CHAPTER IV

RESULTS AND DISCUSSION

A. racemosus is well-known medicinal plants in India and also in Thailand. However, there is not much study on its application as antimicrobial (Mandal, et al., 2000b). In order to investigate for the potential of this medicinal plant as an antifungal agent, we performed the studies as follows.

Extract preparation and quality control

1. Plant extracts

1.1 Preparation of fractional extracts of *A. racemosus* root

The extracts of *A. racemosus* roots were successively extracted with the series of solvents i.e. hexane, ethanol and water provided crude hexane extract (AR-H, a light yellow paste), crude ethanolic extract (AR-E, dark brown paste), aqueous extract-A (AR-A, light brown powder), aqueous extract-B (AR-B, light brown powder) and sediment extract (AR-S, dark brown paste) (Figure 10a-e). The yield of each extract compared to the dried weight of *A. racemosus* root is shown in Table 4.

1.2 Preparation of *A. racemosus* roots saponin enriched extract

Recently, the phytochemical constituents of *A. racemosus* roots have been reported as steroidal saponins such as shatavarins I and IV, The saponin are known to possess various bioactivities including antifungal activity. The saponins from *Allium minutiflorum*, *Dolichos kilimandscharicus* and *Asparagus officinalis* (white asparagus) inhibited the growth of fungi (Elisa, et al., 2007; Marston, et al., 1988; Shimoyamada, et al., 1990). Regarding this reason, we attempted to find the preparation method to provide the high percentage of saponin for improving the bioactivities of *A. racemosus* extract. The roots of *A. racemosus* from Rayong were used as the raw materials. Since, they were obtained from cultivation, it would be easy for scaling up and quality control. On the other hand, the roots from Tak and

Kanchanaburi were collected from wild so the quality of raw materials cannot be assured.

The saponin enriched extract (AR-En) was obtained as a dark brown paste as shown in Figure 11 and the percentage yield is show in Table 4.

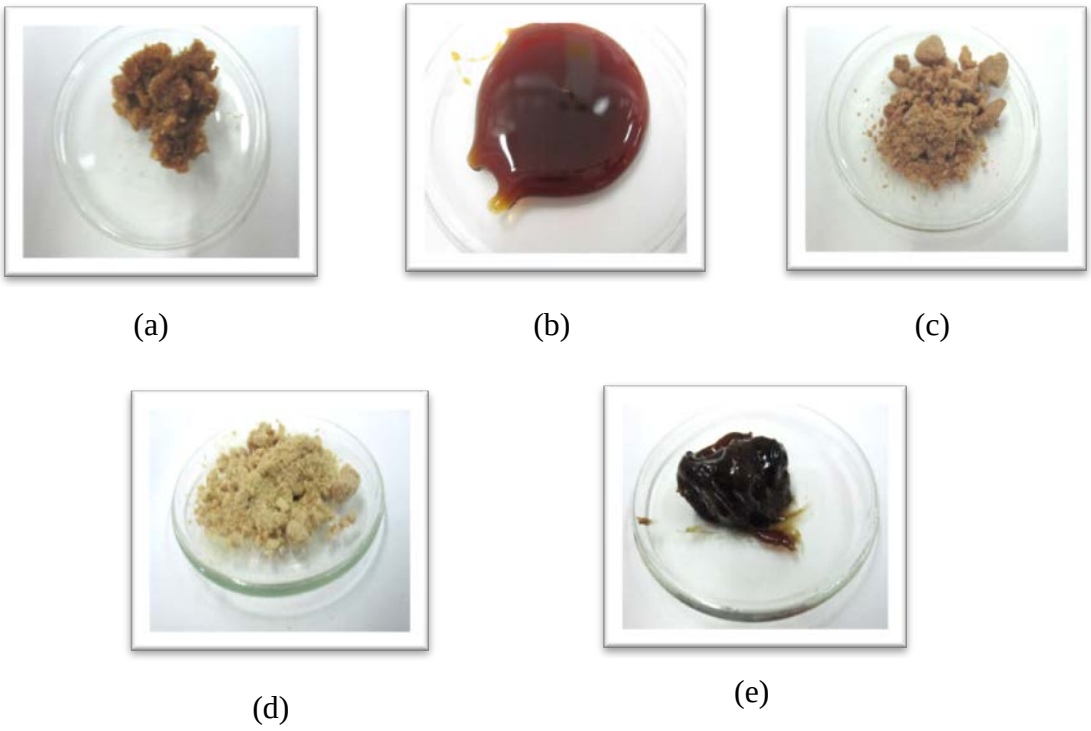


Figure 10 The appearance of *A. racemosus roots* successively extracts
a: hexane extract (AR-H), b: ethanolic extract (AR-E)
c: aqueous extract-A (AR-A), d: aqueous extract-B (AR-B)
e: sediment extract (AR-S)

Figure 11 The appearance of saponin enriched extract of *A. racemosus* roots (AR-En)

2. Qualitative and quantitative analyses

2.1 TLC profiles of *A. racemosus* roots extracts

The chemical constituent patterns of *A. racemosus* extracts were recorded using TLC. Shatavarin IV was used as a standard compound. The saponin glycosides from *A. racemosus* cannot be detected under uv-visible light due to the lack of chromophore, therefore anisaldehyde reagent was used as a TLC spraying reagent.

The TLC profiles of standard shatavarin IV exhibited two yellow spots at R_f 0.3 and 0.38 after sprayed with anisaldehyde reagent and heated. The result indicated that standard of shatavarin IV was contained small amount of other compound. The TLC profiles of *A. racemosus* extracts obtained from different solvents were compared with standard shatavarin IV using ethyl acetate: methanol: water (7.5: 1.5:1) as a mobile phase and anisaldehyde TS as a spraying reagent are shown in Figures 11- 13.

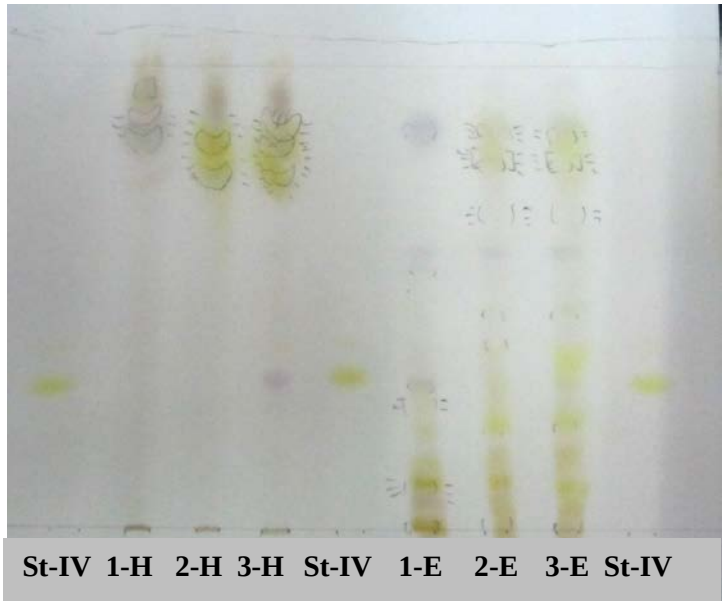


Figure 12 TLC chromatograms of hexane extracts (AR-H) and ethanolic extracts (AR-E) of *A. racemosus*

Note: St-IV: Standard shatavarin IV, 1-H : AR-H from Tak, 2-H: AR-H from Kanchanaburi, 3-H: AR-H from Rayong, 1-E: AR-E from Tak, 2-E: AR-E from Kanchanaburi, 3-E: AR-E from Rayong

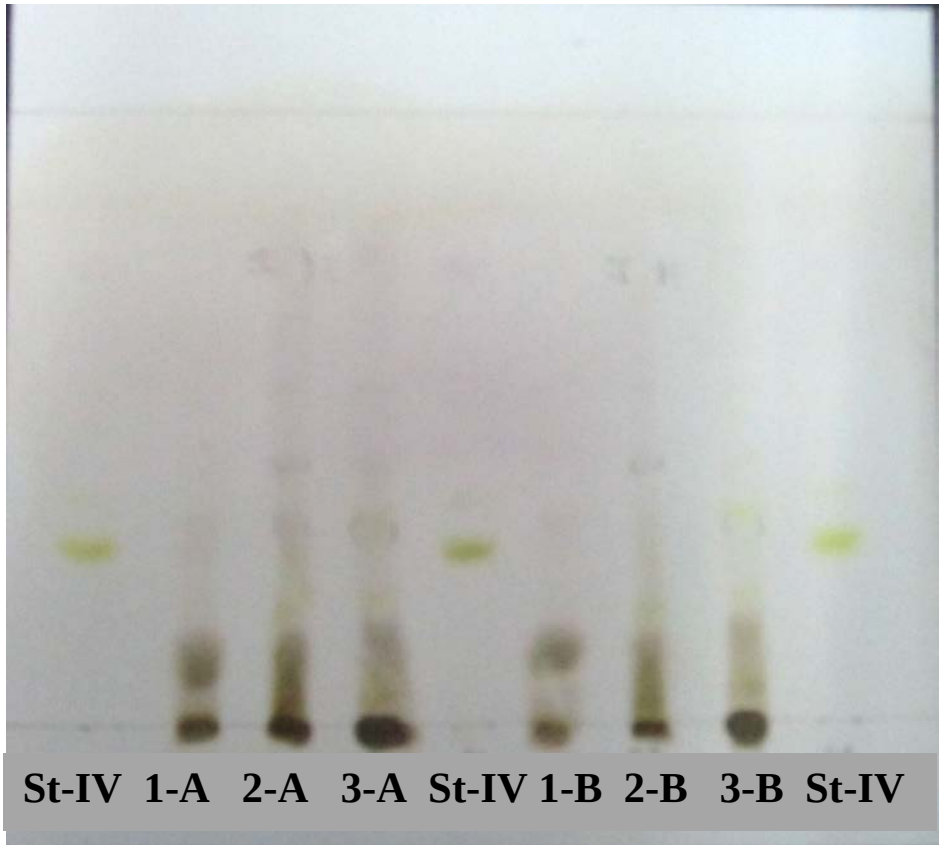


Figure 13 TLC chromatograms of aqueous-A extracts (AR-A) and aqueous-B extracts (AR-B) of *A. racemosus*

Note: St-IV : Standard shatavarin IV, 1-A : AR-A from Tak,
2-A : AR-A from Kanchanaburi, 3-A : AR-A from Rayong,
1-B : AR-B from Tak, 2-B : AR-B from Kanchanaburi,
3-B : AR-B from Rayong

Figures 12-14 show TLC chromatograms of the extracts using different solvents. The chromatograms showed that AR-Hs composed of components with low polarity while AR-Es composed of compounds with higher polarity. The aqueous extracts (AR-A and AR-B) and AR-Ss had higher polar compounds when compared to AR-Es. All of the fractions were compared with standard shatavarin IV. However we did not detect the trace of shatavarin IV in all of the extracts. Therefore, the higher sensitive methods such as LC-MS\MS and ELISA were used to detect shatavarin IV.

TLC fingerprints of the extracts of *A. racemosus* collected from 3 different areas, Tak, Kanchanaburi and Rayong, indicated that the extracts had slightly different profiles of chemical constituents. This might due to the various factors such as environment, locality and ages of the plants. With such variation, the quantitative analysis for saponin contents to standardize the extracts seemed to be necessary.

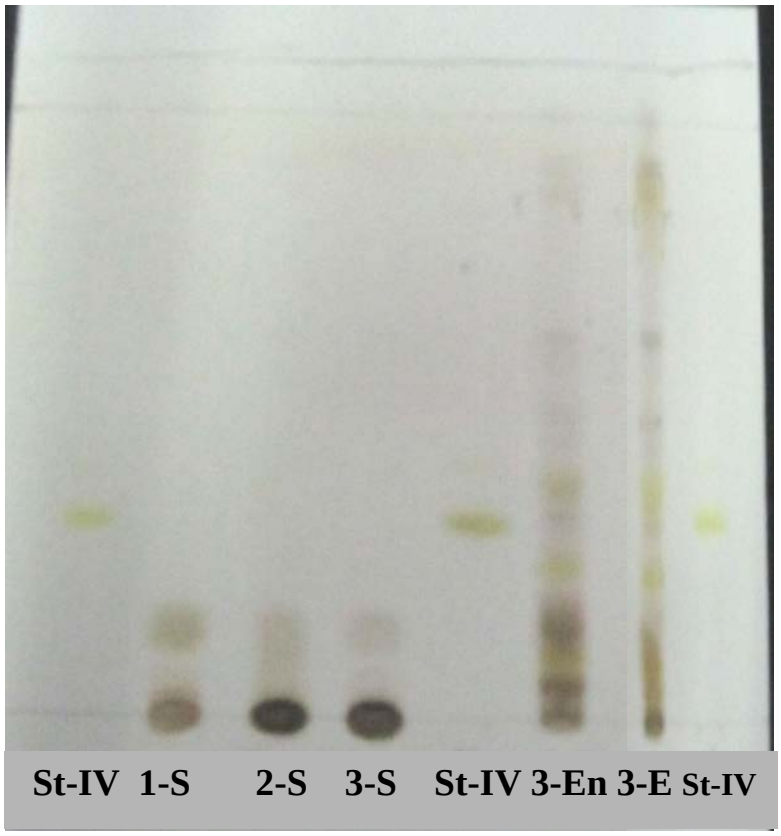


Figure 14 TLC chromatograms of sediment extracts (AR-S), saponin enriched extract (AR-En) and ethanolic extract (AR-E) of *A. racemosus*

Note: St-IV : Standard shatavarin IV, 1-S : AR-S from Tak,
2-S : AR-S from Kanchanaburi, 3-S : AR-S from Rayong,
3-En : AR-En from Rayong, 3-E : AR-E from Rayong

2.2 The quantitative analyses of shatavarin IV in *A. racemosus* root extracts by LC-MS/MS

The LC-MS/MS with MRM mode was used for quantitative analyses of shatavarin IV in *A. racemosus* root extracts. Shatavarin IV was analyzed by LC-MS/MS using MRM mode. Since shatavarin IV can fragment from *m/e* 886 (M^+) to *m/e* of 739 (M-rhamnose-H), 578 (M-rhamnose-glucose) and 723 (M-glucose-H), the MRM pair were set at *m/e* 886/739 886/578 886/723. The structures of shatavarin IV and its fragmentation are shown in Figure 15. LC-MS/MS chromatogram of standard shatavari IV is shown in Figure 16. Shatavarin IV showed peak at 10.00 min. Interestingly, the peaks of *m/e* 886/578 were also found at 8.32 min. They might be from the other compound that has the structure related to that of shatavarin IV. Calibration curve of shatavarin IV detected by LC-MS/MS is shown in Figure 17. Quantity of shatavarin IV in AR-Es from different sources analyzed by LC-MS/MS are shown in Table 5. The other saponin observed at 8.32 min were also determined as equivalent to shatavarin IV (Table 6)

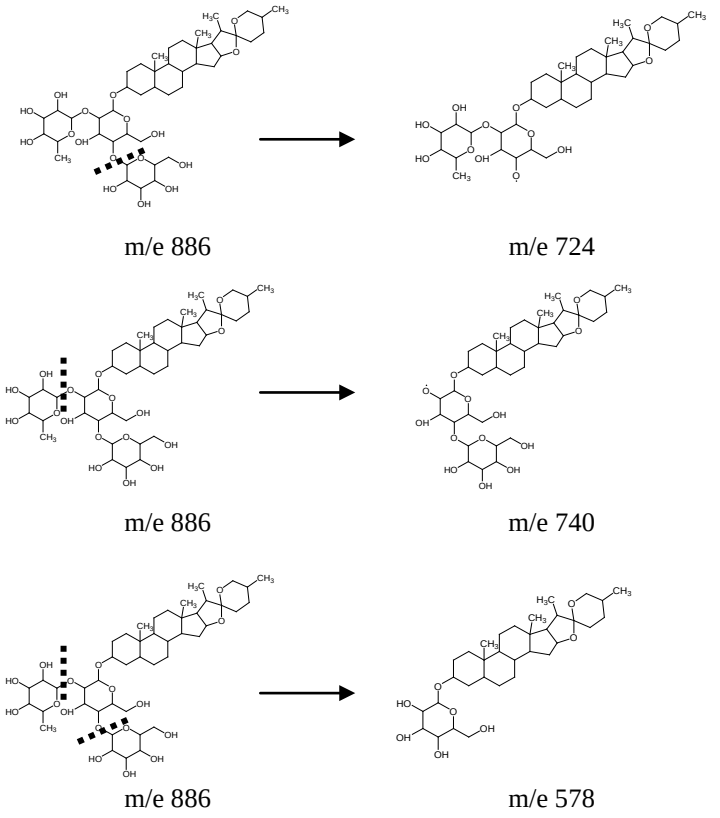


Figure 15 The proposed MS/MS fragmentation structures of shatavarin IV

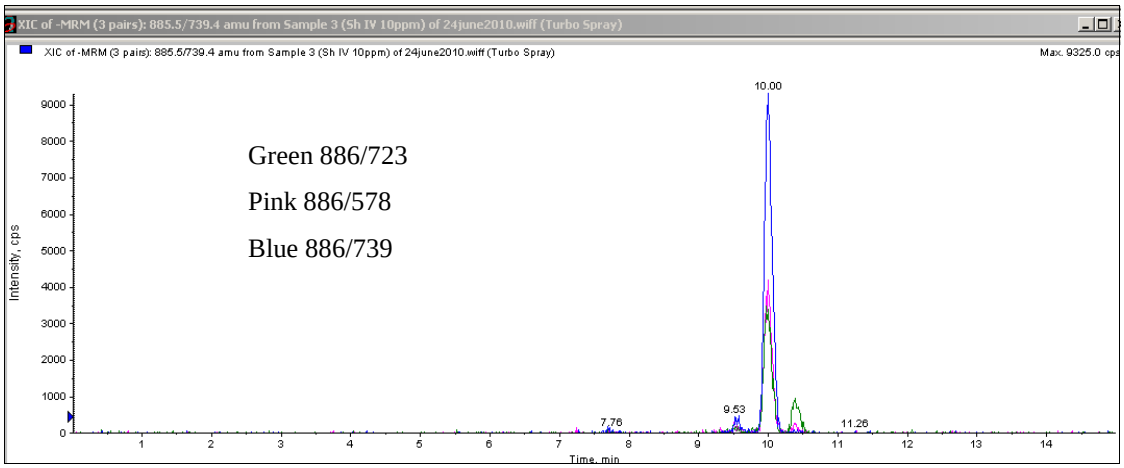


Figure 16 Chromatograms of standard shatavarin IV detected by LC-MS/MS

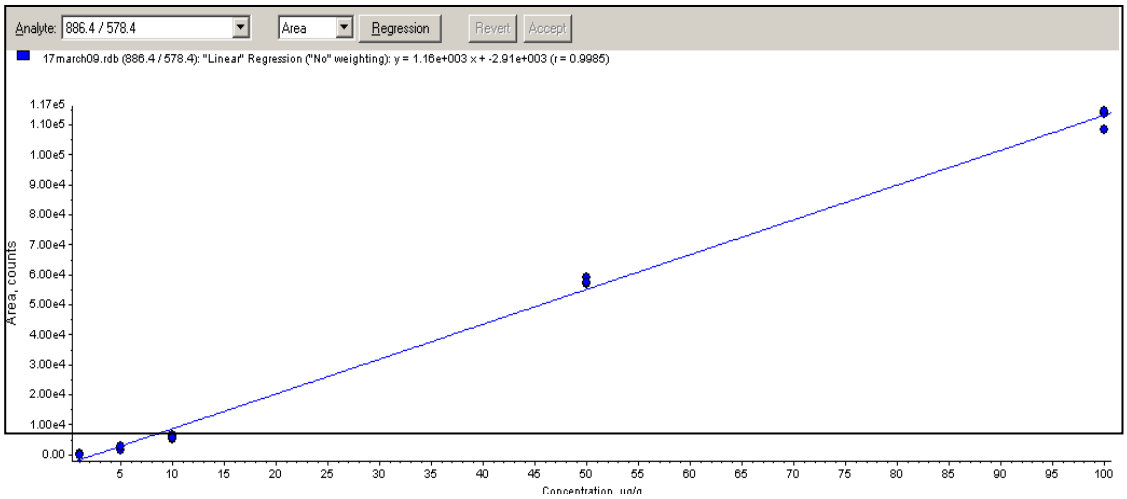


Figure 17 Calibration curve of shatavarin IV detected by LC-MS/MS

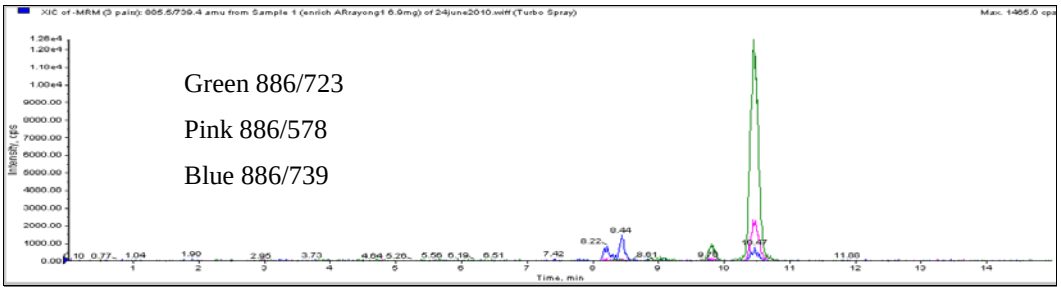
Table 5 Quantity of shatavarin IV in *A. racemosus* ethanolic extracts analyzed by LC-MS/MS at 10.00 min

Code	Sources	Equivalent to SH IV (%)
AR1-E	Tak	Not detected
AR2-E	Kanchanaburi	0.0070±0.0006
AR3-E	Rayong	Not detected
AR3-En	Rayong	Not detected

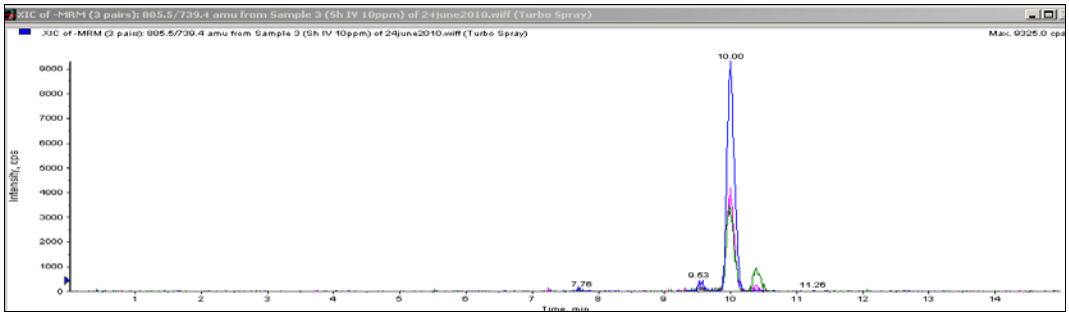
Table 6 Quantity of saponin equivalent to shatavarin IV in AR-E obtained from different sources analyzed by LC-MS/MS at 8.32 min

Code.	Sources	Equivalent to SH IV (%)
AR1-E	Tak	0.0154±0.0020
AR2-E	Kanchanaburi	0.0056±0.0012
AR3-E	Rayong	0.0184±0.0021

From the result of LC-MS/MS, trace amount of shatavarin IV (0.0070%) was only found in AR-E from Kanchanaburi (AR2-E). However, the saponins at 8.32 min were found in all extracts. Chromatogram of AR-En from Rayong (AR3-En) in comparison with chromatogram of shatavarin IV is shown in Figure 18. From the chromatogram of AR3-En, *m/e* 886/723 was found at 10.47 min. It suggested that AR3-En did not contain shatavarin IV but had other compound related to shatavarin IV.



(a)



(b)

Figure 18 Chromatograms of (a): the saponin enriched extract of *A. racemosus* from Rayong (AR3-En) compared with (b): shatavarin IV detected by LC-MS/MS

2.3 Percentage of saponin equivalent to shatavarin IV in the extracts of *A. racemosus* determined by competitive ELISA

ELISA is an immunoassay technique involving the reaction of antigen and antibody *in vitro*. ELISA is sensitive and specific for the detection and quantitation of antigens or antibodies which mostly based on the conversion of a colorless substrate (chromogen) to a colored product. ELISA tests are usually performed in microwell plates. Cross-reactivity may occur with the MAb, resulting in nonspecific signal.

The MAb against shatavarin IV was produced and applied in ELISA for determination of shatavarin IV (Reamyindee et al, 2011). Since the MAb produced showed cross-reactivity with other saponin compounds in the extract, those saponins can be determined as saponin equivalent to shatavarin IV.

The percentages of saponin equivalent to shatavarin IV of *A. racemosus* extracts are shown in Table 7. From all source of AR-E, the results showed the extract from Rayong gave the highest % of saponin equivalent to shatavarin IV (7.4%). Interestingly when compared between AR-E and AR-En from the same source (Rayong), % saponin increased about 5 times by the enrichment method.

Table 7 The percentages of saponin equivalent to shatavarin IV of *A. racemosus* extracts of each sources

Code.	Sources	% saponin equivalent shatavarin IV
AR1-E	Tak	1.466 ± 0.284
AR2-E	Kanchanaburi	3.242 ± 0.414
AR3-E	Rayong	7.428 ± 0.451
AR3-En	Rayong	38.343 ± 1.420

Antimicrobial activity test

Antimicrobial activities of *A. racemosus* extracts were screened by using the paper disc method. And then the MIC 90 and MFC were determined by Broth micro-dilution method.

1. Preliminary screening antimicrobial activity by disc diffusion method

The antimicrobial activities by disc diffusion method were tested in all of *A. racemosus* roots extracts against 6 microorganisms i.e. *E. coli*, *S. aurues*, *P. aeruginosa*, *C. albicans*, *M. furfur* and *M. globosa*. Chloramphenicol, amphotericin B, ketoconazole and zinc pyrithione were used as positive controls. The results from disc diffusion method showed that *A. racemosus* root extracts did not have antibacterial activity against *E. coli*, *S. aurues* and *P. aeruginosa* at concentration 0.1 mg/disc and 1 mg/disc while AR-E and AR-En from all sources showed inhibition effect against *C. albicans*, *M. furfur* and *M. globosa* at concentration of 1 mg/disc (Table 8 and 9). The other extracts of *A. racemosus* showed no inhibitory effects on the disc diffusion method. The extracts that showed inhibition zone in this experiment were, then, determined for the MIC values by broth micro dilution assay.

Table 8 Antimicrobial activities of positive control by disc diffusion test

Microbial	Inhibition zone (mm)*			
	CAP[a]	**AMB[b]**	**KTZ[c]**	**ZPT[c]**
E. coli	18.23 ± 0.29	nd	nd	nd
P. aeruginosa	33.23 ± 0.25	nd	nd	nd
S. aurues	28.06 ± 0.85	nd	nd	nd
C. albicans	nd	14.38 ± 0.25	16.25 ± 0.35	25.13 ± 0.85
M. furfur	nd	nd	46.88 ± 0.63	14.13 ± 0.63
M. globosa	nd	nd	42.33 ± 0.57	12.33 ± 0.29

Note: [a]Chloramphenicol at concentration of 30 µg/disc

[b]Amphotericin B at 1 mg/disc

[c]Ketoconazole and zinc pyrithione at 0.1 mg/disc

Values are mean ± SD (mm) from the experiments in triplicate.

The diameter of the disc (6 mm) was included.

2. Minimal inhibitory concentration and Minimum fungicidal concentration (MIC&MFC) of *A. racemosus* extracts

The MICs of the active extracts against the three yeasts were determined by the broth microdilution method. The results are shown in Table 10. AR-E and AR-En had MIC values ranged from 0.1 mg/ml to 25 mg/ml. The antifungal activity of AR-E from Tak was higher than that from Rayong. Among the tested extracts, AR-En exhibited the strongest antifungal activity with MIC of 0.1 mg/ml for *C. albicans* and 0.2 mg/ml for *M. furfur* and *M. globosa* while the activity of all AR-Es were relatively mild.

The MFC was recorded as the lowest concentration that did not permit any visible fungal colony growth on the agar plate after the period of incubation. The MFCs value were showed in Table 9. AR-En had higher antifungal activity than AR-E for approximately 10 times. As AR-En consisted of higher saponin level than AR-E, it could be possible that saponins are responsible for antifungal activity. The antifungal activity of some saponins in plants was also reported by Zhang et al. (2006). They also explained the mechanism of action that saponin might damage fungal cell membrane.

Table 10 MICs and MFCs against fungi of *A. racemosus* extracts and standard drugs

Samples	MIC (mg/ml)			MFC (mg/ml)		
	C. albicans	***M. furfur***	***M. globosa***	***C. albicans***	***M. furfur***	***M. globosa***
AR 1-E	1.56	3.13	3.13	1.56	3.13	3.13
AR 3-E	1.56	25	25	1.56	25	25
AR 3-En	0.10	0.40	0.20	0.20	0.80	0.40
AMB	0.15	nd[a]	nd	0.62	nd	nd
KTC	0.00128	0.00032	0.00032	0.00256	0.0064	0.0064
ZPT	0.08	0.02	0.02	0.08	0.04	0.04

Note: [a]nd: not detected

Synergistic effect test

Combined effects with ketoconazole

The combined effects of the tested extracts (AR1-E, AR3-E and AR3En) with antifungal drugs (ketoconazole and zinc pyrithione) were evaluated by the checkerboard titer test. The FIC index was calculated by adding both FICs and interpreted as a synergistic effect when it was ≤ 0.5, as additive or indifferent when it was > 0.5 to 4.0, and as antagonistic when it was > 4.0. To explore the possibility of developing a more powerful combination therapy of the extract with antifungal agent, the checkerboard micro-titer tests were performed with combined samples. The FIC index of the combination ranged from 0.75 to 1.50 demonstrated that tested extract showed no synergistic effect when combined with ketoconazole and zinc pyrithione. The FIC and FICI results calculated from the checkerboard micro-titer tests are listed in Table 11.

Table 11 Fractional inhibiting concentrations (FICs) and FIC indices (FICI)

Combinations	*M. furfur*		*M. globosa*	
	FIC	**FICI**	**FIC**	**FICI**
AR1-E	1.00	1.50	0.50	0.75
Ketoconazole	0.50		0.25	
AR1-E	1.00	1.25	1.00	1.25
Zinc pyrithione	0.25		0.25	
AR3-E	0.50	0.75	0.50	0.75
Ketoconazole	0.25		0.25	
AR3-E	0.50	1.00	0.50	1.00
Zinc pyrithione	0.50		0.50	
AR3-En	1.00	1.25	1.00	1.25
Ketoconazole	0.25		0.25	
AR3-En	0.25	1.25	0.50	0.75
Zinc pyrithione	1.00		0.25	

Stability study

The stability of AR-E was studied under accelerated condition employing 50 ^{o}C for 30 days. The change of saponin content was determined by ELISA whereas the change of antifungal activity was determined by disc diffusion method and broth micro dilution method. The appearance of *A. racemosus* extract at day 0 and day 30 are shown in Figure 19. The result showed that the color of AR-E changed to darkened color after 30 day-storage under stress condition.

AR 1-E (Tak) normal **AR 1-E (Tak) 50 °c 30 days**

Figure 19 The appearance of *A. racemosus* extract comparing between Day 0 as a control and Day 30

The quantity of saponin equivalent to shatavarin IV of *A. racemosus* extracts when kept at 50 ºC 30 days compared with control (0 day) are shown in Table 12 and Figure 20. The mean values of the extracts were tested by Pair T-Test at 95% confidence interval (SPSS statistic Program) (Appendix D). The percent remaining of saponin equivalent to shatavarin IV of the extract was measured by ELISA compare with the control (4º C) are shown in Table 13. As shown in Table 12 and Appendix

(D), the results revealed that the percent of saponin equivalent to shatavarin IV in *A. racemosus* extract from Tak and Rayong at 30 days after storage at stress condition were no significant difference compared to the control at p = 0.107 and 0.470 respectively. The control extracts was the initial amount of saponin equivalent to shatavarin IV in the extract and calculated as 100%. However, its saponin level still remained more than 75% (Table 13).

Table 12 The percent of saponin equivalent to shatavarin IV of *A. racemosus* extracts when kept at 50 ºC 30 days compare with control (0 day)

Code.	Sources	% saponin equivalent shatavarin IV
AR1-E 0 Day	Tak	2.06 ± 0.25
AR1-E 30 Days	Tak	1.60 ± 0.64
AR3-E O Day	Rayong	6.22 ± 0.51
AR3-E 30 Days	Rayong	5.31 ± 0.13

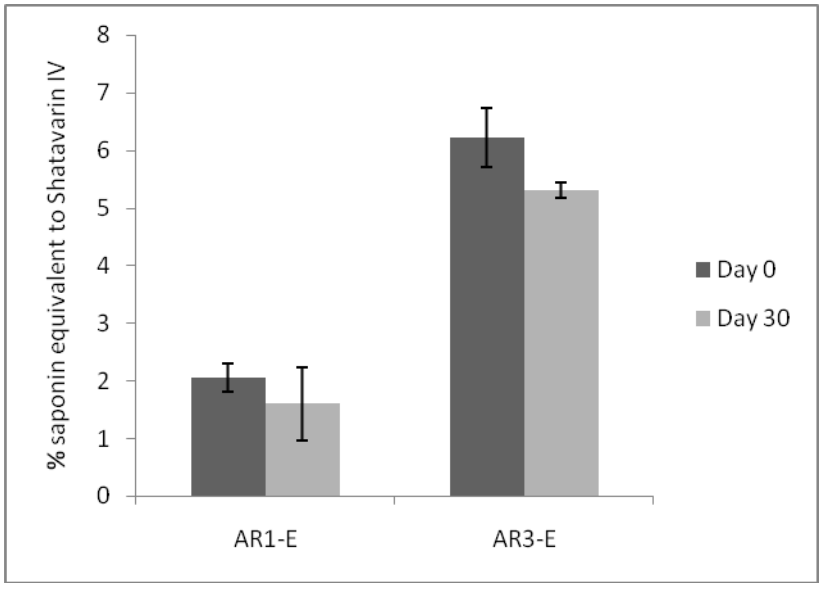


Figure 20 Percentage of saponin equivalent to shatavarin IV in *A. racemosus* extracts compare between days 0 as a control and 30 days as final day (AR1-E: Ethanolic extract from Tak, AR1-E: Ethanolic extract from Rayong)

Table 13 The percentage remaining of saponin equivalent to shatavarin IV in *A. racemosus* extracts after 30 day storage at 50 ºC

Code.	Sources	% remaining of saponin equivalent shatavarin IV
AR1-E	Tak	76.64 ± 24.39
AR3-E	Rayong	85.72 ± 7.60

Tables 14 and 15 show that after storing at 50 ºC for 30 days, the antifungal activities of the extracts evaluated by disc diffusion test and broth microdilution test were not different from the control.

In conclusion, the stability study showed that after storing at accelerate condition for 30 days, the antifungal activity and saponin level of the AR-E still remained in acceptable range. This suggest that the satisfactory stability of AR-E.

Table 14 Comparison of inhibitory activities against *C. albicans* and *M. furfur* by disc diffusion test

Microbial	Inhibition zone (mm)*			
	AR 1-E	**AR 1-E 50 °C**	**AR 3-E**	**AR 3-E 50 °C**
***C. albicans* (ATCC 90028)**	9.25 ± 0.29	9.25 ± 0.35	7.25 ± 0.29	6.75 ± 0.35
***M. furfur* (CBS 1878^{T})**	9.25± 0.29	9.25± 1.06	6.75± 0.28	6.38± 0.18

Note: [a] The extracts at concentration of 1 mg/disc, [b] Amphotericin B at 1 mg/disc and [c] Ketoconazole and zinc pyrithione at 0.1 mg/disc, [d] Value are mean ± SD (mm) from the experiments in triplicate. The diameter of the disc (6 mm) was included.

Table 15 MICs and MFCs against fungi

Agent	MIC 90			MFC		
(mg/ml)	*C. albicans*	*M. furfur*	*M. globosa*	*C. albicans*	*M. furfur*	*M. globosa*
AR 1-E	1.57	3.13	3.13	1.56	3.13	3.13
AR 1-E 50°C	1.57	3.13	3.13	1.56	3.13	3.13
AR 3-E	1.57	25	25	1.56	25	25
AR 3-E 50°C	1.57	25	25	1.56	50	50