CHAPTER 4

RESULTS

1. Comparative study of five methods for α-amylase inhibitor extraction efficiency

Alpha-amylase inhibitor was extracted from the powder of *Parkia speciosa* Hassk. using five methods as described by Grant *et al*. (1995), Pueyo and Delgado-Salinas (1997), Giri and Kachole (1998) and Marshall and Lauda (1975), respectively. According to the method of Grant *et al*. (1995), the α-amylase inhibitor was extracted by either heating or non-heating of the samples as previously described (2.4). Concentration of the sample (mg/ml) that inhibited half of α-amylase activity (IC_{50}) was determined and compared using one-way ANOVA in the SPSS program (version 10.0). Scheffe's test was used to determine the differences between the groups, *p* value less than 0.05 was considered statistically significant. The concentration of crude extracts at IC_{50} was varied among the methods used. As shown in Table 2, the methods of Pueyo and Delgado-Salinas (1997) showed the highest potential in inhibiting half of the α-amylase activity (IC_{50} 1.1 mg powder/ml) because the amount of sample required was less than other methods, which were 1.6, 10.7, 54.6 and 65.8 mg of the powder/ml by methods of Marshall and Lauda (1975), Giri and Kachole (1998), and Grant *et al*. (1995) with heat or without heat, respectively. Although the method of Pueyo and Delgado-Salinas (1997) gave high potency of α-amylase inhibitory activity, it was not selected in this study. This was because it contained β-mercaptoethanol and PMSF which was not safe for future application in health. The method of Marshall and Lauda (1975) was the second efficient method that could extract the inhibitor, but it had no buffer capacity. The third efficient method of Giri and Kachole (1998) contained PVP, a chemical which is not good for health application.

The method of Grant *et al*. (1995) without heating was the chosen one for the extraction of the inhibitor from *Parkia sp*. pericarp powder in further studies. The

original method of Grant *et al*. (1995) (with heating) was not chosen since the resultant extract had less potency in inhibition when compared with the non heat method and also was not stable after 3 months storage.

2. Stability studies of α-amylase inhibitor in crude extract.

Stability of α-amylase inhibitory activity in the crude extracts prepared by five methods was studied by comparing the IC_{50} values determined after the extraction processes with those obtained after three months storage at -20 °C. Table 3 showed that α-amylase inhibitory activity in the crude extract prepared by methods of Pueyo and Delgado-Salinas (1997), Giri and Kachole (1998), and Marshall and Lauda (1975) was more stable than method of Grant *et al*. (1995).

Keeping at -20 °C for 3 months caused no effect in the potency in inhibiting half of α-amylase activity as shown by non significant difference in the concentration at IC_{50} used for the inhibition. However, storage of extract prepared by the method of Grant *et al*. (1995), in which the extract had been heated, showed significant change in the concentration at IC_{50} (Table 3).

Specific inhibitory activity during storage did not change much in all methods. This may be due to degradation of the inhibitory activity was related to the decrease amount of protein.

Table 2. Amount of *Parkia speciosa* powder extracted by 5 different methods for 50% inhibition of α-amylase activity

Extraction methods	IC_{50}	Inhibitory activity at IC_{50}	mg Protein at IC_{50}	Specific inhibitory activity
1. Pueyo and Delgado-Salinas (1997)	1.1 ± 0.1[a]	0.139 ± 0.004	0.004 ± 0.001	34.75 ± 3.35[a]
2. Grant *et al*. (1995) without heat	54.6 ± 0.5[b]	0.139 ± 0.004	0.052 ± 0.002	2.67 ± 0.09[b]
3. Grant *et al*. (1995) with heat	65.8 ± 3.1[c]	0.140 ± 0.006	0.074 ± 0.010	1.85 ± 0.25[b]
4. Giri and Kachole (1998)	10.7 ± 0.5[d]	0.142 ± 0.007	0.008 ± 0.001	17.75 ± 1.47[c]
5. Marshall and Lauda (1975)	1.6 ± 0.1[a]	0.145 ± 0.001	0.004 ± 0.000	36.25 ± 1.76[a]

Data were expressed as mean ± standard error from 4 replicates extracts. Each method was statistically significant difference at *p*-value < 0.05 by Scheffe test. Different characters (a, b, c and d) show significant difference among the comparing mean : IC_{50} (mg *P. speciosa* powder/ ml at 50 % inhibition), Inhibitory activity (mg maltose disappear / 0.1ml at 3' 37^{o}C), Specific inhibitory activity (mg maltose disappear / 0.1ml at 3' 37^{o}C / mg protein)

Table 3. Stability of α-amylase inhibitor in the crude extracts after three months storage at -20°C

Methods of extraction	Month	IC_{50}	Inhibitory activity at IC_{50}	mg protein at IC_{50}	Specific inhibitory activity
1. Pueyo and Delgado- Salinas (1997)	1	1.1 ± 0.1	0.139 ± 0.004	0.004 ± 0.001	34.75 ± 3.35
	3	1.2 ± 0.2	0.138 ± 0.003	0.004 ± 0.000	34.50 ± 2.31
2. Grant *et al*. (1995) without heat	1	54.6 ± 0.5 *	0.139 ± 0.004	0.052 ± 0.002	2.67 ± 0.09
	3	59.4 ± 0.7	0.138 ± 0.003	0.055 ± 0.002	2.51 ± 0.15
3. Grant *et al*. (1995) heat	1	65.8 ± 3.1 *	0.140 ± 0.006	0.074 ± 0.010	1.85 ± 0.25
	3	96.2 ± 0.0	0.138 ± 0.003	0.103 ± 0.011	1.34 ± 0.20
4. Giri and Kachole (1998)	1	10.7 ± 0.5	0.142 ± 0.007	0.008 ± 0.001	17.75 ± 1.47
	3	12.5 ± 0.7	0.138 ± 0.003	0.009 ± 0.001	15.33 ± 1.44
5. Marshall and Lauda (1975)	1	1.6 ± 0.1	0.145 ± 0.001	0.004 ± 0.000	36.25 ± 1.76
	3	1.7 ± 0.3	0.138 ± 0.003	0.004 ± 0.000	34.45 ± 0.80

Data were expressed as mean ± standard error from 4 replicate extracts. IC_{50} of each extract was significantly difference at $*p < 0.05$ by Independent t-test ; IC_{50} (mg *P. speciosa* powder/ ml at 50% inhibition), inhibitory activity at IC_{50} (mg maltose disappear / 0.1ml at 3' 37°C), specific inhibitory activity at IC_{50} (mg maltose disappear / 0.1ml at 3' 37°C / mg protein)

3. Precipitation of amylase inhibitor

Crude extract of α-amylase inhibitor prepared by the method of Grant *et al*. (1995), without heat was separately precipitated with various percentages of ammonium sulfate saturation, ethanol and methanol

3.1 Ammonium sulfate precipitation

In this study the crude extract were each added various percentages of ammonium sulfate saturation. The precipitates were collected and tested for their inhibitory activity against salivary α-amylase. Table 4 showed α-amylase inhibition of the precipitate at 0-80 % saturation, this result showed a non-significant difference of the inhibition (*p*-value >0.05) amongst the percentage used.

3.2 Ethanol precipitation

The crude extract of α-amylase inhibitor was each added various percentages (%) of ethanol and centrifuged to separate precipitate and supernatant. It was found that ethanol below 60% showed no effect in precipitating any parts of the extracts. Precipitate of the extract was observed in the addition of ethanol above 60%. The redissolved precipitate and supernatant solutions were determined for their inhibitory activity, mg protein and specific inhibitory activity against salivary α-amylase. Table 5 revealed that the redissolved precipitate solution from any of % ethanol precipitation had its specific inhibitory activity higher than the supernatants significantly (*p*-value <0.05). The redissolved precipitate solution obtained from 95% ethanol precipitation gave the highest inhibitory activity (at 0.10 unit) while the redissolved precipitate solution obtained from 60% ethanol precipitation gave the least inhibitory activity (at 0.04 unit). Inhibitory activity of the supernatant from any of % ethanol precipitation showed a non significant difference in its inhibitory activity (*p*-value >0.05).

3.3 Methanol precipitation

The crude extract of α-amylase inhibitor was each added various % of methanol and precipitate and supernatant were collected separately after centrifugation. It was found that methanol below 60% showed no effect in precipitating any parts of the extracts. Precipitation of the extract was observed in the addition of methanol above 60%. The redissolved precipitate and supernatant solutions were determined for their inhibitory activity, mg protein and specific inhibitory activity against salivary α-amylase. Table 6 revealed that the redissolved precipitate solution from any % of methanol precipation had its specific inhibitory activity higher than the supernatants, significantly (*p*-value <0.05). The redissolved precipitate solution obtained from 95% methanol precipitation gave the highest inhibitory activity (at 0.20 unit) while the redissolved precipitate solution obtained from 60% methanol precipitation gave the least inhibitory activity (at 0.1 unit). However, the supernatant showed a non significant difference in its inhibitory activity (*p*-value >0.05) among various % methanol used for precipitation.

Table 4. Inhibitory activity of precipitate at different percentage of ammonium sulfate saturation

%Saturation of $AmSO_4$	Precipitate		
	Inhibitory activity	mg protein	Specific inhibitory activity
40	0.07 ± 0.001	0.005 ± 0.001	14.00 ± 0.93
45	0.12 ± 0.019	0.005 ± 0.001	24.00 ± 2.33
50	0.14 ± 0.011	0.006 ± 0.000	23.33 ± 0.67
60	0.07 ± 0.000	0.004 ± 0.000	17.50 ± 0.06
80	0.07 ± 0.001	0.004 ± 0.000	17.50 ± 0.17

Data are expressed as mean ± standard error from 3 replicate assays. Inhibitory activity at each of $\%AmSO_4$ saturation was significantly different at $p > 0.05$ by ANOVA. Inhibitory activity is the amount of the inhibitor that inhibits one unit of α-amylase activity under assay condition (mg maltose disappear / 0.1ml in 3 min at $37^{\circ}C$). Specific inhibitory activity is an inhibitory activity / mg protein.

Table 5. Inhibitory activity of precipitate and supernatant at each percentage of ethanol precipitation

%Ethanol	Precipitate			Supernatant		
	Inhibitory activity	mg protein	Specific inhibitory activity	Inhibitory activity	mg protein	Specific inhibitory activity
60	0.04 ± 0.008	0.0048 ± 0.001	8.33 ± 0.27	0.266 ± 0.000	0.227 ± 0.006	1.17 ± 0.05
80	0.07 ± 0.012	0.0061 ± 0.001	11.48 ± 0.03	0.264 ± 0.002	0.213 ± 0.012	1.24 ± 0.06
95	0.10 ± 0.014	0.0148 ± 0.001	6.76 ± 1.19	0.265 ± 0.002	0.212 ± 0.007	1.25 ± 0.08

Data are expressed as mean ± standard error from 3 replicates. Inhibitory activity of each ethanol percentage was significantly different at $p < 0.05$ for precipitate and $p > 0.05$ for supernatant by ANOVA. Inhibitory activity is the amount of the inhibitor that inhibits one unit of α-amylase activity under assay condition (mg maltose disappear in 3 min at 37^{o}C). Specific inhibitory activity is an inhibitory activity / mg protein.

Table 6. Inhibitory activity of precipitate and supernatant at each percentage of methanol precipitation

%Methanol	precipitate			supernatant		
	Inhibitory activity	mg protein	Specific inhibitory activity	Inhibitory activity	mg protein	Specific inhibitory activity
60	0.10 ± 0.015	0.006 ± 0.001	16.66 ± 1.08	0.265 ± 0.002	0.219 ± 0.01	1.21 ± 0.06
80	0.17 ± 0.002	0.017 ± 0.001	10.00 ± 1.55	0.266 ± 0.001	0.218 ± 0.01	1.22 ± 0.07
95	0.20 ± 0.004	0.024 ± 0.000	8.33 ± 0.12	0.259 ± 0.004	0.202 ± 0.01	1.28 ± 0.09

Data are expressed as mean ± standard error from 3 replicates. Inhibitory activity of each methanol percentage was significantly different at $p < 0.05$ for precipitate and $p > 0.05$ for supernatant by ANOVA. Inhibitory activity is the amount of the inhibitor that inhibits one unit of α-amylase activity under assay condition (mg maltose disappear in 3 min at 37^{o}C). Specific inhibitory activity is inhibitory unit/mg protein.

4. Partial purification of α-amylase inhibitor

4.1 Extraction of α-amylase inhibitor from fresh green pericarp of sataw

A 300 g of fresh green pericarp powder of sataw was weighed and extracted in 0.02 M phosphate buffer pH 6.9 containing 0.15 M NaCl 1,500 ml by the method of Grant *et al*. (1995) without heating. The obtained crude extract of 200 mg/ml was concentrated 15-fold by freeze drying. Crude extract and concentrated crude extract (3,000 mg/ml) gave an IC_{50} values against α-amylase at 2.301 mg/ml and 3.849 mg/ml respectively.

4.2 Precipitation of α-amylase inhibitor from crude extract

Amylase inhibitor was precipitated by adding 95%methanol to concentrated crude extract, and then precipitate and supernatant were collected separately. Precipitate was analyzed for its inhibitory activity and mg protein. Methanol was evaporated from the supernatant and the resultant solid was redissolved and subjected for the determination of its inhibitory activity and mg protein. The precipitate inhibited α-amylase with an IC_{50} value of 11.57 mg/ml, while the supernatant fraction inhibited α-amylase with an IC_{50} value of 7.95 mg/ml.

4.3 Partial purification of α-amylase inhibitor by gel filtration

After loading samples of amylase inhibitor on to Sephadex G-75 column, the column was eluted and each fraction was tested for its inhibitory activity. The elution profiles were shown in Figure 10 and 11 indicating both eluted protein and percentage inhibition peaks.

Figure 10 and 11 showed similar elution profiles of both OD_{280} and % inhibition against α-amylase activity. That is the fourth peak of OD_{280} also contained inhibitory activity.

Figure 12 showed the elution profile of the redissolved precipitate. This chromatogram revealed non of inhibitory peaks and other OD_{280} peaks. Lowry's determination (Lowry, 1951) of each elute fractions provided very low values. Pool fraction of the fourth peaks of crude extract and Aq 95%MeOH showed inhibitory activity at IC_{50} at 0.382 and 0.412 g *P. speciosa* powder /ml.

Figure 13 was TLC study. This figure showed that the fourth peak from Sephadex G 75 fraction of Aq 95%MeOH contained only one spot while that from the Aq 95%MeOH and the crude extract contained other components (peak 1-3). By this reason the pool fraction of Sephadex G-75 from Aq 95%MeOH was used in further studies.

Table 7 presents the summary of purification processes yield and purification fold. Precipitate from methanol 95 % gave specific inhibitory activity of the values 52.42, but with very low recovery at 17.30%. Aq 95% MeOH gave specific inhibitory activity of the values 22.53 with higher total activity in comparison to the precipitate.

After partial purification of the precipitate and Aq 95% MeOH through Sephadex G-75, there were no obtained values, OD_{280} and inhibitory activity for the precipitate as shown in Figure 12,. Aq 95%MeOH had values of specific inhibitory activity 2.641 with 0.107% recovery and 0.25 of purification fold (Table 7).

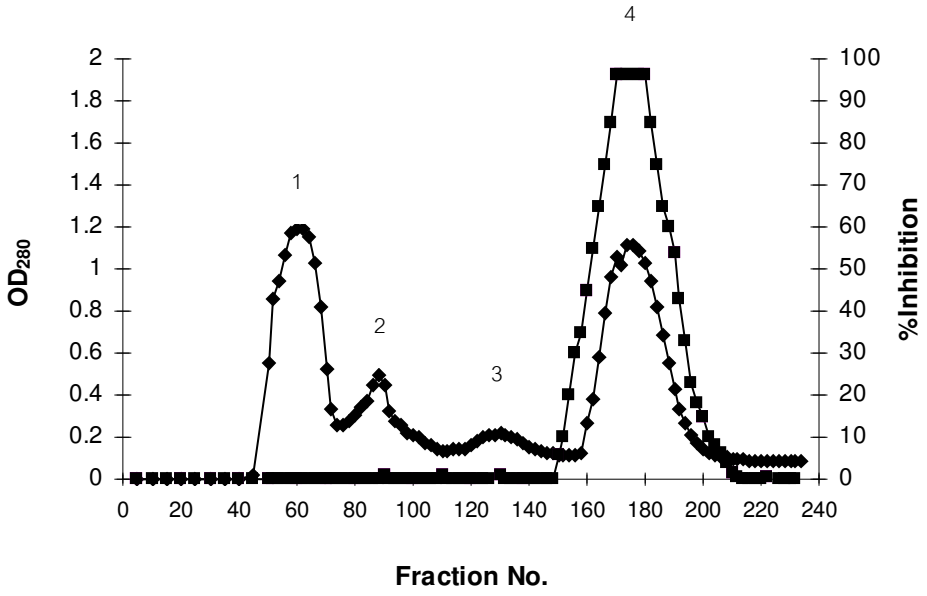


Figure 10. Sephadex G-75 column chromatography (117 x 0.6 cm column) of crude extract. Fractions were eluted with 0.02 M phosphate buffer pH 6.9 containing 0.01 M NaCl at flow rate 30 ml/hour. Diamonds, OD_{280} ; squares,% inhibition ; triangles, mg protein.

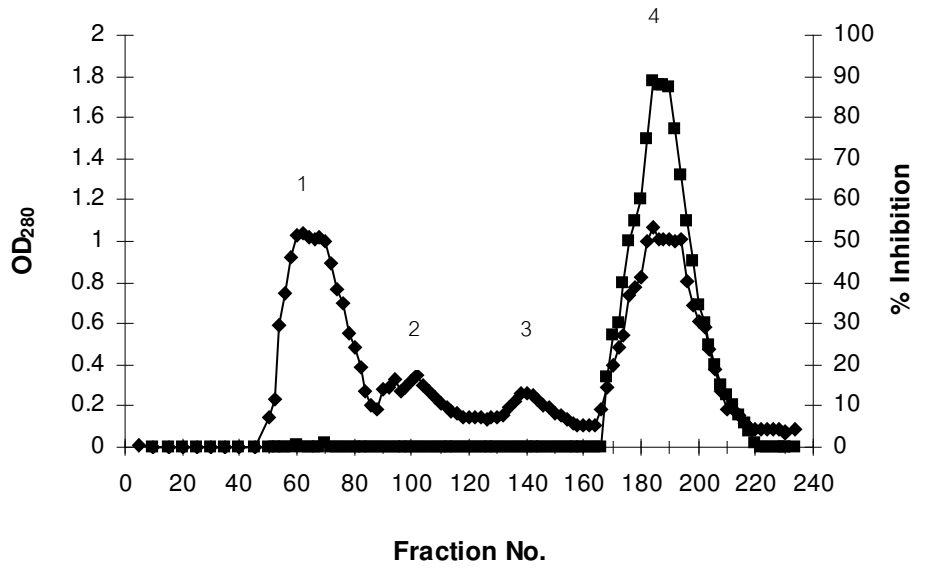


Figure 11. Sephadex G-75 column chromatography (117 x 0.6 cm column) of Aq 95% MeOH. Fractions were eluted with 0.02 M phosphate buffer pH 6.9 containing 0.01 M NaCl at flow rate 30 ml/hour. Diamonds, OD_{280} ; squares,% inhibition ; triangles, mg protein.

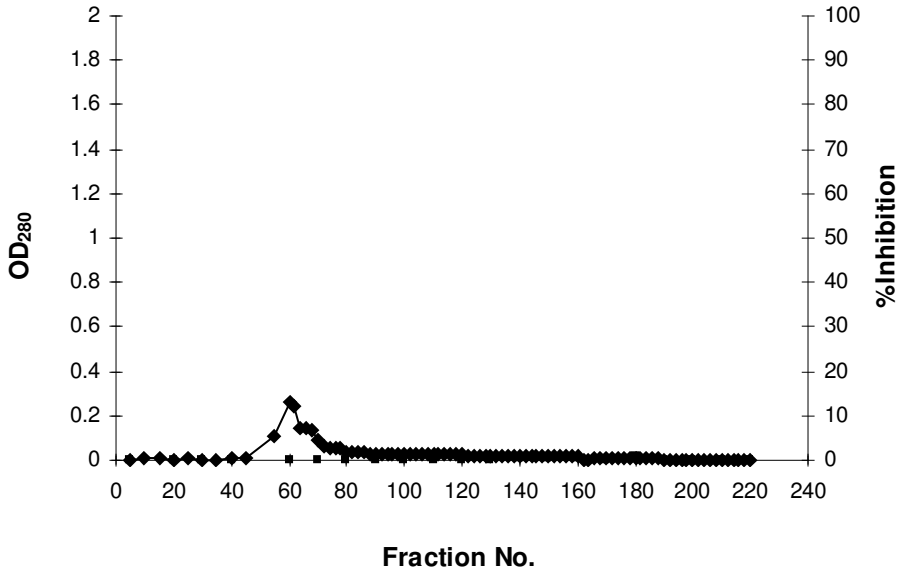


Figure 12. Sephadex G-75 column chromatography (117 x 0.6 cm column) of the redissolved precipitate solution. Fractions were eluted with 0.02 M phosphate buffer pH 6.9 containing 0.01 M NaCl at flow rate 30 ml/hour. Diamonds, OD_{280} ; squares,% inhibition ; triangles, mg protein.

Figure 13. Thin layer chromatography (TLC) of Aq 95%MeOH (lane 1) and Sephadex G-75 fraction (lane 2), the plate was developed with H_2O-Ethyl acetate-Glacial acid (0.5 : 5 : 1 v/v), (A) view under white light (B) under wavelength 366 nm

Table 7. Summary of purification of the α-amylase inhibitor from fresh green pericarp of sataw

Purification step	Total protein* (mg)	Total inhibitory activity*	Specific inhibitory activity	Recovery (%)	Purification (-fold)
crude extract	7299.6	189007.5	25.87	100	1
Methanol precipitation					
- precipitate	624	32,712	52.42	17.30	2.03
- Aq 95%MeOH	2,331.5	52,522.5	22.53	27.79	0.87
Sephadex G-75 chromatography					
- precipitate	-	-	-	-	-
- Aq 95%MeOH	373.3	985.7	2.641	0.107	0.52

Total inhibitory activity unit is mg maltose disappeared in 3 min at 37°C under assay condition; Specific inhibitory activity is an inhibitory activity / mg protein. *All presented values were calculated from the dilution that given IC_{50} multiplied with either protein or inhibitory activity of each purified step.

5. Property studies of amylase inhibitor

Several factors are able to modify the inhibitory activity of α-amylase inhibitor such as temperature, pH and salt (Giri and Kachole, 1998; Kluh, *et al*., 2005 Gibbs and Alli, 1998).

5.1 Effect of temperature on inhibitory activity of the inhibitor

Figure 14 shows effect of temperature on inhibitory activity of AI against α-amylase. Alpha-amylase activity without the inhibitor had constant activity (0.3 Unit) from 4°C to 37 °C and gradually decreased and ceases at 90°C. The addition of inhibitor either crude extract or Sephadex G-75 fraction reduced α-amylase activity from its initial value 0.3 Unit to 0.15 Units. These reductions were at constant level at the temperature range from 4 °C to 40 °C. Above 40 °C to 90°C the inhibitors decreased α-amylase activity in similar pattern to that of enzyme activity without the addition of inhibitors.

5.2 Stability of α-amylase inhibitor in various temperatures

Preincubation of the inhibitors either in the form of crude extract or Sephadex G-75 fraction for 30 min at 4°C, 25°C,30°C and 40°C did not effect on ability of AI in inhibiting α-amylase activity under normal assay condition (described previously) as shown in Figure 15. Preincubation of the inhibitors for 30 min at 50°C and 60°C caused reduction in ability of AI in inhibiting the enzyme activity. Preincubation of the inhibitors at 80°C and 90°C reduced ability of AI drastically as shown in the Figure 15.

5.3 Effect of pH on inhibitory activity of the inhibitor

Inhibitor in the form of crude extract and Sephadex G-75 fraction and enzyme were incubated together at 37°C for 30 min at various pH from pH 5.0, 6.0, 7.0, 8.0 and 9.0. After the solution were adjusted the pHs back to pH 6.9, substrate 0.2% starch solution were added for the assay of amylase activity. Figure 16 shows effect of pH on inhibitory activity of AI against α-amylase. Alpha-amylase activity without the inhibitor sharply increased from pH 5 to 6.9, maximal activity at pH 7.0, and gradually decreased to pH 9. Alpha-amylase inhibitory activity by crude extract or Sephadex G-75 fraction was increased from pH 5.0 to 6.9 with maximal inhibitory activity at pH 7 and gradually decreased from pH 8 to 9.

5.4 Stability of the α-amylase inhibitor in various pH

Preincubation of the inhibitors either in the form of crude extract or Sephadex G-75 fraction for 30 min at various pH, 5.0, 6.0 and 7.0 did not effect to inhibitory activity of inhibitor under normal assay condition as shown in Figure 16. Preincubation of the inhibitors for 30 min at pH 8.0 and 9.0 reduced ability of amylase inhibitor as shown in the Figure 16.

5.5 Effect of salt on inhibitory activity of the inhibitor

Preincubation of the inhibitors either in the form of crude extract or Sephadex G-75 fraction for 30 min in 0.015 M of various salts either sodium chloride, calcium chloride, potassium chloride or magnesium sulfate, had no effect on inhibitory activity of amylase inhibitor as shown in Figure 17.

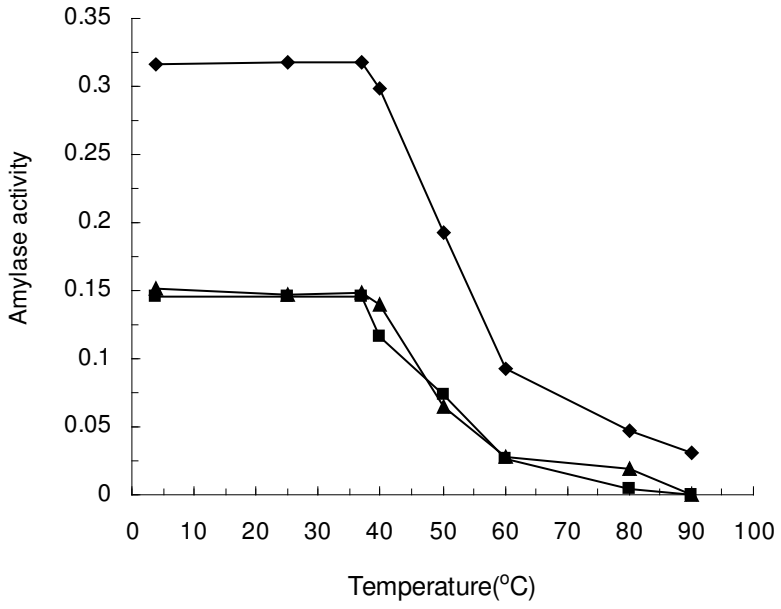


Figure 14. Effect of temperature on inhibitory activity of the inhibitor; Diamonds (α-amylase), squares (α-amylase + crude extract), triangles (α-amylase + Sephadex G-75 fraction), the data were obtained from three replicates.

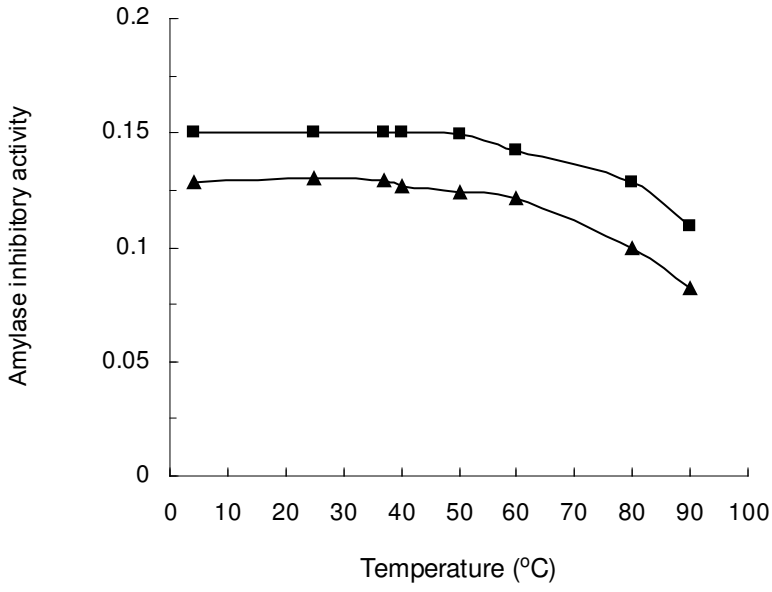


Figure 15. Stability of the inhibitor in various temperatures ; squares (α-amylase + crude extract), triangles (α-amylase + Sephadex G-75 fraction), the data were obtained from three replicats.

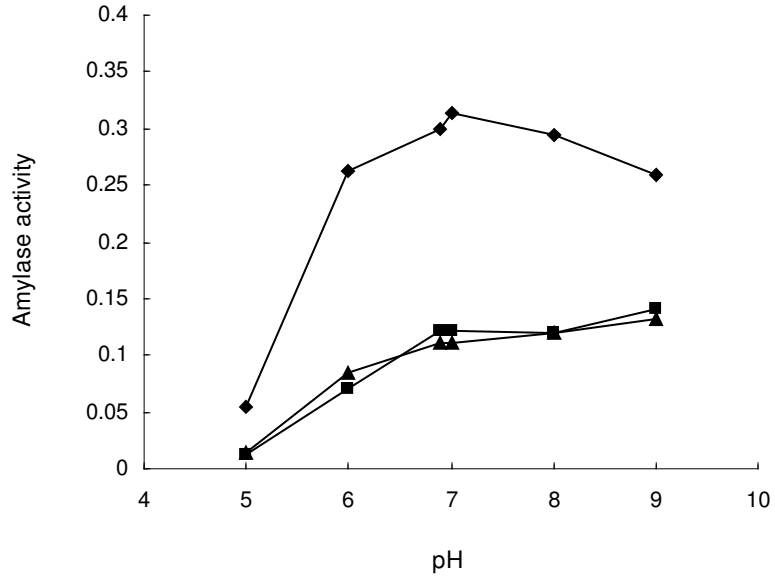


Figure 16. Effect of pH on inhibitory activity of the inhibitor; Diamonds (α-amylase), squares (α-amylase + crude extract), triangles (α-amylase + Sephadex G-75 fraction), the data were obtained from three replicates.

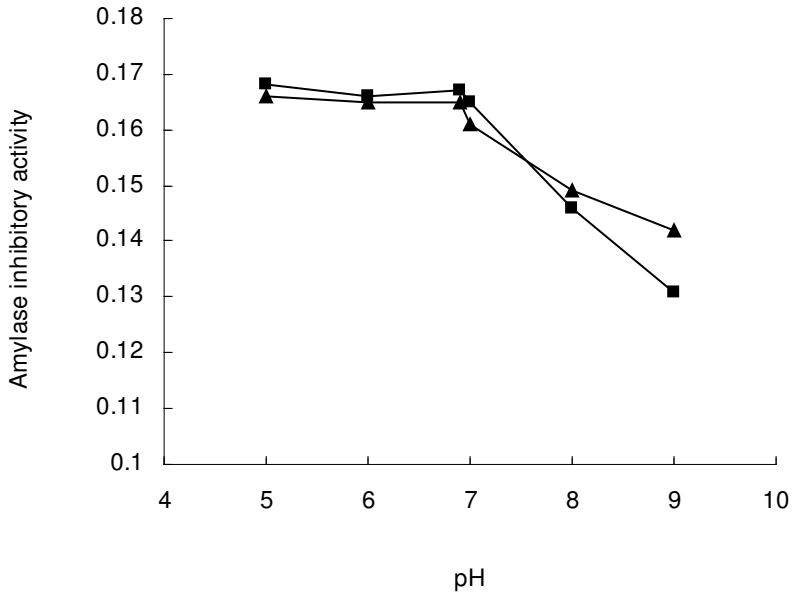


Figure 17. Stability of the inhibitor in various pH; squares (α-amylase + crude extract), triangles (α-amylase + Sephadex G-75 fraction), the data were obtained from three replicates.

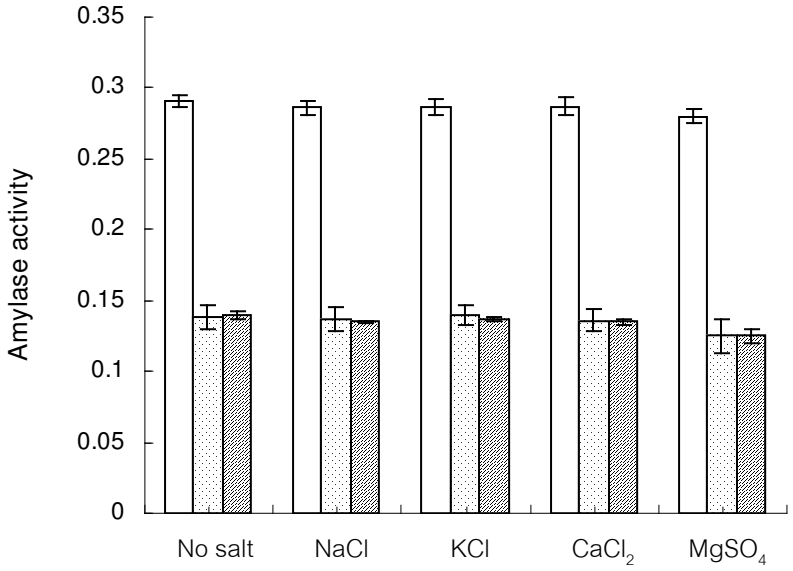


Figure 18. Effect of salt on inhibitory activity of the inhibitor; white bars (α-amylase), mark bars (α-amylase + crude extract), hatched bars (α-amylase + Sephadex G-75 fraction). Values were the average mean ± standard error of three replication.

6. Potential application of α-amylase inhibitor on blood glucose reduction *via* luminal enzymes *i.e.* pancreatic α-amylase, α-glucosidase and sucrase

Table 8 showed the IC_{50} values of several enzymes in the presence of crude extract or Sephadex G-75 fraction. Amylase inhibitor in crude extract have a high potent inhibitory activity on salivary α-amylase, yeast maltase, porcine pancreatic α-amylase, porcine intestinal maltase and yeast sucrase, respectively. Amylase inhibitor from Sephadex G-75 fraction have a high potency in inhibiting salivary α-amylase, yeast maltase, porcine pancreatic α-amylase, yeast sucrase and porcine intestinal maltase, respectively. In addition, crude extract showed higher potency in α-amylase inhibition than Sephadex G-75 fraction since its amount used was less. This result showed a potential application of the inhibitor in controlling blood glucose level *via* digestion of polysaccharides by α-amylase, maltose by α-glucosidase and sucrose by sucrase in the lumen.

Table 8. Potential application of α-amylase inhibitor on blood glucose reduction *via* luminal enzymes

Enzyme	IC_{50}	
	Crude extract	Sephadex G-75 fraction
Salivary α-amylase	2.301 ± 0.000	429 ± 12.00
Porcine pancreatic α-amylase	9.131 ± 0.095	1589 ± 128.00
Porcine intestinal maltase	14.06 ± 0.630	2392 ± 100.00 *
Yeast maltase	3.301 ± 0.118	401 ± 0.00
Yeast sucrase	88.945 ± 11.055	2650 ± 430.00

Data were expressed as mean ± standard error of duplicates. IC_{50} : mg *P. speciosa* pericarp powder/ml at 50% inhibition * The value was estimated from its IC_{25}.

Kinetics of the inhibitor was studied using Lineweaver-Burk plot. Initial velocity (ν) was determined at different substrate concentrations [S] in the presence and absence of a fixed inhibitor concentration. Figure 19 shows the Lineweaver-Burk plot for human salivary α-amylase (0.26U) inhibited by crude extract and Sephadex G-75 fraction. The plot gave straight lines with the intercept in the second quadrant at single point. The inhibition is therefore of the mixed noncompetitive type for both crude extract and Sephadex G-75 fraction with *Km* 3.377 mg/ml for α-amylase; K_i 29.286 mg/ml and K_i' 66.362 mg/ml for Sephadex G-75 fraction; and K_i 0.236 mg/ml and K_i' 0.5126 mg/ml for crude extract.

Figure 20 shows the Lineweaver-Burk plot for yeast sucrase (0.56 U) inhibited by crude extract and Sephadex G-75 fraction. The plot gave straight lines with the intercept in the third quadrant at single point. The inhibition is therefore of the mixed noncompetitive type with *Km* 21.555 mg/ml for sucrase; K_i 269.938 mg/ml and K_i'

124.616 mg/ml for Sephadex G-75 fraction; and K_i 21.783 mg/ml and K_i' 4.214 mg/ml for crude extract.

Figure 21 shows the Lineweaver-Burk plot for yeast maltase (0.56 U) inhibited by crude extract and Sephadex G-75 fraction. The plot gave straight lines with the intercept in the third quadrant at single point. The inhibition is therefore of the mixed noncompetitive type with *Km* 2.881 mg/ml for maltase; K_i 69.349 mg/ml and K_i' 47.495 mg/ml for Sephadex G-75 fraction; and K_i 0.538 mg/ml and K_i' 0.457 mg/ml for crude extract.

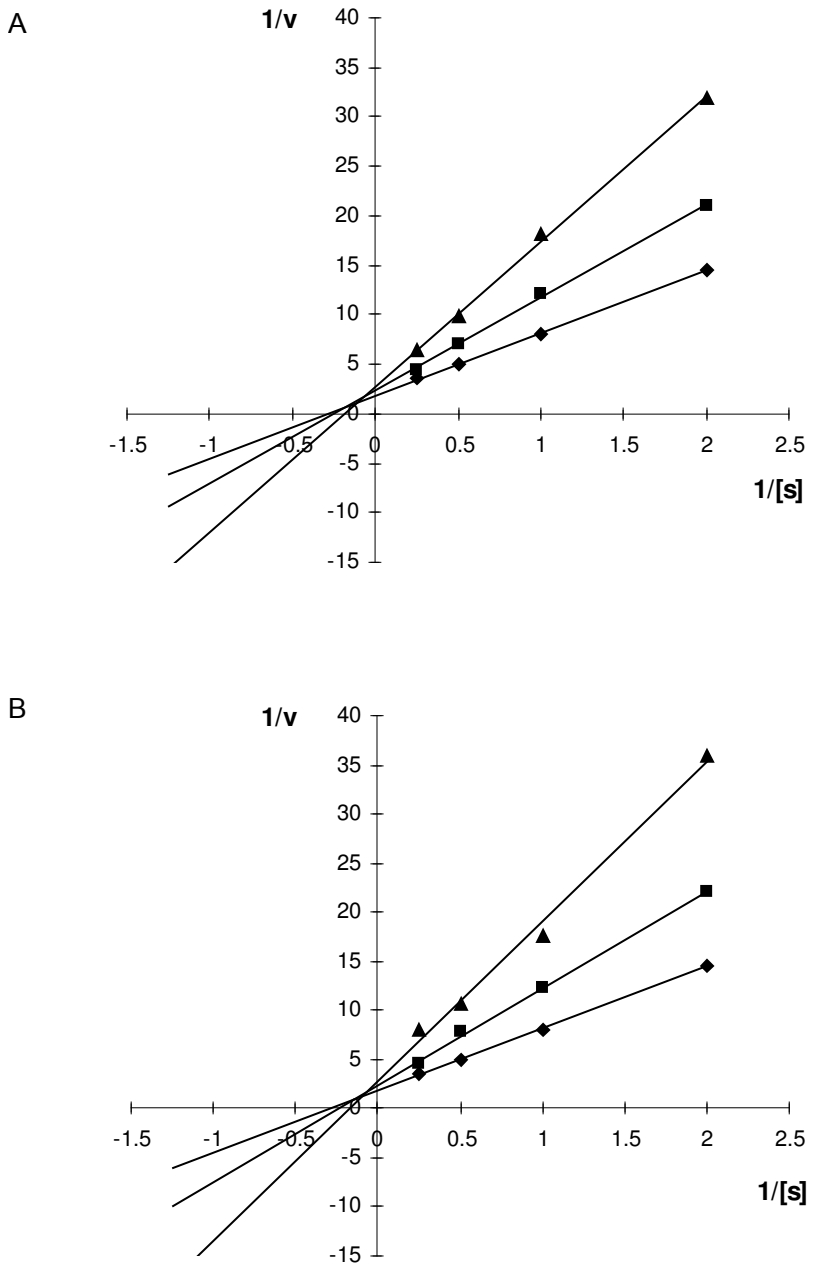


Figure 19. Lineweaver- Burk plot (1/[s] vs 1/v) for humam salivary α-amylase inhibited by crude extract (A) and Sephadex G-75 fraction (B): diamonds, no inhibition; squares, inhibitor at IC_{25} of crude extract and Sephadex G-75 fraction (0.147 or 19.4 mg *P. speciosa* pericarp powder/ml); triangles, inhibitor at IC_{50} of crude extract and Sephadex G-75 fraction (0.23 or 37.5 mg *P. speciosa* pericarp powder/ml); [s] mg starch/ml, [v] mg maltose/ml under assay condition

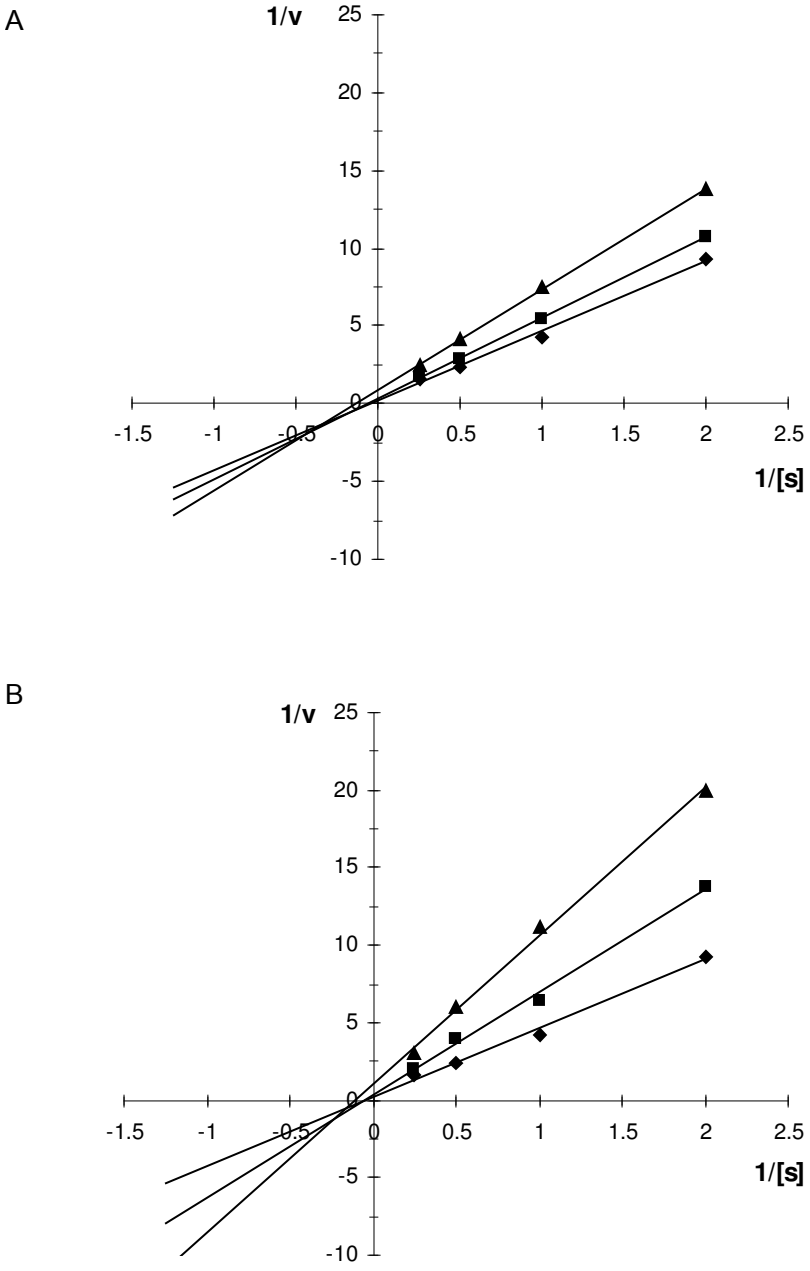


Figure 20. Lineweaver- Burk plot (1/[s] vs 1/v) for yeast sucrase inhibited by crude extract (A) Sephadex G-75 fraction (B) : diamonds, no inhibition ; squares, inhibitor at IC_{25} of crude extract and Sephadex G-75 fraction (3.95 or 105.4 mg *P. speciosa* pericarp powder/ml); triangles, inhibitor at IC_{50} of crude extract and Sephadex G-75 fraction (8.89 or 265.0 mg *P. speciosa* pericarp powder/ml); [s] mg sucrose/ml, [v] mg glucose /ml under assay condition.

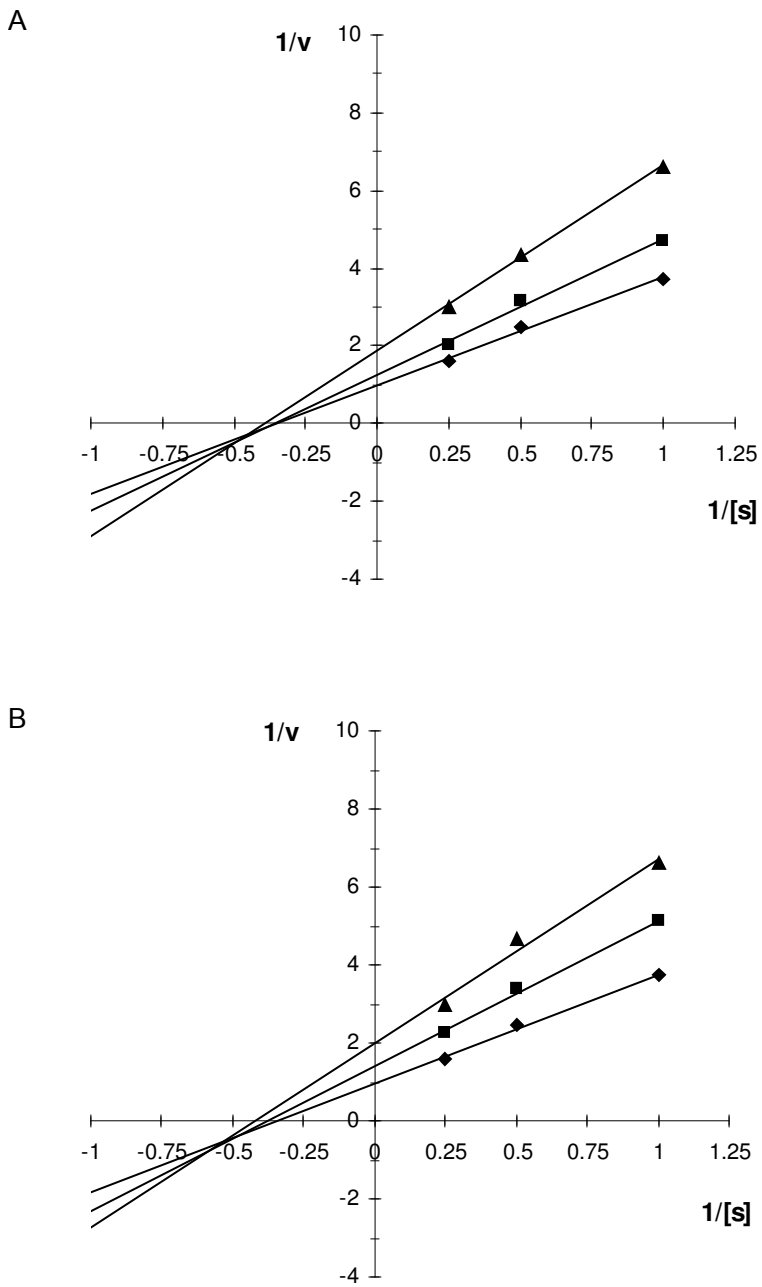


Figure 21. Lineweaver- Burk plot (1/[s] vs 1/v) for yeast maltase inhibited by crude extract (A) Sephadex G-75 fraction (B): diamonds, no inhibition; squares, inhibitor at IC_{25} of crude extract and Sephadex G-75 fraction (0.155 or 268 mg *P. speciosa* pericarp powder/ml); triangles, inhibitor at IC_{50} of crude extract and Sephadex G-75 fraction (0.33 or 40.1 *P. speciosa* pericarp powder/ml); [s] mg maltose/ml, [v] mg glucose /ml under assay condition.

7. Potential application of the inhibitor on pest control

7.1 Effect of the inhibitor on insect α-amylase activity

Inhibition of amylase extracted from three types of insect by crude extract and Sephadex G-75 fraction was presented in Figure 22 and 23. *S. orysea* amylase at 0.26, 0.31 and 0.065 activity units were inhibited by crude extract of sataw pericarp at 85.5%, 74.8% and 58.1% respectively while by Sephadex G-75 fraction were 62.4, 42.4 and 28.4%, accordingly. *C. chinensis* amylase was inhibited to 93.9%, 54% and 32.6 % of its original activity at 0.26, 0.31 and 0.065 respectively by crude extract of sataw pericarp but it was not inhibited by Sephadex G-75 fraction. In contrast amylase from *C. maculatus* was not inhibited by either crude extract or the purified inhibitor (Sephadex G-75 fraction). These results showed the specificity of inhibitors toward the amylases of different insect species.

7.2 Effect of the inhibitor on the growth of insect pests in artificial beans

The study on the effect of an inhibitor on the growth of insect pests (*C. maculatus* and *C. chinensis*) revealed that neither larvae nor adults emerged in artificial beans containing 0.8 mg of a crude extract or 0.105 mg of a Sephadex G-75 fraction per 1g of the bean (Figure 24). This result seems to indicate that the inhibitors did inhibit growth of the insect in the artificial beans. However, the control artificial bean containing buffer A showed a similar results to those obtained from the test samples, with no emerging larvae or insects. This may be due to the hardness of the beans which made its impossible for insects to obtain food. If this is a possible explaination, at this stage is thus not possible to interpret these results. Modification of the preparation of the artificial bean should be further investigated to obtain an artificial bean that is porous with a softor texture to allow for a better possibility for insects to obtain a food supply.

7.3 Effect of the inhibitor on the growth of insect pest in mungbean seeds

When mungbean seeds were coated with 0.8 mg of crude extract and 0.105 mg of Sephadex G-75 fraction, the adults of insect could emerge in equal numbers to those from the control group in which the bean seeds were coated with 0.02 M phosphate buffer containing 0.01 M NaCl and those normal bean seeds (Figure 25). This indicated that the test inhibitors had no effect in controlling the birth of those insect under the assay condition. Increasing the test sample concentration or improvement of the method of coating may show a positive effect, if it was to be tried in the future study.

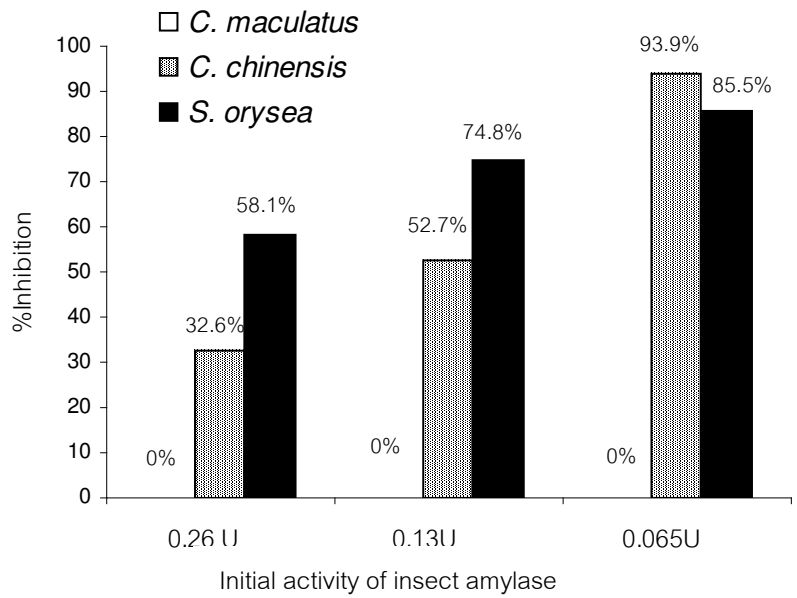


Figure 22. Activity of insect amylases after the addition of crude extract. Percentage (%) indicates percent inhibition of the inhibitor against insect amylase.

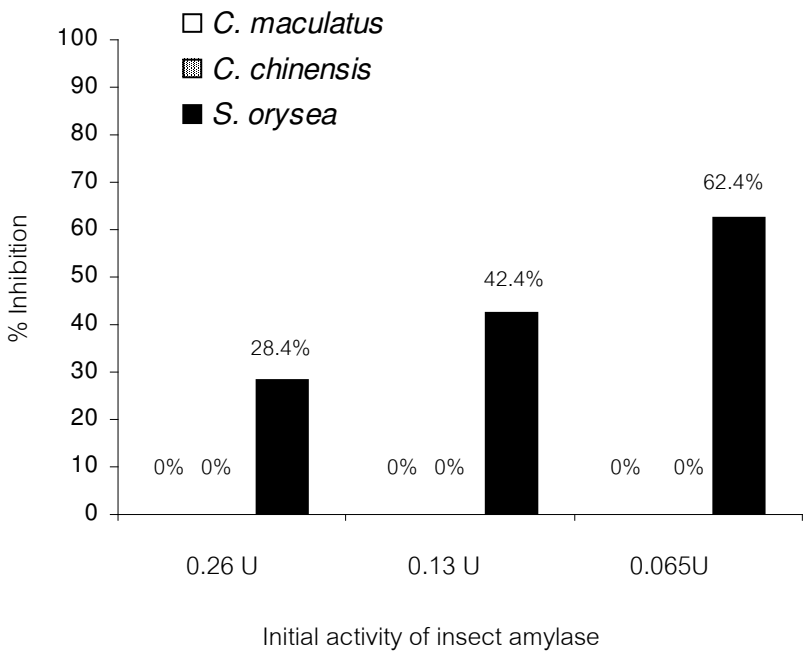


Figure 23. Activity of insect after the addition of Sephadex G-75 fraction. Percentage (%) indicates percent inhibition of the inhibitor against insect amylase.

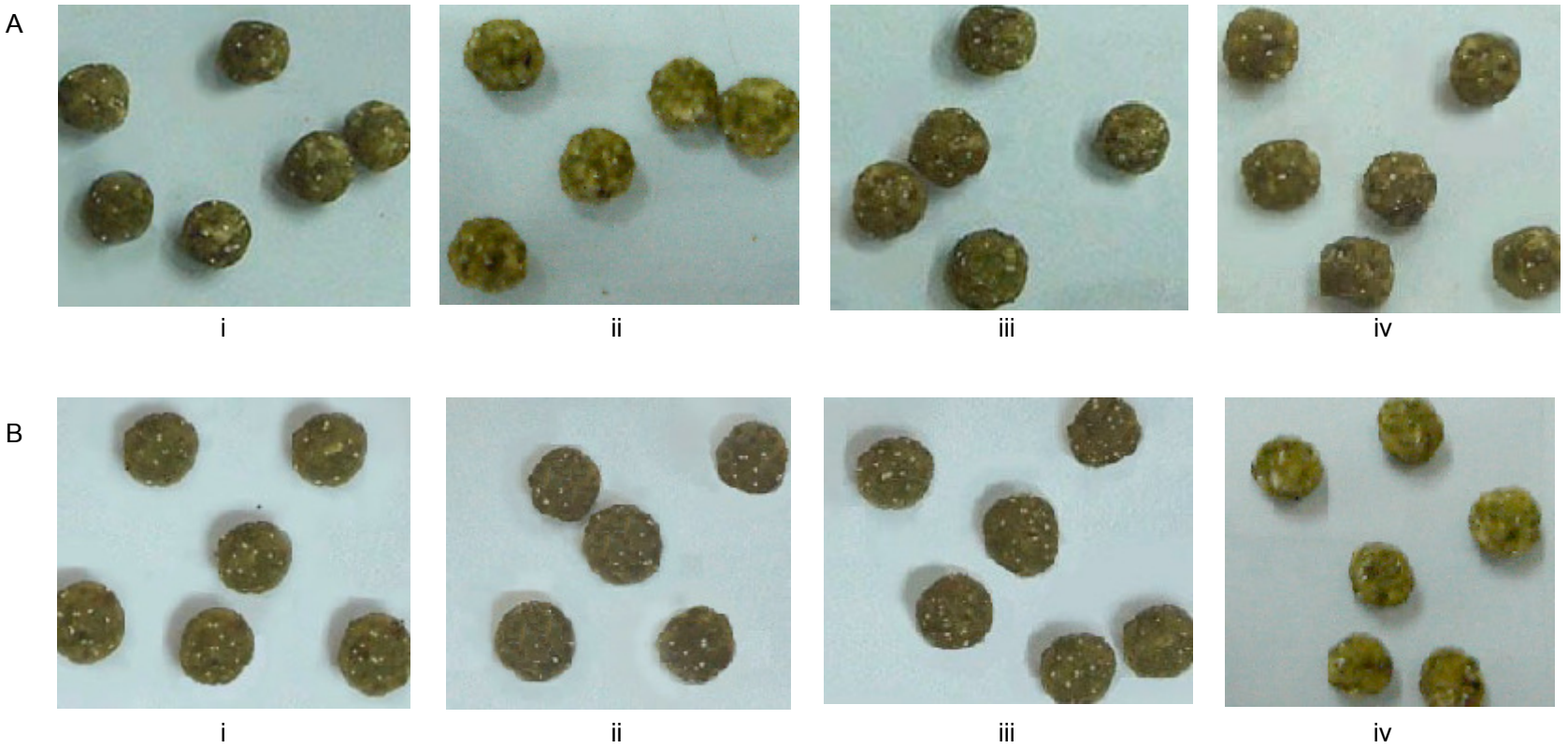


Figure 24. Effect of inhibitor on the growth of insect pest in artificial beans (A) after three days (B) after thirty five days; (i) artificial beans which containing distill water (ii) artificial beans which containing buffer A, (iii) artificial beans which containing crude extract, (iv) artificial beans which containing Sephadex G-75 fraction.

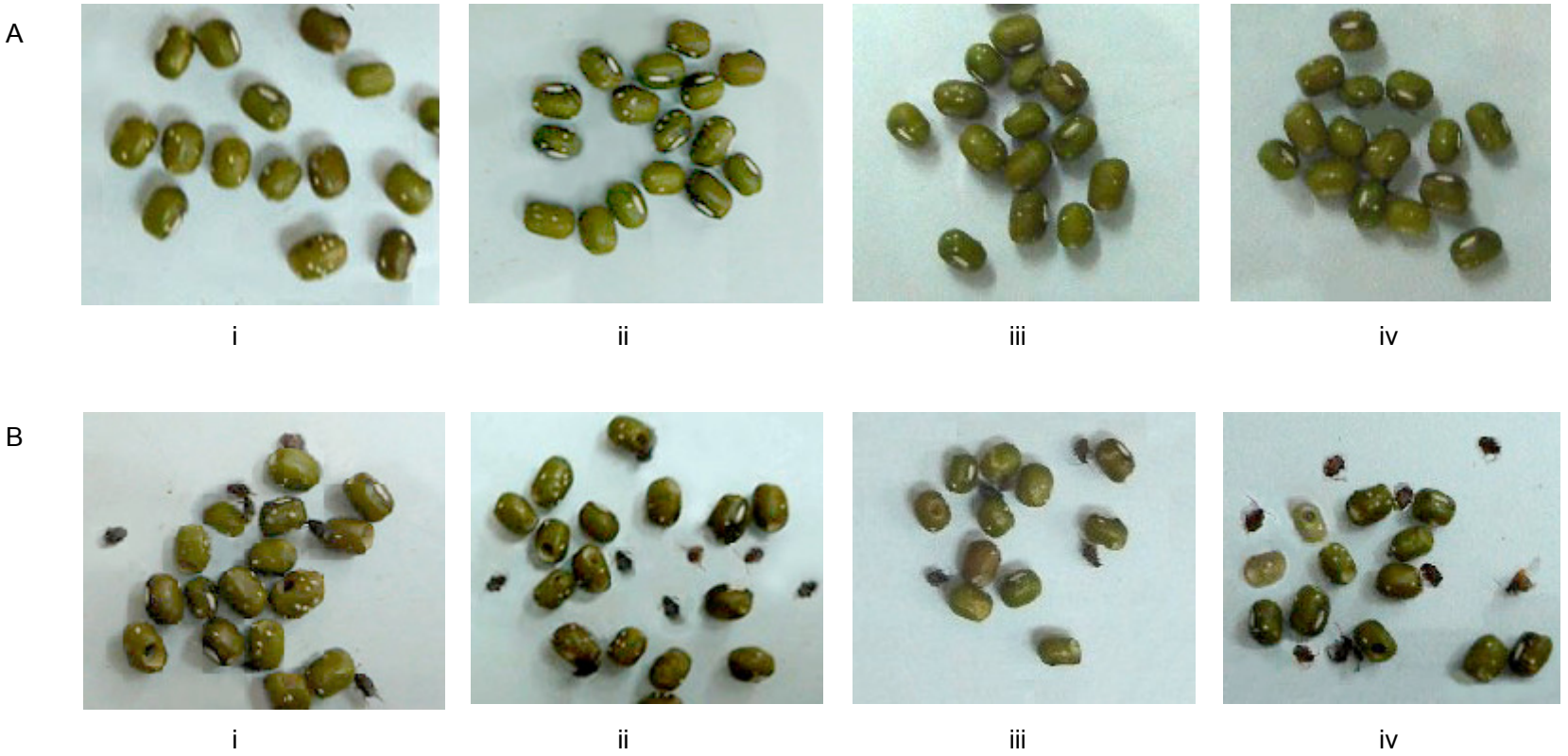


Figure 25. Effect of inhibitor on the growth of insect pest in mungbean(A) after three days (B) after thirty five days; (i) mungbean seeds were coated with distilled water (ii) mungbean seeds coated with buffer A, (iii) mungbean seeds coated with crude extract, (iv) mungbean seeds coated with Sephadex G-75 fraction.

8. Identification of Sephadex G-75 fraction

Identification of Sephadex G-75 fraction with Folin reagent gave a positive blue colour of phenolic compound.

With FTIR, IR absorptions of Sephadex G-75 fraction (Figure 26) were in the region characteristic of aromatic compounds (1100-1600 cm^{-1}). This pattern was also found in an acid hydrolysate of Sephadex G-75 fraction in ether. Sharp peaks attributed to C=O and O-H stretching modes were shown in both samples; at 1617 cm^{-1} and 3399 cm^{-1} for Sephadex G-75 fraction and at 1636 cm^{-1} and 3435 cm^{-1} for the acid hydrolysate respectively. Base on the IR absorption of standard tannic acid and gallic acid and the observe spectrum of the test samples there is a possibility that the test samples contained a structure of aromatic compound with a carboxylic acid and hydroxyl functional groups.

Identification of acid hydrolysate of Sephadex G-75 fraction in ether with thin layer chromatography (TLC) spraying with Folin-Ciocalteu and fuming with ammonia vapour are shown in Figure 27. This identification found that the acid hydrolysate in ether gave a blue color with Folin reagent at R_f = 0.5 for solvent system 1 and 0.7 for solvent system 2 which were equivalent to the migration of standard gallic acid.

On the basis of these three approaches there is a possibility that a nonproteinaceous amylase inhibitor contains a structure of phenolic compound with a carboxylic acid and hydroxyl functional groups in its molecule.

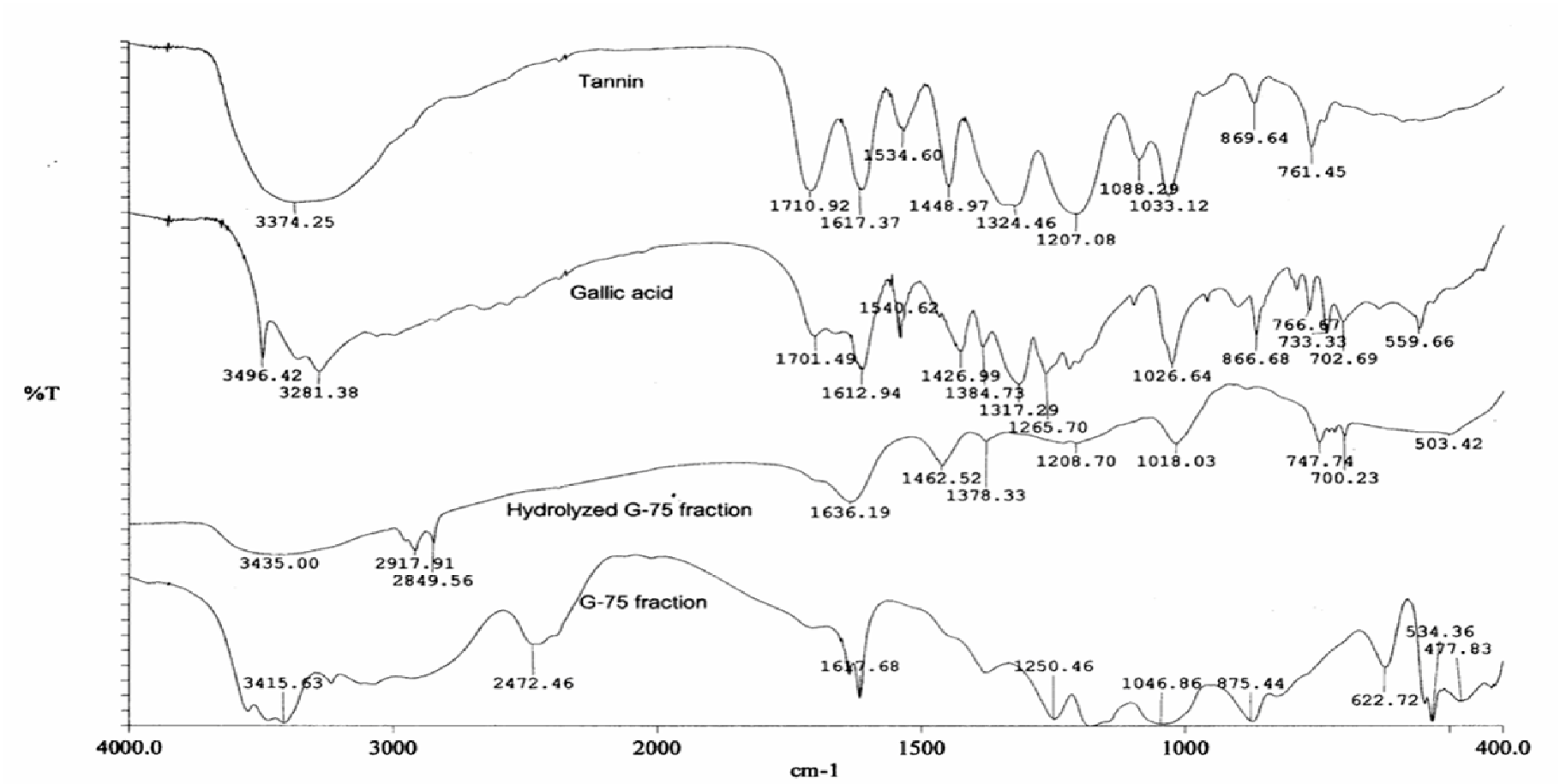
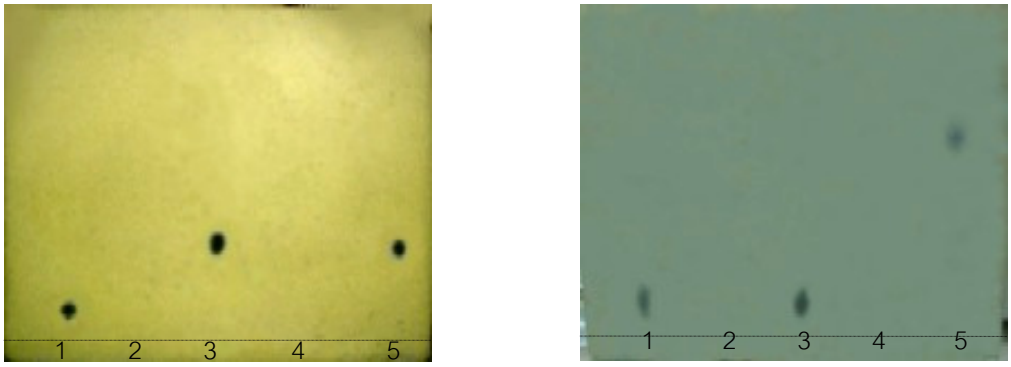
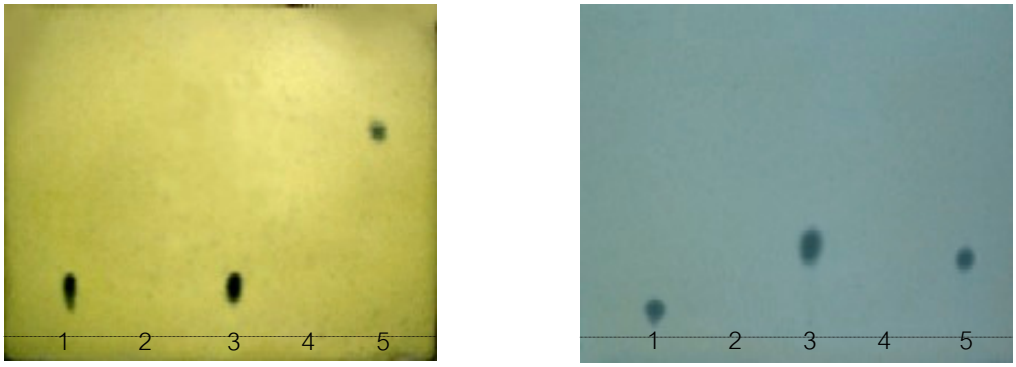


Figure 26. Infrared spectra of Sephadex G-75 fraction, its acid hydrolysate, standards tannic acid and gallic acid. (for detail of each spectra see appendix)

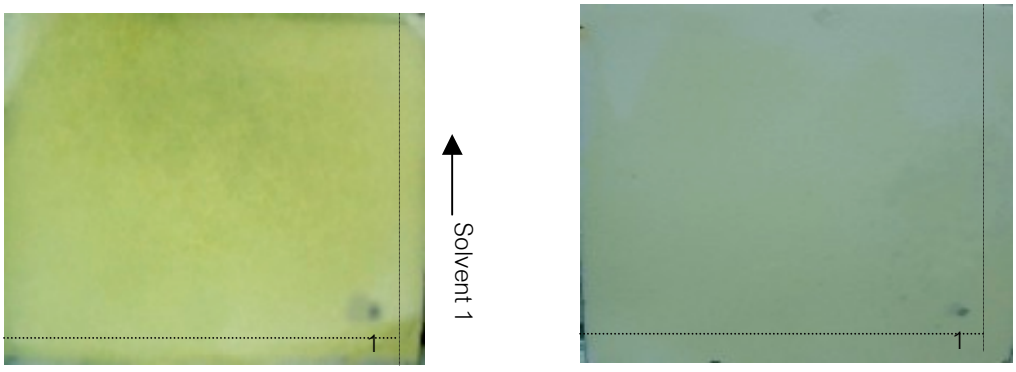
Figure 27. Identification of Sephadex G-75 fraction with thin layer chromatography (TLC): (A) standards separation in solvent system 1 (acetic acid-chloroform 1 : 9 v/v) : lane 1 gallic acid R_f value 0.5, lane 2 *p*-hydroxy benzoic acid, lane 3 2, 5-dihydroxy benzoic acid R_f value 3.2; lane 4 salicylic acid; lane 5 hydroquinone R_f value 2.8), (B) standard separation in solvent system 2 (ethyl acetate-benzene 9 : 11 v/v) : lane 1 gallic acid R_f value 1.0, lane 2 *P*-hydroxy benzoic acid, lane 3 2, 5-dihydroxy benzoic acid R_f value 0.9 ; lane 4 salicylic acid; lane 5 hydroquinone R_f value 10.3), (C) two dimension of sample (R_f value 0.5 and 0.7 for solvent system 1 and 2 respectively; the first column was sprayed with Folin reagent , the second column was fumed with ammonia (NH_4OH) vapour, the third column was sprayed with vanillin reagent.



(A) standards separation in solvent system 1



(B) standard separation in solvent system 2



(C) Two dimension of sample