Chapter 5

Conclusions and Recommendations

The medicinal plant named *Dioscorea membranacea*, was identified by comparison with authentic voucher specimens, SKP 062 04 13 01, and have been kept in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. Microscopic description was also used to identify plant powder and compare its characteristics to the previous report (Itharat, 2002). Its plant powder showed the characteristics of the elliptical shape starch grains, the rhaphide crystal of calcium oxalate in the parenchyma cells, and the dark brown cell wall of cork cells in large quantity. Based on its identical characteristics to those of the previous report (Itharat, 2002), this plant was identified as *Dioscorea membranacea*.

In this study, the extracts of *D. membranacea* were obtained by three extraction methods such as decoction, maceration and soxhlet extraction, using different solvents e.g. hexane, chloroform, methanol, ethanol and water. The qualification and quantification of dioscorealide B as a cytotoxic marker in these extracts was analyzed by the developed HPLC method. Chromatographic separation was performed on a C_{18} column, using a gradient solvent system comprised of water (A)-acetonitrile (B) as followed: 70-55% A at 0-10 min, 55% A for 5 min, 55-30% A at 15-30 min, 30% A for 5 min, 30-70% A at 35-37 min, 70% A for 8 min. The flow rate of the mobile phase was 1.0 ml/min and the injection volume was 10 µl. Chromatographic separations were achieved at room temperature. The UV detector was used to detect the dioscorealide B peak at a fixed wavelength of 270 nm. In this condition, dioscorealide B exhibited a well-defined chromatographic peak at a retention time of 21.93 min. The chemical characteristics shown as HPLC fingerprints of each extract of *D. membranacea* were different depending on extraction methods and the solvents used. These fingerprints were also used for identification and quality evaluation of *D. membranacea* extracts. The HPLC fingerprints of extracts that were obtained from the same solvents exhibited the similar patterns. Every extract displayed the dioscorealide B peak, a standard marker in the chromatograms except water extract. Thus, the developed HPLC fingerprints of *D. membranacea* extracts can be used as standards for quality control. The soxhlet method with both single and polarity sequencing solvent extractions produced significantly higher dioscorealide B than did the maceration prepared in the same way. The reason for this was that heat used in the soxhlet method increased the solubility of dioscorealide B, thus facilitating its extraction from plant materials. Whereas water extract obtained by the decoction did not contain dioscorealide B, hexane, chloroform, methanolic, and ethanolic extracts obtained by the maceration and soxhlet extraction were found to contain different amounts of dioscorealide B. Of these extracts, chloroform extracts obtained by both methods contained the highest absolute amount of dioscorealide B per 1 g of dried plant, which may be due to some similarity in polarity between chloroform and dioscorealide B following the rules of thumb as ‘likes dissolve likes’ (Kenneth, 1994). This statement indicates that a solute will dissolve best in a solvent that has a similar polarity to itself. For a substance to dissolve the cohesive energy of the bonds holding the solid or liquid solute together, and the energy cost of disrupting the solvent-to-solvent bonds must be overcome by the cohesive energy released by the formation of the solute-to-solvent bonds. Thus there are two energy costs (one solute/ solute and one solvent/ solvent) and two energy gains (two solute/ solvent bonds). If these energies are approximately equal, which occurs when the solvent and solute molecules are structurally similar, then the substance will dissolve in the solvent. Hence, the saying: ‘Likes dissolve Likes’. There were significant differences in the amounts of dioscorealide B among the extracts obtained by all extraction methods except those in ethanolic and methanolic extracts obtained from single solvent maceration. Since, the single solvent extraction of soxhlet method using chloroform can be used to extract high amount of dioscorealide B (591.05 ± 3.47 µg/g dried plant) in the shortest time, so it was the most suitable method for extracting the cytotoxic marker, dioscorealide B.

The crude extracts of *D. membranacea* obtained from different extraction methods were tested for their cytotoxicity against a human breast cell line MCF-7 used as a biological characteristics model. According to National Cancer Institute guidelines that consider the extracts with IC_{50} values < 20 µg/ml to be “active”, water, hexane, chloroform and ethanolic extracts prepared by all extraction methods exhibited potent cytotoxic activity against MCF-7 excluding methanolic extracts obtained from single and polarity sequencing solvent macerations (IC_{50} =31.97±1.67 and 21.88±1.70 µg/ml, respectively) and polarity sequencing solvent extraction of soxhlet method (IC_{50} = 64.10±15.07 µg/ml). Hexane extracts (IC_{50} = 5.52 - 6.26 µg/ml), chloroform extracts (IC_{50} = 4.43 - 6.16 µg/ml) and water extract (IC_{50} = 6.56±0.45 µg/ml) showed high cytotoxicity against MCF-7 while methanolic extract from single extraction of soxhlet method (IC_{50} = 14.16±3.67 µg/ml) and ethanolic extracts from maceration (IC_{50} = 14.87±0.22 µg/ml) and soxhlet extraction (IC_{50} = 17.56±2.90 µg/ml) showed moderate activity. The cytotoxic activity against MCF-7 of all extracts was correlated with the amount of dioscorealide B excluding that of water and hexane extracts obtained by maceration. The water extract obtained from decoction exhibited high cytotoxicity against MCF-7 although it did not contain a cytotoxic marker, dioscorealide B. This activity was probably due to effects of other cytotoxic compounds with high polarity in *D. membranacea*. In the same way, hexane extracts from maceration contained a low level of dioscorealide B, but exhibited high cytotoxic activity against MCF-7. That may be produced by other high non-polarity cytotoxic compounds in *D. membranacea*. On the contrary, the chloroform extracts from both maceration and soxhlet extraction showed relationship between the cytotoxic activity against breast cancer cell line and the content of dioscorealide B. The single solvent soxhlet extraction using chloroform exhibited a strong performance in extracting dioscorealide B (591.05±20.26 µg/g dried plant) from *D. membranacea* in a short extraction period of 4 hours and produced high cytotoxic extract against MCF-7 (IC_{50}=5.19±0.79 µg/ml). Thus, it is the method of choice in extracting active ingredient, dioscorealide B from *D. membranacea*.

The results of stability of dioscorealide B in ethanolic extract under accelerated condition at 45ºC and 75% RH for 4 months exhibited that the remaining dioscorealide B was significantly decreased to 64.28±2.42% though the cytotoxicity against MCF-7 of the ethanolic extract was not change. From these stability results, the extract could be stored for two years at room temperature without loss of activity, which met the standards of The Institute of Medical Sciences, Ministry of Public health of Thailand (Ungphaiboon *et al*., 2005). Heat-accelerated conditions at 60, 70, and 80ºC with 75% RH for 1 month, also decreased the remaining dioscorealide B at the end of the exposure time to 61.16±3.72%, 42.72±0.92% and 22.97±2.35%, respectively. However, all extracts obtained by all tested conditions did not show significant changes in cytotoxic activity, which may be due to the presence of other heat-stable cytotoxic agents in extract or the degraded forms of dioscorealide B and other substances in *D. membranacea* induced by these tested conditions. Therefore, it was concluded that the cytotoxic activity of the extract was almost completely stable under high temperature despite the significant decrease in dioscorealide B level.

From all these results, the most effective method for extraction of cytotoxic extracts from *D. membranacea* should be soxhlet and maceration methods using all types of solvent and heat. Moreover, the method for dioscorealide B extraction should be a soxhlet method with non-polar solvents, especially chloroform, and no more than 60°C of heat. Since a high percentage yield of ethanolic extract (3.83±0.39%) with good cytotoxic activity against MCF-7 (IC_{50}=14.87±0.22 µg/ml), was obtained by maceration, this extract was chosen to study its stability under accelerated conditions. Furthermore, the results revealed that its cytotoxic activity was stable under these accelerated conditions that led to conclude that the ethanolic extract could be kept stable at room temperature for two years. Thus, ethanolic and water extracts obtained from *D. membranacea* could be prepared to products for to treat breast cancer patients more effectively. However, this study did not focus on water extract which showed higher cytotoxic activity than ethanolic extract. In addition to soaking in alcohol, traditional doctors always prepare formulations of *D. membranacea* for to treat cancer by boiling the plant material in water. Consequently, future research on cytotoxic markers and stability test of water extract of *D. membranacea* should be continued.