
PHYTOCHEMICAL INVESTIGATION AND
BIOACTIVITIES OF *PISTACIA INTEGERRIMA* AND
DIOSPYROS LOTUS



By

ABDUR RAUF

Dissertation submitted to the University of Peshawar in partial fulfillment of the
requirements for the Degree of Doctor of Philosophy in Chemistry

INSTITUTE OF CHEMICAL SCIENCES

UNIVERSITY OF PESHAWAR, PESHAWAR

PAKISTAN

(DECEMBER, 2014)

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DECLARATIONS

This is to certify that this dissertation prepared by Mr. Abdur Rauf entitled "Phytochemical Investigation and Bioactivities of Pistacia integerrima and Diospyros lotus" is accepted in the present form by the Institute of Chemical Sciences, University of Peshawar as fulfilling this part of the requirements for the degree

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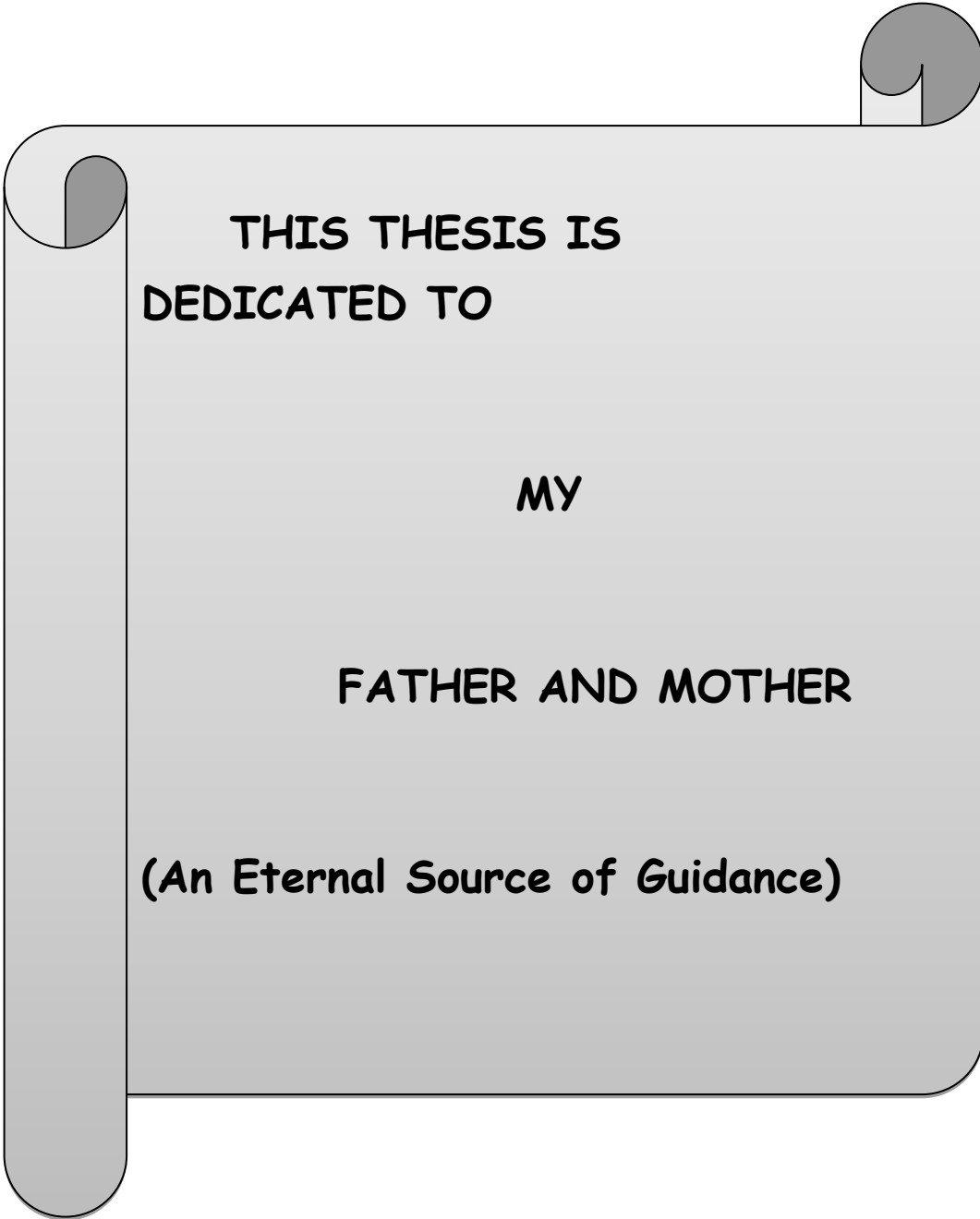
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**THIS THESIS IS
DEDICATED TO**

MY

FATHER AND MOTHER

(An Eternal Source of Guidance)

Table of Contents

Contents	i
Acknowledgments	x
List of Figures	xvii
List of Tables	xix
List of Schemes	xvi
Abstract.....	xvii
List of Abbreviations	xxiv
Chapter 1	Part-A
GENERAL INTRODUCTION	1
Chapter 2	
INTRODUCTION (Part-A).....	3
2.1. Family Ebenaceae	3
2.2. Genus <i>Diospyros</i>	3
2.3. <i>Diospyros lotus</i>	5
2.4. Pharmacological studies of <i>Diospyros</i>	7
2.4.1. Acute toxicity	7
2.4.2. Analgesic activity	7
2.4.3. Antipyretic activity.....	7
2.4.4. Anti-inflammatory.....	7
2.4.5. Hepatoprotective effect	8
2.4.6. Antioxidant activity.....	8
2.4.7. Antibacterial effect	8
2.4.8. Cytotoxic activity	9
2.4.9. Anthelmintic activity.....	9
2.4.10. Tyrosinase inhibiting activity	10
2.4.11. Xanthine oxidase inhibitory activity	10
2.4.12. Collagenase inhibitory effect.....	10
2.4.13. Lipxygenase inhibitor effect.....	10
2.4.14. Protein tyrosine phosphatase 1B	10
2.4.15. Cosmaceutical effect	10

2.4.16. Antihypertensive effect.....	11
2.4.17. Effect on rat skeletal muscles	11
2.4.18. Antidiabetic effect.....	11
2.4.19. Neuropharmacological activities.....	11
2.5. Chemical constituents	13
Chapter 3	
RESULTS AND DISCUSSION (Part-A)	34
3.1. Preliminary phytochemical analysis of <i>D. lotus</i>	34
3.1.1. Phytochemical analysis of hard wood of <i>D. lotus</i>	34
3.1.2. Phytochemical analysis of leaves of <i>D. lotus</i>	35
3.1.3. Phytochemical analysis of bark of <i>D. lotus</i>	35
3.1.4. Phytochemical analysis of roots of <i>D. lotus</i>	36
3.2. Chemical structures of isolated constituents from <i>D. lotus</i> roots	38
3.2.1. New chemical constituents	38
3.2.2. Hitherto unreported chemical constituents	40
3.2.1.1. Di-naphthodiospyrol A (1)	43
3.2.1.2. Di-naphthodiospyrol B (2)	45
3.2.1.3. Di-naphthodiospyrol C (3)	47
3.2.1.4. Di-naphthodiospyrol D (4)	49
3.2.1.5. Di-naphthodiospyrol E (5).....	53
3.2.2.1. Lupeol (6)	56
3.2.2.2. β -Sitosterol (7).....	58
3.2.2.3. Stigmasterol (8)	60
3.2.2.4. Diospyrin (9)	62
3.2.2.5. 8-Hydroxyisodiospyrin (10)	64
3.2.2.6. 7-Methyljuglone (11).....	66
3.2.2.7. Oleanolic acid (12).....	68
3.2.2.8. Ursolic acid (13).....	70
3.2.2.9. Betulinic acid (14).....	72
3.3. Chemical composition of fixed oil from <i>D. lotus</i>	74
3.4. Biological profile of <i>D. lotus</i>	78

3.4.1. <i>In vitro</i> screening.....	78
3.4.1.1. DPPH scavenging effect	78
3.4.1.2. Antibacterial effects of extract and fractions	81
3.4.1.3. Antibacterial effect of isolated chemical constituents	81
3.4.1.4. Lipoxxygenase (LOX) inhibitory activity and molecular docking of diospyrin.....	82
3.4.1.4.1. <i>In vitro</i> lipoxxygenase inhibition assay of diospyrin.....	82
3.4.1.4.2. Molecular docking simulations of diospyrin (9).....	83
3.4.1.4.3. Lipoxxygenase inhibitory activity of 8-hydroxydiospyrin (10).....	85
3.4.1.4.4. <i>In vitro</i> lipoxxygenase inhibition assay	85
3.4.1.4.5. Molecular docking simulations of 8-hydroxydiospyrin (10)	85
3.4.1.5. <i>In vitro</i> anticancer activity	88
3.4.1.5.1. Antiproliferative activity	88
3.4.1.5.2. MDR reversal effect in mouse lymphoma cells	89
3.4.1.6. Enzyme inhibition activities of <i>D. lotus</i>	91
3.5. <i>In vivo</i> screening	93
3.5.1. Antipyretic effects of the crude extract and its fractions.....	93
3.5.2. Acetic acid induced writhing effects of the crude extract and its fractions.....	94
3.5.3. Locomotive effect of <i>D. lotus</i>	97
3.5.4. Muscles relaxant activity of <i>D. lotus</i>	99
3.5.5. Acute toxicity of <i>D. lotus</i>	100
3.5.6. Analgesic effects of <i>D. lotus</i>	100
3.5.6.1. Peripheral analgesic effect in acetic acid induced writhing test	100
3.5.6.2. Central analgesic effect in hot plat test.....	101
3.5.7. Anti-inflammatory effects of <i>D. lotus</i>	103
3.5.8. Sedative effects of <i>D. lotus</i>	107
3.5.9. Muscle relaxants activity of <i>D. lotus</i>	108
3.5.9.1. Chimney animal model.....	108
3.5.9.2. Inclined plane model.....	110
3.5.10. Antipyretic activity of of <i>D. lotus</i>	112
3.5.10.1. Yeast induced pyrexia model	112
3.6. Biological screening of <i>D. lotus</i> fixed oil.....	114

Chapter 4

EXPERIMENTAL (Part-A)	118
4.1. General experimental procedures	118
4.1.1. Chemical reagents and spray	118
4.1.2. Ceric sulphate	118
4.1.3. Dragendorff's reagent	118
4.1.4. Fehling's solution	119
4.2. Plant materials	119
4.2.1. Phytochemical screening of <i>D. lotus</i>	119
4.2.2. Test for alkaloids	119
4.2.3. Test for tannins	119
4.2.4. Test for anthraquinones	120
4.2.5. Test for glycosides	120
4.2.6. Test for reducing sugars	120
4.2.7. Test for saponins	120
4.2.8. Test for flavonoids	120
4.2.9. Test for phlobatanins	120
4.2.10. Test for steroids	121
4.2.11. Test for terpenoids	121
4.3. Present work	121
4.3.1. Extraction and isolation	121
4.4. Chemical structures of new constituents from <i>D. lotus</i> roots	127
4.5. Structure of new source isolated compounds from <i>D. lotus</i> roots	132
4.6. Pharmacological screening of <i>D. lotus</i>	141
4.6.1. <i>In-vitro</i> screening	141
4.6.2. Urease inhibition assay	141
4.6.3. Phosphodiesterase-I inhibition assay	141
4.6.4. Carbonic anhydrase-II assay and inhibition	142
4.6.5. Chymotrypsin inhibition assay	142
4.6.6. DPPH free radical scavenging assay	143

4.6.7. Bacterial strains assortment and preservation	143
4.6.8. Antimicrobial assay against selected bacterial strains	144
4.6.9. Antifungal assay	144
4.6.10. Brine shrimp cytotoxic assay	145
4.6.11. Insecticidal activity	145
4.6.12. Antiproliferative assay	146
4.6.13. Assay for reversal of MDR in mouse lymphoma cells	147
4.7. <i>In vivo</i> screening	148
4.7.1. Analgesic activity	148
4.7.1.1. Acetic acid induced writhing test	148
4.7.1.2. Formalin test	148
4.7.1.3. Hot plat test	149
4.7.2. Anti-inflammatory activity	149
4.7.2.1. Open field test	150
4.7.3. Acute toxicity test	151
4.7.4. Influence on motor coordination in the chimney test	151
4.7.5. Influence on motor coordination in the inclined plane	151
4.7.6. Antipyretic test (yeast induced pyrexia)	152
4.7.7. Statistical analysis	152
4.8. GC-MS analysis	152
4.9. Molecular docking simulation	153
Chapter 5	
Part-B	
INTRODUCTION (Part-B)	154
5.1. Family Anacardiaceae	154
5.1.1. Genus <i>Pistacia</i>	154
5.1.2. <i>Pistacia integerrima</i>	155
5.2. Pharmacological studies	157
5.2.1. <i>In vitro</i> pharmacological studies	157
5.2.2. Antioxidant effect	157
5.2.3. Antimicrobial activity	157
5.2.4. Uric acid lowering effect	157

5.2.5. Wound healing effect	158
5.2.6. Phytotoxicity assay	158
5.2.7. Anticancer effect	158
5.2.8. Anti-Helicobacter pylori effect	158
5.2.10. Anti-inflammatory effect	158
5.2.11. Antidiarrheal effect	159
5.2.12. Toxicity profiling	159
5.2.13. Analgesic profile	159
5.2.14. Antipyretic effect	159
5.2.15. Gastric and duodenal anti-ulcer activity	159
5.2.16. Hepatoprotective effect.....	159
5.2.17. Hypotensive activity	159
5.2.18. Antiemetic effect	160
5.3. Phytochemical studies.....	160
Chapter 6	
RESULTS AND DISCUSSION (Part-B).....	173
6.1. Preliminary phytochemical screening of <i>P. integerrima</i>	173
6.1.1. Phytochemical analysis of <i>P. integerrima</i> galls	173
6.1.2. Phytochemical analysis of <i>P. integerrima</i> leaves.....	174
6.1.3. Phytochemical analysis of the bark of <i>P. integerrima</i>	175
6.1.4. Phytochemical analysis of the roots of <i>P. integerrima</i>	176
6.2. Chemical constituents of <i>P. integerrima</i> (galls, roots and bark).....	178
6.2.1. Structure elucidation of pistagremic acid (15)	181
6.2.2. Structure elucidation of Shikirullaline (16)	184
6.2.3. Structure elucidation of integerrimic acid (17)	189
6.3. Biological screening of <i>Pistacia integerrima</i>	192
6.3.1. Antibacterial effect of <i>P. integerrima</i> galls.....	192
6.3.2. Antibacterial activity of <i>P. integerrima</i> leaves	193
6.3.3. Antimicrobial effect of <i>P. integerrima</i> bark	193
6.3.4. Antimicrobial effect of <i>P. integerrima</i> roots.....	194
6.3.5. Antifungal effect of <i>P. integerrima</i> galls.....	194

6.3.6. Antifungal effect of <i>P. integerrima</i> leaves	195
6.3.7. Antifungal effect of <i>P. integerrima</i> bark	195
6.3.8. Antifungal effect of <i>P. integerrima</i> roots	196
6.3.9. Cytotoxic effect of <i>P. integerrima</i> galls	197
6.3.10. Cytotoxic effect of <i>P. integerrima</i> leaves.....	197
6.3.11. Cytotoxic effect of <i>P. integerrima</i> bark.....	198
6.3.12. Cytotoxic effect of <i>P. integerrima</i> roots.....	198
6.3.13. Effects of galls extracts on DPPH	199
6.3.14. Effects of leaves extracts on DPPH	200
6.3.15. Effects of bark extracts on DPPH	201
6.3.16. Effects of roots extracts on DPPH	202
6.4. Biological screening of pistagremic acid (15).....	204
6.4.1. <i>In vitro</i> biological screening of Pistagremic acid (15).....	204
6.4.1.1. Antibacterial activity of 15.....	204
6.4.1.2. Antifungal activity of 15.....	204
6.4.1.3. Antioxidant activity of 15.....	205
6.4.1.4. Leishmanicidal activity of 15	206
6.4.1.5. Broad spectrum anticancer activity of 15	207
6.4.1.6. Glycosidase inhibitor of 15	211
6.4.2. Enzyme inhibition activities of <i>P. integerrima</i>	213
6.4.2.1. Enzyme inhibition activities of compounds	214
6.5. <i>In vivo</i> biological screening of pistagremic acid (15).....	215
6.5.1. Effect of 15 in acetic acid induced writhing test	215
6.5.2. Effect of 15 in tail immersion test.....	215
6.5.3. Effect of 15 in carrageenan induced paw edema test.....	217
6.5.4. Effect of 15 yeast induced pyrexia test	219
6.5.5. Muscle relaxant effects of 15	220
6.5.6. Effects of 15 in Rota rod.....	220
6.5.7. Effect of 15 in inclined plane	221
6.5.8. Effect of 15 in traction test	222
6.5.9. Effect of 15 in chimney test.....	223

6.5.10. Molecular docking of pistagremic acid (15)	224
6.6. <i>In vivo</i> biological screening of Shakirullaline (16)	225
6.6.1. Anti-inflammatory effect of 16	225
6.6.2. Analgesic effect of Shakirullaline (16)	226
6.6.3. Muscle relaxants activity of Shakirullaline (16)	226
6.6.4. Acute Toxicity of Shakirullaline (16)	227
6.7. <i>In vivo</i> biological screening crude extract and integarrimic acid (17).....	228
6.7.1. Analgesic effects crude extract and integarrimic acid (17)	228
6.8. Molecular docking of integarrimic acid (17)	230
Chapter 7	
(Part-B)	
EXPERIMENTAL (Part-B)	232
7.1. General experimental procedures.....	232
7.1.1. Chemical reagents and spray	232
7.1.2. Plant collection	232
7.2. Phytochemical screening of <i>P. integerrima</i>	232
7.3. Present work	232
7.3.1. Extraction, isolation and purification.....	233
7.3.2. Extraction and isolation of <i>P. integerrima</i> galls	233
7.3.3. Extraction and isolation of <i>P. integerrima</i> bark.....	234
7.4. New constituents of <i>P. integerrima</i>	238
7.5. Pharmacological screening of <i>P. integerrima</i>	243
7.5.1. <i>In-vitro</i> screening	243
7.5.2. Urease inhibition assay	243
7.5.3. Phosphodiesterase-I inhibition assay	243
7.5.4. Carbonic anhydrase-II assay and inhibition	243
7.5.5. Chymotrypsin inhibition assay	243
7.5.6. DPPH free radical scavenging assay	243
7.5.7. Bacterial strains assortment and preservation	243
7.5.8. Antimicrobial assay against selected bacterial Strains	243
7.5.9. Antifungal assay	244
7.5.10. Brine shrimp cytotoxic assay	244

7.5.11. Insecticidal activity	244
7.5.12. Anticancer activity of 15	244
7.5.13. Leishmanicidal activity	245
7.5.14. α -Glucosidase inhibition activity	246
7.5.15. Cytotoxicity assay	246
7.6. <i>In vivo</i> screening	247
7.6.1. Analgesic activity	247
7.6.2. Tail immersion test.....	247
7.6.3. Anti-inflammatory activity	247
7.6.4. Open field test	247
7.6.5. Molecular docking of 15 and 17	247
REFERENCES	249
LIST OF PUBLICATIONS	256

Acknowledgments

In the Holy name of Almighty **Allah** who gave me courage and strength to complete this research work. I am grateful to owe my gratitude to Almighty Allah who provided me the opportunity of exploring two natural indigenous medicinal plants at the biochemical level.

I feel uncountable pleasure in heart to express my inexpressible thanks to my current ever encouraging, enthusiastic and learned supervisor, Prof. Dr. Ghias Uddin (Institute of Chemical Sciences, University of Peshawar), for his personal interest, guidance, valuable suggestions, positive criticism and discussions enabled me to complete this work.

The work personified in this dissertation would have never been accomplished without Prof. Dr. Bina S. Siddiqui (Co-supervisor) for critical attention, continuous encouragement and providing access to excellent facilities at H.E.J. Research Institute of Chemistry: International Center for Chemical and Biological Sciences, University of Karachi, Karachi.

I am also thankful to Prof. Dr. Mohammad Arfan for valuable ideas, discussion, preliminary attention, continuous encouragement, to pursue the research work. I would like to express my gratitude thanks to our collaborator particularly Prof. Dr. Josep Molnar and Dr. Akos Csoka (Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Szeged, Hungary) and Prof. Dr. Thomas J. Simpson (School of Chemistry, University of Bristol, Bristol, UK) for contribution and anticancer study.

My humble thanks go to Prof. Dr. Yousaf Iqbal (Director, Institute of Chemical Sciences) for facilitating my research work by the proper utilization of indigenous fellowship funds. I am also thankful to all faculty members of Institute.

I am highly indebted to the Higher Education Commission (HEC) of Pakistan, for financial support through its indigenous fellowship.

Acknowledgment

Special thanks to Dr. Rasool Khan, Dr. Mumtaz Ali, Dr. Abdul Latif, Mr. Muhammad Akram, Dr. Taj Ur Rehman, Dr. Syed Hamid Hussain, Dr. Ajmal Khan, Dr. Haroon Khan, Dr. Naveed Muhammad, Dr. Waliullah, Dr. Anwar Sadat, Dr. Muhammad Alam and Dr. Asfaq Khan for their encouragement.

I have no words to thank my respectable and loving parent and uncles for their untiring efforts, lots of prayers, encouragement and guidance, moral and financial support. Thanks to my sisters and brothers for their prayers and well wishing.

Abdur Rauf

List of Figures

Figure 1: <i>Diospyros lotus</i> L.....	5
Figure 2: Key HBMC correlations of 1	45
Figure 3: Key HBMC correlations of 2	47
Figure 4: Key HMBC correlations of 3	49
Figure 5: Key HMBC correlations of 4	52
Figure 6: Key n.O.e correlations of 4	52
Figure 7: Key HMBC correlations of 5	55
Figure 8: Key n.O.e correlations of 5	55
Figure 9: Key HMBC correlation of lupeol (6).....	56
Figure 10: Chemical structure of β -sitosterol (7).....	58
Figure 11: Key HMBC correlations of stigmasterol (8).....	60
Figure 12: Key HMBC correlations of diospyrin (9)	62
Figure 13: Key HMBC correlations of 8-hydroxydiospyrin (10).....	64
Figure 14: Key HMBC correlations of 7-methyljuglone (11).....	66
Figure 15: Key HMBC correlations of oleanolic acid (12).....	68
Figure 16: Key HMBC correlations of ursolic acid (13).....	70
Figure 17: Key HMBC correlations of betulinic acid (14).....	72
Figure 18: The effect of extract/fraction of <i>D. lotus</i> in DPPH free radical scavenging assay.	79
Figure 19: The effect of compounds from <i>D. lotus</i> in DPPH free radical scavenging assay.	80
Figure 20: Binding mode of diospyrin inside active site of LOX.....	84
Figure 21: Electrostatic interactions of diospyrin inside active site of LOX..	84
Figure 22: Binding mode of 8-hydroxydiospyrin inside the active site of LOX.	86
Figure 23: Electrostatic interactions of 8-hydroxydiospyrin inside the active site of LOX.....	87
Figure 24: Percent effect of yeast induced pyrexia in hyperthermic mice of extracts.....	94
Figure 25: Percent protection against noxious stimulation induced by acetic acid of extract ...	96
Figure 26: Percent locomotive test effects of crude extracts and isolated fractions	98
Figure 27: Effect of chloroform fraction on muscle coordination in rota rod.....	99
Figure 28: Analgesic effect of chloroform fraction, 9 and 10 in acetic acid induced writhing.	101
Figure 29: Anti-inflammatory effect of CHCl ₃ fraction in carrageenan induced paw edema....	104
Figure 30: Anti-inflammatory effect of 9 in carrageenan induced paw edema in rats..	105
Figure 31: Anti-inflammatory effect of 10 in carrageenan induced paw edema in rats.	106
Figure 32: Percent muscle relaxant effect of 12-14 in chimney test.....	109
Figure 33: Percent muscle relaxant effect of 12-14 in inclined plane test.	111
Figure 34: Induces Pyrexia effect of 12-14	113
Figure 35: Antifungal activity of oil isolated from the roots of <i>D. lotus</i>	114
Figure 36: Antibacterial activity of oil isolated from the roots of <i>D. lotus</i>	115
Figure 37: DPPH radical scavenging activity of oil extracted from <i>D. lotus</i>	115
Figure 38: Antibacterial activity of oil isolated from the roots of <i>D. lotus</i>	117

Figure 39: Antifungal activity of oil isolated from the roots of <i>D. lotus</i>	117
Figure 40: <i>Pistacia integerrima</i> J. L. Stewart ex Brandis	156
Figure 41: Selected HMBC correlations of pistagrrrimic acid (15)	183
Figure 42: X-ray crystallographic image of pistagrrrimic acid (15).....	184
Figure 43: Key HMBC Correlation for compound (16).....	187
Figure 44: Key n.O.e interaction for compound 16	188
Figure 45: X-ray crystallographic image of compound (16).....	188
Figure 46: Selected HMBC correlations of compounds (17).....	191
Figure 47: % Scavenging effect of <i>P. integerrima</i> galls extracts on DPPH at 100 µg/ml	200
Figure 48: % Scavenging effect of <i>P. integerrima</i> extracts leaves on DPPH at 100 µg/ml.....	201
Figure 49: % Scavenging effect of <i>P. integerrima</i> bark extracts on DPPH at 100 µg/ml.....	202
Figure 50: % Scavenging effect of <i>P. integerrima</i> roots extracts on DPPH at 100 µg/ml.....	203
Figure 51: Antibacterial activity of pistagremic acid (15).	204
Figure 52: Antifungal activity of pistagremic acid (15).	205
Figure 53: Antioxidant activity of pistagremic acid (15)	206
Figure 54: Dose response curves of pistagremic acid (15) against all cell lines.....	209
Figure 55: Dose response curves of pistagremic acid (15) against the different cell lines.	210
Figure 56: Binding mode of pistagremic acid inside active site of α -glucosidase..	212
Figure 57: Surface contacts of pistagremic acid inside the catalytic site of α -glucosidase.....	212
Figure 58: Percent effect of pistagremic acid (15) in acetic acid induced writhing test	215
Figure 59: Percent effect of pistagremic acid (15) in tail immersion test	216
Figure 60: Percent effect of pistagremic acid (15) in tail immersion test at after naloxone	217
Figure 61: Percent effect of pistagremic acid (15) in carrageenan induced paw edema test ...	218
Figure 62: Percent antipyretic effect of 15 in yeast induced pyrexia test.....	219
Figure 63: Effect of 15 on muscle coordination in rota rod.	221
Figure 64: Effect of 15 on muscle coordination in inclined plane.....	222
Figure 65: Molecular docking conformation of 15 in the binding pocket of COX-2 enzyme... 224	224
Figure 66: The percent anti-inflammatory activity of shakirullaline (16).....	225
Figure 67: Analgesic effect of shakirullaline (16) in acetic acid induced writing test	226
Figure 68: Analgesic effect of methanolic extract and integarrimic acid (17).....	229
Figure 69: Analgesic effect of crude in tail immersion pain paradigm	229
Figure 70: Analgesic effect of integarrimic acid (17) in tail immersion pain paradigm	230
Figure 71: Docking conformation of integarrimic acid (17) in the binding pocket of COX-2..	231

List of Tables

Table 1: List of chemical constituents isolated from different species of genus <i>Diospyros</i>	13
Table 2: Phytochemical analysis of <i>D. lotus</i> hard wood extract and its fractions.....	34
Table 3: Phytochemical analysis of <i>D. lotus</i> leaves extract and its fractions	35
Table 4: Phytochemical analysis of the extract and fractions of <i>D. lotus</i> bark	36
Table 5: Phytochemical analysis of extract and fractions of <i>D. lotus</i> roots	37
Table 6: ¹³ C and ¹ H-NMR spectral data of 1	44
Table 7: ¹³ C and ¹ H-NMR spectral data of 2	46
Table 8: ¹³ C and ¹ H-NMR spectral data of compound 3	48
Table 9: ¹³ C and ¹ H-NMR spectral data of compound 4	51
Table 10: ¹³ C and ¹ H-NMR spectral data of compound 5	54
Table 11: ¹³ C and ¹ H-NMR spectral data of lupeol (6).....	57
Table 12: ¹³ C and ¹ H-NMR spectral data of β -sitosterol (7)	59
Table 13: ¹³ C and ¹ H-NMR spectral data of stigmasterol (8).....	61
Table 14: ¹³ C and ¹ H-NMR spectral data of compound diospyrin (9).....	63
Table 15: ¹³ C and ¹ H-NMR spectral data of compound 8-hydroxydiospyrin (10)	65
Table 16: ¹³ C and ¹ H-NMR spectral data of 7-methyljuglone (11).....	67
Table 17: ¹³ C and ¹ H-NMR spectral data of oleanolic acid (12).....	69
Table 18: ¹³ C and ¹ H-NMR spectral data of ursolic acid (13).....	71
Table 19: ¹³ C and ¹ H-NMR spectral data of betulinic acid (14)	73
Table 20: Quantification results of fatty acid methyl ester of <i>D. lotus</i> roots	75
Table 21: Quantification results of fatty acid methyl ester (FAMES) from <i>D. lotus</i>	75
Table 22: Quantification results of 36 components internal standard	76
Table 23: Quantification results of fixed oil isolated from <i>D. lotus</i> roots.....	77
Table 24: Antimicrobial effect (zone of inhibition in mm) of <i>D. lotus</i>	81
Table 25: Antimicrobial activity of compounds of <i>D. lotus</i>	82
Table 26: Antiproliferative effect of compounds (1-3)	88
Table 27: Effect of compounds (1-3) on reversal of multidrug resistance on <i>MDR</i> in mouse.....	90
Table 28: Enzymes inhibitory activities of <i>D. lotus</i>	92
Table 29: Enzymes inhibitory activities of compounds (1-10)	92
Table 30: Analgesic effect of chloroform, 9 and 10 assessed by using hot plate model	102
Table 31: Sedative effect of chloroform fraction, 9 and 10	107
Table 32: Cytotoxicity, brine shrimps activity of oil isolated from the roots of <i>D. lotus</i>	116
Table 33: List of compounds isolated from various species of genus <i>Pistacia</i>	160
Table 34: Phytochemical analysis of the crude extract and fractions of <i>P. integerrima</i> galls ...	174
Table 35: Phytochemical analysis of crude extract and its fractions of <i>P. integerrima</i> leaves ..	175
Table 36: Phytochemical analysis of the crude extracts and fractions of <i>P. integerrima</i> bark ..	176
Table 37: Phytochemical analysis of the crude extract and fractions of <i>P. integerrima</i> roots...	177
Table 38: ¹³ C and ¹ H-NMR spectral data of pistagremic acid (15)	182

Table 39: ^{13}C and ^1H -NMR data of Shakirullaline (16)	186
Table 40: ^{13}C and ^1H -NMR spectral data of integerrimic acid (17)	190
Table 41: Antimicrobial activity (zone of inhibition in mm) of <i>P. integerrima</i> galls	192
Table 42: Antimicrobial activity (zone of inhibition in mm) of <i>P. integerrima</i> leaves.....	193
Table 43: Antimicrobial activity (zone of inhibition in mm) of <i>P. integerrima</i> bark.....	193
Table 44: Antimicrobial activity (zone of inhibition in mm) of <i>P. integerrima</i> roots.....	194
Table 45: Antifungal effect of <i>P. integerrima</i> galls	194
Table 46: Antifungal effect of <i>P. integerrima</i> leaves	195
Table 47: Antifungal effect of <i>P. integerrima</i> bark	196
Table 48: Antifungal effect of <i>P. integerrima</i> roots	196
Table 49: Cytotoxic effect of <i>P. integerrima</i> galls.....	197
Table 50: Cytotoxic effect of <i>P. integerrima</i> leaves	197
Table 51: Cytotoxic effect of <i>P. integerrima</i> bark	198
Table 52: Cytotoxic effect of <i>P. integerrima</i> roots	198
Table 53: DPPH radical scavenging activities of <i>P. integerrima</i> galls.....	199
Table 54: DPPH radical scavenging activities of <i>P. integerrima</i> leaves	200
Table 55: DPPH radical scavenging activities of <i>P. integerrima</i> bark	201
Table 56: DPPH radical scavenging activities of <i>P. integerrima</i> roots	203
Table 57: Growth inhibition against all NCI-60 cell-lines exhibited by pistagremic acid (15). 207	
Table 58: Enzymes inhibitory activities of crude extract and isolated fraction of <i>D. lotus</i>	213
Table 59: Enzymes inhibitory activities of compounds (15-17)	214
Table 60: Percent effect of 15 on muscle relaxation (Traction test)	223
Table 61: Percent effect of 15 on muscle relaxation(Chimney test)	223
Table 62: The percent effect of Shakirullaline on muscle relaxation	227

List of Schemes

Scheme 1: Extraction, fractionation and isolation of compounds from <i>D. lotus</i>	124
Scheme 2: Isolation of chemical constituents from chloroform fraction of <i>D. lotus</i>	125
Scheme 3: Isolation of chemical constituents from Fraction SF-6 of <i>D. lotus</i>	126
Scheme 4: Extraction and sequentially partitioned of galls of <i>P. integerrima</i>	235
Scheme 5: Isolation and purification of compounds of <i>P. integerrima</i> galls.....	236
Scheme 6: Isolation of compounds from <i>P. integerrima</i> bark	237

Abstract

The research work in the present thesis deals with the phytochemical investigation, isolation and structure elucidation of *Diospyros lotus* and *Pistacia integerrima*, which is presented in two parts.

PART-A

Part A deals with the study undertaken on the preliminary phytochemical analysis and bioassay-directed isolation of secondary metabolites of *Diospyros lotus* L. The methanolic extract and its fractions exhibited the presence of terpenoids, anthraquinones, saponins, sterols, flavonoids, glycosides reducing sugars and phlobatanins reducing sugars. The chloroform fraction yielded five new dimeric naphthoquinones; di-naphthodiospyrol A (**1**); di-naphthodiospyrol B (**2**); di-naphthodiospyrol C (**3**); di-naphthodiospyrol D (**4**); di-naphthodiospyrol E (**5**) and nine known hitherto unreported compounds lupeol (**6**); 7-methyljuglone (**7**); β -sitosterol (**8**); stigmasterol (**9**); diospyrin (**10**); 8-hydroxydiospyrin (**11**); oleanolic acid (**12**); ursolic acid (**13**); betulinic acid (**14**) by employing column chromatography. Their structures were elucidated by advanced spectroscopic analysis such as ^{13}C -, ^1H -NMR, HSQC, HMBS, NOESY and *J*-resolved. The extract, fractions and constituents were screened for *in-vitro* antiproliferative, reversal of MRD in mouse lymphoma cells, three enzyme ureases (phosphodiesterase-I, carbonic anhydrase-II and α -chymotrypsin inhibitory), antioxidant and antibacterial activities. Among the three new compounds (**1-3**) tested for anticancer activity compound **1** and **2** showed promising antiproliferative activity. In case of reversal of MRD in mouse lymphoma cells the most effective compound was **1**, which increased rhodamine accumulation ten times higher than

the verapamil. It also exhibited such activities against urease enzymes, anti-nociceptive, sedative, anti-inflammatory, antipyretic and acute toxicity. Structure based virtual screening of diospyrin (**9**) and 8-hydroxydiospyrin (**10**) revealed as effective anti-inflammatory agents which were confirmed experimentally. The compounds (**12-14**) showed significant muscle relaxant and antipyretic activity against standard.

PART-B

Part B also comprises phytochemical and bioassay-directed isolation of bioactive chemical constituents from *P. integerrima*. Galls, bark, leaves and roots of the plant showed the presence of terpenoids, alkaloids, tannins and flavonoids. The chloroform fraction of galls and roots furnished three new constituents; pistagremic acid (**15**); Shakirullaline (**16**); integarrimic acid (**17**) along with two known hitherto unreported compounds β -sitosterol (**18**) and stigmasterol (**19**) by column chromatography. Their structures were elucidated by advanced spectroscopic analysis such as ^{13}C -, ^1H -NMR, HSQC, HMBS, NOESY and *J*-resolved. The crude extract, fractions and compounds were screened *in vitro* activities for anticancer, three enzyme ureases (phosphodiesterase-I, carbonic anhydrase-II and α -chymotrypsin inhibitory), antioxidant, antibacterial, antifungal, cytotoxic, phytotoxic activities, which showed significant activity, while the extract and fractions have excellent activity against urease enzymes. The compounds (**15-17**) have promising anti-nociceptive, anti-inflammatory, antipyretic effects and muscle relax activity. Pistagremic acid (**15**) exhibited leishmanicidal potential against omastigotes of leishmania major as compared to amphotericin B. Molecular docking study indicated strong potential of **15** as a possible new α -glucosidase inhibitor while **15** and **17** showed strong potential with COX-2 enzyme.

List of Abbreviations

AcO	Acetate
BB	Broad Band
BCRP	Breast cancer resistance protein
CDCl ₃	Deuterated chloroform
¹³ C-NMR	Carbon-13-nuclear magnetic resonance
COSY	Correlation spectroscopy
CyFlow	Cyflow cytometer
D	Deuterium
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EIMS	Electron impact mass spectrometry
FAR	Fluorescence activity ratio
FSC	Forward scatter count
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
¹ H-NMR	Hydrogen-1-nuclear magnetic resonance
HR-EI-MS	High resolution electrospray ionization mass spectrometry
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
IC ₅₀	Inhibitory concentration 50 percent

List of abbreviations

IR	Infrared
<i>J</i>	Coupling constant
LOX	Lipoxygenase
M/z	Mass to charge ratio
MDR	Multidrug resistance
Mm	Millimole
N. O. E	Nuclear Overhauser differential spectroscopy
NOESY	Nuclear Overhauser effect spectroscopy
Nm	Nanomole
OD	Optical density
Pgp	P-glycoprotein
PTS	Pentacyclic triterpenes
SSC	Side scatter count
μm	Micromole

Medicinal plants are a rich source of producing chemical constituents in the maximum effective way with specific selectivity. From the mid of 19th century, many bioactive chemical constituents have been reported from medicinal plants and numerous of them being used as dynamic constituents in the modern medicine.

The examination for effective, cheaper, quality and easily accessible medicinal molecules is one of the drug finding and design research work in the research institutes throughout the world [1].

Medicinal plants are used by 80% of the world population for their basic health cure. The connection among human, plants and molecules isolated from plants designated the history of mankind. Medicinal plants are the preliminary source of natural drugs containing chemical constituents which have potential to use for the treatment of diseases. Pakistan has countless floras, especially the Khyber Pakhtunkhwa (KPK) province. The country has approximately 6000 wild plants species among them 400-00 are thought to be medicinally significant. During the past era of traditional system of medicine has become a topic of global significance. Currently it has been estimated that throughout the world a great population relies seriously on traditional practitioner for primary health care needs. Herbal drugs have frequently retained popularity for cultural and historic reasons. Presently, numerous people in the advanced countries have initiated to turn to alternate therapies, which include medicinal herbs [2]. Ayurvedic system lacks drug standardization, quality control as well as information about medicinal plants. Ayurvedic medicine is a mixture of several ingredients which is prepared from plants extract but the active chemical constituents when isolated from natural source fail to give the desired activity. In the absence of

pharmacological data on the various plants extracts and constituents which is not possible to standardize the active molecule having desire biological action. Previous studies showed the toxic effects of chemotherapy and radiation in treatment of cancer by decrees by Ayurvedic medication [3].

Naphthoquinones are secondary metabolites that are widely distributed in Genus *Diospyros* and have antitumor and anticancer activity [4]. Quinones predominantly 1,4-naphthoquinones are reported for various biological actions such as antimicrobial, anti-inflammatory, antipyretic properties [5]. Quinones possess the capacity to induce oxidative stress, responsible for start of tissue mutilation selectivity in tumor cells and have approach for directing cancer cells [4, 6].

In continuation to previous work on medicinal plants which have produced a wide range of natural products. Our current study deals with the isolation and characterization of plants derived natural products and their biological potential which is directed to explore the indigenous flora of Pakistan which is a rich source of bioactive molecules of medicinal importance. The aim of this PhD thesis focused on phytochemistry, proximate phytochemical analysis and bioactivity of *D. lotus* and *P. integerrima*.

2.1. Family Ebenaceae

Family Ebenaceae comprises trees and shrubs placed in four genera with 768 species [7] dispersed throughout the tropical and temperate region throughout the world, mostly assorted in the rain forest of Malaysia, Africa and America [8]. The distinguishing feature of Ebenaceae is that flowering plants possess two-ranked leaves which lack teeth but have flat surface and dark color gland on the lower surface of leaves. The flower buds have adpressed brown in color, having T shaped hairs mostly pointed and the petals are attached at the base; having overlap lobes.

2.2. Genus *Diospyros*

The genus *Diospyros* comprises nearly 700 species which are native to Middle-East, South Asia, East Asia especially Japan and China. In Pakistan, two species of *Diospyros* (*D. lotus*, *D. kaki*) are abundantly found in various regions of the country. Since ancient times, various species of *Diospyros* are patronized by traditional healers to cure numerous numbers of disorders. Some of the *Diospyros* species are used in folkloric medicine to treat different ailments such as fungal infections, internal hemorrhage, bedwetting in children, insomnia, hiccough, sedative and diarrhea [9]. Different chemical constituents of phytochemical importance including terpenoids, ursanes, lupanes, polyphenols, tannins, hydrocarbons and lipids, benzopyrones, naphthalene based aromatics, naphthoquinones, oleananes, taraxeranes have been isolated from various species of this genus [10]. The pharmacological and phytochemical evaluation of these medicinally important plants can lead to the identification of novel bioactive compounds which can accelerate the piece of drug discovery [11].

Diospyros is the most important genus, comprising over 350 species of which are deciduous, shrub, small bushes and ever green trees, leaves alternate, flowers genus, axillary,

urceolate/tubular, corolla campanulate, consist lobes (3-7), stamens (4-8), ovary (4-16), large juicy (1-10), staminodia in pistillate flowers, seeded berry fruits, the sap wood and heartwood [12].

Many species of *Diospyros* are of great importances which have edible fruits and valuable timbers. Among various species *D. Kaki* (Japanese persimmon) is cultivated in Japan for many centuries, and is considered the best fruits yielding species. The fruits of *D. kaki* are large orange red berries which are astringent till fully ripened and the tannins content transferred into insoluble crystals. The juice of *D. kaki* fruits is sweet in taste and palatable. Some other species of *Diospyros* including; *D. viraginiana*, *D. ebenaster*, *D. lotus*, *D. mespiliformis* and *D. melanoxylon* are also the significant fruits yielding species [13].

Some of *Diospyros* species are suitable for ornamental purposes and many are used for local ecological importance. Mostly the wood of *D. kaki* is used for ornamental purpose due to extremely smooth surface and a marble like coldness in touch. In Japan, *D. kaki* is valued for ornamental purposes in making boxes, desks and mosaics. Depending on their nature, many species of *Diospyros* are famous as ebony tree which yields useful and valuable wood ebonies. The true ebonies are obtained by stripping off the peripheral light colored wood of taller trees. Among ebonies species include *D. dendo*, *D. mespiliformis*, *D. crassiflora*, *D. ebenum*, *D. melanoxylon*, *D. perrieri* and *D. haplostylis* are used to obtain good ebonies. Moreover the heartwoods of certain *Diospyros* species provide interesting colors, for example *D. chloroxylon*, *D. rubra* and *D. chryophyllos* produce green, red and white colors respectively [10, 14]. Additional constituents found in *Diospyros* are anthraquinones and lignans. These metabolites do not accumulate to any significant extent.

2.3. *Diospyros lotus*

Diospyros lotus belongs to family Ebenaceae which is a deciduous tree, habituated in China and Asia. *D. lotus* has been cultivated for its edible fruits (**Figure 1**) which are considered for its medicinal importance which are used as astringent, febrifuge, sedative, nutritive, antiseptic, anti-diabetic, anti-tumor and laxative [15]. They are also used for the treatment of diarrhea, dry cough and hypertension [16]. Chemical investigation of the fruits led to the identification of fatty acids, sugars, phenolic compounds, and non-volatile acids [17, 18].



Figure 1: *Diospyros lotus* L

Classification (Taxonomy) of *Diospyros lotus* L

Kingdom:	Plantae (Plants)
Sub kingdom:	Tracheobionta (Vascular plants)
Super division:	Spermatophyta (Seed plants)
Division:	Magnoliophyta (Flowering plants)
Class:	Magnoliopsida (Dicotyledons)
Subclass:	Dilleniidae
Order:	Ebenales
Family:	Ebenaceae
Genus:	<i>Diospyros</i> L.
Specie:	<i>Diospyros lotus</i> L.

2.4. Pharmacological studies of *Diospyros*

2.4.1. Acute toxicity

The literature reviews on *Diospyros* showed that the methanolic extract of *D. lotus* at 5g/kg body weight of mice have no toxic effects [19].

2.4.2. Analgesic activity

The *n*-hexane fraction of *D. variegata* has been reported for excellent antinociceptive effect in acetic acid and tail immersion models [20]. The methanol extract of *D. mespiliformis* is also claimed for the relief of pain which has been tested in acetic acid induced and formalin test pain model which poses significant analgesic potential [21]. Taxarene-3-one isolated from *D. mespiliformis* has been published as a promising analgesic against acetic acid induced writhing [22]. *D. cordifolia* crude methanolic extract has been reported for analgesic properties in tail flaking animal model. The crude methanolic extract of *D. lotus* has been proved as central and peripheral analgesic [23].

2.4.3. Antipyretic profile

The *n*-hexane fraction of *D. variegata* displayed important antipyretic effect in brewer's yeast model [20] while methanolic extract of *D. mespiliformis* has been reported to possess significant antipyretic effect in tested animals model [21].

2.4.4. Anti-inflammatory

The *n*-hexane fraction of *D. variegata* revealed promising anti-inflammatory effect in ethyl phenylpropiolate arachidonic acid induced animal model [20]. Similarly the crude methanolic extract of *D. mespiliformis* has also reported as a strong anti-inflammatory agent

[21] while the crude methanolic extract of the leaves of *D. cordifolia* also has anti-inflammatory potential against all phlogistic agent used in a dose dependent manner [24].

2.4.5. Hepatoprotective effect

The hepatoprotective effect of the crude methanolic extract of *D. lotus* against acetaminophen induced liver damage as shown by the reduction of the high level of liver enzymes alanine aminotransferase, aspartate aminotransferase and gamma glutamyltransferase which are raised by acetaminophen [23]. The crude methanol extract of *D. malabarica* bark has also reported to possess important hepatoprotective effect in carbon tetrachloride intoxicated rats [25]. In the literature the hepatoprotective effect of triterpenes have also been documented previously [26].

2.4.6. Antioxidant Activity

Methanolic extract of *D. lotus* has reported to possess antioxidant effects [19]. Antioxidant action of *D. malabarica* bark extract has been studied for antiradical properties on different *in-vitro* models which exhibited good anti-radical properties in dose-dependent manner in all model except in hydroxyl radical inhibition assay. The petroleum ether and methanolic extracts of *D. ebum* and *D. kaki* also showed excellent antioxidant properties [27-28].

2.4.7. Antibacterial effects

Several extracts of *D. ebum* including petroleum ether, ethyl acetate, methanol, and aqueous extracts were tested to assess their anti-bacterial potential against various bacteria strain. All the extracts were found to be potent against *S. aureus* the standard drug (amikacin) except aqueous extract. Furthermore, the crude methanolic extract was more

active against *P. aeruginosa* and *S. typhimurium* than standard drug (amikacin) [27]. Dimeric naphthoquinones have been isolated from *D. piscatorial* which exhibited excellent antibacterial potential [29]. Various extracts of the roots, leaves, stem and bark of *D. mespiliformis* have been reported for their antifungal potential [29]. Plumbagin and diosquinone isolated from the roots of *D. mespiliformis* showed excellent activity against selected human pathogens [30]. The crude methanolic extract and isolated diospyrosides A-D, naphthalene glycosides and naphthoquinones, juglone, and 7-methyljuglone showed activity against periodontal and oral cariogenic bacteria. The methanolic extract of the fruits of *D. peregrina* was scrutinized against some selective bacterial and fungal strains which exhibited highest sensitivity against *E. coli* [31].

2.4.8. Cytotoxic activity

The crude extract and isolated fractions have tested for cytotoxic efficiency which indicated that acetone fraction exhibited an excellent cytotoxic action against human submandibular gland tumor cells and oral squamous cells [32]. The *n*-hexane and methanolic extracts of *D. kaki* have remarkable multidrug resistance (MDR) reversal effect against verapamil [33].

2.4.9. Anthelmintic activity

D. mollis alcoholic extract was found to possess anthelmintic effect on the adults and larvae of the dwarf tapeworm, *Hymenolepis nana*, when screened in mice in comparison with that of flubendazole (anthelmintic drug). Strong effect of *D. mollis* extract were detected on the motility, structure of adults and the number of the adults were reduced in mice with time after administration of the drug 12 days post-infection [34]. The crude methanolic extract of *D. peregrina* fruits has been reported for significant anthelmintic effect [35].

2.4.10. Tyrosinase inhibiting activity

2-Methoxy-4-vinylphenol was isolated from *D. mollis* which showed promising tyrosinase inhibiting activity as compared to the standard drug [36]. Furthermore, polyphenols from *D. kaki* have been reported as potent tyrosinase inhibitors [37].

2.4.11. Xanthine oxidase inhibitory activity

The polyphenols isolated from *D. kaki* were exhibited strong xanthine oxidase inhibition effect at 100 ppm concentrations [37].

2.4.12. Collagenase inhibitory effect

Several polyphenolic constituents from *D. kaki* have been also documented with mild to moderate collagenase inhibition effects [37].

2.4.13. Lipoxygenase inhibitor effect

D. abyssinica aqueous ethanolic and methanolic extracts of *D. abyssinica* exhibited the maximum action as lipoxygenase inhibitor [38].

2.4.14. Protein tyrosine phosphatase 1B

Phytochemical study on methanolic extract of the leaves of *D. kaki* resulted in the isolation of ursanes and oleananes type triterpenoids which were found to inhibit the activity of protein tyrosine phosphatase 1B (PTP1B), whereas triterpenoids with a 3 α -hydroxy moiety were found to be inactive against PTP1B [39].

2.4.15. Cosmaceutical effect

The polyphenolic constituents of *D. kaki* leaves used as additives for human skin due to their beneficial biologic functions, including the antiwrinkle effects and the inhibition of skin problems [37].

2.4.16. Antihypertensive effect

Flavonoids of *D. kaki* leaves extract have been reported for antihypertensive effects and established the angiotensin converting enzyme [40].

2.4.17. Effect on rat skeletal muscles

D. mespiliformis crude extract and its fractions exhibited excellent muscles skeletal effects in a dose dependent manner for better inhibitory activity [41].

2.4.18. Antidiabetic effect

D. peregrina methanolic extract has been reported for its antidiabetic activity in alloxan induced diabetic in rats [42]. *D. kaki* extract has also been reported for antidiabetic potential [43]. The crude extract of *D. lotus* caused excellent decrease in glucose level on administration of different doses [44].

2.4.19. Neuropharmacological activities

D. mespiliformis stem and bark aqueous extracts exhibited promising prolongation of pentobarbital induced sleeping time, spontaneous motor action spontaneous motor action, prolongation of pentobarbital-induced sleeping time, exploratory behavior and motor coordination performance [45]. *D. fischeri* bark ethanolic extract also indicated several neuropharmacological effects while petroleum ether, dichloromethane, methanolic showed low activity [46].

Diospyros species are well-known for the treatment of various ailments in many traditional medicines such as lumbago, carminative, astringent, cure biliousness, sedative, febrifuge, snake bite, urinary discharges and inflammation [47].

The leaf extract of *D. kaki* in combination with jasmine in Japan is used for preparation of anti-tobacco smoking candies. *Diospyros* genus has variety of triterpenoids class which showed excellent anti-inflammatory activity [48, 49] .

2.5. Chemical constituents

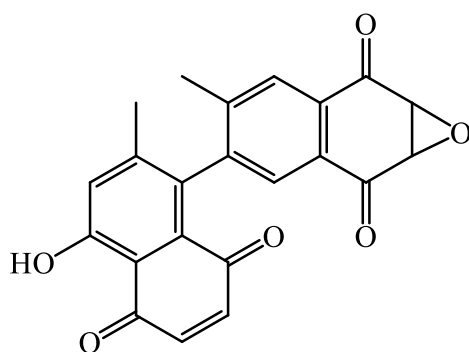
The constituents found in *Diospyros* are naphthoquinones, anthraquinones, terpenoids and lignans. The distributions of different classes of compounds in various *Diospyros* species are summarized in table 1.

Table 1: List of chemical constituents isolated from different species of genus *Diospyros*

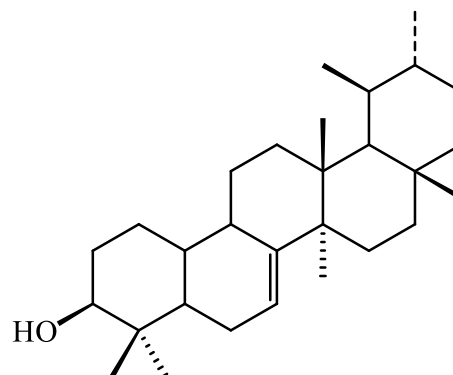
S.No	Name of compounds	Molecular formula	Sources
01	Batocanone	C ₂₂ H ₁₄ O ₇	<i>D. batocana</i> [50]
02	7-Baueren-3-ol	C ₃₀ H ₅₀ O	<i>D. japonica</i> [51]
03	Diospyrososide	C ₂₁ H ₃₂ O ₁₃	<i>D. angustifolia</i> [52]
04	3,3'-Bi[6-hydroxy-5-methoxy-2-methylnaphthoquinone]	C ₂₄ H ₁₈ O ₈	<i>D. melanoxylon</i> [53]
05	3,8'-Bi[5-hydroxy-2-methyl-1,4-naphthoquinone]	C ₂₂ H ₁₄ O ₆	<i>D. maritima</i> [54]
06	Biramentaceone	C ₂₂ H ₁₄ O ₆	<i>D. montana</i> [55]
07	6'',8'-Bisdiosquinone	C ₄₄ H ₂₆ O ₁₄	<i>D. mafiensis</i> [56]
08	Bisisodiospyrin	C ₄₄ H ₂₆ O ₁₂	<i>D. lotus</i> [57]
09	3-Bromo-5-hydroxy-2-methyl-1,4-naphthoquinone	C ₁₁ H ₇ BrO ₃	<i>D. maritima</i> [58]
10	Canaliculatin	C ₂₁ H ₁₄ O ₆	<i>D. canaliculata</i> [59]
11	Celabaquinone	C ₂₃ H ₁₈ O ₆	<i>D. celebica</i> [60]
12	Isocelebaquinone	C ₂₃ H ₁₈ O ₆	<i>D. celebica</i> [60]
13	Chitrane	C ₂₂ H ₁₄ O ₆	<i>D. maritima</i> [61]
14	3-Chloro-5-hydroxy-2-methyl-1,4-naphthoquinone	C ₁₁ H ₇ ClO ₃	<i>D. maritima</i> [58]
15	Crassiflorone	C ₂₁ H ₁₂ O ₆	<i>D. crassiflora</i> [62]
16	Cyclocanaliculatin	C ₂₁ H ₁₂ O ₆	<i>D. canaliculata</i> [63]
17	Cyclodiospyrin	C ₂₂ H ₁₂ O ₆	<i>D. montana</i> [64]
18	5,8-Dihydroxy-2-methyl-4-chromanone	C ₁₀ H ₁₀ O ₄	<i>D. maritima</i> [65]
19	Isoshinanolone	C ₁₁ H ₁₂ O ₃	<i>D. maritima</i> [66].
20	Diospyroside	C ₂₀ H ₂₄ O ₁₃	<i>D. sapota</i> [67]
21	6,6'-Bi[8-hydroxy-3-methyl-1,4-naphthoquinone].	C ₂₂ H ₁₄ O ₆	<i>D. wallichii</i> [68]
22	Diospyric acid D	C ₂₈ H ₄₂ O ₇	<i>D. decandra</i> [69]
23	4,9-Dihydroxy-1,2,6,7,11,12-hexamethylperylene-3,10-quinone	C ₂₆ H ₂₂ O ₄	<i>D. natalensis</i> [70]
24	4,11-Dihydroxy-5-methoxy-2,9-dimethyldinaphtho[1,2-b:2',3'-d]furan-7,12-quinone	C ₂₃ H ₁₆ O ₆	<i>D. melanoxylon</i> [71]

25	2,5,8-Trihydroxy-3-methyl-1,4-naphthoquinone	C ₁₁ H ₈ O ₄	<i>D. maritime</i> [72]
26	5,8-Dihydroxy-2-methyl-1,4-naphthalenedione	C ₁₁ H ₈ O ₄	<i>D. lycioides</i> [73]
27	Diospyric acid A	C ₂₉ H ₄₄ O ₅	<i>D. decandra</i> [69]
28	Diospyric acid E	C ₂₉ H ₄₂ O ₈	<i>D. decandra</i> [69]
29	Diomuscine	C ₁₂ H ₁₂ O ₄	<i>D. wallichii</i> [74]
30	Diosindigo A	C ₂₄ H ₂₀ O ₆	<i>D. maritime</i> [71]
31	Diosindigo B	C ₂₄ H ₂₀ O ₆	<i>D. celebica</i> [71]
32	Diospyrin	C ₂₂ H ₁₄ O ₆	<i>D. lotus</i> [75]
33	8'-Hydroxydiospyrin	C ₂₂ H ₁₄ O ₇	<i>D. lotus</i> [75]
34	Diospyrodin	C ₁₁ H ₂₂ O ₁₀	<i>D. nigra</i> [76]
35	Diospyrol	C ₂₂ H ₁₈ O ₄	<i>D. mollis</i> [77]
36	Diospyrone	C ₄₂ H ₂₆ O ₁₂	<i>D. canaliculata</i> [78]
37	Diospyrosin	C ₂₀ H ₁₈ O ₅	<i>D. quaesita</i> [79]
38	Diosquinone	C ₂₂ H ₁₄ O ₇	<i>D. chamaethamnus</i> [50]
39	Ebenone	C ₂₂ H ₁₆ O ₅	<i>D. ebenum</i> [80]
40	Ehretione	C ₂₂ H ₁₄ O ₆	<i>D. ehretioides</i> [64]
41	Galpinone	C ₃₃ H ₂₀ O ₉	<i>D. galpini</i> [73]
42	Gerberinol	C ₂₁ H ₁₆ O ₆	<i>D. kaki</i> [81]
43	2, 6-Dimethoxy-7-methoxycarbonyljuglone	C ₁₄ H ₁₂ O ₇	<i>D. montana</i> [72]
44	3-Methylplumbagin	C ₁₂ H ₁₀ O ₃	<i>D. maritima</i> [82]
45	5-Hydroxy-3-(2-hydroxyethyl)-2-methyl-1,4-naphthoquinone	C ₁₃ H ₁₂ O ₄	<i>D. maritime</i> [54]
46	Betulinic acid	C ₃₀ H ₄₈ O ₃	<i>D. montana</i> [83]
47	7-Hydroxy-6-methoxycoumarin	C ₁₀ H ₈ O ₄	<i>D. kaki</i> [84]
48	8-Hydroxy-8'-methoxy-3',6-dimethyl-[2,2'-binaphthalene]-1,1',4,4'-tetrone	C ₂₃ H ₁₆ O ₆	<i>D. mollis</i> [85]
49	Diospyrosonaphthoside	C ₂₃ H ₃₀ O ₁₂	<i>D. angustifolia</i> [52]
50	4-Hydroxy-1,5-dimethoxy-2-naphthalenemethanol	C ₁₃ H ₁₄ O ₄	<i>D. wallichii</i> [68]
51	5-Hydroxy-2-methyl-1,4-naphthoquinone	C ₁₁ H ₈ O ₃	<i>D. maritime</i> [86]
52	2,3-Epoxyplumbagin	C ₁₁ H ₈ O ₄	<i>D. maritime</i> [86]
53	5-Methoxy-5-Hydroxy-2-methyl-1,4-naphthoquinone	C ₁₂ H ₁₀ O ₃	<i>D. melanoxylon</i> [87]
54	5-Hydroxy-7-methyl-1,4-naphthoquinone	C ₁₁ H ₈ O ₃	<i>D. usambaraensis</i> [86]
55	8-Methoxy-3-methyl-1,2-naphthoquinone	C ₁₂ H ₁₀ O ₃	<i>D. usambaraensis</i> [86]
56	8-Methoxy-6-methyl-1,2-naphthoquinone	C ₁₂ H ₁₀ O ₃	<i>D. celebica</i> [60]
57	7-Hydroxy-2-nonadecanone	C ₁₉ H ₃₈ O ₂	<i>D. peregrine</i> [88]
58	8-Hydroxy-10-octadecenoic acid	C ₁₈ H ₃₄ O ₃	<i>D. montana</i> [89]
59	Diospyrolide	C ₂₇ H ₄₂ O ₃	<i>D. maritime</i> [90]
60	Diospyrolidone	C ₂₇ H ₄₀ O ₃	<i>D. maritime</i> [90]
61	Ismailin	C ₃₁ H ₂₀ O ₉	<i>D. ismailii</i> [59]
62	Isobatocanone	C ₂₂ H ₁₄ O ₇	<i>D. batocana</i> [50]
63	Isocelabaquinone	C ₂₃ H ₁₈ O ₆	<i>D. celebica</i> [60]

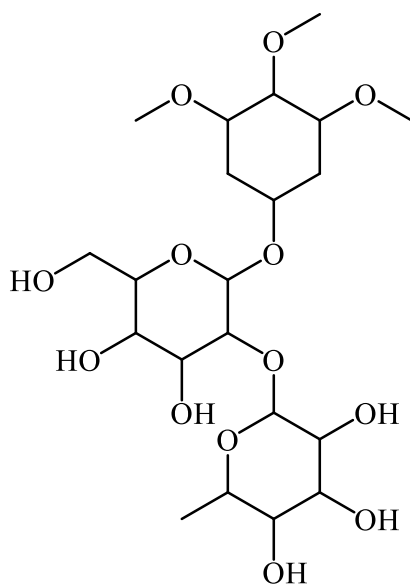
64	Isoxylopyrin	C ₃₃ H ₁₈ O ₉	<i>D. whyteana</i> [91]
65	Isozeylanone	C ₂₂ H ₁₄ O ₆	<i>D. maritime</i> [92]
66	Lemuninol A	C ₂₄ H ₂₀ O ₆	<i>D. lemunihitam</i> [93]
67	Lemuninol B	C ₂₄ H ₂₂ O ₆	<i>D. lemunihitam</i> [93]
68	Lemuninol C	C ₂₅ H ₂₄ O ₆	<i>D. lemunihitam</i> [93]
69	Macassaric acid	C ₁₃ H ₁₄ O ₅	<i>D. celebica</i> [94]
70	Mamegakinone	C ₂₂ H ₁₄ O ₆	<i>D. batocana</i> [57]
71	Maritinone	C ₂₂ H ₁₄ O ₆	<i>D. batocana</i> [77]
72	3,3'-Methylenebiplumbagin	C ₂₃ H ₁₆ O ₆	<i>D. maritime</i> [54]
73	Mutatoxanthin	C ₄₀ H ₅₆ O ₃	<i>D. kaki</i> [95]
74	Neodiospyrin	C ₂₂ H ₁₄ O ₆	<i>D. ismaili</i> [96]
75	Rotundiquinone	C ₂₂ H ₁₄ O ₆	<i>D. ismaili</i> [70]
76	Tetrahydrodiospyrin	C ₂₂ H ₁₈ O ₆	<i>D. montana</i> [97]
77	2,4,4',6-Tetrahydroxyaurone	C ₁₅ H ₁₀ O ₆	<i>D. melanoxylon</i> [98]
78	Whyteone	C ₄₄ H ₂₄ O ₁₂	<i>D. galpinii</i> [70]
79	Xylopyrin	C ₃₃ H ₁₈ O ₉	<i>D. chloroxylon</i> [72]
80	Yerrinquinone	C ₁₄ H ₁₂ O ₇	<i>D. montana</i> [99]
81	Methylgallate	C ₈ H ₈ O ₅	<i>D. lotus</i> [100]
82	Kaempferol	C ₁₅ H ₁₀ O ₆	<i>D. lotus</i> [100]
83	Myricetin	C ₁₅ H ₁₀ O ₈	<i>D. lotus</i> [100]
84	Myricetin 3- <i>O</i> - β -glucuronide	C ₂₁ H ₁₈ O ₁₄	<i>D. lotus</i> [100]
85	Myricetin-3- <i>O</i> - α -rhamnoside	C ₂₁ H ₂₀ O ₁₂	<i>D. lotus</i> [100]
86	Ellagic acid	C ₁₄ H ₆ O ₈	<i>D. lotus</i> [16]
87	Gallic acid	C ₇ H ₆ O ₅	<i>D. lotus</i> [16]
88	Quercetin	C ₁₅ H ₁₀ O ₇	<i>D. lotus</i> [16]

Structures of isolated constituents from the genus *Diospyros*

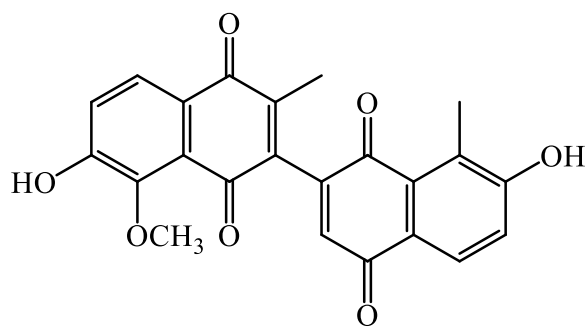
Batocanone [50]



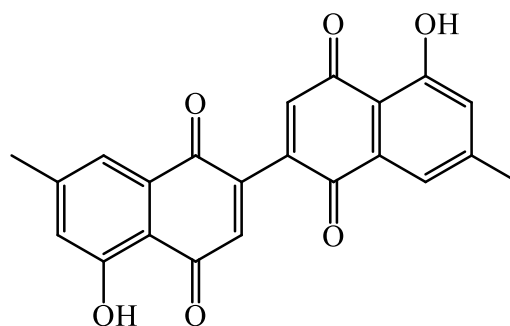
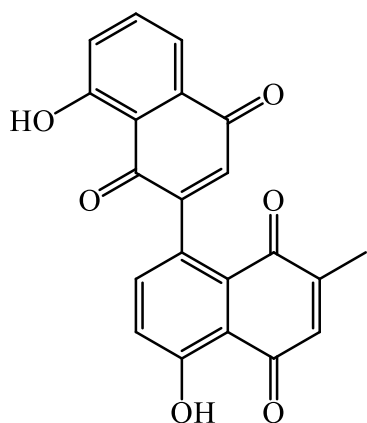
7-Baueren-3-ol [51]



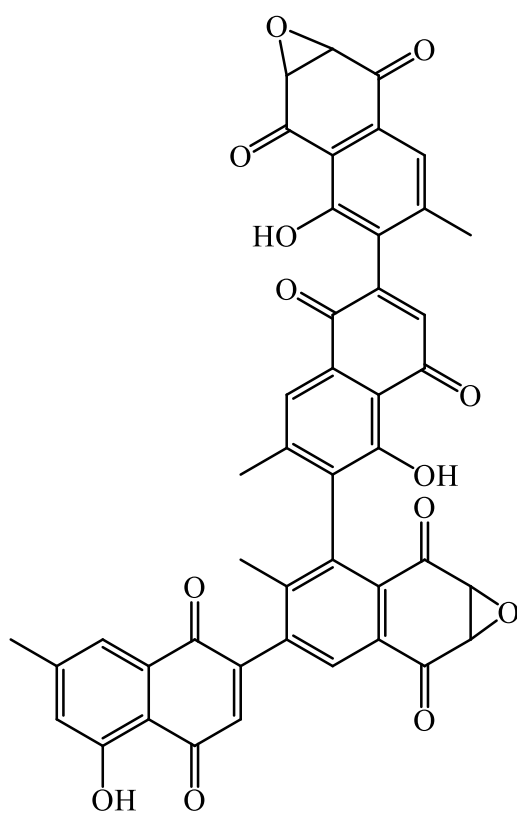
Diospyrososide [52]



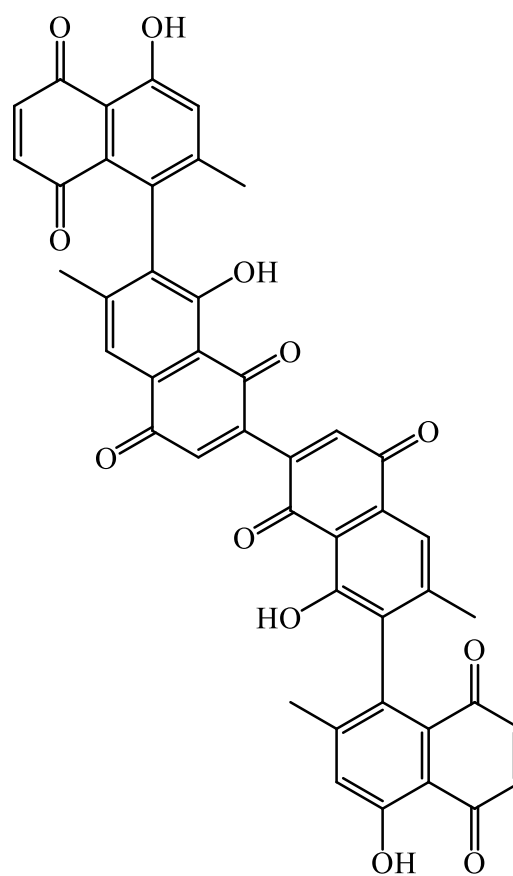
3,3'-Bi[6-hydroxy-5-methoxy-2-methylnaphthoquinone [53]



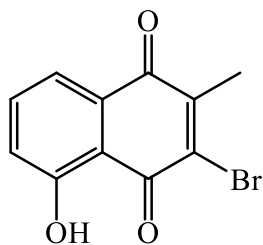
3,3'-Bi[6-hydroxy-5-methoxy-2-methylnaphthoquinone [54] Biramentaceone [55]



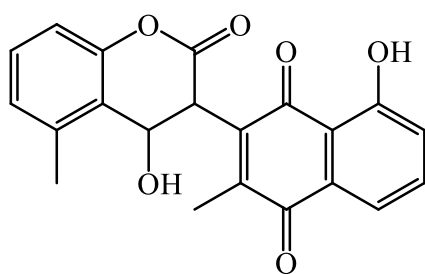
6'',8'-Bisdiosquinone [56]



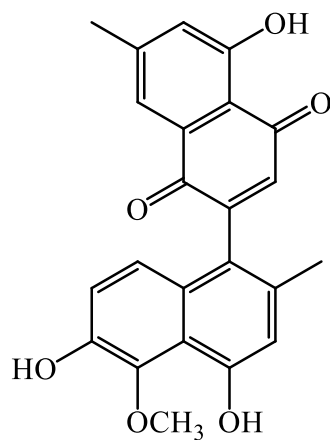
Bisisodiospyrin [57]



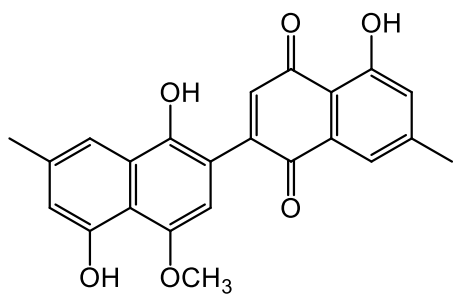
3-Bromo-5-hydroxy-2-methyl-1,4-naphthoquinone [58]



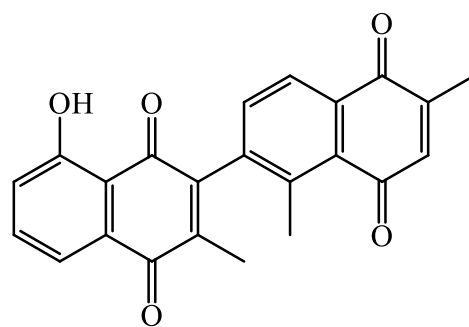
Canaliculatin [59]



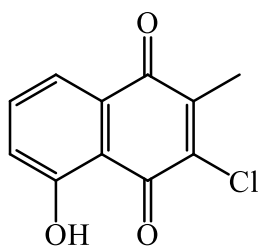
Celebaquinone [60]



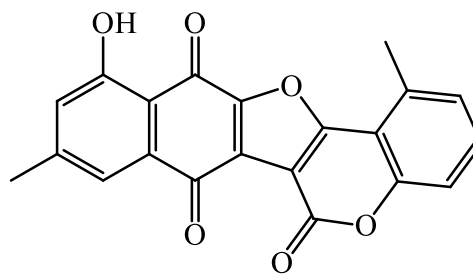
Isocelebaquinone [60]



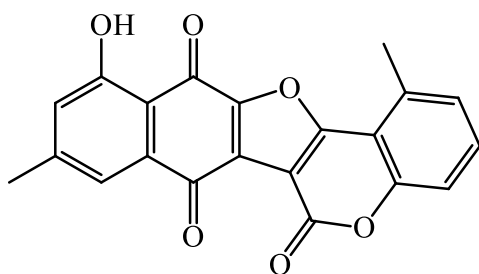
Chitranone [61]



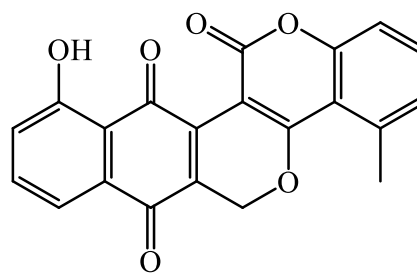
3-Chloro-5-hydroxy-2-methyl-1,4-naphthoquinone [58]



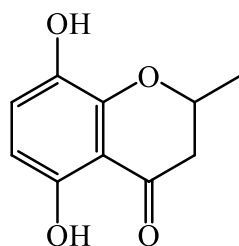
Crassiflorone [62]



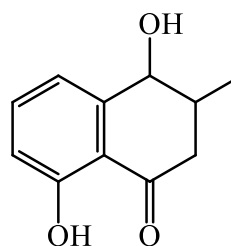
Cyclocanaliculatin [63]



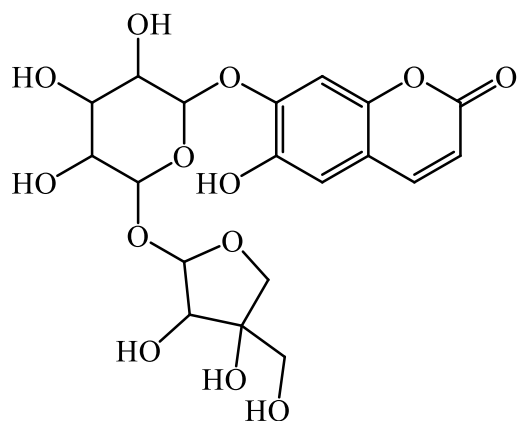
Cyclodiospyrin [64]



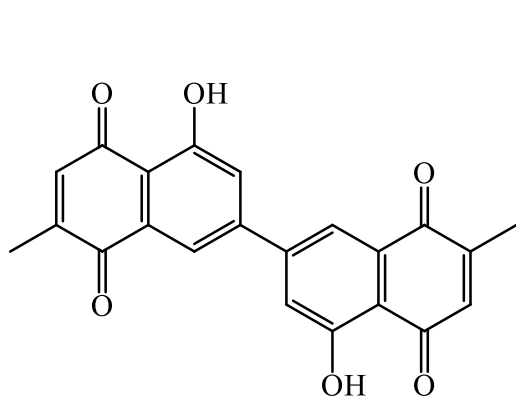
5,8-Dihydroxy-2-methyl-4-chromanone [65]



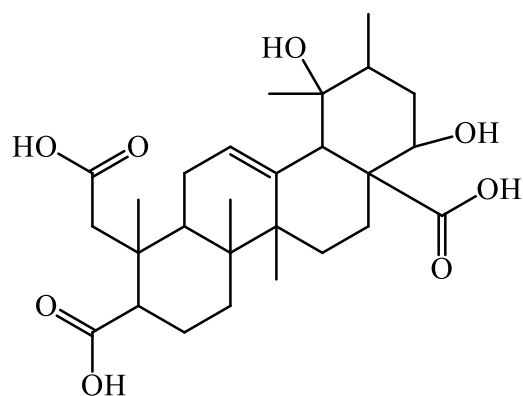
Isoshinanolone [66]



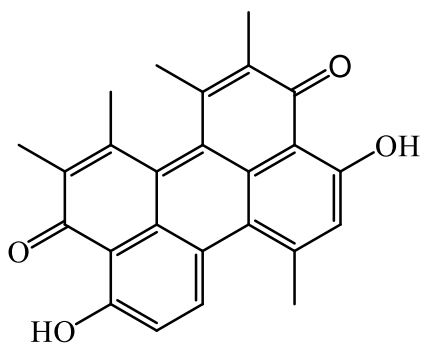
Diospyroside [67]



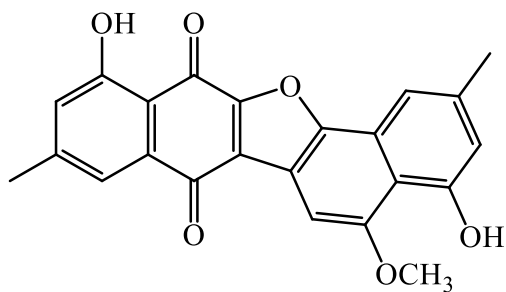
6,6'-Bi[8-hydroxy-3-methyl-1,4-naphthoquinone] [68]



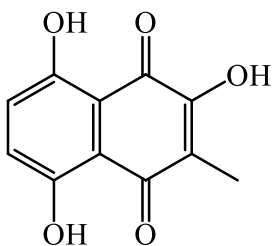
Diospyric acid D [69]



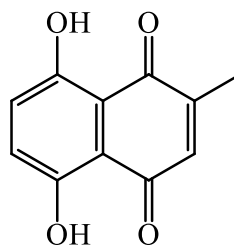
4,9-Dihydroxy-1,2,6,7,11,12-hexamethylperylene-3,10-quinone [70]



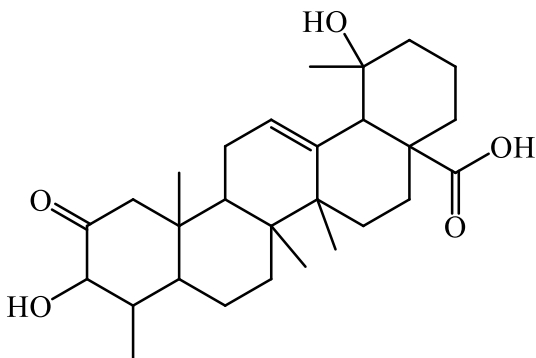
4,11-Dihydroxy-5-methoxy-2,9-dimethyldinaphtho[1,2-b:2',3'-d]furan-7,12-quinon [71]



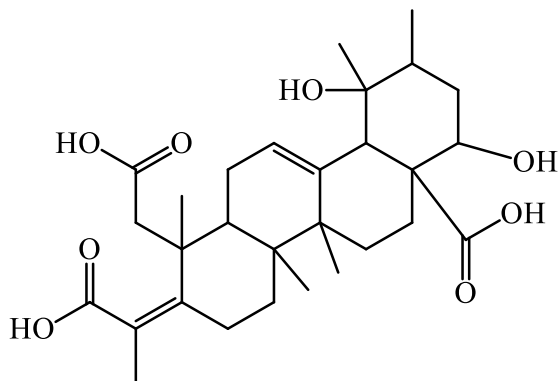
2, 5,8-Trihydroxy-3-methyl-1,4-naphthoquinone [72]



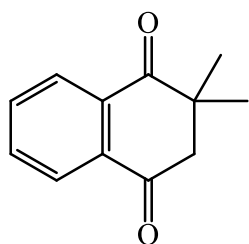
5,8-Dihydroxy-2-methyl-1,4-naphthoquinone [73]



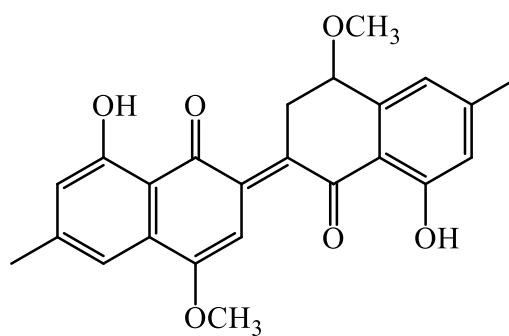
Diospyric acid A [74]



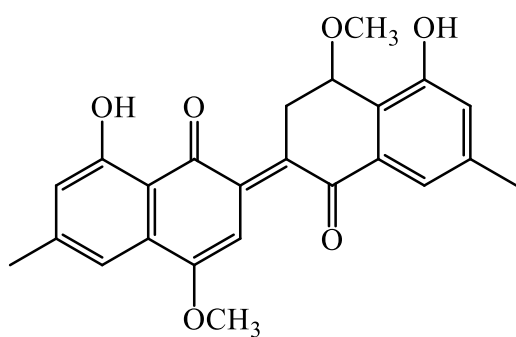
Diospyric acid E [74]



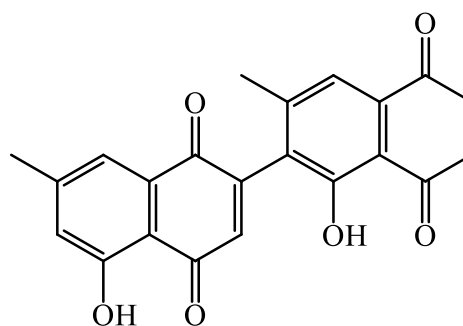
Diomuscine [74]



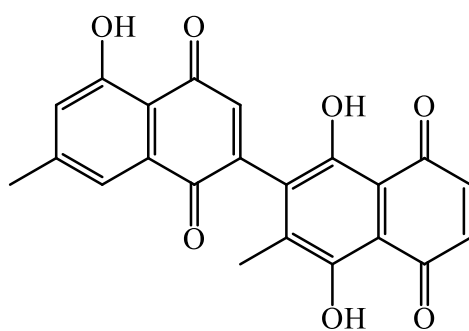
Diosindigo A [71]



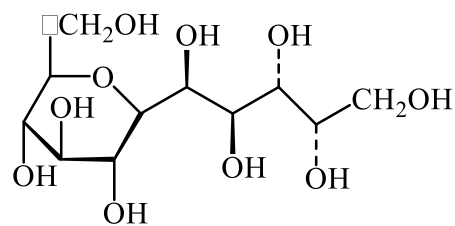
Diosindigo B [71]



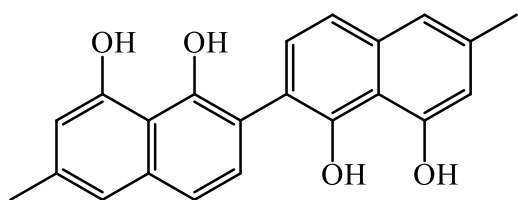
Diospyrin [75]



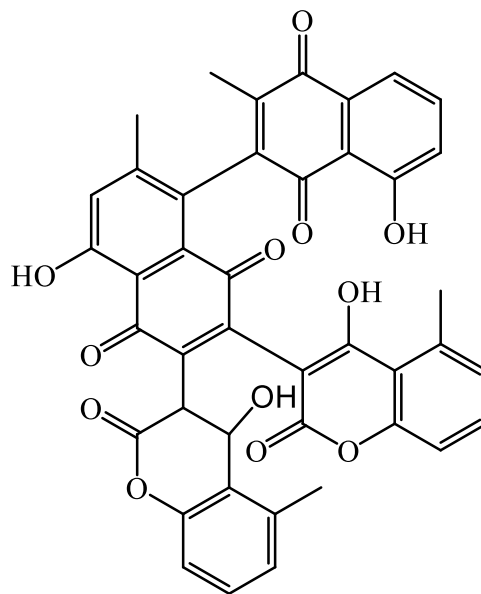
8'-Hydroxydiospyrin [75]



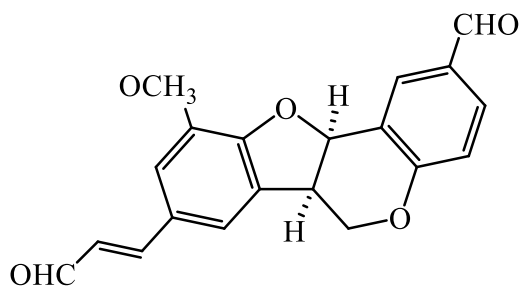
Diospyrodin [76]



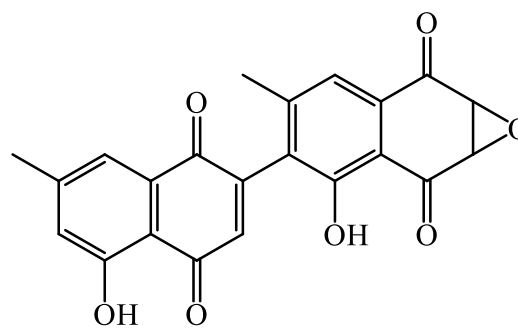
Diospyrol [77]



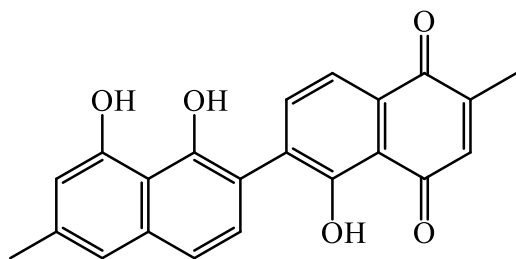
Diospyrone [78]



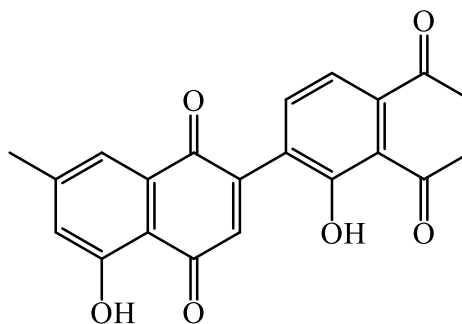
Diospyrosin [79]



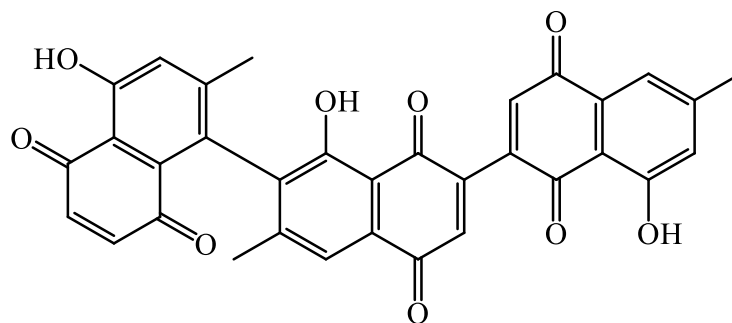
Diosquinone [50]



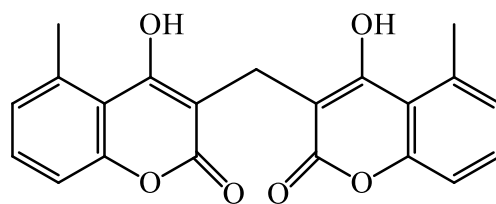
Ebenone [80]



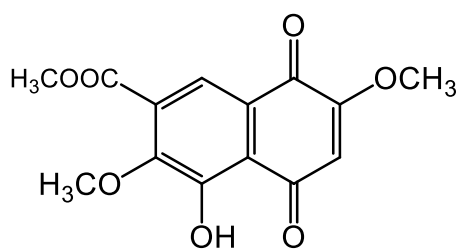
Ehretione [64]



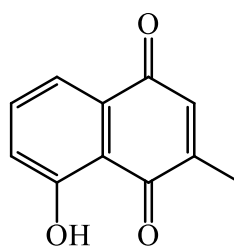
Galpinone [73]



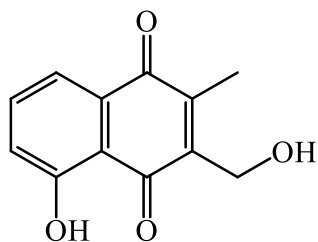
Gerberinol [81]



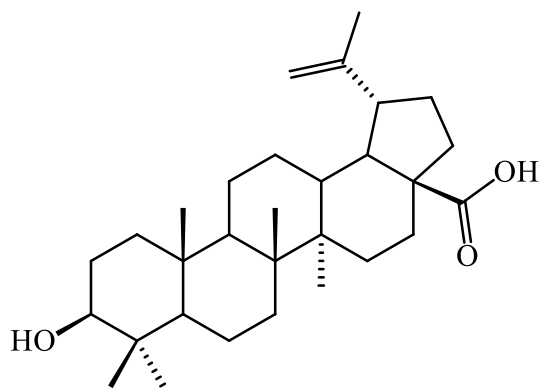
2,6-Dimethoxy-7-methoxycarbonyljuglone [72]



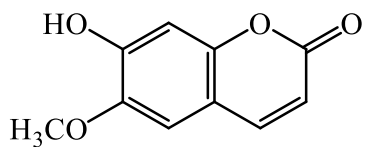
3-Methylplumbagin [82]



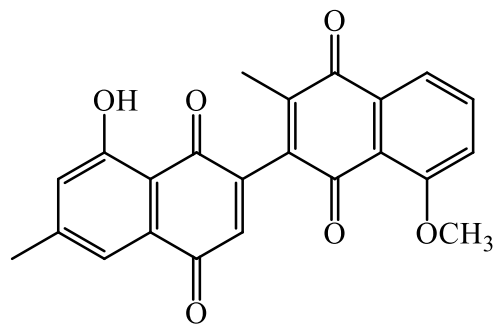
5-Hydroxy-3-(2-hydroxymethyl)-2-methyl-1,4-naphthoquinone [54]



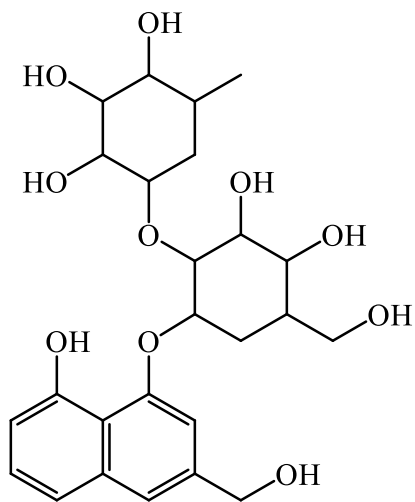
Betulinic acid [83]



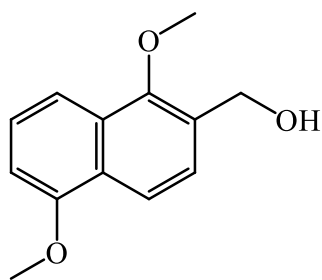
7-Hydroxy-6-methoxycoumarin [84]



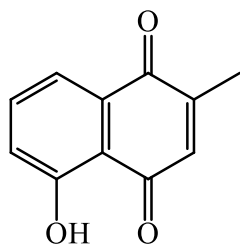
8-Hydroxy-8'-methoxy-3',6-dimethyl-[2,2'-binaphthalene]-1,1',4,4'-tetrone [51]



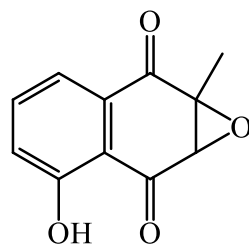
Diospyronaphthoside [52]



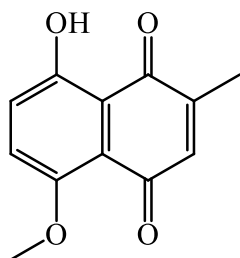
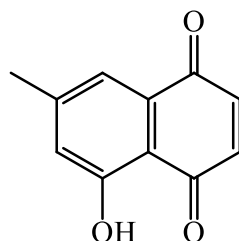
4-Hydroxy-1,5-dimethoxy-2-naphthalenemethanol [68]

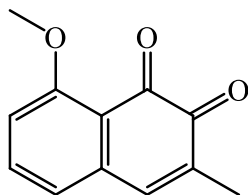


5-Hydroxy-2-methyl-1,4-naphthoquinone [86]

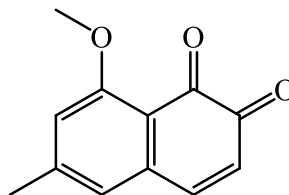


2,3-Epoxyplumbagin [86]

5-Methoxy-8-Hydroxy-2-methyl-
[86]5-Hydroxy-7-methyl-1,4-naphthoquinone
[87]

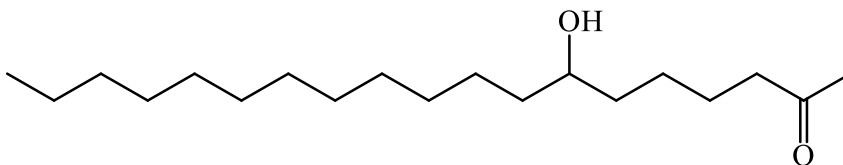


8-Methoxy-3-methyl-1,2-naphthoquinone [86]

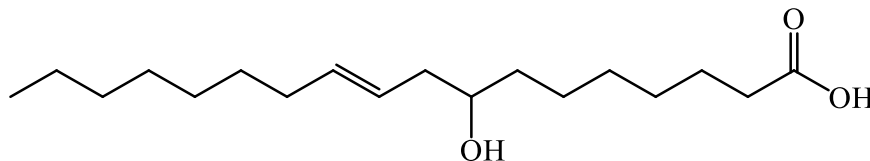


8-Methoxy-6-methyl-1,2-naphthoquinone

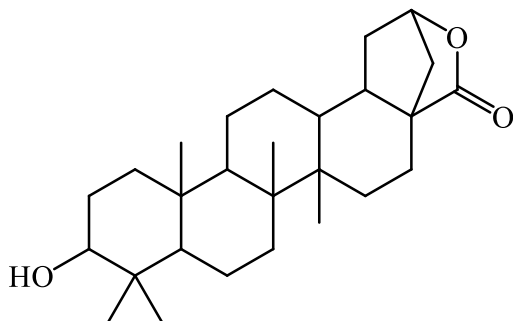
[60]



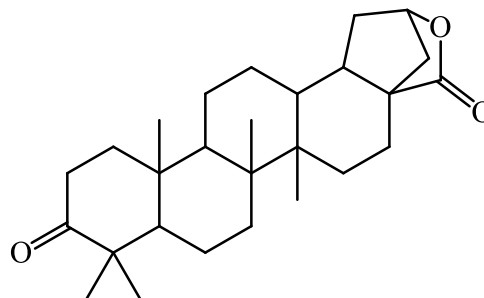
7-Hydroxy-2-nonadecanone [88]



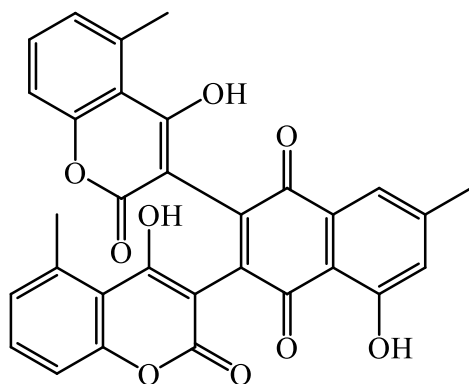
8-Hydroxy-10-octadecenoic acid [89]



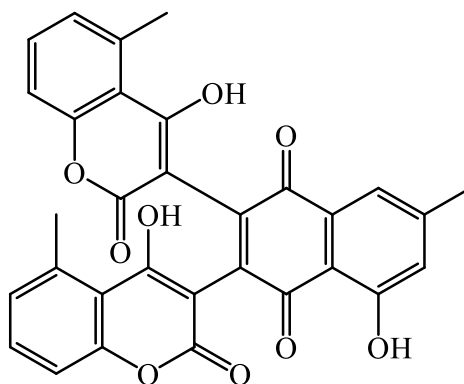
Diospyrolide [90]



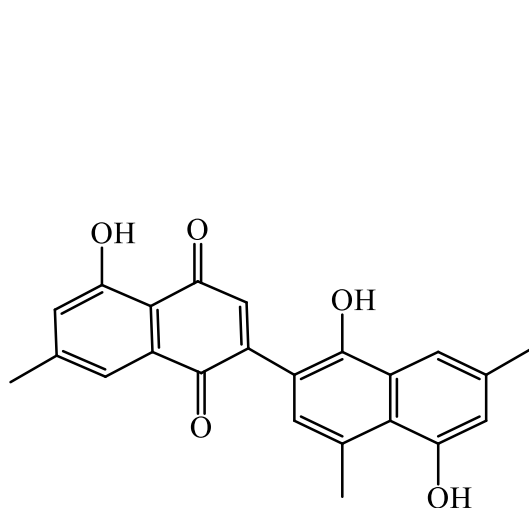
Diospyrolidone [90]



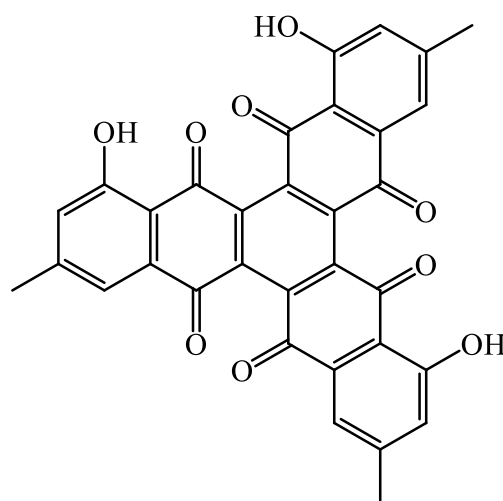
Ismailin [59]



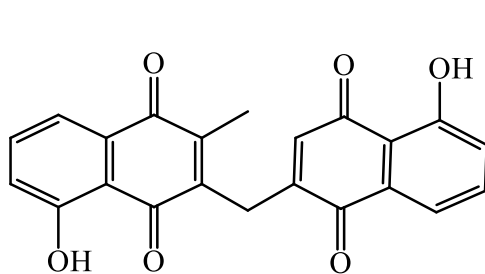
Isobatocanone [50]



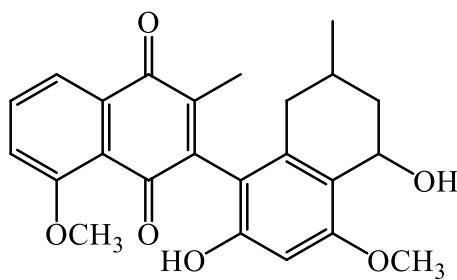
Isocelabaquinone [60]



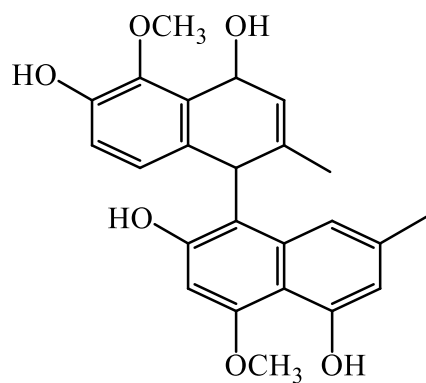
Isoxylopyrin [91]



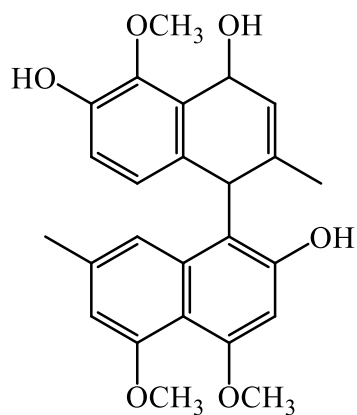
Isozeylanone [92]



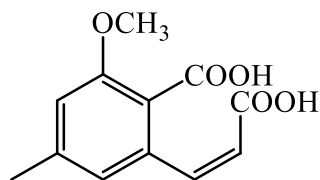
Lemuninol A [93]



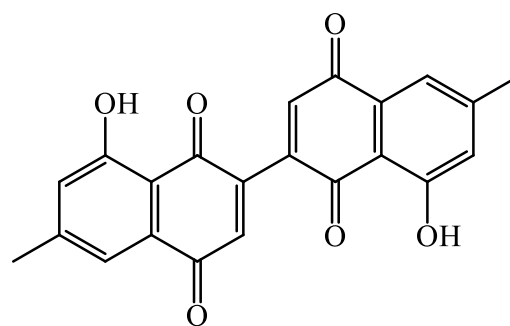
Lemuninol B [93]



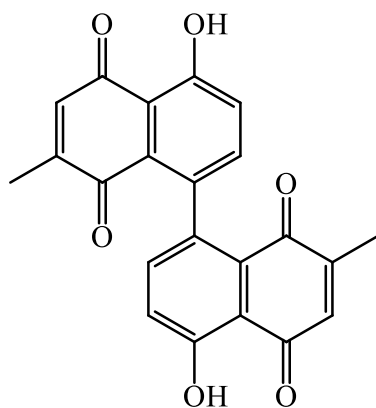
Lemuninol C [93]



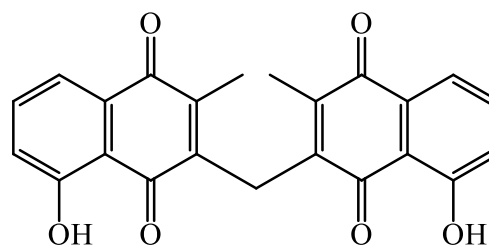
Macassaric acid [94]



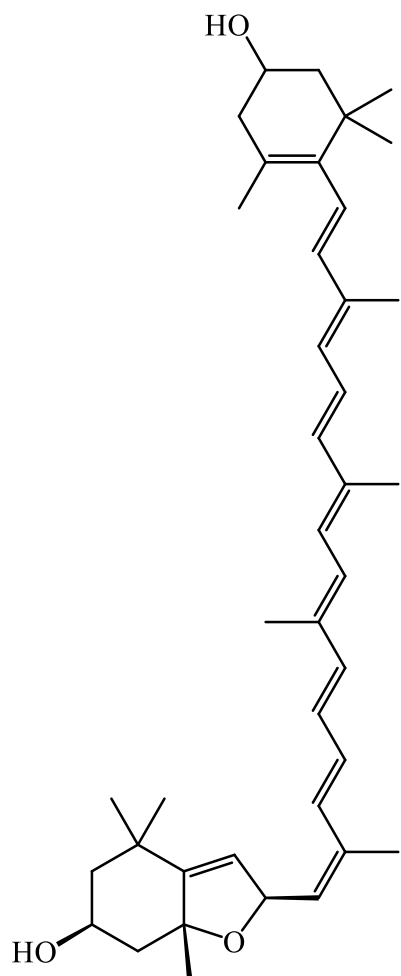
Mamegakinone [57]



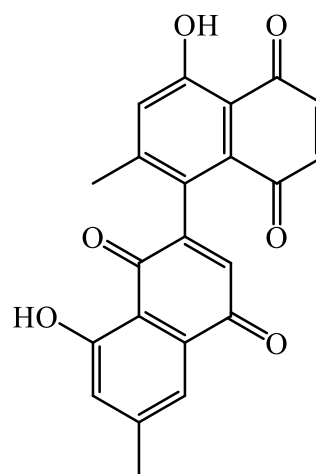
Maritinone [77]



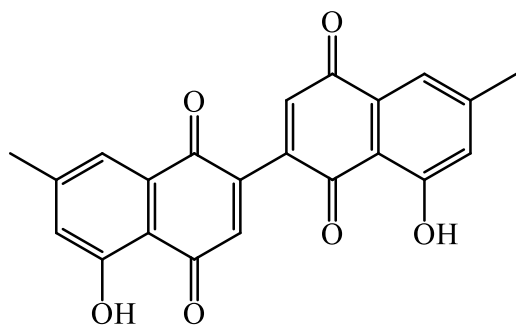
3,3'-Methylenebiplumbagin [54]



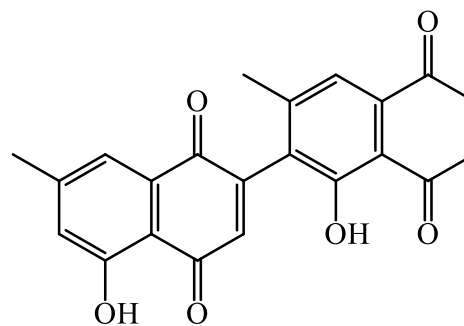
Mutatoxanthin [95]



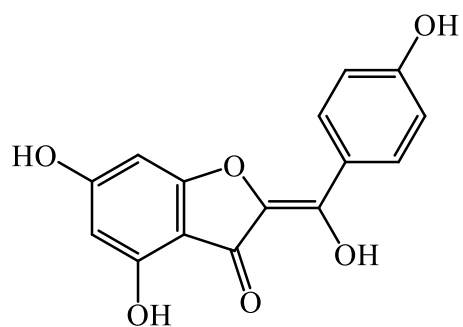
Neodiospyrin [96]



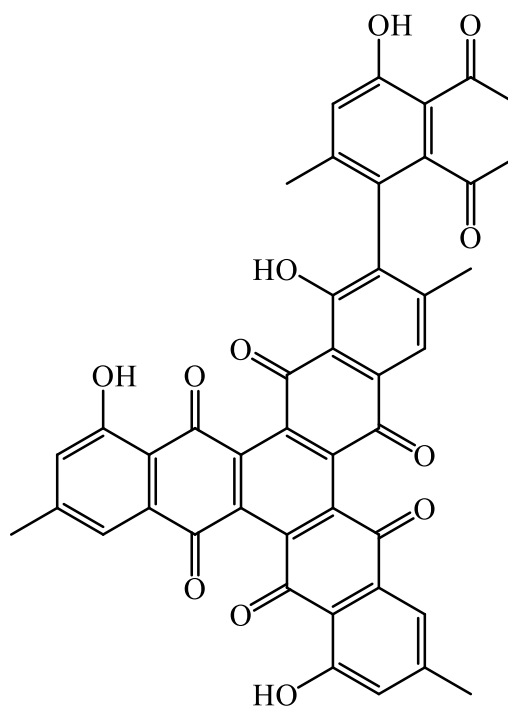
Rotundiquinone [91]



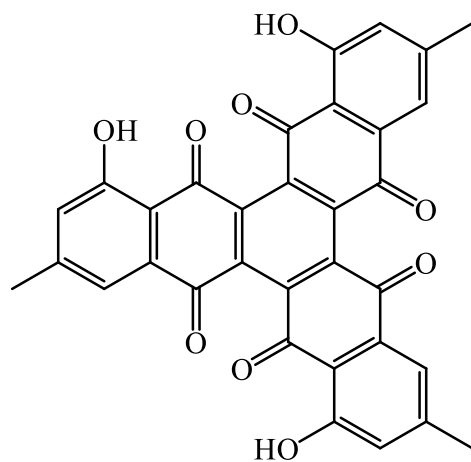
Tetrahydrodiospyrin [97]



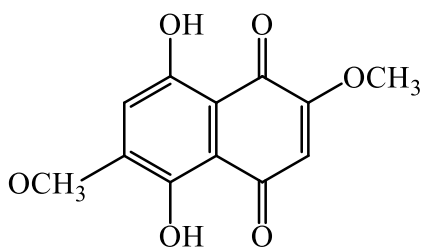
2,4,4',6-Tetrahydroxyaurone [98]



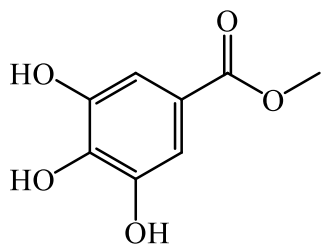
Whyteone [70]



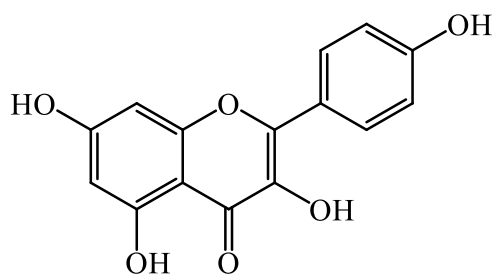
Xylospyrin [72]



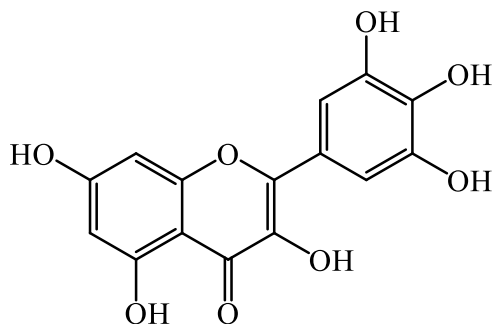
Yerrinquinone [99]



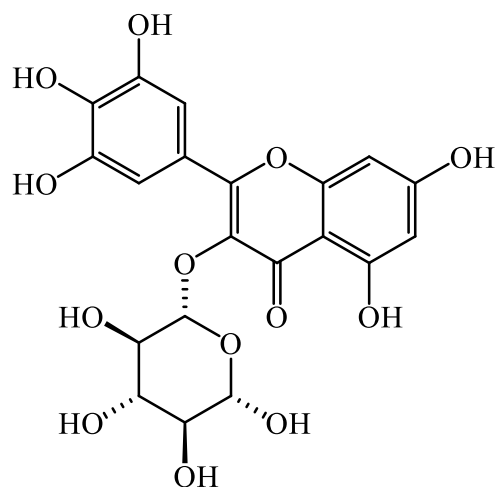
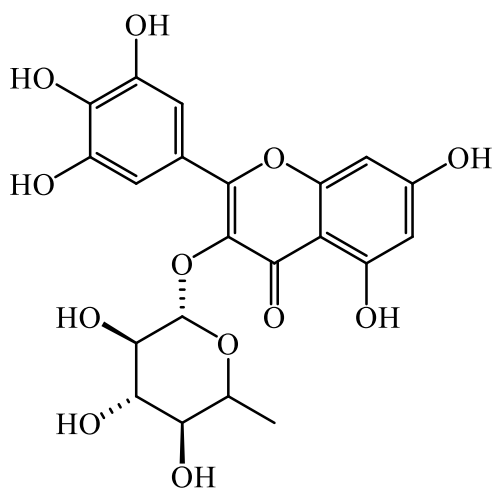
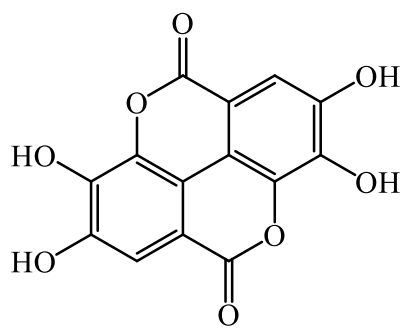
Methylgallate [100]



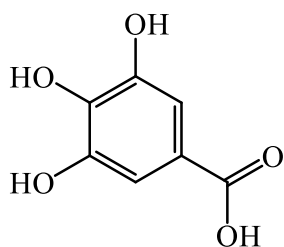
Kaempferol [100]



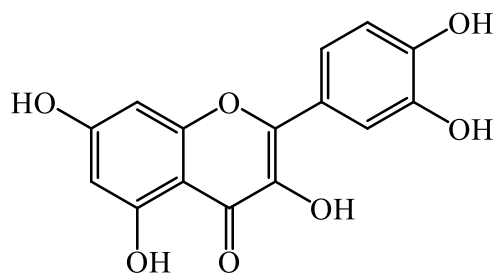
Myricetin [100]

Myricetin-3-O- β -glucuronide [100]Myricetin-3-O- α -rhamnoside [100]

Ellagic acid [16]



Gallic acid [16]



Quercetin [16]

3.1. Preliminary phytochemical analysis of *D. lotus*

Phytochemical screening is an important to know the chemical constituents present in the medicinal plants which lead to isolate of bioactive secondary metabolites. The phytochemical analysis was performed as per standard protocol [1]. The extracts and its fractions (**Scheme 1**) of *D. lotus* (roots, leaves, bark and hard wood) showed the presence of bioactive chemical constituents, such as terpenoids, flavonoids, naphthoquinones and tannins which correlate the medicinal value of the *D. lotus*.

3.1.1. Phytochemical analysis of hard wood of *D. lotus*

The methanolic extract of hard wood of *D. lotus* and its fractions were screened for the determination of bioactive secondary metabolites. The extract and fractions showed the presence of anthraquinones steroids and steroids while *n*-hexane, CHCl_3 and EtOAc have fatty acids and steroids (**Table 2**).

Table 2: Phytochemical analysis of *D. lotus* hard wood extract and its fractions

Test for constituents	<i>n</i> -Hexane	Chloroform	EtOAc	Methanol
Alkaloids	–	–	–	–
Tannins	–	–	–	–
Anthraquinones	–	+	+	+
Reducing sugars	–	+	+	+
Saponins	–	–	–	–
Flavonoids	–	–	–	–
Glycosides	–	–	–	–
Phlobatanins	–	–	–	–
Terpenoids	–	+	+	+
Steroids	–	–	–	–
Protein and amino acid	–	–	–	–
Fatty acids	+	+	–	+
Caumarines	–	–	–	–
Emodines	–	–	–	–
Anthocyanin and betacyanin	–	–	–	–
Carbohydrates	–	–	–	–

3.1.2. Phytochemical analysis of leaves of *D. lotus*

The methanolic extract of leaves of *D. lotus* and its fractions were also tested for the presence of bioactive secondary metabolites. The crude extract, CHCl₃, EtOAc and *n*-hexane fractions indicated the presence of terpenoids, steroids, tannins and fatty acids (Table 3).

Table 3: Phytochemical analysis of *D. lotus* leaves extract and its fractions

Test for constituent	<i>n</i> -Hexane	Chloroform	EtOAc	Methanol
Alkaloids	–	–	–	–
Tannins	–	–	+	+
Anthraquinones	–	–	–	–
Reducing sugars	–	–	–	–
Saponins	–	–	–	–
Flavonoids	–	–	–	–
Glycosides	–	–	–	–
Phlobatanins	–	–	–	–
Terpenoids	–	+	+	+
Steroids	+	+	–	+
Protein and amino acid	–	–	–	–
Fatty acids	+	+	–	+
Caumarines	–	–	–	–
Emodines	–	–	–	–
Anthocyanin and betacyanin	–	–	–	–
Carbohydrates	–	–	–	–

3.1.3. Phytochemical analysis of bark of *D. lotus*

The methanolic extract and its fractions of bark of *D. lotus* were also evaluated for phytochemical screening to identify the presence of bioactive secondary metabolites; the results are summarized in table 4.

Table 4: Phytochemical analysis of the extract and fractions of *D. lotus* bark

Test for constituent	<i>n</i> -Hexane	Chloroform	EtOAc	Methanol
Alkaloids	–	–	–	–
Tannins	–	–	–	–
Anthraquinones	–	++	+	+
Reducing sugars	–	+	+	+
Saponins	–	–	–	–
Flavonoids	–	–	–	–
Glycosides	–	–	–	–
Phlobatanins	–	–	–	–
Terpenoids	–	+	+	+
Steroids	+	+	–	+
Protein and amino acid	–	–	–	–
Fatty acids	+	+	–	+
Caumarines	–	–	–	–
Emodines	–	–	–	–
Anthocyanin and betacyanin	–	–	–	–
Carbohydrates	–	–	–	–

3.1.4. Phytochemical analysis of the roots of *D. lotus*

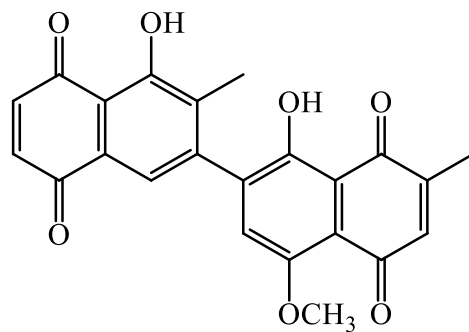
D. lotus extract and its fractions were investigated for phytochemical screening to identify bioactive secondary metabolites and the findings are summarized in table 5.

Table 5: Phytochemical analysis of extract and fractions of *D. lotus* roots

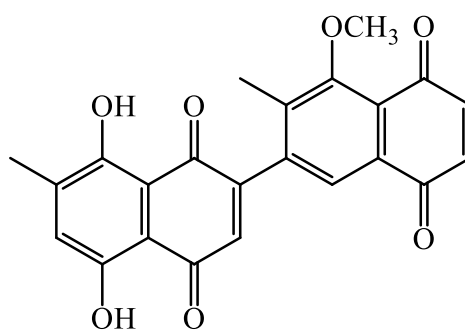
Test for constituent	<i>n</i> -Hexane	Chloroform	EtOAc	Methanol
Alkaloids	–	–	–	–
Tannins	–	–	–	–
Anthraquinones	–	+	+	+
Reducing sugars	–	+	+	+
Saponins	–	–	–	–
Flavonoids	–	–	–	–
Glycosides	–	–	–	–
Phlobatanins	–	–	–	–
Terpenoids	–	+	+	+
Steroids	+	+	–	+
Protein and amino acid	–	–	–	–
Fatty acids	+	+	–	+
Caumarines	–	–	–	–
Emodines	–	–	–	–
Anthocyanin and betacyanin	–	–	–	–
Carbohydrates	–	–	–	–

3.2. Chemical structures of isolated constituents from *D. lotus* roots

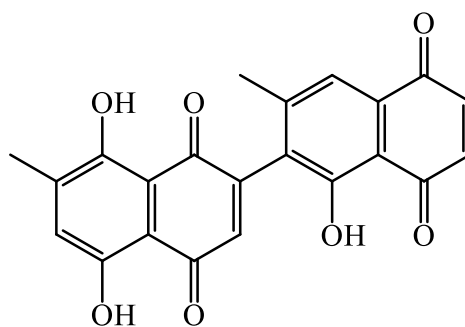
3.2.1. New chemical constituents



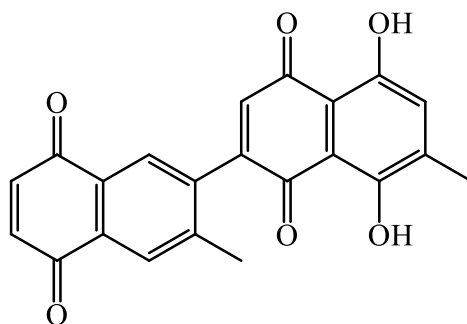
Di-naphthodiospyrol A (1)



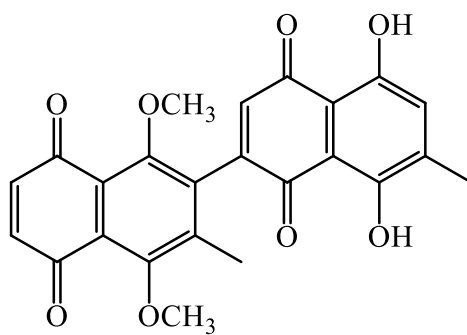
Di-naphthodiospyrol B (2)



Di-naphthodiospyrol C (3)

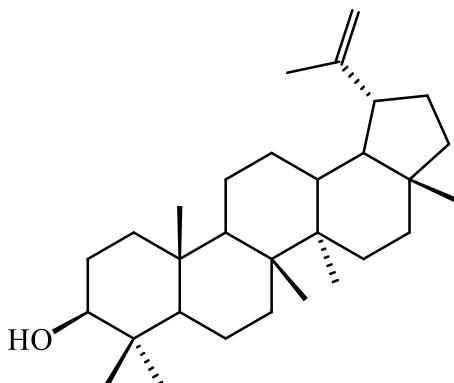


Di-naphthodiospyrol D (4)

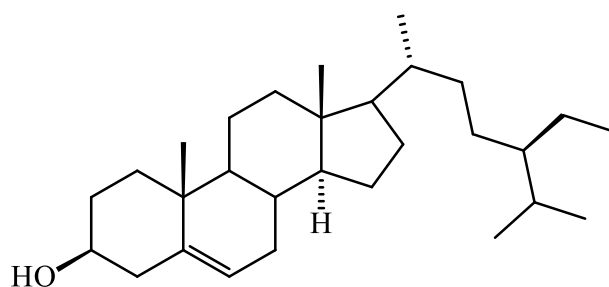
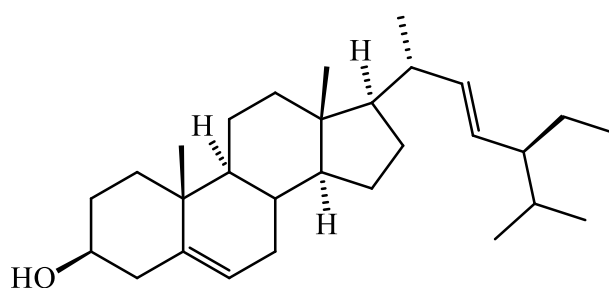


Di-naphthodiospyrol E (5)

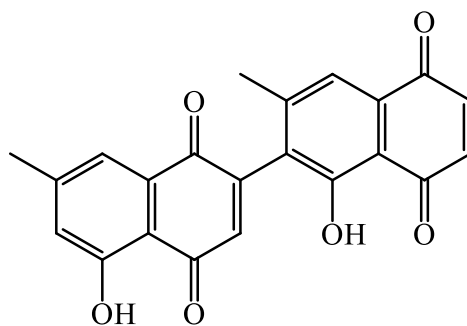
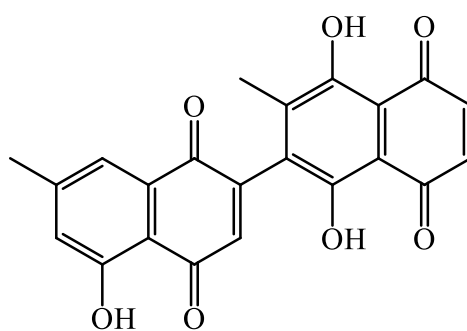
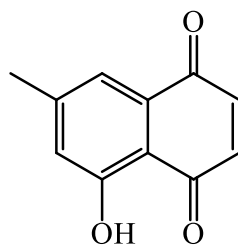
3.2.2. Hitherto unreported chemical constituents

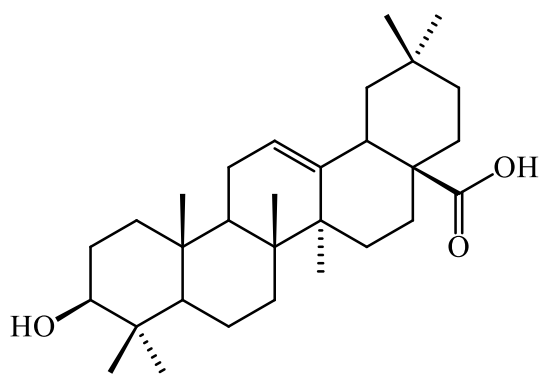
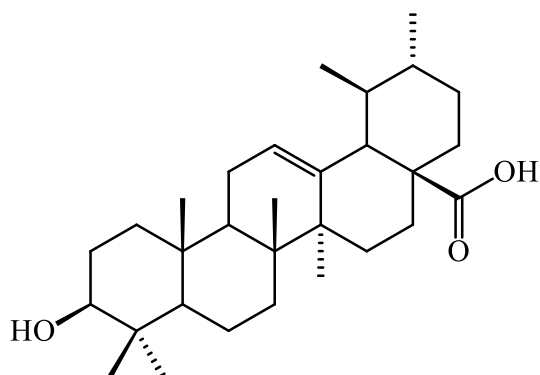
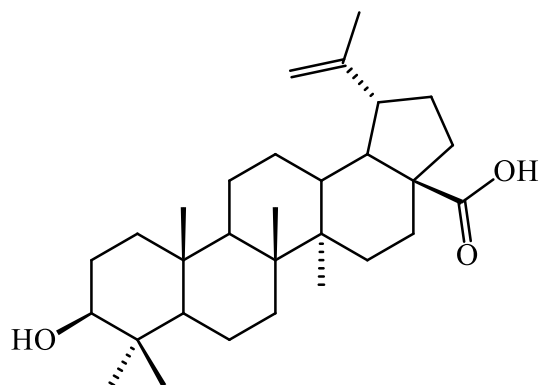


Lupeol (6)

 β -Sitosterol (7)

Stigmasterol (8)

Diospyrin (**9**)Hydroxydiospyrin (**10**)7-Methyljuglone (**11**)

Oleanolic acid (**12**)Ursolic Acid (**13**)Betulinic acid (**14**)

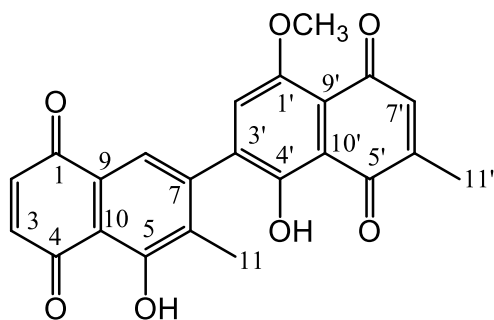
3.2.1.1. Di-naphthodiospyrol A (1)

Compound **1** was isolated as yellow amorphous powder. It exhibited a molecular ions peak at m/z 404.2987 a.m.u. (calcd. 404.2980 a.m.u) corresponding to molecular formula $C_{23}H_{16}O_7$. The IR spectrum (cm^{-1}) showed absorption bands at 3550 for OH stretching, 2924 for CH stretching, 1643 for CO stretching and 1634, 1604, 1460 for CH aromatic stretching. The UV spectrum exhibited absorptions at 253, 296 and 435 nm.

The assignment of protons and carbons were carried out by HMBC, HMQC, ^1H - ^1H -COSY and J -resolved experiments (**Table 6**). The ^1H -NMR spectrum of **1** revealed two tertiary methyl groups (s, δ_H 2.01, 1.97, $2\times 3H$), a methoxy group (s, δ_H 3.91, 3H), three quinoid protons (d, δ_H 6.70, $J=10$ Hz, H-2; d, 6.89, $J=10$ Hz, H-3; s, 6.07, H-7'), two aromatic protons (s, δ_H 7.27, H-8; s, 7.64, H-2'). The ^{13}C (BB and DEPT) spectra (**Table 6**) exhibited 23 carbon signals account for two methyl carbons, one methoxy carbon and 20 carbons for two naphthoquinone units. The quinoid protons resonated as a doublet at δ_H 6.70, (H-2), 6.89, (H-3) which showed HMBC correlation with δ_C 184.8 (C-1) and 190.3 (C-4). The aromatic proton (s, δ_H 7.27) exhibited correlation with (δ_C 129.4 (C-9) and 114.1 (C-10), while proton resonated at (s, δ_H 7.64) showed correlation with δ_C 158.1 (C-1'), 179.5 (C-8') and 108.6 (C-9'). All the assignments were placed with help of HMBC correlations as shown in figure 2. Based on the spectral data of compound **1** is recognized as 5,4'-dihydroxy-1'-methoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,5',8'-tetraone.

Table 6: ^{13}C and ^1H -NMR spectral data of **1**

Carbon No.	δ_{C}	δ_{H} (mult; J , Hz)	Types	HMBC
1	184.8	-	C	-
2	140.1	6.70, d, ($J=10$ Hz)	CH	C-1, C-4
3	137.7	6.89, d, ($J=10$ Hz)	CH	C-1, C-4
4	190.3	-	C	-
5	161.8	-	C	-
6	144.0	-	C	-
7	145.5	-	C	-
8	125.7	7.27, s	CH	C-9, C-10
9	129.4	-	C	-
10	114.1	-	C	-
11	20.6	2.01, s	CH_3	C-6, C-7, C-9
12- OCH_3	56.7	3.91, s	CH_3	-
1'	158.1	7.64, s	C	-
2'	109.4	7.64, s	CH	C-1', C-9', C-8'
3'	139.5	-	C	-
4'	161.1	-	C	-
5'	190.6	-	C	-
6'	139.5	-	C	-
7'	109.4	6.07, s	CH	C-5', C-6', C-8', C-11'
8'	179.5	-	C	-
9'	108.6	-	C	-
10'	112.0	-	C	-
11'	20.2	1.97, s	CH_3	C-5', C-6'

**1**

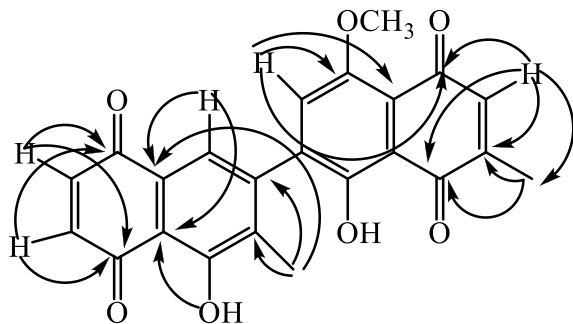


Figure 2: Key HBMC correlations of **1**

3.2.1.2. Di-naphthodiospyrol B (**2**)

Compound **2** was isolated as yellow amorphous powder. The HRMS of **2** exhibited a molecular ions peak at m/z 404.2980 a.m.u (calcd. 404.2972) consistent with the molecular formula $C_{23}H_{16}O_7$. The IR spectrum (cm^{-1}) displayed absorption bands at 3560 for OH stretching, 2925 for CH stretching, 1644 for C=O stretching and 1603 for aromatic proton stretching. The UV spectrum exhibited absorption peaks at 253, 299 and 432 nm. The assignment of protons and carbons was carried out by HMBC, HMQC, ^1H - ^1H -COSY and J -resolved experiments (**Table 7**). The ^1H -NMR spectrum of **2** exhibited the presence of two tertiary methyl groups (s, δ_H 1.98, 1.97) one methoxy group (s, δ_H 3.90), three quinoid protons resonated as a doublet and singlet (d, δ_H 6.70, J = 10.5 Hz, H-2; d, δ_H 6.89, J = 10.5 Hz, H-3; s, δ_H 6.42, H-7'), two aromatic protons (s, δ_H 7.27, H-8, and s, 7.59, H-2'). The ^{13}C -NMR (BB and DEPT) spectra (**Table 7**) exhibited 23 carbon signals for two methyl carbons, one methoxy carbon, and 20 carbons for two naphthoquinone moieties. The quinoid protons at δ_H 6.70 (H-2), 6.70 (H-3) showed strong HMBC correlation with δ_C 184.6 (C-1) and 190.3 (C-4) respectively. The aromatic proton resonated at δ_H 7.27 (H-8) revealed correlation with δ_C 130.4 (C-9) and 114.1(C-10') while the quinoid proton resonated at δ_H 7.59 (H-2') showed

correlation with δ_C 184.1 (C-1') and 137.6 (C-3'). The aromatic proton δ_H 6.42 (H-7') exhibited correlation with δ_C 159.1 (C-8'), 114.1 (C-9') and 184.1(C-1). All substituents were placed with the help of HMBC correlations as shown in figure 3. Compound **2** has been characterized as 5',8'-dihydroxy-5-methoxy-6,6'-dimethyl-7,3'-binaphthyl-1, 4,1',4'-tetraone on the basis by spectral data (**Table 7**).

Table 7: ^1H and ^{13}C -NMR spectral data of **2**

Carbon No.	δ_C	δ_H (mult; J , Hz)	Types	HMBC
1	184.6	-	C	-
2	140.1	6.70, d, (J = 10.5 Hz)	CH	C-1, C-4
3	137.6	6.89, d, (J = 10.5 Hz)	CH	C-1, C-4
4	190.3	-	C	-
5	161.9	-	C	-
6	146.1	-	C	-
7	148.1	-	C	-
8	121.7	7.27, s	CH	C-9, C-9'
9	130.4	-	C	-
10	114.1	-	C	-
11	20.6	1.98, s	CH ₃	C-7, C-8
12-OCH ₃	56.6	3.90, s	CH ₃	-
1'	184.1	-	C	-
2'	125.6	7.59, s	CH	C-1', C-3'
3'	137.6	-	C	-
4'	190.6	-	C	-
5'	161.1	-	C	-
6'	148.1	-	C	-
7'	110.3	6.42, s	CH	C-8',C-9', C-1'
8'	159.1	-	C	-
9'	114.1	-	C	-
10'	118.5	-	C	-
11'	20.5	1.97, s	CH ₃	C-6, C-8

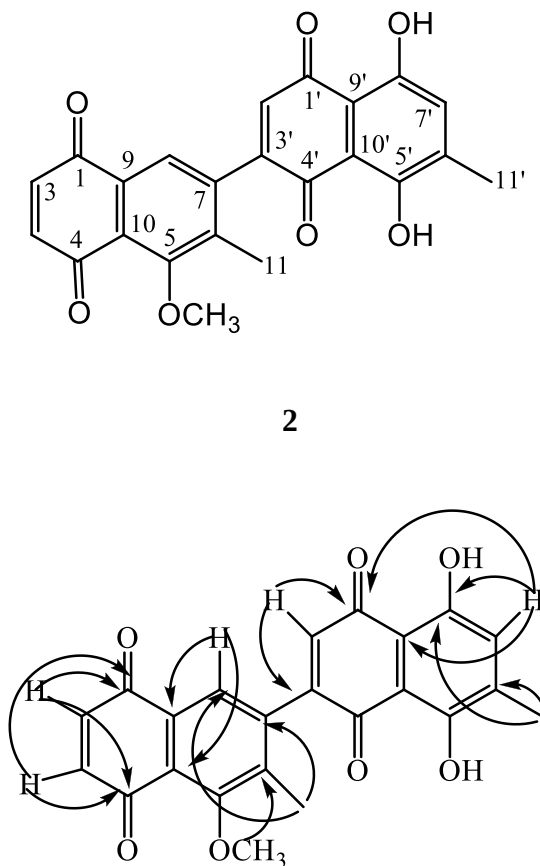


Figure 3: Key HBMC correlations of **2**

3.2.1.3. Di-naphthodiospyrol C (**3**)

Compound **3** was purified as yellow amorphous powder. The HRMS of **3** exhibited a molecular ions peak at m/z 390.0981 a.m.u (calcd. 390.0966) comprising to molecular formula $C_{22}H_{14}O_7$. The IR spectrum (cm^{-1}) displayed absorption bands at 3550 (OH stretching), 2988 (CH stretching), 1642 (C=O stretching) and 1602 (CH aromatic stretching). The UV spectrum exhibited absorption peaks at 250, 301 and 436 nm. The protons and carbons were assignment by HMBC, HMQC, ^1H - ^1H -COSY and J -resolved experiments (**Table 8**). The ^1H -NMR spectrum of **3** showed protons signals indicated the presences of two tertiary methyl groups resonated at δ_H 1.97 (s, H-11) and 2.01 (s, H-11');

three quinoid protons displayed at δ_H 6.66 (d, $J=10$ Hz, H-2), 6.89, (d, $J=10$ Hz, H-3) and δ_H 6.94 (s, H-5) while two aromatic protons singlet at δ_H 7.55 (H-2'), and δ_H 7.27 (H-7'). The ^{13}C -NMR (BB and DEPT) spectra (**Table 8**) exhibited 22 carbons resonance which were identified for two methyl carbons and 20 carbons for two naphthoquinone moieties. The quinoid proton signals (δ_H 6.66, H-2; 6.89, H-3; 7.55, H-2') showed HMBC (**Figure 4**) correlation with δ_C 190.2 (C-1), 195.3 (C-4), 137.6 (C-3'), and 190.1 (C-4') respectively while the aromatic protons signal (δ_H 6.94, H-5; 7.27, H-5) displayed correlation with δ_C 24.7 (C-11), 159.0 (C-8') and 112.0 (C-9') respectively. Based on this spectral data of **3** is characterized as 8,5',8'-trihydroxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone.

Table 8: ^{13}C and ^1H -NMR spectral data of **3**

No	δ_C	δ_H (mult; J , Hz)	Types	HMBC
1	190.2	-	C	-
2	140.0	6.66, d, ($J=10$ Hz)	CH	C-1, C-4
3	139.9	6.89, d, ($J=10$ Hz)	CH	C-1, C-4
4	195.3	-	C	-
5	121.3	6.94, s	CH	C-11
6	149.1	-	C	-
7	148.0	-	C	-
8	162.0	-	C	-
9	130.0	-	C	-
10	114.1	-	C	-
11-CH ₃	24.7	1.97, s	CH ₃	C-6
1'	184.8	-	C	-
2'	125.0	7.55, s	CH	C-3', C-4'
3'	137.6	-	C	-
4'	190.1	-	C	-
5'	161.9	-	C	-
6'	146.5	-	C	-
7'	125.9	7.27, s	CH	C-8', C-9'
8'	159.0	-	C	-
9'	112.0	-	C	-
10'	121.7	-	C	-
11'-CH ₃	20.7	2.01, s	CH ₃	C-4', C-5'

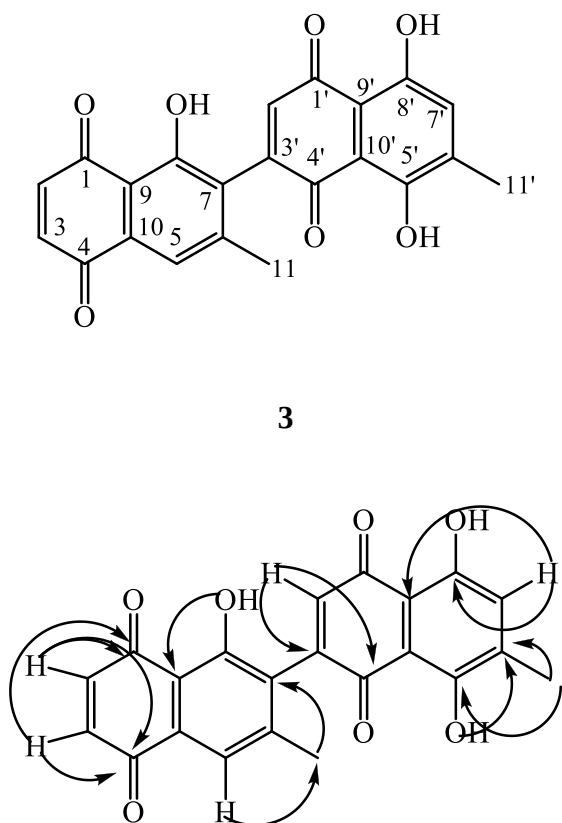


Figure 4: Key HMBC correlations of **3**

3.2.1.4. Di-naphthodiospyrol D (**4**)

Compound **4** was obtained as a red amorphous powder from the chloroform fraction of roots. Its HR-EI-MS exhibited a molecular ions peak at m/z 374.0780 a.m.u. (calcd. 374.0776; $C_{22}H_{14}O_6$) consistent with the molecular formula $C_{22}H_{14}O_6$. The IR spectrum (cm^{-1}) showed absorption bands at 3635 (OH stretching), 2930 (CH stretching 1655), (conjugated CO stretching), 1614, 1603 and 1462 (CH aromatic stretchings). The UV spectrum displayed absorptions at 256, 298 and 437 nm. The protons and carbons were assigned by using HMBC, HMQC, ^1H - ^1H -COSY and J -resolved experiments (**Table 9**). The

types of carbons were identified by ^{13}C -NMR (BB and DEPT). The ^1H -NMR spectrum of **4** revealed; two tertiary methyl groups at δ_{H} 2.01(s, H-11) and 2.03 (s, H11'); three quinoid protons signals at δ_{H} 6.70 (d, $J=10$ Hz, H-2), 6.90 (d, $J=10$ Hz, H-3) and 7.65 (s, H-2'), while three aromatic protons resonated at δ_{H} 7.00 (s, H-5), 7.28 (s, H-8) and δ_{H} 6.93 (s, H-7'). The ^{13}C -NMR (BB and DEPT) spectra (**Table 9**) exhibited 22 carbons signals: two carbons for methyl groups and 20 carbons for two naphthoquinone moieties. The quinoid protons (δ_{H} 6.70, H-2; 6.90, H-3; 7.65, H-2') showed strong HMBC correlation with δ_{C} 190.2 (C-1), 187.5 (C-4) and 161.9 (C-8') while the aromatic proton signal (δ_{H} 7.00, H-5; 7.28, H-8; 6.93, H-7') displayed correlation with δ_{C} 138.7 (C-2), 114.2 (C-9), 114.2 (C-9), 130.0 (C-10), 159.0 (C-5') and 112.0 (C-9'). All the substituents were positioned with the help of HMBC and HMQC correlations as shown in figures 5 and 6. Based on the spectral data of compound **4** is characterized as 5', 8'-dihydroxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone.

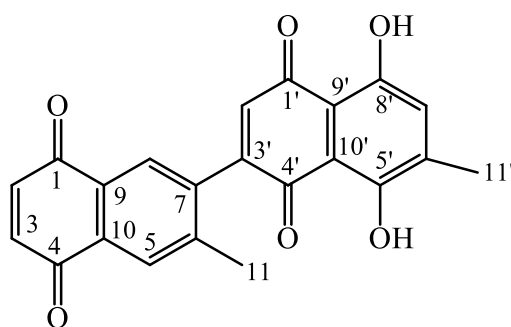
**4**

Table 9: ^{13}C and ^1H -NMR spectral data of **4**

No	δ_{C}	δ_{H} (mult; J , Hz)	Types	HMBC
1	190.2	-	C	-
2	138.7	6.70, d, ($J=10$ Hz)	CH	C-1
3	137.6	6.90, d, ($J=10$ Hz)	CH	C-4
4	187.5	-	C	-
5	121.5	7.00, s	CH	C-7, C-9
6	146.1	-	C	-
7	138.7	-	C	-
8	127.8	7.28, s	CH	C9- C-10
9	114.2	-	C	
10	130.0	-	C	
11	20.6	2.01, s	CH_3	C-6, C-7
1'	184.8	-	C	-
2'	140.0	7.65, s	CH	C-11
3'	148.0	-	C	-
4'	183.2	-	C	-
5'	159.1	-	C	-
6'	148.2	-	C	-
7'	125.7	6.93, s	CH	C-8'
8'	161.9	-	C	-
9'	112.9	-	C	-
10'	132.0	-	C	-
11'	20.4	2.03, s	CH_3	C-5', C-10'

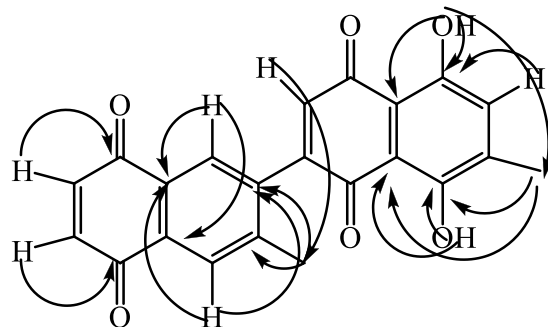


Figure 5: Key HMBC correlation of **4**

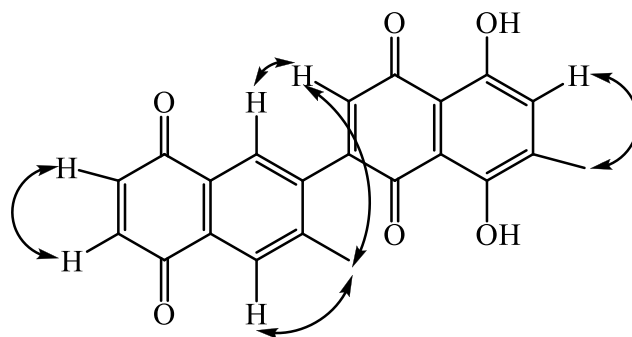
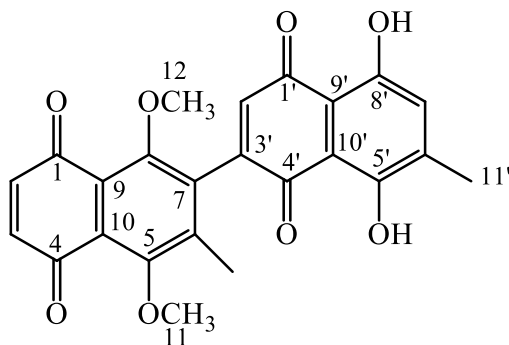


Figure 6: Key n.O.e correlations of **4**

3.2.1.5. Di-naphthodiospyrol E (5)

Compound **5** was isolated as red amorphous powder from CHCl₃ fraction of the roots. It exhibited a molecular ions peak at m/z 433.9998 a.m.u. in the HR-EI-MS (calcd. 434.0126) consistent with the molecular formula C₂₄H₁₈O₈. The IR spectrum (cm⁻¹) showed absorption peaks at 3630 (OH), 2933 (CH stretching) 1660 (conjugated CO stretching) and 1610, 1603, 1462 (CH aromatic stretching). The UV spectrum indicated absorptions at 250, 302 and 436 nm. The protons and carbons were assigning by HMBC, HMQC, ¹H-¹H-COSY and *J*-resolved experiments (**Table 10**). The multiplicity of carbons was identified by ¹³C-NMR (BB and DEPT) spectra. The ¹H-NMR spectrum of **5** revealed two tertiary methyl groups at δ_H 2 (s, H-11), 1.97 (s, H-11'); two tertiary methoxy groups δ_H 3.91 (s, 3H), 3.89 (s, 3H), three quinoid protons signals at δ_H 6.70, (d, *J*=10 Hz, H-2), 6.89 (d, *J*=10 Hz, H-3), 7.63 (s, H-2') and one aromatic proton δ_H 7.20 (s, H-7'). The ¹³C (BB and DEPT) spectra (**Table 10**) exhibited 24 carbons signals account for two methyl carbons, two methoxy carbons and 20 carbons for two naphthoquinone moieties. The quinoid proton displayed doublet (δ_H 6.89, H-2; 6.05, H-3; 7.63, H-2') which showed HMBC correlation with δ_C 190.2 (C-1) and δ_C 187.5 (C-4), δ_C 161.2 (C-8), 137.6 (C-3') and 145.2 (C-7). The aromatic proton signals (δ_H 7.20, H-7') exhibited correlation with δ_C 159 (C-8'), 114.6 (C-10) and 22.7 (C-11'). The assignments were placed with the help of HMBC and HMQC correlations as shown in figures 7 and 8. Based on the spectral data of **5** is characterized as 5',8'-dihydroxy-5,8-dimethoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone.



5

Table 10: ^{13}C and ^1H -NMR spectral data of 5

No	δ_{C}	δ_{H} (mult; $J = \text{Hz}$)	Types	HMBC
1	190.2	-	C	-
2	138.7	6.89, d, ($J=10.5$, Hz)	CH	C-1
3	137.6	6.05, d, ($J=10.5$, Hz)	CH	C-4
4	187.5	-	C	-
5	161.5	-	C	-
6	144.0	-	C	-
7	145.2	-	C	-
8	161.2	-	C	-
9	114.0	-	C	-
10	109.3	-	C	-
11 (CH ₃)	23.0	1.97, s	CH ₃	C-5, C-7
12 (OCH ₃)	56.6	3.91, s	CH ₃	-
13 (OCH ₃)	56.6	3.89, s	CH ₃	-
1'	184.8	-	C	-
2'	125.7	7.63, s	CH	C-1', C-3', C-6
3'	137.6	-	C	-
4'	190.1	-	C	-
5'	158.9	-	C	-
6'	135.5	-	C	-
7'	121.7	7.20, s	CH	C-8', C9', C11'
8'	159.0	-	C	-
9'	114.6	-	C	-
10'	112.3	-	C	-
11'	22.7	2.00, s	CH ₃	C-5'

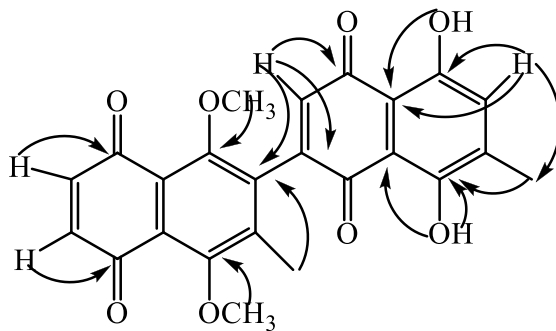


Figure 7: Key HMBC correlations of 5

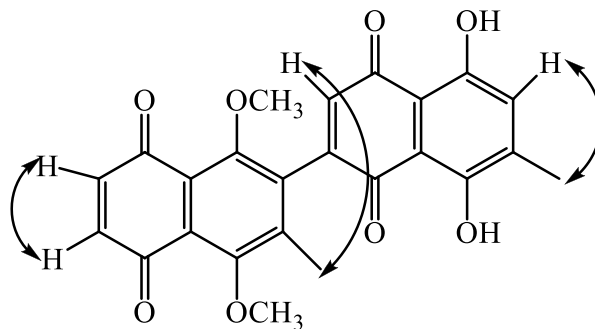
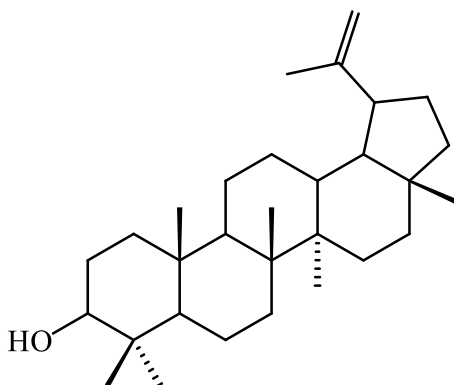


Figure 8: Key n.O.e correlations of 5

3.2.2.1. Lupeol (6)

Lupeol was obtained as a white powder from the chloroform fraction of *D. lotus*. It exhibited a molecular ions peak at m/z 426 a.m.u. which consistent with the molecular formula $C_{30}H_{50}O$. The IR spectrum (cm^{-1}) showed absorption bands at 3530 for (OH), 2932 for CH stretching 2871 and 1665 for double bond conjugated stretching. The UV spectrum displayed absorptions at 245 nm. The ^1H - and ^{13}C -NMR spectral data (**Table 11. vide experimental**) of **6** were found identical with lupeol reported in literature [101, 102] which is further conformed by HMBC correlations as shown in figure 9.



6

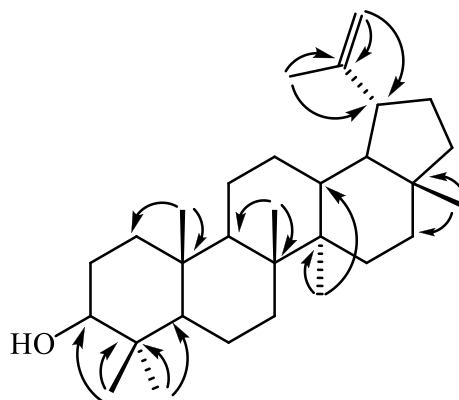


Figure 9: Key HMBC correlations of lupeol (**6**)

Table 11: ^{13}C and ^1H -NMR spectral data of lupeol (**6**)

No	δ_{C}	δ_{H} (mult, J , Hz)	Types	HMBC
1	38.6	1.69, dd, ($J=2.24$)	CH_2	
2	23.8	1.37, m	CH_2	
3	78.7	3.16, dd ($J=11.46, 4.8$)	CH	-
4	38.7	-	C	-
5	55.3	0.66	CH	
6	18.2	1.37, 1.49	CH_2	
7	34.2	1.37	CH_2	
8	40.6	-	C	-
9	50.5	1.24, 1.27, m	CH	
10	37.1	-	C	-
11	27.9	1.55, m; 1.59, m	CH_2	
12	25.2	1.66, m	CH_2	
13	38.3	0.89, m; 1.69, m	CH	-
14	42.5	-	C	-
15	30.9	1.55, d, ($J=4.41$)	CH_2	
16	35.6	1.37, m	CH_2	
17	43.0	-	C	-
18	48.4	1.32, m	CH	
19	48.0	2.38, m	CH	
20	150.9	-	C	-
21	29.8	1.23, s	CH_2	
22	40.0	1.15, s	CH_2	
23	28.0	0.98, s	CH_3	C-4, C-5
24	16.6	0.93, s	CH_3	C-4, C-5
25	16.2	1.02, s	CH_3	C-1, C-10
26	16.0	0.86, s	CH_3	C-8, C-9
27	14.6	0.92, s	CH_3	C-13, C-14
28	18.7	0.84, s	CH_3	C-16, C-17
29	109.3	4.70, brs, 4.58, brs	CH_2	C-19, C-20
30	19.3	1.67, s	CH_3	C-19, C-20

3.2.2.2. β -Sitosterol (7)

β -Sitosterol was obtained as white needle crystals from the chloroform fraction of *D. lotus*. The EI mass spectrum exhibited molecular ions peak at m/z 414 a.m.u which consistent with the molecular formula $C_{29}H_{50}O$. The IR spectrum (cm^{-1}) showed absorption bands at 3500 (OH), 2933, and 2991 for CH stretching, 1675 for double bond stretching. The UV spectrum showed absorptions peaks at 247, 261 and 440 nm. The spectral data (**Table 12** *vide experimental*) of **7** were similar to β -sitosterol [103] which was further conformed by HMBC correlations as shown in figure 10 [103].

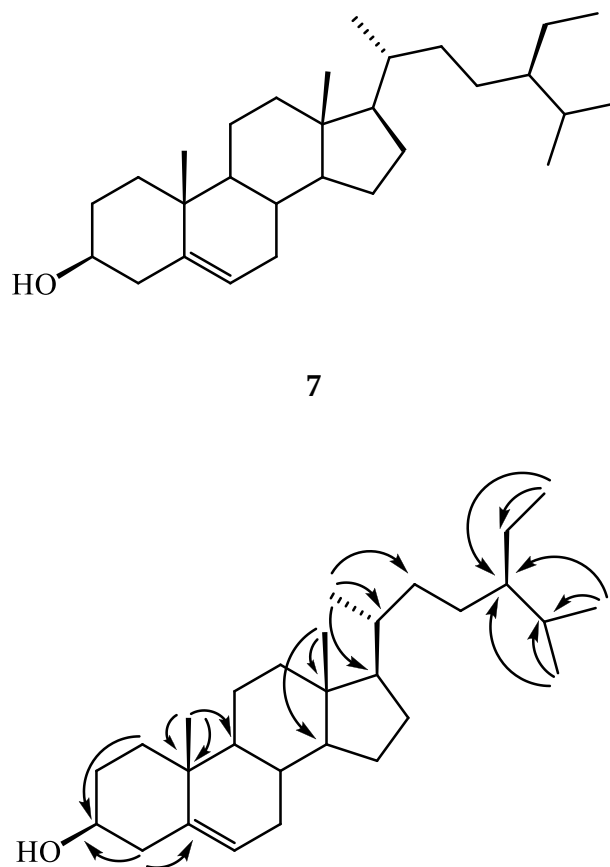


Figure 10: Key HMBC correlations of β -sitosterol (7)

Table 12: ^{13}C and ^1H -NMR spectral data of β -sitosterol (7)

No	δ_{C}	δ_{H} (mult, J , Hz)	Types	HMBC
1	37.3	1.07 dd, ($J=3.5, 6.11$)	CH_2	C-3, C-5, C-19
2	31.8	1.23, 1.48, m	CH_2	-
3	71.9	3.51, m	CH_2	-
4	42.3	2.00, t, ($J=9$); 1.99, (d, $J=3.12$)	CH_2	C-2, C-3, C-5, C-6, C-10
5	140.8	-	C	-
6	121.8	5.34, d, ($J=2.34$)	CH	C-4, C-8, C-10
7	32.2	1.44, m	CH_2	-
8	31.9	1.42, m	CH	-
9	50.5	0.84, m	CH	-
10	37.1	-	C	-
11	20.9	1.49, m, 1.47, m	CH_2	-
12	42.3	2.19, d, ($J=2.1$); 2.25, dd, ($J=2.1, 3$)	CH_2	-
13	39.8	-	C	-
14	56.8	0.96, m	CH	-
15	24.3	1.56, m	CH_2	-
16	28.3	1.23, m	CH_2	-
17	56.1	1.07, dd, ($J=7.8, 3.48$)	CH	-
18	11.9	0.66, s	CH_3	C-12, C-13, C-14, C-17
19	19.4	0.99, s	CH_3	C-1, C-9, C-10
20	36.2	1.34, m	CH	-
21	18.8	0.90, d, ($J=5.26$)	CH_3	C-17, C-20, C-22
22	34.0	1.27, m	CH_2	-
23	26.12	1.14, m	CH_2	-
24	45.90	0.92, m	CH	-
25	29.20	1.25, m	CH	-
26	19.11	0.79, d, ($J=6.8$)	CH_3	C-24, C-25, C-27
27	19.90	0.81, d, ($J=5.58$)	CH_3	C-24, C-25, C-26
28	23.12	1.22, m	CH_2	-
29	12.06	0.82, t, ($J=7.5$)	CH_3	C-24, C-28

3.2.2.3. Stigmasterol (8)

Stigmasterol was obtained as a white needle crystal from the chloroform fraction of *D. lotus*. It exhibited molecular ions peak at m/z 412 a.m.u. which consistent with the molecular formula $C_{29}H_{48}O$. The IR spectrum (cm^{-1}) showed absorption bands at 3500 (OH), 2965 and 2935 (CH stretching) 2870, 1680 (double bond stretching). The UV spectrum showed absorptions peaks at 242 and 261 nm. The ^1H - and ^{13}C -NMR spectral data (**Table 13. vide experimental**) of **8** were compared with stigmasterol [103] which is also supported by HMBC correlations (**Figure 11**).

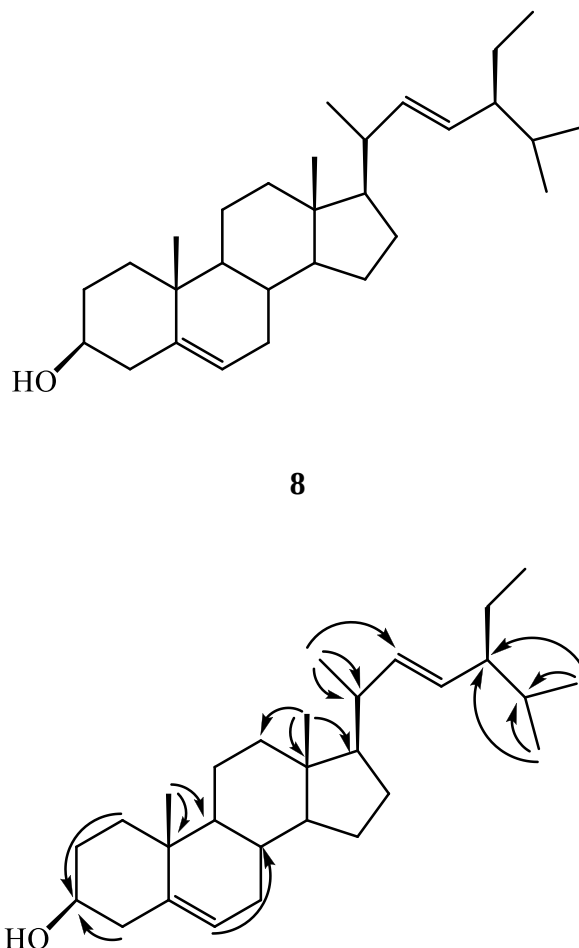


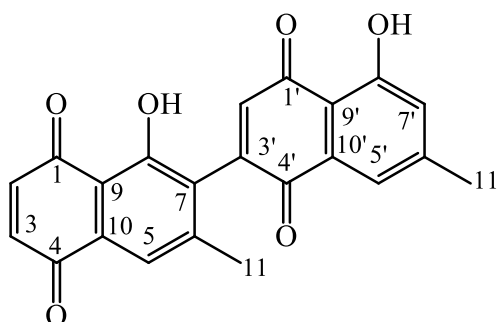
Figure 11: Key HMBC correlations for stigmasterol (**8**)

Table 13: ^{13}C - and ^1H -NMR spectral data of stigmasterol (**8**)

No	δ_{C}	δ_{H} (mult, J , Hz)	Type s	HMBC
1	37.3	1.07, dd, ($J=3.5$, 6.11)	CH_2	C-3, C-5, C-19
2	31.8	1.23, d, ($J=1.48$)	CH_2	-
3	71.9	3.51, m	CH_2	-
4	42.3	2.00, t, ($J=9$); 1.99, d, ($J=3.12$)	CH_2	C-2, C-3, C-5, C-6, C-10
5	140.8	-	C	-
6	121.8	5.34, d, ($J=2.34$)	CH	C-4, C-8, C-10
7	32.2	1.44, m	CH_2	-
8	31.9	1.42, m	CH	-
9	50.5	0.84, m	CH	-
10	37.1	-	C	-
11	20.9	1.49, m, 1.47 m	CH_2	-
12	42.3	2.19, d, ($J=2.1$); 2.25, dd, ($J=2.1$, 3)	CH_2	-
13	39.8	-	C	-
14	56.8	0.96, m	CH	-
15	24.3	1.56, m	CH_2	-
16	28.3	1.23, m	CH_2	-
17	56.1	1.07, dd, ($J=7.8$, 3.48)	CH	-
18	11.9	0.66, s	CH_3	C-12, C-13, C-14, C-17
19	19.4	0.99, s	CH_3	C-1, C-9, C-10
20	36.2	1.34, m	CH	-
21	21.4	1.20, d, ($J=7$)	CH_3	C-17, C-20, C-22
22	139.4	5.03, dd, ($J=15$, 8.5)	CH	-
23	129.6	1.14, dd, ($J=15.1$, 8.5)	CH	-
24	52.9	0.92, m	CH	-
25	30.2	1.25, m	CH	-
26	19.11	0.79, d, ($J=6.8$)	CH_3	C-24, C-25, C-27
27	19.90	0.81, d, ($J=5.58$)	CH_3	C-24, C-25, C-26
28	23.12	1.22, m	CH_2	-
29	12.06	0.82, t, ($J=7.5$)	CH_3	C-24, C-28

3.2.2.4. Diospyrin (9)

Diospyrin (**9**) was isolated as red crystalline solid from the chloroform fraction of *D. lotus*. The EI mass spectrum exhibited molecular ions peak at m/z 374 a.m.u. which consistent with the molecular formula $C_{22}H_{14}O_6$. The IR spectrum (cm^{-1}) showed absorption bands at 3660 for (OH), 2925, and 2916 for CH stretching, and 1672 for CO stretching, 1634, 1460 for aromatic CH stretching. The UV spectrum showed absorptions peaks at 249, 261 and 438 nm. The spectral data (**Table 14. *vide experimental***) of **9** were identical to data reported previously for diospyrin [104] which was further conformed by HMBC correlations (**Figure 12**).



9

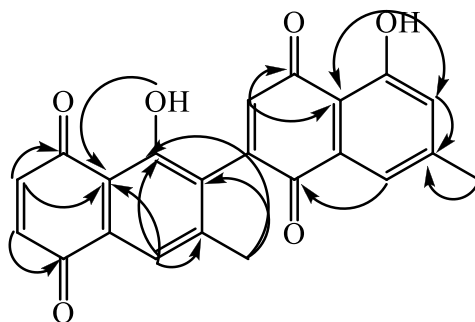


Figure 12: Key HMBC correlations of diospyrin (**9**)

Table 14: ^{13}C - and ^1H -NMR spectral data of diospyrin (**9**)

No	δ_{C}	δ_{H} (mult, J , Hz)	Types	HMBC
1	190.3	-	C	-
2	138.7	6.92, d, ($J=10.5$ Hz)	CH	C-1, C-9
3	137.6	6.70, d, ($J=10.5$ Hz)	CH	C-4
4	190.0	-	C	-
5	139.5	7.28, s	CH	C-6, C-8, C-9
6	130.2	-	C	-
7	128.5	-	C	-
8	158.5	-	C	-
9	114.1	-	C	-
10	135.1	-	C	-
11	20.4	1.99, s	CH_3	C-7, C-8
1'	184.4	-	C	-
2'	140.1	7.59, s	CH	C-1, C-9
3'	145.4	-	C	-
4'	184.8	-	C	-
5'	121.3	6.91, s	CH	
6'	148.1	-	C	-
7'	125.7	7.24, s	CH	C-6
8'	161.9	-	C	-
9'	114.1	-	C	-
10'	130.2	-	C	-
11'	20.6	1.51, s	CH_3	C-7'

3.2.2.5. 8-Hydroxyisodiospyrin (10)

8-Hydroxyisodiospyrin (**10**) was isolated as violet amorphous solid from the chloroform fraction of *D. lotus*. The EI mass displayed molecular ions peak at m/z 390 a.m.u. corresponding to molecular formula $C_{22}H_{14}O_7$. The IR spectrum (cm^{-1}) showed absorption bands at 3610 (OH), 2925, 2848 (CH stretching), and 1690 for (CO stretching), 1634, 1460 for (aromatic CH stretching). The UV spectrum showed absorption peaks at 231, 253 and 437 nm. The spectral data (**Table 15. *vide experimental***) of **10** were coincided well with hydroxydiospyrin [105, 106] which was further conformed by HMBC correlations (**Figure 13**).

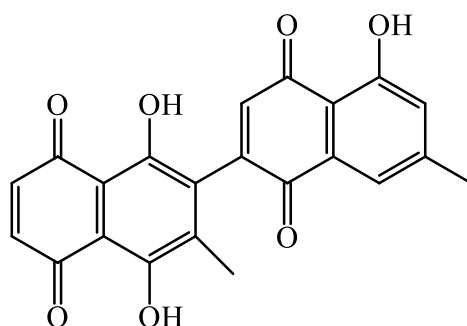
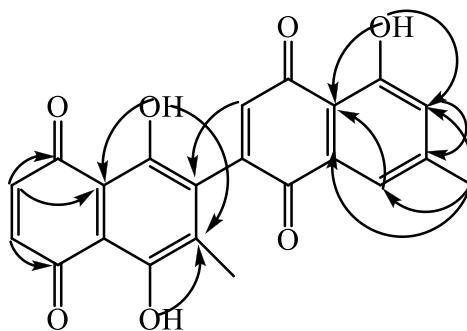
**10****Figure 13:** Key HMBC correlations of 8-hydroxydiospyrin (**10**)

Table 15: ^{13}C - and ^1H -NMR spectral data of 8-hydroxydiospyrin (**10**)

No	δ_{C}	δ_{H} (mult, J , Hz)	Types	HMBC
1	186.5	-	C	-
2	139.7	6.92, d, ($J=10$ Hz)	CH	C-1, C-9
3	137.9	6.75, d, ($J=10.5$ Hz)	CH	C-4
4	190.0	-	C	-
5	158.8	-	C	-
6	129.5	-	C	-
7	140.9	-	C	-
8	158.9	-	C	-
9	112.1	-	C	-
10	112.0	-	C	-
11	22.6	2.17, s	CH_3	C-7, C-8
1'	185.1	-	C	-
2'	125.7	7.26, s	CH	C-7
3'	143.0	-	C	-
4'	184.8	-	C	-
5'	127.6	7.28, s	CH	C-9
6'	158.2	-	C	-
7'	125.7	7.24, s	CH	C-6
8'	162.1	-	C	-
9'	114.0	-	C	-
10'	146.8	-	C	-
11'	20.5	1.83, s	CH_3	C-7'

3.2.2.6. 7-Methyljuglone (11)

7-Methyljuglone (**11**) was isolated as colorless crystals from the chloroform fraction of *D. lotus*. The EI MS exhibited molecular ions peak at m/z 188 a.m.u. which consistent with the molecular formula $C_{11}H_8O_3$. The IR spectrum (cm^{-1}) showed absorption bands at 3615 (OH), 2926, 2880 (CH stretching) and 1680 (CO stretching), 1600, 1412 (aromatic CH stretching). The UV spectrum showed absorption peaks at 220 nm. The spectral data of **11** (**Table 16. vide experimental**) were agreed well with those of reported data [107, 108] and conformed by HMBC correlations (**Figure 14**).

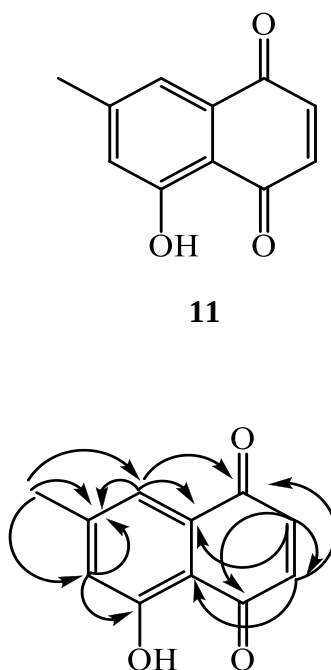


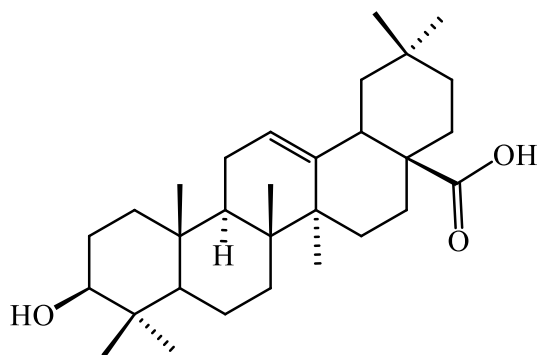
Figure 14: Key HMBC correlations of 7-methyljuglone (**11**)

Table 16: ^{13}C - and ^1H -NMR spectral data of 7-methyljuglone (**11**)

No	δ_{C}	δ_{H} (mult, J , HZ)	Types	HMBC
1	190.1	-	C	-
2	139.2	6.41, d, ($J=10$ Hz)	CH	C-1, C-3, C-9
3	137.3	6.41, d, ($J=10$ Hz)	CH	C-2, C-4, C-10
4	185.0	-	C	-
5	162.0	-	C	-
6	124.5	7.51, s	CH	C-5, C-7, C-8
7	148.9	-	C	
8	120.9	7.11, s	CH	C-6, C-7, C-9, C-1
9	131.8	-	C	-
10	113.3	-	C	-
11	22.6	2.41, s	CH_3	C-6, C-8, C-8

3.2.2.7. Oleanolic acid (12)

Oleanolic acid (**12**) was purified as white crystals from the chloroform fraction of *D. lotus*. The EI MS spectrum exhibited molecular ions peak at m/z 456 a.m.u. consistent with the molecular formula $C_{30}H_{48}O_3$. The IR spectrum (cm^{-1}) showed absorption bands at 3550 (OH), 2932, 2990 (CH stretching), and 1670 (CO stretching). The UV spectrum showed absorption peaks at 254, 264 and 441 nm. The spectral data (**Table 17. *vide experimental***) of **12** were agreed well with oleanolic acid [109] which was further conformed by HMBC correlations (**Figure 15**).



12

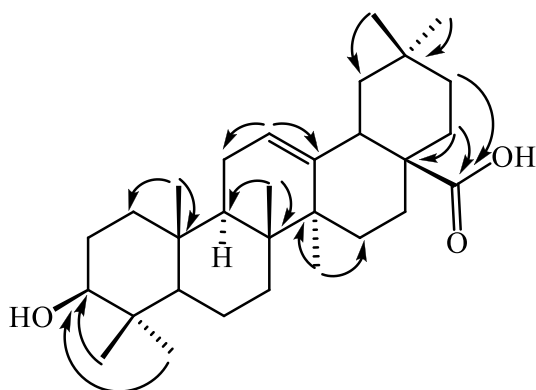


Figure 15: Key HMBC correlations of oleanolic acid (**12**)

Table 17: ^{13}C - and ^1H -NMR spectral data of oleanolic acid (**12**)

No	δ_{C}	δ_{H} (mult, J , Hz)	Types	HMBC
1	38.6		CH_2	
2	27.4		CH_2	
3	78.7	4.64, brs	CH	-
4	38.7	-	C	-
5	55.3		CH	
6	18.2		CH_2	
7	34.2		CH_2	
8	40.6	-	C	-
9	50.5		CH	
10	37.1	-	C	-
11	27.9		CH_2	
12	123.4	5.37, t, ($J=3.5$ Hz)	CH	
13	144.4		C	-
14	42.5	-	C	-
15	30.5		CH_2	
16	32.2		CH_2	
17	56.1	-	C	-
18	47.7	2.51, d, ($J=11$ Hz)	CH	
19	49.1	3.09, t, ($J=10.5$ Hz)	CH_2	C-21, C-22, C-28
20	150.6	-	C	-
21	37.0	1.35, s	CH_2	C-22, C-28
22	29.6	1.51, s	CH_2	C-28
23	27.9	0.87, s	CH_3	C-3, C-4
24	15.2	0.89, s	CH_3	C-3, C-5
25	15.8	0.86, s	CH_3	C-1, C-10
26	16.0	0.96, s	CH_3	C-8, C-9
27	14.6	0.94, s	CH_3	C-8, C-14
28	179.1	-	C	C-13, C-14, C-15
29	33.7	0.94, d, ($J=6.2$ Hz)	CH_3	C-19, C-20
30	24.2	1.03, s	CH_3	C-19, C-20

3.2.2.8. Ursolic acid (13)

Ursolic acid (**13**) was purified as white crystals from the chloroform fraction of *D. lotus*. The EI MS spectrum displayed molecular ions peak at m/z 456 a.m.u. which correspond with the molecular formula $C_{30}H_{48}O_3$. The IR spectrum (cm^{-1}) showed absorption bands at 3540 (OH), 2930, 2995 CH (stretching), and 1660 (CO stretching). The UV spectrum showed absorption peaks at 244, 264 and 441 nm. The spectral data (**Table 18. vide experimental**) of **13** were coincided well with oleanolic acid [109] which was further conformed by key HMBC correlations (**Figure 16**).

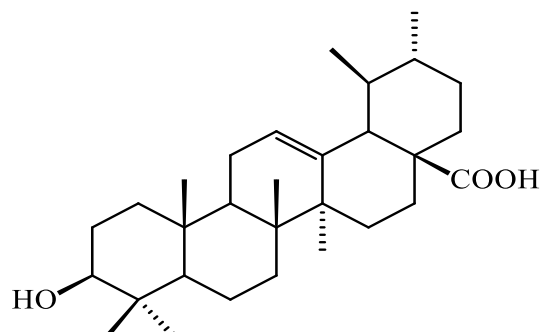
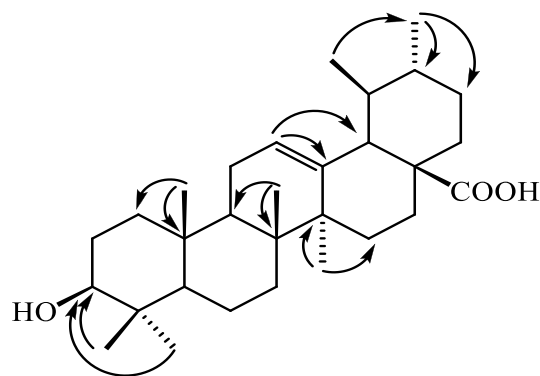
**13****Figure 16:** Key HMBC correlation of ursolic acid (**13**)

Table 18: ^{13}C - and ^1H -NMR spectral data for ursolic acid (**13**)

No	δ_{C}	δ_{H} (mult, J , Hz)	Types	HMBC
1	38.6		CH_2	
2	27.4		CH_2	
3	78.7	4.64, brs	CH	-
4	38.7	-	C	-
5	55.3		CH	
6	18.2		CH_2	
7	34.2		CH_2	
8	40.6	-	C	-
9	50.5		CH	
10	37.1	-	C	-
11	27.9		CH_2	
12	123.4	5.37, t, ($J=3.5$ Hz)	CH	
13	144.4		C	-
14	42.5	-	C	-
15	30.5		CH_2	
16	32.2		CH_2	
17	56.1	-	C	-
18	52.6	2.51, d, ($J=11$ Hz)	CH	
19	30.6	3.09 t, ($J=10.5$ Hz)	CH	C-21, C-22, C-28
20	30.4	-	CH	-
21	27.3	1.35, s	CH_2	C-22, C-28
22	28.4	1.51, s	CH_2	C-28
23	23.5	1.23, s	CH_3	C-3, C-4
24	15.2	1.02, s	CH_3	C-3, C-5
25	15.8	0.92, s	CH_3	C-1, C-10
26	16.0	1.04, s	CH_3	C-8, C-9
27	14.6	1.23, s	CH_3	C-8, C-14
28	179.1	-	C	C-13, C-14, C-15
29	21.0	0.96, s	CH_3	C-19, C-20
30	23.4	0.98, d ($J=3.66$)	CH_3	C-19, C-20

3.2.2.9. Betulinic acid (**14**)

Betulinic acid (**14**) was purified as white crystals from the chloroform fraction of *D. lotus*. The EI MS spectrum indicated molecular ions peak at m/z 456 a.m.u. which consistent with the molecular formula $C_{30}H_{48}O_3$. The IR spectrum (cm^{-1}) showed absorption bands at 3500 (OH), 2933, 2991 (CH stretching), and 1675 (CO stretching). The UV spectrum showed absorption peaks at 247 and 261 nm. The spectral data (**Table 19. *vide experimental***) of **14** were agreed well with reported one [109] which was further conformed by HMBC correlations (**Figure 17**).

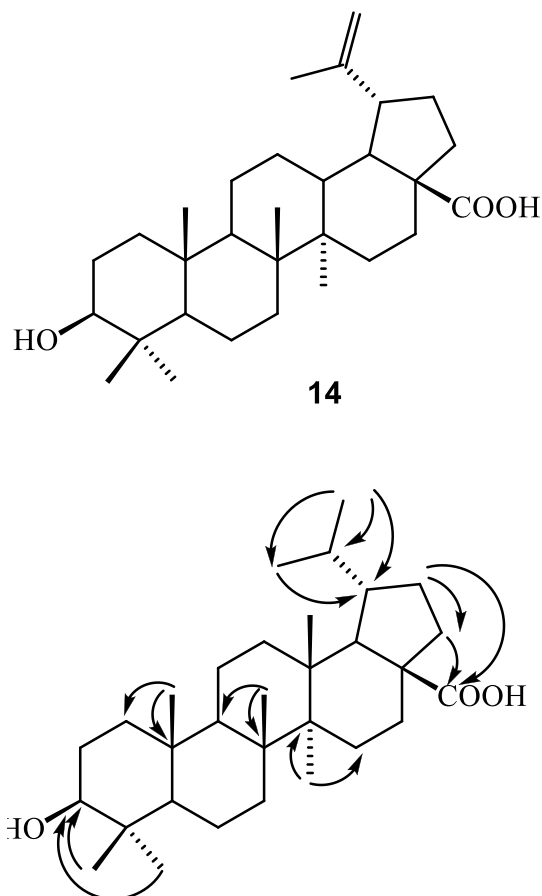


Figure 17: Key HMBC correlations of betulinic acid (**14**)

Table 19: ^{13}C - and ^1H -NMR spectral data of betulinic acid (**14**)

No.	δ_{C}	δ_{H} (mult, J , Hz)	Types	HMBC
1	38.6		CH_2	
2	27.4		CH_2	
3	78.7	4.64, brs	CH	-
4	38.7	-	C	-
5	55.3		CH	
6	18.2		CH_2	
7	34.2		CH_2	
8	40.6	-	C	-
9	50.5		CH	
10	37.1	-	C	-
11	20.8		CH_2	
12	25.4		CH_2	
13	38.2		CH	
14	42.3	-	C	-
15	30.5		CH_2	
16	32.2		CH_2	
17	56.1	-	C	-
18	47.7		CH	
19	49.1	3.09, t, ($J= 10.5$ Hz)	CH	C-21, C-22, C-28
20	150.6	-	C	-
21	37.0	1.35, s	CH_2	C-22, C-28
22	29.6	1.51, s	CH_2	C-28
23	27.9	0.87, s	CH_3	C-3, C-4
24	15.2	0.89, s	CH_3	C-3, C-5
25	15.8	0.86, s	CH_3	C-1, C-10
26	16.0	0.96, s	CH_3	C-8, C-9
27	14.6	0.94, s	CH_3	C-8, C-14
28	179.1	-	C	C-13, C-14, C-15
29	109.4	4.64, s	CH_2	C-19, C-20, C-30
30	19.2	1.61, s	CH_3	C-20

3.3. Chemical composition of fixed oil from *D. lotus*

The isolated fixed oil from the chloroform fraction of *D. lotus* was subjected to GC-MS analysis. The GC-MS analysis showed the chemical composition of fixed oil (**Table 20**), fatty acid methyl ester (**Table 21**) along with 2,3-dimethoxy naphthalene and *trans* squalane of *D. lotus* roots. The main constituents of fixed oil are linoleic acid-methyl ester (44.96%), linoleic acid-ethyl ester (17.85%), palmitic acid-methyl ester (10.82%), *trans* squalane (3%), 2,3-dimethoxy naphthalene (2.98%), oleic acid-methyl ester (2.97%), stearic acid-methyl ester (2.38%) and palmitic acid-methyl ester (2.20%). Rest of the components concentrations are less than 2% against the standard of mixture of 36 components (**Table 20-22**).

Table 20: Quantification results of fatty acid methyl ester of *D. lotus* roots

Peak No.	Name of components	Area	R. Time	Conc. (%)
1	C6:0; Hexanoic acid, methyl ester	8106	3.03	0.41
2	C8:0; Caprylic acid, methyl ester	1946	4.91	0.10
3	C10:0; Capric acid, methyl ester	5171	6.73	0.18
5	C12:0; Lauric acid, methyl ester	22866	8.46	0.31
7	C14:1c; Myristoleic acid methyl ester	62894	10.86	1.18
9	C15:0; Pentadecanoic acid, methyl ester	41415	12.50	0.96
10	C16:0; Palmitic acid, methyl ester	10626	14.48	10.82
12	C16:1c; Palmitoleic acid, methyl ester	7341	15.01	10.71
13	C17:0; Margaric acid, methyl ester	68303	16.27	1.63
14	C17:1;Hepadecenioc acid, methyl ester	9404	17.27	1.02
15	C18:0; Stearic acid, methyl ester	195839	19.42	2.38
16	C18:1c; Oleic acid, methyl ester	208392	19.95	14.79
18	C18:1n7; Octadecenoic acid, methyl ester	32131	20.19	0.11
19	C18:2c; Linoleic acid, methyl ester	109830	21.54	44.96
22	C18:3n3; Linolenic acid, ethyl ester	116610	24.06	17.85
23	C20:0; Arachidic acid, methyl ester	45641	26.94	1.13
27	C21:0; Heneicosanoic acid, methyl ester	22784	30.62	0.57
31	C22:0; Behenic acid, methyl ester	5675	34.57	0.13
34	C23:0;Tricosanoic acid ,methyl ester	11373	37.31	0.28
35	C24:0; Tetracosanoic acid, methyl ester	21115	40.39	0.48

Table 21: Quantification results of fatty acid methyl ester from *D. lotus*

Peak No.	Name of components	Area	R. Time	Conc. (%)
7	C14:1c; Myristoleic acid methyl ester	62891	1.86	0.08
9	C15:0; Pentadecanoic acid, methyl ester	41414	12.50	0.09
10	C16:0; Palmitic acid, methyl ester	10626	14.47	2.20
12	C16:1c; Palmitic acid, ethyl ester	7342	16.50	1.50
13	C17:0; Margaric acid, methyl ester	68301	16.76	0.19
15	C18:0; Stearic acid, methyl ester	19583	19.41	0.44
16	C18:1c; Oleic acid, methyl ester	20839	19.94	2.97
18	C18:1c; Oleic acid, ethyl ester	32132	19.99	2.00
19	C18:2c; Linoleic acid, methyl ester	109830	21.50	3.05
22	C18:3n3; Linolenic acid, ethyl ester	116613	24.06	0.88
23	2,3-dimethyl naphthalene	45645	26.95	2.89
27	<i>Trans</i> -squalane	22781	17.62	3.00

Table 22: Quantification results of 36 components internal standard

Peak No	Name of components	Area	R. Time	Conc. (%)
1	C6:0; Hexanoic acid, methyl ester	2637	3.028	40
2	C8:0; Caprylic acid, methyl ester	43548	4.911	40
3	C10:0; Capric acid, methyl ester	59804	6.743	40
4	C11:0; Undecanoic acid, methyl ester	32300	7.608	20
5	C12:0; Lauric acid, methyl ester	67697	8.493	40
6	C13:0; Tridecanoic acid, methyl ester	35734	9.554	20
7	C14:0; Myristic acid, methyl ester	72212	10.897	40
8	C14:1c; Myristoleic acid, methyl ester	8237	11.466	20
9	C15:0; Pentadecanoic acid, methyl ester	38432	12.548	20
10	C15:1; Pentdecanoic acid, methyl ester	7517	13.234	20
11	C16:0; Palmitic acid, methyl ester	118819	14.533	60
12	C16:1c; Palmitoleic acid, methyl ester	7253	15.066	20
13	C17:0; Margaric acid, methyl ester	34317	16.829	20
14	C17:1; Heptadecenoic acid, methyl ester	7643	17.417	20
15	C18:0; Stearic acid, methyl ester	67063	19.500	40
16	C18:1c; Oleic acid, methyl ester	17841	20.038	40
17	C18:1n9T; Elaidic acid, methyl ester	7232	20.115	20
18	C18:2T; Linoleic acid, methyl ester	8777	21.608	20
19	C18:2C; Octadecadionoic acid, methyl ester	9261	21.856	20
20	C18:3n6; G-linoleic acid, methyl ester	5708	22.762	20
21	C18:3n3; Linolenic acid, methyl ester	6455	24.160	20
22	C20:0; Arachidic acid, methyl ester	66297	27.058	40
23	C20:1; Eicosenoic acid, methyl ester	8757	27.659	20
24	C20:2; Eicosadienoic acid , methyl ester	6480	29.395	20
25	C20:3n6; 8, 11, 14-Eicosatrienoic acid, methyl ester	6128	30.312	20
26	C21:0; Heneicosanoic acid , methyl ester	30613	30.745	20
27	C20:4n6; Arachidonic acid , methyl ester	5846	31.073	20
28	C20:3n3; Eicosatrienoic acid, methyl ester	9586	31.717	20
29	C20:5N3; Eicosapentaenoic acid , methyl ester	6262	33.359	20
30	C22:0; Behenic acid, methyl ester	62867	34.188	40
31	C22:1; Eruccic acid, methyl ester	6847	34.753	20
32	C22:2; Locosadienoic acid, methyl ester	9365	36.323	20
33	C23:0; Tricosanoic acid, methyl ester	29002	37.440	20
34	C24:0; Tetracosanoic acid, methyl ester	60828	40.521	40
35	C22:6n3; Docosahexaenoic acid, methyl ester	5333	40.859	20
36	C24:1; Tetracosenoic acid, methyl ester	9144	41.098	20

The *n*-hexane fraction of *D. lotus* was also subjected to analysis through gas chromatography coupled to mass spectrometer (GC-MS). The results showed that oil comprised both saturated and unsaturated fatty acid (**Table 23**). Table 23 presents the results of GC-MS study indicated the relative concentration of separate fatty acid methyl ester (**FAMES**) which is based on external standard technique.

Table 23: Quantification results of fixed oil isolated from *D. lotus* roots

Peak No	Name of components	Area	R. Time	Conc. (%)
1	C12:0; Lauric acid, methyl ester	2878	8.265	2.21
2	C14:0; Myristic acid, methyl ester	1916	10.989	1.47
3	C16:0; Palmitic acid, methyl ester	75624	14.657	58.07
4	C18:0; Stearic acid, methyl ester	12414	19.670	9.53
5	C18:1c; Oleic acid, methyl ester	13481	20.216	10.35
6	C18:1n 9T; Elaidic acid, methyl ester	845	20.457	0.65
7	C18:2c; Linoleic acid, methyl ester	23066	21.822	17.71

3.4. Biological profile of *D. lotus*

3.4.1. *In vitro* screening

3.4.1.1. DPPH scavenging effect

The crude extract and its fractions were screened for DPPH scavenging properties against vitamin C (standard). The results obtained from the extract and its fractions are summarized in table 24 and figure 18 which exhibited significant quenching effect against DPPH in a concentration dependent manner (**Figure 18a**). The methanolic extract has maximum activity of 76.44% at 100 µg/ml ($IC_{50}=33.16$ µg/ml) while *n*-hexane fraction showed insignificant activity (**Figure 18b**). However, the chloroform and ethyl acetate fractions indicated significant anti-radical activity. The maximum results for both chloroform and ethyl acetate fractions are 97.33% and 85.22% (**Figure 18c and 18d**) with IC_{50} value of 9.34 and 18.90 µg/ml.

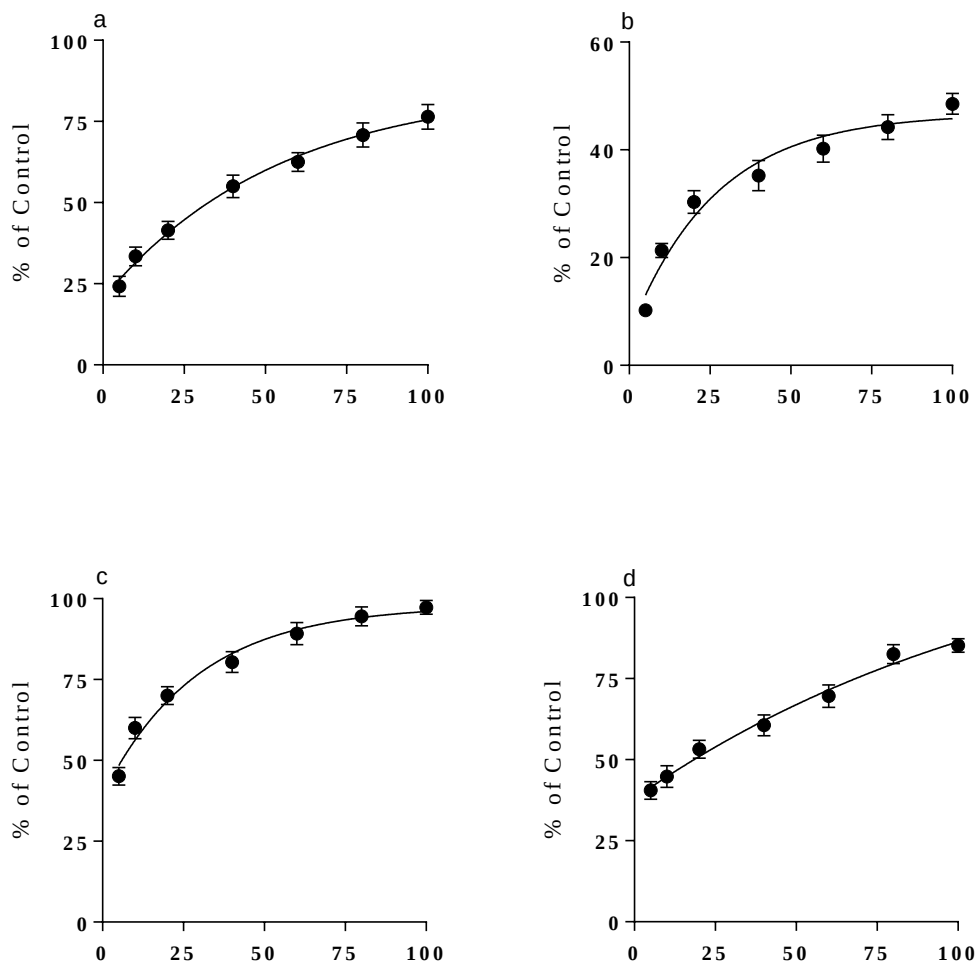


Figure 18: The effect of extract/fractions of *D. lotus* in DPPH free radical scavenging assay (**18a**) crude extract, (**18b**) hexane fraction, (**18c**) chloroform fraction, (**18d**) ethyl acetate fraction. Values are mean $\pm$ SEM of three assays

The isolated constituents were also screened for antioxidant potential against vitamin C. The effect of isolated compounds: lupeol (**6**), 7-methyl juglone (**11**), β -sitosterol (**7**), stigmasterol (**8**), betulinic acid (**14**), diospyrin (**9**) and 8-hydroxydiospyrin (**10**) against DPPH are shown in figure 19. The results revealed that only compounds diospyrin (**9**) and 8-hydroxydiospyrin (**10**) displayed significant quenching effect on free radical, while 7-methyljuglone (**11**) and

betulinic acid (**14**) showed mild effect in a concentration dependent manner (**Figure 19a and 19b**). Diospyrin (**9**) and 8-hydroxydiospyrin (**10**) provoked dose dependent action with maximum activity of 81.44% (IC_{50} 37.19 μ g/ml) and 92.22% (IC_{50} : 37.19 μ g/ml) at 100 μ g/ml (**Figure 19c-d**) respectively while rest of the compounds are inactive.

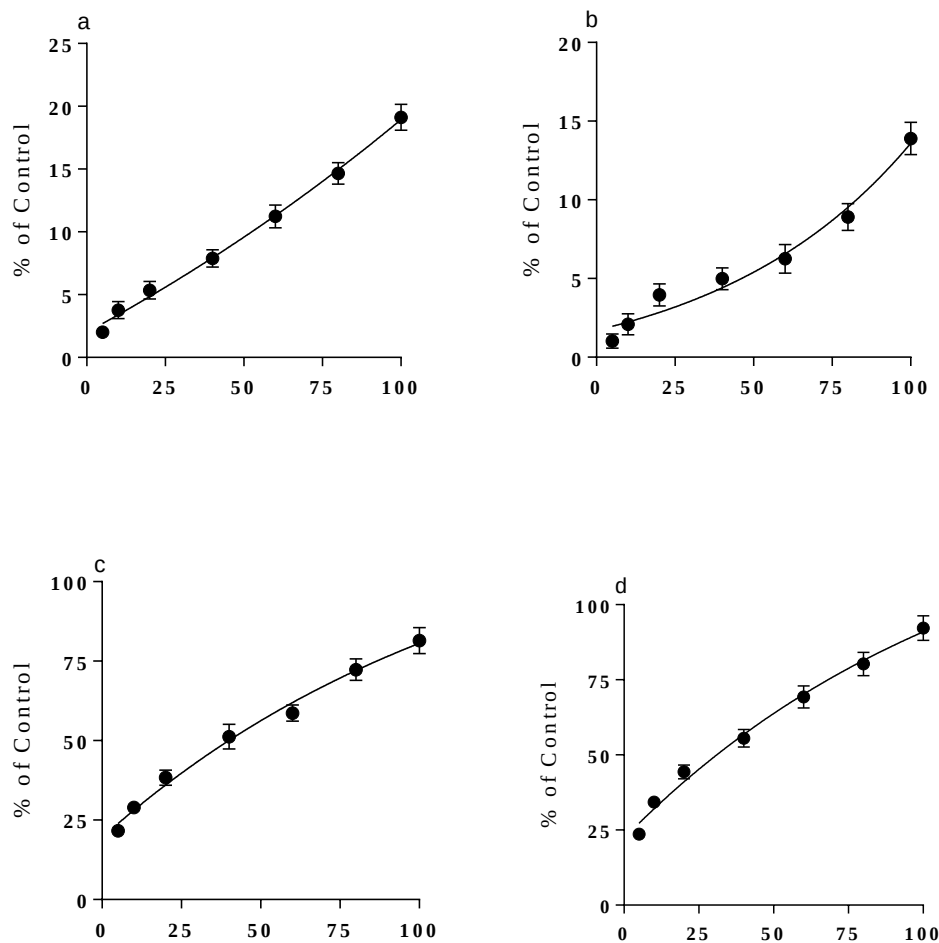


Figure 19: The effect of isolated compounds from *D. lotus* in DPPH free radical scavenging assay (**19a**) Compound **2**, (**19b**) compound **5**, (**19c**) compound **6**, and (**19d**) compound **7**. Values are mean $\pm$ SEM of three assays.

3.4.1.2. Antibacterial effects of extract and fractions

The crude extract of *D. lotus* and its fractions were assessed for antibacterial potential against selected bacterial strain (*B. subtilis*, *S. aureus*, *S. epidermis*, *E. coli* and *K. pneumonia*). The results showed that the plant is susceptible against various bacteria strain (**Table 24**). The crude extract showed antibacterial activity against *B. subtilis*, *S. aureus* and *S. epidermis* while chloroform fraction was the most active against selected Gram-positive bacteria. The ethyl acetate fraction was followed by the similar trend of sensitivity and found active with the exception of *E. coli*. However the *n*-hexane fraction was not significant activity.

Table 24: Antimicrobial effect (zone of inhibition in mm) of *D. lotus*

B. strain	Extrct	<i>n</i> -Hexane	Chloroform	Ethyl acetate	Streptomycin
<i>E. coli</i>	2±0.02	4±0.07	8±0.04	0	30±0.09
<i>K. pneumonia</i>	10±0.04	0	14±0.09	18±0.75	28±0.04
<i>S. epidermis</i>	12±0.20	10±0.25	14±0.50	14±0.99	28±0.92
<i>S. aureus</i>	8±0.09	0	16±0.08	12±0.79	28±0.75
<i>B. subtilis</i>	14±0.06	0	20±0.98	16±0.94	30±0.09

Note: Data are expressed as mean ± SEM of three sets of individual essays in each column. Well size (4 mm), test extract/fractions (2 mg/ml), streptomycin (2 mg/ml)

3.4.1.3. Antibacterial effects of isolated chemical constituents

The isolated constituents of *D. lotus* roots were also assessed for antibacterial properties against selected human pathogens (*B. subtilis*, *S. aureus*, *S. epidermis*, *E. coli* and *K. pneumonia*). Compounds; lupeol (**6**), 7-methyljuglone (**11**), β -sitosterol (**7**), stigmasterol (**8**), betulinic acid (**14**), diospyrin (**9**) and 8-hydroxydiospyrin (**10**) are shown in table 27. The results showed that betulinic acid (**14**) was active against most of the tested bacteria.

However, the rest of the compounds exhibited selectively activity against one or two bacteria (**Table 25**).

Table 25: Antimicrobial activity of compounds of *D. lotus*

Bacterial strain	6	7	8	9	14	10	11	STD
<i>E. coli</i>	0	0	0	0	0	0	0	30±0.09
<i>K. pneumonia</i>	0	0	0	12±0.65	16±0.56	0	0	28±0.04
<i>S. aureus</i>	0	0	0	14±0.92	10±0.08	0	0	28±0.92
<i>S. epidermis</i>	16±0.90	0	0	0	18±0.73	15±0.04	12±0.03	28±0.75
<i>B. subtilis</i>	0	0	0	0	18±0.98	0	0	30±0.09

Note: Data are expressed as mean ± SEM of three sets of individual essays in each column.
STD: Streptomycin (2mg/ml). Well size (4 mm), test drugs (2 mg/ml).

3.4.1.4. Lipoxygenase inhibitory activity and molecular docking of diospyrin

3.4.1.4.1. *In vitro* lipoxygenase inhibition assay of diospyrin

Diospyrin (**9**) was evaluated for lipoxygenase (LOX) inhibitory activity and the results showed significant lipoxygenase inhibitory potential (IC₅₀ value: 31.89±0.14 µmol) against standard drugs; baicalein (IC₅₀ value: 22.1±0.03µM) and tenidap sodium (41.6±0.02 µM).

3.4.1.5. Molecular docking simulations of diospyrin

Diospyrin (**9**) was also evaluated for molecular docking which revealed significant molecular interactions between **9** and LOX showing promising anti-inflammatory potential based on interaction with LOX (His 523, Lle 557 and Leo 770) inhibitors sub-sites. Molecular shape and electrostatic condition of **9** was also identified to be favorably matched with the electrostatic environment of active site inside LOX. Various molecular interactions were observed between the test ligand and LOX. Hydrogen bonding (**Figure 20**) was found between His 518 (2.55 °A) and carbonyl oxygen of quinine moiety. This interaction was further reinforced by hydrogen bonding between Asp 766 (2.79 °A) and phenolic group. However molecular shape of diospyrin (**9**) was incapable to access Ile857 and His 709. Apart from hydrogen bonding, electrostatic environment (**Figure 21**) of active site in LOX further supported the significant molecular interactions of **9** due its matching shapes and electrostatic behavior. Other interaction like π - π interactions between ligand and LOX inside the active site monomer (not bound *via* hydrogen bonding) of **9** which was deeply penetrated inside slightly less polar cavity surrounded by aggregated positive charges. Rotatable bond between both naphthoquinone dimmers provided flexibility to diospyrin (**9**) for penetrating the active with small pockets in directing in different directions.

The Molecular docking studies diospyrin (**9**) showed that compact skeleton of **9** holds strong contcts with the necassery amino acid side chains inside the active place and adjoining with important sites of enzyme therefore stopping its pro inflammatory action. Diospyrin (**9**) which exhibited strong molecular interactions resulted in discovery of a new potential compound **9** which targeting LOX in inflammation and related pathological conditions.

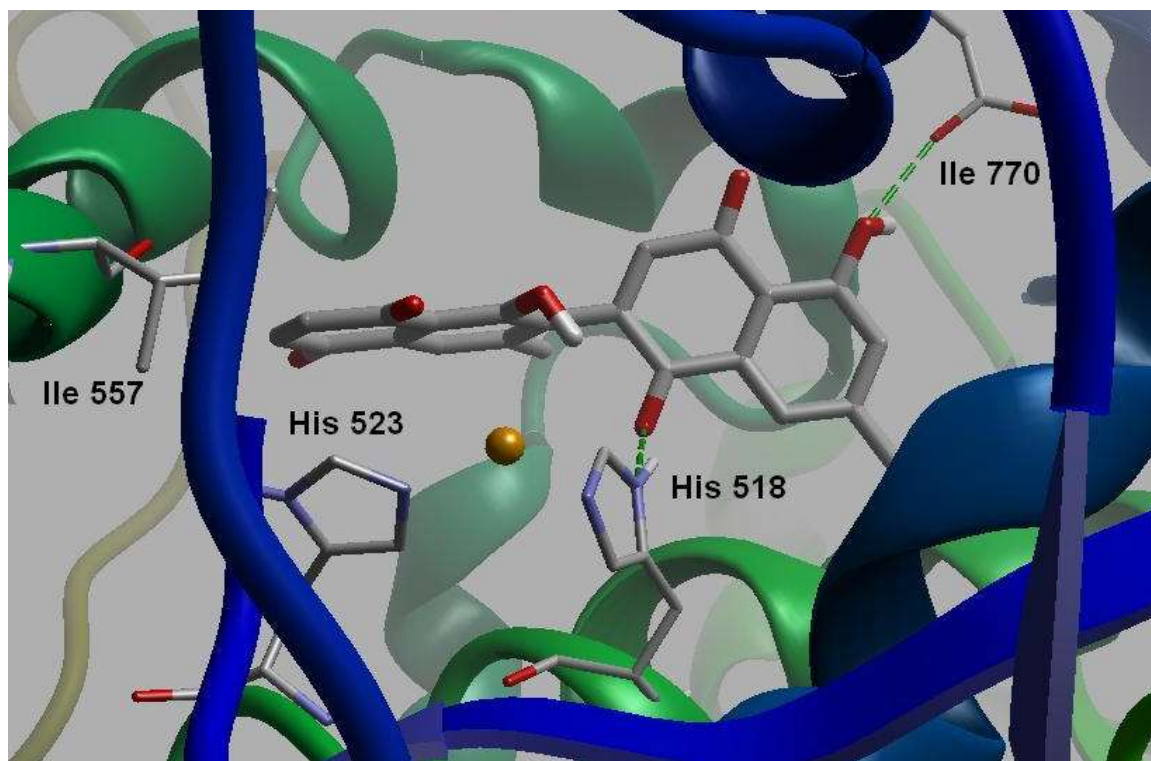


Figure 20: Binding mode of diospyrin (**9**) inside the active site of LOX. Orange colored showed (catalytic iron atom) which is among the His518 and Ile 770 residues. For clarity hydrogen atoms (except polar ones) were omitted.

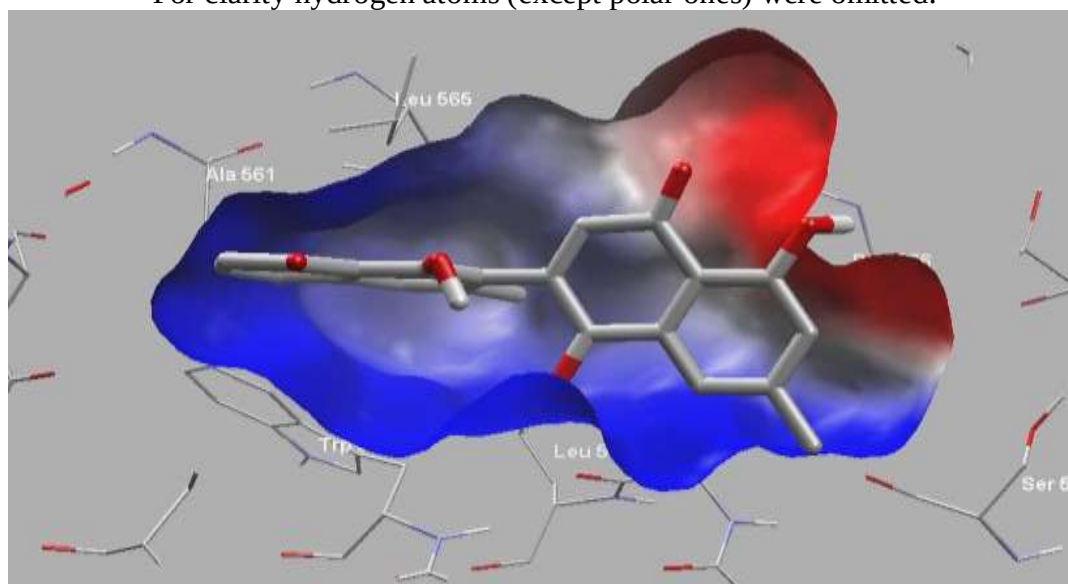


Figure 21: Electrostatic interactions of diospyrin (**9**) inside active site of LOX. Encoding of color (Red area: area with aggregated negative electrostatic potential; blue area: area with aggregated positive electrostatic potential and white area: hydrophobic region).

3.4.1. 6. Lipoxygenase inhibitory activity of 8-hydroxydiospyrin (10)

3.4.1.6.1. *In vitro* lipoxygenase inhibition assay

8-Hydroxydiospyrin (**10**) was isolated from the roots of *D. lotus* and was screened for lipoxygenase effect which showed promising inhibitory activity against lipoxygenase (IC_{50} values of 48.07 ± 0.06) along with standard compounds; baicalein (IC_{50} 22.1 ± 0.03 μM) and tenidap sodium (IC_{50} 41.6 ± 0.02 μM).

3.4.1.6.2. Molecular docking simulations of 8-hydroxydiospyrin (10)

8-Hydroxydiospyrin (**10**) was also screened for molecular docking simulations study which showed various significant interactions with important subsites especially His 513, His 518, Gln 514 and Asp 766, inside the catalytic pocket of LOX. The molecular shape and the electrostatic structure of the test ligand showed a close resemblance with the shape and electrostatic environment of the active site, which further supports the molecular interactions of 8-hydroxydisopyrin with LOX. Phenolic group was found to be responsible for hydrogen bonding (**Figure 22**) with two important amino acids His 518 ($3.52^\circ A$) and Gln 514 ($2.78^\circ A$). This interaction was further reinforced by hydrogen bonding between carbonyl oxygen of quinine moiety and His 513 ($2.37^\circ A$) and that of phenolic group with Asp 766 ($2.96^\circ A$) (**Figure 22**). However, 8-hydroxydiospyrin (**10**) was incapable to access Leu 773 and His 523 (**Figure 23**) instead of parent compound **9** (**Figure 21**). Other interactions like π - π interactions between ligand and LOX inside the active sites were also observed, such as a monomer of 8-hydroxydiospyrin which was deeply penetrated inside slightly less polar cavity surrounded by aggregated positive charges. Rotatable bond between the two naphthoquinone units comforts flexibility to the ligand molecules which aids in penetration, along different directions, the small pockets of the active sites which revealed favorable

interactions based on molecular structure, shape and electrostatic behavior, 8-hydroxydiospyrin emerges as a potential new lead compound having the capability of relieving inflammation and other pathological conditions by targeting LOX.

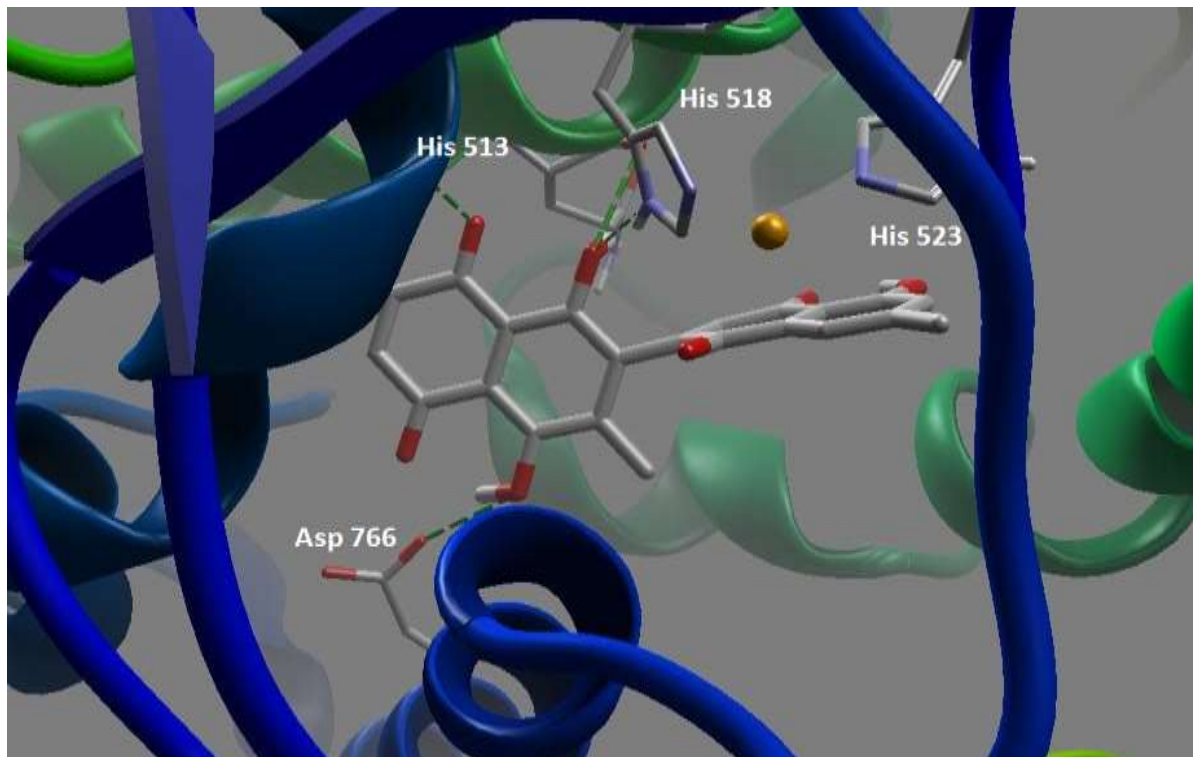


Figure 22: Binding mode of 8-hydroxydiospyrin (**10**) inside the active site of LOX. Orange colored showed (catalytic iron atom). For clarity hydrogen atoms (except polar ones) were omitted.

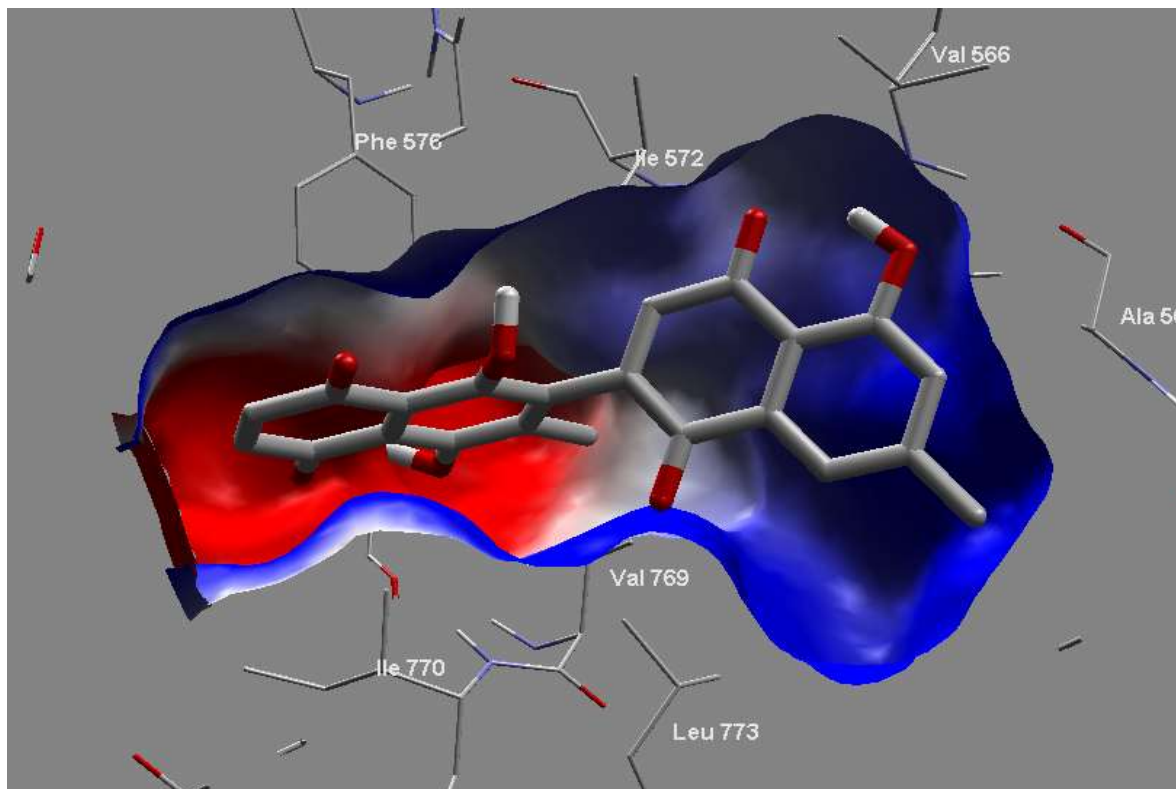


Figure 23: Electrostatic interactions of 8-hydroxydiospyrin (**10**) inside the active site of LOX. Encoding of color (Red area: area with aggregated negative electrostatic potential; blue area: area with aggregated positive electrostatic potential and white area: hydrophobic region).

3.4.8. *In vitro* anticancer activity

3.4.8.1. Antiproliferative activity

The three new dimeric naphthoquinones (**1-3**) of *D. lotus* roots was assessed for antiproliferative effect. The compounds (**1-3**) were first screened for antiproliferative activity on human ABCB1 gene transfected mouse lymphoma cell line (L5178Y) which specifically overexpression of a membrane localized transporter (P-glycoprotein; ABCB1). The antiproliferative effects of compounds (**1-3**) of increasing concentration on compounds (**1-3**) on cell growth were determined by MTT method. The compounds; **1** and **2** showed promising antiproliferative potential with IC₅₀ 0.05 and 0.05 as compare to **3** with IC₅₀ value 0.3 at the same concentration (**Table 26**).

Table 26: Antiproliferative effect of compounds (**1-3**) on the L5178 mouse T-cell lymphoma cell line 2014.03.25.

Compounds no	Concentration (µg/ml)	IC ₅₀ (reading 1-3)	IC ₅₀ mean
1	(1 µg/ml)	0.045	
1	(1 µg/ml)	0.053	
1	(1 µg/ml)	0.054	0.05
2	(1 µg/ml)	0.05	
2	(1 µg/ml)	0.05	
2	(1 µg/ml)	0.04	0.05
3	(1 µg/ml)	0.26	
3	(1 µg/ml)	0.26	0. 26
3	(1 µg/ml)	0.26	

3.4.8.2. MDR reversal effect in mouse lymphoma cells

Dimeric naphthoquinones (**1-3**) were also investigated for their potential properties as multi-drug resistance (MDR) efflux pump modulators in which ABCB1 gene transfected mouse lymphoma cells line and measured intracellular accumulation of rhodamine 123 which is analogue of epirubicine (fluorescent substrate). The fluorescence activity ratio (FAR) value was used to assess the ABCB1 transporter modulating potential (**Table 27**). The values of SSC (side scatter count) and FSC (forward scatter count) were increased in the flow cytometry which showed that the compounds had membrane effect and the granulation of cytoplasm was increased. The results showed a toxic effect on the reversal of multidrug resistance at 1 and $\mu\text{g/ml}$ doses. The FAR values indicated compound **1** was effective MDR modulator while compounds **2** and **3** had no significant effect in a short time experiment (**Table 29**).

Table 27: Effect of compounds (1-3) on reversal of multidrug resistance on MDR in mouse lymphoma cells

S.No.	Samples	Concentrations (µg/ml)	FSC (Forward Scatter Count)	SSC (Side Scatter Count)	Mean fluorescence	FAR (Fluorescence Activity Ratio)	Maximum of fluorescence intensity
1	PAR	-	1880	528	95.3	-	93.1
2	PAR	-	1871	457	79.5	-	77.7
3	MDR	-	1963	628	1.08	-	0.777
	MDR MEAN	-	2045	624,5	0.95	-	-
4	Verapamil	10	1993	613	10	10.5	12
5	1	0.1	2003	606	1.63	1.72	0.673
6	1	1	1982	619	10.1	10.63	17.2
7	2	0.1	2008	609	1.05	1.1	0.806
8	2	1	2024	582	1.24	1.3	0.698
9	3	0.1	2029	606	1.1	1.15	0.806
10	3	1	2013	636	0.801	0.84	0.673
11	DMSO	10 µl	2108	604	0.782	0.82	0.604
12	MDR	-	2127	621	0.816	-	0.723

3.4.9. Enzyme inhibition activities of *D. lotus*

The crude extract and isolated compounds were subjected to various enzyme inhibition activities. The compounds isolated from higher plants have extensive used in the treatment of many diseases such as cancer, stomach disorder and many others. Natural compounds provide a good pharmacophore template for discovery of new drugs. The bio-assay guided isolation of compounds (**1-9**) was performed. In the preliminary step; crude fractions (EtOAc, CHCl₃ and MeOH) were evaluated for *in vitro* enzymes inhibitory assay against urease, phosphodiesterase-I, carbonic anhydrase-II, and α -chymotrypsin. As a result the chloroform and methanol were found significantly active against urease enzyme inhibition assay with IC₅₀ values of 152.2 ± 1.21 and 163.1 ± 2.5 μ M respectively while EtOAc, chloroform and methanol fractions were not active against phosphodiesterase-I, carbonic anhydrase-II, and α -chymotrypsin which showed selectivity towards the urease enzyme (**Table 28**). The chloroform fraction was most active against urease enzyme hence it was further subjected to column chromatography and isolated nine constituents (**1-9**) which showed a varying degree of inhibition against urease enzyme (**Table 29**). Compounds **1** and **2** were significantly active against urease enzyme having IC₅₀ values 260.4 ± 6.37 and 381.4 ± 4.8 μ M respectively, while compounds **3-9** showed less than 50 % inhibition (**Table 29**). Compounds (**1-10**) did not show significant inhibition of phosphodiesterase-I, carbonic anhydrase-II, and α -chymotrypsin. The activity of these compounds (**1-2**) may be due the keto moiety at C-4 along with hydroxyl groups at C-5, which may be chelate to Ni in the active site of urease enzyme. Compound **1** was the most active compound with IC₅₀ values of 260.4 ± 6.37 μ M, which may be due to the di-keto moieties in the other ring and make additional hydrogen bonding at amino acids of active site of urease. Compound **2** with IC₅₀

values of $381.4 \pm 4.8 \mu\text{M}$ has two-hydroxyl groups and ketone moieties which indicated more active.

Table 28: Enzymes inhibitory activities of *D. lotus*

Fractions	Urease		Phosp-I	Carb –II	α -Chym
	% Inhib (0.2 mg/mL)	IC ₅₀ ±S.E.M. (0.2 $\mu\text{g/mL}$)	% Inhib (0.2 mg/mL)	% Inhib (0.2 mg/mL)	% Inhib (0.2 mg/mL)
EtOAc	22.1	NA	12.9	17.8	29.6
CHCl ₃	70.7	152.2±1.21	11.1	21.9	41.3
MeOH	57.6	163.1±2.5	34.8	25.7	39.3
Standard	<u>Thiourea</u> 98.2	<u>Thiourea</u> 21 ±0.11	<u>EDTA</u> 80.1	<u>Acetazolamide</u> 89	<u>Chymostatin</u> 98.6

Note: S. E. M. = Standard error mean, NA = Not active, Phosp-I =Phosphodiesterase-I, Carb –II, Carbonic anhydrase-II, α -Chym= α -Chymotrypsin, % Inhib= % Inhibition

Table 29: Enzymes inhibitory activities of compounds (1-10)

Compounds	Urease		Phosp-I	Carb-II	α -Chym
	% Inhib (0.5 mM)	IC ₅₀ ± S.E.M. (0.5 μM)	% Inhib (0.5 mM)	% Inhib (0.5 mM)	% Inhib (0.5 mM)
1	68.9	260.4 ± 6.37	34.3	11.8	45.2
2	67.7	381.4±4.8	20.5	18.43	14.9
3	36.3	NA	17.2	23.2	18.3
4	44.2	NA	12.1	27.6	14.9
5	17.3	NA	16.2	21.7	12.7
6	2.34	NA	11.3	11.1	2.4
7	4.11	NA	14.8	12.1	11.5
8	2.21	NA	10.5	9.32	10.4
9	31.9	NA	15.2	13.4	13.4
10	40.6	NA	13.1	17.7	2.2
Standard	<u>Thiourea</u> 98.2	<u>Thiourea</u> 21 ±0.11	<u>EDTA</u> 80.1	<u>Acetazolamide</u> 89	<u>Chymostatin</u> 98.6

Note: S. E. M. = Standard error mean, NA = Not active, Phosp-I =Phosphodiesterase-I, Carb –II, Carbonic anhydrase-II, α -Chym= α -Chymotrypsin, % Inhib= % Inhibition

3.5. *In vivo* screening

3.5.1. Antipyretic effect of the crude extract and its fractions

The crude extract and various solvent extracted fractions were subjected for antipyretic properties against paracetamol (acetaminophen) under the yeast induced pyrexia test. The results are presented in figure 24 (A-D). The crude extract strongly ameliorated the induced pyrexia with during various assessment times. The maximum antipyretic effect (60.33%) was observed after 5th of pretreatment of extract at 100 mg/kg i.p (**Figure 24; A**). Upon fractionation, activity differences were noted; *n*-hexane and *n*-butanol did not produce significant effect while chloroform fraction demonstrated outstanding action with maximum reversal of 69.28% after 5th h of drug administration at 100 mg/kg i.p. (**Figure 24; B**). The ethyl acetate fraction was found most active. It had maximum of 72.27% pyrexia control at 100 mg/kg i.p. after 5th h of drug injection (**Figure 24; C**).

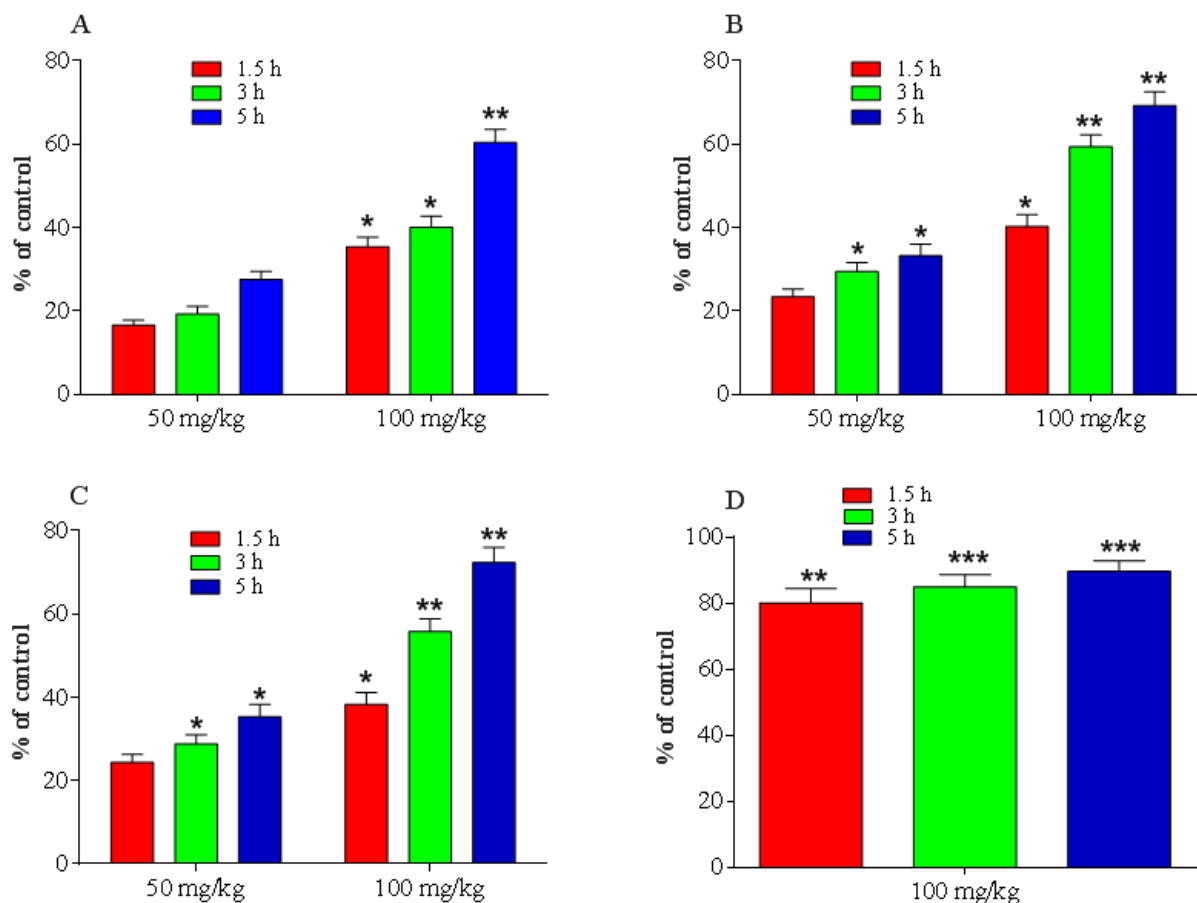


Figure 24: Percent effect of yeast induced pyrexia in hyperthermic mice of extracts. (at 50 and 100 mg/kg) i.p. during various assessment times. Crude extract [A], chloroform fraction [B], ethyl acetate [C] and standard drug, paracetamol [D]. Data are reported as mean $\pm$ SEM (n=6). The data were analyzed by ANOVA followed by Dunnett's test. $P < 0.5$ was considered as statistically significant from control.

3.5.2. Acetic acid induced writhing effect of the crude extract and its fractions

The crude extract and fractions were subjected for analgesic properties against diclofenac sodium. The effects of acetic acid induced writhing test of and various fractions of *D. lotus* in mice are shown in below (**Figure 25; A-F**). The crude extract significantly antagonized

the painful sensation in form of abdominal constriction produced by acetic acid injection up to 61.09% at 100 mg/kg i.p (**Figure 25; A**). The *n*-hexane fraction of *D. lotus* was inactive (**Figure 25; B**) while chloroform fraction exhibited marked anti-nociceptive effect (68.87%) at 100 mg/kg i.p. (**Figure 25; C**). The ethyl acetate fraction most dominantly inhibited (75.89%) noxious stimulation at 100 mg/kg i.p.(**Figure 25; D**) while *n*-butanol fraction; pain inhibition was 55.23% at 100 mg/kg i.p.(**Figure; E**). However, diclofenac was outstanding in the reversal of pain with 84.44% protection at 10 mg/kg i.p. (**Figure 25; F**).

The extract and fractions of *D. lotus* showed marked reduction in the abdominal constriction provoked by the acetic acid in a dose dependent manner.

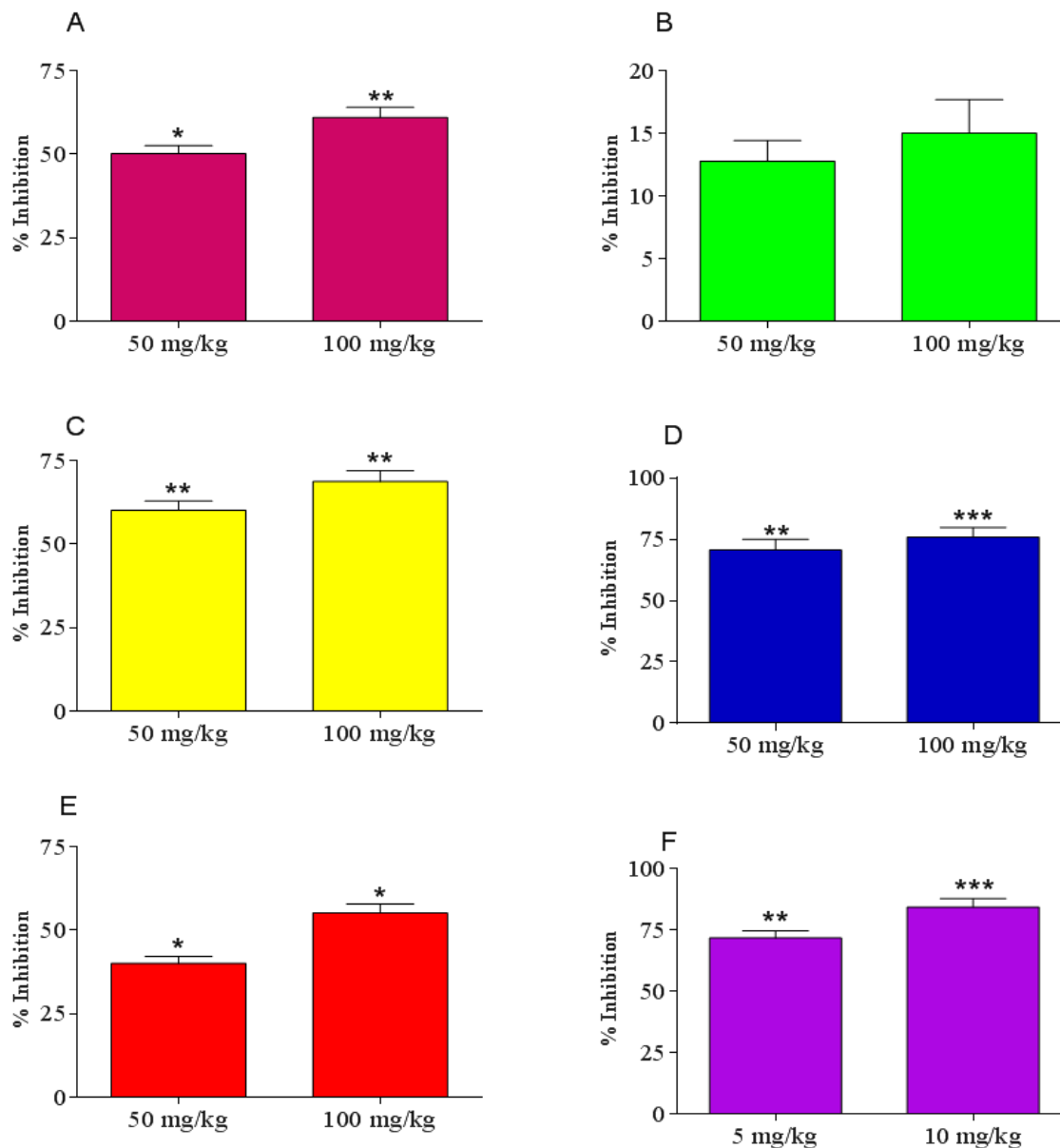


Figure 25: Percent protection against noxious stimulation induced by acetic acid of extracts at 50 and 100 mg/kg i.p. during various assessment times. Crude extract [A], chloroform fraction [B], ethyl acetate [C] and standard drug, paracetamol [D]. Data are reported as mean $\pm$ SEM (n=6). The data were analyzed by ANOVA followed by Dunnett's test. $P < 0.5$ was considered as statistically significant from control.

3.5.3. Locomotive effect of *D. lotus*

The crude extract and subsequent solvent extracted fractions were subjected for locomotive properties against diazepam in mice at dosage of 50 and 100 mg/kg i.p. (**Figure 26**). The methanolic extract and chloroform fraction showed significant activity with 40.43%, 45.98%, 80.01% and 82.67% sedative effects respectively (**Figure 26; A & B**) while *n*-hexane fraction was inactive. The ethyl acetate fraction also showed significant effect with 48.09% and 55.65% activity at 50 and 100 mg/kg i.p. respectively (**Figure 26; C**). 33.98% (50 mg/kg) and 40.87% (100 mg/kg) sedative effect was observed in *n*-butanolic fraction (**Figure 26; D**). However, standard drug exhibited most dominant effect (**Figure 26; E**). Pretreatment of mice with extract and its fractions showed dose dependent reduction in locomotive activity in open field test as compared to control. The reduction in the frequency and amplitude of motion could be attributed to the sedative effect of *D. lotus*. The resulting sedative effect of extract and its fractions of the *D. lotus* were similar to standard drug

(diazepam).

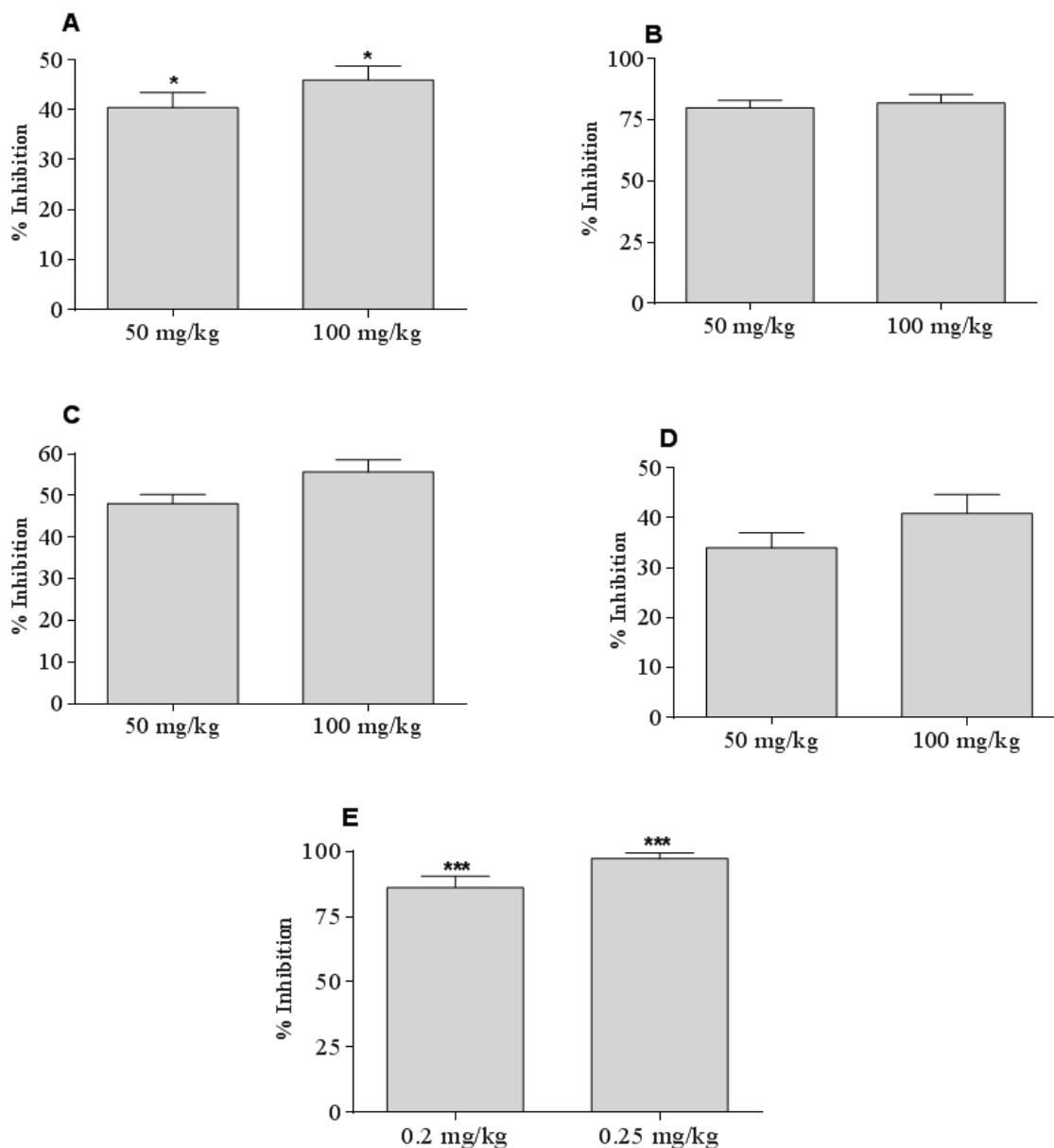


Figure 26: Percent locomotive test effect of crude extract and fractions.

[A] crude extract, [B] chloroform fraction, [C] ethyl acetate fraction [D] butanol and [E] diazepam in locomotive test. Values represent the percent sedative effect. Data presented as mean $\pm$ S.E.M, (n=6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, all compared with control.

3.5.4. Muscle relaxant activity of *D. lotus*

The crude extract of *D. lotus* and its fractions were subjected for muscle relaxant activity in rota rod model. The chloroform fraction provoked significant ($P<0.05$) muscle relaxant effect after 60 and 90 min of drug administration at both doses of 50 and 100 mg/kg i.p. (Figure 27) while ethyl acetate fraction was more effective in its muscle relaxant effect and caused significant effect after 30 min of drug administration at both test doses of 50 and 100 mg/kg i.p. (Figure 27).

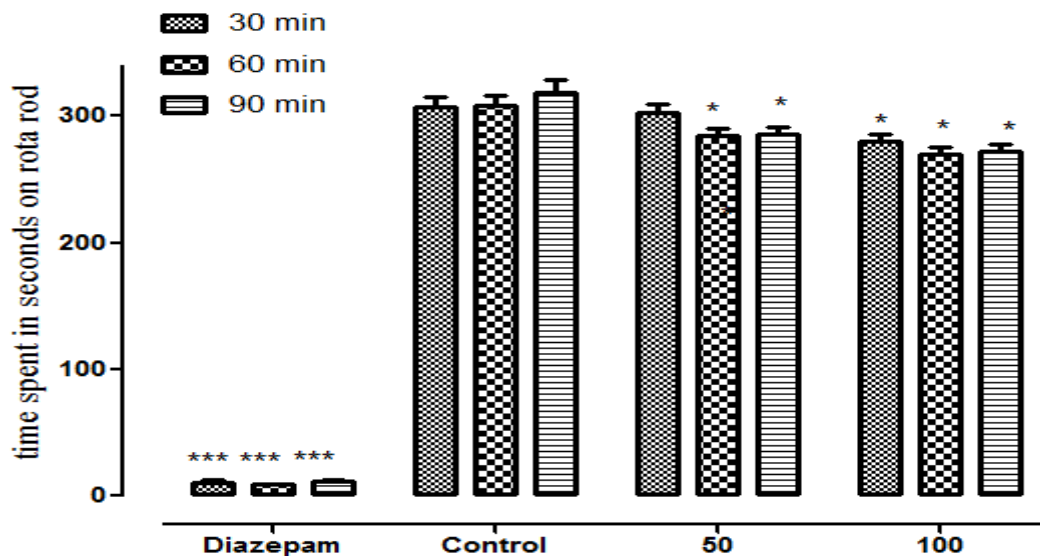


Figure 27: Effect of chloroform fraction on muscle coordination in rota rod, bars represent the time spent in seconds on rota rod, after 30, 60 and 90 min of treatment with distilled water (10 ml/kg), chloroform (50 and 100 mg/kg) or diazepam (0.25 mg/kg). * $P<0.05$ and *** $P<0.001$.

3.5.5. Acute toxicity of *D. lotus*

The acute toxicity of chloroform fraction of *D. lotus* was assessed in dose of 500, 1000 and 2000 mg/kg. During 24 h assessment of the acute behavioral toxicity, no mortality was observed, however the animals exhibited slight sedation after one hour injection of chloroform fraction. The treatment dose range of 25-50 mg/kg of diospyrin (**9**) and hydroxydiospyrin (**10**) exhibited slight sedation with no mortality during 24h of assessment.

3.5.6. Analgesic effect of *D. lotus*

The analgesic potential of chloroform soluble fraction and chemical constituents (**9** and **10**) in various models are given below:

3.5.6. 1. Peripheral analgesic effects in acetic acid induced writhing test

The acetic acid induced writhing was markedly protected by chloroform fraction as presented in figure 28. The chloroform fraction significantly inhibited the writhing dose dependently with the percent inhibition value 33.54 % ($P < 0.05$), 50.87 % ($P < 0.05$), 72.83 % ($P < 0.001$) at the dose of 50, 100, 150 mg/kg respectively. In case of **9** and **10** treatments significant effect at 5 mg/kg (25.98 % and 30.85 % respectively) and 10 mg/kg (40.87 and 65.76% respectively) was observed. However none of the compounds (**9** & **10**) exhibited significant analgesic effect against diclofenac sodium (**Figure 28**).

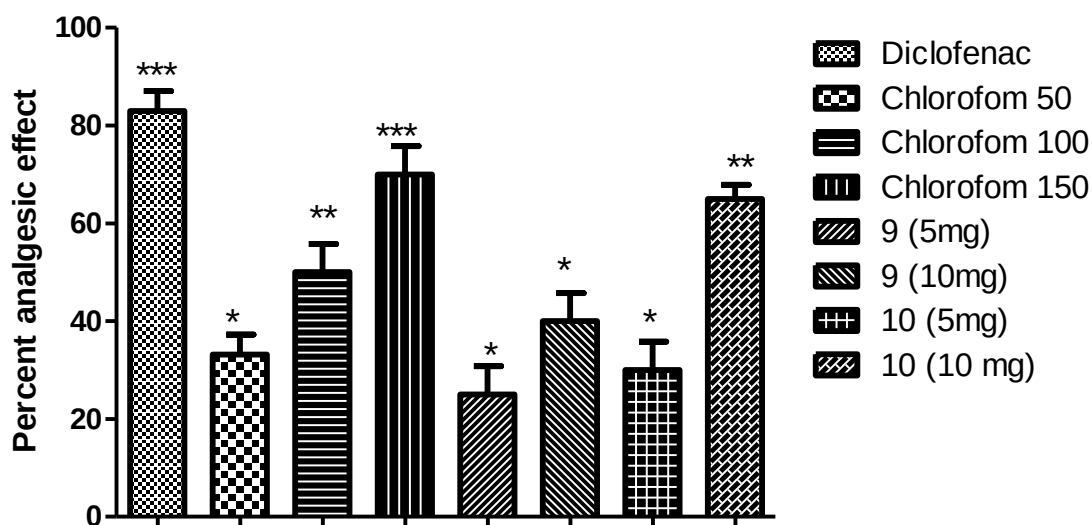


Figure 28: Analgesic effect of chloroform, **9** and **10** in acetic acid induced writhing test.

The values are represents mean $\pm$ S.E.M. for group of six animals ($n = 6$). The data was analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.5.6.2. Central analgesic effect in hot plat model

The crude extract and compounds **9** and **10** demonstrated significant and dose dependent analgesic effect in thermal induced pain model (hot plate) demonstrated in table 30. The chloroform fraction significantly increased ($P < 0.05$) the latency time in tested mice showing the significant analgesic effect in all corresponding doses. The maximum analgesic effect was exhibited after 60 min of post treatment in all treatment groups. The analgesic effect of both **9** and **10** significantly increased ($P < 0.05$) the latency time in a dose dependent manner. The effect of **10** was more than **9** and lesser than chloroform fraction. The analgesic effect was significantly antagonized by the injection of naloxone and the latency time was reversed by naloxone in case of CHCl_3 fraction **9**, **10** and tramadol^R.

Table 30: Analgesic effect of chloroform fraction, **9** and **10** assessed by using hot plate model

Group	Treatment/kg	30min	60 min	90 min	120 min
Saline	10 ml	9.20± 0.08	9.18 ± 0.09	9.20 ± 0.03	9.17 ± 0.11
Tramadol	30 mg	24.67±0.07**	26.08±0.100***	25.77±0.11 ***	25.66±0.45***
Chloroform	50 mg	11.92±2.89*	12.22±2.09*	12.89±2.55*	12.66±2.98*
	100 mg	13.33±2.33*	14.88±2.87**	14.20±2.90**	14.09±1.99**
	150 mg	19.70±2.09*	24.75±2.91**	23.98±1.76**	23.60±2.87**
9	5 mg	11.92±0.44*	12.88±0.90*	12.78±0.87*	12.58±1.00*
	10 mg	12.92±0.44*	15.20±1.11*	15.00±0.77**	14.80±0.99**
10	5 mg	12.00±0.100*	12.90±1.23*	12.80±1.90*	12.70±1.22*
	10 mg	13.00±0.95*	14.89±1.11*	14.70±0.89**	14.60±0.87**
Analgesic effect antagonized by Naloxone (0.5 mg/kg S.C.)					
Chloroform	100 mg	10.90±2.56**	10.80±0.39**	10.95±2.98**	10.98±1.99**
	150 mg	10.99±2.00**	10.80±2.91**	10.85±1.88**	10.87±1.49**
9	10 mg	11.00±0.100*	11.90±1.23*	11.80±1.90*	11.70±1.22*
10	10 mg	12.00±0.78*	13.89±0.98*	13.70±0.39**	13.60±0.57*
Tramadol	30 mg	10.22**±0.05	10.02***±0.09	10.24***±0.03	10.05***±0.00

Values are reported as mean ± S.E.M. for group of six animals. The data was analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The chloroform fraction and compounds (**9** and **10**) exhibited a prominent inhibition of writhing reflex in acetic acid-induced abdominal constriction assay. These findings strongly recommend that the plant have peripheral analgesic activity. Thermal nociception model such as hot plat test was used to evaluate central analgesic activity of *D. lotus* roots. Chloroform fraction showed significant ($P < 0.01$) analgesic effect in both the hot plat test and acetic acid induced writhing test, implicating both spinal and supraspinal analgesic pathways. In these pain paradigms tramadol^R, which is similar to the action of opioid agonists (e.g. morphine), raised the pain threshold level within 30 min of administration. In contrast, chloroform fraction, **9** and **10** showed maximum analgesic effect after 60 min of administration.

3.5.7. Anti-inflammatory effects of *D. lotus*

The anti-inflammatory effects of chloroform fraction and compounds (**9** and **10**) were dose dependent and diclofenac was used as reference drug (**Figure 29**). The maximum percent inhibition (58 %-78.43 %) of paw edema was observed after 3rd hour of administrated of carrageenan injection and chloroform fraction which exhibited significant inhibition ($P < 0.05-0.01$) of paw edema right after the first hour of carrageenan injection (**Figure 29**).

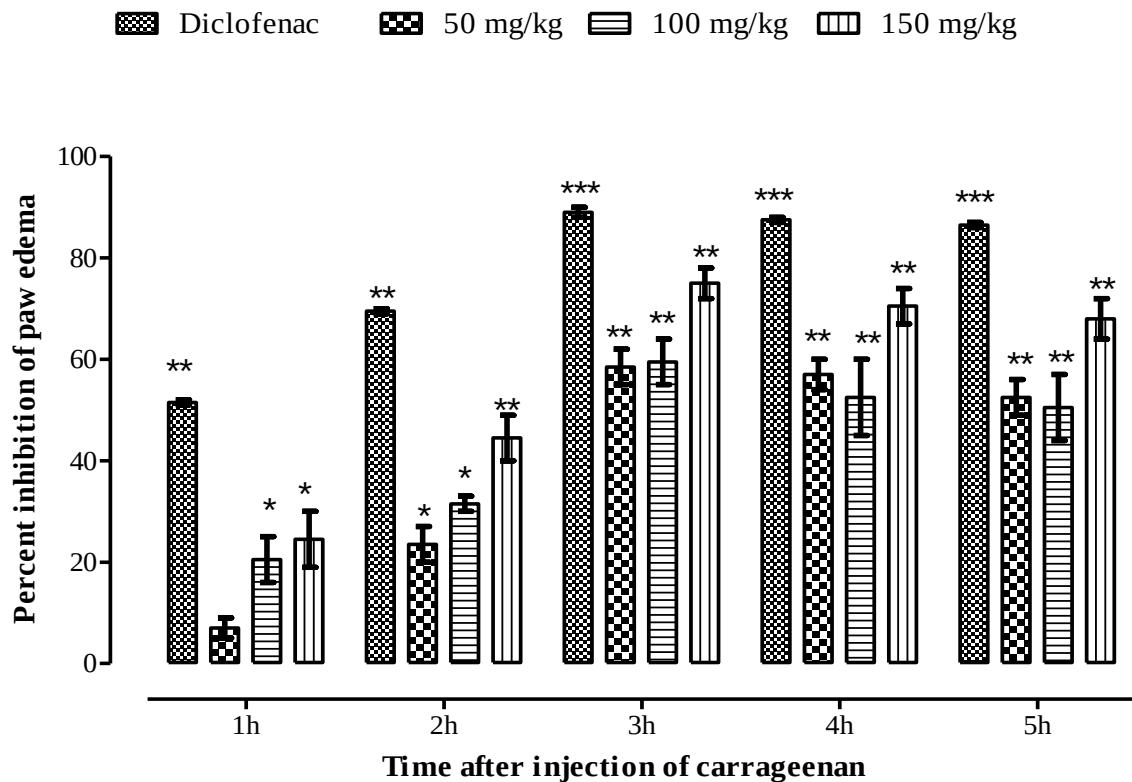


Figure 29: Anti-inflammatory effect of CHCl_3 fraction in carrageenan induced paw edema in rats. Each bar presents percent inhibition of paw edema after 1,2,3,4 and 5h of treatment. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$.

Compound **9** treatment exhibited maximum paw edema inhibition (40 %, 78 % for 5 and 10 mg/kg respectively) at 3rd hour of carrageenan injection (**Figure 30**) which exhibited significant inhibition ($P < 0.05$) at dose dependent manner.

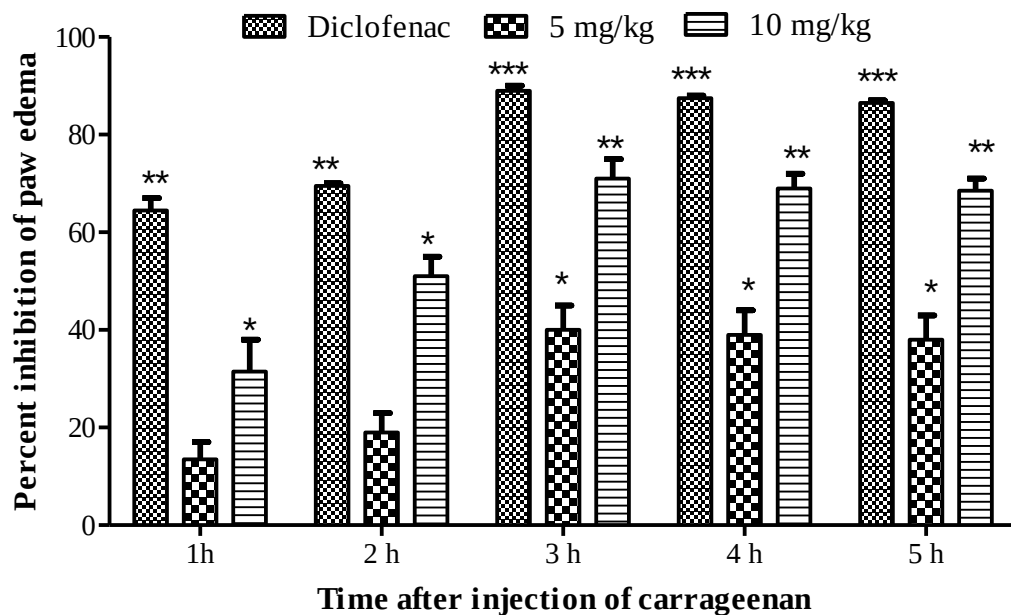


Figure 30: Anti-inflammatory effect of **9** in carrageenan induced paw edema in rats. Each bar presents percent inhibition of paw edema after 1,2,3,4 and 5h of treatment. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$.

Compound **10** was also treated on carrageenan induced paw edema animal's model which exhibited 60 % (5 mg/kg) and 78 % (10 mg/kg) (**Figure 31**). Compound **10** possessed a little greater activity than **9**. It was also noted that the % paw edema effects enhanced with increasing in time interval up to 3 hours after 1 hour of carrageenan injection.

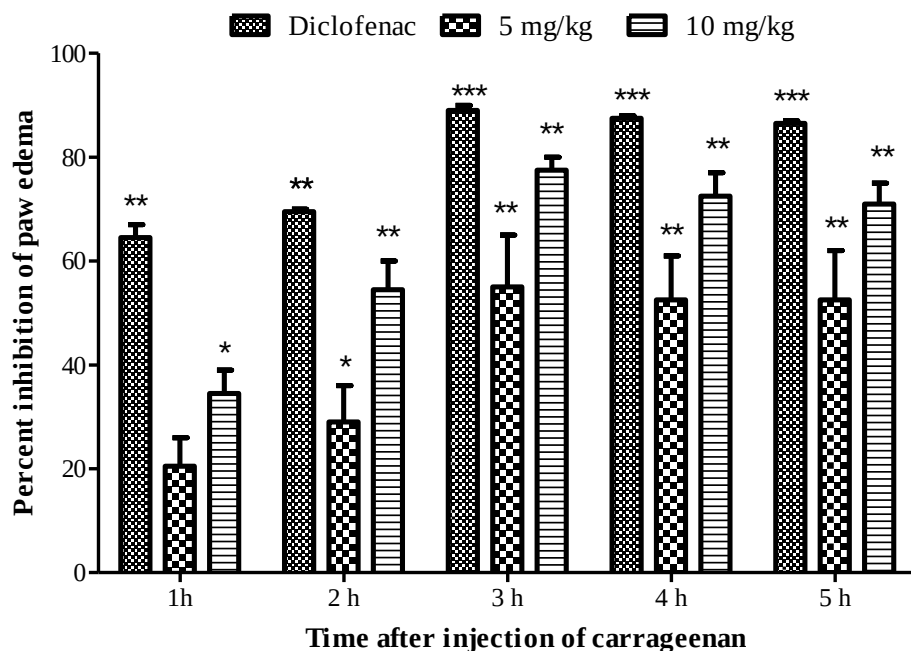


Figure 31: Anti-inflammatory effect of **10** in carrageenan induced paw edema in rats. Each bar presents percent inhibition of paw edema after 1,2,3,4 and 5h of treatment. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

3.5.8. Sedative effects of *D. lotus*

The chloroform fraction and isolated pure compounds (**9** & **10**) were also assessed for sedative activity. The results of open field i.e., sedative effect are presented in below (**Table 31**). Diazepam reference drug exhibited maximum sedation which was significantly ($P < 0.001$) different from saline control group. The chloroform fraction was exhibited mild sedative effect ($P < 0.05$) at the doses of 100 and 150 mg/kg as compared to diazepam. Compounds **9** and **10** also exhibited significant ($P < 0.01$) sedative effect in doses of 5 and 10 mg/kg against standard drug (diazepam) which showed excellent sedative effects then compounds (**9&10**) and chloroform fraction shown in table 31.

Table 31: Sedative effects of chloroform fraction, **9** and **10**

Treatment	Dose mg/kg	No of line crossed in 10 min
Saline	10 ml/kg	130.54 $\pm$ 0.98
Diazepam	0.5	6.87 $\pm$ 0.11***
Chloroform fraction	50	125.34 $\pm$ 2.98
	100	120.90 $\pm$ 2.00*
	150	112.80 $\pm$ 2.90*
9	5	100.34 $\pm$ 0.87***
	10	91.98 $\pm$ 1.09***
10	5	99.89 $\pm$ 1.33***
	10	90.02 $\pm$ 2.98***

The values represent the number of lines crossed by animal in box, 30 minutes after treatment with saline (10 ml/kg, control), diazepam, chloroform fraction, **9** or **10**. Data presented as mean $\pm$ S.E.M for n=6. The asterisk (*) represents the level of significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, all related with control.

3.5.9. Muscle relaxants activity of *D. lotus*

The muscle relaxant activities of isolated pentacyclic triterpenes (**12-14**) of *D. lotus* are given below:

3.5.9.1. Chimney animal model

The pentacyclic triterpenes (**12-14**) isolated from the chloroform soluble fraction were evaluated for their muscle relaxant potential in chimney animal model which possessed moderate activity against reference drug (**Figure 32**). The maximum effect (46.04% and 66.72%) of oleanolic acid (**12**) was observed after 90 min of post treatment of **12** at 5 and 10 mg/kg i.p. respectively (**Figure 32;a**). Ursolic acid (**13**) showed significant muscle relaxant activity (35.88% and 60.21%) at 5 and 10 mg/kg i.p. after 90 min in dose dependent manner (**Figure 32;b**) while betulinic acid produced 27% and 50.77% activity at 5 and 10 mg/kg i.p. after 90 min (**Figure 32;c**). It was observed that the % muscle relaxant effects increased with increasing in interval of time.

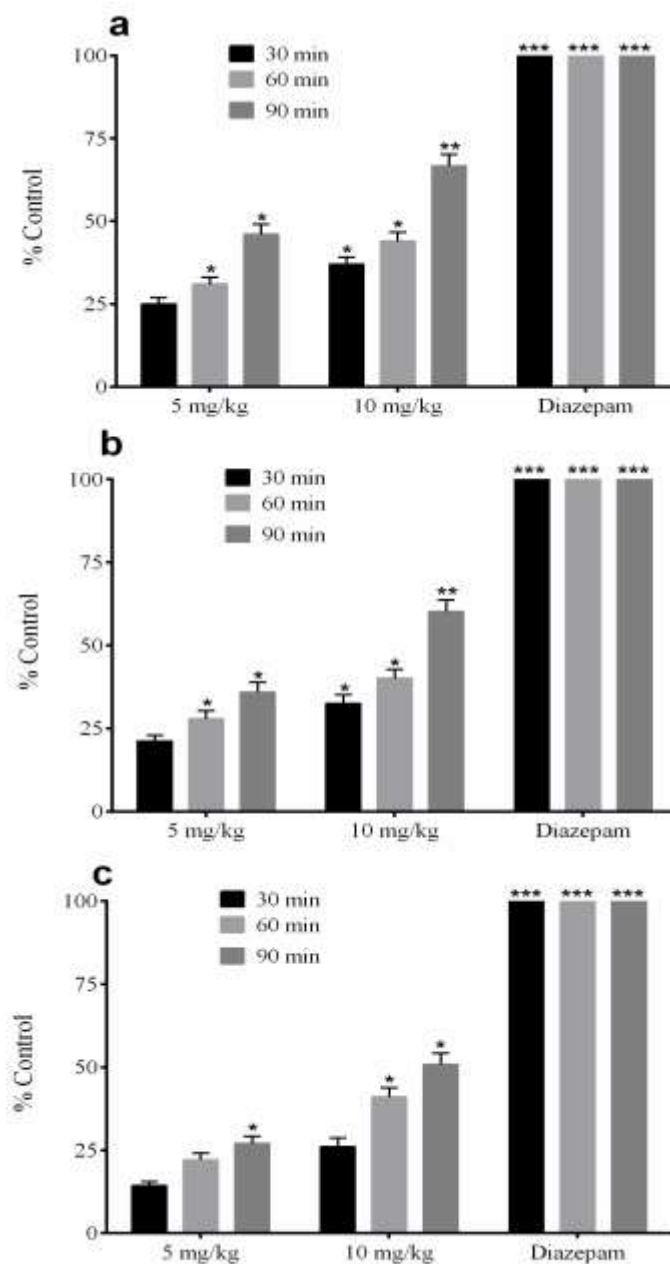


Figure 32: Percent muscle relaxant effects of **12-14** in chimney test; (a) oleanolic acid (**12**), (b) ursolic acid (**13**), (c) betulinic acid (**14**), after 30,60,and 90 min. Data are presented as mean $\pm$ S.E.M (n=6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, all compared with control

3.5.9.2. Inclined plane model

Oleanolic acid (**12**), ursolic acid (**13**) and betulinic acid (**14**) were also evaluated for muscle relaxant effect in inclined plane animal model using diazepam as a standard drug. The effect of tested compounds (**12-14**) are shown in figure 33 in which oleanolic acid (**12**) showed prominent muscle relaxant action during various assessment times (30, 60 and 90 min) and possessed maximum muscle relaxant activity (46.90% and 65.74%) after 90 min at doses of 5 and 10 mg/kg i.p. respectively (**Figure 33; a**). In case of ursolic acid (**13**) which caused also dose dependent action with 35.05% and 59.84% activity muscle relaxant activity at 5 and 10 mg/kg i.p. respectively after 90 min of treatment (**Figure 33; b**) while the post treatment of betulinic (**14**) acid exhibited moderate relaxant activity (27.90% and 51.40%) at 5 and 10 mg/kg i.p. respectively (**Figure 33; c**) after 90 min.

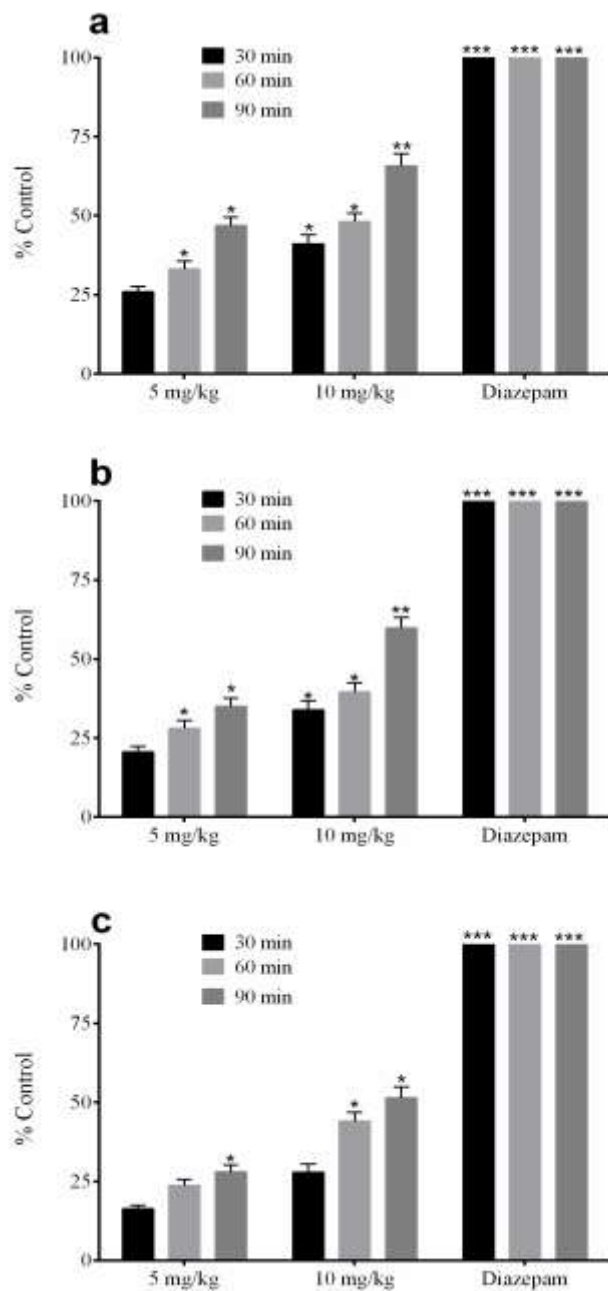


Figure 33: Percent muscle relaxant effects of **12-14** in inclined plane test; (a) oleanolic acid (**12**), (b) ursolic acid (**13**) (c) betulinic acid (**14**), after 30,60,and 90 min. Data are presented as mean $\pm$ S.E.M (n=6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$,all compared with control.

3.5.10. Antipyretic activity of *D. lotus*

The isolated pentacyclic triterpenes were also subjected for antipyretic efficiency in animal model (**Figure 34**).

3.5.10.1. Yeast induced pyrexia model

Oleanolic acid (**12**), ursolic acid (**13**) and betulinic acid (**14**) were also evaluated for antipyretic efficiency in yeast induced pyrexia model using para-acetylamino phenol as a reference drug. The results of pentacyclic triterpenes (**12-14**) are shown in figure 34. Pretreatment of **12** was provoked dose dependent amelioration of induced pyrexia in hyperthermic mice and the effect was significant after 3rd of h treatment at 5 mg kg⁻¹ i.p. while significant effect was observed after 1st h of treatment at 10 mg kg⁻¹ i.p. The percentage anti-hyperthermic effect was exhibited (39.32% and 71.59%) at 5 and 10 at 5 mg kg⁻¹ i.p. respectively after 4th h of treatment (**Figure 34;a**). Ursolic acid (**13**) also showed significant antipyretic activity (**Figure 34; b**) which caused dose dependent attenuation of induced pyrexia in various estimated times (1-5 h). Overall, the effect was significant after 3rd of h treatment at 5 mg kg⁻¹ i.p. and 1st h of treatment at 10 mg kg⁻¹ i.p. of **13**. The maximum antipyretic effect after 4th h of treatment was observed activity (34.32% and 60.99%) at 5 and 10 mg kg⁻¹ i.p. respectively (**Figure34;c**). Betulinic acid (**14**) also evoked dose dependent antipyretic effect at test doses during assessment times. At the dose of 5 mg kg⁻¹ i.p. of **14** was produced significant activity (29.99% and 52.44%) after 3rd/4th h of treatment at 10 at 5 mg kg⁻¹ i.p. respectively (**Figure 34;d**). Generally the standard drug (para-acetylamino phenol) produced more dominant effect is compared to compounds (**12-14**).

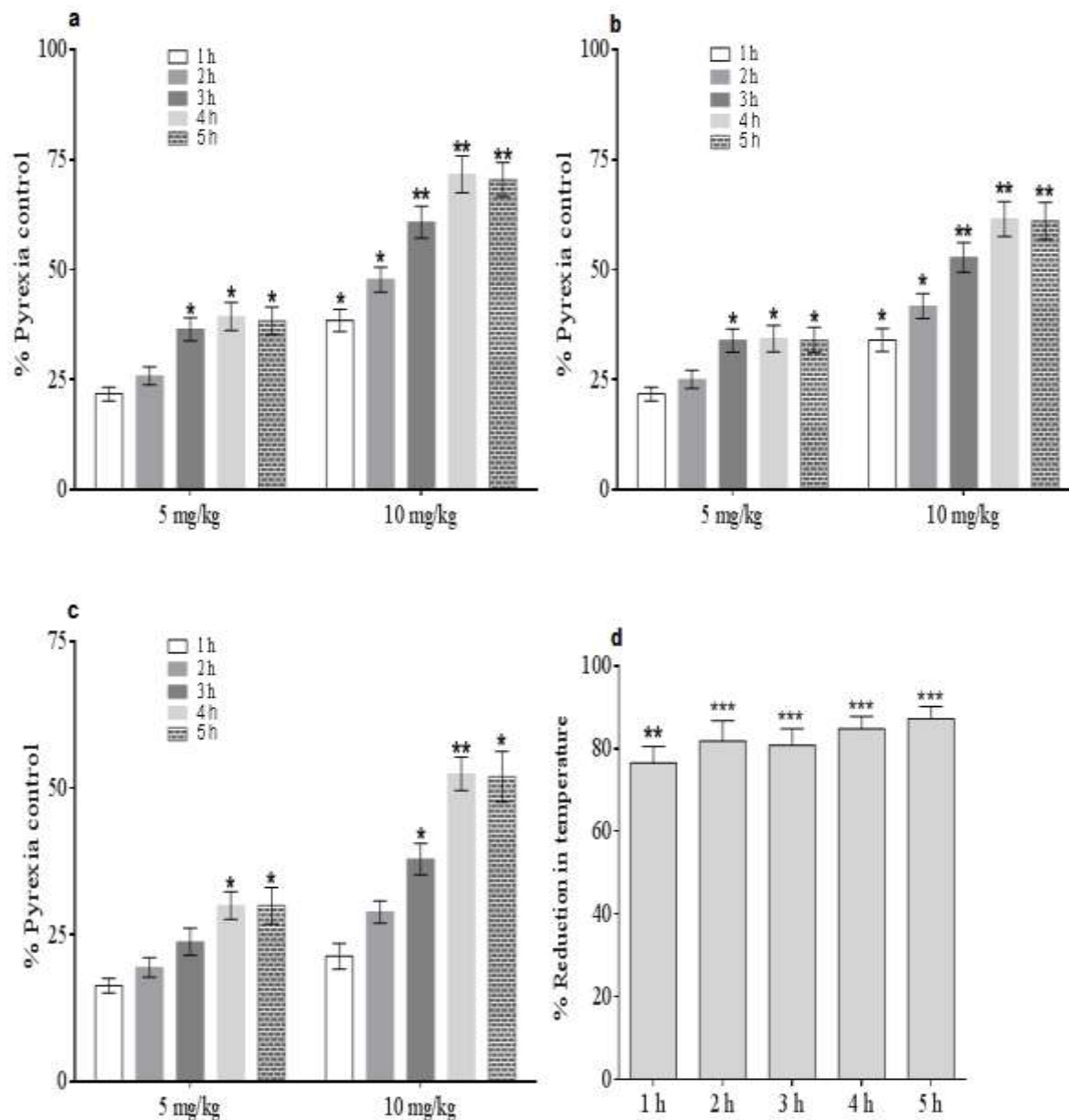


Figure 34: Induces Pyrexia effects of 12-14 (a) oleanolic acid, (b) ursolic acid (c) betulinic acid (d) paracetamol in yeast induced pyrexia test, after 1,2,3,4,5th h. Data are presented as mean $\pm$ S.E.M (n=6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Compared with control.

3.6. Biological screening of *D. lotus* fixed oil

Fixed oil extracted from the chloroform fraction of *D. lotus* roots was subjected to various biological activities like antimicrobial, antioxidant, cytotoxicity and insecticidal assays. The oil was investigated against various selected microbial insects. These investigations showed that roots oil of *D. lotus* revealed considerable antimicrobial activity and (Figure 35 & 36) while low insecticidal activity. The oil of *D. lotus* was also studied for their antioxidant capacity using DPPH radical scavenging assay at different concentration. The assay showed that fixed oil is moderate scavenging activity at low concentration and has good activity at higher concentration (Figure 37). Cytotoxic activity of fixed oil was also evaluated and found almost inactive (Table 32).

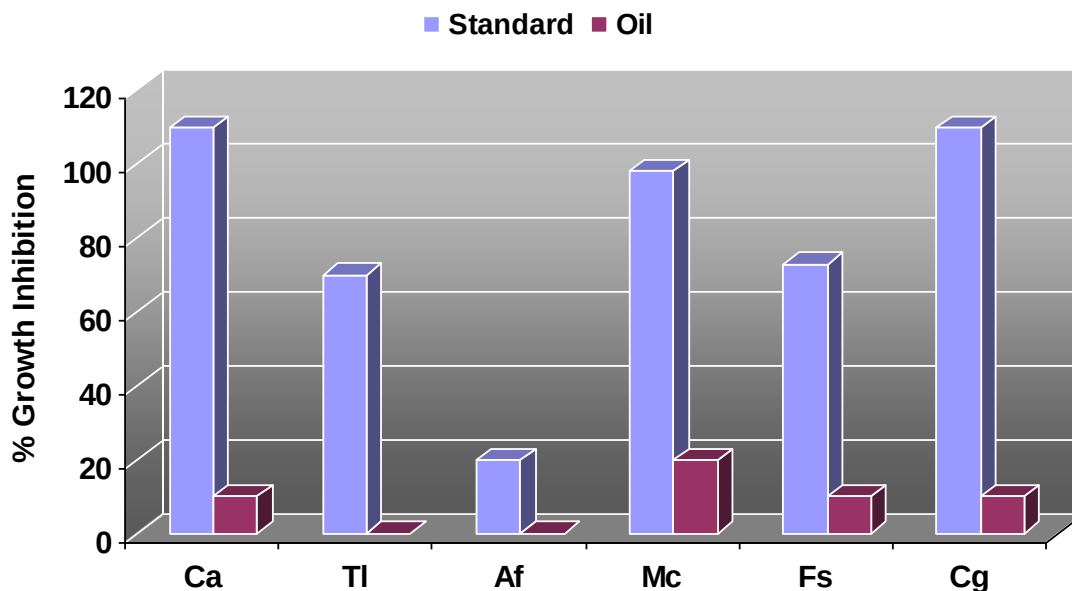


Figure 35: Antifungal activity of oil isolated from the roots of *D. lotus*

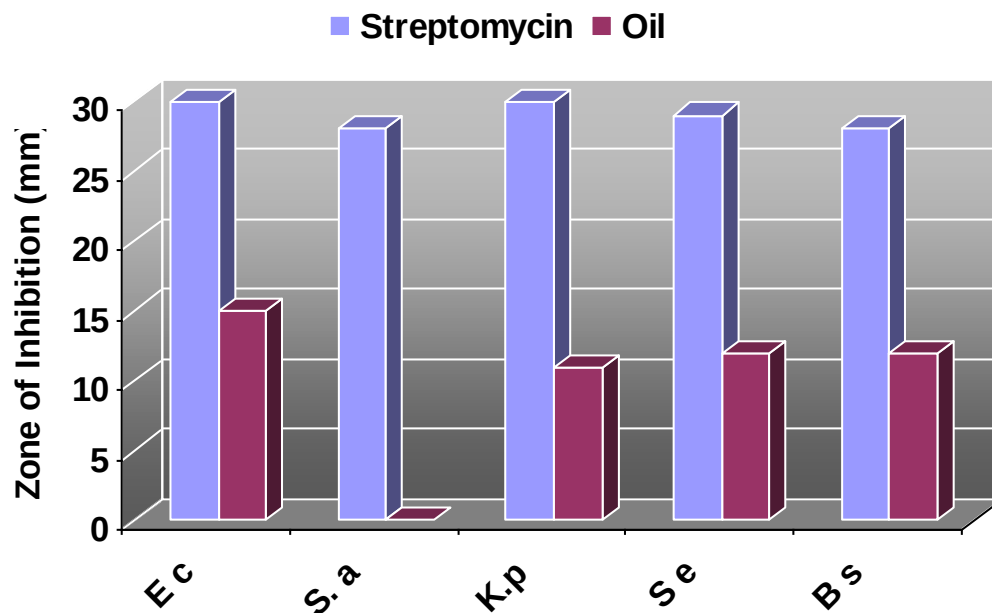


Figure 36: Antibacterial activity of oil isolated from the roots of *D. lotus*

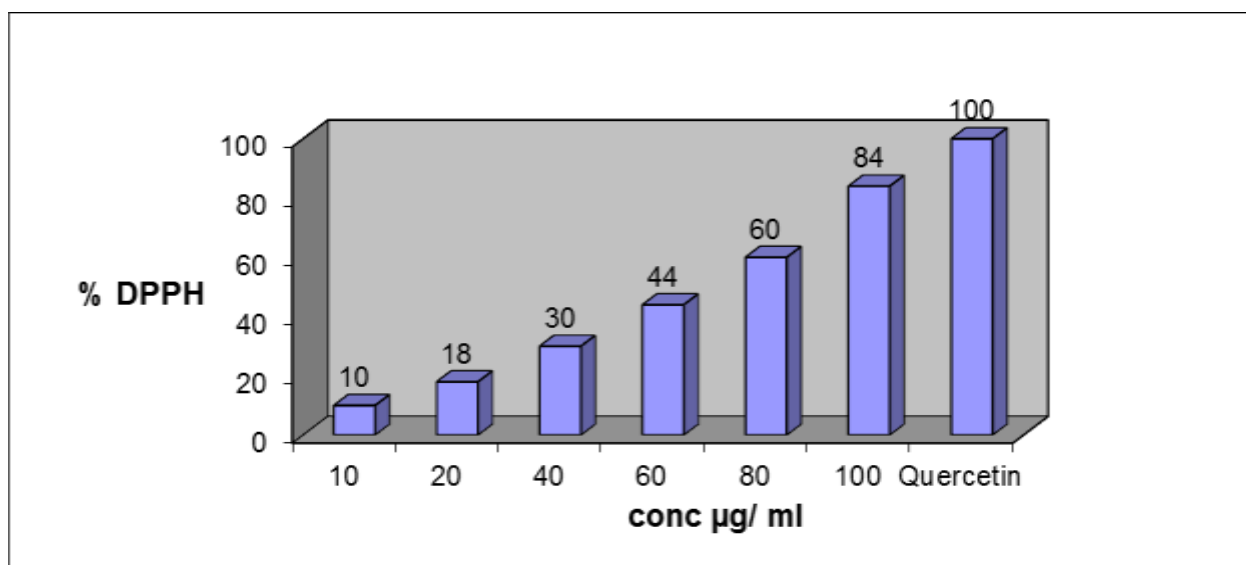


Figure 37: DPPH radical scavenging activity of oil extracted from *D. lotus*

Table 32: Cytotoxicity, brine shrimps activity of oil isolated from the roots of *D. lotus*.

Dose µg/ml	No. of shrimps	No. of survivors	LD ₅₀ µg/ml	STD. Drug	LD ₅₀ µg/ml
1000	30	18	2997.99	Etoposide	7.4625
100	30	23	NC	NC	NC
10	30	27	NC	NC	NC

Upper Limit (Upper Toxic Conc.)...18866

Lower Limit (Lower Toxic Conc.).....578.78

NC: Not calculated

The fixed oil extracted from *n*-hexane fractions was assessed for various biological screening which revealed moderate antibacterial activity of selected bacterial strain (*Staphylococcus aureus*, *Escherichia. coli*) (**Figure 38**) against standard (streptomycin). In case of Antifungal activity of the fixed oil showed low activity of the selected fungal strain (*Candida albicans*, *Aspergillus flavus*, *Microsporum canis*, *Fusarium salani*, *Candida glabrata*) (**Figure 39**). Interestingly oil showed no considerable toxicity in brine-shrimp lethality assay which indicated safety of oils for pharmacological use. The oil was also tested for antioxidant and insecticidal activity but no interesting activity was found.

This study highlighted potential use of fixed oil of *D. lotus* for infection diseases at cellular and molecular levels.

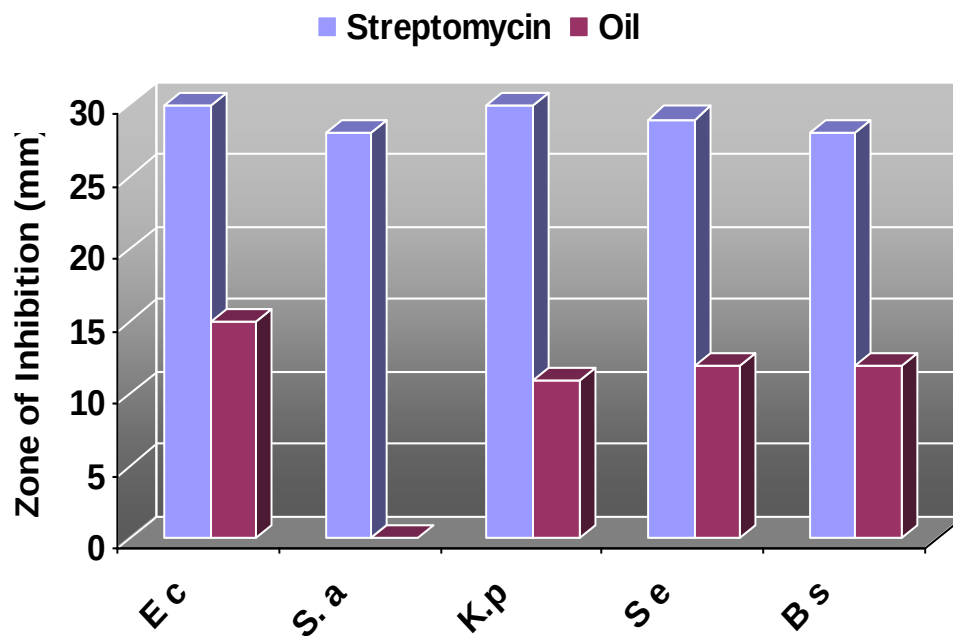


Figure 38: Antibacterial activity of oil isolated from the roots of *D. lotus*

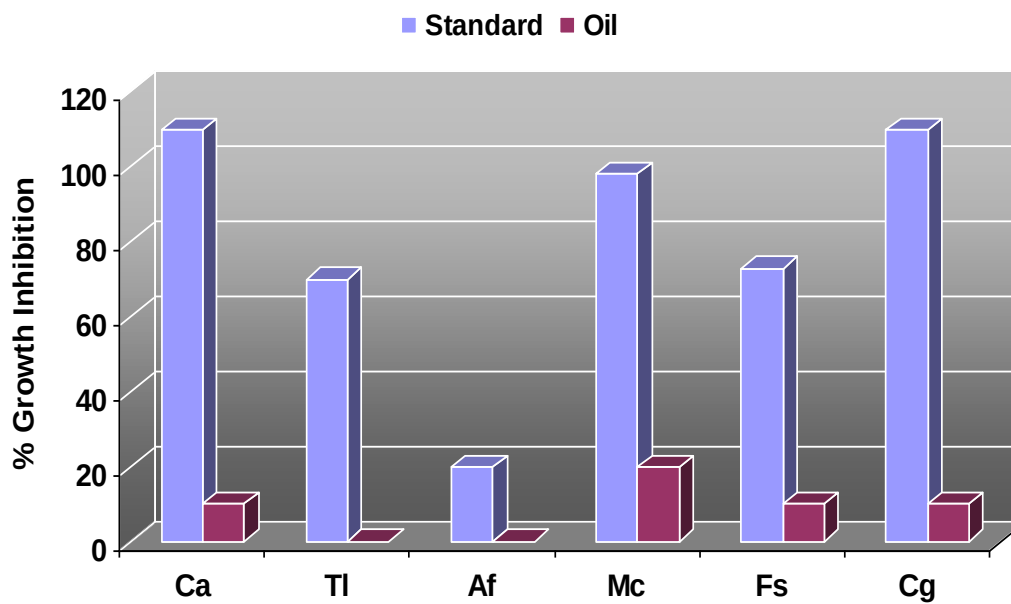


Figure 39: Antifungal activity of oil isolated from the roots of *D. lotus*

4.1. General experimental procedures

Melting points were determined in glass capillaries tubes by Bicote melting point apparatus (Bibby Scientific limited, UK) and are uncorrected. The UV spectra were measured in chloroform by using UV-visible recording spectrometer Model Hitachi-U-3200 (Japan). The IR spectra were recorded on FT-IR Nicolet 380 (Thermo Scientific, UK). ^1H -NMR (600 MHz), ^{13}C -NMR (125 MHz), HMBC (500 MHz) and HSQC (600 MHz) spectra were recorded by AVANCE AV-600 Cryoprob NMR instrument in CDCl_3 . EI-MS, FAB mass and HR-EIMS were recorded on Jeol-JMS-HX-110 mass spectrometer; EI source 70 eV. Column chromatography was performed on Merck silica gel 60 (0.063-0.200 mm) while TLC was carried out on Merck aluminium plates pre-coated with silica gel 60 F₂₅₄ and visualized secondary metabolites by locating reagents.

4.1.1. Chemical reagents and spray

The following reagents were used for the identification of secondary metabolites.

4.1.2. Ceric sulphate

0.6 g of ceric sulphate was dissolved in 10 mL of concentrated H_2SO_4 and then diluted to 100 mL with distilled water in a volumetric conical flask and kept in a spray gun.

4.1.3. Dragendorff's reagent

Dragendorff's reagent was prepared freshly from the mixture of solution A and B. Solution A was prepared by dissolving 1.7 g of bismuth nitrate in 100 mL mixture of distilled water and acetic acid (4:1) while the solution B was prepared by dissolving 40 g potassium iodide in 100 mL distal water. 5 mL of each solution was mixed with 70 mL distal water and 20 mL acetic acid.

4.1.4. Fehling's solution

Fehling's solution was prepared freshly to combine Fehling's solution A and B. Solution A was prepared by dissolving 5 g of copper(II) sulfate in 100 mL distilled water while solution B was prepared by dissolving potassium sodium tartrate in distilled water and NaOH (0.1 M) media.

4.2. Plant materials

Diospyros lotus (roots, stem and aerial parts) were collected from Razagram (Khall), Dir, KPK, Pakistan, in May 2009 and authenticated by Prof. Dr. Abdur Rashid, Taxonomist, Department of Botany University of Peshawar, Pakistan where a voucher specimen (Bot.20036 (PUP) has been deposited at the Herbarium.

4.2.1. Phytochemical screening of *D. lotus*

The phytochemical tests were performed to assess the quantitatives and quantities chemical composition of the crude extract of *D. lotus* and its fractions followed by the standard procedures illustrated precisely for detection of the phytochemicals [110].

4.2.2. Test for alkaloids

0.2 g of each sample was warmed with 2% H₂SO₄ for at least two minutes, and filtered then few drops of Dragendroff's reagent were added. The formation of orange red precipitate which indicates the presence of alkaloids moiety [111,112].

4.2.3. Test for tannins

0.34 g each sample was mixed with distilled water and heated on water bath for a while then filtered few drops of ferric chloride were added. The formation of dark green colour shows the presence of tannins.

4.2.4. Test for anthraquinones

0.5 g of each sample was boiled with 5 mL of 10 % HCl for few minutes on water bath: filtered and allowed to cool. Then 5 mL of CHCl_3 and few drops of 10% ammonia were added. Rose-pink colour was formed which indicates the presence of anthraquinones.

4.2.5. Test for glycosides

0.5 g of each sample was hydrolyzed with HCl (0.2 M) and the neutralized with NaOH (0.1 M) solution. Few drops of Fehling's solution A and B were added to each mixture. Formation of red precipitate indicates the presence of glycosides.

4.2.6. Test for reducing sugars

0.4 g of each sample was shaken with distilled water and filtered. The filtrate was boiled and added few drops of Fehling's solution A and B. Formation of an orange red precipitate indicates the presence of reducing sugars.

4.2.7. Test for saponins

0.2 g of each sample was shaken with 5 mL of distilled water and heated to boiling. The appearance of creamy mass of small bubbles shows the presence of saponins.

4.2.8. Test for flavonoids

0.2 g of each sample was dissolved in 5 mL NaOH (0.1 N) and three drops of conc. HCl were added. The solution became colourless which indicates the presence of flavonoids.

4.2.9. Test for phlobatanins

0.5 g of each sample was dissolved in distilled water and filtered. The filtrate was boiled with 2 % of HCl solution. Red precipitate shows the presence of phlobatanins.

4.2.10. Test for steroids

0.2 g of sample was dissolved in 2 ml of acetic anhydride and added 2 mL of conc. H_2SO_4 .

The colour change from violet to blue or green indicates the presence of steroids.

4.2.11. Test for terpenoids

0.2 g of the each sample was mixed with 2 mL of chloroform and 3 mL of conc. H_2SO_4 were carefully added to form a layer. The formation of a reddish brown coloration at the interface indicates the presence of terpenoids.

4.3. Present work

In view of the folkloric uses and biological implications of *D. lotus* attributed to investigate further their phytochemistry and pharmacological study. As results, five new dimeric naphthoquinones and nine hitherto unreported constituents have been isolated from the roots of *D. lotus*. The biological implications of *D. lotus* were confirmed by biological screening of extracts, fractions and chemical constituents which exhibited potential activity.

4.3.1. Extraction and isolation

Shade-dried roots of *Diospyros lotus* (14 kg) were powdered by local grinder and repeatedly extracted with 60 L, CHCl_3 (x5) at room temperature. The combined extracts were concentrated by evaporating the solvent using rotary evaporator under reduced pressure at a temperature below 50°C to obtain a dark red residue (**Scheme 1**). The crude extract was defatted with *n*-hexane through Soxhlet apparatus (x6), at 50°C to removed *n*-hexane soluble fraction which was subjected to column chromatography (CC); *n*-hexane-EtOAc (100:0 $\rightarrow$ 85:15) eluted gave a reddish oil residue of fatty acids (**Table 20**) the column was further eluted with *n*-hexane-EtOAc (100:0 $\rightarrow$ 85:15) and furnished lupeol (**1**) (m.p. 210-

212 °C), β -sitosterol (**3**) (m.p. 137-140 °C) and stigmasterol (**4**) (m.p. 162-165 °C) (**Scheme 1**).

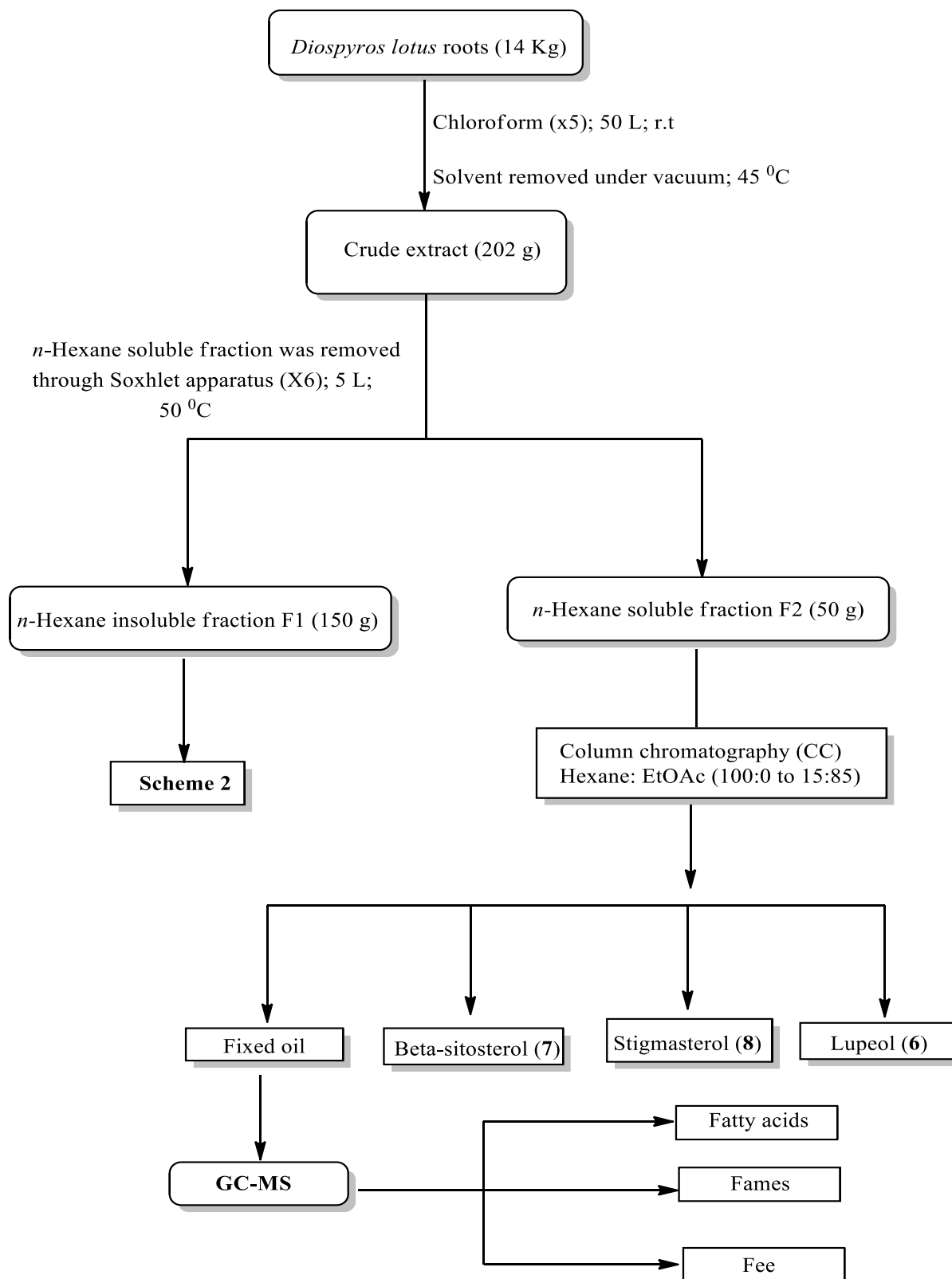
The *n*-hexane insoluble fraction F-1 (30 g) was also subjected to column chromatography on silica gel (Merck silica gel 60 (0.063-0.200 mm), 5×60 cm). The column was eluted with *n*-hexane-ethyl acetate (100:0 → 0:100) as solvent system. A total of 105 fractions, (RF-1 to RF-105) were obtained based on TLC profiles. The fractions RF-1 to RF-10 was combined on the origins of TLC to obtain SF-1 (2 g) which was further subjected to CC with eluate *n*-hexane to yield a reddish residue of fatty acids (**Table 22 & 23**) while the remaining fractions RF-11 to RF-105 was compiled on the basic of TLC profile to give major sub fractions SF-2 (2.34 g), SF-3 (9.89 g), SF-4 (3.25 g) and SF-5 (3.98 g).

Fraction SF-4 (9.89 g), was subjected to column chromatography eluting with *n*-hexane-EtOAc (100:0 → 10:15) and 60 fractions were obtained which combined on the basis of TLC profile yielded three major fractions MF-1 (3.44 g), MF-2 (2.41 g) and MF-3 (1.01 g). Fraction MF-1 (3.44 g) furnished diospyrin (**9**) and 8-hydroxydiospyrin (**10**) followed by pencil column chromatography (**Scheme 2**) while MF-2 (2.41 g) was purified by preparative TLC and furnished three new dimeric naphthoquinones 5,4'-dihydroxy-1'-methoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,5',8'-tetraone (**1**), 5',8'-dihydroxy-5-methoxy-6,6'-dimethyl-7,3'-binaphthyl-1, 4,1',4'-tetraone (**2**) and 8,5',8'-trihydroxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone (**3**) (**Scheme 2**).

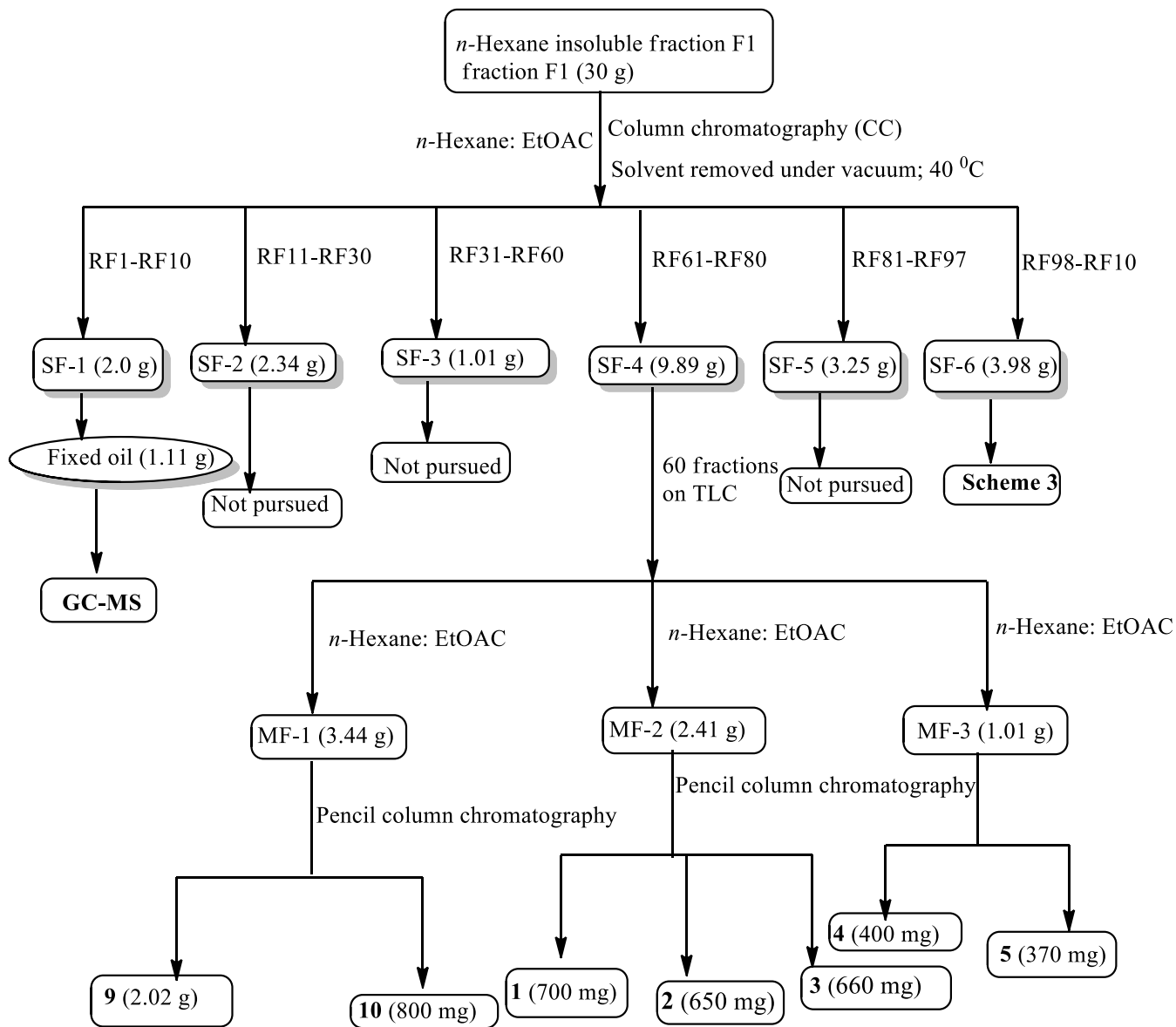
In the same manner MF-3 (1.01 g) was also purified by pencil column chromatography yielded two new dimeric naphthoquinones; 5', 8'-dihydroxy-6,6'-dimethyl-7,3'-binaphthyl-

1,4,1',4'-tetraone (**4**) and 5',8'-dihydroxy-5,8-dimethoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone (**5**) (**Scheme 2**).

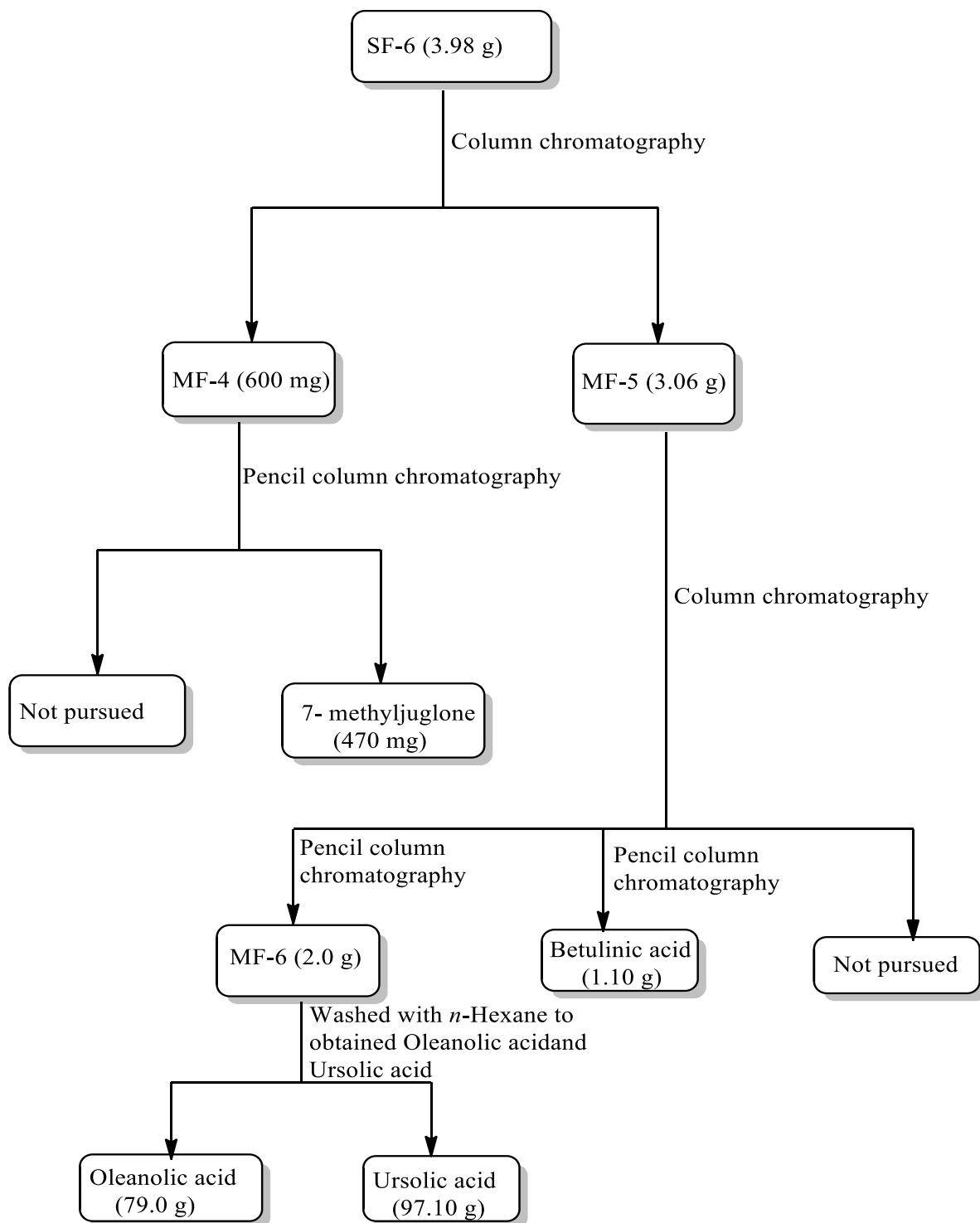
The SF-6 (3.98 g) was yield 27 fractions by CC which was combined on the basis of TLC profile to furnish MF-4 (0.60 g) and MF-5 (3.06 g). Fraction MF-4 (0.60 g) was further purified by pencil column chromatography with *n*-hexane and EtOAC furnished a white crystal of 7-methyljuglone while the MF-5 (3.06 g) gave oleanolic acid (**12**), ursolic acid (**13**) and betulinic acid (**14**) (**Scheme 3**).



Scheme 1: Extraction, fractionation and isolation of compounds from *D. lotus*



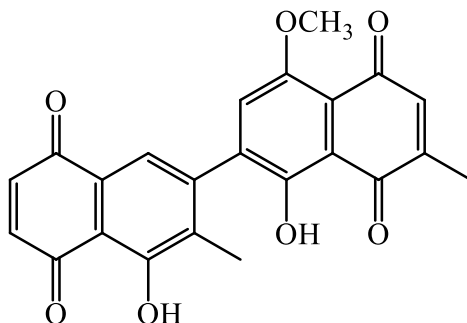
Scheme 2: Isolation of chemical constituents from chloroform soluble fraction of *D. lotus*



Scheme 3: Isolation of chemical constituents from fraction SF-6 of *Diospyros lotus*

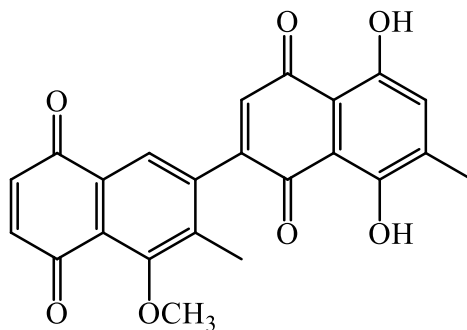
4.4. Chemical structure of new constituents from *D. lotus* roots

• Di-naphthodiospyrol A (1)

5,4'-dihydroxy-1'-methoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,5',8'-tetraone (**1**)

Physical status	Yellow powder
Yield	700 mg
M.P	269-272 °C
IR (cm ⁻¹)	2924, 1643, 1634, 1604 and 1460
Uv $\nu_{\max}$ (nm)	253, 296, 435
HRMS	404.2987
Mol: formula	C ₂₃ H ₁₆ O ₇
IUPAC	5,4'-Dihydroxy-1'-methoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,5',8'-tetraone (1)
¹ H-NMR (CDCl ₃)	Table 6
¹³ C-NMR (CDCl ₃)	Table 6

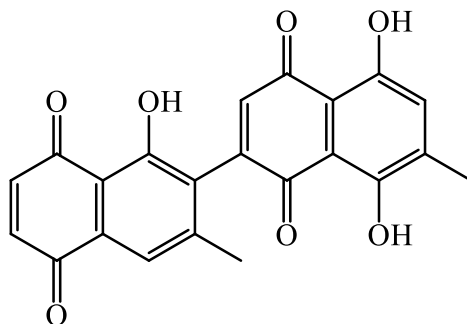
- **Di-naphthodiospyrol B (2)**



5',8'-Dihydroxy-5-methoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone (2)

Physical status	Yellow powder
Yield	650 mg
M.P	264-265 °C
IR (cm ⁻¹)	2925, 1644, 1603
Uv v _{max} (nm)	253, 299, 432
HRMS	404.2980
Mol: formula	C ₂₃ H ₁₆ O ₇ .
IUPAC	5',8'-Dihydroxy-5-methoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone (2)
¹ H-NMR (CDCl ₃)	Table 7
¹³ C-NMR (CDCl ₃)	Table 7

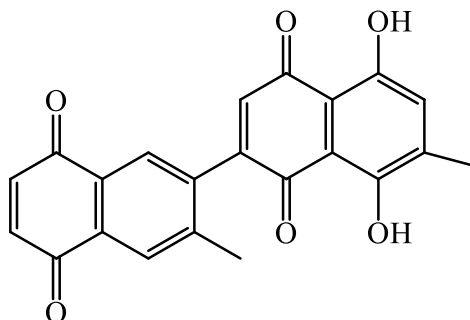
- **Di-naphthodiospyrol C (3)**



8,5',8'-Trihydroxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone (3)

Physical status	Yellow powder
Yield	660 mg
M.P	269-270 °C
IR (cm ⁻¹)	2988, 1642 and 1602
Uv v _{max} (nm)	250, 301, 436
HRMS	390.0981
Mol: formula	C ₂₂ H ₁₄ O ₇
IUPAC	8,5',8'-Trihydroxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone (3)
¹ H-NMR (CDCl ₃)	Table 8
¹³ C-NMR (CDCl ₃)	Table 8

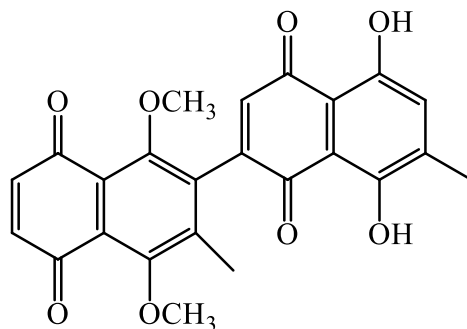
- **Di-naphthodiospyrol D (4)**



5', 8'-Dihydroxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone (4)

Physical status	Yellow powder
Yield	400 mg
M.P	244-246 °C
IR (cm ⁻¹)	2930, 1655 1614, 1603, 1462
Uv v _{max} (nm)	256, 298, 437
HRMS	374.0780
Mol:formula	C ₂₂ H ₁₄ O ₆
IUPAC	5', 8'-Dihydroxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone
¹ H-NMR (CDCl ₃)	Table 9
¹³ C-NMR (CDCl ₃)	Table 9

- **Di-naphthodiospyrol D (5)**

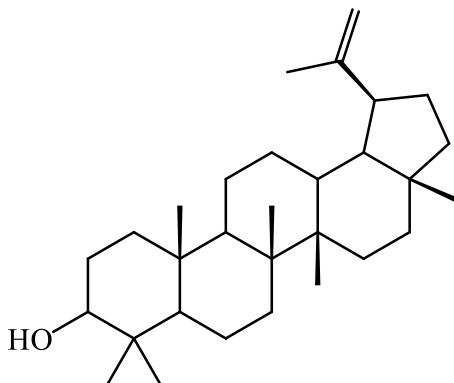


5',8'-Dihydroxy-5,8-dimethoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone (5)

Physical status	Yellow powder
Yield	370 mg
M.P	147-148 °C
IR (cm ⁻¹)	2933, 1660, 1610, 1603, 1462
Uv $\nu_{\max}$ (nm)	250, 302, 436
EIMS	433.9998
Mol: formula	C ₂₄ H ₁₈ O ₈
IUPAC	5',8'-Dihydroxy-5,8-dimethoxy-6,6'-dimethyl-7,3'-binaphthyl-1,4,1',4'-tetraone
¹ H-NMR (CDCl ₃)	Table 10
¹³ C-NMR (CDCl ₃)	Table 10

4. 5. Structure of new source isolated compounds from *D. lotus* roots

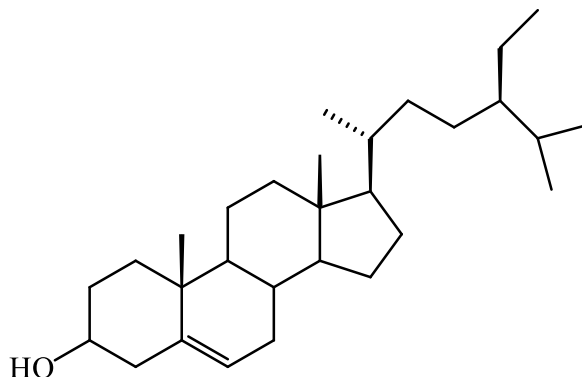
• Lupeol (6)

(3 β ,13 ζ)-Lup-20(29)-en-3-ol

Physical data of compound

Physical status	White needle crystals
Yield	1.25 g
M.P	210-212 °C
IR (cm ⁻¹)	2932, 2871 ,1685
Uv $\nu_{\max}$ (nm)	245
EIMS	426. 7
Mol: formula	C ₃₀ H ₅₀ O
Chemical name	(3 β ,13 ζ)-Lup-20(29)-en-3-ol
¹ H-NMR (CDCl ₃)	Table 11
¹³ C-NMR (CDCl ₃)	Table 11

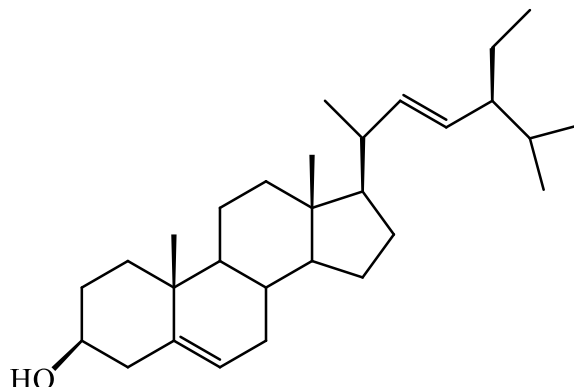
- β -Sitosterol (7)



17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol.

Physical status	White needle crystals
Yield	2.34 g
M.P	137-140 °C
IR (cm ⁻¹)	2968, 2937, 2872, 1686
Uv $\nu_{\max}$ (nm)	255
EIMS	416
Mol: formula	C ₂₉ H ₅₀ O
Chemical name	17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol.
¹ H-NMR (CDCl ₃)	Table 12
¹³ C-NMR (CDCl ₃)	Table 12

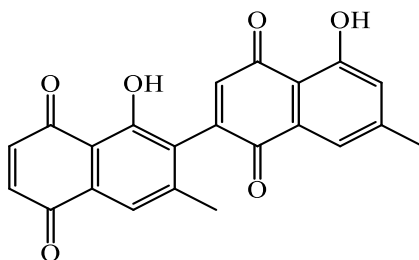
- Stigmasterol (8)



(3S,8S,9S,10R,13R,14S,17R)-17-[(E,2R,5S)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol

Physical status	White needle crystal
Yield	1.98 g
M.P	162-165 °C
IR (cm ⁻¹)	2965, 2935, 2870 and 1680
Uv $\nu_{\max}$ (nm)	260
EIMS	412
Mol: formula	C ₂₉ H ₄₈ O
Chemical name	3S,8S,9S,10R,13R,14S,17R)-17-[(E,2R,5S)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol
¹ H-NMR (CDCl ₃)	Table 13
¹³ C-NMR (CDCl ₃)	Table 13

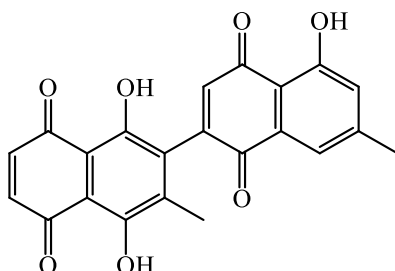
- **Diospyrin (9).**



Diospyrin; Aids108046; aids-108046; 5,5'-dihydroxy 7,7'-binaphthoquinone; 1',5'-dihydroxy-3',7'-dimethyl-2,2'-binaphthalene-1,4,5',8'-tetrone

Physical status	White needle crystal
Yield	800 mg
M.P	252-255 °C
IR (cm ⁻¹)	3660, 2916, 2925, 1672, 1634, 1460
UV v _{max} (nm)	249, 261, 438
EIMS	374
Mol: formula	C ₂₂ H ₁₄ O ₆ ,
Chemical name	Dihydroxy 7,7'-binaphtho-quinone; 1',5'-dihydroxy-3',7'-dimethyl-2,2'-binaphthalene-1,4,5',8'-tetrone
¹ H-NMR (CDCl ₃)	Table 14
¹³ C-NMR (CDCl ₃)	Table 14

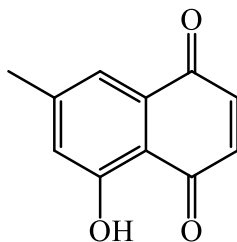
- Hydroxydiospyrin (10)



5,8,8'-trihydroxy 7,7'-binaphtho-quinone;1',5-dihydroxy-3',7-dimethyl-2,2'-binaphthalene-1,4,5',8'-tetrone

Physical status	White needle crystal
Yield	2.02 g
M.P	273-275 °C
IR (cm ⁻¹)	3610, 2925, 2848, 1690
Uv $\nu_{\max}$ (nm)	231, 253, 437
EIMS	3890
Mol: formula	C ₂₂ H ₁₄ O ₇
Chemical name	5,8,8'-trihydroxy 7,7'-binaphtho-quinone;1',5-dihydroxy-3',7-dimethyl-2,2'-binaphthalene-1,4,5',8'-tetrone
¹ H-NMR (CDCl ₃)	Table 15
¹³ C-NMR (CDCl ₃)	Table 15

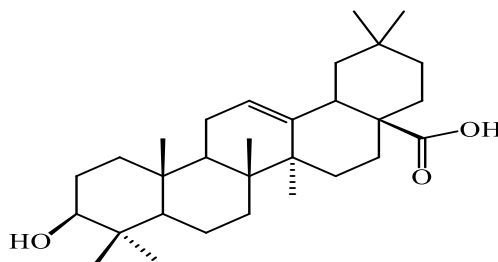
- 7-Methyljuglone (11)



5-Hydroxy-7-methyl-1,4-naphthoquinone

Physical status	White crystals
Yield	470 mg
M.P	113-116 °C
IR (cm ⁻¹)	3615 2926, 2880, 1600, 1412
Uv $\nu_{\max}$ (nm)	220
EIMS	188
Mol: formula	C ₁₁ H ₈ O ₃
Chemical name	5-Hydroxy-7-methyl-1,4-naphthoquinone
¹ H-NMR (CDCl ₃)	Table 16
¹³ C-NMR (CDCl ₃)	Table 16

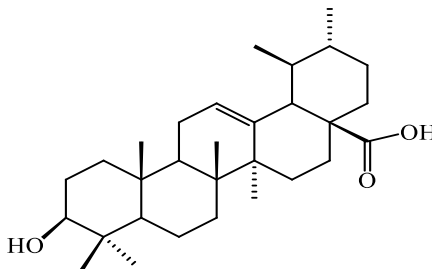
- **Oleanolic acid (12)**



(4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-hydroxy-2,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydronicene-4a-carboxylic acid

Physical status	White needle crystal
Yield	790 mg
M.P	294-297 °C
IR (cm ⁻¹)	2933, 2875, 1688
UV $\nu_{\max}$	247
EIMS	456
Mol:formula	C ₃₀ H ₄₈ O ₃
Chemical name	(4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-hydroxy-2,2,6a-6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydronicene-4a-carboxylic acid
¹ H-NMR (CDCl ₃)	Table 17
¹³ C-NMR (CDCl ₃)	Table 17

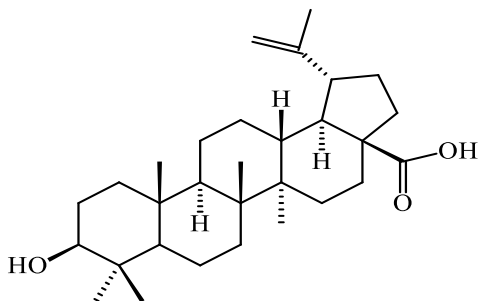
- Ursolic Acid (13)



(1S,2R,4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid

Physical status	White needle crystal
Yield	970 mg
M.P	284-287 °C
IR (cm ⁻¹)	2933, 2875, 1688
Uv v _{max} (nm)	245
EIMS	456
Mol: formula	C ₃₀ H ₄₈ O ₃
Chemical name	(1S,2R,4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid
¹ H-NMR (CDCl ₃)	Table 18
¹³ C-NMR (CDCl ₃)	Table 18

- **Betulinic acid (14)**



(3 β)-3-Hydroxy-lup-20(29)-en-28-oic acid

Physical status	White needle crystal
Yield	1.10 g
M.P	290-293 °C
IR (cm ⁻¹)	2932, 2871 ,1685
Uv max (nm)	254
EIMS	456
Mol: formula	C ₃₀ H ₄₈ O ₃
Chemical name	(3 β)-3-Hydroxy-lup-20(29)-en-28-oic acid
¹ H-NMR (CDCl ₃)	Table 19
¹³ C-NMR (CDCl ₃)	Table 19

4.6. Pharmacological screening of *D. lotus*

4.6.1. *In-vitro* screening

4.6.2. Urease inhibition assay

The urease inhibition action of crude extracts, its fractions and isolated compounds was performed according to standard protocol [113]. The reaction mixture for urease inhibition comprising 25 μ L canavalia ensiformis (Jack bean) and buffer (55 μ L) at pH 6.8, urea (100 mM) and 5 μ L of numerous concentrations of test extracts/compounds (from 0.5 to 0.00625 mM) in 96-well plates the incubation were done for 15 min at 30 $^{\circ}$ C. In kinetics experiments, numerous concentrations of the tested constituents and substrates were used. Then phenol (45 μ L) and alkali 70 μ L reagent were added to each well. Urease inhibition potential were measured by the production of NH_3 according as per standard protocol [113]. The increasing in absorbance at 30 nm was measured after 50 min using micro-plate reader. The reactions were done in triplicate in final volume (200 μ L) [114]. The standard drug used was thiourea and percentage activity were calculated by following formula:

$$\% \text{ inhibition} = 100 - (\text{OD}_{\text{testwell}} / \text{OD}_{\text{control}}) \times 100.$$

4.6.3. Phosphodiesterase-I inhibition assay

The phosphodiesterase-I activity of extracts and compounds were performed by standard procedure. In venom of snake phosphodiesterase-I activity the reaction mixture comprising Tris-HCl buffer (33 mM), magnesium acetate (30 mM) buffer at pH 8.8 and various concentrations of screen constituents and enzyme (0.742 mU/well) were incubated at 37 $^{\circ}$ C for 30 min. A multi-plate reader was used to read the plates, Spectramax plus 384 (Molecular Device, CA, USA) at 410 nm after the addition of 0.33 mM bis-(*p*-nitrophenyl) phosphate [115].

4.6.4. Carbonic anhydrase-II assay and inhibition

The carbonic anhydrase action was performed according as per standard protocol [116]. The experiment was run in the buffer comprising HEPES-Tris solution 20 mM concentration and at pH range from 7.2-7.9. The HEPES-Tris solution (140 μ L) was combine with 20 μ L of recently ready aqueous solution of pure bovine erythrocyte CA-II (0.1-0.2 mg/2000 μ L of the deionized water for 96-well), Fluka MP Biomedicals. The sample were dissolved in 10% DMSO, out of this 20 μ L were combine to the reaction mixture, following by the the adding of 4-NPA (0.8 mM) diluted in ethanol. The reaction was started by addition 4-NPA after 15 min incubation of compound the results were calculated in triplicate. To initiate the reaction, the plate was placed in a microplate reader and the amount of reaction product formed was observed at 1 min interval for 30 min at 400 nm under ambient temperature.

4.6.5. Chymotrypsin inhibition assay

The chymotrypsin inhibition activity was performed according as per standard protocol [117]. The assay was achieved in Tris-HCl buffer (50 mM), CaCl (10 mM) at pH 7.6 according to reported procedure in literature [117]. α -Chymotrypsin (12 U/mL prepared in buffer) and the screen extracts, factions and constituents (0.5 mM prepared in DMSO) were incubated for 25 min at 30 $^{\circ}$ C. The reaction mixture was started by adding N-succinyl-L-phenylalanine-p-nitroanilide (0.4 mM; in buffer). The modification in absorbance was continuously observed at 410 nm. The negative control without enzyme and the positive control without samples or with standard drug were run in corresponding. The % activity was calculated as:

$$\% \text{ inhibition} = 100 - (\text{OD}_{\text{testwell}} / \text{OD}_{\text{control}}) \times 100.$$

4.6.6. DPPH free radical scavenging assay

The antioxidant activity was performed by DPPH radical scavenging assay as per standard protocol [118]. The electron donation or hydrogen atom abilities of the conforming crude extracts, its fractions, constituents, oils and standards were recorded from the lightening of the purple-colored methanol solution of 2,2-diphenyl-1-picrylhydrazyl(DPPH). Experiments were performed in triplicate. 1mM of DPPH radical solution (stock solution) was prepared in methanol and then 1ml of stock solution was combine with 4-4.8 ml of sample (constituents, extracts, fractions) solutions in methanol (10-100 µg/ml) for different fractions while (5-100 µg) for compounds and control. The solution was allowed for 30 min in dark and then absorbance was measured at 517 nm. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. Scavenging of free radicals by DPPH as percent radical scavenging activities (% RSA) was calculated as follows:

$$\% \text{ DPPH} = (\text{OD control} - \text{OD sample}) \times 100 / \text{OD control}$$

Where, OD control is the absorbance of the blank sample, and OD sample is the absorbance of sample or standard sample.

4.6.7. Bacterial strains assortment and preservation

Three selected strains of Gram positive bacteria (*Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Bacillus subtilis*) and two of Gram negative bacteria (*Klebsiella pneumonia* and *Escherichia coli*) were obtained from the stock culture Phytopharmaceutical and Neutraceuticals Research Laboratory (PNRL); Institute of Chemical Sciences, University of Peshawar, Peshawar, Pakistan and stored in Müller-Hinton agar at low temperature (4 °C) prior to subculture.

4.6.8. Antimicrobial assay against selected bacterial strains

The antibacterial activity of the crude extract, fractions and chemical constituents isolated from *D. lotus* was performed as per standard protocol [110]. Agar well diffusion assay was approved to assessment the antibacterial potential of the extract, fractions and chemical constituents by using Muller Hinton agar as medium. The cultures were prepared in triplicates and incubated at 37 °C for a period of 24 to 72 hours and then 0.6 mL of the broth culture of the organism was placed in a sterile Petri-dish and added 20 mL of the sterile molten MHA. Wells were bored in the medium and 0.2 mL of extract/fractions and isolated compounds were introduced to each well using micropipette while the standard drug use was streptomycin. The incubation was achieved 1 hour to confirm the diffusion process of the antimicrobial agent in the the medium. The plates were incubated for 24 hour at 37 °C and the zone of inhibition was measured in millimeters.

4.6.9. Antifungal assay

The antifungal efficacy of the extract and its fractions was investigated according to reported method [119]. Tube dilution technique was performed for antifungal activity of extract/fractions and compounds of various parts *D. lotus*. The samples (22 mg/ ml) were dissolved in DMSO to prepared stock solution. Sabouraud dextrose agar (SDA) (4 mL) was distributed into tubes and then autoclaved at 120 °C for 15 min and allowed to cooled at 15 °C. The non solidified SDA media was also infected with stock solution (66.6 µL) which gives the final concentration of 400 µg of each extract/fractions per mL of SDA. Finally the tubes were allowed to solidify in the slanted position at 25 °C. Then each tubes were inoculated with a piece of 4 mm diameter in inoculums which was removed from a 7 days old culture of fungal strain for non-mycelial growth; an agar surface streak was employed.

Amphotericin-B was used as a standard antifungal drug. Inhibition of fungal growth was recorded after seven days of incubation at room temperature ($28 \pm 1^{\circ}\text{C}$) and comparative humidity (40-50 %). The screening was analyzed for the visible growth of the fungal strain and % antifungal activity was determined.

4.6.10. Brine shrimp cytotoxic assay

In-vitro brine shrimp cytotoxic activity of *D. lotus* the crude extract and its fractions was performed as per to published protocol [120]. The samples (extract/fractions) were prepared in particular solvents of three concentrations (10, 100, and 1000 $\mu\text{g/mL}$). *Artemia salina* Leach (Brine shrimp) nauplii were hatched in a precise tank at 25°C in sea water. 5, 50 and 500 $\mu\text{g/mL}$ from stock solution was injected into 9 vials (3 vials for each dilution). Now each vial comprises 5 mL of brine and 10 shrimps. The shrimps were added with suspension of dry yeast which serve their food and the incubated was achieved under illumination for 24 h. Cytotoxic activity was determined by analysis, the number of live larva in each vial were counted by using magnifying glass and at each dose the percent deaths thus were calculated. The subsequent data were calculated by using Graph Pad Prism version 6. LD_{50} values were the mean of three replicates.

4.6.11. Insecticidal activity

The Insecticidal activity of the extract and its fractions were noted according to previously published protocol [121]. The samples were equipped by dissolving 1019 $\mu\text{g/cm}^2$ of extract/fractions and fixed oil in acetone (3 mL) and subjected in a Petri dish which was covered with the filter papers. After 24 hours duration 10 insects were placed in each plate and incubated for 24 hours at 27°C with 50% comparative humidity in the growth slot. The results were assessed as percentage mortality and calculated with reference to controls

(positive and negative). Permethrin, acetone and insects were used as positive and negative controls while permethrin was used as standard drug. The percentage mortality was calculated according to the following formula formula:

Growth regulation (%) = Number of insect alive in test x 100/ Number of insect alive in control

4.6.12. Antiproliferative assay

Three new compounds (**1-3**) isolated from *D. lotus* roots were also subjected to anticancer activity according to standard protocol [122]. The effects of accumulative concentrations of constituents on cell growth were identified in 96-well flat bottomed micro-titer plates. The constituents **1** and **3** were diluted in volume of 100 µl in McCoy's 5A or RPMI-1640 medium, respectively. For the antiproliferative assay, 6×10^3 cells of mouse T-cell lymphoma cells in 100 µl of medium were introduced to each well. The culture plates were additionally incubated at 37 °C for 72 h and at the end of incubation period, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (20µl) (Sigma, St. Louis, MO. USA) solution (from 5 mg/ml stock) were subjected to all well, then after 4 h, 10% sodium dodecyl sulfate (SDS) (100 µl) (Sigma) and HCl (0.01 M) was measured in each well. The incubation of culture plates was achieved at 37 °C. The cell growth was identified by determining the optical density (OD) at 550 nm (ref. 630nm) with a Multiscan EX ELISA reader (Thermo Labsystem, Cheshire, WA, USA). Cell growth inhibition was calculated by following formula:

$$\% \text{ inhibition} = 100 - \left[\frac{OD_{\text{sample}} - OD_{\text{medium control}}}{OD_{\text{cell control}} - OD_{\text{medium control}}} \right] \times 100$$

4.6.13. Assay for reversal of MDR in mouse lymphoma cells

Compounds (**1-3**) of *D. lotus* roots were subjected to reversal of MDR in mouse lymphoma cells as per reported procedure [122]. The MDR (L5178 and L5178Y) parental cell lines were grown in McCoy's 5A the medium comprise heat inactivated horse serum (10%), which was completed with L-glutamine and antibiotics. The cell lines was familiar to a density of 2×10^6 mL re-suspended in serum-free McCoy's 5A medium and then divided in 0.5 mL aliquots into Eppendorf centrifuge tubes. The compounds (**1-3**) were subjected at numerous concentrations in various volumes (2-20 μ L) of the stock solutions (0.1-1 μ g/mL) and the incubation was done for 10 minutes at room temperature while verapamil was used as a positive control (10 μ g/ml). Next, 10 μ L (5.2 μ M final concentration) of the indicator rhodamine 123 (Sigma, St Louis, MO, USA) was added to the samples and the cells were incubated further 20 minutes at 37 $^{\circ}$ C, washed twice and resuspended in 0.5 mL PBS for analysis. The fluorescence of the cell population was measured with a Partec CyFlow cytometer (Munster, Germany). Verapamil was used as positive control in the rhodamine 123 exclusion experiments (D). The compounds (**1-3**) were dissolved in DMSO, and it was used as solvent control. The treated of MDR and parental cell lines as associated with the untreated cells percentage of mean fluorescence intensity was calculated while the activity proportion R was calculated by using the following equation (E) which is based on the measured fluorescence values:

$$FAR = \frac{MDR_{treated} / MDR_{control}}{parental_{treated} / parental_{control}}$$

4.7. *In vivo* screening

4.7.1. Analgesic activity

4.7.2.1. Acetic acid induced writhing test

BALB/c mice of either sex ($n = 6$) weighing 18-22 g were used. All animals were withdrawn from food 2 h before the start of experiment and were divided in various groups. Group I was injected with normal saline (10 ml/kg) as control, Group II received standard drug diclofenac sodium (10 mg/kg) while the remaining groups were injected with extract/fractions (50, 100 and 150 mg/kg i.p.), and compounds (5 and 10 mg/kg, i.p.) respectively. After 30 min of the above treatment animals were treated i.p. with 1% acetic acid. The number of abdominal constrictions (writhes) was counted after 5 min of acetic acid injection for the period of 10 minutes according to standard protocol [123].

4.7.1.2. Formalin test

The formalin test was performed of the crude extract, fractions and pure constituents. The animals were organized into various groups ($n = 6$) and feed with saline (10 ml/kg), crude extract/fractions (25, 50 and 100 mg/kg i.p.), 7-methylejuglone (5 and 10 mg/kg i.p.). To induce pain 2 % formaldehyde (0.05 ml) was injected into the right hind paw of each mice, after 30 min treatment of all above described animal groups. Analgesic action was measured by the time spent by walking or stands on the right hind; moderately elevated paw; total elevation of injected paw, injected paw licking/biting. The first 0-5 mint was calculated as the first phase (neurogenic) and 25-30 mint as last phase in the assay. Tramadol^R (30 mg/kg i.p.) was used as standard drug according to standard procedure [124].

4.7.1.3. Hot plats test

BALB/c mice of either sex (n=6) weighing 18-22 g were acclimatized to laboratory conditions one hour before the start of experiment with food and water available *ad-libitum*. Animals were then subjected to pre-testing on hot plate (Havard apparatus) maintained at 55 ± 0.1 °C. Animals having latency time greater than 15 seconds on hot plate during pre-testing were rejected (latency time). All the animals were divided in various groups each of six mice. Group I was treated with saline (10 ml/kg), group II was treated with tramadol^R (30 mg/kg i.p) while the remaining groups were injected with extract/fractions (50, 100 and 150 mg/kg i.p), compounds and (5 and 10 mg/kg, i.p.), respectively. After 30 min of the treatment; animals were placed on hot plate and the latency time (time for which mouse remains on the hot plate (55 ± 0.1 °C) without licking or flicking of hind limb or jumping) was measured in seconds. In order to prevent the tissue damage a cut-off time of 30 seconds were imposed for all animals. To find out the opiodergic mechanism in the analgesic activity of chloroform fractions , **9** and **10** some groups were treated with naloxone (0.5 mg/kg s.c.) and after 10 min these groups were treated with extracts (100 and 150 mg/kg i.p), compounds (10 mg/kg, i.p.) and tramadol^R (30 mg/kg i.p.), after 10 min of naloxone injection. The latency time for all groups was recorded at 0, 30, 60, 90 and 120 min. Percent activity was calculated using the following formula [123].

$$\% \text{ Analgesia} = (\text{Test latency} - \text{control latency}) / (\text{Cut} - \text{off time} - \text{control latency}) \times 100$$

4.7.2. Anti-inflammatory activity

The anti-inflammatory activity extracts and its fractions were performed on the mices of both sexes (25-30 g). The animals were distributed in to various groups of each of sex. Group I was feed with normal saline (10 ml/kg), group II with diclofenac sodium (10

mg/kg), while rest of the groups were feed with extract, fractions and compounds at the same dose as in case of analgesic activity. After 30 min intra-peritoneal administration; carrageenan (1%, 0.05 ml) was injected subcutaneously in the sub plantar tissue of the right hind paw of each mouse. The inflammation was measured using plethysmometer (LE 7500 plan lab S.L) immediately after injection of carrageenan and then after 1, 2, 3, 4 and 5 hrs of carrageenan injection. The average foot swelling drug treated animals as well as standard was compared with that of control and the percent inhibition of edema was determined using the formula [123].

Percent inhibition = $A-B/A \times 100$, where A represent edema volume of control and B as paw edema of tested group.

4.7.2.1. Open field test

The apparatus used for this activity was consisted of an area of white wood (150-cm diameter) enclosed by stainless steel walls and divided in 19 squares by black lines. The open field was placed inside a light and sound-attenuated room. BALB/C mice of either sex (n=6) weighing 22 ± 2 g were used in this study. Animals were acclimatized under red light (40 watt red bulb) one hour before the start of experiment in laboratory with food and water available *ad libitum*. The animals were treated with saline, diazepam (reference drug, 0.5 mg/kg i.p.) extract, fractions (50, 100 and 150 mg/kg i.p.) and compounds (5 and 10 mg/kg, i.p.). After 30 min of the treatment each animal was placed in the center of the box and the numbers of lines crossed were counted for each mices [123].

4.7.3. Acute toxicity test

The acute toxicity action of crude extract, fractions and compounds were carried out to assess any probable toxicity. BALB/c mice (n= 6) of both sex were tested by administering different doses (500, 1000 and 2000 mg/kg) of extract, fractions and compounds (25 and 50 mg/kg), while the control group were feed with distilled water (1 mL/kg). All the groups were practical for any gross effect for first 4 hours and then mortality was recorded after 24 hrs [123].

4.7.4. Influence on motor coordination in the chimney test

30 cm long and 3 cm diameter pyrex glass tube was used in this screening. From the base the tube is marked at 20 cm and animals were screened after 30, 60 and 90 min of treatment. Various groups (n=5) were treated with normal saline (10 ml/kg), diazepam (0.5 mg/kg), and test molecules (5 and 10 mg/kg i.p). The animal were introduced at one edge of the tube and then permitted to move up to the marked at 20 cm from the base. When the animal touched the 20 cm mark, then tube was moved directly to the vertical position, the animal tried to climb again to the tube with a backward effort. The mouse which unsuccessful to reach up to the mark within 30 seconds was assumed with relaxed muscles [125] .

4.7.5. Influence on motor coordination in the inclined plane

The plane used in this assay was containing of two play-wood boards in which both boards were associated with each other in such a mode that one board form the base and other is fixed with the base at 65 degrees. Various groups (n=6) were treated with diazepam (0.5 mg/kg), normal saline (10 ml/kg) and test constituents (5 and 10 mg/kg i.p). After 30, 60 and 90 min of treatment the animals were located on the upper part of the inclined plane for 30 seconds to hang or fall [126].

4.7.6. Antipyretic test (yeast induced pyrexia)

The antipyretic effect of the crude extract and its fractions was performed as per standard protocol [127]. The antipyretic activity was determined in BALB/C mice (25-27 g) of both sexes. The animals were separated in five groups (n=5). All groups were fasted overnight and permitted free accesses to drinking water. Group one received saline as control group, second group received para-acetylamino phenol as standard drug while the remaining groups received 50 and 100 mg/kg of extract/fractions. Pyrexia was induced in all mice by injecting aqueous suspension (20%) of Brewer's yeast (10 mL/kg) at room temperature. Rectal temperature was noted after 24 hrs and equivalent groups were injected with various doses. The rectal temperature was noted occasionally at 1.5, 3 and 5 hrs of drugs administration.

4.7.7. Statistical analysis

The results are represented as mean $\pm$ S.E.M. on the one-way ANOVA software, which was used for assessment test of significant changes among groups followed by Dunnett's multiple evaluation posttest as discussed previously [125]. The level of significance ($P < 0.05$) was measured for each assay.

4.8. GC-MS analysis

The GC-MS investigation of fixed oil isolated from *n*-hexane and chloroform fractions followed by reported procedure in which formation of methyl ester (FAMES) by methylation of fatty acids [128]. To prepare sample for GC-MS analysis 25 mg fixed oil was introduced to 0.1 mL internal standard and 1.5 mL of sodium hydroxide solution in methanol (0.5 N), the samples were sealed and then put in water bath to boil for 5 minutes. 2.5 mL of BF_3 was added to the cooled hydrolyzed sample in 10% methanol. The

esterification of fatty acids is achieved by dissolving 5 mL of brine in fixed oil and then extracted three times with 1 mL *n*-hexane. The *n*-hexane soluble portion was filtered through filter paper and injected 1 μ l to GC-MS by auto injector system.

4.9. Molecular docking simulation

Compounds **9** and **10** were also subjected for docking simulation using FRED 2.1 to dock the OMAGA generated conformer [129]. FRED 2.1 approach is thoroughly used to dock/score all potential site of each ligand in the binding place. The comprehensive search was grounded on the rigid spins and translations of the each conformer inside the binding position distinct by box. FRED 2.1 filtered positions collective by rejecting that clash with the protein (LOX) or does not have contact with LOX. The last positions can be then the scored using one or several scoring functions. In our finding, the smooth shaped Gaussain scoring function (shapegauss) was designated to estimate the shape complementarily between each ligand and binding pocket. Defaults FRED procedure was used except for the size of the box defining the binding sites which was an effort to optimized the docking-scoring potential simulation shapegauss a relating the optimization method. The optimization mode contains a systematic solid body optimization of the top ranked poses from the exhaustive docking. Three different boxes were discovered for LOX (PDB ID: 1JNQ). The various simulations were performed with an added value of 8 Å around the reference ligand.

5.1. Family Anacardiaceae

Anacardiaceae, the Cashew family consist nearly 800 species in 82 genera, including primarily trees and shrubs with resin canals. The members of this family are cultivated throughout the world for their edible fruits, seeds and medicinal compounds [130].

5.1.1. Genus *Pistacia*

The genus *Pistacia* is about 80 million years old which comprise approximately 20 species that are native of Africa, Southern Europe, Asia and North America [131]. Four species of *Pistacia* (*P. integerrima*, *P. atlantica*, *P. vera*, *P. khinjuk*) are found in Pakistan. *Pistacia* genus has plants which comprise shrubs and small trees ranging in height from 5 to 15 m [131]. The leaves of *Pistacia* are mostly alternate, compound and pinnate, while certain species of *Pistacia* are evergreen and some are deciduous. The whole genus is dioecious but monoecious individual of *P. atlantica* has been noted [132]. The plants of genus *Pistacia* are dioecious, where male and female flowers are on independent trees. Many plants species have adapted to desert or summer drought climates to tolerate in saline soil, and can grow well in water containing salts [133]. Some species of the genus prefer moderate humidity but cannot grow in high humidity and require a drought period annually for proper development. They can survive in temperature range of -10 °C to 45°C. The growth rate of *Pistacia* genus is very slow and takes 15 to 20 years for development, while bear fruits after 7 to 10 years of development. Only female trees of *Pistacia* genus have fruits and rip during the month of August. The leaves are intense bright green (leathery) and have 3 to 9 leaflets, which are alternate, compound and paripinnate. The unisexual flowers are grouped in clusters. The fruits are drupe, size of pea and colour ranges from red to brown. This difference of colour depends upon the maturity degree. The seeds have no endosperm. The

valuable dispersal sources of the seeds are birds. The other sources of plant increase of roots and shoots. The species can hybridize easily between them, but the hybrid plants are difficult to identify. Some plants have their own specific smell, which is bitter, resinous, medicative, or vary intense and aromatic. Some spices develop galls that occur in the leaves by biting of insects. Recently we have reported the phytochemical profile of different solvent extracted fractions of the whole plant of *P. integerrima* [2, 135] which indicates the presence of different classes of secondary metabolites such as alkaloids, terpenoids, flavonoids and tannins.

5.1.2. *Pistacia integerrima*

P. integerrima belongs to family Anacardiaceae and locally known shaani or kakar singhi; which is distributed in the Himalayan range from Indus to Kumaon as well as in various regions of Indo-Pak [134] at high altitude (12000 to 8000 feet). It is a multi-branched, single stemmed, deciduous tree, up to 25 m tall [135] which is mostly asiatic and shows a preference for dry slopes with shallow soils. It does not tolerate fire and is strongly susceptible to acidic soils area and is wind firm, termite resistant, frost hardy and moderately drought resistant. *P. integerrima* well drained deep entisols and inceptisols and is tolerant to heavy clay soils [2,135] .



Figure 40: *Pistacia integerrima* J. L. Stewart ex Brandis

Classification (Taxonomy) of *Pistacia integerrima* J. L. Stewart ex Brandis.

Kingdom:	Plantae (Plants)
Subkingdom:	Tracheobionta (Vascular plants)
Superdivision:	Spermatophyta (Seed plants)
Division:	Magnoliophyta (Flowering plants)
Class:	Magnoliopsida (Dicotyledons)
Subclass:	Rosidae
Order:	Sapindales
Family:	Anacardiaceae
Genus:	<i>Pistacia</i> Stewart.
Specie:	<i>Pistacia integerrima</i> Stewart

5.2. Pharmacological studies

The genus *Pistacia* is famous for their folkloric uses throughout the world due to the presence of bioactive secondary metabolites such as flavonoids, phenol, terpenoids, alkaloids and steroids. The medicinal uses and pharmacological profile of the genus *Pistacia* are given below.

5.2.1. *In vitro* pharmacological studies

5.2.2. Antioxidant effect

The *n*-butanol, ethyl acetate fractions and ethanolic extract of *P. integerrima* and *P. lentiscus* have been reported for antioxidant effects in literature [136-137].

5.2.3. Antimicrobial activity

The crude extracts of *P. integerrima* and *P. lentiscus* have been reported to inhibit the growth of *Phythium ultimum* and *Rhizoctania solanai* fungus [138]. The efficiency of the crude extract and fractions of *P. lentiscus* have been also reported for antimicrobial potential against *M. cavis*, *T. mentagrophytes* and *T. violaceum*. The literature showed that the leaves extract of *P. lentiscus* has antimicrobial properties [139] while the crude extract and fractions of *P. integerrima* and *P. khinjak* also have been reported for antimicrobial activity against various pathogen [140].

5.2.4. Uric acid lowering effect

The ethyl acetate fraction of *P. integerrima* has been reported for uric acid lowering effect in hyperuricemic mice [141].

5.2.5. Wound healing effect

The crude oils and unsaponifiable oily fraction of *P. lentiscus* have been known for healing of wound and considered as active healing agent which is the current application of *P. lentiscus* [142].

5.2.6. Phytotoxicity assay

The crude methanolic extract of *P. integerrima* and its fractions possess important herbicidal activity however; the outstanding activity was revealed by the ethyl acetate, chloroform and methanol fractions at higher concentration [143].

5.2.7. Anticancer effect

The methanolic extract of *P. integerrima* and various fractions have been reported for anticancer activity against human breast cancer cells line. The ethyl acetate and chloroform fractions have showed anticancer efficiency against Michigan cancer cell line [140].

5.2.8. Anti-Helicobacter pylori effect

The resin (mastic gum) extracted from *P. lentiscus* also possess anti-helicobacter pylori effect, against *Helicobacter pylori* while its resin carried many morphological abnormalities and cellular fragmentation in *H. pylori* cells [144].

5.2.10. Anti-inflammatory effect

P. integerrima leaves and galls extracts have been reported for significant ant-inflammatory effect, however the galls extract possess moderate effect against acute and chronic inflammation [134].

5.2.11. Antidiarrheal effect

The extensively literature studies proved that galls of *P. integerrima* are used as raw for the treatment of diarrhea in folkloric system while the methanolic extract has been reported for anti gastrointestinal tract (GIT) motility influence [145].

5.2.12. Toxicity profile

The literature revealed that bark and galls extract of *P. integerrima* was reported for non-toxic [145].

5.2.13. Analgesic profile

Flavonoids of *P. integerrima* showed excellent analgesic properties [145].

5.2.14. Antipyretic effect

The crude extract of *P. integerrima* bark has been reported for antipyretic activity as compared to aspirin [146].

5.2.15. Gastric and duodenal anti-ulcer activity

The mastic obtained from stem of *P. lentiscus* has been reported for the gastric and duodenal ulcers properties [147].

5.2.16. Hepatoprotective effect

P. lentiscus aqueous extract has been reported for *in-vivo* hepatoprotective effect [148].

5.2.17. Hypotensive activity

P. lentiscus aqueous extract have been reported for excellent hypotensive effect against acetaminophen induced liver damage [149].

5.2.18. Antiemetic effect

P. vera aqueous extracts of the leaves and nuts has been reported for excellent antiemetic activity in young chichens as compared to flubenazole which involved both peripheral and central mechanisms [150].

5.3. Phytochemical studies

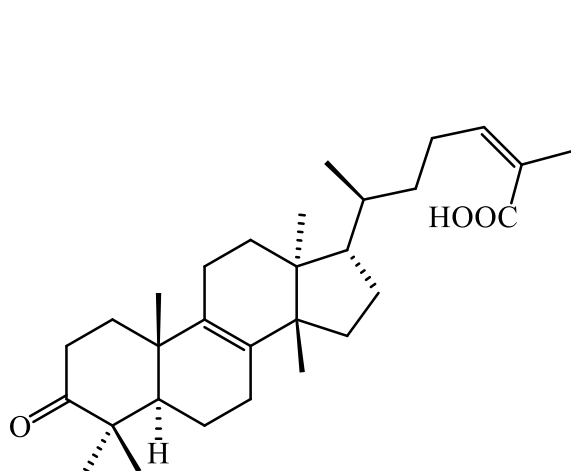
Literature showed genus *Pistacia* is a rich source of different classes of constituents which are summarize in table 33.

Table 33: List of compounds isolated from various species of genus *Pistacia*

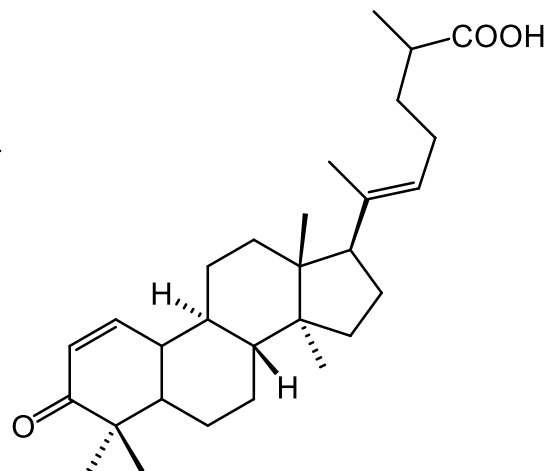
S.No.	Name of compounds	Molecular formula	Sources
1	Pistagremic acid	C ₃₀ H ₄₆ O ₃	<i>P. integerrima</i> [151]
2	Pisticalanstenic acid	C ₃₀ H ₄₆ O ₃	<i>P. integerrima</i> [152]
3	<i>n</i> -Octadecan-9, 11-diol-7-one	C ₁₈ H ₃₆ O ₃	<i>P. integerrima</i> [153]
4	Hydroxydecanyl arachidate	C ₃₀ H ₆₀ O ₃	<i>P. integerrima</i> [153]
5	Pistiphloro-gluciny ester	C ₂₇ H ₃₈ O ₅	<i>P. integerrima</i> [154]
6	Pistaciaphenyl ester	C ₁₇ H ₁₈ O ₅	<i>P. integerrima</i> [154]
7	Pistiphlorogluciny ether	C ₁₄ H ₁₄ O ₅	<i>P. integerrima</i> [154]
8	1-Cyclopropyl-4-methyl-1,3-cyclohexanediol,9Cl.	C ₁₀ H ₁₈ O ₂	<i>P. vera</i> [155]
9	1-Cyclopropyl-4-methyl-3-cyclohexen-1-ol.	C ₁₀ H ₁₆ O	<i>P. vera</i> [156]
10	Masticadienediol	C ₃₀ H ₅₀ O ₂	<i>P. terebinthus</i> [157]
11	3-epimasticadienolic acid	C ₃₀ H ₄₈ O ₃	<i>P. terebinthus</i> [158]
12	Masticadienonic acid	C ₃₀ H ₄₆ O ₃	<i>P. pectigrewianum</i> [158]
13	Isomasticadienonic acid	C ₃₀ H ₄₆ O ₃	<i>P. terebinthus</i> [159]
14	2-Hydroxy-6-(8-tridecenyl) benzoic acid. 6-(8-Tridecenyl) salicylic acid	C ₂₀ H ₃₀ O ₃	<i>P. vera</i> [160]
15	28- <i>nor</i> -20(29)-Lupen-3-one	C ₂₉ H ₄₆ O	<i>P. lentiscus</i> [161]
16	28- <i>nor</i> -12-Oleanen-3-ol	C ₂₉ H ₄₈ O	<i>P. lentiscus</i> [161]
17	3,11,13-Oleananetriol	C ₃₀ H ₅₂ O ₃	<i>P. vera</i> [162]
18	Pistacigerrimone D	C ₃₀ H ₄₂ O ₃	<i>P. integerrima</i> [163]
19	Pistacigerrimone A	C ₃₀ H ₄₄ O ₃	<i>P. integerrima</i> [163]
20	Pistacigerrimone B	C ₃₀ H ₄₄ O ₃	<i>P. integerrima</i> [163]
21	Oleanonic acid	C ₃₁ H ₄₈ O ₃	<i>P. vera</i> [164]
22	Pistacigerrimone E	C ₃₀ H ₄₄ O ₃	<i>P. integerrima</i> [163]
23	Pistacigerrimone C	C ₃₀ H ₄₄ O ₃	<i>P. integerrima</i> [165]

24	Benzenepropanal,9CI,hydrocinnamaldehyde	C ₉ H ₁₀ O	<i>P. lentiscus</i> [166]
25	Pistacimidelor	C ₂₄ H ₃₈ N ₂ O	<i>P. atlantica</i> [167]
26	1,4-Poly- β -myrcene	C ₁₀ H ₁₆	<i>P. lentiscu</i> [168]
27	13,17,21-Polypodatriene-3,8-diol	C ₃₀ H ₅₂ O ₂	<i>P. lentiscus</i> [169]
28	13,17,21-Polypodatriene-8-diol	C ₃₀ H ₅₀ O ₂	<i>P. lentiscus</i> [169]
29	3-Methoxycarpachromene	C ₂₁ H ₁₈ O ₆	<i>P. atlantica</i> [170]
30	Tirucalla-8,24-dien-3-one (Tirucallone)	C ₃₀ H ₄₈ O	<i>P. terebinthu</i> [171]
31	Pistacigerrimone F	C ₃₀ H ₃₆ O ₅	<i>P. integerrima</i> [165]
32	Oleanolic acid	C ₃₀ H ₄₈ O ₃	<i>P. terebinthu</i> [170]
33	Luteolin	C ₁₅ H ₁₀ O ₆	<i>P. atlantica</i> [172]
34	Merulinic acid A	C ₂₄ H ₃₈ O ₄	<i>P. vera</i> [172]
35	3-O-galloylquinic acid	C ₁₄ H ₁₅ O ₁₀	<i>P. weinmannifolia</i> [173]
36	Methyl gallate	C ₈ H ₈ O ₅	<i>P. weinmannifolia</i> [173]
37	Ethyl gallate	C ₉ H ₁₀ O ₅	<i>P. weinmannifolia</i> [173]
38	Penta-O-galloyl- β -D-glucopyranoside	C ₄₆ H ₃₂ O ₂₆	<i>P. weinmannifolia</i> [173]
39	Myricetin 3-O- α -l-rhamnopyranoside	C ₂₁ H ₁₉ O ₁₂	<i>P. weinmannifolia</i> [173]
40	Myricetin-3-O-(3"-O-galloyl)- α -l-rhamnopyranoside	C ₂₉ H ₂₃ O ₁₆	<i>P. weinmannifolia</i> [173]
41	Masticadienonic acid	C ₃₀ H ₄₆ O ₃	<i>P. terebinthus</i> [174]
42	Masticadienolic acid	C ₃₀ H ₄₈ O ₃	<i>P. terebinthus</i> [174]
43	Morolic acid	C ₃₀ H ₄₈ O ₃	<i>P. terebinthus</i> [174]
44	Pistafolian A	C ₂₈ H ₂₄ O ₁₈	<i>P. weinmannifolia</i> [175]
45	Pistafolin B	C ₂₁ H ₂₀ O ₁₄	<i>P. weinmannifolia</i> [175]
46	4-Hydroxy-5-(2-oxo-1-pyrrolidinyl)-benzoic acid	C ₁₁ H ₁₁ O ₄ N	<i>P. chinensis</i> [176]
47	Gallic acid	C ₇ H ₆ O ₅	<i>P. atlantica</i> [177]
48	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	<i>P. atlantica</i> [177]
49	Ellagic acid	C ₁₄ H ₆ O ₈	<i>P. atlantica</i> [177]
50	Sinapic acid	C ₁₁ H ₁₂ O ₅	<i>P. atlantica</i> [177]
51	Protocatechuic acid	C ₇ H ₆ O ₄	<i>P. atlantica</i> [177]
52	Catechin	C ₁₅ H ₁₄ O ₆	<i>P. atlantica</i> [177]
53	Juglone	C ₁₀ H ₆ O ₃	<i>P. atlantica</i> [177]

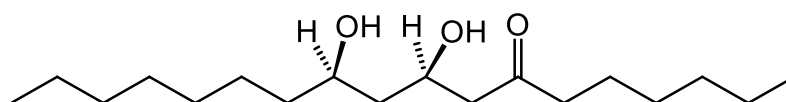
Structures of selected compound's from the genus *Pistacia*



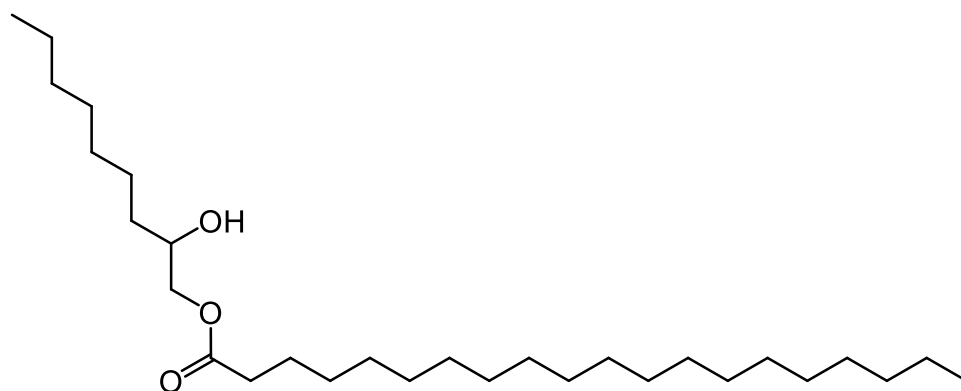
Pistagremic acid [151]



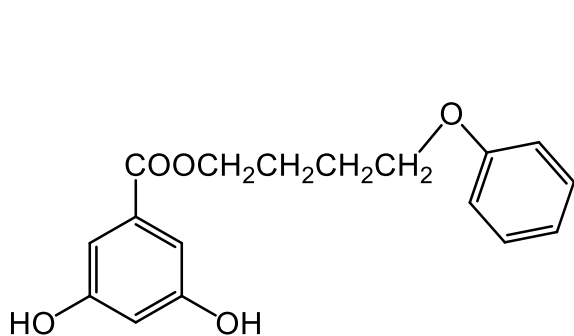
Pisticialanstenic acid [152]



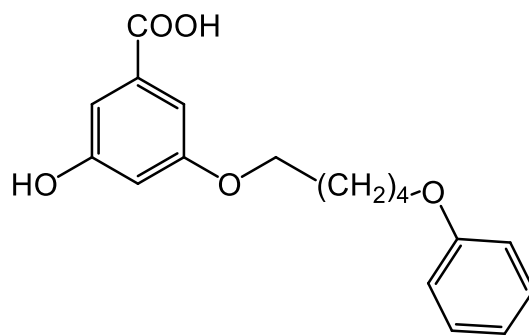
n-Octadecan-9,11-diol-7-one [153]



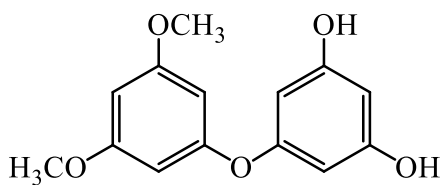
Hydroxydecanyl arachidate [153]



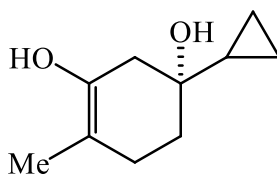
Pistiphloro-glucinyI ester [154]



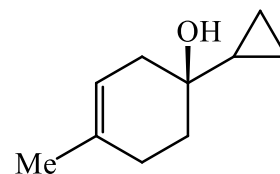
Pistaciaphenyl ether [154]



Pisticphlorogaucynyl ether [154]



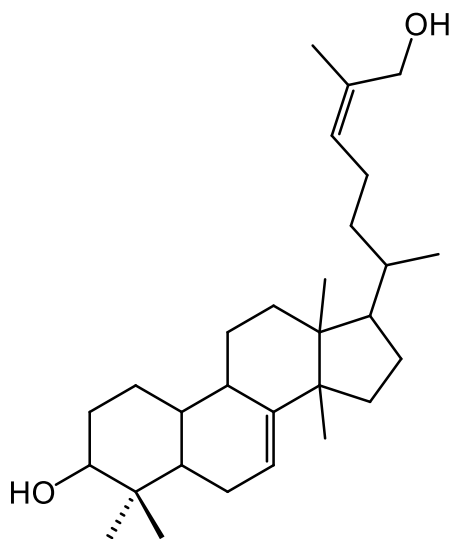
1-Cyclopropyl-4-methyl-1,3-



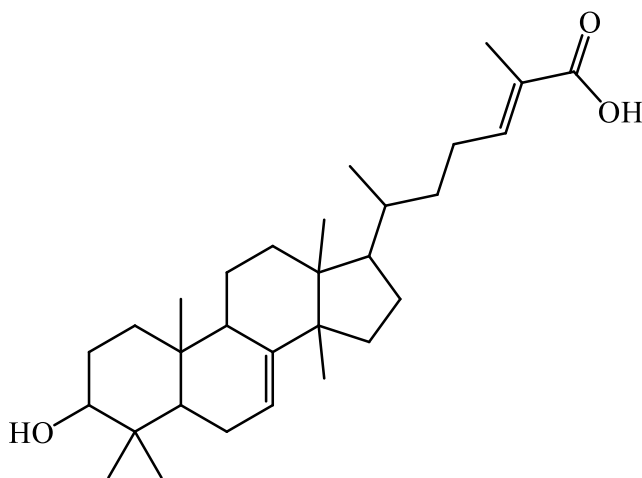
1-Cyclopropyl-4-cyclo

methyl-3-cyclohexanediol,9CI [155]

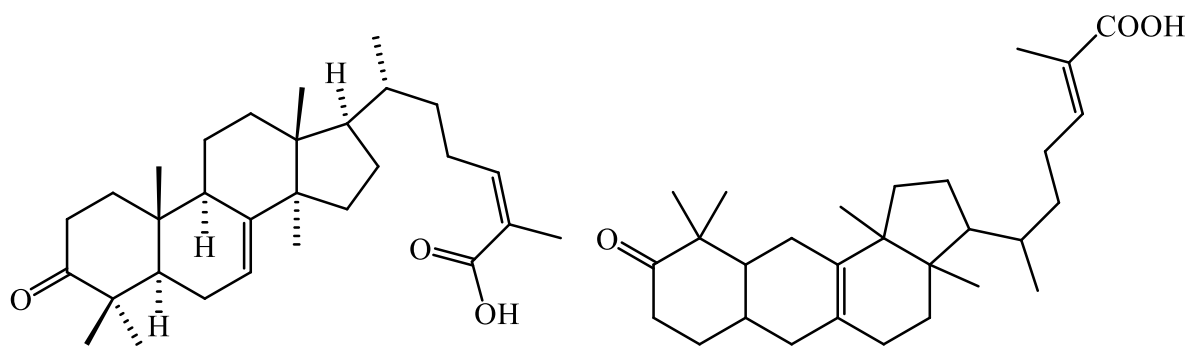
hexen-1-ol,9CI [156]



Masticadienediol [157]

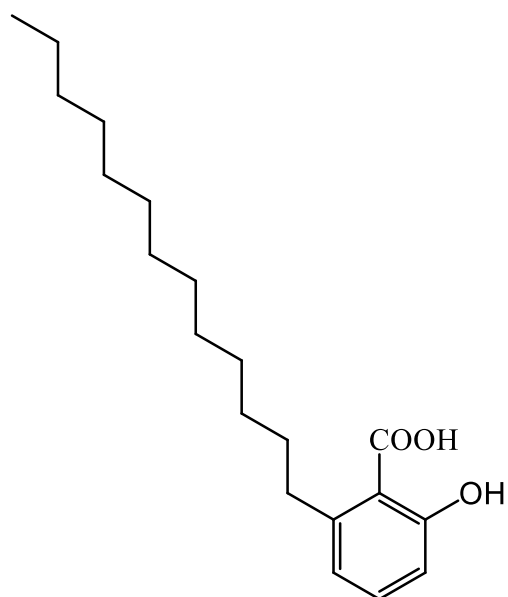


3-Epimasticadienolic acid [158]

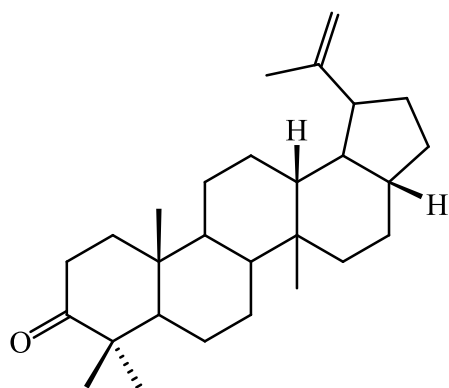


Masticadienonic acid [158]

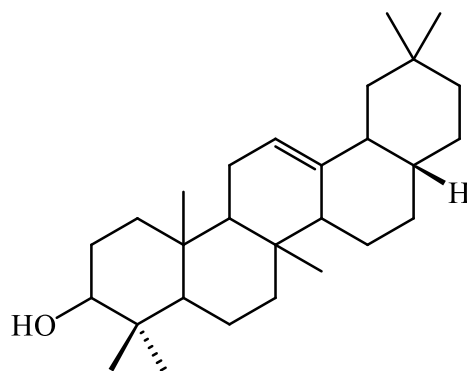
Isomasticadienonic acid [159]



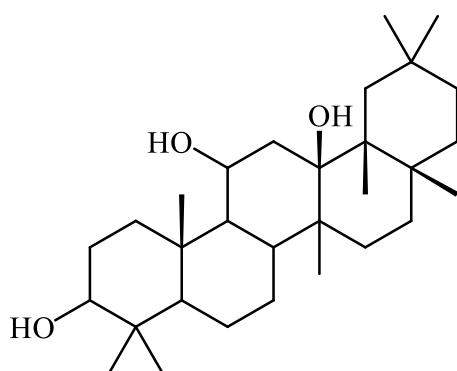
2-Hydroxy-6-(8-tridecenyl) benzoic acid. 6-(8-Tridecenyl) salicylic acid [160]



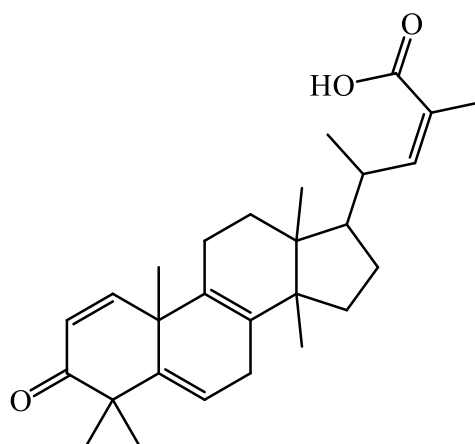
28-nor-20(29)-lupen-3-one [161]



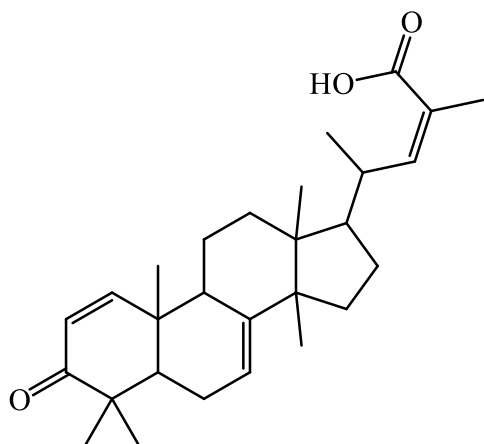
28-nor-12-oleanen-3-ol [161]



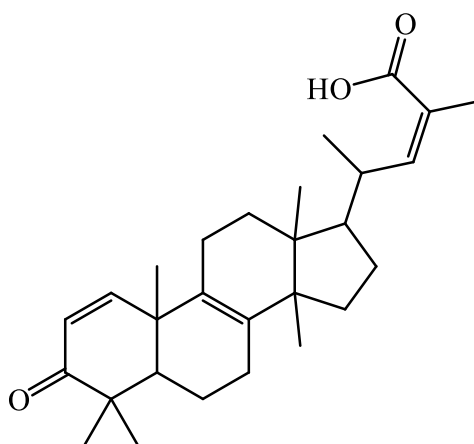
3,11,13-Oleananetriol [162]



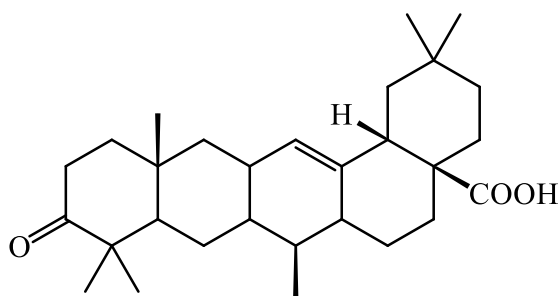
Pistacigerrimone D [163]



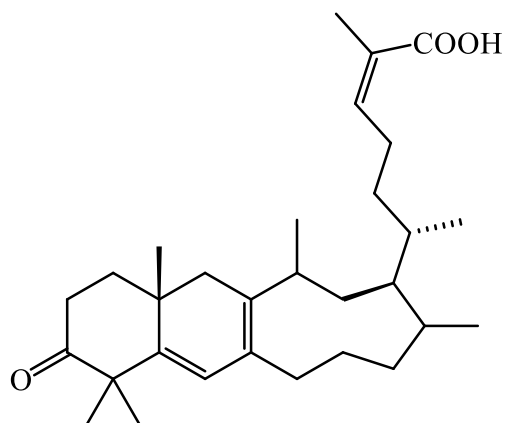
Pistacigerrimone A [163]



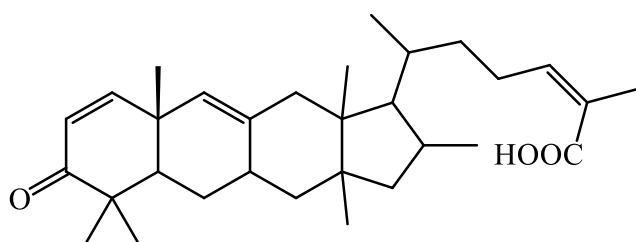
Pistacigerrimone B [163]



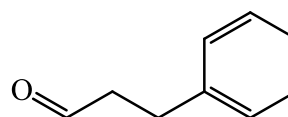
Oleanonic acid [168]



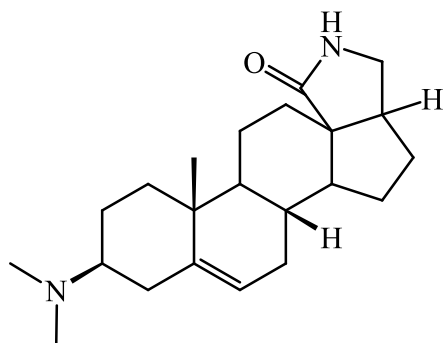
Pistacigerrimone E [163]



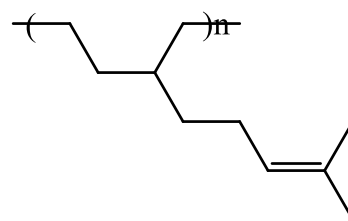
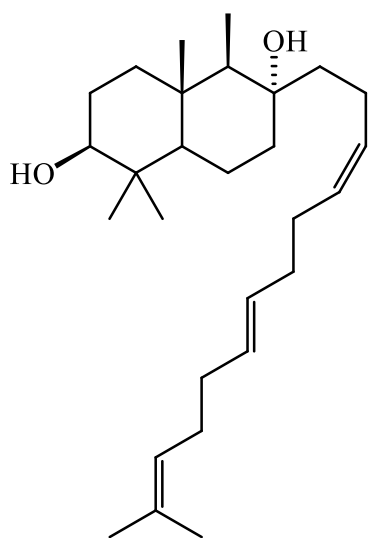
Pistacigerrimone C [165]



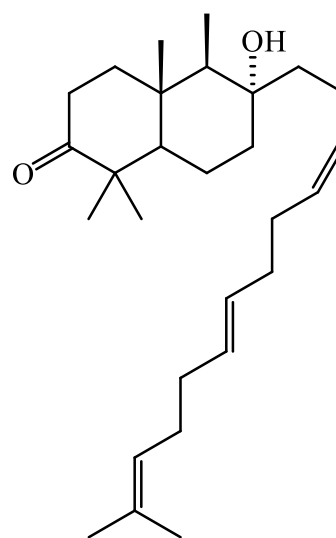
Benzenepropanal [166]



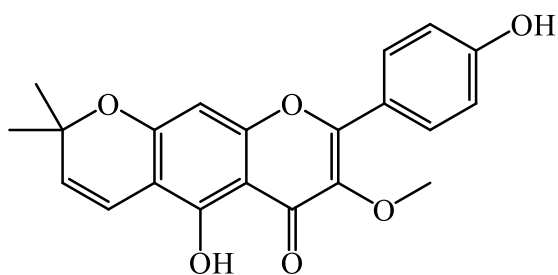
Pistacimidelor [167]

1,4-Poly- β -myrcene [168]

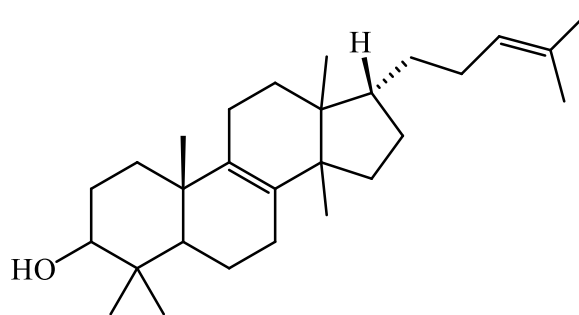
13,17,21-Polypodatriene-3,8-diol [169]



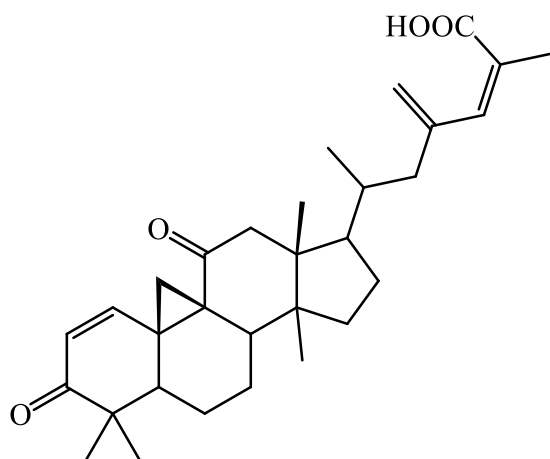
13,17,21-Polypodatriene-8-diol [169]



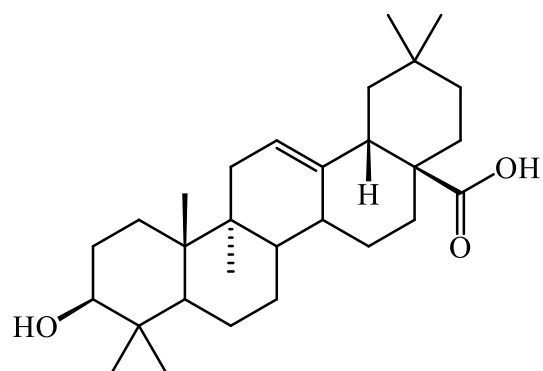
3-Methoxycarpachromene [170]



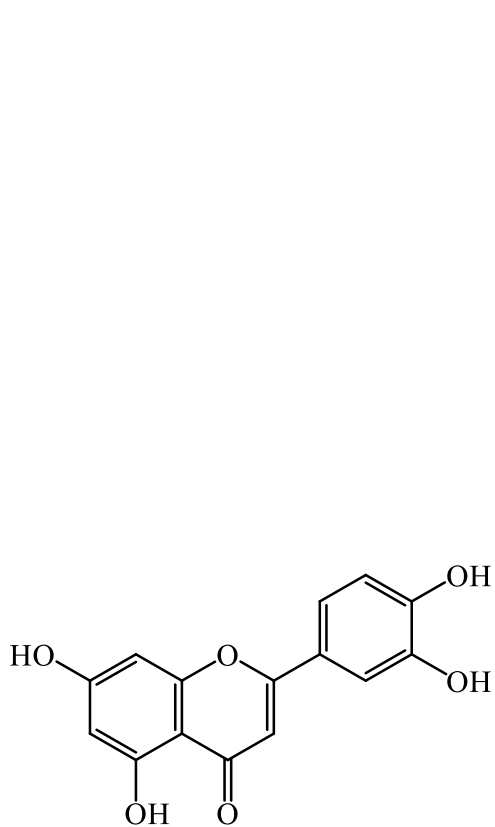
Tirucalla-8,24-dien-3-one.Tirucallone [171]



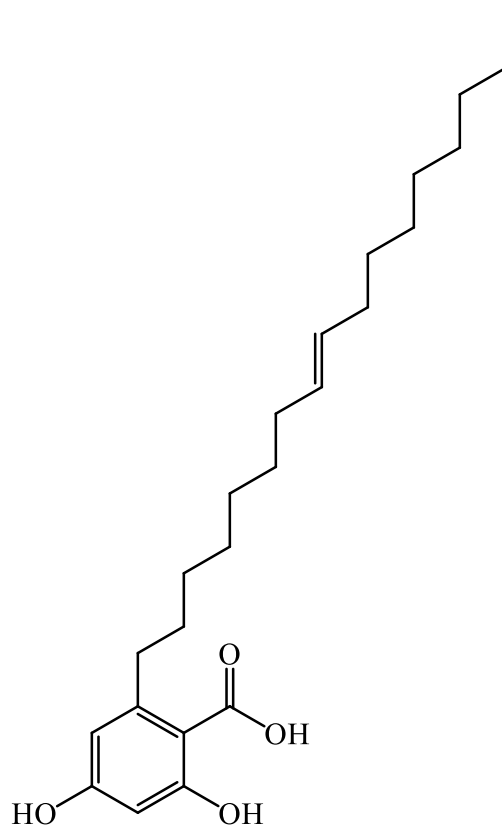
Pistacigerrimone F [165]



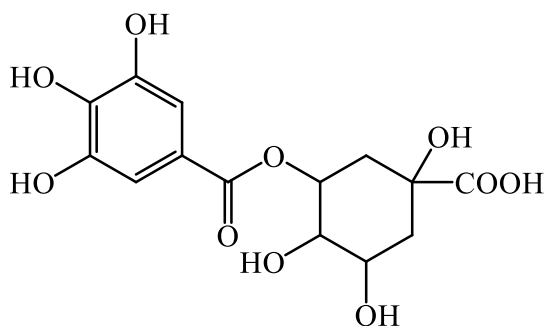
Oleanolic acid [170]



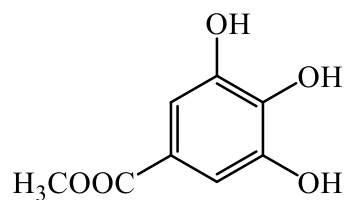
Luteolin [172]



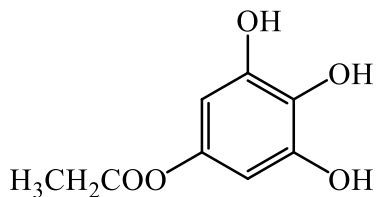
Merulinic acid A [172]



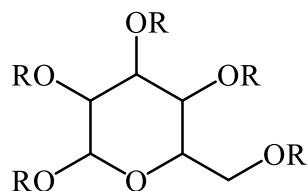
3-O-Galloylquinic acid [173]



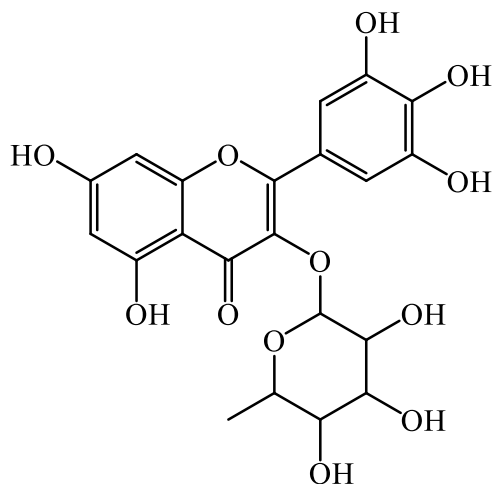
Methyl gallate [173]



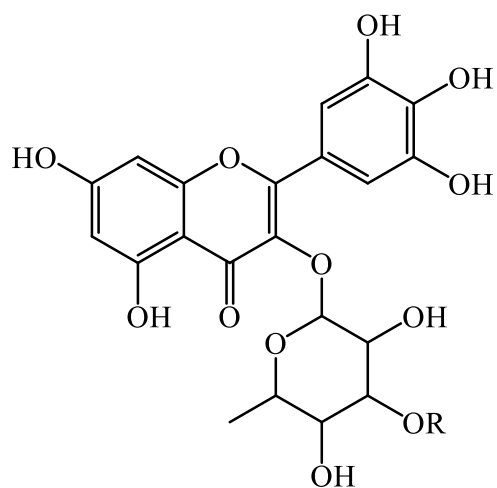
Ethyl gallate [173]



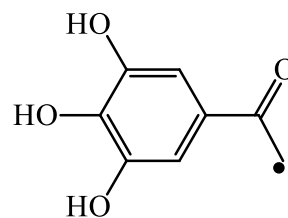
Penta-O-galloyl-B-D-glucopyranoside [173]



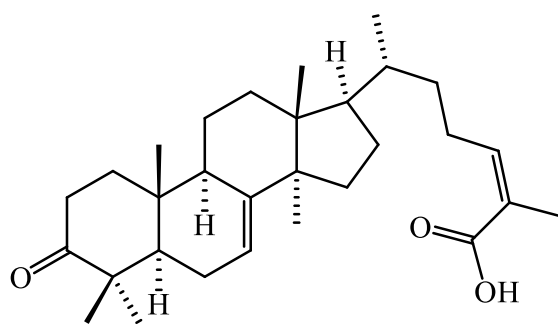
Myricetin 3-O-α-rhamnopyranoside [173]



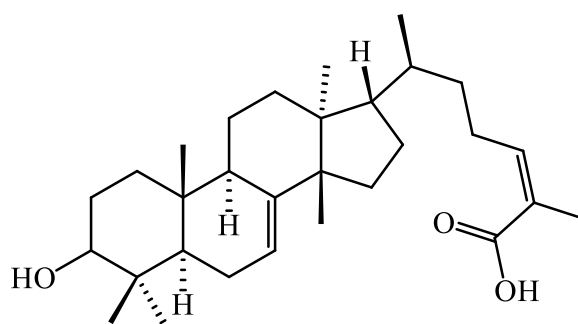
Where **R** is



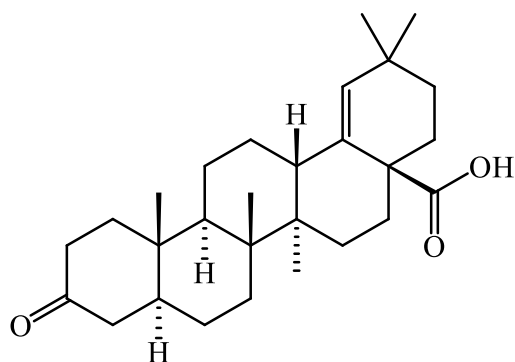
Myricetin- 3-O-(3-O-galloyl)-d-L-rhamnopyranoside [173]



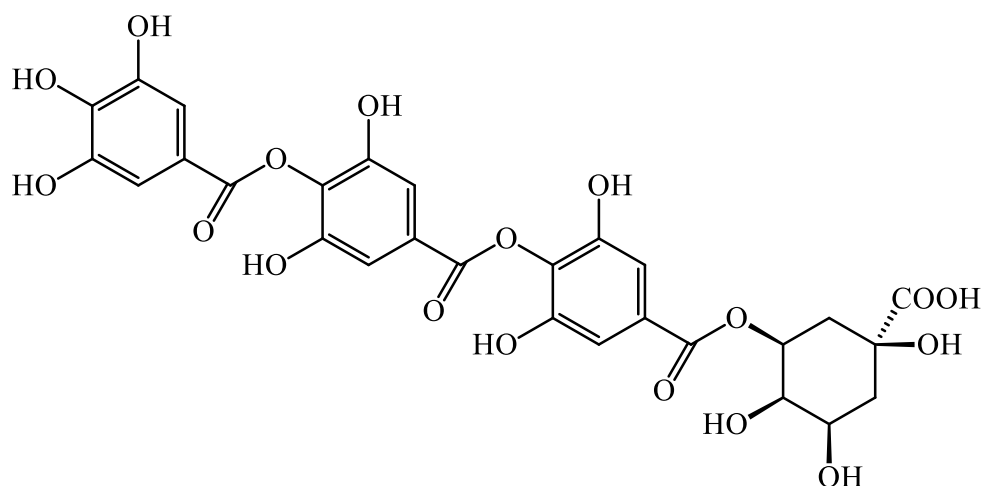
Masticadienonic acid [174]



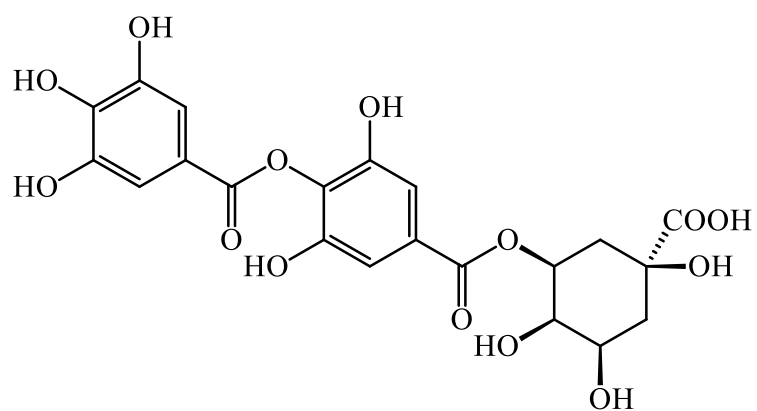
Masticadienolic acid [174]



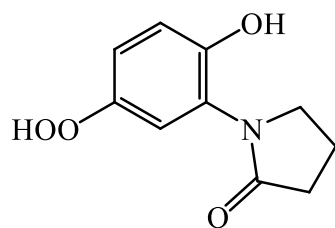
Moronic acid [174]



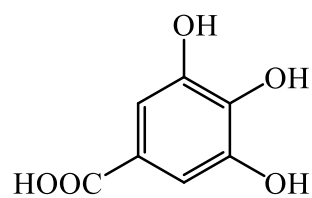
Pistafolian A [175]



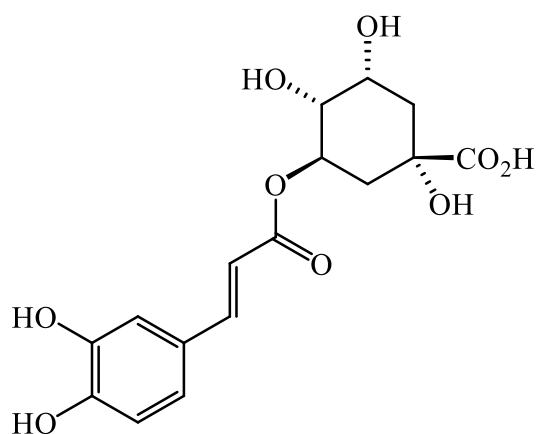
Pistafolin B [175]



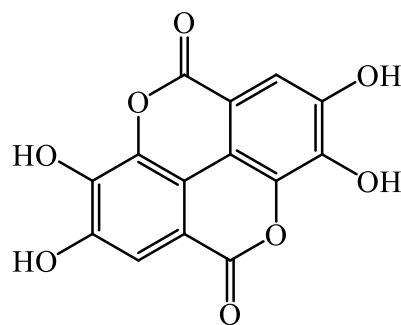
4-Hydroxy-5-(2-oxo-1-pyrrolidinyl)-benzoic acid [176]



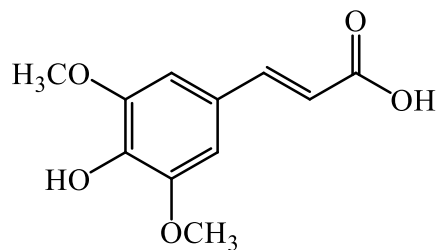
Gallic acid [177]



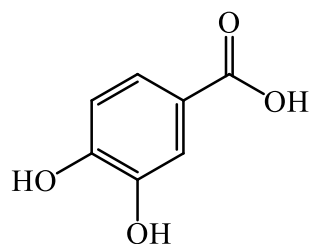
Chlorogenic acid [177]



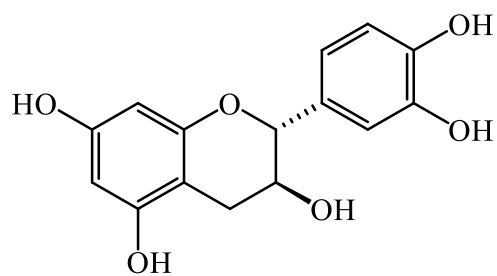
Ellagic acid [177]



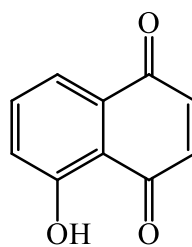
Sinapic acid [177]



Protocatechuic acid [177]



Catechin [177]



Juglone [177]

6.1. Preliminary phytochemical screening of *P. integerrima*

Phytochemical screening is an important step for the isolation of bioactive secondary metabolites from higher plants. The phytochemical analysis of the crude extract and its various fractions were performed as per standard protocol indicated the presence of secondary metabolites, such as alkaloids, terpenoids, flavonoids and reducing sugars. The bio-medicinal importance of *P. integerrima* can be correlated due to the presence of bioactive chemical constituents.

6.1.1. Phytochemical analysis of *P. integerrima* galls

The methanolic extract and various soluble fractions of plant galls were screened for the presence of bioactive secondary metabolites. The methanolic extract, CHCl₃ and EtOAc fractions showed the presence of alkaloids, tannins, reducing sugars, flavonoids, terpenoids, steroids, fatty acids, anthocyanins and betacyanins while the *n*-hexane revealed the presence of fatty acids and steroids (**Table 34**).

Table 34: Phytochemical analysis of the crude extract and fractions of *P. integerrima* galls

Test for constituents	<i>n</i> -Hexane	Chloroform	EtOAc	Methanol
Alkaloids	–	–	+	+
Tannins	–	–	+	+
Anthraquinones	–	–	–	–
Reducing sugars	–	+	+	+
Saponins	–	–	–	+
Flavonoids	–	+	+	+
Glycosides	–	–	–	–
Phlobatanins	–	–	–	–
Terpenoids	–	+	+	+
Steroids	+	+	+	+
Proteins and amino acids	–	–	–	–
Fatty acids	++	+	–	+
Caumarins	–	–	–	–
Emodines	–	–	–	–
Anthocyanins and betacyanins	–	+	+	+
Carbohydrates	–	+	+	+

6.1.2. Phytochemical analysis of *P. integerrima* leaves

The methanolic extract of *P. integerrima* leaves and its fractions were also tested for the bioactive secondly metabolites. The methanolic extract exposed the presence of terpenoids and tannins while the CHCl₃, EtOAc and *n*-hexane fractions indicated the presence of tannins and terpenoids moiety respectively (**Table 35**).

Table 35: Phytochemical analysis of crude extract and its fractions of *P. integerrima* leaves

Test for constituents	<i>n</i> -Hexane	Chloroform	EtOAc	Methanol
Alkaloids	–	–	–	–
Tannins	–	–	+	+
Anthraquinones	–	–	–	–
Reducing sugars	–	–	–	–
Saponins	–	–	–	–
Flavonoids	–	–	–	–
Glycosides	–	–	–	–
Phlobatanins	–	–	–	–
Terpenoids	+	+	+	+
Steroids	–	–	–	–
Proteins and amino acids	–	–	–	–
Fatty acids	–	–	–	–
Caumarins	–	–	–	–
Emodines	–	–	–	–
Anthocyanins and betacyanins	–	–	–	–
Carbohydrates	–	–	–	–

6.1.3. Phytochemical analysis of the bark of *P. integerrima*

The methanolic extract of *P. integerrima* bark and its fractions were also evaluated for phytochemical screening to identify the presence of bioactive natural products. The crude methanolic extract, CHCl₃ and EtOAc exhibited the presence of terpenoids, reducing sugars and steroids while the *n*-hexane fractions showed only the presence of steroids moiety (Table 36).

Table 36: Phytochemical analysis of the crude extract and fractions of *P. integerrima* bark

Test for constituents	<i>n</i> -Hexane	Chloroform	EtOAc	Methanol
Alkaloids	–	–	–	–
Tannins	–	–	–	–
Anthraquinones	–	–	–	–
Reducing sugars	–	+	+	+
Saponins	–	–	–	–
Flavonoids	–	–	–	–
Glycosides	–	–	–	–
Phlobatanins	–	–	–	–
Terpenoids	–	+	+	+
Steroids	+	+	+	+
Proteins and amino acids	–	–	–	–
Fatty acids	–	–	–	–
Caumarins	–	–	–	–
Emodines	–	–	–	–
Anthocyanins and betacyanins	–	–	–	–
Carbohydrates	–	–	–	–

6.1.4. Phytochemical analysis of the roots of *P. integerrima*

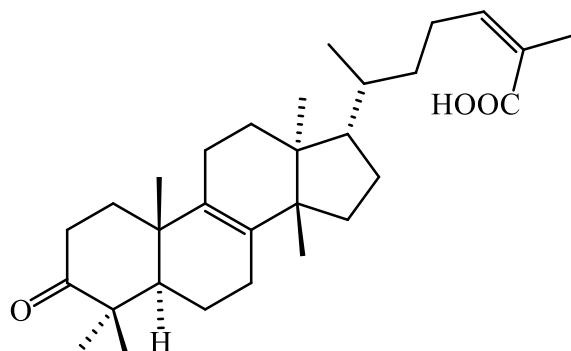
The methanolic extract of *P. integerrima* roots and its fractions were subjected for identification of bioactive natural products. The methanolic extract exhibited the presence of terpenoids, tannins and reducing sugars while CHCl₃ and EtOAc fractions indicated the presence of terpenoids and reducing sugars respectively (**Table 37**).

Table 37: Phytochemical analysis of the crude extract and fractions of *P. integerrima* roots

Test for constituents	<i>n</i> -Hexane	Chloroform	EtOAc	Methanol
Alkaloids	–	–	–	–
Tannins	–	–	–	+
Anthraquinones	–	–	–	–
Reducing sugars	–	+	+	+
Saponins	–	–	–	–
Flavonoids	–	–	–	–
Glycosides	–	–	–	–
Phlobatanins	–	–	–	–
Terpenoids	–	+	+	+
Steroids	–	–	–	–
Proteins and amino acids	–	–	–	–
Fatty acids	–	–	–	–
Caumarins	–	–	–	–
Emodines	–	–	–	–
Anthocyanins and betacyanins	–	–	–	–
Carbohydrates	–	–	–	–

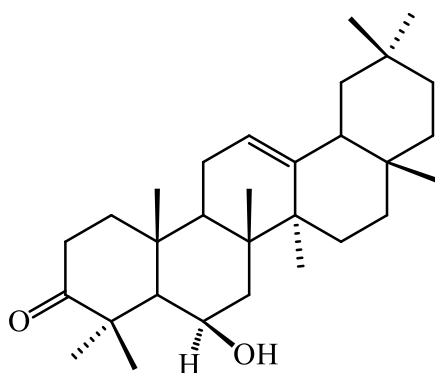
6.2. Chemical constituents of *Pistacia integerrima* (galls, roots and bark)

- New chemical constituents
- Pistagremic acid (15)



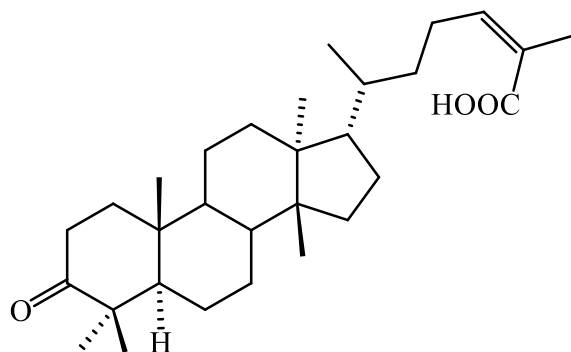
(*E*)-2-methyl-6-(4,4,10,13,14-pentamethyl-3-oxo-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)hept-2-enoic acid

- Shakirullaline (16)



- 3-Keto-6 β -hydroxy- α -amyrin

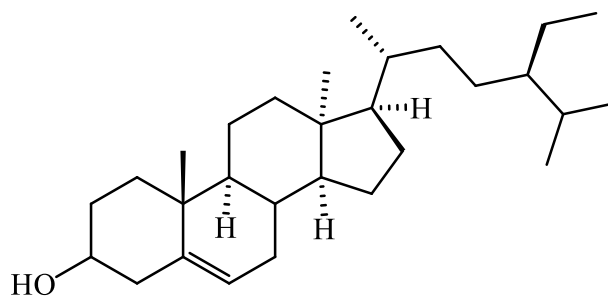
- **Integarrimic acid (17)**



(*E*)-2-methyl-6-(4,4,10,13,14-pentamethyl-3-oxohexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)hept-2-enoic acid

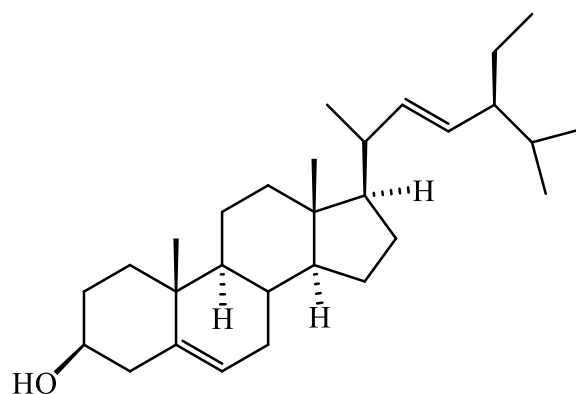
- **Structure of reported chemical constituents**

- **β -Sitosterol (18)**



17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol.

- Stigmasterol (19)



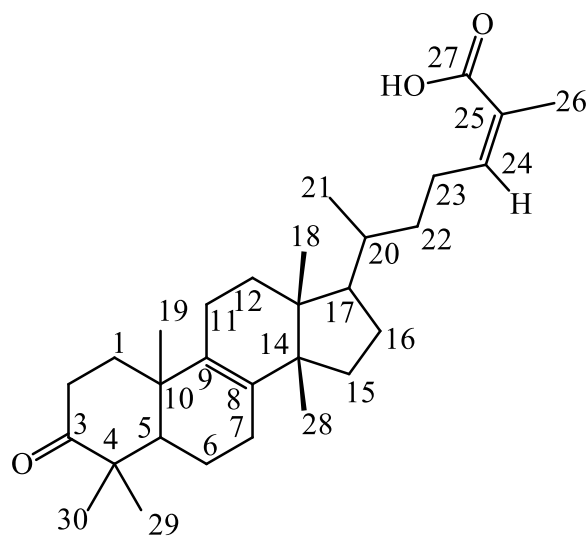
(3S,8S,9S,10R,13R,14S,17R)-17-[(E,2R,5S)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol

6.2.1. Structure elucidation of Pistagremic acid (15)

Compound (**15**) was isolated as white crystalline form the chloroform fraction of *P. integerrima* galls. The IR spectrum (cm^{-1}) of **15** displayed characteristic absorption bands for carbonyl group (1712), carboxylic group (3360), chalcated carboxylic group (1690, 1672) and unsaturation (1612). The UV spectrum showed absorptions at 219, 235 and 265 nm. Its molecular formula was assigned as $\text{C}_{30}\text{H}_{46}\text{O}_3$ based on HR-EIMS (m/z 454.34456; calcd. for $\text{C}_{30}\text{H}_{46}\text{O}_3$, 454.3447). The ^{13}C -NMR (BB, DEPT) spectra (**Table 38**) showed the presence of ketone and carboxylic acid signals at δ_{C} 218.1 and 173.6 ppm, respectively. The ^1H -NMR spectrum (**Table 38**) indicated seven methyl signals; including secondary methyl resonated at δ_{H} 0.91 ($J = 6$, Hz, H-21) and six tertiary methyl singlet at δ_{H} 0.79 (H-4), 0.87 (H-30), 0.98 (H-19), 1.08 (H-28), 1.09 (H-29) and 1.90(H-27). A tri-substituted olefinic proton displayed as a multiplet at δ_{H} 6.06 (1H, H-24) in the proton spectrum of **15**. The structure of **15** was validated through advanced 2D-NMR techniques such as COSY, HSQC, and HMBC. The structure of **15** was confirmed by HMBC correlations (**Figure 41**) which showed cross peaks for H-18 and H-19 to C-13, C-17, C-14 and C-10, C-9, C-5, respectively. Similarly, H-30 of the methyl group exhibited HMBC correlations to C-14, C-8, and C-15. The proton signals of H-1, H-2, H-28 and H-29 possessed HMBC cross peaks for the ketonic carbon assigned to C-3. The olefinic proton (H-24) was correlated to C-23, C-25, and C-26. Furthermore the structure of **15** was confirmed by single x-rays crystallographic technique and related reported structure(**Figure 42**) [134]. Based on the spectral data; compound **15** was assigned as (*E*)-2-methyl-6-(4,4,10,13,14-pentamethyl-3-oxo-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)hept-2-enoic acid.

Table 38: ^{13}C - and ^1H -NMR spectral data for pistagremic acid (**15**)

No	δ_{C}	δ_{H} (mult, J , Hz)	Types	HMBC
1	33.6	1.61, m, 1.71, m	CH_2	C-3
2	34.9	2.50, m	CH_2	C-3
3	218.1	-	C	-
4	47.3	-	C	-
5	52.4	1.69, m	CH	C-2, C-4, C-24
6	21.4	1.72, m, 1.45, m	CH	C-5, C-6
7	26.8	1.92, m, 2.03, m	CH_2	C-5, C-6, C-8, C-14,
8	134.7	-	C	-
9	146.0	-	C	C-10
10	35.6	-	C	-
11	20.3	1.65, m	CH_2	C-6, C-8, C-14, C-26
12	34.6	1.81, m, 1.62, m	CH_2	C-8, C-9, C-11, C-15
13	43.5	-	C	-
14	51.2	-	C	-
15	34.0	1.47, m, 1.50, m	CH_2	C-14, C-17, C-18
16	28.2	1.92, m, 1.23, m	CH_2	C-15, C-17, C-18
17	50.1	1.48, m	CH	-
18	22.0	0.79, s	CH_3	C-13
19	21.6	0.98, s	CH_3	C-18, C-20, C-22, C-29, C-30
20	36.0	1.49, m	CH	-
21	18.2	0.91, d ($J = 6$)	CH_3	C-22
22	35.9	1.60, m, 1.15, m	CH_2	C-17, C-18, C-21
23	26.9	1.20, m, 1.59, m	CH_2	C-3, C-4, C-5
24	147.2	6.06, m	CH	C-23, C-25
25	125.8	-	C	-
26	173.6	-	C	-
27	20.5	1.90, s	CH_3	C-8, C-14
28	26.7	1.08, s	CH_3	C-16, C-17, C-18, C-21, C-22
29	21.1	1.09, s	CH_3	C-20, C-21, C-22
30	24.2	0.87, s	CH_3	C-19, C-20



15

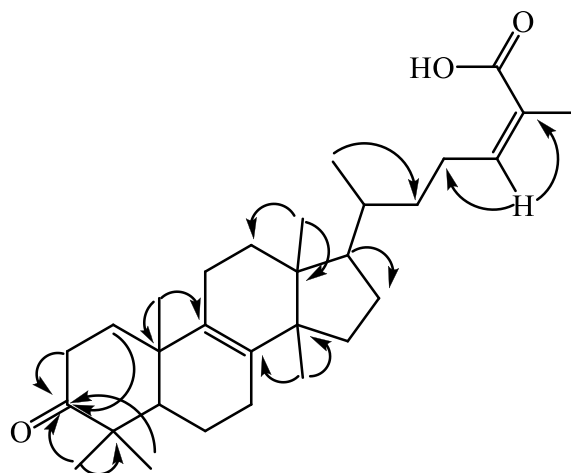


Figure 41: Selected HMBC correlations of pistagrrimic acid (**15**)

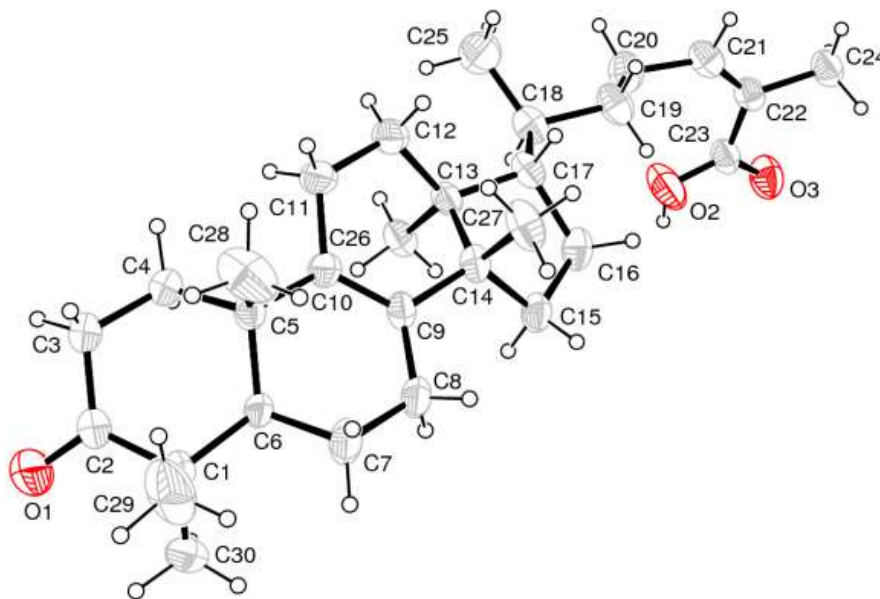


Figure 42: X-ray crystallographic image of pistagrrimic acid (**15**)

6.2.2. Structure elucidation of Shakirullaline (**16**)

Compound **16** was isolated as white crystals from the chloroform fraction of bark of *P. integerrima*. The IR spectrum (cm^{-1}) of **16** displayed characteristic absorption bands for hydroxyl group (3650), carbonyl group (1699), CH stretching saturated (2918) and unsaturation (1559). The UV spectrum exhibited absorptions at 218, 237 and 243 nm. Its molecular formula was determined as $\text{C}_{30}\text{H}_{48}\text{O}_2$ by HREIMS (m/z ; 440.3700 a.m.u; calcd. 440.3710) and ^{13}C -NMR data. The assignment of protons and carbons was carried out by HMBC, HMQC, ^1H - ^1H -COSY and J -resolved experiments (**Table 39** and **Figure 43**).

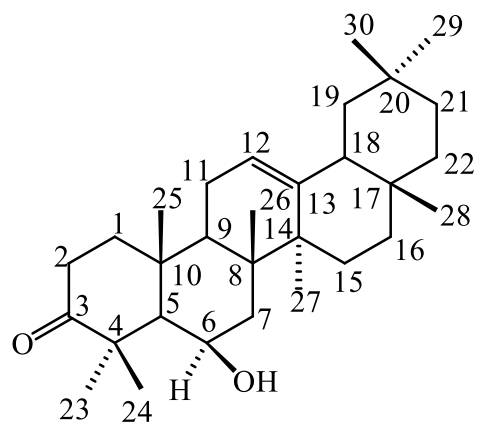
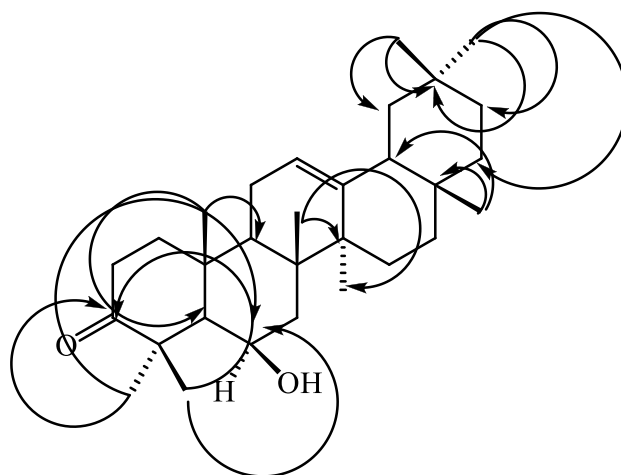
The ^1H -NMR (**Table 39**) of **16** showed a carbinyl proton at δ_{H} 4.49 (H-6) as a broad singlet indicating its equatorial orientation and a vinylic proton at δ_{H} 5.25 (t, $J = 3.5$ Hz; H-12) and eight methyl singlets at δ_{H} 1.15 (Me-23), 1.40 (Me-24), 1.49 (Me-25), 1.32 (Me-

26), 1.08 (Me-27), 0.83 (Me-28), 0.85 (Me-29), and δ_H 0.85 (Me-30). The ^{13}C -NMR data of **16** showed signals at δ_C 216.6 (C=O), 121.2 (C-12), δ_C 144.5 (C-13) and δ_C 69.3 (C-6). In addition five CH, nine CH_2 and eight CH_3 carbons were identified on the basis of DEPT-90 and DEPT-135 experiments. The ^{13}C -NMR also indicated eight quaternary carbon atoms (**Table 39**). These spectral data suggested that compound **16** is an oleanane type of triterpenoid in which the carbonyl group was positioned at C-3 by considering the low field-shifts of ^{13}C -NMR signals of C-2 (δ_C 34.4), C-4 (δ_C 48.7) and C-5 (δ_C 56.5) and biogenetic origin.

The broad singlet of H-6 (equatorial; α) in contrast to a triplet of doublet ($J=10.4, 4.5$ Hz) reported for H-6 (axial; β) [178] was in agreement with the β (axial) orientation of the hydroxyl group proton. This stereochemistry was fully supported by n.O.e difference experiment (**Figure 44**) which showed spatial interactions between H-6 (α) and H-5 (α), H-23 (α) as well as H-27 (α) and between H-24, H-25 and H-26 all lying β . Based on the spectral data (**Table 39**), the structure of Shakirullaline (**16**) is characterized as 3-keto-6 β -hydroxy- α -amyrin which was also confirmed by single X-ray crystallographic techniques (**Figure 45**).

Table 39: The ^1H - and ^{13}C -NMR data of Shikirullaline (**16**)

No.	δ_{C}	δ_{H} (mult, J, Hz)	Types	HMBC
1	40.6	2.22, 2.26	CH_2	C-3
2	34.4	2.73, 2.76	CH_2	C-3
3	216.6	-	C	-
4	48.7	-	C	-
5	56.5	1.64	CH	C-2, C-4, C-24
6	69.3	4.49 br.s	CH	C-5, C-6
7	41.6	1.53, 1.89	CH_2	C-5, C-6, C-8, C-14,
8	42.5	-	C	-
9	47.3	2.06	CH	C-10
10	36.3	-	C	-
11	23.6	1.79, 1.76	CH_2	C-6, C-8, C-14, C-26
12	121.2	5.24 s	CH	C-8, C-9, C-11, C-15
13	144.5	-	C	-
14	39.0	-	C	-
15	26.1	1.62	CH_2	C-14, C-17, C-18
16	26.9	1.98, 1.94	CH_2	C-15, C-17, C-18
17	32.8	-	C	-
18	47.3	1.67	CH	C-13
19	46.7	1.68, 1.65	CH_2	C-18, C-20, C-22, C-29, C-30
20	31.0	-	C	-
21	34.0	1.66	CH_2	C-28, C-29
22	37.0	1.22, 1.25	CH_2	C-17, C-18, C-21
23	25.8	1.15 s	CH_3	C-3, C-4, C-5
24	23.6	1.40 s	CH_3	C-3, C-4, C-5
25	16.5	1.49 s	CH_3	C-5, C-9, C-10
26	18.6	1.32 s	CH_3	C-8, C-14, C-27
27	25.9	1.08 s	CH_3	C-8, C-14
28	28.3	0.83 s	CH_3	C-16, C-17, C-18, C-21, C-22
29	33.3	0.85 s	CH_3	C-20, C-21, C-22
30	23.9	0.85 s	CH_3	C-19, C-20

**16****Figure 43:** Key HMBC Correlation for compound (**16**)

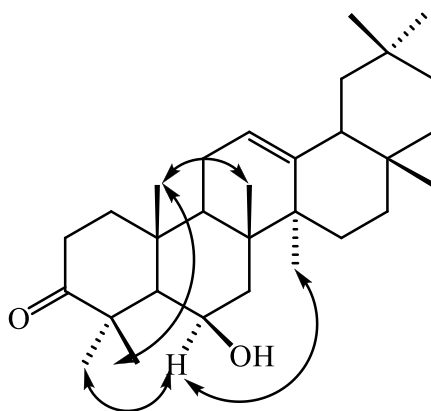


Figure 44: Key n.O.e interaction for compound **16**

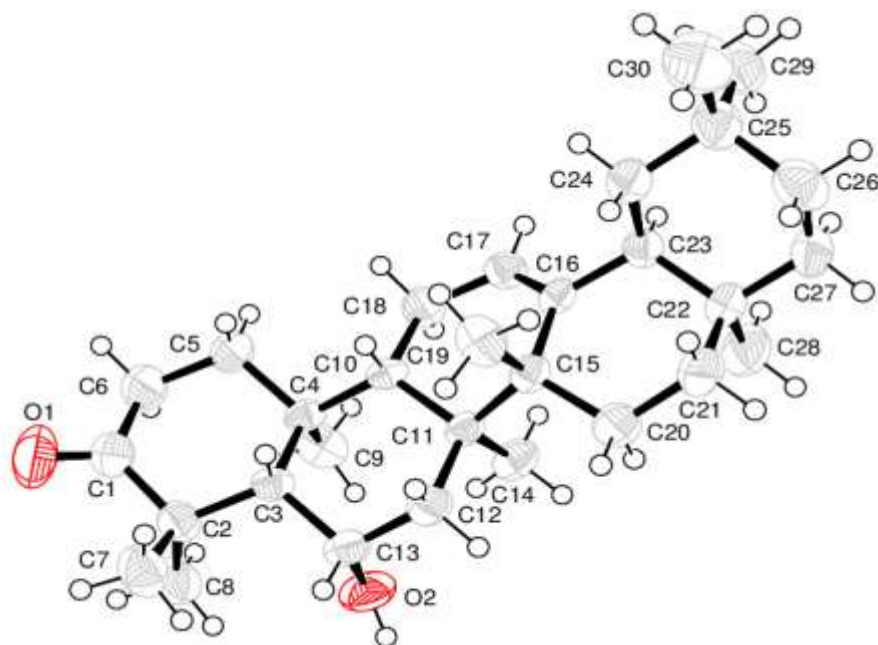


Figure 45: X-ray crystallographic image of compound (**16**)

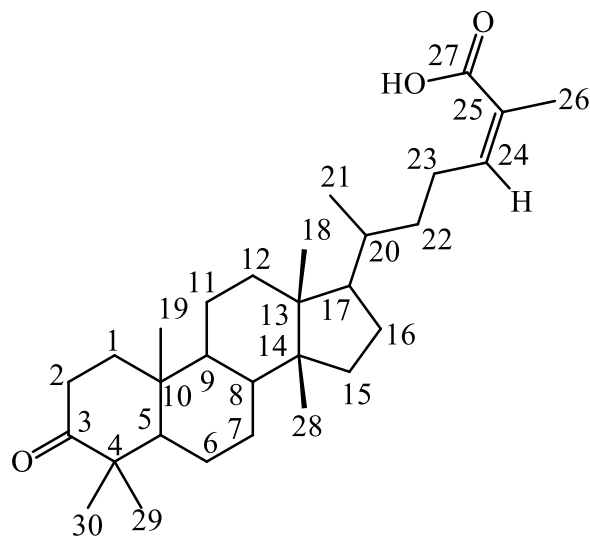
6.2.3. Structure elucidation of integerrimic acid (**17**)

Integerrimic acid (**17**) was isolated as a colorless crystalline from chloroform fraction of barks of *P. integerrima* by column chromatography. IR spectrum (cm^{-1}) of **17** displayed characteristic absorption bands for carbonyl group (1714), carboxylic group (3358) and chelated carboxylic group (1688, 1670). The UV spectrum showed absorption maxima at 217, 230 and 260 nm. Its molecular formula was assigned as $\text{C}_{30}\text{H}_{48}\text{O}_3$ based on HR-EIMS (m/z 456.3432 a.m.u; calcd. for $\text{C}_{30}\text{H}_{48}\text{O}_3$, 456.3438). The ^{13}C -NMR spectrum (**Table 40**) showed the presence of ketone and carboxylic acid at δ_{C} 218.1 and 173.6 ppm, respectively. In ^1H -NMR spectrum (**Table 40**) seven methyl signals were observed, including a secondary methyl at δ_{H} 0.91 ($J = 6.0$ Hz, H-21) and six tertiary methyls at δ_{H} 0.91 (H-18), 0.87 (H-30), 0.98 (H-19), 1.08 (H-28), 1.09 (H-29), and 1.90 (H-27). A trisubstituted olefinic proton resonated at δ_{H} 6.06 (1H, m, H-24) in the proton spectrum of **17**. A comparison of the ^1H and ^{13}C -NMR spectral data of **15** and **17** showed that **17** is closely related to **15** except the addition of unsaturated carbon at position 8 and 9 in **17** which was further conformed by NMR spectral data; $\delta_{\text{C}}/\delta_{\text{H}}$ 38.4/1.41 (C-8/H-8) and $\delta_{\text{C}}/\delta_{\text{H}}$ 39.5/1.40 (C-9/H-9). All these assignments were also confirmed by advanced 2D-NMR techniques such as COSY, HSQC, and HMBC. The HMBC spectrum (**Figure 46**) showed cross peaks for H-18 and H-19 to C-13, C-17, C-14 and C-10, C-9, C-5, respectively. Similarly, H-30 of the methyl group exhibited HMBC correlations to C-14, C-8, and C-15. Similarly the proton signals of H-1, H-2, H-28 and H-29 possessed HMBC cross peaks for the carbonyl carbon assigned to C-3. The olefinic proton (H-24) was correlated to C-23, C-25, and C-26. Based on the above arguments and compared with related compound [179] the structure of **17** is characterized

as (*E*)-2-methyl-6-(4,4,10,13,14-pentamethyl-3-oxohexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)hept-2-enoic acid.

Table 40: ^{13}C - and ^1H -NMR spectral data for integerrimic acid (**17**)

No	δ_{C}	δ_{H} (mult, <i>J</i> , Hz)	Types	HMBC
1	33.6	1.61, m, 1.71, m	CH ₂	C-3
2	34.9	2.50, m	CH ₂	C-3
3	218.1	-	C	-
4	47.3	-	C	-
5	52.4	1.69, m	CH	C-2, C-4, C-24
6	21.4	1.72, m, 1.45, m	CH	C-5, C-6
7	26.8	1.92, m, 2.03, m	CH ₂	C-5, C-6, C-8, C-14,
8	38.5	1.41, m	CH	-
9	39.5	1.40, m	CH	C-10
10	35.6	-	C	-
11	20.3	1.65, m	CH ₂	C-6, C-8, C-14, C-26
12	34.6	1.81, m, 1.62, m	CH ₂	C-8, C-9, C-11, C-15
13	43.5	-	C	-
14	51.2	-	C	-
15	34.0	1.47, m, 1.50, m	CH ₂	C-14, C-17, C-18
16	28.2	1.92, m, 1.23, m	CH ₂	C-15, C-17, C-18
17	50.1	1.48, m	CH	-
18	22.0	0.79, s	CH ₃	C-13
19	21.6	0.98, s	CH ₃	C-18, C-20, C-22, C-29, C-30
20	36.0	1.49, m	CH	-
21	18.2	0.91, d (<i>J</i> = 6)	CH ₃	C-28, C-29
22	35.9	1.60, m, 1.15, m	CH ₂	C-17, C-18, C-21
23	26.9	1.20, m, 1.59, m	CH ₂	C-3, C-4, C-5
24	147.2	6.06, m	CH	C-3, C-4, C-5
25	125.8	-	C	C-5, C-9, C-10
26	173.6	-	C	C-8, C-14, C-27
27	20.5	1.90, s	CH ₃	C-8, C-14
28	26.7	1.08, s	CH ₃	C-16, C-17, C-18, C-21, C-22
29	21.1	1.09, s	CH ₃	C-20, C-21, C-22
30	24.2	0.87, s	CH ₃	C-19, C-20



17

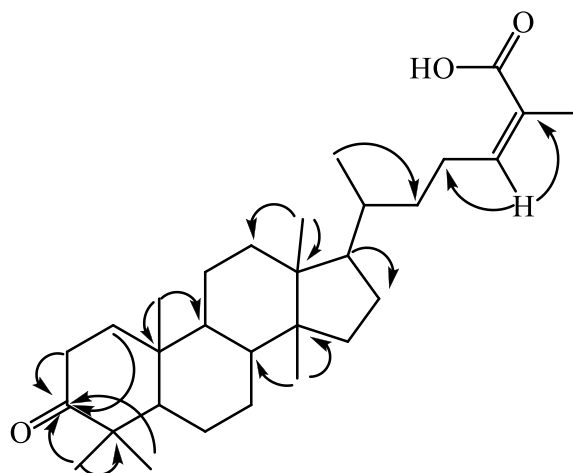


Figure 46: Selected HMBC correlations of compound (17)

6.3. Biological screening of *Pistacia integerrima*

6.3.1. Antibacterial effect of *P. integerrima* galls

The crude methanolic extract and its fractions were screened for antibacterial activity against selected bacterial strain which showed significant activity.

The ethyl acetate fraction possessed activity against *Klebsiella pneumonia*, *Staphylococcus epidermidis* and *Bacillus subtilis* with a zone of inhibition ranging from 10-24 mm, showing its medicinal importance in the treatment of gastroenteritis, and pneumonia. The methanolic extract was active against *Staphylococcus aureus*, *Klebsiella pneumonia*, *S. epidermidis* and *B. subtilis* with a zone of inhibition range from 14-28 mm (**Table 41**). The *n*-hexane extract showed activity against, *K. pneumonia*, *S. epidermidis* and *B. stearothermophilus* with a zone of inhibition range from 10-16 mm while chloroform extract is active against, *K. pneumonia*, *S. epidermidis* and *B. stearothermophilus* with a zone of inhibition range from 18-24 mm (**Table 41**).

Table 41: Antimicrobial activity (zone of inhibition in mm) of *P. integerrima* galls

Bacterial strain	Gram	Streptomycin (2mg/ml)	<i>n</i> -Hexane	CHCl ₃	EtOAc	MeOH
<i>B. stearothermophilus</i>	+	30	10	18	20	24
<i>E. coli</i>	+	30	0	0	0	0
<i>K. pneumonia</i>	-	30	16	22	24	28
<i>S. aureus</i>	+	32	0	0	10	14
<i>S. epidermidis</i>	-	32	15	24	24	28

6.3.2. Antibacterial activity of *P. integerrima* leaves

The methanolic extract of *P. integerrima* leaves and its fractions were also assessed for antibacterial sensitivity which showed moderate activity against the selected fungal strain with zone of inhibition range from 10-14 mm while *n*-hexane fraction showed low activity among the tested samples (**Table 42**).

Table 42: Antimicrobial activity (zone of inhibition in mm) of *P. integerrima* leaves

Bacterial strain	Gram	Streptomycin (2mg/ml)	<i>n</i> -Hexane	CHCl ₃	EtOAc	MeOH
<i>B. stearothermophilus</i>	+	30	10	10	10	10
<i>E. coli</i>	+	30	0	0	0	0
<i>K. pneumonia</i>	-	30	0	12	12	14
<i>S. aureus</i>	+	32	0	0	10	10
<i>S. epidermidis</i>	-	32	0	14	12	14

6.3.3. Antimicrobial effect of *P. integerrima* bark

The results of the crude methanolic extract of *P. integerrima* bark and its fractions are listed in table 5.6 (**Table 43**). The *n*-hexane fraction showed good activity only against *B. stearothermophilus* with zone of inhibition 10 mm. The chloroform, ethyl acetate fractions and crude exhibited good activity as compared to *n*-hexane fraction with zone of inhibition range from 14-22 mm (**Table 43**).

Table 43: Antimicrobial activity (zone of inhibition in mm) of *P. integerrima* bark

Bacterial strain	Gram	Streptomycin (2mg/ml)	<i>n</i> -Hexane	CHCl ₃	EtOAc	MeOH
<i>B. stearothermophilus</i>	+	30	10	14	12	18
<i>E. coli</i>	+	30	0	0	0	0
<i>K. pneumonia</i>	-	30	0	20	16	22
<i>S. aureus</i>	+	32	0	0	10	15
<i>S. epidermidis</i>	-	32	0	18	18	16

6.3.4. Antimicrobial effect of *P. integerrima* roots

The crude methanolic extract and fractions of roots showed activity against selected bacterial strain. The *n*-hexane extract exhibited activity only against *B. stearotheophilus* with inhibitory zone 10 nm. The CHCl_3 , MeOH and EtOAc fractions showed good activity against *S. aureus*, *K. pneumonia*, *S. epidermidis* and *B. stearotheophilus* with zone of inhibition range from 10-14 mm (Table 44).

Table 44: Antimicrobial activity (zone of inhibition in mm) of *P. integerrima* roots

Bacterial strain	Gram	Streptomycin (2mg/ml)	<i>n</i> -Hexane	CHCl_3	EtOAc	MeOH
<i>B. stearotheophilus</i>	+	30	10	10	10	10
<i>E. coli</i>	+	30	0	0	0	0
<i>K. pneumonia</i>	-	30	0	12	12	14
<i>S. aureus</i>	+	32	0	12	10	10
<i>S. epidermidis</i>	-	32	0	14	12	14

6.3.5. Antifungal effect of *P. integerrima* galls

The antifungal activity of the methanolic extract and subsequent solvent fractions of galls are presented in table 45 which showed activity against *Microsporum canis*.

Table 45: Antifungal effect of *P. integerrima* galls

Name of Fungus	% Zone of inhibition (mm)					
	<i>n</i> -Hexane	CHCl_3	EtOAc	MeOH	STD	MIC ($\mu\text{g/ml}$)
<i>A. flavus</i>	-	20	-	-	Amphotericin B	20.20
<i>C. albicans</i>	-	-	-	-	Miconazole	110.8
<i>C. glabrata</i>	-	-	-	-	Miconazole	110.8
<i>F. solani</i>	-	-	-	-	Miconazole	73.25
<i>M. canis</i>	20	35	25	15	Miconazole	98.4
<i>T. logifusus</i>	-	-	-	-	Miconazole	

Results denote the mean of three different experimental readings.

6.3.6. Antifungal effect of *P. integerrima* leaves

The results of antifungal activity are presented in table 46. The *n*-hexane extract exhibited sensitivity against *Aspergillus flavus* and *F. solani* while chloroform fraction showed activity against *M. canis* and *F. solani*. The ethyl acetate fraction showed activity against *Microsporum canis* and *F. solani* while methanolic extract was sensitive against *A. flavus* and *M. canis* among the selected fungal strain (**Table 46**).

Table 46: Antifungal effect of *P. integerrima* leaves

Name of Fungus	% Zone of inhibition (mm)					
	<i>n</i> -Hexane	CHCl ₃	EtOAc	MeOH	STD	MIC (µg/ml)
<i>A. flavus</i>	20	25	-	20	Amphotericin B	20.20
<i>C. albicans</i>	-	-	-	-	Miconazole	110.8
<i>C. glabrata</i>	-	-	-	-	Miconazole	110.8
<i>F. solani</i>	20	-	20	-	Miconazole	73.25
<i>M. canis</i>	-	30	30	20	Miconazole	98.4
<i>T. logifusus</i>	-	-	-	-	Miconazole	

Results represent the mean of three different experimental readings.

6.3.7. Antifungal effect of *P. integerrima* bark

The effects of the crude extract and isolated fractions are presented in table 47. The *n*-hexane extracts showed activity against *Microsporum canis* while chloroform fraction was sensitive against *A. flavus* and *M. canis*. The ethyl acetate and methanolic extract showed activity only against *M. canis* (**Table 47**).

Table 47: Antifungal effect of *P. integerrima* bark

Name of Fungus	% Zone of inhibition (mm)					
	<i>n</i> -Hexane	CHCl ₃	EtOAc	MeOH	STD	MIC (µg/ml)
<i>A. flavus</i>	-	20	-	-	Amphotericin B	20.20
<i>C. albicans</i>	-	-	-	-	Miconazole	110.8
<i>C. glabrata</i>	-	-	-	-	Miconazole	110.8
<i>F. solani</i>	-	-	-	-	Miconazole	73.25
<i>M. canis</i>	20	35	25	15	Miconazole	98.4
<i>T. logifusus</i>	-	-	-	-	Miconazole	

Results represent the mean of three different experimental readings

6.3.8. Antifungal effect of *P. integerrima* roots

The antifungal activity of the crude extract and fractions are illustrated in table 48. The *n*-hexane extract showed activity against *M. canis* while the chloroform extract was found active against *A. flavus* and *M. canis*. The Ethyl acetate fraction and methanolic extract exhibited activity only against *M. canis* (**Table 48**).

Table 48: Antifungal effect of *P. integerrima* roots

Name of Fungus	% Zone of inhibition (mm)					
	<i>n</i> -Hexane	CHCl ₃	EtOAc	MeOH	STD	MIC (µg/ml)
<i>A. flavus</i>	-	20	-	-	Amphotericin B	20.20
<i>C. albicans</i>	-	-	-	-	Miconazole	110.8
<i>C. glabrata</i>	-	-	-	-	Miconazole	110.8
<i>F. solani</i>	-	-	-	-	Miconazole	73.25
<i>M. canis</i>	20	30	25	20	Miconazole	98.4
<i>T. logifusus</i>	-	-	-	-	Miconazole	

Results represent the mean of three different experimental readings.

6.3.9. Cytotoxic effect of *P. integerrima* galls

The crude extract and its solvent fractions were subjected for *in-vitro* cytotoxic activity. The results are shown in (Table 49) which showed significant cytotoxic effect at concentrations of 10, 100 and 1000 µg/ml.

Table 49: Cytotoxic effect of *P. integerrima* galls

Dose	Number of shrimps survived				
	Control	Methanol	<i>n</i> -Hexane	Chloroform	Ethyl acetate
10	0±0.00	6±0.00	8±0.07	6±0.02	7±0.02
100	0±0.00	3±0.08	6±0.02	3±0.01	4±0.02
1000	0±0.00	0±0.00	4±0.00	0±0.00	0±0.00

Data are three mean ± SEM of three different experiments.

6.3.10. Cytotoxic effect of *P. integerrima* leaves

The effects of cytotoxic assay of *P. integerrima* leaves are illustrated in table 50. The results showed inhibition against *A. salina* at concentrations of 10, 100 and 1000 µg/ml.

Table 50: Cytotoxic effect of *P. integerrima* leaves

Dose	Number of shrimps survived				
	Control	Methanol	<i>n</i> -Hexane	Chloroform	Ethyl acetate
10	0±0.00	6±0.00	8±0.07	6±0.02	7±0.02
100	0±0.00	3±0.08	6±0.02	3±0.01	4±0.02
1000	0±0.00	0±0.00	4±0.00	0±0.00	0±0.00

Data are three mean ± SEM of three different experiments.

6.3.11. Cytotoxic effect of *P. integerrima* bark

Regarding cytotoxic effect of crude extract and subsequent solvent fractions of bark of *P. integerrima*, marked mortality was observed against *A. salina* at concentrations of 10, 100 and 1000 µg/ml (**Table 51**).

Table 51: Cytotoxic effect of *P. integerrima* bark

	Number of shrimps survived				
Dose	Control	Methanol	<i>n</i> -Hexane	Chloroform	Ethyl acetate
10	0±0.00	7±0.02	8±0.02	8±0.00	7±0.02
100	0±0.00	4±0.01	6±0.01	6±0.01	3±0.00
1000	0±0.00	0±0.00	4±0.00	4±0.00	0±0.00

Data are three mean ± SEM of three different experiments.

6.3.12. Cytotoxic effect of *P. integerrima* roots

The results of extract and its fractions are shown in figure 52, similar pattern of cytotoxicity was demonstrated by the crude extract and subsequent solvent fractions of roots. The overall effect was in a concentration dependent manner.

Table 52: Cytotoxic effects of *P. integerrima* roots

	Number of shrimps survived				
Dose	Control	Methanol	<i>n</i> -Hexane	Chloroform	Ethyl acetate
10	0±0.00	7±0.02	10±0.00	6±0.01	8±0.00
100	0±0.00	5±0.01	9±0.02	3±0.00	4±0.01
1000	0±0.00	2±0.01	7±0.00	0±0.00	2±0.01

Data are three mean ± SEM of three different experiments.

The results of our study showed that the extracts/fractions of various parts of *P. integerrima* had marked cytotoxic effects on brine shrimp larvae. When tested at various concentrations (10, 100 and 1000 µg/ml), among the different parts of the plant, extracts/fractions of galls

were observed most cytotoxic. The results indicating that the cytotoxic constituents of the plant are concentrated in galls of the plant. Upon fractionation, the *n*-hexane was the less sensitive fraction towards *A. salina* larvae. It suggested that the cytotoxic constituents of the plant in principal are more polar in nature.

6.3.13. Effect of galls extracts on DPPH

The crude extract and various fractions of galls of *P. integerrima* were tested at accumulative concentrations i.e. 10, 20, 40, 60, 80 and 100 µg/ml (**Table 53**) against DPPH. The quenching effect of extract/ fractions was in a concentration dependent mode. The maximum antioxidant effect was observed with ethyl acetate fraction (98.10%) followed by methanolic (96.88%) at 100 µg/ml (**Figure 47**).

Table 53: DPPH radical scavenging activities of *P. integerrima* galls

Conc (µg/ml)	%DPPH				
	Ethanol	<i>n</i> -Hexane	Chloroform	Ethyl acetate	Methanol
10	33.55	8.22	41.21	44.44	39.2
20	47.55	18.12	66.33	77.8	61.43
40	58.33	28.90	77.02	88.21	71.40
60	66.22	35.1	80.4	90.6	79.0
80	70.22	52.9	82.29	92.6	84.54
100	80.55	60.11	94.55	98.10	96.88

Data are shown as mean of three different experiments.

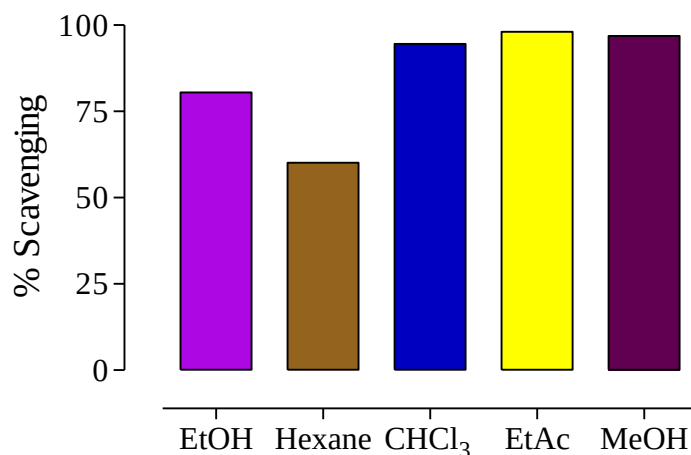


Figure 47: % Scavenging effect of *P. integerrima* galls on DPPH at 100 µg/ml
Results are mean of three different experiments.

6.3.14. Effect of leaves extracts on DPPH

The antioxidant effect of crude ethanolic extract of leaves of *P. integerrima* and its various solvent fractions are presented in table 54. Among the tested samples, maximum anti-radical activity was demonstrated by the methanolic fraction (84.88%) followed by chloroform fraction with 81.14% scavenging at 100 µg/ml (**Figure 48**).

Table 54: DPPH radical scavenging activities of *P. integerrima* leaves

Conc (ppm)	%DPPH				
	Ethanol	<i>n</i> -Hexane	Chloroform	Ethyl acetate	Methanol
10	5.51	3.21	6.31	7.22	6.24
20	10.51	8.13	13.53	16.66	15.44
40	19.31	14.91	20.02	29.11	30.41
60	40.21	29.55	40.55	52.61	55.0
80	49.21	48.11	60.28	73.61	79.51
100	60.51	58.11	69.53	81.14	84.88

Data are shown as mean of three different experiments.

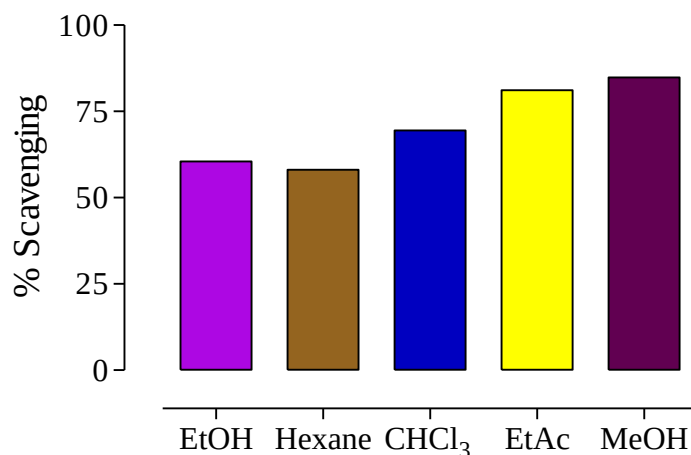


Figure 48: % Scavenging effect of *P. integerrima* leaves extracts on DPPH at 100 µg/ml

6.3.15. Effect of barks extracts on DPPH

The antioxidant profile of the crude ethanolic extract and its various solvent fractions of bark of the plant are illustrated in table 55. In a concentration dependent manner, profound inhibition of DPPH was observed. The maximum antioxidant potential was 96.55% and 90.18% by the chloroform and methanolic fractions respectively (**Figure 49**).

Table 55: DPPH radical scavenging activities of *P. integerrima* bark

Conc (ppm)	%DPPH				
	Ethanol	<i>n</i> -Hexane	Chloroform	Ethyl acetate	Methanol
10	11.51	3.22	20.21	15.41	10.21
20	22.51	6.12	44.33	30.81	19.23
40	44.31	16.90	70.02	44.22	39.22
60	72.21	30.1	80.4	70.62	70.11
80	80.28	40.9	86.29	78.63	80.51
100	88.51	50.11	96.55	82.11	90.81

Data are shown as mean of three different experiments.

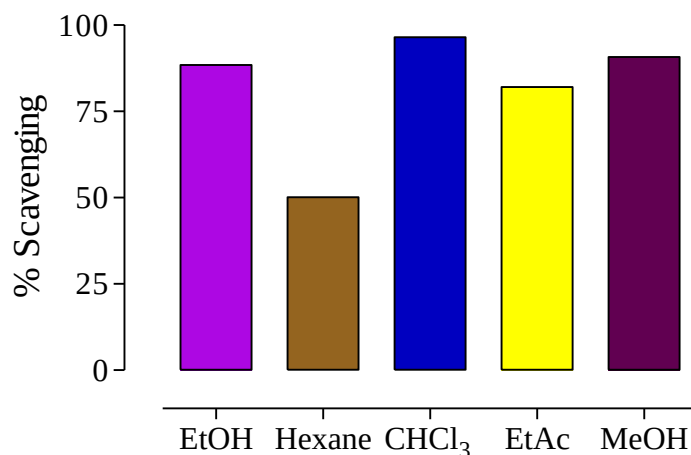


Figure 49: % Scavenging effect of *P. integerrima* bark extracts on DPPH at 100 µg/ml
Results are mean of three different experiments.

6.3.16. Effect of roots extract on DPPH

Table 56 presents the antioxidant profile of the crude ethanolic extract and its various solvent fractions of roots of *P. integerrima*. Results reflected outstanding quenching effect of roots extracts in a concentration dependent manner with maximum scavenging activity of 95.19 and 92.51% by ethyl acetate and chloroform fractions respectively (**Figure 50**).

The results of our study revealed outstanding potential of various fractions of different parts of the plant in a concentration dependent manner. It could therefore be assumed the pharmacological active constituent(s) contained by fractions of the plants of different parts possessed interfered with the activity stable free radical, DPPH.

Table 56: DPPH radical scavenging activities of *P. integerrima* roots

Conc (ppm)	%DPPH				
	Ethanol	<i>n</i> -Hexane	Chloroform	Ethyl acetate	Methanol
10	15.55	6.21	11.32	20.41	18.21
20	22.52	10.13	22.34	44.82	30.41
40	40.32	22.95	40.44	60.23	44.43
60	60.24	30.15	70.45	78.62	58.01
80	72.28	44.99	80.21	90.61	70.52
100	82.51	55.18	92.51	95.19	80.89

Data are shown as mean of three different experiments.

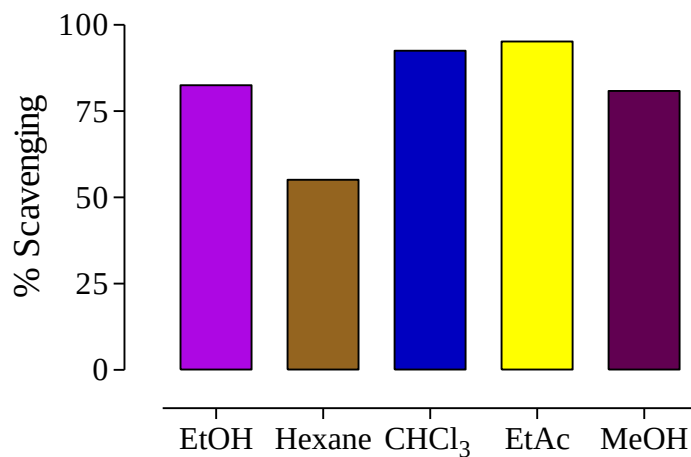


Figure 50: % Scavenging effect of *P. integerrima* roots on DPPH at 100 µg/ml
Results are mean of three different experiments.

6.4. Biological screening of pistagremic acid (15)

6.4.1. *In vitro* biological screening of pistagremic acid (15)

6.4.1.1. Antibacterial activity of 15

Pistagremic acid (**15**) was tested against five selected Gram-positive and Gram-negative bacterial strain as presented in figure 51. Pistagremic acid (**15**) exhibited excellent antibacterial effect against *K. pneumonia*, *S. epidermidis* and *B. stearothermophilu* having zone of inhibition 15, 20 and 14 mm respectively. Streptomycin was used as standard drug (Figure 51).

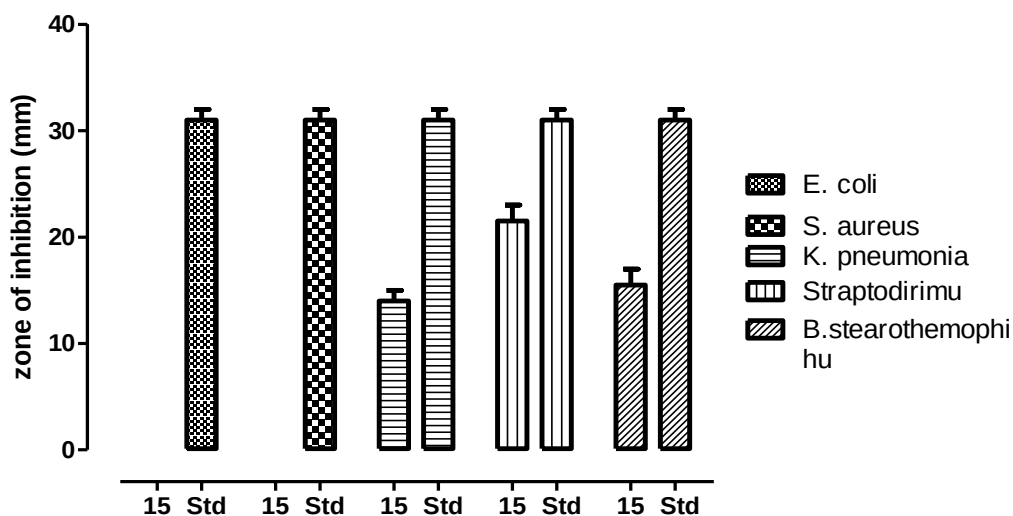


Figure 51: Antibacterial activity of pistagremic acid (**15**).

6.4.1.2. Antifungal activity of 15

Pistagremic acid (**15**) was also tested against *T. longifusus*, *C. albicans*, *A. flavus*, *M. canis*, *F. salani* and *C. glaberata* fungal strain. Among the tested fungus the growth of *C. albicans*, *M. canis*, *F. salani* and *C. glaberata* was inhibited with **15** having zone of inhibition 12, 18, 12 and 14 mm respectively against miconazole (Figure 52).

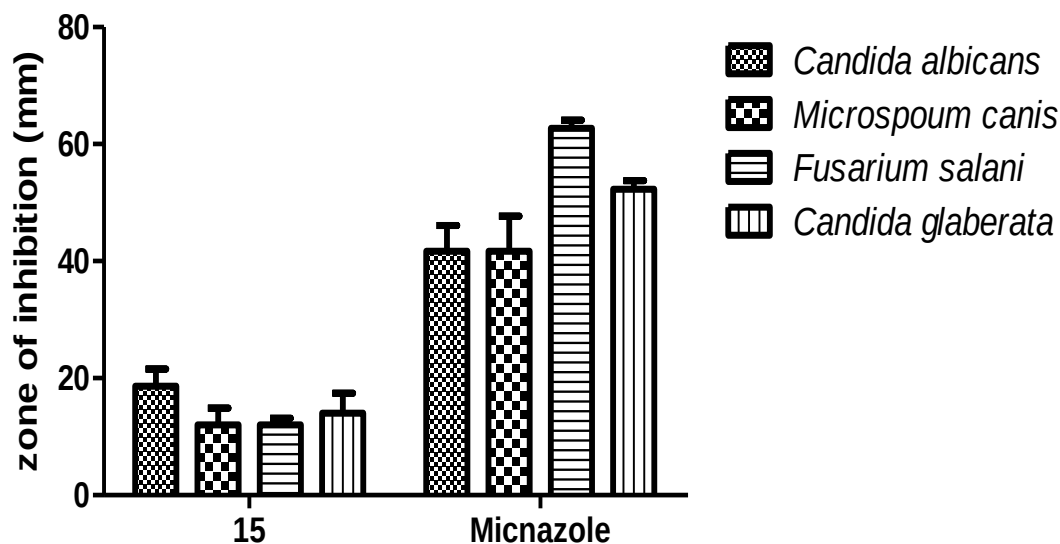


Figure 52: Antifungal activity of pistagremic acid (15)

6.4.1.3. Antioxidant activity of 15

Pistagremic acid (15) showed concentration dependent free radical scavenging potential. The percent DPPH free radical effect of the tested compound was 2.44, 3.33, 6.22, 9.99, 14.55, 18.92 and 30.55 at the tested concentrations of 5-100 $\mu\text{g/ml}$ respectively. The antioxidant effect of the reference drug i.e BTH was better than pistagremic acid as presented in figure 53.

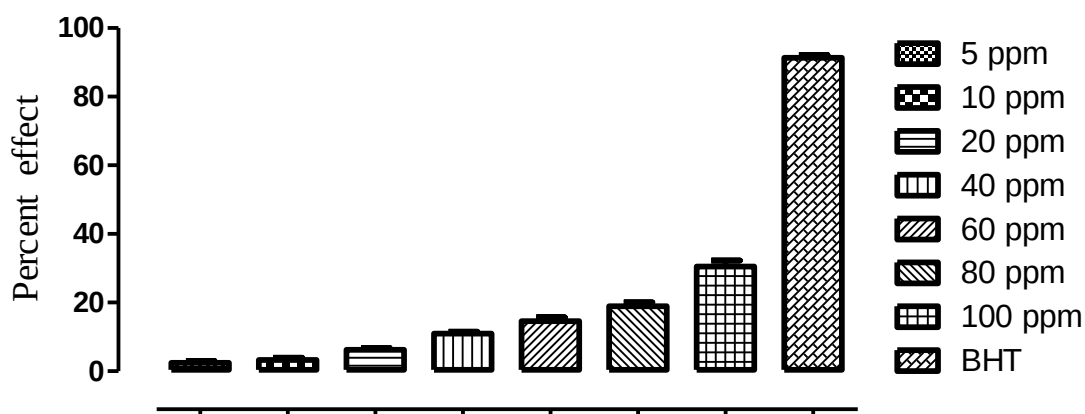


Figure 53: Antioxidant activity of pistagremic acid (**15**)

6.4.1.4. Leishmanicidal activity of **15**

Pistagremic acid (**15**) was screened for leishmanicidal activity which showed substantial activity against amastigotes of *L. major* (IC_{50} : $6.71 \pm 0.09 \mu M$) and comparison amphotericin B (IC_{50} : $0.21 \pm 0.06 \mu M$). Compound **15** which need further investigated and development for the discovery of potential lead drug for the treatment of cutaneous leishmaniasis caused by *L. major*.

6.4.1.5. Broad spectrum anticancer activity of 15

Pistagremic acid (**15**) was evaluated for anticancer potential using *in-vitro* screening assay against NCI-60 DTP human tumor cell lines (**Table 57 & Figure 54 & 55**). The compound **15** was first evaluated at one dose toward 60 cancer cells lines (concentration 2-20 mM) which showed the potent growth inhibition against CNS cancer (SF-295) cell-line at GI_{50} value = 32 nM whereas 193 nM was the highest GI_{50} value showed against non-small cell lung cancer (HOP-62) cell-line. Similarly, in terms of lethal concentration 50% (LC_{50}) 45 nM was the lowest value shown against melanoma (UACC-62) cell-line and over 10000 nM against non-small cell lung cancer (NCI-H226, and NCI-H460) cell-lines. Thus lung cancer cell-lines of **15** showed slight resistance towards this molecule. In dose-dependent response by 10 fold, the typical sigmoidal curves were observed in figure 54 and 55. Pistagremic acid (**15**) showed inhibition against almost all cell-lines. Based on the anti-cancer profile pistagremic acid (**15**) is a broad spectrum anticancer triterpene which exhibited antiproliferative effects at nanomolar range against almost all of the cell lines in the NCI-60 which may serve as a potential lead triterpene for the development of new anticancer drugs.

Table 57: Growth inhibition against all NCI-60 cell-lines exhibited by pistagremic acid (**15**)

Panel/Cell lines	GI_{50} (μ M)	TGI (μ M)	LC_{50} (μ M)
Leukemia			
CCRF-CEM	0.07	0.25	0.73
HL-60(TB)	0.11	0.27	0.64
K-562	0.09	0.27	-
MOLT-4	0.13	0.30	0.69
RPMI-8226	0.07	0.26	-
SR	0.12	0.30	0.76
Non-small cell Lung			
A549/ATCC	0.04	0.18	0.59
EKVX	0.05	0.18	0.54
HOP-62	0.19	0.34	0.62

HOP-92	0.06	0.19	0.46
NCI-H226	0.10	0.37	>10
NCI-H23	0.06	0.21	0.58
NCI-H460	0.07	0.29	>10
NCI-H522	0.07	0.22	0.56
Colon Cancer			
COLO 205	0.17	0.32	0.63
HCC-2998	0.08	0.21	0.48
HCT-116	0.08	0.23	0.61
HCT-15	0.10	0.34	2.34
HT29	0.13	0.28	0.62
KM12	0.07	0.23	0.62
SW-620	0.12	0.31	0.81
CNS Cancer			
SF-268	0.14	0.30	0.64
SF-295	0.03	0.17	0.70
SF-539	0.14	0.29	0.61
SNB-19	0.11	0.26	0.61
SNB-75	0.16	0.30	0.57
U251	0.06	0.21	0.54
Melanoma			
LOX IMVI	0.07	0.22	0.59
MALME-3M	0.14	0.32	0.70
M14	0.09	0.25	0.64
MDA-MB-435	0.11	0.27	0.62
SK-MEL-2	0.11	0.26	0.61
SK-MEL-28	0.12	0.26	0.53
SK-MEL-5	0.07	0.20	0.46
UACC-257	0.16	0.31	0.60
UACC-62	0.05	0.17	0.45
Ovarian Cancer			
IGROV1	0.11	0.25	0.57
OVCAR-3	0.13	0.27	0.55
OVCAR-4	0.13	0.31	0.72
OVCAR-5	0.15	0.30	0.63
OVCAR-8	0.13	0.30	0.69
NCI/ADR-RES	0.12	0.28	0.67
SK-OV-3	0.17	0.30	0.55
Renal Cancer			
786-0	0.10	0.24	0.56
A498	0.07	0.21	0.50
ACHN	0.09	0.22	0.52

CAKI-1	0.05	0.17	0.46
RXF 393	0.13	0.29	0.63
SN12C	0.07	0.22	0.55
TK-10	0.15	0.28	0.55
UO-31	0.10	0.24	0.56
Prostate Cancer			
PC-3	0.10	0.24	0.56
DU-145	0.13	0.28	0.57
Breast Cancer			
MCF7	0.07	0.25	0.69
MDA-MB-231/ATCC	0.14	0.30	0.61
HS 578T	0.11	0.28	0.75
BT-549	0.11	0.24	0.51
T-47D	0.11	0.29	0.75
MDA-MB-468	0.08	0.23	0.57

TGI = Total growth inhibition is the negative $\log_{10}$ minimum concentration that causes total growth inhibition,

GI₅₀ = Multi-dose growth inhibition, determined from dose–response curve.

LC₅₀ = concentration required to reduce total cell count by 50%;

Calculated as $[(T_i - T_0)/T_0] * 100 = 50$,

where T_0 = absorbance at $t = 0$; T_i = absorbance at $t = 48$ hrs.

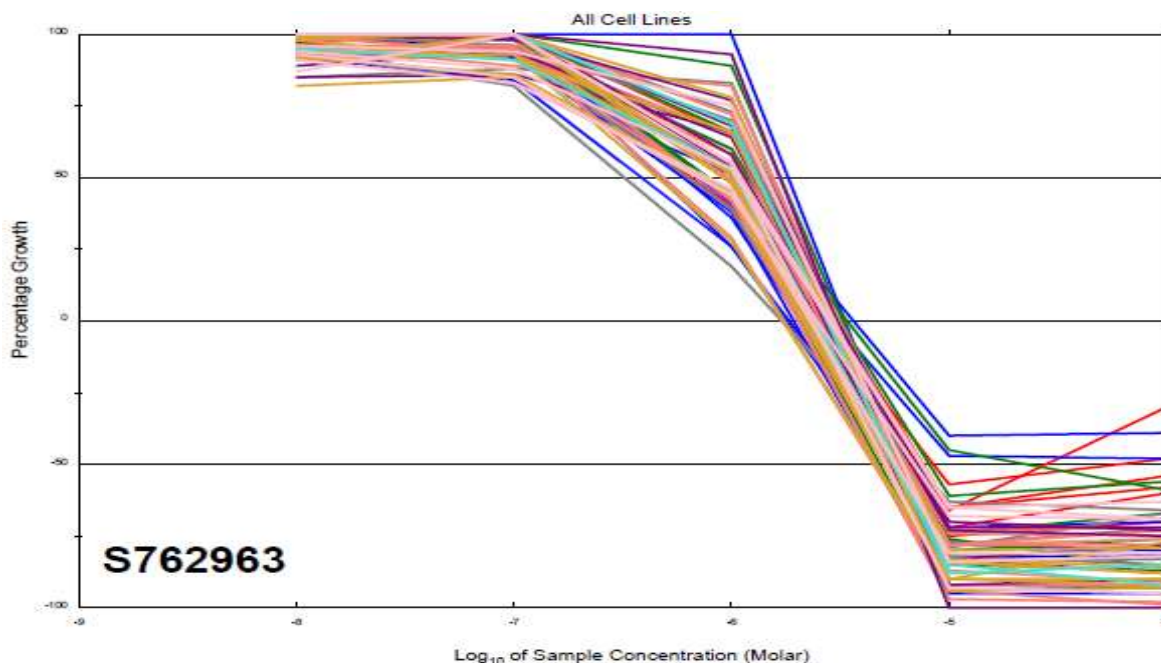


Figure 54: Dose response curves of pistagremic acid (15) against all cell lines

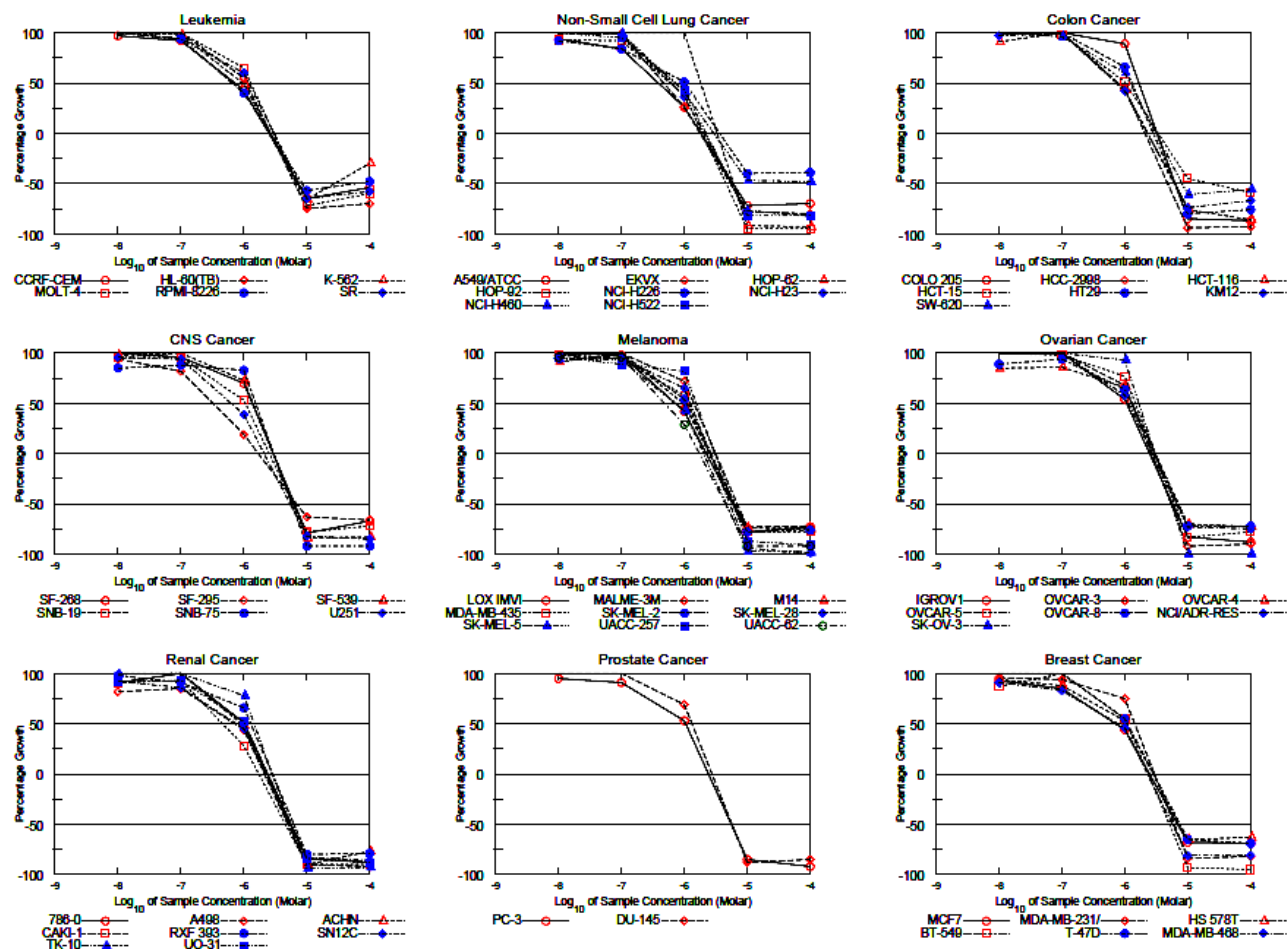


Figure 55: Dose response curves of pistagremic acid (15) against the different cell lines of Leukemia, Non-small cell lung cancer, Colon cancer, CNS cancer, Melanoma, Ovarian cancer, Renal cancer, Prostate cancer and Breast cancer.

6.4.1.6. Glycosidase inhibitor of 15

Pistagremic acid (**15**) was subjected to molecular docking followed by glycosidase inhibitor activity using standard protocol. Molecular docking study indicated strong potential of **15** to be a possible new α -glucosidase inhibitor. Pistagremic acid (**15**) showed good activity (IC_{50} : $89.12 \pm 0.12 \mu M$) against α -glucosidase inhibition. Pistagremic acid (**15**) was also found to be safe based on its cytotoxicity study using MTT assay. Interestingly, pistagremic acid (**15**) showed considerable molecular interactions with amino acid residues surrounding the narrow tunnel leading to catalytic sites of α -glucosidase (**Figure 56**). Both hydrophobic and hydrophilic interactions were identified between the ligand and α -glucosidase. On other side the terminal hydroxyl group revealed hydrogen bonding (at a distance of $2.98 \text{ \AA}$) with Asp 62 lining the inner rim of border surrounding long and narrow catalytic pocket of α -glucosidase while carboxylic group tightly hold pistagremic acid in contact with amino acid residues Arg 69 ($3.04 \text{ \AA}$) and Asp 70 ($3.11 \text{ \AA}$) in terminal pocket *via* hydrogen bonding. In between these two terminal interactions central tetracyclic frame matches the shape of elongated and curved pocket through hydrophobic interactions with comparatively less polar amino acids (**Figure 57**). This combination of both hydrophobic and hydrogen bonding interaction could be the main reason behind the α -glucosidase inhibitory profile of pistagremic acid and has strong potential to be further developed as a potential lead compound targeting α -glucosidase for its therapeutic effects.

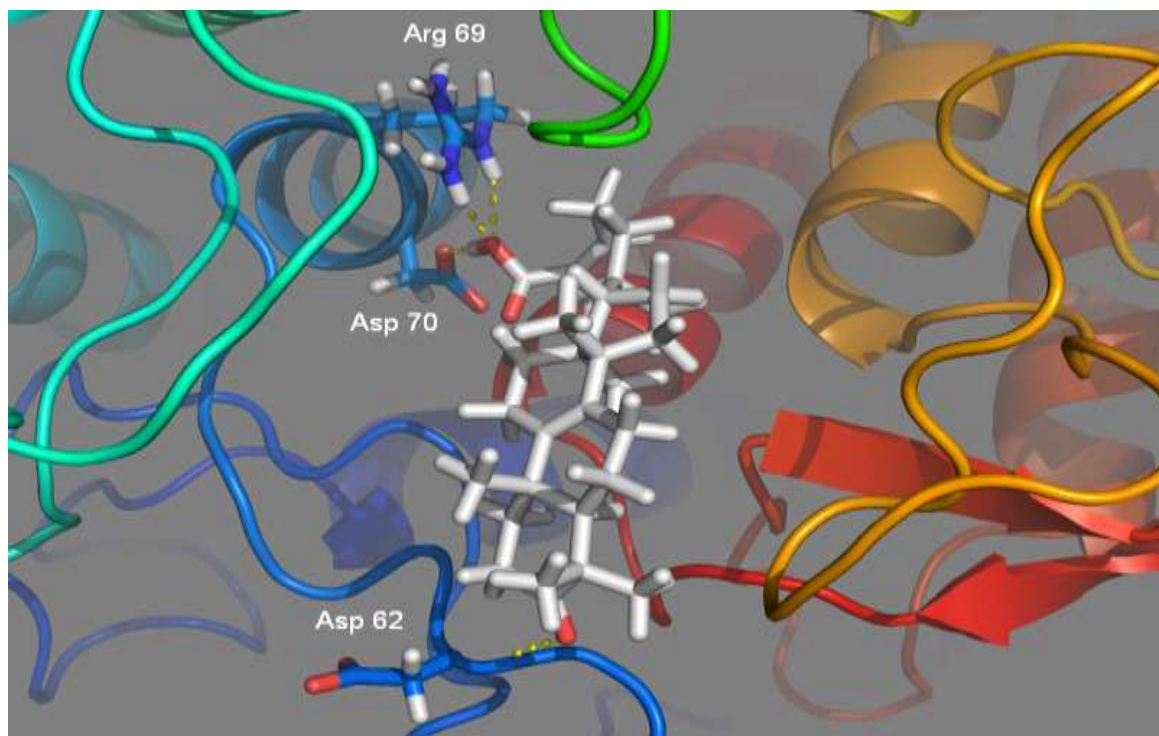


Figure 56: Binding mode of pistagremic acid inside active site of α -glucosidase. Hydrogen atoms (except polar ones) were omitted for clarity. (for interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.

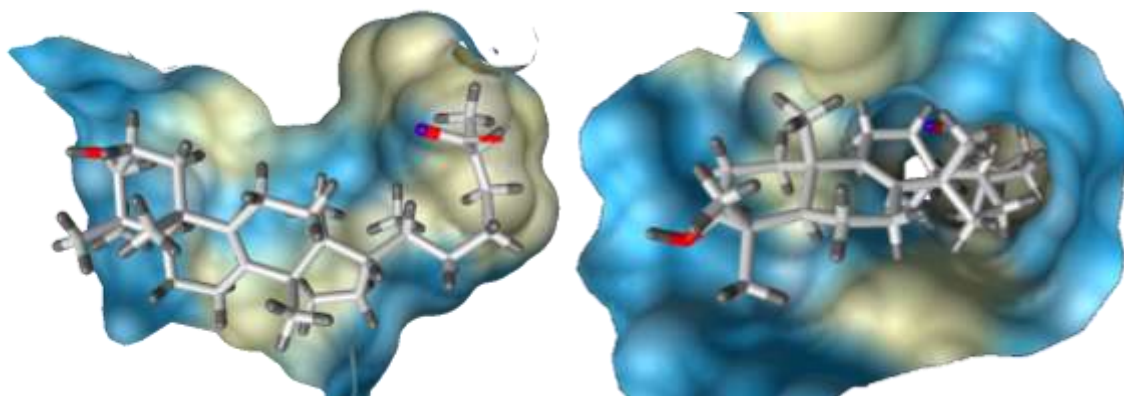


Figure 57: Surface contacts of pistagremic acid inside the catalytic site of α -glucosidase. Hydrophobic regions are represented as yellow colored while hydrophilic regions are represented as blue colored regions. (for interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.

6.4.2. Enzyme inhibition activities of *P. integerrima*

The crude extract and isolated compounds of *P. integerrima* were also subjected to various enzyme inhibition activities. The secondary metabolites purified from higher plants have wide past and present use in the treatment of many diseases. Natural molecules provide a good pharmacophore template for new drugs. In the preliminary step of bio-assay guided isolation of the crude fractions (EtOAc, CHCl₃ and MeOH) were evaluated for *in vitro* enzymes inhibitory assay against urease, phosphodiesterase-I, carbonic anhydrase-II, and α -chymotrypsin. As a result; the chloroform and MeOH were found significantly active against phosphodiesterase-I assay with IC₅₀ values of 141.2±3.46, 112.1±6.55 and 98.5±0.57 μ M respectively, however all the these fractions were not active against urease, carbonic anhydrase-II, and α -chymotrypsin showing selectivity towards the urease enzyme (**Table 58**), and the methanol and chloroform fraction was most active against phosphodiesterase-I enzyme.

Table 58: Enzymes inhibitory activities of the crude extract and isolated fraction of *D. lotus*

Frt	Phosp-I		Urease	Carb –II	α -Chym
	% Inhib (0.2 mg/mL)	IC ₅₀ ± S.E.M (μ g/mL)	% Inhib (0.2 mg/mL)	% Inhib (0.2 mg/mL)	% Inhib (0.2 mg/mL)
EtOAc	57.5	141.2±3.46	8.11	26.4	16.5
CHCl ₃	60.3	112.1±6.55	6.15	30.9	17.3
MeOH	63.4	98.5±0.57	10.66	31.3	19.3
STD	<u>EDTA</u> 80.1	<u>EDTA</u>	<u>Thiourea</u> 98.2	<u>Acetazolamide</u> 89	<u>Chymostatin</u> 98.6

Note: S. E. M. = Standard error mean, Phosp-I =Phosphodiesterase-I, Carb –II, Carbonic anhydrase-II, α -Chym= α -Chymotrypsin, % Inhib= % Inhibition, Frt= fraction, STD= Standard

6.4.2.1. Enzyme inhibition activities of compounds

The compounds (**15-17**) were evaluated against the urease, phosphodiesterase-I, carbonic anhydrase-II, and α -chymotrypsin enzyme inhibition assay, and the results are summarized in table 59 which showed low activity.

Table 59: Enzymes inhibitory activities of compounds (**15-17**)

Compounds	Urease	Phosp-I	Carb –II	α -Chym
	% Inhib (0.5 mM)	% Inhib (0.5 mM)	% Inhib (0.5 mM)	% Inhib (0.5 mM)
1	NA	11.1	NA	16.2
2	NA	11.4	NA	8.4
3	NA	14.7	NA	5.7
STD	Thioure 98.2	EDTA 80.1	Acetazolamide 89	Chymostatin 98.6

Note: S. E. M. = Standard error mean, NA = Not active, Phosp-I =Phosphodiesterase-I, Carb –II, Carbonic anhydrase-II, α -Chym= α -Chymotrypsin, % Inhib= % Inhibition, STD= Standard

6.5. *In vivo* biological screening of pistagremic acid (15)

6.5.1 Effect of 15 in acetic acid induced writhing test

The result of **15** in acetic acid induced writhing test in mice is presented in figure 58. It had a significant inhibition of noxious stimulation in a dose dependent manner with maximum effect of 68% at 10 mg/kg i.p. (**Figure 58**).

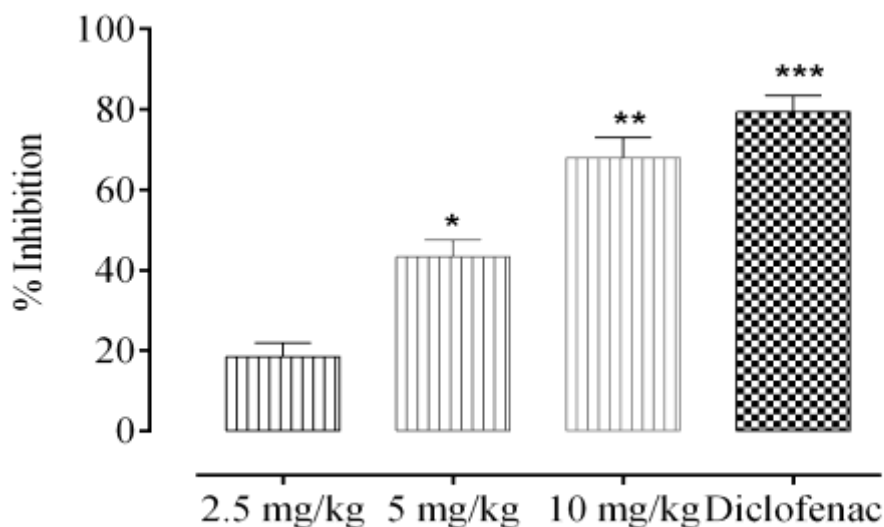


Figure 58: Percent effect of pistagremic acid (**15**) in acetic acid induced writhing test at 2.5, 5 and 10 mg/kg i.p. Values are reported as mean $\pm$ S.E.M. for group of six animals. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

6.5.2. Effect of 15 in tail immersion test

The effect of **15** in tail immersion test in mice is shown in figure 59. The results reflect significant activity during various assessment times in a dose dependent manner. The maximum pain inhibition was 59.46% at 10 mg/kg i.p. after 90 min of **15** treatments. However, the injection was not unable to antagonized the anti-hyperalgesic activity of **15** (**Figure 60**).

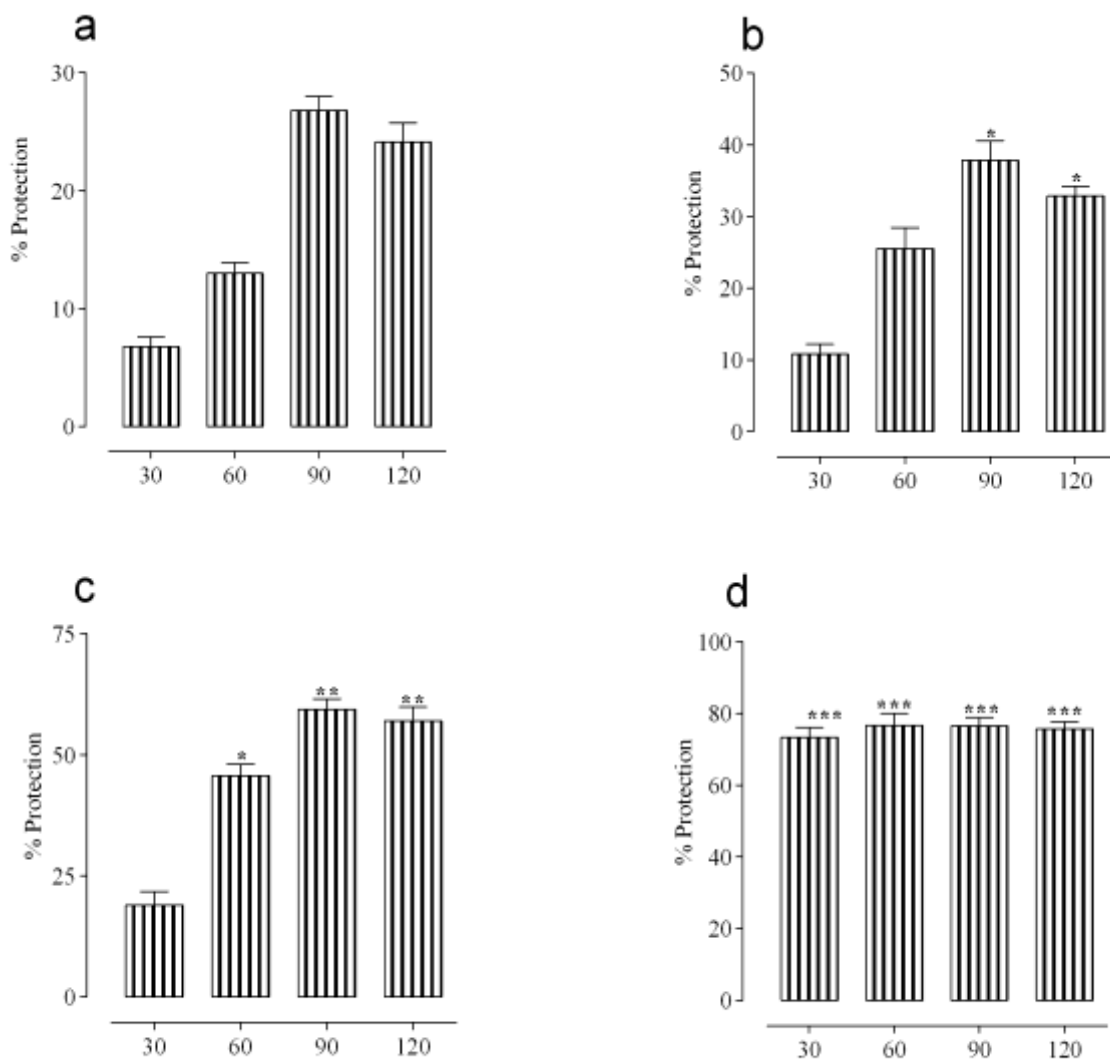


Figure 59: Percent effect of pistagremic acid (**15**) in tail immersion test at [a] 2.5, [b] 5 and [c] 10 [d] paracetamol 100 mg/kg i.p. Values are reported as mean $\pm$ S.E.M. for group of six animals. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

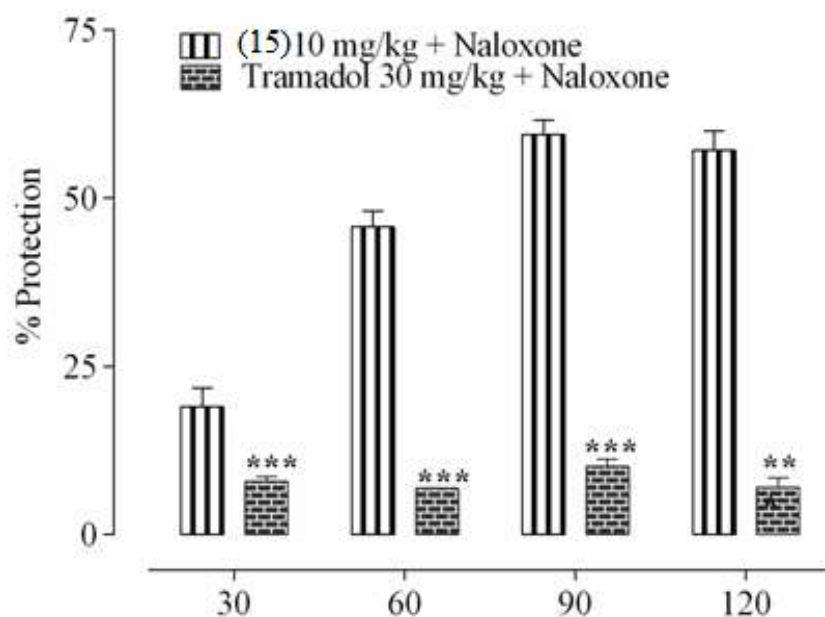


Figure 60: Percent effect of **15** in tail immersion test at after naloxone treatment Values are reported as mean $\pm$ S.E.M. for group of six animals. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$

6.5.3. Effect of **15** in carrageenan induced paw edema test

Pretreatment of **15** produced marked anti-inflammatory effect at both test doses of 5 and 10 mg/kg i.p. (**Figure 61**). The effect was most dominant (60.02%) after 3rd h of drug administration when examined for 5 hrs.

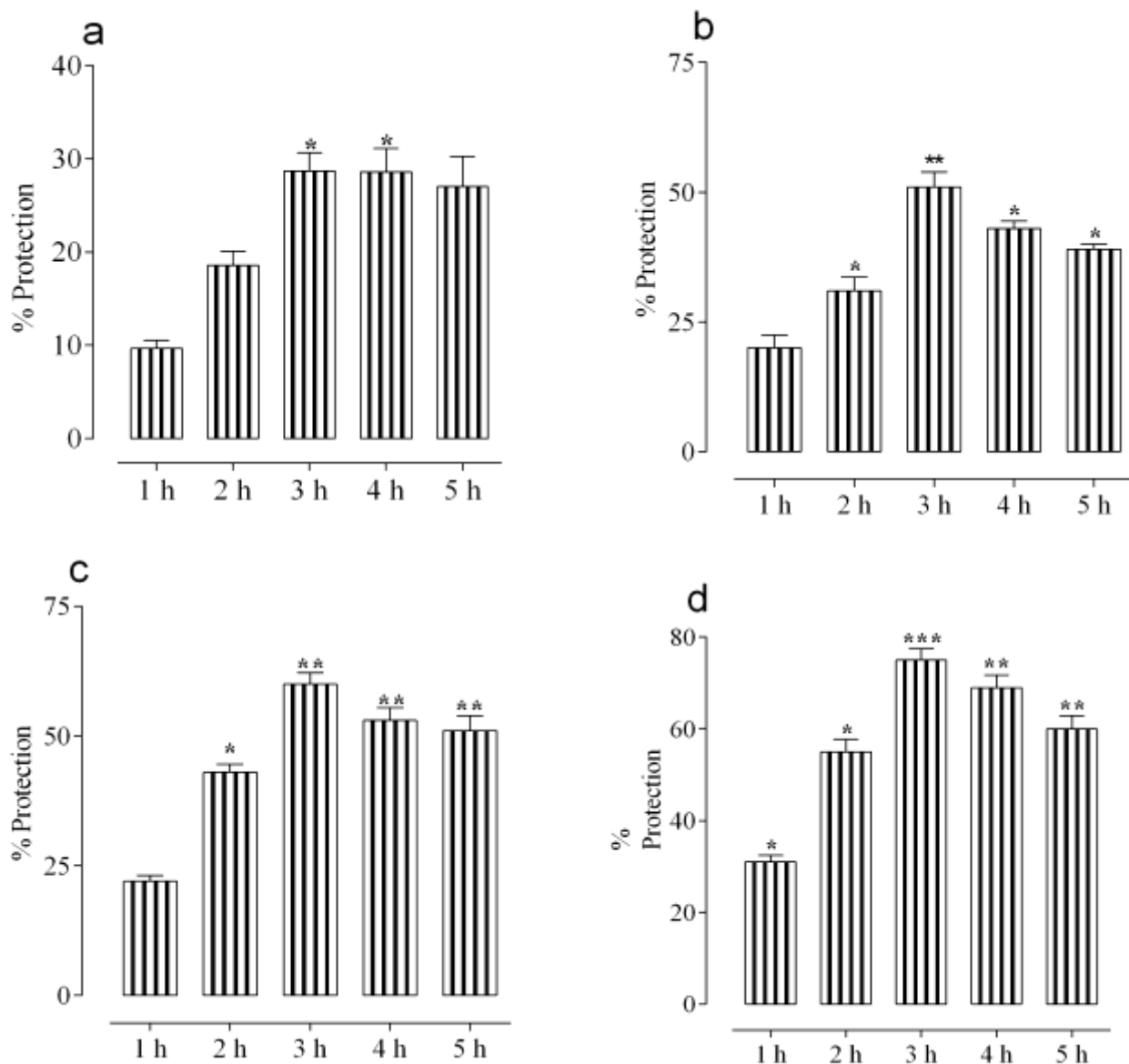


Figure 61: Percent effect of pistagremic acid (15) in carrageenan induced paw edema test at [a] 2.5, [b] 5 and [c] 10 [d] paracetamol 100 mg/kg i.p. Values are reported as mean $\pm$ S.E.M. for group of six animals. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

6.5.4. Effect of 15 in yeast induced pyrexia test

The percent effect of 15 in yeast induced pyrexia test at various doses is demonstrated in figure 62. Post treatment of 15 exhibited significant antipyretic effect in febrile mice during various assessment times (1-5 hrs). The maximum anti-hyperthermic effect (56.69%) was obtained at 10 mg/kg i.p. after 3rd h of drug administration.

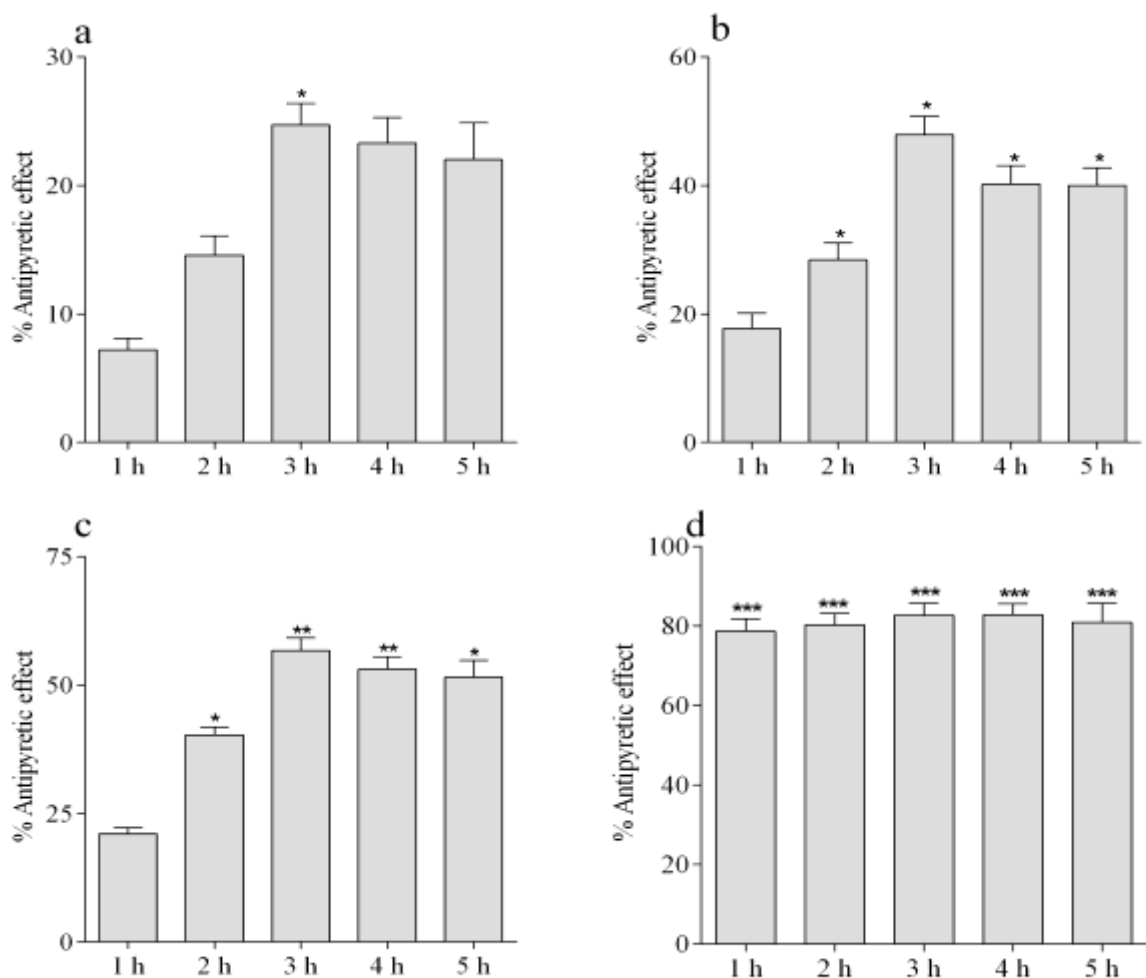


Figure 62: Percent antipyretic effect of 15 in yeast induced pyrexia test at [a] 2.5, [b] 5 and [c] 10 [d] paracetamol 100 mg/kg i.p. Values are reported as mean $\pm$ S.E.M. for group of six animals. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

6.5.5. Muscle relaxant effects of 15

Pistagremic acid (**15**) was also subjected form muscle relaxant potential using various animals model which showed excellent activity.

6.5.6. Effects of 15 in Rota rod

The muscle relaxant property of **15** on Rota rod is shown in figure 63. The time spent on revolving rod was significantly ($P<0.05$) reduced by pretreatment of **15** (5 and 10 mg/kg) with respect to with control.

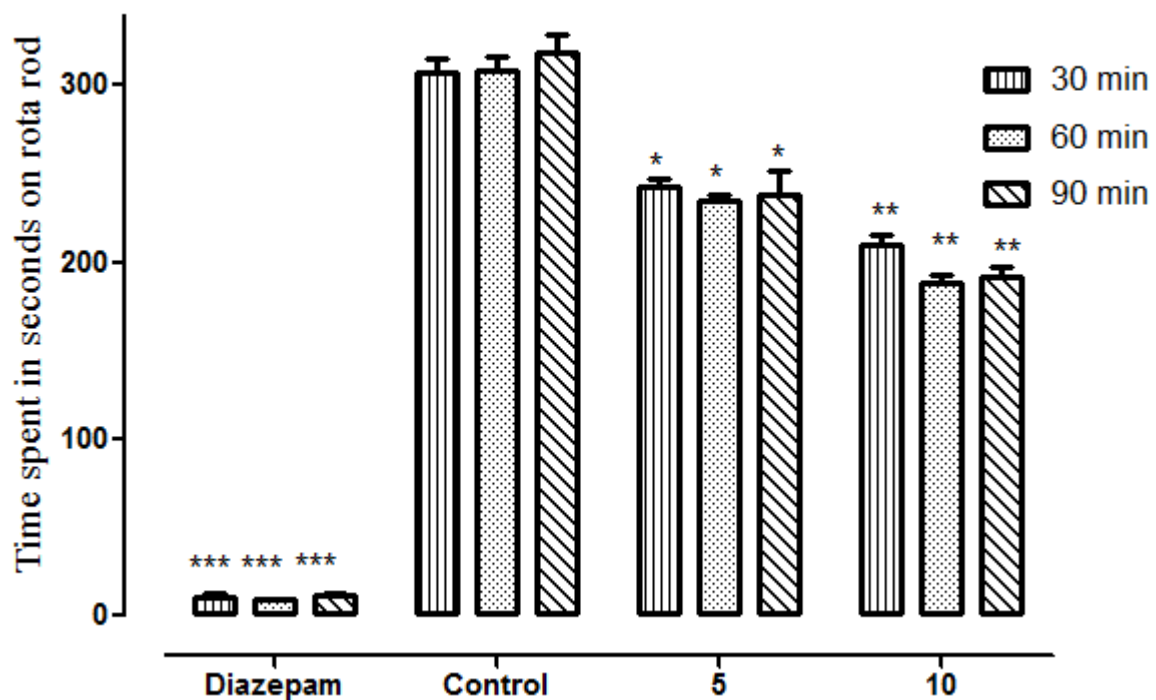


Figure 63: Effect of **15** on muscle coordination in rota rod, bars represent the time spent in seconds on rota rod, after 30, 60 and 90 minutes. * $P < 0.05$ and *** $P < 0.001$.

6.5.7. Effect of **15** in inclined plane

Pistagremic acid (**15**) evoked a dose dependent effect as shown in figure 64. Pretreatment of **15** (5 and 10 mg/kg) caused significant ($P < 0.05$) number of animals fell-down from inclined plan after 30, 60 and 90 min of treatment. The highest activity was achieved after 60 min of administration. A significant number of animals fell down in case of **15** with respect to control. However, standard drug was most dominant.

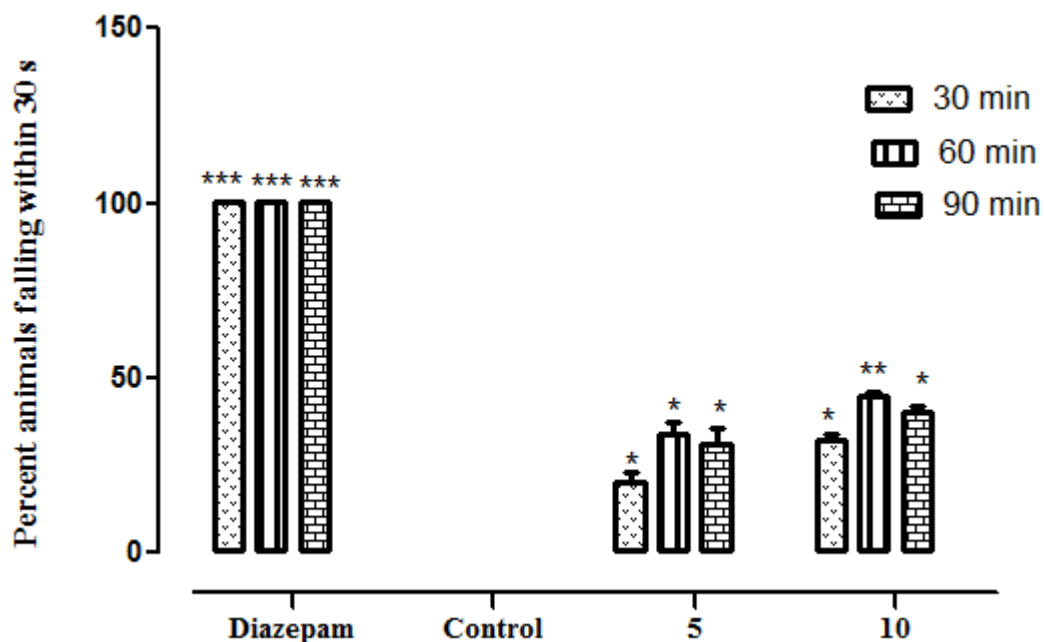


Figure 64: Effect of **15** on muscle coordination in inclined plane, bar represents the percent time spent in seconds by which mice slide off the inclined plane, 30, 60 and 90 min. * $P < 0.05$, *** $P < 0.001$, all with respect to control.

6.5.8. Effect of **15** in Traction test

The percent negative effect in traction test of compound **15** is shown in table 60. The effect of all posttreatment was observed at 30, 60 and 90 min. the maximum effect was shown at 60 min. The effect was dose dependent and the maximum effect was observed at 10 mg/kg (30.11%). The significant ($P < 0.05$) activity was shown by 5 and 10 mg/kg of **15** in comparison with control group.

Table 60: Percent effect of **15** on muscle relaxation (Traction test)

Group	Dose / kg	Traction test (%)		
		30 min	60 min	90 min
Distilled water	10 ml	0± 0.00	0± 0.00	0± 0.00
Diazepam	0.25 mg	100± 0.00***	100± 0.00***	100± 0.00***
15	5 mg	16.34± 1.33*	22.67± 2.11*	21.34± 2.00*

Values represents the percentages of mice (n=6) in traction test, 30, 60, 90 minutes after treatment. All data in table are mean ± S.E.M, (N=6). * $P<0.05$, ** $P<0.01$, all compare with control

6.5.9. Effect of **15** in chimney test

The significant ($P<0.05$) percent negative effect was illustrated by administration of **15** at 5 mg/kg. The muscle relaxant activity was dose dependent as shown in table 61.

Table 61: Percent effect of **15** on muscle relaxation (Chimney test)

Group	Dose / kg	Chimney test (%)		
		30 min	60 min	90 min
Distilled water	10 ml	0 ± 0.00	0± 0.00	0± 0.00
Diazepam	0.25 mg	100± 0.00***	100± 0.00***	100± 0.00***
15	5 mg	15.35± 1.78*	21.56± 2.99*	20.89± 2.00*

Values represents the percentages of mice (n=6) in chimney test, 30, 60, 90 minutes after treatment. All data in table are mean ± S.E.M, (N=6). * $P<0.05$, ** $P<0.01$, all compare with control

6.5.10. Molecular docking of pistagremic acid (15)

Pistagremic acid (**15**) was docked into the active site of COX-2 (PDB ID 1CX2). Ten docking conformations were produced. The top docking conformation was selected for exploring the binding mode of the **15**. The analysis of the docking results showed that the compound fits well in the binding pocket of COX-2 (**Figure 65**). From the ligplot of the top ranked docking conformation of **15** it was observed that **15** established three hydrogen bonds with important active residues R120, Y385 and S530 (**Figure 65**). Besides hydrogen bonding, several hydrophobic interactions between **15** and active residues were observed, e.g., M113, V349, L352, V523, A527 and L531 etc. The hydrogen and hydrophobic interactions of **15** to the active site residues might cause anchoring of the molecule on the active site resulting in activity against the enzyme.

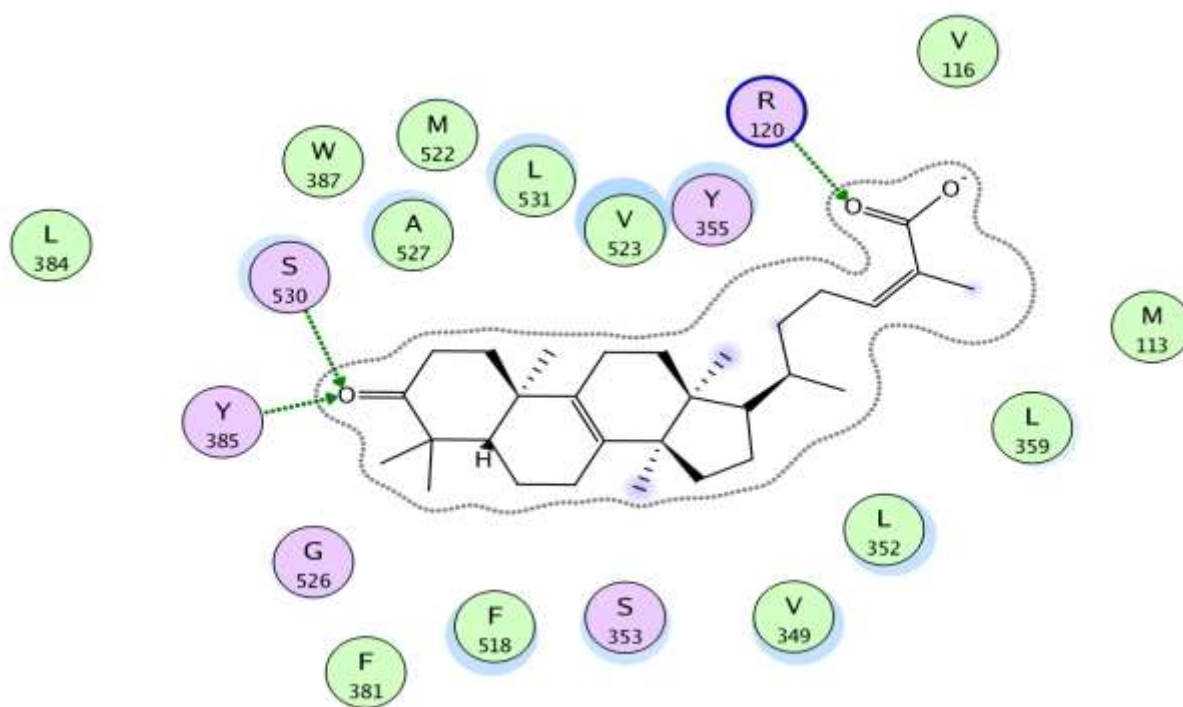


Figure 65: Molecular docking conformation of **15** in the binding pocket of COX-2 enzyme

6.6. *In vivo* biological screening of Shakirullaline (16)

In continuation of our studies aimed to demonstrate the beneficial properties of bioactive chemical constituents of *P. integerrima* for human health, the effects of isolated compound **16** was examined on the following biological activities.

6.6.1. Anti-inflammatory effect

The anti-inflammatory effect of Shakirullaline (**16**) was found to be dose-independent and diclofenac was used as reference drug as demonstrated in figure 76. The percent inhibition value was observed to be increased in dose of 10mg/kg (approx. 80%) comparing with dose of 5 mg/kg (60%). The maximum percent inhibition (58%-78.43%) of paw edema was observed at 3rd hrs of carrageenan injection and onward at 5 mg/kg and 10 mg/kg dosages administrated both group animals (**Figure 66**) and compared with standard drug (diclofenac); as a result Shakirullaline (**16**) exhibited a significant inhibition ($P < 0.01$).

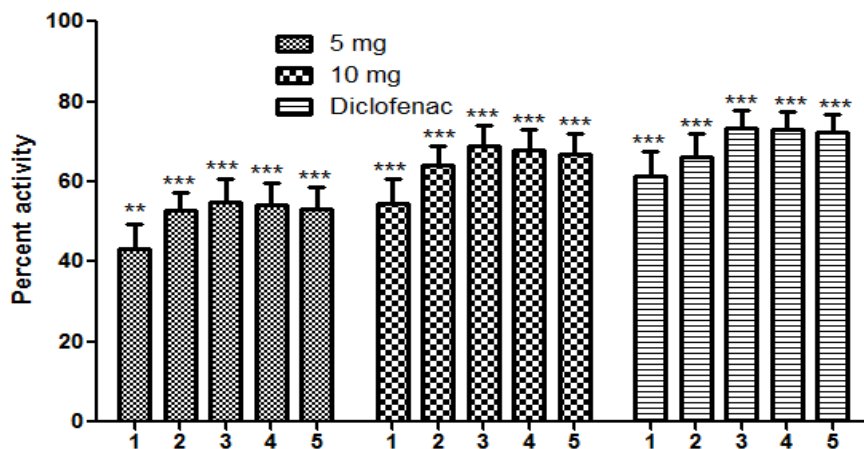


Figure 66: The percent anti-inflammatory activity of Shakirullaline (**16**) in carrageenan induced paw edema in rats. Each bar presents percent inhibition of paw edema after 1,2,3,4 and 5h of treatment. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. ** $P < 0.01$, *** $P < 0.001$.

6.6.2. Analgesic effect of Shakirullaline (16)

The acetic acid induced writhing was markedly protected by **16** as presented in figure 67, which inhibited significantly the writhing dose-independently with the percent inhibition value 65-80 % ($P < 0.05$), at the dose of 5, 10 mg/kg respectively against diclofenac drug.

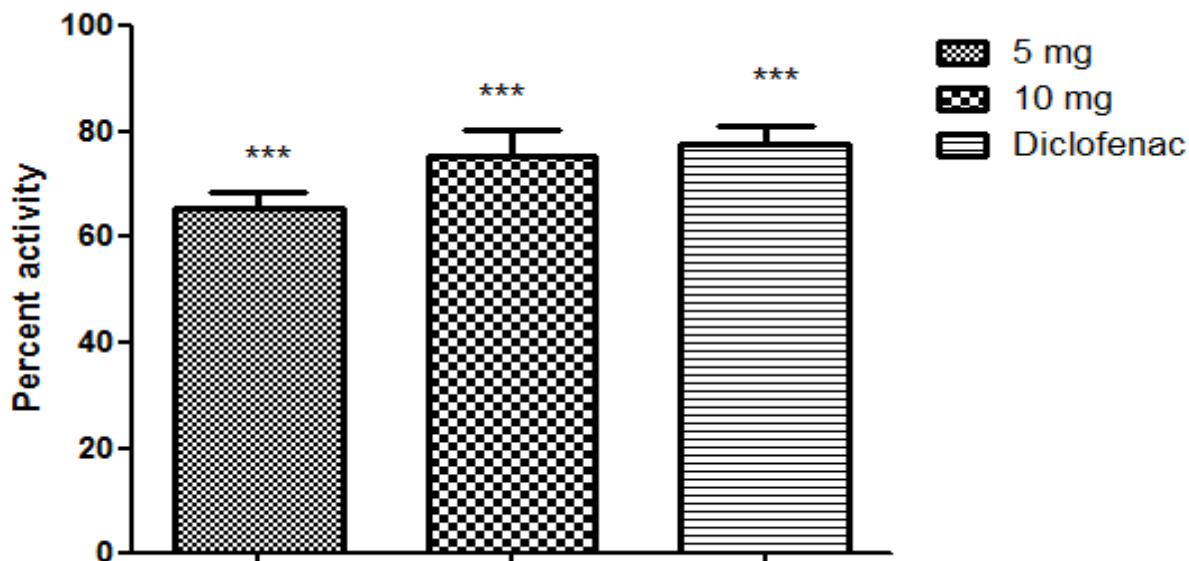


Figure 67: Analgesic effect of Shakirullaline (**16**) in acetic acid induced writhing test in rats. Each bar presents analgesic activity. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. ** $P < 0.01$, *** $P < 0.001$.

6.6.3. Muscle relaxants activity of Shakirullaline (16)

The muscle relaxant effect of **16** was measured by using Chimney and traction test is shown in table 62. The reference drug i.e., diazepam (0.25 mg/kg) exhibited remarkable muscle relaxant activity at measurement time intervals of 30, 60, 90 minutes which was considered 100% muscle relaxant activity. In comparison to the reference drug, the compound **16** at dose of 5 and 10 mg/kg exhibited moderate muscle relaxant activity,

however at high dose (10 mg/kg) the muscle relaxant was slightly increased which was not significantly greater than that 5 mg/kg (**Table 62**).

Group	Dose	Chimney test (%)		
		30 min	60 min	90 min
Distilled water	10 ml/kg	0 ± 0.00	0 ± 0.00	0 ± 0.00
Diazepam	0.25mg/kg	100 ± 0.00**	100 ± 0.00**	100 ± 0.00**
16	5 mg/kg	33.65 ± 2.00*	50.78 ± 1.77**	50.09 ± 1.06**
	10 mg/kg	35.98 ± 2.98*	53.22 ± 1.76**	52.99 ± 2.65**

Table 62: The percent effect of Shakirullaline (**16**) on muscle relaxation.

The percent effect of Shakirullaline (**16**) on muscle relaxation. Values represents the percentages of mice (n=6) showing negative effects in chimney and traction test, 30, 60 and 90 minutes after treatment with distilled water (10 ml/kg), tested compound (5 and 10 mg/kg) or diazepam (0.25 mg/kg). Data presented as mean of percent ± S.E.M, (n=6). * $P < 0.05$, ** $P < 0.01$, all compared with control.

6.6.4 Acute Toxicity of Shakirullaline (**16**)

During 24 hrs assessment of acute toxicity no mortality was observed in the animals treated with the various doses of **16**.

6.7. *In vivo* biological screening crude extract and integarrimic acid (17)**6.7.1. Analgesic effects of the crude extract and integarrimic acid (17)**

The methanolic extract as well as isolated compound (**17**) exhibited a dose dependent analgesic effect in both pain paradigms. Regarding the acetic acid induced writhing test (**Figure 68**) the crude methanolic extract and tested compound significantly ($P<0.01$) attenuated the induced writhing. The percent effect of the crude was 12, 30.34 and 61.85% at the tested doses of 50, 100 and 150 mg/kg respectively, while the effect of (**17**) was 35, 55.23 and 78.46% at the tested dose of 2.5, 5 and 10 mg/kg, respectively. The percent effect of diclofenac sodium was 82.34% which was nearest to our tested compound. The crude methanolic extract demonstrated a dose and time dependent effect (**Figure 69**). The effect was most significant ($P< 0.05$) at the tested dose of 150 mg/kg, and maximum effect was observed after 60 min and remained persistent upto 90 min. The crude methanolic extract (150 mg/kg) exhibited the maximum activity (47.65 and 65.33%) after 60 and 90 min respectively (**Figure 70**). Regarding the analgesic effect of tested compound a promising activity was observed and significantly ($P< 0.05$) increased the latency time of tested animals in hot water.

The methanolic extract and **17** was tested for its analgesic effect using two well established animals paradigm. The acetic acid induced pain and tail immersion methods which are also known as chemical and thermal induced pain models respectively. The chemical induced pain is a well-established protocol in evaluating medicinal agents for their antinociceptive effect.

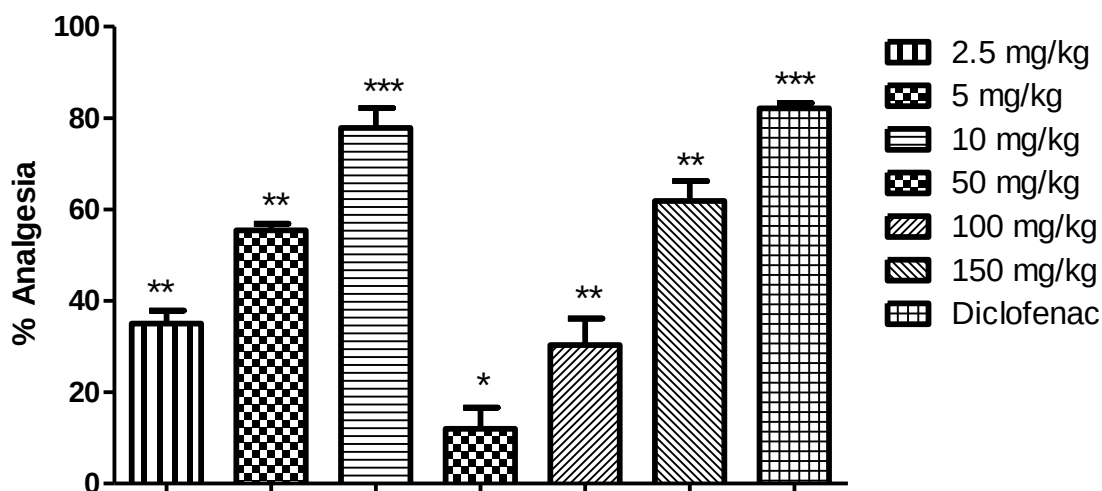


Figure 68: Analgesic effect of methanolic extract and integerrimic acid (17) (50, 100 and 150 mg/kg) extract and isolated compound 1 (2.5, 5 and 10mg/kg) in acetic acid induced writhing test.

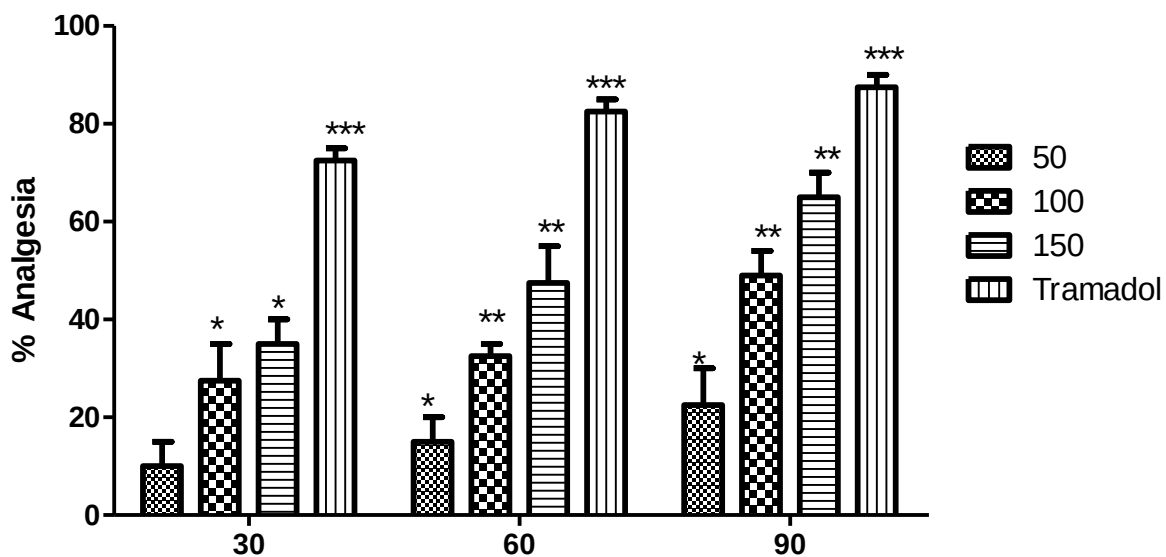


Figure 69: Analgesic effect of crude in tail immersion pain paradigm

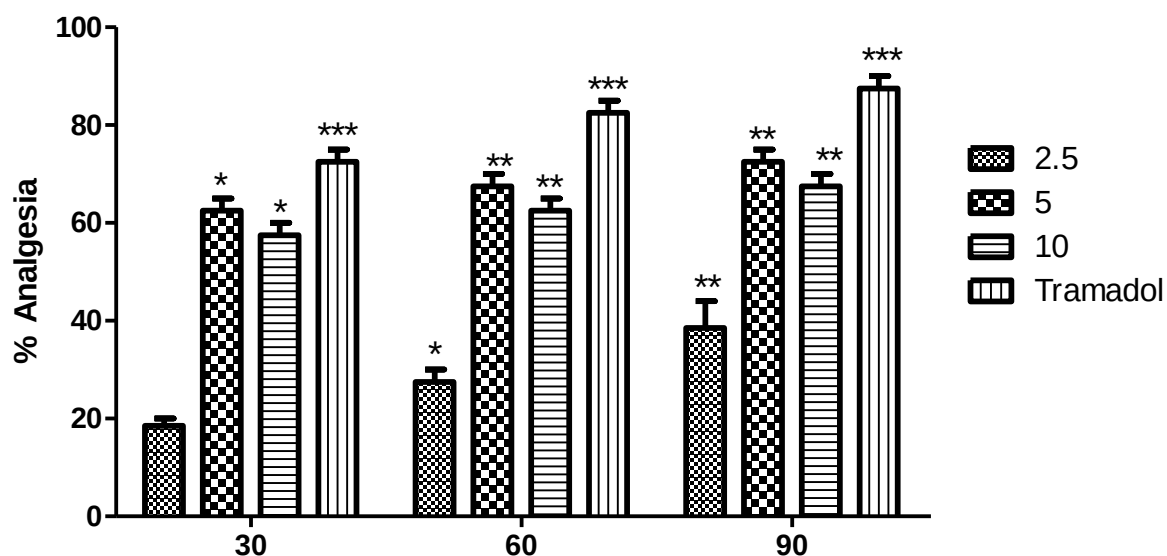


Figure 70: Analgesic effect of integarrimic acid (**17**) in tail immersion pain paradigm

6.8. Molecular docking of integarrimic acid (**17**)

The integarrimic acid (**17**) was docked into the binding pocket of COX-2 (PDB ID 1CX2). Twenty docking conformations were produced for integarrimic acid (**17**). The top docking conformation was selected for exploring the binding mode of the integarrimic acid. The analysis of the docking results showed that the compound fit well in the binding pocket of COX-2 (**Figure 71**). From the top-ranked docking conformation of integarrimic acid it was observed that the integarrimic acid established three hydrogen bonds with important active residues Y355, R513 and E524 (**Figure 71**). Besides hydrogen bonding several hydrophobic interaction between the integarrimic acid (**17**) and active residues were observed, e.g., P86, L93, V116, V349, L384, W387, M522, and V523 etc. The hydrogen and hydrophobic interactions of the **17** to the active site residues of COX-2 might be one of the reasons to anchor in the active site and showed activity against the enzyme.

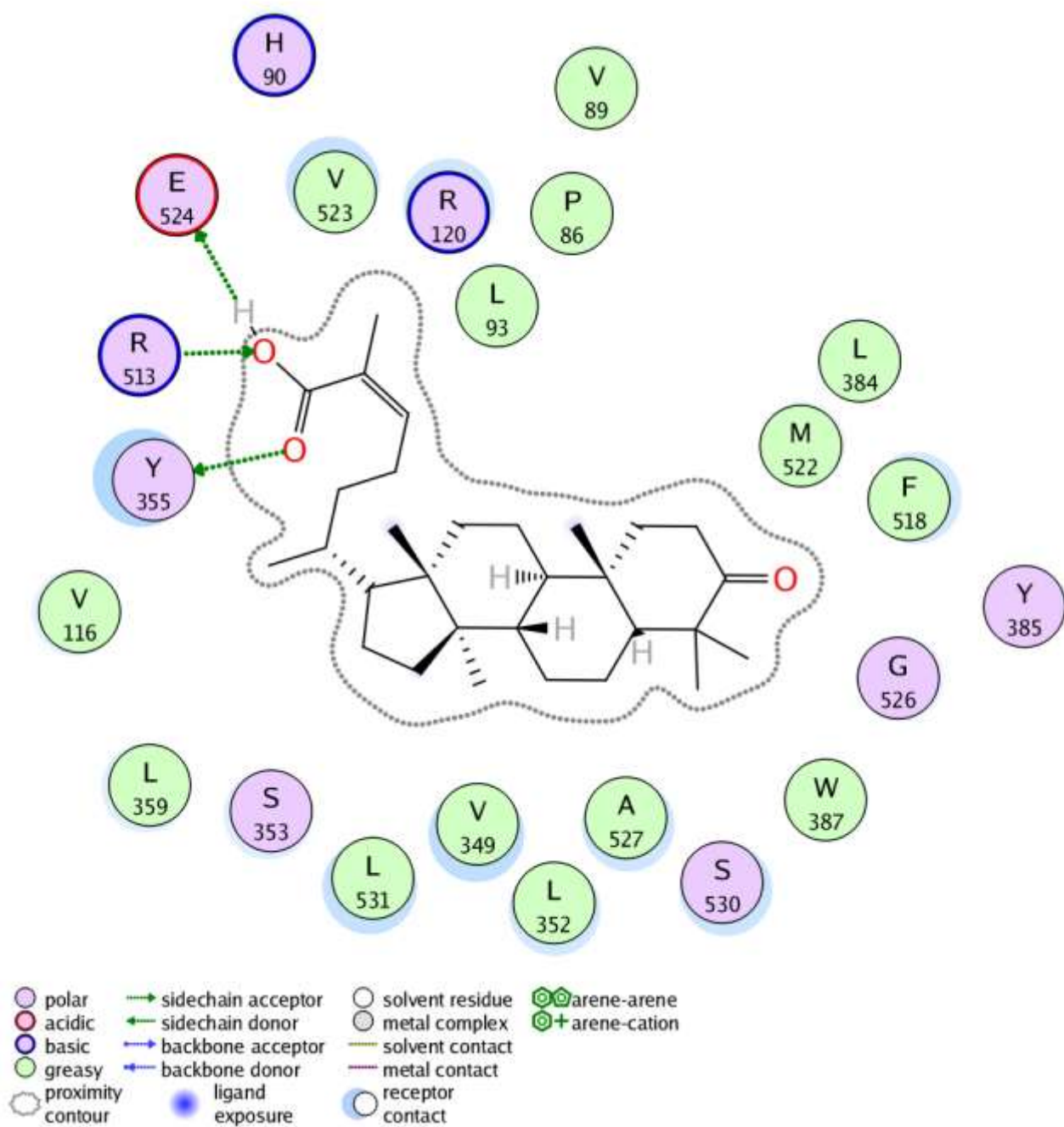


Figure 71: Docking conformation of integerrimic acid (17) in the binding pocket of COX-2 enzyme

7.1. General experimental procedures

General experimental procedures are already discussed in section 4.1 (page 118)

7.1.1. Chemical reagents and spray

All chemicals and reagents are already discussed in section 4.1.1 - 4.1.4 (pages 118,119)

7.1.2. Plant collocation

The plant of *P. integerrima* including; galls, roots, leaves and stem were collected from Toormang, Razagram area of district Dir, Khyber Pukhtunkhawa province of Pakistan during the month of February, 2010. The plant was identified by Prof. Dr. Abdur Rashid; Department of Botany, University of Peshawar, Pakistan. A voucher specimen (BOT. 20036(PUP) was deposited in the botany herbarium.

7.2. Phytochemical screening of *P. integerrima*

Detail experimental procedure for phytochemical screening is already discussed in section 4.2.1-4.2.11 (page 119-121).

7.3. Present work

In view of the folkloric uses in various traditional system and various biomedical implications attributed to *P. integerrima* phytochemical screening, chemical investigation and biological screening of the various parts were undertaken. The current investigations led to the isolation and structural elucidation of three new and two known constituents from the galls of *P. integerrima*. The crude extracts, fractions and chemical constituents were also screened for various *in vitro* and *in vivo* biological study which exhibited potential activity against corresponding standards.

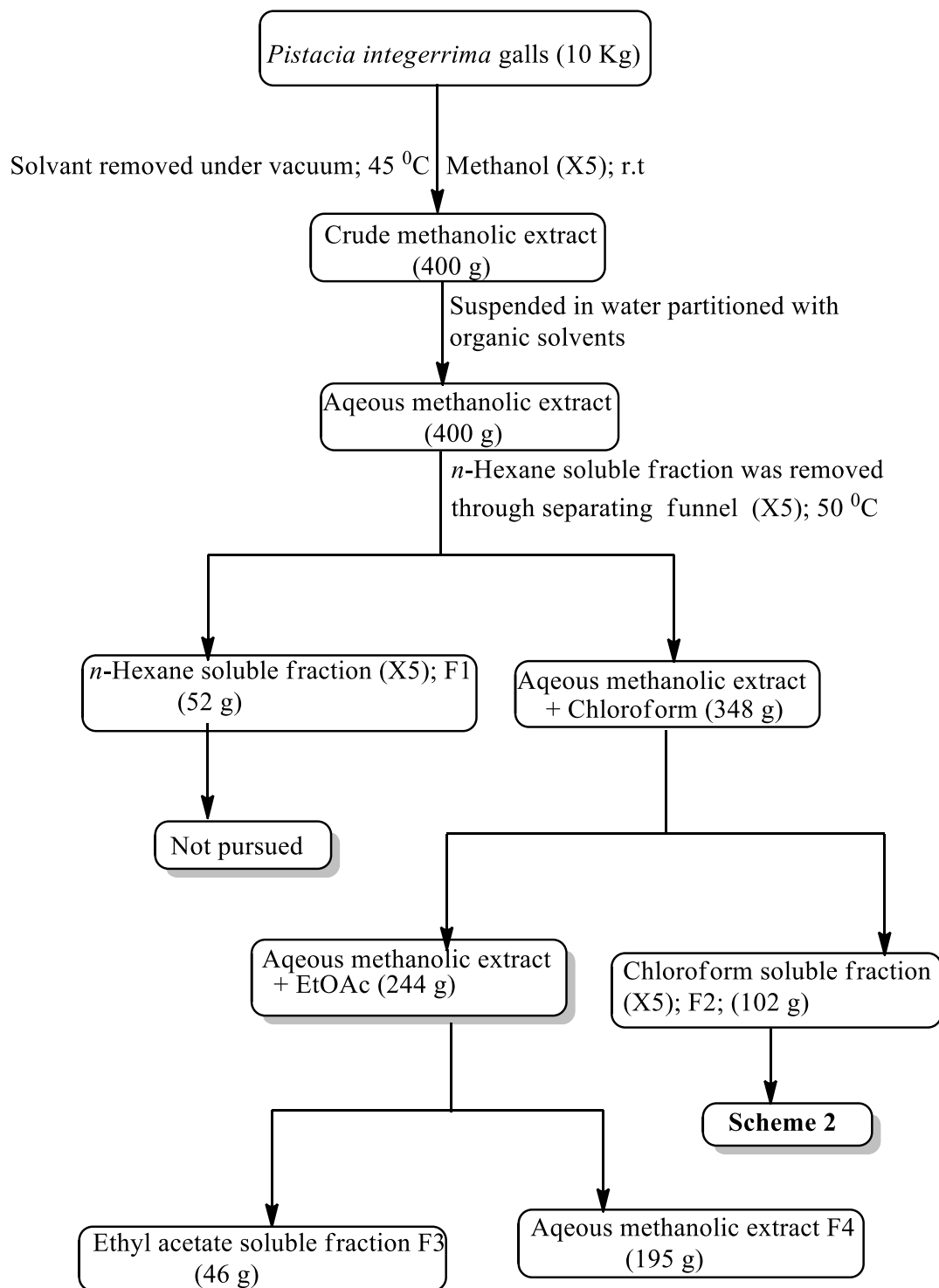
7.3.1. Extraction, isolation and purification

7.3.2. Extraction and isolation of *P. integerrima* galls

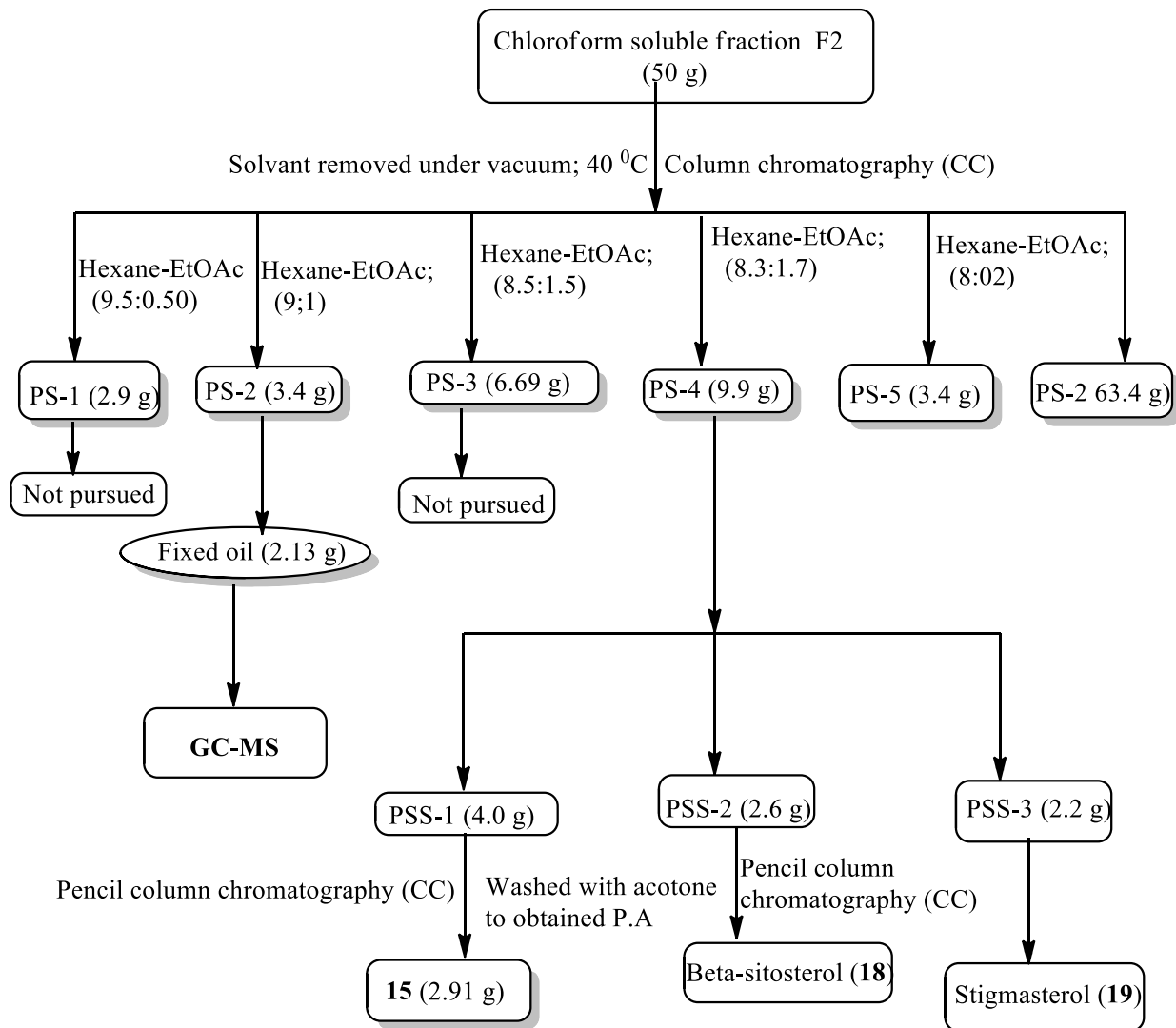
Dried and crushed galls of *P. integerrima* (10 kg) were subjected to cold extraction with MeOH (20 L; x5). The combined extract were concentrated using rotary evaporator under reduced pressure at temperature below 55 °C to obtain a bluish residue (400 g) which was suspended in water and successively partitioned with *n*-hexane, CHCl₃, EtOAc and aqueous fraction (**Scheme 4**). The CHCl₃ fraction (2.91 g) was subjected to column chromatography on silica gel (Merck; silica gel 60 (0.063-0.200 mm), 5×60 cm) and was eluted with *n*-hexane-acetone (100:0 → 0:100). Thirty three fractions (PS-1 to PS-33) were obtained based on TLC profiles, which was combine on the basic of TLC profile to obtain major fractions; PS-1 (2.9 g), PS-2 (3.4 g), PS-3 (6.69 g), PS-4 (9.9 g), PS-5 (3.4 g) and PS-6 (3.4 g). Fractions PS-4 (9.9 g) was further subjected to column chromatography which yielded sub-fractions; PSS-1 (4 g), PSS-2 (2.6 g) and PSS3 (2.2 g). The fraction PSS-1 (4 g) was subjected to CC eluting by *n*-hexane and acetone (100:0 → 15:100) led to the isolation of colorless crystals of various sizes and shapes which was separated from the solution by decantation. The crystals were washed with *n*-hexane for several times and furnished pure crystals which were re-grown in mixture of *n*-hexane-acetone-chloroform (7:2:1) to obtain a pure and large crystals of **15** (50 mg) (**Scheme 5**) while fraction PSS-2 and PSS-3 led to two know compounds; β -sitosterol (**11**) and stigmasterol (**12**).

7.3.3. Extraction and isolation of *P. integerrima* bark

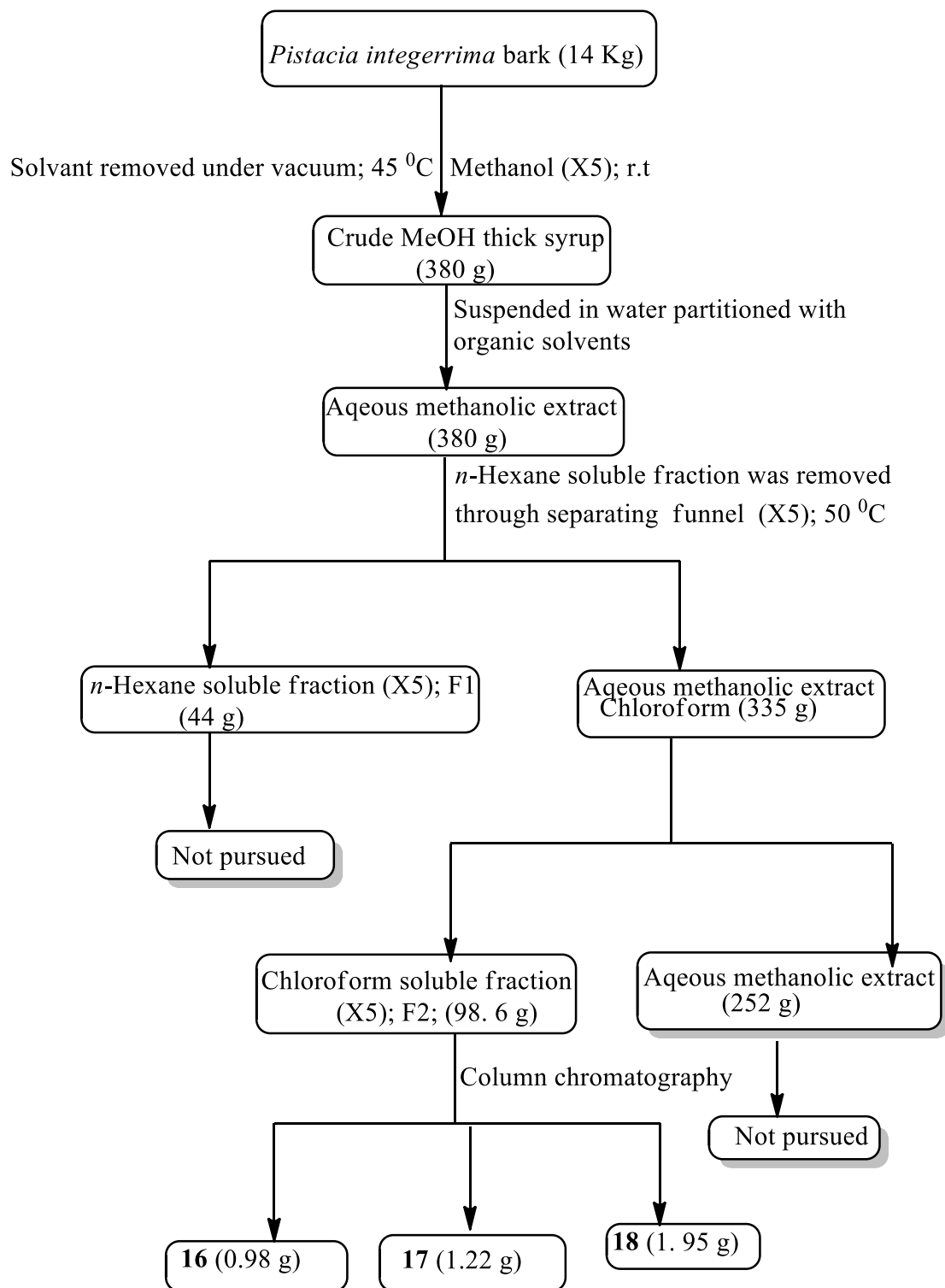
The plant bark was shade-dried at room temperature, grinded into powder and subsequently successively extracted repeatedly with methanol (x 3) at room temperature. The combined methanolic extracts were freed of the solvent under vacuum to obtain thick syrup (380 g) which was successively partitioned between *n*-hexane/water, chloroform/water, ethyl acetate/water and butanol/water. The chloroform fraction was dried (anhyd. Na₂SO₄) concentrated under vacuum where 98.6 g of residue was obtained. A portion (10 g) of this residue was subjected to silica gel column chromatography, eluting with *n*-hexane-ethyl acetate (eluted *n*-hexane-EtOAc, 82:18) in an increasing order of polarity afforded colorless crystals which were separated from the solution by decantation. The crystals were re-crystallized with appropriate solvents (*n*-hexane-acetone, 4:1) which led to the isolation of a new compound Shakirullaline (**16**) while integerrimic acid (**17**) was afforded (**Scheme 6**) as a white amorphous powder (eluted; *n*-hexane-EtOAc, 80:20) along with β -sitosterol (**Scheme 6**).



Scheme 4: Extraction and partitioned of galls of *P. integerrima*



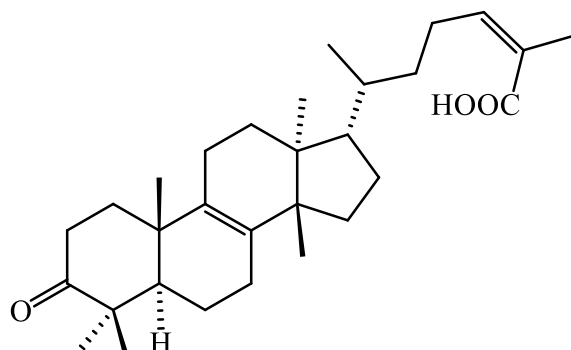
Scheme 5: Isolation and purification of compounds of *P. integerrima* galls



Scheme 6: Isolation of compounds of *P. integerrima* bark

7.4. New constituents of *P. integerrima*

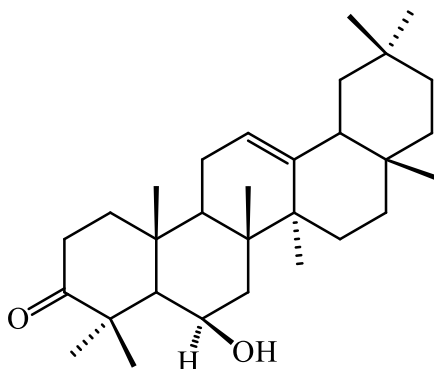
• Pistagremic acid (16)



2-Methyl-6-(4,4,10,13,14-pentamethyl-3-*O*-2,3,4,5,6,7,10,11,12,13,14,15,16,-17-tetradecahydro-1*H*-cyclopenta[*a*]-phenanthren-17-yl) hept-2-enoic acid.

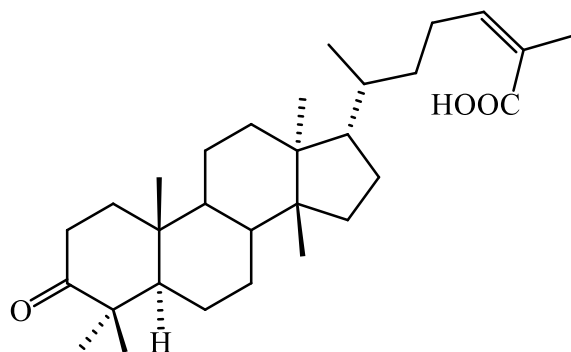
Physical status	White crystal
Yield	1.70 g
M.P	139-141 °C
IR (cm ⁻¹)	3360, 1712, 1690, 1672, 16012
Uv $\nu_{\max}$ (nm)	265, 219
EIMS	454. 3447
Mol: formula	C ₃₀ H ₄₆ O ₃
Chemical name	Pistagremic acid
¹ H-NMR (CDCl ₃)	Table 38
¹³ C-NMR (CDCl ₃)	Table 38

- Shakirullaline (16)

3-keto-6 β -hydroxy- α -amyrin

Physical status	White crystals
Yield	0.98 g
M.P	144-145 °C
IR (cm ⁻¹)	2924, 1643, 1634, 1604, 1460
Uv max (nm)	218, 237 and 243.
EIMS	440.3710
Mol: formula	C ₃₀ H ₄₈ O ₂
Chemical name	3-keto-6 β -hydroxy- α -amyrin
¹ H-NMR (CDCl ₃)	Table 39
¹³ C-NMR (CDCl ₃)	Table 39

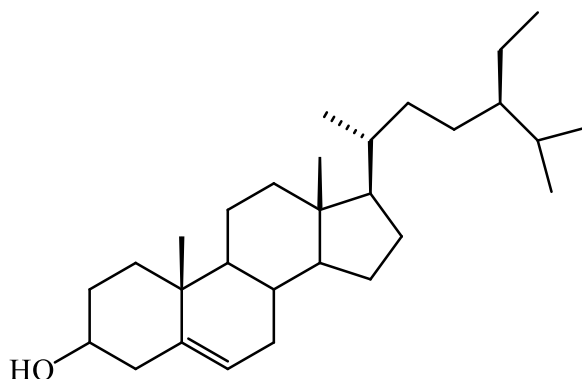
- Integerrimic acid (17)



(Z)-2-methyl-6-(4,4,10,13,14-pentamethyl-3-oxohexadecahydro-1H-cyclopenta[*a*]phenanthr-17-yl)hept-2-enoic acid.

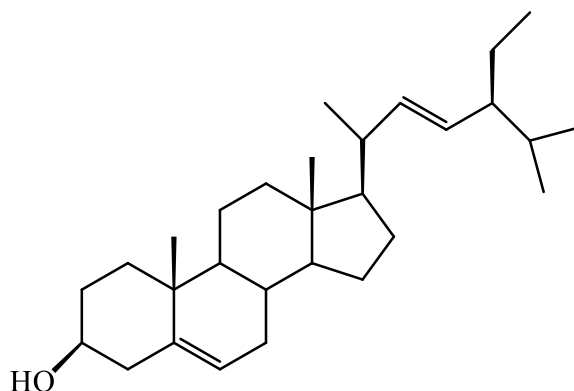
Physical status	white powder
Yield	1.22 g
M.P	148-150 °C
IR (cm ⁻¹)	3358, 1688, 1670
Uv $\nu_{\max}$ (nm)	260, 435
EIMS	456.3432
Mol: formula	C ₃₀ H ₄₈ O ₃
Chemical name	Integerrimic acid
¹ H-NMR (CDCl ₃)	Table 40
¹³ C-NMR (CDCl ₃)	Table 40

Structures of known chemical constituents

***β*-Sitosterol (18)**

17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol.

Physical status	White needle crystals
Yield	2.34 g
M.P	137-140 °C
IR (cm ⁻¹)	2968, 2937, 2872, 1686
Uv $\nu_{\max}$ (nm)	255
EIMS	414
Mol: formula	C ₂₉ H ₅₀ O
Chemical name	17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-,8,9,11,12,-14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol.
¹ H-NMR (CDCl ₃)	Table 12
¹³ C-NMR (CDCl ₃)	Table 12

Stigmasterol (19)

(3S,8S,9S,10R,13R,14S,17R)-17-[(E,2R,5S)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol

Physical status	White needle crystal
Yield	1.98 g
M.P	162-165 °C
IR (cm ⁻¹)	2965, 2935, 2870 and 1680
Uv $\nu_{\max}$ (nm)	260
EIMS	412
Mol: formula	C ₂₉ H ₄₈ O
Chemical name	(3S,8S,9S,10R,13R,14S,17R)-17-[(E,2R,5S)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol
¹ H-NMR (CDCl ₃)	Table 13
¹³ C-NMR (CDCl ₃)	Table 13

7.5. Pharmacological screening of *P. integerrima***7.5.1. *In-vitro* screening****7.5.2. Urease inhibition assay**

The protocol of urease inhibition assay is already discussed in section 4.6.2. (page 137).

7.5.3. Phosphodiesterase-I inhibition assay

Phosphodiesterase-I inhibition assay are already discussed in section 4.6.3. (page 137).

7.5.4. Carbonic anhydrase-II assay and inhibition

Carbonic anhydrase-II assay and inhibition are already discussed in section 4.6.4. (page 138).

7.5.5. Chymotrypsin inhibition assay

Chymotrypsin inhibition assay are already discussed in section 4.6.5. (page 138).

7.5.6. DPPH free radical scavenging assay

DPPH free radical scavenging assay (Antioxidant) is already discussed in section 4.6.6. (page 139).

7.5.7. Bacterial strains assortment and preservation

The procedure for bacterial strains assortment and preservation are already discussed in section 4.6.7. (page 139).

7.5.8. Antimicrobial assay against selected bacterial Strains

Antimicrobial Assay against selected bacterial strains is already discussed in section 4.6.8. (page 140).

7.5.9. Antifungal assay

Antifungal activity is already discussed in section 4. 6.9 (page 140).

7.5.10. Brine shrimp cytotoxic assay

Cytotoxic activity is already discussed in section 4.6.10. (page 141).

7.5.11. Insecticidal activity

Insecticidal activity is already discussed in section 4.6.11. (page 141).

7.5.12. Anticancer activity of 15

The anticancer assay against cancer cell lines was conducted at NCI by using Sulphorhodamine B assay [180]. These cancer cell lines were developed in RPMI-1640 medium, enhanced with 10% fetal bovine serum and 2 mM L-glutamine. The culture flasks was kept in CO₂ (5%) incubator at 37 °C and 100% relative moisture as discussed in literature [181]. The adherent cells were acquired using Trypsin-EDTA solution, followed by adding of 10⁴ cells per mL in each well of 96-wells plate. The plates were incubated in CO₂ incubator for 24 h prior to addition of tested samples. Aliquots of (100 µL) of different dilutions of test extract and compounds was added to the appropriate wells and incubated for 48 h. Then cells were secure *in situ* by the gentle adding of cold 50% (w/v) trichloroacetic acid (TCA) (50 Ml) and incubated for 30 minutes at room temperature. The supernatant was removed and the plates were washed with water and then dried overnight. 100 µL of sulphorhodamine B (SRB) solution (0.4%; w/v) in 1% acetic acid was introduced to each plates (well) and incubated for 30 mint at 25 °C. After staining the unbounded dye from the plates were removed by washing with acetic acid (1%) and then dried in air. Subsequently 24 hrs of ventilation the bounded stain was then solubilized in trizma base (10 mM), and

then absorbance was measured at 545 nm in microplate reader by using a microplate ELISA reader (Spectra Max plus, Molecular Devices, CA, USA). The cytotoxicity effect was noted producing 50% growth inhibition.

Growth inhibition of 50% (GI₅₀) and total growth inhibition (TGI) were calculated by the equation $[(T_i - T_z)/(C - T_z)] \times 100 = 50$ and $T_i = T_z$ respectively while LC₅₀ was calculated as $[(T_i - T_z)/T_z] \times 100 = -50$.

Where, T_z = absorbance measurements at time zero, C= control growth, and T_i = test growth in the presence of drug at the five concentration levels.

7.5.13. Leishmanicidal activity

Leishmania major (DESTO) promastigotes for compound **15** were cultured at 22-25 °C in RPMI-1640 (Sigma) according to standard procedure [182]. The medium was added with heat inactivated fetal bovine serum (FBS) (10%; at 56 °C and 30 min). Promastigote medium in the logarithmic phase of development was centrifuged for 10 min at 2000 rpm, and then washed with saline. The parasites were diluted with fresh culture to a final density of 10⁶ cells/mL. In a 96-well micro titer plate, medium (180 µL) was added in 1st row and 100 µL of medium was introduced in others wells. 20 µL test was introduced in the medium while 100 µL of culture (parasite) was added in each well. Among them one row was used for DMSO (control) which contain medium while other for amphotericin B (standard drugs). All plates were incubated for 72 h at 21-22 °C and finally the numbers of survive parasites were calculated microscopically in Neubauer chamber. The results were measured in triplicate. The 50% inhibitory concentrations (IC₅₀) were measured by EZ-Fit 5.03 Perrella Scientific Software.

7.5.14. α -Glucosidase inhibition activity

α -Glucosidase (E.C.3.2.1.20) from *Saccharomyces* species was obtained from Sigma Aldrich. The activity was measured spectro-photometrically over incessant monitoring of the nitrophenyl formed by the hydrolysis of the substrate *p*-nitrophenyl. 0.7mM α -D-glucopyranoside (PNP-G) and the enzyme (500 mu/mL) used. The enzymatic reaction was done for 30 min at 37 °C as per reported methods [183]. The calculation in absorption at 400 nm, due to the hydrolysis of PNP-G by α --glycosidase, was tested continually on microplate spectrophotometer (Spectra Max, Molecular Devices, USA). Phosphate saline buffer at pH 6.9 was also used, which comprise 50 mM sodium phosphate and 100 mM NaCl while acarbose was used as positive controls.

7.5.15. Cytotoxicity assay

In-vitro cytotoxicity assay was of compound **15** was performed by using mice hepatocytes and LCMK-2 monkey kidney epithelial cells [184] . Compounds **15** were incubated for 24 hrs, and then lastly the cell viability was determined by the MTT procedure. In this bioassay the cells were preserved in RPMI 1640 medium (bought from Gibco BRL) comprising FBS (10%) (Also purchased from Gibco BRL), penicillin sodium salt (110 μ g/mL), sodium bicarbonate solution (2 mg/mL), and streptomycin sulfate (100 μ g/ml). First seeding of the 7.1×10^3 LCMK-2 cells and 8.6×10^3 mice hepatocytes was lead in 96 wells plates. The cells were preserved with test sample at different concentrations and with vehicle (0.2% DMSO) and then incubated for 48 h using by performing MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) procedure (Sigma Chemical Co., St. Louis, MO, USA).

7.6. *In vivo* screening**7.6.1. Analgesic activity**

The protocol of analgesic activity is already discussed in section 4.7.1 (page 144).

7.6.2. Tail immersion test

This protocol are already discussed in section 4.7.2 (page 144)

7.6.3. Anti-inflammatory activity

The protocol of anti-inflammatory activity is already discussed in section 4.7.5 (page 145).

7.6.4. Open field test

Open field test are already discussed in section 4.7.6. (page 146).

7.6.5. Molecular docking of 15 and 17

The molecular docking of COX2 (PDR;1CX2) protiens were done by using MOE-Dock docking software applied in molecular functioning location software package (MOE). Ligplot originated in MOE were used to note the connections among COX-2 and chemical constituents. The MOE-Builder used to contracts the chemical structure of the constituents. The structure energy condensed with nonappearance limits over MOE energy minimization procedure (gradient: 0.05; Force Field: MMFF94X). The protein molecules of cyclo-oxygenase were gained the protein data bank (PDB; code; ICX-2). The molecules of water were isolated and the 3D protonation of protein fragment was performed. The energy of protected saved protein molecules were reduced by with default limits of MOE energy minimization procedure (gradient: 0.05; Force Field; Amber 99). The docking simulation of the ligands was permitted by the MOE-Dock program protection with the defaulting limits. The ligands were then kept elastic to find the precise conformations and stretched with

minimum energy structures. At the end of the docking; the top-ranked conformation of each ligand were studied for their binding communication.

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