Study of Phytoconstituents of Ethnobotanically Valuable Plant *Indigofera linifolia* Retz. by HPLC-DAD-ESI/MS and GC-MS Analyses

A Thesis Presented in Partial Fulfillment of the Requirements for the Degree of Master of Philosophy (M. Phil.) in Organic Chemistry

by

Kashmala Naseem

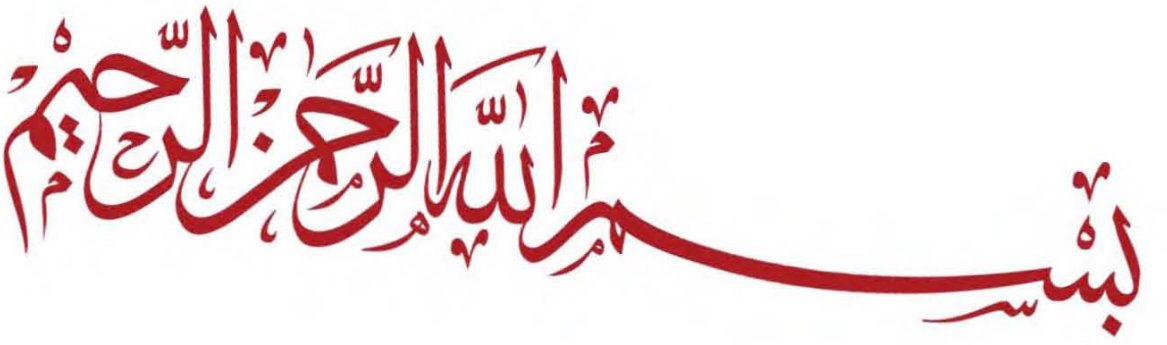
to

The Faculty of Natural Sciences,

Department of Chemistry,

Quaid-i-Azam University, Islamabad.

(2021)


بسم الله الرحمن الرحيم

سَنُرِيهِمْ اٰيٰتِنَا فِي الْاٰفَاقِ وَفِيْٓ اَنْفُسِهِمْ حَتّٰى يَتَبَيَّنَ لَهُمْ اَنَّهُ الْحَقُّ ۭ اَوَلَمْ يَكْفِ بِرَبِّكَ اَنَّهٗ عَلٰي كُلِّ شَيْءٍ شَهِيْدٌ

We will soon show them Our signs in the Universe and in their own souls, until it will become quite clear to them that it is the truth. Is it not sufficient as regards your Lord that He is a witness over all things?

[Al-Quran, Sura Ha-meem Al-Sajda, Verse 53]

ACKNOWLEDGEMENT

I express my gratitude to Allah Almighty for His help in completion of my work. He blessed me with the potential, abilities, and opportunities to achieve my goal. I am grateful to the last Prophet Muhammad (Peace be upon him) who conveyed all that knowledge and principles which make a man successful in life.

I am especially thankful to my supervisor **Dr. Muhammad Farman**, Department of Chemistry, Quaid-i-Azam University Islamabad for his guidance and support to complete my research project. I am grateful for his kindness and sincere concern towards achievement of my goal. He is an inspiration for me in regards of hard-work, discipline, and honesty.

I am grateful to **Prof. Dr. Aamer Saeed**, The respectable Chairman, and **Prof. Dr. Humaira Masood Siddiqi**, Head of Organic section, Department of Chemistry, Quaid-i-Azam University Islamabad. They ensured the provision of all necessary facilities required to complete my project.

I acknowledge my teachers all the way long since grade one for their guidance and hard work. Thanks to my father **Abdul Waheed** and my mother **Tasleem Akhtar** for every struggle they have made for me. I am thankful to my sisters **Mubashira Tasleem** and **Noor-ul-Huda** for their love. I am thankful to my aunt **Shamim Akhtar** for her care.

I am thankful to my friends **Sania Ijaz**, **Sadia Islam**, **Um-e-Habiba**, **Alia Mushtaq** and **Asma Nawaz** for their support and motivation. I also acknowledge my lab fellows **Bibi Rohida, Ayesha Imtiyaz, Muqaddas Zahoor, M. Tashfeen Khan, Arusak Tehreem** and **Amna Razzaq** for their help and cooperation.

Kashmala Naseem

CONTENTS

List of Abbreviations

^{13}C NMR	Carbon 13 Nuclear Magnetic Resonancce
1D NMR	One Dimensional Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{1}H-^{1}H COSY	Proton Correlation Spectroscopy
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
2D-PC	Two-Dimensional Paper Chromatography
2D-TLC	Two-Dimensional Thin Layer Chromatography
3-NP	3-Nitropropionate
6BIO	6-Bromo Indirubin-3′-Oxime
A431 cells	Human Epidermoid Carcinoma Cells
A549	Lung Cancer Cell Line
ABTS	2, 2′-Azinobis- (3-ethylbenzothiazoline-6-sulfonate
Ag-NPs	Silver Nanoparticles
APCI-MS	Atmospheric Pressure Chemical Ionization Mass Spectrometry
BAW	*n*-Butanol-Acetic acid-Water, 4:1:5 (upper layer)
BB	Broad Band spectroscopy
BChE	Cholinesterase
BHT	Butylated Hydroxy Toluene
CFA	Complete Freund′s Adjuvant Induced Arthritic Rat Models
cfu	Colony Forming Units
CML	Chronic Myelocytic Leukemia
COSY	Correlation Spectroscopy
CTLC	Centrifugal Thin Layer Chromatography
DAL	Dalton′s Ascitic Lymphoma
DE-MS	Differential Electrochemical Mass spectrometry
DEN	*N*-Nitrosodiethyl Amine
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EAC	Ehrlich Ascites Carcinoma
EIMS	Electron Impact Mass Spectrometry
ESR	Erythrocyte Sedimentation Rate

EtOH	Ethanol
FIC	Fractional Inhibitory Concentration
FRAP	Ferric Reducing Antioxidant Power Assay
FTIR	Fourier Transform Infrared spectroscopy
GC-MS	Gas Chromatography-Mass spectrometry
GLC	Gas Liquid Chromatography
GSK-3	Glycogen Synthase Kinase
HBV	Anti-Hepatitis B Virus
HDI	Hydrogen Deficiency Index
Hep2-cell	Human Epidermoid Cancer Cell Line
HMBC	Heteronuclear Multiple Bond Correlation
HPLC-ESI-MS	High Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry
HREIMS	High Resolution Electron Impact Mass Spectrometry
HR-FABMS	High Resolution Fast Atom Bombardment Mass Spectrometry
HRMS	High Resolution Mass Spectrometry
HSV	Herpes Simplex Virus
IC_{50}	50% Inhibitory Concentration
ILDH	*Indigofera linifolia S*eed Extract in *n*-hexane
ILDM	*Indigofera linifolia S*eed Extract in MeOH:H_2O (70:30)
ILE	*Indigofera oblongifolia* Leaves
ILLH	*Indigofera linifolia* Leaf Extract in *n*-hexane
ILLM	*Indigofera linifolia* Leaf Extract in MeOH:H_2O (70:30)
ILRH	*Indigofera linifolia* Root Extract in *n*-hexane
ILRM	*Indigofera linifolia* Root Extract in MeOH:H_2O (70:30)
ILSH	*Indigofera linifolia* Stem Extract in *n*-hexane
ILSM	*Indigofera linifolia* Stem Extract in MeOH:H_2O (70:30)
IR	Infrared Spectroscopy
ITP	Immune Thrombocytopenia
LC-MS	Liquid Chromatography Mass Spectrometry
LD_{50}	50% Lethal Dose
LM2	Breast Adenocarcinoma
LOX	Lipoxygenase

LP07	Lung Adenocarcinoma
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MCV	Mouse Corona Virus
MeOH	Methanol
MES	Maximal Electric Shock Models
MIC	Minimum Inhibitory Concentration
MS/MS	Tandem Mass Spectrometry
MTT	Thiazolyl Blue Tetrazolium Bromide salt assay
MW	Molecular Weight
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
OGL	Oral Glucose Loaded
PDT	Photo-Dynamic Therapy
PHP	Polyherbal Preparation
PMR	Proton Magnetic Resonance
Py-silylation	Pyridine silylation
RNA	Ribonucleic Acid
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SK-OV-3	Human Ovarian Cancer Cell Line
TBARS	Thio-Barbituric Acid Reactive Species
TCA	Trans-tetracos-15-enoic acid
UHPLC-MS	Ultra High-Performance Liquid Chromatography Mass Spectrometry
UV	Ultraviolet spectroscopy
VLC	Vacuum Liquid Chromatography

List of Figures

List of Schemes

List of Tables

Abstract

Indigofera is a genus of about 800 plant species that belong to family Fabaceae. Literature survey revealed that numerous structurally diverse organic compounds have been identified in plants of this genus. Several biological activities of immense importance have also been reported as shown by the extracts of these plants.

However, *Indigofera linifolia* was observed as an under-investigated plant of this genus as no previous report was found regarding its phytochemistry. The plant has been associated with cure of various ailments and has great ethnobotanical significance. The present study was carried out to have an insight into the types of phytoconstituents present in *Indigofera linifolia*.

The structural identification of phytoconstituents was carried out by interpretation of data obtained from HPLC-DAD-ESI/MS and GCMS analysis of plant extracts. These are advanced and highly sensitive mass spectrometric techniques commonly used for analytical purposes in research and industry now-a-days. Moreover, a protocol was also established for the isolation of flavonoid from leaves of *Indigofera linifolia*.

Hydromethanolic (MeOH:H_2O 70:30) extracts of various parts (seeds, stem, leaves and roots) of *Indigofera linifolia* were analyzed by HPLC-DAD-ESI/MS. The analyses gave fingerprint chromatograms of these extracts. Moreover, 10 hydrophilic organic constituents were tentatively identified by combining information obtained from retention time, DAD-spectrum, and mass spectrum of each eluent in HPLC. The identified compounds included glycosidic derivatives of coumaric acid, caffeic acid, quinic acid, isoflavone and flavonol.

Fingerprint GC-TIC profiles of *n*-hexane extracts of different parts (seeds, stem, leaves and roots) were obtained by GC-MS analysis. Interpretation of mass spectra led to the identification of various lipophilic constituents present in the plant. It was revealed that methyl hexadecanoate was present in seeds and roots while ethyl hexadecanoate was identified in stem and roots of the plant.

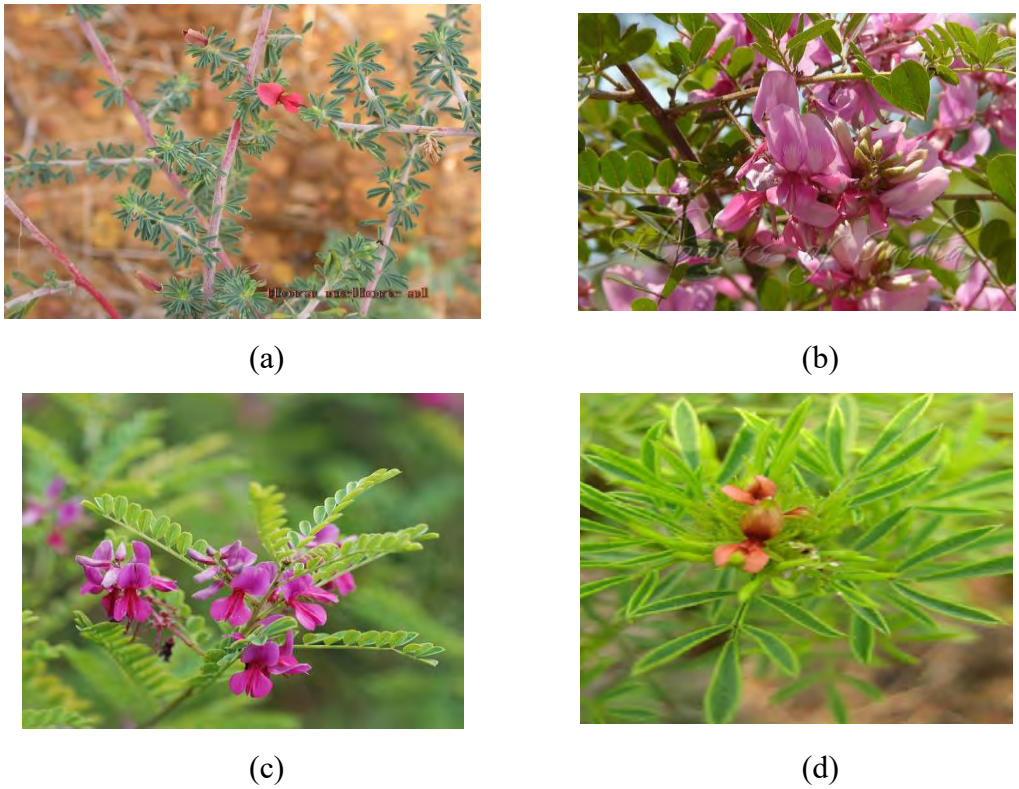
This study constitutes the first report about various phytochemical constituents present in *Indigofera linifolia.*

Chapter 1

Introduction and Literature Review of Genus *Indigofera*

1.1. Introduction

The genus *Indigofera* belongs to family Fabaceae or Leguminosae. It encompasses about 800 species of plants distributed worldwide in the tropical and subtropical areas (**De Kort I. and Thijsse G. 1984)**. Some of the important species of this genus are *I. spinosa* Forssk, *I. suffruticosa* Mill., *I. tinctoria* L., *I. truxellensis* Kunth., *I. zollingeriana* Miq., *I. hebepetala* Benth., *I. jocunda* Schrire, *I. kirilowii* Maxim., *I. lespedezoides* Kunth, *I. longiracemoza* Boivin, *I. microcarpa* Desv., *I. oblongifolia* Forssk., *I. peniculata* Vahl, *I. coerulia* Roxb. and *I. cordifolia* Heyne (**Schrire *et al.*, 2003)**. The morphology of four of the species of genus *Indigofera* has been depicted in Figure 1.1.



(a) (b)

(c) (d)

Figure 1.1. Some important species of genus *Indigofera* (a) *Indigofera aspalathoides* (b) *Indigofera heterantha* (c) *Indigofera tinctoria* (d) *Indigofera pulchra*

1.2. Ethnobotanical significance

There are noteworthy reports regarding the ethnobotanical significance of plants belonging to this genus. For instance, *Indigofera spicata* was orally taken to cure

tonsillitis, evil eye ascariasis, diarrhea, and stomach-ache by people of southern Ethiopia (**Kidane *et al.*, 2014**).

Teklehaymanot (**2017**) also described the different uses of plants of genus *Indigofera* in Ethiopia. Various parts of *Indigofera articulate* Gouan were famous for treatment of jaundice, epilepsy, fire-burn, dyspepsia, epistaxis, snake bite and brucellosis. *Indigofera oblongifolia* Forssk. was used to cure diphtheria, typhoid, herpes zoster, scorpion bite, lung infection, dysentery, breast cancer, angina and pastuerollosis (cattle). The root of *Indigofera spicata* Forssk. was used as body wash or taken through nasal passage to treat jaundice.

According to a survey report, plants of this genus were extensively used by people in past for treatment of malaria (**Asase *et al.*, 2009**). **Chellappandian *et al.*, (2012**) concluded from a quantitative ethnobotanical survey that *Indigofera aspalathoides* was famous for treatment of dermal diseases. *Indigofera heterantha* has been reported to be well known to cure jaw swelling and toothache (**Rahman *et al.*, 2016**).

Al-Fatimi (**2019**) also mentioned ethnopharmacological uses of some plants of genus *Indigofera.* The powdered leaves of *Indigofera articulate* Gouan. were mixed in water and applied on hair to prevent hair fall. Similarly, crushed paste of its leaves was applied on skin to cure inflammation. An infusion of *Indigofera oblongifolia* Forssk. was thought to be effective against kidney stones and urine retention. A paste of its leaves was used to cure eye infections and stem decoction was considered as a source of recovery from diarrhea, gastritis, and abdominal pain. It also cured cough and flu. Infusion of leaves of *Indigofera spinosa* Forssk. was famous to treat urinary tract diseases and respiratory tract ailments like cough and cold. The whole plant was used to treat skin burn. The leaves of *I. spinosa* Forssk. have also been considered as ophthalmic antiseptic agents. Leaves of *Indigofera tinctoria* L. have been reported to be used as remedy against pimples and boils.

1.3. Phytochemistry

1.3.1. Aliphatic alcohols, acids, and esters

The petroleum ether extract of aerial parts of *Indigofera tinctoria* Linn. was fractionated with acetone. This acetone fraction was passed over silica gel column followed by elution with petroleum ether and ethyl acetate (94:4-94:6). A yellow

fraction was obtained which was again subjected to flash column chromatography. Finally, one of the fractions gave a colorless crystalline compound upon recrystallization in methanol. Physical properties and NMR spectroscopy of the compound showed that it was a carboxylic acid with 24 carbon atoms. A trans olefinic bond was present at C_{15} of the main chain (**Singh *et al.*, 2006**). The compound was named as trans-tetracos-15-enoic acid (**1**) (Figure 1.2).

The ethanol extract of leaves of *Indigofera trita* also known as *Indigofera subulata* Vahl. was subjected to GC-MS analysis. A total of fourteen compounds were identified in the plant extract by the aid of their retention times and mass spectra. Two of them (**2**) and (**3**) belonged to the class of carboxylic acids with 18 C long chain while five (**4**-**8**) were aliphatic esters of carboxylic acids having 16 or 18 C long chain **Vinoth *et al.*,** (**2011**) (Figure 1.2).

The GCMS analysis of hydroalcoholic and chloroform extracts of *I. cordifolia* seeds led to the identification of aliphatic carboxylic acids (**9**), (**10**) and (**11**) (**Deshpande *et al.*, 2013**) (Figure 1.2).

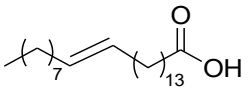
The *n*-hexane extract of *Indigofera aspalathoides* was repeatedly chromatographed using silica gel as stationary phase**.** As a result, an ester of nonadecanoic acid (**12**) and an aliphatic alcohol of 28 C chain length (**13**) was obtained (**Saraswathy *et al.*, 2013**) (Figure 1.2).

Arriaga *et al.*, (**2013**) worked on the ethanol extract of leaves of *Indigofera suffruticosa* and identified an aliphatic unsaturated acid named linoleic acid (**14**). In addition, two aliphatic esters namely, methyl linoleate (**15**) and methyl hexa-decanoate (**16**) were also identified in the extract (Figure 1.2).

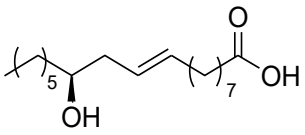
Rahman *et al.*, (**2014a**) worked on the 5% aqueous methanol extract of *I. heterantha* Wall. seeds. A white crystalline compound having molecular formula $C_{32}H_{63}O_2$ as indicated by EIMS was isolated by column chromatography using silica gel as stationary phase. The compound was structurally elucidated as an aliphatic carboxylic acid (**17**) (Figure 1.2).

Rahman *et al.*, (**2014b**) successfully isolated a compound from the 5% aqueous methanol extract of seeds of *Indigofera heterantha.* The molecular formula was found to be $C_{35}H_{70}O_2$. IR analysis confirmed the presence of an ester functionality in the

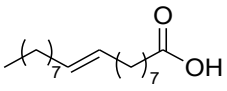
molecule. ^{1}H NMR and ^{13}C NMR studies including BB, DEPT and HMBC correlation studies confirmed the significant structure of the molecule as (**18**) (Figure 1.2).



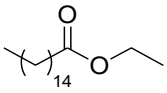
trans-Tetracos-15-enoic acid (**1**)



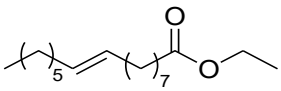
Ricinoleic acid (**2**)



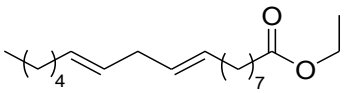
Oleic acid (**3**)



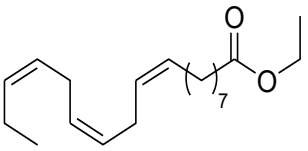
Ethyl hexadecanoate (**4**)



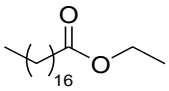
Ethyl hexadec-9-enoate (**5**)



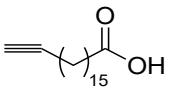
Ethyl 9,12-octadecadienoate (**6**)



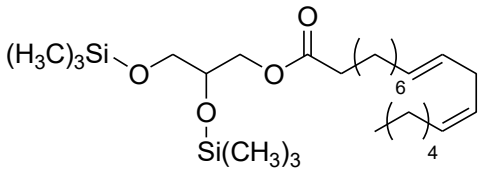
Ethyl 9,12,15-octadecatrienoate (**7**)



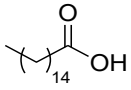
Ethyl octadecanoate (**8**)



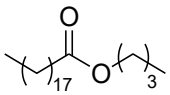
17-Octadecynoic acid (**9**)



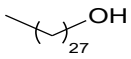
1-Monolinoleoyl glycerol trimethylsilyl ether (**10**)



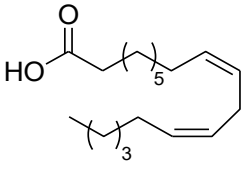
Hexadecanoic acid (**11**)



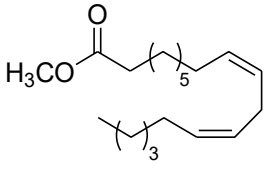
Butyl nonadecanoate (**12**)



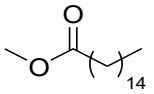
1-Octacosanol (**13**)



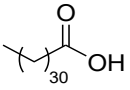
Linoleic acid (**14**)



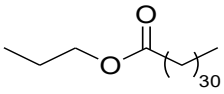
Methyl linoleate (**15**)



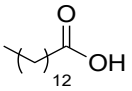
Methyl hexadecanoate (**16**)



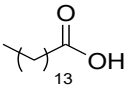
Dotriacontanoic acid (**17**)



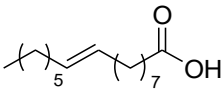
Propyldotriacontanoate (*Indigofera*te) (**18**)



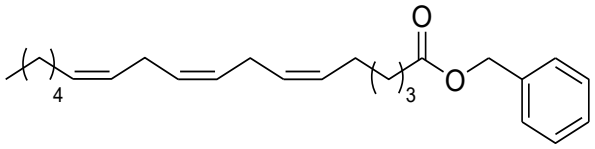
Myristic acid (**19**)



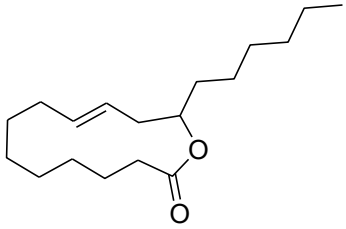
Pentadecanoic acid (**20**)



Palmitoleic acid (**21**)



(6*Z*,9*Z*,12*Z*)-Benzyl octadecatrienoate (**22**)



13-Hexyloxacyclotridec-10-en-2-one (**23**)

Figure 1.2. Aliphatic alcohols, acids, and esters identified in genus *Indigofera*

Yuldasheva *et al.,* (**2016**) investigated the content of different classes of lipids present in seeds of *Indigofera tinctoria.* The study revealed that tri-acyl-glycerides were predominant with 59.89% by mass composition. Both the free fatty acids and tri terpinols came out to be 1.48% each. Similarly, phospholipids, glycolipids and sterols were 11.8, 16.6 and 6.21 % respectively.

Theresa *et al.,* (**2017**) studied the effect of solvent, ultrasonication, moisture content, particle size, temperature, time, and solvent to solid ratio on the yield of oil from roots of *Indigofera colutea.* The optimum conditions for maximum yield were 6% moisture content, 0.15 mm particle size, 65°C temperature, 210 min time duration, and 6:1 solvent-to-solid ratio using *n*-hexane as solvent. The phytochemistry of oil was investigated by FTIR and GCMS analysis. Five fatty acids present in the oil were identified as myristic acid (**19**), pentadecanoic acid (**20**), palmitic acid, palmitoleic acid (**21**) and oleic acid. Oleic acid (**3**) was previously identified by **Vinoth *et al.,*** (**2011**) and palmitic acid or hexadecanoic acid (**11**) by **Deshpande *et al.,*** (**2013**) (Figure 1.2).

Prashanth *et al.,* (**2018**) identified an aliphatic ester in the aqueous extract of *Indigofera tinctoria* leaves using the technique of GC-MS. The ester was a derivative of Octadecatrienoic acid (**22**) (Figure 1.2).

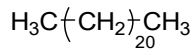
1.3.2. Alicyclic ester

Vinoth *et al.,* (2011) analyzed the ethanol extract of *Indigofera subulata* Vahl. by GC-MS. One of the fourteen identified compounds had MW= 280. Retention time and mass spectrum of the compound matched with standard GC-MS data of 13-hexyloxacyclotridec-10-en-2-one in the NIST library. It is a cyclic ester (**23**) with a double bond at C_{10} and a 6 C long chain present as substituent at C_{13} (Figure 1.2).

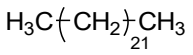
1.3.3. Alkanes and alkenes

Eight types of alkanes (**24-31**) (Figure 1.3) were identified by **Arriaga *et al.,* (2013)** in the ethanol extract of *Indigofera suffruticosa* leaves. The qualitative analysis of extract was carried out by using GC-MS analysis.

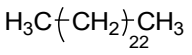
The chloroform and hydro-methanolic extracts of *Indigofera cordifolia* seeds were prepared by Soxhlet extraction. GC-MS analysis revealed the presence of a 16 C alkene (**32**) in chloroform extract **Deshpande *et al.,* (2013)**.



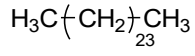
Docosane (**24**)



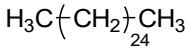
Tricosane (**25**)



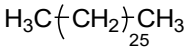
Tetracosane (**26**)



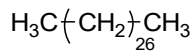
Pentacosane (**27**)



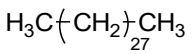
Hexacosane (**28**)



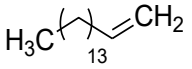
Heptacosane (**29**)



Octacosane (**30**)



Nonacosane (**31**)



1-Hexadecene (**32**)

Figure 1.3. Alkanes and alkenes identified in genus *Indigofera*

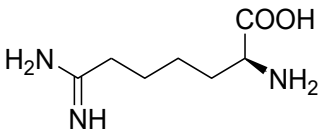
1.3.4. Amino acids and their derivatives

Hegarty and Pound (1970) isolated an amino acid from the aqueous extract of seeds and leaves of *I. spicata* by using preparative paper chromatography on Whatman 3 mm paper. This amino acid of 7 C long chain was indospicine (**33**) (Figure 1.4) which is non-proteinogenic and characteristically present in plants of *Indigofera* genus only.

GC-MS analysis of *Indigofera subulata* Vahl. leaf extract prepared in ethanol led to the identification of an amino acid derivative (**34**) (Figure 1.4) in the extract. The

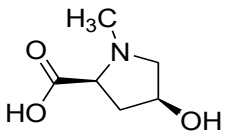
compound (**34**) was proline which was substituted by a methyl group on nitrogen atom and a hydroxyl group at C_4 of the five membered ring (**Vinoth *et al.*, 2011**).

The aqueous and methanol extracts of aerial parts of *Indigofera hirsuta* were analyzed by using HPLC-ESI-MS. An *α*-amino acid with indole as side chain was identified (**Moura *et al.*, 2011**). The amino acid was tryptophan (**35**) (Figure 1.4).

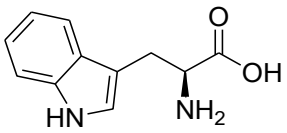


Indospicine

(**33**)



(2*S*,4*S*)-4-Hydroxy-1-methylpyrrolidine-2-carboxylic acid (**34**)



Tryptophan

(**35**)

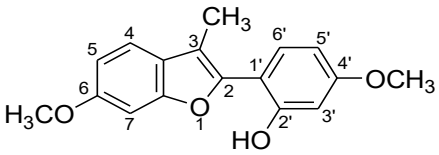
Figure 1.4. Amino acids and their derivatives identified in genus *Indigofera*

1.3.5. Benzofurans

The methanol extract of leaves of *Indigofera microcarpa* was defatted with *n*-hexane followed by solvent extraction with ethyl-acetate. Evaporation of ethyl-acetate gave a brown colored solid which was set to column chromatography over silica gel. Recrystallization successfully led to the isolation of two compounds. Spectroscopic studies by IR, HRMS, UV and ^{1}H NMR for structural characterization revealed that both the compounds contained a benzofuran moiety. In addition, a phenyl ring (substituted by OH and OCH_3 group) was attached to the furan ring of benzofuran unit. The only difference in structure of these two compounds was that benzene ring of benzofuran in (**36**) was substituted by a methoxy group while (**37**) (Figure 1.5) had di-oxy-methylene group (**E Souza *et al.*, 1988**).

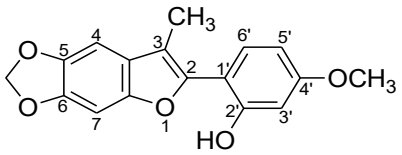
So, literature review indicated that leaves of *I. microcarpa* are a source of two types of 2-arylbenzofurans. Compounds containing benzofuran skeleton are highly valuable in medicinal chemistry due to useful pharmacological activities including antiviral (**Du *et al.*, 2017**), anti-inflammatory (**Baumgartner *et al.*, 2011**), and anticancer activities (**Katsanou *et al.*, 2007**). The work of **E Souza *et al.*, (1988**) also

showed that leaves, and other parts of *I. microcarpa* can be investigated for the presence of more benzofuran derivatives.



2-(2′-Hydroxy-4′-methoxyphenyl)-3-methyl-6-methoxybenzofuran

(**36**)



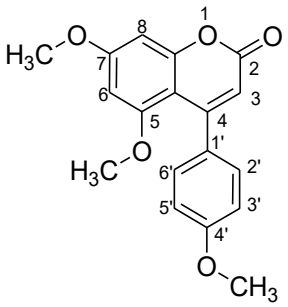
2-(2′-Hydroxy-4′-methoxyphenyl)-3-methyl-5,6-dioxymethyl-ene-benzofuran

(**37**)

Figure 1.5. Benzofurans identified in genus *Indigofera*

1.3.6. Coumarins

Lodha *et al.*, (1998) isolated a constituent from the roots of *Indigofera oblongifolia.* Spectroscopic study showed that the constituent possessed basic coumarin skeleton substituted with methoxylated phenyl ring at position 4. The compound was identified as 5, 7, 4′-trimethoxy-4-phenylcoumarin (**38**) (Figure 1.6).



5,7,4′-trimethoxy-4-phenylcoumarin (**38**)

Figure 1.6. Coumarin identified in genus *Indigofera*

Studies on the coumarins isolated from various natural sources showed them potential candidates for use as lead compounds for drug development due to useful biological activities (**Pinto *et al.,* 2017**), **(Silván *et al.*, 1996), (Widelski *et al.,* 2009**).

1.3.7. Flavonoids

The whole plant of *Indigofera suffruticosa* was extracted with petroleum ether followed by percolation of extract over silica gel column. This compound which was recrystallized as yellowish needles had molecular formula $C_{22}H_{24}O_{50}$. Its structure was elucidated by IR, UV, PMR, C^{13}-NMR, MS and further confirmed through X-ray diffraction. There were three rings forming phenyldihydro-1,4-pyrone unit which is a structural characteristic of all flavanones. The unique feature of this compound was a five membered ring containing ether functionality. It was named as louisfieserone (**39**)

(Figure 1.7). **Domínguez *et al.,* (1978a)**. This work was also reported by **Dominguez *et al.,* (1978b)**.

Hasan *et al.,* (1994) identified the presence of four glycosylated flavonoids (**40-43**) (Figure 1.7) from ethyl acetate fraction of 85% methanolic extract of *Indigofera hebepetala* leaves. The dried ethyl acetate extract was dissolved in methanol and fractionated by 2D-PC (Whatmann No. 1) using BAW (*n*-butanol-acetic acid-water, 4:1:5) and 15% acetic acid as mobile phase. As a result, flavonoid spots were obtained which were eluted and then purified by 2D-TLC (polyamide 6F). The four compounds were analyzed by their R_f values on TLC, UV absorptions using shift reagents and spot color under UV in the absence and presence of NH_3. Acid hydrolysis was carried out with 2N HCl for mono-glycosides and 0.1 N H_2SO_4 for partial hydrolysis of di-glycosides. Thus, sugars were co-chromatographed with standards for identification. The aglycone was kaempferol while allose, arabinose and rhamnose were detected as sugars. Spectroscopic analysis revealed the structure of compounds.

Hasan *et al.,* (1996) isolated two novel flavonol glycosides (**44** and **45**) (Figure 1.7) by working on the *n*-butanol extract of *I. hebepetala* flowers. One of the glycosides was kaempferol-3-*O*-*α*-L–rhamnopyranosyl-(1→2)-*β*-D-galactopyranoside-7–*O*-*α*-L-arabinofuranoside (**44**) and the other was kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-galacto-pyranoside-7-*O*-*α*-L-ribofuranoside (**45**). In addition, two more flavonoid glycosides (**40, 41**) which were previously isolated from *Indigofera hebepetala* leaves by **Hasan *et al.,* (1994)** were again isolated from flowers of this plant. Another known compound but first time isolated from this genus was identified and named as kaempferol-7-rhamnoside (**46**).

Selvam *et al.,* (2004) isolated a flavonoid from the chloroform extract of *Indigofera aspalathoides.* The overall structure was elucidated by the help of NMR data and the compound was named as mucronulatol (**47**) (Figure 1.7).

The ethyl acetate extract of *Indigofera heterantha* yielded two novel flavonoids named as hetranthin A (**48**) and hetranthin B (**49**). Two additional compounds (**50** and **51**) were also isolated which were previously known but first time isolated from genus *Indigofera* (**Rehman *et al.,* 2004**) (Figure 1.7).

A novel isoflavone was isolated from the whole plant of *Indigofera linnae.* Spectroscopic studies showed the presence of a methylenedioxy moiety in the

isoflavone. The overall structure was interpreted as 7, 8-methylenedioxy-4′-methoxyisoflavone (**52**) (**Prasad *et al.*, 2004**).

The column chromatography of chloroform soluble fraction of ethanol extract of *Indigofera heterantha* led to the isolation of five flavonoids (**53-57**) and a flavonoid arabinoside (**58**) (Figure 1.7) which were identified by spectroscopic techniques (**Rehman *et al.*, 2005b**).

The ethanol extract of aerial parts of *Indigofera tinctoria* was fractionated with chloroform and *n*-butanol successively. The chloroform soluble fraction was passed through column packed with silica gel and eluted with different solvents. As a result, three flavonoids (**60**, **61** and **62**) were isolated. Similarly, column chromatography of butanol fraction yielded a flavonoid glycoside (**59**) (**Narender *et al.*, 2006**) (Figure 1.7).

Prakash *et al.*, (2007) identified two flavonoids in the 50 % aqueous methanol extract of flowers of *Indigofera tinctoria* by using HPLC and MS/MS method. The identified flavonoids were kaempferol (**63**) and quercetin (**64**). A flavonoid glycoside named rutin (**65**) was also identified in this extract (Figure 1.7).

A yellow amorphous powder was obtained from the ethyl-acetate soluble fraction of 95% alcoholic extract of *Indigofera aspalathoides* stems. The compound was identified as 5, 4′-dihydroxy-6, 8-dimethoxy-7-*O*-rhamnosyl flavone (**66**) (Figure 1.7) by NMR and EIMS analysis (**Balasubramanian *et al.*, 2007)**.

The ethyl acetate soluble fraction of methanol extract of aerial parts of *Indigofera pulchra* Willd. was subjected to column chromatography over silica gel. As a result, a yellow amorphous compound (**67**) (Figure 1.7) was obtained. NMR and mass spectrometric study showed that it was a chalcone with two free hydroxyl groups and a prenyloxy group as substituents (**Musa *et al.*, 2011**).

The repeated column chromatography of 1-butanol soluble fraction of methyl alcohol extract of *I. zollingeriana* leaves yielded two novel flavonol glycosides (**68** and **69**). One of the glycosides was identified as 3-*O*-*β*-D-(2″-*O*-*β*-D-apiofuranosyl) glucopyranoside-7-*O*-*α*-L-rhamnopyranoside (**68**) and the other was 3-*O*-*β*-D-(2″-*O*-*β*-D-apiofuranosyl-6″-*O*-*α*-L-rhamnopyranosyl)glucopyranoside-7-*O*-*α*-L-rhamnopyranoside (**69**). Four additional glycosides (**70**-**73**) (Figure 1.7) which were

previously known but new to the genus were also isolated by this work (**Hisaeda *et al.*, 2011**).

Ahmadu *et al.*, (2011) worked on the acetone extract of *I. secundiflora.* The extract was subjected to column chromatography over silica gel. Re-chromatography of obtained fractions led to the isolation of three flavonoids. NMR study showed that one of the flavonoids was quercetin (also isolated by **Prakash *et al.*, 2007**) and the other two were (**74**) and (**75**) (Figure 1.7).

Moura *et al.*, (2011) made a study on the water and methanol extracts of *I. hirsuta.* A flavonoid glycoside was identified by using the technique of HPLC-ESI-MS. The aglycone part was a kaempferol attached to a glucose moiety through glycosidic linkage (**76**) (Figure 1.7).

The 80% ethanol extracts of various parts and callus tissues of *Indigofera linnae* and *Indigofera cordifolia* were prepared by using the technique of Soxhlet extraction. Three flavonoids in each case, were isolated by chromatography of ethyl-acetate soluble fraction of ethanol extract while identified by R_f values, melting points, IR, and UV spectroscopic analysis. The flavonoids were apigenin (**77**), kaempferol (**63**) and quercetin (**64**). **Upman *et al.*, (2011)**. Kaempferol and quercetin have already been identified in *Indigofera tinctoria* (**Prakash *et al.*, 2007**) (Figure 1.7).

The ethanolic and aqueous extracts of *Indigofera suffruticosa* were qualitatively and quantitatively analyzed by using HPLC/MS technique. Quercetin (**64**), isoliquiritigenin (**78**) and formononetin (**79**) (Figure 1.7) were identified in both the extracts. However, ethanol extract was richer in these constituents (**Chen *et al.*, 2013**).

Qiu *et al.*, (2013) worked on the ethanol extract of roots of *Indigofera stachyoides.* The ethyl acetate soluble fraction was subjected to repeated column chromatography leading to the isolation of two flavan dimers (**80** and **81**). In addition, a flavonoid having 3-nitropropanoyl moiety (**82**) (Figure 1.7) as substituent was also isolated.

The methanol extract of various parts of *Indigofera spicata* Forssk**.** was prepared and fractionated between *n*-hexane and chloroform. The chloroform soluble fraction was subjected to chromatography. This led to the isolation of seven flavonoids (**83-89**) (Figure 1.7) (**Bueno P. rez *et al.*, 2013**).

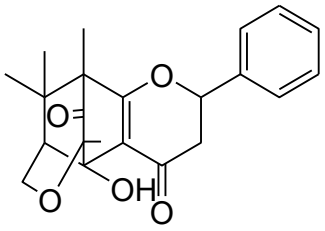
Rahman *et al.,* (**2014a**) worked on the aqueous methanol extract of *Indigofera heterantha* Wall seeds. They succeeded in the isolation of two flavonoids which were identified as quercetin (**64**) and formononetin (**79**) (Figure 1.7). In a previous report, these compounds were identified by **Chen *et al.,*** (**2013**) in *Indigofera suffruticosa.*

Rahman *et al.,* (**2014b**) isolated a tetrasubstituted flavonoid from the 5% aqueous methanol extract of *Indigofera heterantha* seeds using column chromatography. This flavonoid was identified as norartocarpetin (**90**). In addition, a light-yellow compound was also obtained. This compound was a flavonoid which possessed substitution of three hydroxyls and two methoxy groups. It was named as 3, 5, 7-trihydroxy-6, 4′-dimethoxyflavone (**91**). A third compound was also yielded which was identified as 3, 5, 4′-trihydroxy-6, 7-dimethoxyflavone (**92**) (Figure 1.7). The spectral data of later two compounds has also been discussed by **Rehman *et al.,*** (**2016**).

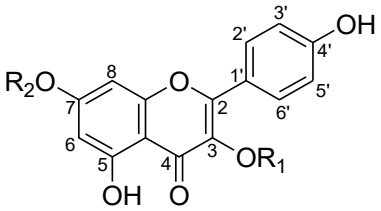
The column chromatography of ethyl-acetate soluble fraction of methanol extract of *Indigofera aspalathoides* yielded three compounds. Spectroscopic data showed that the compounds were kaempferol (**63**), quercetin (**64**), and kaempferol-5-*O*-*β*-D-glucopyranoside (**93**) (Figure 1.7) (**Swarnalatha *et al.,* 2015**). Quercetin was previously isolated from *Indigofera heterantha* Wall. (**Rahman *et al.,* 2014a**). Later, **Bhaskar *et al.,*** (**2016**) isolated this compound from the methanol extract of whole plant of *I. barberi.*

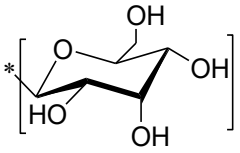
The 5% hydro-methanolic extract of seeds of *Indigofera heterantha* was studied by **Rahman *et al.,*** (**2015**). The extract was fractionated between different solvents and ethyl-acetate soluble fraction was subjected to repeated chromatography over silica gel. An isoflavone (**94**) (Figure 1.7) was isolated which was substituted by an OH group at 7 C and an OCH_3 group at 3′ C.

A flavonol glycoside (**95**) (Figure 1.7) was isolated from the hydro-methanolic extract of *Indigofera tinctoria.* It was found hydroxylated at carbon number 3 and 4 while methoxylated at 3′, 4′ and 7′ positions. The overall structure was elucidated, and compound was named as 3, 6-dihydroxy-(3′, 4′, 7′-trimethoxyphenyl)-chromen-4-one-7-glucoside (**Agarwal and Sharma 2017**).

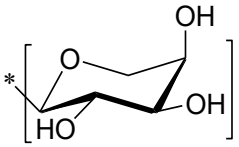


Louisfieserone (**39**)

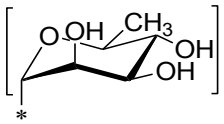




All = Allose

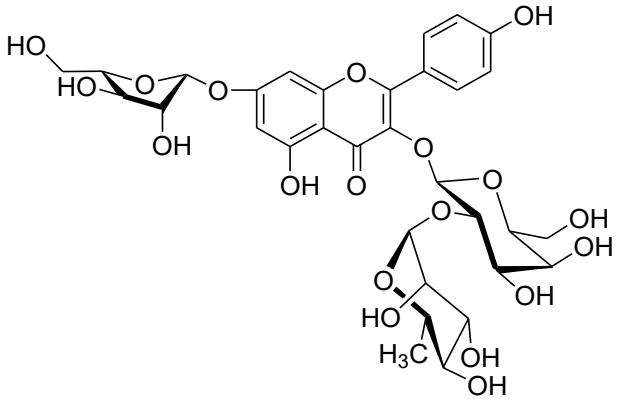


Ara = Arabinose

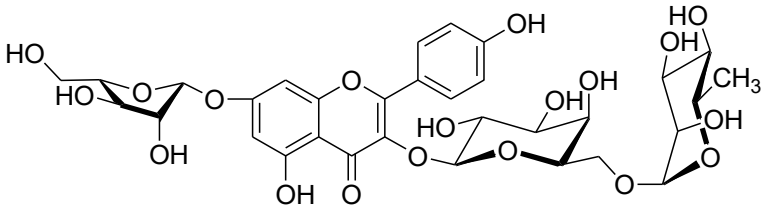


Rha = Rhamnose

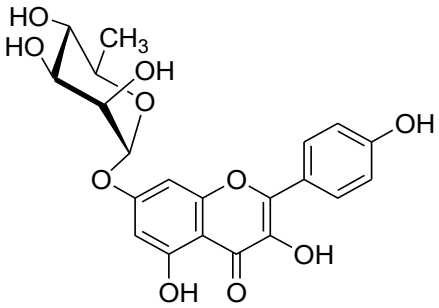
Compound name	R_1	R_2
(**40**) Kaempferol-7-alloside	H	All
(**41**) Kaempferol-3,7-diarabinoside	Ara	Ara
(**42**) Kaempferol-3-arabino-7-rhamnoside	Ara	Rha
(**43**) Kaempferol-7-arabino-3-rhamnoside	Rha	Ara



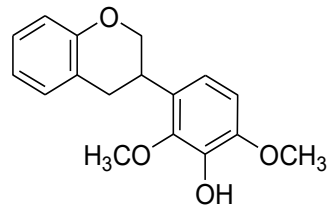
Kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-galactopyranoside-7-*O*-*α*-L-arabinofuranoside (**44**)



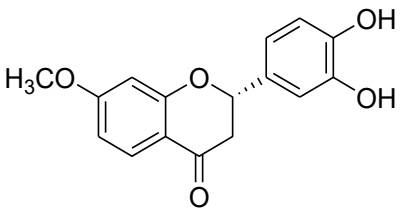
Kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-galacto-pyranoside-7-*O*-*α*-L-ribofuranoside (**45**)



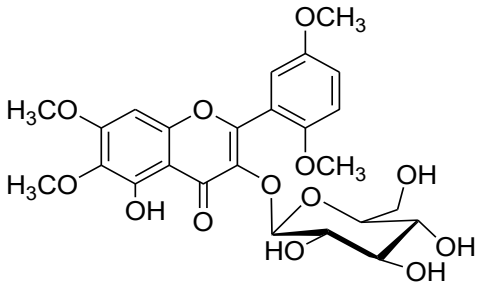
Kaempferol-7-rhamnoside (**46**)



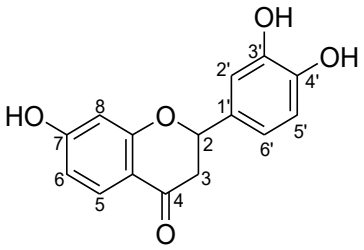
Mucronulatol (**47**)



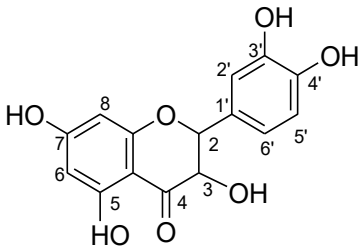
Hetranthin A (**48**)



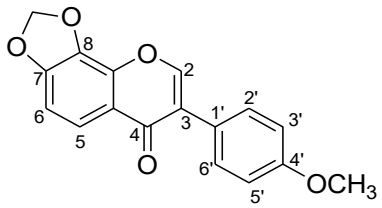
Hetranthin B (**49**)



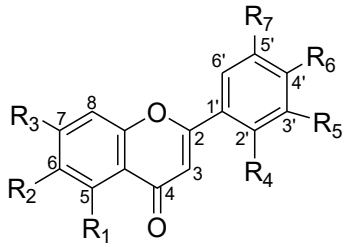
7,3′,4′-Trihydroxyflavanone (**50**)



3,5,7,3′,4′-Pentahydroxyflavanone (**51**)



7,8-Methylenedioxy-4′-methoxyisoflavone (**52**)

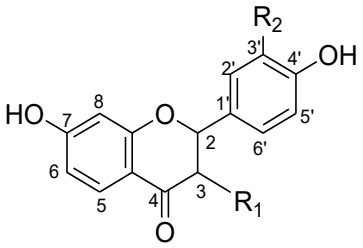


(**53**) 4′-Hydroxy-5,7,3′-trimethoxyflavone

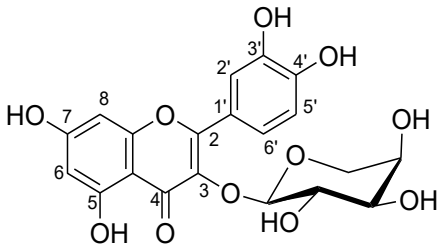
(**54**)5-Hydroxy-2′,4′,5′,7-tetramethoxyflavone

(**55**)3′,5-Dihydroxy-4′,6,7-trimethoxyflavone

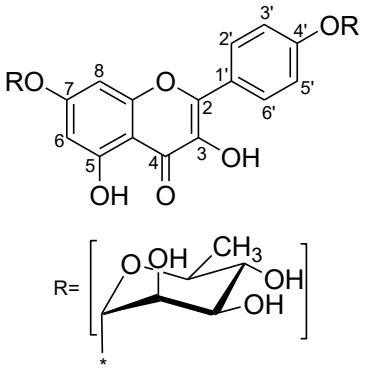
	R_1	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**	**R_7**
	(5)	**(6)**	**(7)**	**(2′)**	**(3′)**	**(4′)**	**(5′)**
(53)	OCH_3	H	OCH_3	H	OCH_3	OH	H
(54)	OH	H	OCH_3	OCH_3	H	OCH_3	OCH_3
(55)	OH	OCH_3	OCH_3	H	OH	OCH_3	H



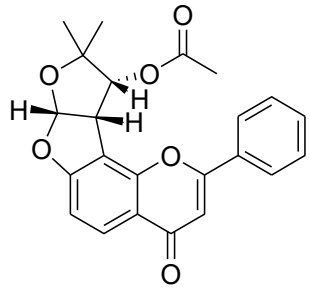
Compound name	**R_1**	**R_2**
56) 4′,7-Dihydroxy-3′-methoxyflavanone	H	OCH_3
57) 3,3′,4′,7-Tetrahydroxyflavanone	OH	OH



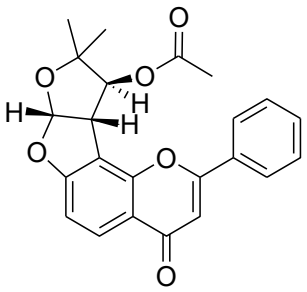
3′,4′,5,7-Tetrahydroxyflavone 3-*O*-*α*-L-arabinoside (**58**)



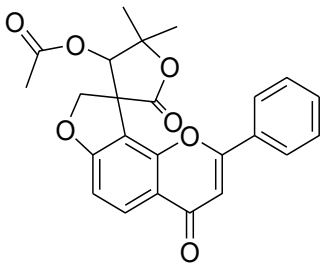
Kaempferol-7,4′-dirhamnoside (**59**)



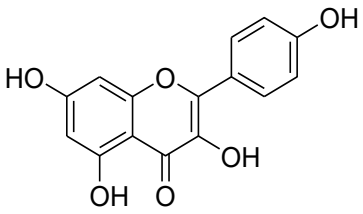
Semiglabrin (a) (**60**)



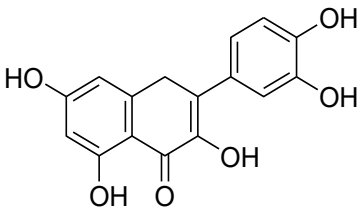
Semiglabrin (b) (**61**)



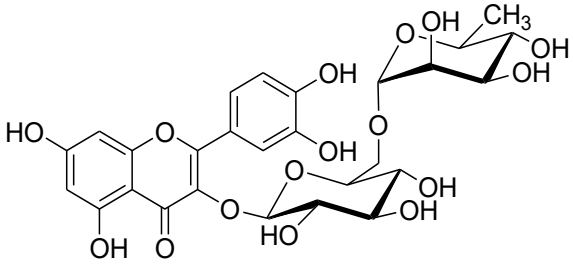
Apollinine (**62**)



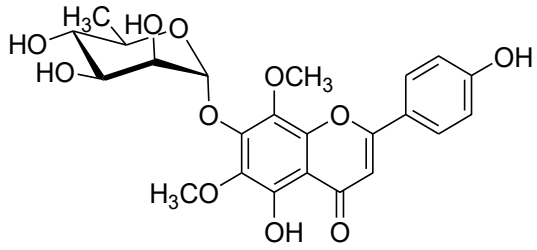
Kaempferol (**63**)



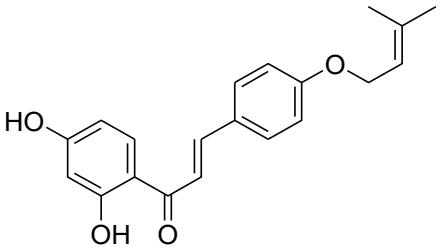
Quercetin (**64**)



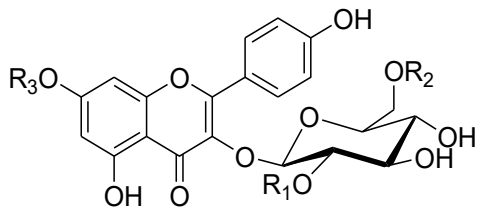
Rutin (**65**)

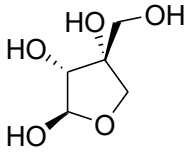


5,4′-Dihydroxy-6,8-dimethoxy-7-*O*-rhamnosyl flavone (**66**)

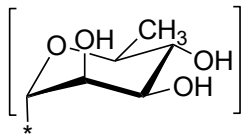


2′,4′-Dihydroxy-4-prenyloxy chalcone (**67**)



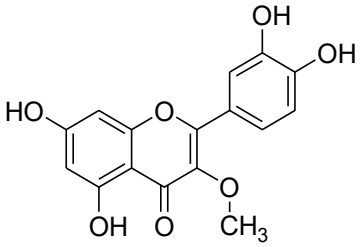


Api= Apiofuranosyl

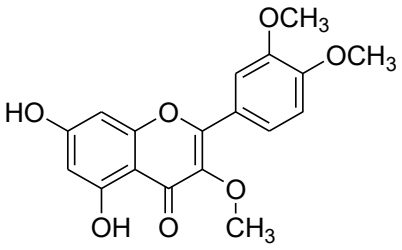


Rha= Rhamnopyranosyl

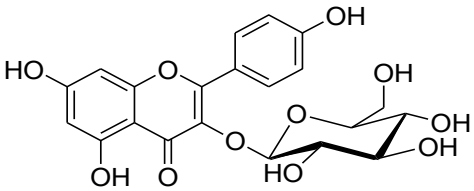
Compound name	R_1	R_2	R_3
(**68**) Kaempferol-3-*O*-*β*-D-(2″-*O*-*β*-D-Apiofuranosyl) glucopyranoside-7-*O*-*α*-L-rhamnopyranoside	Api	H	Rha
(**69**) Kaempferol-3-*O*-*β*-D-(2″-*O*-*β*-D-Apiofuranosyl-6″-*O*-*α*-L-rhamnopyranosyl) glucopyranoside-7-*O*-*α*-L-rhamnopyranoside	Api	Rha	Rha
(**70**) Kaempferol-3-*O*-glucoside-7-*O*-*α*-L-rhamnoside	H	H	Rha
(**71**) Kaempferol-3-*O*-rutinoside	H	Rha	H
(**72**) Kaempferol-3-*O*-*(2″-O-β*-D-apiofuranosyl) rutinoside	Api	Rha	H
(**73**) Kaempferol 3-*O*-rutinoside-7-*O*-*α*-L-rhamonoside	H	Rha	Rha



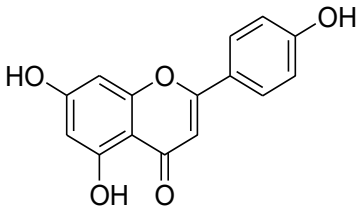
Quercetin 3-*O*-methylether (**74**)



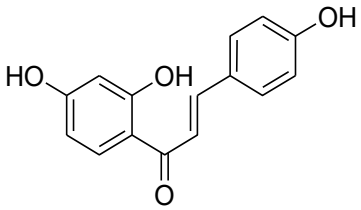
Quercetin-3,3′,4′-trimethylether (**75**)



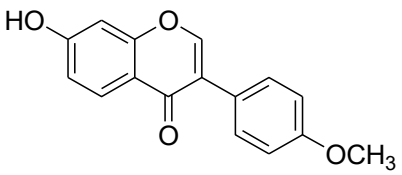
Kaempferol-3-*O*-*β*-D-glucopyranoside (**76**)



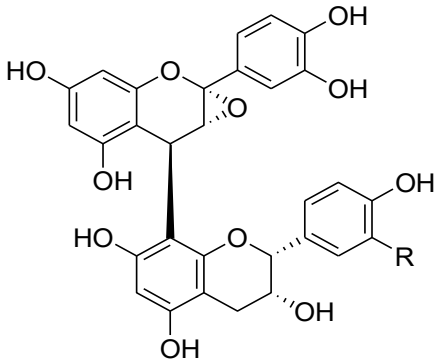
Apigenin (**77**)



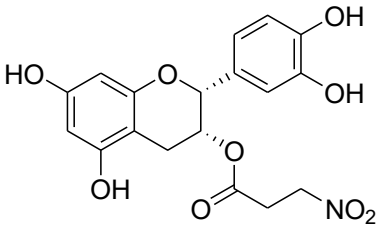
Isoliquiritigenin (**78**)



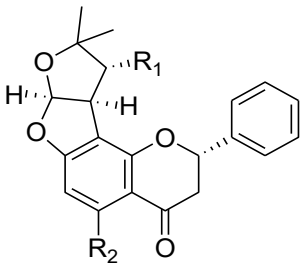
Formononetin (**79**)



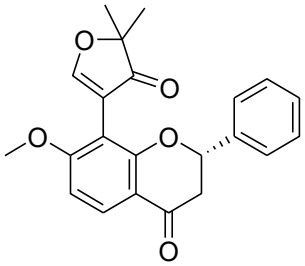
Compound name	R
(**80**) 2*α*,3*α*-Epoxyflavan-5,7,3′,4′-tetraol-(4*β*→8)-flavan-3″,5″,7″,4‴-tetraol	H
(**81**) 2*α*,3*α*-Epoxyfavan-5,7,3′,4′-tetraol-(4*β*→8)-epicatechin	OH



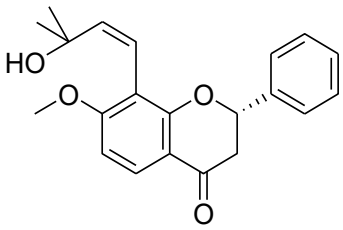
3-*O*-(3-Nitropropanoyl)-2,3-cis-5,7,3′,4′-tetrahydroxyflavan (**82**)



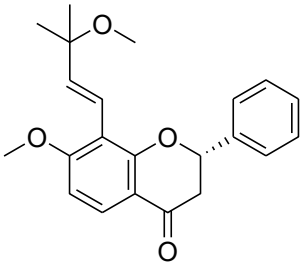
Compound No.	R_1	R_2
(**83**) (+)-5″-Deacetylpurpurin	OH	H
(**84**) (+)-5-Methoxypurpurin	$OCOCH_3$	OCH_3
(**85**) (+)-Purpurin	$OCOCH_3$	H



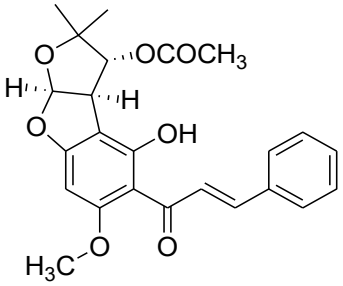
(2*S*)-2,3-Dihydrotephroglabrin (**86**)



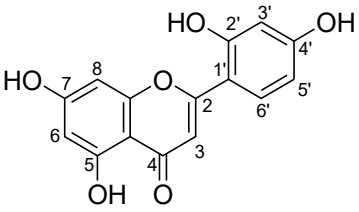
(2*S*)-2,3-Dihydrotephroapolin C (**87**)



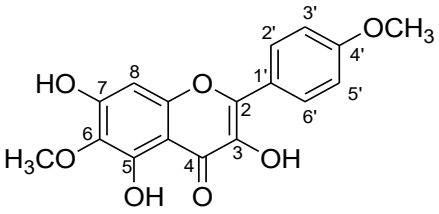
(2*S*)-7-Methoxy-8-(3-methoxy-3-methylbut-1-enyl)flavanone (**88**)



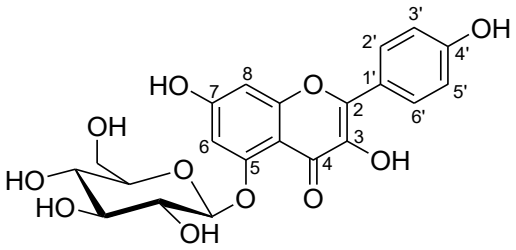
(+)-Tephropurpurin (**89**)



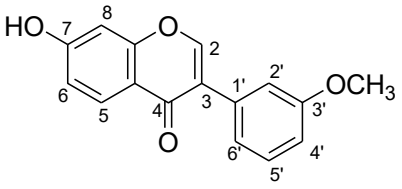
Norartocarpetin (**90**)



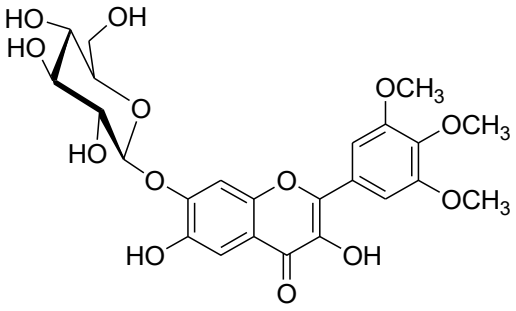
3,5,7-Trihydroxy-6,4′-dimethoxyflavone (**91**)



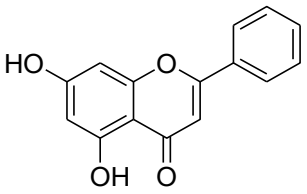
Kaempferol-5-*O*-*β*-D-glucopyranoside (**92**)



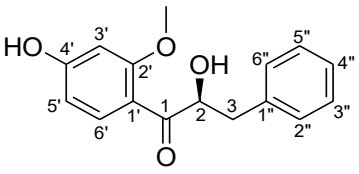
7-Hydroxy-3′-methoxy isoflavone (**93**)



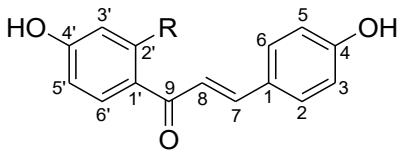
3,6-Dihydroxy-(3′,4′,5′-trimethoxyphenyl)-chromen-4-one-7-glucoside (**94**)



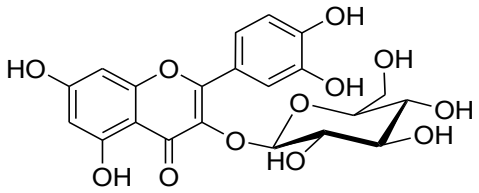
Chrysin (**95**)



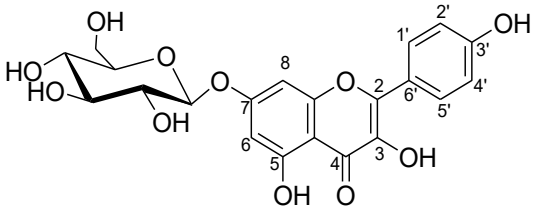
4′-Hydroxy-2′-methoxyphenyl-2-hydroxy-3-phenylpropanone (**96**)



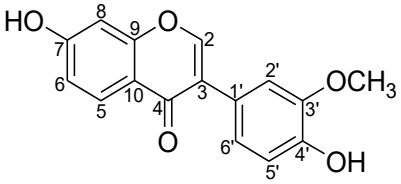
Compound name	R
(**97**) 4,2′,4′-Trihydroxychalcone	OH
(**98**) 4,4′-Dihydroxy-2′-methoxychalcone	OCH_3



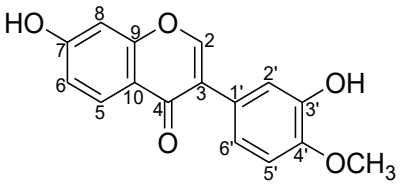
Iso-quercetin (**99**)



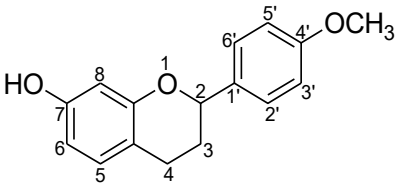
Kaempferol-7-*O*-*β*-D-glucopyranoside (**100**)



7,4′-Dihydroxy-3′-methoxyisoflavone (**101**)



Calycosin (**102**)



7-Hydroxy-4′-methoxyflavan (**103**)

Figure 1.7. Flavonoids identified in genus *Indigofera*

The chloroform extract of *Indigofera tinctoria* was subjected to column chromatography over silica gel. Spectroscopic data and GC-MS analysis revealed that the compound was 5,7-dihydroxyflavone or chrysin (**96**) (Figure 1.7) (**Boothapandi and Ramanibai 2018**).

Zhang *et al.,* (2018) isolated three flavonoids (**97**, **98** and **99**) Figure 1.7) from the ethanol extract of roots of *Indigofera stachyoides.* Two of the compounds (**97**, **98**) belonged to chalcone subclass of flavonoids. They had hydroxyl groups at 4 and 4′ positions but one of them was substituted by methoxy group at 2′ while the other had OH group. They were identified as 4, 4′-dihydroxy-2′-methoxychalcone and 4, 2′, 4′-trihydroxychalcone. The third compound (**99**) was a dihydrochalcone and identified as 4′-hydroxy-2′-methoxypheny-2-hydroxy-3-phenylpropanone.

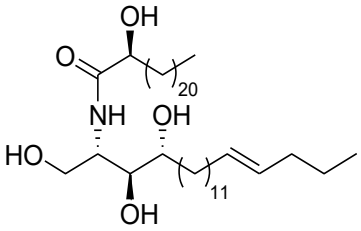
Elmi *et al.,* (2018) isolated three flavonoid glycosides from the methanol extract of fruits of *Indigofera caerulia.* The glycosides were kaempferol-3-*O*-rutinoside (**71**), isoquercetin (**99**) (Figure 1.7) and rutin (**65**). However, there are previous reports for the isolation of kaempferol-3-*O*-rutinoside (**71**) by **Hisaeda *et al.,* (2011)** and rutin (**65**) by **Prakash *et al.,* (2007)**.

Zhou *et al.,* (2020) combined the extracts obtained by treatment of *Indigofrera stachyoides* roots with 95% and 50% ethanol. Chromatographic separation led to the isolation of a flavonoid glycoside (**100**), two iso-flavonoids (**101**, **102**), and a flavanone (**103**).

1.3.8. Indigoferamide

The 5% aqueous methanol extract of seeds of medicinal plant *Indigofera heterantha* was extracted with chloroform followed by extraction with water. The aqueous layer was again fractionated with ethyl-acetate. The ethyl-acetate fraction was extracted with ether: petroleum ether (2:1) and water. As a result, three fractions were obtained. One of these three fractions was subjected to column chromatography over silica gel. Consequently, preparative TLC of fractions obtained from column chromatography led to the isolation of four compounds. One of them was the novel compound *Indigofera*mide-A (**104)** (Figure 1.8)**.** Structural investigation by HR-FABMS, ^{1}H NMR, ^{13}C NMR, and IR showed that the compound had an amide group having nitrogen atom substituted by 20 C long chain. A hydroxyl group was present on each C_1, C_3 and C_4 of this chain (**Rahman *et al.,* 2014a**).

The phytoconstituent (**104**) was proved as effective antibacterial and antidiabetic agent by studies conducted by **Rahman *et al.,* (2014a)** and **Rahman *et al.,* (2019)** respectively.



*Indigofera*mide-A

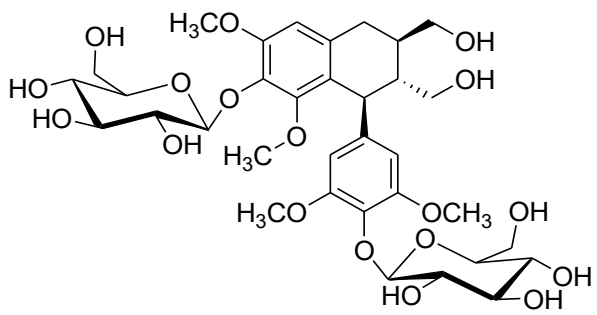
(**104**)

Figure 1.8. Indigoferamide-A identified in genus *Indigofera*

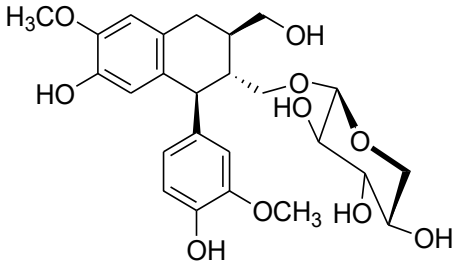
1.3.9. Lignans

The ethyl acetate soluble fraction of methanol extract of whole plant of *Indigofera heterantha* was analyzed by **Rehman *et al.,* (2005a)**. Flash column chromatography of extract over silica gel as stationary phase gave a gummy substance. Spectroscopic data from UV, IR, and NMR along with MS showed that the compound was a lignan-glycoside, and the overall structure was proposed as (**105**).

Zhou *et al.,* (2020) worked on the ethanol extract of *Indigofera stachyoides* roots. Repeated column chromatography yielded a lignan (**106**) in which a 3'-methoxy-4'-hydroxyphenyl moiety was fused to a saturated cyclohexane ring. The same moiety was also present as substituent on this cyclohexane ring. The main core was glycosylated to a xylose unit. Hence, the overall structure of compound was elucidated, and the compound was named as shizandriside.



(+)-Lyoniresinol-4,4′-bis-*O*-*β*-D-glucopyranoside (**105**)



Shizandriside

(**106**)

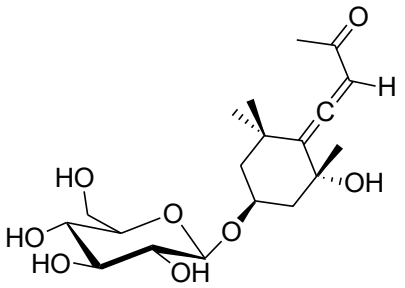
Figure. 1.9. Lignans identified in genus *Indigofera*

It can be deduced that two lignan glycosides have been so far isolated and identified in genus *Indigofera.* Their isolation was successfully obtained by methanol and ethanol extract of *Indigofera heterantha* (whole plant) and *Indigofera stachyoides*

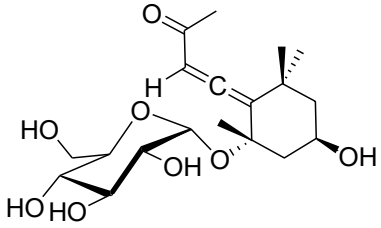
(roots) respectively. The sugars attached to main core increase the hydrophilic character of the constituents increasing their solubility in aqueous environment and hence increased chance for activity in biological environment. The identified compounds (**105**) and (**106**) (Figure 1.9) need to be evaluated for their biological activity.

1.3.10. Megastigmane glycosides

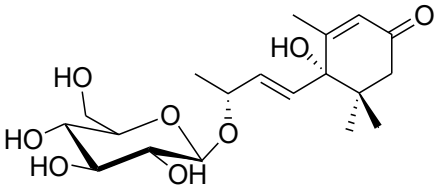
Hisaeda *et al.,* (2011) isolated the following three megastigmane glycosides (**107**), (**108**), (**109**) (Figure 1.10) by working on the methanol extract of *Indigofera zolingeriana* leaves.



Icariside B_1 (**107**)



Citroside A (**108)**



Roseoside (**109**)

Figure 1.10. Megastigmane glycosides identified in genus *Indigofera*

1.3.11. Nitro compounds

Species of *Indigofera* genus characteristically produce nitro compounds. Nitro compounds were quantified in 35 species by **Williams *et al.,* (1981).** The amount of NO_2 compounds in mg/g of dried plant in few of the species analyzed has been reviewed in table 1.1. Overall results showed that all the species examined possessed an amount of nitro compounds between 1-13 mg/g of plant materials. Moreover, qualitative analysis confirmed the presence of nitro compounds in 64 out of total 249 species examined.

The ethyl acetate extract of *Indigofera suffruticosa* roots and stem was passed over silica gel column resulting in the isolation of a colorless compound. NMR data

showed that it was an *α*-glucopyranose substituted by 3-nitropropanoyl group at 2, 3, 4 and 6 carbon atoms (**110**) (Figure 1.11). The presence of sugar unit was also confirmed by GC analysis of the sugar derivative alditol acetate (**Garcez *et al.,* 1989**).

Table 1.1. Amount of NO_2 compounds in mg/g of dried plant in some species of genus *Indigofera*

Species name	Origin of plant species	Nitro-compounds (mg/g of dried plant)
***I. brevipes* Rydb.**	Costa Rica	4.1
***I. echinate* Willd.**	Tanzania	8.8
***I. echinate* Willd.**	Australia	6.6
***I. sabulicola* Benth**	Brazil	10
***I. pseudotinctoria* Matsum**	Japan	4.2

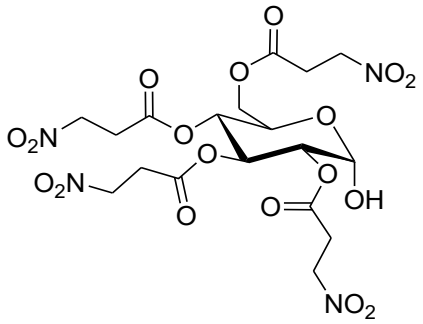
The study on nitro compounds in plants of genus *Indigofera* was advanced by **Majak *et al.,* (1992)**. Methanol extract of aerial parts of *Indigofera linnae* Ali. was passed over silica gel column. The fractions separated by using chloroform: acetone (4:1 and 2:3) as mobile phase were further subjected to centrifugal counter current chromatography. Finally, three nitro compounds were successfully isolated and then identified with the aid of ^{1}H NMR and ^{13}C NMR. One of them, 2,3,4,6-tetra-*O*-(3-nitropropanoyl)-*α*-D-glucopyranose (**110**) was also purified and identified by **Garcez *et al.,* (1989)**. The second nitro compound named 3,4,6-tri-*O*-[3-nitropropanoyl]-*α*-D-glucopyranose (**111**), was identified for the first time in genus. The third compound was 1,2,6-tri-*O*-(3-nitropropanoyl)-*β*-D-glucopyranose (**112**) (Figure 1.11) which was also isolated by **Finnegan and Mueller (1965)**.

Lodha *et al.,* (1997) isolated and identified an acylated glucopyranoside from *Indigofera oblongifolia.* The compound was identified as 2,3,4,6-tetra-*O*-(3-nitropropanoyl)-*α*-D-glucopyranoside (**110**) also isolated by **Garcez *et al.,* (1989)** from *Indigofera suffruticosa***.**

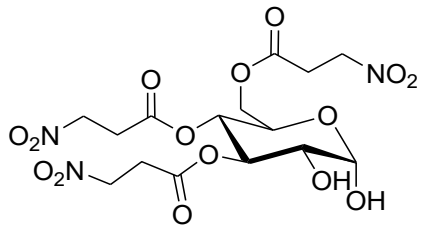
Three new and two previously reported nitro substituted compounds were isolated by **Garcez *et al.,* (2003)**. *Indigofera suffruticosa* roots were extracted with *n*-hexane followed by extraction with ethyl acetate. This ethyl acetate fraction was subjected to column chromatography over silica gel. Isolated compound was 2,3,4,6 -

tetrakis -*O*- [3-nitropropanoyl]-*α*-D-glucopyranose (**110**) (Figure 1.11) (**Garcez *et al.*, 1989**).

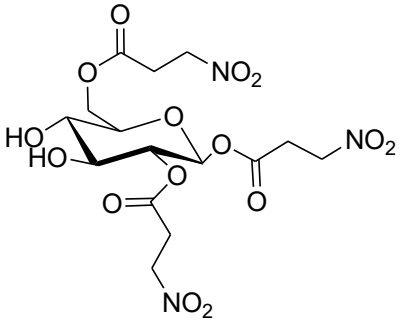
The 95% and 60% ethanol extract of *Indigofera kirilowii* roots was combined and fractionated with petroleum ether followed by extraction with chloroform. This chloroform fraction was passed over silica gel column. Repeated chromatography of obtained fractions led to the isolation of two compounds which were elucidated by IR, ^{1}H NMR, ^{13}C NMR and HR-FABMS. kirilowin A (**116**) possessed a glucose ring substituted with 3-nitropropanoly moiety at 3, 4 and 6 position while the other compound kirilowin B (**117**) (Figure 1.11) had glucose ring only substituted at 3 and 6-position. An acryloyl moiety was present at C_2 of the glucopyranose of both these compounds. This was first report for the presence of acryloyl group in nitro compounds of this genus (**Su *et al.*, 2005**).



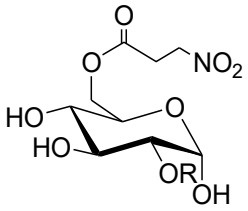
2,3,4,6-Tetra-*O*-(3-nitropropanoyl)-*α*-D-glucopyranose (**110**)



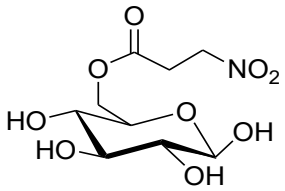
3,4,6-Tri-*O*-[3-nitropropanoyl]-*α*-D-glucopyranose (**111**)



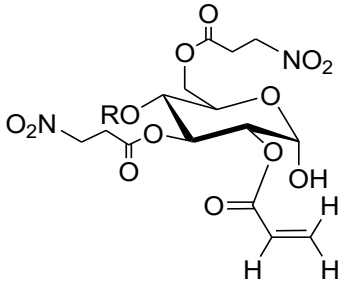
1,2,6-Tri-*O*-(3-nitropropanoyl)-*β*-D-glucopyranose (Karakin) (**112**)



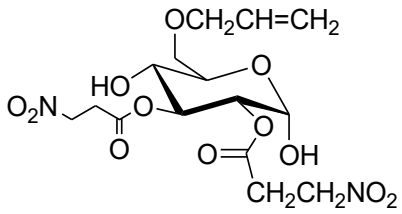
Compound Name	R
(**113**) 6-*O*-(3-Nitropropanoyl)-*α*-D-glucopyranose	H
(**114**) 2,6-Di-*O*-(3-nitropropanoyl)-*α*-D-glucopyranose	$COCH_2CH_2NO_2$



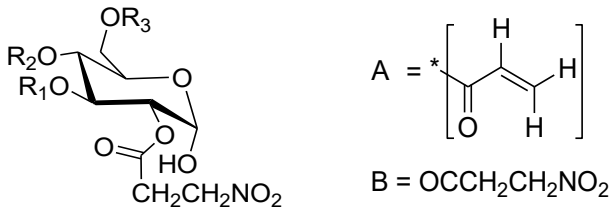
6-*O*-(3-Nitropropanoyl)-*β*-D-glucopyranose **(115)**



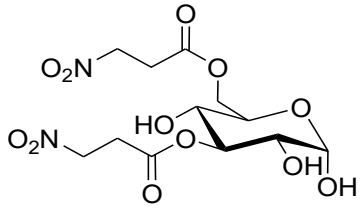
Compound Name	R
(**116**) 2-*O*-Acryloyl-3,4,6-tri-*O*-[3-nitropropanoyl]-*α*-D-glucopyranose (Kirilowin A)	$OCCH_2CH_2NO_2$
(**117**) 2-*O*-Acryloyl-3,6-di-*O*-[3-nitropropanoyl]-*α*-D-glucopyranose (Kirilowin B)	H



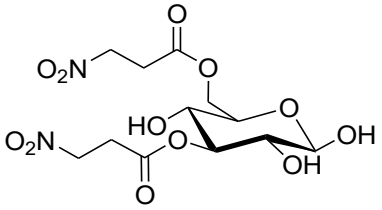
6-*O*-Acryl-2,3-di-*O*-(3-nitropropanoyl)-*α*-D-glucopyranose (**118**)



Compound Name	R_1	R_2	R_3
(**119**) 4-*O*-Acryloyl-2,3,6-tri-*O*-[3-nitropropanoyl]-*α*-D-glucopyranose (Kirilowin C)	B	A	B
(**120**) 3-*O*-Acryloyl-2,6-di-*O*-[3-nitropropanoyl]-*α*-D-glucopyranose (Kirilowin D)	A	H	B
(**121**) 2,3,4-Tri-*O*-[3-nitropropanoyl]-*α*-D-glucopyranose (Kirilowin E)	B	B	H
(**122**) 2,4,6-Tri-*O*-[3-nitropropanoyl]-*α*-D-glucopyranose (Kirilowin F)	H	B	B



3,6-Di-*O*-[3-nitropropanoyl]-*α*-D-glucopyranose (Kirilowin G) (**123**)



3,6-Di-*O*-[3-nitropropanoyl]-*β*-D-glucopyranose (Kirilowin H) (**124**)

Figure 1.11. Nitro compounds identified in genus *Indigofera*

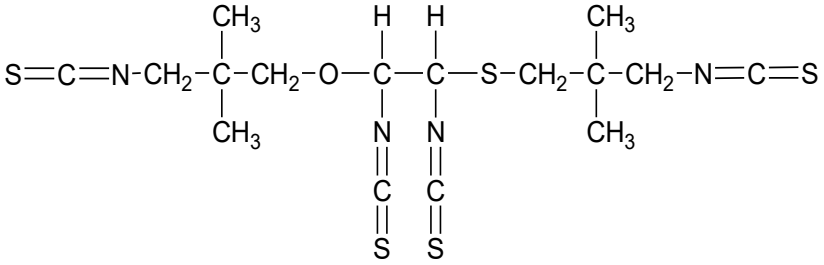
Similarly, **Zhang *et al.,* (2006)** prepared 95% and 60% ethanol extracts of roots of *Indigofera carlesii.* The extracts were combined and successively extracted with petroleum ether, ethyl acetate and *n*-butanol. Column chromatography of ethyl acetate fraction using silica gel as stationary phase resulted in a mixture of two compounds as white needles. Spectroscopic analysis by IR, ^{1}H NMR and ^{13}C NMR revealed that one of the compounds was kirilowin B (**117**) previously isolated from *Indigofea kirilowii* (**Su *et al.,* 2005**). The other compound was 6-*O*-acryl-2,3-di-*O*-(3-nitropropanoyl)-*α*-D-glucopyranose (**118**) (Figure 1.11).

Su *et al.,* (2008) extracted roots of *Indigofera kirilowii* with 95% and 60% ethanol. The combined extract was fractionated with petroleum ether, chloroform, and

ethyl acetate. The chloroform fraction was subjected to silica gel column resulting in the isolation of six new compounds kirilowin C, D, E, F, G and H (**119-124**) (Figure 1.11). Two of the compounds, kirilowin C (**119**) and D (**120**), had acryloyl moiety while the others were substituted by varying number of 3-nitopropanoyl groups.

1.3.12. Organosulphur compounds

Sharma and Singh (**2017**) isolated organo-sulfur compound (**125**) (Figure 1.12) from the aerial parts of *Indigofera tinctoria.* The dried and ground plant sample was extracted with 80% ethanol in a soxhlet extractor. The extract was subjected to column packed with silica gel. Gradual elution was carried out by various solvents used in increasing order of polarity. The fraction containing organo-sulfur compound was further analyzed by LC-MS and UV. Similarly, structure was finally confirmed by using data obtained from IR and ^{1}H NMR.



1-(1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulphanyl)-ethoxyl-3-isothiocyanato-2,2-dimethyl-propane (**125**)

Figure 1.12. Organosulphur compound identified in genus *Indigofera*

1.3.13. Derivatives of benzene

Anuradha *et al.,* (**1987**) studied the distribution pattern of different phenolic compounds in eight *Indigofera* species i.e., *I. dalzellii, I. hirsuta, I. hochstetteri, I. mysorensis, I. oblongifolia, I. prostrata, I. tenuifolia* and *I. tinctoria*. The filtrate obtained from digest of plant material of *Indigofera* plants was analyzed by paper chromatography. The R_f values and color of spots under UV were matched with standard phenolic compounds. Vanillic acid (**126**), *p*-hydroxybenzoic acid (**127**) and *p*-coumaric acid (**128**) (Figure 1.13) were present in all the eight species under study. Gallic acid (**129**) was detected only in *Indigofera hirsuta* while caffeic acid (**130**) was identified in *Indigofera dalzellii* as well as in *Indiofera prostrata* Willd.

Hasan *et al.,* (1989) worked on the isolation of phenolic acids from leaves and flowers of *Indigofera heterantha.* The acid hydrolysis of methanolic extract was followed by isolation of acids by a series of different types of chromatography. Three of the fractions obtained from VLC of leaf extract were further purified by column chromatography over polyamide. Finally, semi-preparative HPLC led to the isolation of three acids. The retention times of acids as recorded by HPLC, and UV spectral studies showed that the acids were protocatechuic acid (**131**) and *p*-methoxy-cinnamic acid (**132**) (Figure 1.13). Similarly, one of the fractions obtained from flash chromatography of methanolic extract of flowers was subjected to CTLC. As a result, two phenolic acids were again isolated and one of them was identified to be protocatechuic acid (**131**).

The GCMS analysis of natural indigo was carried out by **Andreotti *et al.,* (2004**). Natural indigo was prepared from leaves of *Indigofera tinctoria.* The two methods of GCMS analysis (py/silylation/GCMS and DE-MS) led to the identification of compounds (**133-141**) (Figure 1.13).

Rehman *et al.,* (2005a) worked on the ethyl acetate soluble fraction of methanol extract of whole plant of *Indigofera hetrantha.* The ethyl acetate fraction was subjected to flash column chromatography over silica gel. Finally, two compounds were isolated as colorless gummy solids. Structural elucidation by spectroscopic techniques led to the conclusions that they were glycosylated acylphloroglucinols (**142** and **143**) (Figure 1.13).

Sharif *et al.,* (2005) isolated a phenolic acid from the chloroform soluble fraction of methanol extract of whole plant of *Indigofera oblongifolia.* The acid was identified as 3-hydroxybenzoic acid (**144**). Another constituent with molecular formula $C_{30}H_{48}O_4$ was also isolated from the same extract. IR data indicated the presence of a carboxylic group and olefinic double bond. This information combined with UV and NMR data showed that the compound was indigoferic acid (**145**) (Figure 1.13).

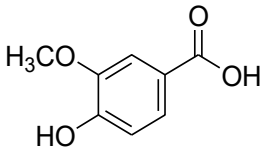
Prakash *et al.,* (2007) identified two phenolic acids in the 50 % aqueous methanol extract of flowers of *Indigofera tinctoria.* HPLC and MS/MS method was applied to identify the composition of extract. The identified acids were caffeic acid (**130**) and gallic acid (**129**) (Figure 1.13).

Moura *et al.,* (**2011**) worked on the aqueous and methanol extracts of aerial parts of *Indigofera hirsuta.* The technique of HPLC-ESI-MS helped in the detection and identification of protocatechuic acid (**131**), methyl gallate (**150**) and gallic acid (**129**) (Figure 1.13) present in the *I. hirsuta* extract.

Three phenolic glycosides were isolated by **Tariq *et al.,*** (**2011**) from the methanol extract of *Indigofera gerardiana* Wall. The compounds were identified as indigoferin A (**151**), B (**152**) and C (**153**) (Figure 1.13).

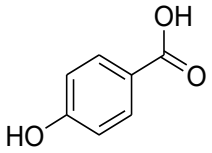
Deshpande *et al.,* (**2013**) analyzed the chloroform and 70 % methanol: water extract of *Indigofera cordifolia* seeds using the technique of GCMS. One of the compounds identified in chloroform extract was a di-ester of benzene-1,2-dicarboxylic acid (**146**) (Figure 1.13).

The chloroform extract of whole plant of *Indigofera aspalathoides* gave *a* phenolic acid by column chromatography. Spectroscopic analysis showed that the compound was salicylic acid (**147**) (Figure 1.13) (**Saraswathy *et al.,* 2013**).



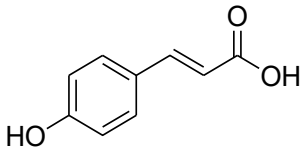
Vanillic acid

(**126**)

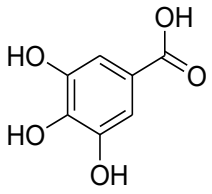


p-Hydroxybenzoic acid

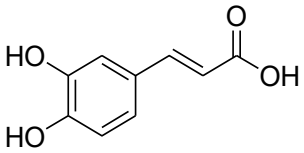
(**127**)



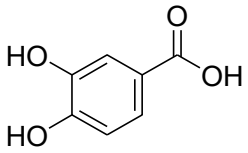
p-Coumaric acid (**128**)



Gallic acid (**129**)

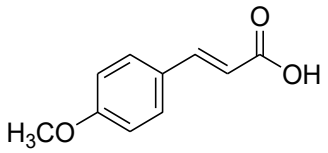


Caffeic acid (**130**)



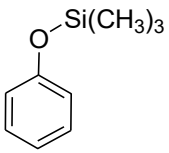
Protocatechuic acid

(**131**)

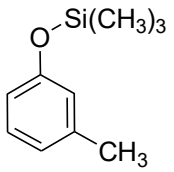


p-Methoxy cinnamic acid

(**132**)

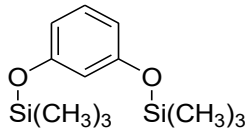


TMS Phenol (**133**)

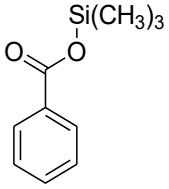


TMS 3-Methylphenol

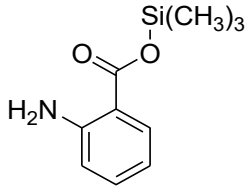
(**134**)



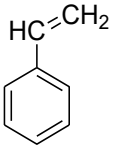
Bis TMS 1,3-Dihydroxybenzene (**135**)



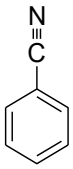
TMS Benzoate (**136**)



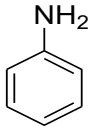
TMS 2-Amino benzoate (**137**)



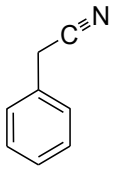
Styrene (**138**)



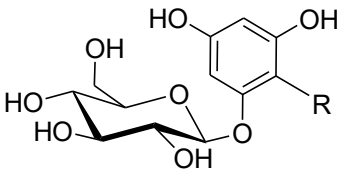
Benzonitrile (**139**)

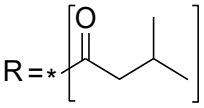


Aniline (**140**)

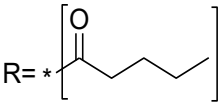


Benzyl nitrile (**141**)

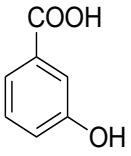




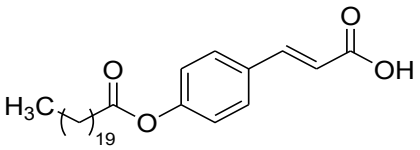
1-[(3-Methylbutanoyl)-phloroglucinyl]-2-*O*-*β*-D-glucopyranoside (**142**)



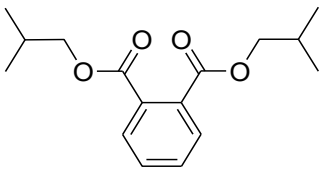
1-[(Pentanoyl) phloroglucinyl]-2-*O*-*β*-*D*-glucopyranoside (**143**)



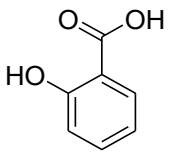
3-Hydroxybenzoic acid (**144**)



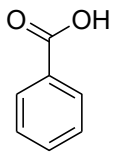
Indigoferic acid (**145**)



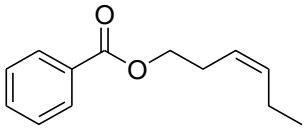
1, 2- Benzene-dicarboxylic acid, bis (2-methylpropyl) ester (**146**)



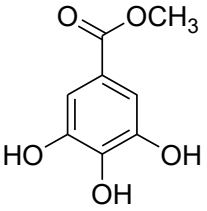
Salicylic acid (**147**)



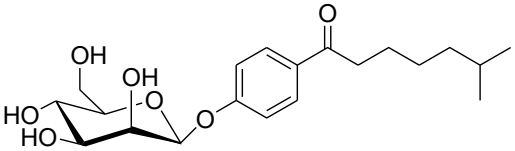
Benzoic acid (**148**)



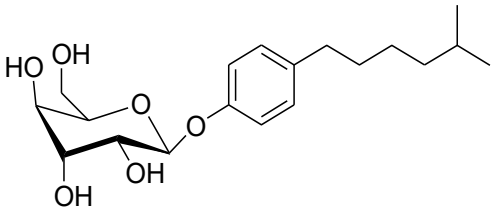
(*Z*)-3-Hexenyl benzoate (**149**)



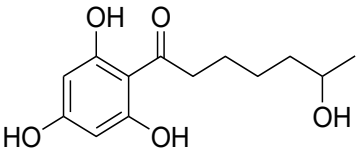
Methyl gallate (**150**)



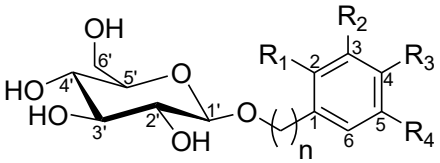
Indigoferin A (**151**)



Indigoferin B (**152**)



Indigoferin C (**153**)



Compound name	R_1	R_2	R_3	R_4	n
(**154**) 3,4,5-Trimethoxyphenyl-*β*-D-glucopyranoside (Koaburside)	H	OCH_3	OCH_3	OCH_3	0
(**155**) 2-Methoxy-4-(2″-hydroxyethyl)–phenol-1-*O*-*β*-D-glucopyranoside	OCH_3	H	CH_2CH_2OH	H	0
(**156**) 2″-(3-Hydroxy-4-methoxyphenyl) ethyl-1″-*O*-*β*-D-glucopyranoside	H	OH	OCH_3	H	2

Figure 1.13. Derivatives of benzene identified in genus *Indigofera*

Arriaga *et al.*, (2013) qualitatively analyzed the ethanol extract of leaves of *Indigofera suffruticosa* Mill. They were successful in the isolation of an aromatic acid by repeated chromatography of ethanol extract of *Indigofera suffruticosa* pods. The

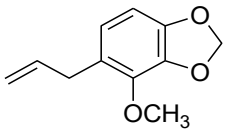
acid was identified as benzoic acid by the help of NMR analysis. Benzoic acid (**148**) was previously identified by **Elmi *et al.,* (2018)** in the methanol extract of fruits of *I. caerulia.* GC-MS analysis of *I. suffruticosa* leaves also revealed the presence of an aromatic ester named (*Z*)-3-hexenyl benzoate (**149**) (Figure 1.13).

Two aromatic acids namely, benzoic acid (**148**) and caffeic acid (**130**) were identified in the methanol extract of fruits of *Indigofera caerulia* by **Elmi *et al.,* (2018**) using RP-HPLC. In addition, gallic acid (**129**) and methyl gallate (**149**) (Figure 1.13) were isolated from the same extract by using column chromatography. Gallic acid (**129**) was previously identified by **Anuradha *et al.,* (1987**) in *Indigofera hirsuta* while caffeic acid (**130**) was identified in *Indigofera dalzellii* as well as in *Indiofera prostrata* Willd.

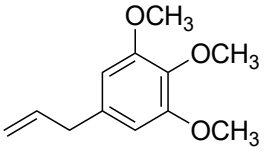
Zhou *et al.,* (2020) examined the combined 95% and 50% ethanol extracts of roots of *Indigofera stachyoides radix.* The extract was subjected to silica gel column chromatography which yielded three kinds of phenolic glycosides (**153**), (**154**) and (**155**) (Figure 1.13).

1.3.14. Phenylpropanoids

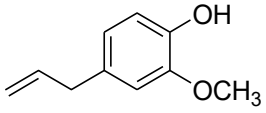
Ten phenyl-propanoids (**157**-**165**) (Figure 1.14) were identified by **Silva *et al.,* (2019)** in the essential oil of *I. suffruticosa* leaves. GCMS analysis showed that this class of compounds constitutes 96.7% of the total composition of the oil obtained.



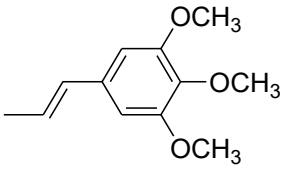
Croweacin (**157**)



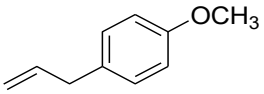
Elemicin (**158**)



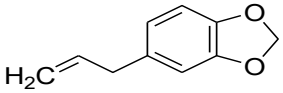
Eugenol (**159**)



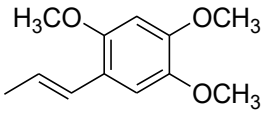
Isoelimicin (**160**)



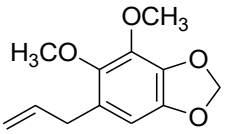
Methyl chavicol (**161**)



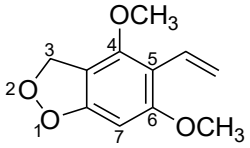
Safrole (**162**)



Z-Asarone (**163**)



Dill apiole (**164**)



4,6-Dimethoxy-5-vinyl-1,2-benzodioxide (**165**)

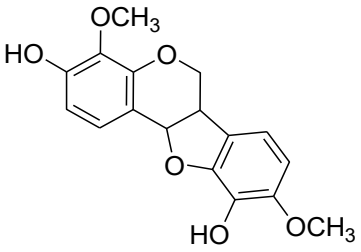
Figure 1.14. Phenylpropanoids identified in genus *Indigofera*

1.3.15. Pterocarpans and their derivatives

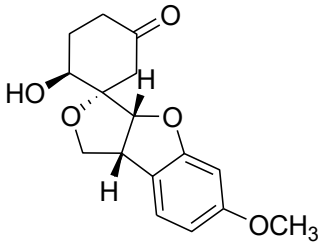
Selvam *et al.,* (2004) isolated a pterocarpan (**166**) (Figure 1.15) by working on the chloroform extract of *Indigofera aspalathoides* Vahl. The extract was fractionated by repeated column chromatography over silica gel and re-chromatography through Sephadex LH-20 column. As a result, a white amorphous compound was obtained. APCI-MS, IR, ^{1}H NMR and ^{13}C NMR data showed that the compound belonged to the class of pterocarpans. Thus, 2D NMR experiments including ^{1}H-^{1}H COSY, HMQC and HMBC helped in establishing the complete structure of this compound. Each of the two benzene rings of pterocarpan unit has a methoxy group present ortho to the hydroxyl group. Presence of phenolic groups was also confirmed by derivatization into pterocarpan diacetate. X-ray diffraction studies confirmed the absolute configuration of molecule.

Investigations made by **Zhang *et al.,* (2018)** revealed that roots of *Indigofera stachyodes* are good source of pterocarpan derivatives. They extracted roots of *I. stachyodes* with 95% ethanol followed by fractionation with ethyl acetate. This Ethyl-acetate soluble fraction yielded a colorless compound with molecular formula $C_{16}H_{18}O_5$. The IR studies indicated presence of an OH, a phenyl ring and a carbonyl group in structure of this molecule. NMR data proved that the compound was a pterocarpan derivative. The special character of its structure is the presence of an unusual spiro-tetrahydrofuran ring. In addition, four more pterocarpans (**167**-**170**) (Figure 1.15) were isolated and identified from the same extract.

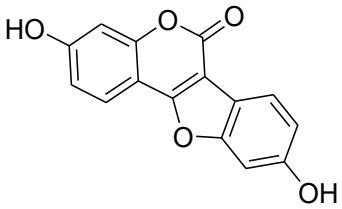
The column chromatography of ethanol extract of roots of *Indigofera stachyodes* Lindl. also yielded a compound with skeletal characteristic of pterocarpans. The compound was identified as maackiain (**171**) (**Zhou *et al.*, 2020**).



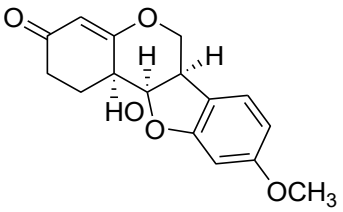
Indigocarpan (**166**)



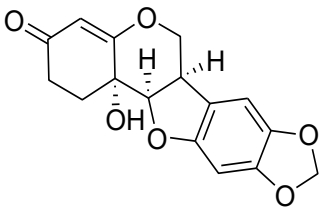
Stachyodin A (**167**)



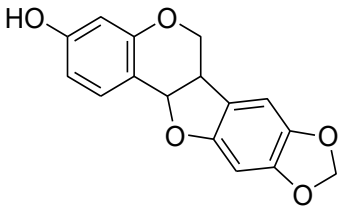
Coumestrol (**168**)



11*b*-Hydroxy-11*b*,1-dihydromedicarpin (**169**)



11*b*-Hydroxy-11*b*,1-dihydromaackiain (**170**)



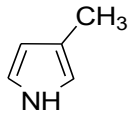
Maackiain (**171**)

Figure. 1.15. Pterocarpans and their derivatives identified in genus *Indigofera*

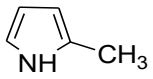
1.3.16. Pyrroles and indoles

Andreotti *et al.*, (**2004**) used GC-MS analysis to characterize the phytoconstituents present in natural indigo prepared from *Indigofera tinctoria* leaves. The identified compounds included two pyrrole (**172**, **173**) and four indole (**174**, **175**, **176** and **177**) derivatives (Figure 1.16).

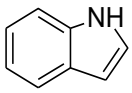
The GCMS analysis of chloroform extract of *Indigofera cordifolia* seeds led to the identification of a significant compound having tetrahydropyrrole ring with 16 C long side chain present as substituent on nitrogen atom (**178**). Another compound identified from the hydro-methanolic extract of *I. cordifolia* seeds had an indole ring attached to a 1, 3-thiazole-moiety (**179**) **Deshpande *et al.*,** (**2013**). (Figure 1.16)



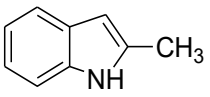
3-Methylpyrrole (**172**)



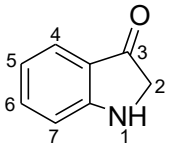
2-Methylpyrrole (**173**)



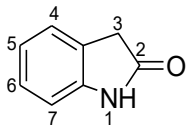
Indole (**174**)



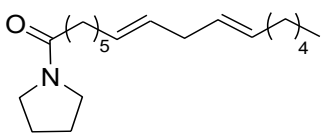
2-Methyl indole (**175**)



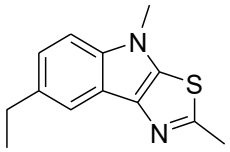
1,2-Dihydro-3*H*-indol-3-one (**176**)



1,3-Dihydro-2*H*-indole-2-one (**177**)



1-(1-Oxo-7,10-hexadeca-dienyl) pyrrolidine (**178**)



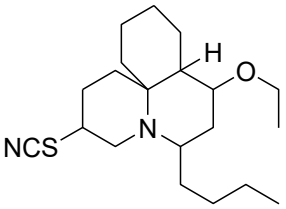
7-Ethyl-2,4-dimethyl-4*H*-thiazolo [5,4-b] indole (**179**)

Figure 1.16. Pyrroles and indoles identified in genus *Indigofera*

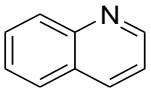
1.3.17. Quinolines

Stems of *Indigofera longeracemosa* have been reported to contain a valuable type of deca-di-hydro-pyridoquinoline (**180**) (Figure 1.17) (**Thangadurai *et al.*, 2001b**). The authors were successful in isolation of this compound by extraction of stems of *I. longercemosa* with dichloromethane followed by extraction with petroleum ether : ethyl acetate (9:1) and then fractionation of extract through HPLC. The data obtained from EIMS, UV, IR, ^{1}H NMR and ^{13}C NMR was used to establish the complete structure of purified compound.

Natural indigo was prepared from leaves of *Indigofera tinctoria.* The components of indigo were analyzed by using methods of Py-silylation/GC/MS and DE-MS **Andreotti *et al.*,** (**2004**). Two of the identified compounds were quinoline (**181**) and a methyl substituted derivative of quinoline (**182**) (Figure 1.17).

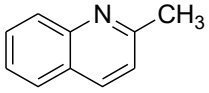


Rel-(3*S*, 5*R*, 6*S*, 8*R*, 8a*R*, 12a*R*)-8-ethoxy-6-butyl-3-isothiocyanatodecahydro-pyrido (2, l) quinoline (**180**)



Quinoline

(**181**)



2-Methylquinoline

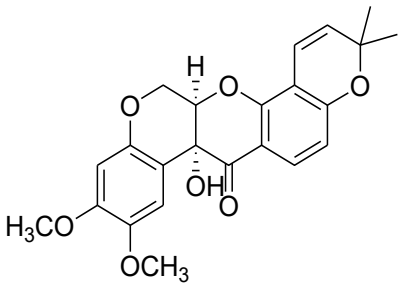
(**182**)

Figure 1.17. Quinolines identified in genus *Indigofera*

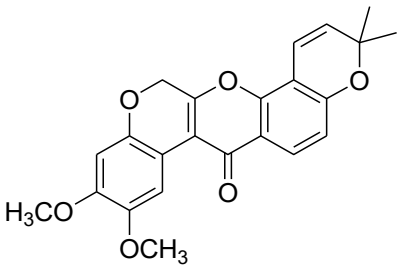
1.3.18. Rotenoids

Six phytoconstituents (**183-188**) (Figure 1.18) were isolated from the *n*-hexane extract of *Indigofera tinctoria* L. UV, IR and GLC showed that the constituents were rotenoids (**Kamal and Mangla, 1993**).

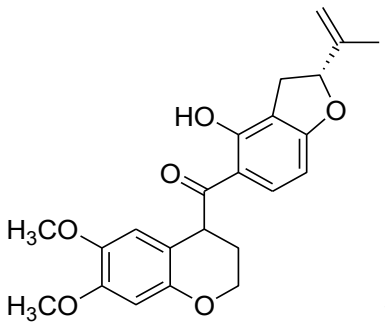
Various parts of *Indigofera spicata* Forssk. were extracted with methanol. The chloroform soluble fraction of *Indigofera spicata* Forssk. gave three rotenoids **189, 190** and **188** (Figure 1.18) including tephrosin (**Bueno P. rez *et al.*, 2013**).



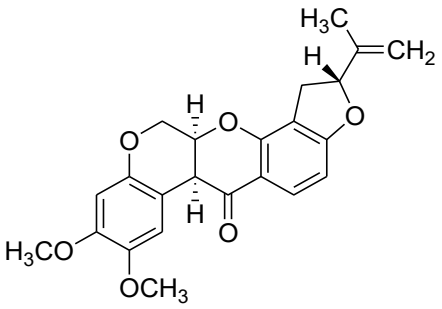
Deguelin (**183**)



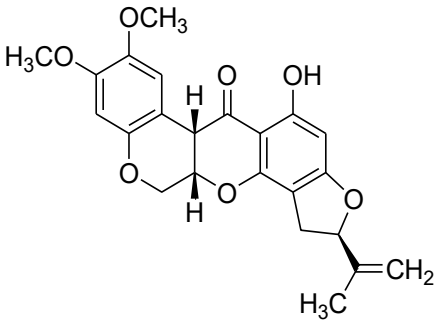
Dehydro-deguelin (**184**)



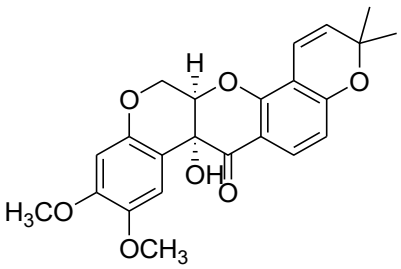
Rotenol (**185**)



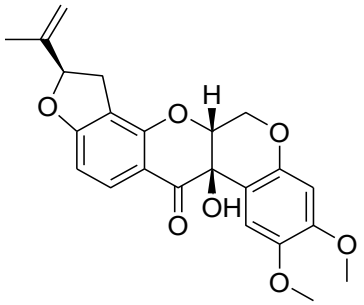
Rotenone (**186**)



Sumatrol (**187**)



Tephrosin (**188**)



cis-(6a*β*,12a*β*)-Hydroxy rotenone (**189**)

Figure. 1.18. Rotenoids identified in genus *Indigofera*

1.3.19. Sterols and steroids

Domínguez *et al.*, (1978a) isolated a sterol from the petroleum ether extract of whole plant of *Indigofera suffruticosa.* It was identified as *β*-sitosterol (**190**) (Figure 1.19).

β-Sitosterol was also isolated from the alcoholic extracts of *Indigofera oblongifolia*, *Indigofera gerardiana* Wall. and *Indigofera suffruticosa* Mill. by **Sharif *et al.*, (2005), Tariq *et al.*, (2011)** and **Arriaga *et al.*, (2013)** respectively.

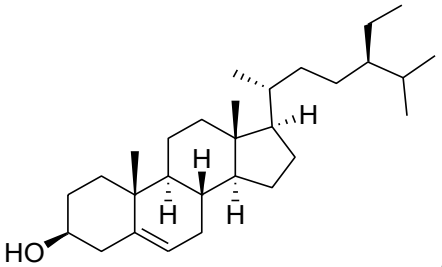
Deshpande *et al.*, (2013) identified two compounds (**191**, **192**) (Figure 1.19) in the chloroform extract of *I. cordifolia* using GC-MS analysis. Both the identified compounds possessed basic sterol skeleton ***i.e.*,** cyclopentano-perhydro-phenanthrene.

The leaves of *Indigofera linnae* Ali were successively extracted with petroleum ether, chloroform, and methanol**.** The chromatography of alcohol extract led to the isolation of a compound identified by spectroscopic analysis as gitoxin (**193**) (Figure 1.19) (**Sandhyavali *et al.,* 2014**). It is a glycoside of gitoxigenin and three units of digitoxose sugar.

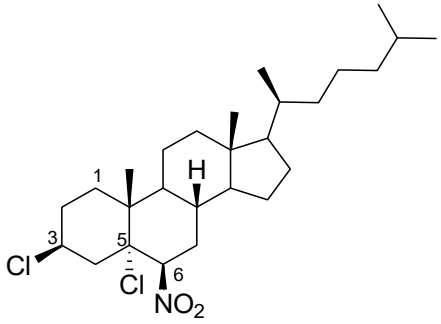
Zeb *et al.,* 2017 studied the ethyl-acetate soluble fraction of methanol extract of *Indigofera heterantha* roots. The concentrated extract was chromatographed over silica gel. Repeated chromatography led to the isolation of *β*-sitosterol (also isolated by **Domínguez *et al.,* 1978a**) and stigmasterol (**194**) (Figure 1.19).

Prashanth *et al.,* (2018) identified a steroid (**195**) (Figure 1.19) in aqueous extract of leaves of *Indigofera tinctoria* by GC-MS analysis.

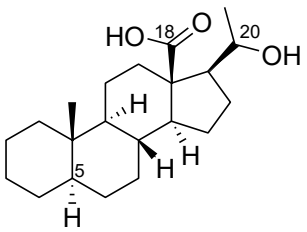
Zhou *et al.,* (2020) made a significant work on the isolation of reputable compounds from roots of *Indigofera stachyoides.* The combined and concentrated extract of roots in 95% ethanol and 50% ethanol was fractionated with petroleum ether, ethyl acetate and *n*-butyl alcohol. Column chromatography and re-chromatography of ethyl acetate fraction led to the isolation of six compounds including sterol and a sterol glycoside. The compounds were stigmasterol (also isolated by **Zeb *et al.,* 2017**) and *β*-sitosterol-D-glucoside (**196**) (Figure 1.19).



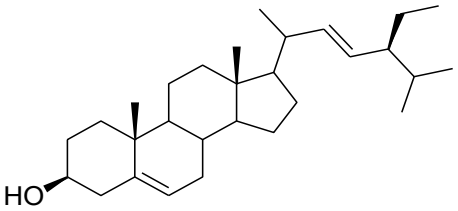
β-Sitosterol (**190**)



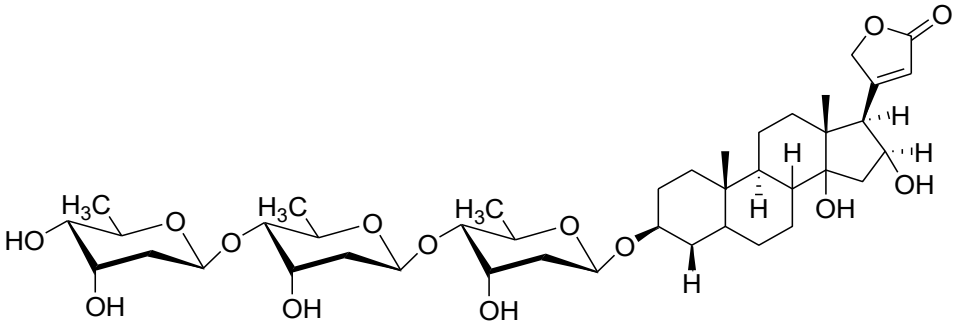
3,5-Dichloro-6-nitro-(3*β*,5*α*,6*β*)- cholestane (**191**)



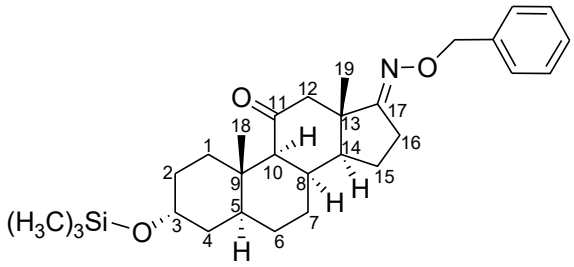
20-Hydroxy-5*α*-pregnan-18-oic acid (**192**)



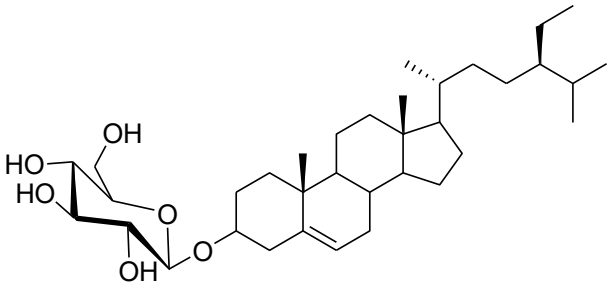
Stigmasterol (**194**)



Gitoxin (**193**)



3-[(Trimethylsilyloxy], 17- [*O*- (phenylmethyl)oxime], (3*α,*5*α*)-androstane, 11,17-dione (**195**)



β-Sitosterol-3-*O*-*β*-D-glucoside (**196**)

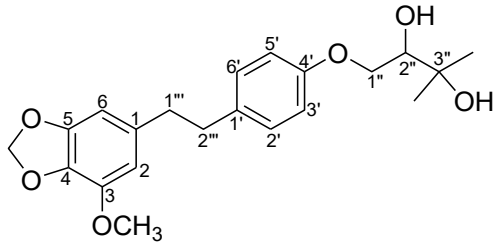
Figure 1.19. Sterols and steroids identified in genus *Indigofera*

1.3.20. Stilbenes and dihydro-stilbenes

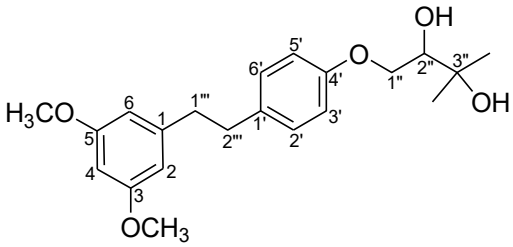
Musa *et al.,* (**2008**) carried out phytochemical analysis on the whole plant of *Indigofera pulchra.* Dried plant material was treated with 80% methanol in soxhlet extractor. The obtained extract was subjected to column chromatography over silica gel

using chloroform and methanol combinations in increasing order of polarity. A subfraction obtained from 10-20% MeOH in $CHCl_3$ was re-chromatographed over Sephadex LH-20. As a result, a mixture of two compounds (**197**) and (**198**) (Figure 1.20) was obtained.

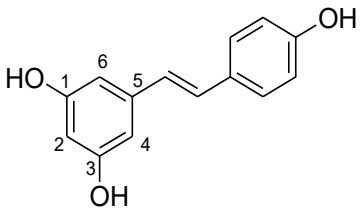
The alcohol extract of leaves of *Indigofera linnae* Ali. gave a compound which was polyhydroxylated and belonged to class of organic compounds called stilbenes (**Sandhyavali *et al.*, 2014**). The compound was identified as resveratrol (**199**) (Figure 1.20), produced by plants in response to pathogenic attack or injury.



3-Methoxy-4,5-methylenedioxy-4′-*O*-(2″,3″-dihydroxy-3″-methylbutyl)-dihydrostilbene (**197**)



3,5-Dimethoxy-4′-*O*-(2″,3″-dihydroxy-3″-methylbutyl)-dihydrostilbene (**198**)



5-[(*E*)-2-(4-Hydroxyphenyl] benzene-1,3-diol (**199**)

Figure 1.20. Stilbenes and dihydro stilbenes identified in genus *Indigofera*

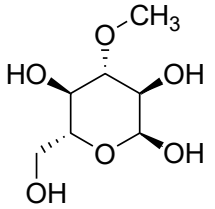
1.3.21. Sugars

Vinoth *et al.*, (2011) identified the presence of glucose (**200**) (Figure 1.21) in ethanol extract of *I. subulata* Vahl. by using the technique of GC-MS. The glucose was substituted by an OCH_3 group at C_3 of the pyranose ring.

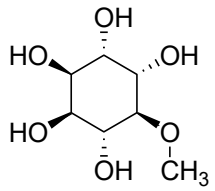
The chromatography of ethanol extract of whole plant of *I. suffruticosa* yielded a compound with molecular formula of $C_7H_{14}O_6$. Spectroscopic study by the help of IR and ^1H NMR showed that the compound was a cyclohexanol. It was finally identified as D (+)-pinitol (**201**) (Figure 1.21) (**Domínguez *et al.*, 1978a**). The same compound

was also isolated from methanol extract of whole plant of *I. barberi* (**Bhaskar *et al.*, 2016**).

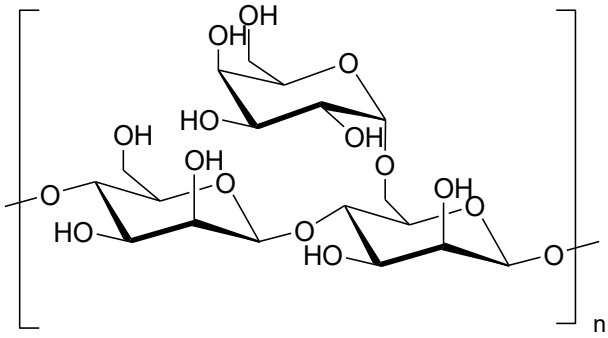
Seeds of *Indigofera tinctoria* were extracted with hot water. A grey powder was precipitated when extract was slowly added into ethanol. The compound was purified by column chromatography over Sephadex G-200. Molecular weight determination followed by GLC analysis, and a series of experimental studies revealed that the compound (**202**) (Figure 1.21) was a polysaccharide. It was a galactomannan of *β*-(1→4) linked D-mannose constituting the main chain substituted by *α*-D-galactose at 6 position of mannose (**Sen *et al.*, 1986**).



3-*O*-Methyl-D-glucose (**200**)



D-Pinitol (**201**)



Galactomannan (**202**)

Figure 1.21. Sugars identified in genus *Indigofera*

1.3.22. Terpenes and terpenoids

Thangadurai *et al.*, (**2002**) isolated and identified a tricyclic diterpenoid (**203**) (Figure 1.22) from the extract of *Indigofera longeracemosa.*

The whole plant of *Indigofera heterantha* was extracted with methanol and fractionated with ethyl acetate. This ethylacetae fraction was subsequently passed over silica gel column leading to the isolation of two compounds. Structural studies were carried out with the aid of spectroscopic techniques including UV, IR, 1D and 2D NMR. Evidence revealed that one of the compounds had molecular formula $C_{18}H_{28}O_8$. The compound was identified as (**204**). The second compound was structurally close to the

first except that it had two glucose units linked together by 1→6 glyosidic linkage. It was finally identified as (**205**) (Figure 1.22) (**Mehmood *et al.,* 2008**).

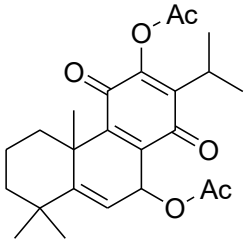
Similarly, the work on isolation of terpenes from *Indigofera* plants was advanced by **Arriaga *et al.,*** (**2008**). The leaves of *Indigofera microcarpa* were extracted by hydro-distillation resulting in a pale-yellow oil. The composition of oil was studied with the aid of GC-MS. It was found that *I. microcarpa* leaves are very rich in sesquiterpenes (**206-214**) (Figure 1.22) while monoterpenes are absent. *β*-caryophyllene is the most abundant hydrocarbon with 56% participation in total composition of the oil.

The ethanol leaf extract of *Indigofera subulata* Vahl. or *I. trita* was analyzed by GC-MS analysis. Comparison of retention time and mass spectra with NIST database led to the identification of a triterpene and a diterpene alcohol which were squalene (**215**) and phytol (**217**) respectively. The compound (**216**) was another terpenoid identified in *I. trita* (**Vinoth *et al.,* 2011**). Later, the GC-MS analysis of ethanol extract of *Indigofera suffruticosa* Mill. also revealed the presence of phytol (**217**) (Figure 1.22) in leaf extract.

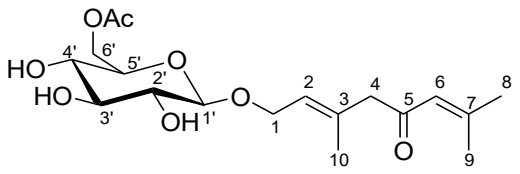
Saraswathy *et al.,* (**2013**) worked on the chloroform extract of whole plant of *I. aspalathoides.* Repeated column chromatography led to the isolation of tricyclic terpenes called erythroxydiol X (**218**) and erythroxydiol Y (**219**) (Figure 1.22).

A phytochemical study on the leaves of *Indigofera suffruticosa* was made by **Silva *et al.,*** (**2019**). The essential oil obtained by hydro-distillation was analyzed by GC-MS analysis and components were identified by comparison of their retention indices with standard retention times reported in literature. Moreover, determination of relative concentration showed that *I. suffruticosa* contains 6.4% sesquiterpenes and 1.4% monoterpenes. Eugenol is the most abundant component comprising 45% composition of the oil. The terpenes present in *I. suffruticosa* leaf oil, were **220-226** (Figure 1.22).

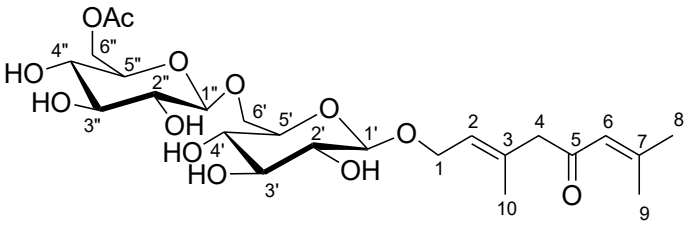
A triterpene was isolated by **Musa *et al.,*** (**2008**) from the methanol extract of whole plant of *Indigofera pulchra.* The terpene was identified as lupeol **227** (Figure 1.22).



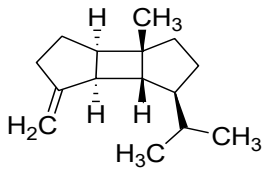
Indigoferabietone (**203**)



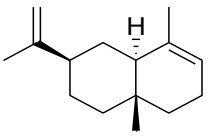
3,7-Dimethyl-(2*E*,6*E*)-octadien-5-one-1-*O*-*β*-D-6′-*O*-acetyl glucopyranoside (**204**)



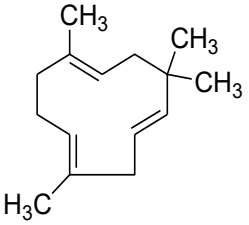
3,7-Dimethyl-(2*E,*6*E*)-octadien-5-one-1-*O*-[*β*-D-6″-*O*-acetylglucopyranosyl (1″→6′)-*β*-D-glucopyranoside (**205**)



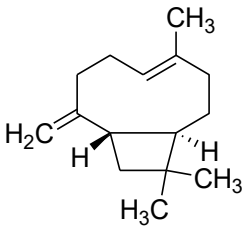
β-Bourbonene (**206**)



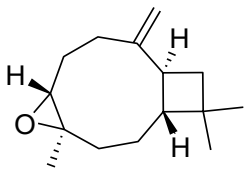
α-Silinene (**207**)



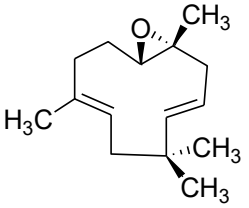
α-Humulene (**208**)



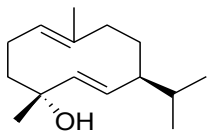
β-Caryophyllene (**209**)



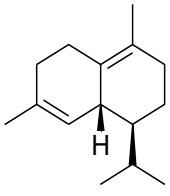
Caryophyllene oxide (**210**)



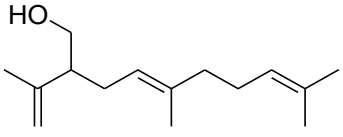
Humulene epoxide II (**211**)



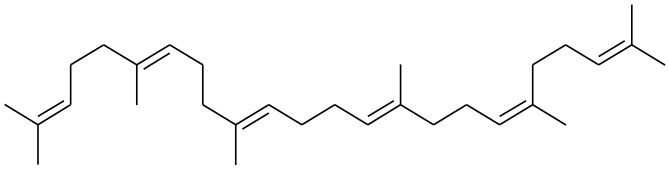
Germacrene D-4-ol (**212**)



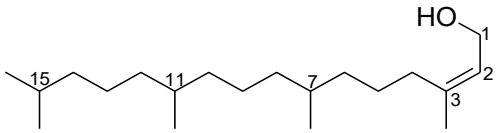
δ- Cadinene (**213**)



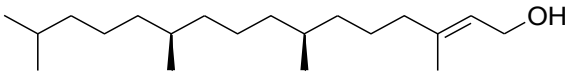
(*E*)-Sesquilavandulol (**214**)



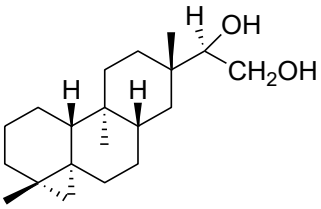
Squalene (**215**)



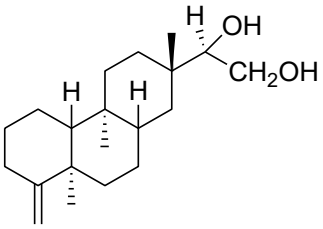
3,7,11,15-Tetramethyl-2-hexadecen-1-ol (**216**)



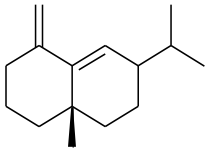
Phytol (**217**)



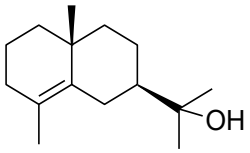
Erythroxydiol X (**218**)



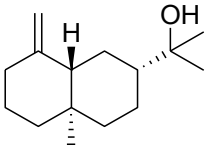
Erythroxydiol Y (**219**)



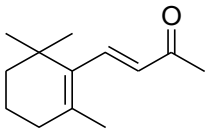
cis-Eudesma-6,11-diene (**220**)



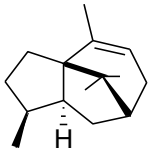
ϒ-Eudesmol (**221**)



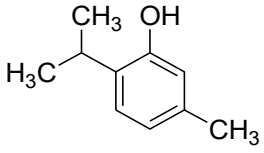
β-Eudesmol (**222**)



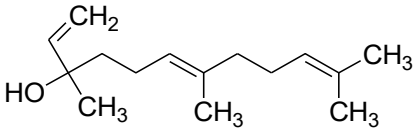
(*E*)-*β*-Ionone (**223**)



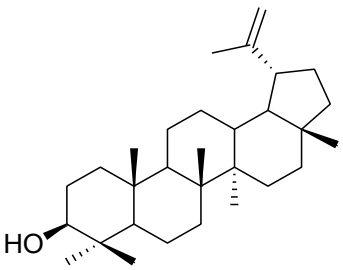
Patchoulene (**224**)



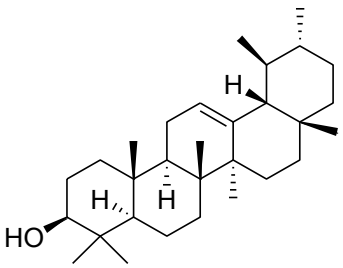
Thymol (**225**)



(*E*)-Nerolidol (**226**)



Lupeol (**227**)



α-amyrin (**228**)

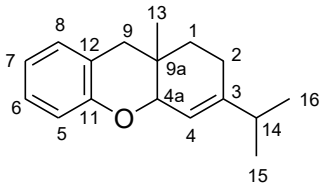
Figure 1.22. Terpenes and terpenoids identified in genus *Indigofera*

The *n*-hexane extract of whole plant of *I. aspalathoides* was chromatographed over silica gel as stationary phase**.** As a result, six compounds were isolated. NMR analysis of one of these compounds showed it was a triterpene and the overall structure was that of α-amyrin (**228**) (Figure 1.22) (**Saraswathy *et al.*, 2013**).

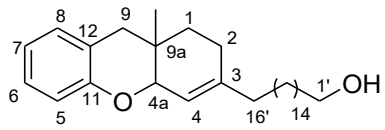
1.3.23. Xanthenes

An important study was carried out by **Thangadurai *et al.*, (2001a)** on the stem of *Indigofera longeracemosa.* Consequently, a colorless substance was isolated. Structural analysis by UV, ^{1}H NMR, ^{13}C NMR, EIMS and HREIMS studies revealed that the colorless substance was a tricyclic compound in which a benzene ring was fused with the pyran ring and the pyran ring was sharing one of its bonds with a cyclohexene ring. The xanthene was novel to the genus because of having a methyl and an isopropyl as substituent. The overall compound was identified as 3-isopropyl-9a-methyl-1, 2, 4a, 9a-tetrahydroxanthene (**229**) (Figure 1.23).

The phytochemical study on methyl alcohol extract of whole plant of *Indigofera oblongifolia* was made by **Sharif *et al.*, (2005**). The methanol extract of dried plant material was fractioned between *n*-hexane and water followed by extraction of aqueous layer with chloroform. This chloroform fraction was submitted to silica gel column. Finally, a white gummy solid was obtained. The spectroscopic data obtained from UV, IR, ^{1}H NMR ^{13}C NMR and HREIMS showed that the gummy solid was a compound of molecular formula $C_{30}H_{48}O_2$, and its structure had three rings fused together. Moreover, a trisubstituted olefinic double bond was indicated. The overall evidence revealed that the molecule had a core structure of xanthene with 16 C chain as substituent, so it was named as 3-(1′-hydroxydecanyl)-9a-methyl-1,2,4a,9a-tetrahydroxanthene or indigin (**230**) (Figure 1.23).



3-Isopropyl-9a-methyl-1,2,4a,9a-tetrahydroxanthene (**229**)

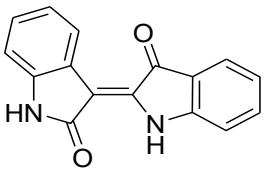


3-(1′-Hydroxydecanyl)-9a-methyl-1,2,4a,9a-tetrahydroxanthene (Indigin) (**230**)

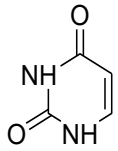
Figure 1.23. Xanthenes identified in genus *Indigofera*

1.3.24. Miscellaneous compounds

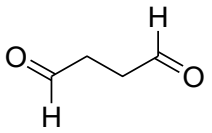
Aourz *et al.*, (2019) prepared DMSO extract of leaves of *Indigofera arrecta.* Column chromatography of the extract over silica gel 60 gave a compound identified as indirubin (**231**) (Figure 1.24).



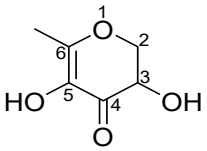
Indirubin (**231**)



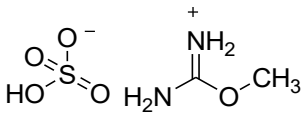
Uracil (**232**)



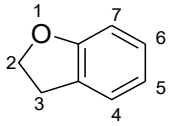
Butanedial (**233**)



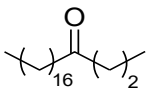
2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-Pyran-4-one (**234**)



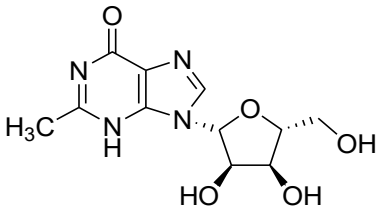
O-Methyl-iso-urea hydrogen sulphate (**235**)



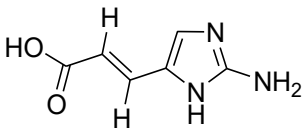
2,3-Dihydrobenzofuran (**236**)



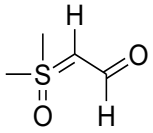
Heneicosan-4-one (**237**)



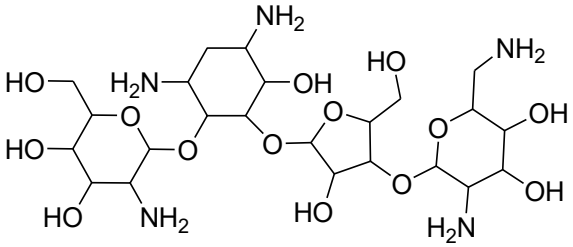
2-Methyl-9-*β*-D-ribofuranosyl hypoxanthine (**238**)



2-Amino-5[(2-carboxy) vinyl]-imidazole (**239**)



Dimethyl-sulfoxonium formyl-methylide (**240**)



5-Amino-2-(aminomethyl)-6-[5-[3,5-diamino-2-[3-amino-4,5-dihydroxy-6(hydroxymethyl)oxan-2-yl] oxy-6-hydroxycyclohexyl] oxy-4-hydroxy-2(hydroxymethyl)oxolan-3-yl] oxyoxane-3,4-diol (**241**)

Figure 1.24. Miscellaneous compounds identified in genus *Indigofera*

The HPLC-ESI-MS analysis of water and methanol extracts of *Indigofera hirsuta* led to the identification of uracil (**232**) (Figure 1.24) which is one of the four nitrogenous bases that are part of RNA (**Moura *et al.*, 2011**).

The phytoconstituents in chloroform and methanol extract of *I. cordifolia* seeds were identified by the help of GC-MS. Four of the total compounds in hydro-methanolic extract were a dialdehyde of 4 C chain length (**233**), a pyran-4-one derivative substituted with a methyl and two hydroxyl groups (**234**), a salt of *O*-methyl-iso-urea with sulfuric acid (**235**) and 2,3-dihydrobenzofuran (**236**) (Figure 1.24) (**Deshpande *et al.*, 2013**).

The chromatographic separation of *n*-hexane extract of whole plant of *Indigofera aspalathoides* gave a compound of 21 C chain length**.** IR, UV, and NMR analysis showed that the compound was a ketone with carbonyl at C_4 of the main chain (**237**) (**Saraswathy *et al.*, 2013**) (Figure 1.24).

The aqueous extract of leaves of *Indigofera tinctoria* was qualitatively analyzed by **Prashanth *et al.*, (2018**). Gas chromatographic analysis of the extract led to the identification of compounds **238**, **239**, **240** and **241** (Figure 1.24).

Various classes of compounds identified in genus *Indigofera* have been summarized in figure 1.25.

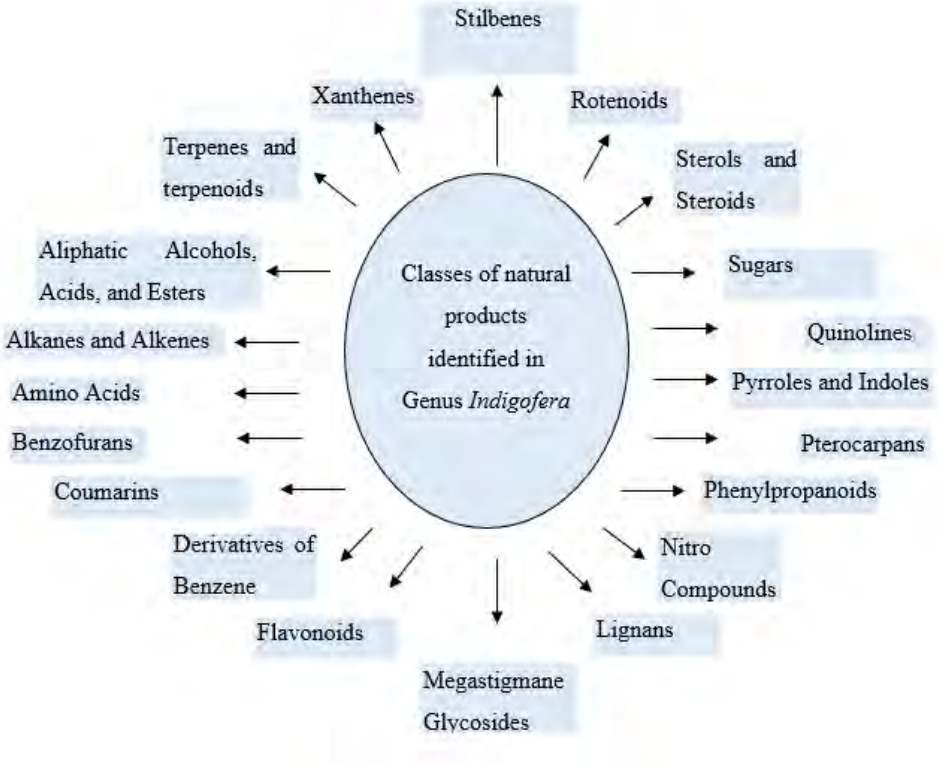
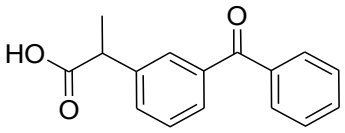


Figure 1.25. Summary of classes of natural products identified in genus *Indigofera*

1.4. Biological activities

1.4.1. Analgesic activity

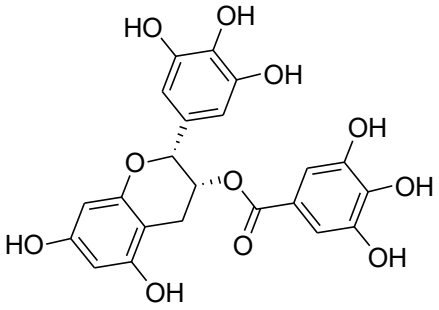
The analgesic or pain-relieving activity of 2′, 4′-dihydroxy-4-prenyloxy chalcone (**67**) was studied by **Musa *et al.*, (2011**). This flavonoid was isolated from the ethyl-acetate fraction of MeOH extract of the whole plant of *I. pulchra.* The analgesic effect shown by 10 mg/kg chalcone against acetic acid induced writhing in mice was 82.7 % that was very close to 88.0 % inhibitory effect produced by 10 mg/kg of ketoprofen (**242**) used as standard analgesic drug in the experiment. The chalcone was proved to be peripheral analgesic agent as it showed no analgesic effect in hot plate method which is used to test central analgesic activity.



Ketoprofen (**242**)

1.4.2. Anti-aging effect

The activity of methanol extract of leaves of *Indigfera tinctoria* L. was studied by **Ranaweera *et al.,*** (**2015**) using spectrophotometry**.** Various concentrations of the extract were investigated *in vitro* for effect against hyaluronidase, an enzyme involved in aging of the skin. It was found that the extract had no effect on this enzyme while the anti-hyaluronidase effect of epigallocatechin gallate (EGCG) used as reference compound, increased with increasing concentration of the compound.



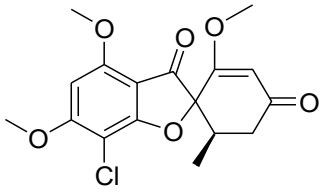
Epigallocatechin gallate (**243**)

It was observed that 12.5 µg/mL of EGCG caused 7.99 % while 200 µg/mL showed 92.9 % inhibition of the enzyme. On contrary, the extract of *I.* tinctoria showed -7.62 to -18.02 % inhibition when 0.19 mg/mL to 3 mg/mL extract was used.

1.4.3. Antibacterial and antifungal activities

Seed plate method was used to check antibacterial potential of protein fractions obtained from defatted leaves of *Indigofera oblongfolia.* The activity was tested against bacterial strains of *E. coli, K. aerogenes, S. aureus, Kl. pneumonia* and *B. subtilis.* Similarly, inhibitory potential of small protein fractions of *I. oblongifolia* leaves was also examined against fungal strains of *A. niger, A. fumigatus, A. flavus* and *P. expansum* by diffusion plate method. It was found that two of the fractions (AP2 and AP4) had moderate antibacterial effect. However, one of the fractions (AP3) completely inhibited the growth of *B. subtilis* (bacterial strain) and *A. niger* (fungal strain) with inhibition zones of 20 mm. The standard antibacterial drug Enaxbid also produced 20 mm inhibition zone against *B. subtilis* and Griseofulvin produced 20 mm inhibition zone against *A. niger*. The fraction AP4 also showed significant inhibition of *A. niger* and *A. flavus* with inhibition zones of 5 mm. It was comparable to 20 mm and 5 mm

inhibition zones produced by Griseofulvin against *A. niger* and *A. flavus* respectively (**Dahot, 1999**).



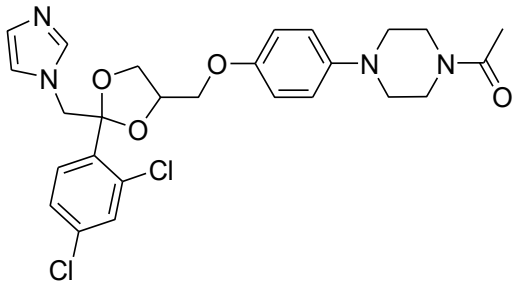
Griseofulvin (**244**)

Esimone *et al.,* (1999) studied the antimicrobial effect of various extracts including fractions of methanol extract of *Indigofera dendroides* leaves. The data obtained by employing disc diffusion method showed that two of the methanol fractions (Mn3 and Mn7) were relatively more active than all the extracts studied against *S. aureus, B. subtilis, E. coli, S. typhimurium, K. pneumoniae, P. aeruginosa, C. albicans* and *A. niger*. It is important to note that the MIC values for extracts determined by agar diffusion method were much higher than Gentamycin taken as positive control. Hence, this study could not establish the antimicrobial significance of *I. dendroides* leaves. The MIC values for Mn3 and Mn7 ranged from 1400 to 4600 µg/mL as against 15 to 17 µg/mL for standard antibacterial drug Gentamycin.

Thangadurai *et al.,* (2002) investigated the antibacterial and antifungal activity of *Indigoferabietone* (**203**), a compound isolated from the extract of *Indigofera longeracemosa.* The compound showed notable effect against *Mycobacterium tuberculosis* and was also proved to be moderately antifungal in action.

Leite *et al.,* (2006) prepared the extracts of leaves of *Indigofera suffruticosa* in water and other organic solvents. The extracts were investigated for activity against various bacterial and fungal strains using agar-solid diffusion method. All the extracts obtained by "maceration" were inactive against tested strains. The aqueous extract prepared by "infusion" showed little inhibition against *Staphylococcus aureus* as compared to chloramphenicol taken as positive control. While the aqueous extract showed significant inhibition against *Microsporum canis* which was comparable to ketoconazole taken as standard antifungal agent. The inhibition zone of 12-14 mm for *Trichophyton rubrum,* a dermato-phytic fungus, with MIC of 2500 µg mL^{-1} extract as compared to 20 mm inhibition zone produced by 1000 µg mL^{-1} of positive control

represents remedial efficacy of aqueous extract of *I. suffruticosa* against skin ailments such as athlete's foot.



Ketoconazole (**245**)

The antibacterial activity of extracts of 21 plants including *Indigofera daleoides* was investigated by **Mathabe *et al.,*** (**2006**). Extracts of various parts of all plants in methanol, acetone, ethanol, and hot water were analyzed by agar well diffusion assay. *I. daleoides* was one of the two plants having antibacterial activity highest of all plants under study. The organic extracts of this plant inhibited growth of all tested bacteria. A low value of MIC= 0.039 mg mL^{-1} showed high activity of ethanol extract of *I. daleoides* against *S. aureus, V. cholera* and *S. dysentery.*

Bhuvaneswari and Balasundaram (**2006**) investigated the efficacy of ethanol extract of five plants including *Indigofera aspalathoides* against bacteria commonly found in fish and named as *Aeromonas hydrophila.* Results showed MBC (minimum bacterial concentration) of 1.00 cfu at 1.29 mg mL^{-1} of *I. aspalathoides* extract against MBC value of 16.33 cfu. shown by 1.29 mg mL^{-1} of terramycin used as positive control. The extract inhibited growth of *A. hydrophila* showing an effect stronger than terramycin, a common drug used to control bacterial strain of *A. hydrophila*.

Inagaki *et al.,* (**2008**) worked on the aqueous extracts of 203 plant species and tested their effect against *C. lagenarium* which causes anthracnose infection in cucumber. *Indigofera pseudotinctoria* was one of the plants which showed more than 90% rate of inhibition against the fungal strain *C. lagenarium.*

The antibacterial effect of two dihydro-stilbenes (**197**, **198**) obtained from the methanol extract of whole plant of *Indigofera pulchra* was studied by **Musa *et al.,*** (**2008**). The study carried out against *S. aureus* by using minimum inhibitory concentration assay with MIC = 128-256 µg mL^{-1} showed that the compounds were

weakly active against the strains of *S. aureus*. The MIC value of standard drug norfloxacin ranged from 1-32 µg mL^{-1} against different strains of *S. aureus*.

Uddin G *et al.,* (2011) studied the antibacterial and antifungal activities of aqueous methanol extract of aerial parts of *Indigofera heterantha* by hot diffusion method. The extract and its fractions in *n*-hexane, methanol and ethyl acetate failed to inhibit growth of *E. coli, B. subtilis, S. flexeneri, S. aureus, P. aeruginosa* and S. typhi bacterial strains. They were also inactive against fungal strains of *T. longifusis, C. albicans, A. flavus, M. canis, F. solani* and *C. glaberata.* Only M_2 fraction of methanol showed little inhibitory potential against *M. canis.*

Renukadevi and Sultana (2011) determined the antibacterial tendency of methanol extract of *Indigofera tinctoria* by agar well diffusion method. Significant zones of inhibition were formed against gram positive bacteria (*B. pumilus, S. aureus* and *S. pyogenes*). Contrarily, there was no inhibitory effect on the growth of gram-negative bacteria (*E. coli* and *P. aeruginosa*). The observed zones of inhibition were 16, 17 and 17 mm against *B. pumilus*, *S. aureus* and *S. pyogenes*, respectively.

The antibacterial activity of various extracts of *Indigofera subulata* Vahl. was evaluated against *B. subtilis, E. coli, K. pneumoniae* and *S. typhi.* Ethanol extract proved to be most active by MIC= 100 µg mL^{-1} against *S. aureus* and *P. aeruginosa.* The zones of inhibition by ethanol extracts of stem and leaves were >10 mm against both the microorganisms (**Vinoth *et al.,* 2011**).

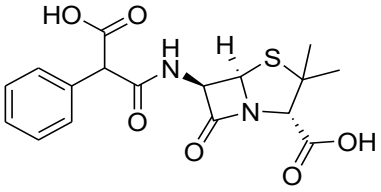
The petroleum ether, dichloromethane, ethanol, and aqueous extracts of roots and leaves of *Indigofera arrecta* were assayed for antibacterial activity by **Madikizela *et al.,* (2013)**. All the extracts showed significant antibacterial potential. However, the ethanol extract of leaves was most active and showed activity with MIC=0.39, 0.39 and 0.78 mg mL^{-1} against bacterial strains of *S. aureus, M. aurum* and *K.* pneumonae, respectively. The value was close to MIC= 0.079 mg mL^{-1} shown by the standard drug neomycin against *S. aureus* and *K. pneumonae*.

The activity of ethanol and chloroform extract of whole plant of *Indigofera linifolia* leaves was studied by **Uvarani *et al.,* (2012)**. Disc diffusion method was employed for this study. The extracts of 200 µg mL^{-1} showed inhibition zones against *S. pneumoniae* and *S. aureus* comparable to that produced by 10 µg mL^{-1} of

Ciprofloxacin. They also showed significant inhibition zones against *E. coli* and *S. typhi.*

Kumar *et al.,* (2013) determined *in vitro* the activity of various extracts of the whole plant of *Indigofera trita* against three gram-positive bacteria and sixteen gram-negative bacteria. Moreover, anti-fungal potential of the extract was also tested against two fungal strains. Results obtained by applying disc diffusion method revealed that all extracts showed significant antibacterial activity. The antifungal activity was less as compared to antibacterial activity.

Rahman *et al.,* (2014a) isolated indigoferamide-A (**104**) from the methanol extract of *Indigofera heterantha* Wall and studied its inhibitory effect on strains of *E. coli* and *B. subtillis*. It was observed that growth of bacteria was decreased with increase in concentration of the compound and concentration of 500 µg mL^{-1} showed maximum inhibitory effect. At higher concentrations, results were close to carbenicillin, a standard antibacterial drug.



Carbenicillin (**246**)

The antibacterial activity of acetone extract of leaves of *Indigofera cylindrica* and eight other plant species was studied by **Dzoyem *et al.,* (2014)**. Results obtained from serial microdilution method showed that *I. cylindrica* showed significant antibacterial activity against *S. aureus, E. faecalis, B cereus, E. coli* and *P. aeruginosa.* The most remarkable inhibitory effect of *I. cylindrica* with MIC= 40 µg mL^{-1} was observed for the bacterial strain, *Salmonella typhimurium.* The result was comparable to MIC= 1.56 µg mL^{-1} for Gentamicin against the same strain.

The antibacterial effect of various extracts of *Indigofera suffruticosa* was evaluated against *Staphylococcus aureus.* All the extracts exhibited antibacterial potency, but acetone extract was most active with MIC_{50} value of 1.56 mg/mL. The acetone and chloroform extracts combined with erythromycin were found to enhance the effect of this antibacterial drug. This was indicated by the average FIC values of

ether, chloroform and acetone extracts combined with standard antibacterial drug erythromycin as 0.81, 0.68 and 0.64 respectively (**Bezerra dos Santos *et al.*, 2015**).

Photo-dynamic therapy (PDT) is a technique in which a photoactive substance having ability to absorb light of required wavelength (600-800nm) accumulates in a target tissue where it reacts with oxygen and generates reactive oxygen species like superoxide (type I mechanism) and singlet oxygen (type II mechanism). As a result, oxidation mechanisms lead to death of microbial cells. The crude extracts (*n*-hexane and methanol), alkaloid fraction and purified compound indigo obtained from aerial parts of *Indigofera truxillensis* were studied for PDT against bacteria and yeast. All of them showed photodynamic action through type I mechanism against gram positive and gram-negative bacteria. They were also effective against *Candida sp* yeast strains but slightly inhibited the growth of *Candida dubliniensis* (**Andreazza *et al.*, 2015**).

Bhaskar *et al.*, (2016) studied the antibacterial effect of crude methanol extract, quercetin (**64**), pinitol (**201**) and chromatographic fractions obtained from the ethyl acetate extract of whole plant of *Indigofera barberi.* The antibacterial efficacy evaluated against Gram negative bacteria like *P. aeruginosa, E. coli, K. pneumonia* and *Citrobacter* sp. by using microdilution doubling method showed that all fractions and compounds were active against bacteria under study. The most potent fraction (IB-E) and **64** showed significant activity against *Citrobacter sp* as indicated by MIC= 62 µg/mL. The MIC value of 62 µg/mL was also shown by standard antibacterial drugs streptomycin and erythromycin against *Citrobacter sp*. Molecular docking study proved strong inhibitory interactions of quercetin with DNA gyrase B present in gram negative bacteria.

Rajagopal *et al.*, (2016) evaluated the antibacterial activity of hydro-methanolic extract of *Indigofera aspalathoides* stem and various parts of nine other plants. The activity was determined against fish pathogen *Aeromonas hydrophila* by agar well diffusion method and agar dilution method. The extract of *I. aspalathoides* showed antibacterial activity with MIC = 2.16 mg mL^{-1} and zone of inhibition=14.01 mm. The effect was stronger than terramycin used as positive control. The zone of inhibition produced by 3.7 mg mL^{-1} of terramycin was 12.17 mm.

The antimicrobial efficacy of aqueous extract of leaves of *Indigofera tinctoria* and its silver and gold nanoparticles had been studied by **Vijayan *et al.*, (2018**). Agar

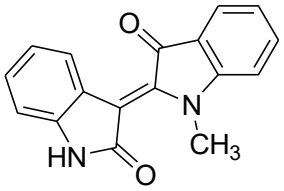
well diffusion method was employed for this work. Significant inhibition zones were produced against strains of fungi, gram-positive and gram-negative bacteria. However, the effect was more pronounced against gram negative bacteria than gram-positive bacteria. Thus *I.* tinctoria extract can be a good source of phytoconstituents with significant antimicrobial potential.

Netala *et al.,* (**2018**) studied antibacterial and antifungal activities of nanoparticles of *Indigofera hirsuta* by disc diffusion method. The nano particles inhibited growth of Gram positive as well as Gram negative bacteria. The highest activity with MIC=3.9 µg mL^{-1} was observed against *B. subtilis*. The effect against *S. aureus*, *P. aeruginosa* and *E. coli* was produced with MIC values of 7.8 µg mL^{-1}, 15.6 µg mL^{-1} and 15.6 µg mL-1, respectively. The zones of inhibition produced by silver nano particles of *I. hirsuta* were 12-17 mm against different microorganisms and those produced by *I. hirsuta leaf* extract were 4-7 mm. The standard drugs streptomycin and voriconazole showed inhibition zones of 18-23 mm.

Reddy *et al.,* (**2019**) prepared aqueous extract of leaves of the *Indigofera barberi* and synthesized silver nanoparticles from it. The tendency of nanoparticles of *I. barberi* to inhibit growth of bacteria was studied by using disc diffusion method. The inhibition zones of 12.3 and 11.2 mm were observed against Gram positive-bacteria *S. aureus* and *B. subtilis*, respectively. Similarly, Gram negative bacteria *E. coli* and *K. pneumonia* were inhibited resulting in zones of 9.8 and 8.7 mm, respectively. These results represent higher potential of Ag-NPs of *I. barberi* extract against Gram-positive bacteria than Gram-negative bacteria.

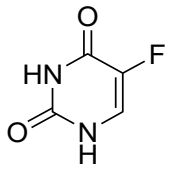
1.4.4. Anticancer activity

Han (**1994**) wrote a review article about anticancer drugs of phytochemical origin in China. According to this article, *Indigofera tictoria* was a part of traditional Chinese pills made from eleven herbs. The pills were used against chronic myelocytic leukemia (CML). The plant was later found to possess strong anticancer effects due to indirubin (**231**) present in it. Indirubin pioneered the synthesis of less toxic, second-generation anticancer drug *N*-methyl isoindigotin.



N-methyl isoindigotin (**247**)

The ethanol extract of whole plant of *Indigofera qspalathoides* was investigated for effect against Dalton′s ascitic lymphoma. The mice bearing DAL-cells were treated with intraperitoneal injection of *I. aspalthoides* extract for ten days while the positive control group was given anticancer drug 5-fluorourasil and the negative control was administered with water only. Study of various parameters led to the conclusions that treatment with extract brought 36.85 % decrease in the cancer cell count of mice relative to the negative control group. Similarly, tumor inhibition, recovery of body weight and life span increase were 25.4, 70.9 and 71.9 % respectively **Christina *et al.,*** (**2003**).



5-Fluorouracil (**248**)

Rajkapoor *et al.,* (**2004**) worked on the ethanol extract of stem of *Indigofera aspalathoides* to investigate its effect against Ehrlich ascites carcinoma (EAC) maintained in Swiss albino mice. The rats were given oral dose of 250 mg/kg/day of *I aspalathoides* extract. It was observed that the extract not only increased the life span of extract treated tumor models but also recovered peritoneal cell count to normal levels and reduced the volume of tumor contradicting to the untreated tumor bearing mice.

The aqueous extracts of leaves of *Indigofera suffruticosa* were examined for antitumor activity by **Vieira *et al.,*** (**2007**). The extracts prepared by infusion and maceration reduced the mean volume of Sarcoma 180 cells by 64.53% and 62.62% respectively when injected with a dose of 50 mg/kg. On contrary, the tumor of control group showed 100% growth. Lack of cytotoxicity against HEp2-cell line proved the selective anticancer potential of *I. suffruticosa* extract against tumor cells over healthy cells.

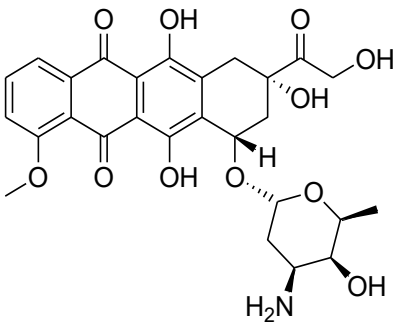
The methanol extract of defatted plant material of *Indigofera aspalathoides* was studied for anticancer activity. Swiss albino mice bearing Ehrlich Ascites Carcinoma

were administered with varying oral doses of the extract up to nine days. As a result, the extract decreased the volume of carcinoma and increased survival time of mice. Other hematological parameters were also improved in extract treated animals compared to untreated or control group. (**Gupta *et al.*, 2007**).

A flavonol glycoside, 5, 4′-dihydroxy-6, 8-dimethoxy-7-*O*-rhamnosyl flavone (**66**) was isolated from the alcoholic extract of *Indigofera aspalathoides* stems. The glycoside was screened *in vitro* for cytotoxic activity against 57 cancer cell lines. Results obtained by using Sulpho-rhodamine B assay showed that the GI_{50} value ranged between 4.06-4.81 μM. The compound was selectively active against cancer cells especially SK-OV-3 and NCI/ADR-RES cell lines of ovarian and breast cancer (**Balasubramanian *et al.*, 2007**).

Kameswaran and Ramanibai (**2008**) evaluated the methanol extract of aerial parts of *Indigofera tinctoria* for activity against human lung cancer cell line A-549. They used MTT assay, flow cytometric analysis and propidium iodide staining for this study. Experiments revealed that flavonoid rich fraction obtained from the methanol extract of *I. tinctoria* inhibited cell cycle in A-549 cells at G0/G1 phase and provoked apoptosis in them.

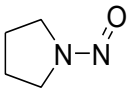
The antitumor effect of alkaloid fraction and indigo obtained from the methanol extract of aerial parts of *Indigofera suffruticosa* was studied by **Lopes *et al.*,** (**2011**). The effect was studied against two tumor cell lines, LM2 (breast adenocarcinoma) and LP07 (lung adenocarcinoma). Alkaloidal fraction and indigo produced significant antitumor effect. The IC_{50} values for alkaloidal fraction against both tumor cell lines were almost equal to that of doxorubicin used as standard antitumor drug in the experiment. IC_{50} values of 0.89 and 1.44 μg mL^{-1} were shown by pure indigo against LM2 and LP07 adenocarcinoma cells. It could be compared to IC_{50} values shown by doxorubicin as 0.83 and 0.52 μg mL^{-1} against LM2 and LP0 cells, respectively.



Doxorubicin (**249**)

The anticancer effect of methanol extract of leaves of *Indigofera tinctoria* was evaluated by **Renukadevi** and **Sultana** (**2011**). The experiment was performed on NCIH69 cancer cell line using MTT assay. It was observed that a concentration of 250 µg mL^{-1} showed 46% cell viability. Overall results proved the dose dependent cytotoxic effect of *I. tinctoria* extract against NCIH69 cells.

Agarwal and Sharma (**2017**) investigated the effect of a flavonoid, 3,6-dihydroxy-(3′,4′,7′-trimethoxyphenyl) chromen-4-one-7-glucoside on events taking place in cancer caused by *N*-nitroso-pyrrolidine. This flavonoid obtained from the hydro-methanolic extract of *Indigofera tinctoria* restored the activity of antioxidant enzymes and biomarkers specific for preventing lung cancer in experimental mice. Thus, it can be considered a promising anticancer agent for future studies on this aspect.



N-Nitroso pyrrolidine

(**250**)

Vijayan *et al.,* (**2018**) prepared silver and gold nanoparticles from aqueous extract of leaves of *Indigofera tinctoria.* Cytotoxic potential was checked against lung cancer cell line A549 using MTT assay. Shrinking of cancer cells proved noteworthy cytotoxic tendency of extract, silver-NPs, and gold-NPs with IC_{50} values of 71.62 µg mL^{-1}, 56.62 µg mL^{-1} and 59.33 µg mL^{-1}, respectively.

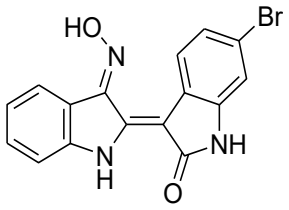
A study was conducted by **Prashanth *et al.,*** (**2018**) on ZnO nanoparticles made from aqueous extract of leaves of *I. tinctoria.* The anticancer activity of nanoparticles using DU-145 and Calu-6 cells and biocompatibility in terms of hemolytic effect was determined. It was demonstrated that nanoparticles of ZnO (MA-2) exhibited high anticancer potential with IC_{50} values of 34.06 µg mL^{-1} on DU-145 cells and 59.67 µg mL^{-1} on Calu-6 cells. The biocompatibility of nanoparticles was also good but at low concentrations.

The cell cycle inhibiting potential of chrysin isolated from chloroform extract of leaves of *Indigofera tinctoria* was studied against human epidermoid carcinoma A431 cells using MTT assay. Chrysin showed selective antiproliferative effect with IC_{50}= 23.52 µg mL^{-1} against A431 cells and caused no harm to normal HaCaT cells.

Further studies revealed that chrysin resulted in death of cancer cells by apoptosis and arrested the cell cycle in G_1 phase (**Boothapandi and Ramanibai, 2018**).

Netala *et al.,* (2018) synthesized nanoparticles of leaves of *lndigofera hirsuta* L. These nanoparticles prepared from aqueous leaf extract remarkably decreased the viability of cancer cells being harmless to normal cells. The IC_{50} values ranged between 65-90 μg mL^{-1} against different cancer cell lines under study.

Plants of *Indigofera* genus are a natural source of indirubin (**231**), an anticancer agent. **Tchoumtchoua *et al.,* (2019)** studied the pharmacokinetics of 6-bromo indirubin-3′-oxime (6BIO) in mice using UHPLC-MS/MS technique. Results obtained by analysis following oral dose of 50 mg kg^{-1} indirubin showed that it was rapidly eliminated from the blood circulatory system and had shown less bioavailability. The half-life time ($t_{1/2}$) was calculated to be 0.72 h.



6-Bromo indirubin-3′-oxime (6BIO) (**251**)

1.4.5. Antidiabetic activity

Aqueous extract of *Indigofera arrecta* was tested for effect against non-insulin dependent diabetes (type II). Experiment was performed on obese-diabetic and non-diabetic mice. Control groups were given water for 90 days while experimental groups were fed with aqueous extract of *I. arrecta*. At intervals of one month, five mice from each group were killed and their blood glucose and body weights were examined and compared. The extract prevented any further increase in blood glucose levels of diabetic mice. But it had no hypoglycemic effect on non-diabetic mice. The extract was also effective in decreasing the body weight of obese diabetic mice (**Addy *et al.,* 1992**).

Sittie *et al.,* (1998) evaluated the safe effect of aqueous extract of leaves of *Indigofera arrecta* on non-diabetic healthy individuals. The individuals were given an oral dose of the extract three times a day up to six weeks. It was revealed that the extract, commonly known to cure diabetes, has no effect on normal glucose levels in healthy individuals. Levels of serum enzymes and metabolites in the body also remained normal

which proves that extract has no harmful effect on liver cells. However, the extract provoked erythrocyte sedimentation rate and lymphocyte count was reduced in blood. There was no apparent toxic effect of the *I. arrecta* extract.

Nyarko *et al.,* (1993) investigated the hyperglycemic effect of aqueous extract of leaves of *Indigofera arrecta* in fasting diabetic and non-diabetic rats. Streptozotocin was used to provoke diabetes in rats and treatment dose of plant extract was scheduled in the form of oral intakes and intra-peritoneal injections. Results showed a 60% reduction in amount of plasma glucose in fasting non-diabetic rats but the amount of glucose in blood was not lowered after an oral intake of large amount of glucose. The dose of plant extract after 17 days of provoking diabetes was ineffective but it showed anti-hyperglycemic effect when given after 7 days of diabetes induction.

Chakrabarti *et al.,* (2006) worked on the ethanol extract of whole plant of *Indigofera mysorensis.* An oral dose of 300 g kg^{-1} was given to diabetic mice non-sensitive to insulin. A noteworthy decrease in plasma glucose, triglyceride and insulin was observed after 10 days. The plasma values of plasma glucose (mg/dl), plasma triglyceride (mg/dl) and insulin (ng/ml) were 207, 101 and 6 for *I. mysorensis* extract, while 308, 63 and 12 for troglitazone, respectively. This showed that antidiabetic effect of *I. mysorensis* extract was even stronger than standard drug troglitazone. The antidiabetic mechanism of action of *I. mysorensis* seems to be acting through insulin sensitization.

The *α*-glucosidase inhibitory activity of ethyl acetate and *n*-butanol extract of *Indigofera stachyodes* roots was evaluated by **Li *et al.,* (2011)**. The observed effect for ethyl acetate (IC_{50} = 14.31 µg mL^{-1}) and *n*-butanol (IC_{50}= 7.01 µg mL^{-1}) extracts was much higher than that for acarbose taken as standard. A seven-day experiment was also conducted to check *in vivo* antidiabetic effect of *I. stachyodes* extracts in alloxan induced diabetic rats. The antidiabetic effect was good as indicated by reduction in blood glucose level, increase in concentration of glycogen in liver, elevation of amounts of superoxide dismutase and lowering of cholesterol, triglyceride and malondialdehyde in diabetic rats as compared with control groups.

Vadivel *et al.,* (2011) processed seeds of various leguminous plants including *Indigofera linifolia* by cooking, oil frying or roasting. The *α*-amylase and *β*-glucosidase inhibition activities of all plants were studied by comparing with commercially

available antidiabetic compound acarbose. It was found that oil fried seeds showed highest activity with α-amylase and β-glucosidase inhibition activities of 19.48% (acarbose = 15.68%) and 41.25% (acarbose = 44.54%) respectively. This represents notable antidiabetic potential of methanol extract of *Indigofera linifolia* seeds.

The antidiabetic effect of *n*-butanol fraction obtained from ethanol extract of *Indigofera cordifolia* seeds was investigated by **Deshpande *et al.,*** (**2014**). The extract was fed to rats having streptozotocin induced diabetes. Blood analysis of rats after fourteen days revealed that all the hormonal changes produced in untreated-diabetic rats were reversed in treated-diabetic rats. Thus, levels of glucose, total cholesterol and hormones which increased to cause hyperglycemia was decreased to normal *in vitro* by plant extract. Moreover, activity of α-amylase and α-glucosidase which are carbohydrate hydrolyzing enzymes, is also retarded.

Birru (**2015**) studied the effect of hydroalcoholic extract of *Indigofera spicata* on amount of glucose in normal, oral glucose loaded (OGL) and diabetic mice. Significant reduction of blood glucose was observed in diabetic mice as compared to normal and OGL models. Also, the anti-diabetic effect was greater in post treated animals than pre-treated group.

Rahman *et al.,* (**2019**) studied *in vitro* the antiglycation effect of *Indigoferamide*-A (**104**) isolated from the methanol extract of *Indigofera heterantha* wall. A 1000 µM solution of *Indigoferamide*-A executed 40% α-glucosidase inhibition activity as compared to 83% inhibition produced by an equal amount of rutin taken as standard. It was concluded from molecular docking study that Thr172 on active site of α-glucosidase develops strong interaction with the compound under study. This α-glucosidase inhibition can decrease blood glucose levels, in turn preventing glucose binding with proteins (glycation) and inhibit diabetes from developing more complexities.

1.4.6. Antidiarrheal activity

The hydro-methanolic extract of roots of *Indigofera spicata* was evaluated for antidiarrheal activity in male Swiss albino mice. Treatment of three groups of castor oil induced diarrheal models with 100, 200 and 400 mg kg^{-1} extract doses, resulted in decreased fluid content in feces. Frequency of defecation was also decreased but antisecretory and antimotility activities were negligible (**Birru *et al.,* 2016**).

1.4.7. Anti-dyslipidemic activity

The anti-dyslipidemic activity of three furano-flavones (**60**, **61**, **62**) and a flavonol glycoside (**59**) isolated from the extract of aerial parts of *Indigofera tinctoria.* was studied by **Narender *et al.,*** (**2006**). Data obtained from the treatment of dyslipidemic hamsters with test compounds at dose of 50 mg/kg by weight showed that two of the furano-flavones (**60**, **61**) were most potent and showed 60% decrease in plasma lipids. The third furano-flavone (**62**) was moderately active. This shows good potential of these compounds to cure cardio-vascular diseases.

1.4.8. Antiepileptic activity

The anti-seizure effect of fluid extracts of various parts of *Indigofera suffruticosa* was studied by **De Alejo *et al.,*** (**1996**). The experimental design included an electric shock model of epilepsy, picrotoxin induced epileptic model, diazepam control group and *Indigofera suffruticosa* control group. The extract was ineffective to picrotoxin induced epilepsy but increased the seizure threshold in electric shock model.

Bu Wong *et al.,* (**1999**) conducted experiments on male Wistar rats to study the effect of *Indigofera suffruticosa* extract on concentration of plasma amino acids associated with development or clarification of epileptic seizures. The amino acids were quantified in non-kindled rats, lidocaine induced kindled rats, ethanol induced kindled rats, rats which were given 0.06 g/kg extract during development of kindling model and rats which were given an oral dose of extract 10 day before and during the development of kindling. Results showed that the effect was not time dependent whether the extract was given before or during induction of lidocaine induced kindling. A protective effect of the extract of *Indigofera suffruticosa* at a dose of 0.06 g kg^{-1} was proved by reduction in the concentration of the inhibitory amino acids, taurine, and glycine and by the increase of the excitatory amino acid glutamic acid while variation in amount of other amino acids studied was not considerable.

Asuntha *et al.,* (**2010**) extracted whole plant of *Indigofera tinctoria* with 95% ethanol and studied the effect of this extract against lithium/pilocarpine provoked status epilepticus in male albino rats. The rats were orally administered with different concentrations of plant extract before injecting pilocarpine. The control group of rats with no anti-epileptic drug depicted stage 4 seizures while the rats treated with diazepam did not show seizures beyond stage 2. The extract was effective in decreasing

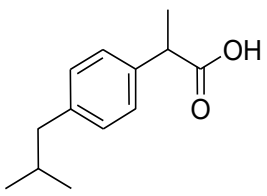
epileptic symptoms and the animals recovered completely after 180 minutes of seizure induction.

The ethanol extract of whole plant of *Indigofera tinctoria* was studied for antiepileptic effect against phenylene tetrazole induced seizures in male albino rats (PTZ) and maximal electric shock models (MES). Treatment of epileptic model animals with various doses of extract showed that it not only delayed the seizures but also reduced their number. In addition, the level of gamma aminobutyric acid (GABA) was increased after administration of 500 or 1000 mg/kg extract. This suggests possible mechanistic pathway of the antiepileptic action of constituents present in the extract (**Garbhapu *et al.*, 2011**).

Leaves of *Indigofera arrecta* were extracted with methanol. This crude extract cured seizures prompted by phenylene tetrazole (PTZ) in zebrafish larvae. Bioassay guided fractionation gave a compound known as "indirubin" (**231**) which showed anticonvulsant action by impeding the activity of glycogen synthase kinase (GSK)-3. Indirubin proved to be effective for decreasing epileptic discharges in zebrafish larvae and rodents taken as model organisms for epilepsy. It is first but most recent report suggesting effectual treatment of epilepsy via GSK-3 target (**Aourz *et al.*, 2019**).

1.4.9. Anti-inflammatory activity

The anti-inflammatory effect of two compounds, indigocarpan (**166**) and mucronulatol (**47**), isolated from the chloroform extract of *I. aspalathoides* was studied by **Selvam *et al.*,** (**2004**). Experiments performed using *in vitro* cyclooxygenase assay showed that the two compounds were significantly active against cyclooxygenase-1 but moderately active against cyclooxygenase-2 enzyme. Indigocarpan showed high inhibitory potential for cyclooxygenase-1 by IC_{50} = 30.5 µM. The compound also resulted in 51.7 % inhibition of rat paw edema at 125 mg/kg dose level as compared to 54.7 % inhibition shown by ibuprofen at dose level of 100 mg/kg. This showed inhibition of carrageenan induced rat paw edema by indigocarpan with higher inhibitory percentage than ibuprofen, standard anti-inflammatory drug used as positive control.

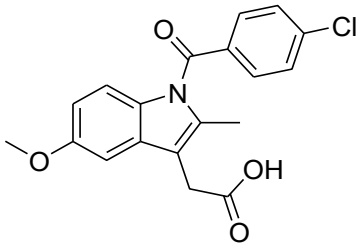


Ibuprofen (**252**)

Rajkapoor *et al.,* (**2009**) investigated the anti-arthritic potential of ethanolic extract of *Indigofera aspalathoides* Vahl stem. The CFA (complete Freund′s adjuvant) induced arthritic rat models were given oral doses of *I. aspalathoides* extract for thirty days. Analysis of rat paw edema at regular intervals revealed that the extract demonstrated anti-inflammatory activity throughout the experimental period. The extract not only prevented development of edema but also reversed the enzymatic changes produced by arthritis contrary to control rats.

Hassan *et al.,* (**2012**) obtained a saponin rich fraction from ethanol extract of leaves of *I. pulchra* Wild. The study conducted on mice by injecting various concentrations of saponin rich extract revealed that LD_{50} was 1264.9 mg/kg which indicates less toxicity. The effect of this extract against inflammation was also studied by rat paw edema test. The ani-inflammatory activity of *I. pulchra* extract (40%) after 4 h was comparable to ketoprofen (63%) taken as positive control.

The ethanol and chloroform extracts of *Indigofera linifolia* leaves were studied for anti-inflammatory activity by **Uvarani *et al.,*** (**2012**). It was found that 200 mg/kg of extract showed inhibition of Carrageenan induced rat paw edema in male Wistar albino rats comparable to that of 10 mg/kg Indomethacin used as standard anti-inflammatory drug.



Indomethacin (**253**)

The methanol extract of *Indigofera aspalathoides* was orally administered to mice bearing carrageenan induced rat paw edema. Biochemical analysis showed that the anti-inflammatory potential of *I. aspalathoides* was very close to indomethacin used as positive control. Likewise, the decrease in lysosomal enzymes and increase in ESR (erythrocyte sedimentation rate) in extract treated animals proves significant anti-inflammatory potential of the *I. aspalathoides* extract (**Bhagavan *et al.,* 2013**).

Chen *et al.,* (**2013**) studied *in vitro* the anti-inflammatory activity of aqueous and ethyl alcohol extracts of *Indigofera suffruticosa.* Lipopolysaccharide (LPS) was

used to induce inflammation in RAW 264.7 macrophage cells. Each of the two extracts proved to be effective by reversing LPS generated increase in the levels of different inflammatory factors such as nitric oxide (NO), tumor necrosis factor-α, pro-interleukin-1β and contraindication of NF-kB activation in these cells. In addition, the extract also stimulated anti-inflammatory factors such as heme oxidase (HO-1) expression in this study.

Seven compounds were isolated from the ethanol extract of roots of *Indigofera stachyodes*. These constituents were assessed for their potential to prevent nitric oxide (NO) generation in lipopolysaccharide-activated BV-2 microglial cells. Three of these compounds which were identified as Stachyodin B, 4, 4′-dihydroxy-2′-methoxy-chalcone and 4, 2′, 4′-trihydroxychalcone showed remarkable antioxidant activity with IC_{50} values of 65 µM, 8.4 µM and 13.2 µM respectively (**Zhang *et al.,* (2018**).

The work of **Boothapandi and Ramanibai** (**2019**) showed that immunomodulatory efficacy of chrysin present in *Indigofera tinctoria* extract is most probably due to its anti-inflammatory action. The expression of pro-inflammatory factors was decreased by chrysin. Thus, translocation of NF-kB protein which causes ROS production, tissue damage and inflammation, was inhibited by chrysin.

The anti-inflammatory efficacy of whole plant of *Indigofera linnae* Ali. was studied *in vitro* by using nitric oxide, lipoxygenase and cyclooxygenase inhibitory assays followed by *in vivo* study on male Wistar rats by rat paw edema test and cotton pellet granuloma method. Oral administration of various concentrations of extract controlled the volume of rat paw edema as well as inhibited the growth of granuloma in a dose dependent manner (**Kumar *et al.,* 2016**).

1.4.10. Anti-malarial activity

Waako *et al.,* (**2007**) studied *in vitro* the antimalarial effect of four plant species, one of them was *Indigofera emarginella.* The effect of plant extracts against two strains of *P. falciparum i.e.,* chloroquine-sensitive D10 and chloroquine-resistant K1 strains was assessed by using nitro-tetrazolium-blue based parasite lactate dehydrogenase assay. The methanol extract of aerial parts of *I. emarginella,* collected in both the dry and wet season, showed significant anti-plasmodial activity with IC_{50} = 22.5 µg mL^{-1} on chloroquine sensitive (D10) strain and 22.4 µg mL^{-1} on chloroquine-

resistant (K1 strain) . However, the activity of plants collected in wet season was higher than that of dry season.

The chloroform, petroleum ether and ethyl alcohol extract of leaves and roots of *Indigofera emerginella* and various parts of nine other plants having antimalarial ethnobotanical significance were studied by **Katuura *et al.,*** (**2007**). The results of *in vitro* study using nitro-tetrazolium blue-based lactate dehydrogenase assay revealed that all extracts of *I. emerginella* showed significant anti-plasmodial activity. Ethyl alcohol extract with IC_{50} = 5.8 µg mL^{-1} proved to be most potent.

The methanolic extract of leaves of *Indigofera oblongifolia* was injected to female mice C57BL/6 inoculated with *Plasmodium chabaudi* infected erythrocytes. The seven-day experiment carried out by a single dose of extract as 100, 200 and 300 mg/kg/day led to the results that parasitemia was remarkably reduced in extract treated groups as compared to the control group (**Lubbad *et al.,* 2015**).

The anti-plasmodial activity of ethyl acetate extract of leaves of *Indigofera tinctoria* was studied *in vitro* by **Kaushik *et al.,*** (**2015**) using SYBR fluorescence assay. It was observed that *I. tinctoria* extract was moderately active with IC_{50} = 57 µg mL^{-1} against chloroquine resistant *Plasmodium falciparum* strain.

The effect of hydromethanolic extract of roots of *Indigofera spicata* was studied against *Plasmodium berghei.* Various doses of extract were administered to male Swiss albino mice experimentally infected with *P. berghei* parasites. Results showed that 600 mg kg^{-1} dose of extract decreased the parasitemia by 53.42%. and increased the mean survival time of mice (**Birru *et al.,* 2017**).

1.4.11. Anti-nociceptive activity

The anti-nociceptive activity of methanol extract of whole plant of *Indigofera linnae* Ali. was studied by **Kumar *et al.,*** (**2016**) on rodent models**.** It was concluded from the results of acetic acid induced writhing test and hot plate experiment that oral doses of extract had shown greater pain killing effect than aspirin used as standard antinociceptive drug.

1.4.12. Antioxidant activity

Gyamfi *et al.,* (**1999**) worked on the aqueous extracts of *Indigofera arrecta* and four other plant species. The extracts were tested for antioxidant efficacy using DPPH assay. The results of study revealed that *I. arrecta* extract did not show any significant DPPH radical scavenging potential.

The antioxidant activity of indigocarpan (**166**) and mucronulatol (**47**) isolated from the chloroform extract of *Indigofera aspalathoides* was studied by **Selvam *et al.,*** (**2004**) by using DPPH radical scavenging assay. Both the compounds showed good antioxidant effect with IC_{50} values of 24.52 µM by **166** and 34.83 µM by **l47**.

The percent antioxidant activity of 50% methanol: water extract of various parts of *Indigofera tinctoria* was evaluated by **Prakash *et al.,*** (**2007**) using linoleic acid and *β*-carotene oxidation assays. The antioxidant activity for leaves, stem and flowers was 67.3%, 35.6% and 60.7% respectively. The flowers were further studied for free radical scavenging activity using different antioxidant assays. The IC_{50} value of flower extract revealed by DPPH assay was 0.20 mg/mL while IC_{50} value calculated by TBARS (thio-barbituric acid reactive species) assay was 0.31 mg/mL. These low IC_{50} values show high free radical capturing tendency of *I. tinctoria* extract.

Bakasso *et al.,* (**2008**) studied antioxidant effect of aqueous acetone extracts of five *Indigofera* species that were *I. colutea, I. macrocalyx, I. nigritana, I. pulchra* and *I. tinctoria.* All the plants showed significant antioxidant activity in response to DPPH assay except *I. pulchra* which depicted IC_{50} = 12.3 µg mL^{-1} and hence relatively lower antioxidant potential. FRAP and ABTS [2, 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonate)] assays showed that highest antioxidant potential was of *I. colutea* demonstrated by values of 2.54 and 3.74 AAE/g respectively.

The leaves of *Indigofera tinctoria* were extracted with methanol and the extract was evaluated for antioxidant activity in relation to effect against alzheimer′s disease**.** Alzheimer′s disease was induced in swiss albino mice by injection of *β*-amyloid peptide (25-35) which decreased the levels of antioxidant enzymes in brain. Treatment of mice with various concentrations of extract recovered the amounts of these enzymes to normal levels, decreased lipid peroxidation and inhibited neuronal loss (**Balamurugan** and **Muralidharan 2010**).

Asuntha *et al.,* (**2010**) studied the antioxidant activity of ethanol extract of *Indigofera tinctoria.* The extract showed significant NO scavenging activity at

concentrations of 0.1-1 µg mL^{-1} against ascorbic acid taken as standard. Likewise, the extract showed DPPH radical scavenging activity as compared to ascorbic acid at concentrations of 0.4 µg mL^{-1} or greater.

The antioxidant potential of *Indigofera stachyodes* root extract was studied by **Li *et al.,* (2011)**. Different types of assays like DPPH scavenging assay, ABTS assay and FRAP assay were performed which indicated that the plant extracts had high antioxidant activity. However, ethyl acetate extract of *I. satachyodes* was more active than *n*-butanol and showed IC_{50} values of 7.94 µg mL^{-1}, 2.73 µg mL^{-1} and 2270.8 µg mL^{-1} with DPPH, ABTS and FRAP assays, respectively. The overall antioxidant effect of both the extracts was significant.

Vadivel *et al.,* (2011) studied the antioxidant activity of methanol extract of raw and differently processed seeds of *Indigofera linifolia* and nine other plants. The antioxidant potential of *I. linifolia* seed extract was comparable to that of butylated hydroxy toluene (BHT) taken as reference compound. The *β*-carotene oxidation assay, DPPH radical scavenging assay and superoxide radical inhibition assay showed the antioxidant activities of sprouted and oil fried *I. linifolia* seeds to be 48.21%, 82.24% and 58.39% respectively. Although the potential value obtained from FRAP assay was 310.89 mmol Fe/g of extract as compared to 1124.72 mmol Fe/g of standard BHT, the overall antioxidant activity was good.

Renukadevi and Sultana (2011) determined the antioxidant potential of methanol extract of *Indigofera tinctoria* leaves. Qualitative DPPH assay gave a positive test for the presence of antioxidants in the extract. The DPPH activity of *I. tinctoria* extract was found to be stronger than ascorbic acid used a reference. An extract of 250 µg mL^{-1} gave IC_{50} value of 51.66 µg mL^{-1} only. The percent inhibition was comparable to 63 % inhibition shown by same amount of ascorbic acid used as standard antioxidant. This shows significant antioxidant tendency of *I. tinctoria* leaf extract.

The antioxidant activity of ethanol extract of *Indigofera suffruticosa* pods was studied by **Arriaga *et al.,* (2013)**. The result of DPPH radical scavenging activity revealed that the *I. suffruticosa* extract is highly antioxidant in effect and has IC_{50} value of about 1.55×10^{-2} mg mL^{-1} as against 4.3×10^{-2} mg mL^{-1} of vitamin C taken as standard.

The ethanol extract of seeds of *Indigofera cordifolia* gave an *n*-butanol soluble fraction which was investigated for antioxidant activity by DPPH and ABTS radical

scavenging assays. Results showed that the antioxidant potential of *I. cordifolia* seeds is significant (**Deshpande *et al.*, 2014**).

The hepatoprotective effect of *Indigofera caerulia* Roxb. against CCl_4 induced liver injury in rats was notable. It was attributed to the antioxidant potential of methanol extract of its leaves. The administration of CCl_4 in rats showed increase in activity of NF-κB and rise in the levels of inflammation producing factors TNF-α and IL-1β. The phytoconstituents in extract were able to inhibit oxidative processes which were producing these pro-inflammatory substances (**Ponmari *et al.*, 2014**).

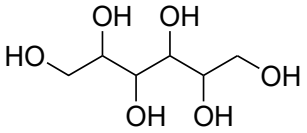
The antioxidant potential of nine plant species including *Indigofera cylindrica* was evaluated by **Dzoyem *et al.*, (2014)**. The extract depicted DPPH and ABTS scavenging activities with IC_{50} values of 22.31 µg/mL and 41.39 µg/mL respectively. These values were comparable to IC_{50} values of 9.71µg/mL and 12.48 µg/mL for DPPH and ABTS scavenging activities respectively for the reference compound Trolox.

Bhaskar *et al.*, (2016) studied the antioxidant activity of methanol extract, chromatographic fractions and compounds obtained from the ethyl-acetate extract of *Indigofera barberi* by using DPPH radical scavenging assay. The DPPH scavenging activity of all fractions and compound-1 ranged between 50-60% at 50 µg/mL concentration except crude methanol extract which showed 30% inhibition at this concentration. The activity increased in a dose dependent manner.

Lubbad *et al.*, (2015) worked to investigate the antioxidant effect of methanol extract of leaves of *Indigofera oblongifolia*. The extract was injected to C57BL/6 mice bearing spleen tissue injury caused by *Plasmodium chabaudi* infection. All the oxidation causing factors, such as increase in levels of malondialdehyde and nitric oxide, were recovered to normal levels by the extract.

The antioxidant potential of ethanolic and hydroethanolic extract of *Indigofera tinctoria* Linn. was studied by **Singh *et al.*, (2015)**. The antioxidant potential of hydro-ethanol extract was higher than that of ethanol extract. The IC_{50} values of hydro-ethanol extract in case of DPPH radical, SO_2 radical, NO_2 radical and OH^- radical scavenging activities were 829 µg/mL, 638.1 µg/mL, 978 µg/mL, and 26.7 µg/mL, respectively. These values were higher than IC_{50} values of respective standards but IC_{50} for OH^- radical scavenging activity of both extracts was much lower than 1044 µg/mL of mannitol used as standard antioxidant. The extracts showed metal chelating activity in

a concentration dependent manner. Similarly, ferrous reducing activity was also shown by hydro-ethanol and ethanol extract of *I. tinctoria.*



Mannitol (**254**)

The aqueous extract of *Indigofera tinctoria* was investigated for antioxidant activity by **Srinivasan *et al.,*** (**2016**) using ascorbic acid as standard**.** The extract at concentration of 250 µg/mL depicted 52.08%, 23.12% and 26.795% antioxidant activity as result of DPPH, nitric oxide, and superoxide anion (O^{2-}) scavenging assays, respectively. Additionally, the IC_{50} values of aqueous extract were 244.7 µg/mL for DPPH, 540.65 µg/mL against NO and 466.59 µg/mL in case of superoxide anion radical scavenging assay. The plant extract also showed concentration dependent reduction potential as result of FRAP assay.

Dkhil *et al.,* (**2016**) worked on the 70% methanolic extract of leaves of *Indigofera oblongifolia.* Oxidative damage was experimentally produced in male Wistar albino rats by injecting lead acetate (20 mg/kg body weight) and extract was examined for antioxidant effect. The activity of antioxidant enzymes was restored in rats pre-treated with *I. oblongifolia* extract (100 mg/kg body weight). The treated group of rats not only showed reduction in noxious effects of lead acetate but less tissue damage than untreated group as well.

The DPPH radical scavenging potential of aqueous extract of *I. tinctoria* leaves and its nanoparticles (NP) was evaluated by **Vijayan *et al.,*** (**2018**) using ascorbic acid as positive control. Considerable antioxidant effect was observed for silver-NP and gold-NP with IC_{50} values of 10.04 and 68.05 µg mL^{-1} respectively. However, the potent antioxidant activity of *I. tinctoria* extract with IC_{50} = 177.52 µg mL^{-1} was lower than that of its nanoparticles.

Netala *et al.,* (**2018**) studied the antioxidant efficacy of nanoparticles of *I. hirsuta.* The nanoparticles showed noteworthy antioxidant efficacy with IC_{50} = 63.43 µg/mL (DPPH radical scavenging assay) and IC_{50}=89.93 µg/mL (H_2O_2 radical scavenging assay).

Elmi *et al.,* (**2018**) studied the antioxidant effect of *n*-hexane, acetone, methanol, and water extracts of fruits of *Indigofera caerulea.* Various *in vitro* antioxidant assays were performed which led to the conclusions that water and *n*-hexane extracts showed very little or no activity. Methanol extract showed more DPPH scavenging potential and FRAP activity than acetone extract. The ABTS radical scavenging action of acetone extract was highest and interestingly more than vitamin C as well.

The antioxidant potential of silver nanoparticles of *I. barberi* leaves extract was evaluated by **Reddy *et al.,*** (**2019**) by using DPPH and H_2O_2 radical scavenging assays. Significant antioxidant potential is indicated by IC_{50} values of 67.37 and 72.04 µg/mL against DPPH and H_2O_2 free radicals, respectively.

1.4.13. Anti-protozoal activity

Ibrahim *et al.,* (**2011**) investigated *in vivo* the effect of methanol extract and *n*-butanol fraction of methanol extract of leaves of *Indigofera pulchra.* The activity was evaluated against *Plasmodium berghei* by taking six groups of mice infected with this protozoan having chloroquine resistivity. Two of the groups were orally treated with methanol extract, two were given *n*-butanol extract, one was chloroquine group, and the last group was untreated one. It was revealed that infected-untreated group had highest concentration of infected red blood cells. The group treated with *n*-butanol fraction showed lowest number of infected red blood cells throughout the experiment. Effect of methanol extract was also noteworthy but lower than that of *n*-butanol fraction. The anemia resulting from *P. berghei* infection was prevented by all treatments of this study.

The effect of methanol extract of *Indigofera oblongifolia* leaves was evaluated against Trypanosoma induced spleen injury. Female Swiss albino mice were infected with *Trypanosoma evansi.* Infection led to weight loss, splenomegaly, anemia, and reduction in levels of certain enzymes like glutathione and catalase. The treatment of infected mice with *I. oblongifolia* extract significantly decreased parasitemia and reversed all the changes produced by Trypanosoma (**Dkhil *et al.,* 2019**).

1.4.14. Anti-viral activity

The hydro-methanolic extract of roots of *Indigofera heterantha* was studied to find mechanism by which it inhibits the replication of HSV-2 virus. So, plant extract was added to Vero cells at various time points (0, 1, 2, 3, 4, 16 and 24 h) after adding HSV-2 viral strain. Experiments were conducted using pretreatment, post-infection, adsorption, attachment, penetration, and envelope glycoprotein assays. It was evident from the results that plant extract was most active against HSV-2 at initial events of infection (0 to 4 h) and rate of antiviral effect was decreased for later stages. The viral plaque formation was completely inhibited at stages of viral attachment and penetration to the cells by extract having concentrations 100-400 µg mL^{-1} and 200 µg mL^{-1} respectively. Loss of immuno-fluorescence in extract treated cells having viral adsorption proved that the extract acts via breaking interaction between host cell surface and glycoprotein D of HSV-2 envelope (**Kaushik *et al*., 2017**).

In 2017**,** a study was carried out to explore anti-hepatitis B virus (HBV) activity of 60 plants. Two of them were *Indigofera caerulea* and *Indigofera tinctoria.* The ethanolic and other organic extracts of all plants were examined for cytotoxic effect on HepG2.2.15 cells. Results revealed that *Indigofera caerulea* had shown marked anti-HBV effect with IC_{50} = 73.21 µg/mL and cytotoxic concentration CC_{50} = 1566 µg/mL (**Arbab *et al.,* 2017**).

The effect of methanol extracts of 30 plants including *Indigofera tinctoria* against 7 different types of viruses was evaluated by **Vimalanathan *et al.,*** (**2009**). The anti-viral activity determined in terms of MIC_{100} was 0.4 µg mL^{-1} for *I. tinctoria* methanol extract against mouse corona virus (MCV) and Herpes simplex virus (HSV). Thus, *I. tinctoria* can prove to be a good source of lead compound for curing certain viral ailments.

1.4.15. Gastroprotective effect

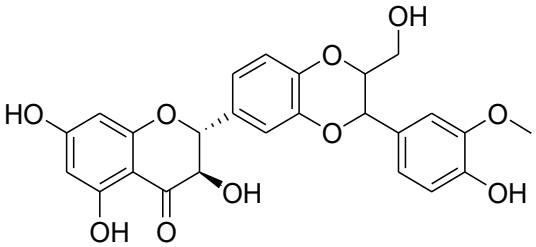
The anti-ulcer activity of water and ethyl acetate soluble fractions of methanol extract of *Indigofera suffruticosa* was studied by **Luiz-Ferreira *et al.,*** (**2011**). Experiments performed on male Unib-WH rats showed that ethanol induced mucosal damage was significantly contradicted by ethyl acetate fraction at dose of 100 mg/kg body weight. The extract also stimulated ulcer healing events in acetic acid induced ulcer model.

1.4.16. Hepatoprotective activity

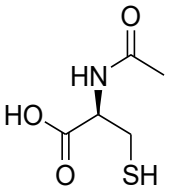
The effect of alcoholic extract of *Indiofera oblongifolia* was studied by **Shahjahan *et al.,*** (**2005**). Different doses of plant extract were given to Wistar rats to cure CCl_4 induced hepatotoxicity in them. After a period of ten days, it was found that levels of marker enzymes in serum were decreased to normal in experimental group. Likewise, the antioxidant status of liver was also maintained in extract treated group as compared to control group.

Rajkapoor *et al.,* (**2005**) worked on the ethanol extract of stems of *Indigofera aspalathoides. N*-nitrosodiethyl amine (DEN), a carcinogen, was administered to male Wistar rats to induce liver tumor. DEN caused increase in liver weight, rise in level of hepatic enzymes, decrease in catalase and changes in liver morphology. Comparison of extract treated mice with control group revealed that all the changes produced by DEN were reversed by *I. aspalathoides*. This proves the high chemo-preventive potential of *I. aspalathoides* extract.

Trans-tetracos-15-enoic acid (TCA), isolated from acetone fraction of petroleum ether extract of aerial parts of *Indigofera tinctoria* Linn., was studied for its hepatoprotective potential. The experiment conducted on albino rats and Swiss albino mice involved CCl_4 and acetaminophen induced liver injury followed by treatment with TCA. The observed ED_{50} values to elevate the level of serum and hepatic parameters in impaired liver ranged from 25 to 45 mg/kg. This study led to the conclusions that TCA had hepatoprotective potential very close to that of silymarin and *N*-acetyl cysteine which are well known hepatoprotective agents (**Singh *et al.,* 2006**).



Silymarin (**255**)



N-acetyl cysteine (**256**)

Mukherjee *et al.,* (**2009**) reviewed the work of **Singh *et al.,* (2001)** and **Shahjahan *et al.,*** (**2005**). The hepatoprotective efficacy of tetra-cos-15-enoic acid obtained from *Indigofera tinctoria* as well as the safe effect of alcoholic extract of *Indigofera oblongifolia* on liver was recapitulated.

Two compounds belonging to the class of flavonoids, one named as 3-*O*-(3-nitropropanoyl)-2,3-cis-5,7,3′,4′-tetrahydroxyflavan (**82**) and the other as 2*α*,3*α*-epoxy-5,7,3′,4′-tetrahydroxyflavan-(4*β*→8)-epicatechin (**81**) were isolated from the EtOH extract of *I. stachyodes.* The compounds were evaluated for hepatoprotective effect against human liver cell line HL-7702. Both the compounds remarkably reversed toxic effects of CCl_4 in liver cell line (**Qiu *et al.,* 2013**).

In another study, hepatoprotective potential of methanol extract of leaves of *Indigofera caerulia* Roxb. was investigated. Acute toxicity study showed the extract was non-toxic up to concentration of 2000 mg/kg. The *in vivo* experiment was carried on Sprague-Dawley rats such that CCl_4 induced hepatic injury was followed by intragastric treatment with methanol extract (100 and 200 mg/kg). It was evident from the results that methanol extract of *I. caerulia* remarkably restored levels of liver specific enzymes in serum which were increased due to hepatic intoxicant CCl_4. Thus, liver injury was minimized by *I. caerulia* extract **Ponmari *et al.,*** (**2014**).

Silva *et al.,* (2014) checked the effect of aqueous extracts of *Indigofera suffruticosa* leaves prepared by infusion and maceration. The Swiss albino mice were administered with tumor, Sarcoma 180. Daily doses of plant extract with concentrations of 50 mg/kg were given to mice up to seven days such that one of the groups was given extract prepared by infusion and the other was given extract prepared by maceration. The pathological and morphometric studies on liver tissues revealed that liver of control group had undergone degradative changes while histology was preserved in extract treated groups. This proves the hepatoprotective effect of *I. suffruticosa.*

The antimalarial effect of 70% methanol extract of leaves of *Indigofera oblongifolia* was studied by **Al-Shaebi *et al.,*** (**2017**). Red blood cells infected with *Plasmodium chabaudi* were intraperitoneally administered to C57BL/6 mice. Three different doses of extract were given to separate groups of infected mice for a week. The analysis of spleen from euthanized mice led to the conclusion that macrophage number, organization and response was increased in extract treated group than untreated control mice.

The 70% methanolic extract of *Indigofera oblongifolia* leaves (ILE) was investigated against liver injury caused by malarial parasite *Plasmodium chabaudi.* An experimental group of adult female-mice G57BL/6 was administered with *P. chabudi-*

infected red blood cells. Half of these mice were given oral dose of ILE for seven days. Augmentation of erythrocyte number, hemoglobin content and antioxidant capacity in the infected mice revealed that the extract had remarkable hepatoprotective potential. Histological analysis showed improved structure of hepatic tissues in infected mice treated with ILE. Most important of all, ILE regulated the gene expression modified by parasite (**Al-Shaebi *et al.,* 2018**).

1.4.17. Immunomodulatory activity

Lopes *et al.,* (2011) investigated the immunomodulatory activity of alkaloid fraction and a compound identified as indigo, isolated from the methanol extract of aerial parts of *Indigofera suffruticosa.* Experiments performed by using cytokine immunoassay on peritoneal macrophages showed that alkaloid fraction stimulated the production of Nitric oxide (NO) but decreased the release of TNF-α in dose dependent manner. Indigo, however, enhanced the production of NO as well as cytokine TNF-α in incubated macrophages. These two factors play positive role in mediating immune system.

Swarnalatha *et al.,* (2015) evaluated the immuno-modulatory activity of Kaempferol-5-*O*-β-D-glucopyranoside isolated from methanol extract of aerial parts of *Indigofera aspalathoides* Vahl ex DC. The experiments were performed on Wistar *albino* rats. Acute toxicity was not observed below 2000 mg/kg of extract. Neutrophil adhesion test revealed that clustering of phagocytic cells around nylon fibers was increased in the presence of compound under study. This indicates increased immuno-modulatory potential of this phytoconstituent.

Zhao *et al.,* (2019) investigated the effect of indirubin on Immune thrombocytopenia (ITP), an ailment resulting from reduction in platelet count due to an upset in number and function of immune modulating CD^{+4} T-cells. This *in vitro* study showed that indirubin, a phytoconstituent of *Indigofera tinctoria* L., restored the number and activity of CD^{+4} regulatory T-cells. It also decreased the activity of effector T-cells to normal level. The same effect was also observed *in vivo* in an active murine model accompanied by noteworthy increase in number of platelets. Thus, the results proved that indirubin has the potential to treat ITP by controlling CD^{+4} T-cells through PD1/phosphatase and tensin homolog/AKT signaling pathway.

One of the recent works on this genus has been carried out by **Boothapandi** and **Ramanibai** (**2019)** to evaluate the immunomodulatory effect of chrysin isolated from leaf extract of *Indigofera tinctoria.* The experiments performed on lipopolysaccharide murine macrophages (RAW 264.7 cell line) revealed that chrysin enhanced the phagocytic activity of macrophages. This effect proves high immunomodulatory potential of *I. tinctoria* extract.

1.4.18. Insecticidal activity

Different parts of *Indigofera tinctoria* were extracted with acetonitrile saturated with *n*-hexane and examined for effect against pulse beetle, *Callosobruchus chinensis* and larvae of mosquito named, *Anopheles stephensi.* The experiments carried out *in vivo* and *in vitro* showed that constituents of *I. tinctoria* extract exhibited greater growth inhibitory potential on *Anopheles stephensi* larvae than on pulse beetle. Moreover, the extract from callus of *Indigofera tinctoria* was more potent against both the insects than extract of any other part of the plant (**Kamal and Mangla, 1993**).

The foraging behavior of an ant, *Solenopsis invicta* Buren and extent of its repellency towards three plant species was studied by **Kaakeh and Dutcher** (**1992**). Choice and no choice experiments revealed that ants preferred *Indigofera hirsuta* L. more than *Sesbania exaltata* but less than *Vigna unguiculata.* The response towards water and ethyl alcohol extracts of *I. hisuta* was also moderate and no significant mortality of ants was observed.

Vieira *et al.,* (2012) investigated the effect of aqueous extract of *Indigofera suffruticosa* leaves on development of eggs and larvae of *Aedes aegypti,* a vector of dengue virus. Experiments performed using various types of bioassays showed that the number of eggs laid by extract treated female mosquitoes was much less than that of control group. Similarly, various concentrations of *I. suffruticosa* extract not only inhibited the eclosion of eggs but also retarded the development of larvae. It was found that 250 µg/mL of the extract stopped growth of 93.3% larvae at instar stage. The extract and its fractions did not show any notable effect against insects. The *n*-hexane, methanol M_1 and residue fractions showed 20% activities against *R. dominicia, Callosbrucuanalis* and *T. castaneum* respectively.

The aqueous methanol extract of aerial parts of *Indigofera heterantha* was studied by **Uddin G. *et al.,*** (**2011**). The extract and its fractions were directly applied

to insects by contact using filter papers. The study was conducted on *Tribolium castaneum, Sitophilus oryzae, Trogoderma granarium, Callosobruchus analis and Rhyzopartha dominica* adults and results were compared with permethrin taken as positive control. The fractions were inactive against insects under study. Only the *n*-hexane and ethyl acetate showed slight activity against *R. dominica* while the methanol extract showed 20% effect against *C. analis*.

An investigation on the acaricidal activity of essential oil of *Indigofera suffruticosa* leaves was carried out by **Silva *et al.,* (2019**). Bioassays were performed to check the effect of oil for eradication of insect, ovicidal activity and oviposition inhibition of *Tetranychus urticae***.** It was concluded that the oil and its components showed noteworthy acaricidal effect and highest potential was shown by thymol and eugenol.

1.4.19. Miscellaneous activities

The aqueous extract of leaves of *Indigofera dendroides* was examined for contraction of smooth muscles of mice, rats, and pigs. It was revealed that contraction of ileal muscles (muscles of ileum, a section of small intestine) of rat and pig proportional to the concentration (0.05-3.2 mg/mL) was produced by *I. dendroids* extract. The extract caused muscle contraction in solution having no Ca^{2+} ions. Lethal dose 50% (LD_{50}) was also calculated and found to be 692.82 mg/kg (**Amos *et al.,* 2003**).

Sharif *et al.,* (2005) studied *in vitro* the lipoxygenase (LOX) and Cholinesterase (BChE) inhibitory effect of two of the three compounds isolated from methanol extract of *Indigofera oblongifolia*. Lipoxygenases and cholinesterase are involved in onset of certain diseases. Results of this experiment showed that the isolated alkyl xanthene and indigoferic acid showed IC_{50} values of 36.5 µM and 49.5 µM respectively against LOX. These values were comparable with IC_{50} value of 22.6 µM for baicalein taken as positive control. Although, the alkylated xanthene showed no activity against BChE, indigoferic acid showed noteworthy anti-cholinesterase effect.

Rehman *et al.,* (2005a) studied the lipoxygenase inhibiting activity of a lignan and two acylphloroglucinols isolated from the methanol extract of whole plant of *Indigofera heterantha.* All the three compounds showed good activity relative to baicalein used as standard lipoxygenase inhibitor. The percent inhibition increased with increasing concentration of the test compound.

The lipoxygenase inhibitory activity of monoterpene glycosides obtained from the ethyl acetate soluble fraction of methyl alcohol extract of whole plant of *Indigofera heteantha* was studied by **Mehmood *et al.,* (2008)**. Lipoxygenase inhibition assay performed by using linoleic acid as substrate showed that both the compounds were moderately active against lipoxygenase.

Tariq *et al.,* (2011) studied *in vitro* the biological activity of four compounds isolated from methanol extract of *Indigofera gerardiana* Wall. The compounds were assayed for urease inhibition activity, an enzyme responsible for growth of microorganisms and cause of several diseases in humans. It was revealed that indigoferin A (**151**) was inactive. Indigoferin B and C (**152**, **153**) showed 91.3 % and 93.3% urease inhibition activities, respectively. The result was comparable to 98.2% inhibition by thiourea taken as standard.

Bhatta *et al.,* (2013) studied *in vitro* the effect of thirty-eight plants including leaves of *Indigofera tinctoria* on fermentation, protozoal population, and methane production in rumen. Results revealed good potential of secondary metabolites of *I. tinctoria* against methanogenesis without any adverse effect on digestibility as protozoal population in the rumen is found to be unaffected.

Pasupula and Pragada, (2018) studied the toxic potential of polyherbal preparation (PHP) made from water extracts of *Mimusops elengi* Linn, *Strobilanthes barbatus* Nees, *Indigofera zollengiriana* Miquel and *Dellenia indica* Linn. The acute toxicity studies conducted on female Wistar rats at various concentrations of PHP ranging from 5 mg/kg to 2000 mg/kg showed that no acute toxicity was observed even at 2000 mg/kg body weight. The preparation also showed no signs of sub-chronic toxicity at concentrations of 250-1000 mg/kg body weight.

Rahman *et al.,* (2019) studied the urease inhibition activity of Indigoferamide-A (**104)** separated and purified from 5% aqueous methanol extract of *Indigofera heterantha* Wall. A 1000 µM solution of **104** showed 29% inhibition as compared with 83% inhibition by equal amount of thiourea taken as standard urease inhibitor. Molecular docking studies revealed that **104** can develop strong interactions with His324 and Asp224 amino acid residues on active site of urease.

1.4.20. Toxicity

(a) Cytotoxicity

The aqueous extracts of leaves of *Indigofera suffruticosa* were examined for cytotoxic activity by **Vieira *et al.*, (2007)**. The extracts obtained by infusion and maceration produced no cytotoxic effect on HEp-2 cell line with concentrations up to 50 µg/mL of extract as assayed by MTT protocol. Moreover, the injection of extracts up to concentrations of 2400 mg/kg did not cause death of Swiss albino mice but symptoms of toxicity were prominent.

The cytotoxic and genotoxic effect of ′indigo naturalis′, a type of powder produced from dried leaves of *Indigofera tinctoria,* was evaluated by **Dominici *et al.*,** (**2010**). The experiments conducted on HepG2 cell line by applying aqueous and dimethyl sulphonyl solutions of *Indigofera tinctoria* leaf powder proved that indigo naturalis has negligible cytotoxic and genotoxic effects.

Uddin G *et al.*, (2011) made an investigation on the cytotoxic effect of aqueous methanol extract of aerial parts of *Indigofera heterantha.* The extract was partitioned between *n*-hexane, chloroform, methanol, and ethyl acetate. The fractions were concentrated, separately dissolved in DMF, and tested for cytotoxic activity using DMF as control. The results of experiment performed on Brine shrimp eggs showed that the extracts had negligible cytotoxicity. An LD_{50} value of 290.74 µg/mL of methanol fraction caused only 13.3% mortality. This lack of cytotoxicity reveals the significant use of *I. heterantha* in ethnopharmacology.

Chen *et al.*, (2013) determined the cytotoxic effect of ethanol and aqueous extracts of *I. suffruticosa* on RAW 264.7 macrophages by using MTT assay. The extracts with all the tested concentrations of ranging from 50 to 1000 $\mu g/mL^{-1}$ showed no adverse effects on cell viability.

Bhaskar *et al.*, (2016) studied the cytotoxic effect of crude methanol extract along-with the fractions and quercetin obtained from ethyl-acetate extract of whole plant of *Indigofera barberi* using MTT assay on DF1 cells and hemolytic assay on sheep erythrocytes. The cytotoxic effect on DF1 cells and hemolytic effect was negligible at 20 µg/mL, moderate for 60 µg/mL and increased at higher concentrations of the extract.

Three of the seven compounds isolated from ethanol extract of roots of *I. stachyodes* showed significant anti-inflammatory effect. The same three compounds showed no cytotoxic effect against BV2 microglial cells when examined by using MTT

assay. This shows their antioxidant potential was not related to cytotoxic action **(Zhang *et al.*, 2018)**.

(b) Embryotoxicity

Leite *et al.*, (2004) worked on the aqueous extract of *Indigofera suffruticosa* to evaluate its toxic effects on pre-implanted mice embryos. The embryos at two-cell stage were removed from reproductive tract of female mice and kept in an *in vitro* biochemical environment containing *I. suffruticosa* extract 5 mg/mL or 10 mg/mL. Results showed that embryos kept in lower concentration of extract had shown significant development while those in higher concentration of plant extract *i.e.,* 10 mg/mL remained at two-cell stage. This result shows that *I. suffruticosa* intake may also be toxic for human embryos.

(c) Haemo-Toxicity

Neto *et al.*, (2001) evaluated the effect of feeding *Indigofera suffruticosa* Mill. to bovines. A daily intake of 10-40 g/kg plant material led to hemoglobinuria accompanied by symptoms of varying intensities among the animals. It did not cause death but one of the bovines was euthanized for experimental examination. The animal had developed anemia, wine-red urine in bladder, swollen kidneys, hepatocytes underwent necrosis and hemoglobin was accumulated in epithelial cells. All these conditions confirm that the animals feeding on *I. suffrticosa* can suffer from anemia characterized by hemoglobinuria.

Salvador *et al.*, (2011) studied the toxic effects of *Indigofera suffruticosa* by giving daily dose of plant to guinea pigs up to fifteen days. The blood analysis of pigs after 2, 4, 6, 8, 10, and 15 days of eating plant material showed that there was a successive decline in concentration of red blood cells with increasing number of days finally resulting in anemia. A turquoise blue pigment present in the urine of pigs due to plant ingestion is probably aniline and is expected to be the cause of anemia. *Indigofera suffruticosa* was thought to be the source of this anemia causing blue pigment.

(d) Hepatotoxicity

Indospicine **(33)** is present 0.8g/100 g of *I. spicata* seeds. The ground *Indigofera spicata* seeds were fed to four groups of rats in concentrations 96, 48, 24 and 15 percent by weight. Each diet group also had a corresponding control group fed on commercial

diet only. Rats from each group were killed after specific days followed by physical and histological analysis of liver. The diets of 96 and 48 percent led to the death of animals in 2 to 6 weeks. Cirrhosis, a chronic liver disease, was developed in rats fed with 15 and 24 percent diet by weeks 16 and 6 respectively. The survival time for rats with diets of higher dose was less. This is probable that **33** as arginine inhibitor can lead to liver enlargement followed by lesions and cirrhosis (**Christie *et al.,* 1975**).

The *in vitro* study by **Alston *et al.,* (1977)** was carried out on mitochondria isolated from mice liver. Mitochondria were added to solution containing succinate, O_2 and 3-Nitropropionate (3-NP), a common toxin in plants of *Indigofera.* It was found that the rate of oxidation of succinate decreased with increasing concentrations of 3-nitropropionate with fist order rate kinetics. This proves that 3-NP is irreversible suicide inhibitor of succinate dehydrogenase present in mitochondria leading to inhibition of Kreb′s cycle.

Williams (1981) investigated the toxic potential of *I. hirsuta, I. echinata, I. pseudotinctoria* and *I. spp*. IRFL 2023, IRFL 2087. The ethanol extract of these plants was dissolved in water, extracted with benzene and the water-soluble portion with final concentration of 2 g/mL was obtained. It was fed to one-week old chicks in dose range of 1-10 g such that each dose level was given to two chicks. The results showed that *I. hirsuta* was non-toxic as it also gave negative test for the presence of nitro compounds. The other four species caused noxious symptoms when 3 to 6 g of extract was fed to the chicks and profound toxic effects were observed when 7 to 10 g of extract dose was given.

Aylward *et al.,* (1987) studied the extent of toxicity of 46 *Indigofera* plants by administering them to mice as diet up to four weeks. This was followed by observation of growth rate, histological analysis of some organs of mice and qualitative analysis of toxic compounds in diet samples. Results showed that growth rates remained unaffected by eight plants while slightly decreased by thirteen plants fed to the mice. *I. spicata and I. nigritana* led to histological changes. Plants which had little effect on growth rate can be considered safe for use as forage.

Toxic effects of water and *n*-hexane extracts of fruit of *Indigofera suffruticosa* Mill were studied by **Ribeiro *et al.,* (1991**). Intraperitoneal administration of extract in

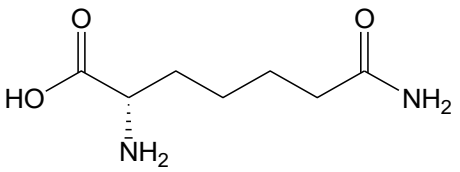
Balb C mice led to cytogenetic effect on liver at low concentrations followed by cytotoxic effects at higher doses.

Pollitt *et al.,* (1999) developed an efficient method to calculate the amounts of hepatotoxin **33** accumulated in the tissues of horses that grazed on *Indigofera linnae.* The method was designed such that the aqueous or 0.01N HCl extracts of horsemeat and serum were ultra-filtered, derivatized with phenyl-iso-thiocyanate and finally **33** was separated on Pico Tag C18 column followed by detection with UV. The recovery of **33** was 87 and 97 % from meat and serum, respectively. This shows high bioavailability of **33**.

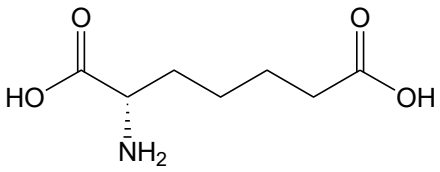
Assis *et al.,* (2009) conducted a survey about plants which proved to be poisonous for cattle in regions of Sertão Paraibano. The farmers and veterinary experts mentioned *Indigofera suffruticosa* to be one of the toxic plants of the region.

Diseased dogs fed on a commercial camel diet were euthanized. The histological study of livers of dogs revealed lesions produced by some hepatotoxin. The compound **33** was detected qualitatively as well as quantitatively in the serum and diets of dogs by using LCMS/MS technique. It was absent in serum of control dogs not administered with camel diet **Fitzgerald *et al.,* (2011)**.

Tan *et al.,* (2017) studied *in vitro* the degradation of hepatotoxic compound **33** in camel and cattle by using UHPLC-MS/MS technique. A 48h experiment conducted by adding foregut fluid samples to medium containing *Indigofea spicata* showed 99% degradation of **33** in camel and 97% in cattle. Time dependent degradation studies showed that **33** was digested within 48 hours and products formed were 2-aminopimelamic acid and 2-aminopimelic acid. The compound **33** is partially degraded in foregut and can accumulate in camel tissues.



2-Aminopimelamic acid (**257)**



2-Aminopimelic acid (**258)**

Sultan *et al.,* (2018a) studied the digestion of compound **33** stored in meat of camel who had been grazing on *Indigofera* plants. The experiment was designed to check digestibility of **33** contained in camel meat by providing *in vitro* environment

analogous to human digestive tract. Studies conducted by using LC-MS analysis showed that **33** was not degraded by microwave cooking of meat and showed complete resistance to digestion. It comes out from camel meat and becomes readily available for absorption across intestine. Thus, it can be transported by blood to different body tissues where it gets stored and may lead to harmful effects in humans.

The toxic effect of **33** on human cells was studied by **Sultan *et al.,* (2018b**). The cytotoxic activity of **33** and its metabolic intermediate 2-aminopimelic acid was examined relative to arginine against three human cell lines Caco-2 (colorectal adrenocarcinoma), HT29-MTX-E12 (colorectal adrenocarcinoma) and HepG2 (hepatocellular carcinoma) cells. It was revealed that amino acid arginine produced minimum toxic effects while **33** with IC_{50} = 727-852 µg mL^{-1} showed highest cytotoxicity. Likewise, IC_{50} value for toxic potential of 2-aminoepimelic acid was 737-1087 µg mL^{-1}. Moreover, HepG2 cells were more sensitive to **33** than caco-2 cells. The rate of uptake of **33** across Caco-2/HT29-MTX-E12 cell lines was also found to be higher than that of arginine. Thus, **33** has high toxic potential than arginine and aminoepimelic acid and it is more likely to cause hepatic toxicity in humans.

Netzel *et al.,* (2019) studied the distribution and metabolism of indospicine (**33**) in camels fed for one month on *Indigofera spicata*. UHPLC-MS/MS analysis of blood plasma of alive camels showed that 2-aminopimelamic acid (**238)**, a foregut metabolite of **33**, was present in low amounts in blood indicating that it can be absorbed across foregut. In euthanized camels, 2-aminopimalemic acid as well as 2-aminopimelic acid (**239)** metabolites of **33** were detected in different tissues and organs of the body showing good tendency for bioaccumulation. Un-euthanized camels had **33** accumulated in the body even after hundred days of withdrawal of *I. spicata* diet. The accumulation period of **33** in body was found higher than its metabolites. Hence, the naturally toxic substance **33** potentially leads to *Indigofera* poisoning by accumulation in the body.

(e) Neurotoxicity

Lima *et al.,* (2012) experimentally poisoned a 7-year horse by allowing it to graze on *Indigofera lespedezioides.* The horse showed symptoms of poisoning on day 44 and died on 59^{th} day from initiation of grazing. Detailed tissue analysis of horse

showed lipofuscinosis in various parts of nervous system. **Riet-Correa *et al.,* (2017)** also enlisted *I. lespedezides* among list of 31 neurotoxic plants in Brazil.

Three horses grazed up to six weeks from a grassland rich with *Indigofera spicata.* They developed symptoms of neurotoxicity. Two of the horses were euthanized and liver analysis revealed serious histological damage. 3-nitropropionic acid (3-NPA) and **33** were determined in plant material of *I. spicata* as 2.66 mg/g and 1.5 mg/g dry matter, respectively. 3-NPA and **33** were also detected in the serum and muscles of one of the horses analyzed by *in vitro* assay. This was the first report on study of poisoning by *I. spicata* (**Ossedryver *et al.,* 2013**).

(f) Phytotoxicity

Uddin G. *et al.,* (2011) performed phytotoxic analysis of aqueous methanol extract of aerial parts of *Indigofera heterantha.* The test conducted on L*emna minor,* an aquatic plant, showed that phytotoxic activity of all extracts of *I. heterantha* was less at 10 µg/mL but increased with increasing concentration. The toxic effect was very strong when 1000 µg/mL concentration of each extract was used. This shows that the *I. heterantha* extract has good potential to act as natural herbicide.

Detoxification

The nitro compounds present in *Indigofera* plants are toxic. 3-nitropropionate irreversibly inhibits succinate dehydrogenase leading to inhibition of ATP production. It is good to know that twenty different kinds of bacteria present in the rumen can detoxify nitro-toxins. *Denitrobacterium detoxificans* is a nitro respiring bacterium which can be manipulated to enhance the rate of metabolism of nitro compounds (**Anderson *et al.,* 2005**).

1.5. Catalytic activity

Vijayan *et al.,* (2018) studied the catalytic effect of silver and gold nanoparticles of leaves of *I. tinctoria.* The reduction of *o*- and *p*-nitroanilines was carried out in the presence of $NABH_4$ and rate of reaction was compared with that in presence of silver-NPs or gold-NPs of *I. tinctoria* extract. Reaction monitoring by UV-vis spectroscopy revealed that kinetic barrier for nitroaniline reduction into phenylenediamine was lower in the presence of nanoparticles of *I. tinctoria.* The $NaBH_4$ carried out reduction of *O*-nitroaniline in 10 minutes and that of *p*-nitroaniline

in 15 minutes. The nanoparticles carried out reduction of *O*-nitroaniline in 8-10 minutes while that of *p*-nitroaniline in 17-18 minutes. Hence, the nanoparticles of *I. tinctoria* extract proved as effective catalysts for reduction of nitroanilines.

1.6. Application as dyes

Chen *et al.,* (2008) dyed cotton fabric with indigo blue obtained from *baphicacanthus cusia, Indigofera tinctoria* and *polygonum tinctorium.* Sodium hydrosulfite and thiourea dioxide proved to be good reducing agents in the process. The optimum time for dyeing was 3-minute and dye showed good UV resistance. A peak of 1624/cm in FTIR spectrum confirmed the indigo dye on fabric.

Splitstoser *et al.,* (2016) reported in an archeological review article that indigo was confirmed on a 6000-year-old cotton fabric obtained from a region of Peru. It was obtained from *Indigofera* spp. Indigo is now commonly known as blue of blue jeans.

Indigofera tinctoria, a source of indigo-blue dye, has use value index UV= 0.6 which is highest of the plants surveyed for dye applications in Roi Et province of Northeast Thailand. Various parts of *I. tinctoria* can be fermented by keeping up to three months followed by extraction with hot or cold water. This extract is reported to be used for dying silk and cotton (**Junsongduang *et al.,* 2017**).

Although the trend has shifted towards commercially produced indigo dye, Li textile weavers and Hainan Miao weavers are internationally recognized indigo producers from plants. They use *Indigofera tinctoria* and *Indigofera suffruticosa* for indigo extraction **Zhang *et al.,* (2019)**.

Conclusions from literature review

Critical review of literature led us to the conclusion of following points about phytochemistry of genus *Indigofera*:

So far, the plants of 26 species of genus *Indigofera* have been studied for phytochemical constituents. They are *I. tinctoria, I. subulata, I. cordifolia, I. aspalathoides, I. suffruticosa, I. heterantha, I. colutea, I. hirsuta, I. microcarpa, I. oblongifolia, I. hebepetala, I. linnae, I. pulchra, I. zollingeriana, I. secundiflora, I. stachyoides, I. spicata, I. caerulia, I. kirilowii, I. dalzelli, I. hochstetteri, I. mysorensis, I. prostrata, I. gerardiana, I. longeracemosa* and *I. arrecta.*

The components identified in *I. tinctoria* include an aliphatic carboxylic acid **1**, an unsaturated aliphatic ester **22**, a flavonoid glycoside **59**, seven flavonoids **60**, **61**, **62**, **63**, **64**, **65, 96,** a flavonol glycoside **95,** an organosulfur compound **125,** five phenolic acids **126**, **127**, **128, 129, 130,** two pyrroles **172, 173,** four indole derivatives **174**, **175**, **176**, **177**, two quinolines **181**, **182,** six rotenoids **183-188**, a steroid **195,** a galactomannan **202** and four other compounds **238**, **239**, **240** and **241**.

The phytoconstituents identified in *I. trita* or *I. sublulata* are two carboxylic acids **2**, **3**, five aliphatic esters **4-8**, a cyclic ester **23**, an amino acid derivative **34**, a sugar **200**, a triterpene **215** and two triterpenoids **216**, **217**.

Studies on *I. cordifolia* led to the identification of various phytoconstituents in it. These include three aliphatic carboxylic acids **9**, **10**, **11**, an alkene **32**, three flavonoids **63**, **64**, **77**, an aromatic ester **146**, a pyrrole derivative **178**, an indole compound **179**, two sterols **191**, **192** and four other compounds **233**, **234**, **235** and **236**.

The natural products identified in *I. aspalathoides* are an aliphatic ester **12**, an aliphatic alcohol **13**, a flavonoid **47, 63, 64**, a flavonoid glycoside **93**, a phenolic acid **147**, a pterocarpan **166**, two terpenoids **218**, **219**, a triterpene **228**, and a ketone **237**.

The secondary metabolites which have been identified in *I. suffruticosa* are an aliphatic carboxylic acid **14**, two aliphatic esters **15**, **16**, alkanes **24-31**, four flavonoids **39**, **64**, **78**, **79**, a nitro derivative **110**, aromatic acid **148**, an aromatic ester **149**, ten phenyl propanoids **156-165**, a sterol **190**, a sugar **201**, a triterpenol **217** and seven terpenes **220-226**.

I. heterantha is known to have constituents including an aliphatic carboxylic acid **17**, an ester **18**, six flavonoids **48**, **50**, **51**, **53**, **54**, **55**, **56**, **57**, **64**, **79**, **90**, **91**, **92**, **94**, two flavonoid glycosides **49**, **58**, an amide **104**, a lignan **105**, two phenolic acids **131**, **132**, a sterol **194** and two terpene derivatives, **204** and **205**.

The extract of *I. colutea* has been known to contain five fatty acids **3**, **11**, **19**, **20** and **21**. An *α*-amino acid **35**, a flavonoid glycoside **76**, phenolic acids **126, 127**, **128**, **129** and a nitrogenous base **232** has been identified in *I. hirsuta.*

Extracts of *I. microcarpa* are known to contain two benzofurans **36**, **37**, and sesquiterpenes **206-214**. The constituents identified in *I. oblongifolia* include a coumarin **38**, an acylated glucopyranoside **110**, phenolic acids **126-129**, **144**, **145,** a

sterol **190 and** a xanthene **230**. The current literature survey also revealed that six glycosylated flavonoids **40-46** are present in *I. hebepetala.*

I. linnae contains an isoflavone **52**, three flavonoids **63**, **64**, **77,** a steroid glycoside **193** and a stilbene **199**. *I. pulchra* has been identified with phytoconstituents, a chalcone **67**, two dihydro-stilbenes **197**, **198** and a triterpene **227**. Six flavonoid glycosides **68-73** have been identified in *I. zollingeriana.*

Three flavonoids **64**, **74**, **75** have been identified in *I. secundiflora*. Two bioflavonoids **80**, **81**, a flavonoid **82**, two chalcones **97**, **98**, a dihydrochalcone **99**, a lignan **106**, three phenolic glycosides **153**, **154**, **155** and a sterol **196** has been identified in *I. stachyoides*. *I. spicata* is known to have an amino acid **33**, seven flavonoids **83-89** and three rotenoids **188**, **189**, **190**.

Three flavonoid glycosides **65**, **71**, **99,** two phenolic acids **148, 130** and an aromatic ester **149** have been identified in *I. caerulia.* Eight nitro compounds **115, 116, 119-124** have been identified in *I. kirilowii.* Four phenolic acids phenolic acids **126**, **127**, **128**, **130** have been identified in *I. dalzellii.* Three phenolic acids **126**, **127**, **128** have been identified in *I. hochstetteri.*

Three phenolic acids **126**, **127**, **128** have been identified in *I. mysorensis.* Four phenolic acids **126**, **127**, **128**, **130** have been identified in *I. prostrata.* Three phenolic glycosides **151**, **152**, **153** have been identified in *I. gerardiana.* A quinoline **180**, a diterpenoid **203**, and a xanthene **229** have been identified in *I. logeracemosa.* An indole dimer **231** has been identified in *I. arrecta.*

Literature review of reported biological activities of plants of Indigofera genus revealed that 2′, 4′-dihydroxy-4-prenyloxy chalcone isolated from *Indigofera pulchra* can act as an analgesic agent. Compounds **197** and **198** isolated from this plant showed weak antibacterial effect against *S. aureus.* The extract of *Indigofera spicata* showed antidiarrheal activity. Compounds **59-62** isolated from *Indigofera tinctoria* were anti-dyslipidemic in action. The callus extract of this plant showed potential insecticidal effect. The methanol extract of whole plant of *Indigofera linnae* showed antinociceptive effect even greater than disprin. The methanol extract of *Indigofera suffruticosa* has healing activity against mucosal damage caused by stomach ulcer.

The extensive literature review of phytochemistry and pharmacological activities of plants of genus *Indigofera* led us to the conclusion that plants of this genus are rich and versatile in both these respects.

The work carried out on biological activities showed that the extracts of these plants show various activities such as analgesic, antibacterial, antifungal, anticancer, antidiabetic, antidiarrheal, anti-dyslipidemic and antiepileptic activities. Similarly, anti-inflammatory, antimalarial, antioxidant, antiprotozoal, antiviral, gastroprotective, hepatoprotective and immunomodulatory activities have also been reported. In addition to pharmacological significance, the insecticidal effect, catalytic activity, and application as dyes makes them economically more valuable.

Although, much work has been done on the phytochemistry of these plants, there are many things of interest to be explored yet. The biological activities of many plants of this genus still need to be studied to confirm basis of their ethnobotanical significance. Plant extracts which are found to be biologically active need identification of their active constituents and those plants from which organic compounds have been isolated and identified, require isolation of more compounds with the aim that they might prove to be a step ahead to the cure of various diseases.

The toxicity of many *Indigofera* plants also compels us for the isolation, identification, and pharmacological study of their components. This will provide awareness regarding the levels and causes of their toxicity. Only then it shall become possible to get benefit from these plants.

Chapter 2

Materials and Methods

2.1. Introduction to *Indigofera linifolia* (Plant of choice for study)

Literature survey revealed the presence of chemically diverse phytoconstituents in plants of genus *Indigofera.* Most of these constituents possess marvellous biological activities. However, *Indigofera linifolia* was found an under-investigated plant of this genus. It was selected as plant of choice for study.

Taxonomy

Kingdom	Plantae
Order	Fabales
Family	Fabaceae
Genus	*Indigofera*
Species	*Indigofera linifolia*

2.1.1. Ethnobotanical significance

The following ethnobotanical uses of *Indigofera linifolia* have been reported in literature.

Toothache: Leaf juice has been used to relieve toothache (**Wagh *et al.,* 2018**). **Cut and wounds**: The plant is known to cure cut and wounds (**Mishra *et al.,* 2015**). The leaves of plant have been used to treat wounds (**Maina *et al.,* 2016**). **Febrile eruptions**: Condition involving fever accompanied by skin allergy is called febrile eruption. *I. linifolia* has been used as skin emollient and to cure skin problems in febrile eruptions (**Iqbal *et al.,* 2020**). **Vermifuge**: The plant is considered as effective vermifuge. (**Gritto *et al.,* 2015**). **Scorpion bite**: *I. linifolia* has been used to treat scorpion bite (**Jahan *et al.,* 2019**). **Liver diseases**: It was also recommended to treat liver diseases (**Jahan *et al.,* 2019**).

Use for treatment of various diseases indicates presence of pharmacologically active phytoconstituents in this plant.

2.1.2. Morphological characteristics

I. linifolia is a branched, prostrate, and annual plant that can be up to 50 cm long. The plant may be erect or perennial. Small hairs are present on the branches. The leaves are stalkless and narrow, so the plant is also called "narrow leaf indigo." The plant has 3-8 mm long flowers, which are pink in color. Small, spherical, and smooth seeds are also characteristic of the plant (Figure 2.1).

(a) (b)

Figure 2.1. Morphological parts of *Indigofera linifolia* (**a**) Branches bearing leaves, flowers, and seeds (**b**) Roots

2.1.3. Habitat

The plant is commonly found in grasslands, on the sandy sides of roads, farmlands, and grassy forests (**De Kort I. and Thijsse G. 1984**).

2.1.4. Vernacular names

The plant is known by various vernacular names in sub-continent. These names include neel gosh, bhangra, ratanjot and torki.

2.2. Aims of study

No research work had been carried out on the phytochemistry of *I. linifolia*. The present study aimed to explore and characterize phytoconstituents present in various parts of this plant.

2.3. Plan of Work

The plan of work was designed, which consisted of the following steps:

- Collection and authentication of the plant
- Drying and cutting
- Extraction with *n*-hexane

- Extraction with hydro-methanolic solvent
- 2D paper chromatographic analysis
- Sugar hydrolysis and identification of sugars
- GC-MS analysis
- LC-MS analysis

2.3.1. Collection and identification of plant

The plant of *Indigofera linifolia* Retz., was collected on October 4, 2019, from the lawn of the Department of Earth Sciences, Quaid-e-Azam University (QAU), Islamabad. Professor Dr. Mushtaq Ahmed identified and authenticated the plant at the Department of Plant Sciences, Quaid-e-Azam University, Islamabad.

2.3.2. Drying, cutting and storage

All the four parts of the plant i.e., roots, stem, leaves, and seeds were separated. The roots and stem were cut into small pieces of approximately 3 mm in size. The finely cut roots and stem together with leaves and seeds were dried in the shade for ten days. The dried parts were crushed into fine particles with the help of mortar and pestle.

Dried and finely crushed plant material was packed into newspaper sheets, labelled, and kept in a safe and dry place.

2.3.3. Preparation of Extracts

a) Extraction with *n*-Hexane

The leaves, stem, seeds, and roots of *I. linifolia* were extracted with *n*-hexane to achieve defatting (removal of non-polar constituents). They were subjected to maceration, a process in which the plant material is soaked in a solvent at room temperature for a specified time.

180 g dried and crushed leaves of *I. linifolia* were soaked in 1200 mL of *n*-hexane. The leaves were kept soaked at room temperature followed by frequent mechanical agitation for up-to four days. The *n*-hexane extract was filtered on the fifth day using Whatman filter paper No.1. This process was repeated for the same plant material thrice by replacing *n*-hexane extract with fresh solvent every fifth day. The extract of three times was combined, filtered, and concentrated on a rotary evaporator at 35 ºC. The concentrated extract was blackish-green solid, transferred to a glass vial, and kept in the refrigerator at 4 ˚C (Figure 2.2).

300 g of the powdered stem was soaked in 1200 mL of *n*-hexane and extracted by the conventional maceration method described above. The combined blackish-green *n*-hexane extract of the stem was also filtered, concentrated, and stored at 4 ˚C.

230 g of dried and crushed *I. linifolia* seeds were treated with 400 mL of *n*-hexane. The extraction was repeated thrice, followed by filtration of the combined extract. The yellowish-black *n*-hexane extract of seeds was also concentrated and stored at low temperature.

Similarly, 50 g of dried roots were extracted thrice with 200 mL of *n*-hexane every time. The concentrated extract of roots was a pale-yellow oily substance transferred to a glass vial and stored in a refrigerator.

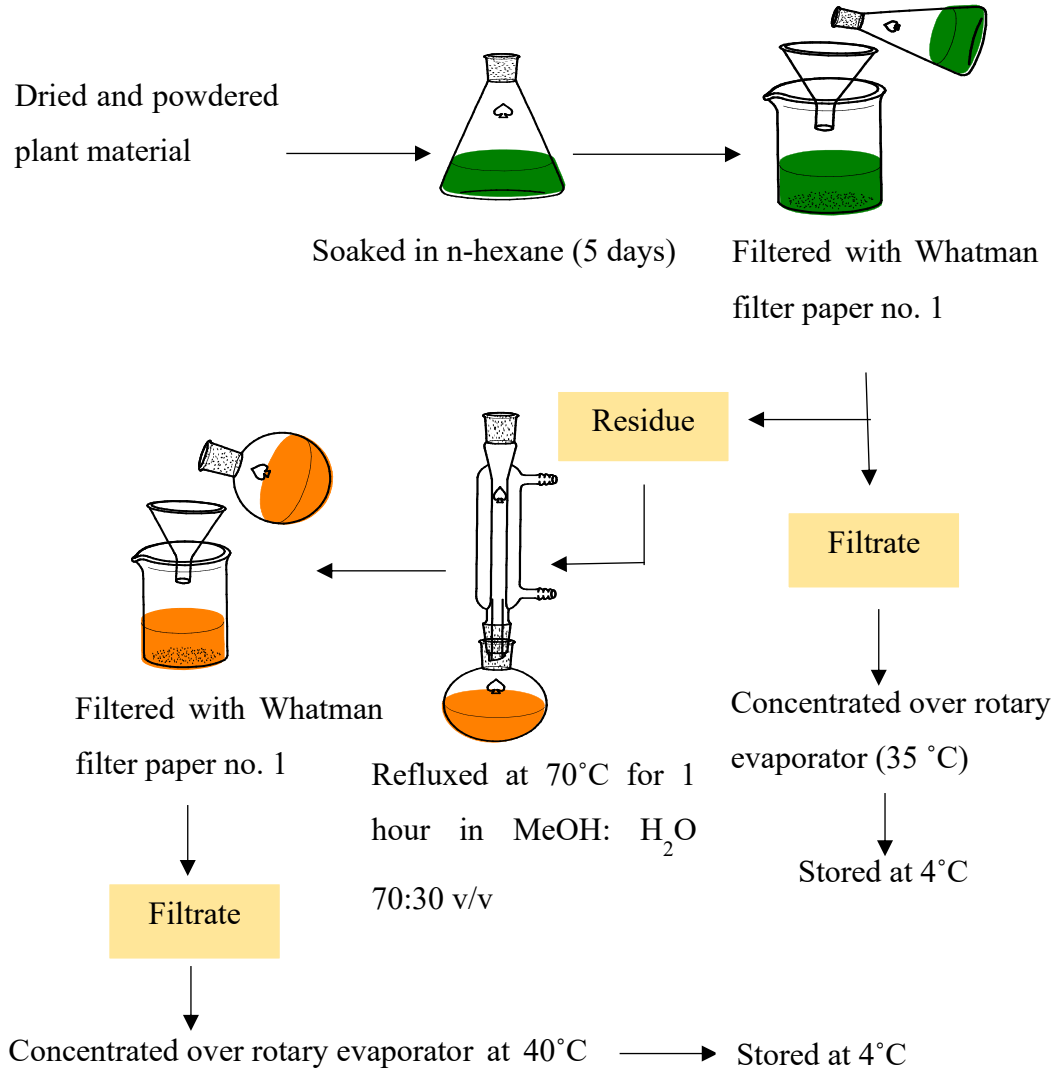


Figure 2.2: Schematic Representation of Steps Involved in Extraction

b) Extraction with hydro-methanol

Defatting of roots, stem, leaves, and fruit with *n*-hexane was followed by treatment with hydro-methanolic solvent (MeOH : H_2O in 70:30 v/v). The plant material was extracted by reflux.

The leaves were set to reflux on a water bath at 70˚C for 1 hour in a round bottom flask. The extract was kept at room temperature for 20 minutes and then filtered with Whatman filter paper No. 1. The process was carried out thrice, and all the three extracts were combined. The hydro-methanolic extract of leaves was orange-brown. The extract (ILLM) was concentrated on a rotary evaporator at 40˚C. The concentrated extract was transferred to a glass vial and stored in a refrigerator at 4˚C (Figure 2.2). The hydro-methanolic extract of the stem (ILSM), seeds (ILDM), and roots (ILRM) was also prepared, filtered, concentrated and kept under the same conditions as for leaves. The color of hydromethanolic extracts of other parts was same as that of hydro-methanolic leaf extract.

The list of eight different types of extracts which were prepared, has been shown in table 2.1.

Table 2.1. List of extracts prepared from various parts of *Indigofera linifolia*

Part of the plant	Mass of dried material (g)	*n*-hexane extract	Hydro-methanolic extract
Leaves	180	ILLH	ILLM
Seeds	230	ILDH	ILDM
Stem	300	ILSH	ILSM
Roots	50	ILRH	ILRM

2.3.4. 2D and 1D Paper chromatographic analysis

a) 2D Paper chromatographic analysis

Materials

BAW (4:1:5), 15% acetic acid, NH_3 solution, UV lamp, chromatographic tank, chromatographic paper (20 x 20 cm, type 3MM Whatman ^{TM}), capillary tube.

Preparation of Mobile phase

BAW (4:1:5)

80 mL of *n*-Butanol was mixed with 20 mL acetic acid, and 100 mL distilled water and kept for 17 hr. Two layers were formed and the upper phase was used as mobile phase.

15 % acetic acid

15 mL of acetic acid was added to a 100 mL volumetric flask containing some distilled water. The flask was shaken well and filled with distilled water up to the mark.

Method

A 20 x 20 cm Whatman chromatographic paper was marked with a lead pencil at 0.5 cm away from the paper's bottom and right side. The ILLM extract was applied as a spot of 0.5 cm at the chromatographic paper's marked corner. The sample spot was dried in the air, and the paper was suspended in a chromatographic tank pre-saturated with BAW (4:1:5) as a mobile phase. After 8 hours, the paper was removed from the chromatographic tank, and the solvent front was marked using a lead pencil. The paper was dried in the air. Then it was rotated at an angle of 90˚ and suspended in a chromatographic tank containing 15 % acetic acid. This development in the second dimension completed in 1 hr. Again, the chromatogram was removed from the tank and dried in the air. The spots on the developed chromatogram were visualized under UV light of wavelength 365 nm. The chromatogram was exposed to ammonia vapors for 05 minutes and readily viewed under a UV lamp to observe color change (**Markham, 1982**).

b) 1D Paper chromatographic analyses

Materials

15% acetic acid, Whatmann filter paper no.1, capillary tube, chromatographic tank.

Method

A 20 x 20 cm Whatmann filter paper no.1 was cut and marked with pencil at four points for ILLM, ILRM, ILDM and ILSM extract. The sample spot of each

concentrated extract was applied at respective position. The paper was suspended in a chromatographic tank pre-saturated with 15% acetic acid. The mobile phase ran over the paper and development was complete in 40 minutes. The paper was dried in fume hood and viewed under UV light 365 nm. The colored spots for each type of extract were marked and R_f value was calculated. The paper was exposed to NH_3 vapours to see any change in color of spots.

2.3.5. Acid hydrolysis

Materials

TLC plate (Silica gel 60 F_{254}), 2M HCl, 0.2M NaH_2PO_4, acetone, distilled water, Aniline hydrogen phthalate reagent, standard sugar solutions.

Preparation

2 M HCl

16.6 mL of 37 % commercially available (12M) HCl was transferred to a 100 mL volumetric flask containing some water. The flask was then filled with distilled water up to the mark.

0.2 M NaH_2PO_4

2.3 g Monosodium dihydrogen phosphate (MW= 119.98 g/mol) was dissolved in 100 mL distilled water in a volumetric flask.

Standard sugar solutions

Standard sugar solutions were prepared by dissolving 100 mg of each sugar in 20 mL of water. 10 % isopropyl alcohol was added as a preservative.

Aniline hydrogen phthalate

0.92 mL aniline and 1.6 g phthalic acid were added to a conical flask containing 49 mL of *n*-Butanol, 49 mL of ether, and 2 mL of water. The flask was agitated to dissolve the acid and aniline. The reagent was then transferred to a spray bottle.

Impregnation of TLC plates

0.2 M NaH_2PO_4 solution was sprayed on TLC plates pre-coated with silica gel and heated in oven at 120 ˚C for 25 minutes.

Method

1 g of ILLM extract was refluxed with 30 mL of 2 M HCl : MeOH (1:1) at 100˚C for 1 hour. The hydrolysate was concentrated on a rotary evaporator, and the residue was dissolved in a minimum amount of MeOH : H_2O (1:1). It was then concentrated on a rotary evaporator such that only the aqueous layer was left behind. The aqueous layer was five times extracted with ethyl acetate in a separating funnel.

A baseline was marked with a lead pencil on a 20 x 20 cm silica gel TLC plate impregnated with 0.2 M NaH_2PO_4. The aqueous layer of hydrolysate was applied as spots of three different types of loadings on the baseline. Spots of standard solutions of galactose, rhamnose, xylose, and arabinose were also applied such that every two spots were 0.5 cm apart. The spots were dried in the air, and the TLC plate was developed in a chromatographic tank containing acetone : water (9:1) as the mobile phase. As the development was complete, the plate was dried in the air and sprayed with aniline hydrogen phthalate to visualize the sugar spots. The plate was kept in the oven at 100 ˚C for 5 minutes. As a result, brown spots of sugars appeared on the chromatogram. Visualization under UV light = 365 nm further enhanced the sensitivity of detection. The sugars in the ILLM extract were identified by comparing their R_f values with that of standard sugars.

2.3.6. GC-MS analysis of *n*-hexane extracts of *Indigofera linifolia*

Sample preparation

1 mg/mL solution of ILLH extract was prepared in *n*-hexane. The solution was filtered by Whatman filter paper. No.1. such that a clear sample solution free of any turbidity or particulate matter was obtained.

Sample solutions for ILSH, ILDH and ILRH were also prepared in the same way.

Instrumentation

The analysis was performed using the Gas Chromatographic system of technology type Agilent 6890N. Chromatographic separation was carried out on

Agilent DB-5 MS column having specific dimensions (30 m x 0.25 mm internal diameter x 0.25 µm film thickness). The column was packed with fused silica containing 5% phenyl methyl poly-siloxane. Highly pure helium gas (99.9 %) was used as the carrier gas at a flow rate of 1.5 mL/min. The sample was injected as 5 µL injection. The system's temperature range was kept at 120-280 ˚C, and the rising temperature rate between this limit was 10 ˚C/min. The mass spectra were recorded in Electron Impact or EI mode at ionization voltage of 70 eV. A mass scan was performed in the range of 50-1000 Da.

2.3.7. LC-MS analysis of hydromethanolic extracts of *Indigofera linifolia*

Sample preparation

200 mL of ILLM extract was concentrated to 2mL on rotary evaporator at 40 ˚C. The extract was filtered by using Whatman filter paper No.1. It was then subjected to LC-MS analysis.

The samples of ILSM, ILDM, and ILRM were prepared by the same procedure.

Instrumentation

The analysis was carried out using Agilent technology HPLC instrument model no. 1200 coupled to 6310 ion trap LC-MS system equipped with G1322A degasser and G1329A ALS autosampler. The separation was performed using a stainless steel HPLC column (4.6 x 150 mm). The stationary phase was Agilent Eclipse XDB C-18 that is octadecyl silica having lesser affinity for more polar compounds. The mobile phase composition was 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The elution was performed in gradient manner such that 0-15 min; 10% B, 16-40 min; 40% B, 41-50 min; 60% B, 51-60 min; 90% B (Table 2.2).

Table 2.2. Gradient elution programme for HPLC

Time (minutes)	Solvent A (Water)	Solvent B (Acetonitrile)
0-15	90	10
16-40	60	40
41-50	40	60
51-60	10	90

The mobile phase flow rate was set as 0.5 mL/min. Formic acid prevents the tailing of chromatographic peaks by suppressing the ionization of aromatic hydroxyl groups. UV chromatograms were recorded at 254, 320 and 380 nm by G1315B DAD detector. The mode of ionization was negative electrospray ionization (-ESI). The sample was introduced as 5µL injection, and the mass scan range was 0-1000 Da.

2.3.8. Fractionation of ILLM extract by preparative paper chromatography

Preparative paper chromatography was carried out by optimized method. The steps involved are described below:

(i) Preparation of sample solution

200 mL of hydro-methanolic (70:30) extract of *I. linifolia* leaves was concentrated on a rotary evaporator to get 2 mL of concentrated extract. The orange-red color of extract became dark brown upon concentration.

(ii) Cutting and marking of chromatographic papers

By using Whatman filter paper no.1 sheets, 500 chromatographic papers (each 20 x 20 cm) were cut. Each paper was marked with a baseline by leaving 1 cm from both sides and 2 cm from bottom side of the paper.

(iii) Drying the papers

The papers were gently dried using hair dryer as source of warm air.

(iv) Sample loading

The ILLM extract was loaded with the help of capillary tube on the baseline marked with pencil. The sample was applied in the form of a 0.3 cm band. The band was dried in air and viewed under a UV lamp at 365 nm to ensure extract's application.

(v) Development

The chromatographic paper with dried band was suspended in chromatographic tank pre-saturated with 15% acetic acid. The development completed in 1 hour.

vi) Drying of chromatograms

After development, the chromatogram was removed from the tank and allowed to dry by hanging in fume hood.

(vii) Marking the bands

When the chromatogram was dried, it was viewed under UV light 365 nm. Variously colored bands were marked using lead pencil. Similarly, all the 500 chromatograms were developed and marked under UV light of 365 nm.

(viii) Cutting of bands

The bands were cut into strips followed by fine cutting using a sharp and clean scissor. Each band was added to a separate flask that was properly labelled.

(ix) Elution

The bands were soaked in MeOH : H_2O (70:30) for five days. During this period, the flasks were mechanically agitated. After five days, the extract from each chromatographic band was filtered, concentrated, and stored in properly labelled vials. The eluted bands were processed further for isolation of phenolic constituents. (Figure 2.3).

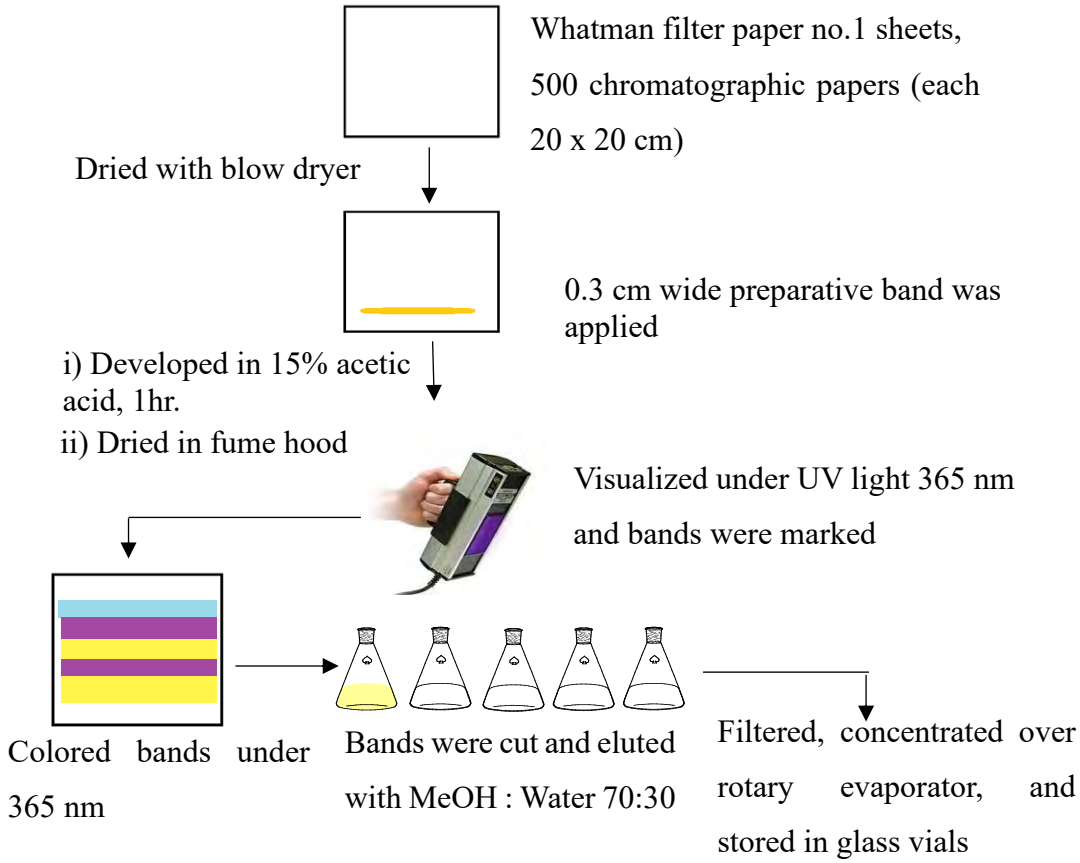


Figure 2.3. Steps involved in fractionation of ILLM extract by paper chromatography

Chapter 3

Results and discussion

3.1. Percentage yields of extraction

The yields of extraction from leaves, stem, fruits and root extracts were calculated using following mathematical equation:

$$Percentage\ yield\ = \frac{mass\ of\ extract\ (g)}{mass\ of\ dried\ plant\ before\ extraction\ (g)} \times 100$$

The yields of various extracts are summarized in table 3.1.

Table. 3.1. Percentage yield of various extracts

Type of Extract	Mass of extract (g)	%Yield	Type of extract	Mass of extract (g)	%Yield
ILLH	2	0.001	ILLM	10	0.05
ILSH	1.5	0.005	ILSM	7	0.02
ILRH	0.1	0.002	ILRM	1	0.02
ILDH	1	0.004	ILDM	12	0.05

3.2. 2D and 1D Paper chromatographic analysis

a) 2D Paper chromatographic anaysis

2D Paper chromatography is a significant source to get pre-liminary information about the types of flavonoids present in a sample. Useful conclusions can be drawn from the number of spots on chromatogram, relative distance travelled by spots in two dimensions and color of spots under UV light (365 nm) in the presence or absence of NH_3 vapours.

The development of chromatogram in one dimension in BAW (4:1:5) resulted in separation of sample spot of ILLM into three spots which were purple, dull yellow and fluorescent sky blue. The R_f values for these spots were 0.06, 0.18 and 0.4 respectively.

The development of this chromatogram in second dimention by using 15 % acetic acid as mobile phase resulted in separation of purple and dull yellow spots into

two spots each. Hence, a total of five spots were obtained in the second dimension such that the R_f values for dull yellow, purple, dull yellow, purple, and fluorescent sky-blue spots were 0.14, 0.20, 0.36, 0.58 and 0.69 respectively (Figure 3.1).

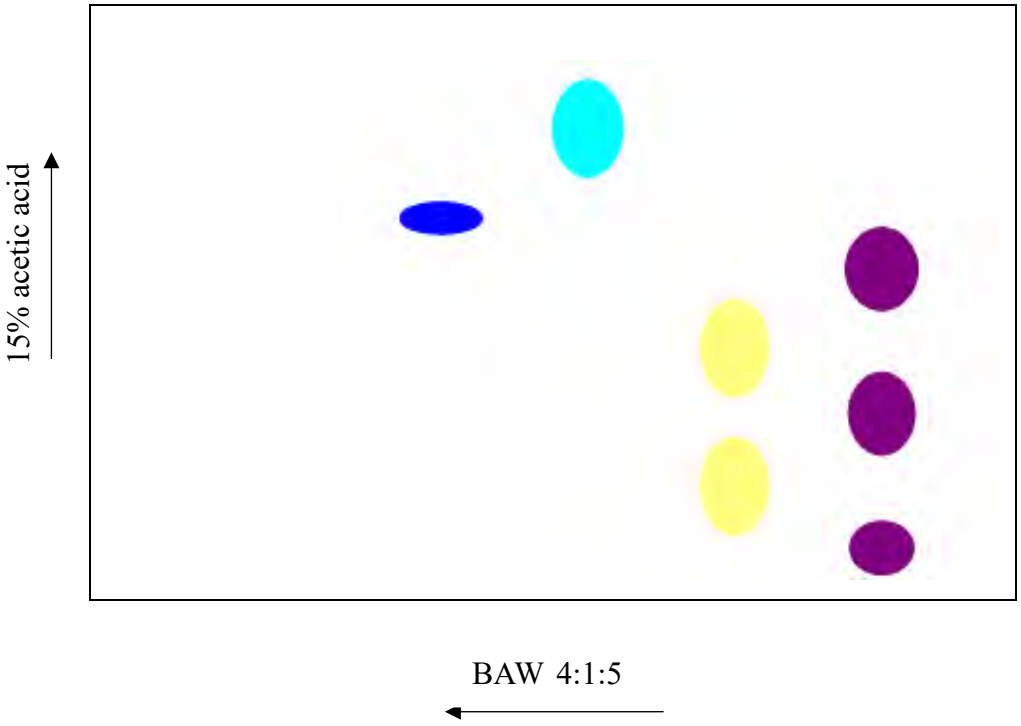


Figure. 3.1. Colored spots obtained by 2D paper chromatography of ILLM extract as viewed under UV light of 365 nm

The information obtained from color of flavonoid spots has been summarized in table 3.2**.** Moreover, the inferences obtained from R_f value of flavonoid spots have been summarized in table 3.3.

b) 1D Paper chromatographic analysis

The 1D-Paper chromatographic profile of ILLM, ILRM, ILSM and ILSD extract was obtained by running the chromatogram in 15 % acetic acid. The chromatogram viewed under UV light 365 nm showed colored spots as shown in figure 3.2. The R_f value for each spot was calculated and is displayed in the profile shown below. Greater R_f value in 15% acetic acid corresponds to higher extent of glycosylation in the flavonoid. The change in color of purple spot to yellow was observed upon exposure to ammonia vapours. The profile can be interpreted in terms of different flavonoid types which may be present in each extract by the help of table 3.2.

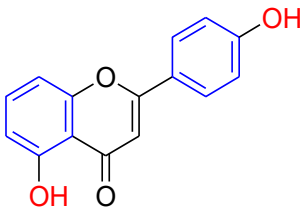
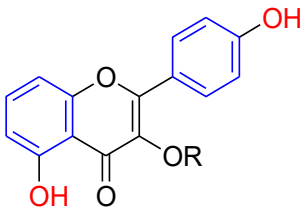
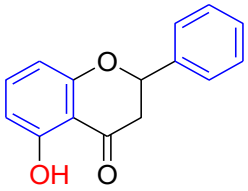
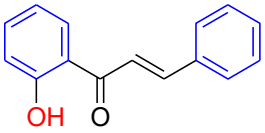
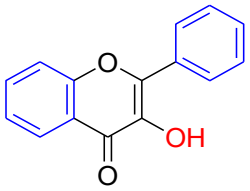
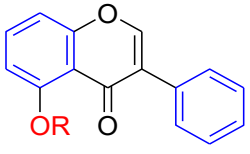
<table>
<tr><td colspan="3">Table 3.2. Information obtained from color of spots with and without NH_3 under UV light 365 nm</td></tr>
<tr><td>Without NH_3</td><td>With NH_3</td><td colspan="2">Inferences</td></tr>
<tr><td rowspan="4">Purple</td><td rowspan="4">Yellow</td><td>  </td><td>  </td></tr>
<tr><td>Flavones with 5-OH free and 4’-OH</td><td>Flavonols 3-O-substituted with 5-OH free and 4’-OH</td></tr>
<tr><td>  </td><td>  </td></tr>
<tr><td>Flavanones with 5-OH free</td><td>Chalcones with 4’-OH, no OH in B ring</td></tr>
<tr><td>Dull Yellow</td><td>Dull Yellow</td><td colspan="2"> 
Flavonols with free 3-OH are indicated</td></tr>
<tr><td>Fluorescent sky blue</td><td>Fluorescent sky blue</td><td colspan="2"> 
Isoflavones with 5-OH absent or substituted are indicated</td></tr>
</table>

Table 3.3. Information obtained from R_f value of spots					
Color of spot	**R_f in BAW**	**Inferences**	**Color of spot**	**R_f in 15% CH_3COOH**	**Inferences**
Purple	0.06	More hydroxylated	Purple	0.01	Aglycone
			Dull Yellow	0.14	Mono-glycosylated
Dull Yellow	0.18	Less hydroxylated	Purple	0.20	Mono or di-glycosylated
			Dull Yellow	0.36	Di or tri-glycosylated
Fluorescent sky blue	0.40	Least hydroxylated	Purple	0.58	Tri-glycosylated
			Fluorescent sky blue	0.69	Tri or tetra-glycosylated

The paper chromatographic profile for hydromethanolic extracts of four parts of *Indigofera linifolia* showed dull yellow, purple, and fluorescent sky-blue spots under UV light 365 nm. The spots remained unchanged upon exposure to ammonia vapours except the purple spot which turned yellow. The interpretation of observations in 1D-PC profile using table 3.2 led to the conclusions that flavones, flavonols and isoflavones are mainly indicated in the extracts. The purple spots indicated a range of flavonoids with free 5-OH. Dull yellow spots observed in all the four parts indicated the presence of flavonols with free 3-OH. Fluorescent sky-blue spots in ILLM and ILSM corresponded to the presence of isoflavones lacking 5-OH or having substituted 5-OH.

3.3. Acid hydrolysis

Hydrolysis is a term which finds its origin in the ancient Greek words, “hydro” meaning “water” and “lysis” meaning “to unbind”. Hydrolysis is the cleavage of a sugar from an aglycone or another sugar by addition of water molecule to break the glycosidic linkage. In present work, sugar hydrolysis was carried out under acidic conditions. Under these conditions, *O*-glycosides can be hydrolyzed in the order rhamnoside >

galactoside = glucoside > glucuronide while *C*-glycosides remain unhydrolyzed (**Markham, 1982**).

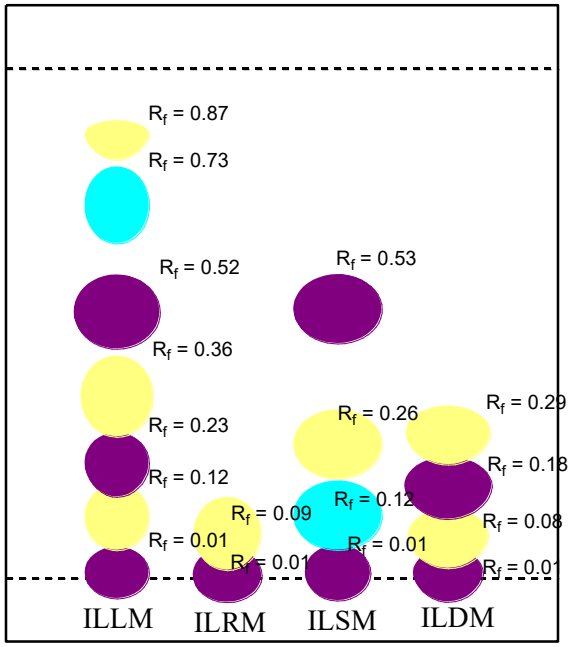


Figure 3.2. 1D-PC profile of extracts in 15% acetic acid (viewed under 365 nm)

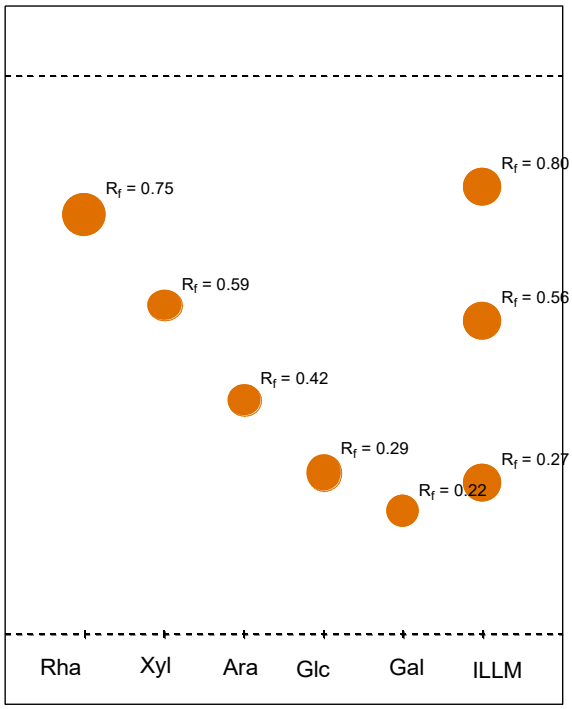


Figure 3.3. Co-chromatographic TLC profile of sugars in ILLM hydrolysate

Co-chromatography of ILLM extract against standard sugar solutions was followed by spraying of chromatogram with Aniline hydrogen phthalate. This reagent is highly selective for reducing sugars and has high sensitivity for pentoses and aldohexoses (**Partridge, 1949**). The reagent resulted in conversion of colorless sugar spots into brown spots (Figure 3.3). The color appearance aided in comparison of R_f value of unknown sugars in ILLM extract with that of standard sugars (Table 3.4).

Table 3.4. R_f value of standard and unknown sugars in the sample

Standard sugars	R_f value	Sugars in sample
Rhamnose (Rha)	0.75	0.8
Xylose (Xyl)	0.59	0.56
Arabinose (Ara)	0.42	absent
Glucose (Glc)	0.29	0.27
Galactose (Gal)	0.22	absent

These observations revealed the presence of rhamnose, xylose and glucose as sugars in the ILLM extract.

3.4. GC-MS analysis of *n*-hexane extracts of *Indigofera linifolia*

GC-MS analyses of various extracts of *Indigofera linifolia* gave TIC profile which showed intensity of each constituent againt retention time. Mass spectra of each component led to the tentative identification of various lipophilic compounds. The result of GC-MS analysis of all the four extracts ILDH, ILSM, ILRM and ILLF has been descussed below.

ILDH extract

TIC profile of ILDH extract

The total ion chromatogram or TIC profile of ILDH extract showed seven signals which corresponded to the presence of volatile and moderately volatile lipophilic costituents in the seed extract (Figure 3.4).

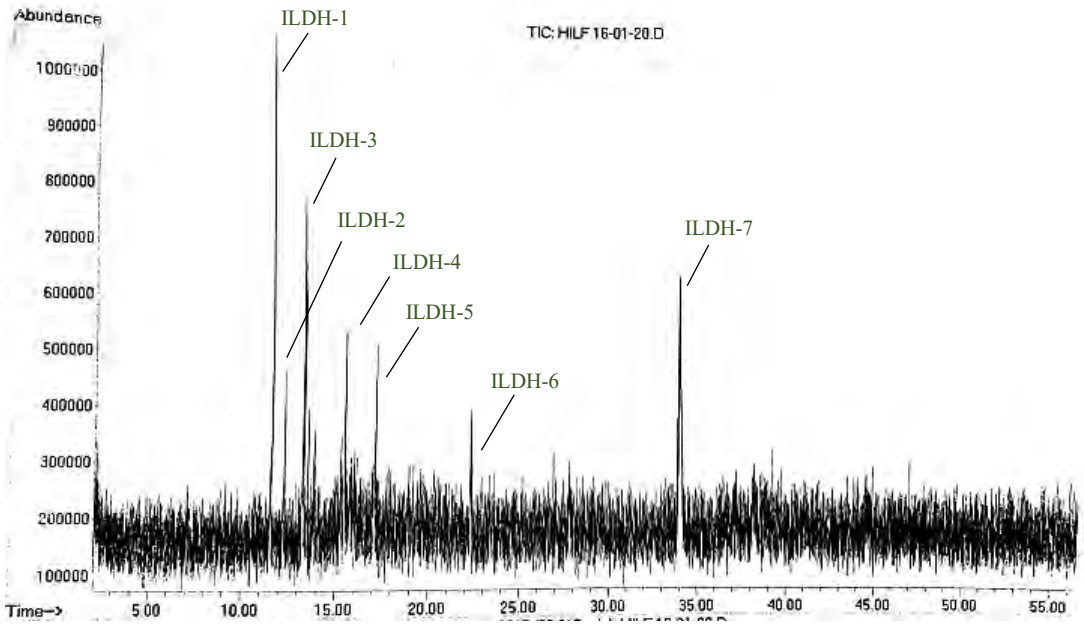


Figure 3.4. TIC profile of ILDH extract

ILDH-1

Retention time of ILDH-1

The component ILDH-1 eluted from GC column at retention time of 11.56 minutes. Moderately low retention time in the gas chromatographic column indicated that the component was volatile in nature.

Mass spectral analysis of ILDH-1

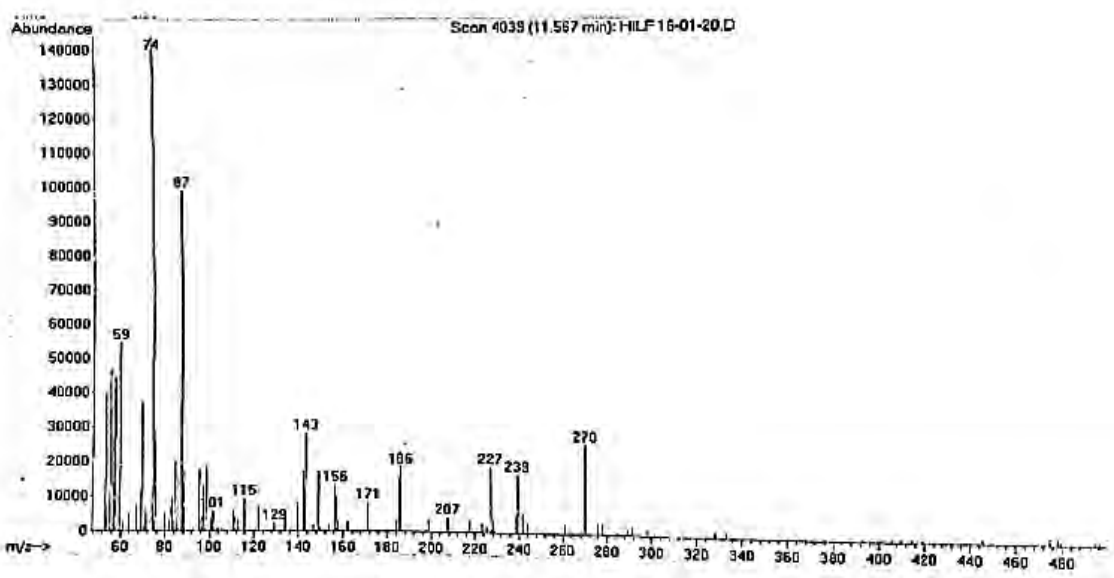
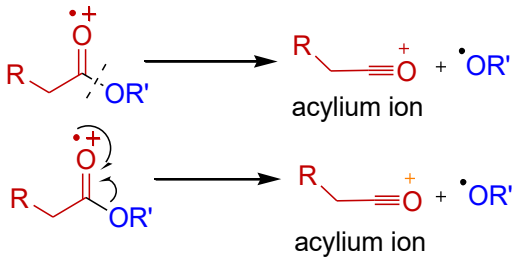
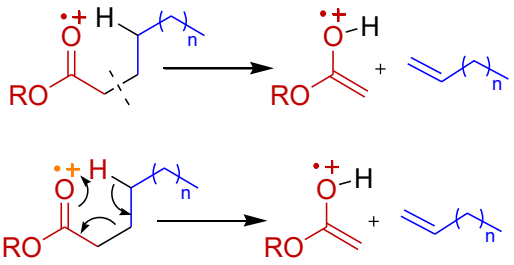


Figure 3.5. Mass spectrum of ILDH-1

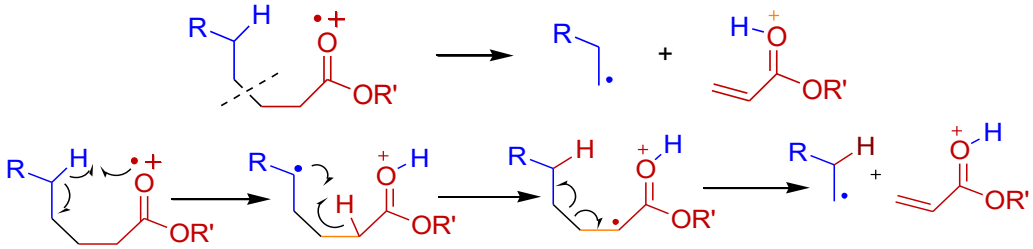
The molecular ion peak appeared at *m/z* 270 which indicated that the molecular weight of the component was 270 amu (Figure 3.5). The base peak at *m/z* 74 suggested that the component ILDH-1 could be a methyl ester. The molecular ion of a methyl ester characteristically undergoes McLafferty rearrangement to produce base peak at *m/z* 74 (**Pavia *et al.*, 2014**).



(a) alpha cleavage **(Pavia *et al.*, 2014)**



(b) McLafferty rearrangement **(Pavia *et al.*, 2014)**



(c)Double hydrogen transfer rearrangement **(Colnaghi Simionato *et al.*, (2007)**

Figure. 3.6. General fragmentation patterns of esters in EI/MS

The base formula for the component was calculated as $C_{20}H_{30}$ by implying rule of thirteen (**Pavia *et al.*, 2014**). This formula was modified to derive molecular formula for the indicated ester as $C_{17}H_{34}O_2$.

HDI (Hydrogen deficiency index) was calculated as below:

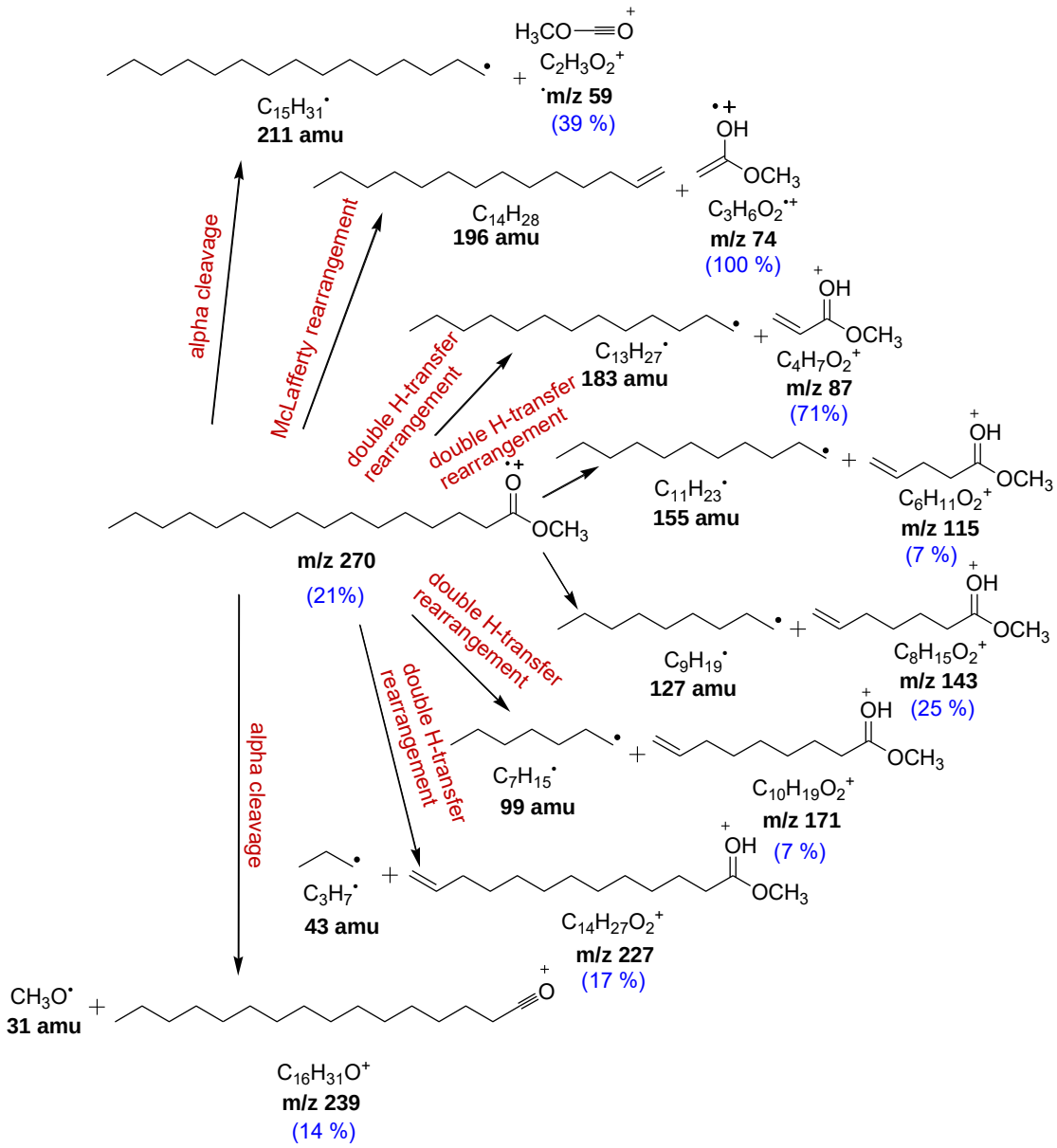
$$HDI = C - \frac{X}{2} + \frac{N}{2} - \frac{H}{2} + 1$$

$$HDI = 17 - \frac{0}{2} + \frac{0}{2} - \frac{34}{2} + 1$$

$$HDI = 1$$

HDI of 1 could be adjusted for one double bond in the molecule that was probably due to carbonyl group of the ester functionality.

General fragmentation patterns of aliphatic esters in EI mass spectrometry are shown in figure 3.6. The mass spectrum of unknown ester was justified based upon these fragmentation patterns.



Scheme 3.1. EI Fragmentation Pattern of ILDH-1

The loss of 31 amu from molecular ion peak resulted in fragment ion (M-31) at *m/z* 239. This was attributed to the loss of OCH_3 group which further supported the identification of compound as methyl ester (**Pavia *et al.*, 2014**). The loss occurred by alpha cleavage of OCH_3 from carbonyl carbon of the ester.

The molecular formula, HDI, observed losses, possible ions and fragmentation pattern supported by references converged the discussion at the point that component (ILDH-1) which eluted at 11.56 minutes by gas chromatography of *n*-hexane extract of *Indigofera linifolia* seeds, is an aliphatic ester named as methyl hexadecanote or methyl palmitate (Scheme 3.1).

Following the same strategy, the retention time and mass spectra of other constituents of ILDH extract were studied. The TIC retention time, m/z values, percentage of NIST library match and name of each putatively identified compound of ILDH extract has been summarized in table 3.5.

Table 3.5. Putatively identified compounds in ILDH extract

Retention time (minutes)	**Ions (m/z)**	**Perecentage of NIST library match**	**Identified constituent**
11.5	270, 227, 143, **74** (100), 53	87	Methyl hexadecanoate
12.2	356, 314, 240, 182, 127, **88** (100), 55	9	(4S)-4-[(t-butoxycarbonyl-(R)-alanyl) amino]-6-methyl-ethyl-2-(E)-heptanoate
13.2	110, **67** (100), 41	64	4-Octyne
15.43	156, 107, 79, **55** (100)	38	5-Nitro-2-furaldehyde p-tosylhydrazone
34	434, 392, 353, 296, 218, 109, **57** (100)	9	Methyl-17-beta-acetoxy-4-oxo-4-propyl-5,6-epoxy-3,4-secoandrostan-3-oate

ILSH extract

TIC Profile of ILSH extract

The TIC profile of ILSH extract showed six peaks indicating the presence of volatile and lipophilic constituents in ILSH extract. The mass spectrum of each

component which appeared in the TIC profile gave useful structural information about respective component (Figure 3.7).

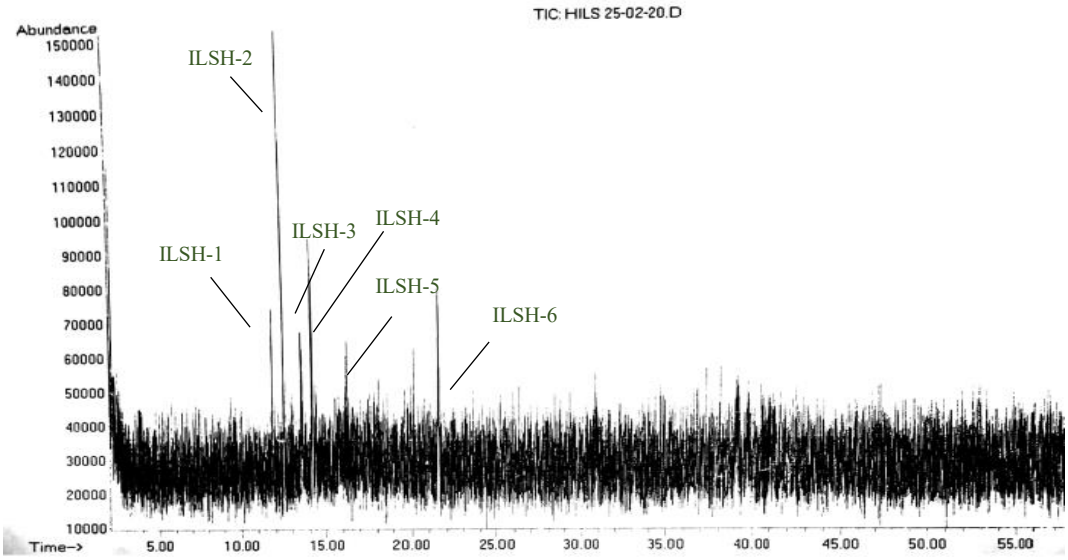


Figure 3.7. TIC profile of ILSH extract

ILSH-2

Retention time of ILSH-2

The retention time of the component ILSH-2 was recorded as 12.43 minutes. This indicated that the component was moderately volatile in character.

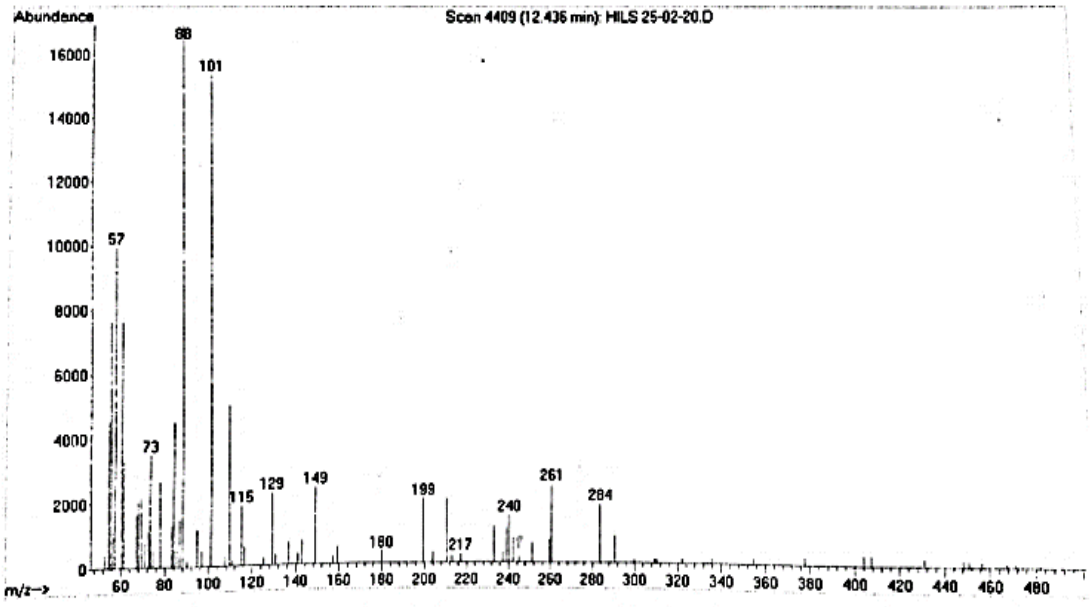
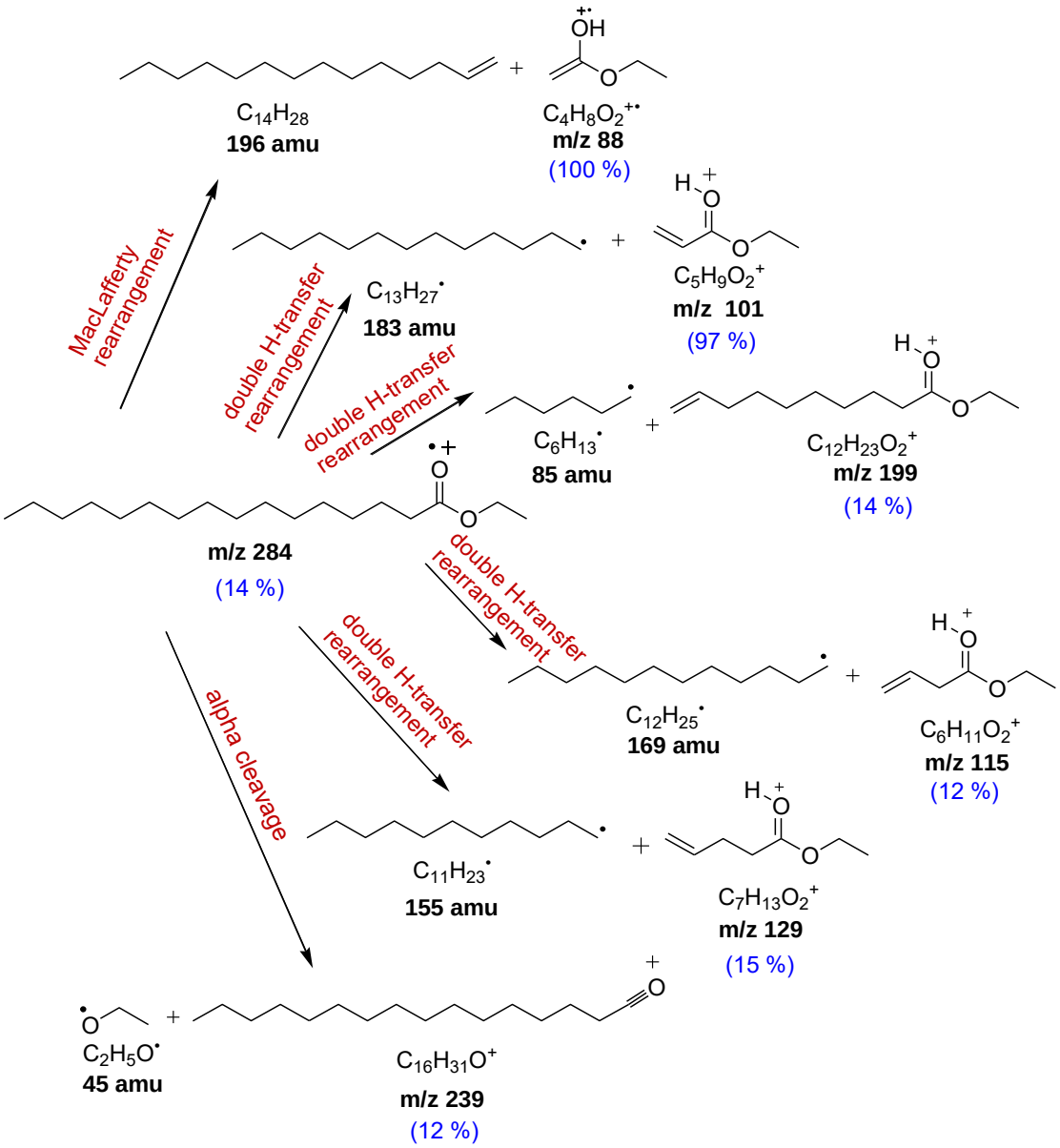


Figure 3.8. Mass spectrum of ILSH-2

Mass spectral analysis of ILSH-2

The mass spectrum of ILSH-2 presented a molecular ion peak at *m/z* 284 (Figure 3.8). Hence the molecular weight of the component was 284 amu. Rule of thirteen was applied to calculate base formula using molecular weight of the component and it was found to be $C_{21}H_{32}$.



Scheme 3.2. EI Fragmentation Pattern of ILSH-2

The base peak suggested that the component could be an ethyl ester because they characteristically undergo McLafferty rearrangement to yield a base peak at *m/z* 88. The molecular formula $C_{18}H_{36}O_2$ was derived from base formula by taking into

consideration the possible number and types of heteroatoms in an ester. HDI index was calculated as below:

$$HDI = C - \frac{X}{2} + \frac{N}{2} - \frac{H}{2} + 1$$

$$HDI = 18 - \frac{0}{2} + \frac{0}{2} - \frac{36}{2} + 1$$

$$HDI = 1$$

The HDI value of 1 indicated the presence of one unsaturation in the component. This was probably due to the double bond of carbonyl carbon of ester functionality.

The M-45 peak was attributed to the loss of OCH_2CH_3 group by homolytic cleavage from the carbonyl carbon. It provided another evidence for the component to be an ethyl ester.

The molecular formula, HDI, observed losses, possible ions and fragmentation pattern led to the tentative identification of ILSH-2 as ethyl hexadecanoate or ethyl palmitate (Scheme 3.2).

Similarly, other constituents of ILSH extract were structurally analyzed based upon TIC retention time, m/z values and NIST library match. The names and GC-MS data of tentatively identified constituents has been tabulated in table 3.6

Table 3.6. Putatively identified compounds in ILSH extract

Retention time (minutes)	**Ions (m/z)**	**Perecentage of NIST library match**	**Identified constituents**
12.43	284, 240, 199, 129, 101, **88** (100), 57	87	Ethyl hexadecanoate
14.05	212, 188, 164, 131, 107, **81** (100), 55	4	2-Cyclooctyl-1,3,2-oxathiaborinane
21.47	548, 411, 386, 149, **69**(100)	5	2,7-Bis-dodecyloxy-fluoren-9-one

ILRH extract

TIC profile of ILRH extract

The TIC profile of ILRH extract showed four intense signals corresponding to the presence of lipophilic constituents in ILRH extract. The retention time of all the constituents was less than 20 minutes. It showed that the components were highy volatile (Figure 3.9).

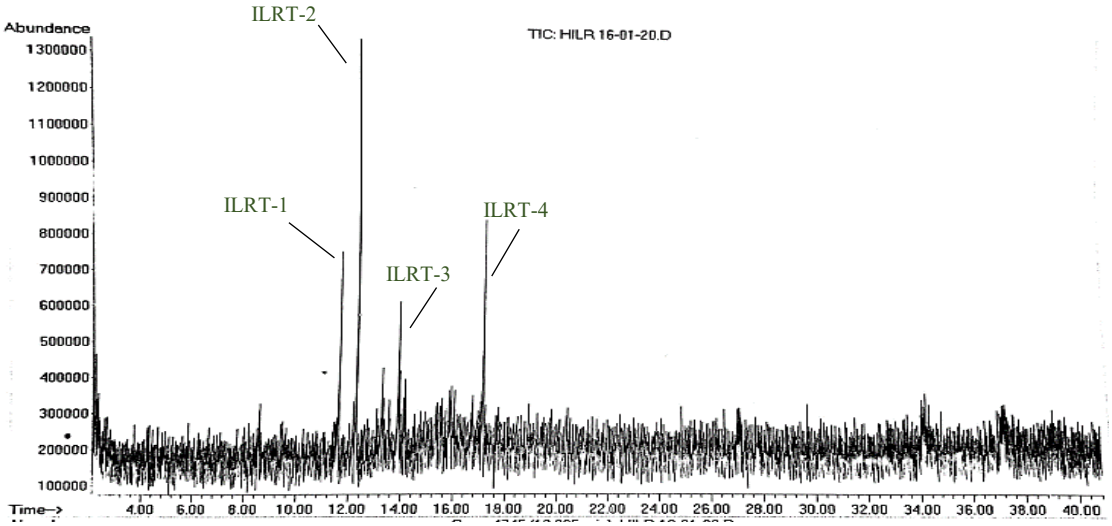


Figure 3.9. TIC profile of ILRH extract

ILRH-1

This component appeared at 11.5 minutes in the TIC profile. The molecular ion peak appeared at m/z 270. The retention time and mass spectral information for ILRH-1 was same as that of ILDH-1. So, the component methyl hexadecanoate was tentatively identified in seeds as well as roots (Scheme 3.1).

ILRH-2

The constituent ILRH-2 appeared at 12.2 minutes in the TIC profile. The mass spectrum showed molecular ion peak at m/z 284. The component was identified as ethyl hexadecanoate. The fragmentation pattern was same as observed for ILSH-1 (Scheme 3.2).

The GC-MS data and percentage of NIST library match for putatively identified constituents in ILRH extract has been summarized in table 3.7.

Table 3.7. Putatively identified compounds in ILRH extract			
Retention time (minutes)	**Ions (m/z)**	**Percentage of NIST library match**	**Identified constituents**
11.5	270, 239, 227, 186, 143, 87, **74** (100), 59	87	Methyl hexadecanoate
12.2	284, 239, 143, 115, **88** (100), 55	87	Ethyl hexadecanoate

ILLH extract

TIC profile of ILLH extract

The TIC profile of ILLH extract showed three signals (Figure 3.10). The retention time and mass spectra of the components were studied. The mass spectral data, percentage of NIST library match and names of putatively identified components have been summarized in table 3.8.

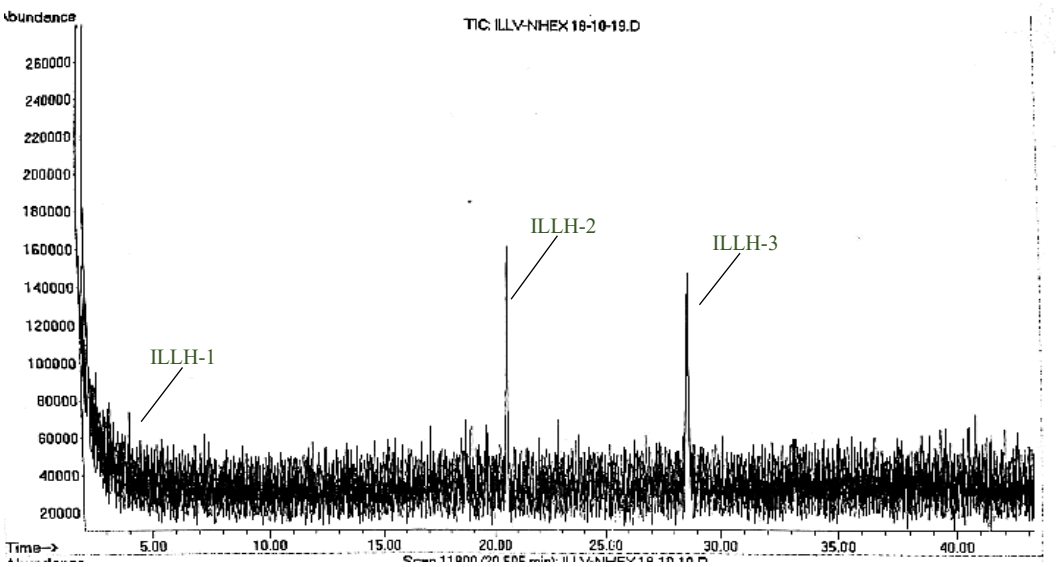


Figure 3.10. TIC profile of ILLH extract

Table 3.8. Putatively identified compounds in ILLH extract			
Retention time (minutes)	**Ions (m/z)**	**Percentage of NIST library match**	**Identified constituent**
2.3	**99** (100), 70, 56	39	1-Methyl-2-pyrrolidinone
20.5	220, 203, 189, 173, 119, 93, **81**(100), 69, 55	37	2-Methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane
28.535	**430**(100), 395, 365, 278, 205, 165, 121, 57	38	Vitamin E

3.5. LC-MS analysis of hydromethanolic extracts of *Indigofera linifolia*

The hydro-methanolic (MeOH : H_2O 70:30 v/v) extracts of the four parts (leaves, seeds, stem, and roots) of *Indigofera linifolia* were subjected to LC-MS analysis for study of hydrophilic constituents present in each extract. Structures of the constituents were proposed on basis of combined evidence obtained from HPLC retention time, DAD-spectra, ESI mass spectra and literature reports on compounds already identified in plants of genus *Indigofera.*

3.5.1. ILDM extract

HPLC Chromatograms of ILDM extract

The separation of constituents in 70 % hydromethanolic extract of *I. linifolia* seeds (ILDM) was recorded by DAD detector hyphenated with reversed phase HPLC column. The chromatograms were recorded at three different wavelengths i.e, 254, 320 and 380 nm to detect constituents showing significant UV absorption at these wavelengths. These wavelengths are particularly used for the targeted analysis of phenolic constituents.

Each chromatogram showed a plot of peak intensity on y-axis versus elution time on x-axis. The components eluted at different retention times depending upon their affinity for mobile phase composition which varied gradually according to gradient elution programme for HPLC (Table 2.2).

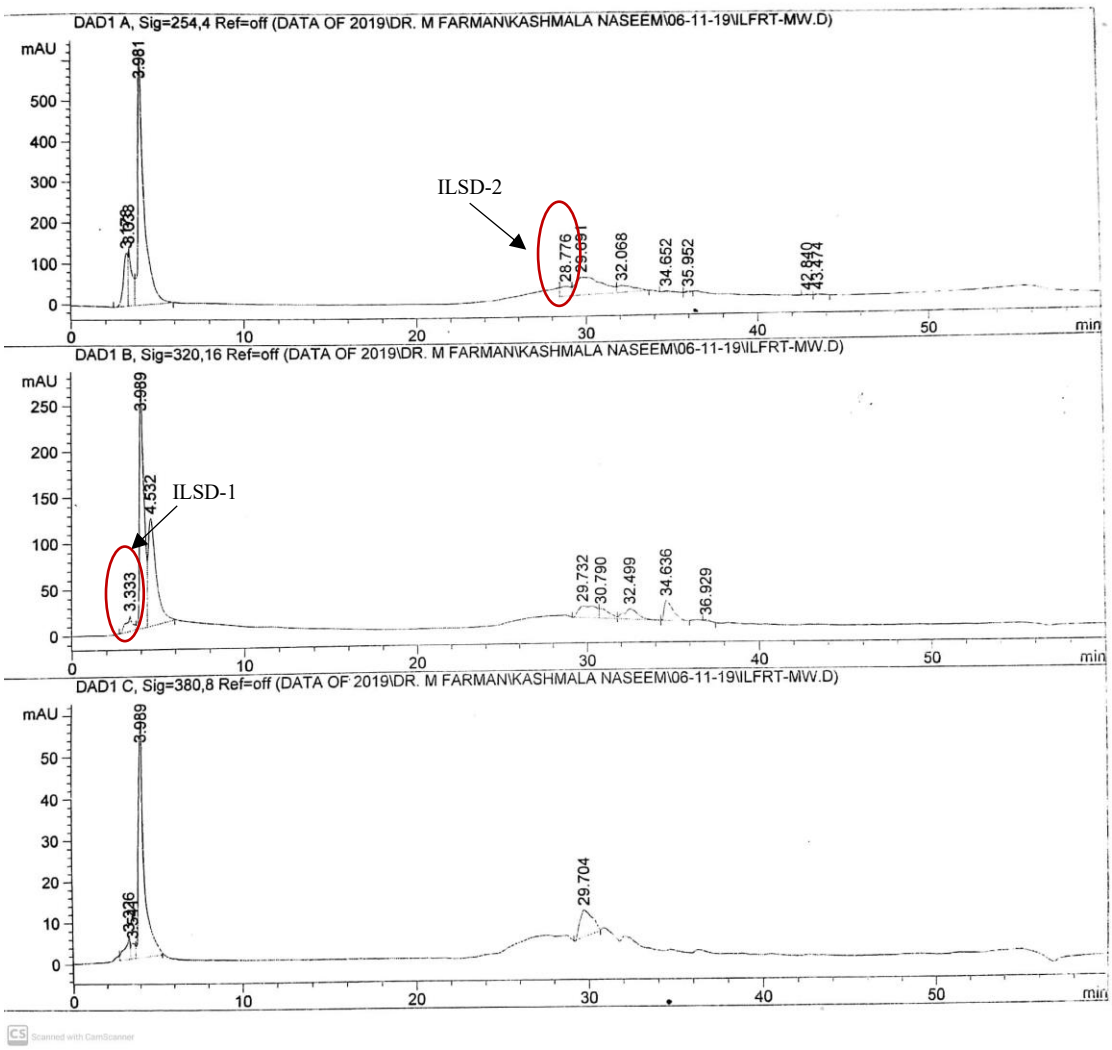


Figure 3.11. Fingerprint HPLC chromatograms of ILDM extract at 254, 320 and 380 nm

Glycosylated phenolic acids possess higher hydrophilic character than flavonoid glycosides or aglycones, so they eluted from the reversed phase HPLC column between 0-10 minutes due to greater affinity with water rich mobile phase. As the time passed during analysis, less hydrophilic constituents appeared on the chromatogram due to increased percentage of organic solvent in the mobile phase. Hence, the observed retention time for phenolic glycosides and flavonoid glycosides was in the following order: phenolic acid glycosides < flavonoid tri-glycosides < flavonoid di-glycosides < flavonoid mono-glycosides < aglycones. The UV spectrum of component corresponding to each peak was recorded to identify the class of constituent. The shape and absorption maxima of UV spectrum were compared with available literature data for tentative identification of the compound.

The DAD chromatogram of ILDM at 254 nm showed 10 peaks indicating that the extract contained significant number and type of phenolic constituents. The chromatogram at 320 nm showed 8 peaks while at 380 nm showed 3 peaks. The component at 3.98 minutes showed highest relative intensity and appeared in all the three chromatograms (Figure 3.11).

TIC Profile of ILDM extract

The TIC (Total ion current) profile of ILDM is shown in figure 3.12. It showed the chromatographic separation of eluting components in the form of peaks. The profile was obtained by mass spectrometer used as detector hyphenated to the HPLC column. The components were recorded as current of pseudo-molecular ions by mass spectrometer operating in the negative ESI/MS mode and appeared as peaks in the TIC chromatogram.

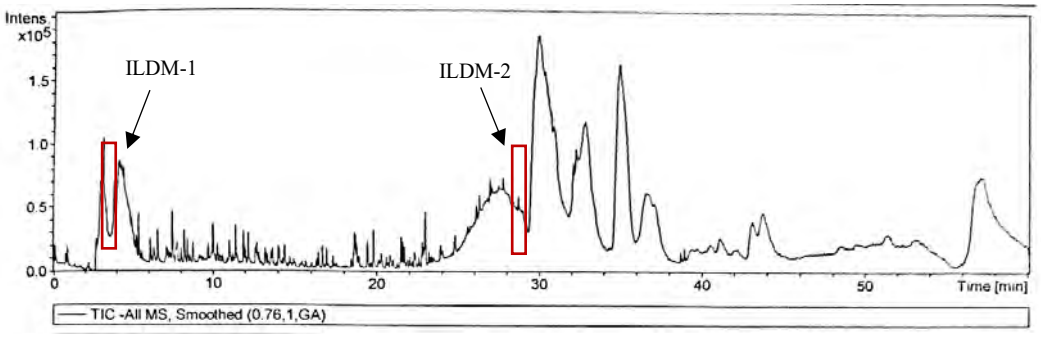


Figure 3.12. TIC profile of ILDM extract in negative ESI/MS mode

ILDM-1

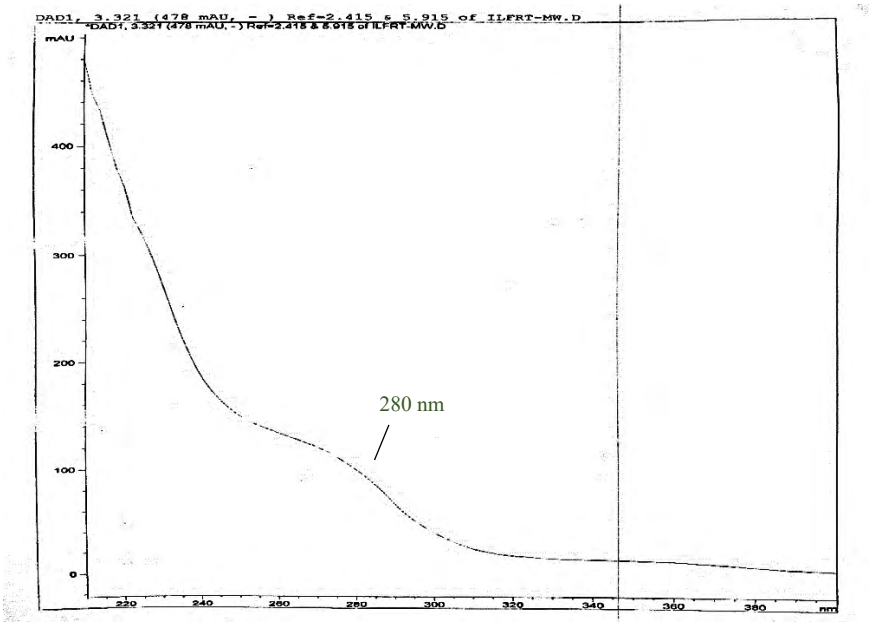
Retention time of ILDM-1

The component eluted from reverse phase HPLC column at 3.32 minutes corresponding to the mobile phase composition 10 : 90 (acetonitrile : water). The elution of ILDM-1 by water rich mobile phase showed strong hydrophilic character of the component.

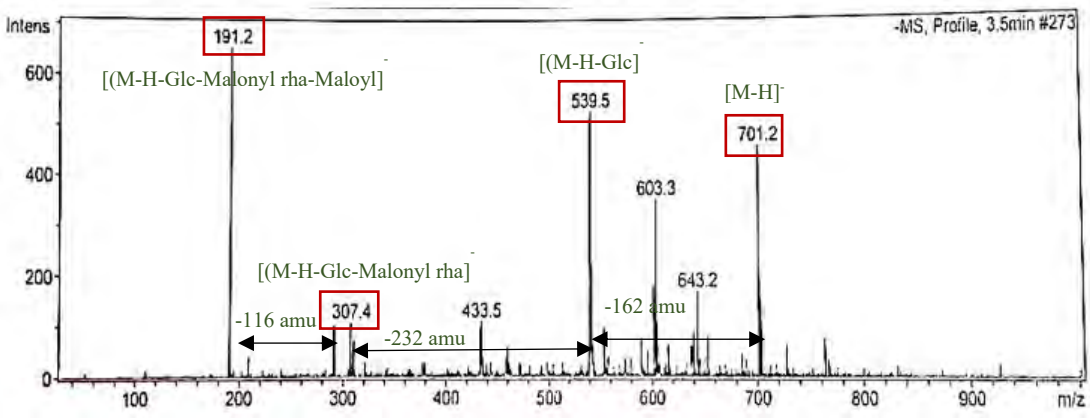
DAD spectrum of ILDM-1

The UV spectrum showed a low intensity shoulder at 280 nm. Such a band may correspond to $n \rightarrow \pi^*$ transitions (ε=15) in carbonyls. The intensity of absorption is low because they are forbidden transitions (**Pavia *et al.*, 2014**). Thus, UV spectrum indicated the presence of carbonyl group in the compound. The lack of prominent

absorption bands above 250 nm also showed absence of conjugated chromophores in the constituent (Figure 3.13 a).



(a)

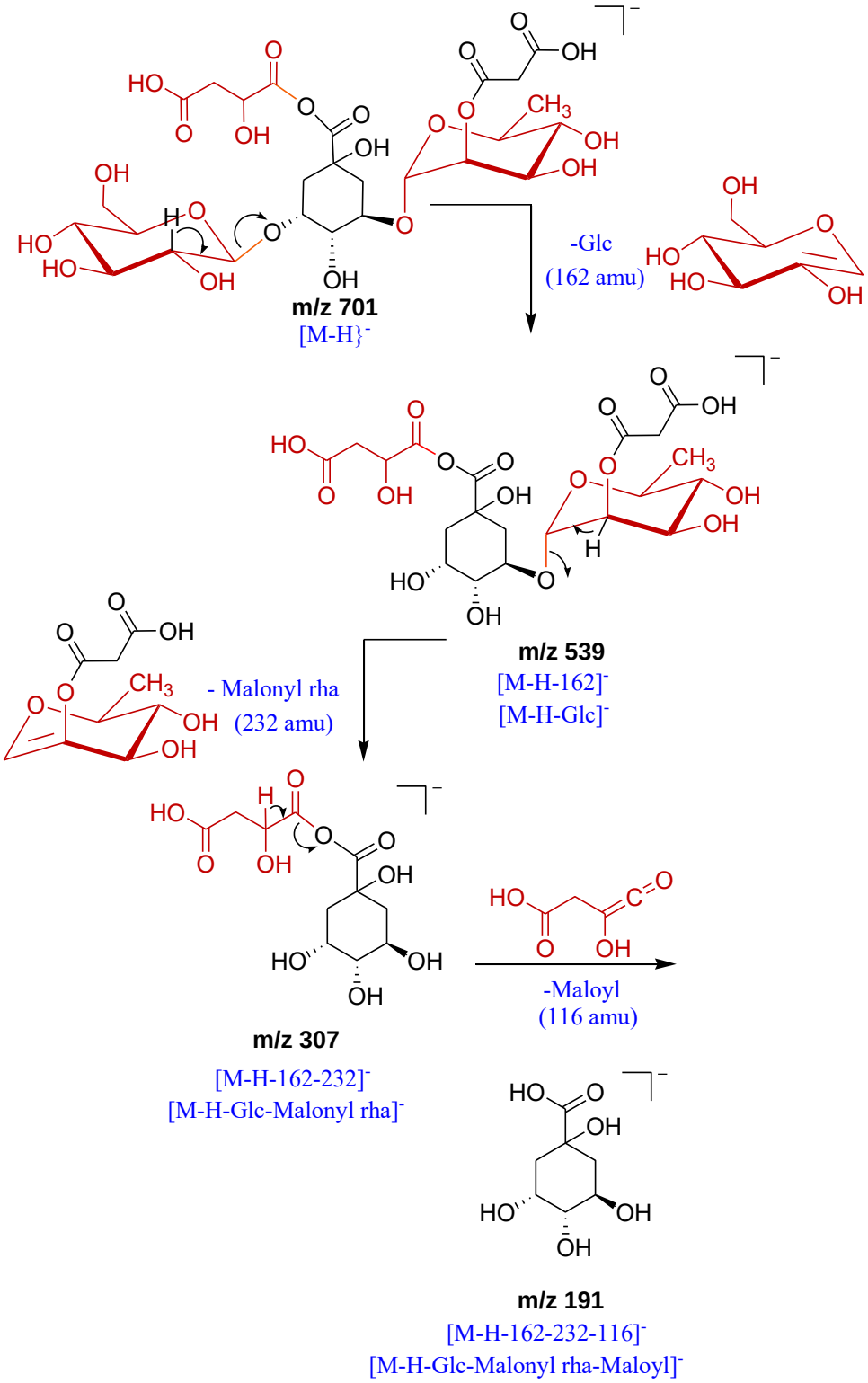


(b)

Figure 3.13.(a) UV spectrum of ILDM-1 (b) Mass spectrum of ILDM-1 in negative ESI/MS

Mass spectral analysis of ILDM-1

The mass spectrum of ILDM-1 recorded in the negative ESI/MS mode presented a peak at *m/z* 701 which corresponded to the deprotonated molecular ion (Figure 3.13 b). This implied that molecular weight of the compound was 702 amu. The fragment ion at *m/z* 539 $[M-H-162]^-$ suggested the fragmentation of *O*-linked hexose moiety which was lost in the form of dehydrated hexose (162 amu) (**Guo *et al.*, 2007**).



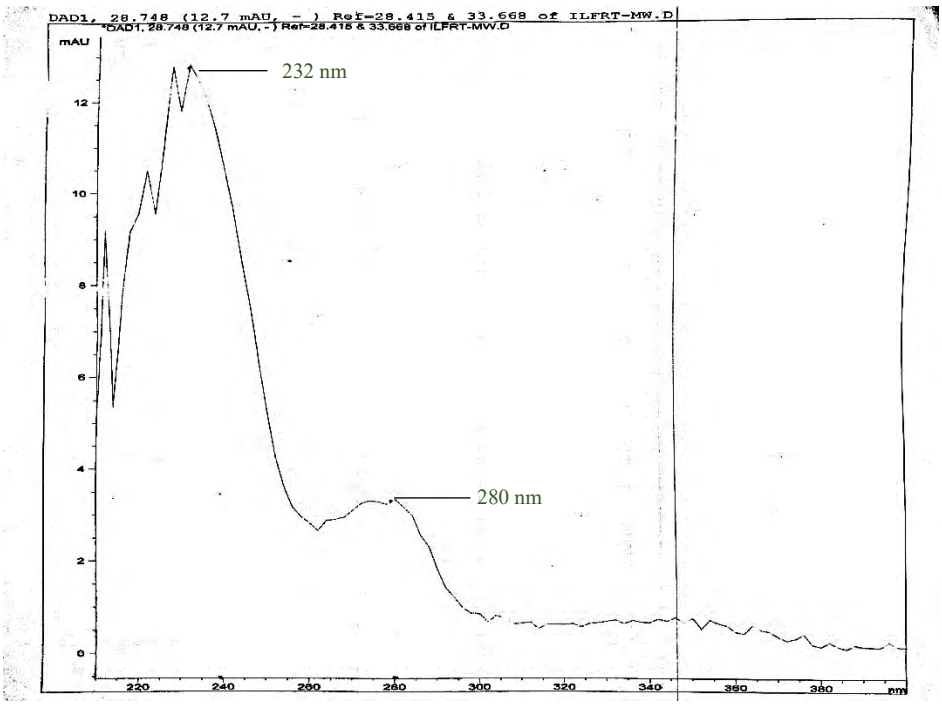
Scheme 3.3. Suggested fragmentation pattern for ILDM-1 in negative ESI/MS mode

Another fragment ion appeared at *m/z* 307 $[M-H-162-232]^-$ or $[M-H-162-(86+146)]^-$ attributing to the removal of malonyl-deoxyhexose residue (232 amu) (**Aaby *et al.*, 2007**). Following this, the elimination of maloyl (116 amu) resulted in fragment ion at *m/z* 191 $[M-H-162-232-116]^-$ (**Said *et al.*, 2017**). The peak at *m/z* 191

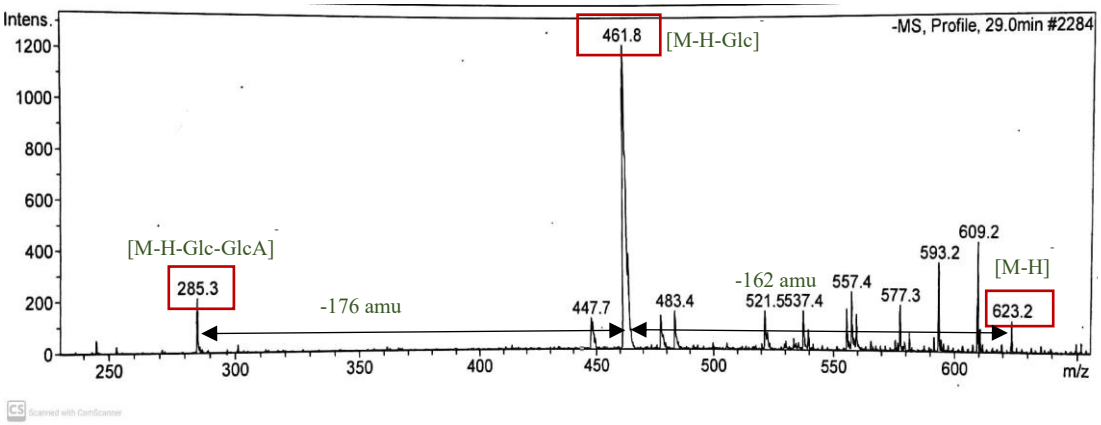
also corresponded to the mass of deprotonated quinic acid (**Miketova *et al.*, 1999**) (Scheme 3.3).

The combined evidence from retention time, UV and mass spectra led to the tetative identification of ILDM-1 as maloyl-[3-*O*-glucopyranosyl-5-*O*-(malonylrhamnopyranosyl)] quinate

ILDM-2



(a)



(b)

Figure 3.14. (a) UV spectrum of ILDM-2 (b) Mass spectrum of ILDM-2 in negative ESI/MS

Retention time of ILDM-2

The eluted component was detected and recorded on HPLC chromatogram at retention time of 28.7 minutes. The elution occurred by mobile phase gradient of 40 % acetonitrile in water which showed partial hydrophilic nature of the constituent. This observation suggested that the eluent could be a flavonoid glycoside.

DAD spectrum of ILDM-2

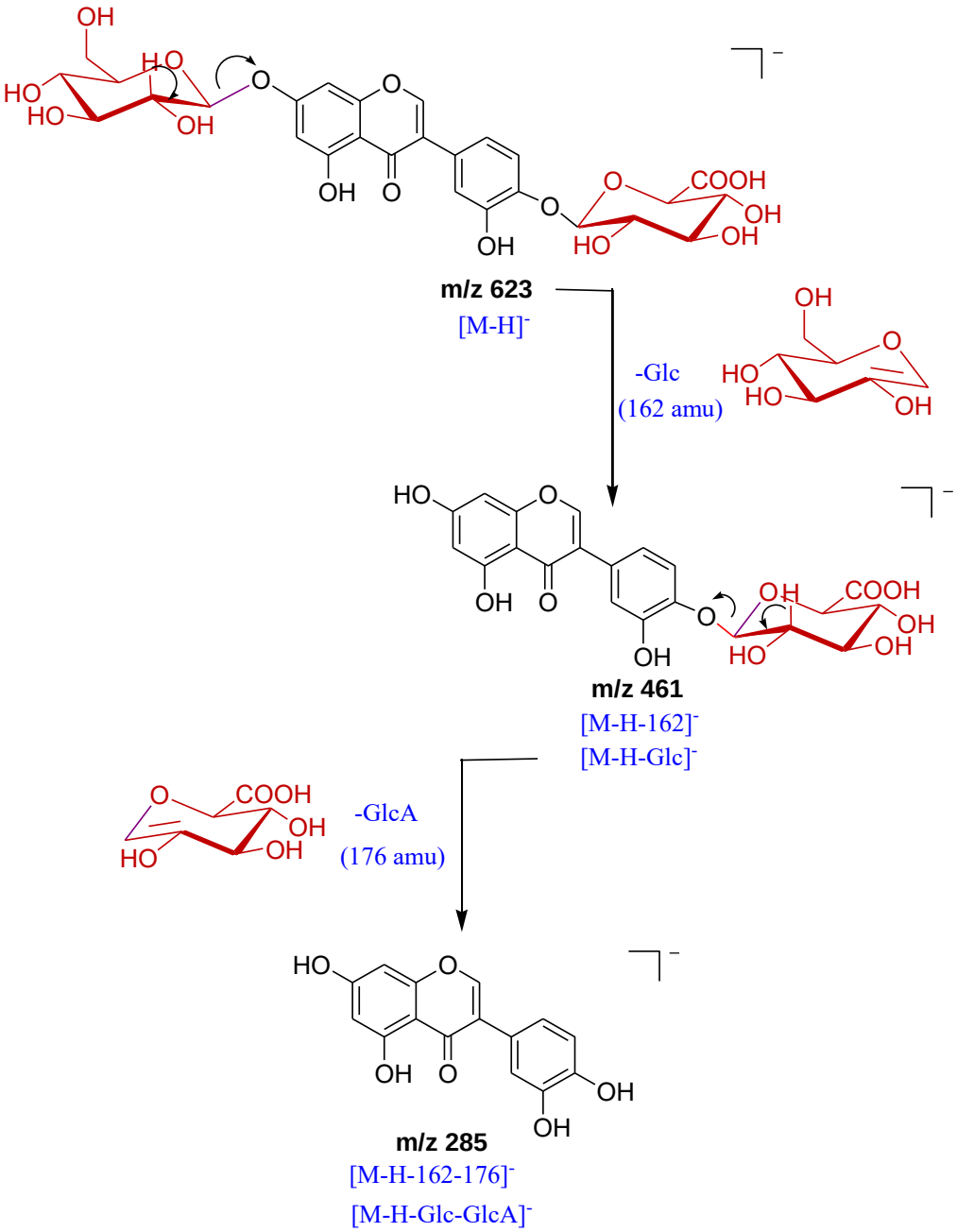
The UV spectrum showed an intense band at 232 nm and shoulder at 280 nm. The shape of spectrum and absorption range indicated the presence of an isoflavone. The UV absorbance pattern matched with those of orobol or 5,7,3′,4′-tetrahydroxyisoflavone and a 30 nm hypsochromic shift in band II was observed which indicated glycosylation in ring A. Moreover, the shoulder was also shifted to lower wavelength than expected indicating glycosylation in ring B (**Mabry *et al.,* 2012**) (Figure 3.14 a).

Mass spectral analysis of ILDM-2

The mass spectrum (Figure 3.14 b) displayed deprotonated molecular ion peak at *m/z* 623 corresponding to $[M-H]^-$. This information provided conclusive evidence for molecular weight of the component to be 624 amu. Fragmentation of $[M-H]^-$ by removal of hexose residue (162 amu) resulted in product ion at *m/z* 461 $[Y_0^7]^-$ $[M-H-162]^-$ (**Cuyckens and Claeys, 2002**). Thus, *O*-hexosyl moiety was indicated in the compound (**Guo *et al.,* 2007**). The ion at *m/z* 285 $[Y_0^3]^-$ $[M-H-162-176]^-$ was generated by cleavage of *O*-glucuronide moiety lost as dehydrated glucuronic acid (176 amu) (**Davis *et al.,* 2006**). The peak at *m/z* 285 suggested the presence of a deprotonated tetrahydroxy flavonoid which could be 5,7,3′,4′-tetrahydroxy isoflavone, orobol or 3′-hydroxygenestein (**Troalen *et al.,* 2014**) (Scheme 3.4).

Flavonoids produce characteristic fragment ions when subjected to ESI/MS fragmentation. A nomenclature for glycoconjugates was suggested by **Domon and Costello (1988)** and modified by **Cuyckens and Claeys (2004)** for flavonoid glycosides. According to this nomenclature, the fragment ion produced may be denoted by $^{0,2}X_1$, $^{0,3}X_1$, $^{0,4}X_1$, $^{0,5}X_1$, Y_2, Y_1, Y_0, Z_2, Z_1, Z_0 etc., depending upon the type of bond broken in fragmentation process.

The component ILDM-2 was tentatively identified as 7-*O*-glucopyranosyl-4'-*O*-glucuronopyranosyl orobol.



Scheme 3.4. Suggested fragmentation pattern for ILDM-2 in negative ESI/MS mode

3.5.2. ILSM extract

HPLC Chromatograms of ILSM extract

The separation of phenolic constituents in ILSM extract was detected by recording DAD-chromatograms at 254, 320 and 380 nm. The components were separated due to difference in their interaction with the varying composition of mobile

phase. More hydrophilic components showed low retention time due to elution with water rich mobile phase gradient. Components with lesser hydrophilic character were eluted at higher retention time when mobile phase was rich in organic solvent.

The DAD-chromatogram of ILSM recorded at 254 nm showed 12 peaks indicating that the extract contained significant number of phenolic constituents. The chromatogram at 320 nm showed 7 peaks while the chromatogram at 380 nm showed 6 peaks. The UV spectrum of each component detected as chromatographic peak provided useful information regarding the class of phenolic constituent. The constituent at 3.7 minutes showed highest relative intensity and appeared in all the three chromatograms (Figure 3.15).

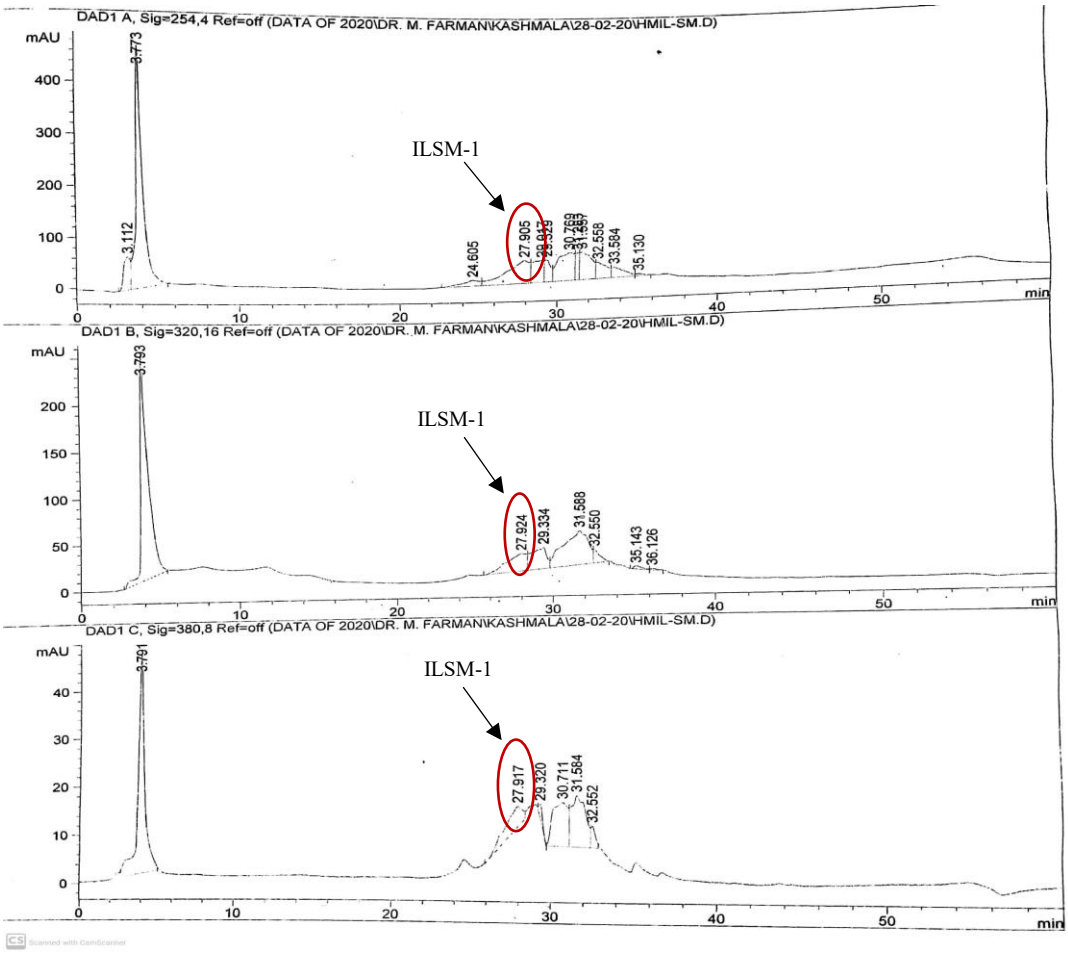


Figure 3.15. Fingerprint HPLC chromatograms of ILSM extract at 254, 320 and 380 nm

TIC Profile of ILSM extract

The TIC (Total ion current) profile of ILSM is shown in Figure 3.16. The TIC profile is a chromatogram recorded by detection of each eluting component from the

HPLC column by hyphenated mass spectrometer operating in the negative ESI/MS mode. The mass spectrum of any peak in TIC profile provides significant structural information about the constituent.

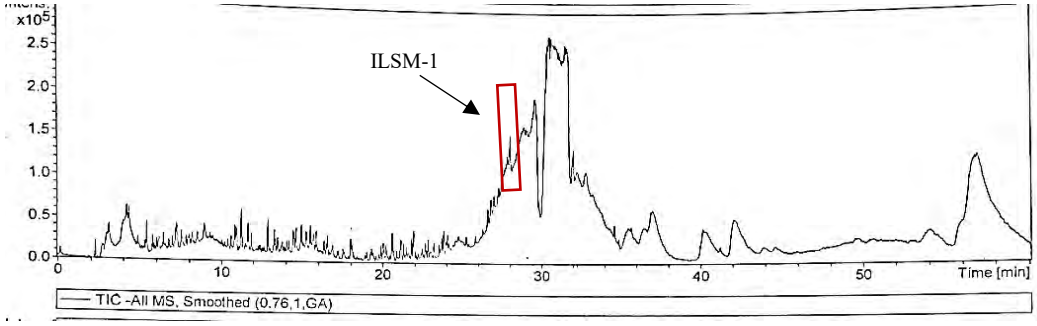


Figure 3.16. TIC profile of ILSM extract

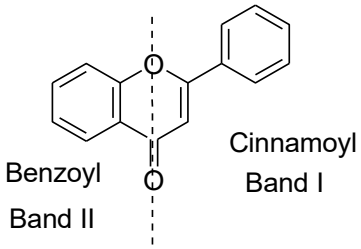
ILSM-1

Retention time of ILSM-1

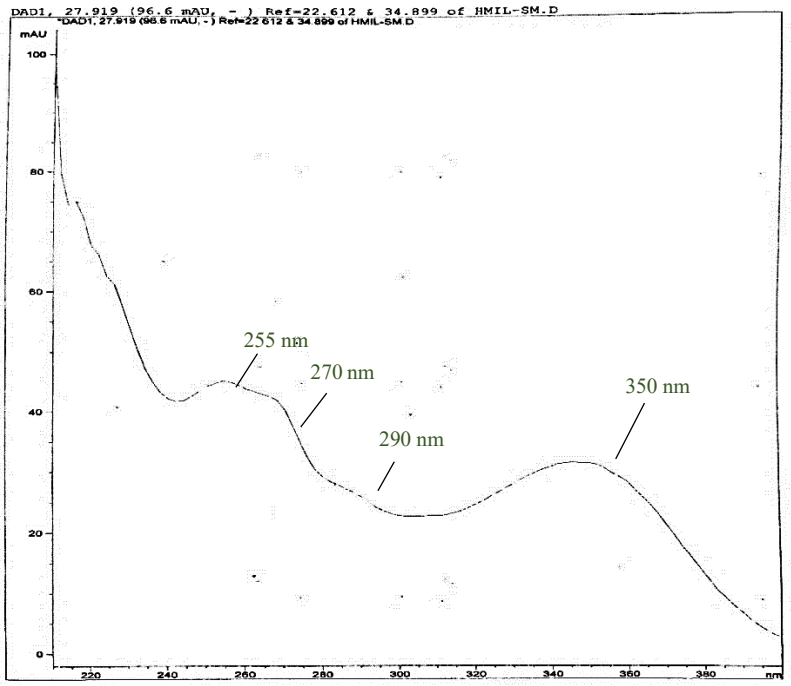
The component appeared on HPLC chromatogram at retention time of 27.9 minutes, when mobile phase gradient was 40 % acetonitrile in water. The elution by this mobile phase gradient showed partial hydrophilic properties of eluent. It suggested that ILSM-1 could be a mono or di-glycosylated flavonoid.

DAD spectrum of ILSM-1

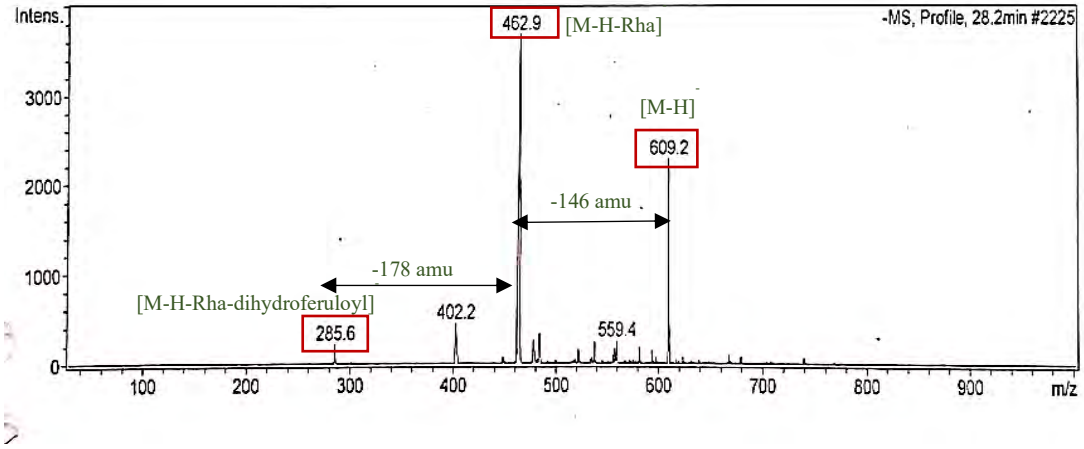
The UV spectrum of ILSM-1 showed two absorption bands. Two prominent absorption bands between 240-400 nm indicated the class of organic compound to be flavone or flavonol. The band I (appearing in the range of 300-380 nm) in flavonoid UV spectra is usually due to absorption by B-ring cinnamoyl system of flavonoid while band II (appearing in the range of 240-280 nm) is due to absorption by A-ring benzoyl system (**Mabry *et al.*, 2012**) (Figure 3.17 a).



The two bands in UV spectrum of ILSM-1 indicated the presence of flavone or flavonol. Low intensity of band I and absorbance at 350 nm indicated 3-*O*-substituted flavonol **(Markham, 1982)**, (**Mabry *et al.*, 2012**).



(a)



(b)

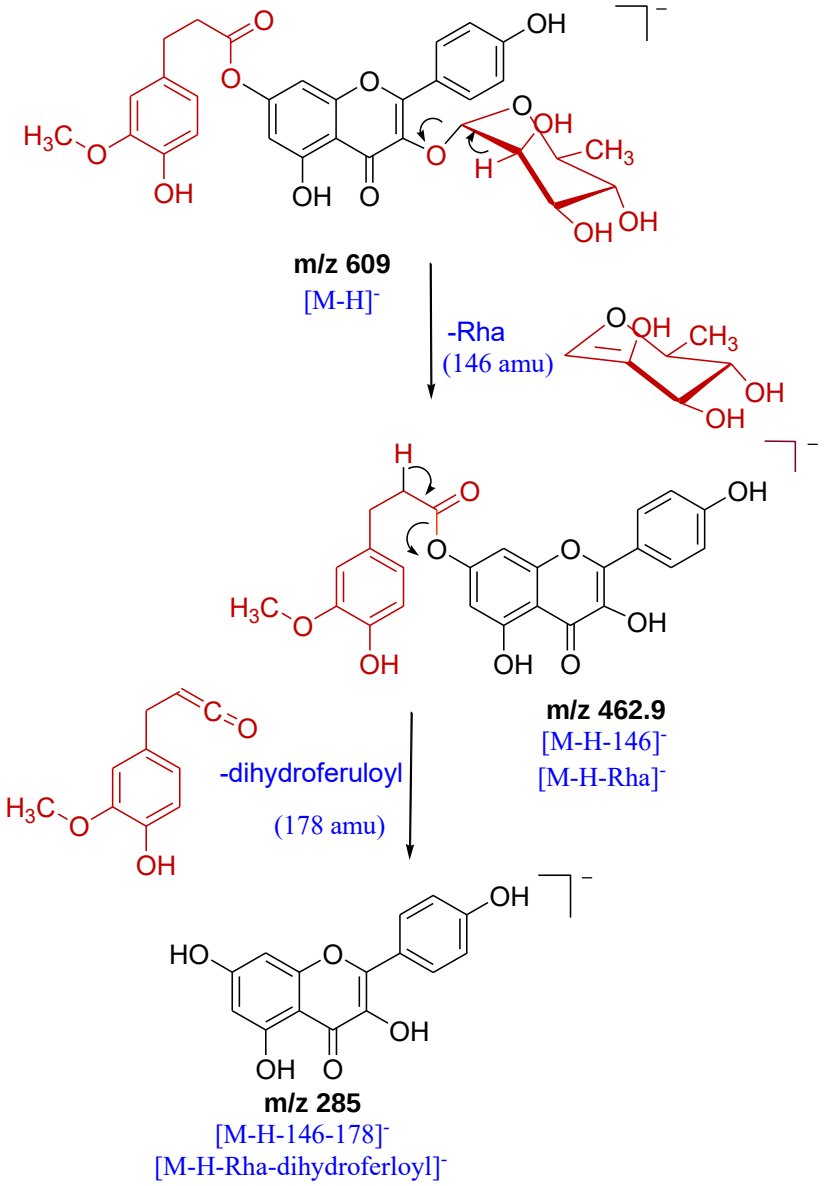
Figure 3.17. (a) UV spectrum of ILSM-1 (b) Mass spectrum of ILSM-1 in negative ESI/MS

Mass spectral analysis of ILSM-1

The mass spectrum (Figure 3.17 b) displayed a deprotonated molecular ion peak at *m/z* 609.2 $[M-H]^-$. This suggested that molecular weight of the component was 610.2 amu. The detection of fragment ion at *m/z* 462.9 $[M-H-146]^-$ was-in-agreement with the loss of *O*-deoxyhexosyl moiety as dehydrated deoxyhexose (146 amu) (**Eklund *et al.*, 2008**). Following this, the removal of dihydro-feruloyl moiety (178 amu) led to the product ion at *m/z* 285.6 $[M-H-146-178]^-$ (**Narváez-Cuenca *et al.*, 2012**). The peak at

m/z 285 corresponded to the presence of deprotonated kaempferol as aglycone moiety (**Truchado *et al.*, 2009**) (Scheme 3.5).

Hence information obtained from retention time, UV spectrum and mass spectrum led to the tentative identification of ILSM-1 as 7-*O*-dihydroferuloyl-3-*O*-rhamnopyranosyl kaempferol.



Scheme 3.5. Suggested fragmentation pattern for ILSM-1 in negative ESI/MS mode

3.5.3. ILRM extract

The chromatograms of ILRM were recorded at 254, 320 and 380 nm for the targeted analysis of phenolic constituents. The chromatogram at 254 nm showed 21 peaks which indicated that the root was highly rich in phenolic constituents. The

number of peaks was almost double than that in the stem and seed extract. The chromatogram at 320 nm showed 11 peaks while at 380 nm showed 2 peaks. The component eluted at 4 minutes showed highest relative intensity and appeared in all the three chromatograms. The UV spectrum of constituent associated with each peak provided useful information for the identification of class of phenolic constituent (Figure 3.18).

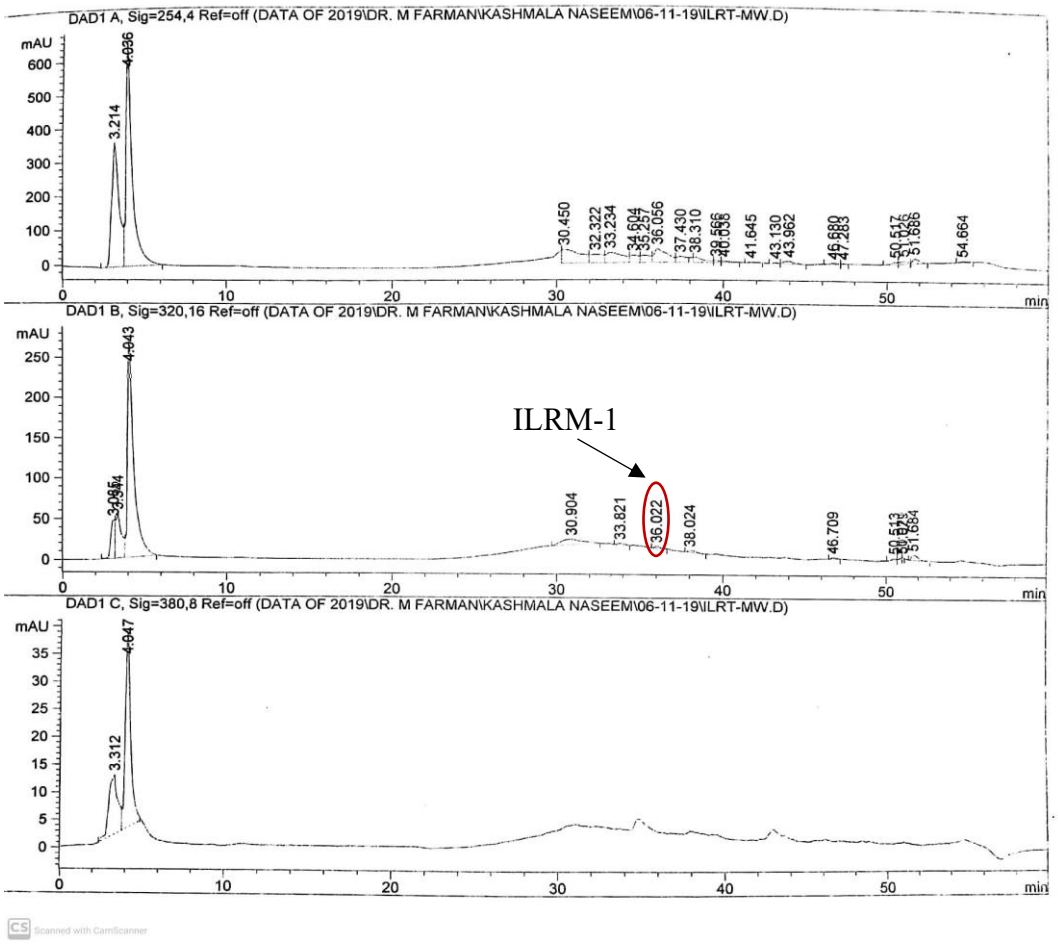


Figure 3.18. Fingerprint HPLC chromatograms of ILRM at 254, 320 and 380 nm

TIC Profile of ILRM extract

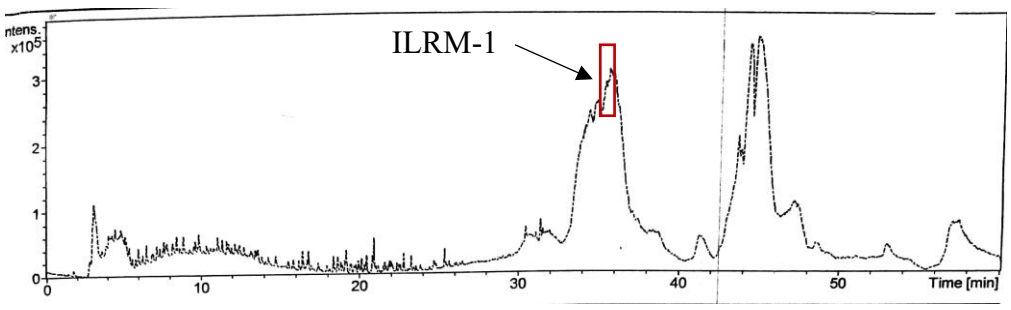
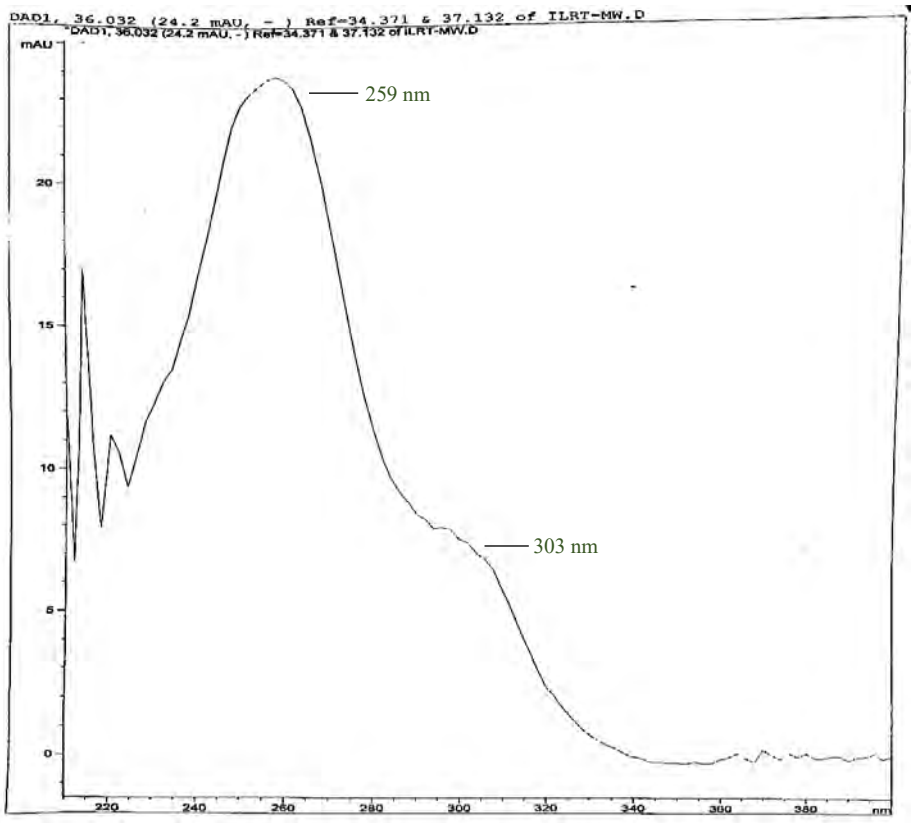


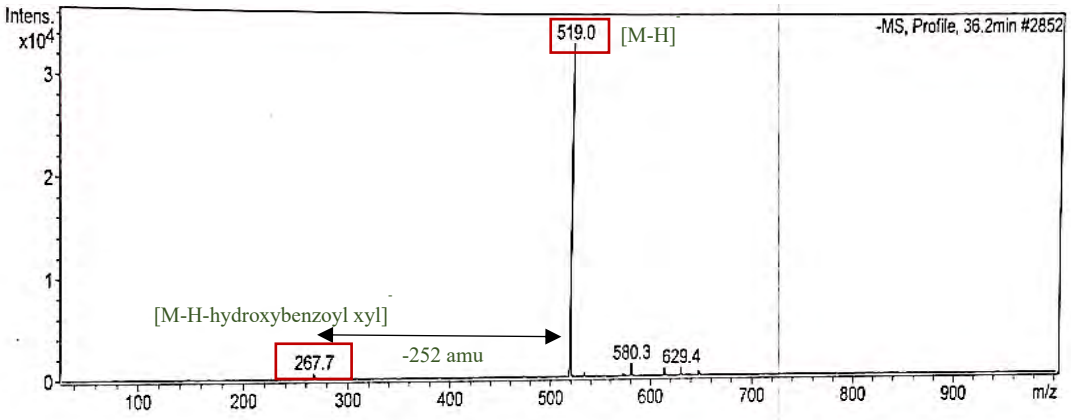
Figure 3.19. TIC Profile of ILRM extract

The TIC (Total ion current) chromatogram of ILRM indicated the separation of constituents as detected by mass spectrometer operating in negative ESI/MS mode (Figure 3.19). It indicated the extent of complexity of the extract. Mass spectrum of constituent for any peak in TIC profile gave useful information regarding structure of the respective constituent.

ILRM-1



(a)



(b)

Figure 3.20. (a) UV spectrum of ILRM-1 (b) Mass spectrum of ILRM-1 in negative ESI/MS

Retention time of ILRM-1

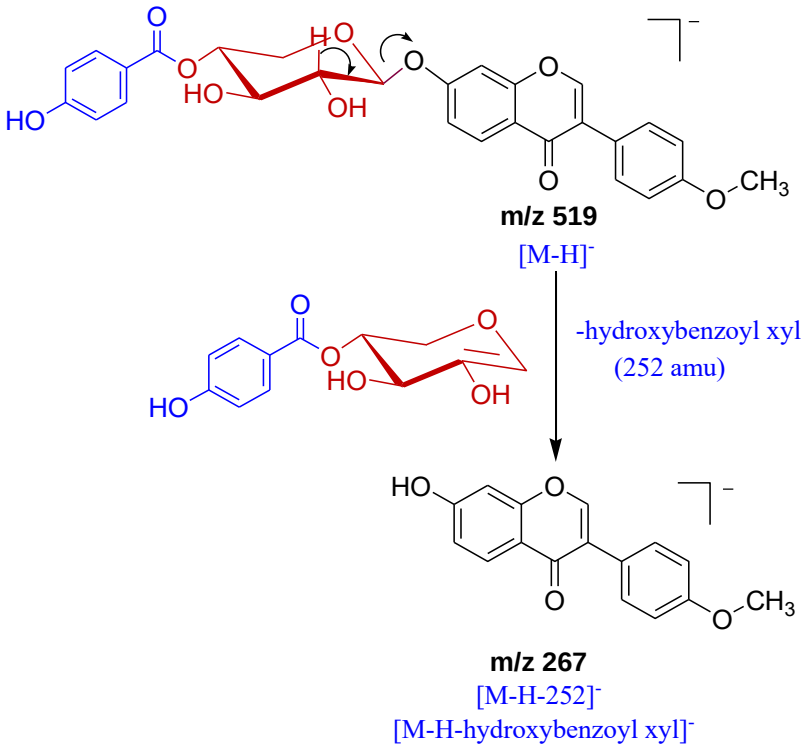
The constituent ILRM-1 eluted and appeared on DAD chromatogram at 36.032 minutes. This elution occurred in the presence of 40 % acetonitrile in water. The elution by this composition of mobile phase showed partially hydrophilic character of the eluting component. It could be a flavonoid mono-glycoside.

DAD spectrum of ILRM-1

The UV spectrum indicated absorbance maxima at 259 nm and a shoulder at 303 nm. The presence of band I as shoulder indicated that class of the constituent was isoflavone. Hence, it could be an isflavone glycoside (**Markham, 1982**) (Figure 3.20 a). The spectrum was closely related to the UV spectrum reported for formononetin-7-*O*-glucoside **(Mabry *et al.*, 2012)**.

Mass spectral analysis of ILRM-1

The deprotonated molecular ion peak appeared at *m/z* 519 $[M-H]^-$ which suggested that molecular weight of the component was 520 amu (Figure 3.20 b).



Scheme 3.6. Suggested fragmentation pattern for ILRM-1 in negative ESI/MS mode

Fragmentation of $[M-H]^-$resulted in fragment ion at *m/z* 267 $[Y_0^7]^-$[M-H-252]$^-$ indicating the loss of hydroxybenzoylpentosyl residue (120+132) amu. The peak at *m/z*

267 was coincident with the mass of deprotonated 7-hydroxy-4′-methoxy isoflavone or formononetin (**Ye *et al.*, 2012**) (Scheme 3.6).

The component ILRM-1 was tentativey identified as 7-*O*-hydroxybenzoylxylopyranosyl formononetin

3.5.4. ILLM extract

HPLC Chromatograms of ILLM extract

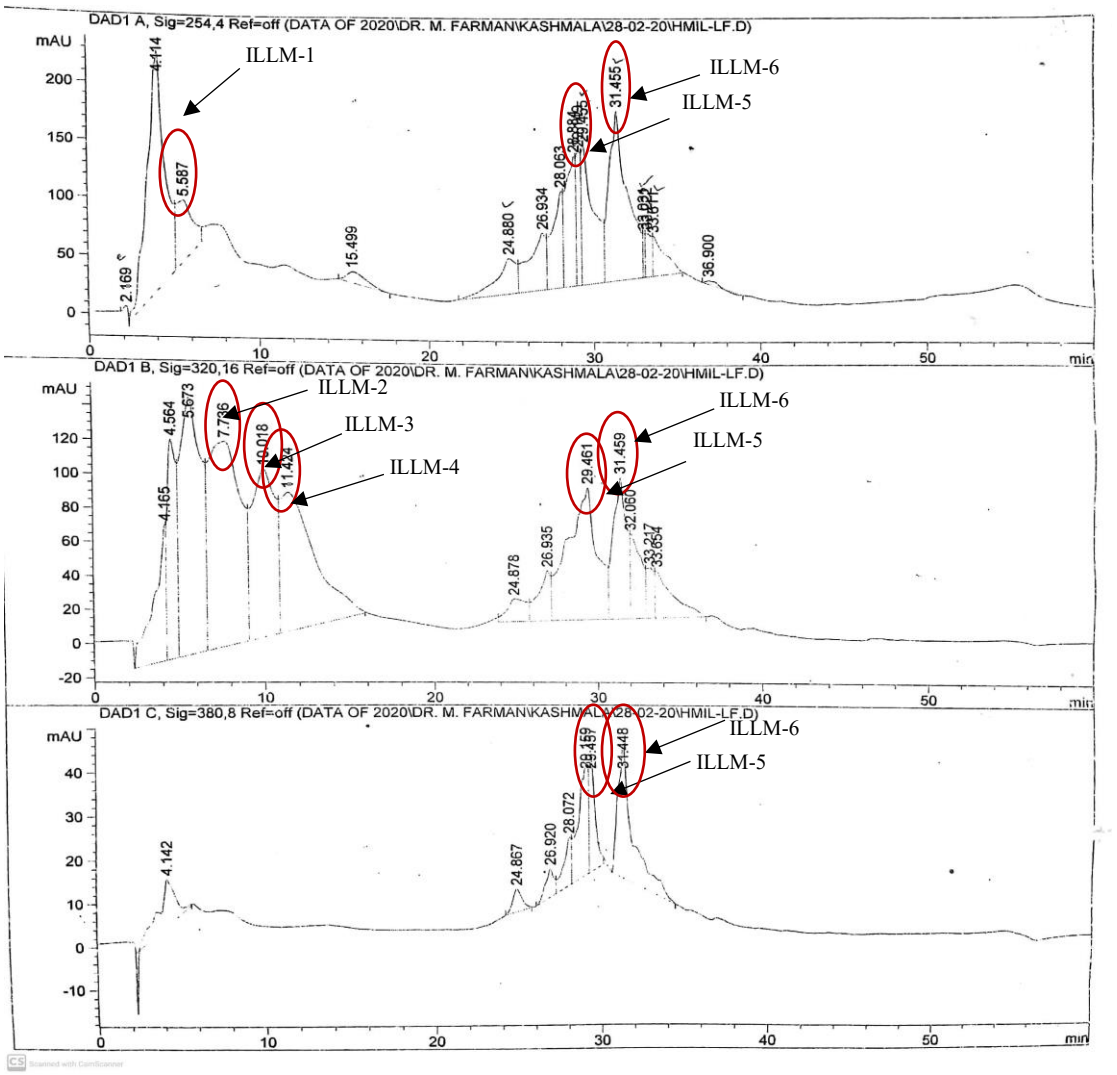


Figure 3.21. Fingerprint HPLC chromatograms of ILLM extract at 254, 320 and 380 nm

The separated constituents of 70 % hydromethanolic extract of *I. linifolia* leaves (ILLM) were detected by DAD detector hyphenated with reversed phase HPLC

column. The chromatograms were recorded at 254, 320 and 380 nm wavelengths suitable for the analysis of phenolic constituents.

The DAD chromatogram of ILLM recorded at 254 nm showed 15 peaks which suggested that the leaves are highly rich in phenolic constituents. Similarly, the chromatograms at 320 and 380 nm showed 13 and 7 peaks, respectively. The components were separated based upon difference in interaction with the mobile phase gradient. More lipophilic components eluted first followed by constituents with lesser hydrophilic character.

The UV spectrum of each separated constituent helped in identification of possible class of phenolic constituent. Comparing the wavelength of absorption bands with literature data of standard compounds led to the suggestion of possible constituent (Figure 3.21).

TIC Profile of ILLM extract

The TIC (total ion current chromatogram) of ILLM showing the separation of components detected by mass spectrometer in the negative ESI/MS mode is shown in Figure 3.22. The number of peaks indicated extent of complexity of the extract. Mass spectrum associated with each peak in TIC profile can provide useful structural information about the respective constituent.

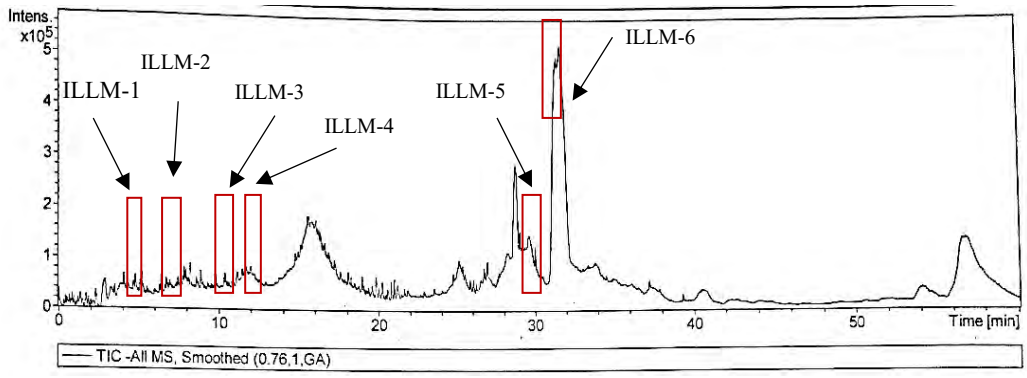
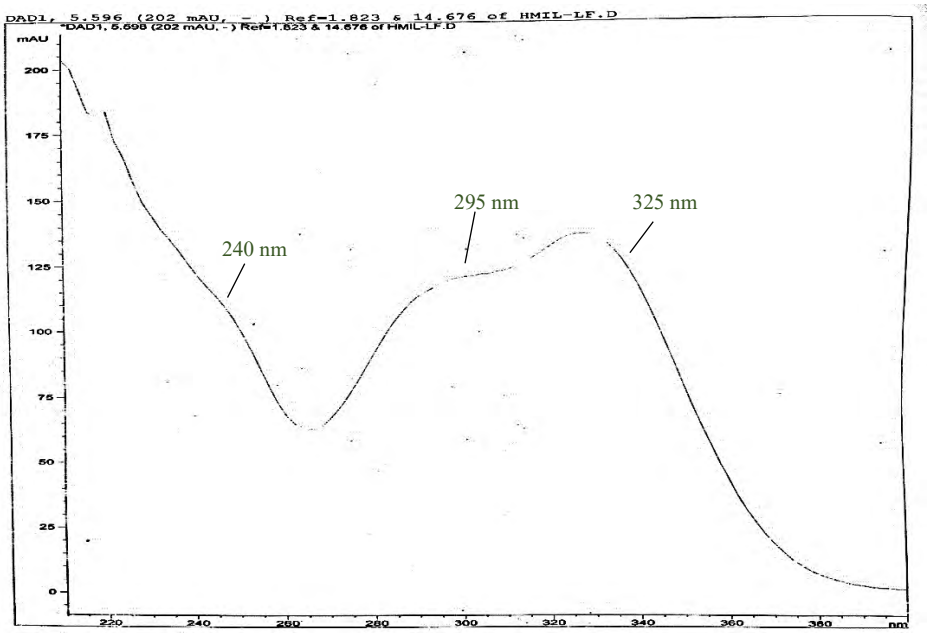


Figure 3.22. TIC profile of ILLM extract

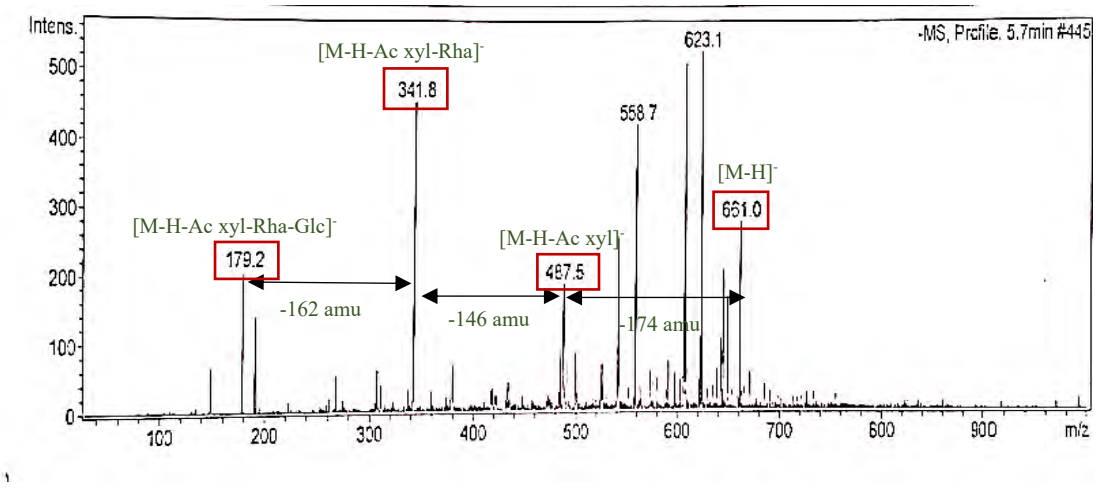
ILLM-1

Retention time of ILLM-1

The eluted component was detected and recorded on DAD chromatogram at relative retention time of 5.5 minutes. The elution at this time was carried by mobile phase gradient of 10 % acetonitrile in water which indicated immense hydrophilic character of the eluent. A glycosylated phenolic acid was indicated by this retention behavior.



(a)



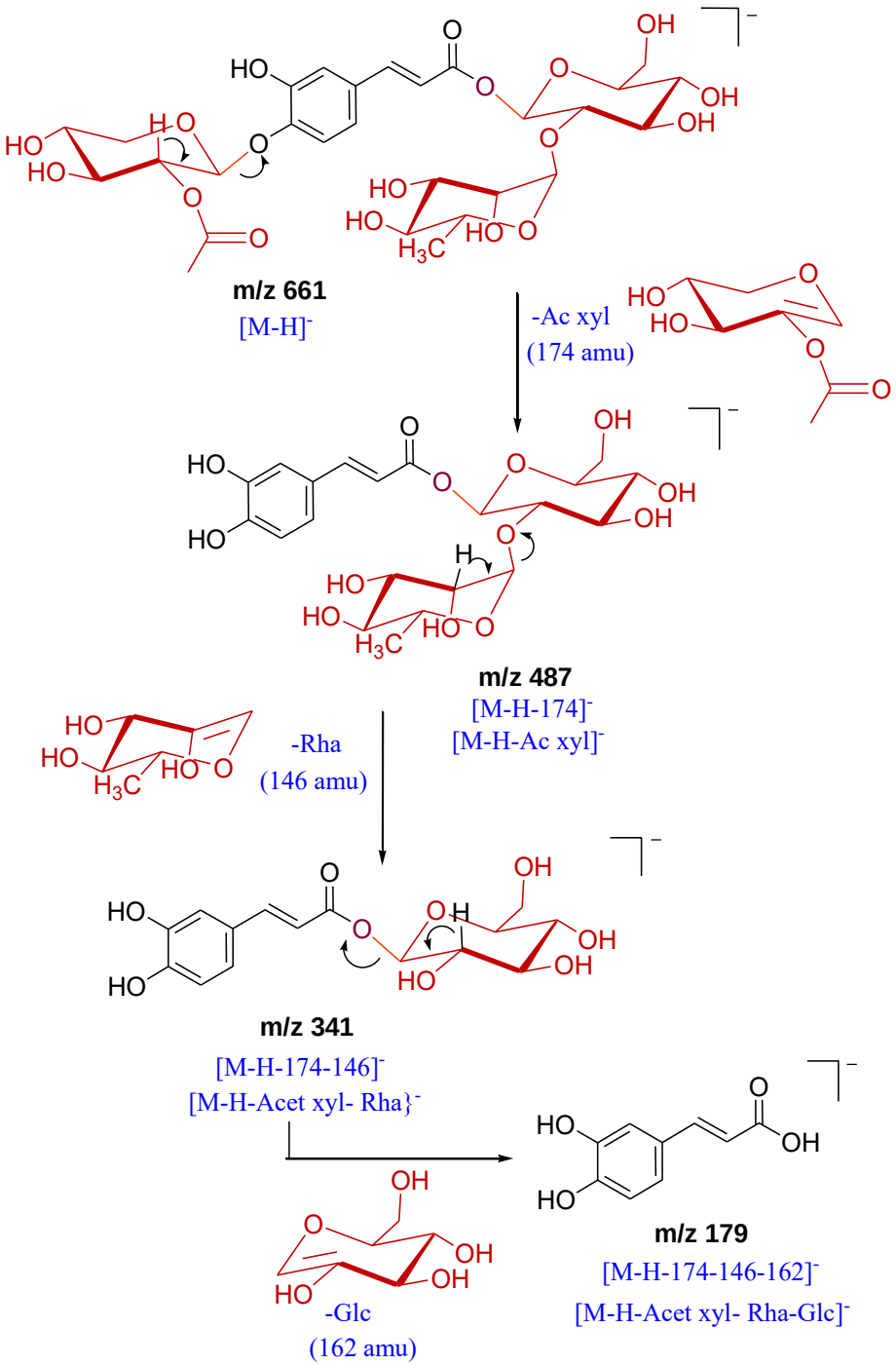
(b)

Figure 3.23. (a) UV spectrum of ILLM-1 (b) Mass spectrum of ILLM-1 in negative ESI/MS

DAD spectrum of ILLM-1

The UV-visible spectrum of ILLM-1 recorded by diode array detector showed two prominent absorptions at 295 and 325 nm. The band at 325 nm with a shoulder at

295 nm is considered as characteristic of 3-(3, 4-dihydroxyphenyl)-propenoic acid or caffeic acid (**Medina-Medrano *et al.*, 2017**). The strongest absorption at 325 nm is observed due to low energy π→π* transitions from caffeic moiety to carbonyl group. The band at 295 nm is attributed to n→π* transitions from carbonyl to carbonyl (**Tošović, 2017**). Hence, UV spectrum indicated the presence of caffeic acid (Figure 3.23 a).



Scheme 3.7. Suggested fragmentation pattern for ILLM-1 in negative ESI/MS mode

Mass spectral analysis of ILLM-1

The mass spectrum acquired in negative ESI/MS mode showed deprotonated molecular ion or $[M-H]^-$ peak at *m/z* 661 (Figure 3.23 b). This observation showed that molecular weight of the component was 662 amu. Another ion detected at *m/z* 487 $[M-H-174]^-$ suggested the loss of acetyl pentose moiety (174 amu) (**Mullen *et al.*, 2003**). Further cleavage resulted in fragment ion at *m/z* 341 $[M-H-174-146]^-$ which indicated the removal of deoxyhexose residue (146 amu) (**Eklund *et al.*, 2008**). The peak at *m/z* 179 $[M-H-174-146-162]^-$provided evidence for the loss of hexose residue (**Guo *et al.*, 2007**). This peak also corresponded to the mass of deprotonated caffeic acid (**Saldanha *et al.*, 2013**) (Scheme 3.7).

The component ILLM-1 was tentatively identified as 4-*O*-acetylxylopyranosyl caffeoyl neohesperidoside.

ILLM-2

Retention time of ILLM-2

The component was eluted from reverse phase HPLC column at 7.7 minutes by 10 % acetonitrile in water used as mobile phase gradient. This low retention time depicted greater affinity of ILLM-2 for water-rich mobile phase and indicated strong hydrophilic properties of the eluent. This observation showed that the component could be a glycosylated derivative of some phenolic acid.

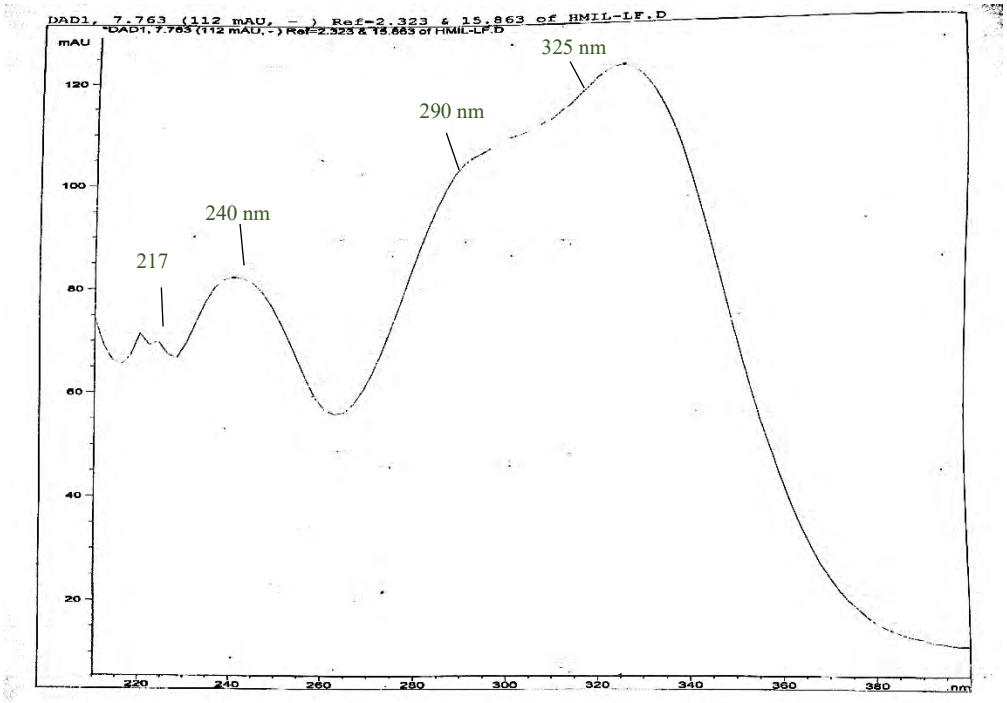
DAD spectrum of ILLM-2

The UV spectrum of eluent showed prominent absorptions at 217, 240, 290sh and 325 nm. These absorptions are characteristic of caffeic acid (**Medina-Medrano *et al.*, 2017**). The band at 325 nm appeared due to π→π* transitions from caffeic moiety to carbonyl. The shoulder at 290 nm corresponded to n→π* transitions from carbonyl to carbonyl. Similarly, π→π* electronic transitions from caffeic moiety to aromatic ring absorb energy at 243 nm. The band at 217 nm appeared due to π→π* transitions from carbonyl to carbonyl (**Tošović, 2017**) (Figure 3.24 a).

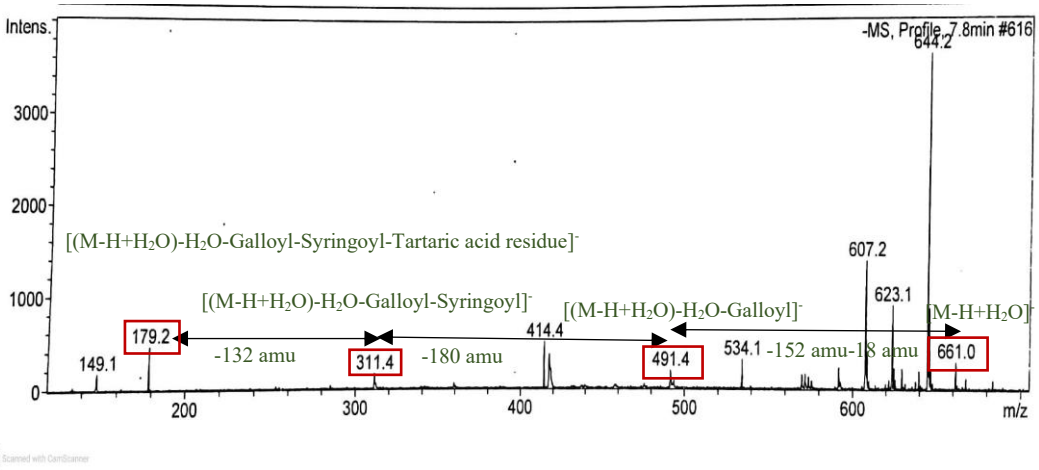
Mass spectral analysis of ILLM-2

The -ESI/MS spectrum of ILLM-2 displayed a peak at *m/z* 661 which represented $[M-H+H_2O]^-$ ion indicating the presence of a H_2O molecule as adduct to

the deprotonated molecular ion (Figure 3.24 b). Thus, the molecular mass of the component was calculated as 644 amu. A peak at *m/z* 491 corresponded to [(M-H+H_2O)-18-152]$^-$ ion formed by the loss of water and galloyl unit (**Miketova *et al.*, 2000**). Another peak appeared at *m/z* 311 amu by the detection of [(M-H+H_2O)-18-152-180]$^-$ ion and indicated the loss of syringoyl residue.



(a)



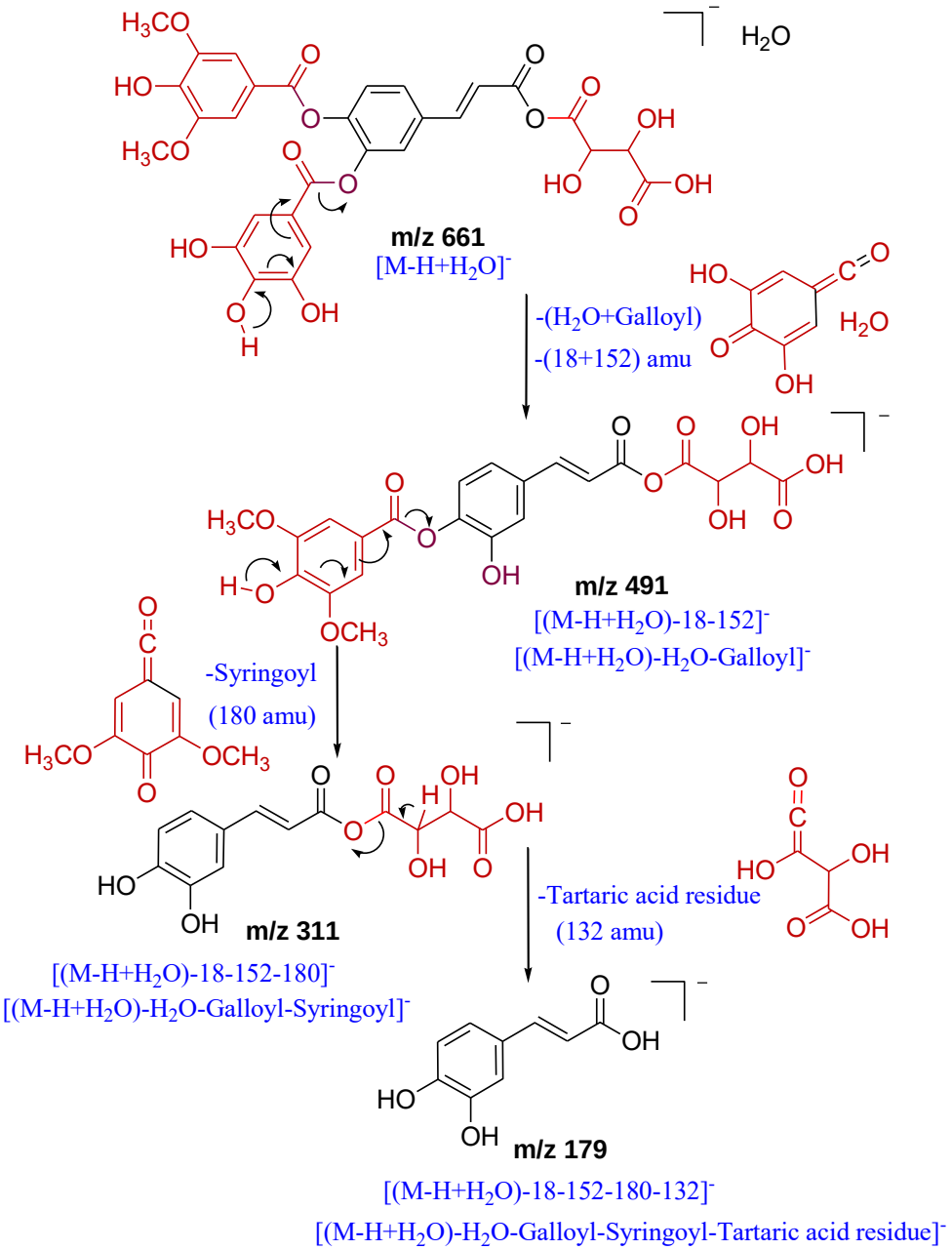
(b)

Figure 3.24. (a) UV spectrum of ILLM-2 (b) Mass spectrum of ILLM-2 in negative ESI/MS

The spectrum showed another peak at *m/z* 179 corresponding to [(M-H+H_2O)-18-152-180-132]$^-$ ion suggesting the loss of a tartaric acid residue (**Hamdan *et al.*,**

2021). The peak at *m/z* 179 represented deprotonated caffeic acid (**Schütz *et al.*, 2005**) (Scheme 3.8).

Hence, ILLM-2 was tentatively identified as 3-*O*-galloyl-4-*O*-syringoyl caffeoyl tartarate.

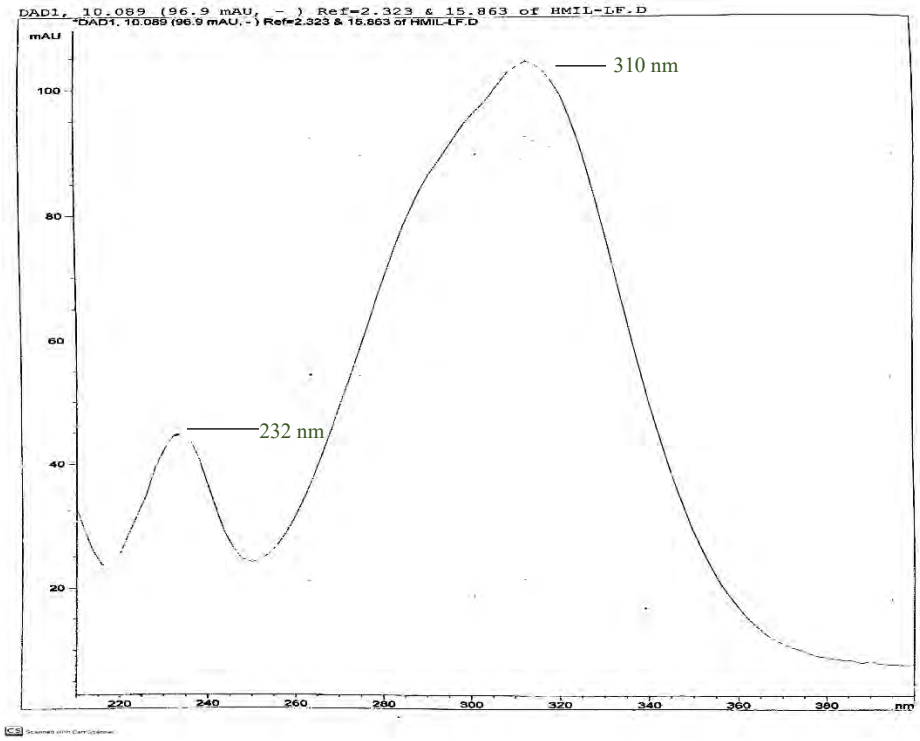


Scheme 3.8. Suggested fragmentation pattern for ILLM-2 in negative ESI/MS mode

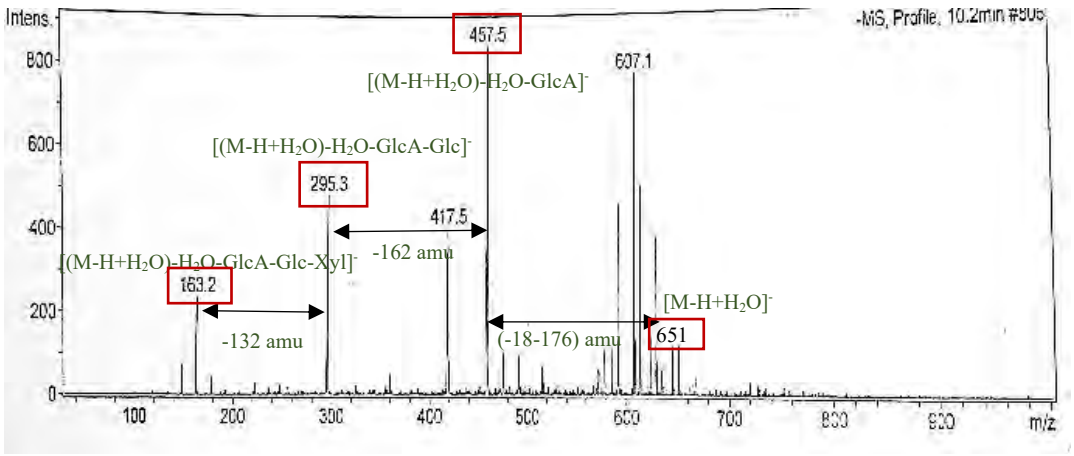
ILLM-3

Retention time of ILLM-3

The component ILLM-3 appeared on HPLC chromatogram at retention time of 10.0 minutes. The mobile phase was a gradient mixture of water containing 10 % acetonitrile in volume fraction. This was an indicative of high hydrophilicity of the eluent. It was deduced that the component could be some glycosylated phenolic acid.



(a)

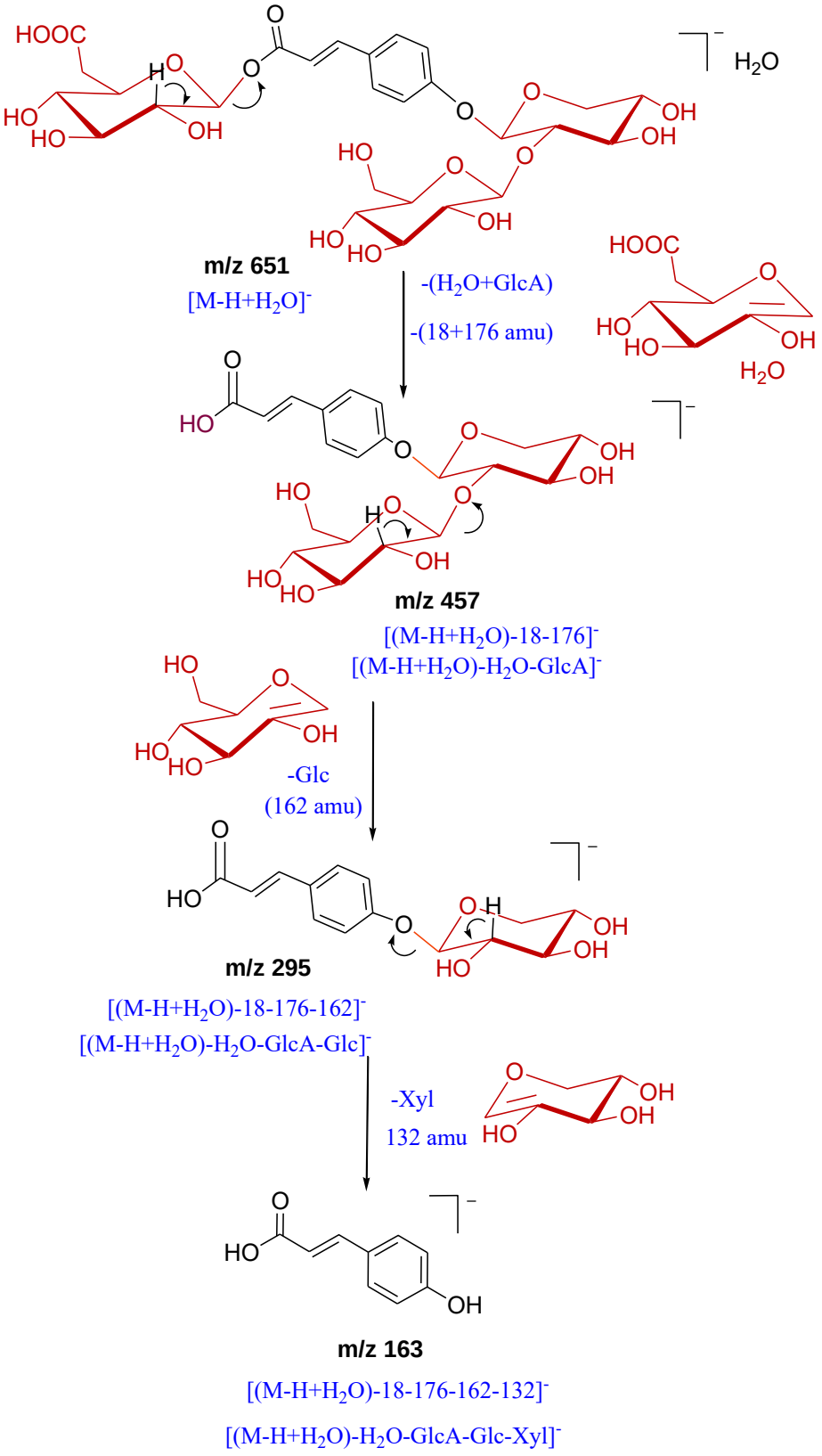


(b)

Figure 3.25. (a) UV spectrum of ILLM-3 (b) Mass spectrum of ILLM-3 in negative ESI/MS

DAD spectrum of ILLM-3

The UV spectrum showed two absorption bands, one being very intense appeared at 310 nm and the other at 232 nm is only ¼th of the first in intensity. The



Scheme 3.9. Suggested fragmentation pattern for ILLM-3 in negative ESI/MS mode

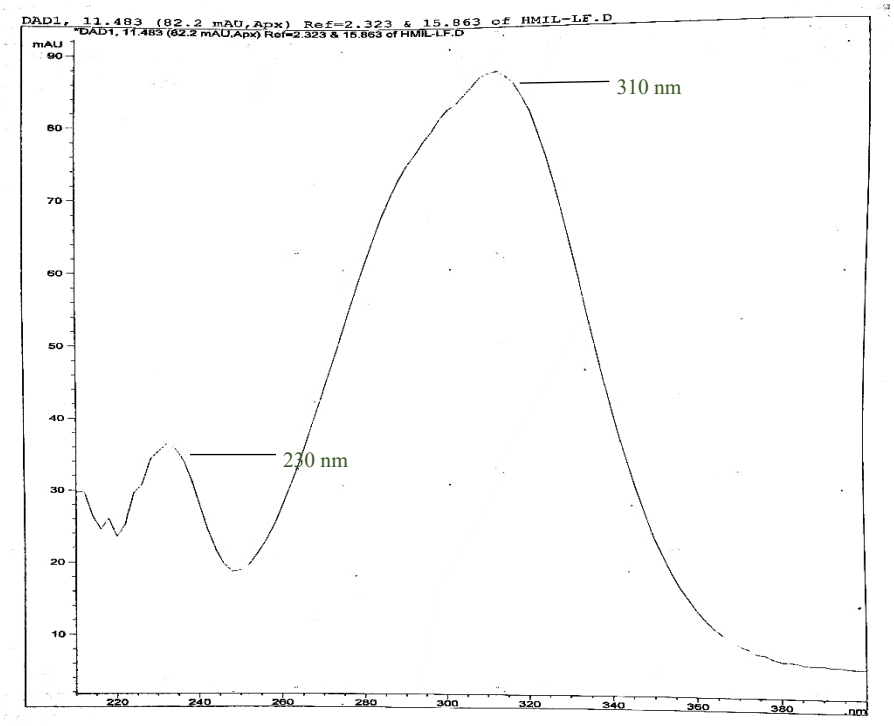
absorption values and relative band intensity were characteristic of trans *p*-coumaric acid (**Istasse *et al.*, 2016**) (Figure 3.25 a).

Mass spectral analysis of ILLM-3

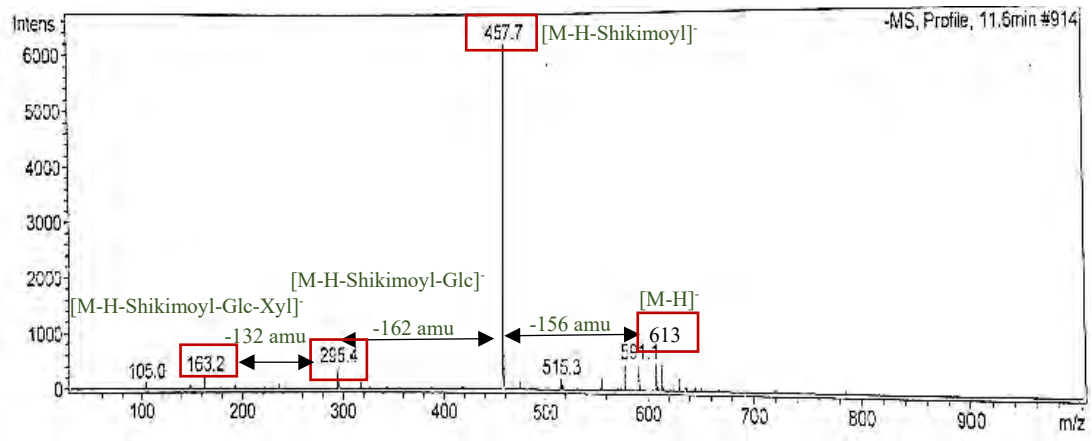
The molecular ion peak appeared at *m/z* 651 and corresponded to $[M-H+H_2O]^-$ ion which indicated the presence of water molecule as adduct to the deprotonated molecular ion (Figure 3.25 b). Thus, the molecular weight of the constituent was calculated as 634 amu. The spectrum showed a peak at *m/z* 457 for $[(M-H+H_2O)-18-176]^-$ ion which indicated the loss of glucuronide moiety with molecular weight 176 amu (**Gao *et al.*, 2007**). Another peak appeared at *m/z* 295 by detection of $[(M-H+H_2O)-18-176-162]^-$ ion being in consistent with the loss of hexose residue (**Said *et al.*, 2017**). The spectrum presented a peak at *m/z* 163 for $[(M-H+H_2O)-18-176-162-132]^-$ ion providing evidence for the loss of pentose residue (**Farag *et al.*, 2019**). The peak at *m/z* 163 corresponded to deprotonated coumaric acid (**Sánchez-Rabaneda *et al.*, 2003**) (Scheme 3.9).

The component ILLM-3 was tentativey identified as coumaroyl 4-*O*-glucopyranosylxylopyranoside glucuronate.

ILLM-4



(a)



(b)

Figure 3.26. (a) UV spectrum of ILLM-4 (b) Mass spectrum of ILLM-4 in negative ESI/MS

Retention time of ILLM-4

The component was eluted with 10 % acetonitrile in water and appeared on HPLC chromatogram at 11.4 minutes. This elution brought about by aqueous-rich mobile phase showed great hydrophilic potential of the eluent. It was probably a glycosylated phenolic acid.

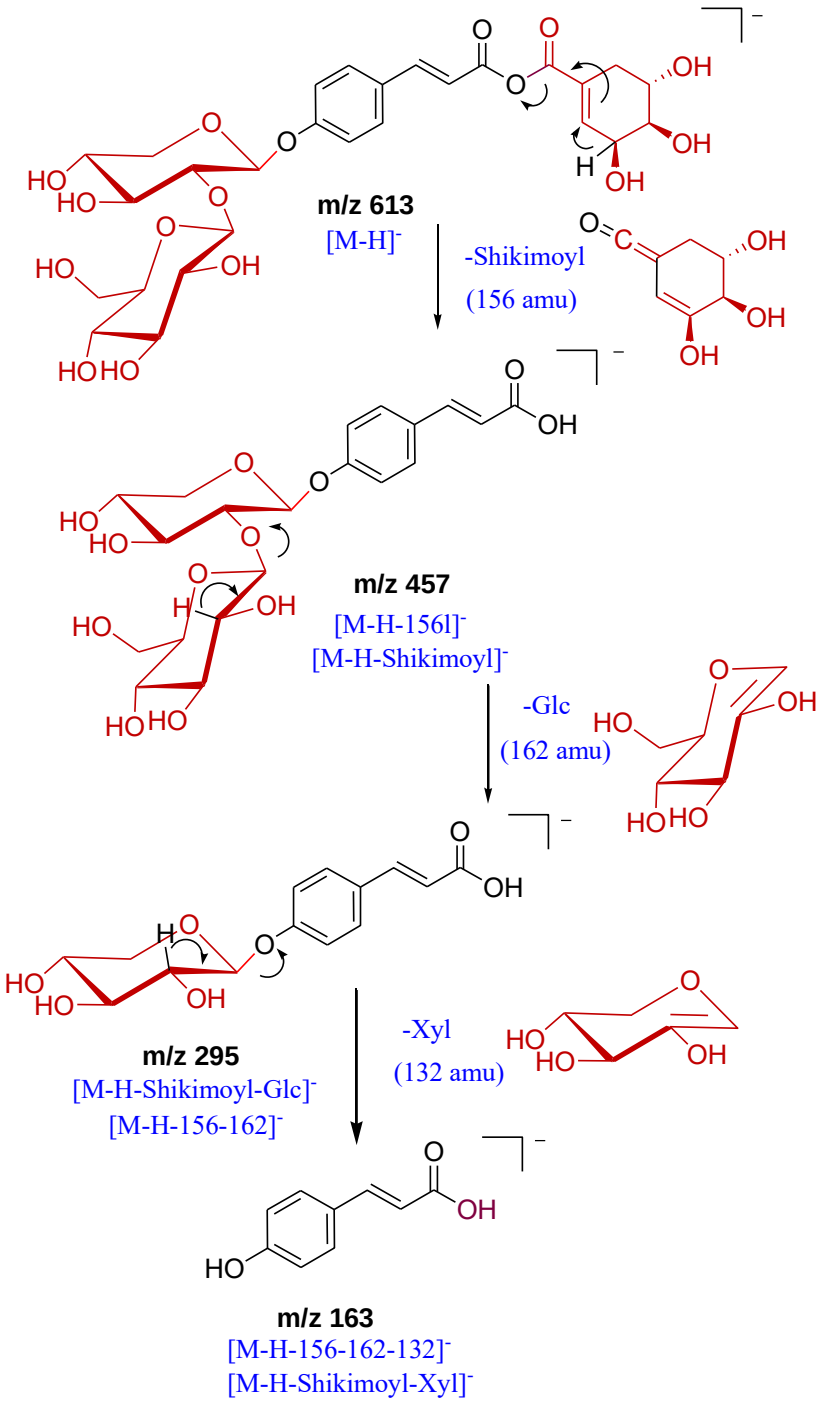
DAD spectrum of ILLM-4

UV spectrum of ILLM-4 showed maximum absorptions at 310 and 230 nm. The band at 310 nm was more intense than at 230 nm. This information provided evidence for the presence of trans *p*-coumaric acid (**Istasse *et al.*, 2016**) (Figure 3.26 a).

Mass spectral analysis of ILLM-4

In the negative ESI/MS, ILLM-4 presented a peak for deprotonated molecular ion $[M-H]^-$ at *m/z* 613 (Figure 3.26 b). The fragmentation of $[M-H]^-$ led to product ion at *m/z* 457 $[M-H-156]^-$ attributing to the loss of shikimoyl (156 amu) (**Otify *et al.*, 2019**). Another cleavage resulted in fragment ion at *m/z* 295 $[M-H-156-162]^-$ suggesting the loss of hexose residue (162 amu) (**Guo *et al.*, 2007**). The removal of dehydrated pentose (132 amu) afforded a secondary ion at *m/z* 163 $[M-H-156-162-132]^-$ (**Farag *et al.*, 2019**). The peak at *m/z* 163 also indicated the presence of deprotonated coumaric acid (**Sánchez-Rabaneda *et al.*, 2003**) (Scheme 3.10).

The evidence obtained from retention time, UV and mass spectra led to the tentative identification of ILLM-4 as trans *p*-coumaroyl 4-*O*-glucosylxyloside shikimate.

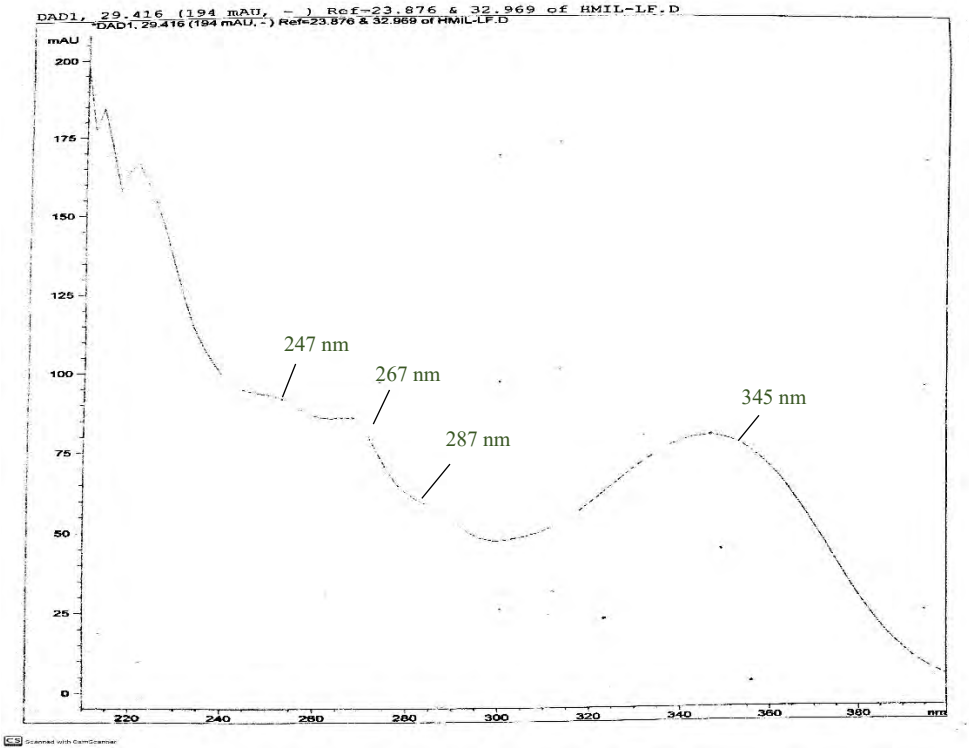


Scheme 3.10. Suggested fragmentation pattern for ILLM-4 in negative ESI/MS mode

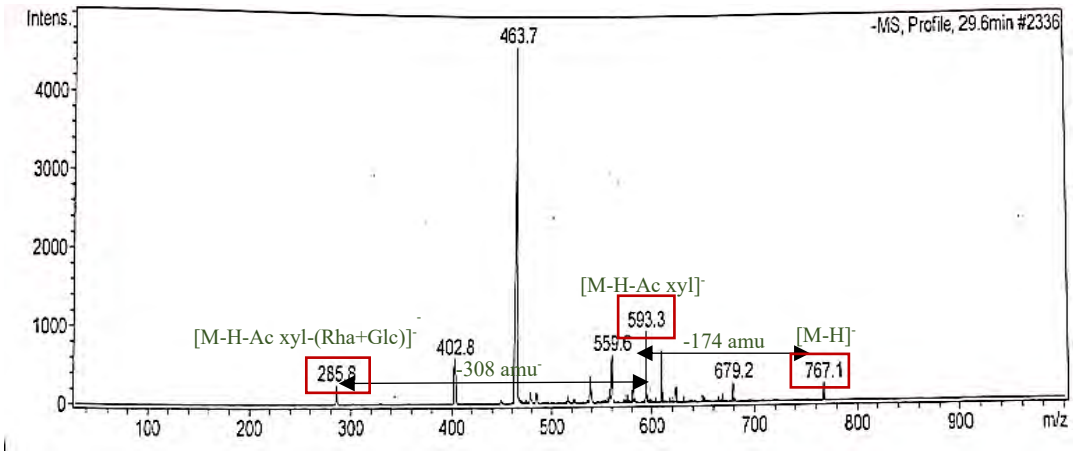
ILLM-5

Retention time of ILLM-5

HPLC chromatogram showed a peak for separation of a component at retention time of 29.4 minutes. The mobile phase gradient applied at this time was 40 % acetonitrile in water, which depicted that the component was partially hydrophilic. It suggested that ILLM-5 could be a mono or di-glycosylated flavonoid.



(a)



(b)

Figure 3.27. (a) UV spectrum of ILLM-5 (b) Mass spectrum of ILLM-5 in negative ESI/MS

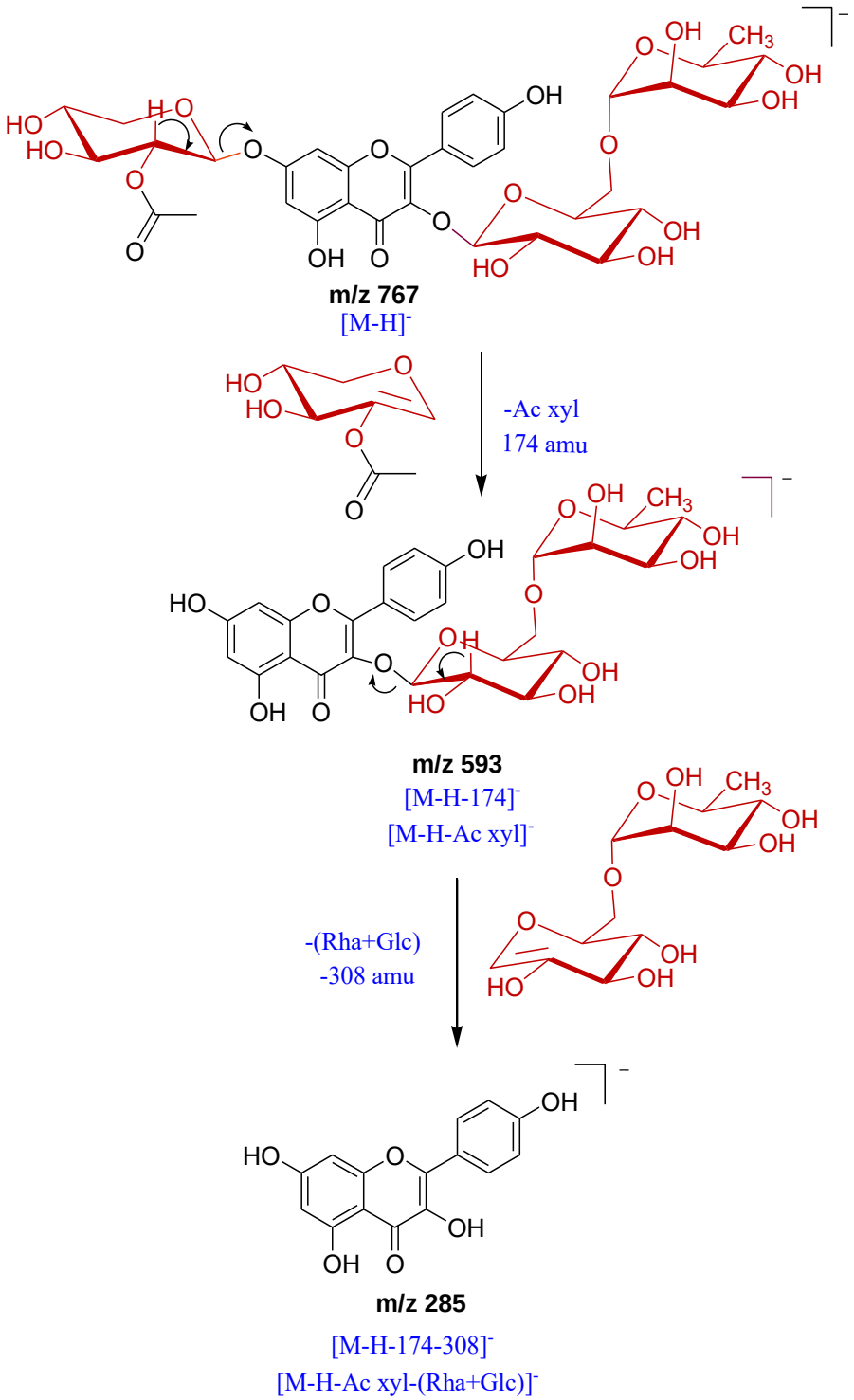
DAD spectrum of ILLM-5

The UV spectrum of this constituent showed absorption bands at 247sh, 267, 287sh and 345 nm. Two prominent absorption bands between 240-400 nm indicated the class of organic compound to be flavone or flavonol. The low intensity of band I and appearance at 345 nm instead of above 350 nm indicated a 3-*O*-substituted flavonol (**Mabry *et al.,* 2012**). The flavones with band I at 345 nm are expected to show high intensity of band I. So, it could be some derivative of kaempferol, a flavonol widely reported in genus *Indigofera* (**Hasan *et al.,* 1996**), (**Prakash *et al.,* 2007**), (**Moura *et al.,* 2011**). The band I for kaempferol usually appears at 367 nm but a 22 nm hypsochromic shift in given spectrum is indicative of substitution at 5-position in the 3-*O*-substituted flavonol (**Mabry *et al.,* 2012**). The UV absorbance matched with kaempferol-3-*O*-rutinoside-7-*O*-rhamnoside (**da Graça Campos & Markham, 2007**) (Figure 3.27 a).

Mass spectral analysis of ILLM-5

The mass spectrum of ILLM-5 recorded in the negative ESI/MS mode presented a peak corresponding to deprotonated molecular ion $[M-H]^-$ at *m/z* 767. It showed that molecular weight of the component to be identified was 768 amu (Figure 3.27 b). The loss of acetyl-pentose (42+132=174 amu) (**Mullen *et al.,* 2003**) resulted in fragment ion $[M-H-174]^-$ or $[Y_0^7]^-$ (**Cuyckens and Claeys, 2004)** which appeared at *m/z* 593. The complete loss of acetyl-pentose indicated that the sugar was attached to the aglycone by *O*-linkage. Since the loss was prior to any other loss from the deprotonated molecule, the sugar was more probably attached at 7-position. This deduction was made in accordance with the sequence of loss of moieties shown by substituents attached at different positions of the aglyone in standard flavonoid glycosides during -ESI/MS analyses. The lost fragment was identified as acetylxylose because co-chromatography of acid hydrolysate of ILLM extract against standard sugars revealed the presence of only one pentose sugar in the extract and it was xylose. The loss of 308 amu corresponded to deoxyhexosyl-hexose or rutinosyl (308 amu) (**Navarro-González *et al.,* 2015**) and led to the fragment ion $[M-H-174-308]^-$ or $[Y_0^7Y_0^3]^-$ (**Cuyckens and Claeys, 2004)** at *m/z* 285. The presence of rutinosyl, a disaccharide of rhamnose and glucose, was supported by indication of rhamnose and glucose sugars in the ILLM extract during sugar analysis by TLC against standard sugars. The peak at *m/z* 285

coresponded to kaempferol **(Truchado *et al.*, 2009)**, a widely distributed flavonol in plants of genus *Indigofera* (Scheme 3.11).



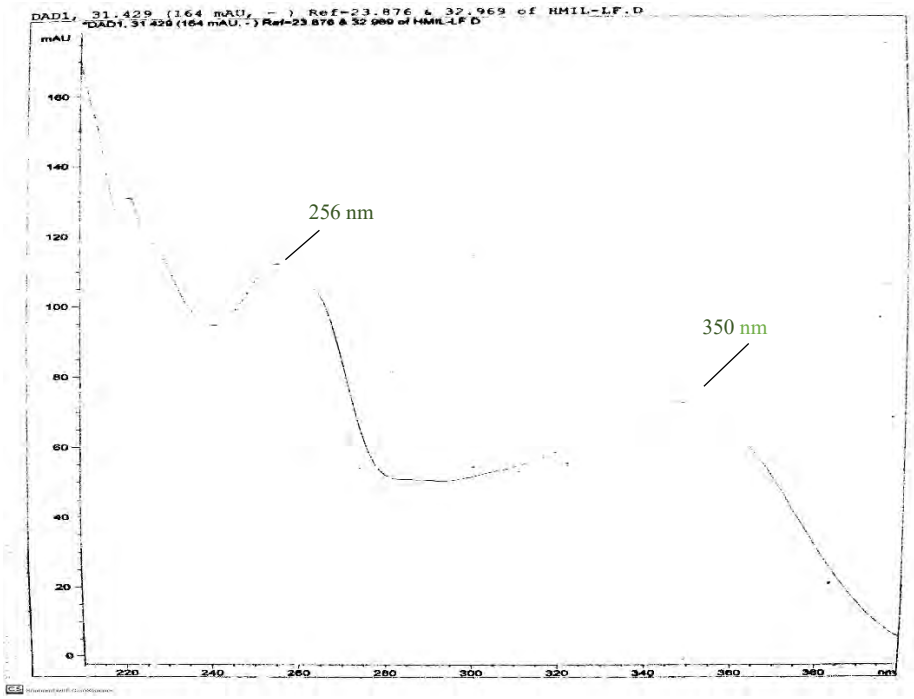
Scheme 3.11. Suggested fragmentation pattern for ILLM-5 in negative ESI/MS mode

The information obtained from retention time, UV and mass spectra led to the tentative identification of ILLM-5 as 3-*O*-ruinosyl-7-*O*-acetylxylopyranosyl kaempferol.

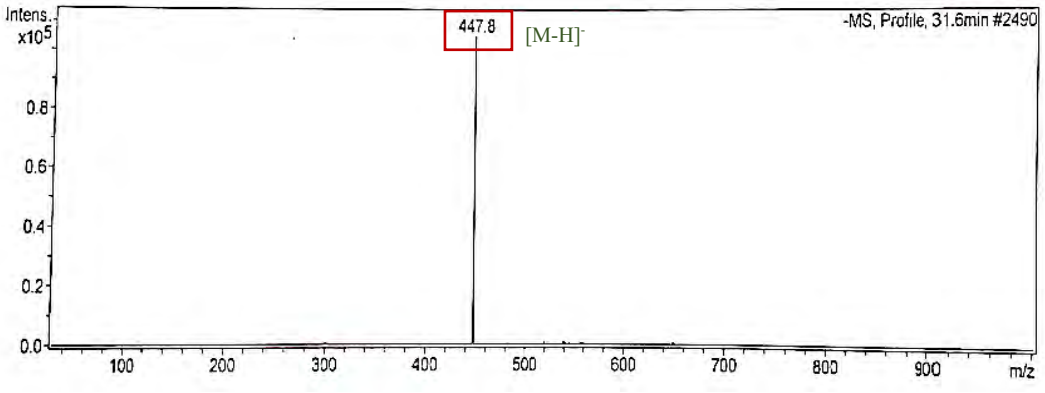
ILLM-6

Retention time of ILLM-6

The phytoconstituent ILLM-6 eluted from reverse phase HPLC column at 31.4 minutes when mobile phase gradient was 40 % acetonitrile in water. The separation of constituent by this composition of mobile phase was clear indicative of the partial hydrophilic character of eluting constituent. This information showed that the component could be a flavonoid mono-glycoside.



(a)



(b)

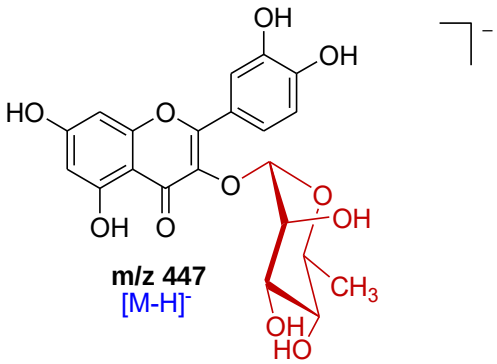
Figure 3.28. (a) UV spectrum of ILLM-6 (b) Mass spectrum of ILLM-6 in negative ESI/MS

DAD spectrum of ILLM-6

The UV spectrum of ILLM-6 showed absorption maxima at 256, 265sh, 301sh and 350 nm. The appearance of two prominent absorption bands between 240-350 nm is characteristic of flavones and 3-*O*-glycosylated flavonols. Intensity of band I was lower which indicated glycosylation at 3-position of flavonol. The spectrum was in-agreement with that reported in literature for standard quercetin-3-*O*-rhamnoside (**Mabry *et al.*, 2012**) (Figure 3.28 a).

Mass spectral analysis of ILLM-6

The mass spectrum showed a single peak at *m/z* 447 corresponding to the deprotonated molecular ion or $[M-H]^-$ (Figure 3.28 b). This suggested that molecular weight of the component was 448 amu. The mass corresponded to quercetin (302 amu) (**Liang *et al.*, 2005**) substituted with deoxyhexose i.e., rhamnose (146 amu) (**Eklund *et al.*, 2008**). The compound was tentatively identified as quercetin-3-*O*-rhamnoside (quercitrin) (Scheme 3.12). Derivatives of quercetin have been previously reported in literature (**Sánchez-Rabaneda *et al.*, 2003**).



Scheme 3.12. Suggested fragmentation pattern for ILLM-6 in negative ESI/MS mode

Conclusions

HPLC-DAD-ESI/MS analysis of hydromethanolic extracts of various parts of *Indigofera linifolia* revealed that the plant is highly rich in phenolic constituents. The present work led to the tentative identification of following hydrophilic constituents in *I. linifolia*.

- Maloyl-[3-*O*-glucopyranosyl-5-*O*-(malonylrhamnopyranosyl)] quinate **(Seeds)**
- 7-*O*-Glucopyranosyl-4′-*O*-glucuronopyranosyl orobol (**Seeds**)
- 7-*O*-Dihydroferuloyl-3-*O*-rhamnopyranosyl kaempferol (**Stem**)
- 7-*O*-Hydroxybenzoylxylopyranosyl formononetin (**Roots**)
- 4-*O*-Acetylxylopyranosyl caffeoyl neohesperidoside. (**Leaves**)
- 3-*O*-Galloyl-4-*O*-syringoyl caffeoyl tartarate (**Leaves**
- Coumaroyl 4-*O*-glucopyranosylxylopyranoside glucuronate (**Leaves**)
- trans *p*-Coumaroyl 4-*O*-glucosylxyloside shikimate. (**Leaves**)
- 3-*O*-Ruinosyl-7-*O*-acetylxylopyranosyl kaempferol. (**Leaves)**
- Quercetin-3-*O*-rhamnopyranoside (**Leaves**)

GC-MS analyses of *n*-hexane extract of *Indigofera linifolia* revealed the presence of various lipophilic constituents in leaves, seeds, stem, and roots of the plant.

- Methyl hexadecanoate is found in seeds as well as root extract of *I. linifolia.*
- Ethyl hexadecanoate has been identified in the stem and roots of this plant.

The study provides an insight into the types of phytoconstituents present in ethnobotanically valuable plant *Indigofera linifolia.* The recorded fingerprint chromatograms are helpful for identification of plant material in future. The plant contains antioxidant and useful anti-inflammatory agents which can be isolated by setting protocols for further studies on this plant.

References

Aaby K., Ekeberg D. and Skrede G. (2007). Characterization of Phenolic Compounds in Strawberry (Fragaria × ananassa) Fruits by Different HPLC Detectors and Contribution of Individual Compounds to Total Antioxidant Capacity. *J. Agric. Food Chem.* vol. 55(11), pp. 4395-4406.

Addy M. E., Addo P. and Nyarko A. K. (1992). *Indigofera arrecta* Prevents the Development of Hyperglycaemia in the db/db Mouse. *Phytother. Res.* vol. 6(1), pp. 25-28.

Agarwal A. and Sharma V. (2017). PUB082 Role of Isolated Flavonoid from *Indigofera tinctoria* in *N*-Nitrosopyrrolidine Induced Biochemical Changes Related to Lung Cancer. *J. Thorac. Oncol.* vol. 12(sup.1), p. S1495.

Ahmadu A. A., Onanuga A. and Ebeshi B. U. (2011). Isolation of Antibacterial Flavonoids from the Aerial Parts of *Indigofera secundiflora*. *Pharmacogn. J.* vol. 3(19), pp. 25-27.

Al-Fatimi M. (2019). Ethnobotanical Survey of Medicinal Plants in Central Abyan Governorate, Yemen. *J. Ethnopharmacol.* vol. 241, p. 111973.

Al-Shaebi E. A., Dkhil M. A. and Al-Quraishy S. (2018). *Indigofera oblongifolia* Regulates the Hepatic Gene Expression Profile Induced by Blood Stage Malaria. *Microb. Pathog.* vol. 119, pp. 170-182.

Al-Shaebi E. M., Taib N. T., Mubaraki M. A., Hafiz T. A., Lokman M. S., Al-Ghamdy A. O., Lubbad M.Y., Bayoumy E. M., Al-Quraishy S. and Dkhil M. A. (2017). *Indigofera oblongifolia* Leaf Extract Regulates Spleen Macrophage Response During *Plasmodium chabaudi* Infection. *Saudi J. Biol. Sci.* vol. 24(7), pp.1663-1666.

Alston T. A., Mela L. and Bright H. J. (1977). 3-Nitropropionate, the Toxic Substance of *Indigofera*, is a Suicide Inactivator of Succinate Dehydrogenase. *PNAS USA*, vol. 74(9), pp. 3767-3771.

Amos S., Binda L., Kunle O. F., Okafor I., Emeje M., Akah P. A., Wambebe C. and Gamaniel K. (2003). Smooth Muscle Contraction Induced by *Indigofera*

dendroides Leaf Extracts may Involve Calcium Mobilization via Potential Sensitive Channels. *Phytother. Res.* vol. 17(7), pp. 792-796.

Anderson R. C., Majak W., Rassmussen M. A., Callaway T. R., Beier R. C., Nisbet D. J. and Allison M. J. (2005). Toxicity and Metabolism of the Conjugates of 3-Nitropropanol and 3-Nitropropionic Acid in Forages Poisonous to Livestock. *J. Agric. Food Chem.* vol. 53(6), pp. 2344-2350.

Andreazza N. L., De Lourenço C. C., Stefanello M. É. A., Atvars T. D. Z. and Salvador M. J. (2015). Photodynamic Antimicrobial Effects of Bis-Indole Alkaloid Indigo from *Indigofera truxillensis* Kunth (Leguminosae). *Lasers Med. Sci.* vol. 30(4), pp.1315-1324.

Andreotti A., Bonaduce I., Colombini M. P. and Ribechini E. (2004). Characterisation of Natural Indigo and Shellfish Purple by Mass Spectrometric Techniques. *Rapid Commun. Mass Spectrom.* vol. 18(11), pp. 1213-1220.

Anuradha S. M. J., Kumar B. V., Radhakrishnaiah M. and Narayana L. L. (1987). Chemosystematics of Some Species of *Indigofera*. *Proc. Indian Acad. Sci. (Plant Sci.)* vol. 97(6), pp. 443-447.

Aourz N., Serruys A. S. K., Chabwine J. N., Balegamire P. B., Afrikanova T., Edrada-Ebel R., Grey A. I., Kamuhabwa A. R., Walrave L. Esguerra C. V., Leuven F. V., De Witte P. A. M., Smolders I. and Crawford A. D. (2019). Identification of GSK-3 as a Potential Therapeutic Entry Point for Epilepsy. *ACS Chem. Neurosci.* vol. 10(4), pp. 1992-2003.

Arbab A. H., Parvez M. K., Al Dosari M. S. and Al Rehaily A. J. (2017). *In Vitro* Evaluation of Novel Antiviral Activities of 60 Medicinal Plants Extracts Against Hepatitis B Virus. *Exp. Ther. Med.* vol. 14(1), pp. 626-634.

Arriaga A. M. C., Andrade-Neto M., Malcher G. T., Gomes T. B. M., Vasconcelos J. N., Rodrigues A. C. P., de Oliveira M. C. F. and Santiago G. M. P. (2008). Composition of the Essential Oil of *Indigofera microcarpa* from the Northeast of Brazil. *Chem. Nat. Compd.* vol. 44(2), pp. 245-246.

Arriaga A. M. C., Lemos T. L. G., Santiago G. M. P., Andrade-Neto M., Braga M. A., De Almeida M. C., Gomes T. B. M., Rodrigues F. E. A., Vasconcelos J. N. and

Alves P. B. (2013). Chemical Composition and Antioxidant Activity of *Indigofera suffruticosa*. *Chem. Nat. Compd.* vol. 49(1), pp. 150-151.

Asase A. and Oppong-Mensah G. (2009). Traditional Antimalarial Phytotherapy Remedies in Herbal Markets in Southern Ghana. *J. Ethnopharmacol.* vol. 126(3), pp. 492-499.

Assis T. S., Medeiros R. M. T., Araújo J. A. S. D., Dantas A. F. M and Riet-Correa F. (2009). Intoxicações por Plantas em Ruminantes e Equídeos no Sertão Paraibano. *Pesqui Vet. Bras.* vol. 29(11), pp. 919-924.

Asuntha G., Prasannaraju Y. and Prasad K. V. S. R. G. (2010). Effect of Ethanol Extract of *Indigofera tinctoria* Linn (Fabaceae) on Lithium/Pilocarpine-Induced Status Epilepticus and Oxidative Stress in Wistar Rats. *Trop. J. Pharm. Res.* vol. 9(2), pp. 149-156.

Aylward J. H., Court R. D., Haydock K. P., Strickland R. W. and Hegarty M. P. (1987). *Indigofera* Species with Agronomic Potential in the Tropics. Rat Toxicity Studies. *Aust. J. Agric. Res.* vol. 38(1), pp. 177-186.

Bakasso S., Lamien-Meda A., Lamien C. E., Kiendrebeogo M, Millogo J., Ouedraogo A. G. and Nacoulma O. G., (2008). Polyphenol Contents and Antioxidant Activities of Five *Indigofera* Species (Fabaceae) from Burkina Faso. *Pak. J. Biol. Sci.* vol. 11(11), pp.1429-1435.

Balamurugan G. and Muralidharan P. (2010). Effect of *Indigofera tinctoria* on *β*-amyloid (25-35) Mediated Alzheimer's Disease in Mice: Relationship to Antioxidant Activity. *Bangladesh J. Pharmacol.* vol. 5(1), pp. 51-56.

Balasubramanian R., Narayanan M., Kedalgovindaram L. and Rama K. D. (2007). Cytotoxic Activity of Flavone Glycoside from the Stem of *Indigofera aspalathoides* Vahl. *J. Nat. Med.* vol. 61(1), pp. 80-83.

Baumgartner L., Sosa S., Atanasov A. G., Bodensieck A., Fakhrudin N., Bauer J., Favero G. D., Ponti C., Heiss E. H., Schwaiger S., Ladurner A., Widowitz U., Loggia R. D., Rollinger J. M., Werz O., Bauer R., Dirsch V. M., Tubaro A. and Stuppner H. (2011). Lignan Derivatives from *Krameria lappacea* Roots Inhibit

Acute Inflammation *In Vivo* and Pro-inflammatory Mediators *In Vitro*. *J. Nat. Prod.* vol. 74(8), pp. 1779-1786.

Bezerra dos Santos A. T., Araújo T. F. D. S., Nascimento da Silva L. C., Silva C. B. D., Oliveira A. F. M. D., Araújo J. M., Correia M. T. D. S. and Lima V. L. D. M. L. (2015). Organic Extracts from *Indigofera suffruticosa* Leaves have Antimicrobial and Synergic Actions with Erythromycin Against *Staphylococcus aureus*. *Front. Microbiol.* vol. 6, pp. 1-7.

Bhagavan N. B., Arunachalam S., Dhasarathan P. and Kannan N. D. (2013). Evaluation of Anti-Inflammatory Activity of *Indigofera aspalathoides* Vahl in Swiss Albino Mice. *J. Pharm. Res.* vol. 6(3), pp. 350-354.

Bhaskar B. V., Mohan A. R., Babu T. M. C., Rajesh S. S., Bhuvaneswar C., Sivaraman T., Gunasekar D. and Rajendra W. (2016). Antibacterial Efficacy of Fractions and Compounds from *Indigofera barberi:* Identification of DNA Gyrase B Inhibitors through Pharmacophore Based Virtual Screening. *Process Biochem.* vol. 51(12), pp. 2208-2221.

Bhatta R., Saravanan M., Baruah L., Sampath K. T. and Prasad C. S. (2013). Effect of Plant Secondary Compounds on *In Vitro* Methane, Ammonia Production and Ruminal Protozoa Population. *J. Appl. Microbiol.* vol. 115(2), pp. 455-465.

Bhuvaneswari R. and Balasundaram C. (2006). Traditional Indian Herbal Extracts Used *In Vitro* Against Growth of the Pathogenic Bacteria-*Aeromonas hydrophila*. *Isr. J. Aquac. – Bamidgeh,* vol. 58(2), pp. 89-96.

Birru E. M. (2015). Evaluation of the Effect of Crude Leaves Extract of *Indigofera spicata* Forssk. (Fabaceae) on Blood Glucose Level of Normoglycemic, Oral Glucose Loaded and Alloxan Induced Diabetic Rodents. *Value Health*, vol. 18(7), p. A704.

Birru E. M., Asrie A. B., Adinew G. M., and Tsegaw A. (2016). Antidiarrheal Activity of Crude Methanolic Root Extract of *Indigofera spicata* Forssk (Fabaceae). *BMC Complement. Altern. Med.* vol. 16(1), pp. 1-7.

Birru E. M., Geta M., and Gurmu A. E. (2017). Antiplasmodial Activity of *Indigofera spicata* Root Extract Against *Plasmodium berghei* Infection in Mice. *Malar. J.* vol. 16(1), pp. 1-7.

Boothapandi M., and Ramanibai R. (2018). Antiproliferative Activity of Chrysin (5, 7-Dihydroxyflavone) from *Indigofera tinctoria* on Human Epidermoid Carcinoma (A431) Cells. *Eur. J. Integr. Med.* vol. 24, pp. 71-78.

Boothapandi M., and Ramanibai R. (2019). Immunomodulatory Effect of Natural Flavonoid Chrysin (5, 7-Dihydroxyflavone) on LPS Stimulated RAW 264.7 Macrophages via Inhibition of NF-KB Activation. *Process Biochem.* vol. 84, pp. 186-195.

Bu Wong M., Rodríguez N. S., Pérez De Alejo J. L., and Fernández Pérez M. (1999). Actividad De La *Indigofera suffruticosa* Mill En La Epilepsia Crónica Experimental Y Su Relación Con Aminoácidos Neurotransmisores. *Rev. Cuba. Plantas Med.* vol. 4(1), pp. 18-21.

Bueno P. rez L., Li J., Lantvit D. D., Pan L., Ninh T. N., Chai H. B., Saejarto D. D., Swanson S. M., Lucas D. M., and Kinghorn A. D. (2013). Bioactive Constituents of *Indigofera spicata. J. Nat. Prod.* vol. 76(8), pp. 1498-1504.

Chakrabarti R., Damarla R. K. B., Mullangi R., Sharma V. M., Vikramadithyan R. K., and Rajagopalan R. (2006). Insulin Sensitizing Property of *Indigofera mysorensis* Extract. *J. Ethnopharmacol.* vol. 105(1-2), pp. 102-106.

Chellappandian M., Mutheeswaran S., Pandikumar P., Duraipandiyan V., and Ignacimuthu S. (2012). Quantitative Ethnobotany of Traditional Siddha Medical Practitioners from Radhapuram *Taluk* of Tirunelveli District, Tamil Nadu, India. *J. Ethnopharmacol.* vol. 143(2), pp. 540-547.

Chen C., Lin C., and Wang H. (2008). The Study on Cotton Fabric Dyeing by Indigo Blue from *Baphicacanthus cusia*, *Indigofera tinctoria* and *Polygonum tinctorium*. *Sen'i Gakkaishi*, vol. 64(10), pp. 297-301.

Chen T. Y., Sun H. L., Yao H. T., Lii C. K., Chen H. W., Chen P. Y., Li C. C., and Liu K. L. (2013). Suppressive Effects of *Indigofera suffruticosa* Mill Extracts on

Lipopolysaccharide-Induced Inflammatory Responses in Murine RAW 264.7 Macrophages. *Food Chem. Toxicol.* vol 55, pp. 257-264.

Christie G. S., Wilson M., and Hegarty M. P. (1975). Effects on the Liver in the Rat of Ingestion of *Indigofera spicata,* a Legume Containing an Inhibitor of Arginine Metabolism. *J. Pathol.* vol. 117(4), pp. 195-205.

Christina A. J. M., Jose M. A., Robert S. J. H., Kothai R., Chidambaranathan N., and Muthumani P. (2003). Effect of *Indigofera aspalathoides* Against Dalton's Ascitic Lymphoma. *Fitoterapia*, vol. 74(3), pp. 280-283.

Colnaghi Simionato A. V., da Silva D. S., Lambais M. R., and Carrilho E. (2007). Characterization of a putative Xylella fastidiosa diffusible signal factor by HRGC-EI-MS. *J. Mass. Spectrom.* vol. 42(10), pp. 1375-1381.

Cuyckens F. and Claeys M. (2002). Optimization of a Liquid Chromatography Method Based on Simultaneous Electrospray Ionization Mass Spectrometric and Ultraviolet Photodiode Array Detection for Analysis of Flavonoid Glycosides. *Rapid Commun. Mass Spectrom.* vol. 16(24), pp. 2341-2348.

Cuyckens F., and Claeys M. (2004). Mass Spectrometry in the Structural Analysis of Flavonoids. *J. Mass Spectrom.* vol. 39(1), pp. 1-15.

da Graça Campos M. & Markham K. R. (2007). Structure Information from HPLC and On-line Measured Absorption Spectra: Flavones, Flavonols and Phenolic Acids. *Imprensa da Universidade de Coimbra/Coimbra University Press*.

Dahot M. U. (1999). Antibacterial and Antifungal Activity of Small Protein of *Indigofera oblongifolia* Leaves. *J. Ethnopharmacol.* vol. 64, pp. 277-282.

Davis B. D., Needs P. W., Kroon P. A. and Brodbelt J. S. (2006). Identification of Isomeric Flavonoid Glucuronides in Urine and Plasma by Metal Complexation and LC-ESI-MS/MS. *J. Mass. Spectrom.* vol. 41(7), pp. 911-920.

De Alejo P., José L., Miranda R. and Rodríguez G. (1996). Actividad Anticonvulsivante (Antiepileptica) Del Extracto Fluido De *Indigofera suffruticosa* (Añil Cimarrón)/Anticonvulsant activity (Antiepileptic) of Fluid Extract from *Indigofera suffruticosa*. *Rev. Cuba. Plantas Med.* vol. 1(2), pp. 7-10.

De Kort I. and Thijsse G. (1984). A Revision of the Genus Indigofera (Legumemosae-Papilionoideae) in Southeast Asia. *Blumea.* vol. 30(1), pp. 89-151.

Deshpande P. S., Khatri D. K. and Juvekar A. R. (2013). Analysis of Bioactive Components from Chloroform and Hydroalcoholic Extracts of *Indigofera cordifolia* Seeds by GC-MS. *World J. Pharm. Pharm. Sci.* vol. 2(5), pp.3320-3328.

Deshpande P., Dhodi J., Gawali N., Kothavade P., Chowdhury A. and Juvekar A. (2014). *Indigofera cordifolia* Seeds Attenuate the Oxidative Stress and Inflammation Associated with Streptozotocin Induced Type 2 Diabetes Mellitus. *Cytokine*, vol. 70(1), p. 37.

Dkhil M. A., Hafiz T. A., Thagfan F. A., Al-Shaebi E. M., Mubaraki M. A., Khalil M., Abdel-Gaber R. and Al-Quraishy S. (2019). *Indigofera oblongifolia* Protects Against Trypanosomiasis-Induced Spleen Injury. *J. Infect. Public Health.* vol. 12(5), pp. 660-665.

Dkhil M. A., Moneim A. E. A. and Al-Quraishy S. (2016). *Indigofera oblongifolia* Ameliorates Lead Acetate-Induced Testicular Oxidative Damage and Apoptosis in a Rat Model. *Biol. Trace Elem. Res.* vol. 173(2), pp. 354-361.

Dominguez X. A., Martinez C., Calero A., Dominguez Jr X. A., Hinojosa M., Zamudio A., de Quimica D., Monterrey I. T., Mexico N. L., Zabel V., Smith W. B. and Watson W. H. (1978b). Louisfieserone, an Unusual Flavanone Derivative from *Indigofera suffruticosa*, Mill. *Tetrahedron Lett.* vol. 19(5), pp. 429-432.

Domínguez X. A., Martínez C., Calero A., Hinojosa M., Zamudio A., Watson W. H. and Zabel V. (1978a). Mexican Medicinal Plants XXXI Chemical Components from "Jiquelite" *Indigofera suffruticosa*. Mill. *Planta Med.* vol. 34(06), pp. 172-175.

Dominici L., Cerbone B., Villarini M., Fatigoni C. and Moretti M. (2010). *In Vitro* Testing for Genotoxicity of Indigo Naturalis Assessed by Micronucleus test. *Nat. Prod. Commun.* vol. 5(7), pp. 1039-1042.

Domon B., and Costello C. E. (1988). A Systematic Nomenclature for Carbohydrate Fragmentations in FAB-MS/MS Spectra of Glycoconjugates. *Glycoconj. J.* vol. 5(4), pp. 397-409.

Du G., Wang Z. C., Hu W. Y., Yan K. L., Wang X. L., Yang H. M., Yang H. Y., Gao Y. H., Liu Q. and Hu Q. F. (2017). Three New 3-Methyl-2-Arylbenzofurans from the Fermentation Products of an Endophytic Fungus *Phomopsis sp.* and their Anti-TMV Activity. *Phytochem. Lett.* vol. 21, pp. 287-290.

Dzoyem J. P., McGaw L. J. and Eloff J. N. (2014). *In Vitro* Antibacterial, Antioxidant and Cytotoxic Activity of Acetone Leaf Extracts of Nine Under-Investigated Fabaceae Tree Species Leads to Potentially Useful Extracts in Animal Health and Productivity. *BMC Complement. Altern. Med.* vol. 14(1), p. 147.

E Souza M. A. D. M., Bieber L. W., Chiappeta A. A., Maciel G. M., De Mello J. F., Delle Monache F. D. and Messana I. (1988). Aryl-benzofurans from *Indigofera microcarpa. Phytochemistry*, vol. 27(6), pp.1817-1819.

Eklund P. C., Backman M. J., Kronberg L. Å., Smeds A. I. and Sjöholm R. E. (2008). Identification of Lignans by Liquid Chromatography-Electrospray Ionization Ion-Trap Mass Spectrometry. *J. Mass Spectrom.* vol. 43(1), pp. 97-107.

Elmi A., Spina R., Abdoul-Latif F., Yagi S., Fontanay S., Risler A., Duval R. E. and Laurain-Mattar D. (2018). Rapid Screening for Bioactive Natural Compounds in *Indigofera caerulea* Rox Fruits. *Ind. Crops Prod.* vol. 125, pp. 123-130.

Esimone C. O., Adikwu M. U. and Muko K. N. (1999). Antimicrobial Properties of *Indigofera dendroides* Leaves. *Fitoterapia*, vol. 70(5), pp. 517-520.

Farag M. A., Tawfike A. F., Donia M. S., Ehrlich A. and Wessjohann L. A. (2019). Influence of Pickling Process on *Allium cepa* and *Citrus limon* Metabolome as Determined Via Mass Spectrometry-Based Metabolomics. *Molecules*, vol. 24(5), p. 928.

Finnegan R. A. and Mueller W. H. (1965). Chemical Examination of a Toxic Extract of *Indigofera endecaphylla*: *The Endecaphyllins. J. Pharm. Sci.* vol. 54(8), pp. 1136-1144.

FitzGerald L. M., Fletcher M. T., Paul A. E. H., Mansfield C. S. and O'Hara A. J. (2011). Hepatotoxicosis in Dogs Consuming a Diet of Camel Meat Contaminated with Indospicine. *Aust. Vet. J.* vol. 89(03), pp. 95-100.

Gao H., Materne O. L., Howe D. L. and Brummel C. L. (2007). Method for Rapid Metabolite Profiling of Drug Candidates in Fresh Hepatocytes Using Liquid Chromatography Coupled with a Hybrid Quadrupole Linear Ion Trap. *Rapid Commun. Mass Spectrom.* vol. 21(22), pp. 3683-3693.

Garbhapu A., Yalavarthi P. and Koganti P. (2011). Effect of Ethanolic Extract of *Indigofera tinctoria* on Chemically Induced Seizures and Brain GABA Levels in Albino Rats. *Iran. J. Basic Med. Sci.* vol. 14(4), pp. 318-326.

Garcez W. S., Garcez F. R. and Barison A. (2003). Additional 3-Nitropropanoyl Esters of Glucose from *Indigofera suffruticosa* (Leguminosae). *Biochem. Syst. Ecol.* vol. 31(2), pp. 207-209.

Garcez W. S., Garcez F. R., Honda N. K. and Da Silva A. J. (1989). A Nitropropanoyl Glucopyranoside from *Indigofera suffruticosa*. *Phytochemistry*, vol. 28(4), pp.1251-1252.

Guo H., Liu A. H., Ye M., Yang M. and Guo D. A. (2007). Characterization of Phenolic Compounds in the Fruits of *Forsythia suspensa* by High-Performance Liquid Chromatography Coupled with Electrospray Ionization Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* vol. 21(5), pp. 715-729.

Gupta M., K Anti Mazumder U., Haldar P. K., Kandar C. C., Manikandan L. and Senthil G. P. (2007). Anticancer Activity of *Indigofera aspalathoides* and *Wedelia calendulaceae* In Swiss Albino Mice. *Iran. J. Pharm. Res.* vol. 6(2), pp. 141-145.

Gyamfi M. A., Yonamine M. and Aniya Y. (1999). Free-radical Scavenging Action of Medicinal Herbs from Ghana: *Thonningia sanguinea* on Experimentally Induced Liver Injuries. *General Pharmacology*: *The Vascular System*, vol. 32(6), pp. 661-667.

Hamdan D. I., Fayed M. A. and Adel R. (2021). *Echinops taeckholmiana* Amin: Optimization of a Tissue Culture Protocol, Biological Evaluation, and Chemical Profiling Using GC and LC-MS. *ACS omega*, vol. 6, pp. 13105-13115.

Han R. (1994). Highlight on the Studies of Anticancer Drugs Derived from Plants in China. *Stem Cells*, vol. 12(1), pp. 53-63.

Hasan A., Ahmad I., Khan M. A. and Chudhary M. I. (1996). Two Flavonol Triglycosides from Flowers of *Indigofera hebepetala. Phytochemistry,* vol. 43(5), pp.1115-1118.

Hasan A., Farman M. and Ahmed I. (1994). Flavonoid Glycosides from *Indigofera hebepetala*. *Phytochemistry*, vol. 35(1), pp. 275-276.

Hasan A., Waterman P. and Iftikhar N. (1989). The Use of New Chromatographic Techniques for the Isolation and Purification of Phenolic acids from *Indigofera heterantha*. *J. Chromatogr. A.* vol. 466, pp. 399-402.

Hassan H. S., Sule M. I., Musa A. M., Musa K. Y., Abubakar M. S. and Hassan A. S. (2012). Anti-inflammatory Activity of Crude Saponin Extracts from Five Nigerian Medicinal Plants. *Afr. J. Tradit. Complement. Altern. Med.* vol. 9(2), pp. 250-255.

Hegarty M. P. and Pound A. W. (1970). Indospicine, A Hepatotoxic Amino acid from *Indigofera spicata:* Isolation, Structure, and Biological Studies. *Aust. J. Biol. Sci.* vol. 23(4), pp. 831-842.

Hisaeda A., Matsunami K., Otsuka H. and Takeda Y. (2011). Flavonol Glycosides from the Leaves of *Indigofera zollingeriana*. *J. Nat. Med.* vol. 65(2), pp. 360-363.

Ibrahim S., Ibrahim M. A., Musa A. M., Aliyu A. B., Haruna N. S. and Okafor A. I. (2011). *Indigofera pulchra* Leaves Extracts Contain Anti-*Plasmodium berghei* Agents. *Bangladesh J. Pharmacol.* vol. 6(2), pp. 69-73.

Inagaki H., Yamaguchi A., Kato K., Kageyama C., Iyozumi H. and Oki Y. (2008). Screening of Weed Extracts for Antifungal Properties Against *Colletotrichum lagenarium*, The Causal Agent of Anthracnose in Cucumber. *Weed Biol. Manag.* vol. 8(4), pp. 276-283.

Iqbal M. S., Dar U. M., M–Khalil T. A., N–Hussain S. A. and Munir S. (2020). Quantitative Analysis of Ethnobotany and Common Remedies Associated with the Threatened Flora of Gujranwala Region, Punjab, Pakistan. *Appl. Ecol. Environ. Res.* vol. 18(6), pp. 7953-7979.

Istasse T., Jacquet N., Berchem T., Haubruge E., Nguyen B. K. and Richel A. (2016). Extraction of Honey Polyphenols: Method Development and Evidence of Cis Isomerization Libertas Academica. *Anal. Chem. Insights,* vol. 11, pp. 49-57.

Jahan I., Rahman M. A. and Hossain M. A. (2019). Medicinal Species of Fabaceae Occurring in Bangladesh and their Conservation Status. *J. Medicinal Plants*, vol. 7(4), pp. 189-195.

Junsongduang A., Sirithip K., Inta A., Nachai R., Onputtha B., Tanming W. and Balslev H. (2017). Diversity and Traditional Knowledge of Textile Dyeing Plants in Northeastern Thailand. *Econ. Bot.* vol. 71(3), pp. 241-255.

Kaakeh W. and Dutcher J. D. (1992). Foraging Preference of Red Imported Fire Ants (Hymenoptera: Formicidae) Among Three Species of Summer Cover Crops and Their Extracts. *J. Econ. Entomol.* vol. 85(2), pp. 389-394.

Kamal R. and Mangla M. (1993). *In Vivo* and *In Vitro* Investigations on Rotenoids from *Indigofera tinctoria* and their Bioefficacy Against the Larvae of *Anopheles stephensi* and Adults of *Callosobruchus chinensis*. *J. Biosci.* vol. 18(1), pp. 93-101.

Kameswaran T. R. and Ramanibai R. (2008). The Antiproliferative Activity of Flavanoidal Fraction of *Indigofera tinctoria* is through Cell Cycle Arrest and Apoptotic Pathway in A-549 cells. *J. Biol. Sci.* vol. 8(3), pp. 584-590.

Katsanou E. S., Halabalaki M., Aligiannis N., Mitakou S., Skaltsounis A. L., Alexi X., Pratsinis H. and Alexis M. N. (2007). Cytotoxic Effects of 2-Arylbenzofuran Phytoestrogens on Human Cancer Cells: Modulation by Adrenal and Gonadal Steroids. *J. Steroid Biochem. Mol. Biol.* vol. 104(3-5), pp. 228-236.

Katuura E., Waako P., Tabuti J. R., Bukenya-Ziraba R. and Ogwal-Okeng J. (2007). Antiplasmodial Activity of Extracts of Selected Medicinal Plants Used by Local

Communities in Western Uganda for Treatment of Malaria. *Afr. J. Ecol.* vol. 45 (Sup. 3), pp. 94-98.

Kaushik N. K., Bagavan A., Rahman A. A., Zahir A. A., Kamaraj C., Elango G., Jayaseelan C., Kirthi A. V., Santhoshkumar T., Marimuthu S., Rajakumar G., Tiwari S. K. and Sahal D. (2015). Evaluation of Antiplasmodial Activity of Medicinal Plants from North Indian Buchpora and South Indian Eastern Ghats. *Malar. J.* vol. 14(1), pp. 1-8.

Kaushik N. K., Guha R. and Thomas B. M. (2017). Antiviral Potential and Mode of Action of *Indigofera heterantha* Against HSV-2 by Targeting the Early Stages of Infection. *Antivir. Ther.* vol. 22(5), pp. 381-391.

Kidane B., Van Andel T., Van Der Maesen L. J. G. and Asfaw Z. (2014). Use and Management of Traditional Medicinal Plants by Maale and Ari Ethnic Communities in Southern Ethiopia. *J. Ethnobiol. Ethnomedicine.* vol. 10(1), pp. 1-15.

Kumar R. S., Moorthy K., Vinodhini R. and Punitha T. (2013). Antimicrobial Efficacy and Phytochemical Analysis of *Indigofera trita* Linn. *Afr. J. Tradit. Complement. Altern. Med*. vol. 10(3), pp. 518-525.

Kumar R. S., Rajkapoor B., Perumal P., Kumar S. V. and Geetha A. S. (2016). Beneficial Effects of Methanolic Extract *of Indigofera linnaei* Ali. On the Inflammatory and Nociceptive Responses in Rodent Models. *Brazilian J. Pharm. Sci.* vol. 52(1), pp. 113-123.

Leite S. P., de Medeiros P. L., da Silva E. C., de Souza Maia M. B., de Menezes Lima V. L. and Saul D. E. (2004). Embryotoxicity *In Vitro* with Extract of *Indigofera suffruticosa* Leaves. *Reprod. Toxicol.* vol. 18(5), pp. 701-705.

Leite S. P., Vieira J. R. C., De Medeiros P. L., Leite R. M. P., De Menezes Lima V. L., Xavier H. S. and de Oliveira Lima E. (2006). Antimicrobial Activity of *Indigofera suffruticosa*. *Evid. Based Complementary Altern. Med.* vol. 3(2), pp. 261-265.

Li Y., Li C., Xu Q. and Kang W. (2011). Antioxidant, α-Glucosidase Inhibitory Activities *In Vitro* and Alloxan-Induced Diabetic Rats' Protective Effect of

Indigofera stachyodes Lindl. Root. *J. Med. Plant Res.* vol. 5(14), pp. 3321-3328.

Liang Q., Qian H. and Yao W. (2005). Identification of Flavonoids and their Glycosides by High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometry and with Diode Array Ultraviolet Detection. *Eur. J. Mass Spectrom.* vol. 11(1), pp. 93-101.

Lima E. F., Riet-Correa F., Gardner D. R., Barros S. S., Medeiros R. M., Soares M. P. and Riet-Correa G. (2012). Poisoning by *Indigofera lespedezioides* in Horses. *Toxicon*, vol. 60(3), pp. 324-328.

Lodha V., Khan H. A. and Ghanim A. (1997). An Acylated Glucopyranoside from *Indigofera oblongifolia*. *J. Indian Chem. Soc.* vol. 74 (5), p. 425.

Lodha V., Khan H. A. and Ghanim A. (1998). 5,7,4'-Trimethoxy-4-phenylcoumarin from Roots of *Indigofera oblongifolia. J. Indian Chem. Soc.* vol. 75(8). p. 485.

Lopes F. C., Calvo T. R., Colombo L. L., Vilegas W. and Carlos I. Z. (2011). Immunostimulatory and Cytotoxic Activities of *Indigofera suffruticosa* (Fabaceae). *Nat. Prod. Res.* vol. 25(19), pp. 1796-1806.

Lubbad M. Y., Al-Quraishy S. and Dkhil M. A. (2015). Antimalarial and Antioxidant Activities of *Indigofera oblongifolia* on *Plasmodium chabaudi*-Induced Spleen Tissue Injury in Mice. *Parasitol. Res.* vol. 114(9), pp. 3431-3438.

Luiz-Ferreira A., Cola M., Barbastefano V., Farias-Silva E., Calvo T. R., de Almeida A. B. A., Pellizzon C. H., Hiruma-Lima C. A., Vilegas W. and Souza-Brito A. R. M. (2011). *Indigofera suffruticosa* Mill. as New Source of Healing Agent: Involvement of Prostaglandin and Mucus and Heat Shock Proteins. *J. Ethnopharmacol.* vol. 137(1), pp. 192-198.

Mabry T., Markham K. R. and Thomas M. B. (2012). *The Systematic Identification of Flavonoids*. Berlin, Heidelberg, New York: Springer Science & Business Media.

Madikizela B., Ndhlala A. R., Finnie J. F. and Staden J. V. (2013). *In Vitro* Antimicrobial Activity of Extracts from Plants Used Traditionally in South

Africa to Treat Tuberculosis and Related Symptoms. *Evid.-Based Complementary Altern. Med*. vol. 2013, pp. 1-8.

Maina V., Kumar R. and Prasad R. (2016). Ethno-Veterinary Plants Used by the Tribal of Dang, Gujarat. *Nelumbo.* vol. 58, pp. 119-125.

Majak W., Benn M., Mcewan D. and Pass M. A. (1992). Three Nitropropanoyl Esters of Glucose from *Indigofera linnaei*. *Phytochemistry*, vol. 31(7), pp. 2393-2395.

Markham K. R. (1982). *Techniques of Flavonoid Identification*. London: Academic Press.

Mathabe M. C., Nikolova R. V., Lall N. and Nyazema N. Z. (2006). Antibacterial Activities of Medicinal Plants Used for the Treatment of Diarrhea in Limpopo Province, South Africa. *J. Ethnopharmacol.* vol. 105(1-2), pp. 286-293.

Medina-Medrano J. R., Mares-Quiñones M. D., Valiente-Banuet J. I., Vázquez-Sánchez M., Álvarez-Bernal D. and Villar-Luna E. (2017). Determination and Quantification of Phenolic Compounds in Methanolic Extracts of *Solanum ferrugineum* (Solanaceae) Fruits by HPLC-DAD and HPLC/ESI-MS/TOF. *J. Liq. Chromatogr. Relat. Technol.* vol. 40(17), pp. 900-906.

Mehmood S., Aziz-Ur-Rahman, Ahmad Z., Afza N., Malik A., Ahmad H. and Choudhary M. I. (2008). Monoterpene Glycosides from *Indigofera hetrantha*. *Nat. Prod. Res.* vol. 22(13), pp. 1189-1195.

Miketova P., Schram K. H., Whitney J., Kearns E. H. and Timmermann B. N. (1999). Mass Spectrometry of 3,5-and 4,5-Dicaffeoylquinic Acids and Selected Derivatives. *J. Mass Spectrom.* vol. 34(12), pp. 1240-1252.

Miketova P., Schram K. H., Whitney J., Li M., Huang R., Kerns E., Valcic S., Timmermann B. N., Rourick R. and Klohr S. (2000). Tandem Mass Spectrometry Studies of Green Tea Catechins. Identification of Three Minor Components in the Polyphenolic Extract of Green Tea. *J. Mass Spectrom.* vol. 35(7), pp. 860-869.

Mishra N. and Pareek A. (2015). Floristic Diversity of Angiosperms with Special Reference to their Medicinal Properties from Kota District of Rajasthan, India. *Int. J. Adv. Res.* vol. 3(12), pp. 994-1007.

Moura A. C. D. S., Vilega W. and Santos L. C. D. (2011). Identificação de Alguns Constituintes Químicos de *Indigofera hirsuta* Linn. (Fabaceae) Por CLAE-IES-EM (TOF) e Avaliação da Atividade Antirradicalar. *Quim. Nova*, vol. 34(7), pp. 1136-1140.

Mukherjee P. K., Sahoo A. K., Narayanan N., Kumar N. S. and Ponnusankar S. (2009). Lead Finding from Medicinal Plants with Hepatoprotective Potentials. *Expert Opin. Drug Discov.* vol. 4(5), pp. 545-576.

Mullen W., Yokota T., Lean M. E. and Crozier A. (2003). Analysis of Ellagitannins and Conjugates of Ellagic acid and Quercetin in Rasberry Fruits by LC–MS^n. *Phytochemistry*, vol. 64(2), pp. 617-624.

Musa A. M., Aliyu A. B., Abdullahi M. I., Yaro A. H., Magaji M. G., Hassan H. S. and Iliya I. (2011). Bioactive Chalcone from *Indigofera pulchra*. *J. Med. Plant Res.* vol. *5*(22), pp. 5444-5449.

Musa A., Haruna A. K., Ilyas M., Ahmadu A., Gibbons S. and Rahman M. M. (2008). Dihydrostilbenes from *Indigofera pulchra*. *Nat. Prod. Commun.* vol. 3(5), pp. 805-808.

Narender T., Khaliq T., Puri A. and Chander R. (2006). Antidyslipidemic Activity of Furano-Flavonoids Isolated from *Indigofera tinctoria*. *Bioorganic Med. Chem. Lett.* vol. 16(13), pp. 3411-3414.

Narváez-Cuenca C. E., Vincken J. P. and Gruppen H. (2012). Identification and Quantification of (dihydro) Hydroxycinnamic Acids and their Conjugates in Potato by UHPLC–DAD–ESI-MS^n. *Food Chem.* vol. 130(3), pp. 730-738.

Navarro-González I., González-Barrio R., García-Valverde V., Bautista-Ortín A. B. and Periago M. J. (2015). Nutritional Composition and Antioxidant Capacity in Edible Flowers: Characterization of Phenolic Compounds by HPLC-DAD-ESI/MS^n. *Int. J. Mol. Sci.* vol. 16(1), pp. 805-822.

Netala V. R., Bukke S., Domdi L., Soneya S., Reddy S. G., Bethu M. S., Kotakdi V. S., Saritha K. V. and Tartte V. (2018). Biogenesis of Silver Nanoparticles Using Leaf Extract of *Indigofera hirsuta* and their Potential Biomedical Applications (3-in-1 System). *Artif. Cells Nanomed. Biotechnol.* vol. 46(1), pp. 1138-1148.

Neto J. D. B., Oliveira C. M. C., Peixoto P. V., Barbosa I. B. P., Ávila S. C. and Tokarnia C. H. (2001). Anemia Hemolítica Causada Por *Indigofera suffruticosa* (Leg. Papilionoideae) Em Bovinos. *Pesqui. Vet. Bras.* vol. 21(1), pp.18-22.

Netzel G., Tan E. T., Yin M., Giles C., Yong K. W., Al Jassim R. and Fletcher M. T. (2019). Bioaccumulation and Distribution of Indospicine and its Foregut Metabolites in Camels Fed *Indigofera spicata*. *Toxins*, vol. 11(3), p. 169.

Nyarko A. K., Sittie A. A. and Addy M. E. (1993). The Basis for the Antihyperglycemic Activity of *Indigofera arrecta* in the Rat. *Phytother. Res.* vol. 7(1), pp. 1-4.

Ossedryver S. M., Baldwin G. I., Stone B. M., McKenzie R. A., Van Eps A. W., Murray S. and Fletcher M. T. (2013). *Indigofera spicata* (Creeping Indigo) Poisoning of Three Ponies. *Aust. Vet. J.* vol. 91(4), pp. 143-149.

Otify A. M., El-Sayed A. M., Michel C. G. and Farag M. A. (2019). Metabolites Profiling of Date Palm (*Phoenix dactylifera* L.) Commercial By-Products (Pits and Pollen) in Relation to its Antioxidant Effect: A Multiplex Approach of MS and NMR Metabolomics. *Metabolomics*, vol. 15(9), pp. 1-17.

Partridge S. M. (1949). Aniline Hydrogen Phthalate as a Spraying Reagent for Chromatography of Sugars. *Nature*, vol. 164(4167), p. 443.

Pasupula R. and Pragada R. R. (2018). Phytochemical and Toxicological Investigation on Aqueous Extract of Polyherbal Preparation. *Int. J. Pharm. Sci. Res.* vol. 9(7), pp. 3083-3093.

Pavia D. L., Lampman G. M., Kriz G. S. and Vyvyan J. A. (2014). Introduction to Spectroscopy. Cengage Learning.

Pinto D. C. and Silva A. (2017). Anticancer Natural Coumarins as Lead Compound for the Discovery of New Drugs. *Curr. Top. Med. Chem*. vol. 17(29), pp. 3190-3198.

Pollitt S., Hegarty M. P. and Pass M. A. (1999). Analysis of the Amino acid Indospicine in Biological Samples by High Performance Liquid Chromatography. *Nat. Toxins,* vol. 7(6), pp. 233-240.

Ponmari G., Annamalai A., Gopalakrishnan V. K., Lakshmi P. T. V. and Guruvayoorappan C. (2014). NF-κB Activation and Proinflammatory

Cytokines Mediated Protective Effect of *Indigofera caerulea* Roxb. On CCl4 Induced Liver Damage in Rats. *Int. Immunopharmacol.* vol. 23(2), pp. 672-680.

Prakash D., Suri S., Upadhyay G. and Singh B. N. (2007). Total Phenol, Antioxidant and Free Radical Scavenging Activities of Some Medicinal Plants. *Int. J. Food Sci. Nutr.* vol. 58(1), pp. 18-28.

Prasad Y. R. and Chakradhar V. (2004). A New Isoflavone from *Indigofera linnaei*. *Indian J. Chem.* vol. 43(8), pp. 1807-1808.

Prashanth G. K., Prashanth P. A., Nagabhushana B. M., Ananda S., Krishnaiah G. M., Nagendra H. G., Sathyananda H. M., Singh C. R., Yogisha S., Anand S. and Tejabhiram Y. (2018). Comparison of Anticancer Activity of Biocompatible ZnO Nanoparticles Prepared by Solution Combustion Synthesis Using Aqueous Leaf Extracts of *Abutilon indicum*, *Melia azedarach* and *Indigofera tinctoria* as Biofuels. *Artif. Cells Nanomed. Biotechnol.* vol. 46(5), pp. 968-979.

Qiu L., Liang Y., Tang G. H., Yuan C. M., Zhang Y., Hao X. Y., Hao X. J. and He H. P. (2013). Two New Flavonols, Including One Flavan Dimer, from the Roots of *Indigofera stachyodes*. *Phytochem. Lett.* vol. 6(3), pp. 368-371.

Rahman I. U., Ijaz F., Iqbal Z., Afzal A., Ali N., Afzal M., Khan M. A., Muhammad S., Qadir G. and Asif M. (2016). A Novel Survey of the Ethnomedicinal Knowledge of Dental Problems in Manoor Valley (Northern Himalaya), Pakistan. *J. Ethnopharmacol.* vol. 194, pp. 877-894.

Rahman T. U., Arfan M., Liaqat W., Uddin G. and Choudhary M. I. (2014a). Isolation of a Novel Indigoferamide-A from Seeds of *Indigofera heterantha* Wall and its Antibacterial Activity. *Rec. Nat. Prod.* vol. 8(4), pp. 412-416.

Rahman T. U., Uddin G., Khattak K. F., Liaqat W., Mohammad G., Choudhary M. I., Wadood A. and Ahmad A. (2014b). Isolation and Characterization of a Novel Ester from Seeds of *Indigofera heterantha* (Wall). *J. Nat. Prod.* vol. 7, pp. 104-112.

Rahman T. U., Uddin G., Nisa R. U., Ludwig R., Liaqat W., Mahmood T., Mohammad G., Chouhadry M. I. and Ayub K. (2015). Spectroscopic and Density Functional Theory Studies of 7-Hydroxy-3′-methoxyisoflavone: A New Isoflavone from

the Seeds of *Indigofera heterantha* (Wall). *Spectrochim. Acta - A: Mol. Biomol. Spectrosc.* vol. 148, pp. 375-381.

Rahman T. U., Zeb M. A., Pu D. B., Liaqat W., Ayub K., Xiao W. L., Mahmood T., Sajid M. and Hussain R. (2019). Density Functional Theory, Molecular Docking and Bioassay Studies on (*S*)-2-Hydroxy-*N*-(2*S*,3*S*,4*R*,*E*)-1,3,4-trihydroxyicos-16-en-2-yl) tricosanamide. *Heliyon*, vol. 5(8), e02038.

Rajagopal B., Narasimman M. and Saravana B. P. (2016). Screening of Selected Medicinal Plants from Tamil Nadu, South India for Antibacterial Activity Against Selected Fish Pathogenic Microbe. *Res. J. Biotechnol.* vol. 11(10), pp. 46-54.

Rajkapoor B., Jayakar B. and Murugesh N. (2004). Antitumor Activity of *Indigofera aspalathoides* on Ehrlich Ascites Carcinoma in Mice. *Indian J. Pharmacol.* vol. 36(1), pp. 38-40.

Rajkapoor B., Kavimani S., Ravichandiran V., Sekhar K., Kumar R. S., Kumar M. R., Paradeepkumar M. Einstein J. W. and Kumar E. P. (2009). Effect of *Indigofera aspalathoides* on Complete Freund's Adjuvant-Induced Arthritis in Rats. *Pharm. Biol.* vol. 47(6), pp. 553-557.

Rajkapoor B., Murugesh N., Chodon D. and Sakthisekaran D. (2005). Chemoprevention of *N*-Nitroso-di-ethylamine Induced Phenobarbitol Promoted Liver Tumors in Rat by Extract of *Indigofera aspalathoides*. *Biol. Pharm. Bull.* vol. 28(2), pp. 364-366.

Ranaweera C. B., Abeysekara W. P., Pathirana R. and Ratnasooriya W. D. (2015). Lack of *In Vitro* Anti-hyaluronidase Activity of Methanolic Leaf Extract of *Indigofera tinctoria* L and Methanolic Stem Bark Extract of *Stereospermum suaveolens* DC. *J. Pharm. Negat. Results*, vol. 6(1), pp. 40-43.

Reddy G. S., Saritha K. V., Reddy Y. M. and Reddy N. V. (2019). Eco-friendly Synthesis and Evaluation of Biological Activity of Silver Nanoparticles from Leaf Extract of *Indigofera barberi* Gamble: An Endemic Plant of Seshachalam Biosphere Reserve. *SN Appl. Sci.* vol. 1(9), p. 968.

Rehman A., Malik A., Mehmood S., Jahan E. and Ahmad H. (2005b). Phytochemical Studies on *Indigofera hetrantha*. *J. Chem. Soc. Pak.* vol. 27(4), pp. 440-442.

Rehman A.U., Malik A., Riaz N., Ahmad H., Nawaz S. A. and Choudhary M. I. (2004). Lipoxygenase Inhibiting Flavonoids from *Indigofera hetrantha. Chem. Pharm. Bull.* vol. 63(2), pp. 359-366.

Rehman A.U., Malik A., Riaz N., Ahmad H., Nawaz S. A. and Choudhary M. I. (2005a). Lipoxygenase Inhibiting Constituents from *Indigofera hetrantha*. *Chem. Pharm. Bull.* vol. 53(3), pp. 263-266.

Rehman T. U., Uddin G., Khattak K. F. and Liaqat W. (2016). Isolation of Flavonoids from *Indigofera heterantha* Seeds. *J. Chem. Pharm. Res.* vol. 8(1), pp. 389-393.

Renukadevi K. P. and Sultana S. S. (2011). Determination of Antibacterial, Antioxidant and Cytotoxicity Effect of *Indigofera tinctoria* on Lung Cancer Cell line NCI-H69. *Int. J. Pharmacol.* vol. 7(3), pp. 356-362.

Ribeiro L. R., Bautista A. R. P. L., Silva R. A., Sales. L. A., Salvadori D. M. F. and Maia P. C. (1991). Toxicological and Toxicogenetic Effects of Plants Used in Popular Medicine and in Cattle food. *Mem. Inst. Oswaldo Cruz*, vol. 86 (sup 2), pp. 89-91.

Riet-Correa F., Medeiros R. M., Pfister J. A. and Mendonça F. S. (2017). Toxic Plants Affecting the Nervous System of Ruminants and Horses in Brazil. *Pesqui Vet. Bras.* vol. 37(12), pp. 1357-1368.

Said A. A. H., Abuotabl E. A. and Raoof G. F. A. (2017). Identification of Constituents from *Pleigynium timorense* (Dc.) Leenh Pericarp and Seeds Using High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometry. *AASCIT J. Chem.* vol. 3(4), pp. 30-36.

Saldanha L. L., Vilegas W. and Dokkedal A. L. (2013). Characterization of Flavonoids and Phenolic Acids in *Myrcia bella* Cambess. Using FIA-ESI-IT-MS^n and HPLC-PAD-ESI-IT-MS Combined with NMR. *Molecules*, vol. 18(7), pp. 8402-8416.

Salvador I. S., Medeiros R. M. T., Pessoa C. R. M., Oliveira D. M., Duarte A. L. A., Fighera R. A. and Riet-Correa F. (2011). Experimental Poisoning of Guinea Pig

(*Cavia porcellus*) with *Indigofera suffruticosa*. *Toxicon,* vol. 57(6), pp. 927-931.

Sánchez-Rabaneda F., Jáuregui O., Casals I., Andr. s-Lacueva C., Izquierdo-Pulido M. and Lamuela-Raventós R. M. (2003). Liquid Chromatographic/Electrospray Ionization Tandem Mass Spectrometric Study of the Phenolic Composition of Cocoa (*Theobroma cacao*). *J. Mass Spectrom.* vol. 38(1), pp. 35-42.

Sandhyavali M. S., Sivakamisundari P., Sharma P. and Murugan V. (2014). Phytochemical Studies on the Aerial parts of *Indigofera linnaei,* Ali. *Pharmacophore*, vol. 5(1), pp. 94-97.

Saraswathy A., Mathuram V. and Allirani T. (2013). Chemical Constituents of *Indigofera aspalathoides* Vahl. Ex. Dc. *J. pharmacogn. phytochem.* vol 2(2), pp. 74-80.

Schrire B. D., Lavin M., Barker N. P., Cortes-Burns H., Von Senger I. and Kim J. H. (2003). Towards a Phylogeny of *Indigofera* (Leguminosae-Papilionoideae): Identification of Major Clades and Relative Ages. *Adv. Legume Syst.* vol. 10, pp. 269-302.

Schütz K., Kammerer D. R., Carle R. and Schieber A. (2005). Characterization of Phenolic Acids and Flavonoids in Dandelion (*Taraxacum officinale* WEB. ex WIGG.) Root and Herb by High-performance liquid Chromatography/Electrospray Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* vol. 19(2), pp. 179-186.

Selvam C., Jachak S. M., Oli R. G., Thilagavathi R., Chakraborti A. K. and Bhutani K. K. (2004). A New Cyclooxygenase (COX) Inhibitory Pterocarpan from *Indigofera aspalathoides*: Structure Elucidation and Determination of Binding Orientations in the Active Sites of the Enzyme by Molecular Docking. *Tetrahedron Lett.* vol. 45(22), pp. 4311-4314.

Sen A. K., Banerjee N. and Farooqi M. I. H. (1986). A Water-Soluble Galactomannan from the Seeds of *Indigofera tinctoria* Linn. *Carbohydr. Res.* vol. 157, pp. 251-256.

Shahjahan M., Vani G. and Devi C. S. S. (2005). Protective Effect of *Indigofera oblongifolia* in CCl_4-Induced Hepatotoxicity. *J. Med. Food*. vol. 8(2), pp. 261-265.

Sharif A., Ahmed E., Malik A., Riaz N., Afza N., Nawaz S. A., Arshad M., Shah M. R. and Choudhary M. I. (2005). Lipoxygenase Inhibitory Constituents from *Indigofera oblongifolia. Arch. Pharm. Res.* vol. 28(7), pp. 761-764.

Sharma V. and Singh R. (2017). Isolation and Structural Elucidation of an Isothiocyanate compound from *Indigofera tinctoria* Linn. Extract. *Curr. Sci.* vol. 113(5), pp. 941-946.

Silva C. B. D., Moraes M. M. D., Da Camara C. A., Ribeiro N. D. C., De Melo J. P., De Lima V. L. and Navarro D. M. (2019). Chemical Composition and Acaricidal Activities of *Indigofera suffruticosa* Essential Oil Against Two-Spotted Spider Mite. *Quim. Nova,* vol. 42(3), pp. 313-318.

Silva I. B. D., Lima I. R., Santana M. A. N., Leite R. M. P. and Leite S. P. (2014). *Indigofera suffruticosa* Mill (Fabaceae): Hepatic Responses in Mice Bearing Sarcoma 180. *Int. J. Morphol.* vol. 32(4). pp. 1228-1233.

Silván A. M., Abad M. J., Bermejo P., Sollhuber M. and Villar A. (1996). Antiinflammatory Activity of Coumarins from *Santolina oblongifolia*. *J. Nat. Prod.* vol. 59(12), pp. 1183-1185.

Singh B., Chandan B. K., Sharma N., Bhardwaj V., Satti N. K., Gupta V. N., Gupta B. D., and Suri K. A. and Suri O. P. (2006). Isolation, Structure Elucidation and *In Vivo* Hepatoprotective Potential of *trans*-Tetracos-15-enoic acid from *Indigofera tinctoria* Linn: *Phytother. Res.* vol. 20(10), pp. 831-839.

Singh B., Saxena A. K., Chandan B. K., Bhardwaj V., Gupta V. N., Suri O. P. and Handa S. S. (2001). Hepatoprotective Activity of Indigtone, A Bioactive Fraction from *Indigofera tinctoria* Linn. *Phytother. Res.* vol. 15(4), pp. 294-297.

Singh R., Sharma S. and Sharma V. (2015). Comparative and Quantitative Analysis of Antioxidant and Scavenging Potential of *Indigofera tinctoria* Linn. Extracts. *J. Integr. Med.* vol. 13(4), pp. 269-278.

Sittie A. A. and Nyarko A. K. (1998). *Indigofera arrecta*: Safety Evaluation of an Antidiabetic Plant Extract in Non-diabetic Human Volunteers. *Phytother. Res.* vol. 12(1), pp. 52-54.

Splitstoser J. C., Dillehay T. D., Wouters J. and Claro A. (2016). Early Pre-hispanic Use of Indigo Blue in Peru. *Sci. Adv.* vol. 2(9), e1501623.

Srinivasan S., Wankhar W., Rathinasamy S. and Rajan R. (2016). Free Radical Scavenging Potential and HPTLC Analysis of *Indigofera tinctoria* Linn. (Fabaceae). *J. Pharm. Anal.* vol. 6(2), pp. 125-131.

Su Y., Li C., Gao Y., Di L., Zhang X. and Guo D. (2005). Acryloylated Glucose 3-Nitropropanoates from *Indigofera kirilowii*. *J. Nat. Prod.* vol. 68(12), pp. 1785-1786.

Su Y., Lü M., Yang F., Li C., Di L., Wu D., Guo Z., Lü J. and Guo D. (2008). Six New Glucose Esters of 3-Nitropropanoic Acid from *Indigofera kirilowii. Fitoterapia*, vol. 79(6), pp. 451-455.

Sultan S., Giles C., Netzel G., Osborne S. A., Netzel M. E. and Fletcher M. T. (2018a). Release of Indospicine from Contaminated Camel Meat Following Cooking and Simulated Gastrointestinal Digestion: Implications for Human Consumption. *Toxins,* vol. 10(9), p. 356.

Sultan S., Osborne S. A., Addepalli R., Netzel G., Netzel M. E. and Fletcher M. T. (2018b). Indospicine Cytotoxicity and Transport in Human Cell Lines. *Food Chem.* vol. 267, pp. 119-123.

Swarnalatha S., Umamaheswari A. and Puratchikody A. (2015). Immunomodulatory Activity of Kaempferol 5-*O*-*β*-D-glucopyranoside from *Indigofera aspalathoides* Vahl Ex Dc. (Papilionaceae). *Med. Chem. Res.* vol. 24(7), pp. 2889-2897.

Tan E. T., Al Jassim R., D'Arcy B. R. and Fletcher M. T. (2017). *In Vitro* Biodegradation of Hepatotoxic Indospicine in *Indigofera spicata* and its Degradation Derivatives by Camel Foregut and Cattle Rumen Fluids. *J. Agric. Food Chem.* vol. 65(34), pp. 7528-7534.

Tariq S. A., Ahmad M. N., Obaidullah, Khan A., Choudhary M. I., Ahmad W. and Ahmad M. (2011). Urease Inhibitors from *Indigofera gerardiana* Wall. *J. Enzyme Inhib. Med. Chem.* vol. 26(4), pp. 480-484.

Tchoumtchoua J., Halabalaki M., Gikas E., Tsarbopoulos A., Fotaki N., Liu L., Nam S., Jove R. and Skaltsounis L. A. (2019). Preliminary Pharmacokinetic Study of the Anticancer 6BIO in Mice Using an UHPLC-MS/MS Approach. *J. Pharm. Biomed. Anal.* vol. 164, pp. 317-325.

Teklehaymanot T. (2017). An Ethnobotanical Survey of Medicinal and Edible Plants of Yalo Woreda in Afar Regional State, Ethiopia. *J. Ethnobiol. Ethnomedicine.* vol. 13(1), p. 40.

Thangadurai D., Ramesh N., Viswanathan M. B. and Prasad D. X. (2001a). A Novel Xanthene from *Indigofera longeracemosa* Stem. *Fitoterapia*, 72(1), pp. 92-94.

Thangadurai D., Viswanathan M. B. and Ramesh N. (2001b). Characterization of a New Deca-hydro-pyrido-quinoline from *Indigofera longeracemosa* Boiv. Ex Baill. (Fabaceae). *Nat. Prod. Lett.* vol. 15(4), pp. 287-290.

Thangadurai D., Viswanathan M. B. and Ramesh N. (2002). Indigoferabietone, A Novel Abietane Diterpenoid from *Indigofera longeracemosa* with Potential Anti-tuberculous and Antibacterial Activity. *Die Pharmazie*, vol. 57(10), 714-715.

Theresa V., Ernest Ravindran R. S., Kumar R. A., Pandian K. and Renganathan S. (2017). Novel Approach to Produce Oil from Non-edible Seeds of *Indigofera colutea*. *Energy Sources A: Recovery Util. Environ. Eff.* vol. 39(13), pp. 1369-1376.

Tošović J. (2017). Spectroscopic Features of Caffeic acid: Theoretical Study. *Kragujev. J. Sci.* vol. 39, pp. 99-108.

Troalen L. G., Phillips A. S., Peggie D. A., Barran P. E. and Hulme A. N. (2014). Historical Textile Dyeing with *Genista tinctoria* L.: A Comprehensive Study by UPLC-MS/MS analysis. *Anal. Methods*, vol. 6(22), pp. 8915-8923.

Truchado P., Ferreres F. and Tomas-Barberan F. A. (2009). Liquid Chromatography–Tandem Mass Spectrometry Reveals the Widespread Occurrence of Flavonoid

Glycosides in Honey, and their Potential as Floral Origin Markers. *J. Chromatogr. A.* vol. 1216(43), pp. 7241-7248.

Uddin G., Rehman T. U., Arfan M., Liaqat W., Rauf A., Khan I., Mohammad G. and Choudhary M. I. (2011). *In Vitro* Pharmacological Investigations of Aerial Parts of *Indigofera heterantha*. *J. Med. Plant Res.* 5(24), pp. 5750-5753.

Upman S. and Sarin R. (2011). Production of Flavonoids from *Indigofera cordifolia* and *I. linnaei In Vivo* and *In Vitro* Tissue Cultures. *Int. J. Nat. Prod. Res.* vol. 1(1), pp. 4-8.

Uvarani M., Pandian R. A., Jenila B., Sutharsingh R. and Thangathirupathi A. (2012). Anti-inflammatory and Antibacterial Studies on *Indigofera linifolia* Retz. *Int. J. Pharm. Sci. Res.* vol. 3(8), pp. 2754-2756.

Vadivel V., Stuetz W., Scherbaum V. and Biesalski H. K. (2011). Total Free Phenolic Content and Health Relevant Functionality of Indian Wild Legume Grains: Effect of Indigenous Processing Methods. *J. Food Compos. Anal.* vol. 24(7), pp. 935-943.

Vieira J. R. C., De Souza I. A., Do Nascimento S. C. and Leite S. P. (2007). *Indigofera suffruticosa*: An Alternative Anticancer Therapy. *Evid.-based Complement. Altern. Med.* vol. 4(3), pp. 355-359.

Vieira J. R. C., Leite R. M. P., Lima I. R., Navarro D. D. A. F., Bianco E. M. and Leite S. P. (2012). Oviposition and Embryotoxicity of *Indigofera suffruticosa* on Early Development of *Aedes Aegypti* (Diptera: Culicidae). *Evid.-based Complement. Altern. Med.* vol. 2012, pp. 1-5.

Vijayan R., Joseph S. and Mathew B. (2018). *Indigofera tinctoria* Leaf Extract Mediated Green Synthesis of Silver and Gold Nanoparticles and Assessment of their Anticancer, Antimicrobial, Antioxidant and Catalytic Properties. *Artif. Cells Nanomed. Biotechnol.* vol. 46(4), pp. 861-871.

Vimalanathan S., Ignacimuthu S. and Hudson J. B. (2009). Medicinal Plants of Tamil Nadu (Southern India) are a Rich Source of Antiviral Activities. *Pharm. Biol.* vol. 47(5), pp. 422-429.

Vinoth S., Rajesh Kanna P., Gurusaravanan P. and Jayabalan N. (2011). Evaluation of Phytochemical, Antimicrobial and GC-MS Analysis of Extracts of *Indigofera trita* LF SPP. *subulata* (Vahl ex Poir). *Int. J. Agric. Res.* vol. 6(4), pp. 358-367.

Waako P. J., Katuura E., Smith P. and Folb P. (2007). East African Medicinal Plants as a Source of Lead Compounds for the Development of New Antimalarial Drugs. *Afr. J. Ecol.* vol. 45 (sup. 1), pp. 102-106.

Wagh V. V. and Jain A. K. (2018). Status of Ethnobotanical Invasive Plants in Western Madhya Pradesh, India. *S. Afr. J. Bot.* vol. 114, pp. 171-180.

Widelski J., Popova M., Graikou K., Glowniak K. and Chinou I. (2009). Coumarins from *Angelica lucida* L.-Antibacterial Activities. *Molecules*, vol. 14(8), pp. 2729-2734.

Williams M. C. (1981). Nitro Compounds in *Indigofera* Species. *Agron. J.* vol. 73(3), pp. 434-436.

Ye M., Yang W. Z., Liu K. D., Qiao X., Li B. J., Cheng J., Feng J., Guo D. A. and Zhao Y. Y. (2012). Characterization of Flavonoids in *Millettia nitida* Var. *hirsutissima* by HPLC/DAD/ESI-MS^n. *J. Pharm. Anal.* vol. 2(1), pp. 35-42.

Yuldasheva N. K., Ul'chenko N. T., Glushenkova A. I. and Ergashev A. (2016). Lipids from Seeds of *Indigofera tinctoria*. *Chem. Nat. Compd.* vol. 52(1), pp. 32-34.

Zeb M., Khan S., Rahman T., Sajid M. and Seloni S. (2017). Isolation and Biological Activity of β-Sitosterol and Stigmasterol from the Roots of *Indigofera heterantha. Pharm. Pharmacol. Int. J.* vol. 5(5), p. 00139.

Zhang L., Wang L., Cunningham A. B., Shi Y. and Wang Y. (2019). Island Blues: Indigenous Knowledge of Indigo-Yielding Plant Species Used by Hainan Miao and Li Dyers on Hainan Island, China. *J. Ethnobiol. Ethnomedicine*, vol. 15(1), p. 31.

Zhang X. X., Zhang Z. X., Chen L. and Su Y. F. (2006). New Aliphatic Nitro-Compounds from *Indigofera carlesii*. *Fitoterapia*, vol. 77(1), pp. 15-18.

Zhang Y. F., Zhu Z. X., Sun H., Yao H. N., Chen X. N., Liu L., Zhang S. L., Zhao Y. F., Tu P. F. and Li J. (2018). Stachyodin A, a Pterocarpan Derivative with

Unusual Spiro-tetrahydrofuran Ring from the Roots of *Indigofera stachyodes*. *Tetrahedron Lett.* vol. 59(51), pp. 4514-4516.

Zhao Y., Han P., Liu L., Wang X., Xu P., Wang H., Yu T., Sun Y., Li L., Sun T., Liu X., Zhou H., Qiu J., Wang L., Peng J., Xu S. and Ming H. (2019). Indirubin Modulates CD4+ T-cell Homeostasis Via PD1/PTEN/AKT Signaling Pathway in Immune Thrombocytopenia. *J. Cell. Mol. Med.* vol. 23(3), pp. 1885-1898.

Zhou W., Lei Z., Lai D., Li G. Q., Liang Y., Zhang X., Cho J. and Hao X. (2020). Natural Glycosides from *Indigofera stachyoides* Radix. *Rec. Nat. Prod.* vol. 14(1), pp. 83-88.