BIOACTIVE CONSTITUENTS FROM *MALLOTUS PHILIPPENSIS* AND *LITSEA MONOPETALA* – SYNTHESIS OF *O*-ALKYLATED BERGENINS AND EVALUATION OF THEIR BIOLOGICAL ACTIVITIES

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DECLARATION

This is to certify that this dissertation prepared by Mr.Hazrat Amin entitled "Bioactive Constituents from Mallotus philippensis and Litsea monopetala - Synthesis of O-Alkylated Bergenins and Evaluation of their Biological Activities" 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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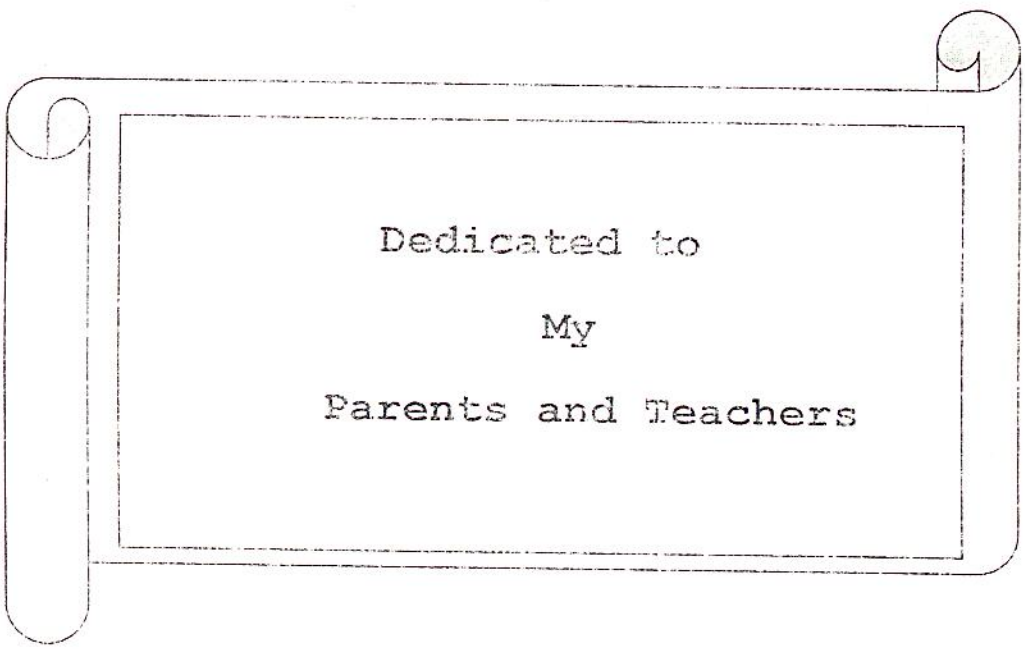
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Dedicated to
My
Parents and Teachers

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ABSTRACT

This doctoral dissertation is comprised of three chapters. Chapter 1 and 2 describe the isolation and pharmacological evaluation of crude extracts, fractions and pure compounds obtained from two medicinal plants namely, *Litsea monopetala* and *Mallotus philippensis*. Chapter 3 is about the synthesis of fifteen new *O*-alkylated bergenins along with their *in vitro* antiinflammatory and antiplasmodial activities. The bergenin used in the synthesis of the series of its derivatives was obtained through extraction, chromatographic separation and purification from the stem of *Mallotus philippensis* with a yield of 16.5% of the crude extract.

Chapter 1 deals with the antioxidant activity of the phenolic fractions of *Litsea monopetala* bark extracts. Four fractions (I-IV) were separated from methanol extracts on a sephadex LH-20 column using methanol as the mobile phase and evaluated for their total antioxidant activity, antiradical activity against DPPH and reducing power. The total phenolic and tannin contents of the fractions were also determined. The fractions exhibited the total antioxidant activities of 1.90 mmol Trolox/g (fraction-I) to 7.06 mmol Trolox/g (fraction-IV). The fraction III was characterized by the highest antiradical activity against DPPH (EC_{50} = 0.01mg). Fraction II and III showed the strongest reducing power. The presence of phenolic compounds was investigated by using RP-HPLC.

Phytochemical investigations of ethyl acetate soluble fraction of *Litsea monopetala* bark has resulted in the isolation of seven compounds, namely lupeol **1**, oleanolic acid **2**, *β*-sitosterol **3**, stigmasterol **4**, (-)-epicatechin **5**, 7-methoxy-(+)-catechin **6** and *β*-sitosterol-3-*O*-*β*-*D*-glucopyranoside **7**. These compounds were reported for the first time from *Litsea monopetala*. The structures of the isolated compounds were ascertained by employing modern spectroscopic tools such as ^{1}H-NMR, ^{13}C-NMR, EI-MS, HREI-MS as well as by comparision with the reported data in the literature.

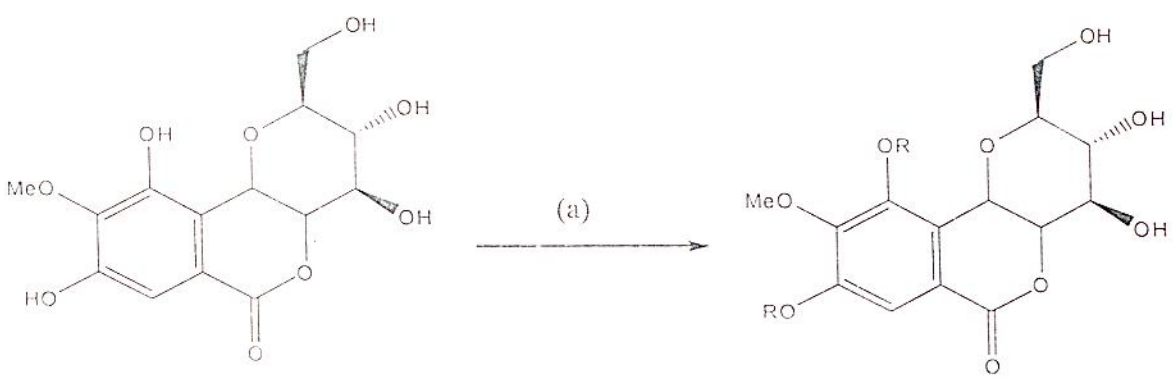
Chapter 2 describes the antioxidant, antiinflammatory, anti-ulcer and antiplasmodial activities of the crude extracts and pure compounds bergenin **13** and 11-*O*-Galloylbergenin **14** isolated from *Mallotus philippensis* stemwood. Extracts of the fruit and bark were evaluated for their total antioxidant activity (TAA), antiradical activity

against DPPH and reducing power. The extract of the bark showed the strongest antiradical activity and reducing power. The phenolic constituents in the extract were detected by revese phase high pressure liquid chromatography (RP-HPLC) using a gradient solvent system with UV-DAD detection.

Phytochemical investigation on the aqueous fraction of *Mallotus philippensis* stemwood resulted in the isolation of six compounds namely, *β*-sitosterol **2**, stigmasterol **3**, 3-acetylaleuritolic acid **11**, (+)-catechin **12**, bergenin **13** and 11-*O*-Galloylbergenin **14**. Leaves, shoots bark and stemwood were analyzed for the bergenin content. The yield of bergenin was 0.3 % in the dried, powdered stemwood, while the shoots and leaves had trace quantities of the said compound. The biological activities and synthetic reactions were carried out on the bergenin isolated from the stemwood.

Bergenin inhibited the *Bacillus pasteurii* urease having IC_{50} value of 21.7± 0.2μm. Thiourea was used as a standard inhibitor having Ki value of 8.41± 0.66 μm. From kinetic data it was found to be a noncompetitive inhibitor having Ki value of 19.2± 0.2 μm. The potential binding mode of bergenin was studied by molecular docking. The comparative antioxidant and antiplasmodial activites of bergenin and 11-*O*-Galloylbergenin were studied. 11-*O*-Galloylbergenin was found to be more active in antioxidant assay as compared to bergenin, which was found to be almost inactive. The EC_{50} values of 11-*O*-Galloylbergenin were 7.45± 0.2 μg/mL and 5.39± 0.28 μg/mL in DPPH antioxidant assay and reducing power assay, respectively. In the total antioxidant phosphomolybdate assay, 11-*O*-Galloylbergenin was found more potent (CATH : 940.04± 115.30) as compared to *α*-tocopherol (CATH : 552.41± 27.9), while EC_{50} value for antiplasmodial assay of both the compounds were less than 2.5 μM. In the *in vivo* activities, 11-*O*-Galloylbergenin showed significant analgesic activity against formalin test in rats. It also exhibited good antiinflammatory activity in carrageenan-induced paw edema model at doses of 10, 20 and 30 mg/kg. The antiulcer effect of the compound was also found to be significant in ethanol-induced ulcers.

Chapter 3 presents the synthesis of *O*-alkylated bergenins and evaluation of their biological activities. The bergenin **13** isolated from *Mallotus philippensis* was subjected to alkylation using various alkyl halides and its derivatives in the presence of base in dimethylformamide at 80° C. During this study, fifteen new alkylated bergenins (**49-63**) were synthesized. The alkyl and its derivative moieties were attached regioselectively to bergenin nucleus by replacing phenolic hydroxyl protons at position C8 and C10. These analogues were evaluated for their *in vitro* antiinflammatory and antiplasmodial activities. Six of the tested compounds **50**, **51**, **55**, **56**, **61** and **63** exhibited high antiinflammatory activities as compared to that of parent compound i.e. bergenin, while interestingly, a few of them **55**, **56**, **61** and **63** displayed more potent antiinflammatory activities as compared to Indomethacin used as a standard drug. All of the derivatives exhibited weak antiplasmodial activity as compared to parent compound **13**. Compound **50** was also studied through X-ray crystallography.



Bergenin (13) — *O*-alkylated bergenins (**49-63**)

(a) K_2CO_3, DMF, RX or derivatives of RX, 80°C, stirring

The 'R' groups in 49 to 63 are listed below:

Compound No.	R
49.	$(CH_3)_2CH$-
50.	$CH_2=CH(CH_2)_2$-
51.	$CH_3(CH_2)_3CH_2$-
52.	CH_3CH_2-
53.	CH_3-
54.	$CH_2=CH(CH_2)_3CH_2$-
55.	$CH_3(CH_2)_5CH_2$-
56.	$CH\equiv CCH_2$-
57.	$CH_2=CHCH_2$-
58.	$N\equiv CCH_2$-
59.	$CH_3(CH_2)_2CH_2$-
60.	$CH_3(CH_2)_6CH_2$-
61.	$CH_3CH_2CH_2$-
62.	$C_2H_5OCOCH_2$-
63.	$(CH_3)_3COCOCH_2$-

CHAPTER-1

BIOACTIVE CONSTITUENTS FROM *LITSEA MONOPETALA*



CHAPTER-1 BIOACTIVE CONSTITUENTS FROM *LITSEA MONOPETALA*

1.1. Introduction

1.1.1. The genus *Litsea*

The genus *Litsea* belongs to the family lauraceae, containing 35 genera and about 2000 species, which are generally dioecious evergreen or deciduous trees or shrubs. These are found in all tropical and sub-tropics except Africa. Pakistan has 4 genera and 8 species of this family.[1] Until now cytotoxic butanolides[2], flavanoides [3,4], sesquiterpenes[5-7], rotunifolides[8], new acetylenic ketone [9], alkaloids[10,11], oblongifolind[12] and phenyl esters [13] have been reported from this genus.

1.1.2. Pharmacological importance of *Litsea*

Anti-diarrheal activity of methanol extract of dried bark and aerial parts of *Litsea polyantha* has been evaluated in mice using different models [14]. The methanolic extracts of *Litsea elliptica* (Blume tree) have exhibited adulticidal activity against adult of *Aedes aegypti* (L.)[15] and the inhibitory potencies of flavonoids isolated from *Litsea japonica* leaves have been reported[4]. Sesquiterpenes and butenolides isolated from the leaves and twigs of *Litsea verticillata* were found to inhibit HIV-1 replication[7]. Phytopesticidal and repellent efficacy of *Litsea salicifolia* against *Aedes aegypti* and *Culex quinquefasciatus* have been reported[16]. Citral refined from *Litsea cubeba* oil has been found to have a strong antifungal activity especially on *Aspergillus flavus*[17]. Both the methanolic extract and its fractions (0.01 mg/mL) from *Litsea cubeba* bark were found to inhibit nitric oxide and prostaglandin E2 production in LPS-activated macrophages without significant cytotoxicity at than 0.01 mg/mL concentration[18]. Methanol extracts of *Litsea elliptica* leaves inhibited the growth of *Helicobacter pylori* strains with minimum inhibitory concentration of 100 μg/mL[19]. The extract of *Litsea petiolata* exhibited significant antimutagenicity[20]. Lignans isolated from *Litsea verticillata* showed moderate anti-HIV activity [21]. The methanol extract of *Litsea verticillata* showed antibacterial activity, comparable to chloramphenicol, against 16 tested microorganisms [22].

1.1.3. *Litsea monopetala*

The presence of the reported biologically and physiologically active classes of compounds in the *Litsea* genus promoted us to carry out the phytochemical study of *Litsea monopetala*. *Litsea monopetala* is a tree up to 7m tall and 60 cm in diameter, distributed in western outer Himalayas, India, west Malaysia and Thailand. The plant material was collected from district Bunner (K.P.K) [1]. The bark of *Litsea monopetala* locally called *Medea sak* is used as nerves and bone tonic, stomachache, stimulant, analgesic and antiseptic in folk medicine in Pakistan and India. The leaves of this tree are used by the local people as fodder for cattle which they claimed to enhance milk production. The bark of this plant is mildy astringent and the powdered bark and roots are used as remedy for bruises and pains. Leaves are used for growing mega silk warm and as cattle fodder[1]. Previously, essential oil has been reported from *Litsea monopetala* [23]. In the present investigation, the ethanolic extract of the bark of *Litsea monopetala* was explored for secondary metabolites and selected bioactivities.

1.1.3. Literature review on the genus *Litsea*

Previous studies on the genus *Litsea* have resulted in the isolation of various compounds including alkaloids, flavanoids, steroids, sesquiterpenes, lactones, phenyl esters, amides, terpenes and acetylenic ketones. An updated review of these compounds is given in table-1.1.

Table-1.1: Compounds isolated from the plants of the genus *Litsea*

S.No.	Compound Name	Mol. Formula	Mol.Wt.	Physical data	Plant & ref.
1	Dehydroxy-methyailanthoidol	$C_{18}H_{16}O_4$	296.10	M.p. 186-187°C Orange prisms	*L. acutivena*[24]
2	Litseakolide D	$C_{16}H_{28}O_4$	285.25	Colorless oil $[\alpha]_D^{25}$=-73.3° (c 0.13, $CHCl_3$)	*L. acutivena*[24]
3	Litseakolide E	$C_{16}H_{28}O_4$	285.25	Colorless oil $[\alpha]_D^{25}$=-83.2° (c 0.20, $CHCl_3$)	*L. acutivena*[24]
4	Isolincomolide	$C_{16}H_{28}O_3$	281.20	Colorless oil $[\alpha]_D^{25}$=54.3° (c 0.49, $CHCl_3$)	*L. acutivena*[24]
5	Akolactone A	$C_{19}H_{32}O_2$	292.00	Colorless oil $[\alpha]_D^{28}$=-13.2° (c 0.10, $CHCl_3$)	*L. akoensis*[2]
6	Akolactone B	$C_{19}H_{28}O_2$	288.00	Colorless oil $[\alpha]_D^{28}$=-10.0° (c 0.10, $CHCl_3$)	*L. akoensis*[2]
7	Litseakolide A	$C_{18}H_{29}O_4$	309.21	Colorless oil $[\alpha]_D^{27}$=-40.0° (c 0.08, $CHCl_3$)	*L. akoensis*[2]
8	Litseakolidee B	$C_{19}H_{30}O_3$	306.00	Colorless oil	*L. akoensis*[2]

9	Indonesiol	$C_{15}H_{26}O$	222.00	M.p. 124-125°C Crystalline Solid $[\alpha]_D$=-165° ($CHCl_3$; c 0.1)	*L. amara*[5]
10	*β*-Sitositerol	$C_{29}H_{48}O$	414.00	M.p. 144°C Crystalline Solid $[\alpha]_D^{25}$=+35.5° (c 0.22, $CHCl_3$)	*L. amara*[5]
11	Stigmasterol	$C_{29}H_{50}O$	412.00	M.p. 170-171°C Crystalline Solid $[\alpha]^{25}{}_D$=-51.5° (c 0.28,$CHCl_3$)	*L. amara*[5]
12	*N*-trans-sinapoylmethoxytyramine	$C_{20}H_{23}NO_6$	373.15	Colorless oil	*L. auriculata*[25]
13	*N*-trans-sinapoyltyramine	$C_{19}H_{21}NO_5$	343.15	Colorless oil	*L. auriculata*[25]
14	*N-trans*-feruapoylmethoxytyramine	$C_{19}H_{21}NO_5$	343.15	Colorless oil	*L. auriculata*[25]

15	*N-trans*-feruloyltyramine	$C_{18}H_{19}NO_4$	313.20	Colorless oil	*L. auriculata*[25]
16	Afzelin	$C_{21}H_{20}O_{10}$	432.00	*	*L. auriculata*[25]
17	Quercitrin	$C_{21}H_{20}O_{11}$	448.00	*	*L. auriculata*[25]
18	Isocurcumol	$C_{15}H_{24}O_2$	236.00	M.p. 106-107°C Cubic Crystal $[\alpha]_D$=-86° (c 0.5,$CHCl_3$)	*L. cassiaefolia*[6]
19	Litseacassifolide	$C_{15}H_{20}O_4$	221.00	M.p. 203-205°C Needle Crystals $[\alpha]_D$=-137° (c 0.3,$CHCl_3$)	*L. cassiaefolia*[6]
20	(-) Litcubine	$C_{19}H_{21}NO_4$	327.15	Amorphous Solid $[\alpha]^{25.5}{}_D$=-111.0° (c 1.0,MeOH)	*L. cubeba*[26]
21	(-) Litcubinine	$C_{18}H_{19}NO_4$	313.02	Amorphous Solid Solid $[\alpha]^{25.5}{}_D$=-144.0° (c 0.5,MeOH)	*L. cubeba*[26]
22	Euosmoside A	$C_{21}H_{24}O_{11}$	475.12	Colorless oil $[\alpha]^{25}{}_D$=-32.03° (c	*L. euosma*[13]

				0.007,MeOH)	
23	Euosmoside B	$C_{22}H_{26}O_{12}$	505.13	Colorless oil $[\alpha]^{25}{}_{D}$=-25.77° (c 0.003,MeOH)	*L. euosma*[13]
24	Valencenediol	$C_{15}H_{26}O_2$	223.00	Orthohombic $[\alpha]_D$=96° (c 0.2,$CHCl_3$).	*L. excelsa*[6]
25	Norboldine	$C_{19}H_{21}NO_4$	327.00	*	*L. glutenosa*[11]
26	Boldine	$C_{19}H_{21}NO_4$	327.00	M.p. 163-164°C	*L. glutenosa*[11]
27	Laurotetanine	$C_{20}H_{23}NO_4$	341.00	*	*L. glutenosa*[11]
28	*N*-Methyllaurotetanine	$C_{21}H_{25}NO_4$	355.00	*	*L. glutenosa*[11]
30	Actinodaphnine	$C_{18}H_{17}NO_4$	311.00	*	*L. glutenosa*[11]
31	*N*-Methylactinodhnine	$C_{19}H_{19}NO_4$	325.00	*	*L. glutenosa*[11]
32	Aporphine	$C_{18}H_{19}NO_4$	313.00	*	*L. glutenosa*[27]

33	Litseglutine A	$C_{18}H_{17}NO_4$	312.12	Brown Solid $[\alpha]^{24}{}_D$= 36.56° (c 0.13,MeOH)	*L. glutenosa*[27]
34	Litseglutin B	$C_{20}H_{23}NO_4$	341.00	Light brown Solid $[\alpha]^{24}{}_D$= 30.5° (c 0.13,MeOH)	*L. glutenosa*[27]
35	Boldine	$C_{19}H_{21}NO_4$	355.00	*	*L. glutenosa*[27]
36	Laurolitsine	$C_{19}H_{21}NO_4$	327.00	*	*L. glutenosa*[27]
37	Litsenolide D1	$C_{17}H_{30}O_3$	282.22	M.p. 49-51°C Prisms form $[\alpha]_D$=-8.4° ($CHCl_3$ c 0.37)	*L. japonica*[28]
38	Litsenolide D2	$C_{17}H_{30}O_3$	282.22	M.p. 33-34°C Prisms form $[\alpha]_D$=-21.6° ($CHCl_3$, c 0.49)	*L. japonica*[28]
39	Litsenolide E1	$C_{19}H_{32}O_3$	308.23	Viscous oil $[\alpha]_D$=-6.0° ($CHCl_3$, 0.40)	*L. japonica*[28]
40	Litsenolide E2	$C_{19}H_{32}O_3$	308.23	Viscous oil $[\alpha]_D$=-42.0°	*L. japonica*[28]

				($CHCl_3$; c 0.05)	
41	Hamabiwalactene A	$C_{17}H_{24}O_2$	260.18	Viscous oil $[\alpha]_D$=3.7° ($CHCl_3$; c 0.30)	*L. japonica*[28]
42	Hamabiwalactene B	$C_{17}H_{26}O_2$	262.19	Viscous oil $[\alpha]_D$=2.2° ($CHCl_3$; c 0.32)	*L. japonica*[28]
43	Tiliroside	$C_{30}H_{26}O_{13}$	595.00	Yellow amorphous Solid $[\alpha]_D$=- 55.9° (c 0.5,MeOH)	*L. japonica*[4]
44	Litsenolide A_1	$C_{17}H_{28}O_3$	300.00	Oil $[\alpha]^{23}_D$=- 2.4±0.7° (c 0.619)	*L. japonica*[29]
45	Litsenolide A_2	$C_{17}H_{28}O_3$	300.00	Oil $[\alpha]^{23}_D$=--40.4±1.2° (c 0.673)	*L. japonica*[29]
46	Litsenolide B_1	$C_{17}H_{26}O_3$	298.00	M.p. 40-41°C Oil $[\alpha]^{23}_D$=- 5.9±0.6° (c 0.712)	*L. japonica*[29]
47	Litsenolide B_2	$C_{17}H_{26}O_3$	298.00	M.p. 42-43°C Oil $[\alpha]^{24}_D$=- 44.9±1.6° (c 0.534)	*L. japonica*[29]
48	Litsenolide C_1	$C_{19}H_{34}O_3$	310.00	M.p. 60-62°C Oil $[\alpha]^{24}_D$=- 9.4±0.8° (c 0.617)	*L. japonica*[29]

49	Litsenolide C_2	$C_{19}H_{34}O_3$	310.00	M.p. 44-45°C Oil $[\alpha]^{26}{}_D$=-45.4±0.9° (c 0.986)	*L. japonica*[29]
50	Litsealactone A	$C_{19}H_{30}O_2$	291.23	Colorless oil $[\alpha]_D^{20}$=-47.5° (c 0.3, $CHCl_3$)	*L. japonica*[30]
51	Litsealactone B	$C_{17}H_{26}O_3$	278.00	Colorless oil $[\alpha]_D^{20}$=-2.9° (c 0.3, $CHCl_3$)	*L. japonica*[30]
52	Noraporphine	$C_{18}H_{19}NO_4$	313.13	*	*L. laeta*[31]
53	(-) Epicatechin	$C_{15}H_{13}O_6$	289.00	M.p. 279-280°C Colorless amorphous solid	*L.rotundifolia*[32]
54	Oblongifolinol	$C_{16}H_{32}O_2$	256.24	Colorless oil $[\alpha]_D^{20}$=-8.6° (c 0.41, $CHCl_3$)	*L.rotundifolia*[12]
55	Rotundifolide A	$C_{17}H_{28}O_3$	280.20	Colorless oil $[\alpha]_D$=11° (c 0.25, dioxane)	*L.rotundifolia*[8]
56	Acetylenic ketone	$C_{14}H_{24}O$	231.00	Colorless oil	*L.rotundifolia*[9]

57	13-Tetradecen-2-one	$C_{14}H_{26}O$	210.00	Colorless oil	*L.rotundifolia*[9]
58	13-Tetradecenoic acid	$C_{14}H_{31}O_2$	231.00	Colorless oil	*L.rotundifolia*[9]
59	11-dodecynoic acid	$C_{12}H_{22}O_2$	198.00	Colorless oil	*L.rotundifolia*[9]
60	13-Tetradecynoic acid	$C_{13}H_{24}O_2$	212.00	Colorless oil	*L.rotundifolia*[9]
61	Rotundifolinol	$C_{18}H_{20}O_6$	323.32	Colorless oil	*L.rotundifolia*[9]
62	Laetine	$C_{18}H_{17}NO_4$	310.11	M.p.296°C (decomp)	*L.salicifolia*[33]
63	Dicentrinone	$C_{16}H_{13}NO_5$	335.10	Light coloured powder	*L.salicifolia*[33]
64	Nordicentrine	$C_{19}H_{19}NO_4$	325.00	Light coloured powder	*L.salicifolia*[33]
65	Sebiferine	$C_{21}H_{26}NO_4I$	356.00	M.p. 112-113°C	*L.sebiferea*[34]

66	Litseferine	$C_{22}H_{21}NO_4$	363.00	M.p. 258°C	*L.sebiferea*[34]
67	Litsomentol	$C_{30}H_{52}O_2$	426.00	M.p. 218-219°C	*L.tomentosa*[35]
68	Laurolitsine	$C_{18}H_{19}NO_4$	313.13	M.p. 133-135°C	*L.tureosa*[36]
69	Litseaverticillol 1	$C_{15}H_{22}O_2$	234.10	Colorless gum $[\alpha]^{20}_D$= 0° (c 0.14, MeOH)	*L.verticillata*[37]
70	Litseaverticillol 2	$C_{15}H_{21}O_3$	271.25	Colorless gum $[\alpha]^{20}_D$= 0° (c 0.14, MeOH)	*L.verticillata*[37]
71	Litseaverticillol 3	$C_{14}H_{21}O_3$	259.15	Colorless gum $[\alpha]^{20}_D$= 0° (c 0.10, MeOH)	*L.verticillata*[37]
72	Litseaverticillol 4	$C_{14}H_{19}O_3$	235.23	Colorless gum $[\alpha]^{20}_D$= 0° (c 1.21, MeOH)	*L.verticillata*[37]
73	Litseagermacrane	$C_{15}H_{22}O_2$	259.17	Colorless gum $[\alpha]_D^{20}$=11°.1(c 0.14, $CHCl_3$)	*L.verticillata*[38]
74	7-Epi-eudesm-4(15)-ene-1α,6β-diol	$C_{15}H_{26}O_2$	238.12	White powder $[\alpha]_D^{20}$=-35.3° (c 0.05, $CHCl_3$)	*L.verticillata*[38]

75	Litseahumulane	$C_{15}H_{24}O_2$	237.19	Colorless gum $[\alpha]_D^{20}$=-34.1° (c 0.09, $CHCl_3$)	*L.verticillata*[38]
76	Litseachromolaevane A	$C_{15}H_{22}O_2$	257.15	Colorless gum $[\alpha]_D^{20}$=6.7° (c 0.23, $CHCl_3$)	*L.verticillata*[38]
77	Litseachromolaevane B	$C_{15}H_{24}O_2$	234.16	Colorless gum $[\alpha]_D^{20}$=0° (c 0.05, $CHCl_3$)	*L.verticillata*[38]
78	Isolitseane A	$C_{15}H_{24}O_2$	254.00	Colorless gum $[\alpha]_D^{20}$=0° (c 0.85, $CHCl_3$)	*L.verticillata*[7]
79	Isolitseane B	$C_{15}H_{24}O_2$	254.00	Colorless gum $[\alpha]_D^{20}$=-7.3° (c 0.15, $CHCl_3$)	*L.verticillata*[7]
80	Isolitseane C	$C_{15}H_{24}O_3$	235.00	Colorless gum $[\alpha]_D^{20}$=-25.0° (c 0.02, $CHCl_3$)	*L.verticillata*[7]
81	Litseabutenolide	$C_{16}H_{26}O_4$	305.00	Colorless gum $[\alpha]_D^{20}$= 0° (c 0.08, $CHCl_3$)	*L.verticillata*[7]
82	Litseaverticillol A	$C_{15}H_{22}O_2$	257.15	Colorless gum $[\alpha]_D^{20}$= 0°	*L.verticillata*[39]
83	Litseaverticillol B	$C_{15}H_{22}O_2$	257.15	Colorless gum	*L.verticillata*[39]

84	Litseaverticillol C	$C_{15}H_{22}O_2$	257.15	Colorless gum	*L.verticillata*[39]
85	Litseaverticillol D	$C_{15}H_{22}O_3$	273.15	Colorless gum	*L.verticillata*[39]
86	Litseaverticillol E	$C_{15}H_{22}O_3$	273.15	Colorless gum	*L.verticillata*[39]
87	Litseaverticillol F	$C_{15}H_{22}O_3$	273.15	Colorless gum	*L.verticillata*[39]
88	Litseaverticillol H	$C_{15}H_{20}O_3$	271.13	Colorless gum	*L.verticillata*[39]
89	Litseane	$C_{15}H_{22}O_2$	235.00	*	*L.verticillata*[39]
90	Glaucine	$C_{21}H_{25}NO_4$	355.00	M.p. 119-120°C $[\alpha]_D^{32}$= 114.5° (EtOH)	*L.wightiana*[10]
91	Norcorydine	$C_{19}H_{21}NO_4$	327.00	M.p. 238-239°C $[\alpha]_D^{30}$= 134.5° (EtOH)	*L.wightiana*[10]

*Not reported in the relevant literature

1.2. Experimental

1.2.1. Material

All the chemicals and solvents used were of analytical grade and were purified before use according to standard methods. IR spectra were recorded on VECTOR22 FT-IR. ^{1}H-NMR and ^{13}C NMR spectra were taken on a Bruker AVANCE AV 300 MHz,400 MHz, 500 MHz in CD_3OD as solvent using TMS as an internal standard, while mass spectra were recorded on MAT 312 instrument. Column chromatography was carried out on silica gel (70-230 mesh). Alumina supported pre-coated TLC plates (MERCK) were used for qualitative analysis. Spots were visualized with ultra violet light and $CeSO_4$/ H_2SO_4. Melting points were determined in open glass capillaries using Buchi 535 melting point apparatus and are uncorrected.

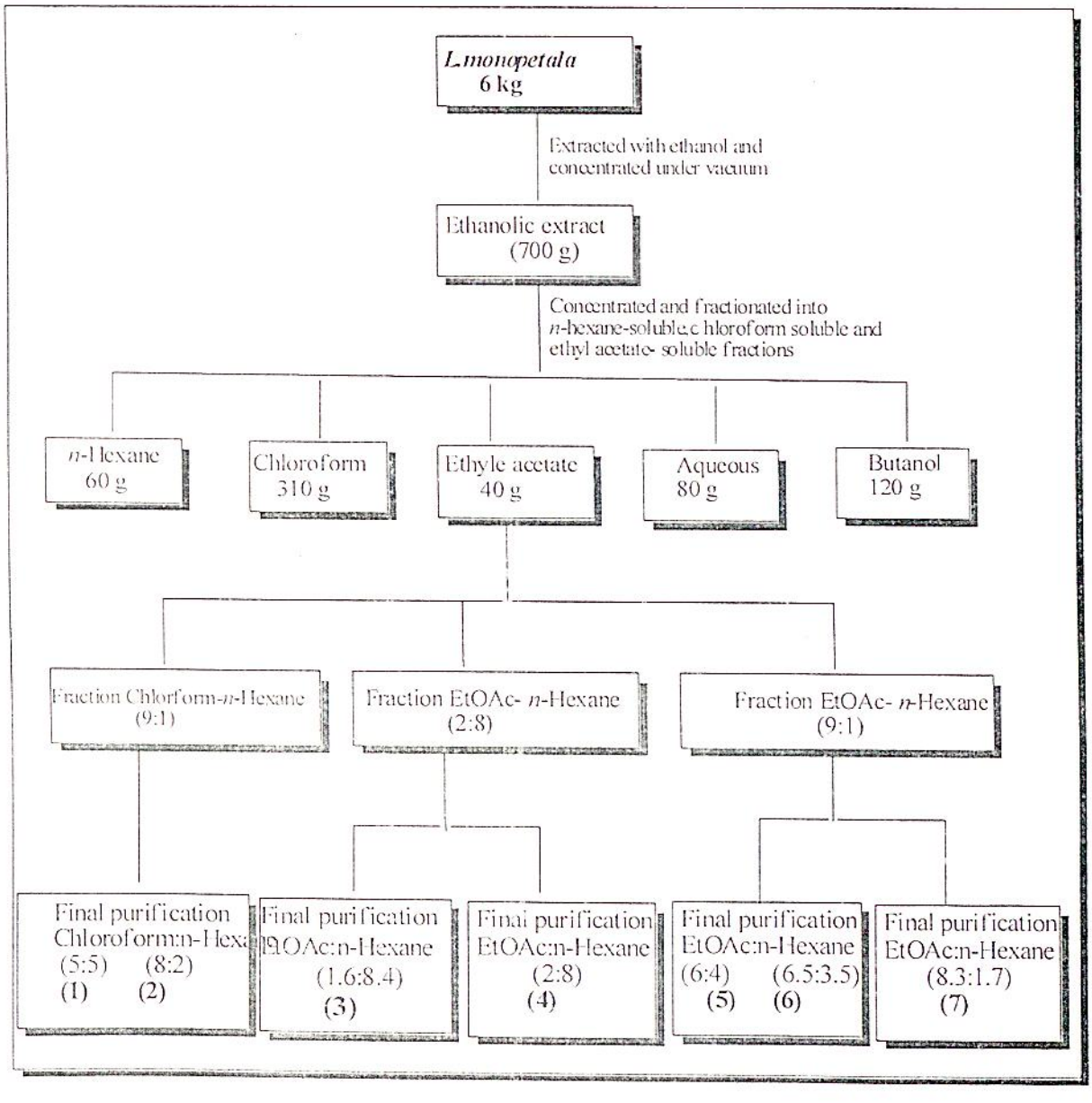
1.2.2. Plant material

The bark of *Litsea monopetala* was collected from district Buner (K.P.K), Pakistan during the month of May 2006 and identified by Prof. Dr. Abdur Rashid, Department of Botany, University of Peshawar. A voucher specimen No. 1014 (pup) was deposited in the herbarium of Department of Botany, University of Peshawar, Peshawar, Pakistan.

1.2.3. Extraction and isolation

The air dried bark (6 kg) under shade was crushed and soaked in commercial ethanol (15L) for 72 h thrice at room temperature. The solvent was evaporated under vacuum to yield a reddish residue (700 g) which was suspended in water and extracted successively with cold n-hexane, chloroform, ethyl acetate and butanol. The ethyl acetate fraction was chromatographed over silica gel (70-230 mesh) using *n*-hexane, chloroform and ethylacetate in increasing order of polarity. The fractions obtained from chloroform: *n*-hexane (9:1) showed two major spots on TLC plate. These two compounds were separated and purified by flash silica gel column chromatography using chloroform: *n*-hexane. The compound **1** (25 mg) was further purified by chloroform: *n*-hexane (5:5), while the compound **2** (30 mg) was purified at (8:2) ratio in the same solvent system. Compounds **3** (40 mg) and **4** (27 mg) were obtained in semi pure form from column

using EtOAc: *n*-hexane (2:8) as eluent.These compounds were further purified by flash silica gel column chromatography with EtOAc: *n*-hexane (1.6:8.4) and (2:8) respectively. The fraction obtained from EtOAc: *n*-hexane (9:1) showed three major spots on TLC. It was resubjected to flash chromatography eluting with the solvent system EtOAc: n-hexane (6:4), (6.5:3.5) and (8.3:1.7) affording three compounds **5** (15 mg), (20 mg) **6** and **7** (30 mg), respectively. **Scheme-1.1**.



Scheme-1.1: Extraction and isolation from *Litsea monopetala* bark

1.2.4. Antioxidant activity (*in vitro*) of the crude fractions of *Litsea monopetala* bark

1.2.4.1. Chemicals

All solvents used for chromatography were of HPLC grade, unless otherwise specified. Ferric chloride, potassium ferricyanide, potassium persulfate, trifluoroacetic acid (TFA) and trichloroacetic acid (TCA) were acquired from the P.O.Ch. Company (Gliwice, Poland). Gallic acid, Folin and Ciocalteau's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH.), 2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma Ltd. (Poznań, Poland).

1.2.4.2. Extraction and fractionation

Phenolic compounds were extracted from the dried and crushed bark of *Litsea monopetala* using methanol (4 L for 12 h; at room temperature). The extraction was repeated twice. The supernatants were combined and methanol was evaporated at 45 ^{0}C using a vacuum rotary evaporator. The crude extract (48 g) was stored at –20^0 C. A 0.5 g portion of the extract so obtained was dissolved in 8 mL of methanol and applied to a chromatographic column (2 × 60 cm) packed with Sephadex LH-20 and eluted with methanol. Fractions containing low molecular weight phenolic compounds (4 mL) were collected using a fraction collector and their absorbance was measured at 280 nm. Eluates were then pooled into major fractions. Organic solvents were evaporated at 45^{0}C using a rotary evaporator. The four fractions (I- IV) collected were subjected to the following antioxidant assays.

1.2.4.3. Total phenolics

The content of total phenolics in the fractions dissolved in methanol was determined according to the procedure described by Amarowicz *et al*[40] using the Folin-Ciocalteau's phenol reagent. Briefly, 1 mL of each of the already prepared extract solution (500µg) was poured in to 50ml volumetric flask and distilled water was added to adjust the volume to 46 ml. The Folin-Ciocalteu reagent (1mL) was added into this mixture and 3

mL of Na_2CO_3 (2%) was added by 3min. Subsequently, the mixture was shaken for 2 h at room temperature and then absorbance was measured at 760 nm. Estimation of the phenolic compounds was carried out in triplicate. The results were recorded as mean values (± standard deviations) and expressed as mg of gallic acid equivalents μg/mg of extract (GAEs) by using an equation that was obtained from the standard curve, is given as : A = 1156C + 0189. Where A is the absorbance and C is the gallic acid equivalent (mg/g).

1.2.4.4. Condensed tannins

The content of condensed tannins in the fractions dissolved in methanol was determined according to a modified vanillin method described by Price *et al.* [41] Briefly, into a 10mL screwcap tube with 6 mL of the acidified butanol (950 mL of *n*-butanol with 50 mL concentrated HCl), 1.0 aliquot of the extract, and 0.2 mL of the iron reagent were added and vortexed. The tube was capped loosely, and placed in a boiling water bath for 50 min. The tube was cooled and the solution was transferred to a volumetric flask and adjusted to 25 mL with acidified butanol. The absorbance was recorded at 500 nm using a Beckman DU 7500 diode array spectrophotmeter. The results were expressed as absorbance units at 500 nm per mg of extract (A_{500}/mg).

1.2.4.5. Total antioxidant activity

The total antioxidant activity (TAA) of the fractions dissolved in methanol was determined according to the Trolox equivalent antioxidant capacity (TEAC) assay described by Re *et al* [42]. A 0.3 mL aliquot of the sample solution in methanol was combined in an Eppendorf tube with 2.7 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). For the blank, 0.3 mL ethanol was mixed with 2.7 mL of the reagent. The tubes were capped and incubated in water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 700 nm against a blank. For samples of unknown composition, water soluble antioxidant capacity was expressed as equivalent of ascorbic acid μmol/mg of extract. The assay was carried out in triplicate and the results are expressed as mean values ± standard deviations. The total antioxidant activity was expressed as mmol Trolox/g of fraction.

1.2.4.6. Radical-scavenging activity

The capacity of the fractions to scavenge the "stable" free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH·) was monitored according the procedure described by Amarowicz *et al* [43]. Briefly, 0.1 mL of methanolic solution of the fractions was mixed with 2 mL of methanol and 0.25 mL methanolic solution (1 mmol/L) of DPPH radical was added. The mixture was vortexed thoroughly for 1 min. Finally, the absorbance of the mixture after standing at ambient temperature for 20 min was read at 517 nm.

1.2.4.7. Reducing power

The reducing power of the fractions was determined by the method of Oyaizu [44]. Briefly, the assay medium contained 0.1–0.5 mg of fraction I and 0.02-0.10 mg of fractions II-IV suspended in 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide. After incubation at 50°C for 20 min, 2.5 mL of 10% (w/v) trichloroacetic acid was added to the mixture followed by centrifugation at 1750×*g* for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL of 0.1% (w/v) ferric chloride and the absorbance of the resultant solution was read at 700 nm.

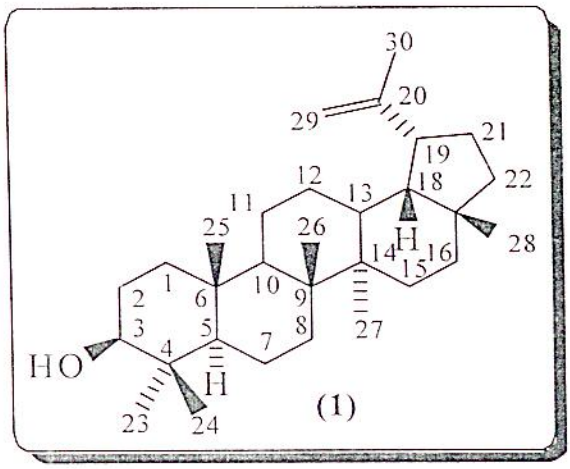
1.2.4.8. HPLC analysis

Phenolic constituents of fraction I were analysed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of two LC-10AD pumps, SCL 10A system controller and SPD-M 10A photodiode array detector. The chromatography was carried out using a pre-packed LiChrospher 100 RP-18 column (4 × 250 mm, 5 µm; Merck, Darmstad, Germany). Elution was employed for 50 min in a gradient system of 5-40% acetonitrile in water adjusted to pH 2.5 with TFA. Detector was set at 280 nm; injection volume was 20 µL and the flow rate was 1 mL/min.

1.2.5. Experimental data of isolated compounds from *litsea monopetala* bark

1.2.5.1. Lupeol (1)

White crystalline solid (25 mg)



MP: 212–213°C

$[\alpha]_D^{25}$ + 26.4° (*c* = 0.19, $CHCl_3$).

IR ($CHCl_3$) λ_{max}: 3400, 1640, 1490, 1382, 1185, 1105, 1040, 984, 943 cm^{-1}.

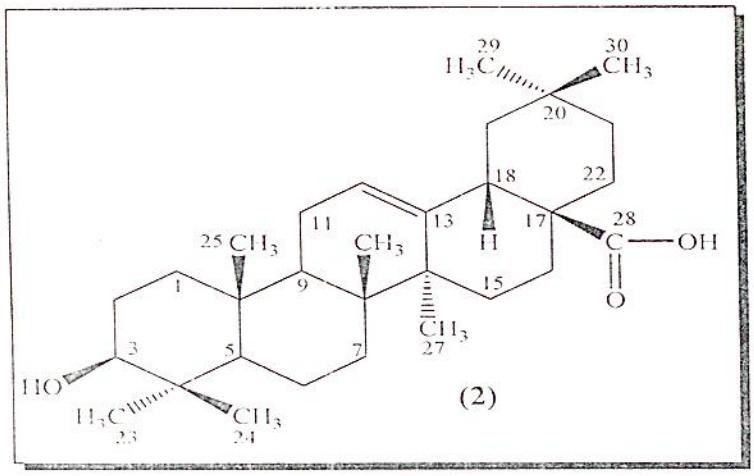
^{1}H-NMR ($CDCl_3$, 400 MHz): δ 4.55, 4.68 (1H each, *m*, H-29a and H-29b), 3.20 (1H, *dd*, *J* = 10.8, 5.4 Hz, H-3α), 2.38 (1H, *ddd*, *J* = 10.6, 10.6, 5.3 Hz, H-19β), 1.68 (3H, *d*, *J* = 1.3 Hz, H-30), 1.03, 0.97, 0.98, 0.85, 0.79 and 0.78 (each 3H, *s*).

^{13}C-NMR ($CDCl_3$, 100 MHz): δ 150.7 (C-20), 109.2 (C-29), 78.8 (C-3), 55.1 (C-5), 50.2 (C-9), 48.1 (C-18), 47.8 (C-19), 42.8 (C-17), 42.6 (C-14), 40.8 (C-8), 39.8 (C-22), 38.7 (C-1), 38.5 (C-4), 38.2 (C-13), 37.2 (C-10), 35.6 (C-16), 34.3 (C-7), 29.7 (C-21), 27.8 (C-23), 27.5 (C-15), 27.4 (C-2), 25.1 (C-12), 20.0 (C-11), 19.3 (C-30), 18.4 (C-6), 17.8 (C-28), 16.2 (C-25), 15.8 (C-26), 15.4 (C-24), 14.6 (C-27).

EIMS: *m/z* $[M]^+$ 426 (14), 229 (5), 207 (23), 161 (10), 121 (36), 95 (64), 55 (100).

HR-EIMS: *m/z* 426.3855 (calcd. for $C_{30}H_{50}O$, 426.3861).

1.2.5.2. Oleanolic acid (2)



(2)

Colourless needles from MeOH (30 mg)

MP: 305-306°C.

$[\alpha]^{25}_{D}$: + 78.9° (c = 0.07, $CHCl_3$).

IR (KBr) ν_{max} cm^{-1}: 3400-2640, 1700, 1660 and 820.

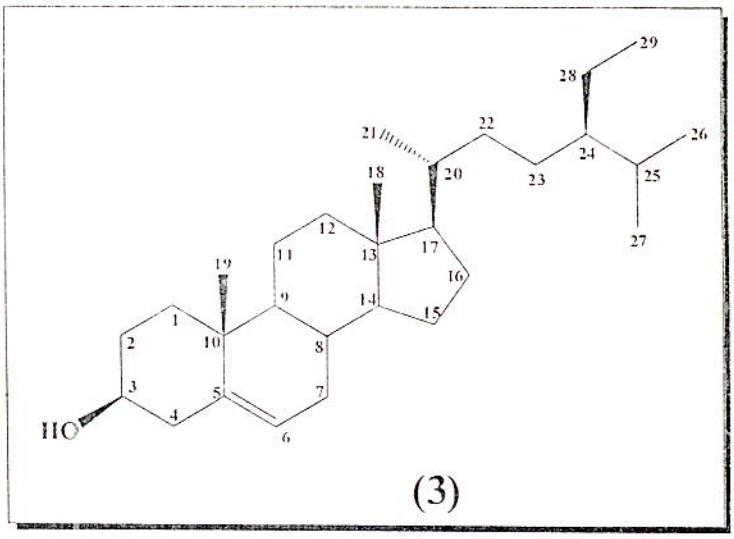
^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.24 (1H, *t*, *J* = 3.45 Hz, H-12), 3.61 (1H, *dd*, *J* = 4.1, 9.9 Hz, H-3), 1.13, 1.04, 0.97, 0.98, 0.91, 0.91 and 0.90 (3H, each *s*, Me).

^{13}C-NMR ($CDCl_3$, 75 MHz): δ 183.3 (C-28), 143.5 (C-13), 122.6 (C-12), 79.1 (C-3), 55.4 (C-5), 47.5 (C-9), 46.6 (C-17), 45.0 (C-19), 41.8 (C-14), 41.1 (C-18), 39.0 (C-8), 38.6 (C-4), 38.6 (C-1), 37.0 (C-10), 33.9 (C-21), 33.1 (C-29), 32.5 (C-7), 32.5 (C-22), 30.5 (C-20), 28.0 (C-23), 27.8 (C-15), 27.3 (C-2), 25.0 (C-27), 23.6 (C-30), 23.3 (C-11), 23.3 (C-16), 18.4 (C-6), 17.2 (C-26), 15.7 (C-24) and 15.4 (C-25).

EIMS *m/z* (rel. int.): 456 $[M]^+$ (4), 248 (98), 208 (12), 203 (60) and 133 (53).

HR-EIMS *m/z*: 456.3610 (calcd. for $C_{30}H_{48}O_3$, 456.3603).

1.2.5.3. *β*-Sitosterol (3)



Crystalline (15 mg).

MP: 144°C

$[\alpha]_D^{25}$: +35.5° (c 0.22, $CHCl_3$).

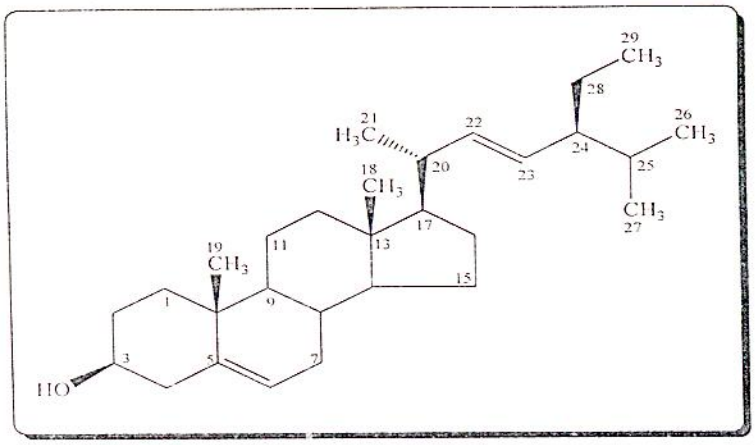
IR (KBr) ν_{max} cm^{-1}:3402, 2901 and 1641.

^{1}H-NMR (400 MHz: $CDCl_3$): δ 5.31 (1H, *m*, H-6), 3.35 (1H, *m*, H-3α), 0.93 (3H, *s*, CH_3-19), 0.89 (3H, *d*, $J_{21, 20}$ = 6.5 Hz, CH_3-21), 0.84 (3H, *d*, $J_{26, 25}$ = 6.5 Hz, CH_3-26), 0.82 (3H, *d*, $J_{27, 25}$ = 6.5 Hz, CH_3-27), 0.78 (3H, *t*, $J_{29, 28}$ = 7.0 Hz, CH_3-29), 0.64 (3H, *s*, CH_3-18).

^{13}C-NMR (100 MHz: $CDCl_3$): δ 140.8 (C-5), 121.8 (C-6), 71.7 (C-3), 56.6 (C-14), 56.1 (C-17), 50.7 (C-9), 50.3 (C-24), 42.5 (C-13), 42.3 (C-4), 40.2 (C-12), 39.6 (C-20), 37.2 (C-1), 36.5 (C-10), 36.2 (C-20), 35.5 (C-8), 34.1 (C-22), 33.1 (C-6), 32.2 (C-7), 32.1 (C-8), 31.7 (C-2), 29.5 (C-23), 28.4 (C-16), 26.3 (C-25), 24.4 (C-15), 23.2 (C-28), 21.2 (C-21), 21.2 (C-11), 19.7 (C-27), 19.5 (C-19), 19.2 (C-21), 18.7 (C-26), 11.8 (C-29), 11.8 (C-18).

HR-EI-MS *m/z*: 414.3857 (calcd. for $C_{29}H_{50}O$, 414.3861).

1.2.5.4. Stigmasterol (4)



(4)

Colorless crystalline solid (15 mg).

MP: 170-171°C

[α] $^{25}_{D}$: -51.5° (*c* = 0.28, $CHCl_3$).

IR ($CHCl_3$) ν_{max}: 3432, 1648 cm^{-1}.

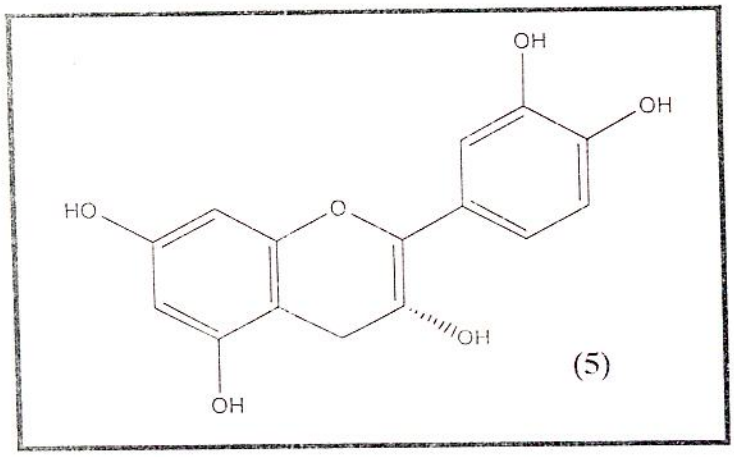
^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.32 (1H, *m*, H-6), 5.16 (1H, *dd*, *J* = 15.1, 8.4 Hz, H-22), 5.03 (1H, *dd*, *J* = 15.2, 8.6 Hz, H-23), 3.27 (1H, *m*, H-3), 0.91 (3H, *d*, *J* = 6.5 Hz, Me-21), 0.84 (3H, *d*, *J* = 6.6 Hz, Me-26), 0.85 (3H, *t*, *J* = 7.0 Hz, Me-29), 0.82 (3H, *d*, *J* = 6.5 Hz, Me-27), 0.81 (3H, *s*, Me-19), 0.66 (3H, *s*, Me-18).

^{13}C-NMR ($CDCl_3$, 100 MHz): δ 140.8 (C-5), 138.5 (C-22), 129.3 (C-23), 121.6 (C-6), 71.8 (C-3), 57.2 (C-14), 56.1 (C-17), 51.2 (C-24), 50.4 (C-9), 42.6 (C-13), 42.3 (C-4), 40.6 (C-20), 39.8 (C-12), 37.6 (C-1), 36.7 (C-10), 32.3 (C-8), 32.1 (C-25), 31.8 (C-7), 31.7 (C-2), 28.8 (C-16), 25.5 (C-28), 24.6 (C-15), 21.3 (C-27), 21.2 (C-21), 21.2 (C-11), 19.5 (C-19), 19.1 (C-26), 12.5 (C-18) and 12.1 (C-29).

EIMS: *m/z* $[M]^+$ 412 (8), 396 (12), 394 (20), 379 (27), 369 (35), 351 (71), 327 (60), 301 (18), 300 (67), 273 (30), 270 (24).

HR-EIMS: *m/z* 412.3920 (calcd. for $C_{29}H_{48}O$, 412.3925).

1.2.5.5. (-)-Epicatechin (5)



Colorless amorphous powder (20 mg).

MP: 279-280°C

$[\alpha]^{25}_{D}$: -14.5° (*c*= 0.3, MeOH).

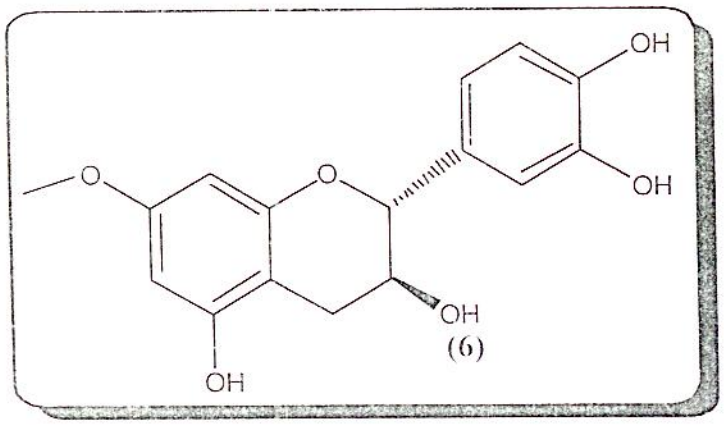
IR (KBr) ν_{max}: 3452, 3044, 1646, 1618, 1595-1550 cm^{-1}.

^{1}H-NMR (CD_3OD): 6 2.67 (lH, *dd*, *J* = 16.0 , 2.9 Hz), 2.77 (lH, *dd*, *J* = 16.0 and 2.9 Hz, H-4a), 4.12 (lH, broad *s*, H-3), 4.81 (lH, submerged H,O, H-2), 5.92 (lH, *d*, *J* = 1.9 Hz, H-6), 5.94 (lH, *d*, *J* = 1.9 Hz, H-81, 6.76 (2H, *m*, H-5' and H6'), 6.85 (lH, *d*, *J* = 1.7 Hz, H-2):

^{13}C-NMR (CD_3OD): δ 77.7 (C-2), 65.2 (C-3), 29.0 (C-4), 156.0 (C-5), 94.8 (C-6), 155.0 (C-7), 93.5(C-8), 155.8 (C-9), 98.0 (C-10), 130.2 (C-1'), 114.4 (C-2'), 144.0 (C-3'), 144.0 (C-4'), 114.4 (C-5'), 117.6 (C-6').

EIMS: *m/z* 308.00 [M^{+}].

1.2.5.6. 7-Methoxy-(+)-catechin (6)



(6)

Colorless amorphous powder (20mg).

MP: 270-272°C

UV MeOH λ_{max}: 270:

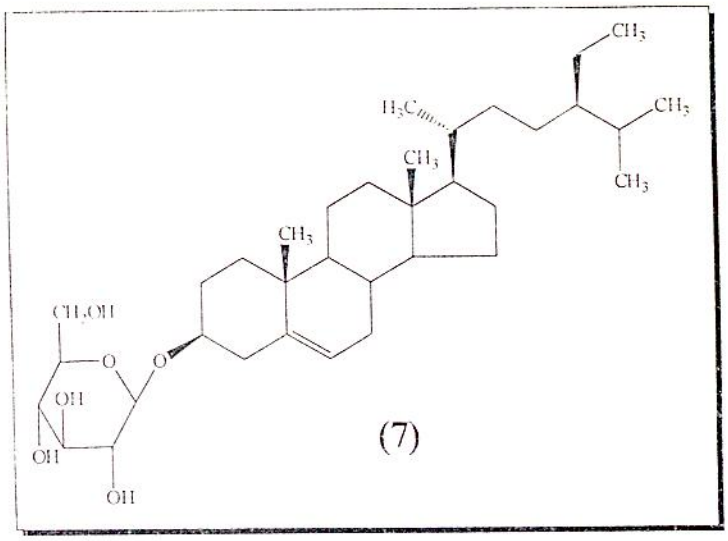
$[\alpha]_D$: +15° (c = 0.6, Me_2 CO).

^{1}H NMR (CD_3OD): δ 2.50 (1H, *dd*, *J* = 16.1 and 8.2 Hz, H-4b), 2.86 (1H, *dd*, *J* = 16.1 and 5.4 Hz, H-4a), 3.95 (3H, *s*, OMe), 3.98 (1H, *m*, H-3), 4.56 (1H, *d*, *J* = 7.5 Hz, H-2), 5.84 (1H, *d*, *J* = 1.9 Hz, H-6), 5.98 (1H, *d*, *J* = 1.9 Hz, H-8), 6.73 (2H, *m*, H-5' and H-6'), 6.83 (1H, *d*, *J* = 1.9Hz, H-2')

^{13}C NMR (CD_3OD): δ 28.3 (C-4), 55.1 (OMe), 68.7 (C-3), 82.7 (C-2), 94.2 (C-8), 94.6 (C-6), 102.8 (C-10), 115.2 (C-2'), 115.9 (C-5'), 120.1 (C-6'), 132.1 (C-1'), 145.2 (C-3' and C-4'), 157.0 (C-5), 156.8 (C-9), 160.7 (C-7).

EIMS: *m/z* 322 $[M + NH_4]^+$ and 305 [M+H].

1.2.5.7. *β*-Sitosterol-3-*O*-*β*-D-glucopyranoside (7)



(7)

Colorless amorphous powder (30 mg).

MP: 279-280°C

$[\alpha]^{25}_{D}$: -14.5° (*c* 0.3, MeOH).

IR (KBr) ν_{max}: 3452, 3044, 1646, 1618, 1595-1550 cm^{-1}

^{1}H-NMR ($CDCl_3$, 300 MHz): δ 5.32 (1H, *d*, *J* = 7.2 Hz, H-1′), 5.13 (1H, br *d*, *J* = 5.4 Hz, H-6), 3.84 (1H, *m*, H-3), 3.81-4.41 (*m*, Glc-H), 1.02 (3H, *s*, Me-19), 0.93 (3H, *d*, *J* = 6.2 Hz, Me-21), 0.85 (3H, *t*, *J* = 7.0 Hz, Me-29), 0.82 (3H, *d*, *J* = 6.5 Hz, Me-26), 0.82 (3H, *d*, *J* = 6.5 Hz, Me-27), 0.67 (3H, *s*, Me-18).

^{13}C-NMR ($CDCl_3$, 100 MHz): δ 142.1 (C-5), 122.2 (C-6), 103.2 (C-1′), 80.8 (C-3), 77.7 (C-3′), 77.5 (C-5′), 75.0 (C-2′), 71.1 (C-4′), 61.8 (C-6′), 56.8 (C-14), 56.3 (C-17), 50.4 (C-24), 49.4 (C-9), 43.7 (C-4), 43.0 (C-13), 40.7 (C-12), 39.4 (C-22), 38.6 (C-1), 37.1 (C-20), 37.0 (C-10), 33.1 (C-7), 32.8 (C-8), 29.8 (C-2), 29.7 (C-16), 29.4 (C-23), 25.7 (C-25), 25.0 (C-15), 23.2 (C-28), 21.4 (C-11), 19.6 (C-27), 19.6 (C-19), 19.2 (C-21), 18.3 (C-26), 12.5 (C-18) and 11.9 (C-29).

EIMS *m/z:* $[M]^+$ 414 (15), 399 (10), 396 (12), 381 (72), 329 (28), 275 (12), 273 (17), 255 (36).

HR-FABMS *m/z:* 576.4386 (calcd. for $C_{35}H_{60}O_6$, 576.4389).

1.3. Results and Discussion

1.3.1. Antioxidant activity (*in vitro*) of the crude fractions of *Litsea monopetala* bark

Four phenolic fractions (I-IV) were separated from the methanolic extract of *Litsea monopetala* bark using Sephadex LH-20 column chromatography **(Figure-1.1).**

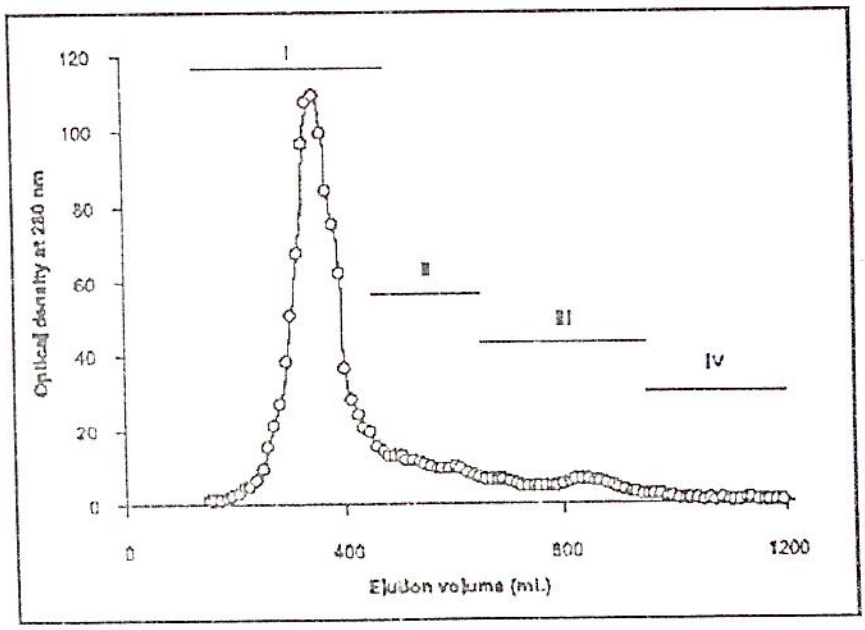


Figure-1.1: Separation of phenolic compounds from *Litsea monopetala* bark extract on a Sephadex LH-20 column with methanol as the mobile phase.

The content of total phenolics in fractions ranged from 169 mg/g (fraction I) to 753 mg/g (fraction II), **(Table-1.2)**. Condensed tannins were detected in fractions II-IV. The highest content of condensed tannins determined using the vanillin/HCl method was found in fraction IV **(Table-1.2)**.

Table-1.2: Total phenolics contents, condensed tannins and total antioxidant activity in the phenolic fractions of *Litsea monopetala* bark extract

Fraction	Total phenolics* (mg/g)	Condensed tannins A_{500}/mg	Total antioxidant activity (mmol Trolox/g)
I	169 ± 5	-	1.90 ± 0.05
II	753 ± 26	0.021 ± 0.002	6.27 ± 0.12
III	695 ± 23	0.972 ± 0.018	6.16 ± 0.10
IV	698 ± 24	1.250 ± 0.027	7.06 ± 0.15

*Reprted as gallic acid equivalents

The content of total phenolics in the fractions separated from the *Litsea monopetala* bark crude extract was high. The results were several times higher than those reported previously for the tannin rich extracts from leguminous seeds oil, almonds, as well as in seeds hulls [45]. The content of total phenolics in fractions of *Mallotus philippensis* bark extract ranged from 54 mg/g to 927 mg/g[46]. Similar content of condensed tannins (from 0.021 to 1.321 absorbance units at 500 nm/mg of fraction) was separated from *Mallotus philippensis* bark extract using the same chromatographic method[47].

Figure-1.2 depicts the concentration-dependent antiradical activity response curves for the fractions of *Litsea monopetala* bark extracts. The results are expressed as the decrease in absorbance of the DPPH• solution at 517 nm. The antiradical activities of the fractions II-IV were several times higher than those of fractions I. The EC_{50} values calculated for fractions I, II, III, and IV were 0.098, 0.013, 0.011, and 0.014 mg/assay. It is worth noting that fraction IV, showing the highest TAA value (**Table-1.2**), was a weaker scavenger of DPPH• as compared to fractions II and III.

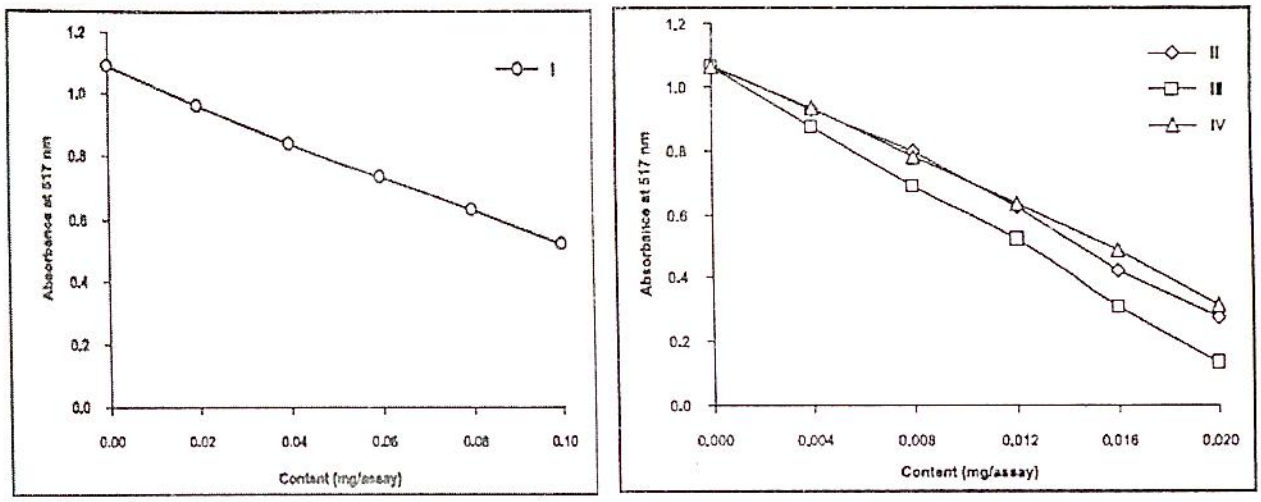


Figure-1.2: Scavenging effect of phenolic fractions of *Litsea monopetala* bark extract on the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) as measured by changes in absorbance at 517 nm.

Fractions II and III showed the strongest reducing power **(Figure-1.3)** which was approximately four times higher than that of fraction I. In the assay used, the presence of reductants (*i.e.* antioxidants) in the fraction caused a reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} state. Therefore, it can be monitored by measuring the formation of Perl's Prussian blue coloration having the maximum absorbance at 700 nm. The strong antioxidant and antiradical properties of the fractions of *Litsea monopetala* bark extracts are in accordance with literature data. The antiradical activity of the pine bark procyanidins were reported.[48,49]. The extracts of pine bark were also active in an emulsion model system[50]. The inhibition of lipid peroxidation in liposomes was caused by *Larus nobilis* bark extract[51]. The antiradical activity against DPPH• was noted for the bark extract obtained from *Betula platyphylla* var. *japonica* [52] and *Uncaria tomentosa* [53, 54].

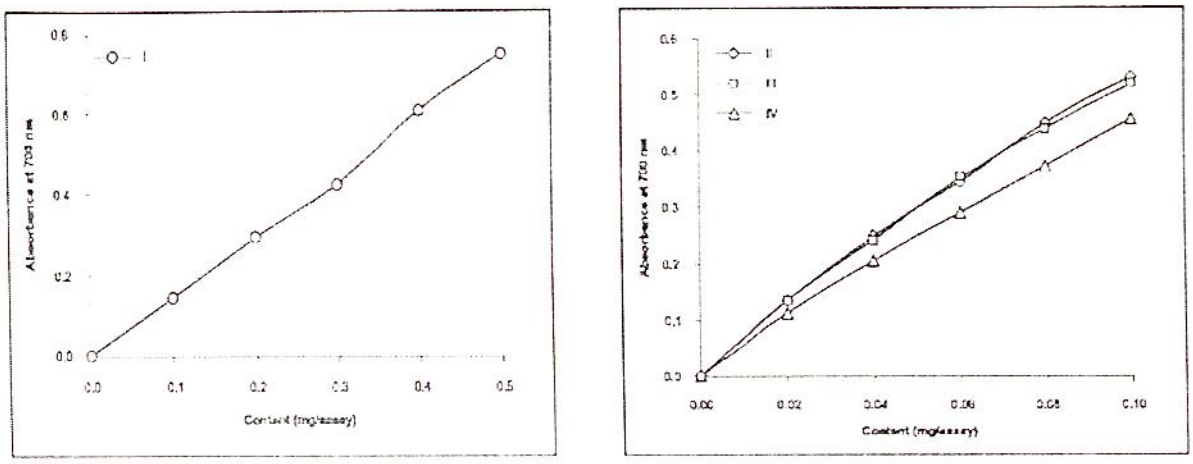


Figure-1.3: Reducing power of phenolic fractions of *Litsea monopetala* bark extract, as measured by changes in absorbance at 700 nm

Fraction I was subjected to RP-HPLC analysis. The RP-HPLC chromatogram recorded at 280 nm was characterised by three dominant peaks with retention times of 29.45 min, 34.06 min, and 40.09 min (**Figure-1.4**). The main phenolic compound from the fraction I of *Litsea monopetala* crude extract was recorded with retention time of 34.06 min (**Figure-1.4**: peak 2). The UV spectrum of the compound giving peak 1 was characterised by λ_{max} at 284 nm (**Figure-1.5**). Compounds 2 and 3 exhibited the same UV spectra with maxima at 282 nm and 301 nm. Interpretation of these results is difficult. Most certainly, in fraction I neither derivatives of hydroxycinnamic acid nor flavonols/flavans were present. The λ_{max} of catechins UV spectra is close to 284 nm but the negative results of vanillin reaction (**Table-1.2**) confirm the absence of flavano-3-ols in fraction I. The same UV spectrum of compounds 2 and 3 can evidence to their similar structure. Therefore, it can be suggested that they can be positional isomers of one phenolic compound. This work has already been published [54a]

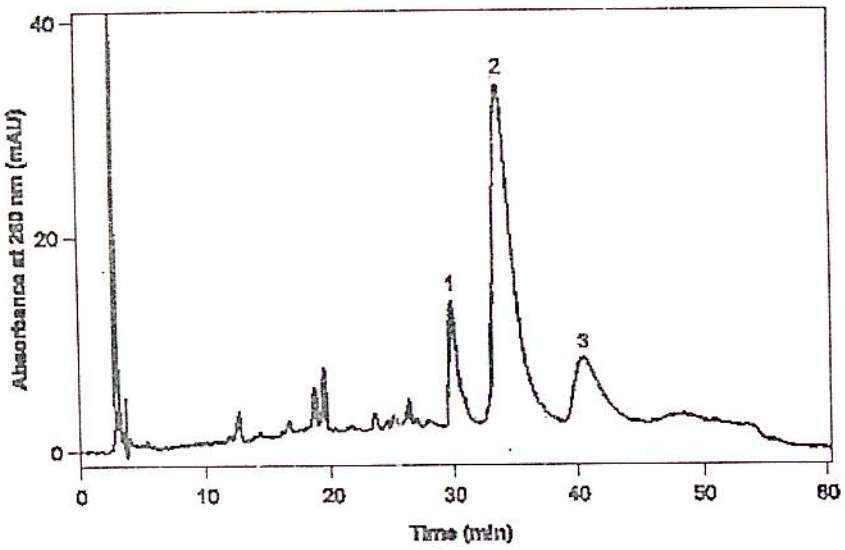


Figure-1.4: HPLC chromatograms of phenolic fraction I of *Litsea monopetala* bark extract

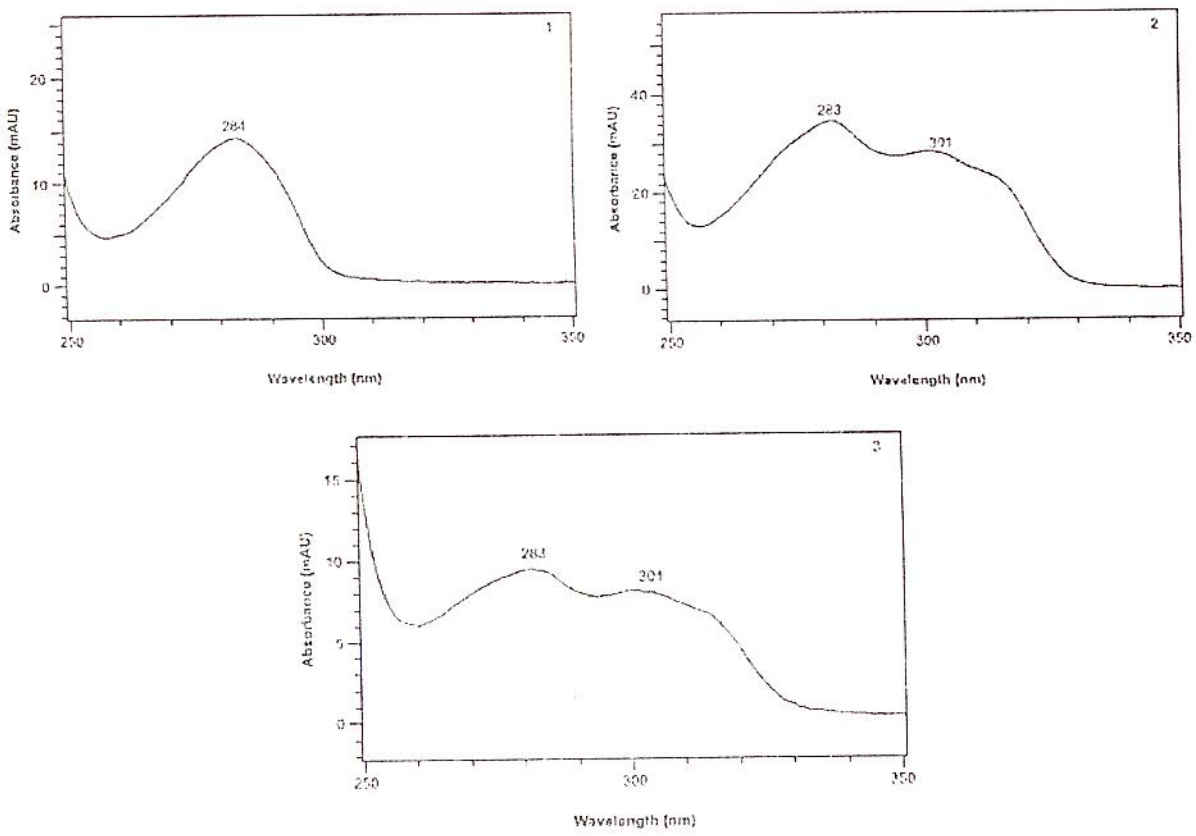
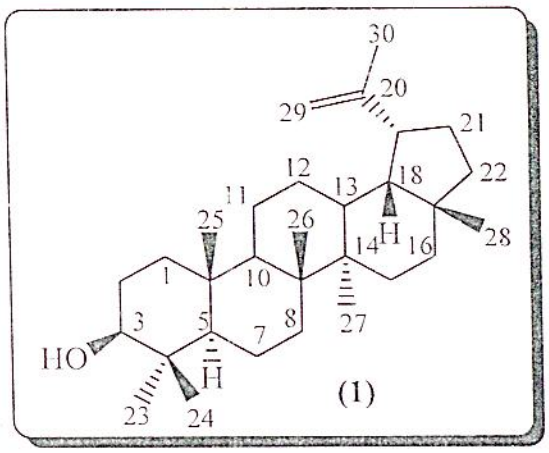


Figure-1.5: UV-DAD spectra of compounds separated from fraction I of the *Litsea monopetala* bark extract using HPLC method; 1-3 numbers of peaks corresponding to figure-1.4

1.3.2. Characterization of isolated compounds from *Litsea monopetala*

1.3.2.1. Lupeol (1)



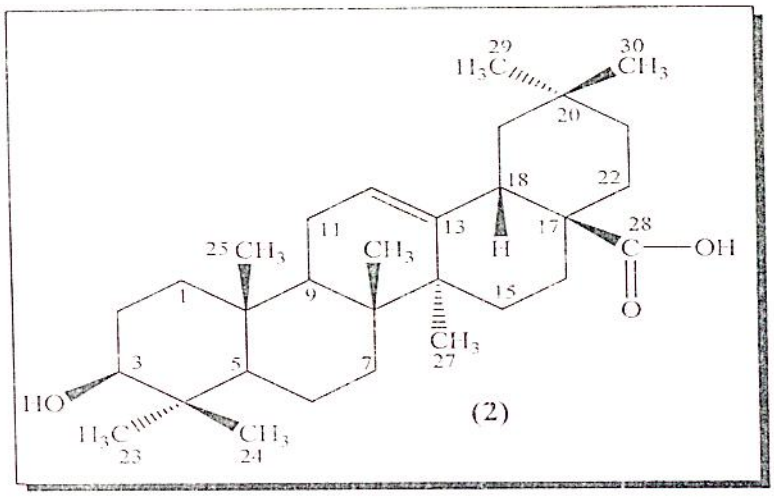
(1)

Compound **1** was isolated as colorless needles from the ethylacetate soluble fraction, subjecting to Column chromatography over silica gel as stationary phase eluting with chloroform*m*: n-Hexane (5:5). The IR spectrum of **1** showed absorption bands at 3400 cm^{-1} and 1640 cm^{-1} due to the hydroxyl and olefinic groups respectively. The molecular formula was established by HR-EIMS *m/z* 426.3855 as $C_{30}H_{50}O$.

The ^{1}H-NMR spectrum of **1** gave seven methyl signals at δ 1.68, 1.03, 0.97, 0.95, 0.83, 0.80 and 0.78, while the characteristic signal for olefinic protons appeared at δ 4.55 and 4.68 (H-29a, 29b). The proton appeared at δ 3.18 as a double doublet was assigned to 3α position.

The ^{13}C-NMR spectrum of **1** showed the presence of seven methyl, eleven methylene and six methine carbons. The chemical shifts of these carbon signals and other physical data were in complete agreement with data for lupeol reported in the literature[55, 56].

1.3.2.2. Oleanolic acid (2)



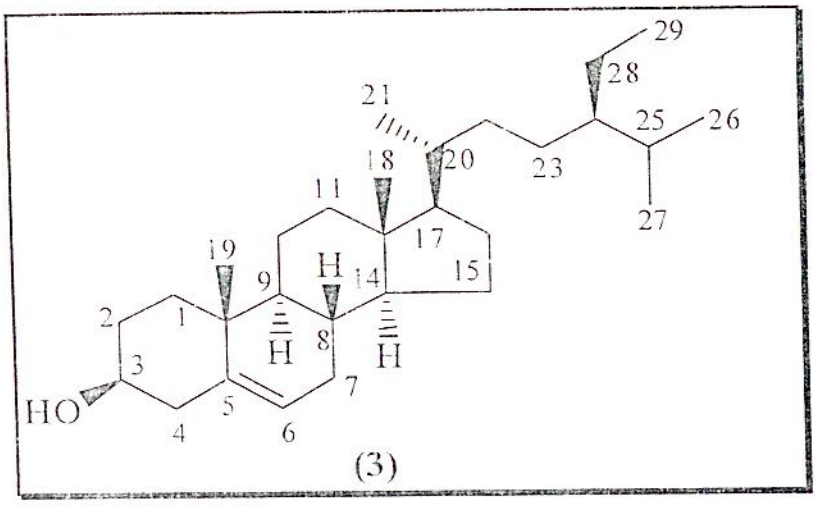
(2)

Compound **2** was isolated as colorless needles type crystals from the ethyl acetate soluble fraction. It gave positive color of triterpene with ceric sulphate spray. The EIMS spectrum showed the molecular ion peak at *m/z* 456.3518, corresponding to molecular formula $C_{30}H_{48}O_3$ (calcd. for $C_{30}H_{48}O_3$, 456.3603). The other molecular ion peaks were observed at *m/z* 248, 203 and 133 which were characteristic for amyrin skeleton. In IR spectrum the bands for hydroxyl group, Carbonyl group and trisubstituted double bond were appeared at (3400-2640 cm^{-1}), 1700 cm^{-1} and (1600-820 cm^{-1}) were respectively.

In ^{1}H-NMR spectrum the signals for seven methyl protons were appeared as singlets at δ 0.88, 0.91, 0.92, 0.96, 0.97, 1.04 and 1.13. The signal for olefinic proton was observed at δ 5.24 (1H, *t*, *J* = 3.4 Hz), while the proton geminal to the hydroxyl group was appeared at δ 3.62 (*dd*, *J* = 4.1, 9.9 Hz).

The ^{13}C-NMR assignments of various carbon atoms were substantiated by DEPT experiments, which showed the presence of seven methyl, ten methylene, five methine and seven quaternary carbon atoms. The physical and spectral data of **2** was in complete agreement to that of olefinic acid reported in previously in the literature[57, 58].

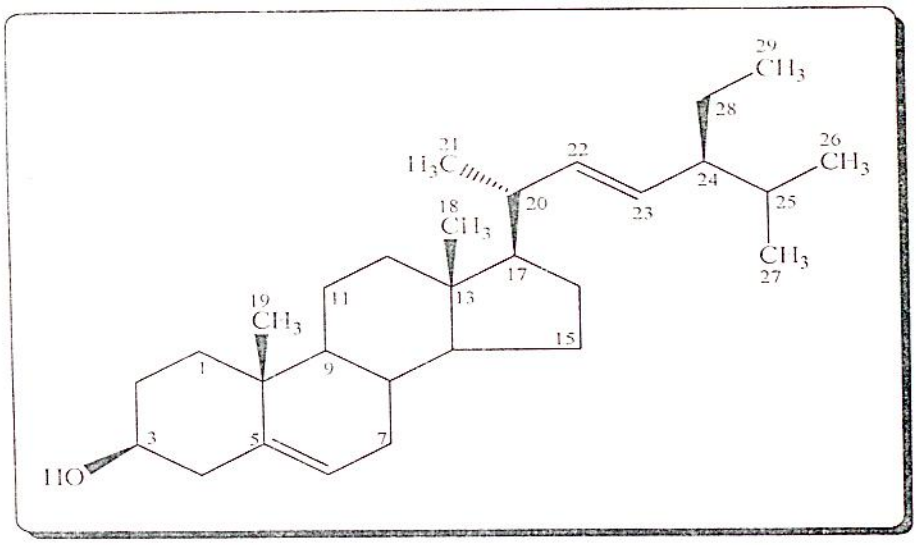
1.3.2.3. *β*-Sitosterol (3)



(3)

Compound **3** was identified as *β*-sitosterol. The EIMS spectrum of **3** showed the characteristic peaks at *m/z* 414.0512. The other prominent peaks were observed at *m/z* 399 $[M^{+}-CH_3]$, 396 $[M^{+}-H_2O]$, 329 $[M^{+}-85]$ and 303 $[M-111]^{+}$. The last two ions were reported to be triggered by the C-5 double bond. The other diagnostic peaks at 273and 231 were due to the loss of C-17 side chain and due to the loss of ring D with the side chain plus extra hydrogen respectively, while their respective dehydration products were appeared at 255 and 213. The above physical data of the compound **3** matched with the [3-*β*-hydroxy-stigmast-5-ene] *β*-sitosterol in the reported literature [60].

1.3.2.4. Stigmasterol (4)



(4)

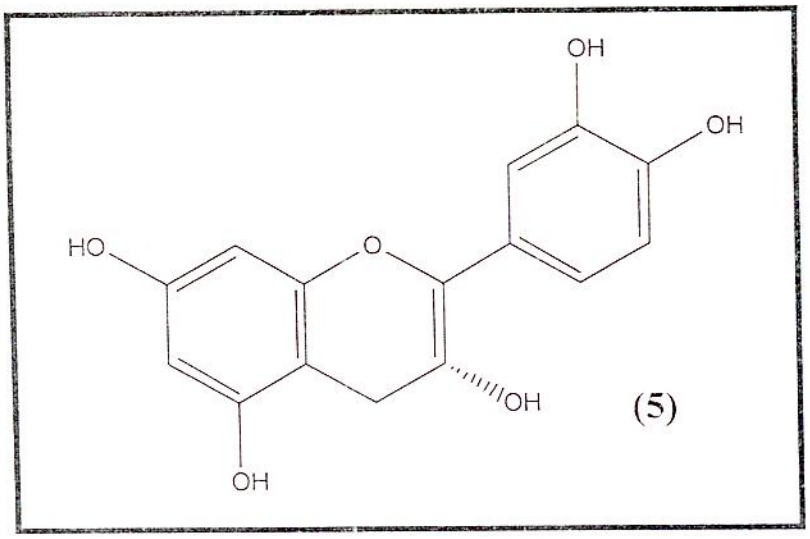
Ethyl acetate soluble fraction yielded compound 81 beside the other compounds. It was isolated as colorless needles crystals by column chromatography using silica gel as stationary phase eluting with *n*-hexane: ethyl acetate (2:8). IR spectrum gave the evidence of hydroxyl group at 3431cm^{-1}. Thus the nature of oxygen was determined by this peak. The molecular formula $C_{29}H_{48}O$ was established through EI-MS showing molecular ion peak $[M]^+$ at *m/z* 412.3921 (calcd. for $C_{29}H_{48}O$, 412.3926). The characteristics fragmentation pattern of sterol was observed in the mass spectrum[61].

The ^{1}H-NMR spectrum of 4 completely matched with the reported data for stigmasterol [62]. Signals for two tertiary methyl groups (δ 0.84, 0.65), two multiplets for three olefinic protons at δ 5.33 (1H) and 5.15 (2H) and a multiplet for the carbinylic proton at δ 3.28 appeared in the ^{1}H-NMR spectrum.

The ^{13}C-NMR spectrum of 4 showed the presence of 29 carbon signals for six methyl, nine methylene, eleven methine and three quaternary carbon atoms.

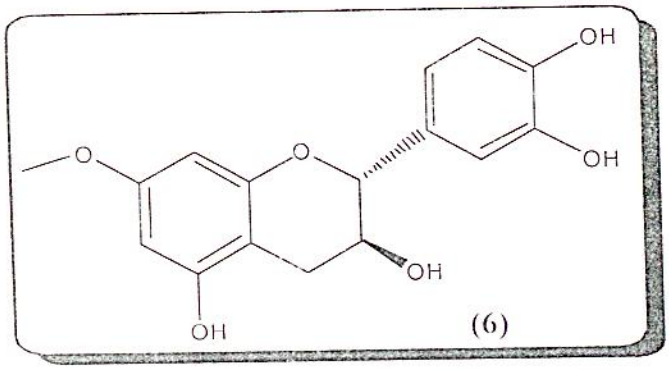
The above data was compared with the literature and showed complete agreement with those for stigmasterol [59].

1.3.2.5. (-)-Epicatechin (5)



Flavonoids give characteristic colour reaction with ceric sulphate spray. Compounds **5** showed this color spot and UV-spectrum of **5** in methanol provided the information about the presence of flavan-3-ol skeleton. The ^{1}H-NMR spectrum confirmed further the above suggestions and indicated the presence of the (-)-epicatechin, **5**. The spectrum was very similar in the aromatic region, the ABX system for ring B were in accordance with a 3',4'-dihydroxylation while the chemical shifts and the coupling constants of H-6 and H-8 indicated that **5** was 5,7-dihydroxylated. In addition, the chemical shifts and *J* couplings of the signals of H-2, H-3 and Hz-4 clearly proved the attachment of the 3-hydroxyl group. The ^{13}C-NMR spectrum confirmed the above conclusions. The experimental data for **5** matched with the data in the literature for epicatechin [63].

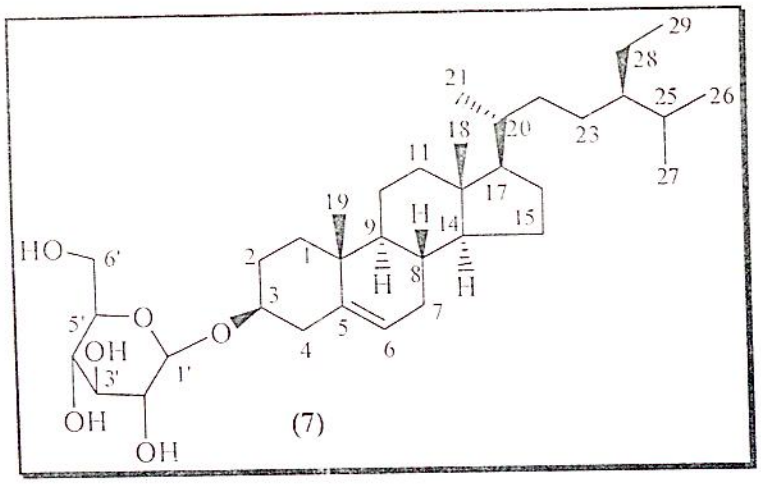
1.3.2.6. 7-Methoxy-(+)-catechin (6)



(6)

Compound 6 gave a molecular ion peak at 304 ppm. In addition to signals attributable to the C ring of a catechin moiety (δ 4.55 (1H, *d*, J =7.6Hz, H-2), at 3.97 (1H, *m*, H-3), and two *dd* (1H each) at δ 2.51 (J = 16.1 and 8.2Hz) and δ 2.85 (J = 16.1 and 5.4 Hz)), the ^{1}H-NMR spectrum revealed signals of five aromatic protons including an AB spin system (δ 5.85 and δ 5.97) with a meta coupling (J = 1.9 Hz, H-6 and H-8) and an ABX system attributable to a 3',4' disubstituted B ring. A singlet at δ 3.96 (3H) was indicative of one aromatic methoxyl group. The linkage of this substituent at C-7 was established by a NOESY experiment (NOE between the signal at 3.96 and the signals of H-6 and H-8) as well as from the ^{13}C NMR spectral data. Compared with the ^{13}C NMR spectrum of catechin, the C-7 peak of compound 6 was shifted downfield by 4.4 ppm, while the signals attributable to the adjacent C-6 and C-8 were shielded of 1.3 ppm. Thus, **6** is 7-methoxy-(+)-catechin[64].

1.3.2.7. *β*-Sitosterol-3-*O*-*β*-*D*-glucopyranoside (7)



Compound 7 was obtained as colorless crystals from the ethyl acetate soluble fraction by subjecting it to column chromatography over flash silica gel and eluting with EtOAc: *n*-hexane (8.3:1.7). The IR spectrum revealed absorption bands for hydroxyl group (3452 cm^{-1}) and trisubstituted double bond (3044, 1646 and 814 cm^{-1}). The HR-FAB-MS showed the molecular ion peak at *m/z* 576.4285 consistent with the molecular formula $C_{35}H_{60}O_6$ (calcd. for $C_{35}H_{60}O_6$, 576.4388). The fragmentation pattern in the mass spectrum of compound 7 was characteristic for sterol with double bond at C-5.

The ^{1}H-NMR data were similar to *β*-sitosterol except additional peaks at δ 5.32 (1H, *d*, *J* = 7.2 Hz) indicating an anomeric proton, and δ 3.81-4.41 for sugar protons geminal to hydroxyl groups.

The ^{13}C-NMR spectrum showed the presence of 35 carbon signals for six methyl, twelve methylene fourteen methine and three quaternary carbon atoms.

The ^{1}H-NMR, ^{13}C-NMR data, co TLC and mixed m.p. with an authentic sample gave the complete evidence for the compound 7 reported previously in the literature as *β*-sitosterol- 3-*O*-*β*-*D*-glucopyranoside[65].

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CHAPTER-2

BIOACTIVE CONSTITUENTS FROM *MALLOTUS PHILIPPENSIS*



CHAPTER 2 BIOACTIVE CONSTITUENTS FROM *MALLOTUS PHILIPPENSIS*

2.1. Introduction

2.1.1. The Genus *Mallotus*

The genus *Mallotus,* which belongs to family Euphorbiaceae, comprises 140 species in worldwide. It is represented in Pakistan by one specie[1].

2.1.2. *Mallotus philippensis*

Mallotus philippensis was collected from the district Bunner in the north of Pakistan in July 2006 and identified void Voucher No. 1013 (pup) by Prof. Dr. Abdur Rashid, department of botany, university of Peshawar, Peshawar, Pakistan.

2.1.3. Pharmacological importance of *Mallotus*

The genus *Mallotus* are a rich source of biologically active compounds such as phloroglucinols, tannins, terpenoids, coumarins, benzopyrans, and chalcons [2,3,4,5,6,7,8,9 10,11,12,13]. The aqueous extract of *Mallotus pelatus* showed a strong antipyretic effect similar to that of standard paracetamol [14, 15]. The phloroglucinol derivatives of *Malotus japonicus* inhibited the pro-inflammatory cytokine production. They exhibited the pharmacological ability to suppress prostaglandin E-2 production by activated macrophages [16], and reduced nitric oxide production by a murine macrophage-like cell line [17]. Bergenin (13) which is the major constituent of *Mallotus japonicas* was reported as hepatoprotective against hepatotoxicity in rats induced carbon tetrachloride [18]. Liphophilic acetylbergenin isolated from *Mallotus japonicus* showed to possess more hepatoprotective activity than the much less lipophilic bergenin[19, 20]. *Mallotus philippensis* Muell. (*Euphorbiaceae*), also known as kamala or kamopollaka, was used as a source of a yellow dye (kamala dye), and as an antioxidant for ghee and vegetable oils [21].

2.1.4. Literature review on genus *Mallotus*

Table-2.3: Compounds isolated from the plants of the genus *Mallotus*

S.No.	Compound Name	Mol. Formula	Mol. Wt.	Physical data	Plant & ref.
1	3-α-Hydroxyhop-22 (29)-ene	$C_{30}H_{50}O$	449.00	M.p.223-224°C White Crystal $[\alpha]^{25.}_{D}=2.5^{o}$ (c 0 1.0, $CHCl_3$)	*M.apelta*[22]
2	Hennadiol	$C_{31}H_{61}O_2$	465.00	M.p.235-237°C White Crystal $[\alpha]^{25.}_{D}=10^{o}$ (c 1.00, $CHCl_3$)	*M.apelta*[22]
3	Friedelin	$C_{30}H_{51}O$	427.29	M.p.265-267°C White Crystal $[\alpha]^{25.}_{D}=24.8^{o}$ (c 1.0, $CHCl_3$)	*M.apelta*[22]
4	Friedelanol	$C_{30}H_{52}O$	451.39	M.p.293-294°C White Crystal $[\alpha]^{25.}_{D}=16.6^{o}$ (c 1.00, $CHCl_3$)	*M.apelta*[22]
5	Epifriedelanol	$C_{29}H_{65}O$	429.00	M.p.280-283°C White Crystal $[\alpha]^{25.}_{D}=22^{o}$	*M.apelta*[22]

				(c 1.00, $CHCl_3$)	
6	Taraxerone	$C_{31}H_{59}O$	447.00	M.p.240-243°C White Crystal $[\alpha]^{25}_D$= 9.6° (c 1.00, $CHCl_3$)	*M.apelta*[22]
7	Epitaraxerol	$C_{31}H_{61}O$	449.30	M.p.270-272°C White Crystal $[\alpha]^{25}_D$= 9.7° (c 1.00, $CHCl_3$)	*M.apelta*[22]
8	Aquillochin	$C_{21}H_{20}O_9$	416.02	*	*M.apelta*[23]
9	Cleomisconsin	$C_{20}H_{18}O_8$	386.00	*	*M.apelta*[23]
10	5'-Demethylaquillochin	$C_{20}H_{18}O_9$	402.09	M.p.245-246°C Pale yellow needles	*M.apelta*[23]
11	Benzopyran A	$C_{26}H_{36}O_5$	428.00	Colorless oil $[\alpha]^{25}_D$=27.3° (c 0.33, $CHCl_3$)	*M.apelta*[7]
12	Benzopyran B	$C_{22}H_{30}O_4$	374.00	Colorless oil $[\alpha]^{25}_D$=18.6° (c 0.75, $CHCl_3$)	*M.apelta*[7]

13	10-Hydroxy-cembrene-5-one	$C_{20}H_{30}O_2$	302.23	*	*M.apelta*[4]
14	6-Hydroxy-cembrene-5,10-dione	$C_{20}H_{28}O_3$	317.00	*	*M.apelta*[4]
15	2α,4β,15,16-tetrahydroxyldolabradane	$C_{20}H_{36}O_4$	322.00	*	*M.apelta*[4]
16	Benzopyran1	$C_{26}H_{36}O_4$	412.26	Yellow oil $[\alpha]^{25}_D$=-1.31° (c 0.57, $CHCl_3$)	*M.apelta*[6]
17	Benzopyran 2	$C_{22}H_{30}O_4$	358.00	Yellow oil $[\alpha]^{25}_D$=0.71° (c 0.42, $CHCl_3$)	*M.apelta*[6]
18	Benzopyran 3	$C_{26}H_{36}O_4$	412.26	Yellow oil $[\alpha]^{25}_D$=-22.0° (c 0.29, $CHCl_3$)	*M.apelta*[6]
19	Benzopyran 4	$C_{22}H_{30}O_4$	358.00	Yellow oil $[\alpha]^{25}_D$=-152.0° (c 0.59, $CHCl_3$)	*M.apelta*[6]
20	Benzopyran 5	$C_{16}H_{20}O_4$	276.00	M.p. 122-124°C Pale yellow	*M.apelta*[6]

				needles $[\alpha]^{25}_{D}$=-92.8° (c 0.13, $CHCl_3$)	
21	Benzopyran 6	$C_{16}H_{20}O_4$	276.00	M.p. 160-162°C Pale yellow needles $[\alpha]^{25}_{D}$=-45.4° (c 0.11, $CHCl_3$)	*M.apelta*[6]
22	Benzopyran 7	$C_{12}H_{14}O_4$	222.00	M.p. 200-202°C Pale yellow needles $[\alpha]^{25}_{D}$=-44.1° (c 0.14, $CHCl_3$)	*M.apelta*[6]
23	Mallophenol A	$C_{25}H_{26}O_{13}$	533.13	Colorless oil $[\alpha]^{25}_{D}$=19.4° (c 0.968, MeOH)	*M.furetianus*[11]
24	Mallophenol B	$C_{26}H_{34}O_{12}$	561.19	Colorless oil $[\alpha]^{25}_{D}$=54.1° (c 1.127, MeOH)	*M.furetianus*[11]
25	Gallic acid	$C_7H_6O_5$	170.00	*	*M.furetianus*[11]

26	3-Hydroxy-4,5 *(R)*-dimethyl-2(5*H*)-furanone	$C_5H_7O_3$	115.00	*	*M.furetianus*[11]
27	Tannin A	$C_{34}H_{26}O_{22}$	785.00	Amorphous powder $[\alpha]^{26}_D$=-71.4° (c 0.5, MeOH)	*M.japonicus*[24]
28	Tannin B	$C_{34}H_{26}O_{22}$	785.00	Amorphous powder $[\alpha]^{26}_D$=-112.0° (c 1.0, MeOH)	*M.japonicus*[24]
29	Mallojaponin	$C_{57}H_{38}O_{37}$	1314.00	Amorphous powder $[\alpha]^{13}_D$=+15.9° (c 0.4, MeOH)	*M.japonicus*[24]
30	Mallonin	$C_{33}H_{28}O_{24}$	808.00	Amorphous powder $[\alpha]^{26}_D$=+13.3° (c 0.9, MeOH)	*M.japonicus*[24]
31	Mallotusinin	$C_{41}H_{26}O_{25}$	918.00	Amorphous powder $[\alpha]^{23}_D$=-20.3° (c 0.9, Acetone)	*M.japonicus*[24]

32	Isomallotolerin	$C_{26}H_{32}O_9$	488.00	M.p. 216-217°C Yellow needles	*M.japonicus*[25]
33	Isomallotochromanol	$C_{24}H_{28}O_9$	460.00	M.p. 150-152°C Yellow needles $[\alpha]^{23}_D = \pm 0^o$ (c 0.27, EtOH)	*M.japonicus*[25]
34	Mallotolerin	$C_{26}H_{32}O_9$	488.00	M.p. 197-198°C Yellow needles $[\alpha]^{23}_D = -0.30^o$ (c 1.0, $CHCl_3$)	*M.japonicus*[26]
35	Mallotochromanol	$C_{24}H_{28}O_9$	460.00	M.p. 216-218°C Yellow needles $[\alpha]^{23}_D = -0.74^o$ (c 1.0, $CHCl_3$)	*M.japonicus*[26]
36	Mallotophenone	$C_{21}H_{24}O_8$	404.00	M.p. 223-225°C Yellow needles	*M.japonicus*[27]
37	Mallotochromene	$C_{24}H_{26}O_8$	442.00	M.p. 190-192°C Yellow needles	*M.japonicus*[27]
38	Trioside	$C_{22}H_{34}O_6$	392.00	Amorphous powder	*M.japonicus*[28]

				$[\alpha]^{24.}{}_{D}$= - 25.8° (c 1.50, MeOH)	
39	Corotoxigenin	$C_{29}H_{42}O_9$	534.00	M.p. 188-192°C Colorless needles $[\alpha]_{.D}$= - 18.5°	*M.japonicus*[29]
40	Coroglucigenin	$C_{29}H_{44}O_9$	536.00	M.p. 275-278°C Colorless needles	*M.japonicus*[29]
41	Uzarigenin	$C_{29}H_{44}O_9$	536.00	M.p. 235-238°C Colorless prisms $[\alpha]_{.D}$= - 37.05°	*M.japonicus*[29]
42	11-Oxouzarigenin	$C_{29}H_{44}O_{10}$	552.00	M.p. 281-283°C Colorless needles $[\alpha]_{.D}$= - 30.52°	*M.japonicus*[29]
43	Mallogenin	$C_{35}H_{52}O_{14}$	696.00	M.p. 197-200°C Colorless plates $[\alpha]_{.D}$= - 24.11°	*M.japonicus*[29]
44	Panogenin	$C_{35}H_{54}O_{14}$	698.00	M.p. 225-228°C Colorless needles $[\alpha]_{.D}$= - 27.00°	*M.japonicus*[29]
45	11-*O*-Galloylbergenin	$C_{21}H_{20}O_{13}$	480.00	M.p. 179°C Amorphous	*M.japonicus*[30]

				powder needles $[\alpha]^{15}_{D}$= 37.6° (c 1.2, EtOH)	
46	4-*O*-Galloylbergenin	$C_{21}H_{20}O_{13}$	480.00	Amorphous powder $[\alpha]^{15}_{D}$= -51° (c 1.0, MeOH)	*M.japonicus*[30]
47	11-*O*-Galloyldemethyl-bergenin	$C_{20}H_{18}O_{13}$	466.00	M.p.217°C Amorphous powder $[\alpha]^{15}_{D}$= 63° (c 0.7, EtOH)	*M.japonicus*[30]
48	Luteolin 7-*O*-(4"-*O*-*(E)*-coumaroyl-*β*-glucopyranoside	$C_{30}H_{25}O_{13}$	593.12	Yellow Amorphous Powder	*M.metcalfianus*[31]
49	Chrysoeriol 7-*O*-(4"-*O*-*(E)*-coumaroyl)-*β*-glucopyranoside	$C_{31}H_{27}O_{13}$	607.14	Yellow amorphous powder	*M.metcalfianus*[31]
50	Hydnocarpin 7-*O*-(4"-*O*-*(E)*-coumaroyl)-*β*-glucopyranoside	$C_{40}H_{35}O_{16}$	771.19	Yellow amorphous powder	*M.metcalfianus*[31]

51	Triterpene A	$C_{30}H_{48}O$	424.00	M.p. 190-195°C $[\alpha]_D$= 45.1° ($CHCl_3$)	*M.nepalensis*[32]
52	Triterpene B	$C_{30}H_{50}O$	426.00	M.p. 216-220°C $[\alpha]_D$= 15.6° ($CHCl_3$)	*M.nepalensis*[32]
53	Mallopallidusol	$C_{24}H_{27}O_8$	443.17	M.p. 208-209°C yellow needles	*M.pallidus*[13]
54	Pallidusol	$CH_{14}H_{20}O_4$	275.12	M.p. 101-102°C	*M.pallidus*[10]
55	Dehydropallidusol	$CH_{14}H_{18}O_4$	273.11	M.p. 75°C	*M.pallidus*[10]
56	Pallidol	$CH_{12}H_{14}O_4$	245.08	M.p. 199-200°C	*M.pallidus*[10]
57	Mallopallidol	$C_{25}H_{30}O_8$	481.19	M.p. 185-186°C	*M.pallidus*[10]
58	Homomallopallidol	$C_{26}H_{32}O_8$	495.10	M.p. 178-179°C $[\alpha]^{20}_D$= 2.83° (c 0.1, MeOH)	*M.pallidus*[10]
59	C-Methylated cinnamoyl chromen	$C_{21}H_{20}O_4$	336.14	M.p. 137-139°C Yellow needles	*M.philippensis*[33]

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				$[\alpha]_D = 0^o$	
60	Rottlerin	$C_{40}H_{38}O_{13}$	726.00	M.p. 199-201°C Red brown needles	*M.philippensis*[33]
61	Rottlerin	$C_{30}H_{28}O_8$	516.00	M.p. 208-210°C Redish brown plates	*M.philippensis*[34]
62	Isoallorottlerin	$C_{30}H_{28}O_8$	516.00	M.p. 178-180°C Yellow prisms	*M.philippensis*[34]
63	Red compound	$C_{21}H_{20}O_4$	336.00	M.p.125-128°C Red needles	*M.philippensis*[34]
64	Yellow compound	$C_{21}H_{20}O_4$	336.00	M.p. 143-145°C Yellow tables	*M.philippensis*[34]
65	Rottlerone	$C_{41}H_{36}O_8$	656.00	M.p.221-222°C Red prisms	*M.philippensis*[34]
66	Isoallorottlerone	$C_{41}H_{36}O_8$	656.00	Yellow amorphous	*M.philippensis*[34]
67	5,7-Dihydroxy-8-methyl-6-prenylflaranone	$C_{21}H_{22}O_4$	338.00	*	*M.philippensis*[34]
68	3'-preylrubranine	$C_{30}H_{33}O_4$	457.00	*	*M.philippensis*[34]

69	Isorottlerin	$C_{30}H_{28}O_8$	516.00	M.p. 208-210°C Redish brown plates	*M.philippensis*[34]
70	Kamalachalcone A	$C_{42}H_{40}O_8$	671.00	Yellow powder $[\alpha]_D$= -51° (c 0.06, MeOH)	*M.philippensis*[3]
71	Kamalachalcone B	$C_{51}H_{48}O_2$	851.00	Orange powder $[\alpha]_D$= -60° (c 0.12, MeOH)	*M.philippensis*[3]
72	3`-Prenylrubranine	$C_{30}H_{34}O_4$	458.00	M.p. 192-194°C Orange red needles $[\alpha]_{546}$= +0.28°, ($CHCl_3$)	*M.philippensis*[35]
73	Mallotus A	$C_{20}H_{18}O_5$	338.00	M.p. 263-264°C Reddish brown needles	*M.philippensis*[35]
74	Mallotus B	$C_{30}H_{30}O_8$	518.00	M.p. 232-234°C Light yellow amorphous solid	*M.philippensis*[35]
75	2,4-Dihrydoxy-6`-methoxy-3`-prenylchalcone	$C_{21}H_{22}O_4$	338.00	M.p. 165-168°C Yellow needles	*M.philippensis*[35]

76	5,7-Dihydroxy-6-methyl-8-prenylflaanone	$C_{21}H_{22}O_4$	338.00	M.p. 182-185°C Colorless crystalline solid	*M.philippensis*[35]
77	Mallotophilippen C	$C_{30}H_{34}O_5$	474.24	Redish yellow plate	*M.philippensis*[36]
78	Mallotophilippen D	$C_{30}H_{34}O_6$	490.00	Redish yellow plate	*M.philippensis*[36]
79	Mallotophilippen E	$C_{30}H_{34}O_6$	490.00	Redish yellow plate	*M.philippensis*[36]
80	Mallotophilippen A	$C_{28}H_{34}O_8$	498.23	Yellow powder	*M.philippensis*[37]
81	Mallotophilippen B	$C_{26}H_{30}O_8$	470.19	Yellow powder	*M.philippensis*[37]
82	4'-Hydroxyisorottleri n	$C_{30}H_{28}O_9$	532.00	Colorless amorphous powder $[\alpha]_D = 0^o$ (c 0.1, MeOH)	*M.philippensis*[38]
				Amorphous pale	

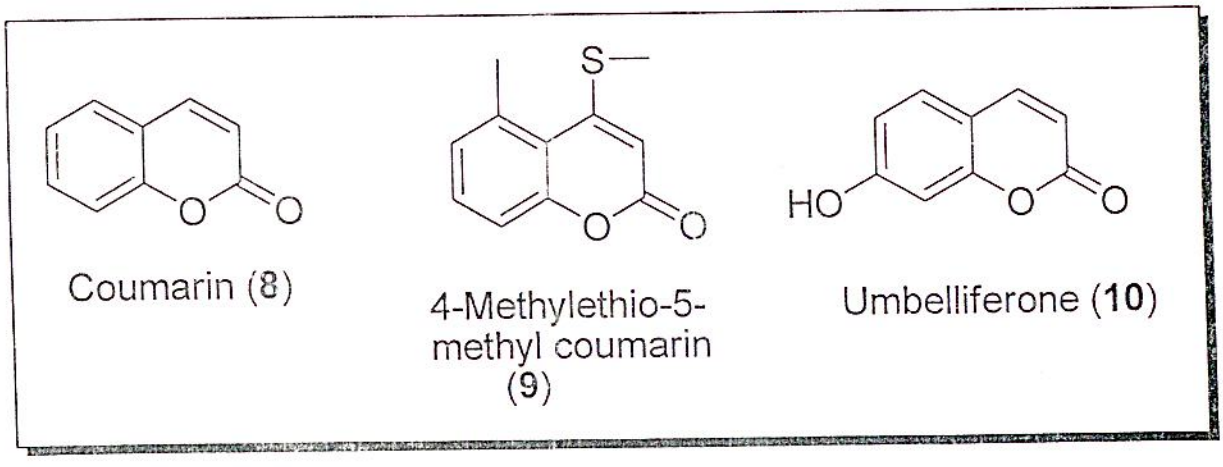
83	Kamalachalcon C	$C_{31}H_{30}O_8$	530.00	yellow $[\alpha]_D = +18^o$ (c 0.07, Acetone)	*M.philippensis*[38]
84	Kamalachalcon D	$C_{65}H_{62}O_{16}$	1097.00	Pale yellow solid $[\alpha]_D = +31^o$ (c 0.13, $CHCl_3$)	*M.philippensis*[38]
85	3α-hydroxy-13α-ursan-28, 12β-olid 3-benzoate	$C_{37}H_{52}O_4$	560.39	M.p = 260-262°C Colorless needles $[\alpha]^{27}_D = -33^o$ (c 1.0, $CHCl_3$)	*M.repandus*[5]
86	3α-hydroxy-28β-methoxoy-13α-ursan-28, 12β-epoxide-3-benzoate	$C_{38}H_{55}O_4$	575.41	M.p = 155-157°C Colorless powder $[\alpha]^{27}_D = 1.79^o$ (c 0.05, $CHCl_3$)	*M.repandus*[5]
88	Mallorepine	$C_7H_6NO_2$	134.05	M.p = 185-186°C Colorless prisms	*M.repandus*[39]
89	δ-Lactone 1	$C_{30}H_{48}O_3$	456.00	M.p = 385-386°C Needles crystals $[\alpha]_D = +17.0^o$	*M.repandus*[40]

90	δ-Lactone 2	$C_{37}H_{52}O_4$	560.00	M.p = 340-342°C Plate crystals	*M.repandus*[40]
91	δ-Lactone 3	$C_{30}H_{48}O_3$	456.00	M.p = 319-21°C $[\alpha]_D$= +9.0°	*M.repandus*[40]
92	3-(1-C-β-*D*-glucopyranosyl)-2,6-dihydoxy-5-methoxybenzoic acid	$C_{14}H_{18}O_{10}$	369.00	M.p. 139-140°C Crystalline solid	*M.roxburghianus*[41]
93	2,4,8,9,10-pentahydroxy-3,7-dimethoxyanthracene-6-*O*-β-*D*-rhamnopyranoside	$C_{22}H_{24}O_{12}$	503.00	M.p. 296-298°C White solid $[\alpha]^{22}_D$= -36° (c 0.02, MeOH)	*M.roxburghianus*[41]
94	Bergenin	$C_{14}H_{16}O_9$	328.00	M.p. 235°C White needles	*M.roxburghianus*[41]
95	4-Hydroxybenzoic acid	$C_7H_6O_3$	138.00	*	*M.roxburghianus*[41]

*Not reported in the relevant literature

2.1.5. Coumarins

Coumarins is a class of compounds which owe their name to coumarou, the vernacular name of the tonka bean (Dipteryxodorata Willd, Fabaceae), from which it was isolated in 1820 [42].Coumarins are found in many dicotyledonous families including the Apiaceae, Asteraceae, Fabaceae, Moraceae, Rosaceae, Rubaceae, Rutaceae and Solanaceae [43,44] as free or heteroside. Many monocotyledonous plants, such as Gramineae and Orchids, also contain large amounts of coumarins. Coumarins occur at the highest levels in the fruit, followed by the roots and stems though it is mainly synthesized in the leaves. Coumarins are a group of compounds known as the form of phenolic, ethereal or glycosidc linkages. Mostly coumarins are oxygenated at C-7. All the natural coumarins bear oxygen atoms at one or more of the six available positions on nucleus except (8) and (9) i.e umbelliferone (10) which acts as the biogenetic precursor of more complex coumarins has oxygen atom[45].



Coumarin (8)

4-Methylethio-5-methyl coumarin (9)

Umbelliferone (10)

2.1.5.1. Pharmacological activities of isocoumarins

Bergenin and 11-*O*-Galloylbergenin isolated from *Mallotus philippensis* in this part of work have also isocoumarin type of nucleus. Isocoumarin are important components in many natural products that exhibit a broad range of biological activities including antiallergic and antimicrobial[46], immunomodulatory[47], cytotoxic[48], antifungal[49], antiinflammatory[50], antiangiogenic[51], anticalmodulin-sensitive cyclic guanosine 30, 50-monophosphate phosphodiesterease[52] effects, and differentiation inducing activity against

leukemic cells[53]. Isocoumarins are useful intermediates in the synthesis of a variety of important compounds including some isoquinoline alkaloids[54].

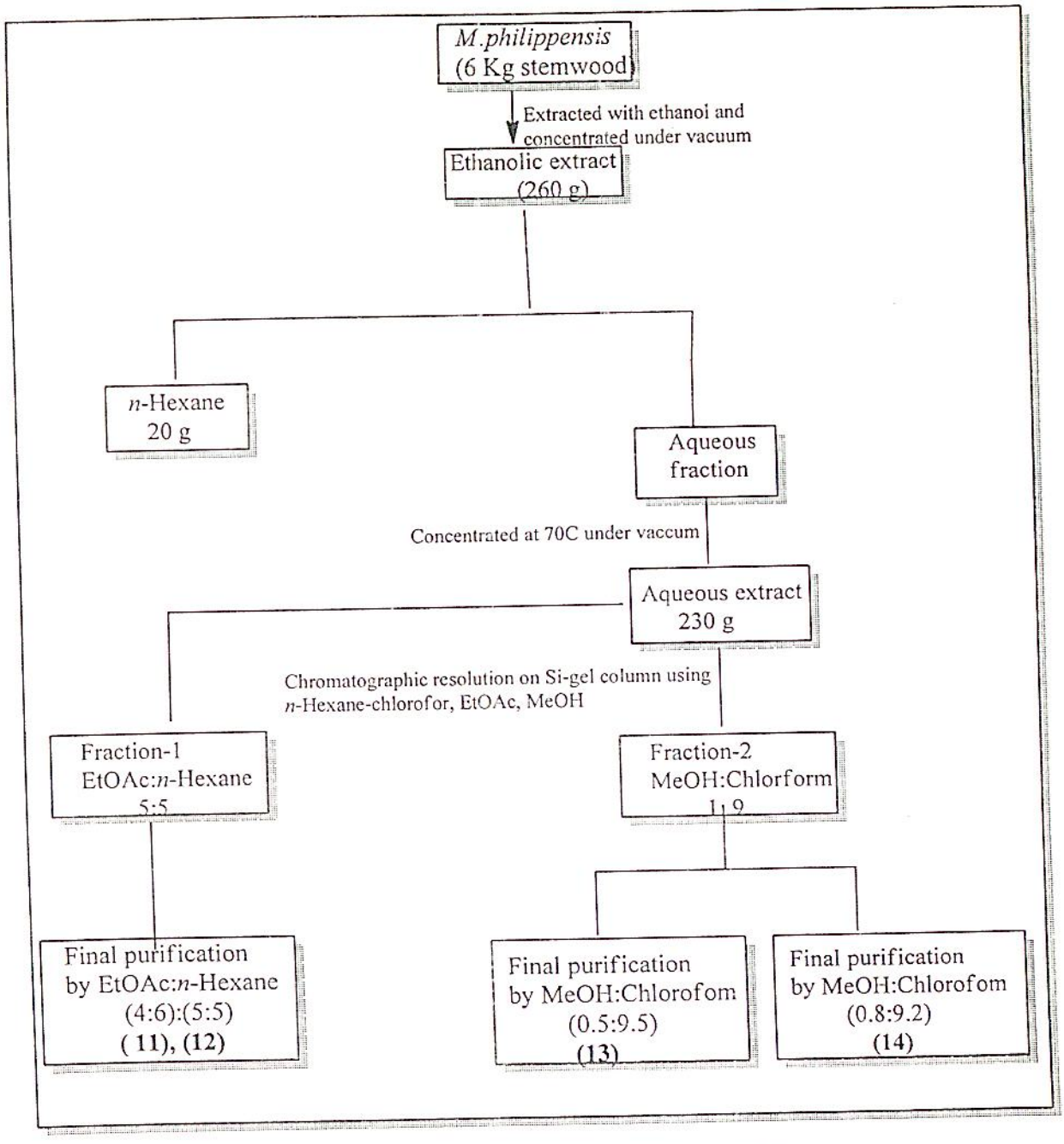
2.2. Experimental

2.2.1. Plant material

Plant material (Stem wood) *of Mallotus philippensis* (Euphorbiaceae) was collected from the district Buner in the north of Pakistan in July 2006 and identified by Prof. Dr. Abdur Rashid, Department of Botany, University of Peshawar, where a voucher specimen No.1013 (pup) has been deposited.

2.2.2. Extraction and isolation

Air dried stem wood of *Mallotus philippensis* (6 kg) was chopped and ground. The grinded material was extracted three times with 20 L commercial ethanol for 72 h and filtrate was evaporated under vacuum, affording 260 g (4.3%). It was dissolved in water and successively extracted with n-hexane which yielded 20 g. The remaining dense reddish solution of water was evaporated under reduced pressure at 70°C to obtain a thick gummy residue (230 g). 50 g of this extract was loaded over silica gel column eluting with ethylacetate: *n*-hexane and methanol: chloroform in increasing order of polarity. The fraction 1 obtained from ethylacetate: *n*-Hexane (5:5) showed two major spots on TLC. It was resubjected to flash chromatography eluting with the solvent system ethylacetate: *n*-hexane (4:6) and (5:5) to afford 3-Acetyl aleuritolic acid **(11)** (10mg) and (+)-catechin **(12)** (20mg). Fraction 2 obtained from methanol: chloroform (1:9) was further subjected to flash silica gel column. Two compounds were purified using methanol: chloroform at (0.5:9.5) and (0.8:9.2) polarity. One was bergenin **(13)** (8.3 g) and the other was its gallic acid derivative, namely 11-*O*-Galloylbergenin **14** (50 mg), **Scheme-2.2**.



Scheme-2.2: Extraction and isolation from *Mallotus Philippensis* stem

2.2.3. Antioxidant activity (*in vitro*) of the crude fractions of *Mallotus philippensis* fruit and bark

2.2.3.1. Materials

All solvents used for chromatography were of high performance liquid chromatography (HPLC) grade, unless otherwise specified. Ferric chloride, potassium ferricyanide, potassium persulfate, trifluoroacetic acid (TFA) and trichloroacetic acid (TCA) were acquired from the P.O.Ch. Company (Gliwice, Poland). (+)-Catechin, (–)-epicatechin, Folin and Ciocalteau's phenol reagent, 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH·) and 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma Ltd. (Poznan′, Poland). The standards of (–)-epigallocatechin gallate, delphinidin, cyanidin and malvidin were obtained from Extrasynthese (Genay Cedex, France). Procyanidin B2 was a gift from Prof. Jan Oszmian′ski (Agricultural University in Wroclaw, Poland). *M. philippensis* fruits and bark were collected from the Buner area, Pakistan. Extracts of phenolic compounds from source materials were obtained according to the flow sheet outlined in **Fig. 2.6**.

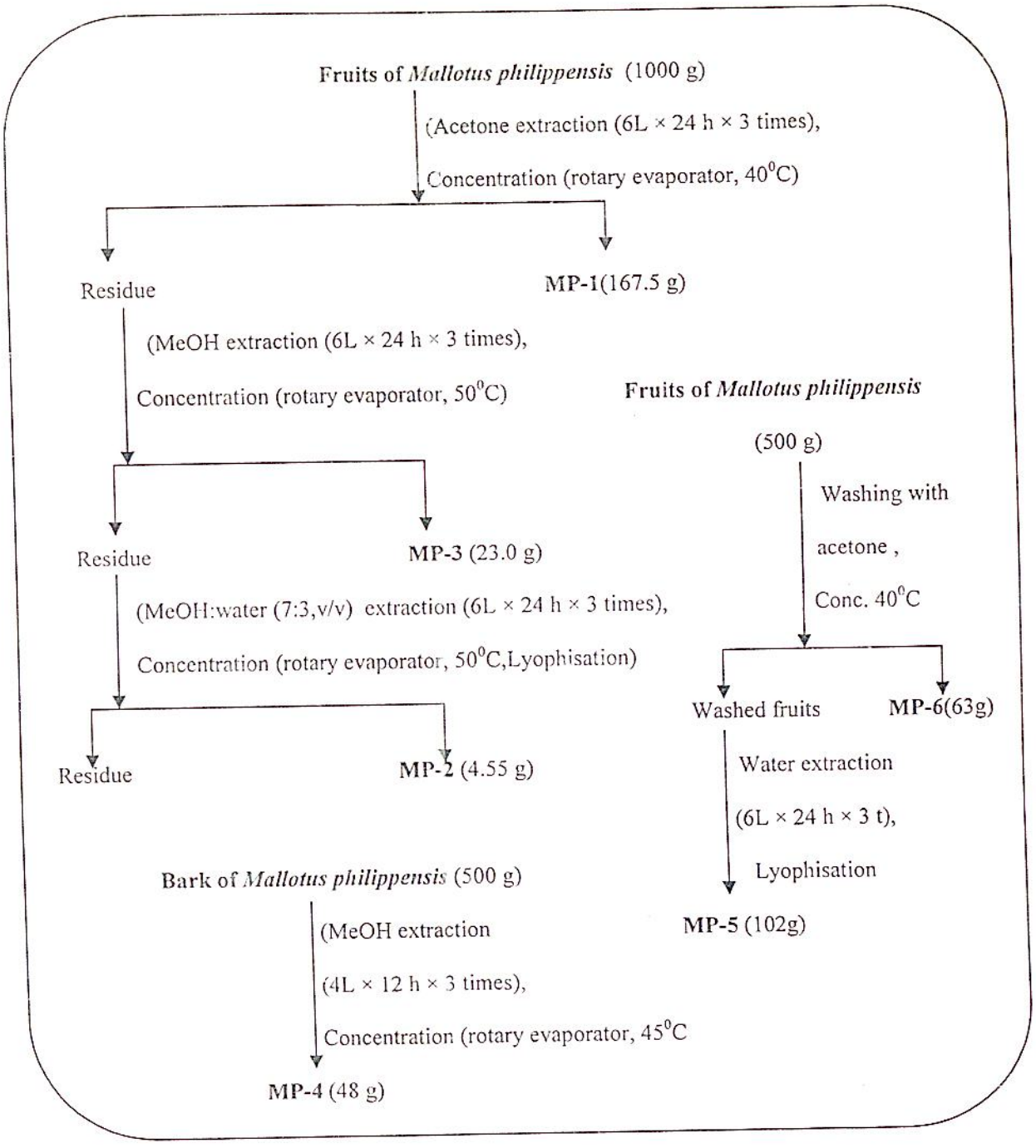


Fig-2.6: Flow diagram for the preparation of extracts from *Mallotus philippensis* fruits and bark

2.2.3.2. Total Phenolics and Condensed Tannins

The content of total phenolics in the extracts was determined as described by Naczk *et al* [55], using the Folin–Ciocalteau reagent. (+)-Catechin was used as a standard in this work (For brief procedure see **1.2.4.3**). The content of condensed tannins in the extracts was determined according to a modified vanillin method described by Price *et al* [56], and expressed as absorbance units at 500 nm per mg of extract (A_{500}/mg).

2.2.3.3. UV Spectra

UV spectra of individual fractions dissolved in methanol were recorded using a Beckman DU 7500 diode array spectrophotometer (Beckman Instruments, Fullerton, CA).

2.2.3.4. Total Antioxidant Capacity

The total antioxidant capacity in the extracts was determined according to the Trolox equivalent antioxidant activity (TEAC) assay described by Re *et al* [57] (For brief procedure see **1.2.4.5**). The total antioxidant capacity was expressed as mmol Trolox equivalents/g of extract and mmol Trolox equivalents/g of starting material.

2.2.3.5. Radical scavenging Activity (RSA) Assay

The capacity of prepared extracts to scavenge the "stable" free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH·) was monitored according to the procedure described by Amarowicz *et al* [58]. Briefly, 0.1 mL of methanolic solution containing 0.02 to 0.1 mg of extract was mixed with 2 mL distilled water, and then 1 mM of 0.25-mL methanolic solution of DPPH radical was added. The mixture was vortexed thoroughly for 1 min. Finally, the absorbance of the mixture after standing at ambient temperature for 30 min was read at 517 nm.

2.2.3.6. Reducing Power

The reducing power of the extracts was determined by the method of Oyaizu[59]. Briefly, the assay medium contained 2.5 mL of the sample extract in 0.2M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide. After incubation at 50^{0}C for 20 min, 2.5 mL of 10% (w/v) TCA was added to the mixture followed by centrifugation at 1,750 ¥ g for 10 min. The supernatant (2.5 mL) was mixed with 2.5mL distilled water and 0.5

mL of 0.1% (w/v) ferric chloride and the absorbance of the resultant solution was read at 700 nm.

2.2.3.7. HPLC Analysis

Phenolic constituents of extracts were analyzed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of two LC-10AD pumps, SCTL 10A system controller and SPD-M 10A photodiode array detector. The chromatography was carried out using a prepacked LiChrospher 100 RP-18 column (4 ¥ 250 mm, 5 mm; Merck, Darmstadt, Germany). Elution for 50 min in a gradient system of 5–40% acetonitrile in water adjusted to pH 2.5 with TFA was employed. The detector was set at 280 nm; the injection volume was 20 mL and the flow rate was 1 mL/min.

2.2.3.8. TLC Analysis

Bark extract was examined on a silica gel TLC plate (Sigma, St. Louis, MO) using the chloroform/methanol/water (65:35:10, v/v/v, lower phase)[60], Vanillin-hydrochloric acid reagent[61] was used for color development reaction of catechins and condensed tannins.

2.2.3.9. Proanthocyanidin Hydrolysis

Proanthocyanidins present in bark extract were hydrolyzed according to the method described by Porter *et al* [62]. To a 10mL screwcap tube with 6 mL of the acid butanol reagent (950 mL of *n*-butanol with 50 mL concentrated HCl), 1.0 aliquot of the extract, and 0.2 mL of the iron reagent were added and vortexed. The tube was capped loosely, and put in a boiling water bath for 50 min.. The tube was then cooled and the solution was transferred to a volumetric flask and adjusted to 25 mL with acidified butanol.

2.2.3.10. HPLC Analysis of Proanthocyanidin Hydrolysate

The same Shimadzu HPLC system was used for analysis of compounds liberated from the bark extract after acidic butanol hydrolysis. The mobile phase was composed of 4% aqueous H_3PO_4/acetonitrile (80:20; v/v) [63], the flow rate was 1.0 mL/min, and the detector was set at 525 nm. The standards of delphinidin, cyanidin and malvidin were injected onto the column under the same conditions.

2.2.3.11. Semipreparative HPLC

The main phenolic compound was separated from the bark extract using a semipreparative Hibar prepacked RT column (10 ¥ 250 mm) with Lichrosorb RP-18 (7 mm) (Merck). The mobile phase water/acetonitrile/acetic acid (88:10:2, v/v/v) was delivered at a rate of 4 mL/min using an injection volume of 500 mL. The detection was monitored at 280 nm [64].

2.2.3.12. ESI-MS

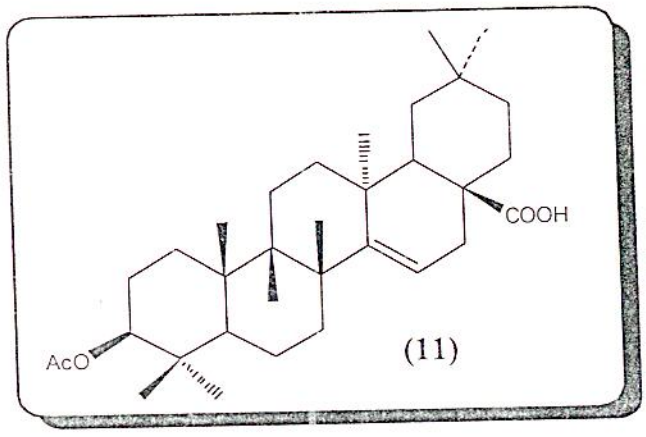
The pure compound separated from the bark extract was dissolved in 80% methanol (v/v) and injected onto a column of Shimadzu liquid chromatograph mass spectrometer LCMS QP8000 system. Conditions of analysis were Luna 3 m C (2) (2 ¥ 150 mm) column (Phenomenex, St. Torrance, CA), mobile phase of 80% (v/v) methanol, flow rate 0.2 mL/min; injection loop of 10 mL; CDL temperature: 230C; CDL voltage: +85 V; probe voltage +4.5 kV, defragmentation voltage +45 V; and nebulizer gas flow of 4.5 mL/min.

2.2.3.13. Statistics

Each extract was considered as a "treatment." All measurements were replicated three times and their means ± SD reported. A P value of <0.05 was used to denote significance of differences among mean values determined by analysis of variance.

2.2.4. Experimental data of isolated compounds of *Mallotus philippensis*

2.2.4.1. 3-Acetylaleuritolic acid (11)



(11)

Crystallised from Methanol: Chloroform (10 mg).

Mp = 304-305°C

$[\alpha]_D^{15}$ + 23.1° (c 0.6, $CHCl_3$).

Rf = 0.35

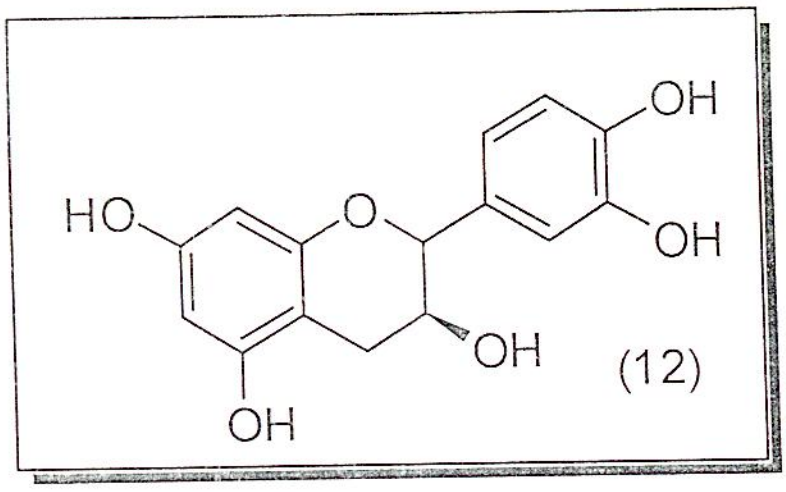
IR ν_{max} (Chloroform) = 3000, 1732, 1087, 820 cm^{-1}

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.46 (1H, J =10.6 Hz), 0.87 (1H, *m*), 5.52 (1H, J =8.4Hz), 0.88 (3H, *s*), 0.86 (3H, *s*), 0.96 (3H, *s*), 0.91 (3H, *s*), 0.93 (3H, *s*), 0.92 (3H, *s*), 2.03 (-$OCOCH_3$)

^{13}C NMR (125 MHz, $CDCl_3$): δ 37.3 (C-1), 23.4 (C-2), 80.8 (C-3), 37.7 (C-4), 55.6 (C-5), 18.8 (C-6), 40.8 (C-7), 39.0 (C-8), 49.0 (C-9), 38.0 (C-10), 17.3 (C-11), 33.3 (C-12), 37.3 (C-13), 160.6 (C-14), 116.9 (C-15'), 31.2 (C-16), 51.5 (C-17), 41.3 (C-i8), 35.3 (C-19), 29.3 (C-20), 33.7 (C-21), 30.7 (C-22), 28.0 (C-23), 16.5 (C-24), 15.7(C-25), 26.2 (C-26), 22.4 (C-27), 184.3 (C-28), 31.9(C-29), 28.7(C-30), 171.0(C-31), 21.3(C-32).

EIMS *m/z* (rel. int.): 498.4 (2) [M^+], 344.3(6), 329(6), 248.1(31), 234.1(100), 189.2(8).

2.2.4.2. (+)-Catechin (12)



Light yellow powder (20 mg)

MP: 279-280° C

$[\alpha]^{25}_{D}$: +14.4° (*c* 0.3, MeOH)

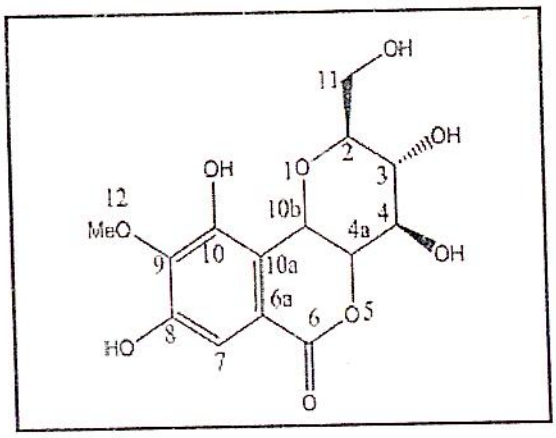
IR (KBr) ν_{max}: 3450, 3042, 1645, 1617, 1594-1552 cm^{-1}

^{1}H-NMR (CD_3OD): δ 2.41 (1H, *dd*, *J* = 16.0, 8.1 Hz, H-4b), 2.75 (1H, *dd*, *J* = 16.1 and 5.5 Hz, H-4a), 3.92 (1H, *m*, H-3), 4.46 (1H, *d*, *J* = 7.4 HZ, H-21, 5.81 (1H, *d*, *J* = 2.1 Hz, H-61, 5.86 (1H, *d*, *J* = 2.1 Hz, H-8), 6.74 (2H, *m*, H-5' and H6'), 6.83 (1H, *d*, *J* = 1.7 HZ,H-2')

^{13}C-NMR (CD_3OD): δ 82 (C-2), 67.3 (C-3), 28.2 (C-4), 156.1 (C-5), 95.0 (C-6), 155.2 (C-7), 93.7 (C-8), 156.4 (C-9), 99.0C-10), 131.5 (C-1'), 114.5 (C-2'), 144.7 (C-3'), 144.8 (C-4'), 115.0 (C-5'), 118.2 (C-6').

EIMS *m/z*: 288 (M^+)

2.2.4.3. Bergenin (13)



(13)

White needles (8.3 g),

M.p= 237 °C

UVλ_{max} (log ε) = 279(4.28).

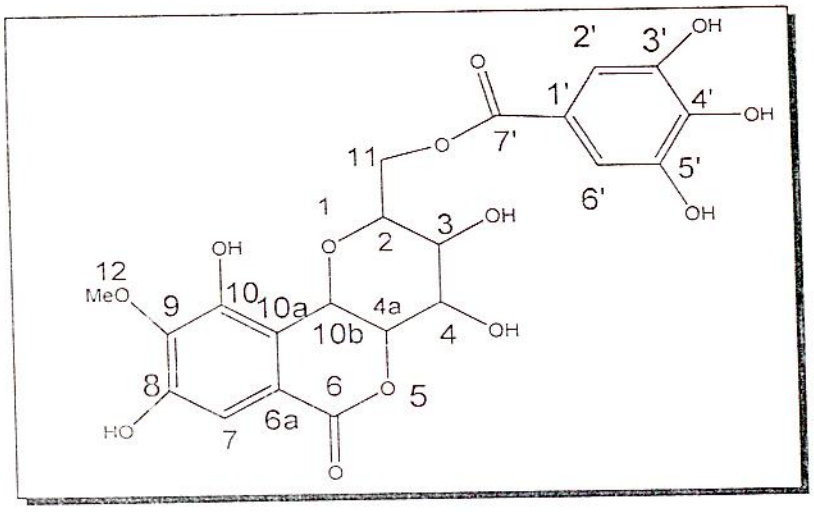
IR(KBr) ν_{max} = 3310, 3350, 1712, 1632, 1609, 1510, 1230 cm^{-1}

^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.97 (1H, *s*, H-7), 5.67 (1H, *d*, H-10b), 4.98 (1H, *dd*, H-4a), 3.89 (1H, *dd*, H-4), 3.81 (2H, *d*, H-11), 3.75 (3H, s, H-12), 3.62 (1H, *m*, H-2), 3.48 (1H, *dd*, H-3).

^{13}C NMR (DMSO- d_6, 100 MHz): δ 60.0 (C-12), 61.1 (C-11), 70.7 (C-3), 72.1 (C-10b), 73.7 (C-4), 79.8 (C-4a), 81.7 (C-2), 109.4 (C-7), 116.0 (C-10a), 118.1 (C-6a), 140.6 (C-9), 148.1 (C-10), 151.0 (C-8), 163.5 (C-6).

EIMS *m/z* (rel. int.): 328 (34), 208 (100), 237 (7), 170 (30).

2.2.4.4. 11-*O*-Galloylbergenin (14)



(14)

White amorphous powder (50 mg).

Mp = 180^{0}C

$[\alpha]_D^{15}$ + 37.6^0 (EtOH; c 1.2)

UV λ_{max} (log ε) = 277(4.22)

Rf = 0.25

IR (KBr) ν_{max} = 3310, 1712, 1632, 1609, 1510, 1230 cm^{-1}

^{1}H NMR (90 MHz, Me_2CO-d): δ 7.21(2H, *s*, gall-H), 7.10 (IH, *s*, H-7), 3.90 (3H, *s*, OMe)

^{13}C NMR (90 MHz, CD_3OD): δ 81.2 (C-2), 72.2 (C-3), 75.7 (C-4), 81.1 (C-4a), 166.5(C-6),120.0, (C-6a), 112.0 (C-7), 153.1 (C-8), 143.3 (C-9), 150.0 (C-10), 117.7 (C-10a), 74.7 (C-10b), 65.1 (C-11), 61.3 (C-12), 121.8 (C-1'), 111.1 (C-2', C-6'),147.4 (C-3', C-5'), 140.0 (C-4'), 169.2 (C-7').

EIMS *m/z* (rel. int.): 480 (32), 328 (34), 208 (100), 237 (7), 170 (30).

2.2.5. Biological activities of isolated compounds

2.2.5.1. Urease inhibition assay (*in vitro*)

Reaction mixture comprising 25 mL (40 units per mL) of enzyme (*Bacillus pasteurii* urease) solution and 55 mL of buffers containing urea (2-24 mM for *Bacillus pasteurii* urease) were incubated with 5mL of bergenin at 30.8 °C for 15 min in 96-well plates. The increased absorbance at 560 nm was measured after 10 min using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 mL. The results (change in absorbance per min) were processed using SoftMax Pro software (Molecular Device, USA). All the assays were performed at pH 6.8 (3mM sodium phosphate buffer) and 7mg of phenol red per mL as indicator. Percentage inhibitions were calculated from 100-($OD_{testwell}/OD_{control}$) *100*. Thiourea was used as the standard inhibitor of urease. [65].

2.2.5.2. Antioxidant activity (*in vitro*)

2.2.5.2.1. DPPH Radical scavenging assay

The DPPH radical scavenging activities of the isolated compounds **13** and **14** were estimated using a slight modified form of the protocol reported by Blois [66, 67]. 1 mM methanolic DPPH radical solution was mixed with 3 ml of ethanolic solution containing the sample (20-100 µg) and control (without sample). Mixture was analyzed after 30 minutes at 517 nm. Decrease in the absorbance given by DPPH solution indicated the increase in DPPH radical scavenging activities. Ascorbic acid, α-tocopherol, quercetin and gallic acid were used as standards. Percentage radical scavenging activity (% RSA) was calculated using equation:

$$\% \text{ RSA} = \text{Control } \frac{\text{absorbance - sample absorbance}}{\text{Control absorbance}} \times 100$$

The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The concentration showing 50% inhibition (EC_{50}) was calculated from the % RSA against concentration. Quercetin, Ascorbic acid, Gallic acid and α-tocopherol used as standards.

2.2.5.2.2. Determination of reducing power

The reducing power of the compounds **13** and **14** were determined by the method of Oyaizu [68]. Briefly, the assay medium contained various concentrations (20 to 100 µg/mL) of the compounds in 0.2-M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide. After incubation at 50°C for 30 min, 2.5 mL of 10% (w/v) Trichloroacetic acid was added to the mixture followed by centrifugation at 1,750 × g for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL of 0.1% (w/v) ferric chloride and the absorbance of the resultant solution was read at 700 nm. Quercetin, ascorbic acid, gallic acid and α-tocopherol used as standards. Higher absorbance indicates higher reducing power. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The concentration providing 0.4 of absorbance (EC_{50}) was calculated from the absorbance at 700 nm.

2.2.5.2.3. Total antioxidant capacity

The total antioxidant capacity of the compounds **13** and **14** were evaluated by the method of Prieto [69]. An aliquot of 0.3 mL of the sample solution (two replicates) was mixed with 1 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The effective concentration of the sample was 50 µg/mL in the reaction mixture. For the blank, 0.1 mL ethanol was mixed with 2.7 mL of the reagent. The absorbance of the test sample was measured at 695 nm. Total antioxidant capacity was expressed as equivalents of ascorbic acid µmole/mg of the compounds.

2.2.5.3. Antiplasmodial assay (*in vitro*)

For antiplasmodial activity the compounds **13** and **14** were tested in duplicate on one occasion against chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10). Continuous *in vitro* cultures of asexual erythrocyte stages of *P.falciparum* were maintained using a modified method of Trager and Jensen [70]. Quantitative assessment of antiplasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase assay using a modified method described by makler [71].

The samples were prepared to a 2 mg/mL stock solution in 10% DMSO and sonicated to enhance solubility. Chloroquine (CQ) was used as the reference drug in all experiments. Compounds were stored at -20°C until use. Test samples were tested at three concentrations which were 10 µM, 5 µM and 2.5 µM. CQ was tested at three concentrations 50 nM, 25 nM and 12.5 nM.

2.2.5.4. Analgesic activity (*in vivo*)

2.2.5.4.1. Animals

To conduct *in vivo* studies, Wistar rats (180–200 g) of both sexes were employed. Animals were kept as 10 per cage under standard and favorable environmental condition with 12 h light dark cycle in addition to their free access to get food and water easily. All the ethical principles as established in 1979 for laboratory animals were strictly followed as described previously[72].

2.2.5.4.2. Formalin test

Formalin test for determining the analgesic activity of test sample was conducted according to the same parameters as reported previously[73]. Briefly, 0.05 ml of formalin solution (2.5% formaldehyde) was administered into the rat hind paw through plantar surface. Initially, animals were treated with various doses of 11-*O*-Galloylbergenin (20, and 40 mg/kg i.p.). After 30 minutes, behavioral responses detected were recorded as scores gained in the following approach: rat either walking or can stand on its injected paw, 0; paw being partially raised, 1; complete elevation of the injected paw, 2; licking or biting of the injected paw, 3. The first phase of analgesia comprised the scores recorded during first 10 minutes after injecting formalin solution. While the late phase of analgesia included the later period between 15 and 60 minutes formalin injection.

2.2.5.5. Antiinflammatory activity (*in vivo*)

2.2.5.5.1. Carrageenan induced oedema

This method was utilized to assess the antiinflammatory potential of test sample via testing its ability to inhibit the carrageenan-induced hind paw oedema[74], as reported earlier[72]. Test samples and the control samples were administered orally in groups to rats. After I hour, acute inflammation at desired site was induced by subplantar injection of 1% suspension of carrageenan (0.1mL) using 2% gum acacia as a suspending agent in normal saline, in the right hind paw of the rats. The paw volume was measured plethysmometrically (Ugo Basile, Italy) at '0' and 3 h after the carrageenan injection. Aspirin 100mg/kg, p.o. suspended in 2% gum acacia was used as positive control. Percent inhibition of the inflammation was determined by applying statistics on raw data followed by the calculation of percent inhibition for each group by comparing with control group. The formula used for comparison was: $\%I=1-(dt/dc)\times100$, where "dt" is the difference in paw volume in the drug-treated group and "dc" is the difference in paw volume in control group. However, "I" stands for inhibition of inflammation.

2.2.5.6. Anti ulcer activity (*in vivo*)

2.2.5.6.1. Absolute ethanol induced ulcer

All the test samples including controls were administered to the rats in groups. The test samples included saline 10 ml/kg body wt.; carbenoxolone 180 mg/kg body wt.; Gal 20, and 40 mg/kg body wt.). One ml of the absolute ethanol was given orally to each animal after 40 minutes of initial administration of the treatments as according to the method[75]. All the animals were sacrificed after 1.5 hours by cervical displacement. Their stomach were separated and opened from the greater curvature. The ulcerative lesion index for all the rats and treatments was determined according to the method[76].

.2.2.5.6.2. Data analysis

Results of the study were expressed as mean ± S.E.M Student *t*-test was used to analyse data between groups and analysis of variance (ANOVA) among groups followed by Dunnet's test for multiple comparisons. $P < 0.05$ was considered significant in all cases.

2.3. Results and Discussion

2.3.1. Antioxidant activity (*invitro*) of the crude fractions of *Mallotus philippensis* fruit and bark

The content of total phenolics in extracts of *M. philippensis* ranged from 27 mg/g (MP-5) to 541 mg/g (MP-4) **Table-2.4**. The highest content was found for the extract prepared from the bark. The washing of fruits with acetone effectively removed a considerable amount of the phenolics. The residue of phenolics in fruits after acetone extraction was found to be very low. The MP-5 possessed only 27 mg of total phenolics per gram. The majority of the phenolics were extracted from the crushed fruits into acetone. The yield of fraction MP-1 was much higher than those for fractions MP-2 and MP-3. (**Fiigure-2.6** provides the informations of each fraction in section **2.2.3.1**)

Table-2.4: Total phenolics contents and condensed tannis in *Mallotus philippensis* extracts, their total antioxidant capacity and spectral data

Extracts	Total phenoloics* (mg/g)	Tannins (A_{500}/g)	Total antioxidant activity (mmol Trolox/g)	λ_{max} (nm)	λ_{sh}†
MP-1	142 ± 4^{A}	-	1.54 ± 0.05^{A}	286;298;344	-
MP-2	50 ± 1^{B}	-	0.38 ± 0.01^{B}	284	346
MP-3	59 ± 1^{C}	-	0.20 ± 0.01^{C}	278	-
MP-4	541 ± 13^{D}	0.412 ±0.009	5.27 ± 0.01^{D}	280	312
MP-5	27 ± 1^{E}	-	0.05 ± 0.01^{E}	-	266
MP-6	133 ± 4^{F}	-	1.79 ± 0.05^{F}	298	282; 340

* Reported as (+)-catechin equivalents.
† Shoulder: averages followed by different letters within the same column are significantly different at $P < 0.05$.

The content of total phenolics in fraction MP-1 was two times higher than those in MP-2 and MP-3. The content of total phenolics in the bark of *M. philippensis* (51.9 mg/g) was six times higher than that of fruits (**Table-2.5**). The extracts MP-1, MP-4 and MP-6 possessed higher contents of total phenolics than extracts of such plant material as beach

pea, fava beans, canola hulls, evening primrose [77], pea [78], vetch [79], rapeseed [80], wheat [81], rye [82] and triticale [83].

Table-2.5: Total phenolics contents, total antioxidant capacity and condensed tannins in *Mallotus philippensis* fruits and bark

Material	Total phenoloics* (mg/g)	Tannins (A_{500}/g)	Total antioxidant capacity (mmol Trolox/g)
Fruits	8.46 ± 0.04	-	0.088 ± 0.005
Bark	51.9 ± 13	0.046 ± 0.001	0.506 ±0.010

* Reported as (+)-catechin equivalents; results were calculated from the data of MP-1, MP-2, MP-3 and MP-4 extracts and yield of extraction

The acetone extract from the bark (MP-4) was found to be a rich source of condensed tannins. Other extracts did not give a positive reaction with the vanillin/HCl reagent **(Table-2.4)**. The content of tannins in MP-4, expressed as absorbance units per mg at 500 nm, was found to be 0.412. This value was much higher than those determined in extracts of leguminous seeds (i.e., from 0.006 absorbance units/mg at 500 nm for broad bean to 0.136 absorbance units/mg at 500 nm for adzuki bean) [84]. The content of condensed tannins in crude extracts of canola and rapeseed hulls ranged from 0.007 to 0.286 absorbance units/mg at 500 nm [85]. Much higher amounts were reported for tannin fractions separated from the crude extracts of beach pea, evening primrose and fava bean using Sephadex LH-20 column chromatography: 0.684, 1.328 and 3.394 absorbance units/mg at 500 nm, respectively [77].

The UV spectra of MP-1 and MP-6 extracts were similar and characterized by maxima at 286, 298 and 344 nm (MP-1), and 298 nm (MP-6) **(Table-2.4)**. The spectra of MP-6 possessed additional shoulders at 282 and 340 nm. Maxima at 284 and 288 nm were noted for MP-2 and MP-3 extracts. The spectrum of the MP-5 extract showed only a shoulder at 266 nm. The MP-4 extract of *M. philippensis* bark exhibited a maximum at 280 nm, which originated from condensed tannins, and a shoulder at 312 nm. Similar UV spectra were reported for the tannin fraction separated from crude extracts of leguminous seeds [78, 91] and almonds [87].

In the method used, the preformed radical cation of 2, 2'-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) was generated by oxidation of ABTS with potassium persulfate prior to the addition of the antioxidant. In the presence of hydrogen-donating antioxidants, the solution of ABTS radical cation decolorizes. The extent of decolorization, measured at 734 nm as percentage inhibition of the $ABTS^{\cdot+}$ solution, was determined as a function of the concentration and calculated relative to the reactivity of Trolox as a standard, under identical conditions[88]. The extract MP-4 obtained from bark exhibited the highest antioxidant capacity of all samples tested (5.27 mmol Trolox/g) (**Table-2.4**). The total antioxidant capacity of bark (0.506 mmol Trolox/g) was ca. 6 times higher than that of the fruits (**Table-2.5**). The extract MP-1 obtained from the fruits using acetone and the extract MP-6 obtained by washing fruits with acetone also exhibited strong total antioxidant capacity of 1.54 and 1.79 mmol Trolox/g, respectively. The capacities of extracts MP-2, MP-3 and MP-5, which were prepared from fruits using methanol, methanol/water and water as solvents after acetone extraction, were much weaker: 0.38, 0.20 and 0.05 mmol Trolox/g. According to the data presented in **Table-2.4**, acetone is a suitable solvent for extraction of the antioxidant constituents from *M. philippensis* fruits. The antioxidant capacity of MP-1, MP-4 and MP-6 extracts proved to be higher or similar when compared with those of plant species used as human food. Characterized by TEAC, the extracts of leguminous seeds ranged from 0.30 mmol Trolox/mg for pea to 1.76 mmol Trolox/mg for adzuki beans[84, 89] reported on methanolic extracts of wheat, barley, rye and oat; the total antioxidant activity in these material ranged from 0.054 to 0.222 mmol Trolox/mg. TEAC of almond extract was 0.24 mmol Trolox/mg[87].The RSA of prepared *M. philippensis* fruits and bark extracts was examined using the DPPH· radical. Freshly prepared DPPH· solution exhibits a deep purple color with an absorption maximum at 517 nm. This color disappears when an antioxidant is added to assayed solution. **Figure-2.7** depicts the concentration-dependent response curves for the RSA of *M. philippensis* extracts; results are expressed as the decrease in absorbance of the DPPH· solution at 517 nm. The RSA of the extract from bark (MP-4), which contained the highest amount of total phenolics, was the highest and thus far superior to any of the other extracts investigated. A scavenging effect was noted when MP-4 was used in the range from 0.01 to 0.05 mg/assay. The extract MP-5 exhibited no

activity. However, the RSA for other extracts was observed for addition level of 0.04 and 0.2 mg/assay. Their RSA was in the order MP-6 > MP- 1 > MP-2 > MP-3. The RSA of MP-4 extract was similar to that of the extract from the leaves of bearberry (*Arctostaphylos-uva-ursi*), which is also a rich source of condensed tannins[90].

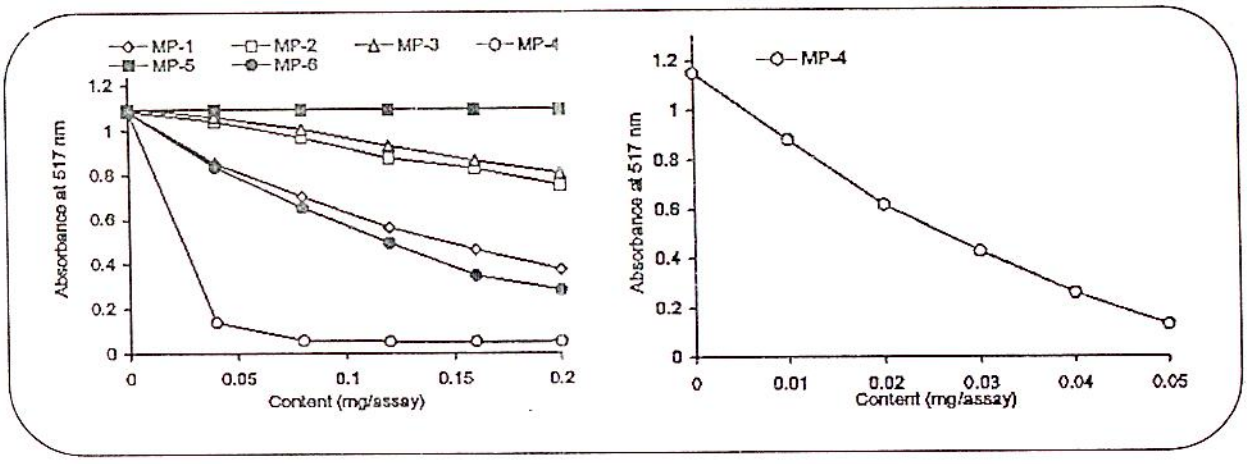


Fig.2.7[a,b]: Scavenging effect of *Mallotus philippensis* fruits and bark extracts on the 2,2-diphenyl 1-picrylhydrazyl radical as measured by changes in absorbance at 517 nm

The RSA of green and black tea extracts reported by[91] was weaker than that of MP-4 and similar to those of MP-6 and MP-1. A strong antiradical activity of tannin extracts against DPPH· has been reported in several studies[92, 93, 77,85,94,78].

The antioxidant and antiradical properties of plant bark were evaluated in several studies. The addition of pine (*Pinus massoniana*) bark extract to a linoleic acid emulsion acted as a strong inhibitor of the peroxidation process [95]. Procyanidins obtained from the same source were found as the scavengers of free radical species[96, 97, 98] noted the inhibition of lipid peroxidation in liposomes, induced by Fe^{2+}/ascorbate system by *Larus nobilis* bark extract. The methanol extract from *Yucca periculosa* demonstrated scavenging properties toward 2, 2, diphenyl-1-picrylhydrazyl in TLC autographic and spectrophotometric assays [99]. The extract of cat's claw (*Uncaria tomentosa*) bark was an effective scavenger of DPPH [100, 101]reported the extract from the dried bark of *Betula platyphylla* var. *japonica* as a strong DPPH scavenger (IC_{50} 2.4 mg/mL) and lipid peroxidation inhibitor (IC_{50} below 4.0 mg/mL). **Figure-2.8** depicts the reducing powers of the prepared extracts.

In this assay, the presence of reductants (i.e., antioxidants) in the extracts causes reduction of the Fe^{3+}/ferricyanide complex to the Fe^{3+} state. Therefore, it can be monitored by measuring the formation of Perl's Prussian blue coloration having a maximum absorbance at 700 nm. The reducing power of MP-4 was far superior to that of any other extracts investigated. In fact, the MP-4 extract was ca. 5 times superior to those of the next most powerful extracts MP-1 and MP-6. The weakest reducing power was noted for MP-2 extract. The reducing power of *M. philippensis* bark extract was higher than that of tea extracts.[102] reported that the reducing powers of green, pouchong, oolong and black tea extracts were 2.47, 2.38, 2.75 and 1.32 at a 1-mg dose. The strong reducing power of condensed tannins extracts in this study was similar to those for beach pea, canola hulls, evening primrose, pea, red bean and blueberry leaves[102,93,77,78,86,94]. The extract of leaves of bearberry (*Arctostaphylos-uva-ursi*) with high content of condensed Tannins and gallotannins were characterized by 2.26 absorbance units at a 1-mg dose[90].

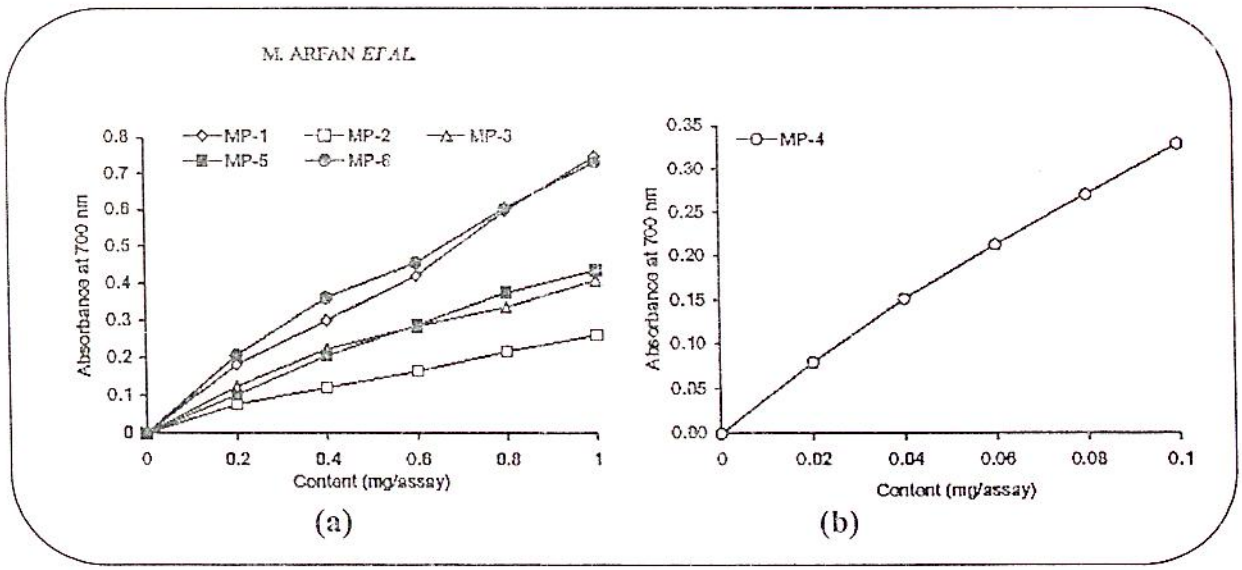


Fig.2.8: [a, b] Reducing power of *Mallotus philippensis* fruit and bark extracts as measured by changes in absorbance at 700 nm

The reversed-phase HPLC chromatogram of the extract of *M. philippensis* bark was characterized by one dominant peak with a retention time of 12.45 min (**Fig. 2.9**; peak 4). The main phenolic compound from the *M. philippensis* fruits was recorded with a retention time of 41.35 min (**Fig. 2.9**; peak 3). The presence of this compound was noted

in MP-1, MP-2, MP-3 and MP-6 extracts obtained from the fruits using acetone (MP-1 and MP-6), methanol/ water (7:3, v/v) (MP-2) or methanol (MP-3). The polar compounds present in extracts MP-2, and MP-3 exhibited HPLC peaks 1 and 2 with retention time of 10.85 and 13.65 min.

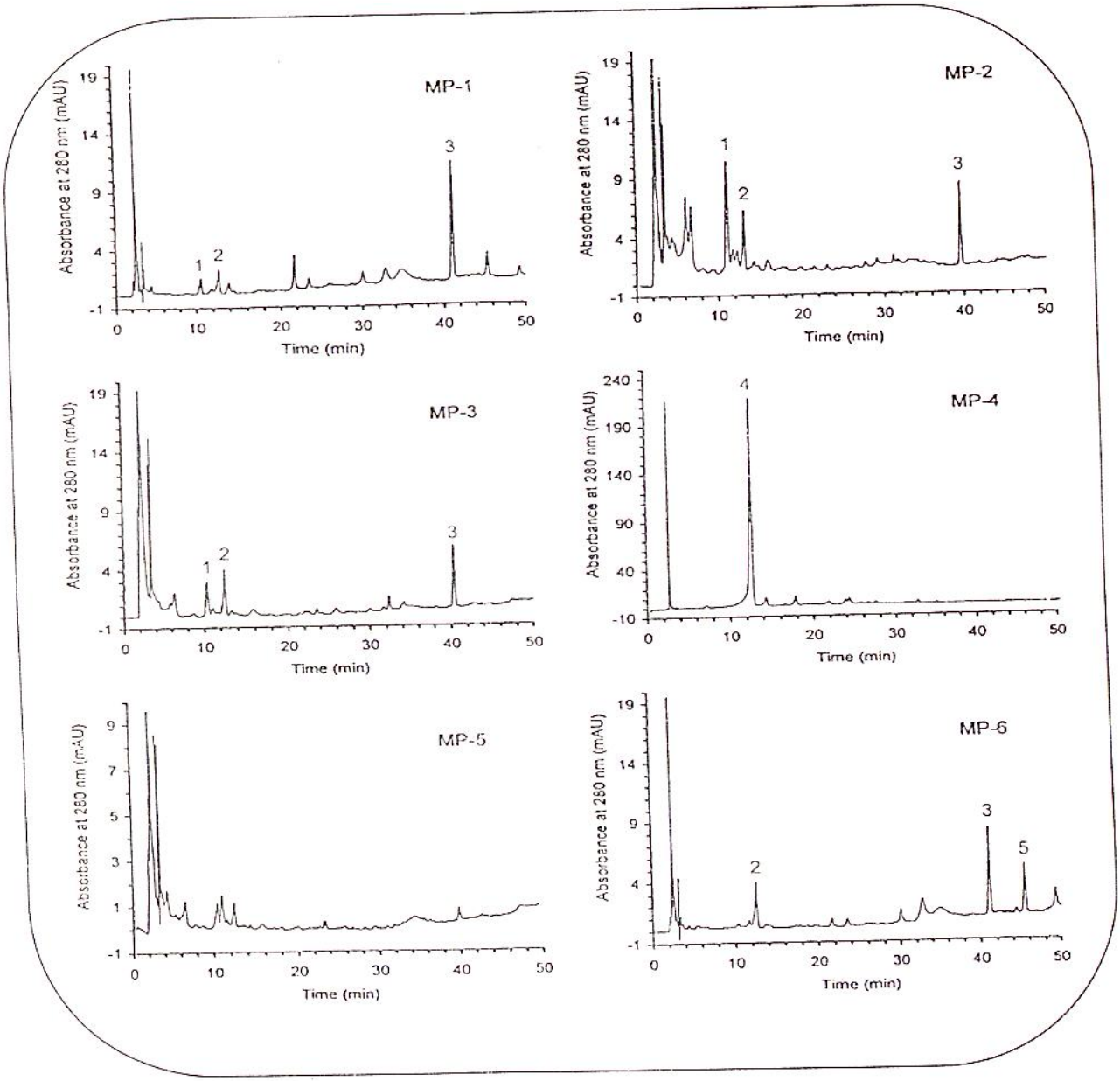


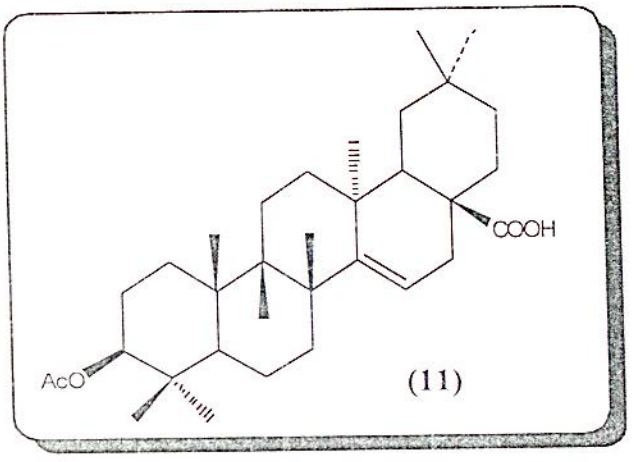
Fig.2.9. High-performance liquid chromatography chromatograms of constituents of *Mallotus philippensis* fruit and bark extracts

An additional peak 5 with a retention time of 45.78 min was noted in the chromatogram of the MP-6 extract obtained by washing of the fruits with acetone. Compounds giving peaks 1 and 2 on the chromatograms from fractions MP-1, MP-2 and MP-3 were characterized by maxima at 268 and 262 nm, respectively. These compounds did not belong to phenolic acids as their UV spectra displayed maxima at 310–330 nm[104]. They can be derivatives of isoflavones; e.g., the UV spectra of 5, 7-dihydroxyisoflavone, 5,7-dimethoxyisoflavone and 2-carboxy-5,7-hydroxyisoflavone showed maxima around 260 nm [105]. Only the small peak in the aromatic region was recorded on the UV-DAD spectra originating from compounds giving peaks 3 and 5. The main phenolic compound present in *M. philippensis* bark extract showed a spectrum with maximum at 264 nm and shoulder at 312 nm. This compound could be a derivate of bergenin, which is the main component of the bark of *M. japonicas* [106]. UV spectra of bergenin are characterized by a maximum at 275 nm[107]. The antiradical activity of bergenin against DPPH radical was noted by Olajide[117].The TLC chromatogram of *M. philippensis* bark extract showed the presence of (–)-epicatechin. In addition, the high polymeric condensed tannins were visible using a vanillin spray reagent. They were more polar than (–) - epigallocatechin gallate (EGCG) and procyanidin B2, and gave on a TLC chromatogram a spot on a start line. On the chromatogram of the *n*-butanol/HCl hydrolysate from the *M. philippensis* bark, the peak with retention time of 5.43 min was recorded. The chemical structure of this compound was similar to that of anthocyanidins. At the same conditions of HPLC analysis, standards of delphinidin, cyaniding and malvidin gave the peaks with retention times of 4.76, 7.29 and 15.72 min, respectively. The absorption spectra of compounds from bark hydrolysates, delphinidin, cyanidin and malvidin were characterized by maxima at 525, 530, 523 and 534 nm, respectively.

The EI-MS spectrum in positive mode of the compound separated from the *M. philippensis* barks using a semipreparative HPLC showed a $[M + H]^+$ at an *m/z* of 479 and dominant peaks of 303 and 304 where the glucuronic acid moiety was cleaved from the glycoside. Mass spectra indicated that the compound has a molecular weight of 478. Molecular weight of the aglycone was calculated as 303. Most probably, this compound was a derivative of bergenin with a molecular weight of 328[108]. This work has also been published [108a].

2.3.2. Characterization of isolated compounds

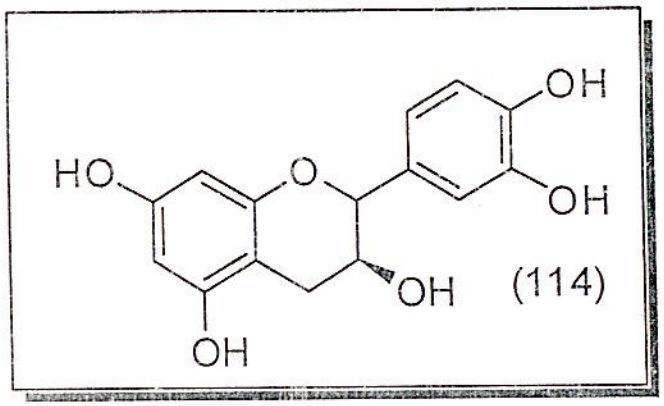
2.3.2.1. 3-Acetylaleuritolic acid (11)



(11)

Compound (11) isolated as a colorless needles crystals and was analysed as $C_{32}H_{50}O_4$ from the mass spectrum data EIMS. The IR spectrum showed strong absorption bands at 1730 and 1240 cm^{-1} for the presence of carboxylic acid group and trisubstituted double bond. ^{1}H-NMR spectrum revealed a characteristics singlet at δ 2.03 assigned to acetoxy protons, a doublet by doublet centered at δ 5.52 (J =8 and 4 Hz) indicating a proton on thr trisubstituted double bond adjacent to methylene fraction.Another doublet by doublet resonated at δ 4.46 (J = 10 and 4 Hz) assigned to the ubiquitous 3 alpha methane protons germinal to the acetoxy function of triterpene skeleton.Seven tertiary methyls appeared as singlets in the region at δ 0.84-0.97. This data suggests that the compound (11) is a mono acetoxy pentacyclic triterpene mono carboxylic acid with a trisubstituted double bond[109].

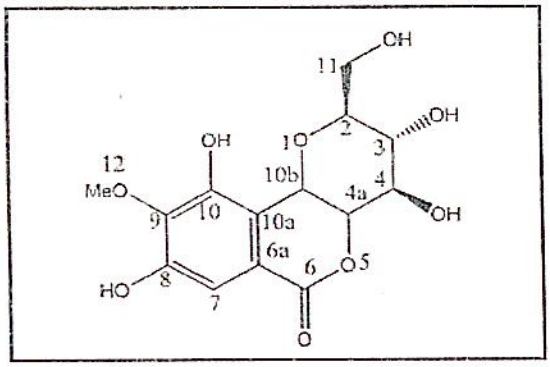
2.3.2.2. (+)-Catechin (12)



(114)

Compounds **12** gave a coloured reaction (red) with the ceric sulphate spray and its UV spectrum in methanol revealed the presence of the flavan-3-01 skeleton. The 'H-NMR spectrum confirmed the above suggestions and evidenced the presence of the (+)-catechin **12**. It spectrum was very similar in the aromatic region, the ABX system for ring B were in accordance with a 3',4'-dihydroxylation while the chemical shifts and the coupling constants of H-6 and H-8 indicated that **12** was 5,7-dihydroxylated. In addition, the chemical shifts and *J* couplings of the signals of H-2, H-3 and H_Z-4 clearly evidenced the orientation of the 3-hydroxyl group. The ^{13}C-NMR spectrum provided the above conclusions and showed the above structure, as reported in the literature[110].

2.3.2.3. Bergenin (13)

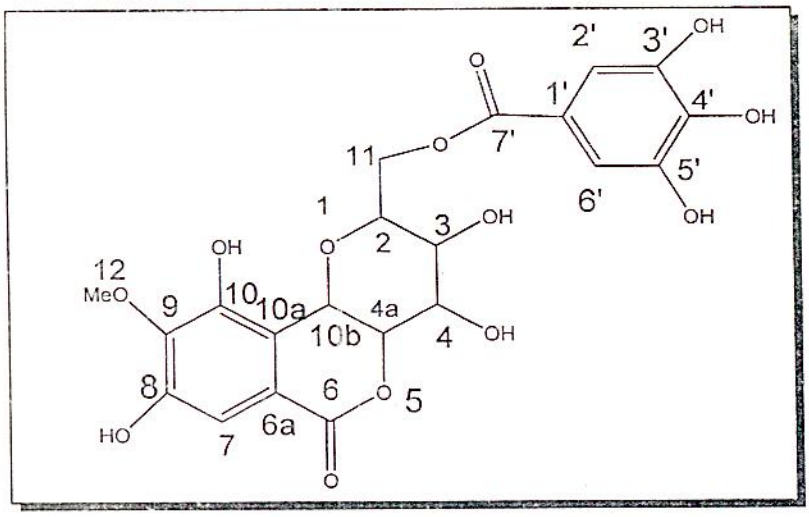


(13)

Compound 13 was obtained as white crystals. The mass spectrum data of the compound 13 gave a molecular ion peak at *m/z* 328 lead to formula $C_{14}H_{16}O_{9,}$ The melting point of 237°C is consistent with the published melting point of bergenin 238°C. The ^{1}H-NMR spectrum showed a signal for one aromatic proton at δ 6.97 (1H, s) and a signal for methoxy protons at δ 3.8 (3H, *s*).

In the ^{13}C NMR spectrum the signals were appeared at δ 163.5 and 60.0 for carbonyl and methoxy grou ps respectively[111].

2.3.2.4. 11-*O*-Galloylbergenin (14)



(14)

Compound **14** was interpreted and analysed as $C_{21}H_{20}O_{13}$ to be monogalloyl ester of bergenin by their 1H-NMR, ^{13}C-NMR and EIMS spectral data, and also by acid hydrolysis, which gave bergenin and gallic acid. The position of the galloyl group in one was established by ^{13}C-NMR spectrum and 2D techniques.The carbon signals other than that of C-11 in the glucose moiety of bergenin, which have not been previously assigned, have now been assigned as follows. The carbon carrying free hydroxyl group (C-3, C-4 and C-11) were unequivocally, distinguished from the others (C-2, C-4a and C-10b) by the deuterium induced differential isotope shift (DIS) measurement [30].

2.3.3. Biological activities of isolated compounds

2.3.3.1. Urease inhibition assay of bergenin (*in vitro*)

Urease is a key enzyme present in many species that catalyzes the hydrolysis of urea to ammonium and carbamate ions, which decompose to carbon dioxide and ammonia. The active catalytic site contains two nickel (II) atoms which are linked by a carbamate bridge; furthermore, two imidazole nitrogen atoms are bound to each nickel atom, and a carboxylate group and a water molecule fill the remaining coordination site of the metal ion[112]. The coordination geometry of the first nickel atom is pseudotetrahedral, while that of second is roughly trigonal bipyramidal. Bergenin displayed potent inhibitory potential having the IC_{50} value of $21.7 \pm 0.2\mu M$ with 68% inhibition against the *B. pasteurii* urease. From molecular docking studies it is evident that bergenin molecule has penetrated deeply into the active site of urease bearing nickel ions preventing the access of urea as substrate to the catalytic site. Principal interactions experienced by the bergenin are hydrogen bonding with the Lys 169 (2.92 Å), Ala 266 (2.71 Å), Arg 339(3.11), Gly 280 (2.47 Å), Asp 363 (2.76 Å) and some hydrophobic interactions with (His 323, Lys 169, His 249, Met 367, His 222 and Ala 366) also stabilized the ligand in the receptor (**Figures-2.10** and **2.11**). This study will be a starting point for further mechanistic studies (e.g. site-directed mutagenesis) of bergenin and its analogs inhibiting the urease enzyme as potential target for their anti-ulcer activities especially against urease producing *H. pylori*. This seems to be a new mechanism involved in its potential anti-ulcer activity against pathogenic GIT ulcers due to urease producing bacteria. Bergenin is established as sufficiently active and safe molecule from previous studies and posses strong potential to be developed as antioxidant and efficient antiulcer drug.

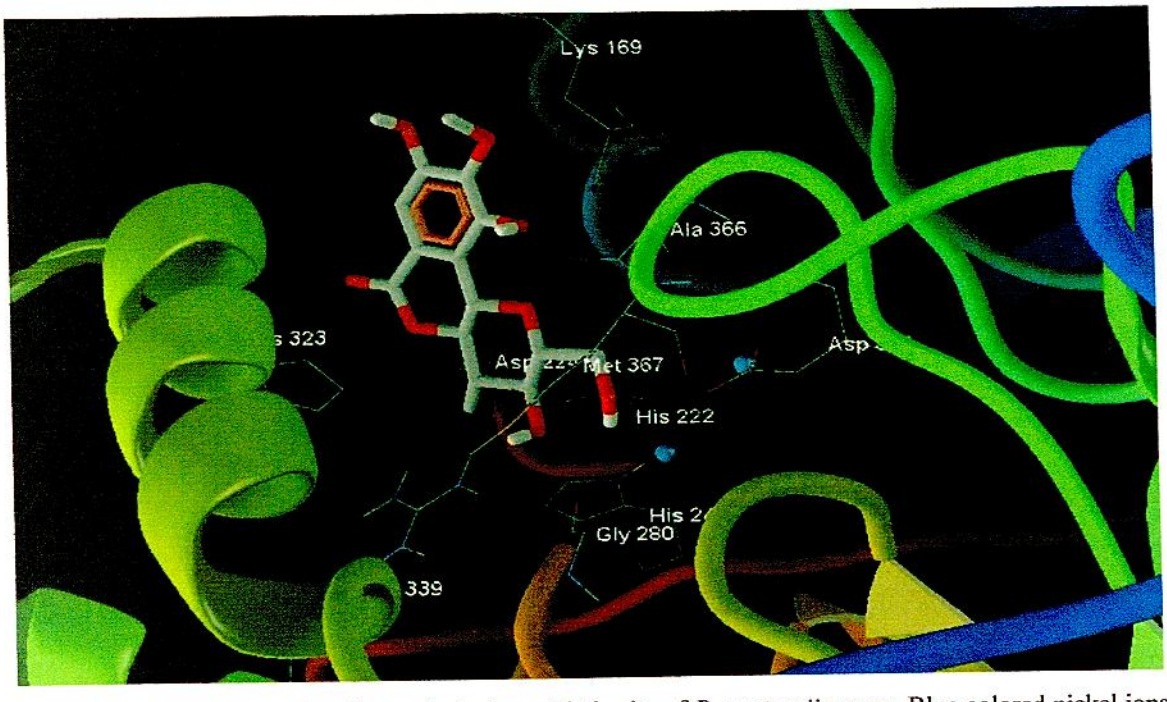


Figure-2.10: Binding mode of bergenin in the catalytic site of *B. pasteurii* urease. Blue colored nickel ions are clearly visible. Hydrogen atoms except polar ones are omitted for clarity.

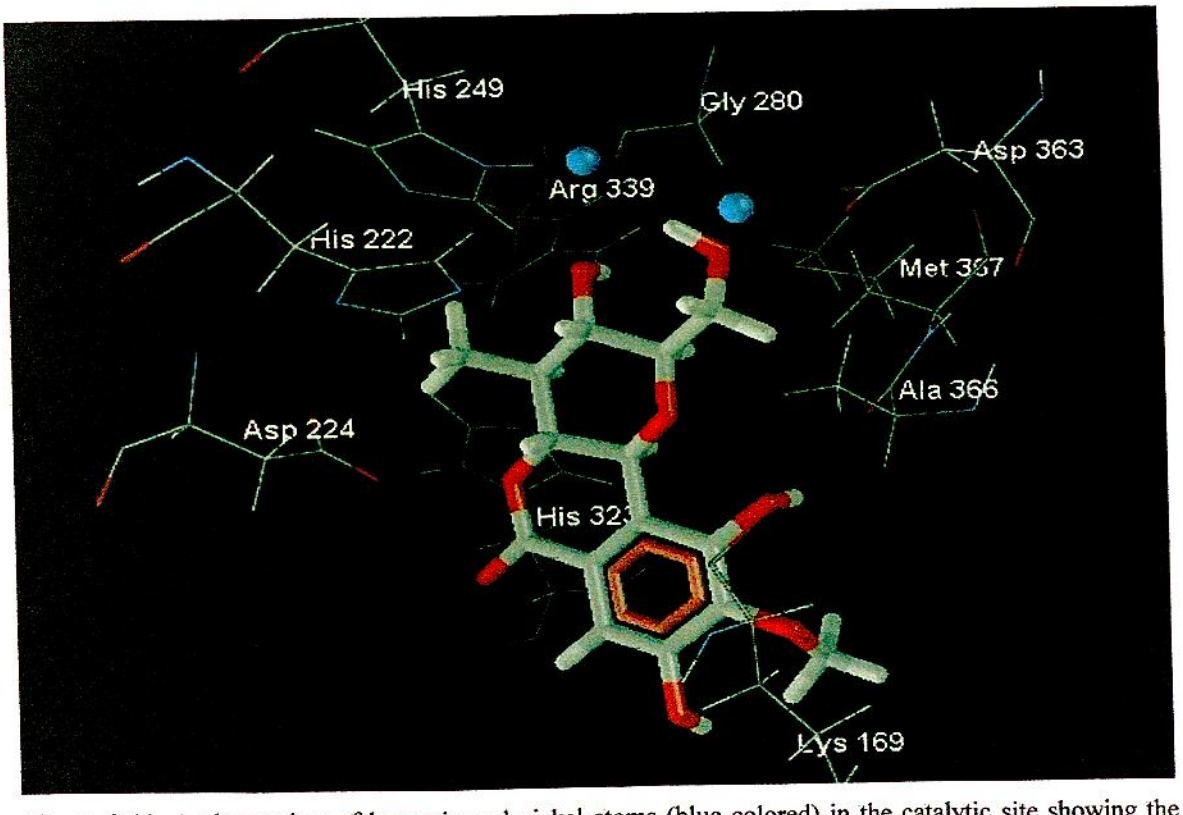


Figure-2.11. A closer view of bergenin and nickel atoms (blue colored) in the catalytic site showing the non competitive behavior due to blocking the access of urea as substrate to the nickel atoms.

2.3.3.2. Comparative antioxidant and antiplasmodial activities of bergenin and 11-*O*-Galloylbergenin (*in vitro*)

The isolated compound bergenin **13** and 11-*O*-Galloylbergenin **14** were characterized by comparing its physical and spectral data with previous literature[30]. 11-*O*-Galloylbergenin was investigated first time from this plant.

In DPPH radical scavenging assay, bergenin and 11-*O*-Galloylbergenin showed 6.44±1.91% and 85.10±0.418% radical scavenging activity at concentration of 100 µg/mL, respectively. The EC_{50} values for 11-*O*-Galloylbergenin in DPPH assay was 7.45±0.2 µg/mL. However the standards were quercetin (98.18 ±0.187 %), ascorbic acid (97.60±0.519%), gallic acid (98.03±0.491%) and α-tocopherol (92.14±0.32%) were found relatively better than the 11-*O*-Galloylbergenin **(Table-2.6)**. The EC_{50} values for standard compounds were 4.19±1.41 µg/mL (quercetin), 6.31±1.03 µg/mL (ascorbic acid), 4.53±0.92 µg/mL (gallic acid) and 32.50±1.58 µg/mL (α-tocopherol) **(Table-2.7)**.

Similarly antioxidant activity in terms of reducing power was found very high for 11-*O*-Galloylbergenin as compared to bergenin. At 25 µg/mL concentration the reducing powers for bergenin, 11-*O*-Galloylbergenin, quercetin, ascorbic acid, gallic acid and α-tocopherol were 0.045±0.003, 1.321±0.035, 1.86±0.031, 3.28±0.045, 1.23±0.024, 21.38±0.069, respectively **(Table-2.6)**. The EC_{50} values for 11-*O*-Galloylbergenin was 5.39±0.28 µg/mL while for standards, these were 1.88±0.031 µg/mL (quercetin), 3.29±0.031 µg/mL (ascorbic acid), 1.23±0.023 µg/mL (gallic acid) and 21.48±0.071 µg/mL (α-tocopherol) **(Table-2.7)**.

Total antioxidant phosphomolybdate assay as ascorbic acid equivalents (CAHT: µmole/mg of test compound), calculated for bergenin, 11-*O*-Galloylbergenin, quercetin, ascorbic acid, gallic acid and α-tocopherol were 48.28±2.46, 940.04±115.30, 2057.51±124.3, 2469.4±149.7, 2176.1±173 and 552.4±27.91, respectively **(Table-2.7)**.

From all the *in vitro* models of antioxidant activities it is evident that 11-*O*-Galloylbergenin is a potent and effective antioxidant especially from CAHT analyisis, it was found that 11-*O*-Galloylbergenin is more active as compared to α-tocopherol. These

promising antioxidant activities of 11-*O*-Galloylbergenin reveals it be a potential lead compound for various pathological conditions associated with the devastating effects of Reactive oxygen species. Furthermore, 11-*O*-Galloylbergenin showed excellent antioxidant activities as compare to bergenin and its synthetic derivatives[113, 108].

Table-2.6: Antioxidant activities of bergenin and 11-*O*-Galloylbergenin

S.No	Sample	[a]DPPH assay % Radical scavenging assay (at100 µg/ml)	[b]Reducing Power (at 25µg/ml)	[c]Total Antioxidant Phosphomolybdate assay as ascorbic acid equivalents (CAHT) (µmole/mg of extract)
1	Bergenin	6.44 ± 1.910	0.045 ± 0.003	48.28 ± 2.46
2	11-O-Galloylbergenin	85.10 ± 0.418	1.321 ± 0.035	940.04 ± 115.30
3	Ascorbic acid	97.60 ± 0.519	3.28 ± 0.045	2469.4 ± 149.7
4	Gallic acid	98.03 ± 0.491	1.23 ± 0.024	2176.1 ± 173
5	Quercetin	98.18 ± 0.187	1.86 ± 0.031	2057.51 ± 124.3
6	A-tocopherol	92.14 ± 0.320	21.38 ± 0.069	552.4 ± 27.91

[a,b,c] The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations

Table-2.7: EC_{50} values[a,b] (µg/ml) in reducing power and DPPH scavenging assays of bergenin and 11-*O*-Galloylbergenin

S.No	Sample	DPPH Radical scavenging assay (EC_{50}[a])	Reducing Power (EC_{50}[b])
1	Bergenin	<100	< 25
2	11-O-Galloylbergenin	7.45 ± 0.2	5.39 ± 0.28
3	Ascorbic acid	6.31 ± 1.03	3.29 ± 0.031
4	Gallic acid	4.53 ± 0.92	1.23 ± 0.023
5	Quercetin	4.19 ± 1.41	1.88 ± 0.031
6	α-tocopherol	32.50 ± 1.58	21.48 ± 0.071

[a] EC_{50} (µg/ml): effective concentration at which 50% of DPPH radicals are scavenged.
[b] EC_{50} (µg/ml): effective concentration at which the absorbance is 0.4.

Compounds 13 and 14 were tested aginst the CQS D10 strain of *P. falciparum*. Both compounds were displayed good activity at low concentration, with IC_{50} values less than 2.5 µg/mL against a value of 12.5 ng/mL for CQ as positive control.

2.3.3.3. Analgesic, Antiinflammatory and Antiulcer activities of 11-*O*-Galloylbergenin (*in vivo*)

The formalin test is considered a model for chronic pain[111], and it is considered as an effective way to measure the inflammatory and non inflammatory pain[115]. In this test, animals presented two distinct nociceptive behavior phases, which probably involved different stimuli. The early phase initiated immediately after formalin injection and lasted about 3–5 min, resulting from chemical stimulation of nociceptors. The late phase initiated 15–20 min after formalin injection, lasted about 20–40 min. The bradykinin was involved in the early phase, while histamine, 5-hydroxytryptamine (5-HT), prostaglandins and bradykinin were involved in the late phase[116]. In this experiment, the analgesic effects of 11-*O*-Galloylbergenin were significant in both the phases.

2.3.3.3.1. Analgesic activity

11-*O*-Galloylbergenin (20mg/kg and 40mg/kg) has significantly reduced the severity of pain as given in **table-2.8**.

Table-2.8: Effect of 11-*O*-Galloylbegenin on formalin-induced noxious pain rats

Treatment	Dose (mg/kg)	Severity of pain	
		Early phase (0-12 min)	Late phase (15-30 min)
Gal 20	20 po	1.4±0.7*	1.2±0.5*
Gal 40	40 po	0.8±0.4*	0.6±0.3*
Aspirin	150	0.2±0.2*	0.2±0.2*
Control	-	2.9±0.4	1.4±0.7

Results are mean±S.E.M. ($n = 4$); $P < 0.05$ vs. control, Student's *t*-test

Carrageenan-induced pawedema as an *in vivo* model of inflammation has been frequently used to assess the antiinflammatory effect of natural products. Carrageenan induced paw edema model is thought to be a biphasic event; in the early phase histamine and serotonin are released, then peak at 180min to release of kinin-like substances, while in the late phase prostaglandins, proteases and lysosymes are released[115]. The obtained results indicated that 11-*O*-Galloylbergenin significantly inhibited the formation of the rat paw edema, in the early and late phases, in fact somewhat more active in the late phase.

2.3.3.3.2. Antiinflammatory activity

11-*O*-Galloylbergenin (10mg, 20 mg and 30 mg/kg) has showed the dose-dependent reduction in carrageenan-induced edema in rats. 20 mg and 30 mg/kg) as revealed in the **figure-2.12**.

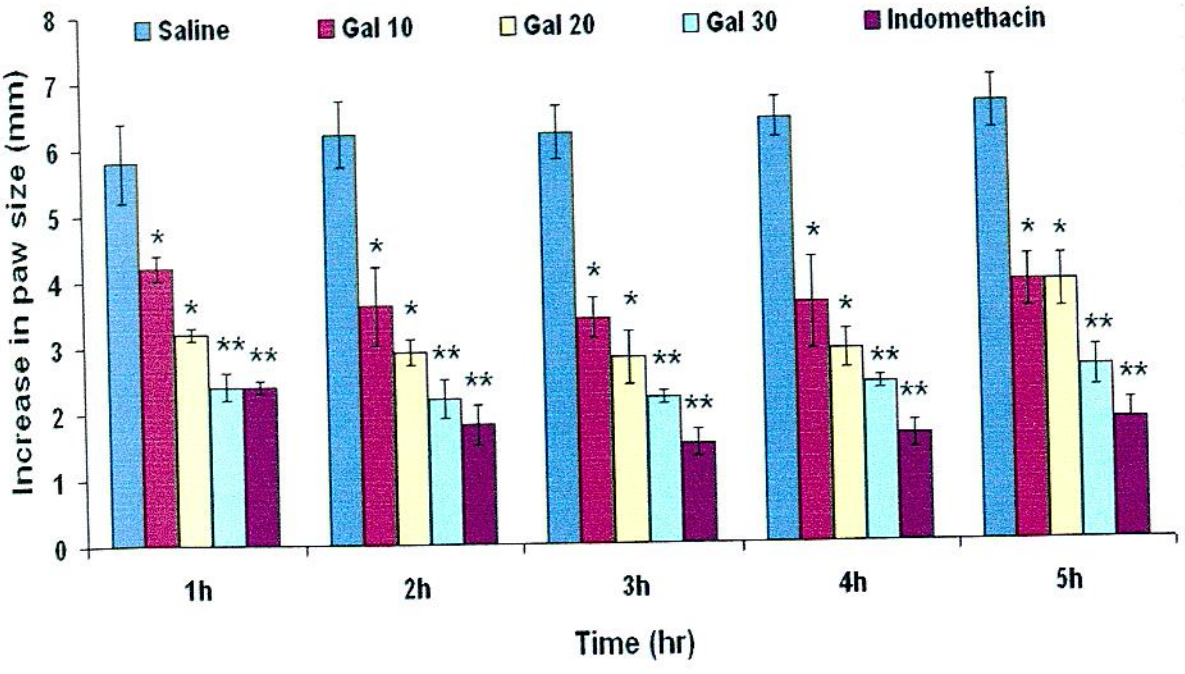


Figure-2.12: (Above) 11-*O*-Galloylbegenin. (Below) Antiinflammatory activity of 11-*O*-Galloylbegenin in carrageenan-induced paw oedema model in rats. ** = $P < 0.01$ vs. control, * = $P < 0.05$ vs. control

Ethanol-induced gastric damage may be due to a direct action of the gastric epithelium causing lipid peroxidation, but this damage can be prevented by prostaglandin administration. Several studies have shown antioxidant activity of flavonoids and another natural compounds[118]. Ethanol treatment induces intracellular oxidative stress and produces mitochondrial permeability transition and mitochondrial depolarization, which precede cell death in gastric mucosal cells. Intracellular antioxidants such as glutathione may have a significant protective action against ethanol in gastric mucosal cells[119]. Ethanol is involved in the formation of free radicals generated extracellularly and/or intracellularly. Because intragastric administration of superoxide dismutase was able to protect the gastric mucosa against the damaging effect of ethanol, this would suggest the involvement of superoxide free radicals in the pathogenesis of ethanol-induced gastric mucosal damage[120, 121].

2.3.3.3.3. Antiulcer activity

In the ethanol-induced ulcer model, used to screen drugs for cytoprotection[75], 11-*O*-Galloylbergenin inhibited the ulcerative lesion index by 77.4 and 88.2% at 20 and 40 mg/kg oral doses, respectively. Results are given in **table-2.9**

Table-2.9: Effect of 11-*O*-Galloylbegenin in absolute ethanol-induced ulcer in rats

Treatment	Route	Dose mg/kg	ULI (± SEM.)	% Inhibition
Gal	Oral	20	34±5.1*	77.45
Gal	Oral	40	18±6.7*	88.2
Carbenoxolone	Oral	100	23±4.6*	84.8
Saline	Oral	-	153±5.9	-

Results are expressed in mean±SEM (n = 4)

In conclusion 11-*O*-Galloylbergenin has the strong potential to be developed as a potential analgesic, antiinflammatory and anti-ulcer lead compound. Extensive study is needed to be conducted and to explore its safety and efficacy. This study has confirmed the folk uses of the plant as a remedy in pain, inflammation and ulcers.

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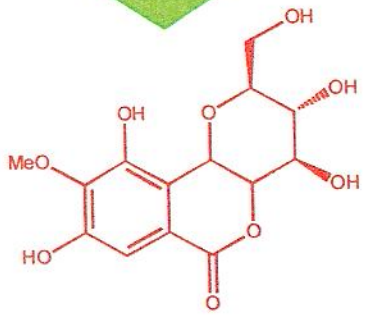
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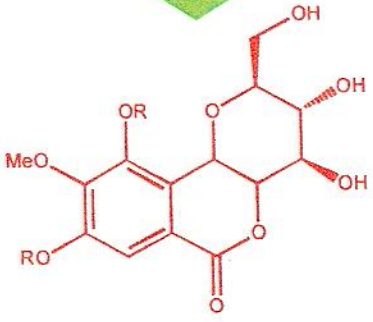
CHAPTER-3

SYNTHESIS OF *O*-ALKYLATED BERGENINS AND EVALUATION OF THEIR BIOLOGICAL ACTIVITIES





Bergenin



O-Alkylated Bergenins

CHAPTER 3 Synthesis of *O*-alkylated Bergenins and Evaluation of their Biological Activities

3.1. Introduction

The synthesis of molecules is an integral part of modern Organic chemistry. The total synthesis of complex natural products are very challenging, but the synthesis of molecules or molecular fragments (natural and synthetic) containing ten carbons or less can also pose great challenges. Examination of many synthetic endeavors, large and small, reveals that the formation of the carbon skeleton through carbon-carbon bond formation requires the most strategic planning. The largest number of actual chemical reactions in a synthesis, however usually involves manipulation of functional groups[1].

Organic synthesis may be categorized broadly into two[2].

1. Total synthesis
2. Method oriented synthesis.

Total synthesis may be of two types.

i. Total synthesis of a natural product.

ii. Total synthesis of a designed molecule.

Method oriented synthesis constitutes the following.

i. New reagent based synthesis.

ii. New catalyst based synthesis.

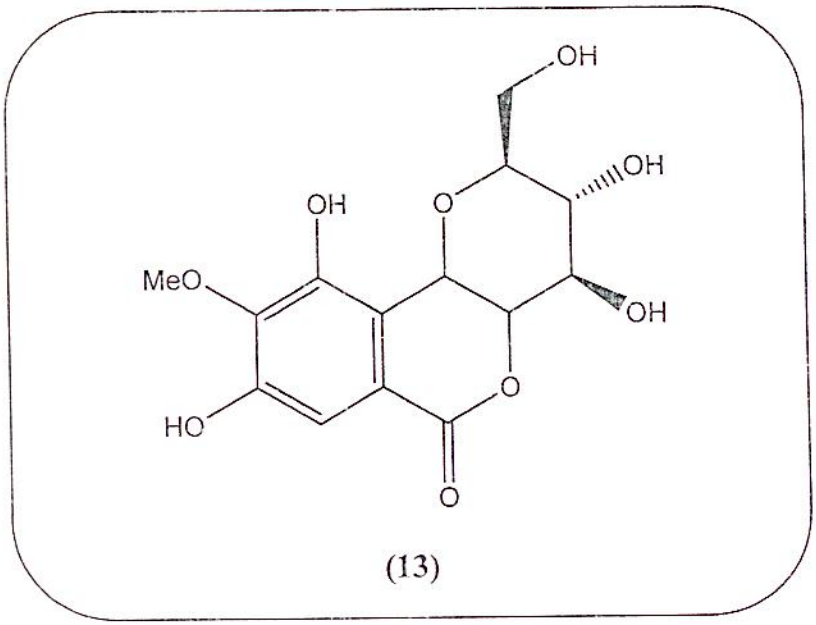
iii. New synthetic strategy based synthesis.

iv. New synthetic tactic based synthesis.

Heterocyclic compounds are cyclic molecules containing one or more atoms other than carbon in their skeleton. These compounds are extensively distributed in nature, and are essential to life in various ways. Most of the sugars and their derivatives, including vitamin C, exist largely in the form of five-membered (furan) or six-membered (pyran) rings containing one oxygen atom. Most members of the vitamin B group possess heterocyclic rings containing nitrogen. For example, vitamin B_6 (pyridoxine) a derivative of pyridine is essential in amino acids metabolism. There are a large number of pharmacologically active heterocyclic compounds, many of which are in regular clinical use. Some of these are natural products, for example, antibiotics such as penicillin and cephalosphorin, alkaloids such as vinblastine and vincristine. However, majority of heterocycles are synthetic in nature and have found widespread uses, for example, as chemotherapeutic agents, drugs, dystuffs and copolymers[3].

3.1.1. Pharmacological activities of bergenin

Bergenin (13) has containg a useful isocoumarin nucleus. Bergenin and the compounds having bergenin type C- glycosides have been disclosed for numerous of biological and pharmacological activities[4]. Bergenin is a gallic acid derived *C*-glycoside that have been isolated from a variety of plants[5]. Bergenin is a bioactive molecule having neuroprotective activity and is the active pharmaceutical ingredients of a Chinese drug described to be effective against cough and bronchitis[6], antitussive[7], hypolipidaemic[8], anti-HIV[9], antiarrhythmic[10], antiinflammatory[11], PTP1B inhibitory activity[12], antimalarial [13] and gastroprotective [14] activities. The exact molecular mechanism is still not clear for these activities. Other important pharmacological activities include inhibitory effect on platelet aggregation and its antioxidant activities are well established and interestingly in one study it was found be as potent antioxidant as ascorbic acid or quercetin[15, 16], inhibitory effect on bovine adrenal tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamine[17]. Its hepatoprotective activity is studied *in vitro* and *in-vivo* in various models such as carbon tetrachloride-intoxicated rats [18] and *D*-galactosamine-induced hepatotoxicity in rats[19]. The antifungal activities of bergenin against seven pathogenic strains were reported[20].



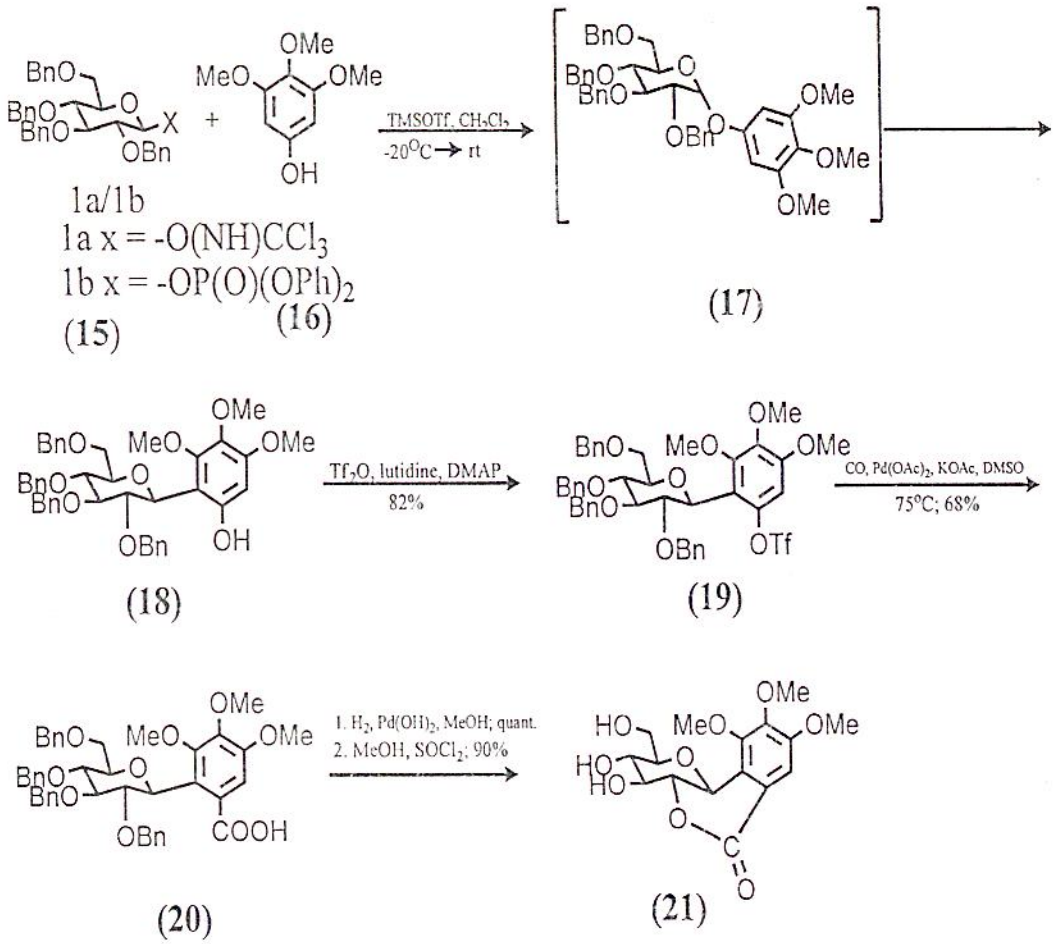
(13)

3.1.2. Synthesis of bergenin scaffold

An efficient method for the synthesis of berginin scaffold was reported by H. Holger *et. al.* This method involves five steps as described in **scheme-3.3.**

Upon activation with trimethylsilyl trifluouromethanesulphonate (TMSOTf), 2,3,4,6-tetra-*O*-benzyl glucopyranosyl trichloroacetimidate (**1a**) and prebenzylated glucosyl diphenyl phosphate (**1b**) react with 3,4,5-trimethoxyphenol to give the β-configured *C*-glycoside (**18**) in 67% and 57% yields, respectively. Then treatment of the phenolic hydroxyl group of (**18**) with triflic anhydride/lutidine resulted in the formation of triflate (**19**). Due to steric hindrance of this hydroxyl group the addition of DMAP as an acylation catalyst proved to be essential. Palladium (0)-catalyzed carbonylation at ambient pressure under careful exclusion of water and oxygen yielded 68% of *C*-glucosyl benzoic acid derivative (**20**). Debenzylation of (**19**) by hydrogenation with pearlman's catalyst in methanol provided the tetrahydroxyl *C*-glucoside (**21**) in quantitative yield. Regioselective lactonization of the C2 hydroxyl group of the glucose scaffold was achieved by treatment with DCC/DMAP in DMF to give (**21**) in 71% yield. A more

efficient method involves the treatment of deprotected (**20**) with $SOCl_2$ in methanol to provide cleanly 8, 10-di-*O*-Methylbergenin in 90% yield.



Scheme-3.3: Synthesis of bergenin scaffold

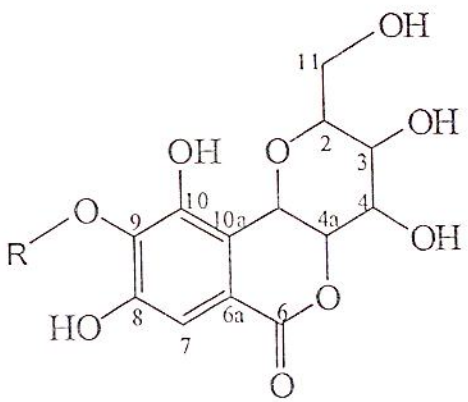
The application of this synthetic route to the preparation of a series of bergenin derivatives is currently under examination[4].

3.1.3. Literature review on bergenin

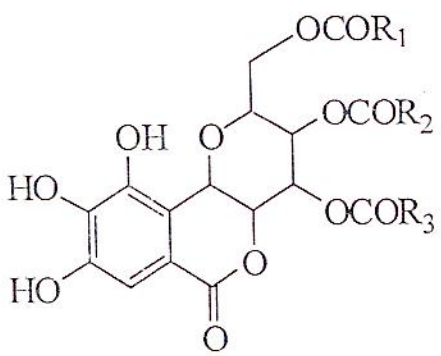
Due to the enormous importance of Bergenin and its derivatives in pharmacological usefulness much attention has been focused on their synthetic derivatives and pharmacological activities.

Holger Herzner *et al*, synthesized 8,10-*D*-*I*-*O*-Methylbergenin **(32)** in five steps. They used the route of stereoselective installation of a *β*-*C*-aryl linkage, a palladium (0)-catalyzed aryl carbonylation and a regioselective lactonization reaction. This pathway should allow access to a host of bergenin analogues[4].

Hironobu Takahashi *et al*, modified bergenin **(13)** to norbergenin **(22)** by acetylation with acetic anhydride and deprotection of the *O*-methyl group with BCl_3 followed by hydrolysis with K_2CO_3 in 71% over three steps. They synthesized a series of **(23-31)** norbergenin derivatives (**fig-2.14**). The three bergenin derivatives **(22)**, **(27)** and **(31)** showed strong antioxidant activity than other. These derivatives were also evaluated for neuroprotective effect which showed this activity[6].



R = Me (13)



Norbergenin (22)

$R_1 = R_2 = R_3 = CH_3$ (23)

$R_1 = R_2 = R_3 = (CH_2)_2\,CH_3$ (24)

$R_1 = R_2 = R_3 = (CH_2)_4\,CH_3$ (25)

$R_1 = R_2 = R_3 = (CH_2)_6\,CH_3$ (26)

$R_1 = R_2 = R_3 = (CH_2)_{14}\,CH_3$ (27)

$R_1 = R_2 = R_3 = (CH_2)_{16}\,CH_3$ (28)

$R_1 = (CH_2)_4\,CH_3$, $R_2 = H$, $R_3 = H$ (29)

$R_1 = H$, $R_2 = (CH_2)_4\,CH_3$, $R_3 = H$ (30)

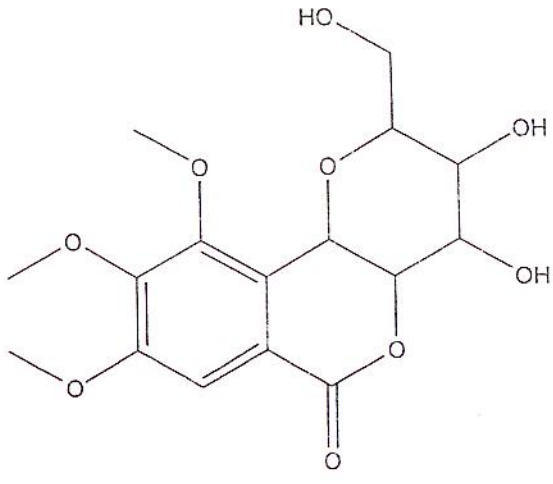
$R_1 = H$, $R_2 = H$, $R_3 = (CH_2)_{14}\,CH_3$ (31)

Fig.- 2.14. Synthesis of norbergenin derivatives

Piegen. X, also reorted the antitussive effect of bergenin [7]. Jahromi *et al*, explored the Hypolipidaemic activity of bergenin in rates isolated from *Flueggen microcarpa*[8].

Sonia piacente *et al.* isolated the bergenin from *Ardisia japonica* and was changed to norbergenin by chemical transformation. Both the compounds were tested for their *in vitro* anti-HIV activity. They showed weak anti-HIV activity[9].

Swarnalakshmi *et al*, reported that bergenin has antiinflammatory effects[11]. Yan-Fang Li *et al*, isolated the bergenin (13) and norbergenin (32) from *Ardsia japonica*[12].



(32)

T.B.C. DA Silva *et al*, determined the *in vitro* as well as *in vivo* anti-malarial assays for bergenin. It was found inactive against *P.falciparum* and weakly active against *P. berghei* (IC_{50} 146.87 mg/kg). These results suggest bergenin is exerting an antimalarial effect by a mechanism other than a dried antiplasmodial action. As the *P. berghei* mouse model does not show an immune component, it is possible that these substances may be converted *in vivo* to an active metabolite (pro drug)[13].

Nighat Nazir *et al,* isolated bergenin from rhizomes of *Bergenia stracheyi* and its *O*-demethylated derivative norbergenin prepared from bergenin. Both the compounds showed the immunomodulatory effect against adjuvant- induced arthritis[15].

Prithiviraj, B. *et al,* reported the antifungal activity of the bergenin[20]. Zhuo chem. *et al,* isolated bergenin from *Saxifrage stolorifera* and studied their inhibitory effect on the tumor cells BGC-823 by MTT assay. However, it did not show significant growth inhibition activity[21].

R.Srinivasan *et al,* reported the antioxidant activity of the crude extract of *Caesalpinia digyna* root and bergenin, isolated from it. Crude extract showed excellent activity while bergenin showed moderate antioxidant activity in all the *in vitro* as well as *in vivo* models[22].

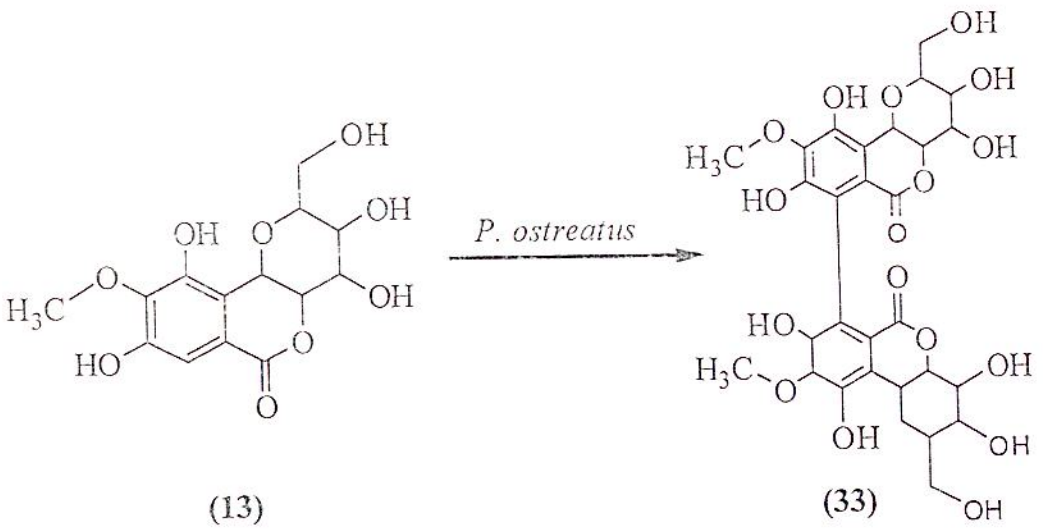
Qian Zhuang *et al,* investigated the electrocatalytic oxidation of bergenin on the surface of the multi-wall carbon nanotubes (MWCNT) modified carbon paste electrode (CPE) using cyclic voltammetry ((CV) and differential pulse votammetry (DPV). The modified electrode showed an excellent electrocatalytic activity in lowering the anddic overpotential and remarkable enhancement of the I_{pa} of bergenin if compared with the electrochemical performances obtained at CPE. The prepared modified electrode is very stable and can be used for at least 3 weeks[23].

Jinghua Chen. *et al,* employed a very sensitive electroanalytical method to determine bergenin in phosphate buffer with a pH of 6.0, bergenin was accumulated at a 4-(2-pyridylazo)-resorcinol (PAR) polymer film modified glassy carbon electrode (GCE)

surface under the condition of open circuit. In the following anodic sweep from -0.4 to 0.8 V, bergenin adsorbed at the PAR polymer film modified electrode surface, was oxidized and yielded a sensitive oxidation peak at 0.595 V. Due to its unique structure and extraordinary properties, the PAR polymer film shows higher accumulation efficiency towards bergenin compared with a bare GCE. Hence, the amount of bergenin at the PAR polymer film modified GCE surface increase significantly, and finally the oxidation peak current improves greatly. This method was successfully demonstrated with bergenin tablets and diluted urine[24].

Zamarud *et al*, Ye *et al*, caldas *et al*, Frick *et al;* extensively studied the bergenin and its analogues through X-ray crystallography[25, 26, 27, 28]. Gabriel N. Folefoe *et al;* reported bergenin from *pentaclethra macrophylla* [29].

Dong wang *et al,* coupled the carbon-carbon atoms of the bergenin compound to form a dimeric bergenin derivative by the way of biotransformation with the help of *Pleurotus ostreatus*. It was noted that the dimeric bergenin derivative **(33)** showed double *in vitro* antioxidant activity as compared to bergenin[30].



(13) (33)

Wantana Reanmongkel *et al,* performed the anti-allergic and antioxidative activities of bergenin along with other compounds isolated from Thai medicinal plants. In both the activities bergenin showed no prominent result[31].

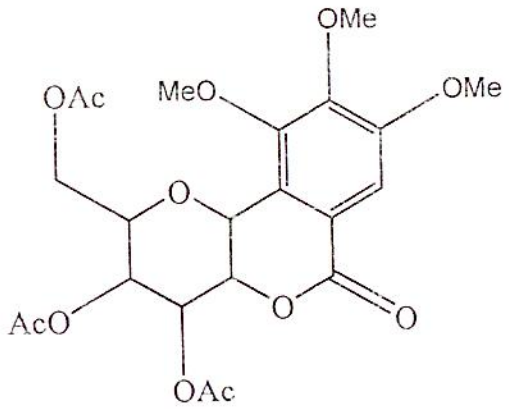
Yang-He Zahng *et al*, reported *in vitro* inhibitory effects of bergenin and norbergenin on Bovine Adrenal Tyrosine Hydroxylase. Both the compounds were isolated from cortex of *Mallotus japonicus*(Euphorbiaceae) see **Table-3.10**.

Table-3.10: The effect of bergenin and norbergenin on Bovine Adrenal Tyrosine Hydroxylase (BATH) activity

	TH activity (n mol/min/mg protein % of control)
Control	1.10 ± 0.04 (100.0)
Bulbocapnine 80	0.62 ± 0.08 (56.4)
Bergenin	
5	0.97 ± 0.08 (87.8)
10	0.92 ± 0.09 (83.9)
20	0.78 ± 0.08 (70.0)
Norbergenin	
5	0.54 ± 0.06 (49.0)
10	0.53 ± 0.04 (48.4)
20	0.51 ± 0.04 (46.6)

The control of TH activity, 1.10 nmol/min/mg protein was taken as 100. The data were expressed as mean ± SEM of 4 experiments. $P < 0.05$ compared with control (students t. test)[32].

Hwa-Kyung Lim *et al*, reported the hepatoprotective effects of bergenin against *D*-galactosamine (GalN)-induced liver damage in rats. This study has demonstrated that bergenin has protective effects against liver damage induced by GalN in rats[33].



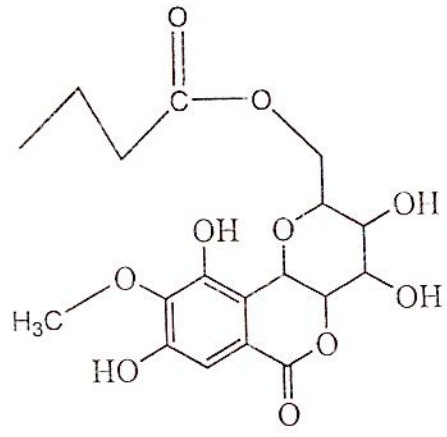
Tri-*O*-methylnorbergenintriacetate

(34)

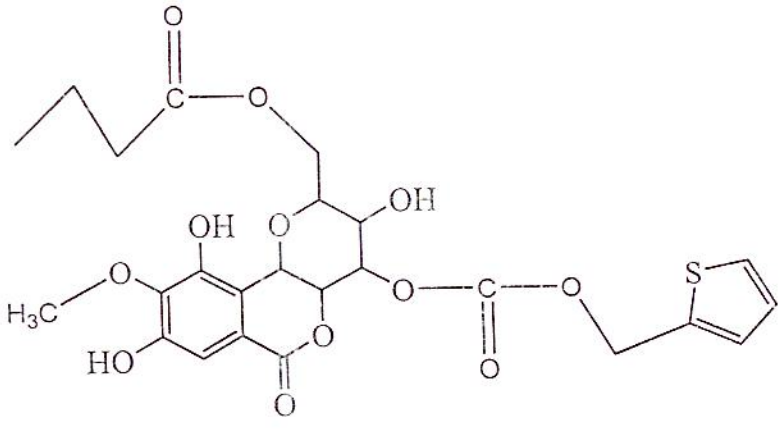
Cyril Rousseau *et al*, tested the way of an Intramolecular *C*-glycosylation reaction for the synthesis of bergenin related natural products. However, attempts to prepare bergenin by such a strategy have failed so far. They synthesized tri-*O*-Methylnorbergenin triacetate as well as *C*-aryl glycoside of Castacrenin B by way of an intramolecular *C*-glycosylation[34].

Hack-Seang Kim *et. al*, also reported the antihepatoxic activity of bergenin on carbon tetrachloride intoxicated hepatocytes[35]. Xiao-Gang Hua *et. al*, reported a convenient formation of aryl *β*-lactones. They reacted *O*-carboxyarylpropargyl bromide with aldehydes mediated by Indium in aqueous medium conveniently generated aryl *β*-lactones. According to them, this convenient 9-aryl lactone formation provides the basis for the synthesis of bergenin and related compounds. Bergenin was first isolated from the roots of *Saxifraga* (*Bergentia*) *Crassifolia* L. and from rhizome of *S. sibiria L. Saxifragaceae*[36].

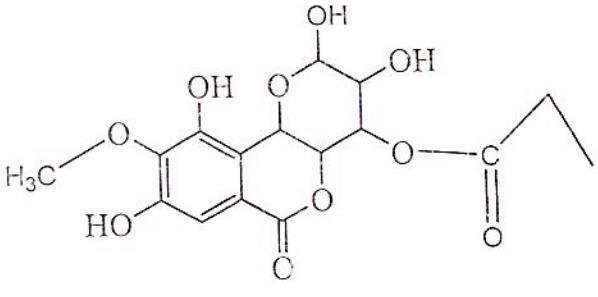
Vadim V. Mozhaev *et al*, reported regioselective enzymatic acylation of bergenin by combinatorial synthesis. They tested over fifty commercially available lipases and proteases as transesterification catalysts in organic solvents using vinyl butyrate as a model acyl donor. By the combinatorial biocatalysis approach they synthesized the following compounds[37]



(35)



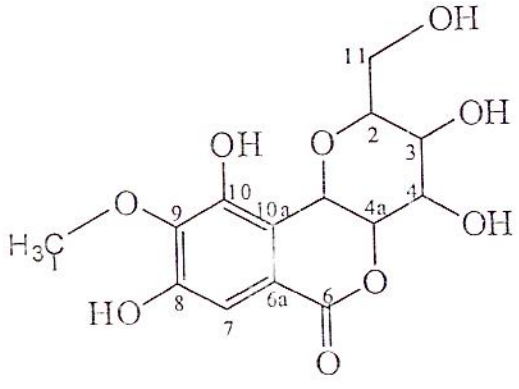
(36)



(37)

Miamta Tandon *et al*, explored the Antifeedant activity of bergenin isolated from *Astilbe rivularis*. The ED_{50} of bergenin was found to the 2432 and 2187ppm for *S.obliqua* and *S. litura*, respectively (% deterrence/log dose regression curves: Y = -7.043 + 3.557. log x and Y = -6.506 + 3.445. log x), respectively. These results exhibited significant antifeedatn activituy against two lepidopterous insects, *S. oblique* and *S. litura*[38].

Jahodar *et al*, reported the complete NMR spectral characteristics of bergenin. For the completion of the spectra, they made use of H, H-COSY, C, H-COSY and Inept techniques. The NMR spectral data with structure is given below in **table-3.11**.



(13)

Table-3.11: ^{1}H and ^{13}C NMR chemical shifts and ^{3}J (H, H) coupling constants of bergenin in DMSO-d6

H/C No	$^{1}H^{a}$ ppm	^{3}J (H,H)[b]	^{13}C ppm
2	3.62 *ddd*	9.1;7.6;1.6	81.93
3	3.26 *dd*	9.1;9.1	70.88
4	3.70 *dd*	9.3;9.1	73.89
4a	4.04 *dd*	10.5;9.3	79.97
6	-	-	163.60
6a	-	-	118.26
7	7.04 *s*	-	109.70
8	-	-	151.15
9	-	-	140.80
10	-	-	148.24
10a	-	-	116.14
10b	5.01 *d*	-	72.32
11	3.89 *dd* 3.49 *dd*	-	61.30
12	3.82 *s*	-	60.50

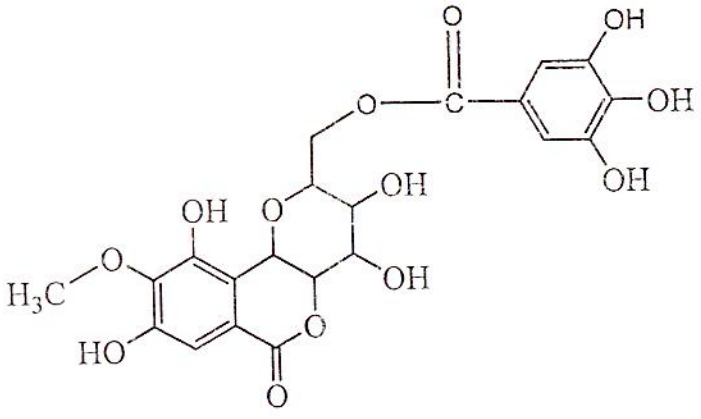
[a] $(OH)_{arom}$ = 9.76, 8.50 ppm : (OH)alif = 5.71, 5.45 and 5.00 ppm

[b] in Hz ± 0.3 Hz

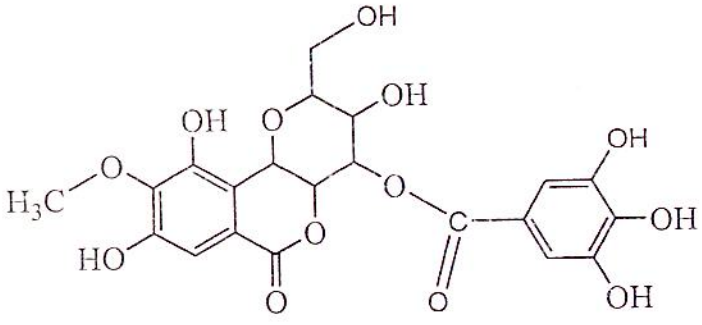
[c] long range coupling from H(7) in INEPT spectrum.

[d] long range coupling from H(10b) in INEPT spectrum[39].

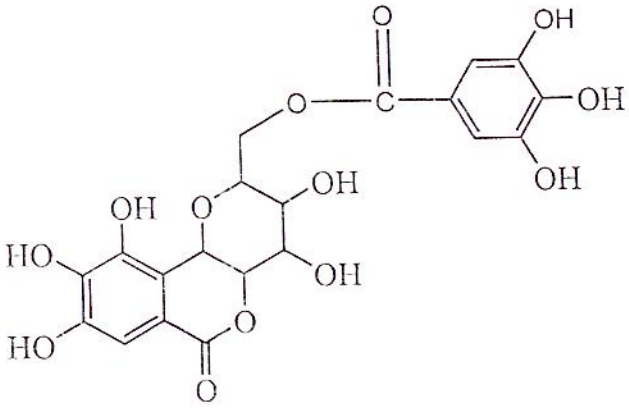
Takashi Yoshida *et al*, reported three new bergenin derivatives from th bark of *Mallotus Japonicus* and determined to be 11-*O*-Galloylbergenin **(14)**, 4-*O*-Galloylbergenin **(38)** and 11-*O*-Galloyldemethylbergenin **(39)**[40].



(14)

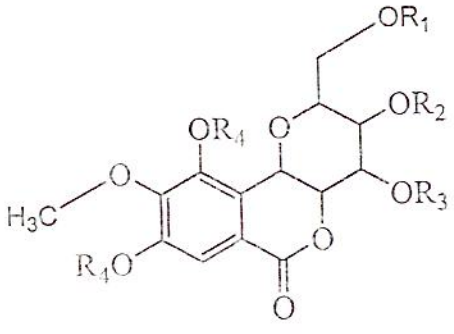


(38)



(39)

P. Atchuta Ramaiah *et al*, explored *Macaranga peltata* and isolated bergenin and its derivatives for the first time from *Macaranga peltata*. These derivatves wre isolated from the bark as well as heart wood of this plant. Following compounds were isolated from *Macaranga* species[41].



$R_1 = R_2 = R_3 = R_4 = H$ (40)

$R_1 = R_2 = R_3 = H, R_4 = CH_3$ (41)

$R_1 = R_4 = Me, R_2 = R_3 = H$ (42)

$R_1 = R_3 = H, R_2 = R_4 = Me$ (43)

$R_1 = R_2 = R_3 = R_4 = Ac$ (44)

$R_1 = R_2 = R_3 = Ac, R_4 = Me$ (45)

$R_1 = R_4 = Me, R_2 = R_3 = Ac$ (46)

$R_1 = R_3 = Ac, R_2 = R_4 = Me$ (47)

$R_1 = R_2 = R_3 = R_4 = Me$ (48)

Okada, T. *et al* and Abe, K. *et al*, reported that bergenin has been used as a folk medicine for treatement and therapy of gastrointestinal diseases such as gastritis gastric ulcer, diarrhea and constipation[42, 43].

3.2. Experimental

3.2.1. Material and methods

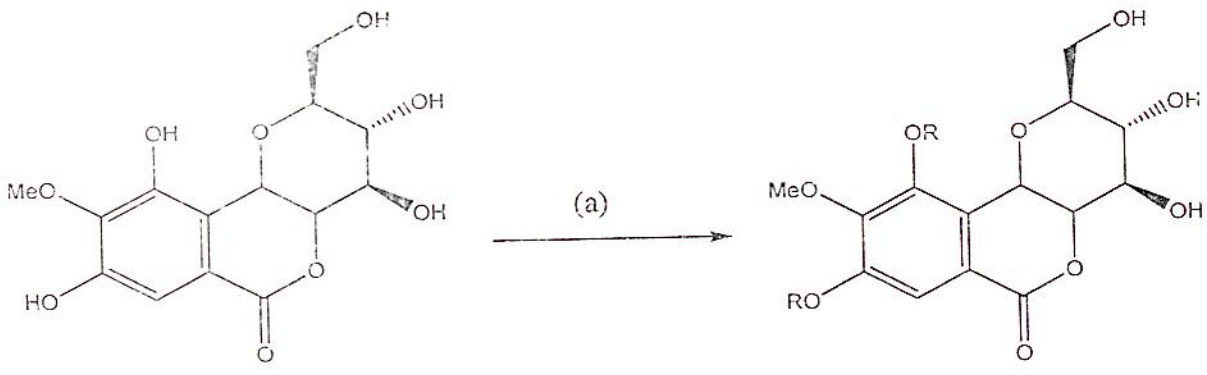
All the chemicals and solvents used were of analytical grade and were purified before use according to standard methods. IR spectra were recorded on VECTOR22 FT-IR. ^{1}H-NMR and ^{13}C NMR spectra were taken on a Bruker AVANCE AV 300 MHz,400 MHz, 500 MHz in CD_3OD as solvent using TMS as an internal standard, while mass spectra were recorded on MAT 312 instrument. Column chromatography was carried out on silica gel (70-230 mesh). TLC analysis was done alumina supported pre-coated TLC plates (Merck). Spots were visualized with ultra violet light and $CeSO_4$/ H_2SO_4. Melting points were determined in open capillary using Gallenkamp melting point apparatus.

3.2.2. Isolation of bergenin

The dried powder of the stemwood of *Mallotus philippensis* (6 Kg) were extracted with 20 L of EtOH at room temperature to give 260 g of crude extract. This extract (50 g) was loaded over silica gel column using CH_3OH: $CHCl_3$ (5:95) as mobile phase. A number of fractions were obtained. After repeated column chromatography, these fractions yielded bergenin (8.3g), which was further purified by recrystallization, was using methanol as a solvent.

3.2.3. General procedure for the synthesis of *O*-alkylated bergenins

A mixture of bergenin (0.328g, 1 mmol) **(13)** and potassium carbonate (0.690g, 5 mmol) were dissolved in 10 mL of dimethylformamide (DMF) **(Scheme-3.5)**. The mixture was stirred for 4-8h in round bottom flask at 80°C and the title reagents (5 mmol) were added drop wise at a stirring interval of 30 minutes. For different stirring times, the TLC profile was taken, using ethyl acetate/hexane (2:1) as solvent system, which showed the complete conversion of bergenin into its respective derivatives. These derivatives were purified by silica gel (CC) using ethyl acetate/hexane (1:1) as mobile phase.



(13) *O*-alkylated bergenins **(49-63)**

(a) K_2CO_3, DMF, RX or derivatives of RX, 80°C, stirring

Scheme-3.5: General scheme for the synthesis of *O*-alkylated bergenins

3.2.3.1. 3, 4-Dihydroxy-2-(hydroxymethyl)-8, 10-diisopropoxy-9-methoxy-2, 3,4, 4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (49)

Appropriate bergenin (0.328g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 2-Bromopropane (5 mmol) was added drop wise. On 5 h stirring, the TLC profile (ethyl acetate: n-hexane 2:1) showed the complete conversion of bergenin into the compound (49). Yield = 69%, m.p = 204^{0}C. R_f = 0.42 (5% methanol/chloroform); λ_{max} nm: 389. EI-MS *m/z* 412.3 (calcd. 412.431 for $C_{20}H_{28}O_9$) ; IR (KBr): 3310 cm^{-1} (OH), 3070 cm^{-1}(aromatic C-H), 1717 cm-1 (-CO-), 1330 cm^{-1} (C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 1.31 (12H, *m*, H-14, 14a, 15, 15a), 3.28 (1H, *dd*, *J* = 9.82, 8.88 Hz, H-3), 3.63-3.85 (2H, *dd*, *J* = 8.85, 4.79 Hz, H-11), 3.65 (1H, *ddd*, *J* = 5.02, 3.83,2.01, H-2), 3.84 (1H, *dd*, *J* = 9.04, 8.86, H-4), 3.86 (3H, *s*, H-12), 4.02 (1H, *dd*, *J* = 9.8, 7.81, H-4a), 4.42 (1H, *m*, H-13), 4.66 (1H, *m*, H-13a), 4.90 (1H, *d*, *J* = 10.2, H-10b), 7.44 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD) : δ 82.4 (C-2), 71.3 (C-3), 75.9 (C-4), 81.7 (C-4a), 166.0 (C-6), 127.8 (C-6a), 113.7 (C-7), 153.0 (C-8), 150.1 (C-9), 151.3 (C-10), 120.3 (C-10a), 77.3 (C-10b), 62.2 (C-11), 61.2 (C-12), 72.9 (C-13), 73.7 (C-13a), 22.2(C-14), 22.6 (C-114a), 22.2 (C-15), 22.8 (C-15a).

3.2.3.2. 8, 10-bis(But-3-enyloxy)-3, 4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (50)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 4-Bromo-1-Butene (5 mmol) was added drop wise. On 5 h and 30 minutes stirring the TLC profile (ethyl acetate: n-hexane 2:1) showed the complete conversion of bergenin into the compound **(50).** Yield = 75%, m.p = 151-153^{0}C. R_f = 0.68 (5% methanol/chloroform); λ_{max} nm: 389. EI-MS *m/z* 436 (calcd. 436.452 for $C_{22}H_{28}O_9$) ; IR (KBr): 3310 cm^{-1} (OH), 3080 cm^{-1}(aromatic C-H), 2933 cm^{-1} (aliphatic C-H), 3080 cm^{-1}(aromatic C-H), 1717 cm-1 (-CO-), 1643 cm^{-1} (C=C), 1330 cm^{-1} (C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 2.55 (4H, *m*), 3.25 (1H, *dd*, *J* = 9.79, 8.23 Hz, H-3), 3.62-3.83 (2H, *dd*, *J* = 8.80, 4.76 Hz, H-11), 3.64 (1H, *ddd*, *J* = 5.04, 3.83,2.04 Hz, H-2), 3.83 (1H, *dd*, *J* = 9.02, 8.85 Hz, H-4), 3.85 (3H, *s*, H-12), 4.03 (1H, *dd*, *J* = 9.8, 7.78 Hz, H-4a), 4.78 (1H, *d*, *J* = 10.2 Hz, H-10b), 5.13 (4H, *m*), 5.94 (2H, *m*), 7.07 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD) : δ 82.5 (C-2), 73.2 (C-3), 75.9 (C-4), 81.7 (C-4a), 165.9 (C-6), 127.9 (C-6a), 111.7 (C-7), 154.2 (C-8), 150.1 (C-9), 151.6 (C-10), 120.3 (C-10a), 75.9 (C-10b), 62.4 (C-11), 61.7 (C-12), 69.5 (C-13), 71.4 (C-13a), 34.6 (C-14), 35.71 (C-14a), 135.78 (C-15), 136.29 (C-15a), 117.04 (C-16), 117.59 (C-16a).

3.2.3.3. 3, 4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8, 10-bis(pentyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (51)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 5-Bromopentane (5 mmol) was added drop wise. On 7 h stirring the TLC profile (ethyl acetate: n-hexane 2:1) showed the complete conversion of bergenin into the compound **(51).** Yield = 71%, m.p = 148-149 ^{0}C. R_f = 0.60 (5% methanol/chloroform); EI-MS *m/z* 468.3 (calcd. 468.537 for $C_{24}H_{36}O_9$); λ_{max} nm: 389. IR (KBr): 3371cm^{-1} (OH), 3050 cm^{-1}(aromatic C-H), 2934 cm^{-1}(aliphatic C-H), 1729cm^{-1} (-CO-), 1332 cm^{-1} (C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 0.95 (6H, *t*, *J* =7.02 Hz, H-17, 17a), 1.40-1.52 (8H, *m*, 16,16a, 15, 15a) 1.74-1.85 (4H, *m*, 14, 14a), 3.29 (3H, *s*, H-12), 3.50 (1H, *ddd*, *J* = 5.02, 3.80,2.01 Hz, H-2), 3.57 (1H, *dd*, *J* = 9.81, 8.87 Hz, H-3),

3.82 (1H, *dd*, *J* = 9.03, 8.86 Hz, H-4), 3.91 (2H, *dd*, *J* = 8.80, 4.78 Hz, H-11), 3.96 (1H, *d*, *J* = 9.70, 7.68 Hz, H-4a), 4.02-4.07 (4H, *m*, 13, 13a), 4.81 (1H, *d*, *J* = 10.3 Hz, H-10b), 7.42 (1H, s, H-7). ^{13}C-NMR (150 MHz, CD_3OD): δ 82.4 (C-2), 71.3 (C-3), 75.9 (C-4), 81.7 (C-4a), 166.0 (C-6), 127.6 (C-6a), 111.5 (C-7), 154.5 (C-8), 150.1 (C-9), 151.8 (C-10), 120.4 (C-10a), 75.9 (C-10b), 62.2 (C-11), 61.6 (C-12), 70.2 (C-13), 75.6 (C-13a), 29.9 (C-14), 31.0 (C-14a), 29.3 (C-15), 29.4 (C-15a), 23.4 (C-16), 23.6 (C-16a), 14.3 (C-17), 14.4 (C-17a).

3.2.3.4. 8,10-Diethoxy-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (52)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 2-Iodoethane (5 mmol) was added drop wise.On 5 h stirring the TLC profile (ethyl acetate: n-hexane 2:1) showed the complete conversion of bergenin into the compound **(52)**. Yield = 72%, m.p = 179-180 ^{0}C. R_f = 0.40 (5% methanol/chloroform); λmax nm: 389. EI-MS *m/z* 384 (calcd. 384.378 for $C_{18}H_{24}O_9$) ; IR (KBr): 3387 cm^{-1} (OH), 3070 cm^{-1}(aromatic C-H), 2978 cm^{-1} (aliphatic C-H) 1709 cm^{-1}(-CO-), 1329 cm^{-1} (C-O-C); ^{1}H NMR (300 MHz, CD_3OD); δ 1.38 (3H, *t*, *J* = 6.98 Hz), 1.44 (3H, *t*, *J* = 6.94 Hz), 3.27 (1H, *dd*, *J* = 9.82, 8.87 Hz, H-3), 3.64 (1H, *ddd*, *J* = 5.03, 3.82,2.02 Hz, H-2), 3.82 (1H, *dd*, *J* = 9.03, 8.84 Hz, H-4), 3.62-3.84 (2H, *dd*, *J* = 8.80, 4.78 Hz, H-11), 3.86 (3H, *s*, H-12), 4.03 (1H, *dd*, *J* = 9.7, 7.68 Hz, H-4a), 4.12 (4H, *m*, H-13, 13a), 4.83 (1H, *d*, *J* = 10.3 Hz, H-10b), 7.43 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD): δ 82.5 (C-2), 71.4 (C-3), 75.9 (C-4), 81.7 (C-4a), 165.9 (C-6), 127.8 (C-6a), 111.8 (C-7), 154.2 (C-8), 150.3 (C-9), 151.5 (C-10), 120.3 (C-10a), 73.3 (C-10b), 62.4 (C-11), 61.4 (C-12), 65.9 (C-13, 13a), 15.0 (C-14), 15.9 (C-14a).

3.2.3.5. 3,4-Dihydroxy-2-(hydroxymethyl)-8,9,10-trimethoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (53)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes Iodomethane (5 mmol) was added drop wise. The compound **(53)** got formed on 5 h stirring Yield = 76%, m.p = 213-215^{0}C. R_f = 0.50 (5% methanol/chloroform); λ_{max} (388) ; EI-MS *m/z* 356 (calcd. 356.325 for $C_{16}H_{20}O_9$) ; IR (KBr): 3564cm^{-1} (OH), 3058cm^{-1} (aromatic C-H), 2946cm^{-1} (aliphatic C-H), 1718cm^{-1} (-CO-), 1338 cm^{-1} (C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 3.30 (9H, *s*, H-13, 13a, 12), 3.38 (1H, *dd*, *J* = 9.83, 8.82 Hz, H-3), 3.64-3.89 (2H, *dd*, *J* = 8.75, 3.92 Hz, H-11), 3.67 (1H, *ddd*, *J* = 4.66, 3.02,1.80 Hz, H-2), 3.80 (1H, *dd*, *J* = 9.32, 8.75 Hz, H-4), 4.06 (1H, *dd*, *J* = 9.92, 6.98 Hz, H-4a), 4.94 (1H, *d*, *J* = 10.38, H-10b), 7.45 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD) : δ 82.6 (C-2), 71.6 (C-3), 75.9 (C-4), 81.7 (C-4a), 165.9 (C-6), 127.8 (C-6a), 110.7 (C-7), 155.0 (C-8), 150.1 (C-9), 152.5 (C-10), 120.4 (C-10a), 73.1 (C-10b), 62.5 (C-11), 62.2 (C-12),56.6 (C-13), 61.5 (C-13a).

3.2.3.6. 8,10-bis(Hex-5-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (54)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 6-Bromo-1-Hexene (5 mmol) was added drop wise The compound **(54)** got formed on 6 h stirring..Yield = 77%, m.p = 151-153^{0}C. R_f = 0.45 (5% methanol/chloroform); λ_{max}: 388 ; EI-MS *m/z* 492.3 (calcd. 492.559 for $C_{26}H_{36}O_9$) ; IR (KBr):3354 cm^{-1} (OH), 3080 cm^{-1} (aromatic C-H) 2936 cm^{-1} (aliphatic C-H), 1732 cm^{-1} (-CO-), 1641 cm^{-1} (C=C) 1331 cm^{-1} (C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 1.60 (4H, *m*, H-18, 18a), 1.81 (4H, *m*, H-17, 17a), 2.14, (4H, *m*), 3.31 (1H, *dd*, *J* = 9.82, 8.88), 3.68-3.89 (2H, *dd*, *J* = 8.87, 4.76 Hz, H-11), 3.68 (1H, *ddd*, *J* = 500, 3.82, 2.02 Hz, H-2), 3.85 (1H, *dd*, *J* = 9.02, 8.82 Hz, H-4), 3.90 (3H, *s*, H-12), 4.06 (1H, *dd*, *J* = 9.8, 7.71 Hz, H-4a), 4.00 (4H, *m*), 4.90 (1H, *d*, *J* = 10.3 Hz, H-10b), 4.99 (4H, *m*), 5.83 (2H, *m*), 7.42 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD) : δ 82.4 (C-2), 71.2 (C-3), 75.9 (C-4), 81.7 (C-4a), 166.0 (C-6), 127.7 (C-6a), 111.7 (C-7), 154.4 (C-8), 150.1 (C-9), 150.1 (C-10),

120.4 (C-10a), 75.4 (C-10b), 62.3 (C-11), 61.6 (C-12), 70.1 (C-13), 75.4 (C-13a), 29.6 (C-14), 30.7 (C-14a), 26.4 (C-15), 26.5 (C-15a), 34.4 (C-16), 34.6 (C-16a), 139.6 (C-17), 139.8 (C-17a), 115.0 (C-18), 115.2 (C-18a).

3.2.3.7. 8,10-bis(Heptyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (55)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 7-Bromoheptane (5 mmol) was added drop wise. The compound **(55)** got formed on 8 h stirring Yield = 74%, m.p = 125 ^{0}C. R_f = 0.55 (5% methanol/chloroform); λ max: 390; EI-MS *m/z* 524.3 (calcd. 524.644 for $C_{28}H_{44}O_9$) ; IR (KBr): 3380 cm^{-1} (OH), 3057 cm^{-1}(aromatic C-H), 2927 cm^{-1} (aliphatic C-H), 1730 cm^{-1} (-CO-), 3131 cm^{-1} (C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 1.91 (6H, *m*, H-19, 19a), 1.33 (12H, *m*, H-18,18a, 17, 17a, 16, 16a), 1.48 (4H, *m*, H-15, 15a), 1.79 (4H, *m*, H-14, 14a), 3.28 (1H, *dd*, *J* = 9.82, 8.87 Hz, H-3), 3.65-3.88 (2H, *dd*, *J* = 8.84, 4.75 Hz H-11), 3.66 (1H, *ddd*, *J* = 5.01, 3.82, 2.02 Hz, H-2), 3.82 (1H, *dd*, *J* = 9.01, 8.82 Hz, H-4), 3.86 (3H, *s*, H-12), 4.00 (4H, *m*, H-13, 13a), 4.04 (1H,*dd*, *J* =9.91, 7.64 Hz, H-4a), 4.83 (1H, d, *J* = 10.3 Hz, H-10b), 7.42 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD): δ 82.5 (C-2), 71.3 (C-3), 75.9 (C-4), 81.7 (C-4a), 166.0 (C-6), 127.6 (C-6a), 111.7 (C-7), 154.5 (C-8), 150.1 (C-9), 151.8 (C-10), 120.4 (C-10a), 73.3 (C-10b), 62.3 (C-11), 61.6 (C-12), 70.3 (C-13), 75.6 (C-13a), 33.0 (C-14), 32.9 (C-14a), 27.0 (C-15), 27.1 (C-15a), 30.1 (C-16), 30.2 (C-16a), 30.3 (C-17), 31.3 (C-17a), 23.6 (C-18), 23.7 (C-18a), 14.3 (C-19), 14.4 (C-19a).

3.2.3.8. 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(prop-2-ynyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (56)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 3-Bromoprop-1-yne (5 mmol) was added drop wise. The compound **(56)** got formed on 4 h stirring. Yield = 80%, m.p = 183-184 °C. R_f = 0.46 (5% methanol/chloroform); λ_{max} nm: 388 ; EI-MS *m/z* 404.1 (calcd. 404.367 for $C_{20}H_{20}O_9$);

IR (KBr): 3397 cm^{-1} (OH) 3288 cm^{-1} (acetylenic C-H), 3060 cm^{-1}(aromatic C-H) 2935 cm^{-1} (aliphatic C-H), 2125 cm^{-2} C≡C, 1708 cm^{-1} (-CO-), 1327 cm^{-1} (-C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 2.97 (2H, *s*, H-15, 15a), 3.02 (2H, *s*), 3.29 (1H, *dd*, J = 9.84, 8.86 Hz, H-3), 3.67 (1H, *ddd*, J = 5.00, 3.84, 2.02 Hz, H-2), 3.68-3.90 (2H, *dd*, J = 8.89, 4.77 Hz, H-11), 3.86 (1H, *dd*, J = 9.02, 8.84 Hz, H-4), 3.91 (3H, *m*), 4.07 (1H, *dd*, J = 9.90, 7.78 Hz, H-4a), 4.73 (1H, *s*)), 4.74 (1H, *s*), 4.90 (1H,*d*, J =10.31 Hz, H-10b), 7.61 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD): δ 82.3 (C-2), 71.2 (C-3), 75.8 (C-4), 81.5 (C-4a), 165.4 (C-6), 128.8 (C-6a), 113.7 (C-7), 152.6 (C-8), 150.0 (C-9), 150.7 (C-10), 120.0 (C-10a), 77.7 (C-10b), 62.6 (C-11), 62.1 (C-12), 57.7 (C-13), 61.8 (C-13a), 77.7 (C-14,14a), 76.7 (C-15, 15a).

3.2.3.9. 8, 10-bis(Allyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (57)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes Allyl bromide (5 mmol) was added drop wise. The compound **(57)** got formed on 4 h stirring. Yield = 82%, m.p = 181-183^{0}C. R_f = 0.60 (5% methanol/chloroform); λ_{max} nm: 389 ; EI-MS *m/z* 408.0 (calcd. 408.399 for $C_{20}H_{24}O_9$) ; IR (KBr): 3319 cm^{-1} (OH), 3085 cm^{-1}(aromatic C-H), 2936 cm^{-1} (aliphatic C-H), 1737cm^{-1} (-CO-), 1647cm^{-1} (C=C), 1329 cm^{-1} (-C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 3.27 (1H, *dd*, J = 9.83, 8.89 Hz, H- 3), 3.65 (1H, *ddd*, J = 5.01, 3.82, 2.01 Hz, H-2), 3.65-388 (2H, *dd*, J = 8.84, 4.75 Hz, H-11), 3.84 (1H, *dd*, J = 9.01, 8.83 Hz, H-4), 3.88 (3H, *s*, H-121), 4.05 (1H, *dd*, J = 9.8, 7.70 Hz, H-4a), 4.58 (1H, *d*, J = 6.38 Hz), 4.81 (1H, *dd*, J = 13.82 Hz,), 4.89 (1H, *d*, J = 10.2 Hz)), 5.34 (4H, *m*), 6.10 (2H, *m*), 7.45 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD): δ 82.5 (C-2), 71.4 (C-3), 75.9 (C-4), 81.7 (C-4a), 165.8 (C-6), 128.1 (C-6a), 112.6 (C-7), 153.9 (C-8), 150.4 (C-9), 151.3 (C-10), 120.3 (C-10a), 76.0 (C-10b), 62.3 (C-11), 61.6 (C-12), 70.9 (C-13), 73.2 (C-13a), 134.1 (C-14), 135.7 (C-14a), 117.8 (C-15), 118.2 (C-15a).

3.2.3.10. 2,2'-(3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetonitrile (58)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 2-bromoacetonitrile (5 mmol) was added drop wise. The compound (58) got formed as colourless solid on 6 h stirring.Yield = 65%, m.p = 180-182^{0}C. R_f = 0.48 (5% methanol/chloroform); λ_{max} nm: 389; EI-MS *m/z* 406.1 (calcd. 406.344 for $C_{18}H_{18}N_2O_9$) ; IR (KBr): 3529 cm^{-1} (OH), 3080.8 cm^{-1}(aromatic C-H), 2992 cm^{-1} (aliphatic C-H), 2230cm^{-1} (CN), 1729cm^{-1} (-CO-), 1320 cm^{-1} (C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 3.28 (1H, *dd*, *J* = 9.83, 8.87 Hz, H- 3), 3.66 (1H, *ddd*, *J* = 5.02, 3.83, 2.00 Hz, H-2), 3.66-387 (2H, *dd*, *J* = 8.84, 4.75 Hz, H-11), 3.83 (1H, *dd*, *J* = 9.02, 8.84 Hz, H-4), 3.88 (3H, *s*, H-121), 4.06 (1H, *dd*, *J* = 9.9, 7.64 Hz, H-4a), 4.90 (1H, *d*, *J* = 10.2 Hz, H-10b), 5.13 (4H, *s*, H-13, 13a), 7.67 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD): δ 82.7 (C-2), 71.6 (C-3), 75.8 (C-4), 81.6 (C-4a), 164.8 (C-6), 130.3 (C-6a), 114.7 (C-7), 151.9 (C-8), 150.1 (C-9), 150.7 (C-10), 120.8 (C-10a), 72.7 (C-10b), 62.4 (C-11), 62.2 (C-12), 55.7 (C-13), 59.8 (C-13a), 116.3 (C-14), 117.4 (C-14a).

3.2.3.11. 8, 10-Dibutoxy-3, 4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2, 3, 4, 4a-tetrahydropyrano[3, 2-c]isochromen-6(10bH)-one (59)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 4-Bromobutane (5 mmol) was added drop wise. The compound **(59)** got formed as colourless solid on 7 h stirring. Yield = 70%, m.p = 168^{0}C. R_f = 0.54 (5% methanol/chloroform) ; EI-MS *m/z* 440.2 (calcd. 440.484 for $C_{22}H_{32}O_9$); λ_{max} nm: 389; IR (KBr): 3312cm^{-1} (OH), 3070 cm^{-1}(aromatic C-H), 2958 cm^{-1} (aliphatic C-H), 1718cm^{-1} (-CO-), 1313 cm^{-1} (-C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 0.99 (6H, *m*, H-16, 16a), 1.53 (4H, *m*, H-15, 15a), 1.78 (4H, *m*, H-14, 14a), 3.28 (1H, *dd*, *J* = 9.82, 8.87 Hz, H-3), 3.66 (1H, *ddd*, *J* = 5.01, 3.82, 2.02 Hz, H-2), 3.65-3.86 (2H, *dd*, *J* = 8.80-4.71, H-11), 3.82 (4H, *dd*, *J* = 9.01, 8.82 Hz, H-4), 3.86 (3H, *s*, H-12), 4.05 (4H, *m*, H-13, 13a), 4.15

(1H, *dd*, *J* = 9.9, 7.64 Hz, H-4a), 4.82 (1H, *d*, *J* = 10.2 Hz, H-10b), 7.67 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD): δ 82.5 (C-2), 71.4 (C-3), 75.9 (C-4), 81.7 (C-4a), 165.8 (C-6), 127.6 (C-6a), 111.7 (C-7), 154.5 (C-8), 150.1 (C-9), 151.8 (C-10), 120.4 (C-10a), 71.8 (C-10b), 62.3 (C-11), 61.6 (C-12), 77.1 (C-13, 13a), 23.5 (C-14), 24.4 (C-14a). 10.1 (C-15), 10.7 (C-15a), 14.3 (C-16), 14.4 (C-16a).

3.2.3.12. 3, 4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8, 10-bis(octyloxy)-2, 3, 4, 4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (60)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes 8-Bromooctane (5 mmol) was added drop wise. The compound **(60)** got formed as colourless solid on 7 h stirring. Yield = 71%, m.p = 147-149^{0}C. R_f = 0.42 (5% methanol/chloroform) ; EI-MS *m/z* 552 (calcd. 552.697 for $C_{30}H_{48}O_9$); λ_{max} nm: 389 ; IR (KBr): 3351 cm^{-1} (OH), 3060 cm^{-1}(aromatic C-H), 2929 cm^{-1} (aliphatic C-H), 1734cm^{-1} (-CO-), 1331 cm^{-1}(-C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 0.89 (6H, *m*, H-20, 20a), 1.41 (16H, *m*, H-19,19a, 18, 18a, 17, 17a, 16, 16a), 1.80 (8H, *m*, H-15, 15a, 14, 14a), 3.28 (1H, *dd*, *J* = 9.83, 8.86 Hz, H-3), 3.66 (1H, *ddd*, *J* = 4.90, 3.76, 1.98 Hz, H-2), 3.69-3.84 (2H, *dd*, *J* = 8.73, 3.78 Hz, H-11), 3.78 (1H, *dd*, *J* = 9.34, 8.78, H-4), 3.88 (3H, *s*, H-12), 4.02 (4H, *m*, 13, 13a), 4.12 (1H, *dd*, *J* = 9.84, 6.62 Hz, H-4a), 4.72 (1H, *d*, *J* =10.26 Hz, H-10b), 7.67 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD): δ 82.5 (C-2), 70.3 (C-3), 75.9 (C-4), 81.7 (C-4a), 166.0 (C-6), 127.6 (C-6a), 111.7 (C-7), 154.5 (C-8), 150.1 (C-9), 151.8 (C-10), 120.4 (C-10a), 75.9 (C-10b), 62.3 (C-11), 61.6 (C-12), 70.3 (C-13), 75.6 (C-13a), 30.2 (C-14), 30.3 (C-14a), 27.1 (C-15), 27.1 (C-15a), 30.5 (C-16), 31.3 (C-16a.), 30.3 (C-17), 30.4 (C-17a), 32.9 (C-18), 33.0 (C-18a), 23.6 (C-19), 23.7 (C-19a), 14.4 (C-20, 20a).

3.2.3.13. 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-dipropoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (61)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C.

After 30 minutes 1-Bromopropane (5 mmol) was added drop wise. The compound **(61)** got formed as colourless solid on 6 h stirring. Yield = 75%, m.p = 179-180^{0}C. R_f = 0.43 (5% methanol/chloroform) ; EI-MS *m/z* 412 (calcd. 412.431 for $C_{20}H_{28}O_9$); λ_{max} nm: 389 ; IR (KBr): 3353 cm^{-1} (OH), 3090 cm^{-1}(aromatic C-H), 2963 cm^{-1} (aliphatic C-H), 1735cm^{-1} (-CO-), 1330 cm^{-1} (-C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 1.06 (6H, *m*, H-15, 15a), 1.82 (4H, *m*, H-14, 14a), 3.29 (1H, *dd*, *J* =9.83, 8.88 Hz, H-3), 3.68 (1H, *ddd*, *J* = 4.91, 3.76, 1.42 Hz, H-2), 3.68-3.87 (2H, *dd*, *J* = 8.73, 3.74, Hz, H-11), 3.79 (1H, *dd*, *J* = 9.34, 8.74 Hz, H-4), 3.90 (4H, *m*, H-13, 13a), 4.03 (1H, *dd*, *J* = 9.85, 6.63 Hz, H-4a), 4.94 (1H, *d*, *J* = 10.38 Hz, H-10b), 7.43 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD) : δ 82.5 (C-2), 71.4 (C-3), 75.9 (C-4), 81.8 (C-4a), 166.0 (C-6), 127.7 (C-6a), 111.7 (C-7), 154.5 (C-8), 150.1 (C-9), 151.8 (C-10), 120.4 (C-10a), 71.8 (C-10b), 62.3 (C-11), 61.6 (C-12), 77.1 (C-13, 13a), 23.5 (C-14), 24.4 (C-14a) 10.7 (C-15), 10.8 (C-15a).

3.2.3.14. Diethyl 2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate (62)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes Ethyl 2-Bromoacetate (5 mmol) was added drop wise. The compound **(62)** got formed as colourless solid on 6 h stirring. Yield = 80%, m.p = 160-162 °C. R_f = 0.45 (5% methanol/chloroform) ; EI-MS *m/z* 500.2 (calcd. 500.450 for $C_{22}H_{28}O_{13}$); λ_{max} nm: 389 ; IR (KBr): 3402 cm^{-1} (OH), 3086 cm^{-1}(aromatic C-H), 2934 cm^{-1} (aliphatic C-H), 1752cm (-CO-), 1376 cm^{-1} (C-O-C); ^{1}H NMR (300 MHz, CD_3OD); δ 1.26 (3H, *t*, *J* = 7.26 Hz, H-16), 1.31 (3H, *t*, *J* = 7.32 Hz, H-16a), 2.85 (2H, *s*, H- 15), 2.98 (2H, *s*, H-15a), 3.28 (1H, *dd*, *J* = 9.82, 8.88 Hz, H-3), 3.64-3.88 (2H, *dd*, *J* = 8.84, 4.75 Hz, H-11), 3.66 (1H, *ddd*, *J* = 5.01, 3.82, 2.01 Hz, H-2), 3.82 (1H, *dd*, *J* = 9.01, 8.82 Hz, H-4), 3.86 (3H, *s*, H-12), 4.05 (1H, *dd*, *J* = 9.9, 7.64, H-4a), 3.97 (4H, *s*, H-13, 13a), 4.82 (1H, *d*, *J* = 10.2, H-10b), 7.38 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD) : δ 82.5 (C-2), 71.5 (C-3), 75.9 (C-4), 81.7 (C-4a), 165.4 (C-6), 128.7 (C-6a), 112.7 (C-7), 153.2 (C-8), 150.5

(C-9), 150.0 (C-10), 120.3 (C-10a), 75.9 (C-10b), 62.2 (C-11), 61.9 (C-12), 66.8 (C-13, 13a), 169.9 (C-14), 170.4 (C-14a), 62.4 (C-15), 62.5 (C-15a), 14.4 (C-16), 14.4 (C-16a).

3.2.3.15. Tert-butyl2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate (63)

Appropriate bergenin (0.328 g, 1 mmol) and potassium carbonate (0.690 g, 5 mmol) were dissolved in 10 mL of DMF. The mixture was stirred in round bottom flask at 80°C. After 30 minutes t- butyl 2- Bromoacetate (5 mmol) was added drop wise. The compound (63) got formed as colourless solid on 5 h stirring. Yield = 78%, m.p = 170^{0}C. R_f = 0.64 (5% methanol/chloroform) ; EI-MS *m/z* 556.3 (calcd. 556.556 for $C_{26}H_{36}O_{13}$); λ_{max} nm: 389 ; IR (KBr): 3430cm^{-1} (OH), 3088 cm^{-1}(aromatic C-H), 2980 cm^{-1} (aliphatic C-H), 1732 cm^{-1} (-CO-), 1370 cm^{-1} (C-O-C) ; ^{1}H NMR (300 MHz, CD_3OD); δ 1.47 (9H, *s*, H-16, 17, 18), 1.51 (9H, *s*, 16a, 17a, 18a), 3.30 (1H, *dd*, *J* = 9.82, 8.87 Hz, H-3), 3.67 (1H, *ddd*, *J* = 4.90, 3.78, 1.40 Hz, H-2), 3.68-3.89 (2H, *dd*, *J* = 8.75, 3.75 Hz, H-11), 3.80 (1H, *dd*, *J* = 9.32, 8.75 Hz, H-4), 3.89 (3H, *s*, H-12), 4.03 (1H, *dd*, *J* = 9.85, 6.64 Hz, H-4a), 4.61 (2H, *s*, H-13), 4.72 (2H, *s*, 13a), 4.94 (1H, *d*, *J* = 10.38, H-10b), 7.34 (1H, *s*, H-7). ^{13}C-NMR (150 MHz, CD_3OD) : δ 82.5 (C-2), 71.5 (C-3), 75.9 (C-4), 81.7 (C-4a), 165.5 (C-6), 128.5 (C-6a), 112.5 (C-7), 153.3 (C-8), 149.9 (C-9), 150.5 (C-10), 120.3 (C-10a), 75.9 (C-10b), 62.4 (C-11), 61.9 (C-12), 67.2 (C-13, 13a), 169.1 (C-14), 170.5 (C-14a), 83.3 (C-15), 83.8 (C-15a), 28.2 (C-16,17,18), 28.3 (C-16a, 17a, 18a).

3.2.4. Bioactivities of the synthetic *O*- alkylated bergenins

3.2.4.1. Antiinflammatory activity (*in vitro*)

The respiratory-burst inhibitory activities of bergenin and its synthetically derived products were determined by mean of the modified assay of *Tan* and *Berridge*[44]. This *in-vitro* assay is based on the reduction of the highly water- soluble tetrazolium salt WST-1 (*Dojindo Laboratories, Kumamoto*, Japan) in the presense of activated human neutrophils. All reagents used were of analytical grade, and *Ficollpaque* was purchased from *Pharmacia Biotech Amersham* (Uppsala, Sweden). Briefly, heparinized fresh venous blood was drawn from healthy volunteers in a local blood bank, and neutrophils

were isolated by a method described previously[45,46]. A total volume of 250µL *MHS* buffer (pH 7.4) was used, containing 1.0 X 10^4 neutrophils/mL, 500µM *WST* –1, and the respective drug to be tested at various drug concentrations. The controls contained buffer, neutrophils, and *WST* –1 only. All compounds were equilibrated at 37°C and the reaction was initiated by adding opsonized zymosan A (*Sigma*; 15mg/mL), which was prepared by mixing with human pooled serum, followed by centrifugation at 3000 r.p.m. The pellet was resuspended in *PBS* buffer (pH 7.4); and the absorbance was measured at 450 nm on a *SpectraMax*-340 micro plate reader (*Molecular Devices*, CA, U.S.A). Indomethacin was used as positive control. IC_{50} values were calculated relative to DMSO (blank), and expressed as percent inhibition of super oxide anions produced.

3.2.4.2. **Antiplasmodial activity (*in vitro*).**

See the details of this activity in **2.2.5.3. (Chapter 2)**

3.3. Results and discussion

3.3.1. Characterization of synthetic *O*-alkylated bergenins

These compounds (49-63) were characterized on the basis of their UV, IR, ^{1}H-NMR, ^{13}C-NMR and MS spectral data as discussed below.

3.3.1.1. 3, 4-Dihydroxy-2-(hydroxymethyl)-8, 10-diisopropoxy-9-methoxy-2, 3, 4, 4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (49)

UV spectrum showed absorptions at λ_{max} nm 390 indicated the presence of aromatic system in the molecule.

IR (KBr): 3310 cm^{-1} (OH), 3070 cm^{-1}(aromatic C-H), 1717 cm-1 (-CO-), 1330 cm^{-1} (C-O-C).

^{1}H-NMR spectrum of the compound (49), showed multiplet for H-14, 14a, 15, 15a at 1.31.The multiplet at δ 4.42 and 4.06 were due to protons at C-13 and 13a. Beside this doublets of doublet (*dd*) in the range of 3.63-3.85 were assigned to protons of H-11. Similarly doublet of doublet at 3.65, doublet of doublet at 3.84 and doublet of doublet at 4.02 were assigned to protons attached to C-2, C-4 and 4a, respectively. The only singlet at 3.65 was assigned to protons of C-12.

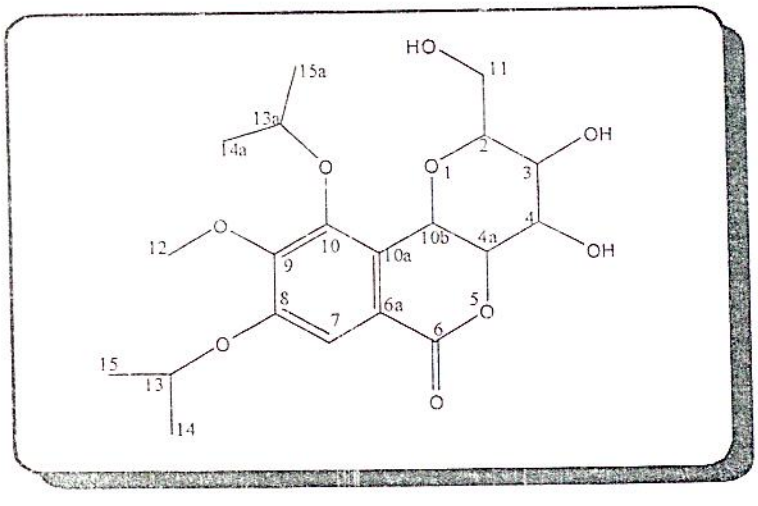


Table-3.12: ^{1}H-NMR (300 MHz, CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-8,10-diisopropoxy-9-methoxy-2,3,4,4a- tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (49)

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-14,14a,15,15a	1.31	12H	*m*	-
H-3	3.28	1H	*dd*	9.82, 8.88
H-11	3.63-3.85	2H	*dd*	8.85, 4.79
H-2	3.65	1H	*ddd*	5.02, 3.83,2.01
H-4	3.84	1H	*dd*	9.04, 8.86
H-12	3.86	3H	*s*	.....
H-4a	4.02	1H	*dd*	9.8, 7.81
H-13	4.42	1H	*m*	....
H-13a	4.66	1H	*m*	.....
H-10b	4.99	1H	*d*	10
H-7	7.44	1H	*s*	...

^{13}C-NMR spectrum of the compound (49), showed the signals at δ 82.4, 71.3, 75.9, 81.7, 166.0, 127.8, 113.7, 153.0, 150.1, 151.3, 120.3, 77.3 and 62.2 for the C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and a signal at 61.29 was assigned to C-12. Those were the characteristics peaks of the basic carbon skeleton of bergenin. Beside those, signals at δ 72.9, 73.7, 22.2, 22.6, 22.2 and 22.8 were assigned to C-13, 13a, C-14, 14a, C-15 and 15a, respectively.

Table-3.13: ^{13}C-NMR (125 MHz, CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-8,10-diisopropoxy-9-methoxy-2,3,4,4a- tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (49)

Assignment	δ (ppm)	Assignement	δ (ppm)
C-2	82.4	C-10a	120.3
C-3	71.3	C-10b	77.3
C-4	75.9	C-11	62.2
C-4a	81.7	C-12	61.2
C-6	166.0	C-13	72.9
C-6a	127.8	C-13a	73.7
C-7	113.7	C-14	22.2
C-8	153.0	C-14a	22.6
C-9	150.1	C-15	22.2
C-10	151.3	C-15a	22.8

EI-MS m/z: 412.3 [M^+] (calcd. 412.431 for $C_{20}H_{28}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure (49) was identified as 3,4-Dihydroxy-2-(hydroxymethyl)-8,10-diisopropoxy-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one

3.3.1.2. 8,10-bis(But-3-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (50)

UV spectrum showed absorptions at λ_{max} nm 389 indicated the presence of aromatic system.

IR (KBr): 3310 cm^{-1} (OH), 3080 cm^{-1}(aromatic C-H), 2933 cm^{-1} (aliphatic C-H), 3080 cm^{-1}(aromatic C-H), 1717 cm-1 (-CO-), 1643 cm^{-1} (C=C), 1330 cm^{-1} (C-O-C).

^{1}H-NMR spectrum of compound (50) showed multiplets at δ 2.55, 5.94 and 5.13 for the protons at C-14, 14a, C-15, 15a, and C-16 and16a, respectively. The doublets of doublets at δ 3.25, 3.83 and 4.03 are due to protons attached to C-3, C-4 and 4a. Similarly the

doublet of doublet (*dd*)and doublet of doublet of doublet (*ddd*) at 3.62-3.83 and 3.64 was due to protons at C-11 and C-2. The only doublet at 4.78 is due to proton at C-10b, while two singlets at δ 3.85 and 7.07 were assigned to protons at C-12 and to aromatic protons at C-7.

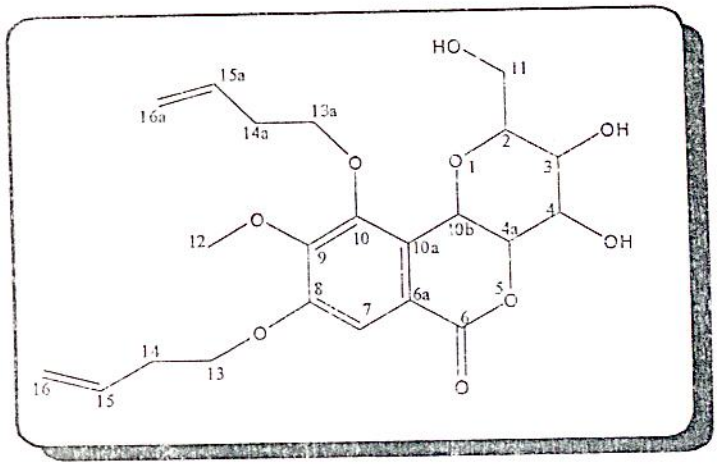


Table-3.14: ^{1}H-NMR (400 MHz, $CDCl_3$+CD_3OD) data of 8,10-bis(But-3-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (**50**)

Assignment	δ (ppm)	Integration	Multiplicity	*J* (Hz)
H-14, 14a	2.55	4H	*m*	.....
H-3	3.25	1H	*dd*	9.79, 8.23
H-11	3.62-3.83	2H,	*dd*	8.80, 4.76
H-2	3.64	1H	*ddd*	5.04, 3.83, 2.04
H-4	3.83	1H	*dd,*	9.02, 8.85
H-12	3.85	3H	*s*	.....
H-4a	4.03	1H	*dd,*	9.8, 7.78
H-10a	4.78	1H	*d*	10.2
H-16, 16a	5.13	4H	*m*	.....
H-15, 15a	5.94	2H	*m*	.....
H-7	7.07	1H	*s*	.....

^{13}C-NMR spectrum of compound (**50**) showed characteristics peaks for the basic carbon skeleton of bergenin nucleus along with some additional signals for the expected moieties attached with oxygen at C-8 and C-10. The signals at δ 69.5 and 71.4 were assigned to C-13 and C-13a. Similarly the signals at δ 34.6, 35.7, 135.7, 136.2, 117.0 and 117.5 were assigned to C-14, 14a, C-15, 15a, C-16 and 16a, respectively. While the remaining signals at δ 82.5, 73.2, 75.9, 81.7, 165.9, 127.9, 111.7, 154.2, 150.1, 151.6, 120.3, 75.9, 62.4 and 61.7 were assigned to C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, C-10b, C-11 and C-12, respectively. These are the characteristics peaks in ^{13}C-NMR spectrum of bergenin nucleus.

Table-3.15: ^{13}C-NMR (125 MHz, CD_3OD) data of 8,10-bis(But-3-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (**50**)

Assignement	δ (ppm)	Assignement	δ (ppm)	Assignement	δ (ppm)
C-2	82.5	C-9	150.1	C-14	34.6
C-3	73.2	C-10	151.6	C-14a	35.7
C-4	75.9	C-10a	120.3	C-15	135.7
C-4a	81.7	C-10b	75.9	C-15a	136.2
C-6	165.9	C-11	62.4	C-16	117.0
C-6a	127.9	C-12	61.7	C-16a	117.5
C-7	111.7	C-13	69.5	.....	.....
C-8	154.2	C-13a	71.4	.....	.....

EI-MS m/z: 436 [M^+] (calcd. 436.452 $C_{22}H_{28}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure (**50**) was identified as 8,10-bis(But-3-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.2.1. X-Ray Crystallography of compound (50)

Along with NMR and mass spectroscopic techniques, The compound was also characterized through single X-ray crystallography, like the XRD analysis of compound **(50)** (**Figure-3.14** and **3.15**) revealed that the molecule is composed of three six membered rings; C5a-C9a, an aromatic ring C5/O5/C4A-C9A/C5A, an annelated lactone ring and C2-C4/C4A/C9B/O4 a glucopyranose ring. The hydroxyls at C4, C3 and hydroxymethyl group at C2 in the glucopyranose ring adopt equatorial conformations. All the three hydroxyl groups acting simultaneously as hydrogen bond donors and acceptors and forming a network through intermolecular hydrogen bonding. The H atoms on C4A and C9B at the junction of rings *B* and *C* are in *trans* positions so the *B/C* junction is trans fused. The conformations of ring *B* and *C* are in good agreement with those reported for bergenin monohydrate[47]. The lactone ring occurs in a half chair conformation while the glucopyranose ring adopts chair conformations. The methoxy group at C8 is out of plane with respect to the attached aromatic ring by making a torsion angle of 82.5(7). The butenyloxy group attached at C7 adopts a fully extended conformation while the other butenyloxy group attached at the C9 position experiences a gauch conformation at its terminal alkene position.**Table-3.16, 3.17, 3.18** and **3.19** show the crystal data, Bond lengths, Torsion angles and hydrogen bonds of the compound **(50).**

Table-3.16: X-ray Crystallography Data of Compound **(50)**

$C_{22}H_{28}O_9$	$D_x = 1.347$ Mg m^{-3}
$M_r = 436.44$	Melting point: 425 K
Monoclinic, $P2_1$	Mo $K\alpha$ radiation, $\lambda = 0.71073$ Å
$a = 12.562$ (3) Å	Cell parameters from 3139 reflections
$b = 5.7377$ (11) Å	$\theta = 2.7$–$26.4°$
$c = 14.956$ (3) Å	$\mu = 0.11$ mm^{-1}
$\beta = 93.70$ (3)°	$T = 158$ K
$V = 1075.7$ (4) Å^3	Plate, colorless
$Z = 2$	0.60 × 0.14 × 0.05 mm
$F_{000} = 464$	.....

Table-3.17: Selected Bond Lengths (Å) of Compound **(50)**

O8—C1'''	1.412 (10)	O7—C1'	1.447 (7)
C1'''—H1G	0.9599	.....	.....
O9—C1''	1.429 (7)	C1'''—H1H	0.9599
C1'—C2'	1.499 (9)	.....	.....
C2'—C3'	1.521 (9)	C1'—H1C	0.9600
C4A—C9B	1.530 (7)	C1'—H1D	0.9600
C2'—H2C	0.9600	C2'—H2B	0.9600
C3'—H3B	0.9600	C3'—C4'	1.281 (13)
C4'—H4D	0.9600	C4'—H4C	0.9600
C1''—C2''	1.481 (10)	C1''—H1''1	0.9600
C1''—H1''2	0.9600	C2''—C3''	1.330 (18)
C2''—H2''1	0.9600	C2''—H2''2	0.9600
C3''—C4''	1.33 (2)	C3''—H3''	0.9600
C4''—H4''1	0.9600	C4''—H4''2	0.9600

Table-3.18: Selected Torsion Angles of Compound **(50)**

C9B—O4—C2—C1	−173.0 (4)	O9—C9—C9A—C5A	−179.3 (5)
C9B—O4—C2—C3	63.4 (5)	C8—C9—C9A—C9B	−175.3 (5)
O4—C2—C3—O2	−175.9 (4)	C1—C2—C3—O2	63.2 (6)
O4—C2—C3—C4	−55.9 (6)	C5—C5A—C9A—C9	−175.9 (5)
C1—C2—C3—C4	−176.8 (5)	C2—C3—C4—O3	173.2 (5)
O2—C3—C4—O3	−66.7 (6)	C6—C5A—C9A—C9B	176.2 (5)
O2—C3—C4—C4A	172.0 (4)	C2—C3—C4—C4A	51.9 (6)
C2—O4—C9B—C9A	173.2 (4)	O3—C4—C4A—O5	60.8 (6)
C2—O4—C9B—C4A	−66.3 (5)	C3—C4—C4A—O5	−179.4 (4)
C9—C9A—C9B—O4	−36.7 (7)	O3—C4—C4A—C9B	−176.5 (4)
C5A—C9A—C9B—O4	148.1 (4)	C3—C4—C4A—C9B	−56.8 (6)
C9—C9A—C9B—C4A	−155.4 (5)	C4—C4A—O5—C5	172.5 (4)
C9B—C4A—O5—C5	51.0 (6)	O5—C4A—C9B—O4	−176.1 (4)
C4A—O5—C5—O6	163.4 (5)	C4—C4A—C9B—O4	64.1 (5)
O5—C4A—C9B—C9A	−54.2 (6)	C4—C4A—C9B—C9A	−174.0 (4)
O6—C5—C5A—C9A	170.0 (5)	O4—C2—C1—O1	−74.4 (6)
O5—C5—C5A—C6	177.3 (5)	C3—C2—C1—O1	48.3 (7)
C5—C5A—C6—C7	176.7 (5)	C6—C7—O7—C1'	−7.9 (8)
C5A—C6—C7—O7	180.0 (5)	C8—C7—O7—C1'	173.1 (5)
C7—C8—O8—C1'''	82.5 (8)	C9—C8—O8—C1'''	−103.7 (8)
C8—C9—O9—C1"	105.2 (6)	C9A—C9—O9—C1"	−75.6 (7)
C6—C7—C8—O8	175.4 (5)	C7—O7—C1'—C2'	−173.9 (5)
O7—C7—C8—C9	−179.4 (5)	O7—C1'—C2'—C3'	174.6 (6)
O8—C8—C9—C9A	−174.8 (5)	C1'—C2'—C3'—C4'	75.0 (11)
C1"—C2"—C3"—C4"	−122.1 (15)	C9—O9—C1"—C2"	−174.7 (8)
O9—C1"—C2"—C3"	48.1 (18)	C7—C8—C9—O9	178.2 (5)

Table-3.19: Selected Hydrogen Bonds (Å)

D—H···*A*	*D*—H	H···*A*	*D*···*A*
O1—H1J···O2^{i}	0.83	1.86	2.627 (6)
O2—H2F···O1^{ii}	0.83	1.87	2.670 (6)
D—H···*A*	*D*—H	H···*A*	*D*···*A*

Symmetry codes: (i) $x, y-1, z$; (ii) $-x, y+1/2, -z+1$; (iii) $-x, y+1/2, -z$.

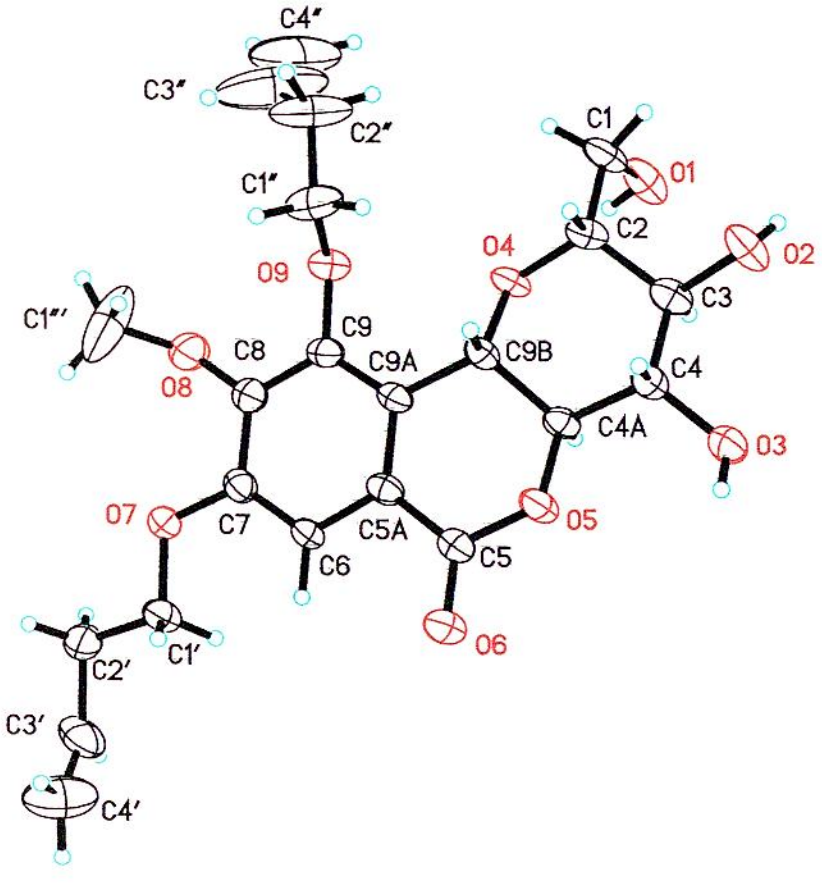


Figure-3.14: A thermal ellipsoid plot with atomic labeling of 7,9-bis (But-3-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-8- methoxy-2,3,4,4a-tetrahydro pyrano[3,2-*c*]isochromen-5(10bH)-one

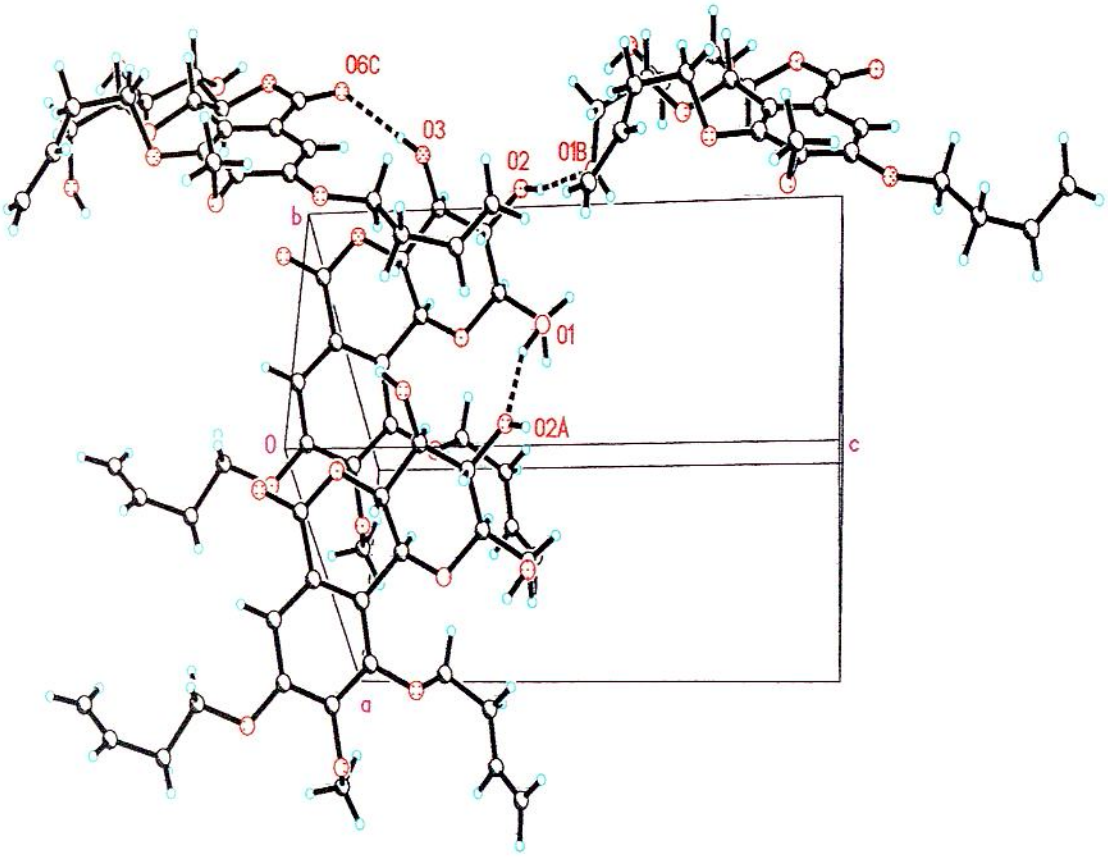


Figure-3.15: Packing diagram of the structure with hydrogen bonds viewed along the *b* axis

3.3.1.3. 3, 4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8, 10-bis(pentyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (51)

UV spectrum showed absorptions at λ_{max} nm 389 indicated the presence of aromatic system in the molecule.

IR (KBr): 3371cm^{-1} (OH), 3050 cm^{-1}(aromatic C-H), 2934 cm^{-1}(aliphatic C-H), 1729cm^{-1} (-CO-), 1332 cm^{-1} (C-O-C).

^{1}H-NMR spectrum of compound **(51)** showed multiplets at δ 1.40-1.52, 1.74 and 4.02 for the protons at C-16, 16a, 15, 15a, C-14,14a and C-13, 13a. The only triplet for CH_3 protons at δ 0.95 was assigned for the C-17, 17a due to presence of methylene protons (-CH_2-) at C-16, 16a. The remaining peaks i.e. doublet of doublet of doublet (*ddd*), doublet of doublet (*dd*), doublet of doublet (*dd*) and doublet of doublet (*dd*) at 3.50, 3.57, 3.82 and 3.91 were assigned to protons at C-2, C-3, C-4 and C-11, respectively. Two doublets (*d*) at δ 3.96 and 4.81 were assigned to protons of C-4a and C-10b, respectively. The singlets at 3.29 and 7.42 were due to C-12 and aromatic protons at C-7. The peaks appeared in the range 3.29-7.42 are the characteristics peaks of bergenin nucleus.

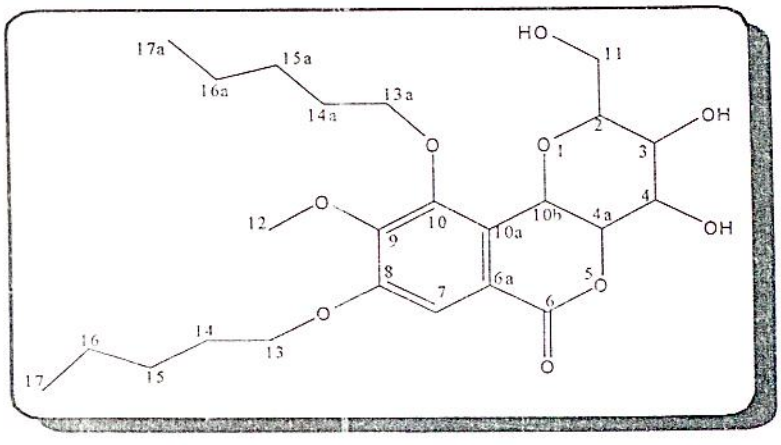


Table-3.20: ^{1}H-NMR (400 MHz, $CDCl_3$ + CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(pentyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(51)**

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-17, 17a	0.95	6H	*t*	7.02
H-16, 16a,15, 15a	1.40-1.52	8H	*m*	.....
H-14, 14a	1.74	4H,	*m*	.....
H-12	3.29	3H	*s*	.....
H-2	3.50	1H	*ddd*	5.02, 3.80,2.01
H-3	3.57	1H	*dd*	9.81, 8.87
H-4	3.82	1H	*dd,*	9.03, 8.86
H-11	3.91	2H	*dd*	8.80, 4.78
H-4a	3.96	1H	*d*	9.70, 7.68
H-13, 13a	4.02	4H	*m*	.....
H-10b	4.81	1H	*d*	10.3
H-7	7.42	1H	*s*	.....

^{13}C-NMR spectrum of compound **(51)** showed the additional signals at δ 70.2, 75.6, 29.9, 31.0, 29.3, 29.4, 23.4, 23.6, 14.3 and 14.4 for the C-13, 13a, C-14, 14a, C-15, 15a, C-16, 16a and C-17, 17a. The remaining signals in the range 82.4-61.6 were assigned to the basic carbon skeleton of bergenin nucleus, The signals at δ 82.4, 71.3, 75.9, 81.7, 166.0, 127.6, 111.5, 154.5, 150.1, 151.8, 120.4, 75.9, 62.2 and 61.2 were assigned to various carbon at C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12.

Table-3.21: ^{13}C-NMR (125 MHz, CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(pentyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(51)**

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.4	C-9	150.1	C-14	29.9
C-3	71.3	C-10	151.8	C-14a	31.0
C-4	75.9	C-10a	120.4	C-15	29.3
C-4a	81.7	C-10b	75.9	C-15a	29.4
C-6	166.0	C-11	62.2	C-16	23.4
C-6a	127.6	C-12	61.6	C-16a	23.6
C-7	111.5	C-13	70.2	C-17	14.3
C-8	154.5	C-13a	75.6	C-17a	14.4

EI-MS m/z: 468 [M^+] (calcd. 468.537 for $C_{24}H_{36}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure **(51)** was identified as 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(pentyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one

3.3.1.4. 8,10-Diethoxy-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (52)

UV spectrum showed absorptions at λ_{max} nm 388 indicated the presence of aromatic system in the molecule.

IR (KBr): 3387 cm^{-1} (OH), 3070 cm^{-1}(aromatic C-H), 2978 cm^{-1} (aliphatic C-H) 1709 cm^{-1}(-CO-), 1329 cm^{-1} (C-O-C).

^{1}H-NMR spectrum of compound **(52)** showed triplets at δ 1.38 and 1.44 9 due to protons at C-14 and 14a. The only multiplet at 4.12 was due to the protons attached to C-13 and 13a. The remaining peaks doublet of doublet (*dd*) at 3.27, doublet of doublet of doublet (*ddd*) at 3.64, doublet of doublet (*dd*) at 3.82, doublet of doublet (*dd*) at 3.62-3.84,

doublet of doublet (*dd*) at 4.03 and doublet (*d*) at 4.83 were assigned to protons attached to carbon 3, C-2,C-4, C-11, C-4a and C-10b, respectively. Similarly the two singlets at 3.86 and 7.43 were assigned to protons at carbon 12 and aromatic proton at carbon 7.

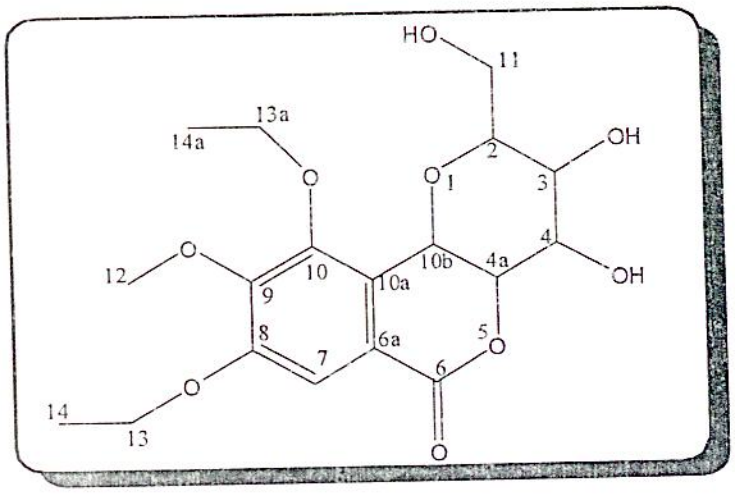


Table-3.22: ^{1}H-NMR (300 MHz, CD_3OD) data of 8,10-Diethoxy-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(52)**

Assignment	δ (ppm)	Integration	Multiplicity	*J* (Hz)
H-14	1.38	3H	*t*	6.98
H-14a	1.44	3H	*t*	6.94
H-3	3.27	1H	*dd*	9.82, 8.87
H-2	3.64	1H	*ddd,*	5.03, 3.82,2.02
H-4	3.82	1H,	*dd*	9.03, 8.84
H-11	3.62-3.84	2H,	*dd*	8.80, 4.78
H-12	3.86	3H,	*s,*	.....
H-4a	4.03	1H,	*dd,*	9.7, 7.68
H-13, 13a	4.12	4H	*m*	.....
H-10b	4.83	1H	*d*	10.3
H-7	7.43	1H	*s*	.....

^{13}C-NMR spectrum of compound **(52)** showed signals at δ 82.5, 71.4, 75.9, 81.7, 165.9, 127.8, 111.8, 154.2, 150.3, 151.5, 120.3, 73.3, 62.4 and 61.4 for the C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12, respectively. Those are the characteristics signals of bergenin nucleus in ^{13}C-NMR spectra. The signals at δ 65.9, 15.0 and 15.9 were the additional signals, which appeared due to C-13, 13a, C-14 and 14a, respectively.

Table-3.23: ^{13}C-NMR (125 MHz, $CDCl_3$) data of 8,10-Diethoxy-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(52)**

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.5	C-7	111.8	C-11	62.4
C-3	71.4	C-8	154.2	C-12	61.4
C-4	75.9	C-9	150.3	C-13,13a	65.9
C-4a	81.7	C-10	151.5	C-14	15.0
C-6	165.9	C-10a	120.3	C-14a	15.9
C-6a	127.8	C-10b	73.3	.....	.....

EI-MS m/z: 384 [M^+] (calcd. 384.378 for $C_{18}H_{24}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure **(52)** was identified as 8,10-Diethoxy-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.5. 3, 4-Dihydroxy-2-(hydroxymethyl)-8, 9, 10-trimethoxy-2, 3, 4, 4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (53)

UV spectrum showed absorptions at λ_{max} nm 388 indicated the presence of aromatic system in the molecule..

IR (KBr): 3564cm^{-1} (OH), 3058cm^{-1} (aromatic C-H), 2946cm^{-1} (aliphatic C-H), 1718cm^{-1} (-CO-), 1338 cm^{-1} (C-O-C).

^{1}H-NMR spectrum of compound **(53)** showed singlet at δ 3.30 for the protons at C-13, 13a and C-12. Similarly doublet of doublet (*dd*) at 3.38, doublet of doublet (*dd*) at 3.64-3.89, doublet of doublet of doublet (*ddd*) at 3.67, doublet of doublet (*dd*) at 3.80, doublet of doublet (*dd*) at 4.06 and doublet at 4.94 were assigned to protons of bergenin nucleus at C-3, C-11, C-2, C-4, 4a and 10b, respectively. The downfield singlet at 7.45 was assigned to aromatic proton at C-7.

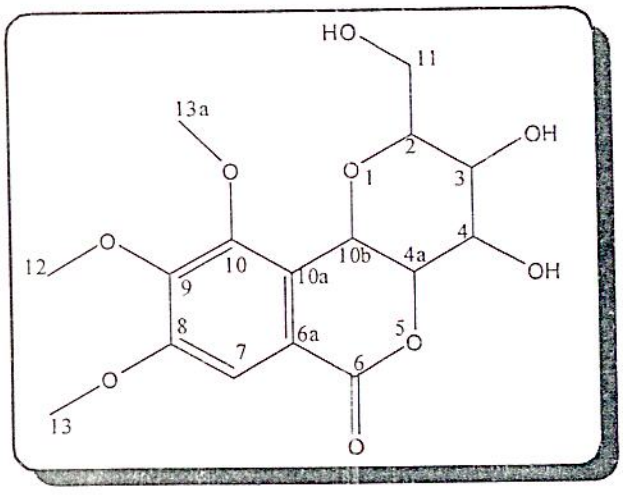


Table-3.24: ^{1}H-NMR (500 MHz, CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-8,9,10-trimethoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(53)**

Assignment	δ (ppm)	Integration	Multiplicity	*J* (Hz)
H-13, 13a, 12	3.30	9H	*s*	.....
H-3	3.38	1H	*dd*	9.83, 8.82
H-11	3.64-3.89	2H	*dd*	8.75, 3.92
H-2	3.67	1H	*ddd*	4.66, 3.02,1.80
H-4	3.80	1H	*dd,*	9.32, 8.75
H-4a	4.06	1H	*dd*	9.92, 6.98
H-10b	4.94	1H	*d,*	10.38
H-7	7.45	1H	*s*	.....

^{13}C-NMR spectrum of compound **(53)** showed signals at δ 82.6, 71.6, 75.9, 81.7, 165.9, 127.8, 110.7, 155.0, 150.1, 152.5, 120.4, 73.1, 62.5 and 62.2 for C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12, respectively. These were the characteristics peaks for bergenin nucleus. The signals at δ 56.6 and 61.5 were the additional signals observed for the attached moeities at C-8 and C-10.

Table-3.25: ^{13}C-NMR (75 MHz, CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-8,9,10-trimethoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(53)**

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.6	C-7	110.7	C-11	62.5
C-3	71.6	C-8	155.0	C-12	62.2
C-4	75.9	C-9	150.1	C-13	56.6
C-4a	81.7	C-10	152.5	C-13a	61.5
C-6	165.9	C-10a	120.4	.....	.....
C-6a	127.8	C-10b	73.1	.....	.....

EI-MS m/z: 356 [M^+] (calcd. 356.325 for $C_{16}H_{20}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure (**53**) was identified as 3,4-Dihydroxy-2-(hydroxymethyl)-8,9,10-trimethoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.6. 8,10-bis(Hex-5-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (54)

UV spectrum showed absorptions at λ_{max} nm 390 indicated the presence of aromatic system in the molecule.

IR (KBr):3354 cm^{-1} (OH), 3080 cm^{-1} (aromatic C-H) 2936 cm^{-1} (aliphatic C-H), 1732 cm^{-1} (-CO-), 1641 cm^{-1} (C=C) 1331 cm^{-1} (C-O-C).

^{1}H-NMR spectrum of the compound (54) showed multiplets at δ 1.60, 1.81, 2.14, 4.00, 4.99 and 5.83 for protons attached to C-15, 15a, C-14, 14a, C-16, 16a, C-13, 13a, C-18, 18a, C-17, and 17a, respectively. Beside these, doublet of doublet (*dd*) at δ 3.68-3.89, doublet of doublet of doublet (*ddd*) at δ 3.68, doublet of doublet (*dd*) at δ 3.85, doublet of doublet (*dd*) at δ 4.06 and doublet (*d*) at δ 4.90 were appeared due to protons at C-11, C-2, C-4, 4a and C-10b, respectively. Similarly the two singlets at 12 and 7 were assigned to three protons of C-12 and aromatic proton at position 7. Those are the characteristics peaks for bergenin nucleus.

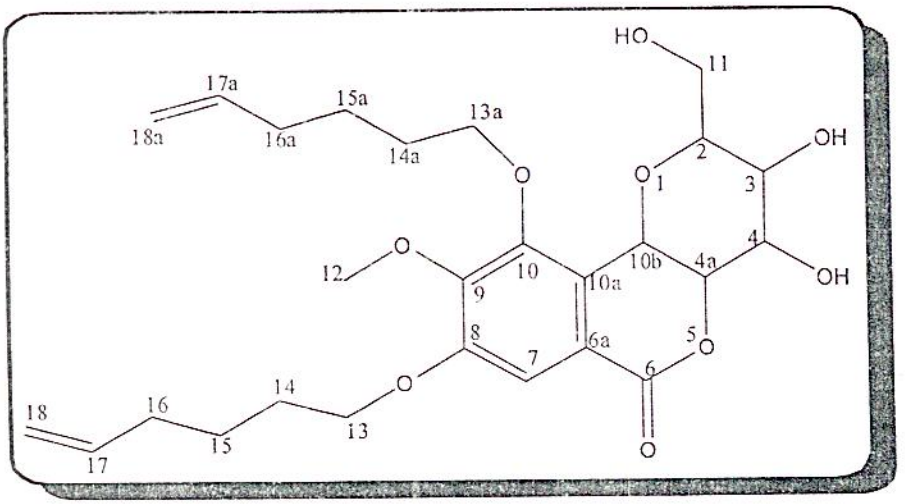


Table-3.26: ^{1}H-NMR (500 MHz, CD_3OD) data of 8,10-bis(Hex-5-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(54)**

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-15, 15a	1.60	4H	*m*	.....
H-14, 14a	1.81	4H	*m*	.....
H-16, 16a	2.14	4H	*m*	.....
H-11	3.68-3.89	2H	*dd*	8.87, 4.76
H-2	3.68	1H,	*ddd,*	5.00, 3.82, 2.02
H-4	3.85	1H	*dd*	9.02, 8.82
H-12	3.90	3H	*s*	.....
H-4a	4.06	1H	*dd*	9.8, 7.71
H-13, 13a	4.00	4H	*m*	.....
H-10b	4.90	1H	*d,*	10.3
H-18, 18a	4.99	4H,	*m,*	.....
H-17, 17a	5.83	2H	*m*	.....
H-7	7.42	1H	*s*	.....

^{13}C-NMR spectrum of compound (54) showed peaks at δ 82.4, 1.2, 75.9, 81.7, 166.0, 127.7, 111.7, 154.4, 150.1, 150.1, 120.4, 75.4, 62.3 and 61.6 for the carbons C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12, respectively, which are the characteristic peaks for bergenin nucleus. The peaks at δ 70.1, 75.4, 29.6, 30.7, 26.4, 26.5, 34.4, 34.6, 139.6, 139.8, 115.0 and 115.2 were assigned to C-13, 13a, C-14, 14a, C-15, 15a, C-16, 16a, C-17, 17a and C-18,18a.

Table-3.27: ^{13}C-NMR (125 MHz, CD_3OD) data of 8,10-bis(Hex-5-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (**54**)

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.4	C-10	150.1	C-15	26.4
C-3	71.2	C-10a	120.4	C-15a	26.5
C-4	75.9	C-10b	75.4	C-16	34.4
C-4a	81.7	C-11	62.3	C-16a	34.6
C-6	166.0	C-12	61.6	C-17	139.6
C-6a	127.7	C-13	70.1	C-17a	139.8
C-7	111.7	C-13a	75.4	C-18	115.0
C-8	154.4	C-14	29.6	C-18a	115.2
C-9	150.1	C-14a	30.7	.....	.....

EI-MS m/z: 492.3 [M^+] (calcd. 492.559 for $C_{26}H_{36}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure (**54**) was identified as 8,10-bis(Hex-5-enyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.7. 8,10-bis(Heptyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (55)

UV spectrum showed absorptions at λ_{max} nm 388 indicated the presence of aromatic system in the molecule.

IR (KBr): 3380 cm^{-1} (OH), 3057 cm^{-1}(aromatic C-H), 2927 cm^{-1} (aliphatic C-H), 1730 cm^{-1} (-CO-), 3131 cm^{-1} (C-O-C).

^{1}H-NMR spectrum of the compound (**55**) multiplets at δ 1.91, 1.33, 1.48, 1.79 and 4.00 were assigned to protons at C-19, 19a, C-18, 18a, C-17, 17a, C-16, 16a, C-15, 15a, C-14,14a and C-13,13a. Those peaks represent the attachment of unsaturated alkyl

branches at C-8 and C-10 on bergenin nucleus. The remaining peaks doublet of doublet (*dd*) at δ 3.65-3.88, doublet of doublet of doublet (*ddd*) at 3.66, doublet of doublet (*dd*) at 3.28, doublet of doublet (*dd*) at 3.82, doublet of doublet (*dd*) at 4.04 and doublet (*d*) at 4.83 were assigned to protons at C-11, C-2, C-3, C-4, 4a and C-10b, respectively. Similarly two singlets at 12 and 7 were assigned to three protons attached to C-12 and aromatic proton at C-7.

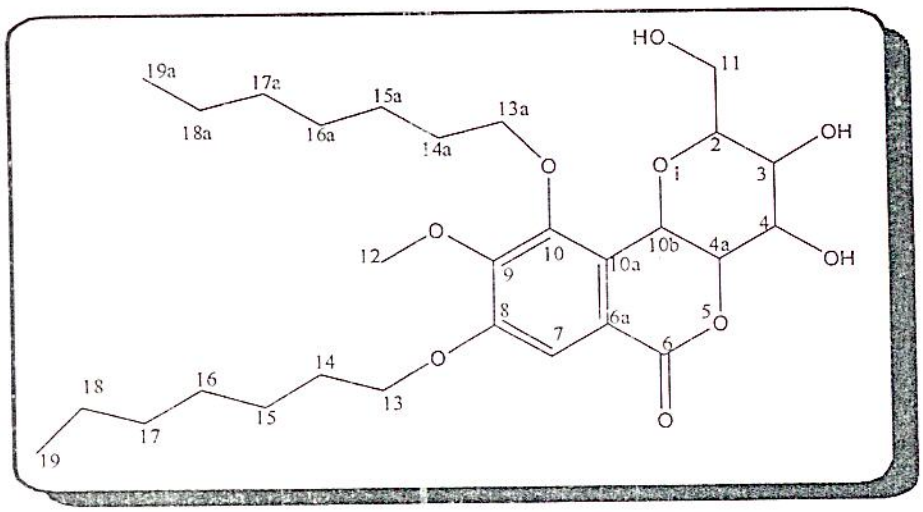


Table-3.28: ^{1}H-NMR (500 MHz, CD_3OD) data of 8,10-bis(Heptyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(55)**

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-19, 19a	1.91	6H	*m*	.....
H-18, 18a, 17, 17a, 16, 16a	1.33	12H	*m*	.....
H-15, 15a	1.48	4H	*m*	.....
H-14, 14a	1.79	4H	*m*	.....
H-3	3.28	1H,	*dd*	9.82, 8.87
H-11	3.65-3.88	2H,	*dd*	8.84, 4.75
H-2	3.66	1H	*ddd,*	5.01, 3.82, 2.02
H-4	3.82	1H,	*dd*	9.01, 8.82
H-12	3.86	3H	*s*	.....
H-13, 13a	4.00	4H,	*m*	.....
H-4a	4.04	1H	*dd,*	9.91, 7.64
H-10b	4.83	1H,	*d*	10.3
H-7	7.42	1H	*s*	.....

^{13}C-NMR spectrum of compound **(55)** showed signals for the basic bergenin carbon nucleus at δ 82.5, 71.3, 75.9, 81.7, 166.0, 127.6, 111.7, 154.5, 150.1, 151.8, 120.4, 73.3, 62.3 and 61.6, along with additional signals at position δ 70.3, 75.6, 33.0, 32.9, 32.9, 27.1, 30.1, 30.2, 30.3, 31.3, 23.6, 23.7, 14.3 and 14.4 for the carbons C-13, 13a, C-14, 14a, C-15, 15a, C-16, 16a, C-17, 17a, C-18, 18a and C-19, 19a, respectively.

Table-3.29: ^{13}C-NMR (125 MHz, CD_3OD+$CDCl_3$) data of 8,10-bis(Heptyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (55)

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.5	C-10a	120.4	C-16	30.1
C-3	71.3	C-10b	73.3	C-16a	30.2
C-4	75.9	C-11	62.3	C-17	30.3
C-4a	81.7	C-12	61.6	C-17a	31.3
C-6	166.0	C-13	70.3	C-18	23.6
C-6a	127.6	C-13a	75.6	C-18a	23.7
C-7	111.7	C-14	33.0	C-19	14.3
C-8	154.5	C-14a	32.9	C-19a	14.4
C-9	150.1	C-15	32.9	.....	.....
C-10	151.8	C-15a	27.1	.....	.....

EI-MS m/z: 524.3 [M^+] (calcd. 524.644 for $C_{28}H_{44}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure (**55**) was identified as 8,10-bis(Heptyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.8. 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(prop-2-ynyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (56)

UV spectrum showed absorptions at λ_{max} nm 390 indicated the presence of aromatic system in the molecule.

IR (KBr): 3397 cm^{-1} (OH) 3288 cm^{-1} (acetylenic C-H), 3060 cm^{-1}(aromatic C-H) 2935 cm^{-1} (aliphatic C-H), 2125 cm^{-2} C≡C, 1708 cm^{-1} (-CO-), 1327 cm^{-1} (-C-O-C).

^{1}H-NMR spectrum of compound **(56)** showed singlets at δ 3.02, 3.91, 4.73, 4.74 and 7.61 for the protons attached to carbons C-15,15a, C-12, C-13, 13a and C-7. The singlet at δ 3.02, 4.37 and 4.37 were the additional peaks assigned for the moieties at C-8 and C-10 in bergenin nucleus. The remaining peaks in the spectra, doublet of doublet (*dd*) at δ 3.27, doublet of doublet of doublet (*ddd*) at δ 3.67, doublet of doublet, doublet (*dd*) at δ 3.68-3.90, doublet of doublet (*dd*) at δ 3.86 and doublet (*d*) at δ 4.90 were assigned to protons at C-3, C-2, C-11, C-4, 4a and C-10b. These are the basic peaks in the ^{1}H-NMR spectra for bergenin.

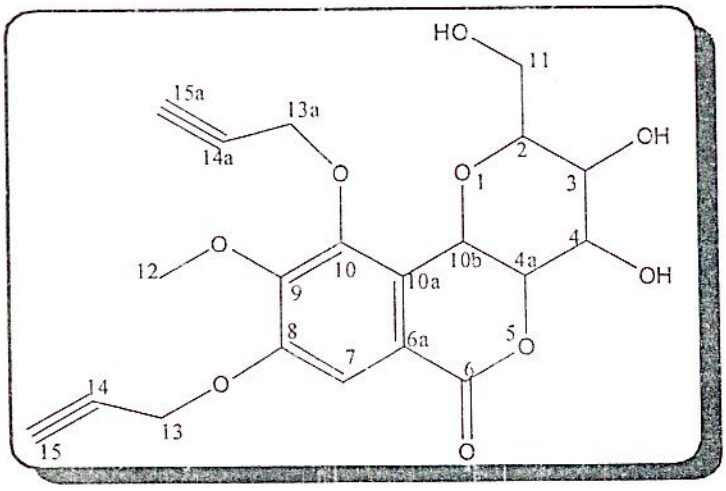


Table-3.30: ^{1}H-NMR (500 MHz, CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(prop-2-ynyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(56)**

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-15, 15a	3.02	2H	*s*	.....
H-3	3.29	1H	*dd,*	9.84, 8.86
H-2	3.67	1H	*ddd*	5.00, 3.84, 2.02
H-11	3.68-3.90	2H	*dd*	8.89, 4.77
H-4	3.86	1H	*dd*	9.02, 8.84
H-12	3.91	3H	*s*	.....
H-4a	4.07	1H	*dd*	9.90, 7.78
H-13	4.73	1H	*s*	.....
H-13a	4.74	1H	*s*	.....
H-10b	4.90	1H	*d*	10.31
H-7	7.61	1H	*s*	.....

^{13}C-NMR spectrum of compound **(56)** the signals at δ 150.0, 150.7, 120.0 and 77.7 were assigned to C-13, 13a, C-14, 14a and C-15,15a. Similarly the signals at δ 82.3, 71.2, 75.8, 81.5, 165.4, 128.8, 113.7, 152.6, 150.0, 150.7, 120.0, 77.7, 62.6 and 62.1 were assigned to the C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12.

Table-3.31: ^{13}C-NMR (125 MHz, CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(prop-2-ynyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (**56**)

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.3	C-7	113.7	C-11	62.6
C-3	71.2	C-8	152.6	C-12	62.1
C-4	75.8	C-9	150.0	C-13	57.7
C-4a	81.5	C-10	150.7	C-13a	61.8
C-6	165.4	C-10a	120.0	C-14,14a	77.7
C-6a	128.8	C-10b	77.7	C-15,15a	76.7

EI-MS m/z: 404.1 [M^+] (calcd. 404.367 for $C_{20}H_{20}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure (**56**) was identified as 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(prop-2-ynyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.9. 8,10-bis(Allyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (57)

UV spectrum showed absorptions at λ_{max} nm 388 indicated the presence of aromatic system in the molecule.

IR (KBr): 3319 cm^{-1} (OH), 3085 cm^{-1}(aromatic C-H), 2936 cm^{-1} (aliphatic C-H), 1737cm^{-1} (-CO-), 1647cm^{-1} (C=C), 1329 cm^{-1} (-C-O-C).

^{1}H-NMR spectrum of the compound (**57**) showed multiplets at position δ 5.34 and 6.10 for protons attached to C-15, 15a and C-14, 14a. The remaining peaks, doublet of doublet (*dd*) at 3.27, doublet of doublet of doublet (*ddd*) at 3.65, doublet of doublet (*dd*) at 3.65-3.88, doublet of doublet (*dd*) at 3.84, doublet of doublet (*dd*) at 4.05, doublet of doublet (*dd*) at 4.81 and doublet (*d*) at 4.89 were assigned to protons attached to C-3, C-2, C-11,

C-4, 4a, C-13, 13a and C-10b. The singlets at δ 3.88 and 7.45 were assigned to protons attached to C-12 and aromatic proton at C-7.

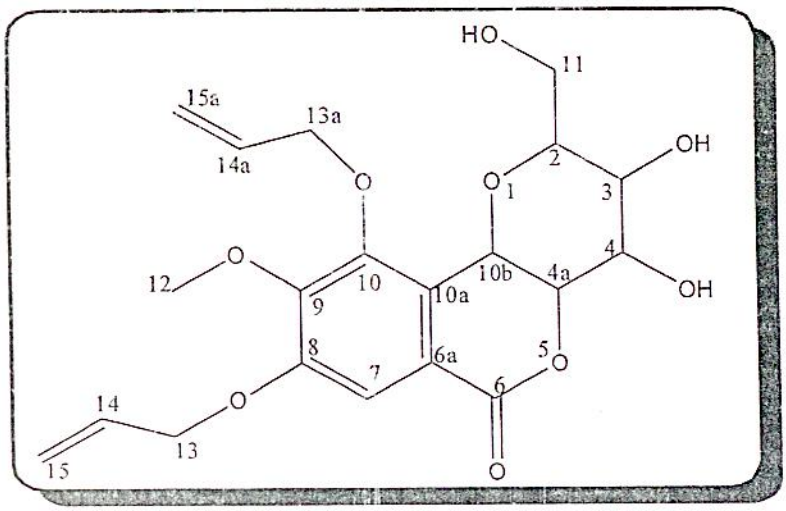


Table-3.32: ^{1}H-NMR (400 MHz, CD_3OD) data of 8,10-bis(Allyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (57)

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-3	3.27	1H	*dd*	9.83, 8.89
H-2	3.65	1H	*ddd*	5.01, 3.82, 2.01
H-11	3.65-388	2H,	*dd*	8.84, 4.75
H-4	3.84	1H	*dd,*	9.01, 8.83
H-12	3.88	3H	*s*	.....
H-4a	4.05	1H	*dd*	9.8, 7.70
H-13	4.81	2H	*dd,*	13.82
H-13a	4.89	2H	*d*	10.2
H-15, 15a	5.34	4H	*m*	.....
H-14, 14a	6.10	2H	*m,*	.....
H-7	7.45	1H,	*s*	.....

^{13}C-NMR spectrum of compound **(57)** showed signals at δ 153.9, 150.4, 151.3, 120.3, 76.0 and 62.3 for carbons at C-13, 13a, C-14, 14a, C-15 and 15a. Similarly the characteristics peaks for beergenin nucleus at δ 82.5, 71.4, 75.9, 81.7, 165.8, 128.1, 112.6, 153.9, 150.4, 151.6, 120.3, 76.0, 62.3 and 61.6 were assigned to C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12.

Table-3.33: ^{13}C-NMR (125 MHz, $CDCl_3$) data of 8,10-bis(Allyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(57)**

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.5	C-8	153.9	C-13	70.9
C-3	71.4	C-9	150.4	C-13a	73.2
C-4	75.9	C-10	151.3	C-14	134.1
C-4a	81.7	C-10a	120.3	C-14a	135.7
C-6	165.8	C-10b	76.0	C-15	117.8
C-6a	128.1	C-11	62.3	C-15a	118.2
C-7	112.6	C-12	61.6	.....	.....

EI-MS m/z: 408.0 [M^+] (calcd. 408.399 for $C_{20}H_{24}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure **(57)** was identified as 8,10-bis(Allyloxy)-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.10. 2,2'-(3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetonitrile (58)

UV spectrum showed absorptions at λ_{max} nm 389 indicated the presence of aromatic system in the molecule.

IR (KBr): 3529 cm^{-1} (OH), 3080.8 cm^{-1}(aromatic C-H), 2992 cm^{-1} (aliphatic C-H), 2230cm^{-1} (CN), 1729cm^{-1} (-CO-), 1320 cm^{-1} (C-O-C).

^{1}**H-NMR** spectrum of compound **(58)** showed singlet at δ 5.13 for C-13 and 13a, while two singlets at 3.88 and 7.67 for the protons attached to C-12 and aromatic proton at C-7 were also observed. Beside this doublet of doublet (*dd*) at 3.28, doublet of doublet of doublet (*ddd*) at 3.66, doublet of doublet (*dd*) in the range of 3.66-3.87, doublet of doublet (*dd*) at 3.83, doublet of doublet (*dd*) at 4.06 and doublet at 4.90 were assigned to the protons C-3, C-2, C-11, C-4, 4a and C-10b, respectively.

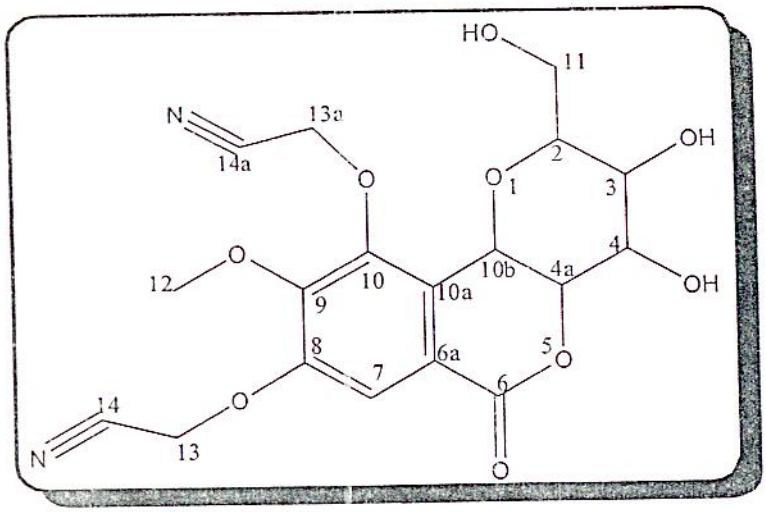


Table-3.34: ^{1}H-NMR (500 MHz, CD_3OD) data of 2,2'-(3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetonitrile **(58)**

Assignment	δ (ppm)	Integration	Multiplicity	*J* (Hz)
H-3	3.28	1H	*dd*	9.83, 8.87
H-2	3.66	1H	*ddd*	5.02, 3.83, 2.00
H-11	3.66-387	2H	*dd*	8.84, 4.75
H-4	3.83	1H	*dd*	9.02, 8.84
H-12	3.88	3H	*s*	.....
H-4a	4.06	1H	*dd*	9.9, 7.64
H-10b	4.90	1H	*d*	10.2
H-13, 13a	5.13	4H	*s*	.....
H-7	7.67	1H	*s*	.....

^{13}C-NMR spectrum of compound **(58)** showed signals at δ 55.7, 59.8, 116.3 and 117.4 for the C-13, 13a, C-14 and 14a. Similarly the signals at δ 82.7, 71.6, 75.8, 81.6, 164.8, 130.3, 114.7 and 151.9 were assigned to C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12 of bergenin nucleus.

Table-3.35: ^{13}C-NMR (125 MHz, CD_3OD) data of 2,2'-(3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetonitrile **(58)**

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.7	C-7	114.7	C-11	62.4
C-3	71.6	C-8	151.9	C-12	62.2
C-4	75.8	C-9	150.1	C-13	55.7
C-4a	81.6	C-10	150.7	C-13a	59.8
C-6	164.8	C-10a	120.8	C-14	116.3
C-6a	130.3	C-10b	72.7	C-14a	.....

EI-MS m/z: 406.1 [M^+] (calcd. 408.344 for $C_{18}H_{18}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure **(58)** was identified as 2,2'-(3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetonitrile.

3.3.1.11. 8,10-Dibutoxy-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (59)

UV spectrum showed absorptions at λ_{max} nm 389 indicated the presence of aromatic system in the molecule.

IR (KBr): 3312cm^{-1} (OH), 3070 cm^{-1}(aromatic C-H), 2958 cm^{-1} (aliphatic C-H), 1718cm^{-1} (-CO-), 1313 cm^{-1} (-C-O-C).

^{1}H-NMR spectrum of compound **(59)** showed a number of multiplets at δ 0.99, 1.53, 1.78 and 4.05 for the protons attached to C-16,16a, C-15, 15a, C-14, 14a and C-13, 13a. The peaks, doublet of doublet (*dd*) at 3.28, doublet of doublet of doublet (*ddd*) at 3.66, doublet of doublet (*dd*) at 3.82, doublet of doublet (*dd*) at 4.15, doublet (*d*) at 4.82 and doublet of doublet (*dd*) at 3.65-3.80 were assigned to protons attached to C-2, C-10, C-4, 4a and C-10b, C-11, respectively.

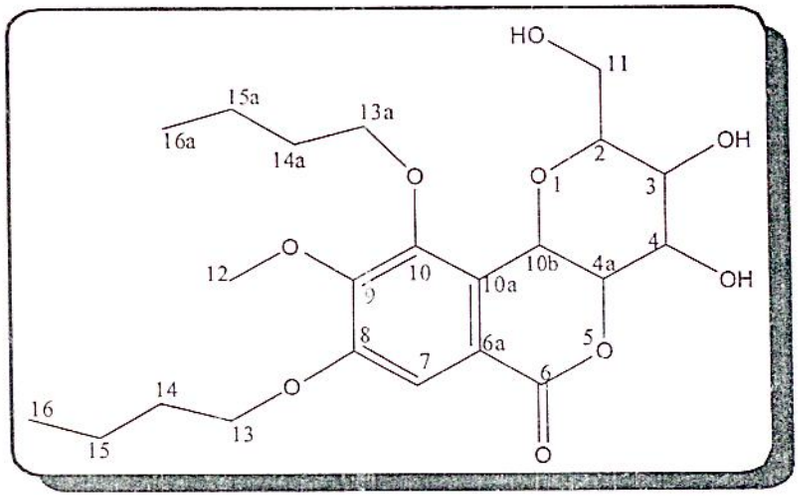


Table-3.36: ^{1}H-NMR (400 MHz, CD_3OD) data of 8,10-Dibutoxy-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (**59**)

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-16. 16a	0.99	6H	*m*	.....
H-15, 15a	1.53	4H	*m*	.....
H-14, 14a	1.78	4H	*m*	.....
H-2	3.28	1H	*dd*	9.82, 8.87
H-11	3.65-3.86	2H	*dd*	8.80-4.71
H-10	3.66	1H	*ddd*	5.01, 3.82, 2.02
H-4	3.82	4H	*dd*	9.01, 8.82
H-12	3.86	3H	*s*	.....
H-13, 13a	4.05	4H	*m*	.....
H-4a	4.15	1H	*dd*	9.9, 7.64
H-10b	4.82	1H	*d*	10.2
H-7	7.67	1H	*s*	.....

^{13}C-NMR spectrum of compound (**59**) showed signals at δ 77.1, 23.5, 24.4, 10.1, 10.7, 14.3 and 14.4 for C-13, 13a, C-14, 14a. C-15, 15a, C-16 and 16a, respectively. Beside these signals at δ 82.5, 71.4, 75.9, 81.7, 165.8, 127.6, 111.7, 154.5, 150.1, 151.8, 120.4, 71.8, 62.3 and 61.6 were assigned to C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12, respectively.

Table-3.37: ^{13}C-NMR (75 MHz, CD_3OD) data of 8,10-Dibutoxy-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (59)

Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.5	C-10b	71.8
C-3	71.4	C-11	62.3
C-4	75.9	C-12	61.6
C-4a	81.7	C-13,13a	77.1
C-6	165.8	C-14	23.5
C-6a	127.6	C-14a	24.4
C-7	111.7	C-15	10.1
C-8	154.5	C-15a	10.7
C-9	150.1	C-16	14.3
C-10	151.8	C-16a	14.4
C-10a	120.4	.....	.....

EI-MS m/z: 440.2 [M^+] (calcd. 440.484 for $C_{22}H_{32}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure (**59**) was identified as 8,10-Dibutoxy-3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.12. 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(octyloxy)-2,3,4,4a- tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (60)

UV spectrum showed absorptions at λ_{max} nm 389 indicated the presence of aromatic system in the molecule.

IR (KBr): 3351 cm^{-1} (OH), 3060 cm^{-1}(aromatic C-H), 2929 cm^{-1} (aliphatic C-H), 1734cm^{-1} (-CO-), 1331 cm^{-1}(-C-O-C).

^{1}H-NMR spectrum of the compound (60) showed multiplets at δ 0.89, 1.41, 1.80 and 4.02 for the protons attached to C-20, 20a, C-19, 19a, C-18, 18a, C-17, 17a, C-16, 16a, C-15, 15a, C-14, 14a and C-13, 13a, respectively. Beside these, doublet of doublet (*dd*) at 3.28, doublet of doublet of doublet (*ddd*) at 3.66, doublet of doublet (*dd*) in the range of 3.69-3.84, doublet of doublet (*dd*) at 3.78, doublet of doublet (*dd*) at 4.12 and a doublet (*d*) at 4.72 were assigned to protons attached to C-3, C-2, C-11, C- 4, 4a and 10b, respectively. The two singlets at δ 3.88 and 7.67 were due to protons at C-12 and single aromatic proton at C-7.

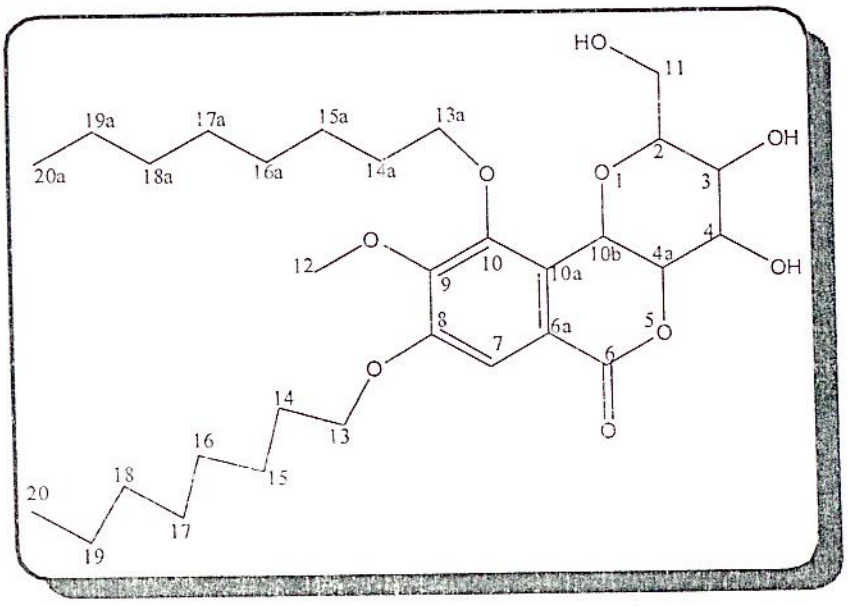


Table-3.38: ^{1}H-NMR (400 MHz, CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(octyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(60)**

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-20, 20a	0.89	6H	*m*	.....
H-19, 19a, 18, 18a, 17, 17a, 16, 16a	1.41	16H,	*m*	.....
H-15, 15a, 14, 14a	1.80	8H	*m*	.....
H-3	3.28	1H	*dd*	9.83, 8.86
H-2	3.66	1H	*ddd*	4.90, 3.76, 1.98
H-11	3.69-3.84	2H,	*dd*	8.73, 3.78
H-4	3.78	1H	*dd*	9.34, 8.78
H-12	3.88	3H	*s*	.....
H-13, 13a	4.02	4H	*m*	.....
H-4a	4.12	1H	*dd*	9.84, 6.62
H-10b	4.72	1H	*d*	10.26
H-7	7.67	1H	*s*	.....

^{13}C-NMR spectrum of compound **(60)** showed signals at δ 70.3, 75.6, 30.2, 30.3, 27.1, 27.1, 30.5, 31.3, 30.3, 30.4, 32.9, 33.0, 23.6, 23.7 and 14.4 for the C-13, 13a, C-14, 14a, C-15, 15a, C-16, 16a, C-17, 17a, C-18, 18a, C-19, 19a and C-20, 20a, respectively. The signals observed at δ 82.5, 70.3, 75.9, 81.7, 166.0, 127.6, 111.7, 154.5, 150.1, 151.8, 120.4, 75.9, 62.3 and 61.6 were due to the carbons of bergenin nucleus C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, C-10b, C-11 and C-12.

Table-3.39: ^{1}C-NMR (125 MHz, CD_3OD) data of 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(octyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(60)**

Assignment	δ (ppm	Assignment	δ (ppm)
C-2	82.5	C-13a	75.6
C-3	70.3	C-14	30.2
C-4	75.9	C-14a	30.3
C-4a	81.7	C-15	27.1
C-6	166.0	C-15a	27.1
C-6a	127.6	C-16	30.5
C-7	111.7	C-16a	31.3
C-8	154.5	C-17	30.3
C-9	150.1	C-17a	30.4
C-10	151.8	C-18	32.9
C-10a	120.4	C-18a	33.0
C-10b	75.9	C-19	23.6
C-11	62.3	C-19a	23.7
C-12	61.6	C-20,20a	14.4
C-13	70.3	.....	.....

EI-MS m/z: 552 [M^+] (calcd. 552.697 for $C_{30}H_{48}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure **(60)** was identified as 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-bis(octyloxy)-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.13. 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-dipropoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (61)

UV spectrum showed absorptions at λ_{max} nm 389 indicated the presence of aromatic system in the molecule.

IR (KBr): 3353 cm^{-1} (OH), 3090 cm^{-1}(aromatic C-H), 2963 cm^{-1} (aliphatic C-H), 1735cm^{-1} (-CO-), 1330 cm^{-1} (-C-O-C).

^{1}H-NMR spectrum of the compound **(61)** showed multiplets at δ 1.06, 1.82 and 3.90 for the protons attached to C-15,15a, C-14,14a C-13, and 13a, respectively. Beside these, doublet of doublet (*dd*) at 3.29, doublet of doublet of doublet (*ddd*) at 3.68, doublet of doublet (*dd*) at 3.68-3.87, doublet of doublet (*dd*) at 3.79, doublet of doublet (*dd*) at 4.03 and doublet at 4.94 were assigned to the protons on bergenin nucleus at position C-3, C-2, C-11, C-4, 4a and C-10b, respectively. The singlets at δ 3.87 and 7.43 were assigned to protons at C-12 and C-7 (aromatic proton).

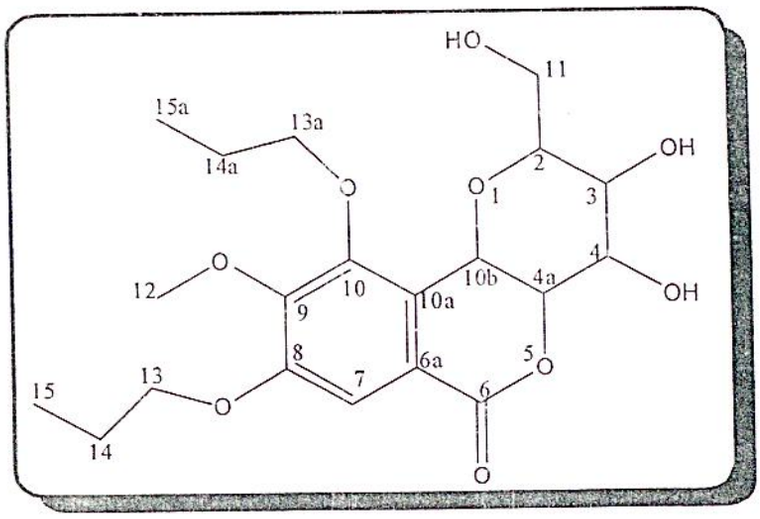


Table-3.40: ^{1}H-NMR (300 MHz, $CDCl_3$) data of 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-dipropoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one **(61)**

Assignment	δ (ppm)	Integration	Muitiplicity	J (Hz)
H-15, 15a	1.06	6H	*m*	.....
H-14, 14a	1.82	4H	*m*	.....
H-3	3.29	1H	*dd*	9.83, 8.88
H-2	3.68	1H,	*ddd*	4.91, 3.76,1.42
H-11	3.68-3.87	2H,	*dd*	= 8.73, 3.74
H-4	3.79	1H	*dd*	9.34, 8.74
H-13, 13a	3.90	4H	*m*	.....
H-4a	4.03	1H	*dd*	9.85, 6.63
H-10b	4.94	1H	*d*	10.38
H-7	7.43	1H	*s*	.....
H-12	3.87	1H	*s*	.....

^{13}C-NMR spectrum of compound **(61)** showed signals at δ 77.1, 23.5, 24.4, 10.7 and 10.8 for the protons at C-13, 13a, C-14, 14a, and C-15, 15a, respectively. Characteristics peaks for bergenin nucleus at δ 82.5, 71.4, 75.9, 81.8, 166.0, 127.7, 111.7, 154.5, 150.1, 151.8, 120.4, 71.8, 62.3 and 61.6 were assigned to C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12.

Table-3.41: ^{13}C-NMR (125 MHz, CD_3OD+$CDCl_3$) data of 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-dipropoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one (**61**)

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.5	C-8	154.5	C-13,13a	77.1
C-3	71.4	C-9	150.1	C-14	23.5
C-4	75.9	C-10	151.8	C-14a	24.4
C-4a	81.8	C-10a	120.4	C-15	10.7
C-6	166.0	C-10b	71.8	C-15a	10.8
C-6a	127.7	C-11	62.3	.....	.....
C-7	111.7	C-12	61.6	.....	.....

EI-MS m/z: 412 [M^+] (calcd. 412.431 for $C_{20}H_{28}O_9$).

On the basis of UV, IR, NMR and mass spectrum, the structure (**61**) was identified as 3,4-Dihydroxy-2-(hydroxymethyl)-9-methoxy-8,10-dipropoxy-2,3,4,4a-tetrahydropyrano[3,2-c]isochromen-6(10bH)-one.

3.3.1.14. Diethyl2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a, 6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate (62)

UV spectrum showed absorptions at λ_{max} nm 389 indicated the presence of aromatic system in the molecule.

IR (KBr): 3402 cm^{-1} (OH), 3086 cm^{-1}(aromatic C-H), 2934 cm^{-1} (aliphatic C-H), 1752cm (-CO-), 1376 cm^{-1} (C-O-C).

^{1}H-NMR spectrum of the compound (**62**) showed the two triplets (*t*) at position δ 1.26 and 1.34 for protons attached with C-16 and 16a, respectively. Beside these, quartet (*q*) at 2.85 and 2.98 were assigned to protons attached to C-15 and 15a, respectively. The singlet at 3.97 was observed due to protons at C-13, 13a. The peaks, doublet of doublet (*dd*) at δ 3.28, doublet of doublet (*dd*) at 3.64-3.88, doublet of doublet of doublet (*ddd*) at

3.66, doublet of doublet (*dd*) at 3.82, doublet of doublet (*dd*) at 4.05 and a doublet (*d*) at 4.82 were assigned to protons attached to C-3, C-11, C-2, C-4, 4a and C-10b, respectively. The two singlets at δ 3.86 and 7.38 were observed due to protons at C-12 and C-7.

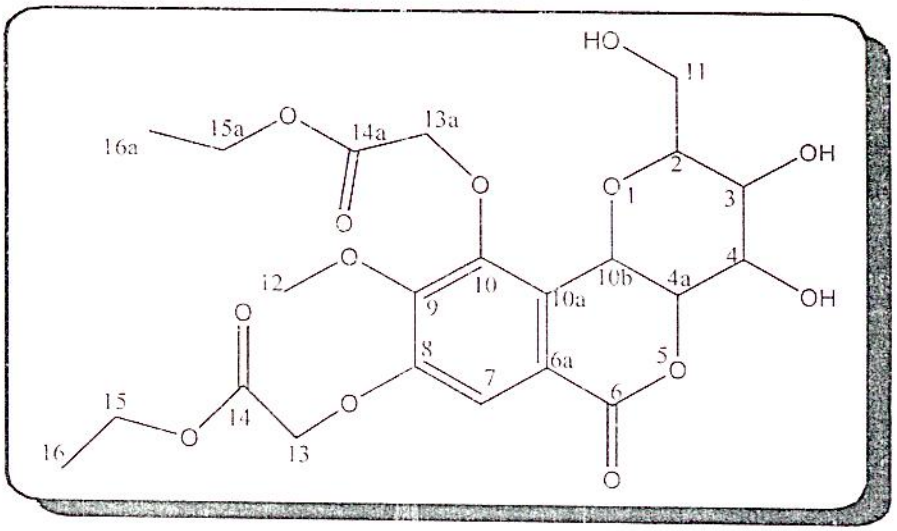


Table-3.42: ^{1}H-NMR (400 MHz, CD_3OD) data of Diethyl 2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate **(62)**

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-16	1.26	3H	*t*	7.26
H-16a	1.31	3H	*t*	7.32
H-15	2.85	2H	*s*	.....
H-15a	2.98	2H	*s*	.....
H-3	3.28	1H	*dd*	9.82, 8.88
H-11	3.64-3.88	2H	*dd*	8.84, 4.75
H-2	3.66	1H,	*ddd*	5.01, 3.82, 2.01
H-4	3.82	1H	*dd*	9.01, 8.82
H-12	3.86	3H	*s*	.....
H-4a	4.05	1H	*dd*	9.9, 7.64
H-13, 13a	3.97	4H	*s*	.....
H-10b	4.82	1H	*d*	10.2
H-7	7.38	1H	*s*	.....

^{13}C-NMR spectrum of compound **(62)** showed signals at δ 153.2, 150.5, 150.0, 120.3, 75.9, 62.2 and 61.9 for carbon C-13,13a, C-14, 14a, C-15, 15a, C-16 and 16a, respectively. The peaks at δ 82.5, 71.5, 75.9, 81.7, 165.4, 128.7, 112.7, 153.2, 150.5, 150.0, 120.3, 75.9, 62.2 and 61.9 were assigned to C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12.

Table-3.43: ^{13}C-NMR (125 MHz, CD_3OD) data of Diethyl 2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate **(62)**

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.5	C-8	153.2	C-13,13a	66.8
C-3	71.5	C-9	150.5	C-14	169.9
C-4	75.9	C-10	150.0	C-14a	170.4
C-4a	81.7	C-10a	120.3	C-15	62.4
C-6	165.4	C-10b	75.9	C-15a	62.5
C-6a	128.7	C-11	62.2	C-16	14.4
C-7	112.7	C-12	61.9	C-16a	14.4

EI-MS m/z: 500.2 [M^+] (calcd. 500.450 for $C_{22}H_{28}O_{13}$).

On the basis of UV, IR, NMR and mass spectrum, the structure **(62)** was identified as Diethyl 2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate.

3.3.1.15. Tert-butyl 2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate (63)

UV spectrum showed absorptions at λ_{max} nm 389 indicated the presence of aromatic system in the molecule.

IR (KBr): 3430cm^{-1} (OH), 3088 cm^{-1}(aromatic C-H), 2980 cm^{-1} (aliphatic C-H), 1732 cm^{-1} (-CO-), 1370 cm^{-1} (C-O-C).

^{1}H-NMR spectrum of compound **(63)** showed singlets at δ 1.47, 1.51, 4.61 and 4.72 for the protons attached to C-16, C-17, C-18, C-16a, C-17a, C-18a, C-13 and 13a, While two singlet at δ 3.82 and 7.34 were observed due to the protons of bergenin nucleus at C-12 and C-7. Beside this doublet of doublet (*dd*) at 3.30, doublet of doublet of doublet (*ddd*) at 3.67, doublet of doublet (*dd*) at 3.68-3.89, doublet of doublet (*dd*) at 3.80, doublet of doublet (*dd*) at 4.03 and a doublet (*d*) at 4.94 were assigned to protons attached to C-3, C-2, C-11, C-4, 4a and C-10b, respectively.

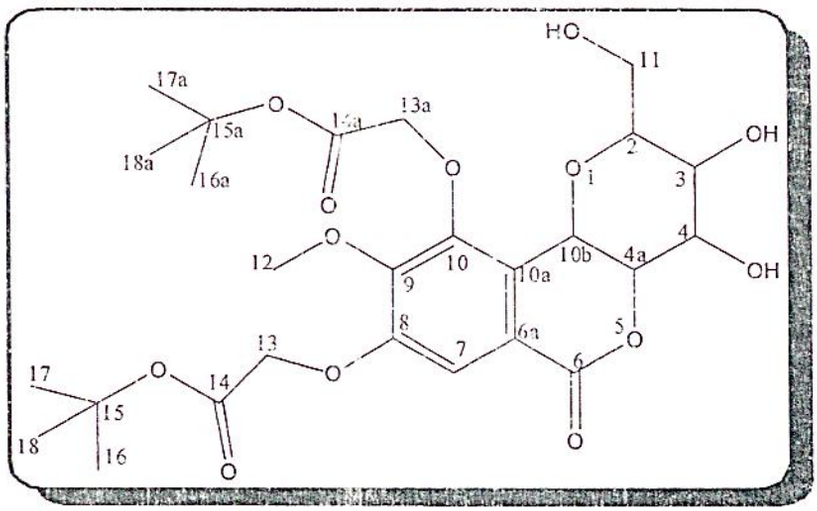


Table-3.44: ^{1}H-NMR (400 MHz, CD_3OD) data of Tert-butyl 2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate (**63**)

Assignment	δ (ppm)	Integration	Multiplicity	J (Hz)
H-16, 17, 18	1.47	9H	*s*	.....
H-16a, 17a, 18a	1.51	9H	*s*	.....
H-3	3.30	1H	*dd*	9.82, 8.87
H-2	3.67	1H	*ddd*	4.90, 3.78, 1.40
H-11	3.68-3.89	2H	*dd*	8.75, 3.75
H-4	3.80	1H	*dd*	9.32, 8.75
H-12	3.89	3H	*s*	.....
H-4a	4.03	1H	*dd*	9.85, 6.64
H-13	4.61	2H	*s*	.....
H-13a	4.72	2H,	*s*	.....
H-10b	4.94	1H	*d*	10.38
H-7	7.34	1H	*s*	.....

^{13}C-NMR spectrum of compound (**63**) showed signals at δ 153.3, 149.9, 150.5, 120.3, 75.9, 62.4 and 61.9 for C-13, 13a, C-14, 14a, C-15, 15a, C-16, C-17, C-18 C-16a, C-17a and C-18a, respectively. Beside these, the signals at δ 82.5, 71.5, 75,9, 81.7, 165.5, 128.5, 112.5, 153.3, 149.9, 150.5, 120.3, 75.9, 62.4 and 61.9 were appeared, which were assigned to C-2, C-3, C-4, 4a, C-6, 6a, C-7, C-8, C-9, C-10, 10a, 10b, C-11 and C-12 in the structure.

Table-3.45: ^{13}C-NMR (125 MHz, CD_3OD+$CDCl_3$) data of Tert-butyl 2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate **(63)**

Assignment	δ (ppm)	Assignment	δ (ppm)	Assignment	δ (ppm)
C-2	82.5	C-8	153.3	C-13,13a	67.2
C-3	71.5	C-9	149.9	C-14	169.1
C-4	75.9	C-10	150.5	C-14a	170.5
C-4a	81.7	C-10a	120.3	C-15	83.3
C-6	165.5	C-10b	75.9	C-15a	83.8
C-6a	128.5	C-11	62.4	C-16,17,18	28.2
C-7	112.5	C-12	61.9	C-16a,17a,18a	28.3

EI-MS m/z: 556.3 [M^+] (calcd. 556.556 for $C_{26}H_{36}O_{13}$).

On the basis of UV, IR, NMR and mass spectrum, the structure (**63**) was identified as Tert-butyl 2,2'-(3,4-dihydroxy-2-(hydroxymethyl)-9-methoxy-6-oxo-2,3,4,4a,6,10b-hexahydropyrano[3,2-c]isochromene-8,10-diyl)bis(oxy)diacetate.

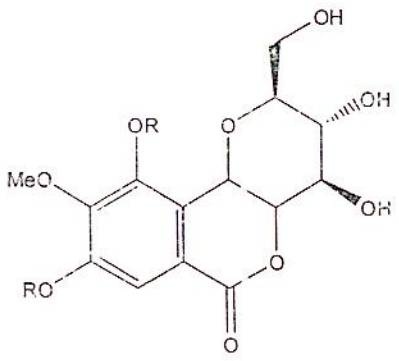
3.3.2. Bioactivities of the synthetic *O*-alkylated bergenins

3.3.2.1. Antiinflammatory activity

Bergenin and all its synthetic derivatives except (54) and (62) exhibited significant *in-vitro* respiratory-burst inhibitory activity model of inflammation (**Table-3.46**). On thorough structural analysis of synthetic bergenin derivatives, structure-activity relationship (SAR) revealed the importance of length as well as saturation of the substituted side chains at C-8 and C-10 phenoxy position of the aromatic ring. Generally for optimal activity, unsaturated propyne group and saturated n-heptyl side chains at C-8 and C-10 phenoxy position were found necessary. The most active derivative was compound (56), having a disubstituted propyne side chain and was found to be more potent than the parent compound bergenin as well as the standard drug indomethacin. The lengths of alkyl chain other than n-heptyl were found to show diverse activity.

Compounds **50** (with 1-butene side chain), **51** (with *n*-pentyl side chain), **55** (with *n*-heptyl side chain), 56 (with propyne group), **61** (with *n*-propyl group) and **63** (with *t*-butyl acetate group) exhibited better potencies than bergenin. Compound (**49**) (with isopropyl side chain), (**50**) (with 1-butene side chain) and (**63**) exhibited relatively better activities than bergenin. Intrestingly, among these **55**, **56**, **61** and **63** exhibited relatively better activities than Indomethacin used as standard drug. Rest of the compounds revealed lower activities e.g. Compound **52** (with ethyl group), **57** (with 1-propene group), 53 (with methyl group), 59 (with n-butyl group), 58 (wjth acetonitrile group) and 60 (with n-octyl group) showed lower activities in a decreasing order, while compound (54) (with 1-butene side chain) and (62) (with ethyl acetate group) were found inactive. In short, we have synthesized a series of bergenin derivatives aimed at increase anti-inflammatory property, which showed the significant antiinflammatory activities. These results indicated that the modification of the phenol hydroxyl groups regioselectively at positions C-8 and C-10 in bergenin with diverse moieties can make contribution to enhancement of antiinflammatory activity. For optimal activity, alkyl chains that are upto 7 carbons in length and other moieties with pi- electron carrier should replace the protons of phenol groups at position C-8 and C-10 of bergenin. This study seems to be a starting

point for designing more potent and effective inhibitor of bergenin as new antiinflammatory agents.



(49-63)

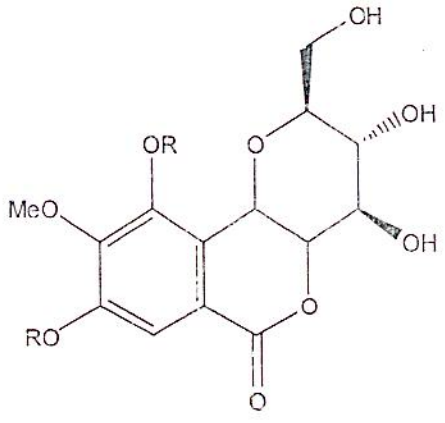
Table-3.46: Antiinflammatory activity of *O*-alkylated Bergenins

Compound #	R	(IC_{50}, (μM)
49	$(CH_3)_2CH$-	303.12±23
50	CH_2=$CH(CH_2)_2$-	288.02 ± 10
51	$CH_3(CH_2)_3$ CH_2-	295.72 ± 63
52	CH_3CH_2-	322.09 ± 31
53	CH_3-	381.58 ± 54
54	CH_2=$CH(CH_2)_3CH_2$-	Inactive
55	$CH_3(CH_2)_5CH_2$ -	269.99 ± 62
56	CH≡CCH_2-	212.95 ± 68
57	CH_2=$CHCH_2$-	333.21 ± 47
58	N≡CCH_2-	415.65 ± 71
59	$CH_3(CH_2)_2CH_2$-	402.93 ± 13
60	$CH_3(CH_2)_6CH_2$-	482.23 ± 23
61	$CH_3CH_2CH_2$-	222.32 ± 01
62	$C_2H_5OCOCH_2$-	Inactive
63	$(CH_3)_3COCOCH_2$-	253.23 ± 60
13	H(Bergenin)	303.12 ± 23
SD*	**Indomethacin**	**271.212±6**

* Standard

3.3.2.2. Antiplasmodial activity (*in vitro*)

In synthetic strategy the phenolic hydroxyl groups at position C-8 and C-10 were modified by different alkyl groups and its derivatives regioselectively. These synthetic derivatives showed lesser antiplasmodial activity than the parent compound bergenin. The decrease in activity of these bergenin derivatives can be structurally correlated to the absence of phenolic hydroxyl groups from their parent moiety. A decrease in activity was also observed when the lengths of alkyl side chains at position C-8 and C-10 were increased. Similarly unsaturated alkyl chains showed no prominent activity like saturated chains. Ester groups also guided the activity to the Lower levels. The **(table-3.47)** shows the complete details of the structure-activity relationship of bergenin and its derivatives.



(49-63)

Table-3.47: Antiplasmodial activity (*in vitro*) of the synthesized *O*-alkylatedbergenins against *P. falciparum* (CQS) D 10 strain

.....	.....	%	Parasite	Survival
Compound No.	**R**	**10 µg/mL**	**5 µg/mL**	**2.5 µg/mL**
49	$(CH_3)_2CH$-	127.19 %	128.73 %	100.81 %
50	CH_2=$CH(CH_2)_2$-	95.44 %	110.61 %	112.14 %
51	$CH_3(CH_2)_3CH_2$-	Not tested (Quantity less)	...	.....
52	CH_3CH_2-	107.30 %	106.72 %	73.95 %
53	CH_3-	106.88 %	112.67 %	111.81 %
54	CH_2=$CH(CH_2)_3CH_2$-	Not tested (Quantity less)	....	.....
55	$CH_3(CH_2)_5CH_2$-	Not tested (Quantity less)	....	.....
56	CH≡CCH_2-	Not tested (Quantity less)	.....	.....
57	CH_2=$CHCH_2$-	126.32 %	128.28 %	109.08 %
58	N≡CCH_2-	Not tested (Quantity less)	.....	.....
59	$CH_3(CH_2)_2CH_2$-	93.84 %	101.37 %	107.19 %
60	$CH_3(CH_2)_6CH_2$-	Not tested (Quantity less)	.....	.....
61	$CH_3CH_2CH_2$-	108.45 %	116.42 %	107.33 %
62	$C_2H_5OCOCH_2$-	139.71%	115.42 %	119.68 %
63	$(CH_3)_3COCOCH_2$-	122.85 %	123.86 %	123.80 %
13	Bergenin	38.28 %	41.27 %	46.01 %
SD*	**CQ**	**50 µg/mL**	**25 µg/mL**	**12.5µg/mL**
.....	**CQ**	**36.62**	**40.18**	**90.95**

*Chloroquine used as standard

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List of Publications

1. Mohammad Arfan, **Hazrat Amin**, Magdalena Karamać, Agnieszk. Kosińska, Wieslaw Wiczkowski and Ryszard Amarowicz. Antioxidant activity of phenolic fractions of *Mallotus philippinensis* bark extract. ***Czech J. Food Sci.*** 2009; 27(2) 109–117.
2. Mohammad Arfan, **Hazrat Amin**, Agnieszka Kosińska, Magdalena Karamać and Ryszard Amarowicz Antioxidant activity of phenolic fractions of *litsea monopetala* (persimon-leaved *litsea*) bark extract. ***Polish Journal of Food and Nutrition Sciences***. 2008; 58(2): 229-233.
3. Mohammad Arfan, **Hazrat Amin**, Magdalena Karamac, Agnieszka Kosinskam, Fereidoon Shahid, Weslaw Wiczkowsk and Ryazard Amarowicz. Antioxidant activity of extracts of *Mallotus philippinensis* fruit and bark. ***Journal of Food Lipids*** 2007; 14: 280-297.
4. Qi Hong, David E. Minter, Scott G. Franzblau, Mohammad Arfan, **Hazrat Amin**, Manfred G. Reinecke. Anti-tubercuIosis Compounds from *Mallotus philippinensis*. ***Natural Product Communications*** 2010; 5 (2): 211-217.
5. Mohammad Arfan, **Hazrat Amin**, Nematullah Khan, Inamullah Khan, Muhammad Saeed, Muhammad Azim Khan, Fazal-ur-Rehman. Analgesic and anti-Inflammatory activities of 11-*O*-galloylbergenin. ***Journal of Ethnopharmacology*** 2010; 131: 502-504.
6. Mohammad Arfan, **Hazrat Amin**, Peter J. Smith, Jamal Rafique, Mumtaz Ali. Comparative antioxidant and antiplasmodial activities of 11-*O*-galloylbergenin and bergenin isolated from *Mallotus philippinensis*. ***Fitoterapia*** (Submitted).
7. Mohammad Arfan, **Hazrat Amin**. Bergenin inhibits urease as a non-competitive inhibitor: New target for old ligand. ***Journal of Enzyme Inhibition and Medicinal Chemistry*** (Submitted).
8. Mohammad Arfan, **Hazrat Amin**, Muhammad Raza Shah, Muhammad Irfan Qadir, Muhammad Iqbal Choudhary. Synthesis and Structure-Activity Relationship of New Bergenin Derivatives as Potent Inhibitors of Inflammation

Based on Neutrophil Respiratory-Burst Activity. ***European Journal of Medicinal Chemistry*** (In process).

9. Mohammad Arfan. **Hazrat Amin**. Jamal Rafique. Viqar Uddin Ahmad. Antifungal and antibacterial activities of *Litsea monopetala* bark. ***Pakistan Journal of Botany*** (Submitted).