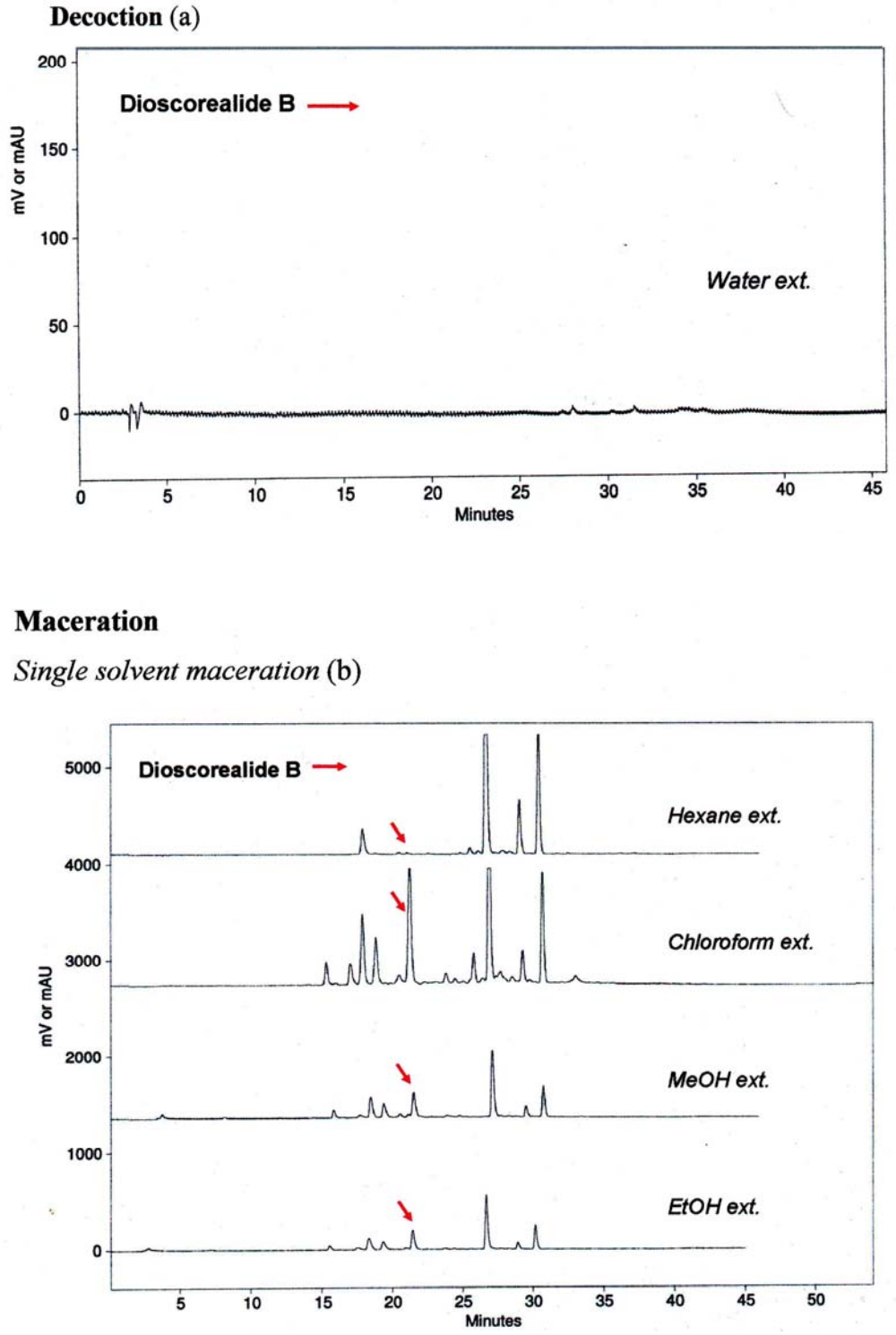
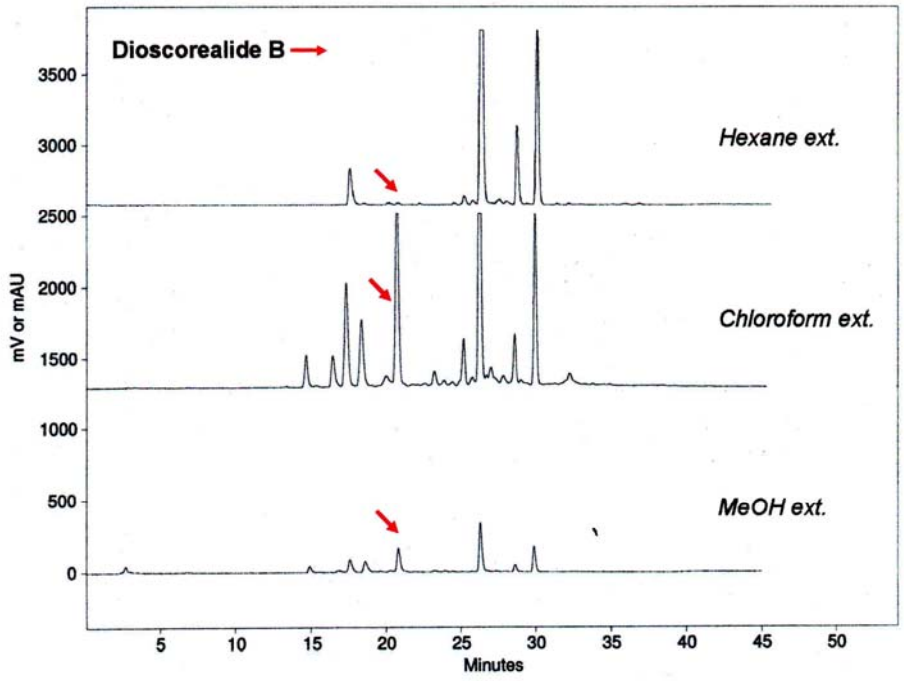



**Figure 4.8**

HPLC fingerprints of *D. membranacea* extracts from different extraction methods; decoction (a), maceration with single (b) and polarity sequencing solvent extractions (c) and soxhlet method with single (d) and polarity sequencing solvent extractions (e)

*Polarity sequencing solvent maceration* (c)




**Soxhlet extraction**

*Single solvent soxhlet extraction* (d)

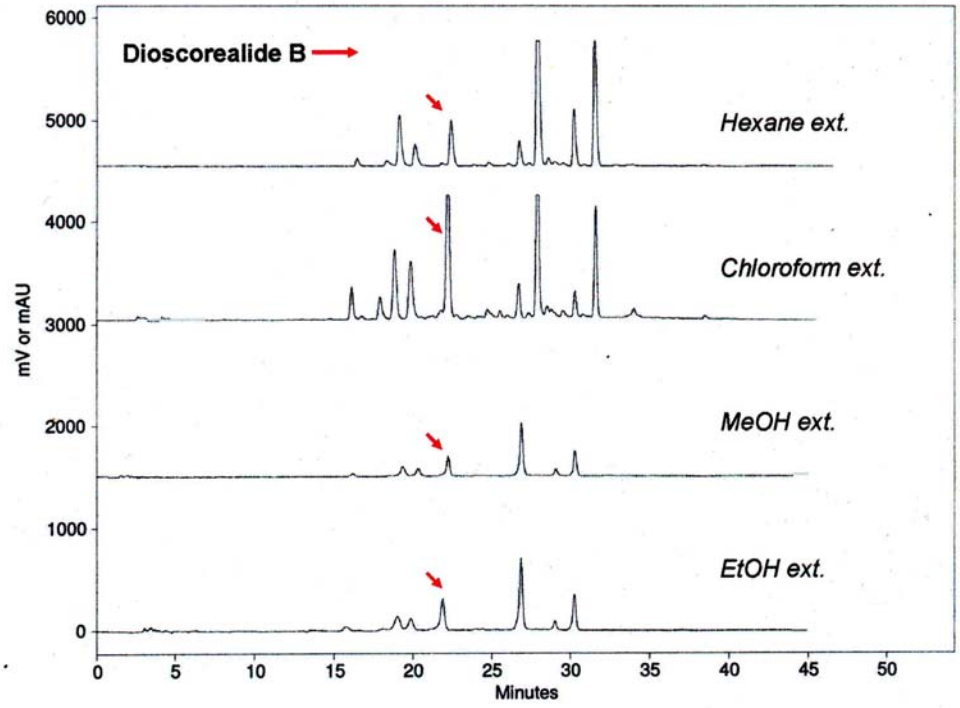



**Figure 4.8** (continued)

*Polarity sequencing solvent soxhlet extraction* (e)

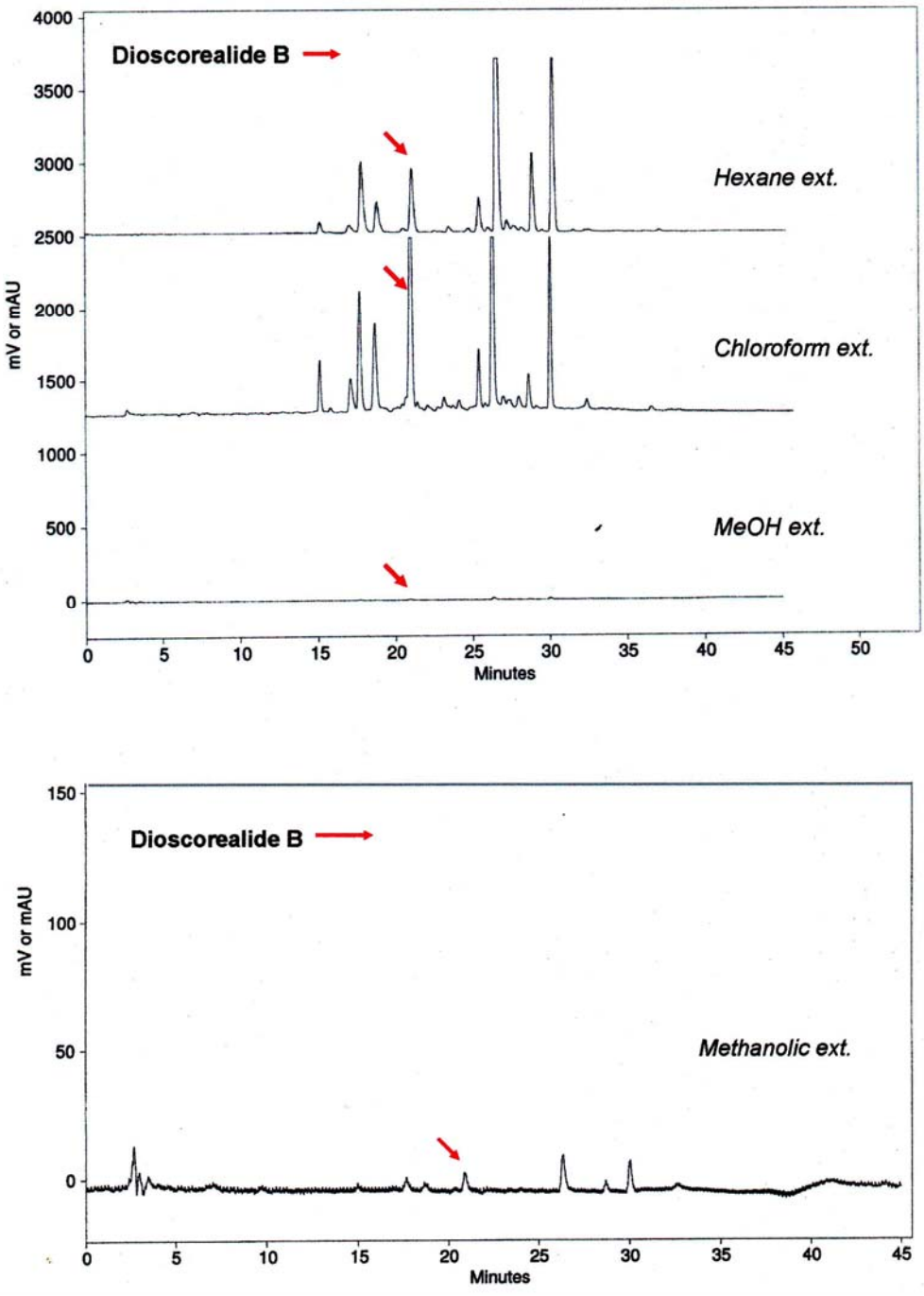



**Figure 4.8** (continued)

The chemical characteristics shown as HPLC fingerprints of each extract of *D. membranacea* (Figure 4.8) 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.

### 4.3 Quantitative determination of dioscorealide B from *D. membranacea* extracts

The amount of dioscorealide B, a cytotoxic marker, in crude extracts was determined by the developed chromatographic condition (Table 4.6). The crude extracts of *D. membranacea* were obtained by different extraction methods such as decoction, maceration and soxhlet extraction, using different solvents e.g. hexane, chloroform, methanol, ethanol and water. 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. 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. 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.

**Table 4.5**

Concentration of dioscorealide B in crude extracts (mg/g) of *D. membranacea* from different extraction methods (n=3)

| Extraction Methods | | Concentration of dioscorealide B in extracts (mg/g) ± SEM | | | | |
|---|---|---|---|---|---|---|
| | | **Water** | **Ethanol** | **Hexane** | **Chloroform** | **Methanol** |
| **Decoction** | | ND | - | - | - | - |
| **Maceration** | Single solvent maceration | - | $^{aa}$6.14 ±0.13 | $^{ab*}$0.33 ±0.05 | $^{ac*}$39.77 ±0.22 | $^{ad}$7.02 ±0.13 |
| | Polarity sequencing solvent maceration | - | - | $^{bb*}$0.33 ±0.05 | $^{bc*}$44.59 ±0.83 | $^{bd*}$4.76 ±0.15 |
| **Soxhlet extraction** | Single solvent soxhlet extraction | - | $^{aa}$10.54 ±0.41 | $^{ab*}$11.46 ±0.67 | $^{ac*}$33.11 ±1.14 | $^{ad}$5.77 ±0.23 |
| | Polarity sequencing solvent soxhlet extraction | - | - | $^{bb*}$11.46 ±0.67 | $^{bc*}$48.49 ±1.42 | $^{bd*}$0.23 ±0.01 |

**. The mean difference is significant at the .05 level.*

ND = Non detect

**Table 4.6**

Total content of dioscorealide B (µg/g dried plant) in crude extracts of *D. membranacea* from different extraction methods (n=3)

| Extraction Methods | | Dioscorealide B content (µg/g dried plant) ± SEM | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Water** | **Ethanol** | **Hexane** | **Chloroform** | **Methanol** | **Total** |
| **Decoction** | | ND | - | - | - | - | - |
| **Maceration** | Single solvent maceration | - | $^{aa*}$262.52 ±5.55 | $^{ab*}$0.56 ±0.08 | $^{ac*}$474.87 ±2.57 | $^{ad*}$237.60 ±4.28 | - |
| | Polarity sequencing solvent maceration | - | - | $^{bb*}$0.56 ±0.08 | $^{bc*}$373.09 ±6.92 | $^{bd*}$154.72 ±4.99 | $^{be*}$528.36 ±5.34 |
| **Soxhlet extraction** | Single solvent soxhlet extraction | - | $^{aa*}$382.45 ±14.88 | $^{ab*}$59.47 ±3.47 | $^{ac*}$591.05 ±20.26 | $^{ad*}$320.2 ±12.66 | - |
| | Polarity sequencing solvent soxhlet extraction | - | - | $^{bb*}$59.47 ±3.47 | $^{bc*}$620.42 ±18.20 | $^{bd*}$10.24 ±0.52 | $^{be*}$690.27 ±21.05 |

**. The mean difference is significant at the .05 level.*

ND = Non detect

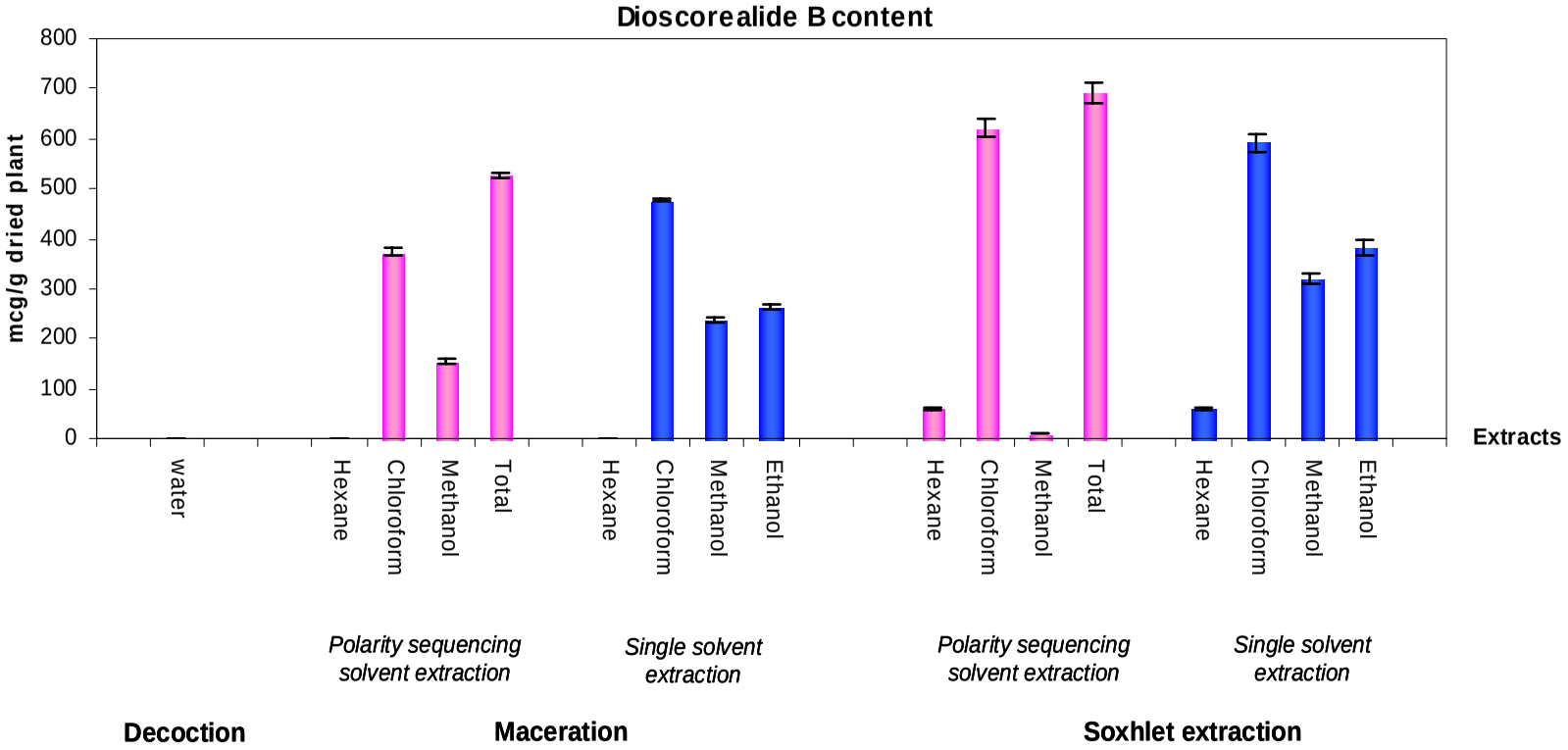



**Figure 4.9**

Total content of dioscorealide B (µg/g dried plant) in crude extracts of *D. membranacea* from different extraction methods (n=3)

**5. *In vitro* assay for cytotoxic activity of *D. membranacea* extracts**

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 activity 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. (Table 4.7)

**Table 4.7**

*In vitro* cytotoxic activity against MCF-7 by SRB assay of *D. membranacea* extracts from different extraction methods (n=2)

| Extraction Methods | | $IC_{50}$ of cytotoxic activity against MCF-7 (µg/ml) ± SEM | | | | |
|---|---|---|---|---|---|---|
| | | **Water** | **Ethanol** | **Hexane** | **Chloroform** | **Methanol** |
| **Decoction** | | 6.56 ±0.45 | - | - | - | - |
| **Maceration** | Single solvent maceration | - | 14.87 ±0.22 | 6.26 ±0.95 | 5.22 ±0.02 | 31.97 ±1.67 |
| | Polarity sequencing solvent maceration | - | - | 6.26 ±0.95 | 4.43 ±0.32 | 21.88 ±1.70 |
| **Soxhlet extraction** | Single solvent Soxhlet extraction | - | 17.56 ±2.90 | 5.52 ±0.70 | 5.19 ±0.79 | 14.16 ±3.67 |
| | Polarity sequencing solvent Soxhlet extraction | - | - | 5.52 ±0.70 | 6.16 ±0.43 | 64.10 ±15.07 |

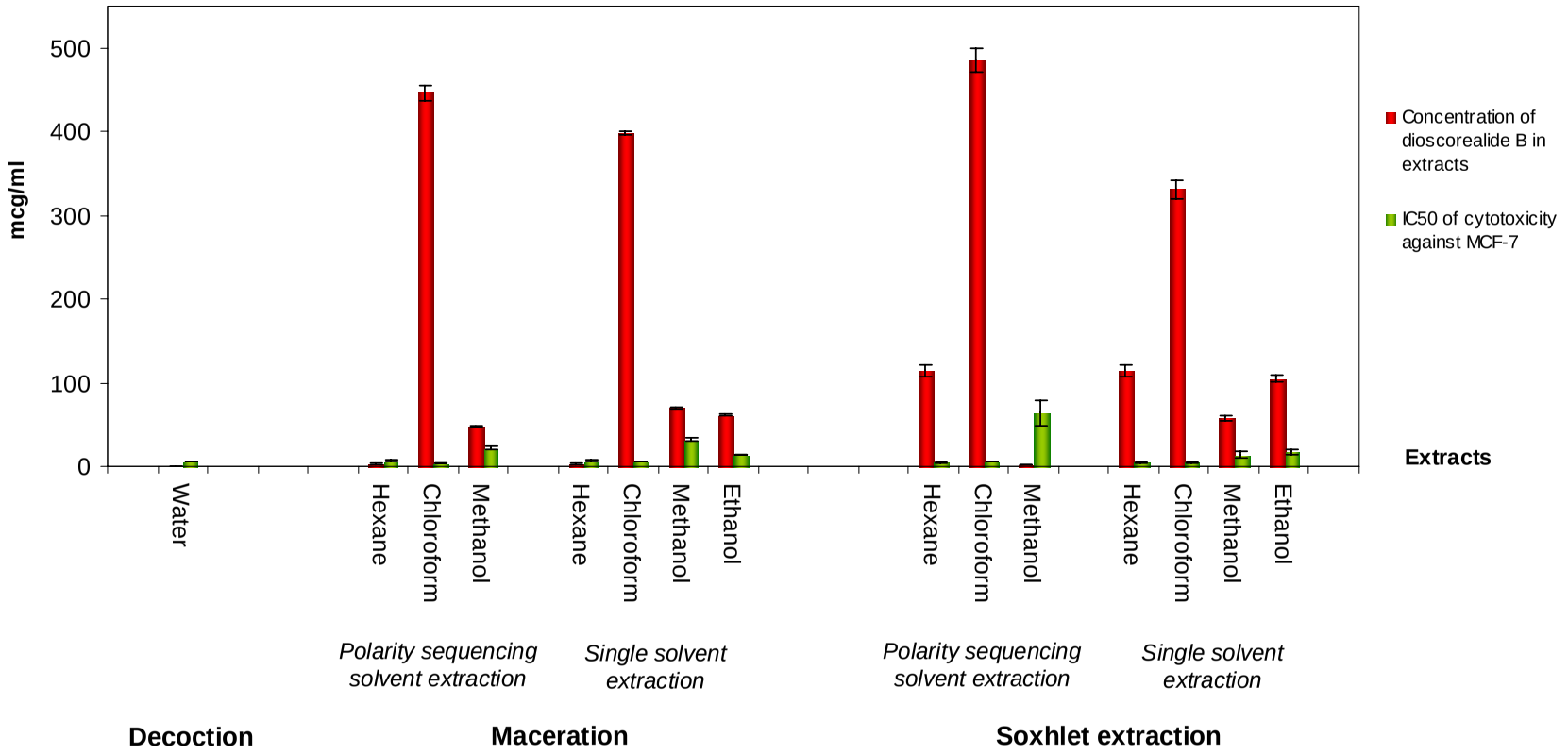



**Figure 4.10**

Concentration of dioscorealide B in crude extracts at 10 mg/ml (n=3) and $IC_{50}$ of cytotoxicity against MCF-7 by SRB assay (n=2) of crude extracts from different extraction methods

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 (Figure 4.10). 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.

## 6. Stability study of ethanolic extract under accelerated conditions

The stability of the ethanolic extract obtained from single solvent maceration was investigated by keeping under heat-accelerated conditions (60, 70, 80$^{o}$C, 75% RH for 1 month) and accelerated condition (45$^{o}$C, 75% RH for 4 months). The samples in screw cap vial were determined the changes of chemical and biological activity characteristics after various storage times. The samples were assayed for dioscorealide B content used as a determination of chemical characteristic change, and assayed for cytotoxic activity against a human breast cancer cell line MCF-7 by SRB assay used as a determination of biological activity characteristic change.

### 6.1 Stability of dioscorealide B in ethanolic extract

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 decreased following with the exposure time period. At the first day, the percentage of dioscorealide B remaining was begun to significantly decrease (85.19 ± 3.31%) and at day120, the dioscorealide B content in ethanolic extract remained 64.28 ± 2.42%. (Table 4.8 and Figure 4.11)

**Table 4.8**

Percentage of dioscorealide B remaining in the ethanolic extract of *D. membranacea* in stability test under accelerated condition at 45°C and 75%RH for 4 months (N=3)

| Day | % Dioscorealide B remaining (Mean ± SEM) |
|---|---|
| 0 | 100.00 ± 0.00 |
| 1 | * 85.19 ± 3.31 |
| 3 | 93.59 ± 5.51 |
| 5 | * 89.56 ± 6.49 |
| 7 | * 84.86 ± 2.52 |
| 9 | * 81.84 ± 2.18 |
| 11 | * 76.95 ± 1.38 |
| 20 | * 86.15 ± 2.82 |
| 30 | * 84.87 ± 1.89 |
| 60 | * 77.54 ± 5.30 |
| 90 | * 70.30 ± 1.80 |
| 120 | * 64.28 ± 2.42 |

**. The mean difference with day 0 is significant at the .05 level.*

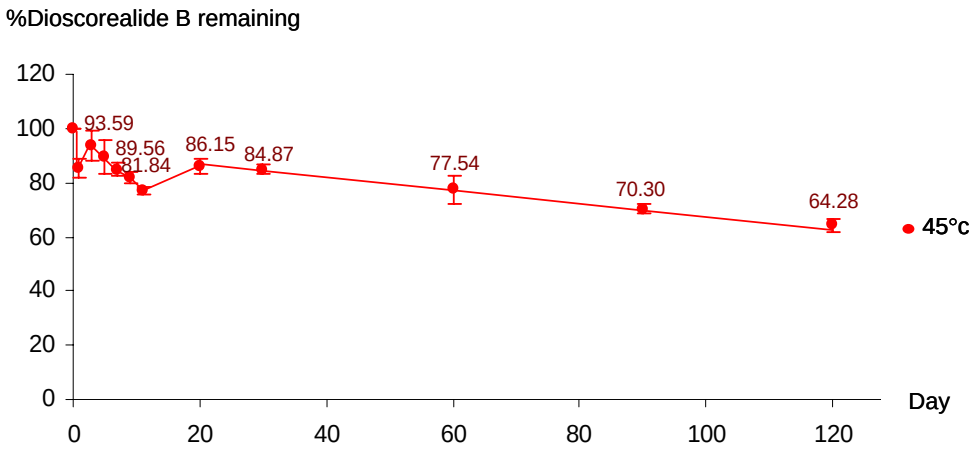



**Figure 4.11**

Percentage of dioscorealide B remaining in the ethanolic extracts of *D. membranacea* in stability test under accelerated conditions at 45°c and 75% RH for 4 months

The stability of dioscorealide B under heat-accelerated conditions, 60, 70 and 80°c with 75% RH for 1 month, was also investigated. The tested ethanolic extract samples possessed lesser dioscorealide B content following with the exposure time period. The dioscorealide B content in samples was begun to significantly decrease in day17 of 60°c condition and in day 2 of both 70°c and 80 °c conditions (Table 4.9). At the end of the exposure time, day 30, dioscorealide B remaining in the ethanolic extract was decreased to 61.16 ± 3.72%, 42.72 ± 0.92% and 22.97 ± 2.35% for 60°c, 70°c and 80°c conditions, respectively. The results exhibited that the heat was the cause of the stability of dioscorealide B. (Table 4.9 and Figure 4.12)

**Table 4.9**

Percentage of dioscorealide B remaining in the ethanolic extract of *D. membranacea* in stability test at 60°c, 70°c and 80°c with 75% for 1 month (N=3)

| Day | % Dioscorealide B remaining (Mean ± SEM) | | |
|---|---|---|---|
| | **60°c** | **70°c** | **80°c** |
| 0 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| 2 | 90.42 ± 5.01 | * 87.89 ± 5.24 | * 78.43 ± 3.44 |
| 4 | 94.49 ± 8.01 | * 78.37 ± 1.93 | * 61.55 ± 1.61 |
| 8 | 87.14 ± 2.52 | * 68.98 ± 2.82 | * 48.43 ± 2.34 |
| 17 | * 69.64 ± 4.70 | * 56.31 ± 2.38 | * 33.79 ± 0.29 |
| 21 | * 65.11 ± 2.18 | * 48.82 ± 2.68 | * 26.87 ± 1.64 |
| 30 | * 61.16 ± 3.72 | * 42.72 ± 0.92 | * 22.97 ± 2.35 |

**. The mean difference with day 0 is significant at the .05 level.*

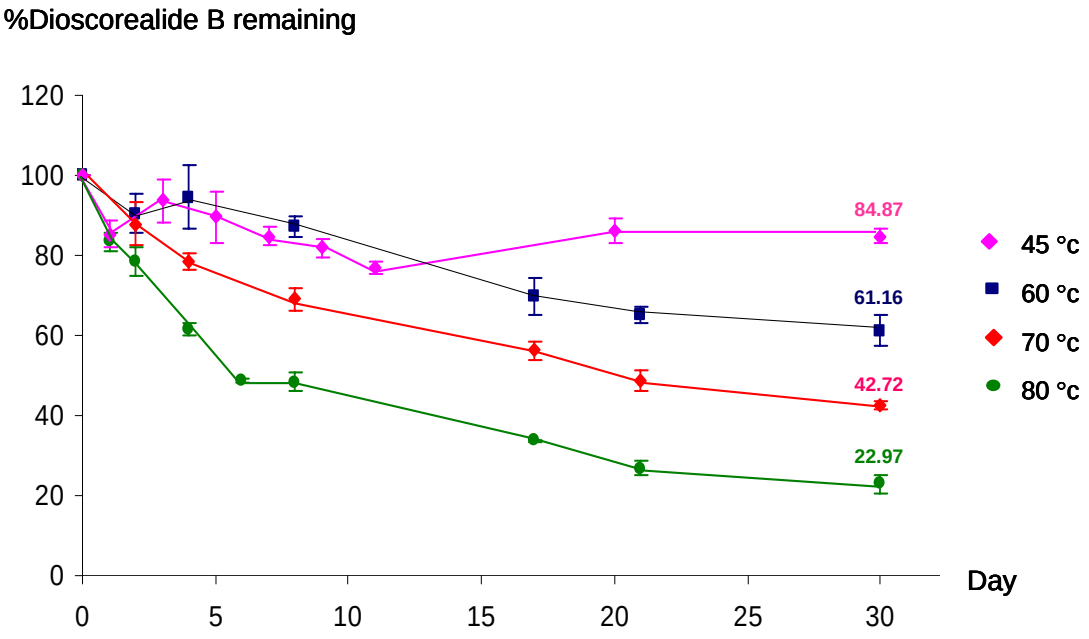



**Figure 4.12**

Percentage of dioscorealide B remaining in the ethanolic extracts of *D. membranacea* in stability test under accelerated conditions at 45°c, 60°c, 70°c and 80°c with 75% for 1 month

### 6.2 Cytotoxic activity change of ethanolic extract

The biological activity characteristic change exhibited as cytotoxic activity against MCF-7 of the ethanolic extract of *D. membranacea* was determined after keeping in the accelerated condition at 45°C and 75%RH for 4 months. The ethanolic extract samples at 10 µg/ml exhibited the %inhibition of cytotoxic activity as 57.20±6.98, 55.26±4.99, 54.35±6.82, 53.46±3.41, 57.28±3.11, 50.68±2.31, 50.99±3.34, 54.38±1.77, 54.77±6.15, 53.61±1.81, 48.14±0.75 and 46.34±1.23 for day 0, 1, 3, 5, 7, 9, 11, 20, 30, 60, 90 and 120, respectively. The results (Table 4.10) showed that the cytotoxicity against MCF-7, a human breast cancer cell line, of the ethanolic extract samples of each various storage time were not different with day 0.

**Table 4.10**

*In vitro* cytotoxic activity against MCF-7 by SRB assay of the ethanolic extract of *D. membranacea* at 10 µg/ml in stability test under accelerated condition at 45°c and 75% RH for 4 months (n = 2)

| Day | % Inhibition on MCF-7 at 10 µg/ml (Mean ± SEM) |
|---|---|
| 0 | 57.20 ± 6.98 |
| 1 | 55.26 ± 4.99 |
| 3 | 54.35 ± 6.82 |
| 5 | 53.46 ± 3.41 |
| 7 | 57.28 ± 3.11 |
| 9 | 50.68 ± 2.31 |
| 11 | 50.99 ± 3.34 |
| 20 | 54.38 ± 1.77 |
| 30 | 54.77 ± 6.15 |
| 60 | 53.61 ± 1.81 |
| 90 | 48.14 ± 0.75 |
| 120 | 46.34 ± 1.23 |

**. The mean difference with day 0 is significant at the .05 level.*

The cytotoxic activity against MCF-7 of ethanolic extract after keeping in the heat-accelerated conditions at 60, 70 and 80 °c with 75% RH for 30 days was also investigated. At 60°c, the ethanolic extract at 10 µg/ml exhibited the %inhibition of cytotoxic activity as 49.66±6.98, 41.86±5.58, 48.76±3.16, 51.95±1.91, 46.21±4.29, 44.88±0.76 and 46.50±0.65 for day0, 2, 4, 8, 17, 21 and 30, respectively. The differences of cytotoxic activity with day0 of all samples in various storage times were not significant. For 70°c condition, the %inhibitions of cytotoxic activity of the extract samples were 46.67±4.77, 45.44±3.63, 38.72±7.58, 38.29±0.65, 38.67±6.63, 34.03±4.71 and 30.59±4.17 for day0, 2, 4, 8, 17, 21 and 30, respectively. The

cytotoxic activity of all samples in each various storage time was not different with day0 excluding that of day 30. In the highest tested temperature condition, 80°c, the %inhibitions of cytotoxic activity were 45.99±5.34, 38.10±1.36, 45.73±2.90, 40.18±5.29, 37.19±0.16, 33.12±3.98 and 36.28±1.90 for day 0, 2, 4, 8, 17, 21 and 30, respectively. The cytotoxic activity of all samples in each various storage time when compared with it of day 0 was not different excluding that of day 21. (Table 4.11)

**Table 4.11**

*In vitro* cytotoxic activity against MCF-7 by SRB assay of the ethanolic extract of *D. membranacea* at 10 µg/ml in stability test under heat-accelerated conditions at 60°c, 70°c and 80°c with 75% RH for 1 month (n = 2)

| **Day** | **% Inhibition on MCF-7 at 10 µg/ml (Mean ± SEM)** | | |
|---|---|---|---|
| | **60°c** | **70°c** | **80°c** |
| 0 | 49.66 ± 6.98 | 46.67 ± 4.77 | 45.99 ± 5.34 |
| 2 | 41.86 ± 5.58 | 45.44 ± 3.63 | 38.10 ± 1.36 |
| 4 | 48.76 ± 3.16 | 38.72 ± 7.58 | 45.73 ± 2.90 |
| 8 | 51.95 ± 1.91 | 38.29 ± 0.65 | 40.18 ± 5.29 |
| 17 | 46.21 ± 4.29 | 38.67 ± 6.63 | 37.19 ± 0.16 |
| 21 | 44.88 ± 0.76 | 34.03 ± 4.71 | *33.12 ± 3.98 |
| 30 | 46.50 ± 0.65 | *30.59 ± 4.17 | 36.28 ± 1.90 |

**. The mean difference with day 0 is significant at the .05 level.*

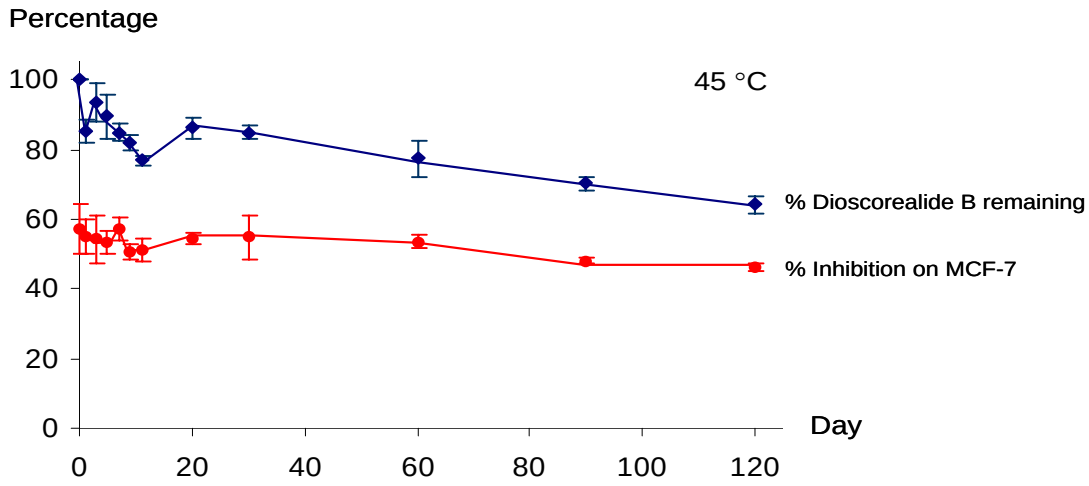



**Figure 4.13**

Stability test of the ethanolic extract of *D. membranacea* under accelerated condition at 45°c, 75% RH for 4 months exhibited as %dioscorealide B remaining (n=3) and %inhibition of cytotoxic activityagainst MCF-7 by SRB assay at 10 µg/ml (n=2)

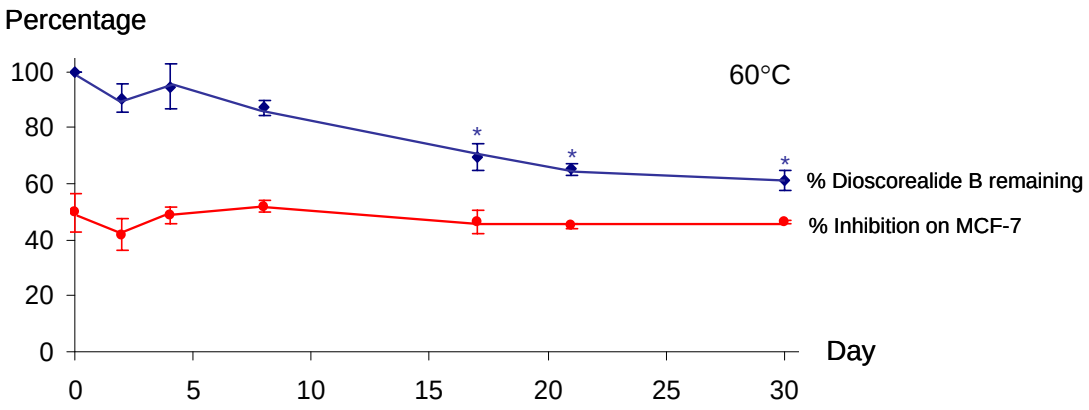



**Figure 4.14**

Stability test of the ethanolic extract of *D. membranacea* under accelerated condition at 60°c, 75% RH for 1 month exhibited as %dioscorealide B remaining (n=3) and %inhibition of cytotoxic activity against MCF-7 by SRB assay at 10 µg/ml (n=2)

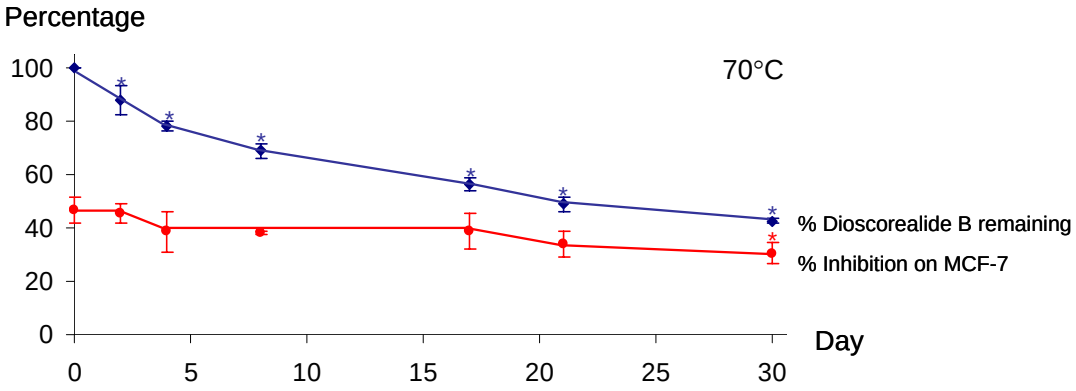



**Figure 4.15**

Stability test of the ethanolic extract of *D. membranacea* under accelerated condition at 70°c, 75% RH for 1 month exhibited as %dioscorealide B remaining (n=3) and %inhibition of cytotoxic activity against MCF-7 by SRB assay at 10 µg/ml (n=2)

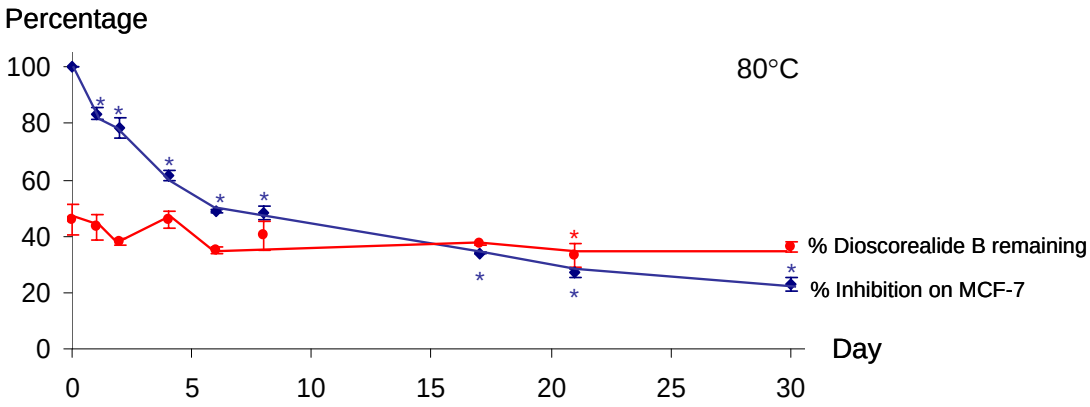



**Figure 4.16**

Stability test of the ethanolic extract of *D. membranacea* under accelerated condition at 80°c, 75% RH for 1 month exhibited as %dioscorealide B remaining (n=3) and %inhibition of cytotoxic activity against MCF-7 by SRB assay at 10 µg/ml (n=2)

From these results, the accelerated conditions caused dioscorealide B content in ethanolic extract of *D. membranacea* decreased significantly while the cytotoxic activity against MCF-7 of extract samples was not change. In the conditions of 45 °C with 75%RH for 4 months and 60°C with 75%RH for 1 month, the cytotoxic activity of extract did not show significant change while the conditions of 70°C and 80°C with 75%RH for 1 month caused cytotoxic activity changed a little bit. These results were suggested that 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. In accelerated condition at 45ºC and 75% RH for 4 months, the remaining dioscorealide B in extract 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 (Ungpaiboon *et al*., 2005). However, the cytotoxic activity of ethanolic extract was nearly stable in high temperature although dioscorealide B content was decreasing.