CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quantitative determination of rhinacanthins in *R. nasutus* leaf extract

We examined the optimal conditions for the simultaneous quantitative determination of rhinacanthin-C, -D and -N in *R. nasutus* leaf extract using isocratic reverse phase HPLC system. As all the three compounds have good absorption at 254 nm, this wavelength was used for the quantitation. Mixtures of methanol and 5 % aqueous acetic acid were examined as the mobile phase and its composition was optimized. The ratio of methanol to 5 % aqueous acetic acid required obtaining a good resolution of the rhinacanthins was 80:20. All the three compounds were eluted within 20 minute with satisfactory resolution (Figure 4.1).

On the basis of the HPLC analysis, rhinacanthin-C was a major rhinacanthin, which the content was up to 1.9 % w/w, while rhinacanthin-D and -N were minor constituents (Table 4.1). The simultaneous quantitative determination of rhinacanthin-C, -D and -N and relatively simple and fast method are the advantage of this method. The previously reported HPLC method was developed to determine only rhinacanthin-C, and validation of the analytical procedure is not yet established (Gotoh *et al*., 2004).

Defining the linearity, accuracy, intraday- and interday-precision and specificity validated the HPLC method. Linearity was evaluated using standard samples over five calibration points with six measurements for each calibration points. Rhinacanthin-C, -D, and -N exhibited good linearity over the evaluated ranges with correlation coefficients 1.0000, 1.0000 and 0.9999, respectively (Table 4.2, Figures 4.2, 4.3, and 4.4).

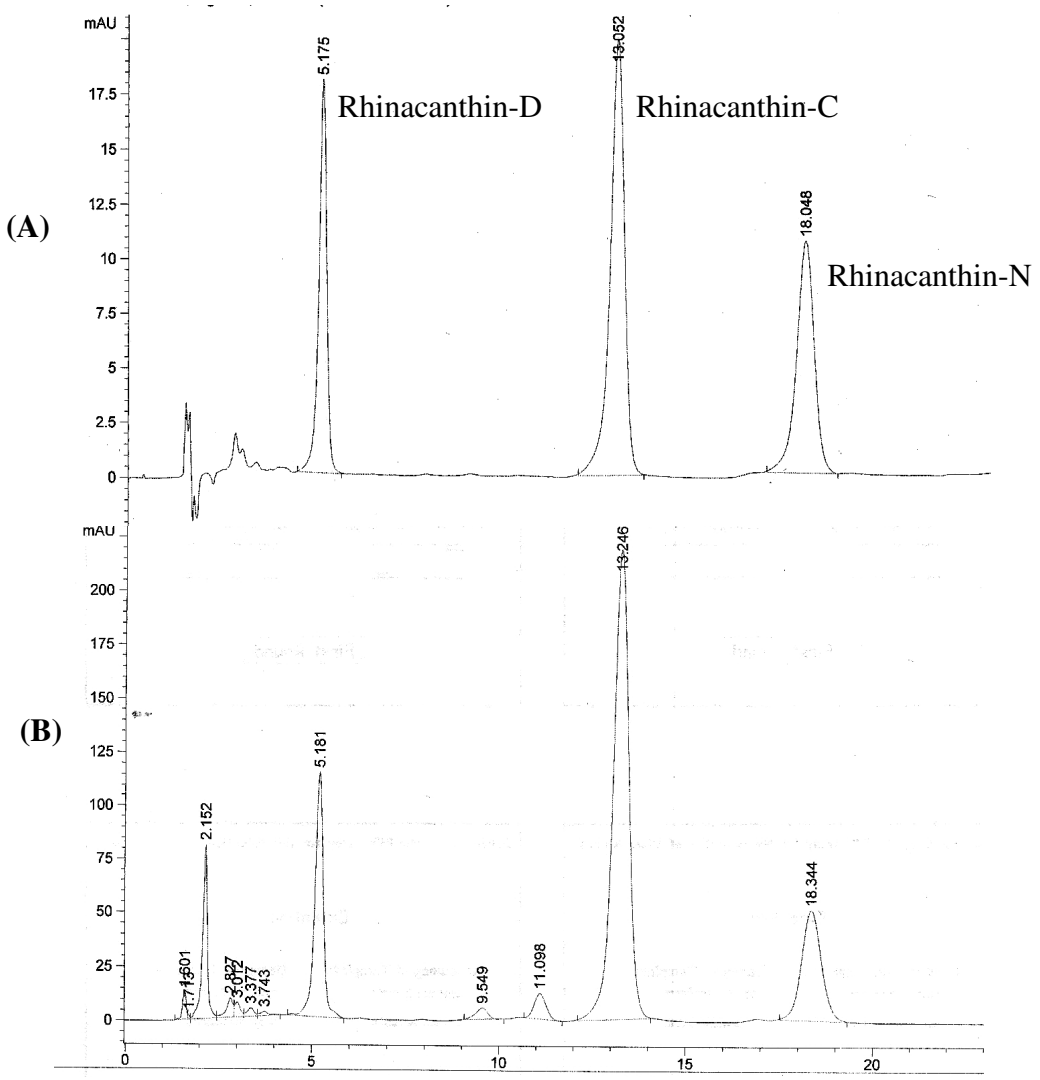


Figure 4.1 HPLC-chromatograms of the authentic rhinacanthin-C, -D, and –N (A) and *R. nasutus* leaf extract (B)

Table 4.1 Rhinacanthin content in *R. nasutus* leaf extract

Compounds	Rhinacanthin content (%w/w ± S.D.)
Rhinacanthin-C	1.91 ± 0.044
Rhinacanthin-D	0.16 ± 0.008
Rhinacanthin-N	0.07 ± 0.001

Table 4.2 Linear ranges and correlation coefficients (r^2) of calibration curves

Constituents	Y = aX + b linear model[a]	r^2	Concentration (µg/mL)
Rhinacanthin-C	Y = 40.569X + 35.263	1.0	12.6 - 201.0
Rhinacanthin-D	Y = 79.615X – 4.168	1.0	3.1 - 51.0
Rhinacanthin-N	Y = 112.810X + 18.292	0.9999	3.1 - 51.0

[a] Y = peak area; X = concentration (µg/mL)

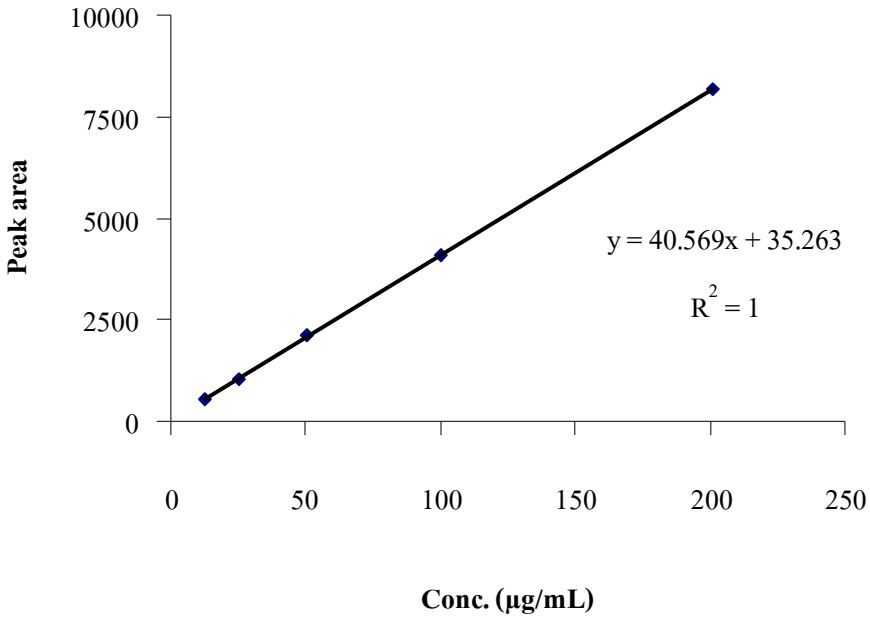


Figure 4.2 Calibration curve of rhinacanthin-C

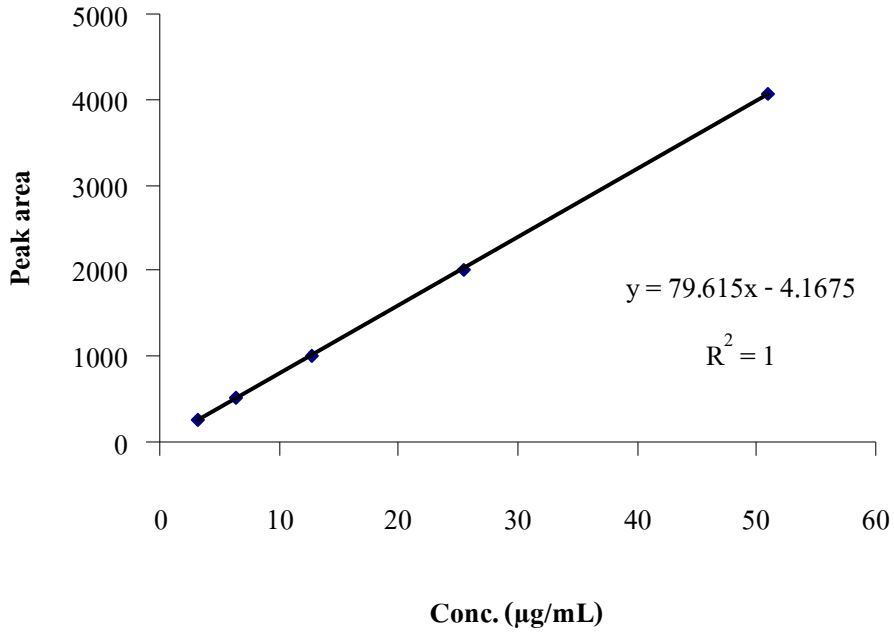


Figure 4.3 Calibration curve of rhinacanthin-D

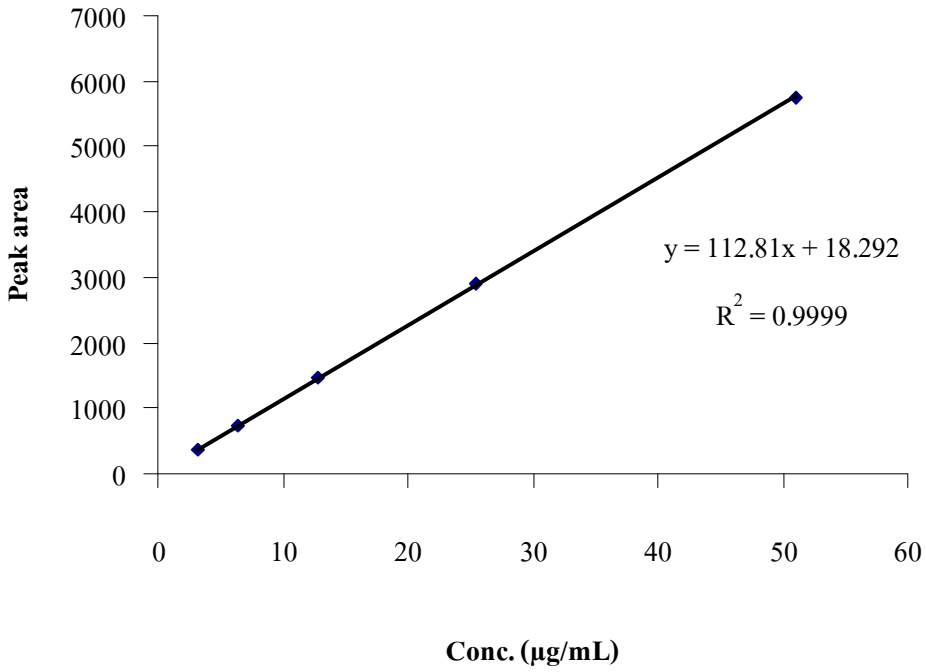


Figure 4.4 Calibration curve of rhinacanthin-N

The precision of the method was assessed by determining %R.S.D. of intraday and interday analysis. The method was shown to be reproducible and reliable with both intraday and interday precision lower than 5 % (Table 4.3).

Accuracy of method was evaluated by analyzing *R. nasutus* leaf extracts spiked a known concentration of the standards. Prior to spiking, the background levels of rhinacanthin-C, -D, and -N in the extracts were determined so as to calculate actual recoveries. Mean recoveries in the range of 94 – 100 % were observed for all compounds (Table 4.3).

Table 4.3 Repeatability, reproducibility and recoveries of rhinacanthin-C, -D, -N from *R. nasutus* leaf extract

Constituents	R.S.D. (%)		% Recovery (Mean ± S.D.)
	Intraday (n = 6)	Interday (n = 18)	
Rhinacanthin-C	0.24	2.32	100.9 ± 0.19
Rhinacanthin-D	2.59	4.94	95.6 ± 2.50
Rhinacanthin-N	0.84	1.68	94.3 ± 0.33

Specificity of the method was evaluated using UV-absorption spectra produced by PDA. The spectra were taken at three points of the peaks. When they were compared with the standard, homogeneity of spectra of all peaks was found (Figures 4.5 - Fig. 4.7).

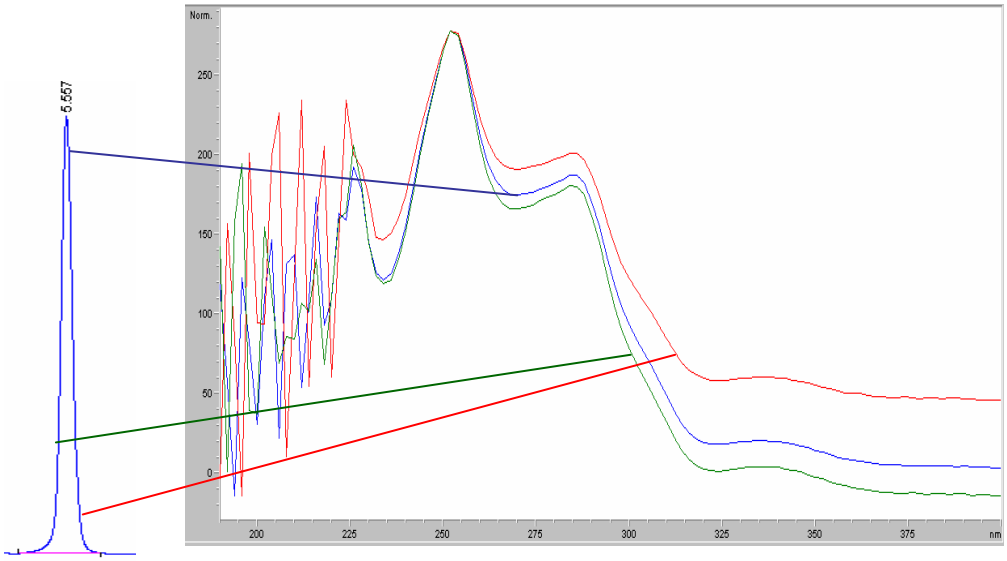


Figure 4.5 UV-absorption spectra of the peak that corresponded to rhinacanthin-D

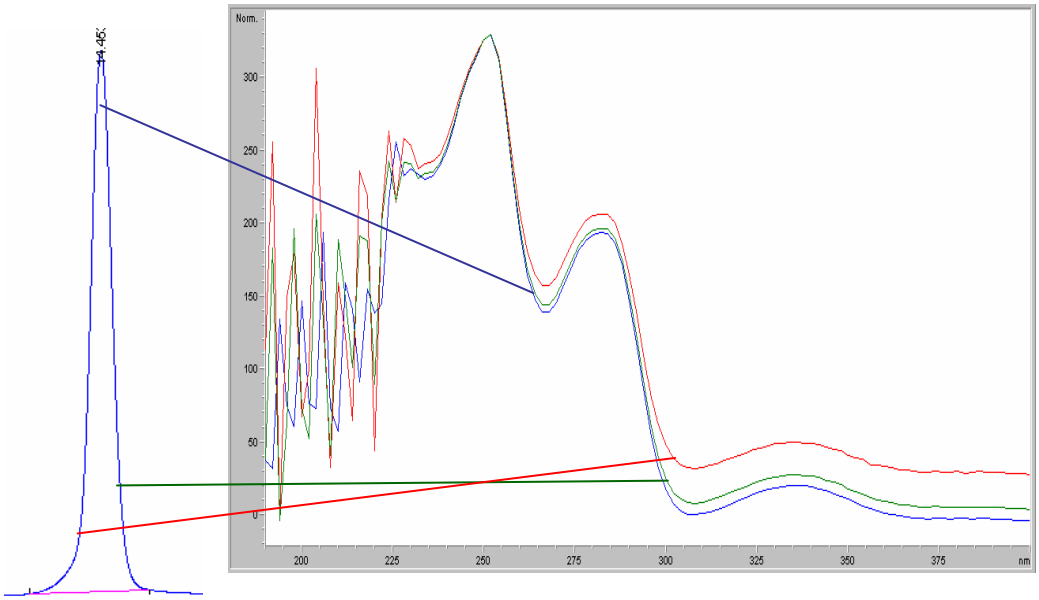


Figure 4.6 UV-absorption spectra of the peak that corresponded to rhinacanthin-C

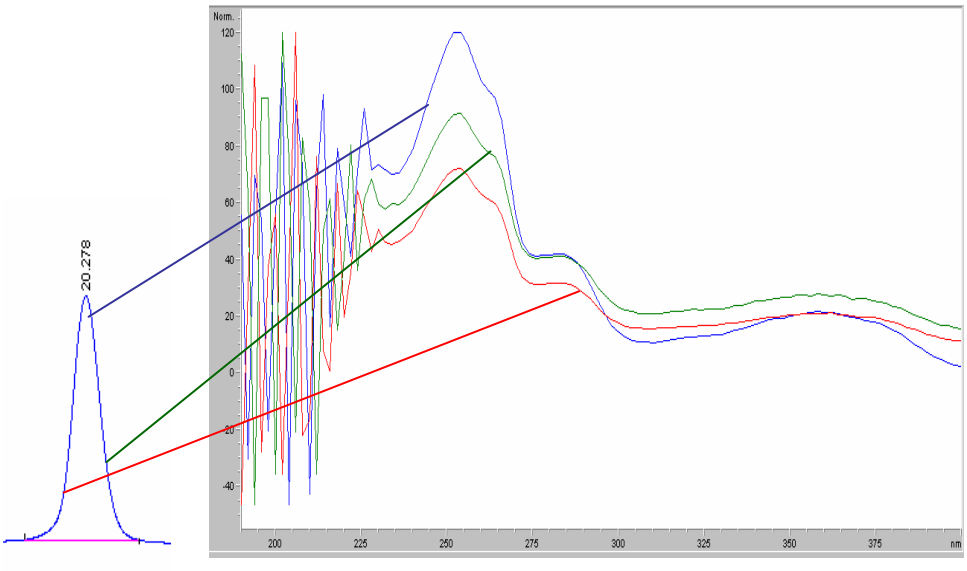


Figure 4.7 UV-absorption spectra of the peak that corresponded to rhinacanthin-N

Finally, it was found that the HPLC method was very sensitive for all rhinacanthins with LOD and LOQ were 0.75 and 3.0 µg/mL, respectively.

4.2 Preparation of rhinacanthin high-yielding *R. nasutus* leaf extract

Ion exchange chromatographic method using Amberlite® IRA-67 column has been developed to improve the rhinacanthin concentration in the extract as well as diminish the interference compounds from the extract (Kongchai and Panichayupakaranat, 2002). In this study, Amberlite® IRA-67 column was capable of improving the total rhinacanthin content in the extract up to 83.61 %w/w (Table 4.4). The interference compounds including chlorophyll and other pigments were markedly excluded from the extract. The weakly basic anion exchanger, Amberlite® IRA-67 was therefore suitable for preparation of rhinacanthin high-yielding *R. nasutus* leaf extract.

Table 4.4 Total rhinacanthin content in the ethyl acetate and rhinacanthin high-yielding extracts of *R. nasutus* leaves

Compounds	Rhinacanthin content (% w/w; Mean ± S.D.)	
	Ethyl acetate extract	Rhinacanthin high-yielding extract
Rhinacanthin-C	22.83 ± 0.04	6.11 ± 0.22
Rhinacanthin-D	2.46 ± 0.04	73.93 ± 1.20
Rhinacanthin-N	1.48 ± 0.06	3.57 ± 0.12
Total rhinacanthins*	26.77 ± 0.12	83.61 ± 1.31

* Total rhinacanthin content was calculated as rhinacanthin-C, -D, and -N

Rhinacanthin high-yielding *R. nasutus* leaf extract was oily dark green liquid (Figure 4.8). The yield of the extracts was 1.68 ± 0.141 %w/w compared to weight of the dried powdered leaves. The extracts contained total rhinacanthin content of 78.37 ± 5.21 %w/w. The percentage recovery of rhinacanthins was 61.69 %w/w when compared with the rhinacanthin content in the dried leaf powder. The result suggested that rhinacanthin-C, -D, and -N had been lost during the extraction and fractionation processes.

Table 4.5 Extraction yield and total rhinacanthin content of rhinacanthin high-yielding *R. nasutus* leaf extract

Extraction (times)	Extract yield (%w/w)	*Total rhinacanthin content (%w/w; Mean ± S.D.)	% Recovery
1	1.84	83.61 ± 1.31	71.89
2	1.73	81.56 ± 0.43	65.93
3	1.63	76.20 ± 1.19	58.04
4	1.51	72.12 ± 1.27	50.89
Average	1.68 ± 0.141	78.37 ± 5.21	61.69 ± 9.16

* Total rhinacanthin content was calculated as rhinacanthin-C, -D, and -N

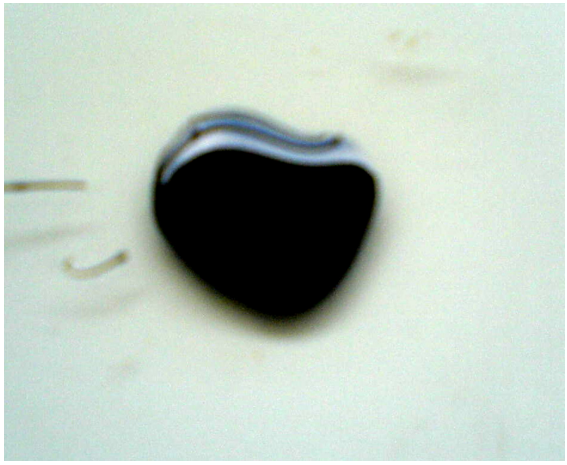


Figure 4.8 Rhinacanthin high-yielding *R. nasutus* leaf extract

4.3 Antifungal activity of the rhinacanthin high-yielding *R. nasutus* leaf extract

The antifungal activity of the rhinacanthin high-yielding *R. nasutus* leaf extract against *T. rubrum*, *T. mentagrophytes* and *M. gypseum* was evaluated and compared with those of the ethyl acetate extract of *R. nasutus* leaves and standard rhinacanthin-C, -D and -N. The result showed that all tested compounds possessed antifungal activity against *T. rubrum*, *T. mentagrophytes* and *M. gypseum* (Table 4.5). This result confirms the traditional therapeutic claims of *R. nasutus* for treatment of ringworm and skin diseases. The antifungal mechanism could be due to the leaks on the cell wall or perhaps some alteration in the membrane permeability, resulting in the loss of the cytoplasm (Darah and Jain, 2001). The antifungal activity of the rhinacanthin high-yielding *R. nasutus* leaf extract was however better than that of the ethyl acetate extract. The antifungal activities of the extracts correlated with the concentration of rhinacanthins in those extracts. The antifungal activity of the rhinacanthin high-yielding *R. nasutus* leaf extract was equal to that of rhinacanthin-C. This may be considered as a synergies effect of rhinacanthin-D, -C, and N. Thus, the rhinacanthin high-yielding *R. nasutus* leaf extract was suitable for the further study of formulation of a topical antifungal cream.

Table 4.6 Antifungal activities of rhinacanthins, EtOAC extract and HRn extract against *M. gypseum*, *T. mentagrophytes*, and *T. rubrum*

Extract/Compound	MIC (μg/mL)		
	M. gypseum	*T. mentagrophytes*	*T. rubrum*
Ethyl acetate extract	500.0	62.5	31.2
Rhinacanthin high-yielding extract	125.0	31.2	7.8
Rhinacanthin-C	125.0	31.2	15.6
Rhinacanthin-D	125.0	31.2	31.2
Rhinacanthin-N	250.0	250.0	62.5
Clotrimazole	0.6	0.6	0.3

4.4 Solubility of the rhinacanthin high-yielding *R. nasutus* leaf extract

The solubility of a substance in any solvents is specified by the saturation mass concentration of the substance in the solvent at a given temperature. The solubility is commonly expressed as units of mass per volume of solution (g/mL). The solubility test of the rhinacanthin high-yielding *R. nasutus* leaf extract is used to estimate the dissolution of the extract in various solvents. It was found that the extract was freely soluble in ethyl acetate and chloroform, soluble in methanol and ethanol, sparingly soluble in dimethyl sulfoxide (DMSO), slightly soluble in hexane, and practically insoluble in water (Table 4.6). The rhinacanthin high-yielding *R. nasutus* leaf extract contains most likely moderate non-polar compounds therefore the suitable solvents for the leaf extract should be a moderate non-polar solvent. The solubility information could be useful to further experiment such as formulation of the antifungal dosage forms.

Table 4.7 Solubility of the rhinacanthin high-yielding *R. nasutus* leaf extract

Solvent	Volume of solvent in ml/g of solute	Level of solubility
Ethyl acetate	8	Freely soluble
Chloroform	9	Freely soluble
Methanol	40	Soluble
Ethanol	35	Soluble
DMSO	95	Sparingly soluble
Hexane	270	Slightly soluble
Water	>10,000	Practically insoluble

4.5 Determination of moisture content

The moisture content of the rhinacanthin high-yielding *R. nasutus* leaf extract was determined prior to further investigation. In general, two methods, gravimetric method (loss on drying) and azeotropic method (toluene distillation), are used for determination of the moisture content of herbals crude drugs and the upper limit of the moisture content of the crude drugs is 8 – 14 %w/w (Panichayupakaranant *et al*., 2006). This limitation may extend the duration of a herbal raw material maintenance. The presence of excess water in herbal drug can promote the growth of microbes and the hydrolysis of the constituents leading deterioration of herbal drugs. In this case, the gravimetric method was applied to determine the moisture content of the rhinacanthin high-yielding *R. nasutus* leaf extract but the upper limit of the moisture content of the herbal extract is not existed. Generally, the moisture content of the herbal extract should be less than its crude drugs. Thus, it was found that all the samples in this study showed very low amount of the moisture content. The moisture content of the rhinacanthin high-yielding *R. nasutus* leaf extract samples were in the range of 0.13 - 0.15 %w/w (the average was 0.14 %w/w) and the standard deviation was 0.012 (Table 4.7). To establish the upper limit of the moisture content of the herbal extract, the several kinds of the herbal extract must be used in the study. For the rhinacanthin high-yielding *R. nasutus* leaf extract, the upper limit of the moisture content was set up from the mean value plus one S.D. It is therefore set as that not more than 0.2 %w/w.

Table 4.8 The moisture content of rhinacanthin high-yielding *R. nasutus* leaf extract

Lot. Number	Loss on drying (%w/w; Mean ± S.D.)
1	0.15 ± 0.025
2	0.13 ± 0.031
3	0.15 ± 0.023
Mean ± S.D.	0.14 ± 0.012

4.6 Determination of ash content

The determination of ash content is a method to measure the amount of residue material after ignition by the method as previously described in the section 3.2.5. This includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological ash", comprising extraneous matters such as sand and soil. For herbal drugs with considerable levels of physiological ash, calcium carbonate in particular, the value of the total ash content alone is not sufficient to reflect the quality of the herbal drugs. Upon treatment with dilute acid and further ignition, the residues left behind are silica materials such as sand and siliceous earth. Therefore, the acid-insoluble ash content serves as another supplementary piece of evidence to illustrate the quality of the plant materials.

4.6.1 Total ash content

The total ash of the rhinacanthin high-yeilding *R. nasutus* leaf extract was determined by using the method according to THP 1995 (Subcommittee on the establishment of the Thai herbal pharmacopoeia, 1995). The result obtained from this determination was used to set up the limitation of the total ash of the *R. nasutus* leaf extract. The results showed that the total ash of the rhinacanthin high-yeilding *R. nasutus* leaf extract was varied in range of 2.09 – 2.33 % w/w (Table 4.8). For the the rhinacanthin high-yeilding *R. nasutus* leaf extract, the limitation of the total ash was set up from the mean value plus one S.D. It is therefore set as that not more than 2.3 %w/w.

The total ash value of the rhinacanthin high-yielding *R. nasutus* leaf extract is rather high, because the plant contains high content of calcium carbonate that accumulated in the lithocyst cells of *R. nasutus* leaves.

Table 4.9 The total ash content of rhinacanthin high-yielding *R. nasutus* leaf extract

Lot. Number	Total ash (%w/w; Mean ± S.D.)
1	2.33 ± 0.326
2	2.09 ± 0.295
3	2.20 ± 0.470
Mean ± S.D.	2.21 ± 0.120

4.6.2 Acid insoluble ash content

After the total ash determination of the rhinacanthin high-yielding *R. nasutus* leaf extract, the ash was subjected to acid insoluble ash determination. The result obtained from this determination was used to set up the limitation of the acid insoluble ash of the leaf extract. The result showed that the acid insoluble ash of the rhinacanthin high-yielding *R. nasutus* leaf extract was not detected (Table 4.9). This result indicates that only physiological ash is found in the rhinacanthin high-yielding *R. nasutus* leaf extract. Thus, the acid insoluble ash value of the rhinacanthin high-yielding *R. nasutus* leaf extract was set up as no acid insoluble ash.

Table 4.10 The acid insoluble ash content of rhinacanthin high-yielding *R. nasutus* leaf extract

Lot. Number	Acid insoluble ash (%w/w; Mean ± S.D.)
1	0
2	0
3	0
Mean ± S.D.	0

4.7 Determination of the microbial contamination

Plant materials, sources of herbal extract, and/or the extract preparation steps could cause a microbial contamination derived from various sources. Thus, estimation of the overall resources provided useful information for the assessment of the herbal extracts' safety and quality. The pour plate method was applicable to the enumeration of the micro-organisms in the herbal extract. In this study the limit for microbial contamination of topical preparations for intact skin are used for the quality control of rhinacanthin high-yielding *R. nasutus* leaf extract. The limit for microbial contamination of topical preparations for intact skin such as creams, lotions, ointments, solutions, and powders are as follow; total aerobic microbial count does not exceed 500 colonies/g (mL), and a one-g (mL) sample is free from Enterobacteria, *Pseudomonas aeruginosa*, *Staphylococus aureus*, yeast, and moulds (Subcommittee on the establishment of the Thai herbal pharmacopoeia, 2000). The results showed that neither bacteria nor fungi contamination was found in the extracts (Table 4.10). The extracts are reached the microbiological requirement for good manufacturing practices. Thus, the microbial limitation of the rhinacanthin high-yielding *R. nasutus* leaf extract was set up as no contamination with aerobic bacteria, *E. coli* and yeast and moulds in 1 g or 1 mL of the extract.

Table 4.11 Determination of microbial contamination in the rhinacanthin high-yielding *R. nasutus* leaf extract

Microbes	Microbial contamination
Aerobic bacteria	not found
Escherichia coli	not found
Yeasts and moulds	not found

4.8 Partition coefficient

The partition coefficient (K) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents, such as *n*-octanol and water. The result is usually given in the form of its logarithm to base ten.

In this study, the shake flask method was applied to determine the partition coefficient (K) of rhinacanthins in the rhinacanthin high-yielding *R. nasutus* leaf extract. The partition coefficient's logarithm (Log K) was calculated and the result was 1.73 ± 0.154 (Table 4.11). Thus, the rhinacanthin high-yielding *R. nasutus* leaf extract was lipophilicity according to its Log K. There are some reports that showed the optimum Log K value of percutaneous drugs and the value was 2.6 (Leo, 1971; Dunn, 1986). Because dermatophytes almost invade the stratum corneum or keratinized structure derived from the epidermis (Duek *et al*., 2004), so the rhinacanthin high-yielding *R. nasutus* leaf extract was suitable designed for topical antifungal drug because it mostly retained onto the infected skin.

Table 4.12 The partition coefficient's logarithm (Log K) of the rhinacanthin high-yielding *R. nasutus* leaf extract

Concentration of the extract (μg/mL)	Log K
200	1.90 ± 0.006
100	1.69 ± 0.008
50	1.60 ± 0.003
Mean ± S.D.	1.73 ± 0.154

4.9 Stability test

4.9.1 Effect of photo on the stability of the extract

The effect of photo on the stability of the rhinacanthin high-yielding *R. nasutus* extract was examined under the condition as described in the section 3.2.9.1. The extracts were kept in the well-closed containers and stored either under fluorescent light or protected from light for a period of 4 months. Physical appearance of the extract was observed as well as the content of rhinacanthin was analyzed at the initial times and every week (or month) in four months period. Physical appearance of the extract after four months was not change from those at the initial time. The total rhinacanthin content of the rhinacanthin high-yielding extract that kept in the light protection container was not decreased through the period of four months. In contrast, the total content of rhinacanthin in the extract that exposed to light was markedly decreased from 75.05 to 48.61 %w/w (Table 4.12 and Figure 4.9). In addition, the content of both rhinacanthin-C and rhinacanthin-N were significantly decreased after 7 days of storing and the content of rhinacanthin-D was significantly decreased after days 21. Based on the HPLC-chromatograms of the extracts, two peaks of the degradation products of rhinacanthins were detected at retention time of 4.33 and 6.03 minutes, respectively (Figure 4.10). This study suggested that rhinacanthin high-yielding *R. nasutus* leaf extract should be kept in well-closed container protected from light to avoid instability of rhinacanthins.

Table 4.13 Rhinacanthin content of the rhinacanthin high-yielding *R. nasutus* leaf extract stored under light and protected from light conditions

Weeks	Rhinacanthin content (%w/w; Mean ± S.D.)					
	Rhinacanthin-D		Rhinacanthin-C		Rhinacanthin-N	
	Protected from light	Light	Protected from light	Light	Protected from light	Light
0	6.52 ± 0.101	6.52 ± 0.104	65.40 ± 0.555	65.40 ± 0.559	3.13 ± 0.052	3.13 ± 0.045
1	6.50 ± 0.113	6.50 ± 0.132	65.29 ± 0.507	64.38 ± 0.521	3.10 ± 0.043	3.09 ± 0.029
2	6.48 ± 0.130	6.43 ± 0.121	65.23 ± 0.604	63.30 ± 0.501*,#	3.07 ± 0.035	2.96 ± 0.021*,#
3	6.48 ± 0.123	6.29 ± 0.142	65.16 ± 0.530	61.72 ± 0.510*,#	3.06 ± 0.026	2.92 ± 0.022*,#
4	6.45 ± 0.114	6.14 ± 0.160*	65.11 ± 0.518	57.48 ± 0.538*,#	3.04 ± 0.032	2.71 ± 0.027*,#
8	6.43 ± 0.128	6.03 ± 0.139*,#	65.10 ± 0.553	52.65 ±0.603*,#	3.01 ± 0.040*	2.50 ± 0.018*,#
12	6.40 ± 0.135	5.72 ± 0.125*,#	65.06 ± 0.512	47.80 ± 0.572*,#	3.04 ± 0.027	2.39 ± 0.030*,#
16	6.39 ± 0.110	5.38 ± 0.151*,#	65.03 ± 0.566	41.26 ± 0.531*,#	3.03 ± 0.024	2.07 ± 0.037*,#

* Significance at $P < 0.05$ compared with the content at initial time

Significance at $P < 0.05$ compared with the content in light protecting condition at the same time

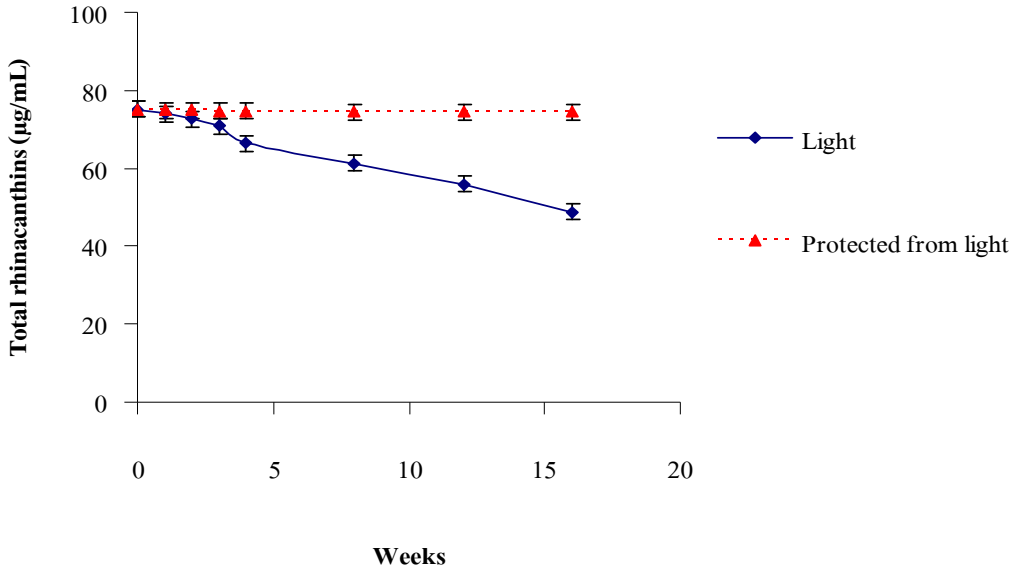
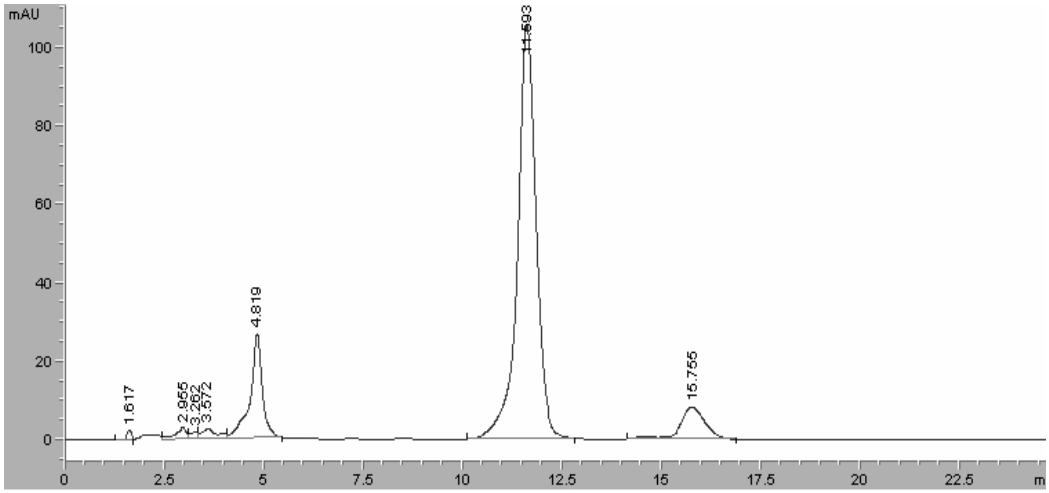


Figure 4.9 Total rhinacanthin content of the rhinacanthin high-yielding *R. nasutus* leaf extract stored under light and protected from light conditions



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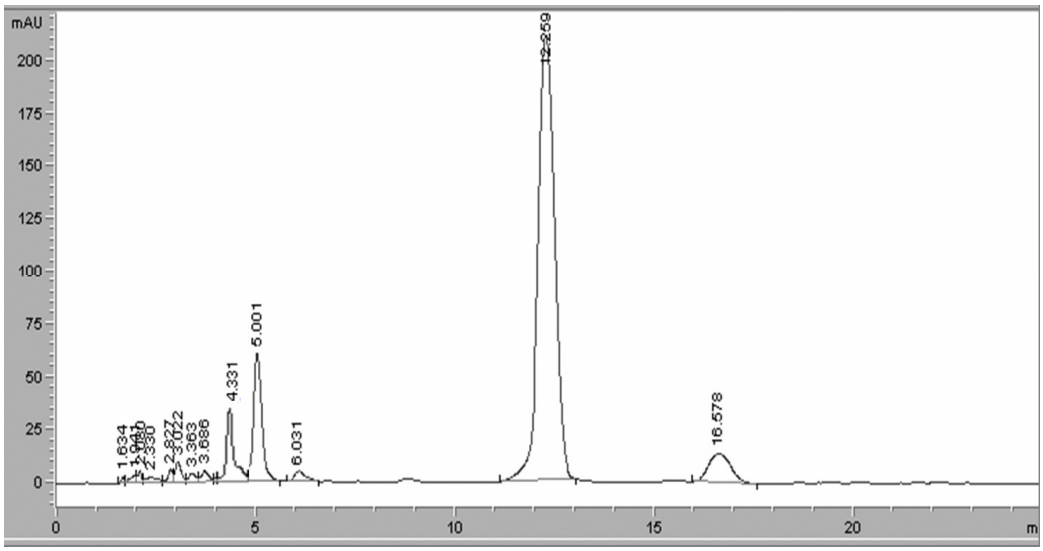

B

Figure 4.10 HPLC-chromatogram of the rhinacanthin high-yielding *R. nasutus* leaf extract at the initial time (A) and after exposed to light for 16 weeks (B)

4.9.2 Effect of temperature on the stability of the extract

The effect of temperature on the stability of the rhinacanthin high-yielding *R. nasutus* leaf extract was examined under two temperatures, 4 ºC and 30 ºC for four months. The extracts were kept in well-closed container protect from light. Physical appearance of the extracts was observed as well as the content of rhinacanthin was analyzed at the initial times and every week (or month) in four-month period. The result showed that both tested temperatures did not affect either the physical appearance of the extracts or rhinacanthin content through the four-month period (Table 4.13 and Figure 4.11). It implies that the rhinacanthin high-yielding *R. nasutus* leaf extract is stable under temperatures of 4 ºC and 30 ºC at least in the period of 4 months.

Table 4.14 Rhinacanthin content of the rhinacanthin high-yielding *R. nasutus* leaf extract stored under 4 ºC and 30 ºC

Weeks	Rhinacanthin content (%w/w; Mean ± S.D.)					
	Rhinacanthin-D		Rhinacanthin-C		Rhinacanthin-N	
	30 °C	4 °C	30 °C	4 °C	30 °C	4 °C
0	6.52 ± 0.103	6.52 ± 0.104	65.40 ± 0.561	65.40 ± 0.562	3.13 ± 0.050	3.13 ± 0.049
1	6.50 ± 0.112	6.51 ± 0.124	65.29 ± 0.514	65.34 ± 0.481	3.10 ± 0.039	3.11 ± 0.042
2	6.48 ± 0.127	6.47 ± 0.109	65.23 ± 0.603	65.27 ± 0.584	3.07 ± 0.042	3.10 ± 0.023
3	6.48 ± 0.120	6.46 ± 0.118	65.16 ± 0.531	65.19 ± 0.562	3.06 ± 0.031	3.08 ± 0.038
4	6.45 ± 0.110	6.44 ± 0.104	65.11 ± 0.524	65.13 ± 0.505	3.04 ± 0.028	3.07 ± 0.030
8	6.43 ± 0.129	6.43 ± 0.122	65.10 ± 0.552	65.08 ± 0.611	3.01 ± 0.037*	3.05 ± 0.024
12	6.40 ± 0.138	6.41 ± 0.142	65.06 ± 0.510	65.07 ± 0.603	3.04 ± 0.031	3.03 ± 0.042
16	6.39 ± 0.114	6.41 ± 0.106	65.03 ± 0.564	65.04 ± 0.575	3.03 ± 0.022	3.02 ± 0.021

* Significance at $P < 0.05$ compared with the content at initial time

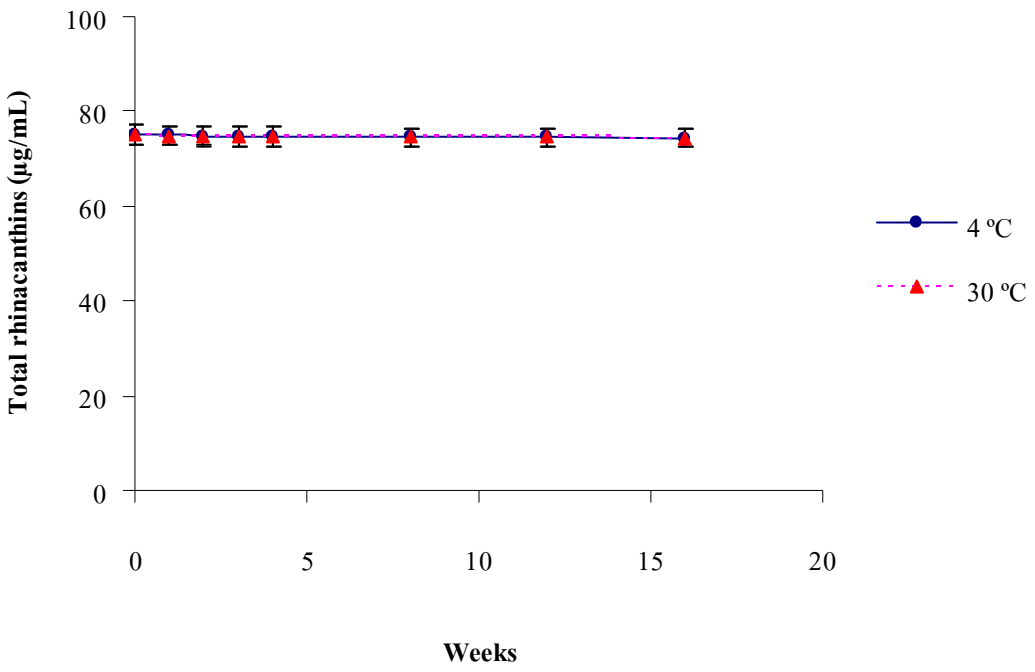

Figure 4.11 Total rhinacanthin content of the rhinacanthin high-yielding *R. nasutus* leaf extract stored under 4 ºC and 30 ºC

4.9.3 Effect of accelerated condition on the stability of the extract

The accelerated stability test of the rhinacanthin high-yielding *R. nasutus* leaf extract was carried out using a stability chamber. The extracts were kept in well-closed containers protected from light and stored in the chamber at 45 ºC with 75 %relative humidity. Physical appearance of the extract was observed as well as the content of rhinacanthin was analyzed at the initial times and every week (or month) in four-month period. The result demonstrated that the physical appearances of the extract were not change even stored under accelerated condition in the period of four months. Unfortunately, the total content of rhinacanthin was significantly decreased from 75.05 to 71.36 %w/w after 12 weeks of storage (Table 4.14 and Figure 4.12). In addition, the content of both rhinacanthin-D and rhinacanthin-C were significantly decreased after 8 weeks and the content of rhinacanthin-N was significantly decreased after 4 weeks. However, the total content of rhinacanthin remained 95.08 % of the content at the initial time. According to the content of rhinacanthin that were still more than 90 % after stability test under accelerated condition, we therefore conclude that the rhinacanthin high-yielding *R. nasutus* leaf extract has been storage for 2 years at normal condition (จุไรรัตน์ รักวาทิน, 2538).

Table 4.15 Rhinacanthin content of the rhinacanthin high-yielding *R. nasutus* leaf extract stored under normal and accelerated conditions

Weeks	Rhinacanthin content (%w/w; Mean ± S.D.)					
	Rhinacanthin-D		Rhinacanthin-C		Rhinacanthin-N	
	30 °C	45 °C	30 °C	45 °C	30 °C	45 °C
0	6.52 ± 0.101	6.52 ± 0.101	65.40 ± 0.563	65.40 ± 0.560	3.13 ± 0.052	3.13 ± 0.054
1	6.50 ± 0.111	6.51 ± 0.103	65.29 ± 0.507	65.25 ± 0.577	3.10 ± 0.043	3.09 ± 0.027
2	6.48 ± 0.134	6.50 ± 0.116	65.23 ± 0.601	65.18 ± 0.511	3.07 ± 0.036	3.05 ± 0.032
3	6.48 ± 0.115	6.45 ± 0.113	65.16 ± 0.529	65.11 ± 0.603	3.06 ± 0.027	3.03 ± 0.041
4	6.45 ± 0.109	6.41 ± 0.118	65.11 ± 0.520	65.10 ± 0.553	3.04 ± 0.033	3.05 ± 0.024
8	6.43 ± 0.132	6.36 ± 0.135	65.10 ± 0.552	64.93 ± 0.555	3.01 ± 0.041*	2.84 ± 0.026*,#
12	6.40 ± 0.140	6.27 ± 0.113*	65.06 ± 0.513	63.84 ± 0.506*,#	3.04 ± 0.031	2.63 ± 0.038*,#
16	6.39 ± 0.113	6.15 ± 0.132*,#	65.03 ± 0.570	62.76 ± 0.539*,#	3.03 ± 0.019	2.45 ± 0.032*,#

* Significance at $P < 0.05$ compared with the content at initial time

Significance at $P < 0.05$ compared with the content in normal condition at the same time

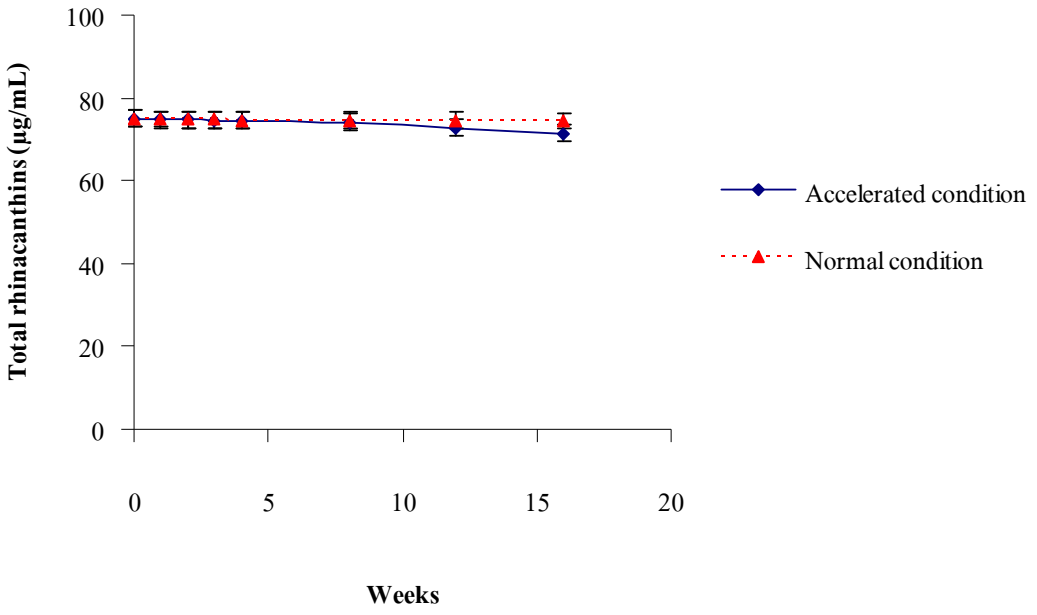

Figure 4.12 Total rhinacanthin content of the rhinacanthin high-yielding *R. nasutus* leaf extract stored under normal and accelerated conditions

4.9.4 Effect of pH on the stability of the extract

The pH stability study of the rhinacanthin high-yielding *R. nasutus* leaf extract in the solution was determined. The extracts were kept in well-closed containers, protected from light and stored at the room temperature (30 ± 2 ºC) for 4 months. An adequate sample was taken at the initial time and every week (or month) for the analysis of the rhinacanthin content. The results showed that under all tested pH, the solutions of the extracts were red color at the initial time (Figure 4.13). Thus, the result suggested that the alteration of rhinacanthin chromophore in *R. nasutus* leaf extract was taken place. In addition, the rhinacanthin content was decreased under all tested pH. Based on the HPLC-chromatograms, the retention times of degraded products were observed at 2.8, 4.7, 6.0, and 6.7 minutes in all tested pH (Figure 4.14). At pH 5.5 and 7.0, the content of rhinacanthin-D was significantly decreased after the third week, and at pH 8.0 it was significantly decreased after the second week (Table 4.15). At pH 5.5, the content of rhinacanthin-C was significantly decreased after the second week and at pH 7.0 and 8.0 was significantly decreased after the first week (Table 4.16). The content of rhinacanthin-N at all test pH conditions was significantly decreased after first week (Table 4.17). It implies that all rhinacanthins are not stable when they are in solution (Figure 4.15). However, rhinacanthins were more stable in the solution at pH 5.5 and 7.0 than at pH 8.0. The instability of rhinacanthins in the solution should be considered as their hydrolysis of the rhinacanthins. Thus, preparation of the *R. nasutus* leaf extract in an aqueous solution should be performed carefully.

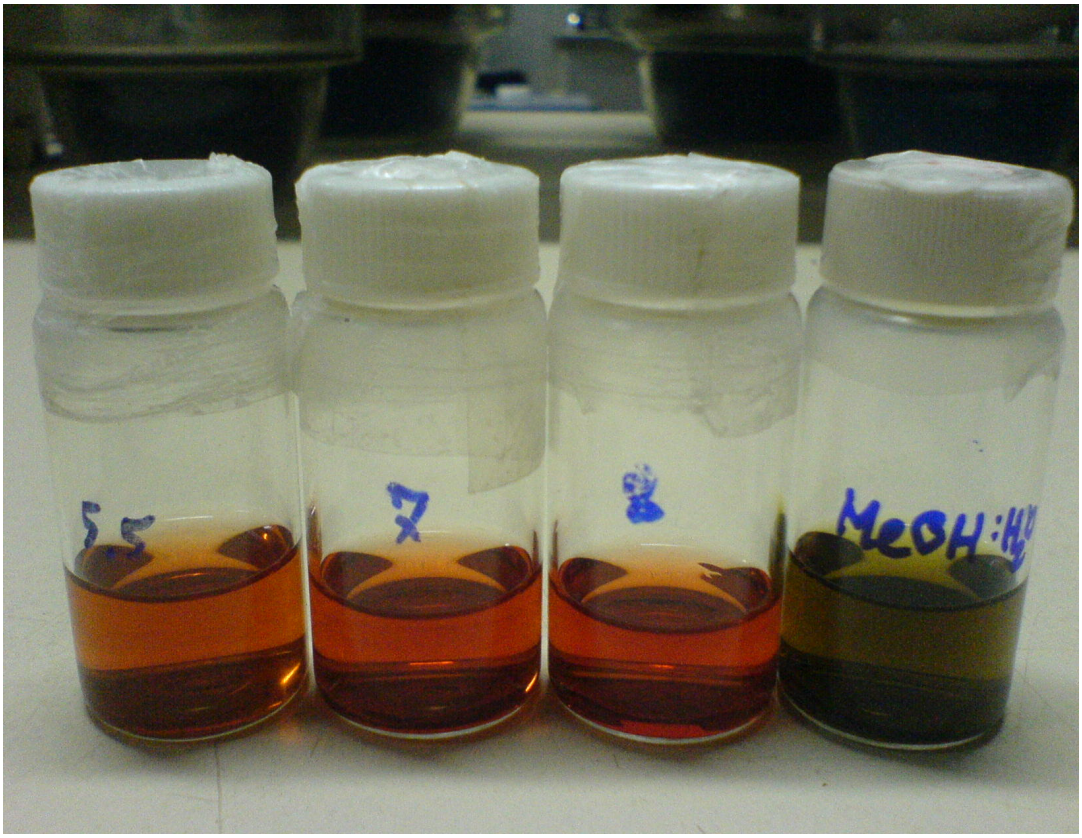

Figure 4.13 The physical appearance of rhinacanthin high-yielding *R. nasutus* leaf extract in the solution of MeOH : H_2O (1 : 1) with phosphate buffer pH 5.5, 7, 8, and no buffer

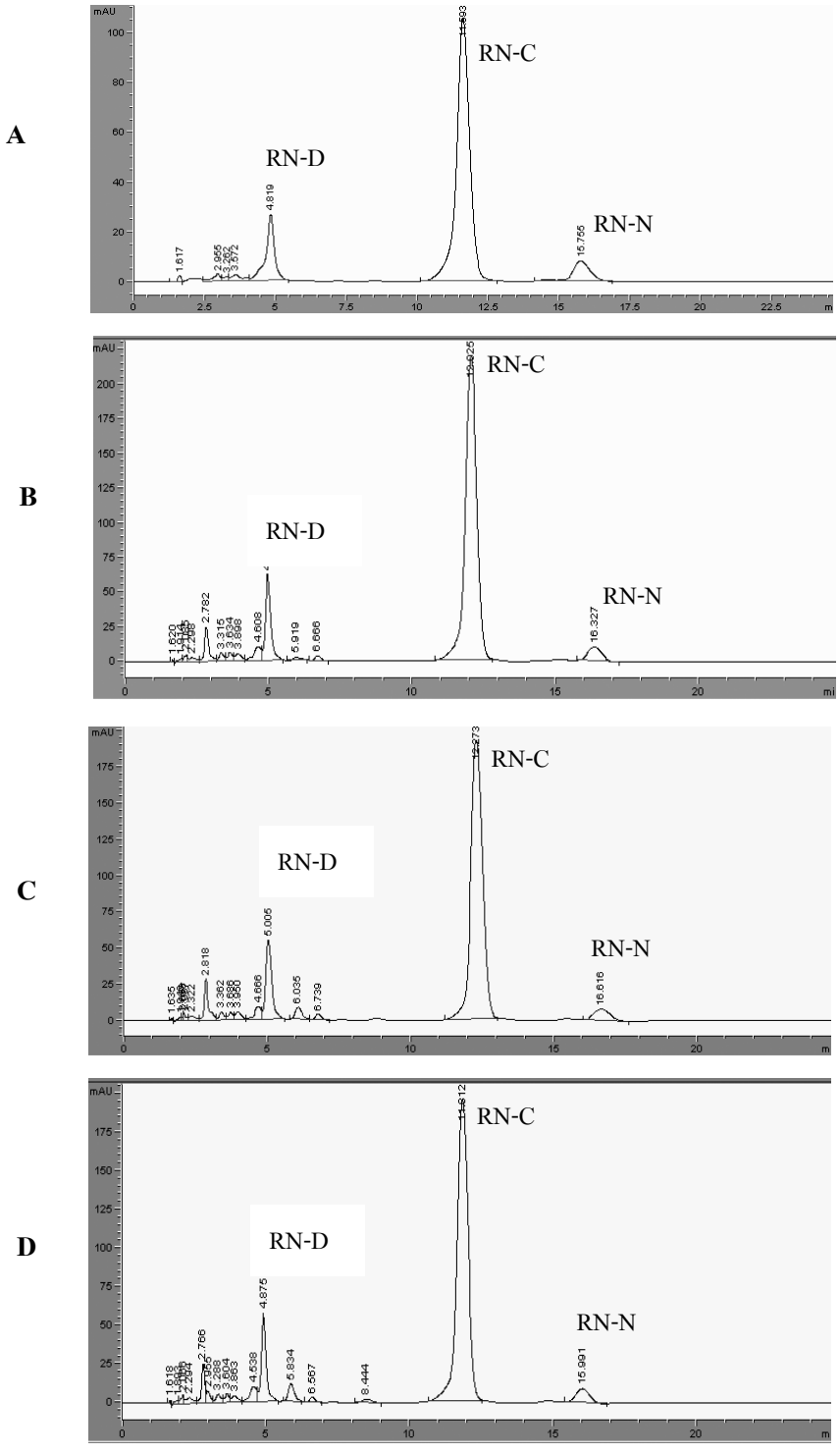

Figure 4.14 HPLC-chromatograms of the rhinacanthin high-yielding *R. nasutus* extract (A) and its solution at pH 5.5 (B), 7.0 (C), and 8.0 (D) after16 weeks

Table 4.16 Rhinacanthin-D content of the rhinacanthin high-yielding *R. nasutus* leaf extract in the solution at pH 5.5, 7.0, and 8.0

Weeks	Rhinacanthin-D content (%w/w; Mean ± S.D.)		
	pH 5.5	pH 7	pH 8
0	5.78 ± 0.102	5.78 ± 0.090	5.80 ± 0.091
1	5.76 ± 0.101	5.66 ± 0.094	5.68 ± 0.110
2	5.59 ± 0.108	5.57 ± 0.118	5.58 ± 0.089
3	5.57 ± 0.127	5.50 ± 0.113	5.42 ± 0.144*
4	5.17 ± 0.135*	5.12 ± 0.131*	5.10 ± 0.121*
8	4.82 ± 0.114*	4.79 ± 0.127*	4.76 ± 0.133*
12	4.60 ± 0.118*	4.55 ± 0.122*	4.48 ± 0.128*
16	4.33 ± 0.104*	4.26 ± 0.114*	4.21 ± 0.113*

* Significance at $P < 0.05$ compared with the content at initial time

Table 4.17 Rhinacanthin-C content of the rhinacanthin high-yielding *R. nasutus* leaf extract in the solution at pH 5.5, 7.0, and 8.0

Weeks	Rhinacanthin-C content (%w/w; Mean ± S.D.)		
	pH 5.5	pH 7	pH 8
0	64.72 ± 0.661	64.85 ± 0.651	64.88 ± 0.657
1	64.32 ± 0.684	63.77 ± 0.662	63.65 ± 0.693
2	63.56 ± 0.622	62.61 ± 0.611*	62.54 ± 0.632*
3	62.92 ± 0.627*	62.33 ± 0.637*	62.24 ± 0.665*
4	58.79 ± 0.640*,#	57.50 ± 0.598*,#	55.61 ± 0.606*
8	53.45 ± 0.615*,#	52.32 ± 0.633*,#	49.83 ± 0.611*
12	48.74 ± 0.601*,#	47.11 ± 0.624*,#	44.07 ± 0.620*
16	43.26 ± 0.593*,#	42.21 ± 0.580*,#	39.12 ± 0.601*

* Significance at $P < 0.05$ compared with the content at initial time

Significance at $P < 0.05$ compared with the content at pH 8 at the same time

Table 4.18 Rhinacanthin-N content of the rhinacanthin high-yielding *R. nasutus* leaf extract in the solution at pH 5.5, 7.0, and 8.0

Weeks	Rhinacanthin-N content (%w/w; Mean ± S.D.)		
	pH 5.5	pH 7	pH 8
0	3.10 ± 0.100	3.09 ± 0.104	3.10 ± 0.076
1	2.94 ± 0.072	2.91 ± 0.093	2.96 ± 0.092
2	2.88 ± 0.093$^{\#}$	2.72 ± 0.111*,$^{\#}$	2.49 ± 0.103*
3	2.81 ± 0.088*,$^{\#}$	2.51 ± 0.089*,$^{\#}$	1.99 ± 0.082*
4	2.50 ± 0.102*,$^{\#}$	2.50 ± 0.083*,$^{\#}$	1.91 ± 0.109*
8	2.30 ± 0.064*,$^{\#}$	2.16 ± 0.045*,$^{\#}$	1.49 ± 0.064*
12	1.95 ± 0.037*,$^{\#}$	1.78 ± 0.036*,$^{\#}$	1.17 ± 0.027*
16	1.69 ± 0.054*,$^{\#}$	1.42 ± 0.031*,$^{\#}$	0.84 ± 0.019*

* Significance at $P < 0.05$ when compared with the content at initial time

$^{\#}$ Significance at $P < 0.05$ when compared with the content at pH 8 at the same time

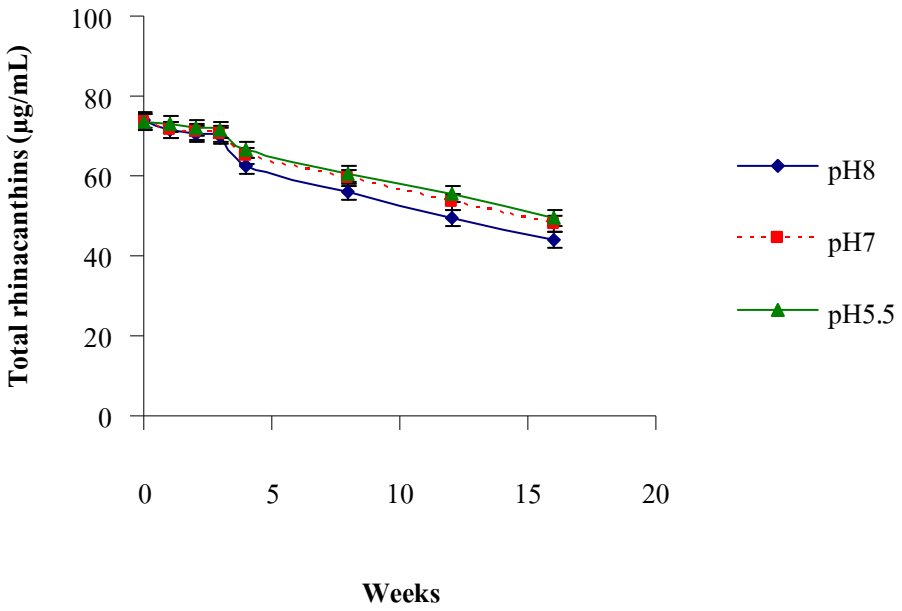

Figure 4.15 Total rhinacanthin content of rhinacanthin high-yielding extract in the solution of MeOH : H_2O (1 : 1) with phosphate buffer pH 5.5, 7, and 8