PHYTOCHEMICAL INVESTIGATION OF *EUPATORIUM ODORATUM* L. FOR CYTOTOXICITY, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES

Ph. D. Thesis

SAJAN AMATYA

Central Department of Chemistry
Institute of Science and Technology
Tribhuvan University, Kirtipur
Kathmandu, NEPAL
2006

PHYTOCHEMICAL INVESTIGATION OF *EUPATORIUM ODORATUM* L.

FOR CYTOTOXICITY, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES

A thesis submitted to the Institute of Science and Technology, **Tribhuvan University,**
for the Degree of **Doctor of Philosophy** (**Ph. D.**) in Chemistry

by

Sajan Amatya, M. Sc.

Central Department of Chemistry
Institute of Science and Technology
Tribhuvan University, Kirtipur
Kathmandu, NEPAL

2006

CERTIFICATE

This is to certify that the work embodied in this thesis entitled "**Phytochemical investigation of *Eupatorium odoratum* L. for cytotoxicity, antimicrobial and antioxidant activities**" has been carried out by **Sajan Amatya** under my guidance and supervision. The work induced in this thesis is all original, unless otherwise stated and has not been submitted elsewhere for a degree.

He has fulfilled all the requirements laid down by the Tribhuvan University, Kirtipur for the submission of the thesis for the award of **Ph. D.** degree.

Sarbajna Man Tuladhar, Ph. D. (Ireland)
Professor
Research Centre for Applied Science and Technology (RECAST)
Tribhuvan University, Kirtipur
Kathmandu, NEPAL

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to Professor Dr. Sarbajna Man Tuladhar, Research Centre for Applied Science and Technology (RECAST), Tribhuvan University (TU), Nepal for his excellent supervision, encouragement, suggestions and criticisms made on my Ph. D. work. Without him this work would not be possible to complete.

I would like to offer my sincere thanks to Dean Office and the research committee of Institute of Science and Technology, Tribhuvan University, Nepal for allowing me to do Ph. D. in Central Department of Chemistry, TU. I am obliged to Professor Dr. Mangala D. Manandhar, the then Head, Central Department of Chemistry, TU, for enrolling me as a Ph.D. student and providing necessary arrangements as well as interactions and invaluable suggestions for me during my work. I am thankful to Professor Dr. Raja R. Pradhananga, the Head, Central Department of Chemistry, TU, for his continued support and encouragement during this study. I am also thankful to Professor Jaya K. Shrestha, Central Department of Chemistry, TU for his suggestion and encouragement.

I would like to give special thanks to Professor Dr. Tulsi Pathak, the then Executive Director of Research Centre for Applied Science and Technology (RECAST), Tribhuvan University, Nepal for providing laboratory and other physical facilities available therein for the accomplishment of this work. I am equally thankful to Professor Dr. Mohan B. Gewali, Executive Director of Research Centre for Applied Science and Technology (RECAST), Tribhuvan University, Nepal for his invaluable suggestions, interactions and his continued support in providing laboratory and physical facilities in RECAST.

University Grant Commission, Nepal is gratefully acknowledged for providing partial financial support. I would like to extend special thank to Science, Technology and Research Institute, Kathmandu, Nepal for providing financial support for the studies on antioxidant activity.

I am deeply indebted to Professor Dr. S. Kadota and Dr. Arjun H. Baskota at Toyama Medical and Pharmaceutical University, Japan for recoding necessary spectra for compound **1** and **2**; Professor Dr. H. Laatsch and Dr. Rajendra Maskey at University of Gottingen, Germany for recording spectra of compounds **4**, **5**, **6** and **7**; Dr. Krishna G. Dongol at Institute of Chemical and Engineering Sciences, Singapore for recording high resolution mass spectrum of compound **1**. I owe much to Dr. Gana B. Bajracharya at Tohoku University,

Japan and Dr. S. Kaluoni, Toyama Medical and Pharmaceutical University, Japan for recording all necessary spectra of compounds isolated from chloroform extract of root. I am also thankful to Professor Dr. Ronald D. McKelvey, Department of Chemistry, University of Wisconsin-La Crosse, USA for recording ^{1}H and ^{13}C-NMR spectra of compound **3** and DEPT of compound **2**.

I would like to thank Dr. Mina Rajbhandari, Lecturer, RECAST, TU, for her interactions, invaluable guidance and encouragement during this study. I am grateful to Associate Professor Dr. Kamal K. Shrestha, Central Department of Chemistry, TU for his suggestion and encouragement. I am also grateful to Dr. Madhav P. Baral, Principal, National College, Kathmandu, Nepal for providing laboratory facilities to conduct antimicrobial test. I would like to thank Professor Dr. Krishna Kumar Shrestha, Central Department of Botany, TU, for the authentification of plant. My colleague, Mr. Deepak Poudel is also thankful for providing me some articles published in international journals.

I would like to share my thanks to the authorities of Natural Product Research Laboratory, Thapathali and Central Library, TU, Kirtipur, Nepal for providing me the library facilities. I owe much to Mr. Lalit R. Shakya, Deputy Instructor, RECAST for memorizing some necessity statistical terms for my works.

I would like to extend sincere appreciation to Father P. T. Augustine, S. J., Campus chief, St. Xavier's College, Kathmandu, Nepal for granting me one year leave in the college and Father Larry Brooks, S.J., Associate Campus Chief, Councellor Department, St. Xavier's College, Nepal for inspection and possible grammatical correction of the paper before publication. I owe my gratitude to all teaching staffs of Computer Department of St. Xavier's College for providing splendid helps and suggestions from beginning till submission of this thesis. I am also much indebted to all members of Department of Chemistry, St. Xavier's college for their encouragement and support at every moment during my Ph. D. work.

Finally I am grateful to my wife Sajani, daughters - Neha, Suzana and my all brothers-in-law for their supports and encouragement which provide tremendous energy to carry out this work.

Sajan Amatya

Kathmandu, Nepal

ABSTRACT

A novel oleanen-type triterpenes, 3*β*-hydroxy-28-carboxyolean-12-ene (**1**), designated as eupatoric acid, together with seven known compounds - poriferasterol (**2**), octadecane (**3**), butyrospermol acetate (**4**), bis(2-ethylhexyl)phthalate (**5**), chrysophanol (**6**), physcion (**7**), palmitic acid (**8**), a mixture of bis(2-ethylhexyl)phthalate (**5**) and chrysophanol (**6**), a mixture of bis(2-ethylhexyl)phthalate (**5**) and physcion (**7**), and a mixture of poriferasterol (**2**) and *β*-sitosterol (**9**) have been isolated from the brine shrimp cytotoxic extracts of the root of *Eupatorium odoratum* L. In addition, anthraquinone (**10**) and hydrocarbon (**11**) were also detected. Structures of all compounds were elucidated on the basis of spectroscopic methods. Compounds **2**-**7** were reported here for the first time from this plant. Compounds **8** and **9**, known to be contained in leaf, were isolated for the first time from the root.

Compounds **1**-**7** were tested for cytotoxicity against brine shrimp. Similarly compounds **1**-**6** and **8** were tested for antibacterial test. An antioxidant activity using *β*-carotene bleaching test and DPPH radical scavenging test on ethanolic extracts of various parts of this plant was also studied.

LIST OF ABBREVIATIONS / ACRONYMS

AnalaR	Analytical reagent
APT	Attached proton transfer
B. subtilis	*Bacillus subtilis*
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene (2, 6-di-*t*-butyl-4-methylphenol)
^{13}C-NMR	Carbon-13 nuclear magnetic resonance
$CDCl_3$	Deuterated chloroform
CD_3OH	Deuterated methanol
CGC	Capillary gas chromatography
DEPT	Distortionless enhancement by polarization transfer
df	Degree of freedom
DMSO	Dimethylsulphoxide
DPPH	1, 1-Diphenyl-2-picrylhydrazyl radical
E. coli	*Escherichia coli*
EIMS	Electron impact mass spectrometer
ESIMS	Electron spraying ionization mass spectrometer
EtOH	Ethanol
Et_2O	Diethyl ether
FABMS	Fast atom bombarding mass spectrometer
GC	Gas chromatography
GCMS	Gas chromatography-mass spectrometer
^{1}H-^{1}H COSY	Proton-proton correlation spectroscopy
^{1}H-NMR	Proton nuclear magnetic resonance
HMBC	Heteronuclear multiple bond coherence
HMQC	Heteronuclear multiple quantum coherence
HRMS	High resolution mass spectrometer
IR	Infrared
Lin.	Linoleic acid
LR	Laboratory reagent
λ_{max}	Wavelength of maximum absorption
m. pt.	Melting point
MBC	Minimum bactericidal concentration

MeOH	Methanol (methyl alcohol)
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
nm	Nanometer
Na_2EDTA	Disodium ethylenediaminetetra acetate
OSB-CoA	*o*-Succinylbenzoic acid coenzyme A
P. aeruginosa	*Pseudomonas aeruginosa*
PAs	Pyrrolizidine alkaloids
PEP	Phosphenol pyruvate
ppmw	Part per million fresh weight
RAA	Relative antioxidant activity
RDA	*retro*-Diels-Alder fragmentation
R_f	Mobility relative to front
RP-HPLC	Reverse phase high performance liquid chromatography
S. aureus	*Staphylococcus aureus*
σ	Standard deviation
SEM	Standard error mean
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
UV-MS	Ultraviolet-mass spectrometer
UV-Vis	Ultraviolet-visible

Other abbreviations and acronyms have their usual significance.

LIST OF FIGURES

Page

Figure 1.1(a): *Eupatorium odoratum* L. 3

Figure 1.1(b): Flower part of *Eupatorium odoratum* L. 3

Figure 1.2: Polyketide pathway for formation of anthraquinones 13

Figure 1.3: Biosynthetic pathway leading to anthraquinones in Rubiaceae 14

Figure 1.4: Cyclization of 2,3-oxidosqualene into different triterpenes products in *Pisum sativum* 15

Figure 1.5: Cyclization of 2,3-oxidosqualene into different sterols products in *Ochromonas malhamensis* 16

Figure 1.6: Autoxidation of a linoleic acid 17

Figure 2.1: Template for uniform placement of paper discs on Petri plate (size:90 mm) 27

Figure 3.1: Structure of compounds isolated from the root of *E. odoratum* L. 40

Figure 3.2: UV spectrum of compound **1** in chloroform 41

Figure 3.3: IR spectrum of compound **1** in KBr disc 41

Figure 3.4: ^{1}H-NMR (400 MHz) spectrum of compound **1** in $CDCl_3$ plus one drop CD_3OD 42

Figure 3.5: (i) ^{13}C-NMR (100 MHz) spectrum of compound **1** in $CDCl_3$ plus one drop CD_3OD 44

(ii) DEPT 135 spectrum data of compound **1** 44

Figure 3.6: HMQC spectrum of compound **1** 47

Figure 3.7: ^{1}H-^{1}H COSY spectrum of compound **1** 48

Figure 3.8: HMBC spectrum of compound **1** 49

Figure 3.9: ^{1}H-^{13}C connectivities in HMBC experiment for compound **1**. Arrows point from proton to carbon. 49

Figure 3.10: Chemical shifts of H-28 in ^{1}H-NMR and C-28 in ^{13}C-NMR for β-amyrin and eupatoric acid (**1**) 50

Figure 3.11: EIMS for compound **1** 51

Figure 3.12: HRMS for compound **1** 52

Figure 3.13: UV spectrum of compound **2** in $CHCl_3$ 54

Figure 3.14: IR spectrum of compound **2** in $CHCl_3$ 54

Figure 3.15: ^{1}H-NMR (400 MHz) spectrum of compound **2** in $CDCl_3$ 55

Figure 3.16: Peaks patterns of six methyl protons in ^{1}H-NMR spectrum of (a) stigmasteryl acetate (b) poriferasteryl acetate and (c) compound **2** in $CDCl_3$ 57

Figure 3.17: (i) ^{13}C-NMR (100 MHz) spectrum of compound **2** in $CDCl_3$ 58
(ii) DEPT spectrum of compound **2** 58
Figure 3.18: ^{1}H-^{1}H COSY of compound **2** 61
Figure 3.19: HMQC spectrum of compound **2** 62
Figure 3.20: HMBC spectrum of compound **2** 63
Figure 3.21: ^{1}H-^{13}C correlations in HMBC experiment of compound **2**. Arrows point from proton to carbon. 64
Figure 3.22: FABMS of compound **2** in glycerol matrix 65
Figure 3.23: GCMS of compound **2** 65
Figure 3.24: GC-MS of compound **3** 67
Figure 3.25: ^{1}H-NMR (100 MHz) spectrum of compound **3** in $CDCl_3$ 68
Figure 3.26: ^{13}C-NMR (75 MHz) spectrum of compound **3** in $CDCl_3$ 68
Figure 3.27: UV spectrum of compound **4** in hexane 70
Figure 3.28: (a) Butyrospermol (the eupha-7, 21-diene skeleton) and (b) Parkeyl acetate (the lanosta-9(11), 24-diene skeleton) 70
Figure 3.29: ^{13}C-NMR (100 MHz) spectrum of compound **4** in $CDCl_3$ 71
Figure 3.30: APT spectrum of compound **4** 73
Figure 3.31: ^{1}H-NMR (300 MHz) spectrum of compound **4** in $CDCl_3$ 73
Figure 3.32: Comparison of ^{1}H-NMR data of compound **4** with butyrospermol 74
Figure 3.33: EIMS of compound **4** 75
Figure 3.34(i): UV spectrum of compound **5** recorded in diethyl ether 76
Figure 3.34(ii): UV spectrum of compound **5** recorded in hexane 76
Figure 3.35: ^{1}H-NMR (300 MHz) spectrum of compound **5** 77
Figure 3.36: ^{1}H-NMR spectrum (400 MHz) of different protons of compound **5** in $CDCl_3$ 77
Figure 3.37: Connectivity between H-1 and H-2 for compound **5** in ^{1}H-^{1}H COSY spectrum of mixture containing compounds **5** and **6** 78
Figure 3.38: Comparison of ^{1}H-NMR data of compound **5** with bis(2-ethylhexyl)phthalate 79
Figure 3.39: ^{13}C-NMR (100 MHz) spectrum of compound **5** 79
Figure 3.40: Comparison of ^{13}C-NMR data of compound **5** with bis(2-ethylhexyl)phthalate 80
Figure 3.41: ^{1}H-^{13}C direct connectivities observed for compound **5** in HMQC spectrum of mixture of compounds **5** and **6** 81
Figure 3.42: Selected HMBC of compound **5**. Arrows point from carbon to proton. 82

Figure 3.43: EIMS of compound **5** 83
Figure 3.44: Mass fragmentation of compound **5** 83
Figure 3.45 (i): GCMS of compound **5** 84
Figure 3.45 (ii): Reported spectrum 84
Figure 3.46: UV-visible spectra of compound **6** recorded in different solvents 85
Figure 3.47: Complexation of chrysophanol with $AlCl_3$ 87
Figure 3.48: Resonances of chrysophanol in presence of alkali 88
Figure 3.49: ^{1}H-NMR spectrum (300 MHz) spectrum of compound **6** in $CDCl_3$ 88
Figure 3.50: Resolved signals of H-5 and H-7 of compound **6** in 400 MHz ^{1}H-NMR spectrum 89
Figure 3.51: Comparison of ^{1}H-NMR data of compound **6** with chrysophanol 90
Figure 3.52: ^{13}C-NMR (75 MHz) spectrum of compound **6** in $CDCl_3$ 90
Figure 3.53: Chemical shifting in ^{13}C-NMR due to effect of H-bonding in C-9 and C-10 position in anthraquinone 92
Figure 3.54: ^{1}H-^{13}C connectivities for compound **6** in HMQC spectrum of mixture of compounds **5** and **6** 93
Figure 3.55: Selected HMBC of compound **6** 94
Figure 3.56: (i) EIMS of compound **6** 95
(ii) GCMS showing molecular ion peak of compound **6** in mixture sample 95
Figure 3.57: UV-visible spectrum of compound **7** in MeOH 96
Figure 3.58: ^{13}C-NMR (100 MHz) spectrum of compound **7** in $CDCl_3$ 97
Figure 3.59: ^{1}H-NMR (300 MHz) spectrum of compound **7** in $CDCl_3$ 99
Figure 3.60: EIMS of compound **7** 99
Figure 3.61: IR spectrum of compound **8** 101
Figure 3.62: ^{1}H-NMR (400 MHz) spectrum of compound **8** in $CDCl_3$ 101
Figure 3.63: ^{13}C-NMR (100 MHz) spectrum of compound **8** in $CDCl_3$ 102
Figure 3.64: ^{1}H-^{1}H COSY spectrum of compound **8** 103
Figure 3.65: HMBC spectrum of compound **8** 104
Figure 3.66: ^{1}H-^{13}C relation revealed in HMBC spectrum. Arrow points carbon to hydrogen. 104
Figure 3.67: GCMS of compound **8** 105
Figure 3.68: ^{1}H-NMR spectrum of steroidal mixture of **2** and **9** 106
Figure 3.69: ^{13}C-NMR spectrum of steroidal mixture of **2** and **9** 106

Figure 3.70: (i) GCMS of compound **2** in steroidal mixture of **2** and **9** 107
(ii) GCMS of compound **9** in steroidal mixture of **2** and **9** 107
Figure 3.71: UV-visible spectra of compound **10** in different solvents 109
Figure 3.72: ^{1}H-NMR spectrum of compound **11** 110
Figure 3.73: GCMS of compound **11** 111
Figure 3.74: Antioxidative effects of methanolic solution of different parts of *E. odoratum* and BHA in the *β*-carotene linoleic acid system at 50 °C 114
Figure 3.75: Comparison of RAA values of different extracts with BHA at 60 and 120 minutes 115
Figure 3.76: Graphical representation of scavenging activity in terms of % inhibition of different extracts on DPPH free radical 116
Figure 3.77: IC_{50} (µg/mL) values of different parts of *E. odoratum* for free radical scavenging activity by DPPH radical. A lower value indicates higher antioxidant activity. 117

LIST OF TABLES

		Page
Table 1.1:	Reported compounds from *E. odoratum*	4
Table 2.1:	General classification of the brine shrimp cytotoxicity test	23
Table 2.2:	Composition of artificial seawater	23
Table 3.1:	Results of phytochemical screening tests of the root	32
Table 3.2:	Percentage yield of root fractions obtained from method I	33
Table 3.3:	Brine shrimp cytotoxicity test for root fractions obtained from method I	34
Table 3.4:	Results of phytochemical screening of cytotoxic and non-cytotoxic fractions of the root obtained from method I	35
Table 3.5:	Brine shrimp cytotoxicity test of root extracts obtained from method II	36
Table 3.6:	Zone of inhibition of root extracts obtained from method II	37
Table 3.7:	^{1}H-NMR (400 MHz) data of compound **1** in $CDCl_3$ plus one drop CD_3OD	43
Table 3.8:	^{13}C-NMR (100 MHz), DEPT data of compound **1** and ^{13}C-NMR literature data of oleanolic acid	46
Table 3.9:	HMQC data of compound **1**	47
Table 3.10:	HMBC data of compound **1**	50
Table 3.11:	Comparison of ^{1}H-NMR data of compound **2** with reported values of stigmasterol and poriferasterol	56
Table 3.12:	^{13}C-NMR (100 MHz) data of compound **2**	59
Table 3.13:	Comparative study of ^{13}C-NMR data of compound **2** with reported data of (a) stigmasterol and (b) poriferasterol	60
Table 3.14:	^{1}H-^{1}H COSY data for compound **2**	61
Table 3.15:	HMQC data for compound **2**	62
Table 3.16:	HMBC data of compound **2**	64
Table 3.17:	Mass fragmentation patterns of isolated poriferasterol (**2**), reported poriferasterol and stigmasterol	66
Table 3.18:	Comparison of melting point of **2** with reported data of poriferasterol and stigmasterol	66
Table 3.19:	^{13}C-NMR (75 MHz) data of compound **3**	69
Table 3.20:	Comparison of ^{13}C-NMR data of olefinic carbons of compound **4** with butyrospermol and parkeyl acetate	71
Table 3.21:	Comparison of ^{13}C-NMR data (100 MHz) of butyrospermol acetate (**4**) with reported butyrospermol in $CDCl_3$	72
Table 3.22:	^{1}H-NMR (300 MHz) data of compound **4** in $CDCl_3$	74

Table 3.23: ^{1}H-NMR (300 and 400 MHz) data of compound **5** in $CDCl_3$ 78
Table 3.24: HMQC data of compound **5** 80
Table 3.25: HMBC data of compound **5** 82
Table 3.26: UV and Visible spectral shifts for compound **6** 86
Table 3.27: ^{1}H-NMR (300 and 400 MHz) data of compound **6** 89
Table 3.28: ^{13}C-NMR (75 MHz) data of compound **6** and chrysophanol 91
Table 3.29: HMQC data of compound **6** 92
Table 3.30: HMBC data of compound **6** 94
Table 3.31: ^{13}C-NMR (75 MHz) data for compound **7** and its reported value 97
Table 3.32: Comparison of ^{1}H-NMR data of compound **7** with physcion 98
Table 3.33: Comparison of ^{1}H-NMR data of compound **7** with przewalskinone 98
Table 3.34: Comparison of mass fragmentation pattern of isolated compound **7** with physcion and przewalskinone 100
Table 3.35: ^{1}H-NMR (400 MHz) data of compound **8** in $CDCl_3$ 102
Table 3.36: ^{13}C-NMR (100 MHz) data of compound **8** in $CDCl_3$ 103
Table 3.37: UV and Visible spectral shifts of compound **10** 108
Table 3.38: Brine shrimp cytotoxicity test of isolates **1**-**7** from the root 112
Table 3.39: Zone of inhibition of compounds 1 – 6 and 8 from the root 112
Table 3.40: Zone of inhibition of hexane extract of the flower 113
Table 3.41: Relative antioxidant activity (RAA) of different parts of E. odoratum 115
Table 3.42: Percentage inhibition of different parts of plant on DPPH radicals 116
Table 5.1: Cytotoxicity of petroleum ether extract (**F1**) of the root against brine shrimp 133
Table 5.2: Cytotoxicity of solid residue (**F2**) of the root against brine shrimp 134
Table 5.3: Cytotoxicity of hexane soluble subfraction (**F2-1**) of **F2** against brine shrimp 135
Table 5.4: Cytotoxicity of dichloromethane soluble subfraction (**F2-2**) of **F2** against brine shrimp 136
Table 5.5: Cytotoxicity of ethyl acetate soluble (**F2-3**), methanol soluble (**F2-4**) subfractions of **F2** and syrupy residue (**F3**) of the root against brine shrimp 137
Table 5.6: Cytotoxicity of berberine (positive control) against brine shrimp 138
Table 5.7: Cytotoxicity of petroleum ether extract (**E1**) of the root against brine shrimp 146
Table 5.8: Cytotoxicity of chloroform extract (**E2**) of the root against brine shrimp 147

Table 5.9: Cytotoxicity of ethyl acetate (**E3**) and ethanol (**E4**) extracts of the root against brine shrimp 148
Table 5.10: Cytotoxicity of bis(2-ethylhexyl)phthalate (**5**) against brine shrimp 170
Table 5.11: Cytotoxicity of chrysophanol (**6**) against brine shrimp 171
Table 5.12: Cytotoxicity of physcion (**7**) against brine shrimp 172
Table 5.13: Cytotoxicity of eupatoric acid (**1),** poriferasterol (**2**) and octadecane (**3**) against brine shrimp 173
Table 5.14: Cytotoxicity of butyrospermol acetate (**4**) against brine shrimp 174
Table 5.15: Cytotoxicity of hexane extract of the flower against brine shrimp 177
Table 5.16: Preparation of solutions corresponding different concentrations for MIC 179
Table 5.17: Cytotoxicity of ethanolic extracts of the stem and leaf against brine shrimp 181
Table 5.18: Absorbance recorded at 15 minutes interval for extracts in *β*-carotene bleaching test 183
Table 5.19: Calculation of RAA of different extracts 184
Table 5.20: Paired *t*-test between ethanolic extract of the leaf and BHA 185
Table 5.21: Paired *t*-test between ethanolic extract of the root and BHA 185
Table 5.22: Paired *t*-test between ethanolic extract of the flower and BHA 186
Table 5.23: Paired *t*- test between ethanolic extract of the stem and BHA 186
Table 5.24: Absorbance recorded for leaf extract 188
Table 5.25: Absorbance recorded for flower extract 188
Table 5.26: Absorbance recorded for stem extract 188
Table 5.27: Absorbance recorded for root extract 189
Table 5.28: Absorbance recorded for BHA (positive control) 189
Table 5.29: Scavenging activity of different parts of plant in terms of % inhibition 189
Table 5.30: Scavenging activity of different extracts of plant in terms of IC_{50} 190

LIST OF SCHEMES

		Page
Scheme 2.1:	General protocol for phytochemical screening	21
Scheme 3.1:	Preparation of root extracts by method I	33
Scheme 3.2:	Preparation of root extracts by method II	36
Scheme 3.3:	Isolation of compounds from petroleum ether extract (**E1**) of the root	38
Scheme 3.4:	Isolation of palmitic acid (**8**) from petroleum ether extract (**E1**) of the root	38
Scheme 3.5:	Isolation of compounds from chloroform extract of the root	39
Scheme 3.6:	Possible mass fragmentation of eupatoric acid (**1**)	53
Scheme 5.3:	Isolation of compounds **1** and **2**	151
Scheme 5.4:	Isolation of compounds **3** and **4** from **R2**	152
Scheme 5.5**:**	Isolation of compounds **5**, **6** and **7** from Fraction **8**	154
Scheme 5.6:	Isolation of compounds from chloroform extract	156
Scheme 5.7:	Isolation of compound **2** from Fraction **5**	158
Scheme 5.8:	Isolation of compounds **2** and **9** in mixture and **10** from **R5**	159
Scheme 5.9:	Isolation of compounds **6** and **11** from **R3**	161

CONTENTS

	Page
Certificate	**i**
Acknowledgements	**ii**
Abstract	**iv**
List of abbreviations / Acronyms	**v**
List of figures	**vii**
List of tables	**xi**
List of schemes	**xiv**
CHAPTER 1: INTRODUCTION	**1-18**
1.1 Plant for investigation	2
1.2 Description for plant	2
1.3 Literature review	4
1.3.1 Ethnopharmacological uses	4
1.3.2 Chemical constituents	4
1.3.3 Biological activities	9
1.4 Rationale of study	12
1.5 Biosynthesis of some plants secondary metabolites	13
1.5.1 Biosynthesis of anthraquinones	13
1.5.2 Biosynthesis of different triterpenes	15
1.5.3 Biosynthesis of sterols	16
1.6 Autoxidation and antioxidants	16
CHAPTER 2: MATERIALS & METHODS	**19-30**
2.1 Materials	20
2.1.1 Solvents and chemicals	20
2.1.2 Chromatographic materials	20
2.1.3 Culture media, chemicals and disc for antibacterial activity	20

2.1.4 Micro-organism 20
2.2 General methods 20
2.2.1 Thin layer chromatography 20
2.2.2 Preparative thin layer chromatography (PTLC) 20
2.2.3 Column chromatography 20
2.2.4 Preliminary phytochemical screening 21
2.2.5 Brine shrimp cytotoxicity or lethality test 22
2.2.5.1 Preparation of artificial seawater 23
2.2.5.2 Hatching the brine shrimp 24
2.2.5.3 Preparation of sample solution for extract/compound 24
2.2.6 Antibacterial activity 24
2.2.6.1 Disc diffusion method 24
2.2.6.2 Two fold serial dilution technique 25
2.2.6.3 Preliminaries for antibacterial test 25
2.2.7 Antioxidant activity 28
2.2.7.1 β - Carotene bleaching test 28
2.2.7.2. DPPH radical scavenging test 29

CHAPTER 3: RESULTS & DISCUSSION 31-170

3.1 Phytochemical screening of the root 32
3.2 Bioassay of the root 33
3.2.1 Preparation of extracts -Method I 33
3.2.1.1 Brine shrimp cytotoxicity test of fractions / subfractions 34
3.2.1.2 Phytochemical screening of cytotoxic (**F1**, **F2**, **F2-1**, **F2-2**) and noncytotoxic (**F2-3**, **F2-4**, **F3**) fractions 34
3.2.2 Preparation of extracts - Method II 35
3.2.2.1 Brine shrimp cytotoxicity test 36
3.2.2.2 Antibacterial test 36
3.3 Isolation of compounds from the root extracts 37
3.3.1 From petroleum ether extract (**E1**) 37
3.3.2 From chloroform extract (**E2**) 39
3.4 Structure elucidation of isolates from the root 41

3.5 Bioassay of isolates from the root 111
3.5.1 Brine shrimp cytotoxicity test 111
3.5.2 Antibacterial test 112
3.6 Bioassay of the flower 113
3.6.1 Brine shrimp cytotoxicity test 113
3.6.2 Antibacterial test 113
3.7 Bioassay of the leaf and the stem 113
3.7.1 Brine shrimp cytotoxicity test 113
3.8 Antioxidant activity of different parts of the plant 114
3.8.1 β-carotene bleaching test 114
3.8.2 DPPH radical scavenging test 115

CHAPTER 4: SUMMARY & CONCLUSIONS 118 - 122

CHAPTER 5: EXPERIMENTALS 123 - 190

5.1 General 124
5.2 Plant materials 124
5.3 Phytochemical screening of the root 125
5.4 Bioassay of the root 130
5.4.1 Preparation of extracts - Method I 130
5.4.1.1 Brine shrimp cytotoxicity test of fractions/subfractions 131
5.4.1.2 Phytochemical screening of cytotoxic (**F1**, **F2**, **F2-1**, **F2-2**) and non-cytotoxic (**F2-3**, **F2-4**, **F3**) fractions 139
5.4.2 Preparation of extracts - Method II 144
5.4.2.1 Brine shrimp cytotoxicity test 144
5.4.2.2 Antibacterial test 149
5.5 Isolation of compounds from the root extracts 150
5.5.1 From petroleum ether extract (**E1**) 150
5.5.2 From chloroform extract (**E2**) 155
5.6 Characterization of isolated compounds and mixtures 162
5.7 Bioassay of isolates from the root 169

5.7.1 Brine shrimp cytotoxicity test 169
5.7.2 Antibacterial test 175
5.8 Bioassay of the flower 176
5.8.1 Preparation of extract 176
5.8.2 Brine shrimp cytotoxicity test 176
5.8.3 Antibacterial test 178
5.9 Bioassay of the leaf and the stem 180
5.9.1 Preparation of extracts 180
5.9.2 Brine shrimp cytotoxicity test 180
5.10 Antioxidant activity of different parts of the plant 182
5.10.1 β-carotene bleaching test 182
5.10.2 DPPH radical scavenging test 187

REFERENCES **191-200**

Annex 1: Preparation of reagents I

Annex 2: Publications / Paper presentation II

Chapter 1

INTRODUCTION

1.1 PLANT FOR INVESTIGATION

Eupatorium odoratum L. (synonym: *Chromolaena odorata* King and Robinson 1970) was selected for present study. *Eupatorium* species (Asteraceae, Eupatorieae) included about 1200 species.[1] Six species are available in Nepal.[2] They are

(1) *Eupatorium cannabinum var. heterophyllum* (DC) Kitam or *E. heterophyluum* DC
(2) *Eupatorium adenophorum* Spreng or *E. glandulosum* Kunth
(3) *Eupatorium odoratum* L. or *Chromolaena odorata* King and Robinson
(4) *Eupatorium capillifolium* Lam. or *E. foeniculaceum* Willd.
(5) *Eupatorium acuminatum* D. Don or *E. vernonia extensa* DC
(6) *Eupatorium wallichii* DC

It is known to have originated from South and Central America and is commonly called Siam weed, triffid weed, bitter bush or jack in the bush. The plant has now naturalized in many countries of the world where it is often considered a threat due to its ability to inhibit growth and may even kill local plants. Therefore, in Nepal this plant is also called '*BANAMARA*' or forest killer.

1.2 DESCRIPTION FOR PLANT[3]

Morphology: erect, softly hairy - more or less aromatic, perennial to 2 m, with many spreading branches, gregarious on wasteland and often forming dense thickets (figure 1.1).
Leaf: rhomboid-ovate or ovate-lanceolate, long-pointed, base wedge-shaped, with 3 main nerves, margins toothed, densely hairy, beneath stalked.
Flower: pale pinkish-mauve in cylindrical heads, 1 cm long arranged in branched terminal clusters. Involucral bracts oblong blunt, white lined with green, margins papery.
Flowering season: December-April
Distribution: Native of America widely naturalized in Central and East Nepal to 1500m.[2]

E. odoratum can be distinguished from *E. adenophorum* (also called *BANAMARA* by local people) in colour of the stem and flower – the former has yellow green stem and pinkish flower while the latter has purple/violet stem and white flower.

Figure 1.1(a): *Eupatorium odoratum* L.

Figure 1.1 (b): Flower part of *Eupatorium odoratum* L.

1.3 LITERATURE SURVEY

Literature survey is based on **Chemical Abstracts** (1918 to 2005), **MEDLINE** Search in the Internet and other bibliography therein.

1.3.1 Ethnopharmacological Uses

The juice of aerial part of plant is used for cuts and wounds healing and a local antiseptic agent.[4 - 6] A decoction of its leaf is valued in native medicine as a cure for malaria and as a cough suppressants.[6, 7] Leaf extract with salt is used as a gargle for sore throats and colds as well as also used to scent aromatic baths.[8] The plant is reported to be used as a herbal tea for health which relieves cough, fever, jaundice, hemorrhoids and stomachache and it also promotes physical strength.[9]

1.3.2 Chemical constituents

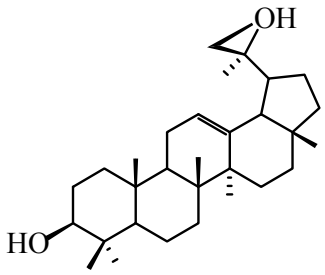
The chemical constituents isolated from different parts of this plant can be broadly divided into (a) triterpenes (b) sterols (c) flavonoids (d) alkaloids (e) essential oil and (f) miscellaneous groups. A list of all the chemical compounds reported so far from this plant is presented in Table 1.1.

Table1.1: Reported compounds from *E. odoratum*

Name of the compound	References
Triterpenes:	
Lupeol, β-amyrin	6, 10, 11
Epoxylupeol	11
Sterols:	
α-sitosterol, γ-sitosterol	12
β-sitosterol	12, 13
Stigmasterol, β-daucosterol	13
Flavonoids:	
Odoratin (2′-hydroxy-4,4′,5′,6′-tetramethoxychalcone)	14-20
Isosakuranetin (5,7-dihydroxy-4′-methoxyflavanone)	7, 14, 15, 20, 21
Acacetin (5,7-dihydroxy-4′-methoxyflavone)	13, 15, 20

Sinensetin (3',4',5,6,7-pentamethoxyflavone)	16
2′-hydroxy-3,4,4′,5′,6′-pentamethoxychalcone	16
Scutellarein tetramethyl ether (4',5,6,7-tetramethoxyflavone)	16
4'-hydroxy-5,6,7-trimethoxyflavanone	16, 17, 20
Isosakuranetin-rhamnoglucoside; Sakuranetin-7-O-arabinoside;	6
3,5,7,3′-tetra-O-methyl quercetagetin;	6
Quercetin (5,7,3',4'-tetrahydroxyflavonol)	6
Sakuranetin (5,4′-dihydroxy-7-methoxyflavanone)	7
Betuletol (5,7-dihydroxy-4',6-dimethoxyflavanol)	6
Kaempferide (5,7-dihydroxy-4'-methoxyflavonol)	6, 7, 21
Quercetagetin-6,4′-dimethyl ether (6,4′-dimethoxyflavonol)	22
2′,5-dihydroxy-5′,7-dimethoxyflavanone	23
Isosakuranetin-7-methyl ether; 4′,5-dihydroxy-3′,7-dimethoxyflavone	18
Tamarixetin (5,7,3′-trihydroxy-4′-methoxyflavanol)	7
5,6,7,4′-tetramethoxyflavanone	20, 23
2′,4-dihydroxy-4′,5′,6′-trimethoxychalcone	16, 17, 19, 20
Marionol (Quercetagetin-5,6,7,3′,4′-pentamethyl ether) or (5,6,7,3′,4′-pentamethoxyflavonol)	24
Naringenin-4′-methyl ether (5,7-dihydroxy-4′-methoxyflavanone);	13
Quercetin-7,4′-dimethyl ether (5,3′-dihydroxy-7,4′-dimethoxyflavonol);	13
Kaempferol-4'-methyl ether (5,7-dihydroxy-4′-methoxyflavanol)	13
Salvigenin (5-hydroxy-4′,6,7-trimethoxyflavone)	10, 11
Persicogenin (5,3′-dihydroxy-7,4′-dimethoxyflavanone)	20
Luteolin (5,7,3′,4′-tetrahydroxyflavone)	20
Pyrrolizidine alkaloids: Intermedine, Rinderine, 3′-Acetylrinderne 7-Angeloylretronecine, 9-Angeloylretronecine	25
Miscellaneous: Linolenic acid, Linoleic acid, Myristic acid, Palmitic acid	33
Amino-butyric acid, Proline, Tyrosine	34
Protocatechuic acid, *p*-Hydroxybenzoic acid, *p*-Coumaric acid, Ferulic acid, Vanillic acid	38
Anisic acid, Trihydric alcohol, $C_{25}H_{34}O_5$	39
Ceryl alcohol	12

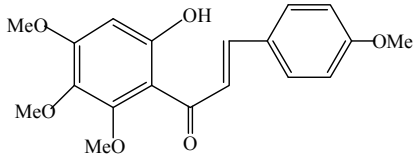
Triterpenes: Two triterpene alcohols, that is, lupeol and *β*-amyrin were isolated from whole plant[10, 11] as well as from the leaf.[6] A new triterpene epoxide, epoxylupeol was isolated from chloroform extract of the whole plant.[11]



Epoxylupeol

Sterols: The steroidal material was isolated from petroleum ether triturates of rectified spirit extract of the leaf and resolved into *α*-sitosterol, *γ*-sitosterol and *β*-sitosterol.[12] Stigmasterol and *β*-daucosterol were reported from aerial part of the plant, in addition to *β*-sitosterol.[13]

Flavonoids: Wide varieties of flavonoids present as either free or glycosides have been isolated by different researchers. Odoratin was isolated first time as a natural product from petroleum ether extract of the leaf of *E. odoratum* growing in India[14] which was reisolated[15] by the same researchers from the leaf. Odoratin was also isolated from the aerial part[16-19] and the flower.[20]

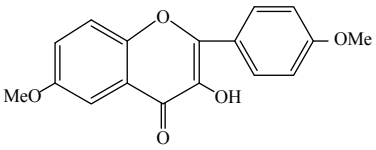


2' -hydroxy-4, 4', 5', 6'-tetramethoxychalcone
(Odoratin)

Isosakuranetin was isolated from the leaf[14, 15, 21], the whole plant[7] as well as from the flower.[20] Acacetin was isolated from the leaf[15], aerial part[13] and flower.[20] Sinensetin, 2′-hydroxy-3,4,4′,5′,6′-pentamethoxychalcone, scutellarein tetramethyl ether and an optically inactive 4′-hydroxy-5,6,7-trimethoxyflavanone were isolated from a dichloromethane extract of aerial part of plant growing in India.[16] Compounds 2′-hydroxy-3,4,4′,5′,6′-pentamethoxychalcone and 4′-hydroxy-5,6,7-trimethoxyflavanone were the first report from a natural source. Compound 4′-hydroxy-5,6,7-trimethoxyflavanone was also isolated from the aerial[17] and flower parts.[20] Isosakuranetin-rhamnoglucoside, quercetin, sakuranetin,

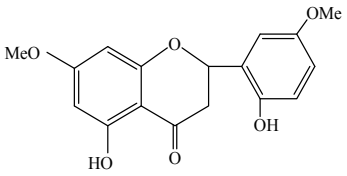
sakuratin-7-O-arabinoside, betuletol and 3,5,7,3′-tetra-O-methyl quercetagetin were reported from the leaf.[6] Isolation of kaempferide was also reported from the leaf[6, 21] and the whole plant.[7]

A rare flavonol quercetagetin-6,4′-dimethyl ether was reported from the leaf.[22]



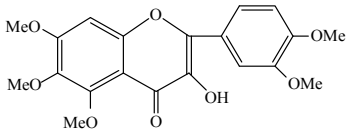
Quercetagetin-6, 4'-dimethyl ether
(3-hydroxy-6, 4'-dimethoxyflavone)

A new flavanone, that is, 2′,5-dihydroxy-5′,7-dimethoxyflavanone was isolated from the aerial part.[23]



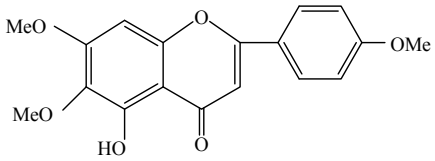
2', 5-dihydroxy-5', 7-dimethoxyflavaone

Isosakuranetin-7-methyl ether and 4′,5-dihydroxy-3′,7-dimethoxyflavone were also reported from its aerial part.[18] Tamarixetin was isolated from the plant growing in Nigeria along with previously reported isosakuranetin, sakuranetin, kaempferide from the aerial part.[7] A compound 5,6,7,4′-tetramethoxyflavanone was reported from the aerial part[17] and flower.[20] A compound 2′,4-dihydroxy-4′,5′,6′-trimethoxychalcone was isolated from the aerial part[16, 17, 19] and the flower.[20] A novel flavonol designated as marionol was isolated from the aerial part of this plant.[24]



Marionol (Quercetagetin -5, 6, 7, 3', 4'-pentamethyl ether
or 3-hydroxy-5, 6, 7, 3', 4'-pentamethoxyflavone)

A chemical investigation of the aerial part further showed the presence of naringenin-4′-methyl ether, quercetin-7,4′-dimethyl ether and kaempferol-4’-methyl ether.[13] A flavanone persicogenin and a flavone luteolin were also reported from the flower.[20] From petroleum ether extract of the whole plant the rarely occurring flavone salvigenin, a first natural product in compositae, was isolated[10] and reisolated.[11]



Salvigenin (5-hydroxy-4', 6, 7- trimethoxyflavone)

Alkaloids: Pyrrolizidine alkaloids (PAs) such as intermedine, rinderine, 3-acetylrinderine, 7-angeloylretronecine and 9-angeloylretronecine were also reported from roots and mature flower heads with highest concentration whereas leaf have almost devoid of alkaloids and nectar have no PAs.[25]

Essential oil: Essential oil was extracted and analyzed from the plant by different researchers using gas chromatography (GC)[5, 26, 27] or capillary gas chromatography (CGC)[28-30] and combined GCMS.[5, 26-30]

Essential oil of leaf obtained by steam distillation was known to contain more than forty compounds where terpenes like geijerene (42.5%) and *β*-cubebene (12.5%) were major compounds.[26] Another group of researchers identified thirty components from volatile oil where terpenoid compounds were major components such as *trans*-caryophyllene (16.5%), *δ*-cadinene (15.8%), *α*-copaene (11.5%), caryophyllene oxide (9.6%), germacrene-D (4.9%), and *δ*-humulene (4.3%).[27]

Essential oil of leaf collected from Houin, France, contained predominantly sesquiterpenes[28]-*β*-caryophyllene (21%), germacrene (15.3%), caryophyllene oxide (8%), *α*-cupaene (7.4%) etc. A study on the essential oil from Nigeria showed that it was mostly composed of hydrocarbons (*α*-pinene, limonene, *p*-cymene, cadinene and *β*-caryophyllene), camphor (15.06%) and cadinol (6.36%).[5] Essential oil of fresh plant obtained from Ivory Coast yielded 3% light yellow oil. Twenty six components were identified and were qualitatively characterized by the presence of pregeijerene (14%), geijerene (5%). The main oxygen containing components were 4-terpineol (0.5%), *β*-eudesmol (0.6%), *γ*-eudesmol (0.3%), *α*-cadinol (0.6%) and *t*-cadinol (0.3%) but absence of camphor. Besides, it was also composed of hydrocarbons (*α*- pinene, *β*-pinene, limonene, α-cadinene, *δ*-cadinene), *β*-caryophyllene and germacrene-D.[5] Similar results like α-pinene (21.1%), pregeijerene (11.6%), *β*-pinene (10.1%), germacrene D (9.5%) etc. were obtained in the oil of fresh leaf collected from Ivory Coast.[29] Essential oil obtained from Shillong, India was characterized by caryophyllene oxide (18.3%), caryophyllene (10.1%), *β*-farnesene (8.0%), *α*-cubebene (6.1%), germacrene D (5.1%), spathulenol (4.8%), *α*-Farnesene (3.5%), *α*-cadinol (3.2 %),

o-phthalic acid (2.7%), junipene (2.1%) and ledol (2.0%).[30] Eupatenes- d and l forms[31] and a sesquiterpene alcohol - eupatol[11] was also isolated from its essential oil.

The yield of essential oil obtained after hydrodistillation of the flower tops was 0.11% and afforded pinene (18.8%), cubebene (13%) and caryophyllene (10.2%) as the major constituents and the light greenish yellow oil thus obtained exhibited $n_D^{30}: 1.503$ and $[\alpha]_D^{29}: -13.55^\circ$.[32]

Miscellaneous: Unsaturated fatty acids – linolenic, linoleic and saturated fatty acids- myristic and palmitic acid were identified by GCMS from flower.[33]

A chemical analysis of the flower's pollen, suspected to play an allergenic role, showed the amount of 0.5% total free amino acids such as amino-butyric acid, proline and tyrosine with 0.09, 0.100 and 0.290 μmol/mg dry weight, respectively.[34] It is also reported that the dried plant material contained high crude protein[35, 36] which was easily degradable and the protein contained about 56% amino acids[35] indicating a good potential for feeding livestock[35, 36] as well useful in human diets.[36] A case study showed that 30% of the dry leaf matter can be fed to rabbits to get weight gained.[35] A study of recently published paper showed the presence of peptides containing cysteine residues from roots, leaf and stems which can bind with mercury indicating the plant has a high potential phytoremediation agent for inorganic mercury. Such type of peptides was isolated and characterized using RP-HPLC and ESIMS.[37]

Phenolic compounds like protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, ferulic and vanillic acids were also identified from ethanolic crude extract of leaf using liquid chromatographed coupled with UV-MS.[38] Besides these, anisic acid[39], ceryl alcohol[12] and trihydric alcohol with molecular formula $C_{25}H_{34}O_5$ [39] were also reported from the leaf.

1.3.3 Biological activities

Anti-inflammatory activity

The aqueous leaf extract of *Eupatorium odoratum* possessed the anti-inflammatory activity in rats which was studied by using the carrageenan-induced oedema, cotton pellet granuloma and formalin-induced oedema methods. The result justifies the traditional uses of the plant in the treatment of wounds and inflammation.[40]

Antimicrobial activity

Chloroform extract of leaf was reported to show maximum antimicrobial activity against *S. aureus, E. coli, B. subtilis, Aspergillus niger* compared to the extracts of petroleum ether, acetone, absolute alcohol and water.[6] Essential oil of Ivory Coast showed antimicrobial activity against gram negative bacteria but no activity towards gram positive bacteria (*S. aureus*).[5] However, essential oil from Nigeria showed positive activity against gram negative species mostly *S. aureus***.**[5] This plant is also found useful for treating fungal and protozoal diseases[41], however, more information were not given since it was patented. Leaf extract also showed ability to inhibit or kill *Neisseria gonorrhoea in vitro*.[42] Quercetin, isolated from this plant, was shown to have specific activity on human Herpes-virus.[6]

Wound healing activity

Eupolin ointment is a topical agent used in the treatment of soft-tissue wounds and burns in Vietnam and is made from an aqueous leaf extract of *Eupatorium odoratum*. This ointment enhanced growth of fibroblasts and endothelial cells at concentrations of 10 and 100 μg/mL of eupolin extract. Toxicity of the extract to fibroblasts was observed at 250 μg/mL in Dulbecco's modified Eagle's medium/0.5% fetal calf serum, but there was no significant damage at this dose to the endothelial cells.[43] The crude ethanolic extract of leaf as well as its purified fractions showed wound healing ability to protect cultured skin cells (fibroblasts and keratinocytes) *in vitro*. Such wound healing ability were investigated using colorimetric and lactate hydrogenase release assay.[38]

Anti-malarial activity

The chloroform extract of the leaf of *E. odoratum* was found to be active *in vitro* against *Plasmodium falciparum* with effective concentration (EC_{50}) values of 3.5 μg/mL. Isosakuranetin, kaempferide and tamarixetin, isolated from chloroform extract of leaf showed activities against *Plasmodium falciparum* at the EC_{50} values of 45.56, 19.86 and 26.20 μM, respectively.[21]

Inhibitory effect on plants, fungi and insect growth

The volatile oil showed the seedling growth inhibition of five test plants in the order of ryegrass > Chinese cabbage > radish > snap been > rice. The inhibitory effect of the oil at concentration of 800 mg/L for *Pyricularia grisea*, *Phytophthora nicotianae* and *Fusarium axysporum* with inhibitory percentage was 61.40, 29.27 and 14.44 mg/L, respectively. The volatile oil at the dose of 10-20 μL/plant had a significant oviposition deterrent effect on

Phyllotreta striolata and *Plutella xylostella*.[27] The essential oil of leaf had shown mortality of *Sitophilus zeamais* (LD_{50} = 6.78%).[44] This showed that oil can be used for insect control in stored products.

Banana aphid repellent

Plant extract also showed repellent effect on banana aphid which was decreased 48 hours after treatment.[45]

Allelopathic effects on tomato and other plants

An aqueous leaf extract and residue material of the plant showed significant allelopathic effects on tomato plants (*Lycopersicum esculentum* Mill) and its seeds. The growth inhibition of tomato plant was accompanied by decreasing in leaf water potential (ψ), and reduced water content.[46] Such effects on water balance might be one of the possible explanations for the inhibition of the tomato and others plants. Inhibition may be due to the effects of allelochemicals present in *E. odoratum*. These chemicals might be phenolic acids present in leaf because *trans*-cinnamic, p-coumaric, vanillic, caffeic, and gallic acids had shown reduction of photosynthetic rate in soybeans.[47] According to the information given by local people of *TINPIPLAY, KAVRE*, from where plant was collected, they uproot the *Eupatorium odoratum* plant from the field as its presence was found to inhibit the growth of maize plant.

Blood coagulation ability

Ethanolic extract of this plant showed the shorter activated partial thromboplastin time reflecting the blood coagulation ability. 4',5,6,7-tetramethoxyflavone, isolated from this plant, was found as blood clotting factor when studied *in vitro*.[48] However, a group of researchers showed the longer activated partial thromboplastin time in aqueous and alcoholic extracts[49] which do not agree with previous report.[48]

Antimycobacterial activity and cytotoxicity

Flavonoids isolated from flower showed moderate to weak antimycobacterial and cytotoxicity.[20] Isosakuranetin exhibited moderate antimycobacterial activity against *Mycobacterium tuberculosis* with the minimum inhibitory concentration (MIC) value of 174.8 μM, whereas compounds 4'-hydroxy-5,6,7-trimethoxyflavanone, acacetin and luteolin exhibited weak activity with the MIC values of 606.0, 704.2 and 699.3 μM respectively. Acacetin showed moderate cytotoxicity against human small cell lung cancer (NCI-H187) cells with the MIC value of 24.6 μM, whereas luteolin exhibited moderate toxicity against

NCI-H187 cells and weak toxicity against human breast cancer (BC) cells with the MIC values of 19.2 and 38.4 μM respectively.[20]

Structure relationship and antimycobacterial activity

According to Suksamrarn *et al*.[20] one of the structural requirements for flavonoids to exhibit antimycobacterial activity was the presence of ring C, either saturated or unsaturated. This was revealed from the active antimycobacterial activity shown by compounds isosakuranetin, 4′-hydroxy-5,6,7-trimethoxyflavanone, acacetin**,** luteolin and inactive activity shown by 2′,4-dihydroxy-4′,5′,6′-trimethoxychalcone and odoratin. Methylation at the 4'-hydroxyl group of 4'-hydroxy-5,6,7-trimethoxyflavanone to yield the 5,6,7,4'-tetramethoxyflavanone resulted in loss of activity. Free 5- and 7-OH groups as well as lack of a 6-methoxyl group seemed to increase biological activity. However, the high activity of isosakuranetin in not explained by the authors.

Compounds like isosakuranetin-7-methyl ether, 4′,5-dihydroxy-3′,7-dimethyloxyflavone and odoratin isolated from this plant, have no significant cytotoxicity in KB and P388 cell lines.[18]

1.4 RATIONALE OF STUDY

Literature survey revealed that a lot of research works were done on the aerial part of *E. odoratum* L. However, no satisfactory phytochemical work has been done on its root portion. Thus, its root portion was selected for the present study to find out new biological activities, search for new compounds or search as a new source of known compounds.

Specific objectives:

(1) Cytotoxicity and Antimicrobial screening of the root of *E. odoratum* L.

(2) Isolation of compounds from the root.

(3) Structural elucidation of the isolated compounds.

(4) Antimicrobial and cytotoxicity of isolated compounds.

(5) Antioxidant activities of different parts of plant

1.5 BIOSYNTHESIS OF SOME PLANT SECONDARY METABOLITES

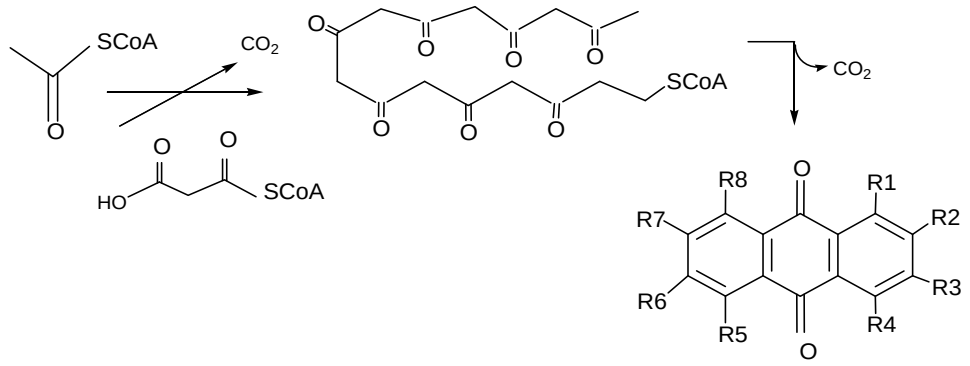
1.5.1 Biosynthesis of anthraquinones:

Anthraquinones constitute an important group of natural products occurring in bacteria, fungi, lichens, and higher plants. In higher plants, they are found in a large number of plants families, including Rubiaceae, Rhamnaceae, Polygonaceae and Leguminosae. There are two main biosynthetic pathways leading to anthraquinones in higher plants: the polyketide pathways and the chorismate/*o*-succinylbenzoic acid pathway.

(i) The Polyketide pathway[50]

In Rumex (Polygonaceae) and Rhamnus (Rhamnaceae), chrysophanol and emodin were shown to be biosynthesized via this pathway (Figure1.2). Here, anthraquinones are formed from one acetyl-CoA unit extended by seven malonyl-CoA units via octaketide chain.

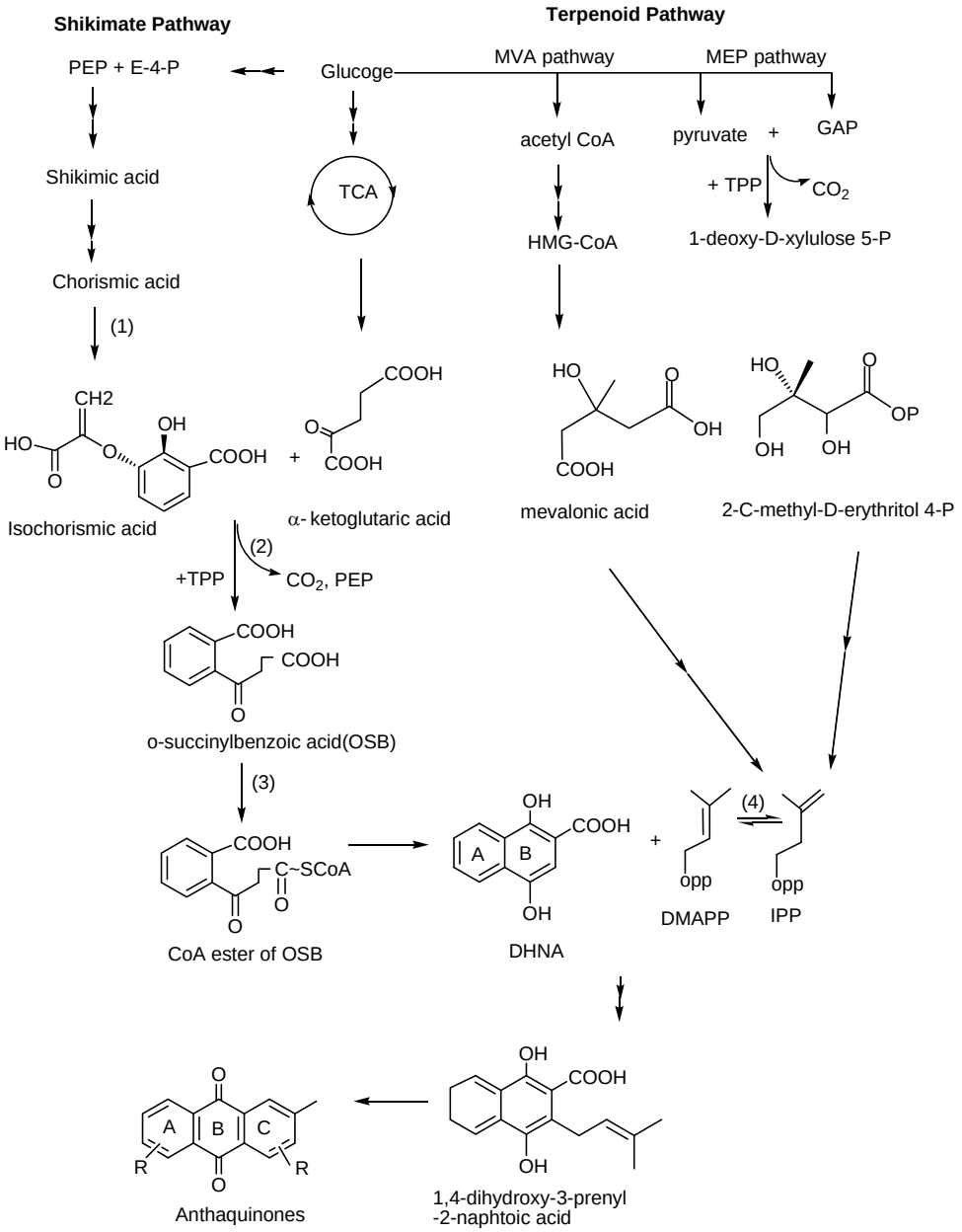
Figure 1.2: Polyketide pathway for formation of anthraquinones



(ii) The chorismate/*o*-succinylbenzoic acid pathway[50]

In Rubia (Rubiaceae), Rubia type anthraquinones were biosynthesized by this pathway (Figure1.3). In this path way, ring A and B are derived from shimkimic acid, α-ketoglutarate via *o*-succinylbenzoic acid (OSB), whereas ring C is formed from isopentenyl diphosphate (IPP) via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, not from mevalonic acid (MVA) pathway, which was thought previously.[50]

Figure 1.3: Biosynthetic pathway leading to anthraquinones in Rubiaceae

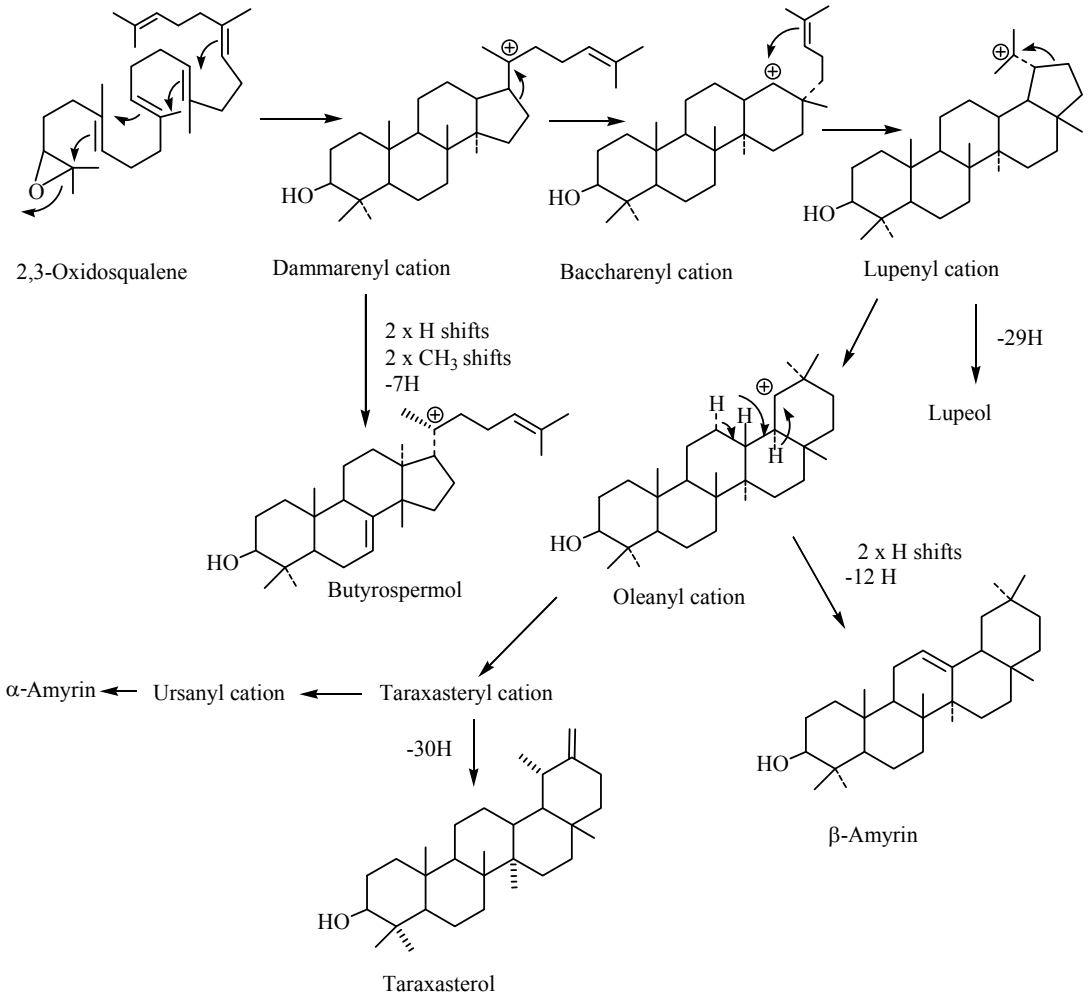


Note: MVA = Mevalonic acid; MEP = 2-C-methyl-D-erythritol 4-Phosphate; DHNA = 1,4-dihydroxy-2-naphthoic acid; DMAPP = 3, 3 -dimethylallyl diphosphate; E-4-P = erythrose 4-phosphate;GAP = glyceraldehyde 3-phosphate; HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A; IPP -isopentenyl diphosphate; TCA - tricarboxylic acid; TPP = thiamine diphosphate. Enzymes: (1) isochorismate synthase (2) *O* - succinylbenzoate synthase (3) OSB:CoA ligase (4) IPP isomerase

1.5.2 Biosynthesis of different triterpenes

Triterpenoids, a large class of isoprenoidal natural products present in higher plants, are biosynthesized from a common precursor 2,3-oxidosqualene. The enzymes triterpene synthases are responsible for the formation of these diverse triterpenes skeletons.[51] Here, cyclization of 2,3-oxidosqualene into different triterpenes in *Pisum sativum* is presented (Figure 1.4).[51, 52] Butyrospermol could be formed from tetracyclic dammarenyl cation through hydride and methyl shifts, and deprotonation. *β*-Amyrin could be formed from oleanyl cation through hydride shift from C-18 to C-19 and deprotonation of H-12.

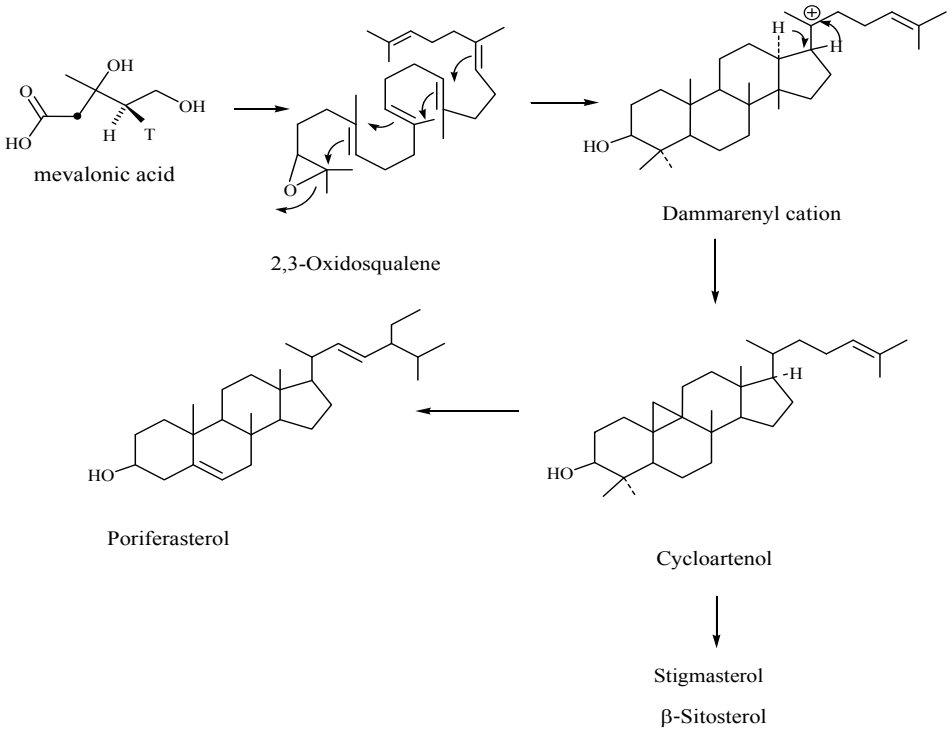
Figure 1.4: Cyclization of 2,3-oxidosqualene into different triterpenes products in *Pisum sativum*



1.5.3 Biosynthesis of sterols

Cycloartenol is the precursor of sterols in higher plants and algae including *Ochromonas malhamensis*. This cycloartenol is resulted from dammarenyl cation where it is expected that H migration from C-17 to C-20 and C-13 to C-17 took place[53] as shown in figure 1.5

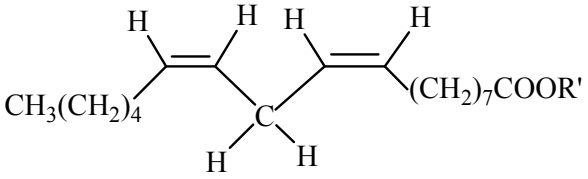
Figure 1.5: Cyclization of 2,3-oxidosqualene into different sterols products in *Ochromonas malhamensis*



1.6 AUTOXIDATION AND ANTIOXIDANTS

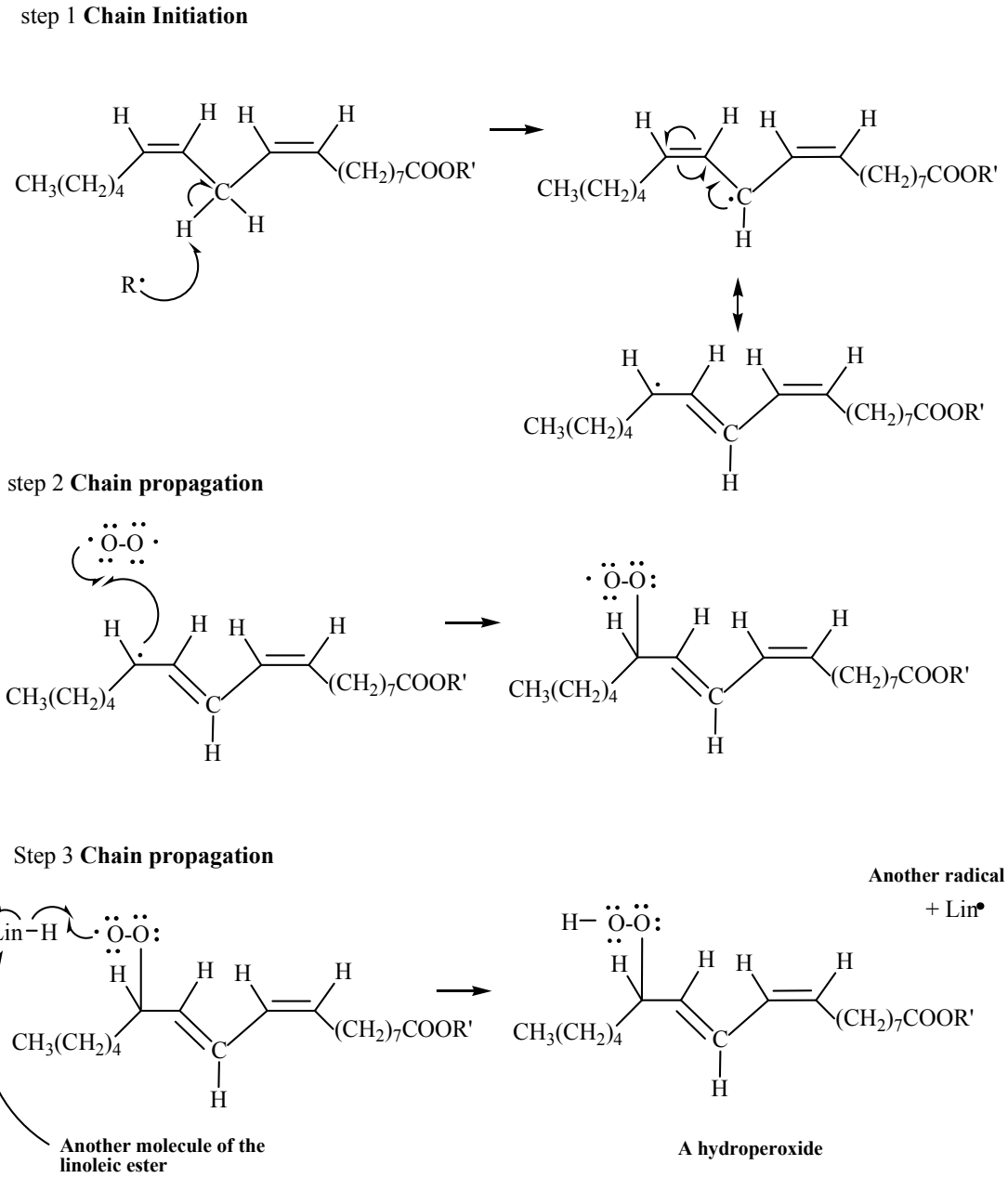
Many foods and cosmetic products contain edible polyunsaturated fats. Linoleic acid is an example of a polyunsaturated fatty acid that occurs as an ester in polyunsaturated fats. They are also widespread in the tissues of the body where they perform numerous vital functions.

An ester of Linoleic acid



The hydrogen atoms of the -CH_2- group located between the two double bonds of linoleic ester (Lin-H) are especially susceptible to abstraction of radicals. Abstraction of one of these hydrogen produces a new radical (Lin•) that can react with oxygen in a chain reaction that belongs to general type of reaction called autoxidation resulting the formation of a hydroperoxide (figure 1.6).[54]

Figure 1.6: Autoxidation of a linoleic acid



Autoxidation is responsible for the development of the rancidity that occurs when fats and oils spoil. Autoxidation also occurs in the body due to metabolic processes. If the generation rates of free radicals are faster than degradation rates under environmental stresses, cells suffer an oxidative stress which has been implicated in diseases like cancer, diabetes, hypertension, inflammation, AIDS,[55] arteriosclerosis and rheumatism.[56] Autoxidation is inhibited when compounds are present that can rapidly trap peroxyl radicals by reacting with them to give stabilized radicals that do not continue the chain.

Vitamin E (α –tocopherol) is capable of acting as a radical trap in this way, and one of the important roles that vitamin E plays in the body may be inhibiting radical reactions that could cause cell damage.[54] Such compound is also called as an antioxidant or antioxidative substance. Antioxidant may be obtained both naturally and synthetically. Synthetic antioxidants such as butylated hydroxytoulene (BHT) and butylated hydroxyanisole (BHA) have restricted use in foods as they are suspected to be carcinogenic.[57] Nowadays, people preferences have shifted the use of synthetic to natural antioxidants. Natural antioxidative substances have a phenolic moiety in their molecular structure. They have been found among flavonoids, tocopherols and catechins. Organic acids, carotenoids, protein hydrolysates and tannins can act as antioxidants or have a synergistic effect when used together with phenolic antioxidants.[57] These antioxidants have been claimed to have potential health functions for reducing aging and possible prevention of cancer and heart diseases. The nutraceutical industries have thus introduced several natural antioxidants as diet supplements.[57]

Chapter 2

MATERIALS & METHODS

2.1 MATERIALS

2.1.1 Solvents and chemicals

Solvents and chemicals of LR and AnalaR grade were purchased from different manufacturers (Qualigens and Merck). Commercial ethyl alcohol purchased in bulk from the local market was redistilled before use. Petroleum ether (40-60 °C) was used throughout the experiment, unless otherwise stated.

2.1.2 Chromatographic materials

Silica gel of 60-120 and 60-200 mesh, Qualigens, was used for column chromatography. Silica gel 60 GF_{254} (Merck or Qualigens) coated TLC plates were used for monitoring the different fractions obtained from column chromatography. Precoated thin layer chromatography plates, Merck Art. 5554 DC-Alufolien Kieselgel 60 F_{254} (20 x 20 cm) were used for measurement of R_f of compounds, unless otherwise stated.

2.1.3 Culture media, chemicals and disc for antibacterial activity

Nutrient broth, Mueller Hinton Agar and Nutrient agar media were procured from HiMedia Laboratories Pvt. Ltd. Gentamycin (10 μg/disc) and sterile paper discs (6 mm diameter, Schleicher and Schuell, D, ref. no. 321860) were purchased from the chemical supplier.

2.1.4 Bacteria

Two gram-positive- *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (NACOL standardized) and two gram-negative- *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) bacteria were obtained from National College (NACOL), Kathmandu, Nepal.

2.2 GENERAL METHODS

2.2.1 Thin layer chromatography

The extracts, fractions and isolated compounds were examined by TLC where microscopic plates were allowed to run using different solvent systems. Spots were visualized in day light, UV light (366 and 254 nm) and iodine vapour. In addition, spraying reagents like 10% methanolic potassium hydroxide solution and 0.01% florescein were used to visualize anthraquinone and fatty acid, respectively on chromatogram.

2.2.2 Preparative thin layer chromatography (PTLC)

Slurry of silica gel GF_{254} made in distilled water was coated over glass plates of size 20 × 20 cm with 0.25 to 0.5 mm thickness, as necessary using TLC applicator. Silica gel glass plates were left at room temperature for overnight. They were activated heating in an oven for 30 minutes at 120 °C before use.

2.2.3 Column chromatography

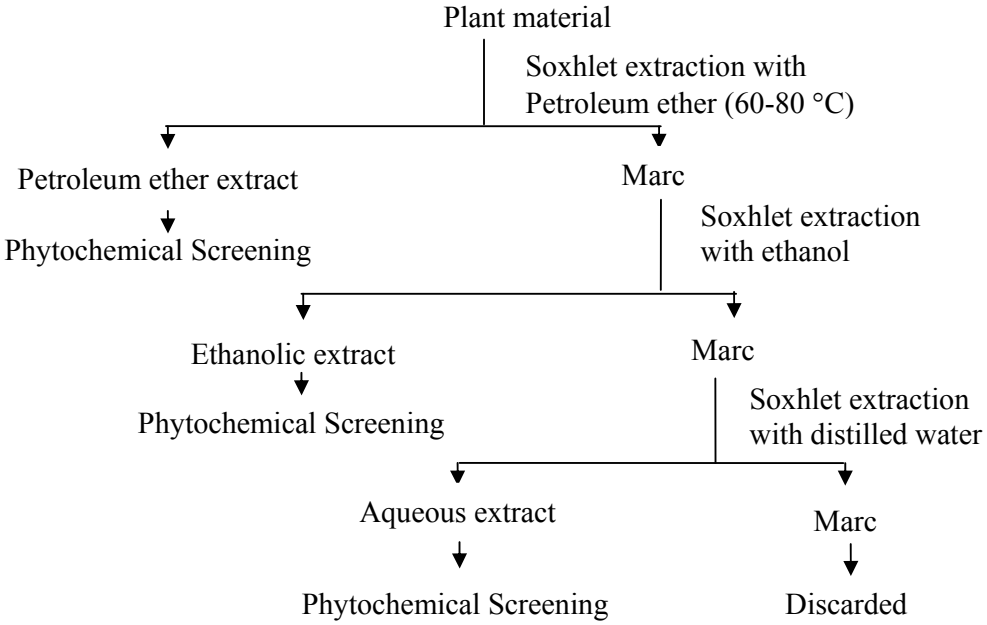
Glass columns of different diameters were used for fractionation and isolation of compounds from the extracts throughout the experiments. Silica gel required for this purposes was discussed in section 2.1.2.

2.2.4 Preliminary phytochemical screening

The method employed for the preliminary phytochemical screening of root is mainly based on the procedure given by I. Ciulei.[58] In this method, the plant materials were extracted successively with the solvent of increasing polarity. Presence of various classes of organic compounds in different extracts was then analyzed by using specific reagents. The flow chart shown in scheme 2.1 refers to the general protocol for phytochemical screening.

Fractions/subfractions of roots, leaves, stems and flowers were tested as outlined in literatures.[59, 60]

Scheme 2.1: General protocol for phytochemical screening



2.2.5 Brine shrimp cytotoxicity or lethality test

Toxicity towards the living cells at a lower dose is considered as cytotoxicity.[61] Brine shrimp cytotoxicity or lethality test is a pre-screen for the antitumor or anticancer assay.[61, 62] Cytotoxicity of compounds, crude extracts or fractions were evaluated by the brine shrimp cytotoxicity or lethality test as described in the literature.[63]

Each extract, fraction or compound was tested at a concentration level of 1000, 100 and 10 µg/mL for the calculation of LC_{50} (lethal concentration required to kill 50% brine shrimps). Berberine was used as positive control since it has been reported as a cytotoxic compound in different cell lines.[64] Any extract or compound with the LC_{50} value lesser than 1000 µg/mL was considered as cytotoxicity whereas the LC_{50} value higher than 1000 µg/mL was considered as non-cytotoxicity.

Calculation

LC_{50} was calculated by Probit analysis [65] using formula as follows.

$$Y = \alpha + \beta X$$

Where Y = 5 (From Probit Transformation Table)

$$\alpha = \frac{1}{n}(\sum y - \beta \sum x)$$

α and β were calculated as follows.

$$\beta = \frac{\sum xy - \left[\frac{\sum x . \sum y}{n}\right]}{\sum x^2 - \left[\frac{(\sum x)^2}{n}\right]}$$

Where
n = no. of doses

$\sum x$ = sum of the log of doses

$\sum y$ = sum of the average responses

$\sum xy$ = sum of the value of xy

Σx^2 = sum of the values of x^2

LC_{50} is given by antilog of X. The 95% confidence interval is the assurance of 95% guarantee for 50% survivor, which is calculated by the following relation.

95% Confidence Interval = $X \pm \frac{t\ \sigma}{\sqrt{N}}$

Where

t = Statistical factor = 4.303 for 2 degree of freedom

σ = Standard deviation

N = no. of experiments repeated

X = Average LC_{50}

Interpretation of data

The magnitude of the cytotoxicity of the plant extracts and compounds were classified according to LC_{50} data as shown in table 2.1, which is a slight modification of the toxicity scale given by Loomis.[66]

Table 2.1: General classification of the brine shrimp cytotoxicity test

LC_{50} (µg/ml)	Classification
< 1	Extreme cytotoxicity
1-100	High cytotoxicity
101-500	Moderate cytotoxicity
501-1000	Low cytotoxicity
> 1000	Non-cytotoxicity

2.2.5.1. Preparation of artificial seawater

Artificial sea water for hatching *Artemia salina* was prepared either by dissolving sea salt (Instant Ocean Aquarium System, Inc., USA) in distilled water (40 g/litre) as available or dissolving different components in doubly distilled water as shown in table 2.2.[67]

Table 2.2: Composition of artificial seawater

Chemicals	Amount (g/L)
NaCl	23.50
Na_2SO_4	4.00
KCl	0.68
H_3BO_3	0.026
$MgCl_2.6H_2O$	10.78
$CaCl_2$ (fused)	1.47
$NaHCO_3$	0.196
Na_2EDTA	0.0003

The pH of the solution was adjusted to 8.0 ± 0.2 by the addition of sodium sulphate or sodium bicarbonate whenever necessary.

2.2.5.2 Hatching the brine shrimp

Brine shrimp egg (Red Jungle brand ^{TM}) was obtained from Ocean Star International, Inc., Snowville, UT 84336 USA. Brine shrimp eggs (0.015 g) were hatched in a small beaker filled with artificial sea water. The eggs were incubated for about 48 hours at 27-30 °C in a water bath. After 48 hours, nauplii were collected with a pasture pipette after by attracting the organisms to one side of glass, the opposite of light source, and transferred to small beaker containing artificial sea water.

2.2.5.3. Preparation of sample solution for extract/compound

Sample solution of extract or compound required for this bioassay was prepared by either of the following two methods:

(1) Dilution Method [63]

(2) Non-dilution method [68]

First method was used only for extracts while second one was used for both extracts and isolated compounds.

2.2.6 Antibacterial activity

Antibacterial activity was performed by disc diffusion method. For quantitative estimation of antibacterial activity shown by an antibacterial agent, dilutions of the antibacterial agent were incorporated into broth or agar medium which was then inoculated with the test organism. They were expressed in terms of minimum inhibitory concentration (**MIC**) and or minimum bactericidal concentration (**MBC**).

2.2.6.1. Disc diffusion method

Zone of inhibition was determined by disc diffusion technique, known as the Kirby-Bauer method.[69] A disc of blotting paper was impregnated with a known volume and appropriate concentration of an antimicrobial substance, and this was placed on a plate of agar inoculated uniformly with the test organism. The antimicrobial diffused from the disc into medium and inhibited the growth of the test organism at a distance from the disc. Strains sensitive to the antimicrobial agent were inhibited at a distance from the disc whereas resistant strains have smaller zones of inhibition or grew up to edge of the disc.

Inhibition zone diameters around disc were measured including 6 mm disc. Inhibition zone shown by positive control, gentamycin (10 µg), was considered as 100% after subtracting disc diameter (6 mm). Inhibition zones shown by antimicrobials were expressed in % with

respect to the standard antibiotic after subtracting disc diameter (6 mm) from all observations.[70]

2.2.6.2 Two fold serial dilution technique

This technique was used for the quantitative estimation of antibiotic activity in which definite concentrations of the test substances were incorporated into the broth tubes. A standardized inoculum of the test organism was then added. After overnight incubation, the **MIC** was reported as the lowest concentration of antimicrobial required to prevent visible growth[71]; if required, the **MBC** was determined by subculturing the all tubes to show visible growth and no growth. The last tube which did not show the growth of tested organism was considered the lowest concentration of antimicrobial required to kill bacteria or to produce sterile culture, after which other tubes showed the growth of organism. In other words, the **MBC** is the lowest concentration of antimicrobial required to kill the bacteria.[71]

2.2.6.3 Preliminaries for antibacterial test

1. Turbidity standard equivalent to McFarland 0.5: This is the barium sulphate standard against which the turbidity of the test and control inocula can be compared. When matched with the standard, the inocula should give confluent or almost confluent growth. The standard should be shaken immediately before use.

Preparation of turbidity standard[71]: A 1% v/v solution of sulphuric acid was prepared by adding 1 mL of concentrated sulphuric acid to 99 mL of water. A 1% w/v solution of barium chloride was prepared by dissolving 0.5 g of dehydrated barium chloride ($BaCl_2.2H_2O$) in 50 mL of distilled water. A volume of 0.6 mL of $BaCl_2$ solution was added to 99.4 mL of the sulphuric acid solution and mixed well. This represents the cell density approximately to 1.5×10^8/mL.

A small volume of the turbid solution was transferred to a capped tube or screw cap bottle of the same type as used for preparing the test and control. The standard was stored in a well sealed container in the dark at room temperature for further uses.

2. Autoclave: All the required glassware, cotton swabs, culture tubes with cotton plugs, Petri plates, paper discs, prepared culture media, micropipette tips except plant extracts were sterilized in autoclave at 121 °C for 15 minutes under 15 psi.

3. Laminar chamber: All the antibacterial activity experiments were accomplished inside laminar chamber. Before using any experiment, UV lamp in laminar chamber was switched on for ten minutes to sterilize the inner environment of chamber. It was then switched off. This chamber was now ready for use. Flame of Bunsen burner or spirit lamp was used through out the experiment for sterilizing loops and forceps.

4. Disinfectant: Ethanol was used as disinfectant. Any cotton swab that was used to streak bacteria in a medium was dipped in ethanol solution. Forceps were also sterilized before and after the disc was transferred on the inoculated plate.

5. Preparation of Media**:** *Nutrient broth*: Nutrient broth powder (1.30 g) was mixed with distilled water (100 mL) in Erlenmeyer flask and boiled with constant shaking. It was cooled and appropriate volume of 3-4 mL was transferred into screw capped tube and autoclaved at 121 °C under 15 psi for 20 minutes.

Mueller Hinton agar (MHA) and Nutrient agar: Mueller Hinton agar powder (3.80 g) and nutrient agar (2.80 g) were separately dissolved with distilled water (100 mL) in Erlenmeyer flasks and autoclaved at 121 °C under 15 psi for 20 minutes. Sterilized media were allowed to cool to 50 °C. They were distributed in the sterile Petri plates of size of 90 mm diameter in the ratio of 20-25 mL/Petri plate aseptically. Plates were left as such for solidification. If water droplets were seen over solidified media, Petri plates were kept in laminar air flow to remove the water droplets.

6. Preparation of standard culture inoculums: Three to five colonies of similar appearance of the organism to be tested were touched using a sterile wire loop. It was transferred to a tube with screw cap containing sterile nutrient broth (3 - 4 mL). It was incubated at 37 °C for 4 hours. When turbidity appeared, tube was compared with turbidity standard equivalent to McFarland 0.5 for antimicrobial activity test, and the density of the test organism suspension was adjusted by adding more bacteria or more distilled water, as necessary.

7. Inoculation of MHA plates: A sterile cotton swab was dipped into the prepared standard working inoculum of organism. Any excess fluid was removed by pressing the swab against the side of the tube above the level of the suspension. This cotton swab of particular organism was streaked all over the surface of the medium in three directions, rotating the plate approximately 60 °C to ensure even distribution. Finally, the swab was passed round the edge of the agar plate. The used cotton swab was discarded by dipping into ethanol solution.

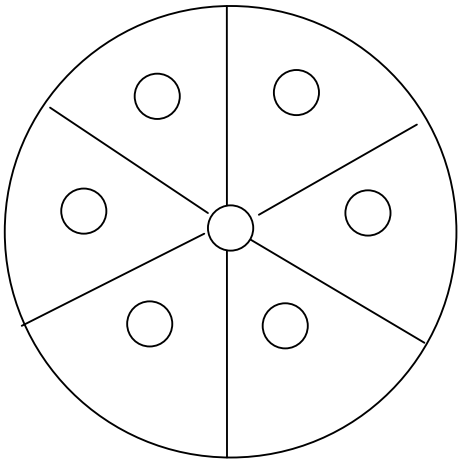
Inoculums were left to dry for a few minutes at room temperature with the lid closed in laminar chamber.

8. Preparation of sample solution: Sample solution of extract was prepared by dissolving 25 mg of extract in DMSO and diluting to 500 µL in a clean sterile Eppendorf tube. This solution corresponded to the concentration of 50 mg/mL.

9. Impregnation of disc by sample solution: Sterile discs of required numbers were arranged in empty sterilized Petri plates with the help of sterilized forceps. These discs were impregnated with definite volume and concentration of the sample solution of each extract or compound using micropipette. Negative control was also prepared by loading same volume of solvent alone separately in blank disc. Solvents in discs were removed by evaporating in a stream of air by placing them inside the laminar chamber for 3-4 hours at room temperature.

10. Transfer of disc into inoculated MHA plates: A paper template (figure 2.1) was used to place the discs above on the inoculated plate by placing the template under it.

Figure 2.1: Template for uniform placement of paper discs on Petri plate (size: 90 mm)



Impregnated discs and negative control disc were placed approximately 15 mm from the edge of the plate. Positive control disc of gentamycin (10 µg) was placed at the centre of template. The forceps was sterilized over spirit lamp or Bunsen burner flame each step before and after the disc was transferred on the inoculated plate and dipped into ethanol contained in a beaker.

11. Incubation of test plates: Four test plates were left for 30 minutes in laminar air flow for diffusion of extract with the lid closed. Then plates were incubated at 37 °C for 18 hours.

2.2.7 Antioxidant Activity

Antioxidant activity was determined by following methods:

(1) β-carotene bleaching test

(2) DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging test.

2.2.7.1 *β* - Carotene bleaching test

Principle

β-Carotene bleaching test involves a reaction between a potential antioxidant, *β*-carotene, and linoleic acid. *β*-Carotene undergoes rapid discoloration due to oxidation by free radicals generated from reaction between oxygen and linoleic acid in the absence of an antioxidant. The presence of antioxidant compounds can hinder the extent of *β*-carotene destruction by neutralizing the linoleate free radical and any other free radicals formed within the system.[72] *β*-Carotene destruction is measured spectrophotometrically at 470 nm against methanol as reference.

Procedure

β-Carotene bleaching test was performed as discussed in literature.[73] BHA was used as a positive control.

Calculation

The antioxidant activity of the samples was evaluated in terms of relative antioxidant activity (RAA)[73] which was calculated by following formula:

$$RAA = \frac{\text{Absorbance of Sample}}{\text{Absorbance of BHA}}$$

Statistical Analysis

Paired-sample Student's *t*-test was performed by following formula for the significant differences between two samples.

$$t = \frac{\bar{d}}{\sqrt{\frac{\sigma^2}{N}}}$$

Where $\bar{d}$ = Mean difference of all the individual differences (d_i) = $\frac{X_A - X_B}{N}$

$\sqrt{\frac{\sigma^2}{N}}$ = Standard Error Mean (SEM)

N = Number of observation

Standard deviation (σ) was calculated as follows.

$$\sigma = \sqrt{\frac{\sum(d_i - \bar{d})^2}{N-1}}$$

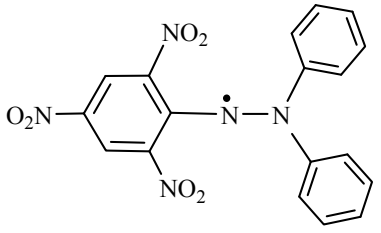
Calculated *t*- value was determined at the (n-1) degree of freedom and expressed this value in terms of probability (*P*). The antioxidant activity of extract was considered not significantly different from BHA when $P > 0.01$ while antioxidant activity was considered significantly different from BHA when $P < 0.01$.[57]

2.2.7.2. DPPH radical scavenging test

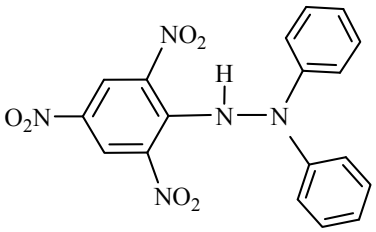
Principle

Radical scavenging activity of plant extracts against stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined spectrophotometrically. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep-violet to light-yellow) were measured at 517 nm on a UV-Visible spectrophotometer. Representing the DPPH radical by $Z^{\bullet}$ and the unsaturated molecules by AH, the primary reaction is

$$Z^{\bullet} + AH = ZH + A^{\bullet}$$



$z^{\bullet}$ = DPPH radical



zH = Diphenylpicrylhydrazine (nonradical)

where ZH is the reduced form and $A^{\bullet}$ is free radical produced in the reaction above. This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorized) by one molecule of the reductant.

Procedure

Radical scavenging activity of extracts was measured by slightly modified method of Brand-Williams *et al.*[74] Procedure in details was discussed in section 5.10.2.

Calculation

Radical scavenging activity in terms of % inhibition was calculated by the following formula[75]:

$$\%\ \text{Inhibition} = \frac{(A_C - A_S)}{A_C} \times 100$$

Where: A_C — absorption of blank sample (t = 0 min.) i.e. 3.9 ml DPPH and 0.1 ml of MeOH; A_S — absorption of sample solution (t = 30 min) i.e. 3.9 ml DPPH and 0.1 ml of methanolic extract or positive control.

Chapter 3

RESULTS & DISCUSSION

3.1 PHYTOCHEMICAL SCREENING OF THE ROOT

The results of phytochemical screening of the root of *E. odoratum* as shown in table 3.1, based on the procedure given by Ciulei,[58] revealed the presence of fatty acids, sterols, polyphenols, reducing compounds, quinones, coumarin derivatives, tannins, saponins, carbohydrates and alkaloids (very likely to contain pyrrolizidine alkaloids).[25] On the other hand, volatile oils, carotenoids, coumarins, flavonoids, cardiac glycosides, emodins, cyanogenic glycosides, anthocyanosides and anthracenosides were not detected (see section 5.3 for details).

Table 3.1: Results of phytochemical screening tests of the root

No.	Class of natural constituents	Name of screening test	Remarks
1	Volatile oils	Spot test	-ve
2	Basic alkaloids	Mayer's test	-ve
		Dragendorff's test	-ve
3	Carotenoids	Sulphuric acid test	-ve
4	Sterols & triterpenes	Lieberman-Burchard test	+ve
5	Fatty acids	Spot test	+ve
6	Coumarins	UV method	-ve
7	Flavone aglycones	Shinoda's test	-ve
		Shibata's test	-ve
8	Emodins	Borntiager's test	-ve
9	Polyphenols/Tannins	Ferric chloride	+ve
10	Reducing compounds	Fehling's test	+ve
11	Alkaloid salts	Mayer's test	Not detected
		Dragendorff's test	+ve
12	Glycosides	Ammonium Hydroxide	-ve
13	Anthocyanosides	Basification method	-ve
14	Anthracenosides	Ammonium Hydroxide	-ve
15	Coumarin derivatives	UV method	+ve
16	Flavonic glycosides	Shinoda's test	-ve
		Shibata's test	-ve
17	Cardiac glycosides	Kedde's test	-ve
18	Saponins	Froth test	+ve
20	Quinones	Ammonium thiocyanate	+ve
21	Carbohydrates	Molisch's test	+ve
22	Alkaloids	Dragendorff's test	+ve
		Mayer's test	Not detected
22	Cyanogenic glycosides	Guignard's test	-ve

3.2 BIOASSAY OF THE ROOT

3.2.1 Preparation of extracts - Method I

Oily yellow residue (**F1**), solid residue (**F2**) and its subfractions - hexane soluble (**F2-1**), dichloromethane soluble (**F2-2**), ethyl acetate soluble (**F2-3**), 80% methanol soluble (**F-4**) and syrupy liquid (**F3**), prepared as shown in scheme 3.1 (see section 5.4.1 for details), yielded 0.31, 0.72, 0.075, 0.17, 0.017, 0.12 and 10.23%, respectively (Table 3.2).

Scheme 3.1: Preparation of root extracts by method I

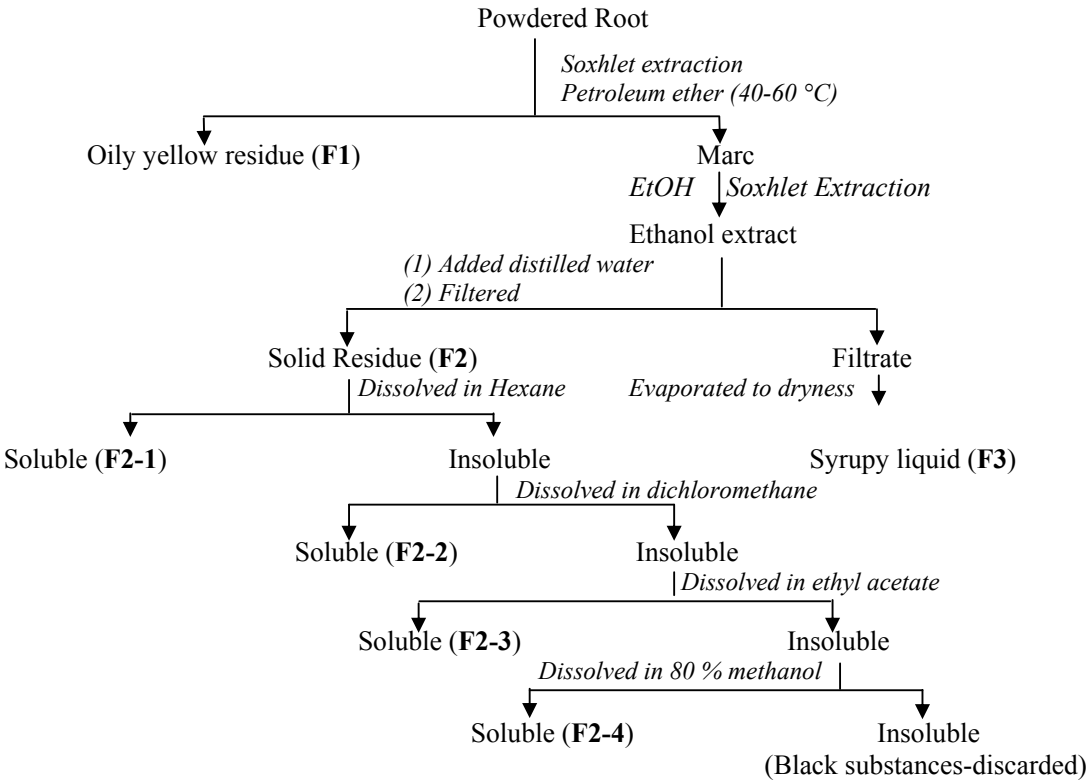


Table 3.2: Percentage yield of root fractions obtained from method I

Fraction		Nature	% yield
Petroleum ether (**F1**)		Yellow oil	0.31
Ethanolic solid residue (**F2**)		Solid	0.72
Ethanolic syrupy liquid (**F3**)		Reddish syrupy liquid	10.23
Subfraction of solid residue (F2)		**Nature**	**% yield**
Hexane soluble	(**F2-1**)	Gummy	0.075
Dichloromethane soluble	(**F2-2**)	Gummy	0.170
Ethyl acetate soluble	(**F2-3**)	Gummy	0.017
80% Methanol soluble	(**F2-4**)	Gummy	0.120

3.2.1.1 Brine shrimp cytotoxicity test of fractions/subfractions

The results of brine shrimp cytotoxicity test as shown in table 3.3 clearly revealed that fractions **F1, F2** and subfractions **F2-1, F2-2** showed LC_{50} lesser than 1000 µg/mL indicating cytotoxicity against brine shrimps while fraction **F3,** subfractions **F2-3** and **F2-4** displayed LC_{50} greater than 1000 µg/mL indicating non-cytotoxicity (see section 5.4.1.1 for details). Table 3.3 also revealed that cytotoxicity of **F2** is greater than that of its own subfractions **F2-1** and **F2-2**. This difference is very much likely to be due to synergetic effect of bioactive hexane soluble (**F2-1**) and dichloromethane soluble (**F2-2**) compounds and therefore they were further investigated (see section 3.2.1.2 for details).

Table 3.3: Brine shrimp cytotoxicity test for root fractions obtained from method I

Extract	LC_{50} (Mean)$^n \pm \sigma$ (µg/mL)	95% Confidence Interval	Remarks
Petroleum ether (**F1**)	237.27 ± 27.14	304.69-169.85	Moderate Cytotoxicity
Solid residue (**F2**)	81.13 ± 20.38	131.76 - 30.50	High cytotoxicity
Syrupy liquid (**F3**)	1.9×10^{11}	n. d.	Non-cytotoxicity
Hexane soluble subfraction (**F2-1**)	263.16 ± 10.61	289.51 - 236.81	Moderate Cytotoxicity
Dichloromethane soluble subfraction (**F2-2**)	207.77 ± 16.69	249.28 - 166.26	Moderate Cytotoxicity
Ethyl acetate soluble subfraction (**F2-3**)	1×10^{18}	n. d.	Non-cytotoxicity
80% methanol soluble subfraction (**F2-4**)	1.6×10^{4}	n. d.	Non-cytotoxicity
Berberine (Positive control)	89.18 ± 4.1	99.36 - 79.0	

n = 3; σ = standard deviation; n. d. = calculation omitted for non-cytotoxic extracts.

3.2.1.2 Phytochemical screening of cytotoxic (F1, F2, F2-1, F2-2) and non-cytotoxic (F2-3, F2-4, F3) fractions

The results of phytochemical screening of cytotoxic (**F1**, **F2**, **F2-1**, **F2-2**) and non-cytotoxic (**F2-3**, **F2-4**, **F3**) fractions were shown in table 3.4 (see section 5.4.1.2 for details).

Table 3.4: Results of phytochemical screening for cytotoxic and non-cytotoxic fractions of the root obtained from method I

Test	**F1**	**F2**	**F3**	**F2-1**	**F2-2**	**F2-3**	**F2-4**
Sterols/triterpenes	+	+	-	+	+	-	-
Polyphenols	-	-	+	n	n	n	n
Reducing sugars	-	+	+	-	-	+	+
Alkaloids	-	+	-	-	+	+	-
Carbohydrates	-	+	-	-	-	-	+
Quinones	+	+	+	+	+	-	-
Flavonoids	-	-	-	-	-	-	-
Tannins	-	-	+	-	n	n	n

(n) = Not tested (–) = absence (+) = presence

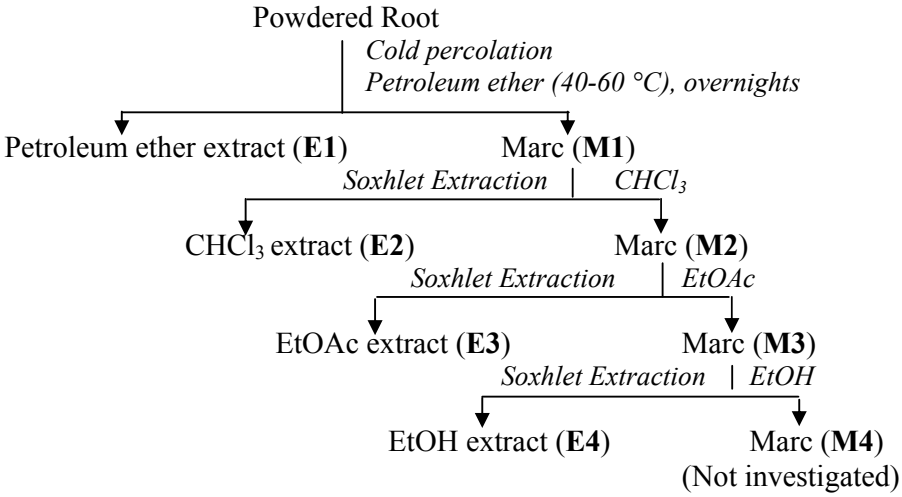
Petroleum ether extract (**F1**) showed the presence of sterols and quinones. Solid ethanolic extract (**F2**) revealed the presence of sterols/triterpenes, reducing sugars, alkaloids, carbohydrates and quinones. Polyphenols and tannins were not detected in both **F1** and **F2** fractions. Syrupy liquid residue (**F3**) showed the presence of polyphenols, reducing sugars, quinones and tannins.

Two subfractions of **F2**, that is, hexane soluble (**F2-1**) and dichloromethane soluble (**F2-2**) showed the presence of sterols/triterpenes and quinones whereas alkaloid was detected in subfraction **F2-2**. It is very much likely that the presence of sterols, triterpenes, alkaloids and quinones made the fractions **F1** and **F2**, subfractions **F2-1** and **F2-2** cytotoxic against brine shrimp. Similarly, the ethyl acetate soluble subfraction (**F2-3**) showed the presence of reducing sugars and alkaloids; 80% methanol soluble subfraction (**F2-4**) showed reducing sugars and carbohydrates but quinones were not detected in both cases above. Flavonoids were not detected in any of the root extracts or fractions.

3.2.2 Preparation of extracts - Method II

Different extracts, **E1**, **E2**, **E3** and **E4** were obtained from sun-dried powdered root (1.99 Kg) as per scheme 3.2 (see section 5.4.2 for details). Yield of different extracts was obtained as 0.24, 1, 2.01 and 7.79 % for **E1**, **E2**, **E3** and **E4**, respectively.

Scheme 3.2: Preparation of root extracts by method II



3.2.2.1 Brine shrimp cytotoxicity test

The results of brine shrimp cytotoxicity test as shown in table 3.5 clearly revealed that extracts **E1** and **E2** have their LC_{50} lesser than 1000 µg/mL indicating moderate cytotoxicity and therefore they were further investigated (see section 3.3). On the other hand, extracts **E3** and **E4** have their LC_{50} greater than 1000 µg/mL indicating non-cytotoxicity and hence not further investigated (see section 5.4.2.1 for details).

Table 3.5: Brine shrimp cytotoxicity test of root extracts obtained from method II

Extract	LC_{50} (Mean)$^n \pm \sigma$ µg/mL	95% Confidence Interval	Remarks
Petroleum ether (**E1**)	279.79 ± 9.78	304.08 - 255.50	Moderate Cytotoxicity
Chloroform (**E2**)	302.54 ± 52.5	432.96 - 172.12	Moderate Cytotoxicity
Ethyl acetate (**E3**)	2.9×10^8	n. d.	Non-cytotoxicity
Ethanol (**E4**)	1×10^{18}	n. d.	Non-cytotoxicity
Berberine (Positive control)	89.18 ± 4.1	99.36 - 79.0	

n = 3; σ = standard deviation; n. d. = calculation omitted for non-cytotoxic extracts.

3.2.2.2 Antibacterial test

The results of antimicrobial activity as shown in table 3.6 revealed that extract **E1** showed very weak antibacterial activity towards the gram-positive bacteria, *S. aureus* (5.5%) and

B. subtilis (5.5%) and no inhibition was observed toward gram-negative bacteria, *P. aeruginosa* and *E. coli* while extracts **E2**, **E3** and **E4** showed no antibacterial activity toward both gram-positive and gram-negative bacteria (see section 5.4.2.2 for details).

Table 3.6: Zone of inhibition for root extracts obtained from method II

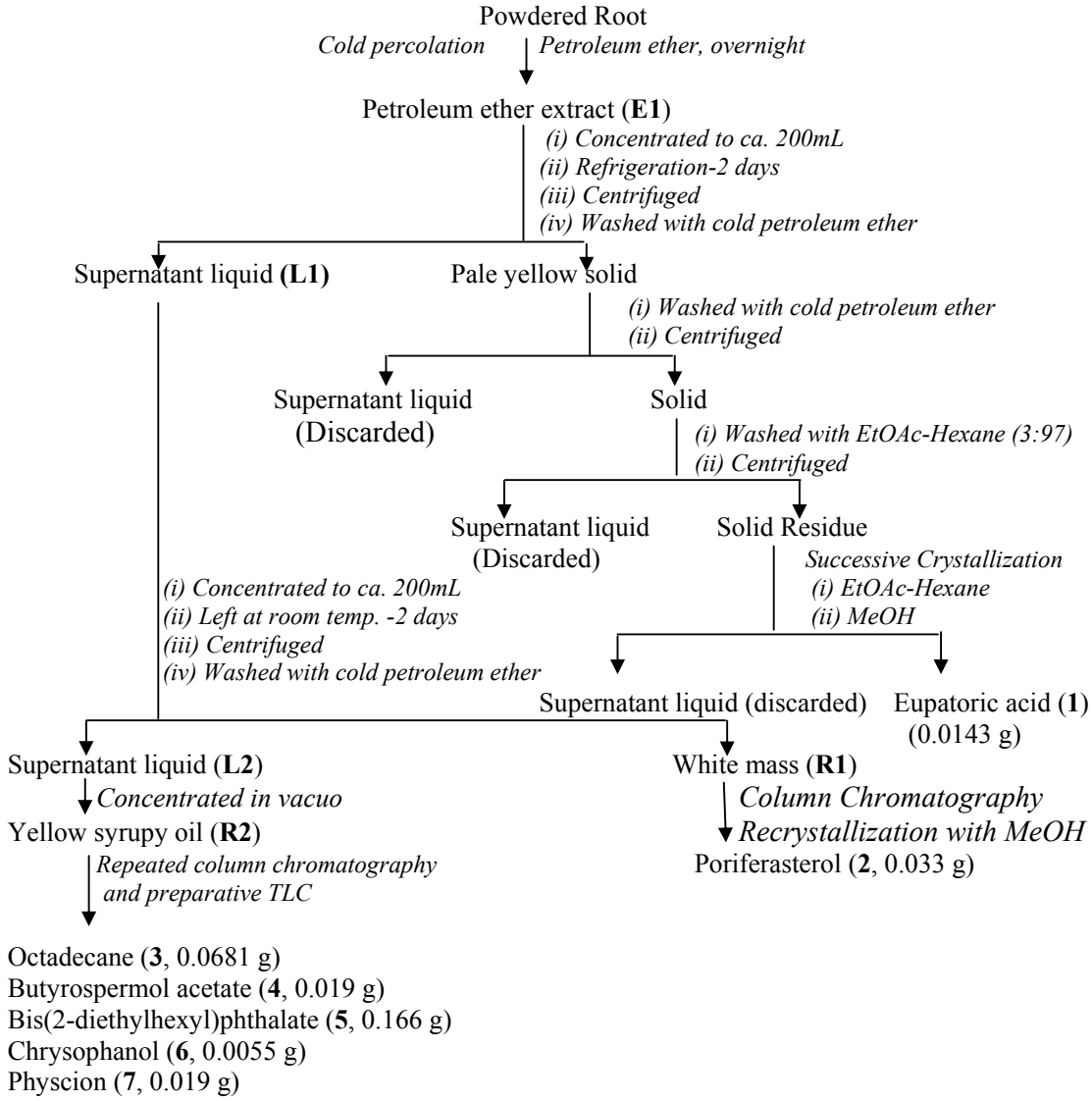
Microorganism →	*S. aureus*		*B. subtilis*		*P. aeruginosa*		*E. coli*	
	Zone of inhibition		Zone of inhibition		Zone of inhibition		one of inhibition	
Extract/drug ↓	mm	%	mm	%	mm	%	mm	%
Gentamycin (10 μg)	24	100	24	100	19	100	18	100
Petroleum ether (**E1**)	7	5.5	8	5.5	6	0	6	0
Chloroform (**E2**)	6	0	6	0	6	0	6	0
Ethyl acetate (**E3**)	6	0	6	0	6	0	6	0
Ethyl alcohol (**E4**)	6	0	6	0	6	0	6	0

3.3 ISOLATION OF COMPOUNDS FROM THE ROOT EXTRACTS

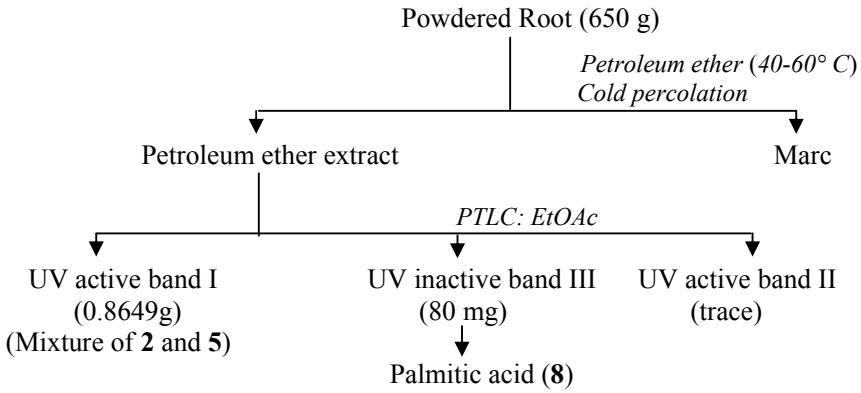
3.3.1 From petroleum ether extract (E1)

Phytochemical investigation on the petroleum ether extract (**E1**) have resulted in the isolation of a novel triterpene, 3*β*-hydroxy-28-carboxyolean-12-ene or eupatoric acid (**1**, 0.0143 g), poriferasterol (**2**, 0.033 g), octadecane (**3**, 0.681 g), butyrospermol acetate (**4**, 0.019 g), bis(2-ethylhexyl)phthalate (**5,** 0.166 g), chrysophanol (**6**, 0.0055 g) and physcion (**7**, 0.019 g) as shown in scheme 3.3; palmitic acid (**8**, 0.080 g) and a mixture of **2** and **5** were obtained as shown in scheme 3.4 (see section 5.5.1 for details).

Scheme 3.3: Isolation of compounds from petroleum ether extract (**E1**) of root



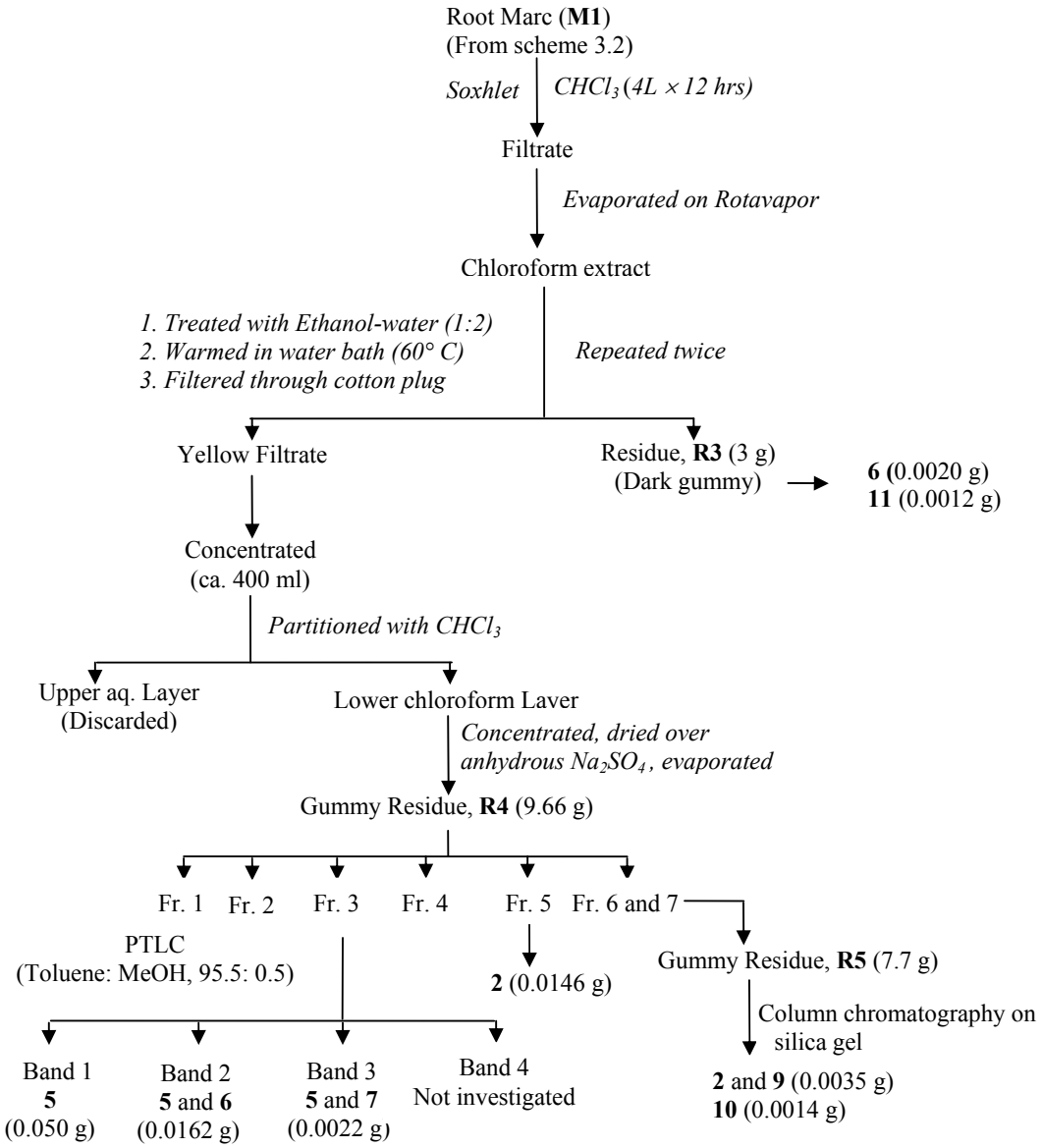
Scheme 3.4: Isolation of palmitic acid (**8**) from petroleum ether extract (**E1**) of root



3.3.2 From chloroform extract (E2)

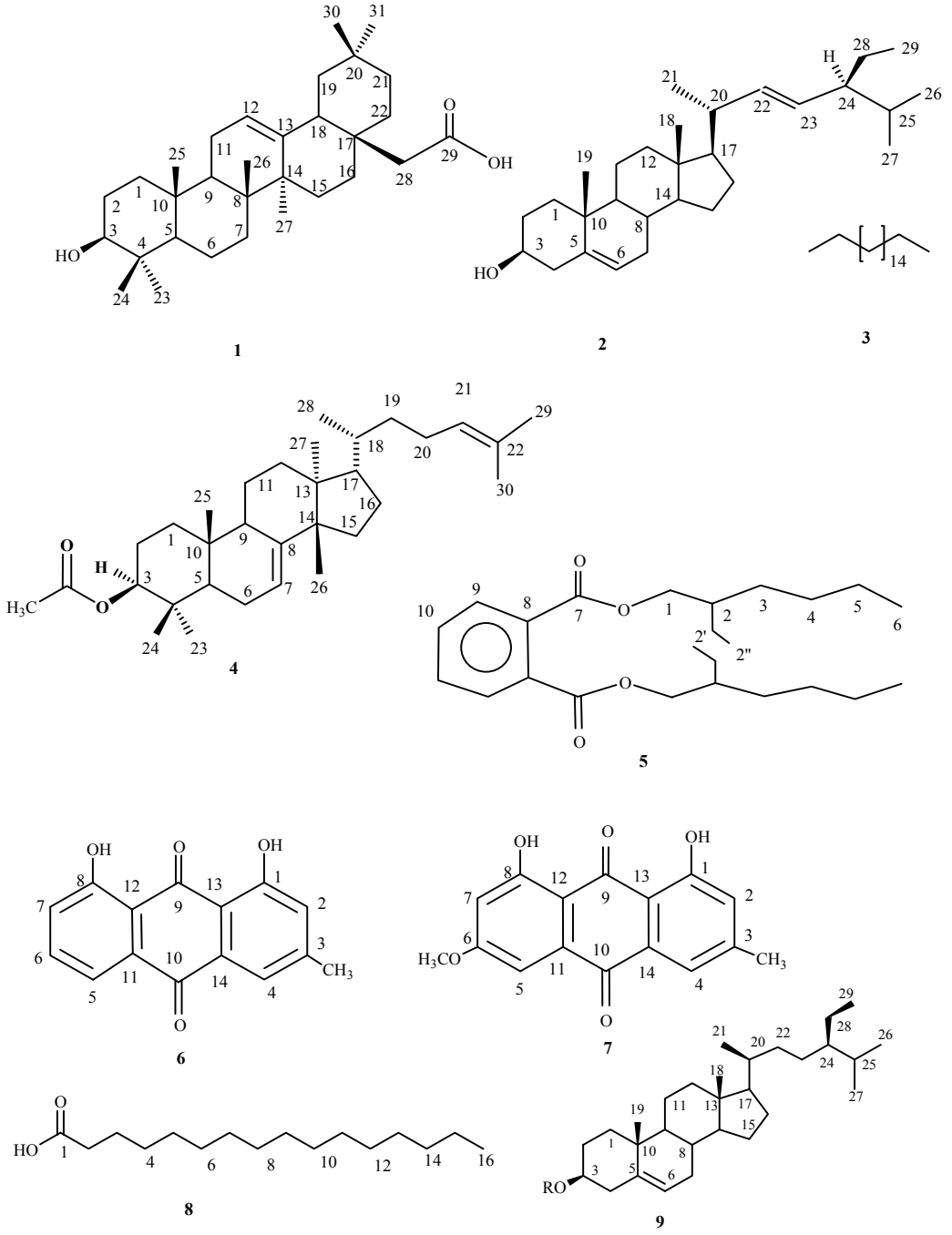
Repeated column chromatography on silica gel and preparative thin layer chromatography techniques on **E2** afforded bis(2-ethylhexyl)phthalate (**5**, 0.050 g), chrysophanol (**6,** 0.002 g), a mixture of bis(2-ethylhexyl)phthalate (**5**) and chrysophanol (**6**) (0.0162 g), a mixture of bis(2-ethylhexyl)phthalate (**5**) and physcion (**7**) (0.0022 g), poriferasterol (**2**, 0.0146 g), a mixture of poriferasterol (**2**) and *β*-sitosterol (**9**) (0.0035 g). In addition, anthraquinone (**10**, 0.0014 g) and hydrocarbon (**11**, 0.0012 g) were also isolated (scheme 3.5) but was not further investigated due to paucity of materials (see section 5.5.2 for details).

Scheme 3.5: Isolation of compounds from chloroform extract of the root



Structure elucidation of isolated compounds (**1-9**) were performed by spectroscopic methods: UV-Visible, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT, APT, ^{1}H-^{1}H COSY, HMQC, HMBC, FABMS, EIMS, GCMS and HRMS, wherever applicable. In addition, melting point, chemical tests and literature comparison were also used for structure elucidation (see section 5.6 for details). Structures of all isolates were shown in figure 3.1.

Figure 3.1: Structure of compounds isolated from the root of *E. odoratum* L.



3.4 STRUCTURE ELUCIDATION OF ISOLATES FROM THE ROOT

3.4.1 Eupatoric acid (1)

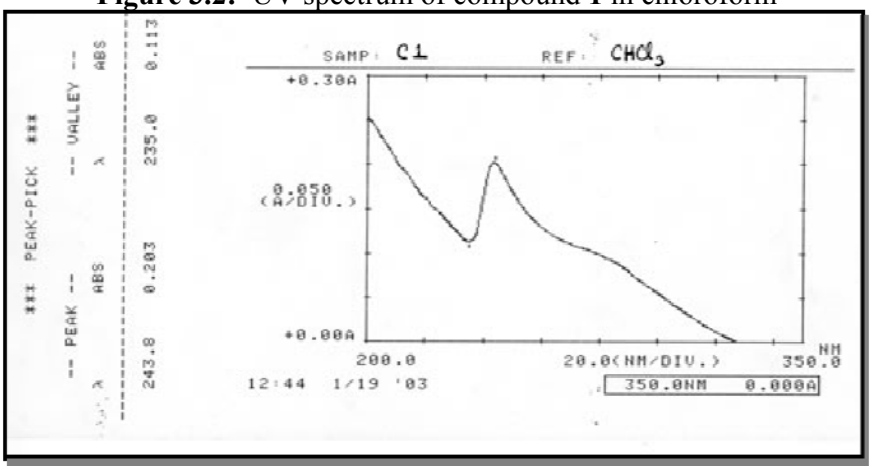
Chemical test

It showed violet color in Liebermann-Burchard test indicating that the compound had a triterpenoid nucleus. It showed false Dragendorff's test.

UV-Visible spectroscopy

UV spectrum (Figure 3.2) showed absorption peak at 243.8 nm in chloroform.

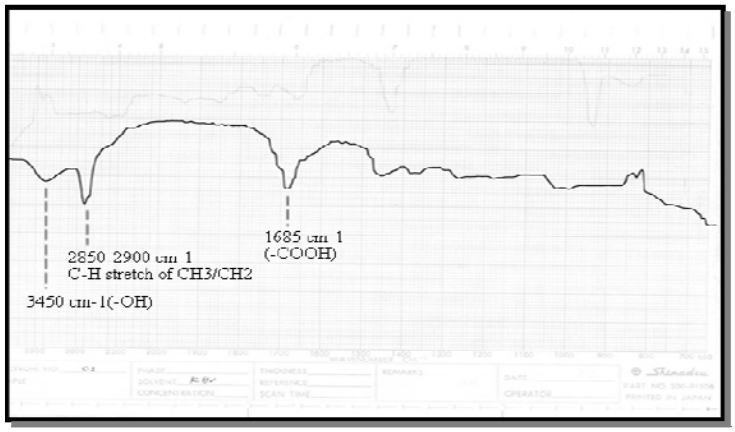
Figure 3.2: UV spectrum of compound **1** in chloroform



IR spectroscopy

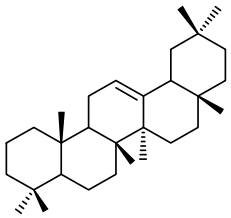
IR spectrum (Figure 3.3) of **1** showed bands at 3450, 2850-2900 and 1685 cm^{-1} suggesting the presence of hydroxyl (-OH), stretching bands of C-H of methyl and methylene and carbonyl of carboxylic acid (-COOH), respectively.

Figure 3.3: IR spectrum of compound **1** in KBr disc



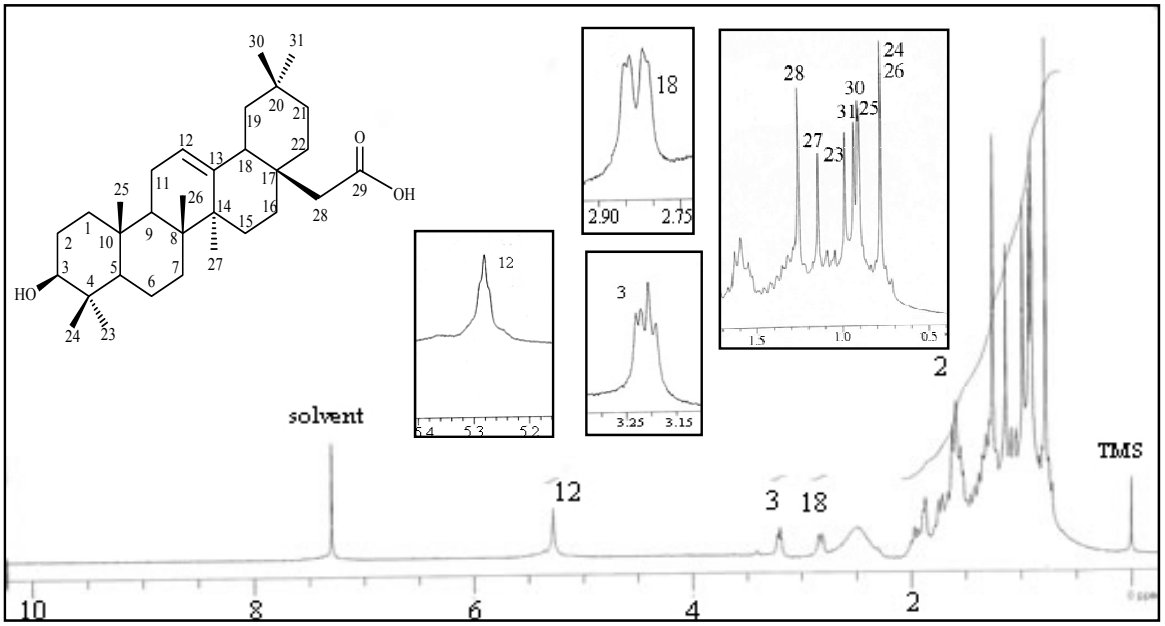
^{1}H-NMR spectroscopy

^{1}H-NMR spectrum of **1** (Figure 3.4) displaced seven peaks to the range of δ 0.75 - 1.30. An intense singlet at δ 0.77 indicated the presence of two different methyl protons, H-24 and H-26. Five singlets at δ 0.90, 0.91, 0.93, 0.98 and 1.13 were assigned to methyl protons, H-25, H-30, H-31, H-23 and H-27, respectively. A singlet at δ 1.25 was assigned to methylene protons, H-28, confirmed by HMQC and DEPT results. A double doublet at δ 2.84, with coupling constants of 14.9 and 4.9 Hz was assigned to methine proton, H-18 due to methylene protons, H-19 indicating *β*-type oleanene skeleton.[76, 77]



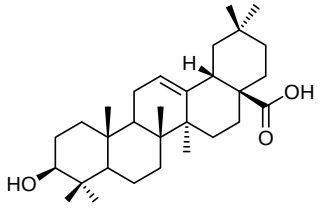
β-type oleanene skeleton

Figure 3.4: ^{1}H-NMR (400 MHz) spectrum of compound **1** in $CDCl_3$ plus one drop CD_3OD



The double doublet at δ 3.22 with coupling constants of 9.9 and 4.9 Hz, due to the axial-axial and axial-equatorial interaction, respectively, was attributed to one carbinolic methine hydrogen, H-3. The signal at δ 3.22 indicated the H-3 at axial (α) position.[78] Literature reviews revealed that if H-3 is equatorial (*β*) position, its chemical shift must appear at δ 3.87.[78] A broad triplet at δ 5.28 with coupling constant of 3.3 Hz was assigned to an

olefinic proton, H-12. These data (Table 3.7) were in close resemblance to that of oleanolic acid[77, 79], with the exception of a singlet at δ 1.25 due to H-28 in **1**. In oleanolic acid, this signal was absent.



Oleanolic acid

In addition, multiplets appearing to the range of δ 1.54 - 1.62 and δ 1.85 -1.90 were assigned to methylene protons, H-2 and H-11, respectively, which were supported by ^{1}H-^{1}H COSY, HMQC and DEPT results.

Table 3.7: ^{1}H-NMR (400 MHz) data of compound **1** in $CDCl_3$ plus one drop CD_3OD

Position of H atom	δ_H	Multiplicity/coupling constant (*J*)
2	1.54 - 1.62	m
3	3.22	dd, *J* = 9.9, 4.9
11	1.85 - 1.90	m
12	5.28	br t, *J* = 3.3
18	2.84	dd, *J* = 14.9, 4.9
23	0.98	s
24	0.77	s
25	0.90	s
26	0.77	s
27	1.13	s
28	1.25	s
30	0.91	s
31	0.93	s

^{13}C-NMR and DEPT spectroscopy

The ^{13}C-NMR (Figure 3.5 i) and DEPT spectra (Figure 3.5 ii) showed 31 signals - seven methyl, eleven methylene, five methine including that of olefinic, seven quaternary and one very less intense carbonyl carbon atoms.

Figure 3.5: (i) ^{13}C-NMR (100 MHz) spectrum of compound **1** in $CDCl_3$ plus one drop CD_3OD

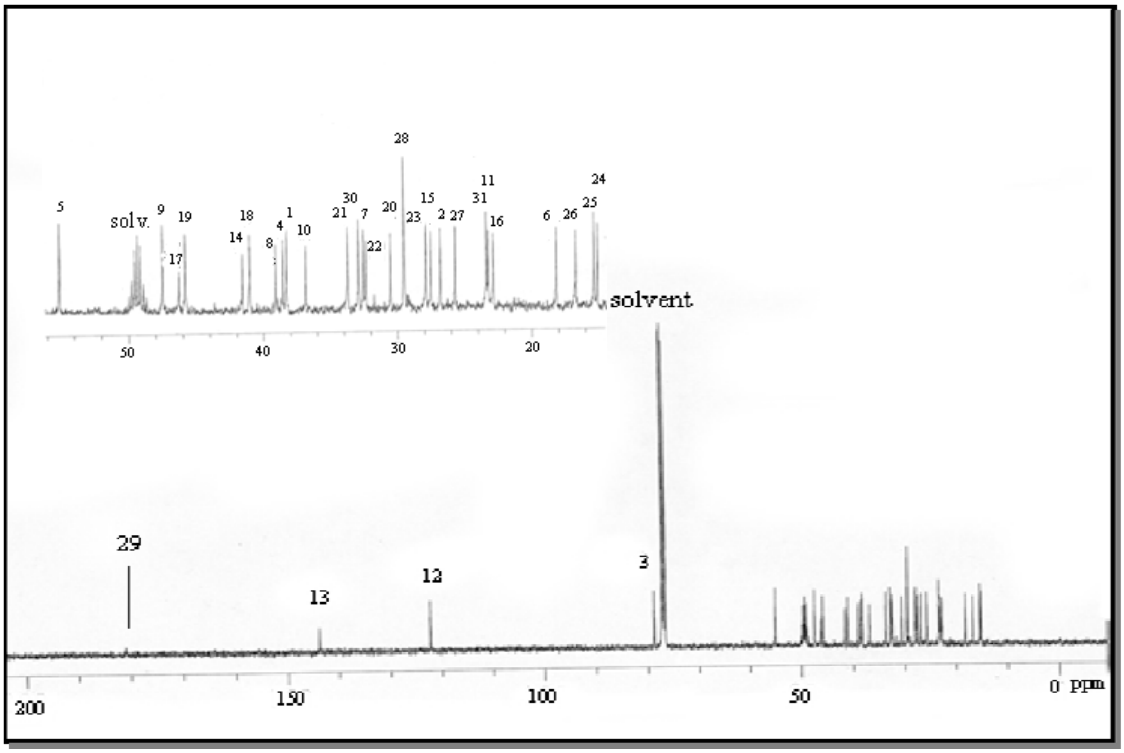
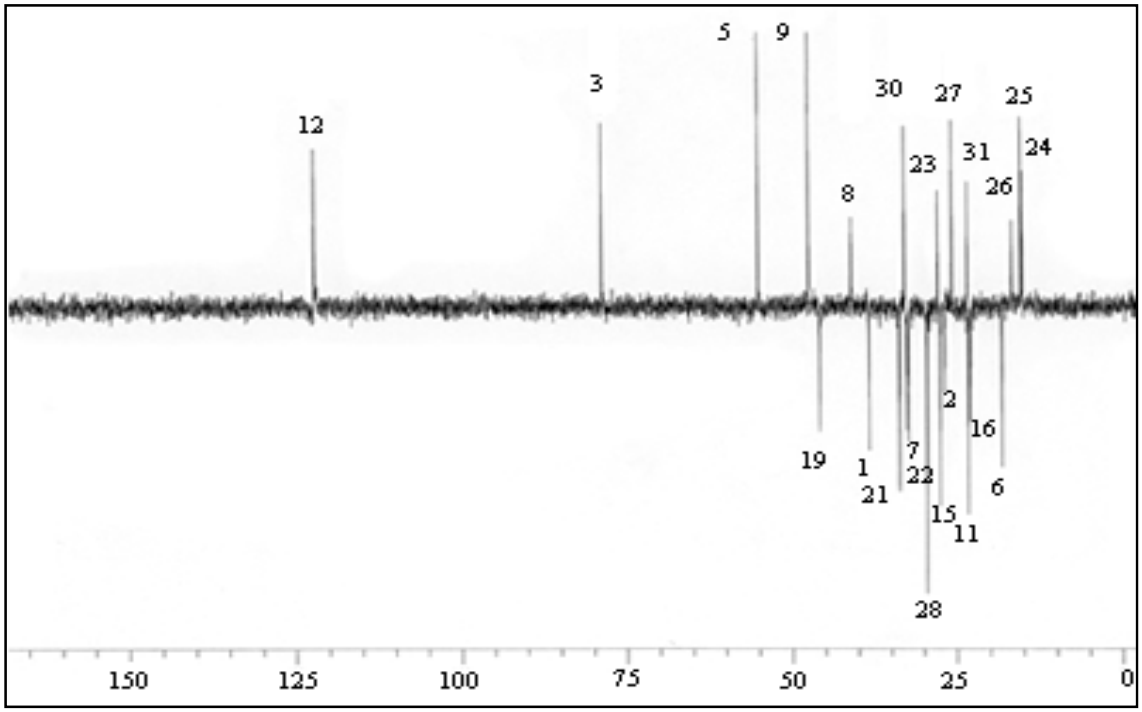


Figure 3.5: (ii) DEPT 135 spectrum data of compound **1**



Two signals at δ 122.20 and 143.77 showed a good correspondence with those of C-12 and C-13 of olean-12-ene derivatives.[80] Therefore, the signals at δ 122.20 and 143.77 were assigned to olefinic secondary carbon C-12 and olefinic quaternary carbon C-13 atoms, respectively, which were confirmed by HMQC and DEPT-135.

With the help of DEPT-135 spectrum, seven methyl signals at δ 15.21, 15.46, 16.79, 23.48, 25.80, 27.96 and 32.98 were assigned to seven methyl carbon atoms C-24, C-25, C-26, C-31, C-27, C-23 and C-30, respectively. Eleven signals resonating at δ 18.26, 22.90, 23.33, 26.92, 27.63, 29.62, 32.45, 32.65, 33.81, 38.39 and 45.91 were assigned to methylene carbon atoms C-26, C-16, C-11, C-2, C-15, C-28, C-22, C-7, C-21, C-1 and C-19, respectively. Four signals at δ 41.10, 47.56, 55.15 and 78.85 were assigned to methine carbon atoms C-18, C-9, C-5 and C-3, respectively. The downfield signal at δ 78.85 was due to attachment of C-3 with more electronegative oxygen atom of -OH group.

Six signals appearing only in ^{13}C-NMR at δ 30.60, 36.97, 38.65, 39.20, 41.65 and 46.30 but not in DEPT-135 spectrum, were assigned to six quaternary carbons C-20, C-10, C-4, C-8, C-14 and C -17, respectively. Less intense signal at δ 181.0 in comparison to others was assigned to carbonyl carbon atom C-29 of -COOH. The less intense signal may be due to longer relaxation time of carbon atom without proton attached.[81]

The chemical shifts shown by thirty carbon atoms of **1** (Table 3.8) were in close agreement with those of oleanolic acid.[82] However, one extra signal of C-28 at δ 29.62 in **1** was absent in oleanolic acid. Assignments of carbon atoms were also confirmed on the basis of ^{1}H - ^{1}H COSY, DEPT and HMQC results.

Table 3.8: ^{13}C-NMR (100 MHz), DEPT data of compound **1** and ^{13}C-NMR literature data of oleanolic acid

Carbon Position	δ_C of **1**	DEPT of **1**	Carbon position of oleanolic acid	δ_C of oleanolic acid[82]
1	38.39	CH_2	1	38.5
2	26.92	CH_2	2	27.4
3	78. 85	CH	3	78.7
4	38.65	C	4	38.7
5	55.15	CH	5	55.2
6	18.26	CH_2	6	18.3
7	32.65	CH_2	7	32.6
8	39.20	C	8	39.3
9	47.56	CH	9	47.6
10	36.97	C	10	37.0
11	23.33	CH_2	11	23.1
12	122.29	CH	12	122.1
13	143.77	C	13	143.4
14	41.65	C	14	41.6
15	27.63	CH_2	15	27.7
16	22.90	CH_2	16	23.4
17	46.30	C	17	46.6
18	41.10	CH	18	41.3
19	45.91	CH_2	19	45.8
20	30.60	C	20	30.6
21	33.81	CH_2	21	33.8
22	32.45	CH_2	22	32.3
23	27.96	CH_3	23	28.1
24	15.21	CH_3	24	15.6
25	15.46	CH_3	25	15.3
26	16.79	CH_3	26	16.8
27	25.80	CH_3	27	26.0
28	29.62	CH_2		
29	181.00	n.o	28	181.0
30	32.98	CH_3	29	33.1
31	23.48	CH_3	30	23.6

n.o. = not observed

Heteronuclear Multiple Quantum Coherence (HMQC) spectroscopy

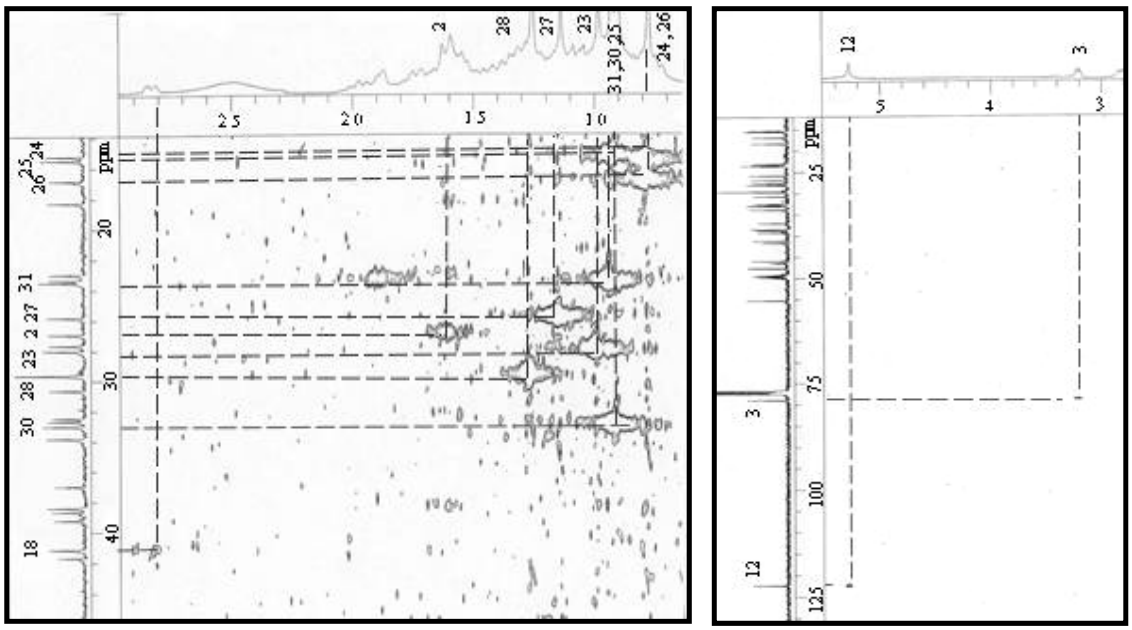
The result of HMQC experiment as shown in table 3.9 revealed that olefinic proton, H-12 at δ 5.28, carbinolic hydrogen, H-3 at δ 3.22 and methine hydrogen, H-18 at δ 2.84 were correlated with unambiguously assigned carbon C-12 (δ 122.29), carbon C-3 (δ 78.85) and

carbon C-18 (δ 41.12), respectively while methylene protons, H-28 at δ 1.25 showed connectivity with carbon C-28 (δ 29.62). Similar connectivities between methyl protons of H-23 at δ 0.98, H-24 at δ 0.77, H-25 at δ 0.90, H-26 at δ 0.77, H-27 at δ 1.13, H-30 at δ 0.91 and H-31 at δ 0.93 with carbon atoms of C-23 (δ 27.96), C-24 (δ 15.21), C-25 (δ 15.46), C-26 (δ 16.79), C-27 (δ 25.79), C-30 (δ 32.98) and C-31 (δ 23.48), respectively, were observed in HMQC experiment (Figure 3.6).

Table 3.9: HMQC data of compound **1**

δ_H	δ_C of Attached C	Assigned C atom	δ_H	δ_C of Attached C	Assigned C atom
5.28	122.29	C-12	0.98	27.96	C-23
3.22	78.85	C-3	0.93	23.48	C-31
2.84	41.12	C-18	0.91	32.98	C-30
1.25	29.62	C-28	0.90	15.46	C-25
1.13	25.79	C-27	0.77	15.21	C-24
			0.77	16.79	C-26

Figure 3.6: HMQC spectrum of compound **1**

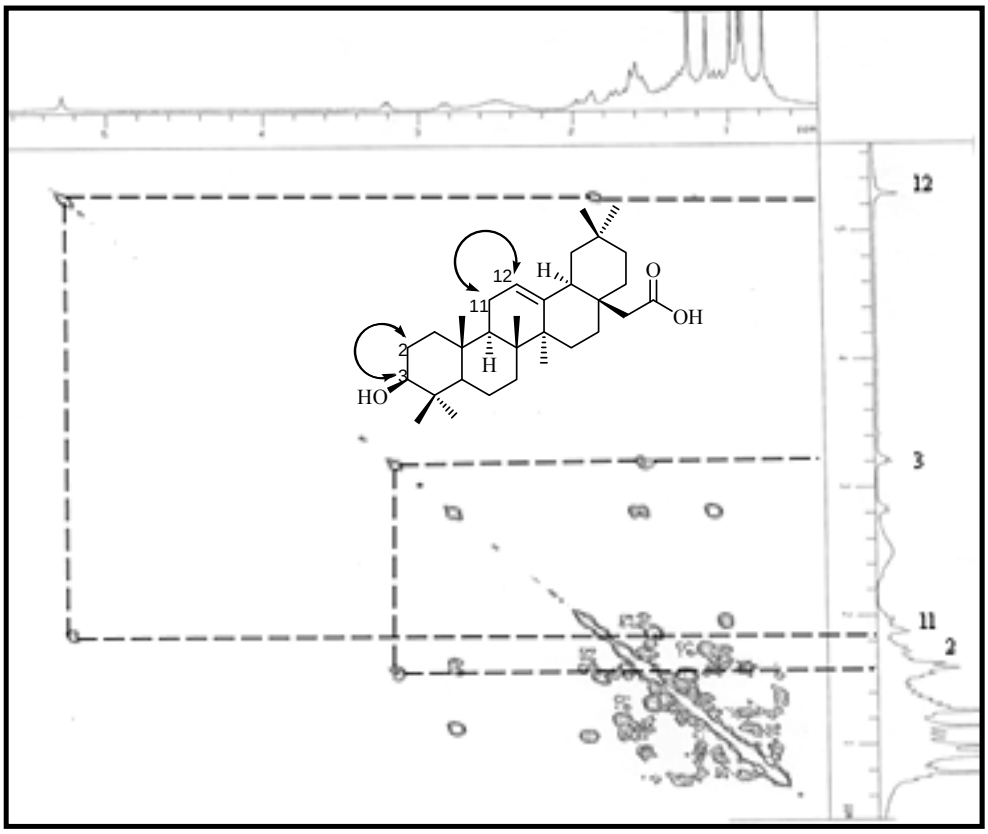


^{1}H-^{1}H Correlation spectroscopy (^{1}H-^{1}H COSY)

The ^{1}H-^{1}H COSY experiment (Figure 3.7) indicated that the olefinic proton H-12 at δ 5.28 was correlated with methylene protons H-11 at δ 1.85 - 1.90, which was correlated by HMQC to a CH_2 signal at δ 23.33, ascribable to position C-11. Similarly, the carbinolic proton H-3 at

δ 3.22 was correlated with methylene protons H-2 at δ 1.54 -1.62, which correlated by HMQC to a CH_2 signal at δ 26.92, ascribable to position C-2.

Figure 3.7: ^{1}H-^{1}H COSY spectrum of compound **1**



Heteronuclear Multiple Bond Coherence (HMBC) Spectroscopy

The HMBC experiment (Figure 3.8) revealed significant cross peaks between H-23 at δ 0.98 and C-3 (δ 78.85), C-5 (δ 55.15) and C-4 (δ 38.65); between H-24 at δ 0.77 and C-3 (δ 78.85), C-5 (δ 55.15), C-23 (δ 27.96) and C-4 (δ 38.65); and between the protons of H-25 at δ 0.90 and C-4 (δ 38.65), C-9 (δ 47.56), C-1(δ 38.39), C-5 (δ 55.15) and C-10 (δ 36.97) as shown in figure 3.9. Similar correlations were appeared between the protons of H-26 at δ 0.77 and C-7 (δ 32.65*)*, C-9 (δ 47.56), C-14 (δ 41.65) and C-8 (δ 39.20); and between the protons of H-27 at δ 1.13 and C-8 (δ 39.20), C-15 (δ 27.63), C-13 (δ 143.77) and C-14 (δ 41.65). Cross-peaks observed between H-30 at δ 0.91 with C-19 (δ 45.91), C-31 (δ 23.48) and C-20 (δ 30.60), and the protons between H-31 at δ 0.93 and C-30 (δ 32.98) and C-20 (δ 30.60).

Figure 3.8: HMBC spectrum of compound **1**

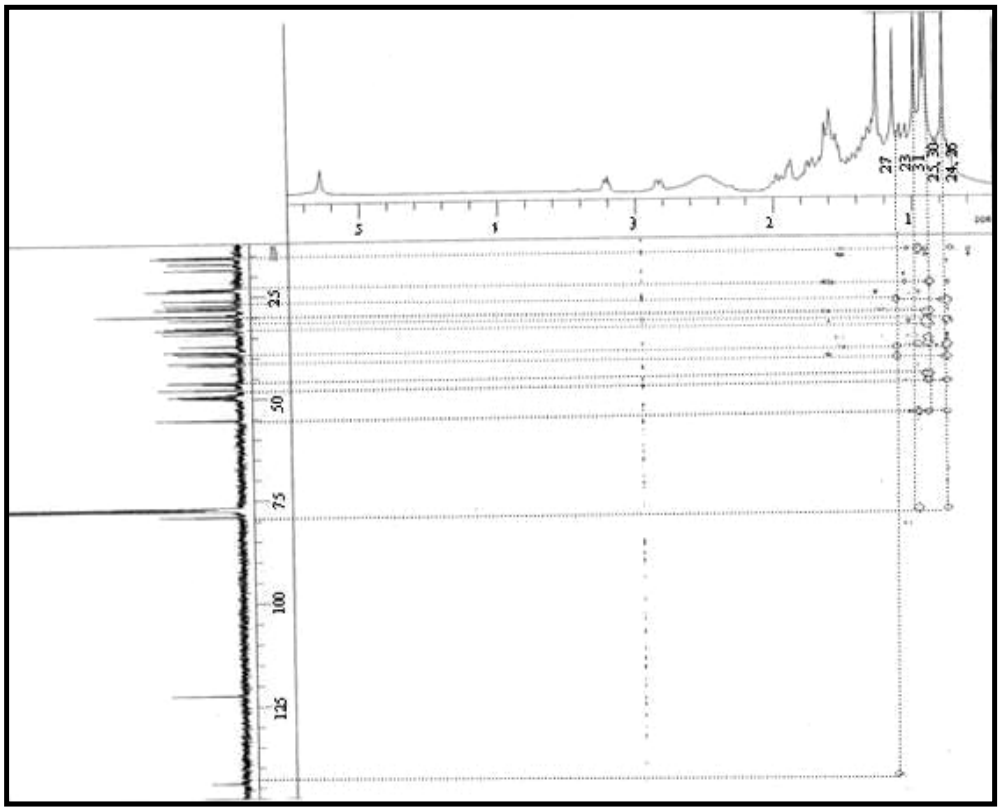
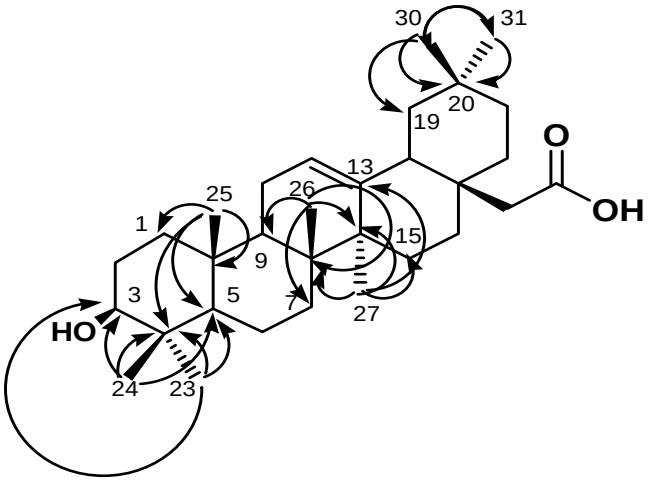


Figure 3.9: ^{1}H-^{13}C connectivities in HMBC experiment for compound **1**. Arrows point from proton to carbon.



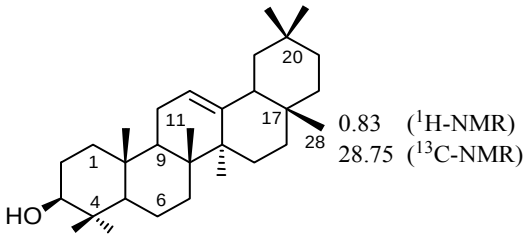
These cross peaks observed were due to two ($^{2}J_{C\text{-}H}$), three ($^{3}J_{C\text{-}H}$) and four ($^{4}J_{C\text{-}H}$) bonds distant correlations (Table 3.10).

Table 3.10: HMBC data of compound **1**

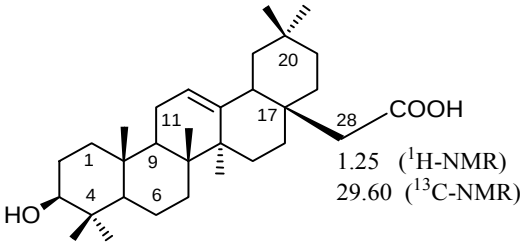
Connected carbon			
Position	$^{4}J_{C\text{-}H}$	$^{3}J_{C\text{-}H}$	$^{2}J_{C\text{-}H}$
H-23		C-3, C-5	C-4
H-24		C-3, C-5, C-23	C-4
H-25	C-4	C-9, C-1,C-5	C-10
H-26		C-7, C-9, C-14	C-8
H-27		C-8, C-15, C-13	C-14
H-30		C-19, C-31	C-20
H-31		C-30	C-20

Furthermore, the chemical shifts of C-28 of compound **1** in ^{1}H-NMR and ^{13}C-NMR spectra were also compared with 3*β*-hydroxyolean-12-ene (*β*-amyrin). In *β*-amyrin, C-28 methyl group occurred as singlet at δ 0.83 in ^{1}H-NMR[52, 83] and δ 28.75 in ^{13}C-NMR.[52] When one of the protons from this C-28 methyl group in *β*-amyrin was replaced by -COOH in eupatoric acid (**1**), chemical shift of C-28 protons (-C<u>H</u>$_2$COOH) was shifted down field to δ 1.25 in ^{1}H-NMR and δ 29.60 in ^{13}C-NMR in favor of the assigned structure of compound **1** as shown in figure 3.10.

Figure 3.10: Chemical shifts of H-28 in ^{1}H-NMR and C-28 in ^{13}C-NMR for *β*-amyrin and eupatoric acid (**1**)



3β –hydroxyolean-12-ene (β–amyrin)



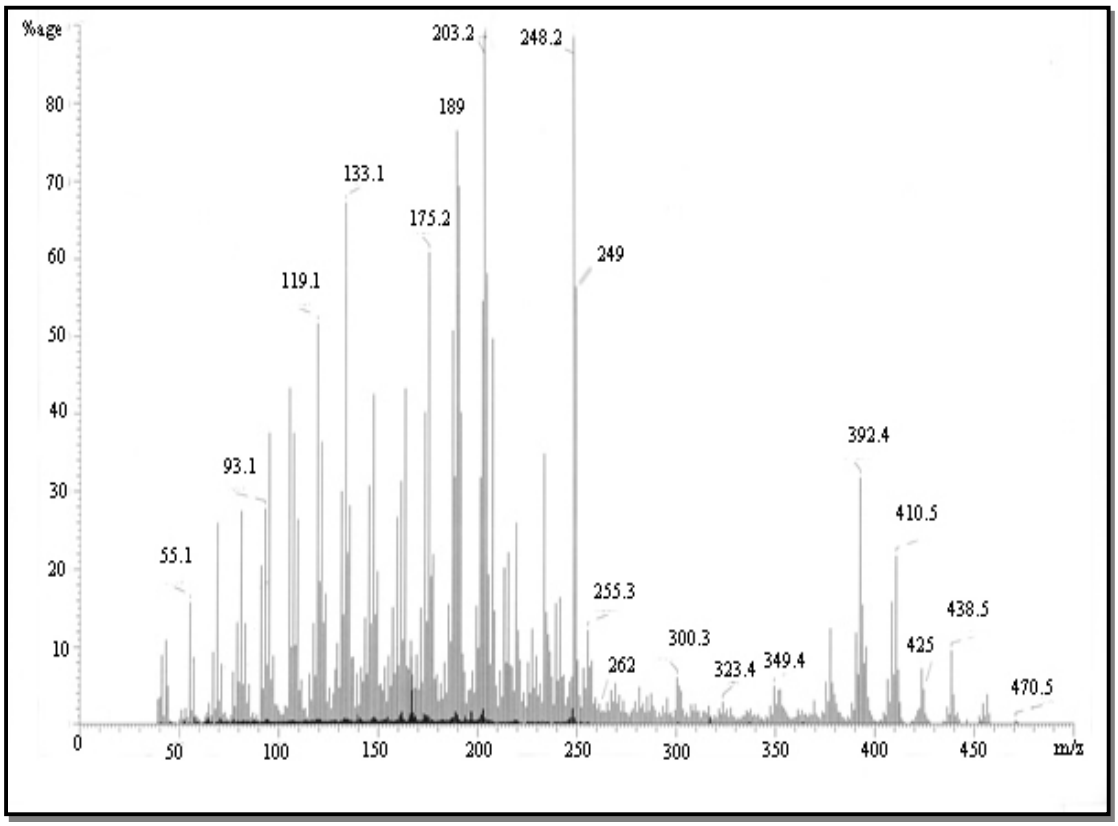
Eupatoric acid (**1**)

Mass spectroscopy

EIMS (Figure 3.11) revealed a molecular ion $[M]^{+}$ at *m/z* 470. The HRMS (Figure 3.12) showed molecular ion at *m/z* 470.3387, accounting for a molecular composition $C_{31}H_{50}O_3$ (calcd. 470.3760). Furthermore, fragment peaks in EIMS at *m/z* 425 and 411 were due to $[M\text{-}COOH]^{+}$ and $[M\text{-}CH_2COOH]^{+}$, respectively. All these spectroscopic studies led to

structure **1** for this new triterpene, 3*β*-hydroxy-28-carboxyolean-12-ene, designated as eupatoric acid.

Figure 3.11: EIMS for compound **1**

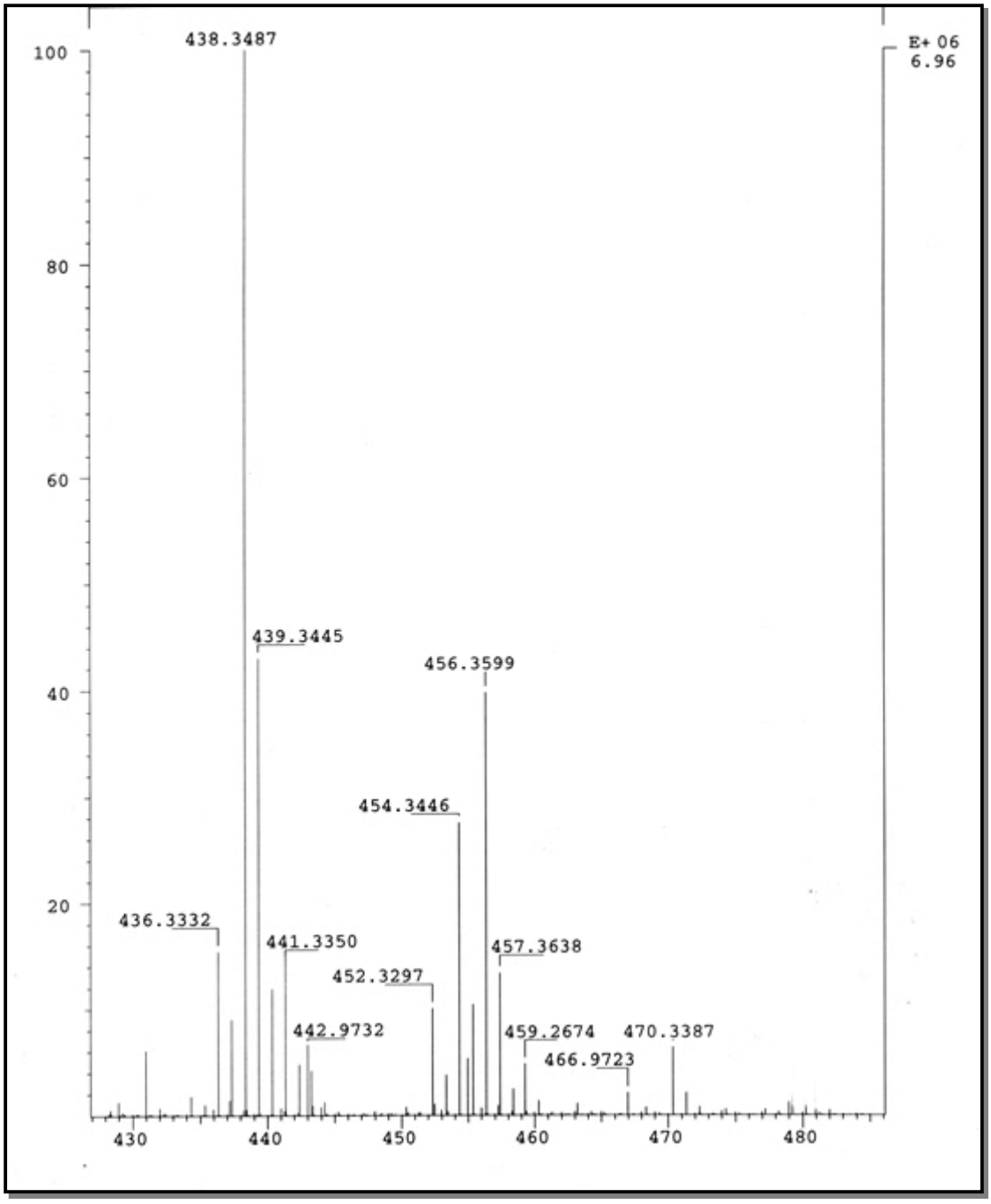


Proposed mass fragmentation pattern for eupatoric acid (1)

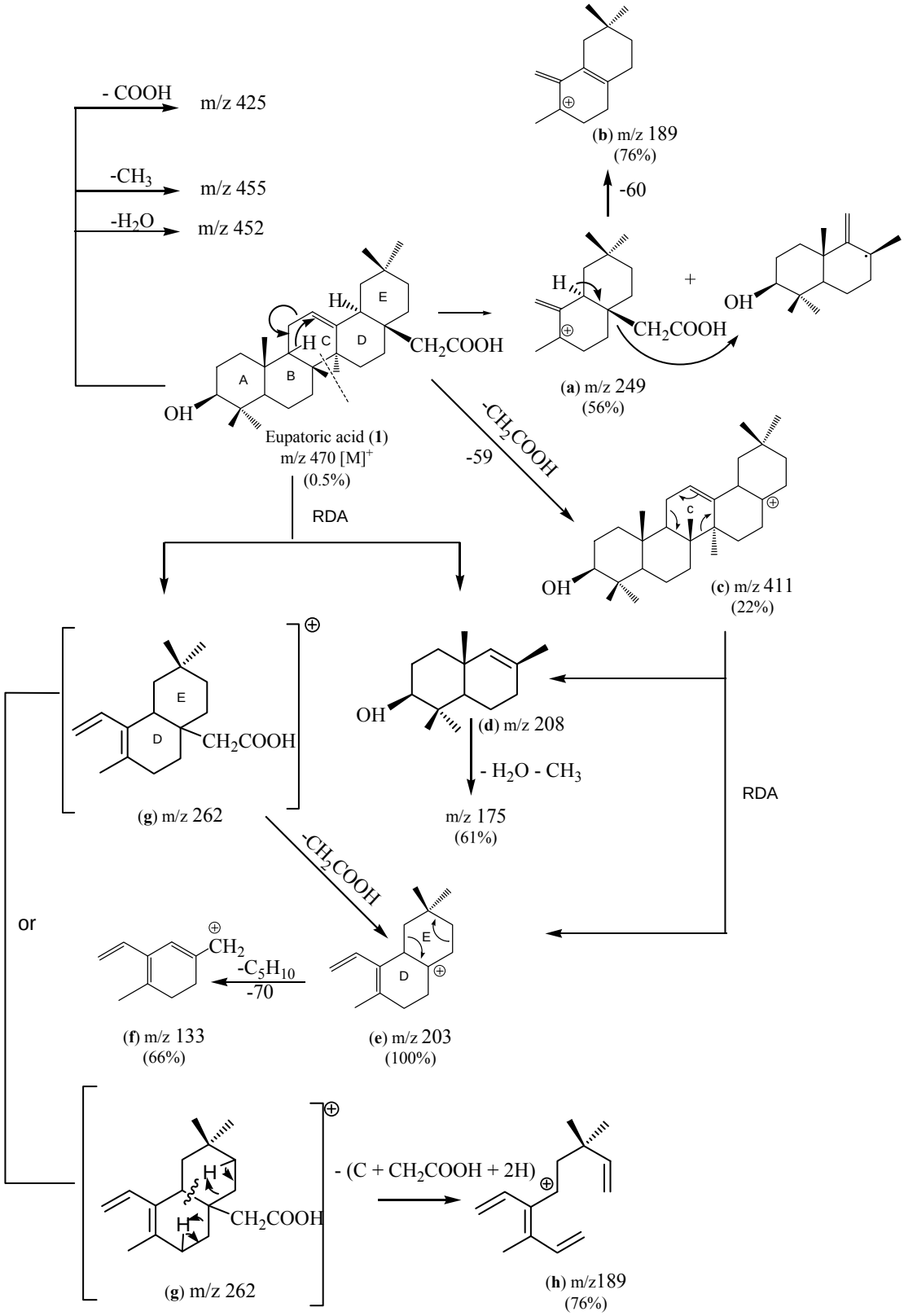
The possible mass fragmentation pattern for **1** was proposed as shown in scheme 3.6. The formation of fragment ion (**a**) with m/z 249 probably involves one hydrogen transfer and cleavage of bonds between C-11 and C-12, C-8 and C-14 of compound **1**.[84] Further, loss of acetic acid from fragment (**a**) can furnish a new fragment ion (**b**) with m/z 189. The compound **1,** under *retro*-Diels-Alder (RDA) fragmentation of the ring C, produces fragments (**g**) at m/z 262 and (**d**) at m/z 208. However, both fragments were difficult to observe in spectra due to overcrowding of peaks. Fragment (**g**) can lead further to the formation of (**e**) and (**h**) by two possible paths. One path leads to the formation of a fragment (**e**) at m/z 203 by losing -CH_2COOH group, which suffers further decomposition by the loss of 70 mass units yielding a fragment (**f**) at m/z 133. This cleavage is probably due to the partial loss of ring E.[84] In another path a highly conjugated cation (**h**) at m/z 189 may be possible due to loss of C-17 and its substituent which necessitates a double hydrogen transfer.[84] Eupatoric acid (**1**) also reveals fragment (**c**) at m/z 411 due to loss of -CH_2COOH

group. Fragment (**c**) under *retro*-Diels-Alder fragmentation in the ring C can also yield fragments (**d**) and (**e**). This reveals that the fragment (**e**) may be formed either through (**g**) or (**c**).

Figure 3.12: HRMS for compound **1**



Scheme 3.6: Possible mass fragmentation of compound **1**



3.4.2 Poriferasterol (2)

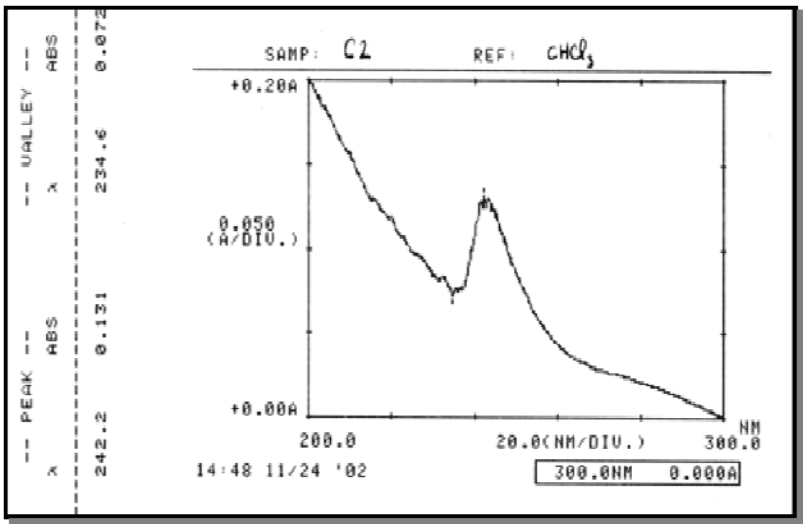
Chemical test

It showed green color in Liebermann-Burchard test indicating that the compound had a steroid nucleus.

UV-vis spectroscopy

UV spectrum (Figure 3.13) showed absorption peak at 242 nm in chloroform.

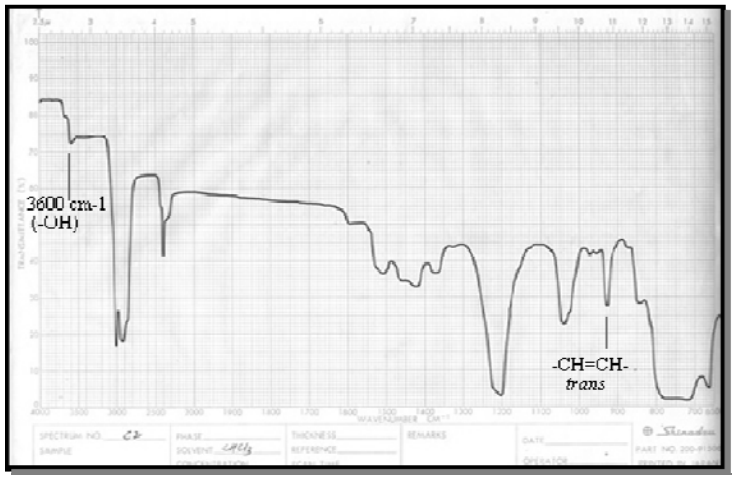
Figure 3.13: UV spectrum of compound **2** in $CHCl_3$



IR spectroscopy

The IR spectrum (Figure 3.14) of the **2** showed the bands at 3600 and 930 cm^{-1} suggesting the presence of hydroxyl group (-OH) and *trans* olefinic protons (-CH=CH-), respectively, in the molecule.

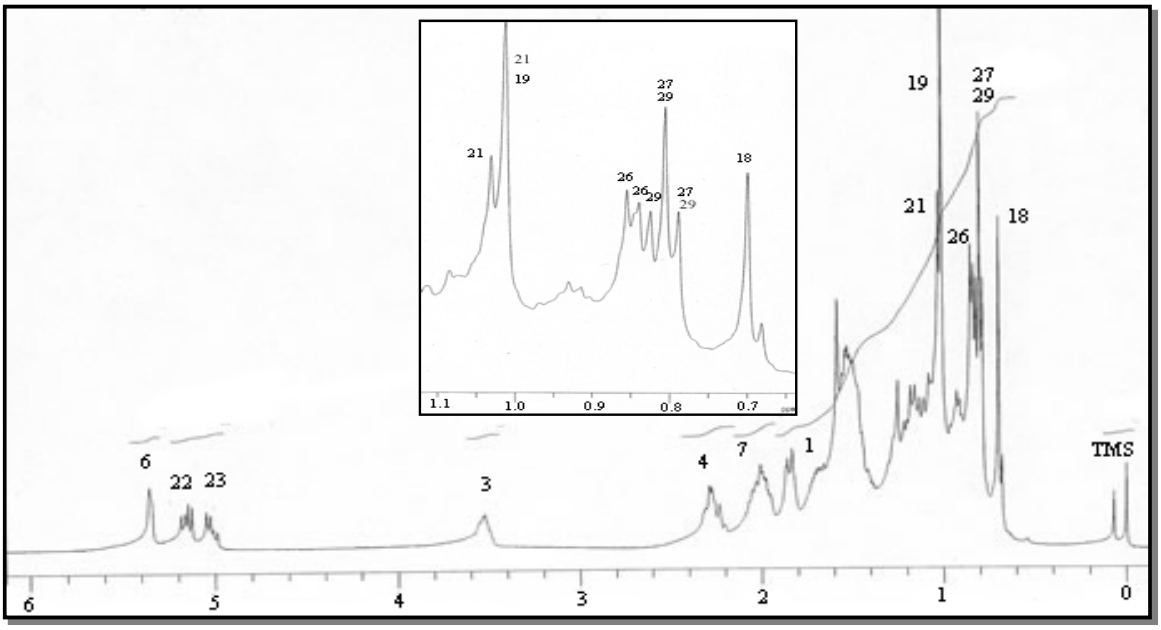
Figure 3.14: IR spectrum of compound **2** in $CHCl_3$



^{1}H-NMR spectroscopy

The ^{1}H-NMR spectrum of **2** (Figure 3.15) revealed the protons of six methyl groups. Two methyl singlets at δ 0.69 and 1.01 were assigned to protons, H-18 and H-19, respectively.

Figure 3.15: ^{1}H-NMR (400 MHz) spectrum of compound **2** in $CDCl_3$

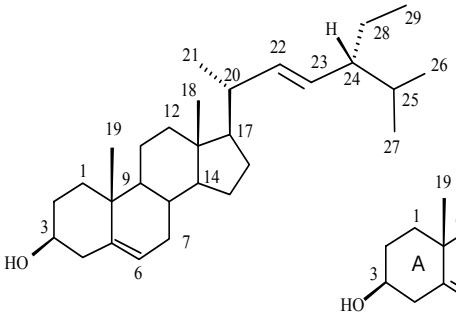


A triplet at δ 0.80 with coupling constant 6.9 Hz was due to methyl proton, H-29. A doublet at δ 0.79 with coupling constant 6.9 Hz was assigned to methyl protons, H-27 of isopropyl group whose one of the wings was overlapped with the central wing of a triplet of H-29. Another doublet at δ 0.84 with coupling constant 5.9 Hz was due to methyl protons, H-26 of isopropyl group. The observed difference in chemical shift between the nonequivalent H-26 and H-27 methyl protons was generated by the C-24 chiral centre, that is, the system CMe_2H-CXYZ.[85] A doublet at δ 1.03 with coupling constant 5.9 Hz was due to methyl protons, H-21 whose one of the wings was overlapped by signal of H-19. A multiplet at δ 2.00 was assigned to proton H-7 which was confirmed from ^{1}H-^{1}H COSY spectrum. A multiplet at δ 3.52 and a broad doublet at δ 5.35 were assigned to carbinolic methine proton, H-3 and olefinic proton, H-6, respectively, which are the characteristic pattern of the Δ^5–sterol skeleton.[86]

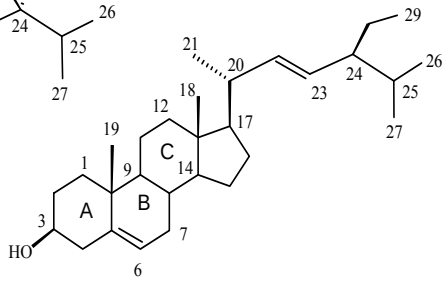
Two double doublets at δ 5.04 and 5.14 with coupling constants of 14.9 and 8.5 Hz for each were assigned to olefinic protons H-23 and H-22, respectively. The coupling constant of 14.9 Hz of each H-22 and H-23 indicated that olefinic protons were in *trans*-positions. Similarly,

the value of coupling constant of 8.5 Hz of each H-22 and H-23 was indicative of free rotation effect of H-24 to H-23, and free rotation effect of H-20 to H-22. Multiplets at δ 1.83, 2.00 and 2.28 were assigned to methylene protons, H-1, H-7 and H-4, respectively. The ^{1}H-NMR data of **2** was in close agreement with reported data[87] of poriferasterol (Table 3.8). However ^{1}H-NMR data of **2** was also in close agreement with reported data[87, 88] of stigmasterol (Table 3.11). Stigmasterol, a diastereomer of poriferasterol, differed only in the orientation of proton at carbon C-24 as shown below.

stigmasterol



poriferasterol (**2**)



Therefore, a difficulty was encountered to identify the compound directly just inspecting the data above.

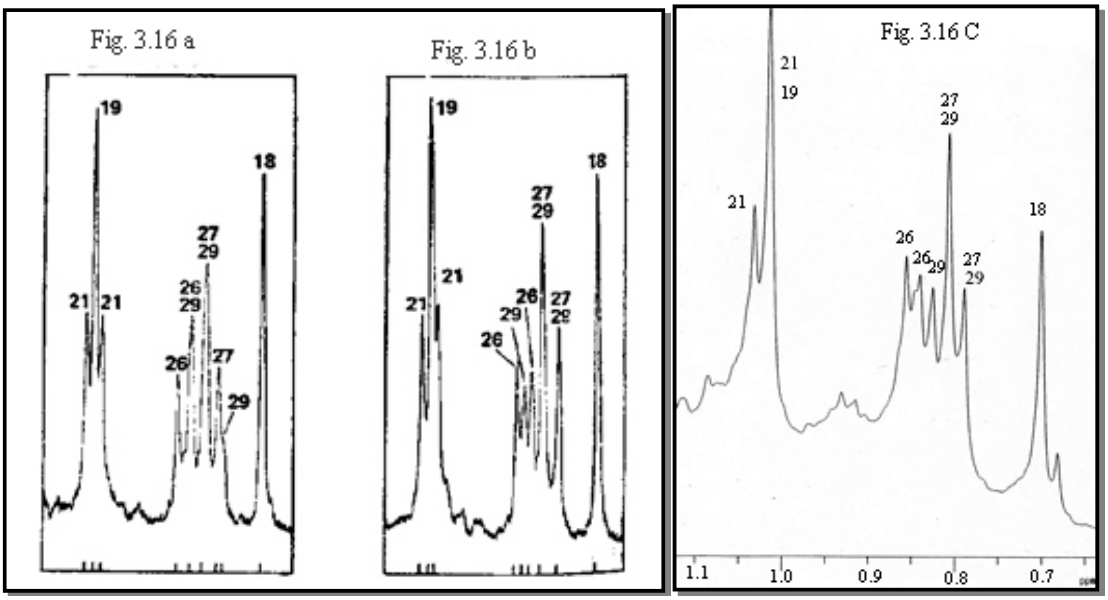
Table 3.11: Comparison of ^{1}H-NMR data of compound **2** with reported values of stigmasterol and poriferasterol

Position of H atom	δ_H of **2**	Multiplicity and coupling constant (*J*)	δ_H stigmasterol[88] (*24 S*)	δ_H stigmasterol[87] (*24 S*)	δ_H poriferasterol[87] (*24 R*)
1	1.83	m			
3	3.52	m	3.46, m	3.505, m	3.505
4	2.28	m			
6	5.35	br d, *J* = 4.2	5.31, br s	5.357, br s	5.357, br s
7	2.00	m	-	-	-
18	0.69	S	0.69, s	0.697, s	0.697, s
19	1.01	S	0.97, s	1.012, s	1.011, s
21	1.03	d, *J* = 5.9	1.00, d, *J* = 6.4	1.022, d, *J* = 6.5	1.024, *J* = 6.5
22	5.14	dd, *J* = 14.9, 8.5	5.11, dd, *J* = 15.5, 8.5	5.159, *J* = 6.5	5.162, *J* = 6.5
23	5.04	dd, *J* = 14.9, 8.5	4.97, dd, *J* = 15.5, 8.5	5.015, *J* = 7.5	5.016, *J* = 7.5
26	0.84	d, *J* = 5.9	0.81, d, *J* = 6.1	0.846, *J* = 6.5	0.843, *J* = 6.5
27	0.79	d, *J* = 6.9	0.76, d, *J* = 6.0	0.795, *J* = 6.6	0.791, *J* = 6.7
29	0.80	t, *J* = 6.9	0.78, t, *J* = 7.5	0.804, *J* = 7.1	0.810, *J* = 7.2

Rubinstein *et al.*[89] showed that stigmasteryl acetate and poriferasteryl acetate have characteristic spectral patterns of six methyl protons as shown in figure 3.16a and figure

3.16b, respectively. The spectral pattern of six methyl protons of compound **2** (Figure 3.16c) revealed three unsymmetrical doublets for protons H-26, H-27 and H-21; a triplet for H-29 protons and two singlets for H-18 and H-19 protons. This pattern was in close agreement with reported peak pattern of six methyl protons of poriferasteryl acetate (Figure 3.16b), reflecting that substituents in ring A have no effect on spectral pattern of six methyl protons.

Figure 3.16: Peaks patterns of six methyl protons in ^{1}H-NMR spectrum of (a) stigmasteryl acetate (b) poriferasteryl acetate and (c) compound **2** in $CDCl_3$



^{13}C-NMR and DEPT spectrum

The ^{13}C-NMR spectrum (Figure 3.17 i) revealed signals of twenty nine carbon atoms at δ 12.04, 12.22, 18.98, 19.39, 21.07, 21.07, 21.21, 24.36, 25.39, 28.90, 31.67, 31.91, 31.91, 31.91, 36.52, 37.27, 39.69, 40.46, 42.23, 42.32, 50.18, 51.24, 55.98, 56.87, 71.80, 121.69, 129.30, 138.30 and 140.76 (Table 3.12).

The DEPT experiment (Figure 3.17 ii) showed eleven methine carbons at δ 31.91, 31.91, 40.46, 50.18, 51.24, 55.98, 56.87, 71.80, 121.69, 129.30, 138.30 and six methyl carbons at δ 12.04, 12.22, 18.98, 19.39, 21.07 and 21.21. Similarly only eight methylene carbons at δ 21.07, 24.36, 25.39, 28.90, 31.67, 37.27, 39.69 and 42.23 were observed in DEPT experiment; however one methylene signal at δ 31.91 was missing in DEPT experiment.

Figure 3.17: (i) ^{13}C-NMR (100 MHz) spectrum of compound **2** in $CDCl_3$

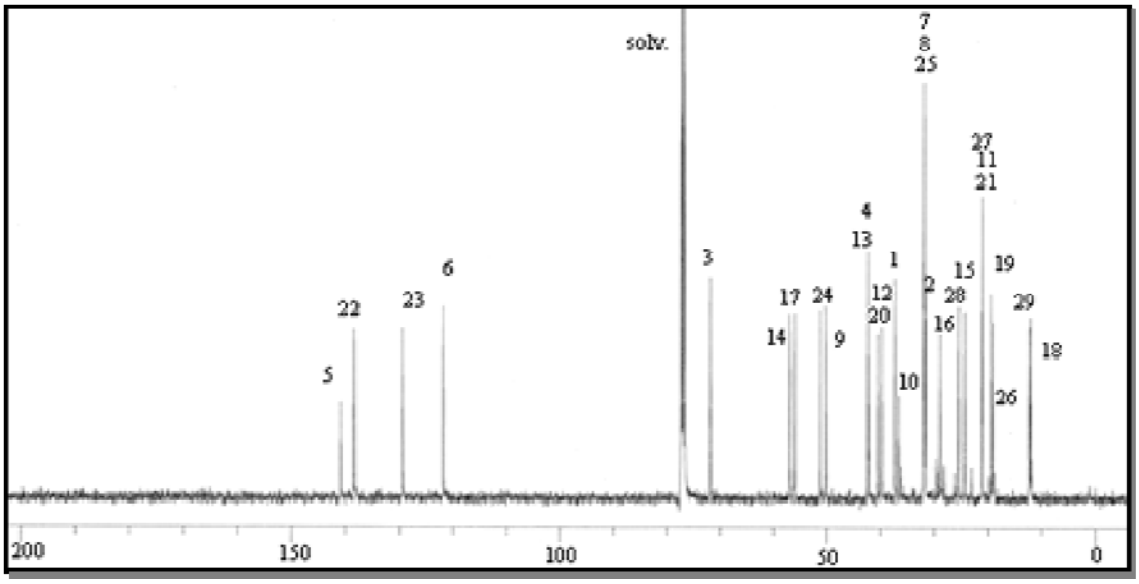
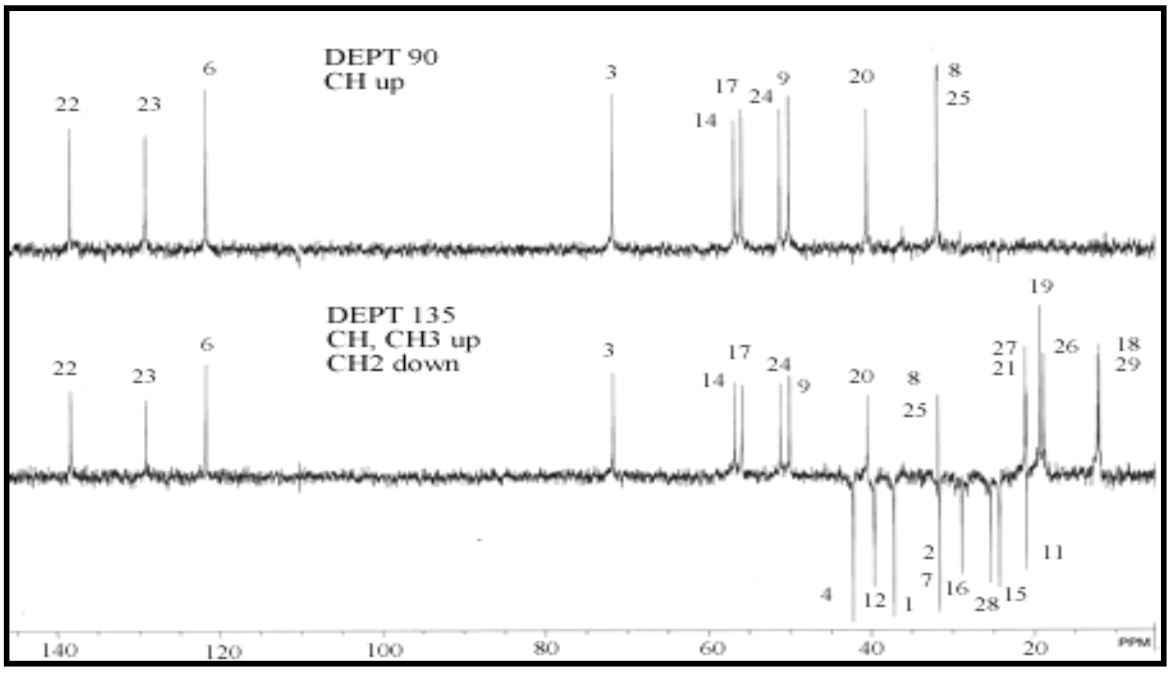


Figure 3.17: (ii) DEPT spectrum of compound **2**



Assignment of proton and carbon atoms of poriferasterol was further confirmed by ^{1}H-^{1}H COSY, HMQC and HMBC experiments.

Table 3.12: ^{13}C-NMR (100 MHz) data of compound **2**

Position of C atom	δ_C	DEPT 90	DEPT 135	Position of C atom	δ_C	DEPT 90	DEPT 135
1	37.27		CH_2	16	28.90		CH_2
2	31.67		CH_2	17	55.98	CH	
3	71.80	CH		18	12.04		CH_3
4	42.23		CH_2	19	19.39		CH_3
5	140.76		C**	20	40.46	CH	
6	121.69	CH		21	21.07		CH_3
7	31.91		CH_2*	22	138.30	CH	
8	31.91	CH		23	129.30	CH	
9	50.18	CH		24	51.24	CH	
10	36.52		C**	25	31.91	CH	
11	21.07		CH_2	26	18.98		CH_3
12	39.69		CH_2	27	21.21		CH_3
13	42.32		C**	28	25.39		CH_2
14	56.87	CH		29	12.22		CH_3
15	24.36		CH_2				

* Missing signal in DEPT spectrum

** Quaternary carbons lost in DEPT 135

Two signals at δ 121.69 and 140.76 indicated the presence of endocyclic double between C-5 and C-6 of sterols, respectively.[90] A comparative study of the ^{13}C-NMR data of **2** with that of reported data[91] of stigmasterol (a) and poriferasterol (b) (Table 3.13) revealed that ^{13}C-NMR data of compound **2** were in close agreement with that of poriferasterol rather than stigmasterol.

It is remarkable that when ^{13}C chemical shifts between compound **2** and its stigmasterol were compared, it is found that C-26 and C-27 showed larger differences (Table 3.13). C-26 of **2** was 2.28 ppm more shielded and C-27 of **2** was 2.19 ppm more deshielded than in stigmasterol. This kind of difference in chemical shift in ^{13}C-NMR between C-26 and C-27 methyl groups results mainly from intrinsic magnetic non-equivalence generated by the C-24 chiral centre.

Table 3.13: Comparative study of ^{13}C-NMR data of compound **2** with reported data of stigmasterol (a) and poriferasterol (b)

C Atom	δ_C of Stigmasterol (a)[91]	δ_C of Poriferasterol (b)[91]	δ_C of (**2**)	DEPT	Δ (b-**2**)	Δ (a-**2**)
1	37.31	37.30	37.27	CH_2	0.03	0.04
2	31.69	31.67	31.67	CH_2	0.02	0.02
3	71.81	71.83	71.80	CH	0.03	0.01
4	42.35	42.27	42.23	CH_2	0.04	0.12
5	140.79	140.79	140.76	C	0.03	0.03
6	121.69	121.72	121.69	CH	0.03	0.00
7	31.94	31.92	31.91	CH_2	0.01	0.03
8	31.94	31.92	31.91	CH	0.01	0.03
9	50.20	50.20	50.18	CH	0.02	0.02
10	36.56	36.55	36.52	C	0.03	0.04
11	21.11	21.11	21.07	CH_2	0.04	0.04
12	39.74	39.73	39.69	CH_2	0.04	0.05
13	42.35	42.33	42.32	C	0.01	0.03
14	56.91	56.90	56.87	CH	0.03	0.04
15	24.39	24.37	24.36	CH_2	0.01	0.03
16	28.96	28.82	28.90	CH_2	- 0.08	0.06
17	56.06	56.06	55.98	CH	0.08	0.08
18	12.07	12.06	12.04	CH_3	0.02	0.03
19	19.42	19.42	19.39	CH_3	0.03	0.03
20	40.54	40.54	40.46	CH	- 0.08	0.02
21	21.11	20.95	21.07	CH_3	- 0.12	0.04
22	138.37	138.28	138.30	CH	0.02	0.07
23	129.32	129.37	129.30	CH	0.07	0.02
24	51.29	51.25	51.24	CH	0.01	0.05
25	31.94	31.87	31.91	CH	- 0.04	0.03
26	21.26	18.97	18.98	CH_3	- 0.01	2.28
27	19.02	21.20	21.21	CH_3	- 0.01	-2.19
28	25.44	25.42	25.39	CH_2	0.03	0.05
29	12.27	12.43	12.22	CH_3	0.21	0.06

^{1}H-^{1}H Correlation spectroscopy (^{1}H-^{1}H COSY)

The direct proton and proton connectivities between H-22 at δ 5.14 and H-23 at δ 5.04 were observed in the ^{1}H-^{1}H COSY of **2** (Figure 3.18), indicating the position of double bond. Furthermore, connectivities of H-6 at δ 5.35 with H-7 at δ 2.00 and connectivities of H-3 at δ 3.52 with H-4 at δ 2.28 were observed. These were presented in table 3.14.

Figure 3.18: ^{1}H-^{1}H COSY of compound **2**

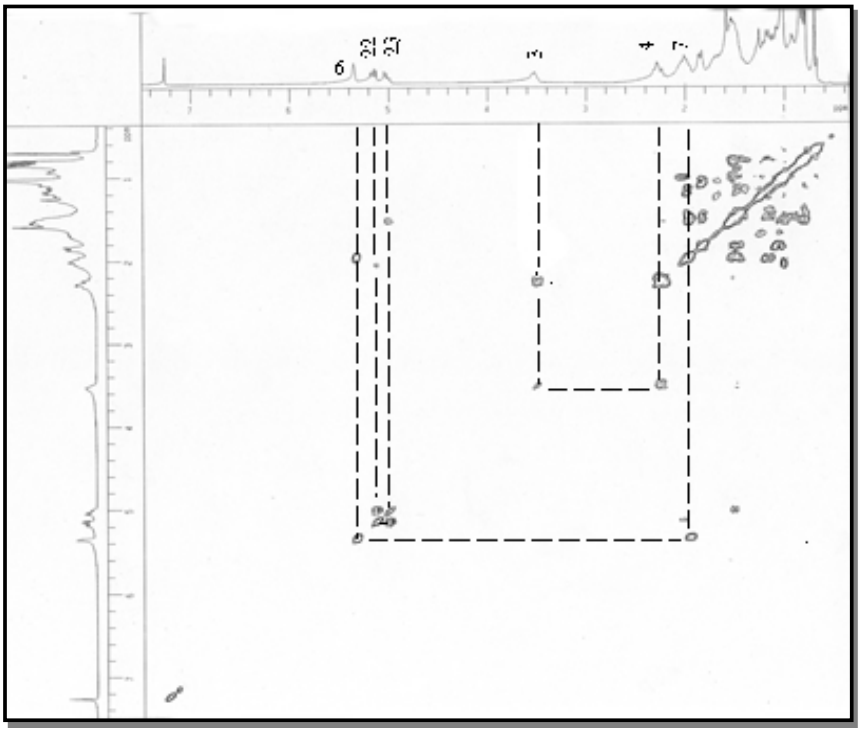


Table 3.14: ^{1}H-^{1}H COSY data for compound **2**

δ_H	Position of H atom	δ_H of correlated protons
5.14	H-22	5.04 (H-23)
5.35	H-6	2.00 (H-7)
3.52	H-3	2.28 (H-4)

Heteronuclear Multiple Quantum Coherence (HMQC) spectroscopy

HMQC spectrum (Figure 3.19) showed connectivities of the carbinolic methine proton H-3 at δ 3.52 with carbon C-3 (δ 71.80); olefinic proton H-6 at δ 5.35 with C-6 (δ 121.69); olefinic proton H-22 at δ 5.14 with carbon C-22 (δ 138.30) and H-23 at δ 5.04 with carbon C-23 (δ 129.30), respectively. Methyl protons H-18 at δ 0.69, H-19 at δ 1.01, H-21 at δ 1.03, H-26 at δ 0.84, H-27 at δ 0.79 and H-29 at δ 0.80 showed connectivities with carbon atoms C-18 (δ 12.04), C-19 (δ 19.39), C-21 (δ 18.98), C-26 (δ 21.07), C-27 (δ 21.21) and C-29 (δ 12.22), respectively. HMQC also revealed the connectivities between methylene protons H-1 at δ 1.83 and C-1 (δ 37.27), between H-4 at δ 2.28 and C-4 (δ 42.23), and between H-7 at δ 2.00 and C-7 (δ 31.91), respectively. These were presented in table 3.15.

Fig 3.19: HMQC spectrum of compound **2**

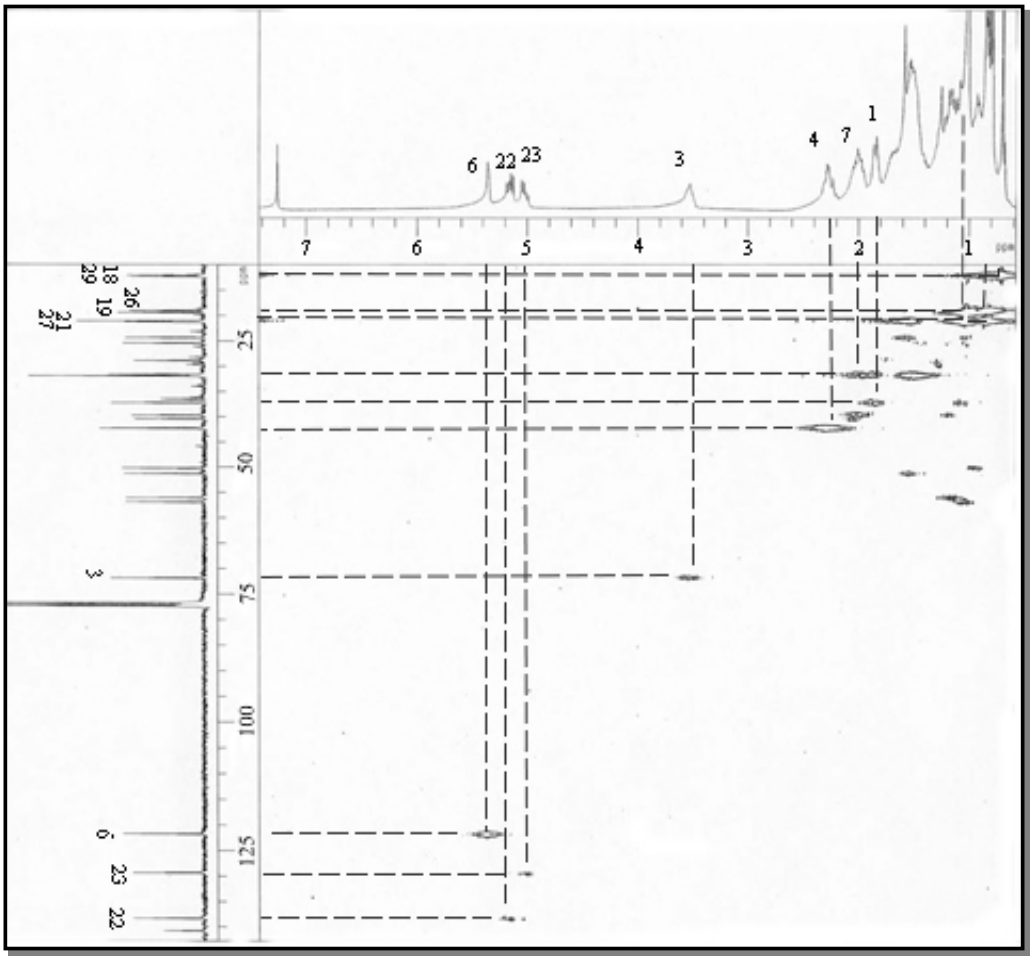


Table 3.15: HMQC data for compound **2**

δ_H	Position of H atom	δ_C	Position of C atom	δ_H	Position of H atom	δ_C	Position of C atom
1.83	H-1	37.27	C-1	1.03	H-21	21.07	C-21
3.52	H-3	71.80	C-3	5.14	H-22	138.30	C-22
2.28	H-4	42.23	C-4	5.04	H-23	129.30	C-23
5.35	H-6	121.69	C-6	0.84	H-26	18.98	C-26
2.00	H-7	31.91	C-7	0.79	H-27	21.21	C-27
0.69	H-18	12.04	C-18	0.80	H-29	12.22	C-29
1.01	H-19	19.39	C-19				

Heteronuclear Multiple Bond Coherence (HMBC) Spectroscopy

The HMBC spectrum of **2** (Figure 3.20) showed significant cross-peaks between H-1 at δ 1.83 and C-3 (δ 71.80), C-5 (δ 140.76), C-10 (δ 36.52); between H-4 at δ 2.28 and C-7

(δ 31.91), C-2 (δ 31.67), C-6 (δ 121.69), C-10 (δ 36.52), C-3 (δ 71.80) and C-5 (δ 140.76); and between the protons of H-6 and C-10 (δ 36.52), C-7 (δ 31.91). Similar correlations appeared between the protons of H-7 at δ 2.00 and C-4 (δ 42.23), C-5 (δ 140.76), C-6 (δ 121.69); between the protons of H-18 and C-12 (δ 39.69), C-13 (δ 42.32) and C-17 (δ 55.98); and between H-19 at δ 1.01 and C-2 (δ 31.67), C-5 (δ 140.76), C-9 (δ 50.18), C-10 (δ 36.52) were observed. Similar connectivity of H-21 at δ 1.03 with C-17 (δ 55.98), C-22 (δ 138.30), C-20 (δ 40.46); connectivity of H-22 at δ 5.14 with C-21 (δ 21.07), C-24 (δ 51.24), C-20 (δ 40.46), C-23 (δ 129.30); connectivity of H-23 at δ 5.04 with C-20 (δ 40.46), C-24 (δ 51.24), C-22 (δ 138.30); connectivities of H-26 at δ 0.84 and H-27 at δ 0.79 with C-23 (δ 129.30); and connectivity of H-29 at δ 0.80 with C-23 (δ 129.30) and C-24 (δ 51.24) were observed. These all connectivities were diagrammatically shown in figure 3.21. The connectivities above were due to two ($^2J_{C-H}$), three ($^3J_{C-H}$) and four ($^4J_{C-H}$) bonds distant correlations (Table 3.16).

Figure 3.20: HMBC spectrum of compound **2**

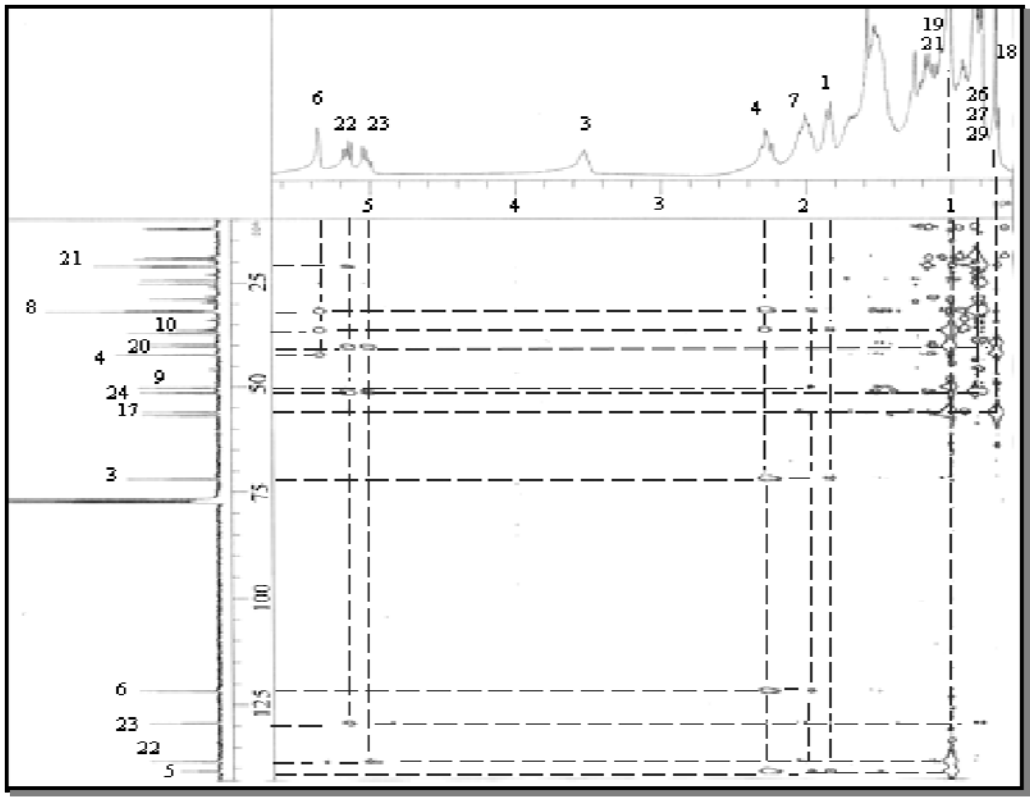


Figure 3.21: ^{1}H-^{13}C correlations in HMBC experiment of compound **2**. Arrows point from proton to carbon.

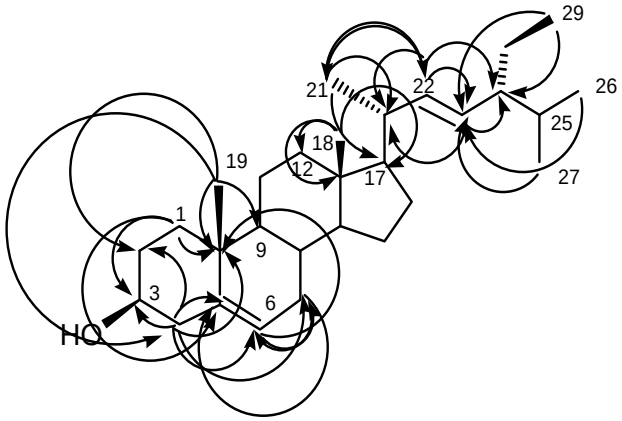


Table 3.16: HMBC data of compound **2**

Position	Connected carbon		
	^4J	^3J	^2J
H-1		C-3, C-5	C-10
H-4	C-7	C-2, C-6, C-10	C-3, C-5
H-6	C-10		C-7
H-7	C-9	C-5, C-8	C-6
H-18	C-20	C-12, C-13, C-17	
H-19	C-2	C-5, C-9, C-10	
H-21		C-17, C-22	C-20
H-22		C-21, C-24	C-20, C-23
H-23		C-20	C-24, C-22
H-26	C-23	C-24	
H-27	C-23	C-24	
H-29		C-24	C-23

Mass spectroscopy

FABMS (Figure 3.22**)** in glycerol matrix showed its molecular ion at m/z 413 $(M+H)^+$. GCMS (Figure 3.23**)** also revealed the molecular ion at m/z 412 $[M]^+$, 413 $[M+1]^+$ in support of the molecular formula $C_{29}H_{48}O$, along with other fragments as shown in table 3.17.

Figure 3.22: FABMS of compound **2** in glycerol matrix

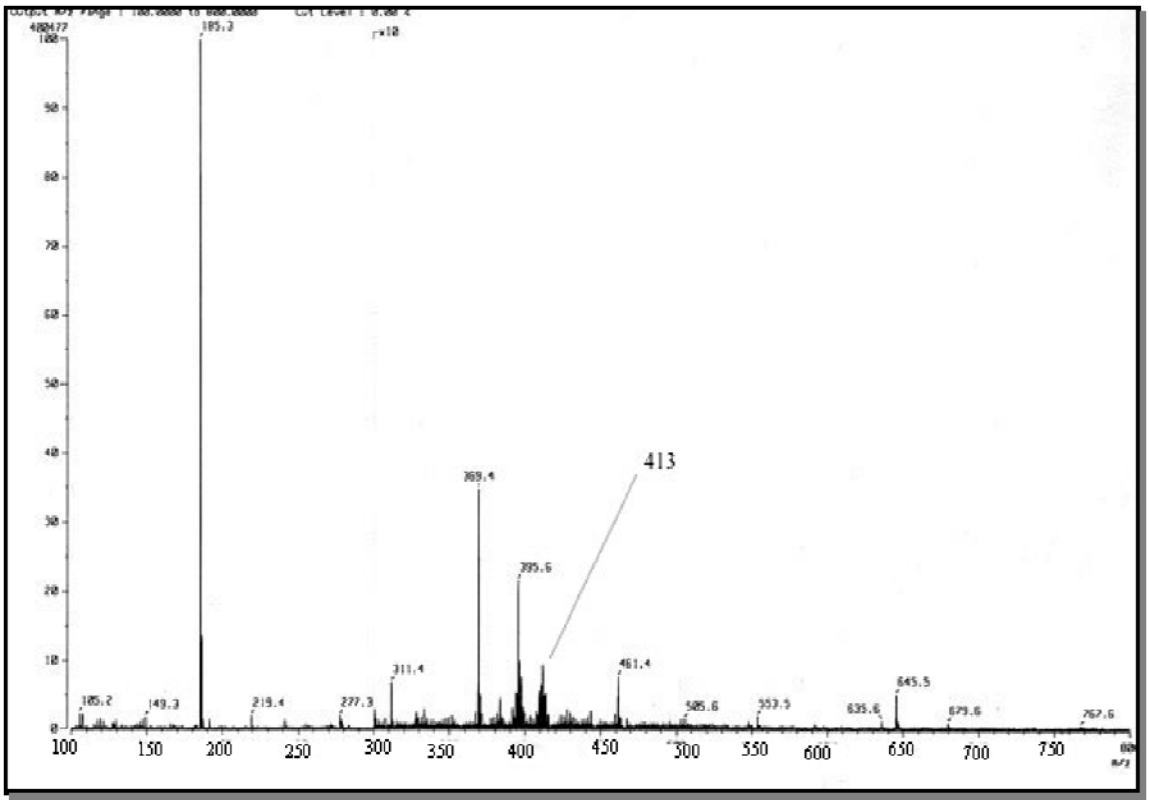
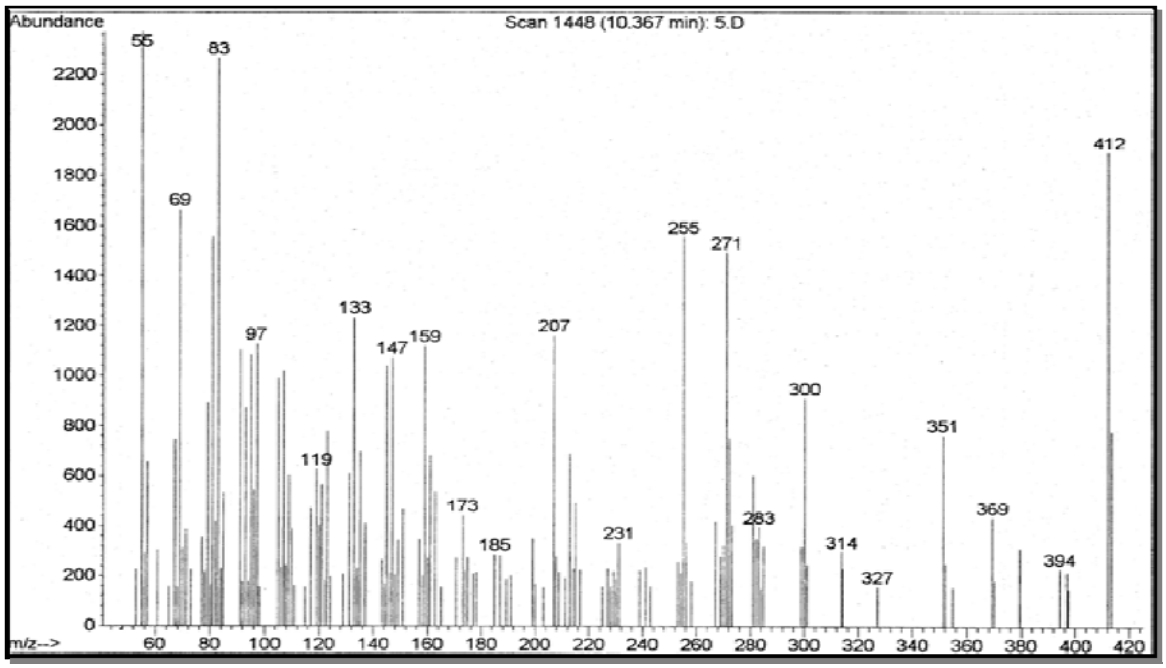


Figure 3.23: GCMS of compound **2**



A comparison of mass fragment patterns of compound **2** with reported fragments of poriferasterol[92] and stigmasterol[92] showed that **2** was in close agreement with poriferasterol due to lack of [M+2] fragment at m/z 414 (Table 3.17).

Table 3.17: Comparison of mass fragmentation patterns of isolated poriferasterol (**2**), reported poriferasterol[92] and stigmasterol[92]

Isolated poriferasterol (**2**) m/z	Reported poriferasterol[92] m/z	Reported stigmasterol[92] m/z
412 $[M]^+$	412 $[M]^+$	412 $[M]^+$
413 $[M+1]^+$	413 $[M+1]^+$	413 $[M+1]^+$
-	-	414 $[M+2]^+$
397 $[M-CH_3]$	397	397
394 $[M-H_2O]$	394	394
379 $[M-CH_3-H_2O]$	379	379
369 [M-isopropyl group]	369	369
351 [M-isopropyl group-H_2O]	351	351
300 [M-112]	300	300
271[M-141]	271	271
255[M-139-H_2O]	255	255

Melting point:

The melting point of compound **2** (154 °C) was in accordance with the literature data of poriferasterol (lit.m.pt.[93] 156 °C). This fact differentiated compound **2** from stigmasterol (lit. m. pt.[94] 165-167 °C) (Table 3.18).

Table 3.18: Comparison of melting point of compound **2** with reported data of poriferasterol and stigmasterol

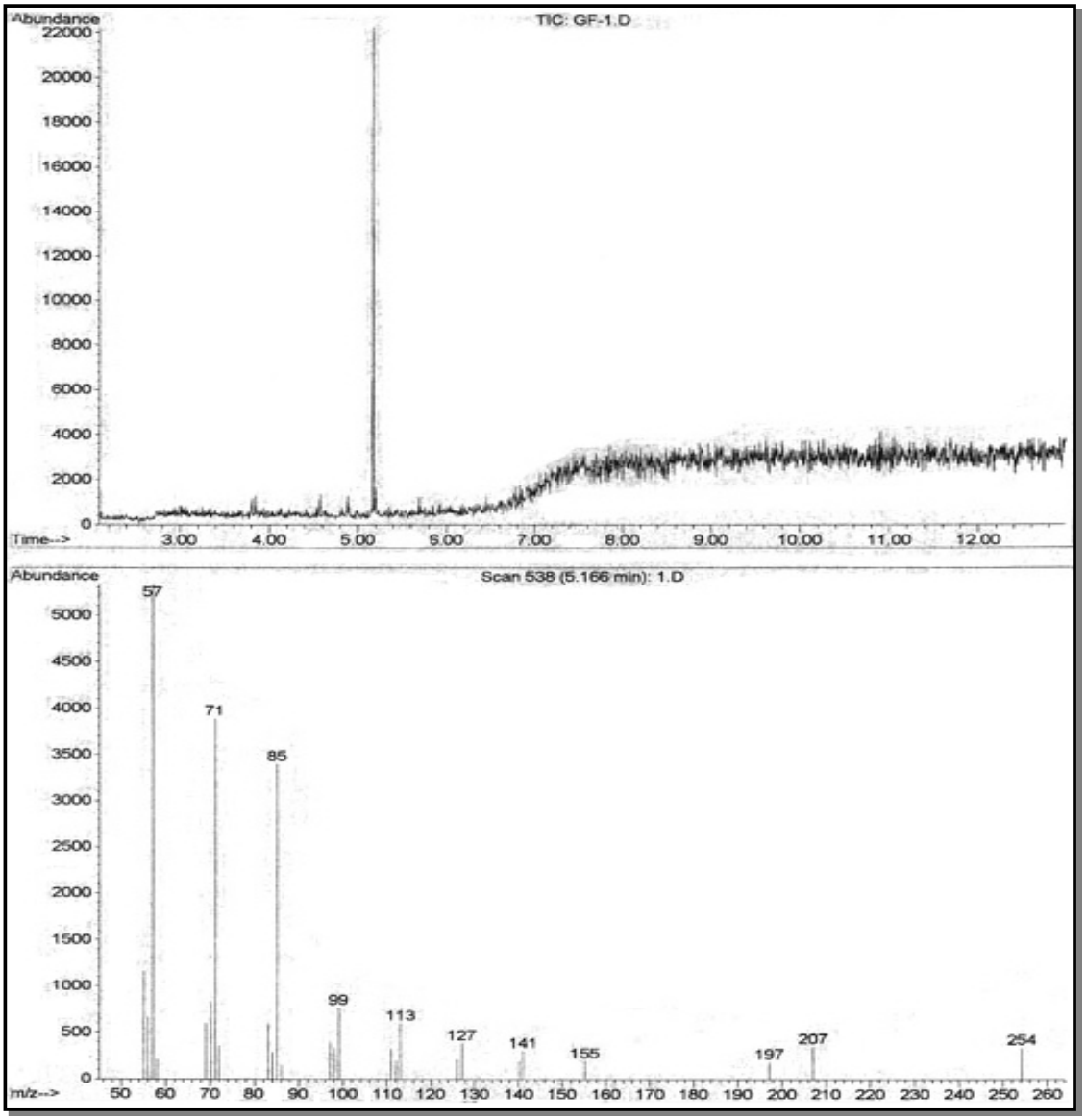
	Compound **2** (Isolated)	Poriferasterol[93]	Stigmasterol[94]
Melting point	154 °C	156 °C	165 -167 °C

3.4.3 Octadecane (3)

Mass spectroscopy

GCMS (Figure 3.24) revealed the molecular ion peak at m/z 254. The peaks at m/z 57 and 113 were due to loss of $C_4H_9^+$ and $C_8H_{17}^+$ giving the fragments of $C_{14}H_{29}^+$ and $C_{10}H_{21}^+$ with mass 197 and 141, respectively. The peak at m/z 127 was due to loss of $C_9H_{19}^+$. The loss of repeated 14 mass units indicated the compound to be long chain hydrocarbon, that is, octadecane, $C_{18}H_{38}$.

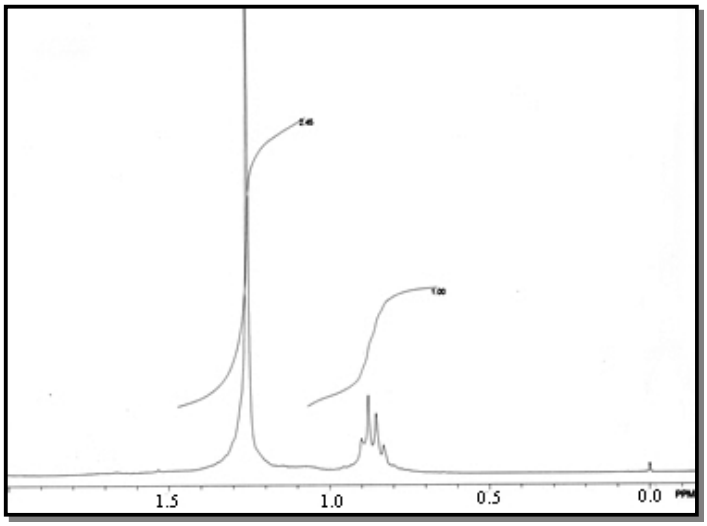
Figure 3.24: GCMS of compound **3**



^{1}H-NMR spectroscopy

A triplet at δ 0.80 - 0.90 was due to two methyl protons, H-1 and H-18. A multiplet at δ 1.20-1.30 was attributed to methylene protons, H-2 to H-17 (Figure 3.25).

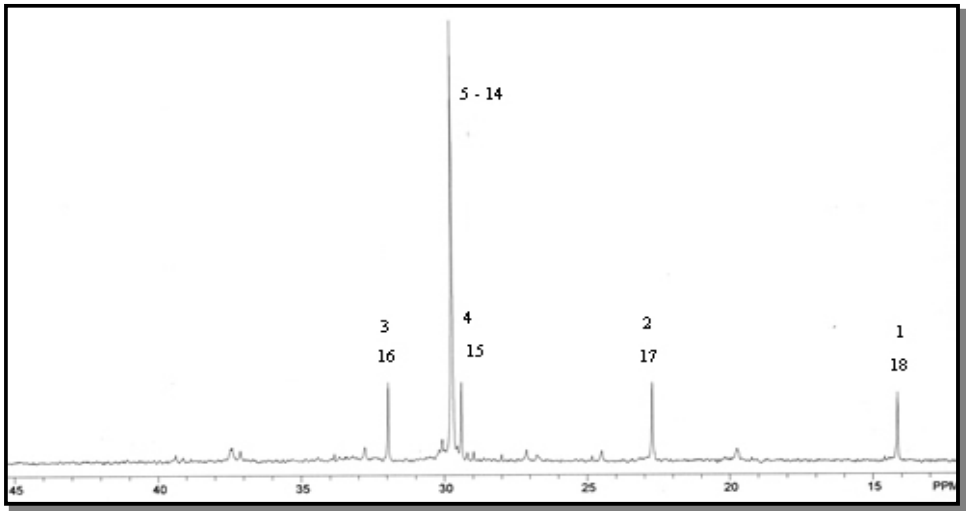
Figure 3.25: ^{1}H-NMR (100 MHz) spectrum of compound **3** in $CDCl_3$



^{13}C-NMR spectroscopy:

In ^{13}C-NMR spectrum (Figure 3.26**)** five distinct signals at δ 14.10, 22.70, 29.39, 29.70 and 31.95 were resonated. One of the signals at δ 29.70 was almost five times intense than others. Since the compound was saturated hydrocarbon containing eighteen carbons as revealed by mass spectrum, each small signal must be two carbon atoms and therefore five times intense signal corresponded ten carbon atoms. Hence, the ^{13}C-NMR conforms to octadecane (**3**).

Figure 3.26: ^{13}C-NMR (75 MHz) spectrum of compound **3** in $CDCl_3$



Signal at δ 14.10 was assigned to C-1 and C-18 methyl carbons. Signal at δ 22.70 was assigned to C-2 and C-17 methylene carbons. Signal at δ 31.95 was assigned to C-3 and C-16 methylene carbons. Similarly signal at δ 29.39 was due to C-4 and C-15 methylene carbons and the signal at δ 29.39 was due to C-5 to C-14 methylene carbons. These assignments (Table 3.19) of **3** were done by comparison of ^{13}C-NMR data obtained from CHEM ULTRA DRAW software.

Table 3.19: ^{13}C-NMR (75 MHz) data of compound **3**

Position of C atom	δ_C	Assignment	Position of C atom	δ_C	Assignment
1	14.10	CH_3	10	29.70	CH_2
2	22.70	CH_2	11	29.70	CH_2
3	31.95	CH_2	12	29.70	CH_2
4	29.39	CH_2	13	29.70	CH_2
5	29.70	CH_2	14	29.70	CH_2
6	29.70	CH_2	15	29.39	CH_2
7	29.70	CH_2	16	31.95	CH_2
8	29.70	CH_2	17	22.70	CH_2
9	29.70	CH_2	18	14.10	CH_3

Melting point

The melting point (23-25 °C) of compound **3** was in accordance with the literature data (lit.m.pt.[95] 28 °C).

3.4.4 Butyrospermol acetate (4)

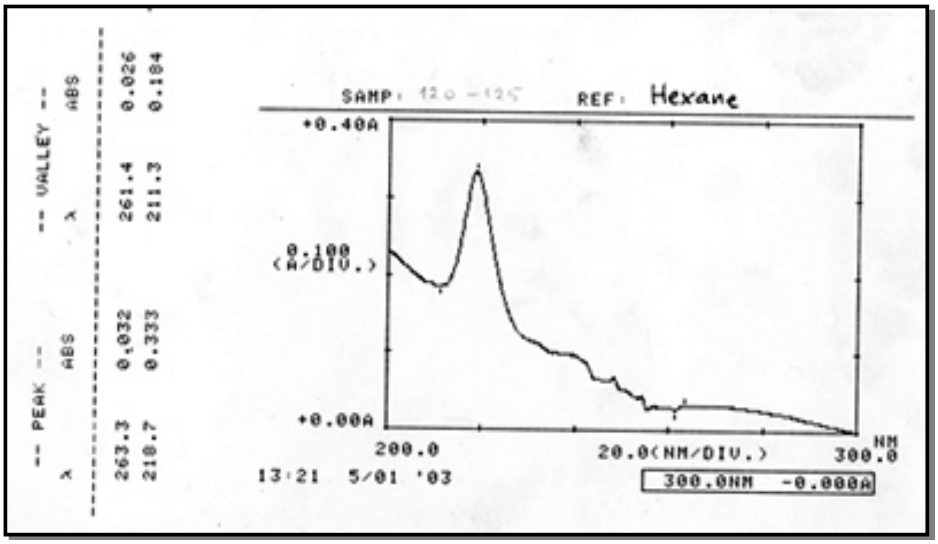
Chemical test:

It showed green color in Liebermann-Burchard test indicating that the compound had a steroid nucleus.

UV-vis spectroscopy:

UV spectrum (Figure 3.27) showed absorption peaks at 218 and 263 nm in hexane.

Figure 3.27: UV spectrum of compound **4** in hexane



^{13}C-NMR and Attached Proton Transfer (APT) spectroscopy

^{13}C-NMR spectrum of **4** showed the presence of thirty two carbon signals. Signals at δ 117.54 and 125.08 revealed olefinic secondary carbon atoms while δ 130.94 and 145.90 revealed olefinic quaternary carbon atoms which were confirmed from APT experiments. Based on information above, compound **4** revealed the presence of either, lanosta-9(11),24-diene or eupha-7,21-diene skeleton. However, a comparison of olefinic carbon signals of **4** with those of the butyrospermol[51] (the eupha-7,21-diene skeleton) (Figure 3.28a) and parkeyl acetate[96] (the lanosta-9(11),24-diene skeleton) (Figure 3.28b) revealed striking resemblance of **4** with butyrospermol (Table 3.20).

Figure 3.28: (a) Butyrospermol (the eupha-7,21-diene skeleton) and (b) Parkeyl acetate (the lanosta-9(11),24-diene skeleton)

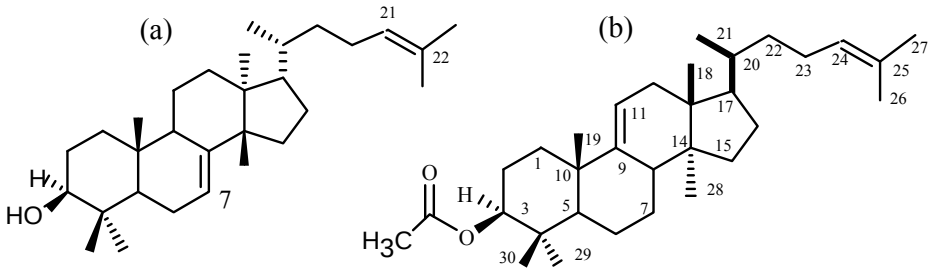


Table 3.20: Comparison of ^{13}C-NMR data of olefinic carbons of compound **4** with butyrospermol and parkeyl acetate

Reported Butyrospermol[51] (a)		Compound **4** (Isolated)	Δ (a-**4**)	Reported Parkeyl acetate[96] (b)		Compound **4** (Isolated)	Δ (b-**4**)
Position	δ_C	δ_C		Position	δ_C	δ_C	
C-7	117.78	117.54	0.24	C-11	115.2	117.54	2.3
C-21	125.12	125.08	0.04	C-24	125.2	125.08	0.2
C-22	130.92	130.94	0.02	C-25	130.9	130.94	0.0
C-8	145.96	145.90	0.06	C-9	148.1	145.90	2.2

Signals at δ 117.54, 125.08, 130.94 and 145.90 were, therefore, assigned to C-7, C-21, C-22 and C-8, respectively, assuming the eupha-7,21-diene skeleton. However, two signals at δ 171.10 and 21.34 indicated the presence of carbonyl and methyl carbons of acetoxy group (CH_3COO-), respectively (Figure 3.29). The remaining signals were in close agreement with those of butyrospermol[51] except for some shifts observed in the regions of C-2, C-3 and C-4, which are very likely due to the effect of the C-3 acetoxy group in compound (**4**) (Table 3.21). These assignments were further supported by APT experiment (Figure 3.30). Based on ^{13}C-NMR/APT data, compound **4** was confirmed as butyrospermol acetate.

Figure 3.29: ^{13}C-NMR (100 MHz) spectrum of compound **4** in $CDCl_3$

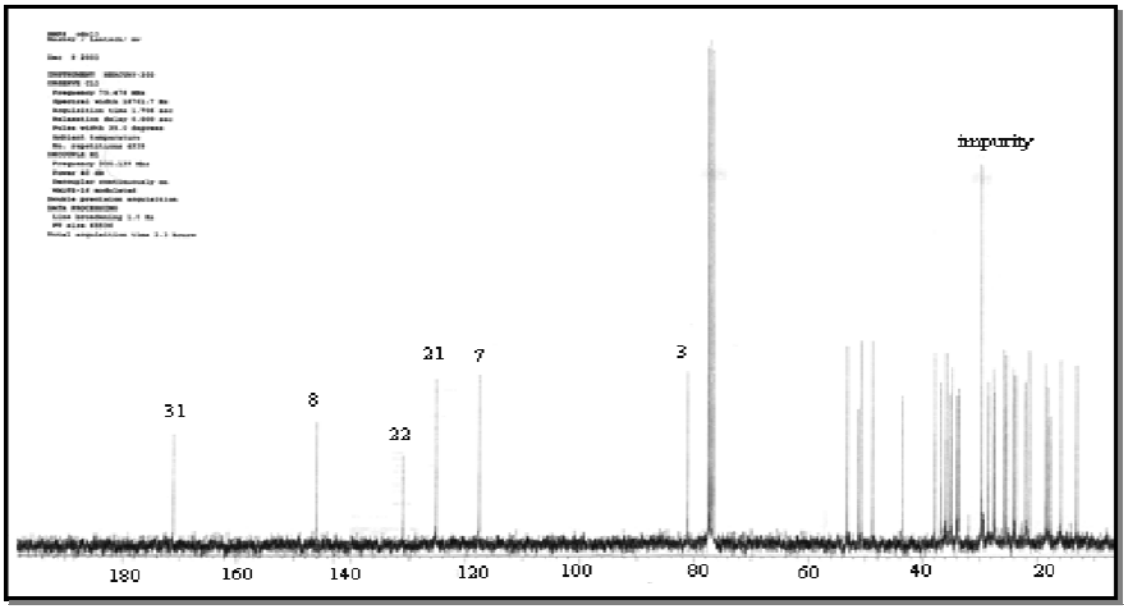
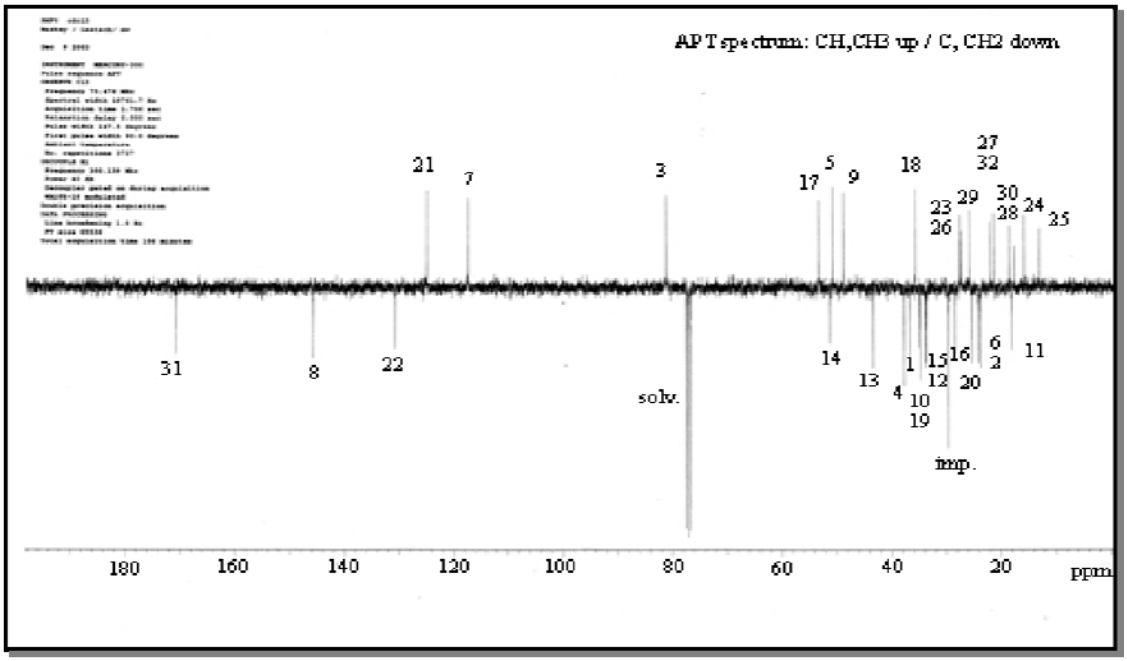


Table 3.21: Comparison of ^{13}C-NMR data (100 MHz) of butyrospermol acetate (**4**) with reported butyrospermol in $CDCl_3$

Carbon Position	APT	δ_C		Δ
		Butyrospermol acetate (**4**) Isolated	Butyrospermol[51] (Reported)	
1	CH_2	36.70	37.18	0.48
2	CH_2	**24.17**	**27.64**	**3.47**
3	CH	**81.11**	**79.25**	**1.86**
4	C	**37.80**	**38.95**	**1.15**
5	CH	50.70	50.62	0.08
6	CH_2	23.73	23.93	0.20
7	CH	117.54	117.78	0.24
8	C	145.90	145.88	0.02
9	CH	48.70	48.91	0.21
10	C	34.70	34.93	0.23
11	CH_2	18.10	18.13	0.03
12	CH_2	33.90	33.81	0.09
13	C	43.46	43.52	0.06
14	C	51.24	51.22	0.02
15	CH_2	33.70	33.94	0.24
16	CH_2	28.45	28.45	0.00
17	CH	53.22	53.22	0.00
18	CH	35.77	35.78	0.01
19	CH_2	35.11	35.15	0.04
20	CH_2	25.33	25.35	0.02
21	CH	125.08	125.12	0.04
22	C	130.94	130.92	0.02
23	CH_3	27.55	27.69	0.14
24	CH_3	15.85	14.71	1.14
25	CH_3	13.11	13.09	0.02
26	CH_3	27.30	27.30	0.00
27	CH_3	22.00	22.06	0.06
28	CH_3	18.56	18.58	0.02
29	CH_3	25.74	25.72	0.02
30	CH_3	17.66	17.66	0.00
31	CO	**171.01**	-	-
32	AcO	**21.34**	-	-

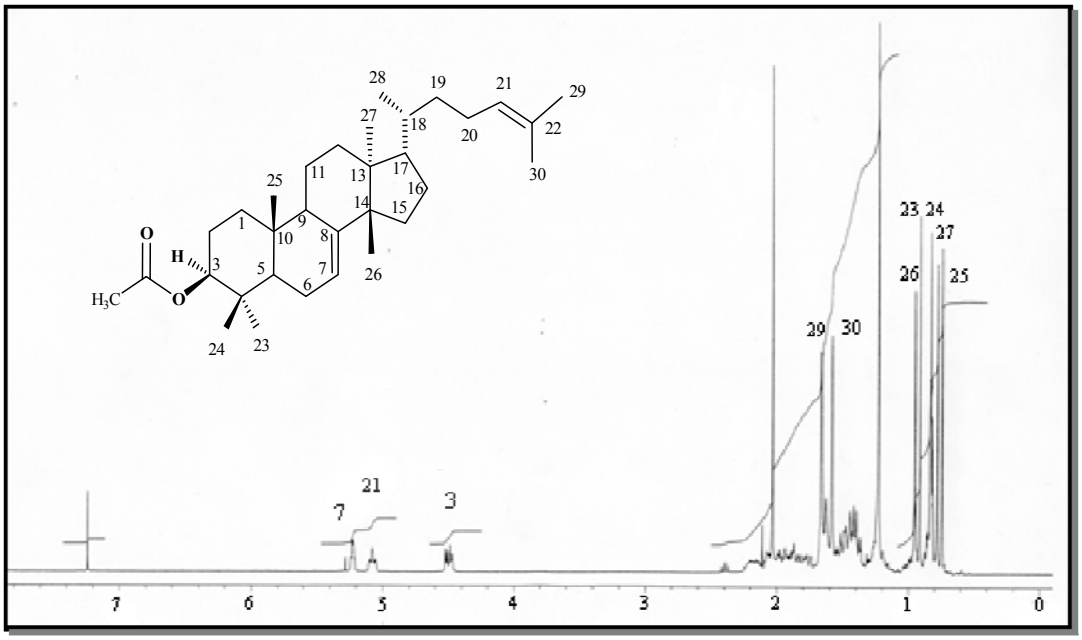
Figure 3.30: APT spectrum of compound **4**



^{1}H-NMR spectroscopy

The ^{1}H-NMR spectrum (Figure 3.31) revealed signals of nine methyl, one methine and two olefinic protons (Table.3.22). Eight methyl singlets at δ 0.74, 0.78, 0.85, 0.92, 0.96, 1.60, 1.63 and 2.03 were assigned to H-25, H-27, H-24, H-23, H-26, H-30, H-29 and methyl proton of acetoxy group (C<u>H</u>$_3$COO-), respectively.

Figure 3.31: ^{1}H-NMR (300 MHz) spectrum of compound **4** in $CDCl_3$

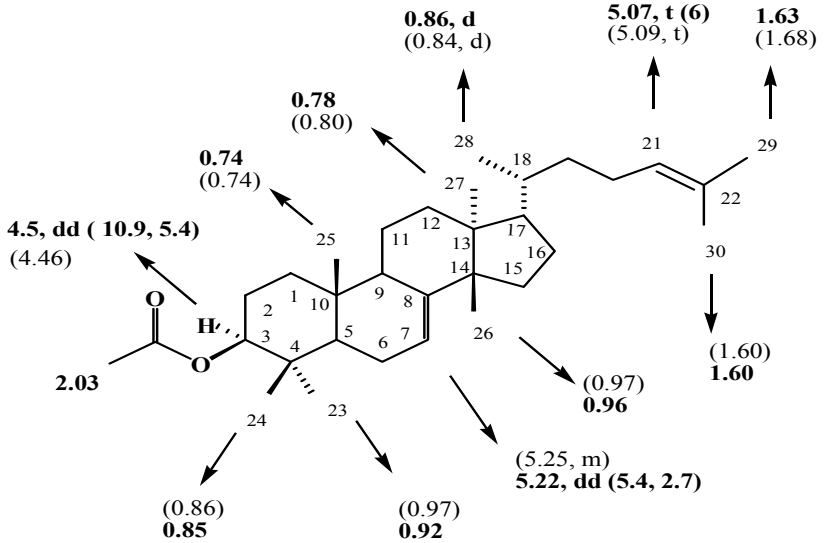


A doublet like signal at δ 0.86 was assigned to methyl protons, H-28. A double doublet at δ 4.50 with coupling constants 10.9 and 5.4 Hz indicating axial-equatorial and axial-axial interaction, respectively, was due to H-3 axial methine proton attached to C-3. A triplet at δ 5.07 with coupling constant of 6 Hz was due to olefinic proton H-21 and a double doublet at δ 5.22 was due to olefinic proton, H-7. These data were in accordance with those of butyrospermol[51] as shown in figure 3.32 except a signal for methyl protons of acetoxy group.

Table 3.22: ^{1}H-NMR (300 MHz) data of compound **4** in $CDCl_3$

δ_H of (**4**)	Multiplicity and coupling constant (*J*)	No. of protons integrated	Assignment	δ_H Butyrospermol[51] (b)	Δ (**4**-b)
0.74	s	3	H-25	0.74, s	0.00
0.78	s	3	H-27	0.78, s	0.00
0.85	s	3	H-24	0.86, s	0.01
0.86	d, *J* couldn't be calculated	3	H-28	0.84, d	0.02
0.92	s	3	H-23	0.97, s	0.05
0.96	s	3	H-26	0.97, s	0.01
1.60	s	3	H-30	1.60, s	0.00
1.65	s	3	H-29	1.68, s	0.03
2.03	s	3	Acetoxy	-	-
4.50	dd, *J* =10.9, 5.4	1	H-3	4.46, dd	0.04
5.07	t, 6.0	1	H-21	5.09, t	0.02
5.22	dd, 5.4, 2.7	1	H-7	5.25, m	0.00

Figure 3.32: Comparison of ^{1}H-NMR data of compound **4** with butyrospermol

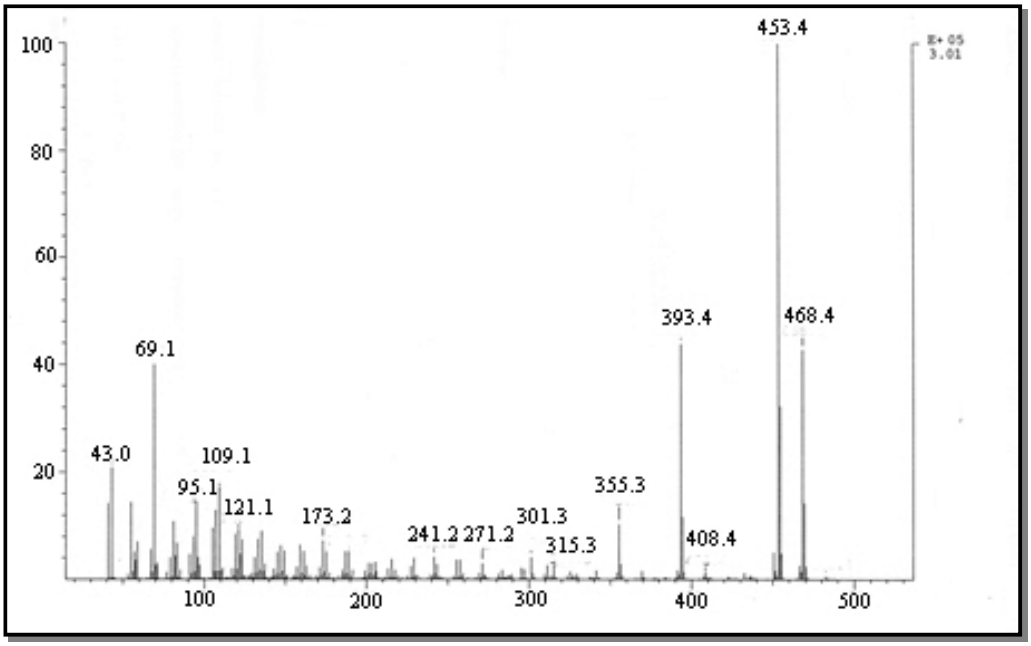


[Note: Bold values are given for isolated compound **4** and values within the parenthesis are for literature data[51] of butyrospermol]

Mass spectroscopy

The EIMS (Figure 3.33) revealed the molecular ion at m/z 468, in favor of molecular formula $C_{32}H_{52}O_2$. The base peak at m/z 453 was due to $[M\text{-}CH_3]^+$. The peak at m/z 408 was due to $[M\text{-}CH_3COO\text{-}]^+$. The prominent peak at m/z 355 was due to the elimination of the side chain and two hydrogen radicals from the molecular ion, that is, $[M\text{-side chain-}2H]^+$. In addition, other fragments appeared at 393 $[M\text{-}CH_3\text{-}CH_3COO]^+$, 355, 341, 315, 301, 271, 255, 241, 87, 121, 109, 95, 69, 55 and 43 $[CH_3CO]^+$ were in close agreement with literature data.[97]

Figure 3.33: EIMS of compound **4**



Melting point

Melting point of isolated butyrospermol acetate (**4**) showed 144 °C which was in accordance with that of reported butyrospermol acetate (lit. m.pt.[97] 143-145 °C). This melting point clearly differentiated **4** from parkeyl acetate (lit.m.pt.[96] 165 °C).

3.4.5 Bis(2-ethylhexyl)phthalate or 2-(diethylhexyl)phthalate (DEPH) (5)

UV-vis spectroscopy

UV spectra showed absorption peaks at 246.2, 273.4 nm in diethyl ether (Figure 3.34i) and peaks at 225.4, 254.2, 273.6 nm in hexane (Figure 3.34ii). Peak at 273.6 or 273.4 nm indicated the presence of the ester.[98]

Figure 3.34 (i): UV spectrum of compound **5** recorded in diethyl ether

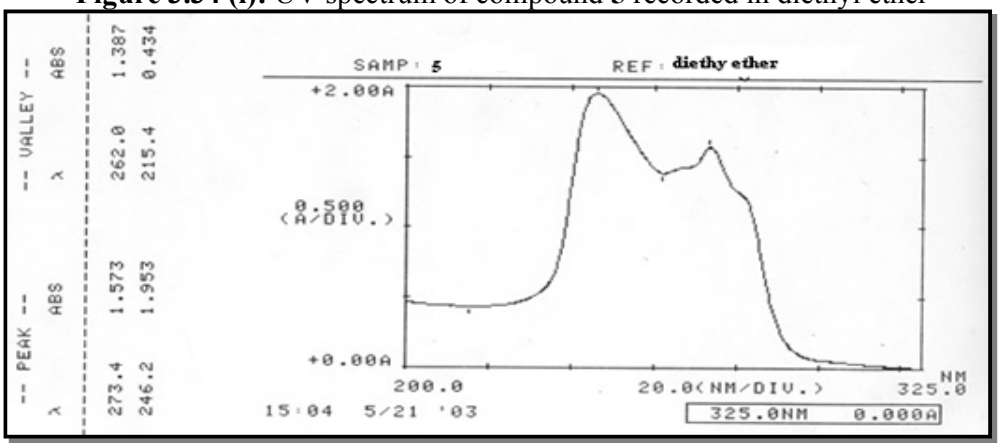
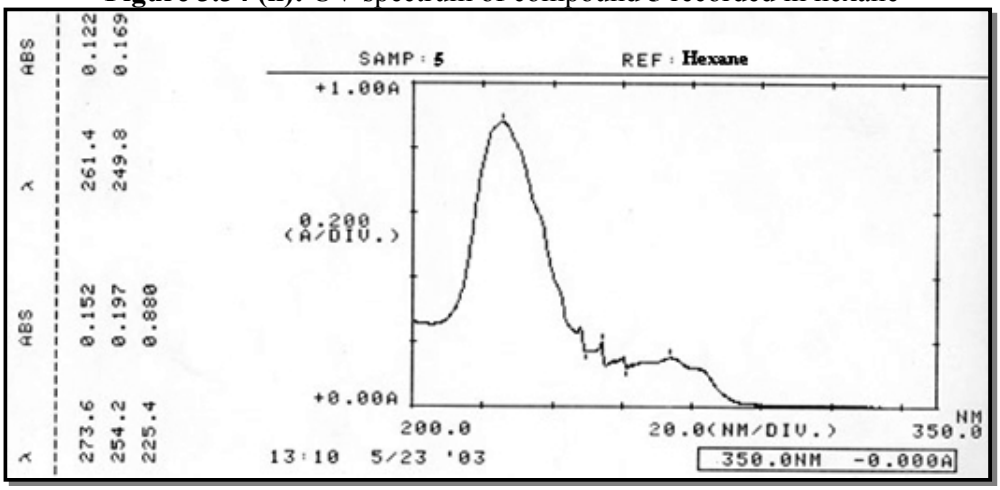


Figure 3.34 (ii): UV spectrum of compound **5** recorded in hexane



^{1}H-NMR spectroscopy

^{1}H-NMR (300 MHz) spectrum (Figure 3.35) showed two types of aromatic-ring protons, H-9 and H-10 at δ 7.68 and 7.51, respectively. The protons at H-9 and H-10 showed double doublets with coupling constants of 3.3 and 6.6 Hz for each, corresponding to *meta*-coupling and *ortho*-coupling, respectively. These double doublets of H-9 and H-10 were resolved better in 400 MHz ^{1}H-NMR spectrum (figure 3.36). Therefore, this aromatic ring must be *ortho*-disubstituted (Table 3.23).

Figure 3.35: ^{1}H-NMR (300 MHz) spectrum of compound **5**

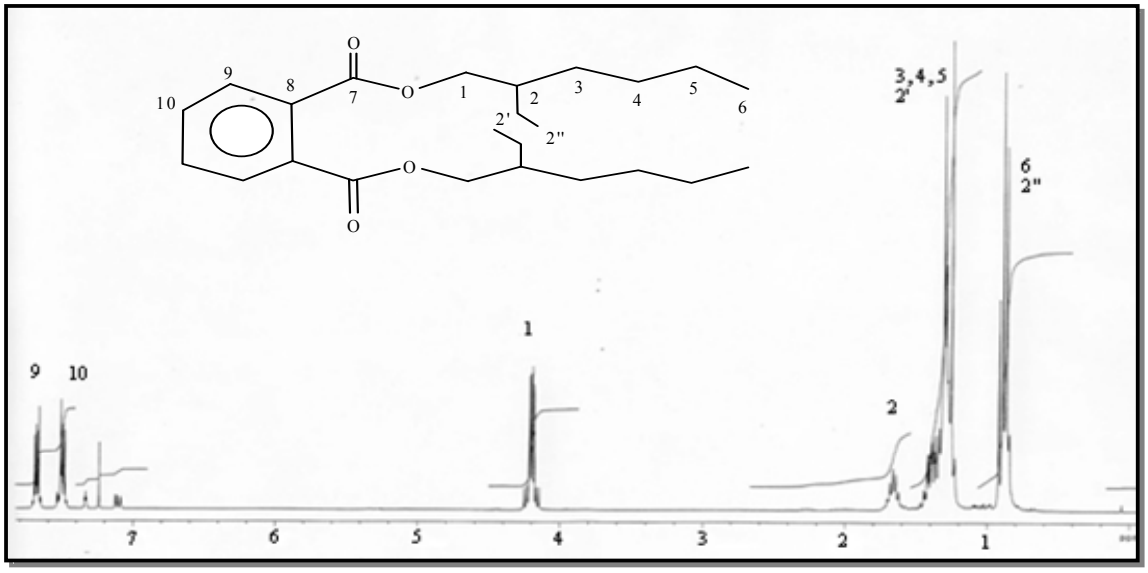
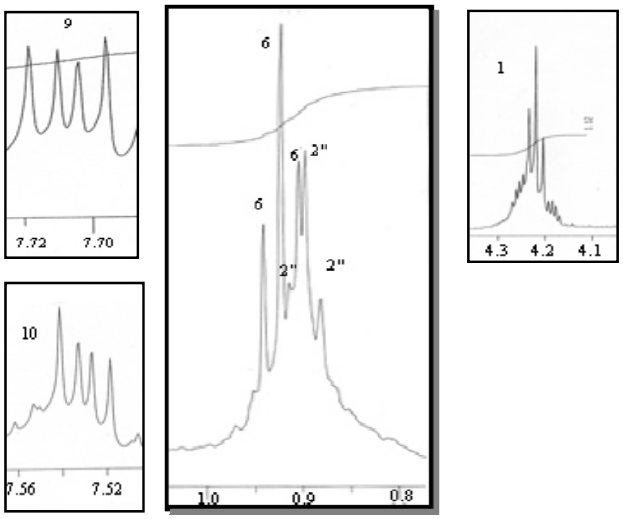


Figure 3.36: ^{1}H-NMR spectrum (400 MHz) of different protons of compound **5** in $CDCl_3$

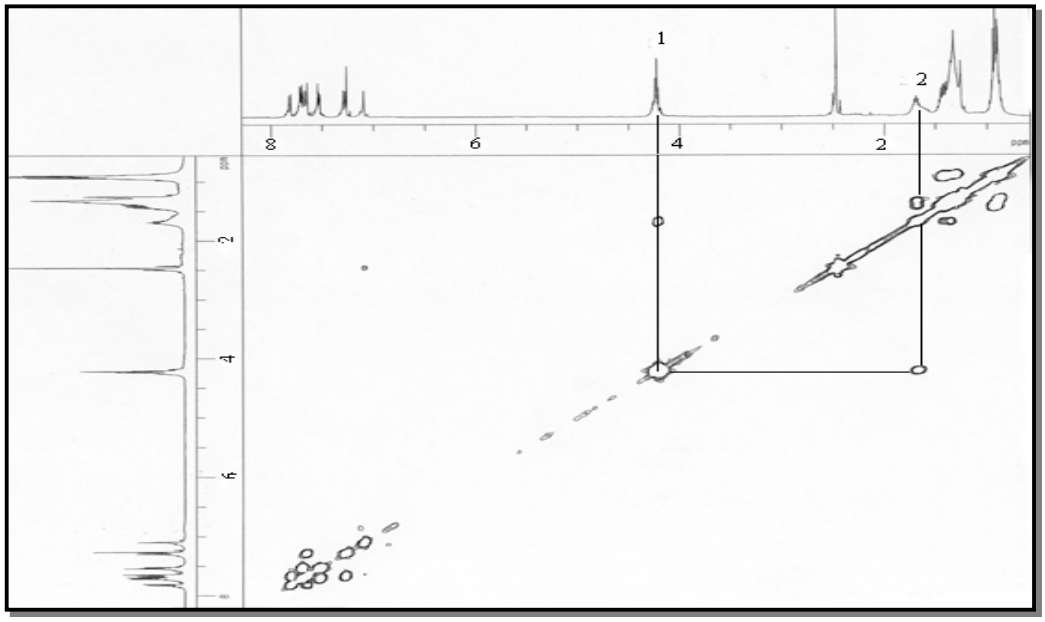


A double doublet at δ 4.20 in 300 MHz ^{1}H-NMR was appeared as a multiplet in 400 MHz ^{1}H-NMR. This was due to methylene protons H-1 attached with oxygen. A multiplet at δ 1.60-1.70 in 300 MHz ^{1}H-NMR was due to H-2 which was supported further by ^{1}H-^{1}H COSY spectrum (Figure 3.37) where connectivity between signals of H-1 and H-2 was observed. A triplet at δ 0.91 with a coupling constant of 6.6 Hz corresponding to six protons due to two methyl groups at C-6 and C-2″ of the type -CH_2-$C\underline{H}_3$ resolved into two sets of triplets in 400 MHz ^{1}H-NMR - one set was observed at δ 0.89 for H-2″ and 0.92 for H-6 with coupling constants of 6.8 and 7.2 Hz, respectively. This fact was further supported by HMQC (see Figure 3.41).

Table 3.23: ^{1}H-NMR (300 and 400 MHz) data of compound **5** in $CDCl_3$

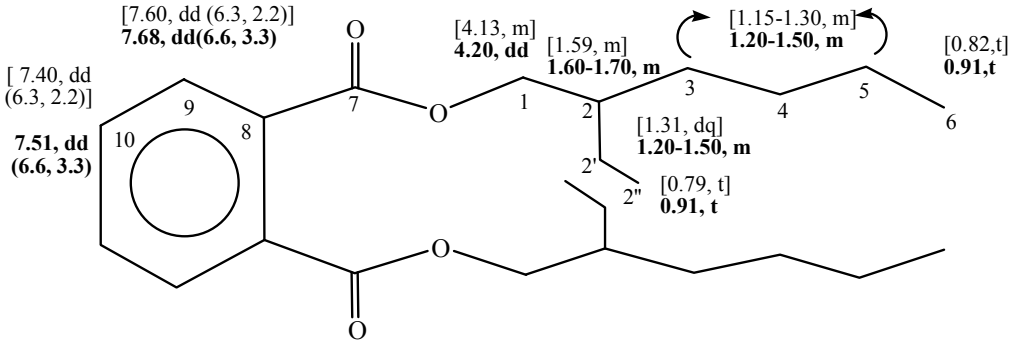
Position of H atom	δ_H (300 MHz)	Multiplicity and coupling constant (J in Hz)	δ_H (400 MHz)	Multiplicity and coupling constant (J)
1	4.20	dd, J = 3.3, 6.6	4.22	m
2	1.60-1.70	m	1.63-1.73	m
3	1.20 -1.50	m	1.20 -1.50	m
4	1.20 -1.50	m	1.20 -1.50	m
5	1.20 -1.50	m	1.20 -1.50	m
6	0.91	t, J = 6.6	0.92	t, J = 6.8
2′	1.20 -1.5	m	1.20 -1.50	m
2″	0.91	t, J = 6.6	0.89	t, J = 7.2
9	7.68	dd, J = 3.3, 6.6	7.71	dd, J = 6, 3.2
10	7.51	dd, J = 3.3, 6.6	7.53	dd, J = 6, 3.2

Figure 3.37: Connectivity between H-1 and H-2 for compound **5** in ^{1}H-^{1}H COSY spectrum of mixtures containing compounds **5** and **6**



Signals at δ 1.20-1.50 were due to combined effect of methylene protons H-3, H-4, H-5 and H-2′. These data were in close agreement with the literature data[99] as shown in figure 3.38.

Figure 3.38: Comparison of ^{1}H-NMR data of compound **5** with bis(2-ethylhexyl)phthalate

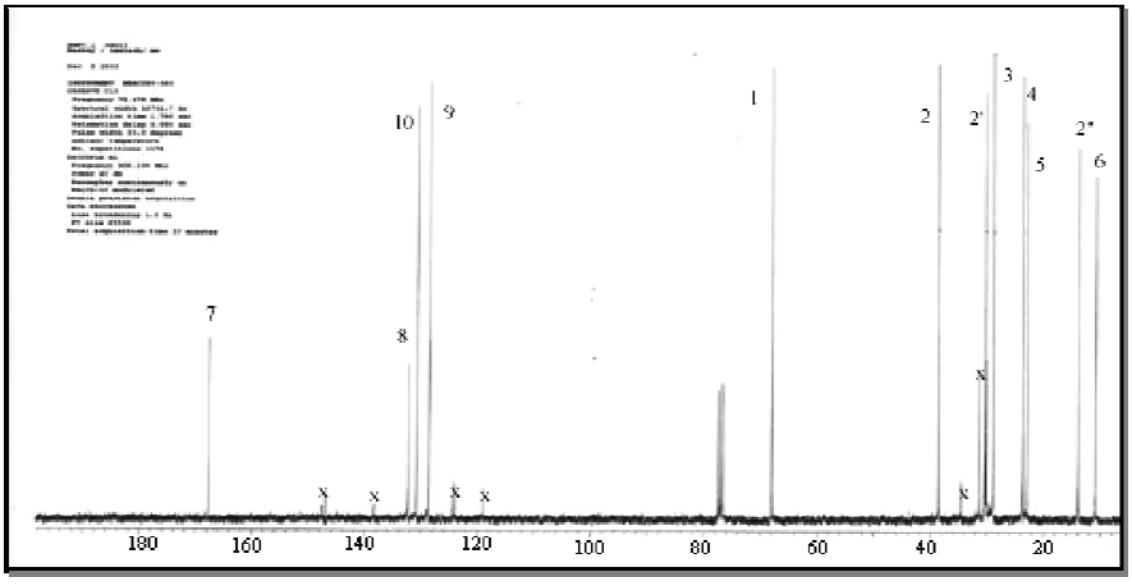


[Note: Bold values are given for compound **5** which was recorded in 300 MHz ^{1}H-NMR and values within the parenthesis are literature data[99]]

^{13}C-NMR spectroscopy

^{13}C-NMR spectrum (Figure 3.39) showed twelve signals. Three aromatic carbon signals at δ 128.72, 130.82 and 132.37 indicated the presence of two substituents at *ortho*-position, confirming the symmetry of the molecule.

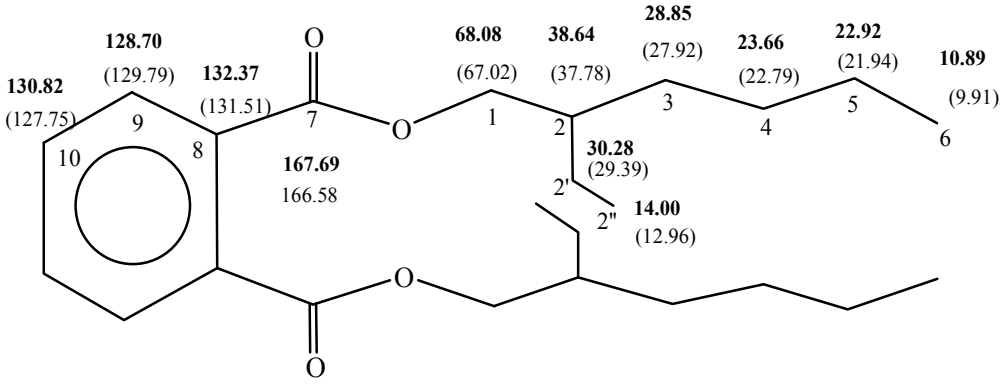
Figure 3.39: ^{13}C-NMR (100 MHz) spectrum of compound **5** in $CDCl_3$



Less intense signal at δ 132.37 was due to two equivalent tertiary aromatic C-8 carbons. Intense signal at δ 130.82 represented two equivalent aromatic C-10 carbons. Similarly, another intense signal at δ 128.70 indicated two equivalent aromatic C-9 carbons. Carbon signal at δ 167.69 corresponded to two equivalent carbonyl C-7 carbons of ester groups.

Carbon signal at δ 10.89 was due to two equivalent methyl groups, C-6. Similarly, signal at δ 14.00 corresponded to two equivalent methyl carbons, C-2″. Signal at δ 22.92 revealed two equivalent C-5 carbons. Signals at δ 23.66, 28.85, 38.64 and 68.04 were due to two equivalent C-4 carbons, two equivalent C-3 carbons, two equivalent C-2 carbons and two equivalent C-1 carbons, respectively. Carbon assignment was confirmed by comparison with literature data[99] (figure 3.40) and HMQC spectrum (figure 3.41).

Figure 3.40: Comparison of ^{13}C-NMR data of compound **5** with bis(2-ethylhexyl)phthalate



[Note: Bold values are given for compound **5** and values within the parenthesis are literature data[99]]

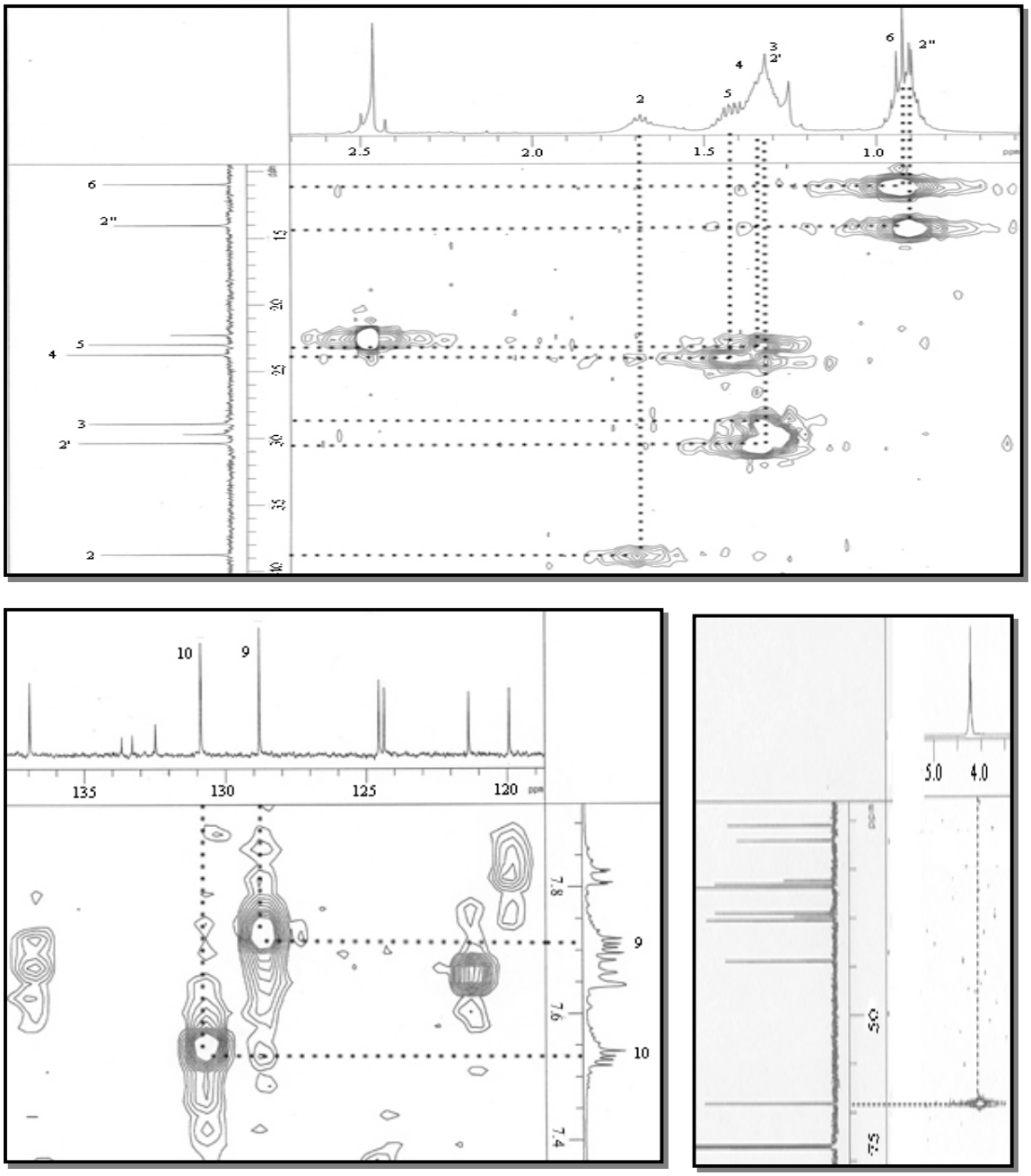
Heteronuclear Multiple Quantum Coherence (HMQC) spectroscopy

Observed connectivities in HMQC spectrum (figure 3.41) were shown in table 3.24. In this spectrum, proton H-1 at δ 4.22 showed connectivity with carbon C-3 (δ 68.04); proton H-2 at δ 1.63-1.73 with C-2 (δ 38.64); proton H-3, H-4, H-5 and H-2′ at δ 1.20-1.50 each with carbons C-3 (δ 28.85), C-4 (δ 23.66) and C-5 (δ 22.92), respectively. In addition, it revealed distinct connectivities between methyl proton H-6 at δ 0.92 and carbon C-2 (δ 10.89), methyl proton H-2″ at δ 0.89 and C-2″ (δ 14.00), aromatic proton H-10 at δ 7.53 and C-10 (δ 130.82), aromatic proton H-9 at δ 7.71 and C-9 (128.70).

Table 3.24: HMQC data of compound **5**

C atom	δ_C	Assignment	δ_H of Connected H	C atom	δ_C	Assignment	δ_H of Connected H
1	68.04	CH_2	4.22	6	10.89	CH_3	0.92, t
2	38.64	CH	1.63-1.73, m	2′	30.28	CH_2	1.20 -1.50, m
3	28.85	CH_2	1.20-1.50, m	2″	14.00	CH_3	0.89, t
4	23.66	CH_2	1.20-1.50, m	9	128.70	CH	7.71, dd
5	22.92	CH_2	1.20-1.50, m	10	130.82	CH	7.53, dd

Figure 3.41: ^{1}H-^{13}C direct connectivities observed for compound **5** in HMQC spectrum of mixture of compounds **5** and **6**



Heteronuclear Multiple Bond Coherence (HMBC) Spectroscopy

The observed correlations between ^{1}H and ^{13}C in HMBC spectrum were shown in figure 3.42. These connectivities were two bonds ($^{2}J_{C\text{-}H}$) to five bonds ($^{5}J_{C\text{-}H}$) distances as shown in table 3.25.

Figure 3.42: Selected HMBC of compound **5**. Arrows point from carbon to proton.

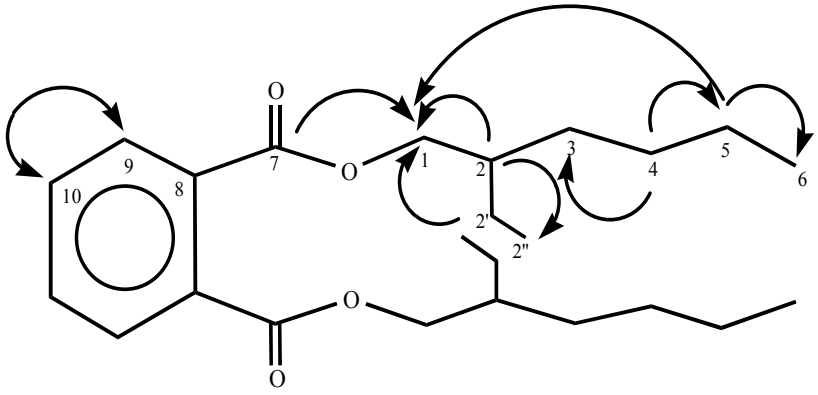


Table 3.25: HMBC data of compound **5**

Position of C atom	HMBC (Connected H atom)		
	$^{2}J_{C\text{-}H}$	$^{3}J_{C\text{-}H}$	$^{5}J_{C\text{-}H}$
2	H-1	H-2″	-
4	H-3, H-5		-
5	H-6	-	H-1
2′	-	H-1	-
7	-	H-1	-
9	H-10	-	-
10	H-9	-	-

Mass spectroscopy

The EIMS (Figure 3.43) showed a less intense molecular ion at m/z 390, corresponding to a molecular formula $C_{24}H_{38}O_4$ and other fragments 279, 167 and a base peak 149, 113. These values were in accordance with reported data.[99] Possible mass fragmentations are shown in figure 3.44.

Figure 3.43: EIMS of compound **5**

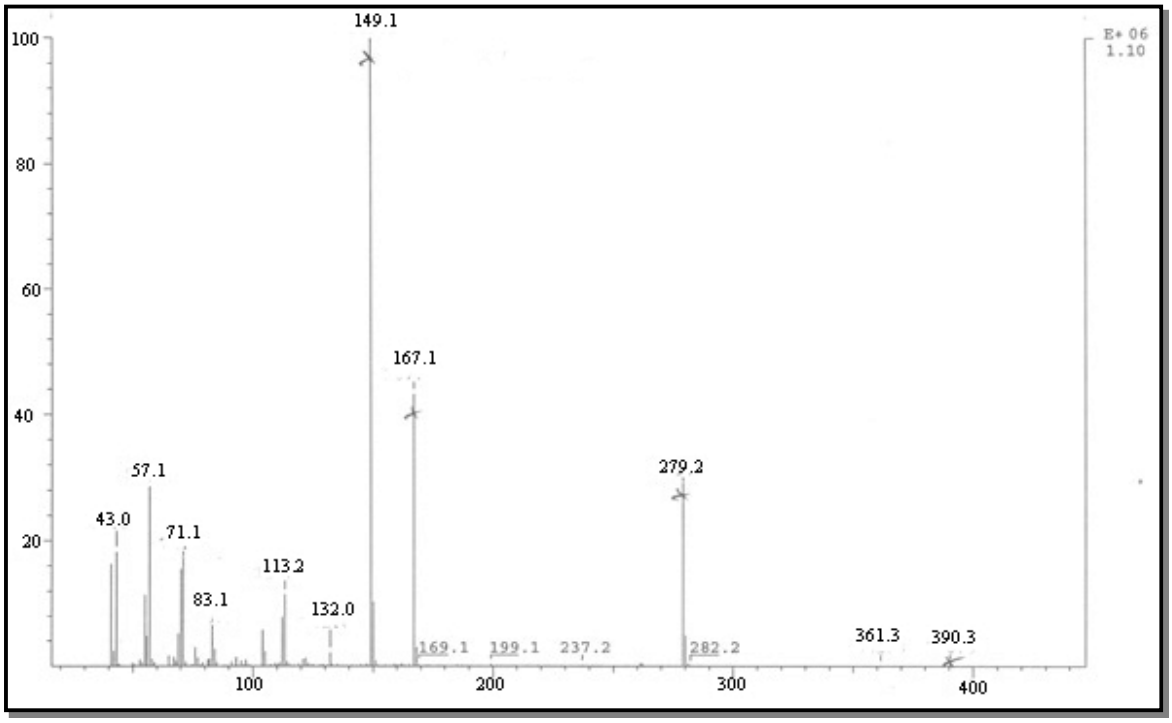
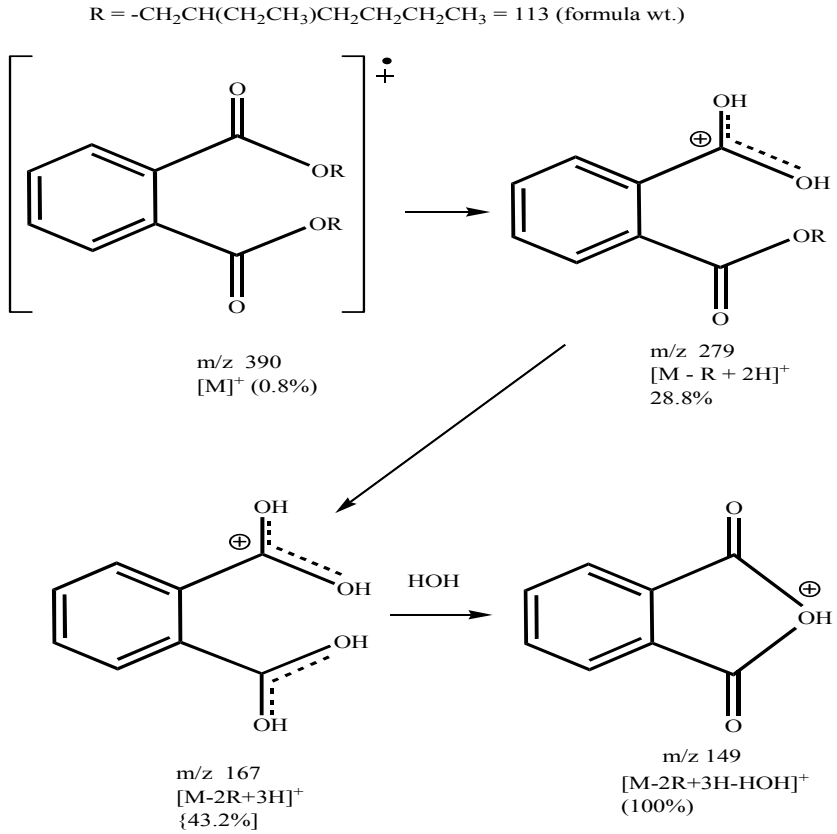


Figure 3.44: Mass fragmentation of compound **5**



GCMS [Figure 3.45(i)] also showed the characteristic peaks at m/z 279, 167, 149, 113, 83, 71 and 57 which were in close agreement with reported spectrum[100] [Figure 3.45 (ii)].

Figure 3.45 (i): GCMS of compound **5**

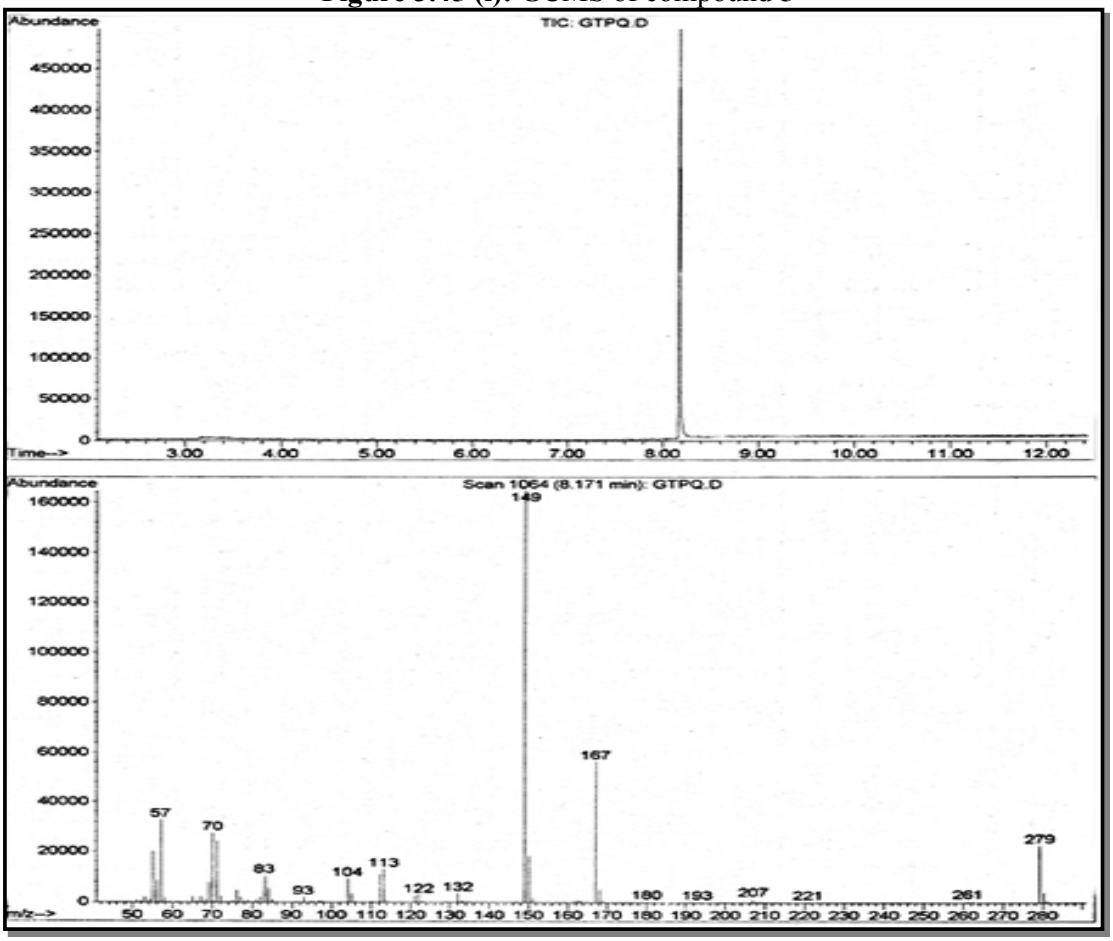
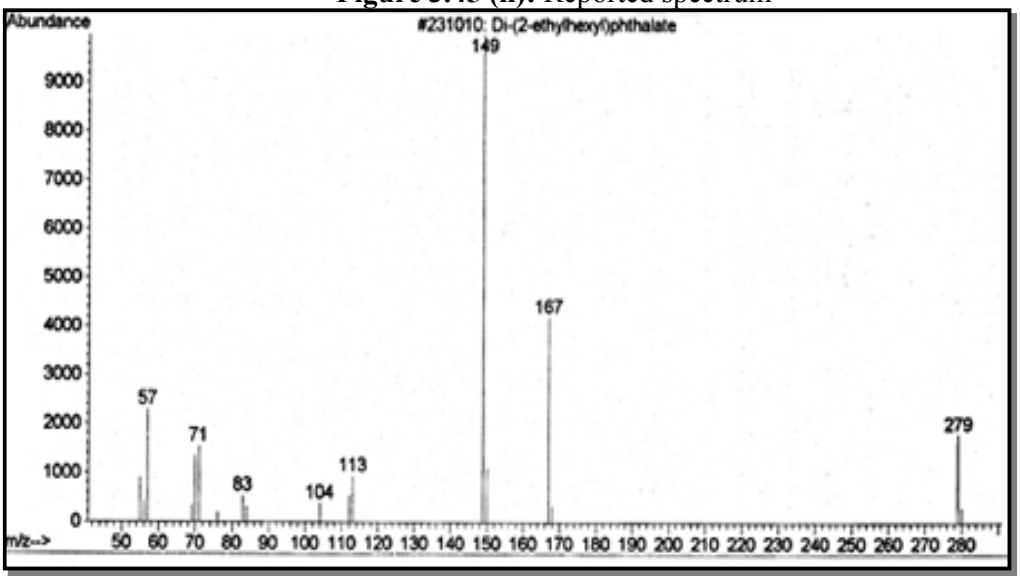


Figure 3.45 (ii): Reported spectrum[100]

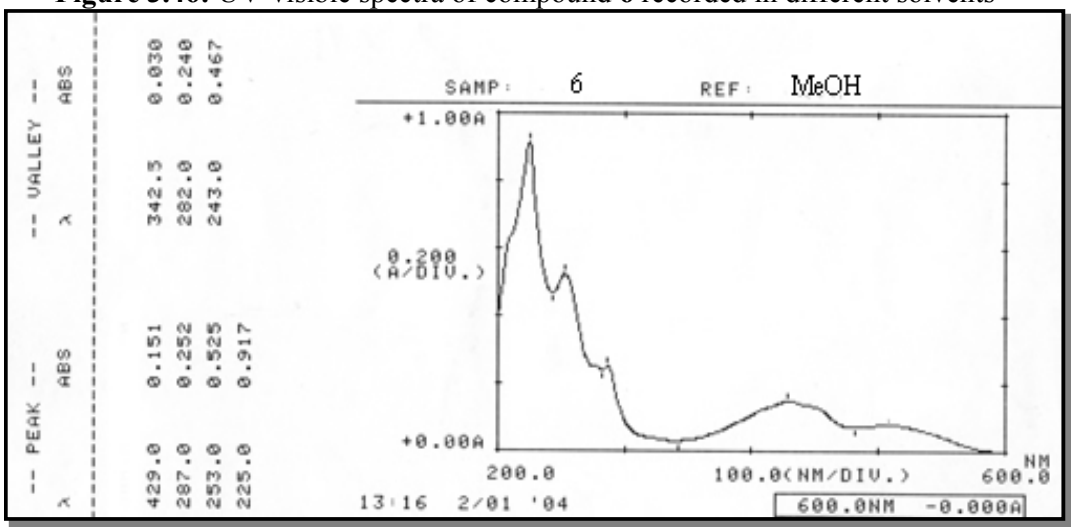


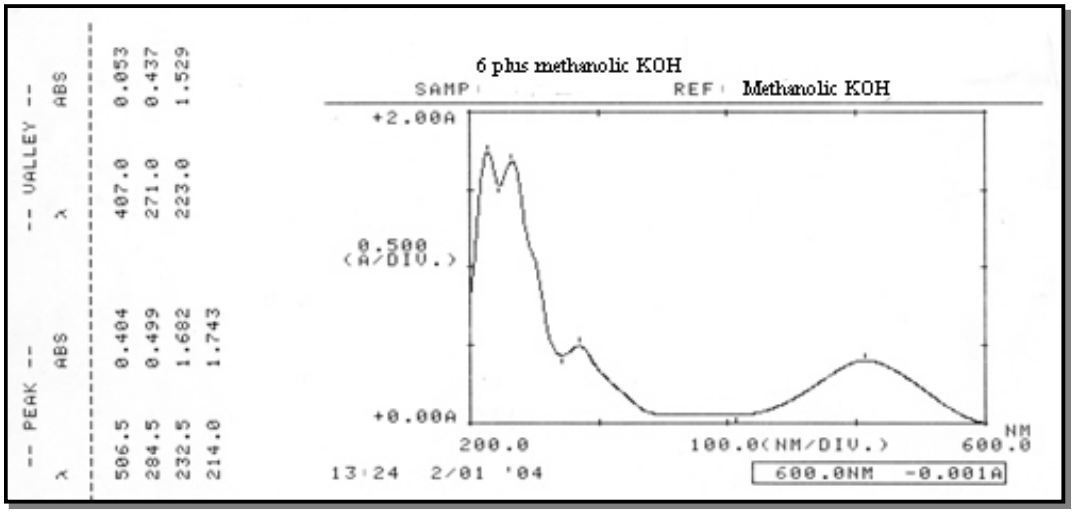
3.4.6 Chrysophanol (6)

UV-Vis spectroscopy

UV-visible spectra (Figure 3.46) recorded in different solvents are presented in table 3.26.

Figure 3.46: UV-visible spectra of compound **6** recorded in different solvents





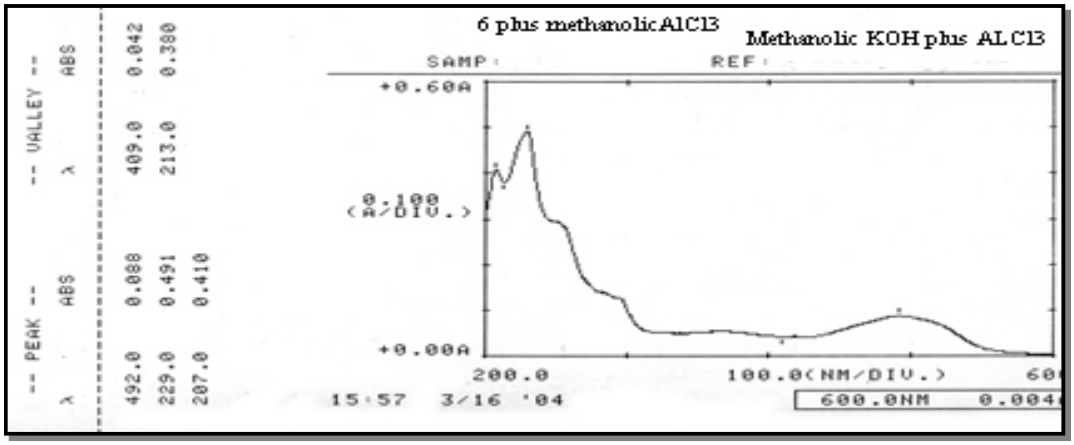


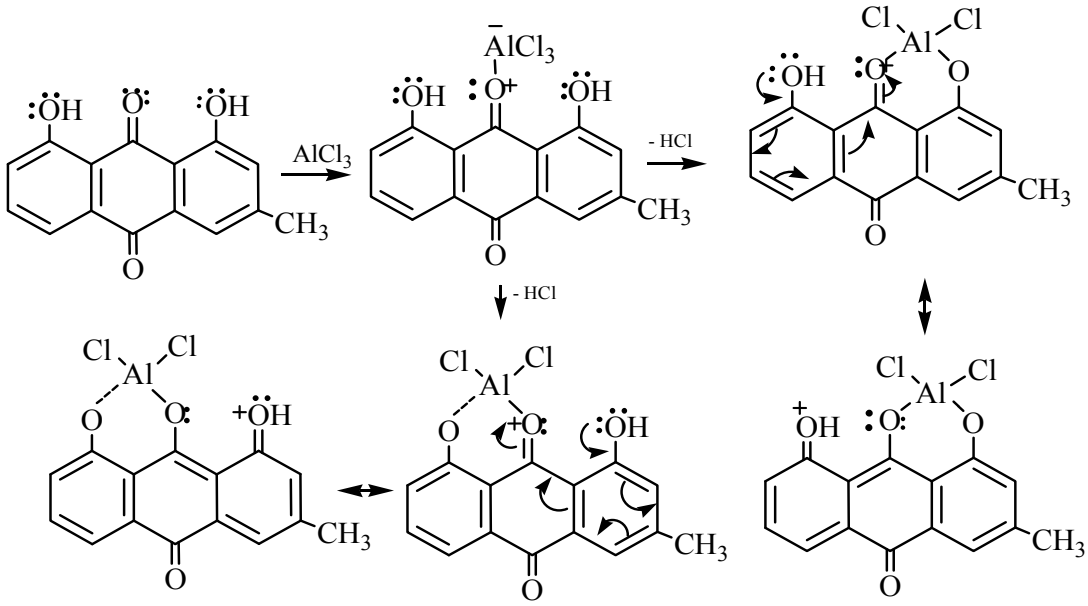
Table 3.26: UV and Visible spectral shifts for compound **6**

Solvent used	Spectral maxima (nm)					Spectral effect	Structural diagnosis
	Band I	Band II	Band III	Band IV	Band V		
Hexane (alone)	225.8	248.4	254.2	260.4	430.5	-	(i) 3-4 intense peaks between 215-300 nm and another one above or about 430 nm is characteristic for anthraquinone.[101] (ii) Peak at about 430 nm is indicative of the presence of at least two -OH in the α- position [102], that is, 1 and 8 position in chrysophanol **6**.
MeOH (alone)	225	253	287	429			(i) 3-4 intense peaks between 215-300 nm and another one above or about 430 nm is characteristic for anthraquinone.[101] (ii) Peak at about 430 nm is indicative of the presence of at least two -OH in the α-position[102], that is, 1 and 8 position in chrysophanol **6**.
MeOH and KOH (2 drops 10% methanolic KOH)	214	233.5	285.5	507		78 nm bathochromic shift (Band IV)	1-OH and 8-OH free
MeOH and $AlCl_3$ (2 drops 5% methanolic $AlCl_3$)	207	229	-	492		63 nm bathochromic shift (Band IV)	1-OH and 8-OH free

UV spectrum in methanol was in close resemblance with literature.[103] Peak at 429 nm in methanol or 430 nm in hexane indicated the presence of two hydroxyl groups at 1 and 8 positions exhibiting weak **n→π*** transition.

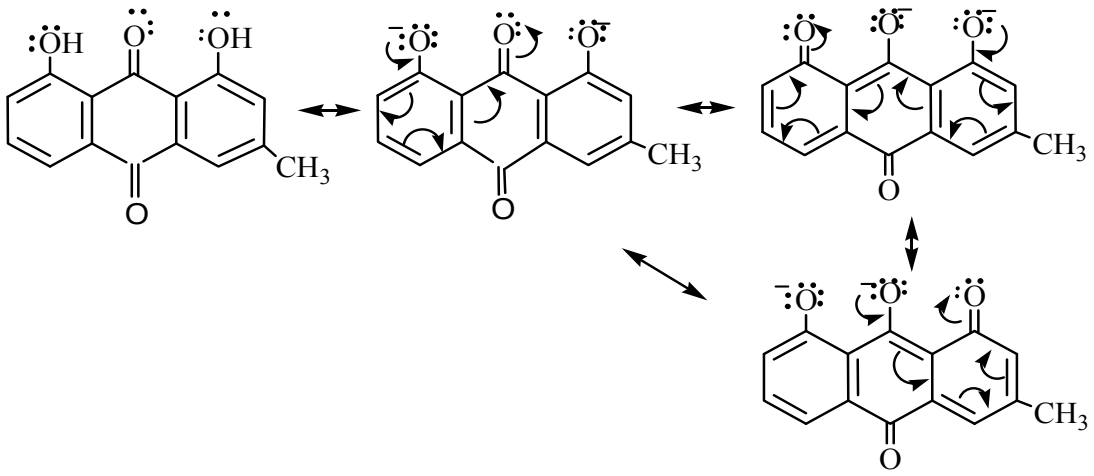
Chrysophanol showed bathochromic shift in alkali as well as in aluminum chloride as revealed from table 3.31. The possible mechanistic pathway of complex formation between chrysophanol and aluminum chloride is illustrated in figure 3.47. Electronic transition observed in such complex due to resonance is not significant to change the color of the solution. Therefore, yellow solution of compound did not change into new color even after addition of $AlCl_3$.

Figure 3.47: Complexation of chrysophanol with $AlCl_3$



The anion formed by chrysophanol in presence of alkali, can attribute bathochromic effect due to extra pair of non-bonding electrons in the anion which interacted with π electron system of the ring as shown in figure 3.48. Consequently, yellow solution was immediately changed into pink.

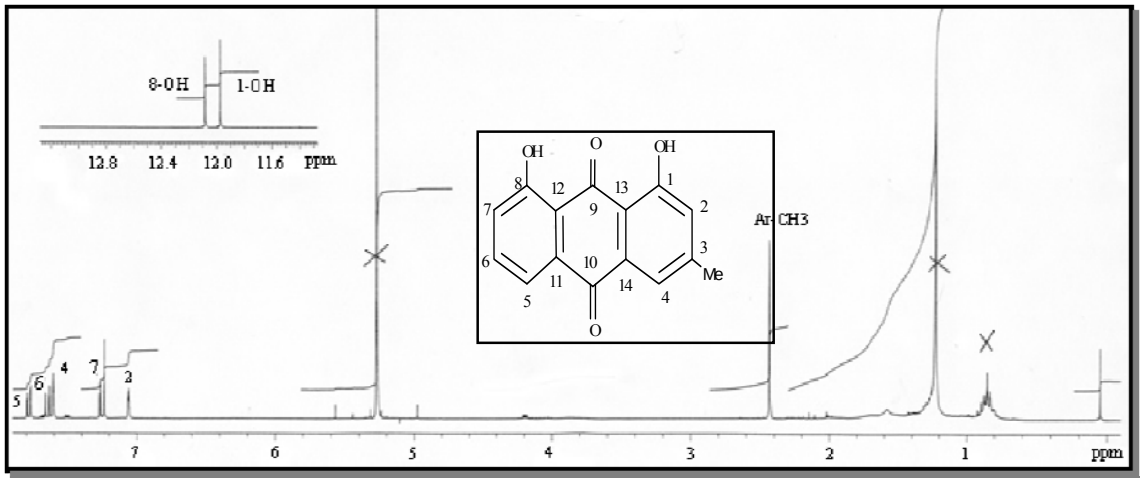
Figure 3.48: Resonances of chrysophanol in presence of alkali



^{1}H-NMR spectroscopy

The ^{1}H-NMR (300 MHz) spectrum (Figure 3.49) displayed signals corresponding to a singlet methyl proton, two singlets of aromatic protons, three sets of double doublet for aromatic protons and two phenolic hydroxyl groups indicating the compound as chrysophanol.

Figure 3.49: ^{1}H-NMR spectrum (300 MHz) spectrum of compound **6** in $CDCl_3$



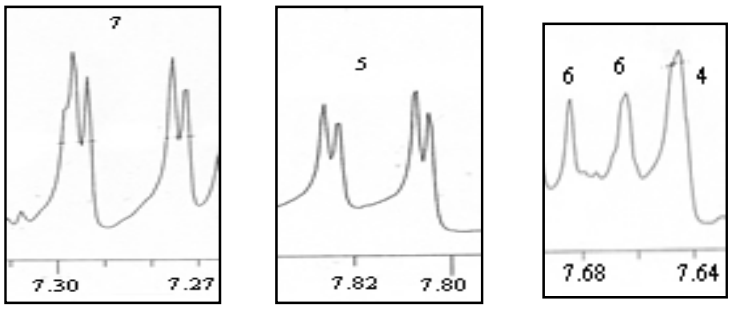
Two singlets at δ 7.06 and 7.60 were assigned to aromatic H-2 and H-4 protons, respectively. A doublet at δ 7.25 with *ortho*-coupling (J = 6.6 Hz) was assigned to H-7 whose one of the wings overlapped with signal of $CDCl_3$. A singlet at δ 2.43 was due to a methyl proton attached to aromatic C-3 carbon. Literature studies revealed that when methyl group is attached to C-2, the chemical shift of methyl proton occurred at slightly downfield at ca. δ 2.30.[104] Two singlets at δ 11.98 and 12.09 were assigned to two phenolic hydroxyl groups attached to C-1 and C-8 carbons, respectively. The *ortho*-coupled H-5 and H-6

protons were observed as two doublets at δ 7.79 and 7.63, respectively, with coupling constants of 6.6 Hz (Table 3.27). However in 400 MHz ^{1}H-NMR, more resolved signals (Figure 3.50) were observed for H-5 and H-7 protons; consequently, a double doublet at δ 7.81 with coupling constants of 8.1 and 1.2 Hz was assigned to H-5 due to *ortho*-coupling with H-6 and *meta*-coupling with H-7, respectively. Similarly a double doublet at δ 7.28 with coupling constants of 8.1 and 1.2 Hz was assigned to H-7 due to ortho coupling with H-6 and *meta*-coupling with H-5, respectively (Table 3.27).

Table 3.27: ^{1}H-NMR (300 and 400 MHz) data of compound **6**

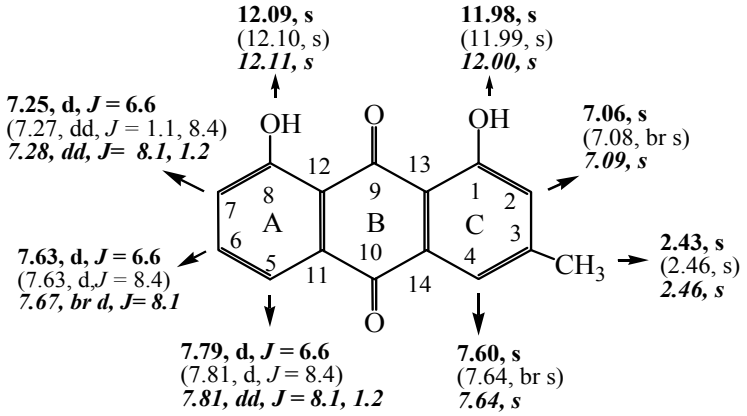
300 MHz		400 MHz		
δ_H	Multiplicity and coupling constant (*J*)	δ_H	Multiplicity and coupling constant (*J*)	Assignment
2.43	s	2.46	s	Ar-CH_3
7.06	s	7.09	s	ArH-2
7.25	d, *J* = 6.6	7.28	dd, *J* = 8.1, 1.2	ArH-7
7.60	s	7.64	s	ArH-4
7.63	d, *J* = 6.6	7.67	br d, *J* = 8.1	ArH -6
7.79	d, *J* =6.6	7.81	dd, *J* = 8.1, 1.2	ArH-5
11.98	s	12.00	s	1-OH
12.09	s	12.11	s	8-OH

Figure 3.50: Resolved signals of H-5, H-7 and H-6 of compound **6** in 400 MHz ^{1}H-NMR spectrum



^{1}H-NMR data of compound **6** was in close agreement with reported values[105] which is illustrated in figure 3.50. Proton assignment was also confirmed by HMQC spectrum (see table 3.29).

Figure 3.51: Comparison of ^{1}H-NMR data of compound **6** with chrysophanol



[Note: Upper bold and lower bold values are given for compound **6** in 300 and 400 MHz, respectively, while values within parenthesis are reported[105] values in 400 MHz.]

^{13}C-NMR spectroscopy

The ^{13}C-NMR (75 MHz) spectrum (Figure 3.52) displaced fourteen aromatic carbon signals at δ 113.67, 115.8, 119.88, 121.32, 124.32, 124.51, 133.20, 133.56, 136.92, 149.31, 162.35, 162.65, 181.92 and 192.46 were assigned to C-12, C-13, C-5, C-4, C-2, C-7, C-14, C-11, C-6, C-3, C-1, C-8, C-10 and C-9, respectively (Table 3.28). Signal at δ 22.25 was assigned to methyl carbon attached to aromatic C-3 carbon.

Figure 3.52: ^{13}C-NMR (75 MHz) spectrum of compound **6** in $CDCl_3$

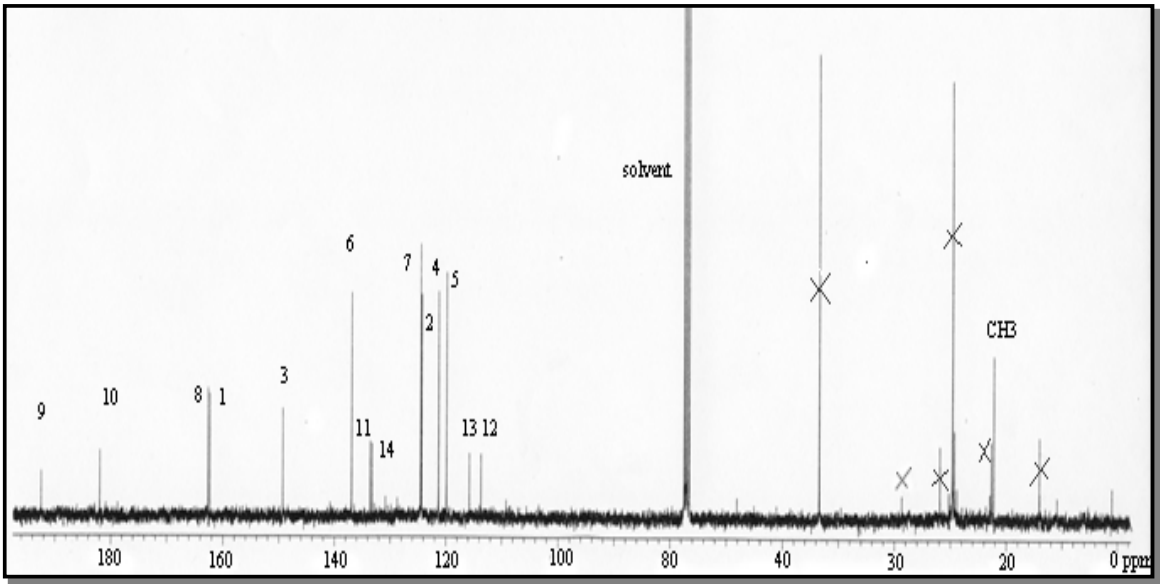


Table 3.28: ^{13}C-NMR (75 MHz) data of compound **6** and chrysophanol

Position of C atom	δ_C of Compound **6**	δ_C of chrysophanol (reported)[105]
C-9	192.46	192.5
C-10	181.92	181.9
C-8	162.65	162.7
C-1	162.35	162.4
C-3	149.31	149.3
C-6	136.92	136.9
C-11	133.56	Missing
C-14	133.20	133.3
C-7	**124.51**	**119.9**
C-2	124.32	Missing
C-4	121.32	121.4
C-5	**119.88**	**124.4**
C-13	115.80	115.9
C-12	113.67	113.8
Ar-CH_3	22.25	22.3

Carbon assignment was done by comparison with literature data[105] and HMQC spectrum. However; HMQC spectrum (see Figure 3.54) revealed that carbon signal at δ 119.88 showed discernible connectivity with H-5 (δ 7.79), carbon signal at δ 124.51 with H-7 (δ 7.25), carbon signal at δ 124.32 with H-2 (δ 7.06), carbon signal at δ 121.32 with H-4 (δ 7.60) and carbon signal at δ 136.92 with H-6 (δ 7.63). Hence, based on HMQC data, the assignment given in the literatures[105, 106] for C-5 at δ 124.51 and C-7 at δ 119.88 had to be reversed; consequently assigning δ 124.51 to C-7 and δ 119.88 to C-5 in the compound **6** as shown in table 3.28. Remaining assignments were in close agreement with reported data.[105]

Literature studies revealed that typical shifts for C-9 and C-10 of anthraquinone (Figure 3.53a) generally occurred at δ 183. However, hydroxyl groups in the *α*-position causes a considerable shift in the C-9 or C-10 signal, ca. 5 ppm due to hydrogen bonding. In the case of hydrogen bonding to one OH group, signal of C-9 (or C-10) is shifted to δ 188 (Figure 3.53b). Similarly, in the case of hydrogen bonding to two OH groups, signal of C-9

(or C-10) is shifted to δ 192 (Figure 3.53c).[107] Hence, two downfield signals observed in compound **6** at δ 181.92 and 192.46 were assigned to C-10 and C-9, respectively.

Figure 3.53: Chemical shifting in ^{13}C-NMR due to effect of H-bonding in C-9 and C-10 position in anthraquinone

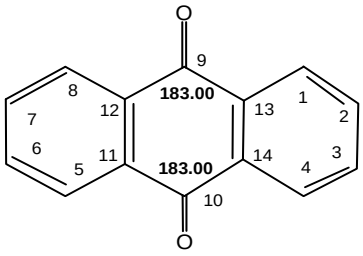


Fig. 3.53 (a)

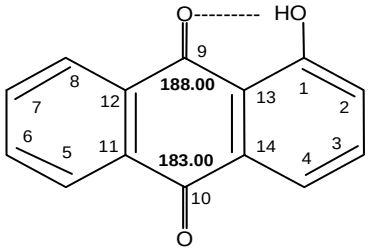


Fig. 3.53 (b)

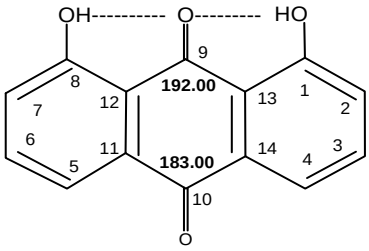


Fig.3.53 (c)

HMQC and HMBC spectroscopy

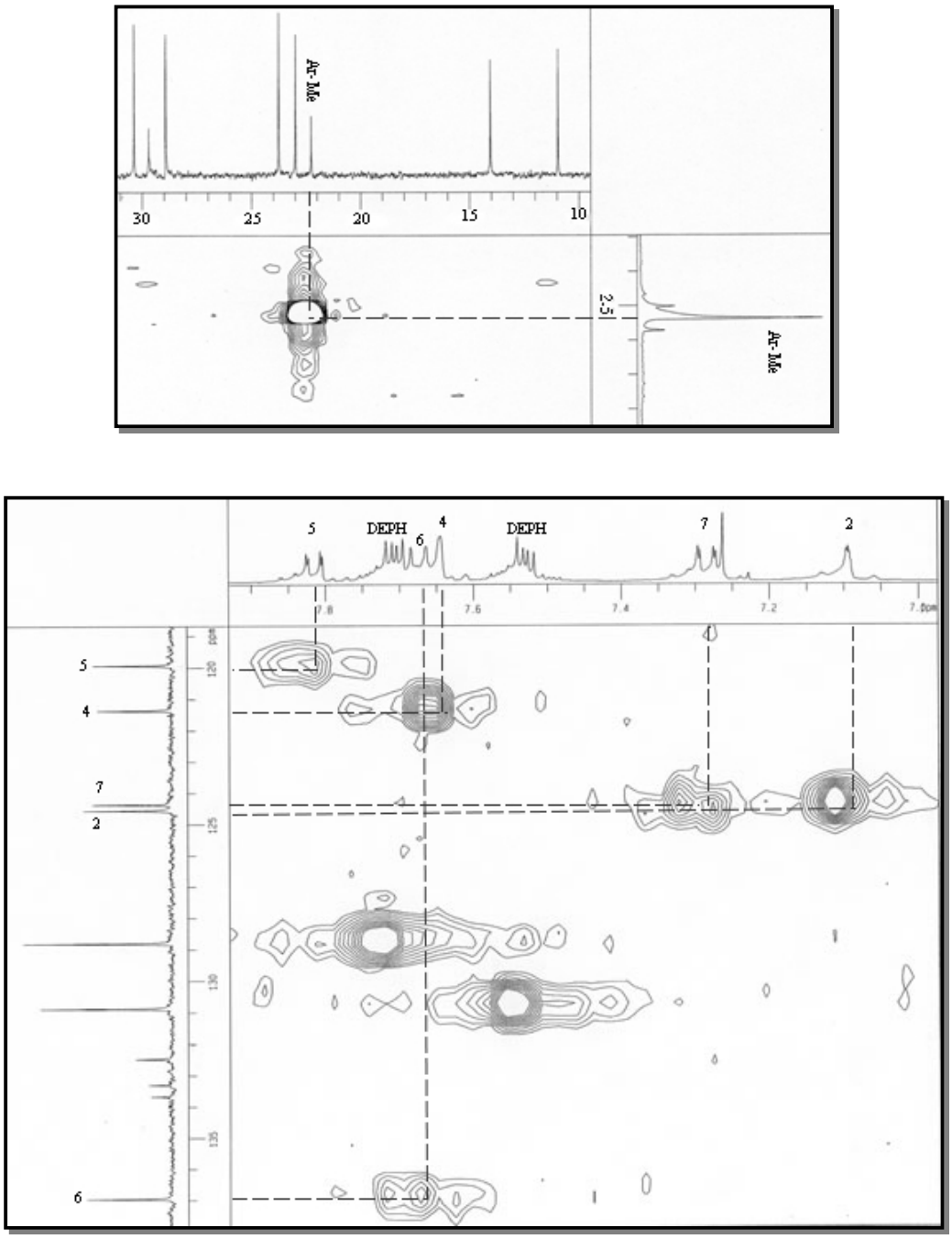
Assignments of proton and carbon atoms were further supported from HMQC and HMBC experiments.

HMQC spectrum (Figure 3.54) revealed the connectivities of C-2 with H-2 (δ 7.06), C-4 with H-4 (δ 7.60), C-5 with H-5 (δ 7.79), C-6 with H-6 (δ 7.63) and C-7 with H-7 (δ 7.25). Similarly, carbon signal at δ 22.27 of methyl group showed the connectivity with a singlet proton at δ 2.43. These are presented in table 3.29.

Table 3.29: HMQC data of compound **6**

Position of C atom	δ_C	Position of H atom	δ_H
2	124.32	H-2	7.06, s
-	22.27	Ar-CH_3	2.43, s
4	121.32	H-4	7.60, d
5	119.88	H-5	7.79, d
6	136.92	H-6	7.63, d
7	124.51	H-7	7.25, d

Figure 3.54: ^{1}H-^{13}C connectivities for compound **6** in HMQC spectrum of mixture of compounds **5** and **6**



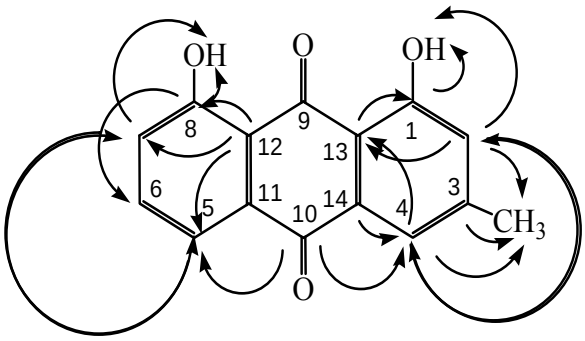
In HMBC experiment (Table 3.30), there were two bond distance connectivities observed between C-1 and proton of -OH group attached at C-1; C-3 and methyl proton attached at C-3; C-8 and proton of -OH group attached at C-8; C-14 and H-4. Similarly, there were three bond distance connectivities observed between C-2 and proton of -OH group attached at C-1,

H-4, methyl proton attached at C-3; C-4 and H-2, methyl proton attached at C-3; C-5 and H-7; C-7 and H-5, proton of -OH attached at C-8; C-8 and H-6; C-10 and H-5, H-4; C-12 and proton of -OH attached at C-8, H-5, H-7; C-13 and proton of -OH attached at C-1, H-4, H-2. These connectivities were illustrated in figure 3.55.

Table 3.30: HMBC data of compound **6**

C atoms	HMBC (Connected H atom)	
	$^{2}J_{C\text{-}H}$	$^{3}J_{C\text{-}H}$
1	OH -1	
2		OH-1, H-4, Ar-CH_3
3	Ar-CH_3	
4		H-2, Ar-CH_3
5		H-7
7		H-5, OH-8
8	OH-8	H-6
10		H-5, H-4
12		OH-8, H-5, H-7
13		OH-1, H-4, H-2
14	H-4	

Figure 3.55: HMBC correlations of compound **6**. Arrow points from carbon to hydrogen



Mass Spectroscopy

The EIMS (Figure 3.56i) displayed a molecular ion $[M]^{+}$ at m/z 254 which was also a base peak; this is one of the characteristic features of the anthraquinone aglycones. In addition to this peak, there were several peaks at m/z 239 $[M\text{-}CH_3]^{+}$, 237 $[M\text{-}OH]^{+}$, 226 $[M\text{-}CO]^{+}$, 198 $[M\text{-}2CO]^{+}$, 197 $[M\text{-}(2CO + H)]^{+}$, 152 $[M\text{-}(CO + OH)]^{+}$, 115 and 57 and 43. The loss of one and two molecules of carbon monoxide from the parent molecular ion is also diagnostic feature of quinones in mass spectrum.[108] GCMS (Figure3.56ii) also showed the molecular ion at m/z 254, often a base peak, supporting for the chrysophanol.

Figure 3.56: (i) EIMS of compound **6**

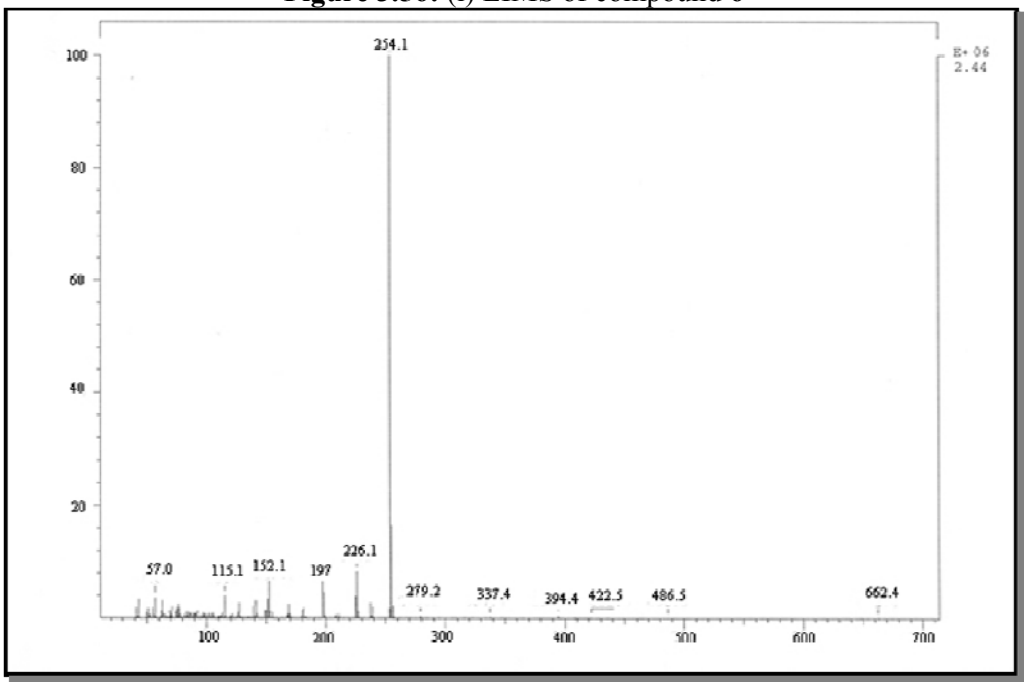
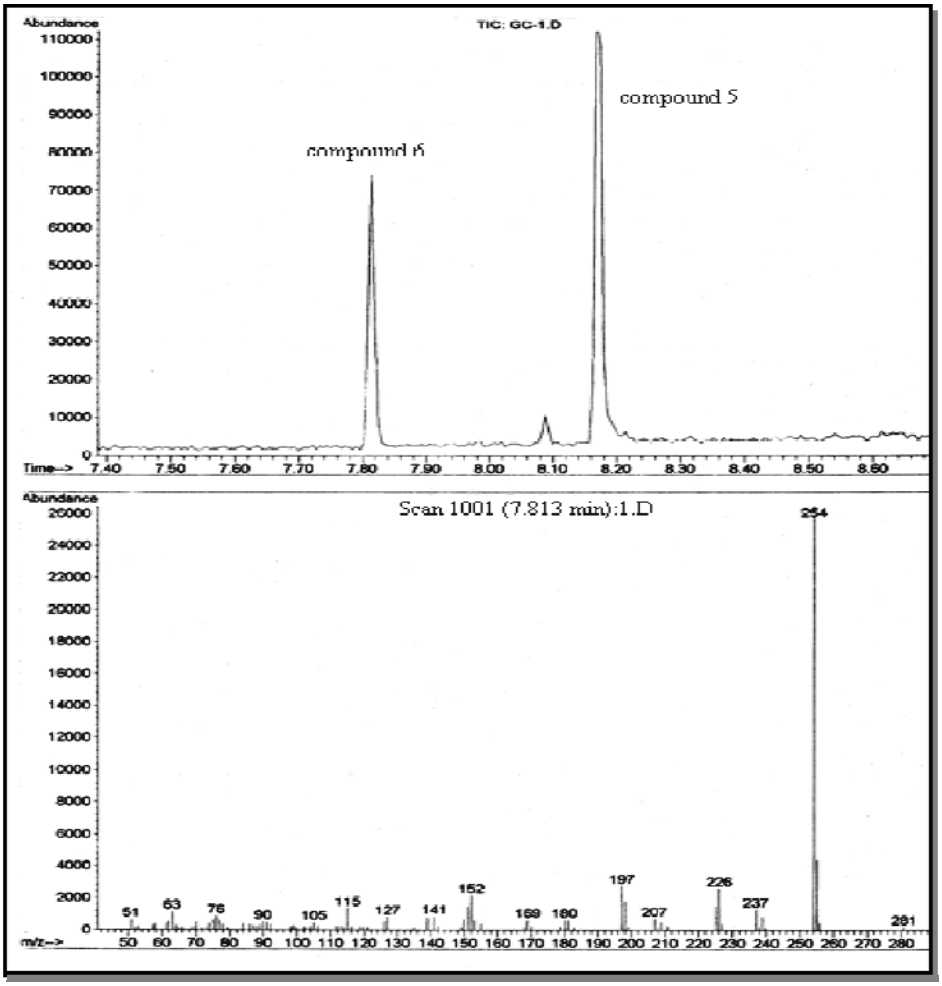


Figure 3.56: (ii) GCMS showing molecular ion peak of compound **6** in mixture sample

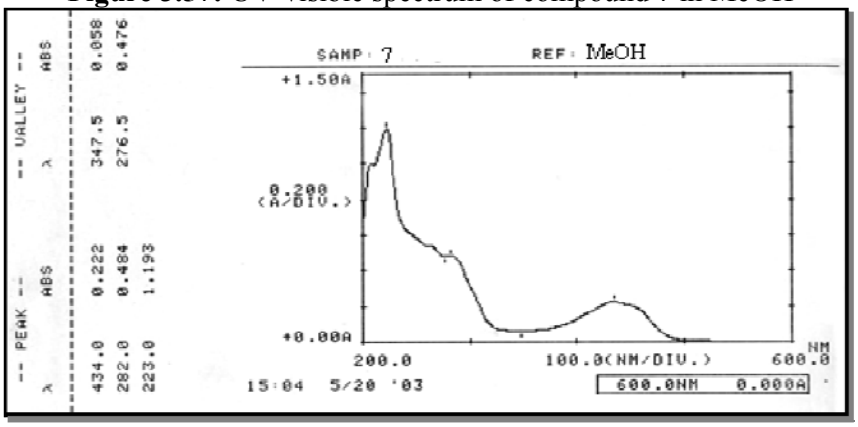


3.4.7 Physcion (7)

UV-Vis spectroscopy

UV-Visible spectrum (Figure 3.57) showed peaks at 223, 282, 434 nm in methanol. Peak at 434 nm indicated the presence of at least two hydroxyl groups at α-positions.[102] Presence of α-hydroxy groups were further supported by the formation of pink spot when methanolic KOH solution was sprayed into a yellow spot on a TLC plate. Similarly, when yellow spot was sprayed with methanolic $FeCl_3$ on a TLC plate, yellow spot was not changed into blue or green indicating the absence of two vicinal hydroxyl groups.[109]

Figure 3.57: UV-visible spectrum of compound **7** in MeOH



^{13}C-NMR spectrum

Six signals resonating in the ^{13}C-NMR spectrum (Figure 3.58) at δ 22.16, 56.08, 106.75, 108.23, 121.3 and 124.52 were assigned to methyl group attached to aromatic carbon C-3, methoxy group attached to aromatic carbon C-6, C-7, C-5, C-4 and C-2. These data were in good agreement with reported[105] data of physcion. Less intense signals observing at δ 149.00, 162.60, 165.80, 166.50, 183.00 and 193.00 were assigned to C-3, C-8, C-1, C-6, C-10 and C-9, respectively, by comparison with reported data[105] of physcion (Table 3.31)**.** The signal of C-9 at δ 193.00 indicated the possibility of hydrogen bonding to two hydroxyl groups, OH-1 and OH-8.[107] Therefore, the signal of C-10 at δ 183.00 ppm indicated the absence of OH group either at C-4 or C-5 position. However, signals of quaternary carbons C-11, C-12, C-13 and C-14 were difficult to observe due to their very long relaxation times.[110]

Figure 3.58: ^{13}C-NMR (75 MHz) spectrum of compound **7** in $CDCl_3$

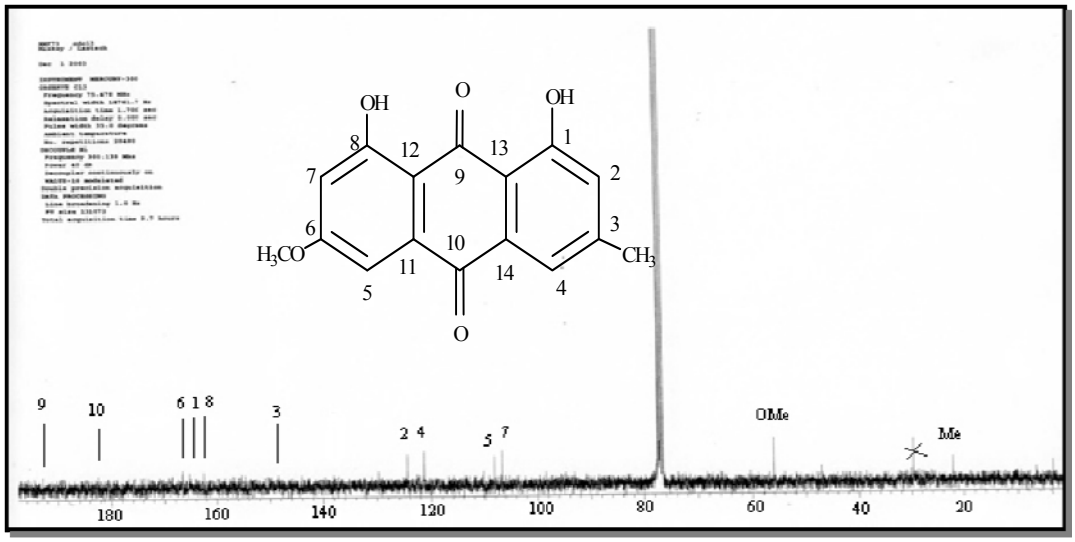


Table 3.31: ^{13}C-NMR (75 MHz) data for compound **7** and its reported value

Carbon position	δ_C of Compound **7**	δ_C of reported value[105]
C-9	193.00	190.8
C-10	183.00	182.0
C-6	166.50	166.6
C-1	165.80	165.2
C-8	162.50	162.5
C-3	149.00	148.5
C-11	*	135.3
C-14	*	133.3
C-2	124.52	124.5
C-4	121.30	121.3
C-13	*	113.7
C-12	*	113.7
C-5	108.23	108.2
C-7	106.75	106.8
OCH_3	56.08	56.1
CH_3	22.16	22.2

* Quaternary carbon difficult to observe due to long relaxation time

^{1}H-NMR spectroscopy

In ^{1}H-NMR spectrum, the presence of C-methyl protons at δ 2.42 and O-methyl protons at δ 3.91, four aromatic protons to the range of δ 6.66-7.62, and the protons of two hydrogen bonded phenolic hydroxyl groups at δ 12.10 and 12.30 revealed the compound either as

physcion[105, 111] (Table 3.32) or przewalskinone[112] (1, 5-dihydroxy-3-methyl-7-methyoxy-9, 10-anthraquinone) (Table 3.33) when compared with reported data.

Table 3.32: Comparison of ^{1}H-NMR data of compound **7** with physcion

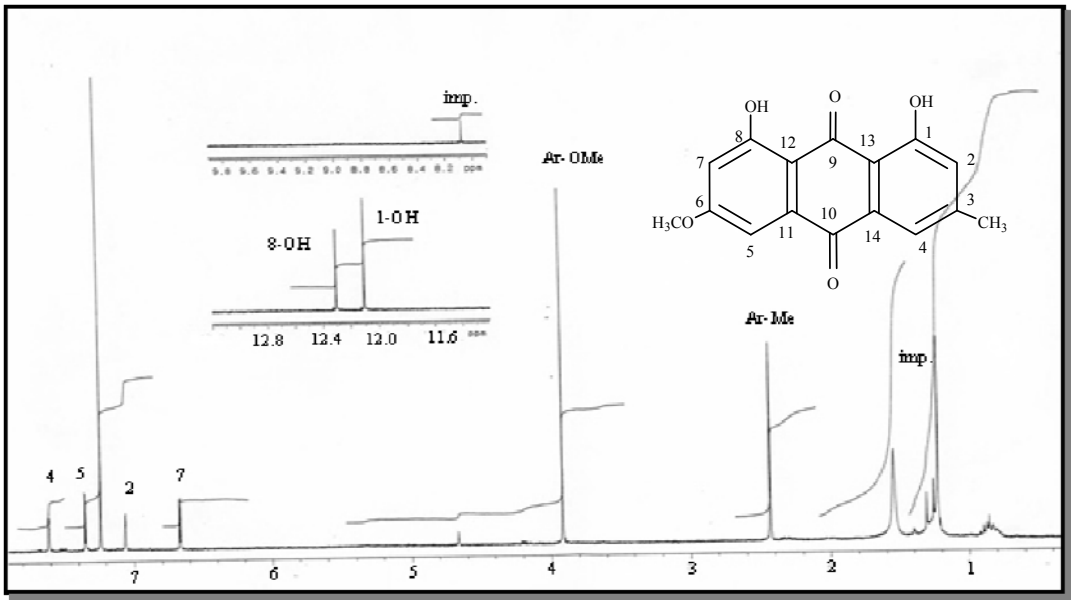
δ_H of **7** (a)	Multiplicity, *J*(Hz)	Assignment	Physcion [111] (b)	Δ (a-b)	Physcion [105] (c)	Δ (a-c)
2.42	s	$ArCH_3$ – 3	2.46, s	0.04	2.45, s	0.03
3.91	s	$ArOCH_3$-6	3.94, s	0.03	3.50, s	0.41
6.66	d, 2.6	ArH-7	6.69, d, 2.4 Hz	0.03	6.69, d, 2.6 Hz	0.03
7.06	d-like	ArH-2	7.09, d-like	0.03	7.08, br s	0.02
7.34	d, 2.6	ArH-5	7.37, d, 2.4 Hz	0.04	7.36 d, 2.6 Hz	0.02
7.62	d-like	ArH-4	7.64, d-like	0.02	7.62, br s	0.00
12.10	s	ArOH-1	12.12, s	0.02	12.10, s	0.00
12.30	s	ArOH-8	12.32, s	0.03	12.31, s	0.01

Table 3.33: Comparison of ^{1}H-NMR data of compound **7** with przewalskinone

δ_H of **7** (a)	Multiplicity, *J*(Hz)	Assignment	Przewalskinone [112] (b)	Δ (a-b)
2.42	S	$ArCH_3$ -3	2.46, br s	0.04
3.91	S	$ArOCH_3$-7	3.94, s	0.03
6.66	d, 2.6	ArH-6	6.69, d, 2 Hz	0.03
7.06	d-like	ArH-2	7.09, br d, 1.6 Hz	0.03
7.34	d, 2.6	ArH-8	7.38, d, 2 Hz	0.04
7.62	d-like	ArH-4	7.64, br d, 1.6 Hz	0.02
12.10	S	ArOH-1	12.13, s	0.02
12.30	S	ArOH-5	12.33, s	0.03

Therefore ^{1}H-NMR data alone (Tables 3.32, 3.33) was inadequate in identifying the corresponding compound. However, the presence of signals at δ 193.00 and 183.00 in ^{13}C-NMR spectrum confirmed the compound **7** as a physcion. Therefore, a singlet at δ 3.91 revealed the presence of methoxy group attached to C-6. Two doublets at δ 6.66 and 7.34 were assigned to aromatic *meta*-coupled H-7 and H-5 protons, respectively, with coupling constants of 2.6 Hz for each. Two doublets like signals at δ 7.06 and 7.62 were assigned to aromatic H-2 and H-4 protons, respectively. Two singlets at δ 12.10 and 12.30 were due to two phenolic hydroxyl groups attached to carbon atoms C-1 and C-8, respectively (Figure 3.59). These data were in close agreement with the reported data[105, 111] of physcion. Signals at δ 0.8 -1.6, 6.3 and 8.14 were likely due to impurities.

Figure 3.59: ^{1}H-NMR (300 MHz) spectrum of compound **7** in $CDCl_3$



Mass spectroscopy

The EIMS (Figure 3.60) afforded a molecular ion $[M]^+$ at m/z 284 as a base peak. In addition, there were presence of several peaks at m/z 255 $[M\text{-}CHO]^+$ (5.6), 241 $[M\text{-}CH_3CO]^+$ (5.6), 227 $[M\text{-}CO\text{-}CHO]^+$ (2.4), 213 $[M\text{-}CH_3CO\text{-}CO]^+$ (3.2), 198 (2.4), 185 $[M\text{-}CH_3CO\text{-}2CO]^+$ (2.4), 128 (4.8), 43 $[CH_3CO]^+$ (8.8). Physcion and przewalskinone, both showed base peaks at m/z 284 corresponding to their respective molecular ion. Therefore, physcion was further confirmed by comparison of its mass fragmentation pattern with reported data of physcion[113] and przewalskinone[112] (table 3.34).

Figure 3.60: EIMS of compound **7**

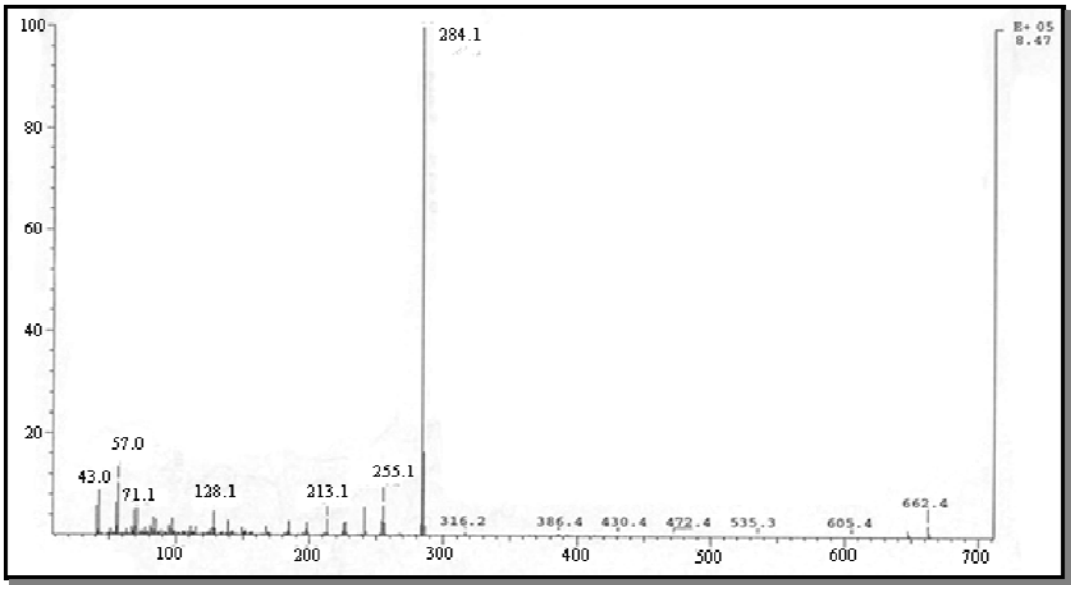


Table 3.34: Comparison of mass fragmentation pattern of isolated compound (**7**) with physcion and przewalskinone

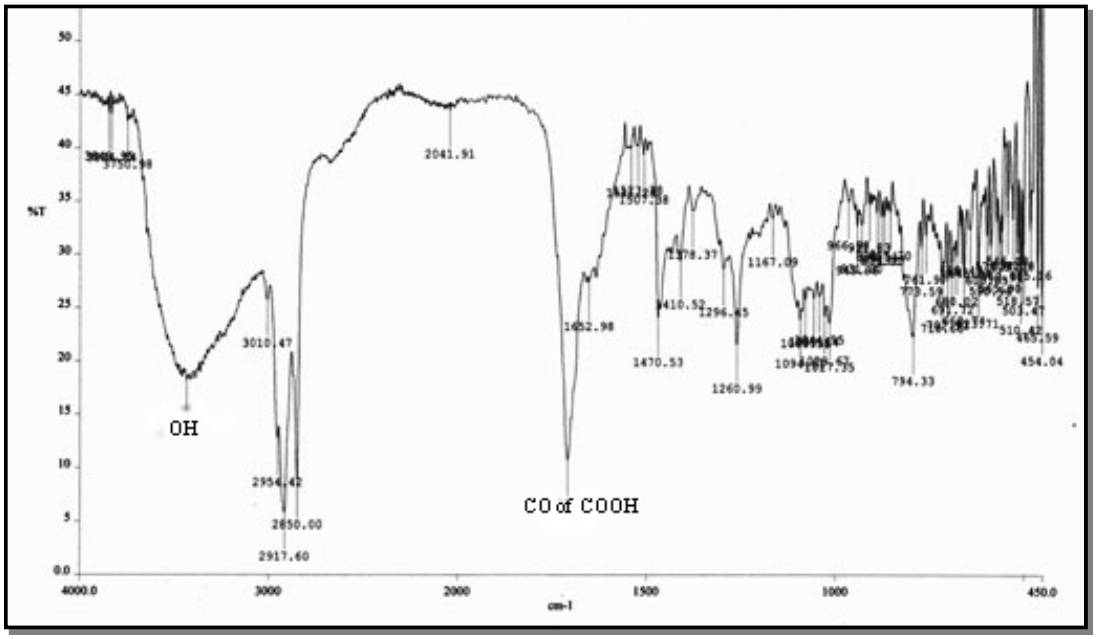
Compound (**7**) (Isolated)	Physcion[113] (Reported)	Przewalskinone[112]
284	284	284
267	267	267
-	-	256
255	255	255
-	-	254
241	241	241
227	227	227
-	-	226
-	-	225
213	213	213
-	-	199
198	198	198
185	185	185
-	-	184
-	-	167
-	-	152
128	128	-
-	-	-
-	-	-
-	-	-
43	43	-

3.4.8 Palmitic acid (8)

IR spectroscopy

IR spectrum (Figure 3.61) showed bands at 3435, 2954, 2917, 2850, 1709, 1470, 1410, 1378, 1296, 1261, 1167, 1094, 943, 794, 718, 691 cm^{-1}. The band at 3435 and 1709 cm^{-1} showed the presence of hydroxyl (-OH) and carbonyl groups (-CO-) of carboxylic acid (-COOH), respectively.

Figure 3.61: IR spectrum of compound **8**



^{1}H-NMR spectroscopy

The ^{1}H-NMR spectrum (Figure 3.62) showed a triplet at δ 0.80 with coupling constant of 6.7 Hz due to methyl protons, H-16. A multiplet at δ 1.10-1.30 was due to combined effects of methylene protons, H-4 to H-15. A quintet at δ 1.50 - 1.60 was assigned to methylene protons, H-3 of the type -$CH_2C\underline{H}_2CH_2COOH$. A downfield triplet at δ 2.27 with coupling constant of 7.5 Hz was attributed to methylene protons, H-2 of the type -$C\underline{H}_2COOH$ (Table 3.35). However, signal of -COOH was not observed in spectrum.

Figure 3.62: ^{1}H-NMR (400 MHz) spectrum of compound **8** in $CDCl_3$

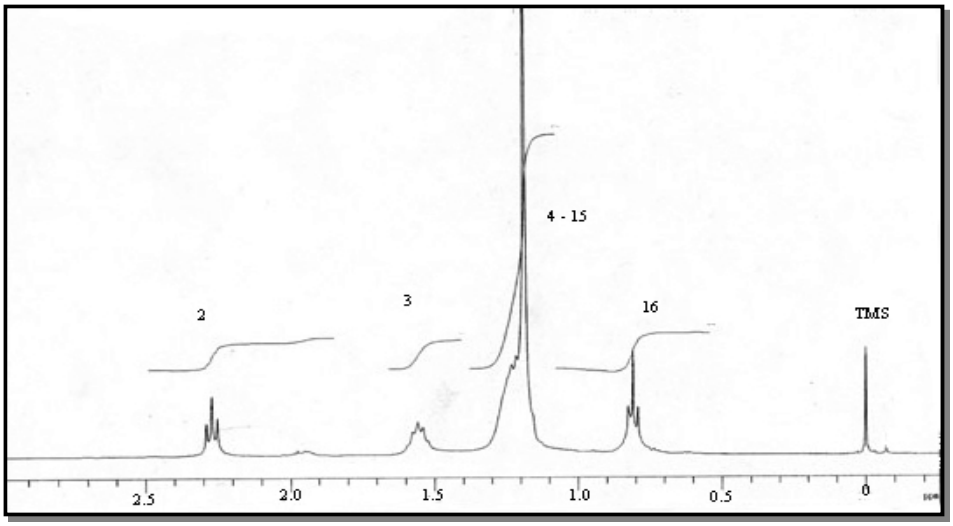


Table 3.35: ^{1}H-NMR (400 MHz) data of compound **8** in $CDCl_3$

Position of H atom	δ_H	Multiplicity and coupling constant (J)	Type of protons
H-16	0.80	t, J = 6.7	$C\underline{H}_3$
H-4 to H-15	1.10-1.30	m	$(C\underline{H}_2)_{12}$
H-3	1.50-1.60	quintet	$-CH_2C\underline{H}_2CH_2COOH$
H-2	2.27	t, J = 7.5	$-C\underline{H}_2COOH$

^{13}C-NMR spectroscopy

The ^{13}C-NMR Spectrum (Figure 3.63) revealed signals of sixteen carbon atoms (table 3.36), of which the signal at δ 29.63 was nearly five times intense than others. These were assigned to methylene carbons C-4 to C-8. Similarly signals at δ 24.63, 29.01, 29.18, 29.29, 29.53, 29.38 and 33.98 were assigned to methylene carbons C-3, C-13, C-12, C-11, C-9, C-10 and C-2 while signal at δ 179.70 was assigned to carboxylic acid group C-1.

Figure 3.63: ^{13}C-NMR (100 MHz) spectrum of compound **8** in $CDCl_3$

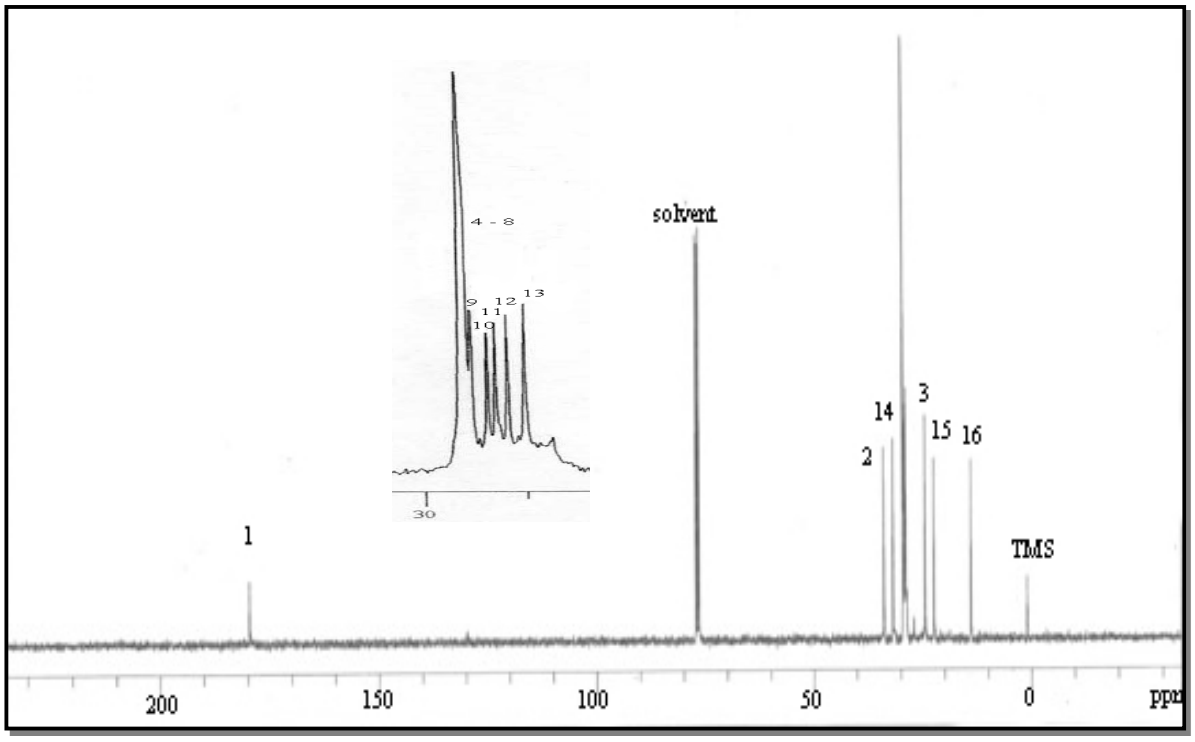


Table 3.36: ^{13}C-NMR (100 MHz) data of compound **8** in $CDCl_3$

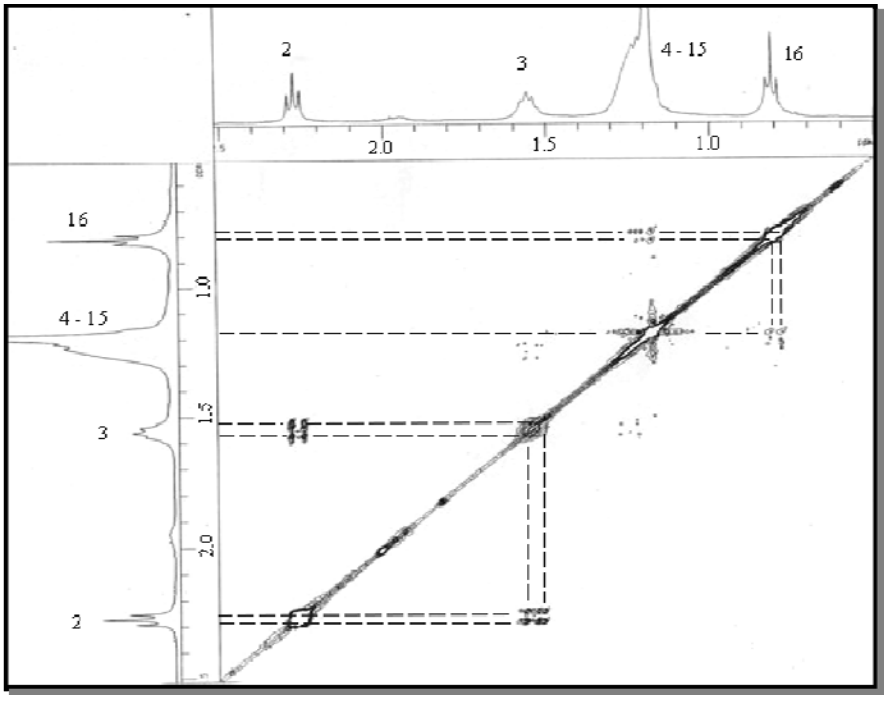
Position of C atom	δ_C	Assignment	Position of C atom	δ_C	Assignment
1	179.70	COOH	9	29.53	CH_2
2	33.98	CH_2	10	29.38	CH_2
3	24.63	CH_2	11	29.29	CH_2
4	29.63	CH_2	12	29.18	CH_2
5	29.63	CH_2	13	29.01	CH_2
6	29.63	CH_2	14	31.86	CH_2
7	29.63	CH_2	15	22.62	CH_2
8	29.63	CH_2	16	14.04	CH_3

This assignment of carbon atoms were also supported by ^{1}H-^{1}H COSY and HMBC experiments as shown below.

^{1}H-^{1}H Correlation spectroscopy (^{1}H-^{1}H COSY)

In ^{1}H-^{1}H COSY, a triplet at δ 2.27 (H-2) showed a distinct cross peak correlation with a quintet at δ 1.50-1.60 (H-3) (figure 3.64).

Figure 3.64: ^{1}H-^{1}H COSY spectrum of compound **8**



HMBC spectroscopy

The HMBC experiment (Figure 3.65) showed two bond distance ($^2J_{C\text{-}H}$) cross-peaks between C-2 at δ 33.98 and H-3 (δ 1.50-1.60), between C-3 at δ 24.63 and H-2 (δ 2.27), between C-15 at δ 22.62 and H-16 (δ 0.80), and between C-1 at δ 179.74 and H-2 (δ 2.27). A three bond distance ($^3J_{C\text{-}H}$) cross peaks appeared between C-14 at δ 31.86 and H-16 (δ 0.80), and between C-1 at δ 179.74 and H-3 (δ 1.50-1.60) (Figure 3.66).

Figure 3.65: HMBC spectrum of compound **8**

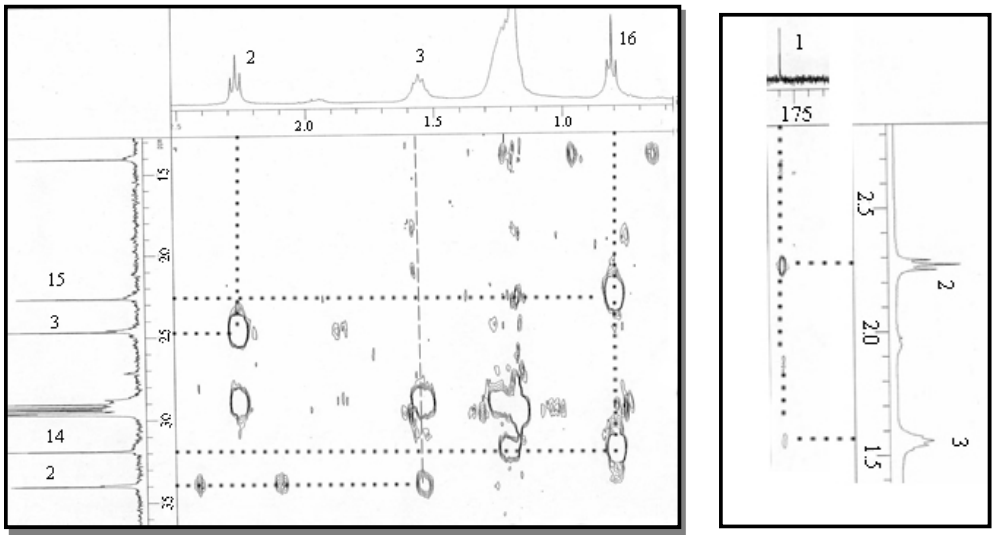
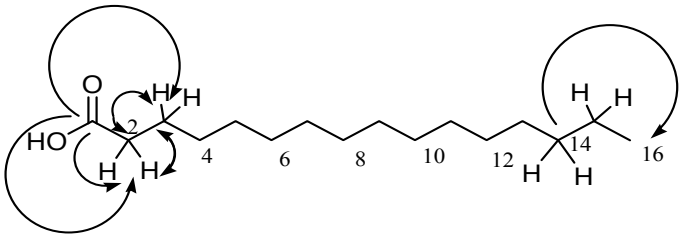


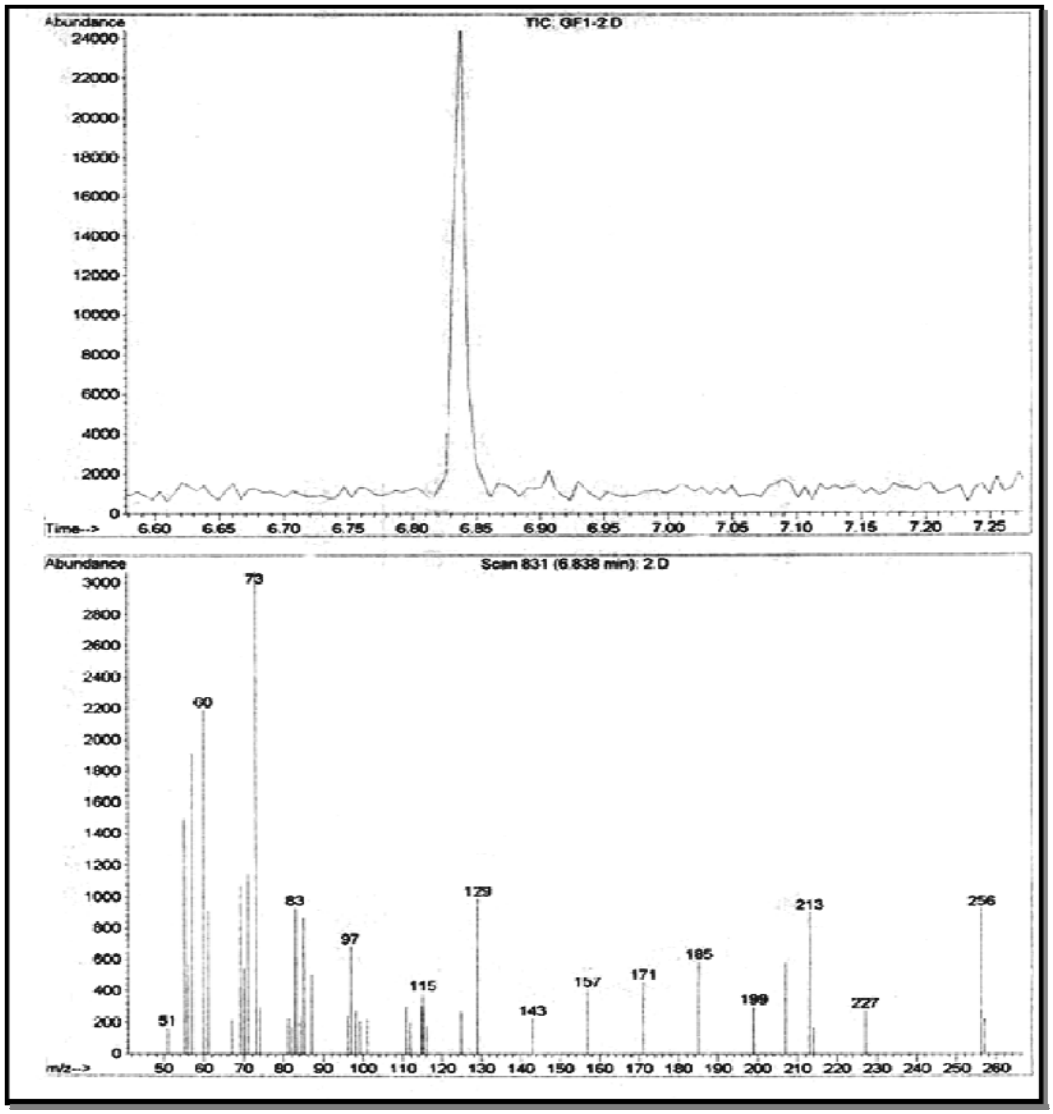
Figure 3.66: ^{1}H-^{13}C relation revealed in HMBC spectrum. Arrow points carbon to hydrogen.



Mass spectroscopy

GCMS (Figure 3.67) showed molecular ion at m/z 256 corresponding to molecular formula $C_{16}H_{32}O_2$. In addition to this peak, there were peaks at m/z 227, 213, 199, 185, 171, 157, 143, 129, 97, 83, 73, 60, 51 which were in close agreement with literature data.[114]

Figure 3.67: GCMS of compound **8**



3.4.9 Steroidal mixture of poriferasterol (2) and *β*-sitosterol (9)

A mixture of poriferasterol (**2**) and *β*-sitosterol (**9**), isolated as white amorphous powder, showed green color in Liebermann-Burchard test indicating that the compound had a steroid nucleus. ^{1}H-NMR spectrum (Figure 3.68) was in close agreement with that of isolated compound **2** except a signal of H-3 (δ 3.51) which appeared as unsymmetrical multiplet while the signal of H-3 (δ 3.52) in compound **2** was symmetrical multiplet as shown in fig.3.15. ^{13}C-NMR spectrum (Figure 3.69) revealed signals in close agreement with that of isolated compound **2**. Melting point determination showed 138 °C. GCMS (Figure 3.70) revealed that it was mixture of two components *viz*. poriferasterol (**2**) and *β*-sitosterol (**9**). Mass fragmentations of **2** were in accordance with reported data[92] while observed mass

fragmentation of **9** (Figure 3.72 ii), that is, 414 $[M]^+$, 399 [M - CH_3], 396 [M-H_2O], 381 [M - CH_3 - H_2O], 329, 303, 275 were in accordance with literature data[115] of *β*-sitosterol.

Figure 3.68: ^{1}H-NMR spectrum of steroidal mixture of **2** and **9**

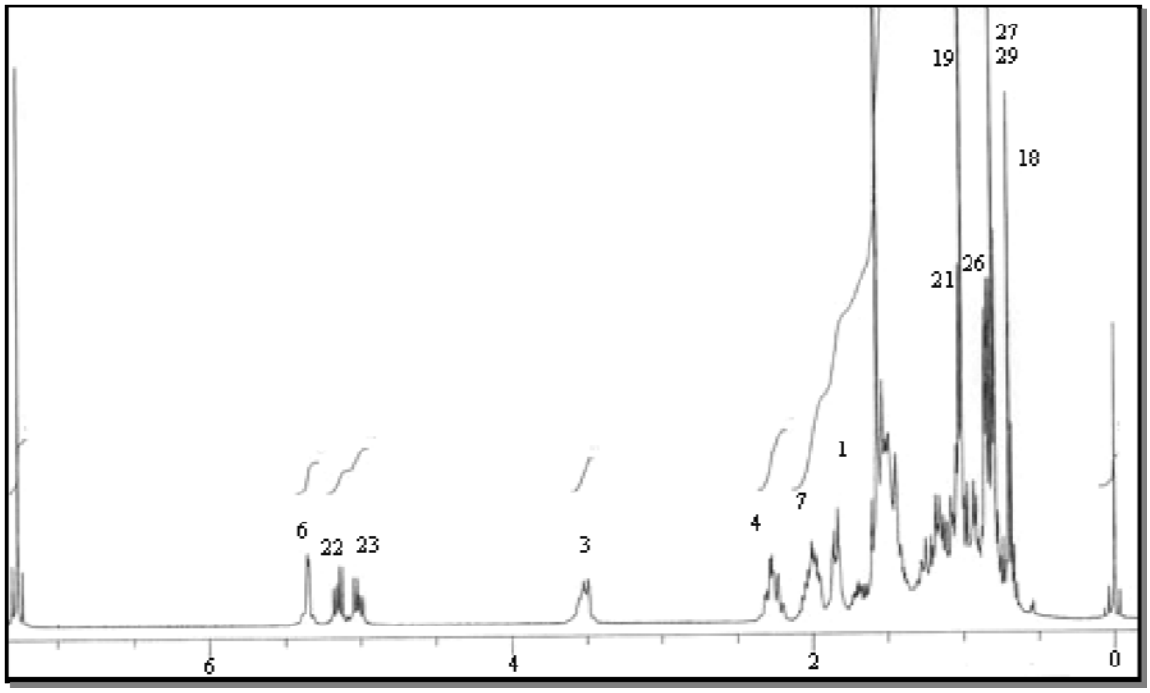


Figure 3.69: ^{13}C-NMR spectrum of steroidal mixture of **2** and **9**

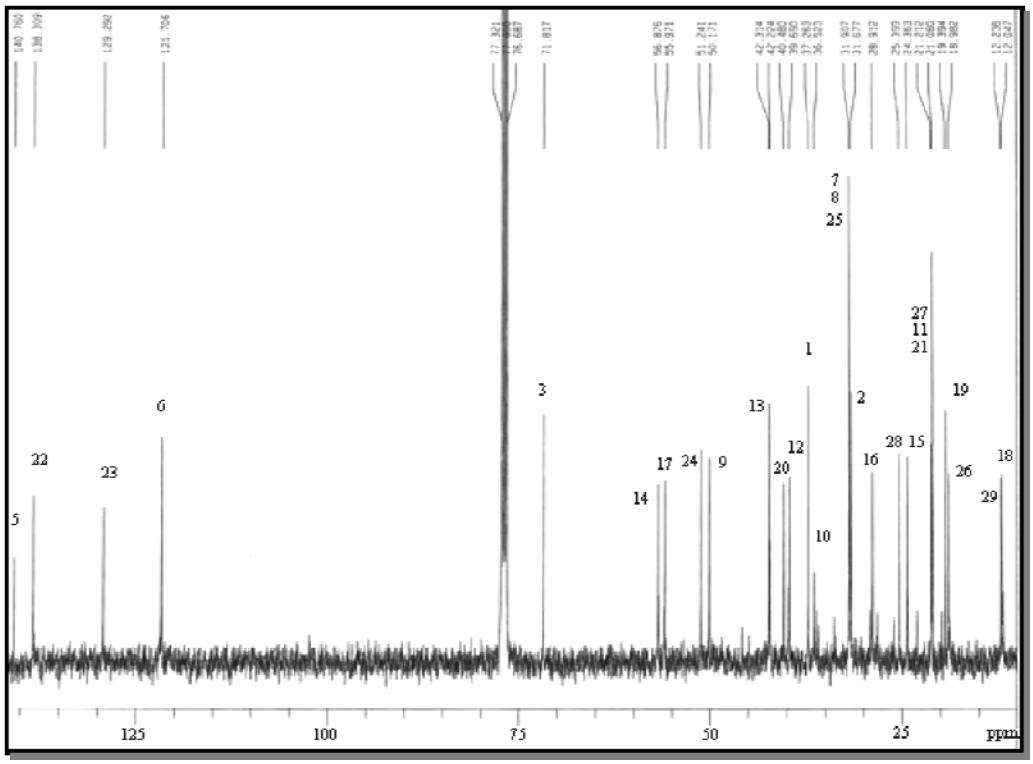


Fig: 3.70: (i) GCMS of compound **2** in steroidal mixture of **2** and **9**

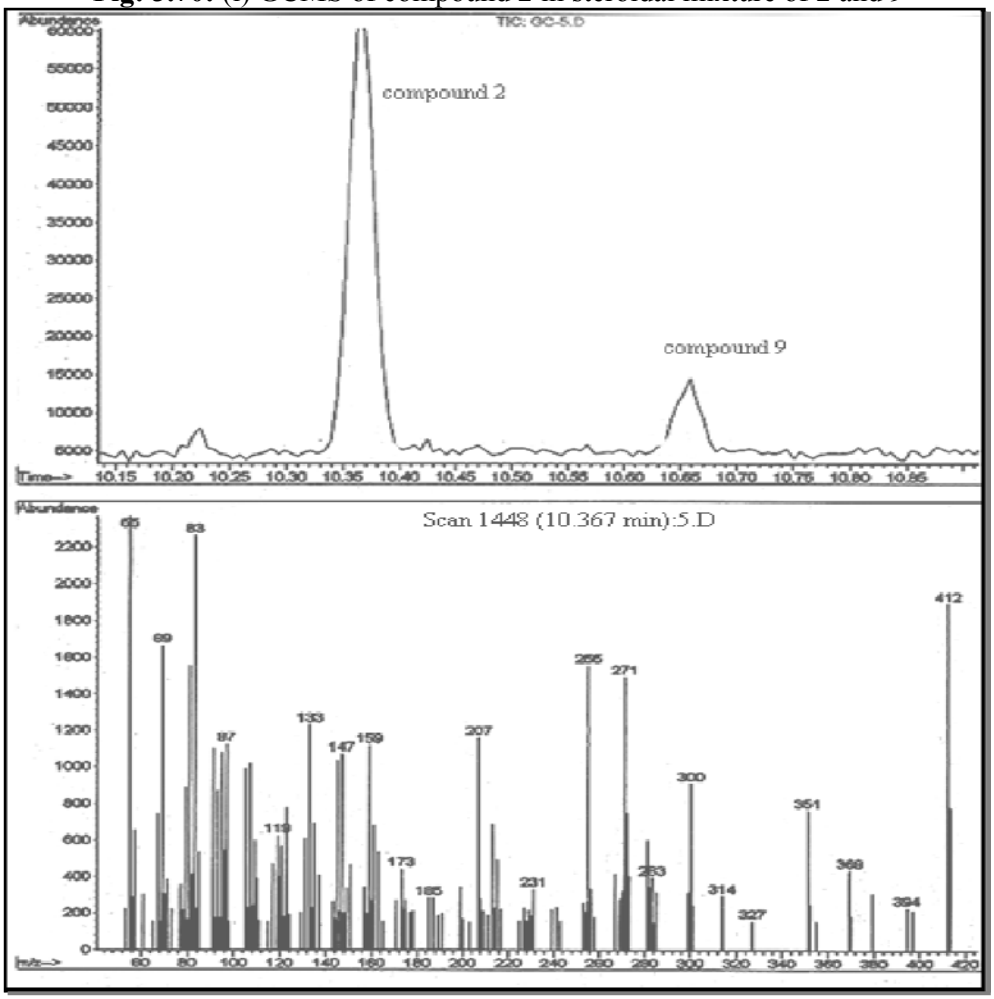
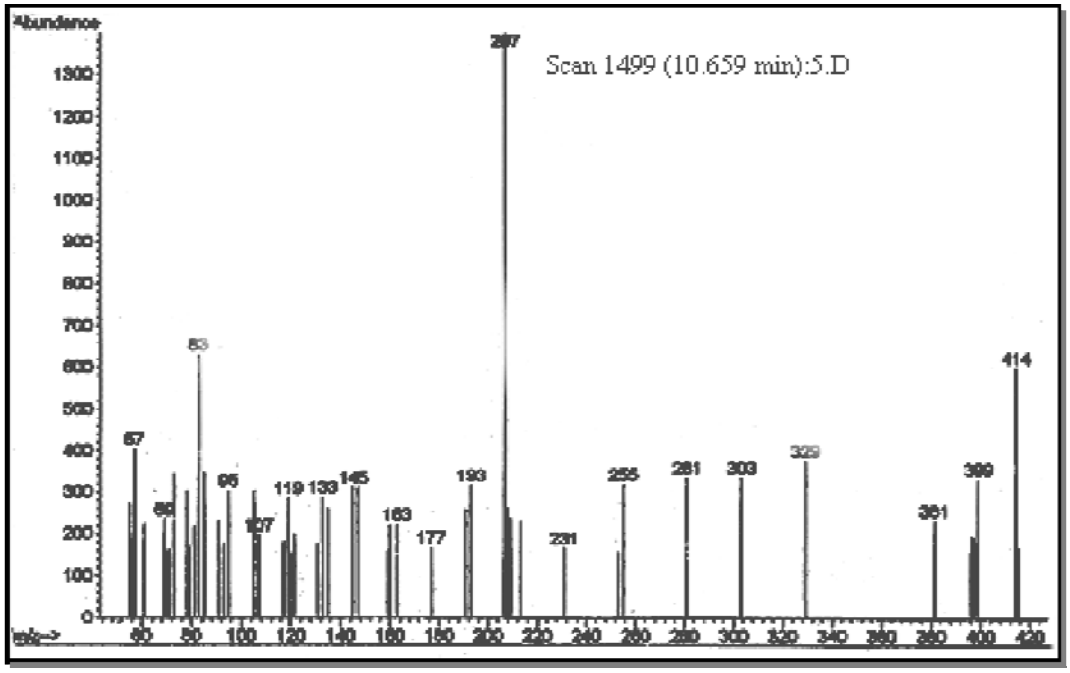


Fig: 3.70: (ii) GCMS of compound **9** in steroidal mixture of **2** and **9**



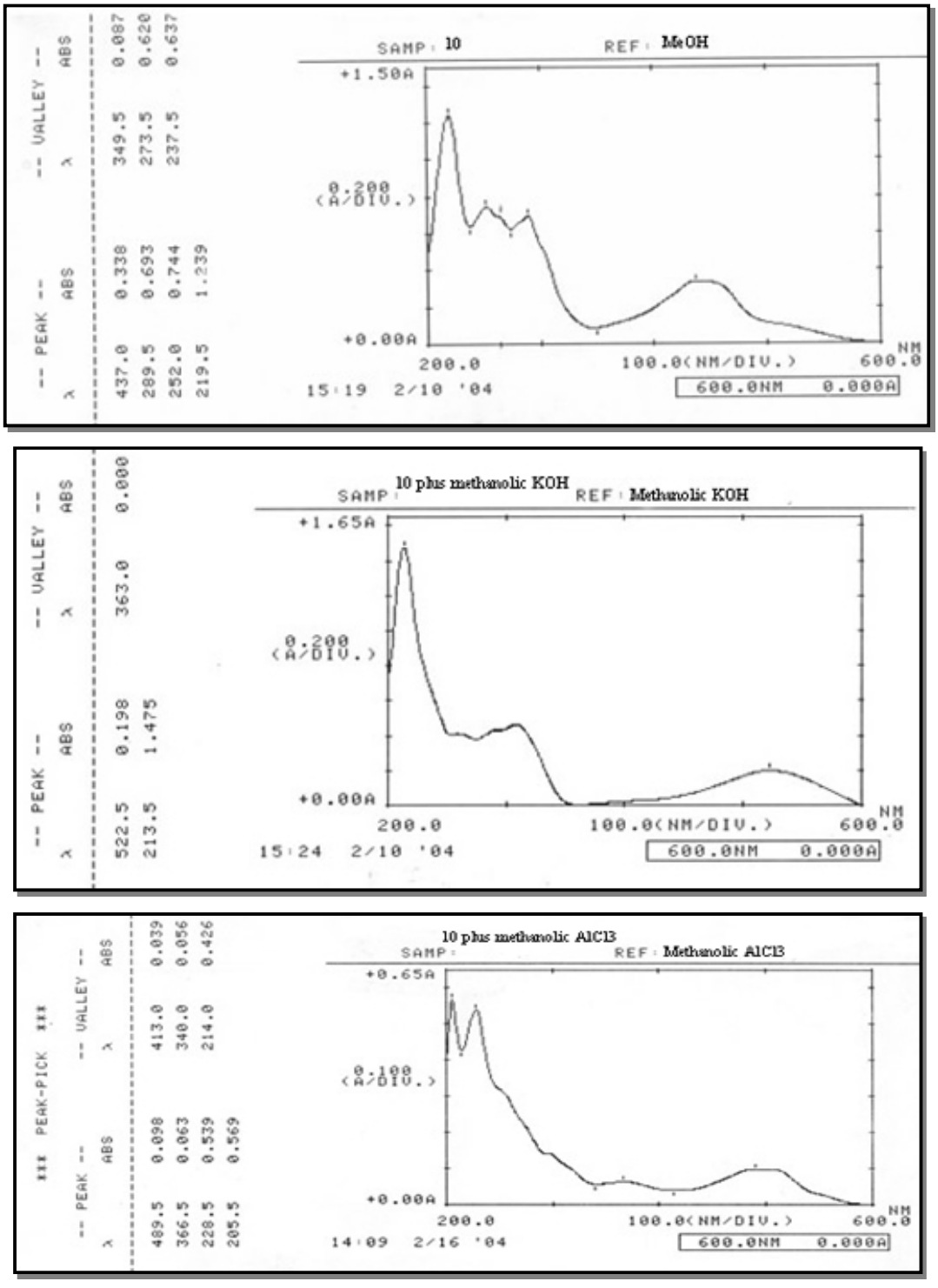
3.4.10 Compound 10

Results of UV-visible spectral data as shown in table 3.37 revealed that compound **10** contained anthraquinone moiety with two hydroxyl groups at α- position, that is, 1 and 8 positions. The observed bathochromic shift in alcoholic KOH for compound **10** was different from that of chrysophanol (Figure 3.71). However the compound was not investigated further due to extremely low yield.

Table 3.37: UV and Visible spectral shifts of compound **10**

Solvent used	Spectral maxima (nm)				Spectral effect	Structural diagnosis
	Band I	Band	Band III	Band IV		
MeOH alone	219.5	252.0	289.5	437.0		(i) 3-4 intense peaks between 215 - 300 nm and another one at about 430 nm or greater (Band IV) is characteristic for anthraquinone skeleton.[101] (ii) Peak at about 430 nm (Band IV) is indicative of the presence of at least two -OH in the α position[102], that is, 1 and 8 position in anthraquinone skeleton.
MeOH + KOH (2 drops 10 % methanolic KOH)	213.5	-	-	522.5	85.5 nm bathochromic shift (Band IV)	1-OH and 8-OH free
MeOH + $AlCl_3$ (2 drops 5% methanolic $AlCl_3$)	205.5	228.5	366.5	489.5	52.5 nm bathochromic shift (Band IV)	1-OH and 8-OH free

Figure 3.71: UV-Visible spectra of compound **10** in different solvents

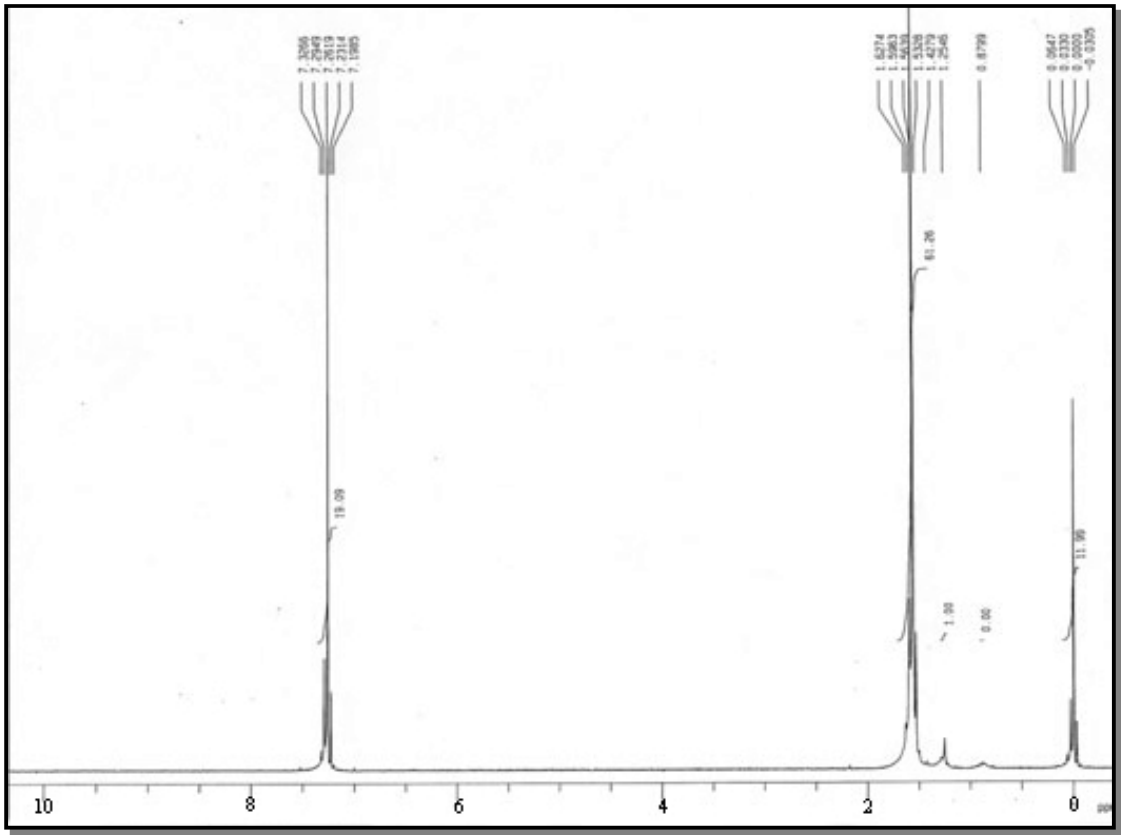


3. 4. 11 COMPOUND 11

^{1}H-NMR spectroscopy

^{1}H-NMR spectrum (Figure 3.72) revealed that the signal at δ 0.87-1.67 was due to methyl and methylene groups and a multiplet signals at δ 7.0-7.5 was due to aromatic protons.

Figure 3.72: ^{1}H-NMR spectrum of compound **11**

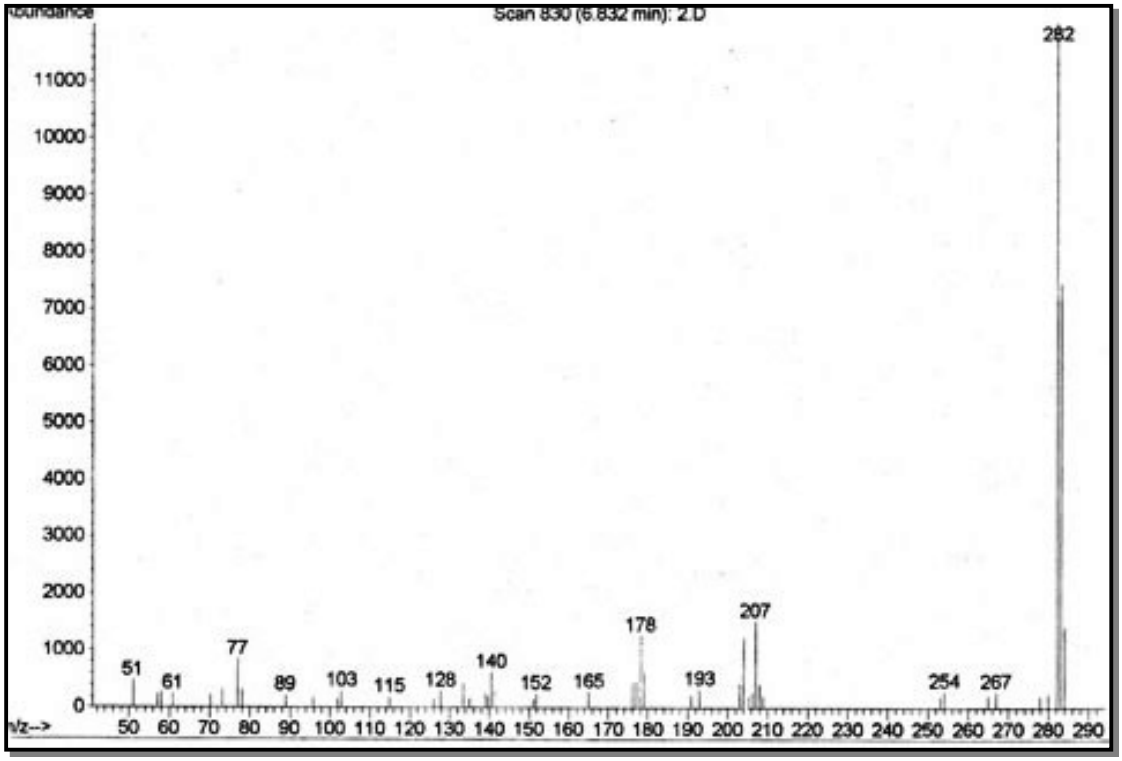


Mass spectroscopy

GCMS (Figure 3.73) showed a prominent molecular ion $[M]^+$ at m/z 282 which was accompanied by two isotopic peaks at m/z 283 $[M+1]^+$ and 284 $[M+2]^+$. Peaks at m/z 267 and 254 were due to elimination of methyl and ethyl groups from molecular ion, respectively. Peaks at m/z 77 was due to phenyl cation $(C_6H_5)^+$ which in turn eliminated C_2H_2 showing the peak at m/z 51 due to $C_4H_3^+$.

Based on ^{1}H-NMR and GCMS, compound **11** must be aromatic hydrocarbon.

Figure 3.73: GCMS of compound **11**



3.5 BIOASSAY OF ISOLATES FROM THE ROOT

Compounds isolated from petroleum ether extract of the root were tested for cytotoxicity and antibacterial activity.

3.5.1 Brine shrimp cytotoxicity test

The results of brine shrimp cytotoxicity test of compounds (**1-7**) as shown in table 3.38 revealed that LC_{50} of bis(2-ethylhexyl)phthalate (**5**)**,** chrysophanol (**6**) and physcion (**7**) were lesser than 1000 µg/mL indicating moderate cytotoxicity against brine shrimps while LC_{50} of eupatoric acid (**1**)**,** poriferasterol (**2**)**,** octadecane (**3**) and butyrospermol acetate (**4**) were greater than 1000 µg/mL indicating non-cytotoxic (see section 5.7.1 for details).

Table 3.38: Brine shrimp cytotoxicity test of isolates **1-7** from the root

Extract	LC_{50} (Mean) ± σ µg/mL	95% Confidence Interval	Remarks
Eupatoric acid (**1**)	n.d.	n.d.	Non-cytotoxicity
Poriferasterol (**2**)	n.d.	n.d.	Non-cytotoxicity
Octadecane (**3**)	n.d.	n.d.	Non-cytotoxicity
Butyrospermol acetate (**4**)	n.d.	n.d.	Non-cytotoxicity
Bis(2-ethylhexyl)phthalate (**5**)	538.15 ± 41.89	642.21 - 434.09	Moderate cytotoxicity
Chrysophanol (**6**)	289.00 ± 55.87	344.87 - 233.13	Moderate cytotoxicity
Physcion (**7**)	158.14 ± 20.70	209.56 - 106.72	Moderate cytotoxicity
Berberine (Positive control)	89.18 ± 4.1	99.36 - 79.0	

σ = standard deviation, n.d. = calculation omitted for non-cytotoxic extracts.

3.5.2 Antibacterial test

Results of antibacterial activity of compounds (**1-6**, **8**) isolated from petroleum ether extract, determined by disc diffusion method, are tabulated in table 3.39. As zone of inhibition was measured including 6 mm disc of paper, diameter of zone with 6 mm value was indicative of no activity. Therefore, compounds (**1-6**, **8**) did not reveal antibacterial activity at 200 µg/disc (see section 5.7.2 for details).

Table 3.39: Zone of inhibition of compounds **1** – **6** and **8** isolated from the root

Microorganism→	*S. aureus*		*B. subtilis*		*P. aeruginosa*		*E. coli*	
	Zone of inhibition		Zone of inhibition		Zone of inhibition		Zone of inhibition	
Compound/drug ↓	mm	%	mm	%	mm	%	mm	%
Gentamycin (10 µg)	24	100	24	100	19	100	18	100
Eupatoric acid (**1**)	6	0	6	0	6	0	6	0
Poriferasterol (**2**)	6	0	6	0	6	0	6	0
Octadecane (**3**)	6	0	6	0	6	0	6	0
Butyrospermol acetate (**4**)	6	0	6	0	6	0	6	0
Bis(2-ethylhexyl)phthalate (**5**)	6	0	6	0	6	0	6	0
Chrysophanol (**6**)	6	0	6	0	6	0	6	0
Palmitic acid (**8**)	6	0	6	0	6	0	6	0

3.6 BIOASSAY OF THE FLOWER

3.6.1 Brine shrimp cytotoxicity test

Hexane extract of the flower (see section 5.8.1 for its preparation), revealed moderately cytotoxic against brine shrimp with LC_{50} of 241.99 µg/mL (see section 5.8.2 for details).

3.6.2 Antibacterial Test:

The result of antibacterial test of hexane extract of the flower as shown in table 3.40 revealed weak activities against *B. subtilis* and *S. aureus* with zone of inhibition 22.2% and 16.6%, respectively, in comparison with gentamycin (10 µg) while it revealed inactive against gram-negative bacteria - *E. coli*, *P. aeruginosa*. The control disks injected with 20 µl of DMSO showed no inhibitory effect against the micro-organisms tested. Hexane extract of the flower revealed MBC value greater than 2500 µg (see section 5.8.3 for details).

Table 3.40: Zone of inhibition of hexane extract of the flower

Microorganism→	*S. aureus*		*B. subtilis*		*P. aeruginosa*		*E. coli*	
	Zone of inhibition		Zone of inhibition		Zone of inhibition		Zone of inhibition	
Extract/drug ↓	Mean	%	Mean	%	Mean	%	Mean	%
Gentamycin (10 µg)	24	100	24	100	19	100	18	100
Negative control	6	0	6	0	6	0	6	0
Hexane extract	9	16.6	10	22.2	6	0	6	0

3.7 BIOASSAY OF THE LEAF AND THE STEM

3.7.1 Brine shrimp cytotoxicity test

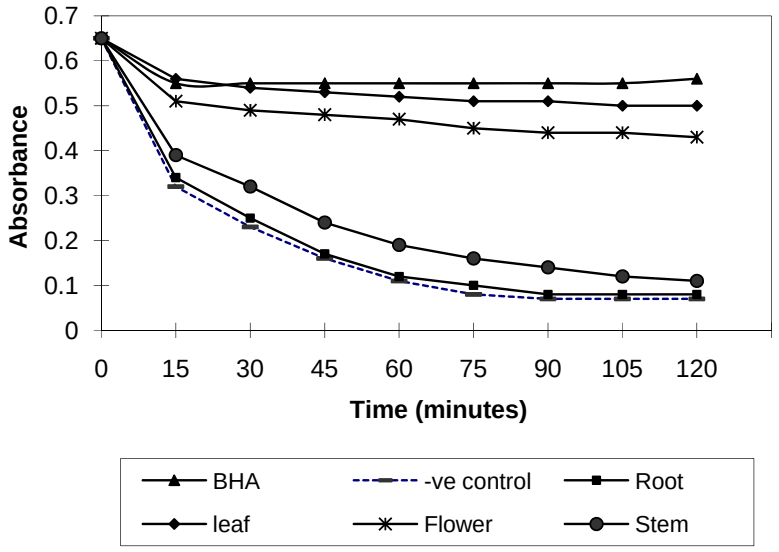
Ethanol extract of the stem and the leaf (see in section 5.9.1 for its preparation) revealed non-cytotoxic against brine shrimp as their LC_{50} values were greater than 1000 µg/mL (see section 5.9 for details).

3.8 ANTIOXIDANT ACTIVITY OF DIFFERENT PARTS OF PLANT

3.8.1 *β*-carotene bleaching test

The performances of ethanolic extracts of the leaf, defatted flower, root and stem of the *E. odoratum* as well as BHA and negative control (linoleic acid + carotene solution) were presented in figure 3.74 (see section 5.10.1 for details). The discoloration process in the model system progressed differently for the various samples which showed antioxidant activity in decreasing order: BHA > leaf > flower > stem > root > -ve control.

Figure 3.74: Antioxidative effects of methanolic solution of different parts of *E. odoratum* and BHA in *β*-carotene linoleic acid model system at 50 °C.



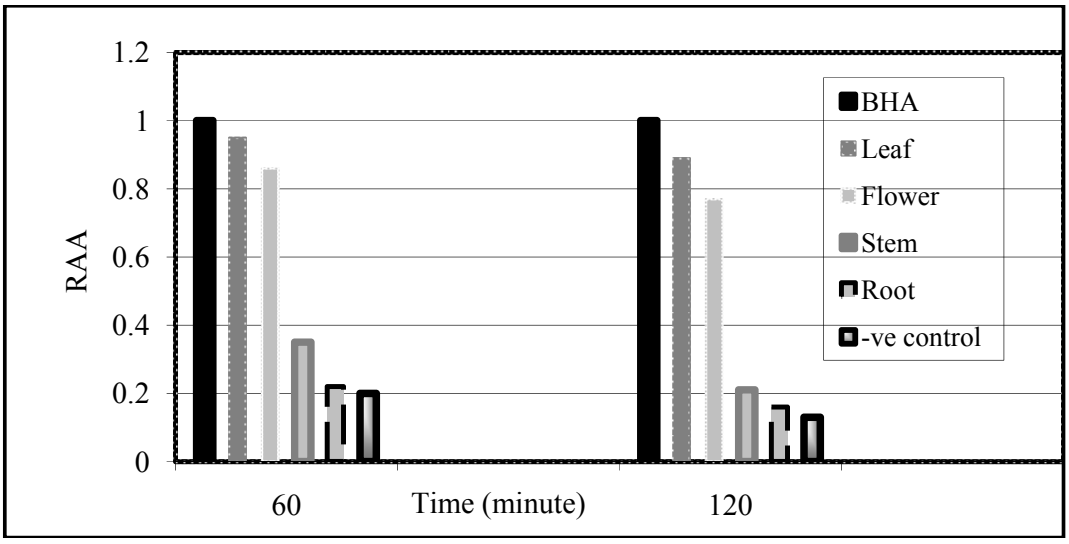
The relative antioxidant activity (RAA) values at 60 and 120 minutes were presented in table 3.41. A comparative presentation of different RAA values is shown in figure 3.73. However, the RAA of root and stem at 60 minutes displayed significantly ($P < 0.01$) lower RAA values than BHA and hence their extracts were considered as very weak antioxidants. After the same period, leaf and flower displayed their RAA values not significantly different ($P > 0.01$) than BHA. In this test, leaf and flower of *E. odoratum* revealed the best results, with RAA values almost equivalent to the positive control (BHA). The RAA values of leaf and flower reduced to 0.89, that is 6.3% and to 0.77 that is 10.4%, respectively, after 120 minutes which were again not significantly different ($P > 0.01$) with comparison to BHA.

Table 3.41: Relative antioxidant activity (RAA) of different parts of *E. odoratum*

Sample	RAA (60 min.)	RAA (120 min.)
Root	0.22 ± 0.015 *	0.16 ± 0.011*
Leaf	0.95 ± 0.029 **	0.89 ± 0.032**
Stem	0.35 ± 0.020 *	0.21 + 0.02*
Flower	0.86 ± 0.050 **	0.77 ± 0.055**
BHA	1.00	1.00
-ve Control	0.20 ± 0.0*	0.13 ± 0.011*

*N = 3. Mean ± SD, * P < 0.01 = significantly different from BHA, **P > 0.01 = Not significantly different from BHA.*

Figure 3.75: Comparison of RAA values of different extracts with BHA at 60 and 120 minutes



3.8. DPPH radical scavenging test

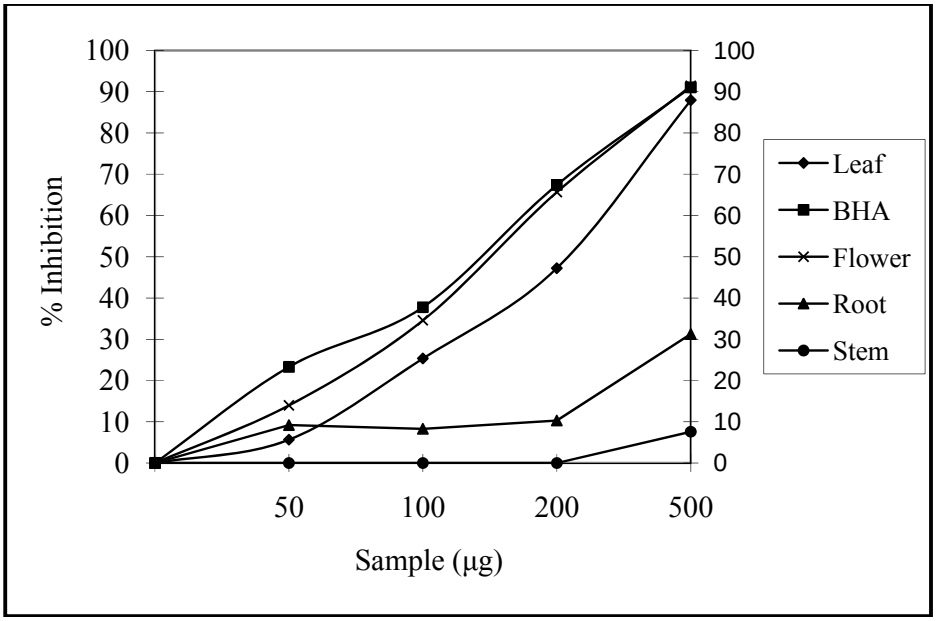
The results of the free radical scavenging effect of ethanolic extracts of leaf, stem, root and defatted flower parts and positive control (BHA) in DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical system were shown table 3.42 and figure 3.76 (see section 5.10.2 for details).

Table 3.42: Percentage inhibition of different parts of plant on DPPH radicals

Concentration (µg)	BHA[a] ± SEM	Leaf ± SEM	Flower ± SEM	Stem ± SEM	Root ± SEM
50	23.29 ± 0.07	5.65 ± 0.33	13.98 ± 1.04	< 0	8.28 ± 0.37
100	37.75 ± 0.40	25.33 ± 0.45	34.57 ± 1.41	< 0	9.18 ± 0.37
200	67.39 ± 0.04	47.21 ± 0.75	65.66 ± 1.25*	< 0	10.29 ± 0.04
500	91.05 ± 0.33	87.93 ± 0.08*	91.40 ± 0.87*	7.59 ± 0.79	31.25 ± 1.01

[a]Positive control: butylated hydroxyanisole; * *P > 0.01 = Not significantly different from BHA.*

Figure 3.76: Graphical representation of scavenging activity in terms of % inhibition of different extracts on DPPH free radical

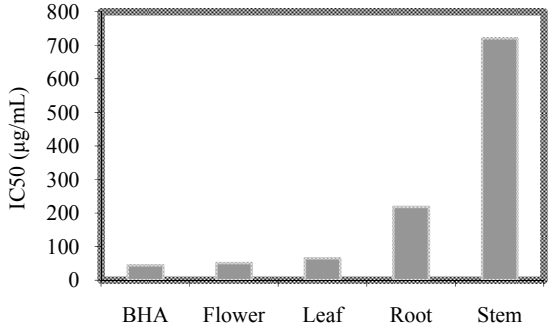


Ethanolic extracts of leaf and defatted flower exhibited a strong scavenging activity on DPPH radical, with values comparable to the positive control (BHA) at 500 µg. Their % inhibitions at 500 µg were significantly not different ($P > 0.01$) from activity shown by BHA of same concentration. Percentage inhibition of leaf and defatted flower at 500 µg can be considered as a full inhibition of DPPH because after completing the reaction the final solution always possesses some yellowish color and therefore its inhibition compared to colorless methanol solution can not reach 100%.[116] Defatted flower extract at 200 µg also revealed good scavenging activity on DPPH radical with 65.66% inhibition. Ethanolic extract of root showed low scavenging activity, while stem extract did not exhibit any scavenging activity.

Furthermore, it should be pointed out that ethanolic extracts of defatted flower and stem were dissolved in methanol, and this procedure could have some effect on the measurements of scavenging activity so far as the extracts were not fully soluble in methanol.

Figure 3.77 shows the amount of each sample needed for 50% inhibition (IC_{50}) of DPPH free radical. IC_{50} of BHA (positive control) was 44.5 μg/mL. IC_{50} of ethanolic extracts of defatted flower, leaf, root and stem were 50.9, 65.4, 218.5 and 720.8 μg/mL, respectively, hence defatted flower and leaf extracts showed the promising scavenging activity.

Figure 3.77: IC_{50} (μg/mL) values of different parts of *E. odoratum* for free radical scavenging activity by DPPH radical. A lower value indicates higher antioxidant activity.



It is noteworthy to mention here that ethanolic extracts of the leaf and defatted flower parts of *E. odoratum* showed the antioxidant activity on both *β*-carotene bleaching test and DPPH radical scavenging test which revealed as the most promising sources of natural antioxidants while stem and root were not significant in both tests. The active compounds for the scavenging activity of leaf could be either the phenolic substance or synergetic effect of phenolic substance and flavonoids. In the case of flower the scavenging activity might be due to the flavonoids. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides.[117]

Chapter 4

Summary & Conclusions

Brine shrimp cytotoxicity test for various root extracts/fractions of *E. odoratum* obtained from method I, revealed that petroleum ether extract (**F1**) and solid residue (**F2**) of ethanol extract were found to exhibit moderate cytotoxicity and high cytotoxicity against brine shrimp with LC_{50} of 237.27 and 81.13 μg/ml, respectively, while syrupy liquid residue (**F3**) of ethanol extract were found to exhibit non-cytotoxicity. On the other hand, hexane soluble subfraction (**F2-1**) and dichloromethane soluble subfraction (**F2-2**) of **F2** were found to have moderate cytotoxicity with LC_{50} of 263.16 and 207.77 μg/ml, respectively, while ethyl acetate soluble subfraction (**F2-3**) and 80% methanol soluble subfraction (**F2-4**) of **F2** were found non-cytotoxic (LC_{50} > 1000 μg/ml). Cytotoxicity of **F2** was found greater than that of its own subfractions **F2-1** and **F2-2**. This difference is very much likely to be due to synergetic effect of hexane soluble and dichloromethane soluble compounds.

Root extracts obtained from method II, namely petroleum ether extract (**E1**) and chloroform extract (**E2**) were found to exhibit moderate cytotoxicity against brine shrimps with LC_{50} of 279.79 and 302.54 μg/mL, respectively, while ethyl acetate extract (**E3**) and ethanol extract (**E4**) were found to exhibit non-cytotoxicity (LC_{50} > 1000 μg/ml).

Furthermore, hexane extract of the flower was found to exhibit moderate cytotoxicity with LC_{50} of 241.99 μg/mL while ethanol extracts of the leaf and the stem showed non-cytotoxicity against brine shrimp.

Root extracts, **E1**, **E2**, **E3** and **E4** obtained from method II were found to have no inhibitory effect against gram-positive bacteria viz *Bacillus subtilis*, *Staphylococcus aureus* and gram-negative bacteria viz. *Escherichia coli*, *Pseudomonas aeruginosa*. However hexane extract of flower was found to have weak activity against *B. subtilis* and *S. aureus* with zone of inhibition 22.2% and 16.6%, respectively, while no inhibitory effect was observed against *E. coli* and *P. aeruginosa*.

Phytochemical screening of the root showed the presence of fatty acids, sterols, polyphenols, reducing compounds, quinones, coumarin derivatives, tannins, saponins, carbohydrates and alkaloids (very likely to contain pyrrolizidine alkaloids).[25] On the other hand, volatile oils, carotenoids, coumarins, flavonoids, cardiac glycosides, emodins, cyanogenic glycosides, anthocyanosides and anthracenosides were not detected in the root.

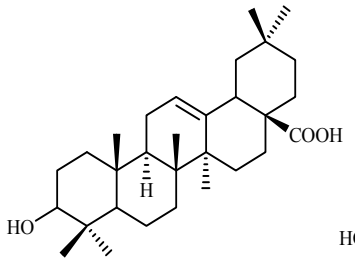
Phytochemical investigation on the petroleum ether extract of the root has resulted in the isolation of a novel triterpene, 3*β*-hydroxy-28-carboxyolean-12-ene which was designated as

eupatoric acid (**1**) along with seven known compounds viz poriferasterol (**2**), octadecane (**3**), butyrospermol acetate (**4**), bis(2-ethylhexyl)phthalate (**5**), chrysophanol (**6**), physcion (**7**) and palmitic acid (**8**) using column chromatography and preparative thin layer column chromatography techniques. Similarly chloroform extract has afforded bis(2-ethylhexyl)phthalate (**5**), chrysophanol (**6**), a mixture of bis(2-ethylhexyl)phthalate (**5**) and chrysophanol (**6**), a mixture of bis(2-ethylhexyl)phthalate (**5**) and physcion (**7**), poriferasterol (**2**), a mixture of poriferasterol (**2**) and *β*-sitosterol (**9**). In addition, anthraquinone (**10**) and hydrocarbon (**11**) were also isolated but was not further investigated due to paucity of material.

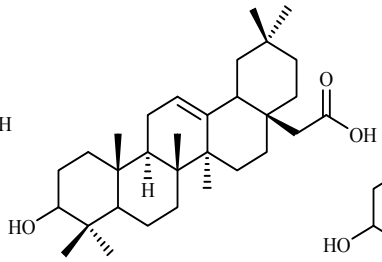
Structure elucidations of compounds (**1-11**) were based on spectroscopic methods, physical and chemical methods as well as literature comparison. Compounds **2-7** were reported here for the first time from this plant. Compounds **8** and **9**, known to be contained in leaf, were also isolated for the first time from the root.

Compounds **6** and **7** were found moderately cytotoxic with LC_{50} of 289.00 and 158.14 µg/mL, respectively, against brine shrimp while **5** showed weak cytotoxicity with LC_{50} of 538.15 µg/mL while compounds **1**, **2**, **3** and **4** were found non-cytotoxic against brine shrimp. Compounds **1**, **2**, **3**, **4**, **5**, **6** and **8** were found to have no antibacterial activity against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*.

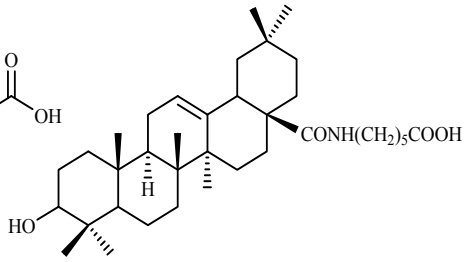
Ma *et al*.[118] reported that oleanolic acid (**12**) showed potent inhibitory activity of HIV protease (PR) with IC_{50} of 8 µM. This HIV PR has been regarded as one of the most promising targets for the development of anti-AIDS agents. Synthesized compound *viz*. N-[3*β*-hydroxyl-12-en-28-oyl]-6-aminohexanoic acid (**13)** showed an inhibitory activity with IC_{50} of 1.7 µM, more than four times the potency of **12**. This can be explained by the highly steric hindrance of the 28-carboxylic acid in **12** hampering the H-bond formation of oleanolic acid (**12**) with enzyme. An introduction of a 28-long acidic chain in **13** greatly reduced the steric hindrance, thus enabling this compound to form a bond with an enzyme stronger than that of **12** though the exact amino acid interacting is not clear at present. Based on this fact, eupatoric acid (**1**) can be expected to have more Anti-HIV-1 Protease activity than oleanolic acid. Hence further study of this compound is required for a better understanding of Anti-HIV-1 Protease activity mechanism.



Oleanolic acid (**12**)
IC_{50} (μM): 8



Eupatoric acid (**1**)
IC_{50} (μM): less than 8
(expected value)



N-[3*β*–hydroxyl-12-en-28-oyl]-6-aminohexanoic acid (**13**)
IC_{50} (μM): 1.7

The antifungal activity of physcion (**7**)[119] and chrysophanol (**6**)[120] and antiviral activity of chrysophanol (**6**)[121] have been reported in the recent past. As these compounds **6** and **7** were present in root portion of this plant further investigation of its root extract for antiviral and antifungal activities is desirable in future.

β-carotene bleaching test showed that ethanolic extracts of different parts of plant were found to be in the order of leaf >flower > stem > root. The RAA values of leaf, flower, stem and root were found to exhibit 0.95, 0.86, 0.35 and 0.22, respectively at 60 minutes. RAA values of leaf and flower were comparable to BHA whose RAA value was considered as 1.

DPPH radical scavenging test also showed that ethanolic extracts of different parts of plant were found to be in the order of flower > leaf > root > stem. Percentage of inhibition for flower, leaf, root and stem were found to exhibit 91.40, 87.93, 31.25 and 7.59, respectively at 500 μg of each plant extracts. These activities were found to decrease as the concentrations of extracts decrease. However, flower was found to have good antioxidant activity with 65.66 percentage inhibition even at 200 μg of its extract. IC_{50} of defatted flower and leaf have shown 50.9 and 65.4 μg/mL, which were comparable to BHA (44.5 μg/mL).

Based on methods viz *β*-carotene bleaching test and DPPH radical scavenging test, we report that leaf and flower parts contained the most promising sources of natural antioxidants while stem and root portions showed very weak antioxidant activity with respect to a synthetic antioxidant, butylated hydroxyanisole (BHA). Internet search also revealed that the plant has been used as herbal tea for health which relieves cough, fever, jaundice, hemorrhoids and stomachache and also promotes physical strength.[9] Hence, leaf and flower can be used as

potent herbal tea for health to prevent and/or cure cancer. This antioxidant activity might be due to various types of flavonoids found in leaf and flower of this plant. Quercetin has been established as a strong antioxidant principle and had been used as standard in antioxidant experiments.[55] Flavonols are known as important compounds in terms of radical scavenging properties.[116] Therefore, further investigation on isolation and identification of active compounds from this plant is required for a better understanding of oxidative mechanism involved and for possible application as dietary supplements.

Literature survey revealed that n-dibutyl phthalate (DBP) showed phytotoxic effect in corn (*Zea mays*) at high dose (>2000 ppmw).[98] Surprisingly, bis(2-ethylhexyl)phthalate (**5**) was isolated from the root of *E. odoratum* during the embodiment of this thesis. This compound might be one of the allelochemicals which diffused from root to surrounding areas inhibiting the growth of other plants. However, Wang *et al*.[122] pointed out that the occurrence of **5** was indicator of environmental pollution since **5** is widely used in plastic industries. But the occurrence of bis(2-ethylhexyl)phthalate (**5**) in a wide variety of natural sources[123-129] indicated a natural phenomenon. About 2.77% of *o*-phthalic acid (1,2-benzenedicarboxylic acid) has been also reported from the leaf of *E. odoratum*[30] which might be formed due to complete hydrolysis of bis(2-ethylhexyl)phthalate. Determination of phthalic acid in *Papaver* and *Vitis*, were made before the widespread use of these compounds.[130] Hence, all these facts are clearly convincing that the occurrence of **5** from the root of *E. odoratum* is natural product, not contamination product from plasticizers.

Chapter 5

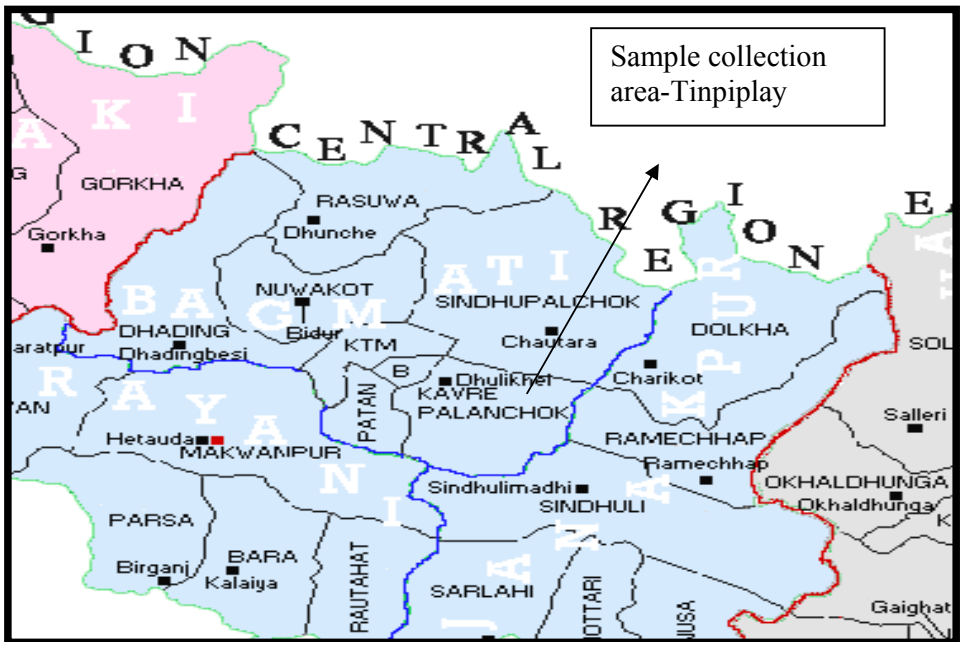
EXPERIMENTALS

5.1 GENERAL

All the melting points reported were uncorrected and determined on either Mettler FP61 instrument or on the Thiele apparatus containing liquid paraffin oil, unless otherwise stated. IR spectra were recorded either in potassium bromide disc or chloroform on Shimadzu spectrophotometer and Perkin Elmer 1310 infrared spectrophotometer. UV spectra were measured on a Chemito UV-VIS 2500 spectrophotometer. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz), DEPT were recorded on either JEOL GX-400 or JEOL AL400 using $CDCl_3$ as solvent.with TMS as internal standard. FAB mass spectrum was recorded on a JEOL LMS-700T Spectrometer with glycerol as matrix. However, ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz), APT as well as EIMS were recorded at the University of Göttingen, Germany. ^{1}H-NMR (100 MHz) and ^{13}C-NMR (25 MHz) were recorded at the University of Wisconsin-La Crosse, USA. HRMS and EIMS for eupatoric acid (**1**) were recorded on Finnigan Mat SSQ 710 (70 eV) and VG7035 instruments respectively at Institute of Chemical and Engineering Sciences (ICES), Singapore. Mass of **1** was calculated from CHEM ULTRA DRAW software 8.0 version. GCMS were recorded on Agilent 5973N plus 6890N using ethyl acetate as a solvent at Tohoku University, Aoba-yama Campus, Japan. The chemical shifts are reported relative to internal standard, tetramethylsilane (TMS) and expressed in δ scale in ppm. Splitting are designated as **s**, singlet; **d**, doublet; **dd**, double doublet; **m**, multiplet. Spin coupling constant values are expressed in hertz (Hz).

5.2 PLANT MATERIALS

Root, leaf, flower and stem of the *Eupatorium odoratum* L. were collected on July 2001 from *TINPIPLAY, KAVRE*. Plant was authenticated in the Central Department of Botany, Tribhuvan University and its voucher herbarium specimen (19-TUCH) was deposited at Tribhuvan University Central Herbarium, Central Department of Botany, Tribhuvan University, Nepal.



5.3 PHYTOCHEMICAL SCREENING OF THE ROOT

5.3.1 Preparation of extracts

Preparation of petroleum ether extract: Air dried and powdered root (25.00 g) was extracted with petroleum ether (60-80 °C) (300 mL) in Soxhlet extractor for 7 hours. The extract solution was concentrated on Rotavapor. Thus concentrated petroleum ether extract was subjected to screening tests. The marc left was air dried and used further for extraction by ethanol.

Preparation of ethanol extract: The air dried marc obtained above was extracted with ethanol (300 mL) in Soxhlet extractor for 7 hours. The extract solution was concentrated on Rotavapor and the concentrated solution was subjected to screening tests. The marc left was again air dried and used further for extraction by water.

Preparation of aqueous extract: The dried marc obtained above was further extracted with water (300 mL) in Soxhlet extractor for 7 hours. The extract solution was concentrated on Rotavapor and the concentrated solution was used for screening tests. The marc left was now discarded.

5.3.2 Screening test of petroleum ether extract

1. Test for volatiles oils: The extract (4 mL) was evaporated to yield a residue. To this residue, methanol (1 mL) was added and shaken vigorously and filtered. Few drops of the filtrate were spotted on a filter paper. Appearance of the spot on the paper after evaporation of solvent indicated the absence of volatile oils.

2. Test for basic alkaloids: The extract (10 mL) was evaporated to yield residue, which was dissolved in 2% (v/v) aqueous hydrochloric acid (3 mL). This solution was equally divided into two test tubes.

a) **Mayer's test:** The first test solution was treated with Mayer's reagent (3 drops). No precipitate appeared indicating the absence of alkaloids.

b) **Dragendorff's test:** The second test solution was treated with Dragendorff's reagent (3 drops). No precipitate appeared indicating the absence of alkaloids.

Saponification of the Petroleum ether extract:

The remaining petroleum ether extract solution was re-extracted thrice with 5% (w/v) aqueous potassium hydroxide (5 mL) in a separating funnel. The combined upper solution (A) and lower alkaline solution were separated. The combined upper layer (A) was divided into two test tubes for the screening test numbers 3 and 4. The lower combined alkaline layer was acidified with concentrated hydrochloric acid until acidic and re-extracted thrice with diethyl ether (10 mL). Thus obtained combined upper ether layer (B) was used for the screening test numbers 5, 6, 7 and 8. The lower acidic layer was discarded.

3. Test for carotenoids: The solution A was concentrated and treated with concentrated sulphuric acid (1 mL). Red colour was not developed on the upper ether layer indicating the absence of carotenoids.

4. Test for sterols and triterpenes (Liebermann-Burchard's test): The solution A was concentrated to yield a residue, which was dissolved in acetic anhydride (1 mL) and chloroform (1 mL). To this solution, concentrated sulphuric acid (2 mL) was added from the side of the test tube without disturbing the contents. Development of green color in upper layer indicated the presence of sterols.

5. Test for fatty acids: The solution B (2 mL) was concentrated and then few drops of concentrated solution B were spotted on a filter paper. Persistence of yellow spot after evaporation indicated the presence of fatty acid.

6. Test for coumarins: The solution B (4 mL) was concentrated to yield residue, which was dissolved in hot water. After cooling, the solution was divided into two test tubes. The first test tube was used as control. To the second test tube, 10% (v/v) ammonium hydroxide solution was added drop by drop until alkaline. It was observed under UV light in 366 nm and the fluorescence was not observed indicating the absence of coumarins.

7. Test for flavone aglycones: The solution B (10 mL) was concentrated to yield a residue, dissolved in ethanol (4 mL). The ethanol solution was equally divided into two test tubes.
a) **Shinoda's test:** The first test solution was treated with a pinch of magnesium powder in presence of concentrated hydrochloric acid (5 drops). No red coloration was observed which indicated the absence of flavone aglycones.
b) **Shibata's test:** The second solution was treated with a pinch of zinc dust in presence of concentrated hydrochloric acid (5 drops). No red coloration was observed which indicated the absence of flavone aglycones.

8. Test for emodins (Borntiager's test): The solution B (2 mL) was treated with 25 % (v/v) ammonium hydroxide solution (1 mL) and shaken vigorously. The test tube was allowed to stand for few minutes to separate two layers. The petroleum ether layer was neither decolorized nor the alkaline layer gained red color indicating the absence of emodins.

5.3.3 Screening tests of ethanol extract

9. Test for polyphenols and tannins (Ferric chloride test): The ethanol extract (1 mL) was mixed with water (1 mL). To this solution, 1% (w/v) ferric chloride solution (3 drops) was added. Development of blue-black (or green or violet) color indicated the presence of tannins or polyphenols.

10. Test for reducing compounds (Fehling's test): The ethanol extract (1 mL) was mixed with water (1 mL). To this solution, Fehling's solution was added. The mixture was heated over a water bath. Appearance of red color precipitate indicated the presence of reducing compounds.

11. Test for alkaloid: The ethanol extract (10 mL) was concentrated to yield residue. To this residue, 2% (v/v) aqueous hydrochloric acid (5 mL) was added and shaken vigorously, then filtered. The filtrate was treated with 10% (v/v) ammonium hydroxide solution until pH 8. The solution was extracted thrice with chloroform (10 mL). The upper alkaline layer was discarded. The lower chloroform layer was concentrated and then added aqueous hydrochloric acid (2% v/v, 5 mL). Thus obtained solution was equally divided into two test tubes.

a) **Mayer's test:** The first test solution was treated with Mayer's reagent (3 drops). No precipitate indicated the absence of alkaloids.

b) **Dragendorff's test:** The second test solution was treated with Dragendorff's reagent (3 drops). Appearance of precipitate indicated the presence of alkaloids.

12. Test for glycosides: The ethanolic extract (4 mL) was concentrated to half of the original volume and then treated with ammonium hydroxide solution (25% v/v, 2 mL) and was shaken vigorously. A cherry red color was not observed indicating the absence of glycosides.

Hydrolysis of the ethanol extract

The remaining ethanol extract was hydrolyzed by refluxing with equal volume of aqueous hydrochloric acid (10% v/v) for 30 minutes. After cooling, the hydrolyzed extract was re-extracted thrice with diethyl ether (10 mL). The lower acidic layer was used for the screening test 13. The upper ether layer was dried over anhydrous sodium sulphate and filtered. The ether filtrate was used for the screening test numbers 14, 15, 16 and 17.

13. Test for anthocyanosides: The lower red acidic layer (4 mL) was treated with solid sodium carbonate until basic to litmus paper. A red color was not discharged indicating the absence of anthocyanosides.

14. Test for anthracenosides: The ether solution (2 mL) was treated with 25% (v/v) ammonium hydroxide solution (1 mL) and was shaken vigorously. The test tube was allowed to stand for few minutes to separate two layers. A red color in the alkaline layer was not developed indicating the absence of anthracenosides.

15. Test for coumarin derivatives: The ether solution (4 mL) was concentrated to yield a residue, which was dissolved in hot water. After cooling, the solution was divided into two test tubes. The first test tube was used as a control. To the second test tube, 10% (v/v)

ammonium hydroxide was added drop by drop until it became alkaline and was observed under UV light. Fluorescence was observed indicating the presence of coumarin derivatives.

16. Test for flavonic glycosides: The ether solution (10 mL) was concentrated to yield a residue, which was dissolved in ethanol (4 mL). The ethanol solution was equally divided into two test tubes.

(a) **Shinoda's test:** The first test solution was treated with a pinch of magnesium powder in presence of concentrated hydrochloric acid (5 drops). No red colouration was seen indicating the absence of flavonic glycosides.

(b) **Shibata's test:** The second test solution was treated with a pinch of zinc dust in presence of concentrated hydrochloric acid (5 drops). Red colouration was not seen indicating the absence of flavonic glycosides.

17. Test for cardiac glycosides (Kedde's test): The ether solution (4 mL) was concentrated to yield a residue, which was dissolved in methanol (2 mL). To this solution, 1% (w/v) methanolic potassium hydroxide (1 mL) and 1% (w/v) methanolic solution of 3,5-dinitrobenzoic acid (3 drops) was added. The mixture was warmed gently. No violet colour was observed showing the absence of cardiac glycosides.

5.3.4 Screening tests of aqueous extract

18. Test for saponins (Froth test): The aqueous extract (2 mL) was shaken vigorously for 30 seconds in a test tube of 1 cm diameter. Persistence of thick froth (about 1 cm) height even after 30 seconds indicated the presence of saponins.

19. Test for polyphenols/tannins (Ferric chloride test): The aqueous extract (2 mL) was treated with 1% (w/v) ferric chloride solution (3 drops). Development of bluish-green color indicated the presence of tannins and or polyphenols.

20. Test for quinones (Ammonium thiocyanate test): The aqueous extract (0.5 mL) was treated with colourless ferrous sulphate solution (0.5 mL) and few grains of ammonium thiocyanate crystals. To this mixture, one drop of concentrated sulphuric acid was added. Persistence of deep red colour indicated the presence of quinones.

21. Test for carbohydrates (Molisch's test): The aqueous extract (1 mL) was treated with Molisch's reagent (0.5 mL) and a few mL of concentrated hydrochloric acid was poured

along the side of the test tube. Appearance of violet ring produced at the junction of the two liquids showed the presence of glycosides or free sugars.

22. Test for alkaloid: The aqueous extract (10 mL) was treated with 10% (v/v) ammonium hydroxide solution until basic and extracted three times with chloroform (5 mL). The upper alkaline layer was discarded. The lower chloroform layer was concentrated and to this, 2% (v/v) aqueous hydrochloric acid (5 mL) was added. Thus obtained solution was equally divided into two test tubes.

a) **Mayer's test:** The first test solution was treated with Mayer's reagent (3 drops). Appearance of turbidity indicated the presence of alkaloids.

b) **Dragendorff's test:** The second test solution was treated with Dragendorff's reagent (3 drops). Appearance of heavy precipitate indicated the presence of alkaloids.

23. Test for cyanogenic glycosides (Guignard's test): Air dried and powdered root (0.005 g) was placed in Erlenmeyer flask with a tight lid. To this, water (5 mL) and chloroform (2 mL) were added. The flask was then firmly corked with a freshly prepared sodium picrate-paper suspended inside enough above the samples. It is incubated at 40 °C for 3 hours and was left at room temperature for 48 hours and re-examined. No development of the red color or the shades on the yellow colored picrate-paper indicated the absence of cyanogenic glycosides.

5.4 BIOASSAY OF THE ROOT

Two methods: method I and method II as shown in scheme 3.1 and 3.2, respectively, were used for the preparation of extracts of root in various solvents.

5.4.1 Preparation of extracts - Method I

Powdered root (50 g × 4) was defatted with petroleum ether (40-60 °C) (300 mL × 4) in a Soxhlet extractor for 7 hours. Extract solutions were combined and evaporated to obtain yellow oil (**F1,** 0.635 g). The dried defatted marc was then extracted with ethanol (500 mL) in Soxhlet extractor for 7 hours in two batches (100 g × 2). Combined extracts were concentrated on Rotavapor at reduced pressure. Thus concentrated ethanol extract was treated with water and filtered off to separate solid residue (**F2,** 1.45 g) and filtrate. The filtrate was evaporated to dryness to yield reddish brown syrupy liquid (**F3,** 20.46 g). The solid residue

(**F2**) was subsequently fractionated by dissolving in succession in hexane, dichloromethane, ethyl acetate and 80% aqueous methanol as depicted in the scheme 5.1. The successive fractionation yielded hexane soluble subfraction (**F2-1**, 0.151 g**)**, dichloromethane soluble subfraction (**F2-2**, 0.341 g), ethyl acetate soluble subfraction (**F2-3**, 0.035 g), and 80% methanol soluble subfraction (**F2-4**, 0.240 g). The black insoluble substance left over did not dissolve in solvents such as methanol and dimethyl sulphoxide (DMSO). Therefore, it was not used further for investigation.

Fractions **F1**, **F2** and its subfractions **F2-1**, **F2-2**, **F2-3**, **F2-4** and fraction **F3** were used for brine shrimp cytotoxicity test.

5.4.1.1 Brine shrimp cytotoxicity test

5.4.1.1a Preparation of sample solutions

Solutions of fractions **F1**, **F2**, **F3** and subfractions **F2-1**, **F2-2**, **F2-3** and **F2-4** were prepared by **non-dilution method**.[68] An amount of 50 mg of each fraction of **F1**, **F2**, **F2-1** and **F2-2** were separately dissolved and diluted to 5 mL solution with chloroform in a 5-mL measuring cylinder. Similarly, 50 mg of each extract of **F2-3**, **F2-4** and **F3** were separately dissolved and diluted to 5 mL solution with methanol in a 5-mL measuring cylinder. Each solution of extracts above corresponded to the concentration of 10 mg/mL.

5.4.1.1b Bioassay procedure

A volume of 500, 50 and 5 μL of each sample solution was transferred to separate test tubes (size: 10 cm × 1). Five replicates were made for each dose level. Controls were also prepared by using respective solvent alone as the volume of sample solution taken for each dose level. The content of each test tube was concentrated on Rotavapor at reduced pressure for about 25 minutes to remove solvents completely at temperature 50 °C. A volume of 4 mL of artificial sea water was added into each test tube above. Ten shrimp were transferred in each test tube *via* a Pasteur pipette and adjusted the volume to 5 mL with artificial sea water. As a result, the final concentration of solution in test tubes with 500, 50 and 5 μL of extract solutions will be 1000, 100 and 10 μg/mL, respectively. Altogether, there were eighteen test tubes. They were kept in room illuminated using 15W bulb maintaining temperature 20-22 °C for 24 hours. The number of survivors was counted after 24 hours and determined the number of deaths in each dose level. Brine shrimp were considered dead if they did not exhibit any movement during 10 seconds of observation. Any brine shrimp death in control test tube was

eliminated and repeated the whole experiment. LC_{50} values in terms of μg/mL were calculated by probit analysis.[65] Experiments were done thrice in total only for that extract which showed LC_{50} lesser than 1000 μg/mL. LC_{50} value of such extract was expressed in μg/mL as mean of three independently performed experiments with 95% confidence interval. Berberine was used as positive control.

Result and calculation: Number of death or survival of shrimp counted after 24 hours and LC_{50} values were shown in table 5.1 to 5.6.

Table 5.1: Cytotoxicity of petroleum ether extract (**F1**) of the root against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	1000 (µg/mL) D	100 (µg/mL) S	100 (µg/mL) D	10 (µg/mL) S	10 (µg/mL) D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
		S	D	S	D	S	D										
	C	10	0	0	0	0	0	Log 10 = 1	0.0	0.0	1						
	1	0	10	10	0	10	0	Log 100 = 2	0.2	0.4	4						
	2	0	10	10	0	10	0	Log 1000 = 3	10.0	30.0	9	- 6.60	5.0	2.32	208.92		
1	3	0	10	10	0	10	0										
	4	0	10	9	1	10	0										
	5	0	10	10	0	10	0										
		ΣD = 50		ΣD = 1		ΣD = 0		Σx = 6	Σy = 10.2	Σx y = 30.4	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	0	10	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	2	0	10	10	0	10	0	Log 100 = 2	0.2	0.4	4	- 5.93	4.5	2.42	263.02	237.27 ± 27.14	304.69-169.85
2	3	0	10	10	0	10	0	Log 1000 = 3	9.0	27.0	9						
	4	3	7	10	0	10	0										
	5	2	8	9	1	10	0										
		ΣD = 45		ΣD = 1		ΣD = 0		Σx = 6	Σy = 9.2	Σx y = 27.4	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	0	10	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	2	0	10	10	0	10	0	Log 100 = 2	0.8	1.6	4	- 5.73	4.5	2.38	239.80		
3	3	0	10	10	0	10	0	Log 1000 = 3	9.0	27.0	9						
	4	0	10	6	4	10	0										
	5	5	5	10	0	10	0										
		ΣD = 45		ΣD = 4		ΣD = 0		Σx = 6	Σy = 9.8	Σx y = 28.6	$\Sigma x^2 = 14$						

Note: Berberine was used as positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.2: Cytotoxicity of solid residue (**F2**) of the root against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL)		100 (µg/mL)		10 (µg/mL)		Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
		S	D	S	D	S	D										
	C	10	0	10	0	0	0	Log 10 = 1	0.0	0.0	1						
	1	1	9	2	8	10	0	Log 100 = 2	8.4	16.8	4	- 3.60	4.8	1.79	61.66		
	2	1	9	2	8	10	0	Log 1000 = 3	9.6	28.8	9						
1	3	0	10	2	8	10	0										
	4	0	10	1	9	10	0										
	5	0	10	1	9	10	0										
		ΣD = 48		ΣD = 42		ΣD = 0		Σx = 6	Σy = 18.4	Σx y = 45.6	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	3	7	3	7	10	0	Log 10 = 1	0.0	0.0	1						
	2	1	9	1	9	10	0	Log 100 = 2	6.6	13.2	4	- 3.26	4.1	2.01	102.32	81.13 ± 20.38	131.76 - 30.50
2	3	1	9	1	9	10	0	Log1000 = 3	8.2	24.6	9						
	4	3	7	3	7	10	0										
	5	1	9	1	9	10	0										
		ΣD = 41		ΣD = 33		ΣD = 0		Σx = 6	Σy = 14.8	Σx y = 37.8	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	1	9	3	7	10	0	Log 10 = 1	0.0	0.0	1						
	2	0	10	3	7	10	0	Log 100 = 2	6.8	13.6	4	- 4.33	4.9	1.90	79.43		
3	3	0	10	2	8	10	0	Log1000 = 3	9.6	28.8	9						
	4	1	9	4	6	10	0										
	5	0	10	4	6	10	0										
		ΣD = 48		ΣD = 34		ΣD = 0		Σx = 6	Σy = 16.4	Σx y = 42.4	$\Sigma x^2 = 14$						

Note: Berberine was used as positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.3: Cytotoxicity of hexane soluble subfraction (**F2-1**) of **F2** against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	1000 (µg/mL) D	100 (µg/mL) S	100 (µg/mL) D	10 (µg/mL) S	10 (µg/mL) D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
		S	D	S	D	S	D										
	C	10	0	0	0	0	0	Log 10 = 1	0.0	0.0	1						
	1	1	9	10	0	10	0	Log 100 = 2	0.0	0.0	4	- 6.13	4.6	2.41	257.03		
	2	0	10	10	0	10	0	Log 1000 = 3	9.2	27.6	9						
1	3	0	10	10	0	10	0										
	4	2	8	10	0	10	0										
	5	1	9	10	0	10	0										
		ΣD = 46		ΣD = 0		ΣD = 0		Σx = 6	Σy = 9.2	Σx y = 27.6	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	0	10	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	2	1	9	10	0	10	0	Log 100 = 2	0.0	0.0	4	- 6.13	4.6	2.41	257.03	263.16 ± 10.61	289.51-236.81
2	3	1	9	10	0	10	0	Log1000 = 3	9.2	27.6	9						
	4	1	9	10	0	10	0										
	5	1	9	10	0	10	0										
		ΣD = 46		ΣD = 0		ΣD = 0		Σx = 6	Σy = 9.2	Σx y = 27.6	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	0	10	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	2	2	8	10	0	10	0	Log 100 = 2	0.0	0.0	4	- 5.53	4.3	2.44	275.42		
3	3	1	9	10	0	10	0	Log1000 = 3	9.2	27.6	9						
	4	2	8	9	1	9	1										
	5	1	9	10	0	10	0										
		ΣD = 44		ΣD = 1		ΣD = 1		Σx = 6	Σy = 9.2	Σx y = 27.6	$\Sigma x^2 = 14$						

Note: Berberine was used as positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.4: Cytotoxicity of dichloromethane soluble subfraction (**F2-2**) of **F2** against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	1000 (µg/mL) D	100 (µg/mL) S	100 (µg/mL) D	10 (µg/mL) S	10 (µg/mL) D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
		S	D	S	D	S	D										
	C	10	0	0	0	0	0	Log 10 = 1	0.2	0.2	1						
	1	0	10	9	1	10	0	Log 100 = 2	0.8	1.6	4	- 6.13	4.9	2.27	190.54		
	2	0	10	9	1	10	0	Log 1000 = 3	10.0	30.0	9						
1	3	0	10	10	0	10	0										
	4	0	10	9	1	10	0										
	5	0	10	9	1	9	1										
		ΣD = 50		ΣD = 4		ΣD = 1		Σx = 6	Σy = 11.0	Σx y = 31.8	$\Sigma x^2 = 14$						
	C																
	1	0	10	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	2	0	10	10	0	10	0	Log 100 = 2	0.4	0.8	4	- 6.40	4.9	2.32	208.92	207.77 ± 16.69	249.28-166.26
2	3	0	10	8	1	10	0	Log 1000 = 3	9.8	29.4	9						
	4	1	9	8	1	10	0										
	5	0	10	10	0	10	0										
		ΣD = 49		ΣD = 2		ΣD = 0		Σx = 6	Σy = 10.2	Σx y = 30.2	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	0	10	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	2	0	10	10	0	10	0	Log 100 = 2	0.0	0.0	4	- 6.53	4.9	2.35	223.87		
3	3	0	10	10	0	10	0	Log 1000 = 3	9.8	29.4	9						
	4	1	9	10	0	10	0										
	5	0	10	10	0	10	0										
		ΣD = 49		ΣD = 0		ΣD = 0		Σx = 6	Σy = 9.8	Σx y = 29.4	$\Sigma x^2 = 14$						

Note: Berberine was used as positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.5: Cytotoxicity of ethyl acetate soluble subfractions (**F2-3**), methanol soluble subfractions (**F2-4**) of **F2** and syrupy residue (**F3**) of the the root against brine shrimp

Experiment	Extract	Replicate	Dose levels (Z)						Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X
			1000 (µg/mL)		100 (µg/mL)		10 (µg/mL)									
			S	D	S	D	S	D								
1	**F2-3**	C	10	0	0	0	0	0	log 10 = 1	0.0	0.0	1	- 0.66	0.5	11.3	1.9×10^{11}
		1	8	2	10	0	10	0	log 100 = 2	0.0	0.0	4				
		2	9	1	10	0	10	0	log1000 = 3	1.0	3.0	9				
		3	9	1	10	0	10	0								
		4	9	1	10	0	10	0								
		5	10	0	10	0	10	0								
			ΣD = 5		ΣD = 0		ΣD = 0		Σx = 6	Σy = 1.0	Σxy = 3.0	$\Sigma x^2 = 14$				
2	**F2-4**	C	10	0	10	0	10	0					- 0.40	0.3	18	1×10^{18}
		1	10	0	10	0	10	0	log 10 = 1	0.0	0.0	1				
		2	9	1	10	0	10	0	log 100 = 2	0.0	0.0	4				
		3	8	2	10	0	10	0	log1000 = 3	0.6	1.8	9				
		4	10	0	10	0	10	0								
		5	10	0	10	0	10	0								
			ΣD = 3		ΣD = 0		ΣD = 0		Σx = 6	Σy = 0.6	Σxy = 1.8	$\Sigma x^2 = 14$				
3	**F3**	C	10	0	10	0	10	0					- 2.20	1.7	4.23	1.6×10^{4}
		1	8	2	9	1	10	0	log 10 = 1	0.0	0.0	1				
		2	4	6	10	0	10	0	log 100 = 2	0.2	0.4	4				
		3	7	3	10	0	10	0	log1000 = 3	3.4	10.2	9				
		4	10	0	10	0	10	0								
		5	4	6	10	0	10	0								
			ΣD = 17		ΣD = 1		ΣD = 0		Σx = 6	Σy = 3.6	Σxy =10.6	$\Sigma x^2 = 14$				

Note: Berberine was used as positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.6: Cytotoxicity of berberine (positive control) against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	D	100 (µg/mL) S	D	10 (µg/mL) S	D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
1	C	10	0	10	0	10	0	Log 10 = 1	1.6	1.6	1	- 0.80	3	1.93	85.11	89.18 ± 4.10	99.36 - 79.0
	1	2	8	3	7	10	0	Log 100 = 2	6.4	12.8	4						
	2	2	8	3	7	8	2	Log 1000 = 3	7.6	22.8	9						
	3	2	8	4	6	8	2										
	4	3	7	5	5	8	2										
	5	3	7	3	7	8	2										
		ΣD = 38		ΣD = 32		ΣD = 8		Σx = 6	Σy = 15.6	Σxy = 37.2	$\Sigma x^2 = 14$						
2	C	10	0	10	0	10	0	Log 10 = 1	1.8	1.8	1	- 0.46	2.8	1.95	89.12		
	1	2	8	3	7	8	2	Log 100 = 2	6.2	12.4	4						
	2	3	7	5	5	6	4	Log 1000 = 3	7.4	22.2	9						
	3	3	7	3	7	10	0										
	4	2	8	4	6	10	0										
	5	3	7	4	6	7	3										
		ΣD = 37		ΣD = 31		ΣD = 9		Σx = 6	Σy = 10.2	Σxy = 36.4	$\Sigma x^2 = 14$						
3	C	10	0	10	0	10	0	Log 10 = 1	2.6	2.6	1	1.06	2.0	1.97	93.32		
	1	2	8	2	8	8	2	Log 100 = 2	6.0	12.0	4						
	2	2	8	3	7	7	3	Log 1000 = 3	6.6	19.8	9						
	3	2	8	4	6	8	2										
	4	6	4	5	5	7	3										
	5	5	5	6	4	7	3										
		ΣD = 33		ΣD =30		ΣD = 13		Σx = 6	Σy = 15.2	Σxy = 34.4	$\Sigma x^2 = 14$						

Note: Berberine was used as positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

Remarks: Fractions **F1, F2** and subfractions **F2-1, F2-2** showed LC_{50} of 237.27, 81.13, 263.16 and 207.77 µg/mL, respectively. Their LC_{50} values were lesser than 1000 µg/mL indicating cytotoxicity against *Artemia salina* (brine shrimp). Fraction **F3,** subfractions **F2-3** and **F2-4** displayed LC_{50} of 1.9×10^{11}, 1×10^{18} and 1.6×10^{4} µg/mL, respectively. Their LC_{50} values were greater than 1000 µg/mL indicating non-cytotoxicity. Berberine, a positive control, displayed LC_{50} of 89.18 µg/mL.

5.4.1.2 Phytochemical screening of cytotoxic (F1, F2, F2-1, F2-2) and non-cytotoxic (F2-3, F2-4, F3) fractions

Different fractions of cytotoxic and noncytotoxic were dissolved in methanol and the soluble portion was used for phytochemical screening. The screening tests were carried out as described in literature[60], in ignition tube unless otherwise it was stated.

Petroleum ether extract (**F1**)

1. **Test for quinones:** Quinones in petroleum ether extract were tested as outlined in Harborne (1973). A solution of extract (**F1**) in chloroform was chromatographed in microscopic glass plate in benzene. TLC plate when sprayed with 10% methanolic potassium hydroxide solution, a red spot (R_f 0.57 in benzene) was appeared indicating the presence of anthraquinone.

2. **Test for sterols and triterpenes (Liebermann-Burchard test):** The filtrate (2 drops) was treated with acetic anhydride (2 drops) and concentrated sulphuric acid (3 drops). Formation of green coloration indicated the presence of sterols.

3. **Test for polyphenols (Ferric chloride test):** The filtrate (2 drops) was treated with ferric chloride (2 drops) solution. No green coloration indicated the absence of polyphenols.

4. **Test for tannins (potassium ferricyanide test):** The filtrate (2 drops) was treated with 25% ammonia (4 drops) and potassium ferricyanide solution (3 drops). No deep red coloration indicated the absence of tannins.

5. **Test for carbohydrate (Molisch's test):** The filtrate (5 drops) was treated with Molisch's reagent (2 drops) and a few drops (5 drops) of concentrated sulphuric acid were poured slowly. No violet ring produced at the junction of the two liquids indicating the absence of glycosides or free sugars.

6. **Test for reducing sugars (Fehling's solution):** The filtrate (0.5 mL) was treated with Fehling's solution (1 mL) in a test tube. The whole mixture was heated strongly on water bath. No red precipitate was formed showing the absence of reducing sugars.

7. **Test for alkaloid (Dragendorff's reagent):** The filtrate (2 drops) was treated with Dragendorff's reagent (1 drop). No distinct precipitate was formed showing the absence of alkaloids.

8. **Test for flavonoids**:

(a) **Shinoda's test:** Filtrate solution (1 mL) was treated with a pinch of magnesium powder in presence of concentrated hydrochloric acid (5 drops). No red colouration was seen indicating the absence of flavonoids.

(b) **Shibata's test:** Extracts solution of leaf and stem were treated with a pinch of zinc dust in presence of concentrated hydrochloric acid (5 drops) separately. No red colouration was observed indicating the absence of flavonoids.

Solid residues of ethanol extract (**F2**)

1. **Test for quinones (Ammonium thiocyanate test):** The filtrate (1 mL) was treated with a colorless solution of ferrous sulphate (1 mL), ammonium thiocyanate crystals (1 or 2 grains) and concentrated sulphuric acid (1 drop) in a test tube. Formation of red coloration showed the presence of quinones

2. **Test for sterols and triterpenes (Liebermann-Burchard test):** Formation of green coloration indicated the presence of sterols.

3. **Test for polyphenols (Ferric chloride):** Neither green nor blue or violet coloration was formed showing the absence of polyphenols.

4. **Test for tannins (potassium ferricyanide test):** No deep red coloration was observed indicating the absence of tannins.

5. **Test for carbohydrate (Molisch's test):** Formation of violet ring at the junction of the two liquids showed the presence of glycosides or free sugars.

6. **Test for reducing sugars (Fehling's solution test):** Red precipitate was formed showing the presence of reducing sugars.

7. **Test for alkaloid (Dragendorff's test):** The filtrate was treated with Dragendorff's reagent. Appearance of heavy precipitate showed the presence of alkaloids.

8. **Test for flavonoids**:

(a) **Shinoda's test:** No red colouration was seen indicating the absence of flavonoids.

(b) **Shibata's test:** No red colouration was observed indicating the absence of flavonoids.

Syrupy liquid residue (**F3**)

1. **Test for quinones (Ammonium thiocyanate test):** Formation of red coloration showed the presence of quinones

2. **Test of sterols and triterpenes (Liebermann-Burchard test):** No green coloration indicated the absence of sterols.

3. **Test of polyphenols (Ferric chloride test):** Green coloration was formed showing the presence of polyphenols.

4. **Test for tannins (Potassium ferricyanide test):** Deep red coloration was observed indicating the presence of tannins.

5. **Test for carbohydrates (Molisch's test):** No violet ring was produced showing the absence of glycosides or free sugars.

6. **Test for reducing sugars (Fehling's solution test):** Red precipitate was formed showing the presence of reducing sugars.

7. **Test for alkaloids (Dragendorff's test):** No precipitate was formed showing the absence of alkaloids.

8. **Test for flavonoids**:

(a) **Shinoda's test:** No red colouration was seen indicating the absence of flavonoids.

(b) **Shibata's test:** No red colouration was observed indicating the absence of flavonoids.

Hexane soluble subfraction (**F2-1**)

1. **Test for quinones (Ammonium thiocyanate test):** Formation of red coloration showed the presence of quinones

2. **Test of sterols and triterpenes (Liebermann-Burchard test):** Green coloration indicated the presence of sterols.

3. **Test for tannins (Potassium ferricyanide test):** Deep red coloration was observed indicating the absence of tannins.

4. **Test for carbohydrates (Molisch's test):** No violet ring was produced showing the absence of glycosides or free sugars.

5. **Test for reducing sugars (Fehling's solution test):** No red precipitate was formed showing the absence of reducing sugars.

6. **Test for alkaloids (Dragendorff's test):** No precipitate was formed showing the absence of alkaloids.

7. **Test for flavonoids**:

(a) **Shinoda's test:** No red colouration was seen indicating the absence of flavonoids.

(b) **Shibata's test:** No red colouration was observed indicating the absence of flavonoids.

Dichloromethane soluble subfraction (**F2-2**)

1. **Test for quinones (Ammonium thiocyanate test):** Formation of red coloration showed the presence of quinones

2. **Test of Sterols and triterpenes (Liebermann-Burchard test):** Green coloration indicated the presence of sterols.

3. **Test for carbohydrates (Molisch's test):** No violet ring was produced showing the absence of glycosides or free sugars.

4. **Test for reducing sugars (Fehling's solution test):** No red precipitate was formed showing the absence of reducing sugars.

5. **Test for Alkaloids (Dragendorff's test):** Heavy precipitate was formed showing the presence of alkaloids.

6. **Test for flavonoids**:

(a) **Shinoda's test:** No red colouration was seen indicating the absence of flavonoids.

(b) **Shibata's test:** No red colouration was observed indicating the absence of flavonoids.

Ethyl acetate soluble subfraction (**F2-3**)

1. **Test for quinones (Ammonium thiocyanate test):** No red coloration showed the absence of quinones.

2. **Test for sterols and triterpenes (Liebermann-Burchard test):** No green coloration indicated the absence of sterols.

3. **Test for carbohydrates (Molisch's test):** No violet ring was produced showing the absence of glycosides or free sugars.

4. **Test for reducing sugars (Fehling's solution test):** Red precipitate was formed showing the presence of reducing sugars.

5. **Test for alkaloids (Dragendorff's test):** Heavy precipitate was formed showing the presence of alkaloids.

Methanol soluble subfraction (**F2-4**)

1. **Test for quinones (Ammonium thiocyanate test):** No red coloration showed the absence of quinones.

2. **Test for sterols and triterpenes (Liebermann-Burchard test):** No green coloration indicated the absence of sterols.

3. **Test for carbohydrates (Molisch's test):** Violet ring was produced showing the presence of glycosides or free sugars.

4. **Test for reducing sugars (Fehling's solution test):** Red precipitate was formed showing the presence of reducing sugars.

5. **Test for alkaloids (Dragendorff's test):** No precipitate was formed showing the presence of alkaloids.

6. **Test for flavonoids**:

(a) **Shinoda's test:** No red colouration was seen indicating the absence of flavonoids.

(b) **Shibata's test:** No red colouration was observed indicating the absence of flavonoids.

5.4.2 Preparation of extracts - Method II

Sun-dried root powder (1.99 Kg) was extracted successively with petroleum ether (6.5 L, 5.5 L and 3.5 L) two overnights by cold percolation and then filtered through cotton plug. The filtrate was evaporated on Rotavapor to obtain yellow residue, **E1** (4.98 g). The marc (**M1**) was dried in air for further investigation.

Thus obtained marc was extracted with chloroform (4 L × 3) in Soxhlet extractor for 12 hours in three batches (650.00, 650.00 and 690.00 g). Extract solution was filtered through cotton plug and evaporated completely on Rotavapor to obtain crude extract, **E2** (20.00 g). The marc (**M2**) was dried in air for further investigation.

The marc, **M2** (750.00 g) was extracted with ethyl acetate (4 L) in Soxhlet extractor for 16 hours. Extract was filtered through cotton and the filtrate was evaporated on Rotavapor to get dark brown gummy residue, **E3** (15.1 g). Marc (**M3**) was dried in air for further investigation.

The marc, **M3** (750.00 g) obtained above was extracted in Soxhlet extractor with ethyl alcohol (4 L) for 12 hours. After filtrating the extract through cotton plug, solvent was evaporated completely on Rotavapor to get dark brown gummy residue, **E4** (58.44 g). The marc, **M4** left over was not subjected to further investigation (see scheme 3.2).

The extracts, **E1, E2, E3** and **E4** were used further for the brine shrimp cytotoxicity assay, antimicrobial tests and isolation of compounds.

5.4.2.1 Brine shrimp cytotoxicity test

5.4.2.1a Preparation of sample solution

Sample solutions of **E1**, **E2**, **E3** and **E4** required for this bioassay was prepared by **dilution method**.[63] An amount of 50 mg of each extract of **E1** and **E2** were separately dissolved and diluted to 5 mL solution with chloroform in a 5-mL measuring cylinder. This was labeled as solution **A** which corresponded to the concentration of 10 mg/mL**.** A volume of 500 µL of solution **A** was transferred to volumetric flask of 10 mL capacity with the help of micropipette and diluted up to mark with solvent used above. This was labeled as solution **B** which corresponded to the concentration of 500 µg/mL. Similarly, solutions **A** and **B** were prepared separately for **E3** and **E4** in methanol as discussed above.

5.4.2.1b Bioassay Procedure

A volume of 500 μL and 50 μL of solution **A** and 100 μL of solution **B** were transferred to separate test tubes (size:10 cm × 1). Five replicates were made for each dose level. Controls were also prepared by using respective solvent alone as the volume of sample solution taken for each dose level. The content of each test tube was concentrated on Rotavapor at reduced pressure for about 25 minutes to remove solvents completely at temperature 50° C. After the solvent was evaporated, 4 mL of artificial sea water was added into each test tube. Ten shrimp were transferred in each test tube *via* a Pasteur pipette and adjusted the volume to 5 mL with artificial sea water. As a result, the final concentration of solution in test tubes with 500 μL, 50 μL of solution **A** and 100 μL of solution **B** will be 1000, 100 and 10 μg/mL, respectively. Altogether, there were eighteen test tubes. They were kept in room illuminated using 15W bulb maintaining temperature 20-22 °C for 24 hours. The number of survivors was counted after 24 hours and determined the number of deaths in each dose level. Brine shrimp were considered dead if they did not exhibit any movement during 10 seconds of observation. Any brine shrimp death in control test tube was eliminated and repeated the whole experiment. LC_{50} values in terms of μg/mL were calculated by probit analysis.[65] Experiments were performed thrice in total only for that extract which showed LC_{50} lesser than 1000 μg/mL. LC_{50} value in μg/mL of such extract was expressed as mean of three independently performed experiments with 95% confidence interval. Berberine was used as positive control.

Result and calculation: Number of death or survival of shrimp counted after 24 hours and LC_{50} values were shown in table 5.7, 5.8 and 5.9.

Table 5.7: Cytotoxicity of petroleum ether extract (**E1**) of the root against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	D	100 (µg/mL) S	D	10 (µg/mL) S	D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
		S	D	S	D	S	D										
	C	10	0	0	0	0	0										
	1	3	7	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	2	3	7	9	1	10	0	Log 100 = 2	0.8	1.6	4						
1	3	0	10	9	1	10	0	Log 1000 = 3	8.4	25.2	9	- 5.3	4.2	2.45	281.83		
	4	0	10	9	1	10	0										
	5	2	8	9	1	10	0										
		ΣD = 42		ΣD = 4		ΣD = 0		Σx = 6	Σy = 9.2	Σx y = 26.8	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	0	10	10	0	10	0										
	2	0	10	10	0	10	0	Log 10 = 1	0.0	0.0	1						
2	3	1	9	9	1	10	1	Log 100 = 2	0.4	0.8	4	-5.73	4.4	2.43	269.15	279.79 ± 9.78	304.08 -255.50
	4	2	8	10	0	10	0	Log 1000 = 3	8.8	26.4	9						
	5	3	7	9	1	10	0										
		ΣD = 44		ΣD = 2		ΣD = 0		Σx = 6	Σy = 9.2	Σx y = 27.2	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	0	10	10	0	10	0										
	2	4	6	10	0	10	0	Log 10 = 1	0.0	0.0	1						
3	3	3	7	10	0	10	0	Log 100 = 2	0.4	0.8	4	- 5.6	4.3	2.46	288.40		
	4	4	9	9	1	10	0	Log 1000 = 3	8.6	25.8	9						
	5	2	8	9	1	10	0										
		ΣD = 43		ΣD = 2		ΣD = 0		Σx = 6	Σy = 9.0	Σx y = 26.6	$\Sigma x^2 = 14$						

Note: Berberine was used as a positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.8: Cytotoxicity of chloroform extract (**E2**) of the root against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	D	100 (µg/mL) S	D	10 (µg/mL) S	D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
1	C	10	0	10	0	0	0					- 6.00	4.5	2.44	275.42	302.54 ± 52.50	432.96 – 172.12
	1	2	8	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	2	0	10	10	0	10	0	Log 100 = 2	0.0	0.0	4						
	3	1	9	10	0	10	0	Log 1000 = 3	9.0	27.0	9						
	4	2	8	10	0	10	0										
	5	0	10	10	0	10	0										
		ΣD = 45		ΣD = 0		ΣD = 0		Σx = 6	Σy = 9	Σx y = 27	$\Sigma x^2 = 14$						
2	C	10	0	10	0	10	0					- 5.00	3.9	2.56	363.07		
	1	2	8	10	0	10	0										
	2	1	9	10	0	10	0	Log 10 = 1	0.2	0.2	1						
	3	1	9	10	0	10	1	Log 100 = 2	0.2	0.4	4						
	4	2	8	10	0	10	0	Log 1000 = 3	8.0	24.0	9						
	5	0	10	10	1	10	0										
		ΣD = 40		ΣD = 1		ΣD = 1		Σx = 6	Σy = 8.4	Σx y = 24.6	$\Sigma x^2 = 14$						
3	C	10	0	10	0	10	0					- 7.40	5.1	2.43	269.15		
	1	2	8	10	0	10	0										
	2	1	9	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	3	2	8	10	0	10	0	Log 100 = 2	0.0	0.0	4						
	4	1	9	10	0	10	0	Log 1000 = 3	8.4	25.2	9						
	5	2	8	10	0	10	0										
		ΣD = 42		ΣD = 0		ΣD = 0		Σx = 6	Σy = 8.4	Σx y = 25.2	$\Sigma x^2 = 14$						

Note: Berberine was used as a positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.9: Cytotoxicity of ethyl acetate (**E3**) and ethanol (**E4**) extracts of the root against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	1000 (µg/mL) D	100 (µg/mL) S	100 (µg/mL) D	10 (µg/mL) S	10 (µg/mL) D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X
		S	D	S	D	S	D								
	C	10	0	10	0	10	0								
	1	10	0	10	0	10	0	Log 10 = 1	0.0	0.0	1				
	2	10	0	10	0	10	0	Log 100 = 2	0.0	0.0	4	- 0.93	0.7	8.47	2.9×10^8
E3	3	10	1	10	0	10	0	Log 1000 = 3	0.8	2.4	9				
	4	9	1	10	0	10	0								
	5	9	2	10	0	10	0								
		ΣD = 4		ΣD = 0		ΣD = 0		Σx = 6	Σy = 0.8	Σx y = 2.4	$\Sigma x^2 = 14$				
	C	10	0	10	0	10	0								
	1	10	0	10	0	10	0								
	2	10	0	10	0	10	0	Log 10 = 1	0.0	0.0	1				
E4	3	9	1	10	0	10	0	Log 100 = 2	0.0	0.0	4	-0.40	0.3	18	1×10^{18}
	4	8	2	10	0	10	0	Log 1000 = 3	0.6	1.8	9				
	5	10	0	10	0	10	0								
		ΣD = 3		ΣD = 0		ΣD = 0		Σx = 6	Σy = 0.6	Σx y = 1.8	$\Sigma x^2 = 14$				

Note: Berberine was used as a positive control (see table 5.6 for detail).S = Survival, D = Death, C = negative control

Remark: Fractions **E1** and **E2** showed LC_{50} of 279.79 and 302.54 μg/ml, respectively indicating moderately cytotoxicity towards brine shrimp as their LC_{50} values were lesser than 1000 μg/mL. Similarly, fractions **E3** and **E4** displayed LC_{50} of 2.9×10^8 and 1×10^{18} μg/mL, respectively. Their LC_{50} values were greater than 1000 μg/mL indicating non-cytotoxicity.

5.4.2.2 Antibacterial Test

Preparation of Extract: Root extracts, **E1**, **E2, E3** and **E4** obtained as shown in Method II (see scheme 3.2), were used for antimicrobial test.

Disc diffusion technique: Zone of inhibition was determined by disc diffusion technique as discussed in section 2.2.6.1.

1. Preparation of media and standard working inoculum: Mueller Hinton agar (MHA) plates and standard working inoculum of the test organisms (equivalent to McFarland 0.5) were prepared as discussed in no. 5 and 6 of section 2.2.6.3.

2. Inoculation of MHA plates: Organisms viz *E.* coli, *S. aureus*, *B. subtilis P. aeruginosa* were streaked in MHA plates as discussed in no. 7 of section 2.2.6.3. The used cotton swab was discarded by dipping into ethanol solution. Inoculums were left to dry for a few minutes at room temperature with the lid closed in laminar chamber.

3. Preparation of sample solution (50 μg/μL): Sample solution of extract was prepared by dissolving 25 mg of extract in DMSO and diluting to 500 μL in a clean sterile eppendorff tube. This solution corresponded to the concentration of 50 mg/mL or 50 μg/μL.

4. Impregnation of disc (1 mg/disc) by sample solution: Sterile discs of required numbers were arranged in empty sterilized Petri plates with the help of sterilized forceps. These discs were impregnated with 20 μL of each stock solution of extracts (equivalent to 1 mg) using micropipette. Four discs were loaded for each extract solution. Four negative controls were also prepared by loading 20 μL of DMSO alone separately in four blank discs. All together, there were twenty discs (16 discs of extracts and 4 of negative controls). Solvent in discs was evaporated by placing them inside the laminar chamber for 3-4 hours at room temperature.

5. Transfer of disc into inoculated MHA plates: With the help of sterilized forceps, positive control disc (gentamycin, 10 μg) and discs impregnated with extract (1 mg/disc) as well as negative control disc (solvent) were transferred on the inoculated plate using paper template

as discussed in no. 10 of section 2.2.6.3. Therefore, total seven discs (five of extracts and two of controls) were loaded. Remaining three inoculate plates were also loaded by discs similar way.

6. Incubation of test plates: Four test plates were left for 30 minutes in laminar air flow for diffusion of extract with the lid closed. Then plates were incubated at 37 °C for 18 hours.

Remarks: Zone of Inhibition was measured including 6 mm disc of paper with the help of ruler after 18 hours of incubation. Data are presented in table 3.39 (see section 3.5.2). Diameter of zone with 6 mm value was indicative of no activity. Percentage was calculated after subtracting disc diameter (6 mm) from all observation with respect to the reference compound, gentamycin. Extract **E1** showed very weak antibacterial activity towards the gram-positive bacteria: *S. aureus* (5.5%) and *B. subtilis* (5.5%) while no activity was detected towards gram-negative bacteria (*P. aeruginosa* and *E. coli*). Extracts **E2**, **E3** and **E4** showed no antibacterial activity towards both gram-positive and gram-negative bacteria.

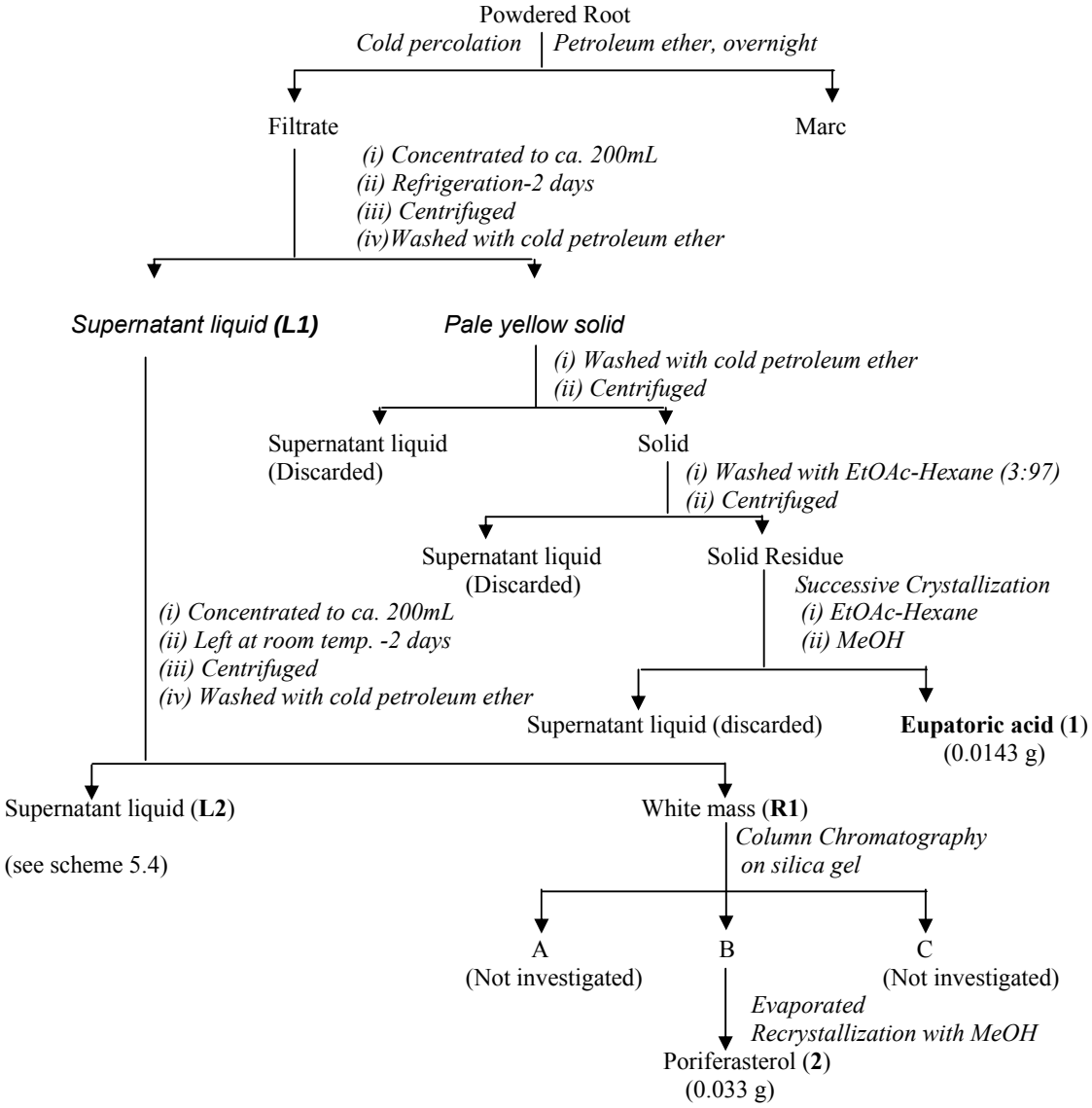
5.5 ISOLATION OF COMPOUNDS FROM THE ROOT EXTRACTS

5.5.1 From petroleum ether extract (E1)

Sun-dried and powdered root (1.99 Kg) was successively extracted with petroleum ether (6.5 L, 5.5 L and 3.5 L) two overnights by cold percolation and then filtered. The filtrate was concentrated to about 200 mL and refrigerated for two nights. The solid obtained was centrifuged and washed with cold petroleum ether (10 mL × 5) to obtain a pale yellow solid and supernatant liquid (**L1**). The pale yellow solid was further washed, followed with a centrifuge with EtOAc-petroleum ether (cold) (3:97, 1 mL × 3), and recrystallized successively from EtOAc-hexane and MeOH, to yield the **eupatoric acid** (**1**, 0.0143 g) with R_f 0.34 (MeOH-$CHCl_3$ 5:95, brown spot in iodine vapour) (scheme 5.3). Supernatant liquid (**L1**) was concentrated to about 200 mL and left two overnights at room temperature, centrifuged and washed with cold petroleum ether (5 mL × 3) to obtain white mass (**R1**, 0.0466 g) and supernatant liquid (**L2**). **R1** was adsorbed on silica gel using dichloromethane and excess solvent was removed on Rotavapor till dry powder was obtained. The resulting powder was loaded in a column of diameter 1.6 cm, packed with slurry of silica gel (60-120 mesh, 15.00 g) made in dichloromethane by eluting with dichloromethane (80 mL) and CH_2Cl_2-EtOAc of increasing polarity of (99:1, 150 mL; 95:5, 50 mL). Eluents were collected

at the rate of 25 mL/min in test tubes. They were pooled together into three fractions (**A**, **B** and **C**) based on TLC characteristics.

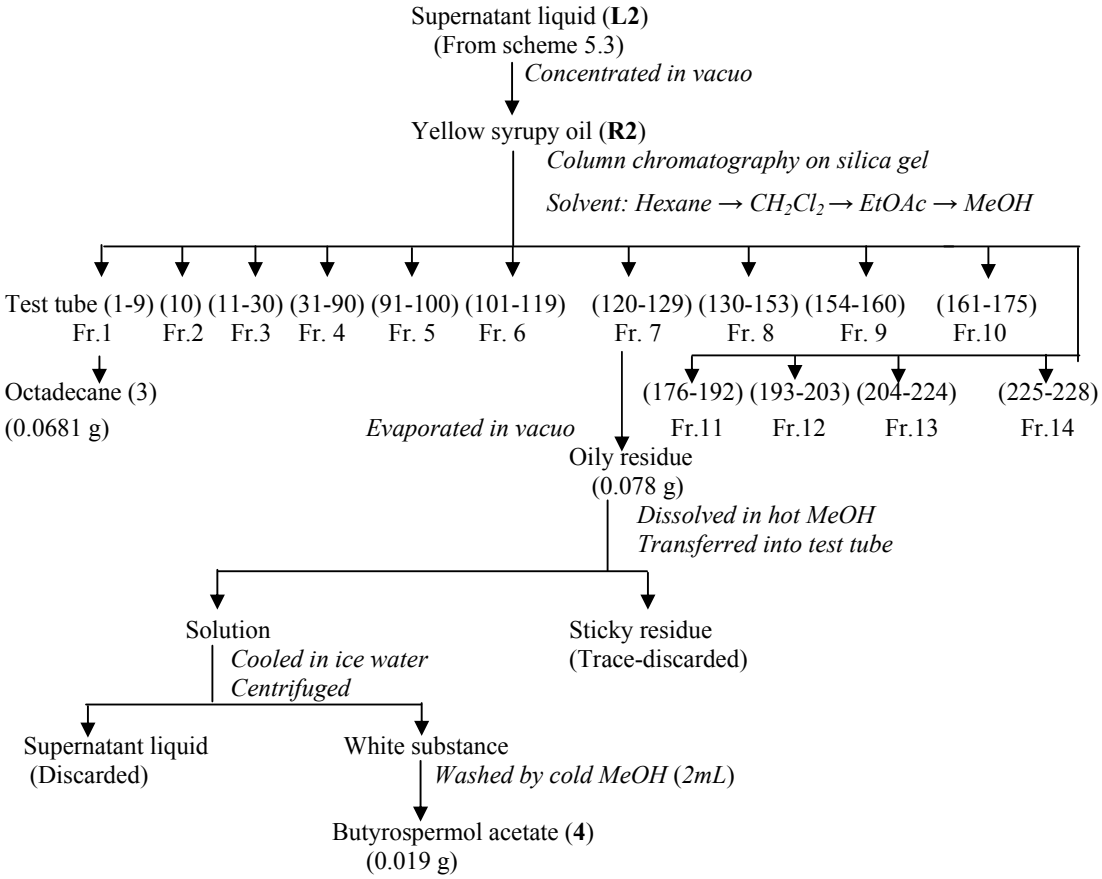
Scheme 5.3: Isolation of compounds **1** and **2**



Fraction **A** (2.9 mg) obtained from dichloromethane contained an array of spots and hence it was not investigated further. Fraction **B** obtained from CH_2Cl_2-EtOAc (99:1), yielded white soft residue on evaporating the solvent to dryness and then recrystallized in methanol to yield white crystals of **poriferasterol** (**2**, 0.033 g) with R_f 0.48 (hexane-EtOAc, 70:30), 0.51 (CH_2Cl_2-EtOAc, 70:30), yellow spot in iodine vapor (scheme 5.3). Fraction **C** obtained from CH_2Cl_2-EtOAc (99:1-95:5) yielded compounds in traces so it was not investigated further.

On evaporating **L2** on Rotavapor yellow syrupy oil residue (**R2**, 4.20 g) was obtained. Thus obtained **R2** was adsorbed on silica gel (9.00 g) using hexane. An excess amount of the solvent was removed on Rotavapor till dry powder was formed. It was chromatographed in a column (60 cm) of diameter 3 cm, packed with slurry of silica gel (145 g) made in hexane by eluting with hexane (1200 mL), hexane-CH_2Cl_2 [97:3, 300 mL; 95:5, 500 mL; 90:10, 200 mL; 80:20, 400 mL; 70:30, 900 mL; 60:40, 100 mL; 50:50, 200 mL; 40:60, 200 mL; 30:70, 200 mL; 10:90, 100 mL], CH_2Cl_2 (200 mL), CH_2Cl_2- EtOAc [95:5, 200 mL; 75:25, 250 mL; 50:50, 250 mL, 25:75, 200 mL], EtOAc (250 mL), EtOAc-MeOH (50:50, 150 mL) and finally with MeOH (50 mL). Eluents were collected at the rate of 20 mL/15 min in each test tube. Test tubes contents having similar TLC characteristics were combined together to provide fractions **1-14** (scheme 5.4).

Scheme 5.4: Isolation of compounds **3** and **4** from **R2**



Fraction **1** obtained by eluting with hexane, yielded **octadecane** (**3**, 0.681 g), R_f 0.74 (hexane-Et_2O, 85: 15, yellow in iodine vapour). Fraction **7** obtained from hexane-CH_2Cl_2 (70:30), yielded oil (0.078 g) after evaporation of solvent on Rotavapor. It was dissolved in hot

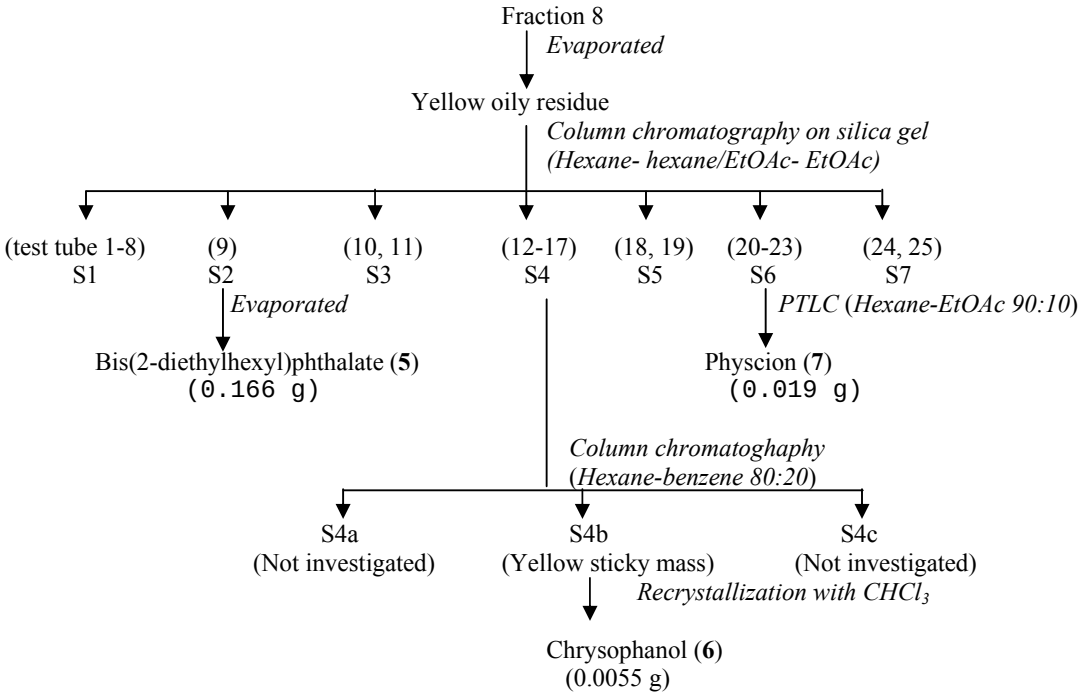
MeOH; soluble portion was transferred to next tube which was allowed to cool over ice water. Thus obtained crystals were separated by centrifuge to get white substance, which was washed by cold MeOH (2 mL). Any solvent left in it was evaporated using Rotavapor to yield **butyrospermol acetate** (**4**, 0.019 g) with R_f 0.28 (CH_2Cl_2-hexane, 30:70, yellow in iodine vapor and blue spot under UV light of 365 nm), 0.48(CH_2Cl_2-hexane, 50:50, yellow in iodine vapor and blue spot under UV light of 365 nm) (scheme 5.4).

Fraction **8** obtained by eluting with hexane-CH_2Cl_2 (70:30-60:40), yielded yellow oil (0.260 g) after evaporation of solvent on Rotavapor. Yellow oil was adsorbed on silica gel (2 g) using dichloromethane and excess solvent was removed on Rotavapor to yield dry powder. Thus obtained powder was subjected to column chromatography in a column (40 cm) of diameter 1.75 cm, packed already with slurry of silica gel (60-120 mesh, 20g) made in hexane. The column above was eluted with hexane, hexane-EtOAc and EtOAc gradients [hexane (40 mL), hexane-EtOAc (97:3, 270 mL), hexane-EtOAc (90:10, 50 mL), hexane-EtOAc (80:20, 50 mL) and EtOAc (25 mL)]. Eluents were collected at the rate of 20 mL/30 min in test tubes. The collected fractions were monitored by TLC and pooled together to seven subfractions- **S1** to **S7**.

Subfraction **S2** obtained by eluting with hexane-EtOAc (97:3), yielded a faint yellow liquid **bis(2-ethylhexyl)phthalate** (**5**, 0.166 g), R_f 0.62 (hexane-EtOAc, 80:20, sky blue under UV light of 366 nm and yellow in iodine vapor), 0.65 on silica gel plate (toluene-MeOH, 99.5: 0.5, sky blue under UV light of 366 nm and yellow in iodine vapor) (scheme 5.5). Subfraction **S4**, obtained by eluting with hexane-EtOAc (97:3) showed yellow spot as a major compound on TLC which yielded a sticky yellow mass (0.073 g). A portion of this sticky yellow mass (0.065 g) was rechromatographed in a column (1 × 48 cm) packed with silica gel (60-120 mesh, 7.00 g) by eluting with hexane-benzene (80:20). Eluents were collected at the rate of 10 mL/20 min in test tubes. They were pooled together into three fractions viz **S4a**, **S4b** and **S4c** on the basis of characteristic of TLC. Fraction **S4b**, gave yellow sticky mass (0.020 g) which on recrystallization with chloroform produced **chrysophanol** (**6**, 0.0055 g), R_f 0.48 (hexane-EtOAc, 70:30), 0.57 (hexane-EtOAc, 70:30), 0.47 (toluene), 0.59 (hexane-EtOAc-MeOH, 1.6:0.2:0.2), 0.58 (benzene) (scheme 5.5). Spots were visualized as yellow under daylight, orange under UV light of 365 nm and pink in 10% methanolic KOH spraying reagent. Yellow spot was also changed into red on heating TLC plate strongly but restored its color after cooling the plate.

Subfraction **S6** obtained by eluting with hexane-EtOAc (90:10-80:20), yielded yellow mass (0.028 g), which was chromatographed by Preparative TLC using hexane-EtOAc (90:10) eluent in five plates (20 × 20 cm) of 0.25 mm thickness. The yellow band corresponding to R_f 0.12 (EtOAc-hexane 1:9) was scrapped out, extracted by warm chloroform and the solution was evaporated on Rotavapor to yield **physcion** (**7,** 0.019 g), R_f 0.12 (EtOAc-hexane, 10:90), 0.54 (benzene) (scheme 5.5). Spots were visualized as yellow under daylight, red under UV light of 365 nm, red in 10% methanolic KOH spraying reagent. Subfractions **S1**, **S3**, **S5** and **S7** were not investigated due to only traces of substances obtained.

Scheme 5.5: Isolation of compounds **5**, **6** and **7** from Fraction **8**



Fraction **11** obtained from CH_2Cl_2-EtOAc (95:5-75:25), was concentrated on Rotavapor and left the solution overnight at room temperature which yielded greenish syrupy liquid (0.198 g). This was dissolved in hot hexane, cooled over ice water to yield white solid, filtered off and washed successively with cold hexane (10 mL × 3) and cold hexane-EtOAc (97:3, 10 mL). Thus obtained white solid was separated by centrifugation and excess solvents were removed on Rotavapor to obtain dried white substance which was identified as **poriferasterol** (**2,** 0.084 g) by co-TLC, R_f 0.48 (hexane-EtOAc, 7:3), 0.51 (CH_2Cl_2-EtOAc, 7:3), melting point, 152 °C (lit. m.pt.[93] 156 °C) and positive result in Liebermann-Burchard test showing green color.

Preparative TLC of petroleum ether extract (E1)

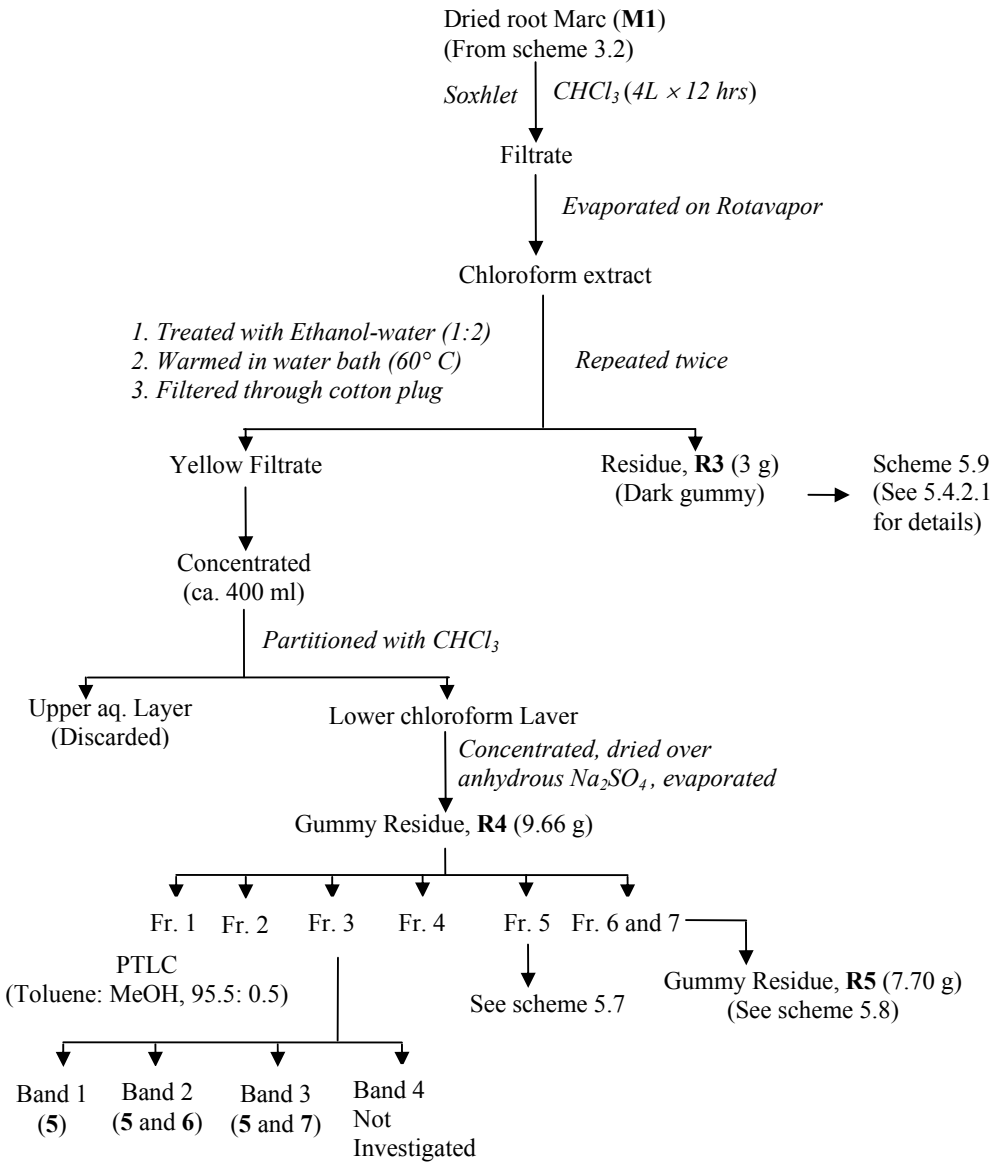
Sun-dried and powdered root (650 g) of *E. odoratum* was extracted with petroleum ether (3 L) by cold percolation for overnight. It was filtered through cotton plug. The marc was further percolated overnight with petroleum ether (1.5 L). The solution was filtered. The filtrate was combined and evaporated the solvent completely on Rotavapor to yield yellow oil (3.037 g). An amount of 2.60 g of thus obtained oil was dissolved in chloroform and streaked in nineteen preparative thin layer chromatography glass plates coated with silica GF_{254} (0.5 mm thickness). Plates were developed in ethyl acetate. After development, plates were allowed to dry in hot air and observed under UV light in 254 nm. Two UV active **bands I** (dark blue) and **II** (light sky blue) were observed at R_f 0.93 (EtOAC) and R_f 0.0 (EtOAC), respectively. **Band II,** R_f 0.0 (EtOAC), on usual work up with ethyl acetate yielded extremly low material and thus it was not further investigated. **Band 1,** R_f 0.93 (EtOAC, dark blue under UV light of 254 nm) was scraped out, extracted with ethyl acetate, filtered off and the filtrate was evaporated to obtain oily residue (0.8649 g) which showed a number of arrays of close spots. The ^{13}C-NMR data of **band 1** showed that it was a mixture of **poriferasterol** (**2**) and **bis(2-ethylhexyl)phthalate** (**5**). The remaining unidentified signals were likely due to some impurities. Hence it was not investigated further. After scrapping out UV active **bands I** and **II**, the remaining part (UV inactive **band III**) of the plates were scraped off, extracted with ethyl acetate, filtered off and dried over desiccator for 3 days to yield the pale yellow solid, **palmitic acid** (**8,** 0.080 g) with R_f 0.7 (hexane-EtOAc, 1:1, brown spot in iodine vapour, yellow in fluorescein spraying reagent) (see scheme 3.4).

5.5.2 From chloroform extract (E2)

Dried marc (**M1**) (1.99 Kg) of the root obtained from scheme 3.2 was extracted with chloroform (4L x 3) in Soxhlet extractor for 12 hours in three batches (650, 650 and 690 g). Thus obtained solution was filtered through cotton plug and solvent was removed on Rotavapor to obtain crude extract (20.00 g). A portion of this crude extract (19.33 g) was treated with ethanol-water (200:400 mL). The heterogeneous mixture was swirled with warming in water bath at 60 °C, allowed to settle and filtered through cotton plug to obtain yellow filtrate and the gummy residue, **R3** (3.00 g) (scheme 5.6). The process was repeated once more. The yellow filtrates from two experiements were combined and concentrated to ca. 400 mL on Rotavapor followed by extraction with chloroform (400 mL × 7) in separating funnel. The lower chloroform layer (red) was separated from the upper aqueous clear layer. The emulsion formed was removed by treating with saturated brine solution (150 mL). The

upper aqueous layer was discarded. The combined chloroform layer was concentrated, dried over anhydrous sodium sulphate, filtered off and finally evaporated completely on Rotavapor to yield dark gummy residue, **R4** (9.66g) (scheme 5.6). The experimental workout for **R3** was discussed in section 5.5.2.1.

Scheme 5.6: Isolation of compounds from chloroform extract



Column chromatography of R4

Slurry of silica gel (138.00 g, 60-120 mesh) made with 25% chloroform in hexane was packed into a column of outer diameter, 5 cm and left overnight. The residue (**R4**, 9.66 g) was adsorbed on silica gel (33.00 g) using chloroform. An excess of solvent was removed on

Rotavapor till dry and fluffy powder was obtained. Thus obtained powder was loaded to column above and eluted with hexane-$CHCl_3$ [75:25, 700 mL; 50:50, 1200 mL, 25:75, 500 mL], $CHCl_3$ (100 mL), $CHCl_3$-MeOH [80:20, 850 mL; 50:50, 500 mL] and finally with MeOH (500 mL). Eluents were collected at the rate of 25mL/5 minutes in each test tube. Test tube contents having similar TLC characteristics were combined together to provide fractions, **1** to **7**.

Fraction **3** obtained from hexane-$CHCl_3$ (25:75), yielded yellowish residue (0.508 g) after evaporation of the solvent on Rotavapor. It was subjected to preparative TLC on silica gel (GF_{254}) glass plates of 0.5 mm thickness using toluene-MeOH (99.5: 0.5) as solvent. Four bands were observed under UV light.

Band 1, on usual work up with chloroform yielded yellowish liquid, **bis(2-ethylhexyl)phthalate** (**5**) (0.0519 g), R_f 0.65 (toluene-MeOH, 99.5: 0.5, sky blue under UV light in 366 nm). This was confirmed by ^{1}H-NMR spectrum recorded in 60 MHz.

Band 2, on usual work up with chloroform yielded yellow soft mass. Thus obtained soft mass was re-chromatographed by repeated Preparative TLC using toluene-MeOH (99.5: 0.5) as eluent to obtain yellow mixture (0.0162 g) of **5** and **6,** R_f 0.54 (toluene-MeOH, 99.5: 0.5, orange under UV light in 366 nm and yellow under UV light in 254 nm, pink in 10% methanolic KOH spraying reagent).

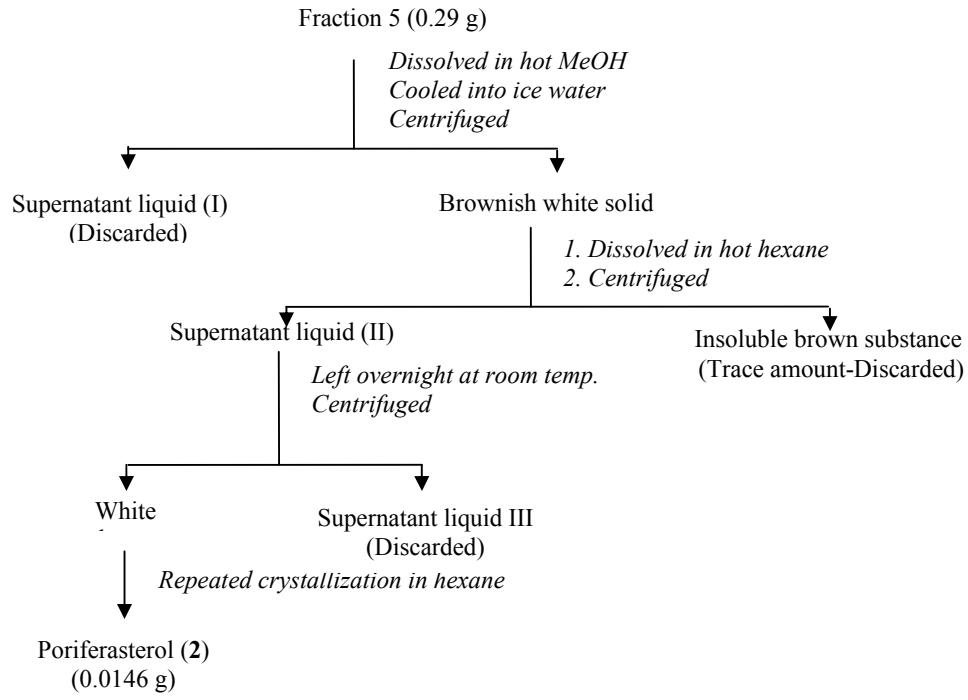
Band 3, on usual work up with chloroform yielded yellow mixture (0.0022 g) of **physcion** (**7**) and **bis(2-ethylhexyl)phthalate** (**5**) with R_f 0.41(toluene-MeOH, 95: 5, yellow under UV light in 366 nm, pink in daylight after spraying 10% methanolic KOH).

Band 4, on usual work up with chloroform yielded only trace of the compound with R_f 0.38 (toluene-MeOH, 95: 5, faint yellow under UV light in 366 nm) and therefore it was not further investigated.

Fraction **5**, obtained from $CHCl_3$-MeOH (80:20) yielded dirty white soft residue (0.29 g) after evaporation of the solvent on Rotavapor. It was dissolved in hot MeOH, cooled in ice water and centrifuged to obtain brownish white solid. Supernatant liquid (I) was discarded. Thus obtained white solid was again dissolved in hot hexane and centrifuged to separate supernatant liquid (II) from the traces of insoluble brown impurities. Supernatant liquid (II) was left overnight at room temperature to yield white substance, which was separated by

centrifugation. Repeated crystallization with hexane yielded white crystals of **poriferasterol** (**2**, 0.0146 g) (scheme 5.7) with melting point 149 °C (lit. m. pt.[93] 156 °C) due to impurities as revealed by ^{1}H-NMR and ^{13}C-NMR spectra.

Scheme 5.7: Isolation of compound **2** from Fraction **5**



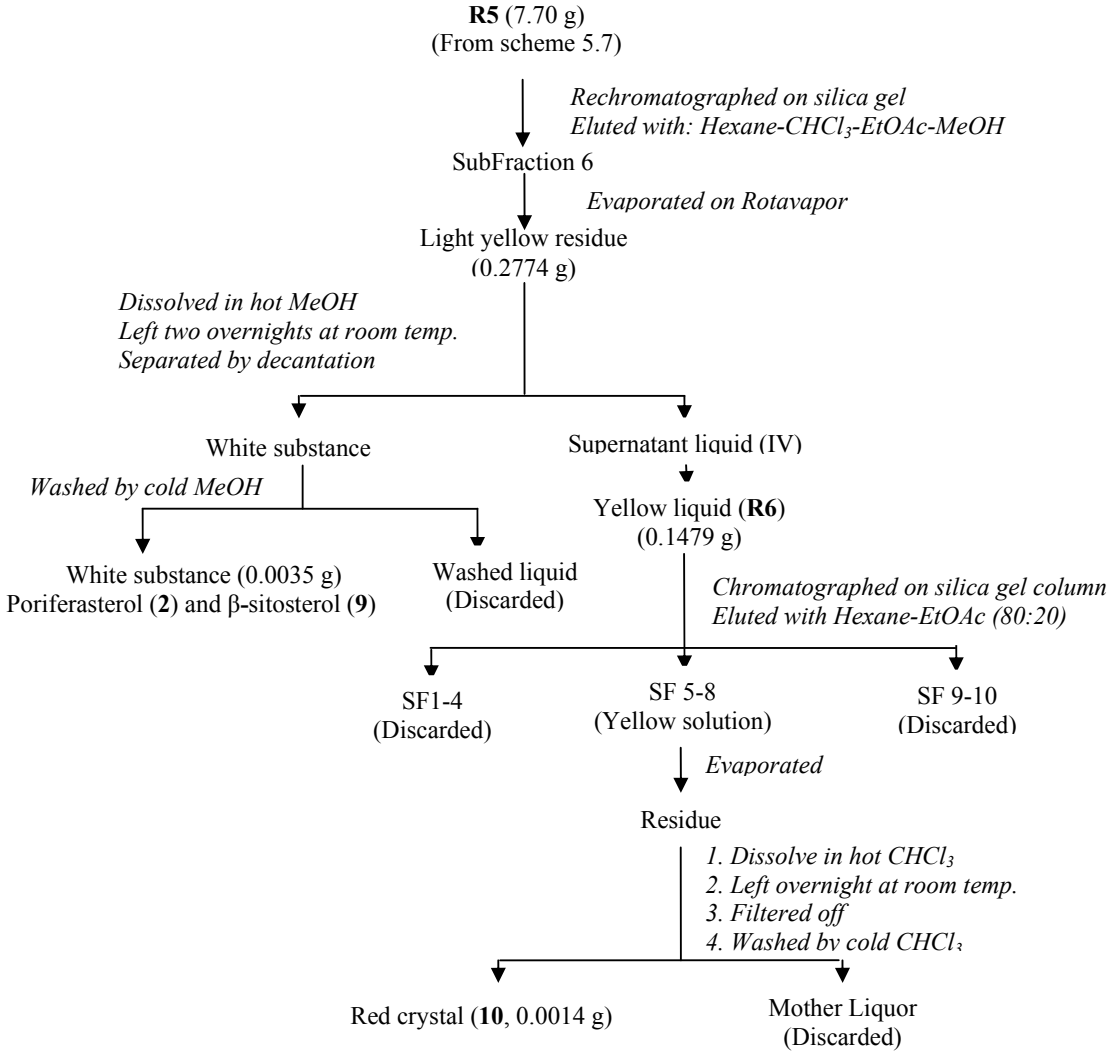
Fractions **6** and **7** were combined since they showed positive test for Dragendorff's reagent. On evaporating the solvent on Rotavapor, brown gummy residue (**R5,** 7.79 g), was obtained.

Column chromatography of R5

Slurry of silica gel (122.00 g, 60-120 mesh) made in hexane was packed in a column of diameter, 5 cm and left overnight. Residue (**R5**, 7.79 g) was adsorbed on silica gel (31.00 g) using chloroform. An excess of solvent was removed on Rotavapor till dry and fluffy powder was obtained. Column was eluted with hexane-$CHCl_3$ (75:25, 350 mL; 65:35, 100 mL; 50:50, 200 mL), hexane-$CHCl_3$-EtOAc (50:50:2.5, 307 mL; 50:50:5, 210 mL; 50:50:12.5, 787 mL; 50:50:17.5, 705 mL; 50:50:25, 500 mL), $CHCl_3$-EtOAc (100:25, 750 mL; 70:30, 100 mL; 60:40, 300 mL; 50:50, 350 mL; 40:60, 280 mL, 20:80, 100 mL), EtOAc (100 mL), EtOAc-MeOH (80:20, 700 mL; 50:50, 500 mL) and finally with MeOH (100 mL). Eluents were collected at the rate of 25 mL/10 minute in each test tube. Test tube contents having similar TLC characteristics were combined together to provide subfractions **S1** to **S8**.

Subfraction **S6** obtained from hexane-$CHCl_3$-EtOAc (50:50:17.5), on evaporation yielded light yellow oil (0.2774 g), with a major spot of R_f 0.25 (hexane-EtOAc-MeOH, 1.3:0.3:0.4, yellow in daylight, red in daylight after spraying with 10% methanolic KOH). Thus obtained yellow oil was dissolved in hot MeOH, left two overnights at room temperature followed by decantation to obtain white substance and supernatant liquid (IV). White substance thus obtained was washed with cold methanol (2 × 2 ml) and dried in vacuum to obtain white mixture (0.0035 g) of **poriferasterol** (**2**) and **β-sitosterol** (**9**) (scheme 5.8).

Scheme 5.8: Isolation of compounds **2** and **9** in mixture and **10** from **R5**



Subfractions **S1** to **S5**, **S7** and **S8** were not further investigated due to absence of anthraquinones as indicated by spray of 10% methanolic KOH on TLC plate. Thus obtained supernatant liquid (IV) was evaporated on Rotavapor to yield yellow liquid (**R6**, 0.1479 g).

Chromatography of R6

Slurry of silica gel (10.00 g, 60-120 mesh) made with hexane was packed into a column of 1cm diameter and was left overnight. Thus obtained **R6** (0.1479 g) was adsorbed on silica gel (1.38 g) using chloroform. An excess of solvent was evaporated on Rotavapor till dry and fluffy powder was obtained. This fluffy powder was loaded to the column above and eluted with hexane-EtOAc (80:20). Eluents were collected at the rate of 10mL/20 minutes. Altogether ten fractions- **SF1** to **SF10** were collected. Fractions **SF1** to **SF4**, **SF9** and **SF10** were discarded due to absence of quinones as revealed from TLC with the help of spraying reagent, 10% methanolic potassium hydroxide solution. Fractions **SF5** to **SF8** were combined together since they showed the presence of quinones and similar TLC characteristics. Solvents were evaporated to get yellow soft mass. Thus obtained soft mass was dissolved with hot chloroform, left overnight at room temperature, filtered off, the residue washed with cold chloroform and dried in air to yield red crystals of anthraquinonoid compound (**10**, 0.0014 g) (scheme 5.8). Yellow mother liquor was not investigated further due to paucity of the material.

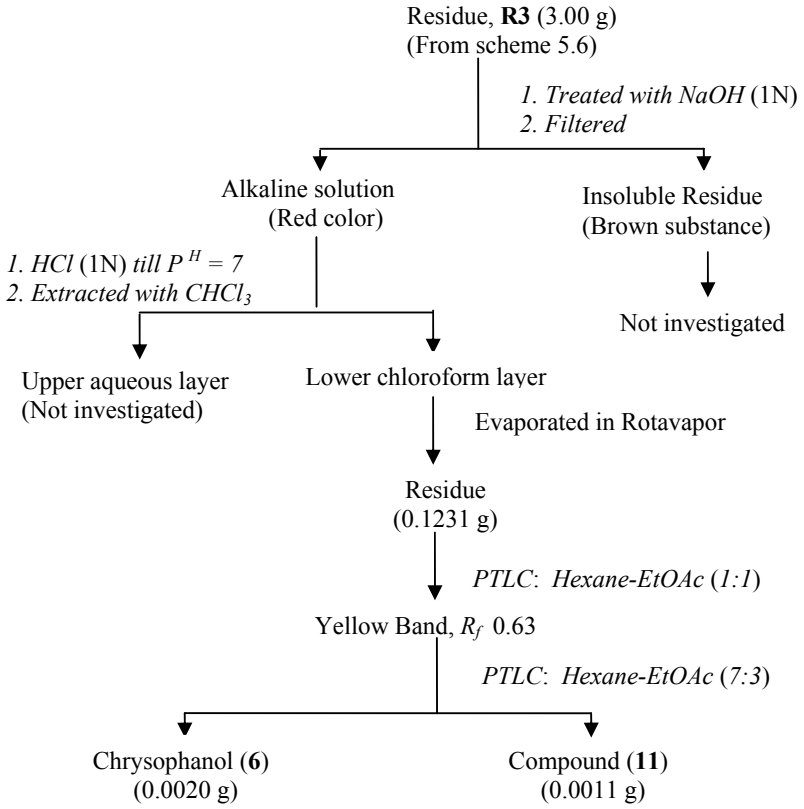
5.5.2.1 Investigation of gummy solid residue (R3)

Gummy residue, **R3** (3.00 g) obtained as discussed in section 5.4.2 was treated with NaOH (1N) solution and filtered off separating red alkaline solution from insoluble brown substance (scheme 5.9). Thus obtained red solution was treated with HCl (1N) till it showed pH 7 in the pH meter. It was extracted thrice with chloroform in a separating funnel. The combined lower chloroform layer was evaporated on Rotavapor to yield residue (0.1231 g) which was chromatographed over two preparative silica gel TLC plates (20 × 20 cm) of 0.25 mm thickness using hexane-EtOAc (35:65). Upper yellow band with R_f 0.63 was worked up as usual with warm chloroform and filtered off. The resulting filtrate was concentrated and rechromatographed over precoated aluminum plate with silica gel (20 × 20 cm) of 0.25 mm thickness using hexane-EtOAc (70:30) as the solvent. Two bands with R_f 0.57 and 0.32 were observed under UV light in 366 nm.

Band 1, on usual work up with warm chloroform yielded **chrysophanol** (**6,** 0.0020 g) with R_f 0.57 (hexane-EtOAc, 70:30, orange under UV light in 366 nm, yellow in daylight, pink in daylight after spraying with 10% methanolic KOH). ^{1}H-NMR and and GCMS inferred the compound as **6**.

Band 2, on usual work up with chloroform yielded light yellow oil of aromatic hydrocarbon (**11**, 0.0011 g), R_f 0.32 (faint yellow under UV light in 366 nm). It was not investigated further due to paucity of material.

Scheme 5.9: Isolation of compounds **6** and **11** from **R3**



5.6 CHARACTERIZATION OF ISOLATED COMPOUNDS AND MIXTURES

Eupatoric acid (1)

Pale yellow amorphous powder, m. pt. > 200 °C, positive Liebermann-Burchard test showing pink color, false Dragendorff's test, soluble in chloroform, dichloromethane and sparingly soluble in methyl alcohol.

UV ($CHCl_3$): λ_{max} = 243.8 nm.

IR (KBr): ν_{max} = 1685 (-COOH), 3450 (-OH) cm^{-1}.

^{1}H-NMR (400 MHz, $CDCl_3$ + one drop CD_3OD): δ = 0.77 (s, 3H, H-24), 0.77 (s, 3H, H-26), 0.90 (s, 3H, H-25), 0.91(s, 3H, H-30), 0.93 (s, 3H, H-31), 0.98 (s, 3H, H-23), 1.13 (s, 3H, H-27), 1.25 (s, 2H, H-28), 1.54-1.62 (m, 2H, H-2), 1.85-1.90 (m, 2H, H-11), 2.84 (dd, 1H, *J* = 14.9, 4.9 Hz, H-18), 3.22 (dd, 1H, *J* = 9.9, 4.9 Hz, H-3), 5.28 (br t, 1H, *J* = 3.3 Hz, H-12).

^{13}C-NMR (100 MHz, $CDCl_3$ + one drop CD_3OD): δ = 15.21 (C-24), 15.46 (C-25), 16.79 (C-26), 18.26 (C-6), 22.90 (C-16), 23.33 (C-11), 23.48 (C-31), 25.80 (C-27), 26.92 (C-2), 27.63 (C-15), 27.96 (C-23), 29.62 (C-28), 30.60 (C-20), 32.45 (C-22), 32.65 (C-7), 32.98 (C-30), 33.81 (C-21), 36.97 (C-10), 38.39 (C-1), 38.65 (C-4), 39.20 (C-8), 41.10 (C-18), 41.65 (C-14), 45.91 (C-19), 46.30 (C-17), 47.56 (C-9), 55.15 (C-5), 78.85 (C-3), 122.29 (C-12), 143.77 (C-13), 181.00 (C-29).

EIMS: *m/z* (%) = 470 $[M]^+$(0.5), 425 $[M\text{-}COOH]^+$ (5), 411 $[M\text{-}CH_2COOH]^+$ (22). HRMS *m/z*: 470.3387 [calcd. for $C_{31}H_{50}O_3$, 470.3760].

Poriferasterol (2)

White soft powder, m. pt. 154 °C (lit. m.pt.[93] 156 °C), positive Liebermann-Burchard test showing green color, soluble in chloroform, diethyl ether, ethyl acetate and hot hexane.

UV ($CHCl_3$): λ_{max} = 242 nm.

IR ($CHCl_3$): ν_{max} = 930 (-CH=CH-, *trans*), 3600 (-OH) cm^{-1}.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.69 (s, 3H, H-18), 0.79 (d, 3H, *J* = 6.9 Hz, H-27), 0.80 (t, 3H, *J* = 6.9 Hz, H-29), 0.84 (d, 3H, *J* = 5.9 Hz, H-26), 1.01 (s, 3H, H-19), 1.03 (d, 3H,

J = 5.9 Hz, H-21), 2.00 (m, 2H, H-7) 3.52 (symmetrical m, 1H, H-3α), 5.04 (dd, 1H, *J* = 14.9, 8.5 Hz, H-23), 5.14 (dd, 1H, *J* = 14.9, 8.5 Hz, H-22), 5.35 (br d, 1H, *J* = 4.2 Hz, H-6).

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 12.04 (C-18), 12.22 (C-29), 18.98 (C-26), 19.39 (C-19), 21.07 (C-11), 21.07 (C-21), 21.21 (C-27), 24.36 (C-15), 25.39 (C-28), 28.90 (C-16), 31.67 (C-2), 31.91 (C-7), 31.91 (C-8), 31.91(C-25), 36.52 (C-10), 37.27 (C-1), 39.69 (C-12), 40.46 (C-20), 42.23 (C-4), 42.32 (C-13), 50.18 (C-9), 51.24 (C-24), 55.98 (C-17), 56.87 (C-14), 71.80 (C-3), 121.69 (C-6), 129.30 (C-23), 138.30 (C-22), 140.76 (C-5).

FABMS (positive ion, glycerol matrix): *m/z* = 413 $[M+1]^+$; GC-MS: *m/z* = 412 $[M]^+$, 413 $[M+1]^+$, 397 $[M-CH_3]$, 394 $[M-H_2O]$, 379 $[M-CH_3-H_2O]$, 369 [M-isopropyl group], 351 [M-isopropyl group-H_2O], 300 [M-112], 271 [M-141], 255 [M-139-H_2O] which were in close agreement with literature data.[92]

Octadecane (**3**)

Clear liquid at room temperature but changed into translucent soft solid on refrigeration, soluble in hexane and chloroform.

^{1}H-NMR (100 MHz, $CDCl_3$): δ = 0.80 - 0.90 (t, 3H × 2), 1.2 - 1.3 (m, CH_2).

^{13}C-NMR (25 MHz, $CDCl_3$): δ = 14.1 (C-1, C-18), 22.7 (C-2, C-17), 31.95 (C-3, C-16), 29.39 (C-4, C-15), 29.70 (C-5 to C-14).

GCMS: *m/z* (%) = 254 $[M]^+$ (6.6), 197 $[M-C_4H_9]^+$ (3.7), 141 $[M-C_8H_{17}]^+$ (5.6), 127 $[M-C_9H_{19}]^+$ (7.5), 113 $[M-C_{10}H_{21}]^+$ (11.2), 57 $[M-C_{14}H_{29}]^+$ (100).

Butyrospermol acetate (**4**)

White amorphous powder, m. pt. 144-145 °C (lit. m. pt.[97] 146 °C), soluble in hexane, chloroform and diethyl ether and boiling methyl alcohol.

UV (hexane): λ_{max} = 218, 263 nm.

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.74 (s, 3H, H-25), 0.78 (s, 3H, H-27), 0.85 (s, 3H, H-24), 0.86 (d like, 3H, H-28), 0.92 (s, 3H, H-23), 0.96 (s, 3H, H-26), 1.60 (s, 3H, H-30), 1.63 (s, 3H, H-29), 2.03 ($C\underline{H}_3COO$-), 4.50 (dd, 1H, *J* = 10.9, 5.4 Hz, H-3), 5.07 (t, 1H, *J* = 6 Hz, H-21), 5.22 (dd, 1H, *J* = 5.4, 2.7 Hz, H-7).

^{13}C-NMR (75 MHz, $CDCl_3$): δ = 13.11 (C-25); 15.85 (C-24), 17.66 (C-30), 18.56 (C-28), 21.34 (OAc), 22.00 (C-27), 23.7 (C-6), 24.1 (C-2), 25.3 (C-20), 25.74 (C-29), 27.30 (C-26), 27.55 (C-23), 28.45 (C-16), 33.70 (C-15), 33.90 (C-12), 34.70 (C-10), 35.11 (C-19), 35.77 (C-18), 36.70 (C-1), 37.80 (C-4), 43.46 (C-13), 48.70 (C-9), 50.70 (C-5), 51.24 (C-14), 53.22 (C-17), 81.10 (C-3), 117.54 (C-7), 125.08 (C-21), 130.94 (C-22), 145.90 (C-8), 171.01 ($CH_3\underline{C}OO$).

EIMS: *m/z* (%) = 468 $[M]^+$ (43.2), 453 $[M\text{-}CH3]^+$ (100), 451 (5.6), 393 (44), 355 [M-side chain-2H]$^+$ (10.4), 315 (3.2), 301 (4), 271 (3.2), 255 (4), 241 (4), 229 (4), 227 (3.2), 187 (5.6), 121 (9.6), 109 (17.6), 95 (14.4), 69 (40), 55 (14.4), 43 $[CH_3CO]^+$ (21.6) which were in close agreement with reported data.[97]

Bis(2-ethylhexyl)phthalate (5)

Light yellow oil, soluble in chloroform, ethyl ether, hexane and carbon tetrachloride.

UV (Et_2O): λ_{max} = 246.2, 273.4 nm; UV (hexane): λ_{max} =225.4, 254.2, 273.6 nm.

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.91 (t, 6H, *J* = 6.6 Hz, H-6, H-2"); 1.20-1.50 (m, H-3, H-4 H-5 and H-2' merged), 1.60-1.70 (m, 2H, H-2), 4.20 (dd like, 2H, H-1), 7.51 (dd, 1H, *J* = 6.6, 3.3 Hz, H-10), 7.68 (dd, 1H, *J* = 6.6, 3.3 Hz, H-9); ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.89 (t, *J* = 7.2 Hz, H- 2″), 0.92 (t, *J* = 6.8 Hz, H-6), 1.20-1.50 (m, H-3, H-4, H-5 and H-2′ merged), 1.63-1.73 (m, H-2), 4.22 (m, H-1), 7.53 (dd, *J* = 6, 3.2 Hz, H-10), 7.71 (dd, *J* = 6, 3.2 Hz, H-9).

^{13}C-NMR (75 MHz, $CDCl_3$,): δ = 10.89 (C-6), 14.00 (C-2"), 22.92 (C-5), 23.66 (C-4), 28.85 (C-3), 30.28 (C-2'), 38.64 (C-2), 68.08 (C-1), 128.70 (C-9), 130.82 (C-10), 132.37 (C-8), 167.69 (C-7). The assignments of C-9 and C-10 in literatures[99, 106] were revised with the help of HMQC.

EIMS: *m/z* (%) = 390 $[M]^+$ (0.8), 279 $[M\text{-}113 + 2H]^+$ (28.8), 167 $[M\text{-}113 + 3H]^+$ (43.2), 149 $[M\text{-}113\text{+}3H\text{-}H_2O]^+$ (100); 132 (2.4), 113 (11.2), 83 (6.4), 71 (18.4); GCMS showed the characteristic peaks at m/z 279 (15), 167 (39), 149 (100), 113 (9), 83 (7), 71(15), 57 (19) which were in close agreement with reported spectrum.[100]

Chrysophanol (6)

Yellow powder, soluble in carbon tetrachloride, chloroform, hexane and MeOH, negative $FeCl_3$ test where no blue or green color change indicating the absence of two vicinal hydroxyl groups[109], distinct smell.

UV (hexane): λ_{max} = 225.8, 248.5, 254.2, 260.4, 430.5 nm; UV (MeOH): λ_{max} = 225, 253, 287, 429 nm. This was in close agreement with literature data.[103] Three to four intense peaks between 215-300 nm and another one above or about 430 nm is characteristic for anthraquinone skeleton.[101] Peak at about 430 nm is indicative of the presence of at least two -OH in the α- position[102], that is, 1 and 8 positions in anthraquinone skeleton.
UV (MeOH + 2 drops 10% methanolic KOH): λ_{max} = 214, 233.5, 285.5, 507 nm. The bathochromic shift from 429 to 507 nm indicated the presence of free OH-1 and OH-8 groups. UV (MeOH + 2 drops 5% methanolic $AlCl_3$): λ_{max} = 207, 229, 492 nm. The bathochromic shift from 429 to 492 nm also indicated the presence of free OH-1 and OH-8 groups.

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 2.43 (s, 3H, Ar-C<u>H</u>$_3$); 7.06, (s, 1H, H-2), 7.25 (d, 1H, *J* = 6.6 Hz, H-7), 7.60 (s, 1H, H-4), 7.63 (d, 1H, *J* = 6.6 Hz, H-6), 7.79 (d, 1H, *J* = 6.6 Hz, H-5), 11.98 (s, OH-1), 12.09 (s, OH-8); ^{1}H-NMR (400 MHz, $CDCl_3$): 2.46 (s, Ar-C<u>H</u>$_3$), 7.09 (s, H-2), 7.28 (dd, *J* = 8.1, 1.2 Hz, H-7), 7.64 (s, H-4), 7.67 (br d, *J* = 8.1 Hz, H-6), 7.81 (dd, *J* = 8.1, 1.2 Hz, H-5), 12.00 (s, OH-1), 12.11 (s, OH-8).

^{13}C-NMR (75 MHz, $CDCl_3$): δ = 22.25 (Ar-CH_3), 113.67 (C-12), 115.80 (C-13), 119.88 (C-5), 121.32 (C-4), 124.32 (C-2), 124.51 (C-7), 133.20 (C-14), 133.56 (C-11), 136.92 (C-6), 149.31 (C-3), 162.35 (C-1), 162.65 (C-8), 181.92 (C-10), 192.46 (C-9). Assignments of C-5 and C-7 in literatures[105, 106] were revised as shown here based on HMQC and HMBC data.

EIMS: *m/z* (%) = 254 $[M]^+$ (100), 239 $[M\text{-}CH_3]^+$ (1.6), 237 $[M\text{-}OH]^+$ (2.4), 226 $[M\text{-}CO]^+$ (8), 198 $[M\text{-}2CO]^+$ (4), 197 $[M\text{-}(2CO + H)]^+$ (6.4), 152 $[M\text{-}CO\text{-}OH)]^+$ (6.4), 115 (4), 57 (3.2), 43 (3.2). GCMS: *m/z* (%) = 254 $[M]^+$ (100)

Physcion (7)

Yellow powder, soluble in carbon tetrachloride, chloroform, partly soluble in MeOH.

UV (MeOH): λ_{max} = 223, 282, 434 nm.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.42 (s, Me-3); 3.91 (s, MeO-6), 6.66 (d, 1H, *J* = 2.6 Hz, H-7), 7.06 (*d*-like, 1H, H-2), 7.34 (d, 1H, *J* = 2.6 Hz, H-5), 7.62 (d-like, 1H, H-4), 12.10 (s, OH-1), 12.30 (s, OH-8). Thses data were in close agreement with the reported data.[105, 111]

^{13}C-NMR (75 MHz, $CDCl_3$): δ = 22.16 (Me), 56.08 (OMe), 106.75 (C-7), 108.23 (C-5), 121.30 (C-4), 124.52 (C-2), 149.00 (C-3), 162.20 (C-8), 165.80 (C-1), 166.20 (C-6), 182.50 (C-10), 193.00 (C-9). However, signals of quaternary carbons C-11, C-12, C-13 and C-14 were missing due to their very long relaxation times.[110]

EIMS: *m/z* (%) = 284 $[M]^+$ (100), 255 $[M\text{-}CHO]^+$ (5.6), 241 $[M\text{-}CH_3CO]^+$ (5.6), 227 $[M\text{-}CO\text{-}CHO]^+$ (2.4), 213 $[M\text{-}CH_3CO\text{-}CO]^+$ (3.2), 198 (2.4), 185 $[M\text{-}CH_3CO\text{-}2CO]^+$ (2.4), 128 (4.8), 43 $[CH_3CO]^+$ (8.8). These data were in close agreement with the reported data.[113]

Palmitic acid (8)

Yellowish white, soft mass, soluble in chloroform.

IR (KBr): ν_{max} = 3435 (-OH), 2954, 2917, 2850, 1709 (-CO-), 1470, 1410, 1378, 1296, 1261, 1167, 1094, 943, 794, 718, 691 cm^{-1}.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.80 (t, 3H, *J* = 6.6 Hz, H-16), 1.1-1.3 (m, 24H, H-4 to H-15), 1.50-1.60 (quintet, 2H, H-3), 2.27 (t, 2H, *J* = 7.6 Hz, H-2).

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 14.04 (C-16), 22.62 (C-15), 24.63 (C-3), 29.01 (C-13), 29.18 (C-12), 29.29 (C-11), 29.38 (C-10), 29.53 (C-9), 29.63 (C-4 to C-8), 31.86 (C-14), 33.98 (C-2), 179.70 (C-1).

GCMS: *m/z* (%) = 256 $[M]^+$ (30.1), 227 (9.4), 213 (40.6), 199 (9.4), 185 (18.8), 171 (14.1), 157 (13.2), 143 (7.5), 129 (32), 97 (21.6), 83 (30.1), 73 (100), 60 (61.2), 51 (5.6) which were in close agreement with literature data.[114] Compound was confirmed from co-TLC.

Mixture of poriferasterol (2) and bis(2-ethylhexyl)phthalate (5)

Yellowish oil, positive Liebermann-Burchard test showing green color. This product gave many signals in ^{13}C-NMR. Of those signals, 29 and 12 signals identical to those of poriferasterol (**2**) and bis(2-ethylhexyl)phthalate (**5**), respectively, were distinguished as major products.

^{13}C-NMR (100 MHz): δ = 12.03 (C-18), 12.22 (C-29), 18.97 (C-26), 19.37 (C-19), 21.06 (C-11), 21.06 (C-21), 21.20 (C-27), 24.35 (C-15), 25.39 (C-28), 28.91 (C-16), 31.64 (C-2), 31.90 (C-7), 31.90 (C-8), 31.90 (C-25), 36.50 (C-10), 37.25 (C-1), 39.68 (C-12), 40.47 (C-20), 42.28 (C-4), 42.32 (C-13), 50.16 (C-9), 51.23 (C-24), 56.77 (C-17), 56.86 (C-14), 71.80 (C-3), 121.69 (C-6), 129.27 (C-23), 138.30 (C-22), 140.74 (C-5) corresponded to those of previously isolated poriferasterol (**2**); δ = 10.89 (C-6), 14.00 (C-2”), 22.92 (C-5), 23.66 (C-4), 28.85 (C-3), 30.28 (C-2’), 38.64 (C-2), 68.15 (C-1), 128.79 (C-9), 130.85 (C-10), 132.45 (C-8), 167.74 (C-7) corresponded to those of bis(2-ethylhexyl)phthalate (**5**).

Mixture of bis(2-ethylhexyl)phthalate (5) and chrysophanol (6)

Yellow substance.

^{13}C-NMR (100 MHz): δ = 10.96 (C-6), 14.05 (C-2"), 22.99 (C-5), 23.76 (C-4), 28.93 (C-3), 30.38 (C-2'), 38.75 (C-2), 68.17 (C-1), 128.81 (C-9), 130.88 (C-10), 132.48 (C-8), 167.76 (C-7) corresponded to those of bis(2-ethylhexyl)phthalate (**5**); δ = 22.27 (CH_3), 113.72 (C-12), 115.89 (C-13), 119.83 (C-5), 121.36 (C-4), 124.37 (C-2), 124.56 (C-7), 133.30 (C-14), 133.67 (C-11), 136.95 (C-6), 149.35 (C-3), 162.45 (C-1), 162.74 (C-8), 182.00 (C-10), 192.55 (C-9) corresponded to those of chrysophanol (**6**).

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 4.22 (m, H-1), 1.60-1.71 (m, H-2), 1.20-1.50 (m, H-3, H-4, H-5, H-2′), 0.92 (t, *J* = 6.8 Hz, H-6), 0.89 (t, *J* = 7.2 Hz, H-2″), 7.71 (dd, *J* = 6, 3.2 Hz, H-9), 7.53 (dd, *J* = 6, 3.2 Hz, H-10) corresponded to compound **5**; δ = 2.46 (s, Ar-CH_3), 7.09 (s, H-2), 7.28 (dd, *J* = 8.1, 1.2 Hz, H-7), 7.64 (s, H-4), 7.67 (br d, 8.1 Hz, H-6), 7.81 (dd, *J* = 8.1, 1.2 Hz, H-5), 12.00 (s, OH-1), 12.11 (s, OH-8) corresponded to those of compound **6**.

GCMS: characteristic peaks at m/z (%) 279 (14.1), 167 (33.9), 149 (100) corresponded to **5**. GCMS: *m/z* (%) = 254 [M^+] (100) corresponded to **6**.

Mixture of bis(2-ethylhexyl)phthalate (5) and physcion (7)

Yellow substance, soluble in chloroform.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.89 (t, H-2″), 0.92 (t, H-6), 1.21-1.49 (m, H-3, H-4, H-5, H-2′), 1.63-1.71 (m, H-2), 4.20 (m, H-1), 7.53 (dd, H-10), δ 7.70 (dd, H-9) corresponded to compound **5**; δ = 2.48 (Ar-CH_3), 3.94 (Ar-OCH_3), 6.69-7.64 (Ar-H × 4), 12.13 (OH-8), 12.33 (OH-1) corresponded to compound **7.**

GCMS: characteristic peaks at m/z (%) 279 (14.1), 167 (33.9), 149 (100) corresponded to **5**.

Steroidal mixture of poriferasterol (2) and *β*-sitosterol (9)

White substance; positive Liebermann-Burchard test showing green color; m. pt. 138 °C.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.69 (s, 3H, H-18), 0.79 (d, 3H, *J* = 6.8 Hz, H-27), 0.80 (t, 3H, *J* = 6.8 Hz, H-29), 0.84 (d, 3H, *J* = 6 Hz, H-26), 1.01 (s, 3H, H-19), 1.02 (d, 3H, *J* = 7.2 Hz, H-21), 3.51 (Unsymmetrical m, 1H, H-3α), 5.01 (dd, 1H, *J* = 14.8, 8.5 Hz, H-23), 5.15 (dd, 1H, *J* = 14.8, 8.5 Hz, H-22), 5.34 (d, 1H, *J* = 4.8 Hz, H-6). The ^{1}H-NMR signals were in accordance with those of **2** except one signal at δ 3.51 which resonated as unsymmetrical multiplet. This may be due to presence of *β*-sitosterol (**9**).

^{13}C-NMR (100 MHz, $CDCl_3$): δ = 12.04 (C-18), 12.22 (C-29), 18.98 (C-26), 19.39 (C-19), 21.07 (C-11), 21.07 (C-21), 21.21 (C-27), 24.36 (C-15), 25.39 (C-28), 28.90 (C-16), 31.67 (C-2), 31.91 (C-7), 31.91 (C-8), 31.91 (C-25), 36.52 (C-10), 37.27 (C-1), 39.69 (C-12), 40.46 (C-20), 42.23 (C-4), 42.32 (C-13), 50.18 (C-9), 51.24 (C-24), 55.98 (C-17), 56.87 (C-14), 71.80 (C-3), 121.69 (C-6), 129.30 (C-23), 138.30 (C-22), 140.76 (C-5). These data were in close agreement with those of compound **2**.

GCMS: *m/z* = 412 $[M]^+$, 413 $[M+1]^+$, 397 $[M-CH_3]$, 394 $[M-H_2O]$, 379 $[M-CH_3-H_2O]$, 369 [M-isopropyl group], 351 [M-isopropyl group-H_2O], 300 [M-112], 271 [M-141], 255 [M-139 -H_2O] which were in accordance with literature data of poriferasterol.[92]

GCMS: *m/z* = 414 $[M]^+$, 399 $[M-CH_3]$, 396 $[M-H_2O]$, 381 $[M-CH_3-H_2O]$, 329, 303, 275 were in accordance with literature data[115] of *β*-sitosterol.

Compound (10)

Red crystal, sparingly soluble in chloroform but soluble in methanol.

UV/Vis (MeOH): λ_{max} =.219.5, 252.0, 289.5, 437.0 nm. Three to four intense peaks between 215-300 nm and another one above or about 430 nm is characteristic for anthraquinone skeleton.[101] Peak at about 430 nm is indicative of the presence of at least two -OH in the *α*-position[102], that is, 1 and 8 positions in anthraquinone skeleton. UV/Vis (MeOH + 2 drops 10% methanolic KOH): λ_{max} = 213.5, 522.5 nm. The bathochromic shift from 437 to 522.5 nm indicated the presence of free OH-1and OH-8 groups. UV/Vis (MeOH + 2 drops 5% methanolic $AlCl_3$): λ_{max} = 205.5, 228.5, 366.5, 489.5 nm. The bathochromic shift from 437 to

489.5 nm also indicated the presence of free OH-1 and OH-8 groups. It was not investigated further due to paucity of material.

Compound (11)

Light yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ = 0.87-1.67 (CH_3 and CH_2 of hydrocarbon), 7.00-7.50 (Ar-H)
GCMS: *m/z* (%) = 282 $[M]^+$ (100), 283 $[M+1]^+$ (62.2), 284 $[M+2]^+$ (16.0), 267 $[M-CH_3]^+$ (1.8), 254 $[M-CH_3CH_2]^+$ (1.8), 77 $(C_6H_5)^+$ (6.6), 51 $(C_4H_3)^+$ (3.7).

5.7 BIOASSAY OF ISOLATES FROM THE ROOT

5.7.1 Brine shrimp cytotoxicity test

Compounds (**1** to **7**) were assayed for cytotoxicity against brine shrimp. Sample solutions were prepared according to **non-dilution method**.[68] An amount of 6 mg of each compound was separately dissolved and diluted to 3 mL with chloroform in a 5-mL measuring cylinder. Each sample solution is equivalent to the concentration of 2 mg/mL. A volume of 500, 50 and 5 μL of thus prepared sample solution of each was transferred by micropipette to three separate eppendorff tubes of 1.5 mL capacity. Five replicates were made for each dose level. Controls were also prepared by using respective solvent alone as the volume of sample solution taken for each dose level. Solvents in each eppendorff tube were evaporated completely on Rotavapor at reduced pressure for about 25 minutes.

A volume of 500 μL of artificial sea water was added to each eppendorff tube. Ten brine shrimp were transferred in it and adjusted the volume to 1 mL with artificial sea water. Altogether, there were eighteen eppendorff tubes for each sample. They were kept in room illuminated using 15W bulb maintaining temperature 20-22 °C for 24 hours. The number of survivors was counted after 24 hours and determined the number of deaths in each dose level. Brine shrimp were considered dead if they did not exhibit any movement during 10 seconds of observation. Any brine shrimp death in control test tube was eliminated and repeated the whole experiment. LC_{50} values were calculated by probit analysis.[65] Experiments were performed thrice in total only for those samples which showed LC_{50} lesser than 1000 μg/mL. The LC_{50} of such sample was expressed in μg/mL as mean of three independently performed experiments with 95% confidence interval. Berberine was used as positive control.

Results and calculation: Number of death or survival of shrimp counted after 24 hours and LC_{50} values were shown in tables 5.10 to 5.14.

Table 5.10: Cytotoxicity of bis(2-ethylhexyl)phthalate (**5**) against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL)		100 (µg/mL)		10 (µg/mL)		Log Z = x	y	x y	x^2	α	β	X	LC_{50} g/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
		S	D	S	D	S	D										
	C	7	3	8	2	9	1										
	1	6	4	9	1	7	2	Log 10 = 1	1.2	1.2	1						
	2	4	6	8	2	10	0	Log 100 = 2	3.6	7.2	4						
1	3	5	5	7	3	10	0	Log 1000 = 3	5.6	16.8	9	-0.93	2.2	2.69	489.77		
	4	6	4	4	6	8	2										
	5	4	6	6	4												
		ΣD = 28		ΣD = 18		ΣD = 6		Σx = 6	Σy = 10.4	Σx y = 25.2	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	5	5	7	3	8	2										
	2	5	5	6	4	10	0	Log 10 = 1	1.0	1.0	1						
2	3	6	4	7	3	9	1	Log 100 = 2	3.6	7.2	4	-1.06	2.2	2.75	562.34	538.15 ± 41.89	642.21 - 434.09
	4	4	6	7	3	9	1	Log 1000 = 3	5.4	16.2	9						
	5	3	7	5	5	9	1										
		ΣD = 27		ΣD = 18		ΣD = 5		Σx = 6	Σy = 10	Σx y = 24.4	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	4	6	6	4	10	0										
	2	4	6	6	4	9	1	Log 10 = 1	0.0	0.0	1						
3	3	3	7	5	5	9	1	Log 100 = 2	0.4	0.8	4	-1.06	2.2	2.75	562.34		
	4	6	4	7	3	9	1	Log 1000 = 3	8.6	25.8	9						
	5	6	4	8	2	8	2										
		ΣD = 27		ΣD = 18		ΣD = 5		Σx = 6	Σy = 9.0	Σx y = 26.6	$\Sigma x^2 = 14$						

Note: Berberine was used as a positive control (see in table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.11: Cytotoxicity of chrysophanol (**6**) against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	D	100 (µg/mL) S	D	10 (µg/mL) S	D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
		S	D	S	D	S	D										
	C	3	7	10	0	10	0										
	1	2	8	8	2	9	1	Log 10 = 1	1.0	1.0	1						
	2	4	6	7	3	9	1	Log 100 = 2	3.2	6.4	4	-2.26	3	2.42	263.02		
1	3	3	7	5	5	9	1	Log 1000 = 3	7.0	21.0	9						
	4	1	9	6	4	9	1										
	5	2	8	8	2	9	1										
		ΣD = 35		ΣD = 16		ΣD = 5		Σx = 6	Σy = 11.2	Σx y = 28.4	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	3	7	7	3	9	1										
	2	3	7	7	3	9	1	Log 10 = 1	1.0	1.0	1						
2	3	4	6	6	4	8	2	Log 100 = 2	3.0	6.0	4	-2.20	2.9	2.48	301.99	289.00 ± 55.87	344.87 - 233.13
	4	4	6	7	3	10	0	Log 1000 = 3	6.8	20.4	9						
	5	2	8	8	2	9	1										
		ΣD = 34		ΣD = 15		ΣD = 5		Σx = 6	Σy = 10.8	Σx y = 27.4	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	2	8	8	2	9	1										
	2	3	7	5	5	9	1	Log 10 = 1	1.0	1.0	1						
3	3	4	6	6	4	9	1	Log 100 = 2	3.0	6.0	4	- 2.20	2.9	2.48	301.99		
	4	4	6	7	3	9	0	Log 1000 = 3	6.8	20.4	9						
	5	3	7	7	3	9	2										
		ΣD = 34		ΣD = 15		ΣD = 5		Σx = 6	Σy = 10.8	Σx y = 27.4	$\Sigma x^2 = 14$						

Note: Berberine was used as a positive control (see in table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.12: Cytotoxicity of physcion (**7**) against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	D	100 (µg/mL) S	D	10 (µg/mL) S	D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
		S	D	S	D	S	D										
	C	10	0	10	0	10	0										
	1	1	9	8	2	8	2	Log 10 = 1	0.8	0.8	1						
	2	2	8	4	6	9	1	Log 100 = 2	3.0	6.0	4	-3.93	4.1	2.17	147.91		
1	3	1	9	8	2	10	0	Log 1000 = 3	9.0	27.0	9						
	4	1	9	7	3	10	0										
	5	10	0	8	2	9	1										
		ΣD = 45		ΣD = 15		ΣD = 4		Σx = 6	Σy = 12.8	Σxy = 33.8	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	0	10	10	0	10	0										
	2	0	10	6	4	9	1	Log 10 = 1	1.0	1.0	1						
2	3	3	7	4	6	9	1	Log 100 = 2	3.2	6.4	4	-3.46	3.9	2.16	144.54	158.14 ± 20.70	209.56 - 106.72
	4	2	8	7	3	9	1	Log 1000 = 3	8.8	26.4	9						
	5	1	9	7	3	10	2										
		ΣD = 44		ΣD = 16		ΣD = 5		Σx = 6	Σy = 13.0	Σxy = 33.8	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	0	10	8	2	9	1										
	2	2	8	8	2	9	1	Log 10 = 1	0.8	0.8	1						
3	3	4	6	10	0	9	1	Log 100 = 2	2.8	5.6	4	-3.60	3.8	2.26	187.97		
	4	1	9	6	6	10	0	Log 1000 = 3	8.4	25.2	9						
	5	1	9	6	4	9	1										
		ΣD = 42		ΣD = 14		ΣD = 4		Σx = 6	Σy = 9.0	Σxy = 31.6	$\Sigma x^2 = 14$						

Note: Berberine was used as a positive control (see in table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.13: Cytotoxicity of eupatoric acid (**1**)**,** poriferasterol (**2**) and octadecane (**3**) against brine shrimp

Compound	Replicate	Dose levels (Z) 1000 (µg/mL) S	D	100 (µg/mL) S	D	10 (µg/mL) S	D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X
	C	10	0	10	0	10	0								
	1	6	4	9	1	8	2	Log 10 = 1	1.0	1.0	1				
	2	5	5	9	1	10	0	Log 100 = 2	1.2	2.4	4	- 1.26	1.75	3.57	3715.3
1	3	5	5	7	3	8	2	Log 1000 = 3	4.5	13.5	9				
	4	6	4	9	1	9	1								
	5	6	4	10	0	10	0								
		ΣD = 22		ΣD = 6		ΣD = 5		Σx = 6	Σy = 6.7	Σx y = 16.9	$\Sigma x^2 = 14$				
	C	10	0	10	0	10	0								
	1	8	2	10	0	10	0								
	2	10	0	9	1	9	1	Log 10 = 1	0.4	1.0	1				
2	3	8	2	8	2	10	0	Log 100 = 2	1.0	2.0	4	- 0.46	0.8	6.8	6.3×10^6
	4	8	2	9	1	9	1	Log 1000 = 3	2.0	6.0	9				
	5	6	4	9	1	10	0								
		ΣD = 10		ΣD = 5		ΣD = 2		Σx = 6	Σy = 3.4	Σx y = 9.0	$\Sigma x^2 = 14$				
	C	10	0	10	0	10	0								
	1	6	4	8	2	10	0								
	2	7	3	8	2	10	0	Log 10 = 1	1.0	1.0	1				
3	3	8	2	7	3	7	3	Log 100 = 2	2.0	4.0	4	- 0.13	0.9	5.7	501187
	4	6	4	9	1	9	1	Log 1000 = 3	2.8	8.4	9				
	5	9	1	8	2	9	1								
		ΣD = 14		ΣD = 10		ΣD = 5		Σx = 6	Σy = 5.8	Σx y = 13.4	$\Sigma x^2 = 14$				

Note: Berberine was used as a positive control (see in table 5.6 for detail). S = Survival, D = Death, C = negative control

Table 5.14: Cytotoxicity of butyrospermol acetate (**4**) against brine shrimp

Compound	Replicate	Dose levels (Z) 1000 (µg/mL)		100 (µg/mL)		10 (µg/mL)		Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X
		S	D	S	D	S	D								
	C	10	0												
	1	10	0	10	0	10	0	Log 10 = 1	0.6	0.6	1				
	2	8	2	9	1	10	0	Log 100 = 2	0.8	1.6	4	-1.2	1.4	4.42	26302
4	3	0	10	9	1	0	10	Log 1000 = 3	3.4	10.2	9				
	4	7	3	10	0	7	3								
	5	8	2	9	1	8	2								
		ΣD = 17		ΣD = 4		ΣD = 3		Σx = 6	Σy = 4.8	Σxy = 12.4	$\Sigma x^2 = 14$				

Note: Berberine was used as a positive control (see in table 5.6 for detail). S = Survival, D = Death, C = negative control

Remarks: Bis(2-ethylhexyl)phthalate (**5**), chrysophanol (**6**) and physcion (**7**) revealed LC_{50} of 538.15, 289.00 and 158.14 μg/mL, respectively. Their LC_{50} values were lesser than 1000 μg/mL indicating cytotoxicity against brine shrimp.

Eupatoric acid (**1**), poriferasterol (**2**), octadecane (**3**) and butyrospermol acetate (**4**) showed LC_{50} of 3715.3, 6.3×10^6, 501187 and 26302 μg/mL, respectively. Their LC_{50} values were greater than 1000 μg/mL indicating non-cytotoxicity.

5.7.2 Antibacterial Test

Compounds (**1-6** and **8**) isolated from petroleum ether extract of root were used for antibacterial activity. Compound **7** was not investigated due to insufficient amount of sample.

5.7.2.1 Disc diffusion technique: Zone of inhibition was determined by disc diffusion technique as discussed in section 2.2.6.1.

1. Mueller Hinton agar (MHA) plates and standard working inoculum of the test organisms (equivalent to McFarland 0.5): They were prepared as discussed in no. 5 and 6 of section 2.2.6.3. Eight MHA plates were prepared.

2. Inoculation of MHA plates: Two MHA plates were inoculated for each organism as discussed in no. 7 of section 2.2.6.3. Therefore, there were four sets of MHA plates.

3. Preparation of working solution: As there was difficulty in dissolving compounds in DMSO, all the compounds (2.00 mg each) were separately dissolved in chloroform (200 μL) to yield the concentration of 10 μg/μL.

4. Impregnation of disc (200 μg /disc) by sample solution: Sterile discs were impregnated with 20 μL solution of each compound (equivalent to 200 μg/disc) with the help of micropipette as described in no. 9 of section 2.2.6.3. Four discs were loaded separately with each compound. Hence, total twenty eight discs of seven compounds were prepared. Four negative controls were also prepared by loading the same volume of chloroform alone in blank sterile discs. Solvents in discs were evaporated in a stream of air inside the laminar chamber for 3-4 hours at room temperature.

5. Transfer of disc into inoculated MHA plates: With the help of sterilized forceps, a positive control (gentamycin, 10 μg) and discs impregnated with solution of compounds **1**, **2**,

3 and **4**, as well as a negative control (solvent) were transferred on one of the inoculated plates of *E. coli* by gently pressing down. Discs impregnated with solution of compounds **5**, **6** and **8** were transferred to another inoculated plate of *E. coli*. A paper template was used to place the discs above on the inoculated plate by placing the template under it. The disc was placed approximately 15 mm from the edge of the plate at one corner of the template (fig. 2.1). The forceps were sterilized over spirit lamp flame each step before and after the disc was transferred on the inoculated plate and dipped into ethanol contained in a beaker. Remaining three sets of inoculate plates were also loaded by discs similar way.

***6. Incubation of test plates*:** Eight plates, with the lid closed were left for 30 minutes in laminar air flow for diffusion of compounds. Then plates were incubated at 37 °C for 18 hours.

Results: Diameter of zone of inhibition around each of the disc (including 6 mm disc) were measured after 18 hours of incubation. Zone of inhibition was measured including 6 mm disc of paper. Diameter of zone with 6 mm value was indicative of no activity. Here, none of the compounds showed antibacterial activity at 200 μg/ disc (table 3.39).

5.8 BIOASSAY OF THE FLOWER

5.8.1 Preparation of extract

The shredded flower (325.00 g) was extracted with hexane (3L) in Soxhlet extractor for 8 hours. Thus obtained yellow solution was filtered and the filtrate was evaporated on Rotavapor to get yellowish brown residue (4.951 g). Thus obtained residue was used for brine shrimp cytotoxicity and antibacterial activity tests.

5.8.2 Brine shrimp cytotoxicity test

Sample solution was prepared by **dilution method**[63] as discusses in section 5.4.2.1a. Brine shrimp cytotoxicity test for this extract was performed as discussed in section 5.4.2.1b. The results were shown as below.

Results and calculation: Number of death or survival of shrimp counted after 24 hours and LC_{50} values were shown in table 5.15.

Table 5.15: Cytotoxicity of hexane extract of the flower against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	1000 (µg/mL) D	100 (µg/mL) S	100 (µg/mL) D	10 (µg/mL) S	10 (µg/mL) D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X	LC_{50} Mean ± σ	95% Confidence Interval
		S	D	S	D	S	D										
	C	10	0	0	0	0	0										
	1	0	10	10	0	10	0	Log 10 = 1	0.0	0.0	1						
	2	2	8	10	0	10	0	Log 100 = 2	0.0	0.0	4						
1	3	0	10	10	0	10	0	Log 1000 = 3	9.2	27.6	9	- 6.13	4.6	2.41	257.03		
	4	2	8	10	0	10	0										
	5	0	10	10	0	10	0										
		ΣD = 46		ΣD = 0		ΣD = 0		Σx = 6	Σy = 9.2	Σx y = 27.6	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	1	9	10	0	10	0										
	2	0	10	10	0	10	0	Log 10 = 1	0.0	0.0	1						
2	3	0	10	9	1	10	0	Log 100 = 2	0.2	0.4	4	- 6.33	4.8	2.36	229.08	241.99 ± 14.09	276.99 - 206.99
	4	1	9	10	0	10	0	Log 1000 = 3	9.6	28.8	9						
	5	0	10	10	0	10	0										
		ΣD = 48		ΣD = 1		ΣD = 0		Σx = 6	Σy = 9.8	Σx y = 29.2	$\Sigma x^2 = 14$						
	C	10	0	10	0	10	0										
	1	2	8	10	0	10	0										
	2	3	7	10	0	10	0	Log 10 = 1	0.0	0.0	1						
3	3	0	10	10	0	10	0	Log 100 = 2	0.8	1.6	4	- 5.73	4.5	2.38	239.88		
	4	0	10	10	4	10	0	Log 1000 = 3	9.0	27.0	9						
	5	0	10	10	0	10	0										
		ΣD = 45		ΣD = 4		ΣD = 0		Σx = 6	Σy = 9.8	Σx y = 28.6	$\Sigma x^2 = 14$						

Note: Berberine was used as a positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

Remarks: Hexane extract of the flower revealed LC_{50} of 241.99 µg/mL indicating cytotoxicity against brine shrimp.

5.8.3 Antibacterial test

5.8.3.1 Disc diffusion technique

Zone of inhibition was determined by disc diffusion technique as discussed in section 2.2.6.1.

Preparation of working solution (50 mg/mL): An amount of 25 mg of extract was taken into clean sterile eppendorff tube, dissolved and diluted to total volume of 500 µL with DMSO.

Impregnation of disc by sample solution: Sterile discs were impregnated with 20 µL of working solution of extract (equivalent to 1 mg) using micropipette as described in section 2.2.6.9. Total of four discs were loaded with this extract solution. Four negative controls were also prepared by loading 20 µL of DMSO alone separately in four blank discs. All together, there were eight discs (4 of extract loaded discs and 4 of negative controls). Solvents in discs were removed by evaporating in a stream of air by placing them inside the laminar chamber for 3-4 hours at room temperature.

Preparation of MHA, standard working inoculum: MHA plates and standard working inoculum of the test organisms (equivalent to McFarland 0.5) and inoculation of MHA plates by organism were prepared as discussed in no. 5, 6 and 7 of section 2.2.6.3. One inoculated MHA plate was prepared for each type of organisms. Therefore, there were altogether four inoculated MHA plates.

Transfer of disc into inoculated MHA plates

In each MHA plate, three discs [extract, positive control (gentamycine, 10 µg) and negative control] were transferred as discussed in no. 10 of section 2.2.6.3.

Incubation of test plates: Four test plates, with the lid closed were left for 30 minutes in laminar air flow for diffusion of compounds. Then plates were incubated at 37 °C for 18 hours.

Remark: Data of antibacterial activity performed for hexane extract of flower were presented in table 3.40. Hexane extract showed weak activity against gram-positive bacteria - *B. subtilis* and *S. aureus* in comparison with gentamycin (10 µg). The gram-negative bacteria - *E. coli*, *P. aeruginosa* were resistant to tested flower extract. The control disks injected with 20 µl of DMSO showed no inhibitory effect against the microorganisms tested.

5.8.3.2 MIC determination

MIC of hexane extract of flower was determined by two fold serial dilution method (see section 2.2.6.2). Eleven screw cap tubes with 1mL of Nutrient broth and one tube with distilled water (1200 µL) were sterilized in autoclave for 15 min and allowed to cool at room temperature. These test tubes were numbered 0 to 10. Test tube labeled 0 (zero) was negative control.

A volume of 300 µL hexane extract solution in DMSO having the concentration of 50 µg/µL was added to test tube containing 1200 µL of autoclaved distilled water to make a final volume of 1500 µL corresponding to 10 µg/µL or 10 mg/ml.

To the 1st test tube containing1 mL nutrient broth, 1 mL of the extract solution (10 mg) was transferred with the help of sterile pipette and mixed thoroughly. After complete homogenization, 1 mL of the contents of 1st tube was again transferred aseptically to the 2nd tube and homogenized thoroughly. Same procedure was adopted for the remaining 9 test tubes. From the 10th test tube, 1 mL mixture was discarded hence all the test tubes contained equal volumes. The corresponding concentrations of each test tube were shown in table 5.16.

Table 5.16: Preparation of solutions corresponding different concentrations for MIC

Test tube Number	0	1	2	3	4	5	6	7	8	9	10
Volume of Nutrient broth	1	1	1	1	1	1	1	1	1	1	1
Volume of extract	0	1	1	1	1	1	1	1	1	1	1
Total volume of broth + extract	1	2	2	2	2	2	2	2	2	2	2
Final volume after transfer to the consecutive test tubes	-	1	1	1	1	1	1	1	1	1	1
Concentration (µg/mL)	-	5000	2500	1250	625	312.5	156.2	78.2	39.1	18.5	9.2

A volume of 100 µL of the test organism standard culture suspension (McFarland 0.5) was added in each test tube except negative control (nutrient broth without extract). Tubes were incubated at 37 °C for 24 hours.

Remark: Due to turbidity in all test tubes, MIC determination was not possible.

5.8.3.3 MBC determination

Two nutrient agar plates were prepared by pouring sterilized molten nutrient agar (25 mL) and allowed to solidify at room temperature. Six sections were marked by black marker pen in bottom of both Petri plates. Sections were labeled by numbers (1-6) in one plate and another was labeled by numbers (7-12). A loop was inserted into each test tube (1-10) and streaked in each section according to number separately in Petri plates. Section numbers 6 and 12 were used for negative controls (nutrient broth without extract). They were incubated at 37 °C for 18 hours. After 18 hours, the last section in which there was no growth was examined.

Remarks: All test tubes (1-10) with definite amount of flower extract showed the growth of *B. subtilis*. Test tube no. 1 containing the highest amount of extract (2500 μg/ml) could not inhibit the growth of tested organism; therefore its MBC must be greater than 2500 μg.

5.9 BIOASSAY OF THE LEAF AND THE STEM

5.9.1 Preparation of extracts

Dried powdered stem (50.00 g) and powdered leaves (50.00 g) were separately extracted with ethanol in Soxhlet extractor for 7 hours. In each case, solvent was evaporated completely on Rotavapor to obtain their respective extracts. The yield of stem extract (liquid) and leaf extract (greenish black gummy solid) were 28.82 and 27.20%, respectively.

5.9.2 Brine shrimp cytotoxicity test

Sample solutions were prepared by **dilution method**[63] as discussed in section 5.4.2.1a. Bioassay was performed as discussed in section 5.4.2.1b.

Results and calculation: Number of death or survival of shrimp counted after 24 hours and LC_{50} values were shown in table 5.17.

Remarks: Ethanolic extract of the stem and leaf revealed LC_{50} of 6.6×10^{13} and 2.1×10^{6} μg/mL, respectively, indicating non-cytotoxicity against brine shrimp.

Table 5.17: Cytotoxicity of ethanolic extracts of the stem and the leaf against brine shrimp

Experiment	Replicate	Dose levels (Z) 1000 (µg/mL) S	D	100 (µg/mL) S	D	10 (µg/mL) S	D	Log Z = x	y	x y	x^2	α	β	X	LC_{50} µg/mL Antilog of X
	C	10	0	0	0	0	0								
	1	8	2	10	0	10	0	Log 10 = 1	0.0	0.0	1				
	2	9	1	10	0	10	0	Log 100 = 2	0.0	0.0	4	-0.53	0.4	13.82	6.6×10^{13}
Stem	3	10	0	10	0	10	0	Log 1000 = 3	0.8	2.4	9				
	4	10	0	10	0	10	0								
	5	9	1	10	0	10	0								
		ΣD = 4		ΣD = 0		ΣD = 0		Σx = 6	Σy = 0.8	Σx y = 2.4	$\Sigma x^2 = 14$				
	C	10	0	10	0	10	0								
	1	8	2	10	0	10	0								
	2	6	4	10	0	10	0	Log 10 = 1	0.0	0.0	1				
Leaf	3	9	1	10	0	10	0	Log 100 = 2	0.0	0.0	4	-1.33	1	6.33	2.1×10^{6}
	4	9	1	10	0	10	0	Log 1000 = 3	2.0	6.0	9				
	5	8	2	10	0	10	0								
		ΣD = 10		ΣD = 0		ΣD = 0		Σx = 6	Σy = 2.0	Σx y = 6.0	$\Sigma x^2 = 14$				

Note: Berberine was used as a positive control (see table 5.6 for detail). S = Survival, D = Death, C = negative control

5.10 ANTIOXIDANT ACTIVITY OF DIFFERENT PARTS OF PLANT

Antioxidant activity of ethanolic extracts of root, stem, defatted flower and leaf of *E. odoratum* was determined by following methods for the present study:

(1) *β*-carotene bleaching test

(2) DPPH radical scavenging test

5.10.1 *β*-carotene bleaching test

5.10.1.1 Preparation of ethanol extract

An amount of 50.00 g of powdered root was extracted with ethanol (400 mL) in a Soxhlet extractor for 7 hours. Similarly, 50.00 g of each of stem powder and leaf powder were separately extracted with ethanol (400 mL) for 7 hours. The shredded flowers were first defatted with hexane. Thus obtained defatted flower (14.00 g) was extracted with ethanol (150 mL) in a Soxhlet extractor for 7 hours. Each extract obtained from plant material above was evaporated completely on Rotavapor to obtain crude extract.

5.10.1.2 Procedure

β-Carotene bleaching test was performed as described in the literature.[73] A solution of *trans*-*β*-carotene (Type I, synthetic, Appox. 95% UV, Sigma, Switzerland) was prepared by dissolving 10 mg in 10 mL of chloroform. A volume of 0.2 mL of this solution was then pipetted into a round-bottom flask containing 20 mg linoleic acid (L, 1376, Sigma, min. 99 %) and 200 mg Tween-40 (Sigma, USA). After removal of chloroform in Rotavapor at 40 °C, 50 mL of distilled water was added to flask and vigorously shaken. An amount of 2 mg of each extract was dissolved in methanol and diluted to 1 mL volume in Eppendorf tube.

Nine test tubes were arranged into three sets-**I**, **II**, **III** with three test tubes in each set. A volume of 0.2 mL methanolic extract solution (2 mg/mL) was pipetted out into each test tube of set **I**. Similarly, a volume of 0.2 mL of butylated hydroxyanisole (BHA) (98% Aldrich, Switzerland) solution (2mg/ml in methanol) was pipetted out into each test tube of set **II** which were used as positive controls. Set **III** was without extract or BHA solution. Then, a volume of 5 mL of emulsion above was added into all test tubes. Set **III** was used as a negative control which contained only emulsion of linoleic acid and carotene. As soon as the emulsion was added to each test tube, the zero time absorbance was measured at 470 nm against same volume of methanol as reference solvent. Subsequent absorbance readings were

recorded at 15 min intervals by keeping the sample in an incubator at 50 °C until the discoloration of *β*-carotene in the negative control tubes disappeared (about 120 min).

The antioxidant activity of the samples was evaluated in terms of relative antioxidant activity (RAA).[73] Results are expressed as mean ± σ (standard deviation). Paired-sample Student's *t*-test was used for the significant differences between two samples as discussed in section 2.2.7.1.

Remarks: Quantitative results of the different extracts of the plant were summarized in table 5.18 and 5.19. Statistically analyzed data using paired-sample Student's *t*-test were presented in table 5.20 to 5.23.

Table 5.18: Absorbance recorded at 15 minutes interval for extracts in *β*-carotene bleaching test

Material	Time: 0 (min.)	15	30	45	60	75	90	105	120
-ve control	0.665	0.336	0.239	0.134	0.109	0.098	0.087	0.080	0.082
	0.660	0.324	0.199	0.170	0.110	0.084	0.067	0.071	0.071
	0.654	0.304	0.272	0.175	0.112	0.087	0.075	0.071	0.071
Mean	**0.659**	**0.321**	**0.236**	**0.159**	**0.110**	**0.089**	**0.076**	**0.074**	**0.074**
BHA	0.665	0.552	0.550	0.587	0.551	0.559	0.561	0.557	0.578
	0.660	0.548	0.552	0.555	0.556	0.549	0.558	0.558	0.578
	0.654	0.555	0.567	0.551	0.543	0.556	0.545	0.550	0.549
Mean	**0.659**	**0.551**	**0.556**	**0.553**	**0.550**	**0.554**	**0.554**	**0.555**	**0.568**
Leaf	0.665	0.562	0.560	0.521	0.542	0.537	0.505	0.498	0.492
	0.660	0.574	0.528	0.524	0.516	0.504	0.500	0.528	0.522
	0.654	0.547	0.532	0.545	0.513	0.510	0.535	0.495	0.488
Mean	**0.659**	**0.561**	**0.540**	**0.530**	**0.523**	**0.517**	**0.513**	**0.507**	**0.500**
Root	0.665	0.340	0.248	0.189	0.113	0.108	0.095	0.079	0.094
	0.660	0.339	0.255	0.163	0.130	0.093	0.090	0.095	0.085
	0.654	0.350	0.253	0.183	0.127	0.110	0.079	0.088	0.080
Mean	**0.659**	**0.343**	**0.252**	**0.178**	**0.123**	**0.103**	**0.088**	**0.087**	**0.086**
Flower	0.665	0.545	0.519	0.519	0.503	0.486	0.458	0.471	0.460
	0.660	0.481	0.462	0.454	0.444	0.428	0.419	0.411	0.398
	0.654	0.525	0.498	0.488	0.481	0.464	0.458	0.448	0.436
Mean	**0.659**	**0.517**	**0.493**	**0.484**	**0.476**	**0.459**	**0.445**	**0.443**	**0.431**
Stem	0.665	0.408	0.336	0.245	0.185	0.161	0.135	0.141	0.126
	0.660	0. 391	0.309	0.250	0.204	0.178	0.155	0.127	0.116
	0.654	0.400	0.323	0.223	0.179	0.157	0.139	0.120	0.109
Mean	**0.659**	**0.399**	**0.322**	**0.239**	**0.189**	**0.165**	**0.143**	**0.129**	**0.110**

Table 5.19: Calculation of RAA of different extracts

Plant material	Absorbance (60 min.)	RAA[a] (Individual)	RAA[a] ± σ (s.d.)	Absorbance (120 min.)	RAA[b] (Individual)	RAA[b] ± σ (s.d.)
Root	0.113	0.113/0.55 = 0.205 = 0.21	0.22 ± 0.015	0.094	0.094/0.56 = 0.167 = 0.17	0.16 ± 0.011
	0.130	0.130/0.55 = 0.236 = 0.24		0.085	0.085/0.56 = 0.151 = 0.15	
	0.127	0.127/0.55 = 0.231 = 0.23		0.094	0.094/0.56 = 0.17	
Flower	0.503	0.91	0.86 ± 0.05	0.46	0.82	0.77 ± 0.06
	0.444	0.81		0.398	0.71	
	0.481	0.87		0.436	0.78	
BHA	0.551	0.55/0.55 = 1	1	0.578	0.56/0.56 = 1	1
	0.556			0.578		
	0.543			0.549		
	Mean = 0.55			Mean = 0.56		
Leaf	0.542	0.542/0.55 = 0.985 = 0.99	0.95 ± 0.032	0.492	0.878 = 0.88	0.89 ± 0.032
	0.516	0.938 = 0.94		0.522	0.932 = 0.93	
	0.513	0.932 = 0.93		0.488	0.871 = 0.87	
-ve Control	0.109	0.109/0.55 = 0.198 = 0.20	0.20 ± 0.099	0.082	0.146 = 0.15	0.13 ± 0.011
	0.112	0.203 = 0.20		0.071	0.126 = 0.13	
	0.112	0.203 = 0.20		0.071	0.126 = 0.13	
Stem	0.185	0.336 = 0.34	0.35 ± 0.020	0.116	0.207 = 0.21	0.21 + 0.02
	0.204	0.37		0.109	0.194 = 0.19	
	0.179	0.325 = 0.33		0.126	0.225 = 0.23	

Statistical Analysis:

Table 5.20: Paired *t*- test between ethanolic extract of the leaf and BHA (Xa = RAA of Extract; Xb = RAA of BHA)

	N	Xa	Xb	Di = |Xa -Xb|	Mean $\bar{d} = \frac{Di}{N}$	Di - $\bar{d}$	(Di - $\bar{d}$)^2	Standard Deviation (σ)	SEM = $\sqrt{\frac{\sigma^2}{N}}$	t	df	Remarks
60 Min.	1	0.99	1.00	0.01	0.046	- 0.036	0.0013	0.031	0.017	-2.70	2	$P > 0.01$
	2	0.93	1.00	0.07		0.024	0.0005					
	3	0.94	1.00	0.06		0.014	0.0002					
				Σ 0.14			Σ 0.0020					
120 Min.	1	0.88	1.00	0.12	0.106	0.014	0.0002	0.031	0.017	6.23	2	$P > 0.01$
	2	0.93	1.00	0.07		- 0.036	0.0013					
	3	0.87	1.00	0.13		0.024	0.0005					
				Σ 0.32			Σ 0.0020					

Table 5.21: Paired *t*-test between ethanolic extract of the root and BHA (Xa = RAA of Extract; Xb = RAA of BHA)

	N	Xa	Xb	Di = |Xa -Xb|	Mean $\bar{d} = \frac{Di}{N}$	Di - $\bar{d}$	(Di - $\bar{d}$)^2	Standard Deviation (σ)	SEM = $\sqrt{\frac{\sigma^2}{N}}$	t	df	Result
60 Min.	1	0.21	1.00	0.79	0.77	0.02	0.0004	0.015	0.008	96.2	2	$P < 0.01$
	2	0.24	1.00	0.76		-0.01	0.0001					
	3	0.23	1.00	0.77		0.00	0.0000					
				Σ 2.32			Σ 0.0005					
120 Min.	1	0.17	1.00	0.83	0.83	0.00	0.0000	0.014	0.008	103.7	2	$P < 0.01$
	2	0.15	1.00	0.85		0.02	0.0004					
	3	0.17	1.00	0.83		0.00	0.0000					
				Σ 2.51			Σ 0.0004					

Note: P > 0.01 = not significantly different from antioxidant property of BHA; $P < 0.01$ = significantly different from BHA

Table 5.22: Paired *t*-test between ethanolic extract of flower and BHA (Xa = RAA of Extract; Xb = RAA of BHA)

	No	Xa	Xb	Di = |Xa -Xb|	Mean $\bar{d} = \frac{Di}{N}$	Di - $\bar{d}$	(Di - $\bar{d}$)^2	Standard Deviation (σ)	SEM = $\sqrt{\frac{\sigma^2}{N}}$	t	df	Result
60 Min.	1	0.91	1.00	0.09	0.136	- 0.046	0.0021	0.050	0.028	4.8	2	$P > 0.01$
	2	0.81	1.00	0.19		0.054	0.0029					
	3	0.87	1.00	0.13		- 0.006	0.0000					
				Σ 0.41			Σ 0.005					
120 Min.	1	0.82	1.00	0.18	0.23	- 0.05	0.0025	0.055	0.031	7.4	2	$P > 0.01$
	2	0.71	1.00	0.29		0.06	0.0036					
	3	0.78	1.00	0.22		0.01	0.0001					
				Σ 0.69			Σ 0.0062					

Table 5.23: Paired *t*-test between ethanolic extract of stem and BHA (Xa = RAA of Extract; Xb = RAA of BHA)

	N	Xa	Xb	Di = |Xa -Xb|	Mean $\bar{d} = \frac{Di}{N}$	Di - $\bar{d}$	(Di - $\bar{d}$)^2	Standard Deviation (σ)	SEM = $\sqrt{\frac{\sigma^2}{N}}$	t	df	Result
60 Min.	1	0.34	1.00	0.66	0.65	0.01	0.0001	0.021	0.012	54.1	2	$P < 0.01$
	2	0.37	1.00	0.63		- 0.02	0.0004					
	3	0.33	1.00	0.67		0.02	0.0004					
				Σ 1.96			Σ 0.0009					
120 Min.	1	0.23	1.00	0.77	0.79	- 0.02	0.0004	0.02	0.011	71.8	2	$P < 0.01$
	2	0.19	1.00	0.81		0.02	0.0004					
	3	0.21	1.00	0.79		0.00	0.0000					
				Σ 2.37			Σ 0.0008					

Note: P > 0.01 = not significantly different from antioxidant property of BHA; $P < 0.01$ = significantly different from BHA

Remark: Leaf and flower ethanolic extracts revealed significant antioxidant activity on the β-carotene bleaching test, with values comparable to the positive control (BHA) while stem and root ethanol extract showed non-significant antioxidant activity with respect to BHA.

5.10.2 DPPH radical scavenging test

5.10.2.1 Preparation of ethanol extracts

Ethanolic extracts of leaf, stem, root and defatted flower parts prepared as discussed in 5.10.1.1 were used for this test.

5.10.2.2 Preparation of DPPH solution

An amount of 0.005 g of DPPH was dissolved and diluted to 200 mL with methanol which corresponded to 6×10^{-5} M concentration. This was prepared daily.

5.10.2.3 Preparation of sample solutions of extracts and positive control

The plant extract (0.5 g) was dissolved in methanol to make 10 mL stock solution. The volumes of 0.1, 0.2, 0.4 and 1 mL of stock solution were pipetted out separately into 10 mL volumetric flasks and diluted further with methanol upto the mark to make four sample solutions corresponding to 0.5, 1, 2 and 5 mg/mL concentrations, respectively. Positive control (BHA) of similar concentrations was also prepared by same method. In the case of ethanolic extract of flower and stem, they were not fully soluble in methanol; therefore, extracts were first treated with methanol and filtered. Thus obtained filtrates were evaporated in Rotavapor to obtain their corresponding residues. After that, stock and sample solutions of flower and stem extracts were prepared from the residues thus obtained, as discussed above.

5.10.2.4 Procedure

Radical scavenging activity of sample solutions was measured by slightly modified method of Brand-Williams *et al*.[74] as shown in the following steps:

(a) A volume of 0.1 mL of each sample solution of various concentrations of 0.5, 1, 2, 5 mg/mL was transferred to separate test tubes which corresponded to 50, 100, 200 and 500 µg extract, respectively. Three replicates were made for each concentration. Therefore, there were twelev test tubes for four concentrations of each extract and also twelve test tubes for four concentrations of positive control.

(b) A blank solution was prepared by mixing 3.9 mL of DPPH solution and 0.1 mL of MeOH in a cuvette whose absorbance was measured at 517 nm using MeOH as

reference. The blank reading was measured every time just before the experiment of each concentration starts in the subsequent steps.

(c) Then immediately, volumes of 3.9 mL DPPH solution was added to three replicates of concentration 50 µg prepared in step (a) and were kept in the dark for 30 minutes at room temperature. This process was repeated for remaining concentrations (100, 200, and 500 µg) of the extract and the positive control (BHA).

(d) After 30 minutes, the absorption was measured at 517 nm using MeOH as reference.

Absorbance measured for different extracts and BHA was shown in table 5.24 to 5.28.

Table 5.24: Absorbance recorded for leaf extract

DPPH + MeOH (Blank)	DPPH + Leaf Extract (500 µg)	DPPH + MeOH (Blank)	DPPH + Leaf Extract (200 µg)	DPPH + MeOH (Blank)	DPPH + Leaf Extract (100 µg)	DPPH + MeOH (Blank)	DPPH + Leaf Extract (50 µg)
0.713	0.087	0.713	0.387	0.715	0.540	0.718	0.677
	0.086		0.372		0.533		0.674
	0.085		0.370		0.529		0.671

Table 5.25: Absorbance recorded for flower extract

DPPH + MeOH (Blank)	DPPH + Flower Extract (500 µg)	DPPH + MeOH (Blank)	DPPH + Flower Extract (200 µg)	DPPH + MeOH (Blank)	DPPH + Flower Extract (100 µg)	DPPH + MeOH (Blank)	DPPH + Flower Extract (50 µg)
0.667	0.068	0.667	0.214	0.670	0.432	0.672	0.591
	0.056		0.239		0.426		0.576
	0.048		0.271		0.457		0.567

Table 5.26: Absorbance recorded for stem extract

DPPH + MeOH (Blank)	DPPH + Stem Extract (500 µg)	DPPH + MeOH (Blank)	DPPH + Stem Extract (200 µg)	DPPH + MeOH (Blank)	DPPH + Stem Extract (100 µg)	DPPH + MeOH (Blank)	DPPH + Stem Extract (50 µg)
0.676	0.631	0.678	0.687	0.680	0.712	0.685	0.745
	0.629		0.672		0.726		0.753
	0.614		0.673		0.724		0.731

Table 5.27: Absorbance recorded for root extract

DPPH + MeOH (Blank)	DPPH + Root Extract (500 µg)	DPPH + MeOH (Blank)	DPPH + Root Extract (200 µg)	DPPH + MeOH (Blank)	DPPH + Root Extract (100 µg)	DPPH + MeOH (Blank)	DPPH + Root Extract (50 µg)
0.754	0.530	0.754	0.676	0.765	0.698	0.760	0.702
	0.521		0.677		0.697		0.697
	0.504		0.675		0.689		0.692

Table 5.28: Absorbance recorded for BHA (positive control)

DPPH + MeOH (Blank)	DPPH + BHA (500 µg)	DPPH + MeOH (Blank)	DPPH + BHA (200 µg)	DPPH + MeOH (Blank)	DPPH + BHA (100 µg)	DPPH + MeOH (Blank)	DPPH + BHA (50 µg)
0.723	0.068	0.729	0.237	0.730	0.460	0.734	0.564
	0.066		0.238		0.453		0.563
	0.060		0.238		0.450		0.562

Calculation for % Inhibition

Percentage inhibition of each sample was calculated as discussed in section 2.7.2 and expressed as mean of three independent experiments ± standard error mean (SEM). Data were shown in table 5.29. Paired-sample Student's *t*-test was used for the significant differences between extract and positive control (BHA of 500 µg) which were computated using SPSS 11.5, 2002 software.

Table 5.29: Scavenging activity of different parts of plant in terms of % inhibition

Concentration (µg)	BHA	Mean ± SEM	Leaf Extract	Mean ± SEM	Stem Extract	Mean ± SEM	Flower Extract	Mean ± SEM	Root Extract	Mean ± SEM
50	23.16	23.29	5.01	5.65	< 0	n.d.	12.05	13.98	7.63	8.28
	23.29	±	6.12	±			14.28	±	8.28	±
	23.43	0.07	5.84	0.33			15.62	1.04	8.94	0.37
100	36.98	37.75	24.47	25.33	< 0	n.d.	35.52	34.57	8.75	9.18
	37.94	±	25.52	±			36.41	±	8.88	±
	38.35	0.40	26.01	0.45			31.79	1.41	9.93	0.37
200	67.35	67.39	45.72	47.21	< 0	n.d.	67.91	65.66*	10.34	10.29
	67.48	±	47.82	±			64.16	± 1.25	10.21	±
	67.48	0.04	48.10	0.75			64.16		10.34	0.04
500	90.59	91.05	87.79	87.93*	6.65	7.59	89.80	91.40*	29.70	31.25
	90.87	±	87.93	± 0.08	6.95	±	91.60	± 0.87	30.90	±
	91.70	0.33	88.07		9.17	0.79	92.80		33.15	1.01

* P > 0.01(significantly not different from BHA of 500 µg); n.d. = not determined

Calculation for IC_{50}

IC_{50} of each sample was calculated by using linear regression equation ($y = a + bx$) at four different concentrations of sample. Here, sample concentrations of 50, 100, 200 and 500 µg corresponded to 12.5, 25, 50 and 125 µg/mL. The constant value, *a* and the regression coefficient, *b* were calculated using SPSS 11.5, 2002 software. After calculating *a* and *b*, x was calculated considering y = 50. The value of x was taken as IC_{50} value.

IC_{50} data in µg/mL of extracts of different parts of plant are presented in table 5.30.

Table 5.30: Scavenging activity of different extracts of plant in terms of IC_{50}

Sample	IC_{50} (µg/mL)
Flower	50.9
Leaf	65.4
Root	218.5
Stem	720.8
BHA	44.5

Remarks

Ethanolic extracts of leaf, defatted flower, root and stem at 500 µg of each showed DPPH scavenging activities with 87.93, 91.40, 31.25 and 7.59 % inhibition, respectively, showing scavenging activity in decreasing order: flower > leaf > root > stem. Such type of observation was also observed at 200, 100 and 50 µg of each extract. The % inhibition of different extracts at various concentrations showed that ethanolic extracts of leaf at 500 µg and defatted flower at 500 and 200 µg showed good scavenging activity on DPPH free radical.

Furthermore, IC_{50} of ethanolic extracts of defatted flower and leaf showed 50.9 and 65.4 µg/mL indicating promising scavenging activity. Meanwhile, root and stem showed IC_{50} higher than 100 µg/mL, revealing low scavenging activity on DPPH free radical.

References

1. http://www.fao.org/Ag/AGA/AGAP/FRG/AFRIS/Data/636.htm accessed in February, 2004.
2. Press JR, Shrestha KK, Sutton DA; Annotated checklist of the flowering plants of Nepal. The Natural History Museum, London, 2000, p 59.
3. Stainton A; Flowers of the Himalaya, A suplement. Oxford University Press, 1977.
4. Taylor RSL, Hudson JB, Manandhar NP, Towers GHN; Antiviral activities of medicinal plants of southern Nepal. *J. Ethnopharmacol*., 1996, **53**, 97 -104.
5. Bamba D, Bessiere J-M, Marion C, Pelissier Y, Fouraste I; Essential oil of Ivory Coast. *Planta Med*., 1993, **59**, 184-185 and references cited therein.
6. Iwu MM, Chiori CO; Antimicrobial activity of *Eupatorium odoratum* extracts. *Fitoterapia*, 1984, **LV** (6), 354-356 and references cited therein.
7. Metwally AM, Ekejiuba EC; Methoxylated flavonols and flavanones from *Eupatorium odoratum*. *Planta Med*., 1981, **42** (4), 403-405.
8. http://www.fs.fed.us/global/iitf/pdf/shrubs/chromolaena odoratum.pdf, accessed in August 2005.
9. http://www.phuketherb.velocall.com, accessed in August 2004.
10. Talpatra SK, Bhar DS, Talpatra B; Flavonoid and terpenoid constituents of *Eupatorium odoratum*. *Phytochemistry*, 1974, **13**, 284-285.
11. Talpatra SK, Bhar DS, Talpatra B; Terpenoids and related compounds: Part XIII-Expoxylupeol, a new triterpenoids from *Eupatorium odoratum*. *Indian J. Chem. Soc*., 1977, **15**B (9), 806-807 and references cited therein.
12. Ahamad M, Nabi MN; Chemical Investigation on the leaves of *Eupatorium odoratum*. *Sci. Res. Dacca*, 1967, **4**(2-3), 154-157. [CA 1968, **68**, 57405u].
13. Ding Z, Zhang X, Liu J, Ding J, Gu K; Chemical constitution from *Eupatorium odoratum*. *Tianran Chanwu Yanijiu Yu Kaifa*, 2001, **13**(5), 22-24. [CA 2002, **136**(26), 398497x].
14. Bose PK, Chakrabarti P, Chakrabarti S, Dutta SP, Barua AK; Flavonoid constituents of *Eupatorium odoratum*. *Phytochemistry*, 1973, **12**(3), 667-668.
15. Bose PK, Chakrabarti P, Chakrabarti S, Dutta SP, Barua AK; Chemical examination of the leaves of *Eupatorium odoratum*. *Phytochemistry*, 1974, **37**(1-2), 25-30.

16. Barua RN, Sharma RP, Thyagarajan G, Hertz W; Flavonoids of *Chromolaena odorata*. *Phytochemistry*, 1978, **17** (10), 1807-1808.

17. Hai MA, Biswas PK, Shil KC, Ahmad MU; Chemical constituents of *Eupatorium odoratum* Linn. *J. Bangladesh Chem. Soc*., 1991, **4** (1), 47-9. [CA 1992, **116**, 148159t].

18. Arene EO, Pettit GR, Ode RH; Antineoplastic agents. Part 49.The isolation of isosakuranetin methyl ether from *Eupatorium odoratum*. *Lloydia*, 1978, **41** (2), 186-189. [CA 1978, **89**, 39397x].

19. Nguyen TDT, Le VNP, Nguyen XD; Chalcones of *Eupatorium odoratum* L. from Vietnam. *Tap. Chi. Hoa. Hoc*., 1993, **31**(2), 79-80. [CA 1995, **122**, 235278y].

20. Suksamrarn A, Chotipong A, Suavansri T, Boongird S, Timsuksai P, Vimuttipong S, Chuaynugul A; Antimycobacterial activity and cytotoxicity of flavonoids from the flowers of *Chromolaena odorata*. *Arch. Pharm. Res*., 2004, **27**(5), 507-11.

21. http://www.grad.mahidol.ac.th/share/thesis/detail.php?ind=5151), accessed in August 2005.

22. Wollenweber E; Doerr M; Muniapan R; Exduate flavonoids in a tropical weed. *Chromolaena odorata*. *Biochem. Syst. Ecol*., 1995, **23**(7/8), 873-4. [CA 1996, **124**(9), 112426d].

23. Hai MA, Saha K, Ahmad MU; Chemical constituents of *Eupatorium odoratum* Linn. *J. Bangladesh Chem. Soc*., 1995, **8**(2), 139-142. [CA 1996, **127**(7), 216990v].

24. Wollenweber E, Roitman JN; Methyl ether of quercetagetin from *Chromolaena odorata*. *Biochem. Syst. Ecol*., 1996, **24** (5), 479-480. [CA 1996, **125**, 297099m]

25. Biller A, Bopre M, Witte L, Hartmann T; Pyrrolizidine alkaloids in *Chromolaena odorata*. Chemical and chemoecological aspects. *Phytochemistry*, 1994, **35**(3), 615-619.

26. Nguyen XD, Bien LK, Leclereq PA; Constituent of the leaf oil of *Chromolaena odorata*. *J. Essent. Oil Res*., 1992, **4**(3), 309-310. [CA 1993, **118**, 209414n]

27. Ling B, Zhang M, Kong C, Pang X, Liang G; Chemical composition of volatile oil from *Chromolaena odorata* and its effect on plant, fungi and insect growth. *Ying Yong Sheng Tai Xue Bao*., 2003, **14**(5), 744-746. [PMID: 12924132].

28. Sohonhloue DK, Dangou J, Djossou GL, Gnonhossou B, Sagbo UA; Chemical composition of essential oil of *Chromolaena odorata* collected in Houin. *J. Soc. Quest-Afr. Chim*., 1996, **1**(2), 75-82. [CA 1997, **127**(22), 305353y].

29. Bedi G, Tonzibo ZF, Chalchat JC, N' Guessan TY; Chemical constituents of *Chromolaena odorata* of essential oil from Abidjan, Ivory Coast. *J. Soc. Quest-Afr. Chim*., 2001, **7**(11), 29-37. [CA 2002, **136** (13), 205160a].

30. Chowdhury AR; Essential oil of the leaves of *Eupatorium odoratum* L. from Shillong (N.E.). *J. Essent. oil-Bearing*, 2002, **5**(1), 14-18. [CA 2003, **138**(4), 44417p].

31. Dhingra SN, Dhingra DR, Bhattacharya SC; Sesquiterpene hydrocarbon from *Eupatorium odoratum*. *Perfumery Essent. Oil Record*, 1956, **47**, 315-316. [CA 1957, **51**, 3513e]

32. Baruah RN, Leclercq PA; Constituents of the essential oil from the flowers of *Chromolaena odorata*. *Planta Med*., 1993, **59**(3), 283.

33. Baruah RN, Pathak MG; Fatty acid composition of *Chromolaena odorata*. *Indian J. Nat. Prod*., 1993, **9**(1), 17-18.

34. Mandal S, Parui S, Mondal AK; Analysis of free amino acids contents in pollen of nine Asteraceae species of known allergenic activity. *Ann. Agric. Environ. Med*., 1998, **5**, 17-20.

35. Bamikole MA, Ikhatua UJ, Osemwenkhae AE; Converting bush to meat: A case of *Chromolaena odorata* feeding to rabbits. *Pak. J. Nutri*., 2004, **3**(4), 258-261 and references cited therein.

36. Ekpa OD; Nutrient composition of three Nigerian medicinal plants. *Food Chem*., 1996, **57**(2), 229-232.

37. Velasco-Alinsug MP, Rivero GC, Quibuyen TAO; Isolation of mercury-binding peptides in vegetative parts of *Chromolaema odorata*. *Z. Naturforsch*. 2005, **60**c, 252-259.

38. Phan TT, Wang L, See P, Grayer RJ, Chan S-Y, Lee ST; Phenolic compounds of *Chromolaena odorata* protect cultured skin cells from oxidative damage: implication for cutaneous wound healing. *Bio. Pharm. Bull*., 2001, **24**(12), 1373-79. [CA 2002, **136**(10), 145191Z].

39. Ahamad M; Chemical Investigation on the leaves of *Eupatorium odoratum* II. *Sci. Res. Dacca*, 1969, **6** (1-2), 37-41. [CA 1970, **72**, 28870u].

40. Owoyele VB, Adediji JO, Soladoye AO; Anti-inflammatory activity of aquoeus leaf extract of *Chromolaena odorata*. *Inflammopharmacol*., 2005, **13**(5-6), 479-484.

41. Okunji CO, Iwu MM, Jackson JE, Tally JD, Bacchi C, Ayafor JF; Plant derived antiparasitic and antifungal compounds and the methods of the extraction the

compounds. PCT int. Apl. W.O. 4, May 2000, 0024, 411(cl. A61K35/78), US Apl. PV 27 Oct. 1998, 105, 888. [CA 2000, **132**(24), P326031k].

42. Caceres A, Menendez H, Mendez E, Cohobon E, Samayoa BE, Jauregni E, peralta E, Carrillo G; Antigonorrheal activity of plants used in Guatemala for the treatment of sexually transmitted diseases. *J. Ethanopharmacol*., 1995, **48**(2), 85-88.
43. Phan TT, Hughes MA, Cherry GW; Enhanced proliferation of fibroblasts and endothelial cells treated with an extract of the leaves of *Chromolaena odorata* (Eupolin), an herbal remedy for treating wounds. *Plast. Reconstr. Surg*., 1998, **101**(3), 756-765. [PMID: 9500394].
44. Bouda H, Tapondjou LA, Fontem DA, Gumedzoe MYD; Effect of essential oils from leaves of *Ageratum conyzoids*, *Lantana camara* and *Chromoleana odorata* on the mortality of *Sitophilius zeamais*. *J. stored Prod. Res*., 2000, **37**(2), 103-109. [CA 2001, **134**(15), 203743c].
45. Lu Y, Liang G, Shao W, Chen P; Effect of plant extracts on banana aphid. *Huazhong Nongye Daxue Xuebao*, 2002, **21**(4), 334-337 (Ch.). [CA 2003, **139**(5), 64795j].
46. Onwugbuta-Enyi J: Allelopathic effects of *Chromolaena odorata* L. (King and Robinson) toxin on tomatoes (*Lycopersicum esculentum* Mill). *J. App. Sci. Environ. Mgmt*., 2001, **5**(1), 69-73.
47. Patterson DT; Effects of allelopathic chemicals on growth and physiological responses of soyabean *(Glycine max)*. *Weed Sci*., 1981, **29**, 309 - 326.
48. Triratana T, Suwannuraks R, Naengchomnong W; Effect of *Eupatorium odoratum* on blood coagulation. *J. Med. Assoc. Thai*., 1991, **74**(5), 283-287.
49. Soogarun S, Wiwanitkit V, Suwansaksri J; Effect of *Chromolaena odorata* on prothrombin time and activated partial thromboplastin time. *Haema*, 2005, **8**(1), 149.
50. Verpoote R, der Heijden RV, Han Y-S; Biosynthesis of anthraquinones in cell cultures of the Rubiaceae. *Plant cell Tiss. Org. Cult*., 2001, **67**, 201-220.
51. Ebizuka Y, Shibuya M., Masuda K, Kushiro T; Mutational studies on triterpene synthases: Engineering lupeol synthases into *β*-amyrin synthases. *J. Am. Chem. Soc*., 2000a, **122**, 6816-6824.
52. Ebizuka Y, Shibuya M, Kushiro T, Masuda K, Morita M; Molecular cloning and fuctional expression of triterpene synthases from pea (*Pisum sativum*). *Eur. J. Biochem*. 2000b, **267**, 3453-3460.

53. Smith ARH, Goad LJ, Goodwin TW; Incorporation of stereospecifically labeled mevalonic acid into poriferasterol by *Ochromonas malhamensis*. *Phytochemistry*, 1972, **11**, 2775-2781.

54. Solomons TWG; Organic chemistry, 6th edition, John Wiley & Sons, Inc., 1996, p 398

55. Aderogba MA, Okoh EK, Adelanwa TA, Obuotor EM; Antioxidant properties of the Nigerian Piliostigma species. *J. Biol. Sci*., 2004, **4**(4), 501-503 and references cited therein.

56. Yepez B, Espinosa M, Lopez S, Bolanos G; Producing antioxidant fractions from herbaceous by supercritical fluid extraction. *Fluid Phase Equilibria*, 2002, **194-197**, 879-884 and references cited therein.

57. Dapkevicius A, Venskutonis R, van Beek TA, Linssen JPH; Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *J. Sci. Food Agric*., 1998, **77**, 140-146 and references cited therein.

58. I. Ciulei; Methods for studying vegetable and drugs, Personal communication.

59. Harborne JB; Phytochemical methods. A guide to techniques of plant analysis. Chapman and Hall, London, New York. 1973, p 34, 85 & 213.

60 Dhoubhadel SP, Shrestha KR; Phytochemical screening of the rhizomes of *Acorus calamus* Linn. *J. Inst. Sci*., 1978, **1**, 43-48.

61. McLaughlin JL, Rogers LL, Anderson JE; The use of biological assays to evaluate botanicals. *Drug Information Journal*, 1998, **32**, 513-524.

62. Ullah SS, Khanal DP; Synthesis, characterization and biological activity of some Titanium complexes. Proceeding of III national conference on science and technology, 1999, March 8-11, p 670-678.

63. Meyer BN, Ferrigin NR, Putnam JE, Jacobsen LB, Nicholas DE, McLaughlin JL; Brine Shrimp: A convenient general bioassay for active plant constituents. *Planta Med*., 1982, **45**, 31-34.

64. Orfila L, Rudriguez M, Colman T, Hasegawa M, Merentes E, Arvelo F; Structural modification of berberine alkaloids in relation to cytotoxic activity *in vitro*. *J. Ethanopharmcol*., 2000, **71** (3), 449-456. [CA 2001, **134**(9), 110092z].

65. Finney DJ; Probit Analysis. 3rd Edn., Cambridge University Press, Cambridge, 1971, p76-80.

66. Loomis TA; Essentials of Toxicity. 2nd Edn., Lea & Febiger, Philadelphia, 1974, p 19. In: Vowles PD, Connell DW; Experiments in environmental chemistry-A laboratory manual, Pergamon Press, 1980, vol. **4**, p 44.

67. Ghimire U; Screening of some Nepalese medicinal plants for the hypoglyceamic activity and the potential cytotoxicity. M. Sc. Thesis, Tribhuvan University, 2000.

68. Ahmad M; Cytotoxic activity of the leaf extract of *Crinum asiaticum* Linn. *Aust*. *J*. *Med*. *Herbalism*., 1996, **8**, 1-6.

69. Bauer AW, Kirby WMM, Sheriss JC, Turck M; Antibiotic susceptibility testing by standardized single method. *Am*. *J*. *Clin*. *Pathol*., 1966, **45**, 493-496.

70. Singh R, Chandra R, Bose M, Luthra PM; Antibacterial activity of *Curcuma longa* rhizome extract on pathogenic bacteria. *Curr*. *Sci*., 2002, **83**(6), 738-740.

71. Brougs MC; District laboratory practice in tropical countries, Cambridge University Press, 2000, p136.

72. Benabadji SH, Wen R, Zheng JB, Dong XC, Yuan SG; Anticarcinogenic and antioxidant activity of diindolylmethane derivatives. *Acta Pharmacol*. *Sin*., 2004, **25** (5), 666-671.

73. Bruni R, Muzzoli M, Ballero M, Loi MC, Fantin G, Poli F, Sacchetti G; Tocopherols, fatty acids and sterols in seeds of four sardinian wild euphorbia species. *Fitoterapia*, 2004, **75**, 50-61.

74. Brand-Williams W, Cuvelier ME, Berset C; Use of free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft und Technologie*, 1995, **28**, 25–30.

75. Yen GC, Duh PD; Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. *J. Agric. Food Chem.*, 1994, **42**, 629-632.

76. Hamzah AS, Lajis NH; Chemical constituents of *Hedyotis herbacea*. ASEAN Review of Biodiversity and Environmental Conservation (ARBEC), 1998 May, Article **II**, 1

77. Chang CI, Kuo CC, Chang JY, Kuo YH ; Three new oleanane-type triterpenes from *Ludwigia octovalvis* with cytotoxic activity against two human cancer cell lines. *J*. *Nat*. *Prod*., 2004, **67**, 91-93.

78. Jackman LM, Sternhell S; Aplication of nuclear magnetic resonance spectroscopy in organic chemistry, 2nd edition, Pergamon Press Ltd. Headington Hill Hall, Oxford. 1969, vol. **5**, p 239.

79. Huang KF, Hsu CJ; Constitution of stem barks of *Erythrina arborescens*. *J*. *Chin*. 2001, **12**(1), 61-67.

80. Doddrell DM, Khong PW, Lewis KG; The sterochemical dependence of ^{13}C chemical shifts in Olean-12-enes and Urs-12-enes an acid to structural assignment. *Tetrahedron lett*., 1974, **27**, 2381-2384.

81. Furniss BS, Hannaford AJ, Smith PWG, Tatchell AR; Vogel's textbook of practical organic chemistry, 5th Edn. ELBS, Longman, 1991, p323.

82. Maillard M, Adewunmi CU, Hosttettmann KA; Triterpene glycoside from the fruits of *Tetrapleura tetraptera*. *Phytochemistry*, 1992, **31**(4), 1321-1323.

83. Takasaki M, Konoshima T, Tokuda H, Masuda K, Arai Y, Shiojima K, Ageta H; Anti-carcinogenic activity of *Taraxacum* plant. II. *Biol*. *Pharm*. *Bull*., 1999, **22**(6), 607.

84. Budzikiewicz H, Wilson JM, Djerassi C; Mass spectrometry in structural and stereochemical problems. XXXII. Pentacyclic triterpenes. *J*. *Am*. *Chem*. *Soc*., 1963, **85**, 3688-3699.

85. Jackman LM, Sternhell S; Aplication of nuclear magnetic resonance spectroscopy in organic chemistry, 2nd edition, Pergamon Press Ltd. Headington Hill Hall, Oxford. 1969, vol. **5**, p 375.

86. Findlay JA, Patil AD; Sterol and other constituents of the brown alga *Desmarestia aculeata*. *Phytochemistry*, 1985, **24**, 366

87. Garg VK , Nes RW; Codisterol and other Δ^5 – sterols in the seeds of C*ucurbita mamima*. *Phytochemistry*, 1984, **23** (12), 2925-2929.

88. Ahmed R, Rashid F, Mansoor S, Ansar N; Constituents of *Prunus armeniaca*. *Pak*. *J*. *Sci*. *Ind*. *Res*., 2004, **47** (2), 142-145.

89. Rubinstien I, Goad LJ, Clague ADH, Mulheirn L; The 220 MHz NMR spectra of phytosterols. *Phytochemistry*, 1976, **15**, 195-200.

90. Kubo H, Ohtant K, Kasai R, Yamasaki K, Nic R, Tanaka O; Curcurbitane glycosides from *Hemsleya panacisscandes* rhizomes. *Phytochemistry*, 1996, **41**, 1169-1174.

91. Wright JLC, McInnes AG, Shimizu S, Smith DG, Walter JA, Idler D, Khalil W; Identification of C-24 alkyl epimers of marine sterols by ^{13}C nuclear magnetic resonance spectroscopy. *Can*. *J*. *Chem*., 1978, **56**, 1898 -1903.

92. Yayali N, Baltaci C; The sterol of *Cyclamen coum*. *Tr*. *J*. *Chemistry* 1996, **20**, 329-334.

93. Heilborn I, Cook A, Bunbury HM, Hey DH, Harris G, Pollock JRA, Stevens R; Dictionary of organic compounds, Eyre and Spottiswoode Ltd., E. and F.N. Spon Ltd., London, 1965a, vol.**5**, p 2773.

94. Aldrich catalog, no. S440-9, 2003-2004, p1689.

95. Furniss BS, Hannaford AJ, Smith PWG, Tatchell AR; Vogel's textbook of practical organic chemistry, 5th Edn. ELBS, Longman, 1991, p1300.

96. Khalid SA; Varga E, Szendrii K, Duddeck H; Lanosta-9 (11), 24-diene -3β-yl acetate from *Leuzea carthamoides*. *J*. *Nat*. *Prod*., 1989, **52**(5), 1136-1138.

97. Murti VVS, Seshadri TR, Sivakumaran S; Cudraniaxanthone and butyrospermol acetate from the roots of *Cudrania javanensis*. *Phytochemistry*, 1972, **11**, 2089-2092.

98. Shea PJ, Weber JB, Overcash MR; Uptake and phytotoxicity of di-n-butyl phthalate in corn (*Zea mays*). *Bull*. *Environm*. *Contam*. *Toxicol*., 1982, **29**, 153-158.

99. Amade P, Mallea M, Bouaïcha N; Isolation, structural identification and biological activity of two metabolites produced by *Penicillium olsonii Bainier* and *Sartory*. *J*. *Antibiot*., 1994, **47**(2), 201-207.

100. WILEY275.L DATABASE, No. 231010.

101. Harborne JB; Phytochemical methods. A guide to techniques of plant analysis. Chapman and Hall, London, New York, 1973, p 18.

102. Das A, Joshi T, Shukla S; Anthraquinones from *Cassia sophera* root bark. *Phytochemistry*, 1984, **23**(11), 2689-2691.

103. Fairbairn JW, El-Muhtadi FJ; Chemotaxonomy of anthraquinones in Rumex. *Phytochemistry*, 1972, **11**, 263-268

104. Wijnsma R, Verpoorte R; Anthraquinone in the *Rubiaceae*. In: Progress in the chemistry of organic natural products. Springer-Verlag, Wien New York, 1986, **49**, p103.

105. Meselhy R; Constituents from Moghat, the roots of *Glossostemon bruguieri* (Desf.). *Molecules*, 2003, **8**, 614-621.

106 Amatya S, Tuladhar SM; Eupatoric Acid: A Novel trierpene from *Eupatorium Odoratum* L. (Asteraceae). *Z*. *Naturforsch*., 2005, **60**b, 1006-1011.

107. Wijnsma R, Verpoorte R; Anthraquinone in the *Rubiaceae*. In: Progress in the chemistry of organic natural products. Springer-Verlag, Wien New York, 1986, **49**, p 86.

108. Harborne JB; Phytochemical methods. A guide to techniques of plant analysis. Chapman and Hall, London, New York, 1973, p 83.

109 Yadav SK, Kalidhar SB; Alquinone: An anthraquinone from *Cassia alata*. *Planta Med*., 1994, **60**, 601

110. Wijnsma R, Verpoorte R; Anthraquinone in the Rubiaceae. In: Progress in the chemistry of organic natural products. Springer-Verlag, Wien New York, 1986, **49**, p 87.

111. Kitanaka S, Takido M; Dimeric hydroanthracenes from the unripe seeds of *Cassia torosa*. *Phytochemistry*, 1982, **21**(8), 2103-2106.

112. Lu XZ, Xu WH, Naoki H; Anthraquinones from *Saliva przewalskii*. *Phytochemistry*, 1992, **31**, 708-709.

113. Tessier AM, Delaveau P, Champion B; New anthraquinones in *Rubia cordifolia* root. *Planta Med*., 1981, **41**, 337-343.

114. Chung I-M, Ali M, Khanh TD, Choung MG, Park HJ, Ahmad A; New stigmastane steroids constituents from rice hulls of *Oryza sativa* and inhibitory activity on radish seed. *Bull. Korean Chem. Soc.*, 2006, **27**(1), 93-97.

115. Lenfant L; Lecompte MF, Farrugies G; Identification des sterols de *Physarum polycephalum*. *Phytochemistry*, 1970, **9**, 2529-2535.

116. Miliauskasa G, Venskutonis PR, van Beek TA; Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chem.*, Corrected proof, Available online 24 October 2003.

117. Osawa T; Novel natural antioxidants for utilization in food and biological systems, in postharvest biochemistry of plant food-materials in the tropics (Eds. Uritani I, Garcia VV, Mendoza EM), Japan scientific societies press, Tokyo, 1994, p 241-251. In: Panovska TK, Kulevanova S, Stefova M; *In vitro* antioxidant activity of some *Teucrium* species (*Lamiaceae*). *Acta Pharm*., 2005, **55**, 207-214.

118. Ma C-M, Nakamura N, Hattori M; Chemical modification of oleanene type triterpenes and their inhibitory activity against HIV-1 Protease dimerization. *Chem*. *Pharm*. *Bull*., 2000, **48** (11), 1681-1688.

119. Manojlovic NT, Solujic S, Sukdolak S, Krstic LJ; Isolation and antimicrobial activity of anthraquinones from some species of the lichen genus *Xanthoria*. *J. Serb*. *Chem*. *Soc*., 2000, **65**(8), 555-560.

120. www.mahidol.ac.th/abstracts/annual1999/0354.htm, accessed in March, 2005.

121. Semple SJ, Pyke SM, Reynolds GD, Flower RLP. *In vitro* antiviral activity of the anthraquinone chrysophanic acid against poliovirus. *Antiviral Res*., 2001, **49**, 169-178.

122. Wang Z-T, Xu G-J, Hattori M, Namba T; Constituents of the roots of *Codonopsis pilosula*. *Shoyakugaku Zasshi*, 1988, **42**(4), 339-342.

123. Valentine FR, Bergmann W; Contribution to the study of marine products, VIII. The sterols of sponges. *J. org. chem.*, 1941, **6**, 452.

124. Williams BL, Goodwin TW, Ryley JF; Sterol content of some protozoa. *J. protozool.*, 1966, **13**(2), 227-230. [CA 1966, **65**, 4315d].

125. Patterson GW, Krauss RW; Sterols of chlorella. *Plant cell physiol.*, 1965, **6**(2), 211-20. [CA 1966, **64**, 3637c].

126. Sofowora EA, Hardman R; Steriods, phthalyl esters and hydrocarbons from *Balanites wilsoniana* stem bark. *Phytochemistry*, 1973, **12**, 403-406.

127. Gusakova SD, Umarov AU; A phthalate and dienoic acid from the oil of *Eremostachys molluceliodes*. *Chem. Nat. Compd.*, 1975, **11**(4), 527.

128. Rao GN, Kismar PM, Dhandapani VS, Krishna TR, Hayashi T; Constituents of *Cassia auriculata*. *Fitoterapia*, 2000, **71**, 82.

129. Morales G, Sierra P, Mancilla A, Paredes A, Loyola LA, Gallardo O, Borquez J; Secondary metabolites from four medicinal plants from northern Chile: Antimicrobial activity and biotoxicity against *Artemia salina*. *J. Chil. Chem. Soc.*, 2003, **48** (2), 1-13

130. Graham PR; Phthalate ester plasticizers- Why and how they are used. Environment health perspect. *Experimental Issue*, 1973, **3**, 3-12.

ANNEX 1

PREPARATION OF REAGENTS

Mayer's reagent

Mercuric chloride (0.679 g) was dissolved in distilled water (20 mL) in a volumetric flask of 50 mL capacity. To this solution, potassium iodide (2.5 g) was added. Thus obtained scarlet red precipitate was dissolved by shaking and diluted further with distilled water up to mark of volumetric flask.

Dragendorff's reagent

Two solutions A and B were prepared as follows

Solution A: Bismuth nitrate (4 g) was dissolved in 5N nitric acid (10 mL) to make solution A.

Solution B: Potassium iodide (13.5 g) was dissolved in distilled water (20 mL) to make solution B.

These solutions A and B were mixed together in a 50 mL volumetric flask. The solution was diluted with distilled water up to mark of volumetric flask.

Sodium picrate solution

Picric acid (0.25 g) was dissolved in distilled water (50 mL) to make aqueous picric acid solution. The solution was neutralized with sodium bicarbonate.

Sodium picrate paper: A strip of Whatman no. 1 filter paper was dipped in the prepared solution above. The paper was dried completely and protected from other contamination. Thus prepared sodium picrate paper was used for cyanogenic glycosides detection.

Molisch's reagent

α-Napthol (5 g) was dissolved in methanol (50 mL).

Alcoholic ferric chloride solution

Ferric chloride (0.5 g) was dissolved in methanol (50 mL).

ANNEX 2

PUBLICATIONS & PAPER PRESENTATION

This thesis has included following papers.

1. Eupatoric Acid: A Novel Triterpenes from *Eupatorium Odoratum* L. (Asteraceae).
   Sajan Amatya and Sarbajna M. Tuladhar
   Z. Naturforsch. 2005, **60**b, 1006-1011.

2. Phytochemical Screening and Cytotoxicity of Root Extracts of *Eupatorium odoratum* Against the Shrimp Nauplii of *Artemia salina*.
   S. Amatya and. S. M. Tuladhar
   Pak. J. Sci. Ind. Res. 2005, **48**(3), 212-215.

3. A review: Chemical and pharmacological constituents of *Chromolaena odorata*
   Sajan Amatya
   Afr. J. Biotech. (Submitted on Feb. 2006, Manuscript No AJB- 06-092)

Paper presentation

Phytochemical investigation of *Eupatorium odoratum* L. for cytotoxicity against *Artemia salina* (Brine shrimp).

Presented in the Chemistry Symposium organized by Nepal Chemical Society on the occasion of 22nd Annual General Meeting held on 14th August, 2004 in Kathmandu, Nepal.

Eupatoric Acid: A Novel Triterpene from *Eupatorium odoratum* L. (Asteraceae)

Sajan Amatya[a] and Sarbajna M. Tuladhar[b]

[a] Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal
[b] Research Centre for Applied Science and Technology, Tribhuvan University, Kirtipur, Kathmandu, Nepal

Reprint requests to Prof. Dr. S. M. Tuladhar. Fax: 977-1-4331303. E-mail: phoenix@wlink.com.np

Z. Naturforsch. **60b**, 1006 – 1011 (2005); received March 21, 2005

Phytochemical studies on the petroleum ether extract of the roots of *Eupatorium odoratum* have resulted in the isolation of a novel triterpene, 3β-hydroxy-28-carboxyolean-12-ene (**1**) along with seven known compounds – poriferasterol (**2**), octadecane (**3**), butyrospermol acetate (**4**), bis(2-ethylhexyl)phthalate (**5**), chrysophanol (**6**), physcion (**7**) and palmitic acid (**8**). Novel compound **1** is designated as eupatoric acid. Compounds **2**–**7** were reported here for the first time from this plant. Palmitic acid (**8**) was also isolated for the first time from this root. The structure of the novel compound was established on the basis of spectroscopic studies. The cytotoxicity of the compounds **1**–**7** was studied using a lethality test against *Artemia salina* (brine shrimp).

Key words: 3β-Hydroxy-28-carboxyolean-12-ene, Eupatoric Acid, *Eupatorium odoratum*, *Artemia salina*

Introduction

Eupatorium odoratum L. (Asteraceae: Eupatoriae), a perennial shrub, grows abundantly in the Central and Eastern regions of Nepal from 400-1500m altitude, being known as "Banmara". The juice of the aerial parts of this plant is used for cuts and wounds to arrest bleeding and promote healing [1]. The plant is suitable for treating fungal and protozoa diseases [2]. The ethanolic extract of the leaf is reported to possess antioxidant activity to protect cultured skin cells [3]. 4',5,6,7-Tetramethoxyflavone isolated from this plant, is found as a blood clotting enhancer factor when studied *in vitro* [4]. Extensive literature searches revealed that very few phytochemical analyses were done on that root [5]. Our preliminary test of the petroleum ether extract showed some cytotoxicity ($LC_{50} < 1000$ μg/ml) against brine shrimp. This encouraged us to perform a phytochemical analysis of the root of *E. odoratum*. We report a new triterpene which has been characterized as 3β-hydroxy-28-carboxyolean-12-ene (**1**) on the basis of spectral analyses and has been designated as eupatoric acid. In addition, we have isolated seven compounds – poriferasterol (**2**), octadecane (**3**), butyrospermol acetate (**4**), bis(2-ethylhexyl)phthalate (**5**), chrysophanol (**6**), physcion (**7**) and palmitic acid (**8**). Compounds **2**–**7** are reported here for the first time from this plant. Palmitic acid (**8**) is isolated for the first time from this root.

Results and Discussion

Compound **1**, eupatoric acid, showed positive on the Liebermann-Burchard test, Ferric chloride test, as well as the false Dragendorff test. Its IR spectrum exhibited bands for hydroxyl (3450 cm^{-1}) and carboxylic (1685 cm^{-1}) groups. The ^{1}H NMR spectrum of **1** showed seven methyls, one olefinic proton and one methine proton indicating an olean-12-ene skeleton. The methyls 23-H_3, 24-H_3, 25-H_3, 26-H_3, 27-H_3, 30-H_3 and 31-H_3 resonated as singlets at $\delta = 0.98, 0.77, 0.90, 0.77, 1.13, 0.91$ and 0.93, respectively. A one-proton broad triplet at $\delta = 5.28$ ($J = 3.3$ Hz) was assigned to the olefinic 12-H. Signals of double doublets at $\delta = 2.84$ (dd, $J = 14.9, 4.9$ Hz) were assigned to the methine proton of 18-H due to 19-H_2. In addition, it was possible to observe a signal typical of 3ax-H at $\delta = 3.22$, (dd, $J = 9.9, 4.9$ Hz) due to the presence of β-OH group at C-3 position. In ^{1}H-^{1}H COSY spectrum of **1**, the olefinic proton 12-H ($\delta = 5.28$) showed connectivity with the methylene group, 11-H_2 at $\delta = 1.85 - 1.90$ and the proton 3-H ($\delta = 3.22$) with the methylene group 2-H_2 at $\delta = 1.54 - 1.62$. The ^{1}H NMR spectrum (Table 1) bore a close resem-

Table 1. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra of Eupatoric acid **1**.

C/H	δ_C [a]	DEPT [b]	δ_H [c] (*J*)	^1H-^1H COSY	HMQC	Cross peaks in HMBC ^2J	^3J	^4J
1	38.3	CH_2						
2	26.9	CH_2	1.54 – 1.62 *m*	H-3				
3	78.8	CH	3.22 *dd* (9.9, 4.9)	H-2	78.8			
4	38.6	C						
5	55.1	CH						
6	18.2	CH_2						
7	32.6	CH_2						
8	39.2	C						
9	47.5	CH						
10	36.9	C						
11	23.3	CH_2	1.85 – 1.9 *m*	H-12				
12	122.2	CH	5.28 *br t* (3.3)	H-11	122.2			
13	143.7	C						
14	41.6	C						
15	27.6	CH_2						
16	22.9	CH_2						
17	46.3	C						
18	41.1	CH	2.84 *dd* (14.9, 4.9)		41.1			
19	45.9	CH_2						
20	30.6	C						
21	33.8	CH_2						
22	32.4	CH_2						
23	27.9	CH_3	0.98 *s*		27.9	C-4	C-3, C-5	
24	15.2	CH_3	0.77 *s*		15.2	C-4	C-3, C-5, C-23	
25	15.4	CH_3	0.90 *s*		15.4	C-10	C-9, C-1, C-5	C-4
26	16.8	CH_3	0.77 *s*		16.8	C-8	C-7, C-9, C-14	
27	25.8	CH_3	1.13 *s*		25.8	C-14	C-8, C-15, C-13	
28	29.6	CH_2	1.25 *s*		29.6			
29	181.0	–						
30	33.0	CH_3	0.91 *s*		33.0	C-20	C-19, C-31	
31	23.4	CH_3	0.93 *s*		23.4	C-20	C-30	

J (in parenthesis) in Hertz; *s*: singlet, *dd*: double doublet, *m*: multiplet, *br t*: broad triplet; [a,c] ^1H NMR and ^{13}C NMR were measured in $CDCl_3$ and one drop CD_3OD, and δ is in ppm; [b] DEPT was measured in $CDCl_3$.

blance to the oleanolic acid [6], with the exception of the one methylene group which appeared at $\delta = 1.25$ as a singlet.

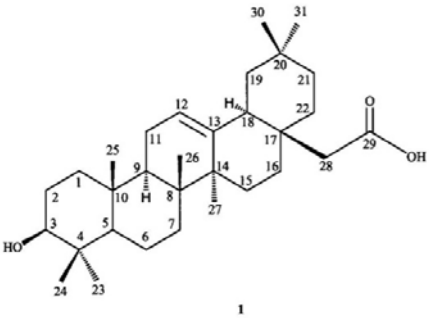
The ^{13}C NMR and DEPT spectra of **1** showed thirty one carbon resonances revealing the presence of seven methyl, eleven methylene, three methine carbons, one carbinol carbon, six quaternary carbons, two olefinic carbons and of one very weak signal for carbonyl carbon of carboxylic group ($\delta = 181.0$) (Table 1). Two olefinic carbons resonated at $\delta = 122.2$ and 143.7 were assigned for C-12 and C-13 respectively [7]. The remaining carbon atoms were assigned by HMQC and a comparison made with reported ^{13}C NMR data [8]. The observed HMQC and HMBC of **1** are presented in Table 1. Its ^{13}C NMR spectrum was in close agreement with that of oleanolic acid [8], except for one methylene group at $\delta = 29.6$, which showed one-bond connectivity with a singlet proton of methylene at $\delta = 1.25$ in the HMQC spectrum. Therefore, this methylene group ($\delta_C = 29.6$, $\delta_H = 1.25$) was seen to connect with quaternary C-17 and C-29. Its number was assigned as C-28 (Fig. 1). The HRMS spectrum of compound **1** showed the molecular ion peak at *m/z* 470.3387, accounting for the molecular composition $C_{31}H_{50}O_3$ (calcd. 470.3760). Furthermore, a small fragment ion in EIMS at *m/z* 425 $[M\text{-}COOH]^+$ and 411 $[M\text{-}CH_2COOH]^+$ confirmed the above results. These spectroscopic studies led to structure **1** for this new triterpene, 3β-hydroxy-28-carboxyolean-12-ene, designated as eupatoric acid.

Compound **2**, previously isolated from various natural sources [9 – 12], was confirmed as poriferasterol by comparison with the reported melting point [13] and ^{13}C NMR [14]. Compound **3** was confirmed as octadecane by ^1H NMR, ^{13}C NMR and GC/MS. In compound **4**, methyl protons and carbon signals were assigned by comparison with those of butyrospermol [15]. Its melting point and mass spectrum were

Table 2. LC_{50} and 95% Confidence interval of isolated compounds (**1**–**7**) from the roots of *E. odoratum* tested at 1000, 100 and 10 μg/ml in the Brine shrimp lethality test.

Compounds	LC_{50} (μg/ml)	95% Confidence intervals
Eupatoric acid (**1**)	> 1000	–
Poriferasterol (**2**)	> 1000	–
Octadecane (**3**)	> 1000	–
Butyrospermol acetate (**4**)	> 1000	–
Bis(2-ethylhexyl)phthalate (**5**)	538.15	642.21 – 434.09
Chrysophanol (**6**)	289.00	344.87 – 233.13
Physcion (**7**)	158.14	209.56 – 106.72
Berberine*	89.12	
Berberine chloride	22.50[e]	

[e] Meyer *et al.* (1982); * positive control.



1

Fig. 1. Eupatoric acid.

in close agreement with those reported in the literature [16]. Compound **5**, previously isolated from plants and microorganism [17–19], was confirmed as bis(2-ethylhexyl)phthalate by comparison with reported ^{1}H NMR, ^{13}C NMR and MS data [19]. Compound **6** and **7** were identified as chrysophanol and physcion, respectively, by comparison with reported ^{1}H NMR, ^{13}C NMR and MS data [20]. However, signals due to quaternary carbons C-11, C-12, C-13 and C-14 in **7** were difficult to observe due to their very long relaxation times [21]. Compound **8** was identified as palmitic acid by co-TLC with an authentic sample and further confirmed from spectroscopic data (GC/MS, ^{13}C NMR, ^{1}H NMR and IR).

Compounds **1**–**7** were tested for cytotoxicity using a brine shrimp lethality test [22]. From Table 2, it was revealed that compounds **1**, **2**, **3** and **4** exhibited non-significant cytotoxicity (LC_{50} > 1000 μg/ml), while **5**, **6** and **7** exhibited significant cytotoxicity (LC_{50} < 1000 μg/ml). LC_{50} values of **5**, **6** and **7** showed 538.15, 289.00 and 158.14 μg/ml, respectively, which were relatively, low as compared to the known cytotoxic compound, berberine chloride (22.5 μg/ml) [22].

Experimental Section

General

The Melting point was determined on Mettler FP61 and was uncorrected. IR spectra were recorded in KBr on Shimadzu spectrophotometer. The UV/vis spectra were measured on a Chemito UV-VIS 2500 spectrophotometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz), DEPT were recorded on a JEOL GX-400 with TMS as internal standard. FAB mass spectrum was recorded on a JEOL LMS-700T Spectrometer with glycerol as matrix. However, ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz), APT as well as EIMS were recorded at the University of Gö ttingen, Germany. ^{1}H NMR (100 MHz) and ^{13}C NMR (25 MHz) were recorded at the University of Wisconsin-La Crosse, USA. HRMS and EIMS for compound **1** were recorded on Finnigan Mat SSQ 710 (70 eV) and VG7035 instruments respectively at Institute of Chemical and Engineering Sciences (ICES), Singapore. GC/MS were recorded on Agilent 5973N plus 6890N at Tohoku University, Aoba-yama Campus, Japan. Silica gels (60–200 and 60–120 mesh) were used for column chromatography. TLC analyses were carried out on microscopic slide coated with silica gel GF_{254}. For PTLC, glass plates (20 × 20 cm) were coated with silica gel GF_{254} with thickness 0.25 mm. Petroleum ether of boiling point (40–60 °C) was used throughout the experiment, unless otherwise stated.

Plant materials

E. odoratum was collected from Tinpipalay (Kabhrepalanchok district), Nepal, in November 2001. A voucher specimen (TUCH 19) was deposited in the Tribhuvan University Central Herbarium, Central Department of Botany, TU, Nepal.

Brine shrimp lethality test

This test was performed as described by Meyer *et al.* [22]. LC_{50} values were calculated by Probit analysis [23]. LC_{50} value was expressed as the mean of three independently performed experiments in μg/ml with 95% confidence interval. Berberine was used as a positive control.

Extraction and isolation

Sun-dried root powder (1.99 kg) was extracted successively with petroleum ether (6.5 l, 5.5 l and 3.5 l) overnights by cold percolation and then filtered. The filtrate was concentrated to about 200 ml and refrigerated for two nights. The solid obtained was centrifuged and washed with cold

petroleum ether (10 ml × 5) to obtain a pale yellow solid and supernatant liquid (L1). The pale yellow solid was further washed, followed by a centrifuge with EtOAc-cold petroleum ether (3:97, 1 ml × 3), and recrystallized successively from EtOAc-hexane and MeOH, to yield the compound **1** [0.0143 g, $R_f = 0.34$ (MeOH-$CHCl_3$ 5:95)]. Supernatant liquid (L1) was concentrated to about 200 ml and left two overnights at room temperature, centrifuged and washed with cold petroleum ether (5 ml × 3) to obtain white mass and supernatant liquid (L2). White mass (0.0466 g) was chromatographed on silica gel column (1.6 × 37 cm, 15 g) by eluting with CH_2Cl_2 and CH_2Cl_2-EtOAc of increasing polarity: [CH_2Cl_2, 80 ml], [CH_2Cl_2-EtOAc (99:1, 150 ml)], [CH_2Cl_2-EtOAc (95:5, 100 ml)]. Fractions were collected at the rate of 25 ml/min in test tubes. They were pooled together into three fractions based on TLC characteristics. Fraction obtained from CH_2Cl_2-EtOAc (99:1), was evaporated to dryness and then recrystallized in methanol to yield white crystals of compound **2** [0.033 g, $R_f = 0.48$ (EtOAc-hexane 3:7), 0.51 (EtOAc-CH_2Cl_2 3:7)]. Yellow syrupy oil (4.2 g) obtained after evaporating supernatant liquid (L2) in Rotavapor, was chromatographed on a silica gel column (3 × 58 cm, 145 g). The column was eluted with hexane-CH_2Cl_2 (0 to 100%), CH_2Cl_2-EtOAc (0 to 100%) and EtOAc-MeOH (0 to 100%) in succession. Fractions were collected at the rate of 20 ml/15 min in test tubes. They were pooled together into fourteen fractions based on TLC characteristics.

Fraction 1, eluted with hexane, yielded compound **3** (0.681 g). Fraction 7 (oil, 0.078 g), eluted with hexane-CH_2Cl_2 (70:30), was dissolved in boiling MeOH, filtered off, allowed to cool over ice water, centrifuged and washed by cold MeOH to yield white amorphous powder **4** [0.019 g, $R_f = 0.48$ (CH_2Cl_2-hexane 50:50), 0.28 (hexane-CH_2Cl_2 70:30)].

Fraction 8 (0.260 g), eluted with hexane-CH_2Cl_2 (70:30 – 60:40) was rechromatographed on silica gel column (1.75 × 40 cm, 60 – 120 mesh, 20 g) by eluting with hexane-EtOAc gradients [hexane (40 ml), hexane-EtOAc (97:3, 270 ml), (90:10, 50 ml), (80:20, 50 ml), EtOAc (25 ml)]. Fractions were collected at the rate of 20 ml/30 min in test tubes. The collected fractions were monitored by TLC and pooled together to seven subfractions (S1 to S7). Subfraction S2 and S4 eluted with hexane-EtOAc (97:3) gave an oily compound **5** (0.166 g) and a sticky yellow mass (0.073 g), respectively after evaporation of solvent completely. Thus sticky yellow mass (0.065 g) was rechromatographed on a silica gel column (1 × 48 cm, 60 – 120 mesh, 7 g) by eluting with hexane/benzene 80:20. Fractions with $R_f = 0.58$ (benzene) were combined to yield a yellow mass (0.020 g), which, on recrystallization with chloroform produced compound **6** [0.0055 g, $R_f = 0.48$ (hexane-EtOAc 7:3)], 0.58 (benzene). Subfraction S6 (0.028 g), eluted with hexane-EtOAc (90:10 – 80:20), was chromatographed by preparative TLC in hexane-EtOAc (90:10) solvent to produce the compound **7** [0.019 g, $R_f = 0.12$ (EtOAc-hexane 1:9), 0.54 (benzene)].

Fraction 11 (greenish syrupy residue, 0.198 g), eluted from CH_2Cl_2-EtOAc (95:5 – 75:25), was dissolved in boiling hexane, cooled over ice water, filtered off and washed successively with cold hexane (10 ml × 3) and cold hexane-EtOAc 97:3 (10 ml) to obtain a white substance (0.084 g). It was confirmed as compound **2** by co-TLC, its melting point as well as the Liebermann-Burchard test.

Isolation of palmitic acid by PTLC from petroleum ether extract

Petroleum ether extract (2.6 g) obtained from root powder of *E. odoratum* (650 g) by cold percolation was dissolved in chloroform and chrmomatographed on PTLC plates with EtOAc solvent. The band with R_f value 0.41 [Hexane: Et_2O (1:1), fluorescein spray (0.01% in ethanol)] on usual work up with EtOAc yielded compound **8** (0.080 g).

Eupatoric acid (**1**): Pale yellow amorphous powder. – UV/vis ($CHCl_3$): λ_{max} = 243.8 nm. – IR (KBr): ν_{max} = 3450 (OH), 1685 (COOH) cm^{-1}. – ^1H NMR and ^{13}C NMR (Table 1). – EIMS: $m/z = 470$ $[M]^+$. HRMS *m/z*: 470.3387 [$C_{31}H_{50}O_3$, calcd. 470.3760].

Poriferasterol (**2**): White soft powder. – M.p. 154 °C. – UV/vis ($CHCl_3$): λ_{max} = 242 nm. – IR ($CHCl_3$): ν_{max} = 3600 (OH), 1040 (C-O stretch), 930 (-CH=CH-, *trans*) cm^{-1}. – ^1H NMR (400 MHz, $CDCl_3$): $\delta = 0.69$ (s, 3H, 18-H), 0.79 (d, 3H, $J = 6.9$ Hz, 27-H), 0.80 (t, 3H, J = 6.9 Hz, 29-H), 0.84 (d, 3H, $J = 5.9$ Hz, 26-H), 1.01 (s, 3H, 19-H), 1.03 (d, 3H, $J = 5.9$ Hz, 21-H), 3.52 (m, 1H, 3α-H), 5.04 (dd, 1H, $J = 14.9$, 8.5 Hz, 23-H), 5.14 (dd, 1H, $J = 14.9$, 8.5 Hz, 22-H), 5.35 (br d, 1H, $J = 4.2$ Hz, 6-H). – ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.04 (C-18), 12.22 (C-29), 18.98 (C-26), 19.39 (C-19), 21.07 (C-11), 21.07 (C-21), 21.21 (C-27), 24.36 (C-15), 25.39 (C-28), 28.90 (C-16), 31.67 (C-2), 31.91 (C-7), 31.91 (C-8), 31.91(C-25), 36.52 (C-10), 37.27 (C-1), 39.69 (C-12), 40.46 (C-20), 42.23 (C-4), 42.32 (C-13), 50.18 (C-9), 51.24 (C-24), 55.98 (C-17), 56.87 (C-14), 71.80 (C-3), 121.69 (C-6), 129.30 (C-23), 138.30 (C-22), 140.76 (C-5). – FABMS (positive ion, glycerol matrix): $m/z = 413$ $[M+1]^+$.

Octadecane (**3**): White translucent solid. – ^1H NMR (100 MHz, $CDCl_3$): $\delta = 0.8 – 0.9$ (t, 3H × 2), 1.2 – 1.3 (m, CH_2). – ^{13}C NMR (25 MHz, $CDCl_3$): $\delta = 14.1$ (C-1, C-18), 22.7 (C-2, C-17), 31.95 (C-3, C-16), 29.39 (C-4, C-15), 29.70 (C-5 to C-14). – GC/MS: $m/z = 254$ $[M]^+$.

Butyrospermol acetate (**4**): White amorphous powder. – M.p. 144 – 145 °C. – UV/vis (hexane): λ_{max} = 218, 263 nm. – ^1H NMR (300 MHz, $CDCl_3$): $\delta = 0.74$ (s, 3H, 25-H), 0.78 (s, 3H, 27-H), 0.85 (d like, 3H, 28-H), 0.86 (s, 3H, 24-H), 0.92 (s, 3H, 23-H), 0.96 (s, 3H, 26-H), 1.60 (s, 3H, 30-H), 1.63 (s, 3H, 29-H), 2.03 (CH_3COO), 4.50 (dd, 1H, $J = 10.9$,

5.4 Hz, 3-H), 5.07 (t, 1H, J = 6 Hz, 21-H), 5.22 (dd, 1H, J = 5.4, 2.7 Hz, 7-H). – ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.1 (C-25); 15.8 (C-24), 17.6 (C-30), 18.5 (C-28), 21.3 (OAc), 22.0 (C-27), 23.7 (C-6), 24.1 (C-2), 25.3 (C-20), 25.7 (C-29), 27.3 (C-26), 27.5 (C-23), 28.4 (C-16), 33.7 (C-15), 33.9 (C-12), 34.7 (C-10), 35.1 (C-19), 35.7 (C-18), 36.7 (C-1), 37.8 (C-4), 43.4 (C-13), 48.7 (C-9), 50.7 (C-5), 51.2 (C-14), 53.2 (C-17), 81.1 (C-3), 117.5 (C-7), 125.0 (C-21), 130.9 (C-22), 145.9 (C-8), 171.0 (CH_3COO). – EIMS: m/z (%) = 468 $[M]^+$ (43.2), 453 [M-CH3]$^+$ (100), 451 (5.6), 393 (44), 355 [M-side chain-2H]$^+$ (10.4), 315 (3.2), 301 (4), 271 (3.2), 255 (4), 241 (4), 229 (4), 227 (3.2), 187 (5.6), 121 (9.6), 109 (17.6), 95 (14.4), 69 (40), 55 (14.4), 43 $[CH_3CO]^+$ (21.6).

Bis(2-ethylhexyl)phthalate (**5**): Light yellow oil. – UV/vis (Et_2O): λ_{max} = 246.2, 273.4 nm. – ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.91 (t, 6H, J = 6.6 Hz, 6-H, 2"-H); 1.20–1.50 (m, 2-H, 3-H, 4-H and 5-H, merged), 1.60–1.70 (q, 2H, 2'-H), 4.20 (dd like, 2H, 1-H), 7.51 (dd, 1H, J = 6.6, 3.3 Hz, 10-H), 7.68 (dd, 1H, J = 6.6, 3.3 Hz, 9-H). – ^{13}C NMR (75 MHz, $CDCl_3$): δ = 10.8 (C-6), 14.0 (C-2"), 23.6 (C-4), 22.9 (C-5), 28.8 (C-3), 30.2 (C-2'), 38.6 (C-2), 68.0 (C-1), 128.7 (C-10), 130.8 (C-9), 132.3 (C-8), 167.6 (C-7). – EIMS: m/z (%) = 390 $[M]^+$ (0.8), 279 (28.8), 167 (43.2), 149 (100), 132 (2.4), 113 (11.2), 83 (6.4), 71 (18.4).

Chrysophanol (**6**): Yellow powder. – UV/vis (MeOH): λ_{max} = 225, 253, 287, 429 nm; – UV/vis (MeOH + KOH): λ_{max} = 214, 233.5, 285.5, 507 nm. – ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.43 (s, 3H, 3-Me); 7.06, (s, 1H, 2-H), 7.25 (d, 1H, J = 6.6 Hz, 7-H), 7.60 (s, 1H, 4-H), 7.63 (d, 1H, J = 6.6 Hz, 6-H), 7.79 (d, 1H, J = 6.6 Hz, 5-H), 11.98 (s, 1-OH), 12.09 (s, 8-OH). – ^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.1 (CH_3), 113.6 (C-12), 115.8 (C-13), 119.8 (C-7), 121.3 (C-4), 124.3 (C-2), 124.5 (C-5), 133.2 (C-14), 133.5 (C-11), 136.9 (C-6), 149.3 (C-3), 162.3 (C-1), 162.6 (C-8), 181.9 (C-10), 192.4 (C-9). – EIMS: m/z (%) = 254 $[M]^+$ (100), 239 [M-CH_3]$^+$ (1.6), 237 (2.4), 226 (8), 198 (4), 197 (6.4), 152 (6.4), 115 (4), 57 (3.2), 43 (3.2).

Physcion (**7**): Yellow powder. – UV/vis (MeOH): λ_{max} = 223, 282, 434 nm. – ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.42 (s, 3-Me); 3.91 (s, 6-OMe), 6.66 (d, 1H, J = 2.6 Hz, 7-H), 7.06 (*d*-like, 1H, 2-H), 7.34 (d, 1H, J = 2.6 Hz, 5-H), 7.62 (*d*-like, 1H, 4-H), 12.10 (s, 1-OH), 12.30 (s, 8-OH). – ^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.1 (Me), 56.0 (OMe), 106.7 (C-7), 108.2 (C-5), 121.3 (C-4), 124.5 (C-2), 149.0 (C-3), 162.2 (C-8), 165.8 (C-1), 166.2 (C-6), 182.5 (C-10), 193.0 (C-9). – EIMS: m/z (%) = 284 $[M]^+$ (100), 255 (5.6), 241 (5.6), 227 (2.4), 213 (3.2), 198 (2.4), 185 (2.4), 128 (4.8), 43 (8.8).

Acknowledgements

One of the authors (S. A.) is grateful to UGC, Nepal for partial fellowship support under the Doctoral Program. We are thankful to Prof. Dr. S. Kadota and Dr. A. H. Banskota, Toyama Medical and Pharmaceutical University, Japan; Prof. Dr. H. Laatsch and Dr. R. Maskey, University of Göttingen, Germany; Dr. K. G. Dongol, Institute of Chemical and Engineering Sciences, Singapore, and Dr. G. B. Bajracharya, Tohoku University, Aoba-yama Campus, Japan for recording all necessary spectra of compounds. We are also thankful to Prof. Dr. K. K. Shrestha, Central Department of Botany, Tribhuvan University, Nepal for identifying the plant.

[1] R. S. L. Taylor, J. B. Hudson, N. P. Manandhar, G. H. N. Towers, J. Ethnopharmacol. **53**, 97 (1996).
[2] C. O. Okunji, M. M. Iwu, J. E. Jackson, J. D. Tally, C. Bacchi, J. F. Ayafor, PCT int. Appl. W.O. 0024 411 cl. A61K35/78 (2000); US Appl. PV 105 888 (1998); Chem. Abstr. **132**(24), P326031k (2000).
[3] T. T. Phan, L. Wang, P. See, R. J. Grayer, S. Y. Chan, S. T. Lee, Biol. Pharm. Bull. **24**(12), 1373 (2001).
[4] T. Triratana, R. Suwannuraks, W. Naengchomnong, J. Med. Assoc. Thai. **74**(5), 283 (1991).
[5] A. Biller, M. Boppre, L. Witte, T. Hartmann, Phytochemistry **35**(3), 615 (1994).
[6] K.-F. Huang, C.-J. Hsu, J. Chin. **12**(1), 61 (2001).
[7] D. M. Doddrell, P. W. Khong, K. G. Lewis, Tetrahedron Lett. **27**, 2381 (1974).
[8] M. Maillard, C. U. Adewunmi, K. Hosttettmann, Phytochemistry **31**(4), 1321 (1992).
[9] G. W. Patterson, R. W. Kraus, Plant Cell Physiol. **6** (2), 211 (1965); Chem. Abstr. **64**, 3637c (1966).
[10] F. R. Valentine, W. Bergmann, J. Org. Chem. **6**, 452 (1941).
[11] B. L Williams, T. W. Goodwin, J. F. Ryley, J. Protozool. **13**(2), 227 (1966); Chem. Abstr. **65**, 4315d (1966).
[12] N. Yayali, C. Baltaci, Turk. J. Chem. **20**, 329 (1996).
[13] I. Heilborn, A. Cook, H. M. Bunbury, D. H. Hey, G. Harris, J. R. A. Pollock, R. Stevens, Dictionary of Organic Compounds, Vol. 5, p. 2773, Eyre and Spottiswoode Ltd., E. and F. N. Spon Ltd., London (1965).
[14] J. L. C. Wright, A. G. McInnes, S. Shimizu, D. G. Smith, J. A. Walter, Can. J. Chem. **56**, 1898 (1978).
[15] Y. Ebizuka, M. Shibuya, K. Masuda, T. Kushiro, J. Am. Chem. Soc. **122**, 6816 (2000).
[16] V. V. S. Murti, T. R. Seshadri, S. Sivakumaran, Phytochemistry **11**, 2089 (1972).
[17] E. A. Sofowora, R. Hardman, Phytochemistry **12**, 403 (1973).
[18] S. D. Gusakova, A. U. Umarov, Chem. Nat. Compd. **11**(4), 527 (1975).
[19] P. Amade, M. Mallea, N. Bouaïcha, J. Antibiot. **47**(2), 201 (1994).
[20] R. Meselhy, Molecules **8**, 614 (2003).

[21] R. Wijnsma, R. Verpoorte, in R. A. Hill, H. Chr. Krebs, R. Verpoorte, R. Wijnsma (eds): Progress in the Chemistry of Organic Natural Products, Anthraquinone in the Rubiaceae, Vol. 49, p. 79, Springer-Verlag, Wien, New York (1986).

[22] B. N. Meyer, N. R. Ferrigin, J. E. Putnam, L. B. Jacobsen, D. E. Nicholas, J. L. McLaughlin, Planta Med. **45**, 31 (1982).

[23] D. J. Finney, Probit Analysis, 3rd edn, p. 76 – 80, Cambridge University Press, Cambridge (1971).

Pak. J. Sci. Ind. Res. 2005 48(3) 212-2215

Phytochemical Screening and Cytotoxicity of Root Extracts of *Eupatorium odoratum* Against the Shrimp Nauplii of *Artemia salina*

S. Amatya[a] and S. M. Tuladhar*[b]

[a] Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal

[b] Research Centre for Applied Science and Technology, Tribhuvan University, Kirtipur, Kathmandu, Nepal

(received August 24, 2004; revised February 28, 2005; accepted March 3, 2005)

Abstract. Extracts of roots of *Eupatorium odoratum* were fractionated using various organic solvents. The fractions so obtained were petroleum ether extract (F1), solid residue left after ethanol extraction (F2), liquid residue left after ethanol extraction (F3), hexane soluble sub-fraction of F2 (F2-1), dichloromethane-soluble sub-fraction of F2 (F2-2), ethyl actate-soluble sub-fraction of F2 (F2-3), and 80% aqueous methanol soluble sub-fraction of F2 (F2-4). The root extract fractions were screened for such phytochemicals as sterols, triterpenoids, polyphenols, carbohydrates and reducing sugurs, alkaloids, flavonoids, quinones, and tannins. These root extract fractions were further tested for their cytotoxicity in the brine shrimp lethality test. The LC_{50} values indicated that F1, F2-1 and F2-2 were significantly cytotoxic against the brine shrimp nauplii. The fractions F3, F2-3 and F2-4 showed no cytotoxicity, whereas F2 was mildly cytotoxic in the brine shrimp lethality test.

Keywords: *Eupatorium odoratum*, brine shrimp lethality test, phytochemical cytotoxicity, *Artemia salina*, shrimp nauplii

Introduction

Eupatorium odoratum is a perennial shrub. In its new habitats it spreads quickly, forms dense thikets and seriously interferes not only with natural vegetation, but also with forestry, pasture and plantation crops. The plant is accordingly known as the forest killer, "banamara", in the Nepali language. The plant is distributed in the central and eastern regions of Nepal from 400-1500 m altitude (Press *et al.*, 2000). Juice of aerial parts of the plant is used on cuts and wounds to arrest bleeding and promote healing (Taylor *et al.*, 1996). The plant is used as fish poison (Talpatra *et al.*, 1977). It is also used as an ingredient in the preparation of antimalarial mixtures and cough suppressants; chloroform extract of leaves has shown antimicrobial activity (Iwu and Chiori, 1984). Okunji *et al.* (2000) have reported that the plant was suitable for treating fungal and protozoan diseases. Essential oil of leaves form Ivory Coast showed notable antimicrobial activity against the gram-negative bacteria (*Klebsiella pneum-oniae, E. coli, Pseudomonas aeruginosa*), but no activity was noted towards gram-positive species (Bamba *et al.*, 1993). Ethanol extract of the leaves showed antioxidant activity to protect cultured skin cells (Phan *et al.*, 2001). Triratana *et al.* (1991) reported that a compound 4,5,6,7-tetramethoxyflavone, isolated from this plant, was found as a blood clotting enhancer factor when studied *in vitro*. Roots of *Eupatorium odoratum* contain the N-oxides of pyrrolizidine alkaloids (Biller *et al.*, 1994). Most plants are toxic to domestic animals and humans (Cheeke, 1989).

*Author for correspondence; E-mail: phoenix@wlink.com.np

Researchers have isolated different classes of compunds from its aerial parts. Some of these are sesquiterpenoids (Chowdhry, 2002; Bedi *et al.*, 2001; Dung *et al.*, 1992), triterpenoids (Ding *et al.*, 2001; Talpatra *et al.*, 1977), pyrrolizidine alkaloids (Biller *et al.*, 1994), flavonoids (Ding *et al.*, 2001; Wollenweber and Roitman 1996; Hai *et al.*, 1995; 1991; Metwally and Ekejiuba, 1981; Barua *et al.*, 1978), fatty acids (Barua *et al.*, 1993), and tannins (Ahmad, 1969).

Very few phytochemical studies have been done on its root portion. The available literature indicates that no previous cytotoxic study has been done on roots of *E. odoratum*. The present study investigated the cytotoxic properties of its root extracts against *Artemia salina* (brine shrimp), along with its phytochemical screening. Brine shrimps have been previously used in bioassay studies for a variety of toxic substances, while this method has been applied to plant extracts in order to facilitate the isolation of biologically active compounds for therapeutics by De Giulio *et al.* (1992). It has been reported that over 300 novel antitumour and natural pesticidal products were isolated using this bioassay technique for prescreening (McLaughlin *et al.*, 1998).

Materials and Methods

Plant material. *E. odoratum* was collected from Tinpipalay, Kabhrepalanchok District, Nepal, in November 2001 and identified by Prof. K.K. Shrestha, a taxonomist in the Central Department of Botany, Tribhuvan University,

Kathmandu, Nepal and a voucher specimen was deposited under number 19-TUCH for record and reference.

Brine shrimp lethality test. This test was performed as described by Meyer *et al.* (1982). Each extract of the fraction solution was tested at a concentration level of 1000, 100 and 10 µg/ml. The eggs of brine shrimp (15 mg, Red Jungle Brand, USA) were hatched in a small beaker, filled with artificial seawater which was prepared from 40 g/l sea salt (Instant Ocean Aquarium System, Inc., USA). The eggs were incubated for about 48 h, at 27-30 °C in a waterbath. After 48 h, nauplii were collected with a pasture pipette by attracting the organisms to one side of the glass beaker, opposite to the light source, and transferred to a small beaker containing artificial seawater. Samples were prepared by dissolving 50 mg of each fraction of the root extract in 5 ml of a suitable solvent (chloroform or mehtanol). Chloroform was used as the solvent for petroleum ether extract (F1), solid residue of ethanol fraction (F2), and sub-fractions: hexane solubles (F2-1) and dichloromethane solubles (F2-2). Methanol was used as the solvent for ethyl acetate soluble fraction (F2-3), and 80% aqueous methanol soluble fraction of F2 (F2-4), as well as the liquid residue of ethanol fraction (F3). From these sample solutions, 500, 50 and 5 µl were transferred to test tubes (10 cm x 1 cm), corresponding to 1000, 100 and 10 µg/ml, respectively. Five replicates were made for each dose level. The contents of each test tube were evaporated completely at a temperature of 50 °C in Rotavapor at reduced pressure. A volume of 4 ml of artificial seawater was added to each test tube as noted above. Ten brine shrimp nauplii were tansferred to each test tube and the volume adjusted to 5 ml by adding more seawater. Solvent controls were also prepared for all the three-dose levels. Test tubes were kept in a room illuminated by using 15 W bulb, maintaining temperature at 20-22 °C for 24 h. The number of survivours were counted after 24 h and deaths of nauplii, in each dose level, were determined. The LC_{50} values were calculated by Probit analysis (Finney, 1971). The LC_{50} values were expressed as mean of three independently performed experiments in µg/ml, with 95% confidence interval.

Extraction process. Root powder (50 g) was first defatted with 300 ml petroleum ether (40-60 °C) for 7 h. The procedure was repeated four times. The extracts were combined and evaporated to obtain yellow oil (F1). The dried defatted marc (100 g) was then extracted with 500 ml ethanol for 7 h. This process was repeated twice. Combined extracts were concentrated in Rotavapor at reduced pressure at 40 °C. The so concentrated ethanol extract was treated with 100 ml water and the supernatant liquid was filtered off through cotton plugs leaving the solid residue in the flask (F2). It was repeated twice. The filtrate was evaporated to dryness to yield reddish brown liquid (F3). The solid residue (F2) was subsequently fractionated by dissolving it in succession with hexane, dichloromethane, ethyl acetate and 70% aqueous methanol. The successive fractionation yielded hexane soluble sub-fraction (F2-1), dichloromethane soluble sub-fraction (F2-2), ethyl acetate soluble sub-fraction (F2-3), and 80% aqueous methanol soluble sub-fraction (F2-4).

Results and Discussion

Phytochemical screening of root extracts. Preliminary chemical tests for the indication of classes of organic compounds were performed on roots of E. *odoratum* extracted in various solvents (F1, F2, F3, F2-1, F2-2, F2-3 and F2-4), as outlined by Harborne (1973) and Dhoubhadel and Shrestha (1978).

Petroleum ether extract (F1) showed the presence of sterols/triterpenoids and quinones (detected by spraying the TLC with 10% alcoholic KOH). Solid ethanolic extract (F2) revealed the presence of sterols/triterpenes, reducing sugars, alkaloids, carbohydrates and quinones. Polyphenols and tannins were not detected in both F1 and F2 fractions. Liquid residue (F3) showed the presence of polyphenols, reducing sugars, quinones and tannins (Table 1). Both hexane-soluble sub-fraction of F2 (F2-1) and dichloromethane-soluble sub-fraction of F2 (F2-2) showed the presence of sterols/triterpenes and quinones, whereas alkaloids were detected in sub-fraction F2-2. Similarly, ethyl acetate-soluble sub-fraction (F2-3) showed the presence of reducing sugars, alkaloids, and the 80% aqueous methanol-soluble sub-fraction (F2-4) showed reducing sugars and carbohydrates, but quinones were not detected in both these cases. Flavonoids were not detected in any of the extracts or fractions (Table 1).

Table 1. Phytochemical screening of root extracts of *Eupatorium odoratum*

Classes of chemicals indicated	Root extact fraction*						
	F1	F2	F3	F2-1	F2-2	F2-3	F2-4
Sterols/triterpenoids	+	+	-	+	+	-	-
Polyphenols	-	-	+	n	n	n	n
Reducing sugars	-	+	+	-	-	+	+
Alkaloids	-	+	-	-	+	+	-
Carbohydrates	-	+	-	-	-	-	+
Flavonoids	-	-	-	n	n	n	n
Quinones	+	+	+	+	+	-	-
Tannins	-	-	+	-	n	n	n

+ present; - absent; n = not tested; * see Brine shrimp lethality test, Extraction process sections, and footnote of Table 2 for details about fractions

The cytotoxicity of root extract was evaluated by the brine shrimps lethality test. Significant activity (LC_{50} < 1000 µg/ml) was exhibited by F1, while F3 (liquid residue) was found to be non-cytotoxic (LC_{50}>1000 µg/ml) (Table 2). The LC_{50} value of petroleum ether extracts (F1) was 237.2 µg/ml and of the solid residue of ethanol fraction (F2) was 81.1 µg/ml, while hexane-soluble sub-fraction of F2 (F2-1) displayed LC_{50} value of 263.1 µg/ml and dichloromethane-soluble sub-fraction of F2 (F2-2) displayed LC_{50} value of 207.7 µg/ml. However, F2-3 and F2-4 sub-fractions of F2 were found to be non-cytotoxic against brine shrimps. It may also be seen from Table 2 that cytotoxicity of F2 was greater than that of its own sub-fractions F2-1 and F2-2. This difference is likely to be due to the synergistic effect of hexane-soluble and dichloromethane-soluble compounds. Phytochemical analysis (Table 1) showed that cytotoxicity of F1, F2, F2-1 and F2-2 may be due to the presence of sterols, triterpenes, alkaloids and quinones. Therefore, the present observations have indicated that root extracts of *E. odoratum* may be used for cytotoxic applications.

Table 2. Brine shrimp *(Artimia salina)* lethality of root extracts of *Eupatorium odoratum*

Extract fraction	Extract yield (%)	LC_{50} (µg/ml)	Confidence interval (95%)
F1	0.31	237.2	304.4-169.8
F2	0.72	81.1	131.7-31.4
F3	10.23	>1000	-
F2-1	0.07	263.1	289.5-236.8
F2-2	0.17	207.7	249.2-166.3
F2-3	0.01	>1000	-
F2-4	0.12	>1000	-

F1: petroleum ether extract; F2: solid residue of ethanol fraction; F3: liquid residue of ethanol fraction; F2-1: hexane-soluble sub-fraction of F2; F2-2: dichloromethane-soluble sub-fraction of F2; F2-3: ethyl aceate-soluble sub-fraction of F2; F2-4: 80% aqueous methanol soluble sub-fraction of F2

Acknowledgement

One of the authors (S.A.) is grateful to the University Grants Commission, Nepal, for financial support.

References

Ahmad, M. 1969. Chemical investigation on the leaves of *Eupatorium odoratum*. *Sci. Res. Dacca* **6:** 37-41.

Bamba, D., Bessiere, J.M., Marion, C., Pelissier, Y., Fouraste, I. 1993. Essential oils of Ivory Coast. *Planta Medica* **59:** 184-185.

Barua, R.N., Pathak, M.G. 1993. Fatty acid composition of *Chromolaena odorata* flower fat. *Indian J. Nat. Prod.* **9:** 17-18.

Barua, R.N., Sharma, R.P., Thyagarajan, G., Hertz, W. 1978. Flavonoids of *Chromolaena odorata*. *Phytochmistry* **17:** 1807-1808.

Bedi, G., Tonzibo, Z.F., Chalchat, J.C., N'Guessan, T.Y. 2001. Chemical consitituents of *Chromolaena odorata* of essential oils from Abidjan, Ivory Coast. *J de La Societ Quest-Africane de Chimie* **7:** 29-37.

Biller, A., Boppre, M., Witte, L., Hartmann, T. 1994. Pyrrolizidine alkaloids in *Chromolaena odorata*. Chemical and chemoecological aspects. *Phytochemistry* **35:** 615-619.

Cheeke, P.R. 1989. Alkaloids. In: *Toxicants of Plant Origin*, P.R. Cheeke (ed.), vol. **1**, p.1, CRC Press, Florida, USA.

Chowdhury, A.R. 2002. Essential oils of the leaves of *Eupatorium odoratum* L. from Shillong (N.E.). *J. Essent. Oil-Bearing Plants* **5:** 14-18.

De Giulio, A., De Rosa, S., Di Vicenzo, G., Strazzullo, G. 1992. Extraction and bioassay-directed fractionation of the cytotoxic metabolites from *Chelidonium majus*. *Phytochem. Analysis* **3:** 122-124.

Dhoubhadel, S.P., Shrestha, K.K. 1978. Phytochemical screening of the rhizomes of *Acorus calamus* Linn. *J. Inst. Sci.* **1:** 43-48.

Ding, A., Zhang, X., Liu, J., Ding, J., Gu, K. 2001. Chemical constituents from *Eupatorium odoratum*. *Tianran Chanwu Yanijiu Yu Kaifa* **13:** 22-24.

Dung, N.X.B., Le, K., Lecereq, P.A. 1992. Constituents of the leaf oil of *Chromolaena odorata*. *J. Essent. Oil Res.* **4:** 309-310.

Finney, D.J. 1971. *Probit Analysis*, pp. 76-80, 3rd edition, Cambridge University Press, Cambridge, UK.

Hai, M.A., Biswas, P.K., Shil, K.C., Ahmad, M.U. 1991. Chemical constituents of *Eupatorium odoratum*. Part XIII. *J. Bangladesh Chem. Soc.* **4:** 47-49.

Hai, M.A., Saha, K., Ahmad, M.U. 1995. Chemical constituents of leaves and stem of *Eupatorium odoratum*. *J. Bangladesh Chem. Soc.* **8:** 139-142.

Harborne, J.B. 1973. *Phytochemical Methods: A Guide to Techniques of Plant Analysis*, pp. 34, 85 and 213, Chapman and Hall, London, UK.

Iwu, M.M., Chiori, C.O. 1984. Antimicrobial activity of *Eupatorium odoratum* extracts. *Fitoterapia* **LV:** 354-356.

McLaughlin, J.L., Rogers, L.L., Anderson, J.E. 1998. The use of biological assays to evaluate botanicals. *Drug Inform. J.* **32:** 513-524.

Metwally, A.M., Ekejiuba, E.C. 1981. Methoxylated flavanols and flavanones from *Eupatorium odoratum*. *Planta*

Medica **42:** 403-405.

Meyer, B.N., Ferrigin, N.R., Putnam, J.E., Jacobsen, L.B., Nicholas, D.E., McLaughlin, J.L. 1982. Brine shrimp lethality: a convenient general bioassay for active plant constituents. *Planta Medica* **45:** 31-34.

Okunji, C.O., Iwu, M.M., Jackson, J.E., Tally, J.D., Bachhi, C., Ayafor, J.F. 2000. Plant Derived Antiparasitic and Antifungal Compounds and the Methods of the Extraction of the Compounds, PCT Patent Int. Appl. WO. 0024 411 (cl. A61K35/78), 2000, US Patent Appl. PV 105 888 1998 [*Chem. Abstr.* 2000, P. 326031K, **132:** (24)].

Phan, T.T., Wang, L., See, P., Grayer, R.J., Chan, S.Y., Lee, S.T. 2001. Phenolic compunds of *Chromolaena odorata* protect cultured skin cells from oxidative damage: implication for cutaneous wound healing. *Biol. Pharmaceut. Bull.* **24:** 1373-1379.

Press, J.R., Shrestha, K.K., Sutton, D.D. 2000. *Annotated Checklist of the Flowering Plants of Nepal,* pp.1-59, The Natural Histroy Museum, London, UK.

Talpatra, S.K., Bhar, D.S., Talpatra, B. 1977. Terpenoids and related compounds. Part XIII. Epoxylupeol, a new triterpenoid from *Eupatorium odoratum. Indian J. Chem. Soc.* **15B:** 806-807.

Taylor, R.S.L., Hudson, J.B., Manandhar, N.P., Towers, G.H.N. 1996. Antiviral activities of medicinal plants of Southern Nepal. *J. Ethnopharmacol.* **53:** 97-104.

Triratana, T., Suwannuraks, R., Naengchomnong, W. 1991. Effect of *Eupatorium odoratum* on blood coagulation. *J. Med. Assoc. Thai.* **74:** 283-287.

Wollenweber, E., Roitman, J.N. 1996. Me-ether of quercetagetin from *Chromolaena odorata. Biochem. Syst. Ecol.* **24:** 479-480.