $^{o}C$ for 2h were utilized in case of separation by preparative TLC technique. Bands were visualized by ultraviolet light either at $\lambda_{max}$ 254 or $\lambda_{max}$ 366 nm. Column chromatography was performed by using silica gel 60H (70-230 mesh ASTM, cat. No. 7734, E. Merck) and vacuum column chromatography was performed by using silica gel 60H (230-400 mesh ASTM, Art. no. 7731, E. Merck).

## 2.2 Plant Materials

The leaves and twigs of *Anogeissus rivularis* were collected fromUbon Ratchathani province of Thailand in July, 2011. A voucher specimen (BKF no. 173843) has been deposited at the Forest Herbarium, Royal Forest Department, Ministry of Agriculture and Cooperative, Bangkok, Thailand.

## 2.3 Extraction procedure for Chemical &Biological Studies

Air-dried and finely powdered leaves and twigs of *Anogeissus rivularis* (21.1kg) were divided to two parts; one part (16.4kg) was subjected to sequential extraction with hexane, ethyl acetate and methanol at room temperature and other part (4.7kg) was subjected to direct methanol extraction.All extracts were submitted to test the biological activities as well as the results were summarized in Scheme2.1.

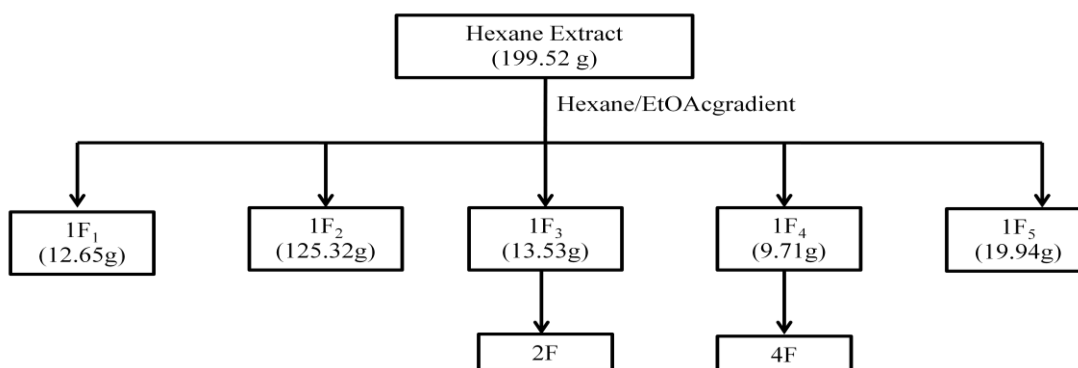



**Scheme-2.1** Extraction procedure of leaves and twigs of *A. rivularis*.

### 2.4 Purification of Hexane extract:

Air-dried and finely powdered leaves and twigs of *Anogeissus rivularis* (16.4kg) was successively macerated with hexane (5 x 10.9 L) at room temperature, followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to give a crude hexane extract (199.52g).

The hexane extract (199.52g) was subjected toflash column chromatography on silica gel[1000 g, Merck Art. No.7736: ∅ 15 x16 cm], eluting with 100% hexanes(4mL), followed by 5%(4L), 10%(6L), 15%(6L), 20%(6L), 25%(6L), 40%(4L), 60%(2L), 80%(2L) ethyl acetate/hexaneand 100% ethyl acetate followed by 100% methanol (300 mL). Fractions (500 mLeach) were collected and combined on the basis of theirTLC pattern and NMR characteristics to give five fractions ($1F_1$–$1F_5$) Scheme 2.2.




**Scheme 2.2** Fractionation of hexane extract of leaves and twigs of *A. rivularis*.

**Fraction $1F_1$** (12.65 g), eluted with 100% hexane and 5% hexane/EtOAc, was obtained as a yellow semi-solid and was found to contain mainly fat, so further purification was not carried out.

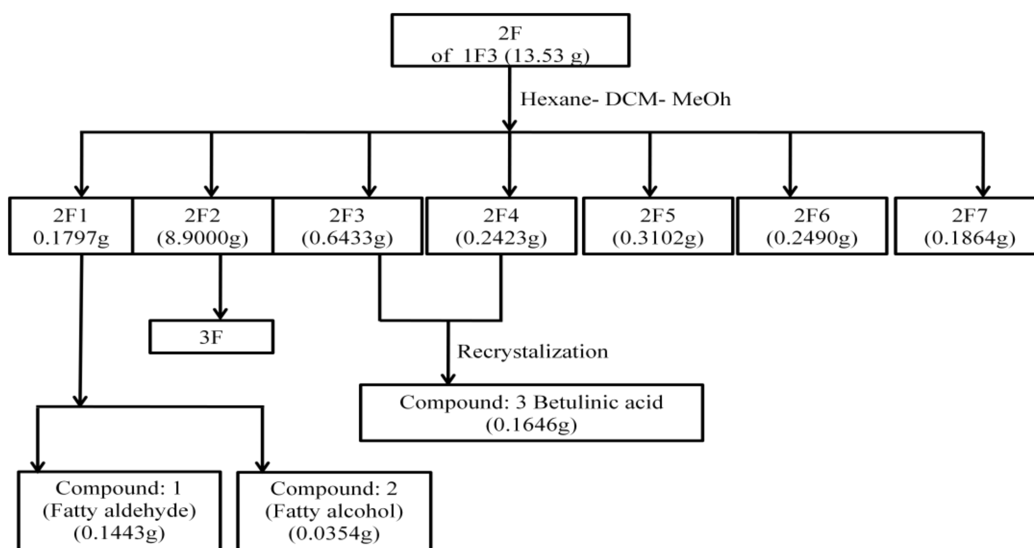
**Fraction $1F_2$** (125.32 g), eluted with 10-15% hexane/EtOAc, was obtained as a greenish yellow semi-solid and was found to contain mainly fat, so further purification was not carried out.

**Fraction $1F_3$**(13.53 g), eluted with 20-25% hexane/EtOAc, was obtained as a green semi-solid. It was separated by column chromatography on silica gel (300 g, Merck Art.No.7734, 7.32 x 20 cm), eluting with hexane/$CH_2Cl_2$, followed by increasing amount of MeOH in hexane/$CH_2Cl_2$and finally with MeOH. Fractions (200 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions$2F_1$–$2F_7$ (Scheme 2.3).

**Fraction $1F_4$**(9.71 g), eluted with 25-40% hexane/EtOAc, was obtained as a green semi-solid. It was separated by column chromatography on silica gel (400 g, Merck Art.No.7734, 7.32 x 24 cm), eluting with hexane, followed by increasing amount of acetoneand finally with MeOH. Fractions (200 mL each) were collected, monitored by TLC

and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $4F_1$–$4F_6$ (Scheme-2.12).

**Fraction $1F_5$** (90.94 g), eluted with 60-100% Hexane/EtOAC and 100% MeOH, was obtained as a greenish yellow semi-solid and was found to contain mainly fat, so further purification was not carried out.



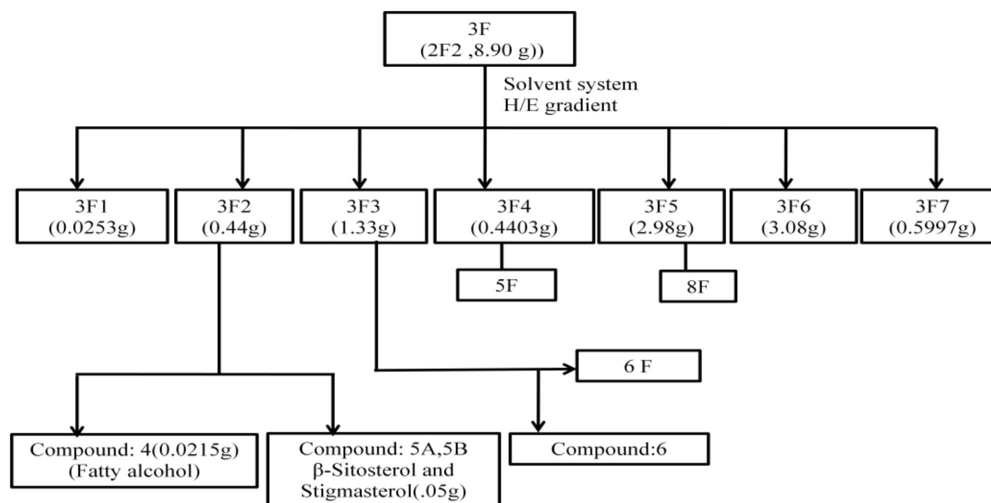

**Scheme 2.3** Fractionationof $1F_3$(2F) of hexane extractobtained from 1$^{st}$ column

**Fraction $2F_1$** (0.1797 g), eluted with 30% hexane/$CH_3Cl_2$, was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out. Pure fatty aldehyde and fatty alcohol(Compound-**1&2**) also found form this fraction.

**Fraction $2F_2$** (8.90 g), eluted with 70:30:2-70:30:2.5; hexane:$CH_2Cl_2$:MeOH was obtained as a greenish yellow semi-solid. It was separated by column chromatography on silica gel (450 g, Merck Art.No.7734, 7.32 x 26 cm), eluting with hexane, followed by increasing amount of EtOAc in hexane and finally with MeOH. Fractions (100 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $3F_1$–$3F_7$ (Schem-2.4).

**Fraction $2F_3$&$2F_4$** (0.8856 g), eluted with 3:30:70-4:30:70; MeOH: $CH_3Cl_2$: hexanes, was obtained as a green semi-solid with crystals. Pure **Betulinic acid (compound 3)** also found form this fraction by recrystalization.

**Fraction $2F_5$, $2F_6$**and **$2F_7$** eluted with 6:30:70; Hexane$CH_2Cl_2$: MeOH to 100% MeOH,was obtained as a greenish yellow semi-solid and was found to contain mainly fat, so further purification was not carried out.




**Scheme 2.4**Fractionationof the 3Fof hexane extract of leaves and twigs of *A. rivularis*.

**Fraction $3F_1$** (0.0253 g), eluted with 5% hexane/EtOAcwas obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Fraction $3F_2$** (0.44 g), eluted with 7-10% hexane/EtOAcwas obtained another fatty alcohol as white powder (**compound4**).Pure **β-sitosterol** and **stigmasterol**(**compound5**) 0.05g also found form this fraction as a mixture.

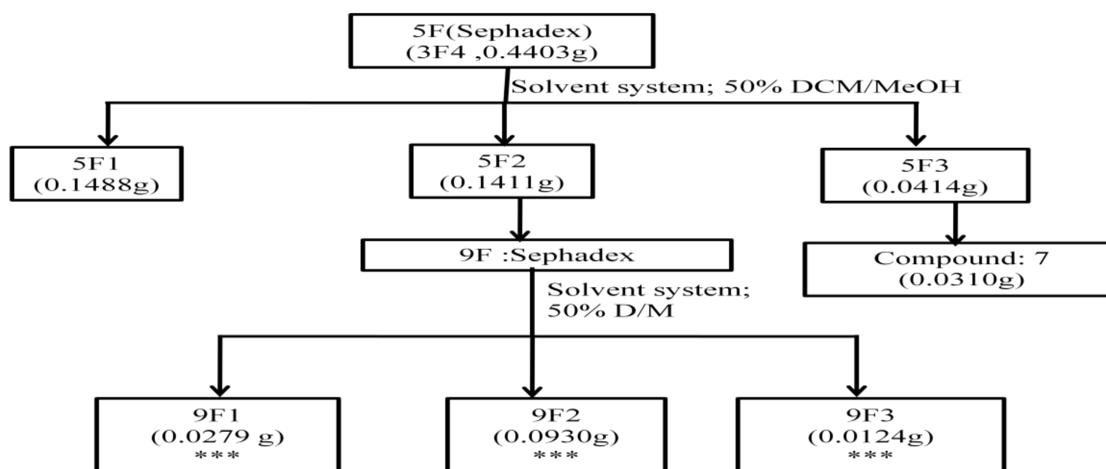
**Fraction $3F_3$** (1.33 g), eluted with 10-14% hexane/EtOAcwas obtained as a green semi-solid with white needle crystal of 3β**-hydroxy-20(29)-en-lupan-30-al (compound 6)**.To get compound -6 more it was separated by column chromatography on silica gel (66 g, Merck Art.No.7734, 3 x 22 cm), eluting with hexane, followed by increasing amount of EtOAc in hexane and finally with MeOH. Fractions (50 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $6F_1$–$6F_6$ (Scheme-2.6).

**Fraction $3F_4$** (0.4403 g), eluted with 16% hexane/EtOAc, was obtained as a green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting

with 50/50; $CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $5F_1$–$5F_3$ (Scheme-2.5).

**Fraction $3F_5$**(2.98 g), eluted with 16% hexane/ EtOAc, was obtained as a greenish brown semi-solid. It was separated by column chromatography on silica gel (165 g, Merck Art.No.7734, 4.4 x 26 cm), eluting with hexane, followed by increasing amount of EtOAc in hexane and finally with MeOH. Fractions (50 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $8F_1$–$8F_7$ (Scheme-2.9).

**Fraction $3F_6$and $3F_7$**, eluted with 16-100 hexane/EtOAc and 100% MeOH, was obtained as a brown semi-solid and was found to contain mainly fat, so further purification was not carried out.




**Scheme 2.5**Fractionationof the 5Fof hexane extract of leaves and twigs of *A. rivularis*.

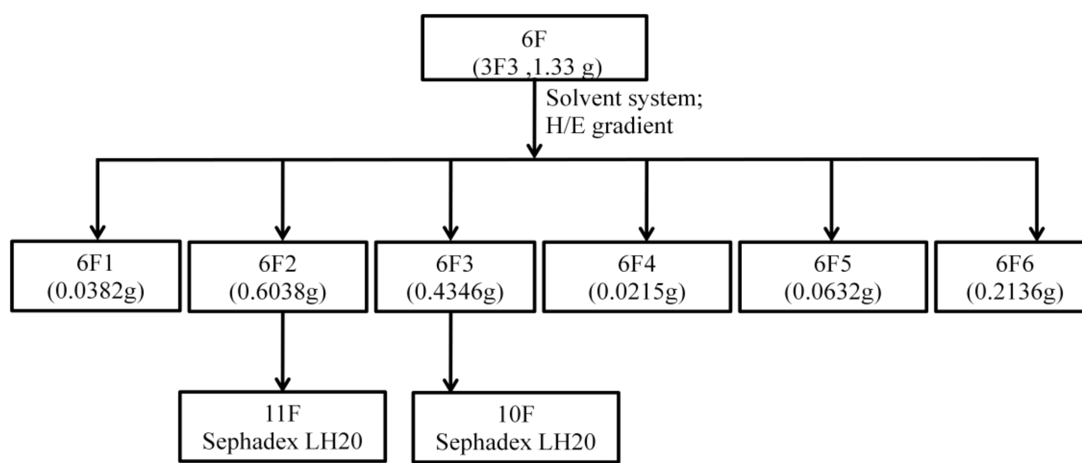
**Fraction $5F_1$**, eluted with 50% $CH_2Cl_2$/MeOH, was obtained as a green semi-solid and was found to contain mainly porphyrine derivatives, so further purification was not carried out.

**Fraction $5F_2$** (0.1411 g), eluted with 50% $CH_2Cl_2$/MeOH,was obtained as a green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% $CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and

combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $9F_1$–$9F_3$ (Scheme-2.5).

**Subfraction $9F_1$, $9F_2$ and $9F_3$**was obtained darkbrown semi-solid and this fraction didn't have interesting NMR peak, so further purification was not carried out.

**Fraction $5F_3$**, eluted with 50% $MeOH/CH_2Cl_2$, was obtained 0.0310g pure white needle crystal of **29-*Nor*-20-oxolupeol** (**Compound7**).



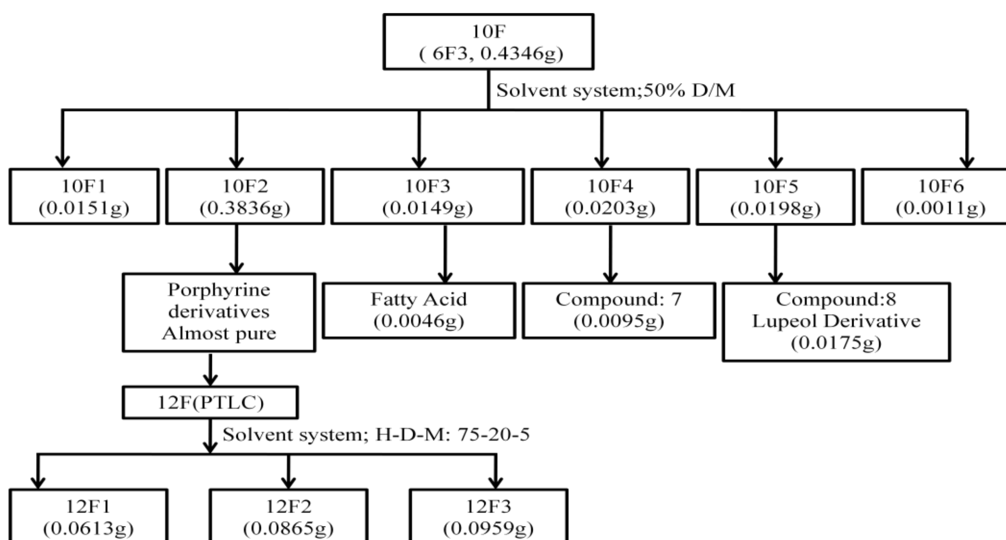

**Scheme 2.6**Fractionationof the 3Fof hexane extract of leaves and twigs of *A. rivularis*.

**Fraction $6F_1$** (0.382 g), eluted with 100% hexane to 10% hexane/EtOAc,was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Fraction $6F_2$** (0.4403 g), eluted with 12-15% hexane/ EtOAc, was obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% $CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $11F_1$–$11F_6$(Scheme 2.8).

**Fraction $6F_3$** (0.4346 g), eluted with 15-18% hexane/EtOAc, was obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% $CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $10F_1$–$10F_6$ (Scheme 2.7).

**Fraction $6F_4$, $6F_5$ and $6F_6$**was obtained dark brown semi-solid and this fraction didn't have interesting NMR signal, so further purification was not carried out.




**Scheme 2.7**Fractionationof the 10F of hexane extract of leaves and twigs of *A. rivularis*.

**Fraction $10F_1$**(0.0151g), eluted with 50% $CH_2Cl_2$/MeOH,was obtained as a green semi-solid and was found to contain mainly porphyrine derivatives, so further purification was not carried out.

**Fraction $10F_2$** (0.383 g), eluted with 50% $CH_2Cl_2$/MeOHwas obtained as a green semi-solid. It was separated by preparative thin layer chromatography (PTLC), eluting with 70:20:5; Hexane:$CH_2Cl_2$:MeOH. The bands were collected to afford subfractions $12F_1$–$12F_3$ (Scheme-2.7).

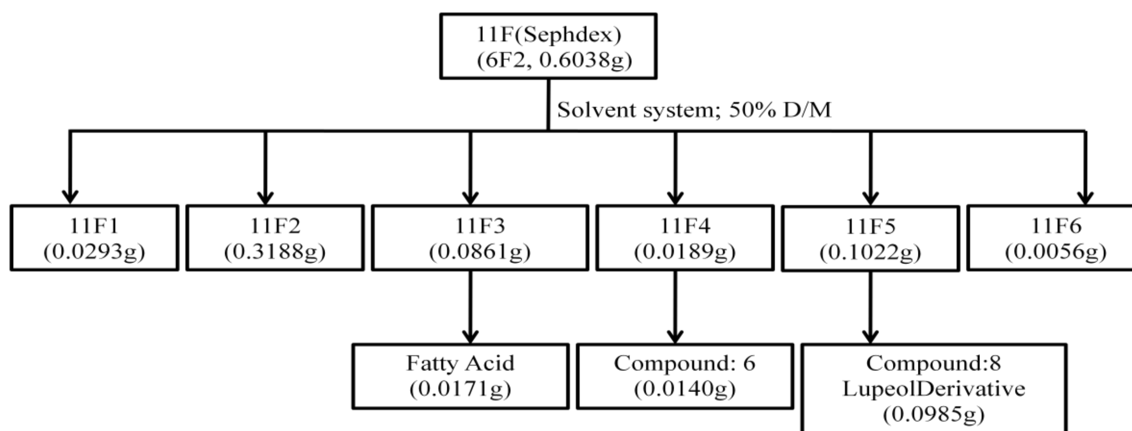
**Subfraction $12F_1$, $12F_2$ and $12F_3$**was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives,so further purification was not carried out.

**Fraction $10F_3$** (0.0046g), eluted with 50% $CH_2Cl_2$/MeOH was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Fraction $10F_4$**(0.0203g), eluted with 50% $CH_2Cl_2$/MeOHwas obtained 0.0095g pure white needle crystal of **29-*Nor*-20-oxolupeol** (**Compound7**).

**Fraction 10F$_5$** (0.0198g) eluted with 50% $CH_2Cl_2$/MeOH,was obtained 0.0175g pure white needle shaped crystal of **3β,6β-Dihydroxylup-20(29)-ene**(**Compound8**).

**Fraction 10F$_6$** (0.0011g), eluted with 50% $CH_2Cl_2$/MeOHwas obtained as a reddish brown solid and was found to contain mainly pigments, so further purification was not carried out.




**Scheme 2.8**Fractionationof 11F of hexane extract of leaves and twigs of *A. rivularis*.

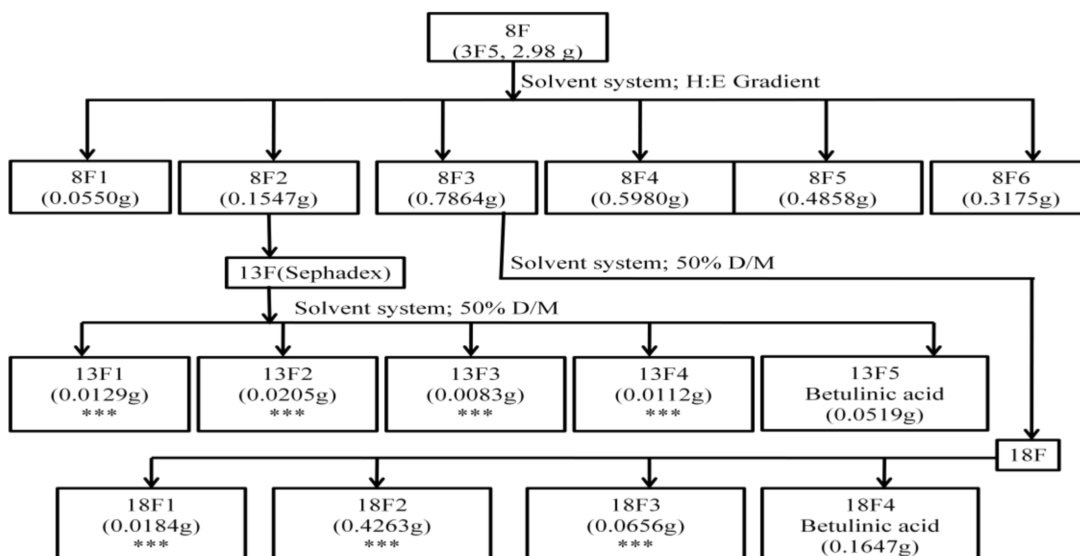
**Fraction 11F$_1$**(0.0293g)&**11F$_2$**(0.3188g), eluted with 50% $CH_2Cl_2$/MeOH,was obtained as a green semi-solid and was found to contain mainly porphyrine derivatives, so further purification was not carried out.

**Fraction 11F$_3$** (0.0861g), eluted with 50% $CH_2Cl_2$/MeOH was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Fraction 11F$_4$** (0.0198g) eluted with 50% $CH_2Cl_2$/MeOH, was obtained 0.0140g white needle crystal of **3β-hydroxy-20(29)-en-lupan-30-al(compound6)**.

**Fraction 10F$_5$** (0.1022g) eluted with 50% $CH_2Cl_2$/MeOHwas obtained 0.0985g pure white needle crystal of **3β,6β-Dihydroxylup-20(29)-ene(Compound8)**.

**Fraction 10F$_6$** (0.0056g), eluted with 50% $CH_2Cl_2$/MeOH, was obtained as a reddish brown solid and was found to contain mainly pigments, so further purification was not carried out.




**Scheme 2.9**Fractionationof 8F of leaves and twigs of *A.rivularis*.

**Fraction $8F_1$** (0.0550 g), eluted with 100% hexane to 10% hexanes/EtOAc, was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Fraction $8F_2$** (0.1547 g), eluted with 10-15% hexane/EtOAc, was obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $13F_1$–$13F_5$(Scheme 2.9).

**Subfraction $13F_1$, $13F_2$, $13F_3$ and $13F_4$**eluted with 50% $CH_2Cl_2$/MeOH was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Subfraction $13F_5$** (0.0519g), eluted with 50% $CH_2Cl_2$/MeOH,was obtained pure white needle crystal of **Betulinic acid (Compound3)**.

**Fraction $8F_3$** (0.7864g), eluted with 15% hexane/EtOAc, was obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% $CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $18F_1$–$18F_4$ (see Scheme 2.9).

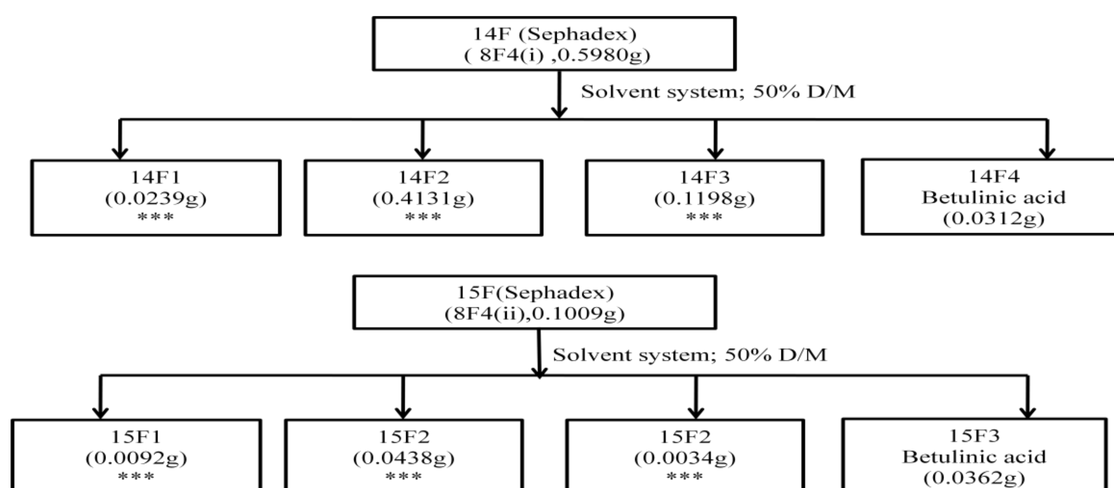
**Subfraction $18F_1$, $18F_2$ and $18F_3$**eluted with 50% $CH_2Cl_2$/MeOH,was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Subfraction $18F_4$** (0.1647g), eluted with 50% $CH_2Cl_2$/MeOH, was obtained pure white needle crystal of **Betulinic acid(Compound3)**.

**Fraction $8F_4$** (0.5980g),eluted with 15-20% hexane/EtOAc,was obtained dark brown semi-solid.It was divided by two parts and separated by column chromatography on sephadex-LH20, eluting with 50% /$CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $14F_1$–$14F_4$ and $15F_1$–$15F_4$ (Scheme-2.10).

**Fraction $8F_5$** (0.4858g),eluted with 20-30% hexane/EtOAc,was obtained dark brown semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% /$CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $16F_1$–$16F_4$ (Scheme-2.11).

**Fraction $8F_6$** (0.3175g), eluted with 50-100 hexane/EtOAc and 100% MeOH, was obtained as a brown semi-solid.It was separated by column chromatography on sephadex-LH20, eluting with 50% $CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $17F_1$–$17F_4$ (Scheme-2.11).



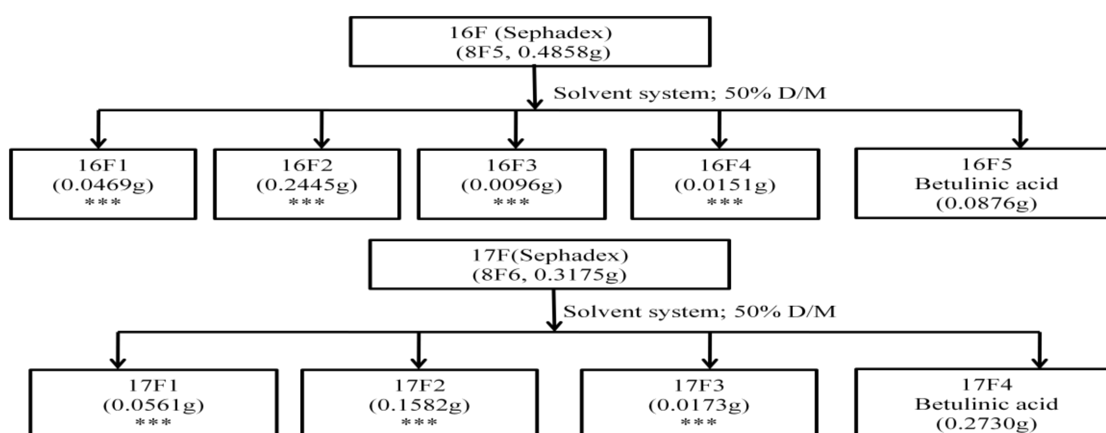

**Scheme 2.10**Fractionationof 14Fand 15F of hexane extract of leaves and twigs of *A. rivularis*.

**Fraction $14F_1$**(0.0239g)**, $14F_2$**(0.4131g) **and $14F_3$**(0.1198g)eluted with 50% $CH_2Cl_2$/MeOH was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction $14F_4$** (0.0312g), eluted with 50% $CH_2Cl_2$/MeOH was obtained pure white needle crystal of **Betulinic acid(Compound-3)**.

**Fraction $15F_1$**(0.0092g)**, $15F_2$**(0.0438g) **and $15F_3$**(0.0.0038g)eluted with 50% $CH_2Cl_2$/MeOH, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction $15F_4$** (0.0362g), eluted with 50% $CH_2Cl_2$/MeOH, was obtained pure white needle crystal of **Betulinic acid(Compound3)**.



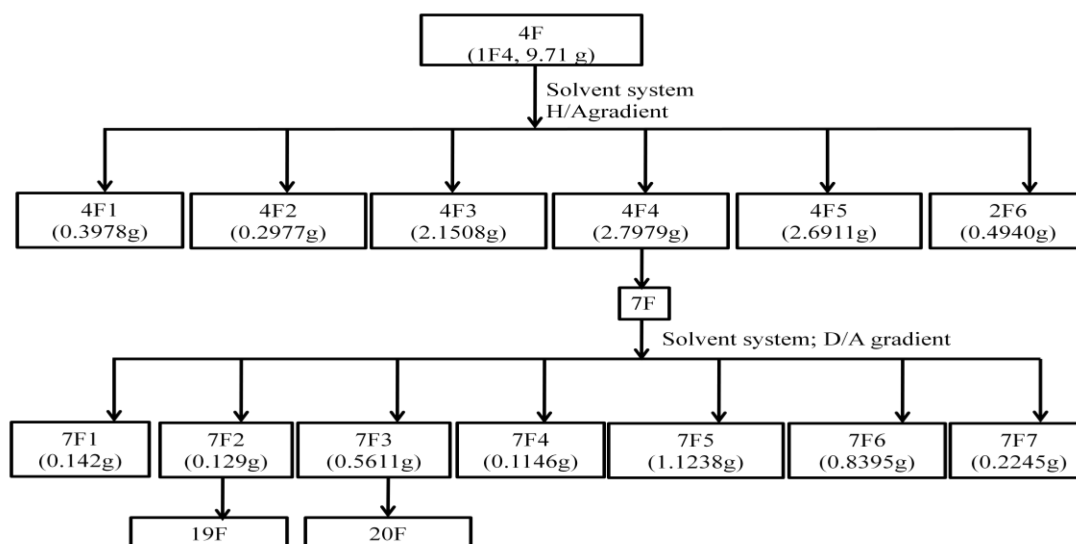

**Scheme 2.11**Fractionationof 16F& 17Fof hexane extract of leaves and twigs of *A. rivularis*.

**Fraction $16F_1$**(0.0469g)**,$16F_2$**(0.2445g)**,$16F_3$**(0.0096g) and **$16F_4$**(0.0151g)eluted with 50% $CH_2Cl_2$/MeOH,was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction $16F_5$** (0.0876g), eluted with 50% /$CH_2Cl_2$/MeOH,was obtained pure white needle crystal of **Betulinic acid(Compound3)**.

**Fraction $17F_1$**(0.0561g)**,$17F_2$**(0.1582g), and **$17F_3$**(0.0173g)eluted with 50% $CH_2Cl_2$/MeOH, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction $17F_4$** (0.2730g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained pure white needle crystal of **Betulinic acid**(**Compound-3**).




**Scheme 2.12**Fractionationof 4F of hexane extrac of leaves and twigs of *A. rivularis*.

**Fraction $4F_1$** (0.3978 g), eluted with 100% hexane to 10% hexane/Acetone, was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Fraction $4F_2$**(0.2977g)&**$4F_3$**(2.1508g), eluted with 10-15% hexane/acetone, was obtained as a green semi-solid and was found to contain mainly porphyrine derivatives , so further purification was not carried out.

**Fraction $4F_4$** (2.7979g), eluted with 15% hexanes/acetone, was obtained as a green semi-solid.It was separated by column chromatography on silica gel (130 g, Merck Art.No.7734, 4.46 x 22.5 cm), eluting with $CH_2Cl_2$, followed by increasing amount of Acetonein $CH_2Cl_2$ and finally with MeOH. Fractions (50 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $7F_1$–$7F_7$ (Scheme-2.12).

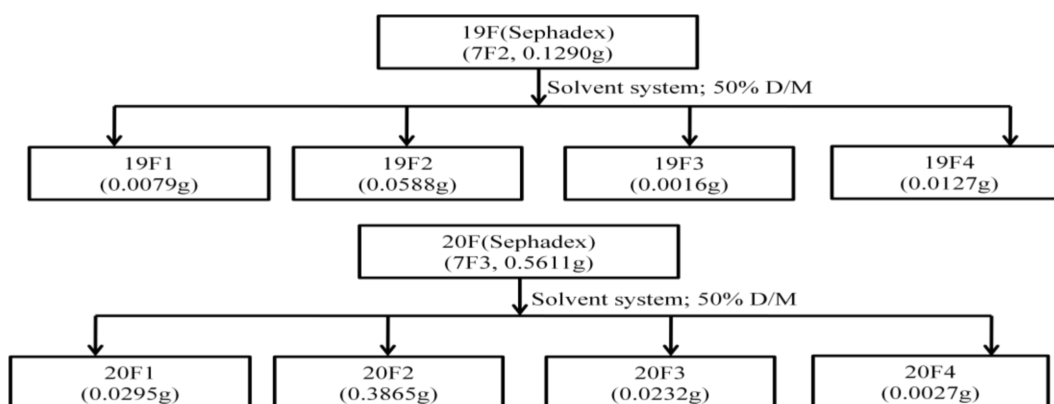
**Subfraction 7$F_1$** (0.1420g), eluted with 100% $CH_2Cl_2$, was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Subfraction 7$F_2$** (0.129g),eluted with 100% $CH_2Cl_2$,was obtained dark brown semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% $CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions 19$F_1$–19$F_4$ (Scheme-2.13).

**Subfraction 7$F_3$** (0.5611g),eluted with 1% $CH_2Cl_2$/acetone, was obtained dark brown semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% $CH_2Cl_2$/MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions 20$F_1$–20$F_4$ (Scheme-2.13).

**Subfraction 7$F_{4,5,6,7}$**eluted with 1% acetone/$CH_2Cl_2$ to 100% acetone,was obtained dark brown semi-solid. This subfraction didn't have interesting NMR signal,so further purification was not carried out.

**Fraction 4$F_5$**(2.6911g) &**4$F_6$** (0.4940g), eluted with 18-100% hexane/acetonewas obtained as a green semi-solid and was found to contain mainly porphyrine derivatives and fatty acid, so further purification was not carried out.




**Scheme 2.13**Fractionationof 19F& 20F of hexane extract of leaves and twigs of *A. rivularis*.

**Fraction 19$F_1$**(0.0079g) &**19$F_2$**(0.0588g), eluted with 50% $CH_2Cl_2$/MeOHwas obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction 19$F_3$**(0.0016g) &**19$F_4$** (0.0127g), eluted with 50% $CH_2Cl_2$/MeOH, was obtained pink semi-solid and was found to contain mainly pigments,so further purification was not carried out.

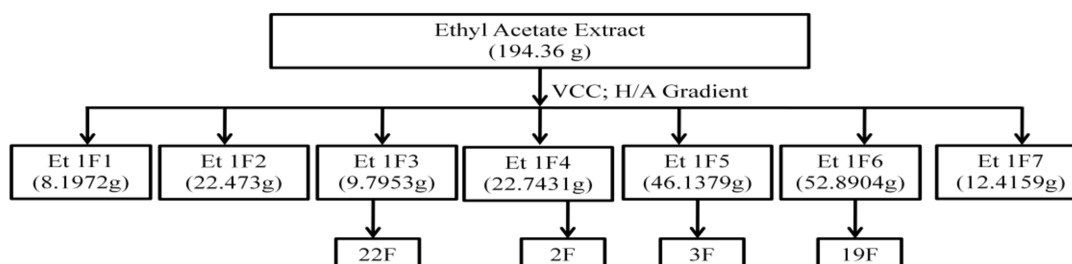
**Fraction 20$F_1$**(0.029g) &**20$F_2$** (0.3865g), eluted with 50% $CH_2Cl_2$/MeOH, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction 20$F_3$**(0.0232g) &**20$F_4$**(0.0027g), eluted with 50% $CH_2Cl_2$/MeOH, was obtained pink semi-solid and was found to contain mainly pigments,so further purification was not carried out.

## 2.5 Purification of Ethyl acetate extracts:

Residue of hexane extract ofleaves and twigs of *A. rivularis* (16.4kg) were successively macerated with ethyl acetate (5 x 10.9 L) at room temperature, followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to give a crude ethyl acetate extract (194.36g).

The ethyl acetate extract (194.36g) was first subjected to vacuum column chromatography on silica gel[202 g, Merck Art. No.7736: ∅ 15x16 cm], eluting with hexane (5000mL), followed by 5%(8L), 10%(4L), 20%(4L), 40%(4L), 60%(4L), 100% acetone/hexane, 50% MeOH/acetone and followed by 100% methanol (300 ml). Fractions (500 mleach) were collected and combined on the basis of their TLC and NMR characteristics to give five fractions (Et1$F_1$–Et1$F_6$) after removal of solvents (Scheme-2.14).




**Scheme-2.14**Fractionation Ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et1F$_1$** (8.1972g), eluted with 100% hexane, was obtained as a yellow semi-solid and was found to contain mainly fat, so further purification was not carried out.

**Fraction Et1F$_2$** (22.473g), eluted with 5% acetone/hexane, was obtained as a green semi-solid and was found to contain mainly porphyine derivatives, so further purification was not carried out.

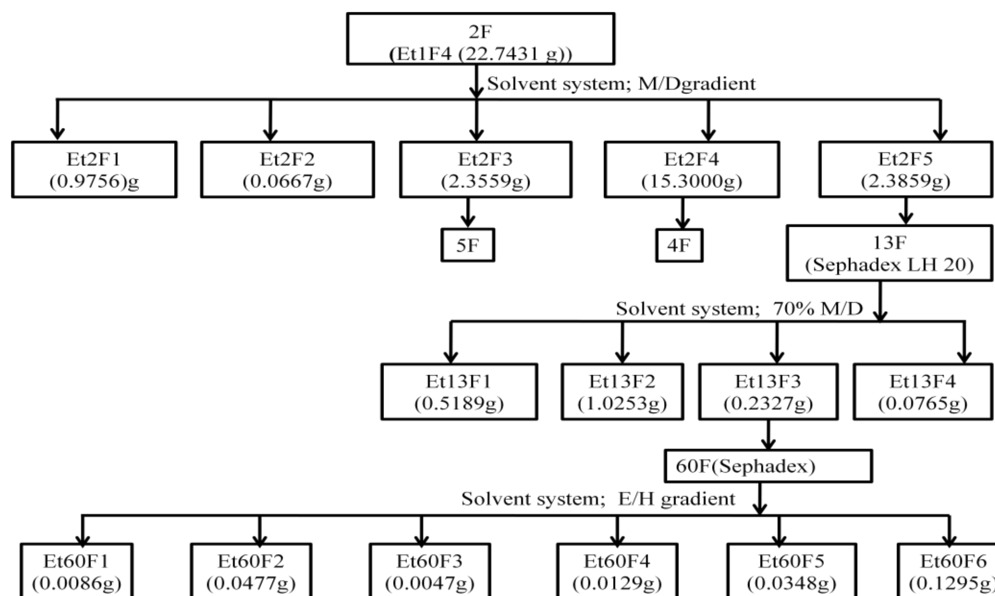
**Fraction Et1F$_3$**(9.7953g), eluted with 10% acetone/hexane, was obtained as a green semi-solid. It was separated by column chromatography on silica gel (305 g, Merck Art. No.7734, 6.5 x 17 cm), eluting with hexane and followed by increasing amount of EtOAc in hexane and finally with MeOH. Fractions (100 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et22F$_1$–Et22F$_7$ (Scheme-2.32).

**Fraction Et1F$_4$**(22.7431g), eluted with 20% acetone/hexane, was obtained as a green semi-solid. It was separated by column chromatography on silica gel (400 g, Merck Art.No.7734, 6 x 25 cm), eluting with $CH_2Cl_2$, followed by increasing amount of MeOH. Fractions (200 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et2F$_1$–Et2F$_5$ (Scheme-2.15).

**Fraction Et1F$_5$**(46.1379g), eluted with 40% acetone/hexane, was obtained as a green semi-solid. It was separated by column chromatography on silica gel (1200g, Merck Art.No.7734, 9.5 x 27 cm), eluting with $CH_2Cl_2$, followed by increasing amount of MeOH. Fractions (200 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et3F$_1$–Et3F$_8$ (Scheme-2.23).

**Fraction Et1F$_6$**(52.8904g), eluted with 60-100% acetone/hexane, was obtained as a green semi-solid. It was separated by column chromatography on silica gel (800g, Merck Art.No.7734, 9.5 x 20 cm), eluting with $CH_2Cl_2$, followed by increasing amount of MeOH. Fractions (200 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et19F$_1$–Et19F$_9$ (Scheme-2.30).

**Fraction Et1F$_7$** (12.4159 g), eluted with 50% MeOH/acetone and 100% MeOH, was obtained as a greenish yellow semi-solid and was found to contain mainly fat, so further purification was not carried out.




**Scheme-2.15**Fractionation Et1$F_4$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et2$F_1$**(0.9756g) &**Et2$F_2$**(0.0667g), eluted with 100% $CH_2Cl_2$ to 1% MeOH/$CH_2Cl_2$, was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Fraction Et2$F_3$** (2.3559g), eluted with 2% MeOH/$CH_2Cl_2$, was obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-$LH_{20}$, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et5$F_1$–Et5$F_7$ (Scheme-2.21).

**Fraction Et2$F_4$**(15.3000g), eluted with 4-6% MeOH/$CH_2Cl_2$, was obtained as a green semi-solid. It was separated by column chromatography on silica gel (400 g, Merck Art.No.7734, 6 x 22.5 cm), eluting with hexane, followed by increasing amount of Acetone in hexane and finally 100% MeOH . Fractions (200 mL each) were collected, monitored by TLC and combined on the basis of their TLC and $^1$H NMR characteristics. The solvents were evaporated to afford subfractions Et4$F_1$–Et4$F_6$ (Scheme-2.16).

**Fraction Et2$F_5$** (2.3859g), eluted with 10% MeOH/$CH_2Cl_2$ to 100% MeOH, was obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-$LH_{20}$, eluting with 70% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected,

monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et13$F_1$–Et13$F_4$ (Scheme-2.15).

**Subfraction Et13$F_1$**(0.5189g), eluted with 70% MeOH/$CH_2Cl_2$was obtained as a greenish brown. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et13$F_2$**(1.0257g), eluted with 70% MeOH/$CH_2Cl_2$was obtained as a greenish brown. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

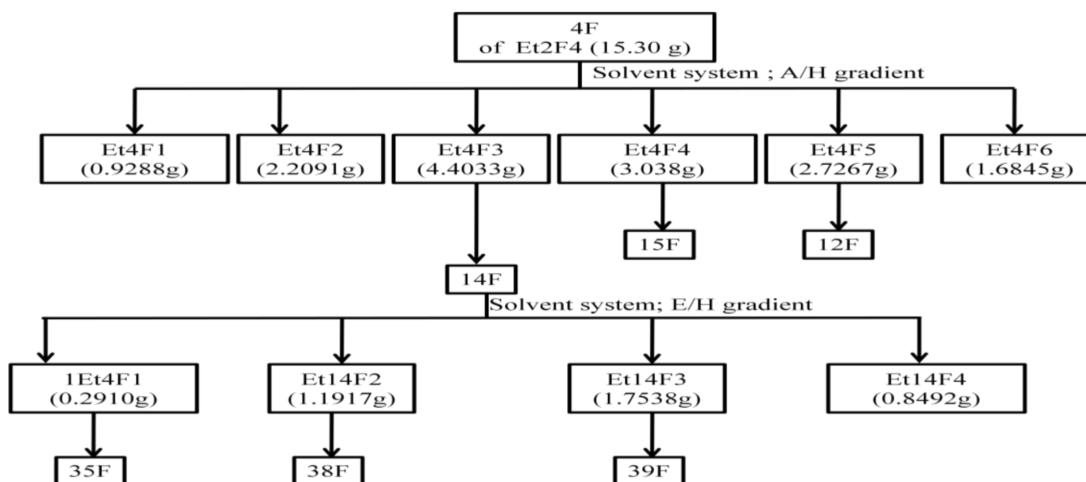
**Subfraction Et13$F_3$**(0.1702g), eluted with 70% MeOH/$CH_2Cl_2$was obtained as a greenish brown. The $^1$H NMR spectrum of this fraction is interesting. It was separated by column chromatography on silica gel (30 g, Merck Art.No.7736, 2 x 15 cm), eluting with Hexane, followed by increasing amount of EtOAc in hexane to 100% EtOAc,after that increaseamount of MeOH in EtOAc and finally 100% MeOH. Fractions (10 mL each) were collected, monitored by TLC and combined on the basis of their TLC and $^1$H NMR characteristics. The solvents were evaporated to afford subfractions Et60$F_1$–Et60$F_6$ (Scheme-2.15).

The $^1$H NMR spectrum of **Et60$F_1$-Et60$F_3$** was not interesting, so further purification was not carried out.

The $^1$H NMRspectrum of **Et60$F_4$ &Et60$F_5$** indicates that it contain simple common compound, so further purification was not carried out.

The $^1$H NMR spectrum of **Et60$F_6$**indicates that it contains pigments, so further purification was not carried out.

**Subfraction Et13$F_4$**(0.0765g), eluted with 70% MeOH/$CH_2Cl_2$was obtained as a greenish brown. The $^1$H NMR spectrum of this fraction was not interesting, so it was not further investigated.




**Scheme-2.16**Fractionation of $Et2F_4$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction $Et4F_1$** (0.9288g), eluted with 100% hexane to 10% acetone/hexane, was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Fraction $Et4F_2$** (2.2091g), eluted with 20% acetone/hexane, was obtained as a green semi-solid and was found to contain mainly porphyine derivatives and the $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction $Et4F_3$**(4.4033g), eluted with 20% acetone/hexane, was obtained as a green semi-solid. It was separated by column chromatography on silica gel (220 g, Merck Art.No.7734, 5 x 27 cm), eluting with 10% EtOAc/hexane, followed by increasing amount of EtOAc in hexane and finally 100% MeOH . Fractions (50ml each) were collected, monitored by TLC and combined on the basis of their TLC and $^1$H NMR characteristics. The solvents were evaporated to afford subfractions $Et14F_1$–$Et14F_4$ (Scheme-2.16).

**Subfraction $Et14F_1$** (0.291g), eluted with 10-30% EtOAc/hexanewas obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 70% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $Et35F_1$–$Et35F_4$ (Scheme-2.17).

**Subfraction Et14F$_2$** (1.1917g), eluted with 40% EtOAc/hexanewas obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et38F$_1$–Et38F$_4$ (Scheme-2.17).

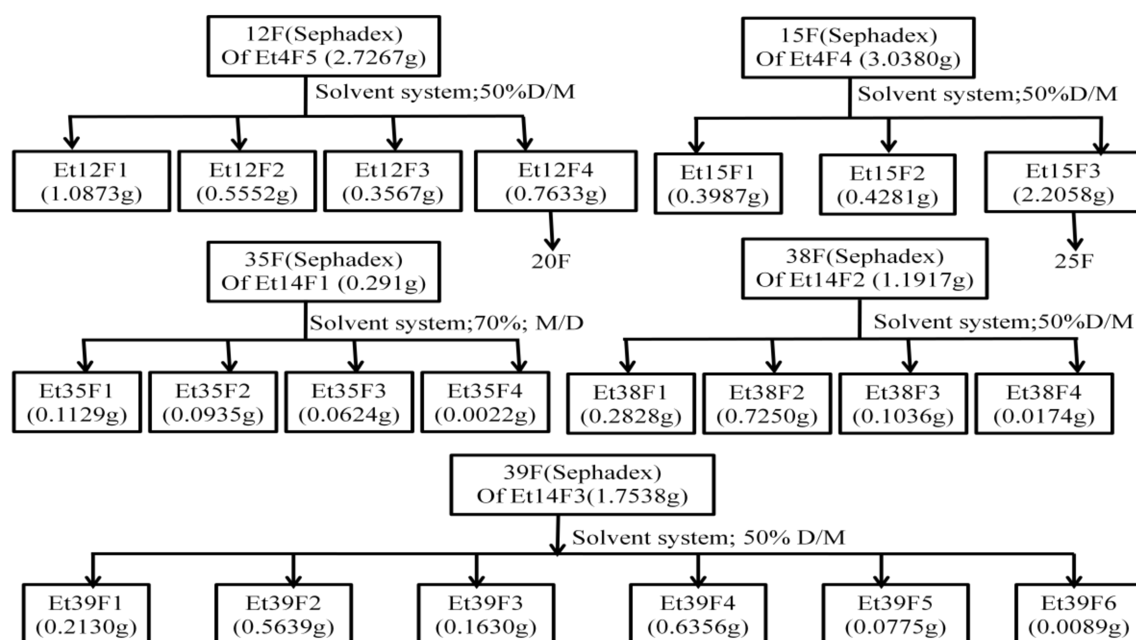
**Subfraction Et14F$_3$** (1.7538g), eluted with 50-85% EtOAc/hexanewas obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et39F$_1$–Et39F$_6$ (see Scheme-2.17).

**Subfraction Et14F$_4$** (0.8492g), eluted with 100% EtOAc and 100% MeOHwas obtained as a dark green semi-solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et4F$_4$** (3.038g), eluted with 25-30% acetone/hexanewas obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et15F$_1$–Et15F$_3$( Scheme-2.17).

**Fraction Et4F$_5$** (2.7269g), eluted with 35-100% acetone/hexanewas obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et12F$_1$–Et12F$_4$ (Scheme-2.17).

**Fraction Et4F$_6$** (1.6845g), eluted with 50% MeOH/acetone and 100% MeOHwas obtained as a greenish brown semi-solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.




**Scheme-2.17**Fractionation of $Et4F_{4,5}$ $Et4F_{1,2,3}$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et12F$_1$**(1.0873g) &**Et12F$_2$**(0.5552g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction Et12F$_3$**(0.3567g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained pink semi-solid and wasfound. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et12F$_4$** (0.6733g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a green semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 70% $MeOH/CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions$Et20F_1$–$Et20F_4$ (Scheme-2.18).

**Fraction Et15F$_1$**(0.3987g), eluted with 50% $MeOH/CH_2Cl_2$,was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction Et15F$_2$**(0.4281g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained pink semi-solid and was found. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et15$F_3$** (02.2058g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a red semi-solid. It was separated by column chromatography on sephadex-LH20, eluting with 50% $MeOH/CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et25$F_1$–Et25$F_6$ (Scheme-2.19).

**Fraction Et35$F_1$**(0.1129g), eluted with 70% $MeOH/CH_2Cl_2$,was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction Et35$F_2$**(0.0935g), eluted with 70% $MeOH/CH_2Cl_2$, was obtained dark brown semi-solid and was found. The $^1H$ NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et35$F_3$**(0.0624g), eluted with 70% $MeOH/CH_2Cl_2$, was obtained brown semi-solid and was found. The $^1H$ NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et35$F_4$**(0.0022g), eluted with 70% $MeOH/CH_2Cl_2$, was obtained brown semi-solid and was found contain mainly pigments. The $^1H$ NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et38$F_1$**(0.2828g), eluted with 50% $MeOH/CH_2Cl_2$,was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction Et38$F_2$**(0.7250g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained dark brown semi-solid and was found. The $^1H$ NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et38$F_3$**(0.1036g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained brown semi-solid and was found. The $^1H$ NMR spectrum of this fraction is not interesting, so further purification was not carried out.

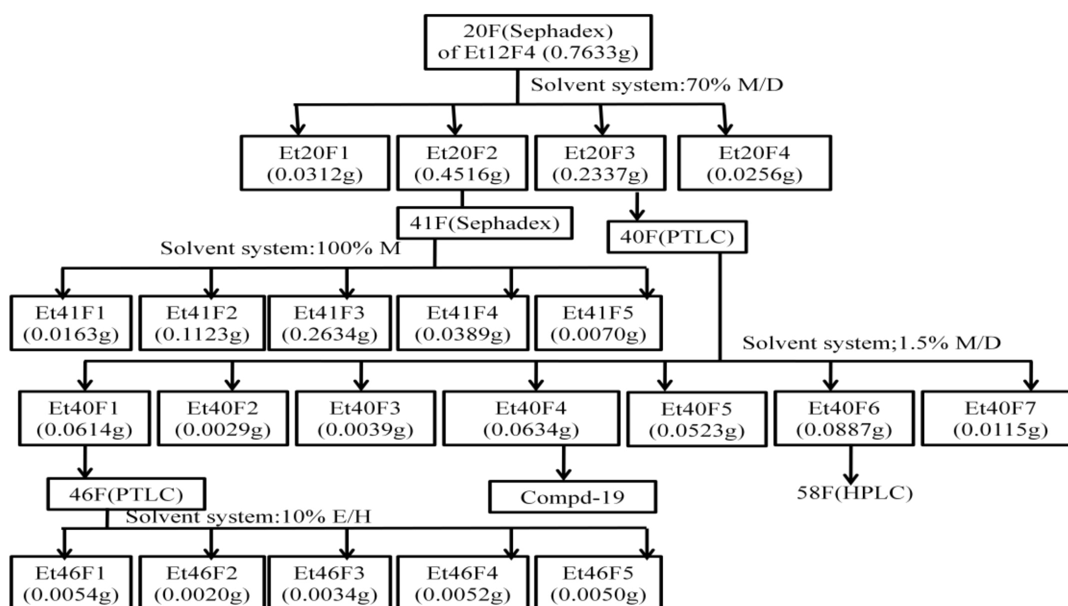
**Fraction Et35$F_4$**(0.0174g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained red semi-solid and was found contain mainly pigments. The $^1H$ NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et39F$_1$**(0.2130g) &**Et39F$_2$**(0.5639g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction Et39F$_3$**(0.1630g) &**Et39F$_4$** (0.6356g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained dark brown semi-solid and was found. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et39F$_5$**(0.0775g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained brown semi-solid and was found. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et39F$_6$**(0.0089g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained yellow powder and was found contain mainly pigments. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.




**Scheme-2.18**Fractionation of Et12F$_4$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et20F$_1$**(0.0312g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction Et20F$_2$** (0.4516g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a red semi-solid. It was separated by column chromatography on sephadex-LH$_{20}$, eluting with 100% MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on

the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et41$F_1$–Et41$F_5$ (Scheme-2.18).

**Subfraction Et41$F_1$**(0.0162g), eluted with 100% MeOH, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Subfraction Et41$F_2$**(0.1123g), eluted with 100% MeOH, was obtained dark brown semi-solid and was found. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et41$F_3$**(0.2634g), eluted with 100% MeOH, was obtained brown semi-solid and was found. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et41$F_4$**(0.0389g), eluted with 100% MeOH, was obtained red semi-solid and was found. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et41$F_5$**(0.0070g), eluted with 100% MeOH, was obtained pink semi-solid and was found contain mainly pigments. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et20$F_3$** (0.2337g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a brown semi-solid. It was separated by preparative thin layer chromatography (PTLC), running with 1.5% MeOH/$CH_2Cl_2$. Seven bands were collected. The solvents were evaporated to afford sub fractions Et40$F_1$–Et40$F_7$ (Scheme-2.18).

**Subfraction Et40$F_1$**(0.0614g), run with 1.5% MeOH/$CH_2Cl_2$, was obtained dark green semi-solid and was found interesting $^1$H NMR spectrum. It was separated by preparative thin layer chromatography (PTLC), running with 10% EtOAc/Hexane. Five bands were collected. The solvents were evaporated to afford sub fractions Et46$F_1$–Et46$F_5$ (Scheme-2.18).

**Subfraction Et46$F_1$**(0.0054g), obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et46$F_2$**(0.0020g), obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et46F$_3$**(0.0034g), obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et46F$_4$**(0.0052g), obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et46F$_5$**(0.0050g), obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et40F$_2$**(0.0029g), run with 1.5% MeOH/$CH_2Cl_2$, was obtained brownsolidwas found. The $^1$H NMR spectrum of this fraction is interesting and almost pure. It can run 2D NMR but small.

**Subfraction Et40F$_3$**(0.0039g), run with 1.5% MeOH/$CH_2Cl_2$, was obtained brownsolidwas found. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

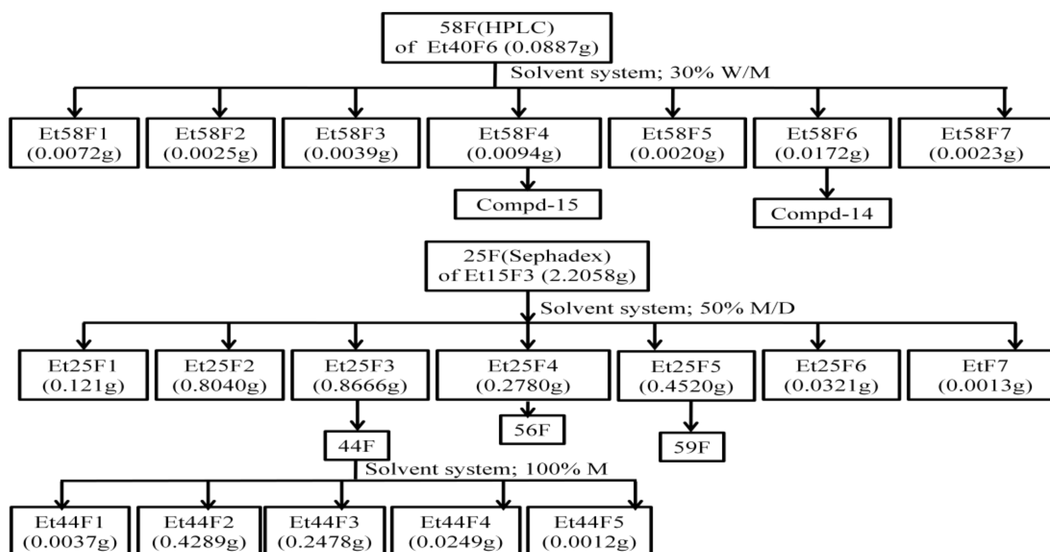
**Subfraction Et40F$_4$**(0.0634g), run with 1.5% MeOH/$CH_2Cl_2$, was obtained pure yellowish**Vanilic acid (3-methoxy 4-hydroxybenzoic acid)**(**compound 19**)was found.

**Subfraction Et40F$_5$**(0.0523g), run with 1.5% MeOH/$CH_2Cl_2$, was obtained pink semi-solid and was found. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et40F$_6$**(0.0887g), run with 1.5% MeOH/$CH_2Cl_2$, was obtained pink semi-solid and was found. The $^1$H NMR spectrum of this fraction is interesting. This fraction was separated by HPLC with solvent system 30% W/M. Twelve peaks were collected. The solvents were evaporated to afford sub fractions Et58F$_1$–Et58F$_7$ (Scheme-2.19).

**Subfraction Et40F$_7$**(0.0115g), run with 1.5% MeOH/$CH_2Cl_2$, was obtained yellow semi-solid and was found. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et20F$_4$**(0.0256g), eluted with 70%; MeOH/$CH_2Cl_2$, was obtained yellow powder and was found contain mainly pigments. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.




**Scheme-2.19**Fractionation of Et12$F_4$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et58$F_1$**(0.0072g), run with 30% $H_2O$/MeOH, obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et58$F_2$**(0.0025g), run with 30% $H_2O$/MeOH, obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et58$F_3$**(0.0039g), run with 30% $H_2O$/MeOH, obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et58$F_4$**(0.0094g), run with 30% $H_2O$/MeOH, obtained pure**2,3-dihydro-*p*-Cumaric acid (Compound 15)** as awhite solid.

**Fraction Et58$F_5$**(0.0020g), run with 30% $H_2O$/MeOH, obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et58$F_6$**(0.0172g), run with 30% $H_2O$/MeOH, obtained pure***p*-Cumaric acid (Compound 14)** as awhite solid.

**Fraction Et58F$_7$**(0.0023g), run with 30% $H_2O$/MeOH, obtained as a white solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et25F$_1$** (0.1210g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction Et25F$_2$** (0.8040g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives. The $^1$H NMR is not interesting,so further purification was not carried out.

**Fraction Et25F$_3$** (0.8666g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a dark green semi-solid. It was separated by column chromatography on sephadex-$LH_{20}$, eluting with 100% MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et44F$_1$–Et44F$_5$ (Scheme-2.19).

**Subfraction Et44F$_1$**(0.0037g), obtained as a brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et44F$_2$**(0.4289g), obtained as a green solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et44F$_3$**(0.2478g), obtained as a yellowish white solid. The $^1$H NMR spectrum of this fraction is interesting and almost pure so need to further purification.

**Subfraction Et44F$_4$**(0.0249g), obtained as a yellowish white solid. The $^1$H NMR spectrum of this fraction is interesting and almost pure so need to further purification.

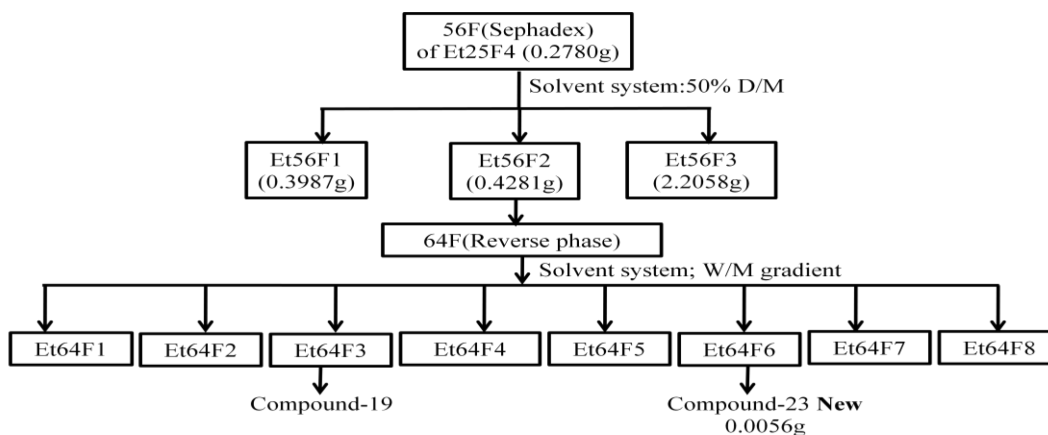
**Subfraction Et44F$_5$**(0.0012g), obtained as a pink solid, contained mainly pigments. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et25F$_4$** (0.2780g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained dark brown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-$LH_{20}$, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected,

monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions $Et56F_1$–$Et56F_3$ (Scheme-2.20).

**Fraction $Et25F_6$** (0.0321g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained pink solid. The $^1H$ NMR is not interesting,so further purification was not carried out.

**Fraction $Et25F_7$**(0.0013g), obtained as a pink solid, contained mainly pigments. The $^1H$ NMR spectrum of this fraction is not interesting, so further purification was not carried out.




**Scheme-2.20**Fractionation of $Et25F_4$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction $Et56F_1$** (0.0073g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained dark brown solid and was found to contain mainly porphyrine derivatives,so further purification was not carried out.

**Fraction $Et56F_2$** (0.2492g), eluted with 50% $MeOH/CH_2Cl_2$. It was separated by reverse phase column chromatography, eluting with 70% /$H_2O$/MeOH to 100% MeOH. Fractions (5 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions $Et64F_1$–$Et64F_8$ (Scheme-2.20).

Due to the limitation of time **Subfraction $Et64F_1$,$Et64F_2$**, was not investigated, so further investigation is needed.

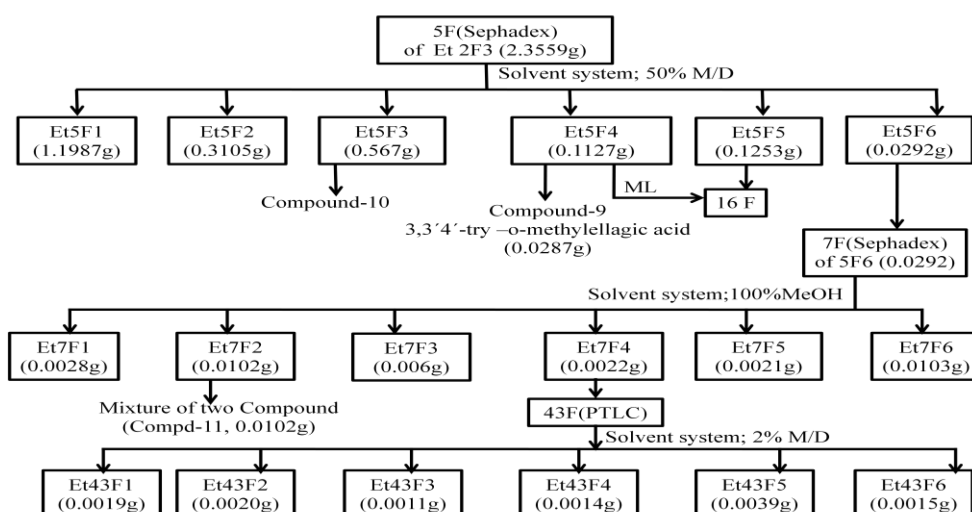
**Subfraction $Et64F_3$**, eluted with 50% $H_2O$/MeOH,**Vanilic acid (compound-19)** was crystal out as a white crystal.

Due to the limitation of time **Subfraction Et64F$_4$,Et64F$_5$**, was not investigated, so further investigation is needed.

**Subfraction Et64F$_6$**, eluted with 50% $H_2O$/MeOH, 0.0056g of**compound 23**,(**New**) was crystal out as a white crystal.

Due to the limitation of time **Subfraction Et64F$_7$,Et64F$_8$**, was not investigated, so further investigation is needed.

**Fraction Et56F$_3$** (0.0042g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained redsolid, contain mainly pigments,so further purification was not carried out.




**Scheme-2.21**Fractionation of Et2F$_3$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et5F$_1$** (1.1987g)&**Et5F$_2$**(0.3105g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained dark green semi-solid and was found to contain mainly porphyrine derivatives and fat,so further purification was not carried out.

**Fraction Et5F$_3$** (0.0567g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained dark brown semi-solid with white crystal of **(Compound10)**,so further purification was not carried out.

**Fraction Et5F$_4$** (0.1127g), eluted with 50% MeOH/$CH_2Cl_2$,**3,3´,4´-tri-*O*-methylellagic acid(compound 9)** was powder out. The $^1$H NMR spectrum of mother liquor is interesting and similar to **Et5F$_5$**, so it's combined with **Et5F$_5$** to run column again.

**Fraction Et5F$_5$** (0.3354g), eluted with 50% MeOH/$CH_2Cl_2$and mother liquor of **Et5F$_4$**, was combined on the basis of $^1$H NMR spectrum. It was separated by column chromatography on sephadex-LH20, eluting with 70% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et16F$_1$–Et16F$_3$ (Scheme-2.22).

**Fraction Et5F$_6$** (0.0292g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained red solid with white crystal. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 100% MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et7F$_1$–Et7F$_6$ (Scheme-2.21).

**Subfraction Et7F$_1$**(0.0028g), obtained as a brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

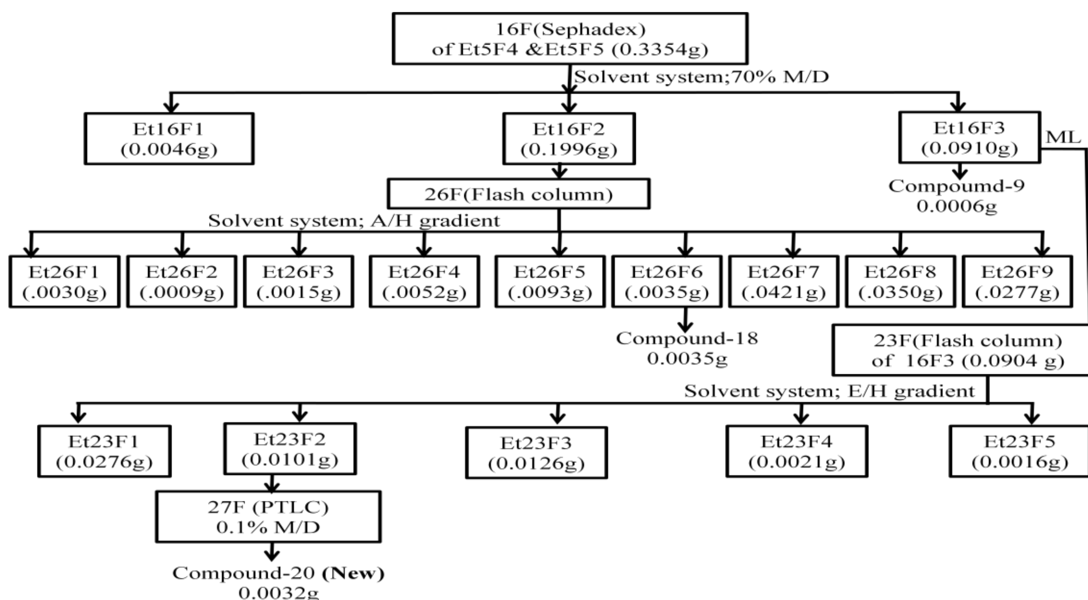
**Subfraction Et7F$_2$**(0.0102g), obtained as a white crystal of two compounds(**Comp$^d$-11**). Both of this are known compound, so further purification was not carried out.

**Subfraction Et7F$_3$**(0.0060g), obtained as a red solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et7F$_4$**(0.0022g), eluted with 100% MeOH, was obtained reddish brown solid and was found interesting $^1$H NMR spectrum. It was separated by preparative thin layer chromatography (PTLC), running with 2% MeOH/$CH_2Cl_2$. Six bands were collected. The solvents were evaporated to afford sub fractions Et43F$_1$–Et43F$_6$ (see Scheme-2.21). The $^1$H NMR spectrum of Et43F$_1$–Et43F$_6$ fraction is not interesting, so further purification was not carried out.

**Subfraction Et7F$_5$**(0.0021g), obtained as a pink solid, contained mainly pigments. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Subfraction Et7F$_6$**(0.0103g), obtained as a off-white powder, contained mainly pigments and fatty acid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.




**Scheme-2.22**Fractionation of Et5F$_4$& Et5F$_5$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et16F$_1$**(0.0046g), eluted with 70% MeOH/$CH_2Cl_2$obtained as a dark brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et16F$_2$**(1.1996g), eluted with 70% MeOH/$CH_2Cl_2$ obtained as a dark brown solid. It was separated by column chromatography on silica gel (30 g, Merck Art.No.7736, 3 x 15 cm), eluting with acetone/hexane gradient , followed by increasing amount of acetone in hexane and finally 100% MeOH . Fractions (5ml each) were collected, monitored by TLC and combined on the basis of their TLC and $^1$H NMR characteristics. The solvents were evaporated to afford subfractions Et26F$_1$–Et26F$_9$ (Scheme-2.22).

The $^1$H NMR spectrum of **Subfraction Et26F$_1$-Et26F$_5$** fraction is not interesting, so further purification was not carried out.

**Subfraction Et26F$_6$**eluted with 20% acetone/hexane, obtained 0.0035g pure**loliolide(Comp$^d$-18)** as awhite crystal.

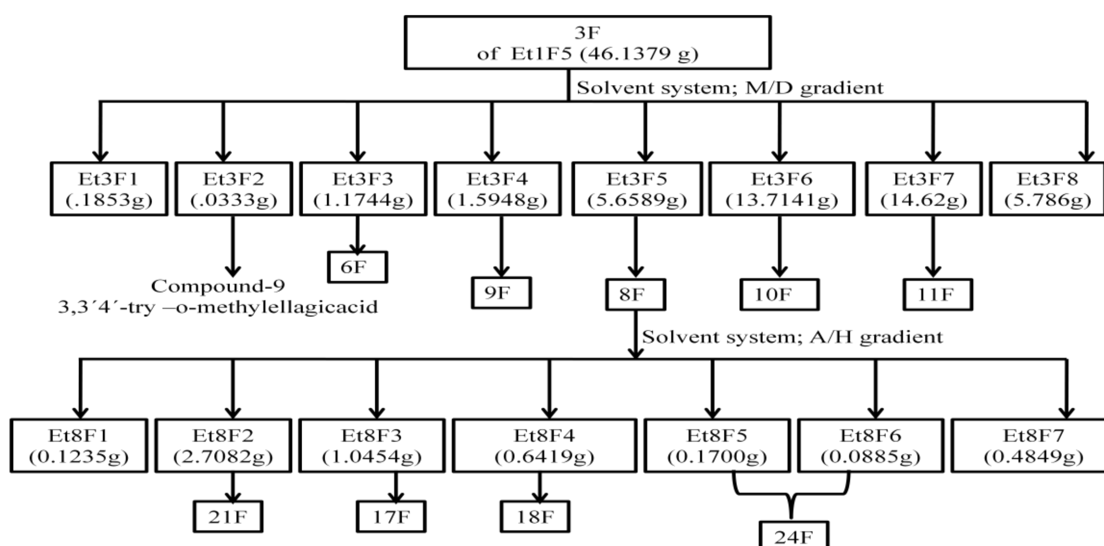
The ^{1}H NMR spectrum of **Subfraction Et26F$_7$-Et26F$_9$** fraction is not interesting, so further purification was not carried out.

**Fraction Et16F$_3$**(0.0910g), eluted with 70% MeOH/$CH_2Cl_2$, pure 0.0006g**3,3´,4´-tri-*O*-methylellagic acid (Compound9)** was powder out. The ^{1}H NMR spectrum of mother liquor is interesting. It was separated by column chromatography on silica gel (20 g, Merck Art.No.7736, 3 x 15 cm), eluting with EtOAc/hexane gradient , followed by increasing amount of EtOAc in hexane and finally 100% MeOH . Fractions (5ml each) were collected, monitored by TLC and combined on the basis of their TLC and ^{1}H NMR characteristics. The solvents were evaporated to afford subfractions Et23F$_1$–Et23F$_5$ (Scheme-2.22).

The ^{1}H NMR spectrum of **Subfraction Et23F$_1$** fraction is not interesting, so further purification was not carried out.

**Subfraction Et23F$_2$**(0.0101g),eluted with 20% EtOAc/hexane, obtained as ared solid. The ^{1}H NMR spectrum of this fraction is interesting and almost pure.. It was separated by preparative thin layer chromatography (PTLC), running with 0.1% MeOH/$CH_2Cl_2$. The bands red with anisoldehyde reagent was collected 0.0032g as a pure **new compound (Compound20)**.

The ^{1}H NMR spectrum of **Subfraction Et23F$_3$-Et26F$_5$** fraction is not interesting, so further purification was not carried out.




**Scheme-2.23**Fractionation of Et1F$_5$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et3F$_1$** (0.1853g), eluted with 100% $CH_2Cl_2$ to 0.5% MeOH/$CH_2Cl_2$, was obtained as a yellow semi-solid and was found to contain mainly fat, so further purification was not carried out.

**Fraction Et3F$_2$** (0.0333g), eluted with 1% MeOH/$CH_2Cl_2$, was obtained as a green semi-solid. **3,3´,4´-tri-*O*-methylellagic acid (compound9)** also powder out as a yellow powder. The mother liquor was found to contain mainly porphyine derivatives, so further purification was not carried out.

**Fraction Et3F$_3$**(1.1744g), eluted with 2% MeOH/$CH_2Cl_2$, was obtained as a green semi-solid. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 70% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et6F$_1$–Et6F$_5$ (Scheme-2.25).

**Fraction Et3F$_4$**(1.5948g), eluted with 3% MeOH/$CH_2Cl_2$, was obtained as a dark green semi-solid. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 70% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et9F$_1$–Et9F$_6$ (Scheme-2.25).

**Fraction Et3F$_5$**(5.6589g), eluted with 5% MeOH/$CH_2Cl_2$, was obtained as a dark green semi-solid. It was separated by column chromatography on silica gel (140 g, Merck Art. No.7734, 4 x 25 cm), eluting with hexanes and followed by increasing amount of Acetone in hexane and finally with methanol. Fractions (100 mL each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et8F$_1$–Et8F$_7$ (Scheme-2.23).

**Subfraction Et8F$_1$** (0.1235g), eluted with 10-20% acetone/hexane, was obtained as a white solid and was found to contain mainly fat, so further purification was not carried out.

**Subfraction Et8F$_2$**(2.7082g), eluted with 30% acetone/hexane, was obtained as a green semi-solid. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml

each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions $Et21F_1$–$Et21F_3$ (Scheme-2.24).

**Subfraction $Et8F_3$**(1.0454g), eluted with 35-50% acetone/hexane, was obtained as a dark green semi-solid. The $^1H$ NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 50% $MeOH/CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions $Et17F_1$–$Et17F_4$ (see Scheme-2.24).

**Subfraction $Et8F_4$**(0.6419g), eluted with 60% acetone/hexane, was obtained as a dark green semi-solid. The $^1H$ NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 70% $MeOH/CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions $Et18F_1$–$Et18F_3$ (Scheme-2.24).

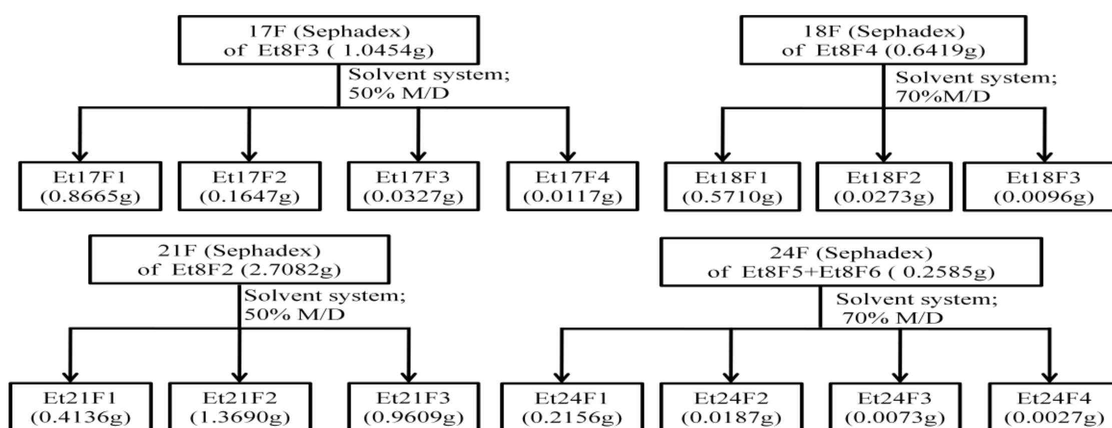
**Subfraction $Et8F_5$**(0.1700g)&**$Et8F_6$**(0.0885g), eluted with 70-80% acetone/hexane, was obtained as a dark green semi-solid. The $^1H$ NMR spectrum is interesting. It was combined and separated by column chromatography on sephadex-LH20, eluting with 70% $MeOH/CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions $Et24F_1$–$Et24F_4$ (Scheme-2.24).

**Subfraction $Et8F_6$** (0.4849g), eluted with 100% acetone and 100% MeOH ,was obtained as a brown solid and was found to contain mainly fatty acid, so further purification was not carried out.

**Fraction $Et3F_6$**(13.7141g), eluted with 5% $MeOH/CH_2Cl_2$, was obtained as a dark brown semi-solid. It was separated by column chromatography on silica gel (350 g, Merck Art. No.7734, 6 x 20 cm), eluting with hexane and followed by increasing amount of EtOAc in hexane and finally with methanol. Fractions (100 mL each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions $Et10F_1$–$Et10F_7$ (Scheme-2.26).

**Fraction Et3F$_7$**(14.62g), eluted with 7-15% MeOH/$CH_2Cl_2$, was obtained as a dark brown semi-solid. It was separated by column chromatography on silica gel (300 g, Merck Art. No.7734, 6 x 16 cm), eluting with hexane and followed by increasing amount of EtOAc in hexane and finally with methanol. Fractions (100 mL each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et11F$_1$–Et11F$_6$ (Scheme-2.26).

**Fraction Et3F$_8$** (5.7860g), eluted with 20-100% MeOH/$CH_2Cl_2$, was obtained as a brown solid and was found to contain mainly fatty acid, so further purification was not carried out.




**Scheme-2.24**Fractionation of Et8F$_2$ toEt8F$_6$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et17F$_1$** (0.8665g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid and was found to contain mainly porphyrine derivatives, so further purification was not carried out.

**Fraction Et17F$_2$** (0.1647g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et17F$_3$** (0.0327g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et17F$_4$** (0.0117g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid, contained mainly pigments. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et18F$_1$** (0.5710g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a green solid and was found to contain mainly porphyrine derivatives, so further purification was not carried out.

**Fraction Et18F$_2$** (0.0273g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et18F$_3$** (0.0096g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a red solid, contained mainly pigments. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et21F$_1$** (0.4136g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid and was found to contain mainly porphyrine derivatives, so further purification was not carried out.

**Fraction Et21F$_2$** (1.3690g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a dark brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

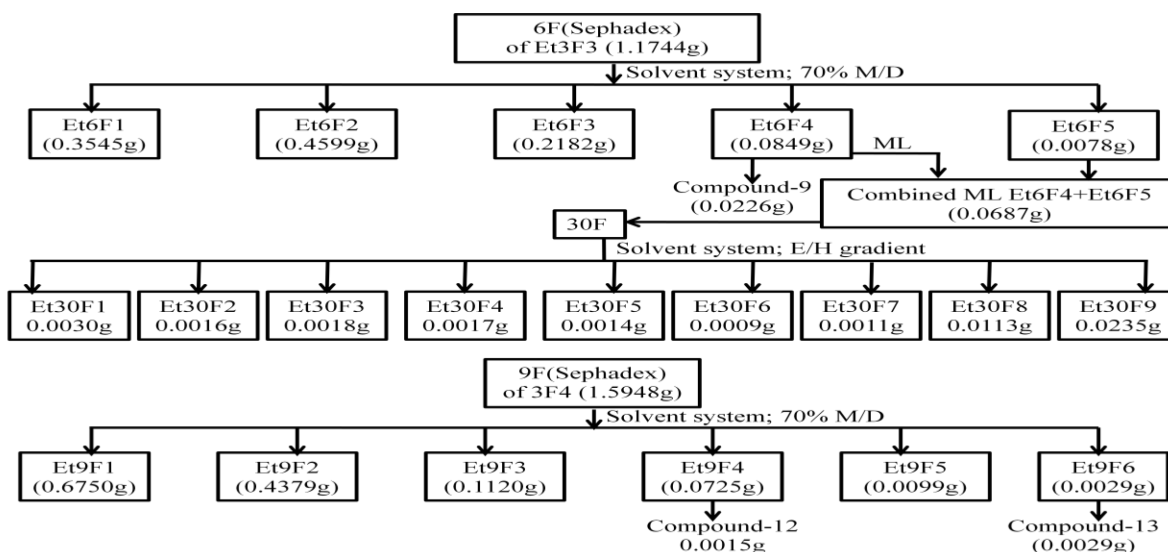
**Fraction Et21F$_4$** (0.9609g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et24F$_1$** (0.2156g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a green solid and was found to contain mainly porphyrine derivatives, so further purification was not carried out.

**Fraction Et24F$_2$** (0.0187g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et24F$_3$** (0.0073g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.

**Fraction Et24F$_4$** (0.0027g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a red solid, contained mainly pigments. The $^1$H NMR spectrum of this fraction is not interesting, so further purification was not carried out.




**Scheme-2.25**Fractionation of Et3F$_3$&Et3F$_4$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et6F$_1$** (0.3545g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a dark green solid and was found to contain mainly porphyrine derivatives, so further purification was not carried out.

**Fraction Et6F$_2$** (0.4599g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et6F$_3$** (0.2182g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a dark brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et6F$_4$** (0.0849g), eluted with 70% MeOH/$CH_2Cl_2$, 0.0226g**3,3´,4´-tri-*O*-methylellagic acid (compound-09)** was powder out. The $^1$H NMR spectrum of mother liquor is interesting and similar to **Et6F$_5$**, so it’s combined with **Et6F$_5$** to run column again.

**Fraction Et6$F_5$** (0.0078g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a red solidand mother liquor of **Et6$F_4$**, was combined on the basis of $^1$H NMR spectrum. . It was separated by column chromatography on silica gel (18 g, Merck Art. No.7736, 2 x 24 cm), eluting with hexane and followed by increasing amount of EtOAc in hexane and finally with MeOH. Fractions (5 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et30$F_1$–Et30$F_9$ (Scheme-2.25).

The $^1$H NMR spectrum of **subfraction Et30$F_1$–Et30$F_9$** is not interesting and the amount was very small, so further purification was not carried out.

**Fraction Et9$F_1$** (0.6750g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a dark green solid and was found to contain mainly porphyrine derivatives, so further purification was not carried out.

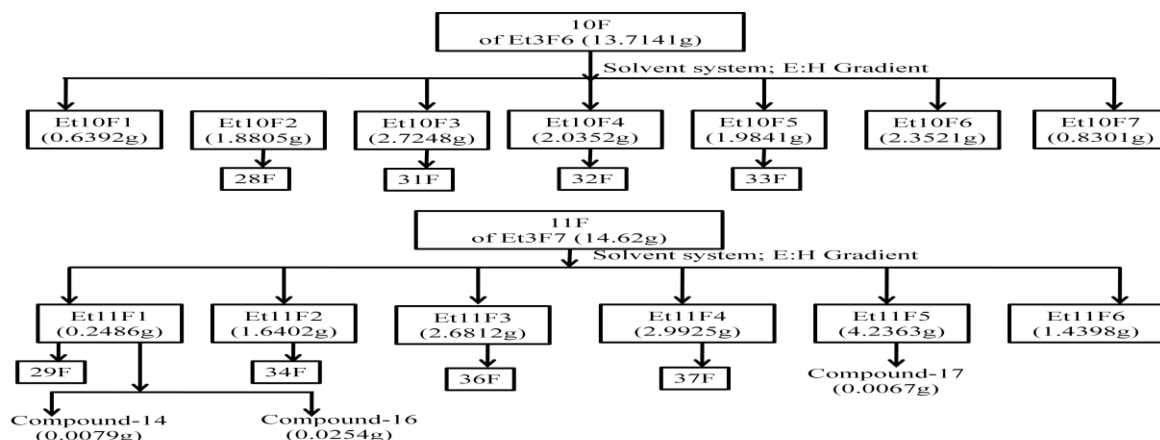
**Fraction Et9$F_2$** (0.4379g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et9$F_3$** (0.1120g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a dark brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et9$F_4$** (0.0849g), eluted with 70% MeOH/$CH_2Cl_2$, 0.0015g**4,4´-di-*O*-methylellagic acid (compound12)** was powder out as a yellow powder.

**Fraction Et9$F_5$** (0.0099g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a red solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et9$F_6$** (0.0029g), eluted with 70% MeOH/$CH_2Cl_2$. Pure 0.0029g**4′,5,7-trihydroxyflavanone (compound13)** was powder out as a yellow powder.




**Scheme-2.26**Fractionation of Et3$F_6$&Et3$F_7$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et10$F_1$** (0.6392g), eluted with 20-50% EtOAc/hexane, was obtained as a white powder and was found to contain mainly fat, so further purification was not carried out.

**Fraction Et10$F_2$**(1.8805g), eluted with 60% EtOAc/hexane, was obtained as a dark green semi-solid. The $^1$H NMR spectrum is interesting. It wasseparated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et28$F_1$–Et28$F_7$ (Scheme-2.27).

**Fraction Et10$F_3$**(2.7248g), eluted with 60-70% EtOAc/hexane, was obtained as a dark green semi-solid. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et31$F_1$–Et31$F_6$ (Scheme-2.28).

**Fraction Et10$F_4$**(2.0352g), eluted with 80% EtOAc/hexane, was obtained as a dark brown semi-solid. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-$LH_{20}$, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et32$F_1$–Et32$F_6$ (Scheme-2.28).

**Fraction Et10F$_5$**(1.9841g), eluted with 100% EtOAc, was obtained as a brown semi-solid. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH$_{20}$, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et33F$_1$–Et33F$_6$ (Scheme-2.28).

**Fraction Et10F$_6$**(2.0352g), eluted with 10% MeOH/EtOAC, was obtained as a dark brown semi-solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et10F$_6$**(0.8301g), eluted with 30-100% MeOH/EtOAC, was obtained as a brown semi-solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et11F$_1$** (0.2486g), eluted with 20-50% EtOAc/hexane, was obtained as a dark brown semi-solid. Pure0.0079g, ***p*-Cumaric acid(compound 14)** and0.0254g, **3,4-dihydroxy benzoic acid(compound16)** was crystal out in vial no. 3 and vial no. 4-5 respectively. The $^1$H NMR spectrum of mother liquor is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et29F$_1$–Et29F$_6$ (Scheme-2.29).

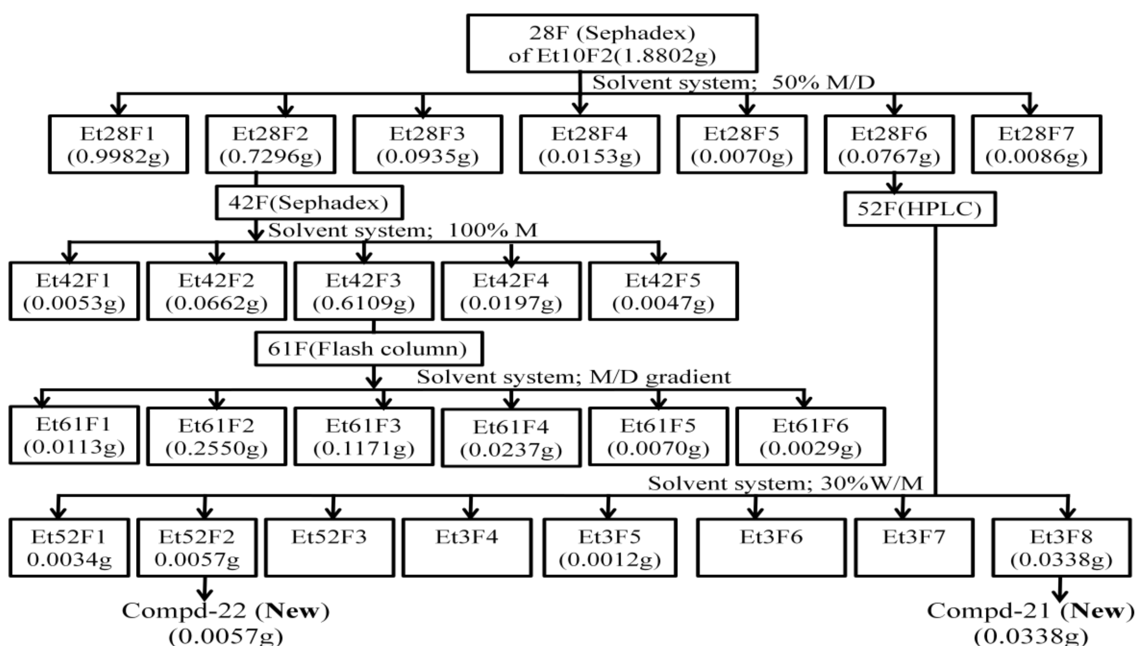
**Fraction Et11F$_2$**(1.6402g), eluted with 60-70% EtOAc/hexane, was obtained as a brown semi-solid. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et34F$_1$–Et34F$_4$ (Scheme-2.29).

**Fraction Et11F$_3$**(2.6812g), eluted with 80% EtOAc/hexane, was obtained as a brown semi-solid. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et36F$_1$–Et36F$_4$ (Scheme-2.29).

**Fraction Et11F$_4$**(2.9925g), eluted with 100% EtOAc to 10% MeOH/EtOAc, was obtained as a brown solid. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et37F$_1$–Et37F$_3$ (Scheme-2.29).

**Fraction Et11F$_5$** (4.2363g), eluted with 10-30% MeOH/EtOAc. Pure 0.0067g,**Stigmast-5-en-3-*O*-β-glucoside (compound 17)** was powder out as a white powder.

**Fraction Et11F$_6$**(1.4398g), eluted with 30-100% MeOH/EtOAc, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.




**Scheme-2.27**Fractionation of Et10F$_2$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et28F$_1$** (0.9982g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et28$F_2$** (0.7296g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a dark green. The $^1$H NMR spectrum is interesting. It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et42$F_1$–Et42$F_3$ (Scheme-2.27).

**Subfraction Et42$F_1$** (0.0053g) &**Et42$F_2$**(0.0662g), eluted with 100% MeOH, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Subfraction Et42$F_3$** (0.0053g), eluted with 100% MeOH, was obtained as a brown solid. The $^1$H NMR spectrum is interesting.It was separated by column chromatography on silica gel (30 g, Merck Art. No.7736, 2 x 15 cm), eluting with $CH_2Cl_2$ and followed by increasing amount of MeOH in $CH_2Cl_2$ and finally with MeOH. Fractions (5 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et61$F_1$–Et61$F_6$ (Scheme-2.27).The $^1$H NMR spectrum of **subfractions Et61$F_1$–Et61$F_6$**are not interesting, so further purification were not carried out.

**Subfraction Et42$F_4$** (0.0197g) &**Et42$F_5$** (0.0046g), eluted with 100% MeOH, was obtained as a red solid, contained mainly pigments. The $^1$H NMR spectrum is not interesting also, so further purification was not carried out.

**Fraction Et28$F_3$** (0.0935g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et28$F_4$** (0.0153g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et28$F_5$** (0.0070g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et28$F_6$** (0.0767g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a pink solid. The $^1$H NMR spectrum of this fraction is interesting.It was separated by HPLC

(C-18, revere phase column), eluting with 30% $H_2O$/MeOH. Eight pick were collected, monitored by TLC. The solvents were evaporated to afford sub fractions Et52$F_1$–Et52$F_8$ (Scheme-2.27).

The $^1$H NMR spectrum of **subfraction 52$F_1$**is not interesting, so further purification was not carried out.

**Subfraction Et52$F_2$**(0.0057g), obtained as a yellow powder, It is pure compound(**compound22, new).**

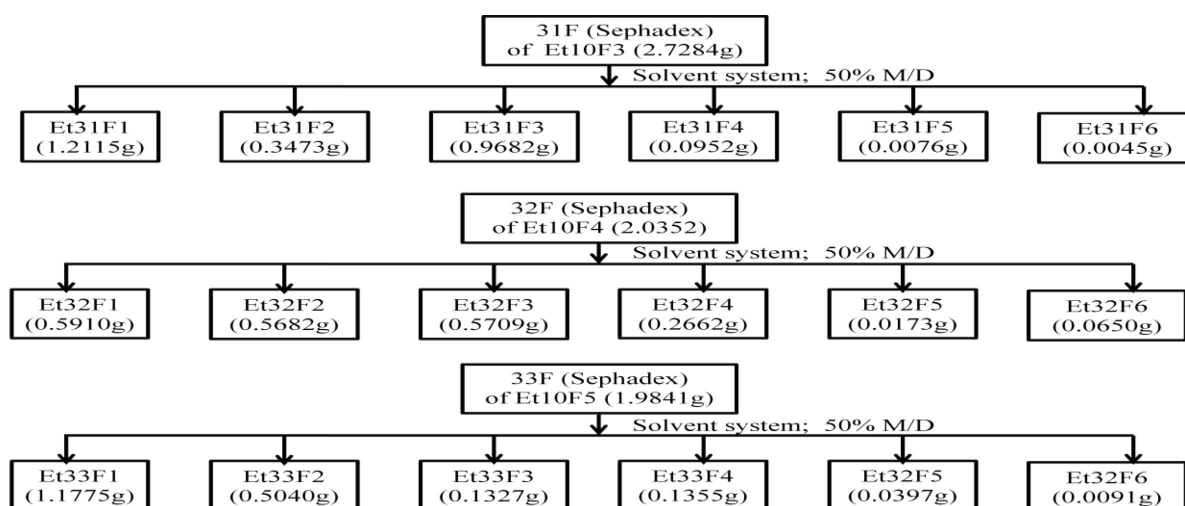
The $^1$H NMR spectrum of **subfraction 52$F_3$ & 52$F_4$**is interesting but very small amount, so further purification was not carried out.

**Subfraction Et52$F_5$**(0.0012g), obtained as a white powderis interesting but very small amount, so further purification was not carried out.

The $^1$H NMR spectrum of **subfraction 52$F_6$ & 52$F_7$**is interesting but very small amount, so further purification was not carried out.

**Subfraction Et52$F_2$**(0.0338g), obtained as a yellow solid, It is pure compound(**Compound 21, new).**

**Fraction Et28$F_7$** (0.0070g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a pink solid, contained mainly pigments. The $^1$H NMR spectrum is not interesting also, so further purification was not carried out.




**Scheme-2.28**Fractionation of Et10$F_{3-5}$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et31F$_1$** (1.2115g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et31F$_2$** (0.3437g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et31F$_3$** (0.9682g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a dark brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et31F$_4$** (0.0952g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et31F$_5$** (0.0076g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a pink solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et31F$_6$** (0.0045g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a pink solid, contained mainly pigments. The $^1$H NMR spectrum is not interesting also, so further purification was not carried out.

**Fraction Et32F$_1$** (0.5910g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a dark green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et32F$_2$** (0.5682g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et32F$_3$** (0.5709g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a dark brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et32F$_4$** (0.2662g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et32F$_5$** (0.0173g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et32F$_6$** (0.0650g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid, contained mainly pigments. The $^1$H NMR spectrum is not interesting also, so further purification was not carried out.

**Fraction Et33F$_1$** (0.1775g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a dark green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

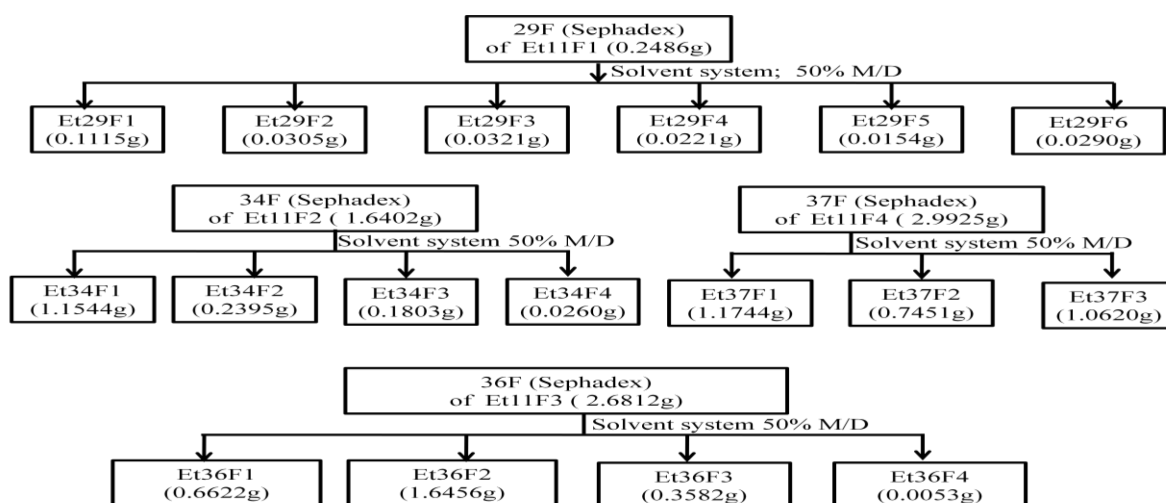
**Fraction Et33F$_2$** (0.5040g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et33F$_3$** (0.1372g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a dark brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et33F$_4$** (0.1355g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et33F$_5$** (0.0397g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et33F$_6$** (0.0091g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid, contained mainly pigments. The $^1$H NMR spectrum is not interesting also, so further purification was not carried out.




**Scheme-2.29**Fractionation of Et10$F_{3-5}$ of ethyl acetate extracts of leaves and twigs of *A. rivularis*.

**Fraction Et29$F_1$** (0.1115g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a green solid, it's contained mainly porphyrine derivatives. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et29$F_2$** (0.0305g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et29$F_3$** (0.0321g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a dark brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et29$F_4$** (0.0221g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et29$F_5$** (0.0154g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a pink solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et29$F_6$** (0.0290g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a pink solid, contained mainly pigments. The $^1$H NMR spectrum is not interesting also, so further purification was not carried out.

**Fraction Et34F$_1$** (1.1544g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. It's contained mainly porphyrine derivatives, so further purification was not carried out.

**Fraction Et34F$_2$** (0.2395g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et34F$_3$** (0.1803g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et34F$_4$** (0.0260g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a pink semi-solid, contained mainly pigments. The $^1$H NMR spectrum is not interesting also, so further purification was not carried out.

**Fraction Et36F$_1$** (0.6622g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. It's contained mainly porphyrine derivatives, so further purification was not carried out.

**Fraction Et36F$_2$** (1.6456g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

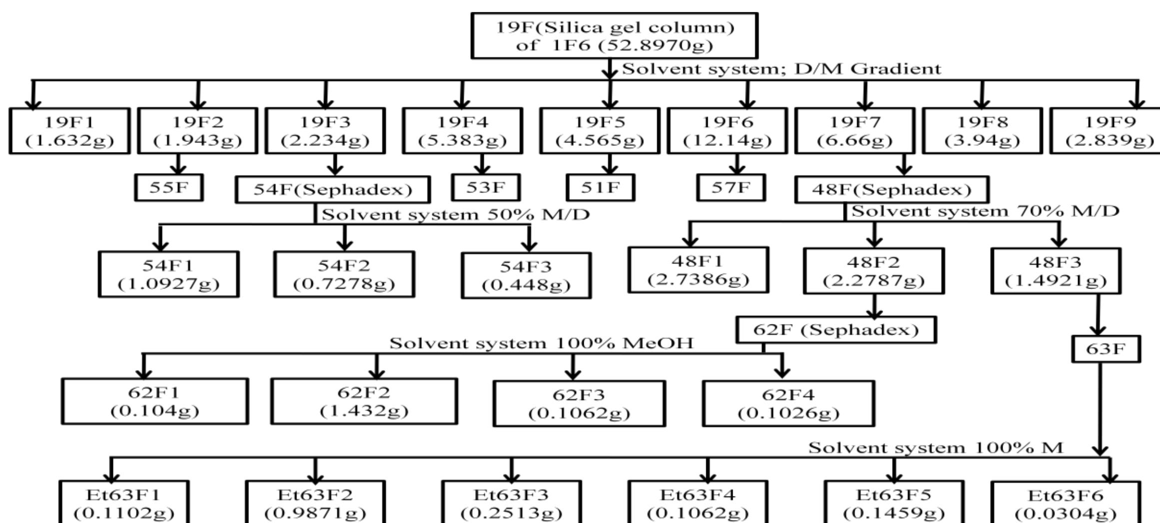
**Fraction Et36F$_3$** (0.3682g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a yellow solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et36F$_4$** (0.0053g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red semi-solid, contained mainly pigments. The $^1$H NMR spectrum is not interesting also, so further purification was not carried out.

**Fraction Et37F$_1$** (1.1744g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. It's contained mainly porphyrine derivatives, so further purification was not carried out.

**Fraction Et37F$_2$** (0.7451g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et34F$_3$** (1.0620g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.




**Scheme-2.30**Fractionation of Et1F$_6$ of ethyl acetate extracts of leaves and twigs of *A. rivularis.*

**FractionEt19F$_1$** (1.6320g), eluted with 100% $CH_2Cl_2$ to 3% MeOH/$CH_2Cl_2$, was obtained as yellow semi-solid and was found to contain mainly fat, so further purification was not carried out.

**Fraction Et19F$_2$**(1.9130g), eluted with 3-6% MeOH/$CH_2Cl_2$,was obtained dark brown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et55F$_1$–Et55F$_5$ (Scheme-2.31).

**Fraction Et19F$_3$**(1.9130g), eluted with 3-6% MeOH/$CH_2Cl_2$, was obtaineddark brown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et54F$_1$–Et54F$_3$ (Scheme-2.30).

**Subfraction Et34F$_1$** (1.0927g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. It’s contained mainly porphyrine derivatives, so further purification was not carried out.

**Subfraction Et54F$_2$** (0.7278g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Subfraction Et54F$_3$** (0.4480g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et19F$_4$** (5.380g), eluted with 9% MeOH/$CH_2Cl_2$, was obtained dark brown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-LH$_{20}$, eluting with 80% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et53F$_1$–Et53F$_3$ (Scheme-2.31).

**Fraction Et19F$_5$** (4.5657g), eluted with 12-15% MeOH/$CH_2Cl_2$, was obtained dark brown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 80% MeOH/$CH_2Cl_2$. Fractions (2 mleach) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et51F$_1$–Et51F$_3$ (Scheme-2.31).

**Fraction Et19F$_6$** (12.1494g), eluted with 20% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is interesting.It was separated by column chromatography on silica gel (300 g, Merck Art. No.7734, 6 x 16 cm), eluting with $CH_2Cl_2$ and followed by increasing amount of MeOH in $CH_2Cl_2$ and finally with MeOH. Fractions (5 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford subfractions Et57F$_1$–Et57F$_7$ (Scheme-2.31).

**Fraction Et19F$_7$** (4.5657g), eluted with 30% MeOH/$CH_2Cl_2$, was obtained dark brown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 70% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et48F$_1$–Et48F$_3$ (Scheme-2.30).

**Subfraction Et48F$_1$** (2.7386g), eluted with 70% MeOH/$CH_2Cl_2$, was obtained as a green solid. It’s contained mainly porphyrine derivatives, so further purification was not carried out.

**Subfraction Et48F$_2$**(2.2787g), eluted with 70% MeOH/$CH_2Cl_2$, was obtainedbrown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 100% MeOH. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et62F$_1$–Et62F$_4$ (Scheme-2.30).

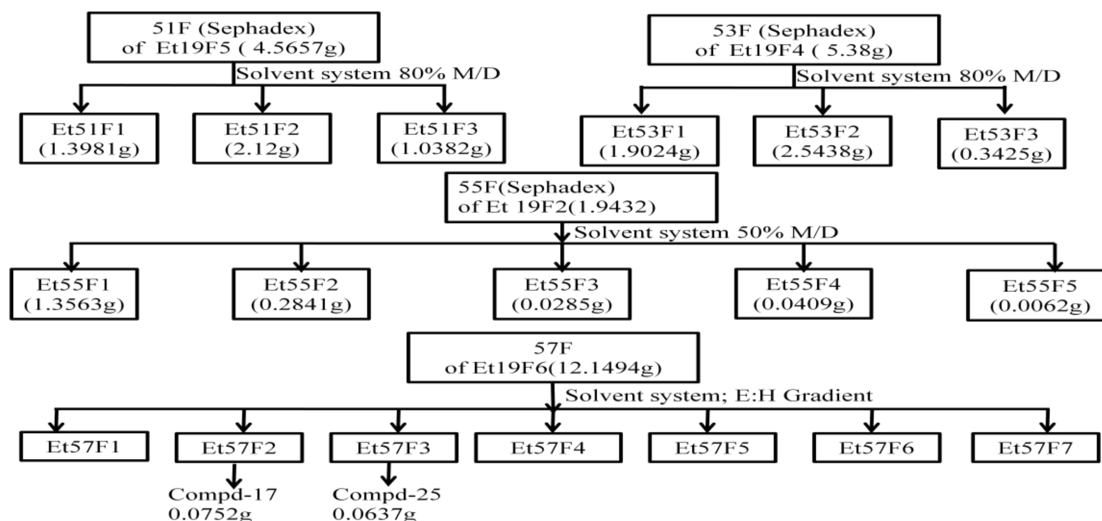
The $^1$H NMR of sub fractions **Et62F$_1$–Et62F$_4$** was not interesting,so further purification was not carried out.

**Subfraction Et48F$_3$**(1.4921g), eluted with 70% MeOH/$CH_2Cl_2$, was obtainedbrown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 100% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et63F$_1$–Et63F$_6$ (Scheme-2.30).

The $^1$H NMR of sub fractions **Et63F$_1$–Et63F$_6$** was not interesting,so further purification was not carried out.

**FractionEt19F$_8$** (3.94g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as reddish brown solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out

**FractionEt19F$_9$** (2.8391g), eluted with 100% MeOH, was obtained as reddish brownand was found to contain mainly fatty acid, so further purification was not carried out.



**Scheme-2.31**Fractionation of $Et19F_{2,4,5,6}$ of ethyl acetate extracts of leaves and twigs of *A. rivularis.*

**Fraction $Et51F_1$** (1.3981g), eluted with 80% $MeOH/CH_2Cl_2$, was obtained as a green solid. The $^1H$ NMR spectrum is not interesting, so further purification was not carried out.

**Fraction $Et51F_2$** (2.12g), eluted with 80% $MeOH/CH_2Cl_2$, was obtained as a dark brown solid. It's need to further purification.

**Fraction $Et51F_3$** (1.0382g), eluted with 80% $MeOH/CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Fraction $Et53F_1$** (1.9024g), eluted with 80% $MeOH/CH_2Cl_2$, was obtained as a green solid. The $^1H$ NMR spectrum is not interesting, so further purification was not carried out.

**Fraction $Et53F_2$** (2.5438g), eluted with 80% $MeOH/CH_2Cl_2$, was obtained as a dark brown solid. It's need to further purification.

**Fraction $Et53F_3$** (1.0382g), eluted with 80% $MeOH/CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Fraction $Et55F_1$** (1.3563g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a green solid. The $^1H$ NMR spectrum is not interesting, so further purification was not carried out.

**Fraction $Et55F_2$** (0.2841g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a dark brown solid. It's need to further purification.

**Fraction $Et55F_3$** (0.0285g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Fraction $Et55F_4$** (0.0409g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Fraction $Et55F_5$** (0.0062g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a reddish brown solid, it contained mainly pigments, so further purification was not carried out.

**Fraction $Et57F_1$**, eluted with 50-80% EtOAc/hexane, was obtained as a green solid and was found to contain mainly fat, so further purification was not carried out.

**Fraction Et57F$_2$**, eluted with 100% EtOAc/hexane to 2% MeOH/$CH_2Cl_2$. Pure 0.0752g,**Stigmast-5-en-3-*O*-β-glucoside (compound17)** was powder out as a white powder.

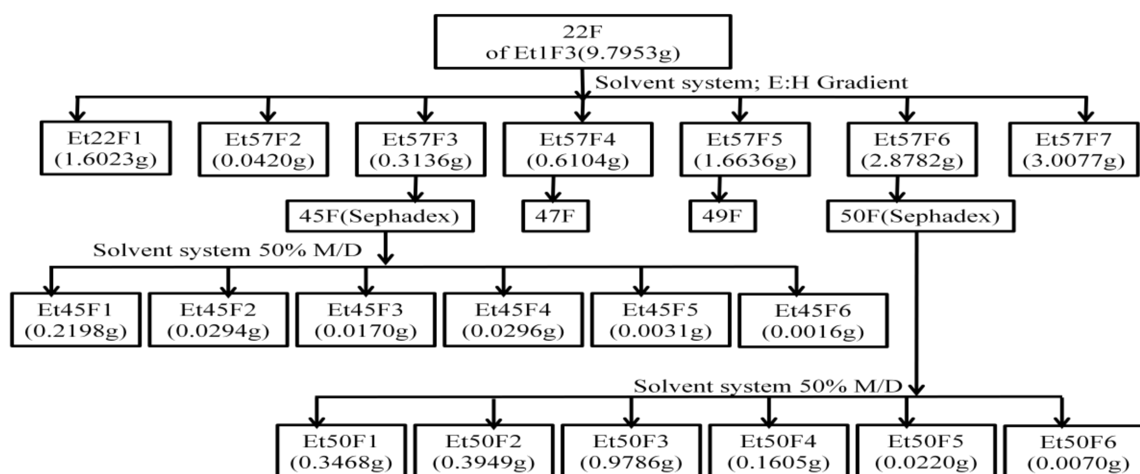
**Fraction Et57F$_3$** , eluted with 5% MeOH/$CH_2Cl_2$, 0.0637g,was powder out as a white powder.

**Fraction Et57F$_4$**, eluted with 10% MeOH/$CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Fraction Et57F$_5$**, eluted with 15-20% MeOH/$CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Fraction Et57F$_6$**, eluted with 30% MeOH/$CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Fraction Et57F$_7$**, eluted with 50% MeOH/$CH_2Cl_2$ to 100% MeOH, was obtained as a brown semi-solid. It's contained mainly fatty acid, so no need to further purification.




**Scheme-2.32**fractionation of Et1F$_3$ of ethyl acetate extracts of leaves and twigs of *A. rivularis.*

**Fraction Et22F$_1$** (1.6032g), eluted with 100% hexane 1% EtOAc/hexane, was obtained as a yellow semi-solid, contained mainly fat, so further purification was not carried out.

**Fraction Et22$F_2$** (0.0420g), eluted with 10% EtOAc/hexane, was obtained as a green semi-solid. The $^1$H NMR spectrum is not interesting, so further purification was not carried out.

**Fraction Et22$F_3$**(0.3136g), eluted with 15% EtOAc/hexane, was obtainedbrown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions Et45$F_1$–Et45$F_6$ (Scheme-2.32).

**Subfraction Et45$F_1$** (0.2198g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid, it contained mainly porphyrine derivatives, so further investigation was not carried out.

**Subfraction Et45$F_2$** (0.0294g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid. The $^1$H NMR spectrum is not interesting, so further investigation was not carried out.

**Subfraction Et45$F_3$** (0.0170g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further investigation was not carried out.

**Subfraction Et45$F_4$** (0.0296g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid, The $^1$H NMR spectrum is interesting. It was separated by reverse phase column chromatography, eluting with MeOH/$H_2O$ and followed by increasing amount of MeOH in $H_2O$ and finally with MeOH. This column needs to subfractionate on the basis of their TLC characteristic.

**Subfraction Et45$F_5$** (0.0031g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid. The $^1$H NMR spectrum is not interesting, so further investigation was not carried out.

**Subfraction Et45$F_6$** (0.0016g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a pink solid, it's contained mainly pigments, so further investigation was not carried out.

**Fraction Et22$F_4$**(0.6104g), eluted with 20% EtOAc/hexane, was obtainedbrown semi-solid. The $^1$H NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 50% MeOH/$CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC

characteristics. The solvents were evaporated to afford sub fractions $Et47F_1$–$Et47F_6$ (Scheme-2.33).

**Fraction $Et22F_5$**(1.6636g), eluted with 20% EtOAc/hexane, was obtainedbrown semi-solid. The $^1H$ NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 50% $MeOH/CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions $Et49F_1$–$Et49F_6$ (Scheme-2.33).

**Fraction $Et22F_6$**(2.8728g), eluted with 25-30% EtOAc/hexane, was obtainedbrown semi-solid. The $^1H$ NMR is interesting.It was separated by column chromatography on sephadex-LH20, eluting with 50% $MeOH/CH_2Cl_2$. Fractions (2 ml each) were collected, monitored by TLC and combined on the basis of their TLC characteristics. The solvents were evaporated to afford sub fractions $Et50F_1$–$Et50F_6$ (Scheme-2.32).

**Subfraction $Et50F_1$** (0.3468g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Subfraction $Et50F_2$** (0.3949g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

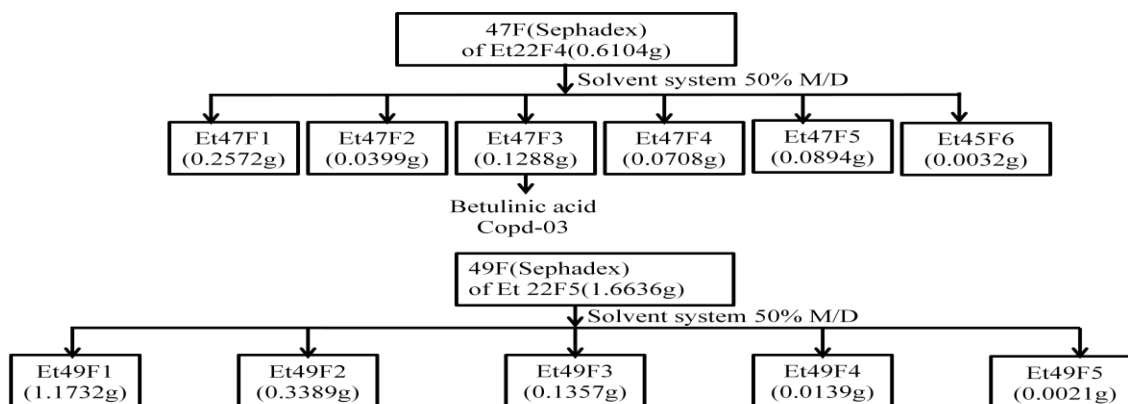
**Subfraction $Et50F_3$** (0.9786g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Subfraction $Et50F_4$** (0.1605g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a brown solid. It's need to further purification.

**Subfraction $Et50F_5$** (0.0220g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a reddish brown solid. It's need to further purification.

**Subfraction $Et50F_2$** (0.0070g), eluted with 50% $MeOH/CH_2Cl_2$, was obtained as a red solid, contained mainly pigments, so no need to further purification.

**Fraction $Et22F_7$**(3.0077g), eluted with 40-100% EtOAc/hexane to 100% MeOH, was obtainedbrown semi-solid. The $^1H$ NMR is interesting, so it needs to further purification.




**Scheme-2.33**Fractionation of Et22$F_{4,5}$ of ethyl acetate extracts of leaves and twigs of *A. rivularis.*

**Fraction Et47$F_1$** (0.2572g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid, it contained mainly porphyrine derivatives, so further investigation was not carried out.

**Fraction Et47$F_2$** (0.0399g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is not interesting, so further investigation was not carried out.

**Fraction Et47$F_3$**(0.1288g), eluted with 50% MeOH/$CH_2Cl_2$. Pure 0.0708g,**Betulinic acid (comp$^d$-03)** was powder out as a white needle crystal.

**Fraction Et47$F_4$** (0.0220g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a reddish brown solid. It's need to further purification.

**Subfraction Et47$F_5$** (0.0894g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid, The $^1$H NMR spectrum is interesting. It was separated by reverse phase column chromatography, eluting with MeOH/$H_2O$ gradient and followed by increasing amount of MeOH in $H_2O$ and finally with MeOH. This column needs to subfractionate on the basis of their TLC characteristic. Vial no. 98-102 has white crystal its need to characterize also.

**Fraction Et47$F_6$**(0.0032g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid, contained mainly pigments, so no need to further purification.

**Fraction Et49F$_1$** (1.1732g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a green solid, it contained mainly prophyrine derivatives, so further investigation was not carried out.

**Fraction Et49F$_2$** (0.3389g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a brown solid. The $^1$H NMR spectrum is interesting, so it's need to further purification.

**Fraction Et49F$_3$** (0.1357g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a reddish brown solid. It's need to further purification.

**Fraction Et49F$_4$** (0.0139g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a reddish brown solid. It's need to further purification.

**Fraction Et47F$_6$**(0.0021g), eluted with 50% MeOH/$CH_2Cl_2$, was obtained as a red solid, contained mainly pigments, so no need to further purification.

## PART-B: Herba Fomulations

### 2.6 Study area and sample collection

Samples of thirteen antidiabetic herbal preparations (ADPHs) as finish commercial pack were purchased randomly from different herbal medicine outlets of Dhaka city. Initially, all the samples were prepared for analysis in the research laboratory,Department of Chemistry, University of Dhaka, Microbiological contamination and heavy metal content were analyzed in the Centre for Advanced Research in Sciences (CARS), University of Dhaka, Bangladesh.

### 2.7 Determination of pH of sample

The pH of different herbal preparation was determined by using microprocessor pH meter (HI 2210; Hanna Instrument, USA)as described earlier [59]. For pH determination, sample solution was prepared by dissolving 12.5 g in 100 mL sterile distilled water with shaking to obtain a homogeneous solution. The pH of the solution of different herbal preparation was measured by microprocessor pH meter and the data are presented as the average of triplicate.

**2.8 Preparation of media**

All the media of microbiological analysis were perpetrated according to the manufactures guidelines and sterilized in an autoclave (CL-32S; ALPCo. Ltd., Japan) at 121℃ for 40 minutes. The sterile media were dispensed or poured into sterilized Petri dishes or test tube as required . The sterility of the prepared media was confirmed by incubating blindly selected plates at 37 ˚C for overnight.

**2.9 Total aerobic bacterial count and total coliform count**

Total aerobic bacterial count was performed to assess the quality and shelf life of the herbal formulation. Twenty five (25) g of each sample were homogenized in 225 ml of sterile saline water. After that, 0.1 ml from two fold diluted samples was spread on a Petridish containing Tryptic Soy agar (TSA)(Oxoid Ltd, Hampshire, England) and incubated at 35ºC for 24 hours for total aerobic bacterial count [60]. To assess the hygiene of the formulations, total coliform count was performed by spreading 0.1 ml of the sample (as used for total aerobic count) on MacConkey agar (Oxoid Ltd, Hampshire, England) and was incubated at 35ºC and 42ºC for 24 hours [61].

**2.10 *Escherichia coli* 0157**

Twenty five (25) g of each sample were homogenized in 225 ml EC medium and incubated at 42ºC for 20 hours. The enriched cultures were streaked onto Sorbitol MacConkeyagar complemented with Cefixime and potassium tellurite supplement and characteristic colonies were subjected to biochemical tests (IMViC). Biochemically confirmed isolates were screened through Caprylate-thallous Agar (CTA) and CHROM Agar. The colonies, which gave characteristic color, were subsequently serotyped by 0157 antisera.

**2.11 *Escherichia coli,* (total)**

Twenty five (25) g of each sample were homogenized in 225 ml *Enterobacteria* enrichment broth-Mossel pre-enrichment medium and incubated at 35ºC for 20 hours. One milliliter aliquots of pre-enriched cultures was mixed with nine milliliters of 2x EC medium and incubated at 35ºC for 20 hours. One loop-full of the culture was inoculated into 10 milliliters 1x EC medium with Durham fermentation tubes and incubated at 42ºC for 20 hours. To isolate *E. coli*, one loop-full of gas produced 1x EC culture broth was streaked on Chromocult agar (CTA) plates and developed typical colonies. The same pre-enrichment culture was used for isolation and characterization of coliform bacteria on Sorbitol MacConkey (SMAC) agar.

**2.12 Salmonella spp.**

Twenty five (25) g of each sample were homogenized in 225 ml of buffered peptone water and incubated at 35ºC for 20 hours. One milliliter pre-enrichment cultures was mixed with nine milliliters of Hanja Tetrathionate Broth and incubated at 35ºC for 20 hours and nine milliliters of Rappaport-Vassiliadis Broth and incubated at 42ºC for 20 hours. The culture broths were subsequently streaked onto Bismuth sulfite ager (BSA). For isolating of each microorganism original solution and $10^{-2}$ (hundred times diluted solution)were used for microbial limit test and $p^H$ of the samples were controlled within the range of 6.9 - 7.9 by adding NaOH or HCL.

**2.13 Qualitative Fungi Counts**

Fungi were identified on Potato dextrose agar (PDA) (Oxoid Ltd, Hampshire, England) after incubation at 30ºC for 5 days. Procedure and dilution were followed as described for total bacterial aerobic count. At the end of 5 days incubation the fungal growth was observed under microscope [62].

**2.14 Sample Preparation and Heavy Metal Analysis**

Heavy metals were analyzed in flame atomizer based Atomic AbsorptionSpectrometer using hollow cathode lamp as a radiation source.Accurately 25g of herbal preparation were transferred into silica crucible and kept in a muffle furnace for ashing at 700ºC for 1 hour. The sample was then cooled down to room temperature and the

heating process was repeated for three times. The ash was then dissolved by adding 5-10 ml of concentrated HCl and finally diluted the sample by 0.1 N $HNO_3$ up to 25 ml. Finally the sample was prepared for heavy metal analysis by filtering through Whiteman filter paper.

For heavy metal analysis, the samples were aspirated through nebulizer and the absorbance was measured against a blank as reference. Specific hollow cathode lamps were used to analyze Copper (wavelength 324.8 nm), Cadmium (wavelength 228.8 nm) and Chromium (wavelength 357.9 nm), Manganese (wavelength 297.5 nm), Lead (wavelength 283.3 nm) and Zinc (wavelength 213.9 nm).Before analysis, the samples were diluted to the appropriate factor according to the detection limit of the Atomic Absorption Spectrophotometer (AAnalyst 200; Perkin Elmer, USA).Calibration curve was obtained using referent standard and all the measurements were run in triplicate for the samples and standards solutions.

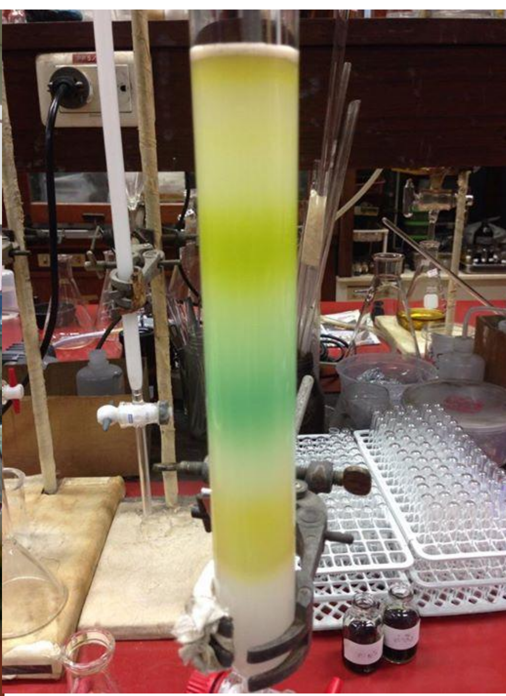
**Table 2.1:** Composition of different antidiabetic herbal preparations (ADHPs) and daily adult dose as indicated on the label of the products.

| **Sample ID** | **Brand Name** | **Composition of the preparation as indicated on the label of finished product** | **Daily adult (70 kg bw) dose in g** | **pH** |
|---|---|---|---|---|
| ADHP-1 | Diacare | *Bambusabambos*(surface of inner skin) *Gymnema Sylvester* (leaf), *Acacia Arabica* (leaf) *Rumexvesicarius*(seeds) | 1.5 | 5.86 |
| ADHP-2 | Ziabit | *Natrumsulfuricum* (a constitutional Homeopathy remedy),Glauber's Salts (Sodium sulphatedeca-hydrate) | 1 | 5.36 |
| ADHP-3 | Insucontrol | *Syzygiumcumini*(seeds),Ferrous sulphate (Salt) *Rumexvesicarius* (seeds) | 3 | 5.44 |
| ADHP-4 | Dolabi | *Gymnemasylvestre* (leaf), *Rumexvesicarius* (seeds) *Bambusabambos*(surface of inner skin),Asphalt | 2.5 | 6.74 |
| ADHP-5 | Diazym | *Gymnema Sylvester* (leaf),Asphalt *Mytilusmargaritiferus* | 2.5 | 6.25 |
| ADHP-6 | Alisa | *Allium sativum,Allium cepa,Mangiferaindica* (leaf) *Myristicafragrance*(dried kernel of the seed) *Syzygiumaromaticum* (flower) | 3.75 | 8.18 |
| ADHP-7 | DaruchiniJa | *Cinnamomumzeylanicum* (bark) | 4.25 | 5.81 |

| | | | | |
|---|---|---|---|---|
| | mseed | *Accacia acuminate* (seeds) | | |
| ADHP-8 | Garlic | *Allium sativum*(bulbs),*Allium cepa*(bulbs)<br>*Syzygiumcumini*(seed),*Mangiferaindica*(leaf)<br>*Myristicafragrance*(dried kernel of the seed)<br>*Syzygiumaromaticum*(flower) | 3.5 | 7.83 |
| ADHP-9 | Methicrash | *Trigonellafoenum-graecum*(seed) | 30 | 6.70 |
| ADHP-10 | Diano | *Bambusaarundinacea*(surface of inner skin)<br>*Rumexvesicarius*(seed),*Gymnemasylvestre*(leaf)<br>Hen's egg shell, Ferrous sulphate, *Mytilusmargaritiferus*, Asphalt | 2.5 | 7.10 |
| ADHP-11 | Azardiracha Indica | *Azadirachtaindica* (leaf extract) | 2.5 | 6.35 |
| ADHP-12 | Cuzium Jam | *Cinnamomumzeylanicum* (bark)<br>*Accacia acuminate* (seeds) | 4 | 5.72 |
| ADHP-13 | Silaraj | *Salvia haematodes* (bark and root),Asphalt<br>Calcined iron oxide,Calcinedstannum | 2.5 | 7.56 |

**Figure-2.1**Some woarking pictures during column chromatography

Flash column



Vacuum Column Column

**Figure-2.2**Some pictures equepments

NMR (400 MHz)

AAS

# CHAPTER III

# RESULTS AND DISCUSSION

## PART-A

## Biological and Chemical Studies of *Anogeissus rivularis* Gannep O. Lecompte

### 3.1 Biological Studies:

The air-dried leaves and twigs of *Anogeissus rivularis (*Gagnep O. Lecompte) were percolated successively with hexane, ethyl acetate and methanol at room temperature, the extracts were collected by filtration. The filtrates were evaporated to dryness under reduce pressure to afford a crude extracts. The crude extracts and the sub-fractions thereof were subjected to biological especially; cytotoxic and anti HIV-1 RT. The results are shown in Table-3.1 to 3.4

**Table-3.1:** Cytotoxic assays of hexane extract and fractions of leaves and twigs of *A. rivularis*

| Extraction/ fractions | Cytotoxicity | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cell lines* | | | | | | |
| | P-388 | KB | HT-29 | MCF-7 | A549 | ASK | Hek 293 |
| Hexane extract | >20 | >20 | 14.31 | >20 | >20 | >20 | >20 |
| 1F-1 | >20 | >20 | >20 | >20 | >20 | >20 | >20 |
| 1F-2 | 10.00 | >20 | >20 | >20 | >20 | >20 | >20 |
| 1F-3 | <4 | 15.29 | 13.94 | 12.31 | 13.93 | >20 | 9.30 |
| 1F-4 | >20 | >20 | >20 | >20 | >20 | >20 | >20 |
| 1F-5 | 3.40 | >20 | >20 | 16.83 | >20 | >20 | >20 |

*Results are expressed as $ED_{50}$ (μg/mL): $ED_{50}$< 20 μg/mL is considered active. P-388 = murine lymphocytic leukemia, KB = human nasopharyngeal carcinoma, HT29 = human colorectal adenocarcinoma, MCF-7 = human breast cancer, A549 = human lung carcinoma, ASK = rat glioma, Hek 293= normal human kidney cell.

The results in Table-3.1 indicated that the crude hexane extract was not toxic in all the cell lines except HT-29 ($ED_{50}$ 14.μg/mL) whereas of its sub-fractions1F-5 1F-3 and 1F-2 were found to be significantly active against P388 cell line [ED50 were 3.40, <4 and 10.00 μg/mL respectively].

Fraction 1F-3 showed was moderately cytotoxic against KB HT-29 MCF-7A549 and Hek293 cell lines and $ED_{50}$were found to be 15.29, 13.94, 12.31, 13.93 and 9.30µg/mL, respectively. Fraction 1F-5 was also moderately cytotoxic against the cell line MCF-7 ($ED_{50}$16.83).

**Table-3.2:** Cytotoxic assays of ethyl acetate extract and fractions of*A. rivularis*

| Extraction/ fractions | Cytotoxicity Cell lines* | | | | | | |
|---|---|---|---|---|---|---|---|
| | P-388 | KB | HT-29 | MCF-7 | A549 | ASK | Hek 293 |
| EtOAc extract | 16.66 | >20 | >20 | >20 | >20 | >20 | >20 |
| Et-1F-1 | >20 | >20 | >20 | >20 | >20 | >20 | >20 |
| Et-1F-2 | 5.40 | >20 | >20 | >20 | >20 | >20 | 11.17 |
| Et-1F-3 | 4.60 | 18.91 | 14.71 | 18.44 | >20 | >20 | 12.78 |
| Et-1F-4 | 6.37 | 16.70 | 12.41 | 16.22 | 14.76 | 16.04 | 12.94 |
| Et-1F-5 | 9.53 | >20 | 17.29 | >20 | >20 | >20 | >20 |
| Et-1F-6 | >20 | >20 | >20 | >20 | >20 | >20 | 19.90 |
| Et-1F-7 | >20 | >20 | >20 | >20 | >20 | >20 | 7.49 |

*Results are expressed as $ED_{50}$ (µg/mL): $ED_{50}$< 20 µg/mL is considered active. P-388 = murine lymphocytic leukemia, KB = human nasopharyngeal carcinoma, HT29 = human colorectal adenocarcinoma, MCF-7 = human breast cancer, A549 = human lung carcinoma, ASK = rat glioma, Hek 293 = normal human kidney cell.

The results in Table-3.2 indicated that the ethyl acetate extract of leaves and twigs of *A. rivularis* was moderately cytotoxic against P-388 cell line ($ED_{50}$ (µg/mL 16.66) but its sub-fraction Et-1F-2 Et-1F-3 and Et-1F-4 possessed high anticancer activity in P388 cell line and $ED_{50}$ were found to be 5.40, 4.60 and 6.37 µg/mL, respectively. Other fractions were moderately cytotoxic against other cell lines.

Cytotoxicity of the extracts and sub-fractions are dependent on the chemical component(s) present in the extracts and sub-fractions. Cytotoxicity assay showed that some of the sub-fractions are potent to isolate cytotoxic compounds which help in anticancer drug discovery program.

**Table-3.3:** Result of Anti-HIV 1RT of Hexane extract and fractions of *A. rivularis*

| Extract/Fraction | Anti-HIV-1 RT** | |
|---|---|---|
| | %inhibition | Activity |
| Hexane extract | 60.50 | M |
| 1F-1 | 1.08 | I |
| 1F-2 | 51.49 | M |
| 1F-3 | 22.56 | I |
| 1F-4 | 3.01 | I |
| 1F-5 | 97.29 | VA |

**RT assay: VA= very active (>70% inhibition), M = moderately active (>50% to 70% inhibition), W = weakly active (30% to 50% inhibition), I = inactive (<30% inhibition).

The results from the Table-3.3 showed that the hexane extract and fraction 1F-2 are moderately active against anti-HIV-1-RT assay with 60.50 and 51.49 % inhibition respectively. Fraction 1F-5 is very active against anti-HIV-1-RT with 97.29 % inhibition.

**Table-3.4:** Results of Anti-HIV–1RT of ethyl acetate extract and fractions of*A. rivularis*

| Extract/Fraction | Anti-HIV-1 RT** | |
|---|---|---|
| | %inhibition | Activity |
| EtOAC extract | 59.31 | M |
| Et-1F-1 | 14.24 | I |
| Et-1F-2 | 26.90 | I |
| Et-1F-3 | 23.84 | I |
| Et-1F-4 | 43.64 | W |
| Et-1F-5 | 32.08 | W |
| Et-1F-6 | 48.54 | W |
| Et-1F-7 | 92.56 | VA |

**RT assay: VA= very active (>70% inhibition), M = moderately active (>50% to 70% inhibition), W = weakly active (30% to 50% inhibition), I = inactive (<30% inhibition).

The results from the Table-3.4 showed that the ethyl acetate extracts are moderately active against anti-HIV-1-RT assay with 59.31 % inhibition. Fraction Et-1F-4, Et-1F-5 and Et-1F-6are weakly active against anti-HIV-1-RTassay with 43.64%, 32.08% and 48.54% inhibition. Fraction Et-1F-7 is very active against anti-HIV-1-RTassay with 92.56 % inhibition.

### 3.2 Chemical Studies

The search for bioactive constituents from the traditionally used medicinal plant sources has led us to initiate bioassay-guided isolation on *Anogeissus rivularis*. Eight compounds (**1**-**8**) were isolated from hexane extract among them **1** was found as a fatty aldehyde, **2** and **4** were fatty alcohol (as the compounds are very simple their structures are not given in the Thesis) and **5** was a mixture of two compounds (β-sitosterol and stigmasterol). From the ethyl acetate extract fifteen compounds (**9**-**23**) including four new compounds (**20**-**23**) were isolated. Compound **10** and **11**were found to be a mixture of two compounds and were not further studied. Isolated known compounds were characterized from their physical properties (melting point, optical rotation), UV, FTIR, high resolution $^{1}$H & $^{13}$C NMR and mass spectral data and comparison with NMR spectral data of known compounds. Isolated four new compounds were characterized from their physical properties (melting point, optical rotation) and extensive spectroscopic studies; UV, FTIR, high resolution $^{1}$H &$^{13}$C NMR (including H-H COSY, HSQC, HMBC and NOSEY) spectral data, high resolution mass and CD spectra.

Compound **3: Betulinic Acid** was obtained as white needle shaped crystal. Melting point was recorded as 295°C and specific rotation was $[\alpha]_D^{25}$= +4.64 (*c* 0.41, pyridine). Reported melting point (290-295°C) and specific rotation were fitted very well {$[\alpha]_D^{25}$=+7.5 (*c*0.37, Pyridine)}[63]with experimental value. The melting point and specific rotation value is low compare to the reported value might be due to the presence of insignificant amount of impurity and or experimental error. The molecular formula was determined as $C_{30}H_{50}O_2$ from high resolution mass spectrum (HRMS-ESI-TOP) at *m/z* 479.3485 $[M+Na]^+$ (Calculated value 479.3496 for $[C_{30}H_{48}NaO_3]^+$) which was also confirmed the molecular formula of compound **3.**

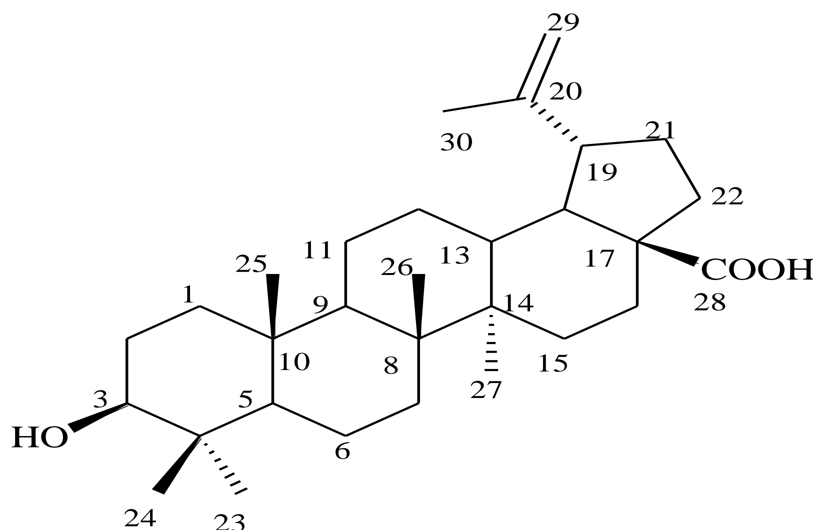



**Figure-3.1 Compound 3: 3β-Hydroxy-19β-hydrogen-lup-20-(29)-en-28-oic acid (Betulinic Acid)**

The $^1$H-NMR spectrum of Compound-**3** (pyridine-$d_5$) showed twenty four signals for protons and $^{13}$C-NMR spectrum of Compound **3** (in pyridine-$d_5$) showed thirty signals for thirty carbons (Table 3.5). The spectral data of Compound **3** was compared with reported NMR spectral data of betulinic acid (Tabla-3.5)[64]. From the physical properties and spectroscopic data and comparison with reported values Compound **3** was identified as Betulinic acid. Betulinic acid is a common constituent of medicinal plants. In this plant it was found as a major compound.

**Table-3.5:** The $^{1}H$ and $^{13}C$ NMR data of Compound **3** in comparison with reported data

| Position | Our data (Pyidine-$d_5$) | | | Reported data (pyridine-$d_5$) [64] | | |
|---|---|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm | | $\delta_C$ ppm |
| | $H_\alpha$ | $H_\beta$ | | $H_\alpha$ | $H_\beta$ | |
| 1 | 0.99 (m) | 1.66 (m) | 39.6 | 0.99 (m) | 1.67 (m) | 39.3 |
| 2 | 1.85 (m) | | 28.6 | 1.85 (m) | | 28.3 |
| 3 | 3.48 (t, 8) | | 78.4 | 3.45 (t, 7.2) | | 78.1 |
| 4 | | | 39.8 | | | 39.5 |
| 5 | 0.82 (m) | | 56.2 | 0.82 (m) | | 56.0 |
| 6 | 1.56 (m) | 1.38 (m) | 19.1 | 1.56 (m) | 1.38 (m) | 18.8 |
| 7 | 1.46 (m) | 1.38 (m) | 35.2 | 1.45 (m) | 1.38 (m) | 34.9 |
| 8 | | | 41.4 | | | 41.1 |
| 9 | 1.38 (m) | | 51.3 | 1.38 (m) | | 51.0 |
| 10 | | | 37.8 | | | 37.6 |
| 11 | 1.43 (m) | 1.21(m) | 21.5 | 1.43 (m) | 1.21(m) | 21.2 |
| 12 | 1.21(m) | 1.95 (m) | 26.4 | 1.21(m) | 1.94 (m) | 26.2 |
| 13 | 2.75 (m) | | 38.9 | 2.74 (m) | | 38.7 |
| 14 | | | 43.2 | | | 42.9 |
| 15 | 1.26 (m) | 1.88 (m) | 30.6 | 1.26 (m) | 1.88 (m) | 30.3 |
| 16 | 1.56(m) | 2.65 (m) | 33.2 | 1.55 (m) | 2.63 (m) | 32.9 |
| 17 | | | 56.9 | | | 56.6 |
| 18 | 1.78 (m) | | 51.1 | 1.77(t, 11.5) | | 49.8 |
| 19 | 3.55 (m) | | 48.1 | 3.52 (m) | | 47.8 |
| 20 | | | 151.6 | | | 151.3 |
| 21 | 1.54 (m) | 2.25 (m) | 31.5 | 1.53(m) | 2.24(m) | 31.2 |
| 22 | 1.57 (m) | 2.28 (m) | 37.9 | 1.57(m) | 2.25(m) | 37.6 |
| 23 | 1.22 (s) | | 29.0 | 1.22 (s) | | 28.7 |
| 24 | 1.03 (s) | | 16.6 | 1.00 (s) | | 16.3 |
| 25 | 0.84 (s) | | 16.7 | 0.83 (s) | | 16.4 |
| 26 | 1.07 (s) | | 16.7 | 1.06 (s) | | 16.4 |
| 27 | 1.09 (s) | | 15.2 | 1.07 (s) | | 14.9 |
| 28 | | | 179.2 | | | 178.8 |
| 29 | 4.97 (s) | 4.79 (s) | 110.2 | 4.95 (s) | 4.77 (s) | 109.9 |
| 30 | 1.81 (s) | | 19.8 | 1.79 (s) | | 19.5 |

Compound **6: 3β-hydroxy-20(29)-en-lupan-30-al** was obtained as white needle shaped crystal. Melting point was recorded as 229$^{\circ}$C and specific rotation was $[\alpha]_D^{25}$=+6.51(*c* 0.14,$CHCl_3$). Reported melting point (284$^{\circ}$C) and specific rotation fitted very close $[\alpha]_D^{25}$ = +5.14 (*c* 0.19, $CHCl_3$) [65]to the experimental value. The melting point value is low compare to the reported value might be due to the presence of insignificant amount of impurity. The difference between reported and recorded value of specific rotation is might be due presence of impurity and or experimental error. The molecular formula was determined as $C_{30}H_{48}O_2$ from high resolution mass spectrum (HRMS-ESI-TOP) at *m/z* 463.3541 $[M+Na]^+$ (Calculated 463.3547 for $[C_{30}H_{48}NaO_2]^+$) which confirmed the molecular formula of Compound-**6.**

**Figure-3.2 Compound 6: 3β-hydroxy-20(29)-en-lupan-30-al**

The $^1$H-NMR spectrum of Compound-**6** ($CDCl_3$) showed twenty four signals for protons and $^{13}$C-NMR spectrum of Compound **6** ($CDCl_3$) showed thirty signals for thirty carbons (Table 3.6). The spectral data of Compound **6** was compared with reported NMR spectral data (Table-3.6) [66]. From the physical properties and spectroscopic data and comparison with reported values [65-66] Compound **6** was identified as a **3β-hydroxy-20(29)-en-lupan-30-al**. Compound-**6** found as a minor compound of this plant.

**Table-3.6:** The $^{1}H$ and $^{13}C$ NMR data of Compound **6** in comparison with reported data

| Position | Our data ($CDCl_3$) | | | Reported data ($CDCl_3$) [66] | | |
|---|---|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm | | $\delta_C$ ppm |
| | $H_\alpha$ | $H_\beta$ | | $H_\alpha$ | $H_\beta$ | |
| 1 | 0.87 | 1.65 | 38.71 | 0.88 | 1.65 | 38.70 |
| 2 | 1.55 | | 27.38 | 1.61 | 1.55 | 27.40 |
| 3 | 3.17 (m) | | 79.00 | 3.18 | | 78.99 |
| 4 | | | 38.86 | | | 38.87 |
| 5 | 0.67 | | 55.29 | 0.67 | | 55.29 |
| 6 | 1.54 | 1.40 | 18.30 | 1.54 | 1.40 | 18.30 |
| 7 | 1.39 | | 34.28 | 1.39 | | 34.28 |
| 8 | | | 40.78 | | | 40.77 |
| 9 | 1.22 | | 50.25 | 1.22 | | 50.24 |
| 10 | | | 37.14 | | | 37.14 |
| 11 | 1.36 | 1.22 | 20.94 | 1.36 | 1.22 | 20.94 |
| 12 | 0.919 | 1.07 | 27.64 | 0.92 | 1.07 | 27.64 |
| 13 | 1.66 | | 37.74 | 1.66 | | 37.73 |
| 14 | | | 42.69 | | | 42.69 |
| 15 | 1.03 | 1.70 | 27.34 | 1.03 | 1.70 | 27.34 |
| 16 | 1.45 | 1.52 | 35.39 | 1.45 | 1.52 | 35.39 |
| 17 | | | 43.28 | | | 43.28 |
| 18 | 1.64 | | 51.18 | 1.64 | | 51.2 |
| 19 | | 2.74 | 36.67 | | 2.75 | 36.7 |
| 20 | | | 157.19 | | | 157.0 |
| 21 | 1.238 | 2.15 | 32.66 | 1.23 | 2.16 | 32.6 |
| 22 | 1.378 | 1.44 | 39.93 | 1.38 | 1.44 | 39.93 |
| 23 | 0.960 | | 27.98 | 0.963 | | 27.99 |
| 24 | | 0.751 | 15.35 | | 0.755 | 15.37 |
| 25 | 0.807 | | 16.05 | 0.811 | | 16.07 |
| 26 | | 1.008 | 15.93 | | 1.013 | 15.94 |
| 27 | 0.919 | | 14.40 | 0.923 | | 14.41 |
| 28 | 0.814 | | 17.78 | | 0.818 | 17.79 |
| 29 | 5.91 | 6.28 | 132.94 | 5.91 | 6.28 | 132.9 |
| 30 | | 9.51 | 195.08 | | 9.51 | 195.08 |

Compound **7: 29-*Nor*-20-oxolupeol** was obtained as white needle shaped crystal. Melting point was recorded 194°C and specific rotation was $[\alpha]_D^{25}$ = -173.80 (*c* 0.02, MeOH), Reported melting point and specific rotation were196°C and $[\alpha]_D^{28}$ = -160 (*c* 0.013, MeOH) [67] respectively. The difference between reported and recorded value of specific rotation is might be due presence of impurity and or experimental error. The molecular formula was determined as $C_{29}H_{48}O_2$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 451.3623 $[M+Na]^+$ (Calculated 451.3547 for $[C_{29}H_{48}NaO_2]^+$) which confirmed the molecular formula of Compound **7**.

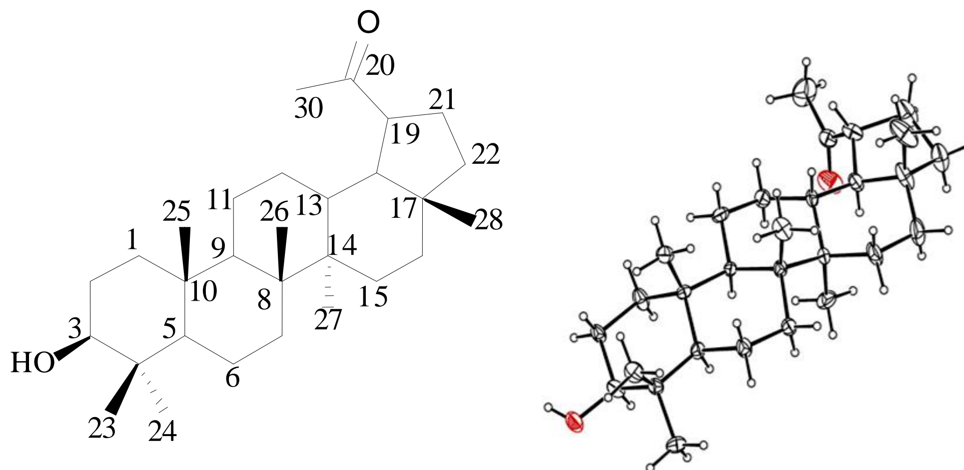



**Figure-3.3 Compound 7: 29-*Nor*-20-oxolupeol**

The $^1H$-NMR spectrum of Compound **7** ($CDCl_3$) showed twenty three signals for protons and $^{13}C$-NMR spectrum showed twenty nine signals for twenty nine carbons. The $^1H$ and $^{13}C$ NMR data of Compound **7** were compared with reference data are shown in Table-3.7. The $^1H$ and $^{13}C$ NMR spectral data and mass of Compound **7** matched with reported data of **29-*Nor*-20-oxolupeol** [68]. The Compound**7** showed negative optical rotation which was also comparable with reported data [67]. Therefore, the Compound **7** was characterized as a **29-*Nor*-20-oxolupeol**. The single crystal X-ray also confirmed the structure of compound **7.** Compound **7** found as a minor compound of this plant.

**Table-3.7:** The $^{1}H$ and $^{13}C$ NMR data of Compound **7** in comparison with reported data

| Position | Our data ($CDCl_3$) | | Reported data ($CDCl_3$) [68] | |
|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm |
| 1 | | 38.64 | | 38.65 |
| 2 | | 27.36 | | 27.31 |
| 3 | 3.20 (dd, 11.37, 4.8) | 78.93 | 3.21 (dd, 11.4, 4.8) | 78.92 |
| 4 | | 38.85 | | 38.85 |
| 5 | 0.68 (brd, 12) | 55.24 | 0.75 (brd, 10.5) | 55.24 |
| 6 | | 18.28 | | 18.28 |
| 7 | | 34.17 | | 34.18 |
| 8 | | 40.72 | | 40.71 |
| 9 | | 50.25 | | 50.25 |
| 10 | | 37.16 | | 37.16 |
| 11 | | 20.89 | | 20.89 |
| 12 | | 27.17 | | 27.17 |
| 13 | | 37.01 | | 37.01[a] |
| 14 | | 43.05 | | 43.05 |
| 15 | | 27.31 | | 27.31 |
| 16 | | 34.96 | | 34.96 |
| 17 | | 42.66 | | 42.66 |
| 18 | 1.84 (t, 11.4) | 49.69 | 1.84 (t, 10.5) | 49.67 |
| 19 | 2.58 (ddd, 11.2, 11.2,5.9) | 52.62 | 2.60 (ddd, 11.4, 11.4, 6) | 52.62 |
| 20 | | 213.00 | | 212.93 |
| 21 | 2.05 (m) | 27.65 | 2.06 (m) | 27.64 |
| 22 | | 39.85 | | 39.84 |
| 23 | 0.97 (s) | 27.98 | 0.99 (s) | 27.98 |
| 24 | 0.77 (s) | 15.38 | 0.79 (s) | 15.36 |
| 25 | 0.82 (s) | 16.08 | 0.85 (s) | 16.07 |
| 26 | 1.01 (s) | 15.90 | 1.04 (s) | 15.90 |
| 27 | 0.97 (s) | 14.46 | 0.99 (s) | 14.46 |
| 28 | 0.76 (s) | 17.98 | 0.78 (s) | 17.98 |
| 30 | 2.15 (s) | 29.18 | 2.16 (s) | 29.15 |

a=C-13 mistake in literature

Compound **8: 3β,6β-Dihydroxylup-20(29)-ene** was obtained as white crystal and melting point was recorded as 198°C and optical rotation was $[\alpha]_D^{27}$= +2.54 (*c* 0.21, $CHCl_3$) but reported value was $[\alpha]_D^{20}$ = +3.2 (*c* 0.19, $CHCl_3$) [69]. The molecular formula was determined as $C_{30}H_{50}O_2$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 465.3702 $[M+Na]^+$ (Calculated 465.3702 for $[C_{30}H_{50}NaO_2]^+$) which confirmed the molecular formula of Compound **8**.

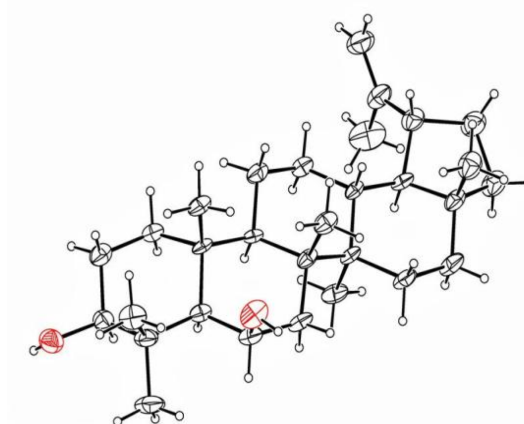


**Figure-3.4 Compound-8: 3β,6β-Dihydroxylup-20(29)-ene**

$^1$H-NMR spectrum of Compound **8** ($CDCl_3$) showed twenty five signals for protons and the $^{13}$C-NMR spectrum showed thirty signals for thirty carbons. The $^1$H and $^{13}$C NMR spectral data of Compound **8** were compared with reference data (Table-3.8). The $^1$H and $^{13}$C NMR spectral data and mass of Compound **8** matched with reported data [69] of **3β,6β-Dihydroxylup-20(29)-ene**. The Compound **8** showed positive optical rotation which was also comparable with reference data [69].This comparison of spectroscopic data confirmed that the Compound **8** was a **3β,6β-Dihydroxylup-20(29)-ene**. The single crystal X-ray confirmed the structure of compound **8**. Compound **8** found as a major compound of this plant.

**Table- 3.8:** The $^{1}H$ and $^{13}C$ NMR data of Compound **8** in comparison with reported data

| Position | Our data ($CDCl_3$) | | Reported data ($CDCl_3$) [69] | |
|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm |
| 1 | 0.91<br>1.66 | 40.69 | 0.91<br>1.67 | 40.7 |
| 2 | 1.60 | 27.52 | 1.59 | 29.7 |
| 3 | 3.13 (t, 7.4) | 79.13 | 3.14 (t, 7.3) | 79.1 |
| 4 | | 39.60 | | 39.6 |
| 5 | 0.68 (d, 1.72) | 55.62 | 0.68 (br s) | 55.6 |
| 6 | 4.52 (br s) | 69.03 | 4.53 (br s) | 69.0 |
| 7 | 1.66 | 42.11 | 1.67 | 42.1 |
| 8 | | 39.90 | | 39.9 |
| 9 | 1.29 | 51.09 | 1.30 | 51.9 |
| 10 | | 36.72 | | 36.7 |
| 11 | | 21.05 | | 21.1 |
| 12 | | 25.30 | | 25.3 |
| 13 | 1.75 | 37.17 | 1.76 | 37.2 |
| 14 | | 43.05 | | 43.0 |
| 15 | | 27.57 | | 27.5 |
| 16 | | 35.52 | | 35.5 |
| 17 | | 43.05 | | 43.1 |
| 18 | 1.38 | 48.38 | 1.38 | 48.4 |
| 19 | 2.39 (ddd, 11,11,5.5) | 47.96 | 2.39 (m) | 48.0 |
| 20 | | 150.92 | | 150.9 |
| 21 | | 29.84 | | 29.9 |
| 22 | | 39.96 | | 40.0 |
| 23 | 1.05 (s) | 27.59 | 1.06 (s) | 27.6 |
| 24 | 1.15 (s) | 16.99 | 1.15 (s) | 16.8 |
| 25 | 1.20 (s) | 17.71 | 1.20 (s) | 17.7 |
| 26 | 1.35 (s) | 16.92 | 1.36 (s) | 16.9 |
| 27 | 0.91 (s) | 14.88 | 0.92 (s) | 14.9 |
| 28 | 0.79 (s) | 17.98 | 0.80 (s) | 18.0 |
| 29 | 4.57 (br s)<br>4.69 (d, 2.04) | 109.92 | 4.59 (br s)<br>4.70 (br s) | 109.4 |
| 30 | 1.68 (s) | 19.34 | 1.69 (s) | 19.3 |

Compound **9: 3,3´,4´-Tri-*O*-methylellagic acid** was obtained as yellow powder, melting point was recorded as 296°C (lit 287-289°C) [70]. The molecular formula was determined as $C_{17}H_{12}O_8$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 345.0611 $[M+H]^+$ and calculated value for the formula is 345.0605 $[C_{17}H_{13}O_8]^+$) that confirmed the molecular formula of compound **9**.

**Figure-3.5 Compound 9: 3,3´,4´-tri-*O*-methylellagic acid**

$^1$H-NMR spectrum of the Compound **9** (in DMSO-$d_6$) showed five signals for protons and $^{13}$C-NMR spectrum gave seventeen signals for seventeen carbons (Table 3.9). The $^1$H and $^{13}$C NMR spectral data were compared with NMR spectral data reported of other compounds (Table-3.9) and the Compound **9** fitted very well with **3,3´,4´-tri-*O*-methylellagic acid** [70] Therefore, it was concluded that the Compound **9** was **3,3´,4´-tri-*O*-methylellagic acid** which is a common constituent of bark of the terrestrial medicinal plants but in the *A. rivularis* plant it was isolated as one of the minor constituents.

**Table-3.9:** The $^{1}H$ and $^{13}C$ NMR data of Compound **9** in comparison with reported data

| Position | Our data (DMSO-$d_6$) | | Reported data (DMSO-$d_6$) [70] | |
|---|---|---|---|---|
| | $\delta_H$ (mult., $J$ (Hz)) ppm | $\delta_C$ ppm | $\delta_H$ (mult., $J$ (Hz)) ppm | $\delta_C$ ppm |
| 1 | | 111.5 | | 111.2 |
| 2 | | 141.3 | | 140.9 |
| 3 | | 140.5 | | 140.3 |
| 4 | | 152.9 | | 152.5 |
| 5 | 7.54 (s) | 111.9 | 7.54 (s) | 111.6 |
| 6 | | 112.2 | | 111.9 |
| 7 | | 158.6 | | 158.3 |
| 1´ | | 112.8 | | 112.4 |
| 2´ | | 141.8 | | 141.5 |
| 3´ | | 141.1 | | 140.8 |
| 4´ | | 154.1 | | 153.8 |
| 5´ | 7.62 (s) | 107.8 | 7.64 (s) | 107.5 |
| 6´ | | 113.7 | | 113.4 |
| 7´ | | 158.8 | | 158.5 |
| 3-OMe | 4.04 (s) | 61.3 | 4.05 (s) | 61.0 |
| 3´-OMe | 4.06 (s) | 61.6 | 4.06 (s) | 61.3 |
| 4´OMe | 4.00 (s) | 57.0 | 4.01 (s) | 56.7 |

Compound **12: 3,3´-Di-*O*-methylellagic acid** was obtained as yellow powder and melting point was more than 300°C (lit >300°C) [70]. The molecular formula was determined as $C_{16}H_{10}O_8$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 331.0467 $[M+H]^+$(Calculated 331.0448 for $[C_{16}H_{11}O_8]^+$) which confirmed the molecular formula of the isolated Compound **12**.

**Figure-3.6 Compound 12: 3,3´-di-*O*-methylellagic acid**

$^1$H-NMR spectrum of Compound **12** (DMSO-$d_6$) showed two signals for protons and in $^{13}$C-NMR spectrum it gave eight signals for sixteen carbons. The $^1$H and $^{13}$C NMR spectral data of the Compound **12** in comparison with reference data are shown in Table-3.10. The $^1$H and $^{13}$C NMR signals and mass of the Compound **12** (in DMSO-$d_6$) was matched with reported data of **3,3´-di-*O*-methylellagic acid** in DMSO-$d_6$ [70]. The melting point of the Compound **12** was also comparable with reference data [70].Therefore, the compound **12** was identified as **3,3´-di-*O*-methylellagic acid**. Compound **12**was found as a minor compound of this plant.

**Table-3.10:** The $^{1}H$ and $^{13}C$ NMR data of Compound **12** in comparison with reported data

| Position | Our data (DMSO-$d_6$) | | Reported data (DMSO-$d_6$) [70] | |
|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm |
| 1 | | 111.7 | | 111.5 |
| 2 | | 141.2 | | 141.1 |
| 3 | | 140.3 | | 140.1 |
| 4 | | 152.2 | | 152.2 |
| 5 | 7.51 (s) | 111.5 | 7.52 (s) | 111.3 |
| 6 | | 112.1 | | 112.1 |
| 7 | | 158.2 | | 158.4 |
| 1´ | | 111.7 | | 111.5 |
| 2´ | | 141.2 | | 141.1 |
| 3´ | | 140.3 | | 140.1 |
| 4´ | | 152.2 | | 152.2 |
| 5´ | 7.51 (s) | 111.5 | 7.52 (s) | 111.3 |
| 6´ | | 112.1 | | 112.1 |
| 7´ | | 158.5 | | 158.4 |
| 3-OMe | 4.03 (s) | 60.9 | 4.05 (s) | 61.0 |
| 3´-OMe | 4.03 (s) | 60.9 | 4.05 (s) | 61.0 |

Compound **13**: **4′,5,7-Trihydroxyflavanone** was obtained as yellowish needle shaped crystal, melting point and specific rotation were found to be 253-255°C and $[\alpha]_D^{25}$ = -17.6 (*c* 0.16, EtOH ),respectively The values were close to the reported values of flavonoid compounds (lit.>300˚C and $[\alpha]_D^{25}$ = -20.7 (*c* 1.0,EtOH) [71,72]. The molecular formula was determined as $C_{15}H_{12}O_5$ from high resolution mass spectrometry (HRMS-ESI-TOP) at *m/z* 295.0565 $[M+Na]^+$ (Calculated 295.0577 for $[C_{15}H_{12Na}O_5]^+$) which confirmed the molecular formula of Compound **13**.

**Figure-3.7 Compound-13: (*S*)-Naringeni (4′,5,7-Trihydroxyflavanone)**

$^{1}$H-NMR and $^{13}$C-NMR spectra of the Compound **13** in Acetone-*d₆* showed nine signals for protons and thirteen signals for fifteen carbons. The $^{1}$H NMR and $^{13}$C NMR spectral data of Compound **13** in comparison with reference data are shown in Table-3.11. The $^{1}$H &$^{13}$C NMR and mass spectra of the Compound **13**(in Acetone-$d_6$) was matched with reported data of **4′,5,7-trihydroxyflavanone** (in Acetone-$d_6$)[73]. The melting point of Compound **13** and negative specific rotation were also comparable with reported data [71-72]. By all physical and spectroscopic data Compound **13** was confirmed as **4′,5,7-trihydroxyflavanone** which isolated for the first time from the plant as a minor constituents.

**Table-3.11** The $^{1}H$ and $^{13}C$ NMR data of Compound **13** in comparison with reported data

| Position | Our data (Acetone-$d_6$) | | Reported data (Acetone-$d_6$) [73] | |
|---|---|---|---|---|
| | $\delta_H$ (mult., $J$ (Hz)) ppm | $\delta_C$ ppm | $\delta_H$ (mult., $J$ (Hz)) ppm | $\delta_C$ ppm |
| 2 | 5.45 (dd,12.88,2.92) | 79.05 | 5.42 (dd,13.1,2.76) | 79.1 |
| 3a | 2.72 (dd,17.1,3) | 42.59 | 2.70 (dd,17.1,2.5) | 42.7 |
| 3e | 3.18 (dd,17.13,12.92) | | 3.20 (m) | |
| 4 | | 196.35 | | 196.4 |
| 5 | 12.18 (s, OH-5) | 164.40 | | 164.2 |
| 6 | 5.94 (d,2.08) | 95.92 | 5.93 (d,2.04) | 96.0 |
| 7 | 9.84 (s, OH-7) | 166.57 | | 166.6 |
| 8 | 5.95 (d,2.08) | 94.96 | 5.94 (s) | 95.0 |
| 9 | | 163.48 | | 163.6 |
| 10 | | 102.22 | | 102.3 |
| 1´ | | 129.85 | | 129.9 |
| 2´ | 7.39 (d,8.48) | 128.11 | 7.36 (d,8.28) | 128.2 |
| 3´ | 6.89 (d,8.57) | 115.29 | 6.87 (d,8.28) | 115.3 |
| 4´ | 8.69 (s, OH-4′) | 157.87 | | 157.9 |
| 5’ | 6.89 (d,8.57) | 115.29 | 6.87 (d,8.28) | 115.3 |
| 6’ | 7.39 (d,8.48) | 128.11 | 7.36 (d,8.28) | 128.2 |

Compound **14: *p*-Cumaric acid** was obtained as white crystal which showed melting point 207°C (**wiki-** 210-213°C). The melting point value is low compare to the reported value might be due to the presence of insignificant amount of impurity. The molecular formula was determined as $C_9H_8O_3$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 187.0370 $[M+Na]^+$ (Calculated 187.0307 for $[C_9H_8NaO_3]^+$) which confirmed the molecular formula of Compound **14**.

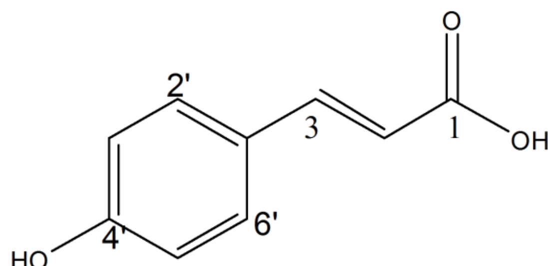



**Figure-3.8 Compound 14: *p*-Cumaric acid ((*E*)-3-(4′-hydroxyphenyl)-2-propenoic acid)**

$^1$H NMR and $^{13}$CNMR spectra of the Compound **14** (in MeOD) showed four signals for protons and seven signals for nine carbons. The $^1$H and $^{13}$C NMR spectral data of compound **14** were with other known compounds (Table-3.12). Spectroscopic data of the Compound **14** and the reported data [74] confirmed that the Compound 14 was *p*-Cumaric acid is also reported first time from this plant.

**Table-3.12:** The $^1$H and $^{13}$C NMR data of Compound **14** in comparison with reported data

| Position | Our data($CD_3OD$) | | Reported data ($CD_3OD$) [74] | |
|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm |
| 1 | | 171,15 | | 171.06 |
| 2 | 6.28 (d,15.89) | 115.77 | 6.28 (d,16) | 115.49 |
| 3 | 7.60 (d.15.92) | 146.78 | 7.60 (d,16) | 146.68 |
| 1′ | | 127.39 | | 127.17 |
| 2′ | 7.45 | 131.22 | 7.45 | 131.06 |
| 3′ | 6.80 | 116.95 | 6.80 | 116.76 |
| 4′ | | 161.29 | | 161.04 |
| 5′ | 6.80 | 116.95 | 6.80 | 116.76 |
| 6′ | 7.45 | 131.22 | 7.45 | 131.06 |

Compound **15: 2,3-Dihydro-*p*-Cumaric acid** was obtained as white crystal, melting point was found to be 127°C (**wiki** 129-131°C). The molecular formula was determined as $C_9H_{10}O_3$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 189.0524 $[M+Na]^+$ (Calculated 189.0522 for $[C_8H_8NaO_4]^+$) which confirmed the molecular formula of compound **15**.

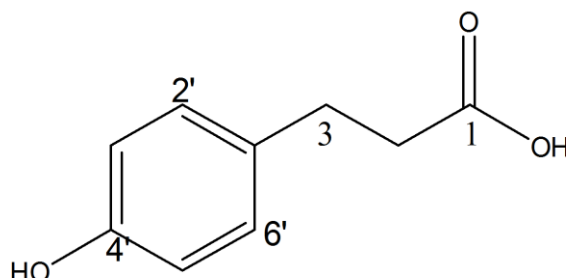



**Figure-3.9 Compound 15: 2,3-Dihydro-p-Cumaric acid; (3-(4′-Dydroxyphenyl)-propanoic acid)**

$^1$H and $^{13}$C NMR spectra of the Compound **15** (in MeOD) showed four signals for protons and seven signals for nine carbons. The $^1$H and $^{13}$C NMR spectral data of Compound **15** in comparison with reported [75] data are shown in Table-3.13. The spectroscopic data of Compound 15 and reported data confirmed that Compound **15** was **2,3-Dihydro-*p*-Cumaric acid** and also isolated from this plant as a minor compound.

**Table-3.13:** The $^1$H and $^{13}$C NMR data of Compound **15** in comparison with reported data

| Position | Our data ($CD_3OD$) | | Reported data ($CD_3OD$) [75] | |
|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm | $\delta_H$ (mult., *J* (z)) ppm | $\delta_C$ ppm |
| 1 | | 177.13 | | 179.11 |
| 2 | 2.53 (t,7.84) | 37.34 | 2.50 (t*) | 38.62 |
| 3 | 2.81 (t, 7.76) | 31.38 | 2.79 (t*) | 31.69 |
| 1′ | | 133.11 | | 133.63 |
| 2′ | 7.03 (d,8.49) | 130.35 | 7.03 (d*) | 130.19 |
| 3′ | 6.69 (d,8.52) | 116.32 | 6.70 (d*) | 116.15 |
| 4′ | | 156.85 | | 156.10 |
| 5′ | 6.69 (d,8.52) | 116.32 | 6.70 (d*) | 116.15 |
| 6′ | 7.03 (d,8.49) | 130.35 | 7.03 (d*) | 130.19 |

d*,t*=pesudodoublet or triplet in AA'XX' spin system.

Compound **16: 3,4-Dihydroxybenzoic acid** was obtained as white needle crystal out which showed melting point 202°C (Ref. 198-200°C) [76]. The molecular formula was determined as $C_7H_6O_4$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 177.0151 $[M+Na]^+$ (Calculated 177.0158 for $[C_7H_6NaO_4]^+$) which confirmed the molecular formula of compound **16**.

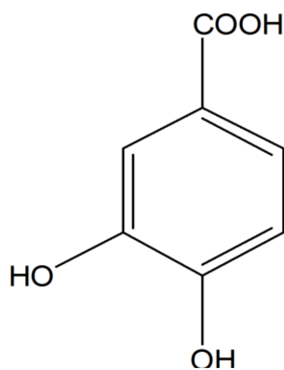



**Figure-3.10 Compound 16: Protocatechuic acid (3,4-Dihydroxybenzoic acid)**

The NMR spectrum of compound **16** in DMSO-$d_6$ showed three signals for protons and seven signals for carbon. The NMR spectral data of compound **16** in comparison with reference data are shown in Table-3.14. The spectroscopic data, melting point and mass of Compound **16** in DMSO-$d_6$ is matched with reported data of **3,4-Dihydroxybenzoic acid** in DMSO-$d_6$ [76]. The comparison of all spectroscopic data with reported confirmed that the compound **16** was **3,4-Dihydroxybenzoic acid**. Compound-**16** found as a minor compound of this plant.

**Table-3.14:** The $^1$H and $^{13}$C NMR data of Compound **16** in comparison with reported data

| Position | Our data (DMSO-$d_6$) | | Reported data (DMSO-$d_6$) [76] | |
|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm |
| 1 | | 121.6 | | 121.6 |
| 2 | 7.33 (1H,d,J=2.0 ) | 116.5 | 7.33 (1H, d, J=1.8) | 116.5 |
| 3 | | 144.9 | | 144.8 |
| 4 | | 150.0 | | 150.0 |
| 5 | 6.78 (1H, d, J=8.2) | 115.1 | 6.79 (1H, d, J=8.5) | 115.1 |
| 6 | 7.28 (1H, dd, J=8.2,2.0) | 121.9 | 7.28 (1H, dd, J=8.5,1.8) | 121.9 |
| 1-COOH | | 167.3 | | 167.2 |

Compound **17: Stigmast-5-en-3-*O*-β-glucoside** was obtained as white needle crystal out which showed melting point 276°C (Ref. 284°C). The molecular formula was determined as $C_{35}H_{60}O_6$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 599.4343 $[M+Na]^+$ (Calculated 599.4282 for $[C_{35}H_{60}NaO_6]^+$) which confirmed the molecular formula of compound **17**.

**Figure-3.11 Compound 17: Stigmast-5-en-3-*O*-β-glucoside**

The NMR spectrum of compound **17** in pyridine-*d5* showed thirty two signals for protons and thirty five signals for thirty five carbons. The spectral data of compound **17** in comparison with reference data are shown in Table-3.15. The spectroscopic data, melting point and mass of compound **17** in pyridine-*d5* is matched with reported data of **Stigmast-5-en-3-*O*-β-glucoside** in pyridine-*d5* [77]. The comparison of all spectroscopic data confirmed that the compound **17** was **Stigmast-5-en-3-*O*-β-glucoside** and was found as a major compound in this plant. **Stigmast-5-en-3-*O*-β-glucoside** was commonly found in almost all plant.

**Table-3.15:** The $^1$H and $^{13}$C NMR data of Compound **17** in comparison with reported data

| Position | Our data (pyridine-*d5*) | | | Reported data (pyridine-*d5*) [77] | | |
|---|---|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm | | $\delta_C$ ppm |
| | $H_\alpha$ | $H_\beta$ | | $H_\alpha$ | $H_\beta$ | |
| 1 | 1.01 (m) | 1.72 (m) | 37.86 | 0.98 (m) | 1.72 (m) | 37.46 |
| 2 | 2.15 (m) | 1.78 (m) | 30.63 | 2.14 (m) | 1.75 (m) | 30.21 |
| 3 | 4.02 (m) | | 78.83 | 3.98 (m) | | 78.38 |

| 4 | 2.76 (br, dd, 2.5, 13.2) | 2.5 (br.t, 11.7) | 39.72 | 2.27 (ddd, 1.98, 4.69, 12.94) | 2.49 (ddd, 1.98, 12.94, 12.94) | 39.31 |
|---|---|---|---|---|---|---|
| 5 | | | 141.31 | | | 140.95 |
| 6 | 5.37 (t,2.4) | | 122.28 | 5.35 (t,2.5) | | 121.90 |
| 7 | 1.58 (m) | 1.94 (m) | 32.55 | 1,60 (ddd, 2.5, 7.0, 16.0) | 1.95 (ddd, 16.0, 2.5, 7.0) | 32.15 |
| 8 | 1.39 (m) | | 32.44 | 1.36 (m) | | 32.04 |
| 9 | 0.89 (m) | | 50.73 | 0.85 (m) | | 50.34 |
| 10 | | | 36.76 | | | 36.08 |
| 11 | 1.45 (m) | 1.44 (m) | 21.66 | 1.42 (m) | 1.42 (m) | 21.26 |
| 12 | 1.98 (m) | 1.10 (m) | 40.33 | 1.98 (dd, 4.3, 12.47) | 1.10 (m) | 39.95 |
| 13 | | | 42.86 | | | 42.47 |
| 14 | 0.95 (m) | | 57.21 | 0.95 (m) | | 56.83 |
| 15 | 1.03 (m) | 1.56 (m) | 24.88 | 1.05 (m) | 1.57 (m) | 24.48 |
| 16 | 1.26 (m) | 1.86 (m) | 28.90 | 1.25 (m) | 1.85 (m) | 28.50 |
| 17 | 1,12 (m) | | 56.64 | 1.10 (m) | | 56.25 |
| 18 | 0.67 (s) | | 12.35 | 0.66 (s) | | 11.95 |
| 19 | 0.95 (s) | | 19.79 | 0.94 (s) | | 19.39 |
| 20 | 1.41 (m) | | 37.30 | 1.40 (m) | | 36.36 |
| 21 | 1.00 (br.d) | | 19.38 | 0.98 (d, 6.5) | | 18.99 |
| 22 | 1.39 (m) | 1.08 (m) | 34.60 | 1.40 (m) | 1.10 (m) | 34.20 |
| 23 | 1.25 (m) | 1.26 (m) | 26.80 | 1.25 (m) | 1.25 (m) | 26.40 |
| 24 | 1.01 (m) | | 46.43 | 1.00 (m) | | 46.04 |
| 25 | 1.68 (m) | | 29.87 | 1.68 (m) | | 29.47 |
| 26 | 0.87 (br.s) | | 19.59 | 0.84 (d, 7.0) | | 19.20 |
| 27 | 0.89 (br.d) | | 20.34 | 0.86 (d, 7.0) | | 19.94 |
| 28 | 1.309 (m) | 1.30 (m) | 23.78 | 1.30 (m) | 1.30 (m) | 23.38 |
| 29 | 0.91 (br.d) | | 12.53 | 0.88 (t, 7.5) | | 12.13 |
| 1′ | 5.09 (d, 7.72) | | 102.96 | 5.04 (d, 7.69) | | 102.57 |
| 2′ | 4.10 (dd, 8.2, 8.8) | | 75.71 | 4.04 (dd, 7.69, 7.50) | | 75.27 |
| 3′ | 4.32 (m) | | 78.97 | 4.27 (t, 7.50) | | 78.54 |
| 4′ | 4.32 (m) | | 72.10 | 4.27 (t, 7.50) | | 71.67 |
| 5′ | 3.98 (m) | | 78.52 | 3.96 (m) | | 78.13 |
| 6′ | 4.60 (dd, 2.2, 11.76) | 4.44 (dd, 5.2,11.78) | 63.23 | 4.55 (dd, 2.5, 11.77) | 4.40 (dd, 5.2, 11.77) | 62.62 |

Compound **18: Loliolide** was obtained as white crystal, melting point 151$^{\circ}$C (Ref. 149.5$^{\circ}$C). The molecular formula was determined as $C_{11}H_{16}O_3$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 219.0984 $[M+Na]^+$ [Calculated 219.0992 for $C_{11}H_{16}NaO_3]^+$) which confirmed the molecular formula of compound **18**.

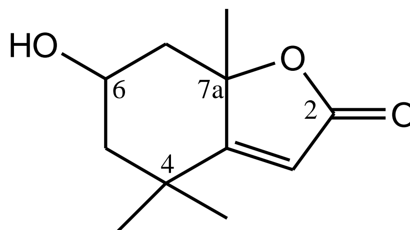



**Figure-3.12 Compound 18: Loliolide (6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydro-4H-benzofuran-2-one)**

$^1$H and $^{13}$C NMR spectra of the compound **18** in $CDCl_3$ showed eight signals for protons and eleven signals for eleven carbons (Table-3.16). The spectral were compared with reference spectral data [78,79], melting point and mass of the Compound **18** (in $CDCl_3$) which matched with **Loliolide**. The comparison of all spectroscopic data confirmed that the compound **18** was **Loliolide**(**6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydro-4H-benzofuran-2-one**).Compound **18** found for the first time as a minor compound of this plant.

**Table-3.16:** The $^1$H and $^{13}$C NMR data of Compound **18** in comparison with reported data

| Position | Our data ($CDCl_3$) | | Reported data [78] | Reported data ($CDCl_3$) [79] | |
|---|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm |
| 2 | | 182.40 | | | 183.00 |
| 3 | 5.70 (1H, s) | 113.09 | 5.71 (1H,s) | 5.69 (1H, s) | 112.43 |
| 4 | | 35.90 | | | 35.99 |
| 5 | 1.54 (dd, 14. 5,2,) | 45.73 | 1.53 (dd, 14.7, 3.7) | 1.4-2.56 (m,4a-H, | 45.52 |
| | 1.98 (broad d, 14.24) | | 1.97 (ddd,14.5, 3.0, 2.3) | 4b-H) | |
| 6 | 4.34 (broad peak) | 66.88 | 4.33 (quintet) | 4.33 (1H, quint.) | 66.38 |
| 7 | 1.79 (broad peak) | 47.36 | 1.78 (dd, 13.5, 3.7) | 1.4-2.56 (m, 6a-H, | 47.14 |
| | 2.47 (broad d) | | 2.46 (ddd, 14.0, 3.2,2.3) | 6b-H) | |
| 8 | | 171.88 | | | 172.11 |
| 7a | | 86.64 | | | 87.12 |
| 7a-$CH_3$ | 1.79 (3H, s) | 30.69 | 1.78 (s) | 1.78 (3H, s) | 30.61 |
| 4-$CH_3$ | 1.47 (3H, s) | 26.52 | 1.47 (s) | 1.47 (3H, s) | 26.40 |
| 4-$CH_3$ | 1.28 (3H, s) | 27.09 | 1.27 (s) | 1.27 (3H, s) | 26.91 |

Compound **19: Vanillic acid** was obtained as white crystal, melting point was recorded as 210°C (Wiki 210-213°C). The molecular formula was determined as $C_8H_8O_4$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 191.0313 $[M+Na]^+$ (Calculated 191.0315 for $[C_8H_8NaO_4]^+$) which confirmed the molecular formula of Compound **19**.

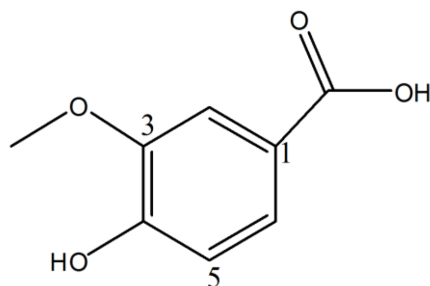



**Figure-3.13 Compound 19: Vanillic acid (3-methoxy 4-hydroxybenzoic acid)**

$^1$H-NMR spectrum of compound **19** (in MeOD) showed three signals for protons and $^{13}$C-NMR spectrum of the compound showed eight signals for eight carbons. The $^1$H and $^{13}$C NMR spectral data of the Compound **19** in comparison with reference data [80,81] are shown in Table-3.17. From melting point, $^1$H, $^{13}$C NMR spectral data and mass of Compound **19** [80] and in comparison with all spectroscopic data confirmed that the compound **19** was **vanillic** acid **(3-methoxy 4-hydroxybenzoic acid**). It was found as one of the as a major compounds of this plant.

**Table-3.17:** The $^1$H and $^{13}$C NMR data of Compound **19** in comparison with reported data

| Position | Our data ($CD_3OD$) | | Reported ($CD_3OD$) data [81] | Reported data ($CD_3OD$) [80] |
|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm | $\delta_C$ ppm | $\delta_H$ (mult., *J* (Hz)) ppm |
| 1 | | 123.3 | 123.1 | |
| 2 | 7.56 (m) | 113.9 | 113.9 | 7.56 (m) |
| 3 | | 148.7 | 148.6 | |
| 4 | | 152.7 | 152.6 | |
| 5 | 6.83 (d, 8.72) | 115.9 | 115.9 | 6.85 (d, 9.0) |
| 6 | 7.56 (m) | 125.4 | 125.3 | 7.56 (m) |
| COOH | | 170.2 | 170.0 | |
| 3-$OCH_3$ | 3.89 (s) | 56.5 | 56.4 | 3.92 (s) |

Compound **20** was obtained as white crystal, melting point was recorded 125°C and specific rotation was $[\alpha]_D^{25}$= +25.2690 (*c* 0.29, MeOH). The molecular formula was determined as $C_{20}H_{22}O_3$ from high resolution mass spectrometry (HRMS-ESI-TOP) at *m/z* 297.1488 $[M+2H-CH_3]^+$ (Calculated 297.1485 for $[C_{19}H_{21}O_3]^+$) which supported the molecular formula of Compound **20**.

**FTIR**(NEAT) shows the absorption bands at $\upsilon_{max}$ 2958, 2922.75 (stretching of -$CH_3$), 2851.93 (stretching of O-$CH_3$), 1605 1554, 1516, 1486, 1463 1432 (C=C stretching of aromatic ring), 1151 (C–O stretching of furan), 972.60 (stretching of CH=CH-trans), 898, 884, 850, 775, 751, 725, 678,653and 620 $cm^{-1}$. The UV spectral data of aromatic showed absorbance at $\lambda_{max}$: 207 (+0.4669), 235 (+0.18103) and 282(+0.08477) nm.

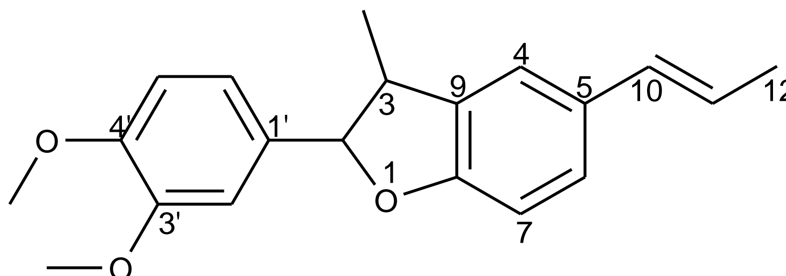



**Figure-3.14 Compound-20: (2-(3′,4′-Dimethoxy-phenyl)-3-methyl-5-propenyl-2,3-dihydro-benzofuran) (New)**

The $^1$H-NMR spectrum of the Compound **20** (in $CDCl_3$) showed eleven signals for fourteen protons and $^{13}$C-NMR spectrum showed twenty signals for twenty carbons. DEPT-90 & DEPT-135 were performed to find out attachment of protons, i.e. primary, secondary, tertiary and quaternary carbons (Table 3.18). In the Compound **20**, four methyl, no methylene, ten methine carbons were found out and the rest were quaternary carbons. H-H COSY spectrum was done and linkages between protons were found out. From HSQC and HMBC all protons to carbons were assigned (Table 3.19). From $^1$H NMR signal coupling constants were measured (Table 3.18) and proton carbon connectivity were found out from HMBC (Table 3.19 and Figure-3.15).

**Table-3.18:** The $^{1}H$, $^{13}C$, , DEPT-90 and DEPT-135 NMR data of compound-**20**

| Position | Our data ($CDCl_3$) | | | |
|---|---|---|---|---|
| | $\delta_H$ (mult., *J* (Hz)) ppm | $\delta_C$ ppm | DEPT90 (CH) | DEPT135 |
| 2 | 5.08 (d, 9.12) | 93.16 | 93.15 | 93.15 |
| 3 | 3.42 (m) | 45.42 | 45.42 | 45.42 |
| 4 | 7.13 (brod. d, 9.44) | 126.49 | 126.49 | 126.49 |
| 5 | | 131.56 | | |
| 6 | 7.13 (brod. d, 9.44) | 120.94 | 120.89 | 120.89 |
| 7 | 6.78 (d, 7.96) | 109.55 | 109.53 | 109.53 |
| 8 | | 158.54 | | |
| 9 | | 133.24 | | |
| 10 | 6.37 (d,15.68) | 130.96 | 130.90 | 130.90 |
| 11 | 6.10 (m) | 123.32 | 123.32 | 123.32 |
| 12 | 1.86 (d, 6.0) | 18.59 | 18.62 | 18.62 |
| 1′ | | 132.57 | | |
| 2′ | 6.96 (brod. d, 7.41) | 109.44 | 109.30 | 109.30 |
| 3′ | | 149.51 | | |
| 4′ | | 149.31 | | |
| 5′ | 6.86 (d, 7.84) | 111.25 | 111.11 | 111.11 |
| 6′ | 6.96 (brod. d, 7.41) | 119.12 | 119.12 | 119.12 |
| 3′-O-$CH_3$ | 3.89 (s) | 56.16 | | 56.12 |
| 4′-O-$CH_3$ | 3.88 (s) | 56.19 | | 56.15 |
| 3-$CH_3$ | 1.45 (d, 6.56) | 17.88 | | 17.82 |

$^{13}C$ NMR DEPT 90 and 135 showed in Compound **20,** 2,3,4,6,7,10, 2′,5′,6′ carbons were -CH- and 3′-*O*-$CH_3$, 4′-*O*-$CH_3$, 3-$CH_3$ were -$CH_3$ and C-11,C-12 were -HC=CH- 5,8,9,1′,3′ 4′ were for quaternary carbons (Table-3.18). The H-10 has *trans* coupling with H-11with *J* value 15.68 Hz indicated that -CH=CH- group in side chain and H-11 gave multiplate signal coupling with H-10 and H-12. The H-3 gave a maltiplete coupling with H-2 & protons of 3-$CH_3$. H-2 gave a doublet coupling with H-3.The protons of 3-$CH_3$ and H-12 (methyl proton) coupled and gave doublet similarly H-3 and H-11 coupled with J values 6.56 Hz and 6.0 Hz. The protons of 3′-*O*-$CH_3$ and 4′-*O*-$CH_3$ gave singlet at $\delta_H$ values 3.89 and 3.88 ppm. The H-4 is *meta* coupling with H-6 and gives doublet with coupling constant 2-4 Hz. Here *para* coupling with H-7 to be zero. For H-6 is

*ortho* coupling with H-7 and *mata* coupling with H-4 and should give *dd* coupling constant 6-8 Hz. The H-4 and H-6 are gave chemical shift at the same region and gave broad doublet at δ 7.3 with coupling constant 9.44 Hz. The H-7 has *ortho* coupling with H-6 and *para* coupling with H-4 is zero and gave doublets with coupling constant 7.96 Hz. The H-2′ is *meta* coupling with H-6′ and *para* coupling with H-5′ is zero. H-6′ is *ortho* coupling with H-5′ and *meta* coupling with H-2′. Both H-2′ and H-6′ are gave chemical shift at the same region and gave broad doublet at δ 9.6 with coupling constant 7.41 Hz. The H-5′ has *ortho* coupling with H-6′ and *para* coupling with H-2′ is zero and gave doublets with coupling constant 7.84 Hz.7.84 Hz (Table-3.18).

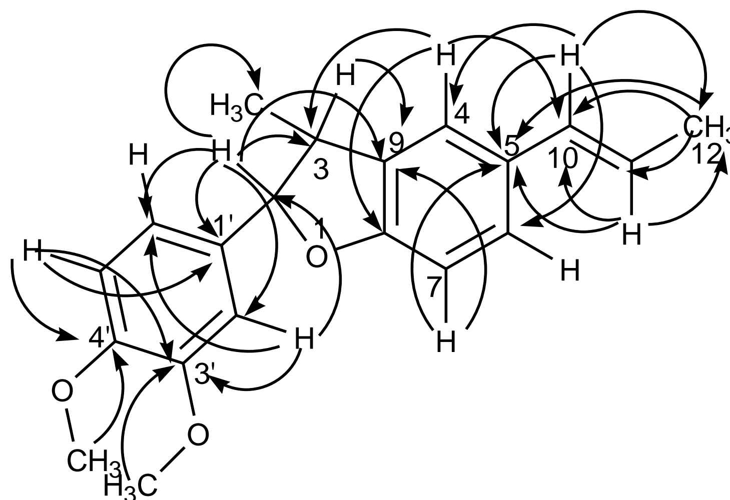



**Figure-3.15** The HMBC correlation of compound-**20**

In HMBC correlations; H-2 (δ 5.08) coupled with C-(3-$CH_3$; δ 17.82), C-3 (δ 45.42), C-9 (δ 133.24), C-1′ (δ 132.57), C-2′(δ 109.30) and C-6′ (δ 119.12). These correlations supported that dimethoxyphenyl group at C-2 position of dihydrobenzofuran. Correlation of H-(3-$CH_3$; δ 1.45) with C-2 (δ 93.15), C-3(δ 45.42), C-9 (δ 133.24), C-1′ (δ 132.57) supported that C-3 position of dihydrobenzofuran contain -$CH_3$ group. Correlation of H-10 (δ 2.75) with C-4 (δ 126.49), C-5 (δ 131.56), C-6 (δ 120.89), C-12 (δ 18.62) supported that propenyl group at C-5 position of dihydrobenzofuran. The H-4 correlated with C-3 (δ 45.42), C-5 (δ 131.56), C-6 (δ 120.89), C-10 (δ 130.90) and the H-2′ Correlated with C-2 (δ 93.15), C-6′ (δ 119.12), C-3′ (δ 149.51) these connectivity also supported the structure of Compound **20** as dihydrobenzofuran derivatives (Table-3.19).

**Table-3.19:** The HSQC and HMBC correlation data of compound-**20**

| Our data ($CDCl_3$) | | |
|---|---|---|
| $\delta_H$ ppm | HSQC correlation | HMBC correlation |
| 5.08 (H-2) | 93.15 (C-2) | 17.82 (3-$CH_3$), 45.42 (C-3), 109.30 (C-2′), 119.12 (C-6′), 132.57 (C-1′), 133.24(C-9) |
| 3.42 (H-3) | 45.42 (C-3) | 132.57(C-1′), 133.24(C-9) |
| 7.13 (H-4) | 126.49 (C-4) | 45.42 (C-3), 131.56 (C-5), 120.89 (C-6), 158(C-8), 130.90(C-10) |
| 7.13 (H-6) | 120.89 (C-6) | 126.49 (C-4), 131.56 (C-5), 158(C-8), 130.90(C-10) |
| 6.78 (H-7) | 109.53 (C-7) | 131.56 (C-5), 158(C-8), 133.24(C-9), |
| 6.37 (H-10) | 130.90 (C-10) | 126.49 (C-4), 131.56 (C-5), 120.89 (C-6),18.62 (C-12) |
| 6.10 (H-11) | 123.32 (C-11) | 131.56 (C-5), 130.90(C-10), 18.62 (C-12) |
| 1.86 (H-12) | 18.62 (C-12) | 131.56 (C-5), 130.90(C-10), 123.32 (C-11) |
| 6.96 (H-2′) | 109.30 (C-2′) | 93.15 (C-2), 119.12 (C-6′), 149.51(C-3′) |
| 6.86 (H-5′) | 111.11 (C-5′) | 132.57(C-1′), 149.51(C-3′), 149.31(C-4′) |
| 6.96 (H-6′) | 119.12 (C-6′) | 93.15 (C-2), 109.30 (C-2′), 149.31(C-4′) |
| 3.89 (H-(3′-O-$CH_3$)) | 56.12 (3′-O-$CH_3$) | 149.51(C-3′) |
| 3.88 (H-(4′-O-$CH_3$)) | 56.15 (4′-O-$CH_3$) | 149.31 (C-4′) |
| 1.45 (H-(3-$CH_3$)) | 17.82 (3-$CH_3$) | 93.15 (C-2), 45.42 (C-3), 133.24(C-9), 132.57(C-1′) |

From H-H COSY H-2 was found to linked with H-3, H-3 with H-(3-$CH_3$), H-7 with H-7, H-10 with H-11, H-11 with H-12 and H-5′ with H-6′. All the correlations supported that they are attached to adjacent carbon atom (Table-3.20). The NOESY correlation is not effective for this compound because all groups are in same plane. From all spectroscopic studies structure of the Compound **20** was elucidated as **[2-(3′,4′-Dimethoxy-phenyl)-3-methyl-5-propenyl-2,3-dihydro-benzofuran].** Literature search including SciFinder showed the Compound 20 is new compound and has not been reported earlier from neither from plant origin nor from synthetic sources.

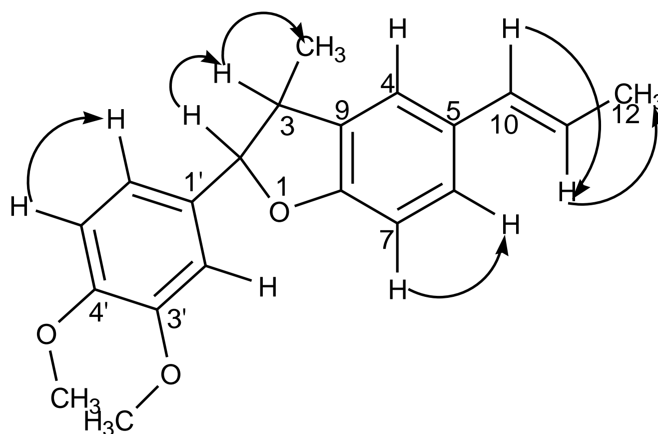



**Figure 3.16:** The COSY correlation of compound-**20**

**Table 3.20:** The COSY correlation data for selected protons of compound-**20**

| Our data ($CDCl_3$) | |
|---|---|
| $\delta_H$ ppm | Protons correlated with $\delta_H$ ppm |
| 5.08 (H-2) | 3.42 (H-3) |
| 3.42 (H-3) | 1.45 (H-(3-$CH_3$)) |
| 7.13 (H-6) | 6.78 (H-7) |
| 6.37 (H-10) | 6.10 (H-11) |
| 6.10 (H-11) | 1.86 (H-12) |
| 6.86 (H-5′) | 6.96 (H-6′) |

Compound **21** was obtained as yellow powder, melting point was 225-227°C and specific rotation was $[\alpha]_D^{25}$ = +192.87 (*c* 0.36, MeOH). The molecular formula was determined as $C_{30}H_{22}O_{10}$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 565.1080 $[M+Na]^+$ (Calculated 565.1105 for $[C_{30}H_{22}NaO_{10}]^+$) which supported the molecular formula of Compound **21**.

**FT-IR** (NEAT) showed the absorption bands at $\upsilon_{max}$ 3197 broad peak for O–H stretching and aromatic =C–H stretching, 1634, 1627 (C=O stretching of ketone), 1616, 1592, 1569, 1515, 1507, 1499, 1480 and 1457 (C=C stretching of aromatic ring), 1151 (C–O stretching of furan), 1077, 1011, (C–O stretching of phenol), 946, 876, 827, 808, 763, 739, 694 $cm^{-1}$. The UV spectral data of aromatic showed absorbance at $\lambda_{max}$: 213 (+0.7506), 229 (+0.56132) and 296 (+0.51057) nm.

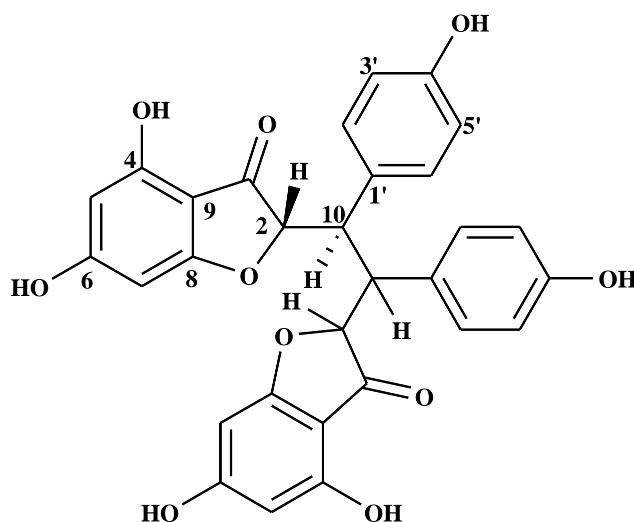



**Figure-3.17 Compound- 21: Dimer of 4,6-dihydroxy-2-(4′-hydroxy-benzyl)-benzofuran-3-one (New)**

The $^{1}H$-NMR spectrum of the Compound **21** (in MeOD) showed six signals for protons and $^{13}C$-NMR spectrum showed thirteen signals for fifteen carbons. DEPT-90 & DEPT-135 were performed to find out attachment of protons, i.e. primary, secondary, tertiary and quaternary carbons (Table 3.21). In the Compound **21**, no methyl, no methylene, eight methine carbons

were found out and the rest were quaternary carbons. H-H COSY spectrum was done and linkages between protons were found out. From HSQC and HMBC all protons to carbons were assigned (Table 3.22). From $^{1}H$ NMR signal coupling constants were measured (Table 3.21) and proton carbon connectivity were found out from HMBC (Table 3.22 and Figure 3.18).

$^{13}C$ NMR DEPT 90 and 135 showed in Compound **21,** 2,5,7,10,2′,3′,5′,6′ carbons were -CH- and 4,6, 4′ were quaternary carbon with -OH group and 8,9,1′ were quaternary carbon and 3 was =CO group (Table-3.21). The H-2 & H-10 gave doublet coupling with each other with J value 11.8 Hz. The H-5 & H-7 gave doublet (J value 2 Hz) coupling with *meta* protons. The H-2′ & H-3′ and H-5′&H-6′ gave doublet (J value 8.6 Hz) coupling with *ortho* protons.(Table-3.21).

**Table 3.21:** The $^{1}H$, $^{13}C$, DEPT-90 and DEPT-135 NMR data of compound-**21**

| Position | Our data (MeOD) | | | |
|---|---|---|---|---|
| | $\delta_H$ ppm (mult., *J* (Hz)) ppm | $\delta_C$ ppm | DEPT-90 (CH) | DEPT-135 |
| 2 | 5.78 (d, 11.8) | 85.09 | 85.10 | 85.10 |
| 3 | | 198.09 | | |
| 4 | | 164.50 | | |
| 5 | 5.79 (d, 2) | 96.24 | 96.24 | 96.24 |
| 6 | | 165.60 | | |
| 7 | 5.88 (d, 2) | 97.39 | 97.40 | 97.39 |
| 8 | | 168.56 | | |
| 9 | | 103.47 | | |
| 10 | 2.75 (d, 11.8) | 51.41 | 51.42 | 51.41 |
| 1′ | | 129.15 | | |
| 2′ | 6.89 (d, 8.6) | 130.42 | 130.42 | 130.42 |
| 3′ | 6.76 (d, 8.6) | 116.45 | 116.65 | 116.64 |
| 4′ | | 159.70 | | |
| 5′ | 6.76 (d, 8.6) | 116.45 | 116.65 | 116.64 |
| 6′ | 6.89 (d, 8.6) | 130.42 | 130.42 | 130.42 |

In HMBC correlation: H-2 (δ 5.78) couple with C-10 (δ 51.41), C-3 (δ 198.09), C-1′(δ 129.15), C-9 (δ 103.47), C-2′and C-6′ (δ 130.42). This correlation supported that hydroxyl benzyl group at C-2 position of benzofuran. Correlation of H-10 (δ 2.75) with C-10 (δ 51.41), C-2 (δ 85.09), C-1′ (δ 129.15), C-3 (δ 198.09) supported that it was a dimer of benzofuran and also supported that hydroxyl benzyl group at C-2 position (Table-3.22 ).

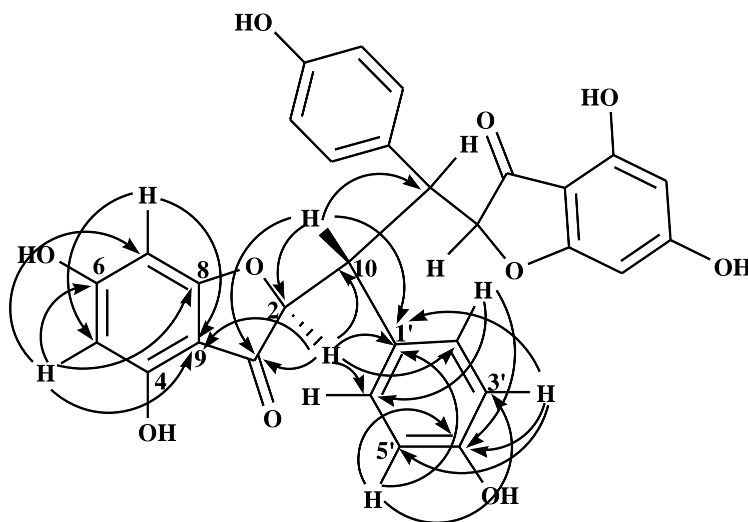



**Figure-3.18** The HMBC correlation of compound-**21**

**Table 3.22:** The HMQC and HMBC NMR data of compound-**21**

| Our data (MeOD) | | |
|---|---|---|
| $\delta_H$ ppm | HMQC correlation | HMBC correlation |
| 5.78 (H-2) | 85.10 (CH-2) | 51.41(C-10), 198.09(C-3), 129.15(C-1′), 103.47(C-9), 130.42(C-2′, C-6′) |
| 5.79 (H-5) | 96.24 (CH-5) | 97.39(C-7), 103.47 (C-9), 165.60(C-6),168.56(C-8) |
| 5.88 (H-7) | 97.40 (CH-7) | 96.24(C-5),103.47(C-9) |
| 2.75 (H-10) | 51.42 (CH-10) | 51.41(C-10), 85.09(C-2), 129.15(C-1′), 198.09(C-3) |
| 6.89 (H-2′) | 130.42 (CH-2′) | 130.42(C-6′), 159.70(C-4′) |
| 6.76 (H-3′) | 116.45 (CH-3′) | 116.45(C-5′), 129.15(C-1′), 159.70(C-4′) |
| 6.76 (H-5′) | 116.45 (CH-5′) | 116.45(C-3′), 129.15(C-1′), 159.70(C-4′) |
| 6.89 (H-6′) | 130.42 (CH-6′) | 130.42(C-2′), 159.70(C-4′) |

From H-H COSY H-2 was found to linked with H-10, H-2′ with H-3′, H-5′ with H-6′. All the correlations supported that they are attached to adjacent carbon atom (Table-3.23). The NOESY correlation is not effective for this compound because all groups are in same plane. From all spectroscopic studies structure of the Compound **21** was elucidated as **[4,6-dihydroxy-2-(4′-hydroxy-benzyl)-benzofuran-3-one].** Literature search including SciFinder showed the Compound 21 is a new compound and has not been reported earlier from neither from plant origin nor from synthetic sources.

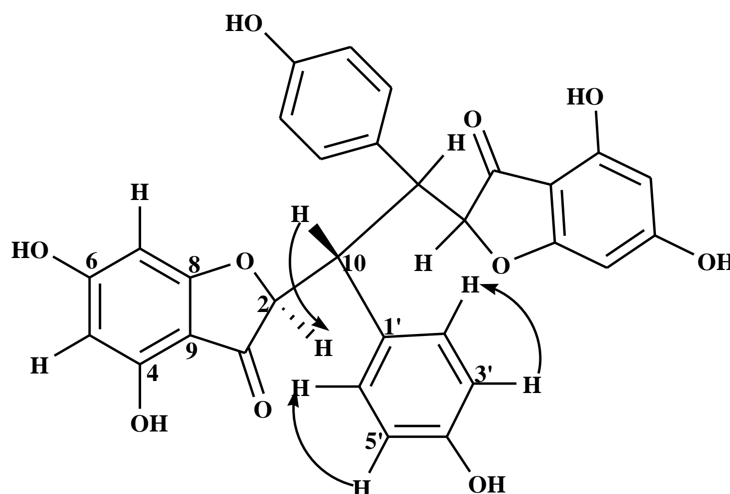



**Figure-3.19** The COSY correlation of compound-**21**

**Table 3.23:** The COSY correlation data for selected protons of compound-**21**

| Our data (MeOD) | |
|---|---|
| $\delta_H$ ppm | Protons correlated with $\delta_H$ ppm |
| 5.78 (H-2) | 2,75 (H-10) |
| 6.89 (H-2′) | 6.76 (H-3′) |
| 6.76 (H-5′) | 6.89 (H-6′) |

Compound-**22** was obtained as yellow powder, melting point was recorded 170-172°C and specific rotation $[\alpha]_D^{25}$ = +19.58 (*c* 0.37, MeOH). The molecular formula was determined as $C_{15}H_{12}O_7$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 327.0478 $[M+Na]^+$ (Calculated 327.0475 for $[C_{15}H_{12}NaO_7]^+$).

**FT-IR** (NEAT) showed the absorption bands at $\upsilon_{max}$ 3422.38 (O–H stretching ), 3132.99 and 2918.35 (aromatic =C–H stretching), 1635.43 (C=O stretching of ketone), 1623, 1616, 1608, 1570, 1521 and 1472 (C=C stretching of aromatic ring), 1250 (C–O stretching of phenol), 1159 (C–O stretching of furan),1136 and 1116 (C–O stretching of alcohol ), 1080, 1018, 949, 858, 810, 778, 703, 666 $cm^{-1}$. The **UV** spectral data of aromatic showed absorbance at $\lambda_{max}$: 206 (+0.36106), 231 (+0.1847) and 289 (+0.11909) nm.

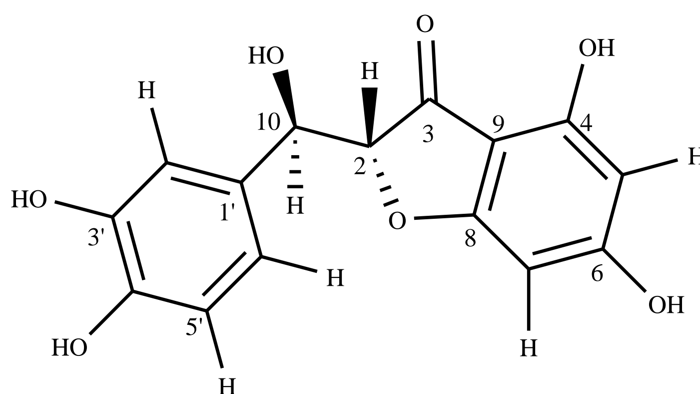



**Figure-3.20 Compound-22, 2-[(3′,4′-Dihydroxy-phenyl)-hydroxy-methyl]-4,6-dihydroxy-benzofuran-3-one (New)**

The $^{1}$H-NMR spectrum of the Compound **22** (in Acetone-*d*$_6$) showed eleven signals for seven protons and $^{13}$C-NMR spectrum showed fifteen signals for fifteen carbons. DEPT-90 & DEPT-135 were performed to find out attachment of protons, i.e. primary, secondary, tertiary and quaternary carbons (Table 3.24). In the Compound **22**, no methyl & methylene, seven methine carbons were found out and the rest were quaternary carbons. H-H COSY spectrum was done and linkages between protons were found out. From HSQC and HMBC all protons to carbons

were assigned (Table 3.25). From $^{1}H$ NMR signal coupling constants were measured (Table 3.24) and proton carbon connectivity were found out from HMBC (Table 3.25 and Figure-3.21).

$^{13}C$ NMR, DEPT 90 and 135 showed in Compound **22,** 2,5,7,10,2′,5′,6′ carbons were -CH- carbon and 4,6, 3′,4′ were quaternary carbon with -OH group and 8,9,1′ were quaternary carbon and 3 was =CO group (Table-3.24). The H-2 gave doublet (J value 11.49 Hz) with coupling with H-10. H-10 gave doublet of doublet (J value 11.48Hz and 3.8Hz) coupling with H-2&10-OH protons respectively. The H-5 & H-7 gave doublet (J value 2.08 Hz.) coupling with *meta* protons. The H-2′ & H-6′ were found in *meta* coupling each other with J value 1.96 Hz and gave doublet. The H-5′&H-6′gave doublet (J value 8.12 Hz) coupling with *ortho* protons and H-6′ gave doublet of doublet (*J* value 8.12 &1.96 hz) coupling with *ortho* and *meta* protons (Table-3.24).

**Table-3.24:** The $^{1}H$, $^{13}C$, DEPT90 and DEPT135 NMR data of compound-**22**

| Position | Our data (Acetone-$d_6$) | | | |
|---|---|---|---|---|
| | $\delta_H$ (Mult., *J* (Hz)) ppm | $\delta_C$ ppm | DEPT90 (CH) | DEPT135 |
| 2 | 5.02 (d, 11.49) | 84.62 | 84.62 | 84.62 |
| 3 | | 198.32 | | |
| 4 | | 165.10 | | |
| 5 | 5.99 (d, 2.08) | 97.16 | 97.16 | 97.16 |
| 6 | | 164.25 | | |
| 7 | 5.95 (d, 2.08) | 96.13 | 96.13 | 96.13 |
| 8 | | 167.99 | | |
| 9 | | 101.62 | | |
| 10 | 4.62 (dd, 11.48,3.8) | 73.26 | 73.26 | 73.26 |
| 1′ | | 129.88 | | |
| 2′ | 7.07 (d, 1.92) | 115.97 | 115.97 | 115.97 |
| 3′ | | 145.89 | | |
| 4′ | | 146.71 | | |
| 5′ | 6.87 (d, 8.12) | 115.86 | 115.87 | 115.87 |
| 6′ | 7.02 (dd, 8.12,1.96) | 120.92 | 120.92 | 120.93 |

In HMBC correlations of H-2 (δ 5.02) coupled with C-10 (δ 73.26), C-3 (δ 198.32), C-1′(δ 129.88), C-2′ (δ 115.97) and C-6′ (δ 120.92). These correlations supported that hydroxyl benzyl group at C-2 position of benzofuran. Correlation of H-10 (δ 2.75) with C-2 (δ 84.62), C-3 (δ 198.32) carbon atom but this proton cannot show any correlation with C-1′(δ 129.88), C-2′ (δ 115.97) and C-6′ (δ 120.92) this indicate that twisting of hydroxyl benzyl group of benzofuran due to H-bonding or OH group. These correlations also supported the structure of Compound **22** as dihydroxybenzofuranone derivatives (Table-3.25).

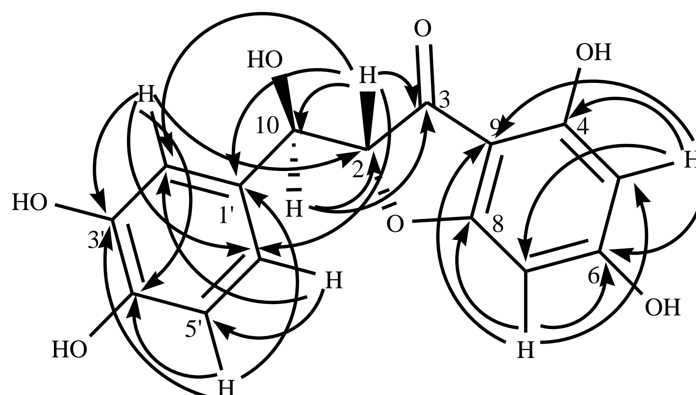



**Figure-3.21** The HMBC correlation of compound-**22**

**Table-3.25:** The $^{1}$H, HMQC and HMBC NMR data of compound-**22**

| Our data (Acetone-$d_6$) | | |
|---|---|---|
| $\delta_H$ (ppm) | HMQC correlation | HMBC correlation |
| 5.02 (H-2) | 84.62 (C-2) | 73.26(C-10), 129.88(C-1′), 115.97 (C-2′), 120.92 (C-6′), 198.32(C-3) |
| 5.99 (H-5) | 97.16 (C-5) | 96.13 (C-7), 101.62(C-9), 167.99(C-8), 164.25(C-6), 165.10 (C-4) |
| 5.95 (H-7) | 96.13 (C-7) | 97.16 (C-5), 101.62(C-9), 165.10 (C-4),164.25(C-6), 167.99 (C-8) |
| 4.62 H-10) | 73.26 (C-10) | 84.62(C-2), 198.32(C-3) |
| 7.07 (H-2′) | 115.97 (C-2′) | 84.62 (C-2), 120.92(C-6′), 145.89(3′), 146.71(C-4′) |
| 6.87 (H-5′) | 115.87 (C-5′) | 129.88(C-1′), 145.89 (3′), 146.71(C-4′), 115(C-2′) |
| 7.02 (H-6′) | 120.92 (C-6′) | 115.87(C-5′), 115.97(C-2′), 146.71(C-4′),84.62(C-2) |

From H-H COSY H-2 was found to linked with H-10′, H-5′ with H-6′. All the correlations supported that they are attached to adjacent carbon atom (Table-3.26). The NOESY correlation is not effective for this compound because all groups are in same plane. From all spectroscopic studies structure of the Compound **22** was elucidated as **[2-[(3′,4′-Dihydroxy-phenyl)-hydroxy-methyl]-4,6-dihydroxy-benzofuran-3-one].** Literature search including SinFinder showed the Compound **22** is a new compound and has not been reported earlier from neither from plant origin nor from synthetic sources.

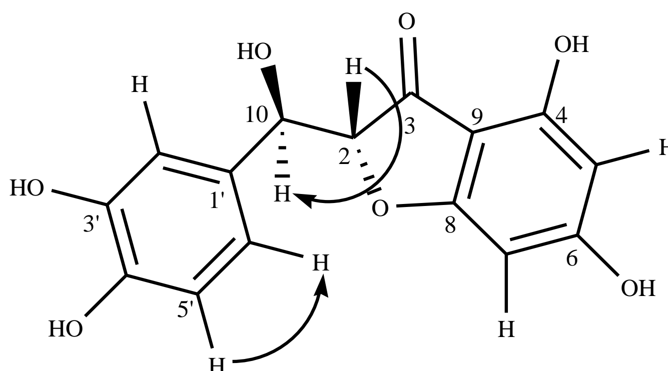



**Figure-3.22** The COSY correlation of compound-**22**

**Table 3.26:** The COSY correlation data for selected protons of compound-**22**

| Our data (Acetone-$d_6$) | |
|---|---|
| $\delta_H$ (ppm) | Protons correlated with $\delta_H$ (ppm) |
| 5.02 (H-2) | 4.62 (H-10) |
| 6.87 (H-5′) | 7.02 (H-6′) |

Compound-**23** was obtained as white needle crystal, melting point was recorded 135-138°C and specific rotation was $[\alpha]_D^{25} = +8.12$ (*c* 0.22, MeOH). The molecular formula was determined as $C_{18}H_{20}O_4$ from high resolution mass spectroscopy (HRMS-ESI-TOP) at *m/z* 323.1261 $[M+Na]^+$ (Calculated 323.1254 for $[C_{18}H_{20}NaO_4]^+$).

**FT-IR** (NEAT) shows the absorption bands at $\upsilon_{max}$ 3293 (O–H stretching ), 2963 and 2927 (C–H stretching), 1613, 1598, 1514, 1443 and 1375 (C=C stretching of aromatic ring), 1261, 1239 (C–O stretching of ether), 1212 (C–O stretching of phenol), 1168 (C–O stretching of furan), 1136, 1107 (C–O stretching of alcohol ), 1107, 1075, 1055, 1019, 987, 947, 901, 835, 825, 805, 782, 755, 716 $cm^{-1}$. The UV spectral data of aromatic showed absorbance at $\lambda_{max}$: 206 (+0.57164), 227 (+0.67019) and 277 (+0.14745) nm.

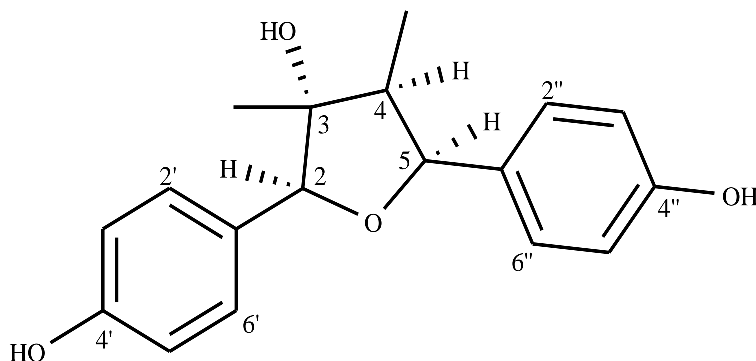



**Figure-3.23 Compound-23 2,5-Bis-[(4′-hydroxy-phenyl)(4″-hydroxy-phenyl)-3,4-dimethyl-tetrahydro-furan-3-ol (New)**

The $^1$H-NMR spectrum of the Compound **23** (in MeOD) showed nine signals for protons and $^{13}$C-NMR spectrum showed fourteen signals for eighteen carbons. DEPT-90 & DEPT-135 were performed to find out attachment of protons, i.e. primary, secondary, tertiary and quaternary carbons (Table 3.18). In the Compound **23**, two methyl, no methylene, eleven methine carbons were found out and the rest were quaternary carbons. H-H COSY spectrum was done and linkages between protons were found out. From HSQC and HMBC all protons to carbons were assigned (Table 3.28). From $^1$H NMR signal coupling constants were measured (Table 3.27) and proton carbon connectivity were found out from HMBC (Table 3.28 and Figure 3.24).

The $^{13}C$ NMR, DEPT 90 and 135 showed in Compound **23**, 2,4,5,2′,3′,5′,6′,2″,3″,5″,6″ carbons were -CH- and C-3,C-1′, C-4′,C1″, C-6′ were quaternary carbon with containing –OH group. The C-3 and C-4 were containing methyl (-$CH_3$) carbon (Table-3.27). The H-4 gave multiplate signal coupling with H-5 & 4-$CH_3$ protons. The H-5 proton gave doublet (J value 7.7 Hz) coupling with H-4 and the H-2 gave singlet. The 3-$CH_3$ gave singlet but 4-$CH_3$ gave doublet (J valu7.5 Hz) coupling with H-4. The protons H-3′,H-3″,H-5′,H-5″ were in almost same chemical environment that's why they gave doublet(J value 8.5Hz) coupling with *ortho* protons. The H-2′&H-6′ and H-2″&H-6″ gave doublet (*J* value 8.5 Hz,) coupling with *ortho* protons. The OH proton gave broad singlet at δ 4.63 ppm (Table-3.27).

**Table-3.27:** The $^{1}H$, $^{13}C$, DEPT90 and DEPT135 NMR data of compound-**23**

| Position | Our data (MeOD) | | | |
|---|---|---|---|---|
| | $\delta_H$ (mult.,*J* (Hz)) ppm | $\delta_C$ ppm | DEPT90 (CH) | DEPT135 |
| 2 | 4.78 (s) | 90.41 | 90.41 | 90.41 |
| 3 | | 82.55 | | |
| 4 | 2.44 (m) | 50.64 | 50.63 | 50.63 |
| 5 | 5.33 (d, 7.7) | 82.49 | 82.49 | 82.49 |
| 1′ | | 131.32 | | |
| 2′ | 7.30 (d, 8.5) | 128.85 | 128.85 | 128.85 |
| 3′ | 6.80 (d,8.5) | 115.97 | 115.97 | 115.97 |
| 4′ | | 158.09 | | |
| 5′ | 6.80 (d, 8.5) | 115.97 | 115.97 | 115.97 |
| 6′ | 7.30 (d, 8.5) | 128.85 | 128.85 | 128.85 |
| 1″ | | 132.10 | | |
| 2″ | 7.23 (d,8.5) | 128.80 | 128.80 | 128.80 |
| 3″ | 6.80 (d, 8.5) | 115.92 | 115.92 | 115.92 |
| 4″ | | 157.76 | | |
| 5″ | 6.80 (d, 8.5) | 115.92 | 115.92 | 115.92 |
| 6″ | 7.23 (d, 8.5) | 128.80 | 128.80 | 128.80 |
| 3-$CH_3$ | 0.78 (s) | 21.14 | | 21.14 (3-$CH_3$) |
| 4-$CH_3$ | 0.58 (d, 7.5) | 11.83 | | 11.83 (4-$CH_3$) |
| -OH | 4.63 (br. S) | | | |

In HMBC correlations; H-2 (δ 4.87) with C-3 (δ 82.55), C-1′(δ 131.32), C-2′ (δ 128.85), C-6′ (δ 128.85) and C-(3-$CH_3$)(δ 21.14). These correlations supported that one hydroxyl phenyl group at C-2 position of tetrahydrofuran. Correlation of H-5 (δ 5.33) with C-3 (δ 82.55), C-1″(δ 132.10), C-2″ (δ 128.80), C-6″ (δ 128.80) and C-(4-$CH_3$) (δ 11.83) supported that another hydroxyl phenyl group at C-5 position of tetrahydrofuran. Correlation of H-4 (δ 2.44) with C-(3-$CH_3$)(δ 21.14), C-(4-$CH_3$) (δ 11.83), C-3 (δ 82.55), C-5 (δ 82.49) and C-1″(δ 132.10) also supported that the hydroxyl phenyl group at C-5 position of tetrahydrofuran. This connectivity also supported the structure of compound 23 as tetrahydrofuran derivative (Table-3.28).

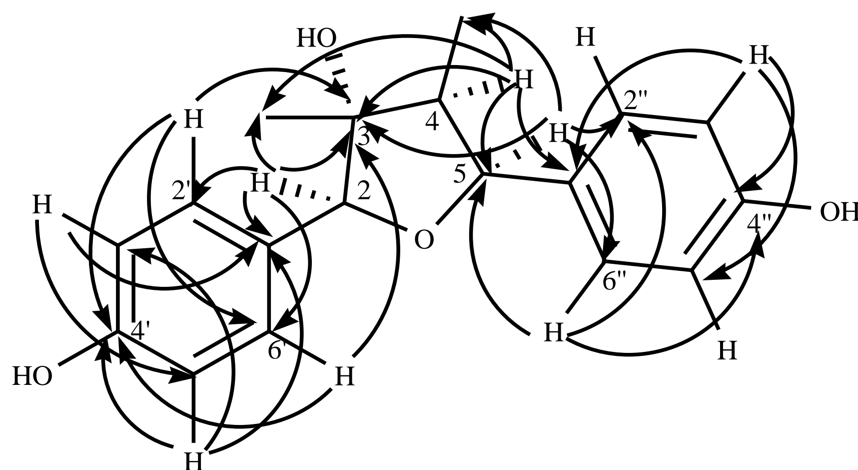



**Figure-3.24** The HMBC correlation of compound-**23**

**Table-3.28:** The $^{1}H$, HMQC and HMBC correlation of compound-**23**

| Our data (MeOD) | | |
|---|---|---|
| $\delta_H$ ppm | HMQC correlation | HMBC correlation |
| 4.78 (H-2) | 90.41 (C-2) | 82.55 (C-3), 21.14 (3-$CH_3$), 131.32(C-1′), 128.85(C-2′), 128.85 (C-6′), |
| 2.44 (H-4) | 50.63 (C-4) | 11.83(4-$CH_3$), 21.14(3-$CH_3$), 82.55(C-3), 82.49(C-5), 132.10(C-1″) |
| 5.33 (H-5) | 82.49 (C-5) | 82.55(C-3), 11.83(4-$CH_3$), 132.10(C-1″), 128.80 (C-2″), 128.80(C-6″) |
| 7.30 (H-2′) | 128.85 (C-2′) | 82.55(C-3), 128.85(C-6′), 158.09(C-4′) |
| 6.80 (H-3′) | 115.97 (C-3′) | 115.97(C-5′), 131.32(C-1′), 158.09(C-4′) |
| 6.80 (H-5′) | 115.97 (C-5′) | 115.97(C-3′), 131.32(C-1′)158.09(C-4′), |
| 7.30 (H-6′) | 128.85 (C-6′) | 82.55(C-3), 128.85(C-2′), 158.09(C-4′) |
| 7.23 (H-2″) | 128.80 (C-2″) | 128.80(C-6″), 157.76(C-4″), 82.49(C-5) |
| 6.80 (H-3″) | 115.92 (C-3″) | 115.92(C-5″), 132.10(C-1″), 157.76(C-4″) |
| 6.80 (H-5″) | 115.92 (C-5″) | 115.92(C-3″), 132.10(C-1″), 157.76(C-4″) |
| 7.23 (H-6″) | 128.80 (C-6″) | 128.80(C-2″), 157.76 (C-4″), 82.49(C-5) |
| 0.78 (3-$CH_3$) | 21.14 (3-$CH_3$) | 90.41(C-2), 50.63(C-4), 82.55(C-3) |
| 0.58 (4-$CH_3$) | 11.83 (4-$CH_3$) | 50.63(C-4), 82.49(C-5), 82.55 (C-3) |

From H-H COSY H-4 was found to linked with H-5 and H-(4-$CH_3$), H-2′ with H-3′, H-5′ with H-6′, H-2″ with H-3″ and H-6″ with H-6″. All the correlations supported that they are attached to adjacent carbon atom (Table-3.29).

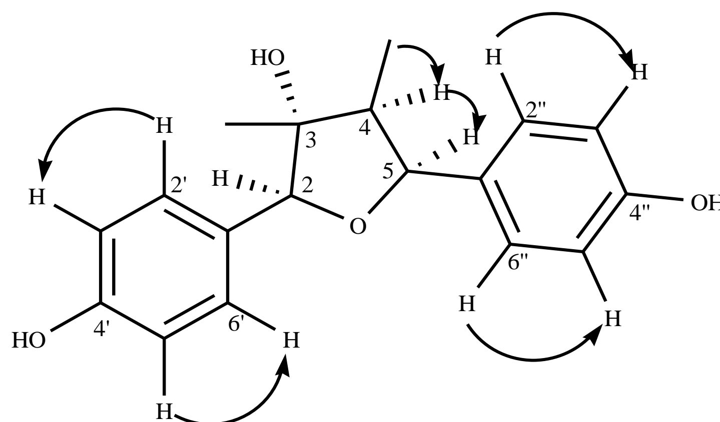



**Figure-3.25** The COSY correlation data for selected protons of compound-**23**

**Table 3.29:** The COSY correlation data for selected protons of compound-**23**

| Our data (MeOD) | |
|---|---|
| $\delta_H$ ppm | Protons correlated with $\delta_H$ (ppm) |
| 2.44 (H-4) | 5.33 (H-5), 0.58 (4-$CH_3$) |
| 7.30 (H-2′) | 6.80 (H-3′) |
| 6.80 (H-5′) | 7.30 (H-6′) |
| 7.23 (H-2″) | 6.80 (H-3″) |
| 6.80 (H-5″) | 7.23 (H-6″) |

The relative stereochemistry of compound 23 was determined on the basis of NOESY experiment. The correlation of H-2 with H-4 and H-5 indicate that they were lying in same plane. The 3-$CH_3$ proton correlates with H-2′ and 4-$CH_3$ protons and 4-$CH_3$ proton correlates with H-2′ and 3-$CH_3$ protons which indicate that two $CH_3$ group and two hydroxyphenyl group were lying in same plane (Table-3.30). From all spectroscopic studies structure of the Compound **23** was elucidated as **2,5-Bis-[(4′-hydroxy-phenyl)(4″-hydroxy-phenyl)-3,4-dimethyl-tetrahydro-furan-3-ol.** Literature search including SciFinder showed the Compound **23** is a new compound and has not been reported earlier from neither from plant origin nor from synthetic sources.

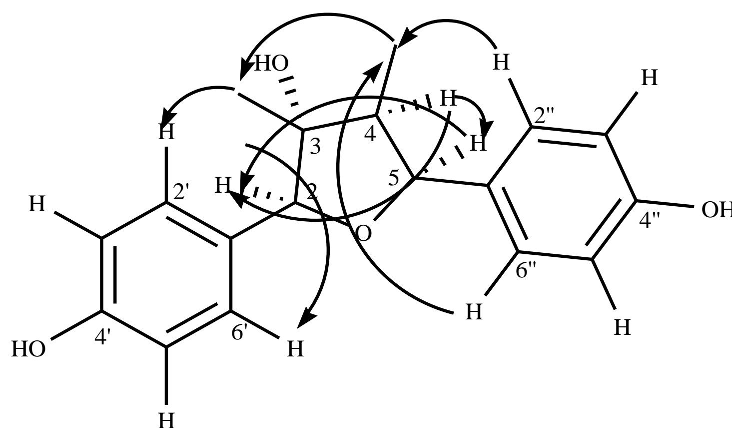



**Figure-3.26:** The NOESY correlation of compound-**23**

**Table 3.30:** The NOESY correlation data for selected protons of compound-**23**

| Our data (MeOD) | |
|---|---|
| $\delta_H$ ppm | Protons correlated with $\delta_H$ (ppm) |
| 4.78 (H-2) | 2.44(H-4), 5.33(H-5) |
| 0.78 (3-$CH_3$) | 0.58(4-$CH_3$), 7.30(H-2′) |
| 0.58 (4-$CH_3$) | 0.78(3-$CH_3$), 7.23(H-2″) |

**PART-B**

**Herbal Formulations**

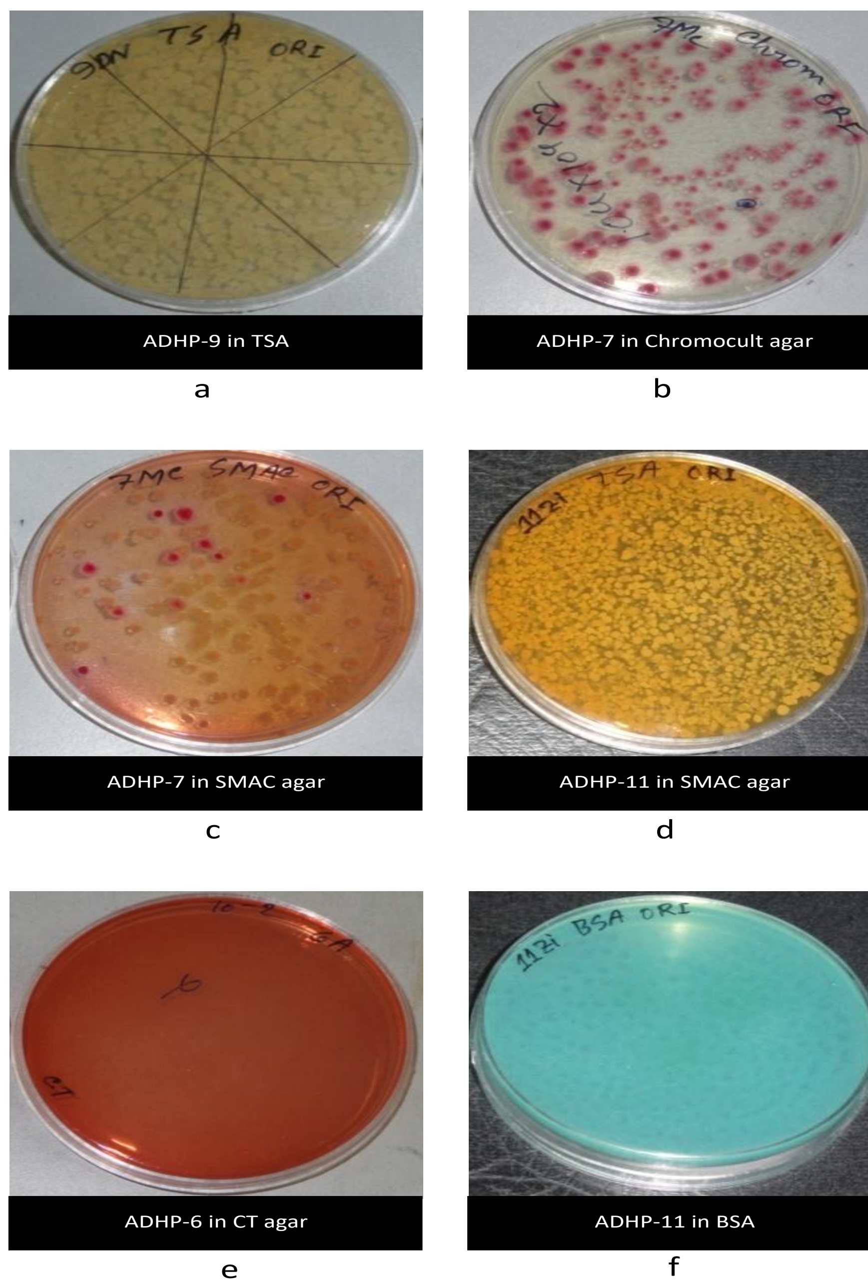
### 3.3 Microbial Contamination

For the evaluation of microbial contamination, total bacterial aerobic, total coliform, total *E. coli*, *E. coli* 0157 and Salmonella spp. count were determined (Figure 3.27). All the preparations showed different levels of total aerobic bacterial count and exceeded the safety limit according to USP (United States Pharmacopoeia) (Tables 3.31 and 3.32) but six of them (ADHP-2, ADHP-4, ADHP-6, ADHP-10, ADHP-11) exceeded the safety limit as indicated by EP (European Pharmacopoeia) and WHO (World Health Organization) guidelines (Table 3.32) whereas two of them (ADHP-5 and ADHP-9) were in marginal level. Total coli form count is the indicator of faecal contamination and found in six of the samples where they exceeded the safety limit (Tables 3.31and 3.32). Total *E. coli* count, a specific Gram negative bacterial species count included in the range of total coli form count, also exceeded the safety limit in fifty percent of the studied preparation. Specific species count such as *E. coli* 0157 and Salmonella spp. were found to be present in around 25% of the preparation (*E. coli* 0157 in ADHP-9, ADHP-11 and ADHP-13 and Salmonella spp. in ADHP-4) (Tables 3.31 and 3.32).

Table 3.31: Microbial assessment of different ADHPs

| Sample Name | Total aerobic bacterial count/ml | Total coliform count/ml | Total *E.coli* count/ml | *E.coli* O157 count/ml | *Salmonella* spp. Count/ml |
|---|---|---|---|---|---|
| ADHP-1 | $2.0x10^4$ | $2.0x10^2$ | Negative | Negative | Negative |
| ADHP-2 | $1.4x10^6$ | Negative | Negative | Negative | Negative |
| ADHP-3 | $4.0x10^4$ | $2.7x10^3$, $1.7x10^3$(w,c) | Negative | Negative | Negative |
| ADHP-4 | $5.72x10^6$ | $8.4x10^3$(P) $8.00x10^2$(W) | $3.1x10^3$(W) $6.0x10^2$(P) | Negative | $2.75x10^3$ |
| ADHP-5 | $2.9x10^5$ | Negative | Negative | Negative | Negative |
| ADHP-6 | $5.7x10^5$ | Negative | Negative | Negative | Negative |
| ADHP-7 | $7.08x10^4$ | $2.08x10^4$ | $2.34x10^4$ | Negative | Negative |
| ADHP-8 | $5.36x10^4$ | Negative | Negative | Negative | Negative |
| ADHP-9 | $2.5x10^5$ | $2.89x10^3$ | $9.5x10^2$ | $4.4x10^2$ | Negative |
| ADHP-10 | $8.3x10^5$ | $8.0x10^2$ | $2.9x10^2$ | Negative | Negative |
| ADHP-11 | $1.38x10^6$ | $3.93x10^4$ | $1.34x10^4$(P) | $2.1x10^3$(P) | Negative |

| | | | $7.8x10^3$(W) | | |
|---|---|---|---|---|---|
| ADHP-12 | $2.03x10^4$ | Negative | Negative | Negative | Negative |
| ADHP-13 | $1.87x10^6$ | $5.0x10^3$ | $5.0x10^2$(P)<br>$1.6x10^3$(W) | $1x10^2$(P) | Negative |




`**Figure-3.27** Incubation of antidiabetic herbal preparations (ADHPs) in different agar media for microbial count. a) ADHP-9 incubated in Tryptic Soya Agar (TSA) plate for total aerobic count, b) ADHP-7 incubated in Chromocult agar plate for total coliform count, c) ADHP-7 and ADHP-11 incubated in Sorbitol MacConkey (SMAC) agar plate for total *E. coli* count, e) ADHP-6 incubated in Caprylate-thallous (CT) agar plate for *E. coli* 0157 count and f) ADHP-11 incubated in Bismuth sulfite agar (BSA) plate for *Salmonella* spp. count.

Almost seventy percent of the total preparation studied (nine preparations) failed to comply with the safety limit at least in one method of microbial contamination evaluation like total microbial counts or specific species count. In this study we counted microorganism in five different ways (total aerobic bacterial count, total coliform count, total *E. coli* count, specific *E. coli* 0157 count and Salmonella spp. count), where ADHP-4 and ADHP-11 exceeded safety limit in four different microbial counting methods. ADHP-9 is in second position in failure the safety limit as it exceeded the safety limit evaluated by three different counts. At least in two different microbial counts, the level of microbial contamination was higher than the safety limit in ADHP-7, ADHP-10 and ADHP-13 as mention in the EP, USP and WHO guidelines (Tables 3.31 and 3.32). From the data, it is found that two of these preparations (ADHP-1 and ADHP-12) only could be able to comply with the safety limit evaluated by all the different microbial counts. If we consider the presence of fungi in the preparation then none of these herbal preparations could comply with different standardizing body for the assurance of safety as all of the thirteen ADHPs have shown positive response in Potato dextrose agar (PDA)(figure 3.28).

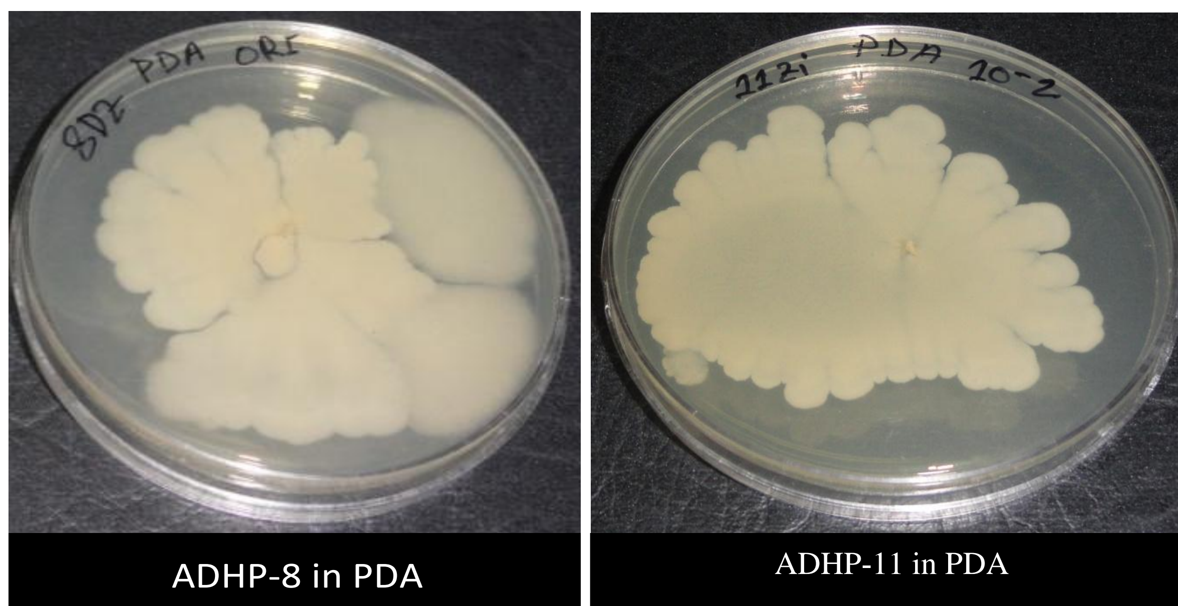



Figure 3.28 Incubation of ADHP-8 and ADPH-11 in Potato Dextrose agar (PDA).

The presence of large numbers of pathogenic bacteria in the studied herbal preparations indicates several windows to consider as a source of contamination. It is worth to mention that pH of all the preparations was within the suitable range (pH 5-8.5) which may appreciates bacterial growth [82]. The contamination could start at the initial phase of raw materials collection as soil influences bacterial growth in several ways. This initial contamination could be carried along to harvesting, drying and storage. Moreover, during the preparation of finished preparation the source of contamination includes personnel, equipments and materials. Therefore, the process of raw material collections, processing of the raw materials and the process of manufacturing for finished preparation should ensure the highest possible level of hygiene to maintain lowest possible level of pathogenic organism in the preparation and thereby assure the quality and safety of herbal preparation.

The level of microbial contamination is mentioned in different standards for publication including EP, USP and WHO guideline to maintain the safety of herbal preparations (Table 3.32). Gram negative bacteria such as Salmonella,Shiegella and *E. coli* should be absent in the preparation. Moreover, the limit for coliforms also mentioned, as it is the most reliable indicator of faecal contamination, which may indicate the possible presence of other harmful disease-causing organisms. The presence of fungi in herbal preparations under certain conditions, may lead to the secretion of toxic metabolites such as mycotoxins, which when ingested, inhaled or absorbed through the skin cause illness or human and animal death [83].

These mycotoxins possess substantial risk of carcinogenic, neurotoxic, immunotoxic and mutagenic effects[53-58]. It is reported that a substantial amount of medicinal plants are contaminated naturally by fungi from soil and environment and thereby may contain mycotoxins[52]. As most of the herbal preparation majorly contains medicinal plants, it is important to assure that the level of mycotoxins are below the safety limit as set by different bodies. For conclusive remark we further need to determine the level of mycotoxins in these herbal preparations.

Table 3.32: Microbial limits for finished herbal/botanical preparations (in colony-forming units/gram (cfu/g) or colony-forming units/ml (cfu/ml)), (Current as of July 2014)

| Reference | EP category C | USP | WHO |
|---|---|---|---|
| Product | Product with Ingredients demonstrated to fail Catergory B w/Processing/ Pretreatment | Containing Botanical Ingredients | Herbal Materials for internal use |
| Total aerobic microbial count | $10^5$ (maximum acceptance limit: $5x10^5$) | $10^4$ | $10^5$ |
| Total combined yeast and mold count | $10^4$ (maximum acceptance limit: $5x10^4$) | $10^3$ | $10^3$ |
| Enterobacterial Count (Bile-tolerant Gram-negative bacteria) | $10^4$ | NA | $10^3$ (other than E. coli) |
| Escherichia coli | Absence in 1 g | Absence in 10 g | 10 in 1 g |
| Salmonella spp. | Absence in 25 g | Absence in 10 g | Absence in 1 g |
| Staphylococcus aureus | NA | NA | NA |
| Clostridia | NA | NA | Absence in 1 g |
| Shigella | NA | NA | Absence in 1 g |

**EP**- European Pharmacopoeia Ed. 8.0, 5.1.8 (Microbiological quality of herbal medicinal products for oral use and extracts used in their preparation), 2013. **USP**- United States Pharmacopeial Convention, USP-NF 37-32, 2014. **WHO**- World Health Organization, WHO Guidelines for Assessing Quality of Herbal Medicines with Reference to Contaminants and Residues, 2007. **NA**- Not Assigned

**3.4 Heavy Metal Content:**

In this study, we determined heavy metal (Cu, Cd, Cr, Mn, Pb and Zn) contents in different ADHPs to identify whether any potential risk of accumulation of these heavy metals leading to toxicity (Table 4). All the thirteen ADHPs containCopper (Cu), Chromium (Cr), Manganese (Mn), Lead (Pb) and Zinc (Zn) in some level with few exceptions (Cr in ADHP-9 and Zn in ADHP-7 and ADHP-12 was in below detection level) (Table 3.33).

Table 3.33: Heavy metal content of investigated ADHP samples

| Sample ID | Zn (ppm) | Cu (ppm) | Mn (ppm) | Cr (ppm) | Cd (ppm) | Pb (ppm) |
|---|---|---|---|---|---|---|
| ADHP-1 | 5.38 | 5.38 | 6.5 | 9.25 | BDL | 8.50¶ |
| ADHP-2 | 3.75 | 10.00 | 9.25 | 4.63 | BDL | 8.50¶ |
| ADHP-3 | 2.00 | 10.50 | 6.63 | 4.38 | BDL | 7.00¶ |
| ADHP-4 | 12.50 | 3.50 | 7.75 | 28.25 | 2.75 *§¶ | 41.38* |
| ADHP- 5 | 2.88 | 4.88 | 1.63 | 8.25 | BDL | 6.63¶ |
| ADHP-6 | 3.75 | 4.50 | 8.50 | 4.00 | BDL | 13.38*¶ |
| ADHP-7 | BDL | 4.13 | 3.00 | 2.88 | BDL | 3.75 |
| ADHP-8 | 2.75 | 3.75 | 0.88 | 6.00 | BDL | 11.50*¶ |
| ADHP-9 | 2.88 | 8.88 | 6.88 | BDL | BDL | 3.88 |
| ADHP-10 | 2 | 7.25 | 8.50 | 11.75 | BDL | 9.88¶ |
| ADHP-11 | 2.38 | 4.50 | 8.00 | 2.13 | BDL | 5.75¶ |
| ADHP-12 | BDL | 4.25 | 4.63 | 2.50 | BDL | 9.38¶ |
| ADHP-13 | 10.50 | 3.13 | 6.00 | 24.63 | 1.38 *§¶ | 33.50*§¶ |

BDL (below detection level); * exceed WHO and US FDA permission limit; § exceed HAS Singapore permission limit; ¶ exceed Chines Pharmacopoeia permission limit

The amount of cadmium was below detection level in all the preparations except ADHP-4 and ADHP-13. There are several regulatory bodies that set specific allowable limit for heavy metal content in herbal and tradition preparations based on different guidelines and this permissiblelimit varies among these regulatory bodies (Table 3.34 and 3.36). It is found that lead content in almost all of the samples (except ADHP7 and ADHP9) exceeded the permissible limit if we consider the stringiest limit of Chines Pharmacopoeia (Table 3.33 and 3.34). Even if we consider a more relax permissible limit for lead (WHO and US FDA guidelines; Table 3.34), one third of the total ADHPs (ADHP4, ADHP6, ADHP8 and ADHP13) failed to comply with the

safety limit. Lead, a highly toxic environmental pollutant, can affect the function of various biomolecules by forming complex with them.

Table 3.34: Permissible limit of heavy metal in herbal drugs

| Heavy/Toxic metal | WHO | US FDA | HSA Singapore | Chinese Pharmacopoeia |
|---|---|---|---|---|
| Cadmium | 0.20 ppm | 0.30 ppm | 0.05 ppm | 0.30 ppm |
| Lead | 10.00 ppm | 10.00 ppm | 20.00 ppm | 5.00 ppm |
| Arsenic | 10.00 ppm | 10.00 ppm | 5.00 ppm | 2.00 ppm |
| Mercury | 1.00 ppm | 1.0 ppm | 0.50 ppm | 0.20 ppm |
| Copper | 20.00 ppm | 20.00 ppm | 150.00 ppm | 20.00 ppm |
| Zinc | 50.00 ppm | 50.00 ppm | -- | -- |

US FDA (United States Food and Drug Administration); HAS (Health Science Authority).

Moreover, excess lead exposure may responsible for poor muscle coordination, gastrointestinal symptoms, brain and kidneys damage, hearing and vision impairments and reproductive defects[84-86].Cadmium content was below detection level in all of the ADHP samples other than ADHP4 and ADHP13. Unfortunately these two (ADHP4 and ADHP13) samples were also failed to comply with safety based on cadmium content (table 3.33 and 3.34).Cadmium toxicity could induces tissue injury [87-89], epigenetic changes in DNA expression [90-92], hypertension [93], diabetes [94], apoptosis [95] and insulin resistance [96, 97].Moreover, excess cadmium may inhibits or up regulates transport pathways [98-100] and heme synthesis [101]. According to JECFA (The Joint FAO/WHO Expert Committee on Food Additives) heavy metal limits for herbal dietary supplements, none of these formulations contains heavy metals in such a level, which could exceed the daily allowable intake (Table 3.35 and 3.36). Considering all of these guidelines, it turned out that only two ADHP samples (ADHP7 and ADHP9) contains heavy metals in safe level. Metals are natural components of soils and some of them (Cu, Mn and Zn) are necessary for micronutrients of plant growth while others (Cd, Cr and Pb) are not but could be accumulated in plants at toxic level [102-104].

Table 3.35: Heavy metal content of investigated ADHP samples and the daily safe intake of different heavy metals

| Sample ID | Cumulative Daily adult dose of preparation* (g) | Daily Adult intake of heavy metal (in µg) as calculated form the dose indicated on the label of the finished product. | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Zn** | **Cu** | **Mn** | **Cr** | **Cd** | **Pb** |
| ADHP-1 | 1.50 | 8.06 | 8.06 | 9.75 | 13.88 | -- | 12.75 |
| ADHP-2 | 1.00 | 3.75 | 10.00 | 9.25 | 4.63 | -- | 8.50 |
| ADHP-3 | 3.00 | 6.00 | 31.50 | 19.88 | 13.13 | -- | 21.00 |
| ADHP-4 | 2.50 | 31.25 | 8.75 | 19.38 | 70.63 | 6.88 | 103.44 |
| ADHP- 5 | 2.50 | 7.19 | 12.19 | 4.06 | 20.63 | -- | 16.56 |
| ADHP-6 | 3.75 | 14.06 | 16.88 | 31.88 | 15.00 | -- | 50.16 |
| ADHP-7 | 4.00 | -- | 16.50 | 12.00 | 11.00 | -- | 15.00 |
| ADHP-8 | 3.50 | 9.63 | 13.13 | 3.06 | 21.00 | -- | 40.25 |
| ADHP-9 | 30.00 | 86.25 | 266.25 | 206.25 | -- | -- | 116.25 |
| ADHP-10 | 2.50 | 5.00 | 18.13 | 21.25 | 29.38 | -- | 24.69 |
| ADHP-11 | 2.50 | 5.94 | 11.25 | 20.00 | 5.31 | -- | 14.38 |
| ADHP-12 | 4.00 | -- | 17.00 | 18.50 | 10.00 | -- | 37.50 |
| ADHP-13 | 2.50 | 26.25 | 7.81 | 15.00 | 61.56 | 3.44 | 83.75 |

* This dose is calculated as indicated on the label of the finished product; BDL (below detection level)

As the major components of these herbal preparations are plants, the presences of heavy metals in ADHPs are very relevant. Some of the identified metals (Zn, Cu, Mn and Cr) have important biological role in the body.

Table 3.36: JECFA heavy metal limits for herbal dietary supplements

| Heavy Metals | Stated Limit (PTWI- weekly) | Calculated Daily Limit (Adult, 70 kg) |
|---|---|---|
| **Arsenic** | 15 µg inorganic arsenic/kg bw | 150 µg |
| **Cadmium** | 7 µg cadmium/kg bw | 70 µg |
| **Lead** | 25 µg lead/kg bw | 250 µg |
| **Mercury** | 1.6 µg methylmercury/kg bw | 16 µg |

JECFA (The Joint FAO/WHO Expert Committee on Food Additives); PTWI (Provisional tolerable weekly intake)

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# REFERENCES


1. Yue-Zhong Shu, (1998), *Journel of Natural product*, 61,1053-1071
2. Van Hensbrock, M.B., Onyiorath, E., Jaffer, S., Schneider, G., Palmer, A., Frenkel, J., Enwere , G., Forck, S., Nusmeijer, A., Bennett, S., Greenwood, B. and Kwiatkowski, D., (1996), *New Eng. J. Med.*, **335**, 69-75.
3. Currens, M.J., Gulakowski, R.j., Mariner, J.M., Moran R.A., Buckheit Jr., Gustafson, K.R., Mcmahon, J.B., and Boyd, M.r., (1996), *J.Pharmacol. Exp. Ther.,* **279,** 645-651.
4. Smitinad, T., (2001), Thai plants name (botanical name-varnacular names) revised edition, Royal Forest Department, Bangkok, Thailand.
5. Aubreville, A., Flore du Camboge, du laos et du Vietnam, Museum National D Histoire Naturelle, Laboratorie de phanerogamine, 16, Rue Buffon, Paris, 1969.
6. http://en. Wikipedia.org./w/index.php? title= Anogeissus & oldid=487094573.
7. Scott, A.J., Arevision of *Anogeissus (Combretaceae*). Kew bulletin 1979, **33,** 555-566.
8. http:/web3.dnp.go.th/botany/detail.aspx?words = %B5 %D0 %0 %A4 %D5 %C2 %B9 %E9 %D3 & typeword= group.
9. Jain, S.K., Tarafder, C.R., Medicinal plant-lore of the sandals, *Econ. Bot.,* 1976, **24**, 241-278.
10. Gobindrarajon, R., Vijayakumar,M., Rao, C.V., Shirwaikar, A., Rawat, A. K. S., Mehrotra, S., Pushpangadan, P., Anti oxidant potential of Anogeissus latifolia, *Biol Pharm. Bul.* 2004, **27(8)**, 1266-1269.
11. Ramachandran, V. S., Nair, N.C., Ethnobotanical observations on irularis of tamil nadu (India), *J. Econ. Tax. Bot.,* 1981, **2**, 183-190.
12. Vasileva, B., Plantes medicinales de Guinee, Republic de Guinee, Conakry, 1969.

13. Baoua, M., Fayyn, J., Bassiere J., Preliminary phytochemical testing of some medicinal plants of Niger, *Plant Med*. 1976, **10**, 251-266.

14. Adigun, J. O., Amuptain, J. O., Kelly, D. R., Isolation and investigation of antimicrobial effect of 3,4,3′-tri-O-methylflavellagic acid and its glycoside from *Anogeissus leocarpus*, Bull. Chem. Soc. Ethiop. 2000, 14(2), 169-174.

15. Fadulu, S. O., The antibacterial properties of the buffer extracts of chewing sticks used in Nigeria, *Planta media*, 1975, **27**, 122-126.

16. El-Hamidia, A., Drugs plants of the Sudan republic in native medicine, *Planta media*, 1970, **18**, 278-280.

17. Almagboul, A. Z., Bashir, A. k., Karim, A., Salih, M., Farouk, A., Khaled, S. A., Antimicrobial activity of certain Sudanese plants used in folkloric medicine, screening foe antifungal activity (VI), *Fiototerapia,*1988, **59**, 393-396.

18. Singh, V.K., Ali, Z. A., Folk medicines in primary health care: common plants used for the treatmentof fevers in India. *Fitoterapia*, 1994, **65(1)**, 68-74.

19. Ramando, A. M., Pezzuto, J. M., Farnsworth, N. R., Santisuk, T., Reutrakul, V., Kawanishi, k., New liglans from Anogeissus acuminata with HIV-1 reverse transcriptase inhibitory activity, *J. Nat. Prod.*, 1994, **57 (7)**, 896-904.

20. Singh, A., Singh, A. V., Nath, L. K., Ghosh, T. K., *Anogeissus latifolia* : aresent update on its chemistry and pharmacological application, *Pharmacologyonline*, 2010, 446-449.

21. Gobindrarajon, R., Vijayakumar,M., Rao, C.V., Shirwaikar, A., Rawat, A. K. S., Mehrotra, S., Pushpangadan, P., Antiulcer and antimicrobial activity of *Anogeissus latifolia*, *Journal of Ethnopharmacology*, 2006, **106,** 57-61.

22. Sahoo, K., Misra, T., Dhal, N. K., Antimicrobial activity of *Anogeissus latifolia* (Robex, ex. DC) Wall. Ex. Guill. And Perr. Environ. Conserv. 2011, **17(2)**, 371-374.

23. Parvati, K. M. M., Ramesh, C. K., Krishna, V., Paramesh, M., Kuppast, I. J., Antihyperglycemic activity of *Anogeissus latifolia* in streptozotocine induced diabetic rats, *J. Chem. Sci,* 2009, **7(3)**, 1974-1982.

24. Parvati, K. M. M., Ramesh, C. K., Krishna, V., Paramesh, M., Kuppast, I. J., Antihyperglycemic activity of *Anogeissus latifolia* bark and leaf extracts, *Asian J. Exp. Sci.* 2009, **23(3)**, 491-495.

25. Konate, K., Kiendrebeogo, M., Ouattara, M, B., Souza, A., Lamien-meida, A.,Non gasida, Y., Baro, N., Millogo-Rasolodimby, J., Nacoulma, O. G., Antibacterial potential of aqueous acetone extracts from five medicinal plant used traditionally to treat infactious diseases in Burkina faco, *Curr. Res. J. Biol. Sci*, 2011, 3(5), 435-442.

26. Mann, A., Banso, A., Clifford, L. C., An anti fungal property of crude plant extracts from *Anogeissus leiocarpus* and *Terminalia avicennioides*, *Tenzania Journel of Health Research,* 2008, **10 (1)**, 34-38.

27. Isimi, C., Obidike, I., Aboh, M., Emeje, M., Anti-plasmodial activity of the mixed stem bark extracts of *Anogeissus leiocapus* and *Prosopis africana* and in vitro evaluation of its tablet dosage form, *J. of. Herbs, Spices & Medicinal Plants*, 2011, **17 (4),** 419-435.

28. Hemalini, K., Gopalakrishnan, S., Phytochemical and antidermatophytic activity of methanolic extracts of *Anogeissus accuminata*, *Pharmacologyonline*, 2010, 507-511.

29. Ramando, A. M., Pezzuto, J. M., Farnsworth, N. R., Santisuk, T., Reutrakul, V., Kawanishi, k., Revision of the NMR assignments of pterostilbene and of Dihydro dehydro-dehydrodiconiferyl alcohol: cytotoxic constituents from *Anogeissus acuminate*, *Nat. Prod. Lett.*, 1994, **4**, 267-272.

30. Bhadoria, B. K., Lata, S., Tandan, S. k., A nonsteroidal anti-inflammatory compound from tree: *Anogeissus pendula, Indian Pat Appl.*, 2011, 20.

31. Srivastava, S., Govindarajon, R., Rawat, A. K. S., Phytochemical and antioxidant studies of two anogeissus species, India, Section B: Biological Science, 2011, **80(1)**, 72-80.

32. Marwah, R. G., Fatope, M. O., Al Mahrooqi, R., Varma, G. B., Al Abadi, H., Al-Burtamani, S., Antioxident capacity of some edible and wound healing plants in Oman, *Food Chemistry*, 2006, **101(2)**, 465-470.

33. Raw, L. R., Raju, R. R., Chemisty of Terminalia species-XI. Isolation of 3,4,3′-tri-O-methyl-flavellagic acid from *Termanila paniculata*, *Roth. Tetrahedron*, 1967, **23**, 879-884.

34. Deshpande, V. H., Patil, A. D., Rao, A. V. R.,Venkataraman, K., 3,3′-di-O-methylellagic *Anogeissus acuminata* bark, *Indian J. Chem*, 1976, **14B**: 641-643.

35. Nduji, A. A., Okwute, S. K., Co-occurrence of 3,3′- tri-O-methylflave llagic acid and 3,3′-di-O-methylellagic *Anogeissus schimperii*, *Phytochemistry,* 1988, **27(5),** 1548-1550.

36. Chaabi, M., Benayache, F., N Gom, S., Triterenes and polyphenols from *Anogeissus leiocarpus*, Biochemical Systematics and Ecology, 2008, **36**, 59-62.

37. Mohammad, S. R., Mohammad, Z. R., Mohammad, A. R., Steriod and triterpenoid from *Anogeissus latifolia, J. Pharm. Sci.,* 2007, **6** (**1**), 47-50.

38. Lin, T-C., Tanaka, T., Nonaka, G., Nishioka, I., Young, T-J., Tanin and Related compounds, *Chem. Pharm. Bull.,* 1991, **39(5)**, 1144-1147.

39. Chaturvedi, S. K., Chabra, B. S., Chemical constituents of *Anogeissus latifolia* , *Asian Journel of Chemistry*, 2005, 17(4), 2850.

40. Lata, S., Bhadoria, B. K., A noval chromone-substituted trimeric dihydroflavanol from *Anogeissus pendula* , *Chem. Nat. Compd.* 2010, **46(5)**, 726-729.

41. Attioua, B., Lagnika, L., Yeo, D., Antheaume, C., Kaiser, M., Weniger, B., Lobstein, A., Senceheau, C. V., In vitro anti-plasmodial and antileishmanial activity of flavonoids from *Anogeissus leiocarpus* , 2010, **11(2)**, 1-6.

42. Arunadevi, R., Lata, S., Bhadoria, B. K., Ramteke, V. D., Kumar, S. K., Sankar, P., Kumar, D., Tandan, S. K., Neuroprtective effect of 5,7 3′,4′,5′-pentahydroxy

dihydroflavanol-3-O-(2″-O-galloyl)-β-D-glypiranoside, a polyphenolic compound in focal cerebral ischemia in rat, *Eur. J. Pharmacol*. 2010, **626**, 205-212.

43. Bent, S., Herbal medicine in the United States: review of efficacy, safety, and regulation: grand rounds at University of California, San Francisco Medical Center. *J Gen Intern Med,* 2008, **23**(6), 854-859.
44. WHO, Traditional Medicine Fact Sheet 134 [WWW Document]. World Health Organization, 2008.
45. Sofowora, A., Research on medicinal plants and traditional medicine in Africa. *J Altern Complement Med,*1996, **2**(3), 365-372.
46. WHO, General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine, WHO/EDM/TRM/2000.1. WHO, 2000.
47. Adeleye, I.A., G. Okogi, and E.O. Ojo, Microbial contamination of herbal preparations in Lagos, Nigeria. *J Health Popul Nutr*, 2005, **23**(3), 296-297.
48. WHO, Research guidelines for evaluating the safety and efficacy of herbal medicines. 1993.
49. Ernst, E., Toxic heavy metals and undeclared drugs in Asian herbal medicines. *Trends Pharmacol Sci*, 2002, **23**(3), 136-139.
50. Wild, S., et al., Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*, 2004, **27**(5), 1047-1053.
51. McCune, L.M. and T. Johns, Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous peoples of the North American boreal forest. *J Ethnopharmacol*, 2002, **82**(2-3), 197-205.
52. Ashiq, S., M. Hussain, and B. Ahmad, Natural occurrence of mycotoxins in medicinal plants: a review. *Fungal Genet Biol*, 2014, **66**, 1-10.
53. Petzinger, E. and K. Ziegler, Ochratoxin A from a toxicological perspective. *J. Vet. Pharmacol Ther.,* 2000, **23**(2), 91-8.

54. Nations, F.F.a.A.O.o.U., Manual on the Application of the HACCP System in Mycotoxin Prevention and Control, FAO Food and Nutrition Paper, Rome, 2001, **73**.

55. Bhatnagar, D., K.C. Ehrlich, and T.E. Cleveland, Molecular genetic analysis and regulation of aflatoxin biosynthesis. *Appl. Microbiol Biotechnol*, 2003, **61**(2), 83-93.

56. Aziz, N.A., et al., Contamination of some common medicinal plant samples and spices by fungi and their mycotoxins. *Botanical Bulletin of Academia Sinica*, 1998, **39**(4), 279-285.

57. Roy, A.K. and S. Kumar, Occurrence of ochratoxin A in herbal drugs of Indian origin - a report. *Mycotoxin Res*, 1993, **9**(2), 94-8.

58. Wu, H.C., et al., Aflatoxin B1 exposure, hepatitis B virus infection, and hepatocellular carcinoma in Taiwan. *Cancer Epidemiol Biomarkers Prev*, 2009, **18**(3), 846-53.

59. Munro, A.L.S., Chapter III Measurement and Control of pH Values, in Methods in Microbiology, J.R. Norris and D.W. Ribbons, , Academic Press, Editors. 1970, 39-89.

60. Maturin, L. and J.T. Peeler, *Chapter 3; Aerobic Plate Count*, B.A. Mannual, FDA, USA. Editor. 2001

61. Feng, P., et al., Chapter 4: Enumeration of Escherichia coli and the Coliform Bacteria, B.A. Manual , Food and Drug Administration, USA, Editor. 2002

62. WHO, *Determination of microorganisms*, in *Quality control methods for herbal materials*, W.W.H. Organization), WHO Press: Switzerland, Editor. 2011,75-84.

63. Lee, S.M., et al., Cytotoxic triterpenoids from the fruits of Zizyphus jujube, *Planta medica,* 2003, **69**(11), 1051-1054.

64. Cichewicz, R.H. and S.A. Kouzi, Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection, *Medicinal Research Reviews*, 2004, **24**(1), 90-114.

65. Khan, M.F., et al., Design and synthesis of lupeol analogues and their glucose uptake stimulatory effect in L6 skeletal muscle cells, *Bioorganic & medicinal chemistry letters*, 2014, **24**(12), 2674-2679.
66. Burns, D., et al., Assignment of 1H and 13C spectra and investigation of hindered side-chain rotation in lupeol derivatives, *Magnetic Resonance in Chemistry*, 2000, **38**(7), 488-493.
67. Ali, M.S., et al., Characterization and bioscreening of a new triterpenoid and a flavanone isolated from Salvia nubicola, *Chemistry & biodiversity*, 2005. **2**(7), 910-916.
68. Kotowicz, C., Cesar A.N. Catalan, Triterpenes and other constituents of Nassauvia axillaris, *Biochemical synthesis and ecology*, 2005, **33**, 737-742.
69. Núñez, M.J., et al., Lupane triterpenoids from Maytenus species, *Journal of natural products*, 2005, **68**(7), 1018-1021.
70. Ye, G., et al., Ellagic acid derivatives from the stem bark of Dipentodon sinicus, *Chemistry of Natural Compounds*, 2007, **43**(2), 125-127.
71. Chen, G.-y., et al., A new flavonol from the stem-bark of Premna fulva, *Arkivoc,* 2010, **2**, 179-185.
72. Olsen, H.T., et al., Isolation of the MAO-inhibitor naringenin from Mentha aquatic L, *Ethnopharmacology*, 2008, **117**(3), 500-502.
73. Yamauchi, K., T. Mitsunaga, and I. Batubara, Isolation, Identification and Tyrosinase Inhibitory Activities of the Extractives from Allamanda cathartica. *Natural Resources*, 2011, **Vol.02, No.03**, 6.
74. Bergman, M., et al., The antioxidant activity of aqueous spinach extract: chemical identification of active fractions. *Phytochemistry*, 2001, **58**(1), 143-152.
75. Owen, R., et al., Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes, *Food and Chemical Toxicology,* 2003, **41**(5), 703-717.
76. Zhang, H.L., et al., Sesquiterpene glycosides from cotton oil cake, *Phytochemistry*, 1998, **48**(4), 665-668.

77. Faizi, S., et al., Complete 1H and 13C NMR assignments of stigma-5-en-3-O-β-glucoside and its acetyl derivative, *Magnetic Resonance in Chemistry*, 2001, **39**(7), 399-405.
78. Kimura, J. and N. Maki, New Loliolide Derivatives from the Brown Alga Undaria pinnatifida. *Journal of Natural Products*, 2002, **65**(1), 57-58.
79. Günter Willuhn, R.-G.W., Loliolide (Calendin) from Calendula officinalis, *Planta medica,* 1987, **53**(3), 304.
80. Sakushima, A., M. Coşkun, and T. Maoka, Hydroxybenzoic acids from Boreava orientalis, *Phytochemistry*, 1995, **40**(1), 257-261.
81. Sang, S., et al., Antioxidative phenolic compounds isolated from almond skins (Prunus amygdalus Batsch), *Journal of Agricultural and Food Chemistry*, 2002, **50**(8), 2459-2463.
82. Lamikanra, A., Essential Microbiology for Students and Practitioners of Pharmacy, *Medicine and Microbiology*, 2nd Edition ed, Amkra Books, 1999
83. Pitt, J.I., What are mycotoxins? *Australian Mycotoxin Newsletter*, 1996, **7**(4), 1.
84. Johnson, F.M., The genetic effects of environmental lead, *Mutat Res*, 1998, **410**(2), 123-40.
85. Kalia, K. and S.J. Flora, Strategies for safe and effective therapeutic measures for chronic arsenic and lead poisoning, *J. Occup. Health*, 2005, **47**(1), 1-21.
86. Pearce, J.M., Burton's line in lead poisoning, *Eur Neurol*, 2007, **57**(2), 118-9.
87. Matovic, V., et al., Cadmium toxicity revisited: focus on oxidative stress induction and interactions with zinc and magnesium, *Arh. Hig. Rada. Toksikol*, 2011, **62**(1), 65-76.
88. Patra, R.C., A.K. Rautray, and D. Swarup, Oxidative stress in lead and cadmium toxicity and its amelioration, *Vet. Med. Int.,* 2011, **2011**, 457327.
89. Cuypers, A., et al., Cadmium stress: an oxidative challenge, *Biometals*, 2010, **23**(5), 927-40.
90. Martinez-Zamudio, R. and H.C. Ha, Environmental epigenetics in metal exposure, *Epigenetics*, 2011. **6**(7), 820-7.
91. Wang, B., et al., Cadmium and its epigenetic effects, *Curr. Med. Chem*, 2012, **19**(16), 2611-20.

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92. Luparello, C., R. Sirchia, and A. Longo, Cadmium as a transcriptional modulator in human cells, *Critical Reviews in Toxicology*, 2011, **41**(1), 73-80.
93. Gallagher, C.M. and J.R. Meliker, Blood and urine cadmium, blood pressure, and hypertension: a systematic review and meta-analysis, *Environ Health Perspect*, 2010, **118**(12), 1676-84.
94. Edwards, J.R. and W.C. Prozialeck, Cadmium, diabetes and chronic kidney disease, *Toxicol Appl. Pharmacol*, 2009, **238**(3), 289-93.
95. Cannino, G., et al., Cadmium and mitochondria, *Mitochondrion*, 2009, **9**(6), 377-84.
96. Satarug, S. and M.R. Moore, Emerging roles of cadmium and heme oxygenase in type-2 diabetes and cancer susceptibility, *Tohoku J. Exp. Med*, 2012, **228**(4), 267-88.
97. Chen, Y.W., et al., Heavy metals, islet function and diabetes development, *Islets*, 2009, **1**(3), 169-76.
98. Thévenod, F., Catch me if you can! Novel aspects of cadmium transport in mammalian cells, *BioMetals*, 2010, **23**(5), 857-875.
99. Wan, L. and H. Zhang, Cadmium toxicity, *Plant Signaling & Behavior*, 2014, **7**(3), 345-348.
100. Van Kerkhove, E., V. Pennemans, and Q. Swennen, Cadmium and transport of ions and substances across cell membranes and epithelia, *Biometals*, 2010, **23**(5), 823-55.
101. Schauder, A., A. Avital, and Z. Malik, Regulation and gene expression of heme synthesis under heavy metal exposure--review, *J. Environ Pathol Toxicol Oncol*, 2010, **29**(2), 137-58.
102. Lasat, M.M., Phytoextraction of toxic metals: a review of biological mechanisms. *J. Environ Qual*., 2002, **31**(1), 109-20.
103. Gaur, A. and A. Adholeya, Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. *Current Science*, 2004, **86**(4), 7.
104. Cho-Ruk, K., et al., Perennial Plants in the Phytoremediation of Lead-contaminated Soils, *Biotechnology(Faisalabad),* 2006, **5**(1), 1-4.

List of publication out of the M.Phil work

1. Rausan Zamir, **Anowar Hosen**, M. Obayed Ullah, Nilufar Nahar,. Microbial and Heavy Metal Contamination of Antidiabatic Herbal Preparations Formulated in Bangladesh., Evidance-Based Complementary and Alternative Medicine, Volume 2015, p. **9**