CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 The chemical components in *S. curtisii*

When using dichloromethane in the extraction process of dried powder of *S. curtisii*, crude extract was obtained in the yield of 2.29 % relative to the weight of dried plant. The extract was then isolated by column chromatography and found that the extract of *S. curtisii*, contained pyridostemin (2.371 %w/w) as a major component with trace amounts of other alkaloids. Pyridostemin could readily be detected by its strong maximum UV absorption at 300 nm (in a mixture of acetonitrile/water/triethylamine), indicative of a conjugated dienone system (Figure 7) (Kaltenegger *et al.*, 2003). The IR spectrum of pyridostemin (KBr) (Figure 8) showed strong absorption bands of C=O stretching of lactone ring at 1740 cm^{-1}, C=C stretching of unsaturated lactone ring at 1618 cm^{-1}, C-O stretching at 1021 and 945 cm^{-1} of furan. The ^1H NMR spectral data of pyridostemin was shown in Figure 9 and Table 6. The data was conformed to the previously reported NMR data of pyridostemin (Mungkornasawakul *et al.*, 2003). Its mass spectrum (Figure 10) exhibited a molecular ion peak [M^+] at *m/z* 347 corresponding to a molecular formula of $C_{19}H_{25}NO_5$ confirming the NMR derived structure.

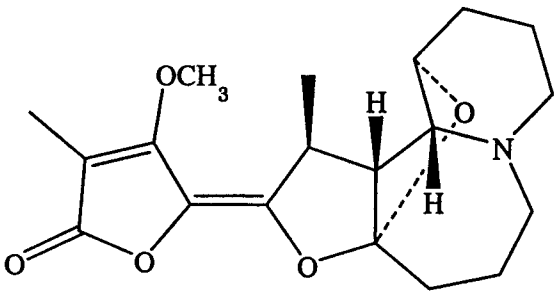

Figure 7. Structure of pyridostemin

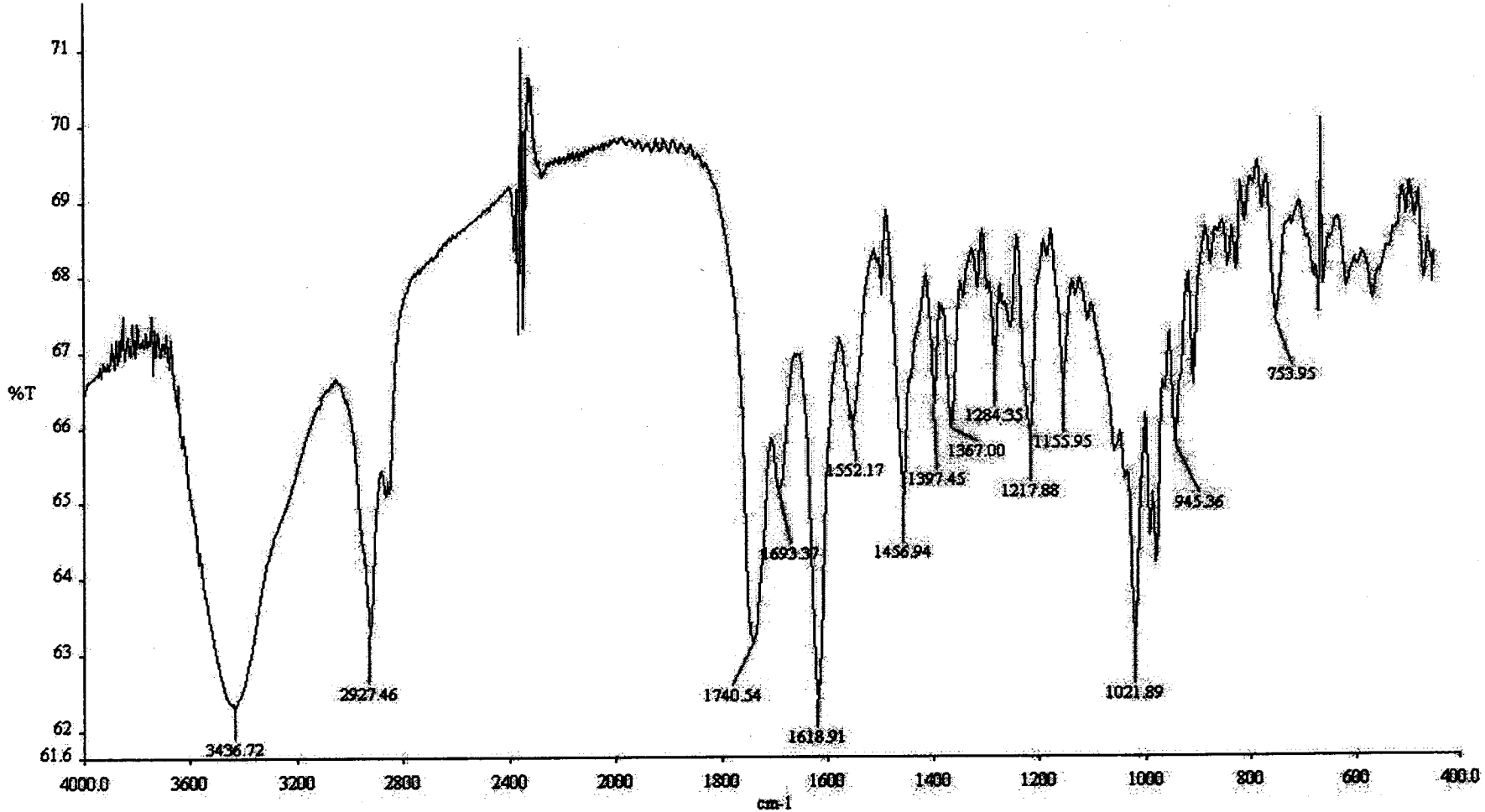

Figure 8. FTIR spectrum of pyridostemin

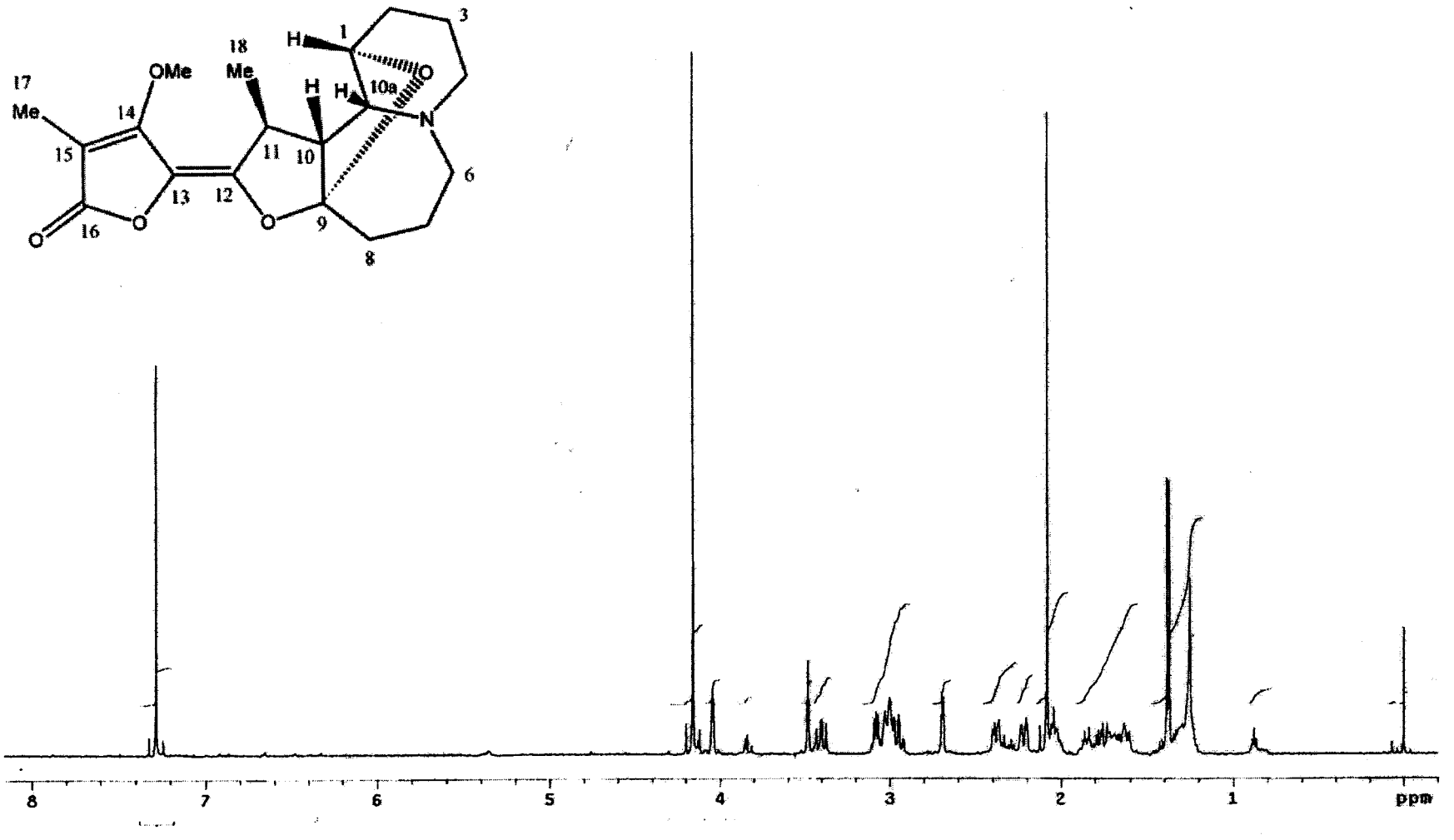

Figure 9. ^{1}H NMR of pyridostemin

Table 6. ^{1}H NMR spectral data of pyridostemin (500 MHz, $CDCl_3$) compared with reference of pyridostemin (500 MHz, $CDCl_3$) (Mungkornasawakul *et al.*, 2003)

Position	Pyridostemin	Reference of pyridostemin
	δ (ppm) [multiplicity, *J* (Hz)]	δ (ppm) [multiplicity, *J* (Hz)]
1	4.04 (s)	4.01 (s)
2	1.62 (m, β)	1.62 (m, β)
	2.22 (d, 14.6, α)	2.21 (d, 14.5, α)
3	1.25 (m, β)	1.21 (d, 13.5, β)
	1.84 (m, α)	1.84 (m, α)
4	2.92 (m, β)	2.87 (m, β)
	3.02 (m, α)	3.02 (m, α)
6	3.40 (dd, 13, 15.5, β)	3.38 (t, 13, β)
	2.96 (m, α)	2.96 (m, α)
7	2.03 (m, α)	2.03 (m, α)
	1.66 (m, β)	1.66 (m, β)
8	2.38 (dd, 4, 13, α)	2.36 (dd, 4.5, 13.5, α)
	1.75 (m, β)	1.75 (m, β)
10	2.69 (d, 5)	2.65 (d, 4.5)
10a	3.48 (s)	3.44 (s)
11	3.07 (quin, 6.5)	3.07 (quin, 6.5)
17	2.08 (s)	2.08 (s)
18	1.38 (d, 7)	1.37 (d, 7)
OCH_3	4.15 (s)	4.15 (s)

3.2 Validation of HPLC method for analysis of pyridostemin in extract

The analysis of chemical components in botanical material is not an easy task. Usually, a sample to be analyzed contains a very complex mixture of many components. Furthermore, the different samples of botanical materials differ substantially in content of the active ingredients and often have different chemical compositions. This diversity of important conditions affecting the quality of botanical remedies requires therefore implementation of stringent, well-designed and closely-monitored standard operating procedures of manufacturing to ensure consistency from batch to batch of a herbal product, followed by application of an appropriate analysis to ensure consistent potency and efficacy (Fu *et al.*, 2002). The development of a method for separation and quantitation of the active compound in plant extract is important in quality control of such preparations (Penissi *et al.*, 2003). Therefore, the quantitative analysis method development and validation were required. HPLC has previously been used for the separation of individual *Stemona* alkaloids from other components (Adams *et al.*, 2005; Ge *et al.*, 2007; Jiang *et al.*, 2006a; Jiang *et al.*, 2006b; Limtrakul *et al.*, 2007; Schinnerl *et al.*, 2007; Zhou *et al.*, 2006).

The HPLC chromatographic conditions for determination of *S. tuberosa* extract have been described by Jiang *et al.* (2006a,b). The HPLC conditions were performed for qualitative analysis to investigate the HPLC fingerprints of *S. tuberosa* extract, not for quantitative analysis. However, the method validation parameters were not reported. Chromatographic separation was carried out on a C_{18} column (150×4.6 mm, 3 μm; Alltech®), using a gradient solvent system consisted of water and acetonitrile containing 0.12% triethylamine (Jiang *et al.*, 2006b). The combination of HPLC and Diode Array UV-Visible detection may give new possibilities in qualitative analysis of *Stemona* alkaloids in plant extracts.

In the present study, a stability indicating HPLC method was developed to give a simple, rapid, sensitive, reproducible and accurate analytical procedure for quantification of pyridostemin in *S. curtisii* extracts or in formulations and subsequently successfully used to study the stability of pyridostemin in the extract or formulations. Chromatographic separation was performed on a C_{18} column, using an isocratic solvent

system comprised of acetonitrile-water (containing 0.12% triethylamine) (30:70 v/v). The UV detection wavelength was fixed at the maximum absorption wavelength of pyridostemin, 300 nm (Figure 11).

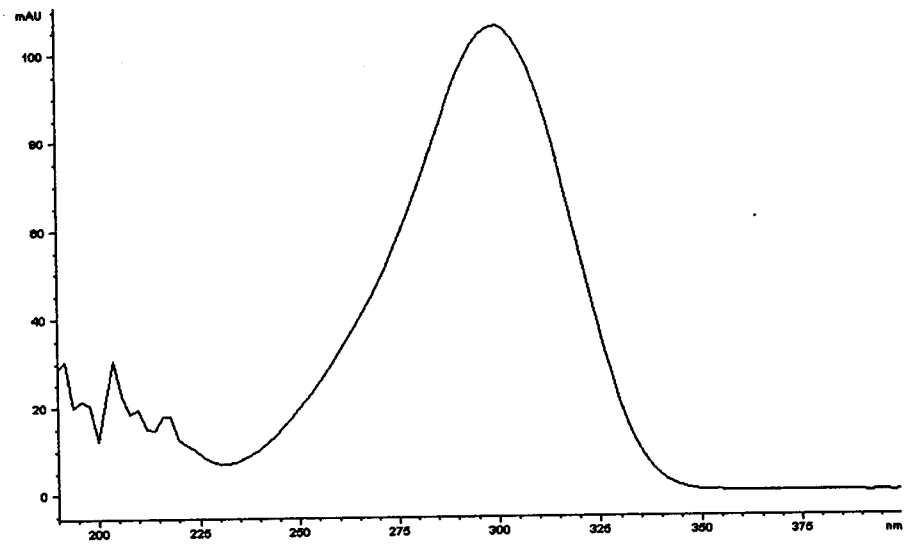

Figure 11. UV spectrum of pyridostemin (in mixture of acetonitrile/water/triethylamine as a solvent)

In the selected optimal experimental conditions, the pyridostemin exhibited a well-defined chromatographic peak with a retention time of 11.2 min. Figure 12 (A) shows the chromatogram obtained by injection of a solution containing only pyridostemin (25 μg/ml). HPLC analysis of the pure pyridostemin gave a single peak. The chromatogram obtained by injection of a solution containing crude extract of *S. curtisii* was shown in Figure 12 (B). The chromatogram shows the pyridostemin peak was clearly separated from the other peaks. This result confirmed the ability of the method to measure the response in the present of its potential impurities.

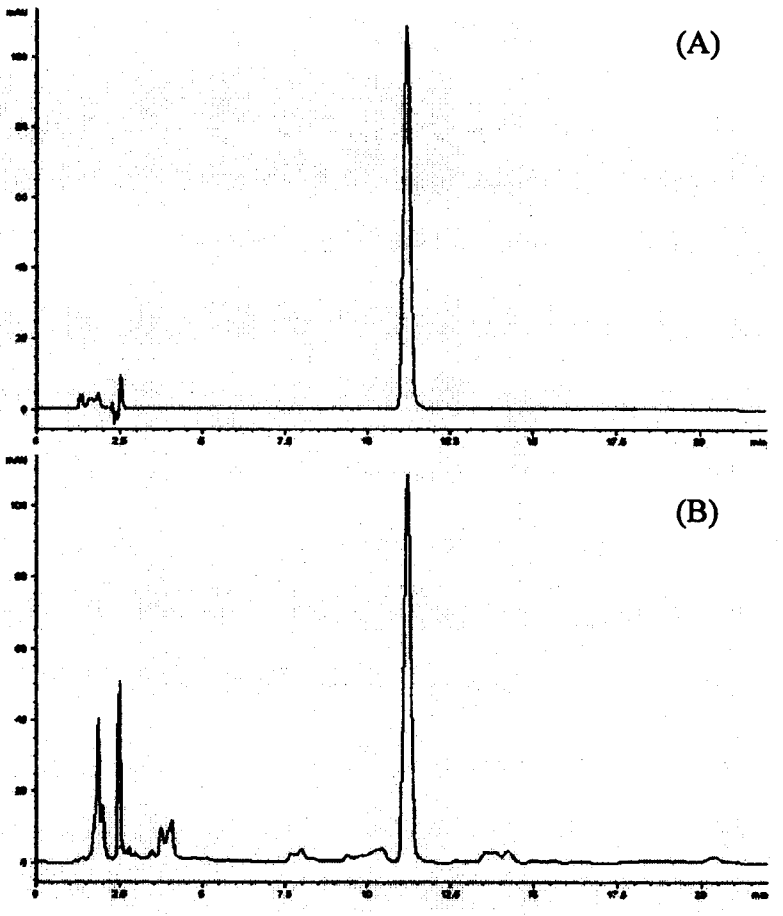

Figure 12. HPLC chromatograms of pure pyridostemin (A) and *S. curtisii* extract (B) using C_{18} reverse phase column with acetonitrile-water-triethylamine (30:70:0.12, v/v/v) using a PDA detector (300 nm); flow rate 1.25 ml/min; injection volume 20 µl

3.2.1 Specificity

Checking peak homogeneity is one of the first and most important steps in chromatographic data evaluation in order to guarantee reliable qualitative and quantitative results (Ebel and Mueck, 1988). The purity of the peak was tested using the PDA detector to ensure that the compound was not co-eluting with an impurity peak. The developed HPLC method for pyridostemin was further tested for its specificity by HPLC analysis of pyridostemin in the presence of the other component in the crude extracts. Furthermore, stress studies were performed for pyridostemin to provide an indication of the stability indicating property and specificity of the developed method.

Peak purity test results confirmed that the pyridostemin peak is homogenous and pure in all the analyzed stress samples (Figure 13). All chromatogram of the stress samples (Figure 14) showed no peak as interferences. This study confirms the stability indicating power of the method and shows that these chromatographic conditions can be used to investigate the stability of pyridostemin both in the plant extract and prepared formulations.

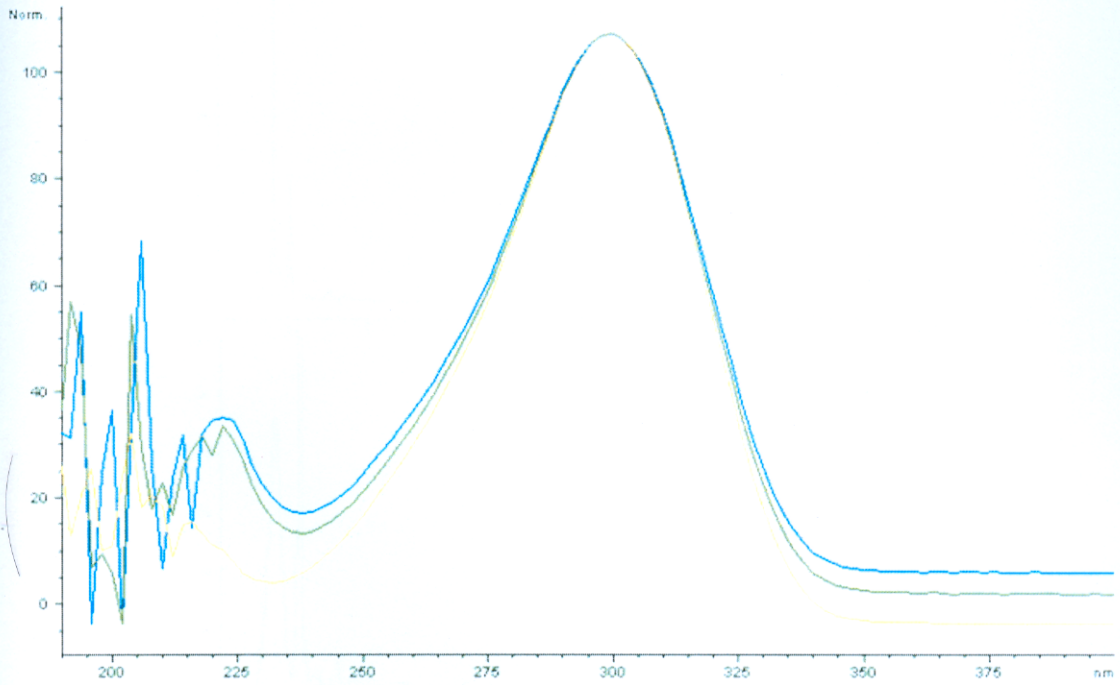

Figure 13. Absorption spectra of the pyridostemin peak at retention time of 11.05 (—), 11.20 (—), and 11.32 (—) min

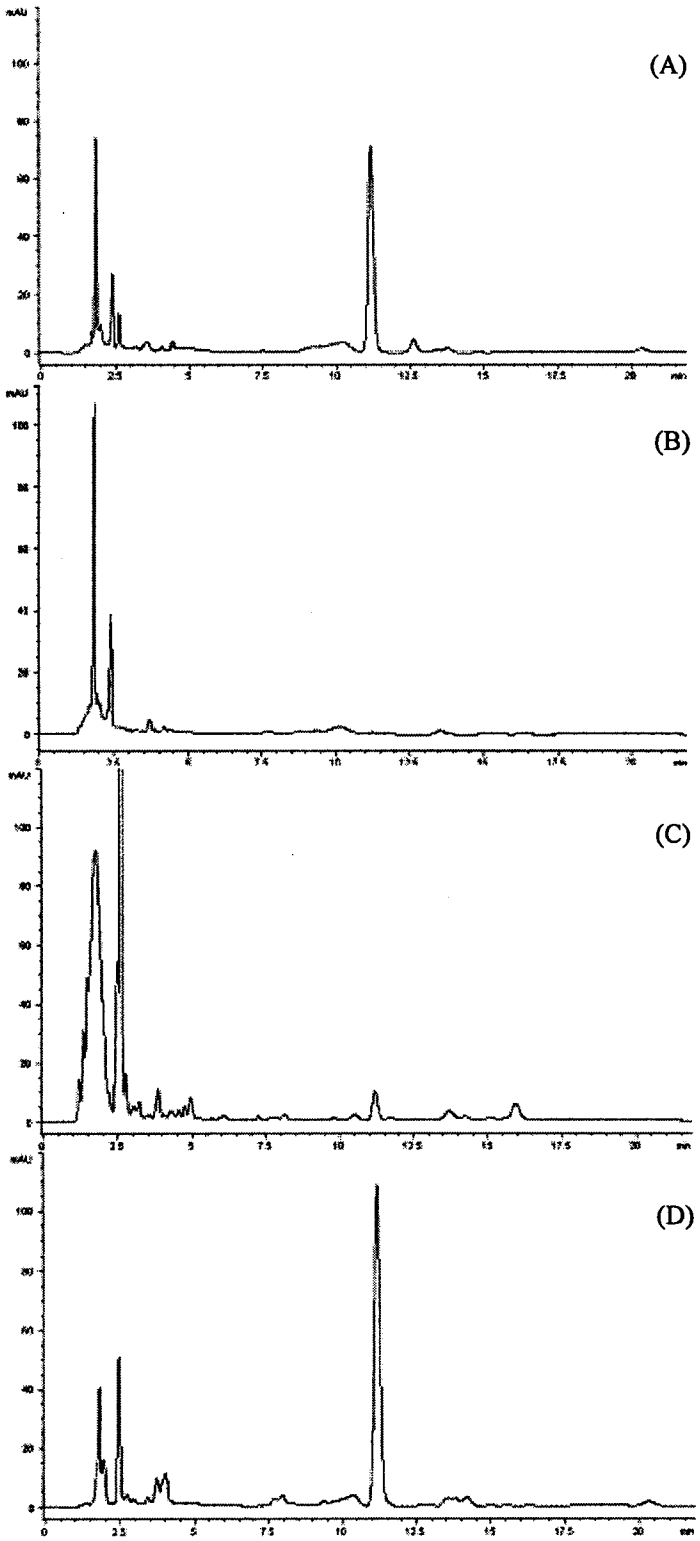

Figure 14. HPLC chromatograms of *S. curtisii* extract in acid hydrolysis (A), base hydrolysis (B), oxidation (C), and reduction (D) conditions for 24 hours (Pyridostemin peak exhibited at retention time of 11.2 min)

Stress testing is an important part of the drug development process as it provides knowledge about the degradation chemistry of active compounds. This knowledge is used primarily to develop stability-indicating analytical methods and also useful for other purposes such as formulation development, package development, and the design of official stability studies (Baertschi and Alsanate, 2005). The peak areas of pyridostemin (retention time 11.2 min) refer to the degradation behaviors of pyridostemin in these conditions. From these HPLC chromatograms indicated that pyridostemin showed the highest stability in the presence of reducing agent, slightly unstable in acid, and unstable in base and in the presence of oxidizing agent.

Decomposition of active ingredients occurs through several pathways, i.e., hydrolysis, oxidation-reduction, racemization, decarboxylation, ring cleavage, and photolysis. The most frequently encountered are hydrolysis and oxidation-reduction reactions (Lachman *et al.*, 1986). Unstable of pyridostemin (Figure 15) could be described by reactions of some functional groups on the structure as follow.

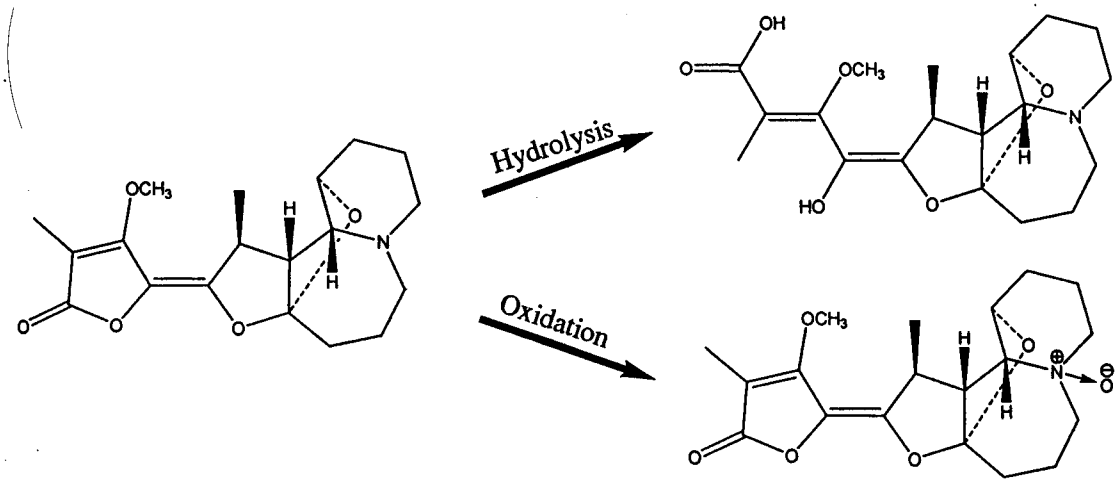

Figure 15. Predicted degradation products of pyridostemin by acid-base hydrolysis and oxidation reactions

Pyridostemin has a functional group of lactone ring (cyclic ester) that could be subjected to general acid or base-catalyzed hydrolysis to form a carboxylic acid and an alcohol (Baertschi and Alsanate, 2005; Bruice, 2007; Morrison and Boyd, 1992). Base-

catalyzed hydrolysis of this functional group is fast with the more powerful attacking -OH nucleophile yielding the salt of the acid (Baertschi and Alsanate, 2005). This reaction is essentially irreversible, since a resonance-stabilized carboxylate anion shows little tendency to react with an alcohol (Morrison and Boyd, 1992). Acid catalyzed hydrolysis is slower and reversible (Baertschi and Alsanate, 2005; Morrison and Boyd, 1992). Acid catalyze the reaction by making the carbonyl carbon more electropositive (by protonation of the carbonyl oxygen) and therefore more susceptible to nucleophillic attack (Baertschi and Alsanate, 2005).

The oxidative unstability of this compound can be focused on the tertiary amine group. Tertiary amine is known for its propensity to oxidize to the amine oxide (*N*-oxide) (Hartauer *et al.*, 2000; Qin and Freeh, 2001; Vermeire and Remon, 1999). That is consistent with finding that oxystemokerrin along with oxystemokerrin-*N*-oxide in *Stemona* extract (Kaltenegger *et al.*, 2003).

Either hydrolysis of lactone ring or oxidation of tertiary amine on the structure may decrease insecticidal activity of pyridostemin based on structure-activity relationships of insecticidal pyrido[1,2-a]azepine alkaloids as previously described by Kaltenegger *et al.* (2003).

3.2.2 Linearity and range

The calibration curve for pyridostemin was constructed by plotting concentrations *versus* corresponding mean peak areas (Figure 16). The chromatographic signals show a linear dependence with the pyridostemin concentration enabling the use of this signal for pyridostemin quantification, according to the following regression equation:

$$Y = 47.5063X - 1.0546$$

$$r^2 = 0.9997 \pm 0.0002$$

(Y = peak area; X = pyridostemin concentration)

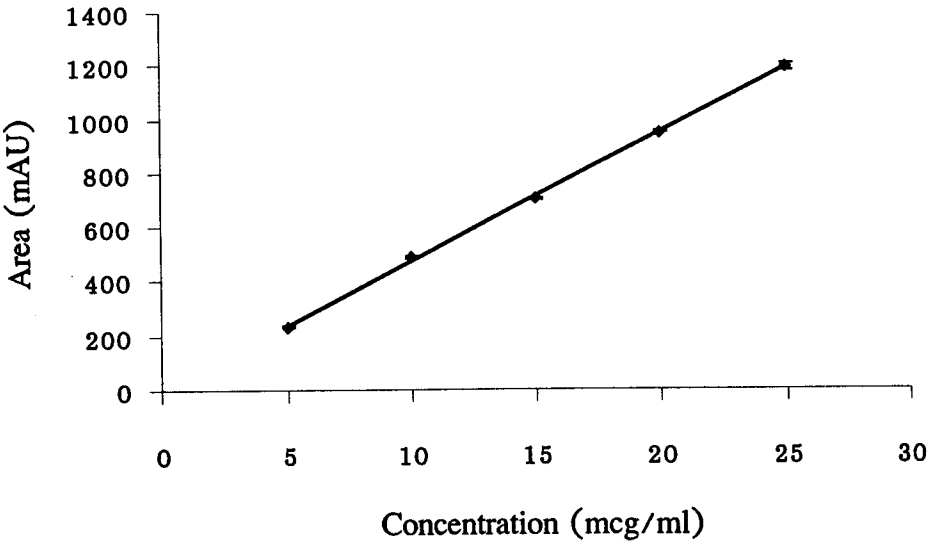

Figure 16. Calibration curve of pyridostemin

Good linearity was observed in the range of 5-25 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 0.52 and 5.70, respectively. The result shows that an excellent correlation existed between the peak area and concentration of the analyte.

3.2.3 Precision

The precision of the method was determined by intra-day repeatability and intermediate precision studies. 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 pyridostemin in the samples are presented in Table 7. The RSD values were lower than 2.0% (between 0.31-1.29%), which shows high precision for the method.

Table 7. RSD (%) of intra-day repeatability and intermediate precision studies

Concentration (μg/ml)	RSD (%)	
	Intra-day repeatability	Intermediate precision
5	0.31	1.29
15	0.90	0.44
25	0.72	0.89

3.2.4 Accuracy

The mean percentage recovery of pyridostemin in the crude extract was ranged from 98.28-102.85 % (Table 8).

Table 8. Recovery (%) of pyridostemin spiked in crude extract at various concentrations

Sample	Recovery (%) on each studied concentration					Recovery (%)
	5 μg/ml	10 μg/ml	15 μg/ml	20 μg/ml	25 μg/ml	(mean±SD)
1	102.85	99.72	98.64	100.46	100.12	100.36±1.55
2	102.41	99.25	99.41	98.80	101.00	100.17±1.50
3	101.51	99.97	98.28	98.84	101.17	99.95±1.41
Average	**102.26**	**99.65**	**98.78**	**99.37**	**100.77**	**100.16±1.38**

The HPLC method validation results showed that determination of pyridostemin in the extract could be performed by validated HPLC method described above with accepted accuracy and precision.

3.3 Comparison of pyridostemin content in *S. curtisii* extract obtained by maceration using different solvents

The amount of the crude extract from dried root powder of *S. curtisii* obtained by maceration using different organic solvents were calculated as the absolute yields of extract (g) obtained from 100 g of dried plant (%w/w). The result was shown in Table 9.

Table 9. Pyridostemin content in *S. curtisii* extract obtained by maceration using different solvent

Solvent for maceration	Weight of extract (g)	Pyridostemin content (%w/w)	
		Per dried plant	Per crude extract
Methanol	2.093±0.067	0.77±0.03	3.70±0.09
Dichloromethane	0.136±0.002	0.25±0.02	18.77±1.22

The extracts obtained by maceration using methanol were found to contain significantly higher absolute amount of pyridostemin per 100 g of dried plant (0.77±0.03 %w/w) than the extract using dichloromethane (0.25±0.02 %w/w). However, pyridostemin content per 100 g of crude extract which extracted by dichloromethane (18.77±1.22 %w/w) was higher than using methanol (3.70±0.09 %w/w). Since, unwanted constituents in crude extract may have an effect in formulation development and partially purification process for the methanol extract was more costly than maceration with dichloromethane, in term of time and cost of solvents and silica gel. Thus dichloromethane was chosen as a suitable extraction solvent for this purpose.

3.4 Determination of pyridostemin content in crude extract and partially purified extract

Although dichloromethane extract contained higher pyridostemin content than methanol extract, the pyridostemin content in the crude extract was still too low for pesticide granule formulation. Therefore, partially purification of the crude extract was required. Partially purification of herbal extract is commonly used in medicinal natural product to improve yielding of active compounds. Partially purification process was applied

in preparation of many natural products such as *Golden Tiara* (Fu *et al.*, 2003; Fu *et al.*, 2002), *Theobroma cacao* (Wollgast, 2004), *Gerbera hydrida* (Yrjonen *et al.*, 2002), *Rhinacanthus nasutus* (Kongchai, 2003), etc. Silica gel vacuum column chromatography is a chromatographic purification technique which has excellent resolving power, easily applied to large scale chromatography and fast in operation. Furthermore, this technique is economically and environmentally friendly due to significant reduction in solvent and the amount of silica gel used (Pedersen and Rosenbohm, 2001).

S. curtisii extract was subjected to partially purification using silica gel vacuum column chromatography using a mixture of dichloromethane, ethyl acetate, and methanol (70:25:5, v/v/v) as eluent resulting in pyridostemin concentrated crude extract in 11.17 %w/w relative to the original crude extract. The partially purified *S. curtisii* extract contained significantly higher pyridostemin content than the crude extract. Pyridostemin contents in crude extract and partially purified extract were 18.77±1.22 and 71.40±5.51 %w/w, respectively. The result from this study reveals that partially purification method of the crude extract is a crucial process that provide higher amount of active compound. Pyridostemin was almost increased four times compared to that of original crude extract. The resulting partially purified extract is now containing a suitable amount of pyridostemin for utilizing in the pesticide formulation.

3.5 Stability of pyridostemin in partially purified extract

The accelerated stability test of pyridostemin in partially purified extract was performed at 3 elevated temperatures; 45, 60, and 70 °C (75 %RH). Upon of the sampling periods from 1 to 84 days, the degradation of pyridostemin was fitted with the first-order kinetic, in which the linearity was best met when the logarithm of the percentage remaining amount of pyridostemin from each temperature were plot against storage time, *t* (day) (Figure 17).

Integrated rate equation of first-order reaction:

$$\ln c = \ln c_0 - kt$$

ln c = logarithm of the percentage remaining amount of pyridostemin

ln c_0 = logarithm of the percentage remaining amount of pyridostemin at t = 0

k = stability rate constant

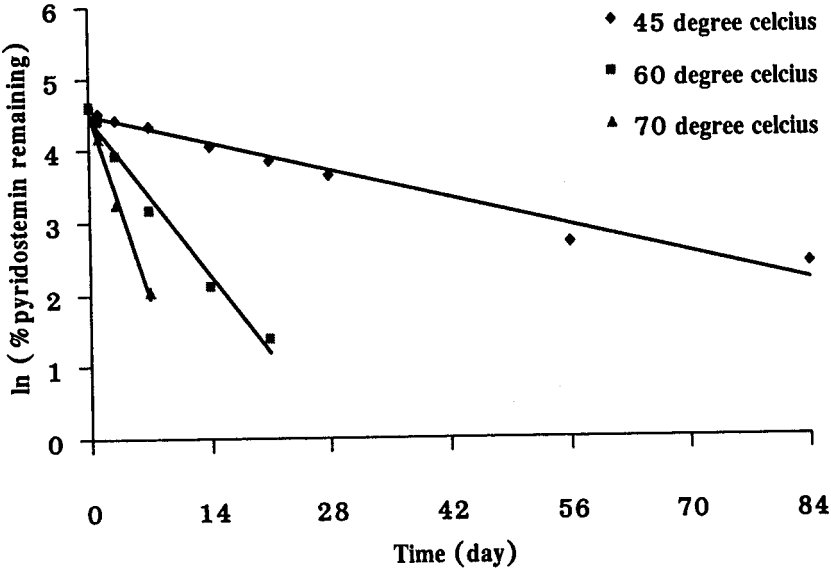

Figure 17. First-order plots of degradation of pyridostemin at 45, 60, and 70 °C (75 %RH). Rate equations of each temperature are expressed as ln c = 4.4857-0.0274t, r^2 = 0.9710; ln c = 4.4497-0.1555t, r^2 = 0.9825; and ln c = 4.5158-0.3656t, r^2 = 0.9893, respectively.

Table 10. Stability rate constants (k) for pyridostemin in partially purified extract

Storage temperature (°C), 75 %RH	ln c_0	Stability rate constant (day^{-1})	r^2
45	4.4857	0.0274	0.9710
60	4.4497	0.1555	0.9825
70	4.5158	0.3656	0.9893

The stability rate constants (Table 10) at each temperature were found as 0.027, 0.156, and 0.366 day^{-1}, respectively. The extrapolation of the Arrhenius plot (Figure 18) obtained from a relation between ln k and reciprocal of absolute temperature (T) led to estimated k at 30 °C ($k_{30°C}$) of 4.83×10^{-3} day^{-1}.

Logarithm form of Arrhenius equation:

$$\ln k = \ln A - \frac{E_a}{RT}$$

A = frequency factor

E_a = activation energy

R = gas constant

T = absolute temperature in Kelvins

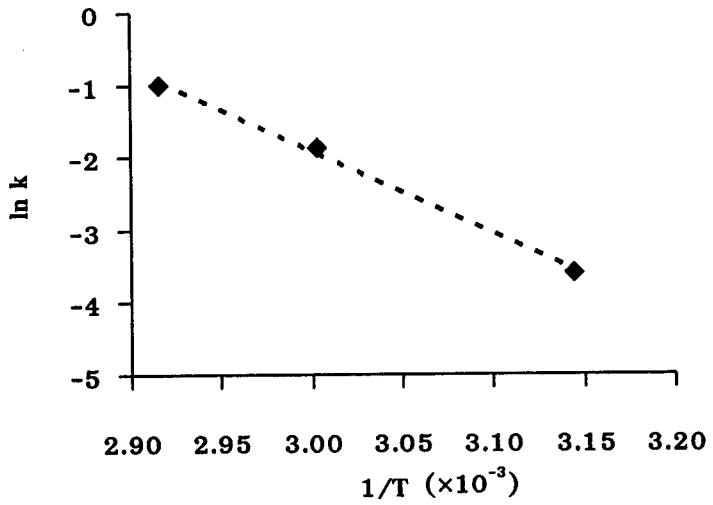

Figure 18. Arrhenius plot of pyridostemin in partially purified extract. The Arrhenius relation of pyridostemin in partially purified extract can be expressed as $\ln k = 32.271 - (11395/T)$; $r^2 = 0.9966$.

The activated energy (E_a) term is a measure of how sensitive the degradation rate of a drug to temperature changes (Yoshioka and Stella, 2000). The E_a calculated from the Arrhenius plot was 95 $kJ \cdot mol^{-1}$. For the experimental condition used here, predicted $t_{1/2}$ and $t_{90\%}$ at 30 °C were 144.2 and 21.9 days, respectively. In addition the long-term stability study of the partially purified extract stored at ambient temperature (30±2 °C) and protected from light for 21 days contained 88.53±0.01 %pyridostemin remaining of the original amount which closed to the predicted shelf-life.

Normal sunlight or room light may cause substantial degradation of drug molecule. The energy from light radiation must be absorbed by the molecule to cause a photolytic reaction. Consequently, compounds such as aromatic hydrocarbons, heterocyclic analogs, aldehydes, ketones, etc. are most susceptible to photolysis (Guillory and Poust, 2002). The stability of partially purified extract stored in light and dark at ambient temperature (30±2 °C) conditions were studied. The result (Figure 19) showed that partially purified extract when kept in dark condition was significantly more stable than in the presence of light. This study indicated that partially purified extract should be kept at low temperature and protected from light to conserve the pyridostemin content. In term of application, this active agent does not leave long-lasting residues on plants due to the biodegradable property when exposed to sunlight. Therefore, more frequent application of natural pesticidal product may be needed to achieve better protection against pest. Moreover, the short lasting residue on plants is an advantage of using *S. curtisii* extract in pest control, since, it is safety to the consumers when applied to edible plants and decrease potential for groundwater contamination (Kenna, 1995).

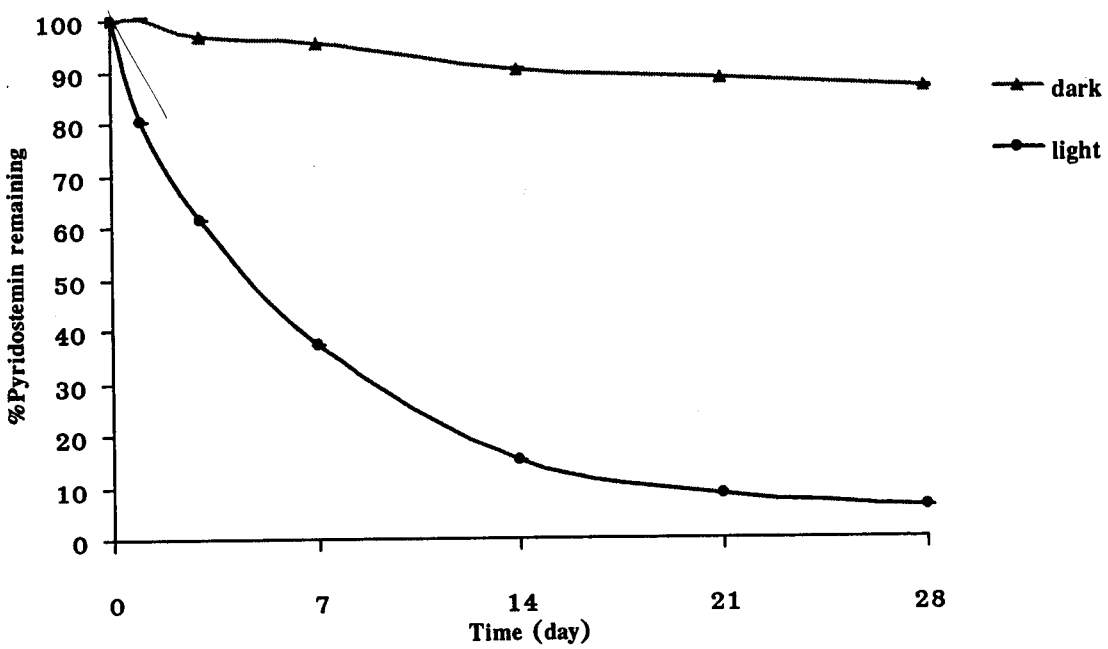

Figure 19. Pyridostemin remaining (%) when storage in light and dark at ambient temperature (30±2 °C) conditions (n = 3)

3.6 Formulation development of *S. curtisii* extract

Pesticides may be formulated into many usable forms for satisfactory storage, effective application, safety to the applicator and the environment, ease of application with readily available equipment, and economy (Kim *et al.*, 2003). In this present study, *S. curtisii* extract was formulated as pesticide in water dispersible granules and emulsifiable concentrate. Development of these formulations was described as follow.

3.6.1 Development of water dispersible granules containing *S. curtisii* extract

The partially purified *S. curtisii* extract was used as an active ingredient due to the high content of pyridostemin. The limitations of using partially purified extract in preparation of pesticidal formulations are the properties of pyridostemin, the active component such as water insolubility, instability under hydrolysis and oxidative conditions. The water dispersible granules are solid formulations that are suitable for natural compound with hydrolysis and oxidative labile. The granules are diluted with water and applied to plants with sprayer. Suspension of the active would be expected to form in the sprayer's tank. Moreover, the water dispersible granules have a number of advantages. The granules can be transferred easily during the mixing and loading process and reduce inhalation hazard to the applicator due to their large particle size (Martin *et al.*, 2004).

The compositions of water dispersible granule formulations were optimized to obtain a suitable formula with fast disintegration and low friability. Varied amounts of PVP K-30, sodium alginate, and lactose were used to prepare granules by wet granulation technique. The granules were evaluated for their physical properties such as friability and disintegration time and the result are displayed in Table 11.

Table 11. Compositions of blank water dispersible granules and their physical properties

Formula	Composition of granule (%w/w)			Disintegration time	Friability index
	PVP K-30	Sodium alginate	Lactose	(min)	(%)
1	-	2.5	97.5	1.42±0.11	94.50±0.51
2	-	5.0	95.0	1.73±0.08	91.29±0.79
3	-	7.5	92.5	1.76±0.16	76.81±0.26
4	2.5	-	97.5	0.55±0.07	95.20±0.11
5	2.5	2.5	95.0	0.46±0.06	93.42±0.81
6	2.5	5.0	92.5	0.99±0.06	95.08±0.25
7	2.5	7.5	90.0	1.71±0.14	94.82±0.44
8	5.0	-	95.0	0.48±0.03	97.88±0.20
9	5.0	2.5	92.5	0.51±0.10	96.33±0.27
10	5.0	5.0	90.0	0.55±0.01	97.43±0.09
11	5.0	7.5	87.5	1.40±0.17	96.30±0.54
12	7.5	-	92.5	0.44±0.02	98.21±0.70
13	7.5	2.5	90.0	0.90±0.09	98.25±0.57
14	7.5	5.0	87.5	1.00±0.07	98.24±0.18
15	7.5	7.5	85.0	1.57±0.19	97.86±0.17

The friability index and disintegration time were affected by amount of PVP K-30 and sodium alginate in the formulations. Disintegration time of granules was dominantly affected by sodium alginate. The granules containing higher amount of sodium alginate delayed for complete disintegration, while amount of PVP K-30 in the granules had less influence on disintegration time. Friability of granules was represented in friability index value. The friability index of granules increased with the addition of PVP K-30 which acts as binder. The short disintegration time (less than 40 seconds) and high friability index (more than 95%) granules were obtained from formula 4, 8, 9, 10, and 12. Nevertheless, viscosity of the obtained suspensions after diluted with water was concerned. In general, more viscosity could retard the settle of particles in suspensions. Therefore, the viscosity of 1 %w/v granules in water was also determined and the results are listed in Table 12.

Table 12. Viscosity of solution of 1 %w/v blank water dispersible granules in water

Formula	Composition of granule (%w/w)			Viscosity (cps)
	PVP K-30	Sodium alginate	Lactose	
4	2.5	-	97.5	0.93±0.2
8	5.0	-	95.0	1.20±0.0
9	5.0	2.5	92.5	1.47±0.1
10	5.0	5.0	90.0	1.67±0.1
12	7.5	-	92.5	0.93±0.1

The result clearly showed that the water dispersible granules which contained sodium alginate gave more viscous solution than the granules without sodium alginate because of its suspending and viscosity increasing properties (Budavari *et al.*, 1999; Rowe *et al.*, 2003). Therefore, the formula 9 and 10 were then chosen to formulate with *S. curtisii* extract and evaluated for their physical properties.

In preliminary study, the granule formulation was prepared using the crude extract, but the resulting damp mass was difficult to be pressed through the sieve no. 14 due to its hardness and stickiness. Moreover, the color of the resulting granules containing the crude extract was dark brown (Figure 20A).

(A) (B)

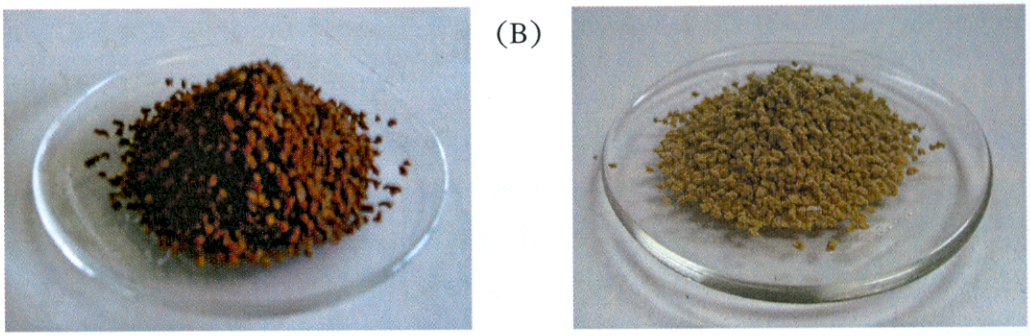

Figure 20. Appearance of water dispersible granules containing *S. curtisii* crude extract (A) and partially purified *S. curtisii* extract (B)

In contrast, the damp mass prepared by using partially purified extract was easy to sieve and resulting in pale brown granules (Figure 20B). These were also the reasons that the partially purified extract was more suitable for this type of formulation than the crude extract.

Furthermore, it was found that the *S. curtisii* extract was difficult to blend with other ingredients to obtain the uniform mixture and the resulting granules were incompletely dispersed in water. These problems could affect to the preparation process, uniformity of product, and may cause strainers and screens to plug. To solve these problems, the solid dispersion technique was applied for improving the solubility of the partially purified extract. The incorporation of poorly soluble drugs into water-soluble carrier in order to increase their solubility or their dissolution rate has been widely studied and extensively reviewed since the first description by Sekiguchi and Obi (1961). This technique provide an efficient method to improve the dissolution rate of drugs (Ford, 1986; Leuner and Dressman, 2000). In solid dispersion systems, a drug may exist as an amorphous form in polymeric carriers, and this may result in improved solubility and dissolution rates as compared with crystalline material (Kim *et al.*, 2006). Three methods of manufacturing solid dispersion are the melting or fusion method, the solvent method, and the melting-solvent method (Chiou and Riegelman, 1971). Macromolecule used to create a solid dispersion are divided into several groups including polyethyleneglycols (PEG) (Guyot *et al.*, 1995), cellulose derivatives such as hydroxypropylmethylcellulose (HPMC) (Kohri *et al.*, 1999; Okimoto *et al.*, 1997), carboxymethylethylcellulose (CMEC) (Kai *et al.*, 1996), and polyvinylpyrrolidones (PVP) (Tantishaiyakul *et al.*, 1996).

In this study, granules in formula 9 and 10 were prepared by wet granulation combined with the solvent method of the solid dispersion technique using PVP K-30 as a hydrophilic carrier. Amount of partially purified extract and PVP K-30 used in the preparation were fixed at the suitable ratio (7:5) for preparing water dispersible granules. In preparation process, partially purified extract was dissolved in dichloromethane and mixed with ethanolic solution of PVP K-30, the solvent was then removed by evaporation to give a solid mixture. After that the solid mixture was levigated by the wetting agent, Tween 80, for improving the coverage of formulation on plant and also the dissolution rate enhancement of partially purified extract via wetting effect of Tween (Veiga

et al., 1993). The mixture was mixed with other excipients to prepare granules by wet granulation method. The physical properties of water dispersible granules containing *S. curtisii* extract formula 9 and 10 were presented in Table 13.

Table 13. Comparison of physical properties of the water dispersible granules containing partially purified *S. curtisii* extract between formula 9 and 10

Physical property	**Formula 9**	**Formula 10**
Disintegration time (min)	3.62±0.24	2.88±0.27
Friability index (%)	99.26±0.45	96.75±1.35
Viscosity (cps) of 1 %w/v granules in water	1.16±0.2	1.6±0.2

Both formulas 9 and 10 showed good physical properties in term of short disintegration time and high friability index (>95 %), in this study, formula 10 was selected for further development and investigation due to its shorter disintegration time and higher viscosity.

The partially purified extract which contained pyridostemin in 71.40±5.51 %w/w was subjected to prepare the water dispersible granules. The pyridotemin content in the granules was determined by validated HPLC method which described in section 2.4.1. The resulting granules contained 2.85±0.11 %w/w pyridostemin which was lower than the equivalent amount added in the formulation (5 %w/w). It is possible that the formulation process could affect the stability of pyridostemin. The wet granulation technique comprised mixture moistening to form damp mass and heating to dry the granules. The two factors (moisture and heat) could promote the pyridostemin degradation.

The resulting granules prepared with partially purified extract content had good appearance with decreased odor when compared to those with the crude extract content. The granules were evaluated for their physical properties such as friability and flowability of granules (Table 14). The granules could be completely disintegrated in water within 3 minutes without any co-solvent. The disintegration time of granules and the physical properties of suspension obtained after dilution were represented in Table 15.

Table 14. Physical properties of water dispersible granules containing partially purified *S. curisii* extract

Physical property	Blank granules	Water dispersible granules containing extract
Friability index (%)	98.66±0.51	96.75±1.35
Angle of repose (°)	24.67±2.53	24.06±0.99

Table 15. Disintegration time of water dispersible granules containing partially purified *S. curisii* extract and the physical properties of suspension after dilution

Physical property	Blank granules	Water dispersible granules containing extract		
	1%w/v	1%w/v	2%w/v	3%w/v
Disintegration time (min)	2.97±0.04	2.88±0.27	2.68±0.16	2.99±0.15
pH	6.61±0.08	7.50±0.01	7.59±0.02	7.55±0.02
Viscosity (cps)	1.8±0.2	1.6±0.2	1.7±0.1	1.9±0.1

No significant difference was observed in friability index, angle of repose, disintegration time, and viscosity between blank granules and granules containing extract (p-value > 0.05). The pH values of suspension prepared from granules containing extract were significantly higher than that of blank granules due to the basicity of alkaloids. The physical evaluations of 1, 2, and 3 %w/v granules containing *S. curtisii* extract showed consistent properties. The high friability index indicated that the granules were less friable when handling or transportation. The excellent flow property was confirmed by the angle of repose which less than 30 degree (Carr, 1965).

A pale brown milky suspension was obtained after dilution with water (Figure 21A). The obtained suspension was stable more than 6 hours. After standing for 12 hours, the sediment was observed (Figure 21B), but it can be easily redispersed to uniform suspension. The physical properties of water dispersible granules containing partially purified *S. cutisii* extract demonstrated their potential for agricultural use.

(A) (B)

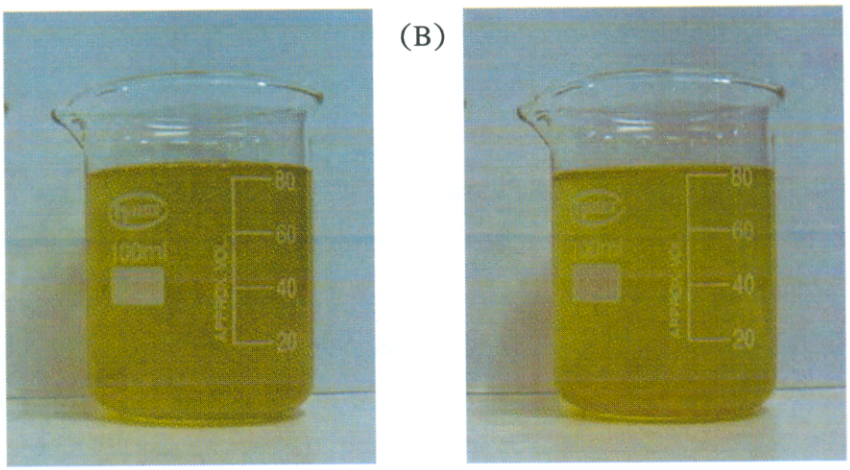

Figure 21. Appearance of suspension of 1 %w/v water dispersible granules containing partially purified *S. curtisii* extract after dilution (A) and stand for 12 hours (B)

3.6.2 Development of emulsifiable concentrate containing *S. curtisii* extract

Self-emulsifying systems have been used extensively in the chemical industry as carriers of concentrated herbicides and pesticides (Eaton, 1962; Hartley, 1967). The systems formed fine oil in water emulsions when introduced into aqueous phases under condition of gentle agitation (Charman *et al.*, 1992). The emulsifiable concentrate was chosen as a suitable formulation for *S. curtisii* extract. The extract was well miscible in the mixture of concentrated oil solution and emulsifier. The hydrolysis degradation of the active ingredient could be avoided due to the absence of water in the formulation. In addition, the oxidative degradation of the active ingredient could be reduced by addition of antioxidant. Furthermore, this concentrated liquid formulation was preferred when compared with emulsion formulation due to ease of handling and delivery.

The formulation compounding was started by ingredient selection. The formulation vehicle for a pesticide could have a significant impact on the stability and performance of the product. In this regard, the crop protection chemical market has seen significant changes in the past decade. The industry is active in developing better products with improved safety for the user, lowered impact on the environment, and more efficient use of products applied in the field (Montemurro *et al.*, 2002). Many different criteria

were applied for the selection of oil phase such as commercial availability, cost, stability, friendly to environment, and non toxic to applicators. The vegetable oils has been previously used to solubilized poorly water soluble drugs such as naproxen (Attama and Enete, 2004), danazol (Cuine *et al.*, 2007), seocalcitol (Grove *et al.*, 2006), and itraconazole (Hong *et al.*, 2006). Soybean oil and coconut oil are vegetable oils that met those criteria. Coconut oil is the most stable among other oils because it is oxidized slowly and thus resistant to rancidity (Devouard *et al.*, 2007a). However, coconut oil still has a limitation for example it consists of about 90% saturated fats and its solidification point is between 22 and 25 °C (Devouard *et al.*, 2007a). Thus, storing the developed emulsifiable concentrate at low temperature (4-6 °C) to preserve the active compound may affected to physical properties of the formulation. In contrast, this problem was not observed in soybean oil, since it consists of about 85% unsaturated fatty acids (Devouard *et al.*, 2007b). Moreover, this oil has been commonly use in pesticidal formulations and some studies showed effective insect repellent property (Barnard and Xue, 2004; Fradin and Day, 2002). The commercial product Bite Blocker® (Homs LLC) contains soybean oil as one active ingredient. Therefore, soybean oil was used as an oil phase in this study. However, soybean oil has a relatively high proportion of oxidation prone linolenic acid (7-10 %), which is an undesirable property (Devouard *et al.*, 2007b), thus, an antioxidant was required to reduce rancidity of the formulation. Tween 80 and Span 80 are nonionic surfactants which were chosen as emulsifiers since they are less toxic, less affected by pH, and help to avoid active ingredient-excipient incompatibles (Constatinides, 1995).

Emulsifiable concentrate formulations should be a clear monophasic liquid at ambient temperature. However, using inappropriate proportions of oil and emulsifiers may result in phase separation. In order to find appropriate formulas, which provide clear homogeneous and stable emulsifiable concentrate, the suitable compositions of oil and emulsifiers were determined by mixing soybean oil with Tween 80 and Span 80 in different proportions.

The result of this experiment showed that the mixtures of soybean oil and Span 80 gave clear solution in several ratios. The mixtures of soybean oil, Span 80, and Tween 80 were miscible in some proportions, while the mixtures of soybean oil and Tween 80 in the absence of Span 80 were immiscible. Since, Span 80 acted as a solvent to

encourage Tween 80 to mix well with soybean oil. The phase diagram was obtained (Figure 22) in which the black domain indicate clear homogeneous solution region.

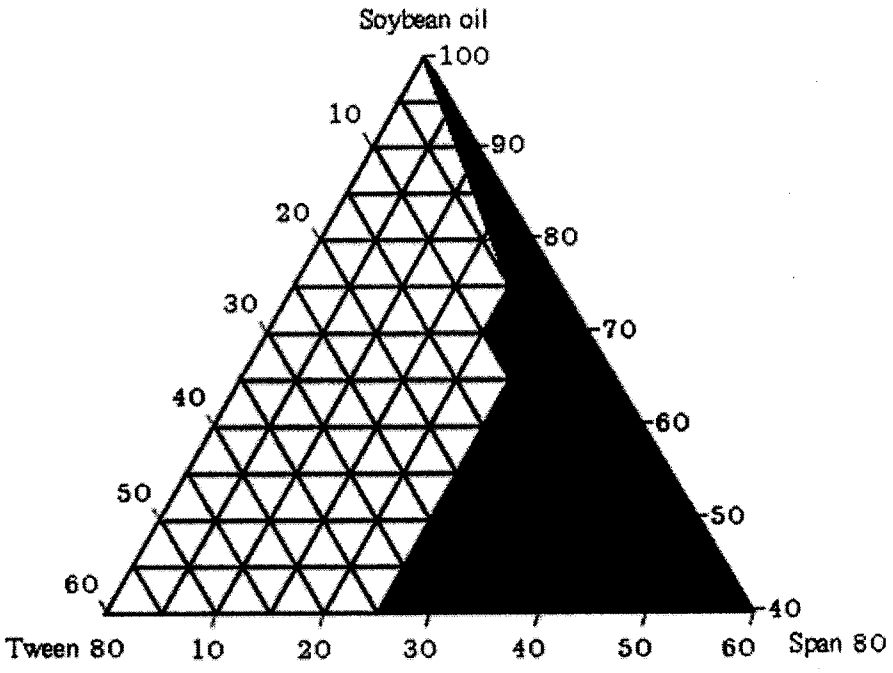

Figure 22. Ternary phase diagram of the mixtures consisting of soybean oil, Tween 80, and Span 80

The mixtures that gave clear homogenous solution was then dispersed in distilled water and observed for emulsion forming. The mixtures consists of soybean oil with both Span 80 and Tween 80 were found to form emulsion, while the mixtures which have only Span 80 could not form emulsion. The results indicated that emulsifiers, Span 80 and Tween 80 are necessary for the emulsifiable concentrate formulations. Moreover, the amounts of each emulsifier in the formulation were found to play an important role in the appearance of obtained emulsion after diluted with water. The resulting emulsions of the formulations comprised higher amount of Tween 80 exhibited more stable emulsions than those with lower amount of this surfactant. Since hydrophilic surfactant such as Tween 80 was an emulsifier which provided a fine and uniform emulsion droplets (Quan *et al.*, 2007). Thus, a large amount of Tween 80 was demanded for promote stable emulsion forming when diluted the emulsifiable concentrate in water.

The appropriate formulas which provided stable 1 %w/v emulsion after dilution of emulsifiable concentrate in water were shown in Table 16.

Table 16. Appropriate formula of emulsifiable concentrate

Formula	Soybean oil (%)	Emulsifiers (%)	
		Tween 80	Span 80
1	50	25	25
2	45	30	25
3	40	35	25

Three emulsifiable concentrate formulations which compose of Tween 80, Span 80, and soybean oil in different ratio were used in this study. For pesticidal formulation, 10 %w/w of soybean oil was replaced by *S. curtisii* extract. The obtained developed emulsifiable concentrates were tested for emulsion forming after dilution with water and only formula 3 gave uniform emulsion. The results from this experiment indicated that the extract required high amount of emulsifiers, especially Tween 80 to disperse oil phase in the large volume of water.

Therefore, the suitable formula for preparation of emulsifiable concentrate containing *S. curtisii* extract consist of Tween 80, Span 80, soybean oil, and *S. curtisii* extract in 35, 25, 29.9, 10 %w/w, respectively. BHT (0.1 %w/w) was used as antioxidant to protect pyridostemin from degradation and also prevent rancidity of soybean oil.

The *S. curtisii* extract which contained pyridostemin in 26.33±2.62 %w/w was subjected to prepare the emulsifiable concentrate. The resulting emulsifiable concentrate was determined for the pyridostemin content and found that the emulsifiable concentrate contained 2.50±0.11 %w/w pyridostemin. The loss of pyridostemin during preparation was not observed; hence the formulation process did not affect the stability of pyridostemin. The emulsifiable concentrate containing *S.curtisii* extract was obtained in dark brown thick viscous liquid (Figure 23).

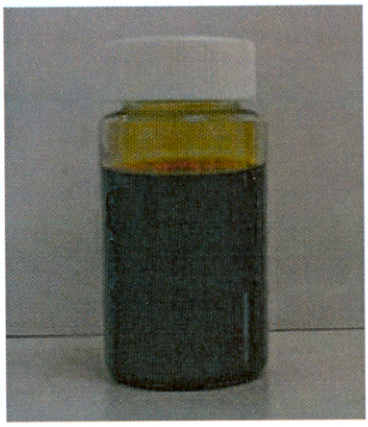

Figure 23. Appearance of emulsifiable concentrate containing *S. curtisii* extract

The emulsifiable concentrate could be completely dispersed in water within 30 seconds to obtain the pale brown milky emulsion (Figure 24A). The obtained emulsion was stable more than 6 hours. After standing for 12 hours, the obtained emulsion was seem to be slightly creaming (Figure 24B), but the emulsion can be easily redispersed to uniform emulsion after shaking.

(A) (B)

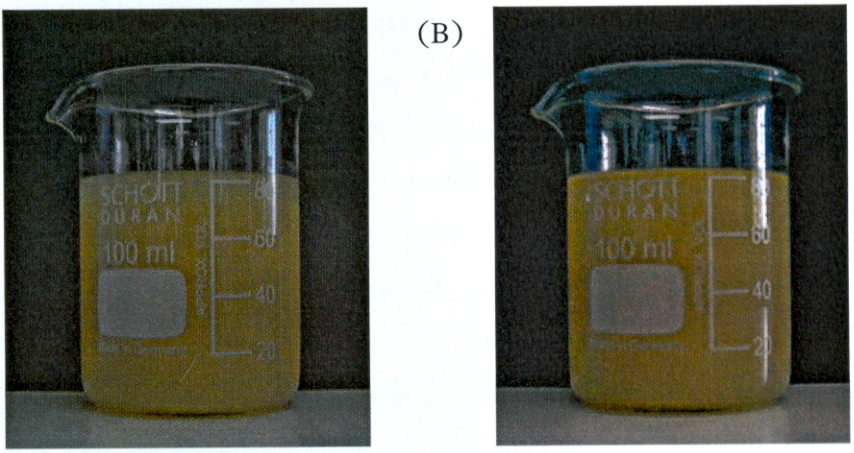

Figure 24. Appearance of emulsion of 1 %w/v emulsifiable concentrate containing *S. curtisii* extract after dilution (A) and stand for 12 hours (B)

Table 17 shows the physical properties of emulsion prepared from the developed emulsifiable concentrate containing *S. curtisii* extract. This formulation can be

diluted with various volume of water in the range of 1-3 %w/v to achieve the suitable concentration for crop pest control.

Table 17. Physical properties of emulsion prepared from emulsifiable concentrate containing *S. curtisii* extract

Physical properties	Blank emulsifiable concentrate	Emulsifiable concentrate containing extract		
	1 %w/v	1 %w/v	2 %w/v	3 %w/v
Mean particle size (μm)	4.20±0.14	1.87±0.12	2.01±0.09	1.97±0.04
pH	6.67±0.07	6.80±0.20	6.44±0.01	6.36±0.05
Viscosity (cps)	1.4±0.2	1.3±0.1	1.5±0.1	1.7±0.1

Mean droplet diameters of the obtained emulsions were less than 5 μm. The emulsifiable concentrate containing *S. curtisii* extract gave smaller mean droplet diameter than that of blank emulsifiable concentrate. The smaller mean inner phase diameter may caused by the crude extract which consisted of many components might affect to emulsion forming. The pH values of emulsion obtained from 1 %w/v blank emulsifiable concentrate and 1 %w/v emulsifiable concentrate containing *S. curtisii* extract were not different. However, the pH values tended to decrease with increasing the percent of emulsifiable concentrate from 1 %w/v to 2 and 3 %w/v, which was in contrast to the result obtained from water dispersible granules containing *S. curtisii* extract. This may be explained by the difference of chemical components of the extract used in each type of formulation. The water dispersible granules were formulated by partially purified extract, while the emulsifiable concentrate was formulated by the crude extract. The several components in the crude extract which removed by partially purification might affect pH values of the obtained emulsion. However, this range of pH would not damage the plant (A&L Canada laboratories, 2002) and the lower pH values gave benefit to the stability of the major compound which most labile in basic condition.

3.7 Stability of developed formulations

Stability testing is performed to ensure that products retain their full efficacy up to the end of their expiration date. The results obtained are used to ensure the quality, efficacy, and safety of the product (Grimm, 1993). This stability testing was determined based on their physicochemical properties.

3.7.1 Chemical stability of developed formulation containing *S. curtisii* extract

Since, the formula used in formulation of pesticidal products consists of a number of ingredients; therefore, it is possible that interaction among the ingredients may cause instability of the product. Different types of compounds have different types of degradation characteristic; hence, the degradation pattern of each ingredient in the mixture should be studied individually in ideal situation. This is, of course, difficult, time-consuming, and cost expensive to accomplish. Fortunately, it is unnecessary for purposes of stability prediction to determine the mechanism of degradation occurrence. In general, it is possible with chemical kinetic reaction orders, the temperature dependency of the degradation can be obtained (Lachman *et al.*, 1986). Therefore, these chemical stability studies were investigated in both water dispersible granules and emulsifiable concentrate using accelerated and real-time stability study.

3.7.1.1 Accelerated degradation of developed formulation containing *S. curtisii* extract

The accelerated stability tests of pyridostemin in water dispersible granules and emulsifiable concentrate were performed in the same conditions used for the partially purified extract investigation. The degradation of pyridostemin in these formulations was also fitted with the first-order kinetic, in which the linearity was best met when the logarithm of the percentage remaining amount of pyridostemin from each temperature were plot against storage time (Figures 25 and 26).

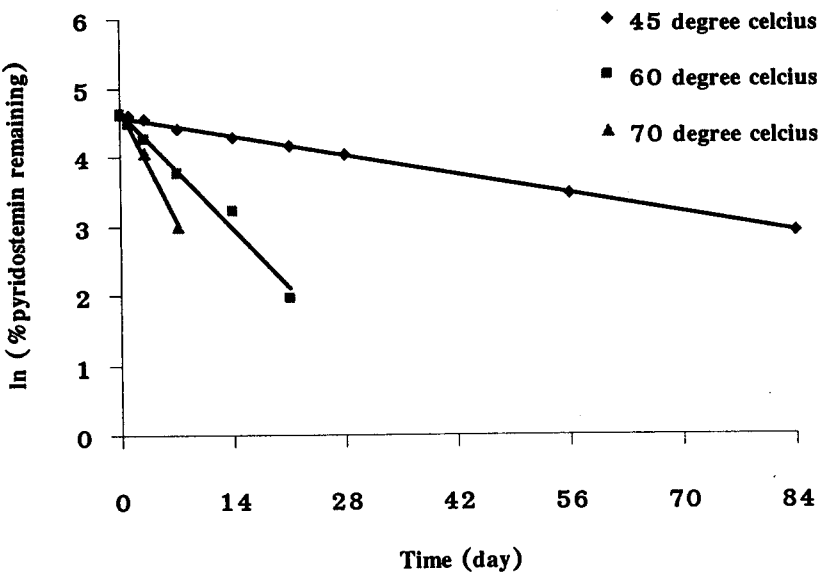

Figure 25. First-order plots of degradation of pyridostemin in water dispersible granules at 45, 60, and 70 °C (75 %RH). Rate equations of each temperature are expressed as $\ln c = 4.6847-0.0199t$, $r^2 = 0.9899$; $\ln c = 4.6339-0.1202t$, $r^2 = 0.9817$; and $\ln c = 4.5778-0.2353t$, $r^2 = 0.9979$, respectively.

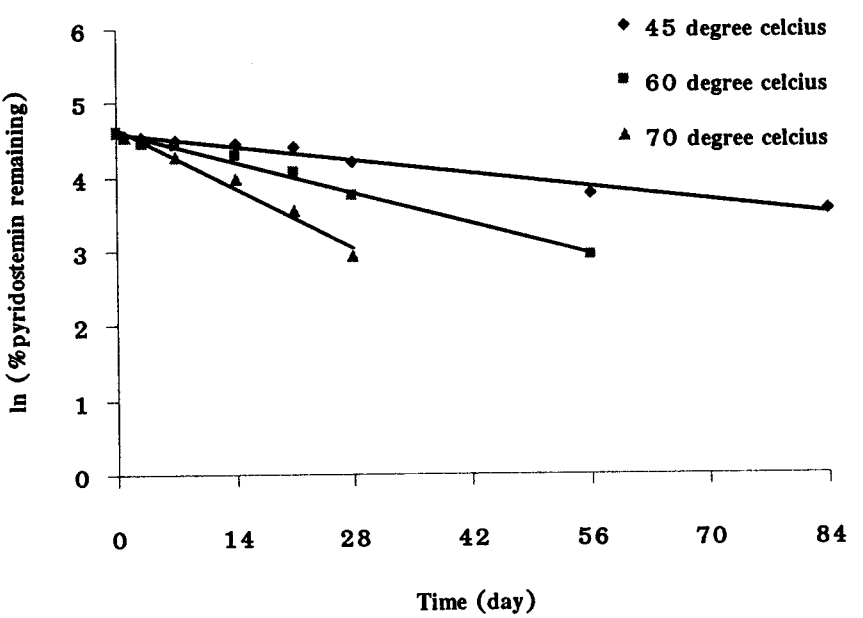

Figure 26. First-order plots of degradation of pyridostemin in emulsifiable concentrate at 45, 60, and 70 °C (75 %RH). Rate equations of each temperature are expressed as $\ln c = 4.6425-0.0130t$, $r^2 = 0.9819$; $\ln c = 4.6066-0.0295t$, $r^2 = 0.9898$; and $\ln c = 4.5876-0.0567t$, $r^2 = 0.9803$, respectively.

The stability rate constants for pyridostemin in water dispersible granules and emulsifiable concentrate were shown in Tables 18 and 19, respectively.

Table 18. Stability rate constants for pyridostemin in water dispersible granules

Storage temperature (°C), 75 %RH	ln c_0	Stability rate constant (day^{-1})	r^2
45	4.6847	0.0199	0.9899
60	4.6339	0.1202	0.9817
70	4.5778	0.2353	0.9979

Table 19. Stability rate constants for pyridostemin in emulsifiable concentrate

Storage temperature (°C), 75 %RH	ln c_0	Stability rate constant (day^{-1})	r^2
45	4.6425	0.0130	0.9819
60	4.6066	0.0295	0.9898
70	4.5876	0.0567	0.9803

The extrapolation of the Arrheniùs plots (Figure 27) obtained from a relation between ln k and reciprocal of absolute temperature led to estimated k at 30 °C ($k_{30°C}$) of 3.87×10^{-3} and 5.20×10^{-3} day^{-1} for pyridostemin in water dispersible granules and emulsifiable concentrate, respectively. The ln A and E_a calculated from the Arrhenius plots were shown in Table 20.

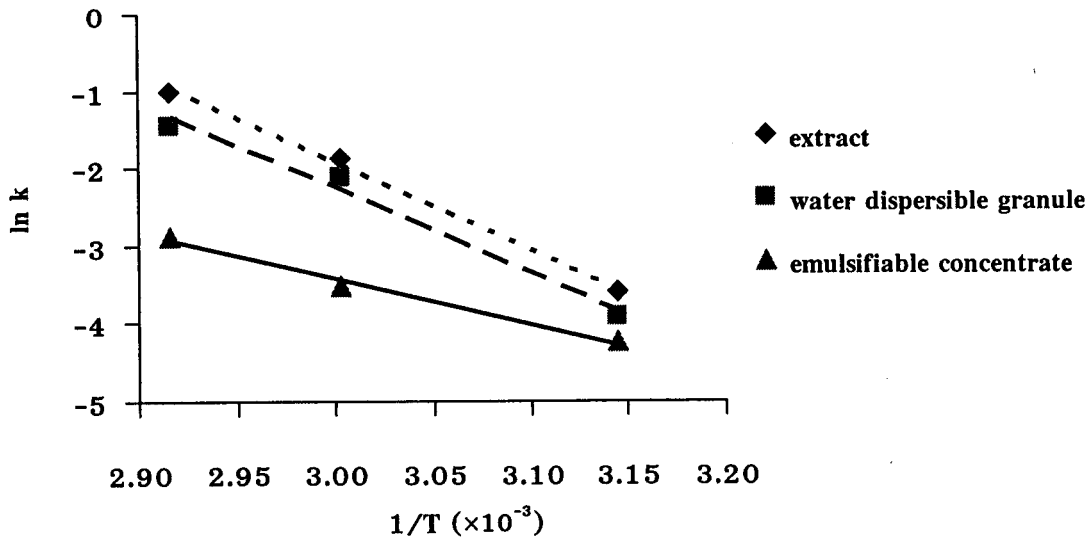

Figure 27. Arrhenius plots of pyridostemin in partially purified extract (♦), water dispersible granules (■), and emulsifiable concentrate (▲). The Arrhenius relation of pyridostemin in granules can be expressed as $\ln k = 30.617 - (10960/T)$; $r^2 = 0.9852$. The Arrhenius relation of pyridostemin in emulsifiable concentrate can be expressed as $\ln k = 15.649 - (6364.7/T)$; $r^2 = 0.9950$.

Table 20. Frequency factor (A) and activation energy (E_a) for pyridostemin calculated from Arrhenius equation

Sample	ln A (mol·day^{-1})	E_a (kJ·mol^{-1})	r^2
Extract	32.271	94.7	0.9966
Water dispersible granules	30.617	91.1	0.9852
Emulsifiable concentrate	15.649	49.3	0.9950

The activation energy (E_a) of a reaction is the amount of energy needed to start the reaction. It represents the minimum energy needed to form an activated complex during a collision between reactants. The E_a value of pyridostemin in partially purified extract was expressed in the same as that in the water dispersible granules. This result indicated that the degradation rate of pyridostemin in partially purified extract was similar to that in water dispersible granules. However, the frequency factor of the pyridostemin in water dispersible granules was less than that in partially purified extract. This showed the

granules may enhance the stability of this compound. While, the E_a value of pyridostemin in the emulsifiable concentrate was less than the E_a value in the extract and water dispersible granules. That indicated that the pyridostemin in emulsifiable concentrate was more sensitive to start the degradation than the other.

3.7.1.2 Real-time stability of developed formulation containing *S. curtisii* extract

The results from accelerated stability study at elevated temperature and humidity gave information on long term stability of the active component that can be performed in short period of time. However, care must be taken in the interpretation of the data as the increase in temperature and humidity could lead to change in physicochemical properties of the compound, producing a modification in the degradation mechanism (Yoshioka and Stella, 2000). In other words, accelerated conditions may not be truly predictive of the real time stability of the system for all cases (Fitzpatrick *et al.*, 2002). It is therefore, any accelerated stability study should be confirmed with real time data to ensure assumptions are valid (Aulton, 1998). In order to compare the stability study correlation between accelerated condition and at ambient temperature, sample sets of developed formulations containing *S. curtisii* extract were stored in glass container at ambient temperature (30±2 °C) and protected from light for 1 month and determined for pyridostemin remaining.

Table 21. Pyridostemin remaining (%) after storage for 1 month at ambient temperature and predicted $t_{90\%}$ of the developed formulations containing *S. curtisii* extract

Formulation	Pyridostemin remaining (%)	Predicted $t_{90\%}$ (day)
Water dispersible granules	71.94±0.04	27.2
Emulsifiable concentrate	89.69±0.12	19.1

Table 21 showed the estimated values from Arrhenius plots were not accordance to real-time information. The sample of water dispersible granules was found to contain 71.94±0.04 %pyridostemin remaining, which lower than estimated value from Arrhenius plot (89.7 %pyridostemin should be remained). In this case, the overestimate of

the rate of degradation from the rate actually observed at ambient temperature could be discussed by physicochemical property change under accelerated conditions (Rees, 1991; Sathivel *et al.*, 2008; Waterman and Adami, 2005).

The sample of emulsifiable concentrate was found to contain 89.69±0.12 %pyridostemin remaining, which higher than estimated value from Arrhenius plot (the predicted $t_{90\%}$ from Arrhenius plot when test samples were kept at 30 °C was 19.1 days). It is possible that the elevated temperature and humidity may enhance the rate of degradation process.

Unreliable Arrhenius equation in predicting pyridostemin in formulations indicated that the stability study of the developed formulations containing *S. curtisii* extract at ambient condition was required. It is known that prolonging the life time of formulated product could be achieved under storage at low temperature. However, the reduction of degradation rate of active compounds in some formulated products after storage at low temperature may not in accordance with the result from accelerated study (Rees, 1991; Savello and Shangraw, 1971).

In order to evaluate the significant of storing conditions of the developed formulations containing *S. curtisii* extract, two sets of samples were stored at 4 °C and at ambient temperature (30±2 °C) in glass containers protected from light. The samples were taken for analysis of the pyridostemin remaining at 0, 1, 2, and 3 months after storage. The results from this study (Figures 28 and 29) indicated that developed water dispersible granules and emulsifiable concentrate when storage at 4 °C could retain the pyridostemin content much better than storage at ambient temperature. After storage at ambient temperature for 3 months, the amount of pyridostemin remaining in the water dispersible granules decreased to 51.25±0.63%. However, storage the water dispersible granules containing *S. curtisii* extract at 4 °C for 3 months was shown to contain 97.98±0.74 %pyridostemin remaining. Therefore, the storage in the cold place (4 °C) is compulsory to conserve the pyridostemin content. Moreover, storage the emulsifiable concentrate containing *S. curtisii* extract at 4 °C for 3 months was shown to contain 94.69±1.05 %pyridostemin remaining. Therefore, the shelf-life of the emulsifiable concentrate

containing *S. curtisii* extract could be prolonged by kept in the cold place (4°C) as recommended storage condition.

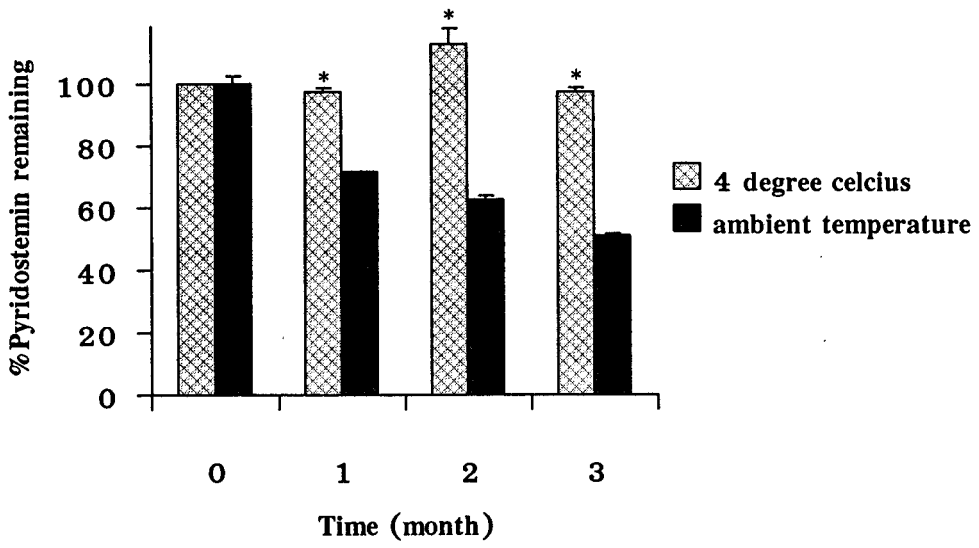

Figure 28. Pyridostemin remaining (%) in water dispersible granules after storage at 4 °C and ambient temperature for 3 months (*significant difference between storing at 4 °C and ambient temperature; p-value < 0.01)

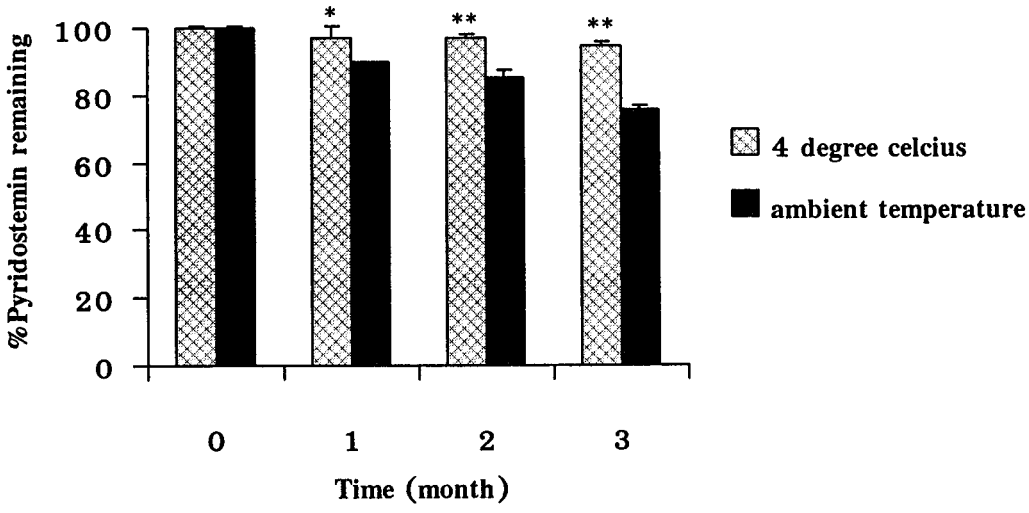

Figure 29. Pyridostemin remaining (%) in the developed emulsifiable concentrate after storage at 4 °C and ambient temperature for 3 months (*significant difference between storing at 4 °C and ambient temperature; p-value < 0.05 and **significant difference between storing at 4 °C and ambient temperature; p-value < 0.01)

To compare the product stability, the developed water dispersible granules and developed emulsifiable concentrate were kept at 4 °C and ambient temperature for 3 months. At 4 °C, no significant difference was found between %pyridostemin remaining in water dispersible granules and emulsifiable concentrate (p-value > 0.05). Whereas, at ambient temperature (Figure 30) %pyridostemin remaining in the developed emulsifiable concentrate was significantly higher than in the developed water dispersible granules (p-value < 0.01).

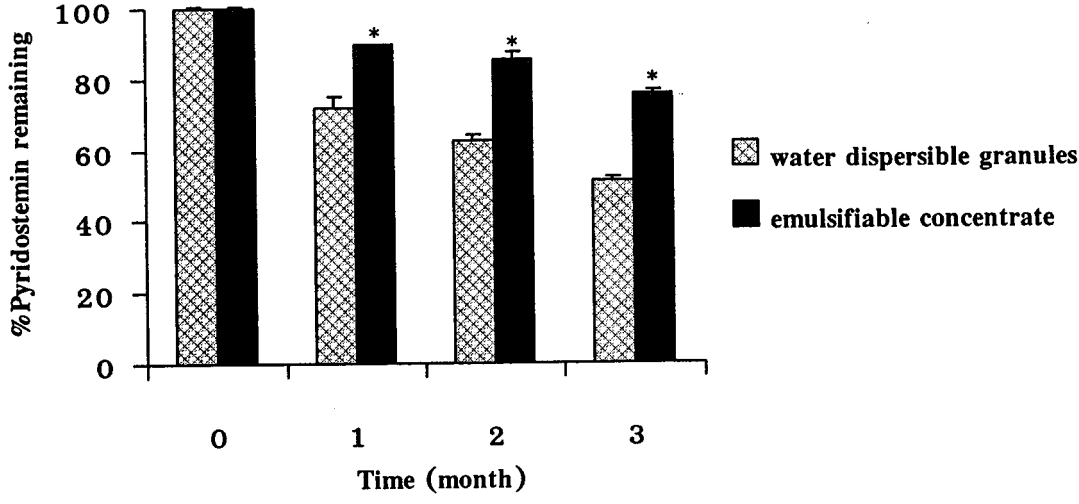

Figure 30. Pyridostemin remaining (%) in the developed water dispersible granules and the developed emulsifiable concentrate after storage at ambient temperature for 3 months (*significant difference between the water dispersible granules and emulsifiable concentrate; p-value < 0.01)

The emulsifiable concentrate could preserve the stability of pyridostemin better than water dispersible granules. After storage for 3 months, the amount of pyridostemin remaining in the emulsifiable concentrate decreased to 75.56±1.28%, while the amount of the same compound in the extract was 56.08±0.04% which showed significantly difference at p-value < 0.01. Since there was an antioxidant (BHT) added to formulation to prevent or reduce oxidation. Moreover, emulsifiers in the formulation (Tween 80 and Span 80) may assist the reduction in degradation of active compound by preventing hydrolysis of poorly water-soluble compound (Rees, 1991; Reigelman,

1960).The results of chemical stability studies demonstrated the suitability of the developed emulsifiable concentrate for the *S. curtisii* extract.

3.7.2 Physical stability of developed formulation containing *S. curtisii* extract

Most studies on drug stability have focused on not only the chemical stability of drug substances, but also the physical stability of formulations (Yoshioka and Stella, 2000). Since, those stabilities may have an effect on the quality and efficacy standpoint. Therefore, in the process of formulation development, determination of chemical and physical stability should be considered (Lachman *et al.*, 1986).

3.7.2.1 Physical stability of water dispersible granules containing *S. curtisii* extract

The water dispersible granules containing partially purified *S. curtisii* extract was developed by using solid dispersion technique which indeed, the major problem of solid dispersions is long-term stability (Kedzierewicz *et al.*, 1995). During storage of the formulation, phase separation of the components can occur. If these macromolecular rearrangements occur during storage, a variation of the mechanical and permeation properties of the materials can be observed (Lovrecich *et al.*, 1996; Sinko *et al.*, 1990). This process is known as physical aging (Guo *et al.*, 1991). The results of physical stability evaluation of this formulation especially disintegration time was required. The evaluations were performed every month for 3 months. The physical properties of water dispersible granules after storage at ambient temperature and 4 °C are shown in Tables 22 and 23, respectively.

Table 22. Physical properties of water dispersible granules containing partially purified *S. curtisii* extract after storage at ambient temperature

Physical property	Storage time (month)			
	0	1	2	3
Disintegration time (min)	2.88±0.27	3.09±0.11	2.79±0.11	2.97±0.22
pH	7.50±0.01	7.32±0.08	7.42±0.04	7.03±0.04
Viscosity (cps)	1.60±0.20	1.67±0.12	1.67±0.23	1.60±0.20
Friability index (%)	96.75±1.35	97.75±0.88	98.26±0.52	98.76±0.55
Angle of repose (°)	24.06±0.99	24.70±0.95	24.38±1.46	24.54±0.98

Table 23. Physical properties of the water dispersible granules containing partially purified *S. curtisii* extract after storage at 4 °C

Physical property	Storage time (month)			
	0	1	2	3
Disintegration time (min)	2.88±0.27	2.95±0.58	3.14±0.24	3.03±0.22
pH	7.50±0.01	7.36±0.05	7.26±0.01	7.30±0.02
Viscosity (cps)	1.60±0.20	1.80±0.20	1.67±0.12	1.80±0.20
Friability index (%)	96.75±1.35	98.64±0.52	98.82±0.24	98.99±0.29
Angle of repose (°)	24.06±0.99	23.91±0.73	24.06±1.11	24.38±0.55

The physical properties such as disintegration time, friability, flowability of granules, and viscosity of formulated suspension at different storage times were not significantly different, except pH values were tend to decrease during storage (p-value < 0.01). This may be caused by pyridostemin degradation during storage at ambient temperature which resulting in product that reduces pH of the formulation. It is worth to note that disintegration times of granules during storage at ambient temperature and 4 °C were not significantly changed (p-value > 0.05). The results from these experiments indicated that this formulation was not affected by physical aging of solid dispersion after 3 months of storage.

The uniform suspensions were obtained after dilution with water and were stable in 6 hour period. After 12 hours, the sediment was observed, but it could be easily redispersed to uniform suspensions.

3.7.2.2 Physical stability of emulsifiable concentrate containing *S. curtisii* extract

The emulsifiable concentrate containing *S. curtisii* extract was investigated for its physical stability. The appearance of emulsifiable concentrate did not change along period of 3 months storage. The emulsifiable concentrate was diluted to 1 %w/v in water and evaluated for particle size, viscosity, and pH every month for 3 months. The physical properties after storage at ambient temperature and 4 °C are demonstrated in Tables 24 and 25, respectively.

Table 24. Physical properties of the emulsifiable concentrate containing *S. curtisii* extract after storage at ambient temperature

Physical property	Storage time (month)			
	0	1	2	3
Mean particle size (μm)	1.87±0.12	1.94±0.11	2.68±0.16	2.85 ±0.25
pH	6.80±0.20	6.59±0.05	6.31±0.03	6.26±0.03
Viscosity (cps)	1.33±0.12	1.40±0.20	1.60±0.20	1.53±0.12

Table 25. Physical properties of emulsifiable concentrate containing *S. curtisii* extract after storage at 4 °C

Physical property	Storage time (month)			
	0	1	2	3
Mean particle size (μm)	1.87±0.12	1.93±0.08	2.20±0.05	2.05±0.01
pH	6.80±0.20	6.71±0.21	6.58±0.07	6.39±0.04
Viscosity (cps)	1.33±0.12	1.47±0.12	1.53±0.12	1.47±0.12

The slightly changes were observed during storage at ambient temperature such as mean particle size and pH of the diluted emulsion (p-value < 0.05). The mean droplet diameters of the sample were slightly increased, but not significantly different because of size distributions of individual samples. Figure 31 displays the oil droplet size ranges of resultant 1 %w/v emulsions at each time of storage.

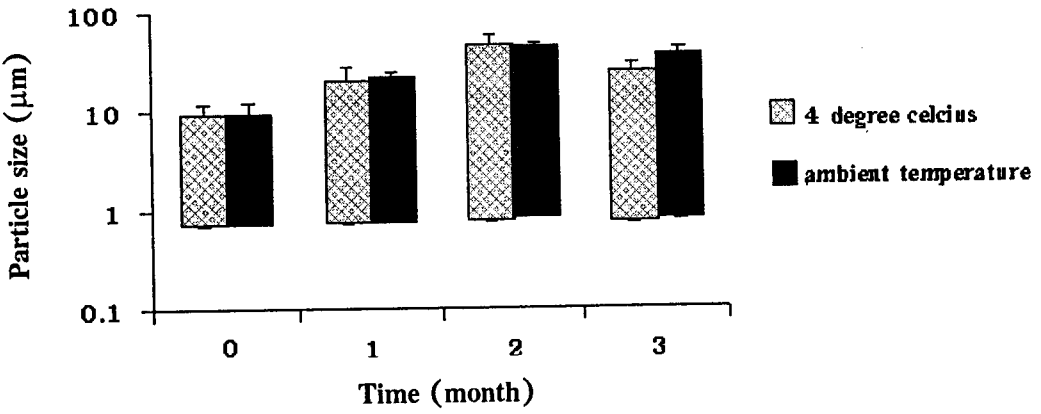

Figure 31. The oil droplet size between 10th and 90th percentile of resultant 1 %w/v emulsions from emulsifiable concentrate containing *S. curtisii* extract after storage at 4 °C and ambient temperature

The results showed the oil droplet sizes of 1 %w/v emulsion were not significantly different either prepared from the developed emulsifiable concentrate containing *S. curtisii* extract stored at 4 °C or from the sample stored at ambient temperature (p-value > 0.05) during storage. Therefore, the temperature change during storage did not affect the oil droplet size. The pH was slightly decreased during 3 months storage in the similar pattern of the water dispersible granules containing *S. curtisii* extract previously described in section 3.7.2.1. The decrease of pH might be attributed to the carboxylic acid formation after hydrolysis of the lactone ring of pyridostemin. The emulsifiable concentrate containing *S. curtisii* extract could be completely dispersed in water within a half of minute. The resultant emulsions were stable more than 6 hours and can be easily redispersed to uniform emulsion. The results indicated that this developed formulation was acceptable for the agricultural applications.