

**BIOASSAY GUIDED ISOLATION AND
CHARACTERIZATION OF SECONDARY
METABOLITES FROM INDIGENOUS *CESTRUM*
SPECIES**



Ph. D Thesis

By

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SECONDARY METABOLITES FROM INDIGENOUS *CESTRUM* SPECIES**



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DECLARATION

The materials contained within this dissertation are my original work and have not been previously submitted to this or any other institution/university.

Humaira Inayat

April, 2016

I owe this work to all my teachers,

from whom I have learnt;

to my dear and loving parents who taught me

the first word in this world

and to my encouraging husband!

CONTENTS		Page
Acknowledgments		i
List of abbreviations.....		iv
List of tables.....		viii
List of schemes		ix
List of figures.....		x
List of graphs.....		xii
ABSTARCT		xiii
CHAPTER 1: INTRODUCTION.....		1
1. General introduction		1
1.1 Family: Solanaceae		3
1.1.1. Genus: <i>Cestrum</i>		4
1.1.2 <i>Cestrum nocturnum</i> L.		4
1.1.3. <i>Cestrum diurnum</i> L.		5
1.1.4. General importance of <i>Cestrum</i> species		6
1.2 Phytochemicals of <i>Cestrum</i>		8
1.2.1. Saponins from <i>Cestrum</i> species		9
1.3 Structure of saponins		10
1.3.1. The saccharide moiety		11
1.3.2. Types of monosaccharides		12
1.4 Physical and chemical properties of saponins		12
1.5 Biological activities of saponins		14
1.5.1. Anti-microbial activity		15
1.5.2. Fungicidal activity		15
1.5.3. Larvicidal activity		15
1.5.4. Pesticidal activity		16
1.5.5. Molluscicidal activity		16

1.5.6.	Insecticidal activity	16
1.5.7.	Anti-feedant activity	17
1.6	Objective	18
1.7	Plan of work	18
CHAPTER 2: RESULTS AND DISCUSSION.....		20
2.1	General	20
2.2	Biological activities	20
2.2.1	Anti-microbial activities	21
2.2.1.1	Anti-microbial activities of <i>Cestrum nocturnum</i> - Aerial part (CN-A)	23
2.2.1.2	Antimicrobial activities of <i>Cestrum diurnum</i> Aerial part- with -out berries (CD-A)	31
2.2.1.3	Antimicrobial activities of <i>Cestrum diurnum</i> fresh green berries	34
2.2.1.4	Antimicrobial activities of <i>Cestrum diurnum</i> , Ripe berries	37
2.2.1.5	Anti-bacterial and anti-fungal activities of crude saponins extract CSE	40
2.2.2	Anti-leishmanial activities	49
2.2.3	Anti-oxidant activities	57
2.3	Phytochemical investigations	61
2.3.1:	Characterization of Hum-V	61
2.3.2	Characterization of Hum-II	80
2.3.3	Characterization of Hum-IV	94
2.4	Conclusions	110
CHAPTER 3 EXPERIMENTAL.....		111
PART A- PHYTO-CHEMISTRY:		
3.1	General Experimental Conditions	111
3.1.1.	Melting points	111

	3.1.2.	UV-lamp	111
	3.1.3.	IR Spectrophotometer	111
	3.1.4.	Mass Spectrometer	111
	3.1.5.	Nuclear Magnetic Resonance	111
	3.1.6.	Solvents	112
3.2		Techniques employed for purification of compounds	112
	3.2.1	Column Chromatography	112
	3.2.2	TLC/PTLC	112
3.3		Spray reagents	113
	3.3.1.	Ceric sulphate	113
	3.3.2.	Anisaldehyde	113
3.4		Plant material	113
	3.4.1.	Extraction and fractionation of <i>Cestrum nocturnum</i>	113
	3.4.2.	Extraction and fractionation of <i>Cestrum diurnum</i> arial portion	116
	3.4.3.	Extraction and fractionation of <i>Cestrum diurnum</i> ripe berries	118
3.5.		Isolation of compounds	120
	3.5.1.	Extraction and isolation of constituents from <i>Cestrum nocturnum</i>	120
	3.5.2.	Extraction and isolation of constituents from CD-A	122
	3.5.3.	Extraction and isolation of constituents from <i>Cestrum diurnum</i> (CD-R)	122

Part B- BIOACTIVITIES

3.6.		Antibacterial assay	125
	3.6.1.	Microorganisms used	125
	3.6.2.	Preparation of agar-plates	125
	3.6.3.	Preparation of stock sample	125
	3.6.4.	McFarland turbidity standard	125
	3.6.5.	Preparation of standards	126

3.6.6.	Application of test	126
	i- Anti-bacterial test	127
	ii- Antifungal assay	127
3.6.7.	Results	128
3.7.	Antioxidant assay	128
3.8.	Cultivation and isolation of parasite	129
3.8.1.	Media preparation	129
3.8.2.	Preparation for leishmania culture	129
3.8.3.	Leishmania parasite collection	129
3.8.4.	Leishmania growth	129
3.8.5.	Experiment Design for chemotherapy	129
CHAPTER 4: REFERENCES.....		131

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LIST OF ABBREVIATIONS

C	<i>Cestrum</i>
CN	<i>Cestrum nocturnum</i>
CD	<i>Cestrum diurnum</i>
CN-A	<i>Cestrum nocturnum</i> Aerial
CD-A	<i>Cestrum diurnum</i> Aerial
CD-G	<i>Cestrum diurnum</i> -Green berries
CD-R	<i>Cestrum diurnum</i> -Ripe berries
CSE	Crude Saponins Extract
^1H	$^1\text{Proton}$
^{13}C	$^{13}\text{Carbon}$
NMR	Nuclear Magnetic Resonance
1-D NMR	one dimensional Nuclear Magnetic Resonance
2-D NMR	one dimensional Nuclear Magnetic Resonance
MS	Mass spectrometry
UV visible spectroscopy	Ultra-violet visible spectroscopy
FT-IR	Fourier transform infra-red spectroscopy
HMBC	Hetero-nuclear Multiple Bond Coherence
Hetero-COSY	Correlation spectroscopy
HMQC	Heteronuclear Multiple Quantum Coherence
FAB-MS	Fast Atom Bombardment- mass spectrometry
m.p.	Melting Point

°C	Degree Celsius
cm	Centi meter
mm	Mili meter
mL	Mili liter
L	Liter
TLC	Thin Layer Chromatography
CC	Column Chromatography
VLC	Vacuum Liquid Chromatography
HPLC	High Performance Liquid Chromatography
HPTLC	high performance thin layer chromatography
GC-MS	Gas chromatography-mass spectrometry
Prep-HPLC	Preparative High Performance Liquid Chromatography
WHO	World Health Organization
%	Percentage
μ	Micro
β	Beta
α	Alpha
δ	Delta
λ	Wave length
<i>m/z</i>	Mass/charge
g	Gram
EC	<i>Escherichia coli</i>
PA	<i>Pseudomonas aeruginosa</i>

SF	<i>Shigella flexneri</i>
SA	<i>Staphylococcus aureus</i>
BS	<i>Bacillus subtilis</i>
KP	<i>Klebsiella pneumonia</i>
EC	<i>Erwinia carotovora</i> ,
ST	<i>Salmonella typhi</i>
BA	<i>Bacillus atrophaeus</i>
CA	<i>Candida albicans</i>
TL	<i>Trichophyton longifusus</i>
AF	<i>Aspergillus flavus</i>
MC	<i>Microsporium canis</i>
FS	<i>Fusarium solani</i>
CG	<i>Candida glabrata</i>
DPPH	2,2-diphenyl-1-picrylhydrazyl
ROS	reactive oxygen species
Pyridine-d ₅	Deuterated pyridine
δ_H	Proton chemical shift
δ_C	Carbon-13 chemical shift
Gal	Galactose
Glc	Glucose
Xyl	Xylose
D	Dextro-rotatory
L	Levo-rotatory

$[M]^+$	Molecular ion peak
MHz	Mega hertz
j	Coupling constant
s	Singlet
d	Doublet
m	Multiplet
MIC	Minimum Inhibition Concentration
ATCC	American Type Culture Collection
RPMI medium	Roswell Park Memorial Institute medium
fig	Figure

List of Tables		Page
Table 2.1	Bacterial and fungal strains used for anti-bacterial and anti-fungal studies.	22
Table 2.2	Anti-bacterial and anti-fungal activities of <i>Cestrum nocturnum</i> aerial part (CN-A).	24
Table 2.3	Minimum inhibitory concentration ($\mu\text{g/ml}$) in the antibacterial and anti-fungal activities of <i>Cestrum nocturnum</i> aerial part (CN-A).	26
Table 2.4	Anti-bacterial and anti-fungal activities of crude extract and fractions of <i>Cestrum nocturnum</i> L aerial part (CN-A).	29
Table 2.5	Anti-bacterial and anti-fungal activities of crude extract and fractions of <i>Cestrum diurnum</i> L (Aerial part).	32
Table 2.6	Anti-bacterial and anti-fungal activities of crude extract and fractions of <i>Cestrum diurnum</i> L (green berries) CD-G.	35
Table 2.7	Anti-bacterial and anti-fungal activities of crude extracts and fractions of <i>Cestrum diurnum</i> L (Ripe berries) CD-R.	38
Table 2.8	Anti-bacterial and anti-fungal activities of crude saponins extracts of CN-A, CD-D, CD-G and CD-R.	41
Table 2.9	Anti-leishmanial activities of <i>Cestrum nocturnum</i> and <i>Cestrum diurnum</i>	54
Table 2.10	The anti-oxidant potentials of CN-A, CD-A CD-G and CD-R	59

Table 2.11	^{13}C and ^1H NMR spectral data of Hum-V from one and two dimensional experiments	67
Table 2.12	^{13}C and ^1H NMR spectral data of Hum-II from one and two dimensional experiments	85
Table 2.13	^{13}C and ^1H NMR spectral data of Hum-IV from one and two dimensional experiments	102

List of Schemes		Page
Scheme 1	Extraction and fractionation of <i>Cestrum nocturnum</i>	115
Scheme 2	Extraction and fractionation of <i>Cestrum diurnum</i>	117
Scheme 3	Extraction and fractionation of <i>Cestrum diurnum</i> berries (CD-R)	119
Scheme 4	Extraction and isolation of compounds from CN-A	121
Scheme 5	Extraction and isolation of compounds from CSE of CD-A	123
Scheme 6	Extraction and isolation of compounds from CD-R, CSE.	124

List of Figures		Page
1.1	<i>Cestrum nocturnum</i> flowers	5
1.2	<i>Cestrum diurnum</i> mature black berries	6
1.3	<i>Cestrum diurnum</i> flowers and pre-mature berries	6
1.4	The genins of some important classes of saponins.	11
2.8	Inhibition by <i>Cestrum nocturnum</i> and <i>Cestrum diurnum</i> against different microbes.	43
2.9	Giemsa stain image showing the amastigote in macrophages	53
2.10	Giemsa stain image showing the epimastigote in macrophages in positive control.	53
2.13	Hum-V, (Nocturnoside A) (25 <i>R</i>)- spirost-5ene-2,3-diol, [2 α , 3 β]3-O- $\{\beta$ -D-glucopyranosyl (1 $\rightarrow$ 3)- β -D-glucopyranosyl(1 $\rightarrow$ 2)- β -D-glucopyranosyl $\{(1\rightarrow3)\text{-}\beta$ -D-xylopyranosyl] (1 $\rightarrow$ 4)- β -D-galactopyranoside	64
2.14	FAB-MS (negative ion mode) Fragmentation pattern of Hum-V, m/z 1210 [M-H] ⁻ (calculated for C ₅₆ H ₈₉ O ₂₈ 1209)	65
2.15	¹³ C NMR (100.61 MHz) chemical shifts δ_C of Hum-V in pyridine	66
2.16	¹ H-NMR chemical shifts δ_H of Hum-V in pyridine-d ₅	77

2.17	HMBC connectivity's of Hum-V (500 MHz, pyridine-d ₅) ¹³ C-NMR chemical shifts are marked in Italics and the arrow indicates the connectivity's from proton to carbon.	78
2.18	Mass fragmentation pattern of Hum-V	79
2.19	¹³ C NMR (100.61 MHz) chemical shifts δ _C of Hum-II in pyridine	84
2.20	FAB-MS (negative ion mode) Fragmentation pattern of Hum-II, m/z 1047 [M-H] ⁻ (calculated for C ₅₆ H ₈₉ O ₂₆)	91
2.21	¹ H-NMR chemical shifts δ _H of Hum-II in pyridine-d ₅	92
2.22	HMBC connectivity's of Hum-II (500 MHz, pyridine-d ₅) ¹³ C-NMR chemical shifts are marked in Italics and the arrow	93
2.23	Hum-IV: 3-O-β-D-xylopyranoside-olean-12-en-28-oic acid-28-O-β-arabinopyranosyl-(1-3)-β-D-galactopyranosyl-(1-2)- β-L-glucopyranosyl-(1-4)- β-L-glucopyranosyl ester.	97
2.24	FAB-MS (negative ion mode) Fragmentation pattern of Hum-IV, m/z 1210 [M-H] ⁻ (calculated for C ₅₈ H ₉₄ O ₂₆ 1206)	98
2.25	¹³ C NMR (100.61 MHz) chemical shifts δ _C of Hum-IV in pyridine	98
2.26	¹ H-NMR chemical shifts δ _H of Hum-IV in pyridine-d ₅	108
2.27	HMBC connectivities of Hum-IV (400 MHz, pyridine-d ₅) ¹³ C-NMR chemical shifts and the arrow indicate the connectivity's from proton to carbon.	109

List of Graphs		page
2.1	The anti-bacterial and antifungal activities of <i>Cestrum nocturnum</i> aerial part (CN-A).	25
2.2	Minimum inhibitory concentration in the antibacterial and anti-fungal activities of <i>Cestrum nocturnum</i> aerial part (CN-A)	27
2.3	The anti-bacterial and anti-fungal activities of crude extract and fractions of <i>Cestrum nocturnum</i> L aerial part (CN-A).	30
2.4	The anti-bacterial and anti-fungal activities of crude extract and fractions of <i>Cestrum diurnum</i> L (Aerial part).	33
2.5	The antibacterial and anti-fungal activities of crude extract and fractions of <i>Cestrum diurnum</i> L (green berries) CD-G.	36
2.6	The anti-bacterial and anti-fungal activities of crude extracts and fractions of <i>Cestrum diurnum</i> L (Ripe berries) CD-R.	39
2.7	The Antibacterial and anti-fungal activities of crude saponins extracts of CN-A, CD-A, CD-G and CD-R	42
2.11	The anti-leishmanial activities of CN-A, CD-A, CD-G and CD-R in percentage.	56
2.12	The anti-oxidant potentials of CN-A, CD-A CD-G and CD-R	60

Abstract

The work reported here deals with the bio-assay guided isolation of secondary metabolites from *Cestrum nocturnum* (leaves) and *Cestrum diurnum* (leaves and berries). First part of the dissertation deals with the biological activities of different parts of the selected plants while second part describes the purification and characterization of the isolated constituents.

Shade dried plant material was extracted with methanol. The concentrated methanolic extract was fractionated with *n*-hexane, chloroform, ethyl acetate and *iso*-butanol and extractive values determined. All the collected extracts were screened for anti-bacterial, anti-fungal, anti-oxidant and anti-leishmanial activities. The anti-oxidant experiment was also conducted with all the parts. Crude extract of aerial part of *C. nocturnum* displayed 57 % antioxidant activity while one of its isolated fractions (ethyl acetate) displayed a maximum of 67 % antioxidant activity. Similarly, the crude extract of aerial parts of CD *i.e.*, aerial part without fruit, green fruit, ripe fruit, exhibited 86, 81 and 90 % antioxidant activities respectively. The hexane fraction of CD green fruit showed higher antioxidant potential (87 %) than its crude methanolic extract. Due to our interest in saponins for further studies, they were selectively isolated and processed in the same way for antioxidant activities in crude form from both the plants. The highest antioxidant activity was found with the CD ripe fruit CS extract (90 %) while the least one was with both the aerial parts of the two plants (approx. 80 %). The results obtained with nine microbial strains were moderate. *S. typhii* was the most resistant strain. On the average gram positive bacteria showed good zone of inhibition with *Cestrum diurnum*. Gram negative strains showed good results mostly with *Cestrum nocturnum*. The crude saponins extracts

of berries were the most active giving zone of inhibition in the range of 8.5-15mm. Incredible anti-leishmanial activities are being reported for the selected plants. All the plant extracts exhibited hundred percent inhibition of the leishmania parasite. Second part of the thesis deals with the isolation and purification of the secondary metabolites of *C. nocturnum* and *C. diurnum*. Hum-V isolated from *C. nocturnum* was characterised using different spectroscopic techniques as (25*R*)- spirost-5ene-2,3-diol, [2 α , 3 β] 3-O- $\{\beta$ -D-glucopyranosyl (1 $\rightarrow$ 4)- β -D-glucopyranosyl (1 $\rightarrow$ 2)- β -D-glucopyranosyl $\}$ [(1 $\rightarrow$ 4)- β -D-xylopyranosyl] (1 $\rightarrow$ 4)- β -D-galactopyranoside). It was reported as nocturnoside A. Another reported compound was also purified and characterized as karatavoiside A, spirost-5-ene-2,3-diol [2 α , 3 β ,25*R*], 3-O- [β -D-glucopyranosyl(1 $\rightarrow$ 2)] $\{-\beta$ -D-xylopyranosyl(1 $\rightarrow$ 3)- β -D-glucopyranosyl(1 $\rightarrow$ 4) $\}$ - β -D-galactopyranoside. A new compound isolated from the same specie, elucidated as 3-O- β -D-xylopyranoside-olean-12-en-28-oic acid-28-O- β -arabinopyranosyl-(1-3)- β -D-galactopyranosyl-(1-2)- β -L-glucopyranosyl-(1-4)- β -L-glucopyranosyl ester.

Chapter 1

INTRODUCTION

1. General Introduction:

The old axiom "we come on this earth as guests of plants" appropriately justifies the services rendered by plants. As a good host they have added in each and every domain of human existence providing food, shelter, clothing, clean environment, *etc.* Another great use is in herbal therapies for curing ailments [1]. They play a vital role in the existence of life on earth.

The relationship among life, disease and plants is as old as the history of mankind itself. Consuming plants for various uses is inherent. Human being started using plants from the very beginning; the primitive humans began to distinguish those plants suitable for nutritional purposes from others which possess definite pharmacological applications e. g., purgative, emetic, stimulant, depressant etc. The knowledge of herbal remedies was passed on to generations as folk medicine. Mankind succeeded in application of herbs for external and internal ailments with herbal, animal and mineral medicines in the ancient times [2]. The animals also have instinct to pick out plants when sick. Dogs and deer uses certain types of grasses when sick or injured [3].

Only 6 % of approximately 600,000 plant species have been subjected to pharmaco-chemical studies. According to W. H. O. estimates, more than half of the world population still depends on the traditional medicines because of their easy availability, cheapness, and socio-cultural background in spite of an increasing shift towards synthetic drugs since early twentieth century [4].

Sifting various plants on the basis of their climate and plausible folklore use, specifies a huge number of species ready to be screened to yield a number of plant derived metabolites having therapeutic significance[1].

Plants produce two types of metabolites as part of their normal metabolism. Substances synthesized by plants essential for their sustenance and fundamental activities are primary metabolites *i.e.*, sugars, proteins, amino acids, chlorophyll *etc.* Secondary metabolites are; distinctive metabolic products of plant species produced in smaller quantities having no significant role in their life cycle but concerned with the process of co-evolution between plants and organisms. Usually they influence other organism's systems, so are termed as active principles of that particular plant [5]. Knowledge about characteristic properties of secondary metabolites is not known sufficiently. They produce toxins and defense molecules used against different predators, some are pheromones, odorants, and color pigments used to attract organisms while others have therapeutic potential for humans and animals. Handling in a proper way, these agents can yield effective drugs [1, 6].

Nowadays synthetic antioxidants, antibiotics and pesticides are massively used in our daily life. Synthetic antioxidants are mainly used in industrial processing to prolong the storage stability of foods [7]. Huge amounts of synthetic pesticides and synthetic fungicides for microbial phytopathogens are developed for agro-industries [8]. The toxicologists and nutritionists through various documented results concluded that chemically treated crops and food have toxic effects on human and animal health, causing pollution and disturbance of ecological balance [9]. Other alternative methods were

developed to circumvent these problems. One of them is the use of bio-chemicals of plant origin, having the advantage of being degradable and less toxic to the life [10].

Since few decades, the essential oils and organic compounds of plants are of great interest as they have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases and for the preservation of foods from the toxic effects of the oxidants [7].

1.1. Family: Solanaceae

Family Solanaceae commonly known as “nightshade” or “potato” family is the third most important taxon economically. The name originates from the Latin word *solari*, meaning "to soothe" referring to soothing properties of some of the psychoactive species of the family. Solanaceae is the most valuable family in terms of vegetable crops and is most variable in terms of agriculture utilities. It includes the tuber-bearing *Solanum tuberosum* (potato), a number of fruit-bearing vegetables (*Solanum lycopersicum*, *solanum melongena* and *Piper longum*), ornamental plants (*Petunia alpicola*, *Nicotiana tabacum* and *Cestrum nocturnum*), plants with edible leaves (*Solanum aethiopicum*, *Solanum macrocarpon*) and medicinal plants (*Hyoscyamun niger* and *Capsicum annuum*) [11-13]. This family is also well known for its medicinal plants like *Solanum nigrum*, *Atropa belladonna*, *Datura fastusa* and *Withania somnifera*, which have proven antirheumatic [14], diuretic[15], antispasmodic [16], sedative, hypnotic [17] and central nervous system [18] stimulant properties. Plants of this family are particularly rich in alkaloids and glycosides, which range in their toxicity from mildly irritating to fatal even in small quantities [19].

1.1.1. Genus: *Cestrum*:

The plants belonging to this genus are shrubs or small trees while some are climbers. Their leaves are alternate in arrangement and entire in shape. The species of this genus have cymose inflorescences that are terminal in position or born axillary and are usually fragrant. Fruits of the genus are berries containing few seeds. There are 175 species belonging to genus *Cestrum* that are native to tropical America and Australia. There are four cultivated species in Pakistan that includes *C. aurantiacum*, *C. diurnum*, *C. nocturnum*, and *C. parqui*[12]. They are commonly known as Cestrum or Jessamine due to their fragrant flowers [20].

The members of the genus mostly love dry soils, tolerate shade and dense allelopathic litter, also they are salt tolerant if protected from heavy-salt spray [21] and over-wash of storms. The flowers and fruits in the genus occur round the year. Their seeds are dispersed by birds resulting in increased population [22].

1.1.2. *Cestrum nocturnum* L.

Cestrum nocturnum is commonly called as night-blooming Cestrum, Raat ki Rani, meaning Lady of the Night, Queen of the Night or Night-blooming jessamine [12]. It is native to the West Indies [23, 24]].

C. nocturnum is a scandent shrub up to 4 m tall and glabrous. Leaves are 5-10 x 3-5 cm, elliptic-ovate in shape with acute margins. Flowers are greenish-yellow that grows in axillary or terminal panicles. Fruit is a berry that is somewhat ovoid in shape

and of white colour. Figure 1 shows flowers of *Cestrum nocturnum*.



Fig. 1.1: *Cestrum nocturnum* flowers

1.1.3. *Cestrum diurnum* L.

Cestrum diurnum is commonly called Day-blooming Cestrum, Day-blooming Jessamine and Din ka Raja (King of the day). It is a branched shrub or small tree up to 4 m tall. The leaves are lanceolate to oblanceolate and 4.5-12.5 x 2.5-4.0 cm. Flowers are white in colour and are born in extra-axillary peduncles. Fruit is a black berry, somewhat globose in shape. The plant blooms from January to April and also during August to October.

Flowers of *C. nocturnum* are fragrant during night whereas those of *C. diurnum* are fragrant during day only; hence locally named as “Raat ki rani” and “Din ka raja”, respectively. Figures 2 and 3 respectively show mature and green berries of *Cestrum diurnum*.



Fig. 1.2: *Cestrum diurnum* mature black berries



Fig. 1.3: *Cestrum diurnum* flowers and pre-mature green berries

1.1.4. General importance of *Cestrum* species:

Phytochemicals from this genus exhibit wide range of pharmacological significances in skin disorders and treating arterial hypotension. They are also used as antiviral, analgesic, abortive, diuretic, antispasmodic, dyspeptic, smooth muscle relaxant,

negative inotropic and chronotropic agent [25-27]. Essential oil and flower extracts of *Cestrum nocturnum* can be substituted for synthetic fungicides in agro industries for the treatment of many microbial phyto-pathogens destroying crops, vegetables and ornamental plants, by affecting spore germination of pathogens. The oil has also demonstrated high disease inhibition efficiency on greenhouse-grown pepper plants [8].

Various parts of the plant including the bark, berries and dried leaves are used in smoking blends as a mild inebriant and flowers as tea or ingested as a hallucinogen. *Cestrum* species are used as food by the caterpillars of several Lepidoptera. These include the Glasswing (*Greta oto*), and *Manduca afflicta* which possibly feeds only on *Cestrum diurnum*. It is known that such Lepidoptera are able to sequester the toxins from the plant, making them noxious to many predators.

The plants of genus *Cestrum*, if eaten, cause severe gastroenteritis. *Cestrum nocturnum* leaf and flower are moderately poisonous. Its ingestion may cause uneasiness in animals [28]. In Chinese folklore, it is used for the treatment of burns and swelling, epilepsy and stupefying charm medicines. *Cestrum parqui* is used as antifebrile, anti-pyritic and inflammation [29, 30]. Alkaloids, saponins, phenolic compounds, tannins, and flavonoids were found to be present in leaves of *Cestrum nocturnum*. [29]

Psychoactive properties of the species are not well documented [31]. Intake of plant parts especially fruits causes respiratory tract sensitivity due to which the asthma patients face difficulty in breathing. Other effects include irritation of the nose and throat, headache, nausea, high temperature, rapid pulse rate, excess salivation and ingestion problems [8]. The plants of genus *Cestrum*, if eaten, cause severe gastroenteritis. *Cestrum nocturnum* leaf and flower are moderately poisonous. Its ingestion may cause uneasiness

in animals [28, 32]. In Chinese folklore, it is used for the treatment of burns and swelling, epilepsy and stupefying charm medicines. *Cestrum parqui* is used for the treatment of fever and inflammation and as antifebrile. Leaves of *Cestrum nocturnum* are used in burns and swellings, curing epilepsy and as stupefying charm medicine for their pharmacological significance. The volatile oil of *Cestrum nocturnum* and *Cestrum diurnum* are efficient mosquito repellent [7].

Cestrum nocturnum is reported to exhibit potent mosquito larvicidal activity against *Aedes aegypti* and showed no toxicity to fish [28, 33, 34]. Essential oils and extracts in different solvents of flower have also shown antioxidant activities using two methods - DPPH radical systems and superoxide radical scavengers. *Cestrum nocturnum* and *Cestrum diurnum* are used to prevent malaria in several African countries [7]. Although *Cestrum diurnum* berries are harmful to the mammals by affecting nervous system, however the fruits of day jasmine are one of the three foods that make up the bulk of the diet of the endangered plain pigeon (*Columba inornata*) in Puerto Rico [35].

Majority of the *Cestrum* species have applications in folk medicine [36]. These are occasionally cultivated as ornamental plants for their sweet scented white flowers. Only few of the species of this genus have been explored chemically [20].

1.2. Phytochemicals of *Cestrum*

The phytochemicals found in this genus include saponins, nicotine type alkaloids, tannins and glycosides, among which saponins are chemo-taxonomically important. Alkaloids, flavonol glycosides, steroidal saponins, fatty acids, essential oils, phenols and various other phytochemicals are documented from different parts of the *C. nocturnum* [7].

Calcinogenic plants are regarded as a significant and economical source of 1,2S-(OH)₂ D, for human and veterinary use. Calcinosis in cattle was reported from the leaves of *Cestrum diurnum* due to a glycoside 1,25-dihydroxycholecalciferol (1,2 S-(OH), D) (a kaurene type diterpene glycoside and carboxyparquin from *C. parqui*). *C. sendtnerianum* poisoning is also reported [37]. Day jasmine leaves (15-30 %) in an animal's diet are the critical amount to lead vitamin D toxicity resulting in elevation and deposition of serum calcium in soft tissue. 1,2S-(OH)₂ D is the most effective hormonal form of vitamin D, maintaining calcium and phosphorus homeostasis [38].

The sapogenin steroids tigo-genine, smilagenine and yuccagenine are isolated from the leaves of *Cestrum nocturnum* [39]. Different constituents including L-arabinitol, isoeugenol, diosgenin and eicosane detected from essential oils and organic extracts of *Cestrum nocturnum* flowers and arial parts were estimated using gas chromatographic-mass spectroscopic (GC-MS) technique [7].

1.2.1. Saponins from *Cestrum* species

The naturally occurring glycosides are classified as phenol glycosides, limonoid glycosides, steroidal glycosides, terpenyl glycosides, terpenoid glycosides sweeteners, amino glycosides (antibiotics), cyanogenic glycosides, saponins, glycosidic bound volatiles, cardiac glycosides, steroidal glycosides, glycoproteins, glycolipids and glucosimnolates.

Due to the surface tension reducing activity, saponins form foaming with water, consequently got the name from Latin word (*sapo*, *saponis*: soap). Saponins or saponosides, due to their diverse range of activities, exhibit industrial and pharmacological applications [6].

Saponins are of plant origin or may be obtained from marine animals. A survey of over 6000 plants representing 208 families and 1307 genera showed the presence of saponins [6]. Different saponins have been reported from various plants including monocotyledons and dicotyledons. The important saponins containing taxa belonging to monocots are *Yucca*, *Trillium*, *Chlorogalum*, *Smilax*, *Nolina*, *agapanthus*, *Agave attenuate*, *Manferda*, *Dioscorea*, *Cestrum parqui*, *Panax ginseng*, *Allium sativum*, *Medicago sativa*, *Saponaria officinalis* and *Glycyrrhiza glabra* [40-43]. In dicots, the saponins have been found in *Digitalis* (Scrophulariaceae), *Solanum*, *Lycopersicon* and *Cestrum* (Solanaceae). The richest sources for commercial uses are *Dioscorea tuber*, and the leaves of *Yucca* and, *Agave*[20].

Saponins are also found in defensive secretions of certain insects and marine animals. Chrysomelidae especially the species of *Platyphora* genus confiscate triterpenic saponins from plant hosts to use them for their own defense while some other saponins are reported from Antarctic starfish and marine sponges (*Ectyoplasia ferox*) [6]. The saponins are considered as chemical defense agents, discouraging predators while in some species they are supposed to have repellent activity against shark [20].

Hepatoprotective effect of leaves of aqueous ethanol extract of *Cestrum nocturnum* against paracetamol-induced hepatotoxicity [44]

1.3. Structure of saponins:

Saponins are constituted of glycone (saccharide) and aglycone (non-saccharide) portion known as genin or the sapogenin. Genin present in the saponins includes triterpenes glycosides, steroid glycosides and steroid alkaloid glycosides. Fig 1.4 represents genins of some important classes of saponins.

In general, the aglycones are hydroxylated at C-3 and certain methyl groups can also be oxidized to hydroxymethyl and aldehyde carboxyl functionalities. Esterification of an acid moiety of aglycone to the sugar leads to the respective glycoside called as ester saponins [20].

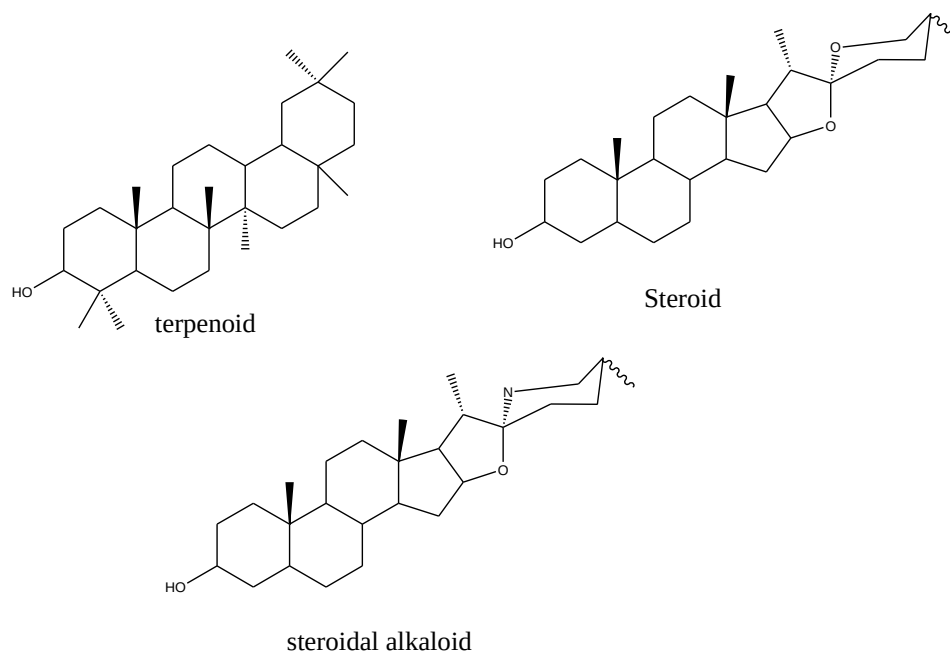


Fig 1.4: The genins of some important classes of saponins.

1.3.1. The saccharide moiety:

In saponins, one or more sugar chains are attached to aglycone. Monodesmosidic saponins have one sugar chain while bidesmosidic saponins have two chains. Both sugar chains attached with ether linkage at C-3, whereas in bidesmosidic saponins the second sugar chain is attached with ester linkage at C-28 (triterpene saponins) [45, 46] or with

ether linkage at C-26 (furostanol saponins). The tridesmosidic saponins having three sugar chains attached are very rare [47].

1.3.2. Types of monosaccharides:

The most frequently found monosaccharide units are D-glucose (Glc), D-galactose (Gal), L-rhamnose (Rha), D-xylose (Xyl), D- glucuronic acid (Glc A), D-galacturonic acid (Gal A), L-arabinose (Ara) and D-fucose (Fuc). Marine organisms also contain D-quinovose (Qui) also written as D-chinovose [20].

Glycosides having more than three monosaccharides are termed as “oligosides”. Oligoside having eleven sugar moieties (Clematoside C) was reported from *Clematis mandshurica*. Saccharide moieties may be linear or branched. Acylated, methylated and sulphated sugar units have also been reported from marine organism. The monosaccharide units are mostly found in pyranose (*p*) or furanose (*f*) form. The configuration of the inter-glycosidic linkages are given by α and β notations [20, 40].

1.4. Physical and chemical properties of saponins:

Saponins act as surface tension decreasing agents due to the presence of both hydrophilic (*water-loving*, polar) and lipophilic (*fat-loving*) properties [48]. While interacting with water, they form foam on shaking which is effective and gentle cleaner. They complexate with cholesterol to form minute opening by affecting the hydrophobic lipophilic balance (HLB) and membrane permeability in red cell (erythrocyte) membranes [20], leading to red cell lysis (hemolysis) on intravenous injection [35]. Saponins are relatively large sized molecules which contain sugars whose degradation is

easier under certain conditions (pH slightly acidic or basic or presence of hydrolysis enzymes [6].

In addition, the amphipathic nature of the class gives them activity as surfactants that can be used to enhance penetration of macromolecules such as proteins through cell membranes [48]. Saponins have also been used as adjuvants in vaccine [49].

The emulsifying property of saponins is the easiest phenomenon to identify saponins qualitatively. When shaken vigorously with water they form foam. Due to this property they are used as surfactants in preference to soaps as they are not affected by alkaline or acidic water. It is not an authentic qualitative method as all saponins do not form foam in water and also some other phytochemicals can also produce froth in water [20].

Saponins are identified to be hypocholesterolaemic. Complexation between saponins and cholesterol was discovered by Windous in 1909. Saponins sometimes form an insoluble complex with cholesterol preventing its absorption by the body especially small intestine. While some other saponins cause elimination of cholesterol indirectly by elevation of the faecal excretion of the bile acid. Dietary saponins provide considerable nutritional and health benefits to humans by controlling plasma cholesterol and nutrient absorption [20].

Saponins are capable of haemolysing blood *in vitro*. Even in very low concentration, the saponins are able to release haemoglobin by lysing erythrocytic membranes. Due to Red Blood Cells (RBCs) sensitivity to saponins, haemolysis of RBCs is used as a method for its quantification [50]. The structure of glycoside significantly affects haemolytic activity. Monodesmosidic steroids specially having least number of

branching or small carbohydrate moieties and increase in branching of a sugar chain enhances haemolytic activity. The steroid aglycone having more affinity for the erythrocytes is better haemolyte than triterpene saponins. Incubation of saponins with cholesterol dramatically inhibit haemolysis of erythrocyte membrane and consistent with the hypothesis that cholesterol is a target of haemolytic activity [20].

1.5. Biological activities of saponins:

Several biological activities are attributed to saponins from *Cestrum* genus, some are known for years *i.e.*, anti-fungal [51] and pesticidal [52] activities while some new are being discovered *i.e.*, anti-sweetness insecticidal and utero-contraction. Steroidal and terpenoid saponins have biological activities as expectorant, diuretic as well as foaming and haemolytic properties, whereas bidesmosidic furostanol saponins are associated with transformations into biologically active spirostanol glycosides, which are only active biologically if hydrolyzed to spirostanol saponins [53].

Saponins degrade under certain conditions like pH (slightly acid or basic) or presence of hydrolysis enzymes leading to the loss of activity, depending completely on the water-soluble sugar chains. sapogenins of *Cestrum parqui* are less active than its saponins [6].

Plant extracts, especially botanical insecticides, are currently studied more and more because of the possibility of their use in plant protection. *Cestrum* saponins are also reported for its insecticidal and pesticidal activities.

1.5.1. Antimicrobial activity

The effects of herbal compounds and phytochemical on pathogenic and economically important bacteria have been well studied [54]. Reports showed that the essential oil of *C. diurnum* flower was comparable to standard antibiotic ampicillin [53]. The Essential oils and plant extracts of *C. nocturnum* can be used to control phytopathogenic fungi in agricultural fields [8, 55].

1.5.2. Fungicidal activity

Saponins are very effective fungicides. Their fungi-toxicity was demonstrated by developing inhibition activities against *C. parqui* saponins for *Fusarium solani* and *Botrytis cinerea*. Crude saponins extract from *C. parqui* showed no activity against phytopathogenic fungi like *F. solani* and *B. cinerea*. It may be due to the presence of detoxifying enzymes [56].

1.5.3. Larvicidal activity

Saponin is the major chemical constituent of *C. nocturnum* leaves. The leaves are reported to have higher larvicidal activity. The researchers attribute this activity to the eight steroidal [57] bioactive compounds responsible for mosquitocidal activity [58].

For the control or abolition of vector-borne health risks, some successful trials have been made to use some bioactive compounds from the leaves of *C. nocturnum*. [29]

The toxicity of *C. parqui* lies in Crude saponins extract. Injection of crude saponins of *C. parqui* to *Spodoptera littoralis*, *Culex pipiens* and *Quinquefasciatus* [59] mosquito larvae, *Schistocerca gregaria*, the Desert locust demonstrated fat body tanning and some narcotic effects at the injection site [60]. Loss of the hydrophilic activity of the saponins

would be faced by the structural modification (acylation and hydrolysis) or degradation. The hydrophily is required for its solubility in the hemolymph of the insects or the culture media. Sapogenins of *C. parqui* are less active than saponins [6, 60]. Steroidal compound from *C. diurnum* are reported to be biologically active showing the LC₅₀ value of 0.70, 0.89, 0.90 and 1.03mg/ 100ml, for first-, second-, third- and fourth-instar larva of *Anopheles stephensi*, respectively [61, 62].

1.5.4. Pesticidal activity

C. parqui saponins are reported to cover large spectrum of action by perturbing its physiology. They have pesticidal toxicity against plant pests like insects, fungus, mollusks and nematodes. The toxicity may be due to cytotoxic activities, as demonstrated histologically on fat body and the gut of *Schistocera gregaria* and *Spodoptera littoralis* or due to the interference with ecdysone metabolism by the abstraction with dietary cholesterol as biologically shown by *Tribolium confuse* [56, 63].

1.5.5. Molluscicidal activity:

Molluscicidal activity of *C. parqui* saponins is observed on *Theba pisana* snails, due to its mucus and water retention activity provoking mortality due to dehydration. CSE from *C. parqui* showed no activity against phytopathogenicfungilike *Fusarium solani* and *Botrytis cinerea*. It may be due to the presence of detoxifying enzymes [56, 64].

1.5.6. Insecticidal activity

Cestrum parqui L'Héritier aqueous extracts had no effects on un-hatched eggs but reduced the reproductive potential of the adult ones. These were screened for toxicity

against *Ceratitis capitata* by of ingesting the toxic compounds of *C. parqui*, demonstrating growth inhibition at 0.6% concentration and $LC_{50} = 0.9\%$. Growth regulation ability of saponins is characterized by disturbing adult emergence duration and biosynthesis failure of ecdysial of larvae [65]. Cholesterol is not absorbed by the digestive track as the lipophylic sites of cholesterol and the saponic aglycone complexat forming insoluble complexes having micelle or spheres structures [6]. Some use *cestrum parqui* to prevent *Epicauta pilme* beetles from potato fields in Southern Chile [66, 67].

1.5.7. Antifeedant activity

Antifeeding activity of Saponins extracted from *Cestrum parqui*, *Solanum laxum*, *Blanites roxburghii*, *Agave cantala* and *Phaseolus vulgaris* were analyzed. Saponins extracted from *Ilex apocea*, alfalfa varieties and several others plants including leguminous plants inhibit the food uptake of *Limantria dispar*, amite species (*Oligonichus illicis*), and two caterpillar's species (*Hyphantria cunea*, *Malacosoma americanum* and *Callosbruchus chinensis*). Saponins inclusion in the diet increases the larvae weight loss and decreased dry food metabolism by larvae. Isolation of triterpenic saponins with two sugars in C-3 position, from Brassicaceae species revealed their important role in inhibition of the food uptake activity. Saponins having the least significant number of sugar chains were most active [6].

For this reason, there is a growing interest in the studies of natural additives as potential antioxidants. The antioxidant properties of many herbs and spices are reported to be effective in retarding the process of lipid per-oxidation in oils and fatty foods and have gained the interest of many research groups. A number of studies on the antioxidant activities of various aromatic plants have been reported over the last 20 years. In the last

decades, the essential oils and organic extracts of plants have been of great interest as they are the sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases and for the preservation of foods from the toxic effects of the oxidants [7].

1.6. Objectives

The Objectives of the work are;

- Isolation, purification and characterization of secondary metabolites from various *Cestrum* species.
- Evaluation of crude extracts and purified compounds for their anti-microbial and enzyme inhibition activities.

Plan of work/ methodology:

Aerial parts of the *Cestrum* species were collected and shade dried. Then this was chopped into fine powder using crushers and pulverizes. For exhaustive extraction, the pulverized plant material was extracted at room temperature with 80% methanol. The crude extract was collected after evaporation of the solvent under reduced pressure.

The dried 80% methanolic crude extract was partitioned between n-hexane and water. The aqueous fraction was further extracted with chloroform, ethyl acetate and finally with n-butanol.

All available classical and non-classical isolation techniques such as column chromatography (CC), thin layer chromatography (TLC), vacuum liquid chromatography

(VLC), high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC) and re-crystallization were used for isolation of compounds.

For the identification and structure elucidation of isolated compounds, all the available modern identification techniques were entertained. The instrumental techniques used may include UV-Visible, IR, H-NMR, ¹³C-NMR, 1-D and 2-D NMR and Mass spectrometry.

The crude extracts, obtained by partitioning between different solvents and isolated compounds will be subjected to different biological activities including anti-oxidant, anti-fungal, anti-microbial and anti-leishmanial.

Chapter 2

RESULTS AND DISCUSSION

2.1 General:

Various aerial parts including the bark, berries and dried leaves of *Cestrum nocturnum* and *Cestrum diurnum* are used in Chinese System of Medicines. Based on the literature and folklore knowledge discussed earlier, *Cestrum nocturnum* and *Cestrum diurnum* (aerial parts, fresh green and ripe berries) were subjected to chemical and biological investigations. In first step, the plant material was exhaustively extracted with methanol. The extract was then partitioned between water and different organic solvents *i.e.*, hexane, chloroform, ethyl acetate and *iso*-butanol to separate various classes of organic compounds. For isolation of pure compounds from the extracts, vacuum liquid chromatographic (VLC), column chromatographic and thin layer chromatographic techniques were used. Crude saponin mixtures were used for further biological and chemical studies.

Some pure compounds from these plants were isolate and characterized using different spectroscopic techniques like UV, IR, ¹H-NMR, ¹³C-NMR, HeteroCOSY, HMBC and HMQC. The toxicity studies are also reported.

2.2 Biological activities:

Anti-bacterial, anti-fungal, antioxidant and anti-leishmanial activities were performed for the above mentioned fractions obtained from *C. nocturnum* and *C. diurnum*. All the activities will be discussed here;

2.2.1 Anti-microbial activities

The use of various antibiotics has increased tremendously in the recent years, due to which there is a swift in multidrug resistant pathogens. Currently, the multidrug resistant bacteria have limited the efficacy as well as the use of the oral antibiotics against infectious pathogens. This study proves that the selected plant's extracts/fractions can be further refined and used for drugs having good results and low risk of side effects.

Agar well-diffusion method [68], discussed in chapter three (3.6) was used for determination of antimicrobial activities [69].

All the cultures were applied uniformly by amending the fresh microbial culture's solution using sterile nutrient broth and adjusting the turbidity to 0.5 McFarland's turbidity standard [70]. The anti-microbial potential was measured for each extract by measuring the zone of bacterial growth inhibition around the well/disc in millimeter (mm). The larger inhibition zones the better bactericidal activity and vice versa. For comparing the activities, standard anti-bacterial and anti-fungal drugs were used. For gram negative bacteria ciprofloxacin while for gram positive bacterial cultures, azithromycin and for fungal strain clotrimazol was used as standard drug. All the tests were applied in duplicate and the average values were tabulated. Table 2.1 enlists the bacterial and fungal cultures used.

Table 2.1: Bacterial and fungal strains used for anti-bacterial and anti-fungal studies.

S. No	Strain	Type	Detail of the stains used
1.	<i>Escherichia coli</i>	Gram negative	ATCC # 25922
2.	<i>Pseudomonas aeruginosa</i>	Gram negative	ATCC # 9721
3.	<i>Salmonella typhi</i>	Gram negative	ATCC 19430
4.	<i>Klebsiella pneumoniae</i>	Gram negative	Clinical isolate, QAU, Islamabad
5.	<i>Erwinia carotovora</i>	Gram negative	ATCC # 39048
6.	<i>Shigella flexneri</i>	Gram negative	Clinical isolate, Hayatabad Medical Complex, Peshawar
7.	<i>Staphylococcus aureus</i>	Gram positive	ATCC # 6538
8.	<i>Bacillus subtilis</i>	Gram positive	Clinical isolate, QAU, Islamabad ATCC 6633
9.	<i>Bacillus atrophaeus</i>	Gram positive	Clinical isolate, QAU, Islamabad
10.	<i>Candida albicans</i>	Fungus	Clinical isolate, Hayatabad Medical Complex, Peshawar. ATCC 2091
11.	<i>Trichophyton longifusus</i>	Fungus	clinical isolate, Hayatabad Medical Complex, Peshawar
12.	<i>Aspergillus flavus</i>	Fungus	ATCC 32611
13.	<i>Microsporium canis</i>	Fungus	ATCC 11622
14.	<i>Fusarium solani</i>	Fungus	ATCC 11712
15.	<i>Candida glabrata</i>	Fungus	ATCC 90030

2.2.1.1 Anti-microbial activities of *Cestrum nocturnum*- Aerial part (CN-A):

The antimicrobial activity of *C. nocturnum* indicates that the crude methanolic extract is a potent inhibitor against the selected pathogens. The crude methanolic extract and *iso*-butanolic fraction were showing promising results against all the gram negative bacterial cultures. Over all, the *iso*-butanol fraction gave better results ranging from 10 mm (lowest) to 14 mm (highest). The inhibition zone of CN-A crude (*Cestrum nocturnum* aerial part-hexane fraction) was 17 mm against *P. aeruginosa*. 16 mm zone was recorded for CN-A chloroform (*Cestrum nocturnum* aerial part-chloroform fraction) against *S. aureus*. Table 2.2 and fig 2.1 shows the anti-bacterial and anti-fungal activities of *Cestrum nocturnum* aerial

MIC's (minimum inhibition concentrations) were ranging from 19-280 µg. The CN-A Chloroform showed least inhibitory concentration *i.e.*, 19 µg/mL against *P. aeruginosa*, 22 µg/mL against *S. aureus* and 75 µg/mL against *B. subtilis*. In general, different fractions of the plant showed significant antibacterial activities against gram positive bacteria *i. e.*, *B. subtilis* and *S. aureus*, and the MIC's were measured from 54 to 75 and 22 to 55µg/mL, respectively. The activities against gram negative bacteria indicated that higher concentration will be required to inhibit the bacterial growth. The MIC's were from 84-280 µg/mL for *E. coli* and 177-220 µg/mL for *S. flexneri*. The activities against gram negative bacteria might be due to the resistance developed by the microbes. The results indicate the plant extract/fractions cover a wide range of infectious diseases and can be worked on to develop drug(s) against different microbial agents. [71, 72]. MIC values are represented of the anti-bacterial and anti-fungal activities of *Cestrum nocturnum* aerial part (CN-A) in table 2.3 and fig 2.2.

Table 2.2: The anti-bacterial and anti-fungal activities* of *Cestrum nocturnum* aerial part (CN-A).

s.no	Sample code	EC	ST	PA	SF	SA	BS	TL	CA	AF	MC	FS	CG
1.	Crude	13	-	17	8	15	12	-	40	-	50	-	30
2.	Hexane	10	-	9	12	-	-	-	-	-	-	-	-
3.	Chloroform	-	-	13	-	16	13	-	60	-	-	-	-
4.	Ethyl acetate	14	-	11	-	13	13	-	-	-	40	-	60
5.	Iso-Butanol	13	-	11	14	14	10	-	65	-	30	-	60
6.	Aqueous residue	9	-	12	-	15	12	-	65	-	50	-	-
7.	Imepenem	24	26	20	28	27	23	-	-	-	-	-	-
8.	Miconazole	-	-	-	-	-	-	100	100	-	100	100	100
9.	Amphotericin	-	-	-	-	-	-	-	-	100	-	-	-

EC= *E. coli*, ST= *S. typhii*, PA= *P. aeruginosa*, SF= *S. flexeneri*, SA= *S. aureus*, BS= *B. subtilis*, TL= *T. longifusus*, CA= *C. albicans*, AF= *A. flavus*, MC= *M. canis*, FS= *F. solani*, CG= *C. glaberata*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

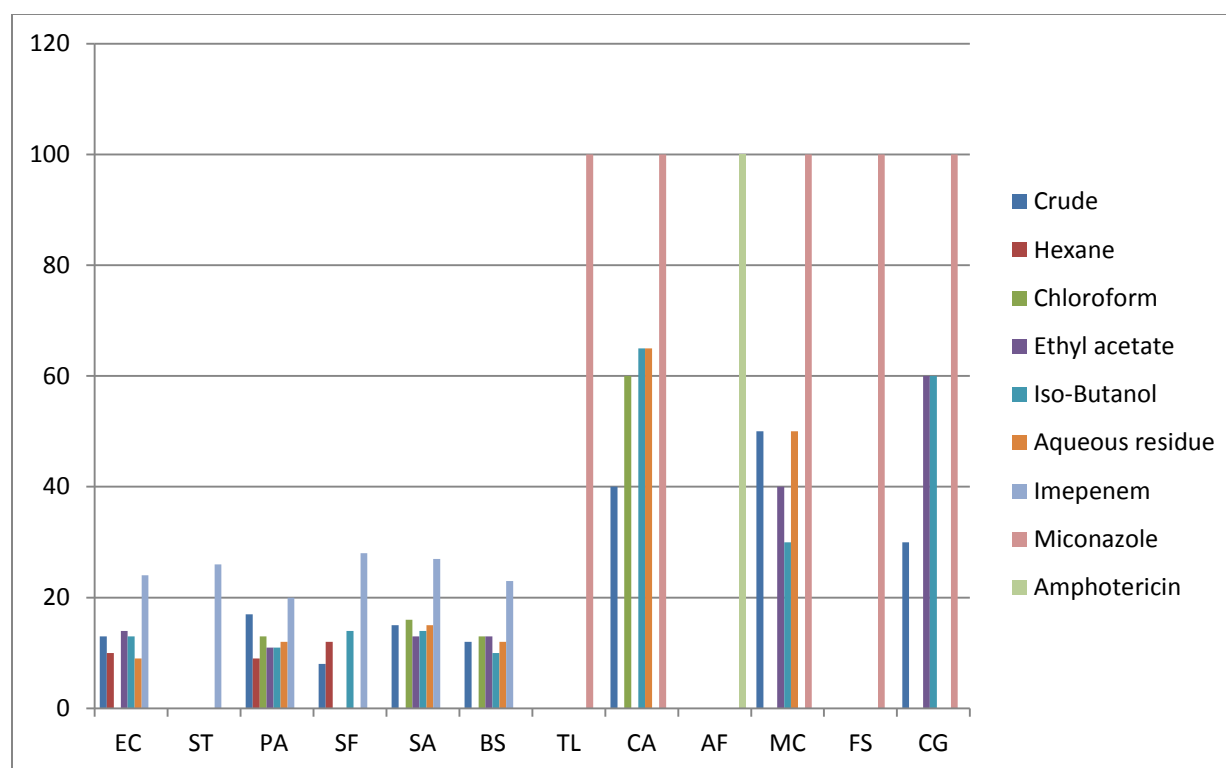


Fig 2.1: The anti-bacterial and anti-fungal activities of *Cestrum nocturnum* aerial part (CN-A).

EC= *E. coli*, ST= *S. typhii*, PA= *P. aeruginosa*, SF= *S. flexeneri*, SA= *S. aureus*, BS= *B. subtilis*, TL= *T. longifusus*, CA= *C. albicans*, AF= *A. flavus*, MC= *M. canis*, FS= *F. solani*, CG= *C. glaberata*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm

Table 2.3: Minimum inhibitory concentration ($\mu\text{g/ml}$) in the antibacterial and anti-fungal activities of *Cestrum nocturnum* aerial part (CN-A).

S.NO	Sample code	EC	ST	PA	SF	SA	BS	TL	CA	AF	MC	FS	CG
1.	Crude	84	-	31	177	36	75	-	210	-	230	-	290
2.	Hexane	195	-	200	220	-	-	-	-	-	-	-	-
3.	Chloroform	-	-	19	-	22	75	-	190	-	-	-	-
4.	Ethyl acetate	95	-	21	-	24	54	-	-	-	290	-	190
5.	Iso-Butanol	175	-	75	180	24	67	-	175	-	250	-	190
6.	Aqueous residue	280	-	62	-	55	65	-	170	-	250	-	-
7.	Imepenem	0.19	0.17	0.31	0.13	0.17	0.22	-		-	-	-	-
8.	Miconazole		-					1.4	1.8	-	1.6	1.1	0.5
9.	amphotericin		-							2.3			-

EC= *E. coli*, ST= *S. typhii*, PA= *P. aeruginosa*, SF= *S. flexeneri*, SA= *S. aureus*, BS= *B. subtilis*, TL= *T. longifusus*, CA= *C. albicans*, AF= *A. flavus*, MC= *M. canis*, FS= *F. solani*, CG= *C. glaberata*

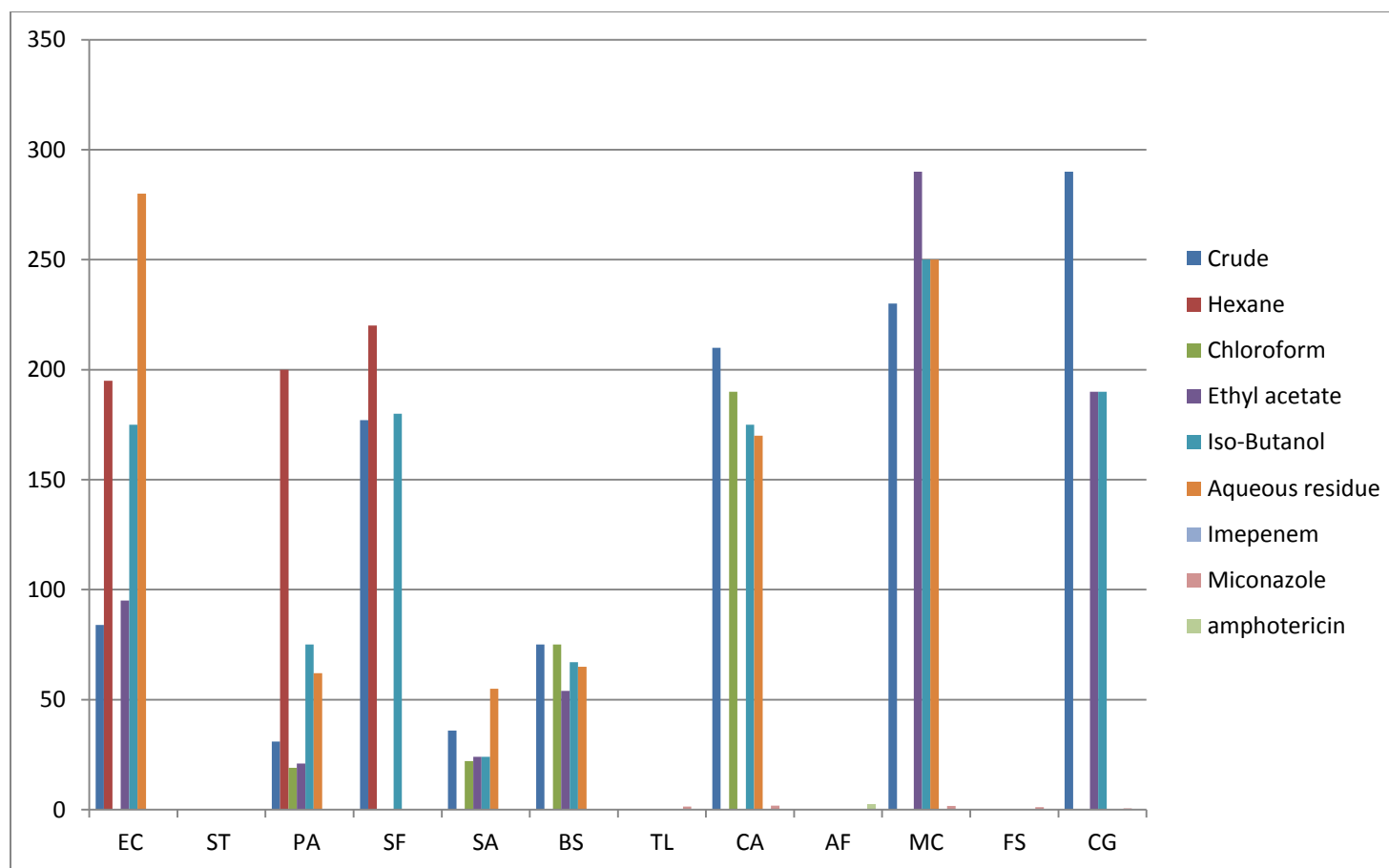


Fig 2.2: Minimum inhibitory concentration in the antibacterial and anti-fungal activities of *Cestrum nocturnum* aerial part (CN-A).

EC= *E. coli*, ST= *S. typhii*, PA= *P. aeruginosa*, SF= *S. flexeneri*, SA= *S. aureus*, BS= *B. subtilis*, TL= *T. longifusus*, CA= *C. albicans*, AF= *A. flavus*, MC= *M. canis*, FS= *F. solani*, CG= *C. glaberata*

This data in table 2.2 was re-done with a little different method given in discussed in chapter in three (3.3.6 (b)). In agar disc diffusion method, a small amount of sample and the standard drug is applied in the test. The inhibited zone was measured in mm after 24 hours.

All the gram negative cultures gave good zone of inhibition except the *S. typhi*. All the extracts/fractions of *C. nocturnum* presented moderate potential against *E. coli* and *E. carotovora*. The larger zone of inhibition amongst them was recorded for crude *iso*-butanolic fraction (14 mm) against *E. carotovora*. From the study, it is revealed that hexane fraction have the potential to inhibit all the tested pathogens. Ethyl acetate and *iso*-butanolic fractions showed marked activities against gram positive bacterium *B. atrophens*.

Overall the crude extract and the fractions showed remarkable activities against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. atrophaeus*. From the microbial activities it is quite evident that the plant may provide a good source for the development of oral antibiotics because it has a broad spectrum of anti-bacterial activities. *S.typhi* is showing strong resistance to all the fractions, this may be due the resistance developed by the strain as it was a clinical isolate.

The tested fungal strain, *Candida albicans* had not shown significant results, *i. e.*, the sensitivity of the fungal strain is not good. Antibacterial potential of crude extract/fractions of CN-A is presented in table 2.4.

Table 2.4: Anti-bacterial and anti-fungal activities* of crude extract and fractions of *Cestrum nocturnum* L aerial part (CN-A).

S.No	Sample Code	EC	KP	PA	ECr	ST	SA	BA	BS	CA
1.	Crude	10	7	8	9	7	8	10	8	8
2.	Hexane	10	7.5	9.5	12	-	8.5	10	9	-
3.	Ethyl acetate	8	8	8.5	10	-	8	12	-	9
4.	<i>Iso</i> -butanol	-	9	-	14	-	-	13	-	-
5.	DMSO	-	-	-	-	-	-	-	-	-
6.	Ciprofloxacin	26	8	16	26	32	-	-	-	-
7.	Azithromycin	-	-	-	-	-	28	29	27	-
8.	Clotrimazol	-	-	-	-	-	-	-	-	25

EC= *E. coli*, KP= *K. pneumonia*, PA= *P. aeruginosa*, ECr= *E. carotovora*, ST= *S. typhi*, SA= *S. aureus*, BA= *B. atrophaeus*, BS= *B. subtilis*, CA= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

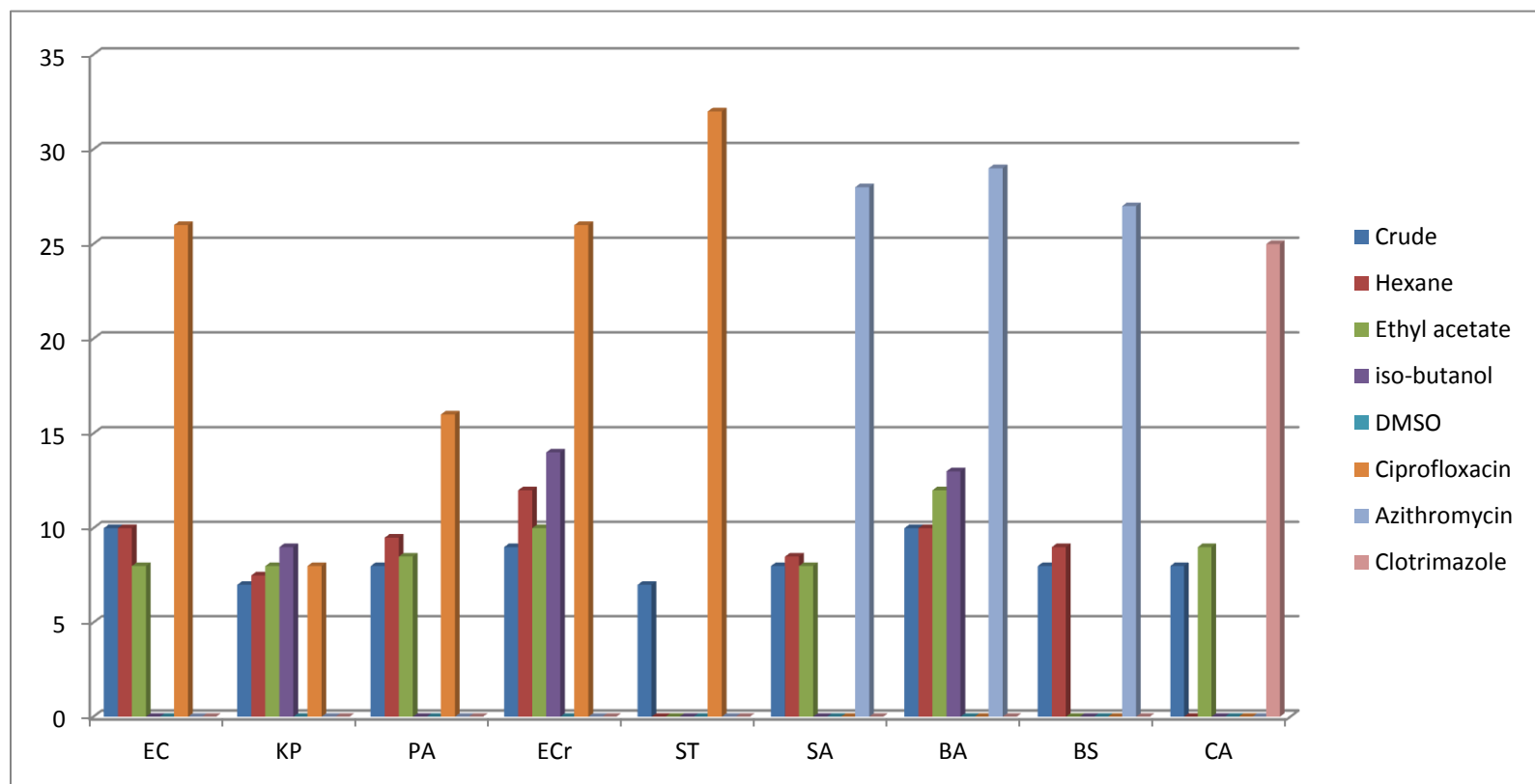


Fig 2.3: Anti-bacterial and anti-fungal activities of crude extract and fractions of *Cestrum nocturnum* L aerial part (CN-A).

EC= *E. coli*, KP= *K. pneumonia*, PA= *P. aeruginosa*, ECr= *E. carotovora*, ST= *S. typhii*, SA= *S. aureus*, BA= *B. atrophaeus*, BS= *B. subtilis*, CA= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

2.2.1.2 . Antimicrobial activities of *Cestrum Diurnum* Aerial (CD-A) without berries:

Like in the *Cestrum nocturnum*, the gram negative bacterial strains were more sensitive than the gram positive bacterial strains for *Cestrum diurnum* plant aerial parts' extracts and fractions. CD-A hexane gave profound results with *K. pneumonia* (13 mm). In *iso*-butanolic fraction, no activity was observed except that it gave 14 mm zone of inhibition against *E. carotovora*. Among gram positive bacteria, the inhibitions of *B. atrophaeus* and *B. subtilis* were comparable *i. e.*, in the range of 9 – 10 mm with the exception that the inhibition for *B. atrophens* was 13 mm around the disc.

The representative fungal strain, *C albicans*, showed very low sensitivities against the plant extracts/fractions. The results are tabulated in table 2.5 and graphically represented in fig 2.4.

Table 2.5: Anti-bacterial and anti-fungal activities* of crude extract and fractions of *Cestrum diurnum* L (Aerial part).

S. No	Sample code	EC	KP	PA	ECr	ST	SA	BA	BS	CA
1.	Crude	9	11	9.5	11	-	9	9.5	9	9.5
2.	hexane	11	13	11	10	-	-	9	-	9.5
3.	ethyl acetate	9	8.5	10	11.5	-	12	13	10	9
4.	iso butanol	0	0	0	14	0	0	0	0	0
5.	Dmso	0	0	0	0	0	0	0	0	0
6.	ciprofloxacin	26	8	16	26	32	0	0	0	0
7.	azithromycin	0	0	0	0	0	28	29	27	0
8	cotrimazole	0	0	0	0	0	0	0	0	25

EC= *E. coli*, KP= *K. pneumonia*, PA= *P. aeruginosa*, ECr= *E. carotovora*, ST= *S. typhii*, SA= *S. aureus*, BA= *B. atrophaeus*, BS= *B. subtilis*, CA= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

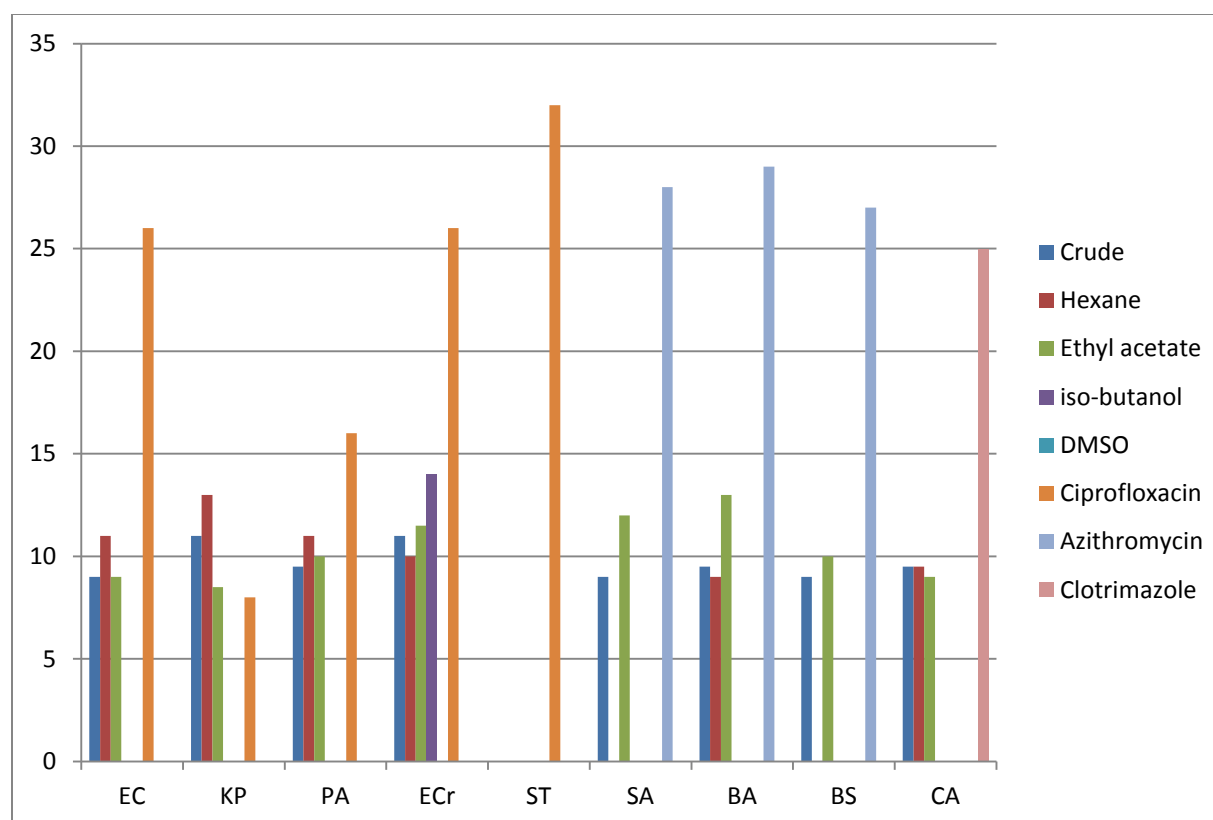


Fig 2.4: Anti-bacterial and anti-fungal activities of crude extract and fractions of *Cestrum diurnum* L (Aerial part).

EC= *E. coli*, KP= *K. pneumonia*, PA= *P. aeruginosa*, ECr= *E. carotovora*, ST= *S. typhi*, SA= *S. aureus*, BA= *B. atrophaeus*, BS= *B. subtilis*, CA= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

2.2.1.3 Antimicrobial activities of *Cestrum diurnum* fresh green berries:

With the crude methanolic extracts and fractions (in various solvents) of *Cestrum diurnum* fresh green berries (CD-G), both the gram negative and gram positive bacteria were found sensitive. Among gram negative bacteria, *E. coli* and *S. typhi* were found most resistant. The berries extracts/fractions were presenting good activities against *K. pneumonia* (11-13 mm) and *E. carotovora* (10-15 mm).

The *B. subtilis* (gram positive bacterium) showed no sensitivity against *iso*-butanolic extract and gave fair results with other extract/fractions, The inhibitory activities of CD-G crude and CD-G *iso*-butanolic fractions against *B. atrophaeus* were good *i.e.*, 14 mm and 13 mm respectively. Moderate bacterial growth inhibition was recorded for *S. aureus*. Table 2.6 represents the anti-bacterial and anti-fungal results CD-G.

Table 2.6: Antibacterial and anti-fungal activities* of crude extract and fractions of *Cestrum diurnum* L (green berries) CD-G.

S. No	Sample Code	EC	KP	PA	ECr	ST	SA	BA	BS	CA
1.	Crude	0	11	8.5	10	0	12	14	10	10
2.	Hexane	9	11	9.5	10	0	10	8.5	8.5	8.5
3.	Iso-butanol	0	11	0	15	0	10	13	0	0
4.	DMSO	0	0	0	0	0	0	0	0	0
5.	Ciprofloxacin	26	8	16	26	32	0	0	0	0
6.	Azithromycin	0	0	0	0	0	28	29	27	0
7.	Clotrimazol	0	0	0	0	0	0	0	0	25

EC= *E. coli*, KP= *K. pneumonia*, PA= *P. aeruginosa*, ECr= *E. carotovora*, ST= *S. typhii*, SA= *S. aureus*, BA= *B. atrophaeus*, BS= *B. subtilis*, CA= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

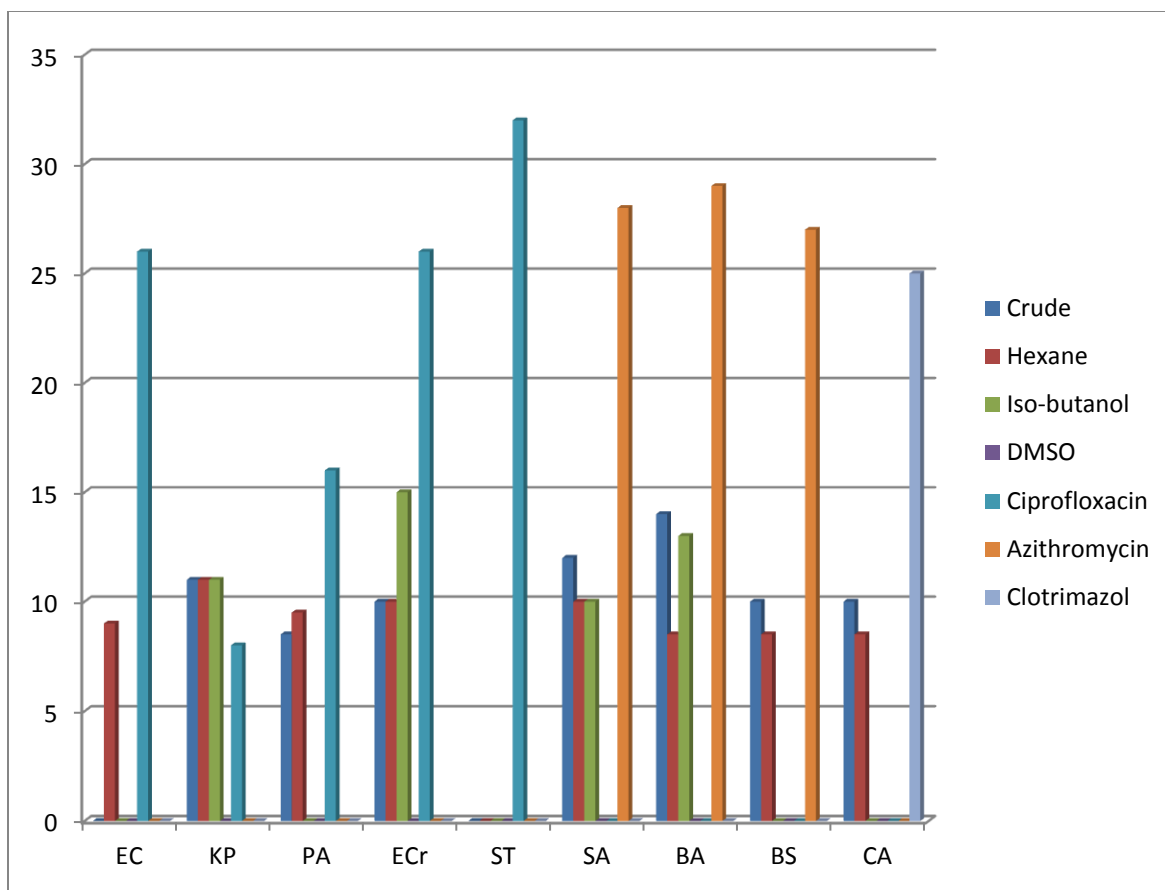


Fig 2.5: Antibacterial and anti-fungal activities of crude extract and fractions of *Cestrum diurnum* L (green berries) CD-G

EC= *E. coli*, KP= *K. pneumonia*, PA= *P. aeruginosa*, ECr= *E. carotovora*, ST= *S. typhii*, SA= *S. aureus*, BA= *B. atrophaeus*, BS= *B. subtilis*, CA= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

2.2.1.4 Antimicrobial activities of *Cestrum diurnum*, Ripe berries:

The activity of *Cestrum diurnum* ripe berries (CD-R) crude extract/fractions was quite low for the tested microbial strains. The largest zone of inhibition (15 mm) was obtained with CD-R crude for *S. aureus*. For all the other bacteria, gram negative and positive, and fungal strain low inhibition potential was observed. The inhibition range was 7 mm to 11.5 mm. Results are tabulated in table 2.7.

Comparing the anti-bacterial and anti-fungal activities of both the un-ripe (green) and ripe berries, it is observed that the potential of inhibition is increased against *E. coli* (8-11.5 mm), *P. aeruginosa* (8-10 mm) and *S. aureus* (9.5-15 mm) while with ripening a decrease in activity was observed in other bacteria. The susceptibility of the fungal strain, *C. albicans*, was also decreased for CD-R crude extract/fractions.

Table 2.7: Anti-bacterial and anti-fungal activities* of crude extracts and fractions of *Cestrum diurnum* L (Ripe berries) CD-R.

S. No	Sample Code	EC	KP	PA	ECr	ST	SA	BA	BS	CA
1.	Crude	8.5	11	10.5	9	0	15	9	7	8
2.	Hexane	11.5	9	8	8.5	0	10.5	9	7.5	8
3.	Iso-butanol	8	11	8.5	10	0	9.5	9	8	8
4.	DMSO	0	0	0	0	0	0	0	0	0
5.	Ciprofloxacin	26	8	16	26	32	0	0	0	0
6.	Azithromycin	0	0	0	0	0	28	29	27	0
7.	Clotrimazole	0	0	0	0	0	0	0	0	25

EC= *E. coli*, KP= *K. pneumonia*, PA= *P. aeruginosa*, ECr= *E. carotovora*, ST= *S. typhii*, SA= *S. aureus*, BA= *B. atrophaeus*, BS= *B. subtilis*, CA= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

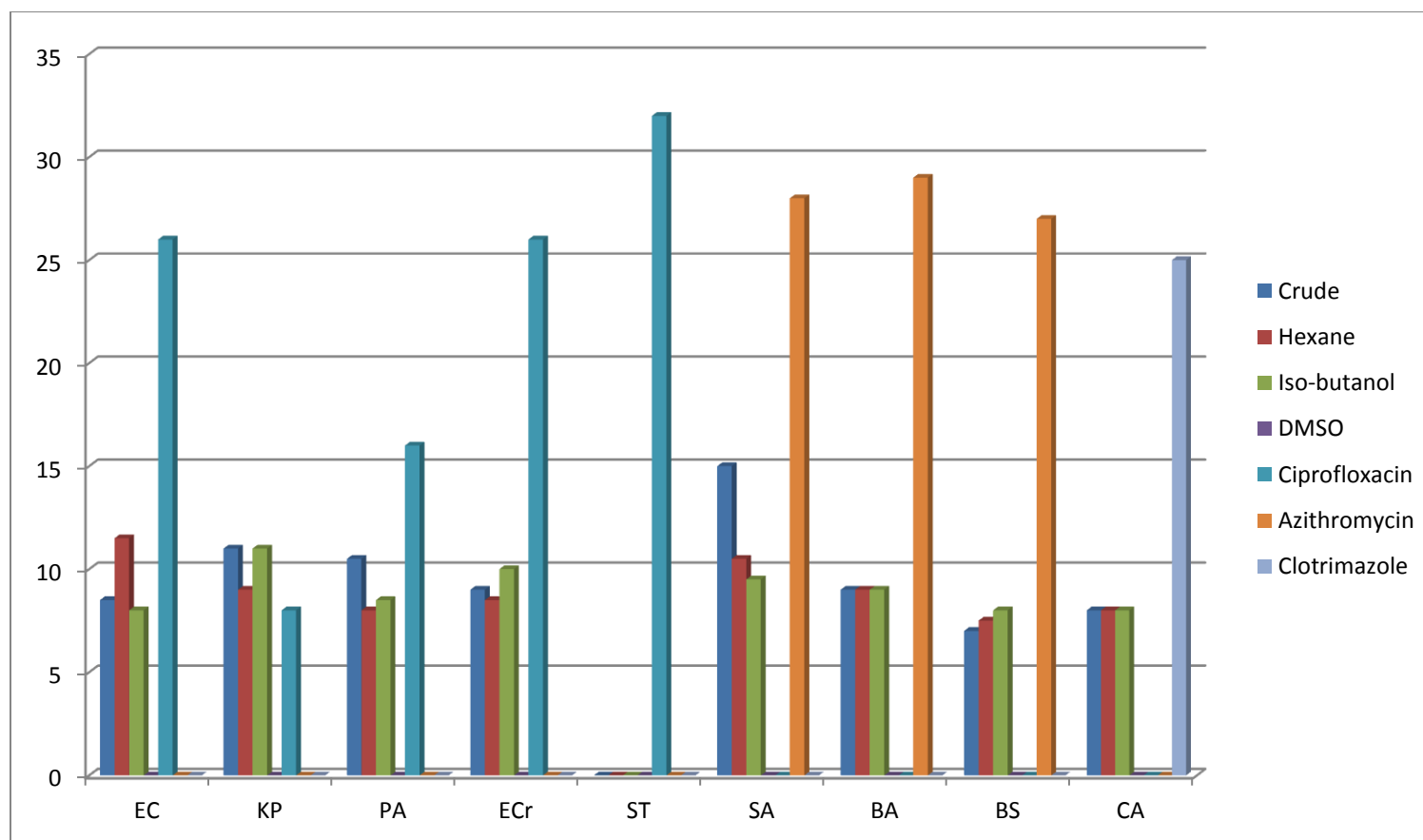


Fig 2.6: Anti-bacterial and anti-fungal activities of crude extracts and fractions of *Cestrum diurnum* L (Ripe berries) CD-R.

EC= *E. coli*, KP= *K. pneumonia*, PA= *P. aeruginosa*, ECr= *E. carotovora*, ST= *S. typhii*, SA= *S. aureus*, BA= *B. atrophaeus*, BS= *B. subtilis*, CA= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

2.2.1.5: Anti-bacterial and anti-fungal activities of crude saponins extract CSE:

The present study is a bioassay guided isolation of active compounds from *Cestrum* species. Saponins are a group of high molecular weight compounds with a large number of –OH groups which attribute very polar nature to them. From literature, it was evident that saponins are present in large quantities in *iso*-butanol fraction of all the plants/plants parts [73]. The anti-microbial activities of the crude extracts/fractions were quite encouraging; therefore, *iso*-butanol fraction was selected for further studies. Crude saponins were extracted from the *iso*-butanol fraction by dissolving the fraction in methanol. With the addition of cold acetone, crude saponins precipitated out. The precipitation process is based on the solubility principle. The solubility of saponins decreases with the addition of acetone in the methanolic extract resulting in precipitation.

The antimicrobial activities of the crude saponin extract (CSE) of CN-A showed good results with *K. pneumonia* (10.5 mm), *E. carotovora* (14 mm) and *B. atrophaeus* (14.5 mm). Crude saponins of CD-A were only active against *E. carotovora* *e.*, 14 mm. The ripe and unripe (green) berries of CD-A were showing sensitivity against the tested strains except *E. coli* and *S. typhii*. The most potent of them was CD-G against *E. carotovora* (15 mm) and *B. atrophaeus* (13.5 mm). CD-R presented moderate anti-microbial potential.

The isolated crude saponins were not good anti-fungal agents as is clear from the results. The results of the activities are listed in table 2.8.

Table 2.8: Anti-bacterial and anti-fungal activities* of crude saponins extracts of CN-A, CD-A, CD-G and CD-R.

S. No	Sample Code	<i>EC</i>	<i>KP</i>	<i>PA</i>	<i>ECr</i>	<i>ST</i>	<i>SA</i>	<i>BA</i>	<i>BS</i>	<i>CA</i>
1.	CN- A CSE	0	10.5	0	14	0	0	14.5	0	0
2.	CD- A CSE	0	0	0	14	0	0	0	0	0
3.	CD- G CSE	0	13	8	15	0	10	13.5	0	0
4.	CD- R CSE	8.5	10	8.5	11	0	9	9.5	8.5	8
5.	DMSO	0	0	0	0	0	0	0	0	0
6.	Ciprofloxacin	26	8	16	26	32	0	0	0	0
7.	Azithromycin	0	0	0	0	0	28	28	27	0
8.	Clotrimazol	0	0	0	0	0	0	0	0	25

EC= *E. coli*, *KP*= *K. pneumonia*, *PA*= *P. aeruginosa*, *ECr*= *E. carotovora*, *ST*= *S. typhii*, *SA*= *S. aureus*, *BA*= *B. atrophaeus*, *BS*= *B. subtilis*, *CA*= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

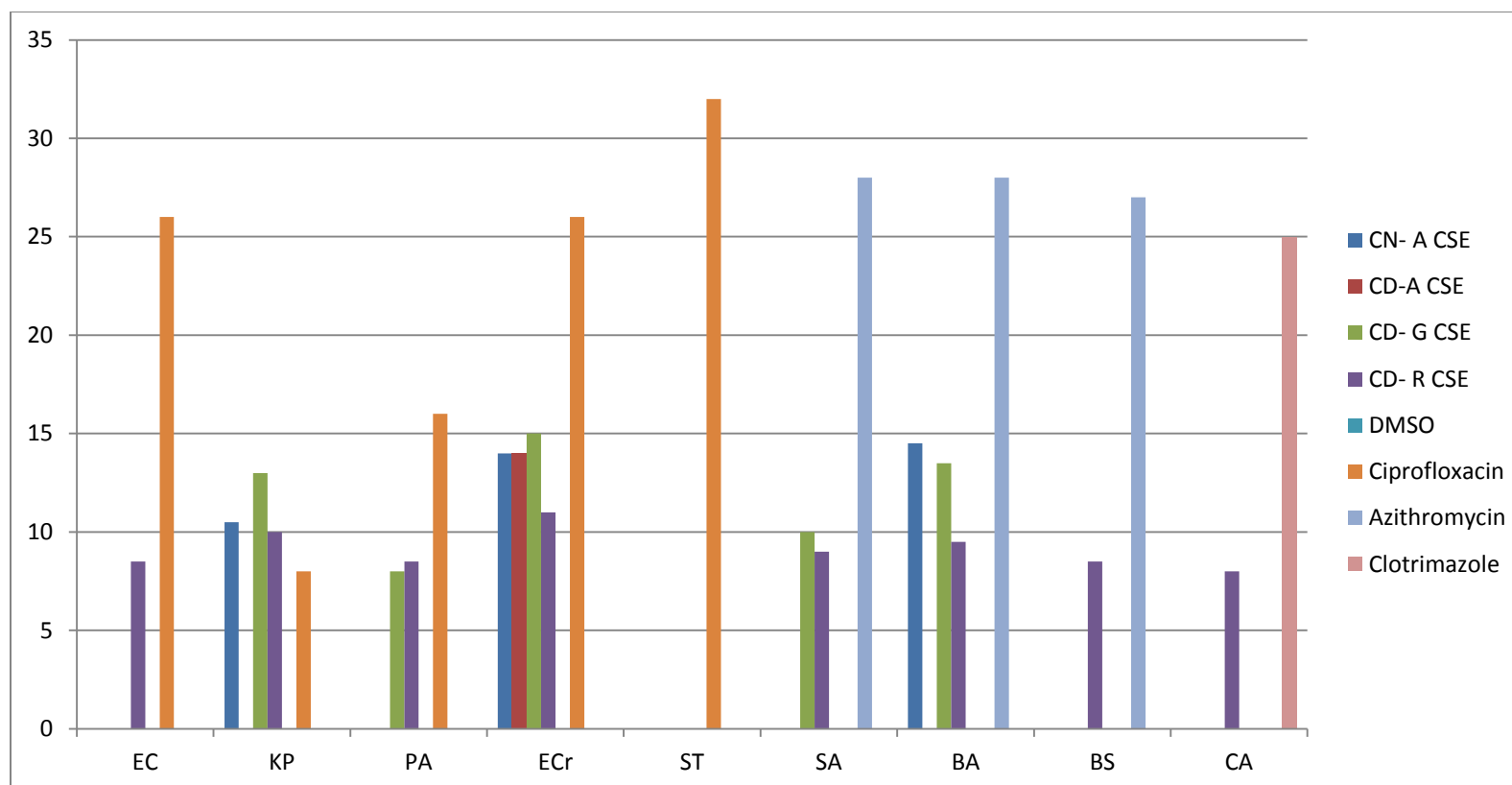


Fig 2.7: Antibacterial and anti-fungal activities of crude saponins extracts of CN-A, CD-A, CD-G and CD-R.

EC= *E. coli*, KP= *K. pneumonia*, PA= *P. aeruginosa*, ECr= *E. carotovora*, ST= *S. typhii*, SA= *S. aureus*, BA= *B. atrophaeus*, BS= *B. subtilis*, CA= *C. albicans*

*The zones of inhibition for anti-bacterial and anti-fungal activities were measured in “mm”.

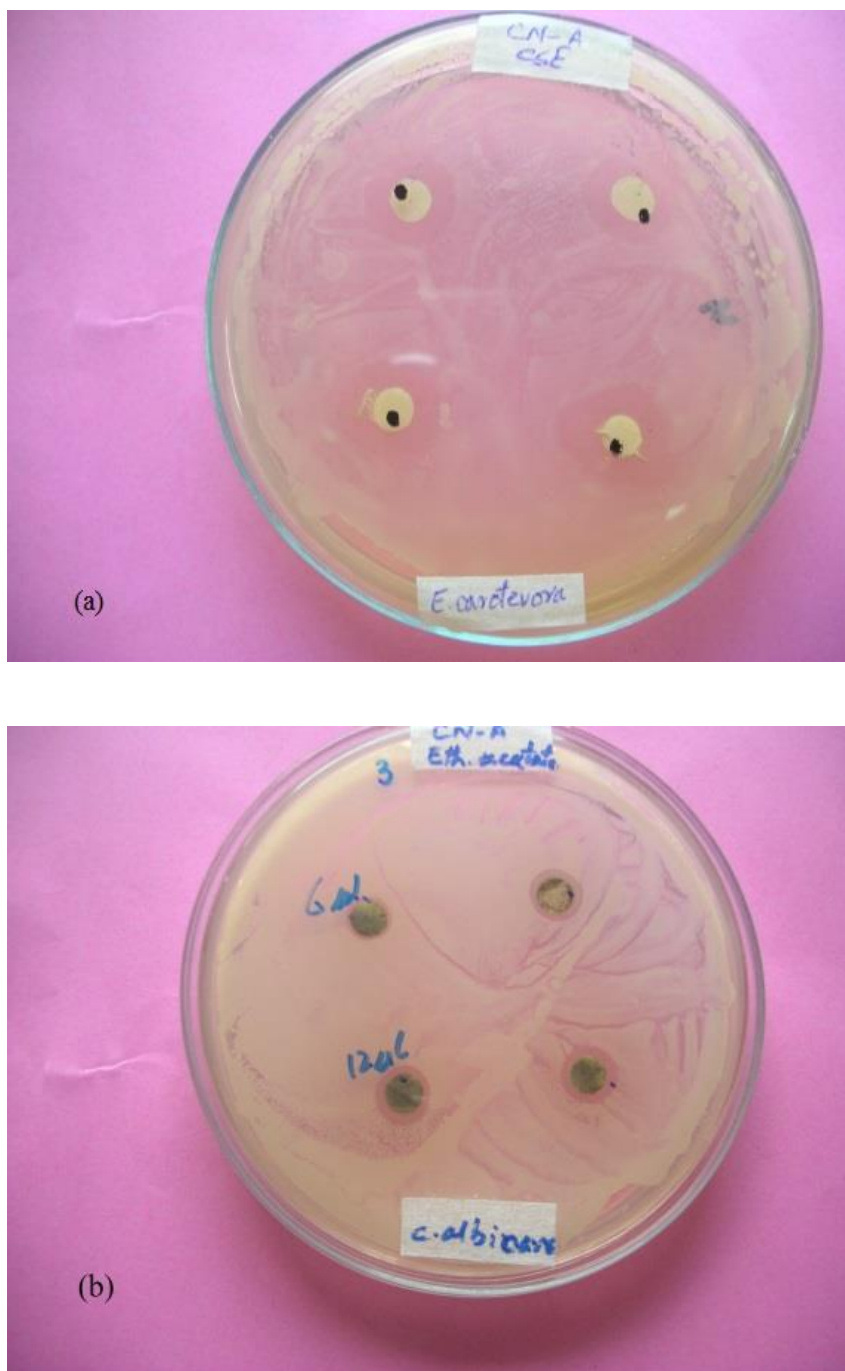


Fig. 2.8: Inhibition by *Cestrum nocturnum* and *Cestrum diurnum* against different microbes.

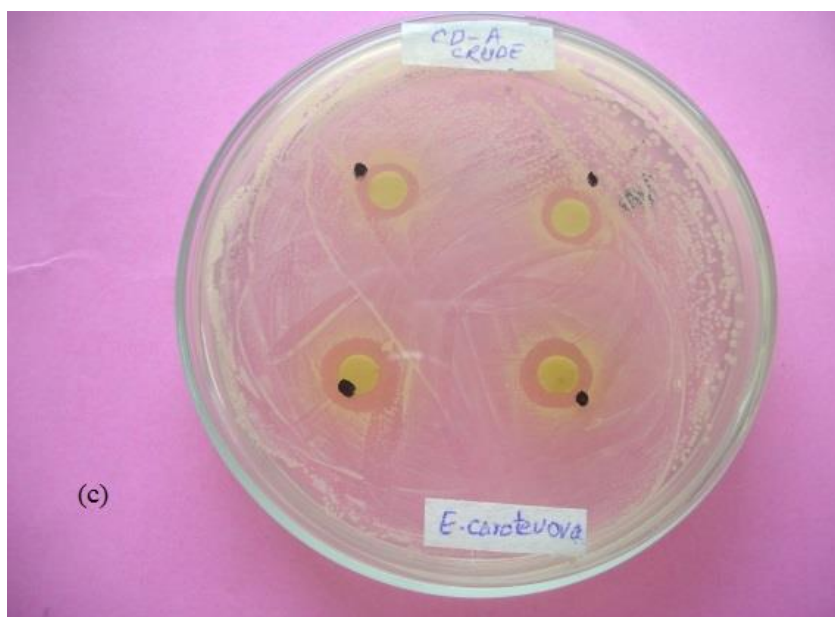


Fig. 2.8: Inhibition by *Cestrum nocturnum* and *Cestrum diurnum* against different microbes.



Fig. 2.8: Inhibition by *Cestrum nocturnum* and *Cestrum diurnum* against different microbes.



(g)



(h)

Fig. 2.8: Inhibition by *Cestrum nocturnum* and *Cestrum diurnum* against different microbes.



Fig. 2.8: Inhibition by *Cestrum nocturnum* and *Cestrum diurnum* against different microbes.

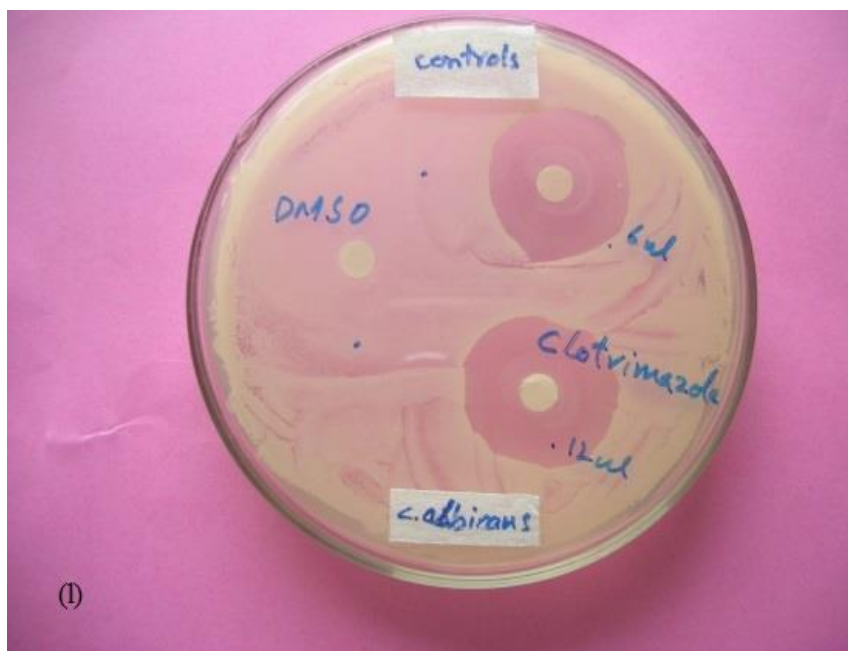


Fig. 2.8: Inhibition by *Cestrum nocturnum* and *Cestrum diurnum* against different microbes.

2.2.2 Anti-leishmanial activities:

Leishmaniasis is a set of infectious protozoal diseases caused by trypanosomal protozoa which are transmitted by a vector, known as sandflies. The common vectors of the disease are mosquitos of *Lutzomia* and *Phlebotomus* genura. It is considered to be a major health risk since 1950's by World Health Organisation (WHO), as it has expanded its hold across the population of the hot and tropical developing countries. [74]. It is threatening a population of 350 million around the world [75]. In 1959, the first anti-leishmanial chemotherapeutic drug (pentavalent antimonial drug) was developed, and is still recommended as a suitable drug for all kinds of the epidemic. Amphotericin B, pentamidine and miltefosine are recently approved alternative drugs for visceral infections. Toxicities to man and the lasting side effects of these synthetic drugs were the major shortcomings. Due to these drawbacks [76] and encouragement by WHO towards the traditional remedies [77], interest was developed to search a drug of plant origin, having less toxicity and high levels of effectiveness [78]. The potent leishmanicidal activities of certain chemically defined molecules isolated from natural sources, present an exciting advance in the search for novel antiprotozoal agents at a time when there is great urge for new and innovative drugs [79].

Anti *leishmania* activities were performed for all the fractions and obtained remarkable results. The present study shows that the crude extracts and fractions of both the plants exhibit interesting *in vitro* anti-leishmanial properties, seeming to validate their use in skin ulcers, wounds and burns *etc*, in folk medicine. All the extracts/fractions obtained from the plants, showed hundred percent results against *leishmania* parasite.

20 μ L *Leishmania tropica* and *Leishmania major* amastigotes were added to each extract/fraction of the plant in laminar flow. These samples were incubated for 96 hours (12 days) and checked for the *leishmanial* parasite presence/growth. Both varieties of the parasite gave similar results for all the extracts. In the negative control no *leishmania* was added and in positive control sample, no extract was added [80].

The culture medium RPMI 64 (Gibco.USA) 0.3 g/30 ml distilled water was distributed in 10 vials and added 10% fetal bovine serum to each vial. Penicillin and kanamycin were also mixed to avoid bacterial contamination. The air tight vials were placed in ice jar. Skin scrapings, from the lesion were directly added to each vial and the medium was incubated at 26°C in incubator. The parasite was kept under observation for 12 days. After each 24 hours, the parasite was stained with Geimsa to check its different life stages during this period under 10 x, 40 x and 100 x magnification of microscope.

To 3 ml RPMI 1640 media, 10% fetal calf serum was supplemented. This was accompanied by penicillin and gentamycin to prevent the secondary bacterial growth, followed by test sample addition at the rate of 25 μ g/ mL. After that 1 ml of *leishmania* culture was added in the vial except in negative control group. A similar process was carried out for all the test extracts/fractions. The cultures were examined on daily basis under the microscopic magnification of 10x, 40x and 100x for 12 days. The experiment was conducted in duplicates for getting more accurate results.

The crude methanolic extracts of CN-A and CD-A were partitioned with hexane, ethyl acetate and *iso*-butanol. The *iso*-butanol fraction was further treated for isolation of crude saponins. All the 10 different samples were investigated for their anti-leishmanial

activities against *leishmania* amastigotes, collected from an infected person and then cultured in laboratory following the reference method [80]. The results of anti-leishmanial activities are tabulated in table 2.9. Amazingly all the tested, ten extract samples checked were giving extra-ordinary results against leishmaniasis showing 90% inhibition on 4th day and hundred per cent activity at the 5th day of the colonization. Studies revealed that the extracts completely subdued the amastigotes at their initial stage. The crude methanolic and hexane extracts of CN-A, CD-A, and CD-R were showing no inhibition at the first day while CD-G exhibited a 10% inhibition at its first day. Up to 60% inhibition was observed for CD-G while the rest ranged for a 40-50-% inhibition on the second day. The minimum activity on the third day was 60% for and maximum was 70% for. On the fourth day, the leishmanial growth was inhibited to 90%. A 100% inhibition was observed on the 5th day of the experiment resulting in no alive leishmania.

The ethyl acetate extracts of CN-A and CD-A were active at the first day showing 10% inhibition activity. Further results were almost all same to those of the above cited fractions. The ethyl acetate fractions gave 100% results on the 5th day while increasing gradually from 40% inhibition at the second day.

As a result of our interest in saponins for our future studies due to their enormously high and diversified bioactivities, crude saponins were first isolated for different bioactivities. Crude saponins were extracted from different parts of the plants by treating their concentrated methanolic extracts with cold acetone. The crude saponins samples of CN-A, CD-A, CD-G and CD-R showed exactly the same results as obtained with crude extracts i.e., 100 % inhibition at amastigote stage on the 5th day. No inhibition

was observed for CN-A CSE and CD-A CSE at the first day while CD-G CSE and CD-R CSE displayed 10% inhibition. Starting with a 50% inhibition for CN-A CSE, CD-A CSE, and CD-G CSE, and 60% for CD-R CSE on second day was recorded. On third day CN-A CSE and CD-A CSE resulted in 60% activity while CD-G CSE and CD-R CSE showed 70% activity. All the CSEs subdued 90% growth at the 4th day whereas a 100% inhibition was observed at the 5th day of experiment.

Although there were no amastigotes observed in the initial stages of the test (at 5th day), still the experiment was proceeded further and checked for the presence/growth of the parasite after every 24 hours as per protocol. The plant fractions inhibited the *leishmanial* parasite survival, leaving no space for the germs to grow. It is obvious from these results that the plants (CD and CN) are potent leishmanicidal. It could be considered as an important milestone for plant nature drug development, having remarkable significance against leishmaniasis.



Amastigote form of
leishmania

Fig. 2.9: Giemsa stain image showing the amastigote in macrophages



epimastigote form of
leishmania

Fig. 2.10: Giemsa stain image showing the epimastigote in macrophages in positive control.

Table 2.9: Anti-leishmanial activities of *Cestrum nocturnum* and *cestrum diurnum*:

S No	sample code	Days.1	Days.2	Days3	Days.4	Days.5	Days.6	Days.7	Days.8	Days.9	Days.10	Days.11	Days.12
1.	CN- A Crude	0%	50%	70%	90%	100%	100%	100%	100%	100%	100%	100%	100%
2.	CN- A Hexane	0%	50%	60%	90%	100%	100%	100%	100%	100%	100%	100%	100%
3.	CN- A Ethyl acetate	10%	40%	50%	90%	100%	100%	100%	100%	100%	100%	100%	100%
4.	CN- A CSE	0%	50%	60%	90%	100%	100%	100%	100%	100%	100%	100%	100%
5.	CD- A Crude	0%	40%	70%	90%	100%	100%	100%	100%	100%	100%	100%	100%
6.	CD- A Hexane	0%	50%	60%	90%	100%	100%	100%	100%	100%	100%	100%	100%
7.	CD- A Ethyl acetate	10%	50	70	90%	100%	100%	100%	100%	100%	100%	100%	100%
8.	CD- A CSE	0%	50	60	90%	100%	100%	100%	100%	100%	100%	100%	100%
9.	CD- G Crude	10%	60	70	90%	100%	100%	100%	100%	100%	100%	100%	100%
10.	CD- G Hexane	10%	50	70	90%	100%	100%	100%	100%	100%	100%	100%	100%
11.	CD- G CSE	10%	50	70	90%	100%	100%	100%	100%	100%	100%	100%	100%

12.	CD- R Crude	0%	40%	60%	90%	100%	100%	100%	100%	100%	100%	100%	100%
13.	CD- R Hexane	0%	50%	70%	90%	100%	100%	100%	100%	100%	100%	100%	100%
14.	CD- R CSE	10%	60%	70%	90%	100%	100%	100%	100%	100%	100%	100%	100%
15.	N.control	—	—	—	—	—	—	—	—	—	—	—	—
16.	Positive Control	++	++	++	++	++	++	++	++	++	++	++	++

++ Indicate the presence of Epimastigote and Amastigote

- Indicates absence of amastigote and epimastigote.

% indicate the mortality of the parasitic stage in observe field

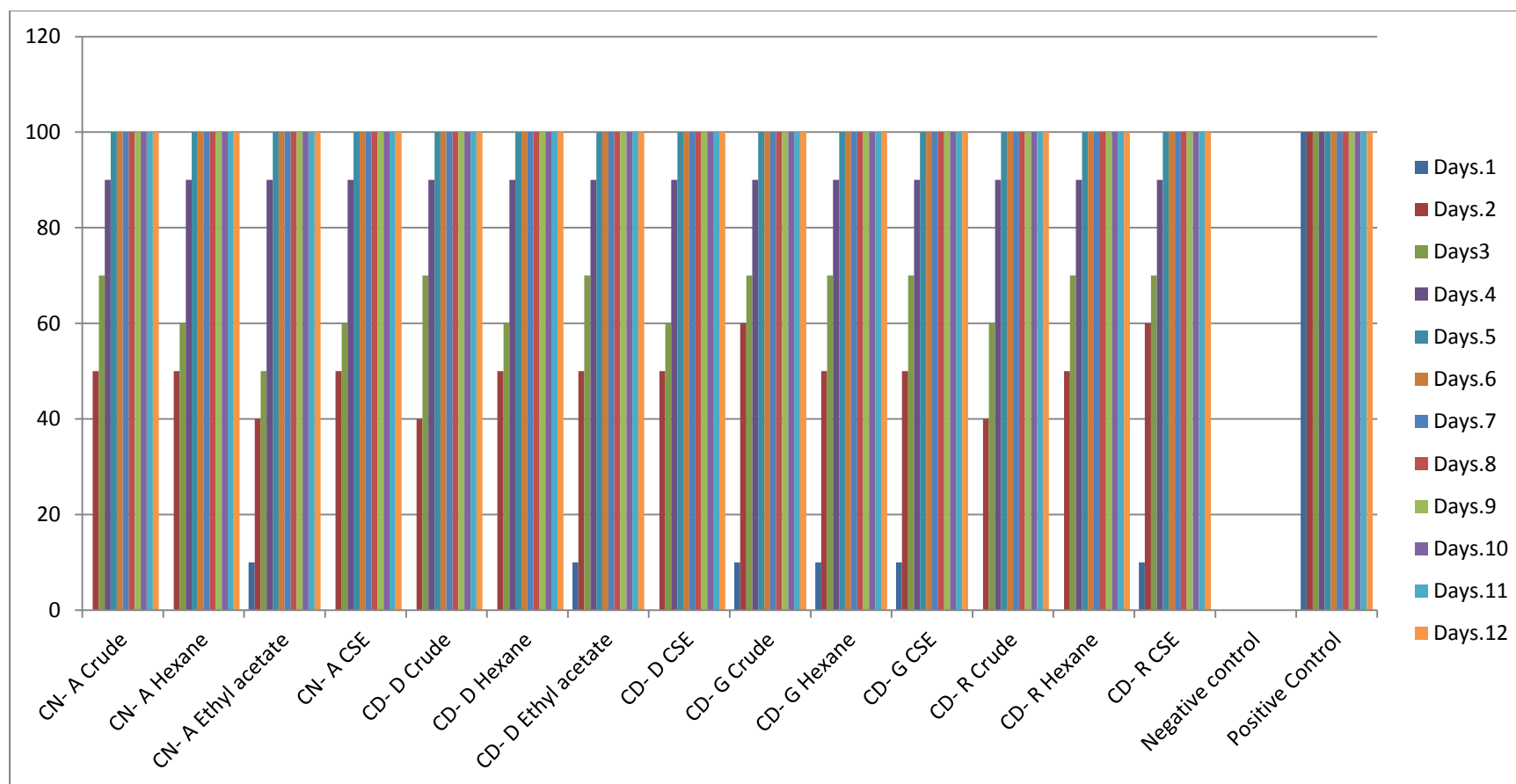


Fig 2.11: Anti-leishmanial activities of CN-A, CD-A, CD-G and CD-R in percentage.

2.2.3 Anti-oxidant activities:

In the recent years, there has been considerable increase in oxidative stress causing serious disorders and damages to human life, due to various environmental factors. The free radicals present on the planet. Effect of these radicals on man is normally in the form of many mutational diseases like various types of cancer, Alzheimer's disease, rheumatoid arthritis, aging process *etc*[81]. Extensive literature has been assembled on the anti-oxidant effect of different natural and synthetic products. The synthetic drugs are not adored by the scientists for their side effects, so search for good natural anti-oxidants has gained profound interest. Natural anti-oxidants which are produced by the plants, have wide range of bio-chemical activities *i. e.*, reserves reactive oxygen species (ROS) generations, scavenging free radicals (directly or indirectly) and adjustment of redox potentials in the cells [82, 83]. Anti-oxidant studies of *Cestrum nocturnum* stem indicated that the plant's 80% methanolic extract had high anti-oxidant potential (86.6%) as compared with DPPH (2,2-diphenyl-1-picrylhydrazyl). The activities could be attributed to the flavonoids, tannins and triterpenoids *etc*[84]. The urosolic acid, from *Cestrum diurnum*, showed good antioxidant activities [85].

In the present study, crude extracts and fractions of *Cestrum nocturnum* and *Cestrum diurnum* arial parts and *Cestrum diurnum* green and ripe berries were subjected to anti-oxidant studies. DPPH was used as a standard. The plant material was extracted with methanol. The methanolic extract was dissolved in water and partitioned with hexane, ethyl acetate and *iso*-butanol. Crude saponins were also precipitated out. All the samples showed high free radical scavenging activities.

The anti-oxidant activities of crude extracts and fractions of *Cestrum nocturnum* aerial part ranged from 18-81 %. The crude methanolic extract exhibited 56.59 % activity while the fractions isolated from the methanolic extract presented an exciting trend in the activity. The anti-oxidant activity exhibited by hexane and ethyl acetate fractions was 17 % and 67 %. The activity was 80.78 % with crude saponin mixture. Better anti-oxidant activity was observed in the fractions compared to the crude methanolic extract that may be due to increase in concentration of the active compound(s) in the fractions.

Crude methanolic extract of *Cestrum diurnum* delivered 85.75 % activity. The hexane fraction showed 63.22 % while the ethyl acetate fraction demonstrated 50.6 % anti-oxidant activity. The crude saponins released 79.7 % oxidative stress in comparison with the DPPH. Results are tabulated in table 2.10.

The anti-oxidant potential of crude methanolic extract of *Cestrum diurnum* green berries was observed to be 81.1 % and 86.75 % for hexane fraction. The crude saponin mixture gave 88.07 % activity. 90.39 % anti-oxidant activity was documented for methanolic extract of *Cestrum diurnum* ripe berries while hexane fraction showed 83.76 % activity. The crude saponins extract gave 90.05 % anti-oxidant activity.

Overall, the crude methanolic and saponin extract of *Cestrum diurnum* ripe berries gave highest anti-oxidant values i. e., 90.05 % and 90.39 % respectively.

Table 2.10: The anti-oxidant potential of CN-A, CD-A CD-G and CD-R

S.No	Sample Code	Free radical scavenging activity (%)
1.	CN- A Crude	56.593
2.	CN- A Hexane	17.826
3.	CN- A Ethyl acetate	67.528
4.	CN- A CSE	80.781
5.	CD- A Crude	85.752
6.	CD- A Hexane	63.220
7.	CD- A Ethyl acetate	50.629
8.	CD- A CSE	79.787
9.	CD- G Crude	81.113
10.	CD- G Hexane	86.746
11.	CD- G CSE	88.071
12.	CD- R Crude	90.390
13.	CD- R Hexane r	83.764
14.	CD- R CSE	90.059
15.	DPPH	100

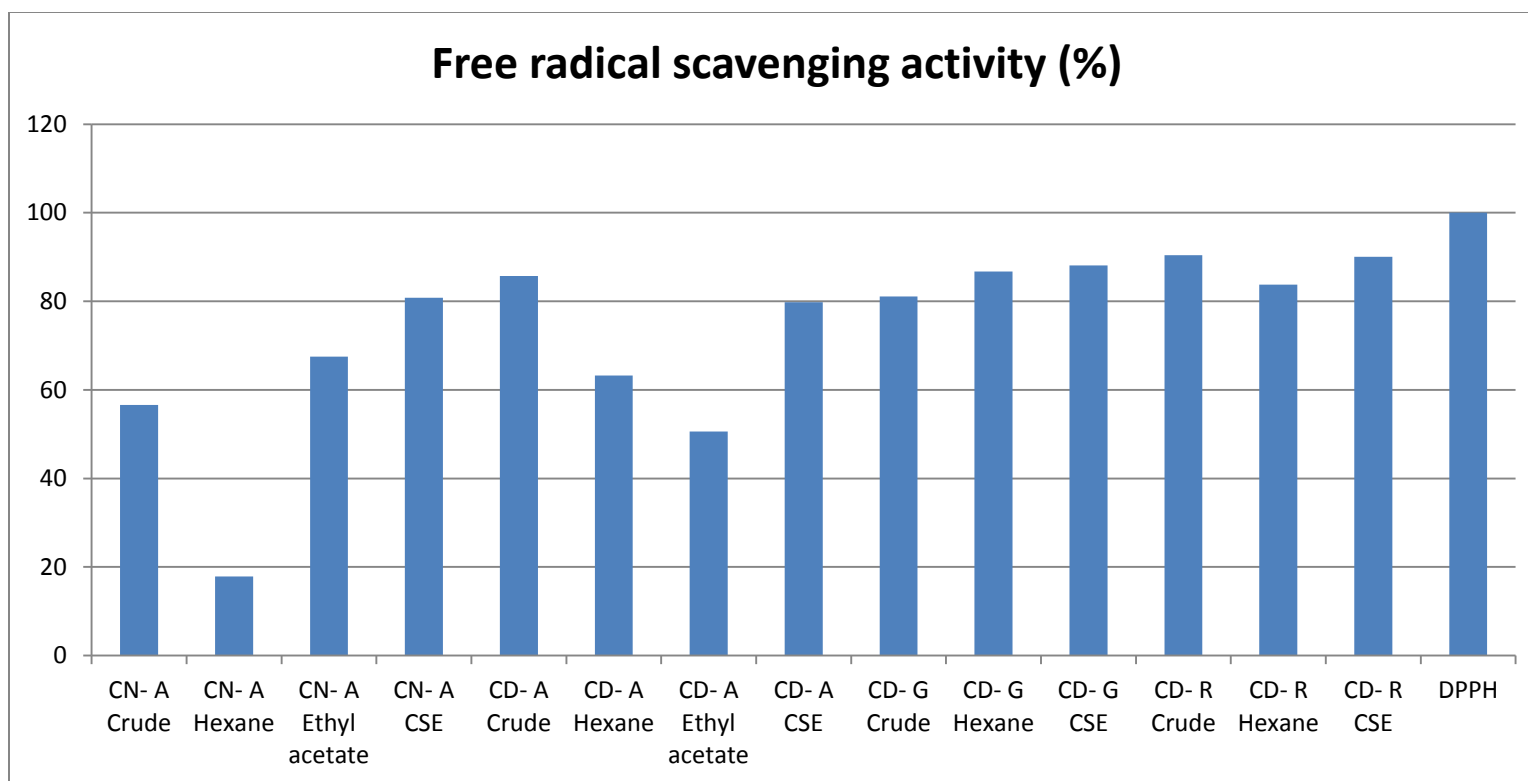


Fig 2.12: The anti-oxidant potential of CN-A, CD-A CD-G and CD-R

2.3: Phytochemical Investigations:

On the basis of biological activities, butanolic fraction was subjected to column chromatography for isolation of pure compounds. From this total ten (10) compounds were isolated. Six (6) compounds were subjected to characterization techniques but only three were identified while four were left behind without characterization due to their very low quantities. Out of the three identified compounds, two compounds were reported previously [86, 87] and the other one was a new compound.

2.3.1: Characterization of Hum-V:

A pure compound labeled as **Hum-V**, was isolated from *iso*-butanol fraction of the crude methanol extract of aerial parts of *Cestrum nocturnum* as white crystals (Fig 2.13).

The FAB negative ion mass spectrum of Hum-V exhibited pseudo-molecular ion peak at m/z 1210 [M-H] (1210 mass was calculated for $C_{56}H_{89}O_{28}$), confirmed that the terminal positions are occupied by pentose (m/z 132) and a hexose (m/z 162) units, giving the fragment ions at m/z 1077 [M-H-132] and 1047 [M-H-162]. The peak at m/z 885 [M-H-2 x 162] is an indication for the branching at the inner hexose attached to the terminal hexose. These attachments were further confirmed by another fragment ion peak at m/z 753 which is due to loss of three fragments *i. e.*, one pentose and two hexose units [M-H-(132 + 2 x 162)]. A peak at 429 showed the aglycone moiety, after the breakage of one more hexose from m/z 591 unit [M-H-(132 + 3 x 162)]. Fig 2.14 represents all the fragments.

Glycosidic nature of Hum-V was indicated by broad absorption bands at 3360 cm^{-1} and 1033 cm^{-1} in IR spectrum which is due to the O-H stretching and C-O stretching frequency, respectively. The band intensity at 927 cm^{-1} is smaller as compared to the band strength at 897 cm^{-1} , indicating the spiroketal chain of the 25*R*-series [20, 88]. The chemical shifts of ^{13}C - NMR in the un-fragmented saponin exhibited the signs that the aglycone is a spirostene type steroid

The ^{13}C NMR (100 MHz) broad band spectrum exhibits 56 carbon resonances. By the application of DEPT technique, the spectra support 34 methine (CH), 14 methylene (CH_2) and 4 methyl (CH_3) groups. Collectively these carbon atoms are 52 and the remaining four carbon atoms are suggested to be quaternary carbon atoms, table 2.11.

The signal at δ_{C} 70.658 was assigned to C-2. It showed a downfield shift of 41.46, due to the electron rich oxygen environment at C-2. The chemical shift value of C-2 appears at δ_{C} 29-32 in the absence of oxygen [89]. Chemical shift at δ_{C} 84.58 was assigned to C-3, indicating β -hydroxyl group at this position. As compared to gitogenin [90], it has a downfield shift of +8.16 (from original value) assuring that glycone moiety is attached to C-3. Comparing the upfield signals at δ_{C} 16.32, 20.42 and 15.00 with literature, it reveals that they are indicating the methyl groups at C-18, C-19 and C-21 positions of the aglycone, respectively. R-orientation of C-27 was confirmed by its upfield signals at δ_{C} 17.30 [91]. The easily recognizable and characteristic signals at δ_{C} 140.08 and 121.93 were assigned to C-5 and C-6, respectively which are linked together in an unsaturated fashion. This downfield shift can be easily observed by comparing the signals for these carbons in saturated saponins [89]. The chemical shift for C-22 appears at δ_{C} 109.01 which is an indication for a ketal type linkage across this carbon. The δ_{C}

31.09, 29.15, 31.82 and 66.58 were indicative chemical shifts for C-23, C-24, C-25 and C-26, respectively. These signals are oftenly observed for spiroketal type structure. The structure of aglycone of Hum-V was confirmed as yuccagenin by comparing all the δ_C with reported steroidal sapogenin/saponin C^{13} NMR shielding results [90] (fig 2.15.)

The intact saponin Hum-V exhibited δ_H 0.67 and 1.1 singlets due to H₃-18 and H₃-19 in the 400 MHz ^1H -NMR spectrum, respectively. Two doublets at δ_H 0.8 and 1.15 were designated to H₃-27 and H₃-21 respectively. The coupling constant for H₃-27 was calculated to be $J = 5.51\text{Hz}$ and for H₃-21 was $J = 6.9\text{Hz}$. For the vinylic H-6 proton, a distorted triplet was observed at 5.28 ppm (fig 2.16.) The interaction of this vinylic proton at δ_H 5.28 ppm with C-6 at δ_C 121.93 was also indicated in hetero-COSY spectrum.

In the heteroCOSY-45° spectrum coupling of H-2 β (δ_H 4.05) was observed with H-1 α (1.3) and H-1 β (2.3) (assignments interchangeable) and H-3 (δ_H 4.92) was coupled with H-2 β (δ_H 4.05) and also with at H-4 α (δ_H 2.55) and H-4 α (δ_H 2.3 β) (assignments interchangeable). Due to the presence of quaternary carbons at C-1 and C-5, further connectivity was not observed at both sides. The vinylic proton at H-6 gave δ_H 5.28 and was coupled with H-7 α (1.80) and H-7 β (1.90) as triplet. The cross links were observed between H-7 and H-8. The assignments for H-7 α (δ_H 1.80) and H-7 β (1.90) were confirmed from its COSY interactions with each other and H-6 (5.28) and H-8 β (1.50). H-8 at δ_H 1.5 showed coupling with 7 α (1.80), 7 β (1.90), 9 α (1.63). Coupling of H-16 at δ_H 4.5 was observed with 15 α (δ_H 1.42), 15 β (δ_H 1.92) and 17 (δ_H 1.75). Another interaction observed in COSY spectrum was between C-26 protons at δ_H 3.50 (26 α) and 3.60 (26 β), which in turn showed connectivity with H-25 at δ_H 1.55.

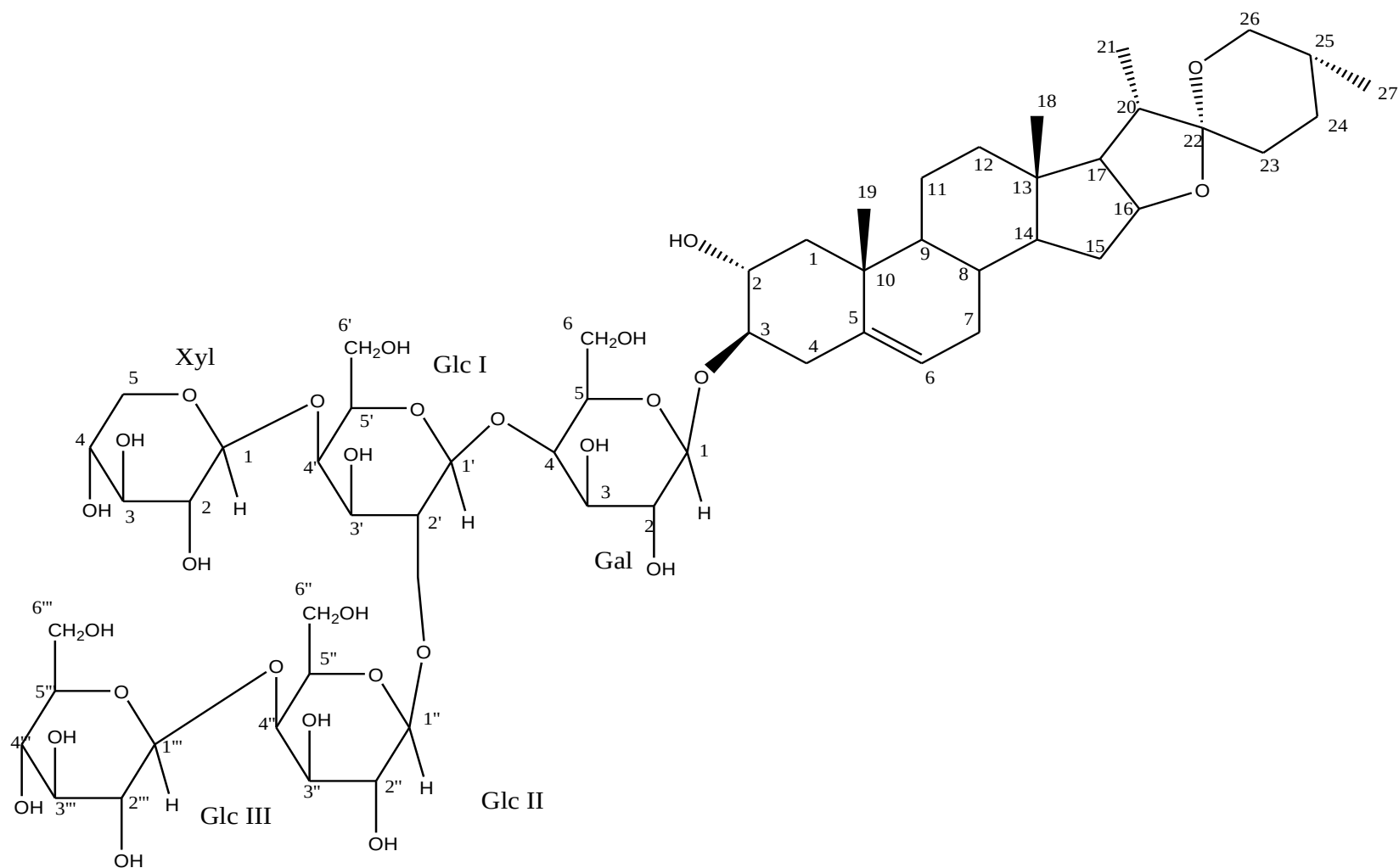


Fig 2.13: Hum-V, (Nocturnoside A) (25R)- spirost-5ene-2,3-diol, [2 α , 3 β] 3-O- $\{\beta$ -D-glucopyranosyl (1 $\rightarrow$ 4)- β -D-glucopyranosyl (1 $\rightarrow$ 2)- β -D-glucopyranosyl $\}$ [(1 $\rightarrow$ 4)- β -D-xylopyranosyl] (1 $\rightarrow$ 4)- β -D-galactopyranoside).

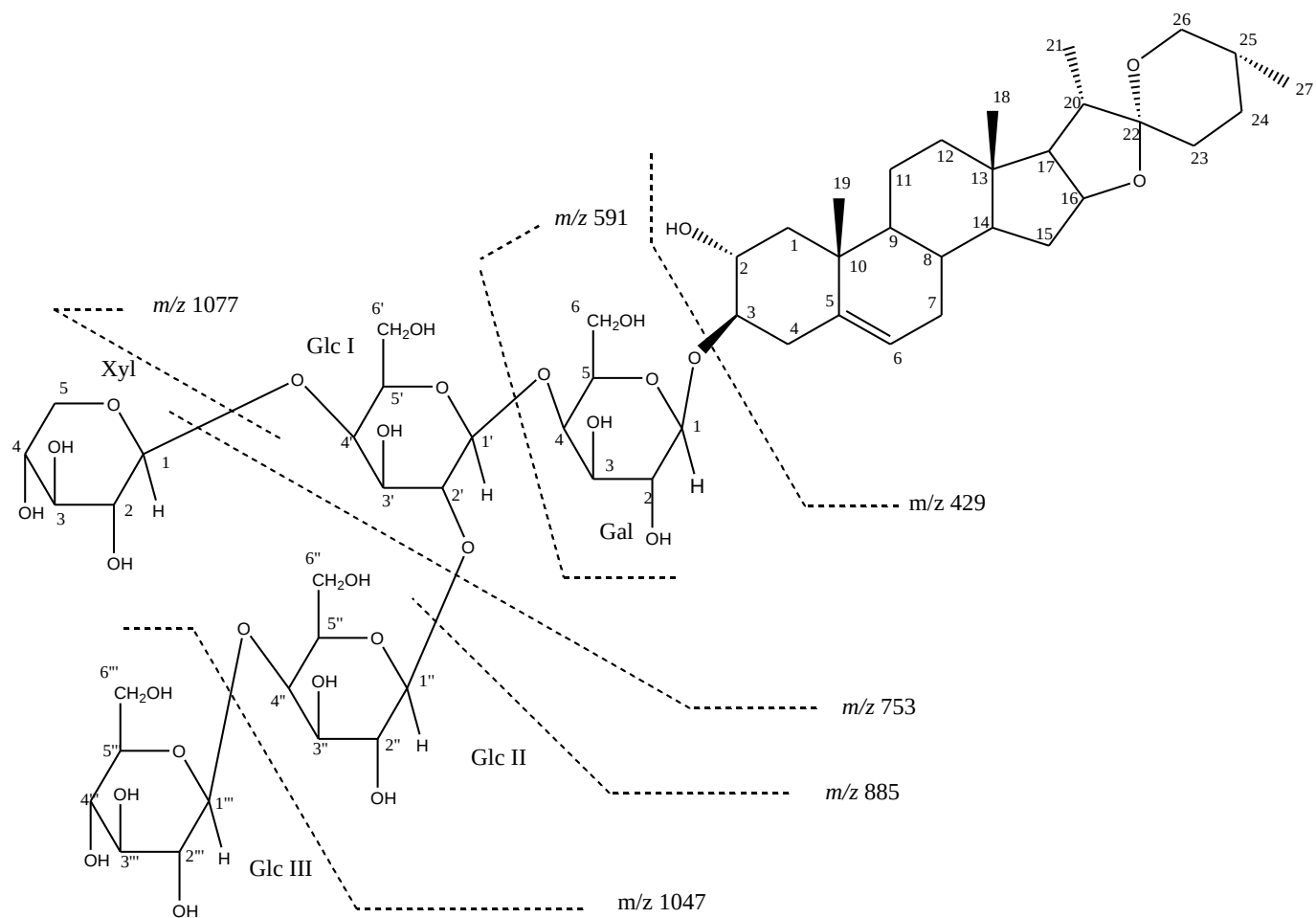


Fig 2.14: **FAB-MS** (negative ion mode) Fragmentation pattern of Hum-V, m/z 1210 $[M-H]^-$ (calculated for $C_{56}H_{89}O_{28}$ 1209)

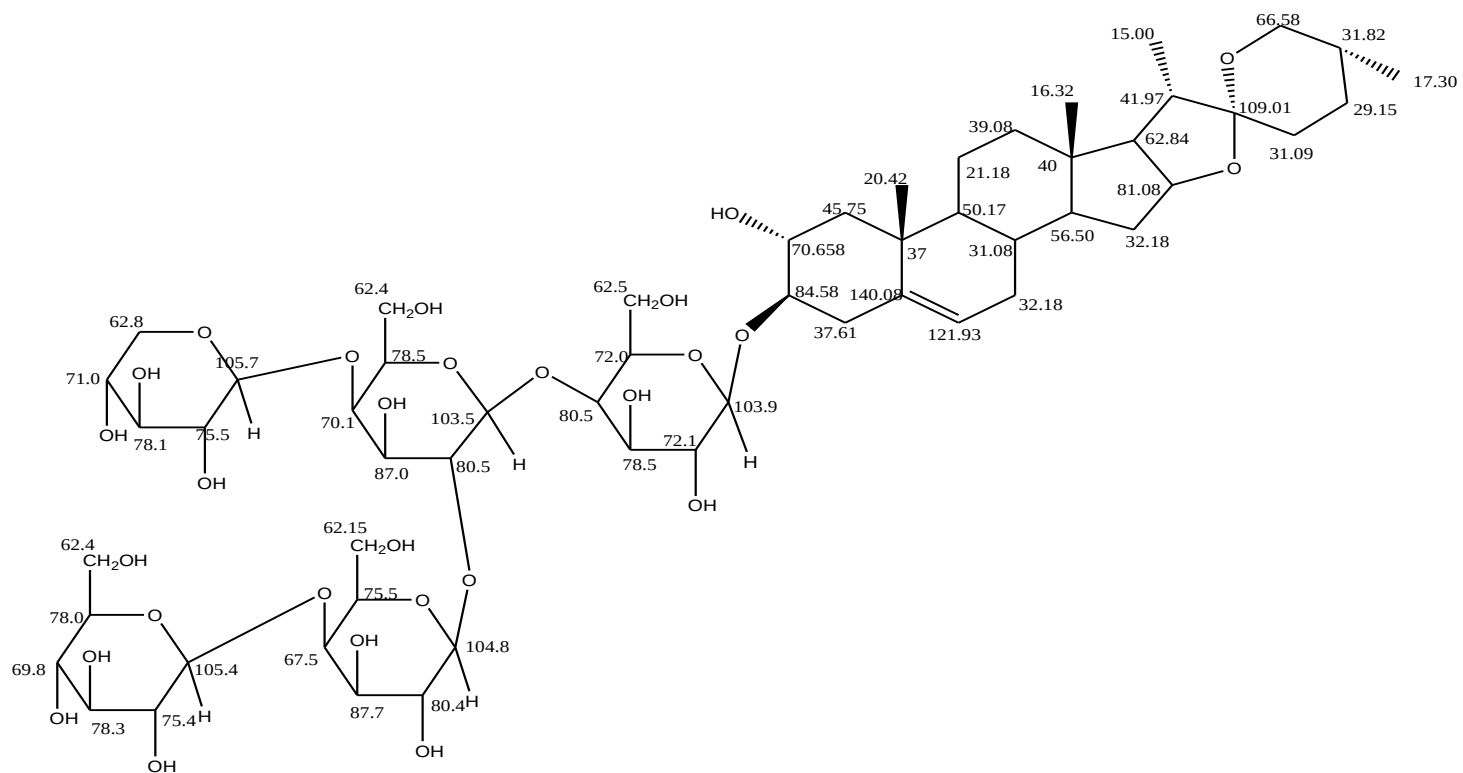


Fig 2.15: ^{13}C NMR (100.61 MHz) chemical shifts δ_{C} of Hum-V in pyridine

Table 2.11: ^{13}C and ^1H NMR spectral data of Hum-V from one and two dimensional experiments

S. No	C	^{13}C Chemical shifts δ_{C}	DEPT	Multip-	^1H Chemical shifts δ_{H}	Connectivity ($^1\text{H}/^1\text{H}$)
1.	1	45.75	CH_2	m	1.3 (1α) 2.3 (1β)	2.3 (1β), 4.05 (2β) 1.3 (1α), 4.05 (2β)
2.	2	70.658	CH	m	4.05 (2β)	2.3 (1β), 1.3 (1α)
3.	3	84.58	CH	m	4.92 (3α)	4.05 (2β), 2.55 (4α), 2.3 (4β)
4.	4	37.61	CH_2		2.55 (4α) 2.3 (4β)	2.3 (4β), 4.92 (3α) 2.55 (4α), 4.92 (3α)
5.	5	140.08	C	--	--	--
6.	6	121.93	CH	distorted t	5.28	1.80 (7α), 1.9 (7β)
7.	7	32.18	CH_2	m	1.80 (7α) 1.90 (7β)	1.90 (7β), 5.28 (6), 1.80 (7α), 5.28 (6) 1.5 ($8\alpha/\beta$)
8.	8	31.08	CH		1.5 ($8\alpha/\beta$)	1.80 (7α), 1.90 (7β), 1.63 (9)

S. No	C	¹³ C Chemical shifts δ_C	DEPT	Multip-	¹ H Chemical shifts δ_H	Connectivity (¹ H/ ¹ H)
9.	9	50.17	CH		1.63	1.5 (8 α / β)
10.	10	37.00	C	--	--	--
11.	11	21.18	CH ₂		1.30 (11 α) 1.1 (11 β)	1.55 (12 α), 1.77 (12 β), 1.63 (9)
12.	12	39.08	CH ₂		1.55 (12 α) 1.77 (12 β)	1.30 (11 α), 1.1 (11 β)
13.	13	40.00	C	--	--	--
14.	14	56.50	CH		??	
15.	15	32.18	CH ₂		1.42 (15 α) 1.92 (15 β)	1.92 (15 β), 4.5 (16 α) 1.42 (15 α), 4.5 (16 α)
16.	16	81.08	CH	q	4.50 (16 α)	1.42 (15 α), 1.92 (15 β), 1.75 (17 α)

S. No	C	¹³ C Chemical shifts δ_C	DEPT	Multip-	¹ H Chemical shifts δ_H	Connectivity (¹ H/ ¹ H)
17.	17	62.84	CH		1.75 (17)	4.50 (16), 1.92 (20)
18.	18	16.32	CH ₃	s	0.67	--
19.	19	20.42	CH ₃	s	1.10	--
20.	20	41.97	CH		1.92	1.75 (17)
21.	21	15.00	CH ₃	d, $J = 6.9\text{Hz}$	1.15	--
22.	22	109.01	C	--	--	--
23.	23	31.09	CH ₂		1.44 (23 α) 1.70 (23 β)	1.52 (24 β), 1.46 (24 α) 1.46 (24 α), 1.52 (24 β)
24.	24	29.15	CH ₂		1.46 (24 α) 1.52 (24 β)	1.44 (23 α), 1.52 (24 β)
25.	25	31.82	CH		1.55	1.46 (24 α), 3.50 (26 α), 3.60

S. No	C	¹³ C Chemical shifts δ_C	DEPT	Multip-	¹ H Chemical shifts δ_H	Connectivity (¹ H/ ¹ H)
						(26 β)
26.	26	66.58	CH ₂		3.50 (26 α) 3.60 (26 β)	1.55 (25)
27.	27	17.30	CH ₃	d, $J = 5.51\text{Hz}$	0.80	--
		Sugar moieties Gal				
28.	1	103.90	CH	d	5.20	Gal H-2 (3.88)
29.	2	72.10	CH		3.88	H-3 (3.92), H-1 (5.20)
30.	3	78.50	CH		3.92	H-4 (4.30)
31.	4	80.50	CH		4.30	H-3 (3.92), H-4 (3.65)
32.	5	72.00	CH		3.65	H-6 (4.10 b), H-6 (4.20 a)

S. No	C	¹³ C Chemical shifts δ_C	DEPT	Multip-	¹ H Chemical shifts δ_H	Connectivity (¹ H/ ¹ H)
33.	6	62.50	CH ₂		4.20 a 4.10 b	H-5 (3.65) H-6 (4.20 a), H-5(3.65)
		Glc I				
34.	1'	103.50	CH	d	5.58	Glc H-2' (4.1)
35.	2'	80.50	CH		4.10	H-3' (4.20), H-1' (5.58)
36.	3'	87.00	CH		4.20	H-4' (3.65), H-2'(4.10)
37.	4'	70.10	CH		3.65	H-5' (3.95)
38.	5'	78.50	CH		3.95	H-5' (3.65), H-6' (4.40 a), H-6' (4.30)
39.	6'	62.40	CH ₂		4.40a 4.30b	H-5' (3.95), H-6' (4.30) H-5' 3.95, H-6' (4.40)
		Glc II				

S. No	C	¹³ C Chemical shifts δ_C	DEPT	Multip-	¹ H Chemical shifts δ_H	Connectivity (¹ H/ ¹ H)
40.	1''	104.80	CH	d	4.99	Glc H-2'' (4.30)
41.	2''	80.40	CH		4.30	H-1'' (4.99), H-3'' (4.04)
42.	3''	87.70	CH		4.04	H-2'' (4.30), H-4'' (3.7)
43.	4''	67.50	CH		3.70	H-5'' (3.90), H-3'' (4.04)
44.	5''	75.50	CH		3.99	H-4'' (3.70), H-6'' (4.55)
45.	6''	62.15	CH ₂		4.55	H-5'' (3.99)
		Glc III				
46.	1'''	105.40	CH	d	5.02	Glc H-2''' (3.90)
47.	2'''	75.40	CH		3.90	H-1''' (5.02), H-3''' (4.05)
48.	3'''	78.30	CH		4.05	H-4''' (3.60)
49.	4'''	69.80	CH		3.60	H-5''' (4.18)
50.	5'''	78.00	CH		4.18	H-6''' (4.56), H-4''' (3.60)

S. No	C	¹³ C Chemical shifts δ _C	DEPT	Multip-	¹ H Chemical shifts δ _H	Connectivity (¹ H/ ¹ H)
51.	6'''	62.40	CH ₂		4.56	H-5''' (4.18)
		Xyl				
52.	1	105.70	CH	d	5.15	Xyl H-2 (3.91)
53.	2	75.50	CH		3.91	H-1 (5.15), H-3 (4.05)
54.	3	78.10	CH		4.05	H-4 (3.83)
55.	4	71.0	CH		3.83	H-3 (4.05), H-5 (4.43)
56.	5	62.8	CH ₂		4.43a 4.55b	H-4 (3.83) H-4 (3.83), H-5a (4.43)

^1H - ^1H COSY interactions in the oligosaccharide moiety:

In the hetero-COSY interactions, cross-peaks among the five anomeric protons at Gal H-I (δ_{H} 5.20), Glc I H-1' (5.58), Glc II H-1'' (4.99), Glc III H-1 (5.02) and Xyl H-1 (5.15) with the adjacent vicinal protons Gal H-2, Glc I H-2', Glc II H-2'' Glc III H-2''' and Xyl H-2 at δ_{H} 3.88, 4.1, 4.30, 3.90 and 3.91 were observed as doublets ($J = 7.5, 7.8, 7.6, 7.8$ and 7.7Hz) respectively, indicating the β -linkage in all the sugars attached [90].

Similarly methylenic protons Gal (H-6) at δ_{H} 4.2 and 4.1 exhibited geminal coupling as well as connectivity with Gal H-5 at δ_{H} 3.65.

The methylenic protons (H-6) of Glc I at δ_{H} 4.40 and 4.30 showed vicinal correlation and connectivity with δ_{H} 3.95 - the proton of Glc I (H-5). The Glc II'' and Glc III''' methylenic protons at δ_{H} 4.55 and 4.56 manifested geminal correlation and cross peaks with respective Glc II H-5'' and Glc III H-5''' vicinal protons at 3.99 and 4.18 respectively.

The methylenic proton pair of Xyl (H₂-5) at δ_{H} 4.43 and 4.55 exhibited geminal cross-peaks apart from connectivity with vicinal proton Xyl H-4 (δ_{H} 3.83).

The remaining protons of the five sugar units' *i. e.*, H-3 protons at δ_{H} 3.92, 4.2, 4.04, 4.05 and 4.05 manifested cross couplings with their respective vicinal protons. The coupling data of all the hydrogens including H-2 and H-4 is given in table 2.11.

The presence of five anomeric carbons was confirmed from the ^{13}C -NMR spectrum of Hum-V at δ_{C} 103.90, 103.50, 104.80, 105.40 and 105.70, showing five sugar units. Hetero-COSY experiments further confirmed these assignments. Using HMQC,

one bond ^{13}C - ^1H chemical shifts of the anomeric carbon atoms and the respective coupled protons was identified. The δ_{C} shifts of Gal C-1, Glc I C-1', Glc II C-1'', Glc III C-1''' and Xyl C-1 delivered cross coupling with respective protons at 5.20, 5.58, 4.99, 5.02 and 5.15 respectively.

Heteronuclear Multiple Bond Coherence:

The Heteronuclear Multiple Bond Coherence (HMBC) spectrum of Hum-V aided further in the confirmation of the structure. The H-3 at δ_{H} 4.92 showed connectivity with the anomeric Gal C-1 (δ_{C} 103.9). The vinylic proton H-6 at δ_{H} 5.28 manifested correlations with C-9 (δ_{C} 50.17).

The singlet at δ_{H} 1.10 observed for H₃-19 showed cross connectivity with C-1 (δ_{C} 45.75), carbon resonating with C-5 at δ_{C} 140.08 and C-10 (δ_{C} 37.0). The carbon at C-10 is quaternary in nature. The methyl protons H₃-18 (δ_{H} 0.67) manifested connectivity with C-12 (δ_{C} 39.08), C-13 (δ_{C} 40.00), C-14 (δ_{C} 56.50) and C-17 (δ_{C} 62.84). The methyl protons H-21 (δ_{H} 1.15) exhibited correlation with C-17 (δ_{C} 62.84) and also showed connectivity with C-20 (δ_{C} 41.97) and quaternary carbon C-22 (δ_{C} 109.01). HMBC correlation of carbon at C-22 (the quaternary) was observed with H-26 β at δ_{H} 3.60. The methyl proton at δ_{H} 0.80 (H₃-27) showed coupling with C-25 (δ_{C} 31.82), C-24 (δ_{C} 29.15) and also with C-26 (δ_{C} 66.58).

By comparing the FAB mass negative ion and ^{13}C NMR spectral data, with the reported data, branching of polysaccharide was determined. Xylose is attached to Glc-I of the sugar moiety. Thus, in consideration with above mentioned details, the structure of Hum-V was suggested to be (25*R*)- spirost-5ene-2,3-diol, [2 α , 3 β] (25*R*)- spirost-5ene-

2,3-diol, [2 α , 3 β] 3-O-{ β -D-glucopyranosyl (1 $\rightarrow$ 4)- β -D-glucopyranosyl (1 $\rightarrow$ 2)- β -D-glucopyranosyl{(1 $\rightarrow$ 4)- β -D-xylopyranosyl] (1 $\rightarrow$ 4)- β -D-galactopyranoside) (figures 2.12 and 2.3). The supposed structure is a reported compound, naming nocturnoside A, from the same plant *Cestrum nocturnum*[92].

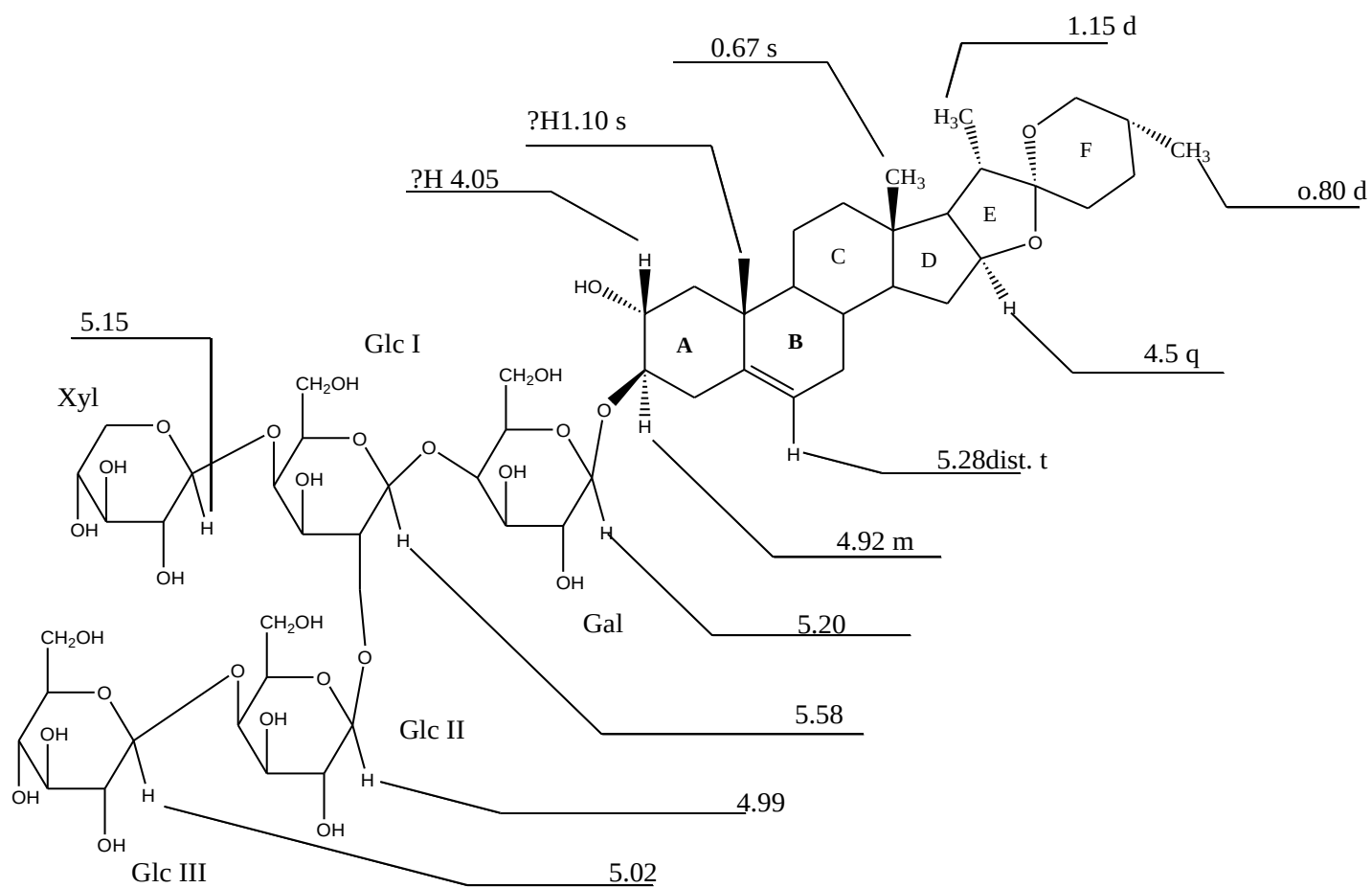


Fig 2.16: ^1H -NMR chemical shifts δH of Hum-V in pyridine-d_5

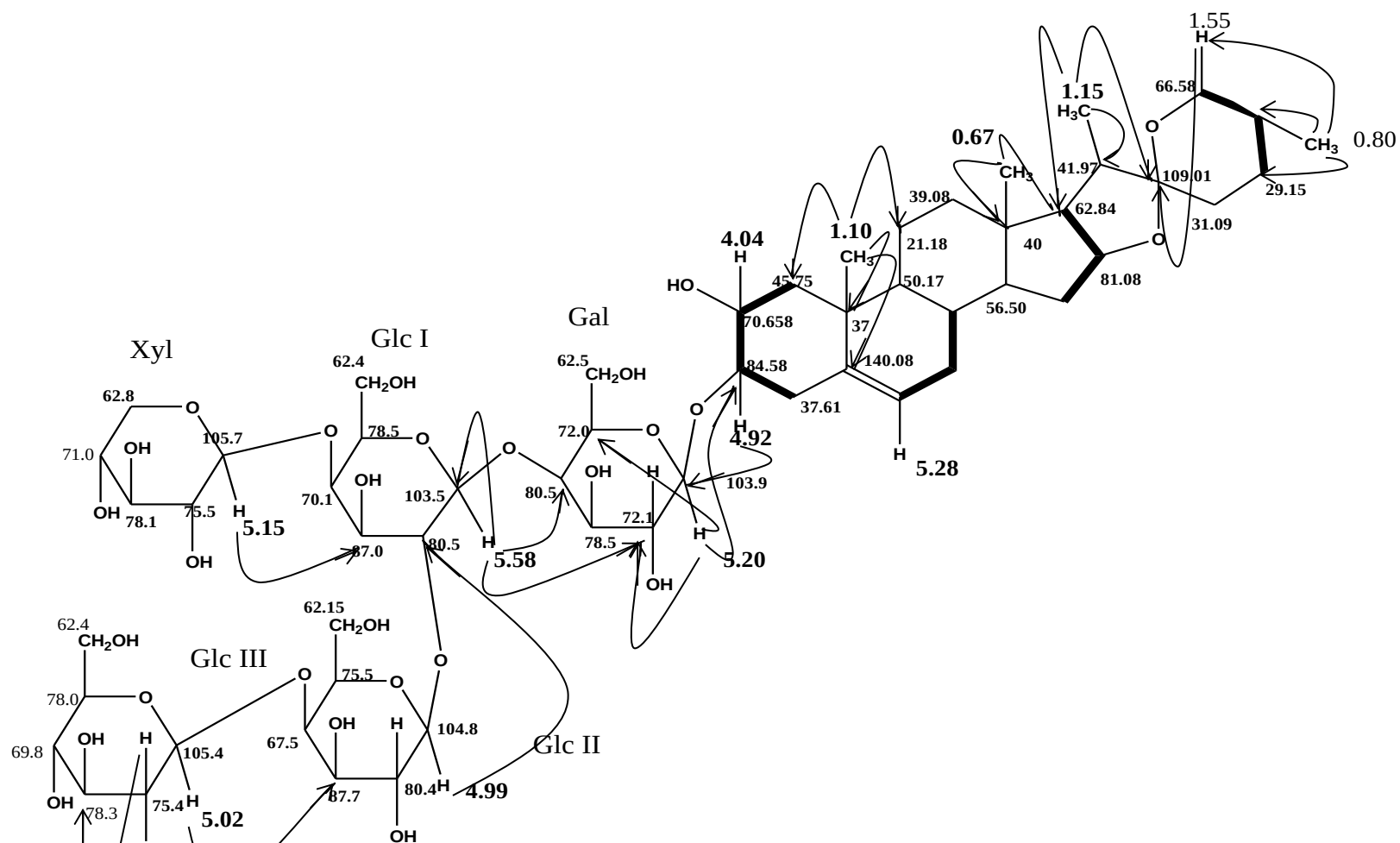


Fig 2.17: HMBC connectivity's of Hum-V (500 MHz, pyridine- d_5), ^{13}C -NMR chemical shifts and the arrow indicates the connectivity's from proton to carbon. The bold bonds indicates the COSY relationships

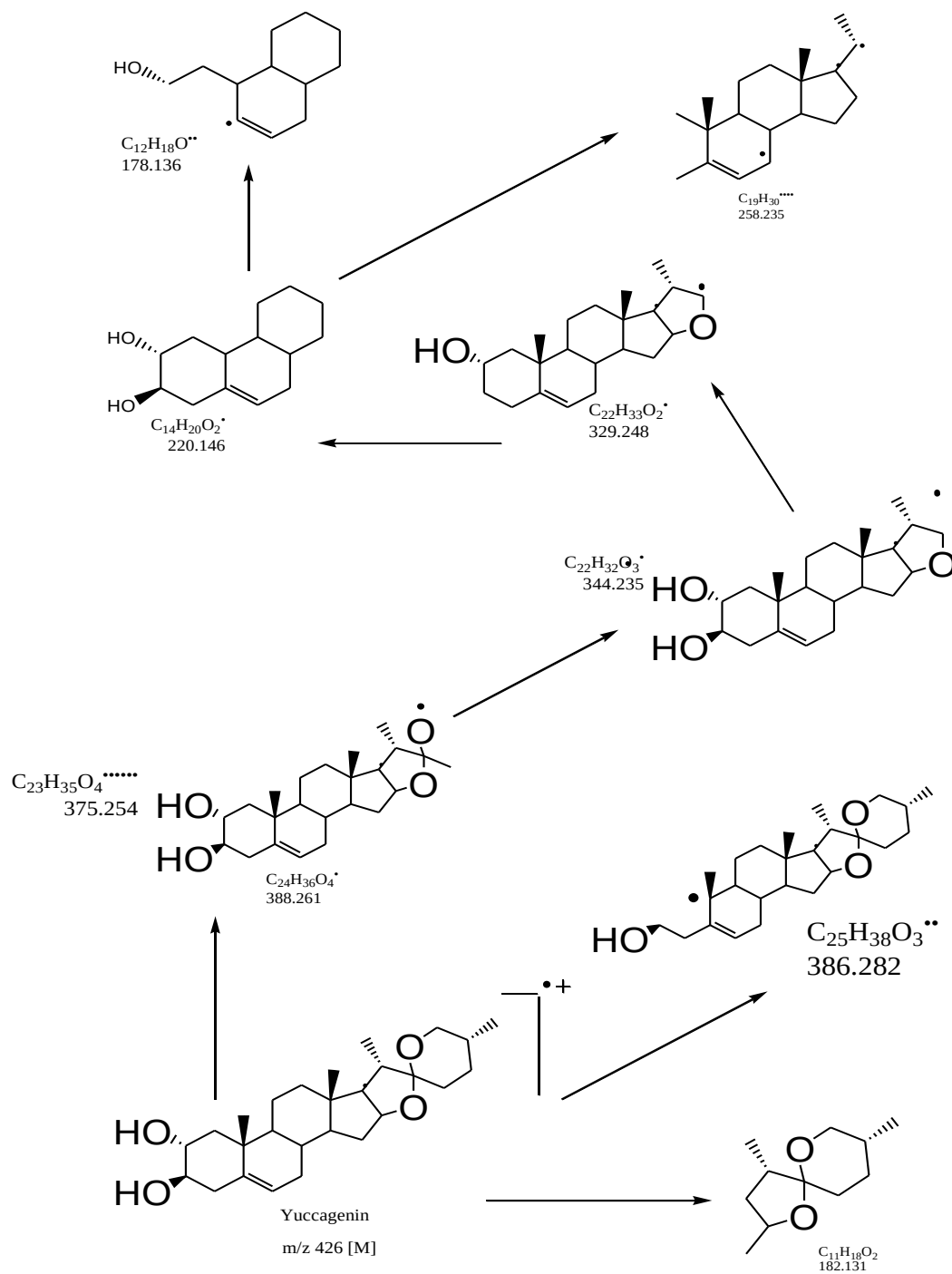


Fig 2.18: Mass fragmentation patron of Hum-V

2.3.2: Characterization of Hum II:

Pure saponin (15 mg) was obtained as white crystals. It was isolated on prep-TLC in butanol: acetic acid: water (12:3:2).

The melting point of the pure crystals was 276-284°C.

The glycosidic nature of Hum-II was showed by broad absorption bands at 3360 cm^{-1} and 1033 cm^{-1} in IR spectrum which is due to the O-H stretching and C-O stretching frequency, respectively. The band intensity at 927 cm^{-1} is smaller as compared to the band strength at 897 cm^{-1} , indicating the spiroketal chain of the 25*R*-series [20, 88].

The negative ion FAB-mass spectrum of the structure (Hum-II) showed psuedu-molecular ion peak at m/z 1047 (molecular mass of $\text{C}_{50}\text{H}_{79}\text{O}_{23}$) [M-H]. Fragment ion peaks at 915 [M-H-132] specify the terminal pentose and 885 [M-H-162] show that a hexose sugar moiety is also at terminal position. These indicate the branched nature of glycone units. The peak at 753 [M-H-(132 + 162)] is showing the loss of one pantose unit. In the spectrum the next fragment ion peak at 591 [M-H-(132 + 162 x2)] is due to the loss of three glycone units *i.e.*, one pentose and two hexoses. The peak at 591 further assures that one hexose moiety is attached to the basic aglycone, giving peak at 429 [M-H-(132 + 162 x 3)] (Fig 2.20).

The ^{13}C NMR (100 MHz) of un-fragmented saponin demonstrated the chemical shifts characteristic of spirostene type steroidal saponin (Table 2.12). The broad band spectrum of the pure compound indicated 50 carbon atoms resonance. The multiplicities of the carbon atoms were assigned by the use of DEPT techniques (DEPT 90 and DEPT 135). It showed sum of thirty four methine (CH), fourteen methylene (CH_2), four methyl

(CH₃) and four quaternary carbon atoms. The olifenic carbons signals were also represented in the spectra at δ_C 140.12 and 121.92. The carbon carbon double bond is present in the ring-B of the skeleton (fig 2.19).

The ¹H NMR spectrum of the Hum-II in pyridine showed olifenic protons signals at δ_H 5.28 ppm (a distorted triplet). Characteristic signals for two secondary methyl groups at δ_H 1.15 d, $J = 5.9$ Hz (H-21) and δ_H 0.67 d, $J = 5.7$ Hz (27) were present in the spectrum. Signals for two tertiary methyl groups at 0.67 s (H-18) and 1.15 s (H-19) were also clearly indicated. The spectrum also revealed the four anomeric protons at δ_H 4.9 (Gal H-1, d , $J = 7.7$ Hz), δ_H 5.21 (Glc H-1', d , $J = 7.9$ Hz), δ_H 5.26 (Glc II, H-1'', d , $J = 7.9$ Hz) and δ_H 5.58 (Xyl H-1, d , $J = 7.75$ Hz) (Fig 2.21).

The structure was further confirmed by two dimensional ¹³C NMR *i.e.*, HMQC and HMBC. At the end COSY-45° experiment was applied to confirm all the assignments finally.

The heteroCOSY-45° spectrum showed clear coupling of H-2 β (δ_H 4.05) with the H-1 α (1.3) and H-1 β (2.3) (assignments interchangeable) and H-3 (δ_H 4.92). The proton at position 3 was coupled with H-2 β (δ_H 4.05) and also with H-4 α (δ_H 2.55) and H-4 β (δ_H 2.3 β) (assignments interchangeable). H-3 α (δ_H 4.92) showed interconnection with H-4 β (2.3) and H-4 α (2.55). Both of the C-4 protons (δ_H 2.55 and δ_H 2.3) interacts with H-3 at δ_H 4.92 giving doublet of doublet at 5.5 and 1.40 Hz. No connectivity was observed across C-5 and C-10 because of quaternary carbon atoms at these positions. A distorted triplet present at δ_H 5.28 (H-6) showing coupling with H-7 α (δ_H 1.80) and H-7 β (δ_H 1.90). The assignments for H-7 α (δ_H 1.80) and H-7 β (1.90) was confirmed from its COSY interactions with each other and H-6 (5.28) and H-8 (1.5). H-8 at δ_H 1.5 showed

coupling with H-7 α (1.80), H-7 β (1.90) and H-9 α (1.63). Coupling of H-16 at δ_H 4.52 was observed with 15 α (δ_H 1.42), 15 β (δ_H 1.92) and 17 (δ_H 1.75). One of the interaction observed in COSY spectrum was between C-26 protons at δ_H 3.50 (26 α) and 3.60 (26 β) (assignments interchangeable), which in turn showed connectivity with H-25 at δ_H 1.5.

^1H - ^1H COSY interactions in the oligosaccharide moiety:

In the hetero-COSY interactions, cross-peaks among the five anomeric protons at Gal H-I (δ_H 4.92), Glc I H-1' (5.21), Glc II H-1'' (5.26) and Xyl H-1 (5.58) with the adjacent vicinal protons Gal H-2, Glc I H-2', Glc II H-2'' and Xyl H-2 at δ_H 4.51, 4.31, 3.93 and 4.01 were observed as doublets ($J= 7.7, 7.9, 7.8, 7.75$) respectively, indicating the β -linkage in all the sugars attached.

Similarly methylenic protons Gal (H-6) at δ_H 4.34 and 4.15 exhibited geminal coupling as well as connectivity with Gal H-5 at δ_H 4.03.

The methylenic protons (H-6) of Glc I at δ_H 4.5 and 4.35 showed vicinal correlation and connectivity with δ_H 3.95 - the proton of Glc I (H-5). The Glc II'' methylenic proton at δ_H 4.55 manifested geminal correlation and cross peaks with respective Glc II H-5'' vicinal proton at δ_H 3.99.

The methylenic proton pair of Xyl (H₂-5) at δ_H 3.60 and 3.8 exhibited geminal cross-peaks apart from connectivity with vicinal proton Xyl H-4 (δ_H 4.08) (fig 22).

The remaining protons of the five sugar units' *i. e.*, H-3 protons at δ_H 3.95, 4.18, 4.03, and 4.05 manifested cross couplings with their respective vicinal protons. The coupling data of H-2 and H-4 and all the other protons is given in table 2.12.

The signals of the aglycone of the structure Hum-II resembles the spectra given by the aglycone of noturnoside A (Hum-V) (table of Hum-v). The ^{13}C NMR spectra of both the structures were compared and observed that all the signals of the aglycone and glycone moieties were similar except that Hum-V has one hexose in excess.

From the molecular ion peak and fragment ion peaks in MS studies, the coupling of protons and the chemical shift values (especially those of anomeric carbons and anomeric protons in the glycone moieties and carbon carbon double bond in the aglycone unit) in the 1D and 2D NMR techniques strongly suggested the structure to be spirost-5-ene-2,3-diol [2 α , 3 β ,25R], 3-O- [β -D-glucopyranosyl(1 $\rightarrow$ 2)] {- β -D-xylopyranosyl(1 $\rightarrow$ 3)- β -D-glucopyranosyl(1 $\rightarrow$ 4)}- β -D-galactopyranoside (fig 17). This compound was also reported by Vollermer Yu. S. *at. el.* in 1978 [87] and by Mimaki, Y. *at. el.* [93, 94], and Baqai, F. T. in 1999 [20]. It was named as karatavoiside A, as isolated for the first from the *Allium karataviense*, family Liliaceae. The phytotoxicity and stimulation at low dose level of this compound was also determined on *actuca sativa* [95].

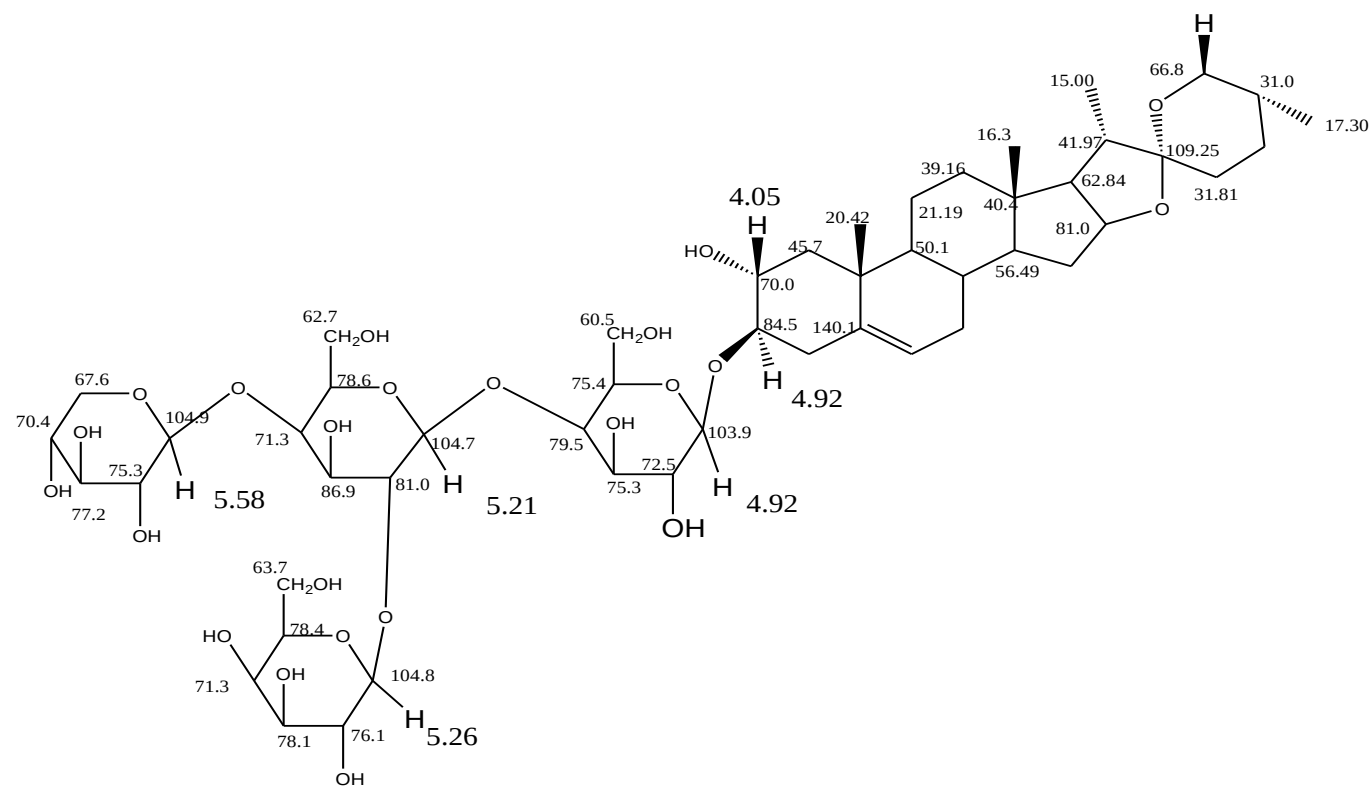


Fig 2.19: ^{13}C NMR (100.61 MHz) chemical shifts δ_{C} of Hum-II in pyridine

Table 2.12: ^{13}C and ^1H NMR spectral data of Hum-II from one and two dimensional experiments

S. No	C	^{13}C Chemical shifts δ_{C}	DEPT	Multip-	^1H Chemical shifts δ_{H}	Connectivity ($^1\text{H}/^1\text{H}$)
1.	1	45.7645	CH_2	dd($j = 4.6, 8.2 \text{ hz}$)	1.3 (1α) 2.3 (1β)	2.3 (1β), 4.5 (2β) 1.3 (1α), 4.05 (2β)
2.	2	70.0	CH	m	4.05 (2β)	2.3 (1β), 1.3 (1α), 2.0 4.05, 4.9
3.	3	84.506	CH	m	4.92 (3α)	4.5, 4.05 (2β), 2.55 (4α), 2.3 (4β)
4.	4	37.9270	CH_2	dd ($J = 5.5, 1.40 \text{ Hz}$)	2.55 (4α) 2.3 (4β)	2.3 (4β), 4.92 (3α) 1.8 2.55 (4α), 4.92 (3α)
5.	5	140	C	--	--	--
6.	6	121.926	CH	dist. t	5.28	1.80 (7α), 1.9 (7β)
7.	7	32.1636	CH_2		1.80 (7α) 1.90 (7β)	1.90 (7β), 5.28 (6), 1.80 (7α), 5.28 (6) 1.5 ($8\alpha/\beta$)
8.	8	32.0	CH		1.5 ($8\alpha/\beta$)	1.80 (7α), 1.90 (7β), 1.63 (9)

9.	9	50.1607	CH		1.63 3,58	1.5 (8 α / β)
10.	10	37.8	C	--	--	--
11.	11	21.1916	CH ₂		1.30 (11 α) 1.0 1.1 (11 β)	1.52 (12 α), 1.70 (12 β), 1.63 (9) 2.23, 1.0
12.	12	32.16	CH ₂		1.52 (12 α) 1.70 (12 β)	1.30 (11 α), 1.0 (11 β)
13.	13	40.4335	C	--	--	--
14.	14	56.492	CH		3.96??	1.3, 3.8, 4.2
15.	15	31.818	CH ₂		1.42 (15 α) 1.92 (15 β)	1.92 (15 β), 4.5 (16 α) 1.42 (15 α), 4.5 (16 α)
16.	16	81.088	CH		4.52 (16 α)	1.42 (15 α), 1.98 (15 β), 1.75 (17 α), 4.1, 4.9
17.	17	62.845	CH	t (J = 6.5 Hz)	1.75 (17)	4.52 (16), 1.92 (20)
18.	18	16.319	CH ₃	S	0.67	1.20 (19)

19.	19	20.422	CH ₃	s	1.20	0.67 (18), 1.92 (20)
20.	20	41.97	CH		1.92	1.75 (17), 1.1
21.	21	15.0001	CH ₃	d (<i>J</i> = 5.9 Hz)	1.15	--
22.	22	109.257	C	--	--	--
23.	23	31.818	CH ₂		1.44 (23 α) 1.70 (23 β)	1.52 (24 β), 1.46 (24 α) 1.46 (24 α), 1.52 (24 β)
24.	24	29.955	CH ₂		1.46 (24 α) 1.52 (24 β)	1.44 (23 α), 1.52 (24 β) 1.7, 1.57, 1.44,
25.	25	30.5967	CH		1.5	1.46 (24 α), 3.80 (26 α), 3.60 (26 β)
26.	26	66.8156	CH ₂	m	3.50 (26 α) 3.60 (26 β)	1.5 (25)
27.	27	17.3071	CH ₃	d (<i>J</i> = 5.7 Hz)	0.80	1.5 (25)
28.		Gal				

29.	1	103.9	CH	d ($J = 7.7$ Hz)	4.92	Gal H-2 (4.51)
30.	2	72.5	CH		4.51	H-3 (3.95), H-1 (4.92)
31.	3	75.3	CH		3.95	H-4 (4.58)
32.	4	79.5	CH	t ($J = 2.5$ Hz)	4.58	H-3 (3.95), H-5 (4.03)
33.	5	75.4	CH		4.03	H-6 (4.15 b), H-6 (4.34 a)
34.	6	60.5	CH ₂		4.34 a 4.15 b	H-5 (4.03) H-6 (4.34 a), H-5(4.03)
35.		Glc I				
36.	1'	104.7	CH	d ($J = 7.9$ Hz)	5.21	Glc H-2' (4.31)
37.	2'	81.0	CH		4.31	H-3' (4.18), H-1' (5.21)
38.	3'	86.9	CH		4.18	H-4' (4.52), H-2'(4.31)
39.	4'	71.3	CH		4.52	H-5' (3.95)
40.	5'	78.6	CH		3.95	H-4' (4.52), H-6' (4.5 a), H-6' (4.35 b)

41.	6'	62.7	CH ₂	Overlapped	4.5a 4.35b	H-5' (3.95), H-6' (4.35 b) H-5' 3.95, H-6' (4.5 a)
42.		Glc II				
43.	1''	104.8	CH	d (<i>J</i> = 7.8 Hz)	5.26	Glc H-2'' (3.93)
44.	2''	76.1	CH		3.93	H-1'' (5.26), H-3'' (4.03)
45.	3''	78.1	CH		4.03	H-2'' (3.93), H-4'' (4.4)
46.	4''	71.3	CH		4.4	H-5'' (3.99), H-3'' (4.03)
47.	5''	78.4	CH		3.99	H-4'' (4.4), H-6'' (4.55)
48.	6''	63.7	CH ₂		4.55 4.40	H-5'' (3.99), H-6'' (4.40 b) H-6'' (4.55 a)
49.		Xyl				
50.	1	104.9	CH	d (<i>J</i> = 7.75 Hz)	5.58	Xyl H-2 (4.01)
51.	2	75.3	CH		4.01	Xyl H-1 (5.58), H-3 (4.05)

52.	3	77.2	CH		4.05	Xyl H-4 (4.08)
53.	4	70.4	CH		4.08	Xyl H-5 (3.60)
54.	5	67.6	CH		3.60 3.8	Xyl H-4 (4.08) H-5 (3.8 b) Xyl H-5 (3.60)

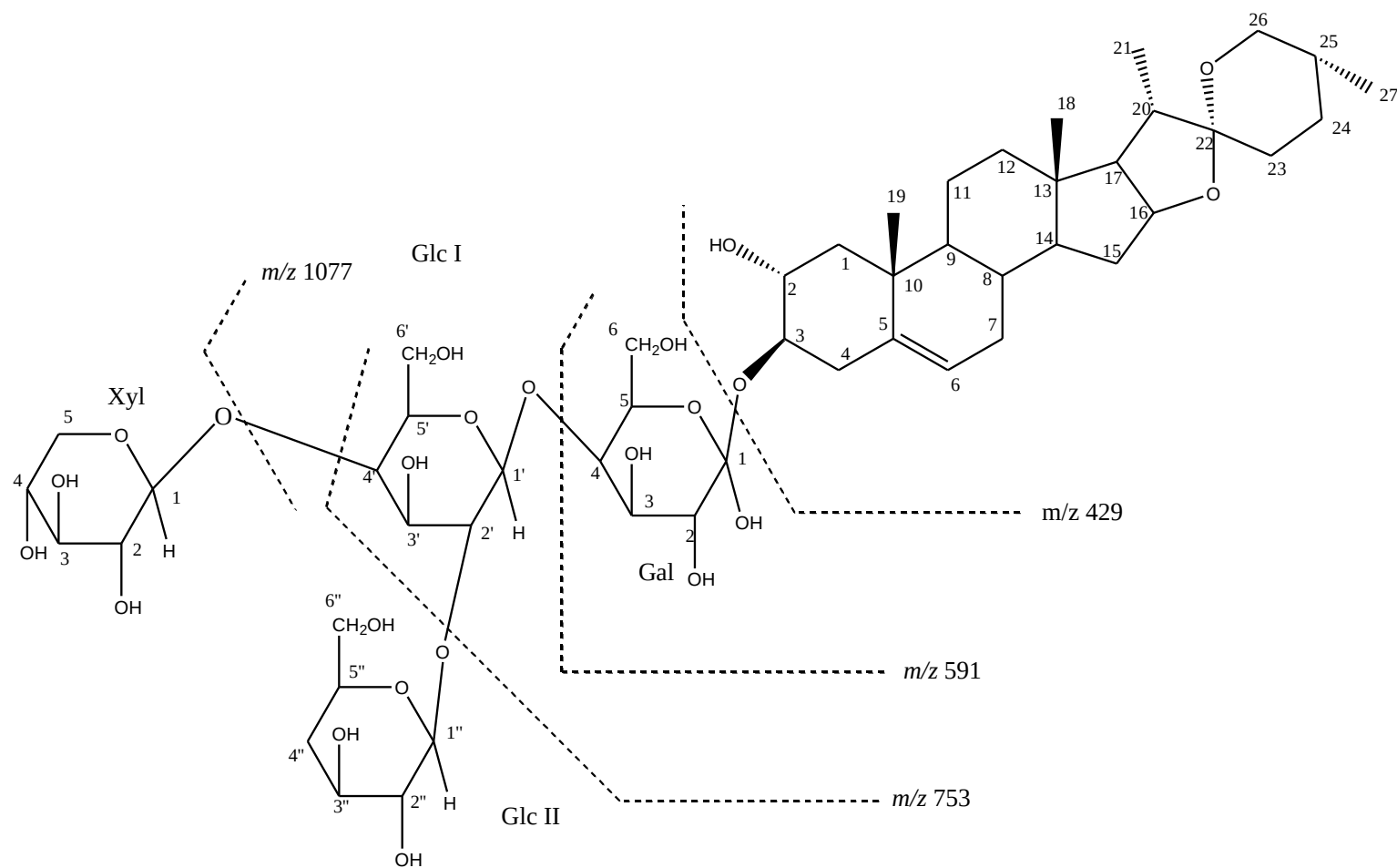


Fig 2.20: FAB-MS (negative ion mode) Fragmentation pattern of Hum-II, m/z 1047 $[M-H]^-$ (calculated for $C_{56}H_{89}O_{26}$)

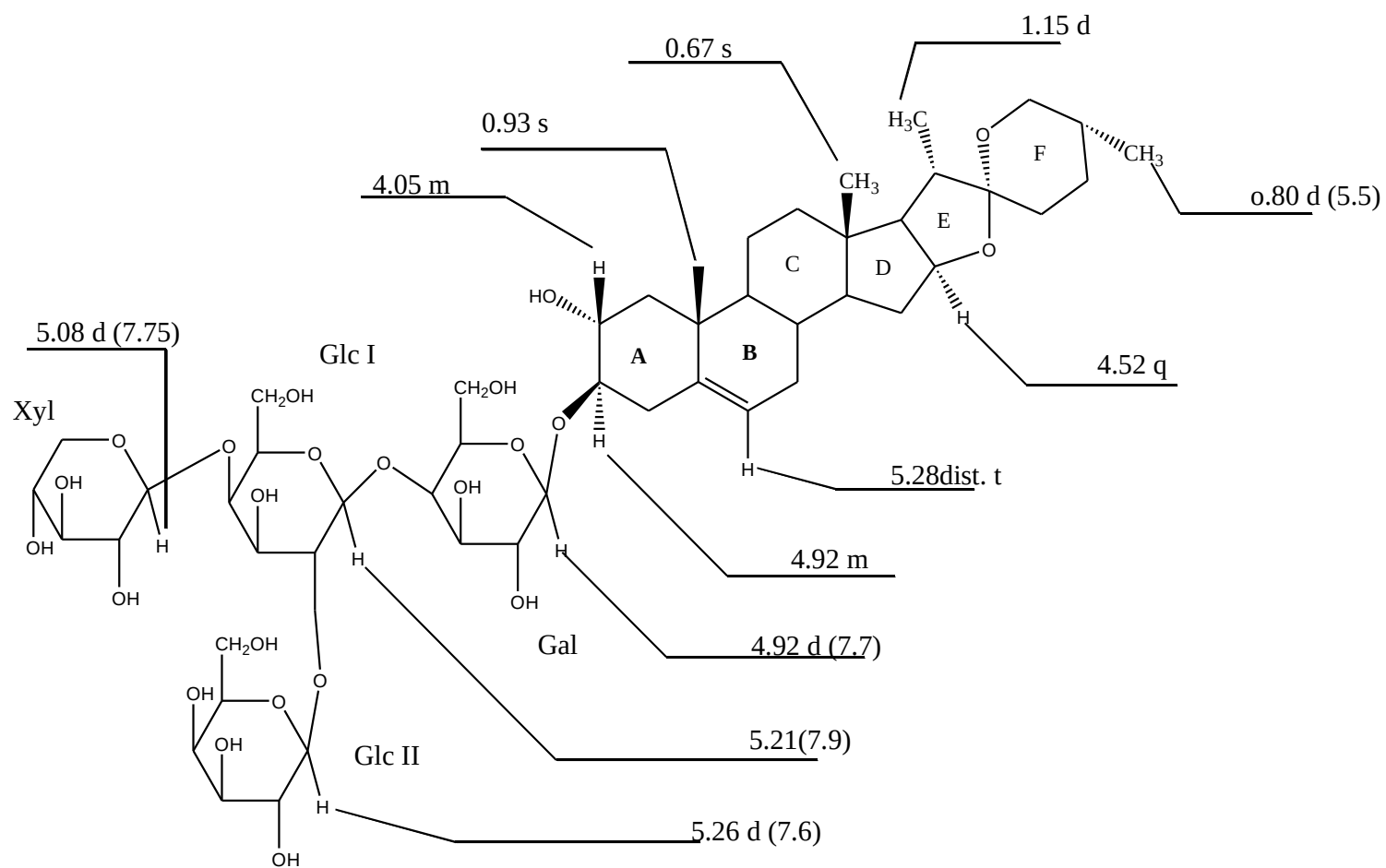


Fig 2.21: ^1H -NMR chemical shifts δH of Hum-II in pyridine-d_5

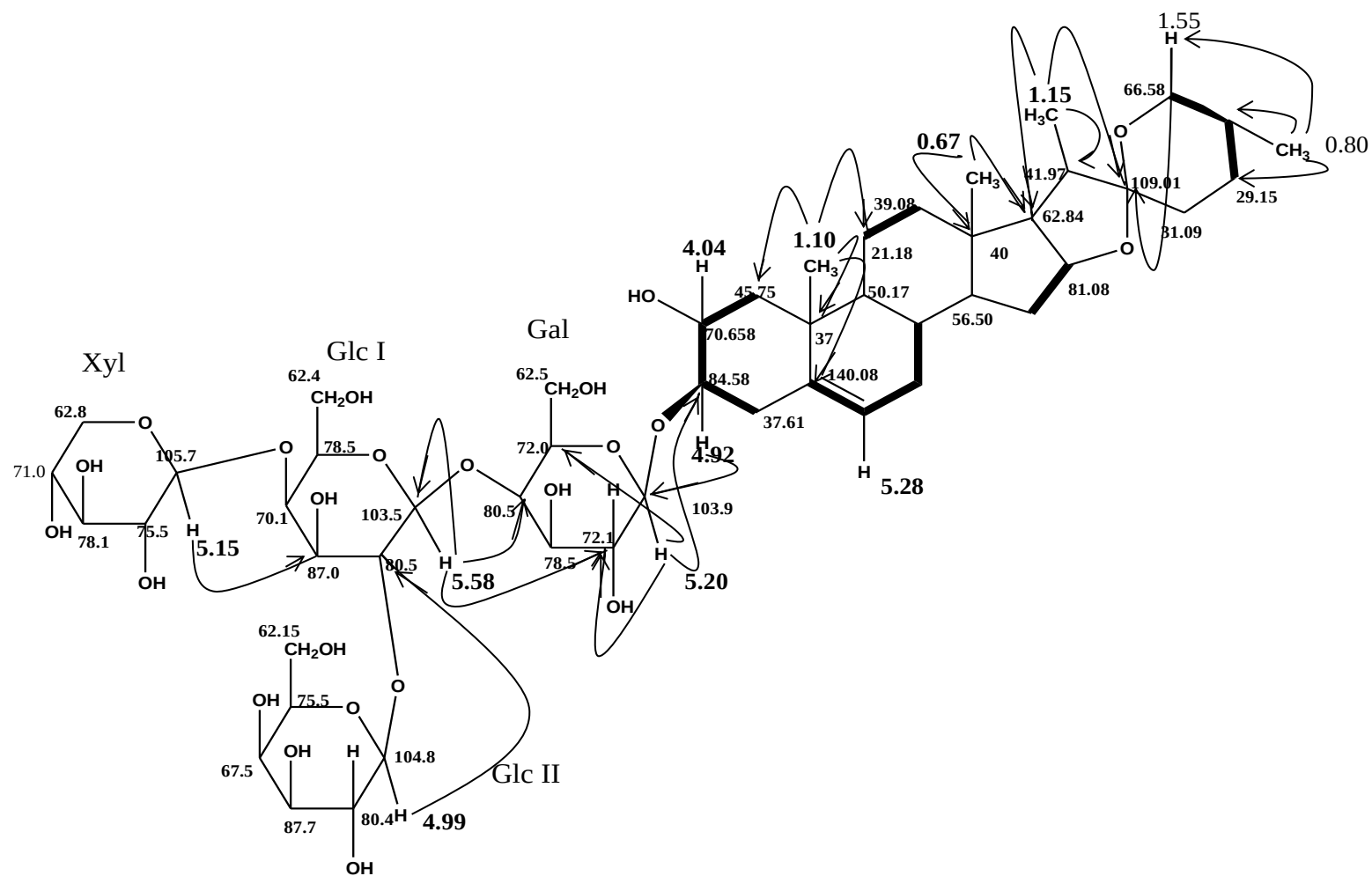


Fig 2.22: HMBC connectivity's of Hum-II (500 MHz, pyridine- d_5) ^{13}C -NMR chemical shifts and the arrow indicates the connectivity's from ^1H to ^{13}C . The bold bonds indicate the COSY relationships.

2.3.2: Characterization of Hum-IV:

The compound **Hum-IV** (32mg) was isolated as white amorphous solid, from *iso*-butanol fraction of *Cestrum nocturnum*, Fig 2.23.

The FAB negative ion mass spectrum confirmed its molecular formula to be $C_{58}H_{94}O_{26}$ (m/z : 1206.35 $[M-H]^-$ for $C_{58}H_{94}O_{26}$ (molecular formula); calculated as 1210). The spectrum showed pseudo-molecular ion peak at m/z 1210 $[M-H]^-$. Five monosaccharide moieties were also confirmed by the prominent fragment ion peaks.

The FAB (Fast Atom Bombardment) mass spectrometry confirmed that the terminal positions are occupied by hexose (m/z 162) units, giving the fragment ions at m/z 1047 $[M-H-162]$ and 885 $[M-H-(162 \times 2)]$. A fragment ion peak at m/z 723 indicates the attachment of another hexose unit to the terminal hexose moiety and a pentose was detected at 591 $[M-H-(162 \times 3) + 132]$. A prominent peak at 459 showed the glycone moiety, after the breakage of one more pentose unit from the molecular ion peak $[M-H-(162 \times 3 + 132)-132]$ (Fig 2.24).

¹H-NMR:

The ¹H NMR (400 MHz) spectrum of the intact pure compound in pyridine- *d*₅ showed five singlets for seven tertiary methyl groups at δ_H 0.70, δ_H 0.90, δ_H 0.94, δ_H 1.05, δ_H 1.25, δ_H 1.48, δ_H 1.45 for H₃-26, H₃-25, H₃-29, H₃-24, H₃-23, H₃-30, H₃-27, respectively. A methine proton at position 3 of the ring A, resonated at δ_H 3.84 as a multiplet. The vinylic H-12 proton signal was also observed at δ_H 5.32 as a multiplet. Fig 2.26 indicates the important protons values.

¹³C NMR:

The broad band ¹³C NMR (100 MHz) spectrum gave 58 carbon resonances. DEPT-90 and DEPT-135 and HMQC techniques distinguished them further as 30 methine (CH), 14 methylene (CH₂) and 7 methyl groups.

The signal at δ_C 75.50 was assigned to C-3, having a hydroxyl group. The methine at C-5 showed a signal at δ_C 62.8. C-12 is resonating at δ_C 121.80. Methyl groups exhibited signals at δ_C 11.10, δ_C 14.17, δ_C 15.00, δ_C 17.30, δ_C 16.32, δ_C 20.41 and δ_C 33.01 for C-23, C-24, C-25, C-26, C-27, C-29 and C-30, respectively. Studying ¹³C broad band spectroscopy, eight quaternary carbons were revealed, and assigned to C-4 (δ_C 30.00), C-8 (δ_C 64.45), C-10 (δ_C 62.84), C-13 (δ_C 140.00), C-14 (δ_C 75.64), C-17 (δ_C 109.29), C-20 (δ_C 29.96) and C-28 (δ_C 167.92). A quaternary carbon at C-13 showed resonance at δ_C 140.00. The signal at 109.25 was assigned to the carbon number 17, where a carbonyl group is attached. The carboxyl attachment at C-17 resonated at δ_C 167.92, as compared to the standard ursolic acid values (δ_C 180-190). In the ¹H-NMR experiment, a distinguished broad singlet at δ_H 10-12 (for the carboxylic acids) was also absent. The upfield chemical shift at C-28 and the absence of the carboxyl proton signal in the spectrum [96]. It was established that the pure compound is a bisdesmosidic glycoside of oleanolic acid with some oligosaccharide units attached at C-3 at δ_C 75.50 through ether bond and remaining to the carboxyl group (carbon 28) through ester linkage. Fig 2.25 shows all the ¹³C chemical shifts.

Examining the ¹H and ¹³C-NMR spectra of the compound, five monosaccharide units were undoubtedly indicated through easily distinguishable signals of the anomeric protons and carbons. The anomeric carbon were resonating at δ_C 105.39, δ_C 103.29, δ_C

103.91, δ_C 104.81 and δ_C 104.28 and the respective anomeric protons were giving clear signals at δ_H 5.12 (d, $J = 7.6$ Hz), δ_H 5.55 (a distorted triplet), δ_H 4.93 (d, $J = 7.1$ Hz), δ_H 5.10 (d, $J = 7.6$ Hz) and δ_H 5.18 (d, $J = 7.7$ Hz). β -linkage was designated to all the anomeric protons configurations by calculating the spin coupling constant values.

The one bond correlation between carbon atoms and the protons of the pure compound were established by the HMQC spectroscopy. All the assignments given to the atoms involved in the structure were reconfirmed by the COSY-45° experimental technique.

Heteronuclear Multiple Bond Coherence:

Very fruitful correlation observations were interpreted from the HMBC spectrum of the compound. The methine proton at position 3 (δ_H 3.84) made strong correlations to C-2 (δ_C 44.00) and to C-24 (δ_C 14.17). Very prominent correlation was detected for the vinylic proton (δ_H 5.32) with carbon 11 (δ_C 32.00). The methyl group at position 24 (δ_H 1.05) displayed correlation with C-4 (δ_C 30.00) and C-3 (δ_C 75.50). Another noticeable interaction was observed among the methyl protons of carbon 25 (δ_H 0.90) and C-1 (δ_C 43.10), C-9 (δ_C 47.50) and C-10 (δ_C 62.84). The methyl protons of carbon 27 (δ_H 1.45) gave helpful correlation interactions with C-13 (δ_C 140.00) and C-15 (δ_C 37.50). The methyl (CH₃) protons at C-29 (δ_H 0.94) and C-30 (δ_H 1.48) showed strong correlation to carbons at position 19 (δ_C 41.50) and 21 (δ_C 38.40). Protons at chemical shift δ_H 1.10 (C-19) was interacting with protons of C-18 (δ_H 1.70) and C-29 (δ_H 0.94) [97].

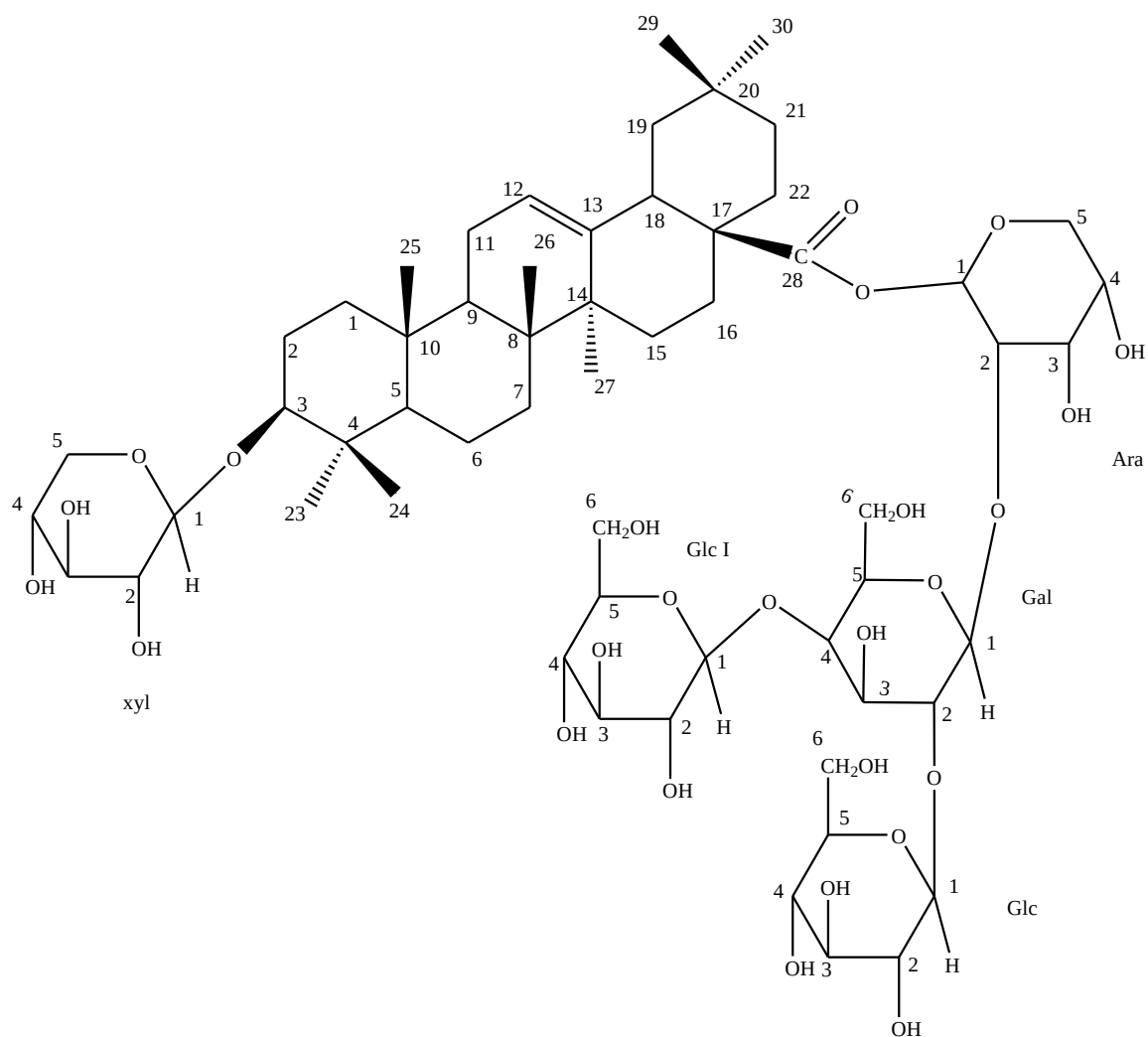


Fig 2.23: Hum-IV: 3-O- β -D-xylopyranoside-olean-12-en-28-oic acid-28-O- β -arabinopyranosyl-(1-3)- β -D-galactopyranosyl-(1-2)- β -L-glucopyranosyl-(1-4)- β -L-glucopyranosyl ester

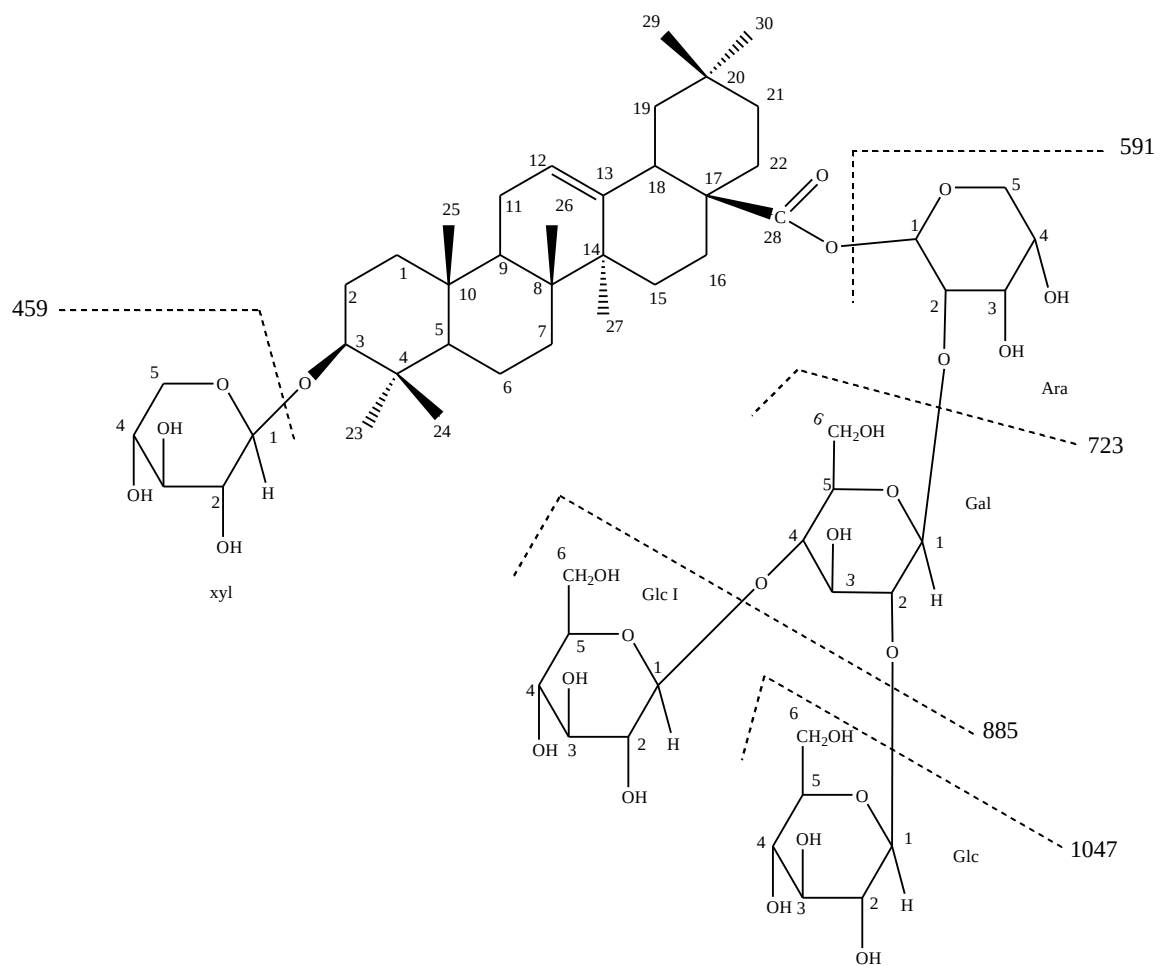


Fig 2.24: FAB-MS (negative ion mode) Fragmentation pattern of Hum-IV, m/z 1210

$[M-H]^-$ (calculated for $C_{58}H_{94}O_{26}$ 1206)

^1H - ^1H COSY interactions:

Supportive correlations were detected in the ^1H - ^1H COSY-45° spectroscopic experimentation. On the spectrum, the coupling of H-1 α (δ_{H} 1.28) was observed with H-2 α (δ_{H} 2.55) and H-2 β (δ_{H} 2.69) (δ_{H} assignments interchangeable), which in turn is linked to H-3 α (δ_{H} 3.84).

The two methylenic protons at H₂-6 (δ_{H} 1.10 and 1.93) showed correlation to H-5 (δ_{H} 1.80) as well as to the protons present at C-7 (H-7 α and H-7 β ; δ_{H} 1.40 and 1.45, interchangeable). The assignments for the vinylic H-12 proton at δ_{H} 5.32 was confirmed by its COSY correlations with H-11 α (δ_{H} 1.48) and H-11 β (δ_{H} 1.83), and in turn the protons at C-11 are showing coupling with H-9 (δ_{H} 1.28). Correlation among H₂-15 and H₂-16 was also observed in the spectrum. The correlation of H-30 (δ_{H} 1.48) was observed with the H-21 α (δ_{H} 1.65) and H-21 β (δ_{H} 1.68), which in turn is extending its coupling with H-22 α and β (1.50 and 1.54). The assignment of proton at position 19 was confirmed by its interactions with H-18 (δ_{H} 2.15) and H₃-29 (δ_{H} 0.94) [98]. Fig 2.25 indicates all the important connectivities.

^1H - ^1H COSY interactions in the oligosaccharide moiety:

In 2D-COSY interactions, cross-relations among the anomeric protons (Xyl H-1 (δ_{H} 5.12), Gal H-1 (4.93), Glc H-1 (5.10) and Glc' H-1 (5.18)) and the adjacent vicinal protons (Xyl H-2, Gal H-2, Glc H-2 and Glc I H-2) were observed as doublets (J = 7.6, 7.1, 7.6 and 7.7hz). A distorted doublet was observed for the arabinose anomeric proton at δ_{H} 5.59.

The methylenic protons at Xyl H-5 at δ_H 3.64 and 4.30 showed coupling with the nearby Xyl H-4 protons at δ_H 4.80. Similarly, the Gal H-6 methylenic protons at δ_H 4.45 and 4.13 exhibited connectivity with Gal H-5 protons at δ_H 4.10. The Glc (δ_H 4.48 and 4.62) and Glc' (δ_H 4.51 and 4.26) methylenic protons manifested correlation and cross links with respective Glc H-5 and Glc' H-5 vicinal protons at δ_H 4.08 and 3.98, respectively.

The methylenic protons of arabinose resonating at δ_H 4.20 and 4.15 showed cross correlation with the adjacent H-4 protons at δ_H 4.38. The protons at position 3 of the oligosaccharide units *i. e.*, at 4.53, 3.78, 4.41, 4.10 and 4.15 revealed coupling with the respective vicinal protons at positions 2 and 4. The detailed coupling data of all the atoms is given in the table 2.13.

^{13}C -NMR spectrum of Hum IV assured the presence of five sugar units by showing five anomeric carbon signals at δ_C 105.39, 103.29, 103.91, 104.81 and 104.28. The chemical shifts of the anomeric carbons and the respective coupled protons were recognised by using HMQC and HMBC experimental techniques. At the end, 2D-COSY experiments authenticated all the assignments allotted to the atoms in the structure.

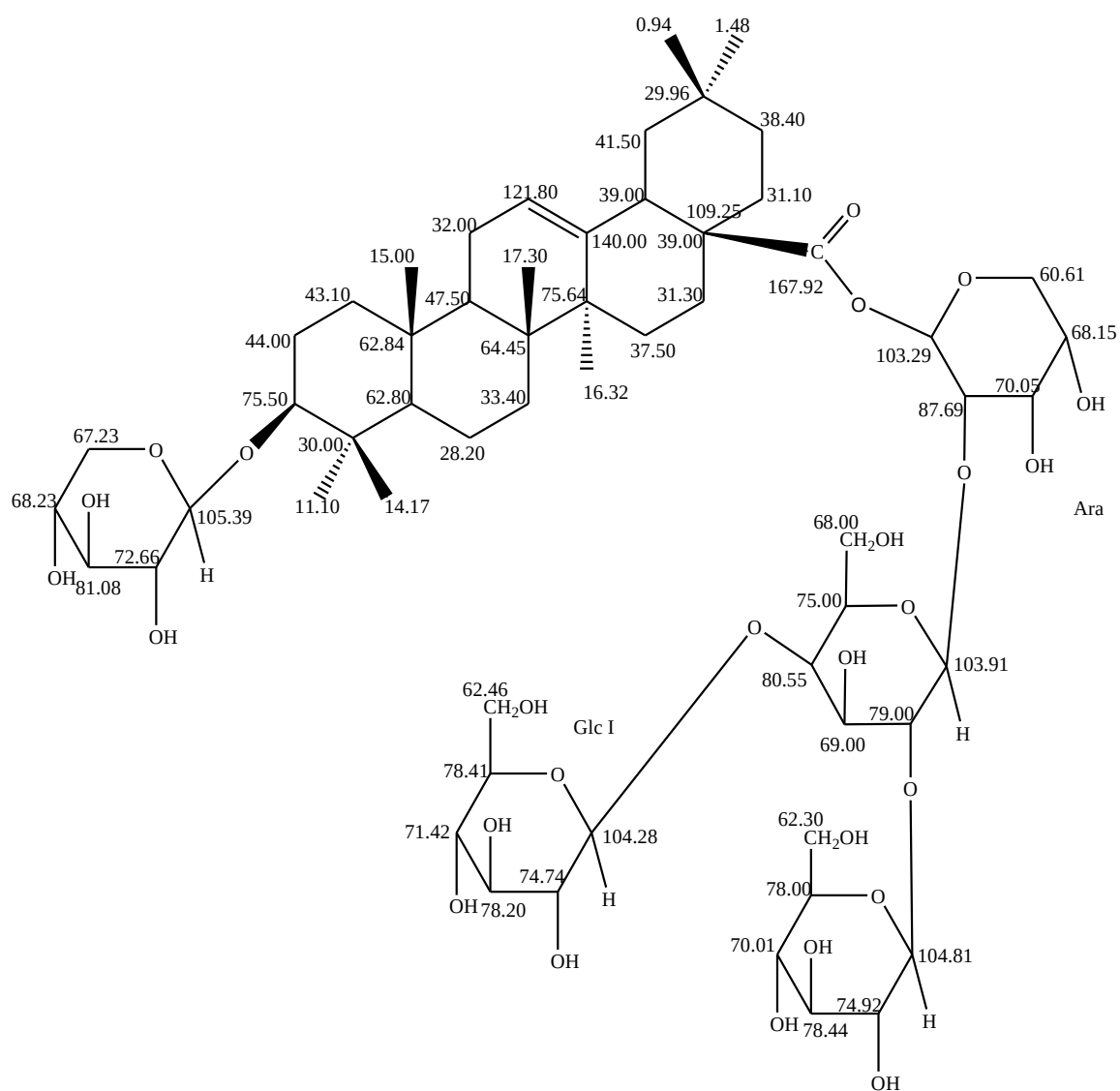


Fig 2.25: ^{13}C NMR (100.61 MHz) chemical shifts δ_{C} of Hum-IV in pyridine

Table 2.13: ^{13}C and ^1H NMR spectral data of Hum-IV from one and two dimensional experiments

S.No	^{13}C	δ_{C}	DEPT	Multiplicity	δ_{H}	Connectivity
1.	1	43.10	CH_2	m	(1 α) 1.28, (1 β) 2.32	2 α (2.55), 2 β (2.69)
2.	2	44.00	CH_2	m	(2 α) 2.55, (2 β) 2.69	1 α (1.28), 1 β (2.32), 3 α (3.84)
3.	3	75.50	CH	m	(3 α) 3.84	2 α (2.55)
4.	4	30.00	C	-----	-----	-----
5.	5	62.8	CH	m	(5 α) 1.80	1.10
6.	6	28.20	CH_2	m	(6 α) 1.10, (6 β) 1.93	(7 α) 1.40, H-5 (1.80)
7.	7	33.40	CH_2	m	(7 α) 1.40, (7 β) 1.45	(6 α) 1.10, (6 β) 1.93
8.	8	64.45	C	-----	-----	-----
9.	9	47.50	CH	m	1.28	-----
10.	10	62.84	C	-----	-----	-----
11.	11	32.00	CH_2	m	(11 α) 1.48, (11 β) 1.83	H-9 (1.28), H-12 (5.32)
12.	12	121.80	CH	m	5.32	11 α (1.48)
13.	13	140.00	C	-----	-----	-----

14.	14	75.64	C	-----	-----	-----
15.	15	37.50	CH ₂	m	(15 α) 2.58, (15 β) 2.68	H-16 α (1.64), 16 β (1.84)
16.	16	31.30	CH ₂	m	(16 α) 1.64, (16 β) 1.84	15 α (2.58)
17.	17	109.25	C	-----	-----	-----
18.	18	41.50	CH	m	2.15	
19.	19	39.00	CH ₂	m	1.70 2.20	H-18 (2.15), H ₃ -29 (0.94)
20.	20	29.96	C	s	-----	-----
21.	21	38.4	CH ₂	m	(21 α) 1.65, (21 β)1.68	H-30 (1.48, H-22 α (1.50)
22.	22	31.10	CH ₂	m	(22 α) 1.50, (22 β)1.54	H-21 α (1.65)
23.	23	11.10	CH ₃	s	1.25	
24.	24	14.17	CH ₃	s	1.05	
25.	25	15.00	CH ₃	s	0.90	
26.	26	17.30	CH ₃	s	0.70	H-7, H-9
27.	27	16.32	CH ₃	s	1.45	H-15, H-13

28.	28	167.92	C	-----	-----	-----
29.	29	20.41	CH ₃	s	0.94	H-30 (1.48), H-21 α (1.65)
30.	30	33.01	CH ₃	s	1.48	H-19 (1.70), H-21 α (1.65), H-21 β (1.68)
	sugar moieties					
	Xyl					
31.	1	105.39	CH	d, $j = 7.6$ hz	5.12	Xyl H-2 (4.52)
32.	2	72.66	CH		4.52	H-3 (4.53), H-1 (5.12)
33.	3	81.08	CH		4.53	H-2 (4.52)
34.	4	68.19	CH		4.80	H-3 (4.53), H-5 β (4.30)
35.	5	67.23	CH ₂		(Xyl H-5 α) 3.64 (Xyl H-5 β) 4.30	H-4 (4.80)
	Arabinose					
36.	1	103.29	CH	d, distorted	5.55	H-2 α (4.13)
37.	2	87.69	CH		4.13	H-1 (5.55), H-3 (3.78)

38.	3	70.05	CH		3.78	H-2 (4.13), H-4 (4.38)
39.	4	68.15	CH		4.38	H-3 (3.78), H-5 α (4.20), H-5 β (4.15)
40.	5	60.61	CH ₂		(H-5 α) 4.20 (H-5 β) 4.15	H-4 (4.38)
	Gal					
41.	1	103.91	CH	d, $J = 7.1$ Hz	4.93	Gal H-2 (4.58), DeRib H-3 (4.15)
42.	2	79.0	CH		4.58	Gal H-1 (4.93), H-3 (4.41), Glc H-1 (5.18)
43.	3	69.0	CH		4.41	Gal H-4 (4.29)
44.	4	80.55	CH		4.29	Gal H-3 (4.41), H-5 (4.10)
45.	5	75.0	CH		4.10	Gal H-6 (4.45)
46.	6	68.0	CH ₂		(Gal H-6 α) 4.45 (Gal H-6 β) 4.13	Gal H-5 (4.10)
	Glc					

47.	1	104.81	CH	d, $J = 7.6$ Hz	5.10	Glc H-2 (4.08), Gal H-4 (4.29)
48.	2	74.92	CH		4.08	Glc H-1 (5.10), Glc H-3 (4.10)
49.	3	78.44	CH		4.10	Glc H-4 (3.88)
50.	4	70.01	CH		3.88	Glc H-3 (4.10)
51.	5	78.00	CH		4.08	Glc H-6 α (4.48), Glc H-6 β (4.62)
52.	6	62.30	CH ₂		4.48 4.62	H-5 (4.08)
	Glc I					
53.	1	104.28	CH	d, $J = 7.7$ Hz	5.18	Gal' H-2 (4.05), Glc H-2 (4.58)
54.	2	74.74	CH		4.05	Glc' H-1 (5.18), Glc' H-3 (4.15)
55.	3	78.20	CH		4.15	Glc' H-4 (4.18)
56.	4	71.42	CH		4.18	Glc' H-5 (3.98)
57.	5	78.41	CH		3.98	Glc' H-6 β (4.26), Glc' H-6 α (4.51)
58.	6	62.46	CH ₂		(Gal' H-6 α) 4.51 (Gal' H-6 β) 4.26	Glc' H-5 (3.98)

By studying the FAB mass negative ion spectrometry, branching of the oligosaccharide was determined. Xylose is attached to position 3 of the aglycone by establishing ethereal-bond and the remaining polysaccharides are attached at the position 28 forming an ester linkage with the main skeleton. Arabinose is attached to the carbonyl group directly. The galactose (Gal) is forming geminal bond with arabinose at carbon 2 of the unit. Glucose (Glc) and Glucose I (Gal I) are linked by a geminal bond to the galactose unit at position 2 and 4, revealing that both the Glc units are at the terminal positions. Considering all the above mentioned detail about Hum-IV, new structure was proposed, that is 3-O- β -D-xylopyranoside-olean-12-en-28-oic acid-28-O- β -arabinopyranosyl-(1-3)- β -D-galactopyranosyl-(1-2)- β -L-glucopyranosyl-(1-4)- β -L-glucopyranosyl ester

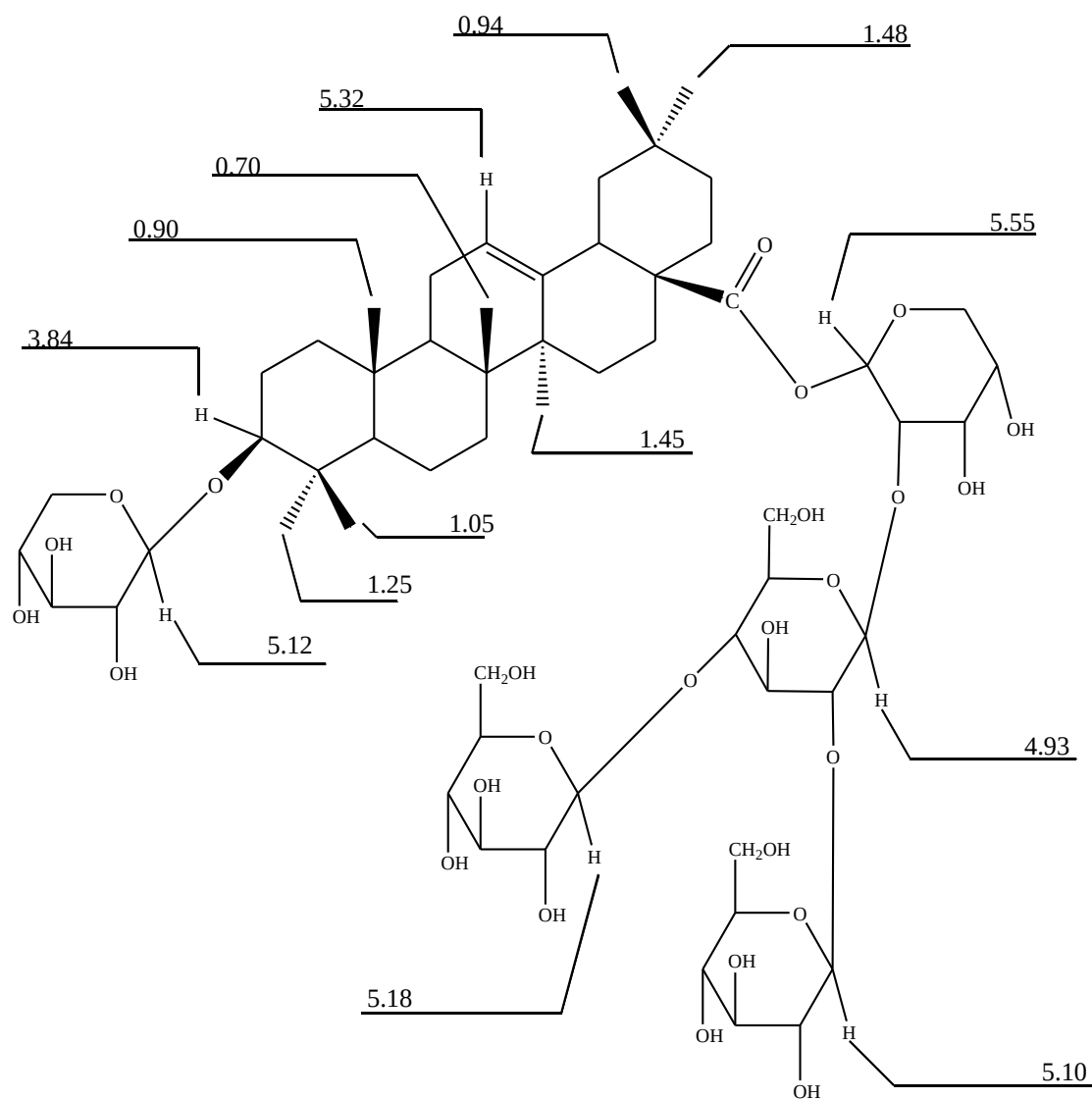


Fig 2.26: ^1H -NMR chemical shifts δ^{H} of Hum-IV in pyridine- d_5

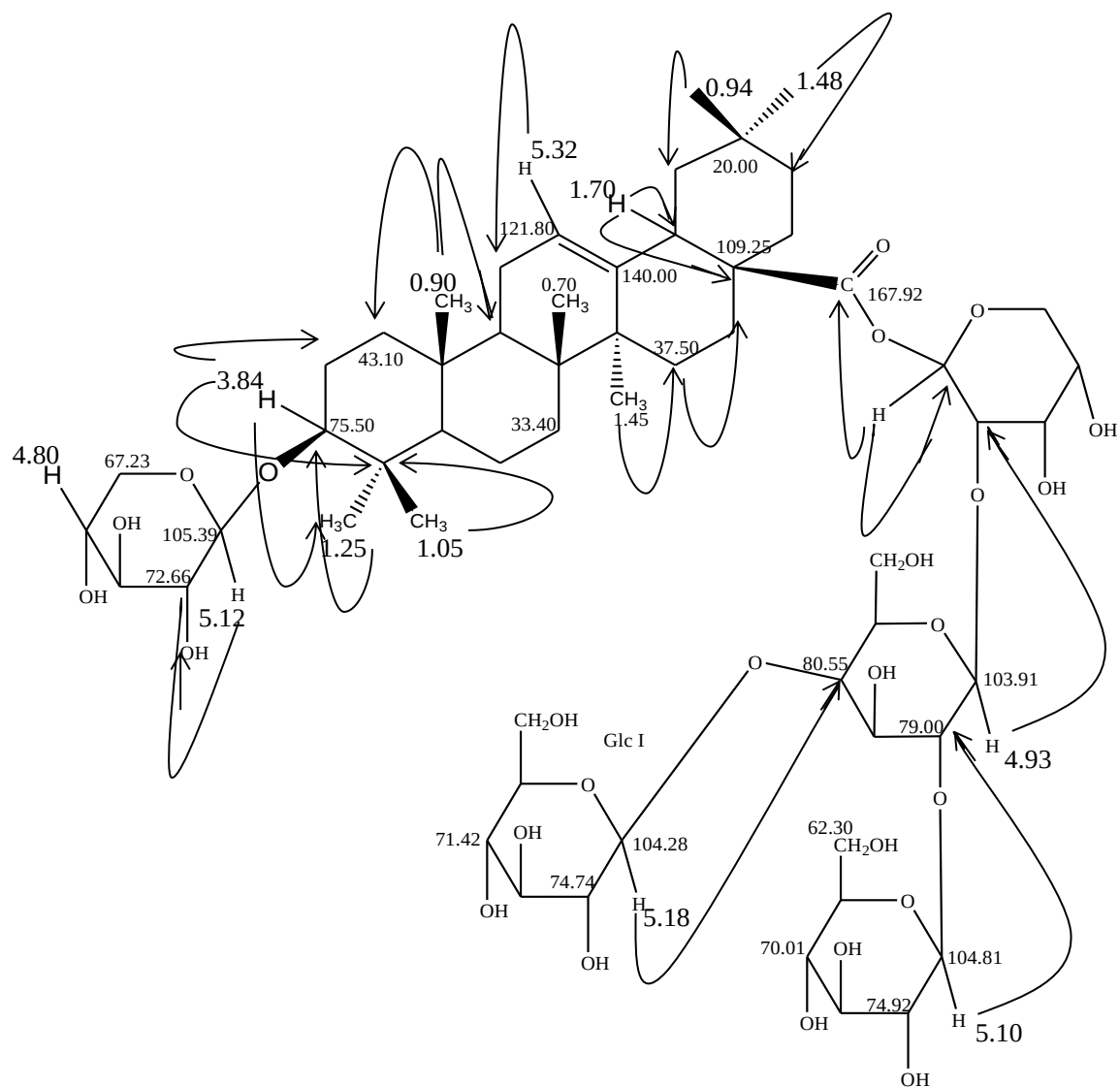


Fig 2.27: HMBC connectivities of Hum-IV (400 MHz, pyridine- d_5) ^{13}C -NMR chemical shifts and the arrows indicate the connectivity's from proton to carbon. The bold bonds indicate the COSY relationships.

2.4 Conclusions:

- CD-G crude and CD-R crude gave 15 and 14 mm zone of inhibition against *S. aureus* and *B. atrophaeus*, respectively. *S. typhii* was the most resistant strain. The anti-bacterial activities of crude saponins extracted from *C. diurnum* green and ripe fruit was good as compared to those extracted from plant's other aerial parts (leaves).
- All the crude methanolic extracts and tested fractions gave hundred percent leishmanial inhibition activities.
- Anti-oxidant activities of CD-R crude methanolic and crude saponins extract was 90 %. Crude saponins extract of all the parts displayed excellent antioxidant activities.
- Hum-V was isolated from *Cestrum nocturnum* and characterized by different spectroscopic techniques, and found to be nocturnoside A, which was a reported compound.
- Hum-II was also isolated from the aerial parts of *Cestrum nocturnum*. After complete characterization availing the modern spectroscopic experiments, the structure of Hum-II was proposed to be a karatavoiside A. it was previously isolated from a member of Liliaceae family.
- Pure Hum-IV, isolated from the most polar butanolic part of *Cestrum nocturnum* was elucidated to be a triterpenoidal glycoside (oleanolic acid derivative). It was proposed to be a new structure, 3-O- β -D-xylopyranoside-olean-12-en-28-oic acid-28-O- β -arabinopyranosyl-(1-3)- β -D-galactopyranosyl-(1-2)- β -L-glucopyranosyl-(1-4)- β -L-glucopyranosyl ester.

Chapter 3

EXPERIMENTAL

PART A- PHYTO-CHEMISTRY:

3.1 General Experimental Conditions:

3.1.1. Melting points:

Melting points were determined on Bock-Monoscop-M melting point apparatus.

3.1.2. UV-lamp:

Components in various fractions and compounds purity were checked by TLC under UV lamp (254 nm and 365 nm)

3.1.3. IR Spectrophotometer

The IR spectra were scanned on Shimadzo IR- spectrophotometer irradiating in the range of 4000-650 cm.

3.1.4. Mass Spectrometer:

Mass spectra were performed on JEOL JMS 600-H machine.

3.1.5. Nuclear Magnetic Resonance:

Proton and C13 NMR spectra (1D and 2D) were recorded on Bruker Avance 500, (500 MHz) Spectrometer using deuterated solvents and all the J-coupling constants were measured, not calculated. Chemical shift values are being reported as parts per million

(ppm). The chemical shift values are relative to the internal NMR standard, tetra methyl saline (TMS).

3.1.6. Solvents

All the solvents (hexane, petroleum ether, chloroform, dichloromethane, ethyl acetate, butanol, methanol and ethanol) used in extraction, fractionation and column chromatography, were purchased from a local distributor, distilled with care and placed in air tight flasks. Solvents used for Thin Layer Chromatography/ preparative Thin Layer Chromatography (TLC/PTLC) (Pyridine, chloroform, methanol, ethyl acetate, butanol, hexane) were purchased from Merck and sigma-Aldrich.

3.2 Techniques employed for purification of compounds:

3.2.1. Column Chromatography

The column chromatography (CC) was carried out by using Merck silica gel 60 (70-230 mesh). Kieselgel 60 PF254 (E. Merck Article No. 21321) was used for Vacuum liquid chromatography.

3.2.2. TLC/PTLC

Thin layer chromatography (TLC) was performed on;

- a) Pre-coated aluminum cards (20 x 20 cm) Kieselgel 60 F₂₅₄ (layer thickness 0.2 mm), Merck Article number 1.05554.0001,
- b) 20 x 20 cm pre-coated glass plates, 0.2 mm thickness, DC-Fertigplatten, Kieselgel 60 F₂₅₄, Merck Article No. 5715,

c) HPTLC Silica gel 60 RP-18, 20 x 10 cm, Merck Article No. 105914.

3.3 Spray reagents:

Various spray reagents were used to observe the spots of the compounds visibly. These include;

3.3.1. Ceric sulphate:

Ceric sulphate (0.1gm) was suspended in 4ml of water, 1gm of trichloroacetic acid was added. The solution was boiled and concentrated sulphuric acid was added drop wise till the disappearance of turbidity. Freshly prepared reagent was used.

3.3.2. Anisaldehyde:

1ml concentrated sulphuric acid was added to a solution of 0.5 ml anisaldehyde in 50ml acetic acid. It was prepared freshly and sprayed on the TLC plate. After heating at 100-105°C for few minutes, spots gave the impression.

3.4 Plant material

Cestrum nocturnum was collected from the premises of University of Peshawar and PCSIR Labs Complex, Peshawar. The plant material was identified by Mr. Shahid Farooq, PSO, PCSIR Labs Complex, Peshawar and voucher sample preserved. It was dried in shade, chopped and pulverized.

3.4.1. Extraction and fractionation of *Cestrum nocturnum*:

Cestrum nocturnum plants aerial part was shade dried, chopped, and exhaustively extracted with methanol. The methanol extract was concentrated and completely dried on

vacuum rotary evaporator at 50°C. The dried extract was dissolved in distilled water and filtered. To the filtrate, petroleum ether (pet. ether) was added to extract pet ether soluble portion. Pet. ether extract was concentrated and dried and weighed. It was named as CN-A, Pet. ether. The aqueous layer was extracted with chloroform (CH₃Cl). The extract was dried, weighed and named as CN-A, CH₃Cl. The remaining aqueous extract, was portioned with ethyl acetate (EtAc) and the organic layer separated and dried and weighed. It was labeled as CN-A, EtAc. At the end the aqueous extract was extracted with iso-butanol. The iso-butanol soluble fraction was also dried after concentration and then weighed, named as CN-A, butanol. Scheme 1 summarizes the above mentioned extraction and labelling process.

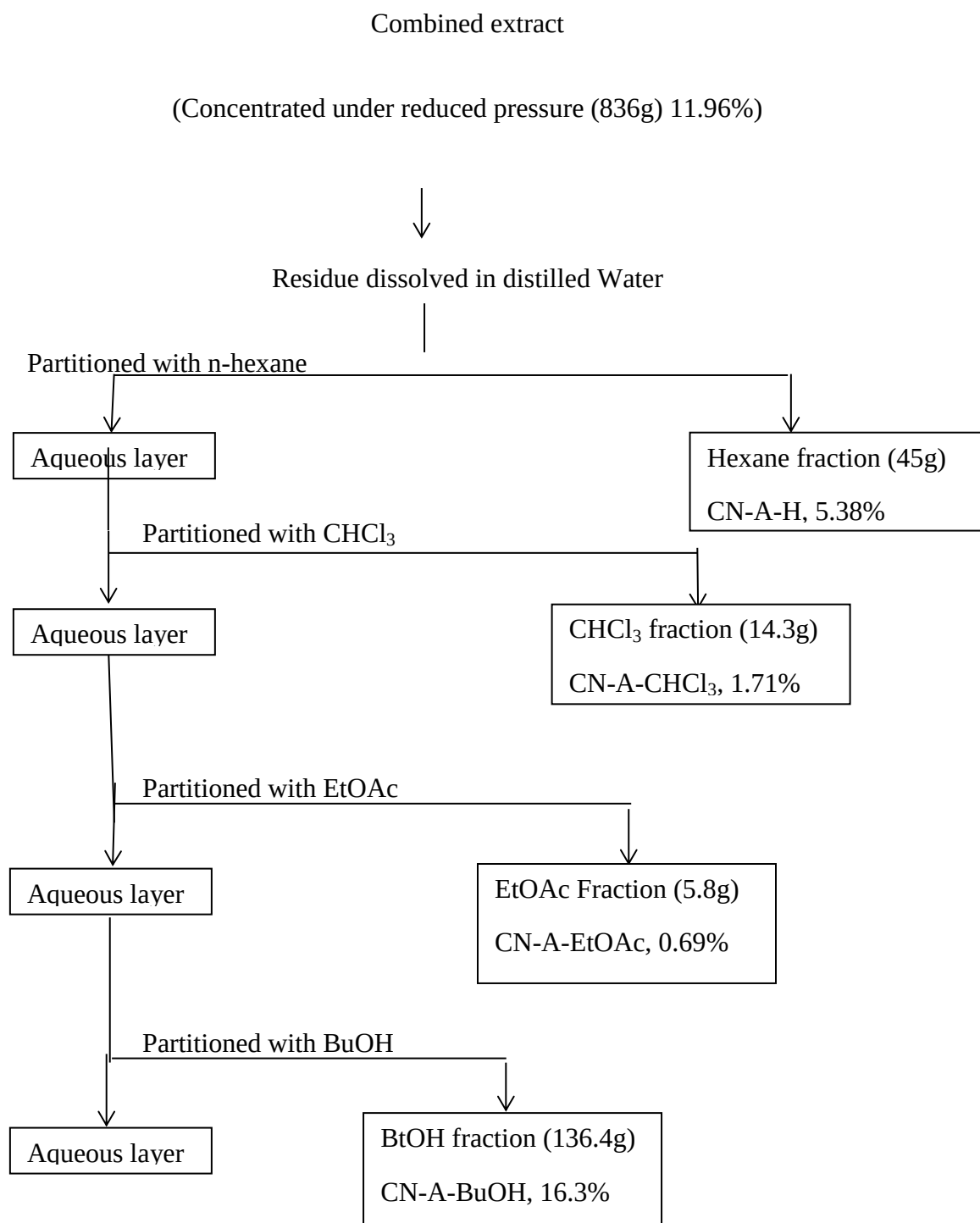
Plant: *Cestrum nocturnum* (CN-A)

Plant part: fresh Leaves and Stems

Weight: 7Kg

Soaked in: Methanol 3x

Duration: 3 days each time



Scheme 1: Extraction and fractionation of *Cestrum nocturnum*

3.4.2. Extraction and fractionation of *Cestrum diurnum* arial portion:

Cestrum diurnum plant's aerial parts (CD-A) were shade dried, chopped, and exhaustively extracted with methanol. The methanol extract was concentrated and completely dried on vacuum rotary evaporator at 50°C. The dried extract was dissolved in distilled water and filtered. To the filtrate, petroleum ether was added to extract pet ether soluble portion. Pet ether extract was concentrated and dried and weighed. It was named as CD-pet ether. To the aqueous layer CHCl_3 was added to extract the CHCl_3 soluble portion. CHCl_3 fraction was dried and weighed and named as CD-A CHCl_3 . The remaining aqueous extract, was portioned with ethyl acetate and the organic layer separated, dried and weighed. It was labeled as CD-A ethyl acetate. At the end the aqueous extract was extracted with iso-butanol. The iso-butanol soluble fraction was also dried after concentration, weighed and named as CD-A butanol.

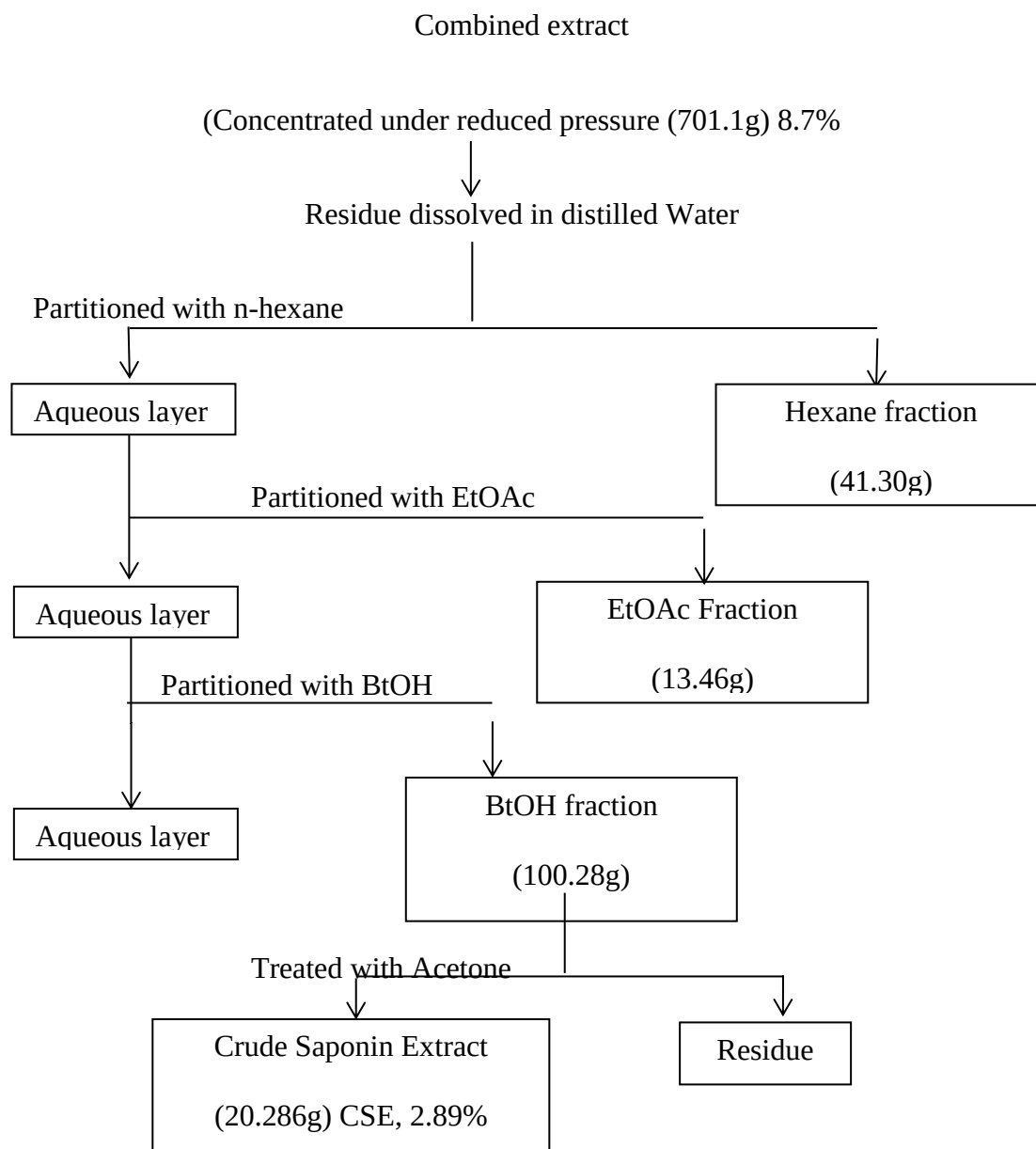
The dried butanol fraction was defatted with hexane and then dissolved in methanol. The extract was filtered and concentrated to one third. Chilled acetone was added to the concentrated methanolic extract slowly and placed in refrigerator for an hour. Precipitated saponins were filtered through buchner funnel, dried at 40°C and weighed. It was named as Crude Saponins Extract (CSE- CD-A). Scheme-2 shows fractionation of *Cestrum nocturnum*.

Plant: *Cestrum diurnum* (CD-A)

Plant part: fresh Leaves and Stems

Weight: 8Kg

Soaked in: Methanol 3x (3 days each time)



Scheme 2: Extraction and fractionation of *Cestrum diurnum*

3.4.3. Extraction and fractionation of *Cestrum diurnum* ripe berries:

Fully mature (black) berries of *Cestrum diurnum* (CD-R) were shade dried. The dried ripe berries were then defatted with hexane and then soaked in distilled methanol. The extract was filtered and concentrated to one third. Cold acetone was added to the concentrated methanolic extract slowly and placed in refrigerator for an hour, undisturbed. Precipitated saponins were filtered through buchner funnel, dried at 40°C and weighed. It was named as Crude Saponins Extract (CSE- CD-R).

Crude saponin extraction is shown in scheme-3.

Plant: *Cestrum diurnum* (CD-R)

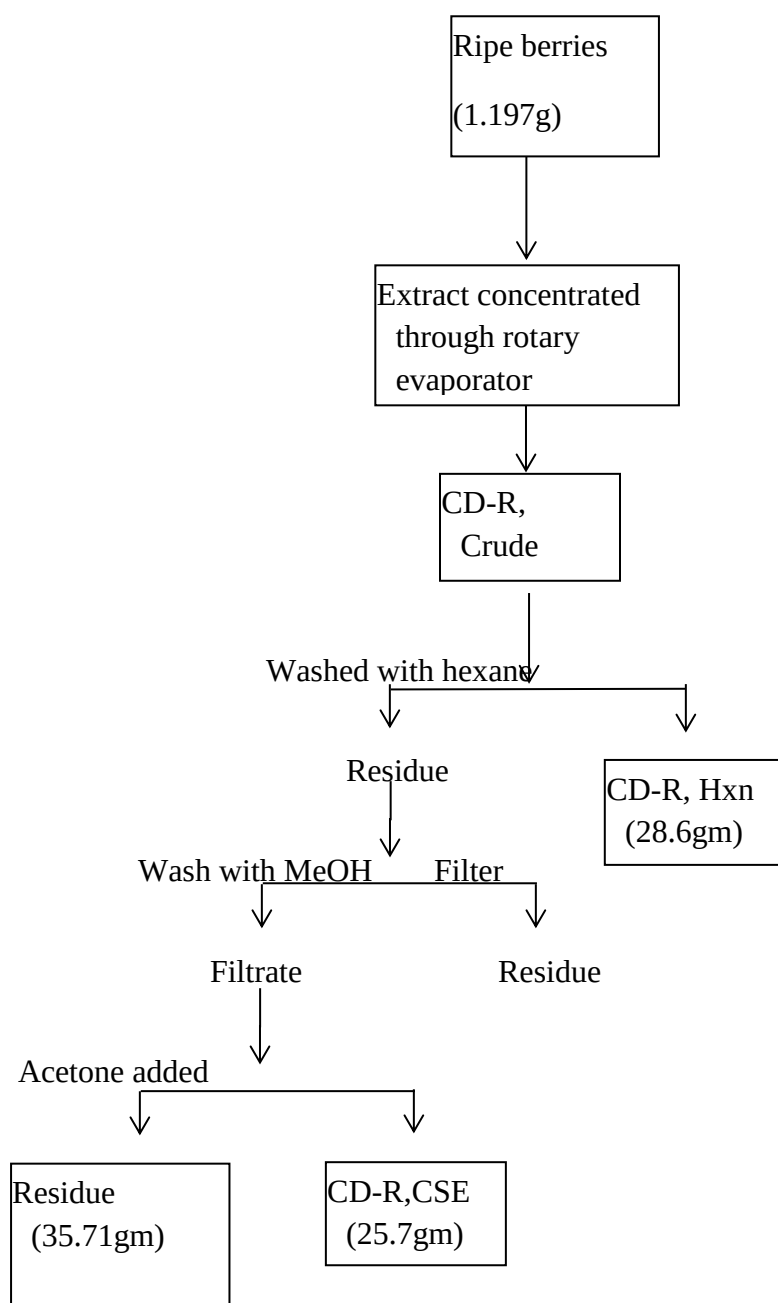
Plant part: ripe berries

Weight: 1.197Kg

Soaked in: MeOH 3x

Duration: 3 days each time

Concentration: rotary evaporator



Scheme 3: Extraction and fractionation of *Cestrum diurnum* berries (CD-R)

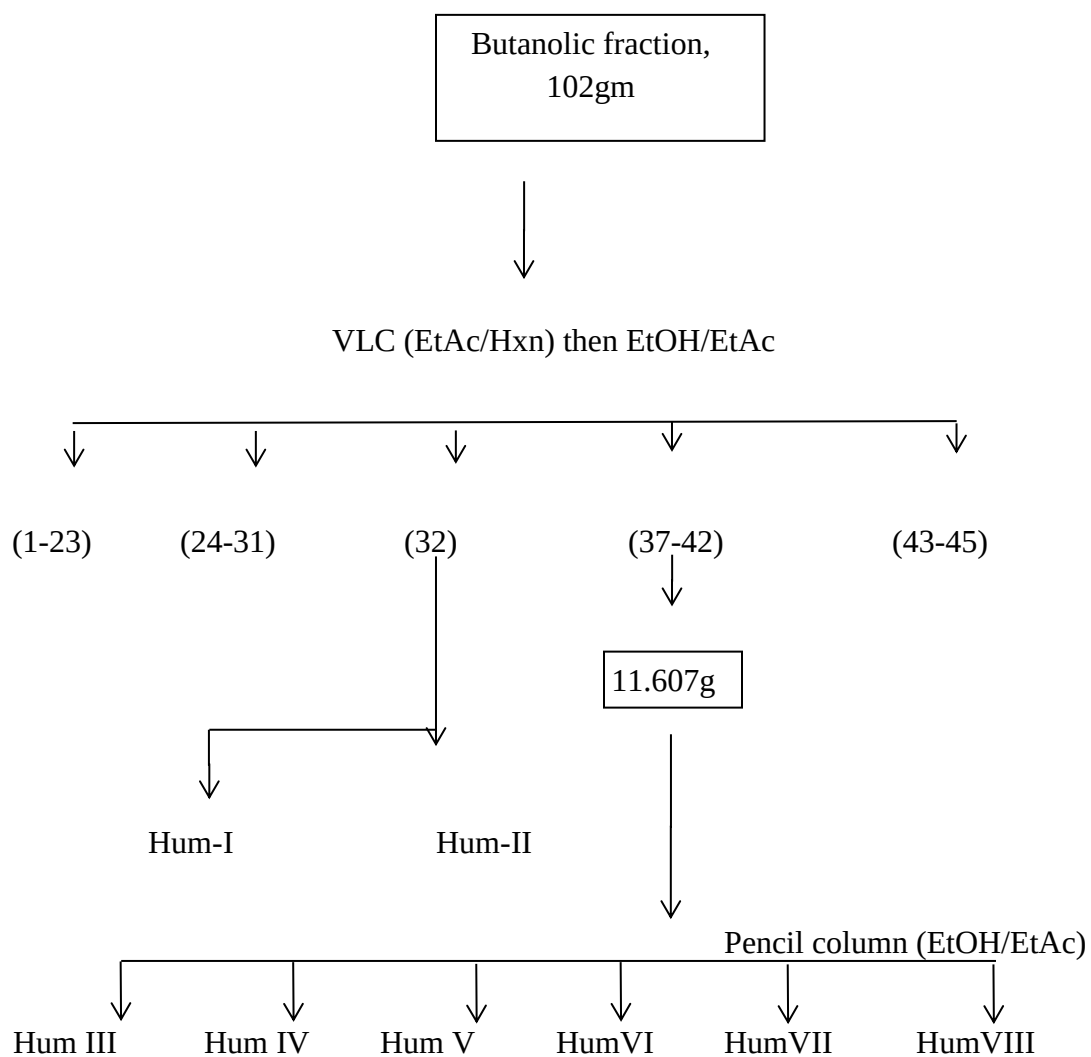
3.5. Isolation of Compounds:

3.5.1. Extraction and isolation of constituents from *Cestrum nocturnum*:

The butanolic fraction was passed through vacuum liquid chromatography, first using 50 % Ethyl Acetate- Hexane (EtAc/Hxn). The eluent was changed to 80% EtAc/Hxn and then to pure EtAc. Elution was continued with EtAc and ethanol (EtOH) mixed in various proportions and different fractions were collected from 17- 45.

With 50% EtOH/EtAc, fraction 32 was obtained, having two spots which are very close to each other and were separated on Prep-TLC in 12:3:2 butanol: acetic acid: water (BAW) system. These were named as Hum-I (57mg) and Hum-II (20mg). Hum-I turned black at 205°C. The melting point of the pure crystals of Hum-II was 260-271°C

Fractions 37-42 were combined (11.607gm) and subjected for column chromatography. Column was eluted with Hexane, EtAc/Hxn, EtAc, EtOH/EtAc, EtOH and MeOH. Fractions from 1- 50 were obtained. Fraction 22 was concentrated having greenish solid and yellowish liquid portion. These were separated on Prep-TLC in 12:3:2 BAW. Hum-III and Hum-IV separated. Fraction 23 gave a pure compound, white ppt. methanol soluble Hum-V. Fraction 24 also had 3 compounds, Hum-VI white ppt. MeOH (warm) and Hum-VII white ppt. insoluble in MeOH Hum-VIII. Scheme-4 shows isolation of various compounds from *Cestrum nocturnum*.



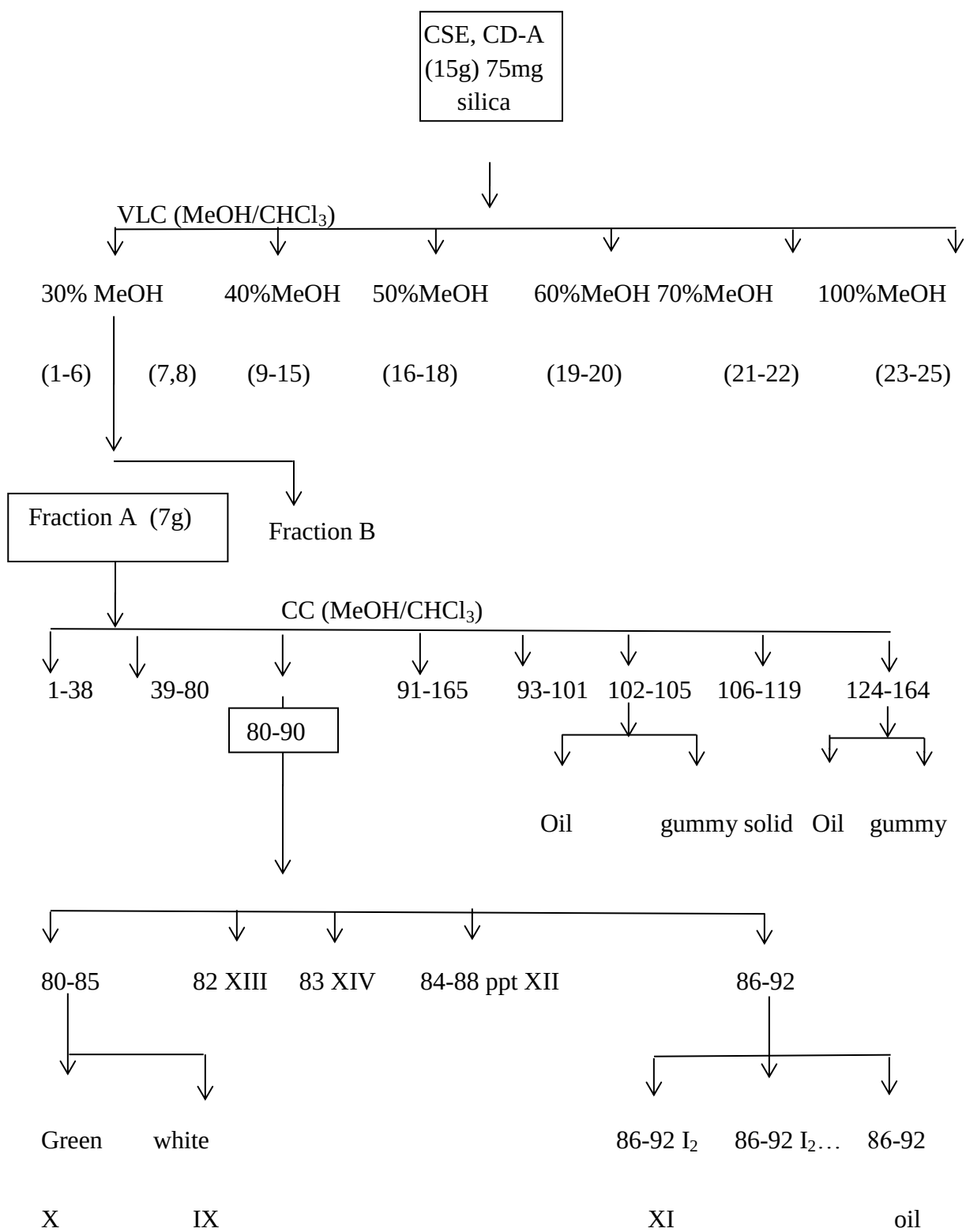
Scheme 4: Extraction and isolation of compounds from CN-A

3.5.2. Extraction and isolation of constituents from CD-A:

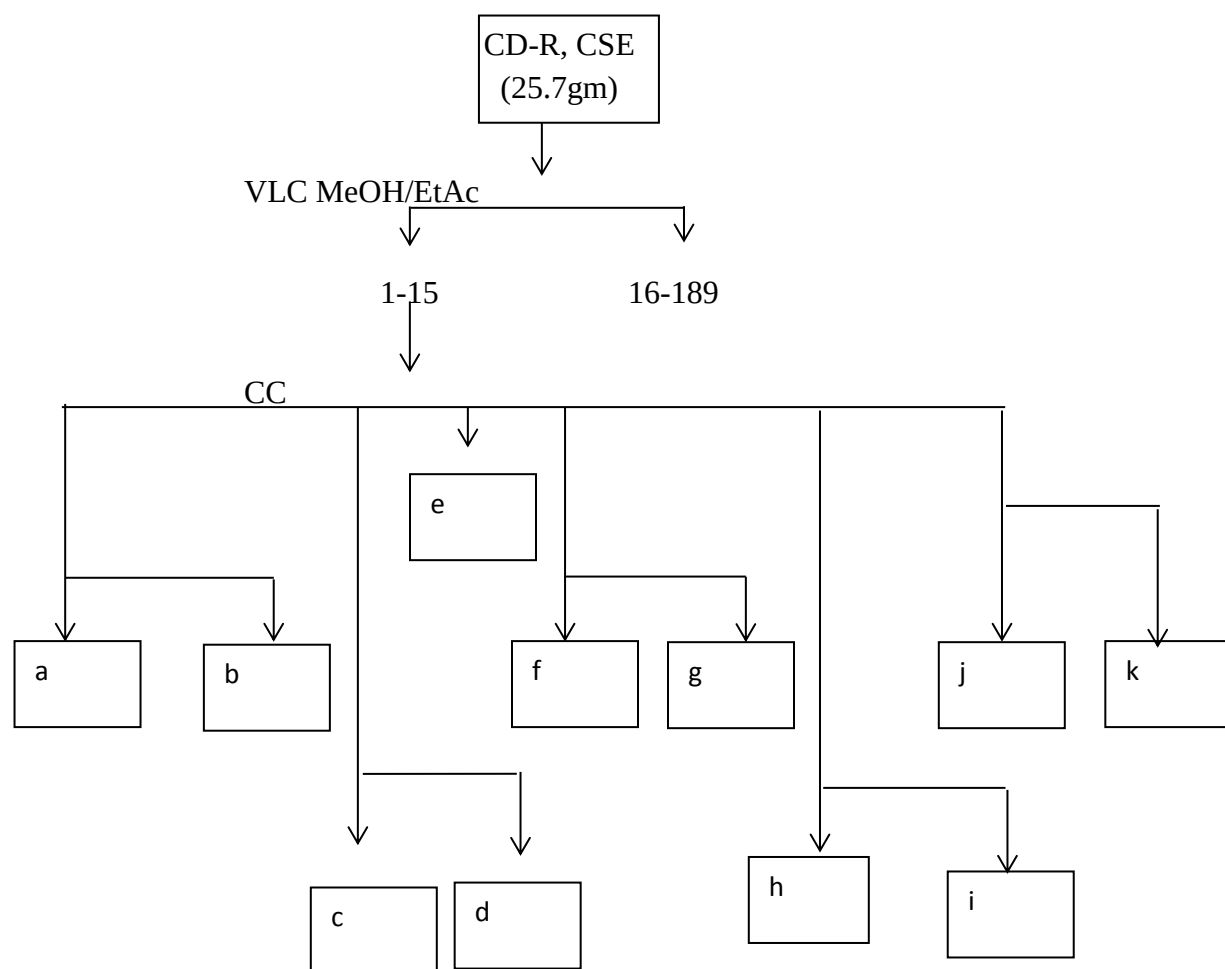
15gm crude saponins extract (CSE) of *Cestrum diurnum* arial part was subjected to vacuum liquid chromatography. The column was eluted with MeOH/CH₃Cl in different ratios. With 30% MeOH/ CHCl₃, a fraction was collected, which was subjected to a small column. With 25% MeOH/ CHCl₃ six (6) compounds were isolated. Hum-IX, Hum-X 1 and Hum-X 2, Hum-XI, Hum-XII, Hum-XIII, Hum-XIV. Isolations from *cestrum diurnum* are given in scheme-5.

3.5.3. Extraction and isolation of constituents from *Cestrum diurnum* (CD-R):

25.6gm crude saponins extract (CSE) was obtained from ripe berries of *Cestrum diurnum* (CD-R). The CSE was subjected to column and eluted with MeOH/EtAc in various concentrations. The initial fifteen fractions were collected and chromatographed over a pencil column. Various compounds were isolated from the fractions. Scheme-6 gives information about isolation from *Cestrum diurnum* ripe berries.



Scheme-5: Extraction and isolation of compounds from CSE of CD-A



Scheme-6: Extraction and isolation of compounds from CD-R, CSE.

Part B- BIOACTIVITIES

3.6. Antibacterial assay:

The crude extract, its various fractions and crude saponins extract in the concentration of 3mg/ml, were screened against various human pathogens by agar well diffusion method [99]

3.6.1. Microorganisms used:

Table 2.1 show the microbial species used in the test; fresh and pure cultures were used in the analysis.

3.6.2. Preparation of agar-plates:

Approximately 45 ml of molten nutrient agar was dispensed on sterilized petri-plates, and was permitted to harden. Nutrient agar was used for bacterial cultures and for fungal strain Sabouraud dextrose agar (SDA) was used.

3.6.3. Preparation of stock sample:

Test material (3 mg/ml) was dissolve in dimethylsulfoxide (DMSO) and placed in dry place.

3.6.4. McFarland turbidity standard:

0.5 Mcfarland standard was prepared by mixing 0.5 ml (1.175 % wt/vol) barium chloride dihydrate ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) solution with 99.5 ml (1 % vol/vol) of sulphuric acid (H_2SO_4). The density of the standard was then confirmed by checking its absorbance at

625nm. The absorbance should be in the range of 0.08 to 1. This turbidity is equal to 10^8 colony forming units / ml (cfu/ml).

3.6.5. Preparation of standards:

Gram +ve:

Positive control = Azithromycin (30µg per disc/well)

Negative control = Solvent media (6µL per disc/well)

Gram –ve:

Positive control = Ciprofloxacin (30µg per disc/well)

Negative control = Solvent media (6µL per disc/well)

For *Candida albicans*:

Positive control = Clotrimazole (30µg per disc/well)

or Fluconazole (20µg per disc/well)

Negative control = Solvent media

3.6.6. Application of test:

Sterilized agar plates and other apparatus were used for the test. The freshly cultured microbial strains were standardized with McFarland's standard so that uniform cultures could be applied for the test.

i- Anti-bacterial test:

a) Bacteria were dispersed on these nutrient agar plates by preparing sterilized soft agar accumulating 100 µl of bacterial culture. 6 mm long sterilized metallic borer was used for wells digging at suitable distance and spotted for identification. Sample (100 µl) was poured into each well, and the plates were incubated at 37°C for 24 hours.

b) In agar disc method, after spreading 70-80µL of standardized bacterial culture, 6mm discs (whatmann filter paper) were placed at a suitable distance from each other. 6µL sample was adsorbed on each disc. The plates were placed for 24 hours in incubator at 37°C.

The antibacterial activity was estimated in terms of inhibition zone. The azithromycin was used as positive control for gram-positive bacteria, ciprofloxacin for gram-negative bacterial strains and DMSO were used as negative control.

ii- Antifungal assay:

a) 100µl of fungal stock culture was dispersed on sterilized Sabouraud dextrose agar. 6mm long sterilized metallic borer was used for wells digging at suitable distance and spotted for identification. Sample (100µl) was poured into each well, and the plates were incubated at 28°C for 24 h.

b) In agar disc method, after spreading 70-80µL of standardized fresh fungal culture, 6mm discs (whatmann filter paper) were placed at a suitable distance from each other. 6µL sample was adsorbed on each disc. The plates were placed for 24 hours in incubator at 28°C.

The antifungal activity was estimated in terms of inhibition zone. DMSO was used as control while fluconazole and clotrimazole as standard drugs [99].

3.6.7. Results:

Results were measured after 24 hours of the application by measuring the zone of inhibition in millimeter. Results are given in table 2.2 to 2.5. These are graphically represented in graphs 2.1 to 2.6

3.7. Antioxidant assay:

The crude methanol extract and subsequent solvent fractions as well as total saponin contents of the whole plant were tested for their antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical.

In this protocol [100], 3.60mg DPPH was dissolved in ethanol (20ml) and the solution was kept in the dark at room temperature. 0.5ml sample solution was added to 1 ml of DPPH solution. This mixture was incubated for 30 min at room temperature. After that, the absorbance was measured at 517 nm. Percentage inhibition of radical scavenging activity was determined by comparison of the results with the control. Ethanol was negative control while ascorbic acid was positive control in the assay. Lower the absorbance of the mixture, higher the free radical scavenging activity. All the analyses were performed in triplicate. Table 2.6 shows the results. Graph 2.7 shows the graphical representation of the antioxidant activities.

$$\% \text{ scavenging DPPH free radical} = 100 \times (1 - \text{AE}/\text{AD})$$

Where AE is absorbance of the solution, AD is the absorbance of the DPPH solution.

3.8. Cultivation and isolation of Leishmania parasite:

3.8.1. Media preparation:

The culture medium RPMI 64 (Gibco,USA) was prepared by dissolving 0.3 g medium in 30 ml distilled water [80].

3.8.2. Preparation for leishmania culture:

Prepared media was dispensed in 10 vials, each having 3 ml medium. 10% fetal bovine serum was added. The antibiotic consisting of penicillin G and kanamycine were mixed to avoid bacterial contamination. The air tight vials were placed in ice jar.

3.8.3. Leishmania parasite collection:

The media in ice jar was taken to the field where samples from the identified suspected person were collected. The skin scrapings were directly mixed from the lesion to each bottle in ice jar. Then the medium was incubated at 26°C in incubator.

3.8.4. Leishmania growth:

For 12 days the parasite was kept under observation. After each 24 hours leishmania parasite was checked for the different life stages with staining Geimsa and identified under 10 x, 40 x and 100 x magnification of microscope.

3.8.5. Experiment Design for chemotherapy

The experiment was conducted in duplicates. 3ml RPMI 1640 media was taken in each vial including negative and positive control vials. These were supplemented with 10% fetal calf serum. Further added the antibiotic i.e. penicillin and Gentamycin to

prevent the secondary bacterial growth. Test samples were added at the rate of 0.025mg/ml in the each vial. After that 1ml of leishmania culture was added in all the vials except in negative control group. The cultures were examined daily under the microscope under the magnification 10x, 40x and 100x for 12 days. Table 2.7 shows the results of the activity.

Figure 2.9 and 2.10 shows the leishmania parasite at different stages.

Chapter 4

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