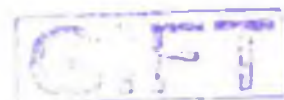


In the name of ALLAH, the Al-mighty

**CYTOTOXIC AND ANTIMICROBIAL
CONSTITUENTS FROM SOME BANGLADESHI
BRIDELIA AND *ERYTHRINA* SPECIES**



**THESIS PRESENTED FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY**

BY

ADEEBA ANJUM

Reg. no. 160, Session 2010-2011

MARC

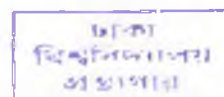
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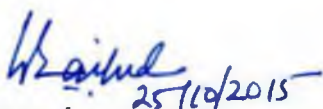


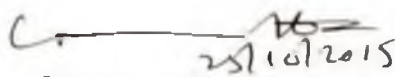
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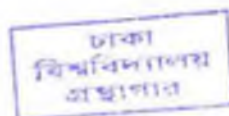
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MY PARENTS

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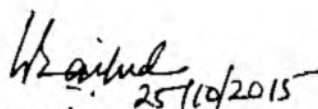
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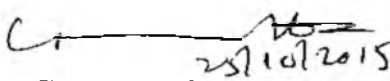
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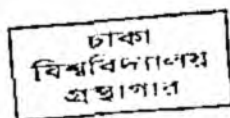
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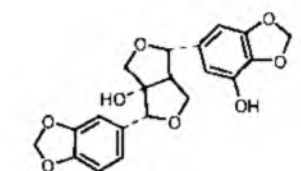
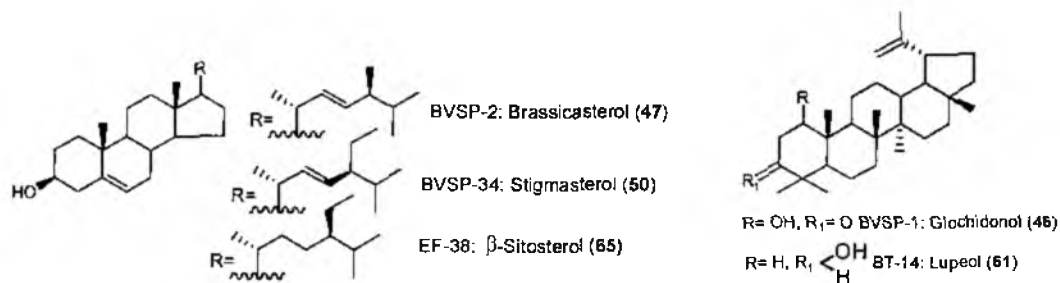


ABSTRACT

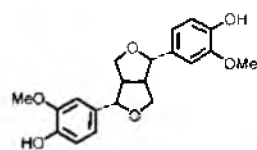
This thesis describes the isolation and structure elucidation of secondary metabolites from four plants *Bridelia verrucosa*, *B. stipularis*, *B. tomentosa* (Family: Phyllanthaceae) and *Erythrina fusca* (Family: Fabaceae) as well as biological studies of the extractives from these plants along with *E. variegata* (Family: Fabaceae). A total of nineteen compounds were isolated of which three appear to be new. The structures of the isolated compounds were elucidated mainly by spectroscopic studies including high field NMR.

The stem bark of *B. verrucosa* afforded eight compounds viz. glochidonol (BVSP-1, **46**), brassicasterol (BVSP-2, **47**), friedelin (BVSP-4, **48**); 5 β -24S-ethylcholestan-3 β -ol (BVSP-6, **49**); stigmasterol (BVSP-34, **50**); 5,5'-dihydroxysemine (BVBC-2, **51**), 3-ketoleanane (**53**) and pinoselinol (BVS-65, **54**). Except stigmasterol (**50**) and pinoselinol (**54**) this is the first report of the isolation of compounds **46-49**, **53** and **54** from the genus *Bridelia* and **51** has been tentatively identified as a new natural product. Phytochemical investigations of methanolic extract of the stem bark of *B. stipularis* provided two triterpenes namely, glut-5(6)-en-3-one (BS-01, **55**), glut-5(6)-en-3 α -ol (BS-06, **56**) and a tentatively identified cholestane type compound (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (BS-11, **57**). All these are the first report of their occurrence from *B. stipularis* while (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (**57**) has been proposed as a tentatively new compound. On the other hand, phytochemical investigation of methanol extract of the stem bark of *B. tomentosa* yielded friedoolean-5(6),14(15)-dien-3-one (BT-1, **58**), β -taraxerol (BT-6, **59**), D₄-stigmasterone (BT-8, **60**) and lupeol (BT-14, **61**). This is the first report of their occurrence from this plant where as friedoolean-5(6),14(15)-dien-3-one (**58**) has been tentatively identified as a new compound. The carbon tetrachloride soluble fractions of a methanol extract of the stem bark of *E. fusca* provided three flavonoids namely, shinpterocarpin (EF-7, **62**), lupinifolin (EF-8, **63**) and 3,9-dihydroxy-4-(3,3-dimethylallyl)[6aR,11aR]-pterocarpin (**64**) and a steroid, β -sitosterol (EF-38, **65**). All these are the first report except **65** from *E. fusca*.

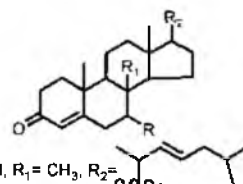
The crude extracts and organic soluble material of the investigated plants and some purified compounds were screened for their antimicrobial activity against a wide range of Gram-positive and Gram-negative microorganisms and fungi by the standardized disc diffusion method. Kanamycin and Griseofulvin were used as reference drugs for the test.



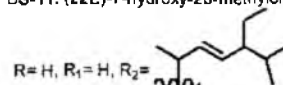
BVBC-2: 5,5'-dihydroxyxysamine (51)



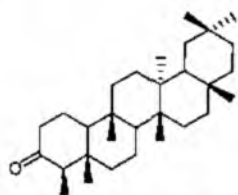
Pinocresinol, BVS-65 (54)



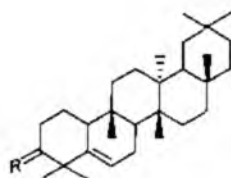
BS-11: (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (57)



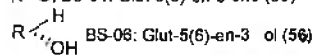
BT-8: D₂-Stigmasterone (60)



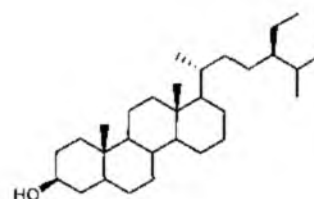
BVSP-4: Friedelin (48)



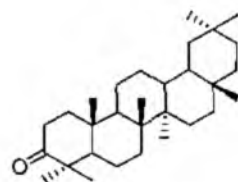
$R = O$, BS-01: Glut-5(6)-en-3-one (55)



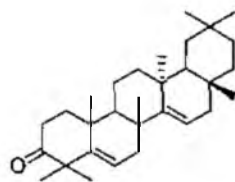
$R = \begin{matrix} H \\ \diagup \end{matrix}$, BS-06: Glut-5(6)-en-3-ol (56)



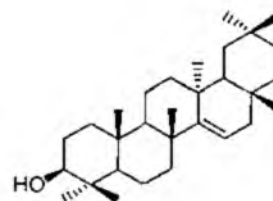
BVSP-6: 5 β -24S-ethylcholestan-3 α -ol (49)



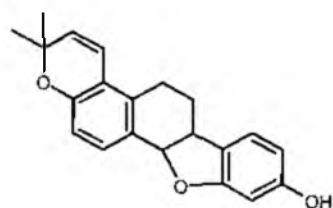
BVSC-8: 3-Ketooleanane (53)



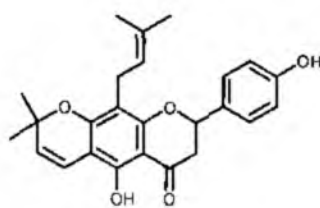
BT-1: Friedoolean-5(6),14(15)-dien-3-one (58)



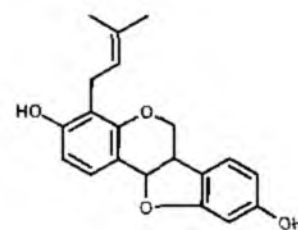
BT-6: β -Taraxerol (59)



EF-7: Shimperocarpin (62)



EF-8: Lupinifolin (63)



EF-12: 3,9-dihydroxy-4-(3,3-dimethylallyl)-[6aR,11aR]-pterocarpan (64)

All the samples showed mild to moderate antimicrobial activity against thirteen Gram positive and Gram negative bacterial strains and three fungi, where the stem bark extract of *B. tomentosa* demonstrated the highest inhibition of growth against *B. cereus* (23.2 mm) and *C. albicans* (17.5 mm). The purified compounds isolated from the investigated plants showed mild to moderate antibacterial activity. BS-01 (**55**) and BS-06 (**56**) revealed strong inhibition of growth of *E. coli* (22.7 mm) and *C. albicans* (20.8 mm), respectively.

The plants were subjected to brine shrimp lethality bioassay for probable cytotoxicity. The aqueous soluble fraction of the leaf of *B. stipularis* revealed the highest cytotoxic activity with LC₅₀ value of 12.59 µg/ml. On the other hand, the *n*-hexane soluble fraction of the stem bark of *E. variegata* revealed the strongest LC₅₀ value of 14.66 µg/ml. The isolated pure compound EF-8 (**62**) displayed the strongest cytotoxic activity with LC₅₀ value of 3.17 µg/ml, as compared to standard vincristine sulfate (LC₅₀ 0.45 µg/ml).

Free radical scavenging activity, total antioxidant capacity and total phenolic content of the extractives were also investigated. Free radical scavenging assay of the methanol extract of the leaf of *B. verrucosa* revealed highest activity having IC₅₀ of 6.35 µg/ml while the chloroform soluble fraction of the leaf of *E. fusca* showed IC₅₀ at 5.50 µg/ml. The IC₅₀ (7.3 µg/ml) of the isolated pure compound BVBC-2 (**51**) indicated strong free radical scavenging activity.

The aqueous and chloroform soluble materials of the leaf of *B. stipularis* demonstrated promising total phenolic content (98.76 and 94.52 mg of GAE/100 g of dried extract). On the other hand, the highest total antioxidant capacity of methanolic extract of the leaf of *B. tomentosa* and *E. fusca* were found as 612.54 and 587.66 mg of ascorbic acid/g of dried extract, respectively.

All the extractives of five plants were also studied for their thrombolytic potential. The carbon tetrachloride soluble fraction and methanol extract of leaf and aqueous soluble material of bark of *B. tomentosa*, methanol extract of bark of *B. stipularis* and carbon tetrachloride soluble partitionate of leaf of *B. verrucosa* exhibited highest thrombolytic activity with clot lysis value of 41.46%, 34.85%, 37.04%, 36.45% and 33.72%, respectively. Standard streptokinase was used as positive control which exhibited 61.50% lysis of clot while the negative control water revealed 2.56% clot lysis.

In conclusion, a total 19 compounds including three new molecules (**51**, **57** and **58**) have been isolated from *B. verrucosa*, *B. stipularis* and *B. tomentosa*, respectively. Also, the methanolic extract of the investigated plants and their Kupchan fractions showed significant antibacterial, cytotoxic, antioxidant and thrombolytic activities. These plants can be further studied in order to find out their unexplored efficacy against other diseases as well as to isolate more chemically interesting and biologically important drug candidates.

CONTENTS

Chapter	Topics	Page No.
	Acknowledgements	I
	Abstract	II
1	Introduction	
1.1	Rationale of the work	1
1.2	Objective of the work	6
1.3	The plant family Phyllanthaceae	7
1.4	Taxonomy of the plant family Phyllanthaceae	8
1.5	Botanical features of plant family Phyllanthaceae	9
1.6	Medicinal uses of Phyllanthaceae species in Bangladesh	11
1.7	The plant genus <i>Bridelia</i> Willd.	13
1.8	Description of the investigated plants of the genus <i>Bridelia</i>	19
1.8.1	Taxonomic hierarchy of <i>Bridelia verrucosa</i> Haines	19
1.8.2	Description of the investigated plant <i>B. verrucosa</i>	19
1.8.3	Taxonomic hierarchy of <i>Bridelia stipularis</i> (L) Blume	21
1.8.4	Description of the investigated plant <i>B. stipularis</i>	22
1.8.5	Taxonomic hierarchy of <i>Bridelia tomentosa</i>	24
1.8.6	Description of the investigated plant <i>B. stipularis</i>	24
1.9	The plant family Fabaceae or Leguminosae	26
1.10	Taxonomy of the plant family Fabaceae	28
1.11	Botanical features of Fabaceae	30
1.12	Medicinal uses of some Fabaceous species growing in Bangladesh	37
1.13	The plant genus <i>Erythrina</i> L.	38
1.13.1	Taxonomic hierarchy of the genus <i>Erythrina</i> L.	39
1.13.2	Some available species of <i>Erythrina</i> L.	40
1.13.3	Medicinal uses	40
1.13.4	Phytochemical data analysis of the genus <i>Erythrina</i> L.	41
1.13.5	Biological activities of some <i>Erythrina</i> species	42
1.14	Description of the investigated plants of the genus <i>Erythrina</i>	46
1.14.1	Taxonomic hierarchy of <i>Erythrina fusca</i> Lour.	46
1.14.2	Description of <i>Erythrina fusca</i> Lour.	46
1.14.3	Taxonomic hierarchy of <i>Erythrina variegata</i> L.	48

2	Phytochemical investigation- Experimental	
2.1	Phytochemical investigation of the investigated plants.	51
2.2	Phytochemical investigation of <i>Bridelia verrucosa</i>	51
2.2.1	Collection and preparation of the plant material	51
2.2.2	Extraction of the plant material	51
2.2.3	Solvent-solvent partitioning of crude extract	52
2.2.4	Gel permeation chromatography of <i>n</i> -hexane soluble fraction of <i>B. verrucosa</i>	54
2.2.5	Analysis of column fractions by TLC	54
2.2.5.1	Isolation of BVSP-1	55
2.2.5.2	Isolation of BVSP-2	55
2.2.5.3	Column chromatography of fractions of 11-18	55
2.2.5.4	Analysis of column chromatography fractions by TLC	56
2.2.5.5	Isolation of BVSP-4	56
2.2.5.6	Isolation of BVSP-6	56
2.2.5.7	Isolation of BVSP-34	56
2.2.6	Column chromatography of carbon tetrachloride soluble fraction of the stem bark of <i>B. verrucosa</i>	57
2.2.7	Analysis of column fractions by TLC	58
2.2.7.1	Isolation of BVSC-8	58
2.2.7.2	Isolation of BVBC-2	58
2.2.8	Column chromatography of chloroform soluble fraction of the stem bark of <i>B. verrucosa</i>	58
2.2.9	Analysis of column chromatography fractions by TLC	59
2.2.9.1	Isolation of BVS-65	59
2.2.10	Properties of the isolated compounds	59
2.3	Phytochemical investigation of <i>Bidelia stipularis</i>	60
2.3.1	Collection and preparation of the plant material	60
2.3.2	Extraction of the plant material	60
2.3.3	Solvent-solvent partitioning of crude extract	61
2.3.4	VLC of the crude extract of stem bark of <i>B. stipularis</i>	61
2.3.5	Analysis of the VLC fractions by TLC	62
2.3.5.1	Isolation and purification of compounds from the selected VLC fractions	63
2.3.5.2	Isolation of BS-01	63
2.3.5.3	Gel permeation chromatography of VLC fractions 9A-11B of the stem bark of <i>B. stipularis</i>	63
2.3.5.3.1	Isolation of BS-06	63
2.3.5.3.2	Isolation of BS-11	64

2.3.6	Properties of the isolated compounds	64
2.4	Phytochemical investigation of <i>Bridelia tomentosa</i>	64
2.4.1	Collection and preparation of the plant material	64
2.4.2	Extraction of the plant material	65
2.4.3	Solvent-solvent partitioning of crude extract	65
2.4.4	Vacuum Liquid Chromatography (VLC) of the crude extract of stem bark of <i>B. tomentosa</i>	65
2.4.5	Analysis of VLC fractions by thin layer chromatography (TLC)	67
2.4.5.1	Isolation and purification of compounds from the selected VLC fractions	67
2.4.5.2	Isolation of BT-1	67
2.4.5.3	Isolation of BT-6	67
2.4.5.4	Isolation of BT-8	68
2.4.5.5	Isolation of BT-14	68
2.4.4.6	Properties of the isolated compounds	68
2.5	Phytochemical investigation of <i>Erythrina fusca</i>	68
2.5.1	Collection and preparation of the plant material	68
2.5.2	Extraction of the plant material	69
2.5.3	Solvent-solvent partitioning of crude extract	69
2.5.4	Column chromatography of carbon tetrachloride soluble fraction of the stem bark of <i>E. fusca</i>	70
2.5.5	Analysis of column fractions by TLC	71
2.5.5.1	Isolation of compound EF-7	71
2.5.5.2	Isolation of compound EF-8	71
2.5.5.3	Isolation of compound EF-12	72
2.5.5.4	Isolation of compound EF-38	72
2.5.5.5	Properties of the isolated compounds	72
2.6	Phytochemical investigation of <i>Erythrina variegata</i>	72
2.6.1	Collection and preparation of the plant material	72
2.6.2	Extraction of the plant material	73
2.6.3	Solvent-solvent partitioning of crude extract	73
3	Phytochemical investigation- Results and discussion	
3.1	Characterization of BVSP-1 as glochidonol (46)	74
3.2	Characterization of BVSP-2 as brassicasterol (47)	82
3.3	Characterization of BVSP-4 as friedelin (48)	86
3.4	Characterization of BVSP-6 as 5 β -24S-ethylcholestan-3 β -ol (49)	98
3.5	Characterization of BVSP-34 as stigmasterol (50)	102
3.6	Characterization of BVBC-2 as 5, 5'-dihydroxy sesamine (51)	105

3.7	Characterization of BVSC-8 as 3-ketooleanane (53)	111
3.8	Characterization of BVS-65 as pinoresinol (54)	115
3.9	Characterization of BS-01 as glut-5(6)-en-3-one (55)	120
3.10	Characterization of BS-06 as glut-5(6)-en-3 α -ol (56)	125
3.11	Characterization of BS-11 as (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (57)	130
3.12	Characterization of BT-1 as friedoolean-5(6),14(15)-dien-3-one (58)	135
3.13	Characterization of BT-6 as β -taraxerol (59)	140
3.14	Characterization of BT-8 as D ₄ -stigmaterone (60)	146
3.15	Characterization of BT-14 as lupeol (61)	150
3.16	Characterization of EF-7 as shinpterocarpin (62)	154
3.17	Characterization of EF-8 as lupinifolin (63)	162
3.18	Characterization of EF-12 as 3,9-dihydroxy-4-(3,3 dimethylallyl) [6aR,11aR]-pterocarpan (64)	168
3.19	Characterization of EF-38 as β -sitosterol (65)	175
4	Antimicrobial screening	
4.1	Introduction	178
4.2	Principle of disc diffusion method	178
4.3	Experimentals	179
4.4	Results and discussion	183
4.4.1	Antimicrobial activity of the stem bark <i>B. verrucosa</i>	183
4.4.2	Antimicrobial activity of the leaf of <i>B. verrucosa</i>	185
4.4.3	Antimicrobial activity of the stem bark of <i>B. stipularis</i>	187
4.4.4	Antimicrobial activity of the leaf of <i>B. stipularis</i>	189
4.4.5	Antimicrobial activity of the stem bark of <i>B. tomentosa</i>	191
4.4.6	Antimicrobial activity of the leaf of <i>B. tomentosa</i>	193
4.4.7	Antimicrobial activity of the stem bark <i>E. fusca</i>	195
4.4.8	Antimicrobial activity of the leaf of <i>E. fusca</i>	197
4.4.9	Antimicrobial activity of the stem bark <i>E. variegata</i>	199
4.4.10	Antimicrobial activity of the leaf of <i>E. variegata</i>	201
4.4.11	Antimicrobial activity of the pure compounds	203
5	Brine shrimp lethality bioassay	
5.1	Introduction	206
5.2	Principle	207
5.3	Material	207
5.4	Procedure	207
5.5	Analysis of data	210

5.6	Results and discussion of the test samples	211
5.7	Statistical analysis	231
5.8	Conclusion	231
6	Evaluation of antioxidant activity	
6.1	Assays for total phenolic contents	232
6.1.1	Principle	232
6.1.2	Standard curve preparation	232
6.1.3	Sample preparation	232
6.2	Antioxidant activity evaluation by DPPH method	232
6.2.1	Principle	232
6.3	Total antioxidant assay by phosphomolybdenum method	232
6.3.1	Principle	232
6.4	Results and discussion	233
6.4.1	Total phenolic content (TPC)	233
6.4.2	Antioxidant activity evaluation by DPPH method	237
6.4.3	Total antioxidant capacity assay by phosphomolybdenum method	253
7	Assay for thrombolytic activity	
7.1	Introduction	257
7.2	Experimentals	258
7.3	Assay for thrombolytic activity	258
7.4	Results and discussion	259
8	Summary	262
9	References	265
10	List of publications	291

List of tables

Table No.	Title	Page No.
1.1	The list of 54 genera from the 2006 revision of Phyllanthaceae.	9
1.2	Medicinal uses of some Phyllanthaceous species growing in Bangladesh.	12
1.3	Some <i>Bridelia</i> species available in Bangladesh.	14
1.4	Ethnomedicinal properties and phytoconstituents of some <i>Bridelia</i> species.	15
1.5	A short synoptic list of genera distributed in various sub families under Fabaceae family.	30
1.6	Medicinal uses of some Fabaceous species in Bangladesh.	37
1.7	Some available species of <i>Erythrina</i> .	40
1.8	Biological activity of some <i>Erythrina</i> species.	43
2.1	Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of <i>B. verrucosa</i> .	54
2.2	Different solvent systems used for gel permeation (Sephadex LH-20) of <i>n</i> -hexane soluble materials of the stem bark of <i>B. verrucosa</i>	54
2.3	List of isolated compounds from <i>n</i> -hexane soluble fraction of the methanolic extract of stem bark of <i>B. verrucosa</i> .	55
2.4	Different solvent systems used for column chromatography of fractions 11-18 from Sephadex (LH-20) column of <i>n</i> -hexane soluble materials of the stem bark of <i>B. verrucosa</i> .	56
2.5	Different solvent systems used for the column chromatographic analysis of the carbon tetrachloride soluble fraction of the stem bark of <i>B. verrucosa</i> .	57
2.6	List of isolated compounds from carbon tetrachloride soluble fraction of the methanolic extract of stem bark of <i>B. verrucosa</i> .	58
2.7	Different solvent systems used for the column chromatographic analysis of the chloroform soluble fraction of the stem bark of <i>B. verrucosa</i> .	59
2.8	List of isolated compounds from chloroform soluble fraction of the methanolic extract of stem bark of <i>B. verrucosa</i> .	59
2.9	Properties of the isolated compounds from stem bark of <i>B. verrucosa</i> .	60
2.10	Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of <i>B. stipularis</i> .	61
2.11	Solvent systems used for VLC analysis of the crude extract of stem bark of <i>B. stipularis</i> .	62
2.12	List of isolated compounds from VLC fraction of the methanolic extract of stem bark of <i>B. stipularis</i> .	62
2.13	Different solvent systems used for gel permeation (Sephadex LH-20) of VLC fractions 9A-11B of the stem bark of <i>B. stipularis</i> .	63
2.14	Properties of the isolated compounds from the stem bark of <i>B. stipularis</i> .	64

2.15	Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of <i>B. tomentosa</i> .	65
2.16	Solvent systems used for VLC analysis of the crude extract of stem bark of <i>B. tomentosa</i> .	66
2.17	List of isolated compounds from VLC fraction of the methanolic extract of stem bark of <i>B. tomentosa</i> .	67
2.18	Properties of the isolated compounds from the stem bark of <i>B. tomentosa</i> .	68
2.19	Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of <i>E. fusca</i> .	69
2.20	Different solvent systems used for the column chromatographic analysis of the carbon tetrachloride soluble fraction of the stem bark of <i>E. fusca</i> .	70
2.21	List of isolated compounds from carbon tetrachloride soluble fraction of the methanolic extract of stem bark of <i>E. fusca</i> .	71
2.22	Properties of the isolated compounds from the stem bark of <i>E. fusca</i> .	72
2.23	Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of <i>Erythrina variegata</i> .	73
3.1	¹ H-NMR spectral data of BVSP-1 (46) and glochidonol (Thu <i>et al.</i> , 2010) in CDCl ₃ .	75
3.2	¹³ C-NMR spectral data of BVSP-1 (46) and glochidonol (Thu <i>et al.</i> , 2010) in CDCl ₃ .	76
3.3	¹ H-NMR spectral data of BVSP-2 (47) and brassicasterol (Jinming <i>et al.</i> , 2001) in CDCl ₃ .	83
3.4	¹ H-NMR spectral data of BVSP-4 (48) and friedelin (Akihisa <i>et al.</i> , 1992) in CDCl ₃ .	87
3.5	¹³ C-NMR spectral data of BVSP-4 (48) and friedelin (Akihisa <i>et al.</i> , 1992) in CDCl ₃ .	88
3.6	¹ H-NMR spectral data of BVSP-6 (49) and 5 β -24S-ethylcholestan-3 β -ol (Luo <i>et al.</i> , 2009) in CDCl ₃ .	99
3.7	¹ H-NMR spectral data of BVSP-34 (50) and stigmasterol (Khan., 1991) in CDCl ₃ .	103
3.8	¹ H-NMR spectral data of BVBC-2 (51) and sesamine (Jayasinghe <i>et al.</i> , 2003) in CDCl ₃ .	106
3.9	¹ H-NMR spectral data of BVSC-8 (53) and 3-ketooleanane (Krishnaveni <i>et al.</i> , 2000) in CDCl ₃ .	112
3.10	¹ H-NMR spectral data of BVS-65 (54) and pinorexinol (Maria <i>et al.</i> , 1991) in CDCl ₃ .	116
3.11	¹ H-NMR spectral data of BS-01 (55) and glut-5(6)-en-3-one (Shazid <i>et al.</i> , 2012) in CDCl ₃ .	121
3.12	¹ H-NMR spectral data of BS-06 (56) and glut-5(6)-en-3 α -ol (Xue-jing <i>et al.</i> , 2012) in CDCl ₃ .	126
3.13	¹ H-NMR spectral data of BS-11(57) in CDCl ₃ .	131
3.14	¹ H-NMR spectral data of BT-1 (58) and other references (Xue-jing <i>et al.</i> , 2012; Shazid <i>et al.</i> , 2012; Fakir <i>et al.</i> , 2009 and Moulisha <i>et al.</i> , 2009) in CDCl ₃ .	136

3.15	¹ H-NMR spectral data of BT-6 (59) and β-taraxerol in CDCl ₃ (Fakir <i>et al.</i> , 2009) in CDCl ₃ .	141
3.16	¹ H-NMR spectral data of BT-8 (60) and D ₄ -stigmaterone in CDCl ₃ (Georges <i>et al.</i> , 2006) in CDCl ₃ .	147
3.17	¹ H-NMR spectral data of BT-14 (61) and lupeol in CDCl ₃ (Jahan <i>et al.</i> , 2010) in CDCl ₃ .	151
3.18	¹ H-NMR spectral data of EF-7 (62) and shinpterocarpin (Kitagawa <i>et al.</i> , 1994) in CDCl ₃ .	155
3.19	¹ H-NMR spectral data of EF-8 (63) and lupinifolin (Mahidol <i>et al.</i> , 1997) in CDCl ₃ .	163
3.20	¹ H-NMR spectral data of EF-12 (64) and and 3,9-dihydroxy-4-(3,3-dimethylallyl)[6aR,11aR]-pterocarpan (Cottiglia <i>et al.</i> , 2005) in CDCl ₃ .	169
3.21	¹ H-NMR spectral data of EF-38 (65) and β-sitosterol (Morales <i>et al.</i> , 2003) in CDCl ₃ .	176
4.1	Test organisms used for screening	179
4.2	Comparison of nutrient agar media	180
4.3	List of test samples and preparation of sample discs for various extracts.	182
4.4	Antimicrobial screening of the stem bark of <i>B. verrucosa</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	184
4.5	Antimicrobial screening of the leaf of <i>B. verrucosa</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	186
4.6	Antimicrobial screening of the stem bark of <i>B. stipularis</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	188
4.7	Antimicrobial screening of the leaf of <i>B. stipularis</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	190
4.8	Antimicrobial screening of the stem bark of <i>B. tomentosa</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	192
4.9	Antimicrobial screening of the leaf of <i>B. tomentosa</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	194
4.10	Antimicrobial screening of the stem bark of <i>E. fusca</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	196
4.11	Antimicrobial screening of the leaf of <i>E. fusca</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	198
4.12	Antimicrobial screening of the stem bark of <i>E. variegata</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	200
4.13	Antimicrobial screening of the leaf of <i>E. variegata</i> extractives (400 μgm/disc) and Kanamycin (30 μgm/disc).	202
4.14	Antimicrobial screening of the some of the pure compounds from <i>B. verrucosa</i> and <i>B. stipularis</i> (50 μgm/disc) and Kanamycin (30 μgm/disc).	204
4.15	Antimicrobial screening of the some of the pure compounds from <i>B. tomentosa</i> and <i>E. fusca</i> (50 μgm/disc) and Kanamycin (30 μgm/disc).	205
5.1	Test samples of experimental plants.	208

5.2	Effect of vincristine sulfate (positive control) on shrimp nauplii.	210
5.3	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	212
5.4	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>B. verrucosa</i> on brine shrimp nauplii.	214
5.5	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), Chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>B. stipularis</i> on brine shrimp nauplii.	215
5.6	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>B. stipularis</i> on brine shrimp nauplii.	217
5.7	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>B. tomentosa</i> on brine shrimp nauplii.	218
5.8	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>B. tomentosa</i> on brine shrimp nauplii.	220
5.9	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	221
5.10	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>E. fusca</i> on brine shrimp nauplii.	223
5.11	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>E. variegata</i> on brine shrimp nauplii.	225
5.12	Effect of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>E. variegata</i> on brine shrimp nauplii.	226
5.13	Effect of pure compounds BVSP-4 (48), BVBC-2 (51) and BVS-65 (54) isolated from the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	228
5.14	Effect of pure compounds BS-01 (55), BS-06 (56) and BT-6 (57) isolated from the stem bark of <i>B. stipularis</i> and <i>B. tomentosa</i> on brine shrimp nauplii.	229
5.15	Effect of pure compounds EF-7 (62), EF-8 (63) and EF-12 (64) isolated from the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	230
6.1	Standard curve preparation by using gallic acid.	233
6.2	Results of the test samples for total phenolic content determination of <i>Bridelia</i> species.	234
6.3	Results of the test samples for total phenolic content determination <i>Erythrina</i> species.	235
6.4	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>B. verrucosa</i> .	238

6.5	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>B. verrucosa</i> .	239
6.6	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>B. stipularis</i> .	241
6.7	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>B. stipularis</i> .	242
6.8	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>B. tomentosa</i> .	243
6.9	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>B. tomentosa</i> .	245
6.10	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>E. fusca</i> .	246
6.11	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>E. fusca</i> .	247
6.12	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of <i>E. variegata</i> .	249
6.13	Free radical scavenging activity of methanol extract (Me), <i>n</i> -hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of <i>E. variegata</i> .	250
6.14	Free radical scavenging activity of the isolated pure compounds BVSP-4 (48), BVSP-6 (49), BVBC-2 (51) and BVS-65 (54) isolated from the stem bark of <i>B. verrucosa</i> .	251
6.15	Free radical scavenging activity of the isolated pure compounds BS-11 (57), EF-7 (62), EF-8 (63) and EF-12 (64) isolated from the stem bark of <i>B. stipularis</i> and <i>E. fusca</i> .	252
6.16	Standard curve preparation by using ascorbic acid.	254
6.17	Total antioxidant capacity of the methanolic extracts and its different soluble fractions of stem bark and leaf of <i>Bridelia</i> species.	254
6.18	Total antioxidant capacity of the methanolic extracts and its different soluble fractions of stem bark and leaf of <i>Erythrina</i> species.	256
7.1	Thrombolytic activity (in terms of % clot lysis) of <i>B. verrucosa</i> , <i>B. stipularis</i> , and <i>B. tomentosa</i> .	260
7.2	Thrombolytic activity (in terms of % clot lysis) of <i>E. fusca</i> and <i>E. variegata</i> .	261

List of figures

Figure No.	Title	Page No.
1.1	Lead compound search and utilization from plants.	5
1.2	Structures of the compounds found in the genus <i>Bridelia</i> .	18
1.3	<i>Bridelia verrucosa</i> Haines flower (1) and leaves with fruits (2).	21
1.4	<i>Bridelia stipularis</i> Blume leaves with fruits (1) and flowers (2).	23
1.5	<i>Bridelia tomentosa</i> Blume leaves with fruits (1) and leaves (2).	26
1.6	<i>Castanospermum austral</i>	30
1.7	<i>Bauhinia galpinii</i>	30
1.8	<i>Albizia distachya</i>	30
1.9	<i>Caesalpinia claibornensis</i> (Caesalpinioideae) fossil legume (fruit) described from the Middle Eocene (40-50 Mya) Claiborne Formation in western Tennessee.	33
1.10	<i>Acacia eocaribbeanensis</i> (Mimosoideae). Specimen showing numerous stamens with long filaments (USNM 458372, holotype) .	33
1.11	<i>Cladrastis</i> subgenus <i>Platycarpa</i> (Papilionoideae) fossil fruit.	33
1.12	<i>Diploptropis claibornensis</i> fossil fruit (IU 15826-7235) from the Middle Eocene Claiborne Formation of the southeastern North America.	34
1.13	Structures of some alkaloids reported from <i>Erythrina</i> species.	41
1.14	Structures of some flavonoids reported from <i>Erythrina</i> species.	42
1.15	Structures of some isoflavonoids reported from <i>Erythrina</i> species.	42
1.16	Structures of some pterocarpan reported from <i>Erythrina</i> species.	42
1.17	<i>Erythrina fusca</i> Lour tree (1) and flowers (2).	48
1.18	<i>Erythrina variegata</i> L. tree (1), leaves (2) and flowers (3).	50
2.1	Schematic representation of the modified Kupchan partitioning of the stem bark of methanolic crude extract of <i>B. verrucosa</i> .	53
3.1	Structure of BVSP-1 (glochidonol, 46).	75
3.2	¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVSP-1 (46).	77
3.3	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVSP-1 (46).	78
3.4	¹³ C-NMR spectrum of BVSP-1 (46) in CDCl ₃ .	79
3.5	Partially expanded ¹³ C-NMR spectrum of BVSP-1 (46) in CDCl ₃ .	80
3.6	Partially expanded ¹³ C-NMR spectrum of BVSP-4 (46) in CDCl ₃ .	81
3.7	Structure of BVSP-2 (brassicasterol, 47).	83
3.8	¹ H-NMR spectrum (500 MHz, CDCl ₃) of BVSP-2 (47).	84
3.9	Partially expanded ¹ H-NMR spectrum (500 MHz, CDCl ₃) of BVSP-2 (47).	85
3.10	Structure of BVSP-4 (friedelin, 48).	87

3.11	¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVSP-4 (48).	89
3.12	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVSP-4 (48).	90
3.13	¹³ C-NMR spectrum of BVSP-4 (48) in CDCl ₃ .	91
3.14	Partially expanded ¹³ C-NMR spectrum of BVSP-4 (48) in CDCl ₃ .	92
3.15	Partially expanded ¹³ C-NMR spectrum of BVSP-4 (48) in CDCl ₃ .	93
3.16	Partially expanded ¹³ C-NMR spectrum of BVSP-4 (48) in CDCl ₃ .	94
3.17	DEPT spectrum of BVSP-4 (48) in CDCl ₃ .	95
3.18	Partially expanded DEPT spectrum of BVSP-4 (48) in CDCl ₃ .	96
3.19	Partially expanded DEPT spectrum of BVSP-4 (48) in CDCl ₃ .	97
3.20	Structure of BVSP-6 (5β-24S-ethylcholestan-3β-ol, 49).	99
3.21	¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVSP-6 (49).	100
3.22	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVSP-6 (49).	101
3.23	Structure of BVSP-34 (stigmasterol, 50).	103
3.24	¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVSP-34 (50).	104
3.25	Structures of BVBC-2 (5,5'-dihydroxysesamine, 51) and sesamine (52).	106
3.26	¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVBC-2 (51).	107
3.27	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVBC-2 (51).	108
3.28	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVBC-2 (51).	109
3.29	¹ H- ¹ H COSY spectrum of BVBC-2 (51) in CDCl ₃ .	110
3.30	Structure of BVSC-8 as 3-ketooleanane (53)	112
3.31	¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVSC-8 (53).	113
3.32	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVSC-8 (53).	114
3.33	Structure of BVS-65 (pinoresinol, 54).	116
3.34	¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVS-65 (54).	117
3.35	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVS-65 (54).	118
3.36	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BVS-65 (54).	119
3.37	Structure of BS-01 (glut-5(6)-en-3-one, 55)	121
3.38	¹ H-NMR spectrum (400 MHz, CDCl ₃) of BS-01 (55).	122
3.39	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BS-01 (55).	123
3.40	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BS-01 (55).	124
3.41	Structure of BS-06 (glut-5(6)-en-3α-ol, 56).	126
3.42	¹ H-NMR spectrum (400 MHz, CDCl ₃) of BS-06 (56).	127
3.43	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BS-06 (56).	128
3.44	Partially expanded ¹ H-NMR spectrum (400 MHz, CDCl ₃) of BS-06 (56).	129
3.45	Structure of BS-11 (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one, 57).	131
3.46	¹ H-NMR spectrum (500 MHz, CDCl ₃) of BS-11 (57).	132

3.47	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of BS-11 (57).	133
3.48	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of BS-11 (57).	134
3.49	Structures of BT-1 (friedoolean-5(6),14(15)-dien-3-one, 58), glut-5(6)-en-3-one (55), glut-5(6)-en-3 α -ol (56), β -taraxerol (59) and friedoolean-14-en,3-one [58(i)].	136
3.50	^1H -NMR spectrum (400 MHz, CDCl_3) of BT-1 (58).	137
3.51	Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BT-1 (58).	138
3.52	Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BT-1 (58).	139
3.53	Structure of BT-6 (β -taraxerol, 59).	141
3.54	ESI-Mass spectrum of BT-6 (59).	142
3.55	^1H -NMR spectrum (500 MHz, CDCl_3) of BT-6 (59).	143
3.56	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of BT-6 (59).	144
3.57	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of BT-6 (59).	145
3.58	Structure of BT-8 (D_4 -stigmaterone, 60).	147
3.59	ESI-Mass spectrum of BT-8 (60).	148
3.60	^1H -NMR spectrum (500 MHz, CDCl_3) of BT-8 (60).	149
3.61	Structure of BT-14 (lupeol, 61).	151
3.62	ESI-Mass spectrum of BT-6 (61).	152
3.63	^1H -NMR spectrum (500 MHz, CDCl_3) of BT-14 (61).	153
3.64	Structure of EF-7 (shinpterocarpin, 62).	155
3.65	^1H -NMR spectrum (500 MHz, CDCl_3) of EF-7 (62).	156
3.66	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-7 (62).	157
3.67	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-7 (62).	158
3.68	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-7 (62).	159
3.69	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-7 (62).	160
3.70	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-7 (62).	161
3.71	Structure of EF-8 (lupinifolin, 63).	163
3.72	^1H -NMR spectrum (500 MHz, CDCl_3) of EF-8 (63).	164
3.73	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-8 (63).	165
3.74	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-8 (63).	166
3.75	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-8 (63).	167
3.76	Structure of EF-12 (3,9-dihydroxy-4-(3,3-dimethylallyl)[6aR,11aR]-pterocarpin, 64).	169
3.77	^1H -NMR spectrum (500 MHz, CDCl_3) of EF-12 (64).	170
3.78	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-12 (64).	171
3.79	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-12 (64).	172
3.80	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-12 (64).	173

3.81	Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-12 (64).	174
3.82	Structure of EF-38 (β -sitosterol, 65).	176
3.83	^1H -NMR spectrum (400 MHz, CDCl_3) of EF-38 (65).	177
5.1	Effect of vincristine sulfate on brine shrimp nauplii.	211
5.2	Effect of crude methanol extract of the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	213
5.3	Effect of <i>n</i> -hexane soluble fraction of the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	213
5.4	Effect of CCl_4 soluble fraction of the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	213
5.5	Effect of chloroform soluble fraction of the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	213
5.6	Effect of aqueous soluble fraction of the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	213
5.7	Effect of crude methanol extract of the leaf of <i>B. verrucosa</i> on brine shrimp nauplii.	214
5.8	Effect of <i>n</i> -hexane soluble fraction of the leaf of <i>B. verrucosa</i> on brine shrimp nauplii.	215
5.9	Effect of CCl_4 soluble fraction of the leaf of <i>B. verrucosa</i> on brine shrimp nauplii.	215
5.10	Effect of chloroform soluble fraction of the leaf of <i>B. verrucosa</i> on brine shrimp nauplii.	215
5.11	Effect of aqueous soluble fraction of the leaf of <i>B. verrucosa</i> on brine shrimp nauplii.	215
5.12	Effect of crude methanol extract of the stem bark of <i>B. stipularis</i> on brine shrimp nauplii.	216
5.13	Effect of <i>n</i> -hexane soluble fraction of the stem bark of <i>B. stipularis</i> on brine shrimp nauplii.	216
5.14	Effect of CCl_4 soluble fraction of the stem bark of <i>B. stipularis</i> on brine shrimp nauplii.	216
5.15	Effect of chloroform soluble fraction of the stem bark of <i>B. stipularis</i> on brine shrimp nauplii.	216
5.16	Effect of aqueous soluble fraction of the stem bark of <i>B. stipularis</i> on brine shrimp nauplii.	216
5.17	Effect of crude methanol extract of the leaf of <i>B. stipularis</i> on brine shrimp nauplii.	217
5.18	Effect of <i>n</i> -hexane soluble fraction of the leaf of <i>B. stipularis</i> on brine shrimp nauplii.	217
5.19	Effect of CCl_4 soluble fraction of the leaf of <i>B. stipularis</i> on brine shrimp nauplii.	217
5.20	Effect of chloroform soluble fraction of the leaf of <i>B. stipularis</i> on brine shrimp nauplii.	218

5.21	Effect of aqueous soluble fraction of the leaf of <i>B. stipularis</i> on brine shrimp nauplii.	218
5.22	Effect of crude methanol extract of the stem bark of <i>B. tomentosa</i> on brine shrimp nauplii.	219
5.23	Effect of n-hexane soluble fraction of the stem bark of <i>B. tomentosa</i> on brine shrimp nauplii.	219
5.24	Effect of CCl ₄ soluble fraction of the stem bark of <i>B. tomentosa</i> on brine shrimp nauplii.	219
5.25	Effect of chloroform soluble fraction of the stem bark of <i>B. tomentosa</i> on brine shrimp nauplii.	219
5.26	Effect of aqueous soluble fraction of the stem bark of <i>B. tomentosa</i> on brine shrimp nauplii.	219
5.27	Effect of crude methanol extract of the leaf of <i>B. tomentosa</i> on brine shrimp nauplii.	220
5.28	Effect of n-hexane soluble fraction of the leaf of <i>B. tomentosa</i> on brine shrimp nauplii.	221
5.29	Effect of CCl ₄ soluble fraction of the leaf of <i>B. tomentosa</i> on brine shrimp nauplii.	221
5.30	Effect of chloroform soluble fraction of the leaf of <i>B. tomentosa</i> on brine shrimp nauplii.	221
5.31	Effect of aqueous soluble fraction of the leaf of <i>B. tomentosa</i> on brine shrimp nauplii.	221
5.32	Effect of crude methanol extract of the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	222
5.33	Effect of n-hexane soluble fraction of the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	222
5.34	Effect of CCl ₄ soluble fraction of the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	222
5.35	Effect of chloroform soluble fraction of the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	223
5.36	Effect of aqueous soluble fraction of the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	223
5.37	Effect of crude methanol extract of the leaf of <i>E. fusca</i> on brine shrimp nauplii.	224
5.38	Effect of n-hexane soluble fraction of the leaf of <i>E. fusca</i> on brine shrimp nauplii.	224
5.39	Effect of CCl ₄ soluble fraction of the leaf of <i>E. fusca</i> on brine shrimp nauplii.	224
5.40	Effect of chloroform soluble fraction of the leaf of <i>E. fusca</i> on brine shrimp nauplii.	224
5.41	Effect of aqueous soluble fraction of the leaf of <i>E. fusca</i> on brine shrimp nauplii.	224
5.42	Effect of crude methanol extract of the stem bark of <i>E. variegata</i> on brine shrimp nauplii.	225
5.43	Effect of n-hexane soluble fraction of the stem bark of <i>E. variegata</i> on brine shrimp nauplii.	225

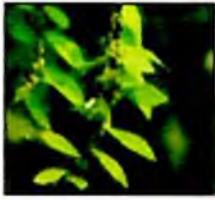
5.44	Effect of CCl ₄ soluble fraction of the stem bark of <i>E. variegata</i> on brine shrimp nauplii.	225
5.45	Effect of chloroform soluble fraction of the stem bark of <i>E. variegata</i> on brine shrimp nauplii.	226
5.46	Effect of aqueous soluble fraction of the stem bark of <i>E. variegata</i> on brine shrimp nauplii.	226
5.47	Effect of crude methanol extract of the leaf of <i>E. variegata</i> on brine shrimp nauplii.	227
5.48	Effect of n-hexane soluble fraction of the leaf of <i>E. variegata</i> on brine shrimp nauplii.	227
5.49	Effect of CCl ₄ soluble fraction of the leaf of <i>E. variegata</i> on brine shrimp nauplii.	227
5.50	Effect of chloroform soluble fraction of the leaf of <i>E. variegata</i> on brine shrimp nauplii.	227
5.51	Effect of aqueous soluble fraction of the leaf of <i>E. variegata</i> on brine shrimp nauplii.	227
5.52	Effect of BVSP-4 from the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	228
5.53	Effect of BVBC-2 from the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	228
5.54	Effect of BVS-65 from the stem bark of <i>B. verrucosa</i> on brine shrimp nauplii.	228
5.55	Effect of BS-01 from the stem bark of <i>B. stipularis</i> on brine shrimp nauplii.	229
5.56	Effect of BS-06 from the stem bark of <i>B. stipularis</i> on brine shrimp nauplii.	229
5.57	Effect of BT-6 from the stem bark of <i>B. stipularis</i> on brine shrimp nauplii.	229
5.58	Effect of EF-7 from the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	230
5.59	Effect of EF-8 from the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	230
5.60	Effect of EF-12 from the stem bark of <i>E. fusca</i> on brine shrimp nauplii.	230
6.1	Standard curve of gallic acid for total phenolic content determination.	233
6.2	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the stem bark of <i>B. verrucosa</i> .	236
6.3	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the leaf of <i>B. verrucosa</i> .	236
6.4	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the stem bark of <i>B. stipularis</i> .	236
6.5	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the leaf of <i>B. stipularis</i> .	236
6.6	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the stem bark of <i>B. tomentosa</i> .	236
6.7	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the leaf of <i>B. tomentosa</i> .	236
6.8	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the stem bark of <i>E. fusca</i> .	237

6.9	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the leaf of <i>E. fusca</i> .	237
6.10	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the stem bark of <i>E. variegata</i> .	237
6.11	Total phenolic content (mg of GAE / gm of extractives) of different extractives of the leaf of <i>E. variegata</i> .	237
6.12	Free radical scavenging activity of the methanol fraction (Me) of the stem bark of <i>B. verrucosa</i> .	238
6.13	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the stem bark of <i>B. verrucosa</i> .	239
6.14	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the stem bark of <i>B. verrucosa</i> .	239
6.15	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of <i>B. verrucosa</i> .	239
6.16	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of <i>B. verrucosa</i> .	239
6.17	Free radical scavenging activity of the methanol fraction (Me) of the leaf of <i>B. verrucosa</i> .	240
6.18	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the leaf of <i>B. verrucosa</i> .	240
6.19	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the leaf of <i>B. verrucosa</i> .	240
6.20	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of <i>B. verrucosa</i> .	240
6.21	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of <i>B. verrucosa</i> .	240
6.22	Free radical scavenging activity of the methanol fraction (Me) of the stem bark of <i>B. stipularis</i> .	241
6.23	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the stem bark of <i>B. stipularis</i> .	241
6.24	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the stem bark of <i>B. stipularis</i> .	241
6.25	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of <i>B. stipularis</i> .	242
6.26	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of <i>B. stipularis</i> .	242
6.27	Free radical scavenging activity of the methanol fraction (Me) of the leaf of <i>B. stipularis</i> .	242
6.28	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the leaf of <i>B. stipularis</i> .	243
6.29	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the leaf of <i>B. stipularis</i> .	243

6.30	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of <i>B. stipularis</i> .	243
6.31	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of <i>B. stipularis</i> .	243
6.32	Free radical scavenging activity of the methanol fraction (Me) of the stem bark of <i>B. tomentosa</i> .	244
6.33	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the stem bark of <i>B. tomentosa</i> .	244
6.34	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the stem bark of <i>B. tomentosa</i> .	244
6.35	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of <i>B. tomentosa</i> .	244
6.36	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of <i>B. tomentosa</i> .	244
6.37	Free radical scavenging activity of the methanol fraction (Me) of the leaf of <i>B. tomentosa</i> .	245
6.38	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the leaf of <i>B. tomentosa</i> .	245
6.39	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the leaf of <i>B. tomentosa</i> .	245
6.40	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of <i>B. tomentosa</i> .	246
6.41	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of <i>B. tomentosa</i> .	246
6.42	Free radical scavenging activity of the methanol fraction (Me) of the stem bark of <i>E. fusca</i> .	246
6.43	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the stem bark of <i>E. fusca</i> .	247
6.44	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the stem bark of <i>E. fusca</i> .	247
6.45	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of <i>E. fusca</i> .	247
6.46	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of <i>E. fusca</i> .	247
6.47	Free radical scavenging activity of the methanol fraction (Me) of the leaf of <i>E. fusca</i> .	248
6.48	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the leaf of <i>E. fusca</i> .	248
6.49	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the leaf of <i>E. fusca</i> .	248
6.50	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of <i>E. fusca</i> .	248

6.51	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of <i>E. fusca</i> .	248
6.52	Free radical scavenging activity of the methanol fraction (Me) of the stem bark of <i>E. variegata</i> .	249
6.53	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the stem bark of <i>E. variegata</i> .	249
6.54	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the stem bark of <i>E. variegata</i> .	249
6.55	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of <i>E. variegata</i> .	250
6.56	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of <i>E. variegata</i> .	250
6.57	Free radical scavenging activity of the methanol fraction (Me) of the leaf of <i>E. variegata</i> .	250
6.58	Free radical scavenging activity of the <i>n</i> -hexane soluble materials (HSF) of the leaf of <i>E. variegata</i> .	251
6.59	Free radical scavenging activity of the CCl ₄ soluble materials (CSF) of the leaf of <i>E. variegata</i> .	251
6.60	Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of <i>E. variegata</i> .	251
6.61	Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of <i>E. variegata</i> .	251
6.62	Free radical scavenging activity of the BVSP-4 (48) isolated from the stem bark of <i>B. verrucosa</i> .	252
6.63	Free radical scavenging activity of the BVSP-6 (49) isolated from the stem bark of <i>B. verrucosa</i> .	252
6.64	Free radical scavenging activity of the BVBC-2 (51) isolated from the stem bark of <i>B. verrucosa</i> .	252
6.65	Free radical scavenging activity of the BVS-65 (54) isolated from the stem bark of <i>B. verrucosa</i> .	252
6.66	Free radical scavenging activity of the BS-11 (57) isolated from the stem bark of <i>B. stipularis</i> .	253
6.67	Free radical scavenging activity of the EF-7 (62) isolated from the stem bark of <i>E. fusca</i> .	253
6.68	Free radical scavenging activity of the EF-8 (63) isolated from the stem bark of <i>E. fusca</i> .	253
6.69	Free radical scavenging activity of the EF-12 (64) isolated from the stem bark of <i>E. fusca</i> .	253
6.70	Standard curve of ascorbic acid for total antioxidant capacity determination.	254
6.71	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the stem bark of <i>B. verrucosa</i> .	255
6.72	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the leaf of <i>B. verrucosa</i> .	255

6.73	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the stem bark of <i>B. stipularis</i> .	255
6.74	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the leaf of <i>B. stipularis</i> .	255
6.75	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the stem bark of <i>B. tomentosa</i> .	255
6.76	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the leaf of <i>B. tomentosa</i> .	255
6.77	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the stem bark of <i>E. fusca</i> .	256
6.78	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the leaf of <i>E. fusca</i> .	256
6.79	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the stem bark of <i>E. variegata</i> .	256
6.80	Total antioxidant capacity (mg of ascorbic acid / gm of dried extract) of different extractives of the leaf of <i>E. variegata</i> .	256



Chapter 1

Introduction

1.1 Rational of the work

Today standing in the twenty-first century, in the age of massive development of modern science, mankind still depends on nature for its survival. This fact is seen from the use of natural herbs to cure diseases and relieve physical sufferings. The importance of herbs dates back to ancient times for their food value as well as invaluable medicinal properties. It is not certain when and how man learned to use plant appropriately but it is thought that; animal was the first guide to show them the use of plants. Because animals naturally know or feel how to get relief from unusual internal conditions and during the long span of time men gradually developed a knowledge of naturally occurring drugs which was transmitted at one time orally, later in written form as papyri, baked clay tablets, parchments, manuscript herbals, printed herbals, pharmacopoeias and other works, and most recently by computerized information-retrieval system (Evans, 1989).

The art of medicine is very ancient. There are countless herbal remedies and cures, which have been passed on by generations. The list of such herbal medications are endless, each unique in their value and origin, some of which we are still unaware of but some of the drugs which have been used in the days of antiquity are still now used, but in a modified form. As far as records from history of pharmacy, it appears that the Babylonians (about 3000 years BC) made clay models of the human body and early writings indicate that, they were aware of medicinal effects of a number of plants (Tyler and Brady, 1985).

Probably the oldest pharmacopoeia of the Emperor Shen Nung (Burger, 1970) of China written between 2730 BC and 3000 BC contains the uses and recipes of hundreds and thousands of Chinese traditional medicines. As evident from the Papyrus Ebers (written in about 1500 BC found in the tomb of a mummy and now kept in the University of Leipzig); the ancient Egyptians possessed a good knowledge of the medicinal properties of hundreds of plants. Many of the plant drugs which are used in present day, like henbane (*Hyoscyamus spp.*); mandrake (*Mandragora officinarum*); opium (*Papaver somniferum* fruit); castor oil (oil of *Ricinus communis* seed) and many others were in common use in Egypt about 4500 years ago (Ghani, 2003).

The Materia Medica of the great Greek physician Hippocrates (406 ~ 370 BC) consists of some 300 to 400 medicinal plants which included opium, mint, rosemary, sage and verbenas. The far-ranging scientific work of Aristotle (384 ~ 322 BC); a Greek philosopher, included an effort to catalogue the properties of the various medicinal herbs known at that time. Theophrastus (Core, 1962) (370 ~ 285 BC); a student of Plato, in his "*Historia plantarum*" described the use of nearly 500 medicinal plants. The encyclopedic work of Dioscorides (1st Century AD)-De Materia Medica was the forerunner of all modern pharmacopoeias and an authoritative text on botanical medicine. The work featured about 600 medicinal plants. Two of the 37 volumes of books written by Pliny De Elder (23 ~ 70 AD) were devoted to medical botany and these included a large number of medicinal plants.

Galen (131 ~ 200 AD); a Greek pharmacist-physician who lived in Rome, described the method of preparing formulas containing plant and animal drugs. He devoted considerable time in compiling this knowledge, which was distributed through out 20 books (Tyler and Brady, 1985).

When the Roman glory began to decline in the Middle Ages, the Arabs contributed much to the vast field of medicine. A canon of medicine by Ibn Sina bears a testimony to this. With the advent of the Muslim conquerors, the Mohammedan system of medicine was introduced in the sub-continent and the system was prevalent during the region of Mughal and Pathan. The Arabian system was superior standard and was fairly advanced for the period. An excellent account of the Indian sub-continent can be seen in the book written by Abu Mansur in that period (Core, 1962).

The discovery of the medicinal plants of the Indian sub-continent was made hundred of years ago (Chopra *et al.*, 1933). Rigveda (4500 ~ 1600 BC); Atharvaveda, Ayurveda, Susruta and Charaka are amongst the oldest treatises on Indian medicinal plants (Chopra *et al.*, 1958).

One of the earliest book containing the description of therapeutic agents were known as the "Herbal" and the oldest herbals were the "Ortus sanitatis" meaning the garden of Health, written in Latin and published at Mainz in 1491 (Wallis, 1967).

Ancient manuscripts, pictures painted on the walls of tombs, plant remains found in caves and burial sites and carving in stones preserved through the ages have given modern scientists at least fragmentary insight into the knowledge of those people about the use of plants. Allopathic and homeopathic system of medicines today are based on the doctrines expatiated by Galen (Sofowra, 1982).

Today, a lot of modern medicines have been discovered as a result of the scientific follow-up of the traditional herbal preparations. A classic example is the latest development of the anti-malarial drug “Quinhasu” from the herb “*Artemisia annua*” which has been used in China against malaria for 2000 years. Withering (1741-1799) introduced digitalis (purple foxglove) as a therapeutic agent for dropsy the active principles from the same material are used in the treatment of heart failure today.

With centuries of efforts scientists have found in plants the remedy of diverse diseases ranging from simple skin diseases to complicated cancer. Based on new scientific developments in isolation, identification and testing technology, the most promising recent contribution of medicinal plants research led to the isolation of anticancer agent taxol (from *Taxus bravifolia*); hypercin (from *Hypericum* species) and anti-AIDS glycyrrhizin (from *Glycyrrhiza* species).

Plants have formed the basis for traditional systems of medicine, which have been used for thousands of years in many countries (Chang, 1986; Kapoor, 1990) and extensively documented (Schultes *et al.*, 1990). These plant-based systems continue to play an essential role in health care and it has been estimated by the World Health Organization (WHO) that approximately 80% of the world’s inhabitants rely mainly on traditional medicines for their primary health care (Arvigo *et al.*, 1993). Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries. In a study it has been shown that at least 119 chemical substances, derived from 90 plant species, can be considered as important drugs that are in use in one or more countries. Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine (Farnsworth *et al.*, 1985).

During the last two decades, the renewed interest in investigating natural products has lead to the advent of several important drugs. For example, recently ricin, a toxin produced by the beans of *Ricinus communis*, has been found to be a very potent antitumor drug (Spalding, 1991; Gupta, 1992). Further, HIV inhibitory activity has been discovered in some novel complex angular coumarins isolated from *Calophyllum lanigerum* (Kashman *et al.*, 1992) and taxol is another example of effective antitumor agent found from *Taxus bravifolia*. Plants containing important secondary metabolites and potential biological activities can be genetically engineered to increase the transcription of enzyme responsible for the production of that particular compound. Thus, knowing the potential resources it is possible to increase the content of important active compounds (Owen *et al.*, 1992) and genes are to be encoded in the host organisms to produce lead compounds from plants in industrial scale.

In fact, plants are the important source of a diverse range of chemical compounds. Some of these compounds possessing a wide range of pharmacological activities are either impossible or trouble some to synthesize in the laboratory. A phytochemist uncovering these resources is producing useful materials for screening programs for drug discovery. Recently developed genetic engineering technology in plants has further increased their importance in the field of medicine, for example, in the production of antibodies by expression of an appropriate gene in the plant. By using this technique it is possible to modify the activity or regulate the properties of the key enzymes responsible for the production of secondary metabolites. Thus, by knowing the potential resources it is possible to increase the content of the important active compounds (Owen *et al.*, 1992) and in the future genes responsible for very specific biosynthetic processes may be encoded into host organism to facilitate synthetic transformation that is produce lead compounds from plants in industrial scale.

Thus, plant is a biosynthetic laboratory and the curative phyto-elements produced inside a plant through a flow of biochemical reactions significantly contribute to the traditional and modern medicines. These interesting active ingredients are nothing but the chemical defense against diseases which can hold back numerous pathological discrepancies and can reset physiological harmony.

The goals of using plants as sources of therapeutic agent are (Daniel *et al.*, 2001)

- To isolate bioactive compounds for direct use as drugs, e.g. digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine;
- To produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics); taxotere, teniposide, verapamil, and miodarone, which are based, respectively, on galegin, Δ^9 - tetrahydrocannabinol, morphine, taxol, podophyllotoxin, khellin, and khellin;
- To use agents as pharmacological tools, e. g., lysergic acid diethylamide, mescaline, yohimbin; and
- To use the whole plant or part of it as a herbal remedy, e.g., cranberry, echinacea, feverfew, garlic, etc.

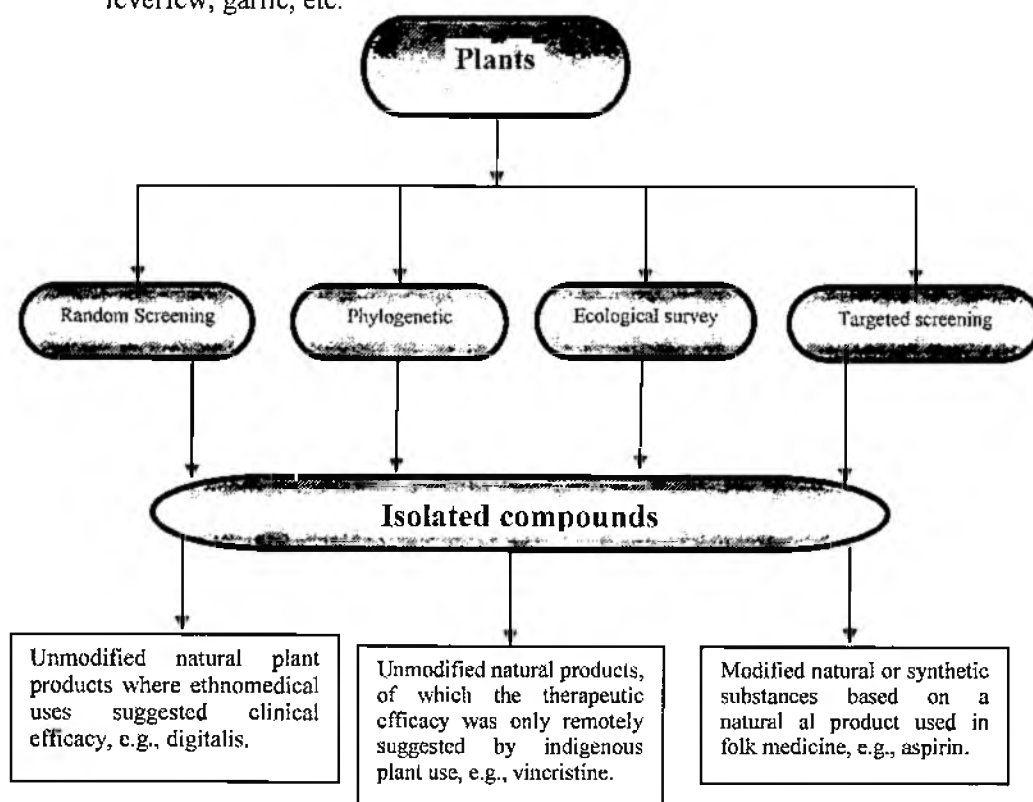


Figure 1.1: Lead compound search and utilization from plants.

There are several familiar approaches to find out lead compounds from plants (Fig 1.1) and the isolated bioactive compounds are utilized in three basic ways (Cox, 1994):

- a) Unmodified natural plant products where ethnomedical uses suggested clinical efficacy, e.g., digitalis.
- b) Unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use, e.g., vincristine.
- c) Modified natural or synthetic substances based on a natural product used in folk medicine, e.g., aspirin.

1.2 Objectives of the work

Nature has been a source of several medicines for treating various types of diseases in humans and animals for many years (Dev, 1997). Natural products are naturally derived metabolites and/or by-products from plants, microorganisms or animals (Baker *et al.*, 2000). In the synthesis or design of new drugs the structure of the chemical substances still play a major role in treatment of diseases either as the drug or as a predecessor. The worlds best known and most universally used medicinal agent is aspirin, which is related to salicin and was originated from the plant genera, *Salix* and *Populus* (Strobel *et al.*, 2004). Examples abound of natural product use, especially in small native populations in a myriad of remote locations on earth. For instance, certain tribal groups in the Amazon basin, the highland people of Papua New Guinea and the Aborigines of Australia, each have identified certain plants to provide relief of symptoms, varying from colds to massive wounds and intestinal ailments (Isaacs, 2002).

Nature has endowed Bangladesh with a lavish gift of medicinal plants most of which grow in jungles, forests and gardens without care. They can also be cultivated in large scale, if desired. Many of these plants have been used in the indigenous system of medicine for centuries without having any knowledge of active principles responsible for therapeutic activities. Researches on some of these plants in the recent years have been much rewarding. As already mentioned, Bangladesh is blessed with numerous medicinal plants belonging to various families including Phyllanthaceae and Fabaceae. Although a large number of plants are included in the above two families and some of the plants have been investigated all over the world, very little is known regarding the chemistry and

pharmacology of *Bridelia* and *Erythrina* species growing in Bangladesh. So, attempts have been taken to study the chemical constituents along with their pharmacological aspects especially cytotoxic and antibacterial profiles of some *Bridelia* and *Erythrina* species available in Bangladesh. The plants selected for investigation include

***Bridelia* species:**

1. *Bridelia verrucosa* Haines.
2. *Bridelia stipularis* (L) Blume.
3. *Bridelia tomentosa* Blume.

***Erythrina* species:**

1. *Erythrina fusca* Lour.
2. *Erythrina variegata* L.

These investigations may provide some remarkable compounds, with promising pharmacological activities. If considerable results are obtained, these can be used as remedy for the treatment of some diseases. Since these plants are available in Bangladesh, these may produce a cost-effective treatment. Thus, the objective was to explore the possibility of developing new drug candidates from these plants for the treatment of various diseases.

1.3 The plant family Phyllanthaceae

Phyllanthaceae is the family of flowering plants in the eudicot order Malpighiales. It is most closely related to the family Picodendraceae. The Phyllanthaceae are most numerous in the tropics, with many in the South Temperate Zone, and a few ranging as far north as the middle of the North Temperate Zone (Hoffman *et al.*, 2006).

The name "Phyllanthaceae" was first validly published by Ivan Ivanovich Martynov in 1820 in a Russian book entitled *Tekhnobotanico Slovar*. A proposal to conserve this name was published in 2007 (Reveal *et al.*, 2007). Martynov's name was rarely used in the 180 years after he published it. During that time, the plants that are now in Phyllanthaceae were placed in the large and heterogeneous family Euphorbiaceae. The monophyly of Euphorbiaceae had long been held in doubt by some, but the first strong evidence of its polyphyly came in 1993 with the first maximum parsimony analysis of DNA sequences of the gene *rbcl* from a large number of seed plants (Chase *et al.*, 1993). Since the 1993 study, all subsequent phylogenetic analyses have shown that the old concept of Euphorbiaceae consisted of several lineages that did not together form a clade in the order Malpighiales. Euphorbiaceae is now defined as a

much smaller family than it had been in the twentieth century (Charles *et al.*, Toru, 2007). Pandaceae, Phyllanthaceae, Picrodendraceae, Putranjivaceae, Peraceae and Centroplacaceae have been removed from it (Stevens, 2001).

However, the obsolete, older concept of Euphorbiaceae, known as Euphorbiaceae *sensu lato*, is sometimes still used for continuity and convenience (Hoffmann *et al.*, 2006). It was the subject of a book and two papers which stood as the standard works on Phyllanthaceae until that family was revised by Hoffmann and co-authors in 2006.

1.4 Taxonomy of the plant family Phyllanthaceae

Taxonomically, the family Phyllanthaceae is divided into two subfamilies: Antidesmatoideae and Phyllanthoideae. Antidesmatoideae is divided into six tribes and Phyllanthoideae is divided into four. The tribe Antidesmateae of Antidesmatoideae and the tribes Brideliaceae and Wielandieae of Phyllanthoideae are further divided into subtribes (Wikipedia).

Phyllanthaceae comprises about 2000 species which are grouped into 54 to 60 genera. Some of the genera are poorly defined, and the number of genera in the family is likely to change as the classification is further refined. The genus *Phyllanthus*, one of the largest genera of flowering plants, with over 1200 species, has more than half of the species in the family (Kathriarachchi *et al.*, 2006). Some of the genera have recently been "absorbed" by others, while other genera have recently been divided. The largest genera and the approximate number of species in each are *Phyllanthus* (1270); *Cleistanthus* (140); *Antidesma* (100); *Aporosa* (90); *Uapaca* (60); *Baccaurea* (50) and *Bridelia* (50) (Stevens, 2001).

Table 1.1: List of 54 genera from the 2006 revision of Phyllanthaceae (Hoffman *et al.*, 2006).

Genera		
▪ <i>Actephila</i>	▪ <i>Didymocistus</i>	▪ <i>Nothobaccaurea</i>
▪ <i>Amanoa</i>	▪ <i>Discocarpus</i>	▪ <i>Oreoporanthera</i>
▪ <i>Andrachne</i>	▪ <i>Distichirrhops</i>	▪ <i>Pentabrachion</i>
▪ <i>Antidesma</i>	▪ <i>Flueggea</i>	▪ <i>Phyllanthus</i>
▪ <i>Apodiscus</i>	▪ <i>Gonatogyne</i>	▪ <i>Plagiocladus</i>
▪ <i>Aporosa</i>	▪ <i>Heywoodia</i>	▪ <i>Poranthera</i>
▪ <i>Ashtonia</i>	▪ <i>Hieronyma</i>	▪ <i>Protomegabaria</i>
▪ <i>Astrocasia</i>	▪ <i>Hymenocardia</i>	▪ <i>Pseudolachnostylis</i>
▪ <i>Baccaurea</i>	▪ <i>Jablonskia</i>	▪ <i>Richeria</i>
▪ <i>Bischofia</i>	▪ <i>Keayodendron</i>	▪ <i>Savia</i>
▪ <i>Bridelia</i>	▪ <i>Lachnostylis</i>	▪ <i>Securinega</i>
▪ <i>Celianella</i>	▪ <i>Leptonema</i>	▪ <i>Spondianthus</i>
▪ <i>Chascotheca</i>	▪ <i>Leptopus</i>	▪ <i>Tacarcuna</i>
▪ <i>Chonocentrum</i>	▪ <i>Lingelsheimia</i>	▪ <i>Thecacoris</i>
▪ <i>Chorisandrachne</i>	▪ <i>Maesobotrya</i>	▪ <i>Uapaca</i>
▪ <i>Cleistanthus</i>	▪ <i>Margaritaria</i>	▪ <i>Wielandia</i>
▪ <i>Croizatia</i>	▪ <i>Martretia</i>	▪ <i>Zimmermannia</i>
▪ <i>Dicoelia</i>	▪ <i>Meineckia</i>	▪ <i>Zimmermanniopsis</i>

1.5 Botanical features of plant family Phyllanthaceae (Hoffman *et al.*, 2006)

Phyllanthaceae is an unusually diverse family for its moderate size. It can be recognized only by a combination of characters because there are a few exceptions to almost everything that is generally true of the family. It is most notable for having two ovules in each locule of the ovary, a trait that clearly distinguishes it from Euphorbiaceae.

1.5.1 Habit and leaf form

The Phyllanthaceae are nearly all trees, shrubs or herbs. A few are climbers or succulents, and one species, *Phyllanthus fluitans* is aquatic. Unlike many of the Euphorbiaceae, none have latex, and only a very few produce a resinous exudates. Any hairs, if present, are almost always simple. Rarely are they branched or scale-like. Thorns and other armament are rare.

Stipules are produced with each leaf, but in some, these fall before the leaf is fully mature. Leaves are present, except for a few species of *Phyllanthus* that have flattened, leaf like stems called cladodes that bear flowers along their edges. The leaves are compound in *Bischofia*, but otherwise simple and usually alternate. Rarely are they opposite, in fascicles, or in whorls around the stem. The leaf margin is almost always entire, rarely toothed. A petiole is nearly always present, often with a pulvinus at its base.

1.5.2 General anatomy

Plants with laticifers (commonly, articulated or non-articulated)

1.5.3 Stem anatomy

Cork cambium present; initially deep-seated (rarely); or superficial. Nodes tri-lacunar, or unilacunar. Primary vascular tissue bicollateral, or centrifugal. Cortical bundles present (occasionally); or absent. Medullary bundles present (occasionally); or absent. Internal phloem present (occasionally); or absent. Secondary thickening developing from a conventional cambial ring (mostly, even in lianes); or anomalous; from a single cambial ring. The secondary phloem stratified into hard (fibrous) and soft (parenchymatous) zones (very rarely. e.g. in *Bridelia*); or not stratified. 'Included' phloem present (occasionally, e.g. *Dalechampia*); or absent. Xylem with libriform fibres. Vessel end-walls scalariform, or simple (usually). Vessels with vestured pits (rarely); or without vestured pits. Wood parenchyma apotracheal, or paratracheal (or very sparse, or absent).

1.5.4 Reproductive type, pollination

Plants monoecious, or dioecious, or hermaphrodite. Pollination entomophilous.

1.5.5 Inflorescences, floral, food and seed morphology

The inflorescences are usually in the axils of leaves, rarely below the leaves or at the ends of stems. In *Uapaca*, the flowers are in a pseudanthium, a tight bundle of flowers that resembles a single flower. Except for four species of *Aporosa*, the flowers are unisexual, the plants being either monoecious or dioecious. The flowers are actinomorphic in form. The sepals are three to eight in number, usually free from each other. Petals may be absent or present. If present, there are usually four to six, and their color is yellow to green, or rarely, pink or maroon. A nectary disk is often present. It may be in the form of a ring, or divided into segments. The stamens are three to ten in number, or rarely more, free or variously fused. The fruit is a schizocarp, drupe, or berry. In some, the schizocarp breaks up explosively. The ovary is superior. The number of locules in the ovary is highly variable, usually from two to five, but sometimes as many as fifteen. The placentation is apical, with a pair of ovules hanging by their funicles from the top of each locule. Often, only one of the ovules will develop into a seed. A single, massive obturator may cover the micropyles of both ovules, or each ovule may have its own thin obturator. The mega gametophyte is of the *Polygonum* type. The style is usually 2-lobed or bifid, sometimes entire, or rarely multifid.

1.5.6 Physiology and biochemistry

Cyanogenic or not cyanogenic. Cyanogenic constituents tyrosine derived or phenylalanine derived or of Hegnauer's 'Group C'. Alkaloids present (commonly); or absent. Iridoids not detected. Proanthocyanidins present (rarely); or absent; when present, cyanidin and delphinidin (in one *Phyllanthus* species). Flavonols present, or absent; kaempferol, or kaempferol and quercetin. Ellagic acid present or absent. Arbutin absent. Saponins/sapogenins present (rarely); or absent. Aluminium accumulation demonstrated (but in relatively few genera); or not found. Sugars transported as sucrose or as sugar alcohols + oligosaccharides + sucrose (e.g. *Phyllanthus*).

1.6 Medicinal uses of Phyllanthaceae species in Bangladesh

Traditional use of some ethno medicinal plants of Phyllanthaceae species are described in Table 1.2 (Ahmed *et al.*, 2009).

Table 1.2: Medicinal uses of some Phyllanthaceae species growing in Bangladesh (Ahmed *et al.*, 2009).

Species	Plant part	Use
<i>Antidesma ghaesembilla</i> Gaertn.	• Bark	Astringent and tonic, jaundice, fever Rheumatoid arthritis, etc.
	• Leaf	Wound healing, severe headache, skin disease, abdominal swelling.
<i>Antidesma acidum</i> Retz.	• Leaf paste	Diabetes, insanity, lumps in throat, remitting fever, lymphoma.
<i>Antidesma roxburghii</i> Wall.	• Whole plant	Hysteria, insanity, epilepsy, dysentery, facial paralysis, febrile convulsion, gout, post natal indigestion, remitting fever, stomach disorders.
<i>Antidesma huius</i> Spreng.	• Leaf	Snakebite, syphilitic catexia.
<i>Antidesma montanum</i> Spreng.	• Leaf	Ulcer and lumber pain.
	• Root	Stomachache.
<i>Phyllanthus acidus</i> L. Skeels.	• Fruit	Astringent, liver tonic, constipation, piles, biliousness.
	• Root & seed	Cathartic.
<i>Phyllanthus emblica</i> L.	• Seed	Asthma, bronchitis and biliousness
	• Fruit	Laxative, diuretic in heart disease, liver complaint, piles, dysentery and diarrhea.
	• Flower	Cooling agent and as snuff in nasal hemorrhage.
<i>Phyllanthus maderaspatensis</i> L.	• Leaf	Expectorant and diaphoretic.
	• Seed	Carminative, laxative, liver tonic and ophthalmic. Bronchitis, pain in ear.
<i>Phyllanthus niruri</i> L.	• Whole plant	Diuretic in gonorrhea and other genitourinary disease.
	• Root	Jaundice.
	• Leaf, bark	Bruises, wounds, scabby infection and purgative.
<i>Phyllanthus reticulatus</i> Poir.	• Whole plant	Smallpox and syphilis.
	• Leaf juice	Dysentery.
	• Fruit	Astringent, inflammation and blood disease.

1.7 The plant genus *Bridelia* Willd.

Bridelia is a plant genus of the family Phyllanthaceae. This genus comprises approximately between 60 and 70 species and found throughout tropical and subtropical regions of the world, mainly in Africa, Australia and Asia. About 50 species are distributed in Tropical Africa, Madagascar, Yemen and in Asia ranging from India and South China throughout Indochina, Malaysia to North Australia and the Solomons and Vanuatu Islands. The species in Southeast Asia are usually a part of the primary and secondary forest vegetation either as large trees or as shrubs/smaller trees in the under storey. Some species are scrambling and one is reported to be a climber. Some different species seem to be restricted to a certain type of habitat, e.g. *Bridelia parvifolia* sand dunes, *Bridelia oligantha* dry savannahs. The species occur from the sea level up to 1800 m. (Ngueyem *et al.*, 2009)

The genus *Bridelia* was named in honor of Samuel Elisée Bridel-Brideri by the German botanist Carl Ludwig Willdenow. *Bridelia* species are used as food plants by the larvae of some *Lepidoptera* species including *Endoclita malabaricus*.

The genus is also known as:

- | | |
|--------------------------------------|---|
| 1. <i>Candelabria</i> Hochst. | 3. <i>Pentameria</i> Klotzsch ex Baill. |
| 2. <i>Gentilia</i> A.Chev. & Beille. | 4. <i>Tzellemtinia</i> Chiov. |

1.7.1 Taxonomic hierarchy of the genus *Bridelia*

- **Domain:** Eukaryota
- **Kingdom:** Plantae
 - **Subkingdom:** Viridaeplantae
 - **Phylum:** Tracheophyta
 - **Division:** Magnoliophyta
 - **Class:** Magnoliopsida
 - **Subclass:** Dilleniidae
 - **Order:** Malpighiales
 - **Family:** Phyllanthaceae
 - **Tribe:** Brideliace
 - **Genus:** *Bridelia*

1.7.2 *Bridelia* species available in Bangladesh

Some available species of *Bridelia* are listed in the table below:

Table 1.3: Some *Bridelia* species available in Bangladesh.

| | | |
|--|--|-------------------------------------|
| 1. <i>B. atroviridis</i> Mull. Arg. | 8. <i>B. grandis</i> Pierre. | 15. <i>B. scleroneuroide</i> Pax. |
| 2. <i>B. balansae</i> Tutchet. | 9. <i>B. ndellensis</i> Beille. | 16. <i>B. scleroneura</i> Mull. Arg |
| 3. <i>B. brideliifolia</i> (Pax)
Fedde. | 10. <i>B. ovata</i> Decne. | 17. <i>B. siamensis</i> Craib. |
| 4. <i>B. cathartica</i> Bertol. | 11. <i>B. pervilleana</i> Baill. | 18. <i>B. stipularis</i> (L) Blume. |
| 5. <i>B. crenulata</i> Roxb. | 12. <i>B. retusa</i> (L) Spreng. | 19. <i>B. tomentosa</i> Blume. |
| 6. <i>B. ferruginea</i> Benth. | 13. <i>B. ripicola</i> Leonard. | 20. <i>B. verrucosa</i> Haines. |
| 7. <i>B. glauca</i> f. <i>balansae</i>
Blume. | 14. <i>B. scandens</i> (Roxb).
Willd. | |

1.7.3 Medicinal uses

Bark, roots and leaves of at least ten *Bridelia* species are used in African and Asian traditional and local medicines for treating several ailments including sexual diseases (*Bridelia atroviridis*); bronchitis (*Bridelia balansae*); anemia (*Bridelia cathartica*); intestine disorders and painful joints (*Bridelia michranta*); dental caries (*Bridelia grandis*); fever, diabetes and diarrhoea (*Bridelia ndellensis*); rheumatism (*Bridelia retusa*); rheumatism, abdominal pain and arthritis (*Bridelia scleroneura*); fever (*Bridelia tomentosa*). The best studied species *Bridelia ferruginea* is used for bladder troubles, diabetes, dysentery, rheumatism pain and for its antimicrobial activity (Ngueyem *et al.*, 2009).

1.7.4 Ethnomedicinal properties and phytoconstituents of some *Bridelia* species

Ethnomedicinal properties and phytoconstituents of some *Bridelia* species are listed below in Table 1.4.

Table 1.4: Ethnomedicinal properties and phytoconstituents of some *Bridelia* species.

| Species and plant part used | Identified phytoconstituents and pharmacological activities | References |
|--|--|--|
| <i>B. atroviridis</i> Mull. Arg.
Bark, leaves | Saponins, tannins, antimicrobial activity, cardiovascular activity | Abbiw, 1990; Agyare <i>et al.</i> , 2006; Corallo <i>et al.</i> , 1997 and Neuwinger, 2000. |
| <i>B. balansae</i> Tut.
Leaves | Balansenate I (9); II (10); bridelone (11); bridelonine (12) | Tsai <i>et al.</i> , 2003. |
| <i>B. brideliifolia</i> Fed.
Twig bark, root, leaves | - | Neuwinger, 2000. |
| <i>B. cathartica</i> Bert.
Roots, leaves, stem bark | Anthocyanins, flavonoids, tannins, anti-anemia for its high amount of iron | Watt and Breyer Brandwijk 1962; Hedberg <i>et al.</i> , 1983; Chhabra <i>et al.</i> , 1990; Ouma <i>et al.</i> , 1997 and Neuwinger 2000. |
| <i>B. crenulata</i> Roxb.
Bark | Luteoforol (3,4,4,5,7-pentahydroxydroxyflavon) (13) | Ramesh <i>et al.</i> , (2001a, b). |
| <i>B. ferruginea</i> Benth.
Fruit, leaves, stem-bark, roots | Tannins, gallocatechin-(4-O-7) epigallocatechin (1); flavonoids and biflavonoids, caffeoyl esters, anti-complement, anti-oxidant, anti-viral, diabetes, anti-inflammatory, anti-microbial activity, effects on rat bladder smooth muscle, quercetin (2); quercetin-3-neohesperidoside (3); rutin (4); myricetin-3-glucoside (5); myricetin-3-rhamnoside (6); 5-demethoxypeltatin-5-O-d-glucopyranoside (7); peltatin-5-O-d-glucopyranoside (8) | Cimanga <i>et al.</i> , 1999, 2001; Tona <i>et al.</i> , 1998; Adebayo and Ishola 2009; Abubakar <i>et al.</i> , 2007; Pieters and Vlietinck 2005; Talla <i>et al.</i> , 2002; Ampofo 1979; Iwu 1980, 1983; Akinpelu <i>et al.</i> , 2000; Olajide <i>et al.</i> , 1999, 2003; Pedersen <i>et al.</i> , 2009; Magassouba <i>et al.</i> , 2007; Onoruvwe <i>et al.</i> , 2001; Akinpelu <i>et al.</i> , 2000; Olajide & Makinde Modupe 2000; Rashid <i>et al.</i> , 2000; Neuwinger 2000 and Oliver-Bever 1986. |

Table-1.4: Ethnomedicinal properties and phytoconstituents of some *Bridelia* species. cont'd

| Species and plant part used | Identified phytoconstituents and pharmacological activities | References |
|--|--|--|
| <i>B. glauca</i> f. <i>balansae</i> Blume.
Leaves | Bridelionoside, bridelioside, (7 <i>R</i> , 8 <i>S</i>)-5-methoxydihydrodehydrodiconiferyl alcohol-4-O-glucopyranoside, deglochi-dioboside | Sueyoshi <i>et al.</i> , 2006, 2007. |
| <i>B. grandis</i> Pierre
Bark, leaves | Antimicrobial activity against oral streptococci, anti-trypanosomal and anti-plasmodial activity | Ngueyem <i>et al.</i> , 2008; Atindehou <i>et al.</i> , 2004 and Neuwinger 2000. |
| <i>B. micrantha</i>
Bark, leaves, root | Taraxerol (14); gallic and ellagic acid, friedelin (15); delphinidin (16); methyl salicylate, anti-diarrhoeal activity, anti-plasmodial activity, weak cytotoxic activity | Watt and Breyer-Brandwijk 1962; Lin <i>et al.</i> , 2002; Steenkamp (2003); Ajaiyeoba <i>et al.</i> , 2006; Gradé <i>et al.</i> , 2009; Gbolade 2009; Abo <i>et al.</i> , 2008; Ssegawa and Kasenene 2007; Clarkson <i>et al.</i> , 2004; Hamill <i>et al.</i> , 2003 and Neuwinger, 2000. |
| <i>B. mollis</i> Hutch.
Root | - | Neuwinger, 2000. |
| <i>B. moonii</i> Thwaites.
Bark | Glochidone (17) | Carpenter <i>et al.</i> , 1980. |
| <i>B. monoica</i> Merr.
Leaves, root, stem | Stigmasterol, sitosterol, friedelan-3-ol (18); glutin-5-en-3-ol, friedelin (15) | Hui and Fung, 1968 and Roosita <i>et al.</i> , 2008. |
| <i>B. ndellensis</i> Beille.
Stem bark | Hypoglycemic effect | Sokeng <i>et al.</i> , 2005 and Neuwinger, 2000. |
| <i>B. ovata</i> Decne.
Leaves, stem bark | 24-methyl-lanosta-9(11);25-dien-3-one (19); 24,24-di methyl-lanosta-9(11)-25-dien-3-one (20); campesterol, stigmasterol, sitosterol, friedelin (15); friedelan-3-ol (18); <i>trans</i> -triacyl-4-hydroxy-3-methoxycinnamate | Boonyaratavej <i>et al.</i> , 1992. |
| <i>B. pervilleana</i> Baill.
Leaves | - | Neuwinger, 2000. |

Table-1.4: Ethnomedicinal properties and phytoconstituents of some *Bridelia* species. cont'd

| Species and plant part used | Chemical compounds identified & pharmacological activities | References |
|---|---|--|
| <i>B. retusa</i> (L.) Spreng
Leaves, root, bark | Isoflavone, 4-[(<i>E</i>)-6-methyl-4-oxohept-2-en-2-yl] benzoic acid (21); 4-[(<i>E</i>)-6-methyl-4-oxohept-2,5-dien-2-yl] benzoic acid, 4-[(<i>R</i>)-6-methyl-4-oxohept-5-en-2-yl] benzoic acid (22); 4-[(<i>R</i>)-6-methyl-4-oxoheptan-2-yl] benzoic acid (23); (-)-isochaminic acid (24); 5-allyl 1,2,3-trimethoxy-benzene (elemicin) (25); (+)-sesamin (26); 4-isopropyl benzoic acid, cyanogenic glycolides, triterpenes, ketone and tannins | Jayasinghe <i>et al.</i> , 2003; Jain <i>et al.</i> , 2004; Ayyanar & Ignacimuthu 2005 and Kshirsagar & Singh, 2001. |
| <i>B. rippicola</i> Lenard
Leaves, bark, plant powder | - | Neuwinger, 2000. |
| <i>B. scleroneura</i> Mull. Arg.
Bark, leaves, root, twig bark | Antinociceptive and anti-inflammatory activity | Dimo <i>et al.</i> , 2006; Tabuti <i>et al.</i> , 2003 and Gradé <i>et al.</i> , 2009. |
| <i>B. scleroneuroides</i> Pax. Root | - | Watt and Breyer-Brandwijk, 1962. |
| <i>B. siamensis</i> Craib. Leaves | (-)-Ovatolide (27) | Delgado and Clardy, 1993. |
| <i>B. stipularis</i> Blume
Leaves, roots | Bridelyl alcohol, phlobatannin, taraxenone | Sengupta and Ghosh, 1963 and Desai <i>et al.</i> , 1976. |
| <i>B. tomentosa</i> Blume
Leaves, root, bark | 24-methyl-lanosta-9(11)-25-dien-3-one (19); 24,24-dimethyl-lanosta-9(11)-25-dien-3-one (20) | Boonyaratavej, 1990. |

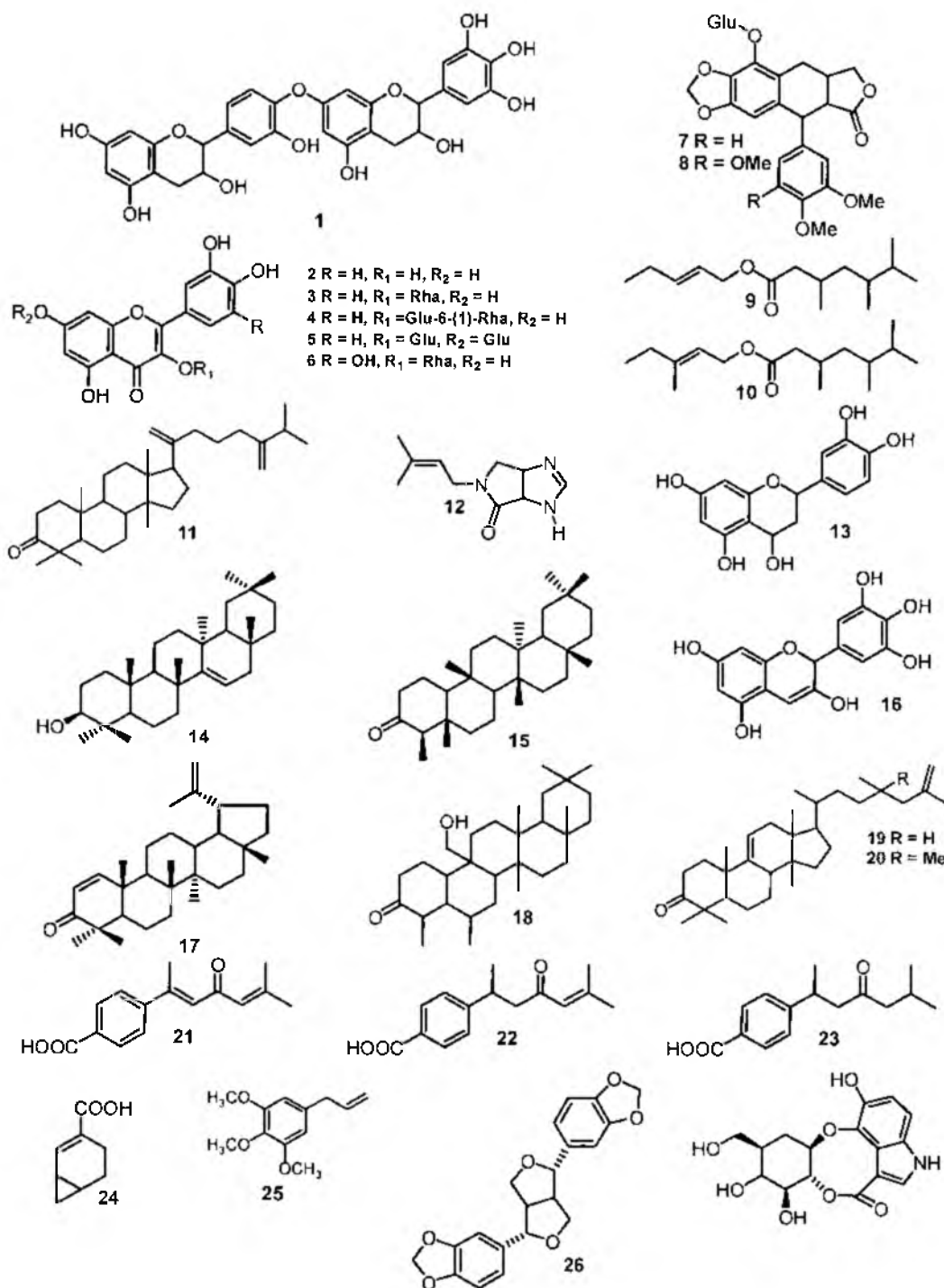


Figure 1.2: Structures of some compounds found in the genus *Bridelia*.

1.8 Description of the investigated plants of the genus *Bridelia*

1.8.1 Taxonomic hierarchy of *Bridelia verrucosa* Haines

- Domain: Eukaryota
- Kingdom: Plantae
 - Subkingdom: Viridaeplantae
 - Phylum: Tracheophyta
 - Division: Magnoliophyta
 - Class: Magnoliopsida
 - Subclass: Dilleniidae
 - Order: Malpighiales
 - Family: Phyllanthaceae
 - Tribe: Brideliaceae
 - Genus: *Bridelia*
 - Species: *B. verrucosa*

1.8.2 Description of the investigated plant *B. verrucosa* (Kirtikar and Basu, 1994)

| | |
|--------------------------|--|
| Taxonomic name | : <i>Bridelia verrucosa</i> Haines |
| Synonyms | : <i>Bridelia montana</i> Willd
<i>Bridelia sikkimensis</i> Gerhmann |
| Common names | : Ghiyai, Gondii, Gayo |
| Vernacular names | : Assam : Kaiso
Bombay : Asano
Nepal : Geio |
| Habit and habitat | : Large shrub or small 3 m strangling tree without thorns, twigs are glabrous and verrucose; occasional in the forest. |
| Leaf | : Leaf-blade elliptic-obovate or broadly elliptic. |
| Perulae | : Pubescent |
| Petioles | : 5 mm long |
| Stipules | : Lanceolate, 2 mm long, acute, glabrous, not persistent. |

- Flowers** : Arranged only in fascicles, which are commonly unisexual, although sometimes bisexual; flowers shortly pedicellate or sessile.
- Male flowers** : Pedicels 1 mm long, glabrous; sepals oblong-lanceolate, 2 mm long, acute, glabrous; petals spatulate-flabelliform, 1 mm long; disc pentagonal, 1 mm diameter; stamina column 1 mm high; anthers 0.5 mm long; pistillode conical, bifid, 0.5 mm long.
- Female flowers** : Sessile; sepals triangular-ovate, 1.5 mm long, sparingly pubescent outside at the base, otherwise glabrous; petals elliptic-oblong, 1 mm long; outer disc roundly pentagonal, inner disc completely enveloping the ovary, only the styles exerted; ovary 1 mm diam., glabrous; styles 2, bifid, 0.5 mm long.
- Fruits** : Ovoid, 5-6 mm long, 4 mm diam. when dried, black; pyrene 1, furrowed, 4 x 3 mm, black.
- Life span** : Perennial.
- Flowering time** : September to November.
- Fruiting time** : October to December.
- Distribution** : Sub-Himalayan Tract, Khasia hills, Northern India from Punjab & Himachal Pradesh to Bihar & Bengal, Nepal, Sikkim, Orissa and Upper Burma.
- Distribution in Bangladesh** : Chittagong hill tracts, especially in Khagrachhori and Panchouri district.
- Medicinal uses** : Anthelmintic and astringent.



1



2

Figure 1.3: *Bridelia verrucosa* Haines flower (1) and leaves with fruits (2).

1.8.3 Taxonomic hierarchy of *Bridelia stipularis* (L) Blume

- **Domain:** Eukaryota
- **Kingdom:** Plantae
 - **Subkingdom:** Viridaeplantae
 - **Phylum:** Tracheophyta
 - **Division:** Magnoliophyta
 - **Class:** Magnoliopsida
 - **Subclass:** Dilleniidae
 - **Order:** Malpighiales
 - **Family:** Phyllanthaceae
 - **Tribe:** Bridelieae
 - **Genus:** *Bridelia*
 - **Species:** *B. stipularis*

1.8.4 Description of the investigated plant *B. stipularis* (Nasir, 2006; Prain, 1963)

| | |
|--------------------------|--|
| Taxonomic name | : <i>Bridelia stipularis</i> (L) Blume. |
| Synonyms | : <i>Bridelia scandens</i> (Roxb.) Willd.
<i>Bridelia dasycalyx</i> Kurz.
<i>Bridelia zollingeri</i> Miq.
<i>Clusia stipularis</i> Linn. |
| Common names | : Harinhara |
| Vernacular names | : Bengali : Patkhowi
Marma : Moo gach, So mui
Tripura : Khaipak |
| Habit and habitat | : Large evergreen scandent shrub attaining 1.5 feet to 2 feet and sometimes more or less climbing shrub; grows in shady, moist forest floors. |
| Leaf | : Leaves 5-20 cm, subcoriaceous, elliptic-obovate or orbicular-oblong. |
| Stipules | : Stipules ovate-triangular, 3-9 × 2-4 mm. |
| Flowers | : Small. in small axillary clusters or long spikes, often subtended by long stipular bracts. Usually 2-6-flowered, sometimes grouped into many-flowered spikes or on terminal small-leaved branches. |
| Male flowers | : Male flowers 0.6-1 cm in diameter; pedicels very short, up to 1 mm; receptacle cup-shaped. |
| Female flowers | : Female flowers up to 12 mm in diam.; pedicels short and stout; receptacle nearly funnel-shaped. |
| Fruits | : Drupes ovoid, 0.7-1.3 × 0.6-1.1 cm, bilocular. Seeds semiovoid, 6-8 × 3-6 mm, yellow to brown, smooth, adaxial surface flattened or slightly concave. |
| Life span | : Perennial. |
| Flowering time | : November to March. |

- Fruiting time** : November to March.
- Distribution** : Nepal, India, Sri-Lanka, Southern China, Indo-China, Malaysia, Indonesia and the Philippines.
- Distribution in Bangladesh** : Forests of Chittagong, Chittagong Hill Tracts, Sylhet, Gazipur, Cox's Bazar, Sayadpur (Rangpur).
- Medicinal use** : Amoebic dysentery, chest pain, constipation, astringent diarrhea, leucoderma and strangury (Nasir, 2006). Decoction of bark is used for cough, fever and asthma. Also hypotensive and hypoglycaemic on animals. Leaves are used for jaundice (Krishnan, 1992). The roots are used as medicine for reducing inflammation. Fruits are used to induce vomiting and as an antitoxic.



1



2

Figure 1.4: *Bridelia stipularis* Blume leaves with fruits (1) and flowers (2).

1.8.5 Taxonomic hierarchy of *Bridelia tomentosa*

- **Domain:** Eukaryota
- **Kingdom:** Plantae
 - **Subkingdom:** Viridaeplantae
 - **Phylum:** Tracheophyta
 - **Division:** Magnoliophyta
 - **Class:** Magnoliopsida
 - **Subclass:** Dilleniidae
 - **Order:** Malpighiales
 - **Family:** Phyllanthaceae
 - **Tribe:** Bridelieae
 - **Genus:** *Bridelia*
 - **Species:** *B. tomentosa*

1.8.6 Description of *B. tomentosa* (Cowan *et al.*, 1929; Krishnan, 1992)

| | |
|--------------------------|--|
| Taxonomic name | : <i>Bridelia tomentosa</i> (Blume) |
| Synonyms | : <i>Bridelia lanceaefolia</i> Roxb.
<i>Bridelia monoica</i> Lour.
<i>Bridelia phyllanthoides</i> W. Fitzg |
| Common names | : Pop-gun seeds |
| Local names | : Khy |
| Vernacular names | : Bengali : Mindri, Sirai |
| Habit and habitat | : Shrubs or small evergreen trees, usually 2 - 5 m tall, rarely up to 12 m tall; bark dark-grey; branches slender and elongate, pubescent when young, glabrous with age; latex absent. Grows in open forest, vine thickets, monsoon forest and the drier types of rain forest. |
| Leaf | : Stipules linear-lanceolate, ca. 7 mm long, usually caducous, pubescent; petioles 3 - 5 mm long, pubescent. |

| | |
|-----------------------------------|--|
| Flowers | : Numerous flowers in each fascicle. Calyx lobes about 1.5-2 x 0.5-0.9 mm. Petals small and inconspicuous, about 0.5-0.6 x 0.4-0.5 mm. |
| Fruits | : Fruits globular, about 6-7 x 4-6 mm, calyx persistent at the base of the fruit. |
| Seeds | : Brown-red, oblong-ovate, 3.5 - 4 mm long and 3 mm wide. |
| Seedlings | : Cotyledons obreniform, wider than long about 7 x 10 mm, apex excavated, base truncate, petiole hairy. |
| Life span | : Perennial. |
| Flowering time | : Almost throughout the year. |
| Fruiting time | : Almost throughout the year. |
| Distribution | : Widespread in shrub lands and wood lands; Malay islands, South China, Taiwan, Philippines, south east Asia, Australia. |
| Distribution in Bangladesh | : Srimangal, Sylhet, Chittagong District and Singhara forest, Dinajpur. |
| Medicinal uses | : Leaves used as herbal medicine for traumatic injury; roots used in epidemic influenza, neurasthenia; barks containing 8% of tannin and used as astringent and colic. |



Figure 1.5: *Bridelia tomentosa* Blume leaves with fruits (1) and leaves (2).

1.9 The plant family: Fabaceae or Leguminosae

Systematic position

Class: Dicotyledonae

Sub-class: Polypetalae

Series: Calyciflorae

Order: Rosales

Family: Fabaceae (From ITIS report)

The Fabaceae or Leguminosae, commonly known as the legume, pea, or bean family, is a large and economically important family of flowering plants. The group is the third largest land plant family with 730 genera and over 19,400 species, behind only the Orchidaceae (orchids) and Asteraceae (daisies, sunflowers) and second only to Poaceae (grasses) in terms of agricultural and economic importance.. It is surpassed in size only by the orchid family (Orchidaceae) with about 20,000 species and the sunflower family (Asteraceae) with about 24,000 species. The largest genera are *Astragalus* (over 2,400 species); *Acacia* (over 950 species); *Indigofera* (around 700 species); *Crotalaria* (around 700 species); and *Mimosa* (around 500 species).

Legumes includes a large number of domesticated species harvested as crops for human and animal consumption as well as for oils, fiber, fuel, fertilizers, timber, medicinals, chemicals, and horticultural varieties (Lewis *et al.*, 2005). In addition, the family includes several species studied as genetic and genomic model systems (e.g., pea, *Pisum sativum*, barrel medic, *Medicago truncatula* and trefoil, *Lotus corniculatus*).

Legumes vary in habit from annual and perennial herbs to shrubs, trees, vines and even a few aquatics. Ranging in size from some of the smallest plants of deserts and arctic or alpine regions to the tallest of rain forest trees, legumes are a conspicuous, and often dominant, component of most of the vegetation types distributed throughout temperate and tropical regions of the world (Rundel, 1989). Legumes are particularly diverse in tropical forests and temperate shrub lands with a seasonally dry or arid climate. This preference for semi-arid to arid habitats is related to a nitrogen demanding metabolism. While many species have the ability to colonize barren and marginal lands because of their capacity to "fix" atmospheric nitrogen via a symbiotic association with root-nodulating bacteria, this is just one of several ways in which legumes obtain high levels of nitrogen to meet the demands of their metabolism (McKey, 1994 and Sprent, 2001).

Over the past 30 years, the study of legume classification and biology has benefited from major advances in our understanding of the morphology, evolution and systematics and ecology of the family (Polhill, 1994; Lewis *et al.*, 2005). Much of this knowledge is recorded in the venerable *Advances in Legume Systematics* series, first published in 1981 by the Royal Botanic Gardens at Kew, United Kingdom. Coming 25 years after publication of the first volumes of *Advances in Legume Systematics* (Polhill and Raven, 1981, parts 1 & 2); the most recent authoritative (and only illustrated) guide to the family, *Legumes of the World*, by Lewis, Schrire, Mackinder, and Lock (2005, Royal Botanic Garden, Kew) continues that tradition.

A number of important agricultural plants, including: *Glycine max* (soybean); *Pisum sativum* (pea); *Cicer arietinum* (chickpeas); *Medicago sativa* (alfalfa); *Arachis hypogaea* (peanut) and *Glycyrrhiza glabra* (licorice) are among the best known members of Fabaceae. A number of species are also weedy pests in different parts of the world,

including: *Cytisus scoparius* (broom); *Ulex europaeus* (gorse); *Pueraria lobata* (kudzu) and a number of *Lupinus* species.

1.10 Taxonomy of the plant family Fabaceae

Taxonomically, Fabaceae has been traditionally divided into three subfamilies, the Caesalpinioideae, Mimosoideae, and Papilionoideae (although sometimes these have been ranked as separate families, as in Caesalpiniaceae, Mimosaceae, and Papilionaceae); and considered most closely related to the Connaraceae and Sapindaceae on the basis of anatomy, morphology, and biogeographic distributions (reviewed in Polhill and Raven, 1981). The recognition of three subfamilies is based on characteristics particularly of the flower, including size, symmetry, aestivation of petals, sepals (united or free); stamen number and heteromorphy, pollen (single or polyads); but also presence of a pleurogram, embryo radicle shape, leaf complexity, and presence of root nodules (reviewed in Lewis *et al.*, 2005). Differences in these characteristics led to the view that the Mimosoideae and Papilionoideae are unique and distinct lineages in the family which arose independently within a paraphyletic "basal" caesalpiniod assemblage (Polhill, 1994).

As Lewis *et al.*, 2005 pointed out, while there has been some disagreement as to whether Fabaceae should be treated as one family (composed of three subfamilies) or three, there is a growing body of evidence from morphology and molecules to support the legumes being one monophyletic family. This view has been reinforced not only by the degree of inter relatedness of taxonomic groups within the legumes compared to that between legumes and its relatives, but also by recent molecular phylogenetic studies (Doyle *et al.*, 2000; Kajita *et al.*, 2001; Wojciechowski, 2003; Wojciechowski *et al.*, 2004) showing strong support for a monophyletic family that is more closely related to Polygalaceae, Surianaceae, and Quillajaceae, which together form the order Fabales (Angiosperm Phylogeny Group, 2003).

So, taxonomically the three recognized subfamilies of Fabaceae are

1. **Papilionoideae**
2. **Caesalpinioideae**
3. **Mimosoideae**

The recognition of three subfamilies is based on characteristics particularly of the flower, including size, symmetry, aestivation of petals, sepals (united or free); stamen number and heteromorphy, pollen (single or polyads); but also presence of a pleurogram, embryo radicle shape, leaf complexity, and presence of root nodules (Lewis *et al.*, 2005).

1.10.1 Papilionoideae:

Members of the subfamily Papilionoideae have true papilionaceous flowers in which the upper petal is outside the lateral petals in the bud. This subfamily includes 476 genera and 14,000 species, most of the members of the Fabaceae family with typical pea-like flowers are *Dalea* (smoke tree); *Lupinus* (lupine); *Lathyrus* (sweet pea); *Erythrina* (coral tree); *Robinia* (black locust) and *Astragalus* (locoweed) etc. One acceptable alternative name for the subfamily Papilionoideae is Faboideae.

1.10.2 Caesalpinioideae:

Members of the subfamily Caesalpinioideae have flowers that are bilateral, typically with five distinct petals, upper petal (banner) enveloped in the bud by the lateral wings. This subfamily includes 153 genera *Cassia* (senna); *Cercis* (redbud); *Bauhinia* (orchid tree); *Cercidium* (palo verde); *Parkinsonia* (Jerusalem thorn); *Caesalpinia* (Brazil wood); *Haematoxylum* (logwood); *Ceratonia* (carob); *Tamarindus* (tamarind) and *Delonix* (royal poinciana) etc.

1.10.3 Mimosoideae:

Members of the subfamily Mimosoideae have flowers with radial symmetry, small, inconspicuous corollas and numerous, showy stamens. The flowers are typically in many flowered heads or spikes. This subfamily includes about 80 genera of which *Acacia* (wattle); *Albizia* (silk tree); *Samanea* (monkey pod); *Prosopis* (mesquite) and *Calliandra* (powder puff) etc are well known.

Table 1.5: A short synoptic list of genera distributed in various sub families under Fabaceae family (Free Dictionary by Farlex).

| Sub family | Some well known genera of the sub family | Image |
|-------------------------|---|---|
| Papilionoideae | <i>Stizolobium, Ononis, Myroxylon, Onobrychis, Ormosia, Oxytropis, Pachyrhizus, Dalea, Parochetus, Phaseolus, Pickeringia, Piscidia, Dalbergia, Glycine, Desmodium, Desmanthus, Crotalaria, Daviesia, Dipogon, Coronilla, Genista, Castanospermum, etc.</i> |  <p>Figure 1.6: <i>Castanospermum australe</i></p> |
| Caesalpinioideae | <i>Acrocarpus, Bauhinia, Senna, Petteria, Parkinsonia, Gleditsia, Delonix, Chamaecrista, Ceretonia, Cassia, etc.</i> |  <p>Figure 1.7: <i>Bauhinia galpinii</i></p> |
| Mimosoideae | <i>Mimosa, Acacia, Adinathera, Albizia, Anadenanthera, Albizzia, Leukaena, Lysiloma, Calliandra, Enterolobium, Pithecolibium, etc.</i> |  <p>Figure 1.8: <i>Albizia distachya</i></p> |

1.11 Botanical features of Fabaceae

1.11.1 Habit

The plants are trees, shrubs, herbs or lianas; resinous, or not resinous. The family includes herbs, shrubs, trees and vines distributed throughout the world, especially the tropical rain forest and even a few aquatics.

Ranging in size from some of the smallest plants of deserts and arctic/alpine regions to the tallest of rain forest trees, legumes are a conspicuous, and often dominant, component of most of the vegetation types distributed throughout temperate and tropical regions of the world. Legumes are particularly diverse in tropical forests and temperate shrub lands with a seasonally dry or arid climate. This preference for semi-arid to arid habitats is related to a nitrogen demanding metabolism. While many species have the ability to colonize barren and marginal lands because of their capacity to "fix" atmospheric nitrogen via a symbiotic association with root-nodulating bacteria, this is just one of several ways in which legumes obtain high levels of nitrogen to meet the demands of their metabolism (Lewis *et al.*, 2005).

1.11.2 Leaf form

Morphologically, Fabaceae is characterized by leaves simple to compound (pinnate, rarely palmate, or bipinnate); unifoliate, trifoliate (*Medicago*, *Trifolium*); sometimes phyllodic (many species of *Acacia*); or reduced to a tendril (as in *Lathyrus*); spirally arranged, with stipules present that are sometimes large and leaf-like (*Pisum*) or developed into spines (*Prosopis*, *Robinia*).

1.11.3 Flowers

Flowers are usually regular or irregular (i.e., actinomorphic to zygomorphic in symmetry, respectively); bisexual, with a single superior carpel (hypogynous to perigynous); pentamerous, arranged singly or in racemes, spikes, or heads.

1.11.4 Fruits

The principal unifying feature of this family is the fruit, the legume (Polhill, 1994). The fruit is technically called a legume or pod. It is composed of a single seed-bearing carpel that splits open along two seams. Legume fruits come in an enormous variety of shapes and sizes, including indehiscent pods that do not split open. With a few exceptions, legumes are typically one-chambered pods (one locule); with parietal placentation along the adaxial suture, ovules two to many, in two alternating rows on a single placenta, typically dry and dehiscent along one or both sutures (legume); occasionally constricted into one-seeded sections (loment) or indehiscent (samara, drupe).

1.11.5 Fossil record

The Fabaceae contains over 19,000 extant species widely distributed throughout the world in many ecological settings, from deserts of high latitudes to seasonally dry and wet tropical forests of equatorial regions (Lewis *et al.*, 2005). Legumes appear to be diversified during the Early Tertiary (Herendeen *et al.*, 1992) to become a ubiquitous feature of modern terrestrial biotas, similar to the timing of diversification of several other modern families of angiosperms (e.g., Fagaceae; Manos and Standford, 2001). Although legumes are now highly diverse in tropical to subtropical Africa and South America, the fossil data alone argue against a moist, warm tropical origin.

The fossil record of the Fabaceae is abundant and diverse, particularly in the Tertiary, with fossil flowers, fruits, leaflets, wood, and pollen known from numerous localities; some examples are shown in the figures below (Crepet and Taylor, 1985, 1986; Crepet and Herendeen, 1992; Herendeen, 1992; Herendeen *et al.*, 1992). Although there are several reports of earlier fossils, Sindora-like pollen (subf. Caesalpinioideae) from the Maastrichtian of Canada, Columbia, and Siberia (Raven and Polhill, 1981) and woods similar to *Cassia* s. l. and Mimosoideae from the same time period (e.g., Müller-Stoll and Mädler, 1967); they cannot be assigned unequivocally to legumes. The first definitive legumes appear during the Late Paleocene (ca. 56 Mya; Herendeen, 2001; Herendeen and Wing, 2001; Wing *et al.*, 2004). Representatives of all three traditionally recognized subfamilies, the caesalpinoids, mimosoids, and papilionoids (Polhill *et al.*, 1981); as well as other taxonomically large clades within these subfamilies (e.g., “genistoids”); are recorded from the fossil record soon afterward, beginning around 50 to 55 Mya (Herendeen *et al.*, 1992). Indeed, the occurrence of diverse assemblages of taxa representing all three subfamilies at multiple localities dating from the middle to upper Eocene, especially the Mississippi Embayment of southeastern North America, suggests that most major lineages of woody legumes (except for the tribe Cercideae) were present and extensive diversification had taken place by this time (Herendeen *et al.*, 1992).

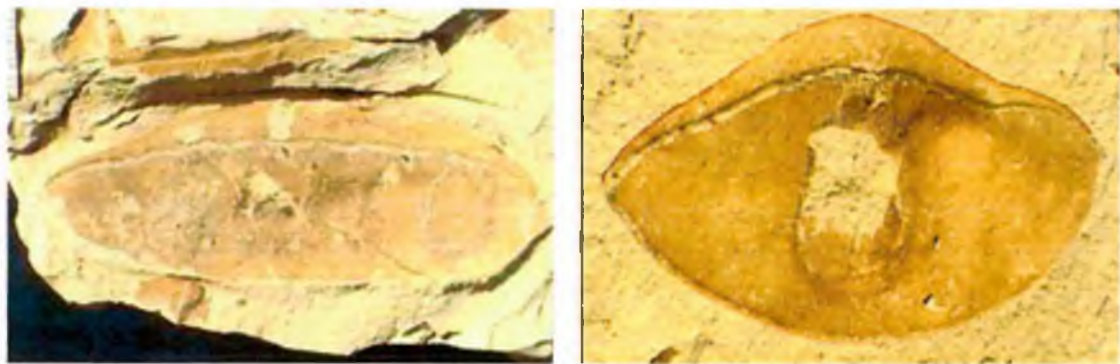


Fig 1.9: *Caesalpinia claibornensis* (Caesalpinioideae) fossil legume (fruit) described from the Middle Eocene (40-50 Mya) Claiborne Formation in western Tennessee.

Left: Holotype (IU 15826-5853) showing outlines of seed chambers. Right: Single-seeded fruit (IU 15826-5848) (Herendeen & Dilcher, 1991).

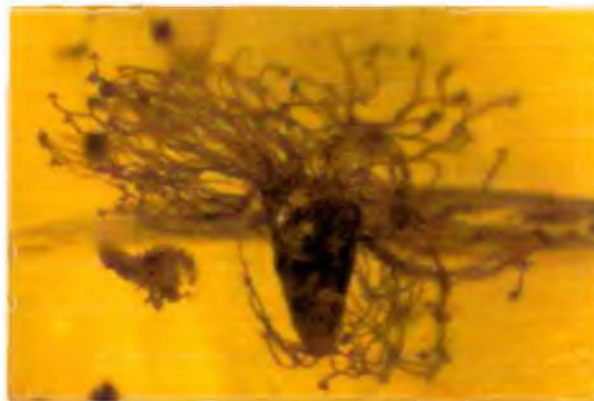


Fig 1.10: *Acacia eocaribbeanensis* (Mimosoideae). Specimen showing numerous stamens with long filaments (USNM 458372, holotype) (Herendeen, Dilcher and Crepet, 1992).



Fig 1.11: *Cladrastis* subgenus *Platycarpa* (Papilionoideae) fossil fruit (Herendeen and Dilcher, 1990).

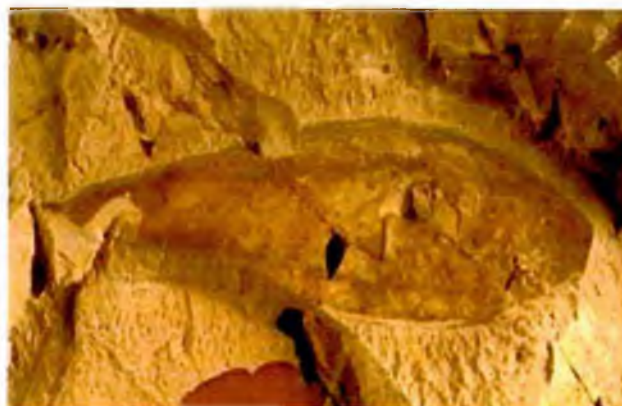


Fig 1.12: *Diplotropis claibornensis* fossil fruit (IU 15826-7235) from the Middle Eocene Claiborne Formation of the southeastern North America (Herendeen and Dilcher, 1990).

Attempts to estimate the age of legumes and diversification in the family, based on molecular sequence data, have been published in recent years. Wikstrom *et al.*, 2001 used a nonmolecular clock based analysis of the three gene data set (plastid *atpB* & *rbcL*, and nuclear 18S rDNA genes; Soltis *et al.*, 2000) with a minimum age of 84 Ma for the split between Fagales and Cucurbitales as an internal calibration point, and estimated an age for Fabaceae of 74-79 Ma. A comprehensive analysis of rates of molecular evolution and estimated ages for crown groups within the legume family has been presented by Lavin *et al.*, 2005. In this study, Tertiary macrofossils that showed distinctive combinations of apomorphic characters or features were used to constrain the minimum age of 12 specific internal nodes to estimate ages of a number of the clades identified in recent family-wide phylogenetic analyses of plastid *matK* (Wojciechowski *et al.*, 2004) and *rbcL* (Kajita *et al.*, 2001) gene sequence data.

Their findings indicate the age of the legume crown clade differs by only 1.0 to 2.5 Ma from the age of the stem clade and the oldest caesalpinoid, mimosoid, and papilionoid crown clades show approximately the same age range of 40 to 59 Ma, findings consistent with a rapid diversification of the family soon after its origin during the Late Paleocene. Remarkably, three large clades that include papilionoids traditionally considered derived (Polhill *et al.*, 1981; Polhill, 1994); the “dalbergioids” (Lavin *et al.*, 2001), “Hologalegina” (Wojciechowski *et al.*, 2000) and “mirbelioids” (Crisp *et al.*, 2000), all

have ages estimates in the 50-Ma time frame or older. One of these, Hologalegina, contains many of the well-known temperate, herbaceous species of legumes grown as food and forage crops (e.g., alfalfa, clovers, peas, and lentils).

1.11.6 Physiology and biochemistry

Nitrogen-fixing root nodules present (seemingly the norm in Papilionoideae and Mimosoideae); or absent (seemingly, from many Caesalpinioideae). Cyanogenic (rarely); or not cyanogenic (mostly). Cyanogenic constituents tyrosine-derived, or phenylalanine-derived, or of Hegnauer's 'Group C', or leucine-derived. Alkaloids present (commonly); or absent. Iridoids not detected. Proanthocyanidins present, or absent; when present, cyanidin, or delphinidin, or cyanidin and delphinidin. Flavonols present (mostly); or absent; kaempferol and quercetin, or quercetin and myricetin, or kaempferol, quercetin, and myricetin. Ellagic acid consistently absent (from 54 species and 41 genera, representing all three subfamilies). Arbutin present, or absent. Aluminium accumulation not found. Sugars transported as sucrose (in numerous species and genera from all three subfamilies). C₃ physiology recorded directly in *Acacia*, *Alysicarpus*, *Amorpha*, *Arachis*, *Astragalus*, *Caragana*, *Cassia*, *Cercidium*, *Crotalaria*, *Dalea*, *Dolichos*, *Genista*, *Gleditsia*, *Glycine*, *Hoffmanseggia*, *Indigofera*, *Lespedeza*, *Lotus*, *Lupinus*, *Medicago*, *Mimosa*, *Olneya*, *Phaseolus*, *Pisum*, *Prosopis*, *Pueraria*, *Robinia*, *Sesbania*, *Spartium*, *Stylosanthes*, *Tephrosia*, *Trifolium*, *Vicia* and *Vigna*. Anatomy non-C₄ type (recorded from numerous genera representing all three subfamilies).

1.11.7 Agricultural and economic importance of legumes

Legumes have demonstrated agricultural importance for thousands of years, beginning with the domestication of lentils (*Lens esculenta*) in Iran dating to 9,500 to 8,000 years ago, their use as a food source during the prehistory of North and South America (beans, more than 3,000 years ago); and their use by the Roman Empire as a food source and for soil improvement (Graham and Vance, 2003). Today legumes are an increasingly invaluable food source not just for humans, accounting for 27% of the world's primary crop production, but also for farm animals (Graham and Vance, 2003). Legumes were grown on more than 13% of the total arable land under cultivation in the world in 2004 (Gepts *et al.*, 2005). Grain legumes alone contribute 33% of the dietary protein nitrogen

needs of humans, while soybeans (*Glycine max*) and peanut (*Arachis hypogaeae*) provide more than 35% of the world's processed vegetable oil and a rich source of dietary protein for the poultry and pork industries (Graham and Vance, 2003).

While they produce nitrogen-containing protein in abundance, legumes are deficient in sulfur containing amino acids and other nutrients needed by people and animals. For this reason, legumes and cereal crops are often raised together to account for the amino acids and other elements they are each deficient in (Gepts *et al.*, 2005). The primary dietary legumes grown, such as bean (*Phaseolus vulgaris*); pea (*Pisum sativum*); chickpea (*Cicer arietinum*); broad bean (*Vicia faba*); pigeon pea (*Cajanus cajan*); cowpea (*Vigna unguiculata*); and lentils (Graham and Vance, 2003); include representatives of each of the four clades within papilionoids, the genistoids, dalbergioids, hologalegina, and phaseoloid/millettoids. Many legumes form root nodules to fix atmospheric nitrogen in a symbiotic relationship with the soil bacteria 'rhizobia'.

Legumes are extremely diverse in their abilities to nodulate, not all species can and there is a wide variety of nodules that form legumes, depending on the species in symbiosis. In addition to their uses as food, legumes are still used as tools in agriculture and forestry as the Romans did. The plants themselves or plant products like leaves and pods can be tilled into the soil as a nitrogen source or legume crops can be rotated with others for soil improvement. These techniques save farmers billions of dollars in the cost of nitrogen fertilizers (Graham and Vance, 2003). Industrially, legumes have many uses in making biodegradable plastics, oils, dyes, and biodiesel fuel.

Legumes are used traditionally in folk medicines, but also demonstrate importance in modern medicine. Isoflavones commonly found in legumes are thought to reduce the risk of cancer and lower cholesterol and soybean phytoestrogens are being studied for use in postmenopausal hormone replacement therapy (Graham and Vance, 2003). Legumes also produce a hypoglycemic effect when eaten, making them a recommended food for diabetics (Gepts *et al.*, 2005).

1.12 Medicinal uses of some Fabaceous species growing in Bangladesh (Ahmed *et al.*, 2009)

Medicinal uses of some plants of Fabaceous species growing in Bangladesh are described in Table 1.6.

Table 1.6: Medicinal uses of some Fabaceous species in Bangladesh.

| Species | Plant part | Use |
|--|-----------------------------------|---|
| <i>Abrus precatorius</i> L. | Bark, leaf | Aphrodisiac, arthritis, rheumatism. |
| <i>Acacia arabica</i> (Lam.) Willd.
Var. <i>indica</i> Benth. | Leaf, root, bark,
flower, seed | Diarrhoea. |
| <i>Acacia catechu</i> (L. f.) Willd. | Leaf, stem | Diarrhoea. |
| <i>Acacia farnesiana</i> (L.) Willd. | Roots | Urinary tract infection, lower
abdominal pain. |
| <i>Adenanthera pavonina</i> L. | Base of trunk | Diarrhoea. |
| <i>Caesalpinia nuga</i> (L.) Aiton. | Plant juice, roots,
fruit | Burning sensation in urinary tract. |
| <i>Cassia alata</i> L. | Leaf, stem | Ringworm, diarrhea. |
| <i>Cassia fistula</i> L. | Root | Diarrhoea. |
| <i>Cassia sophera</i> L. | Whole plant
Roots | Diarrhoea.
Leucorrhea. |
| <i>Cassia tora</i> L. | Leaf | Diarrhoea. |
| <i>Clitoria ternatea</i> L. | Leaf, root | Sexual problems, urinary tract
infections. |
| <i>Desmodium gangeticum</i> (L.) | Leaf, root | Chest pain, sexual problems. |
| <i>Erythrina variegata</i> L. | Leaf | Stomach ache in children. |
| <i>Mimosa pudica</i> L. | Leaf
Bark
Root | Frequent urination, sexual problems,
burning sensations in the vaginal
area, leucorrhoea. |
| <i>Moghania macrophylla</i>
(Willd.) Kuntze | Root | Urinary tract infections, frequent
urination, lack of or infrequent
urination. |
| <i>Pterocarpus santalinus</i> L. | Stem | Conjunctivitis. |
| <i>Senna alata</i> (L) Roxb. | Leaf | Eczema. |
| <i>Tamarindus indica</i> L. | Leaf, Flower | Eye diseases, cataract, rheumatism,
dysentery. |

1.13 The plant genus *Erythrina* L.

Erythrina is a genus of flowering plants in the pea family, Fabaceae. The generic name is derived from the Greek word (*erythros*); meaning "red," referring to the flower color of certain species (Gledhill, D., 2008). It contains about 130 species, which are distributed in tropical and subtropical regions worldwide. Unlike many other genera of forage tree legumes, *Erythrina* is pantropical, consisting of some 112 species, 70 neotropical, 31 African and 12 Asian. Only one species, *E. fusca*, occurs in both the New and Old Worlds. They are trees, growing up to 30 m (98 ft) in height.

Particularly in horticulture, the name coral tree is used as a collective term for these plants. Flame trees is another vernacular name, but may refer to a number of unrelated plants as well. Many species of *Erythrina* have bright red flowers, and this may be the origin of the common name. However, the growth of the branches can resemble the shape of sea coral. Not all species of *Erythrina* have bright red flowers; the Wiliwili (*E. sandwicensis*) has extraordinary variation in its flower colour, with orange, yellow, salmon, green and white all being found within natural populations. This striking colour polymorphism is likely unique in the genus. Other popular names, usually local and particular to distinct species, liken the flowers' red hues to those of a male chicken's wattles, and/or the flower shape to its leg spurs. Commonly seen Spanish names for any local species are *bucaré*, *frejolillo* or *porotillo*, and in Africans some are called *kaffirboom*. *Mullumurikku* is a widespread name in Kerala.

The genus is probably of South American origin but the ability of the seeds to float and retain viability after prolonged immersion in salt water and the probable riverine, coastal or estuarine environments inhabited by the ancestral species have resulted in worldwide distribution. Pollination by birds and a marked ability to hybridise have resulted in a tremendous amount of ecological and morphological diversity, both within and between species, but within rather close cytological and phytochemical relationships. The alkaloids of *Erythrina* are distinct from those of other legumes and they all possess an unusual high activity, low affinity nitrate reductase system distinct from known nitrate reduction patterns in other angiosperms (Neill 1988).

Phytochemical analysis has demonstrated the presence of terpenes in plants from the *Erythrina* genus (Nkengfack *et al.*, 1997); that are also recognized as bioactive alkaloid-rich plants (Ghosal *et al.*, 1971; Barakat *et al.*, 1977) and flavonoids, especially, isoflavones, pterocarpanes, flavanones and isoflavanones (Chacha *et al.*, 2005). Some of these flavonoids have demonstrated a wide variety of biological activities (Table 2). Studies have demonstrated the presence of analgesic and anti-inflammatory effects in extracts obtained from *E. senegalensis*, *E. velutina* and *E. mulungu* (Vasconcelos *et al.*, 2003). In folk medicine, various species are utilized as a tranquilizer, against insomnia and to treat inflammation (Garcia-Mateos *et al.*, 2001).

1.13.1 Taxonomic hierarchy of the genus *Erythrina*

- **Domain:** Eukaryota
- **Kingdom:** Plantae
 - **Subkingdom:** Viridaeplantae
 - **Phylum:** Tracheophyta
 - **Subphyllum:** Euphyllophytina
 - **Class:** Dicotyledone
 - **Subclass:** Polypetaleae
 - **Order:** Fabales
 - **Family:** Fabaceae
 - **Subfamily:** Faboideae
 - **Tribe:** Phaseoleae
 - **Genus:** *Erythrina*

1.13.2 Some available species of *Erythrina*

Some available species of *Erythrina* are listed in Table 1.7.

Table 1.7: Some available species of *Erythrina*.

| | | |
|--------------------------------|------------------------------------|---------------------------------|
| 1. <i>E. abyssinica</i> Lam. | 8. <i>E. humeana</i> Spreng. | 15. <i>E. speciosa</i> Andrews. |
| 2. <i>E. americana</i> Mill. | 9. <i>E. lanceolata</i> Standl. | 16. <i>E. stricta</i> Roxb. |
| 3. <i>E. coralloides</i> D. C. | 10. <i>E. lysistemon</i> Hutch. | 17. <i>E. suberosa</i> Roxb. |
| 4. <i>E. decora</i> Harms. | 11. <i>E. mulungu</i> Diels Mart. | 18. <i>E. tuxtlana</i> Krukoff. |
| 5. <i>E. falcata</i> Benth. | 12. <i>E. polychaeta</i> Harms. | 19. <i>E. velutina</i> Willd. |
| 6. <i>E. fusca</i> Lour. | 13. <i>E. sandwicensis</i> O. Deg. | 20. <i>E. zeyheri</i> Harv. |
| 7. <i>E. herbacea</i> L. | 14. <i>E. schimpffii</i> Diels. | |

1.13.3 Medicinal uses

Erythrina has been used in folk medicine for treatment of insomnia, malaria fever, venereal disease, asthma and toothache. *Erythrina* species are widely prescribed in traditional medicine against frequent diseases from microbial and parasitic origin. The ailments treated are bacterial, fungal, parasitic and viral diseases, gastrointestinal disorders, liver disorders, sexual asthenia, nervous disorders, sterility, eye diseases and kidney pain.

South American Indians used *Erythrina* as a fish poison. In addition, there are reports of its use as a narcotic and anthelmintic. The first compounds isolated from *Erythrina* were alkaloids. β -erythroidine was used for a brief time as a muscle relaxant in surgery and in treatment of schizophrenia. Tea from *Erythrina* flowers is regularly used as a relaxant in Mesoamerica. Subsequently, homoerythrina alkaloids were investigated for their anti-cancer activity. Recently, research involving *Erythrina* has focused on other chemical effects, primarily the antimicrobial action of *Erythrina* lectins and the enzymology of proteinase inhibitors isolated from *Erythrina* (Payne, 1991).

E. abyssinica and *E. senegalensis* are prescribed in ethno veterinary medicine practices against brucellosis, oedema, hygroma, dropsy, bacterial infections, skin diseases (Byavu *et al.*, 2000; Ejobi *et al.*, 2007). *E. vogelii* is used in Cameroon (Ali *et al.*, 2010) against microbial infections. *E. sigmoidea* is prescribed against inflammations (Njamen *et al.*, 2004; Kouam *et al.*, 2007; Udem *et al.*, 2010) and cancer (Watjen *et al.*, 2007).

E. milbraedii is used to prepare remedies against prostate (Tchokouaha *et al.*, 2010). *E. poeppigiana* and *E. fusca* are used against microbial infections (Sato *et al.*, 2003); fever and inflammations (Innok *et al.*, 2009).

All plant parts (leaves, stem barks, roots and flowers) are used for the preparation of remedies in the form of decoction, maceration, infusion, powders or calcinates to treat diseases. The treatments are administered by oral routes and baths.

1.13.4 Phytochemical data analysis of the genus *Erythrina* L.

The phytochemical data analysis showed predominance of alkaloids and flavonoids in the *Erythrina* genus. It is important to note that alkaloids are recognized as markers for plants of this genus in addition to showing a wide array of biological activities, and being important candidates in the development of new drugs.

Some important alkaloids that are distributed within plants from the *Erythrina* genus are erytharbine, erythartine, erysotramidine, erysodine and erysotrine, shown in Figure 1.13. It is noteworthy that a characteristic feature of these alkaloids is the spiro structure in the rings bearing the nitrogen atom.

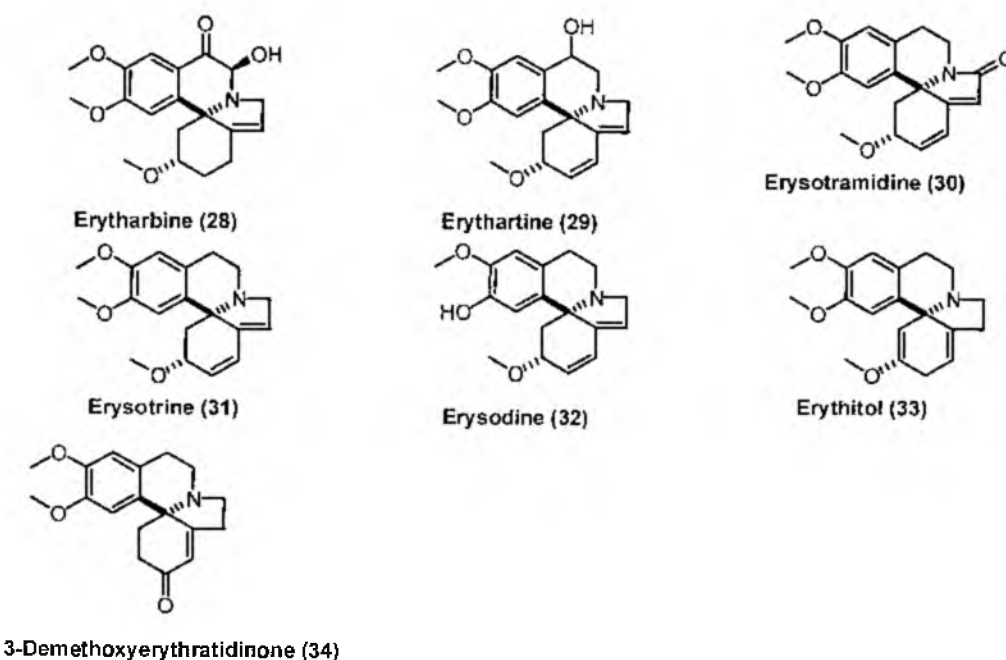


Figure 1.13: Structures of some alkaloids reported from *Erythrina* species.

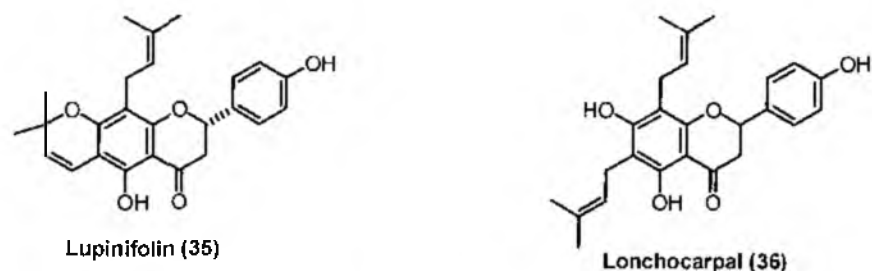


Figure 1.14: Structures of some flavonoids reported from *Erythrina* species.

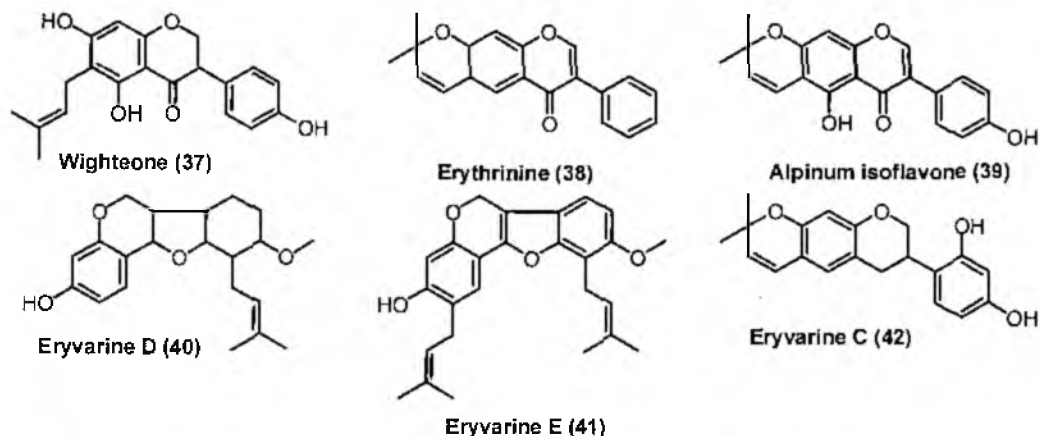


Figure 1.15: Structures of some isoflavonoids reported from *Erythrina* species.

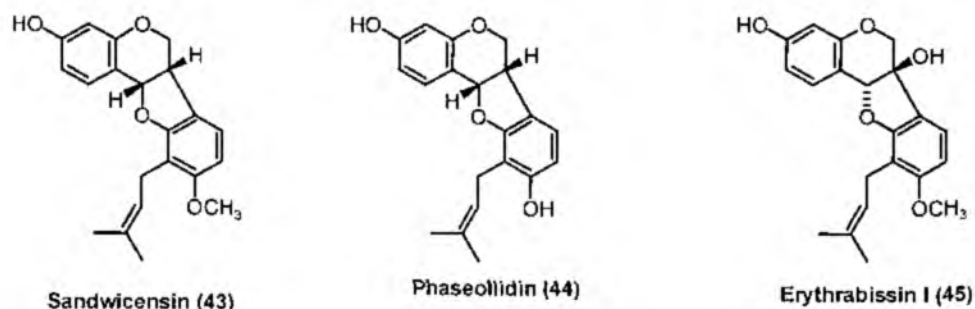


Figure 1.16: Structures of some pterocarpans from *Erythrina* species.

1.13.5 Biological activities of some *Erythrina* species

Analysis of the biological activity data (Table 1.8) shows the wide variety of biological activity of plants from the *Erythrina* genus, and shows too that most of this corroborates with popular knowledge and uses. It is noteworthy to point out that most of these activities, mainly the antibacterial and analgesic properties, confirm the different popular applications of extracts obtained from plants of this genus. Despite the wide array of

available data related to plants of this genus, there is still a need for more research about some of them. The *Erythrina* genus, have the potential to provide new compounds for the development of drugs for the treatment of diseases such as cancer, diabetes and hypertension.

Table-1.8: Biological activity of some *Erythrina* species.

| Species | Plant Part | Biological Activities | Reference |
|---------------------------------------|-----------------------------|--|--|
| <i>E. abyssinica</i> Lam. | Bark, leaf, root, root bark | Mitogenic activity, cell culture, molluscicidal, (<i>Biomphalaria pfeifferi</i>); antibacterial (Gram positive species, <i>Escherichia coli</i>); Anti-yeast (<i>Saccharomyces cerevisiae</i>); antimalárico. | Tachibana <i>et al.</i> , 1993; Kloos, <i>et al.</i> , 1987; Kamat, <i>et al.</i> , 1981; Taniguchi <i>et al.</i> , 1978; Yenesew, <i>et al.</i> , 2004. |
| <i>E. abyssinica</i> Lam. | Bark | Antibacterial (<i>Pseudomonas aeruginosa</i> , <i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i>). | Omer <i>et al.</i> , 1998. |
| <i>E. abyssinica</i> Lam. | Leaf, trunk | Uterine relaxing and stimulant, muscle relaxing and stimulant, peripheric muscle relaxing and stimulant, antidiarrheal, antibacterial (<i>Salmonella typhi</i> , <i>Shigella flexneri</i> , <i>Shigella dysenteriae</i> , <i>Shigella boydii</i> , <i>Shigella sonnei</i>); antiviral, toxic effect in rats. | Chagnon, 1984; Maikere-faniyo <i>et al.</i> , 1989; Vlietinck, <i>et al.</i> , 1995. |
| <i>E. addisoniae</i> Hutch. & Dalziel | Stem bark | Anti-inflammatory. | Talla <i>et al.</i> , 2003. |
| <i>E. americana</i> Mill. | Bark | Plant germination inhibition, molluscicidal. | Dominguez & Alcorn, 1985. |
| <i>E. americana</i> Mill. | Seed | Central nervous system depressor. | Garin-Aguilar <i>et al.</i> , 2000. |
| <i>E. arborescens</i> Roxb. | Leaf, stem | Hypotensive, cytotoxic antispasmodic, uterine stimulant. | Dhar <i>et al.</i> , 1968. |
| <i>E. berteriana</i> Urb. | Leaf | Anti-yeast, antibacterial. | Caceres <i>et al.</i> , 1987. |
| <i>E. brevisflora</i> DC. | Leaf, stem | Cancer induction. | Caldwell & Brewer, 1983. |
| <i>E. caffra</i> Thunb. | Leaf, bark | Antibacterial, COX ₁ inhibitor, | Pillay <i>et al.</i> , 2001. |
| <i>E. cristagalli</i> Linn. | Aerial parts, bark | Anti-inflammatory, analgesic, antibacterial, antifungal. | Mino <i>et al.</i> , 2002; Perez & Anesini, 1994. |

Table-1.8 cont'd: Biological activity of some *Erythrina* species

| Species | Plant Part | Biological Activities | Reference |
|-----------------------------|--------------------------------|--|---|
| <i>E. cristagalli</i> Linn. | Fresh fruit, leaf, stem | Anti-phagocytic. | Yannitsaros, 1996. |
| <i>E. cristagalli</i> Linn. | Leaf, stem | Cytotoxic and antiviral. | Simoes <i>et al.</i> , 1999. |
| <i>E. eriotricha</i> Harms. | Root bark | Antibacterial. | Nkengfack <i>et al.</i> , 1995. |
| <i>E. excelsa</i> Baker | Root bark | Antibacterial, antifungal. | Taniguchi <i>et al.</i> , 1993. |
| <i>E. fusca</i> Lour. | Seeds | Central nervous system depressor. | Widiyanto <i>et al.</i> , 1980. |
| <i>E. glauca</i> Willd. | Bark | Anti-viral | Mc Kee <i>et al.</i> , 1997. |
| <i>E. humeana</i> Spreng. | Bark, leaf | Antibacterial, COX ₁ inhibitor. | Pillay <i>et al.</i> , 2001. |
| <i>E. indica</i> Lour. | Leaf | Antifungal, antibacterial. | Ross <i>et al.</i> , 1980. |
| <i>E. indica</i> Lour. | Leaf | Central nervous system depressor. | Ratnasooriya & Dharmasiri, 1999. |
| <i>E. indica</i> Lour. | Root bark | Anti-mycobacterial, antibacterial. | Waffo <i>et al.</i> , 2000. |
| <i>E. indica</i> Lour. | Stem bark | Cytotoxic. | Nkengfack <i>et al.</i> , 2001. |
| <i>E. latissima</i> Mey. | Bark, leaf | COX ₁ inhibitor, antibacterial. | Pillay <i>et al.</i> , 2001. |
| <i>E. lysistemon</i> Hutch. | Bark, leaf | COX ₁ inhibitor, anti-yeast antibacterial. | Pillay <i>et al.</i> , 2001; Rabe & Van Staden 1997; Motsei <i>et al.</i> , 2003. |
| <i>E. lysistemon</i> Hutch. | Stem bark | Estrogenic, rises seric LDL, bone formation stimulant, anti-diabetic. | Njamen <i>et al.</i> , 2007. |
| <i>E. resupinata</i> Roxb. | Roots | Fetal anti-implantation, uterine stimulant, anti-tumoral, abortive, toxicity evaluation. | Aswal <i>et al.</i> , 1984. |
| <i>E. saculeuxii</i> Hua. | Leaf, root bark | Anti-malarial, cytotoxic. | Gessler <i>et al.</i> , 1994; Gessler <i>et al.</i> , 1995. |
| <i>E. senegalensis</i> DC. | Bark, root, stem bark, flowers | Anti-malarial, analgesic, anti-inflammatory, antibacterial, molluscicidal. | Saidu <i>et al.</i> , 2000; Etkin 1997; Okunji & Iwu 1988; Hussain & Deeni, 1991; Ajaiyeoba <i>et al.</i> , 2004. |
| <i>E. senegalensis</i> DC. | Raiz | Antiviral. | Silva <i>et al.</i> , 1997. |

Table-1.8 cont'd: Biological activity of some *Erythrina* species

| Species | Plant Part | Biological Activities | Reference |
|-------------------------------------|----------------------------|--|---|
| <i>E. sigmoidea</i> Hua. | Bark, root bark, stem bark | Anti-yeast, antibacterial, skeletal muscle relaxing, anti-spasmodic, antifungal. | Biyiti <i>et al.</i> , 1988; Benedicta <i>et al.</i> , 1993; Nkengfack <i>et al.</i> , 1994; Nkeh <i>et al.</i> , 1996. |
| <i>E. stricta</i> Roxb. | Leaf, stem | Spasmolytic, analgesic, diuretic, hypothermic, anti-convulsant, toxicity evaluation, cytotoxic, anti-yeast, anti protozoan, antiviral, antifungal. | Bhakuni <i>et al.</i> , 1988; Dhar <i>et al.</i> , 1968. |
| <i>E. suberosa</i> Roxb. | Leaf, stem bark | Anti-spermatogenic, anti-tumoral, anti-androgen, anti-gonadotropin, cytotoxic, hypo glycaemic, toxicity evaluation of spasm, anti- hypotensive. | Dhar <i>et al.</i> , 1968. |
| <i>E. subumbrans</i> (Hassk.) Merr. | Aerial parts | Fetal anti-implantation, uterine stimulant, anti tumor, abortive effect, toxicity evaluation. | Aswal <i>et al.</i> , 1984. |
| <i>E. ulei</i> Harms. | Bark | Anti-crustacean, DNA linker, anti-oxidant. | Desmarcheilier <i>et al.</i> , 1996. Desmarcheilier <i>et al.</i> , 1997. |
| <i>E. variegata</i> L. | Bark | Anti gastric ulcer. | Muto <i>et al.</i> , 1994. |
| <i>E. variegata</i> L. | Flowers | Anti-yeast, antibacterial. | Avirutnant <i>et al.</i> , 1983. |
| <i>E. variegata</i> L. | Fresh flowers | Anxiolytic. | Flausino <i>et al.</i> , 2007. |
| <i>E. velutina</i> Willd. | Leaf | Anti-inflammatory, analgesic. | Marchioro <i>et al.</i> , 2005. |
| <i>E. vespertilio</i> Benth. | Bark | Inhibition of platelet aggregation, serotonin release inhibition. | Rogers <i>et al.</i> , 2001. |
| <i>E. vogelii</i> Hook.f. | Root bark | Anti-fungal. | Queiroz <i>et al.</i> , 2002. |
| <i>E. zeyheri</i> Harv. | Leaf | Anti-bacterial, COX ₁ inhibitor. | Pillay <i>et al.</i> , 2001. |

1.14 Description of the investigated plants of the genus *Erythrina*

1.14.1 Taxonomic hierarchy of *Erythrina fusca* Lour

- **Domain:** Eukaryota
- **Kingdom:** Plantae
 - **Subkingdom:** Viridaeplantae
 - **Phylum:** Tracheophyta
 - **Subphyllum:** Euphylllophytina
 - **Class:** Dicotyledone
 - **Subclass:** Polypetaleae
 - **Order:** Fabales
 - **Family:** Fabaceae
 - **Subfamily:** Faboideae
 - **Tribe:** Phaseoleae
 - **Genus:** *Erythrina*
 - **Species:** *E. fusca*

1.14.2 Description of *E. fusca* (Orwa *et al.*, 2009)

| | | | |
|--------------------------|--|---|-------------------------------|
| Taxonomic name | : <i>Erythrina fusca</i> Lour. | | |
| Synonyms | : <i>Erythrina atrosanguinea</i> Ridl. | | |
| | <i>Erythrina glauca</i> Willd. | | |
| | <i>Erythrina ovalifolia</i> Roxb. | | |
| Common names | : Pannya mandar, Harikakra, Kanta madar | | |
| Vernacular names | English | : | Coral bean, purple coral tree |
| | Philipino | : | Korung-korung |
| | French | : | Immortelle blanche |
| | Indonesian | : | Cangkring, kane, rase |
| | Thai | : | Thong lang nam |
| Habit and habitat | : Medium to large spreading tree, 10-15 m tall; crown rounded; trunk short, spiny (spines 1-2 cm long); much | | |

- branched, bark brownish-grey or olive-brown, flaky; branches spreading. Most commonly found on the outer margins of swamps on sites that are not permanently flooded.
- Leaf** : Alternate and trifoliate.
- Petioles** : Up to 25 cm long and sometimes sparsely prickly.
- Stipules** : Orbicular and caduceous.
- Flowers** : Pale brick-red or salmon flowers in fascicles scattered along the rachis, covered with deciduous, ferruginous hairs, mostly unarmed peduncle up to 13 cm long.
- Fruits** : Fruit a woody, linear compressed pod, dehiscent, slightly constricted between 3-15 seeds; stipe stout, 1.5 cm long; beak 2 cm long, velvety, ferruginously hairy when young, later glabrescent.
- Seeds** : Oblong-ellipsoid, 12-18 x 5-8 mm, dark brown or black.
- Life span** : Perennial.
- Flowering time** : January to March.
- Fruiting time** : April to June.
- Distribution** : *E. fusca* has the widest distribution of any *Erythrina* species; it grows on coasts and along rivers in tropical Asia (India, Sri Lanka, Myanmar, Indonesia and Bangladesh); Australia, the Mascarene Islands, Madagascar, Africa, and the southern America.
- Distribution in Bangladesh** : Every where in Bangladesh.
- Medicinal uses** : In Thailand, its root, bark and leaves are used as an antipyretic.



(1)



(2)

Figure 1.17: *Erythrina fusca* Lour tree (1) and flowers (2).

1.14.3 Taxonomic hierarchy of *Erythrina variegata* L.

- **Domain:** Eukaryota
- **Kingdom:** Plantae
 - **Subkingdom:** Viridaeplantae
 - **Phylum:** Tracheophyta
 - **Subphyllum:** Euphylllophytina
 - **Class:** Dicotyledone
 - **Subclass:** Polypetaleae
 - **Order:** Fabales
 - **Family:** Fabaceae
 - **Subfamily:** Faboideae
 - **Tribe:** Phaseoleae
 - **Genus:** *Erythrina*
 - **Species:** *E. variegata*

1.14.4 Description of *E. variegata* L. (Website: 1)

| | |
|--------------------------|---|
| Taxonomic name | : <i>Erythrina variegata</i> L. |
| Synonyms | : <i>E. indica</i> Lam.
<i>E. variegata</i> var. <i>Orientalis</i> (L.) Merr |
| Common names | : Tiger's Claw, Indian Coral Tree and Sunshine Tree |
| Vernacular names | : Bengali : Madar, Mandar, Palitamadar
Assam : Modar
Fiji : Drala
Thailand : Thong lang
Vietnam : Vong nem |
| Habit and Habitat | : Thorny deciduous medium to large tree, commonly reaching 15 to 20 m in height in 20 to 25 years; the smooth bark is streaked with vertical lines of green, buff, grey and white; small black prickles cover the stem and branches. It is well adapted to the humid and semi-arid and tropics and subtropics, occurring in zones with annual rainfall of 800 to 1500 mm distributed over a five- to six- month rainy season. |
| Leaf | : The leaves are pinnate with three leaflets. The leaflets are usually variegated, medium to light green, heart shaped, 7 to 12 cm wide and 12 to 18 cm long. |
| Flowers | : Brilliant orange-red flowers emerge in dense, corymbose inflorescences 5 to 7 cm long and 2 to 3 cm wide, usually after the leaves have dropped. Flowering is normally followed by a lavish production of seed. |
| Fruits | : The pods are thick and black-1.5 to 2 cm wide and 15 to 20 cm long. Each contains 5 to 10 egg-shaped seeds. These are glossy brown, red or purple and are 6 to 10 mm in diameter and 12 to 17 mm long. |
| Life span | : Perennial.. |

- Flowering time** : February to May.
- Fruiting time** : April to August.
- Distribution** : Native to the tropical and subtropical regions of eastern Africa, southern Asia, northern Australia, and the islands of the Indian Ocean and the western Pacific Ocean east to Fiji.
- Distribution in Bangladesh** : Forests of Chittagong, Chittagong Hill Tracts, Cox's Bazar and Sylhet, also planted throughout the country.
- Medicinal use** : Leaves are stomachic, anthelmintic, laxative, diuretic, and emmenagogue; applied externally for dispersing venereal buboes, relieve pain of the joints and inflammations; juice is poured into the ear to relief earache and is used as an anodyne in toothache. The bark is astringent, febrifuge anti-bilious and anthelmintic; useful in dysentery and as a collyrium in ophthalmia. The roots are emmenagogue (Yusuf *et al.*, 2009). Total alkaloid fraction from bark showed neuromuscular blocking, smooth muscle relaxant, CNS depressant and anticonvulsant effects (Rastogi & Mehrotra, 1993).



(1)



(2)



(3)

Figure 1.18: *Erythrina variegata* L. tree (1); leaves (2) and flowers (3).



Chapter 2

Phytochemical investigation-Experimental

2.1 Phytochemical Investigation of the experimental plants

Two plant genus and five plant species belonging to the Phyllanthaceae and Fabaceae family were investigated in this study.

| Name of plant | Family | Genus | Plant part |
|----------------------------|----------------|------------------|--------------------|
| <i>Bridelia verrucosa</i> | Phyllanthaceae | <i>Bridelia</i> | Stem bark and leaf |
| <i>Bridelia stipularis</i> | Phyllanthaceae | <i>Bridelia</i> | Stem bark and leaf |
| <i>Bridelia tomentosa</i> | Phyllanthaceae | <i>Bridelia</i> | Stem bark and leaf |
| <i>Erythrina fusca</i> | Fabaceae | <i>Erythrina</i> | Stem bark and leaf |
| <i>Erythrina variegata</i> | Fabaceae | <i>Erythrina</i> | Stem bark and leaf |

The chemical investigations of the first four plants will be discussed here in different sections.

2.2 Phytochemical investigation of *Bridelia verrucosa*

2.2.1 Collection and preparation of the plant material

Leaf and stem bark of *B. verrucosa* was collected from the village of Panchouri, Khagrachhori District in February 2007 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-31376). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature (not more than 40 °C for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.2.2 Extraction of the plant material

The powdered stem bark (550 g) and leaf of *B. verrucosa* (325 g) were separately soaked in 1.5 L and 750 mL of methanol in a clean, round bottomed flask (5 and 3 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 °C) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to get solid residue. The weight of the crude extract obtained from stem bark was 27.5 g and from leaf was 16.25 g.

2.2.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wageningen *et al.* (1993). 5 g of dried methanol extract was triturated with 90 ml of methanol containing 10 ml of distilled water. The crude extract was dissolved completely. This is the mother solution, which was partitioned off successively by three solvents of different polarity; *n*-hexane, carbon tetrachloride and chloroform. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness (Table 2.1) and were used for further analysis.

2.2.3.1 Partitioning with *n*-hexane

The mother solution was taken in a separating funnel. 100 ml of the *n*-hexane was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice; *n*-hexane fractions were collected together and evaporated in a Rotary evaporator.

2.2.3.2. Partitioning with carbon tetrachloride (CCl₄)

To the mother solution left after washing with *n*-hexane, 12.5 ml of distilled water was added and mixed. It was then taken in a separating funnel and extracted with CCl₄ (100 ml X 3). The CCl₄ fractions were collected together and evaporated. The aqueous fraction was preserved for the next step.

2.2.3.3. Partitioning with chloroform (CHCl₃)

To the mother solution that left after washing with *n*-hexane and CCl₄, 16 ml of distilled water was added and mixed uniformly. It was then taken in a separating funnel and extracted with CHCl₃ (100 ml X 3). The CHCl₃ soluble fractions were collected together and evaporated. The aqueous methanolic fraction was preserved as aqueous soluble fraction.

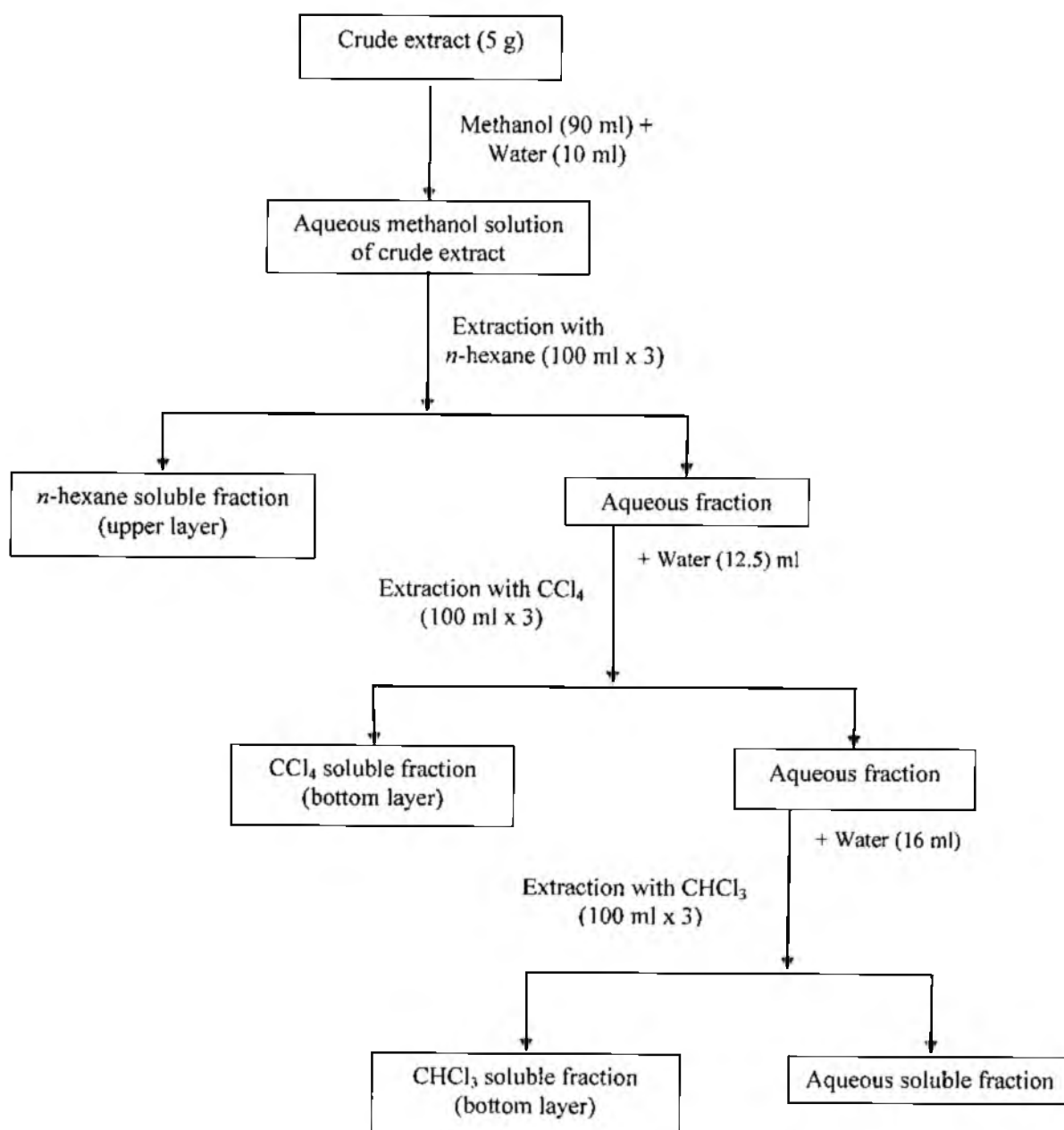


Figure 2.1: Schematic representation of the modified Kupchan partitioning of the stem bark of methanolic extract of *B. verrucosa*.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.1.

Table 2.1: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *B. verrucosa*.

| Plant Part | Weight (g) of methanolic extract for Kupchan partitioning | Fraction code | Weight (g) of soluble fractions |
|------------|---|---------------|---------------------------------|
| Stem bark | 20 | HSF | 3.5 |
| | | CSF | 1.24 |
| | | CHSF | 1.6 |
| | | AQSF | 11.4 |
| Leaf | 10 | HSF | 3.1 |
| | | CSF | 0.62 |
| | | CHSF | 0.62 |
| | | AQSF | 4.0 |

HSF= *n*-hexane soluble fraction; CSF= carbon tetrachloride soluble fraction; CHSF= chloroform soluble fraction; AQSF= aqueous soluble fraction

2.2.4 Gel permeation chromatography of *n*-hexane soluble fraction of the stem bark of *B. verrucosa*

The column was packed with Lipophilic Sephadex (LH-20). At first dried sephadex was soaked in a mixture of solvents with a ratio of *n*-hexane- dichloromethane- methanol (2:5:1) for at least 12 hours for proper swelling. After that, the slurry of sephadex was made and added into a glass column having the length and diameter of 55 cm and 1.1 cm, respectively. When sufficient height of the stationary phase was obtained, a few hundred milliliter of solvent mixture with the same ratio was run through the column for proper packing of the column. The sample (320 mg) was dissolved in this solvent mixture and subsequently applied on top of the column with the help of a Pasteur pipette. The column was then eluted with the same solvent mixture and finally the column was washed with dichloromethane and methanol mixtures of increasing polarity. Solvent systems used as mobile phases in the analysis of extract were listed in Table 2.2.

Table 2.2: Different solvent systems used for gel permeation (Lipophilic Sephadex LH-20) chromatography of *n*-hexane soluble materials of the stem bark of *B. verrucosa*.

| Fraction No. | Solvent systems | Volume collected (mL.) |
|--------------|---|------------------------|
| 1-19 | <i>n</i> -Hexane-dichloromethane-methanol (2:5:1) | 60 |
| 20-22 | Dichloromethane-methanol (9:1) | 25 |
| 23-24 | Dichloromethane-methanol (1:1) | 25 |
| 25-30 | Methanol 100% | 120 |

2.2.5 Analysis of column fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulfuric acid reagent followed by heating at 100-110 °C for 5-10 minutes as well.

Depending on the TLC behavior a number of similar samples were detected and the column fractions were added, these were then purified by employing various techniques. A list of isolated compounds has been summarized in Table 2.3.

Table 2.3: List of isolated compounds from *n*-hexane soluble fraction of the methanolic extract of the stem bark of *B. verrucosa*.

| Column fraction | Mobile Phase | R _f value | Amount (mg) | Code |
|------------------|--------------------------------|----------------------|-------------|---------|
| 11-18 | Toluene- Ethyl acetate (90:10) | 0.71 | 7.23 | BVSP-1 |
| 19-23 | Toluene- Ethyl acetate (95:5) | 0.50 | 6.78 | BVSP-2 |
| Sub column 1-4 | Toluene- Ethyl acetate (95:5) | 0.63 | 5.3 | BVSP-4 |
| Sub column 5-9 | Toluene- Ethyl acetate (95:5) | 0.56 | 7.14 | BVSP-6 |
| Sub column 34-43 | Toluene- Ethyl acetate (95:5) | 0.62 | 6.5 | BVSP-34 |

2.2.5.1 Isolation of compound BVSP-1

The fractions 11 to 18 showed similar TLC feature. So, they were bulked together. After, evaporation of all the solvents, white crystals appeared. The crystals obtained after evaporation of solvents were washed with *n*-hexane to remove the adhered coloring materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound as needles in a sample vial with *n*-hexane and chloroform which was found to be pure by TLC screening. The isolated compound was marked as BVSP-1 (7.23 mg).

2.2.5.2 Isolation of compound BVSP-2

The fractions 19-23 were combined together depending on the identical TLC characterization and subjected to preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated three times for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a purple colored band was visualized. The band was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent afforded a colorless compound BVSP-2 (6.78 mg).

2.2.5.3 Column chromatography (CC) of fractions 11-18

The column was packed with silica gel (Kieselgel 60H, mesh 70-230). Slurry of silica gel was added into a glass column having the length and diameter of 60 cm and 1.5 cm respectively. When sufficient height of the adsorbent bed was obtained, 100 mL of 100% *n*-hexane and then 1% ethyl acetate in *n*-hexane was run through the column for proper packing of the column. The sample was prepared by adsorbing 100 mg of fraction 11-18 onto silica gel (Kieselgel 60H, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with 1% ethyl acetate in *n*-hexane,

followed by mixtures of *n*-hexane and ethyl acetate of increasing polarities. Solvent systems used as mobile phases in the analysis of fraction 11-18 are listed in Table 2.4.

Table 2.4: Different solvent systems used for column chromatography (Kieselgel 60 mesh 70-230) of fractions 11-18 from Sephadex (LH-20) column of *n*-hexane soluble materials of the stem bark of *Bridelia verrucosa*.

| Fraction No. | Solvent Systems | Volume collected (mL)/test tube |
|--------------|---------------------------------------|---------------------------------|
| 1-34 | <i>n</i> -Hexane-Ethyl acetate (99:1) | 20 |
| 35-46 | <i>n</i> -Hexane-Ethyl acetate (98:2) | 20 |
| 47-50 | <i>n</i> -Hexane-Ethyl acetate (95:5) | 20 |

2.2.5.4 Analysis of Column Chromatography fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin-sulfuric acid reagent and then heating at 100-110 °C for 5-10 minutes.

2.2.5.5 Isolation of compound BVSP-4

Fractions 1-4 showing identical spots on TLC were combined in a beaker and was kept at room temperature undisturbed for several days. The crystals obtained after evaporation of solvents were washed with *n*-hexane to remove the adhered coloring materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound designated as BVSP-4 (5.3 mg) as needles.

2.2.5.6 Isolation of compound BVSP-6

The sub-fractions 5-9 were combined together depending on the identical TLC characterization and subjected to preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (95:5) as the developing solvent. The process was repeated five times for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The band was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent afforded a colorless compound BVSP-6 (7.14 mg).

2.2.5.7 Isolation of compound BVSP-34

Column fractions 34-43 gave similar TLC feature and were bulked together. Then it was subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (95:5)]. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating in 110 °C for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray

reagent and eluted with ethyl acetate. Evaporation of the solvent yielded white needles which melted at 163 °C. The compound was analyzed over TLC to check for purity and found to be a pure compound which was termed as BVSP-34 (6.5 mg).

2.2.6 Column chromatography of carbon tetrachloride soluble fraction of the stem bark of *B. verrucosa*

A portion of carbon tetrachloride soluble fraction (270 mg) was subjected to column chromatography (CC) for fractionation. The column was packed with column grade silica gel (Kieselgel 60H, mesh 70-230). Slurry of silica gel in petroleum ether (60-80 °C) was added into a glass column having a length and diameter of 84 and 2 cm, respectively. When the desired height of adsorbent bed was obtained, a few hundred milliliter of solvent was run through the stationary phase for proper packing of the column. After packing, the sample was prepared by adsorbing carbon tetrachloride soluble material onto silica gel (Kieselgel 60H, mesh 70-230) and allowed to dry up to a free flowing powder like mass and subsequently applied on top of the adsorbent layer. Then the column was eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate and then ethyl acetate, finally with ethyl acetate and methanol in order of increasing polarities. The flow was maintained at a constant rate (2 ml/min) for better separation. Solvent system used as mobile phase in CC analysis of carbon tetrachloride soluble material and has been listed in Table 2.5. The eluates were collected in test tubes. A total of 150 fractions were collected each 20 ml.

Table 2.5: Different solvent systems used for the column chromatographic analysis of the carbon tetrachloride soluble fraction of the stem bark of *B. verrucosa*.

| Fraction no. | Solvent systems | Volume collected ml/test tube |
|--------------|--|-------------------------------|
| 1 – 4 | Petroleum ether - Ethyl acetate = (98 : 2) | 20 |
| 5 – 8 | Petroleum ether - Ethyl acetate = (95 : 5) | 20 |
| 9 – 22 | Petroleum ether - Ethyl acetate = (92.5 : 7.5) | 20 |
| 23 – 48 | Petroleum ether - Ethyl acetate = (90 : 10) | 20 |
| 49 – 62 | Petroleum ether - Ethyl acetate = (88 : 12) | 20 |
| 63 – 73 | Petroleum ether - Ethyl acetate = (85 : 15) | 20 |
| 74 – 85 | Petroleum ether - Ethyl acetate = (80 : 20) | 20 |
| 86 – 99 | Petroleum ether - Ethyl acetate = (75 : 25) | 20 |
| 100 – 110 | Petroleum ether - Ethyl acetate = (70 : 30) | 20 |
| 111 – 117 | Petroleum ether - Ethyl acetate = (60 : 40) | 20 |
| 118 – 130 | Petroleum ether - Ethyl acetate = (50 : 50) | 20 |
| 131 – 137 | Ethyl acetate (100%) | 20 |
| 138 – 141 | Ethyl acetate : Methanol = (99 : 1) | 20 |
| 142 – 145 | Ethyl acetate : Methanol = (98 : 2) | 20 |
| 146 – 150 | Ethyl acetate : Methanol = (95 : 5) | 20 |

2.2.7 Analysis of column fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulfuric acid reagent followed by heating at 100-110 °C for 5-10 minutes as well. Depending on the TLC behavior a number of similar samples were detected and the column fractions were added, these were then purified by employing various techniques. A list of isolated compounds has been summarized in Table 2.6.

Table 2.6: List of isolated compounds from carbon tetrachloride soluble fraction of the methanolic extract of the stem bark of *B. verrucosa*.

| Column fraction | Mobile Phase | R _f value | Amount (mg) | Code |
|-----------------|----------------------------------|----------------------|-------------|--------|
| 8-12 | Toluene - Ethyl acetate
90:10 | 0.41 | 7.53 | BVSC-8 |
| 74-76 | Toluene - Ethyl acetate
90:10 | 0.54 | 7.10 | BVBC-2 |

2.2.7.1 Isolation of compound BVSC-8

Fractions 8-12 gave identical TLC features, were combined in a beaker and kept undisturbed at room temperature for several days. Crystals were produced upon evaporation of the solvents. The crystals thus obtained were washed with *n*-hexane to remove adhered color materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound designated as BVSC-8 (7.53 mg) as needles.

2.2.7.2 Isolation of compound BVBC-2

Fractions 74-76 were combined together. Evaporation of solvents yielded a whitish mass which was purified by preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (9:1) as the developing solvent. The process was repeated twice for better separation and isolation of the compound of interest. The desired band was scrapped off and eluted with ethyl acetate to yield BVBC-2 (7.10 mg) as whitish mass.

2.2.8 Column chromatography of chloroform soluble fraction of the stem bark of *B. verrucosa*

A portion of chloroform soluble fraction (500 mg) was subjected to column chromatography (CC) for fractionation. The column was packed with column grade silica gel (Kieselgel 60H, mesh 70-230) and eluted as described in section 2.2.6. Solvent system used as mobile phase in CC analysis of chloroform soluble material has been listed in Table 2.7. The eluates were collected in test tubes. A total of 170 fractions were collected each 20 ml.

Table 2.7: Different solvent systems used for the column chromatographic analysis of the chloroform soluble fraction of the stem bark of *B. verrucosa*.

| Fraction no. | Solvent systems | Volume collected ml/test tube |
|--------------|--|-------------------------------|
| 1 – 3 | Petroleum ether - Ethyl acetate = (98 : 2) | 20 |
| 4 – 6 | Petroleum ether - Ethyl acetate = (95 : 5) | 20 |
| 7 – 18 | Petroleum ether - Ethyl acetate = (92.5 : 7.5) | 20 |
| 19 – 37 | Petroleum ether - Ethyl acetate = (90 : 10) | 20 |
| 38 – 57 | Petroleum ether - Ethyl acetate = (88 : 12) | 20 |
| 58 – 75 | Petroleum ether - Ethyl acetate = (85 : 15) | 20 |
| 76 – 85 | Petroleum ether - Ethyl acetate = (80 : 20) | 20 |
| 86 – 100 | Petroleum ether - Ethyl acetate = (75 : 25) | 20 |
| 101 – 115 | Petroleum ether - Ethyl acetate = (70 : 30) | 20 |
| 116 – 125 | Petroleum ether - Ethyl acetate = (60 : 40) | 20 |
| 126 – 137 | Petroleum ether - Ethyl acetate = (50 : 50) | 20 |
| 138 – 145 | Ethyl acetate (100%) | 20 |
| 146 – 152 | Ethyl acetate - Methanol = (99 : 1) | 20 |
| 153 – 160 | Ethyl acetate - Methanol = (98 : 2) | 20 |
| 161 – 170 | Ethyl acetate - Methanol = (95 : 5) | 20 |

2.2.9 Analysis of column fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulfuric acid reagent followed by heating at 100-110 °C for 5-10 minutes as well. Depending on the TLC behavior a number of similar samples were detected and the column fractions were added, these were then purified by employing various techniques. A list of isolated compounds has been summarized in Table 2.8.

Table 2.8: List of isolated compounds from chloroform soluble fraction of the methanolic extract of the stem bark of *B. verrucosa*.

| Column fraction | Mobile phase | R _f value | Amount (mg) | Code |
|-----------------|------------------------------|----------------------|-------------|--------|
| 59-69 | Chloroform - Methanol (97:3) | 0.42 | 3.54 mg | BVS-65 |

2.2.9.1 Isolation of compound BVS-65

Evaporation of solvents from the fractions 59-69 yielded a yellowish mass which was subjected to preparative TLC over silica gel F₂₅₄ using chloroform-methanol (97:3) as the developing solvent. The desired band was scrapped off on an aluminum foil and eluted initially using a 19:1 mixture of chloroform-methanol (19:1) to yield BVS-65 (3.54 mg).

2.2.10 Properties of the isolated compounds

The physical properties of the isolated compounds and their reactions to vanillin-H₂SO₄ spray reagent are summarized in the Table 2.9.

Table 2.9 Properties of the isolated compounds from stem bark of *B. verrucosa*.

| Isolated compounds | Physical form | Color | Color with vanillin-H ₂ SO ₄ |
|--------------------|---------------|-------------|--|
| BVSP-1 | Crystal | White | Deep purple |
| BVSP-2 | Needles | Colorless | Purple |
| BVSP-4 | Crystal | Transparent | Yellow |
| BVSP-6 | Mass | Whitish | Pink |
| BVSP-34 | Crystal | White | Purple |
| BVSC-8 | Crystal | White | Purple |
| BVBC-2 | Mass | Colorless | Bluish pink which turns into ash |
| BVS-65 | Mass | Yellow | Orange |

2.3 Phytochemical investigation of *Bridelia stipularis*

2.3.1 Collection and preparation of the plant material

Leaf and stem bark of *B. stipularis* was collected from the village of Panchouri, Khagrachhori District in February 2007 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-31378). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature (not more than 40 °C) for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.3.2 Extraction of the plant material

The powdered stem bark (550 g) and leaf (325 g) of *B. stipularis* were separately soaked in 1.5 L and 750 mL methanol in a clean, round bottomed flask (5 and 3 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 °C) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from stem bark was 30 g and from leaf was 15 g.

2.3.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen *et al.*, (1993) as described in section 2.2.3. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness (Table 2.10) and were used for further analysis.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.10.

Table 2.10: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *B. stipularis*.

| Plant Part | Weight (g) of methanolic extract for Kupchan partitioning | Fraction code | Weight (g) of soluble fractions |
|------------|---|---------------|---------------------------------|
| Stem bark | 10 | HSF | 2.75 |
| | | CSF | 0.65 |
| | | CHSF | 0.92 |
| | | AQSF | 4.30 |
| Leaf | 10 | HSF | 3.30 |
| | | CSF | 0.81 |
| | | CHSF | 0.71 |
| | | AQSF | 4.20 |

HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

2.3.4 Vacuum Liquid Chromatography (VLC) of the crude extract of stem bark of *B. stipularis*

2.3.4.1. Packing of Column

The column was packed with fine VLC grade silica gel (Kieselgel 60H). A column having 14 cm length and 10 cm in diameter was packed with the silica gel up to a height of 6 cm under reduced pressure. The column was then washed with petroleum ether to expedite compact packing.

2.3.4.2. Sample Input

The sample was prepared by adsorbing 15 g of crude extract onto column grade silica gel (Kieselgel 60H, mesh 70-230) with continuous stirring under air blower in a porcelain basin. Stirring continued until the sample becomes a free flowing powder. The free flowing sample was then placed on the packed silica into the column. Finally a cotton plug was placed on the sample.

2.3.4.3. Development of Chromatogram

After placing the sample, the column was eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate and finally washed by methanol. Solvent

systems used as mobile phases in the VLC analysis of crude extract were listed in Table 2.11.

Table 2.11: Solvent systems used for VLC analysis of the crude extract of stem bark of *B. stipularis*.

| Fraction no. | Solvent systems | Volume collected (ml) |
|--------------|---|-----------------------|
| 1 | 100% Pet. Ether | 50 |
| 2 | Pet. ether - Ethyl acetate (98 : 2) | 100 |
| 3 | Pet. ether - Ethyl acetate (95 : 5) | 100 |
| 4 | Pet. ether - Ethyl acetate (92.5 : 7.5) | 100 |
| 5 (A+B) | Pet. ether - Ethyl acetate (90 : 10) | 75 + 75 |
| 6 (A+B) | Pet. ether - Ethyl acetate (80 : 20) | 75 + 75 |
| 7 (A+B) | Pet. ether - Ethyl acetate (75 : 25) | 75 + 75 |
| 8 (A+B) | Pet. ether - Ethyl acetate (65 : 35) | 100 + 100 |
| 9 (A+B) | Pet. ether - Ethyl acetate (55 : 45) | 100 + 100 |
| 10 (A+B) | Pet. ether - Ethyl acetate (50 : 50) | 100 + 100 |
| 11 (A+B) | Pet. ether - Ethyl acetate (45 : 55) | 100 + 100 |
| 12 (A+B) | Pet. ether - Ethyl acetate (40 : 60) | 100 + 100 |
| 13 (A+B) | Pet. ether - Ethyl acetate (35 : 65) | 100 + 100 |
| 14 (A+B) | Pet. ether - Ethyl acetate (30 : 70) | 100 + 100 |
| 15 (A+B) | Pet. ether - Ethyl acetate (25 : 75) | 100 + 100 |
| 16 (A+B) | Pet. ether - Ethyl acetate (20 : 80) | 100 + 100 |
| 17 (A+B) | Pet. ether - Ethyl acetate (15 : 85) | 100 + 100 |
| 18 (A+B) | Pet. ether - Ethyl acetate (10 : 90) | 100 + 100 |
| 19 (A+B) | Pet. ether - Ethyl acetate (5 : 95) | 100 + 100 |
| 20 (A+B) | Ethyl acetate (100 %) | 100 + 100 |
| 21 (A+B) | Ethyl acetate - Methanol (98:2) | 50 + 50 |
| 22 (A+B) | Ethyl acetate - Methanol (95:5) | 50 + 50 |
| 23 (A+B) | Ethyl acetate - Methanol (90:10) | 50 + 50 |
| 24 (A+B) | Ethyl acetate - Methanol (80:20) | 50 + 50 |
| 25 (A+B) | Ethyl acetate - Methanol (70:30) | 50 + 50 |

2.3.5 Analysis of VLC fractions by thin layer chromatography (TLC)

All the above VLC fractions were screened by TLC using various solvent systems. The plates were observed under UV light and by spraying with vanillin-sulfuric acid reagent followed by heating at 100-110 °C for 5-10 minutes as well. Depending on the TLC behavior fractions 4 & 5A and 9A-11B were selected for further investigation.

Table 2.12: List of isolated compounds from VLC fraction of the methanolic extract of the stem bark of *B. stipularis*.

| Column fraction | TLC Solvent System | R _f value | Amount (mg) | Code |
|-----------------|---|----------------------|-------------|-------|
| 4 & 5A | <i>n</i> -Hexane - Ethyl acetate (99:1)[3t] | 0.32 | 8.23 | BS-01 |
| 9A - 11B | Toluene - Ethyl acetate (97:3)[2t] | 0.47 | 5.31 | BS-6 |
| 9A - 11B | Toluene - Ethyl acetate (98:2)[5t] | 0.38 | 7.01 | BS-11 |

2.3.5.1 Isolation and purification of compounds from the selected VLC fractions

Five compounds have been isolated and purified from the different VLC fractions by adopting various techniques.

2.3.5.2 Isolation of BS-01

The VLC fractions 4 & 5A were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (99: 1)]. The process was repeated thrice for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of solvent yielded white crystals and was designated as BS-01 (8.23mg).

2.3.5.3 Gel permeation chromatography of VLC fractions 9A-11B of the stem bark of *B. stipularis*

The column was packed as described in section 2.2.4. The sample (450 mg) was dissolved in the solvent mixture (*n*-hexane - dichloromethane - methanol= 2:5:1) and subsequently applied on top of the adsorbent layer with the help of a Pasteur pipette. The column was then eluted with the same solvent mixture and finally the column was washed with dichloromethane and methanol mixtures of increasing polarity. Solvent systems used as mobile phases in the analysis of extract were listed in Table 2.2

Table 2.13: Different solvent systems used for gel permeation (Sephadex LH-20) of VLC fractions 9A-11B of the stem bark of *B. stipularis*.

| Fraction No. | Solvent systems | Volume collected (ml) |
|--------------|---|-----------------------|
| 1-36 | <i>n</i> -Hexane - Dichloromethane - Methanol (2:5:1) | 75 |
| 37-47 | Dichloromethane – Methanol (9:1) | 25 |
| 48-59 | Dichloromethane – Methanol (1:1) | 50 |
| 50-55 | Methanol 100% | 120 |

2.3.5.3.1 Isolation of BS-06

The LH-50 column fractions 20-26 of the VLC column fraction 9A-11B were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (97:3)]. The process was repeated twice for better separation of the desired band. A small portion of

the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The unsprayed portion of the band at stationary phase was scrapped off on aluminium foil and eluted with ethyl acetate. Evaporation of ethyl acetate yielded white needle like crystals which was checked for purity and termed as BS-06 (5.31 mg).

2.3.5.3.2 Isolation of BS-11

The yellowish color of LH-50 column fractions 20-26 of the VLC column fraction 9A-11B was washed with *n*-hexane and due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (98:2)]. The process was repeated five times for better separation of the bands. From the developed plates two yellow colored bands were visualized after spraying with vanillin-sulfuric acid reagent followed by heating in 110° C for 5 minutes. The bands were separately scrapped on to an aluminum foil and eluted with ethyl acetate. Colorless mass was obtained in both cases which were checked for purity and termed as BS-11 (7.01 mg).

2.3.6 Properties of the isolated compounds

The physical properties of the isolated compounds and their reactions to vanillin-H₂SO₄ spray reagent are summarized in the Table 2.14.

Table 2.14 Properties of the isolated compounds from stem bark of *B. stipularis*.

| Isolated compounds | Physical form | Color | Color with vanillin-H ₂ SO ₄ |
|--------------------|---------------|-------|--|
| BS-01 | Crystal | White | Purple |
| BS-06 | Needle | White | Pink |
| BS-11 | Solid | White | Yellow |

2.4 Phytochemical investigation of *Bridelia tomentosa*

2.4.1 Collection and preparation of the plant material

Leaf and stem bark of *B. tomentosa* was collected from the village of Panchouri, Khagrachhori District in February 2007 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-31377). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature (not more than 40 °C for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.4.2 Extraction of the plant material

The powdered stem bark of *B. tomentosa* (575 g) was separately soaked in 1.5 L methanol and 325 g of powdered leaf of was also separately soaked in 750 mL methanol in a clean, round bottomed flask (5 and 3 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 °C) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from stem bark was 35 g and from leaf was 15.5 g.

2.4.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wageningen *et al.*, (1993) as described in section 2.2.3. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness and were used for further analysis.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.15.

Table 2.15: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *B. tomentosa*.

| Plant Part | Weight (g) of methanolic extract for Kupchan partitioning | Fraction code | Weight (g) of soluble fractions |
|------------|---|---------------|---------------------------------|
| Stem bark | 15 | HSF | 3.4 |
| | | CSF | 0.94 |
| | | CHSF | 1.28 |
| | | AQSF | 6.5 |
| Leaf | 10 | HSF | 2.7 |
| | | CSF | 0.83 |
| | | CHSF | 0.69 |
| | | AQSF | 4.5 |

HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

2.4.4 Vacuum Liquid Chromatography (VLC) of the crude extract of stem bark of *B. tomentosa*

2.4.4.1 Packing of Column

The column was packed with fine VLC grade silica gel (Kieselgel 60H). A column having 14 cm length and 10 cm in diameter was packed with the silica gel up to a height

of 6 cm under reduced pressure. The column was then washed with *n*-hexane to speed up compact packing.

2.4.4.2 Sample Input

The sample was prepared by adsorbing 20 g of crude extract onto column grade silica gel (Kieselgel 60H, mesh 70-230) with continuous stirring under air blower in a porcelain basin. Stirring continued until the sample becomes a free flowing powder. The free flowing sample was then placed on the packed silica into the column. Finally a cotton plug was placed sample.

2.4.4.3 Development of Chromatogram

After placing the sample, the column was eluted with *n*-hexane, followed by mixtures of *n*-hexane and ethyl acetate and finally washed by methanol. Solvent systems used as mobile phases in the VLC analysis of crude extract were listed in Table 2.16.

Table 2.16: Solvent systems used for VLC analysis of the crude extract of stem bark of *B. tomentosa*.

| Fraction no. | Solvent systems | Volume collected (ml) |
|--------------|---|-----------------------|
| 1 | 100% <i>n</i> -Hexane | 50 |
| 2 (A+B) | <i>n</i> -Hexane - Ethyl acetate (98 : 2) | 50 + 50 |
| 3 (A+B) | <i>n</i> -Hexane - Ethyl acetate (95 : 5) | 50 + 50 |
| 4 (A+B) | <i>n</i> -Hexane - Ethyl acetate (92.5 : 7.5) | 75 + 75 |
| 5 (A+B) | <i>n</i> -Hexane - Ethyl acetate (90 : 10) | 75 + 75 |
| 6 (A+B) | <i>n</i> -Hexane - Ethyl acetate (80 : 20) | 75 + 75 |
| 7 (A+B) | <i>n</i> -Hexane - Ethyl acetate (75 : 25) | 75 + 75 |
| 8 (A+B) | <i>n</i> -Hexane - Ethyl acetate (60 : 40) | 100 + 100 |
| 9 (A+B) | <i>n</i> -Hexane - Ethyl acetate (55 : 45) | 100 + 100 |
| 10 (A+B) | <i>n</i> -Hexane - Ethyl acetate (50 : 50) | 100 + 100 |
| 11 (A+B) | <i>n</i> -Hexane - Ethyl acetate (45 : 55) | 100 + 100 |
| 12 (A+B) | <i>n</i> -Hexane - Ethyl acetate (40 : 60) | 100 + 100 |
| 13 (A+B) | <i>n</i> -Hexane - Ethyl acetate (30 : 70) | 100 + 100 |
| 14 (A+B) | <i>n</i> -Hexane - Ethyl acetate (25 : 75) | 100 + 100 |
| 15 (A+B) | <i>n</i> -Hexane - Ethyl acetate (20 : 80) | 100 + 100 |
| 16 (A+B) | <i>n</i> -Hexane - Ethyl acetate (15 : 85) | 100 + 100 |
| 17 (A+B) | <i>n</i> -Hexane - Ethyl acetate (10 : 90) | 100 + 100 |
| 18 (A+B) | <i>n</i> -Hexane - Ethyl acetate (5 : 95) | 100 + 100 |
| 19 (A+B) | Ethyl acetate (100 %) | 100 + 100 |
| 20 (A+B) | Ethyl acetate - Methanol (99:1) | 50 + 50 |
| 21 (A+B) | Ethyl acetate - Methanol (97:3) | 50 + 50 |
| 22 (A+B) | Ethyl acetate - Methanol (90:10) | 50 + 50 |
| 23 (A+B) | Ethyl acetate - Methanol (75:25) | 50 + 50 |

2.4.5 Analysis of VLC fractions by thin layer chromatography (TLC)

All the above VLC fractions were screened by TLC using various solvent systems. The plates were observed under UV light and by spraying with vanillin-sulfuric acid reagent followed by heating at 100-110 °C for 5-10 minutes as well. Depending on the TLC behavior fractions 4(A+B), 5B & 6A, 7B & 8A and 12(A+B) & 13A were selected for further investigation.

Table 2.17: List of isolated compounds from VLC fraction of the methanolic extract of the stem bark of *B. tomentosa*.

| Column fraction | TLC Solvent System | R _f value | Amount (mg) | Code |
|-----------------|-------------------------------------|----------------------|-------------|-------|
| 4(A+B) | Toluene - Ethyl acetate (95:5) | 0.345 | 8.04 | BT-1 |
| 5B & 6A | Toluene - Ethyl acetate (95:5) | 0.455 | 5.11 | BT-6 |
| 7B & 8A | Toluene - Ethyl acetate (90:10) | 0.683 | 6.29 | BT-8 |
| 12(A+B) & 13A | Toluene - Ethyl acetate (90:10)[3t] | 0.321 | 6.31 | BT-14 |

2.4.5.1 Isolation and purification of compounds from the selected VLC fractions

Four compounds was isolated and purified from the different VLC fractions by adopting various techniques.

2.4.5.2 Isolation of BT-1

Evaporation of solvents of the VLC fractions 4(A+B) furnished impure white crystalline mass. Repeated washing of these crystalline mass with *n*-hexane and then recrystallizing with *n*-hexane and ethyl acetate yielded white crystals. After TLC with solvent system toluene: ethyl acetate (95:5) a single spot was observed after spraying with vanillin-sulfuric acid reagent followed by heating in 110° C for 5 minutes. These crystals were designated as compound **BT-1** (8.04 mg).

2.4.5.3 Isolation of BT-6

The VLC fractions 5B & 6A were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (95:5)]. A deep purple colored band was visualized after spraying a portion of the developed plate with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes. The unsprayed portion of the band at stationary phase was scrapped off and eluted with ethyl acetate. Colorless mass was obtained which was checked for purity and termed as **BT-6** (5.11 mg).

2.4.5.4 Isolation of BT-8

Again, the VLC fractions 7B & 8A were mixed together due to similar TLC characteristics and subjected to preparative TLC [stationary phase - silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (90:10)], the process was repeated thrice for better separation and isolation of the desired band. From the developed plates a purple colored band was visualized after spraying a small portion of the PTLC plate with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes. The unsprayed stationary phase was scrapped off and eluted with ethyl acetate. After evaporation of the solvent fine tree like crystals were obtained which was termed as BT-8 (6.29 mg).

2.4.5.5 Isolation of BT-14

Again, the VLC fractions 12A & 13B were mixed together due to identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (90:10)]. The process was repeated thrice for better separation and isolation of the desired band. From the developed plates a purple colored band was visualized after spraying a small portion of the PTLC plate with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes. The unsprayed portion of the stationary phase was scrapped off and eluted with ethyl acetate. After evaporation of the solvent fine tree like crystals were obtained which after purity test was termed as BT-14 (6.31 mg).

2.4.6 Properties of the isolated compounds

The physical properties of the isolated compounds and their reactions to vanillin-H₂SO₄ spray reagent are summarized in the Table 2.18.

Table 2.18: Properties of the isolated compounds from the stem bark of *B. tomentosa*.

| Isolated compounds | Physical form | Color | Color with vanillin-H ₂ SO ₄ |
|--------------------|---------------|-----------|--|
| BT-1 | Crystal | White | Violet |
| BT-6 | Amorphous | Whitish | Deep purple |
| BT-8 | Crystals | Tree like | Purplish |
| BT-14 | Crystal | Tree like | Purple |

2.5 Phytochemical investigation of *Erythrina fusca*

2.5.1 Collection and preparation of the plant material

Leaf and stem bark of *E. fusca* was collected from Nandail, Mymensingh District in September 2011 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-

35902). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature (not more than 40 °C) for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.5.2 Extraction of the plant material

The powdered stem bark of *E. fusca* (800 g) was separately soaked in 2.25 L methanol and powdered leaf of (550 g) was also separately soaked in 1.5 mL methanol in a clean, round bottomed flask (7.5 and 5 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 20 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 °C) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from stem bark was 40 g and from leaf was 25 g.

2.5.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen *et al.*, (1993) as described in section 2.2.3. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness and were used for further analysis.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.19.

Table 2.19: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *E. fusca*.

| Plant Part | Weight (g) of methanolic extract for Kupchan partitioning | Fraction code | Weight (g) of soluble fractions |
|------------|---|---------------|---------------------------------|
| Stem bark | 30 | HSF | 8.25 |
| | | CSF | 2.50 |
| | | CHSF | 2.00 |
| | | AQSF | 13.1 |
| Leaf | 15 | HSF | 4.9 |
| | | CSF | 1.23 |
| | | CHSF | 0.95 |
| | | AQSF | 6.7 |

HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

2.5.4 Column chromatography of carbon tetrachloride soluble fraction of the stem bark of *E. fusca*

A portion of carbon tetrachloride soluble fraction (1.4 g) was subjected to column chromatography (CC) for fractionation. The column was packed with silica gel (Kieselgel 60H, mesh 70-230). Slurry of silica gel in petroleum ether (60-80 °C) was added into a glass column having a length and diameter of 84 and 2 cm, respectively. When the desired height of adsorbent bed was obtained, a few hundred milliliter of solvent was run through the stationary phase for proper packing of the column. After packing, the sample was prepared by adsorbing carbon tetrachloride soluble material onto silica gel (Kieselgel 60H, mesh 70-230) and allowed to dry up to a free flowing powder like mass and subsequently applied on top of the adsorbent layer. Then the column was eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate and then ethyl acetate, finally with ethyl acetate and methanol in order of increasing polarities. Solvent system used as mobile phase in CC analysis of carbon tetrachloride soluble material and has been listed in Table 2.20. The eluates were collected in test tubes. A total of 250 fractions were collected each 20 ml.

Table 2.20: Different solvent systems used for the column chromatographic analysis of the carbon tetrachloride soluble fraction of the stem bark of *E. fusca*.

| Fraction no. | Solvent systems | Volume collected ml/test tube |
|--------------|---|-------------------------------|
| 1 - 7 | Petroleum ether - Ethyl acetate = (99 : 1) | 20 |
| 8 - 19 | Petroleum ether - Ethyl acetate = (98 : 2) | 20 |
| 20 - 34 | Petroleum ether - Ethyl acetate = (97 : 3) | 20 |
| 35 - 45 | Petroleum ether - Ethyl acetate = (95 : 5) | 20 |
| 46 - 61 | Petroleum ether - Ethyl acetate = (92.5 : 7.5) | 20 |
| 62 - 76 | Petroleum ether - Ethyl acetate = (90 : 10) | 20 |
| 77 - 86 | Petroleum ether - Ethyl acetate = (87.5 : 12.5) | 20 |
| 87 - 110 | Petroleum ether - Ethyl acetate = (85 : 15) | 20 |
| 111 - 142 | Petroleum ether - Ethyl acetate = (80 : 20) | 20 |
| 143 - 158 | Petroleum ether - Ethyl acetate = (75 : 25) | 20 |
| 159 - 173 | Petroleum ether - Ethyl acetate = (70 : 30) | 20 |
| 174 - 182 | Petroleum ether - Ethyl acetate = (65 : 35) | 20 |
| 183 - 190 | Petroleum ether - Ethyl acetate = (55 : 45) | 20 |
| 191 - 207 | Petroleum ether - Ethyl acetate = (50 : 50) | 20 |
| 208 - 211 | Petroleum ether - Ethyl acetate = (35 : 65) | 20 |
| 212 - 220 | Petroleum ether : Ethyl acetate = (20 : 80) | 20 |
| 221 - 229 | Ethyl acetate (100%) | 20 |
| 230 - 237 | Ethyl acetate - Methanol = (99 : 1) | 20 |
| 238 - 242 | Ethyl acetate - Methanol = (97 : 3) | 20 |
| 243 - 250 | Ethyl acetate - Methanol = (95 : 5) | 20 |

2.5.5 Analysis of column fractions by TLC

All the column fractions were screened by TLC under UV light and by spraying with vanillin sulfuric acid reagent followed by heating at 100-110 °C for 5-10 minutes as well. Depending on the TLC behavior a number of similar samples were detected and the column fractions were added, these were then purified by employing various techniques. A list of isolated compounds has been summarized in Table 2.21.

Table 2.21: List of isolated compounds from carbon tetrachloride soluble fraction of the methanolic extract of the tem bark of *E. fusca*.

| Column fraction | Mobile phase | R _f value | Amount (mg) | Code |
|-----------------|---------------------------------|----------------------|-------------|-------|
| 90-97 | Toluene - Ethyl acetate (97:3) | 0.657 | 6.12 | EF-7 |
| 90-97 | Toluene: Ethyl acetate (97:3) | 0.600 | 8.20 | EF-8 |
| 117-122 | Toluene - Ethyl acetate (90:10) | 0.218 | 4.50 | EF-12 |
| 119-127 | Toluene - Ethyl acetate (85:15) | 0.571 | 6.87 | EF-38 |

2.5.5.1 Isolation of compound EF-7

EF-7 was isolated as yellowish mass by evaporation of solvent from the silica column fractions 90-97 of the carbon tetrachloride soluble portion of methanolic extract of the stem bark of *E. fusca*. The yellowish residue was then subjected to preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated thrice for better separation of the desired band. After excitation with 254 nm UV light, quenching was observed by the desired band as a uniform layer. Then the band was scrapped off on an aluminum foil and eluted with ethyl acetate. Evaporation of the solvent yielded an oily transparent liquid. It was checked for purity and denoted as EF-7 (6.12 mg).

2.5.5.2 Isolation of compound EF-8

Evaporation of solvents from the CC fractions 90-97 of the carbon tetrachloride soluble fraction provided a yellowish mass. The fraction was then subjected to a preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (97:3)]. The process was repeated thrice for better separation of the desired band. The desired band was identified under UV light, then scrapped off and eluted with ethyl acetate to yield EF-8 (8.20 mg) as yellow needles on standing at room temperature.

2.5.5.3 Isolation of compound EF-12

Sub-fractions 90-97 showing identical spots on TLC were combined in a beaker. After drying the solvent a yellowish mass was obtained which was then subjected to preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated five times for better separation of the desired band. A small portion of the developed plate was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded a compound EF-12 (4.5 mg) as amorphous powder.

2.5.5.4 Isolation of compound EF-38

Sub-fractions 119-227 showing identical spots on TLC were combined in a beaker. The fraction was then subjected to a preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (85:15)]. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating in 110 °C for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded white needles. The compound was analyzed over TLC to check for purity and found to be a pure compound which was termed as EF-38 (6.87 mg).

2.5.5.5 Properties of the isolated compounds

The physical properties of the isolated compounds and their reactions to vanillin-H₂SO₄ spray reagent are summarized in the Table 2.22.

Table 2.22: Properties of the isolated compounds from stem bark of *E. fusca*.

| Isolated compounds | Physical form | Color | Color with vanillin-H ₂ SO ₄ |
|--------------------|---------------|-------------|--|
| EF-7 | Oily liquid | Transparent | Magenta |
| EF-8 | Mass | Yellow | Deep orange |
| EF-12 | Powder | White | Pink |
| EF-38 | Needles | White | Scarlet |

2.6 Phytochemical investigation of *Erythrina variegata*

2.6.1 Collection and preparation of the plant material

Leaf and stem bark of *E. variegata* was collected from Savar, Dhaka District in September 2011 and identified in Bangladesh National Herbarium where voucher specimens have been maintained representing this collection (Accession No. DACB-35901). After proper washing, the leaf and stem bark (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at

considerably low temperature (not more than 40 °C) for better grinding. The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka, Bangladesh.

2.6.2 Extraction of the plant material

The powdered stem bark of *E. variegata* (650 g) was separately soaked in 1.75 L methanol and 450 g of powdered leaf of was also separately soaked in 1 L methanol in a clean, round bottomed flask (5 and 3 L, respectively). The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 20 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at low temperature (36-40 °C) and reduced pressure with a Buchii Rotavapour to have a concentrate of the crude extracts. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from stem bark was 30 g and from leaf was 15 g.

2.6.3 Solvent-solvent partitioning of crude extract

Solvent-solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagenen *et al.*, (1993) as described in section 2.2.3. The whole partitioning process is schematically shown in Figure 2.1. All the fractions were evaporated to dryness and were used for further analysis.

The process was repeated for several times and after evaporation the weight of the different soluble fractions obtained is mentioned in Table 2.23.

Table 2.23: Amount of soluble fractions obtained from methanolic extract of the stem bark and leaf of *Erythrina variegata*.

| Plant Part | Weight (g) of methanolic extract for Kupchan partitioning | Fraction code | Weight (g) of soluble fractions |
|------------|---|---------------|---------------------------------|
| Stem bark | 10 | HSF | 2.75 |
| | | CSF | 0.65 |
| | | CHSF | 0.92 |
| | | AQSF | 4.30 |
| Leaf | 10 | HSF | 3.30 |
| | | CSF | 0.81 |
| | | CHSF | 0.71 |
| | | AQSF | 4.20 |

HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction



Chapter 3

Phytochemical investigation- Results and discussion

3.1 Characterization of BVSP-1 as glochidonol (46)

The *n*-hexane soluble portion of the crude methanolic extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain 43 fractions. Fractions 11-18 showing identical spots on TLC were combined in a beaker and it was kept at room temperature undisturbed for several days. The crystals obtained after evaporation of solvents were washed with *n*-hexane to remove the adhering coloring materials and recrystallization from *n*-hexane and ethyl acetate to afforded needle like crystals designated as BVSP-1 (7.23 mg, 46). It was found to be soluble in chloroform, dichloromethane and ethyl acetate.

The crystals of BVSP-1 melted at 227-229 °C which was similar to that reported (228-230 °C) for glochidonol (Thu *et al.*, 2010). The ¹H-NMR spectrum (400 MHz, CDCl₃; Figures 3.2-3.3; Table 3.1) exhibited typical signals for 48 protons including seven tertiary methyl, a carbinol and a terminal exomethylene proton. In the ¹H-NMR of BVSP-1 seven three proton singlets indicated seven tertiary methyl groups at δ 1.03 (H-23), 1.03 (H-24), 0.80 (H-25), 1.01 (H-26), 0.95 (H-27), 0.78 (H-28) and 1.66 (H-30). The spectrum also demonstrated a proton doublet at δ 3.80 (1H, d, *J*= 2.8 Hz) corresponding to oxymethine proton at C-1. It also showed olefinic protons at δ 4.54 (H_a-29) and δ 4.66 (H_b-29, d, *J*= 1.6 Hz).

The ¹³C-NMR (125 MHz, CDCl₃; Figures 3.4-3.6; Table 3.2) displayed a total of 30 carbon resonances including a carbonyl carbon at δ 215.4. The ¹³C-NMR spectrum also showed typical signal at δ 79.0 for the oxymethine carbon at C-1 while two signals at δ 150.8 and δ 109.7 could be assigned to C-20 and C-29, respectively.

All these features are indicative of the presence of a lupane-type triterpenoid type skeleton in BVSP-1.

The melting point and ¹H and ¹³C-NMR spectral data (Tables 3.1 and 3.2, respectively) of BVSP-1 were compared with the published data of glochidonol and found to be identical (Thu *et al.*, 2010). Thus, compound BVSP-1 was characterized as glochidonol (46). This is the first report of isolation of glochidonol from *B. verrucosa*.

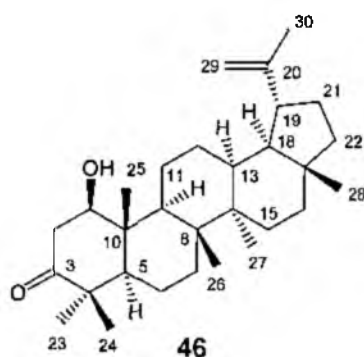


Figure 3.1: Structure of BVSP-1 (glochidonol, 46).

Table 3.1: ^1H -NMR spectral data of BVSP-1 (46) and glochidonol (Thu *et al.*, 2010) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|--------------------|---|---------------------|
| | BVSP-1 (46) | Glochidonol |
| H-1 | 3.80 d (2.8) | 3.89 1H, d (2.8) |
| H _a -2 | 2.99 dd (14.3, 8.1) | 3.00 dd (14.5, 8.0) |
| H _b -2 | 2.38 m | 2.38 m |
| Me-23 | 1.03 s | 1.06 s |
| Me-24 | 1.03 s | 1.06 s |
| Me-25 | 0.80 s | 0.84 s |
| Me-26 | 1.01 s | 1.05 s |
| Me-27 | 0.95 s | 0.98 s |
| Me-28 | 0.78 s | 0.80 s |
| H _a -29 | 4.54 s | 4.56 s |
| H _b -29 | 4.66 d (1.6) | 4.68 d (1.5) |
| Me-30 | 1.66 s | 1.68 s |

Table 3.2: ^{13}C -NMR spectral data of BVSP-1 (46) and glochidonol (Thu *et al.*, 2010) in CDCl_3 .

| Position of carbon | Glochidonol | BVSP-1 (46) |
|--------------------|-------------|-------------|
| C-1 | 79.7 | 79.0 |
| C-2 | 45.2 | 45.3 |
| C-3 | 215.5 | 215.4 |
| C-4 | 47.2 | 47.2 |
| C-5 | 51.5 | 53.3 |
| C-6 | 19.7 | 18.9 |
| C-7 | 33.1 | 33.0 |
| C-8 | 43.0 | 43.0 |
| C-9 | 50.8 | 49.4 |
| C-10 | 43.0 | 43.0 |
| C-11 | 23.2 | 22.5 |
| C-12 | 25.3 | 25.7 |
| C-13 | 38.1 | 38.1 |
| C-14 | 41.3 | 41.5 |
| C-15 | 27.6 | 27.6 |
| C-16 | 35.6 | 35.6 |
| C-17 | 43.0 | 43.1 |
| C-18 | 48.9 | 48.4 |
| C-19 | 48.0 | 48.0 |
| C-20 | 150.7 | 150.8 |
| C-21 | 29.9 | 28.4 |
| C-22 | 40.0 | 39.9 |
| C-23 | 27.9 | 27.9 |
| C-24 | 19.9 | 20.5 |
| C-25 | 11.8 | 11.8 |
| C-26 | 16.0 | 16.4 |
| C-27 | 14.5 | 14.9 |
| C-28 | 18.1 | 18.2 |
| C-29 | 109.5 | 109.7 |
| C-30 | 19.4 | 20.4 |

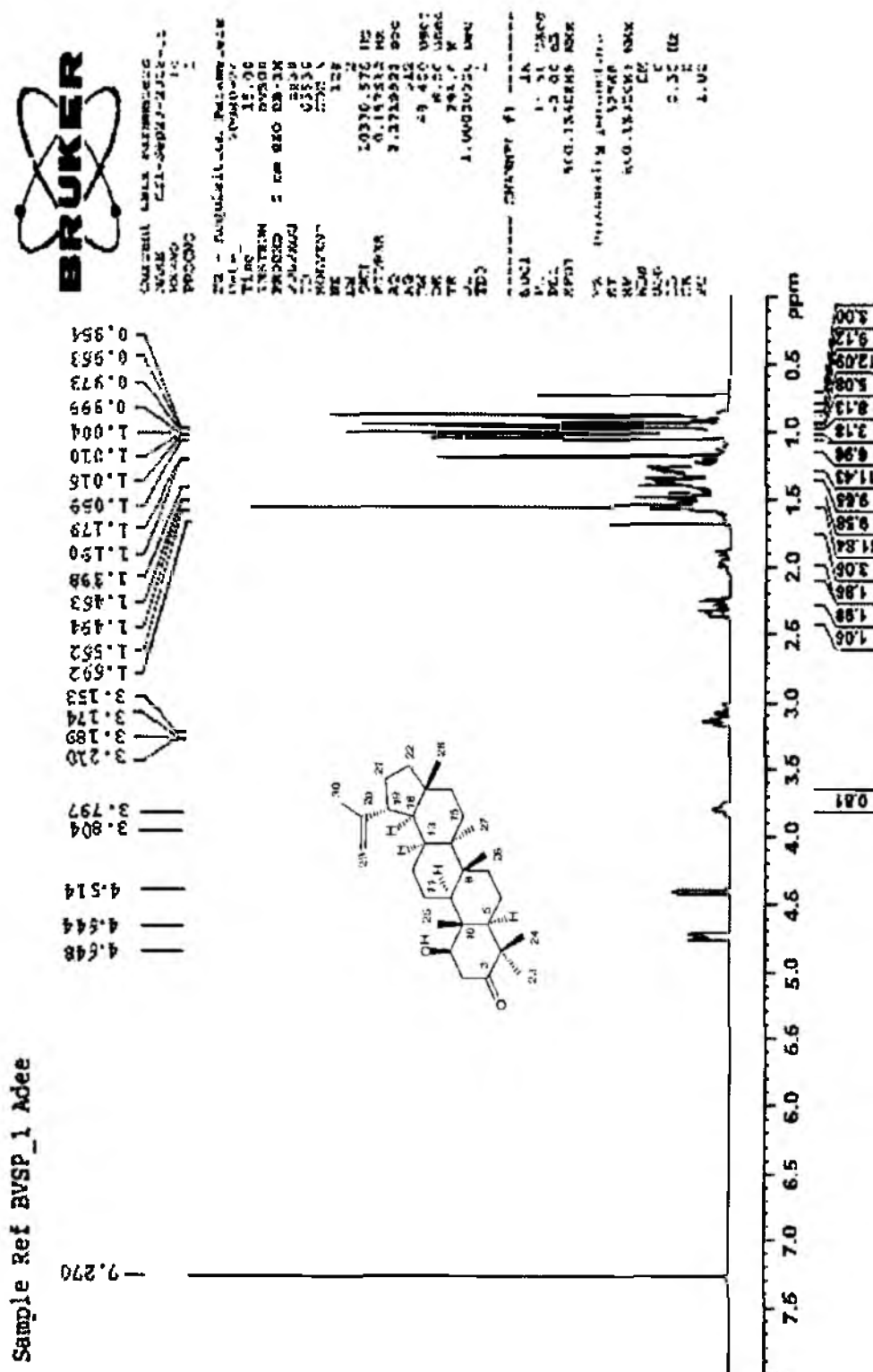


Figure 3.2: ^1H -NMR spectrum (400 MHz, CDCl_3) of BVSP-1 (46).

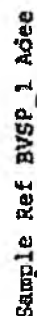


Figure 3.3: Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BVSP-1 (46).

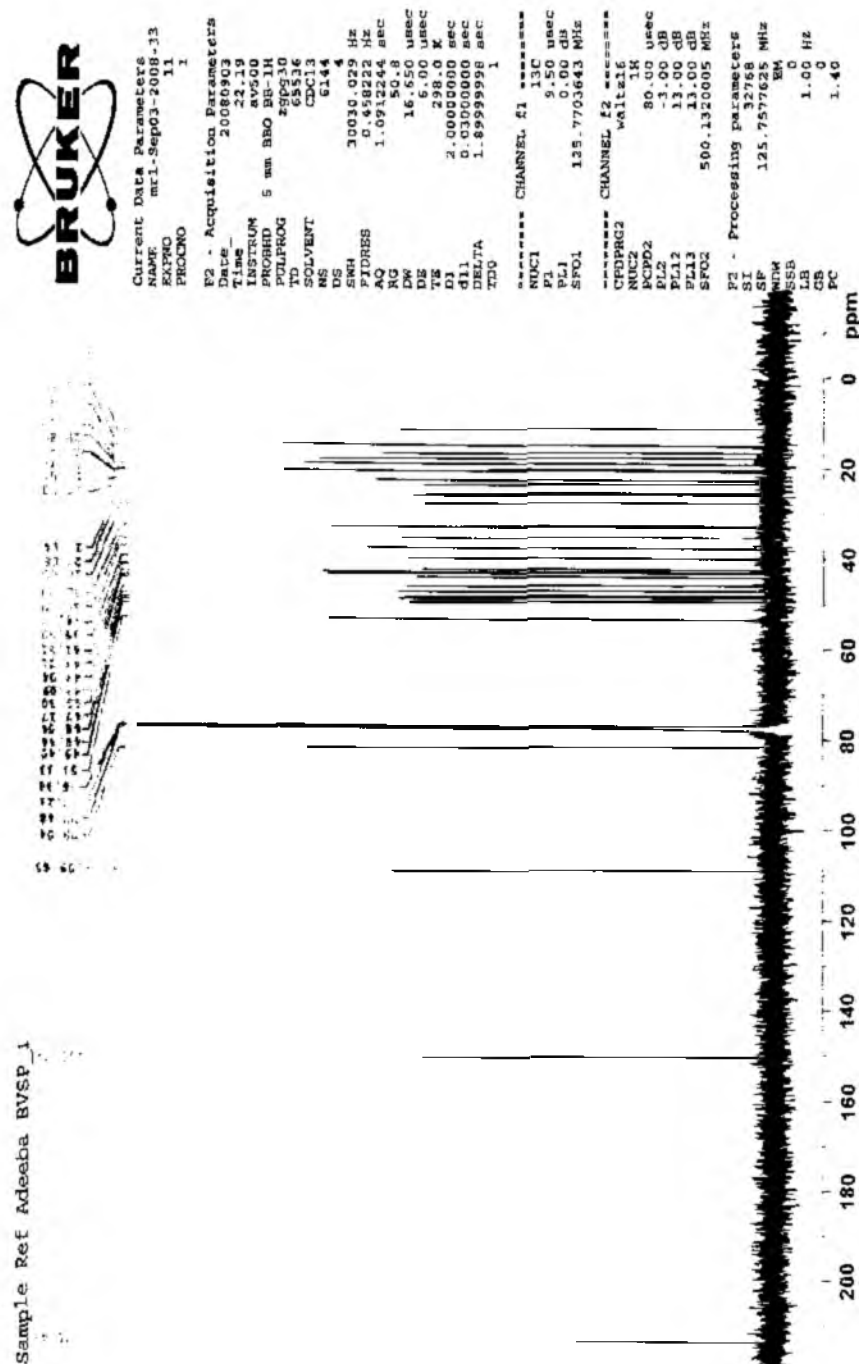


Figure 3.4: ^{13}C -NMR spectrum of BVSP-1 (46) in CDCl_3 .

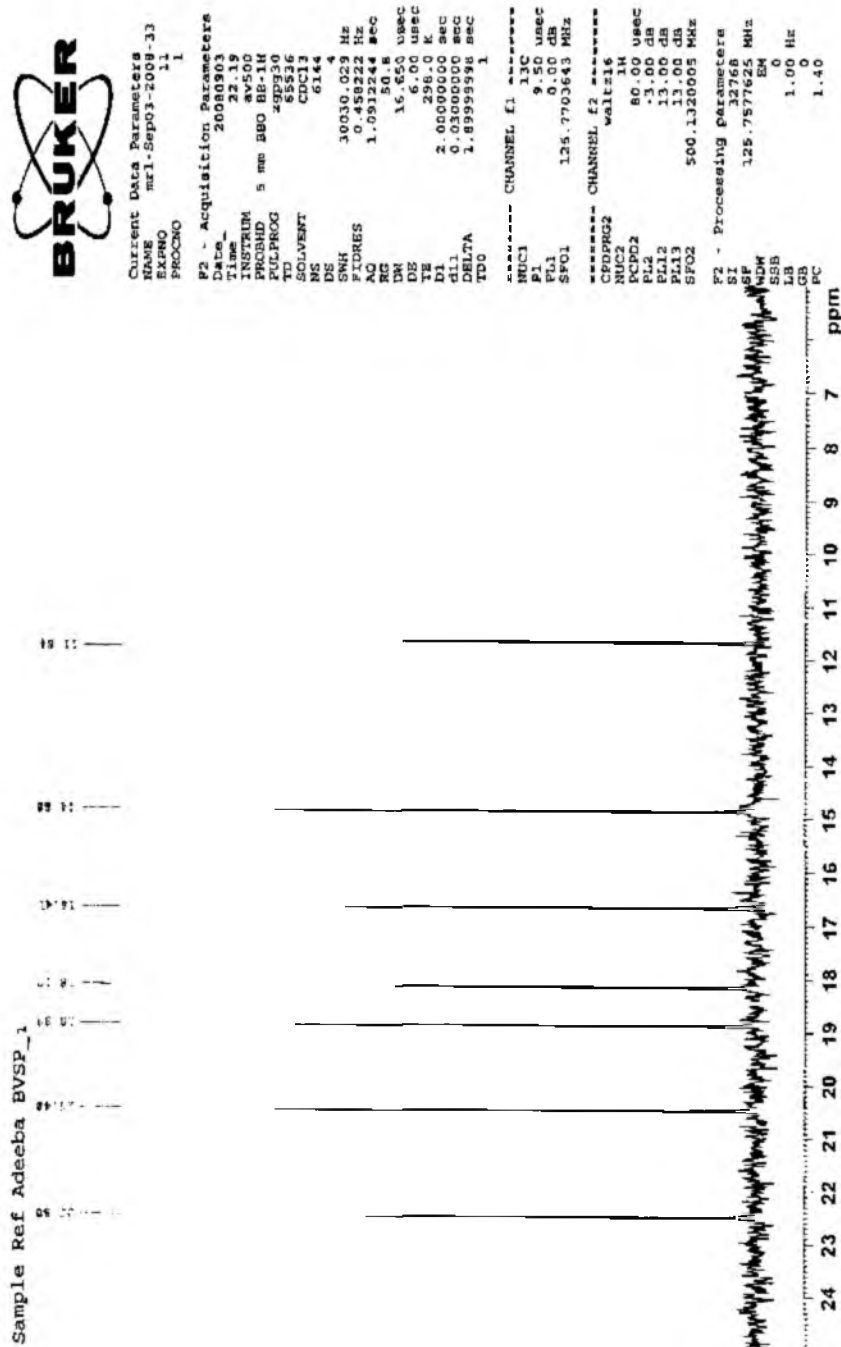
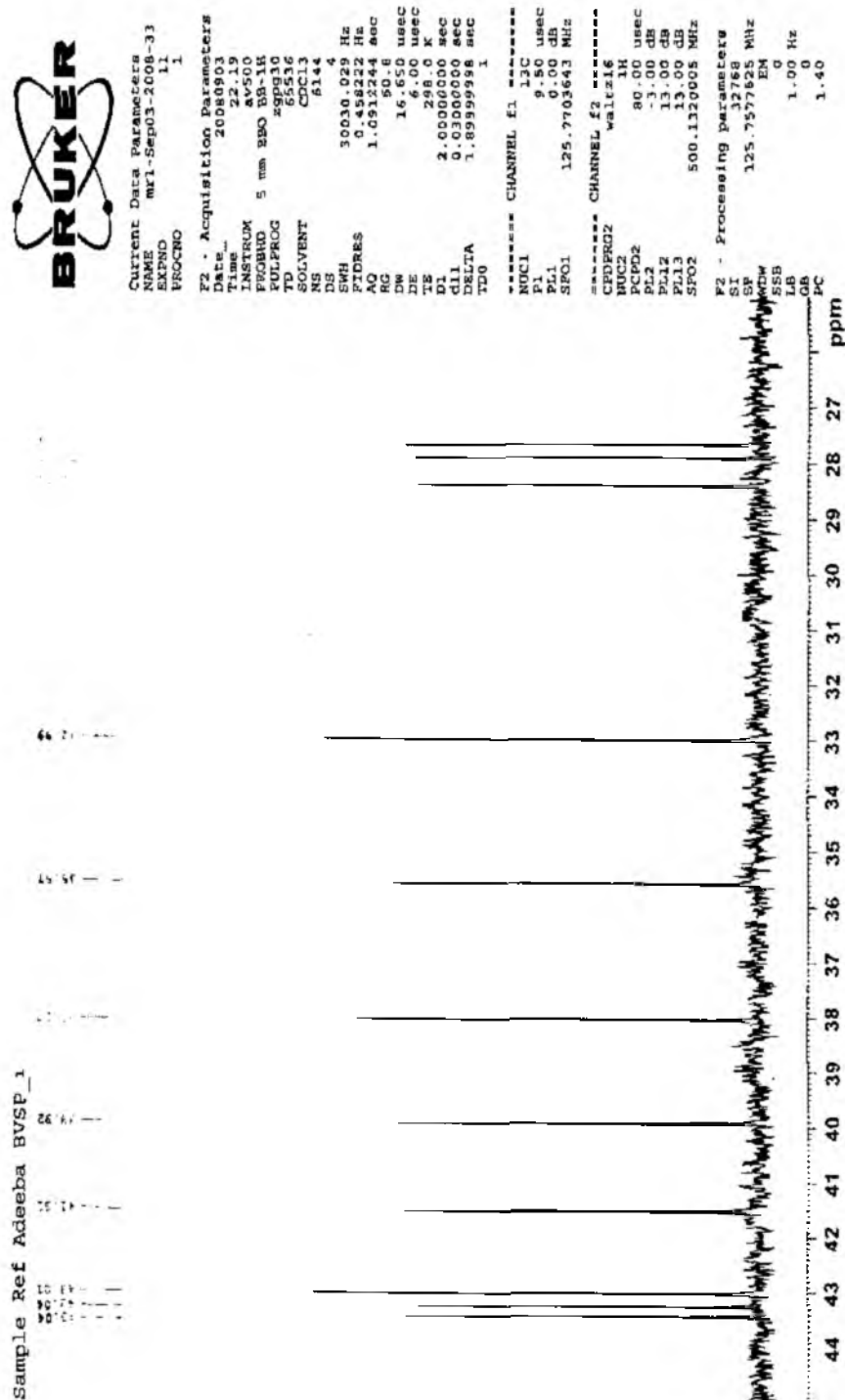


Figure 3.5: Partially expanded ^{13}C -NMR spectrum of BVSP-1 (46) in CDCl_3 .

Figure 3.6: Partially expanded ¹³C-NMR spectrum of BVSP-4 (46) in CDCl₃.

3.2 Characterization of BVSP-2 as brassicasterol (47)

The *n*-hexane soluble portion of the crude methanol extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain forty three fractions. Fractions 19-23 were combined together depending on identical TLC behaviour and subjected to preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated three times for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a purple colored band was visualized. The band was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent afforded colorless needles of BVSP-2 (6.78 mg, 50). The melting point of the crystals of BVSP-2 was found to be at 153-156 °C which was similar to that reported (154-156 °C) for brassicasterol (Jinming *et al.*, 2001). It was soluble in chloroform, dichloromethane and ethyl acetate.

The ¹H-NMR spectrum (500 MHz, CDCl₃; Figures 3.8-3.9; Table 3.3) showed a multiplet of one proton intensity at δ 3.62 for a oxymethine proton at H-3. The typical signal for the olefinic C-6 proton of the steroidal skeleton was evident from a doublet at δ 5.36 (*J*= 6.0 Hz) that integrated for one proton. The *trans*-olefinic protons H-22 and H-23 appeared as characteristic downfield signals at δ 5.17 (2H, m) and δ 5.20 (2H, m) due to couplings with the neighbouring olefinic and methine protons. The spectrum further revealed signals at δ 0.69 and 0.83 (3H each) assignable to two tertiary methyl groups at C-13 (H₃-18) and C-10 (H₃-19), respectively. The ¹H-NMR spectrum also showed four doublets of three proton intensity each at δ 1.00 (3H, d, *J*= 6.5 Hz), 0.84 (3H, d, *J*= 6.5 Hz), 0.93 (3H, d, *J*= 6.5 Hz) and 1.21 (3H, d, *J*= 6.5 Hz) assignable to the methyl groups at Me-21, Me-26, Me-27, Me-28, respectively. All these features are indicative of the presence of a steroidal nucleus.

Furthermore, the melting point and ¹H-NMR spectral data (Table 3.3) of BVSP-2 were compared with the reported values of brassicasterol and found to be identical (Jinming *et al.*, 2001). Thus, BVSP-2 was identified as brassicasterol (50). This is the first report of isolation of brassicasterol from *B. verrucosa*.

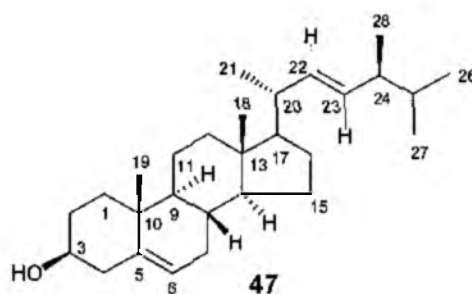


Figure 3.7: Structure of BVSP-2 (brassicasterol, 47).

Table 3.3: ^1H - NMR spectral data of BVSP-2 (47) and brassicasterol (Jinming *et al.*, 2001) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------|---|----------------|
| | BVSP-2 (47) | Brassicasterol |
| H-3 | 3.62 m | 3.53 m |
| H-6 | 5.36 d (6.0) | 5.35 d (5.2) |
| H-18 | 0.69 s | 0.69 s |
| H-19 | 0.83 s | 0.83 s |
| Me-21 | 1.00 d (6.5) | 1.00 d (6.5) |
| H-22 | 5.17 m | 5.17-5.20 m |
| H-23 | 5.22 m | 5.17-5.20 m |
| Me-26 | 0.84 d (6.5) | 0.84 d (6.5) |
| Me-27 | 0.93 d (6.5) | 0.91 d (6.8) |
| Me-28 | 1.21 d (6.5) | 1.17 d (6.5) |

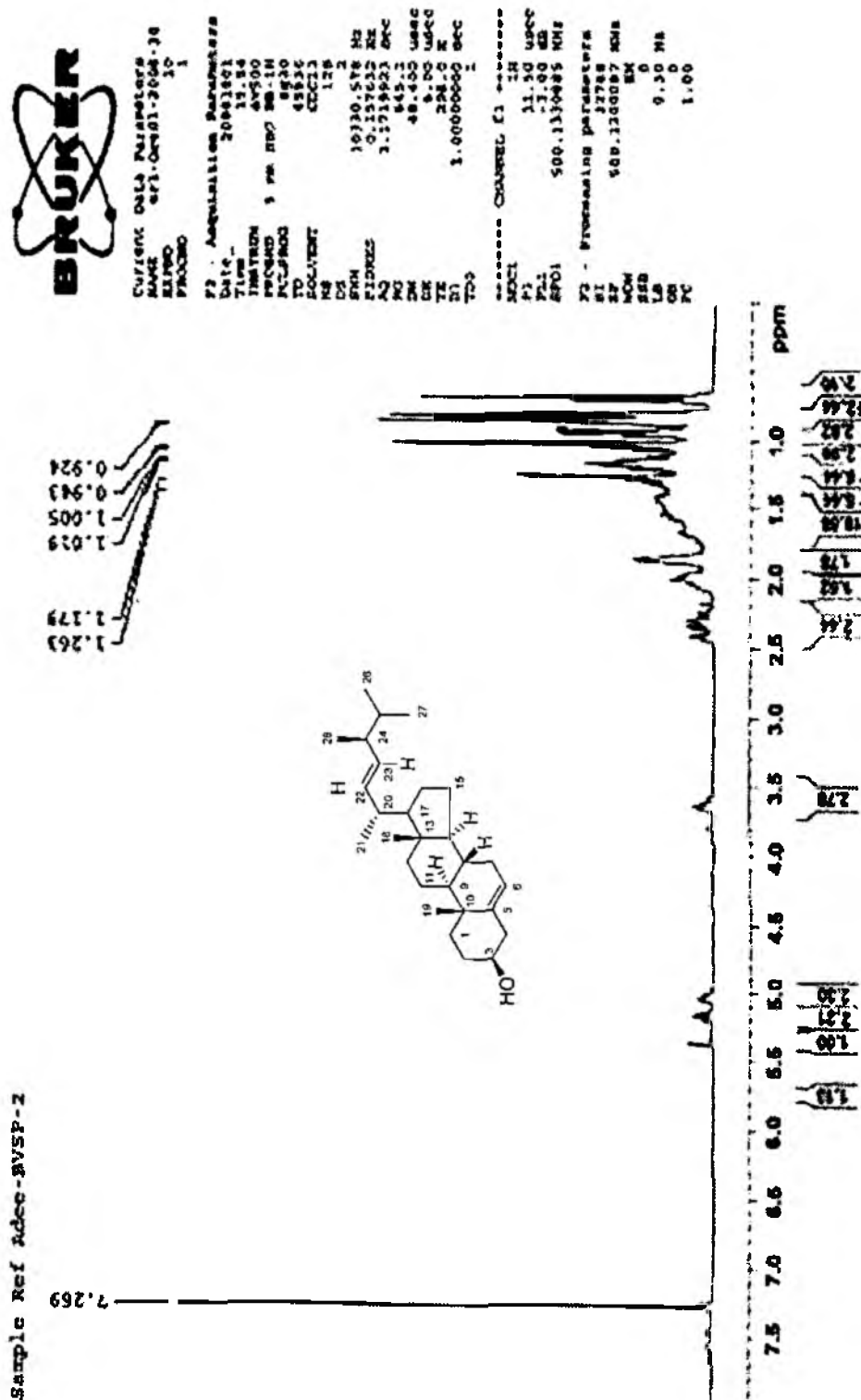


Figure 3.8: ¹H-NMR spectrum (500 MHz, CDCl₃) of BVSP-2 (47).

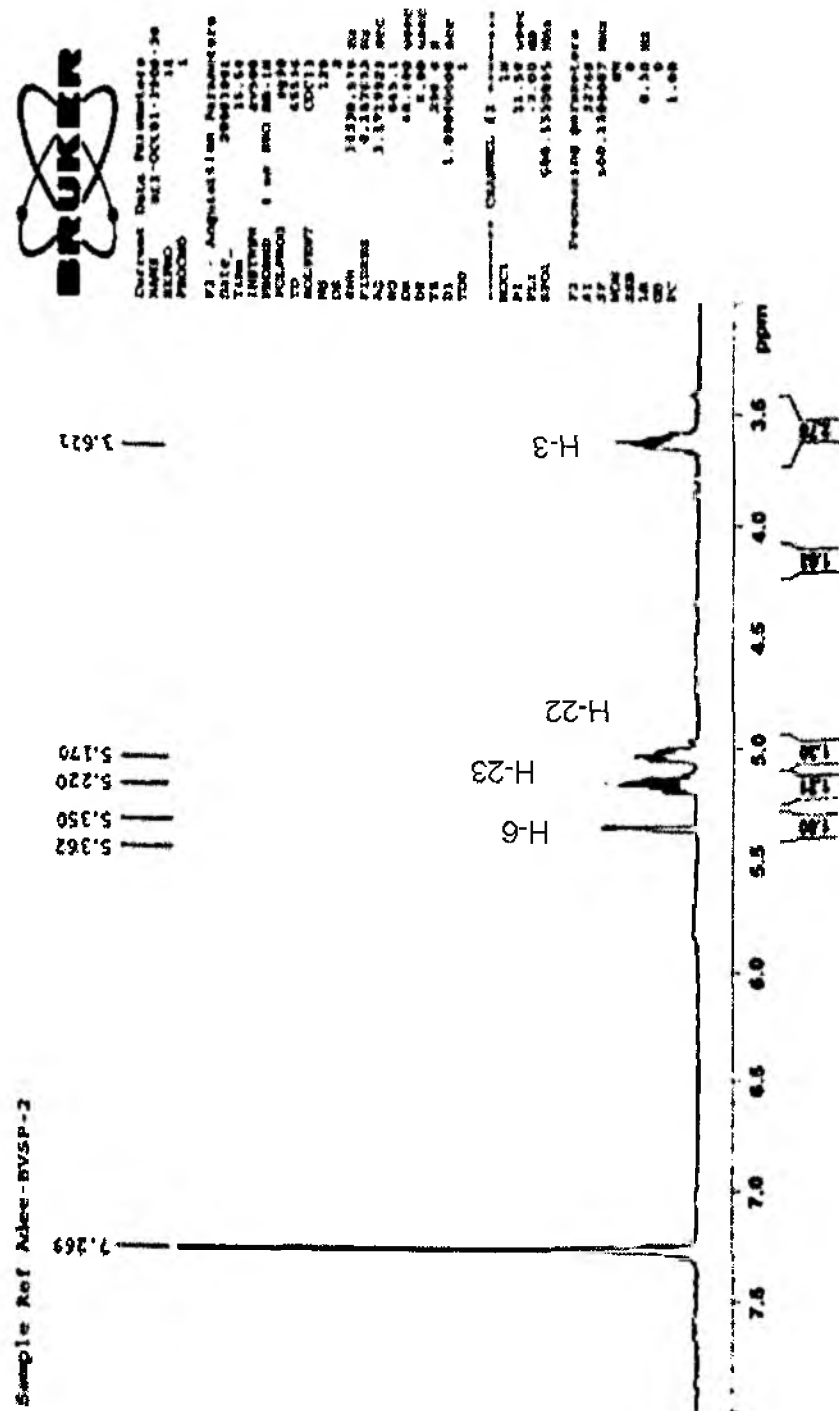


Figure 3.9: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of BVSP-2 (47).

3.3 Characterization of BVSP-4 as friedelin (48)

The *n*-hexane soluble portion of the crude methanolic extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain forty three fractions. Fractions 1-4 showing identical spots on TLC were combined in a beaker and it was kept at room temperature undisturbed for several days. The crystals obtained after evaporation of solvents were washed with *n*-hexane to remove the adhered coloring materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound as needles designated as BVSP-4 (5.3 mg, 48). It was found to be soluble in chloroform, dichloromethane and ethyl acetate.

The crystals of BVSP-4 melted at 268 °C which was identical to that reported (267 °C) for friedelin (Akihisa *et al.*, 1992). The ¹H-NMR spectrum (400 MHz, CDCl₃; Figures 3.11-3.12; Table 3.4) showed seven singlets between δ 0.74-1.19 corresponding to seven methyl groups and a doublet of three proton intensity at δ 0.90 (3H, *J*= 6.4 Hz). These confirmed the presence of eight methyl groups in a pentacyclic type triterpenoid skeleton. However, the lack of any olefinic proton resonance around δ 5.00-5.50 and oxymethine proton signal around δ 3.00 demonstrated the absence of double bond between C-12 and C-13 and the oxymethine proton at C-3.

The ¹³C-NMR (125 MHz, CDCl₃; Figures 3.13-3.16; Table 3.5) displayed 30 carbon resonances including a carbonyl carbon at δ 213.54 ppm. The DEPT spectrum (Figures 3.17-3.19) indicated that twenty three out of the thirty carbons in BVSP-4 had attached protons. The DEPT experiment further revealed the presence of eight methyl, eleven methylene, four methine and seven quaternary carbon atoms. All these features are indicative of the presence of a triterpenoid type carbon skeleton in BVSP-4 having no double bond between C-12 and C-13, and no oxymethine proton at C-3.

The melting point, ¹H and ¹³C-NMR spectral data (Tables 3.4 and 3.5, respectively) of BVSP-4 were compared with the published data of friedelin and found to be identical (Akihisa *et al.*, 1992). Thus, compound BVSP-4 was characterized as friedelin (48). This is the first report of isolation of friedelin from *B. verrucosa*.

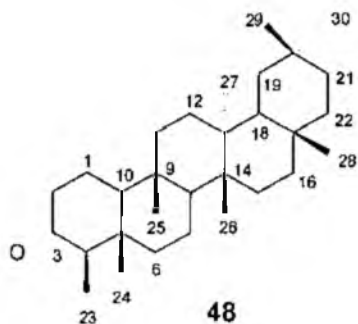


Figure 3.10: Structure of BVSP-4 (friedelin, 48).

Table 3.4: ^1H - NMR spectral data of BVSP-4 (48) and friedelin (Akihisa *et al.*, 1992) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------|---|--------------|
| | BVSP-4 (48) | Friedelin |
| Me-23 | 0.90 d (6.4) | 0.88 d (7.0) |
| Me-24 | 0.74 s | 0.73 s |
| Me-25 | 0.88 s | 0.87 s |
| Me-26 | 1.02 s | 1.01 s |
| Me-27 | 1.06 s | 1.05 s |
| Me-28 | 1.19 s | 1.18 s |
| Me-29 | 0.97 s | 0.95 s |
| Me-30 | 1.01 s | 1.00 s |

Table 3.5: ^{13}C -NMR spectral data of BVSP-4 (48) and friedelin (Akihisa *et al.*, 1992) in CDCl_3 .

| Position of carbon | Type of carbon | Friedelin | BVSP-4 (48) |
|--------------------|----------------------|-----------|-------------|
| C-1 | CH_2 | 22.3 | 22.5 |
| C-2 | CH_2 | 41.5 | 41.5 |
| C-3 | $>\text{C}=\text{O}$ | 213.2 | 213.5 |
| C-4 | CH | 58.2 | 58.5 |
| C-5 | C | 42.1 | 42.4 |
| C-6 | CH_2 | 41.3 | 41.8 |
| C-7 | CH_2 | 18.2 | 18.5 |
| C-8 | CH | 53.1 | 53.3 |
| C-9 | C | 37.4 | 37.7 |
| C-10 | CH | 59.4 | 59.7 |
| C-11 | CH_2 | 35.6 | 35.9 |
| C-12 | CH_2 | 30.5 | 30.1 |
| C-13 | C | 39.7 | 39.2 |
| C-14 | C | 38.4 | 38.5 |
| C-15 | CH_2 | 32.4 | 32.9 |
| C-16 | CH_2 | 36.0 | 36.0 |
| C-17 | C | 30.0 | 30.2 |
| C-18 | CH | 42.8 | 43.0 |
| C-19 | CH_2 | 35.3 | 35.4 |
| C-20 | C | 28.1 | 28.4 |
| C-21 | CH_2 | 32.7 | 32.6 |
| C-22 | CH_2 | 39.2 | 39.5 |
| C-23 | CH_3 | 6.8 | 7.0 |
| C-24 | CH_3 | 14.6 | 14.8 |
| C-25 | CH_3 | 17.9 | 18.1 |
| C-26 | CH_3 | 20.2 | 20.4 |
| C-27 | CH_3 | 18.6 | 18.9 |
| C-28 | CH_3 | 32.1 | 32.0 |
| C-29 | CH_3 | 35.0 | 35.0 |
| C-30 | CH_3 | 31.8 | 31.3 |

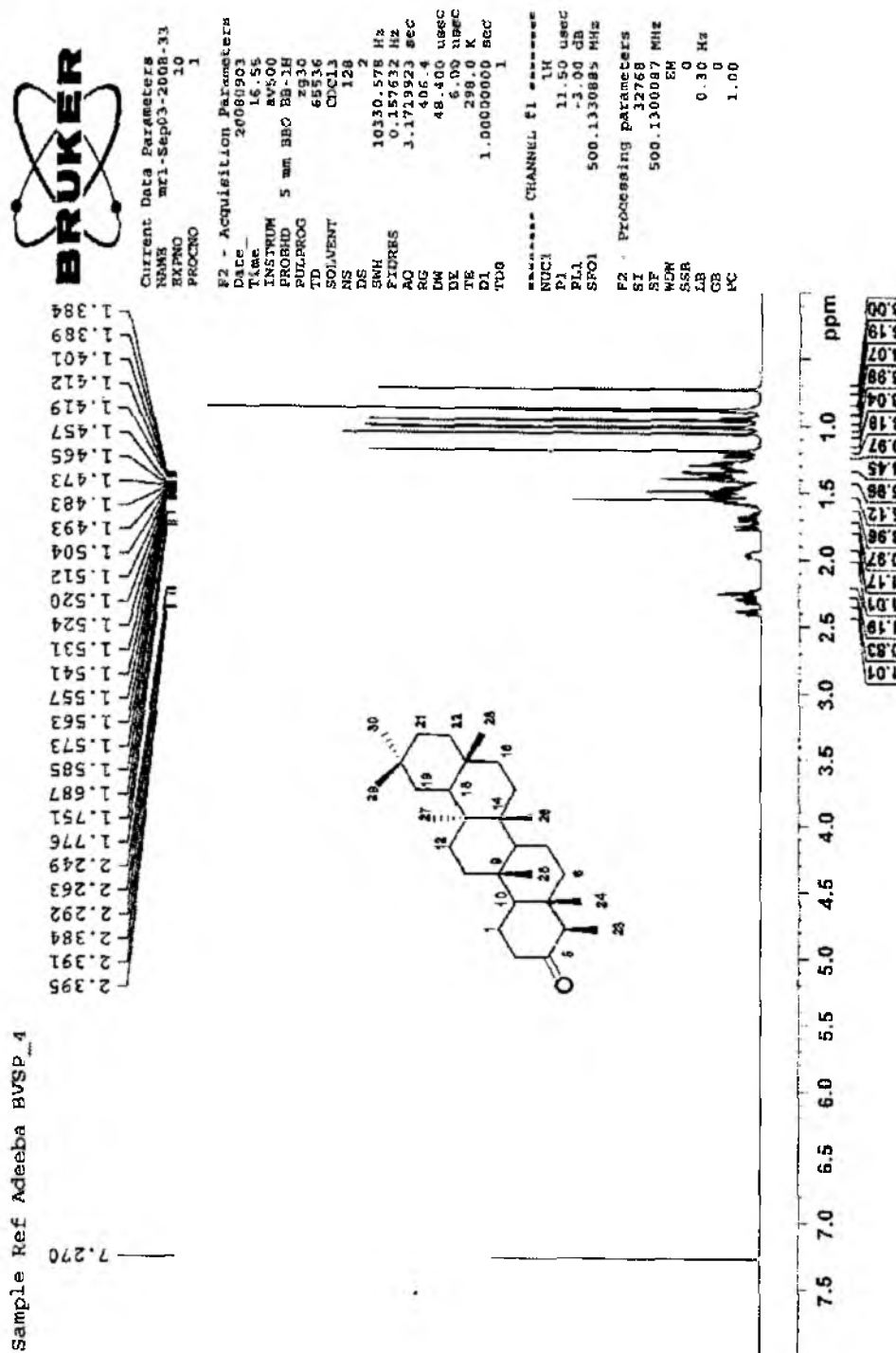


Figure 3.11: ¹H-NMR spectrum (400 MHz, CDCl₃) of BVSP-4 (48).

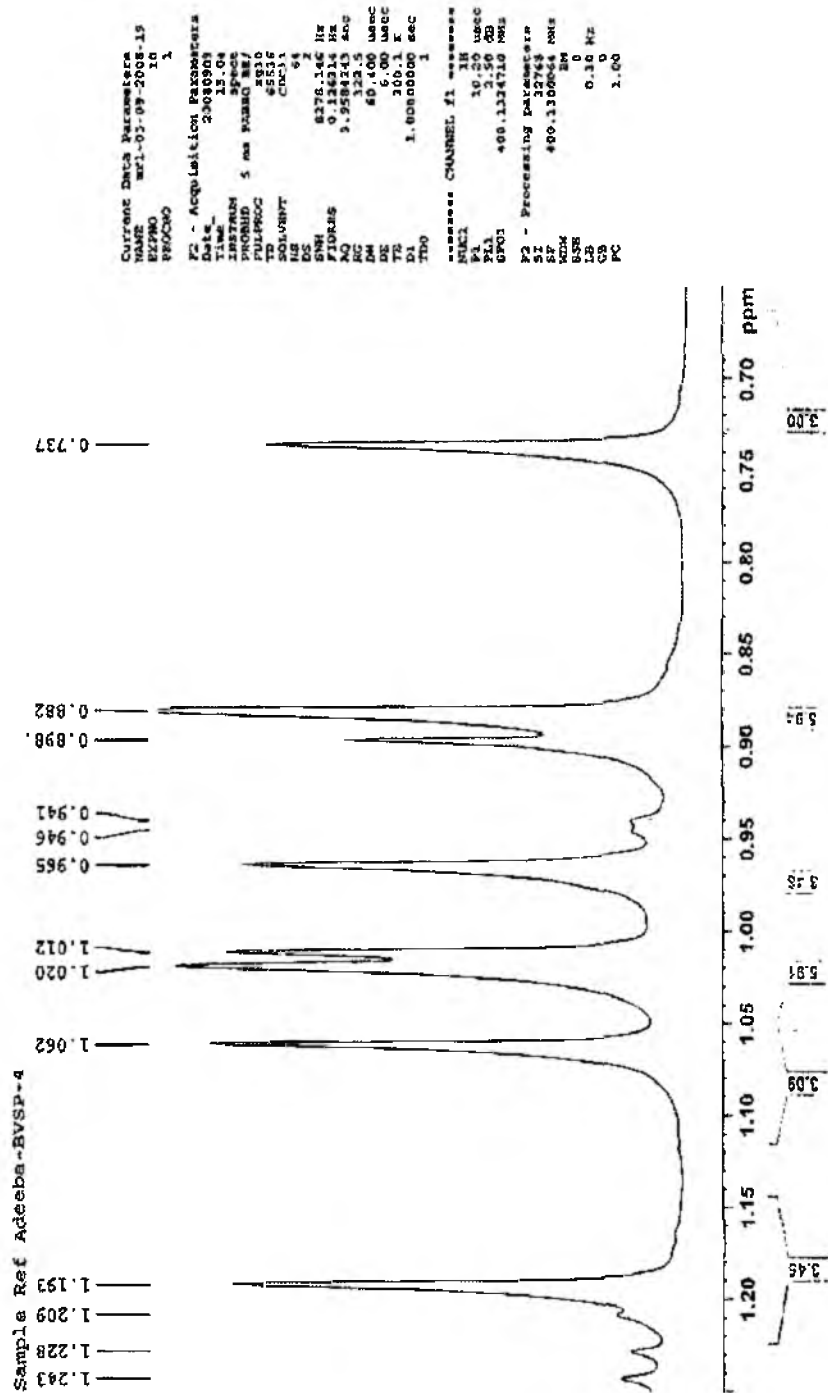
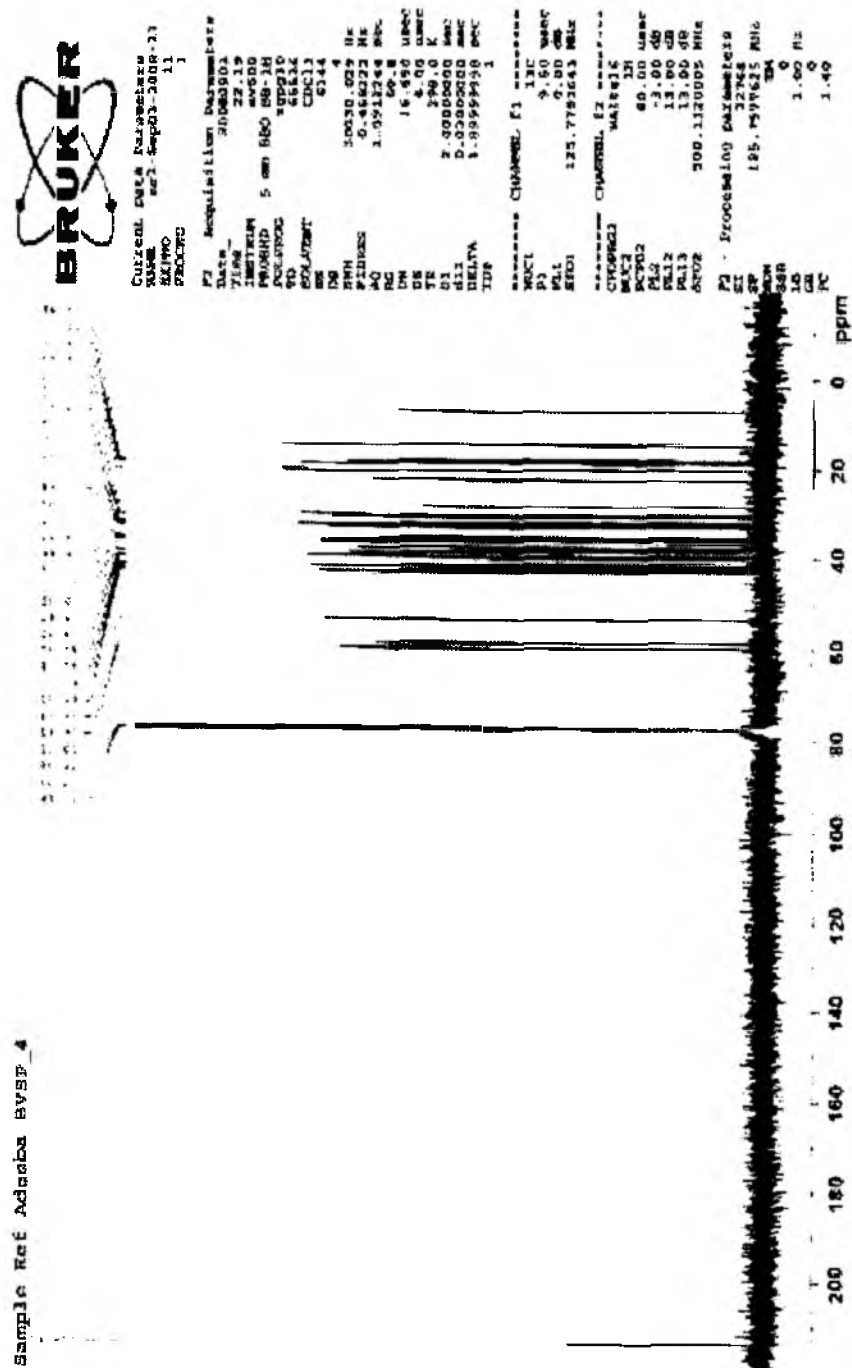


Figure 3.12: Partially expanded ¹H-NMR spectrum (400 MHz, CDCl₃) of BVSP-4 (48).

Figure 3.13: ^{13}C -NMR spectrum of BVSP-4 (48) in CDCl_3 .

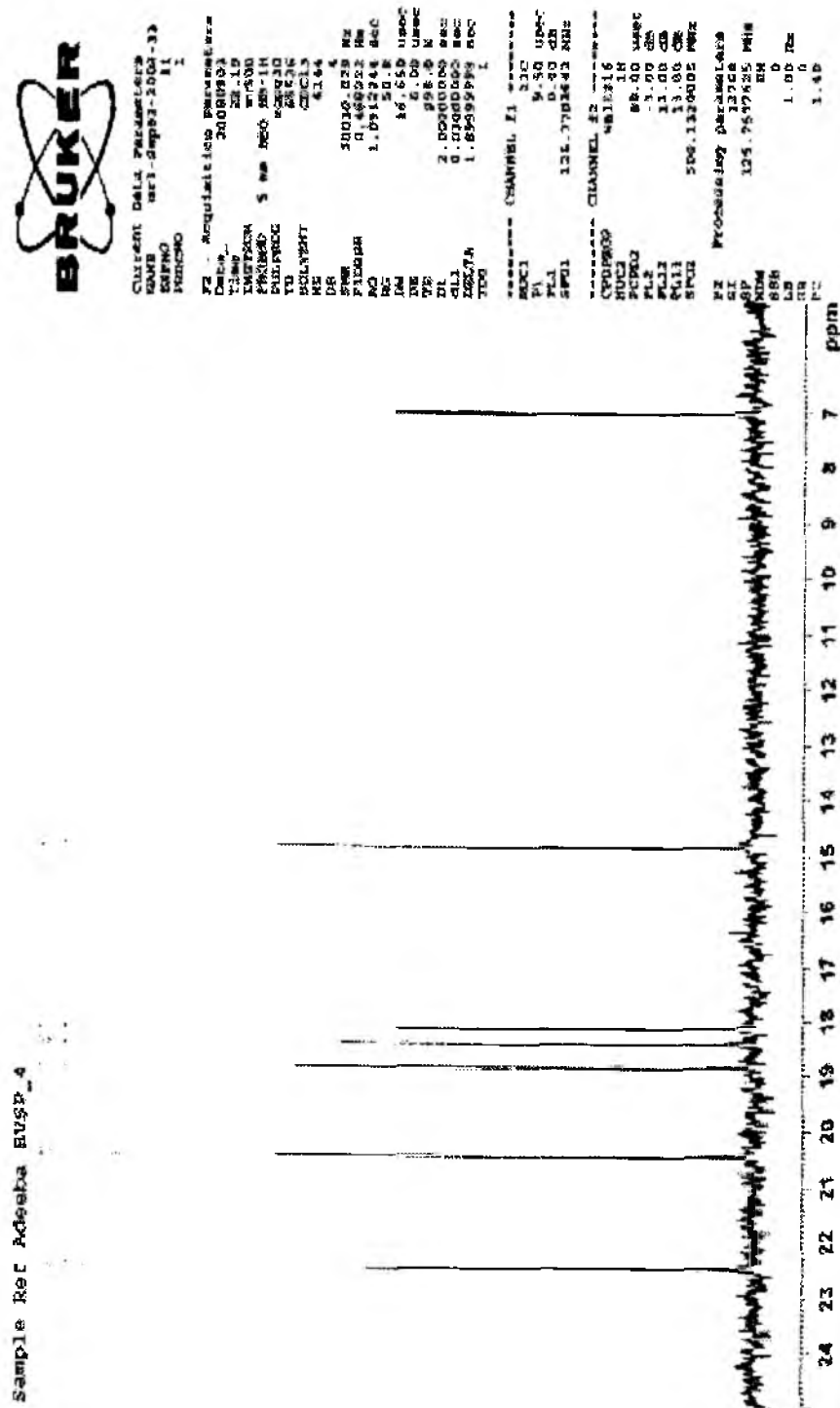


Figure 3.14: Partially expanded ^{13}C -NMR spectrum of BVSP-4 (48) in CDCl_3 .

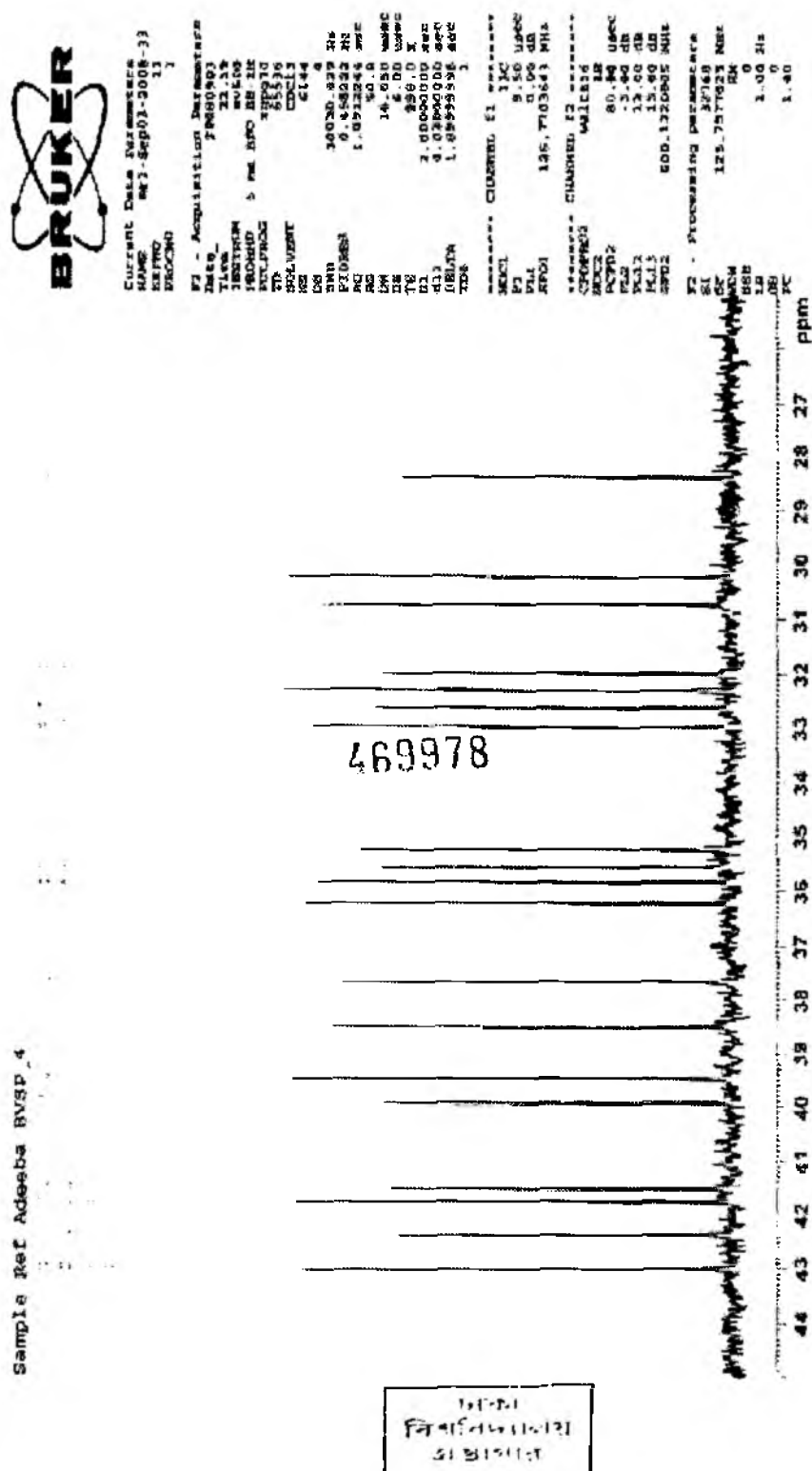
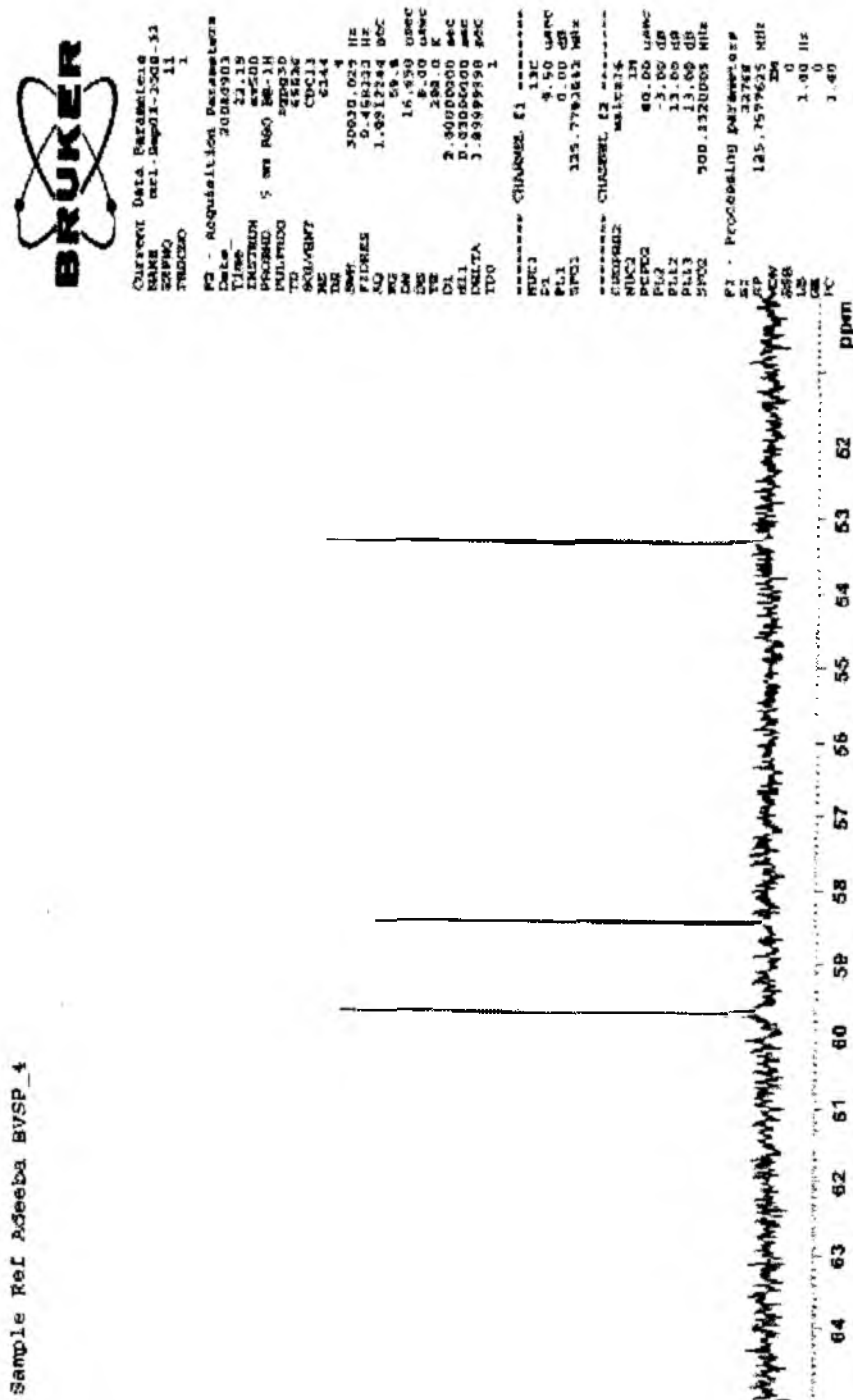


Figure 3.15: Partially expanded ^{13}C -NMR spectrum of BVSP-4 (48) in CDCl_3 .

Figure 3.16: Partially expanded ^{13}C -NMR spectrum of BVSP-4 (48) in CDCl_3 .

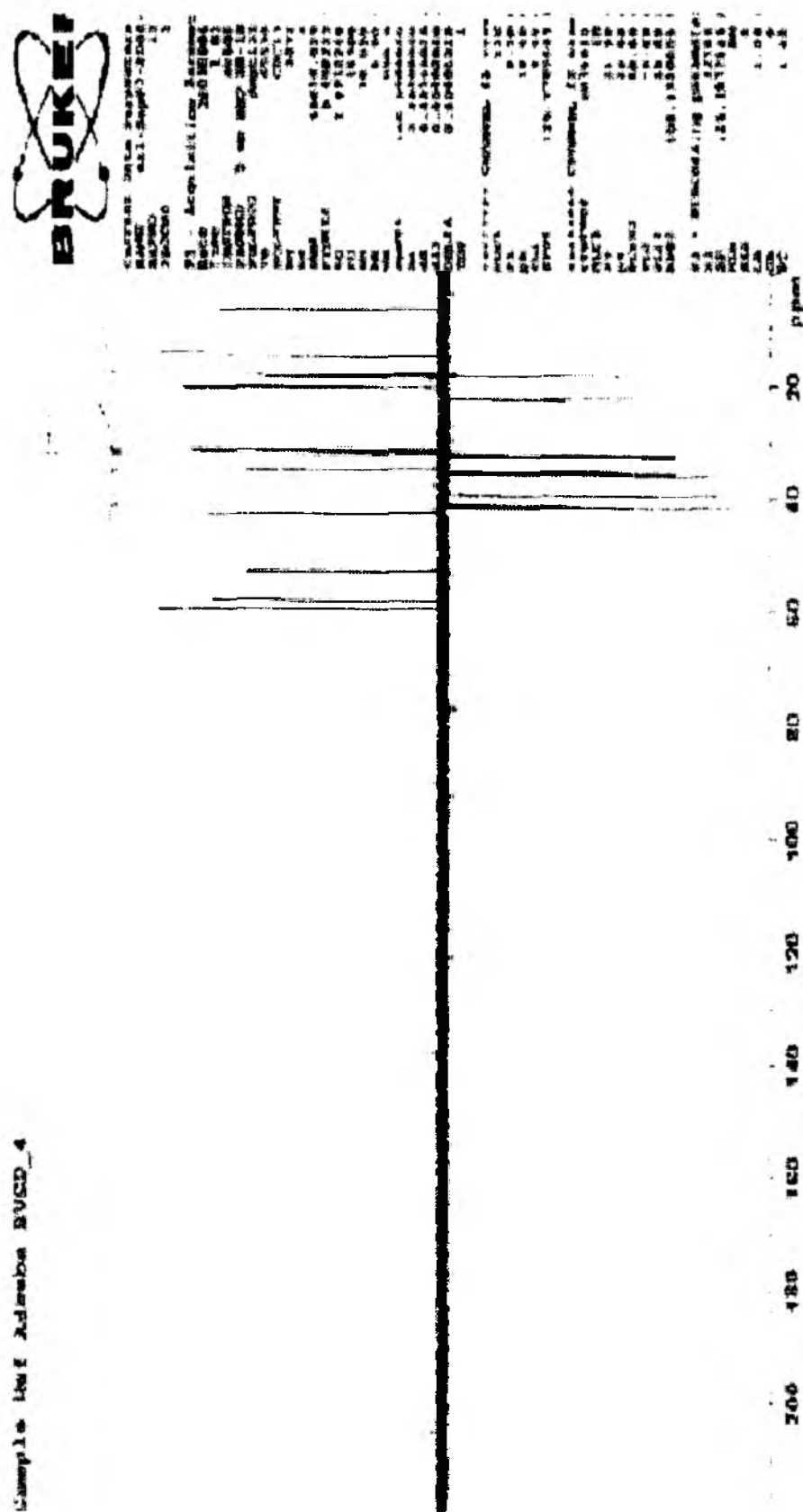


Figure 3.17: DEPT spectrum of BVSP-4 (48) in CDCl₃.

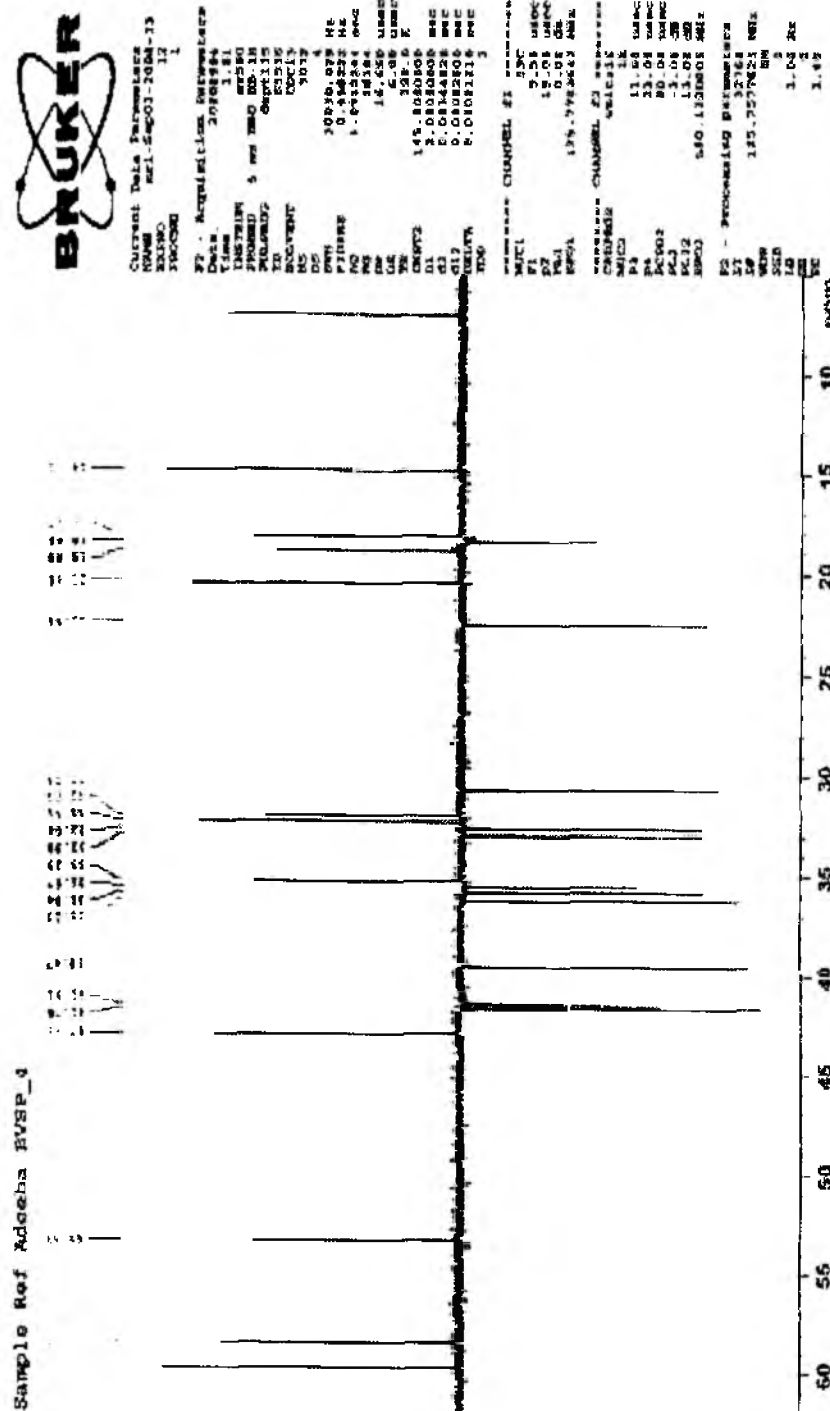


Figure 3.18: Partially expanded DEPT spectrum of BVSP-4 (48) in CDCl₃.

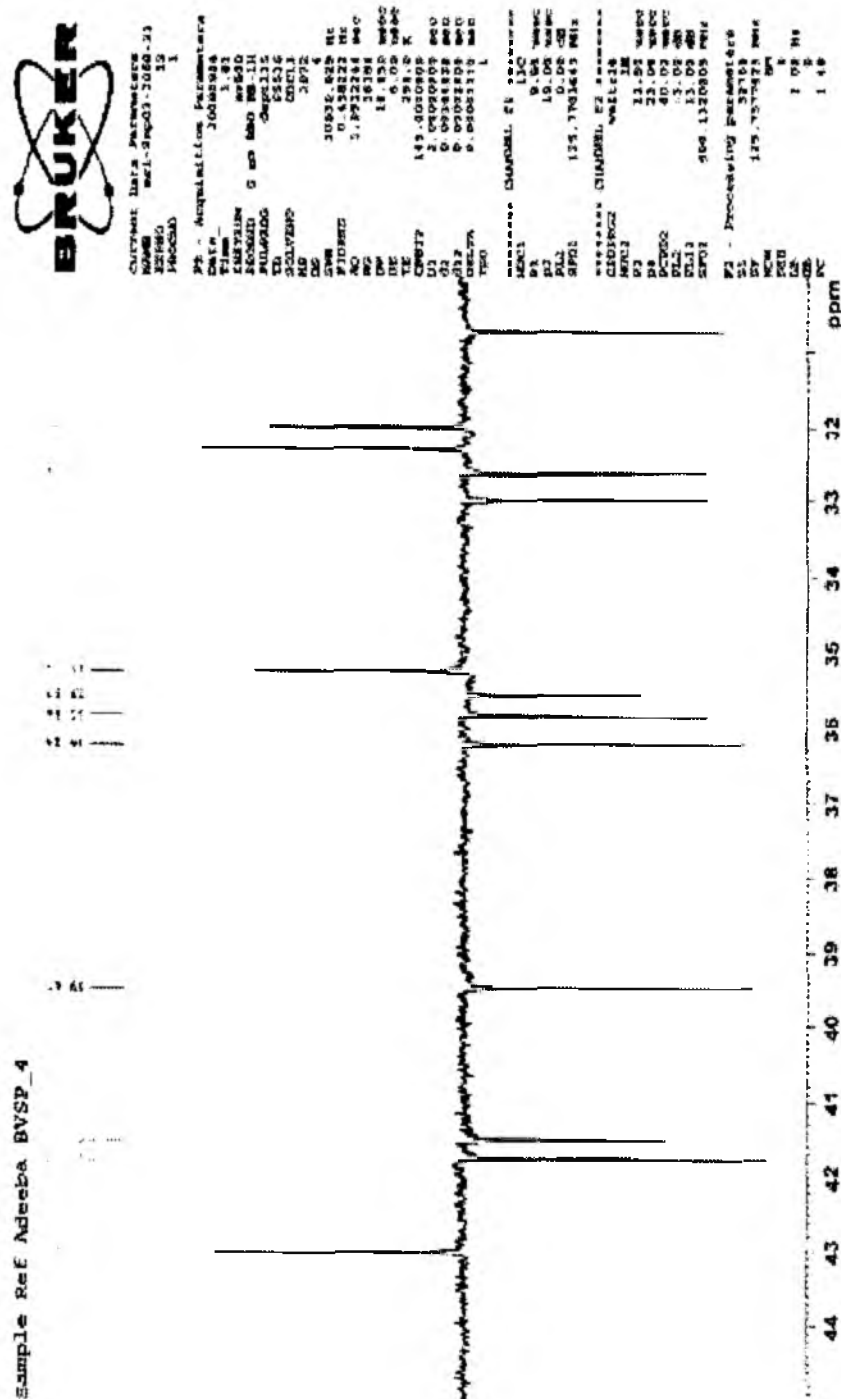


Figure 3.19: Partially expanded DEPT spectrum of BVSP-4 (48) in CDCl₃.

3.4 Characterization of BVSP-6 as 5 β -24S-ethylcholestan-3 β -ol (49)

The *n*-hexane soluble fraction of the crude methanolic extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain 50 sub-fractions. The sub-fractions 5-9 were combined together depending on the identical TLC characterization and subjected to preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (95:5) as the developing solvent. The process was repeated five times for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The band was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent afforded a colorless compound BVSP-6 (7.14 mg) (49). The compound was analyzed over TLC to check its purity and found as a pure compound which was soluble in *n*-hexane, dichloromethane, ethyl acetate and acetone.

The ¹H-NMR spectrum (400 MHz, CDCl₃; Figures 3.21-3.22; Table 3.6) revealed a one proton multiplet at δ 3.75, the position and multiplicity of which was indicative of H-3 in a steroidal nucleus. It also displayed signals of three proton intensity at δ 0.93 and 0.70 indicating two tertiary methyl groups at C-10 and C-13. Again doublets (each 3H intensity) at δ 0.87 (d, *J*= 8.0 Hz) and 0.83 (d, *J*= 7.2 Hz) were observed due to secondary methyl groups at C-25. Another doublet at δ 0.92 (d, *J*= 6.4 Hz) was assigned to a methyl group at C-20. A triplet of three proton intensity at δ 0.89 (*J*= 7.5 Hz) due to primary methyl group to the primary methyl group at C-28. The absence of any signal between δ 5.00 and 6.00 suggested the absence of olefinic proton between C-5 and C-6 as observed in sitosterol and stigmasterol type of steroidal compounds.

These spectral features (Table 3.6) were found to be in close agreement with those of 5 β -24S-ethylcholestan-3 β -ol (49) (Luo *et al.*, 2009). This is the first report of isolation of 5 β -24S-ethylcholestan-3 β -ol from *B. verrucosa*.

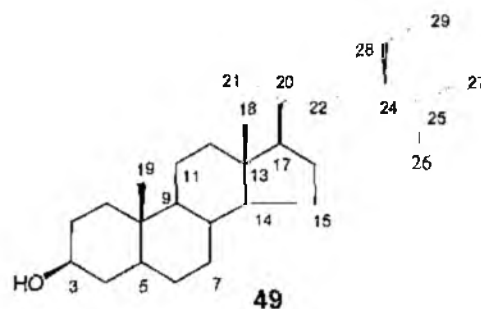


Figure 3.20: Structure of BVSP-6 (5 β -24S-ethylcholestan-3 β -ol, 49).

Table 3.6: ^1H -NMR spectral data of BVSP-6 (49) and 5 β -24S-ethylcholestan-3 β -ol (Luo *et al.*, 2009) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------|---|---|
| | BVSP-6 (49) | 5 β -24S-Ethylcholestan-3 β -ol |
| H-3 | 3.99 m | 4.10 m |
| Me-18 | 0.70 s | 0.64 s |
| Me-19 | 0.93 s | 0.96, s |
| Me-21 | 0.92 d (6.4) | 0.90 d (6.7) |
| Me-26 | 0.87 d (8.0) | 0.82 d (7.7) |
| Me-27 | 0.83 d (7.2) | 0.80 d (7.7) |
| Me-29 | 0.89 t (7.5) | 0.85 t (7.5) |

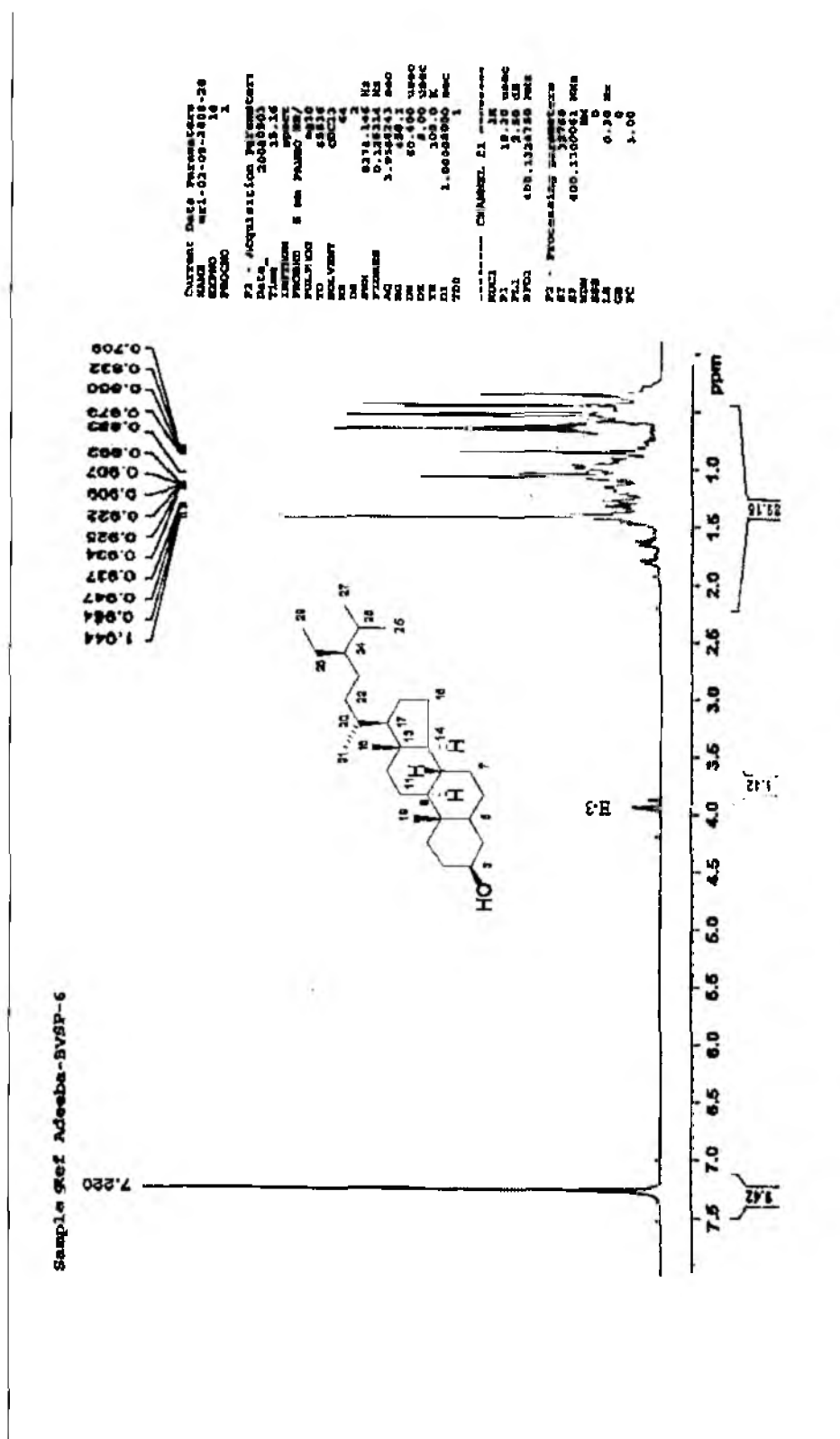


Figure: 3.21: ¹H-NMR spectrum (400 MHz, CDCl₃) of BVSP-6 (49).

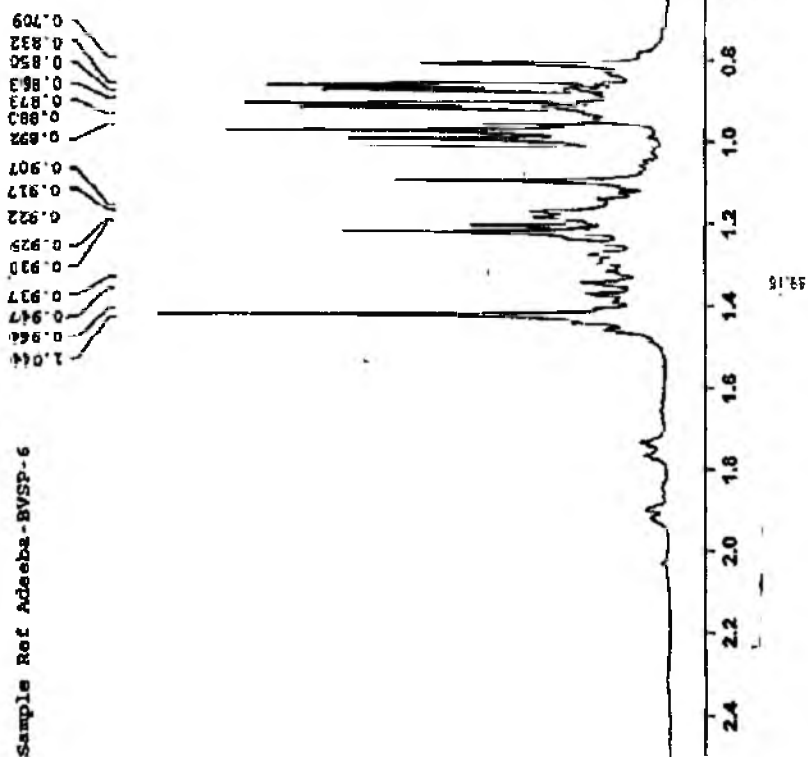


Figure 3.2 : Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BVSP-6 (49).

3.5 Characterization of BVSP-34 as stigmasterol (50)

Fractions 34-43 from silica gel column chromatography of the *n*-hexane soluble portion of the methanol extract of the stem bark of *B. verrucosa* gave similar TLC feature and were combined together. Then it was subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (95:5)]. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating in 110 °C for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded white needles which melted at 163 °C which was identical to that published (163 °C) for stigmasterol (Khan and Mandal, 1991). The compound was analyzed over TLC to check for purity and found to be a pure compound which was termed as BVSP-34 (6.5 mg, 50). It was also found to be soluble in *n*-hexane, dichloromethane, ethyl acetate and acetone.

The ¹H-NMR spectrum (400 MHz, CDCl₃; Figure 3.24; Table 3.7) revealed a one proton multiplet at δ 3.49, the position and multiplicity of which was indicative of H-3 in a steroidal nucleus. The typical C-6 proton was evident as a broad singlet at δ 5.40 that integrated for one proton.

The olefinic protons H-22 and H-23 appeared as characteristic downfield signals at δ 5.14 (dd, *J*= 15.0, 6.5 Hz) and δ 5.04 (dd, *J*= 15.0, 6.5 Hz) which were comparable to the ¹H-NMR data of authentic stigmasterol. It further displayed signals of three proton intensity at δ 0.67 and 1.00 indicating two tertiary methyl protons at C-10 and C-13. Again doublets (each 3H) at δ 0.80 and 0.84 (*J*= 6.0 Hz) were observed due to secondary methyl groups at C-25 and a triplet at δ 0.82 (*J*= 6.5 Hz) was due to primary methyl group. Another doublet near δ 0.90 (*J*= 6.5 Hz) was assigned to a methyl group at C-20. These spectral features (Table 3.4) as well as melting point of BVSP-34 were found to be in close agreement with those of stigmasterol (Khan and Mandal, 1991). Moreover, the spectrum of BVSP-34 was super imposable to the ¹H-NMR spectrum acquired for an authentic stigmasterol previously isolated in our laboratory. So, BVSP-34 was identified as stigmasterol (50). This is the first report of isolation of stigmasterol from *B. verrucosa*.

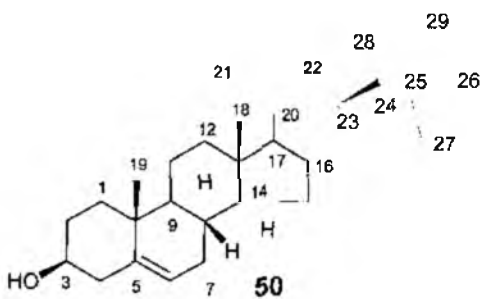


Figure 3.23: Structure of BVSP-34 (stigmasterol, 50).

Table 3.7: ^1H -NMR spectral data of BVSP-34 (50) and stigmasterol (Khan and Mandal, 1991) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------|---|------------------------|
| | BVSP-34 (50) | Stigmasterol |
| H-3 | 3.49 m | 3.52 m |
| H-6 | 5.35 m | 5.32 m |
| Me-18 | 0.67 s | 0.65 s |
| Me-19 | 1.00 s | 1.00 s |
| Me-20 | 0.90 d (6.5) | 0.90 d (6.7) |
| Me-21 | 0.91 d (6.2) | 0.93 d (6.6) |
| H-22 | 5.14 dd (15.0 and 6.5) | 5.17 dd (14.7 and 6.3) |
| H-23 | 5.04 dd (15.0 and 6.5) | 5.04 dd (15.0 and 6.5) |
| Me-25 | 0.84 d (6.0) | 0.84 d (6.0) |
| | 0.80 d (6.0) | 0.80 d (6.0) |
| Me-28 | 0.82 t (6.5) | 0.81 t (6.6) |

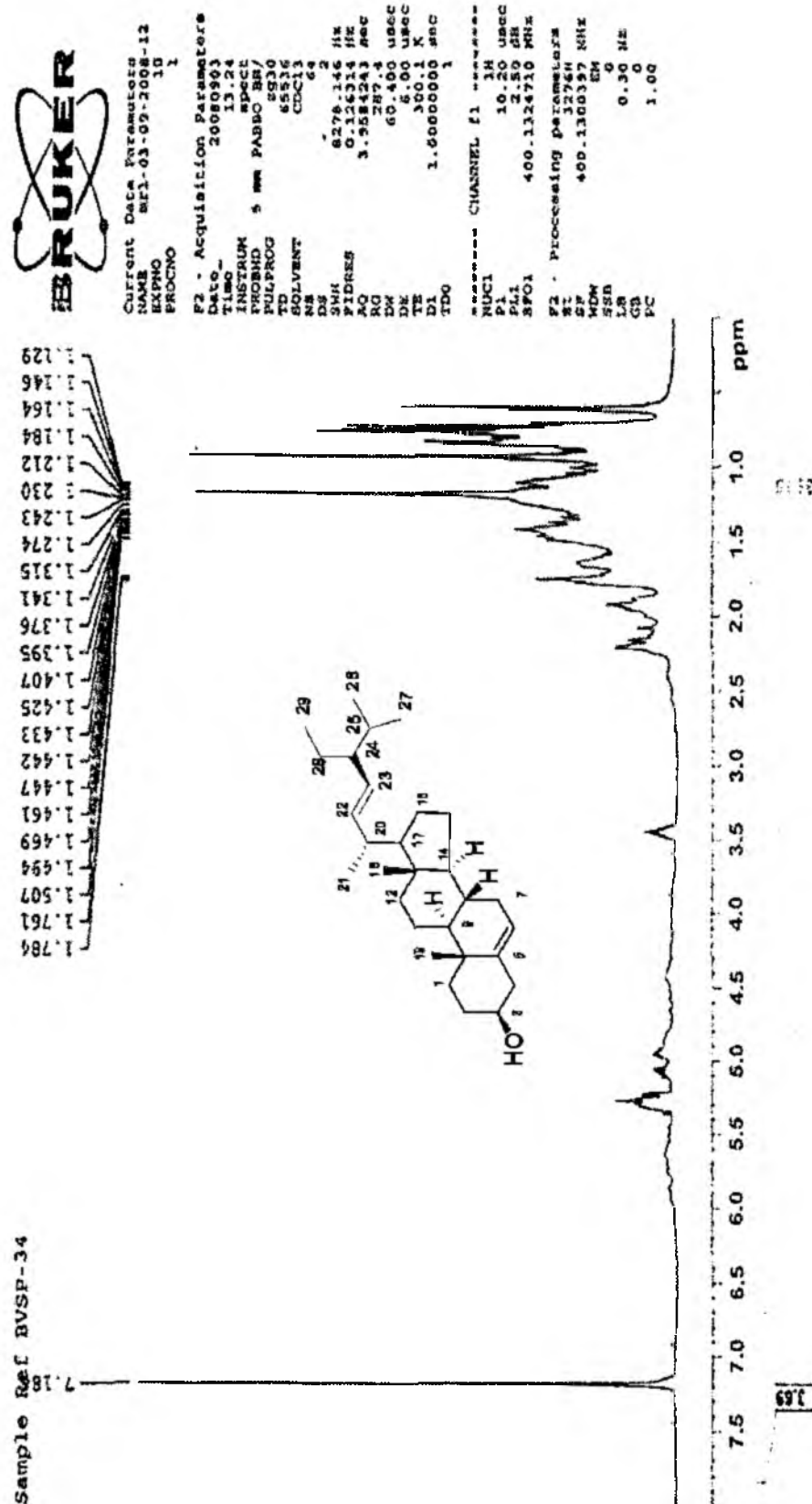


Figure: 3.24: ¹H-NMR spectrum (400 MHz, CDCl₃) of BVSP-34 (50).

3.6 Characterization of BVBC-2 as 5, 5'-dihydroxysesamine (51)

The carbon tetrachloride soluble partitionate (270 mg) of the crude methanolic extract of stem bark of *B. verrucosa* was subjected to silica gel column chromatography [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (9:1)] and 150 fractions were collected. Fractions 74-76 were combined together. Evaporation of solvents yielded a whitish mass which was purified by preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (9:1) as the developing solvent. The process was repeated twice for better separation and isolation of the compound of interest. The desired band was scrapped off and eluted with ethyl acetate to yield BVBC-2 (7.1 mg, **51**) as whitish mass. It was found to be soluble in chloroform, ethyl acetate and methanol.

The ¹H-NMR (400 MHz, CDCl₃; Figures 3.26-3.28; Table 3.8) spectral data of BVBC-2 could be assigned by careful comparison of the data reported for structurally related compound, sesamine (Pelter *et al.*, 1976; Jayasinghe *et al.*, 2003) as well as with the help of ¹H-¹H COSY spectrum (Figure 3.29). It showed a one proton multiplet at δ 3.05 which could be assigned to the methine proton, H-1. In the ¹H-¹H COSY spectrum it was found to couple with a vicinal proton at δ 4.84 (d, J = 5.2 Hz, H-2) and also with a pair of methylene protons at δ 3.85 (dd, J = 9.0 and 6.0 Hz, H-8eq) and δ 4.52 (dd, J = 9.0 and 6.0 Hz, H-8ax). Another pair of methylene protons were observed at δ 4.05 (d, J = 9.6 Hz, H-4eq) and δ 3.92 (d, J = 9.6 Hz, H-4ax) which were geminally coupled to each other only indicating the absence of a vicinal proton at H-5 position. This can further be confirmed as the neighbouring H-6 proton appeared as a singlet at δ 4.84.

Jayasinghe *et al.*, (2003) reported the presence of an aromatic H-5' proton in sesamine (**52**) which coupled with the vicinal aromatic proton H-6' and another proton H-2' to give a double doublet (J = 7.90 and 1.5 Hz, aromatic coupling). But in compound BVBC-2 no ortho coupling for H-6' was observed but due to small meta coupling H-6' appeared as a doublet (J = 1.6 Hz). Hence it is predicted that, there may be an OH group at C-5' position. The remaining spectral data were similar to that of the reported compound sesamine.

On the basis of the above spectral data as well as by comparison with that of sesamine (**52**), the proton chemical shifts of BVBC-2 have been assigned (Table-3.8). So the isolated compound BVBC-2 could be tentatively identified as a new structure, 5,5'-dihydroxysesamine (**51**). However, additional spectral data (MS and ¹³C-NMR) are required to confirm its structure.

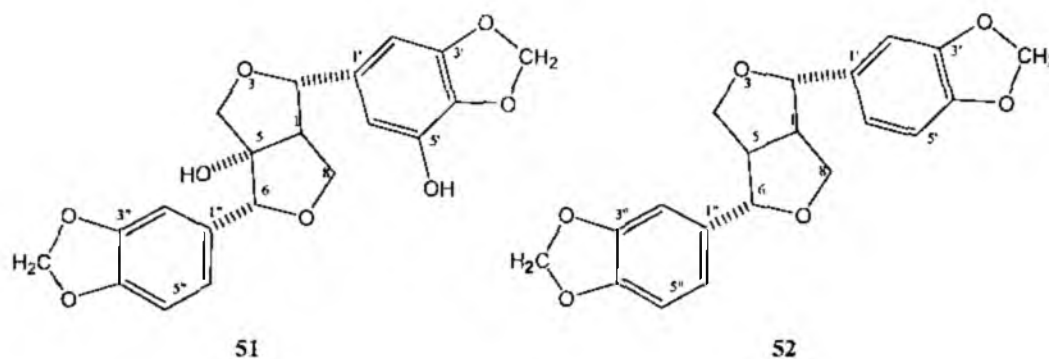


Figure 3.25: Structures of BVBC-2 (5,5'-dihydroxysesamine, **51**) and sesamine (**52**).

Table 3.8: ¹H-NMR spectral data of BVBC-2 (**51**) and sesamine (**52**) (Jayasinghe *et al.*, 2003) in CDCl₃.

| Proton(s) | δ_H in ppm in CDCl ₃ , (<i>J</i> in Hz). | |
|---------------------------------|---|------------------------|
| | BVBC-2 (51) | Sesamine (52) |
| H-1 | 3.05 m | 3.05 m |
| H-2 β | 4.84 d (5.2) | 4.71, d (4.4) |
| H-4eq/ H-4 α | 4.05 d (9.6) | 4.23, dd (9.3 and 6.8) |
| H-4ax/ H-4 β | 3.92 d (9.6) | 3.87, dd (9.3 and 3.7) |
| H-5 | - | 3.05, m |
| H-6 β | 4.84 d (5.2) | 4.71, d (4.4) |
| H-8eq/ H-8 β | 3.85 dd (9.0 and 6.0) | 3.87, dd (9.3 and 3.7) |
| H-8ax/ H-4 α | 4.52 dd (9.2 and 8.0) | 4.23, dd (9.3 and 6.8) |
| H-2' | 6.92 d (1.6) | 6.84 d (1.2) |
| H-5' | - | 6.77 d (7.9) |
| H-6' | 6.95 d (1.6) | 6.80 d (1.5) |
| H-2'' | 6.86 bs | 6.84 d (1.2) |
| H-5'' | 6.80 d (7.6) | 6.77 d (7.9) |
| H-6'' | 6.88 dd (1.6, 0.8) | 6.80 d (1.5) |
| 2 $\times$ -OCH ₂ O- | 5.99 s | 5.95, s |
| | 5.96 s | 5.95, s |

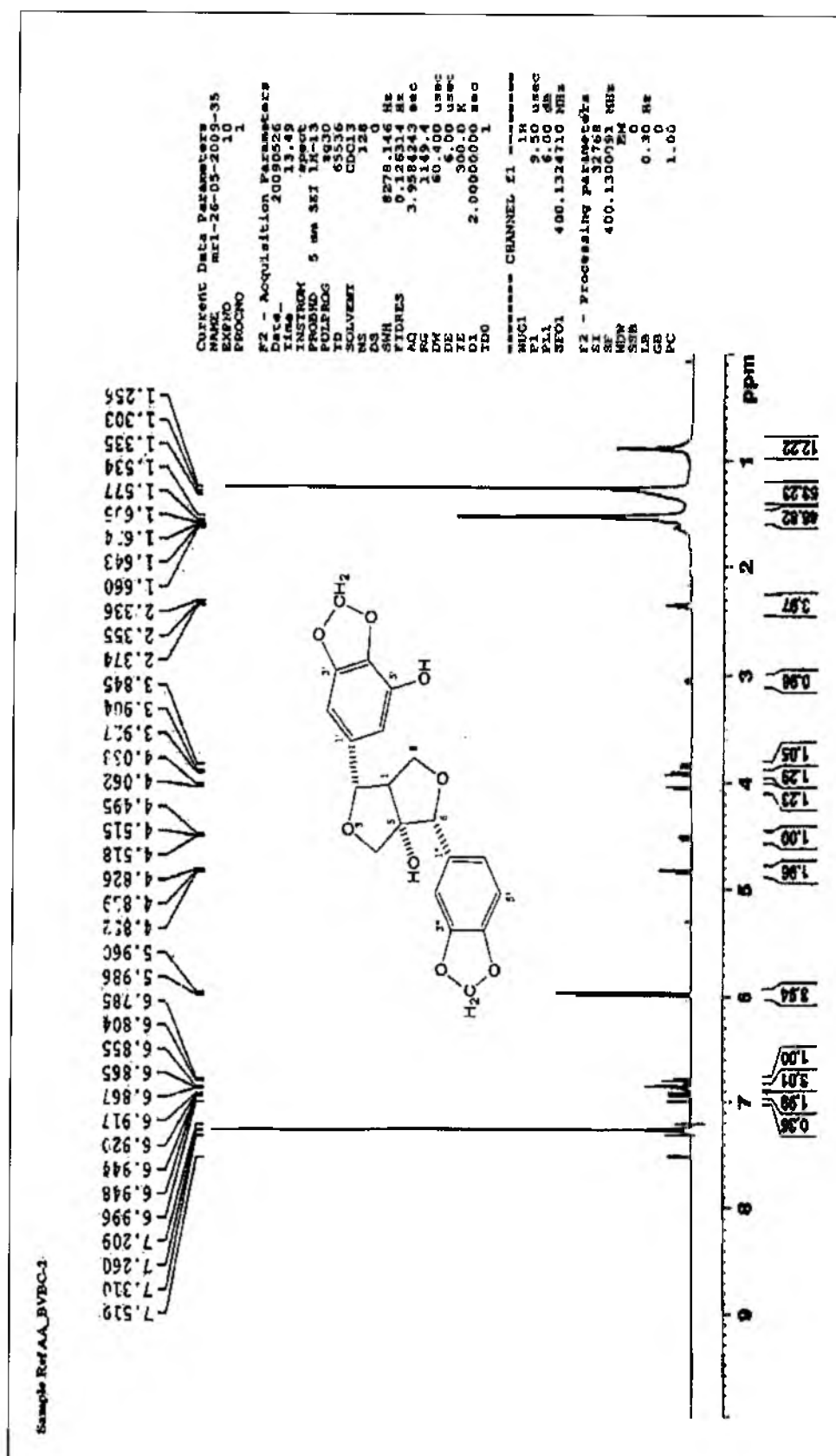


Figure 3.26: ¹H-NMR spectrum (400 MHz, CDCl₃) of BVBC-2 (51).

Sample Ref AA_BVBC-2

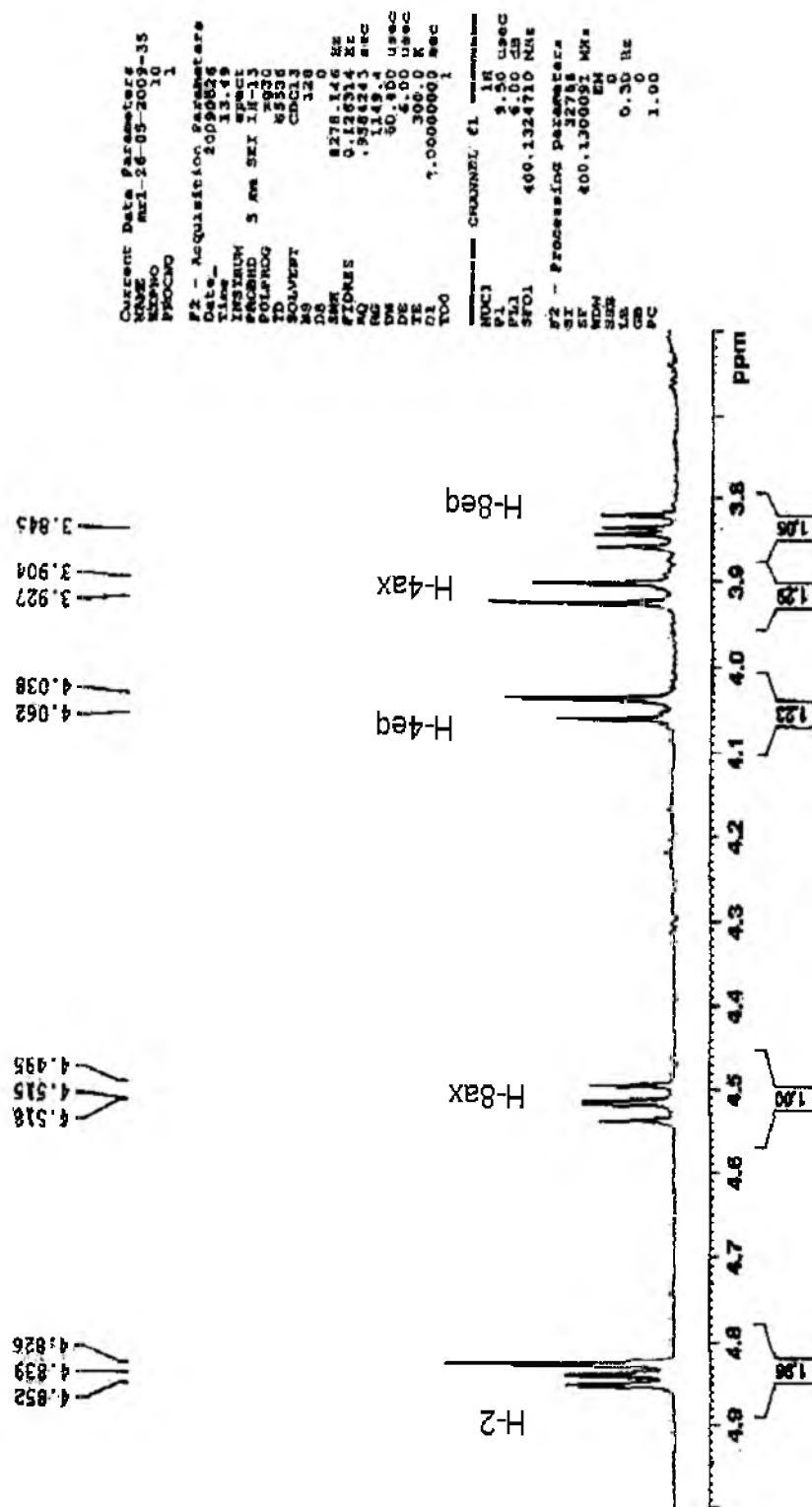


Figure 3.27: Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BVBC-2 (51).

Sample Ref AA_BVBC-2

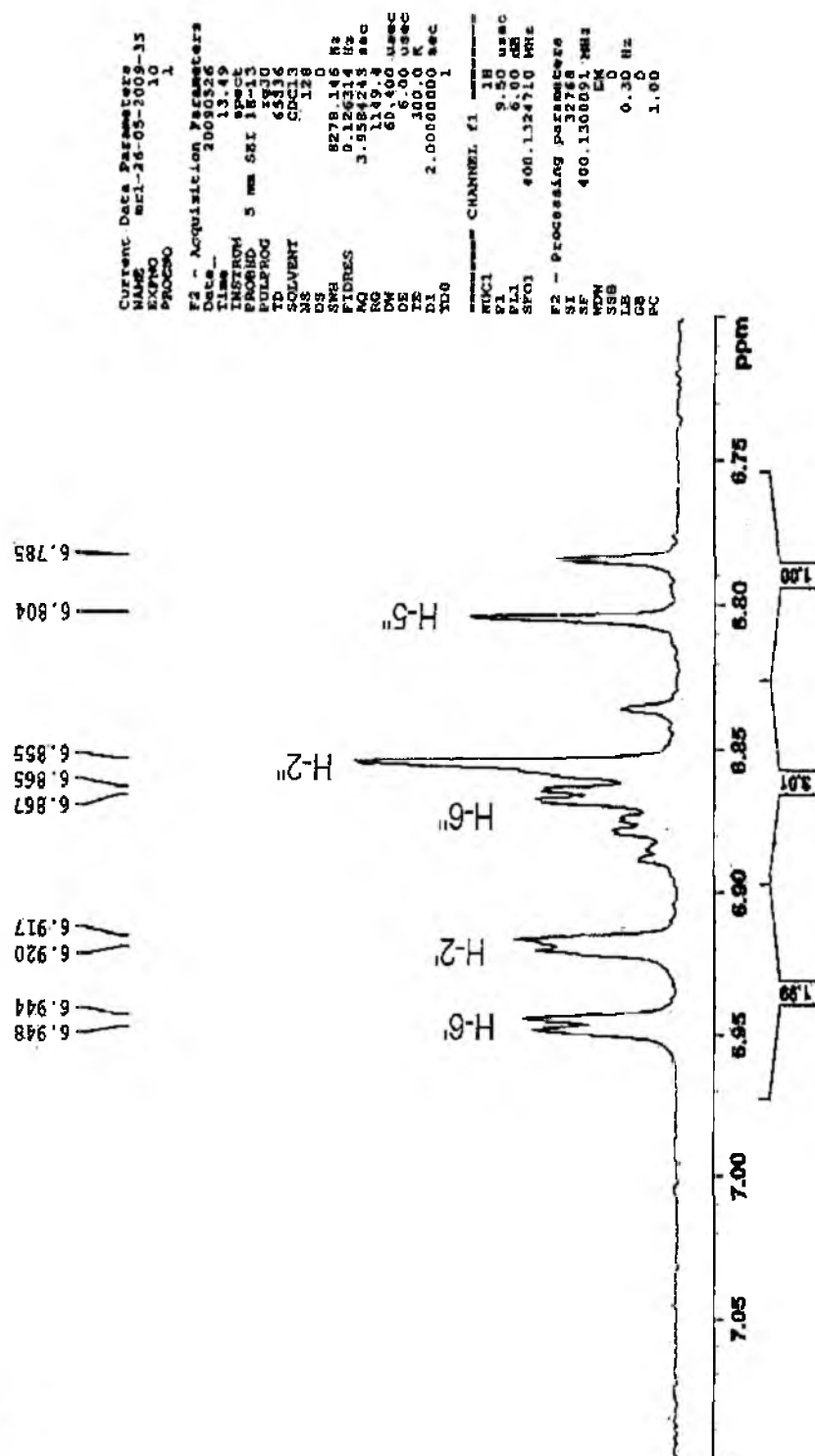


Figure 3.28: Partially expanded ¹H-NMR spectrum (400 MHz, CDCl₃) of BVBC-2 (51).

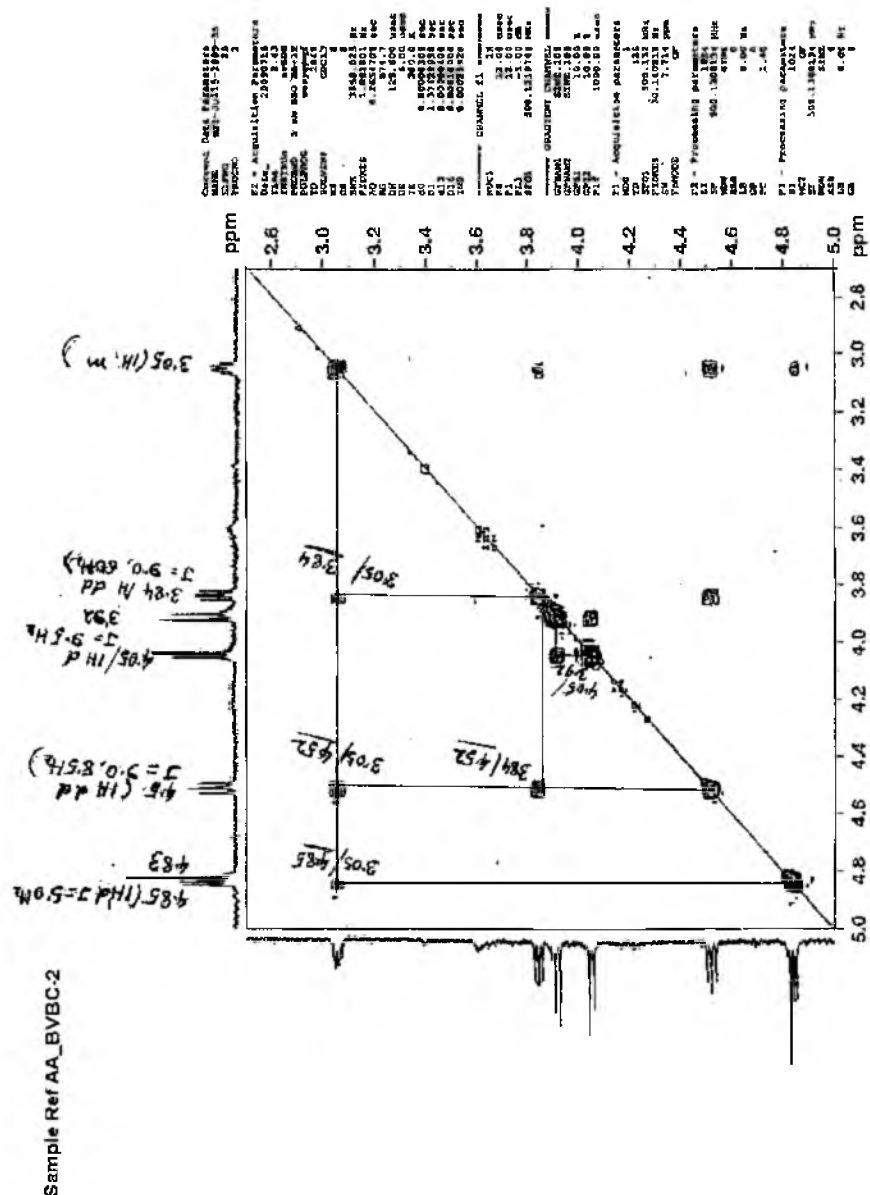


Figure 3.29: ^1H - ^1H COSY spectrum of BVBC-2 (5I) in CDCl_3 .

3.7 Characterization of BVSC-8 as 3-ketooleanane (53)

The carbon tetrachloride soluble portion of the crude methanolic extract of the stem bark of *B. verrucosa* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain one hundred fifty fractions.

Fractions 8-12 showing identical spots on TLC were combined in a beaker and it was kept undisturbed at room temperature for several days. The crystals obtained after evaporation of solvents were washed *n*-hexane to remove adhered coloring materials and recrystallized from *n*-hexane and ethyl acetate to afford a pure compound designated as needles designated BVSC-8 (7.53 mg, **53**) as needles. It was found to be soluble in chloroform, dichloromethane and ethyl acetate.

The crystals of BVSC-8 melted at 235-236 °C which was identical to that reported (233-235 °C) for 3-ketooleanane (Krishnaveni *et al.*, 2000). The ¹H-NMR spectrum (400 MHz, CDCl₃; Figures 3.31 -3.32; Table 3.9) showed eight singlets corresponding to eight methyl groups between δ 0.72-1.25. However, the absence of any olefinic proton resonance near δ 5.00 and oxymethine proton signal around δ 3.00 suggested the absence of double bond between C-12 and C-13, and the oxymethine proton at C-3. All these are indicative of the presence of a pentacyclic triterpenoid type skeleton in BVSC-8 having no double bond between C-12 and C-13.

The melting point and ¹H-NMR spectral data (Tables 3.9) of BVSC-8 were compared with the published data of 3-ketooleanane and found to be identical (Krishnaveni *et al.*, 2000). Thus, compound BVSC-8 was characterized as 3-ketooleanane (**53**). This is the first report of isolation of 3-ketooleanane from *B. verrucosa*.

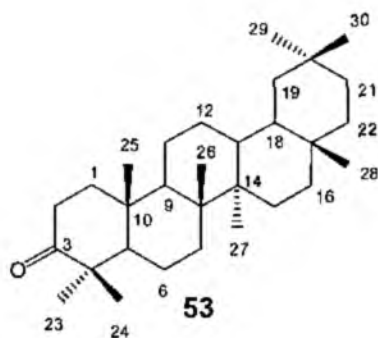


Figure 3.30: Structure of BVSC-8 as 3-ketooleanane (**53**).

Table 3.9: ^1H - NMR spectral data of BVSC-8 (**53**) and 3-ketooleanane (Krishnaveni *et al.*, 2000) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 . | |
|-----------|---|----------------|
| | BVSC-8 (53) | 3-ketooleanane |
| H-3 | - | - |
| H-6 | - | - |
| Me-23 | 1.25 s | 1.25 s |
| Me-24 | 1.18 s | 1.18 s |
| Me-25 | 0.87 s | 0.88 s |
| Me-26 | 0.72 s | 0.75 s |
| Me-27 | 1.05 s | 1.07 s |
| Me-28 | 0.96 s | 0.96 s |
| Me-29 | 1.01 s | 1.02 s |
| Me-30 | 1.00 s | 0.98 s |

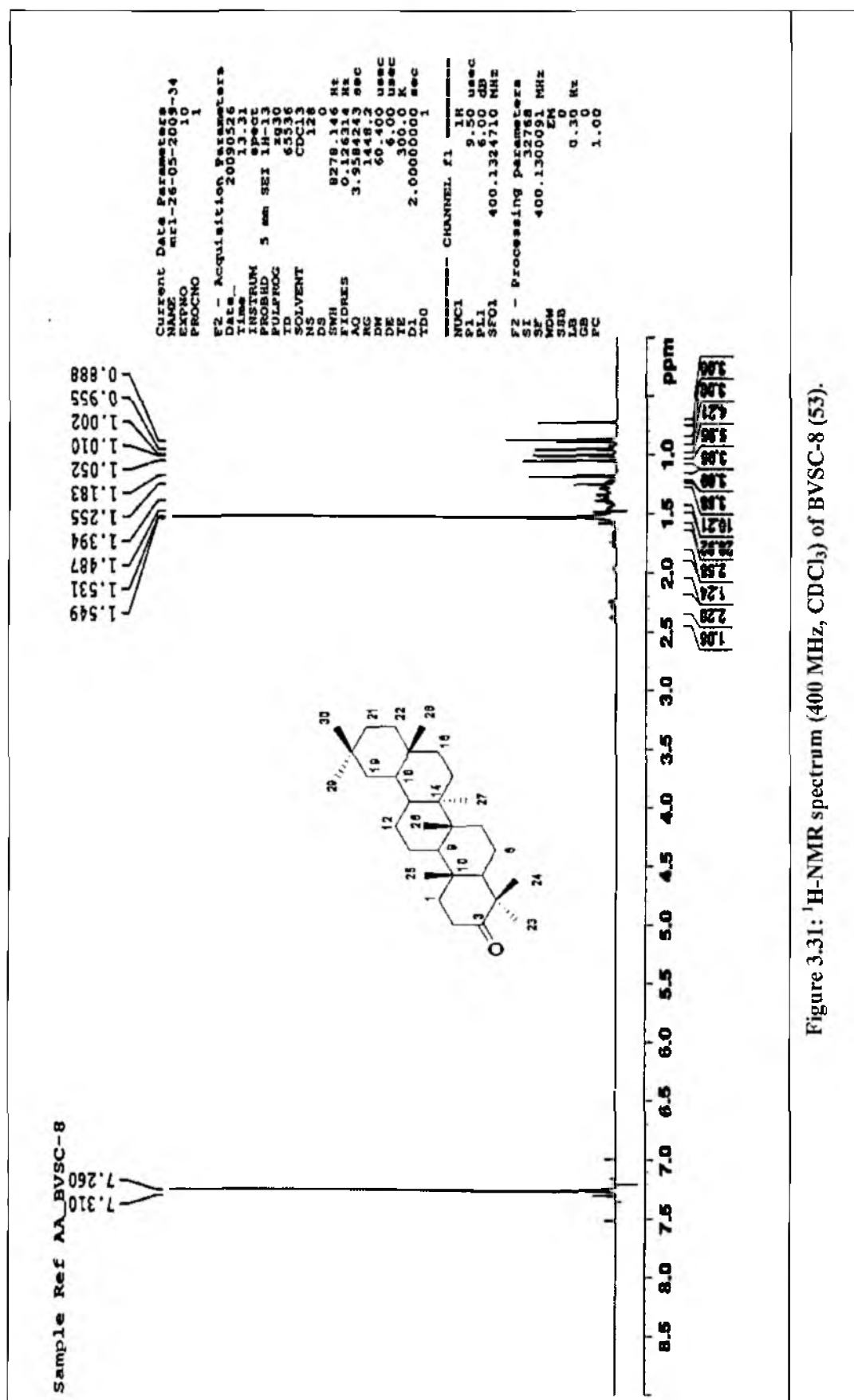
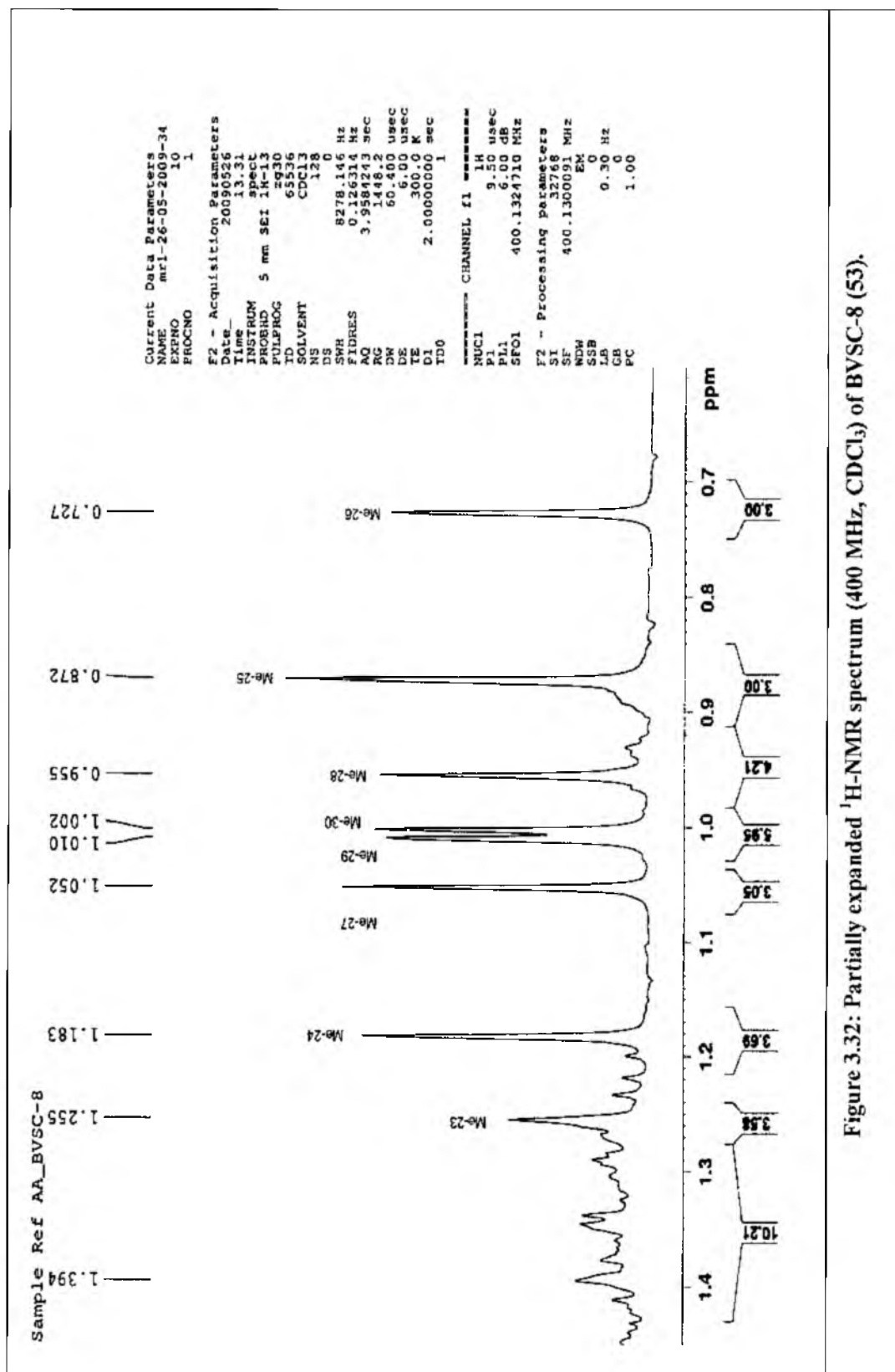


Figure 3.31: ^1H -NMR spectrum (400 MHz, CDCl_3) of BVSC-8 (53).



3.8 Characterization of BVS-65 as pinoresinol (54)

Compound BVS-65 was isolated as yellowish mass from the column chromatographic fractions 59-69 of the chloroform soluble materials of the methanolic extract of the stem bark of *B. verrucosa*. The concentrated fraction was subjected to preparative TLC over silica gel F₂₅₄ using chloroform-methanol (97:3) as the developing solvent. The desired band was scrapped off on an aluminum foil and eluted initially using a mixture of chloroform-methanol (19:1) to yield BVS-65 (3.54 mg, **54**). The compound was found to be soluble in dichloromethane, ethyl acetate, acetone and methanol.

The ¹H-NMR (400 MHz, CDCl₃; Figures 3.34-3.36; Table 3.10) spectrum of BVS-65 indicative of the typical pattern of two 1,3,4-trisubstituted benzene rings displayed signals at δ 6.88 (2H, d, J = 8.0 Hz, H-5', H-5''), δ 6.81 (2H, dd, J = 8.0, 1.6, H-6', H-6'') and δ 6.86 (2H, d, J = 1.6 Hz, H-2', H-2'') and two methoxy groups at δ 3.84 (6H). It also revealed a multiplet of two proton intensity at δ 3.09 (Perez *et al.*, 1995) for H-1 and H-5 and a doublet for H-2 and H-6 at δ 4.72 with J value of 4.00 Hz and another double doublet at δ 4.17 (J = 9.2, 7.2 Hz) that integrated for two protons, which could be attributed to H-4_{eq} and H-8_{eq}. The ¹H-NMR spectrum further showed a double doublet at δ 3.81 (J = 8.8 and 3.6) for two protons assignable to H-4_{ax} and H-8_{ax}.

These ¹H-NMR spectral data indicated that BVS-65 was a lignan of the 2,6-diaryl-3,7-dioxobicyclo [3.3.0] octane type (Pelter *et al.*, 1976). Moreover, all the other proton shifts were similar to that published for pinoresinol (Maria *et al.*, 1991) as shown in Table 3.7. So, the compound BVS-65 was identified as pinoresinol (**54**). This is the first report of isolation of pinoresinol from *B. verrucosa*.

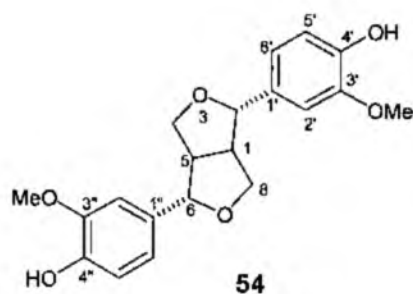


Figure 3.33: Structure of BVS-65 (pinoresinol, **54**).

Table 3.10: ^1H -NMR spectral data of BVS-65 (**54**) and pinoresinol (Maria *et al.*, 1991) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------------------|---|-----------------------|
| | BVS-65 (54) | Pinoresinol |
| H-1, 5 | 3.09 m | 3.1 m |
| H-2, 6 | 4.72 d (4.0) | 4.73 d (4.0) |
| H _{eq} -4, 8 | 4.17 dd (9.2 and 7.2) | 4.25 dd (9.0 and 6.5) |
| H _{ax} -4, 8 | 3.81 dd (8.8 and 3.6) | 3.87 dd (9.0 and 4.0) |
| H-2', 2'' | 6.86 d (1.6) | 6.89 d (2.0) |
| H-4', 4'' | 5.56 s | 5.59 s |
| H-5', 5'' | 6.88 d (8.0) | 6.88 d (8.0) |
| H-6', 6'' | 6.81 dd (8.0 and 1.6) | 6.82 dd (8.0 and 2.0) |
| OMe-3', 3'' | 3.84 s | 3.87 s |

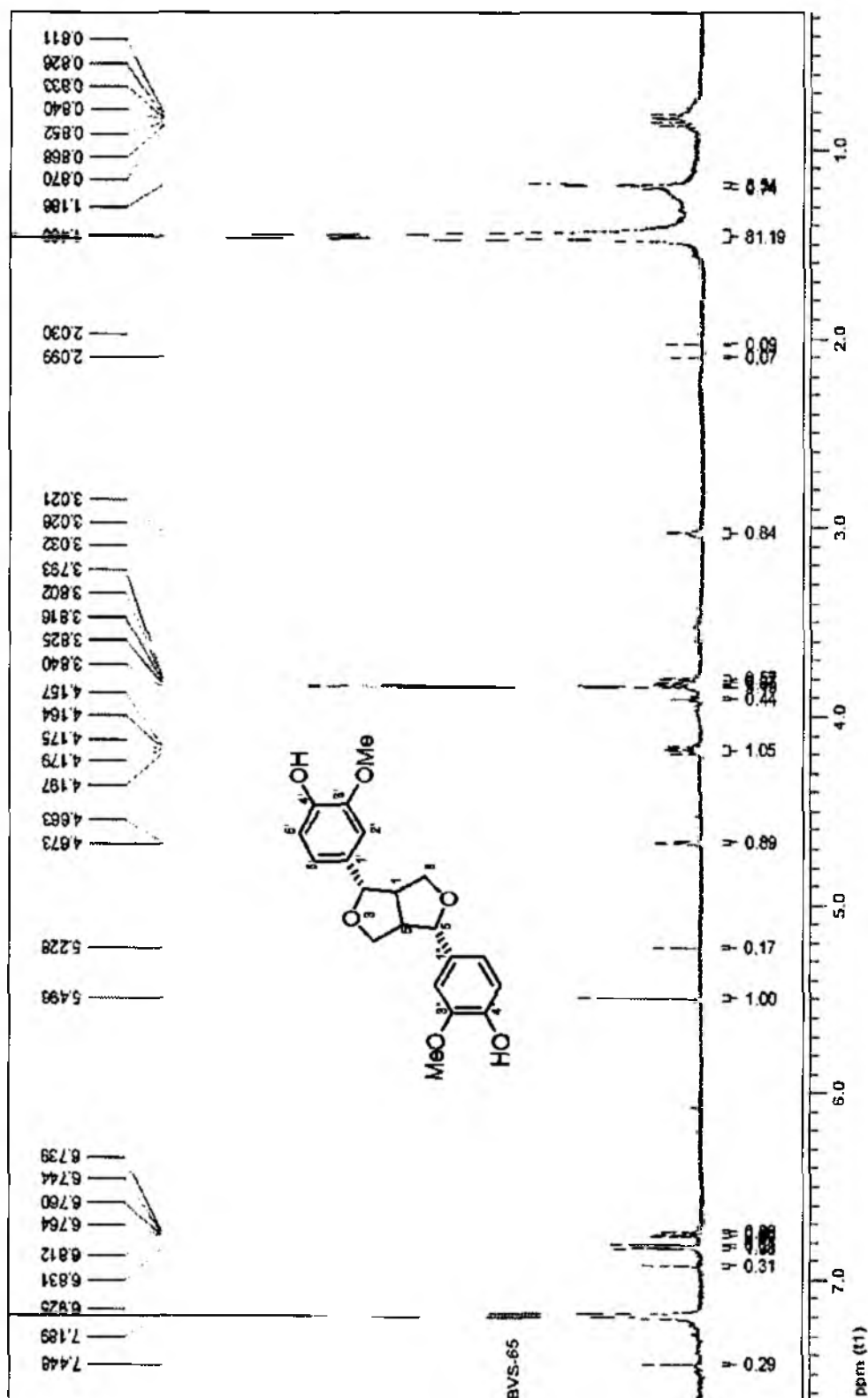


Figure 3.34: ¹H-NMR spectrum (400 MHz, CDCl₃) of BVS-65 (54).

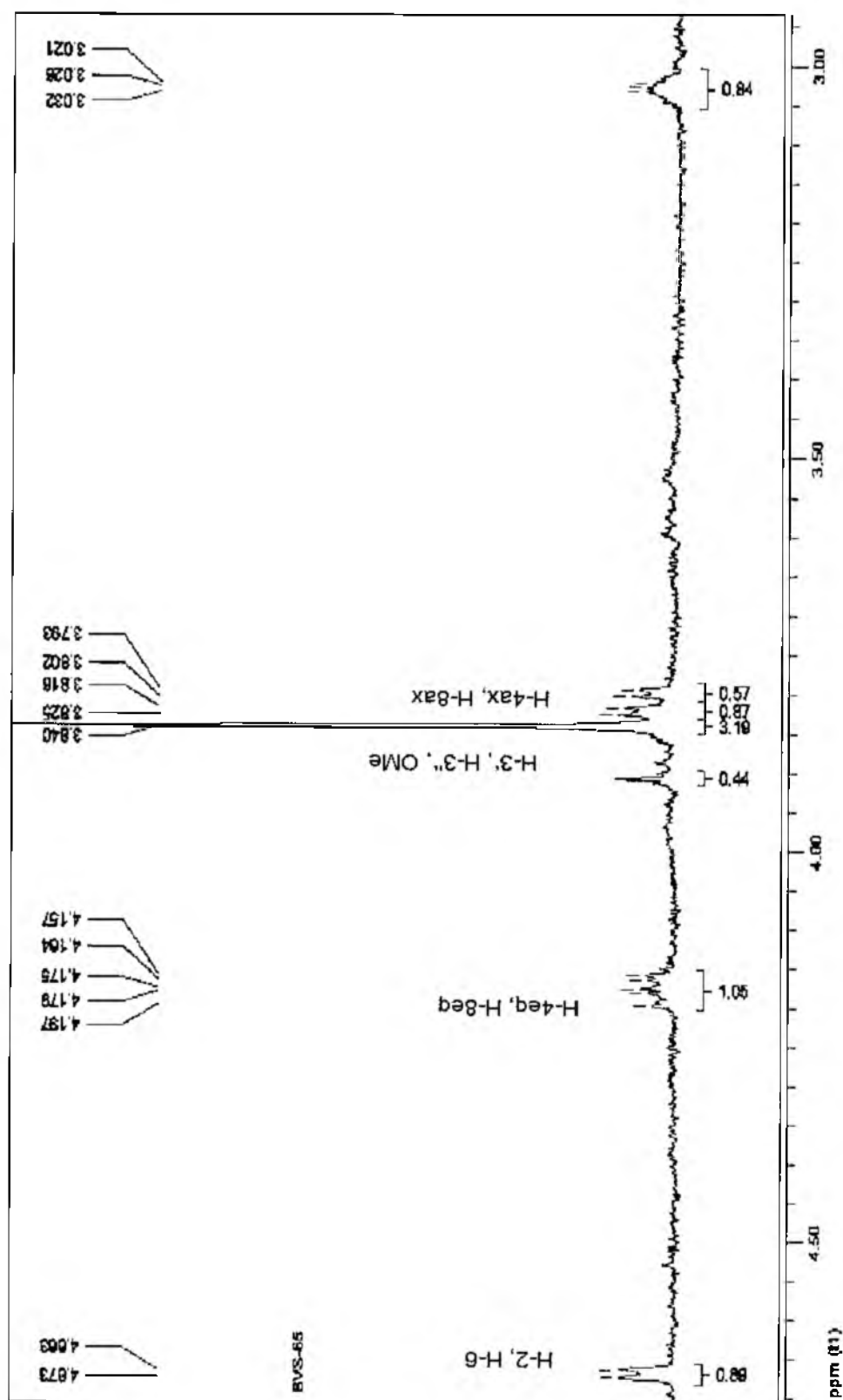


Figure 3.35: Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BVS-65 (54).

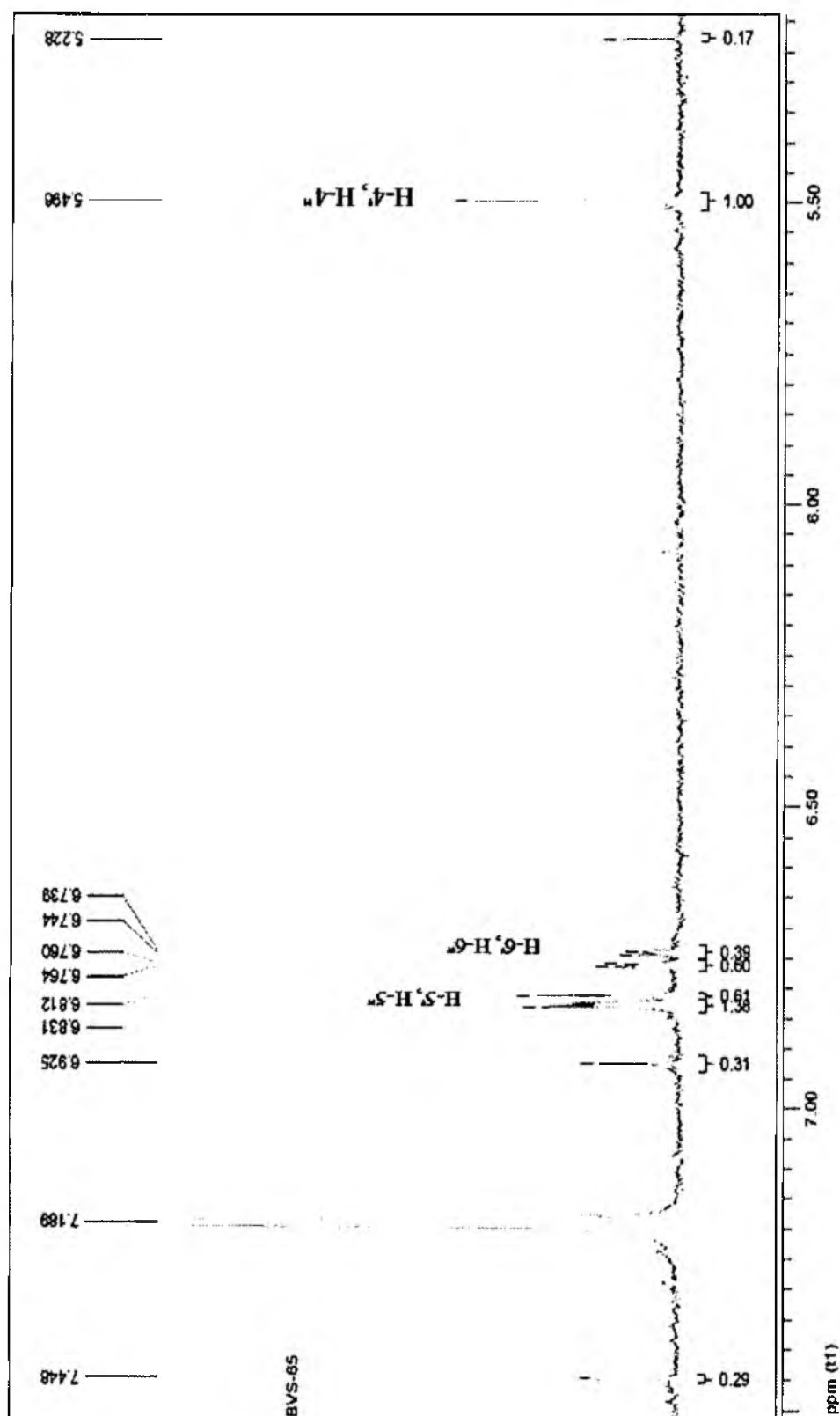


Figure 3.36: Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BVS-65 (54).

3.9 Characterization of BS-01 as glut-5(6)-en-3-one (55)

BS-01 was isolated as white crystals from the VLC fractions 4 and 5A of the crude methanolic extract of the stem bark of *B. stipularis*. These fractions were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (99: 1)]. The process was repeated thrice for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of solvent yielded white crystals and was designated as BS-01 (8.23mg, 55). The crystals melted at 207-210 °C which was identical to that reported (208-210 °C) for glut-5(6)-en-3-one (Shazid *et al.*, 2012). The compound was found to be soluble in dichloromethane, chloroform, ethyl acetate, acetone and methanol.

The ¹H-NMR spectrum (400 MHz, CDCl₃; Figures 3.38-3.40; Table 3.11) of BS-01 displayed an olefinic proton signal at δ 5.61 (1H, m) which could be assigned to H-6 of a pentacyclic triterpenoid-type carbon skeleton. The spectrum also showed eight three proton singlets at δ 0.75, 0.89, 0.93, 0.96, 1.03, 1.10, 1.16 and 1.17 attributable to eight methyl groups present in the molecule.

Comparison of the ¹H-NMR spectral data with reported values of glut-5(6)-en-3-one (Shazid *et al.*, 2012) demonstrated that all the proton shifts were similar as showed in Table 3.11. On this basis BS-01 was characterized as glut-5(6)-en-3-one, which was further approved by co-TLC with an authentic glut-5(6)-en-3-one previously isolated in our laboratory. Therefore, the compound BS-01 was characterized as glut-5(6)-en-3-one (55). This is the first report of isolation of glut-5(6)-en-3-one from *B. stipularis*.

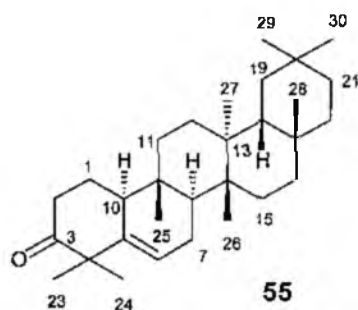


Figure 3.37: Structure of BS-01 (glut-5(6)-en-3-one, **55**).

Table 3.11: ^1H -NMR spectral data of BS-01 (**55**) and glut-5(6)-en-3-one (Shazid *et al.*, 2012) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 . | |
|-----------|---|--------------------|
| | BS-01 (55) | Glut-5(6)-en-3-one |
| H-6 | 5.61 m | 5.69 m |
| Me-23 | 1.16 s | 1.23 s |
| Me-24 | 1.17 s | 1.24 s |
| Me-25 | 0.75 s | 0.82 s |
| Me-26 | 1.03 s | 1.10 s |
| Me-27 | 0.96 s | 1.03 s |
| Me-28 | 1.10 s | 1.17 s |
| Me-29 | 0.89 s | 0.96 s |
| Me-30 | 0.93 s | 0.99 s |

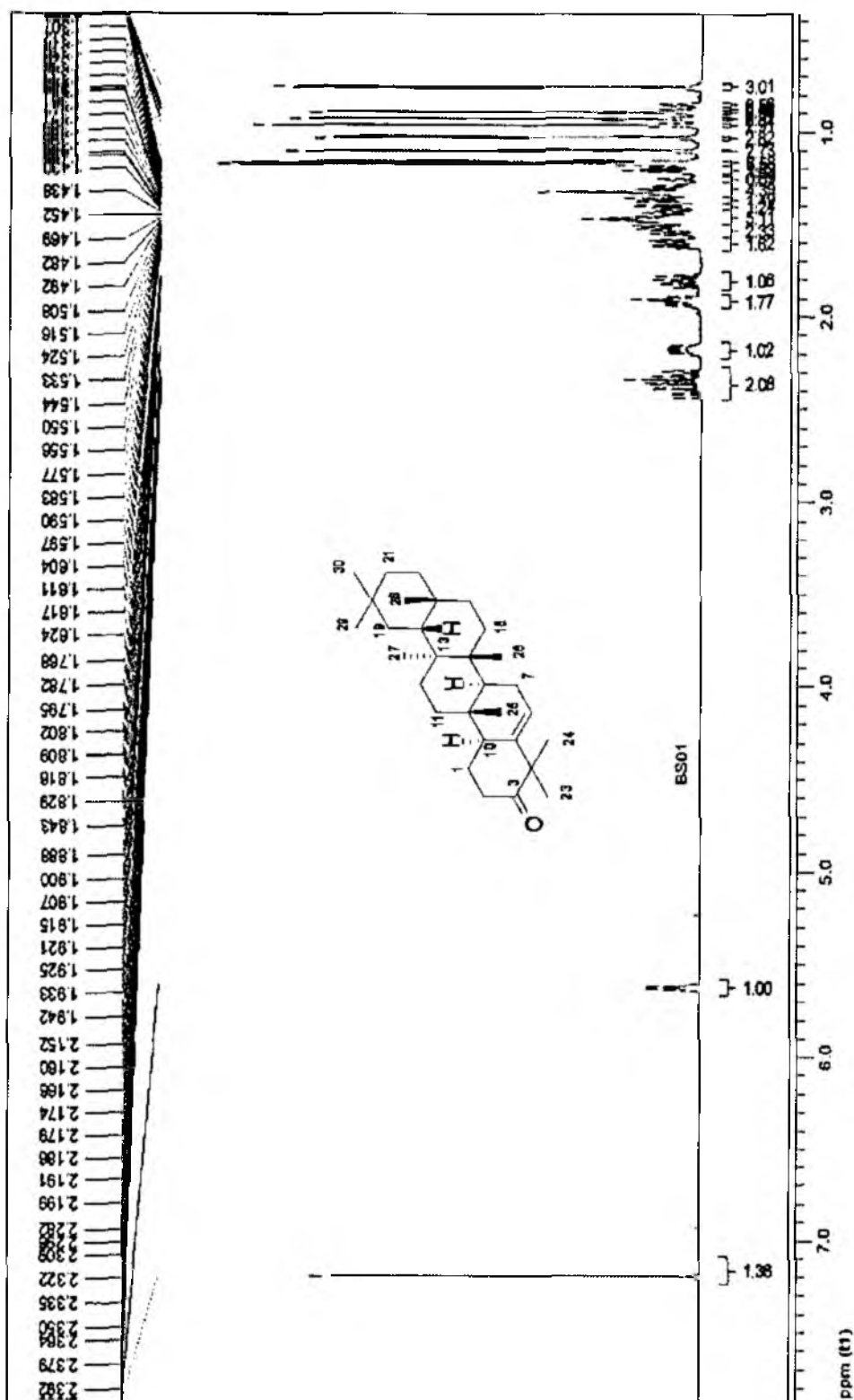


Figure 3.38: ^1H -NMR spectrum (400 MHz, CDCl_3) of BS-01 (55).

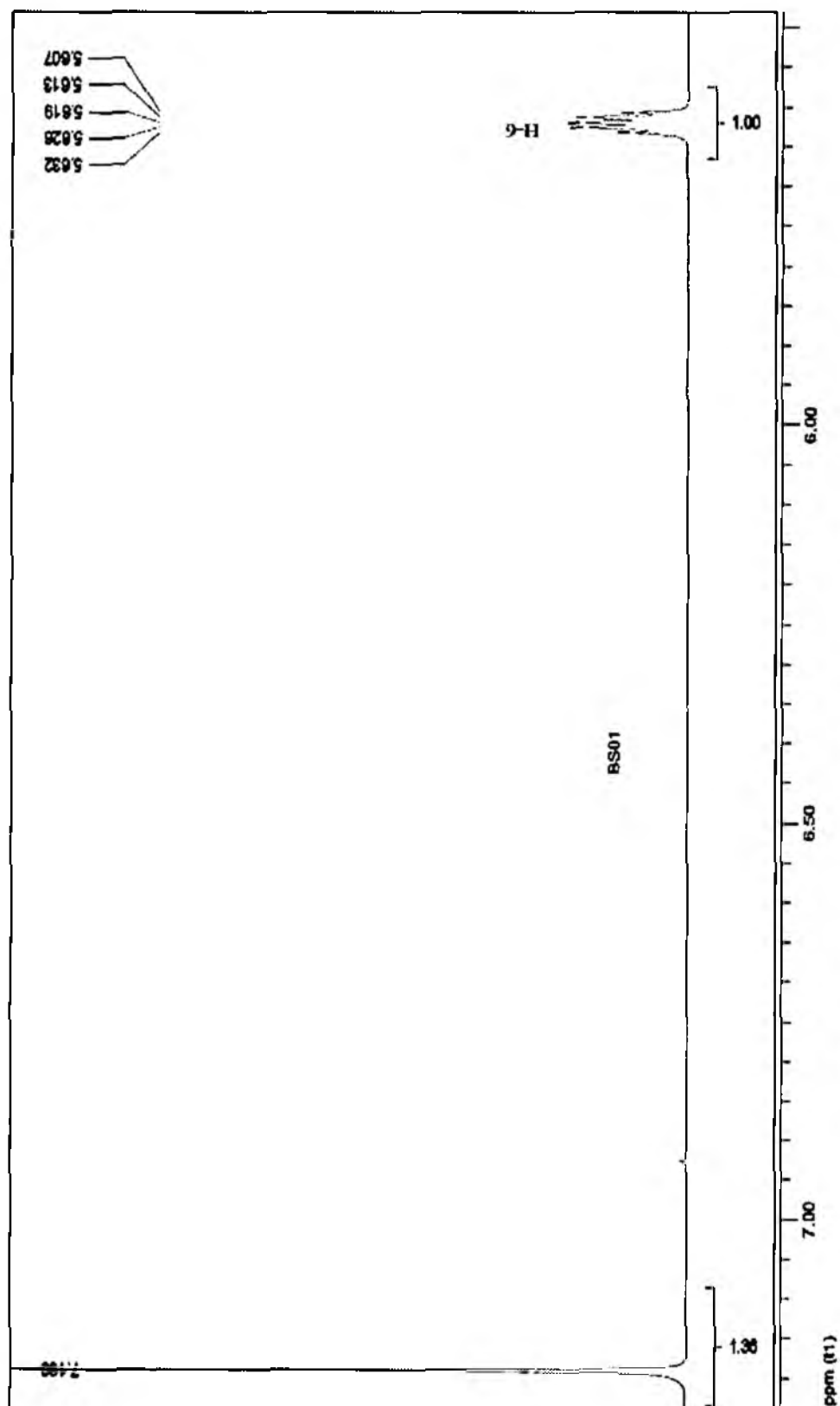


Figure 3.39: Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BS-01 (55).

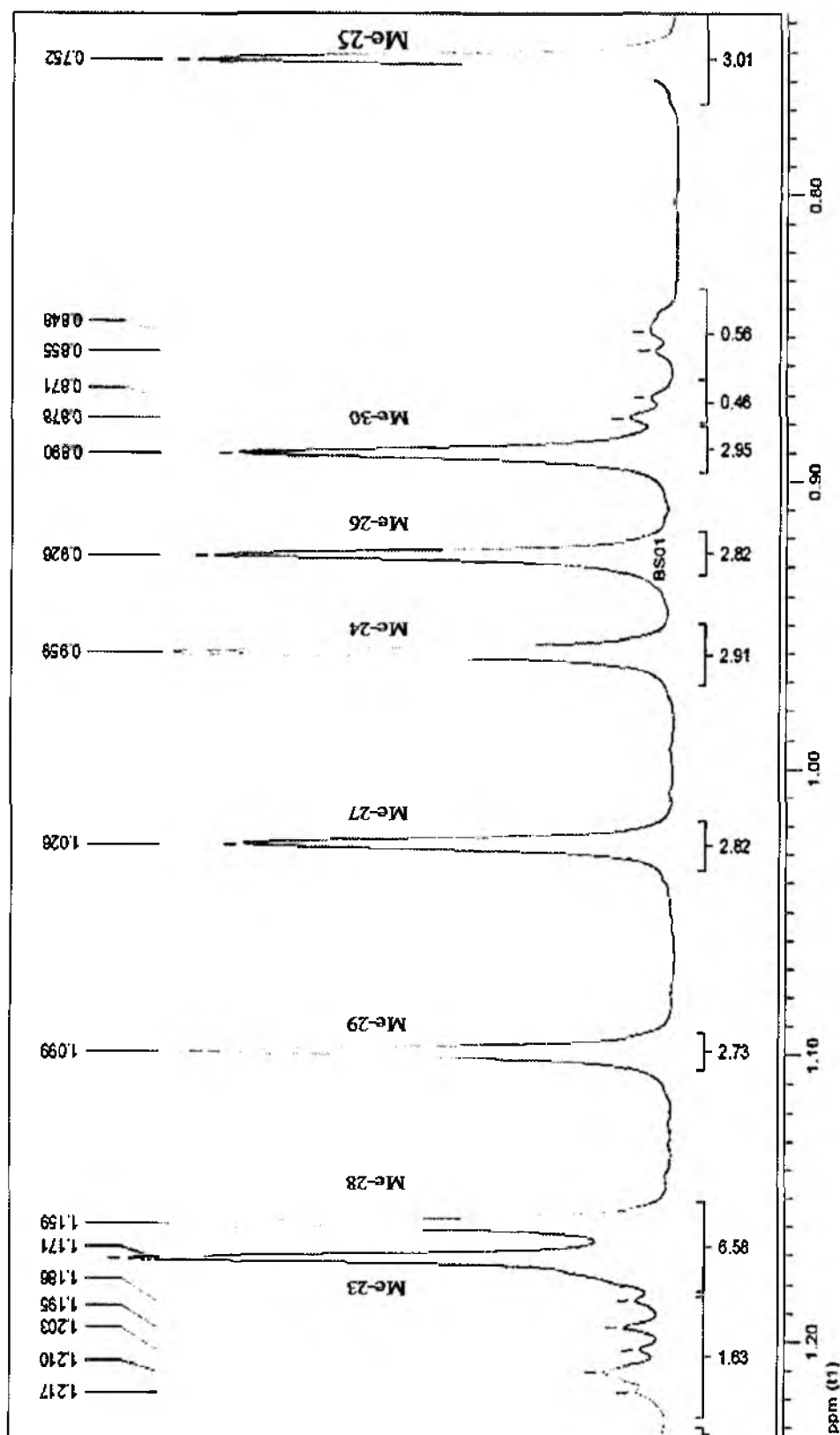


Figure 3.40: Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BS-01 (55).

3.10 Characterization of BS-06 as glut-5(6)-en-3 α -ol (**56**)

Fractions 20-26 of the LH-50 column chromatography obtained from VLC fractions 9A-11B of the crude methanolic extract of the stem bark of *B. stipularis* were mixed together due to their identical TLC spots and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (97:3)]. The process was repeated twice for better separation of the desired band. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The unsprayed portion of the band at stationary phase was scrapped off on aluminium foil and eluted with ethyl acetate. Evaporation of ethyl acetate yielded white needle like crystals which melted at 203 °C. This was identical to the melting point (205 °C) of glut-5(6)-en-3 α -ol (Xue-jing *et al.*, 2012). The compound was checked for purity and termed as BS-06 (5.31 mg, **56**). It was found to be soluble in dichloromethane, chloroform, ethyl acetate and acetone.

The ¹H-NMR spectrum (400 MHz, CDCl₃; Figures 3.42-3.44; Table 3.12) of BS-06 displayed an olefinic proton signal at δ 5.64 (1H, d, J =6.4 Hz) and an oxymethine proton at δ 3.47 (1H, t, J = 2.8 Hz) which were assigned to H-6 and H-3, respectively. The oxymethine proton at δ 3.47 was axial in configuration as confirmed by a triplet with J value 2.8 Hz (Xue-jing *et al.*, 2012). The ¹H-NMR spectrum also showed eight methyl groups resonances as singlets at δ 0.85, 0.95, 0.99, 1.01, 1.05, 1.10, 1.14 and 1.17.

On the basis of the above spectral data, compound BS-06 was characterized as glut-5(6)-en-3 α -ol (**57**) which was confirmed by comparison of its spectral data (Table 3.12) and melting point with reported values (Xue-jing *et al.*, 2012). This is the first report of isolation of glut-5(6)-en-3 α -ol (**56**) from *B. stipularis*.

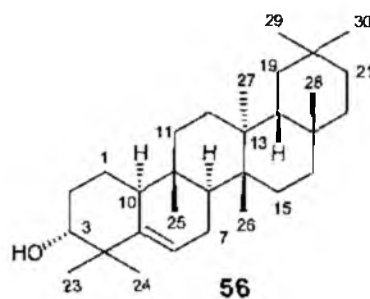


Figure 3.41: Structure of BS-06 (glut-5(6)-en-3 α -ol, **56**).

Table 3.12: ^1H -NMR spectral data of BS-06 (**56**) and glut-5(6)-en-3 α -ol (Xue-jing *et al.*, 2012) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------|---|-----------------------------|
| | BS-06 (56) | Glut-5(6)-en-3 α -ol |
| H-3 | 3.47 t (2.8) | 3.51 t (2.9) |
| H-6 | 5.64 d (6.4) | 5.63 d (6.41) |
| Me-23 | 1.17 s | 1.16 s |
| Me-24 | 1.01 s | 1.00 s |
| Me-25 | 0.85 s | 0.85 s |
| Me-26 | 0.99 s | 0.99 s |
| Me-27 | 1.05 s | 1.05 s |
| Me-28 | 1.14 s | 1.14 s |
| Me-29 | 1.10 s | 1.08 s |
| Me-30 | 0.95 s | 0.95 s |

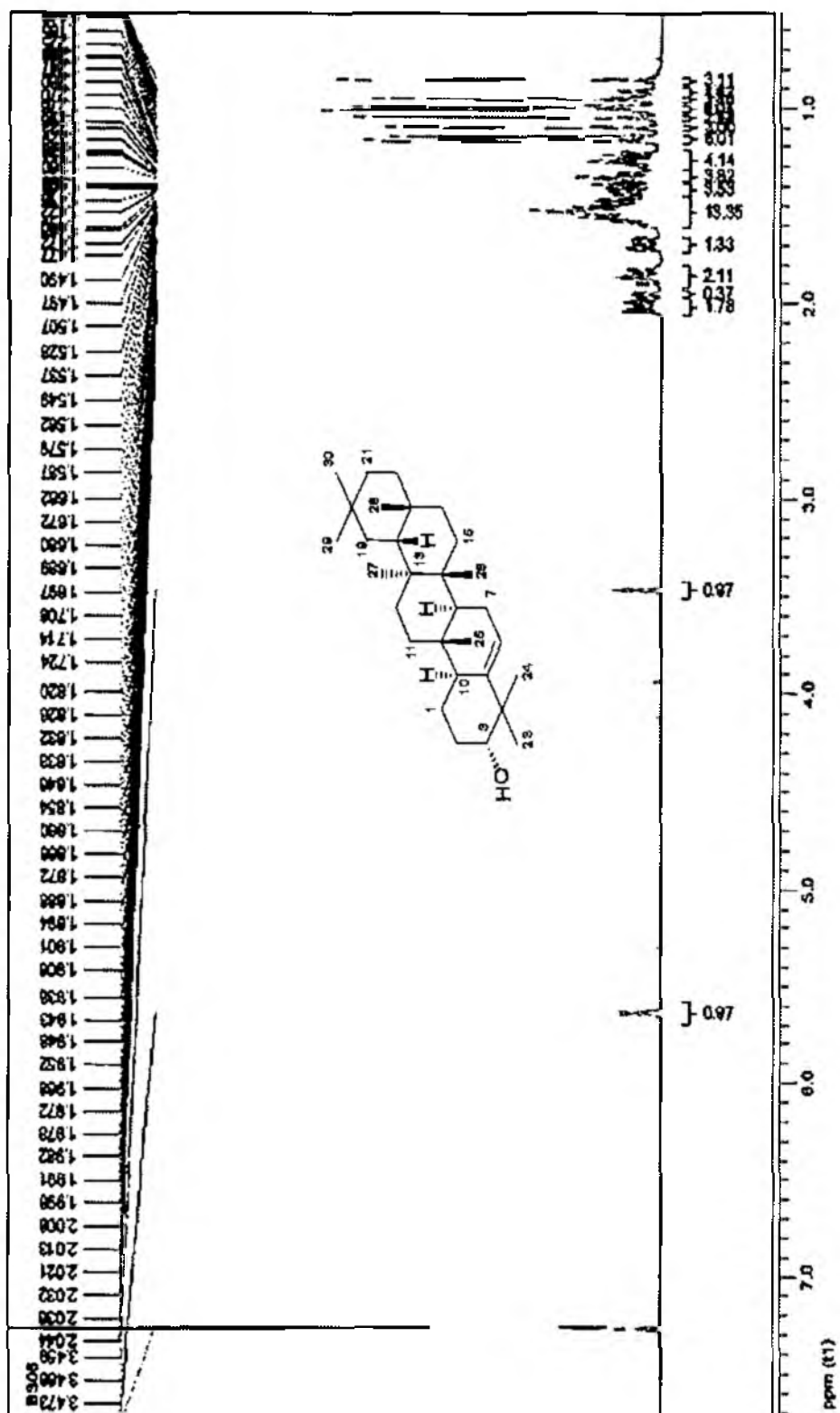


Figure 3.42: $^1\text{H-NMR}$ spectrum (400 MHz, CDCl_3) of BS-06 (56).

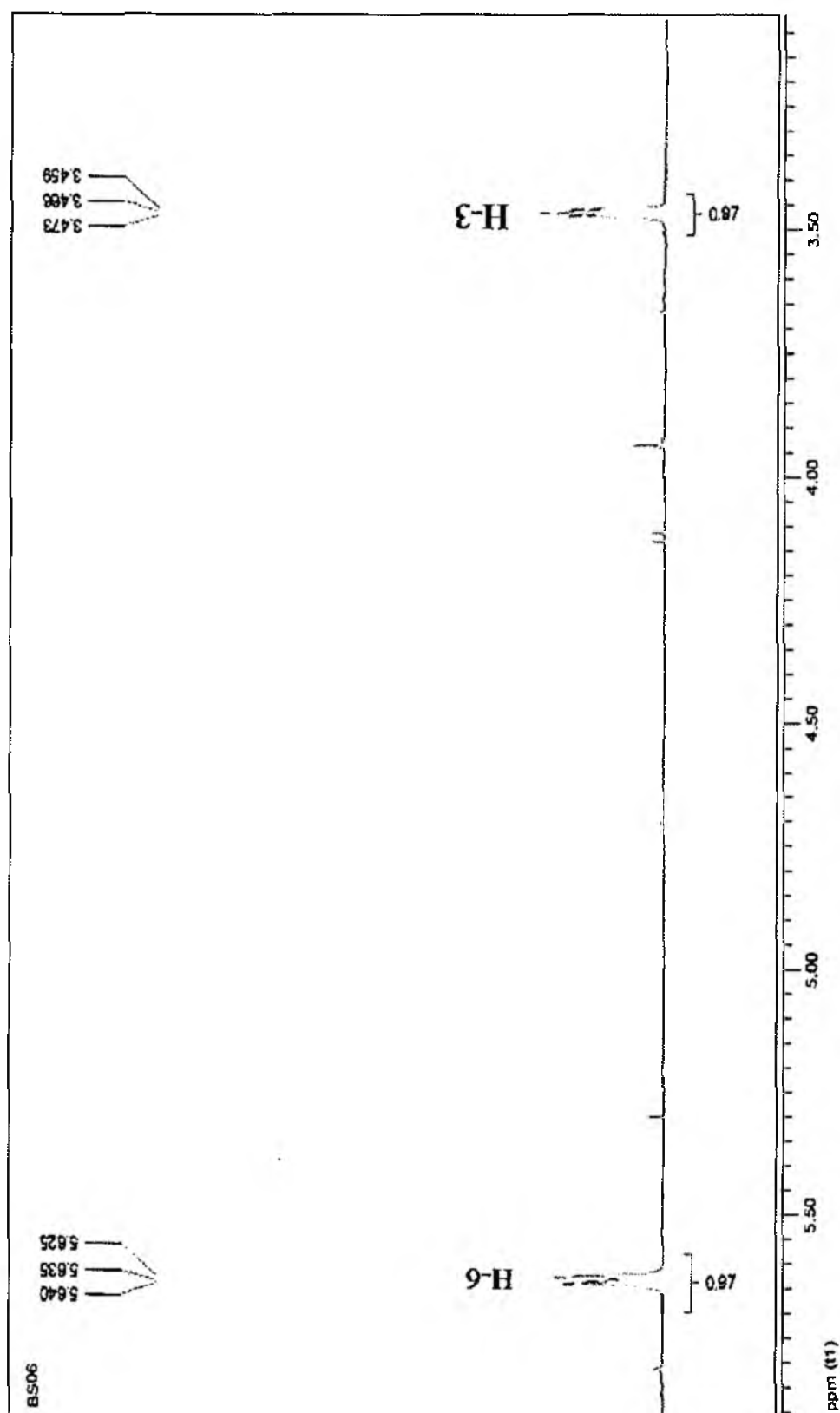


Figure 3.43: Partially expanded ¹H-NMR spectrum (400 MHz, CDCl₃) of BS-06 (56).

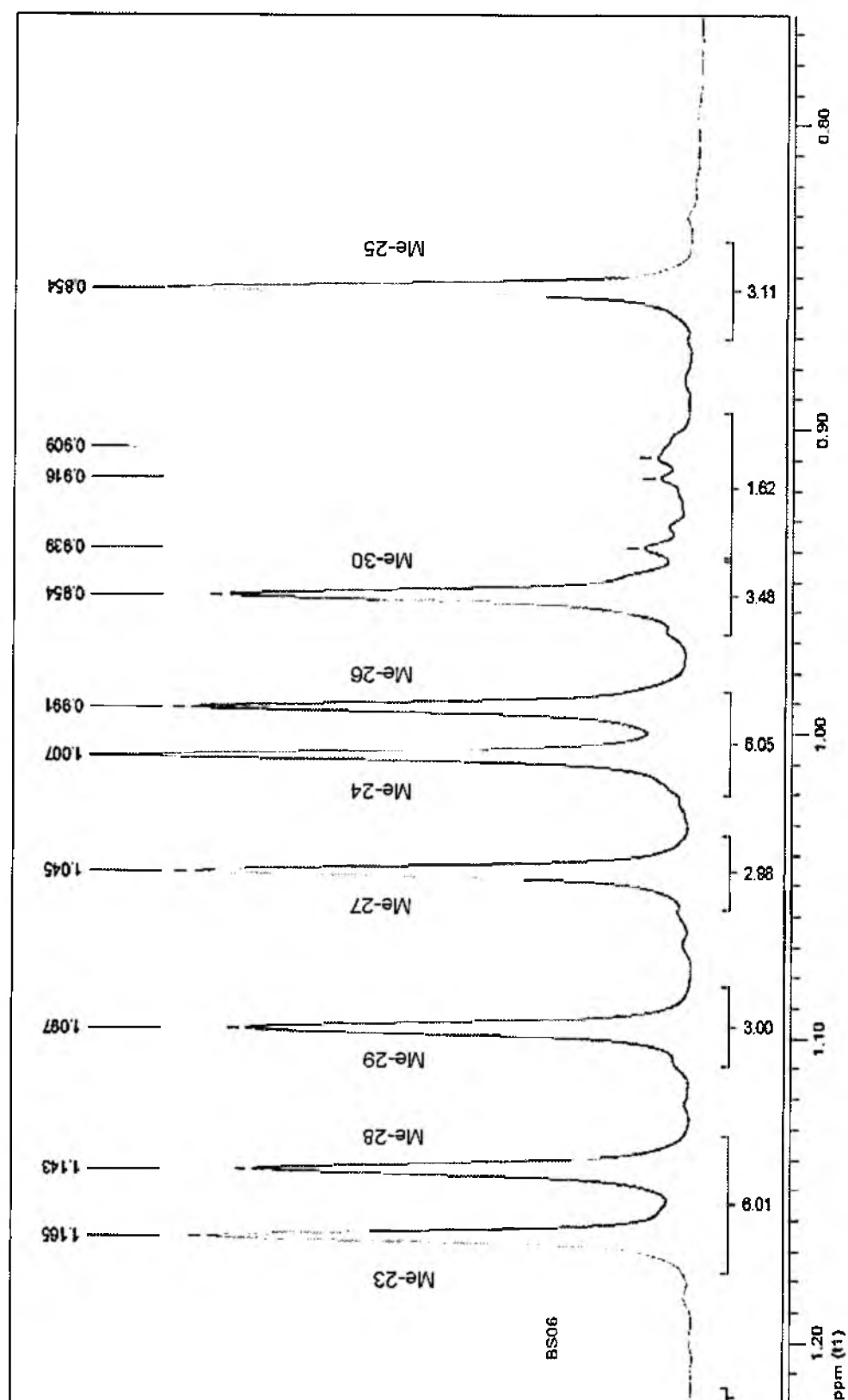


Figure 3.44: Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BS-06 (56).

3.11 Characterization of BS-11 (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (57)

Vacuum liquid column chromatography of the methanolic extract of the stem bark of *B. stipularis* by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities provided 46 fractions. Fractions 9A-11B showing identical spots on TLC were bulked together and subjected to gel permeation chromatography over lipophilic LH-20 to give 55 fractions. Based on the TLC fractions 20-26 of LH-20 were again combined and evaporation of solvents afforded yellowish mass. This was subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (98:2)]. The process was repeated five times for better separation of the desired band, which was scrapped off and eluted with ethyl acetate. Evaporation of solvent yielded a pure white amorphous compound BS-11 (7.01 mg, 57). The compound was soluble in *n*-hexane, dichloromethane, ethyl acetate and methanol.

The ¹H-NMR spectrum of the compound BS-11 (500 MHz, CDCl₃; Figures 3.46-3.48; Table 3.13) displayed signals at δ 0.70 (s, 3H), 0.85 (d, 6H, *J*= 6.8 Hz, overlapped), 1.27 (d, 3H, *J*= 6.7 Hz) and 1.30 (s, 3H) attributable to the methyl groups at C-18, C-26/27, C-21 and C-19, respectively in a cholestane type carbon skeleton. In the ¹H-NMR spectrum, the olefinic protons at H-22 and H-23 appeared as characteristic downfield signals at δ 5.20 (m, 1H) and δ 5.06 (m, 1H) (Aiello *et al.*, 1988). Another olefinic proton signal at δ 5.70 (bs, 1H) was attributed to the trisubstituted double bond conjugated with the ketone group located at C-3, and therefore, the trisubstituted double bond could be assigned at C-4 and C-5 (Mellado *et al.*, 2004). The ¹H-NMR spectrum further displayed signals of a oxymethine proton at δ 3.67 (m, 1H) and a tertiary methyl proton at δ 0.84 indicating a hydroxyl group at C-7 and a methyl group at C-28, respectively (Notaro *et al.*, 1992). In the literature, it was revealed that if the hydroxyl group was situated at C-24, the proton chemical shifts of C-24, C-22 and C-23 moved upfield to δ 3.77, 5.49 and 5.38 which do not match with the resonances present at the ¹H-NMR spectrum of BS-11.

On this basis as well as by comparison of two ¹H-NMR data of with that of chemical shifts of protons of steroidal ketone compounds reported in the literature (Aiello *et al.*, 1988; Mellado *et al.*, 2004), the isolated compound BS-11 could be tentatively identified as a new structure, (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (57). However, additional spectral data (MS and ¹³C-NMR data) are required to confirm its structure.

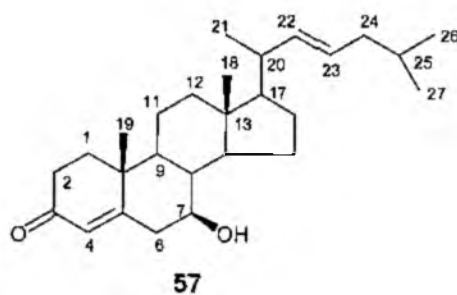


Figure 3.45: Structure of BS-11 (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one, **57**).

Table 3.13: ^1H -NMR spectral data of BS-11 (**57**) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). |
|-----------|---|
| | BS-11 (57) |
| H-4 | 5.70 bs |
| H-7 | 3.67 m |
| Me-18 | 0.70 s |
| Me-19 | 1.33 s |
| Me-21 | 1.27 d (6.7) |
| H-22 | 5.06 (15.5 and 9.0) |
| H-23 | 5.20 (15.0 and 8.5) |
| Me-26 | 0.85 d (6.8) |
| Me-27 | 0.85 d (6.8) |
| Me-28 | 0.84 s |

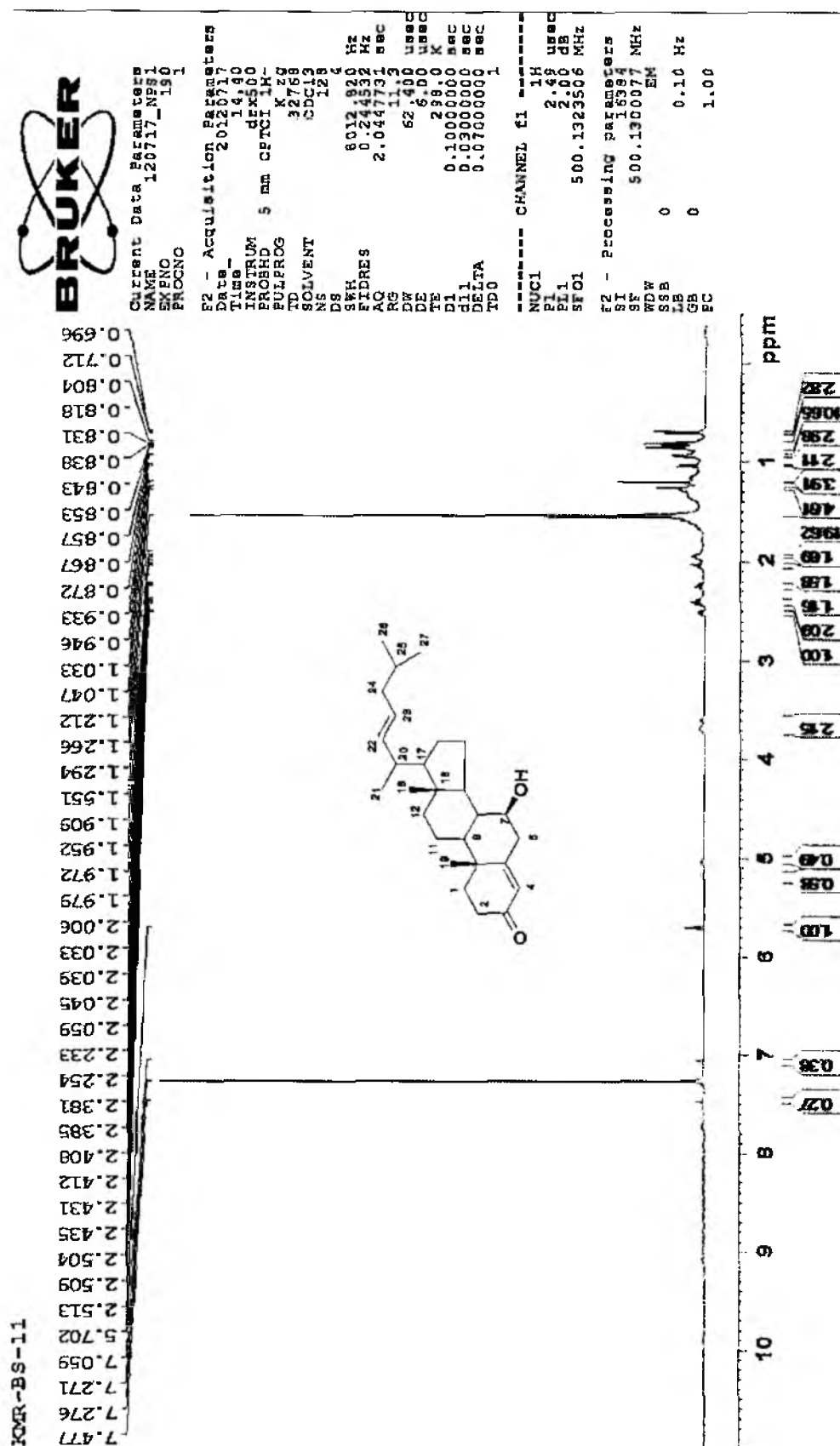


Figure 3.46: ¹H-NMR spectrum (500 MHz, CDCl₃) of BS-11 (57).

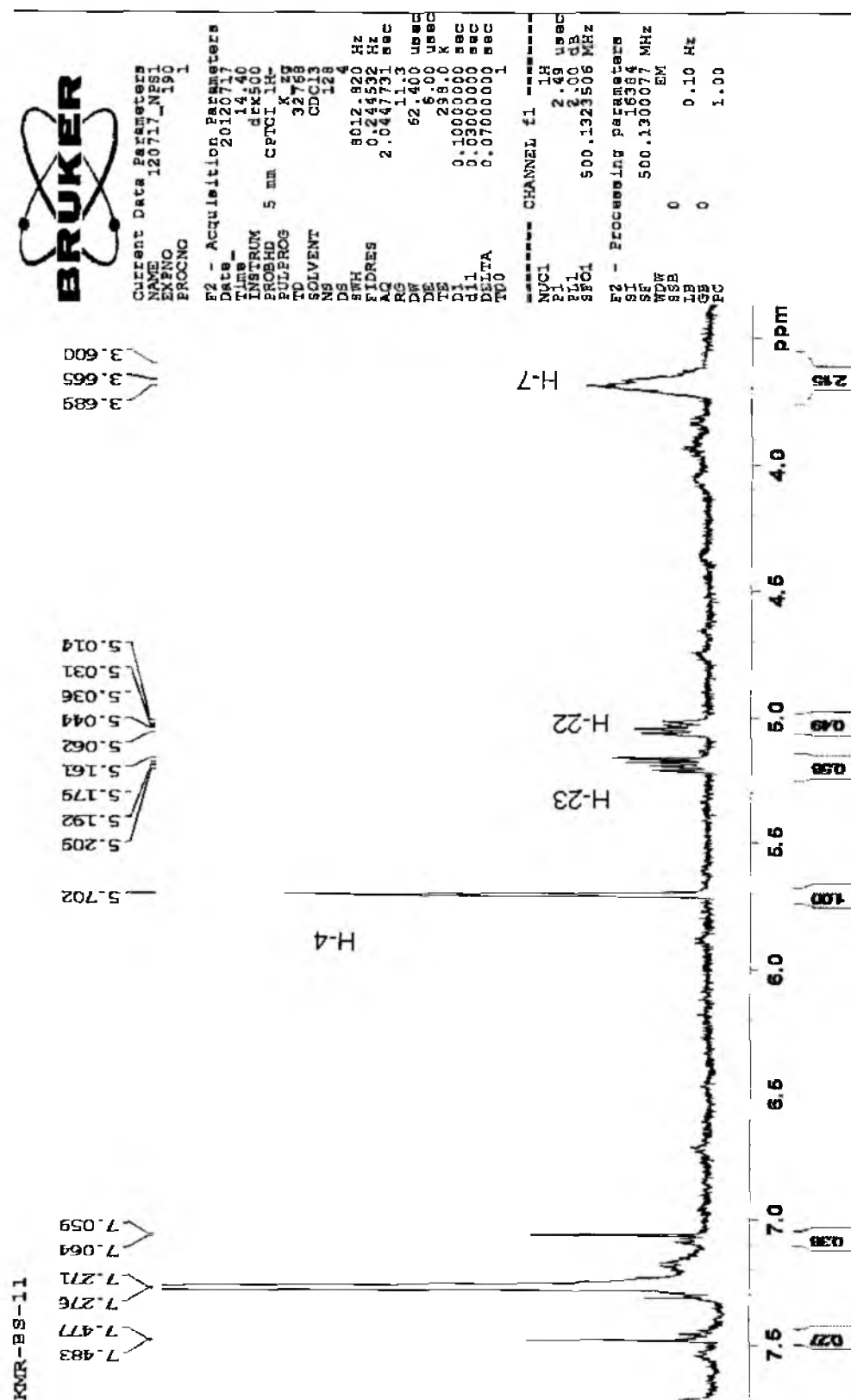


Figure 3.47: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of BS-11 (57).

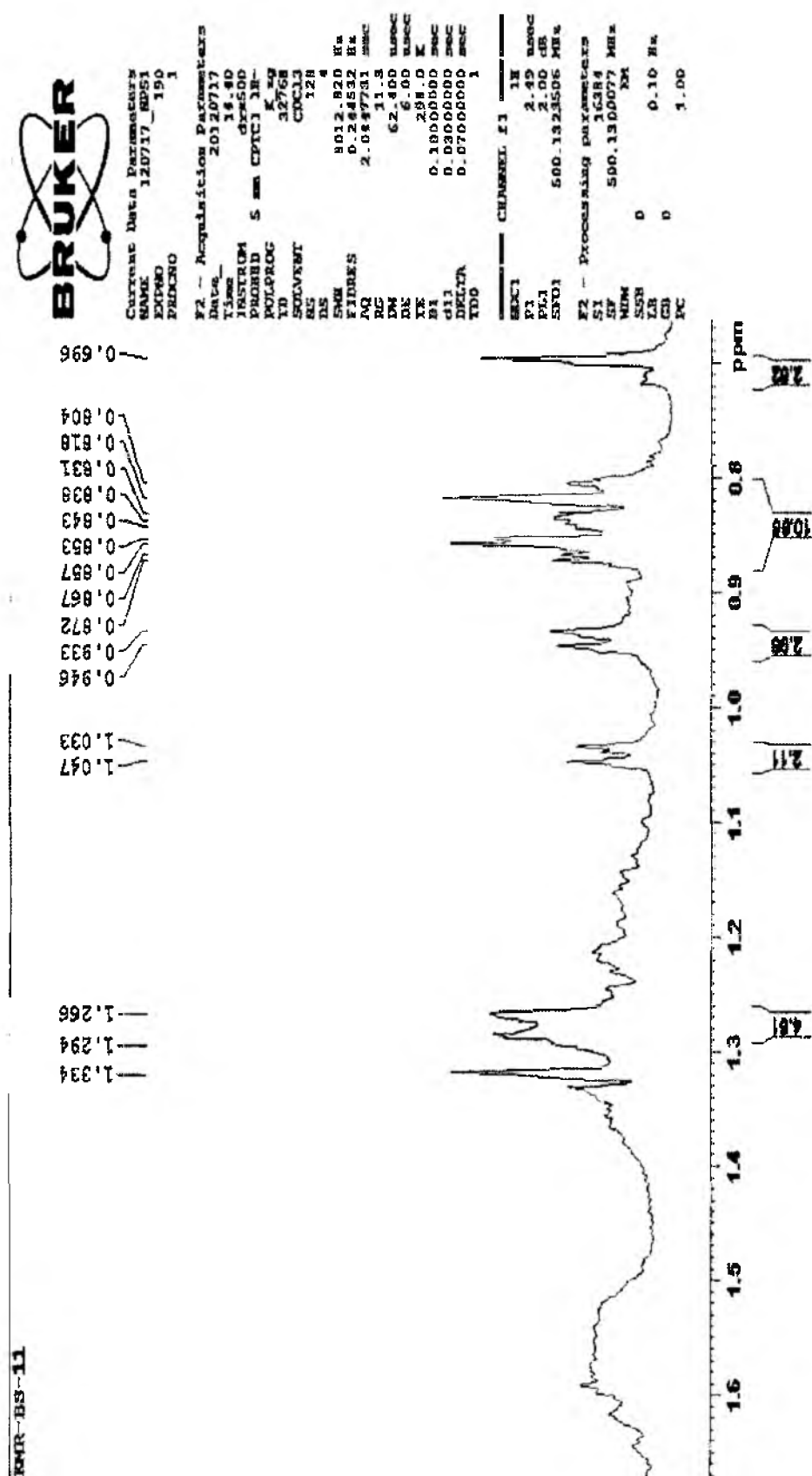


Figure 3.48: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of BS-11 (57).

3.12 Characterization of BT-1 as friedoolean-5(6),14(15)-dien-3-one (58)

VLC fractions 4(A+B) of the methanolic extract of the stem bark of *B. tomentosa* were mixed together and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (95:5)]. A deep purple colored band was visualized after spraying a portion of the developed plate with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes. The unsprayed portion of the band was scrapped off and eluted with ethyl acetate to provide a colorless mass which was checked for purity and termed as BT-1 (8.04 mg, **58**). It was found to be soluble in petroleum ether, chloroform and ethyl acetate.

The ¹H-NMR spectrum (400 MHz, CDCl₃; Figures 3.50-3.52; Table 3.14) of BT-1 showed eight methyl group resonances as singlets at δ 0.81, 0.95, 0.98, 1.02, 1.08, 1.16, 1.22 and 1.23 attributable to eight methyl groups present in the molecule (Xue-jing *et al.*, 2012). The ¹H-NMR spectrum displayed two olefinic proton signals at δ 5.68 (1H, m) and δ 5.56 (1H, dd, *J*= 6.4 and 2.1 Hz) which could be assigned to H-6 and H-15 of a pentacyclic triterpenoid skeleton (Shazid *et al.*, 2012; Fakir *et al.*, 2009; Moulisha *et al.*, 2009).

Comparison of the ¹H-NMR partial spectral data with reported values it was suggested the presence of a typical taraxaren-type pentacyclic triterpene skeleton (Xue-jing *et al.*, 2012; Shazid *et al.*, 2012; Fakir *et al.*, 2009; Moulisha *et al.*, 2009).

On the basis of the above spectral data as well as by comparison with that of chemical shifts of protons of pentacyclic triterpenes reported in the literatures (Xue-jing *et al.*, 2012; Shazid *et al.*, 2012; Fakir *et al.*, 2009 and Moulisha *et al.*, 2009), the isolated compound BT-1 could be tentatively identified as a new structure, friedoolean-5(6),14(15)-dien-3-one (**58**). However, additional spectral data (MS and ¹³C-NMR data) are required to confirm its structure.

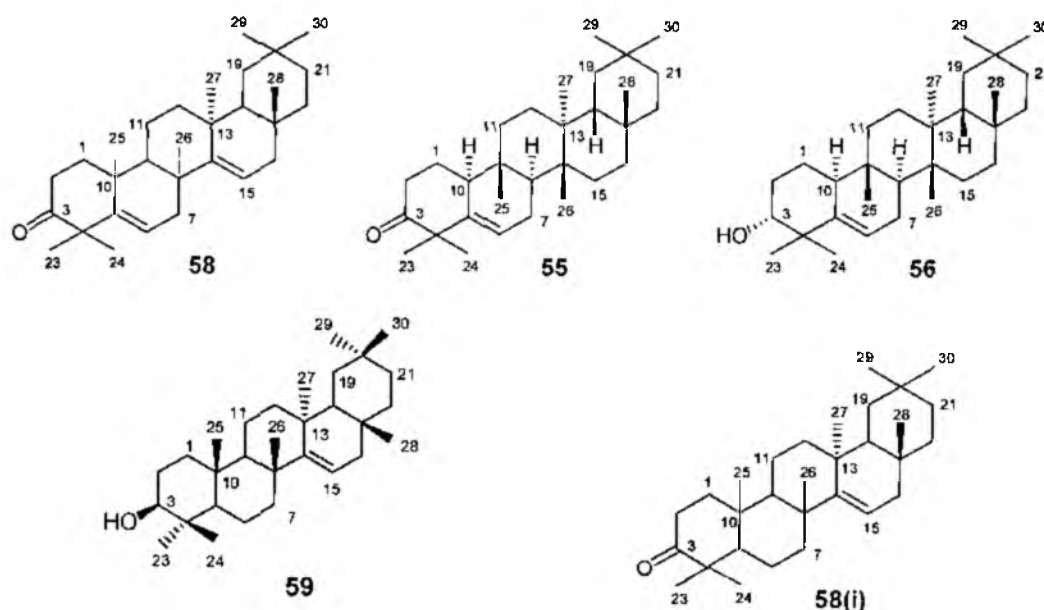


Figure 3.49: Structures of BT-1 (friedoolean-5(6),14(15)-dien-3-one, **58**), glut-5(6)-en-3-one (**55**), glut-5(6)-en-3 α -ol (**56**), β -taraxerol (**59**) and friedoolean-14-en,3-one [**58(i)**].

Table 3.14: ^1H -NMR spectral data of BT-1 (**58**) and other references (Xue-jing *et al.*, 2012; Shazid *et al.*, 2012; Fakir *et al.*, 2009 and Moulisha *et al.*, 2009) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | | | | |
|-----------|---|---|--|-------------------------------------|--|
| | BT-1 (58) | glut-5(6)-
en-3-one
(55) | glut-5(6)-
en-3 α -ol
(56) | β -taraxerol
(59) | friedoolean-14-
en,3-one [58(i)] |
| H-6 | 5.68 m | 5.69 m | - | - | - |
| H-15 | 5.56 dd
(8.2, 3.6) | - | - | 5.57 dd
(8.0, 3.2) | 5.56 dd
(7.8, 2.7) |
| Me-23 | 1.23 s | - | 1.16 s | - | - |
| Me-24 | 1.00 s | - | 1.00 s | - | - |
| Me-25 | 0.81 s | - | 0.85 s | - | - |
| Me-26 | 0.98 s | - | 0.99 s | - | - |
| Me-27 | 1.08 s | - | 1.05 s | - | - |
| Me-28 | 1.22 s | - | 1.14 s | - | - |
| Me-29 | 1.16 s | - | 1.08 s | - | - |
| Me-30 | 0.95 s | - | 0.95 s | - | - |

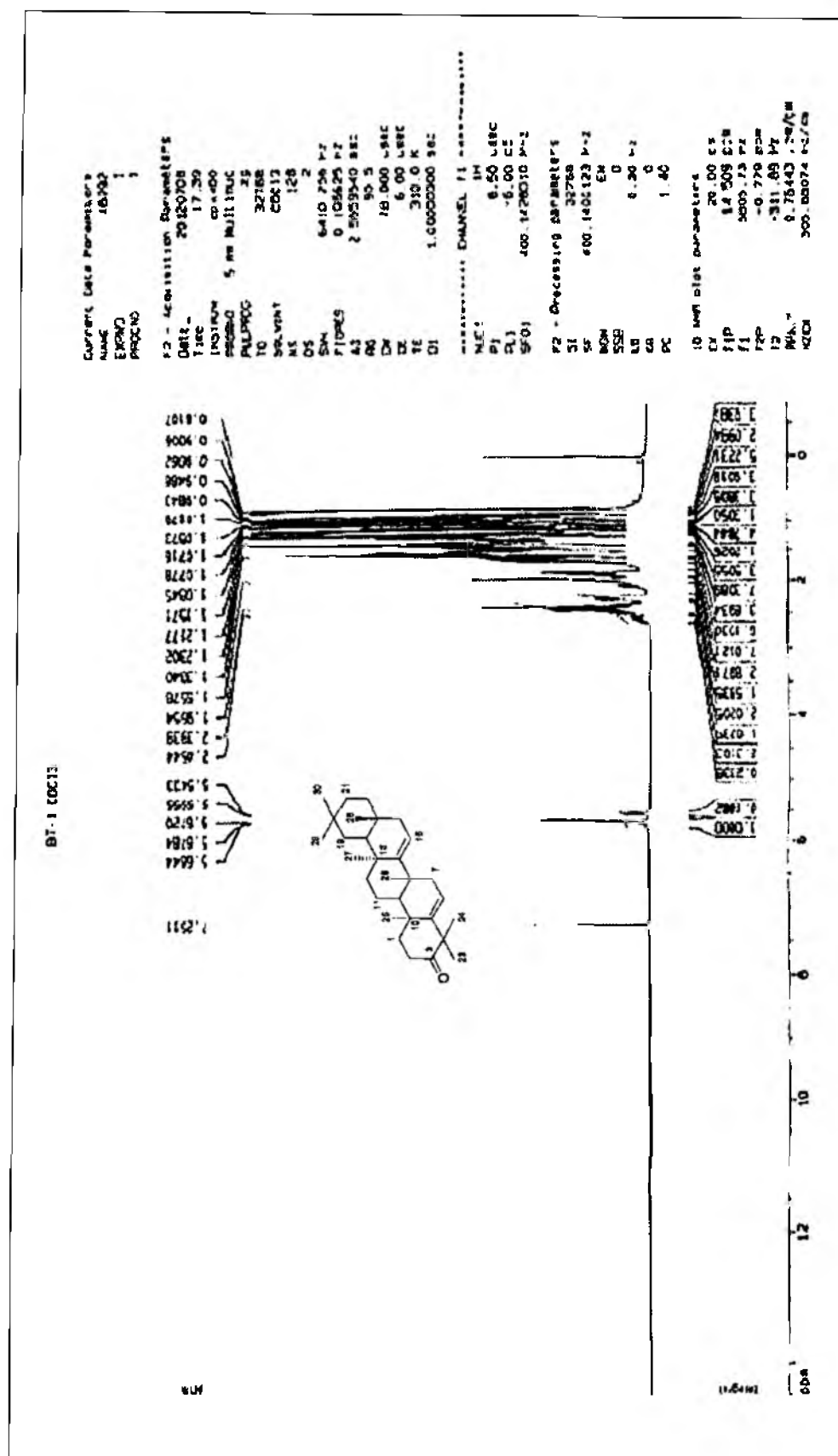


Figure 3.50: ¹H-NMR spectrum (400 MHz, CDCl₃) of BT-1 (58).

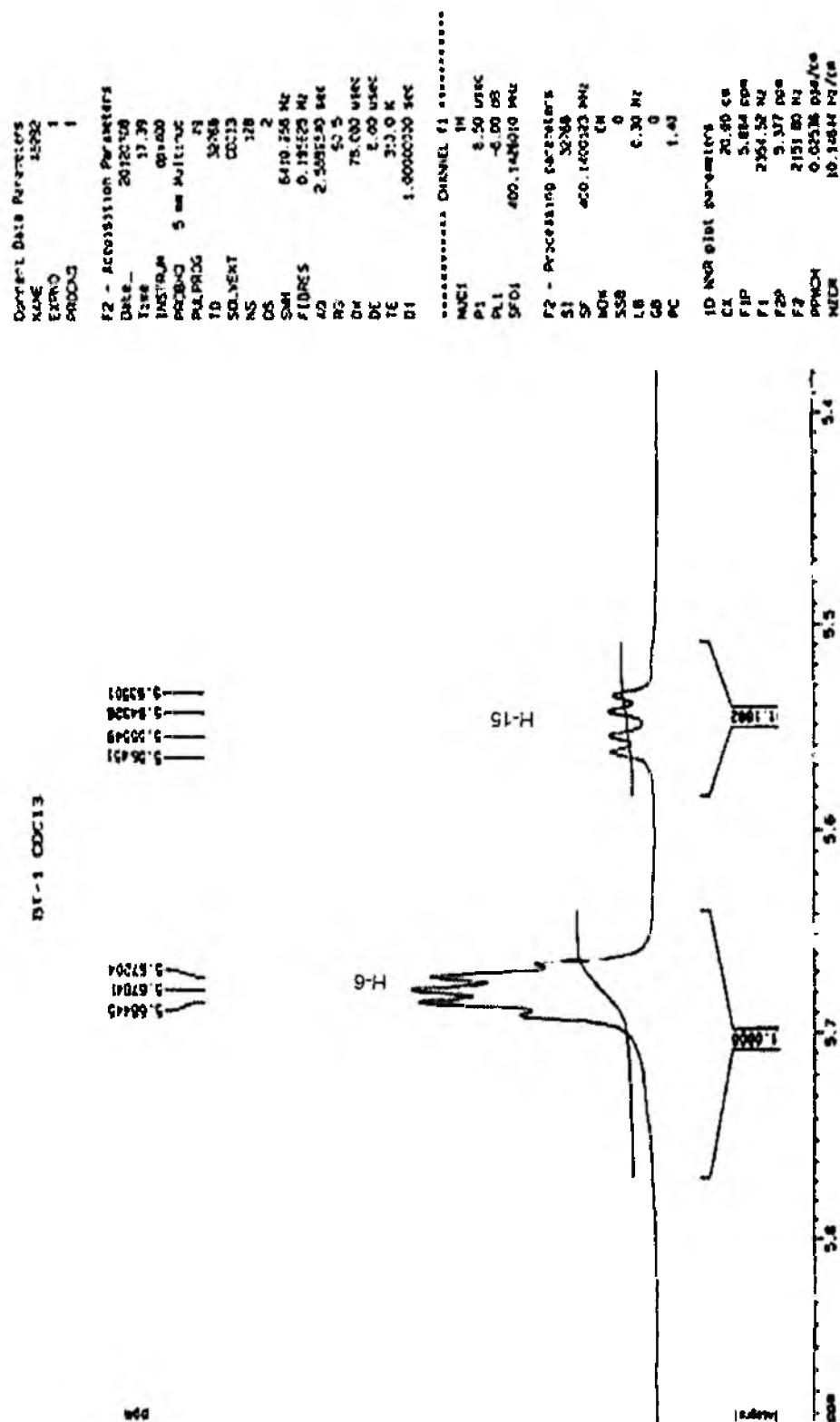


Figure 3.51: Partially expanded ¹H-NMR spectrum (400 MHz, CDCl₃) of BT-1 (58).

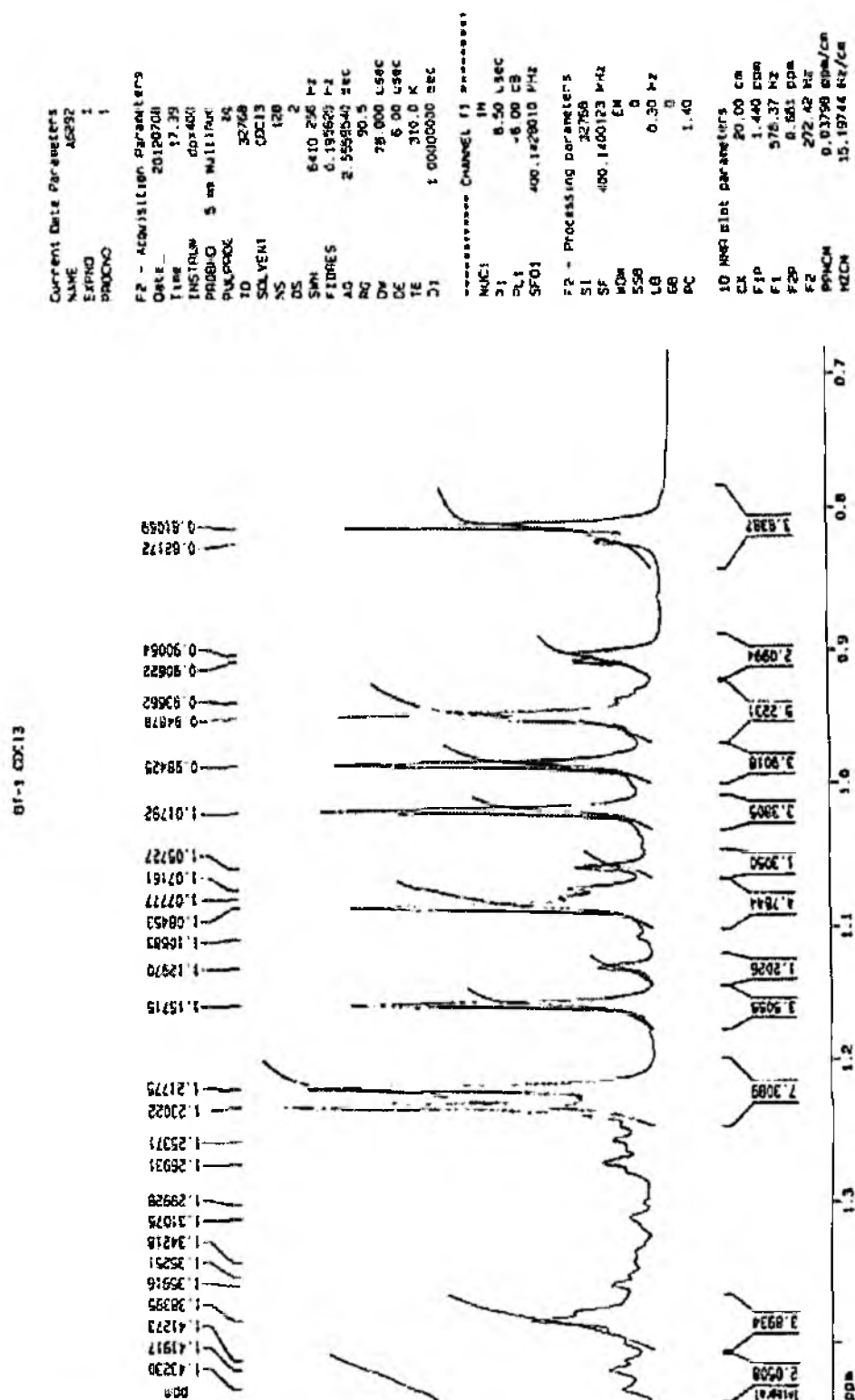


Figure 3.52: Partially expanded ^1H -NMR spectrum (400 MHz, CDCl_3) of BT-1 (58).

3.13 Characterization of BT-6 as β -taraxerol (59)

BT-6 was isolated as colorless mass from the VLC fractions 5B and 6A of the methanolic extract of the stem bark of *B. tomentosa*. Fractions 5B and 6A were mixed together due to their identical TLC spots and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (95:5)]. A deep purple colored band was visualized after spraying a portion of the developed plate with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes. The unsprayed portion of the band on the stationary phase was scrapped off and eluted with ethyl acetate. Colorless mass was obtained which was checked for purity and termed as BT-6 (5.11 mg, **59**). It was found to be soluble in petroleum ether, chloroform and ethyl acetate.

The electrospray ionization mass spectrum (ESI-MS) of BT-6 (Figure 3.54) exhibited a pseudomolecular ion (M+H)⁺ peak at *m/z* 427 which was consistent with molecular ion at 426 and molecular formula of C₃₀H₅₀O. The ¹H-NMR spectrum (500 MHz, CDCl₃; Figures 3.55-3.57; Table 3.15) of BT-6 showed eight three-proton singlets at δ 0.78, 0.80, 0.89, 0.89, 0.91, 0.93, 0.96 and 1.07, which were attributed to the methyl group protons at Me-28, Me-27, Me-23, Me-24, Me-29, Me-30, Me-25 and Me-27, respectively. The double doublet (*J*= 8.0, 3.5 Hz) of one proton intensity centered at δ 5.52 was assigned to the olefinic proton at C-15. Another double doublet (*J*= 10.5, 3.5 Hz) centered at δ 3.17 could be assigned to the oxymethine proton at C-3. The large couplings (*J*= 10.5, 3.5 Hz) of this proton (H-3) with the vicinal methylene protons (H₂-2) suggested a β orientation of the hydroxyl group at C-3. All the above ¹H-NMR signals suggested the presence of a typical taraxaren-type pentacyclic triterpene skeleton.

These spectral features (Table 3.15) were in close agreement to those published for β -taraxerol (Fakir *et al.*, 2009). On this basis, the identity of BT-6 was confirmed as β -taraxerol (**59**). This is the first report of isolation of β -taraxerol from *B. tomentosa*.

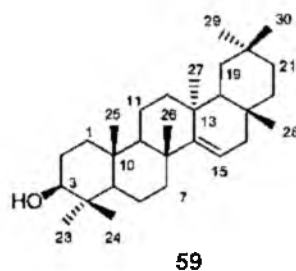


Figure 3.53: Structure of BT-6 (β -taraxerol, 59).

Table 3.15: ^1H -NMR spectral data of BT-6 (59) and β -taraxerol in CDCl_3 (Fakir *et al.*, 2009) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------|---|-----------------------|
| | BT-6 (59) | β -taraxerol |
| H-3 | 3.17 dd (10.5 and 3.5) | 3.19 d (9.2) |
| H-15 | 5.52 dd (8.0 and 3.5) | 5.57 dd (8.0 and 3.2) |
| Me-23 | 0.89 s | 0.90 s |
| Me-24 | 0.89 s | 0.90 s |
| Me-25 | 0.96 s | 0.97 s |
| Me-26 | 1.07 s | 1.08 s |
| Me-27 | 0.80 s | 0.82 s |
| Me-28 | 0.78 s | 0.80 s |
| Me-29 | 0.91 s | 0.92 s |
| Me-30 | 0.93 s | 0.94 s |

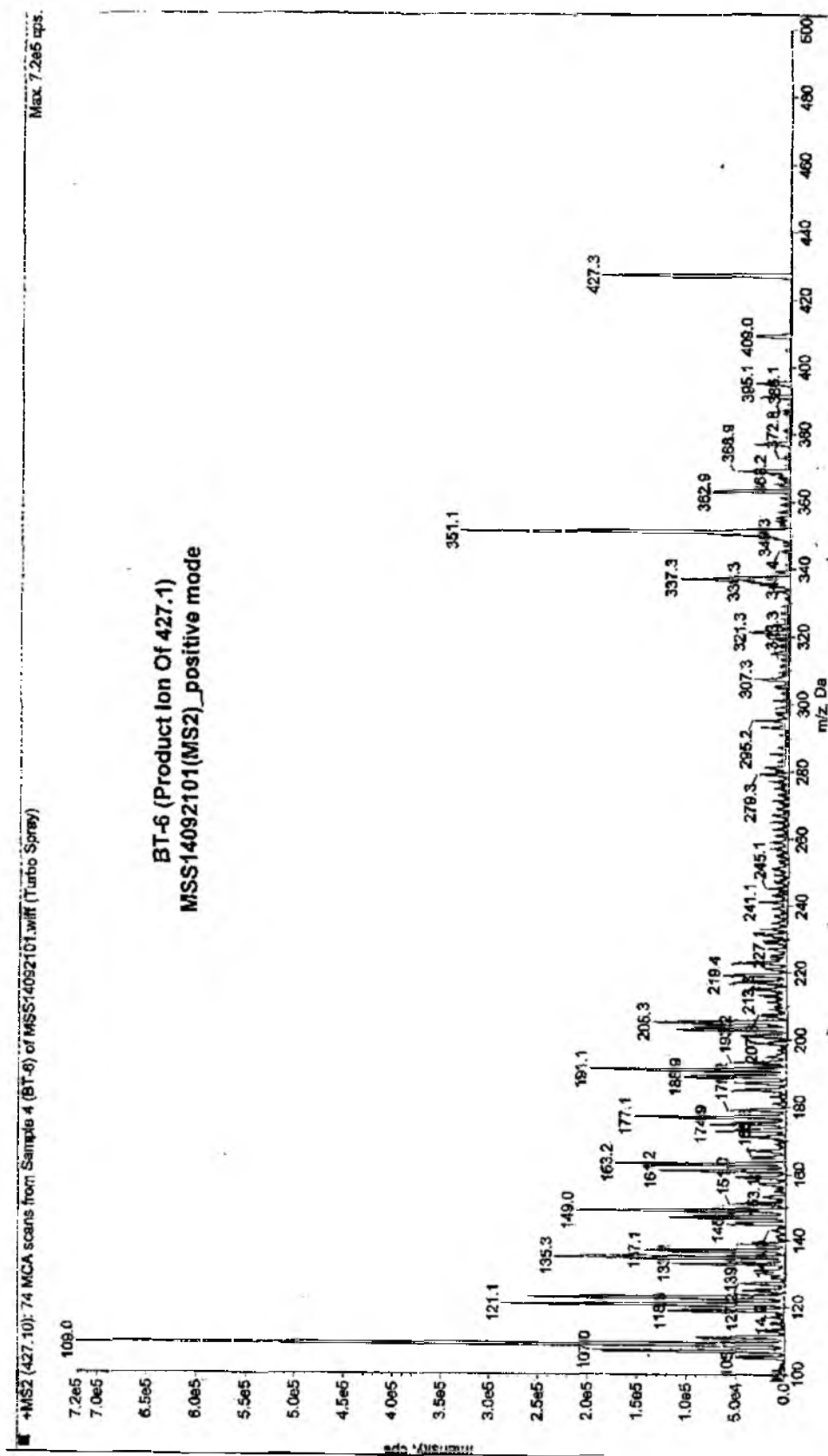


Figure 3.54: ESI-Mass spectrum of BT-6 (59).

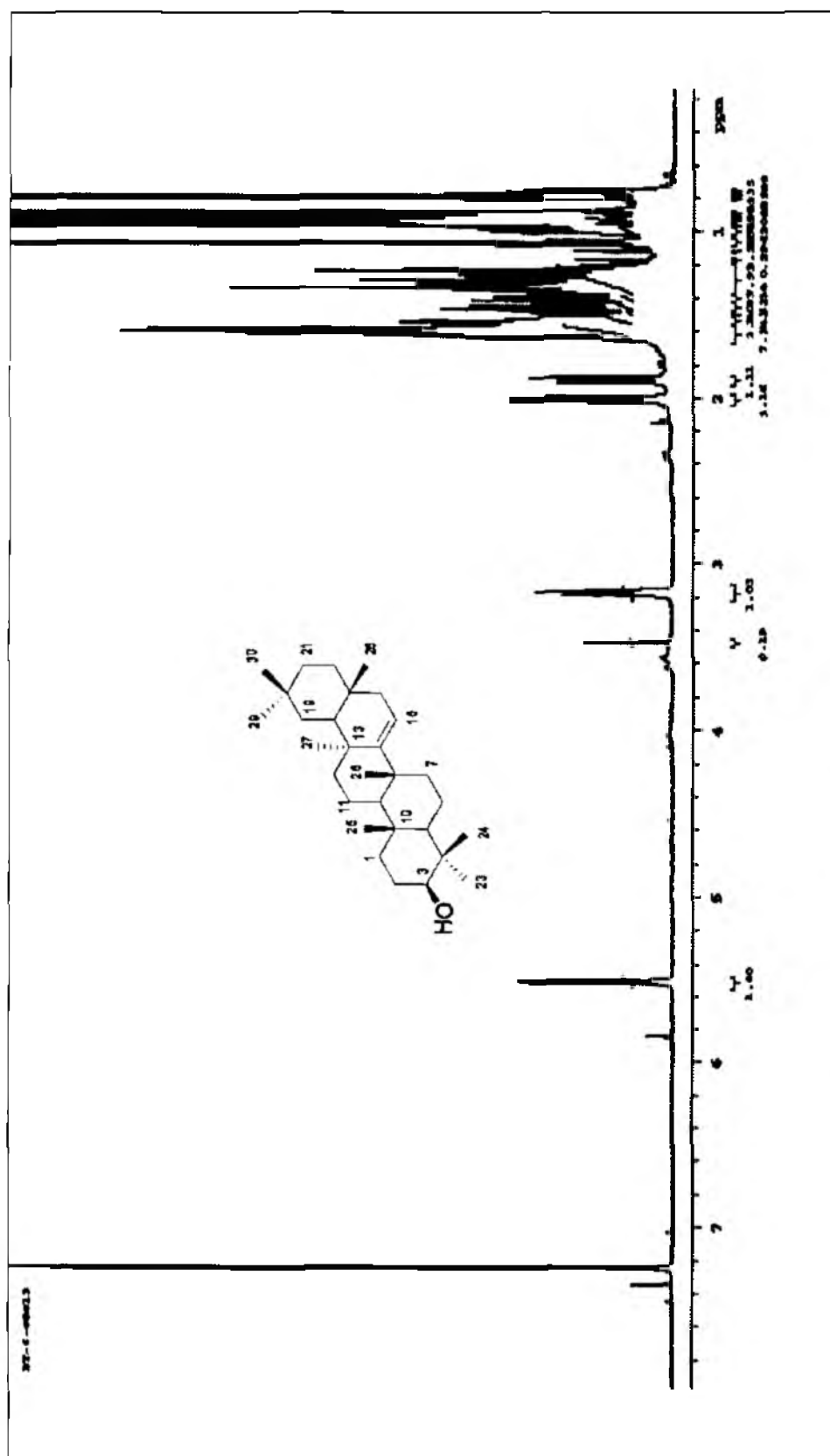


Figure 3.55: ^1H -NMR spectrum (500 MHz, CDCl_3) of BT-6 (59).

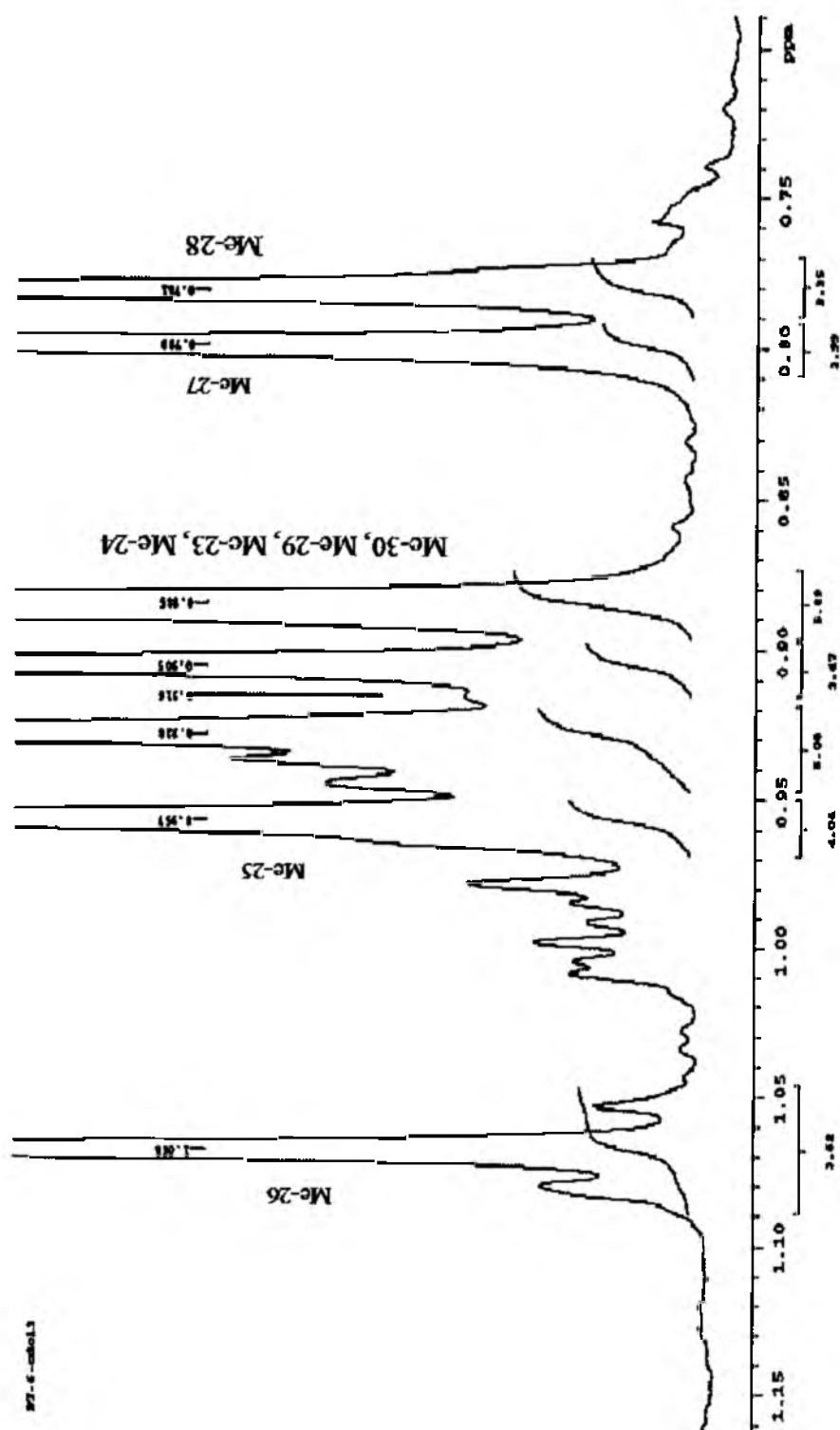


Figure 3.56: Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of BT-6 (59).

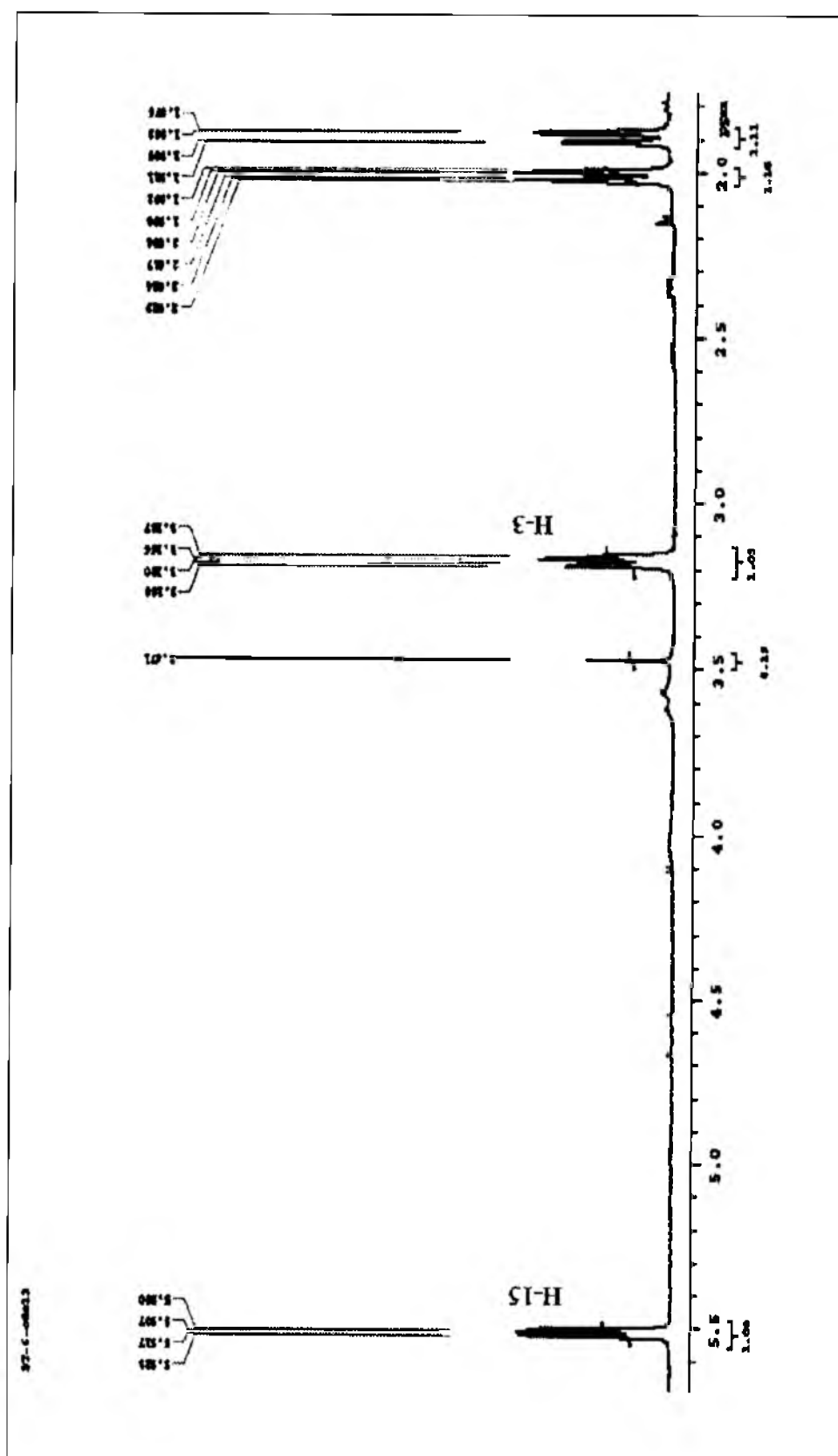


Figure 3.57: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of BT-6 (59).

3.14 Characterization of BT-8 as D₄-stigmasterone (60)

BT-8 was isolated as crystals from the VLC fractions 12(A+B) and 13A of the methanolic extract of stem bark of *B. tomentosa*. VLC fractions 12(A+B) and 13A were mixed together due to their identical TLC characteristics and subjected to preparative TLC (Stationary phase - Silica gel F₂₅₄, Mobile phase - toluene: ethyl acetate = 90: 10), the process was repeated thrice for better separation and isolation of the desired band. From the developed plates a purple colored band was visualized after spraying a small portion of the PTLC plate with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes. The unsprayed stationary phase was scrapped off and eluted with ethyl acetate. After evaporation of the solvent fine tree like crystals were obtained which was termed as BT-8 (6.29 mg, 60). It was found to be soluble in petroleum ether, *n*-hexane, ethyl acetate, chloroform and methanol.

The electrospray ionization mass spectrum (ESI-MS) of BT-8 (Figure 3.59) exhibited a pseudomolecular ion (M+H)⁺ peak at *m/z* 411 which was consistent with molecular ion at 410 that suggested it to be a steroid with molecular formula of C₂₉H₄₆O. The ¹H-NMR spectrum (500 MHz, CDCl₃; Figure 3.60; Table 3.16) showed resonances of six methyl groups, two of which were doublets at δ 0.80 d (*J*= 4.6 Hz) and 0.89 d (*J*= 4.6 Hz), associated to an isopropyl group substituent. Three double bond resonances of methine protons were observed at δ 5.76 as a singlet, 5.13 as a double doublet (*J*= 8.3, 15.1 Hz) and 5.01 also as a double doublet (*J*= 8.5, 15.0 Hz). This is characteristic of H-4, H-22 and H-23 in D₄-stigmasterone. Other spectral features are in close agreement with those of D₄-stigmasterone (Georges *et al.*, 2006), as well as the ¹H-NMR spectrum of BT-8 was super imposable to that of the spectrum acquired for an authentic sample previously isolated in our laboratory. So, the compound BT-8 was identified as D₄-stigmasterone (60). This is first report of isolation of D₄-stigmasterone (59) from *B. tomentosa*.

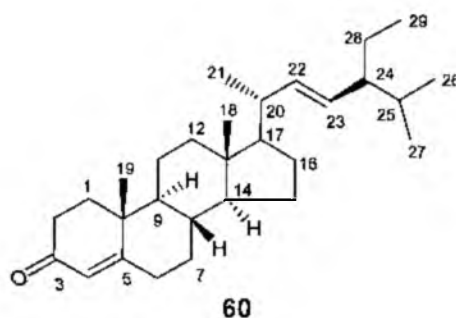


Figure 3.58: Structure of BT-8 (D₄-stigmasterone, 60).

Table 3.16: ¹H-NMR spectral data of BT-8 (60) and D₄-stigmasterone (Georges *et al.*, 2006) in CDCl₃.

| Proton(s) | δ_H in ppm in CDCl ₃ , (<i>J</i> in Hz). | |
|-----------|---|-------------------------------|
| | BT-8 (60) | D ₄ -stigmasterone |
| H-4 | 5.76 s | 5.72 s |
| Me-18 | 0.71 s | 0.73 s |
| Me-19 | 0.85 s | 0.83 s |
| Me-21 | 1.01 s | 1.04 s |
| Me-22 | 5.13 dd (15.1 and 8.3) | 5.15 dd (15.2 and 8.4) |
| Me-23 | 5.01 dd (15.0 and 8.5) | 5.02 dd (15.2 and 8.4) |
| Me-26 | 0.80 d (4.6) | 0.81 d (4.5) |
| Me-27 | 0.89 d (4.6 Hz) | 0.88 d (4.5) |
| Me-29 | 0.81 t (6.6 Hz) | 0.82 t (4.5) |

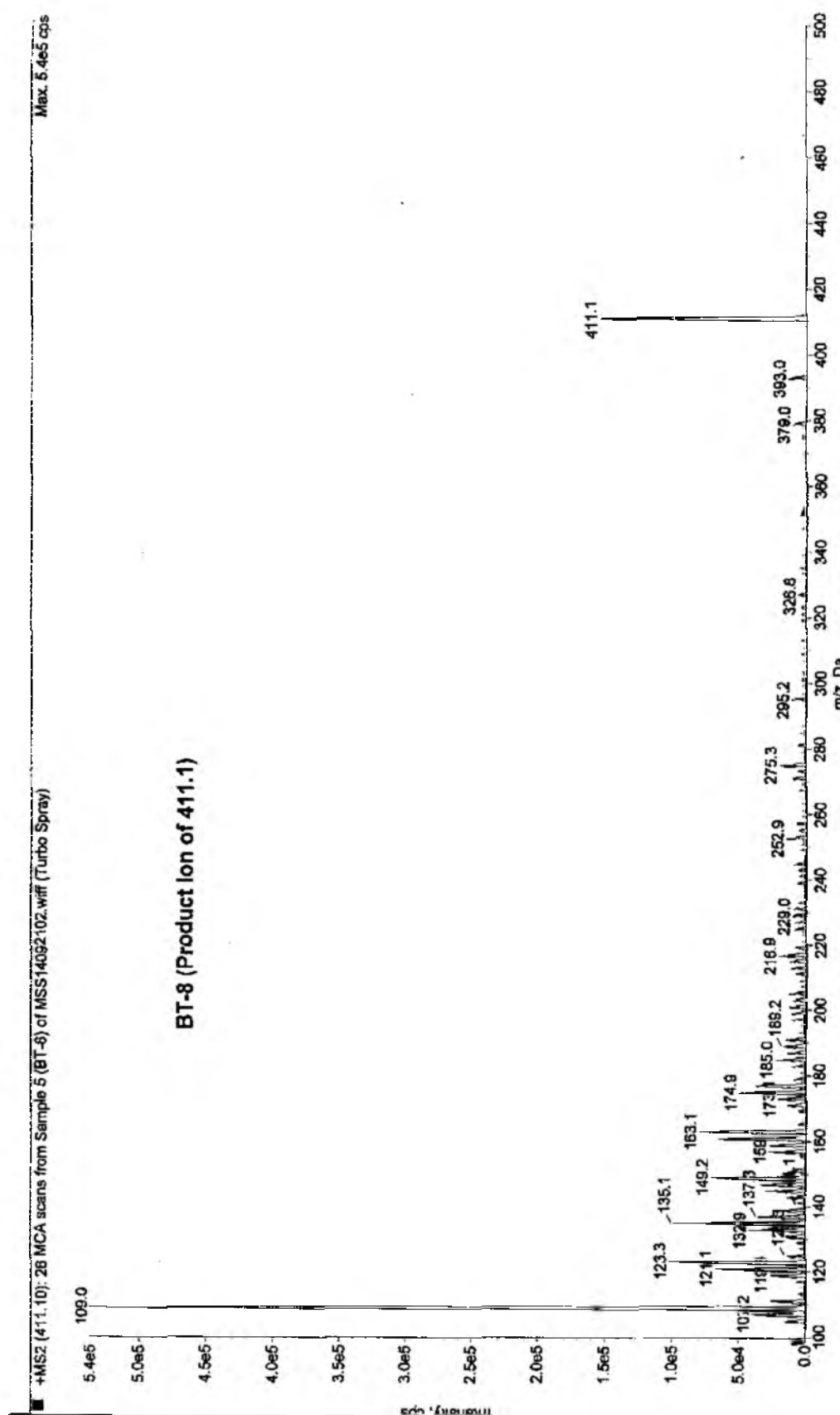


Figure 3.59: ESI-Mass spectrum of BT-8 (60).

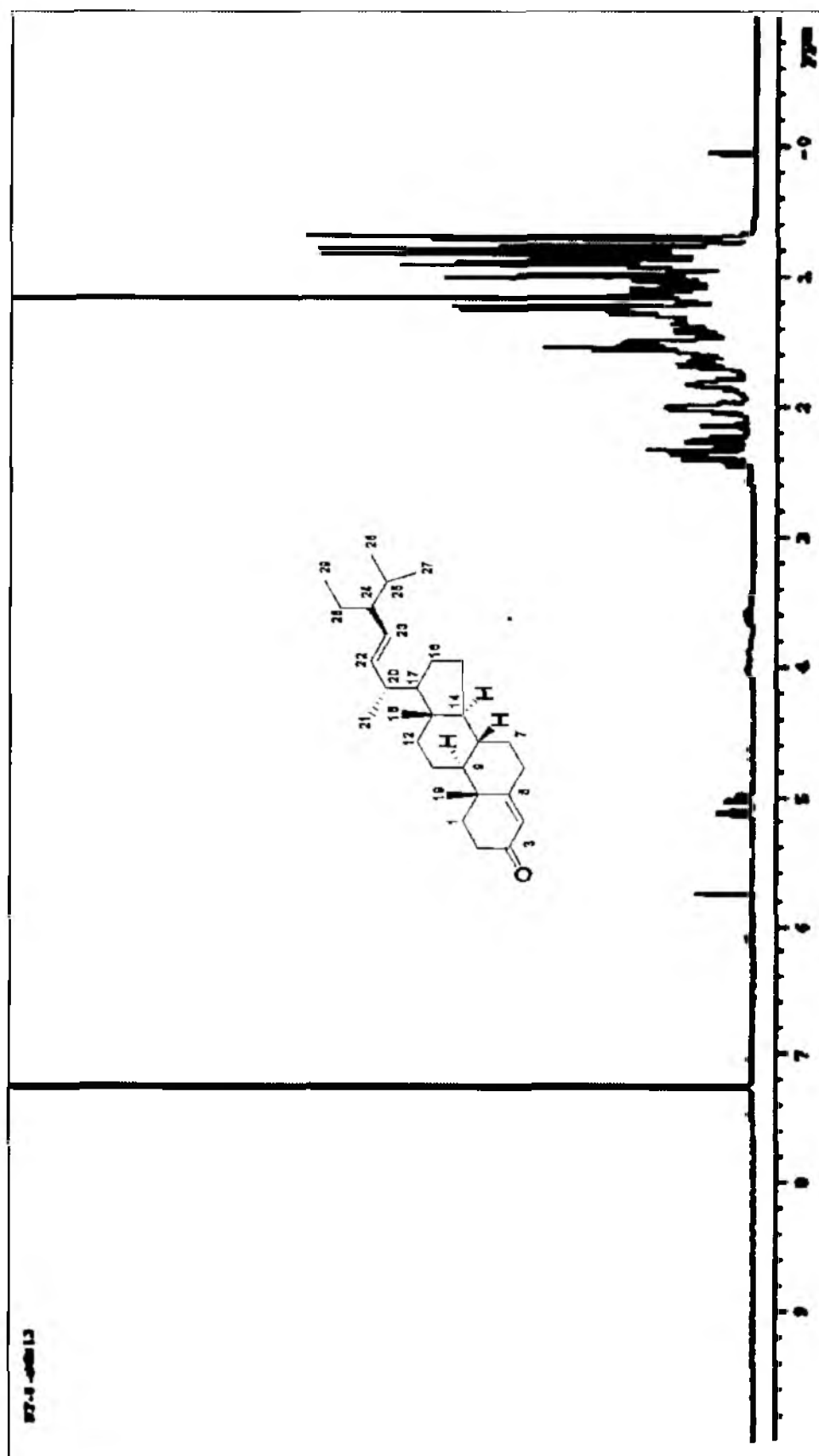


Figure 3.60: ¹H-NMR spectrum (500 MHz, CDCl₃) of BT-8 (60).

3.15 Characterization of BT-14 as lupeol (61)

BT-14 was isolated as crystals from the VLC fractions 12(A+B) and 13A of the methanolic extract of stem bark of *B. tomentosa*. VLC fractions 12(A+B) and 13A were mixed together due to their identical TLC characteristics and subjected to preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (90:10)]. The process was repeated thrice for better separation and isolation of the desired band. From the developed plates a purple colored band was visualized after spraying a small portion of the PTLC plate with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes. The unsprayed portion of the band on the stationary phase was scrapped off and eluted with ethyl acetate. After evaporation of the solvent fine tree like crystals were obtained which after purity test was termed as BT-14 (6.31 mg, **61**).

The crystals were found to be soluble in petroleum ether, *n*-hexane, ethyl acetate, chloroform and sparingly soluble in methanol.

The electrospray ionization mass spectrum (ESI-MS) of BT-14 (Figure 3.62) exhibited a pseudomolecular ion (M+H)⁺ peak at *m/z* 427 which was consistent with molecular ion at 426 that suggested it to be a triterpenoid with molecular formula of C₃₀H₅₀O. The ¹H-NMR spectrum (500 MHz, CDCl₃; Figure 3.63; Table 3.17) of BT-14 showed a double doublet (*J*= 11.2, 4.8 Hz) of one proton intensity at δ 3.20, typical for an oxymethine proton at C-3 in a triterpene. The splitting pattern of this proton revealed the β orientation of the C-3 oxygenated substituent. The spectrum also displayed two broad one proton singlet at δ 4.68 and 4.56 (1H each) assignable to the vinylic protons at C-29. The ¹H-NMR spectrum further showed a characteristic multiplet of one proton intensity at δ 2.36 which could be ascribed to the proton at C-19 and seven singlets at δ 0.96, 0.78, 0.83, 1.02, 0.93, 0.82 and 1.67 (3H each) assignable to the methyl protons at C-4 (H₃-23, H₃-24), C-10 (H₃-25), C-8 (H₃-26), C-14 (H₃-27), C-17 (H₃-28) and C-20 (H₃-30), respectively. The downfield methyl group resonance at δ 1.67 was assigned to the vinylic methyl group at C-20 (H₃-30).

By comparing the ¹H-NMR spectral data (Table 3.17) with previously published values (Jahan *et al.*, 2010) BT-14 was identified as lupeol (**61**). The identity of BT-14 was further substantiated by co-TLC with an authentic lupeol previously isolated in our laboratory. This is the first report of isolation of lupeol from *B. tomentosa*.

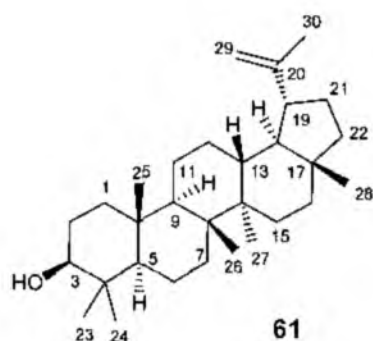


Figure 3.61: Structure of BT-14 (lupeol, 61).

Table 3.17: ^1H -NMR spectral data of BT-14 (61) and lupeol (Jahan *et al.*, 2010) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|---------------------|---|------------------------|
| | BT-14 (61) | Lupeol |
| H-3 | 3.20 dd (11.2 and 4.8) | 3.20 dd (11.5 and 5.0) |
| H-19 | 2.30 m | 2.28 m |
| Me-23 | 0.96 s | 0.95 s |
| Me-24 | 0.78 s | 0.78 s |
| Me-25 | 0.83 s | 0.84 s |
| Me-26 | 1.02 s | 1.02 s |
| Me-27 | 0.94 s | 0.93 s |
| Me-28 | 0.75 s | 0.82 s |
| Me _a -29 | 4.56 s | 4.55 s |
| Me _b -29 | 4.68 s | 4.67 s |
| Me-30 | 1.67 s | 1.68 s |

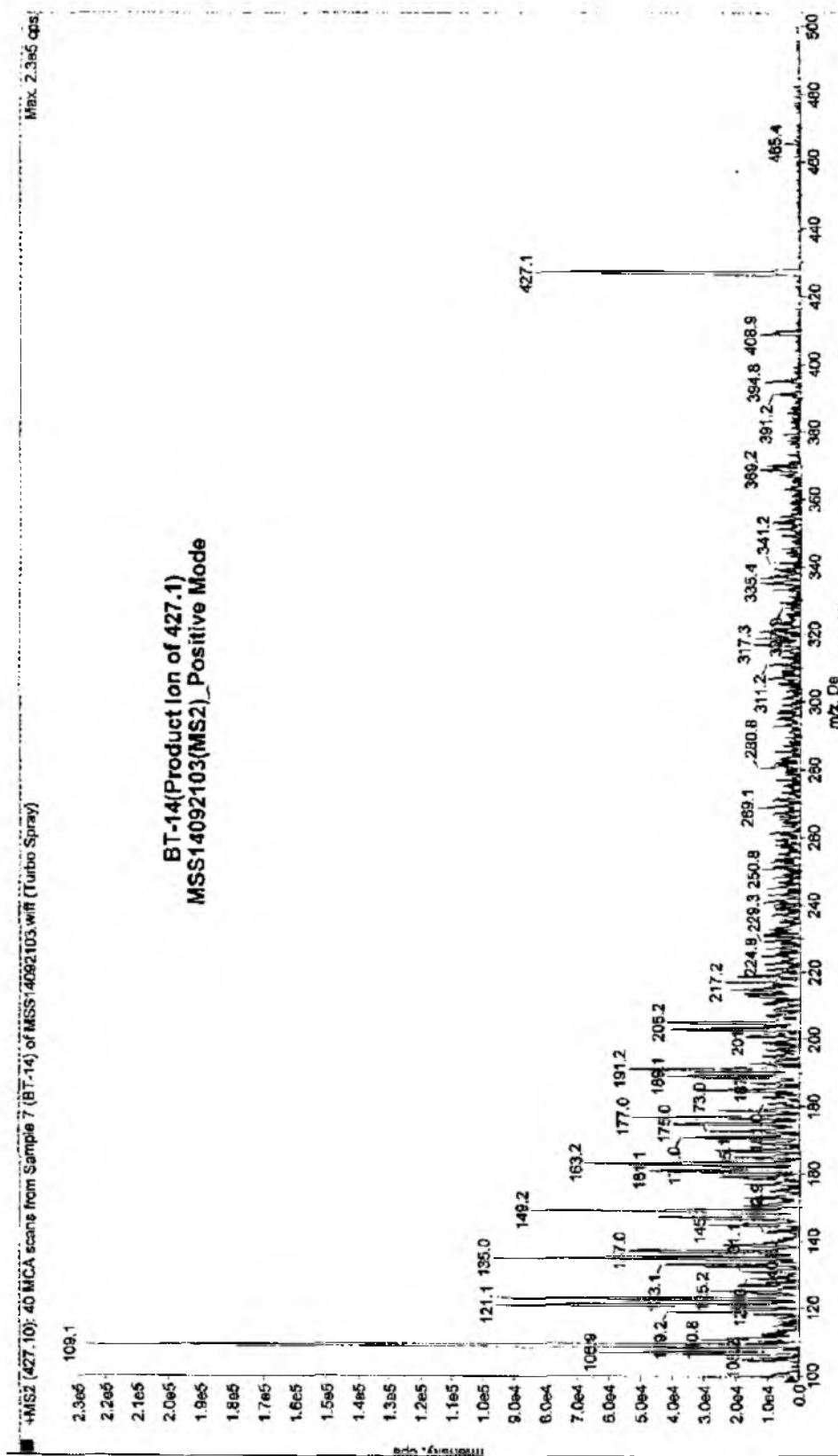


Figure 3.62: Mass spectrum of BT-6 (61).

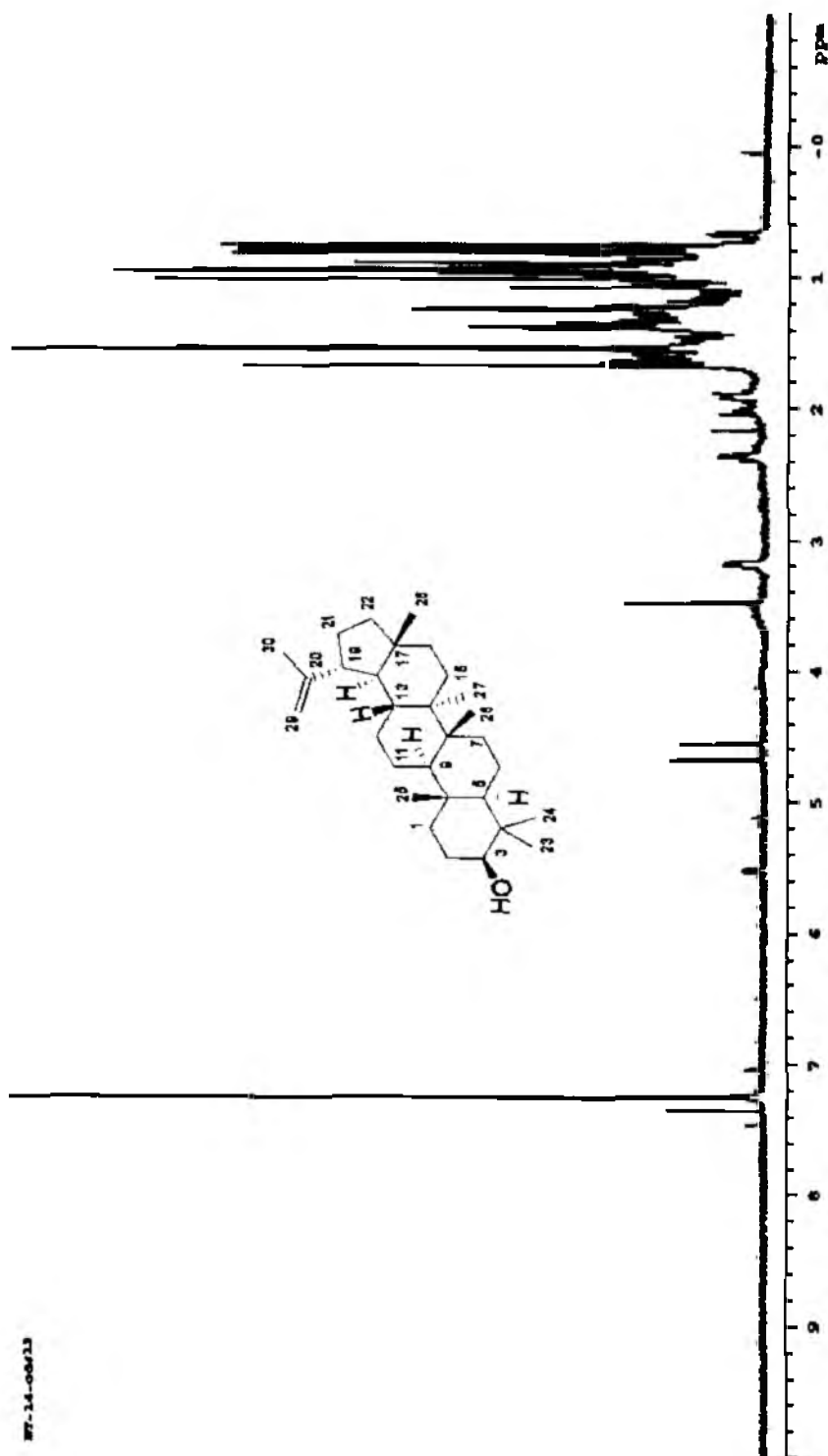


Figure 3.63: ¹H-NMR spectrum (500 MHz, CDCl₃) of BT-14 (61).

3.16 Characterization of EF-7 as shinpterocarpin (62)

EF-7 was isolated as yellowish mass by evaporation of solvent from the silica column fractions 90-97 of the carbon tetrachloride soluble portion of methanolic extract of the stem bark of *E. fusca*. The yellowish residue was then subjected to preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated thrice for better separation of the desired band. After excitation with 254 nm UV light, quenching was observed by the desired band as a uniform layer. Then the band was scrapped off on an aluminum foil and eluted with ethyl acetate. Evaporation of the solvent yielded an oily transparent liquid. It was checked for purity and denoted as EF-7 (6.12 mg, 62). The compound was soluble in *n*-hexane, ethyl acetate, chloroform and methanol.

The ¹H-NMR spectrum of the compound EF-7 (500 MHz, CDCl₃; Figures 3.65-3.70; Table 3.18) showed a one proton multiplet at δ 3.50 (m) due to 6a-H, at δ 5.52 (d, *J*= 6.5 Hz) for 11a-H, two proton signals at δ 3.63 (dd, *J*=11.0, 5.0 Hz) and 4.25 (dd, *J*=11.0, 11.0 Hz) due to 6-H₂ which indicated a pterocarpin skeleton. In the ring A, the presence of two doublets aromatic protons at δ 7.43 (1H, d, *J*= 8.0 Hz) for H-1 and 6.36 (1H, d, *J*= 8.0 Hz) for H-2 indicated that C-3 and C-4 of the ring were substituted. In the D ring, the presence of two doublets with one aromatic proton intensity each at δ 6.97 (1H, d, *J*= 8.0 Hz) for H-7 and 6.44 (1H, d, *J*= 2.0 Hz) for H-8 indicated that the C-9 was substituted with hydroxyl group. The ¹H-NMR spectrum further showed two singlets with three proton intensity at δ 1.45 and 1.44 assignable to the methyl protons at C-6'.

In the ¹H-NMR spectrum of the compound shinpterocarpin (62) the presence of signals of comparatively low intensity were observed. These may be due the presence of another isomer of the compound 62. However, its structure could not be solved.

Comparison of ¹H-NMR data for EF-7 were found to be similar with those for shinpterocarpin (Table 3.18) (Kitagawa *et al.*, 1994). Therefore, the compound EF-7 was identified as shinpterocarpin (62). This is the first report of isolation of shinpterocarpin (62) from *E. fusca*.

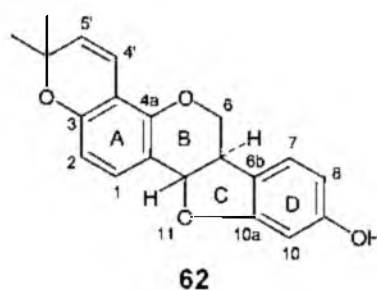


Figure 3.64: Structure of EF-7 (shinpterocarpin, 62).

Table 3.18: ^1H -NMR spectral data of EF-7 (62) and shinpterocarpin (Kitagawa *et al.*, 1994) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------|---|--------------------------------------|
| | EF-7 (62) | Shinpterocarpin |
| H-1 | 7.43 d (8.0) | 7.25 d (8.8) |
| H-2 | 6.36 d (8.0) | 6.48 d (8.8) |
| H-6 | 3.63 dd (11.0 and 5.0) | 3.65 dd (10.5 and 9.4) |
| | 4.25 dd (11.0 and 11.0) | 4.33 dd (10.5 and 4.4) |
| H-6a | 3.5 m | 3.61 m |
| H-7 | 6.97 d (8.0) | 7.13 d (8.3) |
| H-8 | 6.57 dd (8.0 and 2.0) | 6.38 dd (8.3 and 2.2) |
| H-10 | 6.44 d (2.0) | 6.31 d (2.2) |
| H-11a | 5.52 d (6.5) | 5.48 d (6.6) |
| H-4' | 6.52 d (10.0) | 6.60 d (9.9) |
| H-5' | 5.52 d (10.0) | 5.67 d (9.9) |
| 6' methyl | 1.45 s | 1.39 s ($6'\text{-CH}_3 \times 2$) |
| | 1.41 s | |

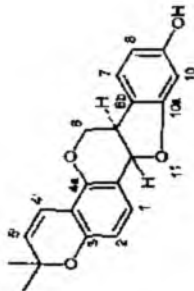
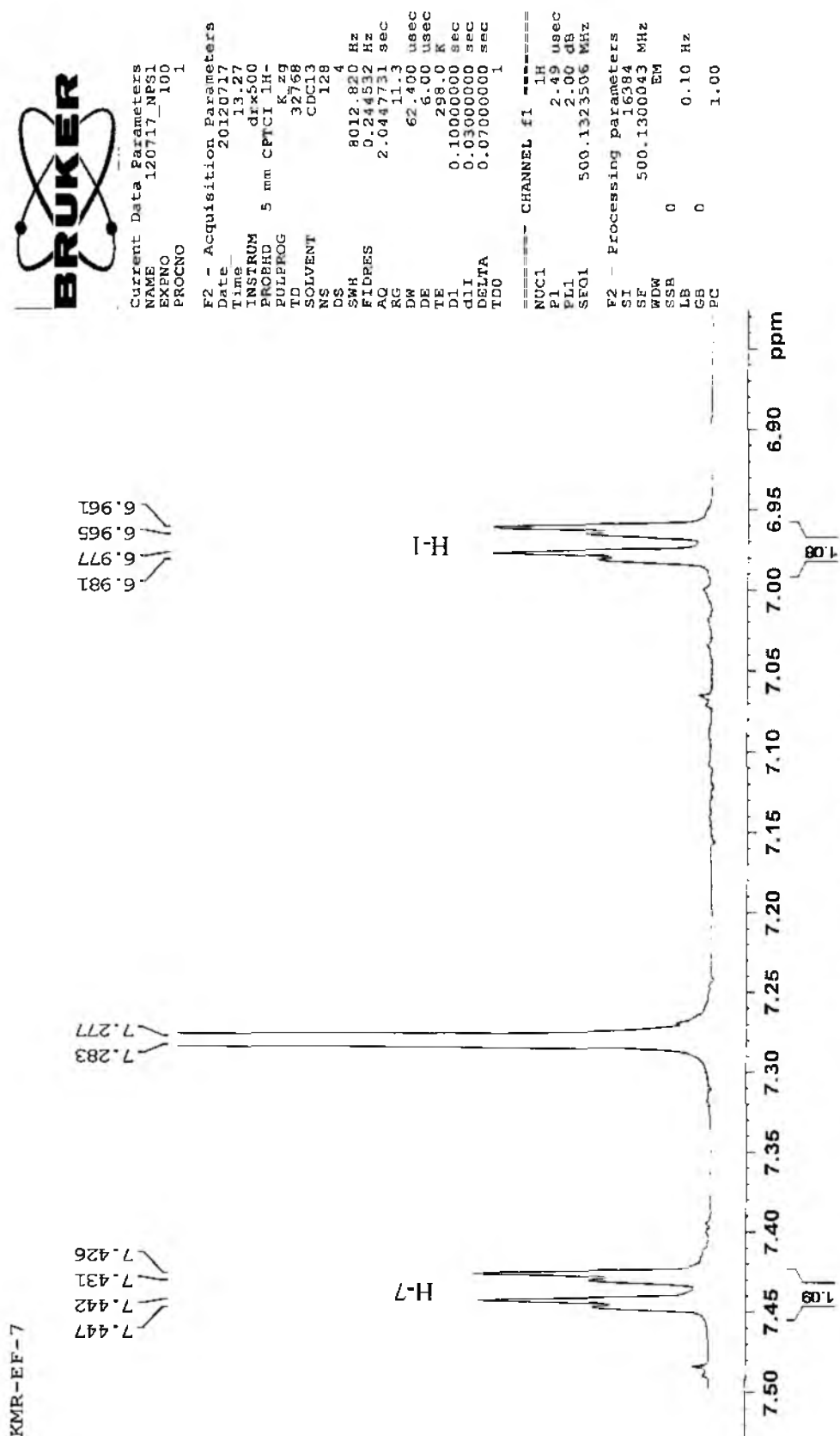


Figure 3.65: ¹H-NMR spectrum (500 MHz, CDCl₃) of EF-7 (62).

Figure 3.66: Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-7 (62).

KMR-EF-7

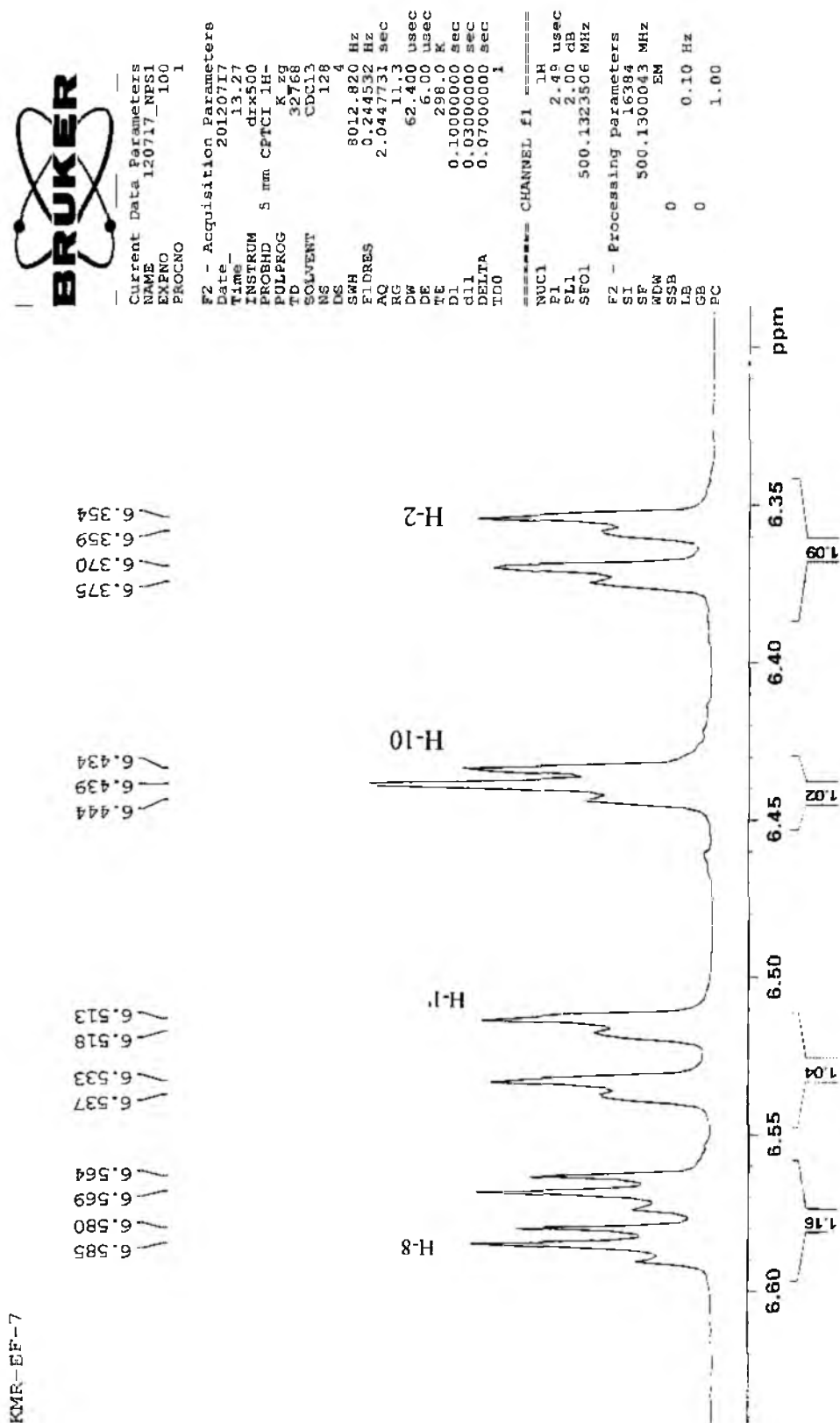


Figure 3.67: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of EF-7 (62).

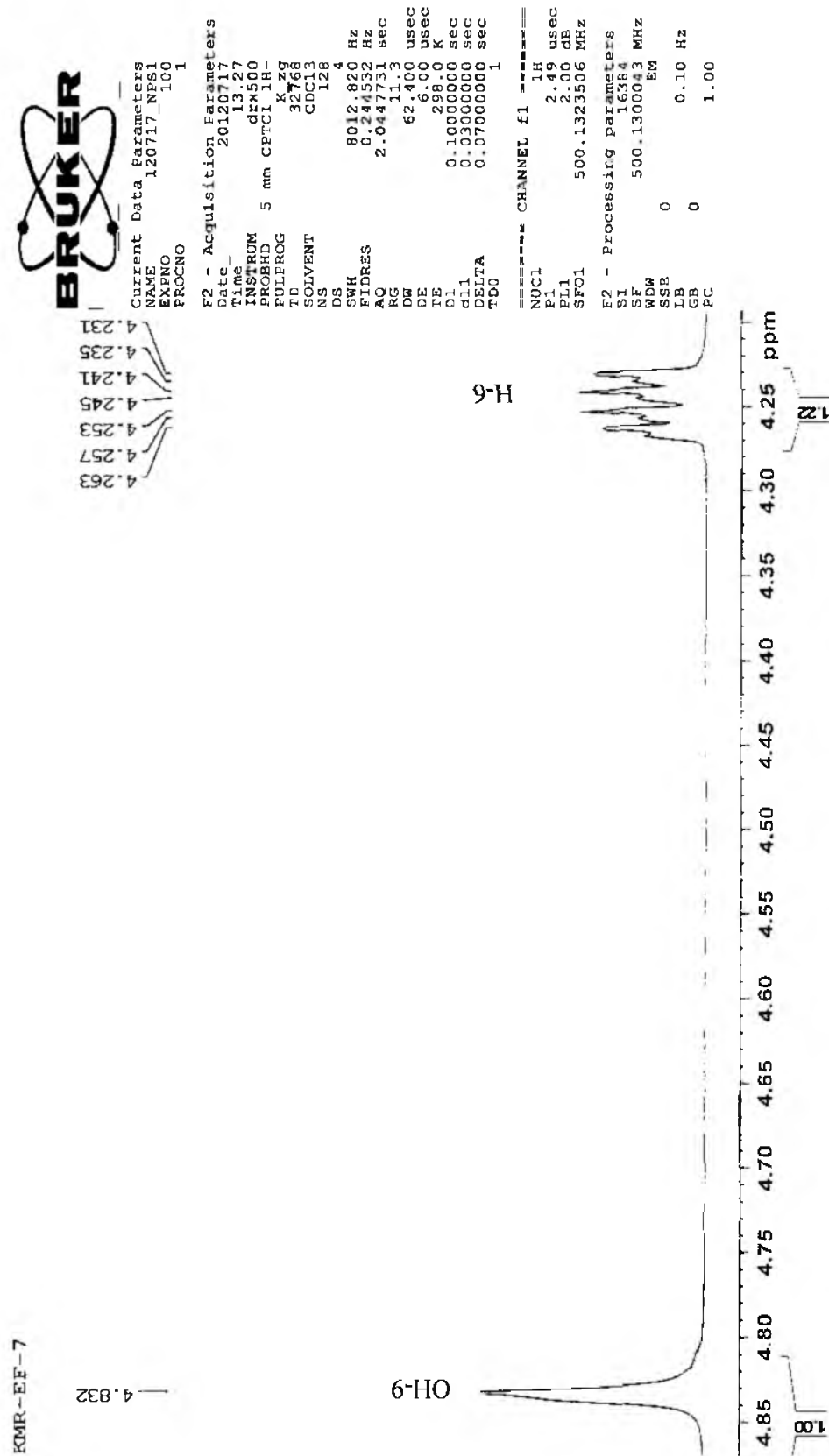
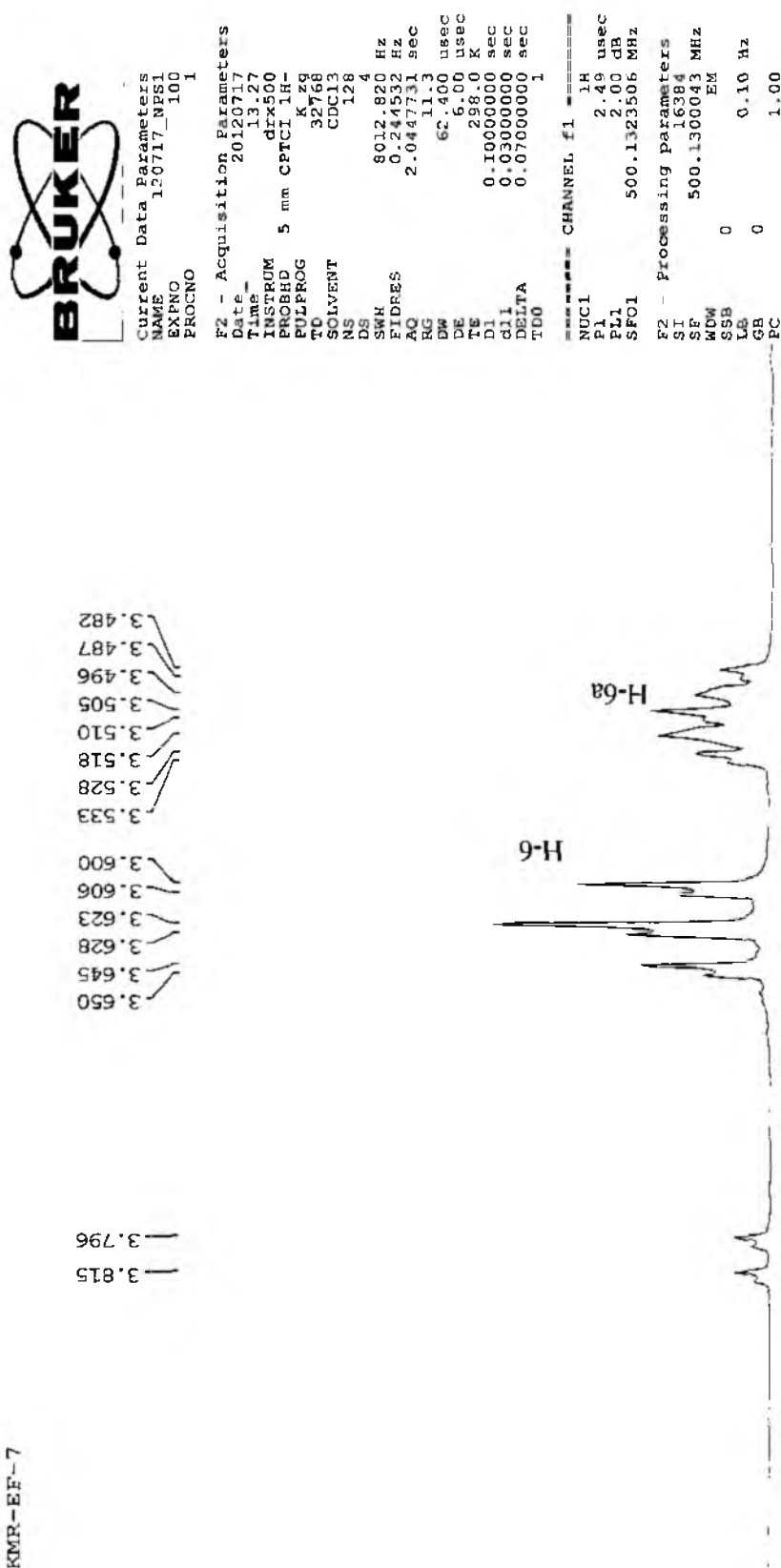


Figure 3.68: Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-7 (62).

KMR-EF-7

Figure 3.69: Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-7 (62).

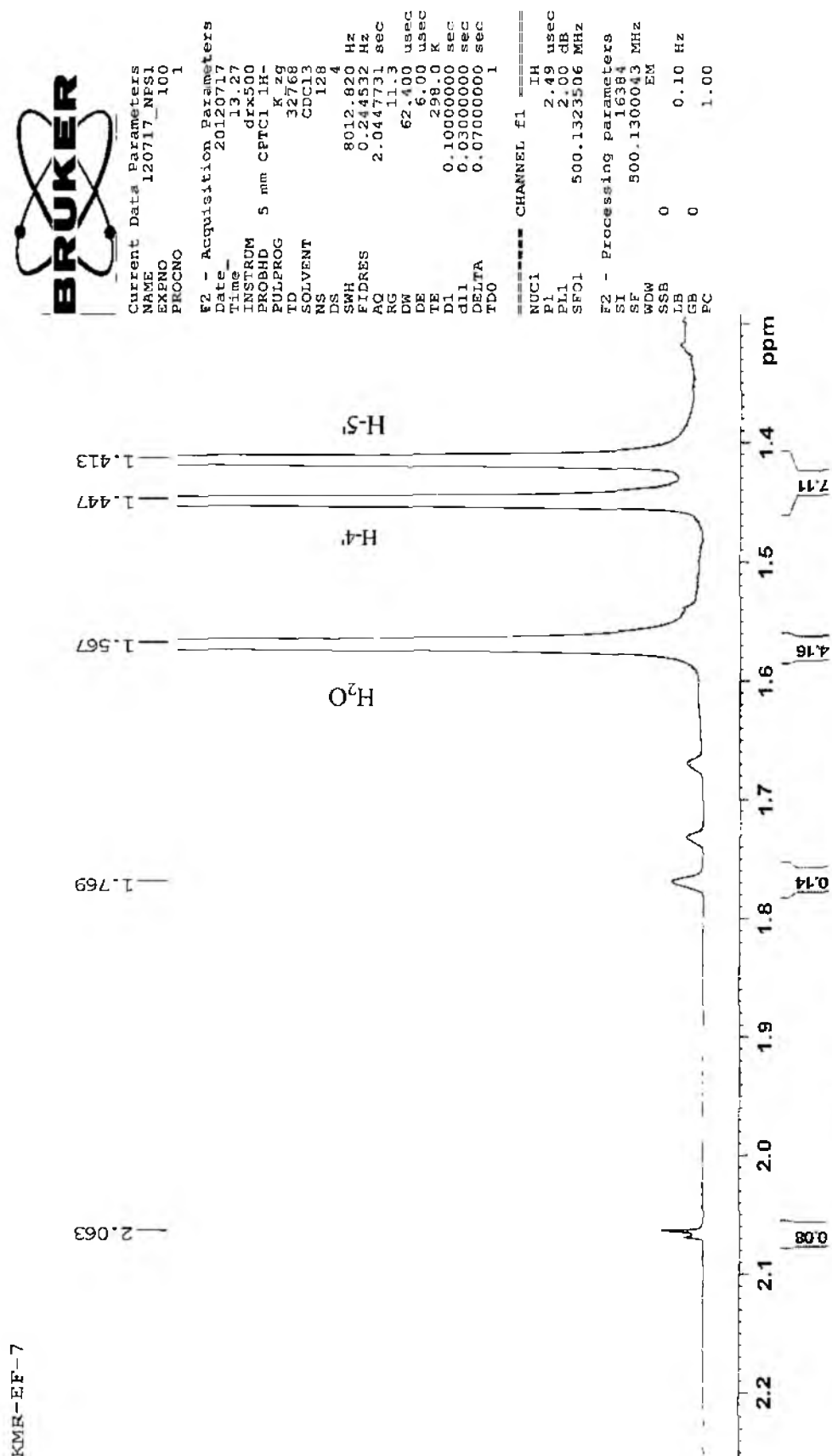


Figure 3.70: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of EF-7 (62).

3.17 Characterization of EF-8 as lupinifolin (63)

EF-8 was obtained as a yellowish mass by evaporation of solvent from the CC fractions 90-97 of the carbon tetrachloride soluble fraction of stem bark of *E. fusca*. The fraction was then subjected to a preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated thrice for better separation of the desired band. The desired band was identified under UV light, then scrapped off and eluted with ethyl acetate to yield EF-8 (8.20 mg, **63**) as yellow needles on standing at room temperature. The compound was soluble *n*-hexane, ethyl acetate, chloroform and methanol.

TLC examination of the compound EF-8 showed a bright yellow colored spot which turned into orange spot when sprayed with vanillin-sulfuric acid reagent indicated the presence of a flavonone type compound.

The ¹H-NMR spectrum (500 MHz, CDCl₃; Figures 3.72-3.75; Table 3.19) of EF-8 revealed a sharp singlet characteristic for the chelated hydroxyl group at C-5 in a pyranoflavonone skeleton at δ 12.25 (1H, s, OH-5). The diagnostic features for a flavonone nucleus were evident δ 3.04 (1H, dd, J = 14.5, 7.3 Hz), 2.80 (1H, dd, J = 13.5, 2.0 Hz) and δ 5.34 (1H, dd, J = 12.0, 2.0 Hz) which could be assigned to H-3 α , H-3 β and H-2 of the flavanone nucleus, respectively. The doublet at δ 5.50 (1H, d, J = 9.5 Hz, H-3'') and δ 6.63 (1H, d, J = 10.0 Hz, H-4''), each equivalent to one proton and the two singlets at δ 1.44 (3H, s) and δ 1.46 (3H, s) were characteristic for the *cis* double bond and *gem*-dimethyl group of a 2,2-dimethyl-chromene moiety, respectively (Rao and Srimannarayana, 1984). Two *ortho*-coupled doublets (J = 8.5 Hz) centered at δ 7.33 (2H) and 6.87 (2H) were assigned to the protons H-2' & H-6' and H-3' & H-5' of the *para*-disubstituted benzene ring (C ring). The presence of an isoprenyl group was inferred from the singlets at δ 1.67 (3H, s, CH₃-4'') and δ 1.65 (3H, s, CH₃-5''), the doublet at δ 3.22 (1H, d, J = 7.0 Hz, H-1'') and the triplet at δ 5.10 (1H, t, J = 7.1 Hz, H-2'').

These ¹H-NMR data (Table 3.19) of compound EF-8 showed similarity with that of lupinifolin (Mahihol *et al.*, 1997). Thus, compound EF-8 was identified as lupinifolin (**63**). It was previously isolated from the bark of the same plant (Innok *et al.*, 2009).

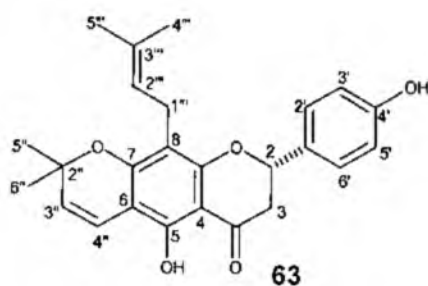


Figure 3.71: Structure of EF-8 (lupinifolin, 63).

Table 3.19: ^1H -NMR spectral data of EF-8 (63) and lupinifolin (Mahidol *et al.*, 1997) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|------------|---|-------------------------|
| | EF-8 (63) | Lupinifolin |
| 2 | 5.34 dd (12.0 and 2.0) | 5.30 dd (12.7 and 3.3) |
| 3 α | 3.04 dd (14.5 and 7.3) | 3.04 dd (17.3 and 12.7) |
| 3 β | 2.80 dd (13.5 and 2.0) | 2.78 dd (17.3 and 3.3) |
| 2', 6' | 7.33 d (8.5) | 7.28 d (8.5) |
| 3', 5' | 6.88 d (8.5) | 6.84 d (8.5) |
| 3'' | 5.50 d (9.5) | 5.48 d (10.0) |
| 4'' | 6.63 d (10.0) | 6.63 d (10.0) |
| 5'' | 1.44 s | 1.44 s |
| 6'' | 1.46 s | 1.44 s |
| 1''' | 3.22 d (7.0) | 3.20 br d (7.0) |
| 2''' | 5.15 t (7.1) | 5.14 t (7.0) |
| 4''' | 1.66 s | 1.64 br s |
| 5''' | 1.67 s | 1.64 br s |
| 5-OH | 12.25 s | 12.20 s |

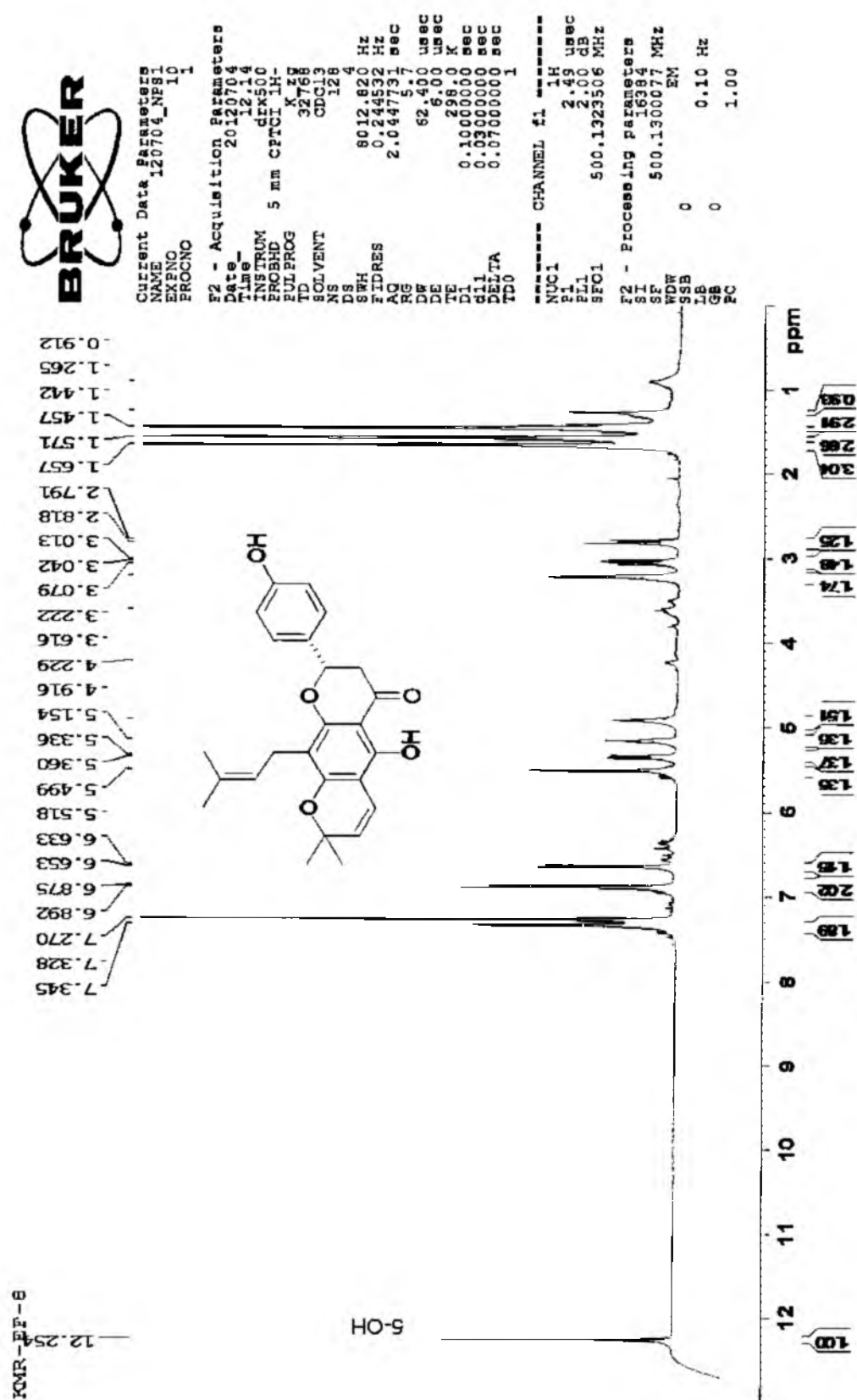


Figure 3.72: ¹H-NMR spectrum (500 MHz, CDCl₃) of EF-8 (63).

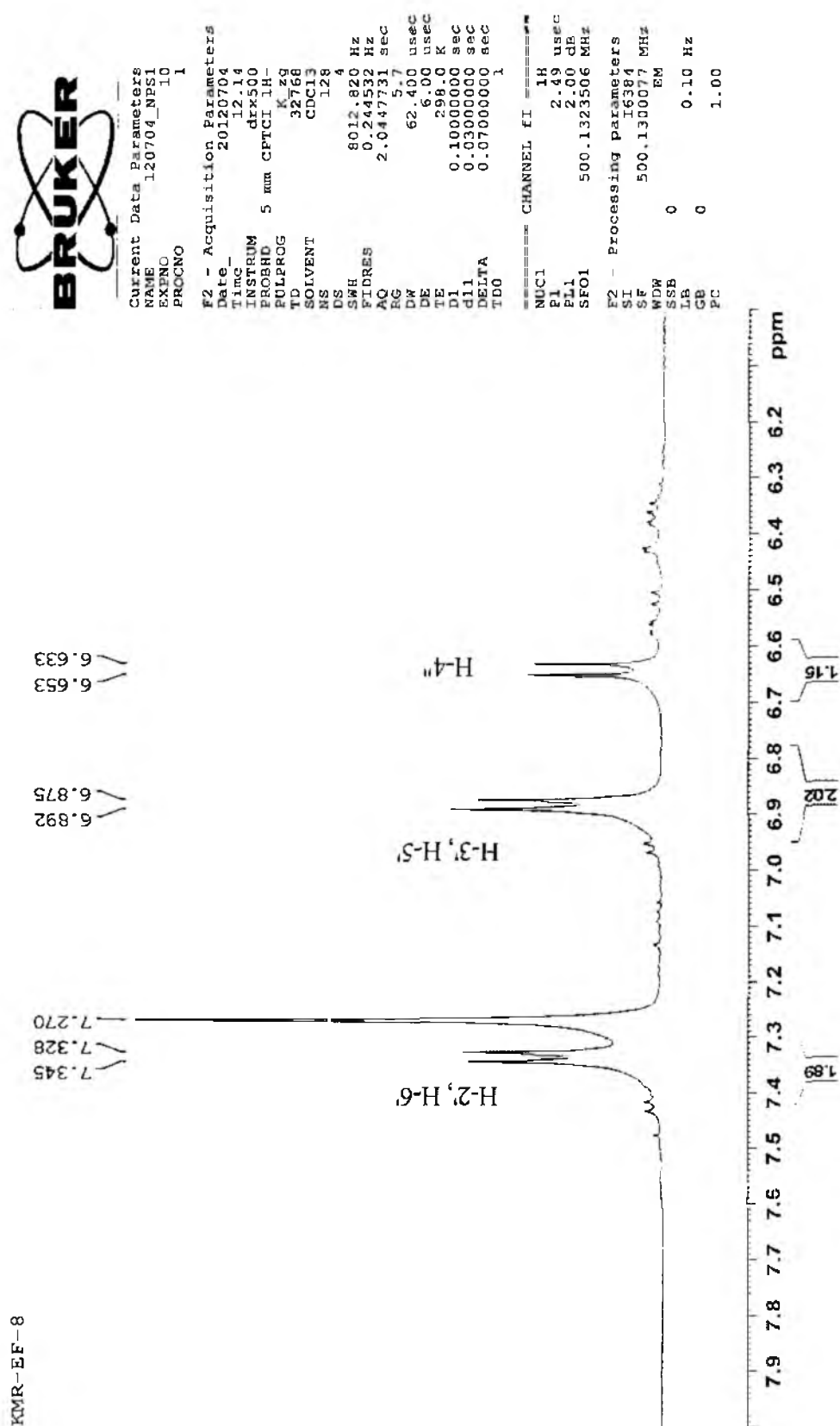


Figure 3.73: Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-8 (63).

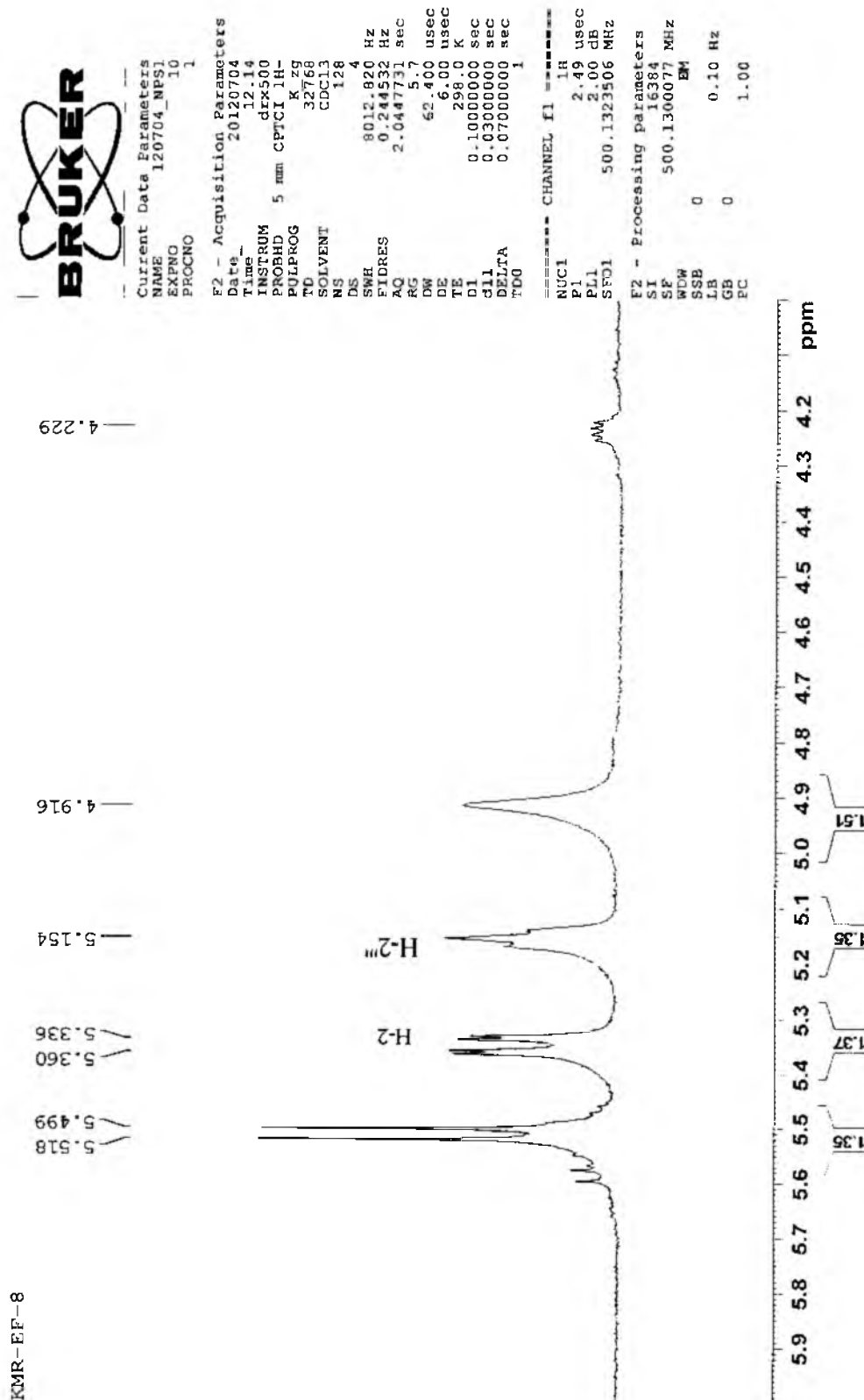


Figure 3.74: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of EF-8 (63).

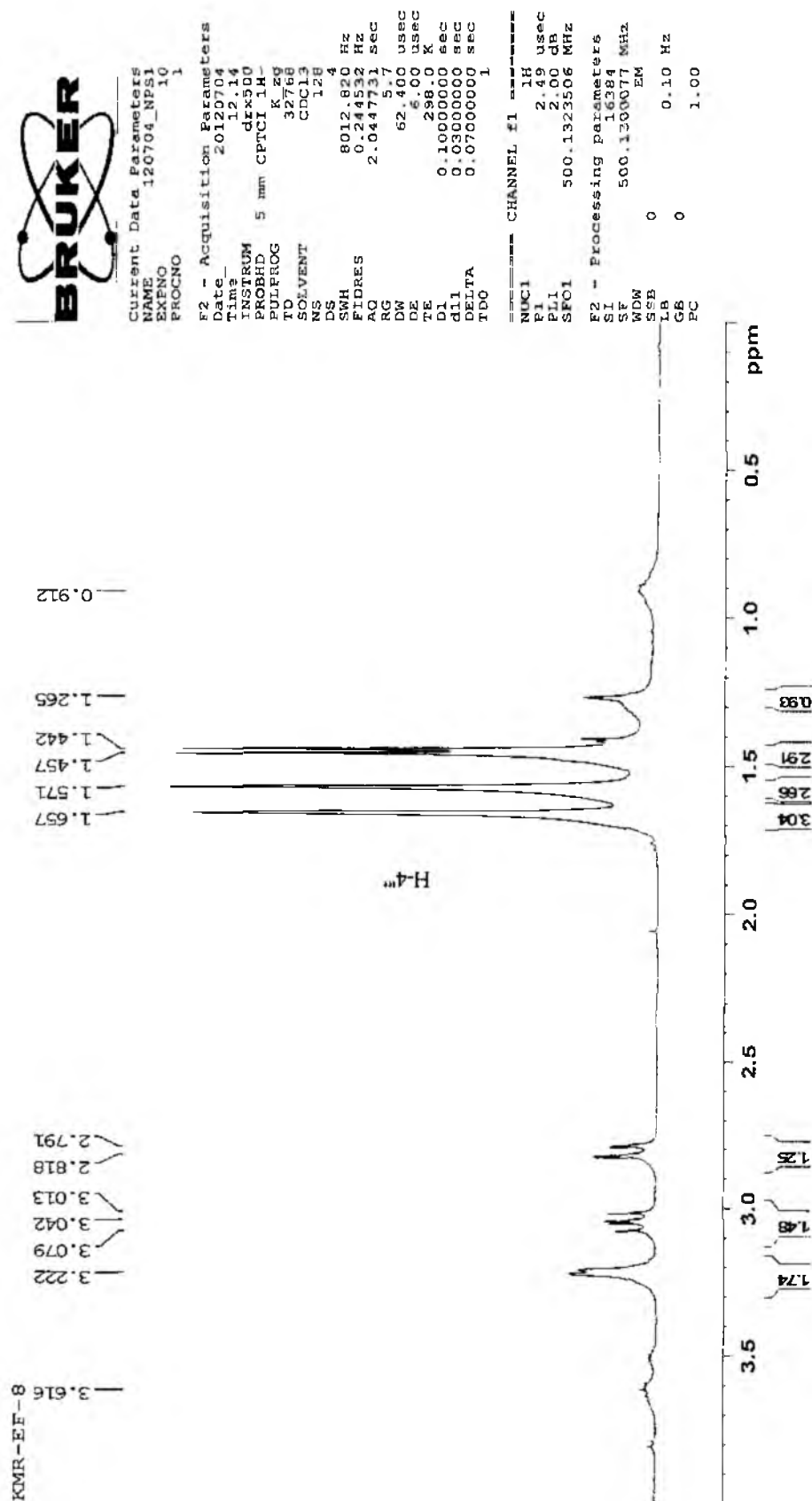


Figure 3.75: Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-8 (63).

3.18 Characterization of EF-12 as 3,9-dihydroxy-4-(3,3 dimethylallyl) [6aR,11aR]-pterocarpan (64)

The carbon tetrachloride soluble fraction of the crude methanolic extract of the stem bark of *E. fusca* was subjected to silica gel column chromatography by using mixtures of *n*-hexane and ethyl acetate in order of increasing polarities to obtain two hundred and fifty sub-fractions. Sub-fractions 90-97 showing identical spots on TLC were combined in a beaker. After drying the solvent a yellowish mass was obtained which was then subjected to preparative TLC over silica gel F₂₅₄ using toluene-ethyl acetate (97:3) as the developing solvent. The process was repeated five times for better separation of the desired band. A small portion of the developed plate was sprayed with vanillin-sulfuric acid reagent followed by heating at 110 °C for 5 minutes and a pink colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded a compound EF-12 (4.5 mg, **64**) as amorphous powder. The compound was analyzed over TLC to check its purity and found a pure compound which was also found to be soluble in *n*-hexane, ethyl acetate, chloroform and methanol.

The ¹H-NMR spectrum of the compound EF-12 (500 MHz, CDCl₃; Figures 3.77-3.81; Table 3.20) showed a set of proton resonances at δ 3.63 (dd, *J*=11.0, 5.0 Hz) and 4.25 (dd, *J*=11.0, 5.0 Hz) due to 6-H₂, at δ 3.51 (m) due to 6a-H and at δ 5.47 (d, *J*= 6.5 Hz) for 11a-H suggested the presence of an -O-CH₂-CH-CH-O- skeleton of B and C rings of a pterocarpan nucleus.

The ¹H-NMR spectrum further revealed the presence of a pair of *ortho*-coupled doublets with one proton intensity each at δ 7.42 (1H, d, *J*=8.5 Hz) (for H-1) and δ 6.39 (1H, d, *J*= 8.0 Hz) (for H-2) in the ring-A showed that C-3 and C-4 of the ring were substituted. The presence of three aromatic protons at δ 6.97 (1H, d, *J*= 8.0 Hz) (H-7), 6.57 (dd, *J*= 8.0, 2.0 Hz) (H-8) and 6.43 (1H, d, *J*= 2.0 Hz) (H-10) indicated ABX spin system and protons of two hydroxyl groups at δ 4.79 and 4.80. Comparison of ¹H-NMR data for EF-12 with that of the published in the literature (Cottiglia *et al.*, 2005) the structure of EF-12 was established as 3,9-dihydroxy-4-(3,3-dimethylallyl)[6aR,11aR]-pterocarpan (**64**). This is the first report of isolation of 3,9-dihydroxy-4-(3,3-dimethylallyl)[6aR,11aR]-pterocarpan (**64**) from *E. fusca*.

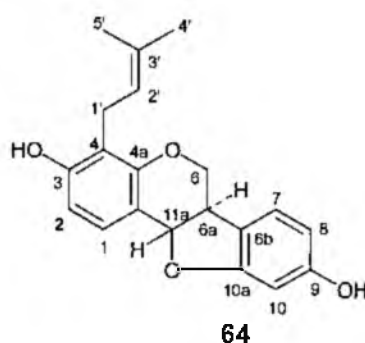


Figure 3.76: Structure of EF-12 (3,9-dihydroxy-4-(3,3-dimethylallyl)[6aR,11aR]-pterocarpan, **64**).

Table 3.20: $^1\text{H-NMR}$ spectral data of EF-12 (**64**) and 3,9-dihydroxy-4-(3,3-dimethylallyl)[6aR,11aR]-pterocarpan (Cottiglia *et al.*, 2005) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------|---|--|
| | EF-12 (64) | 3,9-Dihydroxy-4-(3,3-dimethylallyl)[6aR, 11aR]-pterocarpan |
| 1 | 7.42 d (8.5) | 7.29 d (8.4) |
| 2 | 6.39 d (8.0) | 6.31 d (8.4) |
| 6 | 3.63 dd (11.0 and 5.0) | 3.45 t-like (11.2) |
| | 4.25 dd (11.0 and 5.0) | 4.10 dd (11.2 and 5.2) |
| 6a | 3.51 m | 3.02 m |
| 7 | 6.97 d (8.0) | 6.68 d (8.0) |
| 8 | 6.57 dd (8.0 and 2.0) | 6.21 dd (8.0 and 2.4) |
| 10 | 6.43 d (2.0) | 6.24 d (2.4) |
| 11a | 5.47 d (6.5) | 5.25 d (6.8) |
| 1' | 3.36 m | 3.58 d (8.0) |
| 2' | 5.27 d (4.5) | 5.41 t (8.0) |
| 4' | 1.81 s | 1.61 s |
| 5' | 1.75 s | 1.71 s |
| 3-OH | 4.79 s | 4.71 s |
| 9-OH | 4.80 s | 4.81 s |

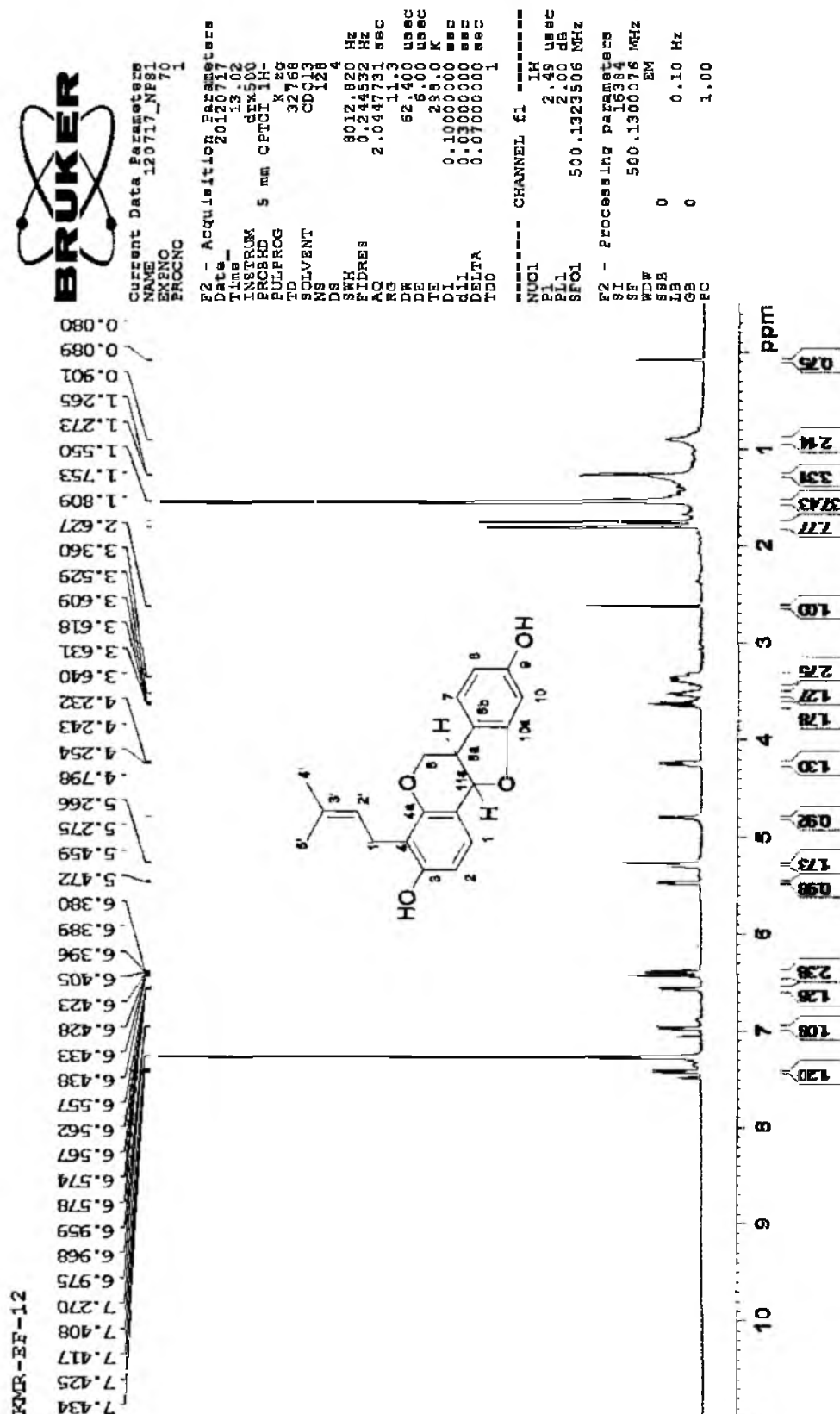


Figure 3.77: ¹H-NMR spectrum (500 MHz, CDCl₃) of EF-12 (64).

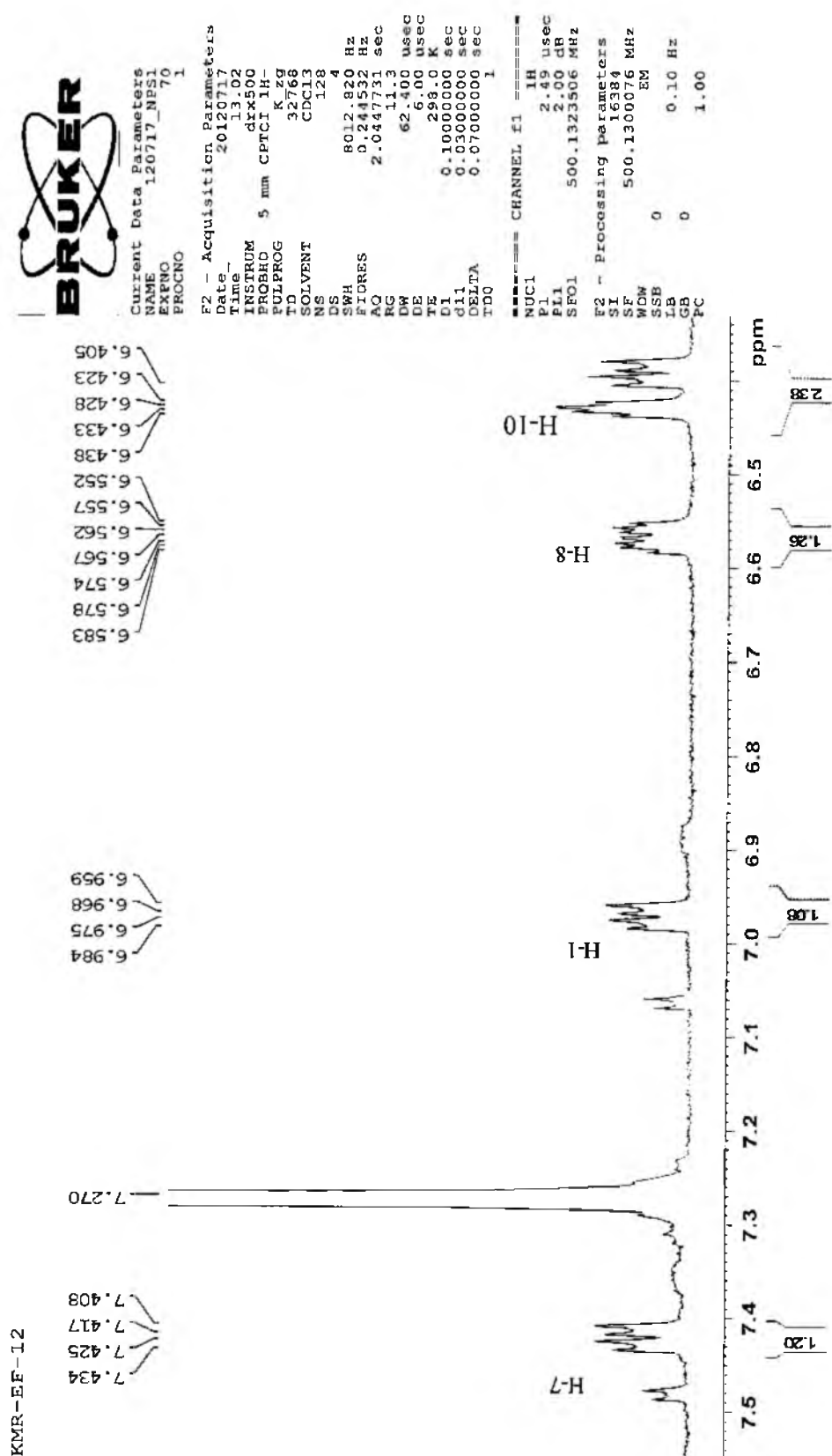


Figure 3.78: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of EF-12 (64).

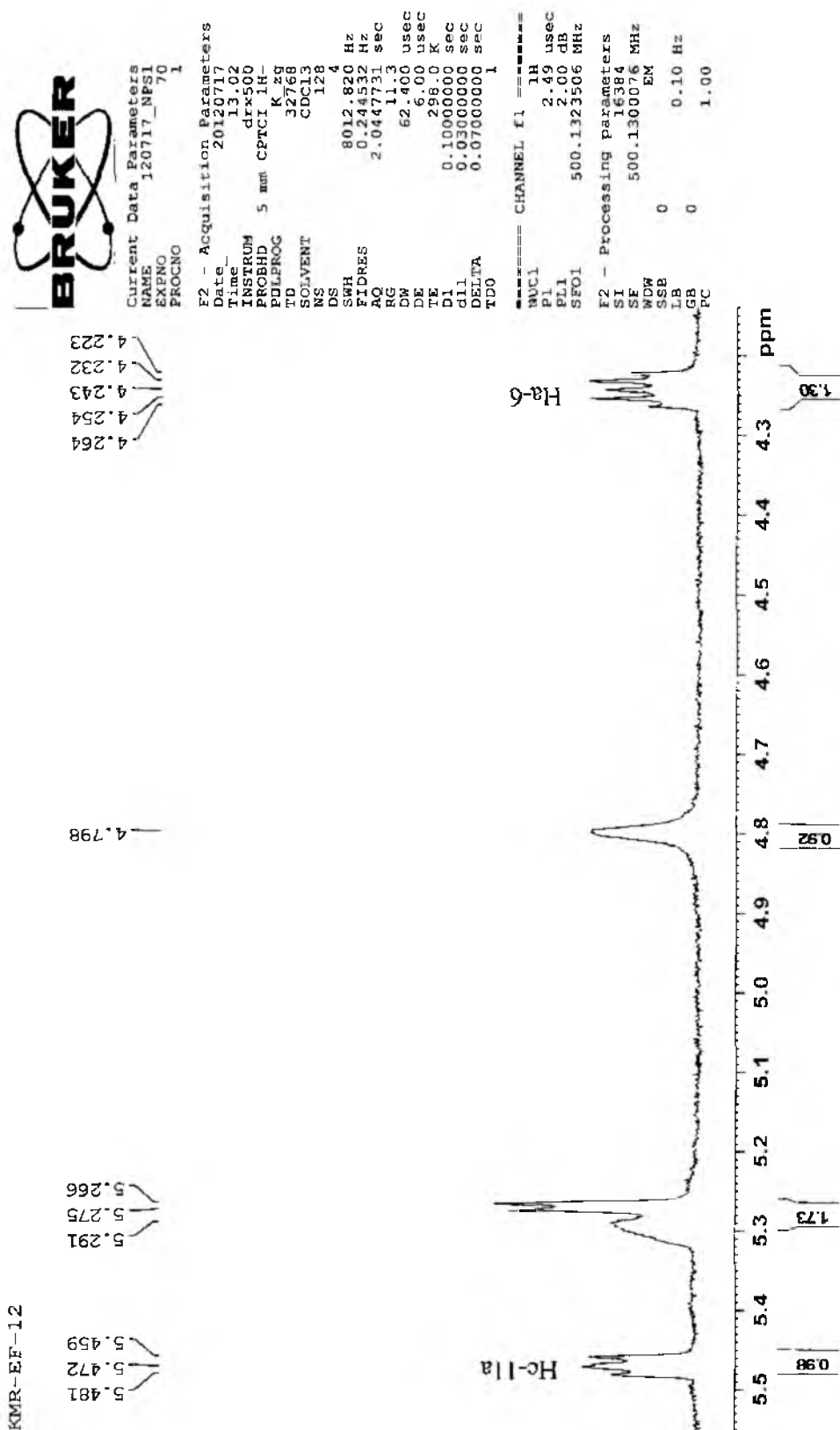


Figure 3.79: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of EF-12 (64).

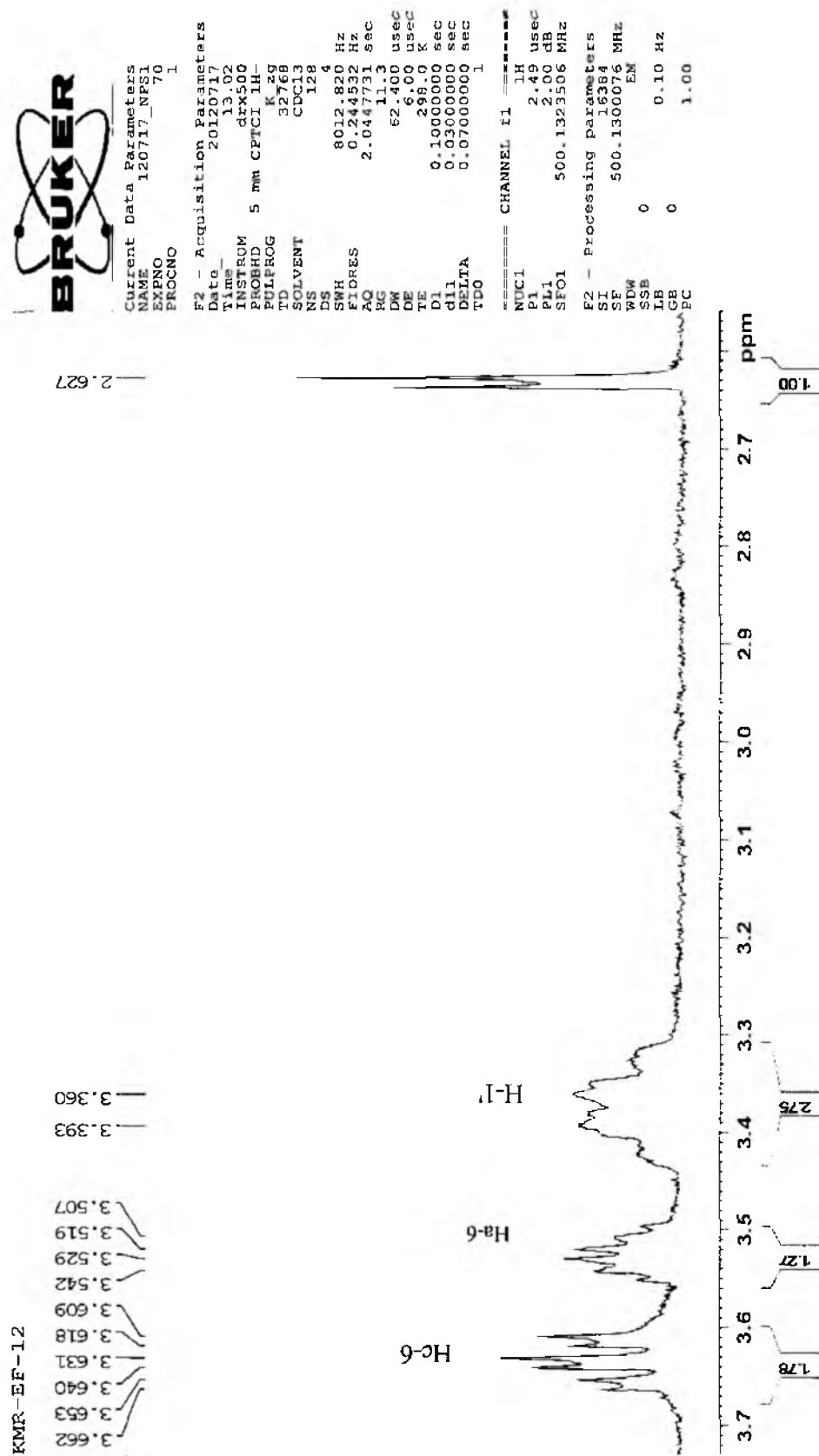


Figure 3.80: Partially expanded ^1H -NMR spectrum (500 MHz, CDCl_3) of EF-12 (64).

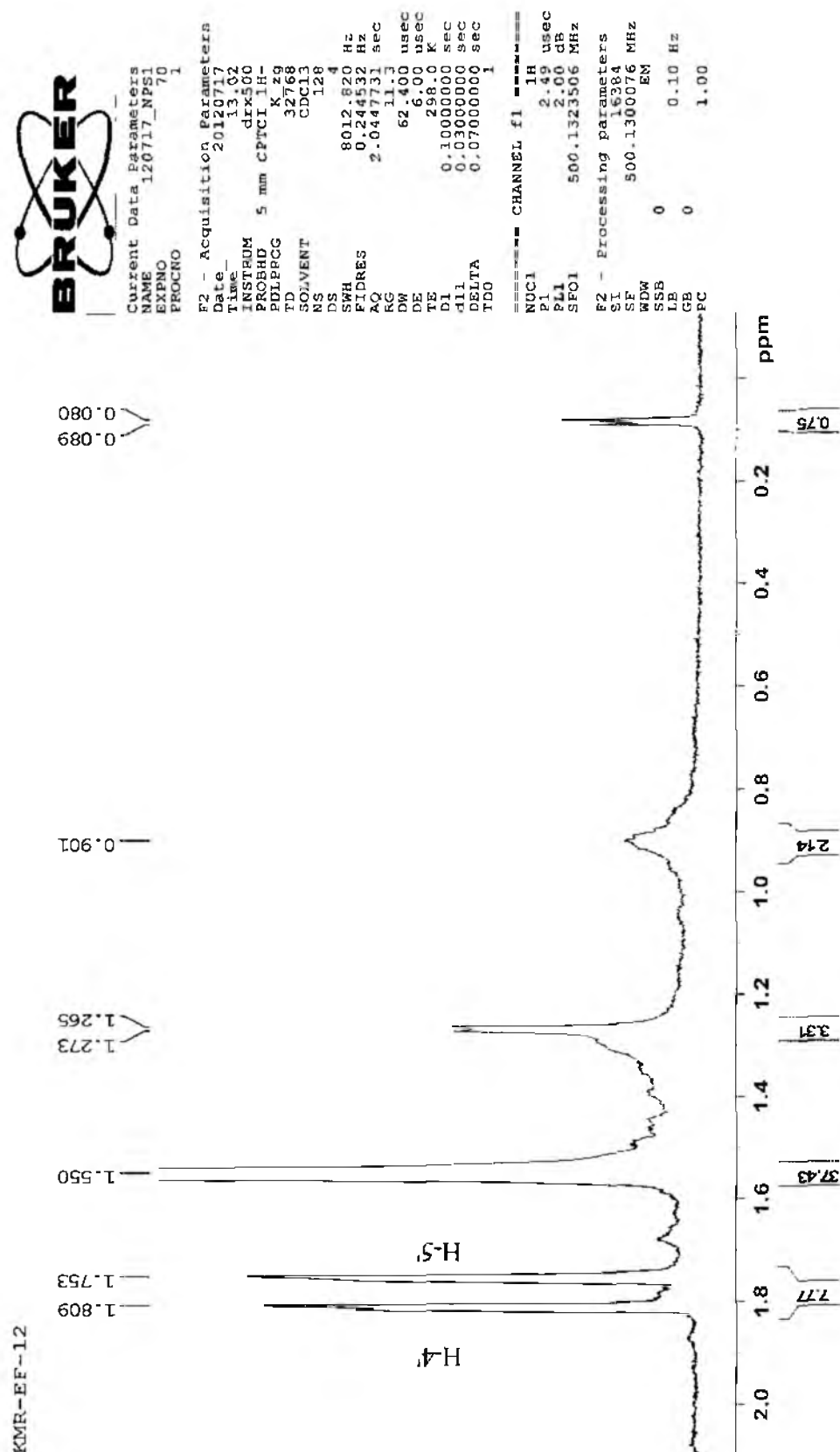


Figure 3.81: Partially expanded ¹H-NMR spectrum (500 MHz, CDCl₃) of EF-12 (64).

3.19 Characterization of EF-38 as β -sitosterol (65)

EF-38 was obtained as crystals by evaporation of solvent from the CC fractions 119-127 of the carbon tetrachloride soluble fraction of stem bark of *E. fusca*. The fraction was then subjected to a preparative TLC [stationary phase: silica gel F₂₅₄, mobile phase: toluene-ethyl acetate (85:15)]. A small portion of the developed plates was sprayed with vanillin-sulfuric acid reagent followed by heating in 110 °C for 5 minutes and a purple colored band was visualized. The stationary phase was scrapped off from the area having no spray reagent and eluted with ethyl acetate. Evaporation of the solvent yielded white needles which melted at 137 °C which was identical to that published for β -sitosterol (Morales *et al.*, 2003). The compound was analyzed over TLC to check for purity and found to be a pure compound which was termed as EF-38 (6.87 mg, 65). It was also found to be soluble in *n*-hexane, dichloromethane, ethyl acetate and acetone.

The ¹H-NMR spectrum of EF-38 (400 MHz, CDCl₃; Figure 3.83; Table 3.21) revealed a one proton multiplet at δ 3.58, the position and multiplicity of which was indicative of H-3 of the steroid nucleus. A triplet at δ 5.35 (J = 7.0 Hz) was evident for the typical olefinic proton at H-6 of the steroidal skeleton. The spectrum further revealed signals at δ 0.73 and δ 1.04 (3H each) assignable to two tertiary methyl groups at Me-18 and Me-19, respectively. The ¹H-NMR spectrum also showed two doublets centered at δ 0.82 (J = 7.0 Hz) and 0.80 (J = 6.9 Hz) which could be attributed to two methyl groups at Me-26 and Me-27, respectively. The doublet at δ 0.93 (J = 6.5 Hz) demonstrated a methyl group at Me-21. On the other hand, the triplet of three-proton intensity at δ 0.86 (J = 7.3) could be assigned to the primary methyl group for Me-29. The above spectral features (Table 3.21) are in close agreement to those observed for β -sitosterol (65) (Morales *et al.*, 2003). The identity of EF-38 was further confirmed by co-TLC with an authentic β -sitosterol previously isolated in our laboratory.

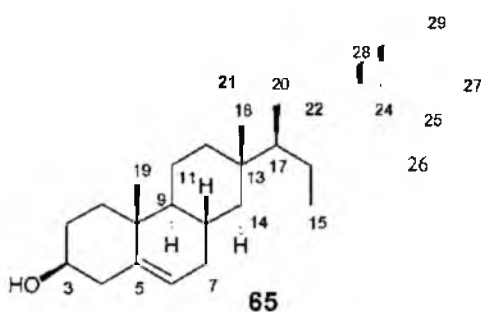


Figure 3.82: Structure of EF-38 (β -sitosterol, 65).

Table 3.21: ^1H -NMR spectral data of EF-38 (65) and β -sitosterol (Morales *et al.*, 2003) in CDCl_3 .

| Proton(s) | δ_{H} in ppm in CDCl_3 , (J in Hz). | |
|-----------|---|---------------------|
| | EF-38 (65) | β -sitosterol |
| H-3 | 3.58 m | 3.52 m |
| H-6 | 5.35 t (7.0) | 5.36 br d (5.3) |
| Me-18 | 0.73 s | 0.70 s |
| Me-19 | 1.04 s | 1.01 s |
| Me-21 | 0.93 d (6.5) | 0.92 d (6.6) |
| Me-26 | 0.82 d (7.0) | 0.84 d (7.0) |
| Me-27 | 0.80 d (6.90) | 0.81 d (7.0) |
| Me-29 | 0.86 t (7.3) | 0.85 t (7.0) |

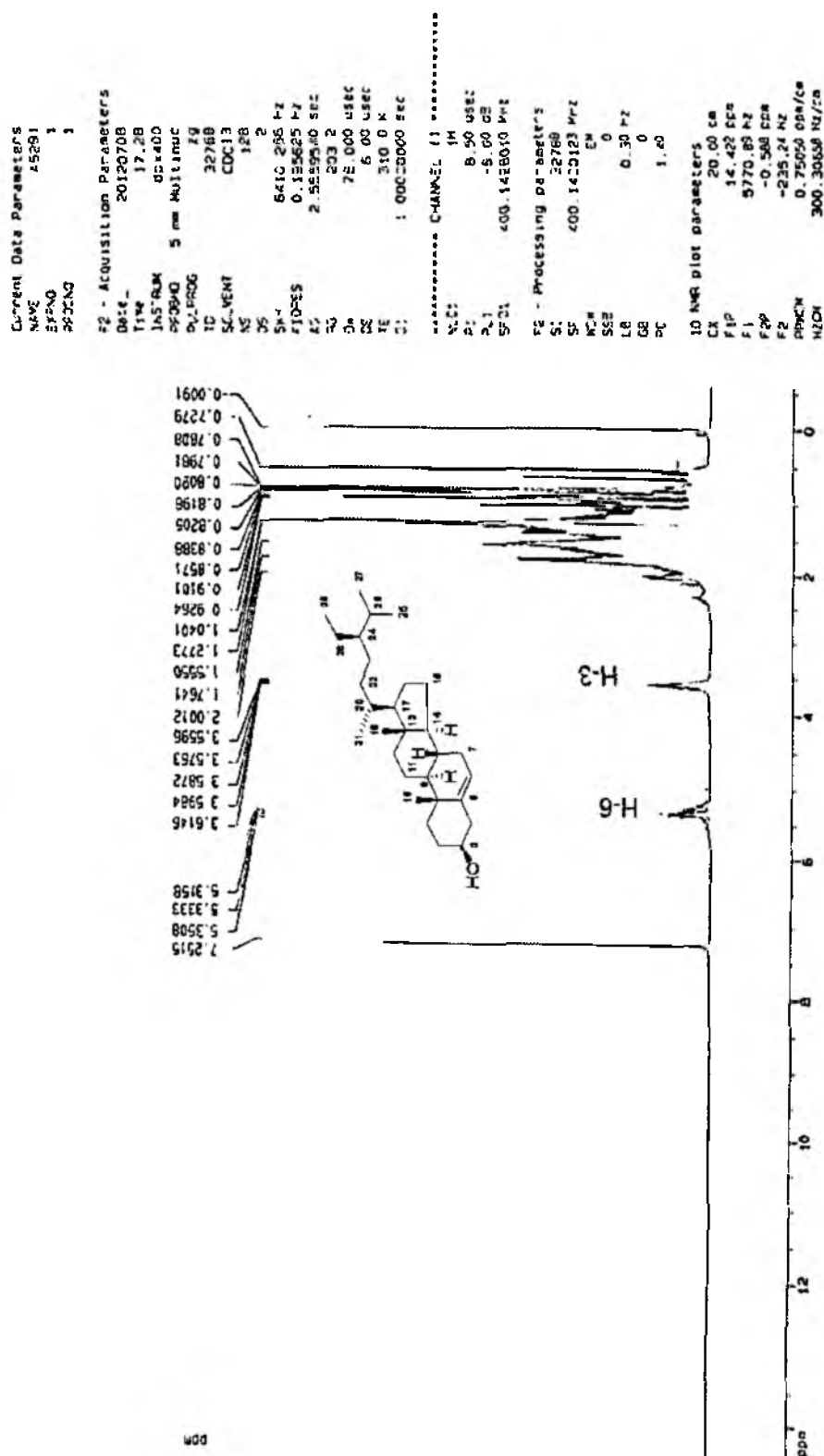
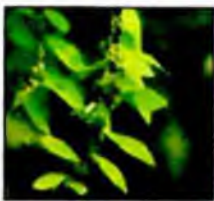


Figure 3.83: ¹H-NMR spectrum (400 MHz, CDCl₃) of EF-38 (65).



Chapter 4

Antimicrobial screening

4.1 Introduction

Bacteria and fungi are responsible for many infectious diseases. The increasing clinical implications of drug resistant bacterial and fungal pathogens have lent additional urgency to antimicrobial drug discovery. The research is performed for antimicrobial drugs to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* bacterial and fungal growth.

The antimicrobial screening of an agent is essential to ascertain its spectrum against various types of pathogenic micro organism. Antimicrobial activity of any plant can be detected by observing the growth response of various micro organism to the plant extract, which is placed in contact with them.

The susceptibility of micro organism to antimicrobial agents can be measured *in vitro* by a number of techniques like

- a. Disc Diffusion method
- b. Dilution method
- c. Bioautographic method

But there is no standardized method for expressing the results of antimicrobial screening (Ayafor *et al.*, 1982). Some investigations use the diameter of zone of inhibition and/or the minimum weight of extractive to inhibit (MIC) the growth of micro organism. However, a great number of factors viz; the extraction methods (Nadir *et al.*, 1986), inoculums volume, culture medium composition (Bauer *et al.*, 1966), pH (Levan *et al.*, 1979), incubation time and temperature (Barry, 1980) can significantly influence the results.

Among these the disc diffusion method (Bauer *et al.*, 1966) is a widely accepted *in vitro* investigation for preliminary screening of test agents, which may possess antimicrobial activity. However no, distinction between bacteriostatic and bactericidal activities can be made by this method (Ronald, 1982).

4.2 Principle of Disc Diffusion Method

Dried and sterile filter paper discs (4 - 6 mm diameter) containing the test compounds are placed on nutrient agar medium seeded with test micro organism. These plates are kept for twelve hours at low temperature (4 °C), to allow maximum diffusion. A number of events take place simultaneously which include:

- a. The dried discs absorb water from the agar medium and the test material is dissolved.
- b. The test material diffuses from the discs to the surrounding medium according to the physical law that controls the diffusion of molecules through agar gel (Barry, 1976).

- c. There is a gradual change of test materials concentration in the agar surrounding each disc.

The plates are then kept in an incubator (37 °C) for 12 - 18 hours to allow the growth of the micro organisms. A test material having any antibacterial activity will inhibit the growth of micro organism giving a clear, distinct zone, called "Zone of Inhibition". Antimicrobial activities of the test samples are expressed by measuring the diameter of zone of inhibition in terms of millimeters (mm). The larger the zone of inhibition, the greater the activity of the test material against the test organism.

The size of the inhibitory zones depends principally on the following factors:

- i. Intrinsic antimicrobial sensitivity of the test sample.
- ii. Growth rate of the test micro organism.
- iii. Diffusion rate of the drug which is related to its water solubility.
- iv. Concentration of the freshly seeded test organisms.
- v. Amount of test samples on the disc.
- vi. Thickness of the test medium in the petridishes.

4.3 Experimentals

In the present study crude extracts, fractions as well as some purified compounds were tested in the laboratory of Biomedical Research Center, Faculty of Pharmacy, University of Dhaka for antimicrobial activity by the disc diffusion method. However, several pure compounds could not be tested due to scarcity of samples.

4.3.1 Test organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food science (INFS), University of Dhaka. Both gram positive and gram negative organisms as listed in Table 4.1 were used for the test.

Table 4.1: Test micro organisms used for screening.

| Gram positive bacteria | Gram negative bacteria | Fungi |
|--|--|--|
| <i>Bacillus cereus</i> (BTCC-19)
<i>B. megaterium</i> (BTCC-18)
<i>B. subtilis</i> (BTCC-17)
<i>Sarcina lutea</i> (BTCC-484)
<i>Staphylococcus aureus</i> (BTCC-483) | <i>Escherichia coli</i> (BTCC-482)
<i>Pseudomonas aeruginosa</i> (BTCC-265)
<i>Salmonella typhi</i> (BTCC-197)
<i>S. paratyphi</i> (BTCC-198)
<i>Shigella boydii</i> (BTCC-498)
<i>Sh. dysenteriae</i> (BTCC-500)
<i>Vibrio mimicus</i> (BTCC-487)
<i>V. parahemolyticus</i> (BTCC-511) | <i>Candida albicans</i> (BTCC-493)
<i>Aspergillus niger</i> (BTCC-504)
<i>Sacharomyces cerevisiae</i> (BTCC-335) |

4.3.2 Culture medium

Nutrient agar medium (Table 4.2) was used for making plates on which antibacterial and antifungal sensitivity tests were carried out. The medium was also used to prepare fresh cultures.

Table 4.2: Composition of nutrient agar medium.

| Ingredients | Amount (gm/l) |
|----------------------|-------------------|
| Bacto peptone | 0.5 gm |
| Sodium chloride | 0.5 gm |
| Bacto yeast extracts | 1.0 gm |
| Bacto agar | 2.0 gm |
| Distilled water q.s. | 100 ml |
| pH | 7.2 ± 0.1 at 25°C |

4.3.3 Preparation of medium

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

4.3.4 Preparation of the test plates

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

4.3.5 Preparation of discs containing samples

Measured amount of each test sample (Table 4.3) was dissolved in specific volume of solvent (chloroform or methanol) to obtain the desired concentration in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs (6 mm diameter) were soaked with solutions of test samples and dried under the Laminar fume hood. Standard kanamycin discs (30 µgm/disc) were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antibacterial agent with that produced by the test

samples and griseofulvin (20 µgm/disc) was used as a standard for antifungal study. Blank discs were used as negative control to ensure that the residual solvents (left over even after air drying) and the filter paper were not active themselves.

4.3.6 Diffusion and incubation

The sample discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. The plates were then kept in a refrigerator at 4 °C for about 24 hours to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37 °C for 24 hours.

4.3.7 Determination of antibacterial activity by measuring the zone of inhibition

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

4.3.8 Statistical Analysis

Three replicates of each sample were used for statistical analysis and the values are reported as mean ± SD.

4.3.9 Test samples

Test samples and preparation of the sample discs of various extracts are listed in Table 4.3.

Table 4.3: List of test samples and preparation of sample discs for various extracts.

| Sample | Extract | Dose
($\mu\text{g}/\text{disc}$) | Amount for 20
discs (mg) |
|--|-------------|---------------------------------------|-----------------------------|
| <i>B. verrucosa</i>
(Stem bark and leaf) | Me | 400 | 8 |
| | HSF | 400 | 8 |
| | CSF | 400 | 8 |
| | CHSF | 400 | 8 |
| | AQSF | 400 | 8 |
| | BVSP-4 (48) | 50 | 1 |
| | BVBC-2 (51) | 50 | 1 |
| | BVS-65 (54) | 50 | 1 |
| <i>B. stipularis</i>
(Stem bark and leaf) | Me | 400 | 8 |
| | HSF | 400 | 8 |
| | CSF | 400 | 8 |
| | CHSF | 400 | 8 |
| | AQSF | 400 | 8 |
| | BS-01(55) | 50 | 1 |
| | BS-06 (56) | 50 | 1 |
| <i>B. tomentosa</i>
(Stem bark and leaf) | Me | 400 | 8 |
| | HSF | 400 | 8 |
| | CSF | 400 | 8 |
| | CHSF | 400 | 8 |
| | AQSF | 400 | 8 |
| | BT-1 (58) | 50 | 1 |
| | BT-6 (59) | 50 | 1 |
| <i>E. fusca</i>
(Stem bark and leaf) | Me | 400 | 8 |
| | HSF | 400 | 8 |
| | CSF | 400 | 8 |
| | CHSF | 400 | 8 |
| | AQSF | 400 | 8 |
| | EF-7 (62) | 50 | 1 |
| | EF-8 (63) | 50 | 1 |
| | EF-12 (64) | 50 | 1 |
| <i>E. variegata</i>
(Stem bark and leaf) | Me | 400 | 8 |
| | HSF | 400 | 8 |
| | CSF | 400 | 8 |
| | CHSF | 400 | 8 |
| | AQSF | 400 | 8 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

4.4 Results and discussion

4.4.1 Antimicrobial activity of the stem bark *B. verrucosa*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *B. verrucosa* showed low to moderate activity with zones of inhibition being between the ranges of 9 to 15 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *B. verrucosa* were ranged from 10-15, 9-14, 11-15, 10-14 and 10-14 mm, respectively (Table 4.3) at a concentration of 400 µg/disc. The carbon tetrachloride soluble materials of the methanol extract showed the highest inhibitory activity against *S. paratyphi* having the zone of inhibition 15 mm. The *n*-hexane soluble materials gave moderate zone size of 14.0, 13.9, 13.5 and 13.6 mm against *S. paratyphi*, *S. typhi*, *V. mimicus* and *V. parahemolyticus*, respectively. At the same time, the carbon tetrachloride partitionate of methanol extract showed mild to moderate activity having zone size of 14.2, 15.0 and 13.6 mm, respectively against *E. coli*, *S. typhi* and *S. dysenteriae*. Again, the chloroform soluble materials showed zone of inhibition of 13.8, 14.0, 14.3 13.5 and 14.1 mm against *B. cereus*, *S. aureus*, *S. lutea*, *V. mimicus* and *V. hemolyticus*, respectively indicating moderate activity. The aqueous soluble materials showed low activity against all the strains ranging from 10-15 mm. The antibacterial activity of test samples of the stem bark of *B. verrucosa* is shown in Table 4.4.

Antifungal screening of the partitionates gave the zone of inhibition was between 10-15 mm indicating mild to moderate activity in contrast to griseofulvin as standard. The carbon tetrachloride soluble materials gave highest activity against *C. albicans* with zone size of 14.2 mm.

Table 4.4: Antimicrobial screening of the stem bark of *B. verrucosa* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|-------------|-------------|-------------|-------------|------------|
| | Me | HSF | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 14.3 ± 1.53 | 9.9 ± 1.15 | 11.6 ± 1.52 | 13.8 ± 2.01 | 10.7 ± 1.37 | 35 |
| <i>B. megaterium</i> | 10.9 ± 1.81 | 12.0 ± 1.72 | 14.9 ± 1.15 | 12.4 ± 1.40 | 11.4 ± 1.36 | 35 |
| <i>B. subtilis</i> | 11.3 ± 2.08 | 9.6 ± 1.53 | 12.3 ± 2.52 | 10.6 ± 2.08 | 11.1 ± 1.60 | 36 |
| <i>S. aureus</i> | 12.3 ± 2.31 | 12.4 ± 1.70 | 11.6 ± 1.26 | 14.3 ± 1.53 | 12.6 ± 1.60 | 32 |
| <i>S. lutea</i> | 10.6 ± 2.52 | 11.7 ± 2.46 | 12.6 ± 2.08 | 14.0 ± 1.70 | 13.6 ± 2.52 | 27 |
| Gram negative bacteria | | | | | | |
| <i>Escherichia coli</i> | 13.7 ± 0.76 | 13.1 ± 2.43 | 14.2 ± 1.67 | 12.6 ± 0.80 | 12.8 ± 1.73 | 25 |
| <i>P. aeruginosa</i> | 14.7 ± 0.50 | 12.8 ± 1.29 | 13.1 ± 0.87 | 13.3 ± 0.78 | 12.9 ± 0.31 | 20 |
| <i>S. paratyphi</i> | 13.1 ± 0.76 | 14.0 ± 1.59 | 14.2 ± 1.67 | 12.6 ± 0.80 | 11.6 ± 0.36 | 27 |
| <i>S. typhi</i> | 13.4 ± 0.79 | 13.9 ± 0.97 | 15.0 ± 1.10 | 13.4 ± 1.12 | 11.3 ± 0.80 | 22 |
| <i>S. boydii</i> | 12.9 ± 1.22 | 13.1 ± 1.11 | 12.8 ± 0.76 | 14.5 ± 1.40 | 11.5 ± 0.35 | 27 |
| <i>S. dysenteriae</i> | 12.8 ± 2.02 | 13.3 ± 0.58 | 13.6 ± 1.26 | 11.1 ± 1.76 | 14.3 ± 0.80 | 25 |
| <i>V. mimicus</i> | 13.1 ± 1.04 | 13.5 ± 2.18 | 12.6 ± 1.15 | 13.5 ± 1.29 | 10.1 ± 0.76 | 25 |
| <i>V. parahemolyticus</i> | 14.8 ± 2.02 | 13.6 ± 1.53 | 12.9 ± 0.31 | 14.1 ± 1.53 | 11.1 ± 1.04 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 12.1 ± 0.76 | 13.1 ± 0.58 | 11.3 ± 0.76 | 12.3 ± 1.04 | 12.4 ± 0.97 | 20 |
| <i>C. albicans</i> | 12.2 ± 0.75 | 12.6 ± 1.53 | 14.2 ± 0.44 | 13.3 ± 2.08 | 13.1 ± 0.76 | 18 |
| <i>S. cerevisiae</i> | 13.6 ± 1.53 | 12.8 ± 1.31 | 10.6 ± 1.22 | 13.4 ± 0.85 | 13.1 ± 0.76 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.2 Antimicrobial activity of the leaf of *B. verrucosa*

On the other hand, the leaf of the methanol extract and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the plant *B. verrucosa* showed mild to moderate activity with zones of inhibition ranging from 12 to 16 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *B. verrucosa* were ranged from 12-16, 12-16, 12-15, 11-16 and 11-13 mm, respectively (Table 4.4) at a concentration of 400 µg/disc. The chloroform soluble materials of the methanol extract showed the highest inhibitory activity against *P. aeruginosa* having the zone of inhibition 15.6 mm. In case of the methanol extract the growth of *B. megaterium* (13.8 mm), *S. lutea* (14.6 mm), *P. aeruginosa* (14.1 mm), *S. paratyphi* (15.1 mm), *S. typhi* (14.1 mm), *S. boydii* (14.3 mm) and *V. parahemolyticus* (13.7 mm) was moderately inhibited. The *n*-hexane soluble materials also gave moderate zone size of 14.7, 14.8, 14.2, 15.1, 15.3, 15.0 and 14.3 mm against *B. cereus*, *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhi* and *S. dysenteriae*, respectively. Also, the carbon tetrachloride partitionate of methanol extract showed mild to moderate activity against *B. cereus*, *S. aureus*, *S. lutea*, *P. aeruginosa*, *S. paratyphi* and *V. mimicus* having zone of inhibition 13.7, 14.1, 14.5, 14.5 and 13.8 mm, respectively. Again, moderate activity was shown by the chloroform soluble materials with zone of inhibition of 14.2, 14.5, 15.6, 14.2 and 14.7 mm against *B. cereus*, *E. coli*, *P. aeruginosa*, *S. typhi* and *V. mimicus*, respectively. The aqueous soluble materials showed low activity against all the strains ranging from 11-13 mm. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 11 mm against *S. aureus* indicating low activity. Table 4.5 shows the antibacterial activity of test samples of the leaf of *B. verrucosa*.

The zone of inhibition was between 11-16 mm indicating moderate to strong activity in contrast to griseofulvin as standard. In case of antifungal screening. The *n*-hexane soluble materials gave highest activity against *C. albicans* with zone size of 15.4 mm.

Table 4.5: Antimicrobial screening of the leaf of *B. verrucosa* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|-------------|-------------|-------------|-------------|------------|
| | Me | HSF | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 12.1 ± 0.76 | 14.7 ± 1.12 | 13.7 ± 0.22 | 14.2 ± 0.85 | 11.7 ± 0.93 | 35 |
| <i>B. megaterium</i> | 13.8 ± 1.42 | 12.7 ± 1.40 | 12.6 ± 0.91 | 11.7 ± 0.67 | 12.5 ± 0.98 | 35 |
| <i>B. subtilis</i> | 13.2 ± 0.87 | 14.8 ± 1.21 | 12.9 ± 1.16 | 13.5 ± 1.33 | 12.4 ± 1.16 | 36 |
| <i>S. lutea</i> | 14.6 ± 1.11 | 12.9 ± 1.25 | 14.1 ± 1.61 | 13.9 ± 1.61 | 12.7 ± 0.36 | 32 |
| <i>S. aureus</i> | 12.9 ± 1.93 | 14.2 ± 1.23 | 13.6 ± 0.75 | 12.4 ± 0.83 | 11.0 ± 0.70 | 27 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 12.6 ± 1.16 | 15.1 ± 0.45 | 13.0 ± 1.68 | 14.5 ± 1.20 | 11.2 ± 0.60 | 25 |
| <i>P. aeruginosa</i> | 14.1 ± 0.60 | 15.3 ± 0.61 | 14.5 ± 0.15 | 15.6 ± 0.50 | 12.2 ± 0.70 | 20 |
| <i>S. paratyphi</i> | 15.1 ± 1.49 | 14.0 ± 0.61 | 14.5 ± 0.68 | 13.1 ± 0.70 | 12.7 ± 0.95 | 27 |
| <i>S. typhi</i> | 14.1 ± 0.87 | 15.0 ± 1.44 | 13.2 ± 1.00 | 14.2 ± 0.45 | 11.5 ± 0.96 | 22 |
| <i>S. boydii</i> | 14.3 ± 0.95 | 14.0 ± 0.70 | 13.6 ± 1.11 | 12.3 ± 0.68 | 11.3 ± 0.70 | 27 |
| <i>S. dysenteriae</i> | 13.4 ± 0.75 | 14.3 ± 1.00 | 13.5 ± 0.79 | 13.9 ± 0.36 | 11.3 ± 0.72 | 25 |
| <i>V. mimicus</i> | 13.5 ± 0.81 | 13.4 ± 0.62 | 13.8 ± 0.38 | 14.7 ± 0.40 | 11.6 ± 0.71 | 25 |
| <i>V. parahemolyticus</i> | 13.7 ± 0.70 | 13.5 ± 0.81 | 13.4 ± 0.78 | 13.5 ± 0.60 | 11.8 ± 0.40 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 13.3 ± 0.56 | 13.7 ± 0.91 | 13.9 ± 0.85 | 14.2 ± 0.61 | 12.5 ± 0.60 | 20 |
| <i>C. albicans</i> | 14.3 ± 0.50 | 15.3 ± 0.51 | 11.2 ± 1.46 | 13.5 ± 0.79 | 11.8 ± 0.85 | 18 |
| <i>S. cerevisiae</i> | 13.6 ± 0.46 | 15.3 ± 0.61 | 13.2 ± 0.56 | 14.2 ± 0.56 | 11.8 ± 0.40 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.3 Antimicrobial activity of the stem bark of *B. stipularis*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *B. stipularis* showed moderate to strong activity against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs with zones of inhibition between the ranges of 11.5 to 23 mm. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *B. stipularis* were ranged from 14-23, 12-19, 13-21, 13-23 and 11-18 mm, respectively (Table 4.5) at a concentration of 400 µg/disc. The methanol extract showed the highest inhibitory activity against *S. aureus* having the zone of inhibition 23 mm. In case of the methanol extract the growth of *B. subtilis* (21.1 mm), *S. aureus* (23 mm), *S. paratyphi* (21.9 mm), *S. typhi* (20.7 mm), *S. boydii* (20.3 mm) and *V. mimicus* (20.3 mm) was strongly inhibited. The *n*-hexane soluble materials gave moderate zone size of 18.7, 18.9, 18.8 and 18.7 mm against *B. megatrium*, *S. paratyphi*, *S. typhi* and *S. boydii*, respectively. At the same time, the carbon tetrachloride partitionate of methanol extract showed strong activity against *B. megatrium* and *E. coli* with zone of inhibition of 21 and 20.8 mm, respectively. It also gave moderate activity against *S. lutea*, *S. paratyphi* and *S. typhi* with zone of inhibition 18.7, 18.1 and 18.8 mm, respectively. Again, the chloroform soluble materials showed zone of inhibition of 20.4, 19.7, 20 and 22.3 mm against *S. aureus*, *S. paratyphi*, *S. dysenteriae* and *V. mimicus*, respectively indicating moderate activity. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 11-18 mm. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 11.9 mm against *V. parahemolyticus* indicating lowest activity. The antibacterial activity of test samples of the stem bark of *B. stipularis* is shown in Table 4.6.

Antifungal screening In case of various extracts of the stem bark of *B. stipularis*, the zone of inhibition was between 9-16 mm indicating mild to strong activity in contrast to griseofulvin as standard. The chloroform soluble materials gave highest activity against *A. niger* with zone size of 15.1 mm.

Table 4.6: Antimicrobial screening of the stem bark of *B. stipularis* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|-------------|-------------|-------------|-------------|------------|
| | Me | HSF | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 18.5 ± 0.82 | 14.8 ± 0.95 | 13.8 ± 0.46 | 16.7 ± 1.15 | 17.2 ± 1.32 | 35 |
| <i>B. megaterium</i> | 17.5 ± 1.32 | 18.7 ± 0.85 | 21.0 ± 0.66 | 17.1 ± 1.36 | 17.4 ± 0.59 | 35 |
| <i>B. subtilis</i> | 21.1 ± 2.05 | 15.7 ± 0.35 | 17.7 ± 1.11 | 19.4 ± 0.50 | 16.9 ± 0.42 | 36 |
| <i>S. lutea</i> | 21.0 ± 1.68 | 17.9 ± 0.74 | 18.7 ± 0.76 | 17.6 ± 0.32 | 16.8 ± 0.42 | 27 |
| <i>S. aureus</i> | 23.0 ± 1.68 | 17.3 ± 0.45 | 15.6 ± 0.76 | 20.4 ± 0.85 | 17.5 ± 1.06 | 32 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 19.1 ± 0.63 | 17.7 ± 0.25 | 20.8 ± 0.60 | 18.5 ± 0.77 | 17.6 ± 0.40 | 25 |
| <i>P. aeruginosa</i> | 14.7 ± 0.50 | 12.8 ± 1.29 | 13.1 ± 0.87 | 13.3 ± 0.78 | 13.0 ± 0.31 | 20 |
| <i>S. paratyphi</i> | 21.9 ± 0.86 | 18.9 ± 0.42 | 18.1 ± 0.67 | 19.7 ± 0.74 | 17.6 ± 1.26 | 27 |
| <i>S. typhi</i> | 20.7 ± 2.09 | 18.8 ± 0.17 | 18.8 ± 0.46 | 16.3 ± 1.40 | 17.3 ± 0.74 | 22 |
| <i>S. boydii</i> | 20.3 ± 1.29 | 18.7 ± 0.21 | 17.4 ± 0.42 | 18.7 ± 0.72 | 16.6 ± 0.46 | 27 |
| <i>S. dysenteriae</i> | 20.1 ± 1.12 | 17.5 ± 0.12 | 15.6 ± 0.47 | 20.0 ± 0.81 | 16.9 ± 0.55 | 25 |
| <i>V. mimicus</i> | 20.3 ± 0.91 | 17.9 ± 0.55 | 17.5 ± 0.51 | 22.3 ± 0.55 | 14.9 ± 1.11 | 25 |
| <i>V. parahemolyticus</i> | 17.4 ± 1.17 | 16.4 ± 0.31 | 16.9 ± 0.38 | 17.8 ± 0.42 | 11.9 ± 0.78 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 13.6 ± 2.07 | 11.9 ± 0.40 | 16.4 ± 0.64 | 15.1 ± 1.02 | 9.4 ± 0.46 | 20 |
| <i>C. albicans</i> | 14.7 ± 0.40 | 13.0 ± 0.42 | 12.4 ± 1.45 | 11.1 ± 0.90 | 13.4 ± 0.66 | 18 |
| <i>S. cerevisiae</i> | 11.7 ± 1.45 | 14.1 ± 1.02 | 12.3 ± 1.62 | 12.7 ± 1.37 | 10.9 ± 0.70 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.4 Antimicrobial activity of the leaf of *B. stipularis*

In comparison to standard Kanamycin discs antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the leaf of *B. stipularis* also showed moderate to strong activity against various Gram positive and Gram negative bacterial strains with zones of inhibition between the ranges of 12 to 22 mm. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *B. stipularis* were ranged from 14-22, 15-22, 14-20, 15-22 and 12-18 mm, respectively (Table 4.6) at a concentration of 400 µg/disc. The methanol extract showed the strongest inhibitory activity against *S. lutea* having the zone of inhibition 21.6 mm and the growth of *B. subtilis* (20.5 mm), *S. lutea* (21.6 mm) and *E. coli* (20 mm) was strongly inhibited. Again, the growth of *S. paratyphi*, *S. boydii* and *V. mimicus* was moderately inhibited with a zone of inhibition of 19.4, 19.4 and 19.6 mm, respectively. The *n*-hexane soluble materials gave strong zone size of 21.2 mm against *S. aureus* and 20 mm against *S. dysenteriae*. The *n*-hexane soluble extract also showed moderate activity with zone size of 19.7, 19.1 and 18.7 mm against *B. subtilis*, *S. paratyphi* and *V. parahemolyticus*, respectively. Simultaneously, the carbon tetrachloride partitionate of the methanol extract showed moderate activity against *S. aureus*, *S. lutea*, *S. typhi* and *V. mimicus* with zone of inhibition 19.4, 19.7, 19.1 and 19.8 mm, respectively. Again, the chloroform soluble materials showed zone of inhibition of 21.3 mm against *B. subtilis* indicating strong activity but it also gave zone size of 18.5, 18.1, 19.4 and 18 mm against *S. aureus*, *E. coli*, *S. paratyphi* and *S. dysenteriae*, respectively indicating moderate activity. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 12-18 mm. The zone of inhibition of *B. subtilis*, *S. aureus* and *E. coli* was 17.9, 17.0 and 17.7 mm, respectively indicating mild activity. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 12.2 mm against *P. aeruginosa* indicating lowest activity. For test samples of the leaf of *B. stipularis* the antibacterial activity is shown in Table 4.7.

In case of antifungal screening, the zone of inhibition was between 10.5-15.5 mm indicating moderate to strong activity in contrast to griseofulvin as standard. The *n*-hexane soluble materials gave highest activity against *C. albicans* with zone size of 15.5 mm.

Table 4.7: Antimicrobial screening of the leaf of *B. stipularis* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|-------------|-------------|-------------|-------------|------------|
| | Me | HSF | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 15.6 ± 0.89 | 18.2 ± 0.50 | 15.6 ± 0.61 | 16.7 ± 0.35 | 14.9 ± 0.78 | 35 |
| <i>B. megaterium</i> | 15.2 ± 0.70 | 17.5 ± 0.87 | 16.1 ± 0.87 | 15.6 ± 0.25 | 15.3 ± 0.76 | 35 |
| <i>B. subtilis</i> | 20.5 ± 1.12 | 19.7 ± 0.85 | 17.6 ± 0.61 | 21.3 ± 1.91 | 17.9 ± 0.65 | 36 |
| <i>S. lutea</i> | 21.6 ± 0.68 | 18.1 ± 0.67 | 19.7 ± 0.57 | 17.5 ± 0.85 | 15.3 ± 0.40 | 27 |
| <i>S. aureus</i> | 19.1 ± 0.80 | 21.2 ± 1.50 | 19.4 ± 0.75 | 18.5 ± 0.80 | 17.0 ± 0.74 | 32 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 20.0 ± 1.52 | 17.8 ± 0.61 | 17.2 ± 0.47 | 18.1 ± 0.38 | 17.7 ± 0.55 | 25 |
| <i>P. aeruginosa</i> | 14.1 ± 0.60 | 15.3 ± 0.61 | 14.5 ± 0.15 | 15.6 ± 0.50 | 12.2 ± 0.70 | 20 |
| <i>S. paratyphi</i> | 19.4 ± 0.35 | 19.1 ± 0.73 | 17.9 ± 1.15 | 19.4 ± 0.70 | 15.9 ± 0.40 | 27 |
| <i>S. typhi</i> | 19.1 ± 0.38 | 17.6 ± 1.25 | 19.1 ± 0.29 | 16.4 ± 1.03 | 15.7 ± 0.56 | 22 |
| <i>S. boydii</i> | 19.4 ± 1.03 | 17.8 ± 0.23 | 18.8 ± 0.31 | 17.6 ± 0.27 | 15.8 ± 0.95 | 27 |
| <i>S. dysenteriae</i> | 19.3 ± 0.87 | 20.0 ± 0.21 | 18.3 ± 0.35 | 18.0 ± 0.61 | 15.9 ± 0.15 | 25 |
| <i>V. mimicus</i> | 19.6 ± 0.78 | 16.2 ± 1.80 | 19.8 ± 2.15 | 15.6 ± 0.70 | 14.8 ± 0.81 | 25 |
| <i>V. parahemolyticus</i> | 17.4 ± 0.42 | 18.7 ± 0.86 | 18.4 ± 1.03 | 16.5 ± 0.49 | 15.3 ± 1.01 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 11.3 ± 0.49 | 11.8 ± 0.40 | 15.6 ± 0.63 | 11.6 ± 1.02 | 11.9 ± 0.78 | 20 |
| <i>C. albicans</i> | 15.4 ± 0.42 | 15.5 ± 0.65 | 11.2 ± 1.46 | 10.5 ± 0.79 | 11.8 ± 0.85 | 18 |
| <i>S. cerevisiae</i> | 14.0 ± 0.61 | 15.4 ± 0.67 | 13.1 ± 0.65 | 14.4 ± 0.46 | 13.1 ± 0.71 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.5 Antimicrobial activity of the stem bark of *B. tomentosa*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *B. tomentosa* also showed moderate to strong activity against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs with zones of inhibition between the ranges of 14 to 23.5 mm. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *B. tomentosa* were ranged from 19-24, 15-20, 15-21, 17-21 and 14-17 mm, respectively (Table 4.7) at a concentration of 400 µg/disc. The methanol extract showed the strongest inhibitory activity against *B. cereus* having the zone of inhibition 23.2 mm and the growth of *B. subtilis* (20.7 mm), *S. aureus* (21 mm), *S. lutea* (21.4 mm), *E. coli* (22.4 mm), *S. paratyphi* (21.1 mm), *S. typhi* (22.3 mm), *S. boydii* (21.3 mm), *S. dysenteriae* (20.9 mm), *V. mimicus* (21.2 mm) and *V. parahemolyticus* (21.3 mm) was strongly inhibited. The *n*-hexane soluble materials gave moderate zone size of 18.1 mm against *S. aureus* and *S. lutea*. The *n*-hexane soluble extract also showed moderate activity with zone size of 18.8 mm against *S. paratyphi*, 19.9 mm against *V. miniscus* and 18.7 mm against *V. parahemolyticus*. At the same time, the carbon tetrachloride partitionate of the methanol extract showed strong activity against *S. typhi* with zone size of 20.5 mm. It also gave moderate activity against *S. aureus*, *E. coli* and *V. mimicus* with zone of inhibition 19.6, 18.9 and 18.2 mm, respectively. Again, the chloroform soluble materials showed zone of inhibition of 20.3 mm against *S. paratyphi* and 20.1 mm against *S. boydii* indicating very strong activity but it also gave zone size of 19, 19.4, 18.9 and 19.5 mm against *B. cereus*, *B. subtilis*, *S. boydii* and *S. dysenteriae*, respectively indicating strong activity. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 12-17 mm. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 12.7 mm against *P. aeruginosa* indicating lowest activity. The antibacterial activity of test samples of the stem bark of *B. tomentosa* is shown in Table 4.8.

For antifungal screening, the zone of inhibition was between 10-16 mm indicating moderate to strong activity in contrast to griseofulvin as standard. The chloroform soluble materials gave highest activity against *S. cerevisiae* with zone size of 15.9 mm.

Table 4.8: Antimicrobial screening of the stem bark of *B. tomentosa* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|-------------|-------------|-------------|-------------|------------|
| | Me | HSF | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 23.2 ± 1.47 | 17.8 ± 0.66 | 16.9 ± 1.10 | 19.0 ± 0.31 | 15.0 ± 0.49 | 35 |
| <i>B. megaterium</i> | 19.1 ± 0.60 | 17.5 ± 0.40 | 16.4 ± 0.50 | 17.3 ± 0.47 | 14.4 ± 0.74 | 35 |
| <i>B. subtilis</i> | 20.7 ± 0.55 | 16.8 ± 0.50 | 18.6 ± 0.61 | 19.4 ± 0.31 | 14.9 ± 0.17 | 36 |
| <i>S. lutea</i> | 21.4 ± 0.50 | 18.1 ± 0.57 | 16.8 ± 0.61 | 18.9 ± 0.78 | 14.8 ± 0.31 | 27 |
| <i>S. aureus</i> | 21.0 ± 0.95 | 18.1 ± 0.35 | 19.6 ± 0.59 | 17.6 ± 0.42 | 15.8 ± 0.46 | 32 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 22.4 ± 0.35 | 17.9 ± 0.21 | 18.9 ± 0.57 | 17.8 ± 0.27 | 15.2 ± 0.51 | 25 |
| <i>P. aeruginosa</i> | 16.6 ± 0.21 | 14.8 ± 0.50 | 13.8 ± 1.01 | 13.8 ± 0.65 | 12.7 ± 0.85 | 20 |
| <i>S. paratyphi</i> | 21.2 ± 0.82 | 18.8 ± 0.50 | 17.5 ± 0.32 | 20.3 ± 0.53 | 15.2 ± 0.51 | 27 |
| <i>S. typhi</i> | 22.3 ± 0.75 | 17.6 ± 0.31 | 20.5 ± 0.38 | 17.3 ± 0.60 | 15.0 ± 0.31 | 22 |
| <i>S. boydii</i> | 21.3 ± 1.40 | 17.7 ± 0.66 | 16.9 ± 0.55 | 20.1 ± 0.45 | 15.4 ± 0.31 | 27 |
| <i>S. dysenteriae</i> | 20.9 ± 0.72 | 17.7 ± 0.56 | 15.9 ± 0.59 | 19.5 ± 1.05 | 16.8 ± 0.36 | 25 |
| <i>V. mimicus</i> | 21.2 ± 1.10 | 19.9 ± 0.47 | 18.2 ± 0.50 | 17.3 ± 0.40 | 14.4 ± 0.50 | 25 |
| <i>V. parahemolyticus</i> | 21.3 ± 0.59 | 18.7 ± 0.55 | 17.1 ± 0.67 | 18.1 ± 0.68 | 13.8 ± 1.29 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 13.6 ± 0.31 | 11.4 ± 0.83 | 10.2 ± 0.42 | 10.8 ± 0.50 | 13.2 ± 0.31 | 20 |
| <i>C. albicans</i> | 13.4 ± 0.91 | 14.2 ± 0.61 | 15.6 ± 0.32 | 13.6 ± 1.14 | 10.2 ± 0.40 | 18 |
| <i>S. cerevisiae</i> | 11.4 ± 0.66 | 14.6 ± 0.31 | 12.6 ± 1.10 | 15.9 ± 0.17 | 12.9 ± 0.72 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.6 Antimicrobial activity of the leaf of *B. tomentosa*

Again, antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the leaf of *B. tomentosa* also showed mild to strong activity against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs with zones of inhibition between the ranges of 13 to 23 mm. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *B. tomentosa* were ranged from 16-23, 14-20, 13-21, 14-20 and 13-17 mm, respectively (Table 4.8) at a concentration of 400 µg/disc. The methanol extract showed the strongest inhibitory activity against *V. parahemolyticus* having the zone of inhibition 22.1 mm and the growth of *B. megaterium* (21.6 mm), *B. subtilis* (20 mm), *E. coli* (20 mm), *S. paratyphi* (20.3 mm), *S. typhi* (20.9 mm), *V. mimicus* (21.3 mm) and *V. parahemolyticus* (22.1 mm) was strongly inhibited. The *n*-hexane soluble materials gave moderate zone size of 18.5, 18.8, 19.6, 18.1 and 18.3 mm against *B. megaterium*, *B. subtilis*, *S. aureus*, *S. paratyphi* and *S. typhi*, respectively. The carbon tetrachloride partitionate of the methanol extract showed strong activity against *B. megaterium* with zone size of 20.1 mm. It also gave moderate activity against *B. cereus*, *B. subtilis*, *S. aureus*, *S. lutea*, *S. boydii* and *S. dysenteriae* with zone of inhibition 19.1, 18.3, 18.8, 18.1, 18.8 and 18.1 mm, respectively. Again, the chloroform soluble materials gave zone size of 18.1, 19.1, 18.3 and 18.1 mm against *B. subtilis*, *E. coli*, *V. mimicus* and *V. parahemolyticus*, respectively indicating moderate activity. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 13-17 mm. The zone of inhibition of *B. megaterium*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. paratyphi* and *S. dysenteriae* was 15.6, 16.7, 16.3, 15.6, 15.6 and 15.7 mm, respectively indicating mild activity. Further of all the samples, the aqueous soluble materials showed lowest zone of inhibition of 13.2 mm against *B. subtilis* indicating lowest activity. Table 4.9 shows the antibacterial activity of test samples of the leaf of *B. tomentosa*.

Using griseofulvin as standard the zone of inhibition was between 10-18 mm indicating moderate to strong activity in case of antifungal screening. The methanol extract gave highest activity against *C. albicans* with zone size of 17.5 mm.

Table 4.9: Antimicrobial screening of the leaf of *B. tomentosa* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|-------------|-------------|-------------|-------------|------------|
| | Me | HSF | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 18.0 ± 1.71 | 16.9 ± 0.75 | 19.1 ± 0.15 | 17.8 ± 0.56 | 15.1 ± 0.70 | 35 |
| <i>B. megaterium</i> | 21.6 ± 0.97 | 18.5 ± 0.87 | 20.1 ± 0.25 | 17.4 ± 0.91 | 15.6 ± 0.57 | 35 |
| <i>B. subtilis</i> | 22.0 ± 0.70 | 18.8 ± 0.35 | 18.3 ± 0.67 | 18.1 ± 0.68 | 13.2 ± 0.42 | 36 |
| <i>S. lutea</i> | 19.4 ± 0.35 | 17.6 ± 0.27 | 18.1 ± 0.40 | 16.0 ± 0.42 | 14.6 ± 0.50 | 27 |
| <i>S. aureus</i> | 19.9 ± 1.12 | 19.6 ± 0.50 | 18.8 ± 0.56 | 16.9 ± 0.68 | 16.7 ± 0.60 | 32 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 20.9 ± 0.71 | 17.4 ± 0.42 | 16.6 ± 0.31 | 19.1 ± 0.25 | 16.3 ± 0.55 | 25 |
| <i>P. aeruginosa</i> | 15.9 ± 0.40 | 14.0 ± 0.60 | 13.5 ± 0.15 | 14.8 ± 0.82 | 15.6 ± 0.63 | 20 |
| <i>S. paratyphi</i> | 20.3 ± 0.96 | 18.1 ± 0.72 | 17.8 ± 0.78 | 17.0 ± 0.56 | 15.6 ± 0.31 | 27 |
| <i>S. typhi</i> | 20.9 ± 0.70 | 18.3 ± 0.85 | 16.9 ± 0.75 | 16.8 ± 0.42 | 14.8 ± 0.50 | 22 |
| <i>S. boydii</i> | 19.8 ± 0.42 | 16.8 ± 0.50 | 18.8 ± 0.42 | 16.8 ± 0.42 | 15.3 ± 0.45 | 27 |
| <i>S. dysenteriae</i> | 20.8 ± 0.76 | 17.5 ± 1.04 | 18.1 ± 0.92 | 17.3 ± 0.59 | 15.7 ± 0.32 | 25 |
| <i>V. mimicus</i> | 21.3 ± 0.72 | 17.2 ± 0.31 | 16.5 ± 0.32 | 18.3 ± 0.78 | 14.4 ± 0.23 | 25 |
| <i>V. parahemolyticus</i> | 22.1 ± 0.72 | 17.9 ± 0.45 | 16.9 ± 0.15 | 18.1 ± 0.55 | 14.2 ± 0.70 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 13.9 ± 0.30 | 11.9 ± 0.32 | 15.8 ± 0.31 | 14.7 ± 0.60 | 13.8 ± 0.71 | 20 |
| <i>C. albicans</i> | 17.5 ± 0.17 | 15.1 ± 0.35 | 11.5 ± 1.60 | 12.2 ± 0.87 | 10.7 ± 1.65 | 18 |
| <i>S. cerevisiae</i> | 13.1 ± 0.72 | 14.2 ± 0.60 | 11.8 ± 0.56 | 13.5 ± 0.38 | 12.8 ± 0.46 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.7 Antimicrobial activity of the stem bark *E. fusca*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *E. fusca* showed low to strong activity with zones of inhibition being between the ranges of 12-19 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *E. fusca* were ranged from 14-20, 13-19, 13-18, 12-20 and 13-19 mm, respectively (Table 4.9) at a concentration of 400 µg/disc. The chloroform soluble fractions of the methanol extract showed the highest inhibitory activity against *B. cereus* having the zone of inhibition 19.3 mm. In case of the methanol extract the growth of *B. megaterium* (18.1 mm), *B. subtilis* (18.4 mm), *S. aureus* (19.1 mm), *S. lutea* (18.3 mm), *S. paratyphi* (19.2 mm) and *V. mimicus* (14.8 mm) was strongly inhibited. The *n*-hexane soluble materials gave strong zone size of 18.6, 18.3 and 18.1 mm against *B. megaterium*, *B. subtilis* and *S. dysenteriae*. On the other hand, the carbon tetrachloride partitionate of methanol extract showed mild to moderate activity against *E. coli*, *S. paratyphi* and *V. mimicus* having zone of inhibition 17.3, 17.6 and 16.9 mm respectively. Again, the chloroform soluble materials showed maximum zone of 19.3 mm against *B. cereus* and zone of 17.6, 17.4, 17.7 16.8 and 16.5 mm against *B. subtilis*, *S. aureus*, *E. coli*, *S. paratyphi* and *S. boydii*, respectively indicating strong to moderate activity. The aqueous soluble materials also showed low to strong activity against all the strains ranging from 12-19 mm. Of all the samples, the chloroform soluble materials showed lowest zone of inhibition of 12.1 mm against *B. cereus* indicating low activity. The antibacterial activity of test samples of the stem bark of *E. fusca* is shown in Table 4.10.

In case of antifungal screening, the zone of inhibition was between 11-17 mm indicating mild to moderate activity in contrast to griseofulvin as standard. The carbon tetrachloride soluble materials gave highest activity against *C. albicans* with zone size of 16.1 mm.

Table 4.10: Antimicrobial screening of the stem bark of *E. fusca* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|---------------|---------------|----------------|---------------|------------|
| | Me | HSF | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 17.5±
0.40 | 16.9±
0.75 | 15.2±
0.55 | 19.3±
0.21 | 14.2±
0.51 | 35 |
| <i>B. megaterium</i> | 18.1±
0.68 | 18.6±
0.70 | 14.9±
0.17 | 12.4±
0.70 | 14.4±
0.74 | 35 |
| <i>B. subtilis</i> | 18.4±
0.75 | 18.3±
0.40 | 16.8±
0.36 | 17.6±
0.42 | 14.8±
0.31 | 36 |
| <i>S. aureus</i> | 19.1±
0.74 | 18.1±
0.55 | 16.7±
0.45 | 17.4±
0.42 | 15.8±
0.56 | 32 |
| <i>S. lutea</i> | 18.3±
0.85 | 17.5±
0.32 | 16.6±
0.31 | 15.6±
0.31 | 17.6±
0.31 | 27 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 17.8±
0.78 | 16.9±
0.75 | 17.3±
0.60 | 17.7±
0.66 | 18.3±
0.85 | 25 |
| <i>P. aeruginosa</i> | 14.4±
0.35 | 13.4±
0.36 | 12.5±
0.69 | 12.1 ±
0.87 | 12.7±
0.85 | 20 |
| <i>S. paratyphi</i> | 19.2±
0.61 | 17.7±
0.56 | 17.6±
0.49 | 16.8±
0.36 | 18.1±
0.93 | 27 |
| <i>S. typhi</i> | 16.2±
0.87 | 17.1±
0.21 | 16.8±
0.36 | 16.2±
0.81 | 15.4±
0.31 | 22 |
| <i>S. boydii</i> | 16.8±
0.36 | 17.8±
0.56 | 17.5±
0.31 | 16.5±
0.35 | 15.4±
0.31 | 27 |
| <i>S. dysenteriae</i> | 16.9±
0.45 | 18.1±
0.55 | 16.7±
0.35 | 15.8±
0.56 | 17.2±
0.31 | 25 |
| <i>V. mimicus</i> | 18.5±
0.53 | 17.6±
0.31 | 16.9±
0.15 | 15.8±
0.31 | 16.1±
0.53 | 25 |
| <i>V. parahemolyticus</i> | 15.8±
0.31 | 15.1±
0.35 | 15.5±
0.74 | 16.1±
0.85 | 15.6±
0.46 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 14.4±
0.42 | 12.2±
0.87 | 13.7±
0.72 | 13.9±
0.57 | 13.5±
0.35 | 20 |
| <i>C. albicans</i> | 15.1±
0.91 | 15.6±
0.31 | 16.1±
0.85 | 14.6±
0.64 | 12.5±
0.32 | 18 |
| <i>S. cerevisiae</i> | 15.5±
0.38 | 12.5±
0.32 | 11.9±
0.72 | 13.2±
0.51 | 12.1±
0.74 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.8 Antimicrobial activity of the leaf of *E. fusca*

Again, the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the leaf of *E. fusca* also showed low to mild activity against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs with zones of inhibition between the ranges of 11-20 mm. At a concentration of 400 µg/disc the zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *E. fusca* were ranged from 13-19, 12-20, 11-18 mm, 12-18 and 13-18 mm, respectively (Table 4.10) The *n*-hexane soluble fraction showed the highest inhibitory activity against *S. lutea* having the zone of inhibition 19.2 mm. In case of the methanol extract the growth of *B. cereus* (18.2 mm), *S. lutea* (18.4 mm), *E. coli* (18.1 mm) and *S. typhi* (18.2 mm) was moderately inhibited. The *n*-hexane soluble materials gave highest zone size of 17.9 mm *B. cereus*, 18.1 mm against *B. megaterium*, 18.1 mm against *V. mimicus* and 18.1 mm against *V. parahemolyticus*. At the same time, the carbon tetrachloride partitionate of methanol extract showed relatively mild activity against *B. cereus*, *E. coli* and *S. typhi* having zone of inhibition 17.4, 16.7 and 16.9 mm respectively. Again, the chloroform soluble materials showed maximum zone of 17.3 mm against *S. dysenteriae* and inhibition zone of 17.2, 16.5, 16.8, and 17.2 mm against *B. megaterium*, *S. paratyphi*, *S. typhi* and *S. boydii*, respectively indicating relatively mild to moderate activity. The aqueous soluble materials also showed low to relatively strong activity against all the strains ranging from 13-18 mm. Out of all samples, the carbon tetrachloride soluble materials showed lowest zone size of 11.5 mm against *P. aeruginosa* indicating low activity. The antibacterial activity of test samples of the leaf of *E. fusca* is shown in Table 4.11.

Yet again, the zone of inhibition for antifungal screening was between 10-16 mm indicating low to strong activity in contrast to griseofulvin as standard. The crude methanol extract gave highest activity against *A. niger* with zone size of 15.5 mm.

Table 4.11: Antimicrobial screening of the leaf of *E. fusca* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|---------------|---------------|---------------|---------------|------------|
| | Me | HSF | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 18.2±
0.83 | 17.9±
0.36 | 17.4±
0.91 | 15.8±
0.40 | 17.1±
0.72 | 35 |
| <i>B. megaterium</i> | 17.1±
0.32 | 18.1±
0.72 | 15.7±
0.65 | 17.2±
0.72 | 15.4±
0.92 | 35 |
| <i>B. subtilis</i> | 17.5±
0.53 | 16.9±
0.35 | 14.7±
0.35 | 16.1±
0.71 | 13.5±
0.32 | 36 |
| <i>S. aureus</i> | 17.9±
0.44 | 17.6±
0.31 | 16.1±
0.55 | 15.1±
0.32 | 15.3±
0.55 | 32 |
| <i>S. lutea</i> | 18.4±
0.97 | 19.2±
0.15 | 16.2±
0.61 | 15.2±
0.51 | 15.6±
0.31 | 27 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 18.1±
0.72 | 17.8±
0.78 | 16.7±
0.40 | 15.1±
0.32 | 16.8±
0.64 | 25 |
| <i>P. aeruginosa</i> | 13.5±
0.25 | 12.1±
0.47 | 11.5±
0.15 | 12.8±
0.75 | 13.8±
0.25 | 20 |
| <i>S. paratyphi</i> | 17.9±
0.74 | 16.9±
0.75 | 14.7±
0.35 | 16.5±
0.35 | 17.3±
0.59 | 27 |
| <i>S. typhi</i> | 18.2±
0.42 | 17.4±
0.36 | 16.9±
0.55 | 16.8±
0.42 | 16.5±
0.35 | 22 |
| <i>S. boydii</i> | 17.3±
0.59 | 17.7±
0.56 | 15.9±
0.32 | 17.2±
0.47 | 16.9±
0.15 | 27 |
| <i>S. dysenteriae</i> | 16.5±
0.35 | 17.5±
0.29 | 16.1±
0.53 | 17.3±
0.59 | 13.8±
0.71 | 25 |
| <i>V. mimicus</i> | 17.8±
0.36 | 18.1±
0.55 | 16.2±
0.36 | 14.5±
0.40 | 13.8±
0.71 | 25 |
| <i>V. parahemolyticus</i> | 16.9±
0.15 | 17.9±
0.45 | 14.2±
0.15 | 15.1±
0.45 | 12.2±
0.70 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 15.5±
0.51 | 14.5±
0.36 | 15.4±
0.31 | 12.3±
0.25 | 13.8±
0.71 | 20 |
| <i>C. albicans</i> | 14.4±
0.35 | 13.8±
0.35 | 10.2±
0.46 | 12.2±
0.61 | 11.9±
0.61 | 18 |
| <i>S. cerevisiae</i> | 14.1±
0.76 | 13.5±
0.15 | 13.5±
0.15 | 14.2±
0.30 | 12.8±
0.46 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.9 Antimicrobial activity of the stem bark *E. variegata*

Antimicrobial screening of the methanol extract along with its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the stem bark of *E. variegata* showed low to strong activity with zones of inhibition being between the ranges of 11-19 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the stem bark of *E. variegata* zones of inhibition ranged from 14-19, 12-18, 11-19, 13-17 and 11-15 mm, respectively (Table 4.11) at a concentration of 400 µg/disc. The crude methanol extract showed the maximum inhibitory activity against *B. cereus* having the zone of inhibition 18.2 mm. In case of the methanol extract the growth of *S. typhi* (17.9 mm), *S. boydii* (17.3 mm), *S. dysenteriae* (17.1 mm) and *V. mimicus* (17.9 mm) was moderately inhibited. The *n*-hexane soluble materials gave moderate zone size for of 17.7 mm against *E. coli*, 17.1 mm against *S. paratyphi* and 17.6 mm against *S. typhi*. The carbon tetrachloride partitionate of methanol extract gave maximum zone of inhibition of 18.1 mm. At the same time, it showed mild to moderate activity against *S. lutea*, *S. paratyphi*, *S. typhi* and *V. mimicus* having zone of inhibition 16.7, 17.4, 16.7 and 17.2, mm respectively. Again, the chloroform soluble materials showed maximum zone of 16.8 mm against *S. typhi* indicating weak activity. The aqueous soluble materials also showed low to moderate activity against all the strains between 11-16 mm. Out of all samples, the chloroform soluble materials showed lowest zone of inhibition of 11.7 mm against *P. aeruginosa* indicating low activity. Table 4.12 shows the antibacterial activity of test samples of the stem bark of *E. variegata*.

For the antifungal screening, the zone of inhibition was between 9-15 mm indicating low to moderate activity in contrast to griseofulvin as standard. The *n*-hexane soluble materials gave highest activity against *C. albicans* with zone size of 14.4 mm.

Table 4.12: Antimicrobial screening of the stem bark of *E. variegata* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|---------------|---------------|---------------|---------------|------------|
| | Me | HSF | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 18.2±
0.68 | 16.8±
0.42 | 15.3±
0.84 | 14.4±
0.74 | 14.9±
0.17 | 35 |
| <i>B. megaterium</i> | 15.2±
0.84 | 16.1±
0.75 | 16.2±
0.23 | 15.3±
0.47 | 13.1±
0.65 | 35 |
| <i>B. subtilis</i> | 14.2±
0.45 | 13.3±
0.45 | 12.1±
0.65 | 13.9±
0.55 | 12.7±
0.45 | 36 |
| <i>S. aureus</i> | 14.9±
0.55 | 12.1±
0.35 | 11.9±
0.55 | 13.9±
0.47 | 11.8±
0.56 | 32 |
| <i>S. lutea</i> | 15.1±
0.32 | 16.8±
0.61 | 16.7±
0.57 | 14.5±
0.38 | 14.3±
0.53 | 27 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 16.6±
0.44 | 17.7±
0.45 | 18.1±
0.72 | 16.5±
0.35 | 15.3±
0.45 | 25 |
| <i>P. aeruginosa</i> | 14.4±
0.35 | 12.2±
0.72 | 12.3±
0.55 | 13.1±
0.35 | 11.7±
0.42 | 20 |
| <i>S. paratyphi</i> | 16.8±
0.42 | 17.1±
0.76 | 17.4±
0.42 | 15.4±
0.61 | 15.2±
0.55 | 27 |
| <i>S. typhi</i> | 17.9±
0.68 | 17.6±
0.42 | 16.7±
0.53 | 16.8±
0.51 | 14.9±
0.20 | 22 |
| <i>S. boydii</i> | 17.3±
0.59 | 16.8±
0.36 | 16.4±
0.42 | 14.4±
0.35 | 15.1±
0.49 | 27 |
| <i>S. dysenteriae</i> | 17.1±
0.67 | 14.7±
0.56 | 16.1±
0.78 | 15.9±
0.55 | 15.1±
0.75 | 25 |
| <i>V. mimicus</i> | 17.9±
0.45 | 15.6±
0.59 | 17.2±
0.56 | 16.3±
0.87 | 13.8±
0.23 | 25 |
| <i>V. parahemolyticus</i> | 15.1±
0.65 | 16.1±
0.32 | 14.2±
0.45 | 14.7±
0.55 | 12.9±
0.78 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 12.9±
0.55 | 13.7±
0.51 | 13.6±
0.42 | 11.8±
0.78 | 9.8±
0.55 | 20 |
| <i>C. albicans</i> | 13.5±
0.49 | 14.4±
0.42 | 12.6±
0.56 | 11.1±
0.35 | 12.6±
0.44 | 18 |
| <i>S. cerevisiae</i> | 13.3±
0.32 | 12.2±
0.56 | 11.4±
0.30 | 12.9±
0.74 | 12.7±
0.85 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.10 Antimicrobial activity of the leaf of *E. variegata*

On the other hand, the leaf of the methanol extract and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials of the plant *E. variegata* showed low to moderate activity with zones of inhibition ranging from 11-18 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The zones of inhibition produced by the crude methanol extracts and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates of the leaf of *E. variegata* were ranged from 11-18, 12-17, 11-17, 12-17 and 11-17 mm, respectively (Table 4.12) at a concentration of 400 µg/disc. The crude methanol extract showed the highest inhibitory activity against *E. coli* having the zone of inhibition 17.8 mm. In case of the methanol extract the growth of *B. cereus* (16.5 mm), *S. typhi* (16.6 mm), *S. dysenteriae* (17.2 mm) and *V. parahemolyticus* (16.5 mm) was strongly inhibited. The *n*-hexane soluble materials also gave strong zone size of 16.9, 16.9, 17.3 and 17.1 mm against *S. lutea*, *E. coli*, *S. typhi* and *S. dysenteriae*. Also, the carbon tetrachloride partitionate of methanol extract showed mild to moderate activity against *E. coli*, *S. paratyphi*, *S. typhi* and *S. dysenteriae* having zone of inhibition of 16.4, 16.5, 16.5, 14.5 and 16.7 mm, respectively. Again, the chloroform soluble materials showed strong zone of inhibition of 17.3 and 16.9 mm against *S. paratyphi* and *S. dysenteriae*, respectively indicating strong activity. But *S. lutea* and *E. coli* had a zone of 15.5 mm each indicating mild inhibition. The aqueous soluble materials showed low to moderate activity against all the strains ranging from 11-17 mm. Out of all samples, the aqueous soluble materials showed lowest zone of inhibition of 11.2 mm against *B. subtilis* indicating low activity and strongest against *S. paratyphi* with a zone of 16.9 mm. The antibacterial activity of test samples of the leaf of *E. variegata* is shown in Table 4.13.

The zone of inhibition was between 11-16 mm indicating moderate to strong activity in contrast to griseofulvin as standard in case of antifungal screening. The *n*-hexane soluble materials gave highest activity against *C. albicans* with zone size of 15.4 mm.

Table 4.13: Antimicrobial screening of the leaf of *E. variegata* extractives (400 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|---------------|---------------|---------------|---------------|------------|
| | Me | HST | CSF | CHSF | AQSF | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 16.5±
0.35 | 15.9±
0.75 | 15.7±
0.57 | 14.8±
0.46 | 14.3±
0.45 | 35 |
| <i>B. megaterium</i> | 13.1±
0.31 | 13.5±
0.25 | 14.4±
0.42 | 15.1±
0.42 | 12.2±
0.96 | 35 |
| <i>B. subtilis</i> | 11.1±
0.74 | 15.8±
0.46 | 12.3±
0.38 | 13.5±
0.26 | 11.2±
0.61 | 36 |
| <i>S. aureus</i> | 12.7±
0.40 | 12.2±
0.42 | 11.8±
0.78 | 14.6±
0.50 | 15.2±
0.36 | 32 |
| <i>S. lutea</i> | 15.3±
0.55 | 16.6±
0.35 | 15.8±
0.66 | 15.5±
0.40 | 14.7±
0.45 | 27 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 17.8±
0.78 | 16.9±
0.61 | 16.4±
0.42 | 15.5±
0.51 | 14.7±
0.45 | 25 |
| <i>P. aeruginosa</i> | 13.1±
0.62 | 13.5±
0.31 | 11.2±
0.68 | 12.2±
0.50 | 12.1±
0.35 | 20 |
| <i>S. paratyphi</i> | 15.4±
0.31 | 14.7±
0.51 | 16.5±
0.25 | 17.3±
0.59 | 16.9±
0.65 | 27 |
| <i>S. typhi</i> | 16.6±
0.31 | 17.3±
0.59 | 16.5±
0.46 | 13.7±
0.56 | 13.3±
0.91 | 22 |
| <i>S. boydii</i> | 15.2±
0.76 | 16.4±
0.36 | 14.6±
0.46 | 14.8±
0.76 | 15.1±
0.76 | 27 |
| <i>S. dysenteriae</i> | 17.2±
0.31 | 17.1±
0.76 | 16.7±
0.35 | 16.9±
0.49 | 14.3±
0.55 | 25 |
| <i>V. mimicus</i> | 15.4±
0.78 | 14.2±
0.31 | 15.5±
0.35 | 13.9±
0.74 | 12.7±
0.45 | 25 |
| <i>V. parahemolyticus</i> | 16.5±
0.55 | 15.8±
0.31 | 13.1±
0.66 | 13.8±
0.71 | 12.9±
0.86 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 13.7±
0.51 | 14.4±
0.36 | 15.3±
0.31 | 12.9±
0.25 | 11.2±
0.71 | 20 |
| <i>C. albicans</i> | 14.3±
0.31 | 13.7±
0.45 | 10.4±
0.55 | 11.8±
0.66 | 9.9±
0.26 | 18 |
| <i>S. cerevisiae</i> | 14.7±
0.47 | 13.7±
0.45 | 13.1±
0.67 | 13.4±
0.44 | 12.4±
0.42 | 19 |

Me= Methanol extract; HST= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

4.4.11 Antimicrobial activity of the pure compounds

Some of the isolated pure compounds from the four investigated plants were also subjected to antimicrobial screening which showed low to strong activity with zones of inhibition being between the ranges of 11 to 23 mm against various Gram positive and Gram negative bacterial strains in comparison to standard Kanamycin discs. The pure compounds BVSP-4 (48), BVBC-2 (51), BVS-65 (54), BS-01 (55), BS-06 (56), BT-1 (58), BT-6 (59), EF-7 (62), EF-8 (63) and EF-12 (64) isolated from the stem bark of *B. verrucosa*, *B. stipularis*, *B. tomentosa* and *E. fusca* produced zone of inhibition ranging from 11-16, 12-16, 11-17, 17-23, 16-20, 16-21, 14-21, 16-18, 14-19 and 15-19 mm, respectively (Table 4.14 and 4.15) at a concentration of 50 µg/disc. The highest zone of inhibition (22.7 mm) was produced by BS-01 (55) against *E. coli*.

In case of antifungal screening, the zone of inhibition was between 10-21 mm indicating mild to strong activity in contrast to griseofulvin as standard. BS-06 (56) gave highest activity against *C. albicans* with zone size of 20.8 mm.

Table 4.14: Antimicrobial screening of the some of the pure compounds from *B. verrucosa* and *B. stipularis* (50 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|---------------|---------------|---------------|---------------|------------|
| | BVSP-4 | BVBC-2 | BVS-65 | BS-01 | BS-06 | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 11.9±
0.33 | 13.9±
0.75 | 13.5±
0.71 | 19.0±
0.60 | 16.1±
0.66 | 35 |
| <i>B. megaterium</i> | 13.1±
0.31 | 14.5±
0.66 | 14.6±
0.41 | 18.4±
0.50 | 16.9±
0.91 | 35 |
| <i>B. subtilis</i> | 12.1±
0.54 | 12.8±
0.46 | 12.3±
0.38 | 17.5±
0.87 | 16.3±
1.05 | 36 |
| <i>S. aureus</i> | 12.9±
0.91 | 12.2±
0.29 | 13.0±
0.78 | 17.8±
0.40 | 18.2±
0.89 | 32 |
| <i>S. lutea</i> | 14.6±
0.55 | 14.5±
0.57 | 11.8±
0.66 | 21.6±
0.87 | 20.7±
1.22 | 27 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 13.2±
0.58 | 12.1±
0.49 | 12.9±
0.71 | 22.7±
0.65 | 18.8±
0.42 | 25 |
| <i>P. aeruginosa</i> | 13.1±
0.62 | 13.5±
0.31 | 14.6±
0.41 | 17.9±
0.76 | 18.4±
0.76 | 20 |
| <i>S. paratyphi</i> | 14.8±
0.31 | 13.1±
0.74 | 14.3±
0.55 | 20.1±
0.65 | 18.4±
0.76 | 27 |
| <i>S. typhi</i> | 12.8±
0.31 | 15.1±
0.76 | 16.5±
0.46 | 22.1±
0.65 | 20.6±
0.86 | 22 |
| <i>S. boydii</i> | 15.2±
0.76 | 16.4±
0.36 | 14.6±
0.46 | 20.2±
1.29 | 18.1±
0.86 | 27 |
| <i>S. dysenteriae</i> | 14.3±
0.55 | 14.8±
0.76 | 16.7±
0.35 | 21.2±
0.36 | 19.2±
0.66 | 25 |
| <i>V. mimicus</i> | 13.3±
0.91 | 14.2±
0.31 | 13.5±
0.35 | 21.3±
0.91 | 16.9±
0.61 | 25 |
| <i>V. parahemolyticus</i> | 13.1±
0.66 | 13.9±
0.74 | 14.6±
0.22 | 20.7±
0.60 | 18.9±
0.59 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 13.7±
0.51 | 14.4±
0.36 | 15.3±
0.31 | 19.1±
0.35 | 18.2±
0.59 | 20 |
| <i>C. albicans</i> | 14.3±
0.31 | 13.7±
0.45 | 10.4±
0.55 | 18.5±
0.60 | 20.8±
0.45 | 18 |
| <i>S. cerevisiae</i> | 14.7±
0.47 | 13.7±
0.45 | 13.1±
0.67 | 18.2±
0.31 | 20.2±
0.83 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc

Table 4.15: Antimicrobial screening of the some of the pure compounds from *B. tomentosa* and *E. fusca* (50 µg/disc) and Kanamycin (30 µg/disc).

| Test bacteria and fungi | Diameter of zone of inhibition (mm) | | | | | |
|-------------------------------|-------------------------------------|---------------|---------------|---------------|---------------|------------|
| | BT-1 | BT-6 | EF-7 | EF-8 | EF-12 | KAN |
| Gram positive bacteria | | | | | | |
| <i>B. cereus</i> | 17.7±
0.57 | 19.1±
0.45 | 17.8±
0.66 | 16.9±
1.10 | 19.0±
0.31 | 35 |
| <i>B. megaterium</i> | 16.1±
0.59 | 16.7±
0.55 | 18.5±
0.87 | 19.1±
0.60 | 15.6±
0.57 | 35 |
| <i>B. subtilis</i> | 18.3±
0.85 | 14.9±
0.96 | 19.4±
0.31 | 18.8±
0.35 | 14.9±
0.17 | 36 |
| <i>S. aureus</i> | 16.9±
0.87 | 16.4±
1.08 | 17.6±
0.27 | 18.1±
0.40 | 61.0±
0.42 | 32 |
| <i>S. lutea</i> | 19.6±
0.83 | 20.8±
0.50 | 16.9±
0.68 | 16.7±
0.60 | 17.6±
0.42 | 27 |
| Gram negative bacteria | | | | | | |
| <i>E. coli</i> | 19.5±
0.21 | 19.1±
0.57 | 17.9±
0.21 | 17.8±
0.27 | 17.4±
0.42 | 25 |
| <i>P. aeruginosa</i> | 15.8±
0.98 | 16.9±
0.68 | 14.8±
0.82 | 14.8±
0.50 | 16.6±
0.21 | 20 |
| <i>S. paratyphi</i> | 21.3±
0.46 | 19.7±
0.70 | 17.5±
0.32 | 17.8±
0.78 | 15.2±
0.51 | 27 |
| <i>S. typhi</i> | 18.9±
0.55 | 20.1±
0.90 | 18.3±
0.85 | 18.8±
0.42 | 17.3±
0.60 | 22 |
| <i>S. boydii</i> | 20.1±
0.92 | 19.8±
0.25 | 16.9±
0.55 | 16.8±
0.42 | 17.7±
0.66 | 27 |
| <i>S. dysenteriae</i> | 20.3±
0.85 | 19.6±
0.70 | 16.8±
0.36 | 17.3±
0.59 | 15.9±
0.59 | 25 |
| <i>V. mimicus</i> | 19.1±
0.87 | 17.2±
0.56 | 17.2±
0.31 | 17.3±
0.40 | 16.5±
0.32 | 25 |
| <i>V. parahemolyticus</i> | 17.2±
0.36 | 18.4±
1.08 | 18.1±
0.55 | 16.9±
0.15 | 17.9±
0.15 | 20 |
| Fungi | | | | | | GRI |
| <i>A. niger</i> | 13.6±
0.55 | 15.8±
0.66 | 13.6±
0.31 | 11.9±
0.32 | 13.2±
0.31 | 20 |
| <i>C. albicans</i> | 12.8±
0.99 | 14.2±
1.23 | 14.2±
0.61 | 13.6±
1.14 | 15.1±
0.35 | 18 |
| <i>S. cerevisiae</i> | 14.2±
0.36 | 12.7±
0.45 | 12.6±
1.10 | 14.2±
0.60 | 14.2±
0.60 | 19 |

Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction; KAN= Kanamycin disc and GRI= Griseofulvin disc



Chapter 5

Brine Shrimp Lethality Bioassay

5.1 Introduction:

Brine shrimp lethality bioassay is a relatively recent development in the bioassay for biologically active crude extracts and pure compounds obtained from natural products and synthetic sources. Here, the simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. Using this bioassay a number of novel anti-tumor and pesticidal natural products have been previously isolated (Meyer *et. al.*, 1982; McLaughlin, 1998). Therefore, the positive result obtained in this assay suggests that the fractionated extracts and pure compounds may contain anti-tumor, anti-bacterial or pesticidal activity. In other words it indicates a wide range of pharmacological activities of the compounds and also it can indicate the general toxicity of the fractionated extracts and pure compounds.

Brine shrimp lethality bioassay (McLaughlin, 1998; Persoone, 1980) is a rapid and comprehensive bioassay for the bioactive compounds of the natural and synthetic origins. Thus, *in vivo* lethality of a simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. Here, the simple zoologic organism is brine shrimp nauplii.

Brine shrimp lethality bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, anti-tumor, antiviral, anticancer, pesticidal etc. actions of the compounds (Meyer *et. al.*, 1982; McLaughlin, 1998). The brine shrimp bioassay has advantages of being rapid (24 hours), inexpensive, simple and require no special equipment or aseptic technique. It utilizes a large number of organisms for statistical validation and a relatively small amount of sample is sufficient. Generally, the LD₅₀ values for cytotoxicities are one tenth of LC₅₀ values in the brine shrimp lethality test.

The brine shrimp bioassay has several advantages such as

- i. Rapid in process (24 hours)
- ii. Inexpensive and simple (e.g. no aseptic technique is required)
- iii. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and relatively small amount of sample is required.
- iv. It does not require animal serum as it is needed for determination of toxicity.

5.2. Principle

The effectiveness or the concentration mortality relationship of plant product is usually expressed as median lethal concentration (LC_{50}) value. This represents the concentration of the chemical agent that produces death in half of the test subjects after a certain exposure period. LC_{90} expresses the concentration of the test sample in which 90% of the test subject dies.

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Test samples are prepared by dissolving in DMSO (Dimethylsulphoxide) and by the addition of calculated volume of DMSO, desired concentration of the test sample is prepared. The nauplii are counted by visual inspection and are taken in vials containing 5 ml of simulated sea water. Then samples of different concentrations are added to the premarked vials through micropipette. The vials are then left for 24 hours. Survivors are counted after 24 hours. These data are processed in a simple program for probit analysis to estimate LC_{50} and LC_{90} values with 95% confidence intervals for statistically significant comparisons of potencies.

The lethal concentration LC_{50} and LC_{90} of the test samples after 24 hour was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis.

5.3 Materials:

- a. *Artemia salina* leach (brine shrimp)
- b. Sea salt (NaCl)
- c. Small tank with perforated dividing dam to hatch the shrimps
- d. Lamp to attract shrimps
- e. Micropipette
- f. Pipette
- g. Magnifying glass
- h. Test samples of experimental plants (Table-5.1)

5.4 Procedure

5.4.1 Preparation of sea water

Sea salt (pure NaCl, 38 gm) was weighed accurately, dissolved in distilled water to make one litre and then filtered off to get a clear solution.

Table 5.1: Test samples of experimental plants

| Name of Plant | Extract | Measured amount (mg) |
|--|-----------------------------------|----------------------|
| <i>Bridelia verrucosa</i>
(Stem bark and leaf) | Crude MeOH extract | 4.0 |
| | <i>n</i> -Hexane soluble fraction | 4.0 |
| | CCl ₄ soluble fraction | 4.0 |
| | Chloroform soluble fraction | 4.0 |
| | Aqueous soluble fraction | 4.0 |
| | BVSP-4 | 1.0 |
| | BVBC-2 | 1.0 |
| | BVS-65 | 1.0 |
| <i>Bridelia stipularis</i>
(Stem bark and leaf) | Crude MeOH extract | 4.0 |
| | <i>n</i> -Hexane soluble fraction | 4.0 |
| | CCl ₄ soluble fraction | 4.0 |
| | Chloroform soluble fraction | 4.0 |
| | Aqueous soluble fraction | 4.0 |
| | BS-01 | 1.0 |
| | BS-06 | 1.0 |
| | BS-11 | 1.0 |
| <i>Bridelia tomentosa</i>
(Stem bark and leaf) | Crude MeOH extract | 4.0 |
| | <i>n</i> -Hexane soluble fraction | 4.0 |
| | CCl ₄ soluble fraction | 4.0 |
| | Chloroform soluble fraction | 4.0 |
| | Aqueous soluble fraction | 4.0 |
| | BT-6 | 1.0 |
| <i>Erythrina fusca</i>
(Stem bark and leaf) | Crude MeOH extract | 4.0 |
| | <i>n</i> -Hexane soluble fraction | 4.0 |
| | CCl ₄ soluble fraction | 4.0 |
| | Chloroform soluble fraction | 4.0 |
| | Aqueous soluble fraction | 4.0 |
| | EF-7 | 1.0 |
| | EF-8 | 1.0 |
| | EF-12 | 1.0 |
| <i>Erythrina variegata</i>
(Stem bark and leaf) | Crude MeOH extract | 4.0 |
| | <i>n</i> -Hexane soluble fraction | 4.0 |
| | CCl ₄ soluble fraction | 4.0 |
| | Chloroform soluble fraction | 4.0 |
| | Aqueous soluble fraction | 4.0 |

5.4.2 Hatching of brine shrimps

Sea water was kept in a hatcher and *Artemia salina* leach (Brine shrimp) eggs were incubated in the hatcher at 28-30°C with strong aeration, under a continuous light regime. Approximately, 24 hours were allowed to hatch the shrimp and to be matured as nauplii. The hatched shrimps were attracted to the lamp through the perforated dam and they were taken for experiment.

5.4.3 Application of brine shrimp nauplii

With the help of a Pasteur pipette 10 living shrimps were added to each of the vials containing 5 ml of sea water.

5.4.4 Preparation of test samples of experimental plants

An aliquot (4.0 mg) of each of the test samples (Table 5.1) was taken and dissolved in 200 μ l of pure dimethylsulphoxide (DMSO) in vials to get stock solutions. Then, 100 ml of solution was taken in test tube each containing 5ml of simulated sea water and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400 μ g/ml. Then a series of solutions of varying concentrations were prepared from stock solution by serial dilution method. In each case 100 μ l sample was added to test tube and fresh 100 μ l DMSO was added to vial. Thus, the concentrations of the obtained solution in the test tubes were as:-

| | | | |
|----|----------------|-----|------------------|
| 1. | 400 μ g/ml | 6. | 12.5 μ g/ml |
| 2. | 200 μ g/ml | 7. | 6.25 μ g/ml |
| 3. | 100 μ g/ml | 8. | 3.125 μ g/ml |
| 4. | 50 μ g/ml | 9. | 1.563 μ g/ml |
| 5. | 25 μ g/ml | 10. | 0.781 μ g/ml |

5.4.5 Preparation of control groups

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used:

i) Positive control

ii) Negative control

5.4.5.1 Preparation of the positive control group

The use of positive control in cytotoxicity study is a widely accepted technique and the result of the test agent is compared with the result obtained for the positive control. Measured amount of the vincristine sulfate was dissolved in DMSO to get an initial concentration of 20 μ g/ml from which serial dilutions were made by using DMSO to get 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125 and 0.0390 μ g/ml, respectively. The positive control solutions were added to the pre-marked vials containing 10 living shrimp nauplii in 5 ml simulated sea water to get positive control group.

5.4.5.2 Preparation of the negative control group

DMSO (100 μ l) was added to each of three pre-marked glass vials containing 5 ml of simulated sea water and 10 shrimps to use as control groups. If the shrimps in these vials show a rapid mortality rate, then the test is considered to be invalid as the nauplii died due to some reasons other than the cytotoxicity of the test compounds.

5.4.6 Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

5.5 Analysis of data

Bioactive compounds are almost always toxic at higher dose. Thus, *in vivo* lethality in a simple zoological organism can be used as a convenient informant for screening and fractionation in the discovery of new bioactive natural products.

In the present bioactivity study all the crude extracts *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fraction along with the methanolic extract of both the leaf and stem bark of the three plants showed positive results indicating the test samples are biologically active. Each of the test samples showed different mortality rates at different concentrations. Plotting of log of concentration versus percent mortality for all test samples showed an approximate linear correlation. The concentration-mortality data were analyzed statistically by using probit analysis and linear regression using a simple IBM-PC program.

Table 5.2: Effect of vincristine sulfate (positive control) on shrimp nauplii.

| Conc. (C)
(μ g/ml) | Log C | % Mortality | LC ₅₀
(μ g/ml) | LC ₉₀
(μ g/ml) |
|----------------------------|----------|-------------|-----------------------------------|-----------------------------------|
| 0.0390 | -1.40894 | 20 | 0.45 | 10.0 |
| 0.078125 | -1.10721 | 30 | | |
| 0.15625 | -0.80618 | 30 | | |
| 0.3125 | -0.50515 | 40 | | |
| 0.625 | -0.20142 | 50 | | |
| 1.25 | 0.09691 | 70 | | |
| 2.5 | 0.39794 | 80 | | |
| 5 | 0.69897 | 80 | | |
| 10 | 1 | 90 | | |
| 20 | 1.30103 | 100 | | |

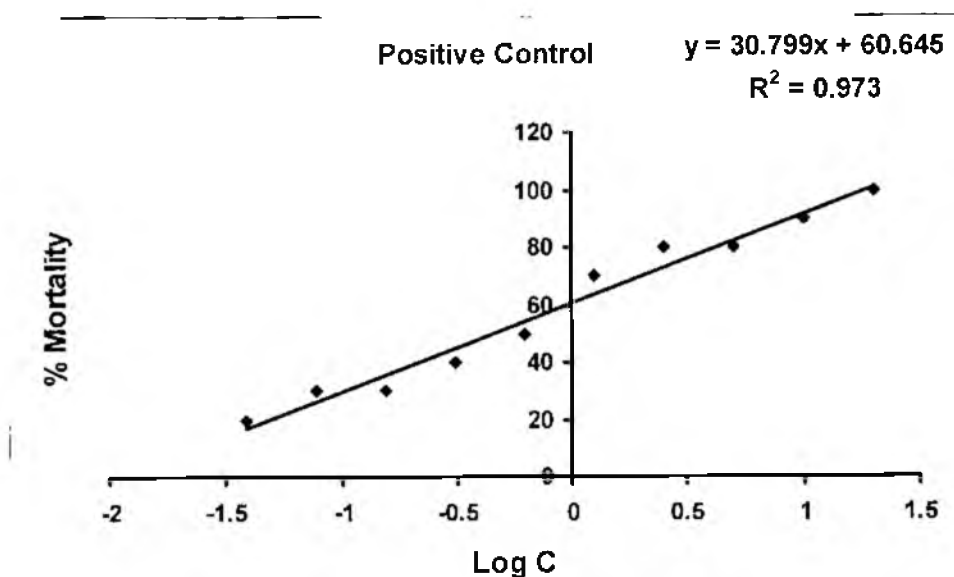


Figure 5.1: Effect of vincristine sulfate on brine shrimp nauplii.

5.6 Results and discussion of the test samples

The crude extracts of the stem bark and leaf of the plants of the genus *Bridelia*; *B. verrucosa*, *B. stipularis* and *B. tomentosa* and *Erythrina*; *E. fusca* and *E. variegata* were screened by brine shrimp lethality bioassay for probable cytotoxic activity in the laboratory of Biomedical Research Center, Faculty of Pharmacy, University of Dhaka, Dhaka. In this bioassay, the crude extract and fraction showed varying degrees of lethality indicating that the extractives and the compounds are biologically active. Each of the test samples showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with increase in concentration of the test samples and a plot of percent mortality versus log concentration on the graph paper produced an approximate linear correlation between them. It is evident that all the test samples were lethal to brine shrimp nauplii.

Table 5.2 gives the results of the brine shrimp lethality after 24 hour exposure to the positive control, vincristine sulfate. The positive control, compared with the negative control (sea water) was lethal, showing significant mortality to the shrimp.

The LC_{50} and LC_{90} values of *n*-hexane, carbon tetrachloride, chloroform, soluble fraction and methanolic extract of both the stem bark and leaf of *B. verrucosa*, *B. stipularis*, *B. tomentosa*, *E. fusca* and *E. variegata* along with the pure compounds isolated from the investigated plants together with the positive control are shown in Table 5.3-5.15 and graphs are in Figures 5.1-5.60. However, varying degree of lethality to *Artemia salina* was observed with exposure to different dose levels of the test samples. The degree of

lethality was directly proportional to the concentration of the extracts ranging from the lowest concentration (0.78125 µg/ml) to the highest concentration (400 µg/ml). Maximum mortalities took place at a concentration of 400 µg/ml, whereas least mortalities were at 0.78125 µg/ml concentration. In other words, mortality increased gradually with the increase in concentration of the test samples.

From the results of the brine shrimp lethality bioassay it can be well predicted that the crude extract and partitionate fraction possess bioactive principles and have considerable cytotoxic potency. Comparison with positive control vincristine sulfate signifies that cytotoxicity exhibited by the crude extracts and further bioactivity guided investigation can be done to find out potent antitumor and pesticidal compounds.

Table 5.3: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *B. verrucosa* on brine shrimp nauplii.

| <i>Bridelia verrucosa</i> (Stem bark) | | | | | | | | | | | |
|---------------------------------------|----------|-------------|-----|-----|------|------|---|----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Conc.
(C)
(µg/ml) | Log
C | % Mortality | | | | | LC ₅₀ , LC ₉₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 400 | 2.602 | 100 | 100 | 100 | 100 | 100 | 6.3 ±
0.25,
170.0 ±
1.33 | 5.1 ±
0.95,
72.4 ±
0.97 | 3.1 ±
0.62,
204.2 ±
0.75 | 0.71 ±
0.14,
43.7 ±
0.95 | 7.08 ±
0.44,
97.7 ±
1.59 |
| 200 | 2.301 | 90 | 100 | 90 | 100 | 100 | | | | | |
| 100 | 2.000 | 90 | 100 | 80 | 100 | 90 | | | | | |
| 50 | 1.699 | 70 | 90 | 70 | 100 | 80 | | | | | |
| 25 | 1.398 | 70 | 80 | 70 | 90 | 80 | | | | | |
| 12.5 | 1.097 | 50 | 70 | 60 | 80 | 70 | | | | | |
| 6.25 | 0.797 | 40 | 50 | 60 | 70 | 40 | | | | | |
| 3.125 | 0.495 | 40 | 40 | 50 | 70 | 40 | | | | | |
| 1.563 | 0.194 | 30 | 30 | 50 | 50 | 20 | | | | | |
| 0.781 | -0.107 | 30 | 20 | 40 | 40 | 10 | | | | | |

In case of *B. verrucosa*, the crude methanol extract of the stem bark as well as its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fraction were subjected to brine shrimp lethality bioassay by using the method of Meyer *et al.*, 1982. The LC₅₀ values were found to be 6.3, 5.1, 3.1, 0.71 and 7.08 µg/ml and LC₉₀ values were found to be 170.0, 72.4, 204.2, 43.7 and 97.7 µg/ml (Table 5.3), respectively. The positive control drug, vincristine sulfate showed LC₅₀ at 0.45 µg/ml and LC₉₀ at 10.0 µg/ml (Table 5.2). The chloroform soluble fraction of methanol extract of the stem bark demonstrated strong cytotoxic activity, while the other three partitionates along with the methanolic extract showed low to moderate cytotoxic activity.

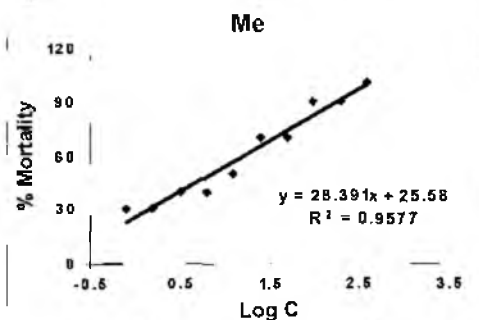


Figure 5.2: Effect of crude methanol extract of the stem bark of *B. verrucosa* on brine shrimp nauplii.

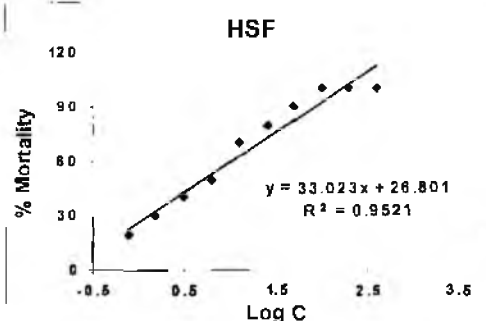


Figure 5.3: Effect of n-hexane soluble fraction of the stem bark of *B. verrucosa* on brine shrimp nauplii.

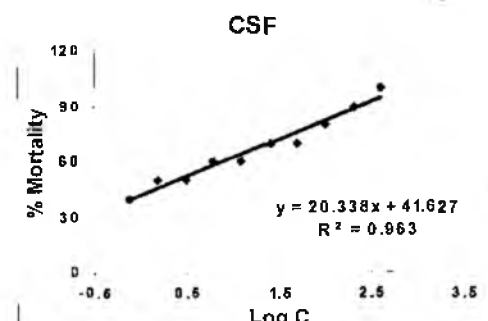


Figure 5.4: Effect of CCl_4 soluble fraction of the stem bark of *B. verrucosa* on brine shrimp nauplii.

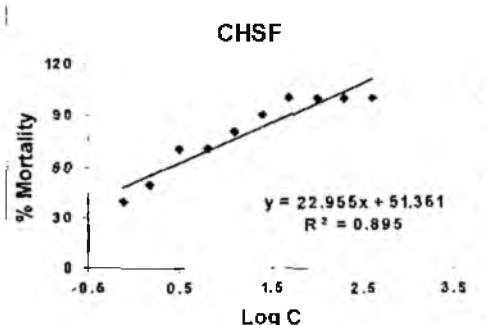


Figure 5.5: Effect of chloroform soluble fraction of the stem bark of *B. verrucosa* on brine shrimp nauplii.

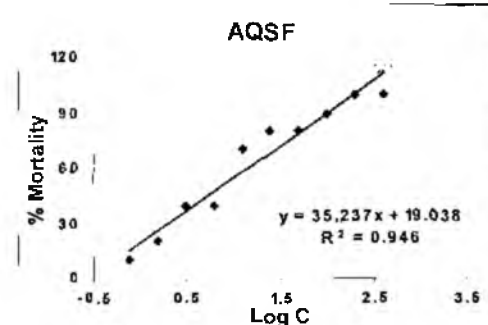


Figure 5.6: Effect of aqueous soluble fraction of the stem bark of *B. verrucosa* on brine shrimp nauplii.

Table 5.4: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *B. verrucosa* on brine shrimp nauplii.

| Bridelia verrucosa (Leaf) | | | | | | | | | | | |
|---------------------------|----------|-------------|-----|-----|------|------|---|----------------------------------|--------------------------------|---------------------------------|----------------------------------|
| Conc.
(C)
(µg/ml) | Log
C | % Mortality | | | | | LC ₅₀ , LC ₉₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Mc | HSF | CSF | CHSF | AQSF |
| 400 | 2.602 | 100 | 100 | 100 | 100 | 100 | 2.51±
0.14,
70.8±
2.35 | 8.13±
0.56,
134.9±
1.31 | 1.2±
0.40,
77.6±
1.18 | 3.13±
0.36,
58.9±
0.40 | 7.94±
0.36,
128.8±
0.52 |
| 200 | 2.301 | 100 | 100 | 100 | 100 | 90 | | | | | |
| 100 | 2.000 | 100 | 90 | 90 | 100 | 80 | | | | | |
| 50 | 1.699 | 100 | 80 | 80 | 100 | 80 | | | | | |
| 25 | 1.398 | 80 | 60 | 80 | 90 | 70 | | | | | |
| 12.5 | 1.097 | 60 | 50 | 70 | 70 | 70 | | | | | |
| 6.25 | 0.797 | 50 | 40 | 70 | 60 | 50 | | | | | |
| 3.125 | 0.495 | 50 | 30 | 60 | 50 | 40 | | | | | |
| 1.563 | 0.194 | 40 | 30 | 50 | 40 | 30 | | | | | |
| 0.781 | -0.107 | 40 | 20 | 40 | 20 | 10 | | | | | |

Again, for brine shrimp lethality bioassay with *B. verrucosa* leaf, the chloroform soluble fraction gave strong cytotoxic activity with LC₅₀ value of 1.2 $\mu\text{g/ml}$ and LC₉₀ value of 77.6 $\mu\text{g/ml}$. The crude methanolic extract and its carbon tetrachloride soluble fraction gave LC₅₀ values of 2.51 and 3.13 $\mu\text{g/ml}$ and LC₉₀ values of 70.8 and 58.9 $\mu\text{g/ml}$, respectively (Table 5.4) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC₅₀ value 0.45 $\mu\text{g/ml}$). The *n*-hexane and aqueous soluble fraction showed low cytotoxic activity with LC₅₀ values of 8.13 and 7.94 $\mu\text{g/ml}$ and LC₉₀ values of 134.9 and 128.8 $\mu\text{g/ml}$, respectively.

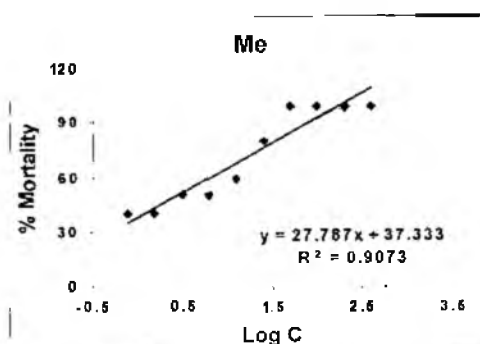


Figure 5.7: Effect of crude methanol extract of the leaf of *B. verrucosa* on brine shrimp nauplii.

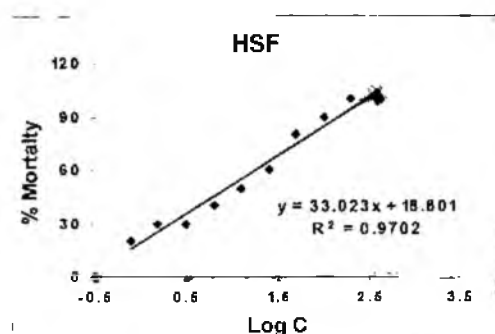


Figure 5.8: Effect of *n*-hexane soluble fraction of the leaf of *B. verrucosa* on brine shrimp nauplii.

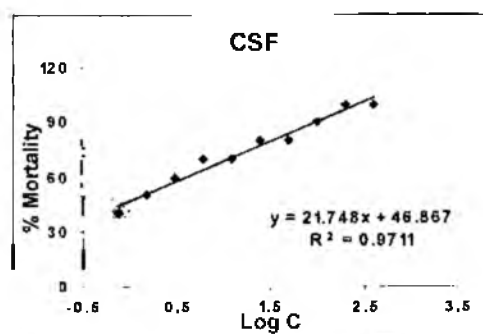


Figure 5.9: Effect of CCl_4 soluble fraction of the leaf of *B. verrucosa* on brine shrimp nauplii.

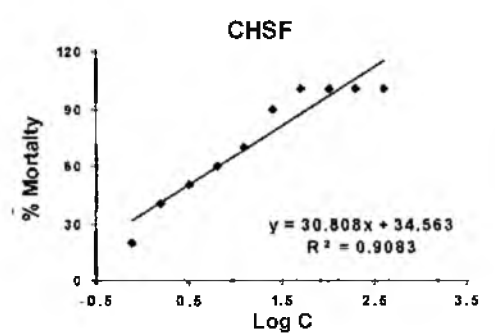


Figure 5.10: Effect of chloroform soluble fraction of the leaf of *B. verrucosa* on brine shrimp nauplii.

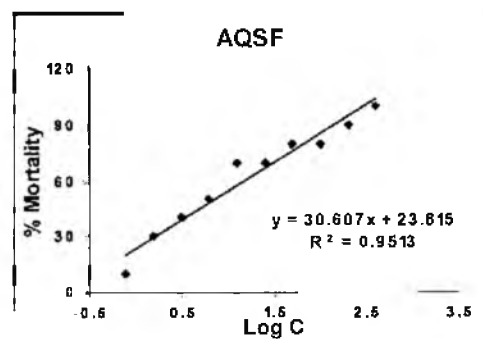


Figure 5.11: Effect of aqueous soluble fraction of the leaf of *B. verrucosa* on brine shrimp nauplii.

Table 5.5: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *B. stipularis* on brine shrimp nauplii.

| <i>Bridelia stipularis</i> (Stem bark) | | | | | | | | | | | |
|--|--------|-------------|-----|-----|------|------|---------------------------------------|-----------------------------------|-----------------------------------|----------------------------------|---------------------------------|
| Conc. (C)
($\mu\text{g/ml}$) | Log C | % Mortality | | | | | LC_{50} ($\mu\text{g/ml}$) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 400 | 2.602 | 100 | 100 | 100 | 100 | 100 | 8.51 $\pm$ 0.19, 199.5 $\pm$ 1.31 | 7.94 $\pm$ 0.43, 138.0 $\pm$ 1.49 | 4.47 $\pm$ 0.65, 131.8 $\pm$ 0.45 | 1.20 $\pm$ 0.51, 112.2 $\pm$ 2.2 | 4.70 $\pm$ 1.08, 70.8 $\pm$ 0.9 |
| 200 | 2.301 | 90 | 90 | 90 | 90 | 100 | | | | | |
| 100 | 2.000 | 90 | 90 | 90 | 90 | 80 | | | | | |
| 50 | 1.699 | 70 | 80 | 80 | 80 | 70 | | | | | |
| 25 | 1.398 | 70 | 60 | 70 | 70 | 70 | | | | | |
| 12.5 | 1.097 | 50 | 60 | 60 | 80 | 60 | | | | | |
| 6.25 | 0.797 | 40 | 50 | 50 | 70 | 50 | | | | | |
| 3.125 | 0.495 | 40 | 30 | 50 | 60 | 40 | | | | | |
| 1.563 | 0.194 | 30 | 30 | 40 | 50 | 40 | | | | | |
| 0.781 | -0.107 | 30 | 10 | 30 | 40 | 20 | | | | | |

The lethality of the crude methanolic extract and its *n*-hexane, carbon tetrachloride and aqueous soluble fraction of the stem bark of *B. stipularis* gave LC₅₀ values of 8.51, 7.94, 4.47 and 4.7 µg/ml and LC₉₀ values of 199.5, 138.0, 131.8 and 70.8 µg/ml respectively (Table 5.5) which exhibited low cytotoxic activity with comparison to the standard vincristine sulfate (LC₅₀ value 0.45 µg/ml). But the chloroform soluble fraction showed high cytotoxic activity with LC₅₀ value of 1.2 µg/ml.

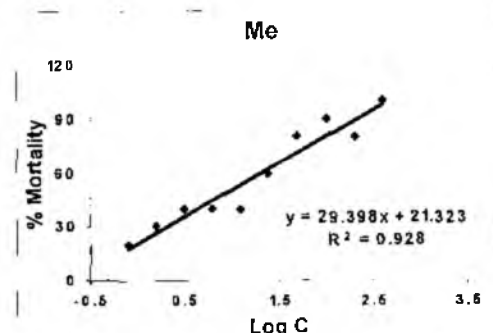


Figure 5.12: Effect of crude methanol extract of the stem bark of *B. stipularis* on brine shrimp nauplii.

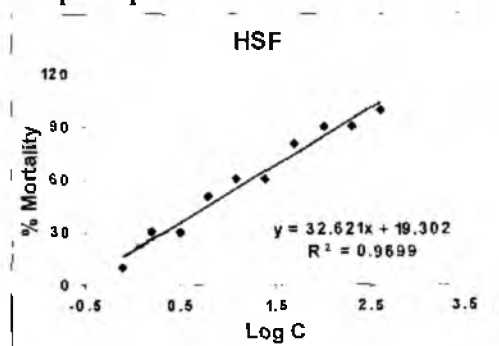


Figure 5.13: Effect of *n*-hexane soluble fraction of the stem bark of *B. stipularis* on brine shrimp nauplii.

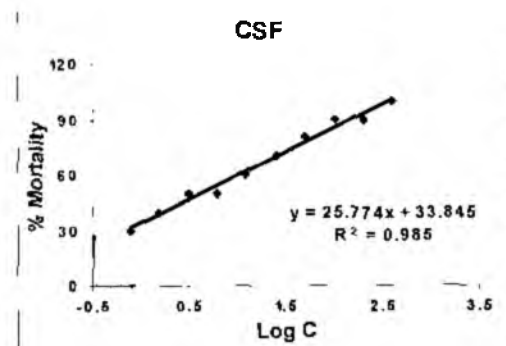


Figure 5.14: Effect of CCl₄ soluble fraction of the stem bark of *B. stipularis* on brine shrimp nauplii.

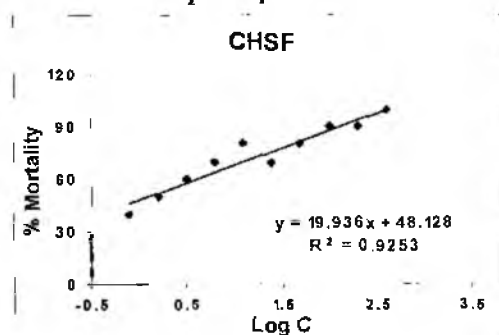


Figure 5.15: Effect of chloroform soluble fraction of the stem bark of *B. stipularis* on brine shrimp nauplii.

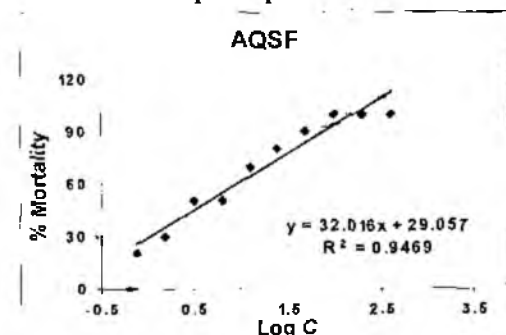


Figure 5.16: Effect of aqueous soluble fraction of the stem bark of *B. stipularis* on brine shrimp nauplii.

Table 5.6: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *B. stipularis* on brine shrimp nauplii.

| Bridelia stipularis (Leaf) | | | | | | | | | | | |
|----------------------------|----------|-------------|-----|-----|------|------|---|------------------------------------|---------------------------------|----------------------------------|----------------------|
| Conc.
(C)
(µg/ml) | Log
C | % Mortality | | | | | LC ₅₀ , LC ₉₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 400 | 2.602 | 100 | 100 | 100 | 100 | 90 | 3.16±
0.18,
91.2±
1.36 | 6.31 ±
0.18,
100.0
± 1.71 | 1.99±
0.25,
95.5±
1.36 | 3.98±
0.98,
102.3±
0.76 | 12.59
± 1.3,
- |
| 200 | 2.301 | 100 | 100 | 90 | 90 | 70 | | | | | |
| 100 | 2.000 | 90 | 100 | 90 | 90 | 70 | | | | | |
| 50 | 1.699 | 80 | 90 | 80 | 90 | 60 | | | | | |
| 25 | 1.398 | 80 | 70 | 80 | 80 | 60 | | | | | |
| 12.5 | 1.097 | 70 | 50 | 70 | 70 | 50 | | | | | |
| 6.25 | 0.797 | 60 | 50 | 70 | 60 | 40 | | | | | |
| 3.125 | 0.495 | 60 | 50 | 70 | 50 | 30 | | | | | |
| 1.563 | 0.194 | 40 | 30 | 40 | 30 | 30 | | | | | |
| 0.781 | -0.107 | 30 | 10 | 30 | 20 | 10 | | | | | |

On the other hand, in case of brine shrimp lethality bioassay with *B. stipularis* leaf, the crude methanolic extract and its *n*-hexane, carbon tetrachloride and chloroform soluble fraction gave LC₅₀ values of 3.16, 6.31, 1.99 and 3.98 $\mu\text{g/ml}$ and LC₉₀ values of 91.2, 100.0, 95.5 and 102.3 $\mu\text{g/ml}$ respectively (Table 5.6) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC₅₀ value 0.45 $\mu\text{g/ml}$). The aqueous soluble fraction showed low cytotoxic activity with LC₅₀ value of 12.59 $\mu\text{g/ml}$.

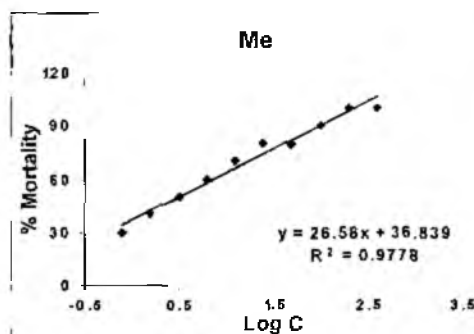


Figure 5.17: Effect of crude methanol extract of the leaf of *B. stipularis* on brine shrimp nauplii.

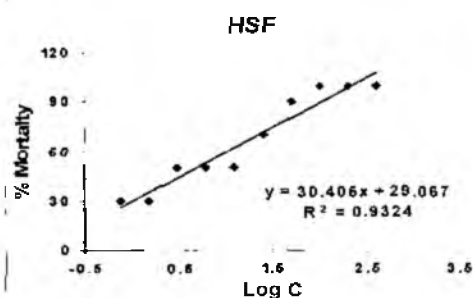


Figure 5.18: Effect of *n*-hexane soluble fraction of the leaf of *B. stipularis* on brine shrimp nauplii.

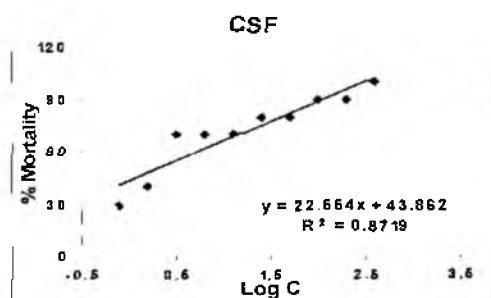


Figure 5.19: Effect of CCl₄ soluble fraction of the leaf of *B. stipularis* on brine shrimp nauplii.

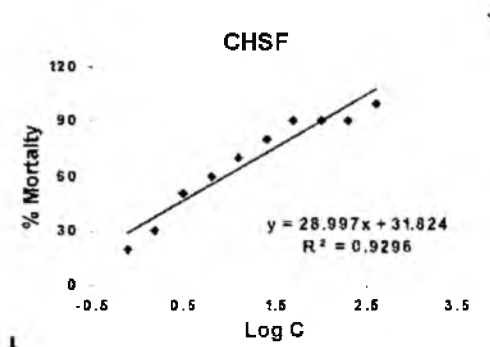


Figure 5.20: Effect of chloroform soluble fraction of the leaf of *B. stipularis* on brine shrimp nauplii.

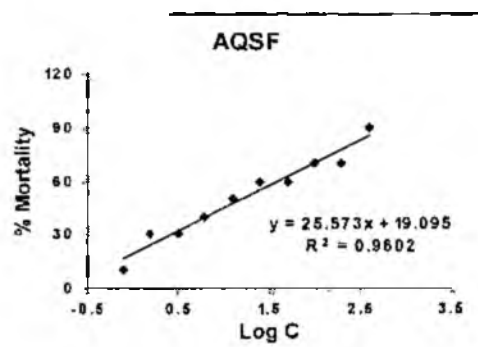


Figure 5.21: Effect of aqueous soluble fraction of the leaf of *B. stipularis* on brine shrimp nauplii.

Table 5.7: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *B. tomentosa* on brine shrimp nauplii.

| Bridelia tomentosa (Stem bark) | | | | | | | | | | | |
|--------------------------------|----------|-------------|-----|-----|------|------|---|----------------------------------|----------------------------------|---------------------------------|---------------------------------|
| Conc.
(C)
(µg/ml) | Log
C | % Mortality | | | | | LC ₅₀ , LC ₉₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 400 | 2.602 | 100 | 100 | 100 | 100 | 100 | 12.02±
0.38,
87.1±
1.85 | 8.13±
0.36,
141.2±
0.65 | 7.08±
1.00,
117.5±
1.31 | 1.59±
0.22,
56.2±
0.23 | 4.47±
0.73,
112.2±
0.4 |
| 200 | 2.301 | 100 | 100 | 90 | 100 | 100 | | | | | |
| 100 | 2.000 | 80 | 100 | 90 | 100 | 90 | | | | | |
| 50 | 1.699 | 80 | 90 | 90 | 80 | 80 | | | | | |
| 25 | 1.398 | 50 | 80 | 70 | 90 | 70 | | | | | |
| 12.5 | 1.097 | 40 | 60 | 70 | 90 | 60 | | | | | |
| 6.25 | 0.797 | 40 | 60 | 30 | 70 | 50 | | | | | |
| 3.125 | 0.495 | 30 | 30 | 30 | 60 | 40 | | | | | |
| 1.563 | 0.194 | 20 | 20 | 30 | 50 | 30 | | | | | |
| 0.781 | -0.107 | 10 | 20 | 20 | 30 | 20 | | | | | |

The brine shrimp lethality bioassay with *B. tomentosa* stem bark, the chloroform soluble fraction showed prominent cytotoxic activity with LC₅₀ and LC₉₀ values of 1.59 and 56.2 $\mu\text{g/ml}$, respectively. The crude methanolic extract and its *n*-hexane and carbon tetrachloride soluble fraction gave LC₅₀ values of 12.02, 8.13 and 7.08 $\mu\text{g/ml}$ and LC₉₀ values of 87.1, 141.2, and 117.5 $\mu\text{g/ml}$, respectively (Table 5.7) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC₅₀ value 0.45 $\mu\text{g/ml}$). The aqueous soluble fraction showed reasonable cytotoxic activity with LC₅₀ value of 4.47 $\mu\text{g/ml}$ and LC₉₀ value of 112.2 $\mu\text{g/ml}$.

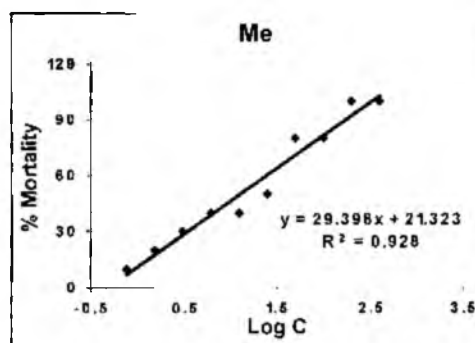


Figure 5.22: Effect of crude methanol extract of the stem bark of *B. tomentosa* on brine shrimp nauplii.

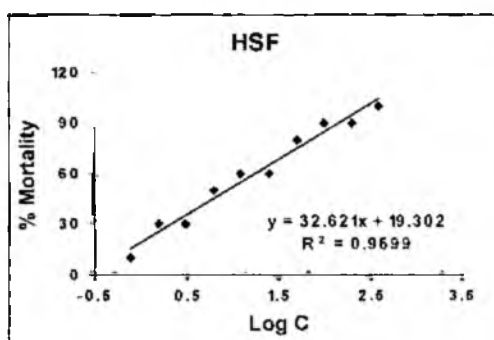


Figure 5.23: Effect of *n*-hexane soluble fraction of the stem bark of *B. tomentosa* on brine shrimp nauplii.

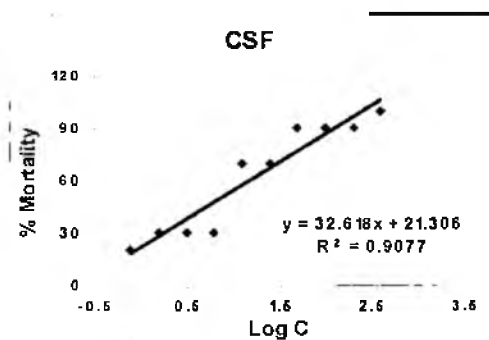


Figure 5.24: Effect of CCl_4 soluble fraction of the stem bark of *B. tomentosa* on brine shrimp nauplii.

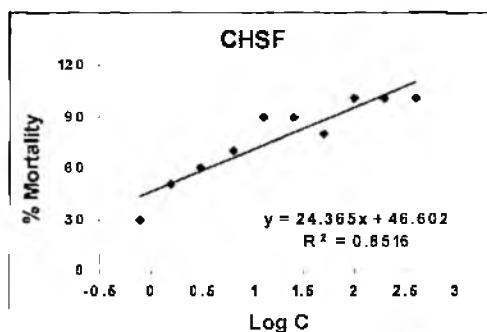


Figure 5.25: Effect of chloroform soluble fraction of the stem bark of *B. tomentosa* on brine shrimp nauplii.

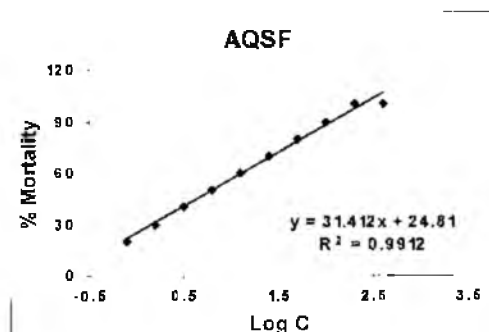


Figure 5.26: Effect of aqueous soluble fraction of the stem bark of *B. tomentosa* on brine shrimp nauplii.

Table 5.8: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *B. tomentosa* on brine shrimp nauplii.

| Bridelia tomentosa (Leaf) | | | | | | | | | | | |
|---------------------------|----------|-------------|-----|-----|------|------|---|---------------------------------|----------------------------------|---------------------------------|----------------------------------|
| Conc.
(C)
(µg/ml) | Log
C | % Mortality | | | | | LC ₅₀ , LC ₉₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 400 | 2.602 | 100 | 100 | 100 | 100 | 100 | 5.75±
0.42,
102.3±
0.75 | 3.55±
0.58,
75.9±
1.93 | 11.22±
0.4,
128.8±
1.12 | 4.37±
0.78,
69.2±
1.24 | 8.51±
1.21,
107.2±
0.41 |
| 200 | 2.301 | 100 | 100 | 90 | 100 | 100 | | | | | |
| 100 | 2.000 | 80 | 100 | 90 | 100 | 90 | | | | | |
| 50 | 1.699 | 80 | 90 | 90 | 100 | 80 | | | | | |
| 25 | 1.398 | 80 | 80 | 80 | 80 | 70 | | | | | |
| 12.5 | 1.097 | 70 | 80 | 50 | 70 | 60 | | | | | |
| 6.25 | 0.797 | 60 | 70 | 30 | 50 | 50 | | | | | |
| 3.125 | 0.495 | 50 | 50 | 20 | 40 | 30 | | | | | |
| 1.563 | 0.194 | 30 | 30 | 10 | 30 | 30 | | | | | |
| 0.781 | -0.107 | 10 | 10 | 10 | 20 | 10 | | | | | |

Yet again, brine shrimp lethality bioassay with *B. tomentosa* leaf, the crude methanolic extract and its *n*-hexane, and chloroform soluble fraction gave LC₅₀ values of 5.75, 3.55 and 4.37 µg/ml and LC₉₀ values of 102.3, 75.9 and 69.2 µg/ml, respectively (Table 5.8) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC₅₀ value 0.45 µg/ml). The carbon tetrachloride and aqueous soluble fraction showed low cytotoxic activity with LC₅₀ value of 11.22 and 8.51 µg/ml and LC₉₀ values of 128.8 and 107.2 µg/ml, respectively.

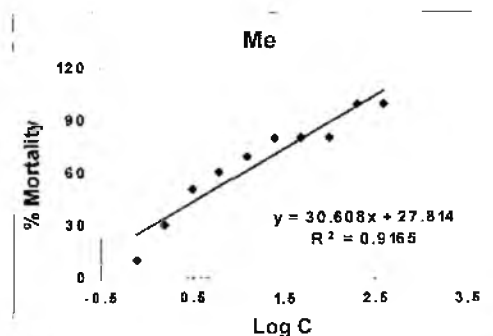


Figure 5.27: Effect of crude methanol extract of the leaf of *B. tomentosa* on brine shrimp nauplii.

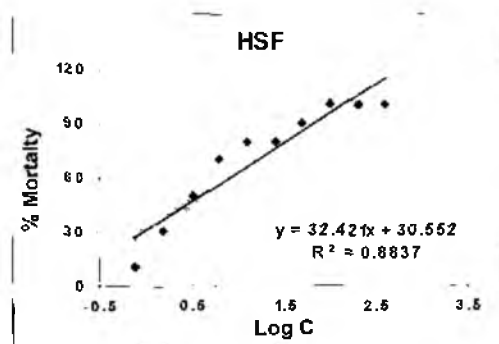


Figure 5.28: Effect of *n*-hexane soluble fraction of the leaf of *B. tomentosa* on brine shrimp nauplii.

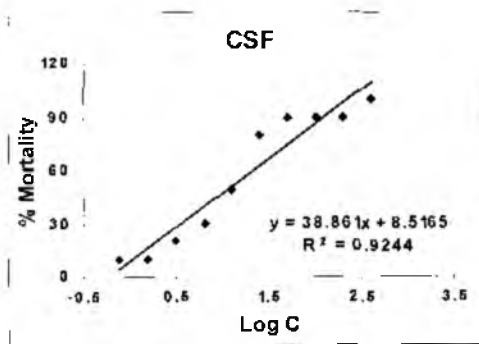


Figure 5.29: Effect of CCl_4 soluble fraction of the leaf of *B. tomentosa* on brine shrimp nauplii.

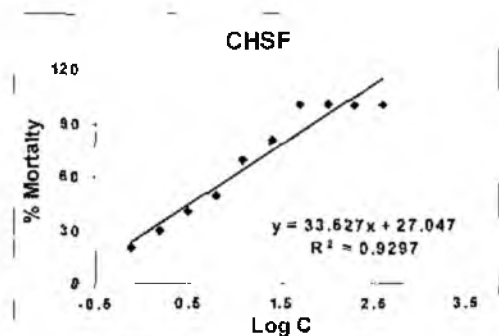


Figure 5.30: Effect of chloroform soluble fraction of the leaf of *B. tomentosa* on brine shrimp nauplii.

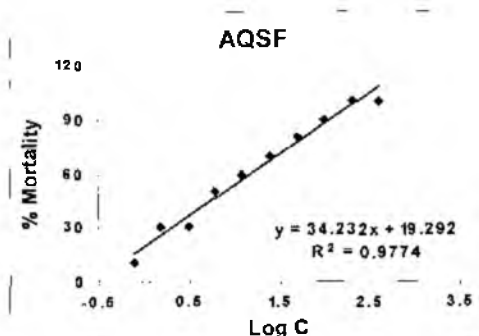


Figure 5.31: Effect of aqueous soluble fraction of the leaf of *B. tomentosa* on brine shrimp nauplii.

Table 5.9: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *E. fusca* on brine shrimp nauplii.

| <i>Erythrina fusca</i> (Stem bark) | | | | | | | | | | | |
|--------------------------------------|----------|-------------|---------|---------|----------|----------|---|---|---|---|---|
| Conc.
(C)
($\mu\text{g/ml}$) | Log
C | % Mortality | | | | | $\text{LC}_{50}, \text{LC}_{90}$ ($\mu\text{g/ml}$) | | | | |
| | | Me | HS
F | CS
F | CHS
F | AQS
F | Me | HSF | CSF | CHSF | AQSF |
| 400 | 2.60 | 10 | 100 | 100 | 100 | 100 | 9.45 $\pm$
0.45,
216.83
$\pm$ 0.43 | 4.96 $\pm$
0.59,
98.66
$\pm$ 0.3 | 1.55 $\pm$
0.3,
65.68
$\pm$ 0.23 | 4.79 $\pm$
0.65,
103.31
$\pm$ 0.49 | 8.95 $\pm$
0.70,
136.28
$\pm$ 0.47 |
| 200 | 2.30 | 70 | 100 | 100 | 90 | 100 | | | | | |
| 100 | 2.00 | 90 | 100 | 90 | 90 | 90 | | | | | |
| 50 | 1.69 | 80 | 80 | 90 | 80 | 70 | | | | | |
| 25 | 1.39 | 80 | 70 | 80 | 80 | 70 | | | | | |
| 12.5 | 1.09 | 70 | 70 | 90 | 70 | 50 | | | | | |
| 6.25 | 0.79 | 40 | 50 | 70 | 70 | 50 | | | | | |
| 3.125 | 0.49 | 40 | 40 | 70 | 60 | 30 | | | | | |

| | | | | | | | | | | |
|-------|-------|----|----|----|----|----|--|--|--|--|
| | 5 | | | | | | | | | |
| | 0.19 | 40 | 40 | 50 | 20 | 30 | | | | |
| 1.563 | 4 | | | | | | | | | |
| 0.781 | 0.197 | 30 | 20 | 20 | 10 | 10 | | | | |

The brine shrimp lethality bioassay with *E. fusca* stem bark, the carbon tetrachloride soluble fraction showed prominent cytotoxic activity with LC₅₀ and LC₉₀ values of 1.55 and 65.68 µg/ml. The *n*-hexane and chloroform soluble fraction gave LC₅₀ values of 4.96 and 4.79 µg/ml and LC₉₀ values of 98.66, and 103.31 µg/ml, respectively (Table 5.9) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC₅₀ value 0.45 µg/ml). The crude methanolic extract and aqueous soluble fraction showed reasonable cytotoxic activity with LC₅₀ value of 9.45 and 8.95 µg/ml, respectively and LC₉₀ value of 216.83 and 136.28 µg/ml, respectively.

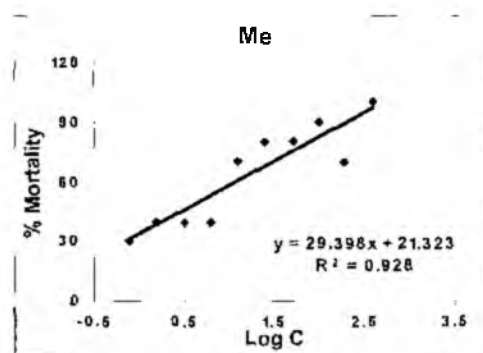


Figure 5.32: Effect of crude methanol extract of the stem bark of *E. fusca* on brine shrimp nauplii.

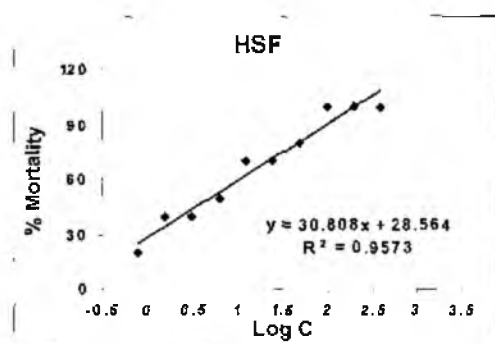


Figure 5.33: Effect of *n*-hexane soluble fraction of the stem bark of *E. fusca* on brine shrimp nauplii.

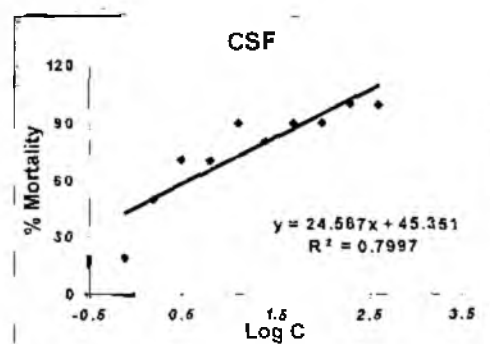


Figure 5.34: Effect of CCl₄ soluble fraction of the stem bark of *E. fusca* on brine shrimp nauplii.

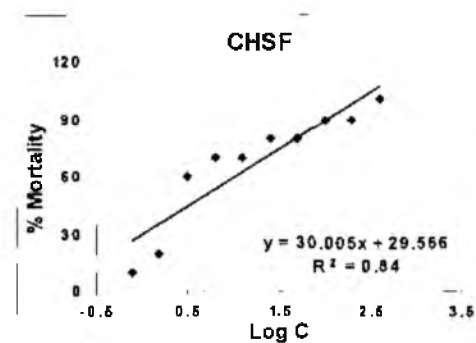


Figure 5.35: Effect of chloroform soluble fraction of the stem bark of *E. fusca* on brine shrimp nauplii.

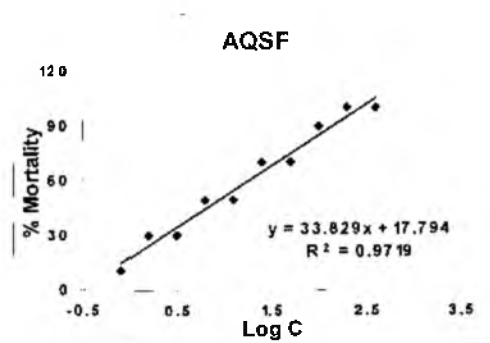


Figure 5.36: Effect of aqueous soluble fraction of the stem bark of *E. fusca* on brine shrimp nauplii.

Table 5.10: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *E. fusca* on brine shrimp nauplii.

| Erythrina fusca (Leaf) | | | | | | | | | | | |
|-------------------------|----------|-------------|-----|-----|------|------|---|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Conc.
(C)
(µg/ml) | Log
C | % Mortality | | | | | LC ₅₀ , LC ₉₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 400 | 2.602 | 100 | 100 | 100 | 100 | 100 | 7.34±
0.40,
137.7±
0.08 | 7.96±
0.19,
113.7±
0.38 | 6.82±
0.67,
163.4±
0.22 | 7.63±
0.16,
220.3±
0.33 | 7.89±
0.52,
116.2±
0.69 |
| 200 | 2.301 | 100 | 100 | 90 | 80 | 100 | | | | | |
| 100 | 2.000 | 80 | 90 | 80 | 80 | 90 | | | | | |
| 50 | 1.699 | 80 | 70 | 80 | 70 | 80 | | | | | |
| 25 | 1.398 | 70 | 80 | 70 | 70 | 70 | | | | | |
| 12.5 | 1.097 | 50 | 60 | 60 | 50 | 60 | | | | | |
| 6.25 | 0.797 | 50 | 60 | 50 | 50 | 50 | | | | | |
| 3.125 | 0.495 | 50 | 30 | 40 | 60 | 30 | | | | | |
| 1.563 | 0.194 | 30 | 20 | 30 | 40 | 30 | | | | | |
| 0.781 | -0.107 | 10 | 10 | 20 | 0 | 10 | | | | | |

Again, brine shrimp lethality bioassay with *E. fusca* leaf, the crude methanolic extract and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fraction gave LC₅₀ values of 7.34, 7.96, 6.82, 7.63 and 7.89 µg/ml and LC₉₀ values of 137.70, 113.77, 163.41, 220.35 and 116.29, respectively (Table 5.10) which exhibited low cytotoxic activity with comparison to the standard vincristine sulfate (LC₅₀ value 0.45 µg/ml).

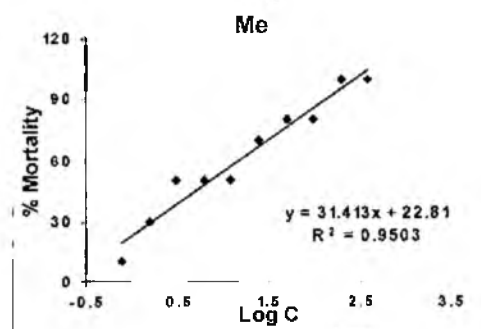


Figure 5.37: Effect of crude methanol extract of the leaf of *E. fusca* on brine shrimp nauplii.

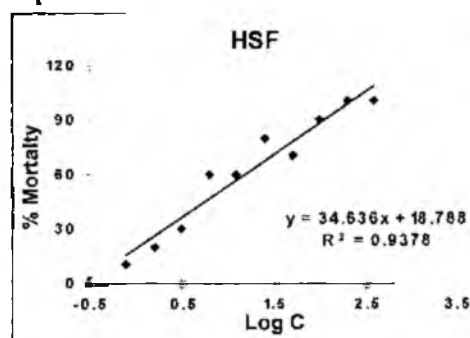


Figure 5.38: Effect of *n*-hexane soluble fraction of the leaf of *E. fusca* on brine shrimp nauplii.

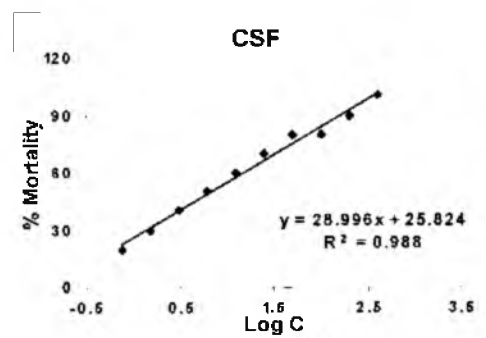


Figure 5.39: Effect of CCl_4 soluble fraction of the leaf of *E. fusca* on brine shrimp nauplii.

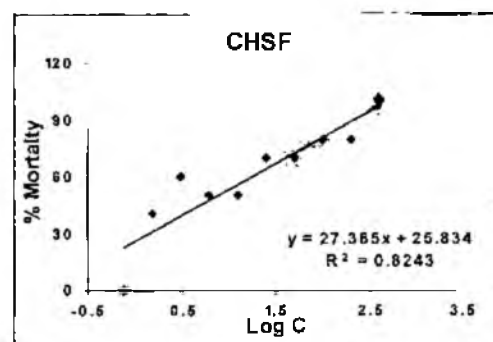


Figure 5.40: Effect of chloroform soluble fraction of the leaf of *E. fusca* on brine shrimp nauplii.

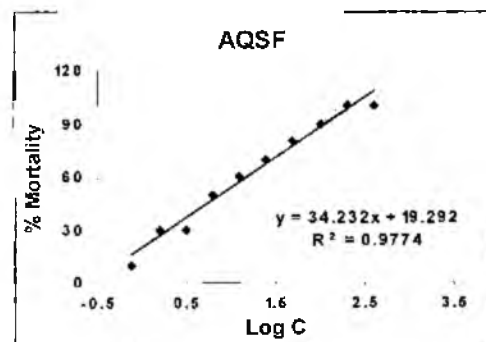


Figure 5.41: Effect of aqueous soluble fraction of the leaf of *E. fusca* on brine shrimp nauplii.

Table 5.11: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the stem bark of *E. variegata* on brine shrimp nauplii.

| <i>Erythrina variegata</i> (Stem bark) | | | | | | | | | | | |
|--|----------|-------------|-----|-----|------|------|--|--|--|---|--|
| Conc.
(C)
($\mu\text{g/ml}$) | Log
C | % Mortality | | | | | LC ₅₀ , LC ₉₀ ($\mu\text{g/ml}$) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 400 | 2.602 | 100 | 100 | 100 | 90 | 100 | 9.98 $\pm$
0.14,
174.1 $\pm$
0.86 | 14.66 $\pm$
0.75,
178.5 $\pm$
0.7 | 7.86 $\pm$
0.45,
150.2 $\pm$
1.19 | 12.87 $\pm$
0.99,
308.4 $\pm$
0.46 | 5.13 $\pm$
0.21,
94.42 $\pm$
0.63 |
| 200 | 2.301 | 90 | 90 | 100 | 80 | 100 | | | | | |
| 100 | 2.000 | 80 | 90 | 80 | 80 | 90 | | | | | |
| 50 | 1.699 | 70 | 70 | 70 | 70 | 80 | | | | | |
| 25 | 1.398 | 80 | 60 | 70 | 50 | 80 | | | | | |
| 12.5 | 1.097 | 50 | 50 | 60 | 60 | 70 | | | | | |
| 6.25 | 0.797 | 40 | 30 | 40 | 40 | 60 | | | | | |
| 3.125 | 0.495 | 30 | 10 | 50 | 40 | 50 | | | | | |
| 1.563 | 0.194 | 30 | 20 | 30 | 30 | 30 | | | | | |
| 0.781 | -0.107 | 10 | 10 | 10 | 0 | 10 | | | | | |

The lethality of the crude methanolic extract and its *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fraction of the stem bark of *E. variegata* gave LC₅₀ values of 9.98, 14.66, 7.86, 12.87 and 5.13 $\mu\text{g/ml}$ and LC₉₀ values of 174.13, 178.56, 150.25, 308.42 and 94.42 $\mu\text{g/ml}$ respectively (Table 5.11) which exhibited low cytotoxic activity with comparison to the standard vincristine sulfate (LC₅₀ value 0.45 $\mu\text{g/ml}$).

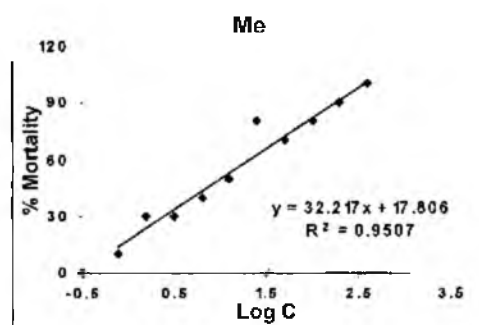


Figure 5.42: Effect of crude methanol extract of the stem bark of *E. variegata* on brine shrimp nauplii.

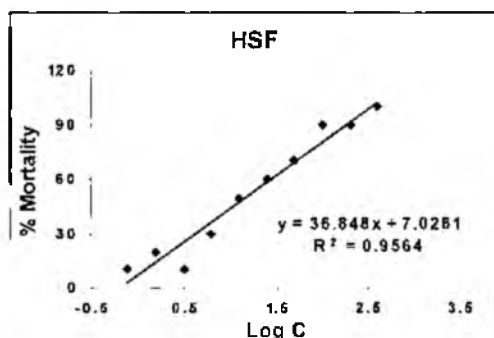


Figure 5.43: Effect of *n*-hexane soluble fraction of the stem bark of *E. variegata* on brine shrimp nauplii.

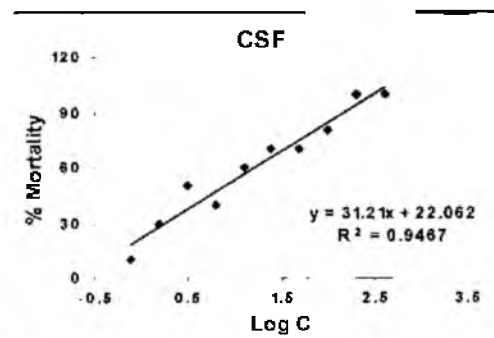


Figure 5.44: Effect of CCl_4 soluble fraction of the stem bark of *E. variegata* on brine shrimp nauplii.

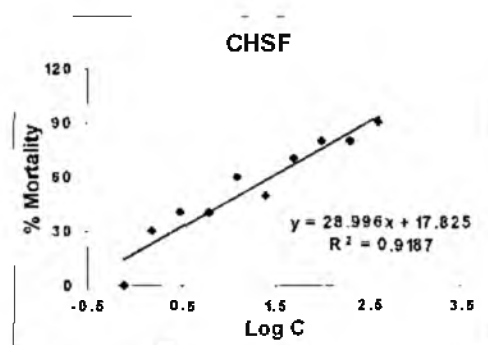


Figure 5.45: Effect of chloroform soluble fraction of the stem bark of *E. variegata* on brine shrimp nauplii.

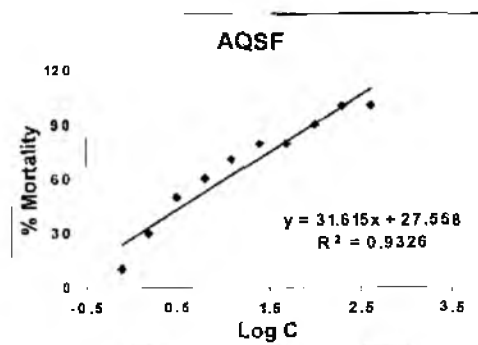


Figure 5.46: Effect of aqueous soluble fraction of the stem bark of *E. variegata* on brine shrimp nauplii.

Table 5.12: Effect of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fractions of the leaf of *E. variegata* on brine shrimp nauplii.

| Erythrina variegata (Leaf) | | | | | | | | | | | |
|--------------------------------------|----------|-------------|-----|-----|------|------|--|--|---|--|--|
| Conc.
(C)
($\mu\text{g/ml}$) | Log
C | % Mortality | | | | | LC ₅₀ , LC ₉₀ ($\mu\text{g/ml}$) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 400 | 2.602 | 100 | 100 | 100 | 100 | 100 | 6.61 $\pm$
0.41,
136.6 $\pm$
0.52 | 4.89 $\pm$
0.52,
85.19 $\pm$
0.50 | 10.81 $\pm$
0.92,
126.4 $\pm$
0.74 | 4.40 $\pm$
0.30,
96.79
$\pm$ 1.23 | 7.66 $\pm$
0.41,
124.5 $\pm$
0.32 |
| 200 | 2.301 | 90 | 100 | 90 | 100 | 90 | | | | | |
| 100 | 2.000 | 80 | 90 | 90 | 90 | 90 | | | | | |
| 50 | 1.699 | 80 | 90 | 80 | 90 | 80 | | | | | |
| 25 | 1.398 | 80 | 80 | 80 | 80 | 70 | | | | | |
| 12.5 | 1.097 | 70 | 70 | 60 | 60 | 70 | | | | | |
| 6.25 | 0.797 | 50 | 60 | 40 | 50 | 50 | | | | | |
| 3.125 | 0.495 | 40 | 50 | 20 | 50 | 40 | | | | | |
| 1.563 | 0.194 | 20 | 30 | 10 | 40 | 20 | | | | | |
| 0.781 | -0.107 | 20 | 10 | 10 | 20 | 10 | | | | | |

On the other hand, in case of brine shrimp lethality bioassay with *E. variegata* leaf, the crude methanolic extract and its *n*-hexane, chloroform and aqueous soluble fraction gave LC₅₀ values of 6.61, 4.89, 4.40 and 7.66 $\mu\text{g/ml}$ and LC₉₀ values of 136.65, 85.19, 96.79 and 124.58 $\mu\text{g/ml}$ respectively (Table 5.12) which exhibited low cytotoxic activity with comparison to the standard vincristine sulfate (LC₅₀ value 0.45 $\mu\text{g/ml}$). The carbon tetrachloride soluble fraction showed very low cytotoxic activity with LC₅₀ and LC₉₀ value of 10.81 and 126.47 $\mu\text{g/ml}$.

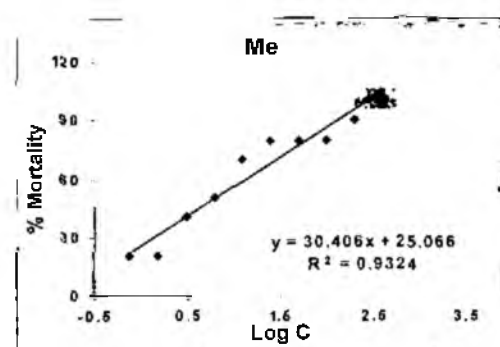


Figure 5.47: Effect of crude methanol extract of the leaf of *E. variegata* on brine shrimp nauplii.

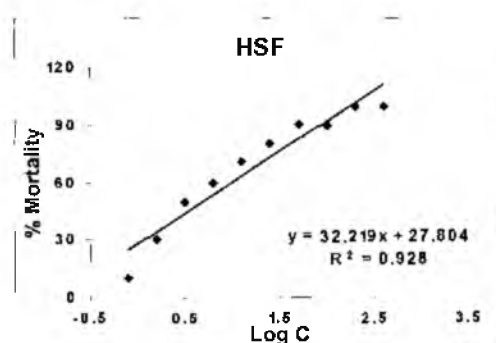


Figure 5.48: Effect of *n*-hexane soluble fraction of the leaf of *E. variegata* on brine shrimp nauplii.

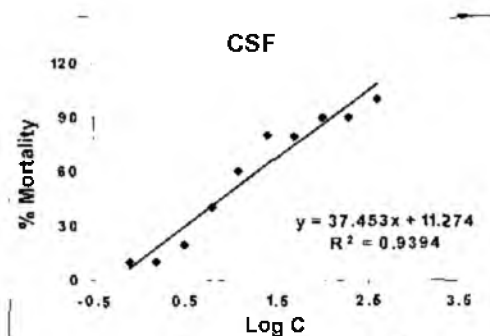


Figure 5.49: Effect of CCl_4 soluble fraction of the leaf of *E. variegata* on brine shrimp nauplii.

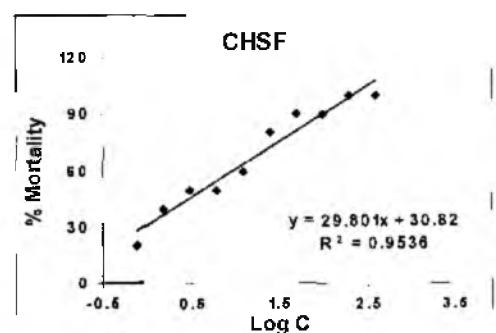


Figure 5.50: Effect of chloroform soluble fraction of the leaf of *E. variegata* on brine shrimp nauplii.

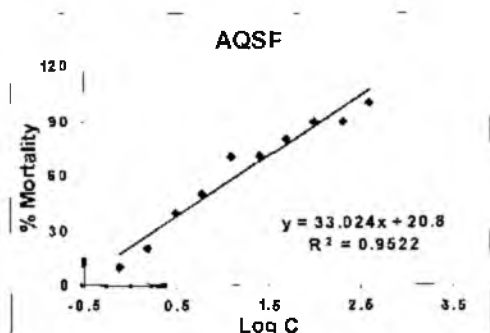


Figure 5.51: Effect of aqueous soluble fraction of the leaf of *E. variegata* on brine shrimp nauplii.

Table 5.13: Effect of pure compounds BVSP-4 (48), BVBC-2 (51) and BVS-65 (54) isolated from the stem bark of *B. verrucosa* on brine shrimp nauplii.

| <i>Bridelia verrucosa</i> (Stem bark) | | | | | | | |
|---------------------------------------|--------|-------------|-------------|-------------|---|--------------|---------------|
| Conc. (C) (µg/ml) | Log C | % Mortality | | | LC ₅₀ , LC ₉₀ (µg/ml) | | |
| | | BVSP-4 (48) | BVBC-2 (51) | BVS-65 (54) | BVSP-4 (48) | BVBC-2 (51) | BVS-65 (54) |
| 400 | 2.602 | 100 | 100 | 100 | 13.80, 213.79 | 8.11, 109.14 | 10.12, 164.82 |
| 200 | 2.301 | 90 | 100 | 100 | | | |
| 100 | 2.000 | 80 | 90 | 80 | | | |
| 50 | 1.699 | 70 | 80 | 70 | | | |
| 25 | 1.398 | 60 | 80 | 60 | | | |
| 12.5 | 1.097 | 40 | 60 | 60 | | | |
| 6.25 | 0.797 | 30 | 40 | 40 | | | |
| 3.125 | 0.495 | 30 | 40 | 30 | | | |
| 1.563 | 0.194 | 20 | 20 | 30 | | | |
| 0.781 | -0.107 | 10 | 10 | 10 | | | |

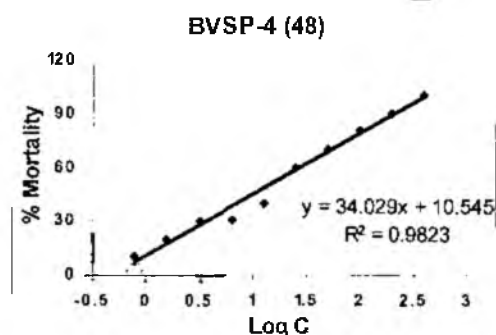


Figure 5.52: Effect of BVSP-4 (48) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

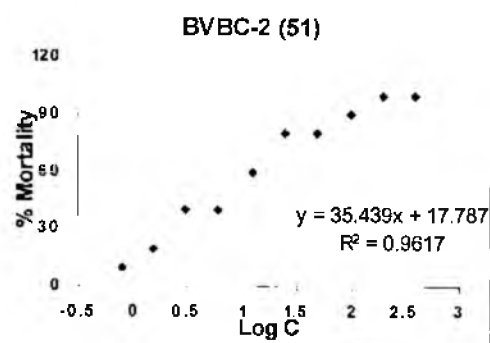


Figure 5.53: Effect of BVBC-2 (51) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

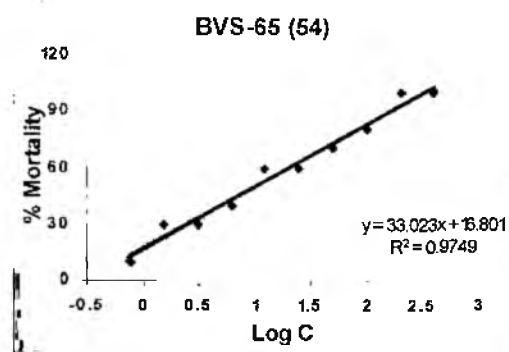


Figure 5.54: Effect of BVS-65 (54) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

Table 5.14: Effect of pure compounds BS-01 (55), BS-06 (56) and BT-6 (59) isolated from the stem bark of *B. stipularis* and *B. tomentosa* on brine shrimp nauplii.

| <i>Bridelia stipularis</i> and <i>B. tomentosa</i> (Stem bark) | | | | | | | |
|--|--------|-------------|------------|-----------|---|--------------|-------------|
| Conc. (C) (µg/ml) | Log C | % Mortality | | | LC ₅₀ , LC ₉₀ (µg/ml) | | |
| | | BS-01 (55) | BS-06 (56) | BT-6 (59) | BS-01 (55) | BS-06 (56) | BT-6 (59) |
| 400 | 2.602 | 100 | 90 | 100 | 7.64, 101.16 | 8.53, 218.27 | 7.71, 99.31 |
| 200 | 2.301 | 100 | 90 | 100 | | | |
| 100 | 2.000 | 90 | 80 | 90 | | | |
| 50 | 1.699 | 80 | 70 | 90 | | | |
| 25 | 1.398 | 80 | 70 | 80 | | | |
| 12.5 | 1.097 | 70 | 60 | 60 | | | |
| 6.25 | 0.797 | 50 | 50 | 50 | | | |
| 3.125 | 0.495 | 30 | 40 | 20 | | | |
| 1.563 | 0.194 | 20 | 30 | 30 | | | |
| 0.781 | -0.107 | 10 | 10 | 10 | | | |

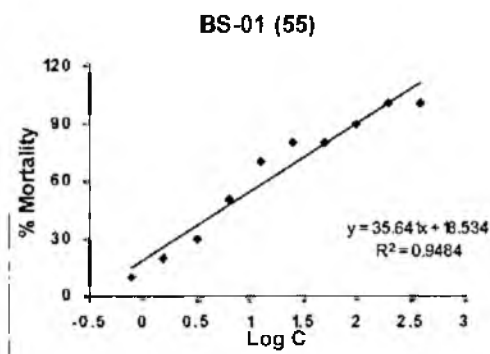


Figure 5.55: Effect of BS-01 (55) from the stem bark of *B. stipularis* on brine shrimp nauplii.

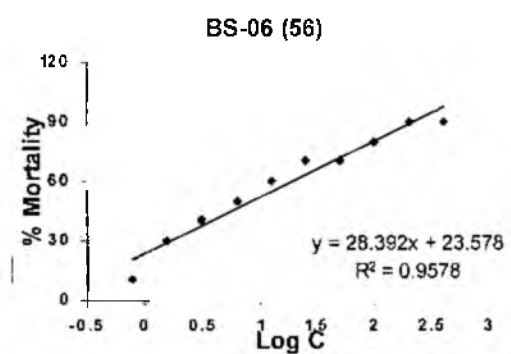


Figure 5.56: Effect of BS-06 (56) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

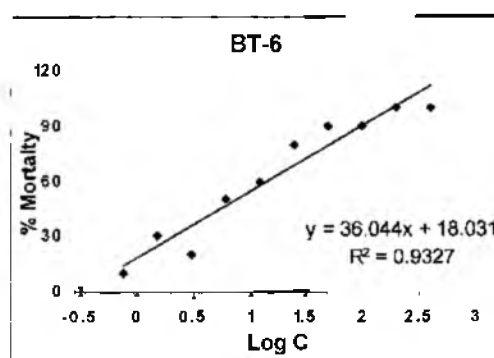


Figure 5.57: Effect of BT-6 (59) from the stem bark of *B. verrucosa* on brine shrimp nauplii.

Table 5.15: Effect of pure compounds EF-7 (62), EF-8 (63) and EF-12 (64) isolated from the stem bark of *E. fusca* on brine shrimp nauplii.

| Conc.
(C)
(µg/ml) | Log C | <i>Erythrina fusca</i> (Stem bark) | | | LC ₅₀ , LC ₉₀ (µg/ml) | | |
|-------------------------|--------|------------------------------------|-----------|------------|---|----------------|----------------|
| | | % Mortality | | | EF-7 (62) | EF-8 (63) | EF-12 (64) |
| | | EF-7 (62) | EF-8 (63) | EF-12 (64) | | | |
| 400 | 2.602 | 100 | 100 | 100 | 4.70,
129.12 | 3.17,
62.95 | 4.81,
86.90 |
| 200 | 2.301 | 90 | 90 | 100 | | | |
| 100 | 2.000 | 90 | 90 | 90 | | | |
| 50 | 1.699 | 70 | 90 | 90 | | | |
| 25 | 1.398 | 80 | 80 | 80 | | | |
| 12.5 | 1.097 | 60 | 70 | 70 | | | |
| 6.25 | 0.797 | 70 | 40 | 60 | | | |
| 3.125 | 0.495 | 50 | 40 | 50 | | | |
| 1.563 | 0.194 | 40 | 30 | 20 | | | |
| 0.781 | -0.107 | 10 | 10 | 20 | | | |

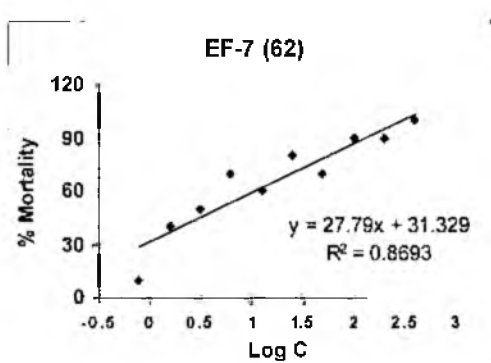


Figure 5.58: Effect of EF-7 (62) from the stem bark of *E. fusca* on brine shrimp nauplii.

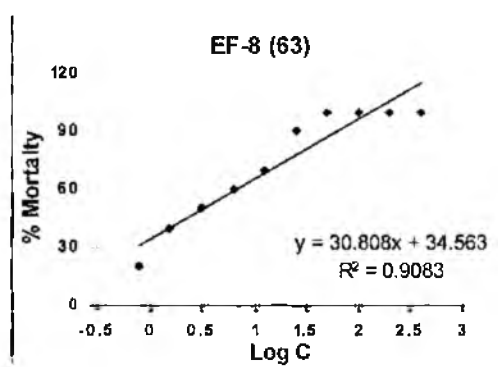


Figure 5.59: Effect of EF-8 (63) from the stem bark of *E. fusca* on brine shrimp nauplii.

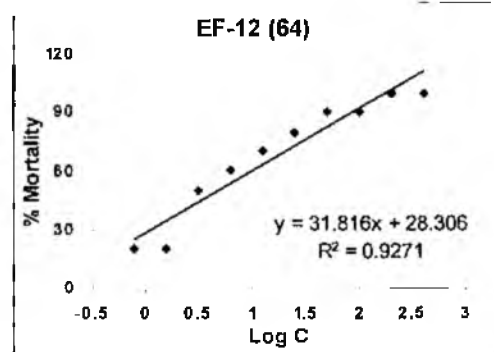


Figure 5.60: Effect of EF-12 (64) from the stem bark of *E. fusca* on brine shrimp nauplii.

The brine shrimp lethality bioassay with pure compounds showed moderate to mild cytotoxic activity. The LC_{50} and LC_{90} values of the pure compound EF-8 (**62**) isolated from the stem bark of *Erythrina fusca* gave promising LC_{50} and LC_{90} values of 3.17 and 62.95 $\mu\text{g/ml}$, respectively. The other isolated pure compounds BVSP-4 (**48**), BVBC-2 (**51**), BVS-65 (**54**), BS-01 (**55**), BS-06 (**56**) and BT-6 (**59**) gave LC_{50} values of 13.8, 8.11, 10.12, 7.64, 8.53 and 7.71 $\mu\text{g/ml}$ and LC_{90} values of 213.79, 109.14, 164.82, 101.16, 218.27 and 99.31 $\mu\text{g/ml}$, respectively (Table 5.13 and 5.14) which exhibited moderate cytotoxic activity with comparison to the standard vincristine sulfate (LC_{50} value 0.45 $\mu\text{g/ml}$). The other two pure compounds EF-7 (**61**) and EF-12 (**63**) showed reasonable cytotoxic activity with LC_{50} values of 4.70 and 4.81 $\mu\text{g/ml}$ and LC_{90} value of 129.12 and 86.90 $\mu\text{g/ml}$, respectively (Table 5.15).

5.7 Statistical Analysis

Three replicates of each sample were used for statistical analysis and the values are reported as mean $\pm$ SD.

5.8 Conclusion

The crude extracts of the plants, *B. verrucosa*, *B. stipularis*, *B. tomentosa*, *E. fusca* and *E. variegata* and their Kupchan fractions and some purified compounds were screened by brine shrimp lethality bioassay for probable cytotoxic activity. In this bioassay, the crude extract and compounds showed lethality indicating that the compounds are biologically active. Each of the test samples showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with increase in concentration of the test samples and a plot of percent mortality versus log concentration on the graph paper produced an approximate linear correlation between them. It is evident that all the test samples were lethal to brine shrimp nauplii.

From the results of the brine shrimp lethality bioassay and comparison with positive control vincristine sulfate signifies that cytotoxicity exhibited by the crude extracts and further bioactivity guided investigation can be done to find out potent antitumor and pesticidal compound. However, this can not be confirmed without further studies and specific tests.



Chapter 6

Evaluation of Antioxidant Activity

6.1 Assays for total phenolic contents

6.1.1 Principle

Total phenolic content of *B. verrucosa*, *B. stipularis*, *B. tomentosa*, *E. fusca* and *E. variegata* extractives were measured employing the method as described by Skerget *et al.*, 2005 involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard (Majhenic *et al.*, 2007).

6.1.2 Standard curve preparation

Gallic acid (standard) solution of different concentration ranging from 100 µg/ ml to 0 µg / ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na₂CO₃ (7.5% w/v) solution was added to 0.5 ml of gallic acid solution. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 760 nm. A graph was constructed by plotting the absorbance in ordinate against the concentration in abscissa where a linear relationship was obtained which was used as a standard curve for determination of the total phenolic content of the samples.

6.1.3 Sample preparation

1 mg of each of the extractives was taken and dissolved in the distilled water to get a sample concentration of 250 µg/ml in every case.

6.2 Antioxidant activity evaluation by DPPH method

6.2.1 Principle

The free radical scavenging activity (antioxidant capacity) of the plant extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was estimated by the method developed by Brand-Williams *et al.*, 1995.

6.3 Total antioxidant assay by phosphomolybdenum method

6.3.1 Principle

The phosphomolybdenum method was based on the reduction of molybdenum, Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate-Mo (V) complex with a maximal absorption at 695 nm. As it being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extracts (Prieto *et al.*, 1999).

6.4 Results and discussions

6.4.1 Total phenolic content (TPC)

The methanolic extract of the stem bark and the leaf of the three *Bridelia* species, *B. verrucosa*, *B. stipularis*, *B. tomentosa* and also two *Erythrina* species, *E. fusca* and *E. variegata* along with their different partitionates i.e. *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble materials were subjected to total phenolic content determination. Based on the absorbance values of the various test solutions, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid (Table 6.1), results of the colorimetric analysis of the total phenolics are given in Table 6.2 and 6.3 Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent)/100 g of extractives.

Table 6.1: Standard curve preparation by using gallic acid.

| SL. No. | Conc. Of the standard (µg/ml) | Absorbance | Regression line | R ² |
|---------|-------------------------------|------------|------------------------|----------------|
| 1 | 100 | 1.620 | $y = 0.0162x + 0.0215$ | 0.9985 |
| 2 | 50 | 0.866 | | |
| 3 | 25 | 0.450 | | |
| 4 | 12.5 | 0.253 | | |
| 5 | 6.25 | 0.120 | | |
| 6 | 3.125 | 0.059 | | |
| 7 | 1.5625 | 0.034 | | |
| 8 | 0.78125 | 0.022 | | |
| 9 | 0.3906 | 0.020 | | |
| 10 | 0 | 0.011 | | |

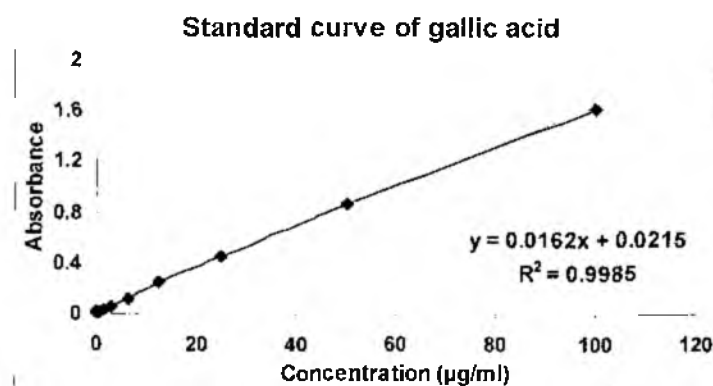


Figure 6.1: Standard curve of gallic acid for total phenolic content determination.

Table 6.2: Results of the test samples for total phenolic content determination of *Bridelia* species.

| Sample | Conc. (µg/ml) | Mean Absorbance | Gallic acid equivalent
(mg of GAE/100 g of dried extract) |
|----------------------------------|---------------|-----------------|--|
| <i>B. verrucosa</i> (Stem bark) | | | |
| Me | 250 | 0.069±0.004 | 2.95±0.22 |
| HSF | 250 | 0.98±0.008 | 59.15±0.46 |
| CSF | 250 | 1.043±0.011 | 63.03±0.65 |
| CHSF | 250 | 1.125±0.098 | 68.14±6.02 |
| AQSF | 250 | 1.174±0.010 | 89.14±0.62 |
| <i>B. verrucosa</i> (Leaf) | | | |
| Me | 250 | 0.144±0.013 | 7.54±0.77 |
| HSF | 250 | 1.045±0.006 | 63.18±0.37 |
| CSF | 250 | 0.763±0.003 | 45.79±0.16 |
| CHSF | 250 | 0.074±0.011 | 3.26±0.65 |
| AQSF | 250 | 1.328±0.012 | 80.63±0.74 |
| <i>B. stipularis</i> (Stem bark) | | | |
| Me | 250 | 0.324±0.030 | 18.67±1.84 |
| HSF | 250 | 1.059±0.032 | 63.90±1.99 |
| CSF | 250 | 0.282±0.020 | 16.08±1.22 |
| CHSF | 250 | 0.349±0.018 | 20.22±1.11 |
| AQSF | 250 | 1.424±0.009 | 86.59±0.58 |
| <i>B. stipularis</i> (Leaf) | | | |
| Me | 250 | 0.562±0.022 | 33.38±1.33 |
| HSF | 250 | 0.764±0.013 | 45.85±0.77 |
| CSF | 250 | 0.843±0.018 | 50.71±1.13 |
| CHSF | 250 | 1.432±0.204 | 87.31±0.59 |
| AQSF | 250 | 1.621±0.021 | 98.76±1.27 |
| <i>B. tomentosa</i> (Stem bark) | | | |
| Me | 250 | 0.517±0.011 | 30.59±0.68 |
| HSF | 250 | 0.813±0.013 | 48.86±0.81 |
| CSF | 250 | 1.259±0.068 | 76.39±4.20 |
| CHSF | 250 | 1.233±0.265 | 74.78±1.33 |
| AQSF | 250 | 1.574±0.041 | 95.81±1.54 |
| <i>B. tomentosa</i> (Leaf) | | | |
| Me | 250 | 0.437±0.016 | 25.65±0.99 |
| HSF | 250 | 0.752±0.012 | 45.09 ±0.71 |
| CSF | 250 | 0.637±0.004 | 38.01±0.23 |
| CHSF | 250 | 0.426±0.012 | 24.97±0.74 |
| AQSF | 250 | 1.432±0.020 | 87.05±1.24 |

Values are expressed as average±SD (n=3); Me = Methanolic extract; HSF = *n*-hexane soluble fraction; CSF = carbon tetrachloride soluble fraction; CHSF = chloroform soluble fraction and AQSF = aqueous soluble fraction.

The amount of total phenolic content was found differ in different extractives compared with gallic acid equivalent. The amount of total phenolic content in case of the three *Bridelia* species varied for different partitionates ranging from 2.95 mg to 98.76 mg of GAE/100 g of dried extract. The highest total phenolic content was found from aqueous soluble fraction of the leaf of *B. stipularis* (98.76 mg of GAE/100 g of dried extract) and

the lowest from the stem bark of the methanolic extract of *B. verrucosa* (2.95 mg of GAE/100 g of dried extract).

Table 6.3: Results of the test samples for total phenolic content determination *Erythrina* species.

| Sample | Cone. (µg/ml) | Mean Absorbance | Gallic acid equivalent
(mg of GAE / 100 g of dried extract) |
|---------------------------------|---------------|-----------------|--|
| <i>E. fusca</i> (Stem bark) | | | |
| Me | 250 | 0.187±0.008 | 10.22±0.505 |
| HSF | 250 | 0.768±0.008 | 46.11±0.463 |
| CSF | 250 | 0.164±0.006 | 8.82±0.351 |
| CHSF | 250 | 0.458±0.013 | 26.92±0.829 |
| AQSF | 250 | 1.158±0.008 | 70.17±0.463 |
| <i>E. fusca</i> (Leaf) | | | |
| Me | 250 | 0.380±0.010 | 22.13±0.617 |
| HSF | 250 | 0.207±0.010 | 11.47±0.600 |
| CSF | 250 | 0.232±0.003 | 12.97±0.189 |
| CHSF | 250 | 0.522±0.003 | 30.90±0.223 |
| AQSF | 250 | 1.545±0.016 | 94.02±1.004 |
| <i>E. variegata</i> (Stem bark) | | | |
| Me | 250 | 0.172±0.014 | 9.31±0.840 |
| HSF | 250 | 0.564±0.015 | 33.51±0.916 |
| CSF | 250 | 0.087±0.012 | 4.02±0.747 |
| CHSF | 250 | 1.247±0.005 | 75.65±0.327 |
| AQSF | 250 | 1.553±0.048 | 94.52±2.981 |
| <i>E. variegata</i> (Leaf) | | | |
| Me | 250 | 1.157±0.023 | 70.11±1.448 |
| HSF | 250 | 0.504±0.007 | 29.78±0.432 |
| CSF | 250 | 0.842±0.018 | 50.65±1.123 |
| CHSF | 250 | 0.065±0.006 | 2.66±0.372 |
| AQSF | 250 | 1.33±0.020 | 80.77±1.235 |

Values are expressed as average±SD (n=3); Me = Methanolic extract; HSF = *n*-hexane soluble fraction; CSF = carbon tetrachloride soluble fraction; CHSF = chloroform soluble fraction and AQSF = aqueous soluble fraction.

Similarly, for the two *Erythrina* species the total phenolic content varied from 2.66 g to 94.52 g of GAE/100 g dried extract). The highest total phenolic content was found from aqueous soluble fraction of the stem bark of *E. variegata* (94.52 g of GAE/100 g of dried extract) and the lowest from the leaf of chloroform soluble fraction of *E. variegata* (2.66 g of GAE/100 g of dried extract).

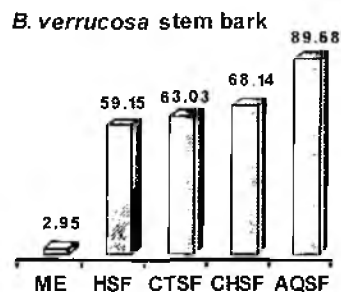


Figure 6.2: Total phenolic content (mg of GAE/gm of extractives) of different extracts of the stem bark of *B. verrucosa*.

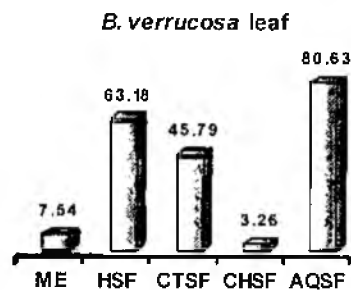


Figure 6.3: Total phenolic content (mg of GAE/gm of extractives) of different extracts of the leaf of *B. verrucosa*.

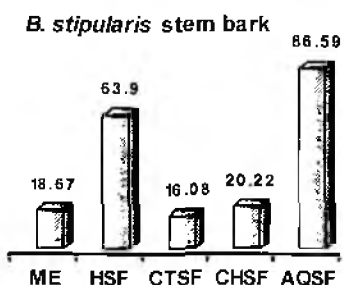


Figure 6.4: Total phenolic content (mg of GAE/gm of extractives) of different extracts of the stem bark of *B. stipularis*.

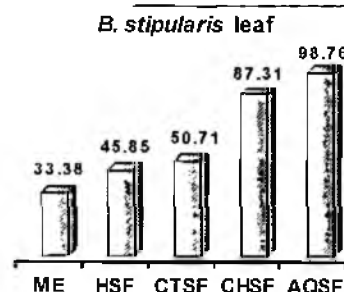


Figure 6.5: Total phenolic content (mg of GAE/gm of extractives) of different extracts of the leaf of *B. stipularis*.

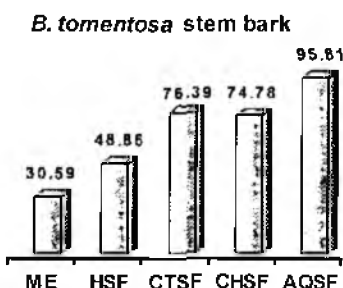


Figure 6.6: Total phenolic content (mg of GAE/gm of extractives) of different extracts of the stem bark of *B. tomentosa*.

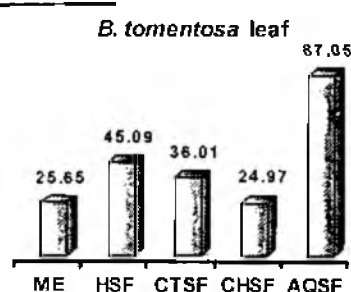


Figure 6.7: Total phenolic content (mg of GAE/gm of extractives) of different extracts of the leaf of *B. tomentosa*.

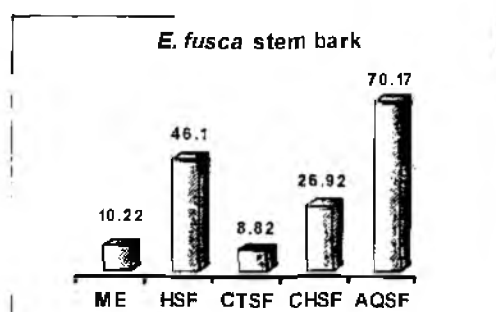


Figure 6.8: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the stem bark of *E. fusca*.

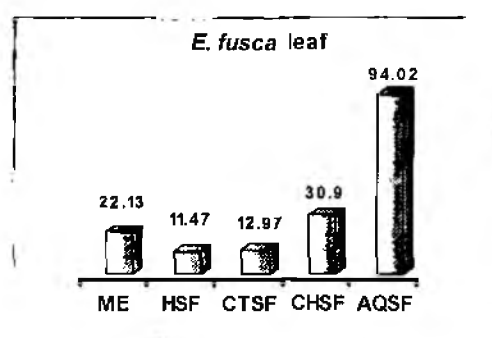


Figure 6.9: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the leaf of *E. fusca*.

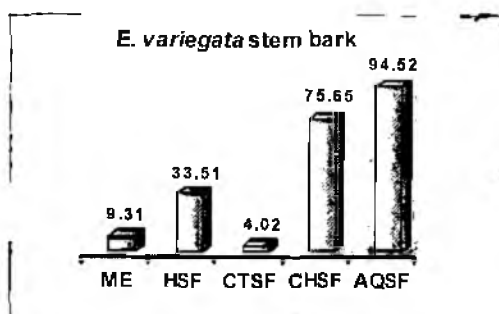


Figure 6.10: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the stem bark of *E. variegata*.

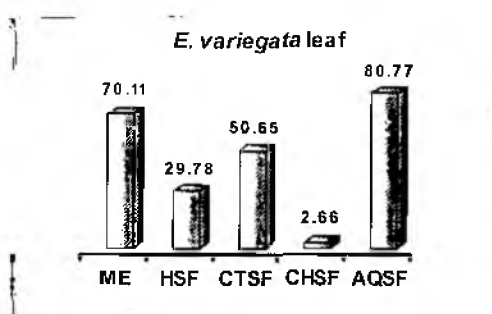


Figure 6.11: Total phenolic content (mg of GAE/gm of extractives) of different extractives of the leaf of *E. variegata*.

6.4.2 Antioxidant activity evaluation by DPPH method

The methanolic extract of the three *Bridelia* species, *B. verrucosa*, *B. stipularis* and *B. tomentosa* and two species of *Erythrina* species, *E. fusca* and *E. variegata* available in Bangladesh (Me) and different partitionates i.e. *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous (AQSF) soluble fractions were subjected to free radical scavenging activity by the method of Brand-Williams *et al.*, 1995. Smaller IC_{50} value corresponds to a higher activity of the plant extracts (Maisuthisakul *et al.*, 2007). Here, *tert*-butyl-1-hydroxytoluene (BHT) and ascorbic acid (ASA) were used as reference standard.

In this investigation, the free radical scavenging activity of the methanolic extract of the leaf of *B. verrucosa* was found to be highest having IC_{50} value of 6.35 μ g/ml and lowest activity was shown by the aqueous soluble fraction of the leaf of *B. tomentosa* with IC_{50} being 26.4 μ g/ml. The other soluble fractions of the three plants demonstrated high to moderate free radical scavenging activity with the IC_{50} value ranging from 7.34 μ g/ml to 21.3 μ g/ml, as compared to the standards, i.e. *tert*-butyl-1-hydroxytoluene (BHT),

(IC_{50} = 24.35 μ g/ml) and ascorbic acid, ASA (IC_{50} = 5.80 μ g/ml). Again, for the *Erythrina* species free radical scavenging activity of the chloroform soluble fraction of the leaf of *E. fusca* was highest with IC_{50} value of 5.5 μ g/ml and lowest activity was shown by the chloroform soluble fraction of the leaf of *E. variegata* with IC_{50} being 21.3 μ g/ml. Other soluble fractions of the two plants demonstrated high to moderate free radical scavenging activity with the IC_{50} value ranging from 5.5 μ g/ml to 21.3 μ g/ml, as compared to the standards. The IC_{50} (7.3 μ g/ml) of the isolated pure compound BVBC-2 (**51**) indicated strong free radical scavenging activity. All the results are shown in Tables 6.4-6.15 and Figures 6.12-6.65.

Table 6.4: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *B. verrucosa*.

| <i>B. verrucosa</i> (Stem bark) | | | | | | | | | | | |
|---------------------------------|---------------------|--------------|-------|-------|-------|-------|-------------------------|---------------------|---------------------|--------------------|--------------------|
| Blank | Conc. (μ g/ml) | % Inhibition | | | | | IC_{50} (μ g/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 0.395 | 500 | 85.06 | 90.63 | 92.66 | 90.63 | 92.91 | 11.6 $\pm$
0.39 | 10.51 $\pm$
0.43 | 11.65 $\pm$
0.80 | 15.8 $\pm$
0.15 | 15.5 $\pm$
0.36 |
| 0.395 | 250 | 75.95 | 90.38 | 88.61 | 85.57 | 87.59 | | | | | |
| 0.395 | 125 | 57.22 | 85.06 | 82.53 | 82.53 | 74.94 | | | | | |
| 0.395 | 62.5 | 58.48 | 82.03 | 76.46 | 81.01 | 70.89 | | | | | |
| 0.395 | 31.25 | 65.57 | 83.54 | 73.16 | 65.82 | 60.51 | | | | | |
| 0.395 | 15.625 | 52.91 | 72.91 | 55.19 | 49.62 | 49.87 | | | | | |
| 0.395 | 7.813 | 46.08 | 31.39 | 38.48 | 32.15 | 44.05 | | | | | |
| 0.395 | 3.906 | 36.46 | 23.8 | 11.65 | 30.13 | 25.06 | | | | | |
| 0.395 | 1.953 | 23.8 | 12.41 | 8.61 | 7.85 | 20.51 | | | | | |
| 0.395 | 0.0977 | 12.41 | 4.05 | 4.56 | 3.54 | 15.95 | | | | | |

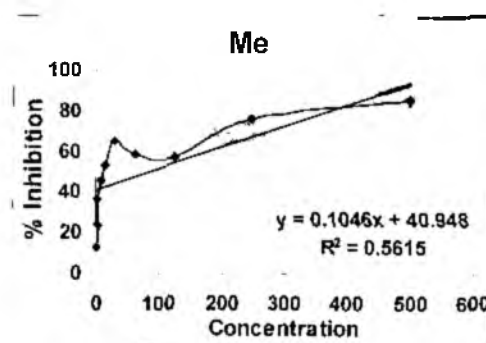


Figure 6.12: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *B. verrucosa*.

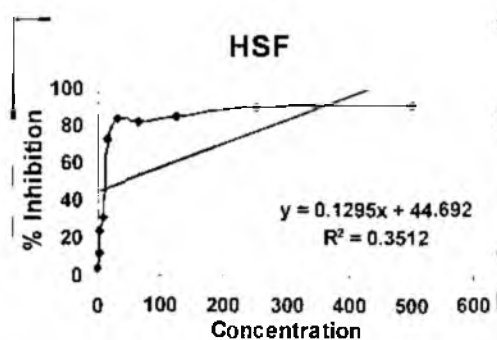


Figure 6.13: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *B. verrucosa*.

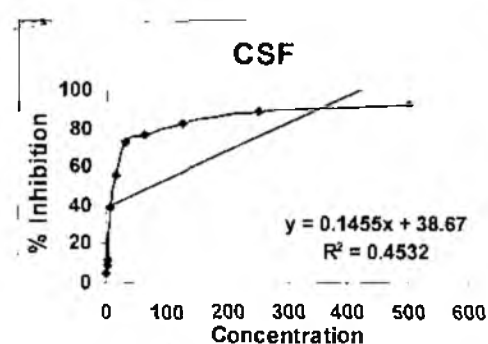


Figure 6.14: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the stem bark of *B. verrucosa*.

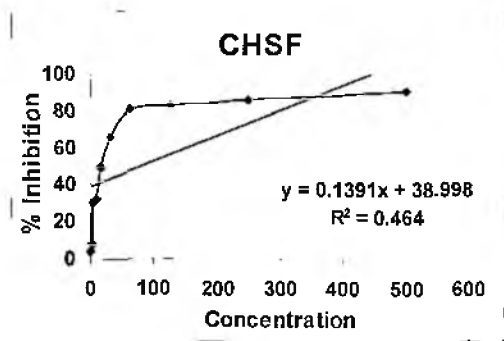


Figure 6.15: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *B. verrucosa*.

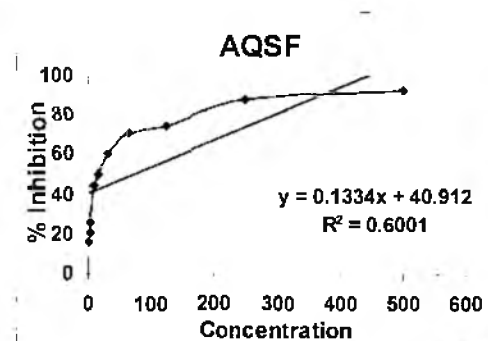


Figure 6.16: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *B. verrucosa*.

Table 6.5: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *B. verrucosa*.

| B. verrucosa (leaf) | | | | | | | | | | | |
|---------------------|---------------|--------------|-------|-------|-------|-------|--------------------------|---------------|--------------|---------------|---------------|
| Blank | Conc. (µg/ml) | % Inhibition | | | | | IC ₅₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 0.395 | 500 | 89.11 | 90.38 | 90.63 | 93.67 | 76.46 | 6.35±
0.80 | 25.8±
0.21 | 5.9±
0.85 | 5.25±
0.10 | 15.8±
0.40 |
| 0.395 | 250 | 85.57 | 87.09 | 91.9 | 90.63 | 75.44 | | | | | |
| 0.395 | 125 | 85.57 | 76.2 | 85.82 | 86.84 | 70.38 | | | | | |
| 0.395 | 62.5 | 84.3 | 65.32 | 75.7 | 80 | 69.37 | | | | | |
| 0.395 | 31.25 | 81.27 | 52.15 | 67.59 | 77.97 | 57.22 | | | | | |
| 0.395 | 15.625 | 69.87 | 42.28 | 65.82 | 67.09 | 50.13 | | | | | |
| 0.395 | 7.813 | 51.65 | 26.84 | 64.05 | 65.32 | 43.54 | | | | | |
| 0.395 | 3.906 | 42.28 | 23.8 | 25.06 | 37.72 | 24.81 | | | | | |
| 0.395 | 1.953 | 33.16 | 17.22 | 23.54 | 30.13 | 20.51 | | | | | |
| 0.395 | 0.0977 | 28.86 | 4.3 | 9.87 | 16.71 | 12.15 | | | | | |

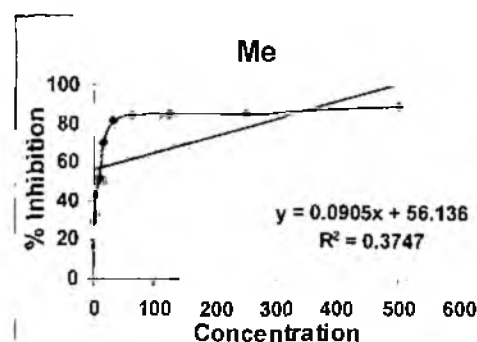


Figure 6.17: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *B. verrucosa*

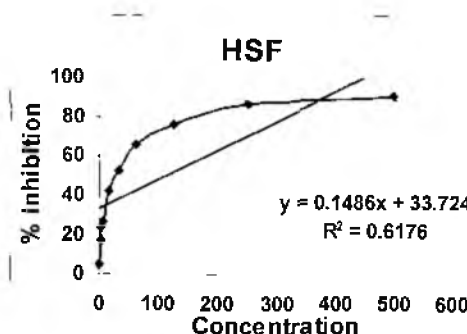


Figure 6.18: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *B. verrucosa*

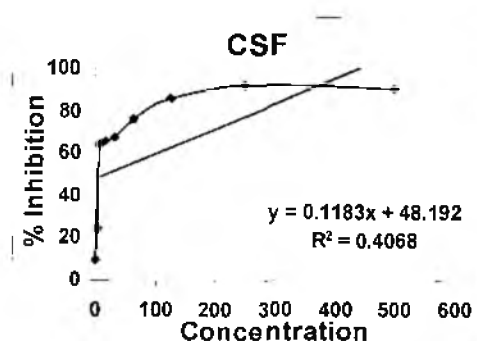


Figure 6.19: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the leaf of *B. verrucosa*

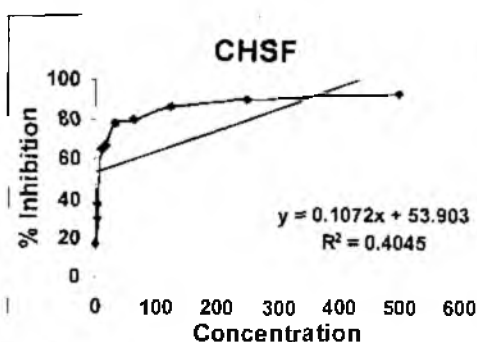


Figure 6.20: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *B. verrucosa*.

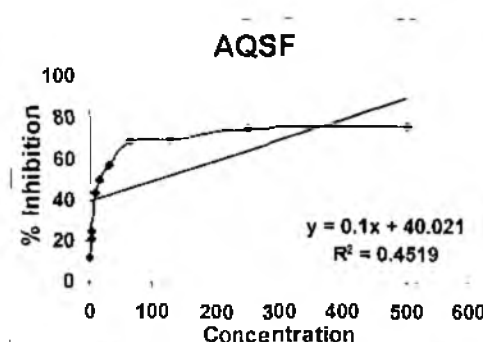


Figure 6.21: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *B. verrucosa*.

Table 6.6: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *B. stipularis*.

| <i>B. stipularis</i> (stem bark) | | | | | | | | | | | |
|----------------------------------|---------------|--------------|-------|-------|-------|-------|--------------------------|----------------|----------------|---------------|---------------|
| Blank | Conc. (µg/ml) | % Inhibition | | | | | IC ₅₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 0.395 | 500 | 94.68 | 90.38 | 86.58 | 94.68 | 87.59 | 9.10±
0.40 | 10.20±
0.55 | 15.60±
0.50 | 13.2±
0.10 | 21.3±
0.40 |
| 0.395 | 250 | 92.15 | 85.06 | 78.23 | 92.15 | 80.25 | | | | | |
| 0.395 | 125 | 86.58 | 80.25 | 65.06 | 86.84 | 70.63 | | | | | |
| 0.395 | 62.5 | 77.97 | 71.39 | 61.01 | 82.53 | 56.2 | | | | | |
| 0.395 | 31.25 | 75.7 | 70.13 | 54.94 | 76.46 | 49.87 | | | | | |
| 0.395 | 15.625 | 71.9 | 68.61 | 49.62 | 52.91 | 45.32 | | | | | |
| 0.395 | 7.813 | 43.29 | 37.47 | 23.54 | 42.03 | 39.49 | | | | | |
| 0.395 | 3.906 | 25.82 | 23.54 | 17.47 | 30.13 | 24.81 | | | | | |
| 0.395 | 1.953 | 14.43 | 10.63 | 11.65 | 21.27 | 13.16 | | | | | |
| 0.395 | 0.0977 | 6.58 | 1.52 | 2.03 | 13.67 | 9.87 | | | | | |

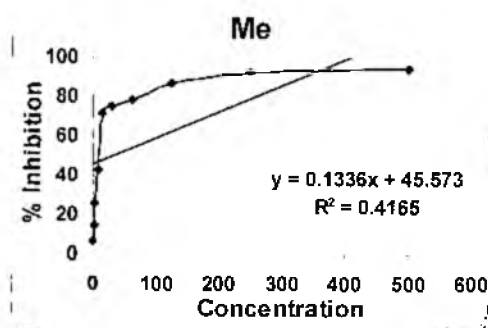


Figure 6.22: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *B. stipularis*.

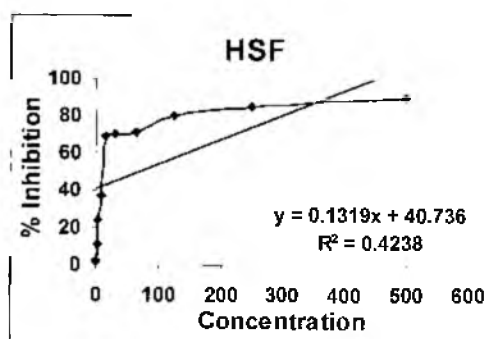


Figure 6.23: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *B. stipularis*.

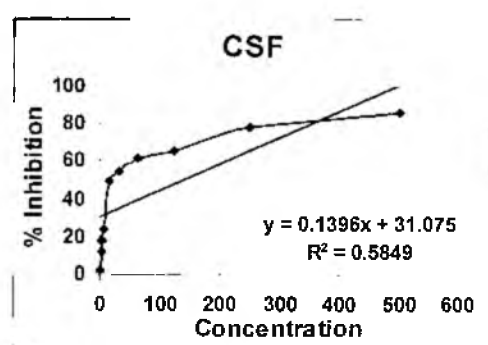


Figure 6.24: Free radical scavenging activity of the CCl₄ soluble materials (CSF) of the stem bark of *B. stipularis*.

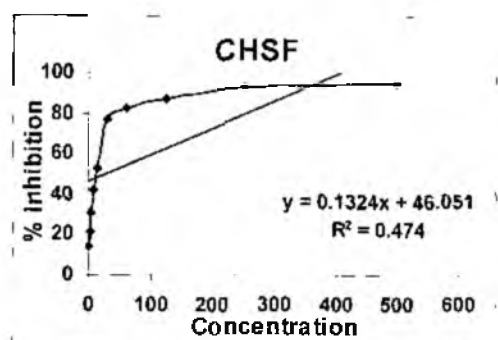


Figure 6.25: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *B. stipularis*.

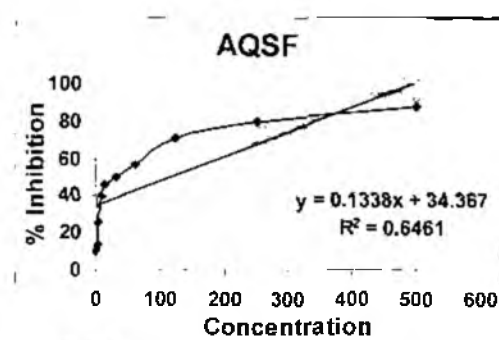


Figure 6.26: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *B. stipularis*.

Table 6.7: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *B. stipularis*.

| <i>B. stipularis</i> (leaf) | | | | | | | | | | | |
|-----------------------------|---------------|--------------|-------|-------|-------|-------|--------------------------|---------------|---------------|---------------|---------------|
| Blank | Conc. (µg/ml) | % Inhibition | | | | | IC ₅₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 0.395 | 500 | 87.59 | 79.24 | 90.13 | 93.42 | 85.82 | 13.9±
0.80 | 11.8±
0.55 | 9.75±
0.74 | 7.34±
0.46 | 21.3±
0.60 |
| 0.395 | 250 | 84.05 | 76.71 | 84.05 | 90.63 | 77.97 | | | | | |
| 0.395 | 125 | 75.19 | 73.67 | 75.19 | 87.09 | 76.46 | | | | | |
| 0.395 | 62.5 | 70.13 | 71.65 | 69.87 | 80.76 | 69.87 | | | | | |
| 0.395 | 31.25 | 63.04 | 65.06 | 66.58 | 70.63 | 52.91 | | | | | |
| 0.395 | 15.625 | 51.14 | 52.15 | 60.25 | 60.00 | 46.33 | | | | | |
| 0.395 | 7.813 | 42.03 | 46.33 | 42.78 | 50.63 | 34.68 | | | | | |
| 0.395 | 3.906 | 30.13 | 26.84 | 26.84 | 31.9 | 28.35 | | | | | |
| 0.395 | 1.953 | 21.77 | 19.24 | 17.72 | 17.72 | 10.89 | | | | | |
| 0.395 | 0.0977 | 16.71 | 5.82 | 7.09 | 10.38 | 6.08 | | | | | |

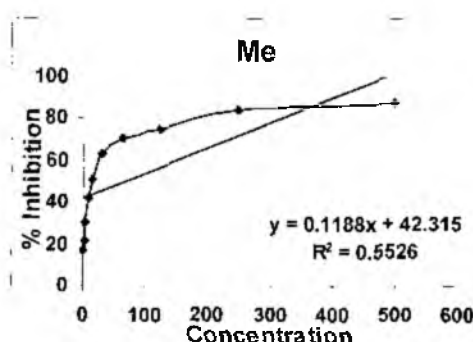


Figure 6.27: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *B. stipularis*.

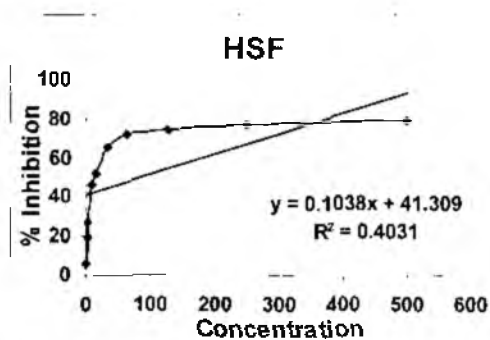


Figure 6.28: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *B. stipularis*.

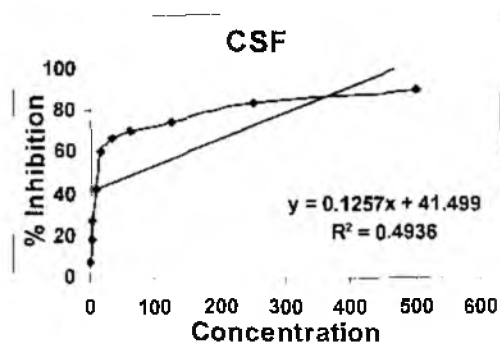


Figure 6.29: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the leaf of *B. stipularis*.

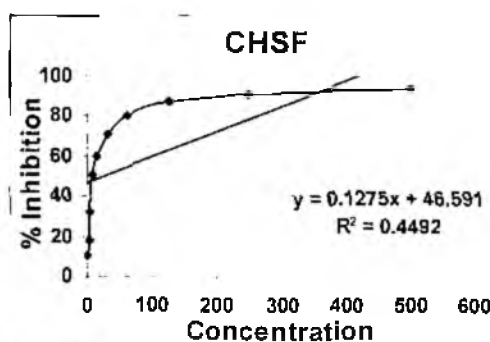


Figure 6.30: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *B. stipularis*.

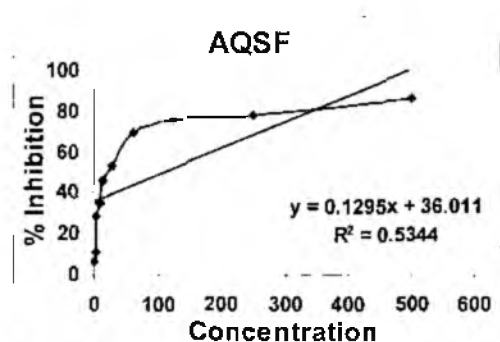


Figure 6.31: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *B. stipularis*.

Table 6.8: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *B. tomentosa*.

| B. tomentosa (stem bark) | | | | | | | | | | | |
|--------------------------|---------------|--------------|-------|-------|-------|-------|--------------------------|------------|------------|-----------|------------|
| Blank | Conc. (µg/ml) | % Inhibition | | | | | IC ₅₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | IISF | CSF | CIISF | AQSF |
| 0.395 | 500 | 90.63 | 93.16 | 90.63 | 92.15 | 91.39 | 7.55±0.27 | 10.35±0.48 | 10.50±0.44 | 7.35±0.40 | 17.60±0.33 |
| 0.395 | 250 | 89.37 | 88.1 | 89.62 | 86.33 | 87.09 | | | | | |
| 0.395 | 125 | 86.33 | 89.62 | 81.77 | 84.56 | 75.44 | | | | | |
| 0.395 | 62.5 | 75.44 | 84.05 | 75.70 | 79.49 | 68.86 | | | | | |
| 0.395 | 31.25 | 67.85 | 78.99 | 76.46 | 72.91 | 56.2 | | | | | |
| 0.395 | 15.625 | 61.27 | 69.37 | 58.73 | 61.52 | 47.85 | | | | | |
| 0.395 | 7.813 | 49.62 | 35.95 | 41.52 | 50.38 | 32.41 | | | | | |
| 0.395 | 3.906 | 32.41 | 20.76 | 19.75 | 28.86 | 17.22 | | | | | |
| 0.395 | 1.953 | 21.27 | 15.44 | 11.14 | 22.28 | 6.08 | | | | | |
| 0.395 | 0.0977 | 7.59 | 2.03 | 1.27 | 10.38 | 2.53 | | | | | |

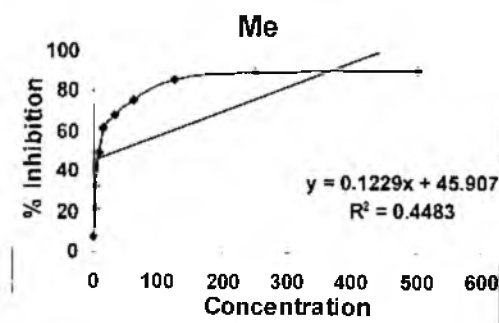


Figure 6.32: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *B. tomentosa*.

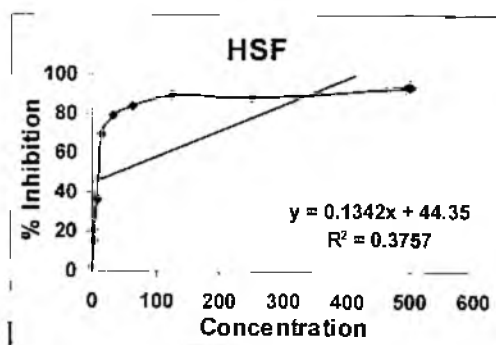


Figure 6.33: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *B. tomentosa*.

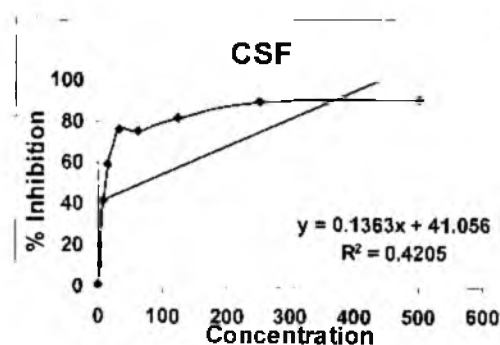


Figure 6.34: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the stem bark of *B. tomentosa*.

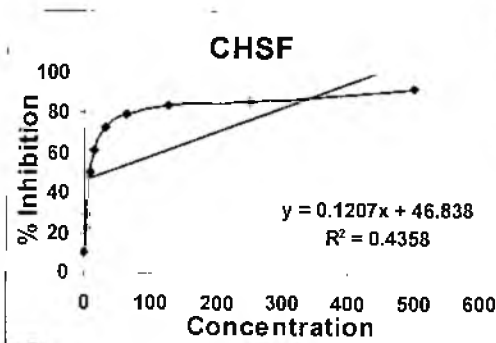


Figure 6.35: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *B. tomentosa*.

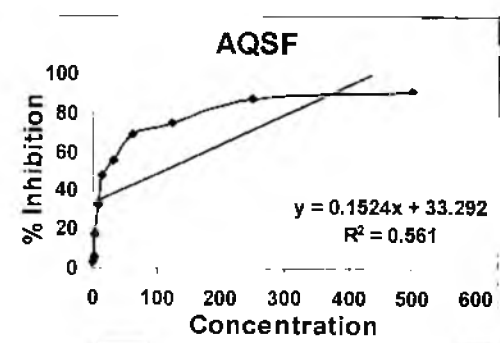


Figure 6.36: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *B. tomentosa*.

Table 6.9: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *B. tomentosa*.

| <i>B. tomentosa</i> (leaf) | | | | | | | | | | | |
|-----------------------------|------------------|--------------|-------|-------|-------|-------|--------------------------|----------------|--------------|--------------|---------------|
| Blank | Conc.
(µg/ml) | % Inhibition | | | | | IC ₅₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 0.395 | 500 | 93.16 | 92.91 | 94.18 | 91.39 | 85.06 | 9.70±
0.25 | 11.20±
0.50 | 8.8±
0.10 | 3.9±
0.80 | 26.4±
0.45 |
| 0.395 | 250 | 90.13 | 88.1 | 90.38 | 87.09 | 77.97 | | | | | |
| 0.395 | 125 | 87.09 | 84.56 | 87.59 | 90.38 | 67.85 | | | | | |
| 0.395 | 62.5 | 82.53 | 76.96 | 81.52 | 87.09 | 61.52 | | | | | |
| 0.395 | 31.25 | 75.44 | 63.8 | 78.48 | 83.04 | 53.67 | | | | | |
| 0.395 | 15.625 | 61.01 | 58.73 | 69.37 | 76.46 | 32.41 | | | | | |
| 0.395 | 7.813 | 43.54 | 36.46 | 45.06 | 69.37 | 23.04 | | | | | |
| 0.395 | 3.906 | 30.63 | 24.3 | 23.54 | 50.38 | 19.75 | | | | | |
| 0.395 | 1.953 | 20.76 | 16.2 | 13.67 | 28.35 | 11.14 | | | | | |
| 0.395 | 0.0977 | 1.52 | 7.09 | 1.52 | 18.73 | 4.3 | | | | | |

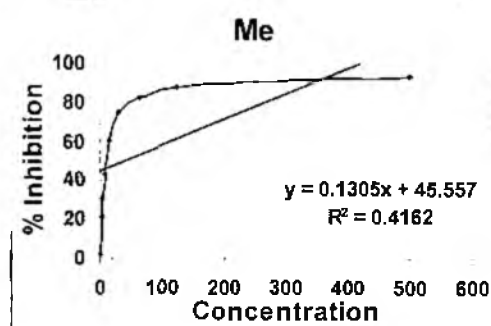


Figure 6.37: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *B. tomentosa*.

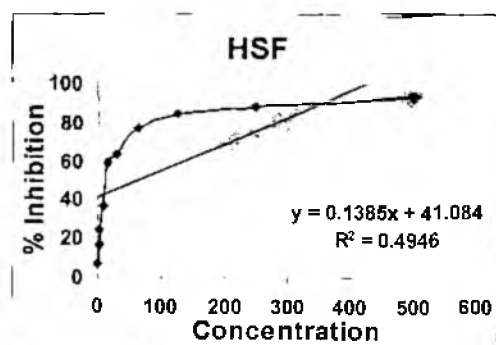


Figure 6.38: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *B. tomentosa*.

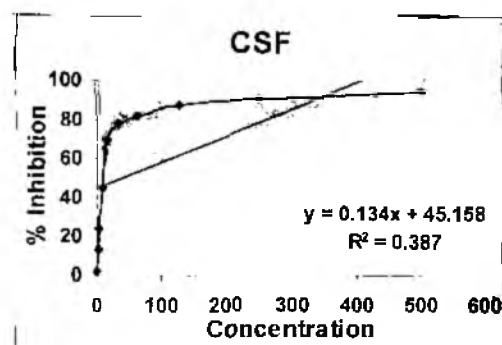


Figure 6.39: Free radical scavenging activity of the CCl₄ soluble materials (CSF) of the leaf of *B. tomentosa*.

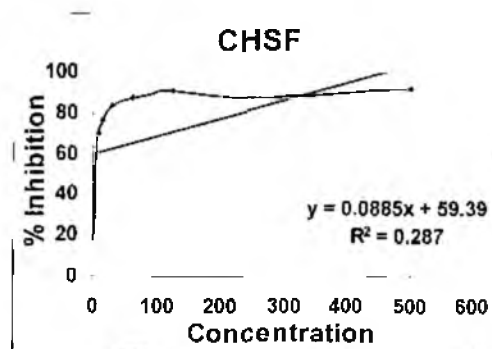


Figure 6.40: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *B. tomentosa*.

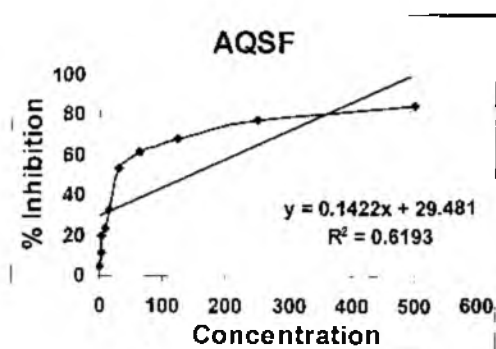


Figure 6.41: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *B. tomentosa*.

Table 6.10: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *E. fusca*.

| <i>E. fusca</i> (stem bark) | | | | | | | | | | | |
|-----------------------------|---------------|--------------|-------|-------|-------|-------|--------------------------|--------------|---------------|--------------|---------------|
| Blank | Conc. (µg/ml) | % Inhibition | | | | | IC ₅₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 0.395 | 500 | 94.68 | 90.38 | 91.39 | 86.58 | 89.62 | 11.8±
0.35 | 7.7±
0.95 | 13.1±
0.75 | 8.8±
0.95 | 12.1±
0.45 |
| 0.395 | 250 | 91.90 | 85.32 | 85.82 | 80.76 | 81.77 | | | | | |
| 0.395 | 125 | 83.29 | 81.52 | 76.46 | 73.67 | 74.43 | | | | | |
| 0.395 | 62.5 | 75.44 | 75.95 | 69.37 | 68.86 | 70.13 | | | | | |
| 0.395 | 31.25 | 65.06 | 66.84 | 57.22 | 62.78 | 59.75 | | | | | |
| 0.395 | 15.625 | 53.67 | 61.27 | 50.13 | 54.68 | 52.15 | | | | | |
| 0.395 | 7.813 | 42.78 | 50.89 | 43.54 | 47.85 | 45.06 | | | | | |
| 0.395 | 3.906 | 27.85 | 24.81 | 24.81 | 34.94 | 35.19 | | | | | |
| 0.395 | 1.953 | 24.30 | 16.71 | 20.51 | 19.24 | 19.24 | | | | | |
| 0.395 | 0.0977 | 18.73 | 6.08 | 12.15 | 5.82 | 13.67 | | | | | |

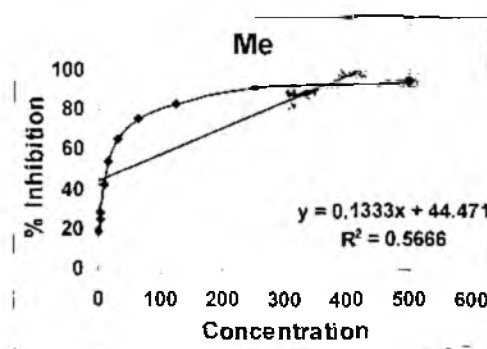


Figure 6.42: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *E. fusca*.

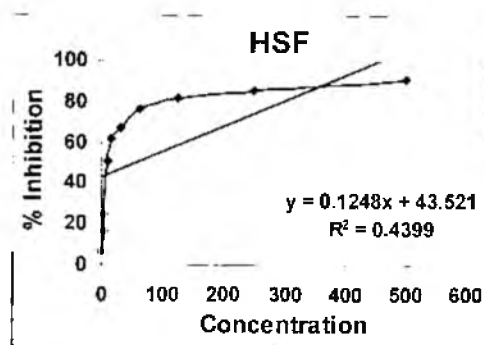


Figure 6.43: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *E. fusca*.

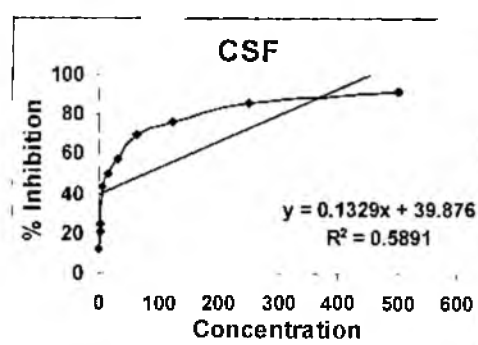


Figure 6.44: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the stem bark of *E. fusca*.

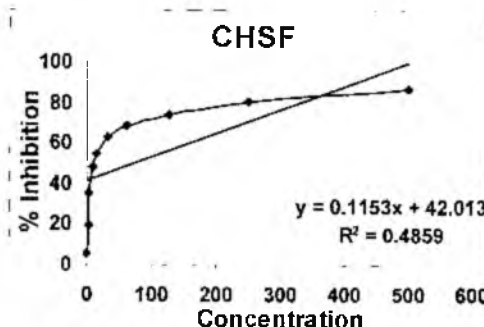


Figure 6.45: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *E. fusca*.

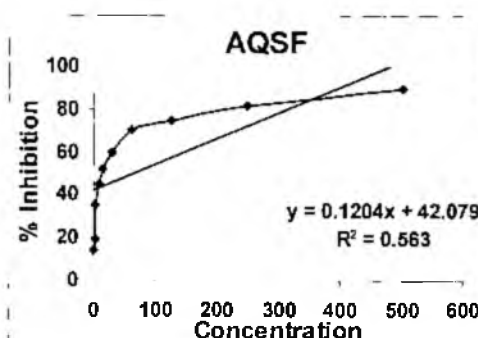


Figure 6.46: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *E. fusca*.

Table 6.11: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *E. fusca*.

| <i>E. fusca</i> (leaf) | | | | | | | | | | | |
|------------------------|---------------|--------------|-------|-------|-------|-------|--------------------------|--------------|---------------|--------------|---------------|
| Blank | Conc. (μg/ml) | % Inhibition | | | | | IC_{50} (μg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 0.395 | 500 | 93.16 | 91.9 | 88.61 | 94.18 | 95.7 | 9.9±
0.95 | 7.9±
0.70 | 19.9±
0.95 | 5.5±
0.60 | 12.6±
0.20 |
| 0.395 | 250 | 89.37 | 88.1 | 81.52 | 88.10 | 92.15 | | | | | |
| 0.395 | 125 | 81.01 | 87.09 | 73.67 | 82.53 | 85.57 | | | | | |
| 0.395 | 62.5 | 76.46 | 78.99 | 62.78 | 75.44 | 81.52 | | | | | |
| 0.395 | 31.25 | 67.85 | 70.63 | 52.91 | 71.65 | 75.95 | | | | | |
| 0.395 | 15.625 | 53.42 | 62.28 | 47.34 | 66.08 | 52.66 | | | | | |
| 0.395 | 7.813 | 46.08 | 49.11 | 39.49 | 52.66 | 44.81 | | | | | |
| 0.395 | 3.906 | 29.62 | 37.22 | 29.87 | 46.08 | 26.84 | | | | | |
| 0.395 | 1.953 | 14.94 | 14.18 | 16.96 | 34.94 | 16.71 | | | | | |
| 0.395 | 0.0977 | 9.62 | 8.61 | 7.34 | 13.42 | 10.38 | | | | | |

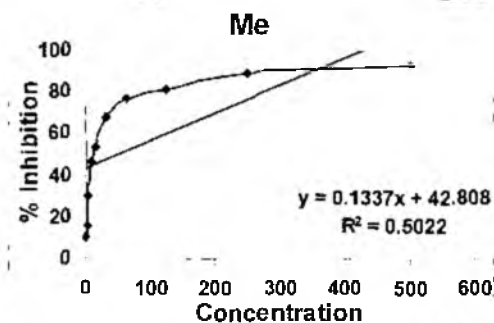


Figure 6.47: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *E. fusca*.

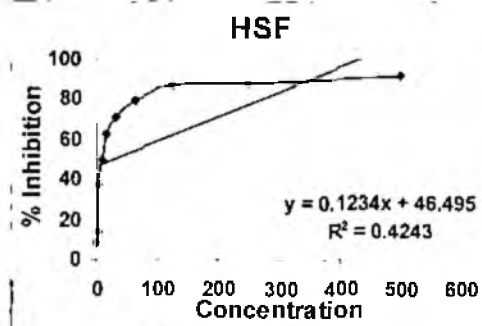


Figure 6.48: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *E. fusca*.

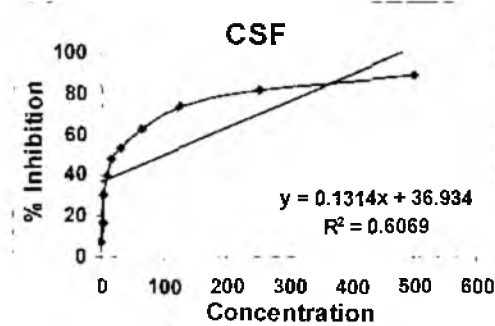


Figure 6.49: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the leaf of *E. fusca*.

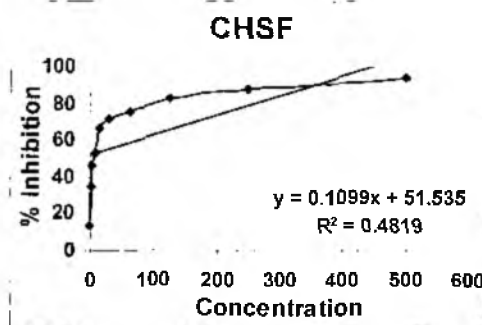


Figure 6.50: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *E. fusca*.

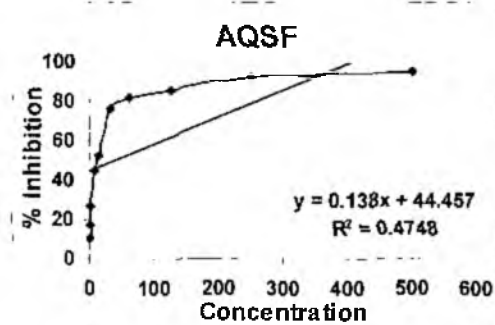


Figure 6.51: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *E. fusca*.

Table 6.12: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the stem bark of *E. variegata*.

| <i>E. variegata</i> (stem bark) | | | | | | | | | | | |
|---------------------------------|---------------|--------------|-------|-------|-------|-------|--------------------------|---------------|---------------|--------------|--------------|
| Blank | Conc. (µg/ml) | % Inhibition | | | | | IC ₅₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 0.395 | 500 | 91.90 | 91.39 | 92.91 | 95.19 | 92.66 | 6.6±
0.25 | 13.2±
0.61 | 16.5±
0.40 | 7.9±
0.80 | 7.3±
0.50 |
| 0.395 | 250 | 83.80 | 87.09 | 88.1 | 91.39 | 88.35 | | | | | |
| 0.395 | 125 | 75.19 | 77.72 | 76.96 | 85.57 | 90.13 | | | | | |
| 0.395 | 62.5 | 72.41 | 70.38 | 64.81 | 77.47 | 81.52 | | | | | |
| 0.395 | 31.25 | 69.11 | 62.53 | 53.42 | 73.92 | 75.95 | | | | | |
| 0.395 | 15.625 | 61.01 | 50.63 | 48.61 | 65.57 | 67.59 | | | | | |
| 0.395 | 7.813 | 53.42 | 44.81 | 43.54 | 48.86 | 51.39 | | | | | |
| 0.395 | 3.906 | 48.86 | 35.95 | 23.8 | 29.37 | 32.66 | | | | | |
| 0.395 | 1.953 | 34.43 | 16.96 | 18.73 | 19.75 | 6.58 | | | | | |
| 0.395 | 0.0977 | 21.01 | 8.35 | 12.15 | 6.08 | 1.77 | | | | | |

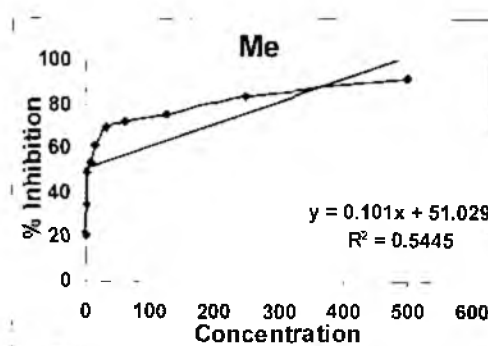


Figure 6.52: Free radical scavenging activity of the methanol fraction (Me) of the stem bark of *E. variegata*.

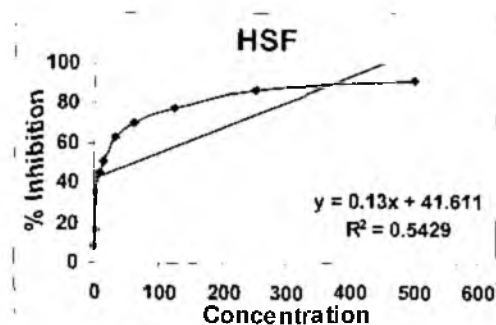


Figure 6.53: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the stem bark of *E. variegata*.

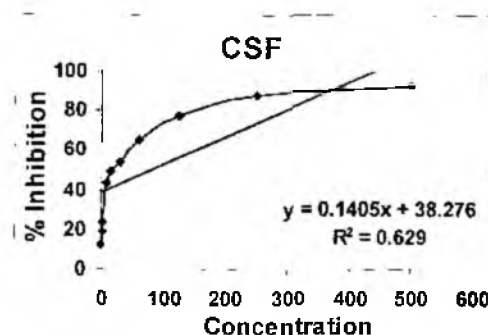


Figure 6.54: Free radical scavenging activity of the CCl₄ soluble materials (CSF) of the stem bark of *E. variegata*.

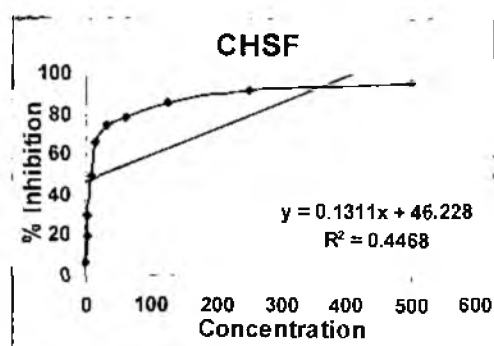


Figure 6.55: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the stem bark of *E. variegata*.

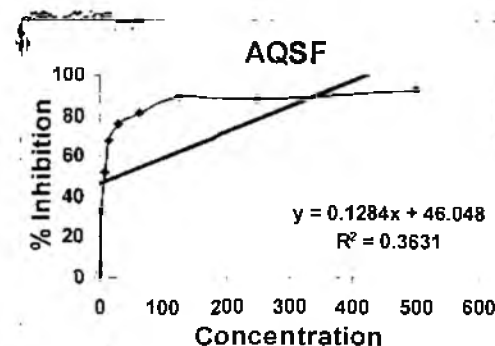


Figure 6.56: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the stem bark of *E. variegata*.

Table 6.13: Free radical scavenging activity of methanol extract (Me), *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous soluble (AQSF) fraction of the leaf of *E. variegata*.

| <i>Erythrina variegata</i> (leaf) | | | | | | | | | | | |
|-----------------------------------|---------------|--------------|-------|-------|-------|-------|--------------------------|----------------|---------------|---------------|---------------|
| Blank | Conc. (µg/ml) | % Inhibition | | | | | IC ₅₀ (µg/ml) | | | | |
| | | Me | HSF | CSF | CHSF | AQSF | Me | HSF | CSF | CHSF | AQSF |
| 0.395 | 500 | 90.13 | 90.89 | 93.92 | 85.82 | 93.42 | 14.7±
0.85 | 10.45±
0.65 | 14.3±
0.25 | 21.3±
0.50 | 7.34±
0.45 |
| 0.395 | 250 | 87.59 | 84.56 | 91.14 | 77.97 | 90.63 | | | | | |
| 0.395 | 125 | 77.72 | 79.49 | 86.61 | 76.46 | 87.09 | | | | | |
| 0.395 | 62.5 | 83.04 | 70.89 | 81.52 | 69.87 | 80.76 | | | | | |
| 0.395 | 31.25 | 65.06 | 69.37 | 75.44 | 52.91 | 70.63 | | | | | |
| 0.395 | 15.625 | 50.38 | 67.85 | 51.39 | 46.33 | 59.75 | | | | | |
| 0.395 | 7.813 | 46.08 | 36.46 | 40.76 | 34.68 | 50.63 | | | | | |
| 0.395 | 3.906 | 16.96 | 22.53 | 28.86 | 28.35 | 31.9 | | | | | |
| 0.395 | 1.953 | 11.65 | 9.62 | 20.25 | 10.89 | 17.72 | | | | | |
| 0.395 | 0.0977 | 1.01 | 2.03 | 12.41 | 6.08 | 10.38 | | | | | |

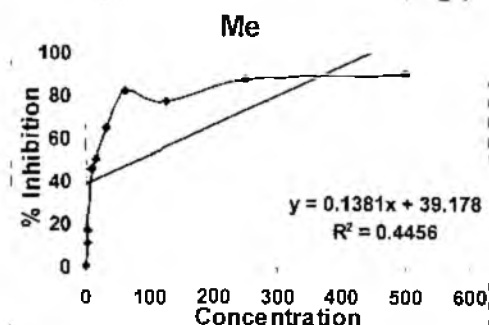


Figure 6.57: Free radical scavenging activity of the methanol fraction (Me) of the leaf of *E. variegata*.

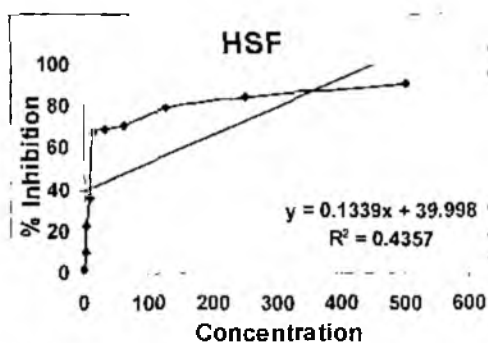


Figure 6.58: Free radical scavenging activity of the *n*-hexane soluble materials (HSF) of the leaf of *E. variegata*.

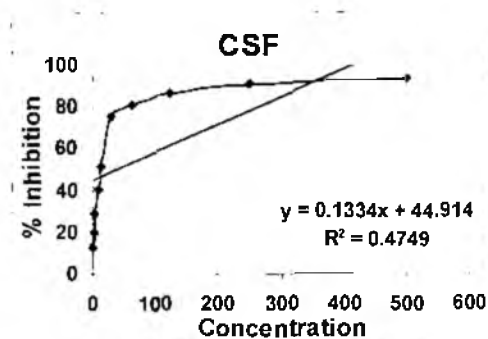


Figure 6.59: Free radical scavenging activity of the CCl_4 soluble materials (CSF) of the leaf of *E. variegata*.

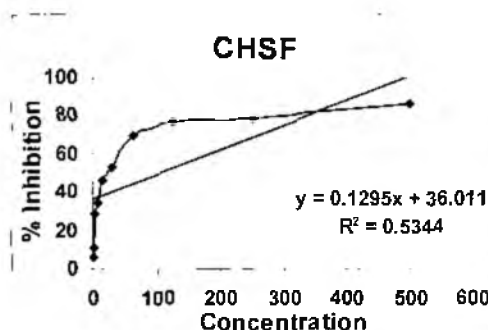


Figure 6.60: Free radical scavenging activity of the chloroform soluble materials (CHSF) of the leaf of *E. variegata*.

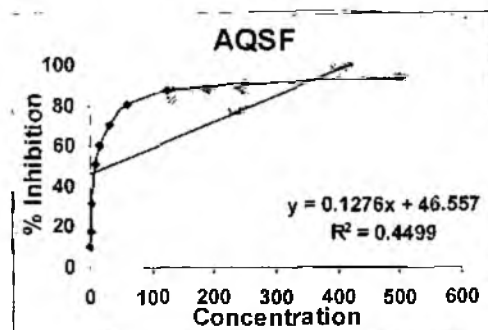


Figure 6.61: Free radical scavenging activity of the aqueous soluble materials (AQSF) of the leaf of *E. variegata*.

Table 6.14: Free radical scavenging activity of the isolated pure compounds BVSP-4, BVSP-6, BVBC-2 and BVS-65 isolated from the stem bark of *B. verrusosa*.

| Blank | Conc.
($\mu\text{g/ml}$) | % Inhibition | | | | IC_{50} ($\mu\text{g/ml}$) | | | |
|-------|-------------------------------|--------------|--------|--------|--------|---------------------------------------|--------|--------|--------|
| | | BVSP-4 | BVSP-6 | BVBC-2 | BVS-65 | BVSP-4 | BVSP-6 | BVBC-2 | BVS-65 |
| 0.395 | 500 | 85.82 | 90.13 | 92.66 | 97.72 | 17.9 | 14.7 | 7.3 | 8.4 |
| 0.395 | 250 | 80.25 | 87.59 | 88.35 | 94.68 | | | | |
| 0.395 | 125 | 76.46 | 77.72 | 90.13 | 90.13 | | | | |
| 0.395 | 62.5 | 69.87 | 83.04 | 81.52 | 82.78 | | | | |
| 0.395 | 31.25 | 53.67 | 65.06 | 75.95 | 75.95 | | | | |
| 0.395 | 15.625 | 47.09 | 50.38 | 67.59 | 56.20 | | | | |
| 0.395 | 7.813 | 32.15 | 46.08 | 51.39 | 49.62 | | | | |
| 0.395 | 3.906 | 19.75 | 16.96 | 32.66 | 35.95 | | | | |
| 0.395 | 1.953 | 9.62 | 11.65 | 6.58 | 21.27 | | | | |
| 0.395 | 0.0977 | 4.56 | 1.01 | 1.77 | 8.35 | | | | |

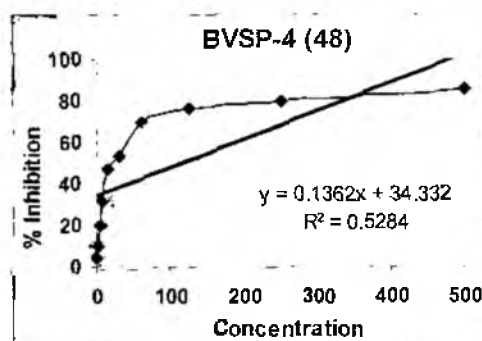


Figure 6.62: Free radical scavenging activity BVSP-4 (48) isolated from the stem bark of *B. verrucosa*.

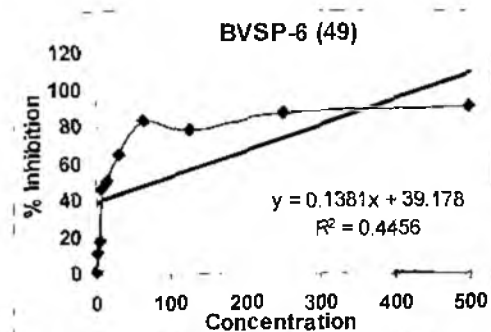


Figure 6.63: Free radical scavenging activity BVSP-6 (49) isolated from the stem bark of *B. verrucosa*.

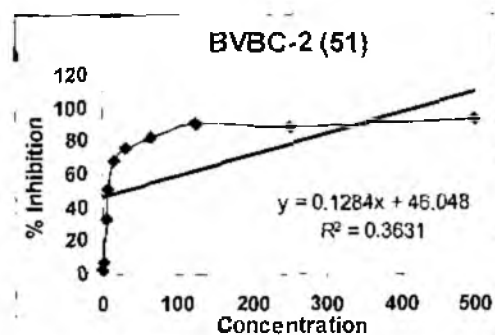


Figure 6.64: Free radical scavenging activity BVBC-2 (51) isolated from the stem bark of *B. verrucosa*.

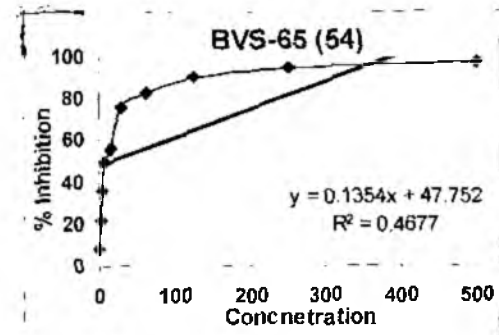


Figure 6.65: Free radical scavenging activity BVS-65 (54) isolated from the stem bark of *B. verrucosa*.

Table 6.15: Free radical scavenging activity of the isolated pure compounds BS-11, EF-7, EF-8 and EF-12 isolated from the stem bark of *B. stipularis* and *E. fusca*.

| Pure compounds | | | | | | | | | |
|----------------|------------------|--------------|-------|-------|-------|--------------------------|------|------|-------|
| Blank | Conc.
(µg/ml) | % Inhibition | | | | IC ₅₀ (µg/ml) | | | |
| | | BS-11 | EF-7 | EF-8 | EF-12 | BS-11 | EF-7 | EF-8 | EF-12 |
| 0.395 | 500 | 95.7 | 90.38 | 87.59 | 95.19 | 12.6 | 8.8 | 7.7 | 7.9 |
| 0.395 | 250 | 92.15 | 87.09 | 91.14 | 91.39 | | | | |
| 0.395 | 125 | 85.57 | 80.51 | 75.19 | 85.57 | | | | |
| 0.395 | 62.5 | 81.52 | 75.44 | 80.51 | 77.47 | | | | |
| 0.395 | 31.25 | 75.95 | 67.85 | 65.57 | 73.92 | | | | |
| 0.395 | 15.625 | 52.66 | 53.67 | 49.87 | 65.57 | | | | |
| 0.395 | 7.813 | 44.81 | 47.34 | 49.11 | 48.86 | | | | |
| 0.395 | 3.906 | 26.84 | 24.56 | 20.76 | 29.37 | | | | |
| 0.395 | 1.953 | 16.71 | 12.41 | 9.62 | 19.75 | | | | |
| 0.395 | 0.0977 | 4.3 | 0.76 | 1.77 | 6.08 | | | | |

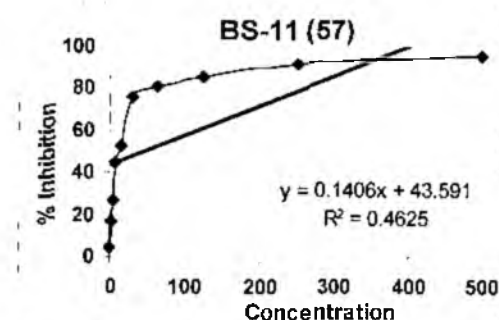


Figure 6.66: Free radical scavenging activity BS-11 (57) isolated from the stem bark of *B. verrucosa*.

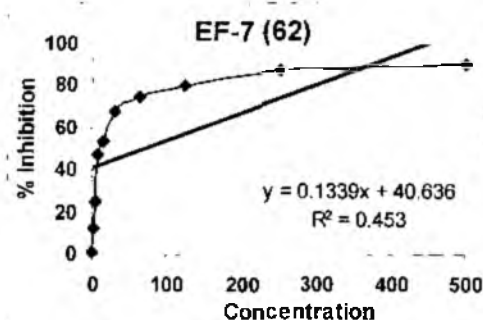


Figure 6.67: Free radical scavenging activity EF-7 (62) isolated from the stem bark of *E. fusca*.

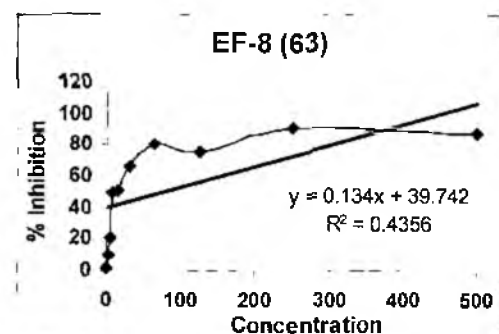


Figure 6.68: Free radical scavenging activity EF-8 (63) isolated from the stem bark of *E. fusca*.

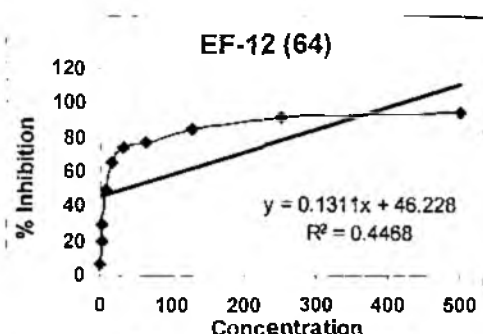


Figure 6.69: Free radical scavenging activity EF-12 (64) isolated from the stem bark of *E. fusca*.

6.4.3 Total antioxidant capacity assay by the phosphomolybdenum method

Total antioxidant capacity in terms of absorbance values of the methanol extract (Me) of *B. verrucosa*, *B. stipularis* and *B. tomentosa*, *E. fusca* and *E. variegata* available in Bangladesh and fractions generate from the crude extract i.e. *n*-hexane (HSF), carbon tetrachloride (CSF), chloroform (CHSF) and aqueous (AQSF) soluble partitionates were determined by the phosphomolybdenum method and were expressed as equivalents of ascorbic acid as standard (Table 6.15 and 6.16). In case of *Bridelia* species, the total antioxidant capacity of the methanolic extract of the leaf of *B. tomentosa* was highest (612.54 mg of ascorbic acid/g of dried extract) and *n*-hexane soluble fraction of the stem bark of *B. stipularis* being the lowest (142.66 mg of ascorbic acid/g of dried extract). The fractions of the three plants demonstrated varying degrees of antioxidant capacity as compared to the standard ascorbic acid. Again, for the *Erythrina* species antioxidant capacity of the methanol extract of the leaf of *E. fusca* was highest being 587.66 mg of ascorbic acid/g of dried extract and lowest capacity was shown by the chloroform soluble fraction of the leaf of *E. variegata* being 96.66 mg of ascorbic acid/g of dried extract. The

other remaining fractions of the two plants demonstrated high to moderate antioxidant capacity as compared to the standard.

Table 6.16: Standard curve preparation by using ascorbic acid.

| SL. No. | Cone. Of the standard (µg/ml) | Absorbance | Regression line | R ² |
|---------|-------------------------------|------------|-----------------------|----------------|
| 1 | 100 | 0.298 | $y = 0.003x + 0.1057$ | 0.9845 |
| 2 | 200 | 0.641 | | |
| 3 | 400 | 1.465 | | |
| 4 | 600 | 2.1 | | |
| 5 | 800 | 2.54 | | |
| 6 | 1000 | 3 | | |

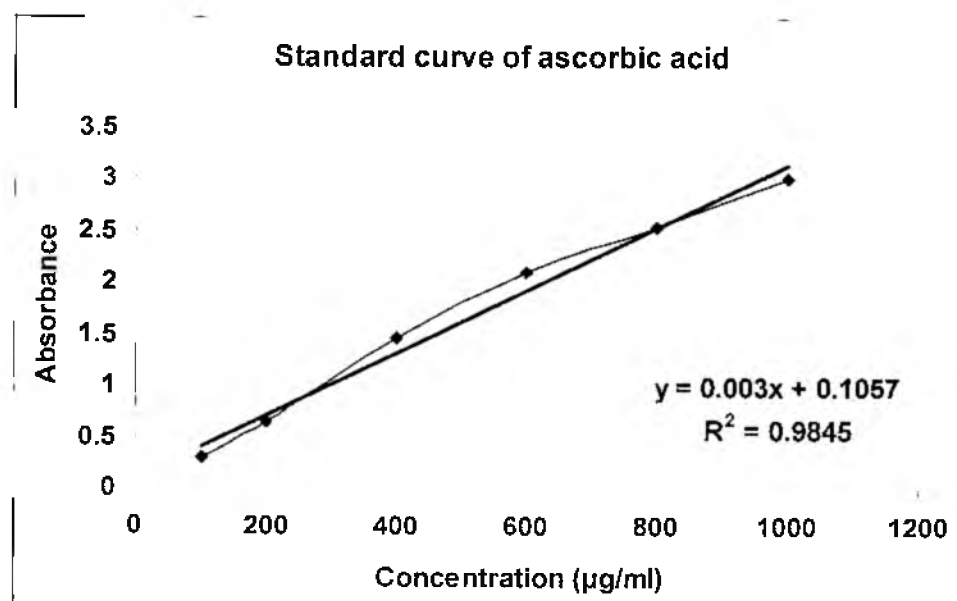


Figure 6.70: Standard curve of ascorbic acid for total antioxidant capacity determination.

Table 6.17: Total antioxidant capacity of the methanolic extracts and its different soluble fractions of stem bark and leaf of *Bridelia* species.

| Soluble fractions | <i>B. verrucosa</i> | | <i>B. stipularis</i> | | <i>B. tomentosa</i> | |
|-------------------|--|---|--|---|--|---|
| | Stem bark (mg of ascorbic acid/g of dried extract) | Leaf (mg of ascorbic acid/g of dried extract) | Stem bark (mg of ascorbic acid/g of dried extract) | Leaf (mg of ascorbic acid/g of dried extract) | Stem bark (mg of ascorbic acid/g of dried extract) | Leaf (mg of ascorbic acid/g of dried extract) |
| Me | 542.88±1.61 | 484.43±1.11 | 248.99±0.75 | 392.54±2.32 | 442.77±3.93 | 612.54±1.79 |
| HSF | 217.32±0.91 | 161.88±1.02 | 142.66±0.63 | 243.54±0.73 | 254.32±0.83 | 210.99±1.38 |
| CSF | 257.1±1.36 | 208.43±0.77 | 226.32±0.4 | 192.77±1.32 | 211.66±0.98 | 185.77±0.61 |
| CHSF | 151.88±0.76 | 148.43±0.85 | 181.21±1.08 | 226.32±0.95 | 153.77±0.65 | 152.21±1.24 |
| AQSF | 462.88±2.65 | 279.99±1.83 | 285.66±0.30 | 293.32±2.40 | 254.32±0.81 | 287.10±0.54 |

Values are expressed as average±SD (n=3); Me = Methanolic extract; HSF = *n*-hexane soluble fraction; CSF = carbon tetrachloride soluble fraction; CHSF = chloroform soluble fraction and AQSF = aqueous soluble fraction.

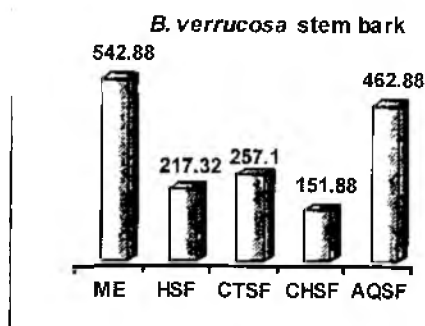


Figure 6.71: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *B. verrucosa*.

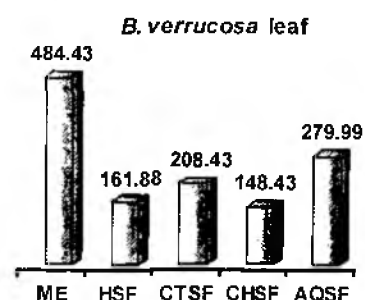


Figure 6.72: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *B. verrucosa*.

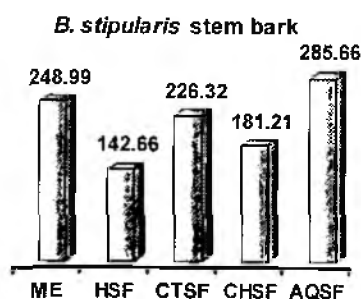


Figure 6.73: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *B. stipularis*.

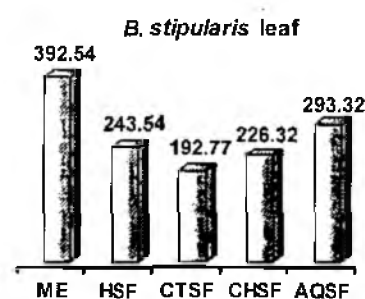


Figure 6.74: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *B. stipularis*.

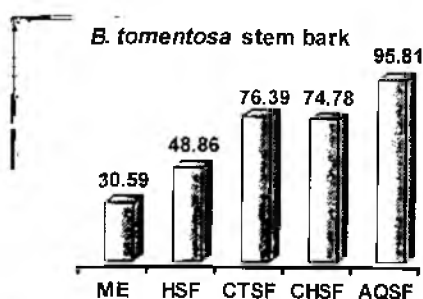


Figure 6.75: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *B. tomentosa*.

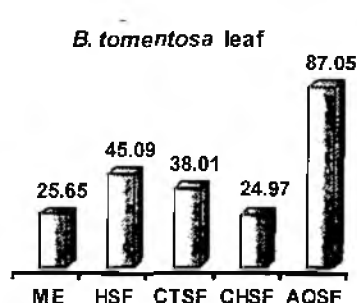


Figure 6.76: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *B. tomentosa*.

Table 6.18: Total antioxidant capacity of the methanolic extracts and its different soluble fractions of *Erythrina* species.

| Soluble fractions | <i>E. fusca</i> | | <i>E. variegata</i> | |
|-------------------|---|--|---|--|
| | Stem bark
(mg of ascorbic acid/g of dried extract) | Leaf
(mg of ascorbic acid/g of dried extract) | Stem bark
(mg of ascorbic acid/g of dried extract) | Leaf
(mg of ascorbic acid/g of dried extract) |
| Me | 472.21±2.78 | 587.66±0.85 | 454.43±0.42 | 289.21±1.06 |
| HSF | 175.66±0.63 | 142.21±0.52 | 175.99±0.73 | 120.66±0.58 |
| CSF | 125.99±0.29 | 106.10±0.89 | 221.99±0.46 | 256.10±0.60 |
| CHSF | 216.54±0.47 | 260.77±0.19 | 146.54±0.77 | 96.66±0.92 |
| AQSF | 415.10±2.12 | 153.77±0.63 | 191.10±1.35 | 251.77±0.74 |

Values are expressed as average±SD (n=3); Me = Methanolic extract; HSF = *n*-hexane soluble fraction; CSF = carbon tetrachloride soluble fraction; CHSF = chloroform soluble fraction and AQSF = aqueous soluble fraction.

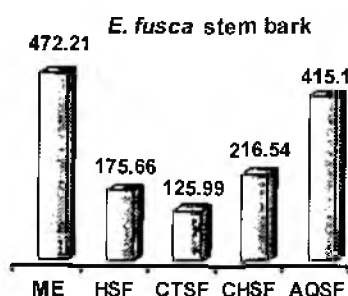


Figure 6.77: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *E. fusca*.

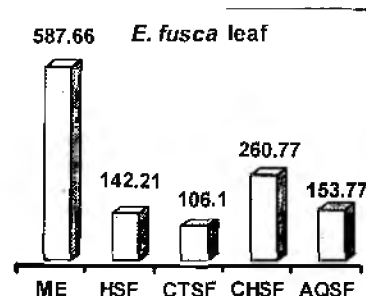


Figure 6.78: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *E. fusca*.

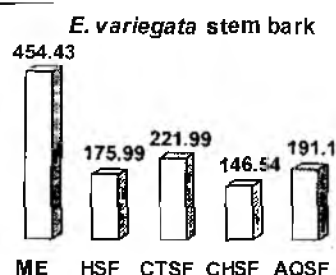


Figure 6.79: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the stem bark of *E. variegata*.

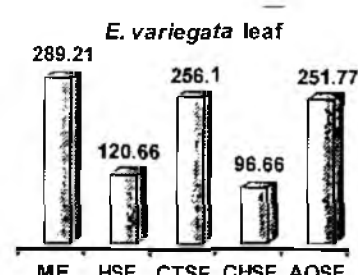


Figure 6.80: Total antioxidant capacity (mg of ascorbic acid/g of dried extract) of different extractives of the leaf of *E. variegata*.



Chapter 7

Assay for Thrombolytic Activity

7.1 Introduction

One of the major causes of cardiovascular disease is lack of proper blood circulation due to formation of clots. Thrombi or emboli can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction (infarction), or even death of the tissues (necrosis) in that area. A blood clot (thrombus) is formed from fibrinogen by thrombin and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). Fibrinolytic drugs have been used to dissolve thrombi in acutely occluded coronary arteries thereby to restore blood supply to ischaemic myocardium, to limit necrosis and to improve prognosis (Laurence, 1992).

Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic alteplase in most infarct patients while having the advantages of being much less expensive. Tissue-type Plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs (Nicolini *et al.*, 1992; Adams *et al.*, 1991; Lijnen *et al.*, 1991; Marder, 1993; Wu *et al.*, 2001). Heparin and Aspirin are only moderately efficient for acceleration of lysis and prevention of reocclusion, but are safe. More selective thrombin inhibitors and antiplatelet agents are more potent, but their safety remains to be confirmed. Continued investigation in this area will provide new insights and promote progress toward the development of the ideal thrombolytic therapy, characterized by maximized stable coronary arterial thrombolysis with minimal bleeding (Collen, 1990).

For the treatment of several diseases herbal preparations have been used since ages and are often perceived as safe because they are “natural” (Gesler, 1992). In India, in recent years, there is increased research on traditional ayurvedic herbal medicines on the basis of their known effectiveness in the treatment of ailments for which they have been traditionally applied. Considerable efforts have been directed towards the discovery and development of natural products from various plant and animal sources which have antiplatelet (Briggs *et al.*, 2000; Demrow *et al.*, 1995), anticoagulant (Leta *et al.*, 2002; Zhguang *et al.*, 2000), antithrombotic (Rajapakse, 2005) and thrombolytic activity. Epidemiologic studies have provided evidence that foods with experimentally proved

antithrombotic effect could reduce risk of thrombosis. Herbs showing thrombolytic activity have been studied and some significant observations have been reported (Yamamoto *et al.*, 2005).

The aim of present study was to investigate the thrombolytic activity of methanol extracts and its different soluble fractions of stem bark and leaf of three *Bridelia* and two *Erythrina* species available in Bangladesh.

7.2 Experimentals

7.2.1 Preparation of sample

The thrombolytic activity of all extractives was evaluated by a method using streptokinase (SK) as standard substance. 10 mg of each plant extract (crude methanol extract and its kupchan fractions of the stem bark of *B. verrucosa*, *B. stipularis*, *B. tomentosa*, *E. fusca* and *E. variegata*) was suspended in 1 ml distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept over night and decanted to remove the soluble supernatant, which was filtered through a 0.22 micron syringe filter. Then this solution is ready for the study to check *in vitro* thrombolytic activity (Prasad, *et al.*, 2007).

7.2.2 Streptokinase (SK)

Commercially available lyophilized Altepare (Streptokinase) vial (Beacon Pharmaceuticals Ltd) of 15,00,000 I.U., was collected and mixed well with 5 ml sterile distilled water. This suspension was used as a stock from which 100 μ l (30,000 I.U) was used for *in vitro* thrombolysis (Prasad, *et al.*, 2007).

7.2.3 Blood Sample

Whole blood (n=10) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1 ml of blood was transferred to the previously weighed sterile vials and was allowed to form clots.

7.3 Assay for Thrombolytic activity

Aliquots (5 ml) of venous blood were drawn from healthy volunteers which were distributed in ten different pre weighed sterile micro centrifuge tube/Eppendorf tube (1 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each vial having clot was again weighed to determine the clot weigh (clot weight = weight of clot containing tube – weight of tube alone).

To each vial containing pre-weighed clot, 100 μ l aqueous solution of different partitionates along with the crude extracts was added separately. As a positive control,

100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control vials. All the vials were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed and vials were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

$$\% \text{ of clot lysis} = (\text{wt of released clot} / \text{clot wt}) \times 100$$

7.4 Results and Discussion

As a part of discovery of cardio protective drugs from natural sources, the extractives of stem bark and leaf of the three *Bridelia* and two *Erythrina* species available in Bangladesh were subjected to assays for thrombolytic activity in the phytochemical laboratory of the State University of Bangladesh and the results are presented in Table 7.1 and 7.2. Addition of 100µl SK, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37 °C, showed 66.7% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot (8.12%). The mean difference in percentage of clot lysis between positive and negative control was found to be significant.

In this study, the methanol extract of the stem bark and leaf of *B. verrucosa*, *B. stipularis* and *B. tomentosa* exhibited thrombolytic activity ranging from 20.94% to 36.45%. The *n*-hexane soluble fraction (HSF) from the stem bark and leaf of three *Bridelia* species showed 10.43% to 28.21%. The carbon tetrachloride soluble fraction (CSF) exhibited clot lysis activity from 22.06% to 41.46%, the chloroform soluble fraction (CHSF) from 8.94% to 23.07% and the aqueous soluble fraction (AQSF) revealed thrombolytic activity between 9.24% to 37.04%.

Again, in this study, the methanol extract of the stem bark and leaf of *E. fusca* and *E. variegata* demonstrated thrombolytic activity ranging from 12.45% to 15.65% and 12.28% to 24.48%. The *n*-hexane soluble fraction (HSF), carbon tetrachloride soluble fraction (CSF), the chloroform soluble fraction (CHSF) and the aqueous soluble fraction (AQSF) of the methanolextract of the stem bark and leaf of *E. fusca* and *E. variegata* exhibited clot lysis ranging from 14.27% to 15.33% & 12.65% to 23.54%, 8.02% to 22.75% & 5.62% to 15.78%, 6.34% to 12.19% & 9.46% to 46.73, respectively.

Table 7.1: Thrombolytic activity (in terms of % clot lysis) of *B. verrucosa*, *B. stipularis* and *B. tomentosa*.

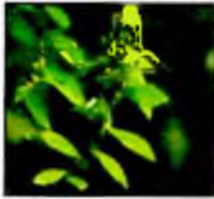
| <i>B. verrucosa</i> (stem bark) | | | | |
|---|----------------|----------------|----------------|--------------|
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.8956 ± 0.15 | 5.3520 ± 0.06 | 5.2165 ± 0.02 | 29.69 ± 0.97 |
| HSF | 4.8682 ± 0.00 | 5.4435 ± 0.10 | 5.3428 ± 0.09 | 17.50 ± 0.54 |
| CSF | 4.8954 ± 0.17 | 5.2189 ± 0.04 | 5.1467 ± 0.02 | 22.32 ± 0.95 |
| CHSF | 4.8154 ± 0.05 | 5.2185 ± 0.08 | 5.1255 ± 0.01 | 23.07 ± 0.86 |
| AQSF | 4.7817 ± 0.18 | 5.3107 ± 0.08 | 5.2130 ± 0.13 | 18.47 ± 2.24 |
| <i>B. verrucosa</i> (leaf) | | | | |
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.8756 ± 0.08 | 5.3386 ± 0.03 | 5.2271 ± 0.01 | 24.08 ± 0.68 |
| HSF | 4.8682 ± 0.06 | 5.4435 ± 0.00 | 5.3428 ± 0.04 | 17.50 ± 0.41 |
| CSF | 4.9762 ± 0.09 | 5.2781 ± 0.10 | 5.1763 ± 0.02 | 33.72 ± 1.05 |
| CHSF | 4.8813 ± 0.10 | 5.3892 ± 0.03 | 5.3438 ± 0.09 | 8.94 ± 0.22 |
| AQSF | 4.6689 ± 0.11 | 5.2056 ± 0.07 | 5.1560 ± 0.13 | 9.24 ± 1.86 |
| <i>B. stipularis</i> (stem bark) | | | | |
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.7870 ± 0.10 | 5.1582 ± 0.04 | 5.0229 ± 0.08 | 36.45 ± 0.66 |
| HSF | 4.7747 ± 0.09 | 5.2446 ± 0.10 | 5.1151 ± 0.04 | 27.56 ± 0.73 |
| CSF | 4.8069 ± 0.01 | 5.2529 ± 0.06 | 5.1196 ± 0.01 | 30.06 ± 0.55 |
| CHSF | 4.6709 ± 0.10 | 5.0970 ± 0.09 | 5.0039 ± 0.02 | 21.85 ± 0.47 |
| AQSF | 4.7420 ± 0.08 | 5.3244 ± 0.10 | 5.1721 ± 0.01 | 28.73 ± 0.86 |
| <i>B. stipularis</i> (leaf) | | | | |
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.9863 ± 0.12 | 5.4876 ± 0.05 | 5.3486 ± 0.04 | 27.73 ± 0.92 |
| HSF | 4.8854 ± 0.11 | 5.3218 ± 0.06 | 5.1987 ± 0.01 | 28.21 ± 0.82 |
| CSF | 4.8639 ± 0.11 | 5.3465 ± 0.07 | 5.2188 ± 0.03 | 26.46 ± 0.73 |
| CHSF | 4.9769 ± 0.07 | 5.4712 ± 0.05 | 5.3982 ± 0.02 | 14.77 ± 0.51 |
| AQSF | 4.8792 ± 0.11 | 5.3478 ± 0.03 | 5.2168 ± 0.03 | 27.96 ± 0.79 |
| <i>B. tomentosa</i> (stem bark) | | | | |
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.8708 ± 0.11 | 5.4598 ± 0.05 | 5.3391 ± 0.01 | 20.49 ± 0.72 |
| HSF | 4.7375 ± 0.10 | 5.2563 ± 0.09 | 5.2020 ± 0.02 | 10.43 ± 0.48 |
| CSF | 4.7678 ± 0.10 | 5.1586 ± 0.06 | 5.0724 ± 0.01 | 22.06 ± 0.32 |
| CHSF | 4.7813 ± 0.09 | 5.2239 ± 0.03 | 5.1609 ± 0.03 | 16.13 ± 0.43 |
| AQSF | 4.8321 ± 0.03 | 5.2587 ± 0.05 | 5.1007 ± 0.01 | 37.04 ± 0.78 |
| <i>B. tomentosa</i> (leaf) | | | | |
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.7583 ± 0.09 | 5.2786 ± 0.03 | 5.0973 ± 0.01 | 34.85 ± 1.00 |
| HSF | 4.8953 ± 0.17 | 5.3865 ± 0.11 | 5.2804 ± 0.08 | 21.60 ± 1.37 |
| CSF | 4.8537 ± 0.00 | 5.2895 ± 0.09 | 5.1087 ± 0.06 | 41.46 ± 0.98 |
| CHSF | 4.9765 ± 0.07 | 5.4487 ± 0.15 | 5.3786 ± 0.02 | 14.85 ± 1.11 |
| AQSF | 4.8569 ± 0.04 | 5.2786 ± 0.19 | 5.1874 ± 0.01 | 21.63 ± 2.33 |

W₁ = Weight of vial alone; W₂ = Weight of clot containing vial; W₃ = Weight of clot containing vial after clot disruption; Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction

Table 7.2: Thrombolytic activity (in terms of % clot lysis) of *E. fusca* and *E. variegata*.

| <i>E. fusca</i> (stem bark) | | | | |
|---------------------------------|----------------|----------------|----------------|--------------|
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.7355 ± 0.12 | 5.1508 ± 0.01 | 5.0991 ± 0.09 | 12.45 ± 1.31 |
| HSF | 4.8124 ± 0.18 | 5.2631 ± 0.10 | 5.1988 ± 0.03 | 14.27 ± 0.98 |
| CSF | 4.8242 ± 0.34 | 5.2458 ± 0.07 | 5.1499 ± 0.06 | 22.75 ± 0.59 |
| CHSF | 4.7377 ± 0.25 | 5.1211 ± 0.02 | 5.0968 ± 0.12 | 6.34 ± 0.47 |
| AQSF | 4.8599 ± 0.09 | 5.2391 ± 0.01 | 5.1905 ± 0.01 | 12.82 ± 0.35 |
| <i>E. fusca</i> (leaf) | | | | |
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.8122 ± 0.16 | 5.2743 ± 0.01 | 5.2035 ± 0.02 | 15.65 ± 0.94 |
| HSF | 5.2202 ± 0.21 | 5.7155 ± 0.01 | 5.6368 ± 0.10 | 15.33 ± 0.32 |
| CSF | 5.1043 ± 0.13 | 5.6606 ± 0.06 | 5.6160 ± 0.14 | 8.02 ± 1.78 |
| CHSF | 4.8928 ± 0.23 | 5.2941 ± 0.02 | 5.2452 ± 0.04 | 12.19 ± 1.07 |
| AQSF | 4.9631 ± 0.17 | 5.2375 ± 0.18 | 5.1954 ± 0.08 | 15.35 ± 0.35 |
| <i>E. variegata</i> (stem bark) | | | | |
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.8759 ± 0.09 | 5.4632 ± 0.22 | 5.3194 ± 0.03 | 24.48 ± 1.97 |
| HSF | 4.7659 ± 0.20 | 5.3174 ± 0.02 | 5.1876 ± 0.02 | 23.54 ± 0.60 |
| CSF | 4.8216 ± 0.17 | 5.3267 ± 0.13 | 5.2885 ± 0.06 | 5.62 ± 0.90 |
| CHSF | 4.7953 ± 0.09 | 5.1876 ± 0.03 | 5.0278 ± 0.05 | 40.73 ± 1.67 |
| AQSF | 4.8712 ± 0.09 | 5.3718 ± 0.07 | 5.2368 ± 0.03 | 26.97 ± 0.94 |
| <i>E. variegata</i> (leaf) | | | | |
| Fractions | W ₁ | W ₂ | W ₃ | % of lysis |
| Me | 4.7761 ± 0.15 | 5.2738 ± 0.07 | 5.2127 ± 0.10 | 12.28 ± 0.18 |
| HSF | 4.8766 ± 0.18 | 5.3470 ± 0.06 | 5.2875 ± 0.07 | 12.65 ± 0.54 |
| CSF | 4.7760 ± 0.07 | 5.2246 ± 0.05 | 5.1538 ± 0.04 | 15.78 ± 0.40 |
| CHSF | 4.9860 ± 0.11 | 5.5124 ± 0.20 | 5.4626 ± 0.19 | 9.46 ± 1.44 |
| AQSF | 4.8460 ± 0.19 | 5.5023 ± 0.01 | 5.2213 ± 0.01 | 42.82 ± 1.43 |

W₁ = Weight of vial alone; W₂ = Weight of clot containing vial; W₃ = Weight of clot containing vial after clot disruption; Me= Methanol extract; HSF= *n*-hexane soluble fraction; CSF= Carbon tetrachloride soluble fraction; CHSF= Chloroform soluble fraction; AQSF= Aqueous soluble fraction



Chapter 8

Summary

SUMMARY

This dissertation describes the isolation and structure elucidation of secondary metabolites from four plants *Bridelia verrucosa*, *B. stipularis*, *B. tomentosa* (Family: Phyllanthaceae) and *Erythrina fusca* (Family: Fabaceae) as well as biological studies of the extractives from these plants along with *E. variegata* (Family: Fabaceae). A total of nineteen compounds were isolated of which three appear to be new. The structures of the isolated compounds were elucidated mainly by spectroscopic studies including high field NMR.

Kupchan partitioning method was used for partitioning the crude methanolic extract and the various chromatographic techniques were used to isolate the pure compounds from the investigated plants. For the *n*-hexane soluble materials of the stem bark of *B. verrucosa*, gel permeation (Lipophilic Sephadex LH-20) chromatography was used followed by column and preparative thin layer chromatography (PTLC). The stem bark of *B. verrucosa* afforded eight compounds viz. glochidonol (BVSP-1, **46**), brassicasterol (BVSP-2, **47**), friedelin (BVSP-4, **48**); 5 β -24S-ethylcholestan-3 β -ol (BVSP-6, **49**); stigmasterol (BVSP-34, **50**); 5,5'-dihydroxysesamine (BVBC-2, **51**), 3-ketoleanane (**53**) and pinoresinol (BVS-65, **54**). Except stigmasterol (**50**) and pinoresinol (**54**) this is the first report of the isolation of compounds **46-49**, **53** and **54** from the genus *Bridelia* and **51** has been tentatively identified as a new natural product.

In case of the stem bark of *B. stipularis* and *B. tomentosa* vacuum liquid chromatography was used for fractioning the methanolic extract followed by gel permeation chromatography and then PTLC was done to isolate two triterpenes namely, glut-5(6)-en-3-one (BS-01, **55**), glut-5(6)-en-3 α -ol (BS-06, **56**) and a cholestane type compound (22E)-7-hydroxy-28-methylcholesta-4,22-dien-3-one (BS-11, **57**). All these are the first report of their occurrence from *B. stipularis* while **57** has been proposed as a tentatively new compound. On the other hand, phytochemical investigation of methanol extract of the stem bark of *B. tomentosa* yielded friedoolean-5(6),14(15)-dien-3-one (BT-1, **58**), β -taraxerol (BT-6, **59**), D₄-stigmasterone (BT-8, **60**) and lupeol (BT-14, **61**). This is the first report of their occurrence from this plant and **58** has been tentatively identified as a new compound.

Again, for the stem bark of *E. fusca* column chromatography of the carbon tetrachloride soluble materials was carried out. The fractions were then subjected to PTLC to afford three flavonoids namely, shinpterocarpin (EF-7, **62**), lupinifolin (EF-8, **63**) and 3,9-

dihydroxy-4-(3,3-dimethylallyl)[6a*R*,11a*R*]-pterocarpan (**64**) and a steroid, β -sitosterol (EF-38, **65**). All these are the first report from *E. fusca*.

The crude extracts and organic soluble material of the investigated plants and some purified compounds were screened for their antimicrobial activity against a wide range of Gram-positive and Gram-negative microorganisms and fungi by the standardized disc diffusion method. Kanamycin and Griseofulvin were used as reference drugs for the test.

All the samples showed mild to moderate antimicrobial activity against thirteen Gram positive and Gram negative bacterial strains and three fungi, where the stem bark extract of *B. tomentosa* demonstrated the highest inhibition of growth against *B. cereus* (23.2 mm) and *C. albicans* (17.5 mm). The purified compounds isolated from the investigated plants showed mild to moderate antibacterial activity. BS-01 (**55**) and BS-06 (**56**) revealed strong inhibition of growth of *E. coli* (22.7 mm) and *C. albicans* (20.8 mm), respectively.

The plants were subjected to brine shrimp lethality bioassay for probable cytotoxicity. The aqueous soluble fraction of the leaf of *B. stipularis* revealed the highest cytotoxic activity with LC₅₀ value of 12.59 μ g/ml. On the other hand, the *n*-hexane soluble fraction of the stem bark of *E. variegata* revealed the strongest LC₅₀ value of 14.66 μ g/ml. The isolated pure compound EF-8 (**62**) displayed the strongest cytotoxic activity with LC₅₀ and LC₉₀ values of 3.17 and 62.95 μ g/ml, respectively as compared to standard vincristine sulfate (LC₅₀ 0.45 μ g/ml and LC₉₀ 10 μ g/ml).

LC₅₀ (median lethal concentration) is determined by using brine shrimp lethality bioassay which indicates cytotoxicity as well as a wide range of pharmacological activities (e.g. anticancer, antimicrobial, insecticidal, pesticidal, etc.) of the compounds. The assay is considered as a useful tool for preliminary assessment of toxicity and it has been used for the detection of degree of toxicity. If plant extract shows the cytotoxic effect, it is due to the toxic compounds present in the extract that possess ovicidal and larvicidal properties. These properties attribute synergistic effect of zone of inhibition against growth of microorganisms i.e. the antimicrobial activities or MIC (minimum inhibitory concentration). But if the plant extract or compound possess less cytotoxic activity but more antimicrobial activities (higher zone of inhibition or less MIC) it indicate that the plant or compound is suitable for the use to human being. In some cases compounds showing low LC₅₀ value showed higher zone of inhibition in the antimicrobial screening

[e. g. pure compounds EF-7 (62), EF-8 (63) and EF-12 (64) having less LC_{50} value 4.7, 3.17 and 4.81 $\mu\text{g/ml}$ gave high zone of inhibition of 19.4, 19.1 and 19.0 mm against *Bacillus subtilis*, *B. megaterium* and *B. cereus*, respectively]. However this could not be observed in some other cases [e. g. pure compounds BVBC-2 (51) and BVS-65 (54) having high LC_{50} value 8.11 and 10.12 $\mu\text{g/ml}$ showed low zone of inhibition of 12.2 and 11.8 mm against *Staphylococcus aureus* and *Sarcina lutea*, respectively].

Free radical scavenging activity, total antioxidant capacity and total phenolic content of the extractives were also investigated. Free radical scavenging assay of the methanol extract of the leaf of *B. verrucosa* revealed highest activity having IC_{50} of 6.35 $\mu\text{g/ml}$ while the chloroform soluble fraction of the leaf of *E. fusca* showed IC_{50} at 5.50 $\mu\text{g/ml}$. The IC_{50} (7.3 $\mu\text{g/ml}$) of the isolated pure compound BVBC-2 (51) indicated strong free radical scavenging activity.

The aqueous and chloroform soluble materials of the leaf of *B. stipularis* demonstrated promising total phenolic content (98.76 and 94.52 mg of GAE/100 g of dried extract). On the other hand, the highest total antioxidant capacity of methanolic extract of the leaf of *B. tomentosa* and *E. fusca* were found as 612.54 and 587.66 mg of ascorbic acid/g of dried extract, respectively.

All the extractives of five plants were also studied for their thrombolytic potential. The carbon tetrachloride soluble fraction and methanol extract of leaf and aqueous soluble material of bark of *B. tomentosa*, methanol extract of bark of *B. stipularis* and carbon tetrachloride soluble partitionate of leaf of *B. verrucosa* exhibited highest thrombolytic activity with clot lysis value of 41.46%, 34.85%, 37.04%, 36.45% and 33.72%, respectively. Standard streptokinase was used as positive control which exhibited 61.50% lysis of clot while the negative control water revealed 2.56% clot lysis.

In conclusion, a total 19 compounds including three new molecules (51, 57 and 58) have been isolated from *B. verrucosa*, *B. stipularis* and *B. tomentosa*, respectively. In case of biological investigation, the methanolic extract of the investigated plants and their Kupchan fractions showed significant antibacterial, cytotoxic, antioxidant and thrombolytic activities. These plants can be further studied in order to find out their unexplored efficacy against other diseases as well as to isolate more chemically interesting and biologically important drug candidates.



Chapter 9

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Chapter 10

List of Publications

LIST OF PUBLICATIONS

1. Adeeba Anjum, Mohammad R. Haque, Mohammad S. Rahman, Choudhury M. Hasan, Md. Ekramul Haque, Mohammad A. Rashid. 2011. *In vitro* antibacterial, antifungal and cytotoxic activity of three Bangladeshi *Bridelia* species. *International Research of Pharmacy and Pharmacology*. 1(7), 149-154. ISSN 2251-0176.
2. Adeeba Anjum, M. A. Sikder, Mohammad R. Haque, Choudhury M. Hasan, Mohammad A. Rashid. 2013. *In vitro* antioxidant and thrombolytic activities of *Bridelia* species growing in Bangladesh. *J. Sci. Res.* 5(2), 343-351.

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