Chapter 4

Findings and Results

1. Identification of plant material

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 Science, 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 28-48 x 32-100 μm (width x length) elliptical shape starch grains (Figure 4.1), the rhaphide crystal of calcium oxalate in the parenchyma cells (Figure 4.2), 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*.

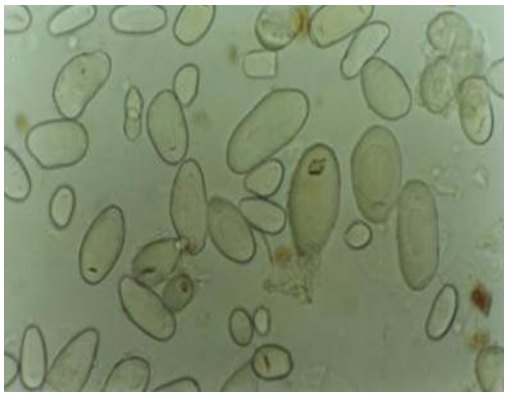

Figure 4.1

Starch grains of *D. membranacea* rhizome under microscope (x375)

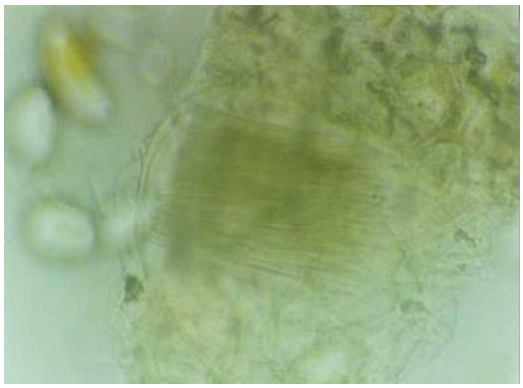

Figure 4.2

Calcium oxalate crystals of *D. membranacea* rhizome under microscope (x375)

2. Preparation of plant extracts

2.1 Percentage of yield of *D. membranacea* extracts

The crude extracts from dried powder of rhizome 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. All extracts were calculated as the absolute yields of extract (g) in 100 g of dried plant (%w/w). The percentage of yield of each extracts was shown in Table 4.1.

Table 4.1

Percentage of yield of crude extracts of *D. membranacea* from different extraction methods (n=3)

Extraction Methods		% Yield of extracts ± SD				
		Water	**Ethanol**	**Hexane**	**Chloroform**	**Methanol**
Decoction		41.45 ±2.62	-	-	-	-
Maceration	Single solvent maceration	-	3.83 ±0.39	0.18 ±0.01	1.17 ±0.02	3.87 ±0.44
	Polarity sequencing solvent maceration	-	-	0.18 ±0.01	0.89 ±0.04	3.45 ±0.18
Soxhlet extraction	Single solvent soxhlet extraction	-	4.45 ±1.16	0.49 ±0.05	1.45 ±0.48	6.41 ±1.23
	Polarity sequencing solvent soxhlet extraction	-	-	0.49 ±0.05	1.39 ±0.16	4.16 ±0.53

Extraction methods of *D. membranacea* rhizomes

1. Decoction

Dried plant material (5 kg) was boiled in distilled water (3 L) at 70°c for 30 minutes, filtered and dried by using a lyophillizer. This part was called as water extract.

2. Maceration

Dried plant material (300 g) was soaked with solvent (1 L) for 3 days by single solvent maceration and polarity sequencing solvent maceration. The extracts from each solvent were filtered and dried by an evaporator.

2.1 Single solvent maceration

The 1.2 kg dried plant powder was divided to be 4 portions. Each portion of plant powder was macerated by hexane, chloroform, methanol, and 95% ethanol. Thus, we obtained hexane, chloroform methanolic and ethanolic extracts.

2.2 Polarity sequencing solvent maceration

Dried plant powder (300 g) was macerated by solvents continuously from non-polar solvent to polar solvent (hexane, chloroform and methanol, respectively) of the same plant powder. Thus, we obtained hexane, chloroform and methanolic extracts, respectively.

3. Soxhlet extraction

Soxhlet extractor was used to extract dried plant material (75 g) by single solvent soxhlet extraction and polarity sequencing solvent soxhlet extraction for 4 hours of each solvent. The extracts from each solvent were filtered and dried by an evaporator.

3.1 Single solvent soxhlet extraction

The 300 g plant powder was divided to be 4 portions. Each portion of plant powder was extracted by hexane, chloroform, methanol, and 95% ethanol. So, we obtained hexane, chloroform methanolic and ethanolic extracts.

3.2 Polarity sequencing solvent soxhlet extraction

The plant powder (75 g) was extracted by soxhlet apparatus using solvents continuously from non-polar solvent to polar solvent (hexane, chloroform and methanol, respectively) of the same plant powder. We obtained hexane, chloroform and methanolic extracts.

3. Isolation of dioscorealide B from *D. membranacea* by column chromatography

3.1 Isolation of dioscorealide B

The ethanolic extract (40 g) obtained from rhizome of *D. membranacea* by maceration was separated by column chromatography to yield 21.05 mg of pure compound (yield = 0.053%) that was expected to be dioscorealide B. So, the isolated pure compound was identified by comparison its TLC chromatogram characteristic with dioscorealide B standard's and its proton NMR spectrum with authentic proton NMR spectrum of dioscorealide B.

3.2 Identification of isolated compound

3.2.1 Identification of isolated compound by TLC

The identification of isolated compound was performed by TLC using 3 solvent systems [Ethylacetate:Hexane (7:3 and 1:1) and $CHCl_3$] and compared the identical behavior with the dioscorealide B standard which kindly provided from Assoc. Prof. Arunporn Itharat (Figure 4.3). The developed spot of isolated compound was single at the same position of dioscorealide B spot in all solvent systems. Its behavior on TLC examination was the same as dioscorealide B standard's behavior. Both spots were positive to UV absorption at short wave (254 nm) as brown spot and also positive to anisaldehyde spraying reagent as green spot.

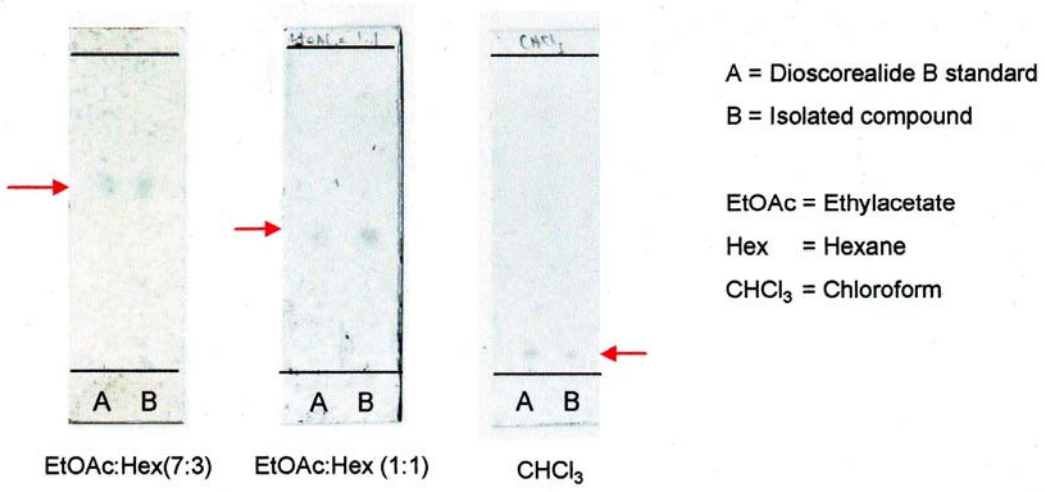

Figure 4.3

TLC chromatograms of isolated compound and dioscorealide B standard using 3 solvent systems [Ethylacetate:Hexane (7:3 and 1:1) and $CHCl_3$]

3.2.2 Identification of isolated compound by ^{1}HNMR

The ^{1}H NMR spectrum of isolated compound (Figure 4.5) was examined by comparison with the previous ^{1}H-NMR data of dioscorealide B (Itharat *et al*., 2003) showed as Table 4.2. The result showed the same ^{1}H NMR spectrums between isolated compound and dioscorealide B standard. Thus, it was strongly supported that isolated compound was dioscorealide B. The structure was shown in Figure 4.4.

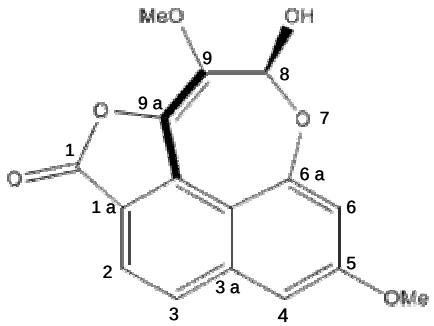

Figure 4.4

Chemical structure of dioscorealide B

Table 4.2

^{1}H-NMR spectral data of isolated compound and the previous data of dioscorealide B (Itharat *et al*., 2003)

Atom	δ_H (mult., *J* in 300 MHz)[c] of Isolated compound	δ_H (mult., *J* in 500 MHz)[c] of dioscorealide B in previous report
1	-	-
1a	-	-
1b	-	-
2	7.70 (d, 8.4)	7.76 (d, 8.5)
3	7.77 (d, 8.4)	7.8 (d, 8.5)
3a	-	-
3b	-	-
4	7.07 (d, 2.4)	7.36 (d, 2.5)
5	-	-
6	6.93 (d, 2.4)	6.88 (d, 2.5)
6a	-	-
8	5.99 (s)	6.03 (s)
8a	-	-
9	-	-
5-OMe	3.93 (s)	3.96 (s)
9-OMe	4.27 (s)	4.13 (s)

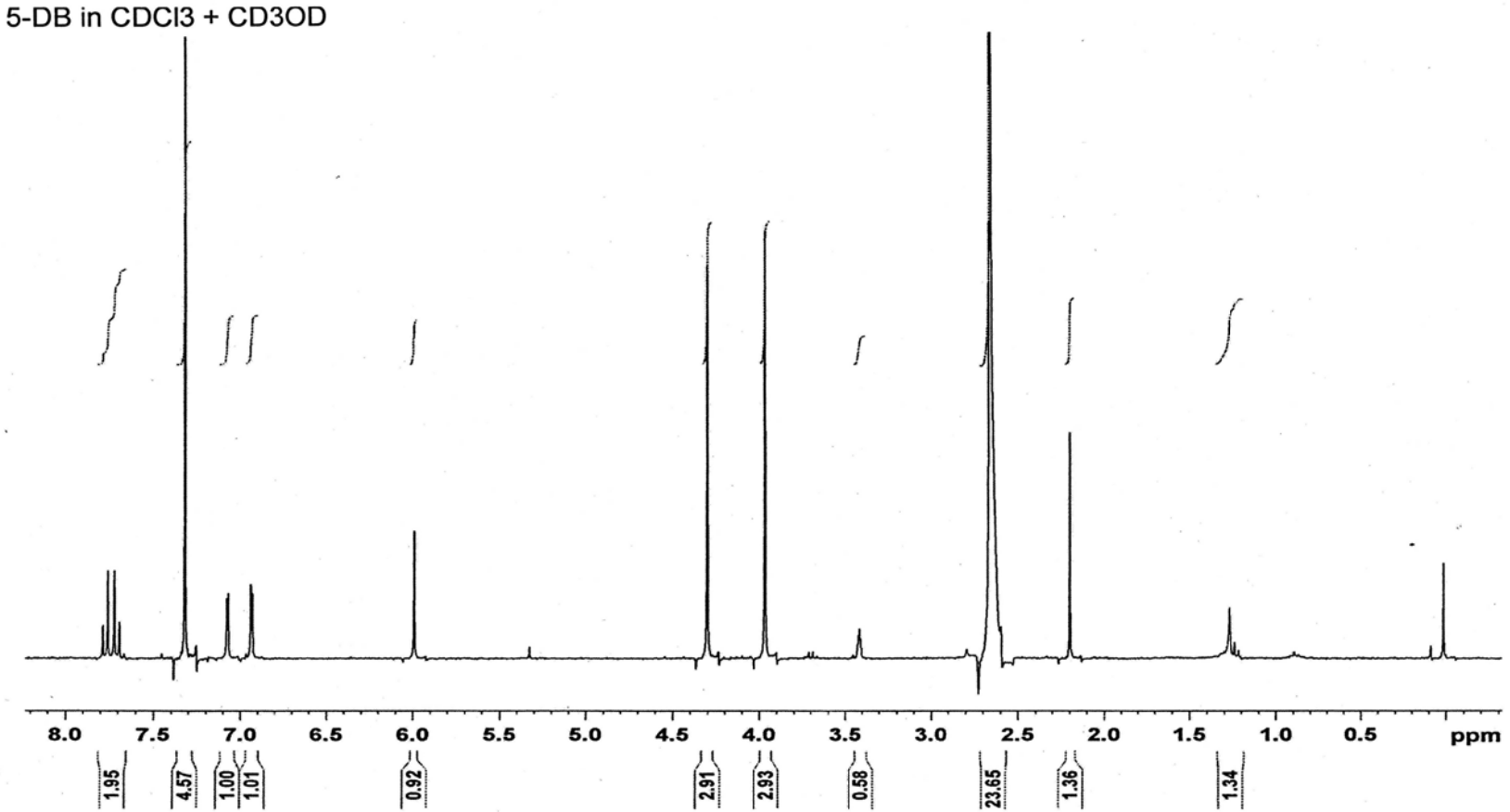

Figure 4.5
^{1}HNMR spectrum of isolated dioscorealide B

4. Chemical fingerprint analysis of *D. membranacea* extracts by HPLC technique

Herbal medicines have been widely used for health needs over many centuries, and become more and more popular worldwide during the last decade. However, due to the fact that in those herbs there may be hundreds of complex active components of which we have limited knowledge, it is almost impossible to identify all these substances and to carry on quantitative analysis. Generally, only a few effective components are determined by high-performance liquid chromatography (HPLC) for evaluating the quality of herbal medicines. According to the theory of traditional Chinese medicine (TCM), the therapeutic actions of herbal medicines are based on integral interaction of many kinds of ingredients combined rationally. The fingerprint chromatographic technology was introduced and accepted by the WHO as a strategy for identification and quality evaluation of herbal medicine. In 2000, the State Drug Administration (SDA) of China began to develop the fingerprints of TCM as the standard of quality control. Chromatographic methods were highly recommended for developing fingerprints of TCMs and their preparations. Since then, increasing interest in HPLC fingerprint analysis can be observed, not only in China but also in other countries all over the world. (Ji *et al*., 2005)

The obtained fingerprints are usually complex chromatograms, and their application relies on a comparison of profiles. Hence, fingerprint development has a stringent demand on resolution and peak capacity in the separation process. However, it is really difficult to separate all active components of herbal medicines in a single chromatographic run. Therefore, the development and optimization of a methodology to create fingerprints seems to be very important. It should be pointed out that the obtained chromatographic fingerprints would be used to authenticate and identify herbal medicines by the verification institute or by quality control laboratories. Hence, the developed fingerprint should feature good selectivity, reproducibility and feasibility. (Ji *et al*., 2005)

The chemical characteristics of each extract of *D. membranacea* that obtained from different extraction methods were studied using HPLC technique as the

HPLC fingerprints of each extract and the content of dioscorealide B, a cytotoxic marker of *D. membranacea* (Itharat *et al*., 2003).

4.1 Validation of HPLC method for dioscorealide B content analysis

The development of a method for separation and quantitation of the active compound in plant extract is very important for quality control of such preparations (Penissi *et al*., 2003). Therefore, the quantitative analysis method development and validation were required. Method validation is supposed to be the finishing touch on a proper method development process. The critical performance indicators of the method need to be investigated thoroughly during the development phase of the method. At validation, there should be no surprises toward failures on the method performance. Usually method validation is covered by standard procedure approaches and is executed within the shortest possible time. (Jimidar *et al*., 2007)

In the present study, HPLC method was developed to give a simple, rapid, sensitive, reproducible and accurate analytical procedure for determine the quantification of dioscorealide B, cytotoxic marker, in *D. membranacea* extracts. 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 peak of dioscorealide B with the fixed wavelength at 270 nm (Sirikatitham *et al*., 2007). In the selected optimal experimental conditions, the dioscorealide B exhibited a well-defined chromatographic peak with a retention time of 21.93 min. Figure 4.6 shows the chromatogram obtained by injection of a solution containing only dioscorealide B (100 µg/ml).

4.1.1 Specificity

Specificity is the ability to find and quantify the compound of interest also in the presence of other compounds. This refer to chromatographic methods where the analyze can be separated with sufficient resolution from all accompanying

peaks and that it can be detected with a suitable instrument (Meyer, 2004). The specificity of the developed HPLC method for dioscorealide B was carried out in the presence of the other components in the crude extract.

The peak of dioscorealide B in HPLC chromatogram can be separated from other compounds in *D. membranacea* extracts by used chromatographic condition. (see Figure 4.6 and 4.8)

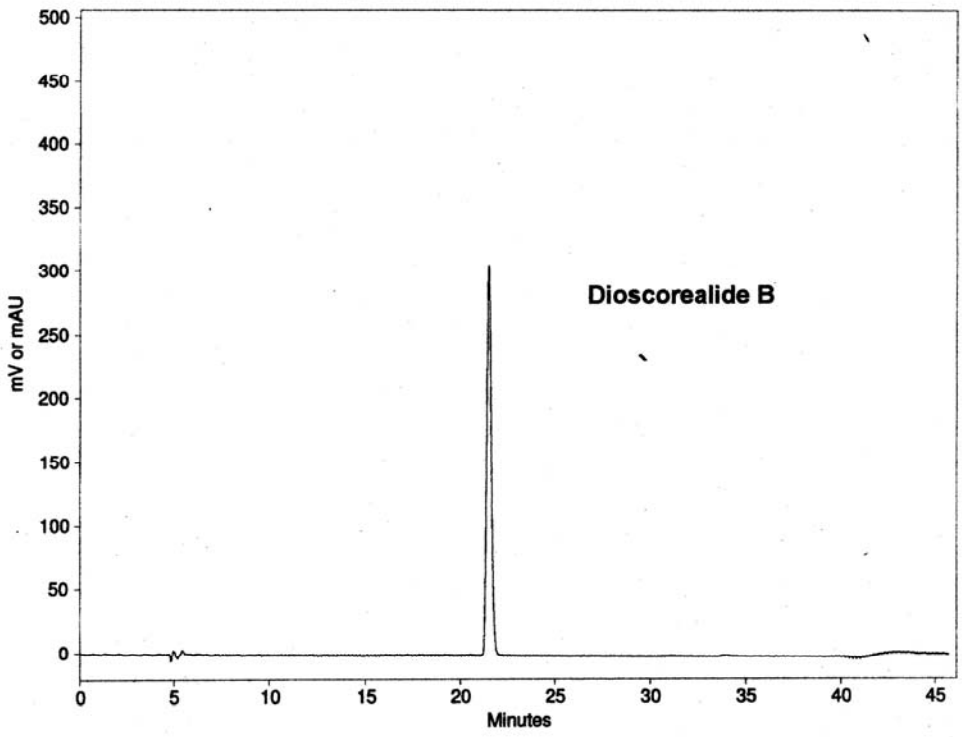

Figure 4.6

HPLC chromatogram of dioscorealide B

4.1.2 Linearity and range

The calibration curve of dioscorealide B was created by plotting concentrations versus corresponding mean peak areas (Figure 4.7). The chromatographic signals indicated a linear dependence with the concentration of dioscorealide B. Therefore, the dioscorealide B concentration was able to calculate from regression equation:

$Y = (51394.6667 \pm 651.7149) X + (39834.6667 \pm 12045.1380)$

$r^2 = 0.9997 \pm 0.0002$

(Y = peak area; X = dioscorealide B concentration)

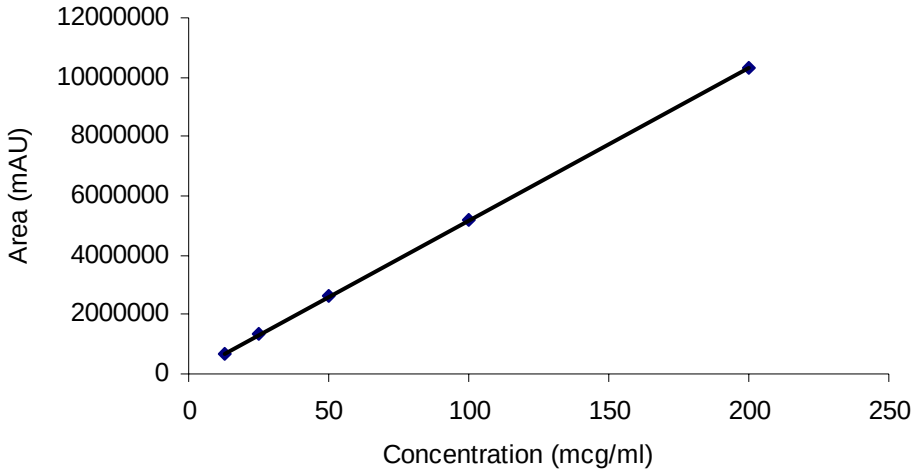

Figure 4.7

Calibration curve of dioscorealide B

The linearity was observed in the range of 12.5-200 μg/ml. A linear simple regression by the least squares method was applied and showed excellent correlation coefficient (R^2) greater than 0.9995. Linearity was checked for the assay method over the same concentration range for 3 consecutive days. The SD values of the slope and Y-intercept of the calibration curves were 51394.67 and 39834.67, respectively. The result exhibited that the correlation between the peak area and the concentration of dioscorealide B was excellent.

4.1.3 Precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions (Analytical Methods Technical Committee, 1994). It is the ability to re-run an analysis with low standard deviation (Meyer, 2004). Thus. it is necessary to distinguish between intermediate precision (inter-day precision) and repeatability (intra-day precision).

The precision of the method was expressed as relative standard deviation (RSD) of a series of measurements. The experimental values obtained in the determination of dioscorealide B in the samples are presented in Table 4.3. The RSD values were lower than 2.0% (between 0.49-1.94%), indicating the high precision of this method.

Table 4.3

RSD (%) of intra-day repeatability and intermediate precision studies

Concentration (μg/ml)	RSD (%) ± SD	
	Intra-day repeatability	Intermediate precision
12.5	0.49	0.92
50	1.52	0.82
200	1.16	1.94
Average	1.06 ± 0.52	1.23 ± 0.62

4.1.4 Accuracy

Accuracy is the ability to run an analysis with low difference between the 'true' and found value. The mean percentage recovery of dioscorealide B in ethanolic extract sample was ranged from 98.78-103.70 % (Table 4.4), which shows high accuracy of the method.

Table 4.4

% Recovery of dioscorealide B in the ethanolic extract of *D. membranacea*

Sample	Recovery (%) on each studied concentration (μg/ml)					Mean recovery (%) ± SD
	12.5	25	50	100	200	
1	103.70	99.47	102.03	100.56	103.34	101.82 ± 1.80
2	101.10	99.16	101.42	98.78	100.05	100.10 ± 1.16
3	103.43	100.10	101.91	102.38	102.74	102.11 ± 1.25
Average	102.74	99.58	101.79	100.57	102.04	101.35 ± 1.26

HPLC was employed to determine the content of dioscorealide B, a cytotoxic marker compound, in the extracts of *D. membranacea*. The separation was performed on a C_{18} column by gradient elution with water (A) and acetonitrile (B) system (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) at a flow rate of 1.0 ml/min and with UV detection at 270 nm. Regression equation showed good linear relationship between the peak area of marker and its concentration. The recovery was 101.35%. The repeatability (1.06%) and intermediate precision (1.23%) indicated that the method is suitable for quantitative analysis.

4.2 HPLC fingerprints of *D. membranacea* extracts

Recently, chromatographic fingerprint technique, as a more meaningful formulation for controlling the quality of herbal samples or their products, has been attracting more and more people's attention because the fingerprint technique emphasizes on the systemic characterization of compositions of samples and focus on identifying and assessing the stability of the plants. Fingerprint analysis has been introduced and accepted by WHO as a strategy for the assessment of herbal medicines. And it is also required by the Drug Administration Bureau of China to standardize injections made from traditional Chinese medicines (TCM) and their raw materials. The chromatographic methods involving fingerprint include TLC, high-performance liquid chromatography (HPLC), X-ray, CE, etc. Because of its advantages and popularization, HPLC fingerprint analysis has been regarded as the first choice. (Zhao *et al*., 2005)

The chromatographic condition described above was used to analyze the chemical characteristics of the crude extracts of *D. membranacea* as the HPLC finger prints. Retention time of the dioscorealide B (21.93 min) and spiking technique were used to identify the bioactive substance, dioscorealide B.