Discussion

CHAPTER 5

DISCUSSION

5.1 Bioactivity of *Diospyros sp*

5.1.1 Brine Shrimp Lethality Bioassay

The major goal of using the brine shrimp lethality bioassay is to isolate novel bioactive phytochemicals that have useful potential applications or can serve as lead compounds for synthetic modifications. It is simple yet effective bench-top bioassay that can lead us to isolate numerous bioactive compounds with potential uses as anti-tumour agents. This assay has the advantage of being rapid (24 hours), inexpensive and simple (no aseptic technique required). It easily utilizes a large number of organisms for statistical validations and requires no special equipment and a relatively small amount of test samples (2-20 mg or less). Furthermore, it does not require animal serum as needed for cytotoxicity assay. Animal rights advocates have not yet objected to the use of these invertebrates in experimental work (McLaughlin, 1970).

The brine shrimp lethality bioassay has shown good correlation with P-388 cytotoxicity (Meyer *et. al.*, 1982) and its utility as a prescreen for anti-tumour activity was recently confirmed in a blind comparison with *in vivo* cytotoxicity and 3-PS (*in vivo* murine leukaemia) activity (Anderson *et. al.*, 1991). McLaughlin *et. al.* (1970) has also described ten recent examples for their successful application of this assay to yield some interesting

and potentially useful bioactive compounds. For example, they have successfully isolated piceatannol from the seed of *Euphorbia lagascae* Spreng (Euyphorbiaceae) which gave LC_{50} value of 278 ppm. This compound was further used as a potent inhibitor of protein-tyrosine kinases. They also found two new acetogenins which showed LC_{50} values of 234 ppm and 123 ppm. Both compounds has been used as herbicidal and plant-growth promoter respectively.

In the present study, 15 crude extracts obtained from the stem and stem bark of three selected *Diospyros sp* namely *Diospyros graciliflora, D. discolor* and *D. lanceifolia* have been tested using the brine shrimp lethality bioassay. The crude PE extract of of *D. discolor* stem and stem bark, and the crude PE extract of *D. lanceifolia* stem indicated strong bioactivities with LC_{50} values of 122.4 ppm, 146.7 ppm and 196.5 ppm respectively. The LC_{50} value of crude PE extract of *D. graciliflora* stem was 301.7 ppm which was considered to indicate weak bioactivity. All the other extracts tested did not exhibit significant lethality values. The four crude PE extract were further investigated for their anti-tumour promoter activities. These extracts were also screened for their phytochemical properties.

5.1.2 Anti-tumour Promoter Activity

Various methods were used in previous studies to detect the presence of anti-tumour promoter activity that naturally occurred in the environment. Among them was the short term assay of inhibition of EBV activation induced by TPA in cell lines (Koshimizu *et al.*, 1988). In the present study, the method used to screen anti-tumour promoter activity in three selected *Diospyros sp* was the short term asay of inhibition of EBV-EA activation induced by TPA in Raji cells, a human lymphoblastoid cell line. The inhibitory activity on TPA enhanced EBV-EA activation by anti-tumour promoters *in vitro* seemed to correlate well with the ability to inhibit tumour promotion *in vivo* (Nishino *et al.*, 1984).

For a long time, TPA has been used in research as a potent tumour promoter (Boutwell, 1974). Therefore, it was choosen to induced EBV-EA activation in the present study. The inhibition of TPA induced EBV-EA activation in Raji cells was detected using the indirect immunofloresence assay (Henle *et al.*, 1966). This technique was simple and the result obtained was reproducible. Under this technique, the expressing cells were detected as fluorescing cells when observed using a UV microscope. Raji cells induced by TPA and further treated with *Diospyros sp* extracts that exhibited the anti-tumour promoter activity were found to have less or none of these fluorescing cells. The approximate IC_{50} values of the anti-tumour promoter activity that showed the potency as anti-tumour promoters were obtained from the dose response cuves that were plotted from the concentration range used in the present study.

A cytotoxicity assay was also carried out to determine the cytotoxic effect of all extracts on Raji cells. This is to ensure that the anti-tumour promoter activity demonstrated by the extracts in Raji cells were not false readings caused by cell death. Therefore, the present study was focused on extracts demonstrating high anti-tumour promoter activity but lacked cytotoxicity.

In the pesent study, three crude PE extracts of *D. graciliflora*, *D. discolor* and *D. lanceifolia* stem, crude PE extract of *D. discolor* stem bark and three pure compounds namely lupeol, betulin and betulinic acid were tested for their anti-tumour promoter activities.. Among the four samples of crude PE extract of *D. discolor*, *D. graciliflora* and *D. lanceifolia* stem and stem bark, the crude PE extract of *D. discolor* demonstrated the highest anti-tumour promoter activity. 70.2% inhibition of EBV EA activation was observed at concentration of plant extract 160 μg/ml. Cytotoxicity effect on Raji cells at this concentration was as low as 7.3%. This extract also demonstrated approximate IC_{50} of anti-tumour promoter activity at concentration 16.43 μg/ml. The IC_{50} values of other samples cannot be calculated from the same dose response curve. This result clearly showed that crude PE extract of *D. discolor* stem possesses potent anti-tumour promoter activity. As was reported in previous sstudies, the leaves of *D. discolor* has been used as therapeutic agents in the treatment of swellings, leprosy and eye and skin disease (Siddiqui *et al.*, 1988). The leaves also possesses cardiotonic and anti-bacterial properties.

Among the three pure compounds isolated from the crude PE extract of *D. discolor* stem, only lupeol demonstrated anti-tumour promoter activity. Cytotoxicity of this compound was observed at a low range i.e 0.5% at concentration 0.02 µg/ml to 29.9% at concentration 160 µg/ml. Furthermore, complete EBV EA inhibition was observed at concentration ranging from 20 µg/ml to 160 µg/ml. The approximate IC_{50} value observed was 0.009 µg/ml. The result showed that lupeol possesses the strongest anti-tumour promoter activity. In an earlier study done by Pant and Chaturvedi (1989), lupeol was successfully isolate from the latex of *Calotropsis procera*. The latex has been extensively used in Indian medicine and employed as an narrow poison by the natives of African and Columbia (Shukla and Krishna, 1971).

Lupeol was found ubiquitous in many *Diospyros sp* (Muhamad *et al.*, 1984). However the crude PE extract of *D. graciliflora* and *D. lanceifolia* stem, also the crude PE extract of *D. discolor* stem bark were negative for anti-tumour promoter activity. In these crude extracts, lupeol might be present in small quantities. Since only crude extracts were tested, the activity were not detected.

The crude PE extracts of five plants from the Euphorbiaceae family have been used in the present study in place of TPA as a tumour promoter. The results showed that among the three pure compounds, lupeol again posesses the highest anti-tumour promoter activity. The approximte IC_{50} values given by lupeol were 0.012, 0.013, 0.014, 0.016 and 0.015 µg/ml when the Raji cells were induced with crude PE extracts of *E. hirta, E. tirucalli,*

E. splendes, *J.podagrica* and *P. tithymaloides* respectively. The IC_{50} values of betulin and betulinic acid could not be calculated from the same dose response curve because the cytotoxicity observed were all at low ranges of 1.7-3.9%, 3.5 to 4.5%, 1.0 to 3.0%, 1.4 to 4.0% and 3.7 to 4.8% when Raji cells were activated with crude PE extracts *E. hirta*, *E. tirucalli*, *E. splendes*, *J. podagrica* and *P. tithymaloides* respectively. These results obtained clearly showed that lupeol again possesses the strongest anti-tumour activity. The study also showed that the crude PE extract of the five Euphorbiaceae plants possesses strong tumour promoter activity and could be used to induce EBV EA in Raji cells in place of the classical tumour promoter, TPA.

Plant diterpene esters in the Euphorbiaceae and Thymalaeceae plants have been found to exert EBV-inducing activity (Ito *et al.*, 1981). In an earlier study done by Norhanom and Yadav (1995), 20% of the Euphorbiaceae plants screened (13 out of 48 species) were found to be positive for tumour-promoting activity when tested at concentrations of 10 to 40 μg/ml cell culture medium. Among the Malays, these plant extracts are either taken internally as decoction or concoction or used externally as a poultice. Medicinal plants with tumour-promoting activity could well be an important actiological factor in the promotion of tumours among Malays who used these plants regularly in folk medicine.

5.2 Thin-layer Chromatographic Screenings

In this study, three selected *Diospyros sp* were investigated. Thin-layer chromatographic screenings were done to the light PE, chloroform and methanol extract of *D. graciliflora*, *D. discolor* and *D. lanceifolia* stem. The screening was also done to the extracts of *D. discolor* stem bark.

5.2.1 TLC Screening of *D. graciliflora* Stem

Preliminary screenings of the light PE extract indicated the presence of seven compounds comprising mainly of terpenes. Dg P5 appeared as a distinct blue under UV-365 but meagrely stained with vanillin-H_2SO_4 and iodine. Thus, this could be a coumarin present in trace amount. The chloroform extract also presented seven compounds comprising mainly of terpenes. The methanol extract yielded six compounds of four terpenes.

5.2.2 TLC Screenings of *D discolor* Stem

Preliminary screenings of the light PE extract presented eight compounds with the majority of them were from the class of terpenes. A spot of coumarin present in trace amount was detected which imparted an intense blue under UV-365. The chloroform extract indicated the presence of eight compounds mainly of terpenes. All terpenes (Dd C2, Dd C3, Dd C5, Dd C6 and Dd C7) appeared negatively under UV-365. Dd C2, Dd C3 and Dd C5 were stained strongly with iodine, Dd C6 stained moderately while Dd C7 was stained weakly. Dd C2, Dd C3 and Dd C7 showed intense violet colour when sprayed with Dragendorff reagent while Dd C5 and Dd C6 imparted a brown and blue colour under the same treatement. The methanol extract yielded seven compounds with

the majority five of them were terpenes.

5.2.3 TLC Screenings of *D. discolor* Stem Bark

Preliminary screenings of the light petroleum ether extract revealed the presence of seven compounds consisiting of three terpenes and a coumarin. Dds P4 imparted an intense blue under UV-365 indicating a possible coumarin compound. Although Dds P6 responded negatively to vanillin-H_2SO_4, it stained strongly with iodine, elaminating the possibility of Dds P6 being a terpene.

The chloroform extract indicated the presence of eight compounds comprising mainly of terpenes. The methanol extract yielded seven compounds with six of them were terpenes.

5.2.4 TLC Screenings of *D. lanceifolia* Stem

Preliminary screenings of PE extract presented six compounds mainly from the class of terpene. Dl P3 responded negatively under UV-365 but stained strongly with iodine and stained blue with vanillin-H_2SO_4, elaminating the possibility of Dl P3 being a terpene.

The chloroform extract yielded seven compounds comprising mainly or terpenes. Compound Dl C4 and Dl C5 were stained blue and brown with vanillin-H_2SO_4 and also stained strongly with iodine eventhough they both responded negatively under UV-365, thus confirmed the presence of terpene in both compounds. The methanol extract presented six compounds , all from the class of terpenes. Compounds Dl M2 and Dl M3

detected negatively under UV-365. However they stained moderately with iodine and imparted intense brown and violet colours when sprayed with vanillin-H_2SO_4.

An investigation of the stem bark, wood and/or fruit of nine Asian *Diospyros sp* has been done by Muhamad *et al.* (1984) which revealed a range of triterpenes and naphtoquinones. In addition to lupeol, betulin and betulinic acid which were present in all samples, seven other triterpenes were isolated and identified. Six of the nine species also yielded naphtoquinones. An examination of two samples of stem bark of *Diospyros manii* was done by Jeffreys *et al.* (1983). Investigation on the extraction of the stem bark of this plant yielded three naphtoquinones and three triterpenes.

5.3 Structure Elucidation of Isolated Compounds of *Diospyros discolor*

5.3.1 Isolated Compound Dd P2

Dd P2 was collected from fraction 29-40 after scrapping and purifying by repeated washing with chloroform. The TLC screening showed an R_f value of 0.75 in solvent mixture of PE : $CHCl_3$ (30 : 70), 0.79 in $CHCl_3$ and 0.88 in solvent mixture of MeOH : $CHCl_3$ (5 : 95). It gave an intense blue spot when sprayed with vanillin-H_2SO_4 reagent which suggested that it could be a terpene. This compound was obtained as a white needle-shaped crystals with a melting point of 215 °C.

The IR spectrum of this compound projected absorption bands at 1635 cm^{-1} and 870 cm^{-1} which have been considered as a characteristic of 20 : 29 double bond of a pentacyclic triterpene (Cole *et al.*, 1965). Proton NMR values δ (ppm) for this compound in $CHCl_3$ revealed signals at δ 4.66, 4.56, 3.16, 2.34, 1.66, 1.01, 0.94, 0.92, 0.81, 0.77 and 0.74. The signals at δ 4.66 and 4.56 could be due to the two hydrogen at C-29. The multiplet signals at δ 3.20 was assigned to the presence of hydroxyl group at C-3 position.

From the data of ^{13}C nuclear magnetic resonance (^{13}C-NMR), C-3 appeared at δ 79.0 due to the presence of OH group (Solichin *et al.*, 1980). Double bond were found at C-20 (δ 150.9) and C-29 (δ 109.3). A triplet signal (-CH2-) at δ 35.6 was assigned to C-16 from the calculated value derived from the semi empirical equation reported by Bierbeck *et al.* (1980). Other triplets at δ 29.9 and δ 40.0 were assigned to C-21 and C-22 respectively.

This compound also showed a molecular ion peak at m/z 426 which is corresponded to the molecular formula of $C_{30}H_{50}O$. The mass spectrum of this compound was characteristic of a pentacyclic triterpene of the lupane series with an intense peak at m/e 189 irrespective of the nature of substitution in ring A, B, D and E (Figure 5.2) (Budzikiewicz *et al.*, 1963). The peak at m/z 189, 207, and 218 could be the contribution of the fragmentation process shown in Figure 5.1. This process is common for a saturated pentacyclic (Budzikiewicz *et al.*, 1963). Based on the fragmentation pattern and other spectral data obtained for this sample the most probable compound for this sample is lupeol or lup-20(29)en-3β-ol. Accordingly, this compound has been assigned the structure given in Figure 5.2.

Lupeol was found abundantly in Asian *Diospyros sp* (Muhamad *et al.*, 1984). According to Pant and Chaturvedi (1989), lupeol which was isolated from the latex of *Calotropsis procera* was used extensively in Indian medicine and employed as an arrow poison by the natives of African and Columbia (Shukla and Krishna, 1971).

Figure 5.1 : Fragmentation process of compound labelled as Dd P2

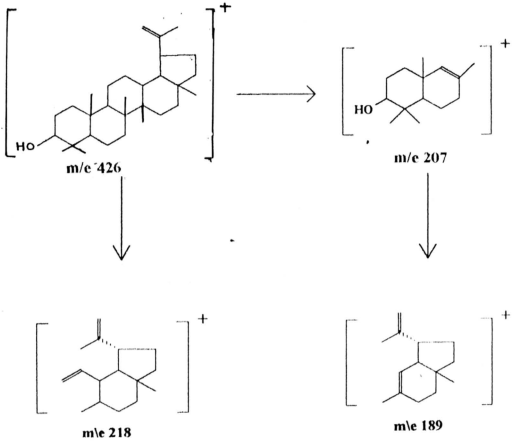
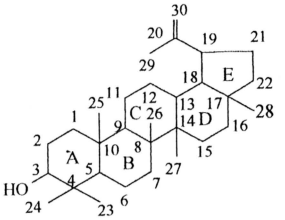


Figure 5.2 : Structure of lupeol



5.3.2 Isolated Compound Dd P3

Fraction 48-55 of light PE extract *of D. discolor* stem revealed Dd P3. In solvent mixture of PE : $CHCl_3$ (30 : 70), the R_f value given by this compound was 0.71. It gave an R_f value of 0.74 in 100% $CHCl_3$ while in solvent mixture of MeOH : $CHCl_3$ (5 : 95), the R_f value was 0.84. An intense blue spot was showed when sprayed with vanllin-H_2SO_4 suggesting that this compound could be from the class of terpene. The melting pont of this compound was 256-257 °C.

Five methyl singlets that were found in ^{1}H-NMR spectrum at δ 0.76, 0.82, 0.79, 0.98 and 4.51 (H-30), and two doublets at δ 4.68 and 1.68 can be assigned to H-29 and the bands in IR spectrum at 1645 and 890 cm^{-1} clearly showed that it is a lupane type of triterpenoid (Siddiqui *et al.*, 1988). One of the hydroxyl function was placed at C-3, the β orientation of which was supported by a double doublet at δ 3.17 (H-3). Another hydroxyl group is presented as CH_2OH was obvious from a set of doublet at δ 3.81 and 3.35.

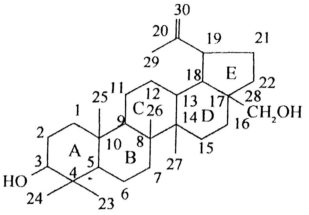
The olefinic carbon signal for C-20 and C-30 which was identified on the basis of chemical shifts and multiplicities (Solichin *et al.*, 1980). The remaining singlet was displaced downfield by +4.9 ppm (compared to ^{13}C-NMR value of Dd P2) due to hydroxy-substitution at C-28 could be assigned to C-17 quartenary carbon. A triplet signal at δ 29.2 was assigned to C-16 by an upfield shift (-6.4 ppm) due to the same process. Other triplets at δ 29.8 and 34.0 were assigned to C-21 and C-22 respectively.

The latter signal was shielded by -6.0 ppm. The signal of Dd P3 was strongly de-shielded to δ 42.6 ppm and was assigned to C-28.

The molecular formula $C_{30}H_{50}O_2$ of this compound was determined through peak matching of the molecular ion at m/z 442. The principle peak of m/e 207 and 189 were a characteristic of a pentacyclic triterpene of the lupane series. Based on the data collected for this compound, it was determined as betulin. Thus, the compound is assigned as shown in Figure 5.3.

According to Erich *et al.* (1989), betulin contining plants are systematically widespresd within the Angiosperms. Reports on the occurance of betulin are mainly dealing with the orders of Buxales, Dilleniales, Ebenales and Lamiales. The only occurance of betulin in Gymnosperms was reported from the American *Abies balsamea.* The birch bark of *Oleum ruscii* which contained 10 to 14% of betulin has been used as an antiseptic and in folk medicine against skin disease, malaria, dropsy and gout. Similarly, birch tar of the same species was used to treat rheumatism and gout as well as a nematocide antiseptic and against colic and manage in veterinary medicine. The birch bark oil is used as an antirheumatic and disinfectant.

Figure 5.3 : Structure of betulin



5.3.3 Isolated Compound Dd P6

Dd P6 was obtained from fraction 92-99 of light PE extract of *D. discolor* stem. R_f value of 0.5 was given by this compound in solvent mixture of PE : $CHCl_3$ (30 :70). It gave an intense R_f value of 0.57 in 100% $CHCl_3$ while in solvent mixture of MeOH : $CHCl_3$, the R_f value was 0.68. An intense violet spot was detected when sprayed with vanillin-H_2SO_4 reagent. Again, this suggested that Dd P6 could be from the class of terpene. The melting point given by this compound was 314 °C.

The IR spectrum of Dd P6 showed a strong peak at 1685 cm^{-1}, suggestive of the presence of an acid enolised β–diketone (Pakrashi *et al.*, 1968). The band at 1640 and 880 cm^{-1} indicated the presence of a methylene group that was confirmed by the signals at δ 4.60 and 4.73 in the NMR spectrum of this compound which also exhibited peak at δ 1.68 for the methyl of an isopropenyl group. It exhibited additional absorption band at 3480 and 1043 cm^{-1} attributed to the terminal methylene group (Nakanishi, 1984).

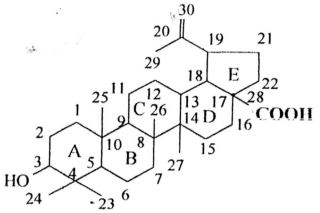
The ^{13}C NMR assignment showed the olefinic carbon signal for C-20 and C-30 which was identified on the basis of chemical shift and multiplicities (Solichin *et al.*, 1980). The remaining shift was found at C-17 quaternary carbon (δ 56.3). This value was displaced downfield by +13.3 ppm (compared to ^{13}C NMR value of Dd P2. Due to substitution-induced shift of carbixyl group at C-28. A triplet signal at δ 2.6 was assigned to C-16 by an upfield shift (-3.0 ppm) due to the same process. Other triplets at δ 29.9 and 37.3

were assigned to C-21 and C-22 respectively. The latter signal was shielded by -2.7 ppm. The signal of Dd P6 at δ 178.9 was strongly deshielded to δ 160.9 and was assigned to C-28.

Molecular weight of 456 as observed from the mass spectrum indicated the molecular formula to be $C_{30}H_{48}O_3$. The mass spectrum of this compound was also characteristic of a pentacyclic triterpene of the lupane series (Budzikiewicz *et al.*, 1963). The principal peaks were m/e 207, 189 and 248. Other fragments characteristic of the lupane skeleton were found at m/e 219 and 220. On the basis of the above data, compound Dd P6 was determined as betulinic acid. Thus, the structure of this compound is shown in Figure 5.4.

In the previous study which was done by Muhamad *et al.* (1984), betulinic acid was detected in all plant parts investigated (stem bark and wood) of *nine Diospyros sp* from the Asian origin. However, the medicinal properties of this compound has not been documented.

Figure 5.4 : Structure of betulinic acid



5.4 Conclusions

In the present study, crude PE extract of *D. discolor* stem which is commonly used in traditional medicine was found to have anti-tumour promoter activity. A pure compound namely lupeol was isolated from this extract and lupeol demonstrated the strongest anti-tumour promoter activity when tested using the TPA induced EBV EA activation assay in Raji cells. The present study also showed that natural phorbol esters obtained from the Euphorbiaceae plants could be used in place of TPA as a strong tumour promoter. The mode of action or mechanisme of lupeol as an anti-tumour promoter was however not studied.

The present investigation by an *in vivo* short term assay strongly suggested that lupeol may be a valuable anti-tumour promoter. Since the distribution of lupeol is ubiquitous in the plant kingdom, it is evisaged that this compound can play a role in future cancer chemoprevention. In the case of lupeol, this is advantagous because to date, lupeol has not been reported to show any significant biological activtities including cell cytotoxicity. Lupeol also occurs as free, esterified and also aglycon of triterpene saponins in many plants. Since it is not toxic, daily intake of plants with lupeol may have long-term protective effects against neoplastic disease.

In Malaysia, good epidemiological studies regarding an association between the use of plants with anti-tumour promoting activity and the prevalance of tumours are clearly needed. More plants should be screened for this activity in Malaysian plants are nescessary.

For the development of anti-cancer control strategies, there is a need to investigate various plants including Ebenaceae species which posseses anti-tumour promoter activity.