Results

CHAPTER 4

RESULTS

4.1 Biological Activity Bioassays

4.1.1 Brine Shrimp Lethality Bioassay

Brine shrimp lethality is a preliminary and general bioassay which is indicative of bioactivity. It is inexpensive and easy to perform in the laboratory. This assay was done on all the 15 crude extracts of stem and stem bark of *Diospyros sp.* The data collected was analysed with the Finney computer program with 95% confidence intervals for statistically significant comparison of potencies. The cut-off value of LC_{50} was 200 ppm (McLaughlin, 1990). Extracts with LC_{50} values of less than 200 ppm were considered to indicate bioactivity.

The crude findings of the assays are tabulated in Apendix 1 to Appendix 5. Table 4.1 summerized the LC_{50} values of the crude extracts *of D. graciliiflora, D. discolor and D. lanceifolia.* Crude petroleum ether extract of *D. discolor* stem and stem bark and *D .lanceifolia* stem indicated strong bioactivity with LC_{50} values of 122.4 ppm, 146.7 ppm and 196.5 ppm respectively. The LC_{50} value of crude petroleum ether extract of *D. graciliflora* stem was 301.7 ppm which was considered to indicate weak bioactivity.

Table 4.1 : LC_{50} Values for Brine Shrimp Lethality Bioassay of Various Crude Extracts of *Diospyros sp*

Samples	Extracts		
	Petroleum Ether	Chloroform	Methanol
Stem of *Diospyros graciliflora*	•301.7 ppm	5 942.3 ppm	10 104.5 ppm
Stem bark of *Diospyros graciliflora*	5 413. 7ppm	7 957.3 ppm	3 877.0 ppm
Stem of *Diospyros discolor*	*122.4 ppm	26 338.4 ppm	56 462.4 ppm
Stem bark of *Diospyros discolor*	*146.7 ppm	4 555.7 ppm	5 413.7 ppm
Stem of *Diospyros lanceifolia*	*196.5 ppm	5 396.3 ppm	661 705.1 ppm

Note :

*Indicate strong bioactivity

• Indicate weak bioactivity

4.1.2 Anti-Tumour Promoter Activity Bioassay

Three crude PE extracts, namely stem and stem bark of *D. discolor*, and stem of *D. lanceifolia* with significant lethalities towards brine shrimp larvae were tested for their anti-tumour promoter activity via the inhibition of EBV EA expression in Raji cells. The crude PE extract of *D. graciliflora* was also tested since it demonstrated low bioactivity on the brine shrimps. The crude findings of the assays are tabulated in Appendix 6 to Appendix 9. The IC_{50} values of these extracts were calculated from the dose response curve in Figure 4.1. Only the crude PE extract *D. discolor* stem showed an inhibitory effect with IC_{50} of 16.43 μg/ml. No inhibitory effect were observed with the other crude extracts as the IC_{50} values cannot be calculated from the same dose response curve.

Three pure compounds identified as lupeol, betulin and betulinic acid, isolated from the crude PE extract of *D. discolor* stem were also tested for their anti-tumour promoter activities. The crude results were detailed in Appendix 10 to Appendix 12. The IC_{50} values of these compounds were calculated from the dose response curve shown in Figure 4.2. Only lupeol exhibited significant inhibitory activity with IC_{50} of 0.009 μg/ml.

Natural phorbol esters were isolated from the five plants of Euphorbiaceae family. These phorbol esters were used in place of TPA in the EBV EA expression in Raji cells. Experimental findings of anti-tumour promoter activities of the three pure compounds with the Raji cells treated with the natural phorbol esters are shown in Appendix 13 to Appendix 27. Their IC_{50} values were obtained from the dose response curve in Figure 4.3

to 4.7. Of the three compounds tested, only lupeol showed strong anti-tumour promoter activity with an IC_{50} value of 0.012, 0.014, 0.013, 0.016 and 0.015 μg/ml after being treated with crude PE extract of *E. hirta, E. tirucalli, E. splendes, J. podagrica* and *P. tithymaloides* respectively.

Figure 4.1 : Dose response curve of crude PE extract of *Diospyros sp* stem and stem bark on EBV EA expression induced by TPA in Raji cells

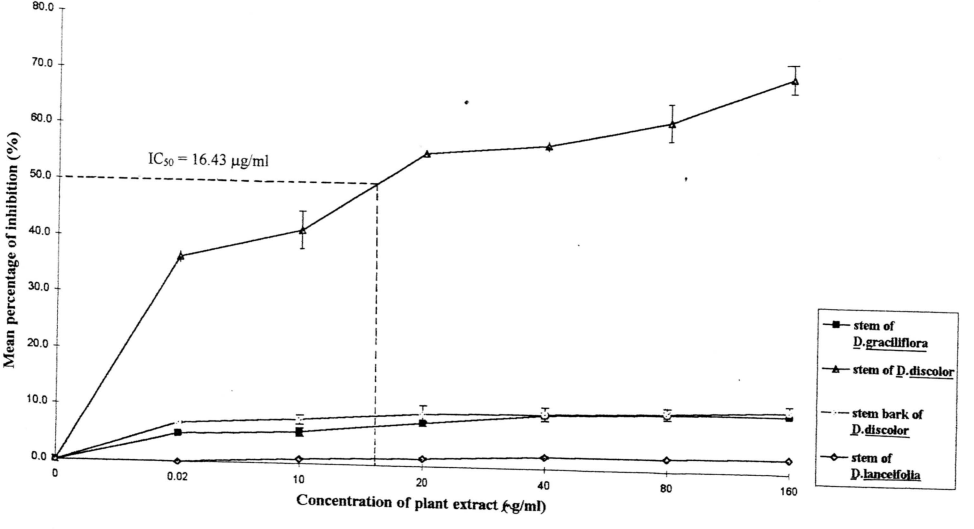


Figure 4.2 : Dose response curve of lupeol, betulin and betulinic acid on EBV EA expression induced by TPA in Raji cells

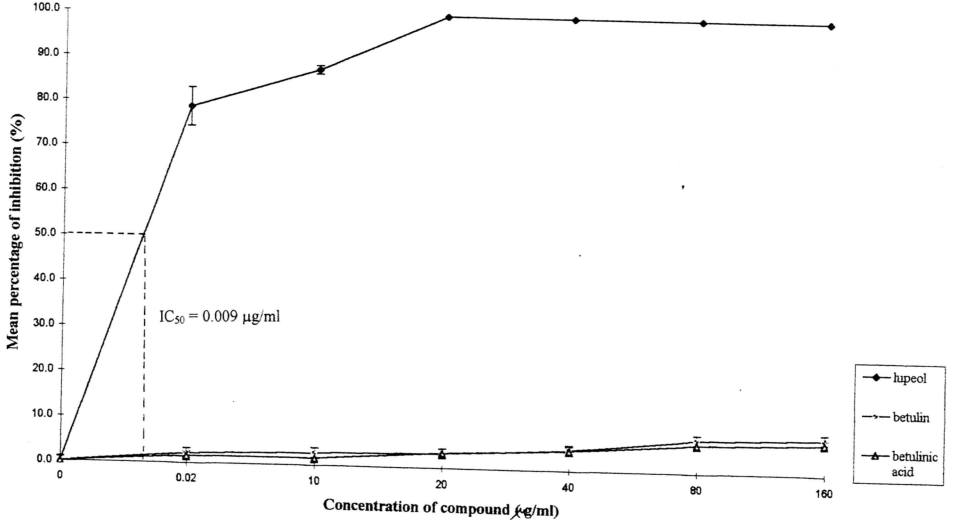


Figure 4.3 : Dose response curve of lupeol, betulin and betulinic acid on EBV EA expression induced by PE extract of *E. hirta* in Raji cells

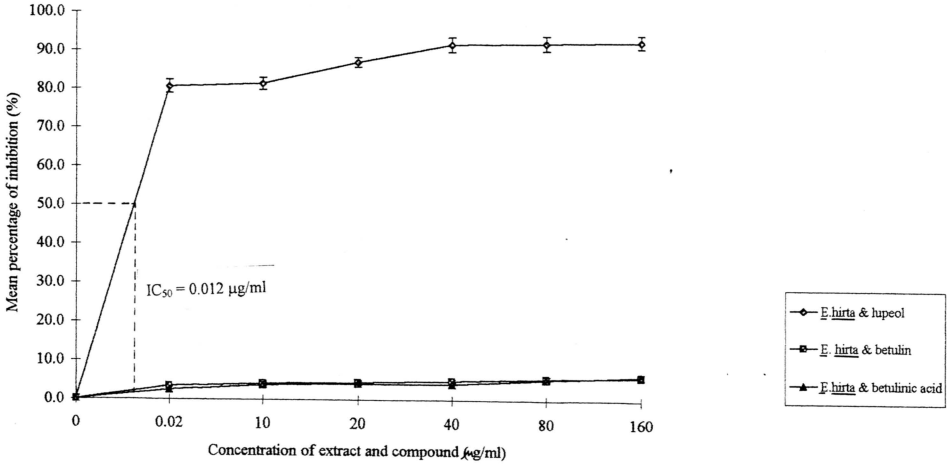


Figure 4.4 : Dose response curve of lupeol, betulin and betulinic acid on EBV EA expression induced by petroleum ether extract of *E.tirucalli* in Raji cells

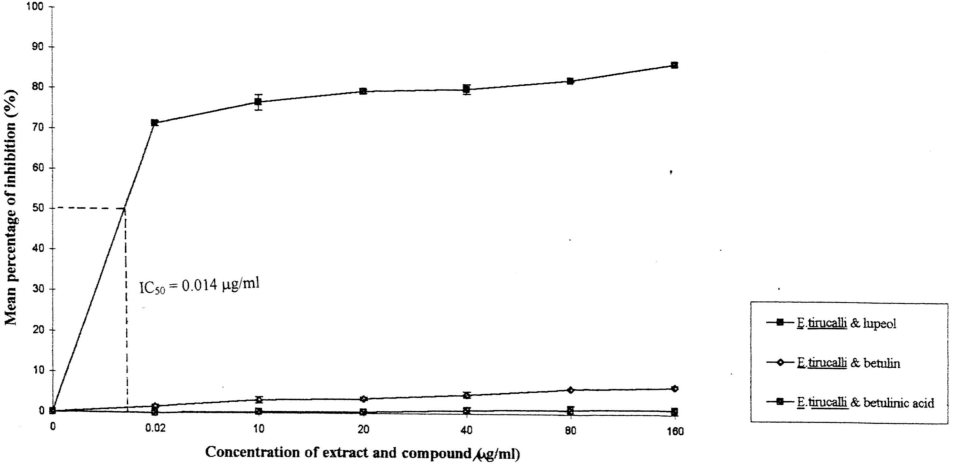


Figure 4.5 : Dose response curve of lupeol, betulin and betulinic acid on EBV EA expression induced by petroleum ether extract of *E. splendes* in Raji cells

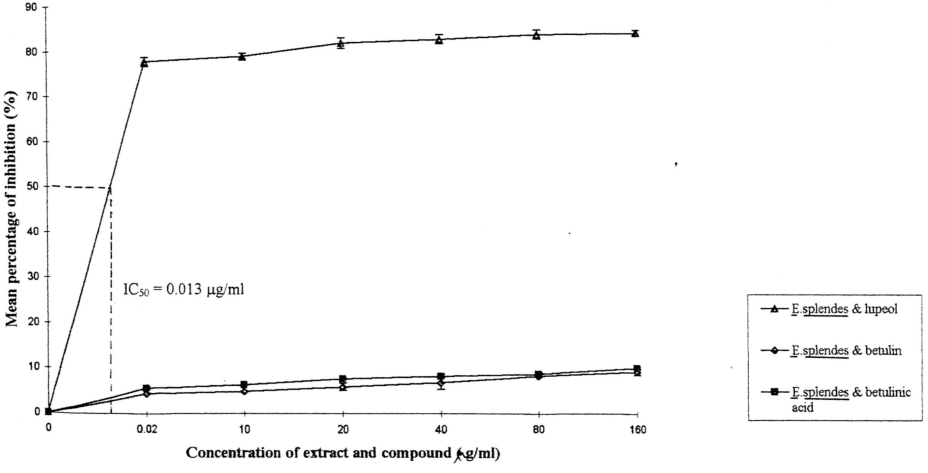


Figure 4.6 : Dose response curve of lupeol, betulin and betulinic acid on EBV EA expression induced by petroleum ether extract of *J. podagrica* in Raji cells

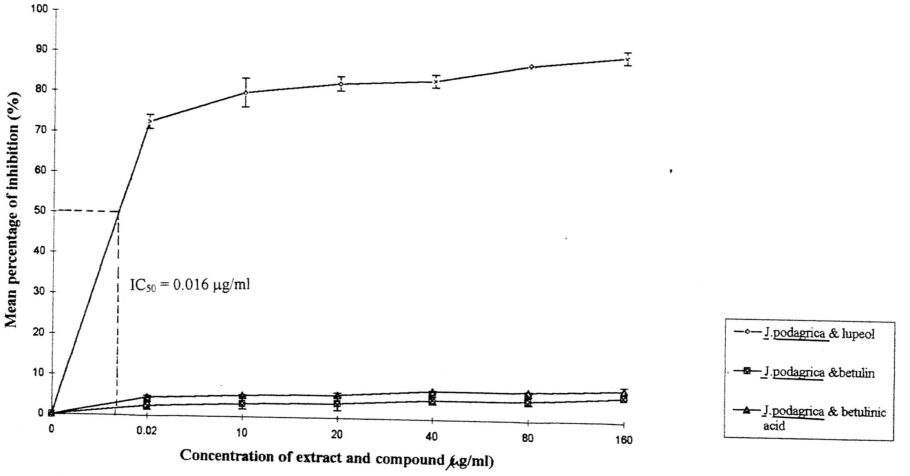
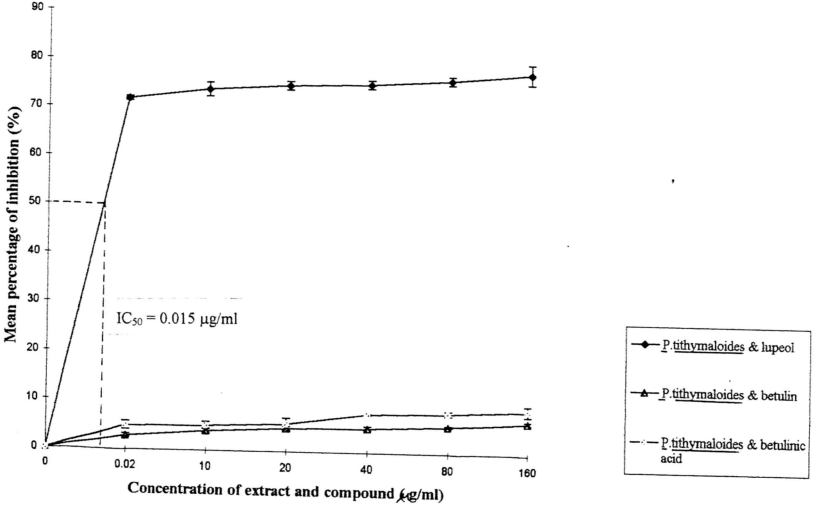


Figure 4.7 : Dose response curve of lupeol, betulin and betulinic acid on EBV EA expression induced by PE extract of *P. tithymaloides* in Raji cells



4.1.3 Cytotoxicity of *Diospyros sp* in Raji Cells

Raji cells were incubated with various crude PE extracts of *Diospyros sp* and the Euphorbiaceae family, also the pure compounds at concentrations ranging from 0.02 µg/ml to 160 µg/ml. In each experiment, the control that consisted of Raji cells treated with the combination of 20 µg/ml of TPA and 4 mM/ml sodium n-butyrate showed no signs of cytotoxicity (0% of cytotoxicity). This control had dividing Raji cells at a density of 1 X 10^6 cells/ml. The percentage of killing shown in Appendix 6 to Appendix 27 are relative to their controls. Plant extracts that caused cell death relative to controls were considered cytotoxic.

As shown in Appendix 6, the crude PE extract of D. graciliflora stem demonstrated the killing of Raji cells ranging from 19.3% at 0.02 µg/ml to 49.9% at 160 µg/ml. The crude PE extract of *D. discolor* stem showed cytotoxic effect on Raji cells ranging from 11.1% at 0.02 µg/ml to 49.8% at 160 µg/ml (Appendix 7). The range of cytotoxic effect on Raji cells for both crude PE extract of *D. discolor* stem bark and *D. lanceifolia* stem were 26.9% at 0.02 µg/ml to 48.0% at 160 µg/ml and 28.4% at 0.02 µg/ml to 47.3% at 160 µg/ml respectively (Appendix 8 and 9 respectively)

The pure compounds namely lupeol, betulin and betulinic acid were found to demonstrated the range of Raji cells killing from 0.5% to 29.9%, 15.1% 35.5% and 30.7% to 47.8% respectively at concentration range of 0.02 to 160 µg/ml (Appendix 10, 11,12 respectively).

Crude PE extract of *E. hirta* with lupeol, betulin and betulinic acid demonstrated cytotoxicity at all concentrations used. The range of Raji cells killing of crude PE extract of *E. hirta* was found to be 17.1% at concentration 0.02 µg/ml to 39.5% at concentration 160 µg/ml (Appendix 13). The killing range of 40.6% to 47.6% and 35.5% to 48.2% at concentation 0.02 µg/ml to 160 µg/ml were demonstrated by the crude PE extract of *E. hirta* with betulin and betulinic acid respectively (Appendix 14 and 15).

As shown in Appendix 16, the crude PE extract of *E. tirucalli* with lupeol was found to have cytotoxic effect on Raji cells at a range of 35.7% at concentration 0.02 µg/ml to 45.7% at concentration 160 µg/ml. Whereas the same extract with betulin and betulinic acid demonstrated the killing range of 32.2% to 48.2% and 39.0% to 48.3% respectively (Appendix 17 and 18) at concentration 0.02 µg/ml to 160 µg/ml.

The same pattern of cytotoxic effect on Raji cells were demonstrated by other crude PE extract of *E. splendes*, *J. podagrica* and *P. tithymaloides* with lupeol, betulin and betulinic acid. Crude PE extract of *E. splendes* with lupeol, betulin and betulinic acid demonstrated cytotoxiciy at the range of 10.0% to 30.0% (Appendix 19), 38.1% to 46.8% (Appendix 20) and 36.4% to 49.5% (Appendix 21) respectively. The crude PE extract of *J. podagrica* with lupeol, betulin and betulinic acid were found to demonstrated cytotoxicity at the range of 14.8% to 40.1% (Appendix 23) and 6.3%to 31.0% (Appendix 24) respectively. Wheeeas the crude PE extract of *P. tithymaloides* with lupeol, betulin and betulinic acid were found to have cytotoxic effect on Raji cells at a range of 37.8%

25), 23.9 to 39.8% (Appendix 26) and 39.1% to 49.2% (Appendix 27) respectively.

4.2 Phytochemical Screening of Secondary Metabolites of *D. graciliflora*, *D. discolor* and *D .lanceifolia*

Finely ground dried stems of *D. graciliflora* (50 g), *D. discolor* (50 g) and *D. lanceifolia* (50 g), also finely ground dried stem barks of *D graciliflora* (50 g) and *D. discolor* (50 g) were successively extracted with 100 ml light petroleum ether, 100 ml chloroform and 100 ml methanol. The extract were concentrated in vacuo and weighted (Table 4.2).

Table 4.2 : Dry Weight of Crude Extracts of *Diospyros sp* Stem and Stem Bark

Plants	Light Petroleum Ether Extract (% w/w)	Chloroform Extract (% w/w)	Methanol Extract (% w/w)
D. graciliflora stem	0.38	1.90	4.52
D. graciliflora stem bark	0.41	0.95	3.47
D. discolor stem	0.25	2.54	5.15
D. discolor stem bark	0.31	3.85	6.15
D. lanceifolia stem	0.54	1.88	3.27

Each of the concentrates was thoroughly screened using TLC for the presence of secondary metabolites. The screening were carried out under three different solvent systems of varying polarities. The compounds were identified using specific spraying reagents and the results were tabulated in Table 4.3 to 4.14.

Table 4.3 : TLC Analysis of Petroleum Ether Extract of *Diospyros graciliflora* Stem

R_f in solvent system				Detection				Label	Comments
PE : $CHCl_3$ 30 : 70	$CHCl_3$	MeOH : $CHCl_3$ 5 : 95	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.85	0.87	0.92	Brown	-ve	Violet +++	-ve	+++	Dg P1	Terpene
0.63	0.67	0.75	Yell-green	Purple	Violet +++	-ve	+++	Dg P2	Terpene
0.55	0.58	0.68	Pink	-ve	Violet +++	-ve	++	Dg P3	Terpene
0.43	0.46	0.55	-ve	Red	-ve	-ve	++	Dg P4	Chlorophyll
0.37	0.40	0.51	-ve	Blue	Violet +++	-ve	+++	Dg P5	Coumarin
-	0.23	0.30	-ve	Red	Brown	-ve	++	Dg P6	Chlorophyll
-	0.12	0.20	-ve	Violet	Blue	-ve	+++	Dg P7	Terpene

Abbreviations :
+++ Strong
++ Moderate
\+ Weak

D'dorff = Dragendorff

Table 4.4 : TLC Analysis of Chloroform Extract of *Diospyros graciliflora* Stem

R_f in solvent system			Detection					Label	Comments
$CHCl_3$	$CHCl_3$: MeOH 90 : 10	MeOH : $CHCl_3$ 20 : 80	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.68	0.78	0.87	Brown	Red	-ve	-ve	++	Dg C1	Chlorophyll
0.60	0.72	0.80	-ve	Green	-ve	-ve	++	Dg C2	Chlorophyll
0.54	0.62	0.76	-ve	-ve	Brown +	-ve	-ve	Dg C3	Terpene
0.41	0.46	0.56	-ve	-ve	Brown ++	-ve	+++	Dg C4	Terpene
0.22	0.25	0.30	-ve	-ve	Violet	-ve	++	Dg C5	Terpene
0.10	0.15	0.19	Brown	Red	-ve	-ve	+++	Dg C6	Chlorophyll

Abbreviations :
+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.5 : TLC Analysis of Methanol Extract of *Diospyros graciliflora* Stem

R_f in solvent system			Detection					Label	Comments
$CHCl_3$	$CHCl_3$: MeOH 90 : 10	MeOH : $CHCl_3$ 20 : 80	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.81	0.89	0.96	-ve	Purple	Violet +++	-ve	+	Dg M1	Terpene
0.72	0.80	0.82	-ve	Green	-ve	-ve	++	Dg M2	Chlorophyll
0.65	0.74	0.81	-ve	Blue+	Brown+	-ve	+++	Dg M3	Terpene
0.43	0.47	0.57	-ve	Red	-ve	-ve	++	Dg M4	Chlorophyll
0.28	0.31	0.41	-ve	-ve	Brown+	-ve	+	Dg M5	Terpene
0.22	0.24	0.27	-ve	-ve	Blue+	-ve	++	Dg M6	Terpene

Abbreviations :
+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.6 : TLC Analysis of Petroleum Ether Extract of *Diospyros discolor* Stem

R_f in solvent system			Detection					Label	Comments
PE : $CHCl_3$ 30 : 70	$CHCl_3$	MeOH : $CHCl_3$ 5 : 95	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.87	0.90	0.95	-ve	Violet	Violet +++	-ve	+++	Dd P1	Terpene
0.75	0.79	0.88	-ve	-ve	Blue +++	-ve	++	Dd P2	Terpene
0.71	0.74	0.84	-ve	-ve	Blue +++	-ve	++	Dd P3	Terpene
0.65	0.70	0.79	Brown	Blue	Violet ++	-ve	+	Dd P4	Coumarin
0.54	0.58	0.72	-ve	Purple	Violet+	-ve	+++	Dd P5	Terpene
0.50	0.57	0.68	-ve	Purple	Violet +++	-ve	++	Dd P6	Terpene
-	0.40	0.55	-ve	-ve	Blue ++	-ve	++	Dd P7	Terpene
-	0.22	0.34	-ve	Red	Brown	-ve	++	Dd P8	Chlorophyll

Abbreviations :
+++ Strong
++ Moderate
\+ Weak

D'dorff = Dragendorff

Table 4.7 : TLC Analysis of Chloroform Extract of *Diospyros discolor* Stem

R_f in solvent system			Detection					Label	Comments
$CHCl_3$	$CHCl_3$: MeOH 90 : 10	MeOH : $CHCl_3$ 20 : 80	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.81	0.89	0.95	Brown	Red	-ve	-ve	+	Dd C1	Chlorophyll
0.73	0.83	0.90	-ve	-ve	Violet +++	-ve	+++	Dd C2	Terpene
0.65	0.77	0.85	-ve	-ve	Violet +++	-ve	+++	Dd C3	Terpene
0.62	0.76	0.84	Brown	Red	-ve	-ve	+	Dd C4	Chlorophyll
0.54	0.60	0.65	-ve	-ve	Brown +++	-ve	+++	Dd C5	Terpene
0.43	0.50	0.57	-ve	-ve	Blue	-ve	++	DdC6	Terpene
0.21	0.24	0.26	-ve	-ve	Violet +++	-ve	+	Dd C7	Terpene
0.18	0.20	0.22	-ve	Green	-ve	-ve	+	Dd C8	Chlorophyll

Abbreviations :

+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.8 : TLC Analysis of Methanol Extract of *Diospyros discolor* Stem

R_f in solvent system			Detection					Label	Comments
$CHCl_3$	$CHCl_3$: MeOH 90 : 10	MeOH : $CHCl_3$ 20 : 80	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.79	0.86	0.92	-ve	Red	-ve	-ve	+++	Dd M1	Terpene
0.71	0.78	0.87	-ve	Violet+	Violet+	-ve	++	Dd M2	Terpene
0.60	0.68	0.78	-ve	Purple ++	Violet +++	-ve	+	Dd M3	Terpene
0.52	0.58	0.65	-ve	Red	-ve	-ve	+	Dd M4	Chlorophyll
0.49	0.53	0.58	-ve	-ve	Brown+	-ve	++	Dd M5	Coumarin
0.37	0.39	0.42	-ve	Violet ++	Blue ++	-ve	+++	Dd M6	Chlorophyll
0.21	0.22	0.25	-ve	-ve	Brown+	-ve	+	Dd M7	Terpene

Abbreviations :
+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.9 : TLC Analysis of Petroleum Ether Extract of *Diospyros discolor* Stem Bark

R_f in solvent system			Detection					Label	Comments
PE : $CHCl_3$ 30 : 70	$CHCl_3$	MeOH : $CHCl_3$ 5 : 95	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.85	0.87	0.95	-ve	Red	Brown ++	-ve	+++	Dds P1	Chlorophyll
0.71	0.74	0.82	-ve	Purple	Blue +++	-ve	++	Dds P2	Terpene
0.51	0.55	0.67	-ve	Purple	Blue +++	-ve	++	Dds P3	Terpene
0.49	0.52	0.63	-ve	Blue	-ve	-ve	+	Dds P4	Coumarin
-	0.43	0.58	Brown	Red	-ve	-ve	+++	Dds P5	Chlorophyll
-	0.29	0.49	-ve	-ve	-ve	-ve	+++	Dds P6	Terpene
-	0.21	0.39	Brown	Red	-ve	-ve	+	Dds P7	Chlorophyll

Abbreviations
+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.10 : TLC Analysis of Chloroform Extract of *Diospyros discolor* Stem Bark

R_f in solvent system			Detection					Label	Comments
$CHCl_3$	$CHCl_3$: MeOH 90 : 10	MeOH : $CHCl_3$ 20 : 80	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.65	0.77	0.85	-ve	Blue	Brown	-ve	+++	Dds C1	Terpene
0.63	0.75	0.83	-ve	Purple+	Violet +++	-ve	++	Dds C2	Terpene
0.51	0.58	0.65	-ve	Green	-ve	-ve	+	Dds C3	Chlorophyll
0.49	0.54	0.63	-ve	Green	-ve	-ve	+	Dds C4	Chlorophyll
0.33	0.39	0.44	Brown	Red	-ve	-ve	++	Dds C5	Chlorophyll
0.21	0.25	0.31	-ve	Blue+	Brown ++	-ve	+	Dds C6	Terpene
Chlorophyll	0.22	0.28	Brown	Red	-ve	-ve	+	Dds C7	Chlorophyll
0.07	0.12	0.18	-ve	Purple+	Violet +++	-ve	+	Dds C8	Terpene

Abbreviations :
+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.11 : TLC Analysis of Methanol Extract of *Diospyros discolor* Stem Bark

R_f in solvent system			Detection					Label	Comments
$CHCl_3$	$CHCl_3$: MeOH 90 : 10	MeOH : $CHCl_3$ 20 : 80	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.81	0.90	0.6	Brown	-ve	Violet +++	-ve	+++	Dds M1	Terpene
0.65	0.73	0.78	Yell-green	Purple	Violet +++	-ve	+++	Dds M2	Terpene
0.53	0.61	0.64	Pink	-ve	Violet +++	-ve	++	Dds M3	Terpene
0.41	0.47	0.1	-ve	Red	-ve	-ve	++	Dds M4	Terpene
0.38	0.43	0.45	-ve	Blue	Violet++	-ve	+++	Dds M5	Terpene
0.26	0.33	0.35	-ve	Red	Brown	-ve	++	Dds M6	Terpene
0.11	0.15	0.16	-ve	Red	-ve	-ve	+++	Dds M7	Chlorophyll

Abbreviations :
+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.12 : TLC Analysis of Petroleum Ether Extract of *Diospyros lanceifolia* Stem

R_F in solvent system			Detection					Label	Comments
PE : $CHCl_3$ 30 : 70	$CHCl_3$	MeOH : $CHCl_3$ 5 : 95	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.81	0.84	0.93	-ve	Violet	Blue+	-ve	+++	Dl P1	Terpene
0.75	0.77	0.89	Brown	Red	Brown-	-ve	+	Dl P2	Chlorophyll
0.63	0.64	0.79	-ve	-ve	Blue++	-ve	+++	DlP3	Terpene
0.54	0.56	0.64	Brown	Red	Brown ++	-ve	+	Dl P4	Chlorophyll
-	0.41	0.47	-ve	Purple++	Violet+	-ve	++	Dl P5	Terpene
-	0.23	0.33	-ve	-ve	Violet++	-ve	+++	Dl P6	Terpene

Abbreviations :
+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.13 : TLC Analysis of Chloroform Extract of *Diospyros lanceifolia* Stem

R_f in solvent system			Detection					Label	Comments
$CHCl_3$	$CHCl_3$: MeOH 90 : 10	MeOH : $CHCl_3$ 20 : 80	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.79	0.90	0.98	-ve	Violet	Violet---	-ve	---	Dl C1	Terpene
0.65	0.74	0.81	-ve	Purple	Brown-	-ve	--	Dl C2	Terpene
0.51	0.59	0.67	Brown	Red	-ve	-ve	-	Dl C3	Chlorophyll
0.42	0.48	0.53	-ve	-ve	Blue--	-ve	---	Dl C4	Terpene
0.33	0.40	0.44	-ve	-ve	Brown --	-ve	---	Dl C5	Terpene
0.24	0.27	0.29	-ve	Blue	Violet-	-ve	--	Dl C6	Terpene
0.19	0.22	0.26	-ve	Violet-	Violet-	-ve	--	Dl C7	Terpene

Abbreviations

+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.13 : TLC Analysis of Chloroform Extract of *Diospyros lanceifolia* Stem

R_f in solvent system			Detection					Label	Comments
$CHCl_3$	$CHCl_3$: MeOH 90 : 10	MeOH : $CHCl_3$ 20 : 80	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.79	0.90	0.98	-ve	Violet	Violet--	-ve	---	Dl C1	Terpene
0.65	0.74	0.81	-ve	Purple	Brown-	-ve	--	Dl C2	Terpene
0.51	0.59	0.67	Brown	Red	-ve	-ve	-	Dl C3	Chlorophyll
0.42	0.48	0.53	-ve	-ve	Blue--	-ve	---	Dl C4	Terpene
0.33	0.40	0.44	-ve	-ve	Brown --	-ve	---	Dl C5	Terpene
0.24	0.27	0.29	-ve	Blue	Violet-	-ve	--	Dl C6	Terpene
0.19	0.22	0.26	-ve	Violet-	Violet-	-ve	--	Dl C7	Terpene

Abbreviations

+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

Table 4.14 : TLC Analysis of Methanol Extract of *Diospyros lanceifolia* Stem

R_f in solvent system			Detection					Label	Comments
$CHCl_3$	$CHCl_3$: MeOH 90 : 10	MeOH : $CHCl_3$ 20 : 80	Visible	UV 365	Vanillin-H_2SO_4	D'dorff	Iodine		
0.85	0.87	0.92	Brown	-ve	Violet +++	-ve	+++	Dg P1	Terpene
0.63	0.67	0.75	Yell-green	Purple	Violet +++	-ve	+++	Dg P2	Terpene
0.55	0.58	0.68	Pink	-ve	Violet +++	-ve	++	Dg P3	Terpene
0.43	0.46	0.55	-ve	Red	-ve	-ve	++	Dg P4	Chlorophyll
0.37	0.40	0.51	-ve	Blue	Violet +++	-ve	+++	Dg P5	Coumarin
-	0.23	0.30	-ve	Red	Brown	-ve	++	Dg P6	Chlorophyll
-	0.12	0.20	-ve	Violet	Blue	-ve	+++	Dg P7	Terpene

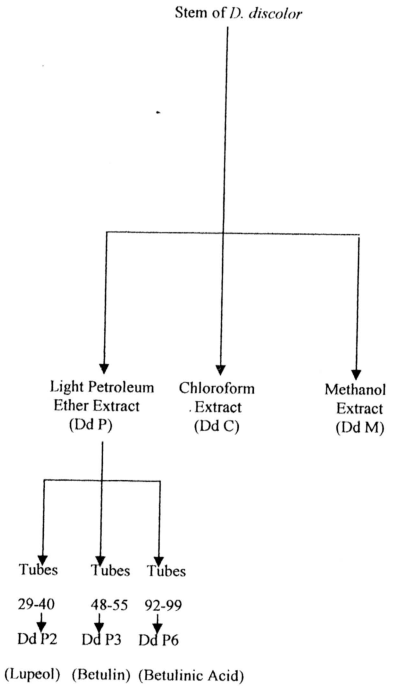
Abbreviations :
+++ Strong
++ Moderate
+ Weak

D'dorff = Dragendorff

4.3 Phytochemical Investigation of PE Extract of *D.discolor* Stem

The schematic flow of the extraction and isolation procedures of *D. discolor* was depicted in Figure 4.8.

Figure 4.8 : Schematic Isolation of *D. discolor*



4.3.1 Isolation of Compound Labelled Dd P2

The light petroleum ether extract of *D. discolor* stem (1.0 g) was chromatographed over a column of silica gel in chloroform : hexane (90 : 10, v/v) as the initial eluent. A hundred and twenty (120) fractions of 5 ml each were collected and monitered using TLC with chloroform as the developing system. Fractions were combined on the basis of their chromatographic similarity.

Fraction 29-40 was separated on preparative TLC plates with chloroform : hexane (80 :20, v/v) as the developing system. Compound Dd P2 was obtained from this fraction after scrapping and purifying by repeated washings with chloroform.

This compound exhibited the following spectral properties :

Dd P2 (5.5 mg) White needle-shaped crystals; m.p. 215°C [lit. 215 °C (Fish *et al*, 1978)]; IR (γ_{max}, KBR, cm^{-1}) : 3420, 3040, 1635, 1010, 980, 870; ^1H NMR ($CDCL_3$, δ) : 0.74, 0.77, 0.81, 0.92, 0.94, 1.01 (each s, 3-H),1.66 (broad s, 3-H), 2.34 (1H, dt, J=11.0, 5.6 Hz), 3.16 (1H, dd, J=11.0, 5.6 Hz), 4.56, 4.66 (each broad s, 1-H); ^{13}C NMR ($CDCL_3$, δ) : (C-1) 38.7, (C-2) 27.5, (C-3) 79.0, (C-4) 38.9, (C-5) 55.3, (C-6) 18.3, (C-7) 34.3, (C-8) 40.9, (C-10) 37.2, (C-11) 21.0, (C-12) 25.2, (C-13) 38.1, (C-14) 42.9, (C-15) 27.5, (C-16) 35.6, (C-17) 43.0, (C-18) 48.0, (C-19) 48.3, (C-20) 150.9, (C-21) 29.9, (C-22) 40.0, (C-

23) 28.0, (C-24) 15.3, (C-25) 16.1, (C-26) 16.0, (C-27) 14.6, (C-28) 18.0, (C-29) 109.3, (C-30) 19.3.

MS (m/z, %) : 426 (M^+,12), 411 (6), 218 (42), 207 (60), 203 (38), 189 (100), 175 (37), 135 (60), 121 (60).

4.3.2 Isolation of Compound Labelled Dd P3

Compound Dd P3 was isolated from fraction 48-55 of the PE extract of stem of *D. discolor* as previously described (Section 4.2.1). Repeated washings with chloroform gave a white needle-shaped crystals which exhibited the following spectral properties :

Dd P3 (0.8 mg) White needle shaped crystals; m.p. 256-257 °C [lit. 256-257 °C (Tezuka *et al.*,1973)]; IR (γ_{max}, KBR, cm^{-1}): 3400, 3080, 1645, 1450, 1380, 1010, 890; ^1H NMR ($CDCl_3$, δ, ppm): 4.68 (m, 1H, 29-H), 4.58 (m, 1H, 29-H), 3.81 (d, J=10.3Hz, 1H, 28-H), 3.35 (d, J=10.3 Hz, 1H, 28-H), 3.17 (m,1H,3-H), 2.38 (m, 1H, 19-H), 1.05-2.05 (complex, CH_2, CH),1.68 (m, 3H, 30-H), 1.02, 0.98, 0.97, 0.82, 0.76, (all s, 15H, 5xCH_3); ^{13}C NMR ($CDCl_3$,δ) : (C-1) 38.7, (C-2) 27.4, (C-3) 79.1, (C-4) 38.8, (C-5) 55.3, (C-6) 18.3, (C-7) 34.3, (C-8) 41.0, (C-9) 50.5, (C-10) 37.3, (C-11) 20.9, (C-12) 25.2, (C-13) 37.2, (C-14) 42.8, (C-15) 27.1, (C-16) 29.2, (C-17) 47.9, (C-18) 47.9, (C-19) 48.8, (C-20) 150.5, (C-21) 29.8, (C-22) 34.0, (C-23) 28.1, (C-24) 15.4, (C-25) 16.1, (C-26) 16.1, (C-27) 14.8, (C-28) 60.6, (C-29) 109.7, (C-30) 19.1.

MS (m/z, %) : 442 (M^+, 16), 411 (32), 381 (15), 234 (34), 207 (65), 189 (100), 175 (43), 147 (44), 121 (74), 107 (81).

4.3.3 Isolation of Compound Labelled Dd P6

This compound was isolated through the previously described method in Section 4.3.1 from fraction 92-99. White needle-crystals was obtained after repeated washings with chloroform. The spectral properties of this compound is given below :

Dd P6 (2.0 mg) White needle-shaped crystals; m.p. 314 °C [lit. 300 °C (Tesuka *et. al.*, 1973)}; IR (γ_{max}, KBR, cm^{-1}) : 3480, 1685, 1043, 1640, 880; ^1H NMR ($CDCl_3$, δ) : 4.60 (m, 1H, 29-H), 4.73 (m, 1H, 29-H), 3.19 (m, 1H, 3-H), 2.97 (m, 1H, 19-H), 1.68 (m, 3H, 30-H), 0.97, 0,96, 0.93, 0.82, 0.75 (all s, 5H, 5 X CH_3); ^{13}C NMR ($CDCL_3$-C_5D_5N (1:1), δ) : (C-1) 39.0, (C-2) 27.6, (C-3) 78.2, (C-4) 39.0, (C-5) 55.5, (C-6) 18.4, (C-7) 34.5, (C-8) 40.8, (C-9) 50.7, (C-10) 37.3, (C-11) 21.0, (C-12) 25.6, (C-13) 38.2, (C-14) 42.5, (C-15) 30.8, (C-16) 32.6, (C-17) 56.3, (C-18) 47.1, (C-19) 49.4, (C-20) 150.9, (C-21) 29.9, (C-22) 37.3, (C-23) 28.2, (C-24) 15.6, (C-25) 16.1, (C-26) 16.1, (C-27) 14.7, (C-28) 178.9, (C-29) 109.4, (C-30) 19.4.

MS (m/z, %) : 456 (M^+, 14), 430 (18), 318 (25), 248 (48), 220 (39), 219 (45), 207 (63), 189 (100), 165 (24), 121 (39), 105 (30).