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Chemical and Biological Studies on Antidiabetic Single Plants and Composite Herbal Preparations

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University of Rajshahi

CHEMICAL AND BIOLOGICAL STUDIES ON ANTIDIABETIC SINGLE PLANTS AND COMPOSITE HERBAL PREPARATIONS

THESIS SUBMITTED FOR THE FULFILLMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY

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Certification

The whole work has been submitted by Md Ranzu Ahmed as a thesis entitled "**Chemical and Biological Studies on Antidiabetic Single Plants and Composite Herbal Preparations"** to the Department of Applied Chemistry & Chemical Engineering, University of Rajshahi for the degree of Doctor of Philosophy (Ph.D.) under our direct supervision. This study has been carried out in the Department of Chemistry, Bangladesh University of Health Sciences (BUHS), Dhaka, Department of Chemistry, University of Dhaka, Dhaka Laboratory, BCSIR, Dhaka, Dept of Pharmacology, BIRDEM, Dhaka and Department of Applied Chemistry & Chemical Engineering, University of Rajshahi. A part of the work was carried out in the Laboratory of Natural Products, Institute of Bioscience, University Putra Malaysia, Kula Lampur, Malaysia. No part of the work referred to in the thesis has been submitted in supporting of an application for another degree or qualification of this or any other university or other institutes of learning.

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I, Md Ranzu Ahmed declare that this thesis and work entitled as "Chemical and Biological Studies on Antidiabetic Single Plants and Composite Herbal Preparations" presented in it are my own and have been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly while in candidature for the Doctor of Philosophy (Ph.D.) degree at this University;

2. Where I have consulted the published work of others, this is always clearly attributed;

3. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

4. I have acknowledged all main sources of help;

5. Where this thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

6. Any part of this thesis has not been submitted for a degree or any other qualification at this University or any other institution previously.

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ABBREVIATIONS OR ACRONYMS

ABTS	[2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)]
ADHP	Antidiabetic Herbal Preparation
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
ALP	Alkaline Phosphates
BC	Before Christ
BUHS	Bangladesh University of Health Sciences
b.p.	Boiling point
b.w.	Body Weight
CAM	Complementary and Alternative Medicine
CC	Column Chromatography
CFU	Colony-forming unit
COSY	^{1}H-^{1}H Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
DM	Diabetes Mellitus
DNPH	Dinitrophenylhydrazine
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediamine tetra acetic acid
eg	Exempli gratia (for example)
et al.	et alliori (All others)
FAB MS	Fast Atom Bombardment Mass Spectrum
FTIR	Fourier Transformed Infrared spectroscopy
GHz	Gigahertz
GI	Glycemic Index
HB	H-bonding interaction
Hb	Hemoglobin
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HM	Herbal Medicine
HMBC	Heteronuclear Multiple Bond Connectivity

HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Pressure Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
Hz	Hertz
IDDM	Insulin dependent diabetes mellitus
IC	Inhibitory Concentration
i.p.	Intra-peritoneal
ID	Identity
IR	Infrared
IU	International Unit
kcal	Kilocalorie
kg	Kilogram
Ki	Inhibition Constant
LC	Liquid chromatography
LD_{50}	50% Lethal Dose
LDL	Low Density Lipoprotein
m.p.	Melting point
m/z	Mass to charge ratio
MHz	Mega Hertz
MIC	Minimum Inhibitory Concentration
MS	Mass Spectroscopy
MW	Molecular weight
n-BuOH	n-Butanol
NIDDM	Non-Insulin dependent diabetes mellitus
ng	nanogram
nm	Nanometer
nM	Nanomolar
NMR	Nuclear magnetic resonance
ns	Statistically non-significant values
OGTT	Oral Glucose Tolerance Test
PCV	Packed cell volume
PDB	Protein data bank

PG	Propylene Glycol
PNPG	p-nitrophenyl-α-D-glucopyranose
PTLC	Preparative Thin Layer Chromatography
RBC	Red Blood Cells
R_f	Retention factor / Retardation factor
rpm	Revolutions per minute
SD	Standard Deviation
SEM	Standard Error Mean
SGOT	Serum Glutamate Oxaloacetate Transaminase
SGPT	Serum Glutamate Pyruvate Transaminase
STZ	Streptozotocin
TLC	Thin Layer Chromatography
TM	Traditional Medicine
T2DM	Type 2 Diabetic Model Rats
UV	Ultra violet
w/v	Weight/Volume
w/w	Weight/Weight
WHO	World Health Organization
ad libitum	Sufficient quantity as required
λmax	Absorption maxima
μg	Microgram
μL	Microlitre
μM	Micromolar
μmol	Micromole
cm^{-1}	Per centimeter
^{1}H-NMR	Proton Nuclear Magnetic Resonance
^{13}C-NMR	Carbon Nuclear Magnetic Resonance

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SUMMARY

Eleven antidiabetic herbal preparations (ADHPs) were collected from the local herbal drug stores in Dhaka city. Among these ADHPs, four were tablet, three were capsule and rest of the ADHPs were in liquid form. Eleven ADHPs were screened at BIRDEM for their efficacy in reducing blood glucose in type 2 diabetic Long Evans model rats produced at BIRDEM and BUHS. Seven drugs showed positive response in acute tests. These seven drugs were fed to diabetic model rats for 28 days and some of them showed significant hypoglycemic activity. One with the significant activity had only one plant *Salvia haematodes* Lin in its composition. This plant (roots) was taken for chemical studies.

The flowers of the plant *Phlogacanthus thyrsiflorus* Nees is used for controlling blood glucose in South Eastern Indian people. The flowers were used to study the antidiabetic effect on Type 2 diabetic rats.

The powdered root (5 kg) of *Salvia haematodes* Linn was extracted with n-hexane, DCM, EtOAc and methanol at room temperature, respectively. Dried and powdered flowers (300 g) of *P thyrsiflorus* was extracted with DCM, EtOAc and methanol at room temperature, respectively. This flower also was extracted with aq 80% ethanol to get 144 g extract. The ethanol extract (144 g) was suspended in water and was partitioned with DCM followed by 1-butanol. The DCM and 1-butanol soluble part was 49.87 g and 54 g, respectively.

The extracts of ADHPs and *Pthyrsiflorus* flowers were directly tested for their antidiabetic effects, alpha-glucosidase inhibitor activity, antioxidant activity, SPF testing, microbial contamination, insulin secratory activity, isolation of active compound (s), toxic metal analysis, OCPs testing, identification & quantification of synthetic drug and antioxidant.

ADHP-2, ADHP-4 and ADHP-6 showed significant results of lowering the blood glucose level and lipid profile. The body weight of the treated T2DM rats were unchanged during the experimentation. Ethanol extract of flowers of *P thyrsiflorus* Nees possesses

hypoglycemic and hypolipidemic properties, which reduces the oxidative changes induced by STZ administration.

The results of the current study revealed that roots and flower of *S haematodes* & *P thyrsiflorus,* respectively, exhibited wide range of α-glucosidase inhibitory activities. For the root of *S haematodes*, n-haxane part of MeOH extract showed more significant (IC_{50} = 0.5226 µg/mL) activity which was better than positive control quercetin (IC_{50} = 1.544 µg/mL). Another fraction, root's of *S haematodes*, EtOAc (EA) part of MeOH extract showed significant (IC_{50} = 1.318 µg/mL value) activity compare to the positive control quercetin (IC_{50} = 1.544 µg/mL). n-Hexane part of EtOAc extract of the same plant also showed significant (IC_{50} = 1.184 µg/mL) activity compared to the positive control quercetin (IC_{50} = 1.544 µg/mL). From the flower of *P thyrsiflorus*, EtOAc (EA) part showed significant activity (IC_{50} = 1.539 µg/mL) which is very similar to the positive control quercetin (IC_{50} = 1.544 µg/mL).

Methanol extracts of ADHP-1, ADHP-7, ADSP-8 and ADSP-9 exihibited 94.8 ± 0.0312%, 91.88 ± 0.0534%, 94.76 ± 0.0.0045% and 94.37 ± 0.0466%, respectively, at concentration 400 µg/ml when compared with standard sample (ascorbic acid, 97.15 ± 0.0502%). The IC_{50} value of the methanol extracts of ADHP-1 (17±0.0243 µg /ml) was significant when compared to that of the standard Ascorbic acid, 2 ± 0.0156, respectively.

Methanol extracts of ADHP-1, ADHP-3, ADHP-6, ADHP-7, ADSP-8 and ADSP-9 was found to 98.38 ± 0.0156%, 95.54 ± 0.0321%, 94.34 ± 0.0371%, 95.90 ± 0.0551 % , 96.03 ± 0.0151% and 96.03 ± 0.0212% free radical scavenging activity, respectively at concentration 400 µg/ml when control sample (ascorbic acid) exhibited 99.90 ± 0.0145%. IC_{50} values for the methanol extract of ADHP-1 (21±0.0251 µg /ml) was significant when compared with control sample (ascorbic acid) exhibited IC_{50} at 10 ± 0.0156 µg/ml.

Methanol extracts of different ADHP's and ADSP's sample exhibited good activity for reducing power assay in the present study. Where standard ascorbic acid gives absorbance 1.250 ± 0.0075 at 700 nm. The present study reveals, ADHP-6, ADSP-8 and ADSP-9 extracts exhibited 715.54±0.0485, 830.48±0.0365 & 850.74±0.0854 absorbances, respectively for total antioxidant activity.

The plant extract which possess potential antioxidant activity and UV absorption capacity can prevent photo-aging and skin cancer (Ganesan *et. al.,* 2013). The present study showed that the *S haematodes* Linn has significant UV absorption capacity, especially the chloroform and ethyl acetate fractions with the SPF value 10.18 and 9.63, respectively. On the other hand, another plant's flower *(P thyrsiflorus* Nees)* showed the SPF value 5.82 for methanol extract.

Out of 07 antidiabetic solid and liquid samples, except ADHP-3, *Bacillus subtilis* (3.5 – 4.0 x 10^4 cfu/g) was identified from solid ADHPs and *Enterococcus spp.* (1.0 x10^4 cfu/ml) was identified from liquid ADHP, but all samples were free from fungi (yeasts and moulds). *Bacillus subtillis* has been identified 3.5 x 10^4 cfu/g, 3.7 x 10^4 cfu/g, 3.2 x 10^4 cfu/g, 3.8 x 10^4 cfu/g, 4.0 x 10^4 cfu/g in the ADHP-1, ADHP-4, ADHP-5, ADHP-6 and ADHP-7, respectively. *Enterococcus spp.* (1.0 x10^4 cfu/ml) was identified from liquid ADHP-2. ADHP-3 was free from all kinds of microbial contaminations.

Compounds **1** and **4** of *P thyrsiflorus* showed significant effect on glucose-stimulated insulin secretion from MIN6 cells.

Using repeated column and high performance liquid chromatography (HPLC) eight compounds were isolated from the dichloromethane, chloroform and 1-butanol soluble part of methanol extracts of flower and root of the plants. The structures of the isolated compounds were elucidated by extensive spectroscopic studies. Three compounds β-sitosterol (**1**), stigmasterol (**2**) and 7-hydroxy coumarin (**3**) were isolated from the DCM and Chloroform part of roots of *S haematodes*. Morever, five compounds β- sitosterol (**4**), stigmasterol (**5**), 8(17),13-labdadien-15,16-olide-19-oic acid (**6**), 19-hydroxy-8(17),13-labdadien-15,16-olide (**7**) and 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone (luteolin) (**8**) were isolated from the dichloromethane extract and 1-butanol part of methanol extract of flowers, respectively. Among these eight compounds, compound (**4**) and (**7**) showed significant effect on glucose-stimulated insulin secretion from MIN6 cells.

Toxic metal content particularly, Nickel (Ni), Copper (Cu), Manganese (Mn), Chromium (Cr), Cadmium (Cd), Lead (Pb), and Arsenic (As) were tested in the thirteen ADHPs. The amount of nickel was below detection level in all the preparations when the detection

limit was 0.02 ppm. The copper concentrations varied in a wide range in all ADHPs between 0.48 to 39.58 ppm except ADHP 5 & 6. Cadmium content was detected some of the ADHP samples except ADHP-2, 4, 5, 6 & 7. Unfortunately, ADHP-3, 8, 9 & 10 samples the cadmium level were more than the level of all permissible limit (WHO, US FDA, HAS Singapore and Chinese Pharmacopoeia, Tables 3.3.1 and 3.3.2). Even if we consider a more relax permissible limit for cadmium (WHO and US FDA guidelines; Table 3.3.2), one-third of the total ADHPs (ADHP-3, 8, 9 & 10) failed to comply with the safety limit. The content of lead (Pb) in the analyzed samples ranged from less than 1.0 to 18.44 ppm. All the ADHP's samples showed the value of Pb metal below detection level (DL=0.00005 ppm) except ADHP-3 and 8. The maximum concentrations of lead in ADHP- 3 and ADHP-8 were 0.08 ppm and 18.44 ppm, respectively.

Arsenic (As) content in the analyzed samples ranged from 0.05 to 0.75 ppm. All the ADHP's samples showed the value of As metal with the highest value in ADHP-7 (0.75 ppm) & ADHP-8 (0.74 ppm) and lowest value in ADHP-6 (0.05 ppm) as shown in Table 3.8.1. The US FDA, WHO, HSA Singapore & Chinese Pharmacopiea maximum permissible limit of As in consumed medicinal herbs is 10 ppm, 10 ppm, 5 ppm and 2 ppm respectively (Table: 3.8.2). The obtained results showed that all the analyzed ADHP samples the As contents were below the permissible limits.

Four organochlorine pesticide's residue analysis of ADHPs standards namely 4,4-DDE; 4,4-DDD; 2,4-DDT & 4,4-DDT were used to quantify the pesticides present in 07 Antidiabetic Herbal Preparations (ADHPs). 4,4-DDE was found in ADHP-1, ADHP-2, ADHP-6 & ADHP-7 amounting to 0.203 ng/g, 0.588 ng/g, 1.413 ng/g & 1.838 ng/g, respectively. 4,4-DDD amounting to 0.373 ng/g was found only in ADHP-2. 2,4-DDT was found in ADHP-2 and ADHP- 6 amounting to 1.937 ng/g and 0.756 ng/g, respectively. And 4,4-DDT was found to be present in ADHP-1, ADHP-2, ADHP-6 and ADHP-7 amounting to 0.971 ng/g, 8.608 ng/g, 3.377 ng/g and 2.917 ng/g, respectively. ADHP-03, 04, 05 were free from organochlorine pesticide residues.

Small amount of metformin HCl was found in all samples of ADHP. Metformin HCl was quantified 2.25%, 2.62%, 1.28%, 1.58%, 1.47%, 1.35% & 2.47% in ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6 and ADHP-7, respectively. Large amounts of glaclazide (5.74%)

and glibenclamide (3.82%) were quantified and isolated from ADHP-6 and ADHP-7, respectively, indicating deliberate addition.

A new HPLC method to analyze seventeen antioxidant compounds simultaneously was developed and validated for, linearity, accuracy, stability and precision. All of the ADHPs and ADSPs cotainined good amount of different antioxidants. Especially ADHP-1, ADHP-2, ADHP-4, ADHP-5, ADHP-6, ADHP-7, and ADSP-8, 9 showed significat amount of antioxidants which is known to play benifical role for humans (Table-3.11.11).

1. INTRODUCTION

1.0 INTRODUCTION

1.1 GENERAL INTRODUCTION (HERBAL MEDICINE AND PLANT)

Herbal Medicine (HM) plays a very important role in primary health care of millions of people around the globe. It is almost indispensable in poorer third world countries where accessibility and affordability of modern medicine is limited. Interestingly, HM is increasingly becoming more popular in wealthier developed nations in the recent past.

The World Health Organization (WHO) survey indicated that about 70-80% of the world population particularly in the developing countries rely on non-conventional medicines mainly of herbal origins for their primary health care. This is because herbal medicines are relatively accessible and cheaper than the synthetic drugs (Abba *et. al*., 2009). In line with the increasing importance of traditional medicine in various healthcare systems around the world, the WHO Traditional Medicine Strategy has recently been updated. 'The goals of the strategy for the next decade (2014–2023) are to support Member States in (a) harnessing the potential contribution of traditional medicine to health, wellness and people-centered health-care; and (b) promoting the safe and effective use of traditional medicine by regulating, researching and integrating traditional medicine products, practitioners and practice into health systems where appropriate' (WHO, 2013).

Throughout the ages, human race have fulfilled its basic needs from nature, such as foodstuffs, shelters, clothing, fertilizers, flavors and fragrances. Progressively they started to use parts of plants (leaves, stems, roots, rhizomes, etc.), and some animal parts, along with minerals, for the treatment of diseases. History of medicine thus started with the genesis of human civilization. The modern medicine or allopathy is a result of continuous scientific and observational development of traditional medicines and practices.

Traditional medicine systems which have been in use for years are mainly depending on plants. The first record, which was written at about 2,600 BC on clay tablets in cuneiform, is from Mesopotamia. Oils of *Cedrus species* (cedar) and *Cupressus sempevirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora species* (myrth), and *Papaver somniferum* (poppy juice) are some examples amongst approximately 1,000

plant-derived substances which people have used in ancient times and are still in use for the treatment of diseases such as cough and cold, parasitic infections and inflammation (Newman *et. al.,* 2000). Nearly all ancient cultures have used plants as a source of medicine. Egyptian pharmaceutical record Ebers Papyrus at 1,500 BC includes 700 drugs, mostly plants, though animal organs and some minerals were also used (Newman *et. al.,* 2000).

Ayurveda is an ancient system of medicine which is still widely practiced in South Asia and has a sound philosophical and experimental basis. Atharvaveda (around 1,200 BC), Charak Samhita and Sushrut Samhita (1,000 - 500 BC) are the classic texts that give detailed descriptions of over 700 herbs used in Ayurveda (Patwardhan *et. al.,* 2004). The Chinese Materia Medica, which was first compiled in 1,100 BC has been extensively revised over the centuries. This was followed by recorded history such as the Shennong Herbal (~100 BC; 365 drugs) and the Tang Herbal (659 AD; 850 drugs) (Newman *et. al.,* 2000).

The Greeks have contributed significantly in the development of the herbal drugs in prehistoric western world. Theophrastus (A Philosopher and Natural Scientist) reported the medicinal properties of many plants and changes in their characteristics through cultivation, in his book 'History of Plants,' (300 BC). Dioscorides, a Greek Physician (100 AD), reported the method of collection, storage, and use of many medicinal herbs. Galen (130-200 AD), who was a teacher of pharmacy and medicine in Rome, published nearly 30 books on prescription and formulae of herbal drugs (Newman *et. al.,* 2000).

In many developing countries, people are still depending on traditional medicines for their healthcare. In developed countries too, people are now turning to herbal remedies for multiple reasons. Also many modern medicines contain substances of plant origin. The World Health Organization (WHO) estimates that about 80% inhabitants of developing countries rely almost entirely on traditional medicines for their primary healthcare needs. Over 3 billion people in the developing countries utilize plant-based medicines on a regular basis. Natural pharmaceuticals (Naturaceuticals), nutraceuticals and cosmeceuticals have tremendous significance in global healthcare regime. Plants possess an immense chemical diversity which can be used in different types of drug

discovery, such as antimicrobial, cardiovascular, immunosuppressive and anticancer drugs. According to the WHO survey, the international market of herbal products or botanicals is estimated to be US$ 62 billion per annum. It is estimated to grow to US$ 5 trillion by the year 2050. (Govil, *et. al.,* 2006). The nutraceutical marketplace in Europe is estimated to be US$ 9 billion, while in US marketplace it is about US$ 10-12 billion. It is expanding at a rate of more than 20% per year. (Patwardhan *et. al.,* 2004 and Govil *et. al.,* 2006)

Natural products chemistry actually began with the isolation of morphine from opium (*Papaver somniferum*) by Serturner. This was followed by the isolation of quinine from Cinchona tree in 1860. A German chemist Carl Koler isolated cocaine from *Erythroxylon coca*, the chemical responsible for paralyzing the nerve endings responsible for transmitting pain. Reserpine, an anti-hypertensive alkaloid was obtained from *Rauwolfia serpentine*. (Patwardhan *et. al.,* 2004).

Secondary metabolites which are produced by living organisms (microbes and plants) for the purpose of their own survival have a major importance in drug discovery. Secondary metabolites with therapeutic indications include cyclosporine (immunosuppression), mevinolin (hypercholesterolaemia), avermectin (parasitic disease), artemisinin (malaria), vinblastine, vincristine and taxol (cancer) (Adhikari 2014).

Today, natural product-based drug discovery competes with the combinatorial chemistry, which has given a significant complementary way for drug development. It produced thousands of compounds of almost similar structures in a single experiment, which are immediately available for bio-assay screening. In contrast, plants and microorganisms have the potential to synthesize novel chemicals in unpredictable ways. The genes and metabolic pathways by which plants and microbes produce novel structures are now understood in many cases and this knowledge has been used in drug discovery. Plants and microbes used same technique as in combinatorial chemistry to biosynthesize secondary metabolites. Natural product biosynthesis is mostly catalyzed by enzymes, which result from an enormous gene pool that has evolved over billions of years. (Nisbet & Moore 1997)

Recent improvements in instrumentation, robotics, and bioassay technology have increased the speed of bioassay-guided isolation and structure elucidation of natural products significantly. These improvements have allowed natural product research to be more competitive and cost effective, as compared to synthetic medicinal chemistry.

Between 2005 & 2008, thirteen natural product (NP)-derived drugs have been lunched in the market (Table-1.1), in addition to the 37 late stage clinical development NP-based candidates (6 in registration and 31 in phase III). The traditional strengths of NPs in oncological and transmittable diseases is still explored with 19 (51%) compounds being evaluated for the treatment of cancer and 10 (27%) for the treatment of bacterial infections. The remaining compounds are for the treatment of metabolic diseases, pain and multiple sclerosis. A large number of NP-derived compounds are in various stages of clinical development which indicates that natural products are still the major feasible source of new drug candidates. In absence of NP-derived compounds, it is predicted that there would be a considerable therapeutic shortage in a number of important areas, such as neurodegenerative and cardiovascular diseases, most solid tumors and immune-inflammatory diseases (Butler 2008).

A. BIOLOGICAL INVESTIGATIONS

1.2 DIABETES MELLITUS (DM)

1.2.1 General Information

Diabetes Mellitus (DM) is a principal cause of morbidity and mortality in human populations (Steppan *et. al.,* 2001). It is a syndrome characterized by hyperglycemia, polydipsia and polyuria and causes complications to the eyes, kidneys, and nerves. It is also associated with an increased incidence of cardiovascular disease (Pickup & Williams, 1991). The number of people with diabetes is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity. The prevalence of diabetes mellitus, estimated as 382 million people in 2013, is predicted to increase to 592 million people by the year 2035 (IDF, Atlas 6th ed, 2013). In addition, to a possible genetic predisposition, other environmental factors, have been identified as

contributing to the DM epidemic in Asia. These includes rapid socioeconomic change, urbanization, sedentary lifestyle, and changes in dietary patterns. Recent epidemiological studies have shown an increased prevalence of DM in India (12.1%), Pakistan (11.1%), and China (6.1%) (Ramachandran *et. al*., 2001, Shera *et. al*., 1999 and Dong *et. al.,* 2005). In recent years, Bangladesh has experienced rapid urbanization (Laskar *et al*., 1996 and Bangladesh Bureau of Statistics, 2004 & 2006) increasing the risk of increased number of diabetic population.

Table. 1.1:   NP-derived drugs launched between 2005 & 2008. (Butler, 2008).

Year	Trade name	Lead compound	Classification	Disease area
2005	Dronabinol/Cannabidol (Sativex®)	Dronabinol/ Cannabidol	NPs	pain
2005	Fumagilin (Flisint®)	fumagillin	NP	antiparasitic
2005	Doripenem (Finibax®/ DoribaxTM)	thienamycin	NP-derived	antibacterial
2005	Tigecycline (Tygacil®)	tetracycline	Semi-synthetic NP	antibacterial
2005	Ziconotide (Prialt®)	ziconotide	NP	pain
2005	Zotarolimus (Endeavor	sirolimus	Semi-synthetic NP	Cardiovascular
2006	Anidulafungin (Eraxis TM / Ecalta TM)	Echinocandin B	Semi-synthetic NP	antifungal
2006	Exenatide (Byetta TM)	Exenatide-4	NP	diabetes
2007	Lisdexamfetamine (Vyuanse TM)	amphetamine	NP-derived	ADHD
2007	Retapamulin (Altabax TM/Altargo TM)	pleuromutilin	Semi-synthetic NP	antibacterial
2007	Temsirolimus (Torisel	sirolimus	Semi-synthetic NP	oncology
2007	Trabectedin (Yondelis	trabectedin	NP	oncology
2007	Ixabepilone (Ixempra	Epothilone B	Semi-synthetic NP	oncology

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is due to deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia (ADA, 2009).

Diabetes is the common term for several metabolic disorders in which the body no longer produces insulin or ineffectively uses the insulin it produces. It is a common condition and is characterised by abnormally high blood sugar levels. Diabetes is known as *"diabetes mellitus"* - where diabetes comes from the Greek word for siphon, which describes the excessive thirst and urination of this condition, and mellitus is the Latin word for honey. Diabetes mellitus (DM) is one of the most prevalent and devastating chronic non-communicable disease having serious health, economic and social consequences (IDF, 2011). It is a lifelong disease for which there is not yet a cure. It is one of the commonest endocrine and metabolic disorders affecting mankind all over the world. People of the developing countries are the worst victims of such life-long diseases because of the lack of organized health care delivery. An increasing worldwide prevalence of diabetes has been acknowledged by several authorities and today the situation in several areas of the Third World is considered an epidemic (Zimmet *et. al.,* 1996). According to recent estimates of the International Diabetes Federation (IDF), not only will the largest increases in prevalence of diabetes occur in developing regions of the world, but it will also affect younger age groups (Mohan *et. al.,* 2010).

Diabetes has been known to mankind for several thousands of years. The history of it is supposedly started in approximately 1550 BC. The Egyptian papyrus of Ebers described the appearance of diabetes in succeeding generations and mentioned the dietary use to remedy of this disease (Copenhagen *et. al.,* 1937, Williams and Pickup, 2004). This is thought to be the first reference to the disease. The sweet, honey-like taste of urine in polyuric states, which attracted ants and other insects, was reported by Hindu physicians

such as Sushrut (Susruta) during the 5th and 6th century AD. The word 'diabetes' was first used by the Greek Physician Aretaeus of Cappadocia in the 2nd century AD , which was derived from the Greek work '*diabainein*' meaning 'to run through a siphon.' and the Latin word mellitus (honey) was applied much later (Ackernecht, 1955 and Williams & Pickup, 2004).

Diabetes mellitus is defined as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. A number of pathogenic processes are involved in the development of diabetes including autoimmune destruction of β-cells. Diabetes is known as a disease (serious complex condition) which can affect the entire body. It is described as a 'chronic' condition, meaning that it lasts a long time, often for someone's whole life. Diabetes requires daily self care and if complications develop, quality of life is reduced. (Diabetes Australia, 2015, https://www.diabetesaustralia.com.au/what-is-diabetes).

1.2.2 Epidemiology of Diabetes:

The incidence of diabetes is increasing in an alarming rate. International Diabetes Federation (IDF) estimated that in 2010 the five countries with the largest numbers of people (in million) with diabetes are India (50.8), China (43.2), the United States (26.8), Russia (9.6) and Brazil (7.6). It also reported that five countries with the highest diabetes prevalence in adult population are Pacific island nation of Nauru (30.9%), the United Arab Emirates (18.7%), Saudi Arabia (16.8%), Mauritius (16.2%) and Bahrain (15.4%) (Shaw *et.al,* 2001). According to IDF, latest diabetes figures paint grim global picture (IDF, 2009). The average rate was found to be 14.8 in a population aged over 20 years. (Abdella *et al.,* 1998).

Proportional and absolute increase will occur in developing countries, where the prevalence will rise from 4.2 to 5.6% (National Service Framework for Diabetes, 2011). India and China accounts for the largest number of people suffering from diabetes mellitus.
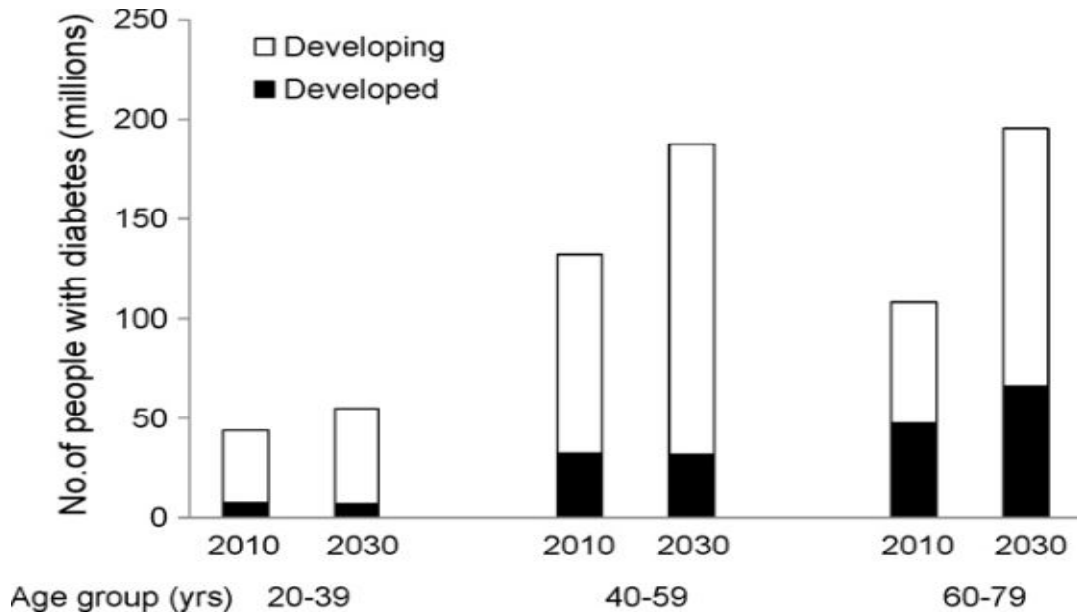
Figure-1.1: Numbers of adults with diabetes in developed and developing countries in 2010 and 2030 (projected), according to age group.

Type 2 diabetes mellitus is less common in non-Western countries where typical diets contain fewer calories and daily caloric expenditure is higher. However, as people in these countries adopt Western lifestyles, weight gain and type 2 diabetes mellitus are becoming virtually epidemic. Rates of diabetes are increasing worldwide. The top 10 countries in number of people with diabetes are currently India, China, the United States, Indonesia, Japan, Pakistan, Russia, Brazil, Italy, and Bangladesh. The greatest percentage increase in rates of diabetes will occur in Africa over the next 20 years. The prevalence of type 2 diabetes mellitus varies widely among various racial and ethnic groups. Type 2 diabetes mellitus is more prevalent among Hispanics, Native Americans, African Americans, and Asians/Pacific Islanders than in caucasians. Indeed, the disease is becoming virtually pandemic in some groups of Native Americans and Hispanic people. The risk of retinopathy and nephropathy appears to be greater in blacks, Native Americans, and Hispanics. It was narrated in the news letter (2012) for preventive health screening for diabetes that the greatest percentage of diabetes will occur in Africa over the next 20 years. Unfortunately, at least 80% of people in Africa with diabetes are undiagnosed and many in their 30s to 60s will die from diabetes there. (Newsletter, 2012, http://www.yourdiagnosis.com/yourdiagnosis/newsletter/diabetesscreening.htm).

With the prevalence of T2DM rising alarmingly throughout the world, prevention of these diseases is becoming increasingly important. The management of T2DM has often been unsatisfactory; partly because of the limited efficacy of the oral hypoglycemic agents, is the most common cause of death in diabetics. The prevention of T2DM and control of its complications will require an integrated, international approach for significant reduction in the huge premature morbidity and mortality they cause (Paul *et. al.,* 2001). An ideal oral treatment would be a drug that not only controls the glycemic level but also prevents the development of oxidative stresses and other complications of diabetes. Unfortunately, among the currently available drugs, the choice is very limited. Morever, besides having a number of side effects, none of the antihyperglycemic agents have been successful in maintaining hyperglycemia and controlling long term micro and macrovascular complications of DM. As a result a growing need for new antihyperglycemic drugs as an alternative therapy exists in type-2 diabetes mellitus (T2DM).

In contrary to the conventional drugs, plants are well known in traditional herbal medicine for their various pharmacological activities. Dependence on herbs as medicines in the treatment of disease is common among a large proportion of population of the rural populace because of its availability and affordability. Due to the increasing awareness of the importance of traditional medicine in human and animal healthcare, researches into the efficacy of some of the herbs used in the treatment of some illness would be worthwhile. WHO supports the use of effective and safe remedies and accepts traditional medicine as a valuable resource for primary healthcare.

There has been increasing demand for the use of plant products with antidiabetic activities due to low cost, easy availability and lesser side effects (Vivek *et. al.,* 2010). The use of traditional plants treatment for DM dates from Papyrus Ebers of 1500 BC (Bailey *et. al.,* 1989). Many herbs, spices and other plant materials had since been described for the treatment of diabetes, but their use declined in occidental societies following the introduction of insulin and the antihyperglycemic drugs. Herbal medicines are currently enjoying a revival in popularity in west and are of course the primary form of medicine, required evolution of standard and specific methods to be utilized to their

full effects. During the last few decades a resurgence of interest has been noticed in plants as a source of medicines and as novel lead molecules.

"Health for all" is a dream and a goal which humanity at large shares and strives for. Unfortunately, it has now been proven without doubt that modern pharmaceuticals are and will remain out of reach for a large proportion of the human population for the foreseeable future. This has created an appreciation and a need for the use of other sources of human knowledge to provide common health benefits. Alternative and traditional medicines, largely herbal in nature, are now regarded as important but underutilized tools against disease. The World Health Organization (WHO) recognized this fact in the early 1970s and encouraged governments to effectively utilize local knowledge of herbal medicines for disease prevention and health promotion (Mosihuzzaman *et. al*., 2008).

1.2.3 Classification of Diabetes:

According to WHO, Diabetes Mellitus is classified as Type 1, Type 2, and other specific types (Hussain *et. al.* 2005).

Etiologic classification of diabetes mellitus

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)

> A. Immune mediated

> B. Idiopathic

II. Type 2 Diabetes may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance.

III. Gestational diabetes mellitus (GDM)

Gestational diabetes mellitus (GDM) (or, gestational diabetes) is a condition in which women without previously diagnosed diabetes exhibit high blood glucose (blood sugar) levels during pregnancy (especially during their third trimester). Gestational diabetes is caused when insulin receptors do not function properly. This is likely due to pregnancy-related factors such as the presence of human placental lactogen that interferes with susceptible insulin receptors. This in turn causes inappropriately elevated blood sugar

levels. Gestational diabetes is formally defined as "any degree of glucose intolerance with onset or first recognition during pregnancy". (Metzger & Coustan, 1998).

Gestational diabetes generally has few symptoms and it is most commonly diagnosed by screening during pregnancy. Diagnostic tests detect inappropriately high levels of glucose in blood samples. Gestational diabetes affects 3-10% of pregnancies, depending on the population studied (Moore *et al*, 2005).

IV. Other specific types

Some other uncommon specific syndromes which may be classified as diabetes. Example: Genetic defects of β-cell function, Genetic defects in insulin action, Diseases of the exocrine pancreas, Endocrinopathies, Drug- or chemical-induced and Infections.

1.2.4 Etiopathogenesis of Diabetes Mellitus (DM)

1.2.4.1 Type 1 Diabetes

Although the precise cause of type 1 diabetes is unknown, it is believed to be caused by one or more of the following: genetic susceptibility, a diabetogenic trigger and/or exposure to a driving antigen (WHO, 1999) .The main causes of type 1 diabetes mellitus are:

- Genetic
- Environment
- Drugs

1.2.4.2 Type 2 Diabetes

Type 2 diabetes is due primarily to lifestyle factors and genetics (Knip *et al.,* 2005). A number of lifestyle factors are known to be important to the development of type 2 diabetes, including: obesity (defined by a body mass index of greater than thirty), lack of physical activity, poor diet, stress, and urbanization (Riserus *et al.,* 2009). Excess body fat is associated with 30% of cases in those of Chinese and Japanese descent, 60-80% of cases in those of European and African descent, and 100% of Pima Indians and Pacific

Islanders. Those who are not obese often have a high waist–hip ratio (Williams's textbook of endocrinology, 12th ed, 2012) with a high risk of diabetes.

1.2.5 Pathophysiology of type 2 Diabetes Mellitus

Type 2 diabetes is a more complex condition than type 1 diabetes because there is a combination of resistance to the actions of insulin in liver and muscle together with impaired pancreatic β-cell function leading to 'relative' insulin deficiency. Insulin resistance appears to come first, and leads to elevated insulin secretion in order to maintain normal blood glucose levels. However, in susceptible individuals the pancreatic β cells are unable to sustain the increased demand for insulin and a slowly progressive insulin deficiency develops (Schofield & Sutherland, 2012).
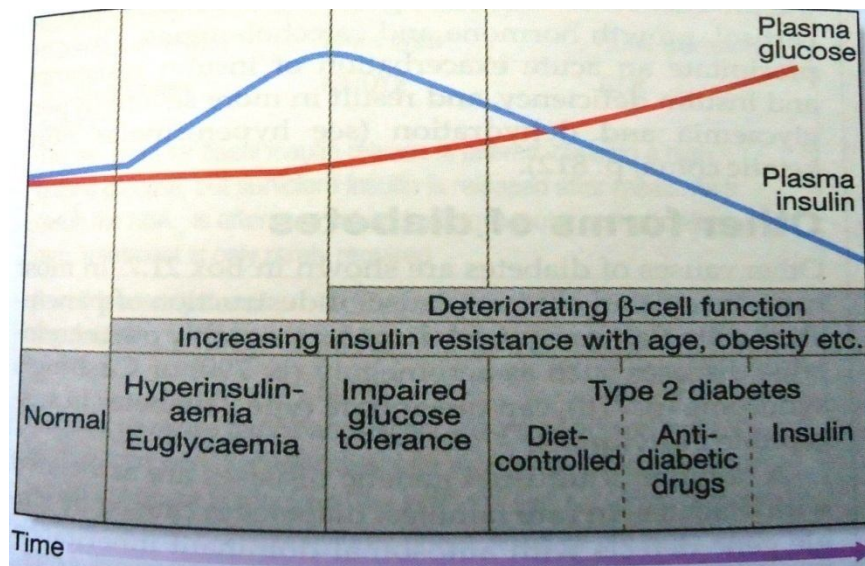
Figure-1.2: Natural history of type 2 diabetes.

In the early stage of the disorder the response to progressive insulin resistance is an increase in insulin secretion by the pancreatic cells, causing hyperinsulinaemia. Eventually the β cells are unable to compensate adequately and blood glucose rises, producing hyperglycaemia. With further β–cell failure (type 2 diabetes) glycaemic control deteriorates and treatment requirements escalate.

1.2.6 Signs and Symptoms of Diabetes Mellitus

The classic symptoms of untreated diabetes are loss of weight, polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger) (Lambert &

Bingley, 2002). Symptoms may develop rapidly (weeks or months) in type 1 diabetes, while they usually develop much more slowly and may be subtle or absent in type 2 diabetes.

Prolonged high blood glucose can cause glucose absorption in the lens of the eye, which leads to changes in its shape, resulting in vision changes. Blurred vision is a common complaint leading to a diabetes diagnosis. A number of skin rashes that can occur in diabetes are collectively known as diabetic dermadromes.
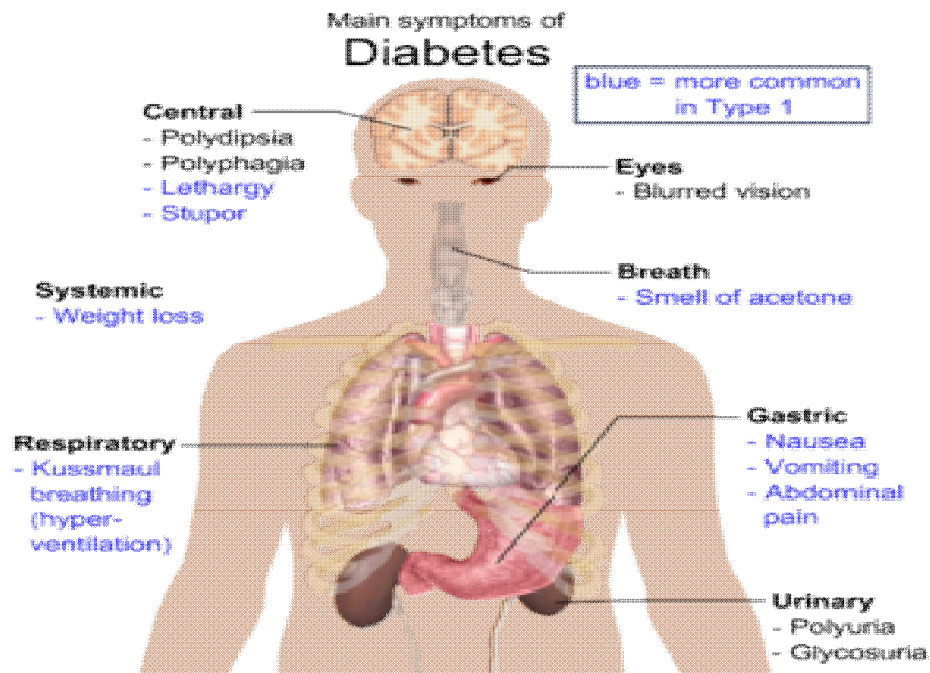
Figure-1.3: Overview of the most significant symptoms of diabetes (Lambert & Bingley, 2002)

1.2.7 Complication of Type 2 Diabetes

All forms of diabetes increase the risk of long-term complications. These typically develop after many years (10–20), but may be the first symptom in those who have otherwise not received a diagnosis before that time. The major long-term complications relate to damage to blood vessels. Diabetes doubles the risk of cardiovascular disease (Cooke & Plotnick, 2008). The main "macrovascular" diseases (related to atherosclerosis of larger arteries) are ischemic heart disease (angina and myocardial infarction), stroke and peripheral vascular disease.

Diabetes also damages the capillaries (causes microangiopathy) (Emerging Risk Factors Collaboration: The Lancet, 2010). Diabetic retinopathy, which affects blood vessel formation in the retina of the eye, can lead to visual symptoms including reduced vision and potentially blindness. Diabetic nephropathy, the impact of diabetes on the kidneys, can lead to scarring changes in the kidney tissue, loss of small or progressively larger amounts of protein in the urine, and eventually chronic kidney disease requiring dialysis.

Another risk is diabetic neuropathy, the impact of diabetes on the nervous system - most commonly causing numbness, tingling and pain in the feet, and also increasing the risk of skin damage due to altered sensation. Together with vascular disease in the legs, neuropathy contributes to the risk of diabetes-related foot.

1.2.8 Management of Diabetes

Diabetes mellitus is a chronic disease, for which there is no known cure except in very specific situations. Management concentrates on keeping blood sugar levels as close to normal ("euglycemia") as possible, without causing hypoglycemia. This can usually be accomplished with diet, exercise, and use of appropriate medications (insulin in the case of type 1 diabetes; oral medications, as well as possibly insulin, in type 2 diabetes).

Patient education, understanding, and participation is vital, since the complications of diabetes are far less common and less severe in people who have well-managed blood sugar levels (Emerging Risk Factors Collaboration: The Lancet, 2010 and Boussageon *et al.* 2011). The goal of treatment is an $HbA1_C$ level of 6.5%, but should not be lower than that, and may be set higher. Attention is also paid to other health problems that may accelerate the deleterious effects of diabetes. These include smoking, elevated cholesterol levels, obesity, high blood pressure, and lack of regular exercise (The effect of intensive Annals of Internal Medicine, 2012). Specialized footwear is widely used to reduce the risk of ulceration, or re-ulceration, in at-risk diabetic feet. Evidence for the efficacy of this remains equivocal, however (NIHCE, Type 2 diabetes, London; 2008).

In 2013, the American Association of Clinical Endocrinologists (AACE) issued a comprehensive new type 2 diabetes treatment algorithm--the first to incorporate obesity,

prediabetes, and cardiovascular risk factor management. (Tucker, 2013 and Garber *et. al.,* 2013).

Obesity management was incorporated into the algorithm because it is now clear that weight loss also reduces blood glucose. The authors suggest that obesity management can be considered first-line treatment for people with prediabetes. The prediabetes section of the algorithm considers cardiovascular risk factors and the options of antihyperglycemic or antiobesity therapy, though without making a recommendation regarding which form of treatment is better.

As in the AACE's (American Association of Clinical Endocrinologists) earlier glycemic-control algorithm, the level of treatment depends on the initial hemoglobin A1c (HbA1c). (Lifestyle modification, including weight loss, is a component of all treatments.) Whereas the earlier algorithm recommended an HbA1c of 6.5% or lower as the goal for most patients, the current algorithm refines this advice, recommending an HbA1c of 6.5% or lower for healthy patients without concurrent illness and at low risk for hypoglycemia but individualized target HbA1c values greater than 6.5% for patients with concurrent illness and those who are at risk for hypoglycemia.

1.2.9 Treatment of Diabetic Mellitus:

1.2.9.1 Metformin

Metformin is the preferred initial agent for monotherapy and is a standard part of combination treatments. Advantages of metformin include the following:

- Efficacy
- Absence of weight gain or hypoglycemia
- Generally low level of side effects
- High level of patient acceptance
- Relatively low cost

The dose of metformin is titrated over 1-2 months to at least 2000 mg daily, administered in divided doses [during or after meals to reduce gastrointestinal (GI) side effects]. Exercise increases metformin levels and interferes with its glucose-lowering effect. (Boule *et. al*., 2011).

Treatment of Type 2 Diabetes Mellitus

	monotherapy*	add	add
obese	metformin	sulfonylurea	exenatide or insulin or glitazone
non-obese	sulfonylurea or metformin	metformin or sulfonylurea	exenatide or insulin or glitazone
elderly	low dose secretagogue	switch to simple insulin regimen	----
Asians	glitazone	metformin	sulfonylurea or insulin or exenatide**

*for symptomatic patients, may initially use secretagogue or insulin to rapidly decrease glucose
**exenatide not approved for use with glitazone

Figure-1.4: Treatment of type 2 diabetes mellitus.

1.2.9.2 Triple-drug therapy

If 2 drugs prove unsuccessful after 2-3 months, the next step is triple therapy. The third drug may be an oral agent from a third class of antidiabetic drugs, basal insulin (typically at bedtime), or the injectable drug exenatide. The expense and adverse effect profile of Thiazolidinediones (TZDs or glitazones) make their use in an oral triple therapy approach less desirable.

The addition of exenatide to 1 or 2 oral agents (eg, metformin and/or a sulfonylurea) is attractive because of its simplicity (ie, only 2 possible doses of exenatide, with easy titration compared with insulin); although expensive, it avoids hypoglycemia. If basal insulin is used, the insulin dose is titrated to the fasting glucose concentration, which the patient can measure at home.

1.2.9.3 Glucose values

For patients trying to achieve near euglycemia, premeal glucose values of 80-120 mg/dL are the goal, with the patient going to sleep at night with a value at least 100 mg/dL. In patients with less stringent glycemic goals (eg, because of advanced age, advanced complications, or severe concomitant disease), preprandial glucose values of 100-140 mg/dL are desired. Because of the limitations of therapies, essentially no patient is able to achieve these goals all the time if, in fact, insulin is needed to treat their disease. For patients who primarily have fasting hyperglycemia, basal insulin is the easiest way to correct this abnormality. Basal insulin is typically scheduled at bedtime but can be given at suppertime if that is more convenient for the patient.

The goal of a combined daytime oral agent plus once-a-day insulin is to lower the fasting glucose level to 100 mg/dL by titrating the insulin. When this target is achieved, the oral agents can be effective in maintaining preprandial and postprandial blood glucose levels throughout the day. If a regimen combining oral agents and insulin fails to lower glucose levels into the normal range, patients should be switched to a daily multiple-injection schedule with a premeal rapid-acting insulin and a longer-acting basal insulin.

1.2.10 Plants Products or Natural Products in the Management of Diabetes Mellitus:

The use of plants in medicine is an age-long practice in various parts of the globe for both preventive and curative purposes. Several warnings have been issued over lack of quality control, scientific evidence for the efficacy, and potential adverse effects of herbal remedies including hepatotoxicity, nephrotoxicity, cardiotoxicity, and reproductive toxicity among others. Despite all of these, reliance on herbs as medicine for the management of diabetes mellitus is still much practiced by a large proportion of the world population because it is readily available and affordable with perceived reduced toxicity. Therefore, with the upsurge of interests in medicinal plants, there is need for thorough scientific investigations of these plants for both efficacy and potential toxicity. (Hindawi, 2015, http://www.hindawi.com/journals/ecam/si/785194/cfp/)

A number of reviews on medicinal plants used in the management of diabetes in different parts of the world (Bailey & Day, 1989, Marles & Farnsworth,1995), as well as those

used specifically in certain regions, such as in West Africa (Bever, 1980), Central America (Andrade-Cetto & Heinrich, 2005) and Asia (Grover *et. al.,* 2002) exist. It also recognizes traditional medicine as 'an accessible, affordable and culturally acceptable form of healthcare trusted by large numbers of people, which stands out as a way of coping with the relentless rise of chronic non-communicable diseases in the midst of soaring health-care costs and nearly universal austerity' (WHO, 2013).

1.3 ALPHA-GLUCOSIDASE INHIBITOR

1.3.1 General Information

Free radicals are the molecular species that possess a single unpaired electron in the outermost atomic orbit. In spite of different types of free radicals, important free radicals in biological system are the reactive oxygen species (ROS) which include hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite (Valko *et. al*., 2007). Human body has antioxidant defense system to cope with the activity of free radicals formed in the body. However, the imbalance between the free radical production and the antioxidant defense arises when the free radicals are overproduced in the body (Bahorun *et. al*., 2006). This may result in the accumulation of oxidative stress in the body and cause damage to macromolecules, such as lipid, protein and nucleic acid. Oxidative damage to these molecular species leads to the development of various diseases, such as cardiovascular disease, cancer, cataract, diabetes mellitus and Parkinson's disease (Lobo *et. al.*, 2010, Mayne, 2003).

Diabetes in turn will enhance the oxidative stress in the body of diabetic patient. The possible mechanisms leading to enhanced oxidative stress in diabetic patients include compromised antioxidant defenses, glucose autoxidation, formation of advanced glycated end products and a change in the glutathione redox status (Davison *et. al*., 2002). The enhanced oxidative stress in diabetes leads to its complication with cardiovascular disease (Jay *et. al.*, 2006). Hence, consumption of antioxidant rich foods is beneficial in relieving diabetes particularly the type 2 diabetes.

Other than the oxidative damage, diabetes also might arise because of the release of glucose from carbohydrate in the diet resulting in a high postprandial blood glucose level in diabetic patients. The carbohydrate-hydrolyzing enzyme, α-glucosidase in the digestive tract hydrolyzes the carbohydrate, releasing glucose and cause the raised postprandial blood glucose level. Hence, the inhibition of the activity of this enzyme can effectively reduce the postprandial blood glucose level. α-Glucosidase inhibitors combine with the intestinal α-glucosidase and inhibit the release of glucose from the carbohydrate and hence inhibit the uptake of postprandial blood glucose. There are many synthetic drugs available to prevent α-glucosidase activity or treat diabetes such as acarbose, vogibose and miglitol. However, they may usually cause hepatic disorders and other negative gastrointestinal symptoms (Murai *et. al.,* 2002). With the diverse species of flora, it is expected that there are valuable medicinal plants in the tropical rainforest in Bangladesh and India. The environmental variations in the growing area of the plants are known to affect the metabolism of the plants. Such variations include fluctuations in sunlight, water stress, temperature, intensity of rain, restrictions on nutritive components and air humidity (Ahmed *et. al.,* 2012). Variations in these parameters affect both the primary and secondary metabolism of the plant and hence giving to the different bioactivity of the plants (Hong *et. al.,* 2008).

1.3.2 Natural alpha-glucosidase inhibitors

There are a large number of natural products with alpha-glucosidase inhibitory action (Benalla *et. al*., 2010 and Fang *et al*., 2010). For example, research has shown the culinary mushroom Maitake (*Grifola frondosa*) has a hypoglycemic effect (Konno, *et. al.,* 2001, Hong, *et. al*., 2007 and Hui-Chen. *et. al.*, 2008). The reason Maitake lowers blood sugar is because the mushroom naturally contains an alpha-glucosidase inhibitor (Matsuura *et. al.,* 2002).

1.3.3 Role in clinical use

 α-Glucosidase inhibitors are used to establish greater glycemic control over hyperglycemia in diabetes mellitus type 2, particularly with regard to postprandial hyperglycemia. They may be used as monotherapy in conjunction with an appropriate diabetic diet and exercise, or they may be used in conjunction with other anti-diabetic

drugs. α-Glucosidase inhibitors may also be useful in patients with diabetes mellitus type 1; however, this use has not been officially approved by the Food and Drug Administration.

1.3.4 Mechanism of action

α-Glucosidase inhibitors are saccharides that act as competitive inhibitors of enzymes needed to digest carbohydrates: specifically α-glucosidase enzymes in the brush border of the small intestines. The membrane-bound intestinal α-glucosidases hydrolyze oligosaccharides, trisaccharides, and disaccharides to glucose and other monosaccharides in the small intestine.

Acarbose also blocks pancreatic alpha-amylase in addition to inhibiting membrane-bound α-glucosidases. Pancreatic alpha-amylase hydrolyzes complex starches to oligosaccharides in the lumen of the small intestine. Inhibition of these enzyme systems reduces the rate of digestion of carbohydrates. Less glucose is absorbed because the carbohydrates are not broken down into glucose molecules. In diabetic patients, the short-term effect of these drugs therapies is to decrease current blood glucose levels: the long-term effect is a small reduction in hemoglobin A1c level (Venable & Samantha, 2010, ISBN 0-7817-4839-9).

1.3.5 Human health effects & precautions

Since α-glucosidase inhibitors prevent the degradation of carbohydrates into glucose, the carbohydrates will remain in the intestine. In the colon, bacteria will digest the complex carbohydrates, thereby causing gastrointestinal side effects such as flatulence and diarrhea. Since these effects are dose-related, it is generally advised to start with a low dose and gradually increase the dose to the desired amount. If a patient using an α-glucosidase inhibitor suffers from an episode of hypoglycemia, the patient should eat something containing monosaccharides, such as glucose tablets.

1.3.6 Alpha-glucosidase inhibitors for the treatment of type 2 diabetes mellitus

Diet and exercise is the first step in the treatment of T2DM. But if these measures alone fail to sufficiently control blood glucose levels, starting oral drug therapy is recommended *(Rutten et. al., 2006)*. To date, 6 classes of oral antihyperglycemic drugs

are available: biguanides (metformin), sulphonylurea (eg, tolbutamide), glinidines (eg, repaglinide), thiazolidinediones (eg, pioglitazone), dipeptidyl peptidase IV inhibitors (eg, sitagliptin) and alpha-glucosidase inhibitors (AGIs; eg, acarbose) (Nathan, 2007).

AGIs reversibly inhibit a number of alpha-glucosidase enzymes (eg, maltase), consequently delaying the absorption of sugars from the gut (Campbell *et. al.,* 1996). In a recent study among healthy subjects it was suggested that the therapeutic effects of AGIs are not only based on a delayed digestion of complex carbohydrates, but also on metabolic effects of colonic starch fermentation (Wachters-Hagedoorn *et. al*., 2007). Acarbose (Glucobay) is the most widely prescribed AGI.

The other AGIs are miglitol (Glyset) and voglibose (Volix, Basen). AGIs might be a reasonable option as first-line drug in the treatment of patients with T2DM as it specifically targets postprandial hyperglycemia, a possible independent risk factor for cardiovascular complications (Ceriello *et. al.,* 2005). Although rare cases of hepatic injury were described, AGIs are expected to cause no hypoglycemic events or other life-threatening events, even at overdoses, and cause no weight gain (Chiasson *et. al.*, 2003).

1.4 ANTIOXIDANTS

1.4.1 General Information

Antioxidants are molecules which inhibit the oxidation of organic molecules. They are very important, not only for food preservation, but also for the defense of living systems against oxidative stress (Masuda *et. al.,* 2003). Antioxidants are nutrients (vitamins and minerals) as well as enzymes. They are believed to play a role in preventing the development of such chronic diseases as cancer, heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis, and cataracts.

 In biological systems, the antioxidants are defined as "any substance that when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate." This covers all oxidizable cellular substrates, i.e., above-mentioned lipids, proteins, DNA, and carbohydrates (Frankel *et. al.,* 2000). The

concept of antioxidants relates to what are collectively termed reactive oxygen species (ROS).

Reactive oxygen species (free radicals) are a natural by-product of human metabolism. Because oxygen abounds in each of our cells, it can sometimes pick up electrons from the enzymes that are naturally present to break down nutrients from our food, giving rise to the various types of ROS. These species are called "reactive" because of the ease with which they react with and damage crucial cellular molecules including DNA, proteins and fats. This is where antioxidants come in: they can neutralize ROS. They are able to achieve this by interacting with ROS and becoming oxidized themselves, generating a harmless oxygen compound (like O_2 or water) in the process. The oxidized antioxidant can then be recycled (by reduction) or simply disposed of (Sies *et. al.,* 1997).

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (Kumpulainen *et. al.,* 1999, Cook *et. al.,* 1996). Free radicals can easily damage the structural and functional components of the cells such as lipids, proteins, carbohydrates, DNA and RNA. Oxidation process is one of the most important routes for producing free radicals in food, drugs and even living systems. They are generally by products of various endogenous processes that can be stimulated by external factors such as air pollution, irradiation, smoking, stress and toxins present in food and/or drinking water.

The data have shown that oxidative stress arises from an imbalance of oxidant/ antioxidant ratio in the human body and contributes to the etiology of many chronic diseases of high prevalence (Simonetti *et. al.,* 1997) such as brain dysfunction (Aruoma *et. al.,* 1994), cancer, and cardiovascular diseases (Hertog *et. al.,* 1995). Antioxidants can prevent/retard the oxidation caused by free radicals and sufficient intake of antioxidants is supposed to protect against these diseases.

1.4.2 History of Antioxidants

Use of substances to enhance quality of food by means of delaying lipid oxidation has been in practice for centuries, although it was not chemically defined or understood. The first recorded scientific observation on oxidation inhibitors came from Berthollet in 1797

(Moureu & Dufraisse, *et. al.,* 1926). Their theory was described as ''catalyst poisoning'' in oxidative reactors, and this was well before the free radical theory of peroxidation had been proposed. The earliest reported work on the use of antioxidants to retard lipid oxidation appeared in 1843, in which Deschamps showed that an ointment made of fresh lard containing gum benzoin (contains vanillin) or populin (from polar buds, contains saligenin and derivatives) did not become rancid as did the one with pure lard (Moureu & Dufraisse, *et. al.,* 1926).

Interestingly, the first reports on antioxidants employed for food lipids were about using natural sources; in 1852, Wright (Wright, 1852) reported that elm bark was effective in preserving butterfat and lard. Moureu and Dufraise (Moureu & Dufraisse, 1922a); (Moureu & Dufraisse, 1922b), first reported the possibility of using synthetic chemicals, especially phenolic compounds, to retard oxidative decomposition of food lipids. Their work provided the basic information leading to theories of lipid oxidation and antioxidants, which they referred to as ''inverse catalysis.'' Systematic investigation of antioxidant activity based on the chemistry of radical chain peroxidation of ''model'' chemicals was reported by Lowry and his collegues (Lowry *et. al.,* 1933) and Bolland and tenHave (Bolland & tenHave, 1947) of the British Rubber Producers Research Association.

Antioxidant synergism in food was first reported by H. S. Olcott and H.. A. Mattill (Olcott & Mattill, 1936), and this was significant in achieving oxidative stability in food by using a combination of antioxidants found in the unsaponifiable fraction of oils. They described the antioxidants as inhibitors and grouped them into acid type, inhibitols, and hydroquinone and phenolics. Since the early 1960s, the understandings of autoxidation of unsaturated lipids and antioxidative mechanisms have advanced significantly as a result of development of effective analytical tools.

Around the world a revival is seen in studying the natural antioxidants in foods and the potential health benefits of natural antioxidants in relation to prevention and therapy of oxidative stress and related diseases. Enough scientific evidences have already been accumulated in relation to these conditions with free radicals and reactive oxygen

species. The quest for understanding the oxidation of lipids and its prevention and control has continued since historical times and is still on.

1.4.3 Classification of Antioxidants

Antioxidants may be broadly grouped according to their mechanism of action:

    a) Primary or chain breaking antioxidants and
    b) Secondary or preventive antioxidants

Classification of antioxidants (primary and secondary antioxidants) is discussed below.

1.4.4. Primary Antioxidants

Primary antioxidants are also referred to as type 1 or chain-breaking antioxidants. Because of the chemical nature of these molecules, they can act as free radical acceptors/scavengers and delay or inhibit the initiation step or interrupt the propagation step of autoxidation. Below the figure illustrates possible events that primary antioxidants may interfere along the lipid autoxidation pathway. Primary antioxidants cannot inhibit photosensitized oxidation or scavenge singlet oxygen.
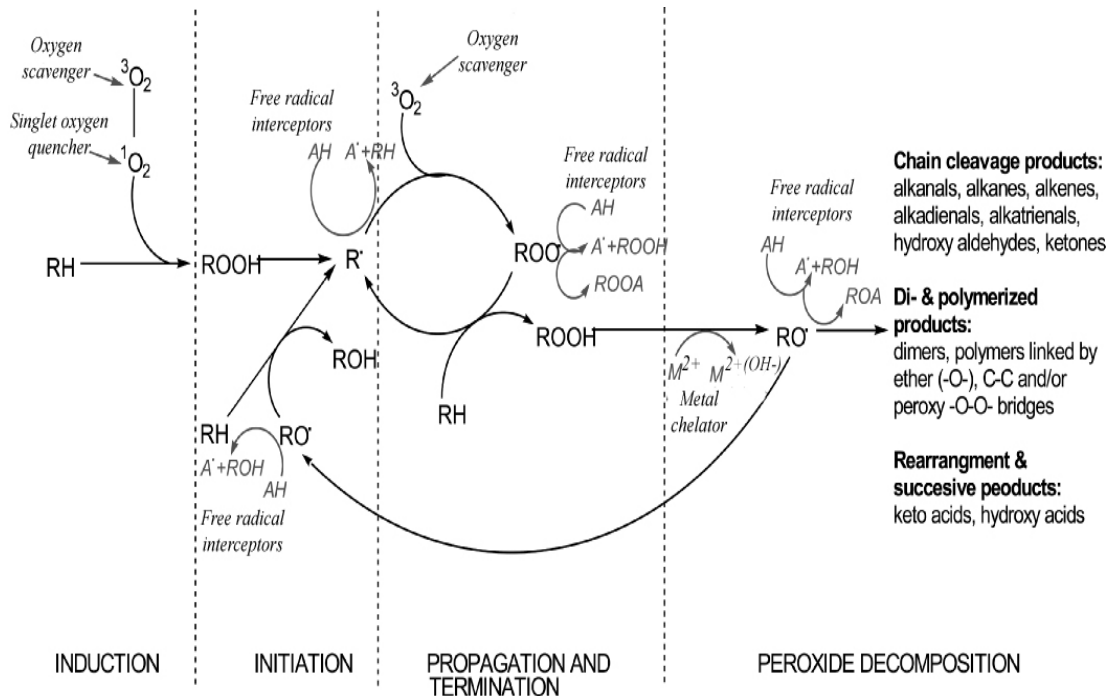
Figure-1.5: Possible interactions of primary and secondary antioxidants with lipid oxidation pathway in foods. (Figure mentioned in Science, Technology, and ApplicationsChapter · July 2005, P. K. J. P. D. Wanasundara&F. Shahidi)

I

$$RH \rightarrow R\bullet + H\bullet \qquad\qquad (1)$$

$$R\bullet + {}^3O_2 \rightarrow ROO\bullet \qquad\qquad (2)$$

$$ROO\bullet + RH \rightarrow ROOH + R\bullet \qquad\qquad (3)$$

$$ROOH \rightarrow RO\bullet + OH\bullet \qquad\qquad (4)$$

$$RO\bullet + RH \rightarrow ROH + R\bullet \qquad\qquad (5)$$
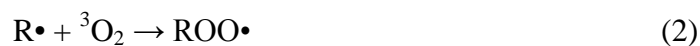
Figure-1.6: Possible reactions of the autoxidation process.

"R" is an alkyl group of an unsaturated lipid molecule. "H" is an α-methylenic hydrogen. "RO•" is alkoxy radical, "ROO•" is peroxy radical, and "I" is an initiator.

$$ROO\bullet + AH \rightarrow ROOH + A\bullet \qquad\qquad (6)$$

$$R\bullet + AH \rightarrow RH + A\bullet \qquad\qquad (7)$$

$$ROO\bullet + A\bullet \rightarrow ROOA \qquad\qquad (8)$$

$$RO\bullet + AH \rightarrow ROH + A\bullet \qquad\qquad (9)$$

$$RO\bullet + A\bullet \rightarrow ROA \qquad\qquad (10)$$

$$A\bullet + A\bullet \rightarrow AA \qquad\qquad (11)$$

Figure-1.7: Mechanism of primary antioxidant activity. AH is an antioxidant molecule.

The first kinetic study of antioxidant activity was conducted by Boland and tenHave (Bolland *et al.,* 1947) who postulated the Equations 6 and 7. The primary antioxidants (AH) react with lipid and peroxy radicals (ROO•) and convert them to more stable, nonradical products as shown in (Figure-1.7), Equations 8 and 9. These antioxidants are capable of donating a hydrogen atom to lipid radicals and produce lipid derivatives and antioxidant radicals (A•) that are more stable and less readily available to participate in propagation reactions (Equation 7). Primary antioxidants have higher affinities for peroxy radicals than lipids and react predominantly with peroxy radicals.

The compounds that exhibit primary antioxidant activity include polyhydroxyphenolics aswell as the hindered phenolics. There are several synthetic ringsubstitutedphenolics as well as naturally occurring phenolic compounds that may perform via the primary antioxidant mechanism. The common feature of all of these antioxidants is that they are mono or polyhydroxy phenols with various ring substitutes (Figure-1.8). Substitution with an electron-donating group/s ortho and/or para to the hydroxyl group of phenol increases the antioxidant activity of the compound by an inductive effect (e.g., 2,6-di-tert-butyl-4-methylphenol or BHA). Thus, the presence of a second hydroxyl group in the 2-(ortho) or the 4-position (para) of a phenol increases the antioxidant activity (e.g., TBHQ). In the dihydroxybenzene derivatives, the semiquinoid radical produced initially can be further oxidized to a quinone by reacting with another lipid radical (Figure-1.11). The formed antioxidant radical is stabilized by delocalization of the unpaired electron around the phenol ring to form a stable resonance hybrid (Figure-1.10) and as a result attained low-energy levels. Table-1.2 provides examples of some of compounds that exhibit primary antioxidant activity commonly used in food.
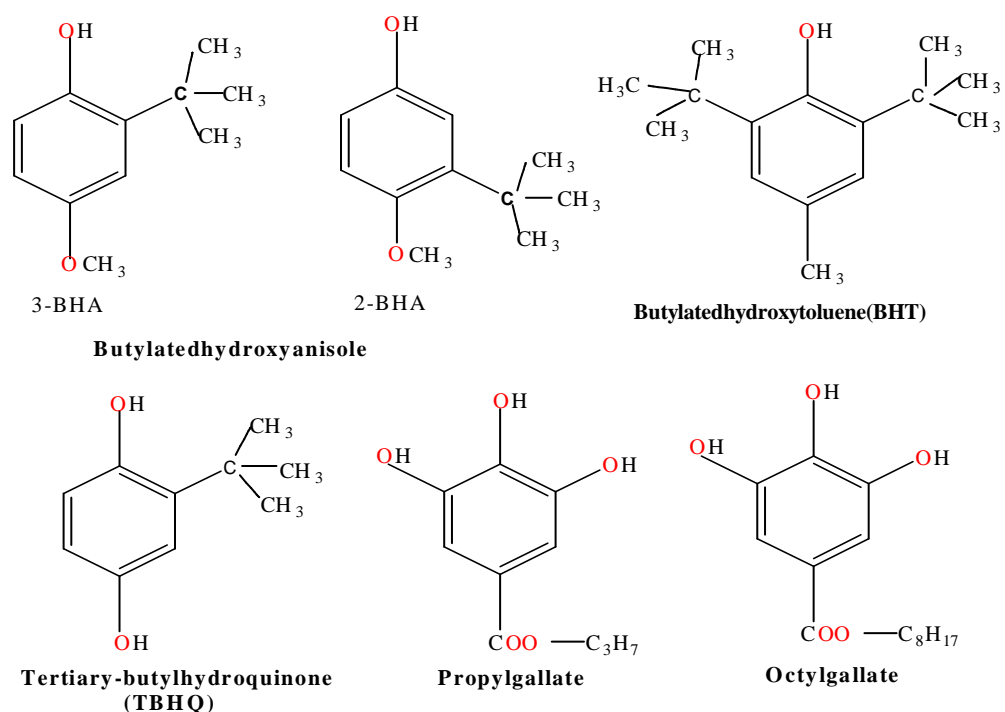
Figure-1.8: Chemical structures of synthetic phenolic antioxidants commonly used in fats and oils.
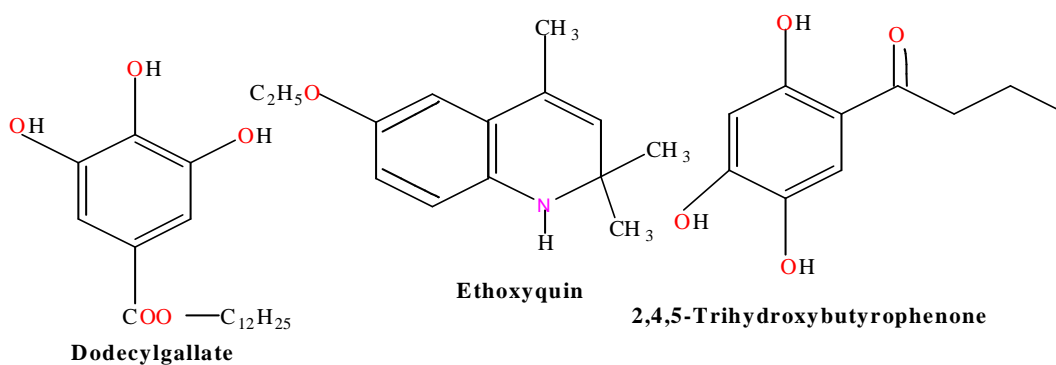
Figure-1.9: Chemical structures of synthetic phenolic antioxidants commonly used in fats and oils.
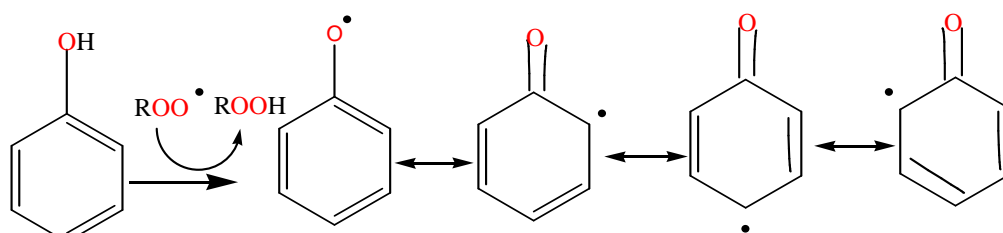
Figure-1.10: Stable resonance hybrids of phenoxy radical of phenolic antioxidant
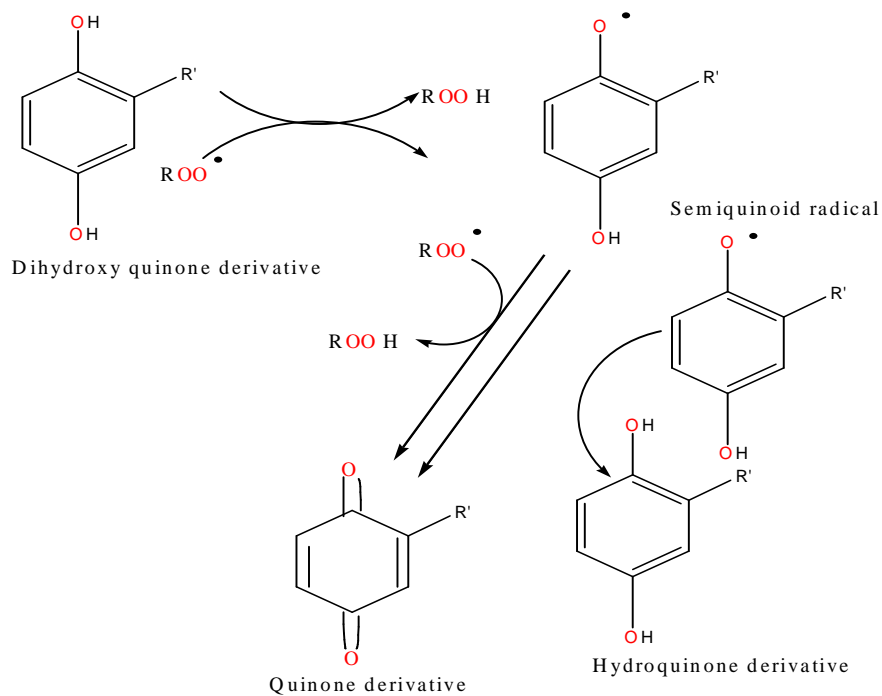
Struc-27 Possible mechanism of antioxidant activity of dihydroxybenzene derivative.

Figure: 1.11: Possible mechanism of antioxidant activity of dihydroxybenzene

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Table-1.2: Primary Antioxidants that are commonly used in Foods

Natural	Synthetic
Carotenoids	Butylatedhydroxyanisole (BHA)
Flavonoids	Butylatedhydroxytoluene (BHT)
Phenolic acids	Ethoxyquin, Propyl gallate (PG)
Tocopherols and tocotrienols	Tertiary-butylhydroquinone (TBHQ)

1.4.5: Secondary Antioxidants

Secondary antioxidants are also classified as preventive or class II antioxidants. They offer their antioxidant activity through various mechanisms to slow the rate of oxidation reactions. The main difference with primary antioxidants is that the secondary antioxidants do not convert free radicals into stable molecules. They act as chelators for prooxidant or catalyst metal ions, provide H to primary antioxidants, decompose hydroperoxide to nonradical species, deactivate singlet oxygen, absorb ultraviolet radiation, or act as oxygen scavengers. They often enhance the antioxidant activity of primary antioxidants. Table-1.3 provides examples of some of these compounds that exhibit secondary antioxidant activity

Table-1.3: Compounds that Exhibit Secondary Antioxidant Activity

Mode of Activity	Compounds in use
Metal chelation	Cirtic, Malic, Succinic and Tartaric acids Ethylene diamine tetraacetic acid, Phosphates
Oxygen scavenging and reducing agents	Ascorbic acid, Ascorbylpalmitate, Erythorbic acid, Sodium erythorbate, Sulfites
Singlet oxygen quenching	Carotenoids (b-Carotene, Lycopene and Lutein

1.5 SUN SKIN PROTECTION FACTOR (SPF)

Photo ageing generally occurs due to continuous exposure to UV radiation which stimulates the discharge of free radicals in the skin. The free radicals activate the Nuclear factor kB (NF-kB) and activator protein 1 (AP-1) pathways and in reverse inhibit the transforming growth factor-beta (TGF-β) pathway, finally promoting the expression of matrix metalloproteinases (MMPs) and inflammatory cytokines (Chen *at al*., 2012). About 95% of the sun's UV-radiation reaching the earth's surface is long wave UV radiation (UVA, 320-400 nm). UVA radiation is able to penetrate the deeper layers of the epidermis and dermis skin, resulting in wrinkle formation and premature ageing (Balakrishnan *et. al.,* 2011). A lower percentage of UV radiation (UVB, 280-320 nm) reaches the earth surface but the radiation is more intense, enough to cause skin reddening and sunburns. Preventing or reducing exposure to UVA and UVB radiation will reduce signs of ageing, deep wrinkles, solar elastosis, coarse textures, telangiectasias and skin cancer (Chen *et. al*., 2012, Balakrishnan *et. al.,* 2011). Numerous synthetic organic compounds that absorb UV radiation have been developed to protect skin from the damaging effects of sunlight. These synthetic compounds are either UVA- or UVB-absorbing compounds and hence are used in combination to provide a broad-spectrum UV screen. The necessity to provide high sun protection factor (SPF) and blocking efficiency against both UVA and UVB wavelengths has led to the development of sunscreen formulations with multiple added sunscreen chemicals (Vilela *et. al.,* 2011). Most chemical compounds used in sunscreen products are active in the UVB region while only a few chemicals block the UVA region. It is also a point of growing concern that the safety of many of these compounds has not been established, especially for longterm human use. For example, although broad-spectrum protection is achievable using titanium dioxide, zinc oxides or iron oxides, these are promoted on the basis that they may be less harmful than organic sunscreen absorbers. It should be noted that microfine (nanoparticles) titanium dioxide as a sunscreen product also has no long-term safety data (Sambandan & Ratner, 2011). Hence, there is a need to search for alternative source of effective and safer photoprotective agents that can be utilized in sunscreen products as well as in cosmetic preparations. In general, whole plant extracts have shown better potential as photoprotective agents due to their complex chemical composition and

broad UV absorption spectra as well as their antioxidant power. Although they have not completely replaced the dominance of synthetic materials, the use of these botanical extracts is becoming more common. For example, green tea and black tea have been reported to ameliorate adverse skin reactions following UV exposure, while *Aloe vera* gel assists in cell regeneration (Yusuf *et. al*., 2007, ATudose *et. al.,* 2009 and Kumar *et al*., 2010). Interests in adding natural ingredients in sunscreen formulation are mainly driven by the 'back to nature' movement and the promise of equal or greater efficiency with lesser side effects by using these materials. Furthermore, there is also a growing interest in natural antioxidants present in medicinal plants (Arulselvan *et. al*., 2013) or herbal extracts that can reduce oxidative damage for use in cosmetic science as beauty products and to maintain the physiological balance of the human skin (WV-Magalhães *et. al*., 2011).

1.6 POSSIBLE MICROBIAL CONTAMINATION OF ADHPs

Bauer (1998) showed that the quality criteria for herbal drugs are based on a clear scientific definition of the raw materials. It is difficult to establish comprehensive quality criteria for herbal drugs due to 'professional secrecy' of herbalists, but in order to improve the purity and safety of the products, observation of basic hygiene during preparation, standardization of some physical characteristic such as moisture content, pH and microbiological contamination levels are desirable. Previous studies have confirmed the presence of potential contaminants in herbal preparations *(*De-Smet*,* 1999*)*. The contaminants that present serious health hazard are pathogenic bacteria such as *Salmonella, Escherichia coli, Staphylococcus aureus, Shigella* spp and other Gram positive and Gram negative strains of bacteria (Arias *et. al.,* 1999, Erich *et. al.,* 2001, Wolfgang *et al.*, 2002, Adeleye *et al.,* 2005 and Okunlola *et. al.,* 2007). A greater number of residents in Bangladesh are believed to depend on traditional herbalists for their medical needs. Unfortunately, no researches (to the best of our knowledge) have been carried out to determine the microbiological safety of these herbal products in this developing country. In the present work, the level of contamination of powdered herbal products marketed in Dhaka with selected pathogenic bacteria and the susceptibility of these contaminants to commonly prescribed antibiotics in the metropolis are studied.

B. CHEMISTRY OF ADHPs AND ADSPs

1.7 TOXIC METAL ANALYSIS

The nature, full of living organism and non-living objects, is the best gift of Creator for human. The nature is nicely decorated with different types of extrusive flowers, plants and animals. The Creator created these things in such a way that everything is depending on each other. Human, the best of the creations, always depend on trees/plants for preparing their foodstuffs, clothing, shelter and healthcare.

Herbal remedies are widely used for the treatment of various illnesses. They often contain highly active pharmacological components including minerals and trace metals (Fabricant & Farnsworth, *et. al.,* 2001). In developing countries, it is estimated that 70–80% of the population somehow relay on nonconventional medicines mainly of herbal origins for their primary health care (WHO, 2008), as they are cheap and easily accessible (Sofowora, *et. al.,* 1996). Herbal preparations are produced from any raw or processed part of a plant, which includes leaves, stems, flowers, roots, and seeds, and in most of the cases it is a complex mixture of organic chemicals from natural sources (Bent, 2008; WHO, 2000; Adeleye. *et., al.* 2005). 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 parts of herbs (Adeleye. *et., al.* 2005), (Table 1.4). Moreover, 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 (Table 1.5). In general, most common contaminants are heavy metals, pesticides, microbes, and mycotoxins (WHO, 1993; Ernst, *et. al.,* 2002).

The range of the usage (Wild *et. al.,* 2004) of herbal preparations is vast as they are frequently used in the treatment of several chronic diseases including type 2 diabetes (diabetes mellitus). Diabetes is a noncommunicable heterogeneous group of disorder and affects approximately 200 million individuals globally. Moreover, it is predicted that over 300 million people will be diabetic by 2015 (Wild *et. al.,* 2004; Mc-Cune & Johns, 2002). In general, this poses challenges to the health care and social welfare but, in particular, it is a huge challenge to a developing country like Bangladesh because of its limited resources and weak economy.

Table 1.4: 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	Amount of main ingredients	Daily adult (70 kg bw) in g or mL
ADHP – 1	Cap Sijijium Jamb	*Syzgium cumini*	Not Mentioned	4.25
ADHP–2	Syp ABC-3	*Allium sativam, Allium cepa, Syjium cumini, Suitania mahaguni, Gymnema sylvester*	Not Mentioned	20-30 (ml)
ADHP–3	Syp Gambadya Rista	*Syzgium cumini, Ficus carica*	0.19mg/5 mL 0.19mg/5 mL	20-40 (ml)
ADHP–4	Tab Alisa Garlitab	*Allium sativam Allium cepa Syzygium cumini Magnifera indica Myristica fragrans Syzygium aromaticum*	30 mg 20 mg 20 mg 20 mg 10 mg 10 mg	3.75
ADHP–5	Cap Silaraj	*Salvia Haematodes*	71.43 mg/ 500 mg	2.5
ADHP–6	Tab Diagym	*Gymnema sylvestre*	56.65 mg/ 500 mg	2.5
ADHP–7	Cap Dicare	*Bambusa bambos Rumex vasicarius Acacia arabica Gymnema sylvester*	146.57mg/ 500 mg 97.72 mg/ 500 mg 26.67 mg/ 500 mg 65.15mg/ 500 mg	1.5
ADHP-8	Tab Dolabi	*Gymnema sylvestre Bambusa bambus Rumex vasicarious*	42.49 mg /500 mg 95.60 mg /500 mg 63.73 mg/500 mg	2.5
ADHP-9	Syp Diabetes China Gold	*Papaver somniferum*	35.71 mg /500 mg	20-30 (ml)
ADHP-10	Diabeno Herbal Tea	*Lagerstroemia Speciosa*	Not Mebtioned	2.5
ADHP-11	Tab Ziabit	*Bambusa bambos Rumex vasicarius Gymnema sylvester*	191.24 mg/500 mg 127.5 mg/500 mg 85.00 mg/500 mg	1

Table 1.5: Role of Different Heavy Metals in Human Biology (WHO, 2004)

Name of Metal	Features	Normal function in body	Disorder related to deficiency	Disorder related to excess accumulation then normal limit
Zinc (Z_n)	Presenting in all tissue	Catalyst for more than 200 enzymes, important for immune function , wound healing	Growth retardation, loss of appetite, and impaired immune function, hair loss, diarrhea	Nausea, vomiting, loss of abdominal cramps, diarrhea
Copper (C_u)	Presenting in all tissue	Cofactors for enzymes, cellular respiration , peptide amidation , neurotransmitter biosynthesis	Hernias, aneurysms, blood vessel breakage manifesting as bruising or nosebleeds.	Nausea, vomiting, abdominal pain and cramps, headache.
Manganese (M_n)	Presenting in all tissue	Essential for normal metabolism of amino acid, lipid, protein and carbohydrate, ATP generation	Malformation of bones, altered macromolecular metabolism, reduce fertility, weakness.	Trigger toxicity in Central Nervous System (CNS).
Chromium (C_r)	Proposed to be an essential element for mammals	Potentiates the actions of Insulin, plays a role in glucose, fat, and protein metabolism, Chromium supplementation increases muscle gain and fat loss	No clinically defined state of chromium deficiency , but diabetes has been shown, increases the cardiovascular risks	DNA damage , Kidney failure
Cadmium (C_d)	Exposed to human body from the ingestion	No known biological function	No known complication related to Cadmium deficiency	Cadmium toxicity induces tissue injury , epigenetic changes in DNA expression
Lead (P_b)	A Potent occupational toxin	No known biological function	No known complication related to Lead(P_b)deficiency	Primarily affects the central nervous, hematopoietic , hepatic

The trend of uses of antidiabetic herbal preparations (mostly 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 different contaminants including microbial contaminants and heavy metals (particularly in Ayurvedic preparation). In 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 (Bent, 2008). Although in several countries herbal medicine (a part of complementary and alternative medicine) is the officially approved system, there is no guidelines and regulations for assuring the safety of these preparations. 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 metals beyond the limit could be toxic. Therefore, it is important to evaluate the safety of these antidiabetic herbal preparations based on relevant scientific investigation. This research focused on the safety of antidiabetic herbal preparations available in Bangladesh particularly related to heavy metal contamination.

In Bangladesh, several antidiabetic herbal preparations are readily available and are being used, but studies of heavy metal content in locally produced herbal preparations are limited. Therefore, it is necessary to evaluate the heavy metal content in some locally produced and widely used herbal preparations. In this study, the level of heavy metal content present in antidiabetic herbal preparations widely used and formulated in Bangladesh have been investigated.

1.8 ORGANOCHLORINE PESTICIDES (OCPS)

1.8.1 General Information

Pesticides are a group of chemicals made for the purpose of killing or otherwise deterring "pest" species. The word pesticide may refer to insecticides, herbicides, fungicides, or other pest control formulations. Pesticides are inherently toxic and often associated with adverse health effects in non-target organisms (USEPA, 2009).

Organochlorine pesticides are a large class of multipurpose chlorinated hydrocarbon chemicals. Organochlorine pesticides break down slowly in the environment and accumulate in the fatty tissues of animals. Thus, they stay in the environment and food web long after the being applied (Swackhamer & Hites, 1988). DDT, now banned in most countries including Bangladesh because of its harm to the health of wildlife and people, is a notable example of an organochlorine pesticide.

Many organochlorine pesticides are endocrine disrupting chemicals, meaning they have subtle toxic effects on the body's hormonal systems *(*Lemaire*, 2004).* Endocrine disrupting chemicals often mimic the body's natural hormones, disrupting normal functions and contributing to adverse health effects. Examples of organochlorine pesticides are dichlorodiphenyltrichloroethane (DDT), hexachlorocyclo hexane (HCH), hexachloro benzene (HCB), chlordane, aldrin, dieldrin, endrin, heptachlor, heptachlor epoxide, endosulfan, methoxychlor, mirex and trans-nonachlor

1.8.2 Organochlorine Pesticides in Human Bodies

Organochlorine pesticides are ubiquitous environmental contaminants because they break down very slowly. According to the Centers for Disease Control and Prevention (CDC), most people have organochlorine pesticides present in their bodies (NCEH, 2005). Many organochlorine pesticides are persistent organic pollutants (POPs), a class of chemicals known to break down very slowly and bio-accumulate in lipid rich tissue such as body fat (Swackhamer & Hites. 1988). Exposure to low concentrations of organochlorine chemicals over a long period may eventually lead to a substantial body burden of toxic chemicals (Quintana, *et. al.,* 2004). Chemicals in a maternal body burden may be transferred to fetuses in utero via the placenta and to children through breast feeding. This means that children who are born years after maternal exposure to toxic organochlorine pesticides can still be affected (Jurewicz & Hanke, 2008).

1.8.3 How Are Human Exposed?

Because of the multiple types and uses of organochlorine pesticides, there are many ways people can be exposed to these chemicals. Wind and rain may move pesticides away from where they were used, causing contamination of surface waters, groundwater and/or soil (Bouman, *et. al.,* 2002; Shomar, 2005). Using pesticides in your home or on your

land may increase your exposure to these chemicals, and living or working close to where pesticides are used is also a risk factor (Fenske, *et. al.,* 2002).

Organochlorine pesticides are not often used in personal products because of their toxicity, but some products, such as lice shampoos, may be a source of exposure (Hall RCW, 1999). Exposure may also occur through consumption of contaminated foods. Organochlorine pesticides are carried long distances via atmospheric and oceanic currents from where they are manufactured and used, and build up in the fatty tissues of animals (Bentzen, *et. al.,* 2008). Many studies have linked organochlorine pesticide exposure with consumption of contaminated animal products, mostly meat, dairy, fish, and marine mammals (Fitzgerald, *et. al.,* 2001; Hagmar., *et. al.,* 2001; Mwevura., *et. al.,* 2002; Bradman, *et. al.,* 2007). Fetuses and children may be exposed to pesticides in utero as well as through breast milk (Jurewicz & Hanke, 2008).

1.8.4 What Does Exposure to Organochlorine Pesticides Mean for Human Health?

The presence of environmental chemicals in the human body does not necessarily imply that they are causing adverse health effects; however, environmental chemical exposures can and do affect human health. It is important to note that both the dosage and the timing of exposure have significant effect on any potential health outcome. The health effects of organochlorine pesticide exposure depend on the specific pesticide, the level of exposure, the timing of exposure and the individual. Different pesticides result in a range of health symptoms.

1.8.4.1 Cancer

Numerous studies have linked organochlorine pesticide exposures with cancers and other health effects. Exposure to DDT has been linked to pancreatic cancer and non-Hodgkin's Lymphoma (Garabrant *et. al.,* 1992, Cantor *et. al.,* 1992). Exposure to DDT early in life is associated with an increased breast cancer risk later in life. Many other organo-chlorine pesticides, such as mirex, chlordane and toxaphene, are known to be carcinogenic as well (Dich *et. al.,* 1997). A study of women from an agricultural area in India showed that women with breast cancer had much higher total organochlorine pesticide concentrations in their blood. The women had average total pesticide concentrations of 7,468 ± 771 ppb (ng/mL) in their blood (Mathur, *et. al.,* 2002).

1.8.4.2 Neurodevelopmental Outcomes

There is clear evidence that exposure to organochlorine pesti-cides disrupts normal development. In one study, exposure to DDT shortly after birth created a lifelong sensitivity to other pesticide exposures in mice, and permanent behavioral changes upon secondary exposure through food (Johansson *et. al.,* 1995). Prenatal exposure to the organochlorine pesticide chlordane has been linked to reduction of testosterone in adult female rats and behavioral changes in both sexes (Cassidy, *et. al.*, 1994). Rats prenatally exposed to varying levels of DDT showed behavioral alterations that lasted into adulthood (Palanzaa *et. al.,*1999).

Organochlorine pesticide exposure is associated with neuro-developmental health effects in humans. Exposure to organochlorine pesticides has been linked to decreased psychomotor function and mental function, including memory, attention, and verbal skills in children (Jurewicz & Hanke, 2008; Korrick & Sagiv, 2008). Children born in agricultural areas where pesticides were applied were found to have lower performance on numerous neurobehavioral assessments when compared to children not born in an agricultural region (Ribas-Fito *et. al.,* 2006.). There is also some evidence that organochlorine pesticide exposure is associated with the development of autism, although this is based on limited research (Roberts. *et. al.,* 2007).

1.8.4.3 Reproductive Effects

In humans, maternal concentrations of DDE (a metabolite of DDT) above 10 ppb (μg/L) are associated with preterm birth and babies' size. The higher the concentration of DDE in the mother's blood, the more likely she was to have a preterm birth and the baby was more likely to be small for its gestation age (Longnecker *et. al.,* 2001). Maternal exposure to beta-HCH, a byproduct of lindane manufacture, is associated with preterm births. Women in the study had beta-HCH average concentrations of 9.83 ppb (ng/mL) in their blood (Pathuk, *et. al.,* 2009).

1.8.4.4 Thyroid Disruption

Organochlorine compounds, including pesticides, have been found to alter levels of maternal thyroid hormones during pregnancy. Women with hexachlorobenzene

concentrations that ranged from 7.5-841.0 ppb (ng/mL) had altered thyroid hormone levels (Chevrier *et. al.,* 2008*).* Another study found that dieldrin exposure was associated with decreased T4 levels. Women with decreased thyroid hormone had average dieldrin concentrations of 5380 ppb (ng/mL) (Rathore, *et. al.,* 2002).

Many organochlorine chemicals, including DDT, endosulfan and lindane, are known to produce anti-thyroid effects (Román, 2007). Thyroid hormones are critical for normal growth and development in fetuses, infants, and small children. (Glinoer, 2007). Thyroid deficiencies during pregnancy and post partum are known to cause altered development, retardation, decreased intellectual capacity, psychomotor delays, and deafness. Additionally, there is speculation that thyroid disrupting chemicals may play a role in the development of autism. (Roman, 2007)

1.8.4.5 Neurodegenerative Disease

Neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease are more common in people with general pesticide exposures, including organochlorine pesticide exposure. (Kamel & Hoppin, 2004)

In the present study 04 organochlorine pesticides namely 4, 4 DDE; 4, 4 DDD; 2, 4 DDT & 4, 4 DDT in the 7 ADHP (s) were studied.

4, 4-DDE   *4, 4-DDD*

2,4-DDT   *4,4DDT*
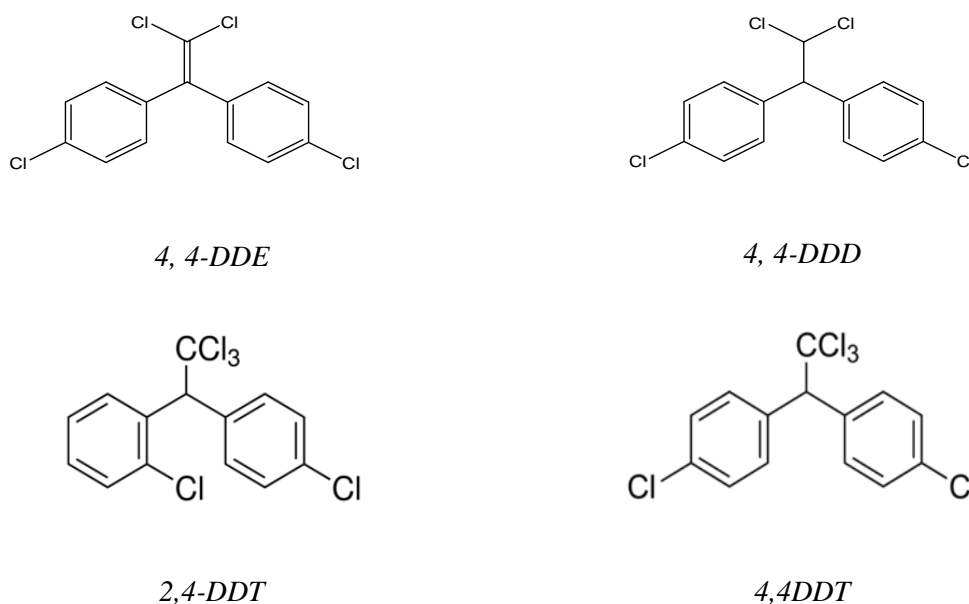
Figure-1.12: Structure of different oreganochlorine pesticides

1.9 SYNTHETIC DRUG CONTAMINATION

1.9.1 General Information

The use of Antidiabetic herbal medicines (ADHPs) has recently increased, thereby enhancing the market for herbal products worldwide because of their low risk factor (Bodeker & Kronenberg, 2002, Zollman & Vickers, 1999). Herbal medicines are marketed in pharmacies, drugstores and directly by local practitioners, with advertising either in the printed media or, increasingly, from international internet suppliers, with direct delivery (Morris & Avorn, 2003, Eisenberg *et. al.,* 1998, Tindle *et. al.,* 2005, De-Smet, 2004). Many products are delivered as different formulations, without any adequate declaration of the ingredients, or information on the recommended dosages or side effects. Translations of product information are often missing from foreign products (Routledge, 2008, Foster, 2005, Ernst E, *et. al.,* 2002). One of the greatest risks to human health related to HMPs arises from economically motivated adulteration. Such adulteration can occur through the addition of undeclared pharmaceutical drugs or, in illicit attempts to evade detection of adulteration, their analogues (Ernst E*, et. al.,* 2002). Examples of HMP types commonly adulterated include sleeping aids that have been adulterated with benzodiazepines such as estazolam or clonazepam (Health Canada Sleep Supplement Found to Contain Habit-Forming Drug, 2007), weight loss products adulterated with sibutramine or fenfluramine (Corns & Metcalfe, 2002, Jung, *et. al.,* 2006 and Yuen *et. al.,* 2007), erectile dysfunction or sexual enhancement products adulterated with sildenafil, tadalafil, vardenafil, or their analogues (Li, *et. al.,* 2009, Singh, *et. al.,* 2009) remedies for diabetes adulterated with glibenclamide (Padinjakara *et. al.,* 2009) and bodybuilding products adulterated with androgenic steroids (U.S. FDA, 2009).

Bangladesh is the source of many medicinal plants and a number of herbal medicinal producers are producing different kinds of herbal preparations. People of this developing country are not aware about the safety of these herbal medicines. The producers are not good at the soil, environments and use of pesticides for the products or of the raw materials of herbal medicines. Some people claimed that synthetic drugs like metformin hydrochloride, glipizide, gliclazide, glibenclamide and glimepiride may be mixed with the herbal preparations used for diabetes in the local market of Bangladesh.

The work presented in this research brings into light use of synthetic antidiabetic standards drugs, metformin hydrochloride, glipizide, gliclazide, glibenclamide and glimepiride admixed with some traditionally used herbal preparations for diabetes which are sold in the local market of Dhaka city in Bangladesh. For evaluation the adulteration of synthetic drugs, five standard reference samples used for the treatment of Diabetes were compared with the 07 ADHPs by using HPLC. The following standard reference samples were used:

I. Metformin (Hundal, 2003)

II. Glipizide (Glucotrol, 2005)

III. Gliclazid (Del *et. al*. 2007)

IV. Glibenclamide (Marble, 1971)

V. Glimepiride (Davis, 2004)

I. Metformin

Metformin is an oral antidiabetic drug in the biguanide class. It is the first-line drug of choice for the treatment of type 2 diabetes, in particular, in overweight and obese people and those with normal kidney function (ADA, 2009). Its use in gestational diabetes has been limited by safety concerns. It is also used in the treatment of polycystic ovary syndrome, and has been investigated for other diseases where insulin resistance may be an important factor. Metformin also works by suppressing glucose production by the liver (Online clinic, http://www.onlineclinic.co.uk/metformin.html).

II. Glipizide

Glipizide is an oral rapid and shortacting antidiabetic drug from the sulfonylurea class. It is classified as a second generation sulfonylurea, which means that it undergoes enterohepatic circulation. Second generation sulfonylureas are both more potent and have shorter half-lives than the first generation sulfonylureas.

III. Gliclazide

Gliclazide is an oral hypoglycemic (antidiabetic drug) and is classified as a sulfonylurea. Its classification has been ambiguous, as literature uses it as both a first generation

(Ballagi-Pordány, *et. al.,* 1990) and second generation (Shimoyama, *et. al.,* 2006) sulfonylurea.

IV.   Glibenclamide

Glibenclamide is an antidiabetic drug in a class of medications known as sulfonylureas, closely related to sulfa drugs. It was developed in 1966 in a cooperative study between Boehringer Mannheim (now part of Roche) and Hoechst (now part of Sanofi-Aventis) (Marble, 1971).

V.   Glimepiride

Glimepiride is a medium to long-acting sulfonylurea antidiabetic drug. It is sometimes classified as either the third-generation sulfonylurea (Hamaguchi, *et. al.,* 2004), or as second generation (Davis, 2004).

1.
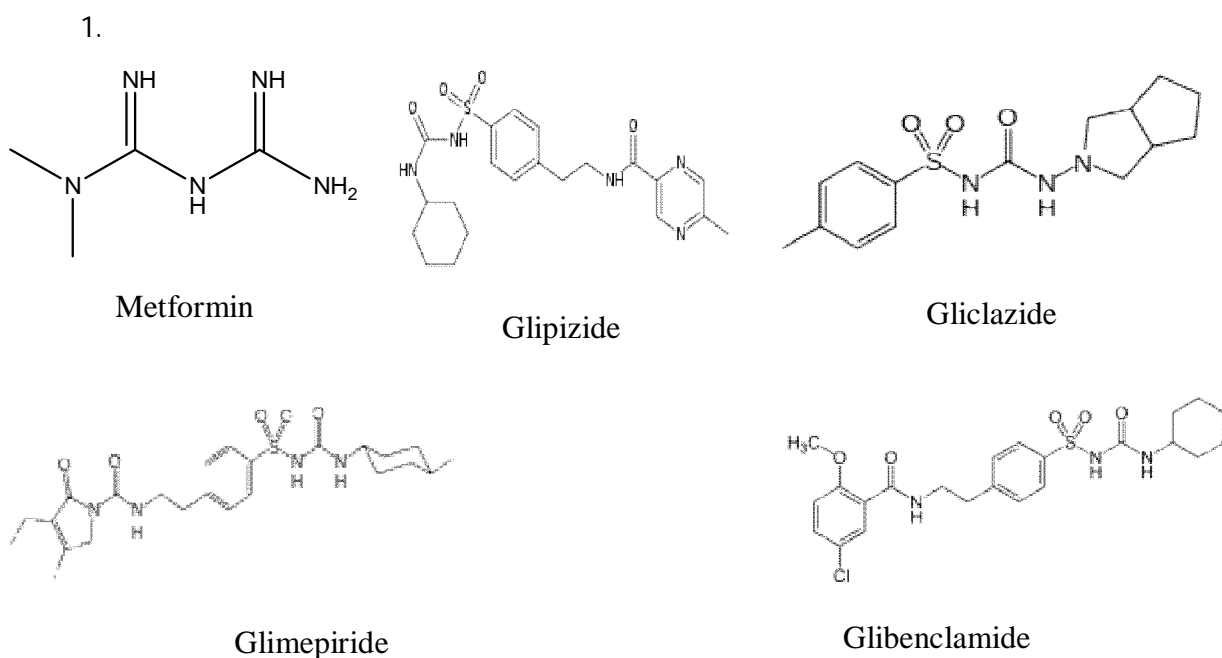
Figure-1.13: Structure of standard of synthetic drugs

1.10 TRADITIONAL USES AND PHARMACOLOGY OF PLANTS IN THE TREATMENT OF DIABETES MELLITUS

More than 400 plant species having hypoglycemic activity have been available in literature, however, searching for new antidiabetic drugs from natural plants is still attractive because they contain substances which demonstrate alternative and safe effects on diabetes mellitus. Most of plants contain glycosides, alkaloids, terpenoids, flavonoids, carotenoids, *etc.*, that are frequently implicated as having antidiabetic effect (Pate *et. al.,* 2012). Some plants which have been studied by our research group are briefly described here.

The seeds of *Trigonella foenumgraecum* (fenugreek) are more widely recommended (Swanston-Flatt *et. al.,* 1989) for Type 2 patients. Trigonelline, the N-methyl derivative of nicotinic acid isolated from *T. foenumgraecum*, has a weak and transient hypoglycemic effect when administered orally to diabetic patients (Shani *et. al.,* 1974, Mishinisky *et al.,* 1967). The whole powder of *T. foenumgraecum* seeds and its extracts were investigated (Mosihuzzaman *et al.,* 1995) for their hypoglycemic effect on normal and diabetic model rats. The methanol extract and the residue remaining after methanol extraction showed significant hypoglycemic effect when fed simultaneously with glucose. The water extract of the methanol extractive-free residue of the seed powder also showed significant hypoglycemic activity at different prandial states. They have also tested the soluble dietary fiber (SDF) fraction that showed significant hypoglycemic effect in Type 2 model rats when fed simultaneously with glucose.

Rhizome of *Costus speciosus*, tuber of *Nephrolepsis tuberosa*, and bulb of *Stephania hernandifolia*, used by the local people and traditional healers in the Eastern Himalayan belt, were studied (Mosihuzzaman *et. al.,* 1994) for their effects on serum glucose levels in nondiabetic and diabetic rat models at different prandial states. In non-diabetic rat juice extract of *C speciosus* and *N tuberosa* had no significant effect in the fasting or post prandial state (when the juice extract was fed simultaneously with glucose). However, when the juice extract was fed 30 min before the glucose load, both juice extracts showed hypoglycemic effect. In Type 2 model rats, *N tuberos's* juice extract opposed rise in serum glucose level when it was fed 30 minutes before the glucose load, whereas juice

extract of *S hernandifolia* had a tendency to raise the serum glucose level. In Type 1 model rats none of these juice extracts showed any effect in the fasting state, whereas *C. specious* showed significant hypoglycemic effect when the juice extract was fed simultaneously with glucose load and *S hernandifolia* showed significant hypoglycemic effect in both the stages (fed simultaneously with, and 30 min before the glucose load) of prandial states.

Hypoglycemic effect of raw *Momordica charantia* fruit and its aqueous extract has been reported (Akhtar *et. al.,* 1981, Sharma *et. al.,* 1960) in healthy and alloxan-induced diabetic animals. Oral consumption of *M. charantia* did not enhance insulin release, although its aqueous extract has been shown (Welihinda *et. al.,* 1982) to stimulate insulin release from normal isolated islets *in vitro*. The antihyperglycemic activity of *M. charantia* fruit juice was found (Welihenda *et. al.,* 1986) when administered orally to human adult (100 ml juice 30 min before the oral glucose load) which improved glucose tolerance of 73% of the patients investigated.

Studied of the different parts of *M. charantia* for their hypoglycemic activity Ali *et. al*. (1993*)* showed that *M. charantia* fruit pulp juice lowered fasting blood glucose levels in normal rats. The pulp juice also had a significant hypoglycemic effect in the glucose-fed normal rats. In the Type 2 model rats, saponin free methanol extract of the pulp juice produced a significant hypoglycemic effect in fasting and in postprandial states.

Raw onion bulbs (*Allium cepa*) and garlic cloves (*Allium sativum*) have been reported to lower blood glucose level of nondiabetic (Jain *et. al.,* 1973) and diabetic (Shela and Augustia, 1992) animals. Our research group recently investigated (Rokeya *et al*. 1999) *A. cepa* and *A. sativum* for their hypoglycemic effects in nondiabetic and diabetic model rats. The result showed that in nondiabetic rats, freeze-dried juice of *A. cepa* significantly reduced fasting blood glucose level at 60 min and 120 min. Freeze-dried juice of *A. cepa* had no effect on T2DM rats but it had significant blood glucose lowering effect on Type 1 model rats when the material was fed simultaneously with glucose load. Freeze-dried juice of *A. sativum* had no effect on fasting nondiabetic model rats but it had significant effect in nondiabetic, Type 2 and Type 1 model rats when the material was fed simultaneously with glucose load.

Recent studies (Rokeya *et al.* 1999) by our group showed that *Plantago ovata* (methanol extract of husk), *Gymnema sylvestre* (methanol extract of leaves) and *A sativum* (juice extract) had antihyperglycemic effect in Type 2 and Type 1 model rats when the samples were fed simultaneously with glucose. These three plants did not show any significant hypoglycemic activity in any of the model rats under fasting condition. But with glucose load, *P. ovata* rapid effect (significant blood glucose lowering effect) was observed at 30 and 75 min ($p<0.05$) and *G. sylvestre* showed significant hypoglycemic effect only at 75 min ($p<0.05$). When the extracts were fed 30 min before glucose load *P. ovata* showed significant hypoglycemic effect at 105 min; *G. sylvestre* and *S. platensis* showed significant antihypoglycemic effect at 60 and 105 min. A pure compound isolated by our group from *G. sylvestre*, showed significant hypoglycemic effect ($p< 0.04$) on diabetic model rats.

Coccinia indica, *Musa paradisiaca* and *Syzizium cumini* were also investigated (Nahar *et al.,* 2000) by our research group for their hypoglycemic effects in nondiabetic and diabetic model rats. The results showed that *C. indica* was effective in fasted as well as in Type 1 model rats when the extracts were fed 30 min prior to glucose load. *M. paradisiaca* was effective in normal, Type 2 and Type 1 model rats when the extracts were fed simultaneously with glucose load and the efficacy of *S. cumini* was found in Type 2 and Type 1 model rats when the extracts were fed simultaneously with glucose load.

The studies of *Pterospermum acerifolium* on normal, Type I and Type 2 diabetic model rats (Mamun *et al.*, 2001) showed that methanol extract of bark of *P. acerifolium* had a significant anti-hyperglycemic effect on nondiabetic ($p<0.01$), Type 2 ($p<0.05$) and Type 1 ($p<0.01$) diabetic model rats when it was fed simultaneously with glucose and also when the extract was fed 30 min before glucose. Chloroform extract of leaves of *P. acerifolium* showed significant anti-hyperglycemic effect ($p<0.05$) on Type 1 diabetic model rats.

1.11 ANTIDIABETIC AGENTS REPORTED FROM MEDICINAL PLANTS

More than 200 pure compounds from plant sources have been reported (Marles & Farnsworth, *et. al.,* 1995) to show blood-glucose lowering activity. Table 1.6 provides a summary of the chemical classes of those compounds.

Table 1.6: Hypoglycemic Natural Products (Marles and Farnsworth, *et. al.,* 1995):

Chemical class	Number active	Chemical class	Number active
Alkaloids	38	Peptides and amines	15
Carbohydrates	66	Phenolics (simple)	4
Coumarins	4	Phenolpropanoids	1
Cyanogenic glycosides	1	Steroids	7
Flavonoids	7	Stilbenes	1
Glycopeptides	20	Sulfur compounds	2
Inorganic salts	3	Terpenoids	17
Iridoids	4	Vitamins	2
Lipids	6	Xanthenes	1

The wide variety of chemical classes indicates that a variety of mechanisms must be involved in the lowering of blood-glucose level.

Plant growth regulators such as indole-3-acetic acid (**14**) and its analogs such as indole-3-butyric acid, indole-3-propionic acid, L-tryptophan (**15**) and p-chlorophenoxyacetic acid (**16**), inhibit (Mirsky *et al.,* 1956) insulinase *in vitro* and are hypoglycemic *in vivo* in normal rats. Trigonelline (**17**), a hypoglycemic alkaloid isolated (Mirsky *et. al.,* 1956) from *Trigonella foenumgraecum* is a plant growth inhibitor that produces dormancy. Salicylic acid (**18**), is also a plant growth inhibitor as well as a hypoglycemic agent (Oliver-Bever & Zahnd, *et. al.,* 1979).
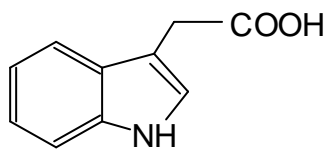
Indole-3-acetic acid (**14**)
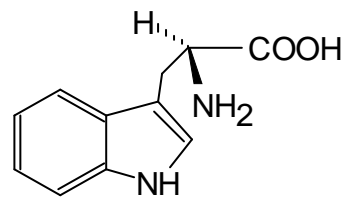
L-Tryptophan (**15**)
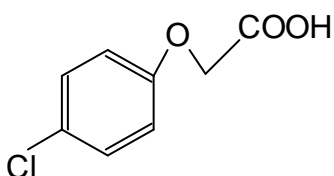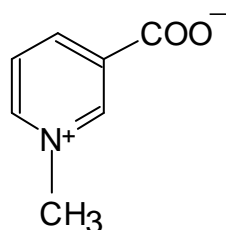
p-Chlorophenoxyacetic acid (**16**)   Trigonelline (**17**)   Salicylic acid (**18**)
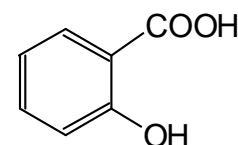
Several active compounds have been isolated from *Momordica charantina* and some of their mechanistic studies have also been done. Polypeptide-p, a 17-amino acid, 166-residue polypeptide isolated from the fruits, seeds and tissue culture seedlings was shown (Khanna *et. al.,* 1981) to be "insulinomimetic" when administered subcutaneously in rodent and primate experimental assays and in limited clinical trial with both juvenile- and maturity-onset diabetic patient. Charantin, a mixture of *β*-sitosterol-3-*O*-*β*-D-glucoside (**19**) and 5,25-stigmastadien-3-*O*-*β*-D-glucoside (**20**) isolated from *Momordica charantina* and *Momordica foetida* was found (Lotlikar & Rao, *et. al.,* 1966) to show hypoglycemic activity in normal rabbits, rats and cats.
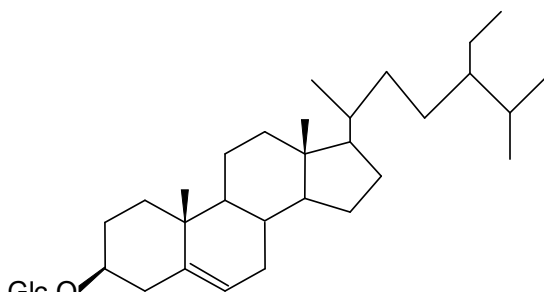
β-Sitosterol-3-*O*-D-glucoside (**19**)   5,25-Stigmastadien-3-*O*-*β*-D-glucoside (**20**)
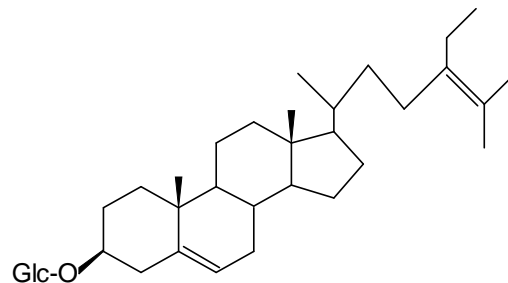
A number of alkaloid isolated (Svoboda *et. al.,*1964) from *Catharanthus roseus*, such as catharanthine (**21**), leurosine, lochnerine (**22**), tetrahydroalstonine (**23**), vindoline and

vindolinine (**24**) possess hypolycemic activity. Leurosine sulfate and vindolinine hydrochloride was found to be more hypoglycemic than the commercial antidiabetic sulfonylurea when administered orally in a dose of 100 mg/kg body weight.

The sulfur containing compounds, allyl propyl disulfide (**25**) and diallyl disulfide oxide (**26**) isolated (Oliver-Bever & Zahnd, *et. al.,* 1979; Augustia *et al.*, 1974) from *Allium cepa* and *Allium sativum* were active in normal and alloxan-diabetic animals and patients with Type 2, but not in pancreatectomized animals.

The cyclopropanoid amino acids, hypoglycin A (**27**) and its γ-L-glutamyl dipeptide, hypoglycin B (**28**), isolated (Oliver-Bever & Zahnd, *et. al.,* 1979; Feng & Patrick, *et. al.,* 1958) from *Blighia sapida* were also found to be potent hypoglycemics which appeared to act by inhibiting β-oxidase enzymes, thus blocking oxidation of long-chain fatty acids.

A lot of compounds from the plant kingdom have been reported to have hypoglycemic activity but no plant-derived compound has yet been used as a therapeutic agent. Therefore, concerted efforts should be made to isolate new hypoglycemic agent(s) from natural source, which may be used as drug.
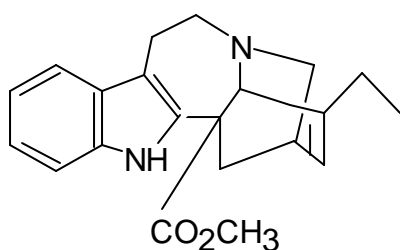
Catharanthine (**21**)
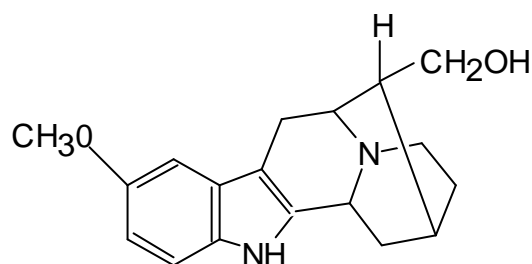
Locherine (**22**)

Tetrahydroalstonine (**23**)

Vindolinine (**24**)

CH$_2$=CH-CH$_2$-S-S-CH$_2$-CH$_2$-CH$_3$ CH$_2$=CH-CH$_2$-S(→O)-S-CH$_2$-CH=CH$_2$
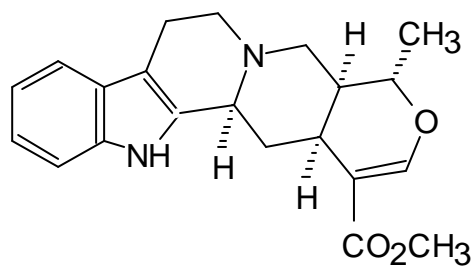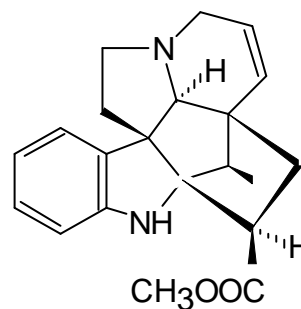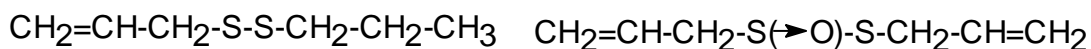
Allyl propyl disulfide (**25**)   Diallyl disulfide oxide (**26**)
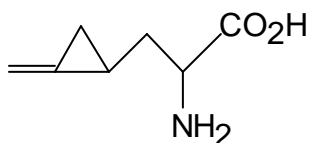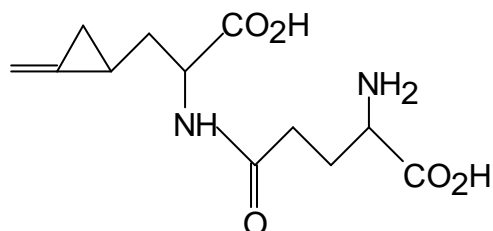
Hypoglycin A (**27**)   Hypoglycin B (**28**)

1.12 DRUGS AVAILABLE FOR THE TREATMENT OF DIABETES

Insulin is the most widely used modern drug in the treatment of diabetes mellitus which is being used as a regimen of daily injection for Type 1 diabetes. Several different preparation of bovin, porcine and human insulin (**29**) are now available in the market. Whereas Type 2 diabetic patients are treated by changing their lifestyle and diet, physical exercise and the use of oral hypoglycemic agents. Sometimes insulin is also used for the treatment of Type 2 diabetic patients. There are two chemical classes of hypoglycemic agent, sulfonylureas and biguanides. Tolbutamide (**30**), tolazamide, chlorpropamide (**31**), gliclazide (**32**), glipizide, glyburide, etc. belong to sulonylurea group and phenformin (**33**), metformin (**34**) etc. belongs to biguanide group. Another type of oral hypoglycemic agent known as acarbose (**34a**) (precose) is used to treat type 2 (noninsulin-dependent) diabetes when high blood sugar levels cannot be controlled by diet alone. Precose works by slowing the body's digestion of carbohydrates so that blood sugar levels would not surge upward after a meal.

gly-ile-val-glu-gln-cys-cys-thr-ser-ile-cys-ser-leu-tyr-gln-leu-glu-asn-tyr-cys-asn

8 9 10

phe-val-asn-gln-his-leu-cys-gly-ser-his-leu-val-glu-ala-leu-tyr-leuval-cys-gly-glu-arg-gly-phe-phe-tyr-thr

pro-lys-thr
30
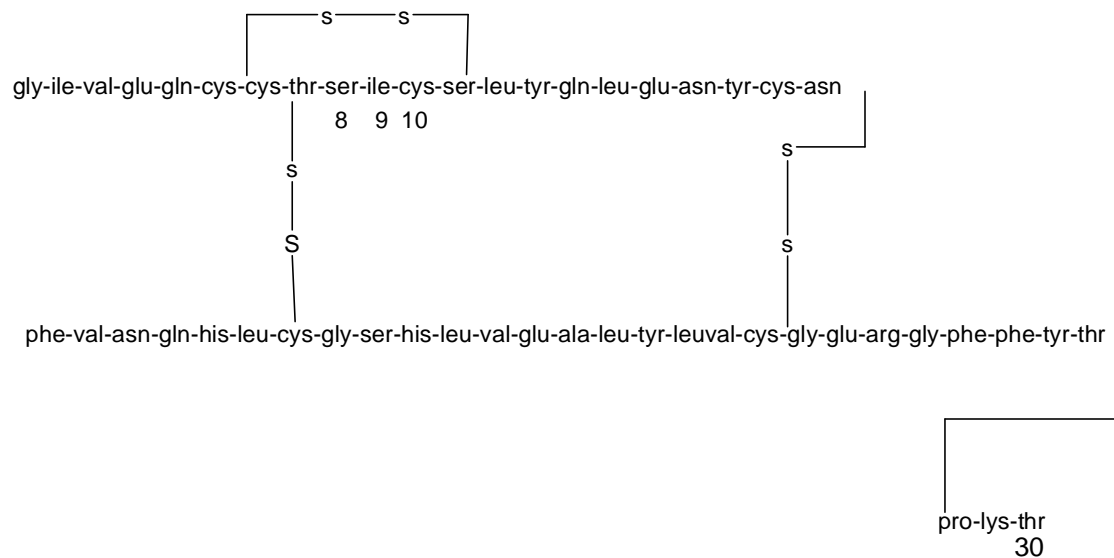
	8	9	10		30
Bovine insulin:	ala	ser	val		ala
Porcine insulin:	thr	ser	ile		ala

Insulin (**29)**
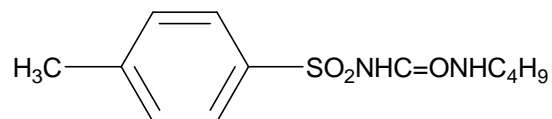
Tolbutamide (**30**)
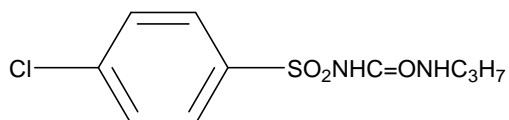
Chlorpropamide (**31**)
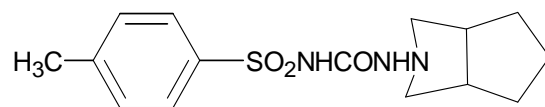
Gliclazide (**32**)
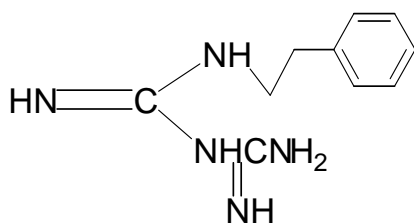
Phenformin (**33**)
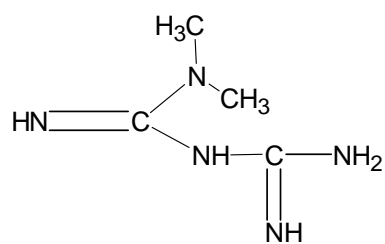
Metformin (**34**)
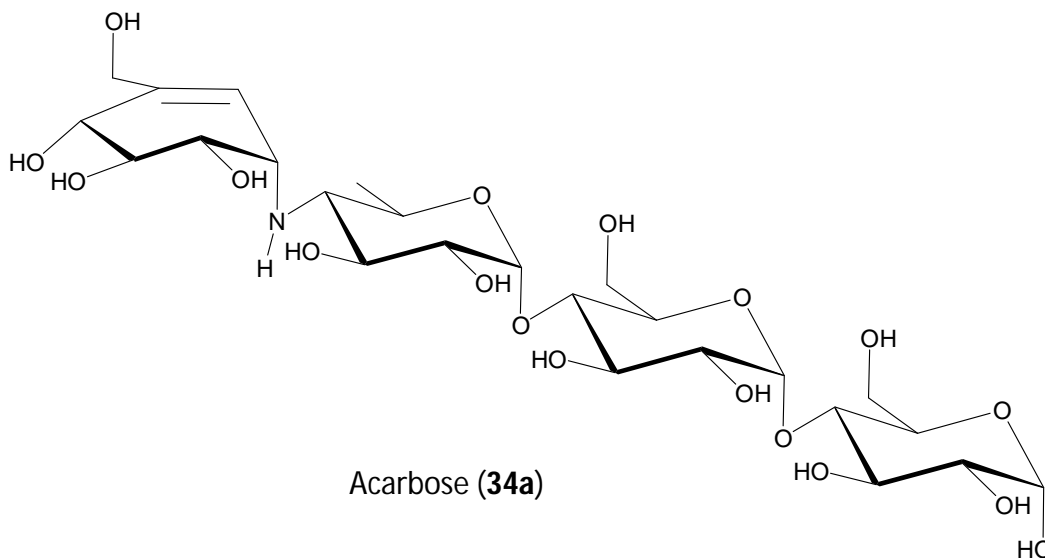
Acarbose (**34a**)

1.13 ANTIDIABETIC HERBAL PREPARATIONS (ADHPs)

Herbal Medicine is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization. Primitive man observed and appreciated the great diversity of plants available to him. The plants provided food, clothing, shelter and medicine. Much of the medicinal use of plants seems to have been developed through observations of wild animals and by trial and error (Yoganarsimhan, 2000). As time went on, each tribe added the medicinal power of herbs in their area to its knowledge base. They methodically collected information on herbs and developed well-defined herbal pharmacopoeias. Indeed, well into the 20th century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native people (Yoganarsimhan., 2000). Many conventional drugs originate from plant sources: a century ago, most of the few effective drugs were plant-based (Vickers *et. al.,* 2001).

The World Health Organization (WHO, 2008) survey indicated that about 70–80% of the world population particularly in developing countries rely on non-conventional medicines mainly of herbal origins for their primary health care. This is because herbal medicines are accessible and cheap (Sofowora, 1996). Therefore, the quality and safety of herbal preparations are also of great concern. The World Health Organization (WHO, 1993) explained that quality is the basis of reproducible efficacy and safety of herbal drugs, and to ensure the standard of research on herbal medicines, the quality of the plant materials or preparations is of utmost importance.

Eleven ADHPs (Table-1.4) and two single plants (Table-1.7) were chosen for this study.

Table-1.7: Name and code of 02 single plants

Sl No	Name of the single plants	Code
1	*Phlogacanthus thyrsiflorus* Nees (flowers)	ADSP-8
2	*Salvia haematodes* Linn (roots)	ADSP-9

1.14 *Phlogacanthus thyrsiflorus* Nees (ADSP-08)

Phlogacanthus thyrsiflorus is a gregarious shrub which belongs to the family Acanthaceae. This plant has long orange-red tubular flowers, appearing in upright spikes at the end of branches. It is commonly known as Rangabahaka or Teeta phool in Assamese and Lal basak in Bengali and Hindi. The plants can be seen growing mostly during Dec-April and is distributed throughout the tropics and in the entire North East Region of India. An evergreen shrub upto 2.4 m high, branchlets quadrangular, leaves are 13-35 cm long, oblanceolate, elliptic oblong, acute or acuminate, entire. Flowers are in terminal elongated, thyrsoid panicles, upto 30cm long. Capsule is 3.8 cm long, linear clavate. In early spring the plant becomes showy with its dense cylindrical spikes of brick red velvety flower. Calyx lobe is 6.8 mm, bristly haired. Bracts are 6 to 12 mm long. Seeds are disc like. Flowering occurs in the month of February to April (Tamang, *et. al.,* 2005). Fruits and leaves are taken by the Karbi tribes of Assam after burning them as a specific treatment for fever (Patwari, 1992). Medicinal salt extracted from the ash of whole plant is used in cases of indigestion, gastritis, pharyngitis, cough, asthma and checked acidity. The paste of root is used in case of chronic leucorrhoea. Flowers are antidote to pox, prevents skin diseases like sore, scabies etc. It has also been used in jaundice (Khanikar, 2005). It is very commonly used as a folk medicine in Assam. It is used as an anti-allergic. Curry prepared from aerial portion is given orally with rice once daily until cure (Kalita & Bora, 2008). It is also used in curing coughs and cold, chronic bronchitis, asthma and rheumatism. Different parts of the plant have been used as an anti-septic and also as a good insecticide. Leaf extract is administered orally in gout and rheumatism in Chakma community of Bangladesh (Roy, *et. al.,* 2008). Decoction of leaves is also beneficial in liver and spleen diseases (Khare, 2007). There are reports on the use of different parts of the plant as anti-septic, insecticide (Chanu KV *et. al.,* 2012) and as an anti-allergic. It is use in traditional herbal vapour therapy in Manipur to cure dry cough and pneumonia (Ningthoujam, *et. al.,* 2013). Jaintia tribe of Meghalaya uses fruit and leaf ash of *Phlogacanthus thyrsiflorus* Nees mixed in equal amount to treat fever (Jaiswal & Vidhan, 2010). The causation of several diseases of known and unknown etiologies such as Rheumatoid Arthritis, Cancer, Diabetes etc are due to generation of free radicals and compounds that can scavenge free radicals have great potential in

ameliorating these disease processes. *Phlogacanthus thyrsiflora* Nees has prominent free radical scavenging property (Kshirsagar & Upadhyay, 2009). *Phlogacanthus thyrsiflorus* Nees is used in herbal recipe during 'Bohag Bihu', the main festival of Assam (Begum & Gogoi, 2007). Fresh extract of the leaf, 2-3 tea spoons early in the morning is traditionally use by local people of Kokrajhar District of Bodoland Territorial Council, India for treatment of diabetes (Swargiary, et. al., 2013). The flower of the plant is eaten as vegetable by the ethnic communities of Tinsukia district of Assam, which is said to be useful in rheumatism, anemia and cough (Buragohain, 2011).

The leaves are reported to contain diterpene lactone, Phlogantholide, flavanoids and saponins (Khare, 2007, Singh & Singh, 2010). A new diterpene glucoside, phloganthoside has been isolated from *Phlogacanthus thyrsiflorus* and its structure has been established as phlogantholide-A-19-*O*-β-dglucopyranoside (Barua *et. al.,* 1985). Another diterpene lactone Phlogantholide-A isolated from the leaves of *Phlogacanthus thyrsiflorus* has been determined as 2β, 15,18 -trihydroxy-*ent*-labd-8(17),13-dien-16-oic lactone by chemical and spectroscopic means (Barua, *et. al.,* 1985). It was found that powdered stem bark of *Phlogacanthus thyrsiflorus* on extraction with methanol and then partioned between chloroform and petroleum ether, two labdane diterpenes namely 19 – hydroxy-labda 8(17),13-diene-15,16-olide and ent-labd-8(17), 13-dien-15,16-olide-19-oic acid (pinusolidic acid) and one triterpene betulin were isolated *(Singh & Singh, 2010).* Aqueous extracts of the plant show the presence of tannin, saponin, flavonoid, steroid, triterpenoid and phenol (Barua *et. al.,* 1985). Due to presence of this phytochemicals the plant is of medicinal value.

Phlogacanthus thyrsiflorus Nees has good Hypoglycemic activity (Ilham, *et. al.,* 2012; Chakravarty & Kalita, 2012), Hypolipidaemic activity (Tassa *et. al.,* 2012), Hepatoprotective activity (Chakravarty & Kalita, 2012), Anti-nociceptive activity (Ilham, *et. al.,* 2012), Antioxidant activity (Tassa *et. al.,* 2012), Analgesic activity (Mukherjee, *et. al.,* 2009), Antibacterial activity (Ahmed *et. al.,* 2010) and Anti-asthmatic activity (Paharia & Pandurangan, 2013).

Phlogacanthus thyrsiflorus Nees **,** locally known as Titaphool or Bagha tita is available at different places of Assam in India. It is also distributed in the different parts of

Bangladesh and India. In Bangladesh, it has been recorded from forests of Chittagong, Bandarban and Sylhet districts (Encyclopedia of Flora and Fauna of Bangladesh). It is a shrub grows in May to August. The leaves are elliptical. The Flowers are yellow in colour. Flowers taste bitter when it is taken after frying. It grows in forest and regenerated from seeds (Kar & Bortakur, 2008).

This botanical herbaceous plant is widely used as verdant foods, medicine practice and traditional medicines. Both the fresh and dry flower of the plant have been claimed to possess antidiabetic properties.

Figure-1.14: Flowers of Phlogacanthus thyrsiflorus Nees

Bengali Name : Baghatita , Ram Basak , Tamrapuspi Basak

It is an evergreen shrub upto 2.4m high , branchlets quandrangular . Leaves are 13-35 cm long , oblanceolate , elliptic – oblong , acute or acuminate , entire . Flowers are terminal elongated , thyrsoid panicles , upto 30 cm long ; corolla tubular , curved , orange or brick red villous . Capsule 38 cm long, linear – clavate . (Medicinal Plants of Bangladesh: http://www.mpbd.info/plants/phlogocanthus-thyrsiflorus.php)

Scientific Classification of *Phlogacanthus thyrsiflorus* Nees

Kingdom-*Plantae*

Phylum: *Tracheophyta*

Class: *Magnoliopsida*

Order: *Scrophulariales*

Family: *Acanthaceae*

Genus: *Phlogacanthus*

Specific epithet: *thyrsiflorus* - Nees

Botanical name: - *Phlogacanthus thyrsiflorus* Nees

Folk Names : Dieng –soh kajut (Meghalaya), Chuhai (Bihar), Titaphool (Assam)

Medicinal use:

Fruits and leaves are taken after burning as a specific treatment for fevers. Leaves are used as an expectorant in cough, bronchitis and asthma and worms. Leaf extracts are also used as external application in scabies. Medicinal uses are same as *Adhatoda zeylanica* (Yusuf *et. al.,* 2009). *Phlogacanthus thyrsiflorus* Nees is used in herbal recipeduring 'Bohag Bihu', the main festival of Assam. (Begum & Gogoi., 2007). The pharmacological studies performed on the plant revealed therapeutic potential in the treatment of inflammation, diabetes, jaundice, diarrhea, asthma, infectious diseases (e.g., due to, Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli) and prevention of oxidative stress. The scientific research on suggest a huge biological potential of this plant **(**Gogoi *et. al.,* 2013). The flower of the plant is eaten as vegetable by the ethnic communities of Tinsukia district of Assam, which is said to be useful in rheumatism, anaemia and cough. (Buragohain *et. al.,* 2011).

Antidiabetic property

Fresh extract of the leaf, 2-3 tea spoons early in the morning is traditionally use by local people of Kokrajhar District of Bodoland Territorial Council, India for treatment of diabetes. (Swargiary, *et. al.,* 2013). Decoction of bark and *Zingiber Officinale* rhizome is used by Loi community of the Thoubal district of Manipur, Northeast India (Khan & Yadava, 2010).

Abdominal disorders

One table spoon of inflorescence juice is taken after meal twice daily for 3 days to protect against intestinal worm (Kar & Bortakur, 2008).

Lungs & Airways

Flowers mixed with fish curry are considered good for relieving cough. Flowers are edible (Srivastava & Nyishi, 2010).

Parts used: Flowers

Chemical Composition of *Phlogacanthus thyrsiflorus* Nees:

The plant contains flavonoids, tannins, phytosterols, phenol, glycosides, fatty acids, galactoglycero lipid and volatile oil. (Barnali Gogoi *et. al.,* 2013)

Figure-1.15: Dry flowers of Phlogacanthus thyrsiflorus Nees.

The leaves are reported to contain diterpene lactone, Phlogantholide, flavanoids and saponins *(*Khare., 2007 and Singh & Singh., 2010). A new diterpene glucoside, phloganthoside has been isolated from *Phlogacanthus thyrsiflorus* and its structure has been established as phlogantholide-A-19-*O*-β-dglucopyranoside (Barua., *et. al.,* 1987).
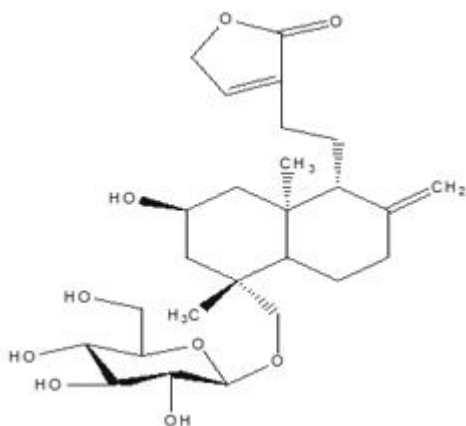
(Phloganthoside)
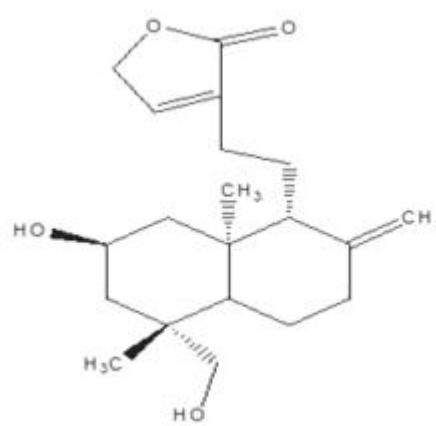
(Phlogantholide)

Another diterpene lactone Phlogantholide-A isolated from the leaves of *Phlogacanthus thyrsiflorus* has been determined as 2β, 15,18 –trihydroxy-*ent*-labd-8(17),13-dien-16-oic lactone by chemical and spectroscopic means (Barua. *et. al.,* 1985).

It was found that powdered stem bark of *Phlogacanthus thyrsiflorus* on extraction with methanol and then partioned between chloroform and petroleum ether, two labdane diterpenes namely 19 –hydroxy-labda 8(17),13-diene-15,16-olide and ent-labd-8(17), 13-dien-15,16-olide-19-oic acid (pinusolidic acid) and one triterpene betulin were isolated (Singh & Singh., 2010).

(Bitulin)

(Pinusolidic acid)

Aqueous extracts of the plant show the presence of tannin, saponin, flavonoid, steroid, triterpenoid and phenol *(Barua., et. al., 1987).* Due to presence of this phytochemicals the plant is of medicinal value.

Beta-sitosterol (Fig.13), lupeol (Fig.14), and betulin(Fig.15), were also isolated from the leaves of ***Phlogacanthus thyrsiflorus* Nees.** *(Ghani, 2003)*
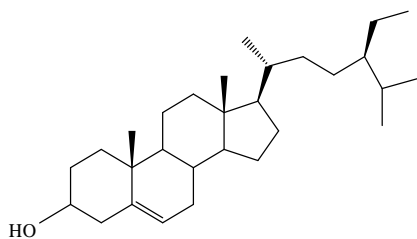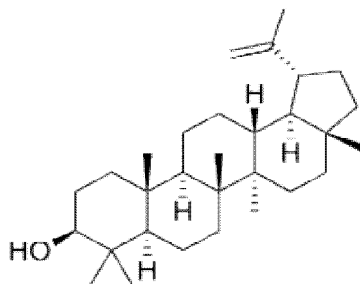
β Sitosterol   Lupeol   Betulin
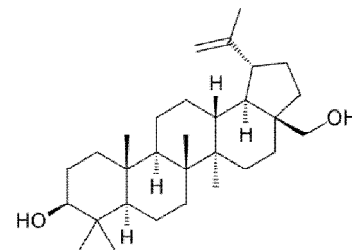
Distribution Zone:

Sub –tropical Himalayas, Upper Gangetic Plain, Bihar, North Bengal and Assam in India (Gogoi et al. 2013) and occur in the forests of Sylhet in Bangaldesh (Ghani, 2003).

1.15 *Salvia haematodes* **Linn (ADSP-09)**

The genus Salvia, one of the most important genus of Lamiaceae family, is widely used in flavouring and folk medicine all around the world (Zargari, 2003). Fifty-eight species of this genus are documented in the Flora of Iran; 17 of them are endemic (Rustayan, *et. al.,* 1999). Salvia is an important genus consisting of about 900 species in the Lamiaceae family (Rechinger & Salvia, 1982). They are several reports that some Salvia genesis has effects on the central nervous system (CNS). *Salvia haematodes* has CNS-depressant, antinociceptive and anticonvulsant activities *(*Akbar, *et. al.,* 1984 & Akbar, *et. al.,* 1985). These plants has also multiple pharmacological effects such as analgesic and anti-inflammatory (Hernandez-perez, et. al., 1995), antioxidant (Cuppett & Hall, 1998), hepatoprotective (Wasser, et. al., 1998), hypoglycemic activities (Jimenez, et. al., 1996), and anti-ischemia (Akbar, et. al., 1985; Yu, 1994).

Sage (*Salvia* species) has been used as a herb with beneficial healing properties for millennia. The name itself comes from the Latin word for health (*salvare* or heal). Ancient authors called it *elelisphakon.* This term most likely refers to several species, such as *Salvia fruticosa* Mill., *Salvia officinalis L. and Salvia pomifera* L. (Rivera *et. al.,* 1994). A tenth century Salerno School called it *Salvia salviatrix,* whereas the Spanish call it *ierba buena* or "good herb". Both terms admire feats attributed to sage. A proverb assures us, that a man who has sage in his garden needs no doctor. Sage became very popular also in China in the eighteenth century where the merchants would exchange two crates of best tea for a crate of sage (Toussaint- Samat, 1996).

Salvia haematodes Linn is an herbaceous perennial in the family Lamiaceae. Salvia is the largest genus in this family and constitutes almost one quarter of the Lamiaceae. Several species are used to treat microbial infections, cancer, malaria, inflammation, loss of memory and to disinfect homes after sickness. (Kamatou *et. al.,* 2008).

Cultivar   :   Pink Forms

Bloom Color   :   Pink

Bloom Time   :   Mid Spring, Late Spring or Early Summer

Bangla Name   :   Lal Behman

English Name   :   Red Sage, Garden, Sage, Dalmation Sage, Common Sage

Family   :   lamiaceae

Life cycle   :   perennial (Z3-10)

Flowers   :   blue-purple

Size   :   3'

Light   :   sun-part shade

Common name   :   meadow sage

Latin name   :   Salvia haematodes

Figure-1.16 Flower and steam of Salvia haematodes Linn

Plant *Salvia haematodes* Linn, Locally known as Lal bahman

Arabic Name   : Naa'ima, Saalbiya, Saalfiyaa, Bahman Abyad

Bengali Name   : Lal Behman

Chinese Name   : Dan shen

English Name   : Red Sage, Garden Sage, Dalmation Sage, Common Sage

French Name   : Sauge Rouge

German Name   : Wiesensalbei

Hindi Name   : Lal Bahaman

Latin name   : Salvia haematodes Linn.

Persian Name   : Behman Surkh

Urdu Name   : Behman Surkh

Scientific Classification of *Salvia Haematodes* Linn

Kingdom   : Plantae

(unranked)   : Angiosperms

(unranked)   : Eudicots

(unranked)   : Asterids

Order   : Lamiales

Family   : Lamiaceae

Genus   : *Salvia*

Species   : *S. haematodes*

Botanical Name *Salvia Haematodes* Linn

Medicinal Use:

S haematodes known as red sage, was found to posses significant CNS depressant (anticonvulsant) properties (Akbar *et. al.,* 1985). Further pharmacological screening revealed a broad variety of pharmacological effects. When tested in animal models, the ethanolic extract of red sage showed anti-inflammatory and analgesic effects, hypothermic response in non-pyretic rats and enhancement of the wound healing process (Akbar, 1984). The ethanolic extract of *S haematodes* had significant inotropic and chronotropic effects on isolated rabbit hearts. It also had a parasympathomimetic effect on isolated rabbit duodenum. Unfortunately, active substances responsible for these effects have been so far unknown, although different constituents are probably involved.

In South Africa, the majority of Salvia species are distributed predominantly in the Cape region. Salvia species are used in many parts of the world to treat various conditions. Many sages, if not all, form an integral part of traditional healing in South Africa particularly in regions where they occur in abundance. (Kamatou , *et. al.,* 2008).

Parts used: Root

Chemical Constituents:

Actually there is no more research had been done on this particular plant. But another five plants including Salvia lachnocalyx, Salvia indica, Salvia grossheimii, Salvia chloreleuca, Salvia cerathophylla, were collected from natural habitats of the same species (Sefid *et. al.*, 2008). The essential oils of aerial parts were extracted and then their quantities and qualities analyzed. The essential oils were identified by GC, GC/MS and the computer libraries.

Figure-1.17 Root of
Salvia Haematodes Linn

The aerial parts of *Salvia cerathophylla* were collected from Tehran province, Fasham disticts in May 1385 (Sefid *et. al.*, 2008) and its essential oil was obtained in yields of 0.1% (w/w). The major components were aromadendron (19.7%), germacrene (10.7%), and bicyc1ogermacrene (25.9%). The aerial parts of Salvia chloreleuca were collected from Golestan province-Shah koh in May 1384 and its essential oil was obtained in yields

of 0.2% (w/w). The major components were â –ocimene(13.5%), â-pinene (6.8%) and a-pinene (4.1%). The aerial parts of Salvia grossheimii were collected from Azarbayejan province-koh Mishodagh in May 1385. Its essential oil was obtained in yields of 0.4% (w/w) and the main components were germacrene (45.4%), â- caryophyllene (22.4%) and bicyclogermacrene(7.1%). The aerial parts of Salvia indica were collected from Lorestan province-Farah Kash in April 1385 and its essential oil was obtained in yields of 0.8% (w/w). Its main components were golobolol (24.35%), aromadanderon (10.1%) and germacrene (10.4%). The aerial parts of Salvia lachnocalyx were collected from Fars province-west of Abadeh in April 1384. Its essential oil was obtained in yields of 0.3% (w/w) and the major components were a-pinene (13.2%), â -pinene (10.3%), sabinene (11.7%). (Sefid *et. al.*, 2008).

Five new N-containing compounds, neosalvianen, salvianen, salvianan, salviadione and 5-(methoxymethyl)-1H-pyrrole-2-carbaldehyde were isolated from *Salvia miltiorrhiza*. Their structures were mainly established by spectroscopic methods. Neosalvianen and its analogues were synthesized for spectroscopic data comparison. Compounds (neosalvianen, salvianen, salviadione), were evaluated for their cytotoxic activities against selected cancer cell lines. Among these components, salvianen exhibited the most potent cytotoxicity with a CD_{50} range of 30.4-39.5 microM against HeLa (cervical epitheloid carcinoma), HepG2 (hepatocellular carcinoma), and OVCAR-3 (ovarian adenocarcinoma) cell lines in a dose-dependent manner. The cytotoxicities of the tested compounds were not specific and showed similar activities to the selected cancer cell lines (Don *et. al.*, 2005)

Distribution Zone:

Salvia is an annual or perennial species from labiatae family distributed all over in Iran which 17 species from 58 species existed in Iran are endemic (Sefid *et. al.,* 2008) and also grows in different area of India. (Herbalveda, https://www.herbalveda.co.uk/ index.php?dispatch=products.view&product_id=30641).
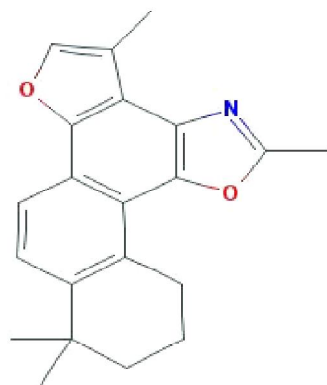
Neosalvianen
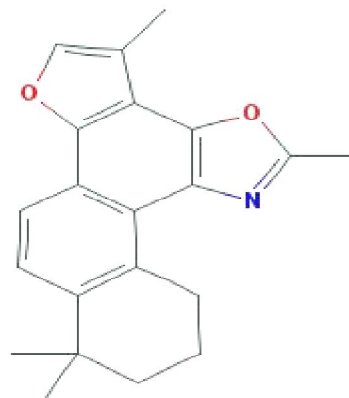
Salvianen
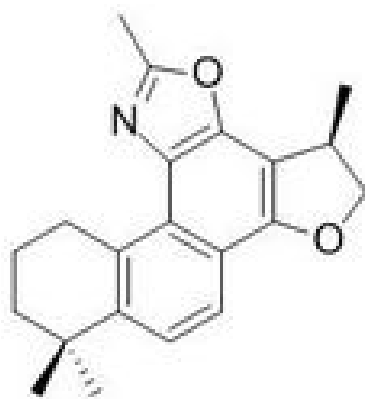
Salvianan
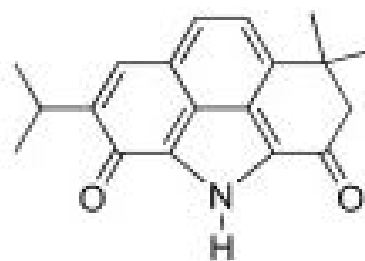
Salviadione
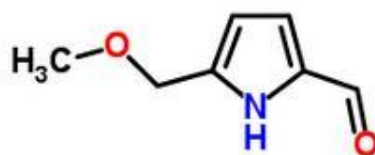
5-(methoxymethyl)-1H-pyrrole-2-carbaldehyde

1.16 OBJECTIVES OF THE PRESENT STUDY

1.16.1 General Objectives

The nature is the main source of novel chemical compounds. Plants have a complex chemical defense system, which is based on the production of a large number of chemically diverse compounds. Natural products are capable of providing the complex molecules that would not be accessible by other way. Around 5-15% of the total higher plants have been investigated and a large number of plants and marine species remain uninvestigated. In addition, only less than 1% of bacterial and 5% of fungal species are currently known (Crag & Newman, 2000). Microorganisms have a great potential to yield novel bio-active compounds, because they survive in extreme and competitive environments.

The use of traditional plant for the treatments of diabetes mellitus dates from the Papyrus Ebers of 1500 BC that recommended a high fiber diet of wheat grains and ochre (Bailey *et al.,* 1989). Many herbs, spices, and other plant materials had since been described for the treatment of diabetes. Traditional plant treatments remain the major form of therapy for diabetes. A total of more than 400 species were reported to display hypoglycemic effects, but few of them have been investigated scientifically (Bailey et. al., 1989). The ethnobotanical information reports about 800 plants that may possess anti-diabetic potential (Alarcon-Aguilara *et al.*, 1998). So far, 90 plants for hypoglycemic properties have been screened from Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) and neighboring countries. Among them *Trigonella foenumgraecum, Allium cepa, Hemidesmus indicus, Syzigium cumini, Murraya koegin*, *Aloe vera* etc are worth mentioning.

Bangladesh has a rich treasure of plants, herbs and trees. A lot of plants are being used for the management of diabetes by the traditional healers in the country. There are many herbal producers producing many herbal medicines for different kinds of diseases. The Research Group at BIRDEM and BUHS have been working for several years on plant materials having hypoglycemic activity and to isolate new hypogycemic agents which may be used for therapeutic purpose.

Some herbal preparations which are used for antidiabetic effect in the local market in Bangladesh were selected for the present studies for (for chemical and biological) evaluating their safety and efficacy. The plant material in the most active ADHP will be chosen for chemical and biological studies. Any of the single material reported to be used as antidiabetic remedy in the folklore will also be studied.

1.16.2 Specific Objectives

A. Biological:

1. Hypoglycemic effects on T2DM rats
    a) To examine the glycemic status by determining serum fasting blood glucose level.
    b) To assess the insulinemic status by determining the serum insulin level.
    c) To determine the status of glycogenesis by estimating the liver glycogen content.
    d) To examine the lipidemic status by determining serum total cholesterol (Chol), Triglyceride (TG) and high density lipoprotein-cholesterol (HDL).
    e) To determine the oxidation status by estimating levels of plasma malondialdehyde (MDA) and erythrocyte reduced glutathione (GSH).
    f) Estimation of fasting serum creatinine by Enzymatic Colorimetric (GOD-POD) Method.

2. Alpha glucosidase inhibitor activity test

3. Sun skin protection factor test

4. Antioxidant activity test of ADHPs and plant materials.

5. Test for possible bacterial contamination

6. Insulin secratory activity

B. Chemical:

1. Isolation of some compounds from two different plant materials.

2. Toxic metal analysis

3. Organochlorine pesticides testing

4. Analysis of deliberately added synthetic drugs (if any)

5. Quantification of antioxidant components like total phenolic content, total flavonoid content, total tanin content.

6. Identification and quantification of antioxidants

1.17 EXPECTED OUTCOMES

Herbal medicines (HMs) include herbs, herbal materials, herbal preparations and finished herbal products. Such medicinal preparations have been used since ancient times to treat a wide range of diseases. The World Health Organization (WHO) survey indicated that about 70-80% of the world population particularly in the developing countries rely on non-conventional medicines mainly of herbal origins for their primary health care. This is because herbal medicines are relatively accessible and cheaper than the synthetic drugs. Once the above mentioned biological & chemical parameters are determined, it is expected that concrete evaluation of the HMs and plants will be evaluated. A big proportion of people directly and indirectly depend on herbal medicines and plants for their primary healthcare. But these HMs are not scientifically examined and free from toxic metals in all the developing countries especially in Bangladesh.

A number of HMs manufacturers are producing bulk amount of finished products with the basis of non significant formulations. Antidiabetic effectiveness and safety of some antidiabetic herbal preparations (ADHPs) will be clarified from this study. The ADHPs will be bought from the local market of Dhaka city in Bangladesh. Some biological activity like antidiabetic activity on T2DM rats, α-glucosidase inhibitor activity test, antioxidant activity test, SPF test, microbial activity test by these ADHPs and insulin secratory activity of isolated compounds from plants will be evaluated. After the biological activity evaluation of ADHPs and plants and active preparations & plant will be selected for the chemical studies.

Medicinal plants from time immemorial have been used frequently for the treatment of diabetes. Plants will be selected from the active formulations for the active compound

isolation from different extracts. Some other chemical analysis like toxic metal analysis, organochlorine pesticides analysis, deliberately added synthetic drugs analysis and identification and quantification of antioxidants will be evaluated for the human being. After confirming the chemical and biochemical evaluation some ADHPs and plant (s) they may be designated as safe or unsafe for using as an antidiabetic agent, which will be a big achievement. The results will be benificial for the people of Bangladesh as well as the developing countries.

2. MATERIALS AND METHODS

2.0 MATERIALS AND METHOD

A. BIOLOGICAL INVESTIGATIONS

2.1 ANTIDIABETIC ACTIVITY

2.1.1. Collection of Antidiabetic Herbal Preparations (ADHPs) and ADSPs

In this study, several antidiabetic herbal preparations were collected from local herbal drug stores in Dhaka City. Eleven ADHPs were screened at BIRDEM for their efficacy in reducing blood glucose in type 2 diabetic Long Evans model rats produced at BIRDEM and BUHS. Seven drugs showed positive response in acute tests. These seven drugs were fed to diabetic model rats for 28 days and some of them showed significant hypoglycemic activity. From the given label of these seven active drugs, only one plant material *Salvia haematodes* Linn was used in one of the drugs. So, this plant was chosen for further studies and the root of this plant was collected from Hamdard Laboratories (wafq) Bangladesh, Meghnaghat, Sonargaon, Narayangonj, Dhaka, Bangladesh.

Another plant *Phlogacanthus thyrsiflorus* Nees is used for blood glucose control in South Eastern India. The dried flower of *Phlogacanthus thyrsiflorus* Nees was collected from Assam, India. The collected materials were dried in a dryer at 40^0C and pulverized to powder.

Roots of *Salvia haematodes* Linn and flower of *Phlogacanthus thyrsiflorus* Nees were identified by taxonomist of Bangladesh National Herbarium (BNH), Mirpur, Dhaka. The accession number for *S haematodes* root is **42993** and for *P thyrsiflorus* flower is **42992.**

Some antidiabetic herbal predations (ADHPs) are producing by the different herbal medicines producers in Bangladesh. A short description of these ADHPs is given in Table 1.4 (page 33).

ADSP-08 (*Phlogacanthus thyrsiflorus* Nees)

Phlogacanthus thyrsiflorus Nees has good hypoglycemic activity (Ilham S, *et. al.,* 2012; Chakravarty & Kalita, 2012)*,* hypolipidaemic activity (Tassa, *et. al.,* 2012), hepatoprotective activity (Chakravarty & Kalita, 2012)*,* anti-nociceptive activity (Ilham, *et. al.,* 2012), antioxidant activity (Tassa, *et. al.,* 2012), analgesic activity (Mukherjee , *et. al.,* 2009), antibacterial activity (Ahmed, *et. al.,* 2010) and anti-asthmatic activity (Paharia & Pandurangan , 2013).

**ADSP-09 (*Salvia haematodes* Linn*)*

S. haematodes, known as red sage, was found to posses significant central nervous system (CNS) depressant (anticonvulsant) properties (Akbar *et. al.,* 1985). Further pharmacological screening revealed a broad variety of pharmacological effects. When tested in animal models, the ethanolic extract of red sage showed anti-inflammatory and analgesic effects, hypothermic response in non-pyretic rats and enhancement of the wound healing process (Akbar *et. al.,* 1984). The ethanolic extract of *S. haematodes* had significant inotropic and chronotropic effects on isolated rabbit hearts. It also had a parasympathomimetic effect on isolated rabbit duodenum. Unfortunately, active substances responsible for these effects have been so far unknown, although different constituents are probably involved.

2.1.2 Antidiabetic Study on T2DM Rats

2.1.2.1. Animals

Adult Long Evans rats weighing 190-260 g were included in the study. The animals were bred at Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) and BUHS animal house, Dhaka, Bangladesh, maintained at a constant room temperature of 22 ± 5^{0}C with humidity of 40-70 % and the

natural 12 hours day-night cycle. The rats were fed on a standard laboratory pellet diet and water supplied *ad libitum*. Standard rat pallet contained wheat (40%), wheat bran (20%), rice polishings (5%), fish meal (10%), oil cake (10%), gram (3.9%), pulses (3.9%), milk (3.8%), soyabean oil (1.5%), molasses (0.95%) and salt (0.95%). Embavit GS (vitamin mixture) 250 g was added per 100 kg of rat food. The influence of circadian rhythm was avoided by starting all experiments at 7:30 am. The experiments were conducted according to the ethical guidelines approved by the Bangladesh Association for Laboratory Animal Science.

2.1.2. 2. Place of the Study and Study Period

The study was conducted in the Department of Pharmacology, Biomedical Research Group (BMRG), BIRDEM and Department of Pharmacology, Bangladesh University of Health Sciences, Dhaka, Bangladesh. The study was done during the period of 2011 to 2015.

2.1.3.1.Preparation of ADHPs Extract

For the preparation of ADHPs extract, solid (Tablet & Capsule) ADHPs were ground to powder and mixed with water. For the liquids (syrup), they were fed directly in the original form according to the established procedure.

2.1.3.2 Preparation of Ethanol Extract of *P thyrsiflorus* flowers.

In the laboratory, dried *P thyrsiflorus* flowers were heated at 40^0C for 10 minutes. The weight of the flowers was recorded before and after heating. After that the flowers were grinded and weighed which was about 1 kg.

One kg fine flowers powder was dissolved in 4.5 liters of aq 80% ethanol at 1:6 ratio and kept overnight in freezing condition. This suspension was filtered with thin and clean cloth and then by filter paper and 2.4 liter filtrate was collected. Using BUCHI Rota vapor R-114, the main extract of *P thyrsiflorus* was retrieved. The BUCHI water bath was maintained at 55^0C to evaporate ethanol which was distilled in the receiving flask. As a result, a total of 220 mL of extract was obtained.

The semi-dried extract was further dried in a freeze drier (HETOSICC, Heto Lab Equipment Denmark) at -55^{0}C temperature and stored in a reagent bottle at -8^{0}C in a freezer. The dried extract was finally weighed using Digital balance GIBERTINI E 42-B and 100 g of ethanol extract of *P thyrsiflorus* flowers was obtained.

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┌─────────────────────────────────────────────────────┐
│   Grinding of dried flowers of P. thyrsiflorus Nees   │
└─────────────────────────────────────────────────────┘
                         │
                         ▼
┌─────────────────────────────────────────────────────┐
│         Extraction using aq 80% ethanol solvent        │
└─────────────────────────────────────────────────────┘
                         │
                         ▼
┌─────────────────────────────────────────────────────┐
│      Concentrated at low temperature under reduced     │
│              pressure rotary evaporation               │
└─────────────────────────────────────────────────────┘
                         │
                         ▼
┌─────────────────────────────────────────────────────┐
│                    Freeze drying                       │
└─────────────────────────────────────────────────────┘
                         │
                         ▼
┌─────────────────────────────────────────────────────┐
│            Ethanol extract of dried flowers            │
└─────────────────────────────────────────────────────┘
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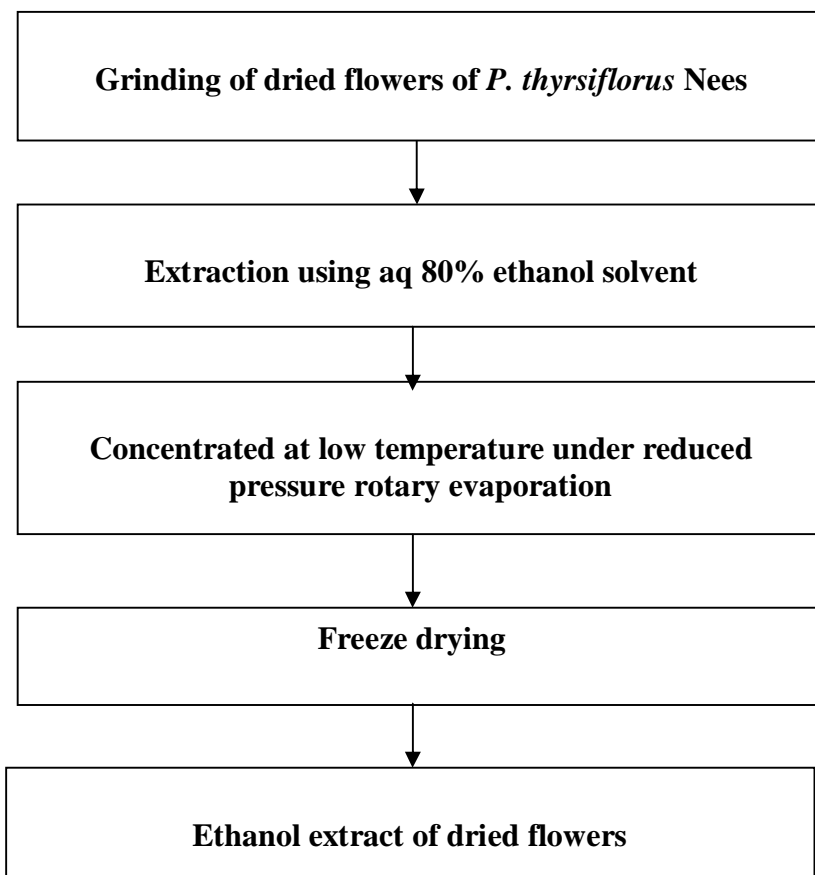
Figure- 2.1.1. Protocol for preparation of ethanol extract of P thyrsiflorus flowers

2.1.3.3 Preparation of Type 2 Diabetes Model Rats

Type 2 diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) in citrate buffer (10 mL), at a dose of 90 mg/kg body weight to the rat pups (48 hours old, average weight 7 g) as described by (Bonner *et. al.,* 1981) (Figure 2.1.2). Experiments were carried out 3 months after STZ injection. Model rats were selected for the experiment after scrutiny by the oral glucose tolerance test (OGTT). Diabetic model rats having blood glucose level 7-9 mmol/L at fasting condition were taken to carry out the experiments.
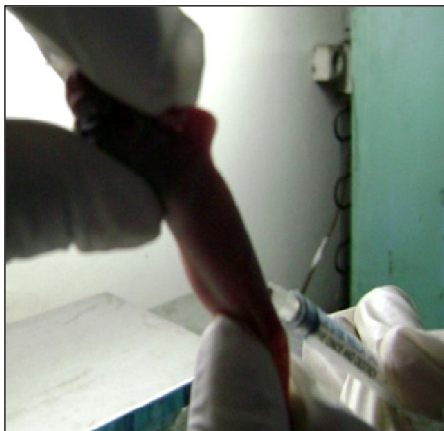
Figure-2.1.2 Injection of Streptozotocin for Type 2 Model Rats

2.1.3.4. Formulation of Glibenclamide and extract dose

For all the pharmacological studies, the drug glibenclamide was prepared at a dose of 5 mg per 10 mL of solvent (9.9 ml H_2O + 0.1 mL Tween 20)/kg body weight of T2DM rat models. The formulation of the extract involves mixing of 1.25 g extract per 10 mL of H_2O per kg body weight of T2DM rats.

2.1.3.5 Dose and Route of Administration

For the evaluation of antidiabetic activity, screening was done with a single feeding simultaneously with glucose load or 30 minutes before glucose load (500 mg/kg bw) with the water extracts of 11 ADHPs at a calculated dose of 1.25 g/kg bw per 10 mL. For chronic test water extract of seven ADHPs and the ethanol extract of flowers of *P thyrsifllorus* were administrated orally to the T2DM rats for 28 days at a dose of 1.25 g/kg body weight (Figure 2.1.3). For all the pharmacological studies, the drug glibenclamide was administrated orally at a dose of 5 mg/10 mL (9.9 mL H_2O + 0.1 mL Tween 20)/kg body weight for type 2 model rats. For the control groups, 10 mL water was administrated per kg body weight.

2.1.3.6. Experimental design for 11 ADHPs

A total 215 type 2 diabetic model rats were used in acute and chronic (28 days) experimental period.They were divided in 3 groups shown in Table 2.1.2 amd 2.1.3.

Figure-2.1.3 Administration of extract to the T2 DM Rats

Table 2.1.1 Acute experimental groups

Group-1 (n=20)	Type 2 water control group
	[10 mL water/per kg body weight]
Group -2 (n=20)	Type 2 glibenclamide group
	[5mg/10 mL (9.9 mL water+0.1mL Tween 20)/kg body weight]
Group -3 (n=80)	11 ADHP's extracts treated group
	(500 mg/kg body weight)

Table 2.1.2 Chronic experimental groups

Group-1 (n=13)	Type 2 water control group
	[10 mL water/per kg body weight]
Group -2 (n=13)	Type 2 glibenclamide group
	[5mg/10 mL(9.9 mL water+0.1 mL Twin 20)/kg body weight]
Group -3 (n=69)	07 ADHP's extracts treated group
	(1.25g/kg body weight)

2.1.3.7. Chronic experimental protocol for administration of 07 ADHPs extracts

It was a case control intervention study and was conducted according to following protocol:

ADHP's extracts (n=38)

Water (n=13)

Glibenclamide (n=13)

Oral administration each day, from 0th to 28th

Subchronic Rat model of T2DM

STZ injection

0 day 2nd day 90th day

Recording of body weight on 0, 7th, 14th, 21st and 28th days

Birth of pups

0 day 7th day 14th day 21st day 28th ay

Blood collection from tail tips

Sacrifice

Determination of fasting serum glucose, cholesterol, TG, HDL-C and insulin.

Determination of fasting serum glucose.

Cervical venous blood collection.

Collection of rat liver, kidney and spleen.

Determination of fasting serum glucose, cholesterol, TG, HDL-C and insulin.

Estimation of plasma MDA and erythrocyte GSH.

Estimation of liver glycogen.

Statistical analysis

Figure-2.1.4 Experimental design of the study

2.1.3.8. Experimental design for *P thyrsifllorus*

A total 18 T2DM rats were used in this 28 days experimental period. They were divided in 3 groups shown in Table 2.1.4.

Table 2.1.3 Experimental groups

Group-1(n=6)	Type 2 water control group
	[10 mL water/per kg body weight]
Group -2(n=6)	Type 2 glibenclamide group
	[5mg/10 mL (9.9 mL water + 0.1 mL Tween 20)/kg body weight]
Group -3(n=6)	Ethanolic extract of *P.thyrsiflorus* Nees flowers treated group
	(1.25 g/kg body weight)

2.1.3.9. Body Weight Measurement

All the rats were kept at constant environmental conditions and were provided with enough food and water throughout the experiment. The body weight of each rat was measured at seven days interval (Figure 2.1.5) and accordingly they were fed with their respective treatment.

Figure-2.1.5 Body Weight Measurement of T2 DM Rat

2.1.3.10. Experimental protocol for administration of *P thyrsiflorus* flowers extract

It was a case control intervention study and was conducted according to following protocol:

Ethanol extract (n=6)

Water (n=6)

Glibenclamide (n=6)

Oral administration each day, from 0th to 28th

Subchronic Rat model of T2DM

STZ injection

Recording of body weight on 0, 7th, 14th, 21st and 28th days

0 day 2nd day 90th day

Birth of pups

0 day 7th day 14th day 21st day 28th day

Blood collection from tail tips

Sacrifice

Determination of fasting serum glucose, cholesterol, TG, HDL-C and insulin.

Determination of fasting serum glucose.

Cervical venous blood collection.

Collection of rat liver, kidney and spleen.

Determination of fasting serum glucose, cholesterol, TG, HDL-C and insulin.

Estimation of plasma MDA and erythrocyte GSH.

Estimation of liver glycogen.

Statistical analysis

Figure- 2.1.6 Experimental design of the study

2.1.3.11 Blood Collection Procedure for Biochemical Analysis

The rats were kept fasting 12 hours for both (Acute & Chronic) kinds of experimentations. For the acute study blood were collected at several times (0, 30, 75 and 120 Mins) from T2DM rats. Extracts were fed simultaneously or 30 minutes before glucose load.

The rats were kept fasting for 12 hours on the 0 and 14th day. Blood were collected from these fasting rats by amputation of the tail tip under diethyl ether anesthesia (Figure 2.1.6).
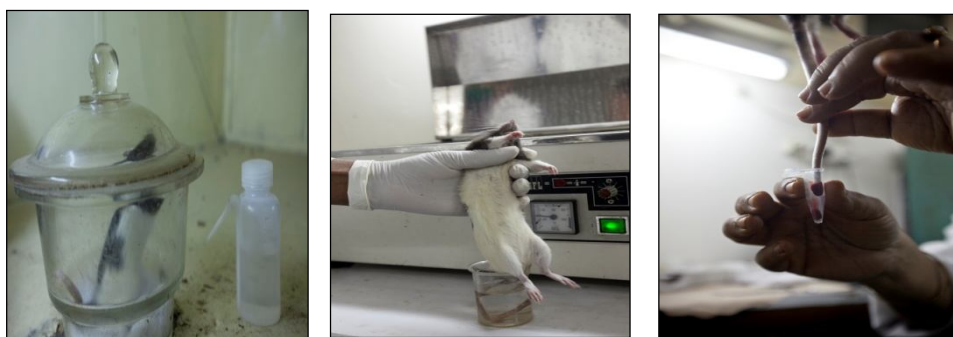
Figure- 2.1.7 Blood collection procedure

Just before the amputation, the tail was immersed into warm water (40^0C) for approximately 20-30 seconds for vasodialation. After cutting the tail tip, about 0.2 mL blood was taken cautiously in eppendorf tube to avoid haemolysis. On the 28th day the animals were decapitated and their blood was collected from heart (Figure: 2.1.7).

Figure-2.1.8 Blood collection from heart

The collected blood samples were centrifuged at 2500 rpm for 15 minutes and the serums were separated into another eppendorf tube (Figure 2.1.8). Serum was aliquoted and kept frozen at -20^{0}C until analysis.

Figure-2.1.9 Separation of serum after centrifugation from the blood.

2.1.4. Biochemical Analysis

The following parameters of type 2 diabetic model rats were measured for the anti-diabetic effect of *P thyrsiflorus* Nees flowers extract

- Serum glucose was measured by Glucose Oxidase (GOD-POD) method using micro-plate reader (Bio-Tec, ELISA).

- Serum total cholesterol by enzymatic colorimetric (Cholesterol Oxidase /Peroxidase, CHOD-PAP) method (Randox Laboratories Ltd., UK), using autoanalyzer, AutoLab.

- Serum triglyceride (TG) by enzymatic colorimetric (GPO-PAP) method (Randox Laboratories Ltd., UK) using autoanalyzer, AutoLab.

- Serum insulin by rat insulin enzyme linked immunosorbent assay (ELISA) method. (Crystal Chem Inc., USA).

- Measurement of glycogen from rat liver by standard Anthrone-sulphuric acid method.

- Estimation of hemoglobin in blood by hemoglobincyanide method. (Van-Kampen and Zijlstra, 1961)

- Estimation of plasma Malondialdehyde (MDA) level by thiobarbituric acid method (Janero, 1990).

- Estimation of reduced Glutathione (GSH) concentration in erythrocytes by Ellman's method. (Ellman, 1959)

2.1.4.1 Serum Glucose Estimation by Glucose Oxidase peroxidase (GOD-POD) Method (Boehringer- Mannheim Gmbh)

Principle

The aldehyde group of β-D-glucose is oxidized by glucose oxidase to produce gluconic acid and hydrogen peroxide. Hydrogen peroxide is further broken down to water and oxygen in the presence of peroxidase and in presence of an oxygen acceptor (i.e., phenol) to produce a colored compound. The reaction of GOD-POD reagent with glucose produces 4-aminophenazone, a red colored compound (Barham *et al.,* 1972).

$$\text{β-D-glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone- mono-imino) phenazone} + 4H_2O.$$
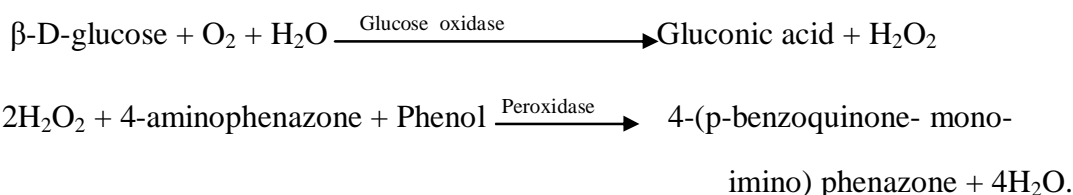
Reagents:

(a) Buffer : Phosphate buffer (0.1 mol/l, ph 7.0),
Phenol (11mol/l)

(b) GOD-POD Reagent : 4-aminophenazone (0.77 mmol/L),
Glucose oxidase ($\geq$ 1.5 kU/l)

Peroxidase ($\geq$1.5 kU/l)

(c) Standard Glucose (5.55 mmol/L).

Preparation of the standard glucose solution:

A sample of 36 mg of glucose was taken in a clean stopper test tube and then dissolved in 10 mL of water to make a stock solution of 20 mmol/L. The stock solution was diluted to produce different concentration (20, 16, 12, 8, 4, 2 and 1 mmol/L) of standard glucose solutions with deionized water.

Procedure:

While pipetting into the wells, the first two wells were kept blank and the 7 standard glucose solutions (5 μL) of each concentration were pipetted in the next 7 wells of the micro-plate with duplicates. The serum samples (5 μL) (Figure 2.1.9) were pipetted in the remaining micro-wells of the plate, each of them were pipeted twice. GOD-PAP reagent (250 μL) was next added in all the wells (Figure 2.1.10). The plate was then incubated in Labsystems iEMS Shaker incubator (Figure 2.1.11) for proper dilution for 15 minutes at 37^0C and, using the Ultra micro-plate ELISA Reader (Bio-Tek ELx 808, USA), the absorbance of the samples (Figure 2.1.12) were read at 512 nm.

Figure- 2.1.10 Serum samples of rats.

Figure 2.1.11 GOD-POD reagents being added to all the samples for determination of glucose.

Two duplicate experiments were carried out for each sample. Thus, a calibration curve was obtained for the absorbance vs. concentration of the standard solutions against a reagent blank (Figure 2.1.13). Based on the calibration curve, the unknown concentrations of glucose in the serum sample were measured maintaining the same mixing and incubation conditions as for the standard solutions. The standard curve was drawn parallel on every experimental day. Reading paper was collected from Epson LP-300+.

Figure-2.1.12 Lab system iEMS incubator.
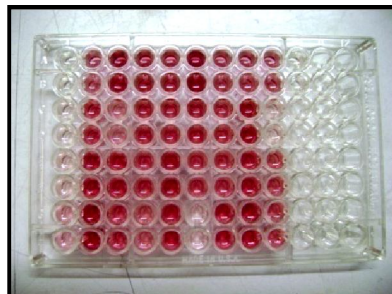
Figure- 2.1.13 Microtiter plate with samples.
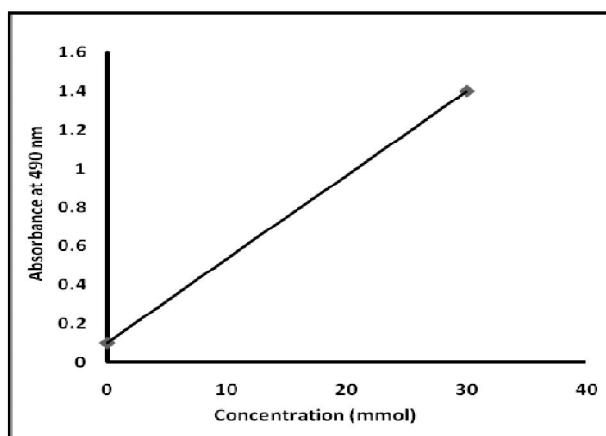
Figure-2.1.14 Standard curves for glucose.

Calculation:

In this GOD-POD method (Boehringer-Mannheim GmbH), the concentration was calculated by using the kinetic calculation program for Ultra microplate ELISA reader (Bio-Tek Elx-808, USA).

Calculation of result for unknown sample was as follows:

$$\text{Conc. of unknown sample} = \frac{\text{Optical density of unknown sample}}{\text{Optical density of standard}} \times \text{Standard Conc.}$$

$$\text{Glucose concentration (mmol/L)} = \frac{A_{sample}}{A_{standard}} \times 5.55$$

$$\text{Glucose concentration (mg/dl)} = \frac{A_{sample}}{A_{standard}} \times 100$$

Normal values: Serum, 4.2-6.4 mmol/L or 75-115 mg/dl (Teuscher *et. al.,* 1971).

2.1.4.2 Estimation of Serum Total Cholesterol by Enzymatic Colorimetric (Cholesterol Oxidase / Peroxidase) Method

Principle:

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine was formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase (Trinder, 1969).

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

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimmine} + 4H_2O$$
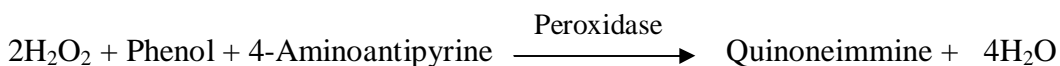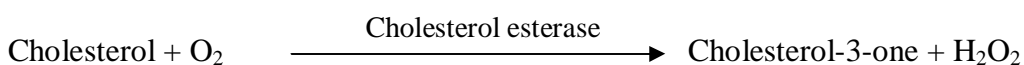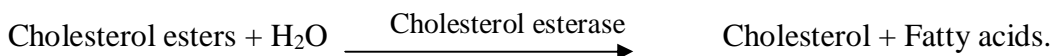
Reagent Composition:

All reagents were purchased from RANDOX, UK for the determination of the levels of cholesterol in human and rat serum samples.

Content	Initial Concentration Of Solution
Reagent	
4-Aminoantipyrine	0.30 mmol/L
Phenol	6 mmol/L
Peroxidase	>0.5 U/mL
Cholesterol esterase	>0.15U/mL
Cholesterol Oxidase	>0.1 U/mL
Pipes Buffer	80 mmol/L; pH 6.8
Standard	5.17 mmol/L (200 mg/dl)

Materials:

Micro-centrifuge tube

Micropipettes and pipettes

Automatic Analyzer (Boehringer Mannheim, 704; HITACHI)

Procedure:

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the Automatic Analyzer. 5 µl sample and 500 µL reagent were mixed and incubated at 37^0C for 5 min within the Auto Analyzer. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 min.

Calculation of result:

Concentration of cholesterol in sample was calculated by using software program with the following formula and expressed in mg/dl.

Cholesterol concentration (mg/dl) = $\dfrac{\Delta A \, \text{sample}}{\Delta A \, \text{standard}} \times$ Concentration of standard

2.1.4.3 Estimation of serum Triglyceride (TG) by Enzymatic Colorimetric Method

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of Randox Laboratories Ltd., UK.

Principle:

Sample triglycerides incubated with a lipoprotein lipase liberate glycerol and fatty acids. Glycerol is converted to glycerol-3-phosphate by glycerol kinase and ATP. Glycerol-3-phosphate oxidase (GPO) oxidizes glycerol-3-phosphate into dihydroxy acetone phosphate and H_2O_2. In the presence of peroxidase, hydrogen peroxide oxidizes the chromogen-4-aminoantipyrine and 4-chlorophenol to a violet colored complex. The

quinone formed is proportional to the amount of triglycerides present in the sample. The principle is based on the following reaction system (Fossati *et al.,* 1982).

Triglyceride $+H_2O$ $\xrightarrow{\textit{Lipase}}$ glycerol + Fatty acids

Glycerol + ATP $\xrightarrow[\textit{Kinase}]{\textit{Glycerol}}$ glycerol-3-phosphate + ADP

Glycerol-3-phosphate $+O_2$ $\xrightarrow[\textit{Oxidase}]{\substack{\textit{Glycerol}\\\textit{Phosphate}}}$ dihydroxy acetone phosphate$+ H_2O_2$

$2H_2O_2$ + 4-aminophenazone +4- chlorophenol $\xrightarrow{\textit{Peroxidase}}$ quinoneimine + HCl +4 H_2O
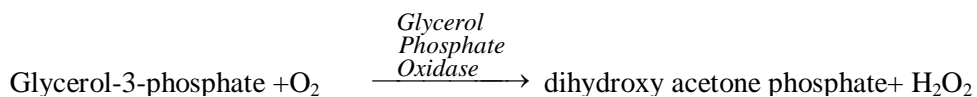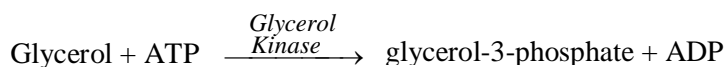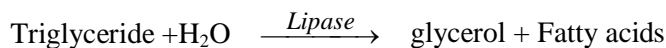
Reagents:

Content	Concentrations in the Test
Buffer	
Pipes Buffer	40 mmol/L, pH 7.6
4-choloro-phenol	5.5 mmol/L
Magnesium-ions	17.5 mmol/L
ATP	1.0 mmol/L
Lipases	>150 U/mL
Glycerol-3-phosphate oxidase	1.5 U/mL
Peroxidas	0.5 U/mL
Standard	2.29 mmol/L (200 mg/dl)

Materials:

- Micropipettes and pipettes
- Disposable tips
- Auto analyzer

Procedure:

Serum and reagents were taken in specific cup. They were arranged serially. Then ID number for test was entered in the analyzer. Five (5) μl sample and 500 μL reagent were mixed and incubated at 37^0C for 5 min within the cell. Reading was taken at 500 nm.

Calculation of result:

Triglyceride concentration was calculated by following formula: Triglyceride concentration (mg/dl) = $\dfrac{A_{Sample}}{A_{S\tan dard}} \times$ Concentration of standard

2.1.4.4 Determination of Serum HDL-Cholesterol

Principle

HDL lipoproteins are assayed, after precipitation of LDL and very low density lipoproteins (VLDL) with PEG (Polyethylene glycol) 6000, measuring their content of cholesterol or phospholipids. This measurement seems to be more reliable than high density proteins one.

Composition of precipitating reagent:

Solution of PEG 6000 at 14.5%; preservatives and surfactant.

Procedure:

50 μL of serum and 100 μL of precipitating reagent were pippeted in to an eppendorf tube. The mixture was mixed well and centrifuged at 4000 rpm for 10 min. After centrifugation 5 μL of supernatant was taken and further procedure was followed by the procedure of cholesterol estimation.

The result was multiplied by 3 (The dilution factor of the sample).

Calculation:

$$\text{Serum HDL-C (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard} \times \text{dilution factor.}$$

2.1.4.5 Determination of Serum LDL-Cholesterol

Serum LDL-cholesterol was determined from the values of serum total cholesterol, triglyceride and HDL-cholesterol by using the following equation:

Serum LDL-cholesterol= Serum total cholesterol – (HDL-cholesterol + Triglyceride/5).

2.1.4.6 Estimation of Serum Insulin

Serum insulin was estimated by the Rat Insulin ELISA (Enzyme Linked Immunosorbant Assay) kit of Crystal Chem Inc. USA.

Principle of the assay (Kratzsch *et. al*., 1990):

Rat insulin ELISA Kit reagents:
Contents of the kit:

1. Antibody-coated Micro-plate, Lyophilized: 2 packs. (One pack contains 6x8 well modules, i.e., 48 wells in each pack).

2. Rat Insulin Standard, Lyophilized: 2 ng/vial (for 200 μL).

3. Sample Diluent 1, Lyophilized: 1bottle (for 50 mL).

4. Sample Diluent 2, Lyophilized: 1bottle (for 30 mL).

5. Guinea pig Anti-insulin, Lyophilized Serum: 2 vials (for 3 mL each).

6. Anti-Guinea pig Antibody Enzyme Conjugate, Lyophilized: 2 vials (for 6 mL each).

7. O-Phenylenediamine (OPD) tablet: 2 tablets (10 mg/tablet).

8. Enzyme Substrate Diluent (phosphate-citrate buffer containing H_2O_2): 1 bottle (30 mL).

9. Enzyme Reaction Stopping Solution (1N Sulfuric acid): 1 vial (6 mL).

10. Phosphate Buffered Saline (PBS) powder: 1 bottle (for 500 mL).

20% Tween 20: 1 vial (1.25 mL).

11. Frame for fixing the micro-plate well module 1 piece.

12. Plastic micro-plate covers 1 piece.

Materials required:

- Micropipettes and disposable tips

- Volumetric pipettes and volumetric cylinders
- Distilled water (or deionized water)
- Polypropylene test tubes
- Vortex mixer
- Aspirator for washing procedure
- Micro-plate reader (Measuring wave length: 492 nm, Subtracting wave length: 630 nm)

Preparation of reagents:

Prior to use, all reagents were brought to room temperature (18-25^{0}C) and stored at 2-8^{0}C immediately after use. Rat insulin stock solution was kept at -20^{0}C after reconstitution.

Antibody-coated Micro-plate, Lyophilized: The micro-plate well modules were taken out from the sealed foil pouch after the pouch had been equilibrated to room temperature. Although some wells appeared slightly white material in the well, it did not affect the performance of the assay. The micro-plate was used immediately after opening the pouch.

Rat insulin stock solution: Rat Insulin Standard, Lyophilized was reconstituted with the careful addition of 200 μL of distilled or deionized water into a vial. The vial was inverted gently until the contents dissolved completely. This stock solution contains 10 ng/mL rat insulin. The reconstituted rat insulin stock solution is stable for one month at -20^{0}C (Repeated freezing and thawing were avoided)**.** Rat insulin stock solution was diluted to prepare the working rat insulin standards for each assay.

Washing buffer: Phosphate Buffered Saline (PBS) powder and 20% Tween 20 were dissolved with distilled or deionized water and filled up to 500 mL. In order to avoid foaming, PBS powder was dissolved completely with 450 mL water and 1.25 mL of 20% Tween 20 was added just before filling up to 500 mL. It was mixed well to provide the homogenous solution. This washing buffer is stable for one month at 2-8^{0}C.

Diluent 1: Sample Diluent 1, Lyophilized, was reconstituted with 50 mL washing buffer. It was mixed well to provide homogenous solution. Diluent 1 is stable for one week at

$2\text{-}8^0C$.

Diluent 2: Sample Diluent 2, Lyophilized, was reconstituted with 30 mL Diluent 1. It was dissolved completely to provide homogenous solution. Diluent 2 is stable for one week at $2\text{-}8^0C$.

Guinea pig Anti-insulin:

For assaying the serum or plasma sample: Guinea pig Anti-insulin, Lyophilized, serum was reconstituted with 3 mL Diluent 2. It was mixed well to provide homogenous solution. Guinea pig Anti-insulin was used immediately just after reconstitution.

Anti-Guinea pig Antibody Enzyme Conjugate: Anti-Guinea pig Antibody Enzyme Conjugate was used on the second day of the assay.

Anti-Guinea pig Antibody Enzyme Conjugate, Lyophilized, was reconstituted with 6 mL Diluent 2. It was mixed well to provide homogenous solution. Anti-Guinea pig Antibody Enzyme Conjugate was used immediately just after reconstitution.

Enzyme Substrate Solution: Enzyme Substrate Solution was used on the second day of the assay. It was protected from the exposure of light.

One O-Phenylenediamine (OPD) tablet was dissolved with 10 mL Enzyme Substrate Diluent. It was mixed well to provide homogenous solution. Enzyme Substrate Solution was prepared just before use and was used within 60 min after preparation.

Assay Procedure:

1. General remarks:

a. All insulin standards were included in each assay.

b. Each standard and sample was assayed in duplicate to ensure the confidence in values obtained.

c. The assay accuracy was predominantly depending especially on the precision of pipetting,

d. Pipetting the small sample volume such as 5 μL. Accordingly, pipetting was done

accurately in order to minimize the variability. The droplets clinging to the outside of the pipette tip results the inaccuracy; tips were wiped off very carefully every time.

e. In order to prevent the well from drying, the sample and reagents were provided quickly.

f. The dispensing time for each plate was not exceeded 10 min.

g. The same sequence was kept in all procedures.

h. Complete removal of well contents was essential for good performance.

i. Washing procedure was done completely in order to minimize the background.

2. The assay procedure for assaying the serum sample:

A. Preparation of the serum sample.

Serum: Blood was collected, allowed to clot and prepare the serum sample by centrifugation.

Note: Avoided hemolysis during the preparation. Turbid serum was not used as the sample. Turbid serum was centrifuged to provide clear solution.

B. Preparation of the working standards of rat insulin

a. Pipetting of 30 μL of Diluent 2 and 30 μL of rat insulin stock solution into a polypropylene micro-tube was labeled 5000 pg/ml, and then mixed thoroughly.

b. Five polypropylene micro-tubes were labeled to 156, 313, 625, 1250 and 2500 pg/ml respectively and dispensed 30 μL of Diluent 2 into all tubes.

c. Pipetting of 30 μL of 5000 pg/mL standard into 2500 pg/mL tube, and it was mixed thoroughly.

d. 30 μL of 2500 pg/mL standard was transferred into 1250 pg/mL tube, and it was mixed thoroughly.

e. This dilution was repeated successively on remaining tubes.

f. One polypropylene micro-tube was labeled to 0 pg/mL of Diluent 2

	Insulin concentration (pg/ml)						
	5000	2500	1250	625	313	156	0
RISS	30 µL						
D 2	30 µL	30 µL	30 µL	30 µL	30 µL	30 µL	60 µL
		30 µL	30 µL	30 µL	30 µL	30 µL	
Total	60 µL	60 µL	60 µL	60 µL	60 µL	60 µL	60 µL

RISS: Rat insulin stock solution, D 2: Diluent 2

Note: The working insulin standards were prepared using the polypropylene test tube because polypropylene test tube shows the minimum adsorption of insulin. Working insulin standards were discarded after use.

C. **Assay procedure**:

 First reaction:

a). The microplate well module was taken out from the sealed foil pouch after the pouch has been equibrated to room temperature. Then it was set in a fixing frame.

b). Each well was washed with 300 µL washing buffer and then the buffer was aspirated. Washing procedure was repeated again. After the last wash, remaining buffer was removed by inverting the plate on a clean paper towel.

c). To each well, 50 µL Guinea pig Anti-insulin reconstituted by Diluent 2 was dispensed.

d). After dispensing of 45 µL of Diluent 2, pipetting of 5 µL sample (or 0, 156, 313, 625, 1250, 2500, 5000 working insulin standards and 10000 pg/mL Rat insulin stock solution) to the well. Total volume of the first reaction became 100 µL.

e). The micro-plate was covered with a plastic micro-plate cover, and kept to stand overnight (16-20 hours) at 4^0C.

Second reaction:

f). The well contents were aspirated and washed three times with 300 µL washing buffer. After the last wash, remaining solution was removed by inverting the plate on a clean paper towel.

g). Dispensed 100 µL of Anti-Guinea pig Antibody Enzyme Conjugate.

h). The micro-plate was covered with a plate cover and incubated for 3 hours at room temperature.

Enzyme reaction:

Note: Enzyme substrate solution was prepared just before the termination of second reaction.

i). The well contents were aspirated and washed five times with 300 µL washing buffer. After the last wash, remaining solution was removed by inverting the plate on the clean paper towel.

j). Immediately, 100 µL of Enzyme substrate solution was dispensed and allowed to react for 30 min at room temperature. During enzyme reaction, exposuring of the micro-plate from light was avoided.

k). Providing 50 µL of Enzyme Reaction Stopping Solution stopped enzyme reaction.

l). The absorbance was measured by plate reader within 30 min (Measuring wave length: 450 nm, Subtracting wave length: 630 nm).

m). Insulin concentration was calculated from the standard curve.

3. Determination of the insulin concentration:

1. Average of the absorbance of each set of duplicate standards or samples were used for determination of concentration.

2. On a semi-log section paper, the insulin standard was constructed by plotting the mean absorbance value for each standard on Y-axis (logarithmic).

Note: A standard curve was generated for each day.
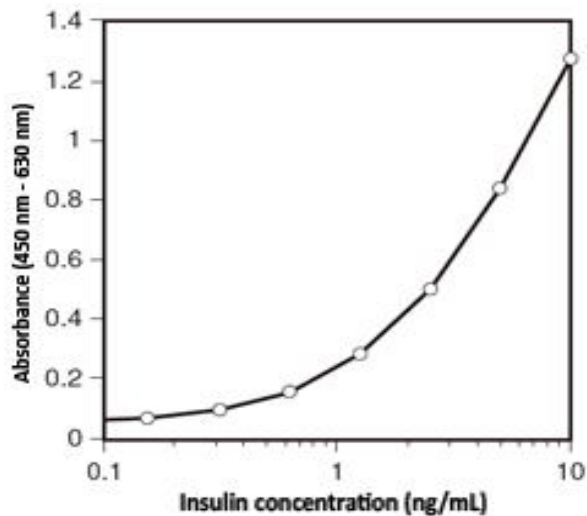
Figure-2.1.15: Standard curve for insulin

3. The insulin concentration of the sample was determined by the standard curve using the mean absorbance value of each sample.

Note: The insulin concentration of normal rat is 0.5-2.0 ng/mL.

Summary of the assay:

Each well of the micro-plate was washed twice with 300 μL washing buffer

↓

Guinea pig anti-insulin serum was added (reconstituted by Diluent2) per well

↓

45μL Diluent 2 and 5μL sample or 45μL Diluent 2 and 5μL working insulin standard was added (reconstituted by Diluent 2) per well

↓

The micro-plate was incubated overnight at 4^0C for 16-20 hours

↓

The well was washed three times with 300 μL washing buffer

↓

100μl Anti-Guinea Pig Antibody Enzyme Conjugate was added per well

↓

The micro-plate was incubated 3 hours at room temperature

↓

The well was washed five times with 300 μL washing buffer

↓

100 μL Enzyme Substrate solution was added per well

↓

The micro-plate was incubate 30 mins at room temperature in dark

↓

Adding 50 μL Enzyme Reaction Stopping Solution per well stopped the reaction

↓

The absorbance was measured at 490 nm subtracting that at 630 nm

↓

The insulin concentration was calculated for the standard curve

2.1.4.7 Measurement of glycogen from rat liver

Liver glycogen levels were estimated by Anthrone-sulphuric acid method (Seifter *et. al.,* 1950).

First step: Preparation of standard glucose solution

Second step: Preparation of anthrone solution (Glucose stock 0.009375 gm/50 mL).

1 Preparation of serial dilution from glucose stock

<u>Concentration of glucose</u>	<u>Glucose</u>	<u>Water</u>
0.3375 mg/100 mL	200 μL	5.8 mL
0.75 mg/100 mL	400 μL	5.6 mL
1.5 mg/100 mL	800 μL	5.2 mL
3.0 mg/100 mL	1.6 mL	4.4 mL
4.5 mg/100 mL	2.4 mL	3.6 mL
6.0 mg/100 mL	3.2 mL	2.8 mL
6.0 mg/100 mL	4.0 mL	2.0 mL

2. Anthrone solution

100 mg anthrone was dissolved in 50 mL concentrated sulfuric acid.

3. Procedure

   a) After sacrification of rats on 28th day (different groups) 200mg liver was taken from each rat.

   b) Then it was homogenized with 10ml 5% TCA (Trichloro acetic acid). First 100 μL of TCA was added and grinded very well. After that rest of the TCA was gradually added and grinded, and made it 10 mL. The solution was filtered with a filter paper.

c) 1ml of the filtrate was taken in 2 mL 10N KOH in a test tube (pyrex) and was mixed by shaking. For standard and blank 1 mL of 5% TCA in 2 mL 10N KOH was taken.

d) Both sample and standard were kept in boiling water (100^0C) for 1 hour.

e) After that they were made cool by using ice-cold water to stop the reaction.

f) In the next step 1 mL glacial acetic acid was added in each [sample, standard, blank (2 blanks and 7 standards were used)] test tube and made each solution as 10 mL with deionized water. For standard, glucose solution was added and then made each solution to 10 mL. Shaking mixed all the solutions very well.

g) 1 mL of this solution was added in 2 mL of anthrone slowly and was mixed by shaking carefully as it became hot. All the test tubes were kept on ice.

h) Again the test tubes were kept in boiling water exactly for 10 min.

i) The test tubes were made cool on ice-cold water to stop the reaction.

j) 200 µL solutions from each tube were added in a micro-titer plate and took the absorbance at 650 nm.

k) Using liner-liner graph paper, a standard curve was created by plotting the absorbance (Y) of each Reference Standard against its corresponding concentration (X) in mg/100 mL.
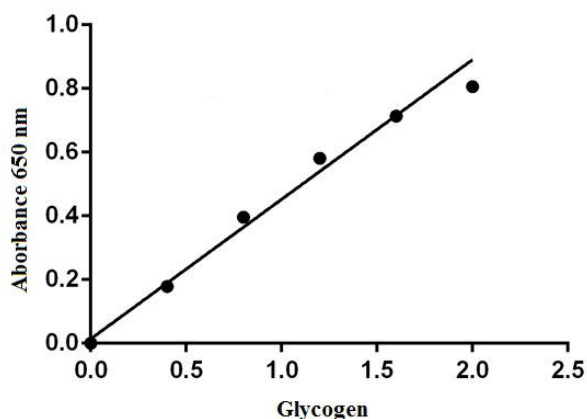
Figure-2.1.16: Standard curve for glycogen.

l) The average absorbance was used of each serum sample to determine the corresponding glucose value by simple interpolation from this standard curve (multiply the value by the initial sample dilution, if necessary).

2.1.4.8 Estimation of Malondialdehyde and reduced Glutathione

Collection of blood for Hemoglobin, MDA and GSH

About 2 ml of blood from each rat was collected separately in a test tube by cervical decapitation. For Hb and GSH estimation, blood was taken in test tubes containing 3 units of heparin.

Preparation of Erythrocyte hemolysate

1. After 5 minutes, heparinized blood was centrifuged at room temperature at 1200 rpm for 5 min. A white fibrin clot formed in the upper layer of plasma. Large forcep was used to squeeze the clot to leave a stringy, white fibrin mass.

2. The tube was spinned again at 1200 rpm for 5 minutes. An upper phase of clear serum was separated from red blood cells by fibrin clot.

3. Serum and fibrin clot were removed by sterile Pasteur pipette and the packed RBCs were washed with five volumes of 0.9% saline. Then the mixture was gently agitated for two minutes and centrifuged to separate the washed cells. The former step was repeated three times.

4. Then cleaned RBCs were analysed with five volumes of distilled deionized water.

Figure-2.1.17 Serum and fibrin clot removed by sterile pasteur pipette

Figure-2.1.18 Packed RBCs washed with five volumes 0.9% saline

Figure-2.1.19 Washed cells separated after centrifugation

2.1.4.8.1 Estimation of Hemoglobin in blood

Method

Hemoglobin in blood was estimated by using reagent kit (Human, Germany) by Photometric Colorimetric test by Cyanmethemoglobin method (Van Kampen, 1961). Absorbance and the concentration of enzyme were measured in Spectrophotometer (Micro Flow Cell Photometer AE-100F ERMA Inc.).

Test Principle

Hemoglobin from a whole blood sample is released from the erythrocytes and is oxidised by ferricyanide to methemoglobin. The absorbance of cyanmethemoglobin is measured at 540 nm and is directly proportional to the hemoglobin concentration in the sample.

Requirements for setting and measurements

Wavelength of the spectrophotometer	: 540 nm
Optical path	: 1 cm
Incubation	: 3 min.
Temperature	: 20 - 25^{0}C
Measurement	: Against reagent blank
Specimen	: Capillary blood, EDTA-venous blood

Reagents

1. Reagent A	2. Reagent B
Potassium hexacyanoferrate (III)	Potassium cyanide
Potassium bicarbonate	Potassium bicarbonate

Preparation of the working reagent

1. The reagents were allowed to attain the room temperature.

2. To prepare the working reagents, one bottle Reagent A (25 ml) was mixed with one bottle Reagent B (25 ml) and 40 ml deionized water was added to it.

3. Working reagent stored in a closed dark amber colored glass container and labeled properly.

4. Working Reagents is stable for 12 months at 15 - 25^{0}C in the dark, but no longer than the stated expiry date of the test kit.

5. Contamination was avoided.

Procedure

1. The sample (blood) and the working reagent were allowed to attain room temperature prior to use.

2. Five ml test tubes were cleaned properly, dried in air and then labeled as sample (S).

3. The test tubes were put in a rack and 5 mL of the working reagent was taken in the test tubes labeled with the help of micropipette.

4. 20 µl of the sample (blood) was added to the test tubes, mixed thoroughly and incubated at room temperature for 3 min.

5. Pipette was rinsed several times with the working reagent.

6. Then the contents of the test tubes were mixed thoroughly and incubated at room temperature for 3 min.

7. The absorbance was read at the earliest against reagent blank.

The hemoglobin was calculated followed standard formula

Calculation of the Hemoglobin Concentration

$$\text{Hemoglobin (g/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Conc of standard} \times \frac{\text{Dilation Factor (251)}}{1000}$$

This is simplified as,

Hemoglobin (g/dl) = 36.8 X Absorbance of the Sample (Blood)

2.1.4.8.2 Method for estimation of plasma malondialdehyde (MDA) level

Estimation of MDA level by using the thiobarbituric acid reactive substances (TBARS) method. (Yagi, 1994, cited by Ates *et al.,* 2004).

Principle

Lipid peroxidation, defined as the oxidative deterioration of polyunsaturated fatty acids (PUFAs), is a free radical induced phenomenon. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. PUFAs peroxides generate MDA upon decomposition.

The measurement of thiobarbituric acid reactive substances (TBARS) is a well established method for screening and monitoring lipid peroxidation. The principle is based on the fact that MDA reacts with 2-thiobarbituric acid (TBA) under acidic conditions, to yield a pink colored complex, measured spectrophotometrically at 532 nm.

Preparation of reagents for plasma MDA determination

a. Preparation of 10% trichloro acetic acid (TCA): In a volumetric flask, 20 mg of TCA was taken and total volume was brought upto 100 mL by addition of deionized water. The solution was stirred and mixed properly.

b. Preparation of 0.05 M H_2SO_4: Concentrated H_2SO_4 often comes from the factory at an 18.0 molar concentration. This means that there are 18 moles of H_2SO_4. This means that 1 mL of reagent contains 0.018 moles of H_2SO_4. Therefore,

$$\frac{1 \text{ mL}}{0.018 \text{ moles}} = \frac{X \text{ mL}}{1 \text{ mole}}$$

Solving for x, we find that we need 55.6 ml of H_2SO_4 reagent to make 1 molar H_2SO_4 solution.

So, for 0.05 mole,

$$\frac{1\,mL}{0.018\ moles} = \frac{X\ mL}{1\ mole}$$

Thus, x= 2.8 mL of H_2SO_4.

So, 2.8 mL of H_2SO_4 to 1 liter of deionized water is needed to make 0.05M H_2SO_4.

Procedure

1. Plasma was mixed with 2.5 ml 20% TCA.

2. The mixture was centrifuged at 3500 rpm for 10 min and the supernatant was decanted.

3. The precipitate was washed once with 0.5 mL 0.05M H_2SO_4 and then 2.5 mL 0.05M H_2SO_4 acid and 3 mL TBA (TBA 2g/L in 2M sodium sulfate solution) were added.

4. The mixture was kept in a boiling water bath for 30 min, and cooled in running tap, which result in development of pink color.

5. Then in the test tube 4 mL n-butanol was added and vortex mixing was done.

6. The mixture was centrifuged at 3500 rpm for 10 min.

7. The butanol extract was measured at 532 nm against a butanol blank.

8. The concentration of TBA reactive material was calculated by using 1, 1, 3, 3 tetraethoxy propane as standard.

9. The result was expressed as µmol MDA/L plasma
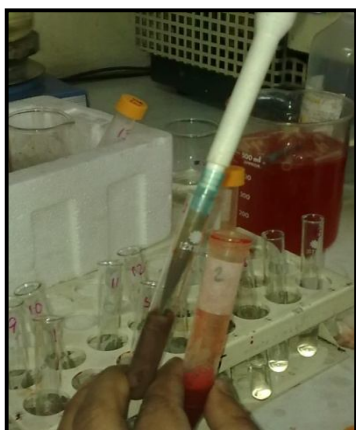
Figure-2.1.20 5% TCA added to the erythrocyte hemolysate.

Figure -2.1.21 Supernatant separated after centrifugation.

Figure-2.1.22 Pink solutions produced after boiling showing the different concentration of MDA.

Standard curve for estimation of plasma MDA

Standard MDA calibrator was prepared by dissolving 1 mL MDA standard in 1 mL deionized water. MDA was dissolved thoroughly by inverting the measuring cylinder repeatedly. From this solution different strength (0, 0.50, 0.75 and 1.25 and 2.5 μmol/mL) calibrators were prepared. The absorbance of MDA was measured by spectrophotometer at 532 nm and the reading was placed in a graph paper to produce a calibration curve.
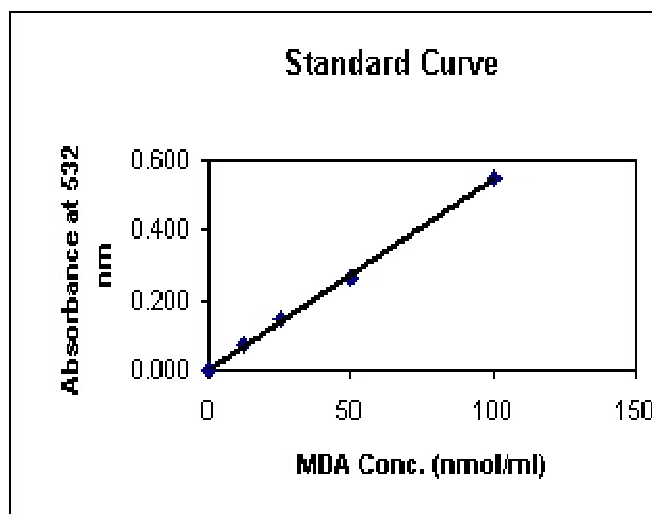
Figure 2.1.23 Standard Curve for estimation of plasma MDA

2.1.4.8.3 Estimation of Reduced Glutathione (GSH) Concentration in Erythrocyte

Reduced glutathione was assayed by Ellman's method (Ellman, 1959).

Principle

5,5-Dithiobis-2-nitrobenzoic acid (DTNB) is reduced by –SH groups and form 1 mole of 2-nitro-5-merceptobenzoic acid per mole of –SH. DTNB is a disulfide chromogen that is readily reduced by –SH compounds to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412 nm by spectrophotometer and is directly proportional to the GSH concentration.

Reagents

- 5.5% Trichloroacetic acid.

- 4.25% Disodium hydrogen phosphate.

- 0.04% 5,5-Dithiobis-2-nitobenzoic acid.

- Reduced glutathione standard (1 mg/mL).

Setting measurements

Wavelength	:	412 nm
Optical path	:	1 cm
Temperature	:	37°C
Measurement	:	Against reagent blank

Procedure

1. 1 mL erythrocyte hemolysate and 1 mL of 5.5% TCA were added to the labeled test tube.

2. The mixture was vortexed and centrifuged at 1725 rpm for 5 min.

3. Supernatant poured into another test tube. 2250 µL of supernatant, 2 mL Na_2HPO_4 and 250 µL of DTNB were added.

4. The mixture was allowed to stand for approximately 15 minutes, forming a yellow substance. The optical density was measured at 412 nm by spectrophotometer before measuring the density.

Figure 2.1.24 Yellow solutions produced showing the different concentration of GSH Calculation

The concentration of GSH in erythrocyte was calculated by using the following formula:

$$\text{Concentration of GSH} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the standard}} \times \text{Concentration of Standard Hb (mg/dl)}$$

1 ml of erythrocyte hemolysate +	← Centrifuged at 1725 xg for 5 mins.

↓

Supernatant was obtained

↓

250 µl of cleared solution + 2 ml of Na_2HPO_4	← After 10 mins of addition of DNTB.

↓

Absorbance was measured at 412 nm by spectrophotometer
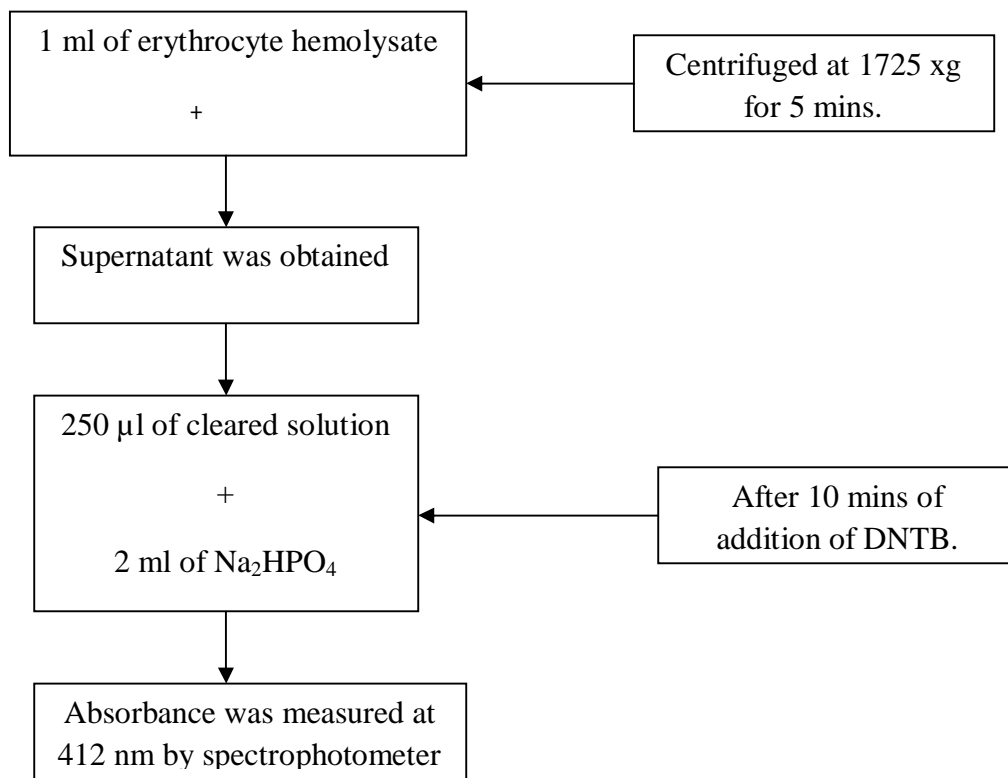
Figure-2.1.25 Procedure of estimation of GSH concentration in erythrocyte

Standard curve for estimation of reduced glutathione

Standard glutathione calibrator was prepared by dissolving 1 mg reduced glutathione in 1 ml deionized water. Glutathione was dissolved thoroughly by inverting the measuring cylinder repeatedly. From this solution different strength (0, 0.25, 0.50, 0.75 and 1.00 mg / mL) calibrators were prepared. The absorbance of reduced glutathione was measured by spectrophotometer at 412 nm and the reading was placed in a graph paper to produce a calibration curve.

A linear curve was produced when the absorbance and the estimated concentration of reduced glutathione were plotted along the Y and X axis, respectively. Each point represents the mean of five recordings.
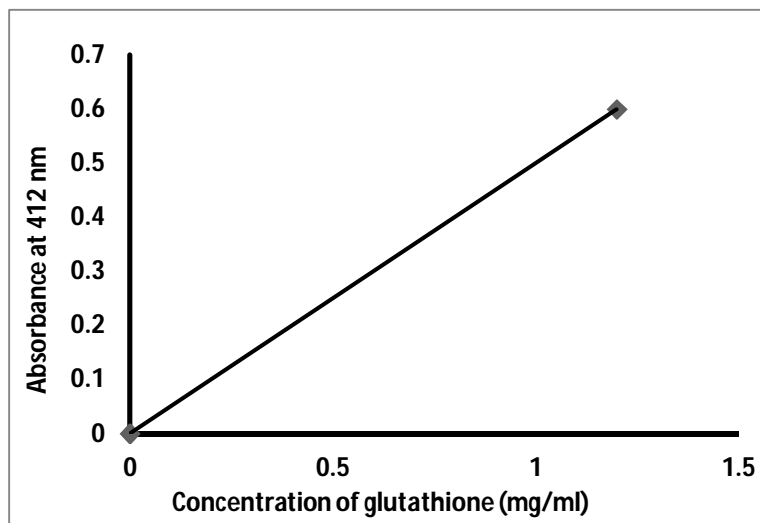
Figure 2.1.26 Standard Curve for estimation of GSH concentration in erythrocyte

2.1.5 Statistical analysis

Data from the biological experiments were analyzed using the Statistical Package for Social Science (SPSS) software for windows SPSS version 16 (SPSS Inc., Chicago, Illinois, USA). Variables were expressed as mean±SD (standard deviation) or as Median (Range) as appropriate. Pre-treatment and after treatment values were statistically analyzed by Student's t-test (paired and unpaired) or ANOVA (analysis of variance) followed by Bonferroni post hoc test or Mann Whitney (U) test. The differences were considered statistically significant at *p<0.05*

2.2 ALPHA-GLUCOSIDASE INHIBITOR ACTIVITY ASSAY

2.2.1 Plant materials

The *S haematodes* roots and *P thyrsiflorus* flowers were cut into small pieces and dried separately in ventilated drying oven at 40^0C for 24 hours. They were then ground to fine powder (particle size 0.70 mm) and stored in air-tight containers until further use.

2.2.2 Chemical reagents

Methanol, sodium carbonate, Folin-Ciocalteu reagent, acarbose, quercetin, phosphate buffer, α-glucosidase enzyme, glycine and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were brought from E. Merck (Darmstadt, Germany) while p-nitrophenyl-α-D-glucopyranose (PNPG) was obtained from Sigma Aldrich (Germany).

2.2.3 Plant extraction

After the grinding step, the roots and flowers were extracted using methanol. The extraction was performed by weighing 20 g of the plants materials, and then soaked in 500 mL of methanol and subjected to sonication (at controlled temperature) in sonicator (Nexul Ultrasonic Cleaner, NXP 1002) for 1 hour. The mixture was then filtered using filter paper and the filtrates were concentrated separately using rotary vacuum evaporator and then fridge dried to yield the crude extract. The crude extracts were stored at 4^0C until further analysis.

2.2.4 Alpha-glucosidase inhibition assay

The α-glucosidase inhibition activity was determined as described by Lee *et. al., (2014)*. The α-glucosidase enzyme and substrate PNPG were prepared separately by dissolving them in 50mM phosphate buffer, which was first adjusted to pH 6.5 to simulate the intestinal fluid. To test the inhibitory effect of the extracts, each well was loaded with 100 µl of 30mM phosphate buffer, 10 µL of the extract in six different concentrations (3.13, 6.25, 12.5, 25, 50 and 100 mg/mL) and 15 µL of enzyme. The extract was allowed to interact with the enzyme for 5 min at room temperature before the reaction was started by the addition of 75 µL substrate. The total volume in each well was 200 µl. After 15 min of incubation at room temperature, the reaction was stopped by adding 50 µL of glycine

(adjusted to pH 10). The plate was read on a spectrophotometer (SPECTRAmax PLUS) at a wavelength of 405 nm. As a blank sample, the reagent mixture above was used without enzyme; the enzyme was replaced by another 15 µL of 30mM phosphate buffer. As a negative control, the reagent mixture without sample was used; the 10 µL plant extract was replaced with 10 µL of solvent. For the blank substrate, a reagent mixture of 115 µL of 30mM phosphate buffer and 10 µL solvent was used. The glycine was replaced with distilled water for the blank substrate. The α-glucosidase inhibition activity of the test sample was expressed as the percentage (%) of inhibition and was calculated as the % inhibition of sample $= [(a_n-a_s)/a_n] \times 100 \%$, where a_n is the difference in the absorbance of the negative control and the blank substrate and a_s is the difference in the absorbance of the sample and the blank sample. The α-glucosidase inhibition activity was plotted against the concentration to determine the concentration of extract required to inhibit the enzyme by 50%. The results were reported as (IC_{50}).
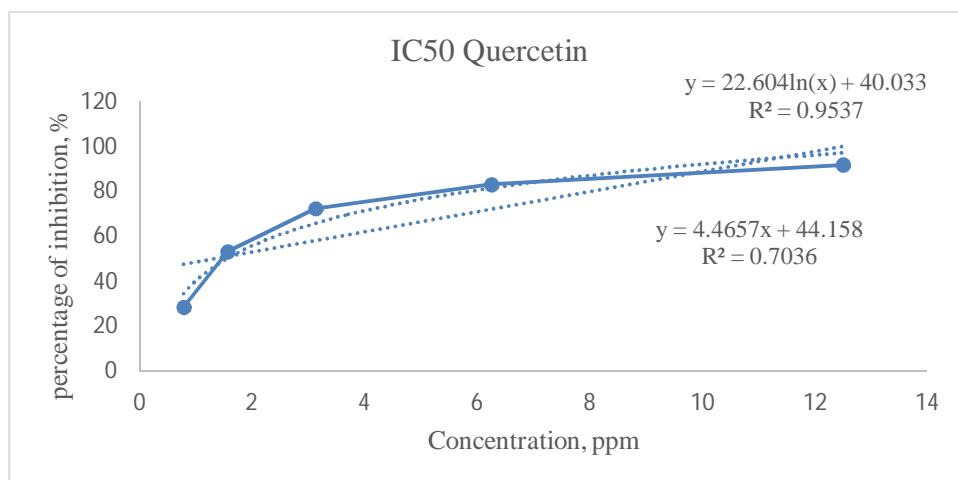
Figure-2.2.1: Percentage of inhibition vs concentration (ppm) of positive control quercetin.

2.2.5 Statistical analysis

The results were expressed as mean ± standard deviation of three replicates. ANOVA was used to execute the analysis of significant difference. MS Excel and Minitab 14 software (Version 14, Minitab Inc, State College, PA, USA) were used for statistical calculation. Pearson correlation test was also performed using Minitab 14 software. For Pearson correlation and the IC_{50} was converted to $1/IC_{50}$ to invert the relation between absorbance and the activity.

2.3 ANTIOXIDANT ACTIVITY TESTING

2.3.1. ADHPs and Plant extracts

Extracts from ADHPs and extracts of *S haematodes* & *P thyrsiflorus* were screened for the presence of phytochemical groups in the methanol extracts. Significant presence of flavonoids, phenolic & Tannin were observed. It is known that flavonoids, phenolic & Tannin etc. are antioxidant compounds. So, determination of the antioxidant activity of the 07 ADHPs and 02 plants samples were performed. To evaluate the antioxidant activity four different parameters were studied.

> ➤ DPPH scavenging activity.
> ➤ Reducing power assay.
> ➤ ABTS scavenging activity.
> ➤ Total antioxidant capacity determination.

2.3.2: Samples preparation for antioxidant screening and reducing power assay

Sample Extraction

For Solid Samples of ADHPs

Tablets or Capsules of ADHPs (ADHP-1, ADHP-4, ADHP-5, ADHP-6 and ADHP-7) were opened up & grinded by the Mortar & Pestle to powder. The powdered ADHPs were extracted with methanol (MeOH) at room temperature using a special glass tank for three days by changing MeOH each day. The MeOH extract was filtered and the filtrate was concentrated. The concentrated extracts were combined, evaporated to dryness and finally dried in a freeze-dryer to give MeOH extract. The extracts were stored at -22^{0}C in a refrigerator.

For Liquid Samples of ADHPs

Liquid ADHPs (ADHP-2 and ADHP-3) were evaporated by rotary evaporator and dried by freeze dryer. The freeze dried material was extracted with MeOH at room temperature using a special glass tank for three days by changing MeOH each day. The MeOH extract

was filtered and the filtrate was concentrated. The concentrated extracts were combined, evaporated to dryness and finally dried in a freeze-dryer to give MeOH extract. The extracts were stored at -22^0C in a refrigerator.

Extraction Scheme: 2.3.1

```
                    ┌──────────────────┐
                    │ Sample of ADHPs/ │
                    │     ADSPs        │
                    └──────────────────┘
                             │         ┌──────────────────────────────────────┐
                             │         │ Sample soaked in MeOH for three days by│
                             │         │ changing MeOH each day                 │
                             │         └──────────────────────────────────────┘
                             │
        ┌────────────────────┴──────────────────────────────────┐
        │                                                        │
┌──────────────────┐                                    ┌──────────────────┐
│  MeOH Extract of │                                    │    Residue of    │
│   ADHPs/ ADSPs   │                                    │   ADHPs/ADSHs    │
└──────────────────┘                                    └──────────────────┘
        │
┌──────────────────────────┐
│ Evaporation of MeOH at   │
│ reduced pressure & low   │
│ temperature and          │
│ concentrated.            │
└──────────────────────────┘
        │
┌──────────────────┐      ┌──────────────────┐
│  Freeze drying   │─────▶│ Dry Extract of   │
│                  │      │ ADHPs/ ADSPs (Wt)│
└──────────────────┘      └──────────────────┘
```
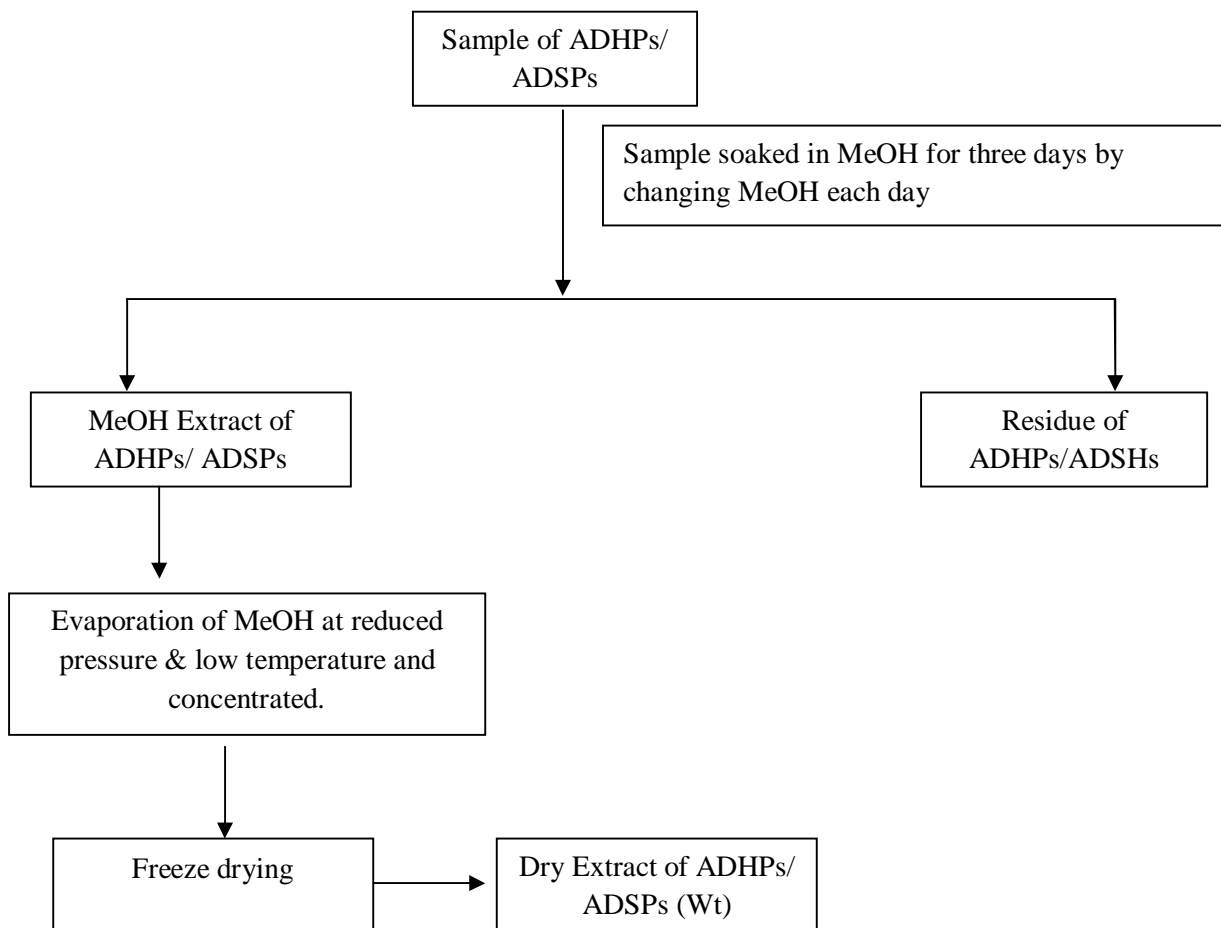
3.3.3 Sample Preparation for antioxidant analysis:

HPLC grade MeOH was used to prepare 1000 ppm stock solutions for the particular extracts. The concentrations of these solutions were 1 mg/mL. Samples were prepared in respective solvent systems from which serial dilutions were carried out to obtain the concentrations of 2.5, 5, 10, 20, 40, 60, 80, 100, 200 & 400 µg/mL and used as stock solutions for the antioxidant screening & reducing power assay. These diluted extracts were carried out for determining antioxidant activity.

3.3.4: Chemicals Used

HPLC grade methanol, Folin-Ciocalteu's reagent, Na_2CO_3 (75 g/mL), gallic acid, $AlCl_3$ solution, sodiuma acetate, quercetin, H_2SO_4 (0.6M), Na_3PO_4 (28mM), Ammonium molybdate (4mM), DPPH, Ascorbic acid, ABTS (7mM), potassium persulfate (2.45mM), phosphate buffer (0.2M, pH (6.6), potassium fcrricyanide (1%), trichloro acetic acid (10%), $FeCl_3.6H_2O$ (0.1%), Na_2CO_3 (35%), Tannic acid were either procured or prepared.

A double beam Analykjena UV/Visible spectrophotometer was used for the phytochemical screening and determination of antioxidant activity.

Figure-2.3.1: Double beam Analykjena UV/Visible spectrophotometer (Model 205, Germany)

2.3.5 Test for phytochemical screening

Phytochemical screening tests for all the extract samples were done in the three methods: determination of total phenolic contents, determination of total flavonoids content and determination of total tannin content.

2.3.5.1 Total phenolic content determination:

The total phenolic content was determined by the modified Folin-Ciocalteu method (Wolfe *et. al.,* 2003). One mL of each extract (1 mg/mL) was mixed with 5 mL Folin-Ciocaltu reagent (1:10 v/v distilled water) and 4 mL (75 g/L) of sodium carbonate. The

mixture was then vortexed for 15 second for the development of color and the mixture was allowed to stand for 30 min at 40^0C. Then the absorbance was read at 765 nm with the same spectrophotometer (cintar-6, double beam UV-visible Spectrophotometer). Total phenolic content was calculated as mg of gallic acid equivalent per gram using the equation obtained from a standard gallic acid calibration curve y = 0.008x + 0.038, R^2=0.998.
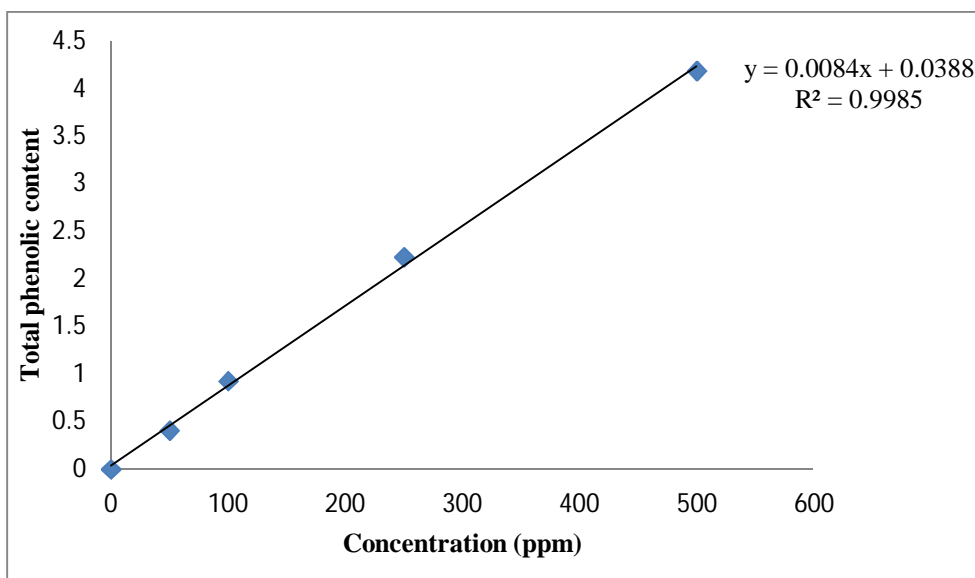
Figure-2.3.2: Standard gallic acid calibration curve for total phenolic content

2.3.5.2. Total flavonoids content determination:

Aluminium chloride colorimetric method (Chang *et. al.,* 2002) was used for determination of total flavonoids concentration in the samples of ADHPs and ADSPs extracts. Five mL of each extract was individually mixed with 2.5 mL of aluminium chloride ($AlCl_3$) solution. They were allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 430 nm with a double beam spectrophotometer (cintar-6, double beam UV-visible Spectrophotometer). Total flavonoids content was determined as mg of quercetin equivalent per gram using the equation calibration curve, y = 0.0356x + 0.0769, R^2 = 1.
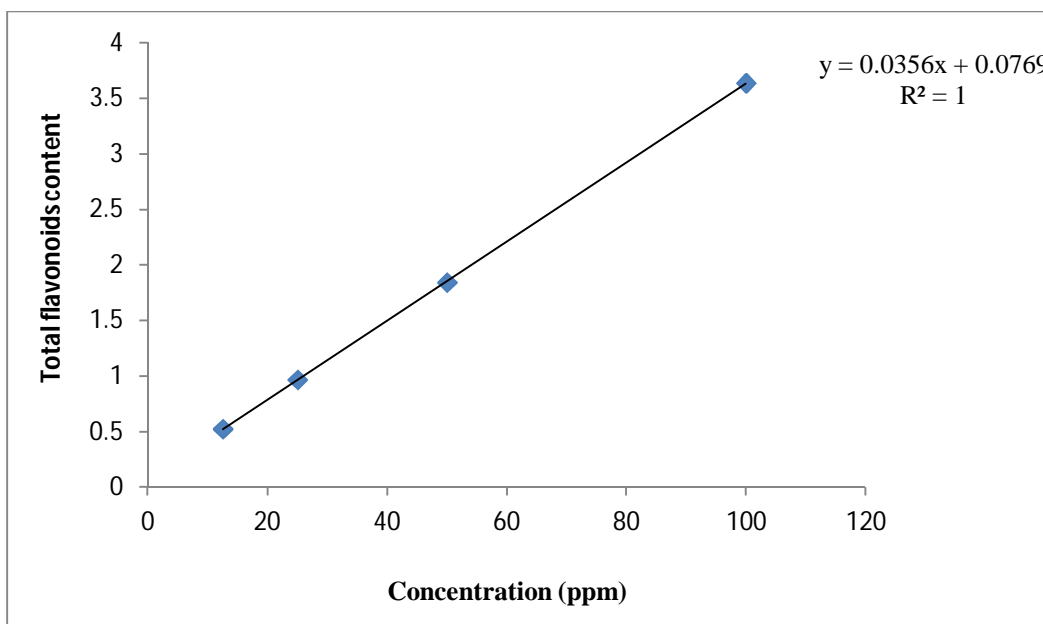
Figure-2.3.3: Standard quercetin calibration curve for total flavonoids content.

2.3.5.3. Determination of Total Tannins content:

The total tannin content was determined by the modified Folin-Ciocalteu phenol reagent method of Meghashri (Meghashri, *et. al.,* 2010). One mL of sample was added to 7.5 mL distilled water. Then 0.5 mL of Folin Ciocalteu Phenol reagent was mixed with the sample solution.   One mL of 35% sodium carbonate was then added to the reaction mixture. The mixture was shaken well and kept at room temperature for 30 min. After 30 min, absorbance was read against a blank at 725 nm with a double beam UV/Visible spectrophotometer (cintar-6, double beam UV-visible Spectrophotometer). Blank was prepared with methanol instead of the sample. A set of standard solutions of gallic acid was treated in the same manner as described earlier and read against a blank. The results of tannins are expressed in terms of gallic acid mg/g of extract using the equation obtained from a standard calibration curve, $y = 0.32x – 0.12$, $R^2=0.997$.
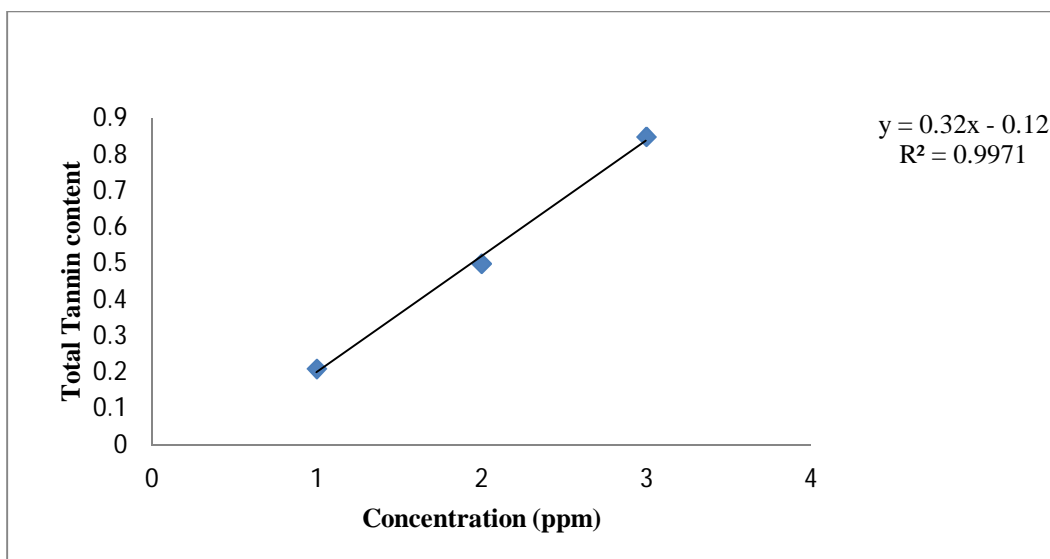
Figure-2.3.4: Standard gallic acid calibration curve for total tannin content.

2.3.6: Methods Used for Determination of Antioxidant Activity

There are various well known methods which can be followed to determine the antioxidant properties of plant extracts. The antioxidant activity of different extracts of 07 ADHPs and 02 ADSPs obtained was evaluated by the following methods:

- ➢ Total antioxidant capacity determination
- ➢ DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity.
- ➢ ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging activity.
- ➢ Reducing power assay.

2.3.6.1. Total antioxidant capacity determination

The total antioxidant capacity was evaluated by the phosphomolybdenum assay method (Prieto *et. al.,* 1999) which is based on the reduction of Mo (VI) to Mo (V) and the subsequent formation of a green phosphate-Mo (V) complex in acidic condition. Each extract (0.3 mL) was allowed to mix with 3.0 mL of the reagent solution (0.6M H_2SO_4, 28mM Na_3PO_4 and 4mM ammonium molybdate). This reaction mixture was incubated at 95^0C for 90 min. After letting the solution cool back to room temperature, the absorbance was measured at 695 nm using a spectrophotometer against a blank solution. The total

antioxidant capacity was determined and expressed as mg ascorbic acid equivalents per gram of dry extract using the equation obtained from a standard ascorbic acid calibration curve, $y = 0.001x + 0.003$, R^2:0.999.
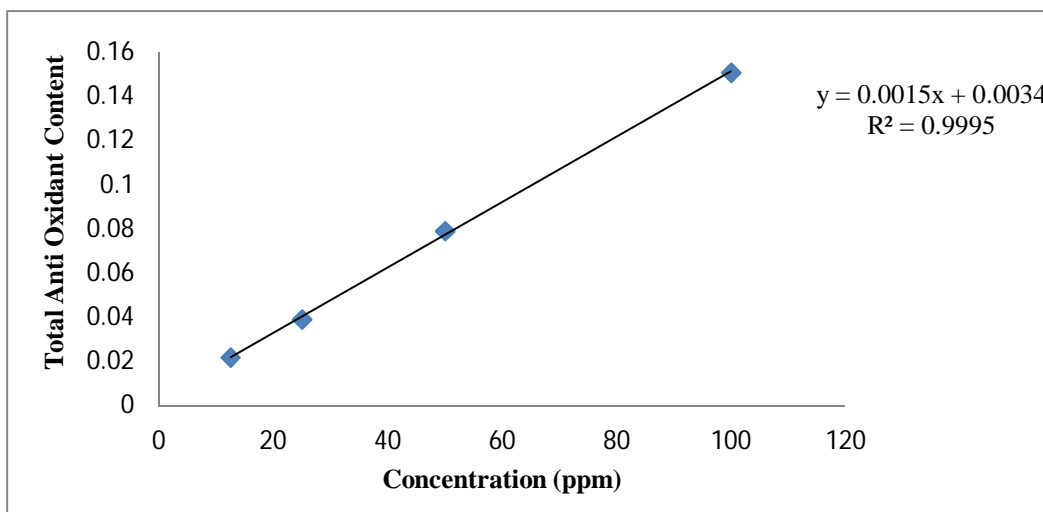
Figure-2.3.5: Standard ascorbic acid calibration curve for total anti-oxidant Content

2.3.6.2: DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical Scavenging Activity

DPPH radical serves as the oxidizing radical to be reduced by the antioxidant (AH) and is the indicator for the reaction.

DPPH• + AH → DPPH-H + A• (Frankel and Meyer, 2000).

DPPH (free radical)

DPPH (non radical)

Inhibition of polymer chain, R, by DPPH
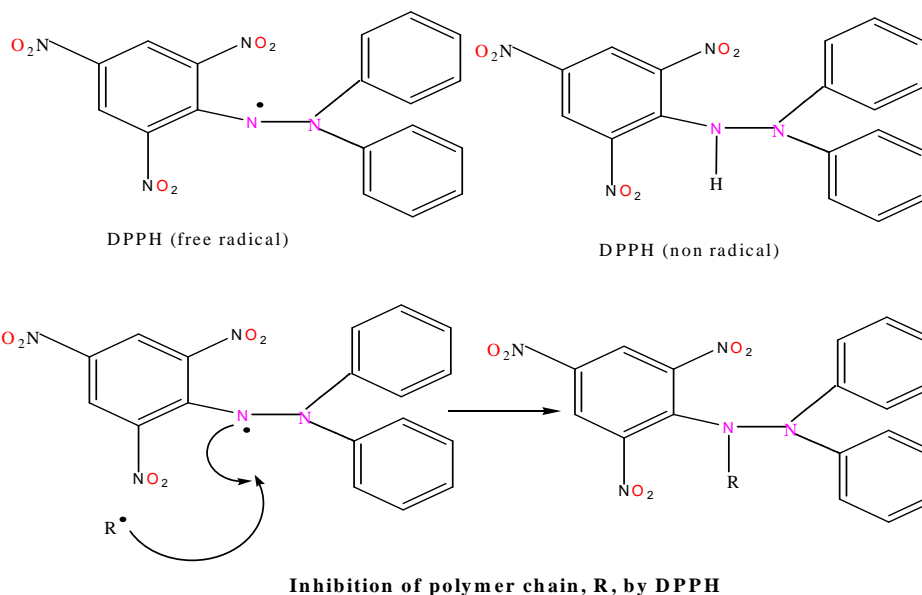
Structure-33: DPPH radical , non radical & inhibition mechanism.

Figure-2.3.6: DPPH radical, non radical& inhibition mechanism.

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The method of Govindarajan (Govindarajan, *et. al.,* 2003) was used for performing the DPPH radical scavenging activity. Each sample extracts (2 mL) and 2 mL DPPH (0.1mM) solution were mixed. The mixture was vortex and allowed to stand at the dark place for 30 min. The absorbance of the mixture was read against a blank at 517 nm using a spectrophotometer. The radical scavenging activity was expressed as the inhibition percentage (I %) and calculated as per the equation: $I\,(\%) = (A_{blank} - A_{sample} / A_{blank}) \times 100$, where A_{blank} is the absorbance of the control (containing all the reagents except the testing compound), and A_{sample} is the absorbance of the experimental sample with all reagents. The IC_{50} value (the concentration of a sample required to scavenge 50% DPPH radical) was calculated from the plot of inhibition (%) against the concentration of the extract. All determinations were carried out in triplicate and their average was noted. Ascorbic acid was used as the standard antioxidant.

2.3.6.3. ABTS radical scavenging activity:

ABTS radical scavenging activity of samples was determined by the Fan YJ and coworkers method (Fan *et. al.,* 2009). ABTS radical cation was produced by reacting 7mM ABTS solution with 2.45mM potassium per sulfate and the mixture was allowed to stand in the dark at room temperature for 16 hours. At the moment of use, the ABTS solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Each sample (1 mL) with various concentrations (2.5 to 400 µg/mL) were added to 1 ml of ABTS solution and mixed vigorously. The reaction mixture was allowed to stand at room temperature for 6 min and the absorbance at 734 nm was immediately recorded. The ABTS scavenging effect was calculated as follows: ABTS scavenging effect = $(A_{blank} - A_{sample} / A_{blank}) \times 100$, where A_{blank} is the absorbance of the control (containing all the reagents except the testing compound), and A_{sample} is the absorbance of the experimental sample with all reagents. Ascorbic acid was used as the standard antioxidant.

2.3.6.4. Reducing power:

The reducing power of ADHP and ADSP samples was determined according to Dehpour and Nabavi method (Dehpour, *et. al.,* 2009). Different concentrations (2.5-400 µg/mL) of the extract (1 mL) of each sample was mixed with 2.5 mL phosphate buffer (0.2M, pH

6.6) and 2.5 ml of potassium ferricyanide [$K_4Fe(CN)_6$] (1%). The mixture was then incubated at 50^0C for 20 min. After 20 min the solution was cooled and 2.5 mL of 10% solution of trichloroacetic acid was added. It was then centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of 0.1% $FeCl_3$ and the absorbance of the mixture were measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. All the determinations were carried out thrice and the average of the results were taken. Ascorbic acid was used as the standard reference compound in this study.

2.4 SUNSCREEN PROTECTION FACTOR TESTING

2.4.1 Chemicals and Reagents

All chemicals used were of analytical grade reagent obtained from Sigma -Aldrich (USA). Solvents used for extraction were all pre-purified by distillation.

2.4.2 Preparation of experimental samples

The *S haematodes* root was cut into small pieces, dried and ground into fine powder (5 kg) using a Wiley mill. The powdered material was then extracted with methanol using ultrasound-assisted extraction technique (Zhang *et. al.,* 2009). The extract was filtered and dried under vacuum at 40-50^{0}C yielding 152 g of crude extract. The crude extract was then resuspended in methanol and subjected to liquid-liquid partitioning into organic solvents of varying polarities, starting with n-hexane, chloroform, ethyl acetate and 1-butanol. The resultant solvent fractions were dried under vacuum and lyophilized to yield n-hexane, chloroform, ethyl acetate and 1-butanol fractions, all of which were stored individually at -20^{0}C prior to analysis.

2.4.3 Sun Protection Factor (SPF) Measurements

The *in-vitro* SPF value was obtained by the following method. Briefly, the absorbance of a methanolic solution (100 μg/mL) of the test sample was determined on a UV-Visible spectrophotometer at 290-320 nm. Methanol was used as a blank and measurements were made in triplicates. The SPF value was then calculated by using the formula (Santhanam *et. al.,* 2013):

$$\text{SPF spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where: EE (λ) is the erythemal effect spectrum

I (λ) is the solar intensity spectrum

EE (λ) x I(λ) are constants.

Abs (λ) is the absorbance of test sample

CF is the correction factor (= 10)

2.4.4 Measurement of UVA/UVB Absorption Spectrum

The UV absorption spectrum for each test sample (100 μg/mL in methanol) was measured by a UV-Visible spectrophotometer using 1 cm quartz cell, over a wavelength range of 200-400 nm. The absorption spectrum of the test samples was compared to that of Epigallocatechingallate (EGCG) prepared at the same concentration (Patil *et. al.,* 2009).

2.5 MICROBIAL CONTAMINATION TESTING

2.5.1 Sample Collection and Study Area

A total of 07 different antidiabetic herbal preparations (ADHPs) were analyzed for microorganisms in the Department of Microbiology, Bangladesh University of Health Sciences (BUHS), Mirpur, Dhaka.

2.5.2 Chemical and Reagent

All chemicals and reagents were of analytical grade and procured from oxoid ltd, UK. Experiments were done carefully with appropriate control. Chemicals and reagents used were:

For culture: Blood agar, Mac Conkey agar, Chocolate agar and Saboraud's dextrose agar.

For Gram's stain: 1% Crystal violet, Lugol's iodine, Acetone, Dilute carbol fuchsin.

Others: Hydrogen peroxide, Bile esculin, 6.5 % sodium chloride in trypticase soy broth and normal saline.

2.5.3 Bacteriological analyses

Blood agar, MacConkey agar, Chocolate agar and Saboraud's dextrose agar were used (Oxoid) for culturing and isolation of bacteria and fungus (Prescott *et. al.,* 1999). Identification of organisms were done as standard ways (Texeira *et. al.,* 2011 and Logan *et. al.,* 2011).

2.5.4 Preparation of media

All dehydrated media were prepared according to manufacturer's instructions. The sterile media were dispensed or poured into sterilized petri dishes and allowed to cool. The sterility of the prepared media was checked by incubation of blindly selected plates at 37^0C for 24 hours.

2.5.5 Total aerobic bacterial plate count

The method as mentioned by Brown, Poxton and Wilkinson was used (Brown *et. al.,* 1989). For liquid drugs 1:10 dilutions were made. For tablets and capsules 1 g was dissolved in 10 mL normal saline and then 1:10 dilutions were made. The dilute sample solutions (100 µL each) were placed on the surface of plates and spread widely with sterile inoculation wire. The count was calculated from average colony count/plate (Brown *et. al.,* 1989).

2.6 INSULIN SECRETORY ACTIVITY

2.6.1. Insulin Secretion Activity of Isolated compounds from *Phlogacanthus thyrsiflorus* Nees

The cell culture and insulin secretion assay were done at the Dr. Panjwani Center for Molecular Medicine and Drug Research (PCMD), International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan under a collaboration arrangement.

2.6.2. MIN6 Cell Culture and Insulin Secretion Assay

Mouse insulinoma pancreatic beta cells (MIN6) were cultured as described by Miyazaki *et. al.*, (1990). MIN6 cells were cultured in Dulbecco's modified Eagle's medium containing 25mM glucose supplemented with 12% fetal bovine serum (FBS), 2mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 5 µL β-mercaptoethanol at 37^0C in a humidified atmosphere of 5% CO_2 / 95% air. Cells of passage number 24-30 were used for insulin secretion assay.

MIN6 cells were seeded onto 24-well plates at a density of 5×10^5 cells per well. After 24 hours of plating, cells were washed twice and pre-incubated with Krebs-Ringer HEPES buffer containing 119mM NaCl, 4.7mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 10mM HEPES (pH 7.4), 2mM glucose and 0.1% BSA for 60 min at 37^0C. These cells were then incubated at 37^0C in Krebs-Ringer HEPES buffer containing 2 or 20mM glucose in the presence or absence of test compound. After 30 min of incubation, 100 µL supernatant was collected, centrifuged at 1000 rpm, and stored at -40^0C until insulin assay. Secreted insulin in supernatant was measured using an ultra sensitive mouse insulin ELISA kit (Crystal Chem Inc., IL, USA).

2.6.3 Toxicity Assay

Cytotoxicity activity of compounds **1**, **3** and **4** of *P thyrsiflorus* were evaluated on MIN6 cell lines by using the standard MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colorimetric assay according to Mosmann (1983).

B. CHEMICAL ANALYSIS

2.7 ISOLATION OF COMPOUNDS FROM TWO PLANTS

2.7.1 Solvents and chemicals

All solvents and chemicals used in the present work were procured from E. Merck (Germany) and BDH (England); and these were either meant for laboratory use or were of analytical reagent grade. Commercial grade of chloroform, methanol, ethyl acetate and absolute alcohol were distilled in glass distillation system before use. Some solvents were of gradient grade for using in HPLC.

2.7.2 Evaporation

All evaporations were performed at reduced pressure using rotary vacuum evaporator (Eyela N-1100, Japan) at water bath (Eyela –SB-1100) temperature not exceeding 40^0C (Pump: Eyela A-1000 S). Small volume of non-aqueous solvent was concentrated by flushing with nitrogen at room temperature.

2.7.3 Freeze-drying

All freeze-drying were carried out with a ilShin Biobase (Korea) model FDS5508 freeze-dryer and the aqueous solution of the samples were first frozen in round bottomed flask in a Shell freezer at - 30^0C.

2.7. 4 Chromatographic techniques:

Various traditional and modern chromatographic techniques were executed successfully for the isolation and purification of compounds from plant extracts and antidiabetic herbal preparations (ADHPs) in the present study. Silicagel and Sephadex LH-20 were used as stationary phase for column chromatography. Two types of chromatographic techniques such as thin layer chromatography (TLC) and column chromatography (CC) were used.

2.7.4.1 Thin layer chromatography (TLC)

For thin layer chromatography (TLC), precoated aluminium sheets or glass plates (TLC grade silica gel 60 F_{254}; Merck, Germany), both normal and reverse phase were used. Some of the solvent systems used in this work are given below:

For the less polar fractions and compounds binary solvent systems were used *e.g.*,

(A) Hexane : chloroform   (in different ratio)

(B) Hexane : ethyl acetate   (in different ratio)

(C) Ethyl acetate : methanol   (in different ratio)

(D) Dichlomethane : methanol   (in different ratio)

and for more polar fractions and compounds trinary and tetranary solvent systems were used *e.g.*,

 (E) Ethylacetate : acetic acid : water   (8: 2 : 1)

(F) Ethylacetate : 1-butanol : methanol : water   (4: 2 : 1: 0.1)

The plates were developed by one of the following methods to detect the position of the spots.

i)   Examination under a UV light source with two different wave lengths (254 nm and 350 nm).

ii)   The plates were exposed to iodine vapour.

iii)   The plates were sprayed with vanillin-sulphuric acid reagent followed by heating at 120^0C for 15 min.

iv)   The plates were sprayed with aqueous 50% H_2SO_4 followed by heating at 120^0C for 15 min.

2.7.4.2 Development of TLC plates.

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

plate was taken out and dried. The solvent front was not allowed beyond the end of silica-coated surface.

2.7.4.3 Detection of Spots

For the location of the separated compounds, the plates were examined under UV light in different wave lengths, 254 nm and 365 nm and/ or the plates were developed by the sulfuric acid spray reagent followed by heating in an oven at 80^0C for 5 min.

2.7.4.4 Preparation of vanillin– sulfuric acid reagent

To prepare vanillin-sulfuric acid reagent, one gram of vanillin was dissolved in 100 mLof concentrated sulfuric acid. The prepared solution was then kept in a glass made sprayer. Irrigated TLC plates were sprayed and heated at 120^0C for 10 minutes.

2.7.4.5 The R_f value

Retardation factor (R_f) is the ratio of the distance that the compound travels and the distance that the solvent front moves.

$$Rf = \frac{\text{Distance travelled by a compound}}{\text{Distance traveled by a solvent}}$$

Usually, the R_f value is constant for any given compound for a given solvent or solvent mixture and it corresponds to a physical property of that compound.

2.7.5 High-performance liquid chromatography (HPLC)

HPLC used for Identification:

High Performance Liquid Chromatography (HPLC) was carried out with a Shimadzu HPLC system using two pumps (SHIMADZU Prominence LC 20AT) which were coupled with a column oven (CTO-20AT) and a detector (PDF-20AT). The detecting signal was processed by a data processor. C18 (250 mm x 4.6 mm) analytical and semi-preparative columns were used. The pumps, detector and column oven were controled by a system controller (SCL-20AT).

Flow Rate: 1.0 mL/ min

Solvent System (Mobile Phase): ACN: Buffer = 52::48

Column: C18 column (250 mm x 4.6 mm)

Detector: PDA

Wavelength: 230 nm

HPLC used for Separation and Isolation:

High performance liquid chromatography (HPLC) was carried out in Shimadzu HPLC system using single pump (SCL-10A vp) which was coupled with a column oven (CTO-10 AS vp) and a detector (SPD-10A vp). The detecting signal was processed by a Class 10 Avp software. Luna 5 µm packs of RP-C18 and DB Silica analytical and semi-preparative column were used. The pumps, detector and column oven were controlled by a system controller (LC- 10Avp).

Flow Rate: 1.0 mL/ min

Solvent System (mobile phase): ACN : H_2O = 70 :: 30 and different ratio of MeOH : H_2O, IPA : n-hexane were used.

Column: C18 column (250 mm x 4.6 mm) and DB Silica (250 mm x 4.6 mm) columns were used.

Detector: UV-Vis

Wavelength: 210 nm, 230 nm and 254 nm etc

2.7.6 Ultraviolet spectroscopy (UV)

The UV absorbance was measured in ethanol or MeOH using a PERKIN ELMER LAMBDA-25, UV/VIS spectrophotometer by selecting the particular wavelength for the particular fraction / compound.

2.7.7 Infrared spectroscopy (IR)

A Shimadzu FT-IR, 8400 S, IR-Prestige spectrometer was used for recording infrared spectrum. Major bands (Vmax) were recorded in wave number (cm^{-1}) as potassium bromide (KBr) pellets.

2.7.8 Nuclear magnetic resonance (NMR) spectroscopy

1D and 2D NMR spectra were recorded on a BRUKER NMR DBX-400 MHz instrument, with chemical shift data reported in ppm relative to TMS. The spectra were taken by using $CDCl_3$, CD_3OD, DMSO-d_6 and D_2O with tetra methyl silane (TMS) as standard reference.

2.7.9 NMR Techniques

Both 1D and 2D NMR techniques were used extensively in the structure elucidation of the compounds isolated in this study. FT techniques involves irradiating the sample with a single radio frequency pulse of a few microseconds duration (Sadler, 1988), this is normally enough to excite all of the nuclei of a given magnetic isotope and results in an emission signal from the excited nuclei. The signal is known as the free induction decay (FID). Fourier Transformation (FT) of this decay (or more frequently, of an accumulation of a set of successively obtained and combined decays) yields the NMR spectrum. Different types of comparatively modern NMR techniques, which were applied in this study, will be described briefly.

2.7.9.1 One – Dimensional (1D) NMR Spectroscopy

The simple ^{1}H-and ^{13}C-NMR spectra were acquired for all the isolated compounds. In some cases, the information was inadequate and therefore required 2D NMR experiments either for confirmation or to complete a structure. With ^{13}C-NMR spectroscopy the J-modulated ^{13}C spectrum was preferred to the ^{13}C off-resonance because it was more informative than the latter (Coll and Bowden, 1986).

2.7.9.2 J-modulated ^{13}C NMR spectroscopy

The J-modulated ^{13}C experiments simplifies the ^{13}C-NMR spectrum without losing the structural information provided by the multiplicity of ^{13}C resonances. In this technique non-protonoted or quarternary carbon (C) and methylene (-CH_2-) carbon nuclei appear positive in contrast to negative methane (CH_4) and methyl (-CH_3) signals. By varying the delay time it is possible to increase the sensitivity of some desired carbons (Sadler, 1988)

2.7.9.3 Two-dimensional Homonuclear Correlation Spectroscopy

^{1}H-^{1}H Correlation Spectroscopy (^{1}H-^{1}H COSY).

The ^{1}H-^{1}H COSY technique (Edward and Stocker, 1967; Edward and Stocker, 1968,Stech, 1967) indicates ^{1}H-^{1}H connectivities that are geminal, vicinal or in a W-relationship of the H atoms in a molecule. Every ^{1}H-^{1}H coupling interaction can be indentified in the ^{1}H-^{1}H COSY contour plot by two diagonal signals and the cross signals of the coupling patterns, which form the four corners of a square. The coupling partner (cross signal) of a particular proton generates a signal on the vertical or horizontal line from the relevant ^{1}H signal.

2.7.9.4 Two Dimensional Heteronuclear Correlation Spectroscopy HMBC and HSQC

 In these experiments, two-dimensional spectra are obtained with ^{13}C chemical shifts on one axis and ^{1}H chemical shifts on the other. In the HSQC experiment (Bax, 1983), a correlation is achieved between ^{13}C and the proton to which it is directly attached (i.e., one bond correlation ^{1}J) whereas long range ^{1}H-^{13}C correlation (^{2}J, ^{3}J) are observed from the HMBC experiment (Bax & Summers, 1986) and provide a wealth of structural information which is particularly helpful in the structure elucidation of complex compounds.

2.7.10 Mass spectroscopy

The mass spectra were recorded at 70 eV with a helium flow rate of 2.5 mLmin^{-1} with a Finnigan 4021 instrument in BCSIR, Bangladesh and GC-MS QP2010 Ultra (Shimadzu) in University Putra Malaysia. The fast atom bombardment mass spectrum (FAB+MS) was recorded as a positive ion mode with m/z ranging between 0.0020-1000.0000.

2.7.11 Preparation of silica gel flash column

Glass columns of different size, varying from a large glass tube (30 cm × 14 cm, i.d., fitted with a rotaflow) to a small burette like tube (30 cm × 1 cm, i.d., fitted with teflon rotaflow) were used for chromatographic separations. Column grade silica gel of different

mesh (70-230; 230-400 µm) were used as stationary phase. To prepare a particular column the required amount of silica gel was swelled into a selected solvent (e.g., hexane, chloroform or ethyl acetate or a mixture of different solvents in different ratio) for a while and then poured into the column with continuous flow of the solvent and occasional tapping of the column for homogeneous packing of the silica gel. The column was equilibrated with 3-4 bed volumes of solvent by a fish aquarium air pump in order to settle the column bed.

2.7.11.1 Application of the sample into the column

The crude extract or sub-fraction thereof was applied into the column either as a solution or in a powdered form with the solid phase. To apply the sample in powder form the sample was dissolved in a particular solvent or a mixture of solvents and silica gel (sample: gel, 1:2 w/w) was added to the sample solution and the mixture was evaporated to dryness. The dried material was ground thoroughly in a mortar to make a fine powder and the powder was then applied onto the column.

To apply the sample in the form of solution it was dissolved in minimum volume of the column equilibrating solvent and was applied into the column. After application of the sample, some glass wool or some sand was placed on the top of the silica gel bed so that the surface of the bed was not affected during solvent application.

2.7.11.2 Fractionation and monitoring procedure

After application of the sample, the columns were eluted with the equilibrating solvent and the polarity of the mobile phase was increased gradually by adding more polar solvent such as DCM, ethyl acetate and methanol. The eluted samples were collected in conical flasks/test tubes and the fractions were monitored by TLC on the basis of their R_f values, using different solvent systems. The fractions were also monitored on the basis of their low-resolution ^{1}H-NMR spectra where applicable.

2.7.12 Preparation of Sephadex LH-20 column (Gel filtration)

Gel filtration or size exclusion chromatography was mainly used for the separation of small hydrophobic compounds from their larger contaminants, usually chlorophylls and other pigments either from the extracts or from column fractions. Normal glass columns

packed with Sephadex LH-20 (particle size 25-100 μm, Pharmacia) and developed by MeOH : H_2O mixtures of different ratios were used. At last, the column was washed with pure MeOH to clean it for further use.

2.7.13 Melting point

The melting point (m.p.) of compounds were determined by using an electro-thermal melting point apparatus (Stuart Scientific SMP3, UK).

2.7.14 Extractions of root (underground part) of *Salvia haematodes* Linn with different solvents (Scheme 2.7.1)

The dried and powdered roots (5 kg) of *S haematodes* was extracted with dichloromethane (DCM) (5 X 3=15 L) at room temperature using a special glass tank for three days by changing DCM each day. The DCM extract was filtered and the filtrate was concentrated. The concentrated extracts were combined, evaporated to dryness and finally dried in a freeze-dryer to give 48 g of DCM extract. The extracts were stored at -22^0C in a refrigerator. The residue, after DCM extraction of root of *S haematodes* was extracted with ethyl acetate (4 X 3=12 L) following the same procedure as described above. The extract was filtered and the filtrate was concentrated by a vacuum rotary evaporator. The concentrated extracts were combined, evaporated to dryness and finally freeze-dried to give 19 g extract.

Then the residue, after ethyl acetate extraction was extracted with methanol (5 X 4=20 L) following the same procedure as described above. The extract was filtered and the filtrate was concentrated by a vacuum rotary evaporator. The concentrated extracts were combined, evaporated to dryness and finally freeze-dried to give 152 g extract.

Scheme 2.7.1

Salvia haematodes **Linn**

| Root dried powder 5 kg |

Extraction with DCM
3 times (total 14 L)

| DCM soluble part (48 g) | Extraction with ethyl acetate 3 times (total 10 L) | Residue |

| Ethyl acetate soluble part (19 g) | Extraction with methanol 4 times (total 17 L) | Residue |

| Methanol soluble part (152 g) | Residue 4.5 kg |
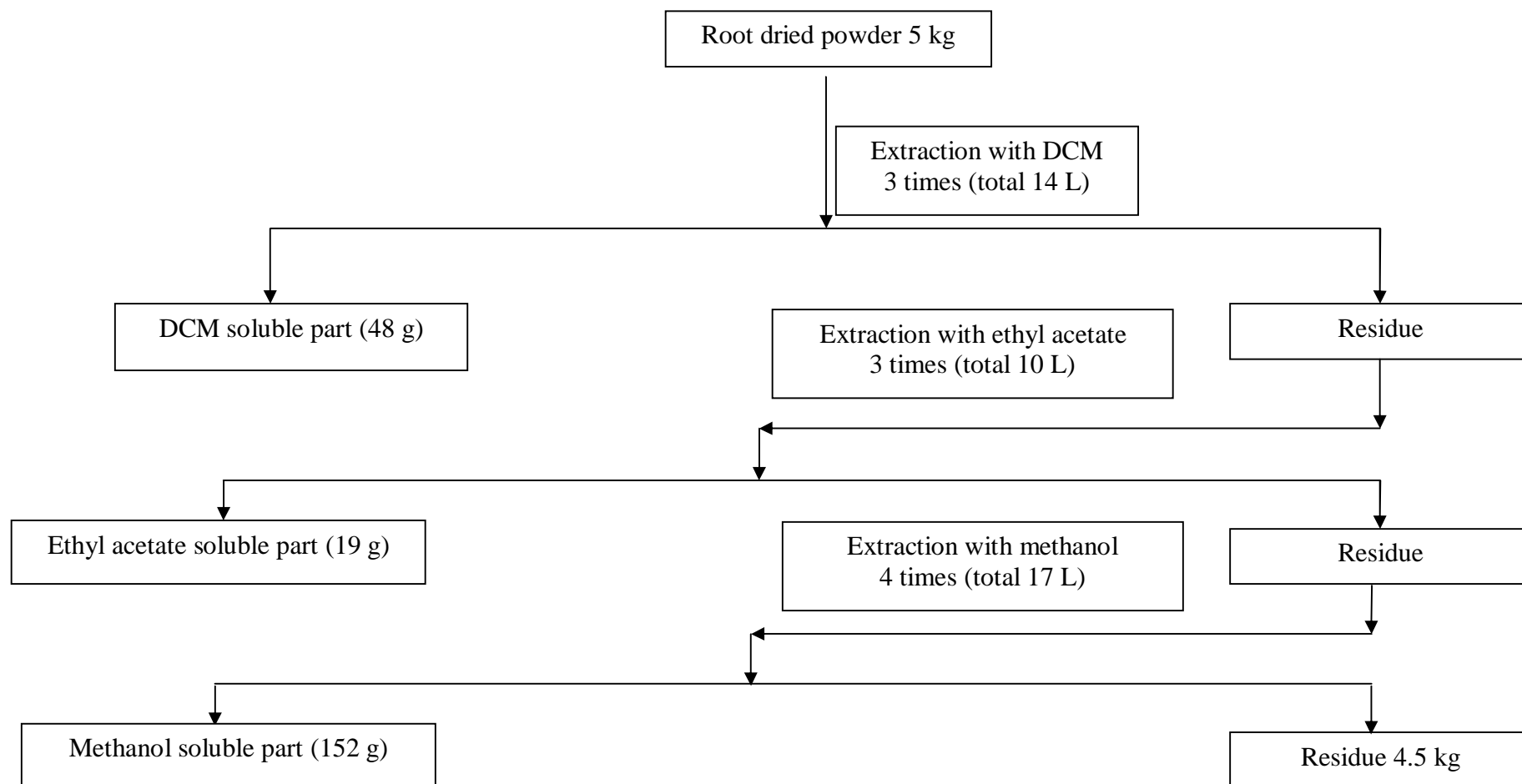
2.7.15.1 Extraction of flower of *Phlogacanthus thyrsiflorus* Nees with different solvents (Scheme 2.7.2)

The dried and ground powder of *P thyrsiflorus* flower (300 g) was soaked in DCM (2 L X 4) at room temperature using a special stainless steel tank for six days by changing solvent (DCM) each day. The extract was filtered and the filtrate was concentrated. The concentrated extracts were combined, evaporated to dryness and finally dried in a freeze-dryer to give 8.1 g of DCM extract. The extract was stored at -22^0C in a refrigerator.

The residue, after DCM extraction of *P thyrsiflorus* flowers was extracted with ethyl acetate (EtOAc) (2 L x 4) following the same procedure as described above. The extract was filtered and the filtrate was concentrated by a vacuum rotary evaporator. The concentrated extracts were combined, evaporated to dryness and finally freeze-dried to give 3 g extract. The extract was stored at -22^0C in a refrigerator.

After EtOAc extraction the residue was extracted with methanol (2 L x 5) following the same procedure as described above. The extract was filtered and the filtrate was concentrated by a vacuum rotary evaporator. The concentrated extracts were combined, evaporated to dryness and finally freeze-dried to give 16 g extract. The extract was stored at -22^0C in a refrigerator. The residue (259 g) was dried and stroed.

Scheme 2.7.2

Phlogocanthus thyrsiflorus **Nees**

```
                        ┌─────────────────┐
                        │ Dried Powder of │
                        │  Flower 300 g   │
                        └─────────────────┘
                                │
                        ┌──────────────────────────┐
                        │   Extraction with DCM     │
                        │ 4 times (2+1.5+1.5 + 1.5 L)│
                        └──────────────────────────┘
          ┌─────────────────────┐              │
          │ DCM soluble part     │         ┌──────────┐
          │ (8.1 g)              │         │ Residue  │
          └─────────────────────┘         └──────────┘
                    ┌───────────────────────────┐   │
                    │ Extraction with ethyl acetate│
                    │ 4 times (2 L each time)     │
                    └───────────────────────────┘
   ┌───────────────────────────┐        ┌──────────┐
   │ Ethyl acetate soluble part │       │ Residue  │
   │ (3 g)                      │       └──────────┘
   └───────────────────────────┘            │
              ┌───────────────────────┐
              │ Extraction with methanol│
              │ 5 times (10 Liters)    │
              └───────────────────────┘
   ┌───────────────────────────┐       ┌────────────────────┐
   │ Methanol soluble part (16 g)│      │ Residue    (259 g) │
   └───────────────────────────┘       └────────────────────┘
```
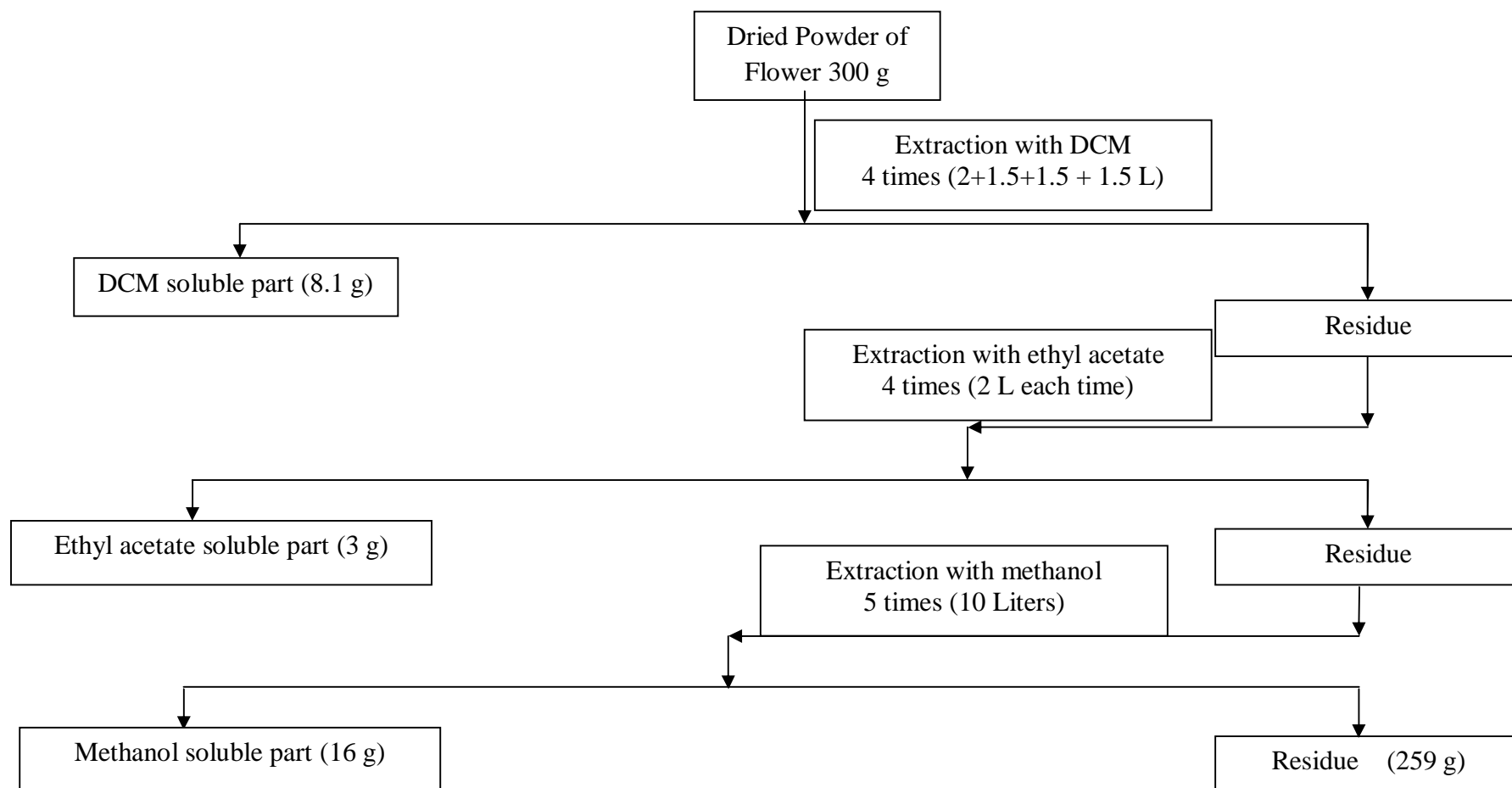
2.7.15.2 Extraction of Flower of *Phlogacanthus thyrsiflorus* Nees with different solvents (Scheme 2.7.3)

The flower powder (800 g) of *P thyrsiflorus* was extracted with aq 80% ethanol (3L X 6) at room temperature using a special stainless steel tank. The extract was filtered and the filtrate was concentrated. The concentrated extracts were combined, evaporated to dryness and finally dried in a freeze-dryer to give 144 g of ethanol extract. Then 02 gm aq 80% EtOH was kept in freeze and rest of the extract (142 g) was used for solvent-solvent extraction.

2.7.15.2.1 Extraction of residue with DCM:
The residue, after aq 80% ethanol extraction was extracted with DCM (2 L X 5) at room temperature for five days by changing DCM each day. The extract was filtered and the filtrate was evaporated to dryness and finally freeze-dried to give 13 g DCM extract and this amount of DCM were mixed with another DCM part. So the total amount of DCM part is 50 g.

2.7.15.2.2. Solvent-solvent partition of ethanol extract of the flower

The aq 80% ethanol extract of flower (142 g) was suspended in water (500 mL), ultrasonicated and partitioned with DCM (500 mL X 3). The DCM soluble part was concentrated, evaporated to dryness and added with another DCM part from residue of aq 80% EtOH. Finally dried in a freeze - dryer to give 49.87 g of DCM soluble part. Then this DCM soluble part was suspend with aq 90% MeOH and partition 3 times with n-hexane and collected both fractions. n-hexane soluble part was evaporated to dryness and finally dried in a freeze-dryer to give (11.95 g) of n-hexane soluble part. The aqueous 90% MeOH was evaporated to dryness and finally freeze-dried to give 34.96 g of aq 90% MeOH soluble part. From these two parts, ^{1}H-NMR was taken and stored the fractions for isolation of compound(s).

The H_2O part aq 80% of EtOH (after partition with DCM) was again partition with 1-BuOH for three times and collected both of these parts in different conical flask. Then both of these parts were evaporated to dryness and finally freeze-dried to give 54 g of 1 BuOH soluble part and 53.55 g of aqueous part. From these two parts, ^{1}H-NMR was taken and stored the fractions for isolation of compound(s).

Scheme 2.7.3

Phlogacanthus thyrsiflorus **Nees**

Dried Fresh Powder of Flower 800 g

Extraction with aq 80% EtOH 6 times

Aq 80% EtOH Soluble Part (144 g)

Save 2 g for other assay

Residue

Extraction with DCM

Suspend in H₂O (Ultra sonication) add DCM

Aqueous Part

DCM Soluble Part (36.590 g) after freeze dry

DCM Soluble Part (13 g) after freeze dry

Residue (630) g (SAVE)

1- BuOH

Combined

Aqueous Part

1- BuOH part 54 g

Total DCM Soluble Part (49.872 g)

Save 2 g for other assay

Freeze Dry

¹H-NMR

Concentrated to remove water and Dry with Freeze dryer

H₂O part 53.553 g SAVE

Isolation of Compounds

Aqueous 90% MeOH and Partition with n-hexane

134

```
                        ┌──────────────────────┐                              ┌──────────────────────────────┐
                        │    n-hexane  Part     │                              │   Aqueous 90% MeOH  part     │
                        └──────────────────────┘                              └──────────────────────────────┘
                                  │    ┌──────────────┐                                   │    ┌──────────────┐
                                  │    │  Freeze Dry  │                                   │    │  Freeze Dry  │
                                  │    └──────────────┘                                   │    └──────────────┘
                                  ▼                                                       ▼
                        ┌──────────────────────────────┐              ┌──────────────────────────────────────┐
                        │ Dry n-Hexane part 11.947 gm  │              │ Dry Aqueous 90% MeOH  part  34.960 g │
                        └──────────────────────────────┘              └──────────────────────────────────────┘
                                  │    ┌──────────────┐                                   │    ┌──────────────┐
                                  │    │  ¹H-NMR      │                                   │    │  ¹H-NMR      │
                                  │    └──────────────┘                                   │    └──────────────┘
                                  ▼                                                       ▼
                        ┌──────────────────────────┐                      ┌──────────────────────────┐
                        │  Isolation of Compounds  │                      │  Isolation of Compounds  │
                        └──────────────────────────┘                      └──────────────────────────┘
```

n-hexane Part

Freeze Dry

Dry n-Hexane part 11.947 gm

^{1}H-NMR

Isolation of Compounds

Aqueous 90% MeOH part

Freeze Dry

Dry Aqueous 90% MeOH part 34.960 g

^{1}H-NMR

Isolation of Compounds

2.7.15.2.3 Sephadex Column LH-20

Washing Procedure

```
┌─────────────────────────────┐
│    100 g Sephadex LH-20      │
└─────────────────────────────┘
                │        ┌──────────────┐
                │        │     Wash     │
                ▼        └──────────────┘
┌─────────────────────────────┐
│           Acetone            │
└─────────────────────────────┘
                │
                ▼
┌─────────────────────────────┐
│        100 % Methanol        │
└─────────────────────────────┘
                │
                ▼
┌─────────────────────────────┐
│   50% MeOH + 50% DI H2O      │
└─────────────────────────────┘
                │
                ▼
┌─────────────────────────────┐
│      100 % DI $H_2O$         │
└─────────────────────────────┘
```
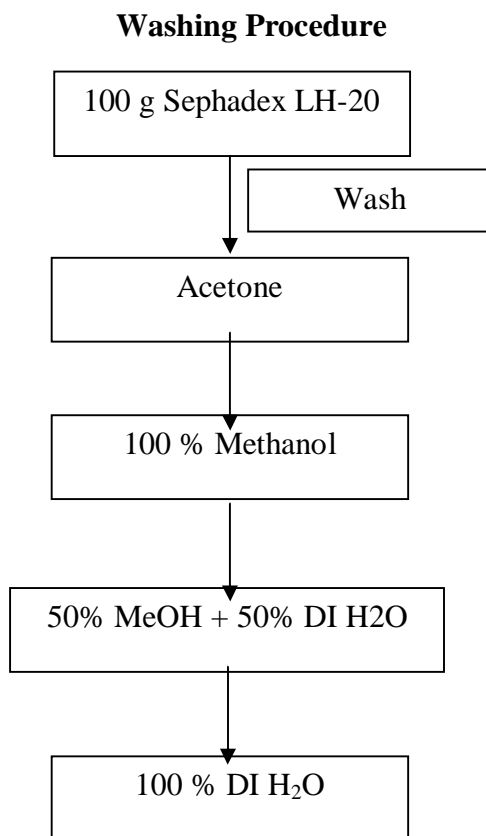
2.7.16 Isolation of Compounds 1-3 from DCM and EtOAc extracts of *Salvia haematodes* Linn (Schemes 2.7.4 and 2.7.5)

A part of the DCM extract of root of *S haematodes* (48 g) was chromatographed over silica gel (Scharlau G-60, spain) column and eluted gradually with hexane, DCM, EtOAc and methanol mixtures of increasing polarity which gave eighteen fractions. The fifth fraction was again fractionation over a silica gel column and eluted gradually with n-hexane and the polarity was increased by adding DCM and EtOAC in different proportions. The needle shaped crystalline sixth fraction (1.03 g) was purified by washing with n-hexane and methanol to give Compound **1** (Scheme 2.7.4).

The eleventh fraction of 3rd column (1.66 g) upon rechromatography over silica gel Scharlau G-60 (spain) using 50% n-hexane in DCM as the mobile phase to give six fractions. Compound **2**, was isolated as white needles from the fifth fraction of the second column, after washing with DCM and methanol (Scheme 2.7.4).

About 1 g of ethyl acetate (EtOAc) extract was dissolved with aqueous 10% MeOH and partition with n-hexane, Chloroform and EtOAc, successively. Then collected four fractions were evaporated to dryness and finally freeze-dried to give 355 mg of n-Hexane, 350 mg of $CHCl_3$, 180 mg of EtOAc and 135 mg of aqueous part.

By the $CHCl_3$ fraction (271 mg) of EtOAc extract, was chromatographed over silica gel Scharlau G-60 (spain) column and eluted gradually with n-hexane, EtOAc and methanol mixtures of increasing polarity which gave seventeen fractions. Fractions-10 and 12 were purified by washing several times with n-hexane and MeOH and Compound **3**, was obtained from the fraction-10 (09 mg) (Scheme 2.7.5).

Scheme 2.7.4

DCM extract of SH-RT

1st column

F1-F4   F5   F6-F10   F11   F12- F14   F15-F18

2nd column

3rd column

F1-F5   F6   F4   F1-F4   F5   F6

Compound 1   **Compound 2**   137

Scheme 2.7.5

EtOAc extract of SH-RT

Suspend in aq 10% MeOH and partition with n-hexane, Chloroform, EtOAc and MeOH successively

n-HX part   CHCl$_3$ part   EtOAc part   Aqueous part

Normal Phase CC

F1-F2   F4-F6   F7   F8-F9   F10   F11   F12   F13-14   F15-F16
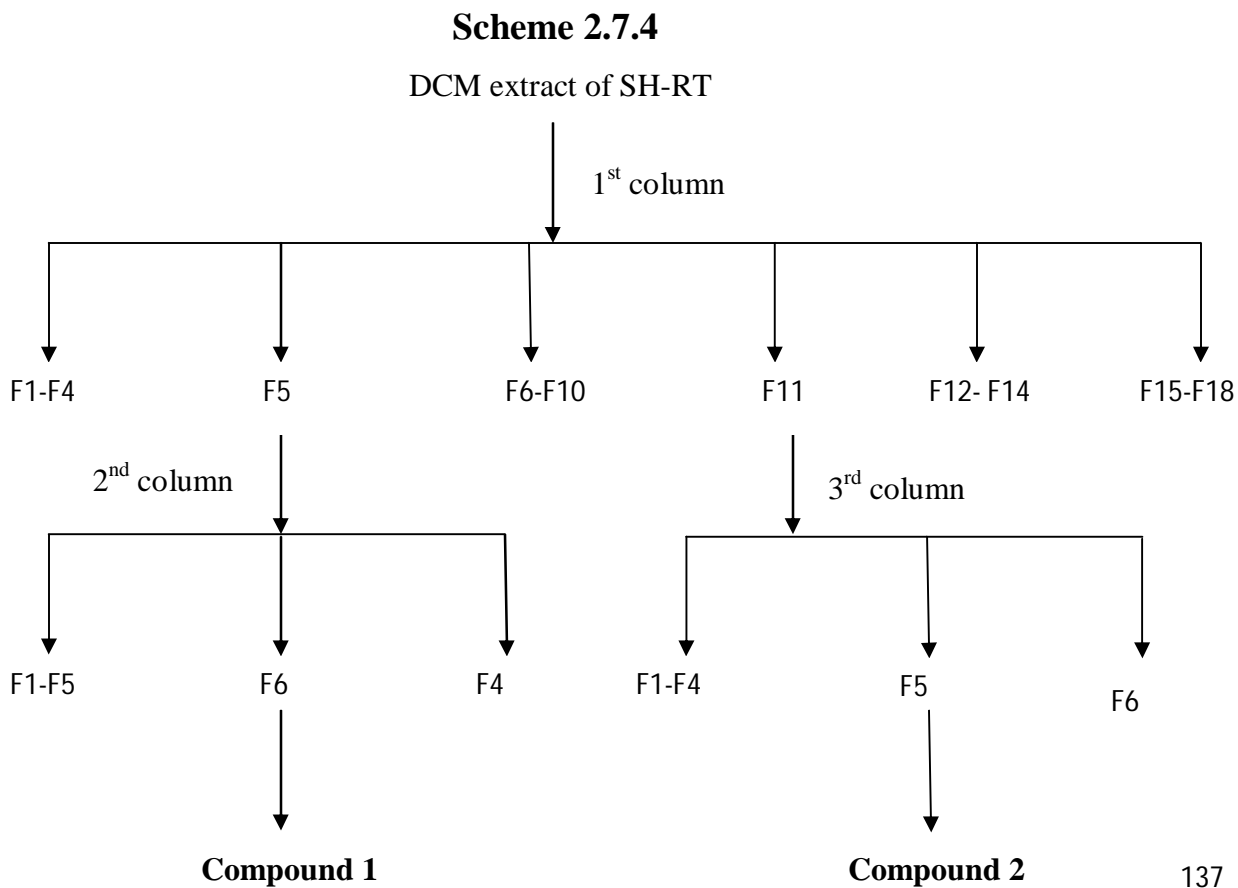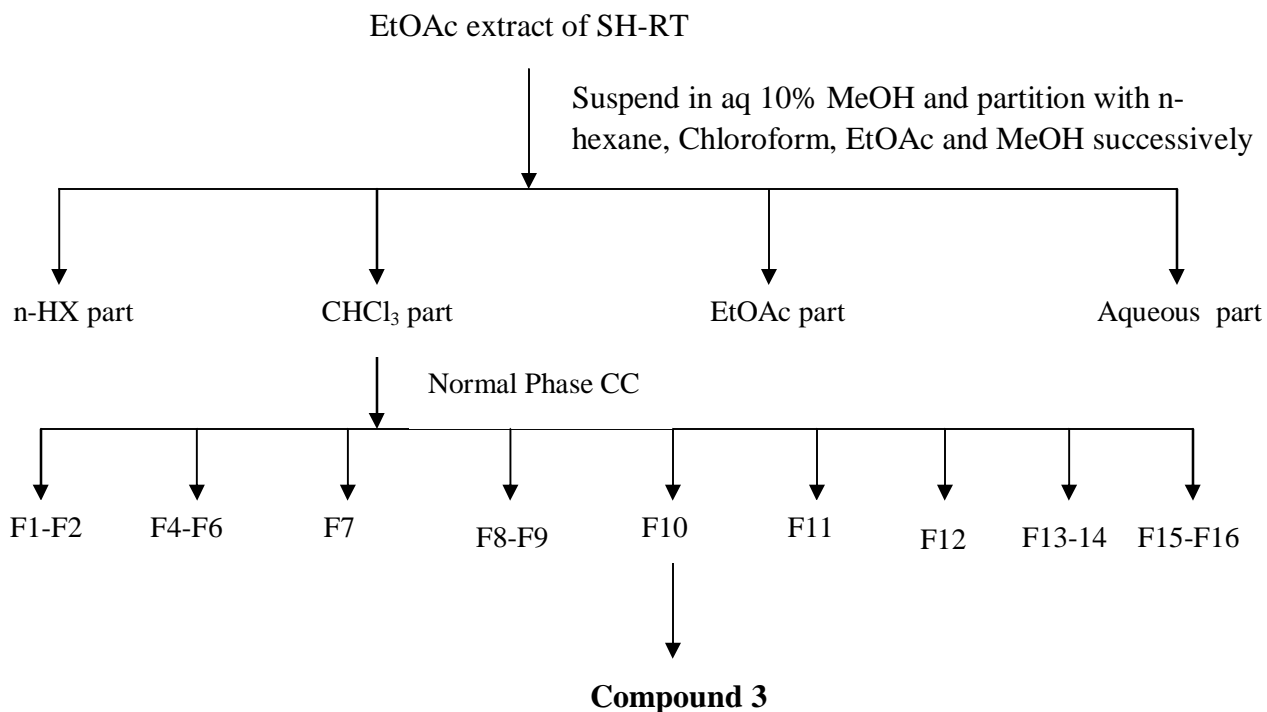
Compound 3

2.7.17 Isolation of Compounds 4-7 from DCM soluble material of flower extract of *Phlogacanthus thyrsiflorus* Nees (Schemes 2.7.6)

First column

The DCM extract of flower of *P thyrsiflorus* Linn (8.1g) was chromatographed over silica gel [Scharlau G-60 (spain)] column and eluted gradually with n-hexane, EtOAc and methanol mixtures of increasing polarity which gave fourteen fractions.

The fifth fraction of the first column was again fractionated over a normal phase silica gel column (2nd column) and eluted gradually with n-hexane and the polarity was increased by adding EtOAc & methanol in different proportion. The fifth column fractions showed needle shaped crystals. Compound **4** was isolated by washing the crystal with n-hexane and methanol.

Column fractions 6-12 of 2nd column was again fractionation over a silica gel column (3rd column) and eluted gradually with n-hexane and the polarity was increased by adding EtOAc & methanol in different proportion. The fifth column fraction showed needle shaped crystals. Compound **5** was isolated by washing the crystal with n-hexane and methanol.

The eleventh (F11) fraction of 1st column showed brownish crystals. Then the fraction was again fractionation over a silica gel column (4th column) and eluted gradually with n-hexane and the polarity was increased by adding EtOAc & methanol in different proportions. Fifth fraction of 4th column showed fatty shaped crystals. Compound **6** was isolated by washing the fatty crystals with n-hexane and methanol.

The eighth fraction of the 4th column fraction also showed white powder. Compound **7** was isolated by washing the powder with n-hexane and methanol.

Scheme 2.7.6

DCM extract of *P thyrsiflorus*

Column Chromatography-1

1st column

F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12 F13 F14

2nd and 3rd column

4th column

F1-4 F5 F6-12 F1-4 F5 F6 F7 F8 F9 F10 F11-14

Compound 4

Compound 5

Compound 6   **Compound 7**

139
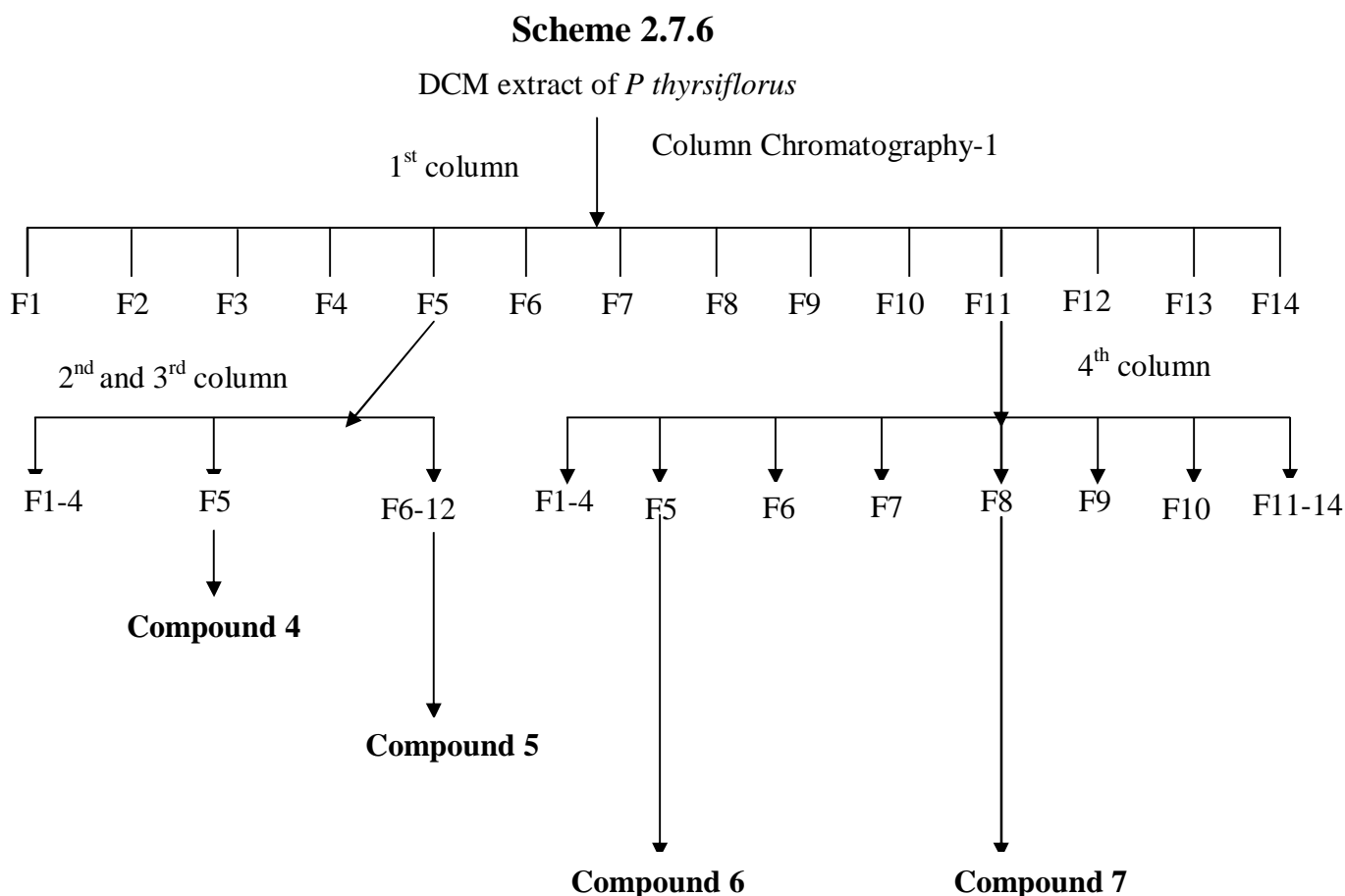
2.7.18. Isolation of compounds 8 from BuOH soluble material of flower extract of *Phlogacanthus thyrsiflorus* Nees (Scheme 2.7.7)

The 1-BuOH extract of flower of *P thyrsiflorus* (52g) was chromatographed over Sephadex LH-20 column and eluted gradually with DI H_2O and MeOH mixtures of decreasing polarity which was collected in different 71 conical flasks.

Then fraction (F35) was passed into the RP-C18 cartridge. Then the sample was injected in HPLC system.

Flow Rate: 1.0 ml/ min

Solvent System (Mobile Phase): MeOH : H_2O = 3 : 2

Column: C18 column (length: 250 mm   diameter: 4.6 mm   particle size: 5 µm)

Detector: UV-Vis

Wavelength: 254 nm

There are several small peaks were shown in the HPLC chromatogram and the major peak was collected as Compound **8** (19 mg). Then NMR spectra were run of the compound **8** and was recorded using 400 MHz NMR Spectrophotometer.

Scheme 2.7.7

1-BuOH soluble part of P thyrsiflorus flowers

Sephadex LH 20 Column

F1	F2	F3	F4	F5	F6
1-19	20-29	30-38	39-48	49-58	59-71

Column Fraction-35

Wash by using Cartridge and filtered

Filtered

HPLC

Peak-1   Peak 2   Peak 3   Peak 4
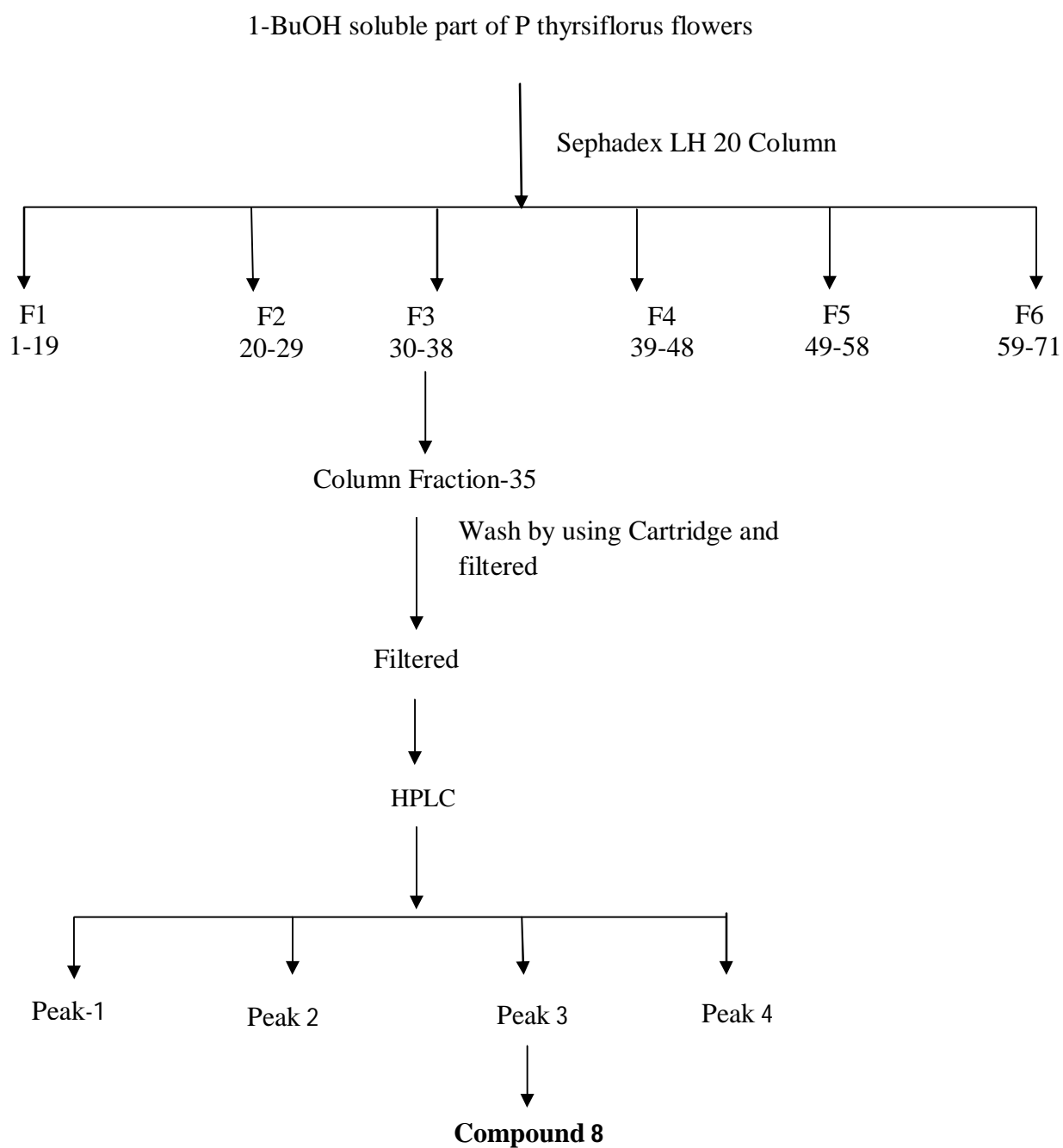
Compound 8

2.7. 19 Characterization of Compounds (1-8)

2.7. 19.1. Compound 1

Its melting point was 137-38^{0}C. R_f value was 0.5 (30% EtOAc in n-hexane). The ^{1}H- and ^{13}C-NMR spectral data (CDCl$_3$) are presented in Table 3.7.1.

2.7. 19.2. Compound 2

The compound was needle shaped crystals. The m.p. of the compound was 162^{0}C. The crystal of this compound was soluble in CHCl$_3$. It showed a purple colored single spot on TLC plate when visualized with anisaldehyde/sulphuric acid spray reagent with R_f value 0.55 (n-hexane:EtOAc). From the ^{1}H- and ^{13}C-NMR spectral data (CDCl$_3$) are presented in Table 3.7.2.

2.7. 19.3. Compound 3

Compound **3** was yellowish white crystalline powder. It gave single spot on TLC (n-hexane : ethyl acetate :: 17:3) under UV light at 365 nm observed as blue color with R_f = 0.59. Melting point of the compound was 230^{0}C and molar mass was 162 g/mol. MS: m/z(M); 163.15. ^{1}H-NMR (400 MHz, CDCl$_3$) had signals at δ d 2.49 (s, 3H, C4–CH$_3$), 6.31 (s, 1H, C3–H), 6.92 (d, 1H, C6–H, J = 9.0 Hz), 6.94 (s,1H, C8–H) and 7.57 (d, 1H, C5–H, J = 9.0 Hz). The ^{1}H-NMR and GC-MS spectral data (CDCl$_3$) are presented in Figure 3.7.7 & 3.7.8, respectively.

2.7. 19.4. Compound 4

Compound **4** was needle shaped crystals with m.p. 137-138^{0}C. The ^{1}H- and ^{13}C-NMR spectral data (CDCl$_3$) are presented in Table 3.7.1.

2.7. 19.5. Compound 5

Compound **5** was needle shaped crystals with m.p. 162.5^{0}C. The crystal of the compound was soluble in CHCl$_3$. From the ^{1}H- and ^{13}C-NMR spectral data (CDCl$_3$) are presented in Table 3.7.2.

2.7. 19.6. Compound 6

Compound **6** was long fatty shaped crystals. It gave single spot on TLC with R_f value 0.53 (n-hexane : ethyl acetate; 3:7). GCMS m/z 332 [M+H$^+$]. FTIR (KBr) ν_{max}: 2940,

1691, 1528, 669 cm^{-1}. The ^{1}H- and ^{13}C-NMR spectral data (CDCl$_3$) are presented in Table 3.7.3.

2.7. 19.7 Compound 7

Compound **7** was white powder. It gave single spot on TLC with R$_f$ value 0.50 (n-hexane : ethyl acetate; 4:6). GCMS m/z 334 [M+H$^+$]. FTIR (KBr) ν_{max}: 2935, 1714, 1528, 670 cm^{-1}. The ^{1}H- and ^{13}C-NMR spectral data (CDCl$_3$) are presented in Table 3.7.4.

2.7. 19.8. Compound 8

Compound **8** was brownish powder. It gave single peak in HPLC (MeOH : DI H$_2$O). From the ^{1}H- and ^{13}C-NMR spectral data (CDCl$_3$) are presented in Table 3.7.5.

2.8 TOXIC METAL ANALYSIS

2.8.1. Study Area

The thirteen ADHPs procured for the present study were prepared for chemical analysis in the research laboratory of Bangladesh University of Health Sciences (BUHS), Dhaka, Bangladesh. Toxic metals content was done in the Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka Laboratories, Bangladesh.

2.8.2. Sample Preparation and Toxic Metal Analysis

Toxic metals were analyzed in flame atomizer based atomic absorption spectrometer using hollow cathode lamp as a radiation source. Accurately, 25 g of herbal preparation was transferred into silica crucible and kept in a muffle furnace for ashing at 700°C for 1 hour. The sample was then cooled down to room temperature and the heating process was repeated for three more times. The ash was then dissolved by adding 5–10 mL of concentrated HCl and finally diluted the sample by $0.1N\ HNO_3$ up to 25 mL. Finally, the sample was prepared for toxic metal analysis by filtering through ash trace Whatman filter paper.

For toxic metal analysis, the samples were aspirated through nebulizer and the absorbance was measured against a blank as a reference. Specific hollow cathode lamps were used to analyze Nickel (wavelength 232.0 nm) Copper (wavelength 324.8 nm), Cadmium (wavelength 228.8 nm), Chromium (wavelength 357.9 nm), Manganese (wavelength 297.5 nm), Lead (wavelength 283.3 nm), and Arsenic (wavelength 193.7 nm). Before analysis, the samples were diluted to the appropriate concentration according to the detection limit of the Atomic Absorption Spectrophotometer (AA 7000; SHIMADZU). Calibration curve was obtained by using standards and all the measurements were run in triplicate for the samples and standard solutions.

2.9 ORGANOCHLORINE PESTICIDES (OCPs) TESTING

2.9.1 Sample preparation:

About 10 g of ADHP's sample was taken separately in a conical flask containing 10 g silica and 5 g anhydrous Na_2SO_4 and was mixed thoroughly in a mortar. About 50 mL ethyl actate was added and filtered. The extraction with ethyl acetate was repeated three times. The filtrates were combined and concentrated to 5 mL. The concentrated filtrate was reconstituted to 2 mL with the help of n-hexane in a screw-capped vial. About 2 mL of concentrated sulfuric acid was added and inverted 40-50 times. The total solution was centrifuged and 1 mL of upper layer was collected for analyzing pesticide residues by Gas Chromatography [GC- 2010 (SHIMADZU)]. The same procedure was followed for each of the ADHP sample.

Procedure of work Scheme: 2.9.1

Extraction Procedure

| Herbal preparation (10 g) |

↓

| Sample 10 g + 10 g silica sand (solid dispersion method) |

↓

| Add 5-10 g anhy Na_2SO_4 and mix thoroughly in a morter |

↓

| Free flowing powder |

| Solid-liquid extraction 3 times with ethyl acetate (50, 40 and 30 mL) |

| Filtered with cotton + anhydrous Na_2SO_4 |

↓

| Filtrate, 120 mL |

| Concentrated by using rotary evaporator |

↓

| Concentrated to 5 mL |

| Reconstitute with n-hexane |

↓

| 2 mL |

| 2 mL conc. H_2SO_4 |

| Inversion (40 – 50 times) |

↓

| Upper part (n-hexane) GC -ECD | | Lower part |

Figure: 2.9.1 Gas Chromatograph

Gas Chromatograph (GC)-2010 (SHIMADZU)

Injection Temperature	:	220^0C
Injection Mode	:	Spliteless
Column gas flow	:	1 mL/min
Column	:	HP – 5 ms (Length - 30 m, Film thickness – 0.25 µm and 0.25 mm ID)

Oven Temp	Rate	Temp	Hold
	---	120^0C	1.0 min
	15^0C	185^0C	4.0 min

Detector Temperature	:	290^0C

2.10 ANALYSIS OF DELIBERATELY ADDED SYNTHETIC DRUGS (if any)

2.10.1 Sample collection

Several antidiabetic herbal preparations (ADHPs) were collected from the local market of Dhaka city in Bangladesh which is producing by the different herbal medicines producing companies.

2.10.2 Reagents and materials

The working standards of metformin hydrochloride (99.4%), glipizide (99.3%), gliclazide (99.0%), glibenclamide (99.3%), and glimepiride (99.1%), were obtained from different medicine producer companies like Square, Beximco and ACI Pharmaceutical in Bangladesh. The triethylamine was of AR grade (Merck, Germany), ortho-Phosphoric acid was GR grade (Merck, Germany), ethanol was of GR grade (Merck, Germany), methanol was of HPLC grade (Merck, Germany) and acetonitrile was of HPLC grade (Merck, Germany). Water purified with Millipore water system (Elix 10 C model) was used for the preparation of buffer. Cellulose acetate filter (0.45 micron – Sartorium stedim) was used for the filtration of the mobile phase.

2.10.3 Instrumentation

A SHIMADZU Prominence LC 20AT HPLC well equipped system with auto injector, thermostatted column compartment and PDA detector and software was used. Thermo Hypersil C18 column (Hypersil BDS column of 250 mm x 4.6 mm i.d., 5 µ particle size) was used for the analysis.

2.10.4 Mobile Phase

A mixture of buffer and acetonitrile in the ratio of 42:58 (v/v) was used as the mobile phase. The buffer for mobile phase was prepared by diluting 5 mL of triethylamine to 1000 mL with water, adjusting the pH to 3.5 ± 0.05 using ortho-phosphoric acid. The buffer and acetonitrile mixture was degassed by sonication and filtered through 0.45 µ cellulose acetate membrane filter.

2.10.5 Standard Stock Preparation

About 100 mg of each of working standards of metformin hydrochloride, glipizide, gliclazide, glibenclamide and glimepiride were weighed and transferred into a 100 mL volumetric flask. About 70 mL of methanol was added and sonicated to dissolve the substances. The volume was made up to 100 mL with methanol and mixed well.

2.10.6 Standard Preparation

Out of standard stock solution, 10 mL of the solution was dilute to 100 mL with mobile phase. The resulting solution (10 mL) was diluted to 100 mLl with mobile phase to obtain a concentration of 10 μg/mL of each of metformin hydrochloride, glipizide, gliclazide, glibenclamide and glimepiride.

3.10.7 Mixed Standard Preparation

For mixed standard sample's stock solutions, 2 mL were taken from the 05 different volumetric flasks and mixed another volumetric flask. Then this mixed standard sample was sonicated and filtered for using in HPLC and keep in GC vial.

2.10.8 Sample Extraction (Scheme-2.10.1)

2.10.8.1 For Solid Samples of ADHPs

Tablets or Capsules of ADHPs (ADHP-1, ADHP-4, ADHP-5, ADHP-6 and ADHP-7) were opened up & grinded by the Mortar & Pestle and got powder. The powdered of ADHPs were extracted with Methanol (MeOH) at room temperature using a special glass tank for three days by changing MeOH each day. The MeOH extract was filtered and the filtrate was concentrated. The concentrated extracts were combined, evaporated to dryness and finally dried in a freeze-dryer to give MeOH extract. The extracts were stored at -22^{0}C in a refrigerator.

2.10.8.2 For Liquid Samples of ADHPs

Liquid ADHPs (ADHP-2 and ADHP-3) were evaporated by Rotary and dried into Freeze Dryer and got solid samples. Then extracted with Methanol (MeOH) at room temperature using a special glass tank for three days by changing MeOH each day. The MeOH extract

was filtered and the filtrate was concentrated. The concentrated extracts were combined, evaporated to dryness and finally dried in a freeze-dryer to give MeOH extract. The extracts were stored at -22^0C in a refrigerator.

Figure of Extraction: Scheme-2.10.1

```
                    ┌─────────────────────┐
                    │  Sample of ADHPs/   │
                    │       ADSPs         │
                    └─────────────────────┘
                              │      ┌──────────────────────────────────┐
                              │      │ Sample soaked in MeOH for three  │
                              │      │ days by changing MeOH each day   │
                              │      └──────────────────────────────────┘
            ┌─────────────────┴───────────────────────────────┐
            ▼                                                  ▼
  ┌─────────────────────┐                          ┌─────────────────────┐
  │  MeOH Extract of    │                          │     Residue of      │
  │   ADHPs/ ADSPs      │                          │    ADHPs/ADSHs      │
  └─────────────────────┘                          └─────────────────────┘
            │
            ▼
  ┌──────────────────────────┐
  │ Evaporation of MeOH at   │
  │ reduced pressure & low   │
  │ temperature and          │
  │ concentrated.            │
  └──────────────────────────┘
            │
            ▼
  ┌─────────────────┐        ┌─────────────────────┐
  │   Freeze Dry    │───────▶│ Dry Extract of ADHPs/│
  │                 │        │    ADSPs (Wt)       │
  └─────────────────┘        └─────────────────────┘
```
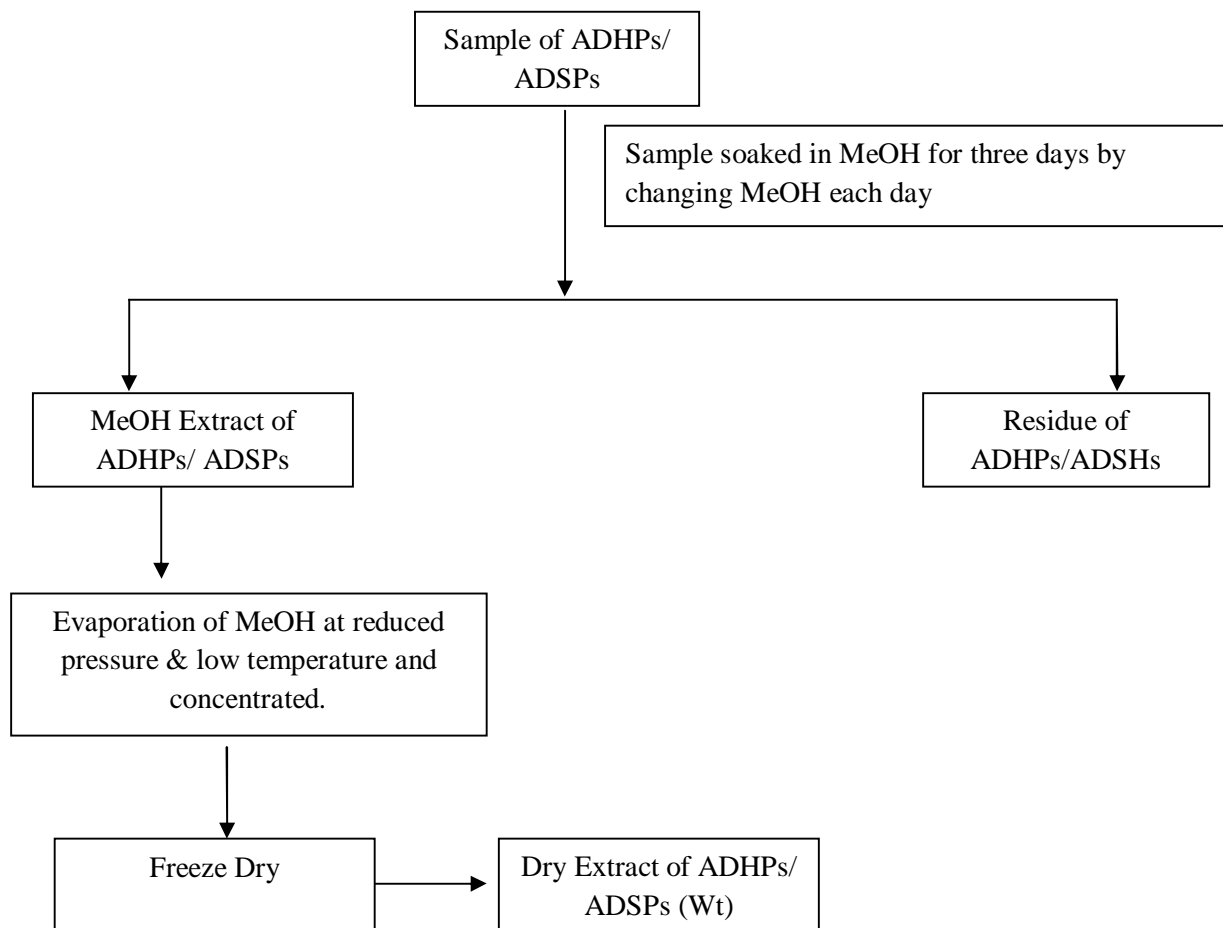
2.10.8.3 Sample Preparation:

HPLC grade MeOH was used to prepare 1000 ppm stock solutions for the particular extracts in different volumetric flask. The concentrations of these solutions were 1 mg/mL (sample was taken 25 mg in 25 mL HPLC grade MeOH). Then the samples were taken 3 mL from each volumetric flask and filtered & kept in vial for HPLC analysis. These samples were used to determin the presence of synthetic drugs.

2.10.9 Chromatographic parameters

For HPLC studies, a flow rate of 1.0 mL/minute and detection wavelength of 230 nm was used. The sample injection volume was 20 μL and the column was maintained at ambient temperature. The run time for each injection was 12 minutes.

2.10.10 Method validation

The method validation was performed in accordance with the current guidelines.

2.11 IDENTIFICATION AND QUANTIFICATION OF ANTIOXIDENTS

2.11.1 Chemicals

Gallic acid (GA), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), syringic acid (SA), (-)-epicatechin (EC), vanillin (VL), *p*-coumaric acid (PCA), *trans*-ferulic acid (FA), rutin hydrate (RH), ellagic acid (EA), benzoic acid (BA), rosmarinic acid (RA), myricetin (MC), quercetin (QH), *trans*-cinnamic acid (TCA), and kaempferol (KF) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol was obtained from Merck (Darmstadt, Germany).

Methanol was used for sample preparation. Acetonitrile (solvent A), Acetic acid solution pH 3.0 (solvent B), and methanol (solvent C) and 5% IPA (solvent D) were used as mobile and stationary phases of HPLC.

2.11.2 Standard preparation

A stock standard solution (100 µg/mL) of each phenolic compound was prepared in methanol by weighing out approximately 0.0050 g of the analyte into 50 mL volumetric flask. The mixed standard solution was prepared by dilution the mixed stock standard solutions in methanol to give a concentration of 5 µg/ml for each polyphenols except (+)-catechin hydrate, caffeic acid, rutin hydrate (4 µg/mL) and quercetin (3 µg/mL). All standard solutions were stored in the dark at 5^0C and were stable for at least three months.

The calibration curves of the standards were made by a dilution of the stock standards (five set of standard dilutions) with methanol to yield 1.0 - 5.0 µg/mL for GA, CH, VA, CA, SA, EC, VL, PCA, FA, RH, EA, BA, RA, MC, QH, TCA and KF. The calibration curves were constructed from chromatograms as peak area vs. concentration of standard.

2.11.3 Sample preparation

A solution of the extract was prepared in methanol having the concentration of 5 mg/ml (5000 ppm). Prior to HPLC analysis, all the solutions (mixed standards, sample, and

spiked solutions) were filtered through 0.20 µm syringe filter (Sartorius, Germany) and then degassed in an ultrasonic bath (Hwashin, Korea) for 15 min.

2.11.4 HPLC system

Chromatographic analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), coupled to a quaternary rapid separation pump (LPG-3400RS), Ultimate 3000RS autosampler (WPS-3000) and rapid separation diode array detector (DAD-3000RS). Phenolic compounds were separated on a Acclaim® Polar Advantage II (PAII) C18 (4.6 x 250 mm; 5µm; 120 A°) column (Dionix, USA) which was controlled at 30^0C using a temperature controlled column compartment (TCC-3000). Data acquisition, peak integration, and calibrations were performed with Dionix Chromeleon software (Version 6.80 RS 10).

Figure-2.11.1: High-performance liquid chromatography (HPLC)

2.11.5 HPLC detection and quantification of antioxidant compounds

Detection and quantification of selected phenolic compounds in the ethanol extract were determined by HPLC-DAD analysis as described by (Islam *et. al.,* 2014) with some modifications. It was carried out on a Dionex UltiMate 3000 system equipped with quaternary rapid separation pump (LPG-3400RS) and photodiode array detector (DAD-3000RS). Separation was performed using Acclaim® C_{18} (5µm) Dionex column (4.6 x 250 mm) at 30 ºC with a flow rate of 1 mL/min and an injection volume of 20 µL. The

mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C) with the gradient elution program of 5% A/ 95% B (0-9 min), 10% A/ 90% B (10-14), 10% A/ 85% B/ 5% C (15-19), 15% A/ 70% B/ 15% C (20-29 min), 20% A/ 60% B/ 20% C (30-34 min), 30% A/ 40% B/ 30% C (35-38) and 100% A (39-40 min). The PDA detector was set to 280 nm for 25.0 min, changed to 320 nm for 26.0 min, again change to 280 nm for 31.0 min, changed to 320 nm for 26.0 min to 33.0 min, and finally to 380 nm for 40.0 min and held for the rest of the analysis period while the diode array detector was set at an acquisition range from 200 nm to 700 nm. For the preparation of calibration curve, a standard stock solution was prepared in methanol containing each of all the standards at the described concentration.

2.11.6 Statistical Analysis:

In order to see the average behavior and discursion of data, Mean and Standard Deviation (SD) were calculated by microsoft exel. One way ANOVA test was performed to see which are significantly different and which are similar by Statistical Software SPSS (Statistical Package for Social Science).

Data acquisition, peak integration, and calibrations were calculated with Dionex Chromeleon software (Version 6.80 RS 10).

3. RESULTS AND DISCUSSION

3.0   RESULTS AND DISCUSSION

A. BIOLOGICAL INVESTIGATIONS:

3.1 ANTIDIABETIC ACTIVITY

3.1.1 General Information

A total number of eleven antidiabetic herbal preparations (ADHPs) and one single plant were tested on Type 2 diabetic model rats (T2DM) for evaluating the antidiabetic effect. The study was performed in three batches as follows according to the availability of the ADHPs.

1. In the 1st batch (ADHP-3, ADHP-5, ADHP-6, ADHP-8, ADHP-9 and ADHP-10)
2. In the 2nd batch (ADHP-1, ADHP-2, ADHP-4, ADHP-7, and ADHP-11) and
3. In the 3rd batch (*Phlogacanthus thyrsiflorus* Nees)

Six and five herbal medicines were tested in the 1st batch and 2nd batch, respectively and the single plant was tested in the last batch, and the results are presented accordingly.

3.1.2 Results for acute antidiabetic effect of six Antidiabetic Herbal Preparations

Table 3.1.1 shows the effect of six ADHPs (ADHP-3, ADHP-5, ADHP-6, ADHP-8, ADHP-9 and ADHP-10) on blood glucose levels of Type 2 diabetic model rats when the HMs were fed simultaneously with glucose load. It is evident from the table that in all the groups of rats, simultaneous glucose load caused a sharp rise in serum glucose level at 30 min. Compared to 0 minute value the rise was 187.41%, 169.98%, 196.96%, 176.73%, 187.41%, 169.98%, 180.76% and 191.70% in water control, ADHP-3, ADHP-5, ADHP-6, ADHP-8, ADHP-9 and ADHP-10 treated groups respectively. Among the studied herbal medicines ADHP-6 showed significant antihyperglycemic effect at 30 min (p<0.05). As

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expected at 75 minutes serum glucose level decreased significantly in Glibenclamide treated group (p<0.01) compared to water control group.

Table 3.1.1: Acute effects of Herbal Medicines (ADHP-3, ADHP-5, ADHP-6, ADHP-8, ADHP-9 and ADHP-10) on blood glucose levels of Type 2 rats when fed simultaneously with glucose load.

Group	Min 0 (mmol/L)	Min 30 (mmol/L)	Min 75 (mmol/L)
Water Control (n=14)	8.50±1.60 (100%)	15.93±1.70 (187.41%)	15.91±2.64 (187.17%)
Glibenclamide (n=14)	8.86±1.26 (100%)	15.06±2.68 (169.98%)	12.58±3.17* (141.99%)
ADHP-3 (n=6)	9.63±0.73 (100%)	16.37±1.51 (169.98%)	16.40±2.10 (170.30%)
ADHP-5 (n=7)	8.51±1.35 (100%)	15.04±2.96 (176.73%)	15.20±3.10 (178.61%)
ADHP-6 (n=6)	8.95±1.45 (100%)	13.92±2.10* (187.41%)	12.16±2.33 (135.68%)
ADHP-8 (n=7)	7.92±1.65 (100%)	15.60±1.30 (196.96%)	15.45±1.29 (195.08%)
ADHP-9 (n=8)	8.42±1.21 (100%)	15.22±0.93 (180.76%)	15.88±1.40 (188.60%)
ADHP-10 (n=8)	8.43±2.22 (100%)	16.16±2.37 (191.70%)	15.79±2.37 (187.30%)

Data are expressed as mean ±SD; n, number of animals in each group. Statistical analysis was done by using one way ANOVA with Bonferroni post hoc test. ADHP=Antidiabetic Herbal Preparation.

Table 3.1.2 represents the effect on postprandial blood glucose levels of Type 2 rats when Herbal formulations (ADHP-3, ADHP-5, ADHP-6, ADHP-8, ADHP-9 and ADHP-10) were fed 30 minutes before glucose load. It is seen that ADHP-6 significantly opposed the rise of serum glucose arising out of the glucose load at both time points i.e at 60 and 105 minutes (p<0.01 for both time points). Glibenclamide treated group also opposed significantly (p<0.01) the rise of serum glucose level at 60 and 75 minutes. Since oral administration of ADHP-6 with simultaneous glucose was associated with a significant decline in serum glucose level compared to control rats, therefore it may be assumed that better tissue utilizing capacity of ADHP-6 treated type 2 diabetic rats.

Table 3.1.2: Acute effects of Herbal Medicines (ADHP-3, ADHP-5, ADHP-6, ADHP-8, ADHP-9 and ADHP-10) on blood glucose levels of Type 2 rats when fed 30 minutes before to glucose load.

Group	Min 0 (mmol/L)	Min 60 (mmol/L)	Min 105 (mmol/L)
Water Control (n=14)	8.55±1.34 (100%)	15.32±2.43 (179.18%)	15.42±2.31 (180.35%)
Glibenclamide (n=14)	9.68±1.18 (100%)	12.88±2.81* (133.05%)	12.58±3.17* (107.65%)
ADHP-3 (n=6)	9.07±1.64 (100%)	16.91±2.02 (186.44%)	15.98±3.24 (176.18%)
ADHP-5 (n=7)	8.52±0.85 (100%)	15.08±1.40 (196.99%)	14.15±2.04 (166.07%)
ADHP-6 (n=6)	8.20±1.17 (100%)	9.84±3.89** (120.00%)	9.94±2.99** (121.21%)
ADHP-8 (n=7)	7.37±1.41 (100%)	14.13±2.12 (191.20%)	13.38±2.27 (181.06%)
ADHP-9 (n=8)	8.82±1.71 (100%)	16.34±1.23 (185.26%)	16.31±1.87 (184.92%)
ADHP-10 (n=8)	9.82±1.53 (100%)	16.97±1.19 (172.82%)	16.85±0.93 (171.59%)

Data are expressed as mean ±SD; n, number of animals in each group. *Statistical analysis was done by using one way ANOVA with Bonferroni post hoc test., ADHP=Antidiabetic Herbal Preparation)*

After the acute experiments with 6 ADHPs on T2DM rats, ADHP-5 and ADHP-6 were selected to persue chronic experiments due to their better results on glucose lowering effect

Table 3.1.3 shows the effect of Herbal formulations (ADHP-5 and ADHP-6) on body weight changes of type 2 diabetic model rats during 28 days of chronic administration. Body weight of each rat was taken at seven days interval. As it is evident from the table there was a gradual increase in body weight in all of the groups although the increase was nonsignificant. The increase in body weight denotes that ADHP-5 and ADHP-6 have apparently no toxic effects.

Table 3.1.3: Effect of different doses of ADHP-5 and ADHP-6 on body weight of type 2 model rats.

Group	Body Weight (g)				
	Day_0	Day_7	Day_14	Day_21	Day_28
Water Control (n=14)	226±23 (100%)	215±28 (95.13%)	228±30 (100.88%)	217±35 (96.02%)	233±30 (103.10%)
Glibenclamide (n=14)	217±19 (100%)	211±27 (97.24%)	226±31 (104.15%)	214±29 (98.62%)	221±23 (101.85%)
ADHP-5 (200 mg/kg) (n=8)	210±30 (100%)	205±28 (97.62%)	214±35 (101.90%)	209±35 (99.52%)	220±34 (104.76%)
ADHP-5 (400 mg/kg) (n=8)	203±35 (100%)	201±29 (99.01%)	216±36 (106.40%)	207±30 (101.97%)	219±31 (107.88%)
ADHP-6 (200 mg/kg) (n=7)	218±34 (100%)	216±30 (99.09%)	229±32 (105.05%)	217±29 (99.54%)	229±31 (105.05%)
ADHP-6 (400 mg/kg) (n=8)	197±26 (100%)	191±29 (96.95%)	206±37 (104.57%)	194±31 (98.48%)	206±31 (104.57%)

Data are expressed as mean ±SD; n, number of animals in each group. Statistical analysis was done by using one way ANOVA with post hoc Bonferroni test. ADHP=Antidiabetic Herbal Preparation.

Results of fasting serum glucose (FSG) levels of the studied rats at baseline (before onset of feeding i.e 0 day, at the middle i.e 14th day and 28 day of feeding of different doses of ADHP-5 and ADHP-6 is presented in Table 3.1.4. At baseline there was no significant difference in FSG level among the different study groups. Fourteen days of consecutive feeding of the two herbal drugs led to some reduction of serum glucose levels. However, a 19% increase was found in water control group. Twenty eight days of chronic feeding of the herbal drugs resulted in further decrease in serum glucose level. As it is seen FSG level decreased by 30%, 7%, 16%, 16%, and 3% in Glibenclamide, ADHP-6 200 mg/kg bw, and 400 mg/kg bw, ADHP-5 200 mg/kg bw and 400 mg/kg bw treated groups, respectively compared to 0 day value. ADHP-5 200 mg/kg bw decreased the fasting serum glucose level, however, the decrease remained just outside the significance level. ADHP-6 at the dose of 400 mg/kg bw significantly ($p < 0.05$) decreased serum glucose

level of type 2 diabetic rats. As expected glibenclamide also reduced FSG significantly (p=0.039).

Table 3.1.4: Chronic effect of ADHP-5 and ADHP-6 with different doses on fasting serum glucose levels of type 2 model rats.

Group	Day_0 (g)	Day_14 (g)	Day_28 (g)
Water Control (n=14)	8.01±1.31 (100%)	9.55±1.06 (119.23%)	8.97±1.65 (111.99%)
Glibenclamide (n=14)	8.68±1.39 (100%)	7.09±1.16 (81.68%)	6.07±0.53 (69.93%)
ADHP-5 200 mg/kg (n=8)	8.891±1.29 (100%)	7.30±1.52 (82.11%)	7.46±1.90 (83.91%)
ADHP-5 400 mg/kg (n=8)	7.77±0.74 (100%)	8.45±1.63 (108.75%)	7.57±1.22 (97.43%)
ADHP-6 200 mg/kg (n=7)	8.47±0.85 (100%)	9.14±0.98 (107.91%)	7.90±0.86 (93.27%)
ADHP-6 400 mg/kg (n=8)	7.97±1.11 (100%)	7.75±1.86 (97.24%)	6.71±0.53* (84.19%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis between group was done by using one way ANOVA with post hoc Bonferroni test. ADHP=Antidiabetic Herbal Preparation)

Chronic effect of different doses of ADHP-5 and ADHP-6 on serum cholesterol and serum triglycerides level is presented in Table 3.1.5. From this table it is seen that in glibenclamide treated group total cholesterol levels was decreased by 6% on final day. There was 1% increase in total cholesterol level in ADHP-6 200 mg/kg treated group. Serum total cholesterol remained almost unchanged with different doses of ADHP-5. ADHP-6 at a dose of 400 mg/kg decreased total cholesterol level by 6% compared to 0 day value. Serum were decreased by 13%, 10%, 3%, 13% and 2 % in glibnclamide, ADHP-6 200 mg/kg bw, ADHP-6 400 mg/kg bw, ADHP-5 200 mg/kg bw and ADHP-5 400 mg/kg bw treated groups, respectively on final day in comparison to baseline values. Lowering of triglycerides levels by the two doses of ADHP-5 and ADHP-6 is beneficial for health. The reduction in serum triglycerides may be due to the enhanced activity of lipoprotein lipase that is known to be associated with accelerated clearance of triglyceride-rich lipoproteins (TRL) and high fasting HDL- cholesterol.

Table 3.1.5: Chronic effect of ADHP-5 and ADHP-6 at different doses on serum lipid levels of type 2 model rats.

Group	Chol Day_0 mg/dl)	Chol Day_28 mg/dl)	TG Day_0 mg/dl)	TG Day_28 mg/dl)
Water Control (n=14)	79±11 (100%)	72±50 (91.14%)	66±10 (100%)	62±70 (93.94%)
Glibenclamide (n=14)	78±30 (100%)	74±60 (94.87%)	67±12 (100%)	58±70 (86.57%)
ADHP-5 200mg/kg (n=8)	75±70 (100%)	75±50 (100%)	70±11 (100%)	61±12 (87.14%)
ADHP-5 400 mg/kg (n=8)	72±10 (100%)	73±50 (101.39%)	67±14 (100%)	66±12 (98.51%)
ADHP-6 200 mg/kg (n=7)	73±10 (100%)	74±10 (101.37%)	63±14 (100%)	57±12 (90.48%)
ADHP-6 400 mg/kg (n=8)	82±10 (100%)	79±60 (96.34%)	69±18 (100%)	67±15 (97.10%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis between group was done by using paired sample t test. Chol=Serum total cholesterol, TG= Serum triglycerides, .ADHP=Antidiabetic Herbal Preparation.

Chronic effect of different herbal formulations on HDL and LDL-cholesterol levels of type 2 diabetic model rats were also determined. As it is seen from Table 3.1.6 there were 8 % and 2% increase in HDL & cholesterol level of ADHP-6 200 mg and 400 mg treated groups on 28 day compared with 0 day value respectively. More importantly LDL-cholesterol levels were decreased by 17% by ADHP-6 400 mg treated group on final day when it was compared to the baseline value. No change was found in HDL level by the chronic treatment of both the doses of ADHP-5. However, paradoxical result was found in case of LDL level which was found to be increased by 14% and 10% by 200 mg and 400 mg of ADHP-5, respectively which is not beneficial.

Table 3.1.6: Chronic effect of different doses of ADHP-5 and ADHP-6 on serum lipid levels of type 2 model rats.

Group	HDL Day_0 (mg/dl)	HDL Day_28 (mg/dl)	LDL Day_0 (mg/dl)	LDL Day_28 (mg/dl)
Water Control (n=14)	42±30 (100%)	38±50 (90.48%)	23±11 (100%)	23±70 (100%)
Glibenclamide (n=14)	44±30 (100%)	38±50 (86.36%)	20±50 (100%)	25±70 (125%)
ADHP-5 200 mg/kg bw (n=8)	40±40 (100%)	39±40 (97.50%)	21±50 (100%)	24±90 (114.29%)
ADHP-5 400 mg/kg bw (n=8)	38±50 (100%)	38±30 (100%)	20±10 (100%)	22±60 (110%)
ADHP-6 200 mg/kg bw (n=7)	37±60 (100%)	40±20 (108.11%)	23±80 (100%)	23±10 (100%)
ADHP-6 400 mg/kg bw (n=8)	39±40 (100%)	40±60 (102.56%)	30±60 (100%)	25±50 (83.33%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis between group was done by using one way ANOVA with post hoc Bonferroni test and Pair sample 't' test. (HDL→ High-density lipoprotein, LDL→ Low-density lipoprotein, ADHP=Antidiabetic Herbal Preparation.)

Chronic effect of the herbal medicines comprising the 2nd batch (ADHP-1, ADHP-2, ADHP-4, ADHP-7, ADHP-11) on body weight changes is depicted in Table 3.1.7. It is evident that consecutive feeding for 28 days of these herbal medicines to type 2 diabetic rats did not alter body weight significantly. A rising trend in body weight of the rats by the effect of the studied plants were noted. This was due to improvement in glycemic control. Therefore, it may be assumed that the herbal medicines of the 2nd batch also had no harmful effect on body weight.

Table 3.1.7. Effect of some herbal drugs on the body weight of type 2 diabetic model rats

Groups	Body weight (g)				
	0 day	**7 day**	**14 day**	**21 day**	**28 day**
WC (n=6)	199±26 (100%)	196±30 (98%)	200±29 (100%)	206±33 (103%)	213±31 (107%)
Gliben (n=6)	195±13 (100%)	187±18 (96%)	193±16 (99%)	200±13 (102%)	197±21 (101%)
ADHP-1 (n=7)	197±22 (100%)	210±22 (106%)	210±28 (106%)	219±31 (111%)	216±27 (109%)
ADHP-2 (n=8)	194±28 (100%)	183±22 (94%)	186±21 (96%)	194±24 (100%)	198±31 (102%)
ADHP-4 (n=8)	181±21 (100%)	175±19 (97%)	185±20 (102%)	186±21 (103%)	188±22 (104%)
ADHP-7 (n=8)	196±18 (100%)	189±20 (96%)	190±18 (97%)	199±24 (102%)	205±26 (106%)
ADHP-11 (n=7)	193±22 (100%)	202±22 (104%)	197±31 (102%)	213±25 (110%)	203±33 (104%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis between group was done by using one way ANOVA with post hoc Bonferroni test. (WC = Water Control; Gliben = Glibenclamide treated group ADHP=Antidiabetic Herbal Preparation.)

Table 3.1.8 shows blood glucose levels of water control, glibenclamide and herbal medicine treated rats simultaneously treated with glucose load (2.5 g/kg bw). Type 2 rats treated with glibenclamide and herbal medicine showed a nonsignificant decrease in blood glucose levels at 30 min, 75 min and 120 min compared to water control group. The administration of herbal medicines and glibenclamide tend to decrease the glucose level arising out of the glucose load. As expected glibenclamide opposed the rise in blood glucose level maximally compared to herbal medicines. However, none of the ADHP significantly reduced serum glucose level arising out of the glucose load.

Table 3.1.8. Acute effect of some herbal drugs on the blood glucose levels of T2DM rats when the herbal medicines were fed simultaneously with glucose load on the 0 day.

	Serum Glucose [mmol/L, Mean (±SD)]			
Groups	**0 min**	**30 min**	**75 min**	**120 min**
WC (n=8)	8.27±0.91 (100%)	15.39±2.06 (186%)	15.62±1.32 (189%)	14.75±1.55 (178%) P=0.288 (c)
Gliben (n=8)	8.17±0.52 (100%)	14.07±3.03 (172%)	13.69±2.78 (168%)	11.27±3.28 (138%)
ADHP-1 (n=7)	6.67±0.87 (100%) P=0.042 (a) P=0.075 (b)	14.89±1.89 (223%)	14.49±2.00 (217%)	12.93±2.28 (194%)
ADHP-2 (n=8)	8.64±0.83 (100%)	15.56±2.31 (180%)	15.00±3.42 (174%)	14.03±3.78 (162%)
ADHP-4 (n=8)	8.53±0.98 (100%)	15.74±2.05 (1.85%)	15.04±2.59 (176%)	13.69±2.15 (160%)
ADHP-7 (n=8)	8.44±1.01 (100%)	15.52±2.29 (1.84%)	15.72±2.44 (186%)	13.43±3.22 (159%)
ADHP-11 (n=7)	8.16±1.39 (100%)	15.39±1.39 (189%)	13.43±3.23 (165%)	13.64±1.75 (167%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis between group was done by using one way ANOVA with post hoc Bonferroni test. (WC = Type 2 Water Control; Gliben = Type 2 Glibenclamide treated group, a= WC Vs ADHP-1 Ext; b= Gliben Vs ADHP-1 Ext; c= WC Vs Gliben.

Table 3.1.9 depicts the blood glucose levels of water control, glibenclamide and herbal medicine treated rats simultaneously treated with glucose load after 14 days of consecutive treatment. Here it is also seen that compared to water control group, groups treated with glibenclamide and herbal medicine opposed the rise in blood glucose level.

Table 3.1.9 Acute effect of some herbal drugs on the blood glucose levels of T2DM rats when the herbal medicines were fed simultaneously with glucose on the 14 day

Groups	Serum Glucose [mmol/L, Mean (±SD)]			
	0 min	30 min	75 min	120 min
WC (n=8)	7.98±0.96	14.76±2.04	14.48±2.33	15.02±1.36
	(100%)	(185%)	(181%)	(188%)
Gliben (n=8)	7.13±1.02	14.84±2.85	14.19±3.15	12.96±2.87
	(100%)	(208%)	(199%)	(182%)
ADHP-1 (n=7)	7.01±0.47	14.99±1.30	13.69±3.12	10.96±3.25
	(100%)	(214%)	(195%)	(156%)
ADHP-2 (n=8)	8.04±0.94	13.75±2.89	13.76±4.35	12.11±4.17
	(100%)	(171%)	(171%)	(150%)
ADHP-4 (n=8)	7.15±0.79	14.92±2.47	14.42±3.38	13.13±2.77
	(100%)	(209%)	(202%)	(184%)
ADHP-7 (n=8)	8.02±0.99	16.16±1.65	15.74±1.90	13.61±2.11
	(100%)	(201%)	(196%)	(170%)
ADHP-11 (n=7)	7.77±0.87	14.23±1.71	13.25±1.94	11.29±1.79
	(100%)	(183%)	(171%)	(145%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis between group was done by using one way ANOVA with post hoc Bonferroni test. (WC = Type 2 Water Control; Gliben = Type 2 Glibenclamide treated group)

Table 3.1.10 illustrates fasting blood glucose levels of type 2 diabetic rats, water control, glibenclamide and herbal medicine groups treated for 28 days. At baseline there was no significant difference in FSG level among the different study groups except ADHP-11. Fasting serum glucose level was significantly low in ADHP-11 compared to Glibenclamide and ADHP-4 groups (p=0.011 and p=0.010 respectively). Consecutive feeding for 14 days of the 2nd batch of herbal medicines led to some reduction of serum glucose levels. As it is seen fasting blood glucose level decreased by 15%, 7%, 16% and 4% in ADHP-1, ADHP-2, ADHP-4, ADHP-7 and treated groups. respectively compared to 0 day value. On the other hand ADHP-11 increased 17 % fasting blood glucose level. Treatment with ADHP-4 caused significant reduction in serum glucose level on 14th day (p<0.05).

Table 3.1.10. Effect of some herbal drugs on fasting serum glucose level of type-2 diabetic rats

	Glucose Level (mmol/L)		
Groups	**0 day**	**14 day**	**28 day**
WC (n=8)	8.10±1.01 (100%)	7.77±1.09 (96%)	7.14±1.26 (88%)
Gliben (n=8)	8.05±0.62 (100%)	7.02±1.02 (87%)	6.77±1.52 (84%) P=0.025
ADHP-1 (n=7)	8.21±1.32 (100%)	7.00±0.47 (85%)	7.49±1.18 (91%)
ADHP-2 (n=8)	8.64±0.83 (100%)	8.04±0.94 (93%)	7.48±1.27 (87%) P= 0.029
ADHP-4 (n=8)	8.53±0.98 (100%)	7.15±0.79 (84%)	6.49±0.79 (76%) P=0.001
ADHP-7 (n=8)	8.44±1.01 (100%)	8.09±0.92 (96%)	7.93±1.86 (94%)
ADHP-11 (n=7)	6.62±0.86 (100%)	7.76±0.87 (117%)	7.09±1.29 (107%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis between group comparison was done by using one way ANOVA with post hoc Bonferroni test and within group comparison was done by paired samples t test. (WC = Type 2 Water Control; Gliben = Type 2 Glibenclamide treated group, ADHP=Antidiabetic Herbal Preparation).

Further decrease in serum glucose level was noticed after 28th days treatment. Blood glucose level decreased by 9%, 13%, 24% and 6%, in ADHP-1, ADHP-2, ADHP-4, and ADHP-7 treated groups, respectively compared to 0 day value. But blood glucose level increased 7% in ADHP-11. Significant reduction in blood glucose level was observed by the treatment of ADHP-2 and ADHP-4 ($p < 0.029$ and $p < 0.001$, respectively) compared to 0 day value). Glibenclamide treatment also significantly reduced the increased blood glucose level compared to 0 day level ($p < 0.011$ and $p < 0.0025$ on 14th day and 28th day, respectively). Significant lowering of fasting glucose level by ADHP-2 and ADHP-4 indicates that these herbal preparations might contain some hypoglycemic principles which act probably by stimulation of insulin secretion from beta cells of islets or acting at the gut level or at the peripheral tissues.

The effect of Herbal Medicines on insulinemic status of type 2 diabetic model rats was observed (Table 3.1.11). On 0 day, water control, glibenclamide, ADHP-4, ADHP-1

group showed almost similar level of serum insulin. ADHP-7 and ADHP-11 had higher serum insulin level at the beginning compared to other groups. After 28 days study period, there was 47% decrease in serum insulin level in water control group. In case of ADHP-1 and ADHP-2 treated group there was 152% and 30% increase in the insulin level respectively at the end of the study period. Glibenclamide treated group showed a slight decrease in serum insulin level. ADHP-7 and ADHP-11 tend to decrease serum insulin level nonsignificantly. Increase in serum insulin level by ADHP-2 may be partly responsible for significant lowering of fasting glucose level by ADHP-2 in T2 rats.

Table 3.1.11 Effect of some herbal drugs on the Serum Insulin of Type-2 Diabetic Model Rats.

Groups	Insulin (ng/ml)	
	0 Day	**28 Day**
WC (n=8)	0.357±0.15 (100%)	0.190±0.13 (53%)
Gliben (n=8)	0.247±1.41 (100%)	0.187±0.13 (76%)
ADHP-1 (n=7)	0.137±0.51 (100%)	0.345±0.05 (252%)
ADHP-2 (n=8)	0.162±0.56 (100%)	0.212±0.19 (130%)
ADHP-4 (n=8)	0.169±0.39 (100%)	0.106±0.18 (63%)
ADHP-7 (n=8)	0.623±0.20 (100%)	0.490±0.28 (79%)
ADHP-11 (n=7)	0.679±0.35 (100%)	0.261±0.22 (38%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis was done by paired samples t test. WC = Type 2 Water Control; Gliben = Type 2 Glibenclamide treated group, ADHP=Antidiabetic Herbal Preparation.

Effect of the studied drugs on serum total cholesterol and triglycerides is shown in Table 3.1.12. Among the herbal drugs treated groups, there was a tendency to decrease total cholesterol level on 28th day while only ADHP-7 showed a rising tendency. In case of serum triglyceride (TG) level, water control group increased serum TG level. All the other treated groups decreased serum TG level after 28 days study period. ADHP-1 reduced serum TG level significantly when compared to 0 day value ($p < 0.014$).

Table 3.1.12. Effect of some herbal drugs on total serum cholesterol and Triglycerides level of type-2 diabetic model rats (Batch-2).

Groups	Chol (mg/dl)		TG (mg/dl)	
	0 Day	28 Day	0 Day	28 Day
WC (n=8)	69±8	65±12	63±16	64±13
	(100%)	(94%)	(100%)	(102%)
Gliben (n=8)	68±9	64±10	61±14	58±17
	(100%)	(94%)	(100%)	(95%)
ADHP-1 (n=7)	70±7	66±6	80±11	61±7
	(100%)	(94%)	(100%)	(76%)
				P=0.014
ADHP-2 (n=8)	69±9	66±10	55±15	50±21
	(100%)	(96%)	(100%)	(91%)
ADHP-4 (n=8)	67±8	66±8	63±13	64±15
	(100%)	(99%)	(100%)	(102%)
ADHP-7 (n=8)	69±11	72±14	61±12	60±14
	(100%)	(104%)	(100%)	(98%)
ADHP-11 (n=7)	60±3	60±4	81±9	75±16
	(100%)	(100%)	(100%)	(93%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis was done within group comparison was done by paired samples t test. WC =Type 2 Water Control; Gliben = Type 2 Glibenclamide treated group, Chol= Serum total cholesterol, TG= Serum triglycerides.

The effects of 28 days treatment with herbal drugs on the serum HDL and LDL cholesterol levels of type 2 diabetic model rats are summarized in Table 3.1.13. It was found that HDL level decreased slightly in all test groups at the end of the experimental period. Only ADHP-4 showed a nonsignificant rise in serum HDL level at the end of the study period in Type 2 rats which was a beneficial effect. The control groups showed no change. In case of LDL-cholesterol, the level was decreased by the treatment of the herbal drugs ADHP-2 and ADHP-4 which was beneficial. However opposite results were obtained with the treatment of ADHP-1, ADHP-7 which increased serum LDL level. ADHP-11 did not change serum LDL level. Therefore, only ADHP-2 and ADHP-4 showed beneficial effect on dyslipidemia of T2 model rats.

Table 3.1.13. Effect of some herbal drugs on HDL-Cholesterol and LDL-Cholesterol level of type-2 diabetic model rats.

Groups	HDL (mg/dl)		LDL (mg/dl)	
	0 Day	28 Day	0 Day	28 Day
WC (n=8)	41±5	37±7	15±8	16±14
	(100%)	(90%)	(100%)	(107%)
Gliben (n=8)	40±6	36±6	16±7	16±9
	(100%)	(90%)	(100%)	(100%)
ADHP-1 (n=7)	39±5	34±5	15±6	20±39
	(100%)	(87%)	(100%)	(133%)
ADHP-2 (n=8)	34±6	34±4	24±9	22±10
	(100%)	(100%)	(100%)	(92%)
ADHP-4 (n=8)	32±6	33±6	22±5	20±6
	(100%)	(103%)	(100%)	(91%)
ADHP-7 (n=8)	37±5	35±5	20±10	25±12
	(100%)	(94%)	(100%)	(125%)
ADHP-11 (n=7)	36±4	35±3	9±4	10±7
	(100%)	(97%)	(100%)	(111%)

Data are expressed as mean ±SD. n, number of animals in each group. Statistical analysis between group comparison was done by using one way ANOVA with post hoc Bonferroni test and within group comparison was done by paired samples T test. HDL= High-density lipoprotein, LDL=Low-density lipoprotein WC = Type 2 Water Control; Gliben = Type 2 Glibenclamide treated group,

Effect of the herbal drugs on hepatic glycogen content of type 2 diabetic model rats is illustrated in Table 3.1.14. Glibenclamide treated group and herbal drugs treated groups showed an increase in liver glycogen content of Type 2 rats compared to water control group. Both herbal drugs and glibenclamide treatment elevated the reduced liver glycogen content although nonsignificantly in Type 2 rats which suggest an improvement in the liver glycogenesis. It is well established that glycogen is the primary intracellular storable form of glucose and its levels in various tissues are a direct reflection of insulin activity as insulin promotes intracellular glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase (Yki-Jarvinen *et. al.,* 1987, Groop & Ortho-Melander, 2008)

Table 3.1.14. Effect of some herbal drugs on hepatic glycogen content of type 2 diabetic model rats.

Group	Glycogen (mg/g) [M±SD]
WC (n=6)	8.47±7.37 (100%)
Gliben (n=6)	14.33±7.21 (169%)
ADHP-1 (n=7)	8.92±3.23 (105%)
ADHP-2 (n=8)	13.31±7.39 (157%)
ADHP-4 (n=8)	10.77±7.72 (127%)
ADHP-7 (n=8)	12.25±9.57 (145%)
ADHP-11 (n=7)	9.21±3.61 (109%)

Data are expressed as Mean ±SD. n, number of animals in each group. Statistical analysis between group comparison was done by using independent sample test. WC = Type 2 Water Control; Gliben = Type 2 Glibenclamide treated group.

ADHP-5 and ADHP-6 exhibited better results on T2 DM rats in the acute study of 1st batch. From the given label of ADHP-5 & ADHP-6, it was seen that *Salvia haematodes* Linn and *Gymnema sylvetre* were the ingredient of these drugs, respectively. Since lots of research has been done on *Gymnema sylvestre* therefore attention has been paid to Salvia haematodes and chosen for further studies.

Another plant *Phlogacanthus thyrsiflorus* Nees used for blood glucose control in Assam, India and it was chosen for the chemical and biological studies.

3.1.3 Chronic effect of the Single plant (*Phlogacanthus thyrsiflorus* Nees) flower comprising the 3rd batch of the biological investigation.

3.1.3.1 Effect of *P thyrsiflorus* flower on fasting serum glucose level of Type 2 diabetic model rats

Fasting serum glucose (FSG) levels of type 2 diabetic models rats of the three experimental groups were almost similar on 0 day (Table 3.1.15 and Figure 3.1.1). After oral administration of respective treatment to the type 2 diabetic model rats of different groups for 28 days of experimental period, it was found that the FSG level of type 2 rats

treated with ethanol extract of *P thyrsiflorus* flowers showed a significant decrease while comparing within groups (p=0.042). As expected, glibenclamide also ameliorated the diabetic condition on 28th day (p=0.037). Moreover, ethanol extract treated group showed a reduction of 28% of FSG level compared to base line value. Glibenclamide significantly (p=0.002) reduced fasting glucose on 28th day when compared to base line value. There was a 13% increase in the fasting level of Type 2 control group on 28th day of experimental period.

Table 3.1.15. Effect of *P thyrsiflorus* flower extract on fasting blood glucose level of STZ-induced Type 2 diabetic model rats.

Group	Glucose(mmol/L) 0 day	Glucose(mmol/L) 14 day	Glucose(mmol/L) 28 day
T2WC **(n=6)**	7.57±.52 (100%)	8.30±.88 (110%)	8.52±1.43 (113%)
Gliben **(n=6)**	7.99±1.03 (100%)	6.59±1.30 (82%)	6.39±1.14 * (80%) p=0.037 (a)
Ethanol ext **(n=6)**	8.86±.79 (100%)	7.17±1.22 (81%)	6.42±1.32 * (72%) p=0.042 (b)

*Data are expressed as Mean ±SD. n, number of animals in each group. Statistical analysis within groups was done using paired 't'- test and between groups comparison was done using one-way ANOVA with post Hoc Bonferroni test. *= p<0.05; **= p<0.005. T2WC= Type 2 Water Control, Gliben= Glibenclamide.a= T2WC vs. Gliben, b= T2WC vs. Ethanol*

Figure 3.1.1 Effect of P thyrsiflorus flower extract on fasting blood glucose level of   STZ-induced Type 2 diabetic model rats.

3.1.3.2 Effect of *P thyrsiflorus* flower on serum insulin level of STZ-induced Type 2 diabetic model rats

The effect of *P thyrsiflorus* extract on insulinemic status of type 2 diabetic model rats was observed (Table 3.1.16 and Figure 3.1.2). At the beginning of the study period, all groups showed almost similar serum insulin level. After 28 days consecutive feeding glibenclamide treated rats showed more than fivefold increase in serum insulin level which was highly significant (p < 0.001). Ethanol extract showed a 23% increase in serum insulin level at the end of the study period compared to baseline level. On the contrary, T2WC group showed a 48% reduction compared to baseline value.

Table 3.1.16. Effect of *P thyrsiflorus* flower on the serum insulin level of STZ-induced Type 2 diabetic model rats

Group	Insulin (ng/ml)	
	0 day	**28 day**
T2WC	0.46±0.29	0.24±0.07
(n=5)	(100%)	(52%)
Gliben	0.19±0.04	1.03±0.31**
(n=5)	(100%)	(542%)
		p=0.0001 (a)
Ethanol ext	0.30±0.13	0.37±0.21**
(n=5)	(100%)	(123%)
		p=0.001(b)

Data are expressed as Mean ±SD. n, number of animals in each group. Statistical analysis within groups was done using one-way ANOVA with post Hoc Bonferroni test. T2WC= Type 2 Water Control, Gliben= Glibenclamide.a= T2WC vs. Gliben, b= Gliben vs. Ethanol ext
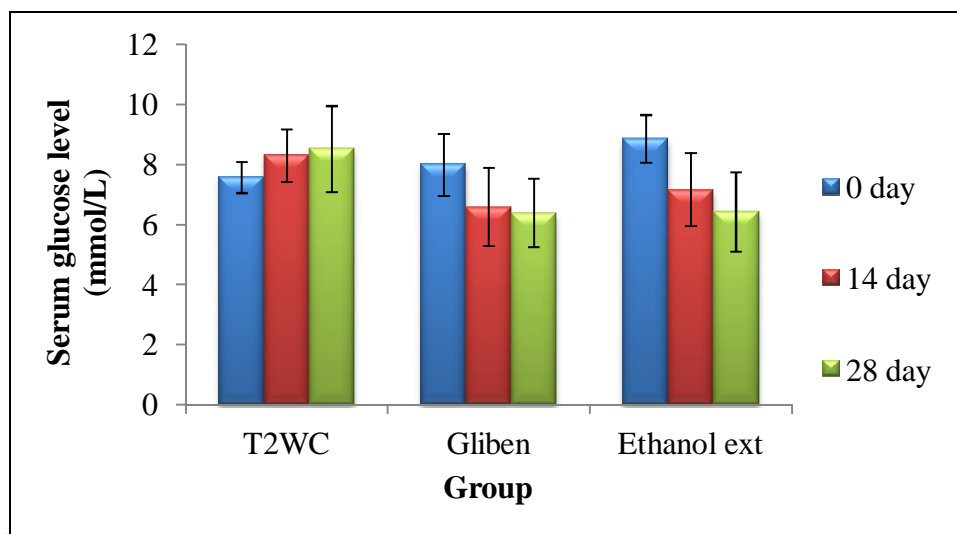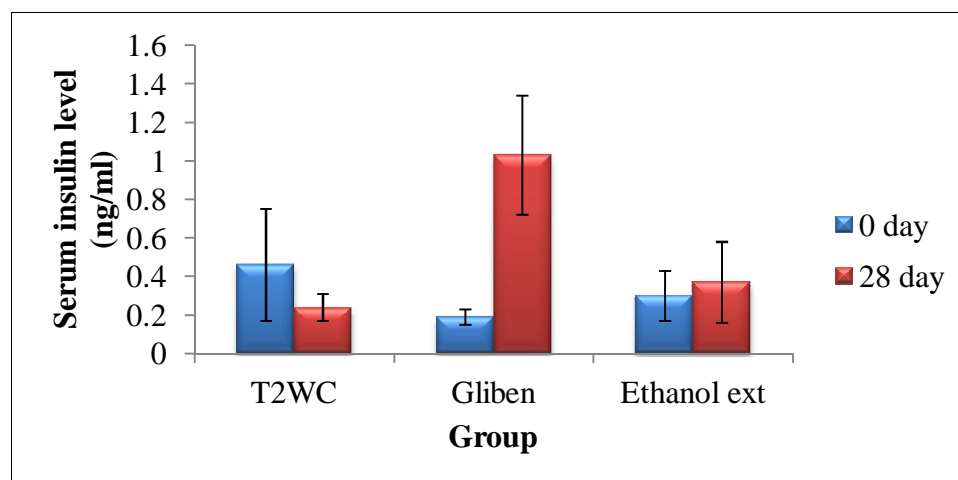

Figure 3.1.2 Effect of P thyrsiflorus flower on the serum insulin level of STZ-induced Type 2 diabetic model rats.

3.1.3.3 Effect of *P thyrsiflorus* flower extract on the body weight of Type 2 diabetic model rats

The effect of *P thyrsiflorus* extract on body weight of type 2 diabetic model rats was observed during 28 days study period. Body weight of each rat was taken at seven days interval. No significant change was found in body weight in any group after 28 days study (Table 3.1.17 and Figure 3.1.3).

Table 3.1.17. Effect of *P thyrsiflorus* flower extract on the body weight of Type 2 diabetic model rats

Group	Body weight (g)				
	0 day	**7 day**	**14 day**	**21 day**	**28 day**
T2WC	192±21	185±28	188±32	182±29	193±34
(n=6)	(100%)	(96%)	(98%)	(95%)	(101%)
Gliben	182±13	189±25	183±8	182±7	182±16
(n=6)	(100%)	(104%)	(101%)	(101%)	(100%)
Ethanol ext	202±7	193±19	200±18	195±20	198±18
(n=6)	(100%)	(96%)	(99%)	(97%)	(98%)

Data are expressed as Mean ±SD. n, number of animals in each group. Statistical analysis within groups was done using one-way ANOVA with post Hoc Bonferroni test. T2WC= Type 2 Water Control, Gliben= Glibenclamide.

Figure 3.1.3 Effect of P thyrsiflorus flower extract on the body weight of Type 2 diabetic model rats

3.1.3.4 Effect of *P thyrsiflorus* flower on the liver glycogen content of Type 2 diabetic model rats

Effect of *P thyrsiflorus* extract on liver glycogen content of type 2 diabetic model rats are presented in Table 3.1.18 and Figure 3.1.4. There was no significant change in liver glycogen content between the water control and ethanol extract treated groups. Glibenclamide treated group showed higher glycogen content in comparison to T2WC and ethanol extract treated group. However, the increase was not significant.

Table 3.1.18. Effect of P *thyrsiflorus* flower on the liver glycogen content of Type 2 diabetic model rats

Group	Glycogen(mg/g) 28 day
T2WC (n=6)	15.58±13.93
Gliben (n=6)	20.13±7.98
Ethanol ext (n=6)	15.1±8.2

Data are expressed as Mean ±SD. n, number of animals in each group. Statistical analysis within groups was done using one-way ANOVA with post Hoc Bonferroni test. T2WC= Type 2 Water Control, Gliben= Glibenclamide

Figure 3.1.4. Effect of P thyrsiflorus flower on liver glycogen content of Type 2 diabetic model rats

3.1.3.5 Effects of *P thyrsiflorus* flower on serum cholesterol and triglyceride levels of STZ-induced Type 2 diabetic model rats

Effect of *P thyrsiflorus* flower on serum cholesterol level was presented in Table 3.1.19 and Figure 3.1.5.. Ethanol extract of *P thyrsiflorus* flower caused some reduction in total cholesterol level on 28th day [Serum cholesterol (M±SD) mg/dl, 0 day (80±11.21) vs. 28 day (71±8.96)] (p < NS), Glibenclamide decreased serum cholesterol level by 20% when compared to the base line level. Type 2 control also showed a 8% decrease of serum cholesterol level. In case of serum triglyceride (TG) level, there was a reduction of 23% in glibenclamide treated groups after 28 days study period. Ethanol extract treated group also showed a reduction in serum TG level by 16% (Table 3.1.19 and Figure 3.1.6 and 3.1.7).

Table 3.1.19. Effects of *P thyrsiflorus* flower on serum cholesterol and triglyceride levels of STZ-induced Type 2 diabetic model rats

Group	Cholesterol (mg/dl)		TG(mg/dl)	
	0 day	28 day	0 day	28 day
T2WC	79±5.93	73±8.28	70±4.12	59±8.56
(n=6)	(100%)	(92%)	(100%)	(84%)
Gliben	81±8.87	65±5.78	77±7.53	59±11.85
(n=6)	(100%)	(80%)	(100%)	(77%)
Ethanol ext	80±11.21	71±8.96	70±9.09	54±7.52
(n=6)	(100%)	(89%)	(100%)	(77%)

Data are expressed as Mean ±SD. n, number of animals in each group. Statistical analysis within groups was done using one-way ANOVA with post Hoc Bonferroni test. T2WC= Type 2 Water Control, Gliben= Glibenclamide.
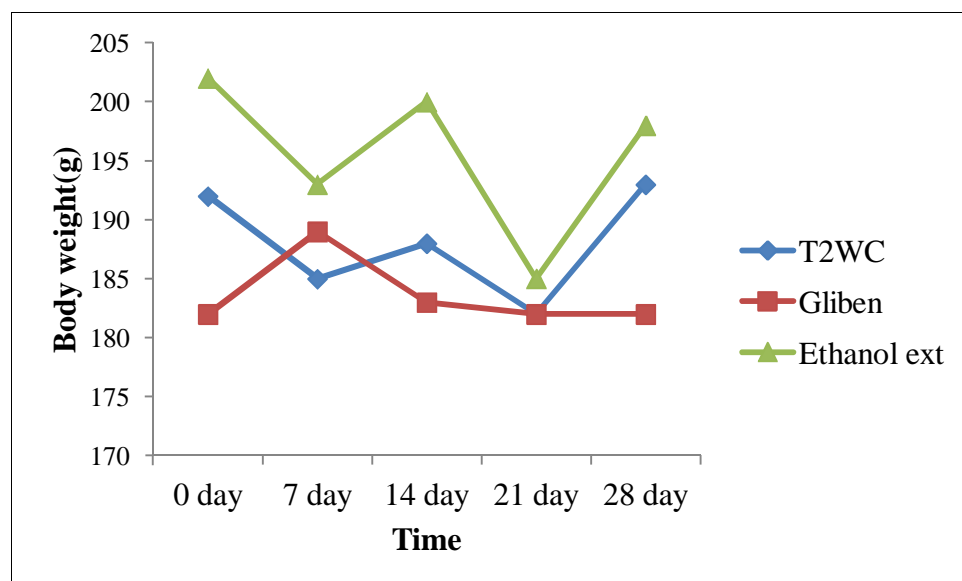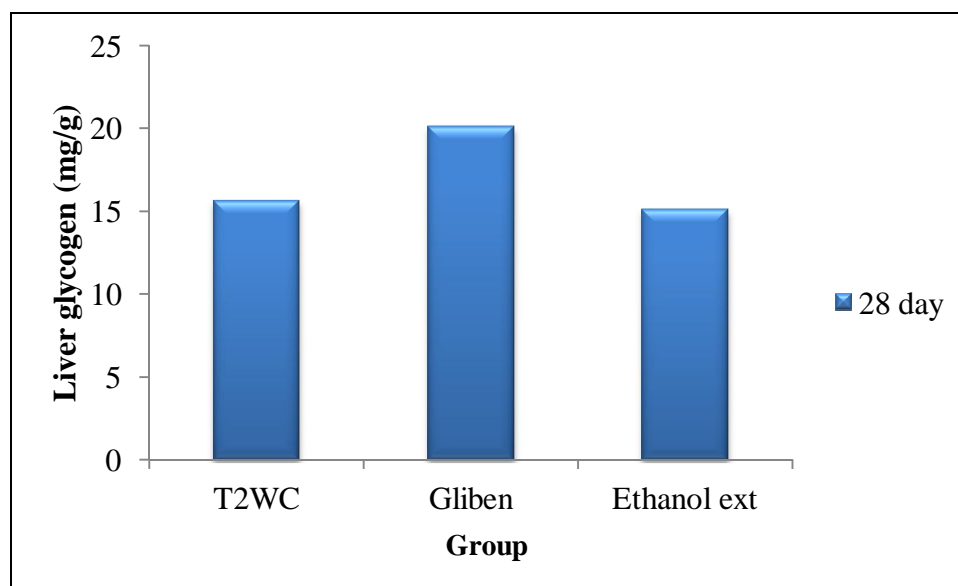

Figure 3.1.5 Effect of P thyrsiflorus flower on serum cholesterol level of STZ- induced Type 2 diabetic model rats
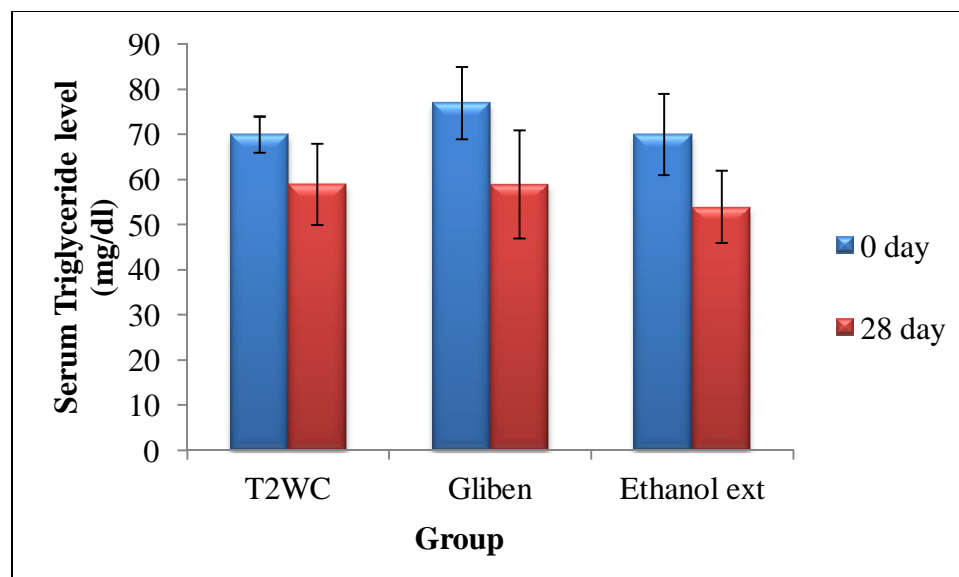
Figure-3.1.6 Effect of P thyrsiflorus flower on serum triglyceride level of STZ- induced Type 2 diabetic model rats

3.1.3.6 Chronic effects of *P thyrsiflorus* flower on serum HDL-C and LDL-C levels of STZ-induced Type 2 diabetic model rats

The effects of chronic treatment with extract of *P thyrsiflorus* flower on the serum HDL- and LDL-cholesterol levels of type 2 diabetic model rats are summarized in Table 3.1.20, Figure 3.1.8 and Figure 3.1.9. It was found that HDL level decreased in control by 17%, glibenclamide treated group showed 10% decrease in serum HDL level at the end of 28 days study period when compared to the baseline level. HDL level remained unchanged in ethanol extract treated group during the 28 days study period. [Glibenclamide also decreased LDL level by 35% compared to the initial value. On the otherhand, control group showed a 12% increase in serum LDL which is harmful for health].

In case of atherogenic LDL-cholesterol, the level was decreased by the treatment of ethanol extract of *P thyrsiflorus*. 24% (25-19 mg/dl) reduction was noticed by the ethanol extract treated group (Table 3.1.20) at the end of the study period compared to baseline level.

Table 3.1.20 Effect of *P thyrsiflorus* flower on the serum HDL-C and LDL-C levels of STZ-induced Type 2 diabetic model rats.

Group	HDL (mg/dl)		LDL (mg/dl)	
	0 day	28 day	0 day	28 day
T2WC **(n=6)**	40±2.81 (100%)	33±3.25 (83%)	25±6.98 (100%)	28±10.62 (112%)
Gliben **(n=6)**	40±3.08 (100%)	36±6.26 (90%)	26±7.07 (100%)	17±9.70 (65%)
Ethanol extract **(n=6)**	41±3.20 (100%)	41±3.39 * (100%) p=0.031(a)	25±13.60 (100%)	19±9.05 (76%)

Data are expressed as Mean ±SD. n, number of animals in each group. Statistical analysis within groups was done using paired 't'- test and between groups comparison was done using one-way ANOVA with post Hoc Bonferroni test. a= T2WC vs. Ethanol ext. T2WC= Type 2 Water Control, Gliben= Glibenclamide. *= p<0.05; **= p<0.005.
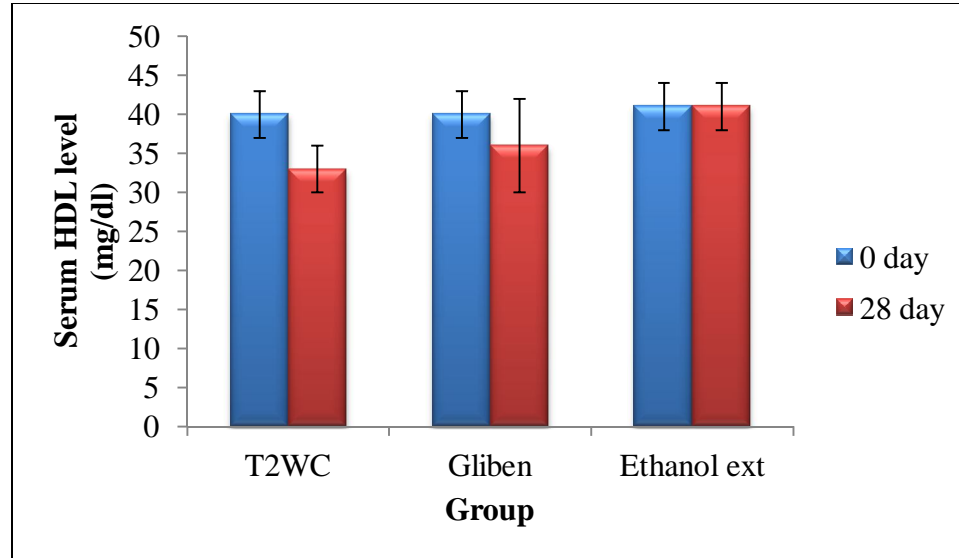
Figure-3.1.7. Effect of P thyrsiflorus flower on the serum HDL-C level of STZ-induced Type 2 diabetic model rats.
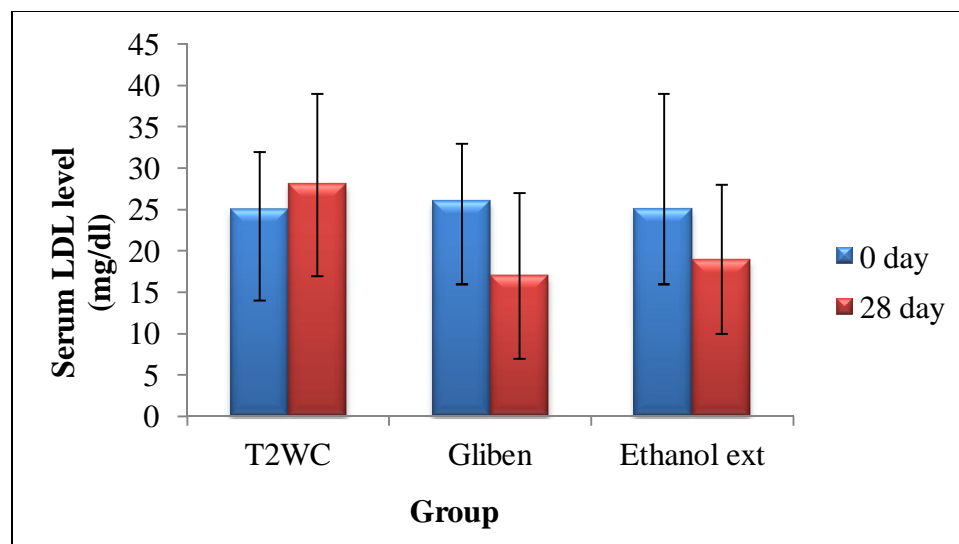
Figure-3.1.8. Chronic effect of P thyrsiflorus flower on the serum LDL-C level of STZ-induced Type 2 diabetic model rats

In case of atherogenic LDL-cholesterol, the level was decreased by the treatment of *Phlogacanthus thyrsiflorus* Nees. About 24% (25-19 mg/dl) reduction was noticed by the ethanol extract treated group (Table 3.1.20).

3.1.3.7 Effects of extract of *P thyrsiflorus* flower on Erythrocyte Malondialdehyde (MDA) and reduced Glutathione (GSH) levels of Type 2 Diabetic Model rats

Table 3.1.21, Figure 3.1.9 shows that the concentration of erythrocyte lipid peroxidation products i.e. malondialdehyde (MDA) and reduced Glutathione (GSH) in different groups of rats after 28 days of the study period.

Lipid peroxidation is determined by thiobarbuturic acid reactive (TBAR) substances level. Jain et al (1999) reported that there is a significant increased membrane lipid peroxidation in diabetic erythrocytes compared with normal erythrocytes (Jain & McVie, 1999). On this basis, the present study evaluated MDA (TBAR) levels in erythrocyte homolysate to determine the degree of oxidative damage in diabetic rats. After 28 days study period as expected, type 2 diabetic water control rats showed the highest MDA level (median=3.24 nmol/g Hb), when compared with other groups. Administration of extract to type 2 rats tends to normalize the erythrocyte MDA level (median = 1.83 nmol/g Hb) compared to control group.

As it is seen from the table, the levels of the major cellular antioxidant GSH was lower in type 2 control rats (median=15.34 mg/g Hb). The decrease of GSH content contributes to the pathogenesis of complications associated with chronic diabetic state. GSH level increased in *Phlogacanthu sthyrsiflorus* Nees extract treated group (median=18.19 mg/g Hb) but it is just outside the significant level (P=.074). Glibenclamide treated rats also had a higher GSH level.

Table 3.1.21 Effects of extract of *P thyrsiflorus* flower on Erythrocyte Malondialdehyde (MDA) and reduced Glutathione (GSH) levels of Type 2 Diabetic Model rats.

Group	GSH(mg/g Hb)	MDA(nmol/g Hb)
T2WC (n=5)	15.34 (13.54 - 18.24)	3.24 (1.28 - 4.05)
Gliben (n=5)	17.66 (10.18 - 21.54)	2.00 (1.59 - 2.70)
Ethanol ext (n=5)	18.19 (16.32 - 20.69)	1.83 (1.40 -. 3.04)
Mann-Whitney U/ P value		
Group	**GSH**	**MDA**
T2WC vs. Gliben	8.00/.344	7.00/.248
T2WC vs. ethanol ext	4.00/.074	7.00/.248
Glibenvs.ethanolext	10.00/.599	12.00/.916

Data are expressed as Median (Range). n, number of animals in each group. Statistical analysis between group comparisons was done using Mann -Whitney U test. T2WC= Type 2 Water Control, Gliben= Glibenclamide.
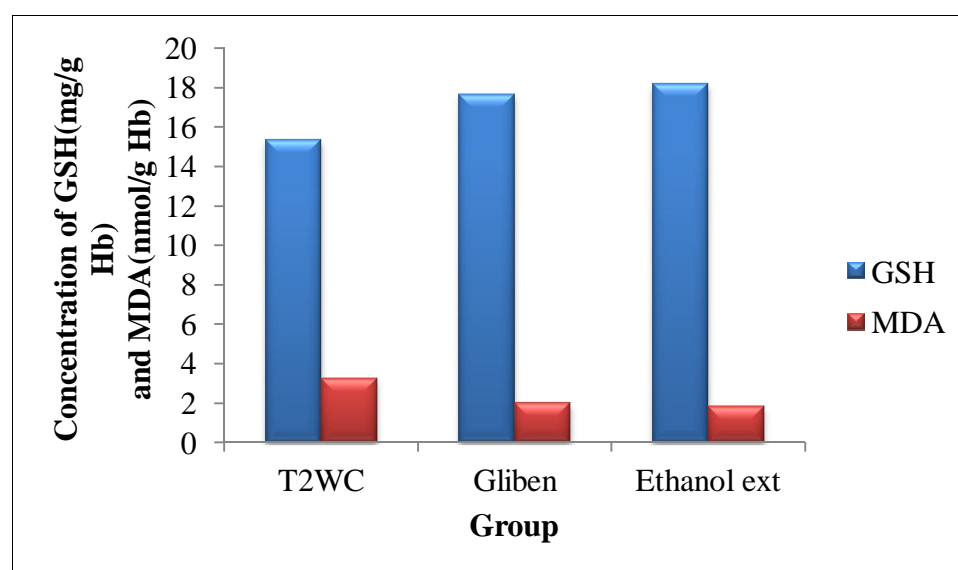
Figure-3.1.9. Effects of extract of P thyrsiflorus flower on Erythrocyte Malondialdehyde (MDA) and reduced Glutathione (GSH) levels of Type 2 Diabetic Model rats.

From the obtained results it is seen that, flowers of *P thyrsiflorus* possesses hypoglycemic and to some extent hypolipidemic properties. The obtained antidiabetic activity by the ethanolic extract of *P thyrsiflorus* flowers may be due to the presence hypoglycemic compounds, the activities of which remain to be explored in future.

3.2 ALPHA-GLUCOSIDASE INHIBITOR ACTIVITY

3.2.1 α-Glucosidase inhibitory activity of the plant extracts

The α-glucosidase enzyme is one of the key enzymes involved in dietary carbohydrate digestion in human. It hydrolyzes the carbohydrate, releasing glucose and cause the raised postprandial blood glucose level. Inhibition to this enzyme can effectively descent the postprandial blood glucose level. This is especially beneficial for diabetic patients. There are many synthetic drugs available to inhibit α-glucosidase such as acarbose, vogibose and miglitol. However, they usually can cause hepatic disorders and other negative gastrointestinal symptoms (Murai *et al.*, 2002). Hence, α-glucosidase inhibitors from natural source is preferable as a means to treat diabetes. In this study, the α-glucosidase inhibitory activity of the extracts of *Salvia haematodes* Linn roots and *Phlogacanthus thyrsiflorus* Nees flowers were evaluated and the results are reported as (IC_{50}) shown in Table 3.2.1. Ethyl acetate and methanol extracts of the plant materials were used for the tests.

Among the above mentioned fractions following 04 exhibited good results. The positive control was used quercetin and the IC_{50} was found to be 1.544. n-Hexane part of the methanol fraction (IC_{50} = 0.5226); n-hexane part of the ethyl acetate fraction (IC_{50} = 1.184); ethyl acetate part of methanol fraction (IC_{50} = 1.318) of *S haematodes* roots and ethyl acetate part (IC_{50} = 1.539) of *P thyrsiflorus* flowers were exhibited significant results. The results showed (Table: 3.2.1) that IC_{50} for both the root of *S haematodes* and flower of *P thyrsiflorus* possessed the potent α-glucosidase inhibitory activity. For the root of *S haematodes*, n-haxane part of the methanol extract showed significant (IC_{50} = 0.5226 μg/mL) activity which was better than the positive control quercetin (IC_{50} = 1.544 μg/mL).

Table 3.2.1. Percentage of α-glucosidase inhibition and the IC$_{50}$ values

Sl No		Sample	IC$_{50}$ (µg/mL)
1		Quercetin (positive control)	1.544[**]
Phlogacantus thyrsiflorus **Nees**			
2		EtOAc extract	1.539[**]
3		MeOH extract after hexane, DCM and EtOAc extraction.	2.348
4		Direct MeOH extract	2.542
Salvia haematodes **Linn**			
5		EtOAc extract	8.511
	a)	n-Hexane part	1.184[**]
	b)	Chloroform part	6.507
	c)	EtOAc part	4.105
	d)	n-Butanol part	636.3
	e)	Water part	2844
6		MeOH extract	4.483
	a)	n-Hexane part	0.5226[***]
	b)	EtOAc part	1.318[**]
	c)	n-Butanol part	13.98
	d)	Water part	6.100

3.3 ANTIOXIDANT ACTIVITY TESTING

3.3.1. Antioxidant Activity Testing

The present study was carried out to determine of phytochemical parameters e.g. total phenolic content, total flavonoid content and total tannin content. The antioxidant activity e.g. total antioxidant capacity, DPPH (2,2-diphenyl-1-picrylhydrazyl) inhibitory activity, ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] inhibitory activity and reducing power inhibitory activity of methanol extract of the samples.

3.3.2. Total Phenolic Content

The total phenolic content of methanol extracts of the samples ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADSP-8, ADSP-9, were found to be 591.21± 0.1530, 400.52±0.1452, 260.35±0.1542, 180.24±0.1785, 340.36±0.1259, 469.65±0.1425, 518.52±0.1654, 562.74±0.1257 and 630.5±0.1745 mg/g of gallic acid equivalent, respectively. The phenolic content was found to be highest in ADSP-9 and lowest in ADHP-4. According to the results of this study, it can be revealed that the highest amount of phenolic contents in ADSP-9 (630.5±0.1745 mg/g) might be due to the high concentration of phenolic compounds present in this plant. Phenolic compounds are known to be good natural antioxidants.

Table-3.3.1: Total Phenolic Content in different samples.

Samples ID	Total Phenolic Content (mg/g)
ADHP-1	597.21± 0.1530
ADHP-2	400.52±0.1452
ADHP-3	260.35±0.1542
ADHP-4	180.24±0.1785
ADHP-5	340.36±0.1259
ADHP-6	469.65±0.1425
ADHP-7	518.52±0.1654
ADSP-8	562.74±0.1257
ADSP-9	630.5±0.1745

ADHP→Antidiabetic Herbal Preparation, ADSP→ Antidiabetic Single Plant
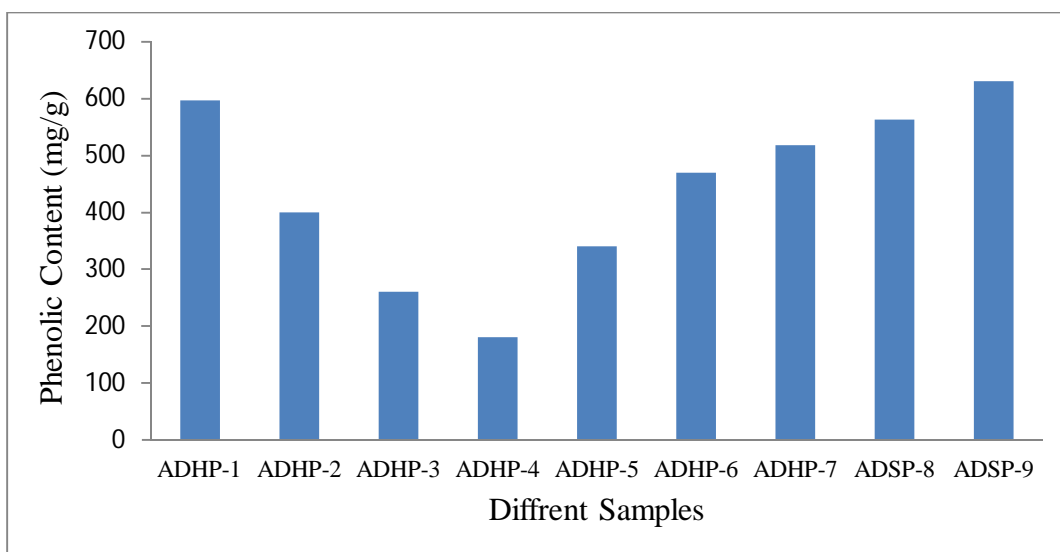
Figure-3.3.1: Total Phenolic Content of different samples of ADHPs and ADSPs

3.3.3. Total Flavonoid Content.

Flavonoid content of methanol extracts of ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADSP-8, ADSP-9, were found to be 14.64± 0.131, 14.5± 0.201, 22.15± 0.123, 13.75± 0.141, 18.03± 0.211, 27.39± 0.174, 30.67± 0.132, 97.8± 0.184 and 20.98± 0.145 mg quercetin/ g of dry extract respectively. A significant amount of total flavonoid (97.8± 0.184 mg quercetin/ g of dry extract) was observed to be present in the ADSP-8, and ADHP-4 was found to be possess lowest amount of total flavonoid. It is to be noted that ADSP-8 also had the highest amount of phenolic compounds indicating it to be a potent antioxidant agent.

Table-3.3.2: Total Flavonoid content in different samples of *ADHPs and ADSPs*.

Samples ID	Flavonoid content (mg/g)
ADHP-1	14.64 ± 0.131
ADHP-2	14.5 ± 0.201
ADHP-3	22.15 ± 0.123
ADHP-4	13.75 ± 0.141
ADHP-5	18.03 ± 0.211
ADHP-6	27.39 ± 0.174
ADHP-7	30.67 ± 0.132
ADSP-8	97.8 ± 0.184
ADSP-9	20.98 ± 0.145

ADHP→Antidiabetic Herbal Preparation, ADSP→ Antidiabetic Single Plant
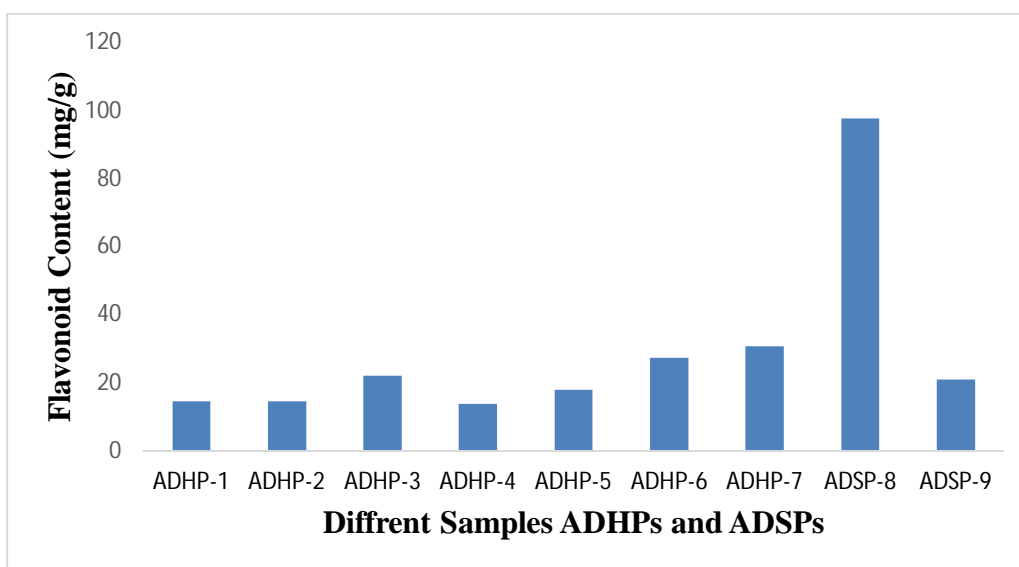
Figure-3.3.2: Flavonoid content of different samples of ADHPs and ADSPs

3.3.4. Total Tannin Content

Total tannin content of methanol extracts of the samples ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADSP-8, ADSP-9 were 40.10±0.1254, 14.3±0.1945, 11.9±0.1524, 8.3±0.1784, 10.0±0.0145, 27.1±0.1235, 26.0±0.1658,

67.6±0.1478 and 104.4±0.1524 mg/g of tannic acid equivalent, respectively. From the table it was observed that total tannin content was highest in ADSP-9.

Table-3.3.3: Total Tannin Content in different *ADHPs and ADSPs* samples

Samples ID	Total Tannin Content (mg/g)
ADHP-1	40.1±0.1254
ADHP-2	14.3±0.1945
ADHP-3	11.9±0.1524
ADHP-4	8.3±0.1784
ADHP-5	10.0±0.0145
ADHP-6	27.1±0.1235
ADHP-7	26.0±0.1658
ADSP-8	67.6±0.1478
ADSP-9	104.4±0.1524

ADHP→Antidiabetic Herbal Preparation, ADSP→ Antidiabetic Single Plant
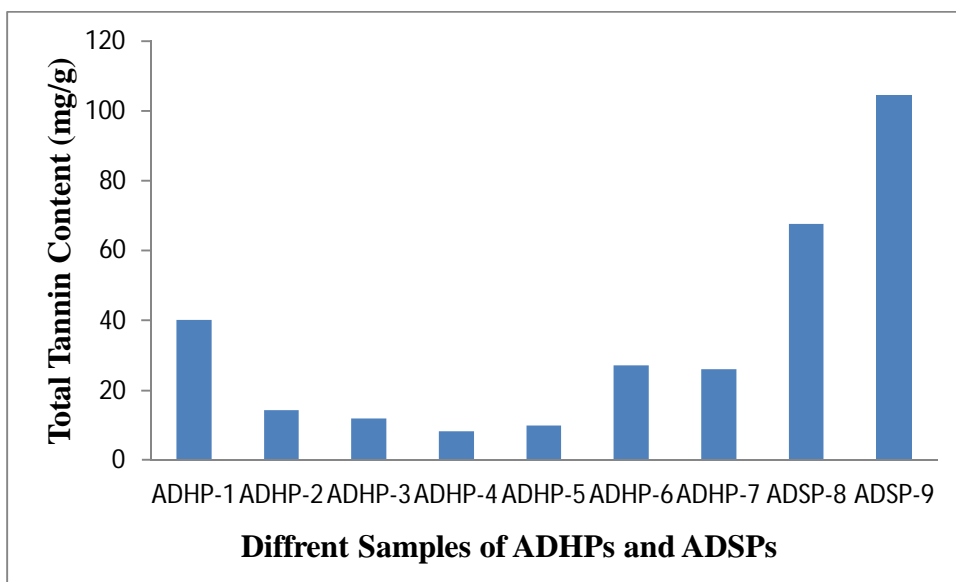
Figure-3.3.3: Total Tannin Content of different ADHPs and ADSPs samples

3.3.5. Total Antioxidant Activity

Total antioxidant activity of methanol extracts of the samples ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADSP-8, ADSP-9, were found to be 535.54±0.0124, 455.21±0.0451, 487.55±0.0875, 275.84±0.0451, 255.12±0.0687,

715.54±0.0485, 330.85±0.0784, 830.48±0.0365 and 850.74±0.0854 mg/g of ascorbic acid equivalent. This study reveals that the antioxidant activity of the ADHP-6, ADSP-8 and ADSP-9 extracts were higher than the other extracts (Table & Figure 3.3.4). Thus, the extracts demonstrated electron donating capacity, may act as radical chain terminators' transforming reactive free radical species into stable non reactive products (Singleton & Rossi. 1965, Prieto *et. al.,* 1999) and act as antioxidant.

Table-3.3.4: Total Antioxidant Activity in different *ADHPs and ADSPs* samples.

Samples ID	Total Antioxidant Activity (mg/g)
ADHP-1	535.54±0.0124
ADHP-2	455.21±0.0451
ADHP-3	487.55±0.0875
ADHP-4	275.84±0.0451
ADHP-5	255.12±0.0687
ADHP-6	715.54±0.0485
ADHP-7	330.85±0.0784
ADSP-8	830.48±0.0365
ADSP-9	850.74±0.0854

ADHP→Antidiabetic Herbal Preparation, ADSP→ Antidiabetic Single Plant
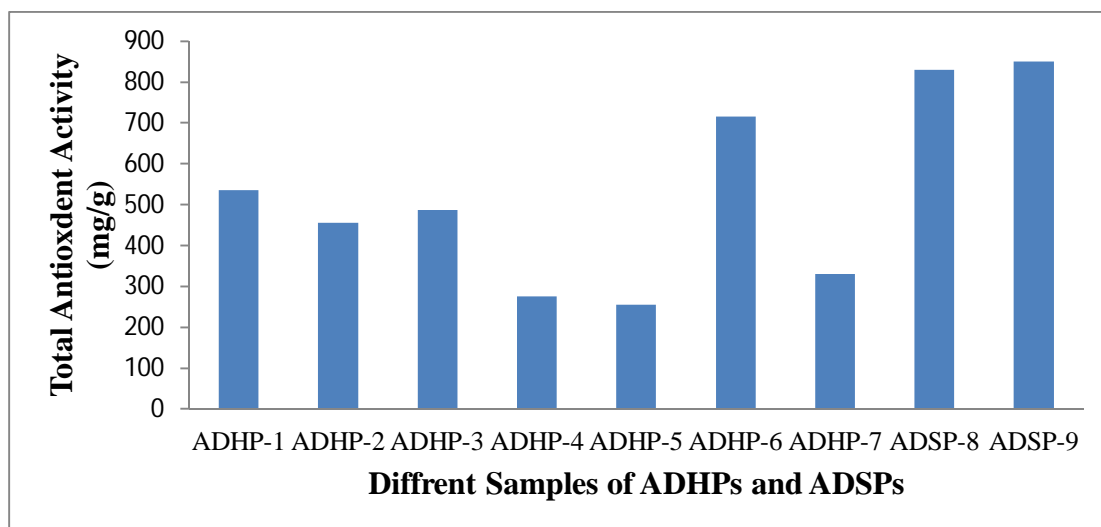
Figure-3.3.4: Total Antioxidant Activity of different ADHPs and ADSPs samples.

3.3.6. DPPH free radical scavenging activities.

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activities of the samples ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADSP-8 and ADSP-9 varied from 9.53± 0.0442 to 94.8± 0.0312, 5.59± 0.0632 to 93.4± 0.0530, 7.35± 0.0325 to 93.7± 0.0156, 1.3± 0.0267 to 38.41± 0.0106, 0.44± 0.0332 to 42.94±0.0123, 2.93± 0.0435 to 86.25± 0.0023, 3.44± 0.0165 to 91.88± 0.0534, 13.63± 0.0104 to 94.76±0.0045 and 16.65± 0.0323 to 94.37± 0.0466 % of inhibition, respectively with concentration. On the other hand standard ascorbic acid varied between 40.5± 0.0387 to 97.15± 0.0502 % of inhibition, respectively with same concentration change (Table & Figure 3.3.5). Among the nine extracts, four were found to have reasonable DPPH free radical scavenging activity such as ADHP-1, ADHP-7, ADHP-8 and ADHP-9. Increase of concentration increased the inhibition percentage. Which is very comparable to the activity of the standard anti oxidant ascorbic acid (AA). The IC_{50} values of the methanol extracts of the extracts were also comparable to that of ascorbic acid.
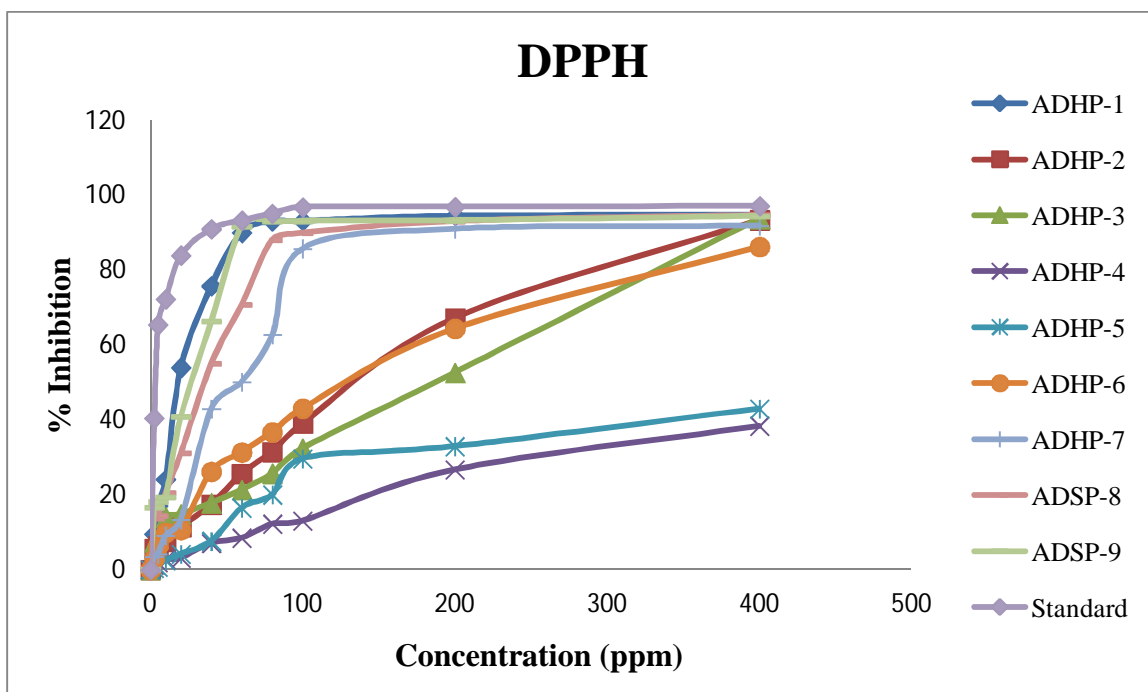
Figure-3.3.5: DPPH free radical % inhibition vs concentration of methanol extracts of different ADHP and ADSP samples.

Table-3.3.5: Percent inhibition at different concentration of methanol extracts of different ADHPs and ADSPs samples.

Conc. ppm	ADHP-1	ADHP-2	ADHP-3	ADHP-4	ADHP-5	ADHP-6	ADHP-7	ADSP-8	ADSP-9	Standard
0	0	0	0	0	0	0	0	0	0	0
2.5	9.53± 0.0442	5.59± 0.0632	7.35± 0.0325	1.30± 0.0267	0.44± 0.0332	2.93± 0.0435	3.44± 0.0165	13.63± 0.0104	16.65± 0.0323	40.5± 0.0387
5	17.06±0.0214	6.26±0.0545	8.29±0.0325	1.89±0.0254	0.73±0.0254	6.36±0.0315	4.16±0.0218	14.38±0.0142	18.08±0.0340	65.44±0.0341
10	24.15±0.0487	7.52±0.0589	14.02±0.0235	2.7±0.0298	2.44±0.0145	9.88±0.0241	9.06±0.0271	20.57±0.0150	19.4±0.0348	72.29±0.0453
20	54.00±0.0465	11.39±0.0574	14.84±0.0258	3.15±0.0274	4.13±0.0172	10.66±0.0352	13.42±0.0341	31.2±0.0158	40.87±0.0345	83.96±0.0548
40	75.78±0.0345	17.4±0.0512	17.79±0.0345	7.06±0.0185	7.62±0.0245	26.18±0.0367	42.95±0.0412	55.03±0.0089	66.36±0.0435	90.98±0.0460
60	90.02±0.0382	25.6±0.063	21.32±0.0278	8.49±0.0165	16.51±0.0259	31.34±0.0285	50.19±0.0159	70.76±0.0192	91.73±0.0398	93.28±0.0572
80	93.12±0.0427	31.44±0.0601	25.67±0.0296	12.23±0.0157	19.98±0.0243	36.73±0.0291	62.73±0.0137	88.14±0.0240	92.99±0.0350	95.15±0.0487
100	93.18±0.0496	39.05±0.0532	32.34±0.0304	13.03±0.0241	29.66±0.0148	43.07±0.0173	85.77±0.0436	89.9±0.0137	93.11±0.0421	96.86±0.0360
200	94.54±0.0387	67.20±0.0541	52.57±0.0189	26.71±0.0215	32.94±0.0139	64.4±0.0128	91.03±0.0205	93.09±0.0180	93.29±0.0465	96.92±0.0461
400	94.8± 0.0312	93.4± 0.0530	93.7± 0.0156	38.41± .0106	42.94±0.0123	86.25± .0023	91.88± .0534	94.76±0.0045	94.37± 0.0466	97.15± 0.0502
IC_{50}	17± 0.0243	131±0.2886	210 ±0.2594	NA	NA	124.5±0.2309	59±0.2016	34±0.0512	26± 0.0265	3.25 ± 0.0209

ADHP→Antidiabetic Herbal Preparation, ADSP→ Antidiabetic Single Plant, IC_{50} Values are expressed in mg /ml.

3.3.7. ABTS free radical scavenging activity of *ADHPs and ADSPs*:

The ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging activity of methanol extracts of given as % inhibition ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADHP-8 and ADHP-9 were found to vary between 3.55±0.0231 to 98.38±0.0156, 1.7±0.0289 to 70.03±0.0134, 1.52±0.0451 to 95.54±0.0321, 1.42±0.0233 to 40.5±0.0211, 0.5±0.0286 to 35.5±0.0243, 1.2±0.0287 to 94.34±0.0371, 1.15±0.0233 to 95.9±0.0551, 2.75±0.0125 to 96.03±0.0151 and 2.81±0.0451 to 96.93±0.0212 %, respectively with change of concentration from 2.5 to 400 ppm. On the other hand standard ascorbic acid which varied from 12.5±0.0155 to 99.9±0.0145 % inhibition at the same concentration range. From the Table & Figure 3.3.6 it may be seen that ABTS free radical scavenging activity of most of the extracts showed good activity at high concentration. The activity was found to be increased with the increase of concentration of the extract. The IC$_{50}$ values of the extracts were reasonably good when compared to that of the standard ascorbic acid. ABTS free radical scavenging property reflects the ability of an antioxidant species to donate electron and hydrogen atom to inactivate these radical species (Roy *et. al.,* 2006, Preethi *et. al.,* 2006).
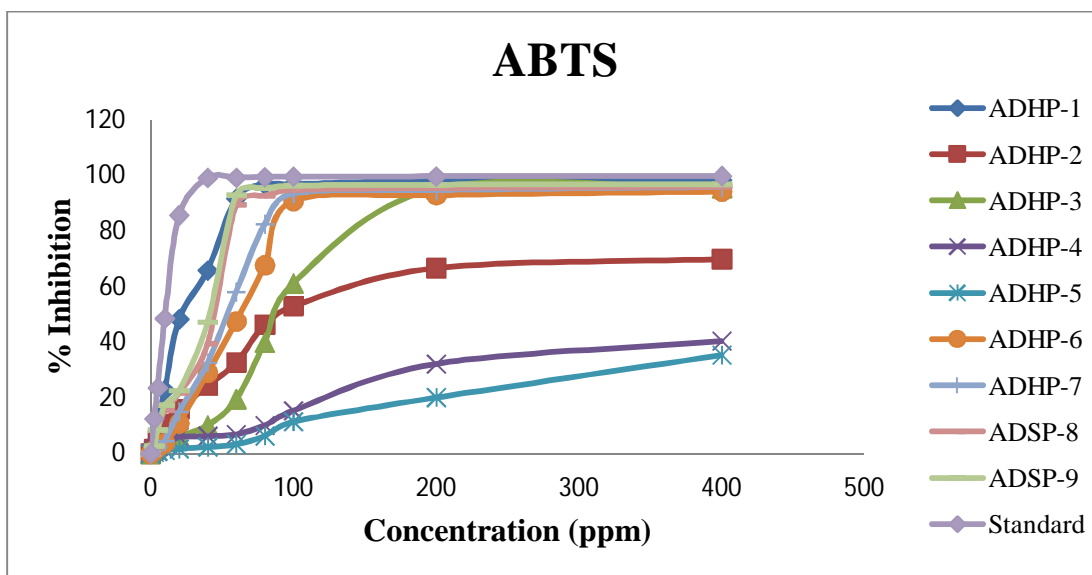
Figure-3.3.6: Concentration vs % inhibition of the methanol extracts of different ADHPs and ADSHs samples.

Table-3.3.6: Percent inhibition at different concentration of the methanol extracts of different ADHPs and ADSPs samples

Conc. Ppm	ADHP-1	ADHP-2	ADHP-3	ADHP-4	ADHP-5	ADHP-6	ADHP-7	ADSP-8	ADSP-9	Standard
0	0	0	0	0	0	0	0	0	0	0
2.5	3.55±0.0231	1.7±0.0289	1.52±0.0451	1.42±0.0233	0.50±0.0286	1.20±0.0287	1.15±0.0233	2.75±0.0125	2.81±0.0451	12.50±0.0155
5	12.91±0.0215	4.81±0.0278	3.13±0.0308	2.50±0.0215	0.87±0.0251	2.30±0.0351	2.33±0.0306	8.53±0.0142	8.62±0.0241	23.67±0.0140
10	22.83±0.0235	10.66±0.0246	4.74±0.0462	3.81±0.022	1.20±0.0205	3.90±0.0306	4.50±0.0425	15.56±0.0250	17.50±0.0261	48.60±0.0185
20	48.37±0.0198	15.97±0.0248	6.36±0.0372	5.97±0.0245	1.75±0.0263	10.89±0.0253	15.11±0.0256	21.91±0.0261	22.70±0.0258	85.79±0.0160
40	65.93±0.0142	24.46±0.0173	10.20±0.0412	6.31±0.0209	2.44±0.0248	29.16±0.0290	32.78±0.0402	39.56±0.0280	47.33±0.0268	99.19±0.0185
60	91.76±0.0169	32.82±0.0249	19.60±0.0389	6.98±0.0247	3.40±0.0201	47.61±0.0254	58.15±0.0176	89.58±0.0210	92.96±0.0273	99.25±0.0105
80	96.77±0.0241	46.47±0.0215	39.97±0.0351	10.20±0.0231	6.39±0.0289	67.77±0.0261	82.56±0.0390	92.85±0.0241	95.48±0.0361	99.53±0.0149
100	96.87±0.0120	53.12±0.0191	61.28±0.0407	15.49±0.0253	11.50±0.0236	90.91±0.0381	93.48±0.0150	94.92±0.0261	96.41±0.0354	99.68±0.0170
200	98.06±0.0219	66.81±0.0204	95.28±0.0435	32.29±0.0259	20.20±0.0245	93.05±0.0250	94.77±0.030	95.60±0.0238	96.77±0.0381	99.72±0.0195
400	98.38±0.0156	70.03±0.0134	95.54±0.0321	40.50±0.0211	35.50±0.0243	94.34±0.0371	95.90±0.0551	96.03±0.0151	96.93±0.0212	99.90±0.0145
IC_{50}	21±0.251	88±0.2523	87±0.1126	NA	NA	62.5±0.1629	54±0.1014	44.7±0.0852	41.5±0.0624	10.30±0.1194

ADHP→Antidiabetic Herbal Preparation, ADSP→ Antidiabetic Single Plant, IC_{50} Values are expressed in mg /ml.

3.3.8. Reducing Power:

The reducing power of the samples as given by absorbance against concentration ADHP-1, ADHP-2, ADHP-3, ADHP-4, ADHP-5, ADHP-6, ADHP-7, ADSP-8 and ADSP-9 were varies from 0.008±0.0001 to 0.348± 0.0070, 0.006±0.0005 to 0.088±0.0046, 0.005±0.0002 to 0.065±0.0051, 0.003±0.0001 to 0.110±0.0085, 0.004± 0.0001 to 0.150±0.0076, 0.006±0.0002 to 0.200±0.0080, 0.007±0.0003 to 0.295±0.0041, 0.009±0.0001 to 0.327±0.0085 and 0.001± 0.0010 to 0.366±0.0095 nm, respectively with concentration from 25 to 400 ppm. On the other hand standard ascorbic acid showed absorbance from 0.325±0.0040 to 1.25± 0.0075 nm, respectively with concentration change (Table 3.3.7 and Figure 3.3.7).
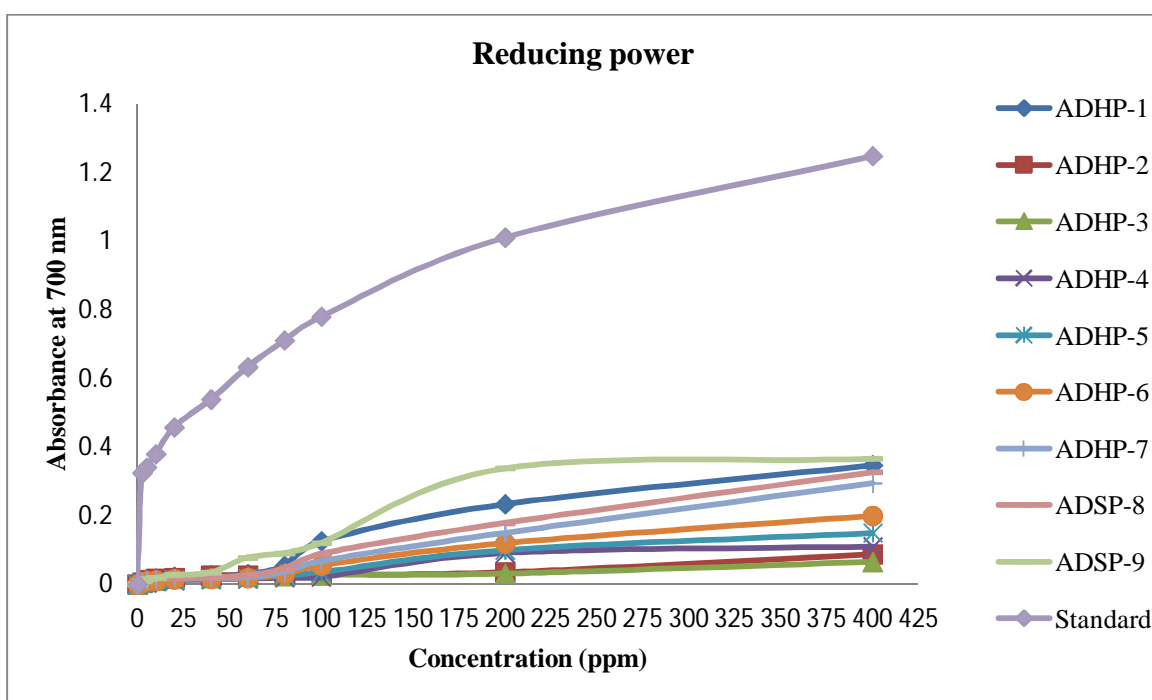
Figure-3.3.7: Reducing power of different samples   of ADHPs and ADSPs.

Table-3.3.7: Percent inhibition at different concentration of the methanol extracts of different ADHP's and ADSP's samples

Conc. Ppm	ADHP-1	ADHP-2	ADHP-3	ADHP-4	ADHP-5	ADHP-6	ADHP-7	ADSP-8	ADSP-9	Standard
0	0	0	0	0	0	0	0	0	0	0
2.5	0.008±0.0001	0.006±0.0005	0.005±0.0002	0.003±0.0001	0.004±0.0001	0.006±0.0002	0.007±0.0003	0.009±0.0001	0.011±0.0010	0.325±0.0040
5	0.015±0.0010	0.012±0.0007	0.007±0.0006	0.005±0.0006	0.009±0.0002	0.010±0.0005	0.012±0.0004	0.014±0.0003	0.015±0.0006	0.341±0.0026
10	0.017±0.0014	0.016±0.0013	0.012±0.0013	0.008±0.0004	0.010±0.0004	0.011±0.0015	0.014±0.0010	0.016±0.0002	0.019±0.0020	0.380±0.0081
20	0.022±0.0015	0.019±0.0017	0.016±0.0020	0.011±0.0010	0.013±0.0010	0.015±0.0026	0.017±0.0016	0.019±0.0020	0.028±0.0019	0.458±0.0064
40	0.026±0.0017	0.025±0.0019	0.019±0.0025	0.014±0.0012	0.015±0.0040	0.017±0.0035	0.019±0.0024	0.020±0.0019	0.034±0.0030	0.540±0.0026
60	0.030±0.0010	0.025±0.0024	0.022±0.0046	0.016±0.0025	0.017±0.0053	0.019±0.0024	0.020±0.0010	0.027±0.0040	0.078±0.0045	0.634±0.0056
80	0.054±0.0016	0.026±0.0010	0.025±0.0003	0.019±0.0032	0.025±0.0023	0.030±0.0050	0.035±0.0040	0.046±0.0016	0.091±0.0026	0.712±0.0075
100	0.126±0.0020	0.029±0.0020	0.027±0.0014	0.021±0.0015	0.035±0.0041	0.055±0.0065	0.068±0.0036	0.089±0.0061	0.121±0.0057	0.781±0.0092
200	0.234±0.0046	0.035±0.0035	0.031±0.0030	0.091±0.0024	0.100±0.0025	0.120±0.0010	0.150±0.0054	0.179±0.0076	0.339±0.0087	1.012±0.0046
400	0.348±0.0070	0.088±0.0046	0.065±0.0051	0.110±0.0085	0.150±0.0076	0.200±0.0080	0.295±0.0041	0.327±0.0085	0.366±0.0095	1.250±0.0075

ADHP→Antidiabetic Herbal Preparation, ADSP→ Antidiabetic Single Plant

3.3.8. Correlation between bioactive compounds and antioxidant capacity

Table-3.3.8: Pearson's correlation coefficients (r) between bioactive compounds and antioxidant capacity (IC$_{50}$) of 07 ADHPs and 02 ADSPs

	Total Phenolic Content	Flavonoid Content	Total Tannin Content	Total Antioxident Activity	DPPH	ABTS	Reducing Power
	r/p-value	*r/p-value*	*r/p-value*	*r/p-value*	*r/p-value*	*r/p-value*	*r/p-value*
Total Phenolic Content	1						
Flavonoid Content	0.351/0.354	1					
Total Tannin Content	0.782[*]/0.013	0.402/0.284	1				
Total Antioxident Activity	0.679[*]/0.044	0.531/0.141	0.826[**]/0.006	1			
DPPH	-0.976[**]/0.001	-0.295/0.521	-0.702/.079	-0.365/.420	1		
ABTS	-0.893[**]/0.007	-0.203/.663	-0.614/.143	-0.369/.415	0.902[**]/0.005	1	
Reducing Power	0.909[**]/0.001	0.393/0.295	0.804[**]/0.009	0.574/0.106	0.954[**]/0.001	-0.942[**]/0.001	1

Correlation is significant at the 0.05 level (2-tailed)[*]. Correlation is significant at the 0.01 level (2-tailed)[**]. DPPH → 2,2-diphenyl-1-picrylhydrazyl, ABTS →[2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)]

It was important to examine the correlation content of the main antioxidant components and the antioxidant capacity of the studied extracts. A significant correlation was found in this study between bioactive compounds and antioxidant capacity of methanol extracts of 07 ADHPs & 02 ADSHs (Table: 3.3.8). These data were in accordance with previous research (Gorinstein *et. al.,* 2003, Maisuthisakul *et. al.,* 2007), which have shown that high total polyphenols content increases antioxidant activity and there was a linear correlation between phenolic content and antioxidant activity. Phenolic compounds have been extensively investigated in the past 30 years. They have one or more aromatic rings bearing hydroxyl groups that are potentially able to act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers (Mettei *et. al.,* 1998; Rice-Evans et. al., 1996). However, flavonoids, carotenoids, tannin and total antioxidant content also have significant correlation with antioxidant properties of these herbal preparations and single plants. Thus the sample of ADHPs and ADSPs expressed the highest antioxidant capacities because these samples had high phenolic, flavonoids and tannin contents. There are significant correlation were between total tannin content & total phenolic content, total antioxidant with total phenolic and total tannin content, DPPH with phenolic content, ABTS with phenolic content and DPPH, reducing power with phenolic content, tannin content, DPPH and ABTS. No significant correlation was observed between the flavonoids and tannin, antioxidant content, DPPH, ABTS & reducing power.

3.4 SUNSCREEN PROTECTION FACTOR TESTING

3.4.1 Sun Protection Factor (SPF) Value

The effectiveness of a sunscreen is measured as a function of their SPF value, defined as the ratio of the least amount of ultraviolet energy required to produce minimal erythema or burning on sunscreen protected skin to the amount of energy required to produce the same erythema on unprotected skin (Rai & Srinivas, 2007). Thus the SPF value indicates the ability of a sunscreen product to reduce UV-induced erythema.

The *in vitro* SPF value was determined by a spectrophotometric method using the UVB (280-320 nm) region which is considered to be the region of greatest incidence during the day in which people are exposed longer to the sun's radiation.

From Figure 3.4.1, it is found to be observed that the SPF value of chloroform and ethyl acetate fractions of *S haematodes* root's chloroform extract and ethyl acetate extract showed higher SPF values (10.18 ± 0.12) & (9.63 ± 0.08) than the rest of the solvent fractions, at a test concentration of 100 μg/mL. The *P thyrsiflorus* flower extracts showed SPF value of 5.82 ± 0.05 for MeOH extract and 4.16 ± 0.04 for ethyl acetate fraction at 100 μg/mL concentration. Thus it is clearly observed that the SPF value of the chloroform and ethyl acetate fractions of methanol extract of *S haematodes* has appreciably enhanced sun-blocking properties. The methanol and ethyl acetate fractions of *P thyrsiflorus* flower however, showed lesser ability in sun-blocking, with lower SPF values. S. haematodes root can be a potential source of cosmeticutical for sun-protecting formulation.

Concentration 100µg/ml
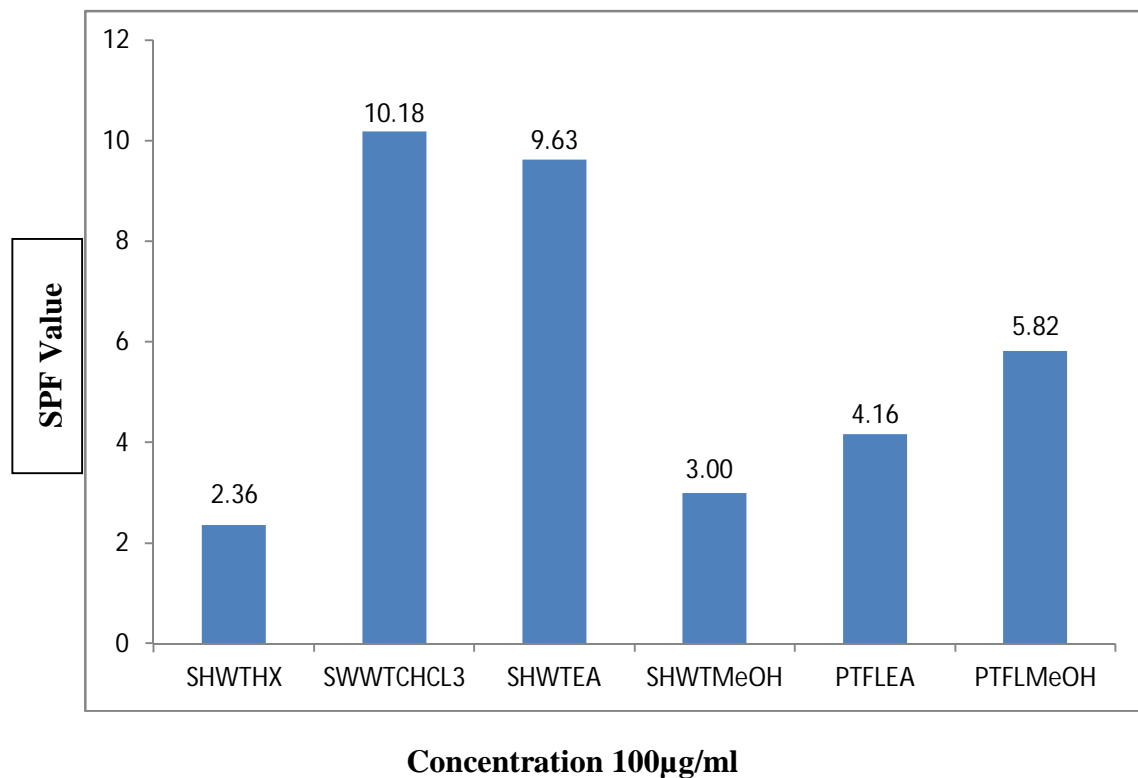
Figure 3.4.1. Sunscreen Protection Factor values of various fractions of S haematodes root and P thyrsiflorus flower fractions. Data are expressed as mean± standard deviation.(n=3) (SHWTHX→ n-hexane extract of S haematodes; SHWTCHCL3→chloroform extract of S haematodes; SHWTEA →ethyl acetate extract of S haematodes; SHWTMeOH→ methanol extract of S haematodes; PTFLEA→ ethyl acetate extract of P thyrsiflorus; PTFLMeOH→ methanol extract of P thyrsiflorus).
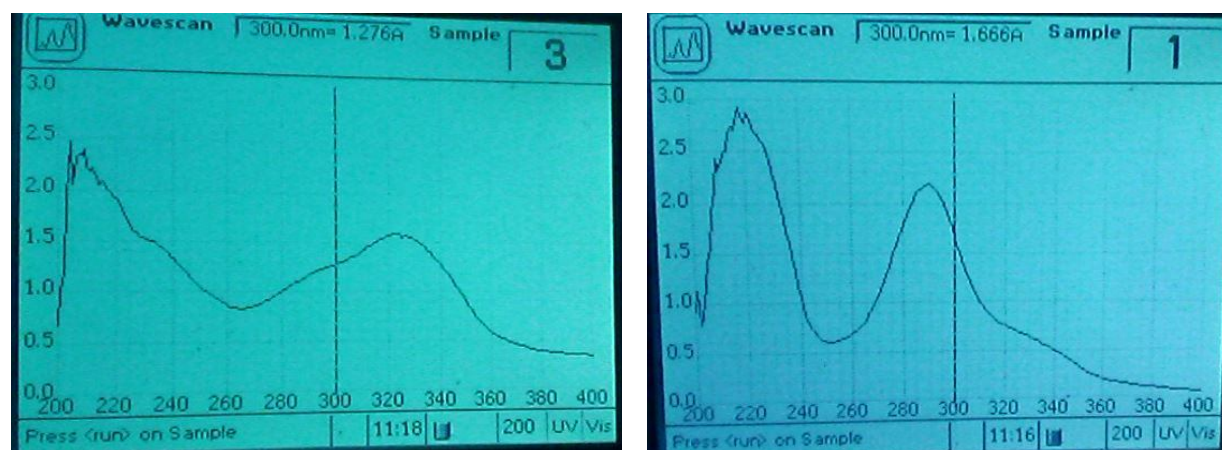
Figure 3.4.2. UV Absorption spectra of chloroform and ethyl acetate fractions of *S haematodes* root solvent fractions at 100 µg/ml of (Wavelength (nm) vs Abs)

3.4.2. Sun screen Protection Factor (SPF) of different fractions of *S haematodes* and *P thyrsiflorus*.

Table-3.4.2.1: SPF value for the Flower of *Phlogacanthus thyrsiflorus* Nees

Wavelength (nm)	MeOH extract after hexane, DCM and EtOAc extraction.	Direct MeOH extract	EtOAc extract after hexane and DCM extraction.	EE X I	MeOH extract after hexane, DCM and EtOAc extraction.	Direct MeOH extract	EtOAc extract after hexane and DCM extraction.
290	0.883	0.813	0.794	0.015	0.013245	0.012195	0.01191
295	0.824	0.758	0.733	0.0817	0.0673208	0.0619286	0.0598861
300	0.789	0.725	0.657	0.02874	0.02267586	0.0208365	0.01888218
305	0.765	0.703	0.558	0.3278	0.250767	0.2304434	0.1829124
310	0.795	0.725	0.531	0.1864	0.148188	0.13514	0.0989784
315	0.766	0.712	0.415	0.0839	0.0642674	0.0597368	0.0348185
320	0.889	0.843	0.473	0.018	0.016002	0.015174	0.008514
					0.58246606	0.5354543	0.41590158
				SPF	**5.8246606**	**5.3**	**4.1590158**

Table-3.4.2.2: SPF value for the Root of *Salvia haematodes* Linn

Wave length (nm)	MeOH extract after hexane, DCM and EtOAc extraction.	n-hexane part of EtOAc extract	Chloroform part of EtOAc extract	EtOAc part of EtOAc extract	n-butanol part of EtOAc extract	EtOAc extract after hexane and DCM extraction.	EE X I	MeOH extract after hexane, DCM and EtOAc extraction.	n-hexane part of EtOAc extract	Chloroform part of EtOAc extract	EtOAc part of EtOAc extract	n-butanol part of EtOAc extract	EtOAc extract after hexane and DCM extraction.
290	0.677	0.315	0.968	2.266	0.636	0.685	0.015	0.010155	0.004725	0.01452	0.03399	0.00954	0.010275
295	0.586	0.345	1.106	2.080	0.603	0.687	0.0817	0.047876	0.028186	0.0903602	0.169936	0.049265	0.056127
300	0.496	0.333	1.281	1.704	0.530	0.627	0.02874	0.014255	0.009570	0.0368159	0.048972	0.015232	0.018019
305	0.400	0.302	1.351	1.293	0.447	0.547	0.3278	0.13112	0.098995	0.4428578	0.423845	0.146526	0.179306
310	0.353	0.322	1.461	1.052	0.408	0.522	0.1864	0.065799	0.060020	0.2723304	0.196092	0.076051	0.097300
315	0.304	0.336	1.571	0.901	0.376	0.512	0.0839	0.025505	0.028190	0.1318069	0.075593	0.031546	0.0429568
320	0.268	0.341	1.635	0.825	0.348	0.515	0.018	0.004824	0.006138	0.02943	0.01485	0.006264	0.00927
								0.299535	0.235826	1.0181212	0.9632810	0.334425	0.413257
							SPF	2.995350	2.358267	**10.181212**	**9.632816**	3.344255	4.1325708

3.5 MICROBIAL CONTAMINATION TESTING

3.5.1 Microbial Contamination Testing of 07 ADHPs

In the perspective of Bangladesh, where many people rely upon the herbal medicines for medication, the assessment of pathogens in these medicines is urgently required. Based on this urge, identification and quantification of the pathogenic microorganisms in the ADHPs randomly collected from local markets were performed.

After overnight incubation, the colony count was made from each plate and average was made for each drug. In case of no growth the culture plate was incubated for up to 48 hours. If there is no growth after 48 hours the culture is taken as "no growth". The result is given in Table no. 3.5.1. It was noted that "no growth" of any bacteria was found in one drug and no growth of any fungi was found in all drugs. In one drug, Enterococcus (*Streptococcus faecalis*) was found and in others *bacillus subtilis* was identified.

Table-3.5.1. Selected bacteria identified in the antidiabetic herbal preparations (ADHPs)

Name of Drug	Name of Bacteria isolated	Colony count	Name of fungus isolated
ADHP – 1	*Bacillus subtillis*	3.5×10^4/g	N/G
ADHP–2	Enterococcus (*streptococcus faecalis*)	1.0×10^4/ml	N/G
ADHP–3	N/G	00	N/G
ADHP–4	*Bacillus subtillis*	3.7×10^4/g	N/G
ADHP–5	*Bacillus subtillis*	3.2×10^4/g	N/G
ADHP–6	*Bacillus subtillis*	3.8×10^4/g	N/G
ADHP–7	*Bacillus subtillis*	4.0×10^4/g	N/G
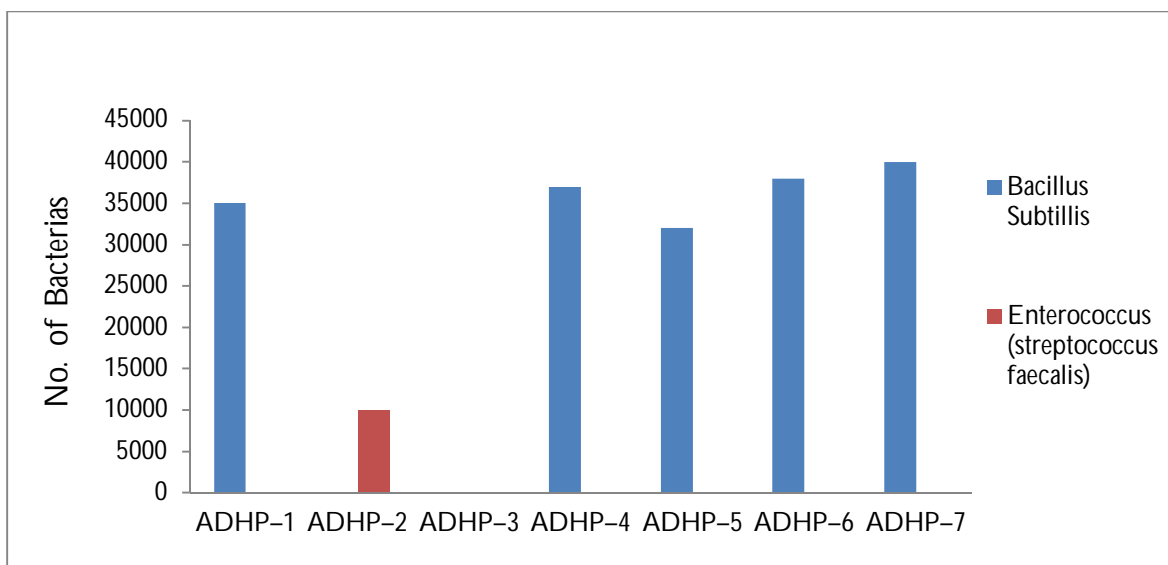
Figure-3.5.1. Average bacterial count of 07 ADHPs

In the present study 7 ADHPs were studied of which one showed no growth of any bacteria or fungi. The organisms found in our study were *Bacillus subtilis* in (71.43% of total samples) 5 ADHPs and Enterococcus in one (14.29% of total samples) and were free from *Salmonella, Shigella, Escherichia coli,* other coliforms and fungi (yeast and mould). In one study (Abba *et. al.,* 2009), organisms were identified in all 150 herbal preparations. *Salmonella typhi* was found in 70 (46.67% of total samples), *Shigella sp*. in 29 (19.33% of total samples), *Escherichia coli* in 88 (58.67% of total samples), and *Staphylococcus aurues* in 98 (65.33% of total samples). However, Noor *et. al.*, (2013) in their study found coliform in one (1.18% of total samples), fungus in10 (11.76% of total samples) but no *Salmonella* or *Shigella sp.* in any sample. Like the present study, Shah and Pokhrel (2012) found predominantly *Bacillus subtilis* in herbal medicine samples.

Bacterial load the present study was *Bacillus subtilis* $3.5 - 4 \times 10^4$ cfu/g in solid ADHPs and *Enterococcus sp.* 1×10^4 cfu/mL in liquid ADHP. However, the counts were very much within the standard limit of microbial contamination according to British Pharmacopeia (2004) where the limits of microbial contamination are given as: total aerobic bacteria 10^5 cfu/g or mL, yeasts and moulds 10^4 cfu/g or mL, *Enterobacteriaceae* and other gram negative organism and *E. coli* and *Salmonella* should be absent (British Pharmacopoeia Commission, 2004) at 10^3 cfu/g.

3.6 INSULIN SECRETORY ACTIVITY

3.6.1 Insulin secretory activity of compounds 1 and 4 of *Phlogacanthus thyrsiflorus* Nees.

Compounds **1**, **3** and **4** were tested for their cytotoxicity in MIN6 cells using MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) colorimetric assay (Mosmann, 1983). In this assay, compound **1** (IC_{50} >200μM) and **4** (IC_{50} >200μM) showed no toxicity; however, compound **3** showed toxic effect in MIN6 cells. Considering the toxicity data, compounds **1** and **4** were studied further for their insulin secretory activity in MIN6 cells (Fig. 3.6.1). It has been reported that, MIN6 cells secrete insulin in response to glucose and other secretagogues (Thams and Capito, 1999). After 30 min incubation with 2 and 20mM glucose, insulin secretion was 10.1 ± 1.2 and 32.4 ± 2.0 ng/5×10^5 cells, respectively at basal (2mM) and stimulatory (20mM) glucose concentration. The value are in good agreement with reported data (Le *et. al.*, 1997). At 2mM glucose, compound **1** and **4** has little to no effect on insulin secretion (data not shown). However, at 20mM glucose, both compound **1** and **4** stimulated insulin secretion from MIN6 cells. At 20mM glucose, the dose 50μM of compound **1** has little effect on stimulation; however, significant (P <0.05) stimulation of insulin secretion was observed in 100μM dose (56.4 ± 5.3 ng/5×10^5 cells) compared to the insulin secretion by 20mM glucose alone. The dose 200μM more efficiently (P <0.001) stimulated insulin secretion compared to the dose 100μM. No more insulin secretion was observed above the 200μM dose. Similar effect was noted for the compound **4**. Compound **4** stimulated insulin secretion from MIN6 cells in a dose-dependent manner and the dose 50μM significantly (P <0.05) stimulated insulin secretion (53.6 ± 3.3 ng/5×10^5 cells) at 20mM glucose. More potent (P <0.01) insulin secretion was observed at 100μM dose and maximum (P <0.001) stimulation was found at 200μM dose (105.7 ± 8.2 ng/5×10^5 cells). No further increase in insulin secretion was observed at 400μM dose. MIN6 cells also responded well to the non glucose secretagogue L-arginine (50mM), used as a positive control. The insulin secretion by L-arginine was 115.5 ± 6.2 ng/5×10^5 cells, a 3.5-fold increase compared to 20mM glucose alone. Compound **1** (β- sitosterol) and compound **4** (19-Hydroxy-8 (17),

13-labda dien-15, 16-olide) may be considered for drug development as insulin secretagogue.
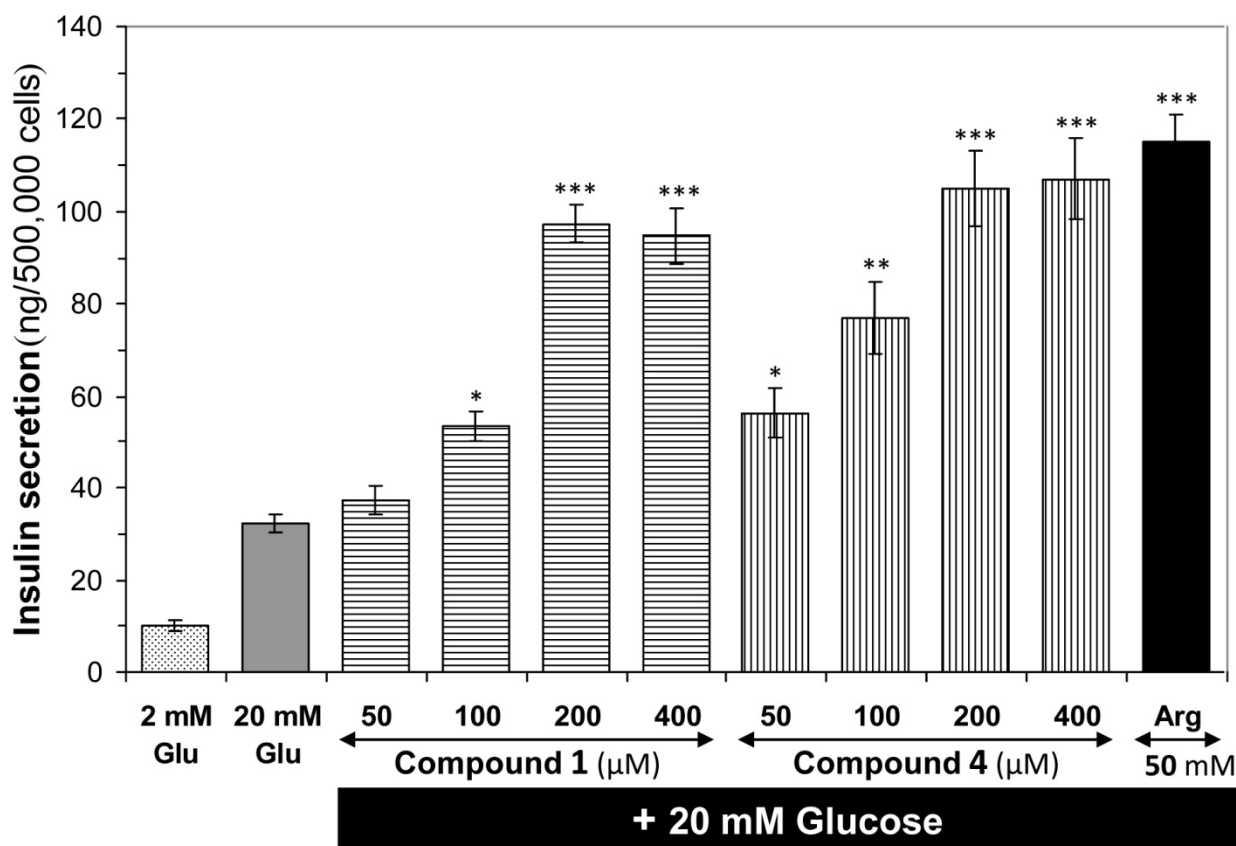
Figure Legend

Fig. 3.6.1: Effect of compounds 1 and 4 on glucose-stimulated insulin secretion from MIN6 cells. MIN6 cells (5×10^5) were incubated at 37 ºC for 30 min in KRB buffer containing 2 mM or 20 mM glucose in the absence or presence of test compound. Values are mean ± SEM from 5 independent experiments. *p <0.05, **p <0.01, ***p <0.001, compared with the value for 20 mM glucose alone. Glu, glucose; Arg, L-arginine.

B. CHEMICAL ANALYSIS

3.7 ISOLATION OF COMPOUNDS FROM TWO PLANTS

3.7.1 General Information of Antidiabetic Single Plants (ADSPs)

Some herbal preparations which have been being used for the management of diabetes in the local market in Bangladesh were selected for the present studies for evaluating their safety and efficacy. Seven ADHPs were found to possess reasonable antidiabetic activity among the 11 ADHPs studied with acute feeding of the drugs. In the chronic studies by feeding for 28 days on T2DM rats ADHP-5 showed good activity just outside the significant level and ADHP-6 showed significant blood glucose lowering activity. From the label of ADHP-5, it was seen that the drug contained only one plant material which was *Salvia haematodes* Linn roots. Therefore, *S haematodes* roots were studied for its chemical constituents. It was seen that *Gymnema sylvestre* leaf was one of the plant material of ADHP-6 from the label of the finish product. But extensive researches were performed on this plant material, so this plant was not selected for the present study. *S haematodes* known as red sage was found to possess significant CNS depressant (anticonvulsant) properties (Akbar *et al.,* 1985). It also showed anti-inflammatory and analgesic effects, hypothermic response in non-pyretic rats and enhancement of the wound healing process (Akbar, 1989).

Flower of another plant *Phlogacanthus thyrsiflorus* Nees are used by the local people in Assam, India for the management of diabetes (Chakravarty *et. al.,* 2014; Ningombam & Singh, 2014). The flower of the plant is also eaten as vegetable by the ethnic communities of Tinsukia district of Assam, which is said to be useful in rheumatism, anaemia and cough. (Buragohain *et. al.,* 2011). The flowers were obtained through a research fellow and was studied both for its biological properties and chemical constituents.

Three compounds from the root extracts of *S haematodes* and five compounds from the flower extracts of *P thyrsiflorus* were isolated. All the compounds of *P thyrsiflorus* were

isolated for the first time from this plant's flower. Characterization of the compounds are discussed below.

3.7.2 Compounds from *Salvia haematodes* Linn roots

3.7.2.1 Compound 1

Compound **1** was isolated from the DCM extract of the roots of *S haematodes* as needle shaped crystals. It gave single spot on TLC. The m.p. of the compound was 137-38^{0}C. The 1-olefinic proton resonance at δ 5.35 and 1-proton connected to hydroxyl resonance at δ 3.52. The ^{13}C-NMR (Table. 3.7.1 and Figure 3.7.1 & 3.7.2) spectrum of the crystals in CDCl$_3$ showed 29 carbons including an oxymethine carbon signal at 71.8 (C-3) and two olefinic carbons at δ 140.80 & δ 121.74 (C-6). The double bonded unsaturation at δ 140.80 & 121.74 was characteristics of spirostene (Agrawal, *et. al.,* 1985). The DEPT-135 experiment confirmed that this compound was having six methyl groups at δ 11.88 (C-18), 19.41 (C-19), 19.07 (C-21), 19.83 (C-26), 21.12 (C-27) and 12.01 (C-29), eleven methylene (C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-22, C-23 & C-28), nine methane (C-3, C-6, C-8, C-9, C-14, C-17, C-20, C-24 & C-25) and three quaternary (C-5, C-10 & C-13) carbons. The ^{1}H-& ^{13}C-NMR spectral data of compound **1** are similar to the reported data for β-sitosterol (Morales *et. al.,* 2003 & Motloubi *et. al.,* 2007). Therefore, the structure of compound **1** was characterized as β-sitosterol (Fig 3.7.3). β-Sitosterol is a very common steroidal compound present in almost all kinds of plants.

Table-3.7.1: ¹H and ¹³C NMR spectral data for compound 1 (400 MHz, δ Value in CDCl₃)

Position	¹³C (δ ppm)	¹H (δ ppm)
1	37.3 t	-
2	31.7 t	-
3	71.8 d	3.52 (1H, m)
4	42.3 t	-
5	140.8 s	-
6	121.7 d	5.35 (1H, brs)
7	31.9 t	-
8	31.9 d	-
9	51.2 d	-
10	36.5 s	-
11	21.1 t	-
12	39.8 t	-
13	42.2 s	-
14	56.8 d	-
15	24.3 t	-
16	28.2 t	-
17	56.1 d	-
18	12.0 q	0.68 (3H,s)
19	19.4 q	1.01(3H,s)
20	36.1 d	-
21	18.8 q	0.92 (3H, d, 6.4)
22	34.0 t	-
23	26.1 t	-
24	45.9 d	-
25	29.2 d	-
26	19.0 q	0.81 (3H, d, 6.5)
27	19.0 q	0.83 (3H, d, 6.5)
28	29.1 d	-
29	12.2 q	0.85 (3H, t, 7.5)

Coupling constant *J*, are in Hz

Figure-3.7.1: ¹H-NMR of Compound 1

Figure-3.7.2: ^{13}C-NMR of Compound 1
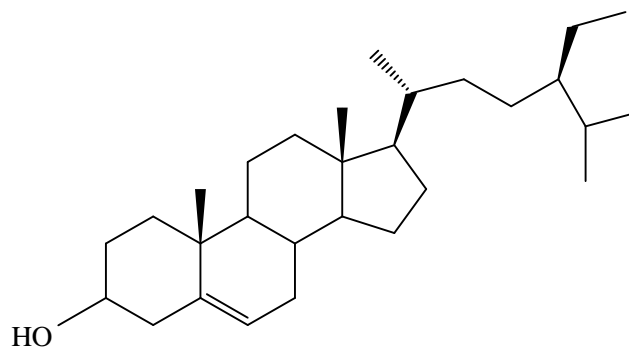
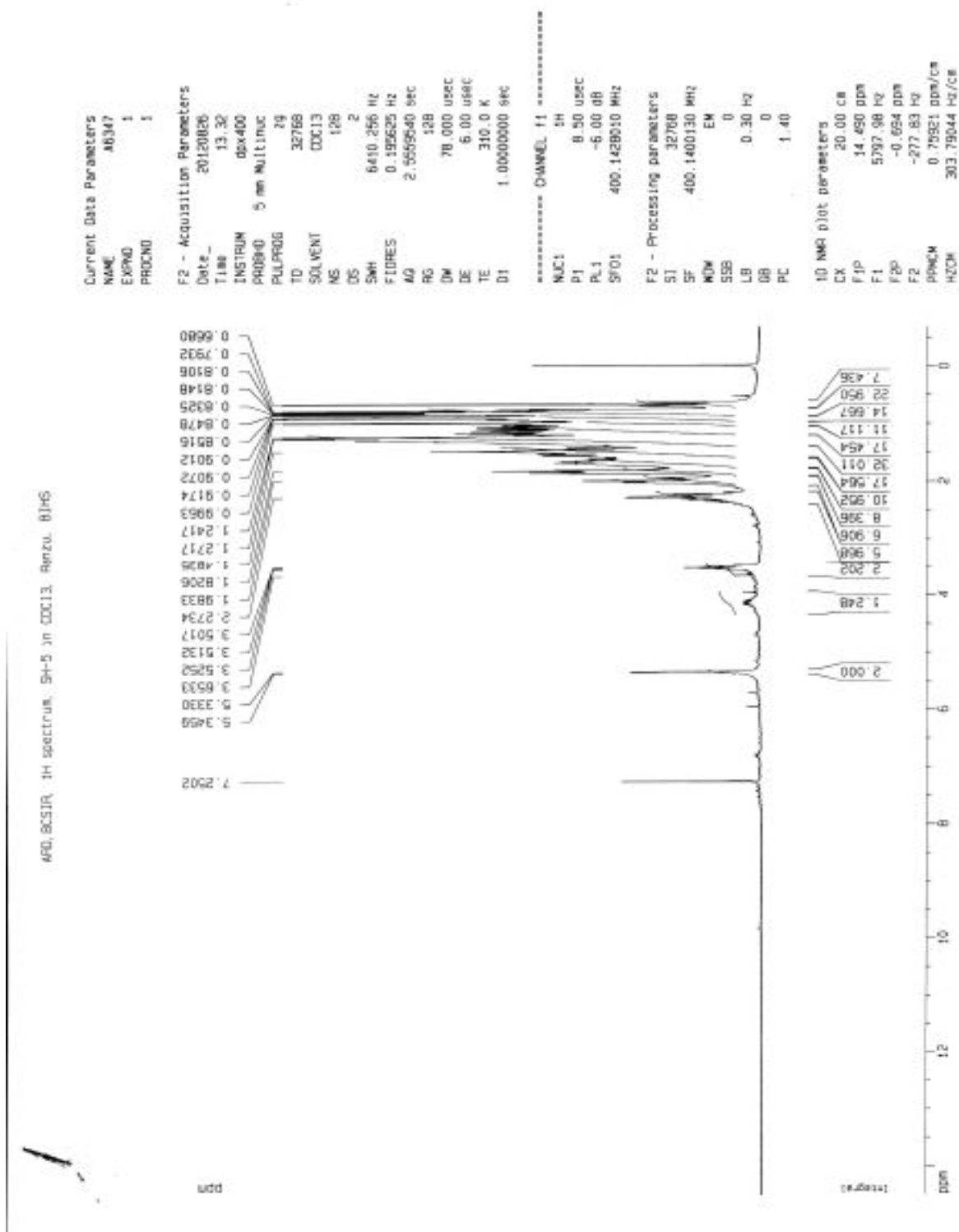*Figure-3.7.3: Structure of Compound 1 (β- sitosterol)*

3.7.2.2 Compound 2

Compound **2** was also isolated from the DCM extract of the roots of *Salvia haematodes* Linn as needle shaped crystals. It gave single spot on TLC. The m.p. of the compound was 162^0C. The crystal of this compound was soluble in CHCl$_3$. It showed purple colored single spot on TLC plate when visualized with anisaldehyde/sulphuric acid spray reagent with R_f value 0.55 (n-HX:EtOAc). The ^{1}H-NMR spectrum of compound **2** (Figure 3.7.4 and Table 3.7.2) revealed multiplet signal for an oxymethine proton at δ_H 3.52. The olefinic proton resonance at δ 5.35 was charecterististics of 5-steroids (Agrawal *et. al.,* 1985). The spectrum revealed signal at δ 0.68 and 1.01 (3H each) assignable to two tertiary methyl groups at C-18 C-19, respectively. The signals of two further secondary methyl groups at δ 0.84 (*J*=7.3 Hz) and δ 0.83 (*J*=7.3 Hz) could be attributed to two methyl groups at C-26 and C-27, respectively. The doublet at δ 0.92 (d, *J*=8 Hz, H-21) was demonstrative of a methyl group at C-21. On the other hand, the triplet of three proton intensity at δ 0.81 could be assigned to the primary group at C-29. The ^{1}H-, ^{13}C- and DEPT-NMR spectra (Fig. 3.7.4 & 3.7.5) of compound **2** were similar to the reported data of stigmasterol (Kamboj and Saluja, 2010). Stigmasterol is a very common steroidal compound present in many plants but this compound was isolated from this plant for the first time according to the literature search.

Table-3.7.2: ^{1}H-and ^{13}C-NMR spectral data for compound 2 (500 MHz, δ Value in CDCl$_3$)

Position	^{13} C (δ ppm)	^{1}H (δ ppm)
1	37.3 t	
2	31.7 t	
3	71.8 d	3.52 (1H, m)
4	42.3 t	
5	140.8 s	
6	121.7 d	5.35 (1H, brs)
7	31.9 t	
8	31.9 d	
9	51.2 d	
10	36.5 s	
11	21.1 t	
12	39.7t	
13	42.2 s	
14	56.8 d	
15	24.3 t	
16	28.2 t	
17	56.1 d	
18	11.8 q	0.70 (3H,s)
19	19.8 q	1.01 (3H,s)
20	40.4 t	
21	21.2 q	1.02 (3H, d, J=7.5 Hz)
22	138.3 s	5.14 (dd, J=8.4 Hz, J=8.8 Hz)
23	129.3 d	5.02 (dd, J=8.4 Hz, J=8.4 Hz)
24	50.2 d	-
25	31.7 d	-
26	21.0 q	0.79 (3H, d. J=6.5 Hz)
27	19.0 q	0.85 (3H, d, J=6.5 Hz)
28	25.4 d	-
29	12.0 q	0.80 (3H, t, J=7.5 Hz)

Coupling constant *J*, are in Hz
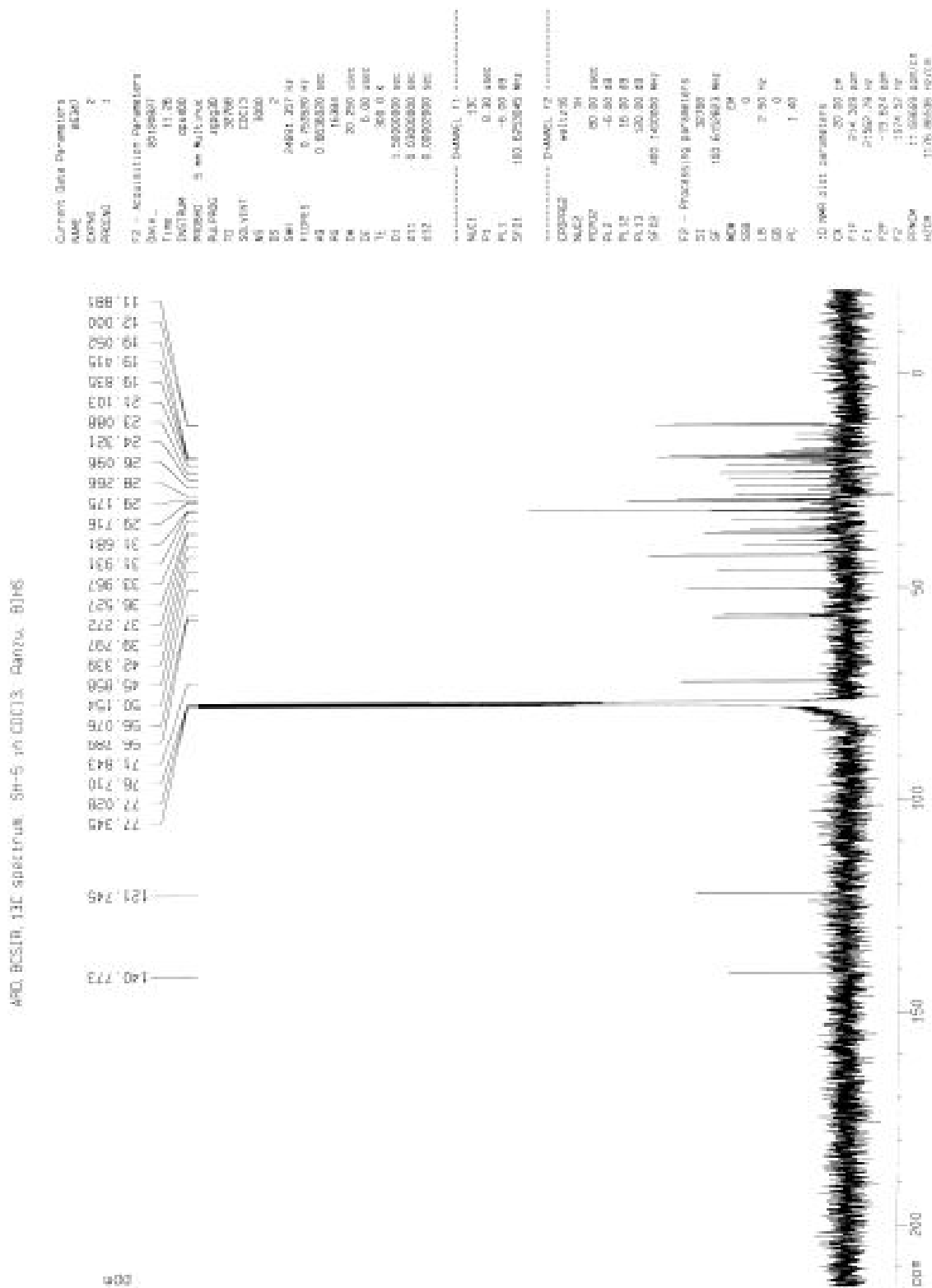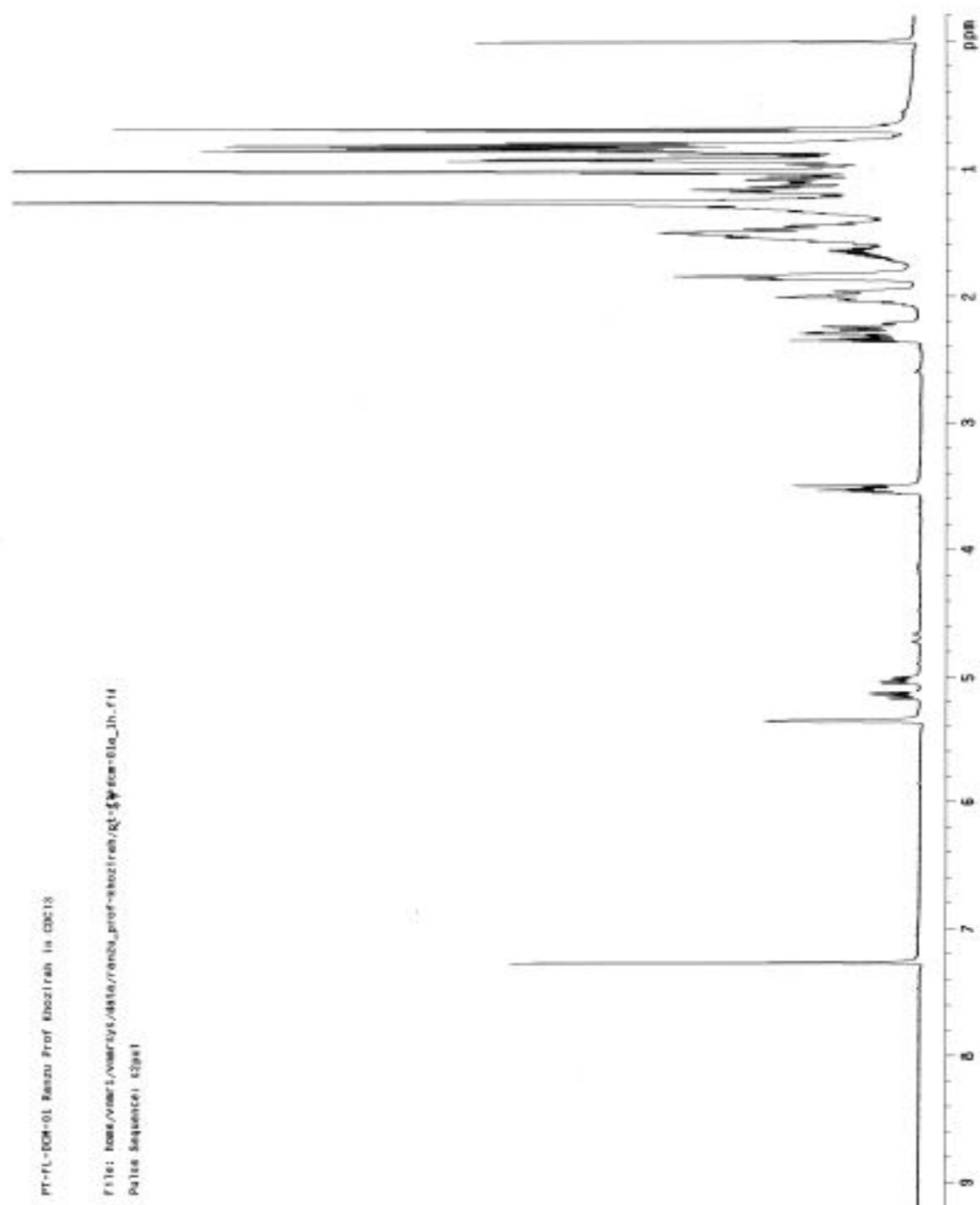
Figure-3.7.4: ¹H-NMR of Compound 2
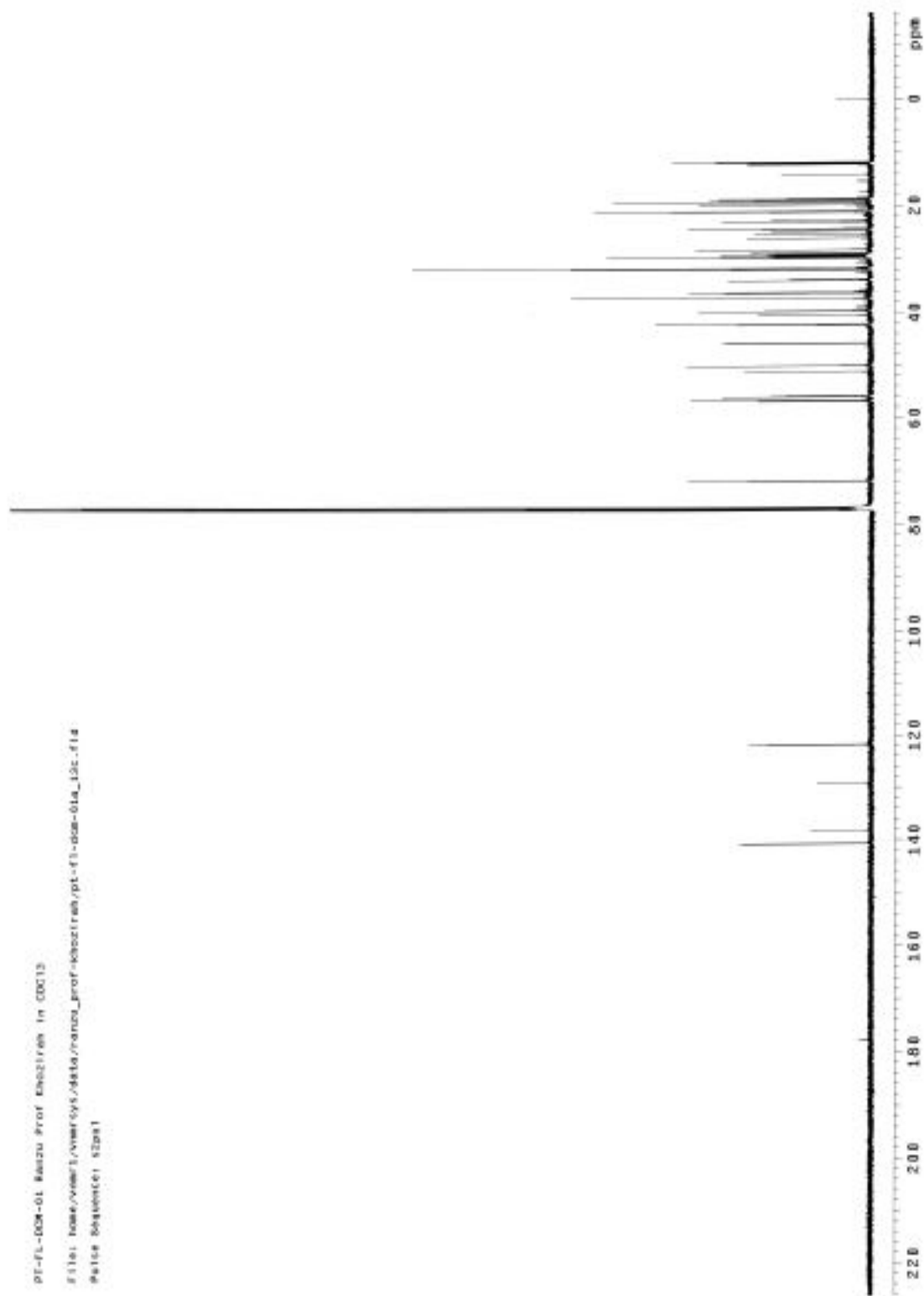
Figure: 3.7.5 ^{13}C-NMR of Compound 2
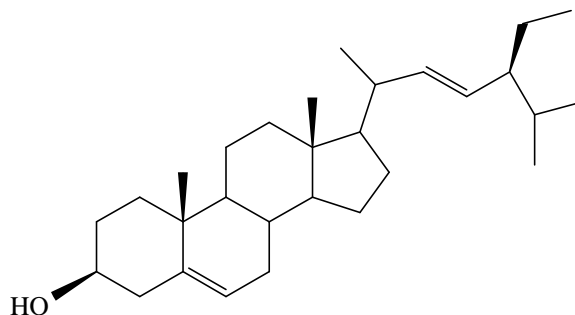
Figure - 3.7.6: Structure of Compound 2 (Stigmasterol)

3.7.2.3 Compound 3

Compound **3** was isolated from the chloroform fraction of EtOAc extract of the roots of *Salvia haematodes* Linn as yellowish white crystalline powder. It gave single spot on TLC under UV light at 365 nm observed as blue color (R_f = 0.59). Melting point of the compound was 230^0C and molar mass was 162 g/mol (Figure. 3.7.8), IR (KBr,m max): 3,423 (–OH), 1,733(–CO-), 1,555 (–C=C–) cm-1. MS: m/z(M); 163.15. ^{1}H-NMR (400 MHz, CDCl$_3$) had signals at δ d 2.49 (s, 3H, C4–CH$_3$), 6.31 (s, 1H, C3–H), 6.92 (d, 1H, C6–H, J = 9.0 Hz), 6.94 (s,1H, C8–H) and 7.57 (d, 1H, C5–H, J = 9.0 Hz) (Fig. 3.7.7). From the mass and NMR spectral data the molecular formula of compound **3** was given at C$_9$H$_6$O$_3$. The spectral data corresponded very well with the reported data of 7-hydroxy coumarin (Lari *et. al.,* 2009). Therefore, compound **3** was characterized as 7-hydroxy coumarin. This compound is isolated for the first time from *S haematodes*.
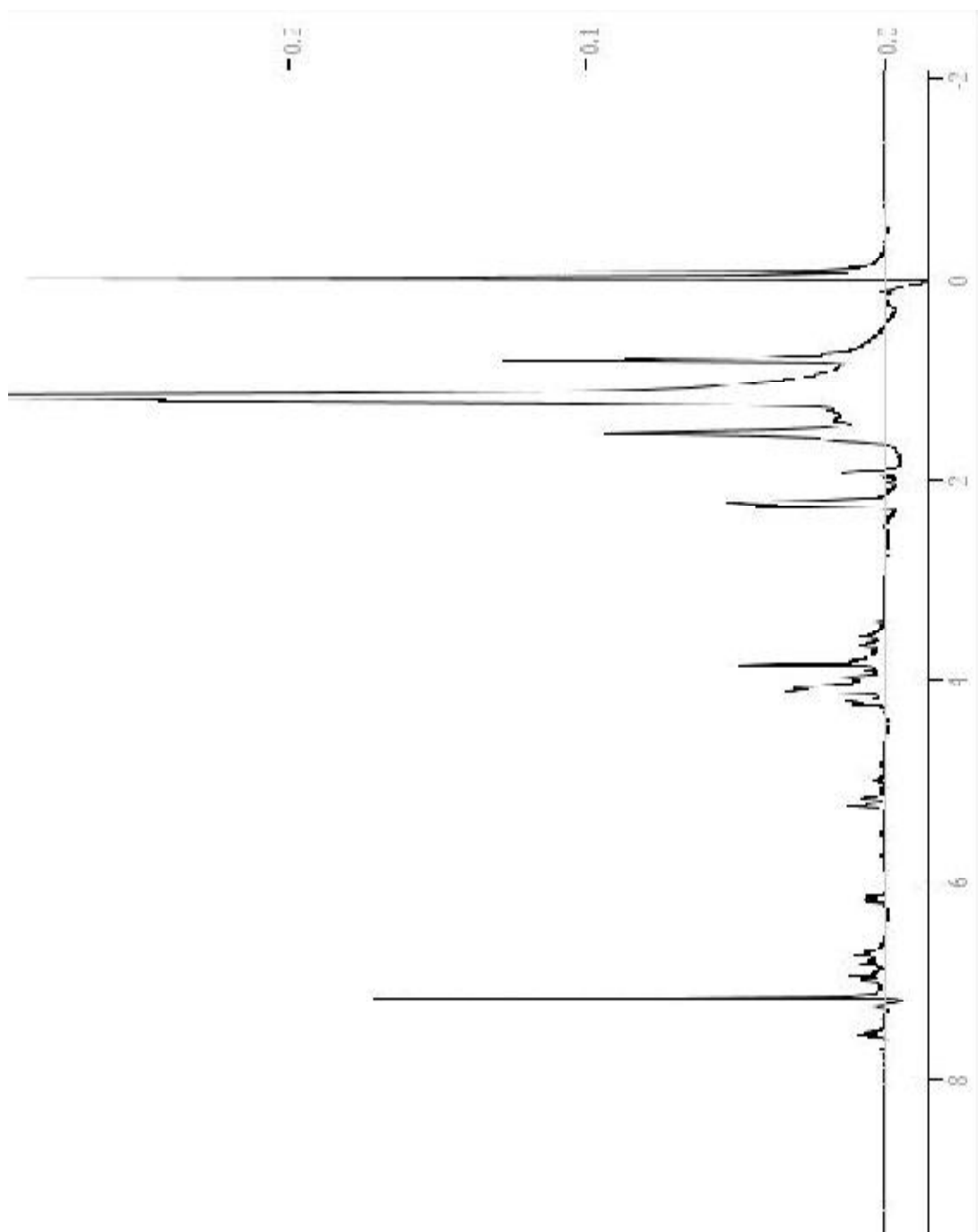
Figure-3.7.7: ^{1}H-NMR of Compound 3
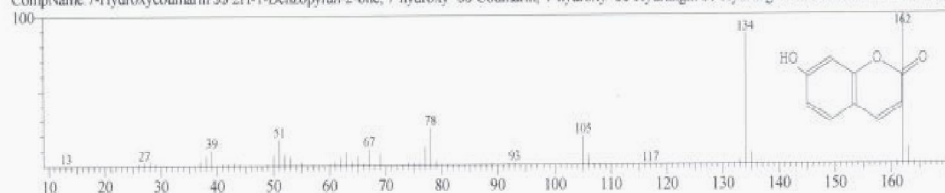
Figure- 3.7.8: GC MS spectra and value of Compound 3
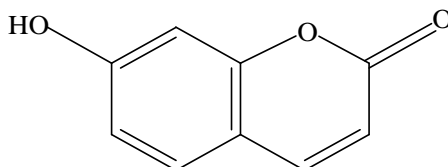
Figure- 3.7.9: Structure of Compound 3

3.7.3 Compound from *Phlogacanthus thyrsiflorus* Nees

3.7.3.1 Compound 4

Compound **4** was isolated from the DCM extract of the flower of *P thyrsiflorus* flower as needle shaped crystals. It gave single spot on TLC. The m.p. of the compound was 137-38^{0}C. The 1-olefinic proton resonated at δ 5.35 and 1-proton connected to hydroxyl resonated at δ 3.52. The ^{13}C-NMR (Table-3.7.1 and Figure 3.7.2) of compound **4** showed 29 carbons including an oxymethine carbon signal at 71.8 (C-3) and two olefinic carbons at δ 140.80 & δ 121.74 (C-6). The double bonded unsaturation at δ 140.80 & 121.74 was characteristics of spirostene (Agrawal *et. al.,* 1985).

The DEPT-135 experiment confirmed that this compound was having six methyl group at δ 12.0 (C-18), 19.41 (C-19.4), 18.8 (C-21), 19.0 (C-26), 19.0 (C-27) and 12.2 (C-29), eleven methylene (C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-22, C-23 & C-28), nine methyne (C-3, C-6, C-8, C-9, C-14, C-17, C-20, C-24 & C-25) and three quaternary (C-5, C-10 & C-13) carbons. It is evident from ^{1}H-& ^{13}C-NMR spectral data that compound **4** is similar to the reported data for β-sitosterol (Kamboj and Saluja, 2010). Therefore, the structure of compound **4** is characterized as β-sitosterol (Figure 3.7.3). From the best of my knowledge this compound was isolated first time from the flower of this plant.

3.7.3.2 Compound 5

Compound **5** was also isolated from the DCM extract of the flower of *P thyrsiflorus* flower as needle shaped crystals. It gave single spot on TLC. The m.p. of the compound was 162.5^{0}C. The crystal of this compound was soluble in CHCl$_3$. It showed purple colored single spot on TLC plate when visualized sulphuric acid spray reagent with R$_f$ value 0.56 (n-HX:EtOAc). The ^{1}H-NMR spectrum of compound **5** (Figure: 3.7.4) revealed multiplet signal for an oxymethine proton at δ$_H$ 3.52. The olefinic proton resonated at δ 5.35 was charecterististics of 5-steroids (Agrawal *et. al.,* 1985). The spectrum revealed signal at δ 0.68 and 1.01 (3H each) assignable to two tertiary methyl groups at C-18 C-19, respectively. The signals of two further secondary methyl groups δ 0.84 (J=7.3 Hz) and δ 0.83 (j=7.3 Hz) which could be attributed to two methyl groups at C-26 and C-27. The doublet at δ 0.92 (d, J=8 Hz, H-21) was demonstrative of a methyl

group at C-21. On the otherhand, the triplet of three proton intensity at δ 0.81 could be assigned to the primary group at C-29. The ^{1}H-and ^{13}C-NMR (Figure. 3.7.4 & 3.7.5) of compound **5** are similar to the reported data of stigmasterol (Kamboj and Saluja, 2010). Stigmasterol is a very common steroidal compound present in many plants but this compound was isolated from the flower of this plant for the first time according to the literature search.

3.7.3.3 Compounds 6

Compound **6** was isolated from the DCM extract of the flower of *P thyrsiflorus* as long fatty shaped crystals. It gave single spot on TLC with R_f value 0.53 (n-HX:EtOAc 3:7). IR (KBr) v: 2940 (-OH), 1691 (>C=O), 1528, 669 cm^{-1}. In the proton NMR spectrum three CH$_2$ proton appeared at δ 1.63 (m), 1.67(m), 1.78 (m), two H appeared at δ 4.70 (s) and 4.72 (s) for the CH$_2$ of the furan ring. Two olefinic H of 17 carbon appeared at 4.90 (s) and 4.49 (s). Another olefinic H of furan ring came at 5.86(s). In the ^{13}C-NMR spectrum the carboxylic functional group containing carbon appeared at δ 184.8, furan ring containing quarternary carbon at δ 170.9 and cyclic carbonyl carbon at δ 174.2. The ^{1}H- and ^{13}C-NMR spectra (Fig. 3.7.10 & 3.7.11) of compound **6** are similar to the reported data of 8 (17), 13-labda dien-15,16-olide-19-oic acid) (Singh and Singh, 2010). Therefore Compound **6** was characterized as 8 (17), 13-labda dien-15,16-olide-19-oic acid. This compound was first time isolated from the flower of this plant according to the literature survey.

Table-3.7.3: ^{1}H-and ^{13}C-NMR spectral data for compound PT FL DCM-06 (400 MHz, δ Value in CDCL$_3$)

Position	^{13}C (δppm)	^{1}H (δppm)
1	38.0 t	1.78 (m)
2	18.3 t	1.63 (m)
3	37.0 t	1.67 (m)
4	47.4 s	-
5	49.4 d	1.97 (d; J=11.65 Hz)
6	26.8 t	1.53 (dddd/dq, J=4.15, 12.90, 25.65 Hz) 1.41 (d?; J=10.75 Hz)
7	37.7 t	2.40 (d; J=12.75 Hz) 2.08 (ddd/dt; J=4.12, 12.35, 16.80, 24.7 Hz)
8	147.1 d	-
9	56.2 d	1.73
10	39.0 s	-
11	21.2 t	1.82 (m) 1.67 (m)
12	27.5 t	2.29 (m) 2.59 (sept/m; J=3.35, 5.80, 8.65, 14,45 Hz)
13	170.9 s	-
14	115.2 s	5.86 (s)
15	174.2 s	-
16	73.1 t	4.72 (s) 4.70 (s)
17	107.2 t	4.90 (s) 4.49 (s)
18	16.3 q	1.16 (s)
19	184.8 s	-
20	14.7 q	0.74 (s)

Coupling constant J, are in Hz
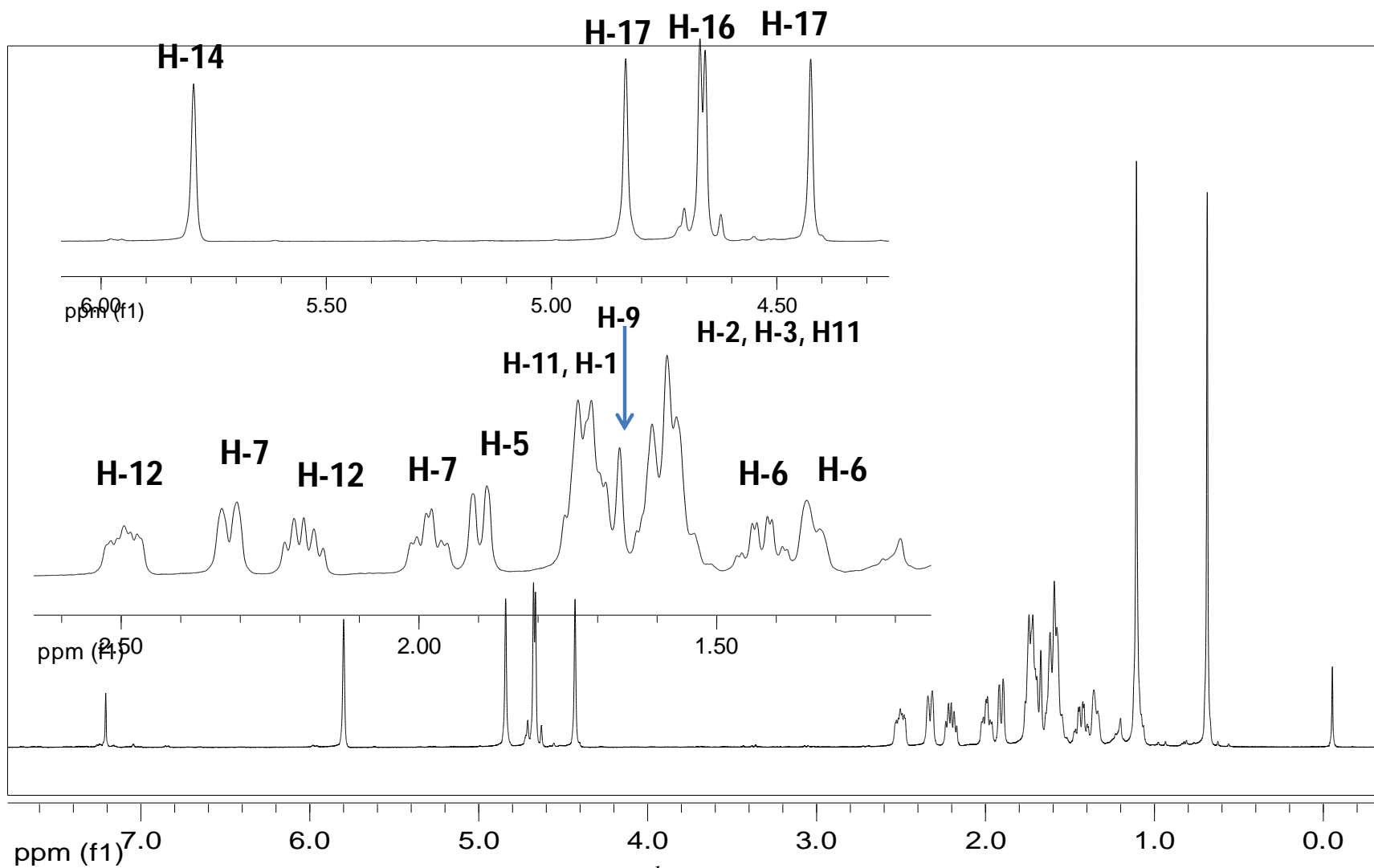
Figure- 3.7.10: ¹H-NMR of Compound 6
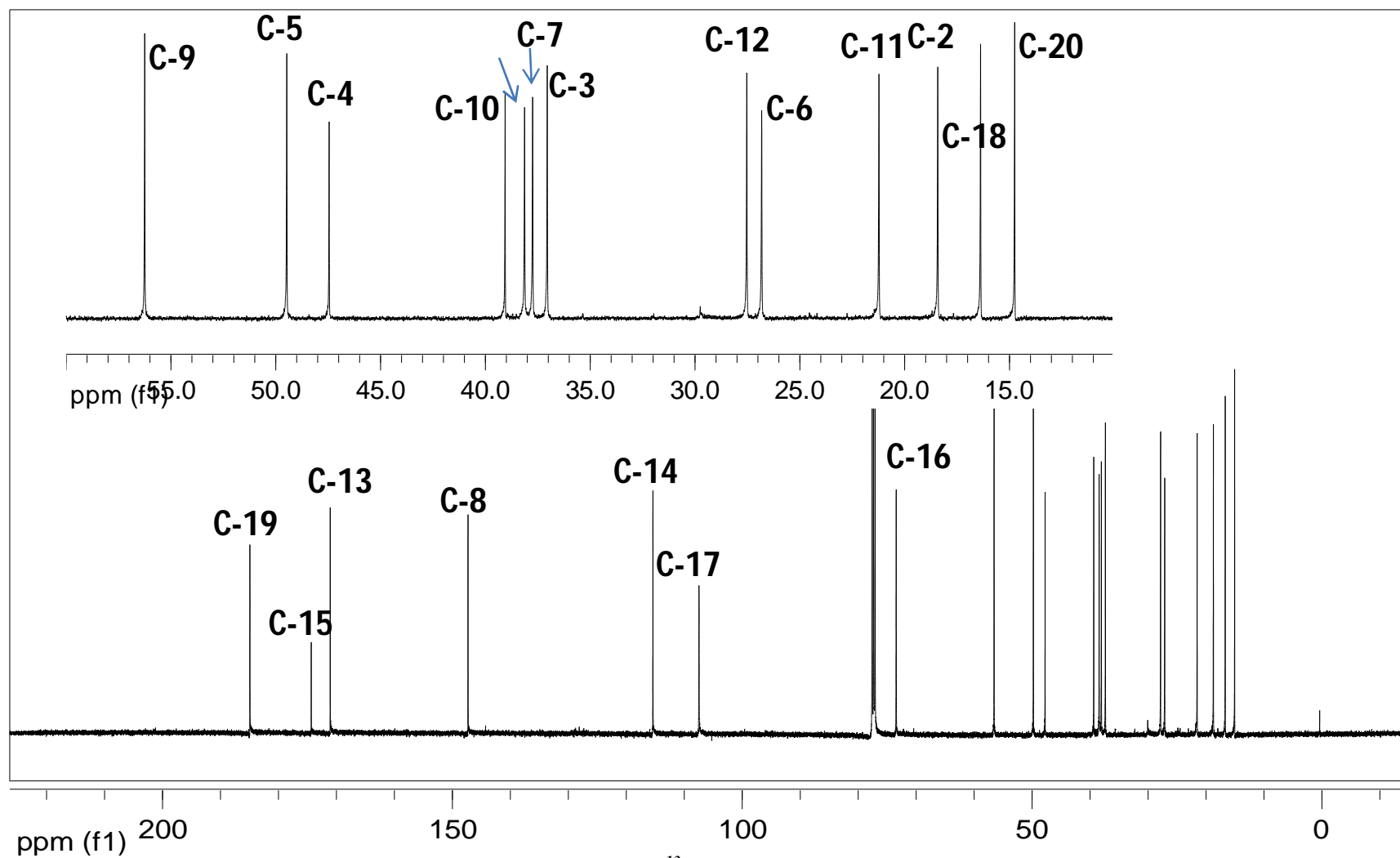
Figure- 3.7.11: ^{13}C-NMR of Compound 6

GCMS
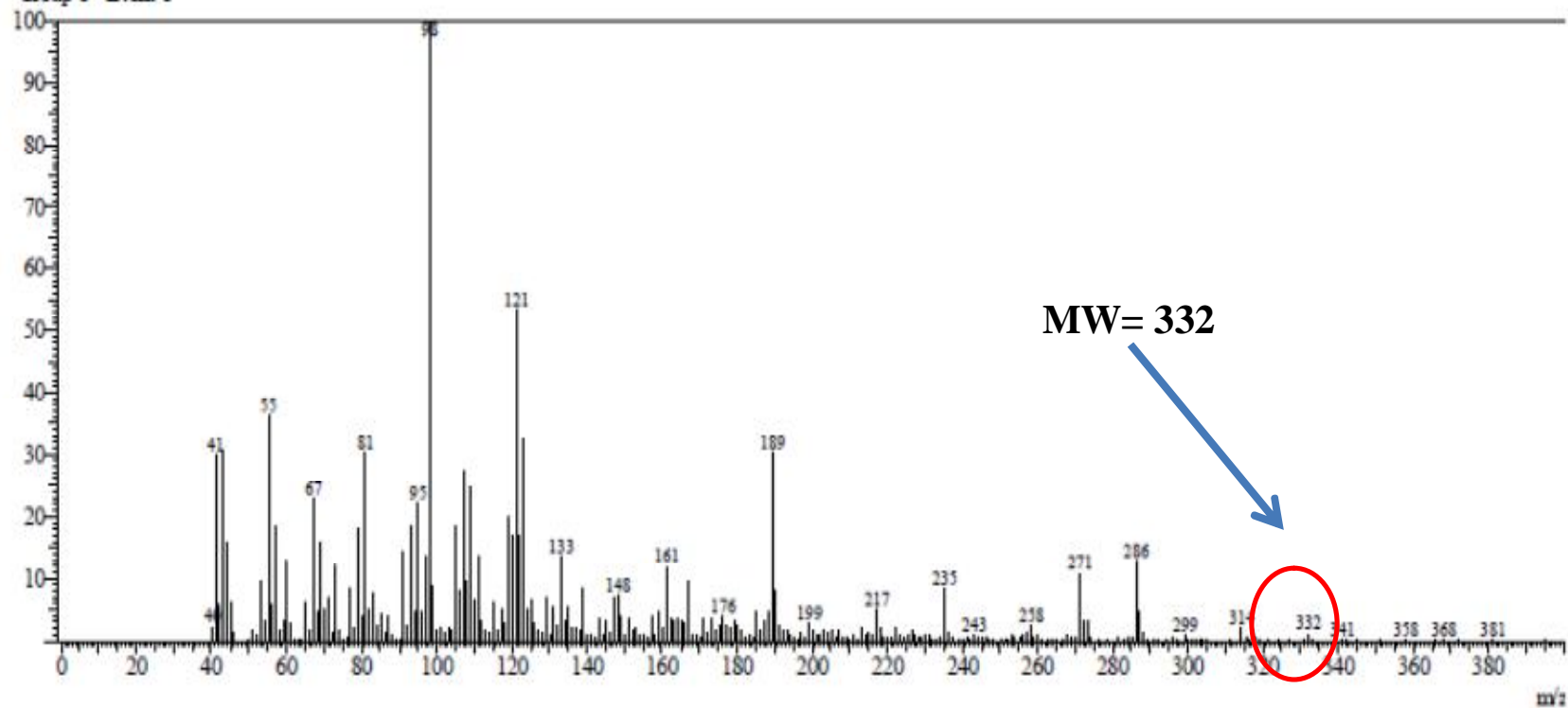
Figure- 3.7.12: Molecular weight of Compound 6
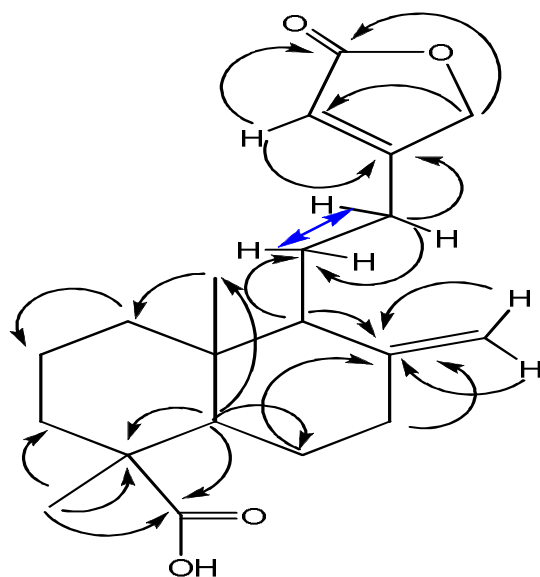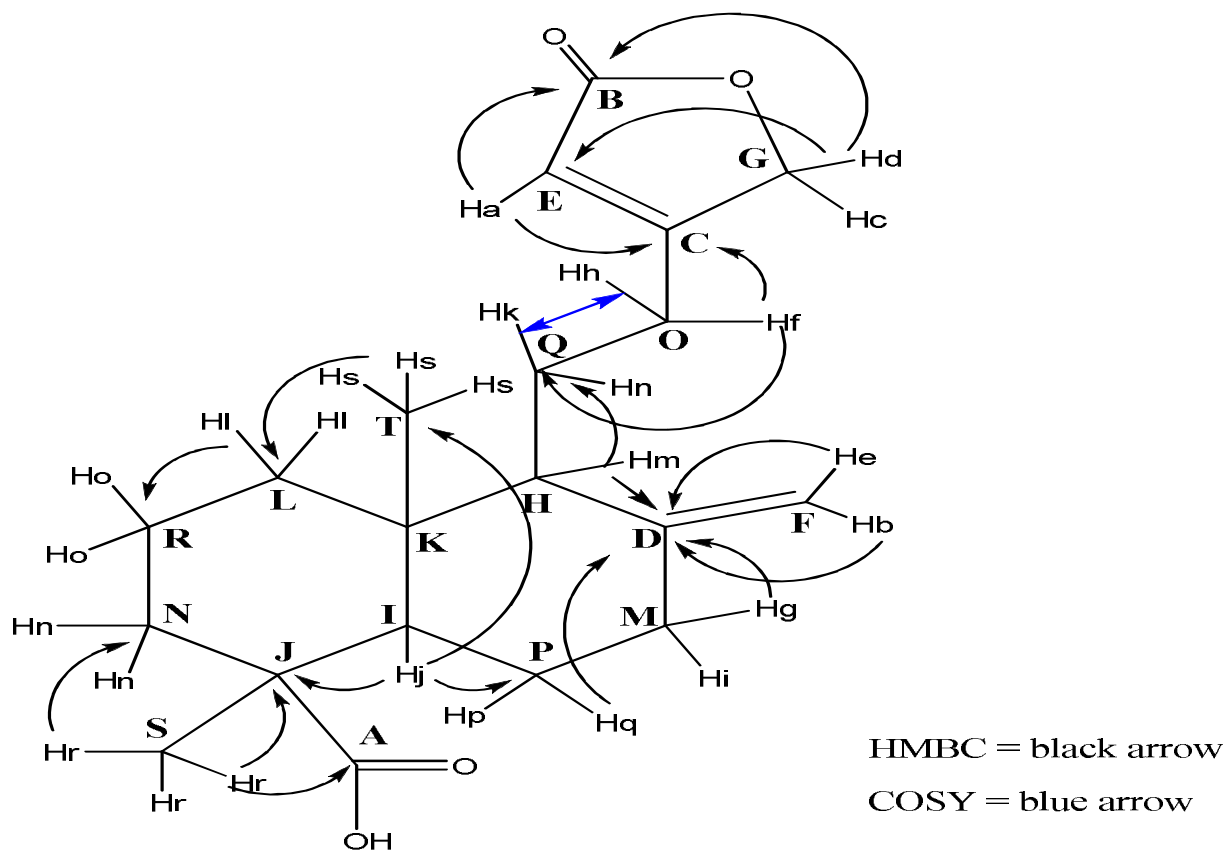
Figure- 3.7.13: HMBC and COSY Correlations of Compound 6
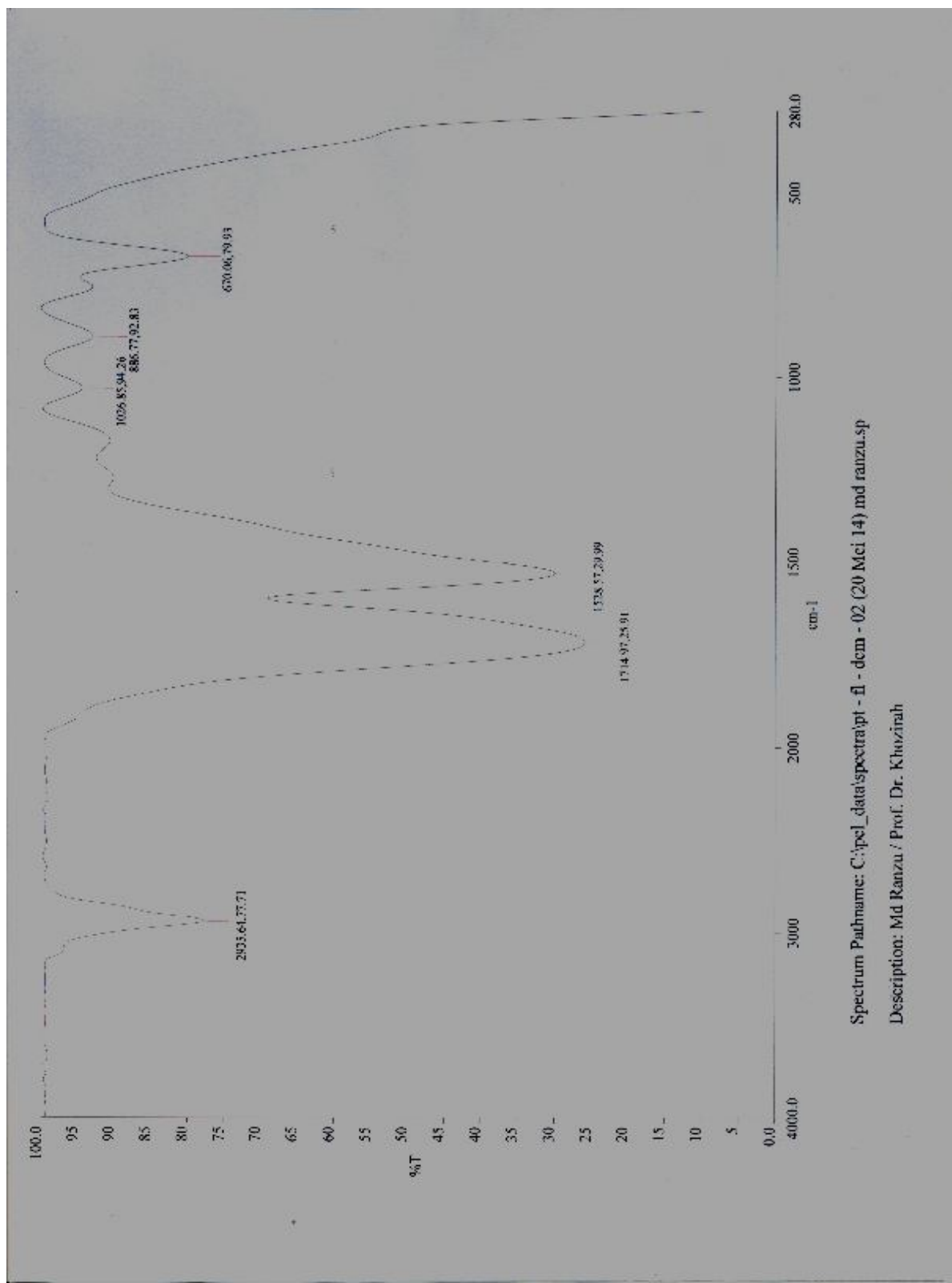
Figure- 3.7.14: FTIR Spectra of Compound 6
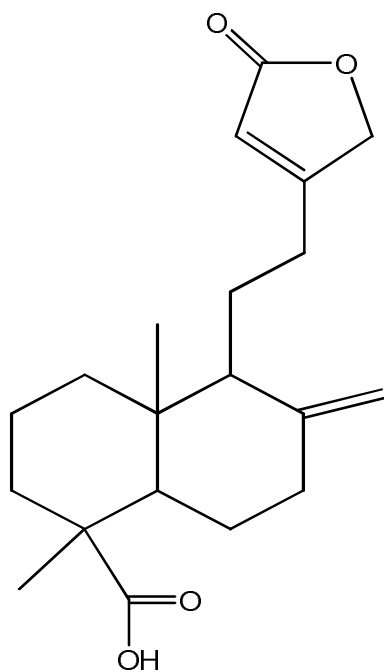
Chemical Formula: $C_{20}H_{28}O_4$
Molecular Weight: 332.43
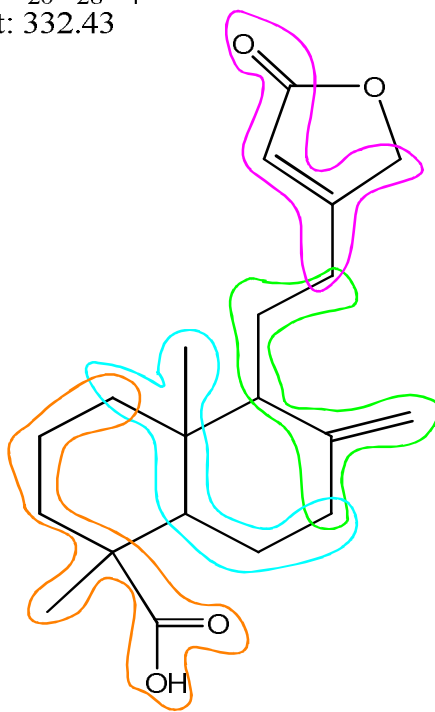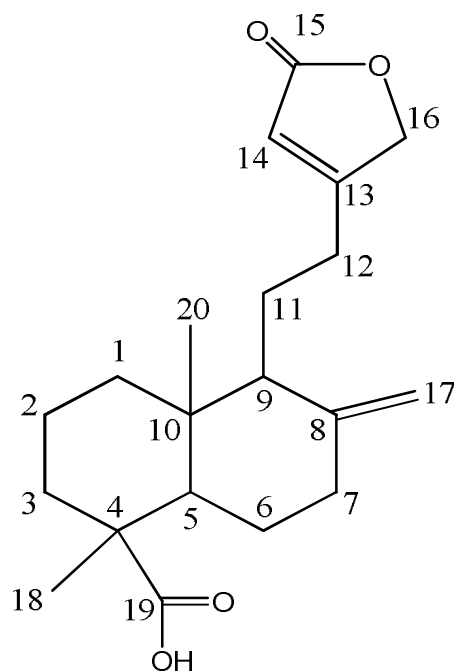
Figure- 3.7.15: Structure of Compound 6   [8 (17), 13-labda dien-15, 16-olide-19-oic acid]

3.7.3.4. Compound 7

Compound **7** was isolated from the DCM extract of the flowers of *P thyrsiflorus* as microcrystal. It gave single spot on TLC with R_f value 0.59. Compound **7** has three CH_2 protons appearing at δ_H 1.65 (m), 1.69 (m) and 1.78 (m). Two H appears at δ_H 4.71 (s) and 4.73 (s) for the CH_2 of furan ring. Two olefinic H of 17 carbon appears at δ_H 4.88 (s) and 4.77 (s). Another olefinic H of furan ring comes at δ_H 5.85 (s). Hydroxyl functional group containing carbon appears at δ_C 174.2, furan ring containing quarternary carbon at δ_C 147.5 and cyclic carbonyl carbon at δ_C 171.0. The ^{1}H-, ^{13}C- (Table 3.7.4), and DEPT-NMR spectra of compound **7** are similar to the reported data of 8(17),13-labdadien-15,16-olide-19-oic acid (Barua, *et. al.,* 1985) except the carboxylic acid functional group reduced to hydroxyl group. The molecular formula $C_{20}H_{30}O_4$ of compound **7** was deduced from GCMS data. Therefore, the structure of compound **7** is characterized as 19-hydroxy-8(17),13-labdadien-15,16-olide. This is first report of 19-hydroxy-8(17),13-labdadien-15,16-olide from the flowers of this plant.

Table-3.7.4: ^{1}H-and ^{13}C-NMR spectral data for compound PT FL DCM-7 (400 MHz, δ Value in CDCL$_3$)

Position	^{13}C (δppm)	^{1}H (δppm)
1	39.2 t	1.78 (m)
2	21.5 t	1.65 (m)
3	35.4 t	1.69 (m)
4	56.3 s	-
5	56.4 s	1.96 (d; J=11.65 Hz)
6	27.2 t	1.53 (dddd/dq, J=4.15, 12.90, 25.65 Hz) 1.40 (d?; J=10.75 Hz)
7	38.6 t	2.40 (d; J=12.75 Hz) 2.24 (ddd/dt; J=4.12, 12.35, 16.80, 24.7 Hz)
8	115.3 s	-
9	65.1 s	1.73
10	39.7 s	-
11	24.5 s	1.82 (m) 1.66 (m)
12	27.5 t	2.29 (m) 2.57 (sept/m; J=3.35, 5.80, 8.65, 14,45 Hz)
13	147.5 s	-
14	115.3 t	5.85 (s)
15	171.0 s	-
16	73.2 d	4.73 (s) 4.71 (s)
17	106.8 t	4.88 (s) 4.77 (s)
18	19.0 q	1.1 (s)
19	174.2 t	-
20	15.3 d	0.69 (s)

Coupling constant J, are in Hz
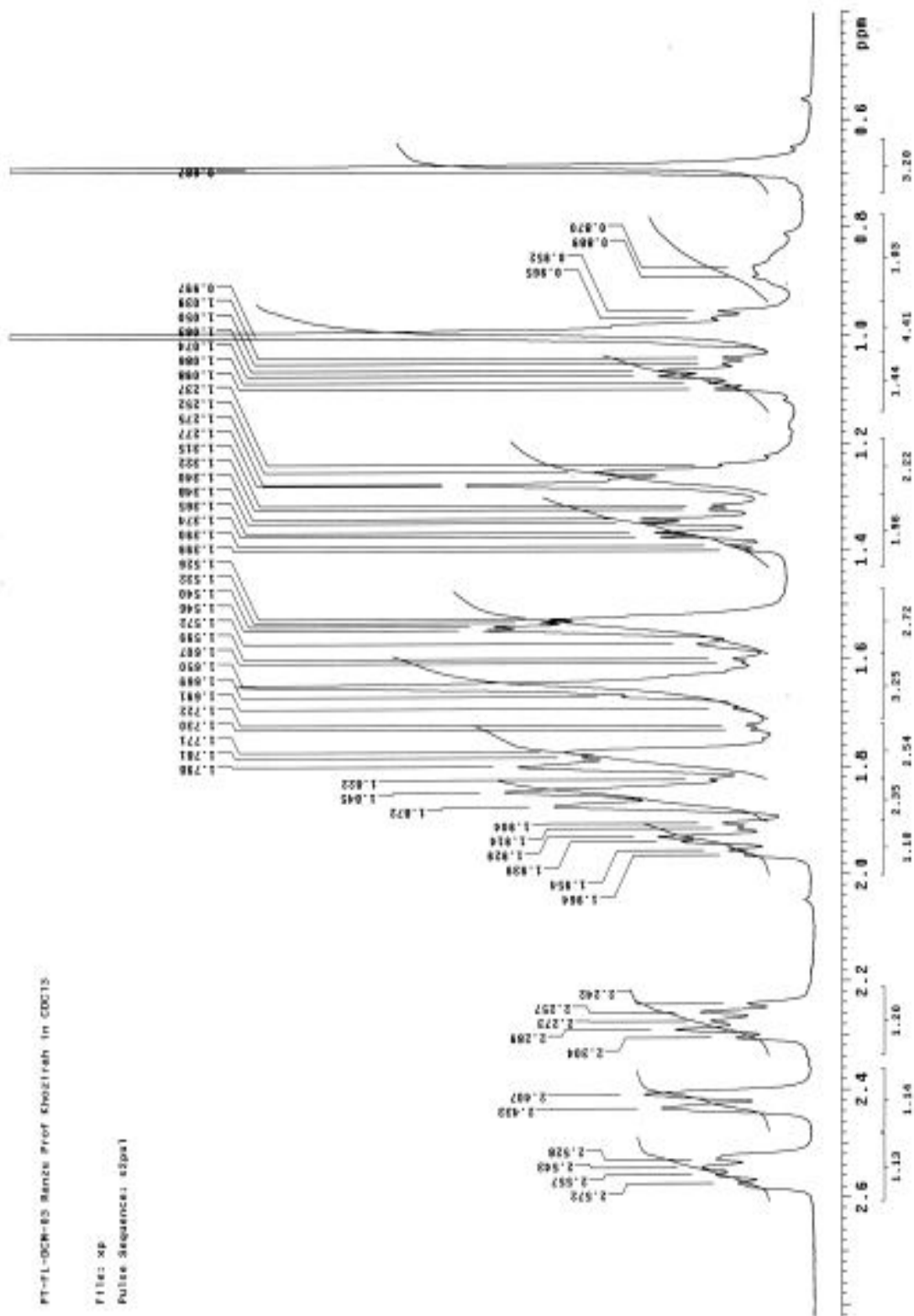
Figure- 3.7.16.1: ¹H-NMR of compound 7
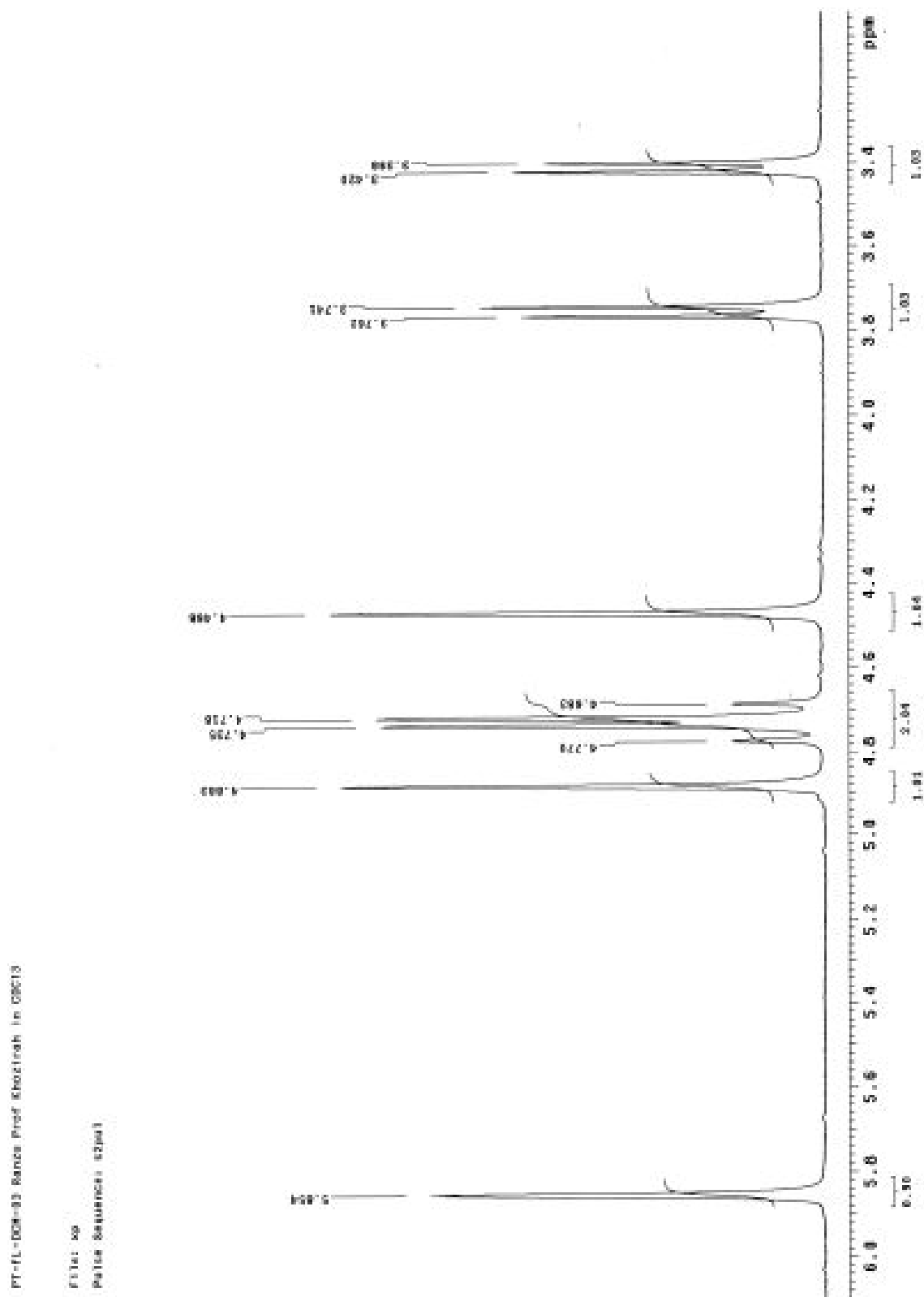
Figure- 3.7.16.2: ¹H-NMR of compound 7
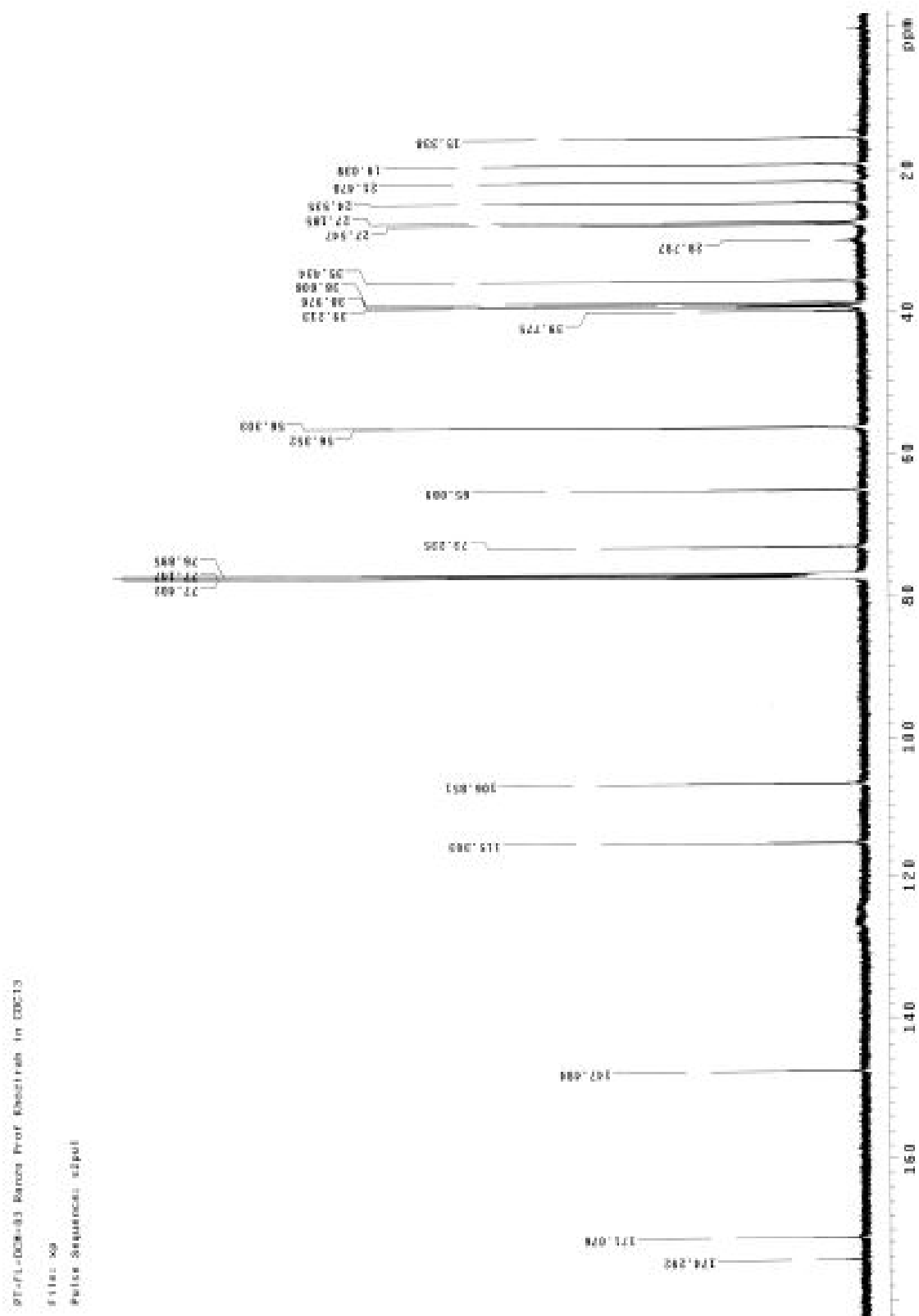
Figure- 3.7.17: ^{13}C-NMR of compound 7

GCMS
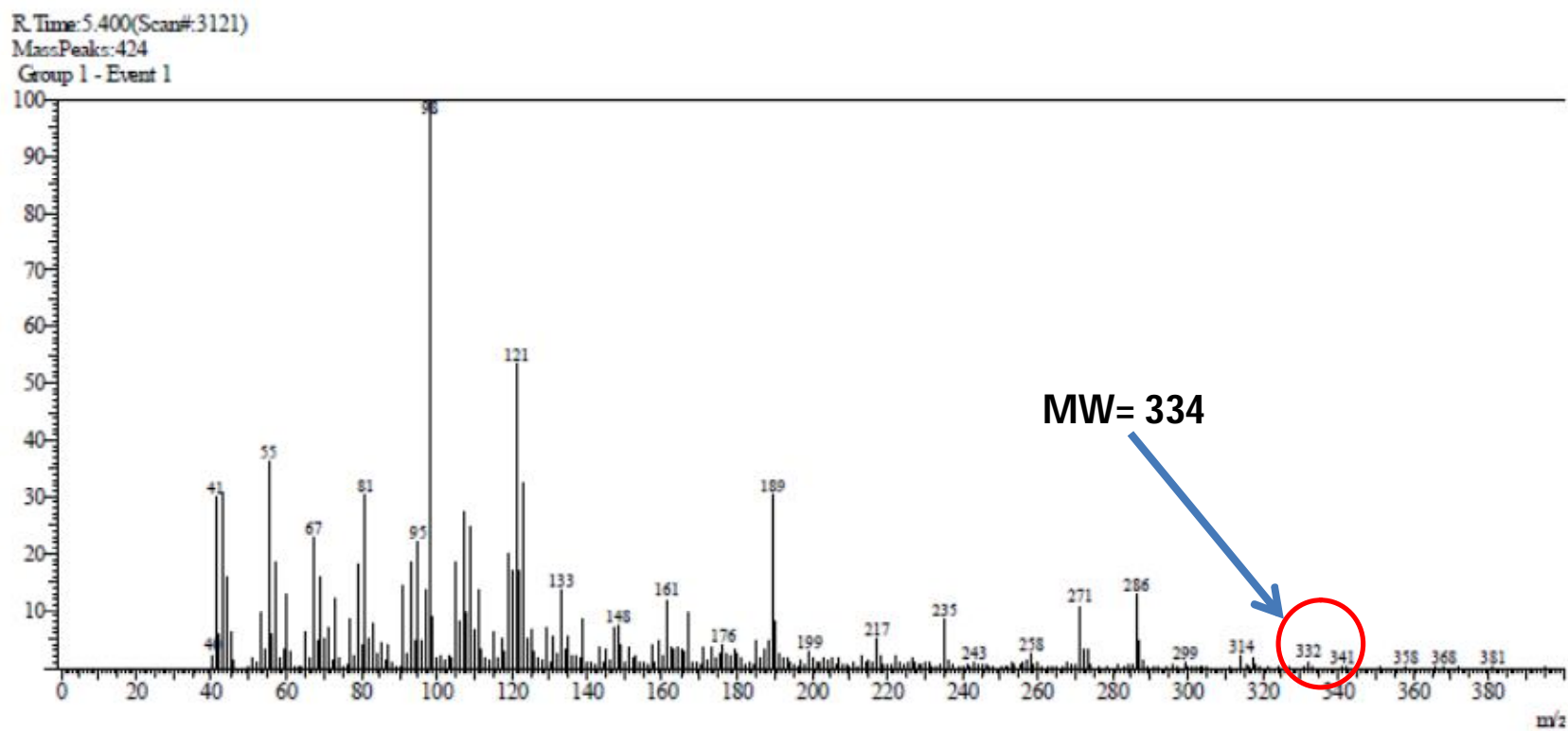
Figure-3.7.18: Molecular weight of Compound 7
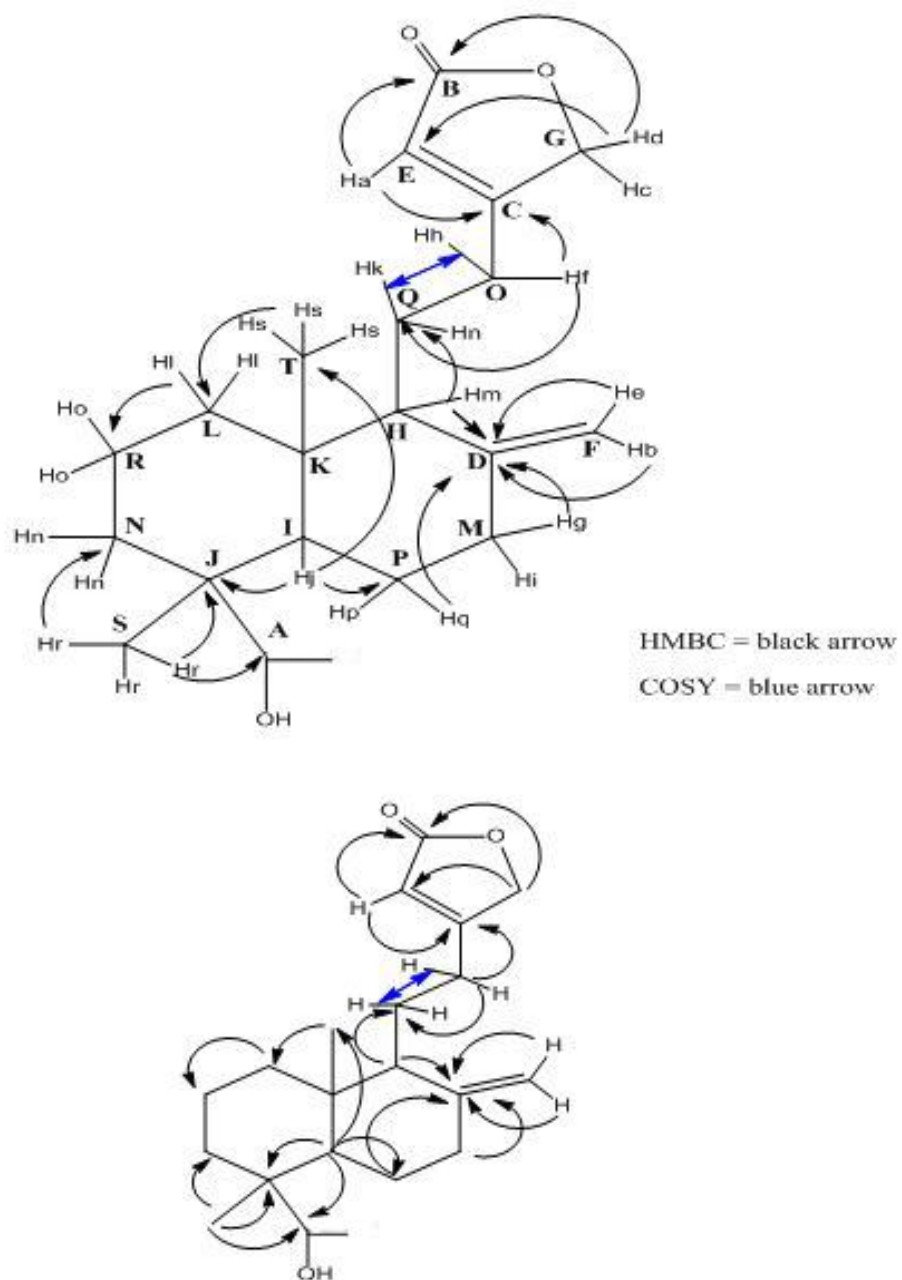
Figure- 3.7.19: HMBC and COSY correlations of Compound 7
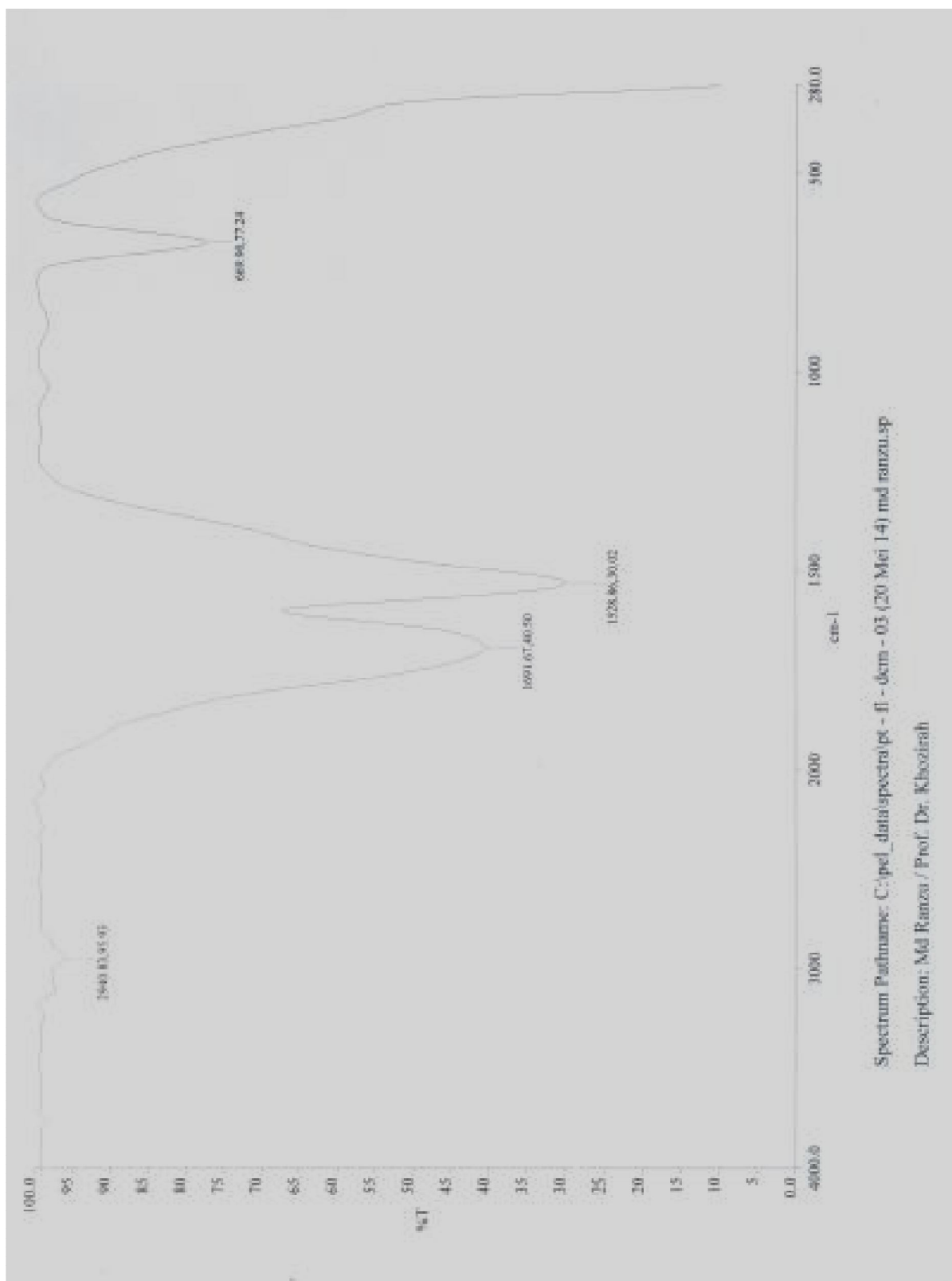
Figure-3.7.20:   FTIR Spectra of Compound 7

Chemical Formula: $C_{20}H_{28}O_4$
Molecular Weight: 332.43
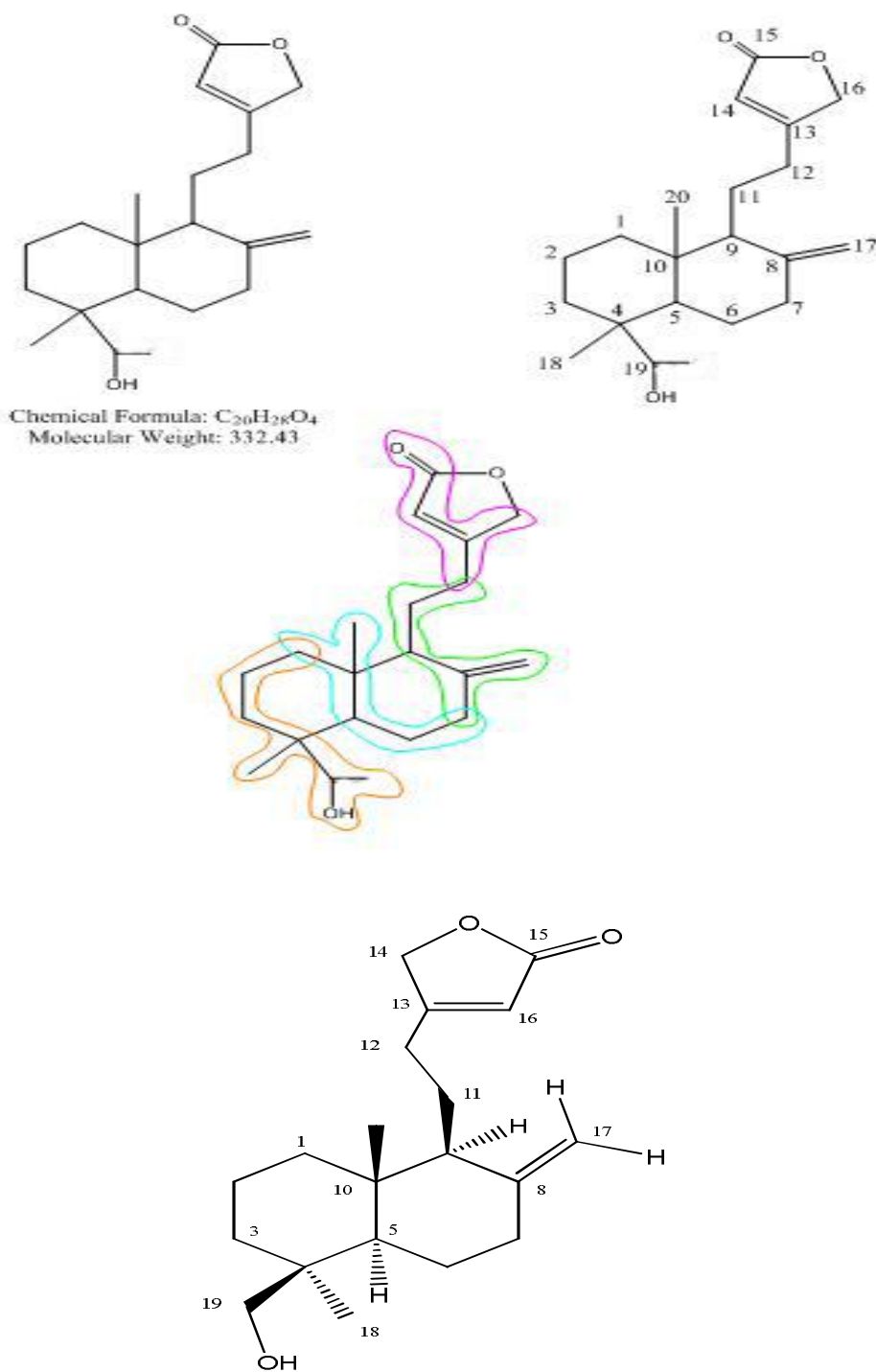
*Figure- 3.7.21: Structure of compound 7 (**19-Hydroxy-8 (17), 13-labda dien-15, 16-olide**)*

3.7.3.5. Compound 8

Compound **8** was isolated from the BuOH extract of the flowers of *Pthyrsiflorus* Nees as brownish powder. It gave single spot on TLC and also single peak in HPLC. The molecular formula $C_{15}H_{10}O_6$ was determined from 1D and 2D NMR data of compound **8**. The ^{13}C-NMR spectrum of **8** displayed 15 carbon atoms, which is characteristic of a flavonoid type skeleton. The molecular mass of this compound was 286 g/mol. The ^{1}H-, ^{13}C- (Table 3.7.5) and DEPT-NMR data of compound **8** are similar to the reported data of luteolin (Peters *et. al.,* 1986). On the basis of all the spectral data, the compound **8** is characterized as luteolin (Figure 3.7.24). Luteolin is a known compound and its IUPAC name is 2-(3,4-dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone. Compound **8** is isolated for the first time from this plant.

Table-3.7.5: ^{1}H-and ^{13}C- NMR spectral data for compound 08 (400 MHz, δ Value in CD₃OD)

Position	^{13}C (δppm)	^{1}H (δppm)
1	O	-
2	123.8	-
3	103.8	(6.53, s)
4	183.9	-
5	166.7	-
6	100.3	(6.43, br s)
7	166.4	-
8	95.1	(6.2. br s)
9	159.4	-
10	105.2	-
2′	114.1	-
3′	147.0	-
4′	151.0	-
5′	116.8	(6.89, d, J-12 Hz)
6′	120.4	(7.38, d, J-12 Hz)

Coupling constant J, are in Hz
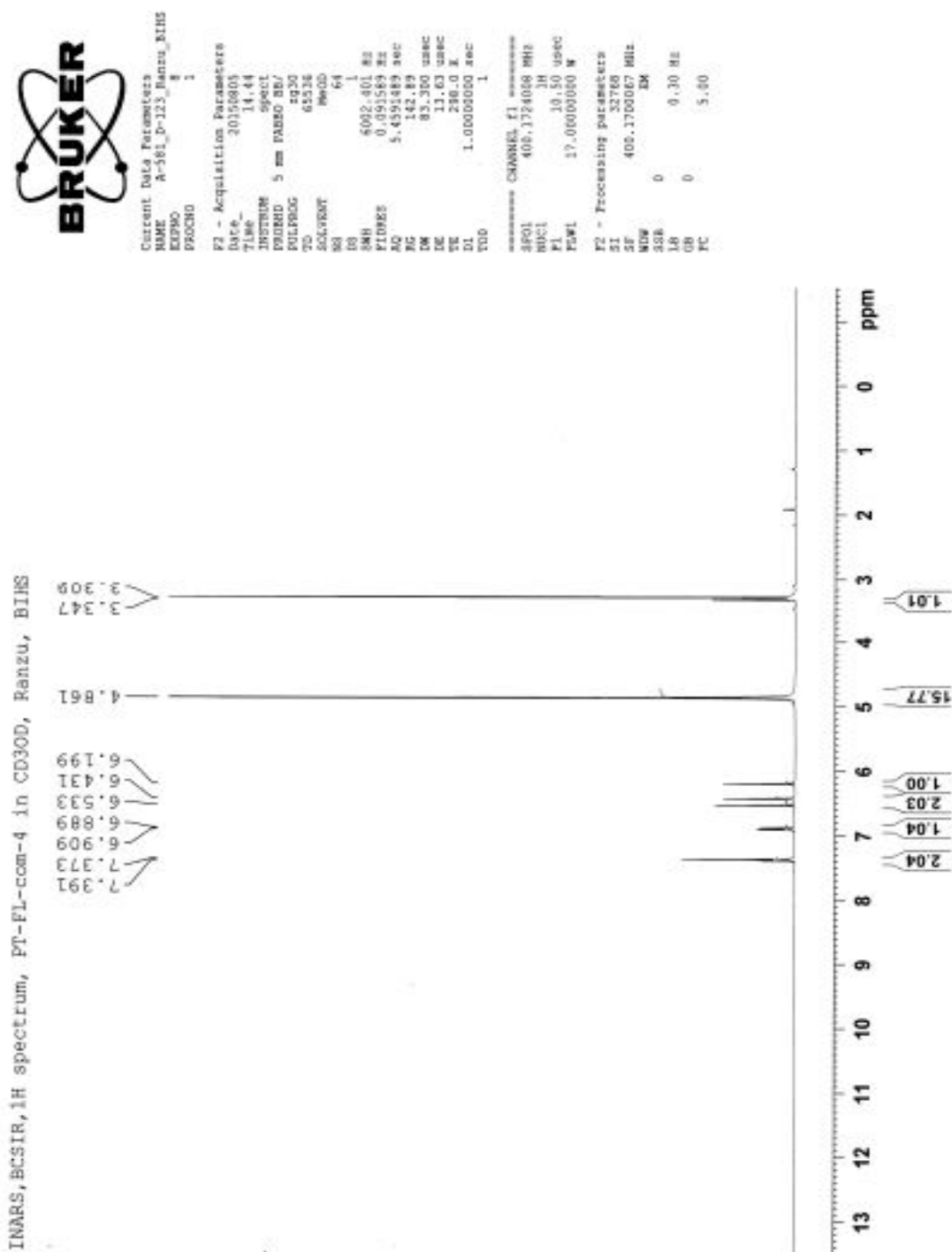
Figure- 3.7.22: ¹H-NMR spectrum of Compound 8
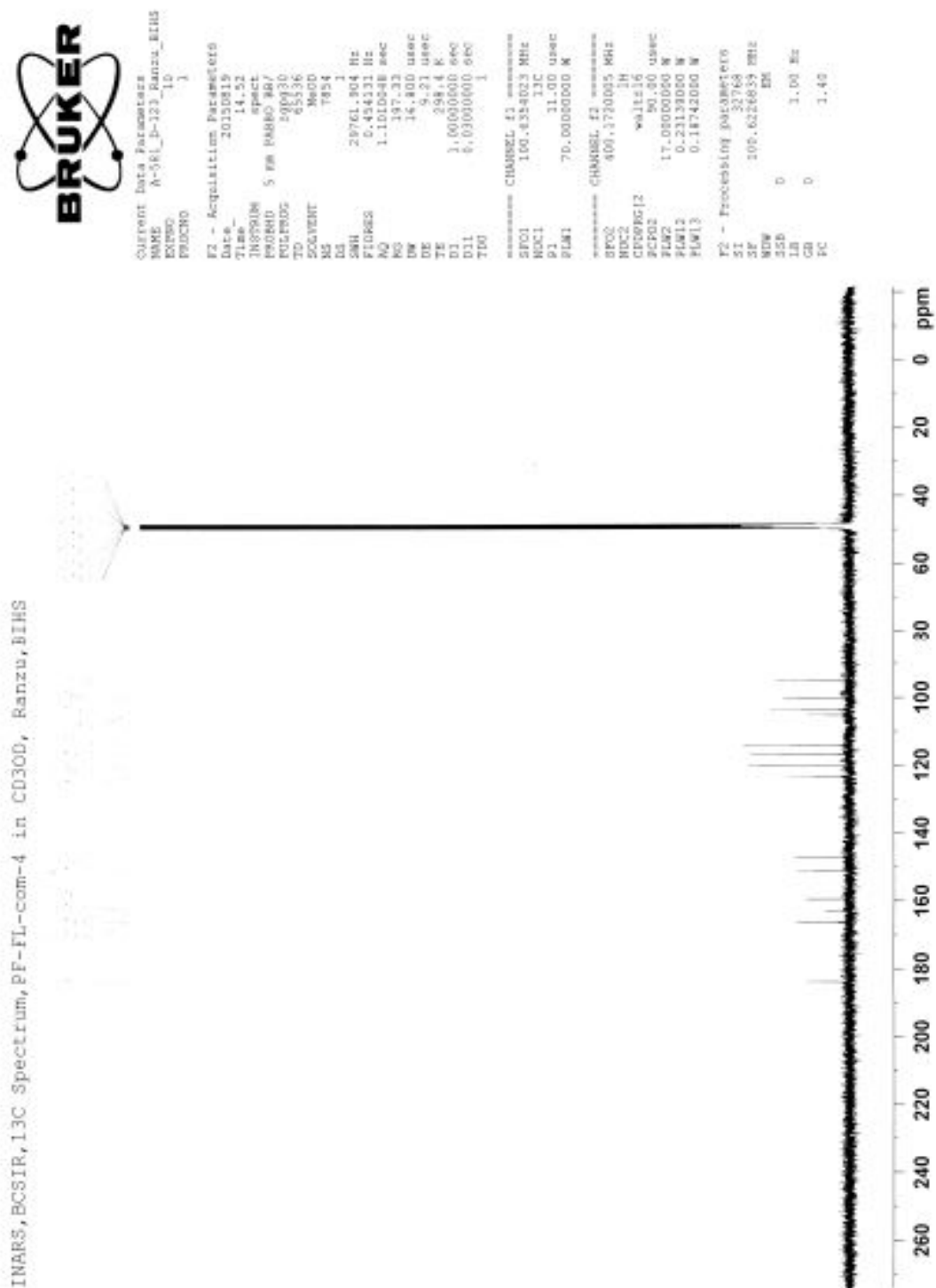
Figure- 3.7.23: ^{13}C-NMR spectrum of Compound 8
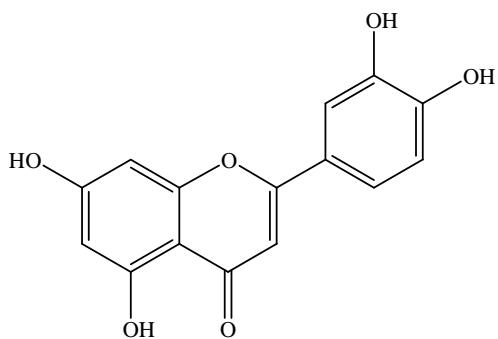
Figure- 3.7.24: Structure of Compound 8 [2-(3,4-Dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone]

3.8 TOXIC METAL ANALYSIS

3.8.1. Toxic Metal Content

As the study of safety of commercial herbal medicine was a prionary goal of the present work, all the 11 ADHPs were analysis for the presence of toxic metals like Ni, Cu, Mn, Cr, Cd, Pb, and As. Presence of one or more of these metals in ADHPs may indicate potential risk of the accumulation of these toxic metals leading to toxicity. (Table 3.8.1). All the eleven ADHPs contained copper (Cu), manganese (Mn), chromium (Cr), cadmium (Cd), lead (Pb) and arsenic (As) in some levels with exceptions of Cu in ADHP 5 & 6, Mn in ADHP 5 & 6, Cr in ADHP-2, 4 & 11, Cd in 2, 4, 5, 6 & 7 and Pb in ADHP 1, 2, 4, 5, 6, 7, 9 &11, were below detection level (Table 3.8.1). There are several regulatory bodies that set specific allowable limit for toxic metal content in herbal and traditional preparations based on different guidelines and this permissible limit varies among these regulatory bodies (Tables 3.8.2 and 3.8.4). It was found that lead content in almost all of the samples were below the permissible limit except ADHP 8 and ADHP 10 if we consider the stringent limit of Chinese Pharmacopoeia (Tables 3.8.1 and 3.8.2).

Table 3.8.1: Toxic metal content investigated ADHP samples.

Sample ID	Ni (ppm)	Cu (ppm)	Mn (ppm)	Cr (ppm)	Cd (ppm)	Pb (ppm)	As (ppm)
ADHP 1	BDL	7.88	101.42	0.36	0.02	BDL	0.49
ADHP–2	BDL	5.67	15.45	BDL	BDL	BDL	0.50
ADHP–3	BDL	1.58	5.26	11.51	$0.52^{*,\bullet,\P}$	0.08	0.05
ADHP–4	BDL	5.87	15.88	BDL	BDL	BDL	0.36
ADHP–5	BDL	BDL	BDL	14.63	BDL	BDL	0.54
ADHP–6	BDL	BDL	BDL	8.25	BDL	BDL	0.05
ADHP–7	BDL	11.53	121.32	4.03	BDL	BDL	0.75
ADHP-8	BDL	$27.01^{*,\bullet}$	98.69	28.25	$3.74^{*,\bullet,\P}$	$18.44^{*\P}$	0.74
ADHP-9	BDL	0.48	2.19	2.88	$0.48^{*,\bullet,\P}$	BDL	0.06
ADHP-10	BDL	$39.58^{*\P}$	317.55	11.75	$0.99^{*,\bullet,\P}$	0.37	0.11
ADHP-11	BDL	15.62	141.76	BDL	$0.27^{\P}$	BDL	0.40

BDL: below detection level; [*]exceed WHO and US FDA permissible limit; [•]exceed HAS Singapore permissible limit; [¶]exceeds Chinese Pharmacopoeia permissible limit. Detection level (DL) in ppm: Ni = 0.02, Cu = 0.01, Mn = 0.01, Cr = 0.01, Cd = 0.003, Pb = 0.00005 and As = 0.0001.

Table 3.8.2: Permissible limit of toxic metal in herbal drugs.

Toxic/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	—	—

WHO: World Health Organization; US FDA: United States Food and Drug Administration; HAS: Health Science Authority.

3.8.1.1 Nickel: Nickel is a toxic metal and it plated faucets contaminating water and soil, mining or smelting waste-water production, cooking with nickel–steel alloy house wares, and eating in nickel-pigmented dishes. The amount of nickel was below detection level in all the preparations.

3.8.1.2 Copper: The copper (Cu) concentrations varied in a wide range in all ADHPs between 0.48 to 39.58 ppm except ADHP 5 & 6. The maximum concentrations of copper in ADHP 1, 2, 3, 4, 7, 8, 9, 10 and 11 were 7.88, 5.67, 1.58, 5.87, 11.53, 27.01, 0.48, 39.58 and 15.62 ppm, respectively. ADHP-8 and 10 have exceeded the regulatory limits of the WHO/FAO and Chinese Pharmacopoeia. HAS Singapore had set limits for copper in medicinal plants at 150 ppm (Ulla *et. al.,* 2012). In this study, none of the samples exceeded the HAS Singapore of 150 ppm, where two samples (ADHP-8 and 9) showed a value above 20 ppm limit of WHO/FAO and Chinese Pharmacopoeia. Copper is an essential component of many enzymes, therefore playing a significant role in a wide range of physiological processes including iron utilization, free radicals elimination, bone and connective tissues development, melanin production, and many others. Nevertheless, excessive intake of copper can cause dermatitis, irritation of the upper respiratory tract, abdominal pain, nausea, diarrhea, vomiting, and liver damage (Martin and Griswold, 2009 and Ulla, *et. al.,* 2012).

3.8.1.3 Manganese: Manganese is a naturally occurring element and an essential nutrient. Comprising approximately 0.1% of the earth's crust, it is the twelfth most

abundant element and the fifth most abundant metal. Manganese does not exist in nature as an elemental form, but is found mainly as oxides, carbonates, and silicates in over 100 minerals with pyrolusite (manganese dioxide) as the most common naturally-occurring form. Excess exposure to manganese may be revealed by tests to detect heightened levels in body fluids as well as in hair samples.

Manganese was found in all ADHP's samples except ADHP 5 and 6. The Manganese (Mn) concentrations varied in a wide range in all ADHPs between 2.19 to 317.55 ppm. The maximum concentrations of copper in ADHP 1, 2, 3, 4, 7, 8, 9, 10 and 11 were 101.42, 15.45, 5.26, 5.87, 15.88, 121.32, 98.69, 39.58, 2.19, 317.55 and 141.76 ppm, respectively. The regulatory limits of the WHO/FAO have not been established yet for the manganese in herbal medicines (Ulla et. al., 2012).

Normal ranges of manganese levels in body fluids are 4–15 μg/L in blood, 1–8 μg/L in urine, and 0.4–0.85 μg/L in serum. Excess manganese in the body characteristically accumulates in the brain region known as the basal ganglia (Toxicological profile for manganese, http://www.atsdr.cdc.gov/toxprofiles/tp151-c2.pdf). Although low levels of manganese intake are necessary for human health, exposures to high manganese levels are toxic.

Adequate Intake (AI) for Manganese (Ref: http://www.atsdr.cdc.gov/toxprofiles/tp151-c2.pdf)

Life stage	Age	Males (mg/day)	Females (mg/day)
Infants	0–6 Months	0.003	0.003
Infants	7–12 Months	0.6	0.6
Children	1–3 Years	1.2	1.2
Children	4–8 Years	1.5	1.5
Children	9–13 Years	1.9	1.6
Adolescents	14–18 Years	2.2	1.6
Adults	19 Years and older	2.3	1.8
Pregnancy	All ages	—	2.0
Lactation	All ages	—	2.6

Source: FNB/IOM 2001

Manganese toxicity can result in a permanent neurological disorder known as manganism with symptoms that include tremors, difficulty in walking, and facial muscle spasms.

3.8.1.4 Cromium: Chromium is available in two ionic forms, that is, trivalent and hexavalent. The trivalent chromium is present in food and is utilised by humans because of its safety. Chromium participates in glucose metabolism. The information to set the recommended dietary allowance for chromium is not enough. However, the food and nutrition board set out an adequate intake level based on chromium contents in normal diet, that is, for children 11 - 25 µg/day and for adult 30 -35 µg/day. Exposure to high level of chromium causes lungs cancer and dermatitis (FNB, 2001; ATSDR, 2004).

Chromium was found in all ADHP's samples except ADHP 2, 4 and 11. The Chromium (Cr) concentrations varied in a wide range in all ADHPs between 0.36 to 28.25 ppm. The maximum concentrations of copper in ADHP 1, 3, 5, 6, 7, 8, 9 and 10 were 0.36, 11.51, 14.63, 8.25, 4.03, 28.25, 2.88 and 11.75 ppm, respectively. According to the level of Food and Nutrition Board (FNB) and Agency for Toxic Substances and Disease Registry (ATSDR) for the chromium contents, all the ADHPs are safe to use except ADHP-3, 5, 8 and 10 (Table: 3.8.4).

3.8.1.5 Cadmium: Cadmium content was detected in all of the ADHP samples except ADHP 2, 4, 5, 6 & 7. Unfortunately, ADHP 3, 8, 9 & 10 samples level were much more than the level of all permissible limit (WHO, US FDA, HAS Singapore and Chinese Pharmacopoeia, Tables 3.3.1 and 3.3.2). Even if we consider a more relaxed permissible limit for cadmium (WHO and US FDA guidelines; Table 3.3.2), one-third of the total ADHPs (ADHP 3, 8, 9 & 10) failed to comply with the safety limit. Cadmium toxicity could induce tissue injury (Matović, *et. al,* 2011, Patra *et. al.*, 2011 and Cuypers *et. al.,* 2010) epigenetic changes in DNA expression (Martinez-Zamudio and Ha, 2011, Wang *et. al.,* 2012 and Luparello *et. al.,* 2011), hypertension (Gallagher & Meliker, 2010), diabetes (Edwards & Prozialeck, 2009), apoptosis (Cannino *et. al.,* 2009) and insulin resistance (Satarug and Moore, 2012 and Chen *et. al.,* 2009). Moreover, excess cadmium may inhibit or upregulate transport pathways (Thévenod, 2010, Wan and Zhang, 2012 and Kerkhove, *et. al.,* 2010), and heme synthesis (Schauder *et. al.,* 2010). Considering the JECFA (The Joint FAO/WHO Expert Committee on Food Additives) toxic metal limits for herbal dietary supplements, none of ADHP contained toxic metals in such a level, which could exceed the daily allowable intake (Tables 3.8.3 and 3.8.4).

3.8.1.6 Lead: Lead, a highly toxic environmental pollutant, can affect the function of various biomolecules by forming complex with them. Moreover, excess lead exposure may be responsible for poor muscle coordination, gastrointestinal symptoms, brain and kidney damage, hearing and vision impairments, and reproductive defects (Johnson, 1998, Kalia & Flora, 2005 and Pearce 2007).

The content of lead (Pb) in the analyzed samples ranged from less than 1.0 to 18.44 ppm. All the ADHP's samples showed the value of Pb metal under detection level except ADHP-3 and 8 & 10. The maximum concentrations of lead in ADHP- 3, 8 & 10 were 0.08, 18.44 & 0.37 ppm, respectively. The FAO/WHO maximum permissible limit of lead in consumed medicinal herbs is 10 ppm (WHO, 2006 and WHO, 2005). The obtained results showed that only one of the analyzed sample exceeded this limit.

3.8.1.7 Arsenic: Elemental arsenic is found naturally in the earth's crust at concentrations of 2–5 ppm (Tamaki & Frankenberger 1992). Most arsenic release into the environment is inorganic and accumulates by binding to organic soil matter (Smedley & Kinniburgh 2005). Soils with high arsenic concentrations can yield foods with exceedingly elevated arsenic levels. Diet is the largest source of exposure for nonoccupationally exposed individuals, with an average total (inorganic and methylated) arsenic intake of 40 μg/day. U.S. dietary intake of inorganic arsenic has been estimated to range from 1 to 20 μg/day (Schoof *et. al*., 1999). Because of high arsenic concentration in algae and marine micro-organisms, seafood is the highest dietary source of arsenic (Tao & Bolger, 1999). Arsenic concentrations for fish and seafood average 4–5 ppm (Bennett, 1981), significantly higher than concentrations found in grains and cereals, with an average of 0.02 ppm (Gartrell et al. 1986). Although chronic low-level exposure to arsenic does occur from dietary sources, it is usually significantly below toxic levels. The tragedy of acute and chronic arsenic poisoning from contamination of water in Bangladesh, West Bengal, and elsewhere in the world has recently been described (Mead, 2005).

Table 3.8.3: Toxic metal content investigated of ADHP samples and the daily safe intake of different Toxic metals.

Sample ID	Cumulative daily adult dose of preparation * (g)	Daily adult intake of Toxic metal (in µg) as calculated from the dose indicated on the label of the finished product						
		Ni	Cu	Mn	Cr	Cd	Pb	As
ADHP – 1	4.25	BDL	33.49	431.42	1.53	0.09	BDL	2.08
ADHP–2	25 (ml)	BDL	141.75	386.25	BDL	BDL	BDL	12.5
ADHP–3	30 (ml)	BDL	47.40	157.80	345.3	15.60	2.40	1.5
ADHP–4	3.75	BDL	22.01	59.55	BDL	BDL	BDL	1.35
ADHP–5	2.5	BDL	BDL	BDL	36.57	BDL	BDL	1.35
ADHP–6	2.5	BDL	BDL	BDL	20.62	BDL	BDL	00.12
ADHP–7	1.5	BDL	17.29	181.98	6.04	BDL	BDL	1.12
ADHP-8	2.5	BDL	67.52	246.72	70.62	9.35	46.10	1.85
ADHP-9	25 (ml)	BDL	12	54.75	72.00	12	BDL	1.50
ADHP-10	2.5	BDL	98.95	793.87	29.37	2.47	0.92	0.275
ADHP-11	1	BDL	15.62	141.76	BDL	0.27	BDL	0.40

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

Table 3.8.4: JECFA Toxic metal limits for herbal dietary supplements.

Toxic 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.

Arsenic (As) content in the analyzed samples ranged from 0.05 to 0.75 ppm. All the ADHP's samples showed the value of As metal with the highest value in ADHP 7 (0.75 ppm) & ADHP 8 (0.74 ppm) and lowest value in ADHP 6 (0.05 ppm) in Table 3.8.1. The US FDA, WHO, HSA Singapore & Chinese Pharmacopiea maximum permissible limit of As in consumed medicinal herbs is 10 ppm, 10 ppm, 5 ppm and 2 ppm respectively (Table: 3.8.2). The obtained results showed that all the analyzed ADHP samples were below the permissible limits (PL).

Considering all of these guidelines, it turned out that six ADHP samples (ADHP 1, ADHP 2, ADHP 4, ADHP 5, ADHP 6 and ADHP 7) contain toxic metals in safe level. Metals are natural components of soils and some of them (Cu & Mn) are necessary for micronutrients of plant growth while others (Ni, Cd, Cr, Pb and As) are not but could be accumulated in plants at toxic level (Lasat, 2002, Gaur & Adholeya, 2004 and Cho-Ruk, *et. al.,* 2006). As the major components of these herbal preparations are plants, the presence of toxic metals in ADHPs is very relevant. Some of the identified metals (Cu, Mn, and Cr) have important biological role in the body.

3.9 ORGANOCHLORINE PESTICIDES (OCPs) TESTING

3.9 General Information

Organochlorine pesticides are used in plant protection. Therefore, the herbal formulations may contain harmful pesticides. The organochlorine pesticides were identified and quantified using the gas chromatographic facilities at the Department of Chemistry, University of Dhaka. Four standards 4, 4-DDE, 4, 4-DDD, 2, 4-DDT and 4, 4-DDT were run on GC (Figure: 3.9.1). The ADHP samples were extracted (Section: 2.9.1) and the extracts were analysed by GC
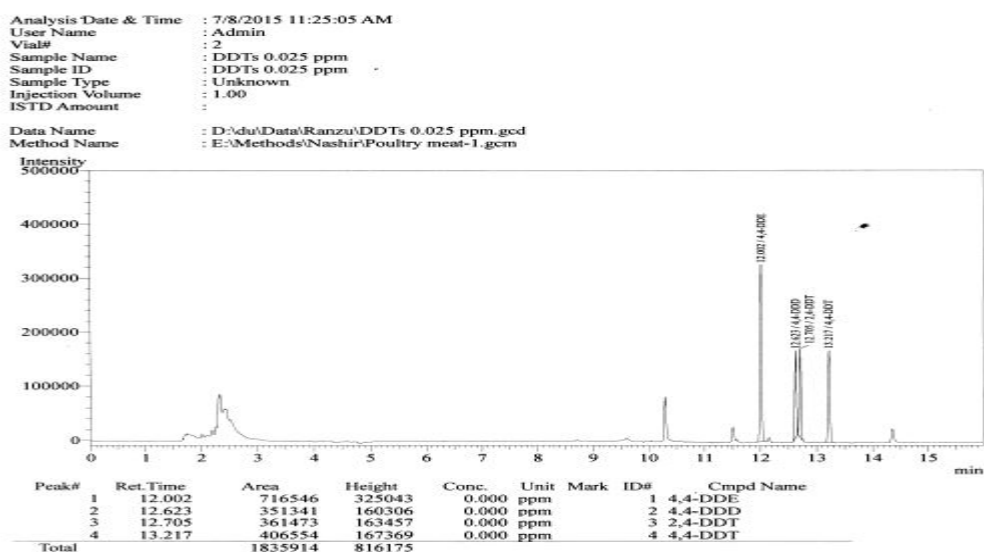
Figure-3.9.1: GC chromatogram of the four standards.

Analysis Date & Time : 9/8/2015 2:37:12 PM
User Name : Admin
Vial# : 1
Sample Name : Pesticides in Herbal products
Sample ID : ADHP-1
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\du\Data\Ranzu\ADHP-1.gcd
Method Name : E:\du\Methods\Nashir\Poultry meat-1.gcm

Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	11.992	29103	14100	0.000	ppm		1	4,4-DDE
2	13.200	78942	35641	0.000	ppm		4	4,4-DDT
Total		108045	49741					

Figure-3.9.2: GC chromatogram for analysis of pesticide resides in ADHP-1

Calculation of results for ADHP-1

4, 4-DDE $= \dfrac{29103 \times 0.025 \times 2}{716546 \times 10} = 0.203\ ng/g$

4, 4-DDT $= \dfrac{78942 \times 0.025 \times 2}{406554 \times 10} = 0.971\ ng/g$

Analysis Date & Time : 9/8/2015 2:58:02 PM
User Name : Admin
Vial# : 2
Sample Name : Pesticides in Herbal products
Sample ID : ADHP-2
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\du\Data\Ranzu\ADHP-2.gcd
Method Name : E:\du\Methods\Nashir\Poultry meat-1.gcm

Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	12.017	84233	36332	0.000	ppm		1	4,4-DDE
2	12.641	26206	13870	0.000	ppm		2	4,4-DDD
3	12.717	140078	69158	0.000	ppm		3	2,4-DDT
4	13.225	699978	290649	0.000	ppm		4	4,4-DDT
Total		950495	410009					

Figure-3.9.3: GC chromatogram for analysis of pesticide resides in ADHP-2

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Calculation of results for ADHP-2

4, 4-DDE $= \dfrac{84233 \times 0.025 \times 2}{716546 \times 10} = 0.588\,ng/g$

4, 4-DDD $= \dfrac{26206 \times 0.025 \times 2}{351341 \times 10} = 0.373\,ng/g$

2, 4-DDT $= \dfrac{140078 \times 0.025 \times 2}{361473 \times 10} = 1.937\,ng/g$

4, 4-DDT $= \dfrac{699978 \times 0.025 \times 2}{406554 \times 10} = 8.608\,ng/g$

```
Analysis Date & Time   : 9/8/2015 3:18:52 PM
User Name              : Admin
Vial#                  : 3
Sample Name            : Pesticides in Herbal products
Sample ID              : ADHP-3
Sample Type            : Unknown
Injection Volume       : 1.00
ISTD Amount            :

Data Name              : D:\du\Data\Ranzu\ADHP-3.gcd
Method Name            : E:\du\Methods\Nashir\Poultry meat-1.gcm
```
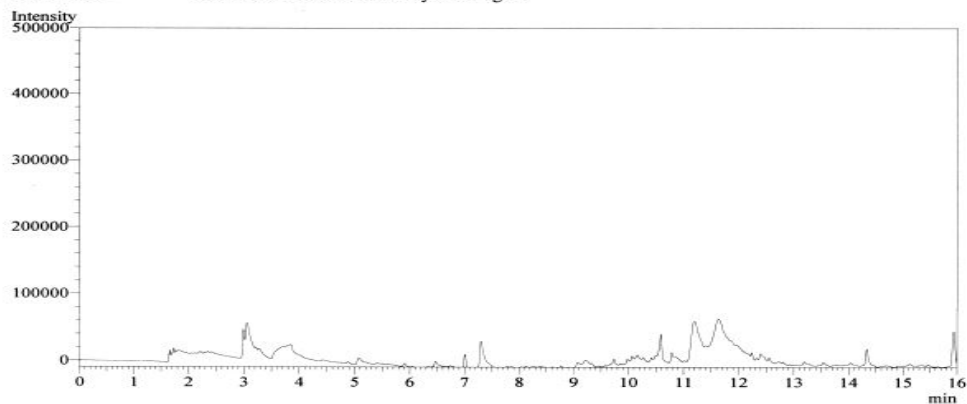
Figure-3.9.4: GC chromatogram for analysis of pesticide resides in ADHP-3

No organochlorine pesticide peak was detected is this antidiabetic herbal preparation.

Analysis Date & Time : 9/8/2015 3:39:42 PM
User Name : Admin
Vial# : 4
Sample Name : Pesticides in Herbal products
Sample ID : ADHP-4
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\du\Data\Ranzu\ADHP-4.gcd
Method Name : E:\du\Methods\Nashir\Poultry meat-1.gcm
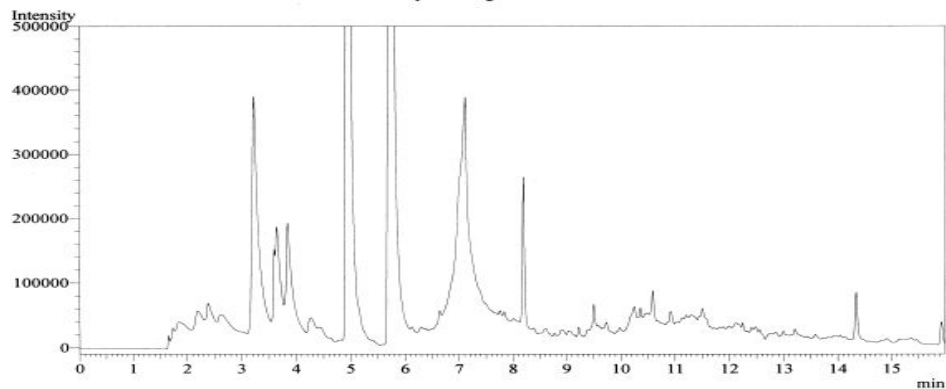
Figure-3.9.5: GC chromatogram for analysis of pesticide resides in ADHP-4

No organochlorine pesticide peak was detected is this antidiabetic herbal preparation.

Analysis Date & Time : 9/8/2015 4:00:36 PM
User Name : Admin
Vial# : 5
Sample Name : Pesticides in Herbal products
Sample ID : ADHP-5
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\du\Data\Ranzu\ADHP-5.gcd
Method Name : E:\du\Methods\Nashir\Poultry meat-1.gcm
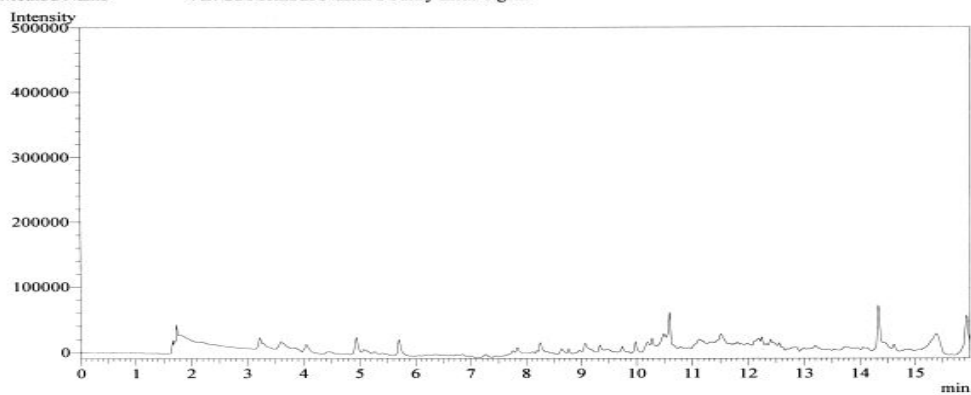
Figure-3.9.6: GC chromatogram for analysis of pesticide resides in ADHP-5

No organochlorine pesticide peak was detected is this antidiabetic herbal preparation.

Analysis Date & Time : 9/8/2015 4:21:30 PM
User Name : Admin
Vial# : 6
Sample Name : Pesticides in Herbal products
Sample ID : ADHP-6
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\du\Data\Ranzu\ADHP-6.gcd
Method Name : E:\du\Methods\Nashir\Poultry meat-1.gcm

Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	11.994	202480	96101	0.000	ppm		1	4,4-DDE
2	12.694	54650	27713	0.000	ppm		3	2,4-DDT
3	13.205	274584	119531	0.000	ppm		4	4,4-DDT
Total		531714	243345					
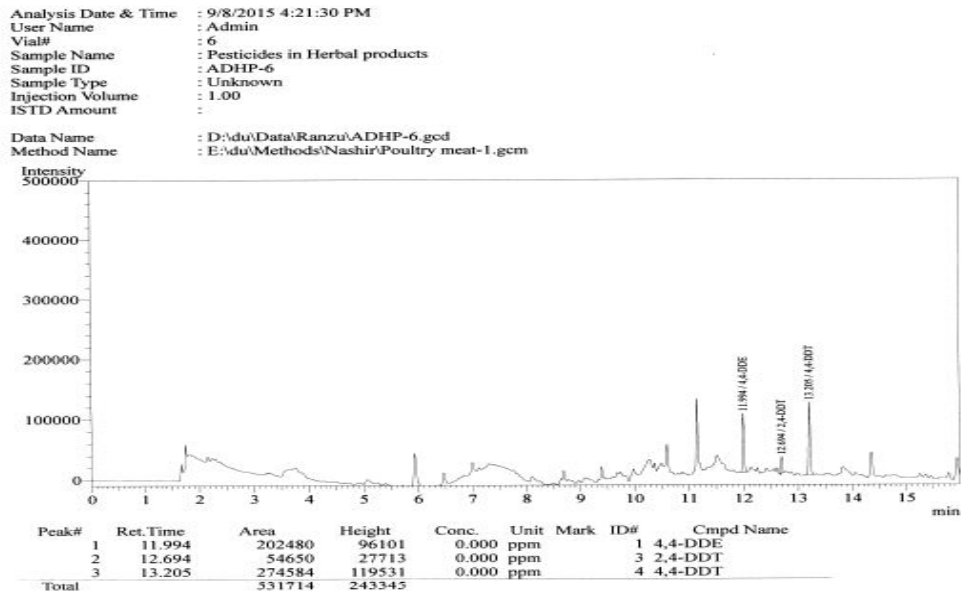
Figure-3.9.7: GC chromatogram for analysis of pesticide resides in ADHP-6

Calculation of fesults for ADHP-6

4, 4-DDE $= \dfrac{202480 \times 0.025 \times 2}{716546 \times 10} = 1.413\,ng/g$

2, 4-DDT $= \dfrac{54650 \times 0.025 \times 2}{361473 \times 10} = 0.756\,ng/g$

4, 4-DDT $= \dfrac{274584 \times 0.025 \times 2}{406554 \times 10} = 3.377\,ng/g$

Analysis Date & Time : 9/8/2015 5:03:05 PM
User Name : Admin
Vial# : 1
Sample Name : Pesticides in Herbal products
Sample ID : ADHP-7
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\du\Data\Ranzu\ADHP-07.gcd
Method Name : E:\du\Methods\Nashir\Poultry meat-1.gcm

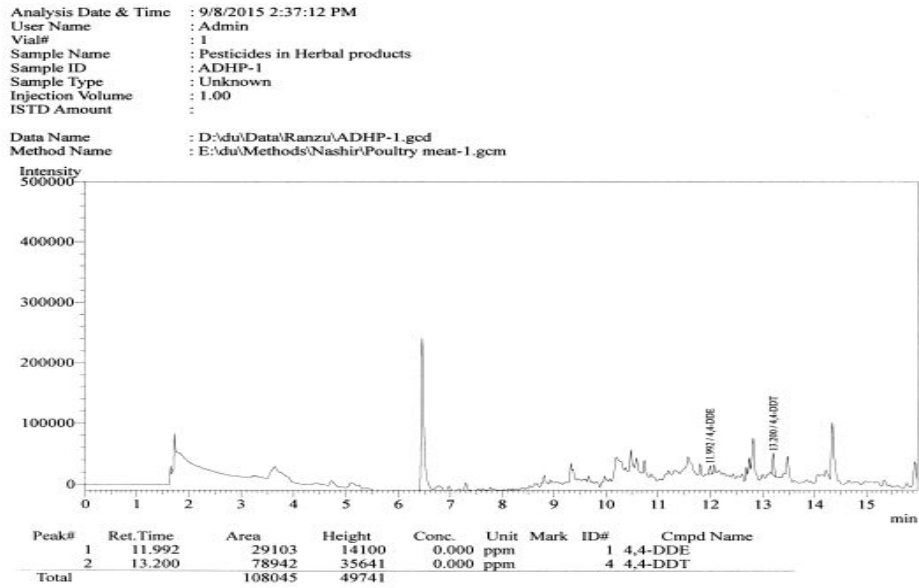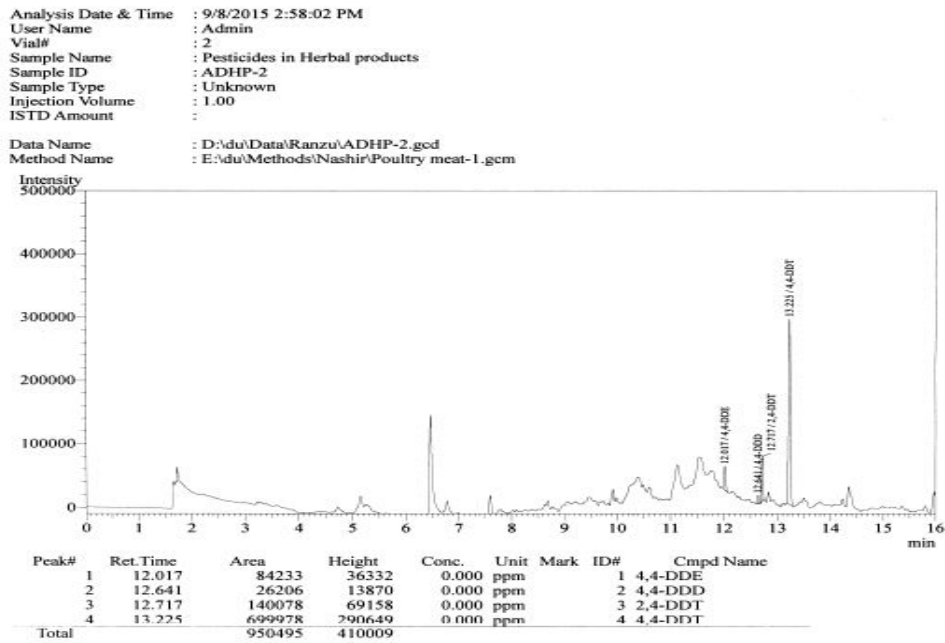| Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	12.014	263451	113199	0.000	ppm		1	4,4-DDE
2	13.235	237216	85222	0.000	ppm		4	4,4-DDT
Total		500667	198421					
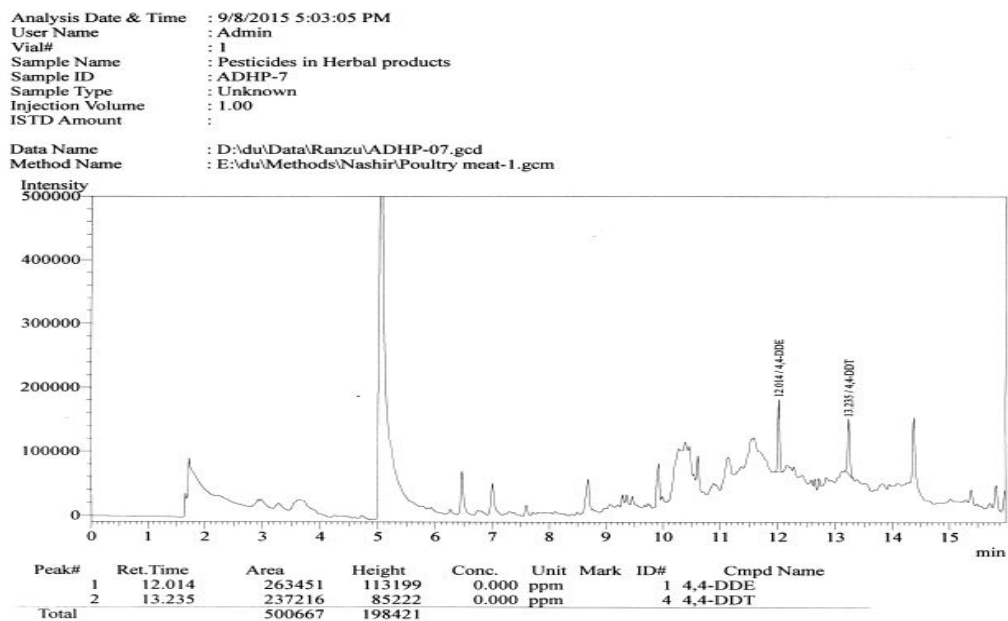
Figure-3.9.8: GC chromatogram for analysis of pesticide resides in ADHP-7

Calculation of results for ADHP-7

4, 4-DDE $= \dfrac{263451 \times 0.025 \times 2}{716546 \times 10} = 1.838 \, ng/g$

4, 4-DDT $= \dfrac{237216 \times 0.025 \times 2}{406554 \times 10} = 2.917 \, ng/g$

3.10 ANALYSIS OF DELIBERATELY ADDED SYNTHETIC DRUGS (if any)

3.10.1. Deliberately added synthetic drugs

Metformin HCl was identified in all ADHPs by using HPLC. But two major peaks were identified in ADHP-6 and ADHP-7 where retention times are matched to that of the gliclazid and glibenclamide respectively. Another peak was also found at the retention time of 7.99 with low intensity which may compare with gliclazid. The major ingredient was detected in the ADHP-6 and was identified as gliclazid (Figure 3.10.3) comparing with HPLC chromatogram.

The presence of gliclazide and glibenclmide in ADHP-6 and ADHP-7 respectively were also confirmed through isolation of these two compounds from these two ADHPs by HPLC. The structure of the isolated compounds were confirmed as gliclazide and glibenclmide through their ^{1}H-NMR study (Table: 3.10.2 & 3.10.3 and Figure: 3.10.9, 3.10.10, 3.10.11 & 3.10.12). These isolated compounds and the reported compound's NMR value are similar. So, it was shown that gliclazid and glibenclamide were used in the ADHP-6 & ADHP-7, respectively. Table-3.10.1.

HPLC analysis of the 07 ADHPs were done to investigate the presence of synthetic drugs adulteration. The adulterated drugs analyzed were metformin hydrochloride, glipizide, gliclazide, glibenclamide and glimepiride. Figure: 3.10.1 showed the chromatogram of 05 standards of the drugs for the treatment of Diabetes Mellitus.

Figure: 3.10.2 to 3.10.8 revealed the analytical results chromatogram for HPLC analysis of adulteration of synthetic drugs in ADHPs. From these HPLC analysis it is observed that all the ADHP's (ADHP-1 to 7) contain the synthetic drug metformin hydrochloride (1.28% to 2.62%) within the retention time 2.19 to 2.24.

It also observed that a peak for glibenclamide was found to be present in ADHP-2 (0.23%) (Figure: 3.10.3) and ADHP-7 (3.82%) (Figure: 3.10.8).

ADHP-1 was also found to contain glimperide 0.074% (Figure: 3.10.2). On the other hand in ADHP-6, glyclazide was observed to be present at significant amount 5.74 % (Figure 3.10.7).

Table-3.10.1. Amount of different types of antidiabetic drug standards in methanol extract of ADHPs were analysed by using HPLC.

Sl No	Metformin HCl (%)	Glipizide (%)	Glyclazide (%)	Glibenclamide (%)	Glimeperide (%)
ADHP-1	2.25	ND	ND	ND	ND
ADHP-2	2.62	ND	ND	0.23	0.74
ADHP-3	1.28	ND	ND	ND	ND
ADHP-4	1.58	ND	ND	ND	ND
ADHP-5	1.47	ND	0.054	ND	ND
ADHP-6	1.35	ND	**5.74**	ND	ND
ADHP-7	2.47	ND	0	**3.82**	ND

ND= Not Detected; Detection Limit=0.05 ppm
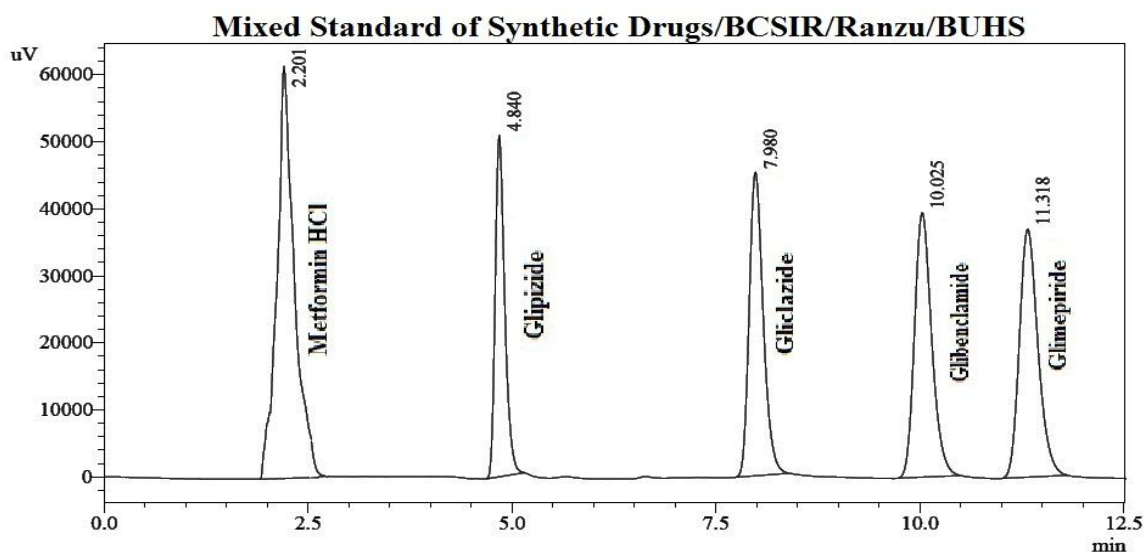
Figure-3.10.1: Chromatogram of synthetic drug's mixed standard.

HPLC analysis conditions:

SHIMADZU Prominence LC 20AT
Flow Rate: 1.0 ml/ min
Solvent System (Mobile Phase): ACN: Buffer = 52 :: 48
Column: C18 column (250 mm x 4.6 mm)
Detector: PDA
Wavelength: 230 nm
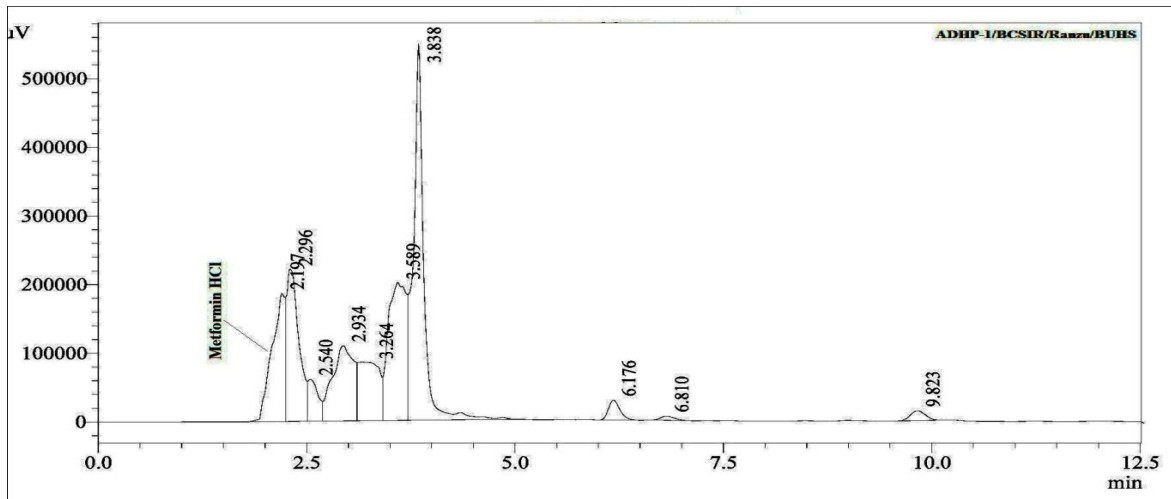
Figure-3.10.2: HPLC analysis of adulterated synthetic drugs in ADHP-1.
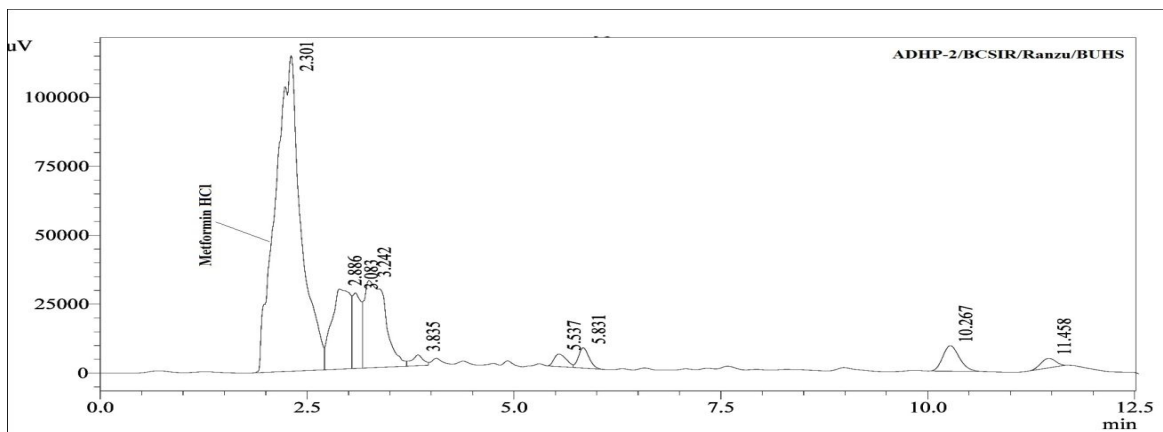
Figure-3.10.3: HPLC analysis of adulterated synthetic drugs in ADHP-2.
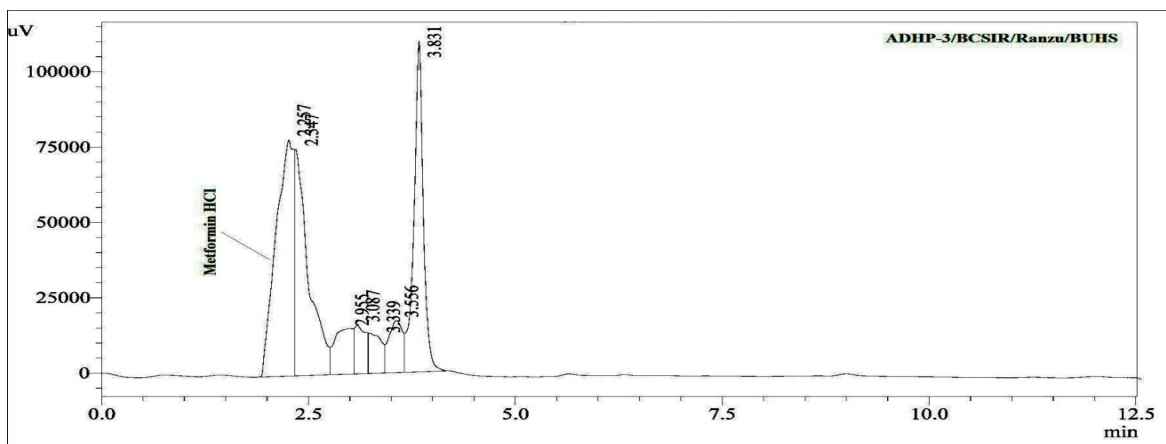
Figure-3.10.4: HPLC analysis of adulterated synthetic drugs in ADHP-3.
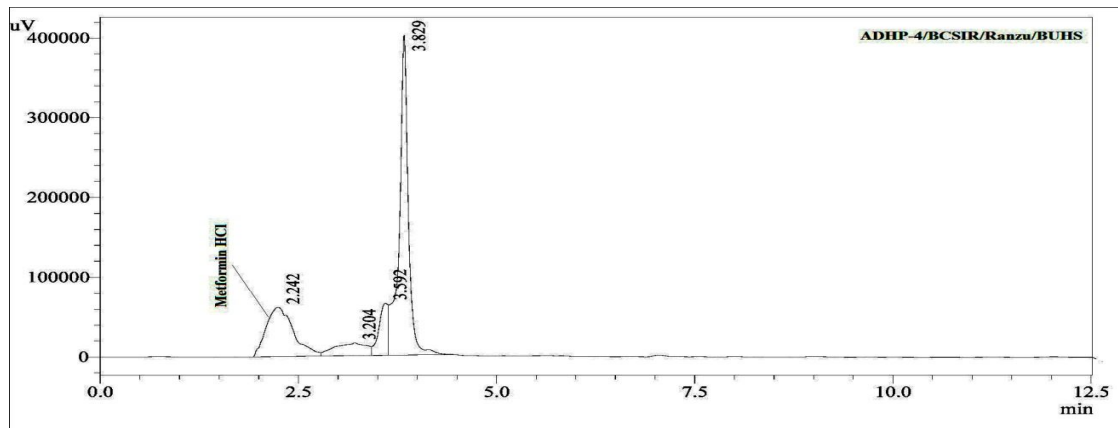
Figure-3.10.5: HPLC analysis of adulterated synthetic drugs in ADHP-4.
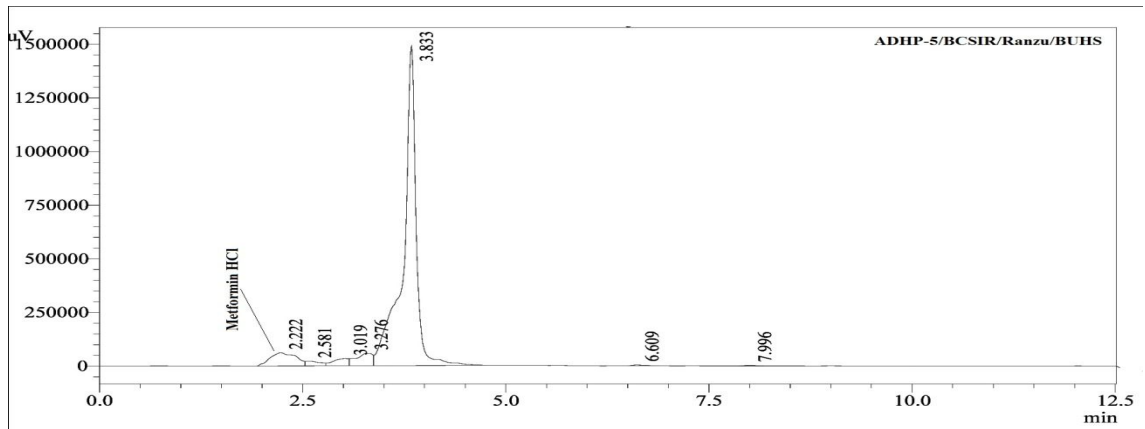
Figure-3.10.6: HPLC analysis of adulterated synthetic drugs in ADHP-5.
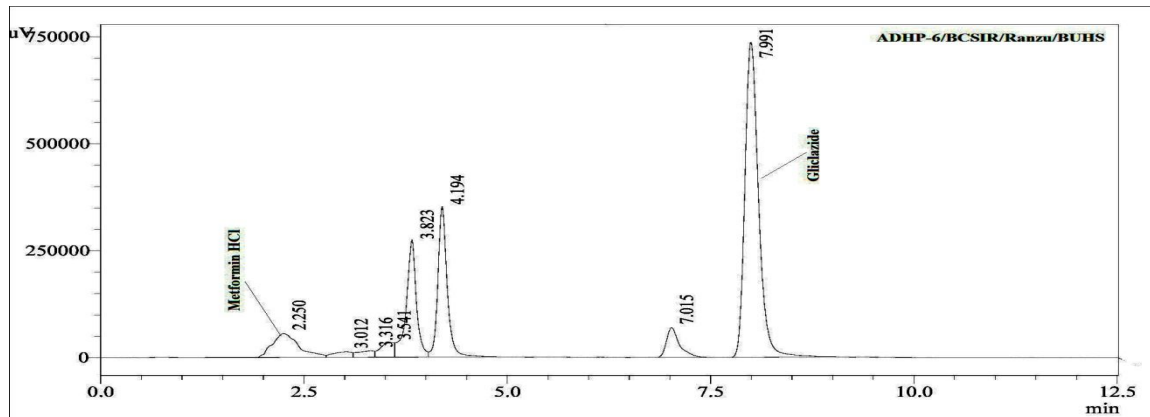
Figure-3.10.7: HPLC analysis of adulterated synthetic drugs in ADHP-6.
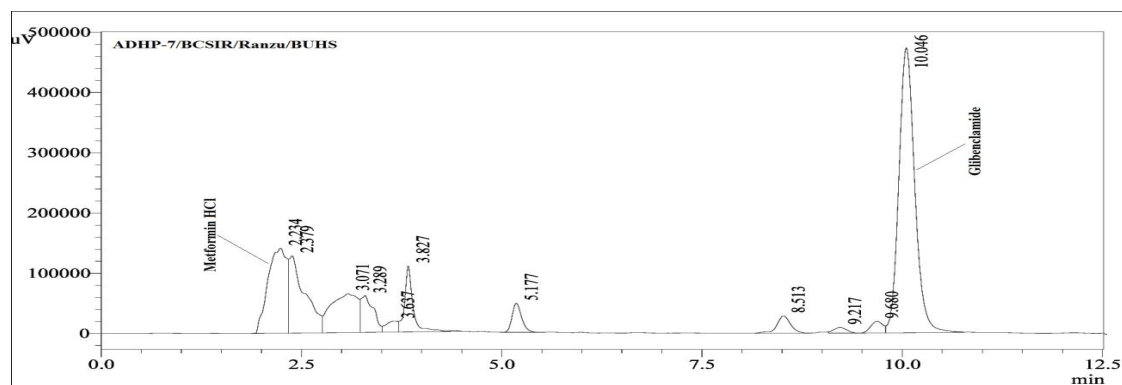
Figure-3.10.8: HPLC analysis of adulterated synthetic drugs in ADHP-7.

Many herbal medicines and synthetic drugs are useful at therapeutic doses but toxic at high concentrations. Co-administration of herbs and drugs can enhance or decrease the pharmacological or toxicological effects of drugs. Synergistic therapeutic effects may also complicate long-term medication. Prescribing these types of herbal drugs without knowing the history of a patient may lead to serious side effects due to overdosing and co-administration with other synthetic medicines (Kua *et. al.,* 2003). Metformin hydrochloride, glipizide, gliclazide, glibenclamide and glimepiride. are oral anti-hyperglycemic drugs used in the management of type 2 diabetes. It improves glucose tolerance in patients with type 2 diabetes (NIDDM), lowering both basal and postprandial plasma glucose.

Table 3.10.2: ^{1}H-NMR spectral data Standard gliclazide and main peak of ADHP-06 (400 MHz, δ Value in CD$_3$OD)

Position	^{1}H (δppm) of Standard Gliclazid	^{1}H (δppm) Main peak of ADHP-06
1	1.461	1.455
2	1.660	1.679
3	2.435	2.435
4	2.606	2.607
5	3.310	3.310
6	4.877	4.858
7	7.373	7.371
8	7.393	7.391
9	7.876	7.872
10	7.895	7.891
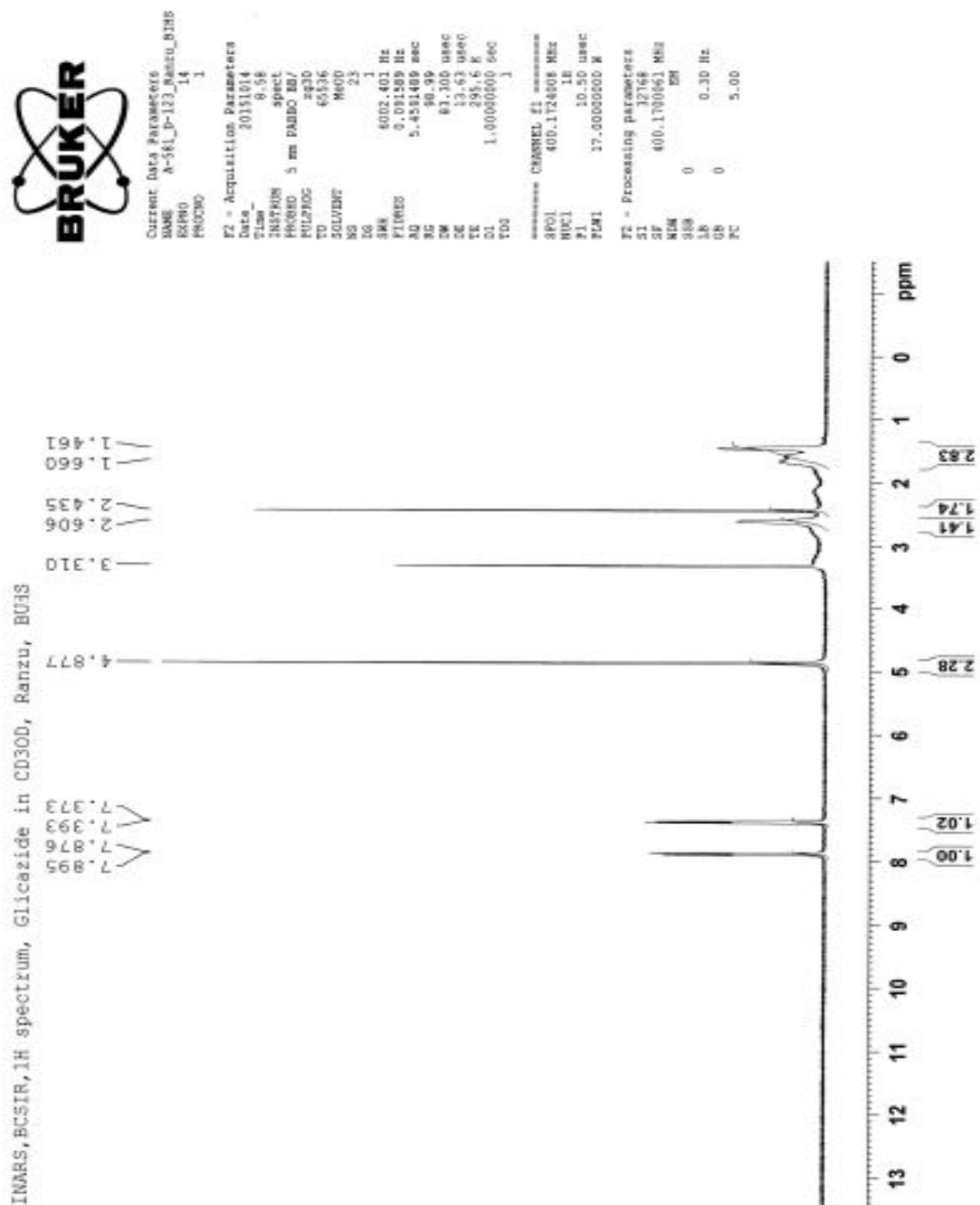
Figure-3.10.9: ¹H NMR of synthetic drug's standard gliclazide
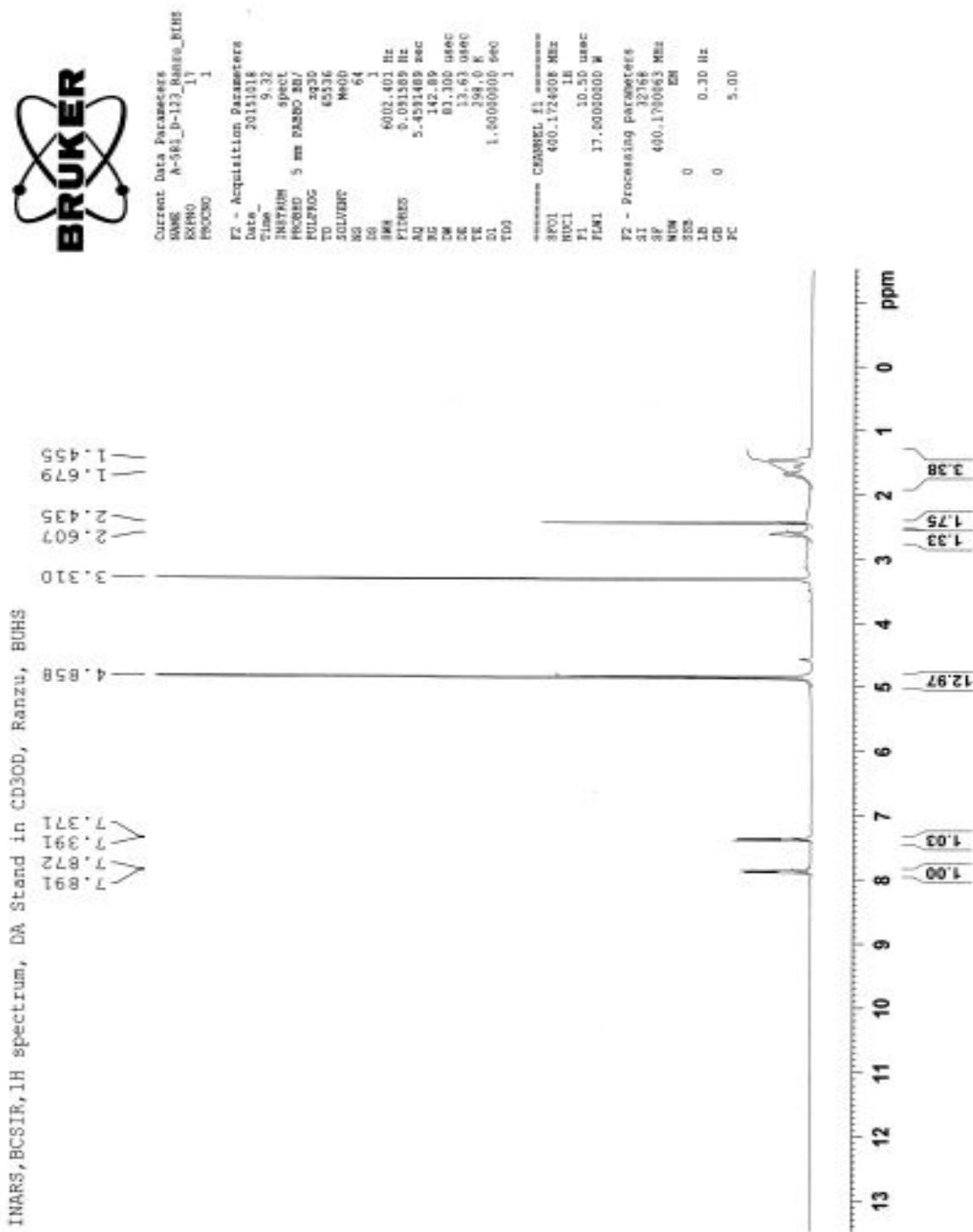
Figure-3.10.10: ^{1}H-NMR of main peak in ADHP-6

Table 3.10.3: ^{1}H-NMR spectral data standard glibenclamide and main peak of ADHP-07 (400 MHz, δ Value in CD$_3$OD)

Position	^{1}H (δppm) of Standard Glibenclamide	^{1}H (δppm) Main peak of ADHP-07
1	1.206	1.108
2	1.235	1.136
3	1.288	1.288
4	1.346	1.334
5	1.644	1.642
6	1.766	1.758
7	3.013	2.983
8	3.309	3.310
9	3.482	3.662
10	3.676	3.679
11	3.693	3.695
12	3.829	3.813
13	4.860	4.883
14	7.088	7.081
15	7.110	7.103
16	7.439	7.390
17	7.461	7.409
18	7.493	7.436
19	7.513	7.458
20	7.808	7.827
21	7.905	7.869
22	7.925	7.888
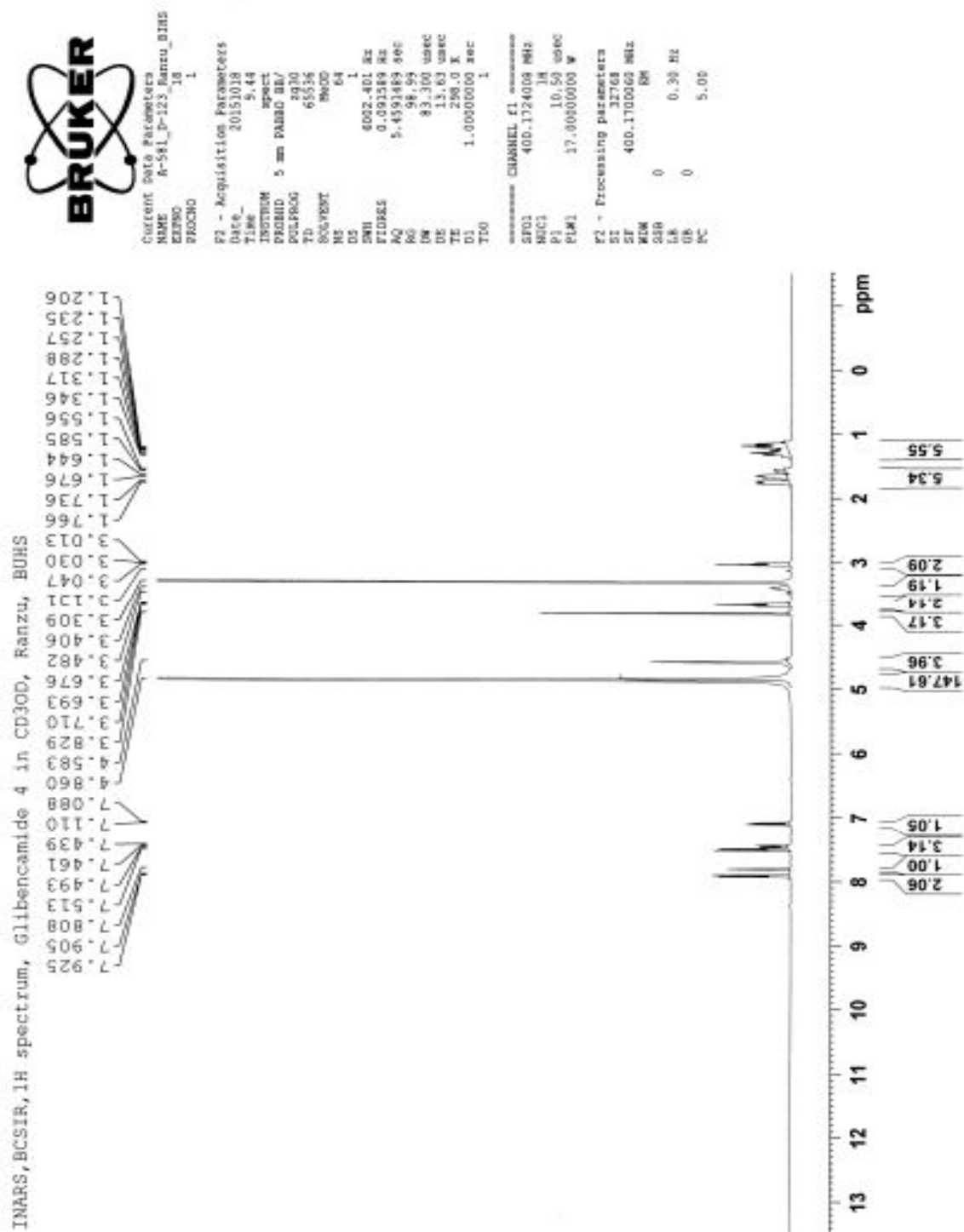
Figure-3.10.11: ¹H-NMR of synthetic drug's standard glibenclamide.
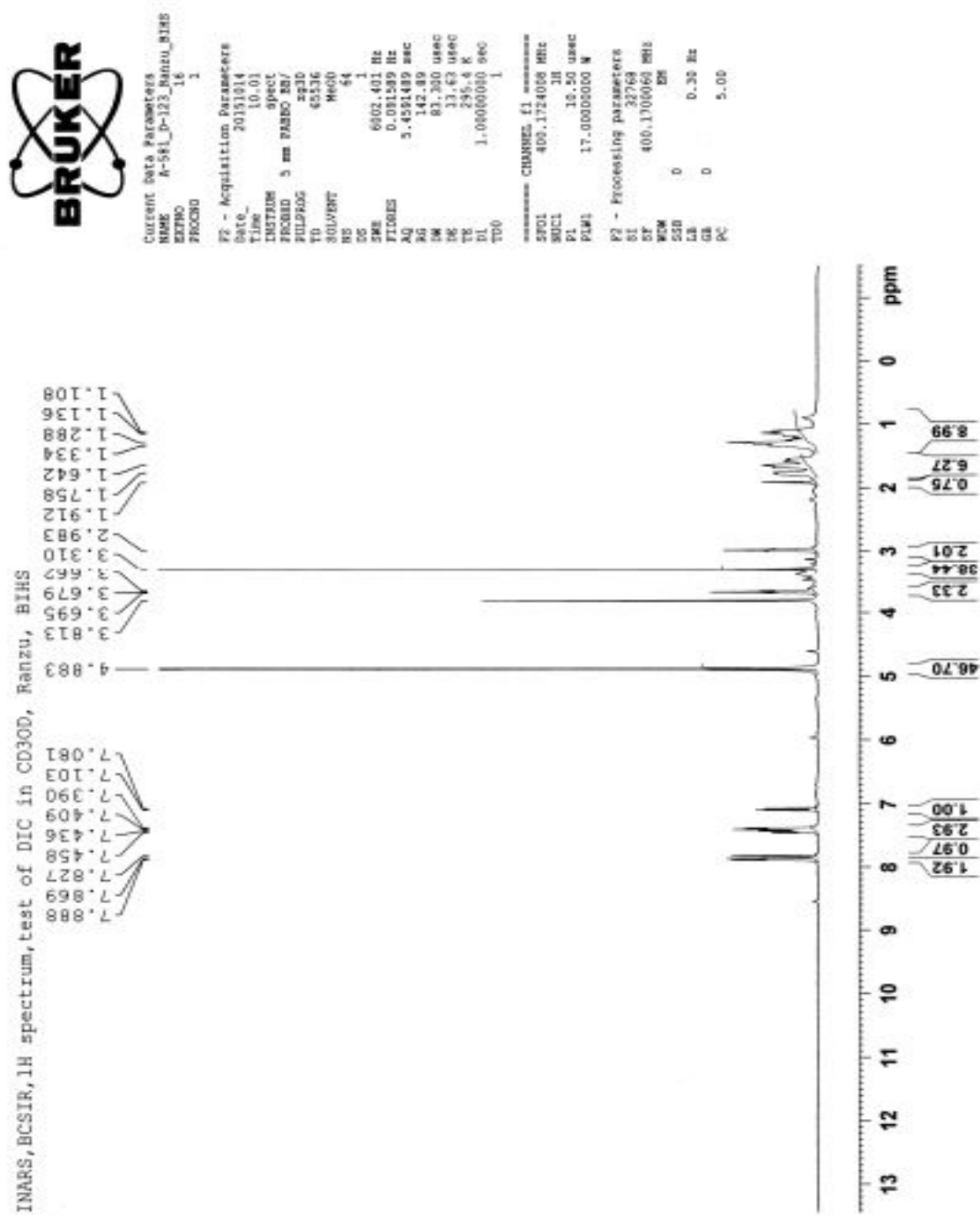
Figure-3.10.12: ^{1}H-NMR of main peak in ADHP-07

3.11 IDENTIFICATION AND QUANTIFICATION OF ANTIOXIDENTS

3.11.1 Analysis of methanolic extracts ADHPs and ADSPs by HPLC

Identification and quantification of antioxidants in the methanolic extracts of and seven antidiabetic herbal preparations (ADHP) and two antidiabetic single plant (ADSP) were analysed by HPLC. The chromatographic separations of antioxidants in standard and methanolic extracts of ADHPs and ADSPs are shown in Figures 3.11.1 - 3.11.10, respectively. The content of each phenolic compound found in the methanolic extracts of ADHPs and ADSPs calculated from the corresponding calibration curve and presented as the mean of five determinations as shown in Table 3.11.2 - 3.11.10, respectively.

3.11.2 Peak characterization and quantification

The compounds were identified by comparing with standards shown in Figure-3.11.1. Each compound was identified using the retention time, the absorbance spectrum profile and also by running the samples after the addition of pure standards. Quantification was performed by establishing calibration curves for each compound determined, using the standards. Linear calibration curves for standards (peak area vs. concentration) were constructed with R^2 exceeding 0.995. Data are reported as means ± standard deviations of triplicate independent analyses.
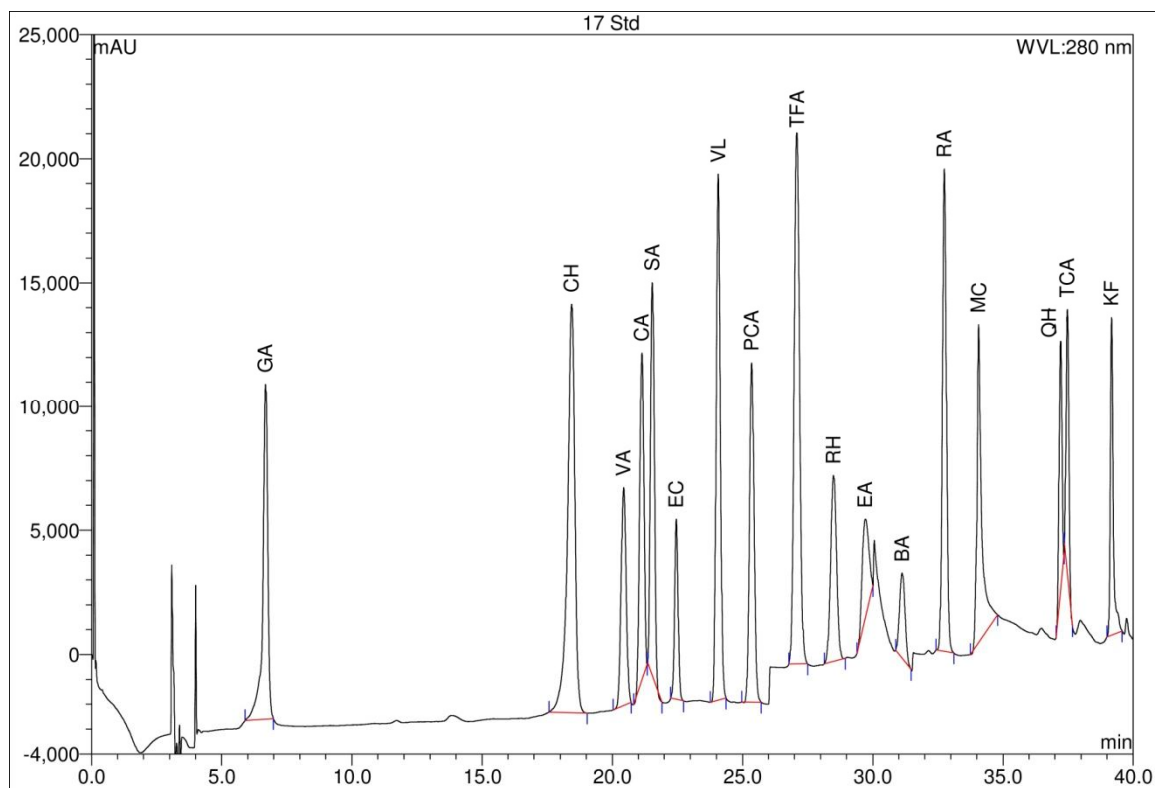
Figure-3.11.1: HPLC chromatogram of 17 standards mixture of antioxidant compounds.

Table 3.11.1: List of the name of standards used for identification and quantification of antioxidant from ADHPs and ADSPs.

Sl No	Name of the standard	Symbol	Sl No	Name of the standard	Symbol
1	gallic acid	GA	10	rutin hydrate	RH
2	(+)-catechin	CH	11	ellagic acid	EA
3	vanillic acid	VA	12	benzoic acid	BA
4	caffeic acid	CA	13	rosmarinic acid	RA
5	syringic acid	SA	14	myricetin	MC
6	(−)-epicatechin	EC	15	quercetin	QH
7	vanillin	VL	16	trans-cinnamic acid	TCA
8	p-coumaric acid	PCA	17	kaempferol	KF
9	trans-ferulic acid	TFA			

Figure-3.11.2: Specific antioxidant content's HPLC chromatogram in ADHP-1

Table 3.11.2: Contents of antioxidant compounds in the methanol extract of ADHP-1 (n=5).

Antioxidant compound	Methanol extract of **ADHP-1**	
	Content (mg/100 g of dry extract)	% RSD
GA	586.34	0.42
BA	1025.02	0.63
RA	73.16	0.05
TCA	132.12	0.04

RSD: Relative Standard Deviation

The experimental results indicated that methanol extract of ADHP-1 contained an especially high concentration of benzoic acid, gallic acid and trans-cinnamic acid (1025.02 mg, 586.35 mg and 132.12 mg per 100 g of dry weight, respectively). It was also shown that rosmarinic acid (73.16 mg/100 g of dry weight) was present moderately in this ADHP.

Figure-3.11.3: Specific antioxidant content's HPLC chromatogram in ADHP-2

Table-3.11.3: Contents of antioxidant compounds in the methanol extract of ADHP-2 (n=5).

Antioxidant compound	Methanol extract of **ADHP-2**	
	Content (mg/100 g of dry extract)	% RSD
GA	294.56	0.42
CH	21.18	0.19
VL	18.86	0.14
PCA	7.34	0.11
TFA	17.33	0.04
EA	380.98	0.56
RA	19.52	0.06

RSD: Relative Standard Deviation

The results of this experimentation indicated that methanol extract of ADHP-2 contained high concentration of gallic acid and ellagic acid (294.56 mg, and 380.98 mg per 100 g of dry weight, respectively). It was also shown that small amounts of (+)-catechin, vanillin, p-coumaric acid, trans-ferulic acid and rosmarinic acid were present in this ADHP.

Figure 3.11.4: Specific antioxidant content's HPLC chromatogram in ADHP-3

Table-3.11.4: Contents of antioxidant compounds in the methanol extract of ADHP-3 (n=5).

Antioxidant compound	Methanol extract of **ADHP-3**	
	Content (mg/100 g of dry extract)	% RSD
GA	513.51	0.42
SA	17.24	0.19
BA	1067.04	0.84

RSD: Relative Standard Deviation

In ADHP-3, the experimental results indicated that methanol extract contained an especially high concentration of benzoic acid and galic acid (1067.04 mg and 513.51 mg per 100 g of dry weight, respectively). Smaller amount of syringic acid (17.24 mg/100 g of dry weight) was also shown in this ADHP.
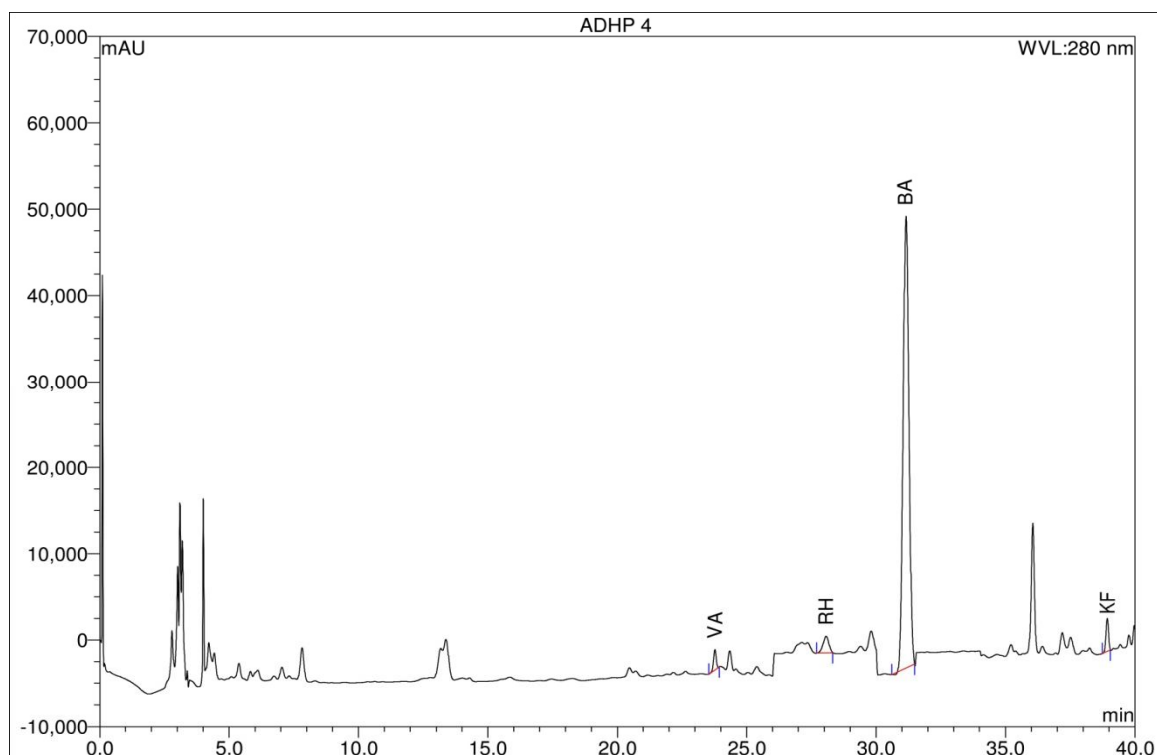
Figure-3.11.5: Specific antioxidant content's HPLC chromatogram in ADHP-4

Table-3.11.5: Contents of antioxidant compounds in the methanol extract of ADHP-4 (n=5).

Antioxidant compound	Methanol extract of **ADHP-4**	
	Content (mg/100 g of dry extract)	% RSD
VL	11.44	0.42
RH	60.61	0.19
BA	1160.82	0.64
KF	31.80	0.11

RSD: Relative Standard Deviation

The experimental results indicated that methanol extract of ADHP-4 contained prominent amount of benzoic acid (1160.82 mg per 100 g of dry weight). Rutin hydrate (60.61 mg per 100 g of dry weight) was present in moderately and very small amount of vanillin & kaempferol (11.44 mg and 31.80 mg per 100 g of dry weight, respectively) were also present in this ADHP.

266
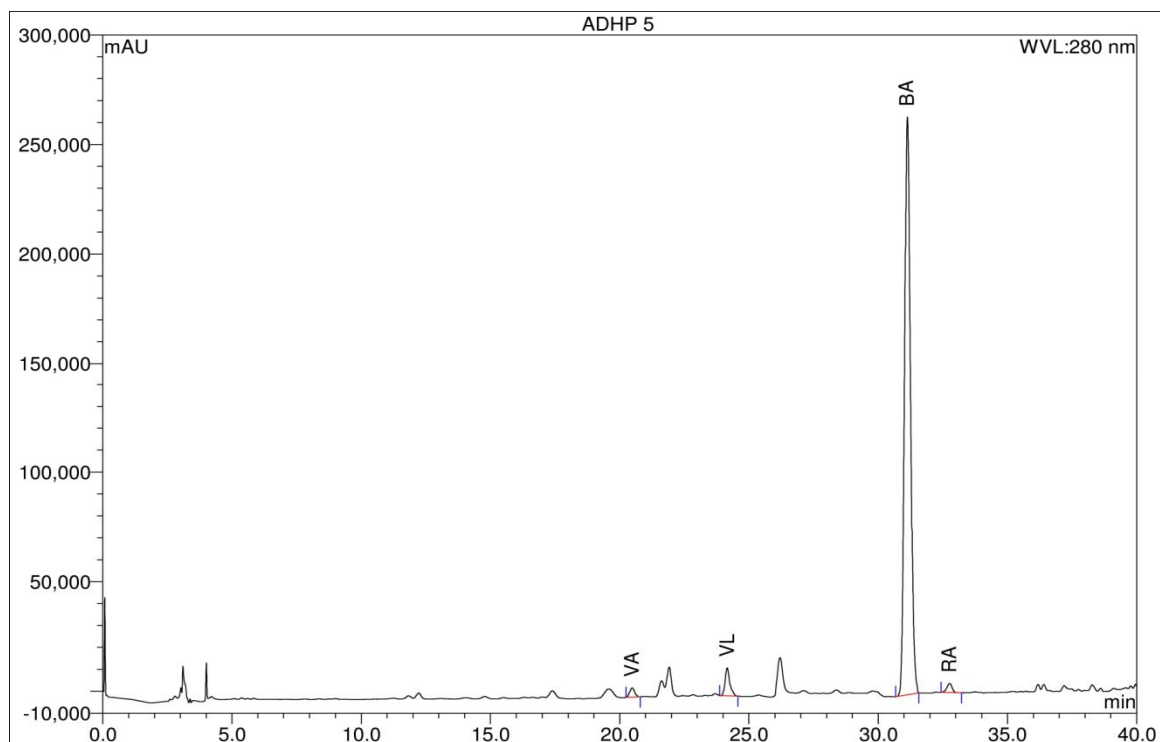
Figure-3.11.6: Specific antioxidant content's HPLC chromatogram in ADHP-5

Table-3.11.6: Contents of antioxidant compounds in the methanol extract of ADHP-5 (n=5).

Antioxidant compound	Methanol extract of **ADHP-5**	
	Content (mg/100 g of dry extract)	% RSD
VA	77.44	0.06
VL	111.18	0.03
BA	2035.83	0.75
RA	44.03	0.04

RSD: Relative Standard Deviation

In ADHP-5, the experimental results indicated that methanol extract contained an especially high concentration of benzoic acid (2035.83 mg per 100 g of dry weight). Good amount of vanillic acid & vanillin (77.44 mg and 111.18 / 100 g of dry weight) and small amount of rosmarinic acid (44.03 mg/100 g of dry weight) were also present in this ADHP.

267
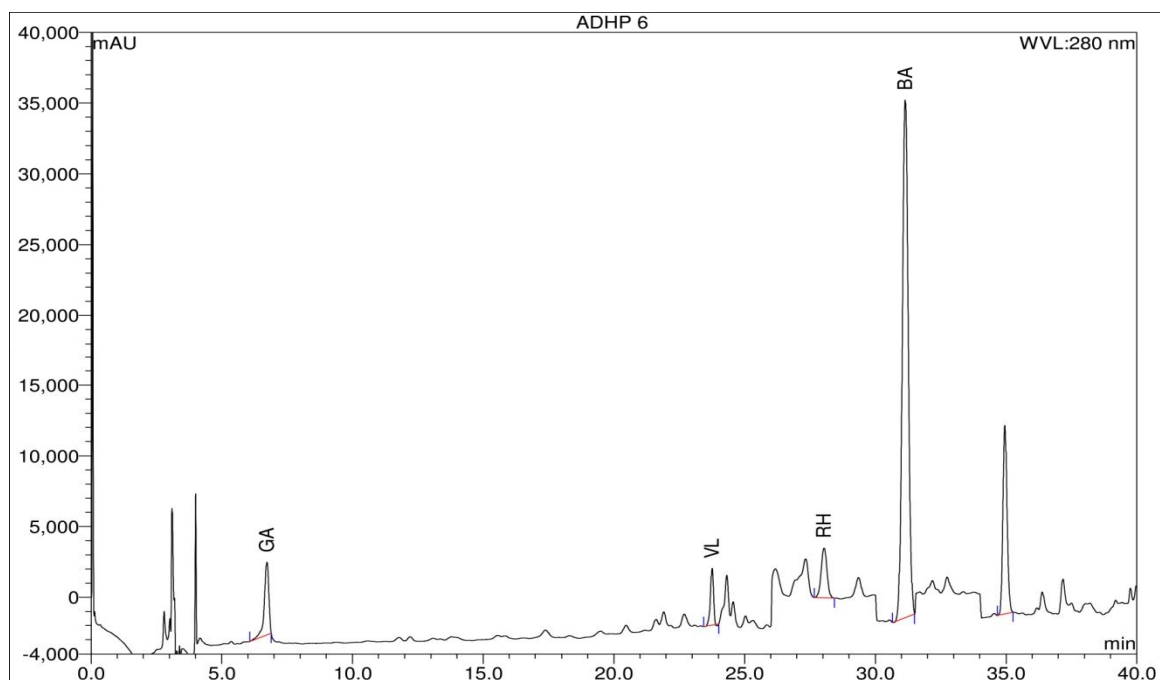
Figure-3.11.7: Specific antioxidant content's HPLC chromatogram in ADHP-6

Table-3.11.7: Contents of antioxidant compounds in the methanol extract of ADHP-6 (n=5).

Antioxidant compound	Methanol extract of **ADHP-6**	
	Content (mg/100 g of dry extract)	% RSD
GA	55.35	0.06
VL	21.73	0.03
RH	108.71	0.05
BA	633.91	0.54

RSD: Relative Standard Deviation

The experimental results indicated that methanol extract of ADHP-6 contained prominent amount of benzoic acid and rutin hydrate (638.91mg and 108.71 mg per 100 g of dry weight, respectively). Gallic acid (55.35 mg per 100 g of dry weight) was present in moderately and very small amount of vanillin (21.73 mg per 100 g of dry weight) was also shown in this ADHP.
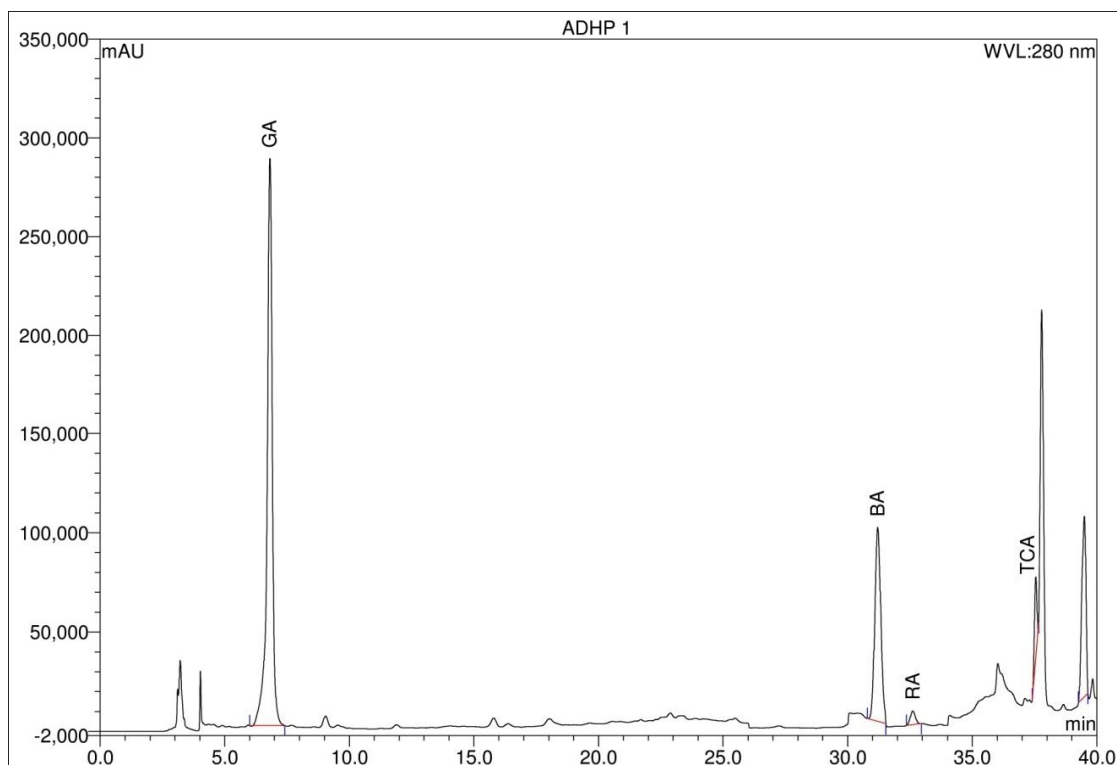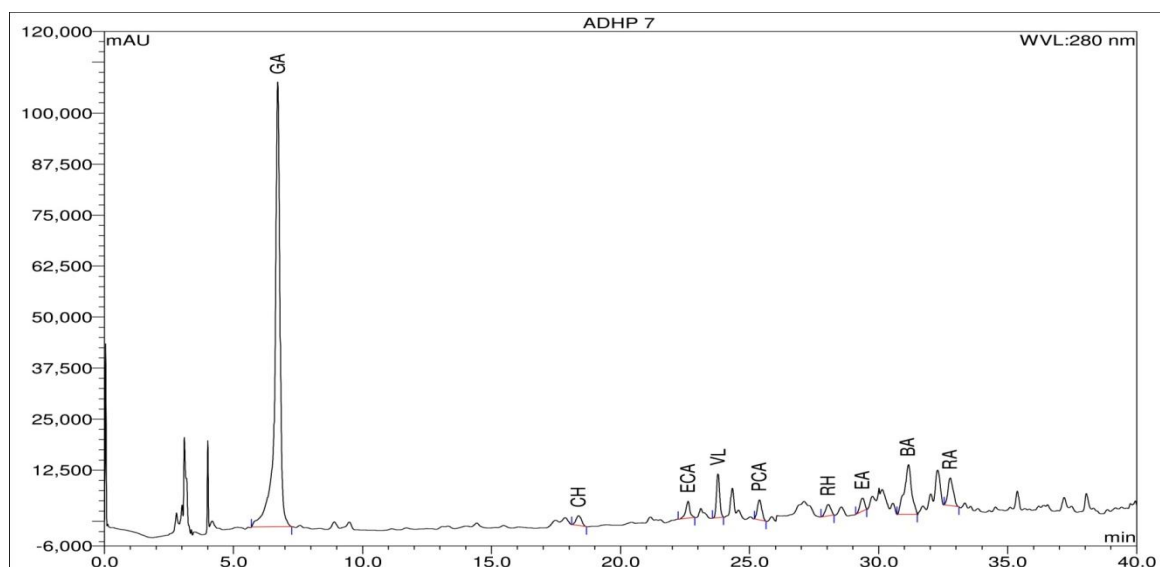
Figure-3.11.8: Specific antioxidant content's HPLC chromatogram in ADHP-7

Table-3.11.8: Contents of antioxidant compounds in the methanol extract of ADHP-7 (n=5).

Antioxidant compound	Methanol extract of **ADHP-7**	
	Content (mg/100 g of dry extract)	% RSD
GA	686.67	0.42
CH	105.86	0.19
EC	134.85	0.14
VL	60.64	0.11
PCA	27.95	0.04
RH	79.15	0.16
EA	530.10	0.26
BA	1016.23	0.33
RA	71.82	0.05

RSD: Relative Standard Deviation

In ADHP-7, the experimental results indicated that methanol extract contained especially high concentration of benzoic acid, gallic acid and ellagic acid (1016.23 mg, 686.67 mg and 530.10 mg per 100 g of dry weight, respectively). Prominent amount of (+)-catechin & (-)-epicatechin (105.86 mg and 134.85 mg / 100 g of dry weight, respectively), good amount of vanillin, rutin hydrate and rosmarinic acid (60.64 mg, 79.15 mg and 71.82 mg

/ 100 g of dry weight) and small amount of *p*-coumaric acid (27.95 mg/100 g of dry weight) were also present in this ADHP.
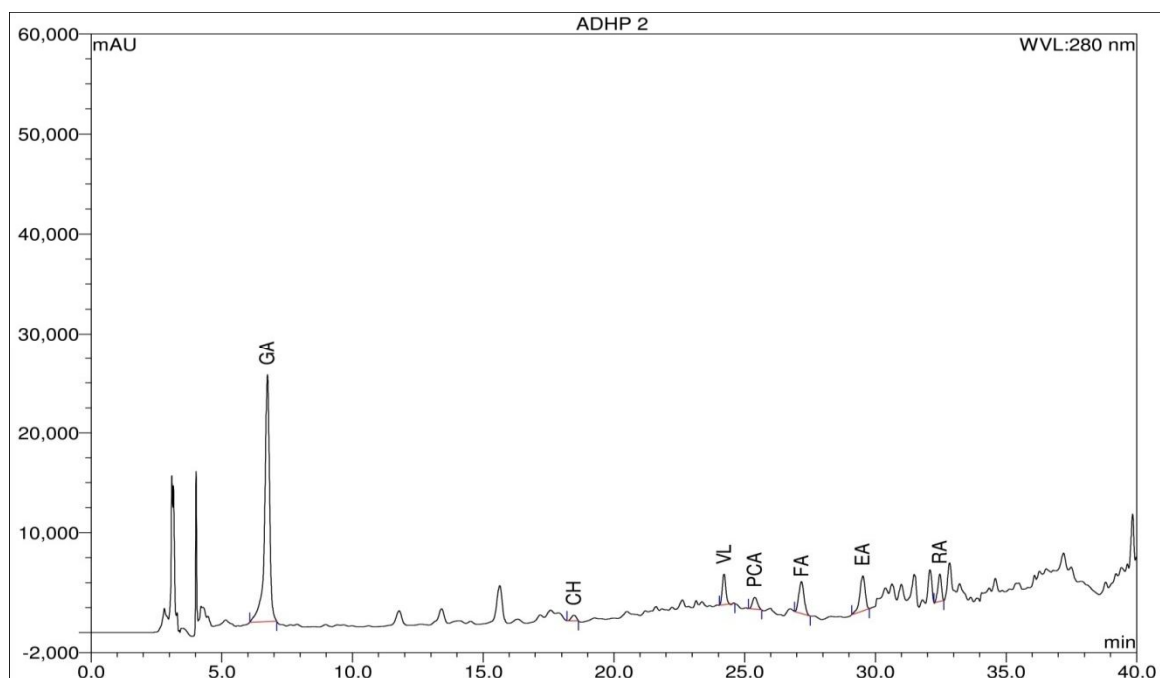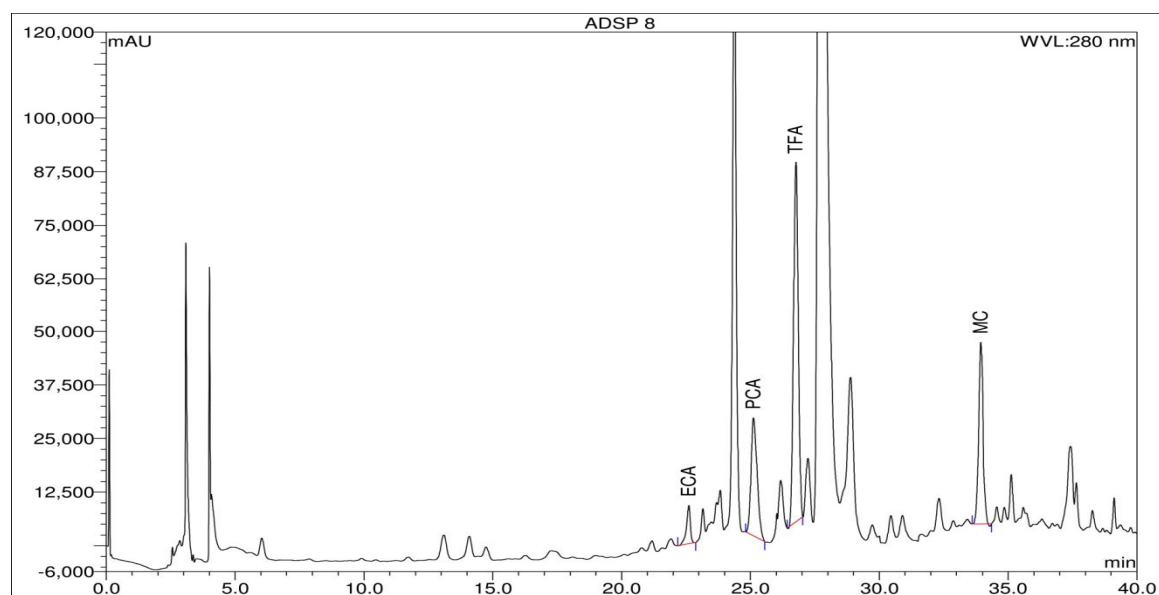

Figure-3.11.9: Specific antioxidant content's HPLC chromatogram in ADSP-8

Table-3.11.9: Contents of antioxidant compounds in the methanol extract of ADSP-8 (n=5).

Antioxidant compound	Methanol extract of **ADSP-8**	
	Content (mg/100 g of dry extract)	% RSD
EC	268.63	0.16
PCA	214.69	0.06
TFA	220.84	0.03
MC	190.07	0.05

RSD: Relative Standard Deviation

The experimental results indicated that methanol extract of ADSP-8 contained prominent amount of (-)-epicatechin, p-coumaric acid, trans-ferulic acid and myricetin (268.63 mg 214.69 mg, 220.84 mg and 190.07 mg per 100 g of dry weight, respectively).
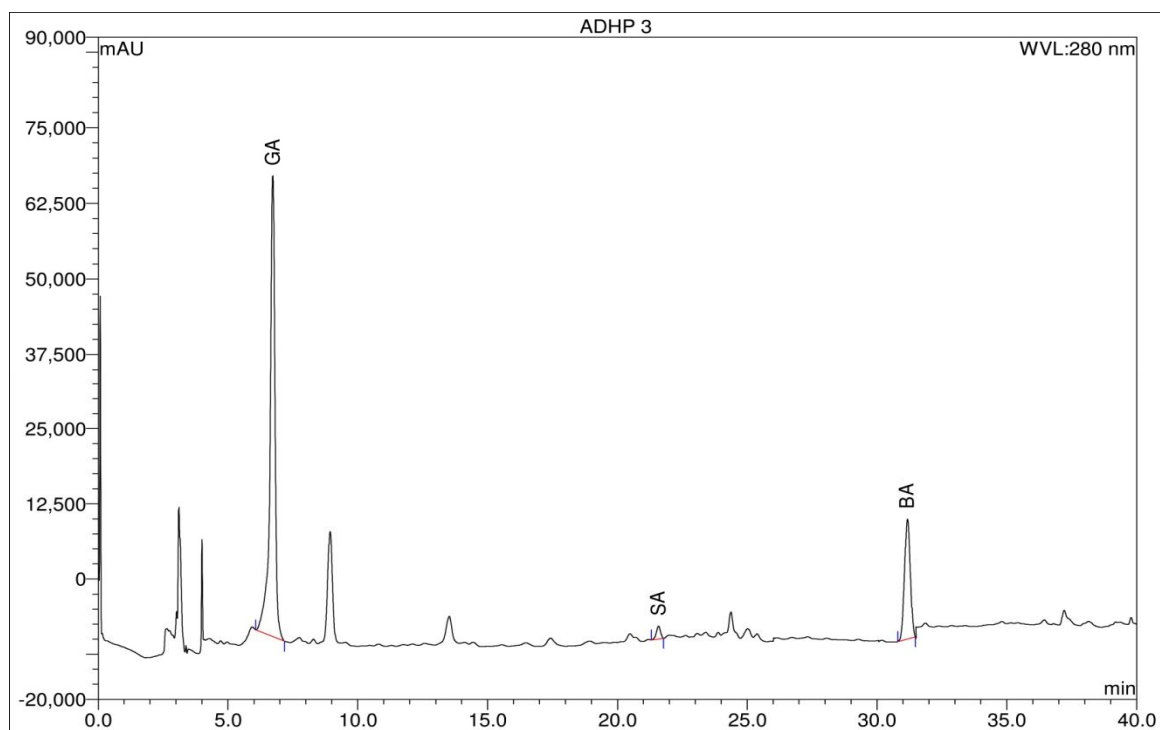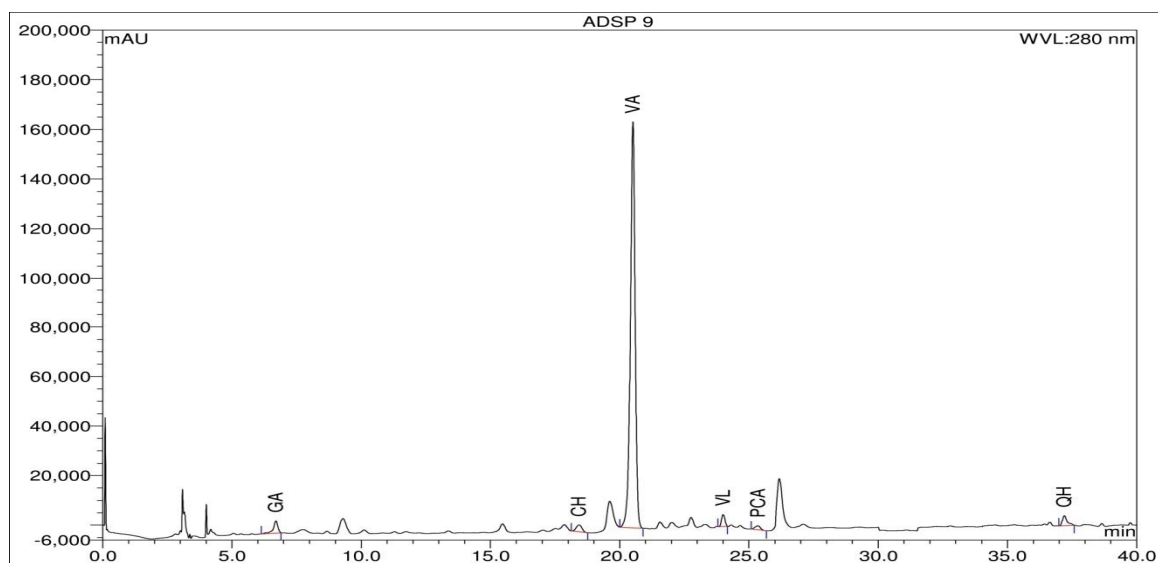
Figure-3.11.10: Specific antioxidant content's HPLC chromatogram in ADSP-9

Table-3.11.10: Contents of antioxidant compounds in the methanol extract of **ADSP-9** (n=5).

Antioxidant compound	Methanol extract of **ADSP-9**	
	Content (mg/100 g of dry extract)	% RSD
GA	56.47	0.42
CH	126.65	0.19
VA	895.33	0.44
VL	28.54	0.11
PCA	10.77	0.08
QH	54.53	0.12

RSD: Relative Standard Deviation

In ADSP-9, the experimental results indicated that methanol extract contained especially high concentration of vanillic acid and prominent amount of (+)-catechin (895.33 mg, and 126.65 mg per 100 g of dry weight, respectively). Good amount of gallic acid, quercetin hydrate (56.47 mg and 54.53 / 100 g of dry weight) and small amount of vanillin, *p*-coumaric acid (28.54 mg & 10.77 mg /100 g of dry weight) were also present in this ADSP.

Table 3.11.11: Comparative study of different antioxidant contents in ADHPs and ADSPs

Code	GA	CH	VA	CA	SA	EC	VL	PCA	TFA	RH	EA	BA	RA	MC	QH	TCA	KF
ADHP-1	586.34											1025.02	73.16			132.12	
ADHP-2	294.56	21.18					18.86	7.34	17.33		380.98		19.52				
ADHP-3	513.51				17.24							1067.04					
ADHP-4							11.44			60.61		1160.82					31.80
ADHP-5			77.44				111.18					2035.83	44.03				
ADHP-6	55.35						21.73			108.71		633.91					
ADHP-7	686.67	105.86				134.85	60.64	27.95		79.15	530.10	1016.23	71.82				
ADSP-8						268.63		214.69	220.84					190.07			
ADSP-9	56.47	126.65	895.33				28.54	10.77							54.53		

ADHP→ Antidiabetic Herbal Preparation, ADSP→ Antidiabetic Single Plant.

Gallic Acid: Gallic acid, a naturally occurring plant phenol with antioxidant activity (Inoue *et. all.,* 1994). It and its derivatives have a large number of applications in various fields of science. In nature, these compounds are widely distributed in plants and fruits, and thus they are being used as food stuffs, preservatives, etc. directly or indirectly by human community. They have also been implicated as anticarcinogenic, antimicrobial, antimutagenic, antiangiogenic and anti-inflammatory agents besides their use in treating critical diseases like depression, cancer, microbial infections, lipid-related diseases, etc (Choubey *et. al,.* 2015). In the present study gallic acid has been identified in all ADHPs and ADSP except ADHP-4, 5 and ADSP-8.

(+)-Catechin: Catechin is a flavonoid (flavan-3-ol) found, for example, in tea, wine, some fruits, vegetables and chocolate. It has been proved that catechin has radical scavenging metal-chelating and antiproliferative effects (Maria, *et. al.,* 2016). It also has been demonstrated that (+)-catechin has the ability to inhibit HIV-1 reverse transcriptase *in vitro* (Moore, 1992). ADHP-7 and ADSP-9 had recognisable amount of (+)-catechin.

Vanillin: Vanillin contains strong antioxidant and antibacterial properties, and even cancer or tumor fighting abilities (Diet Health Club, n.d.). ADHP-2, 4, 5, 6, 7 and ADSP-9 contained good amount of vanillin.

***p*-Coumaric acid:** *p*-Coumaric acid has antioxidant properties and is believed to reduce the risk of stomach cancer (Ferguson, et. al., 2005) by reducing the formation of carcinogenic nitrosamines (Kikugawa et. al., 1983). p-Coumaric acid also have anti-inflammatory (Fernández *et. al.,* 2006), antioxidant (Arrieta-Baez *et al.,* 2012) sedative (Nugroho et al., 2012). ADHP-2, 7 and ADSP-8 (significant amount), ADSP-9 showed the presence of *p*-coumaric acid.

Rutin hydrate: Rutin hydrate have antioxidant (Gao et al., 2003), analgesic (both central and peripheral) (Gene et al., 1996, Song *et al.,* 1996), antiinflammatory (Selloum et al., 2003, Gene *et al.,* 1996) and sedative activities (Shariati et al., 2008). ADHP-4, 6 and 7 had good amount of rutin hydrate.

Ellagic acid: Various simple phenolics including gallic acid, caffeic acid, flavonoids, which give rise to tannins, are also known to exert anthelmintic action. Ellagic acid and related antioxidants are excellent acceptor of free radical and delocalise them in the

phenolic ring. Thus, they can also quench electrons from various biological systems including that of electron transport system (ETS). Disruption of electron flow in ETS results in the inhibition of oxidative phophorylation (Vattem & Shetty, 2005). Ellagic acid was identified in ADHP-2 & 7 in small amounts.

Benzoic Acid: Benzoic acid occurs naturally in many plants and it was used as an expectorant, analgesic, and antiseptic in the early 20th century. (Lillard Benjamin, 1919). 2-hydroxy-4-methoxy benzoic acid has also showed antioxidant effect on ethanol induced hepatoxicity in rats (Saravanan *et. al.,* 2007). All ADHPs (except ADHP-2) showed the presence of significant amount of benzoic acid. Benzoic acid may also be generated from sodium benzoate used as a preservative.

Rosmarinic Acid: Rosmarinic acid is a polyphenol similar to caffeic acid (found in coffee) and is in high levels in perilla oil and rosemary, from which it draws its name. It is a general antioxidant and health compound, and seen as 'healthy'. (Lee *et. al.,* 2013 and Examine.com, https://examine.com/supplements/rosmarinic-acid/).

4. CONCLUSION

AND

RECOMMENDATION

4.0 CONCLUSION AND RECOMMENDATION

4.1 CONCLUSION

The obtained results revealed that:

Among the studied eleven ADHPs, ADHP-2, ADHP-4 and ADHP-6 showed significant blood glucose lowering effect in type 2 diabetic and some improvement in lipid profile of type 2 diabetic model rats. ADHP-1, ADHP-3, ADHP-6 and ADHP-7 samples showed significant antioxidant activity.

All the tested ADHPs were within acceptable limit of microbial contamination.

Most of the ADHPs contained small amount of toxic metals but some of ADHPs (ADHP-3, ADHP-8, ADHP-9 and ADHP-10) crossed the safety limit set by WHO, US FDA and HAS Singapore permissible limit.

Some organochlorine pesticides were found to be present in ADHP-1, ADHP-2, ADHP-6 and ADHP-7 which are not expectable.

All the tested ADHPs contained antidiabetic drug metformin hydrochloride in small amounts. Large amount of gliclazide and glibenclamide were identified and quantified in ADHP-6 and ADHP-7, respectively.

The roots of *S haematodes* were studied for alpha-glucosidase inhibition activity, antioxidant activity & sunscreen protection activity and it showed significant results in all aspects. Two compounds namely β- sitosterol (**1**) & stigmasterol (**2**) were isolated from the dichloromethane extract and 7 hydroxy coumarin (**3**) were isolated from the chloroform fraction of EtOAc extract of root of this plant.

The flowers of *P thyrsiflorus* possessed hypoglycemic and to some extent hypolipidemic properties. β- Sitosterol (**4**), stigmasterol (**5**), 8(17),13-labdadien-15,16-olide-19-oic acid (**6**), 19-hydroxy-8(17),13-labdadien-15,16-olide (**7**) were isolated from the dichloromethane extract and 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone (luteolin) (**8**) was isolated from the 1-butanol part of methanol extract of the *P*

thyrsiflorus flowers. The obtained antidiabetic activity by the ethanolic extract of *P thyrsiflorus* flowers due to the presence of compound (**4**) and Compound (**7**) was justified by insulin secratory activity test on mice.

4.2 RECOMMENDATION:

The present study showed that some toxic metals and organochlorine pesticides are found to be present in ADHPs. In general, this contamination may come from raw materials, during processing of raw materials and manufacturing of finished products due to the production environment. In a nutshell, finished products should reach consumers with zero contamination; quality has to be maintained throughout the process beginning from the selection of raw material to the final product. On the other hand some synthetic antidiabetic agents were added to increase the activity of the herbal preparations which are not desirable. Taking these facts into consideration, regulatory agencies should come forward and take the necessary measures to ensure the safety of finished herbal preparations. It is evident that there is an urgent need to implement a regular monitoring and testing program on the quality of the local and imported herbal medicine sold in the Bangladeshi market.

5. REFERENCES

5.0 REFERENCES

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6.0   LIST OF PUBLICATIONS

Publications from PhD thesis works.

1. **Md Ranzu Ahmed,** Begum Rokeya, AK Azad Khan, Md. Rausan Zamir, M Abu Sayeed and M Mosihuzzaman, Efficacy Appraisal of Some Antidiabetic Herbal Preparations Available in Bangladesh. *British Journal of Pharmaceutical Research, 8(3): 1-6, 2015, Article no.BJPR.19870 ISSN: 2231-2919;* SCIENCEDOMAIN international.

2. **Md Ranzu Ahmed,** S. M. Z. H. Asna, M. S. H. Khan, Begum Rokeya, M. Mosihuzzaman and M. Abu Sayeed, Microbial Contamination of Some Antidiabetic Herbal Preparations Available in Bangladesh. *European Journal of Medicinal Plants,* 13(1): 01-05, 2016, Article no.EJMP.23556, ISSN: 2231-0894, NLM ID: 101583475, SCIENCEDOMAIN international.

3. **Md Ranzu Ahmed**, Tania Sultana, R Routary, MSH Khan, M Abu Sayeed, M Mosihuzzaman and Begum Rokeya. Chemistry and antidiabetic effects of *Phlogocanthus thyrsiflorus* Nees flowers. (Manuscript has been prepared for submitting in the journal of Fitoterapia, Elsevier).

Publications from PhD thesis related works.

4. Hemayet Hossain, Abdullah-Al-Mamun, Sanjida Akter, Umme Sara, and **Md. Ranzu Ahmed,** Evaluation of anti-inflammatory activity and total tannin content from the leaves of *bacopa monnieri* (linn.) (IJPSR/RA-3267/11-13, Vol. 5, Issue 04; April, 2014 Issue of International Journal of Pharmaceutical sciences and Research (An Official Publication of Society of Pharmaceutical sciences and Research).

5. Ismet Ara Jahan, Proity Nayeeb Akbar, Mohammad Enayetullah, Nazir Ahmmad, Mohammad Nuruddin and **Md Ranzu Ahmed,** 2015/EJMP/20312 (Entitled: "Elemental And Fatty Acid Content Of Four Medicinal Plants: Kaiempferia Rotunda, Cuscuta Reflexa, Centella Asiatica And Asparagus Racemosus.") published in European Journal of Medicinal Plants, *10(4): 1-10, 2015, Article no.EJMP.20312, ISSN: 2231-0894, NLM ID: 101583475,* SCIENCEDOMAIN *international*

6. Ismet Ara Jahan, Proity Nayeeb Akbar, Mohammad Enayetullah, Nazir Ahmmad, and **Md Ranzu Ahmed** (2015/IRJPAC/20449) (Entitled: Elemental and Fatty Acid Content of the Tubers of *Ipomoea Digitata* at Different Ages of the Plant) has been accepted for publication in International Research Journal of Medicinal Plants.

7.0   PUBLISHED ABSTRACTs

1. Evaluation of chemical and antidiabetic properties of some herbal preparations on diabetic model rats in 2nd Indian National Seminar ' PLANTS IN DIABETES: PROSPECTS & CHALLENGES, Phanidhar Dutta Seminar Hall, Guwahati University, Assam, India, 08 - 09 April, 2011.

   M R Ahmed, B Rokeya, L Ali, S Murshed, R Zamir, MA Sayeed & M Mosihuzzaman.

2. Antidiabetic properties of some herbal preparations on diabetic model rats (Abstract number: P-1174, IDF -2011, World Trade Centre, Dubai, UAE, 04-08 December, 2011)

   M Ahmed, MA Sayeed, B Rokeya, S Murshed, R Zamir & M Mosihuzzaman.

3. Chemical and Bilogical Studies on Antidiabetic Composite Herbal Preparations (17th "Diabetes & Endocrine conference , BIHS, Mirpur, Dhaka-1216, 13-15 December, 2011

   MR Ahmed, M Mosihuzzaman, MA Sayeed, L Ali, S Murshed, R Zamir & B Rokeya

4. Biological Studies on Antidiabetic Composite Herbal Preparations (ISE-2012, Kolkata, INDIA, 17-19 February, 2012)

   MR Ahmed, R Zamir, L Ali, S Murshed, MA Sayeed, B Rokeya and M Mosihuzzaman.

5. Studies of Harmful Constituent in Antidiabetic Herbal Preparations Available in Bangladesh (ISE-2012, Kolkata, INDIA, 17-19 February, 2012)

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6. Chemical Studies of Herbal Preparations Found to Have Antidiabetic Effects (3rd Indian National Seminar, RG Kar Medical College & Hospital, Kolkata, India, 14-16 September, 2012)

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