# CHAPTER 3

## RESULTS

### 3.1 Mutagenicity study of shallot (Allium ascalonicum Linn.)

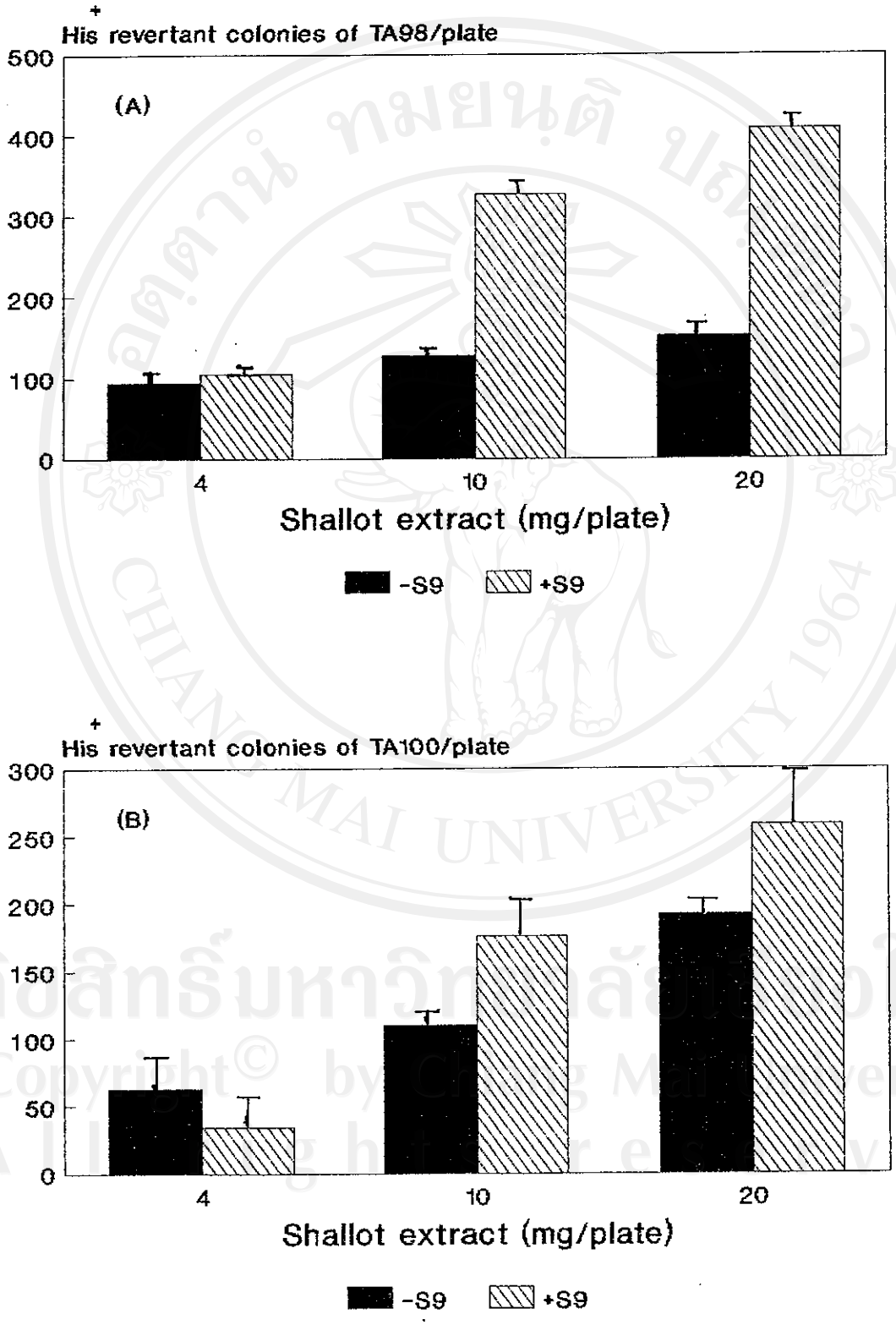
The mutagenicity response of a methanol extract of shallot TA98 and TA100 is shown in Table 1. Mutagenicity to TA98 was stronger than to TA100 both with and without metabolic activation. The mutagenic activity of a methanol extract of shallot was dose-dependent manner to both TA98 and TA100 as shown in Fig. 5. It was observed that the methanol extract of shallot might contain direct mutagenic compound(s) to the tester strains; after metabolic activation by rat-liver enzymes (S9 mix) the mutagenicity was increased in both.

Table 1 Mutagenicity of methanol extract of shallot (His$^+$ revertant colonies/plate)

| Amount of shallot extract (mg) | *S. typhimurium* TA98 (His$^+$ revertant colonies) | |
|---|---|---|
| | +S9 | -S9 |
| 4 | 105 | 94 |
| 10 | 327 | 128 |
| 20 | 408 | 152 |

| Amount of shallot extract (mg) | *S. typhimurium* TA100 (His$^+$ revertant colonies) | |
|---|---|---|
| | +S9 | -S9 |
| 4 | 34 | 63 |
| 10 | 176 | 110 |
| 20 | 258 | 192 |

Fig. 5 Mutagenicity of methanol extract of shallot to TA98(A) and TA100(B) with and without S9 mix.

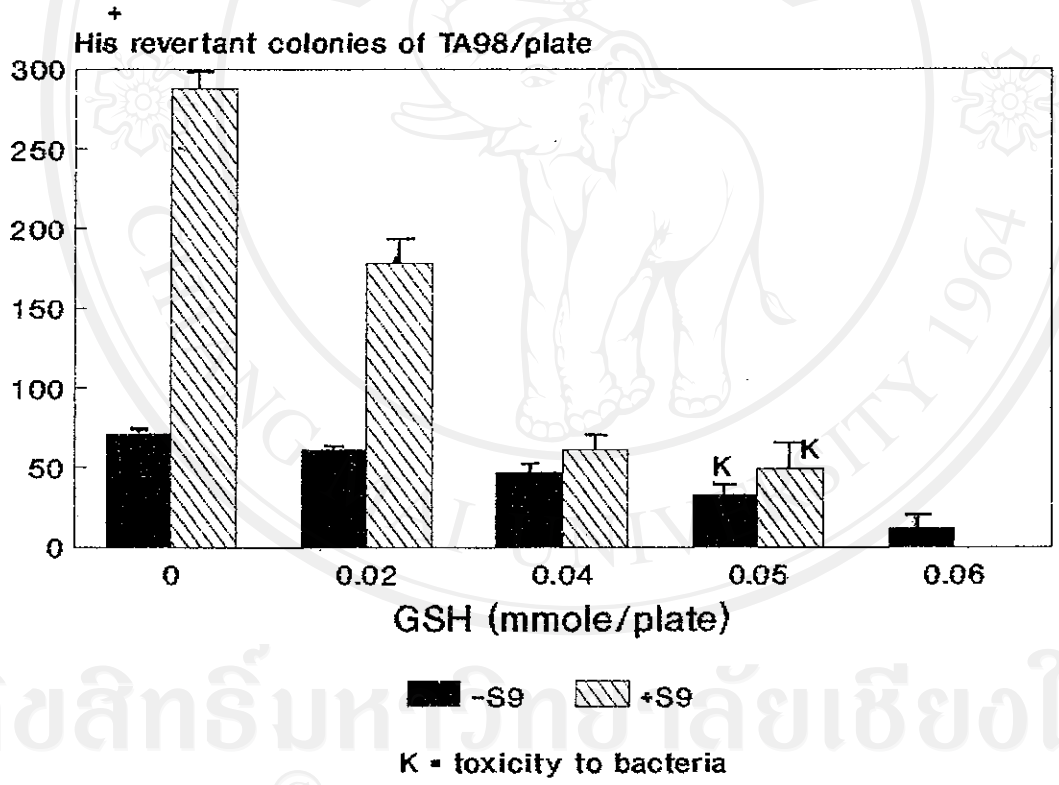


3.2 Modulation of shallot mutagenesis

3.2.1 Effect of glutathione (GSH)

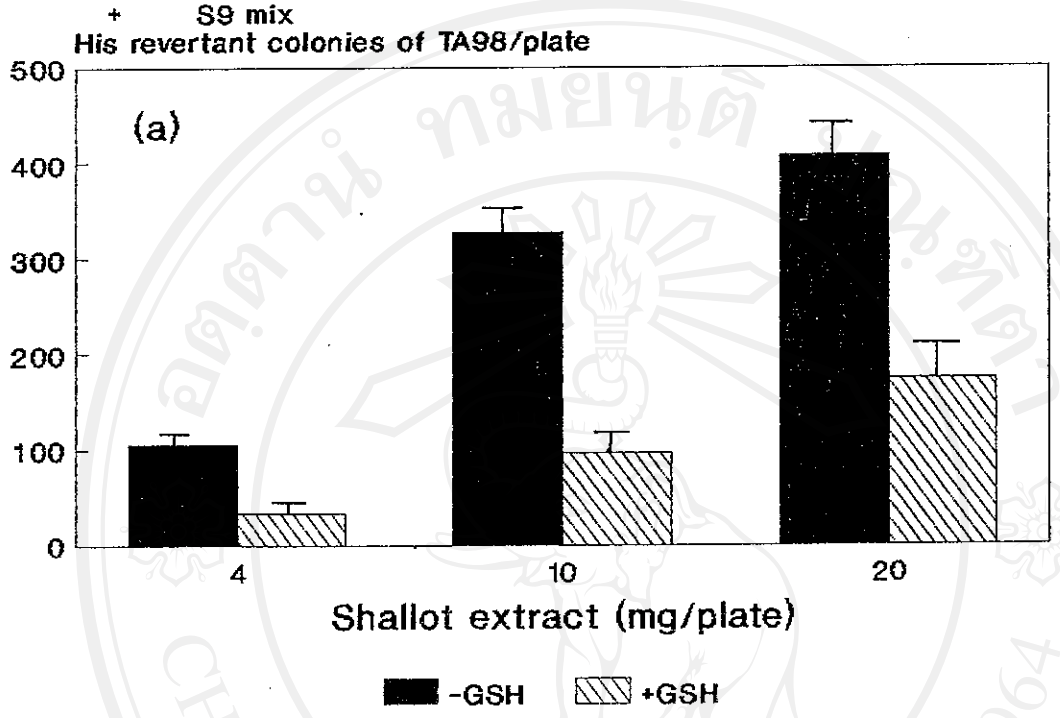
Various concentrations of glutathione ($2 \times 10^{-2}$ to $6 \times 10^{-2}$ mmole) were studied for their ability to modify the mutagenicity of the methanol extract of shallot. The concentration of GSH of more than $4 \times 10^{-2}$ mmole was toxic to bacteria as shown by a clear background lawn of the bacteria under stereomicroscope. Concentrations of GSH up to $4 \times 10^{-2}$ mmole can decrease mutagenicity of shallot extract. The decrease of the mutagenicity was clearly demonstrated in the presence of S9 mix as shown in Fig. 6. To examined the ability of GSH at $4 \times 10^{-2}$ mmole to decrease the mutagenicity of shallot extracts, different amounts of shallot extracts were mixed with GSH and the mutagenicity was examined. It was demonstrated that an addition of GSH can suppress the mutagenicity of shallot extract both with and without S9 mix(Fig. 7). The reduction of shallot mutagenesis by GSH was statistically significantly ($\alpha = 0.05$, by ANOVA test).

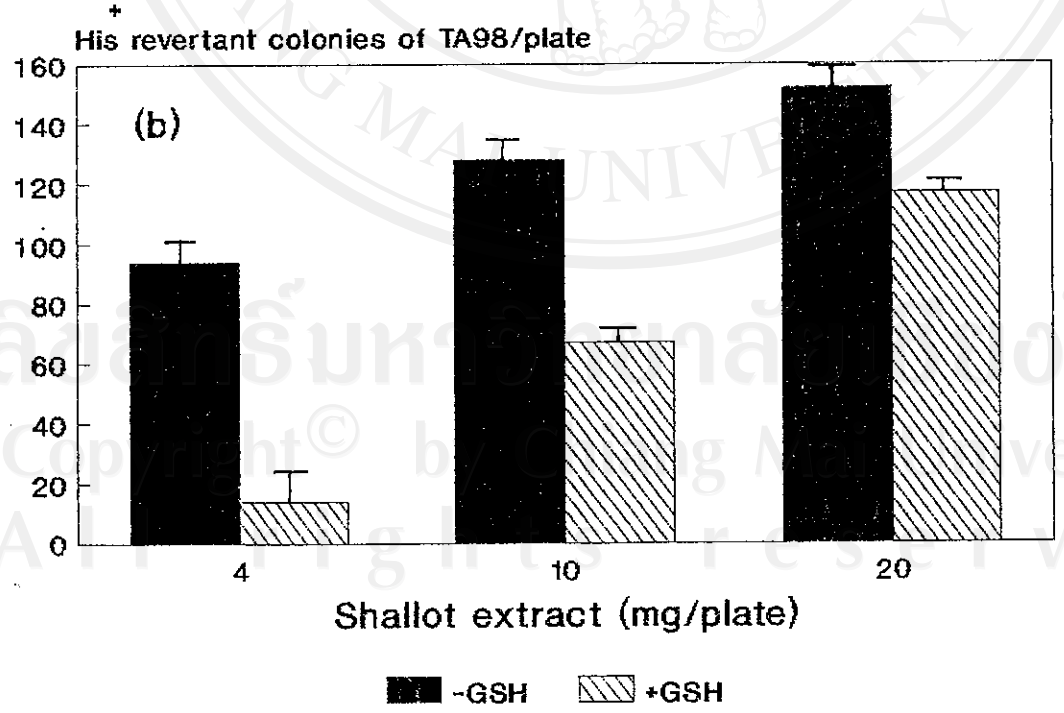
Fig. 6 Decreased mutagenicity of shallot extract with GSH.




K = toxicity to bacteria

Fig. 7 Suppression of shallot extract mutagenicity by GSH when tested with TA98 in the presence(a) and absence(b) of S9 mix



3.2.2 Effect of dithiothreitol (DTT)

Dithiothreitol could exhibit a suppress the mutagenicity of shallot extract, the suppression was dose-dependent in manner, as shown in Fig. 8. The ability of dithiothreitol at $2.5 \times 10^{-2}$ mmole to decrease the mutagenicity of various amounts of shallot extracts was demonstrated in Fig. 9. DTT decreased the mutagenicity of shallot extracts to a greater extent in TA100 than in TA98 (Fig. 9 and Fig. 10). An addition of S9 mix did not change the modification of shallot mutagenesis by DTT toward TA100, but the mutagicity to TA98 after DTT treatment was still evident in the presence of S9 mix.

Fig. 8 Suppression of the mutagenicity of shallot extract with DTT.

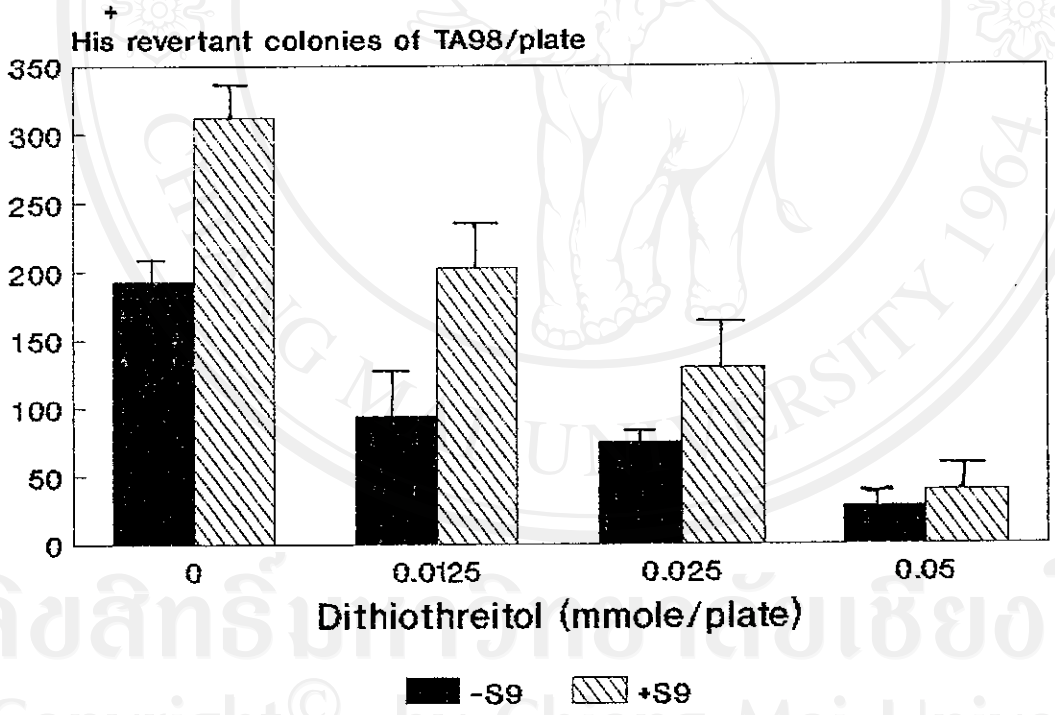
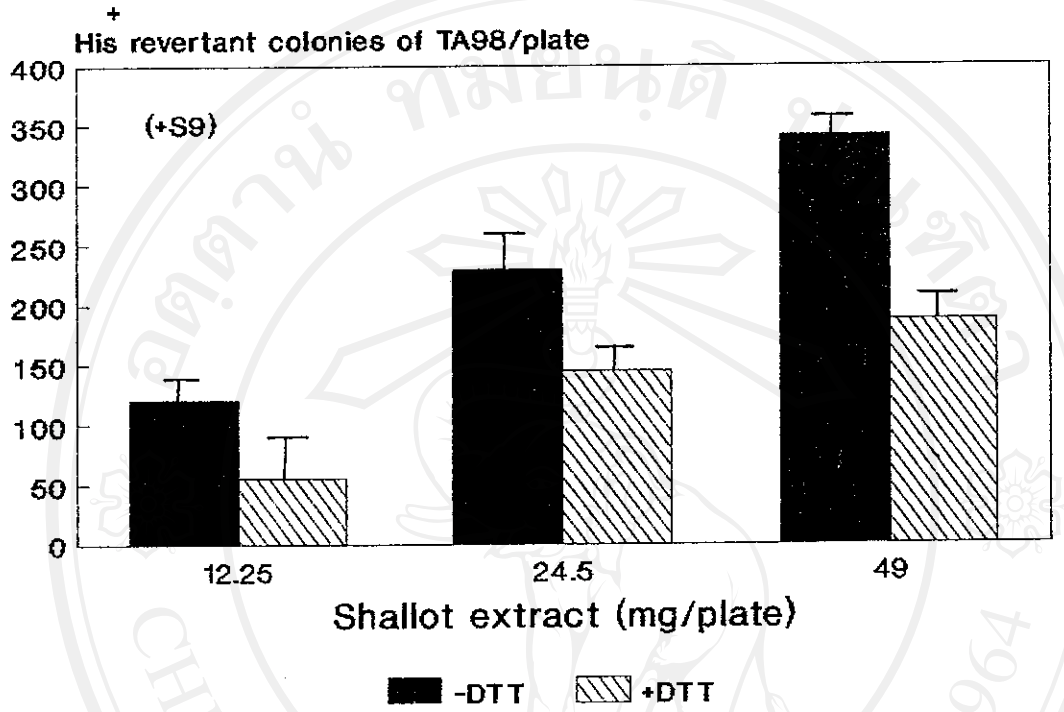


Fig. 9 Supression of shallot extract mutagenicity by DTT when tested with TA98 with and without S9 mix.



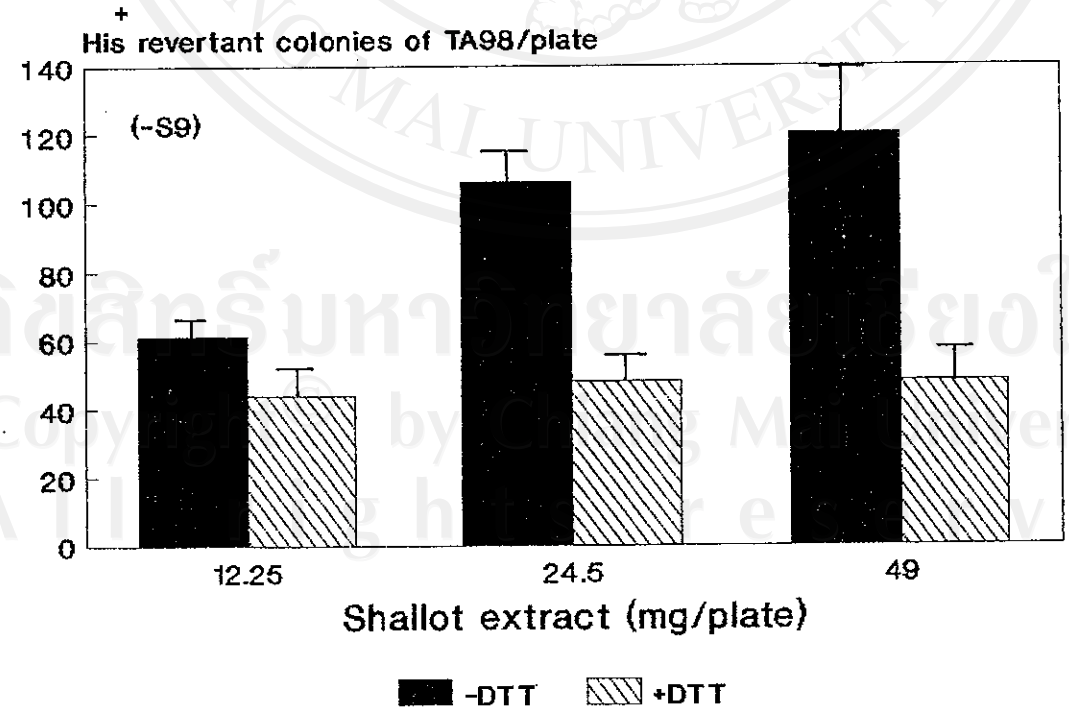
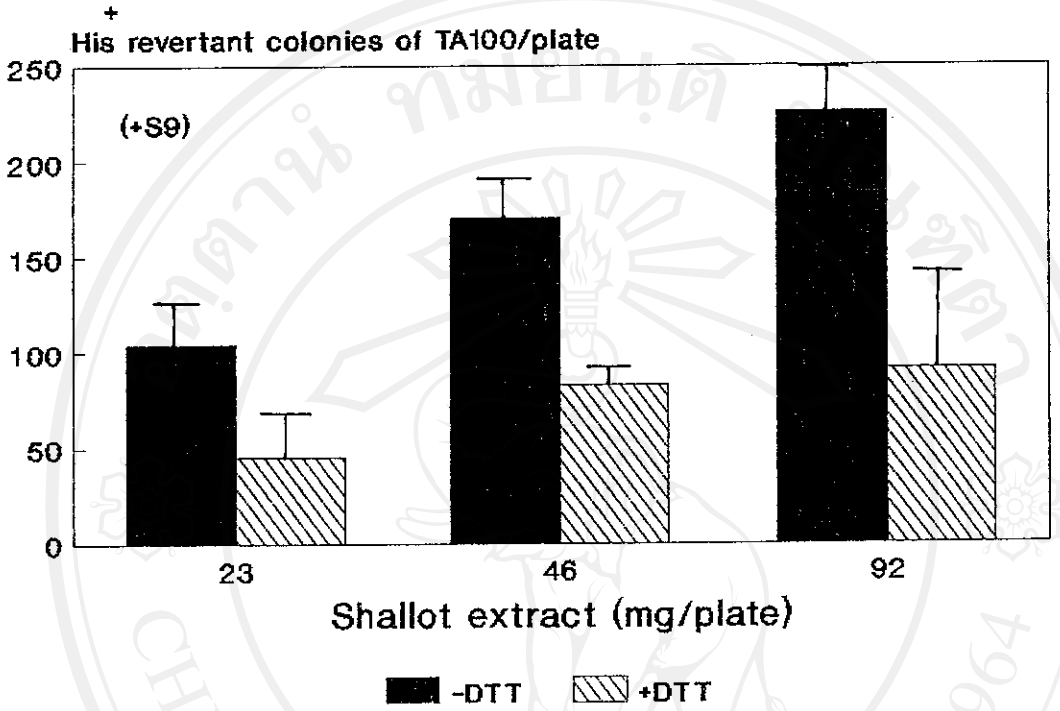



Fig. 10 Suppression of shallot extract mutagenicity by DTT in TA100 with and without S9 mix.



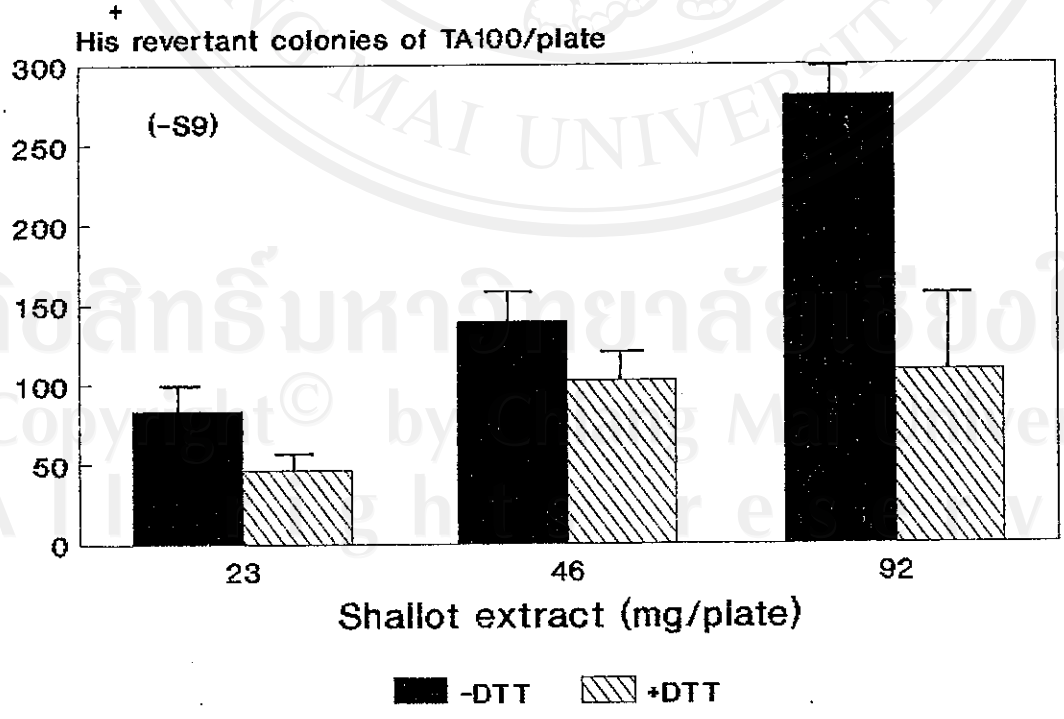



Table 2 Inhibition of mutagenicity of shallot extract by GSH and DTT

| Amount of shallot extract (mg/plate) | Inhibited by 0.04 mmole GSH(%) | |
|---|---|---|
| | +S9 | -S9 |
| 4 | 69 | 85 |
| 10 | 71 | 48 |
| 20 | 57 | 23 |

| Amount of shallot extract (mg/plate) | Inhibited by 0.025 mmole DTT(%) | |
|---|---|---|
| | +S9 | -S9 |
| 12.25 | 54 | 28 |
| 24.50 | 37 | 55 |
| 49 | 45 | 60 |

3.2.3 Effect of uridine-5 -diphosphoglucuronic acid (UDPGA)

It was demonstrated that UDPGA had no ability to modify the mutagenicity of shallot extract in an absence of S9 mix(Fig.11B), an addition of S9 mix, UDPGA might conjugate with mutagenic substances in shallot extract and reduced theirs mutagenicity as shown in Fig.11A.( $\alpha$ = 0.05, by ANOVA test)

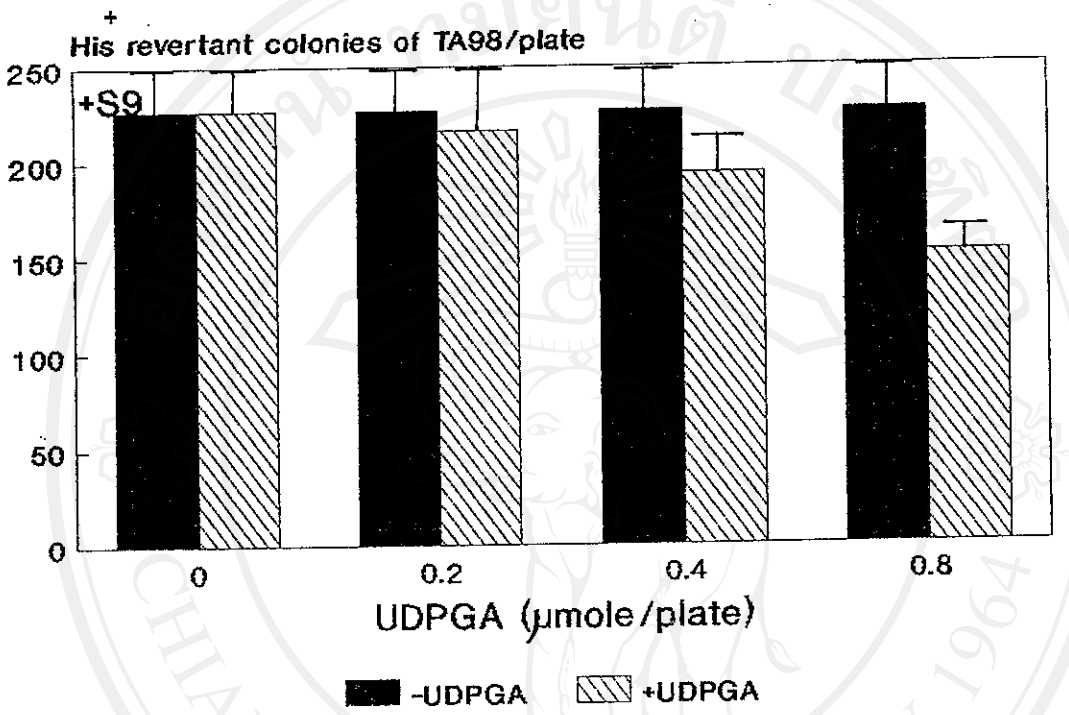
3.2.4 Effect of retinoic acid

As shown in Fig.12 and Fig.13. Retinoic acid could not modify the mutagenicity of shallot extract in both TA98 and TA100, with and without metabolic activation.( $\alpha$ = 0.05, by ANOVA test)

3.2.5 Effect of ascorbic acid

Vitamin C (ascorbic acid) at the concentration up to 20 uM could not suppress the mutagenicity of shallot extract in the bacteria TA98 and TA100 (Fig.14 and Fig.15).( $\alpha$ = 0.05, by ANOVA test)

Fig.11 Suppression of shallot extract mutagenicity by low concentration of UDPGA in TA98 with and without S9 mix.



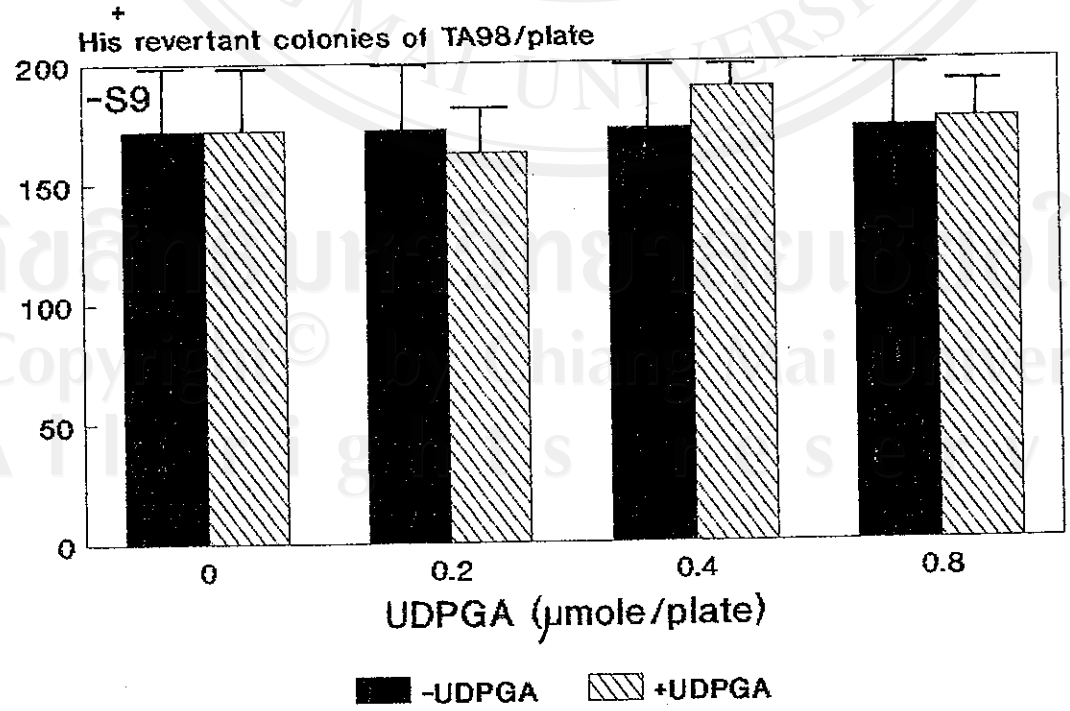
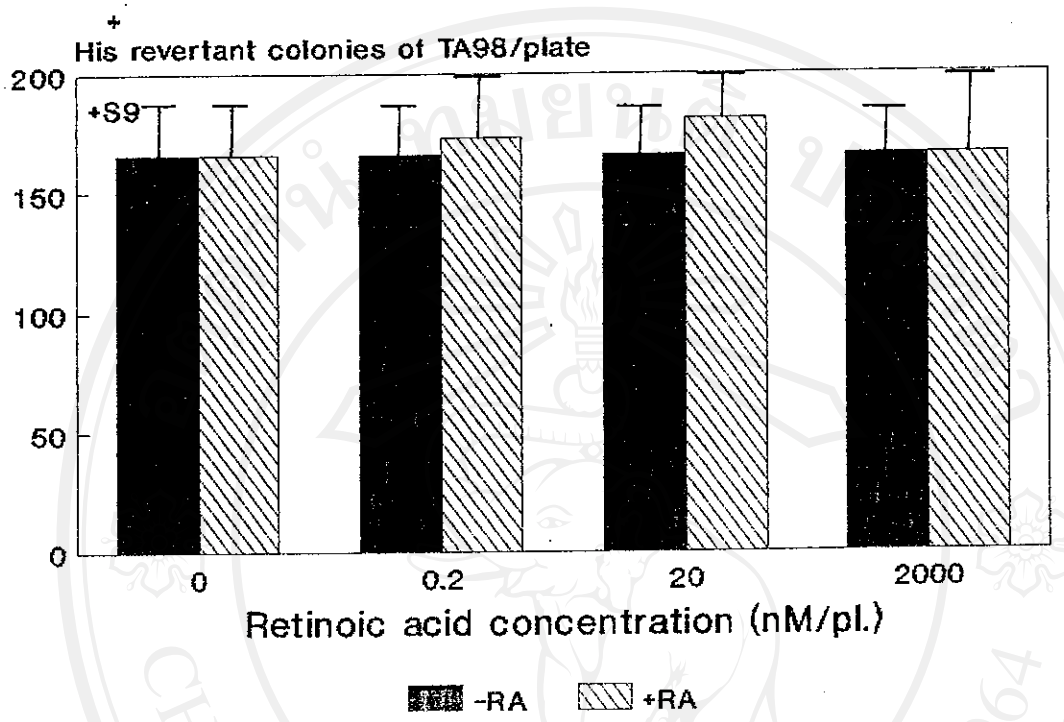
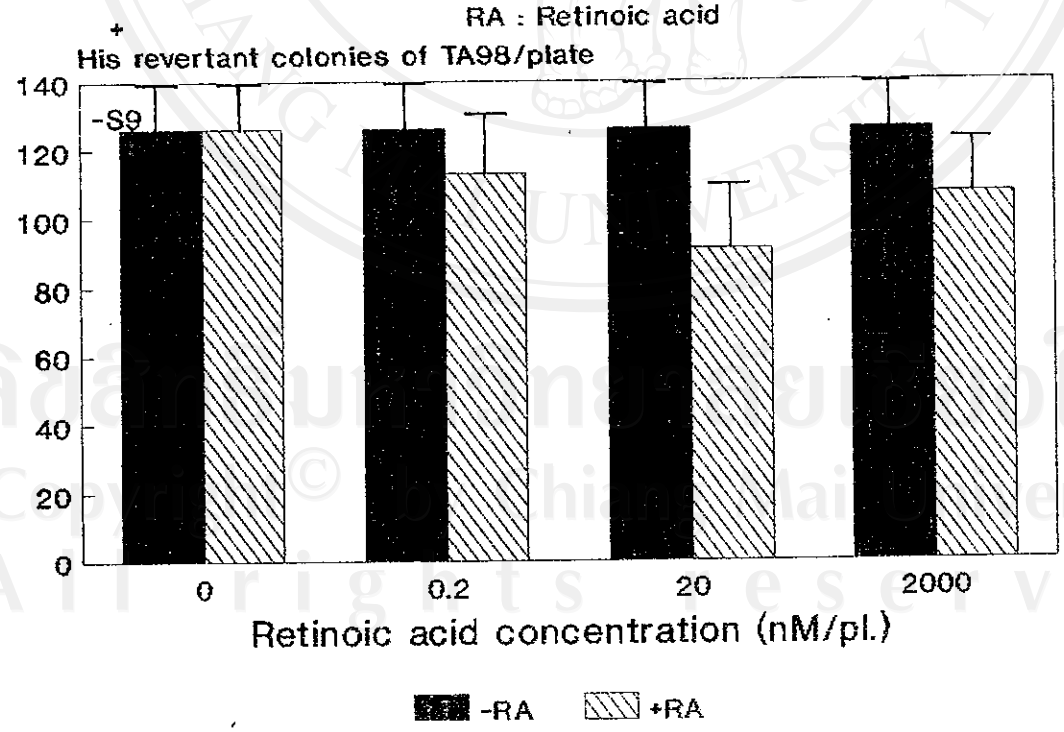



Fig.12 Effect of retinoic acid on the mutagenicity of shallot extract in TA98 with and without S9 mix.

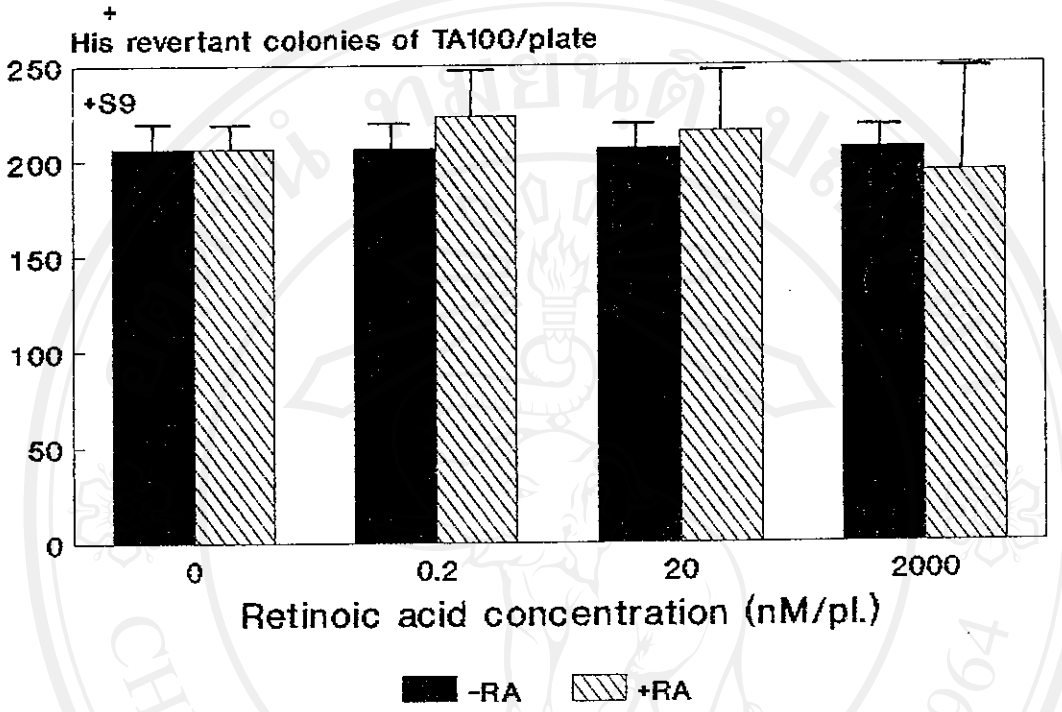



RA : Retinoic acid

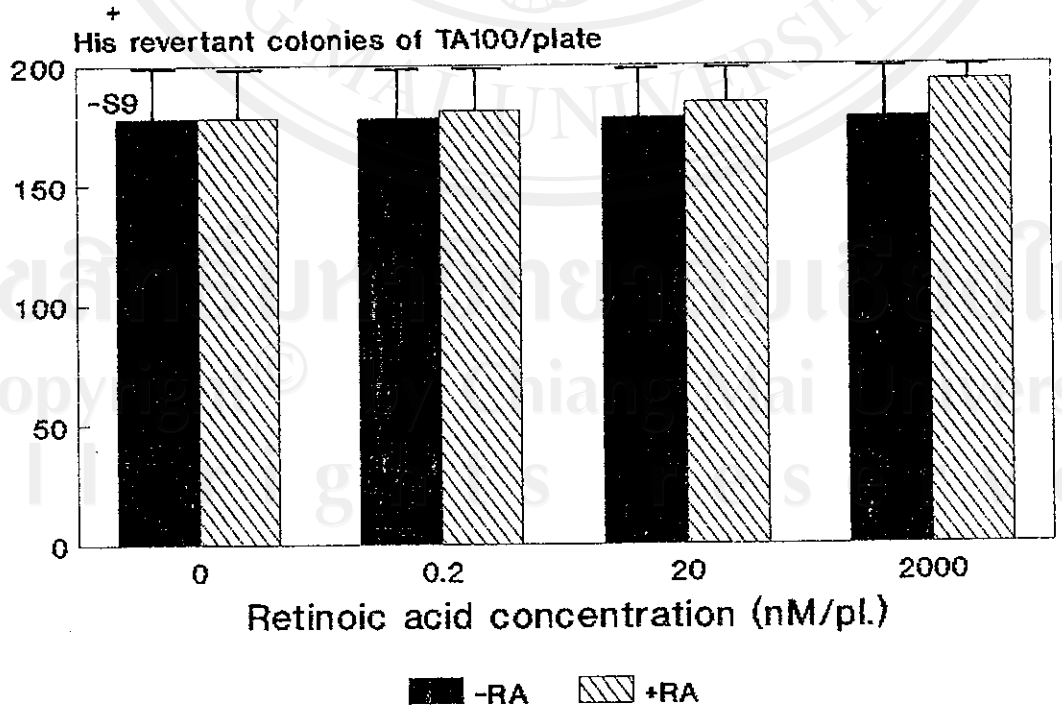



RA : Retinoic acid

Fig.13 Effect of retinoic acid on the mutagenicity of shallot extract in TA100 with and without S9 mix.

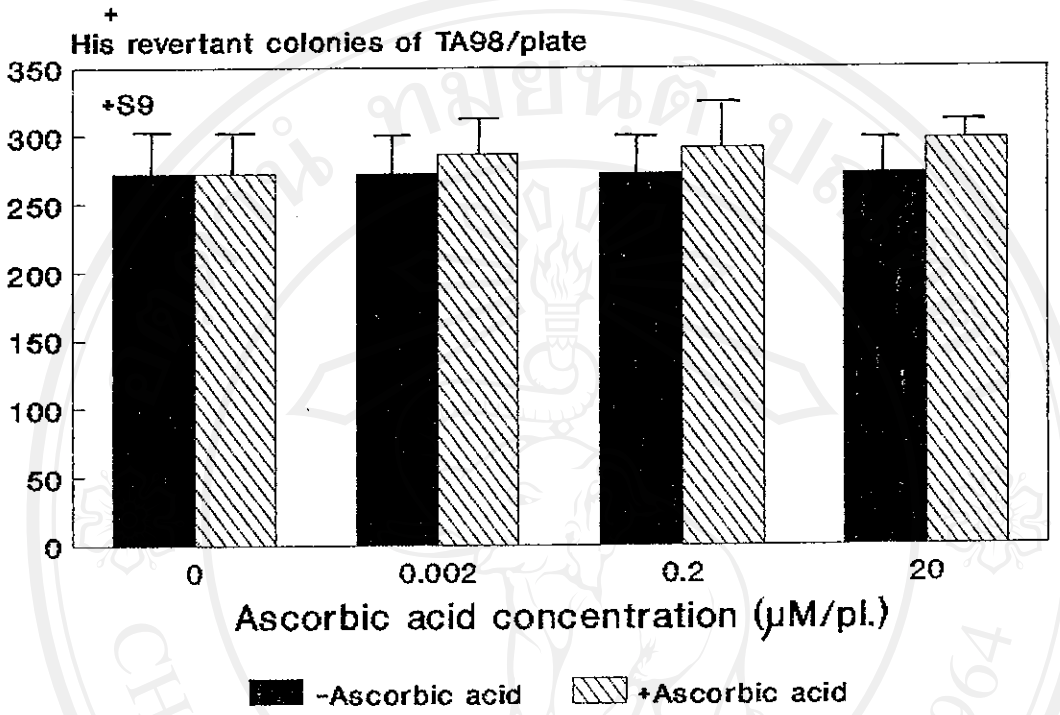



RA : Retinoic acid




RA : Retinoic acid

Fig.14 Effect of ascorbic acid on the mutagenicity of shallot extract in TA98 with and without S9 mix.



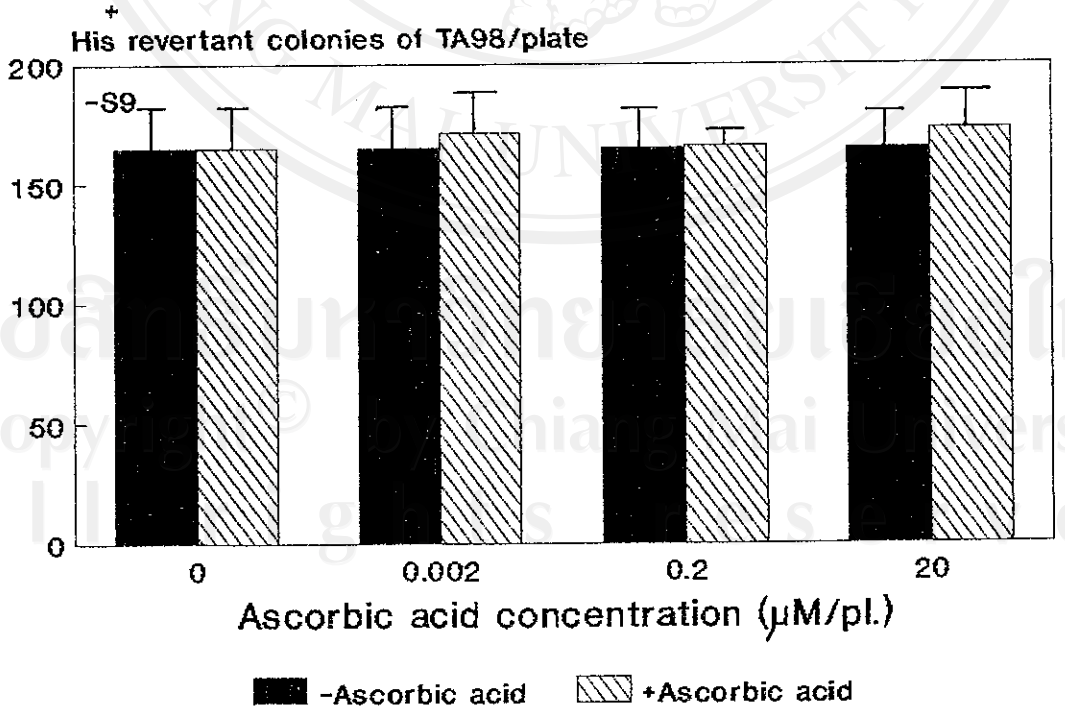
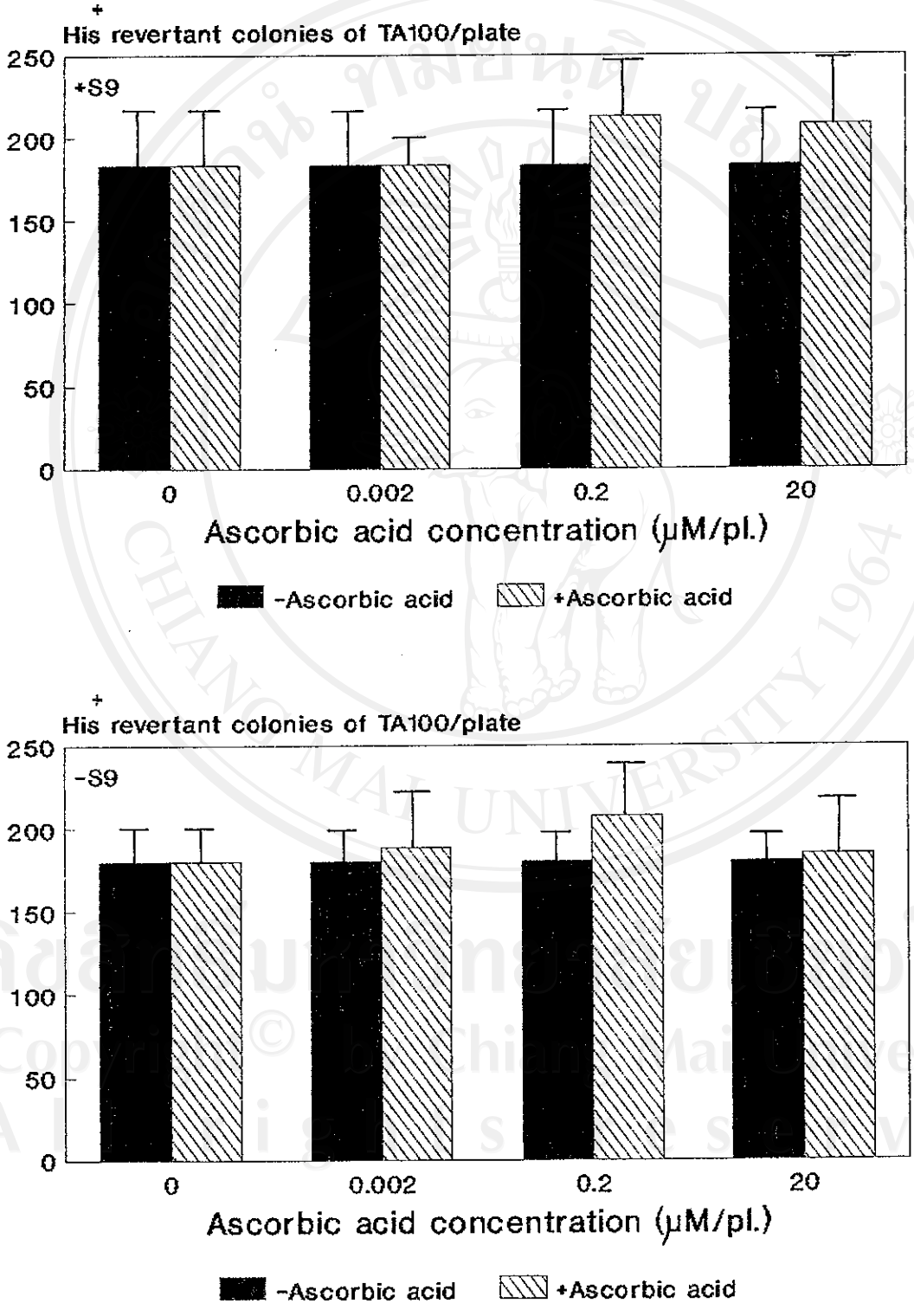



Fig.15 Effect of ascorbic acid on the mutagenicity of shallot extract in TA100 with and without S9 mix.



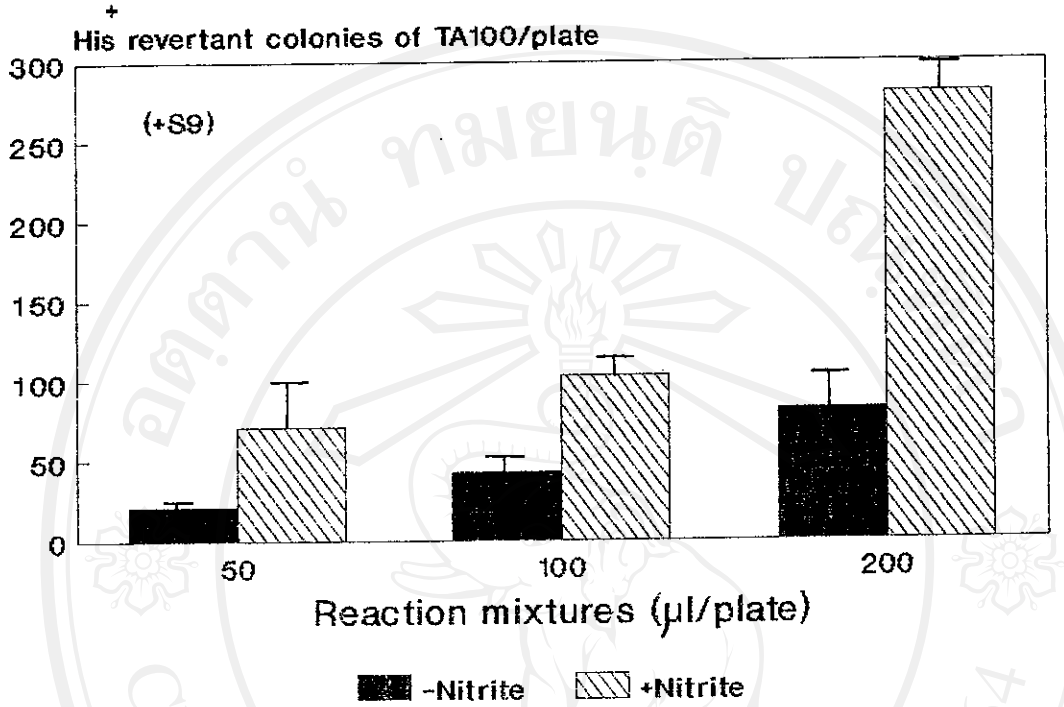
3.2.6 Effect of sodium nitrite

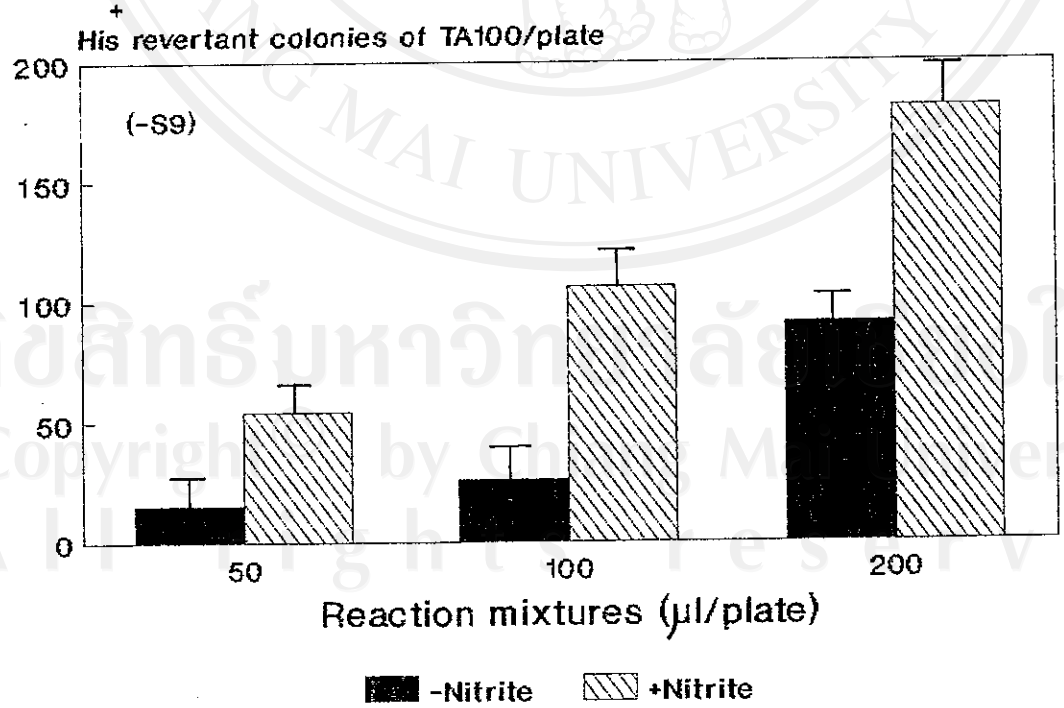
After nitrite treatment of shallot extract, the presumed nitrosation products did not show mutagenic activity to TA98 in either the presence or absence of S9 mix (Table 2 ). On the contrary nitrite-treated products showed mutagenicity to TA100 in the presence and absence of S9 mix (Fig.16).

Table 3 Lack of mutagenicity of presumed nitrosation products to TA 98 ($His^+$ revertant colonies/plate)

| Amount of reaction mixture (µl) | + S9 | | -S9 | |
|---|---|---|---|---|
| | Without nitrite | Nitrite treatment | Without nitrite | Nitrite treatment |
| 50 | 37 | 23 | 6 | 22 |
| 100 | 44 | 29 | 7 | 32 |
| 200 | 78 | 35 | 11 | 42 |

Fig.16 Mutagenicity of nitrite-treated products of shallot extract toward TA100.

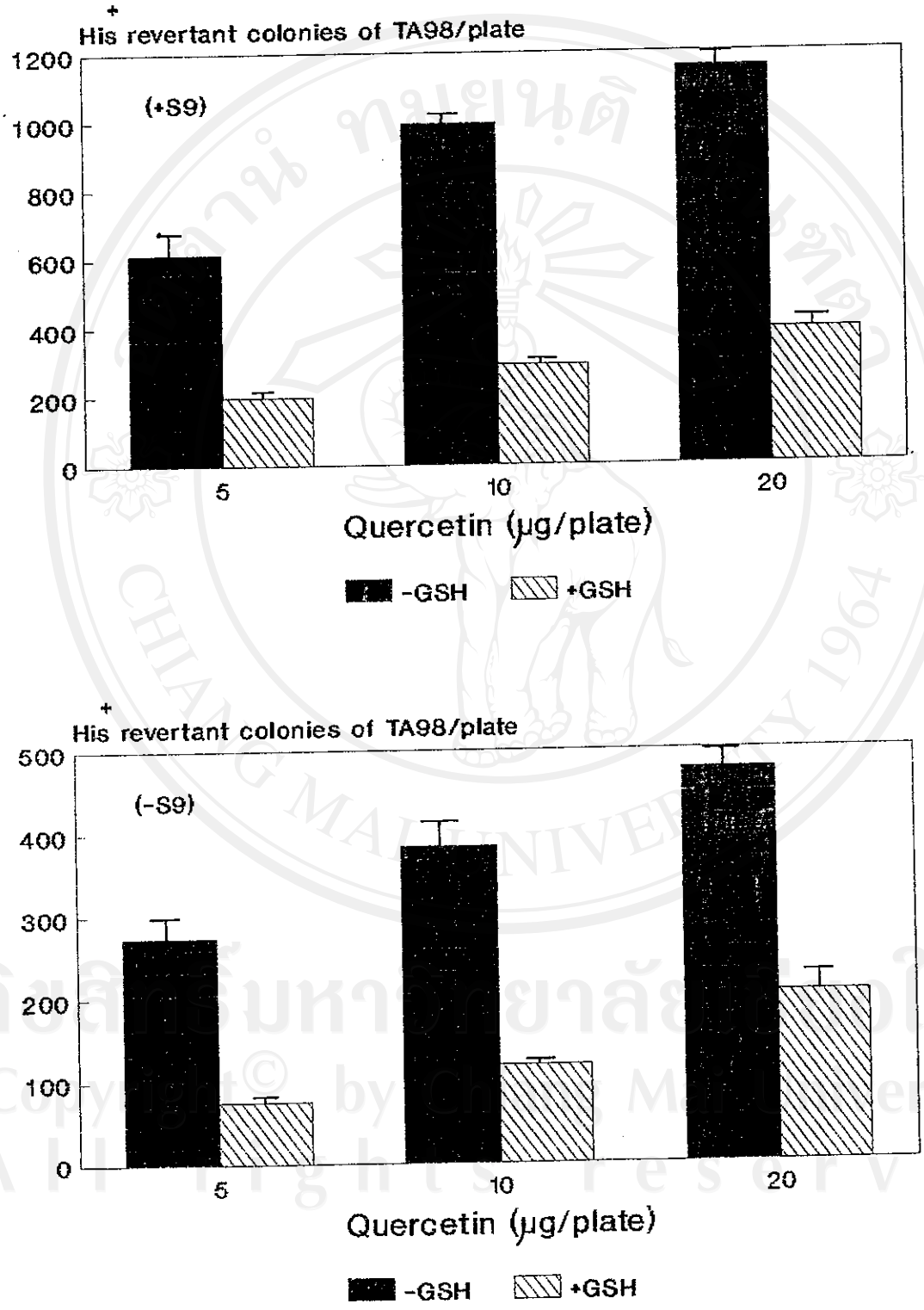


3.3 Effect of GSH on the mutagenicity of quercetin

Quercetin was reported to be present in shallot extract (Purintrapiban, J., 1989, and Vinitketkumnuen, U., 1991). Therefore, the modulation of quercetin mutagenicity by GSH was studied. It was demonstrated in Fig. 17 that, GSH could suppress the mutagenicity of quercetin. ( $\alpha$ = 0.05, by ANOVA test)

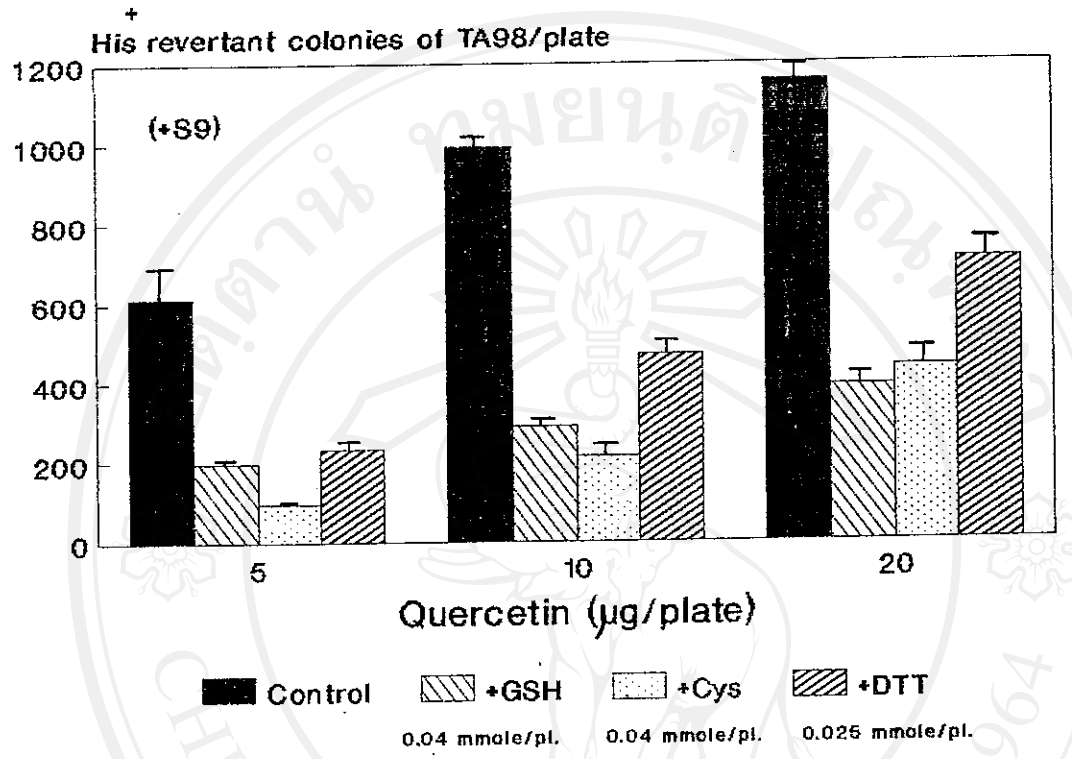
Fig.17 Suppression of quercetin mutagenicity by GSH.



3.3.1 Modulators of quercetin mutagenicity

As shown in Fig.18 glutathione, cysteine and dithiotheitol can modify the mutagenic activity of quercetin to TA98. The modification was demonstrated both with and without metabolic activation. ($\alpha$= 0.05, by ANOVA test)

Fig.18 Comparision of the mutagenicity of quercetin after treatment with different chemicals.



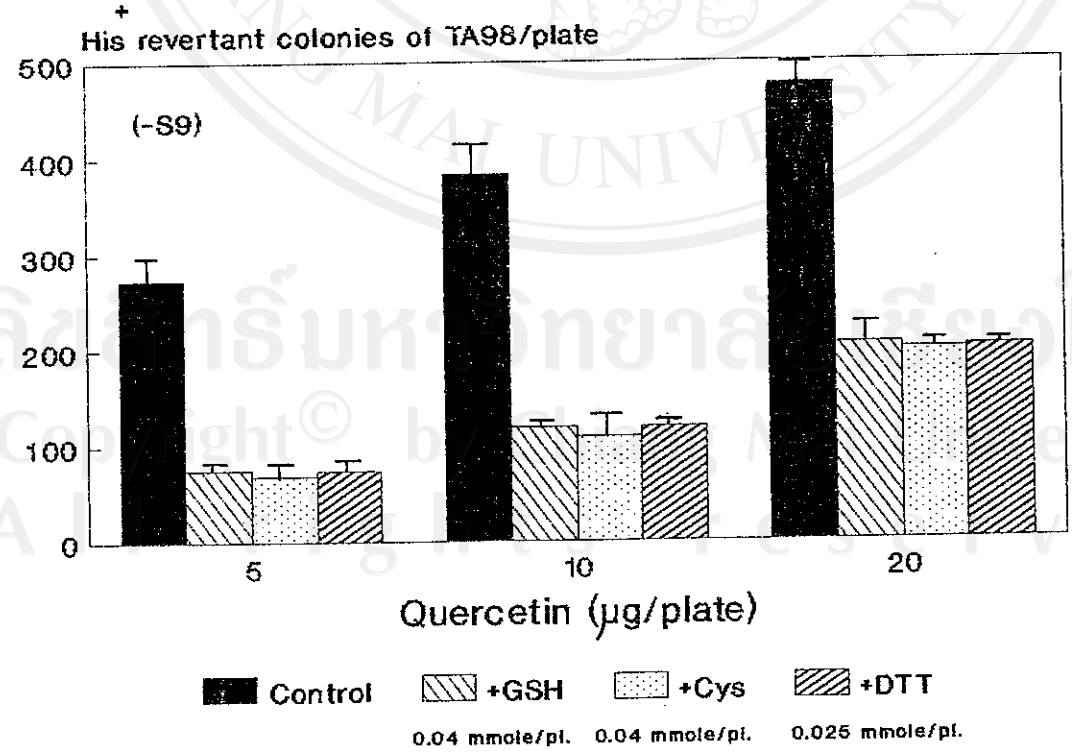
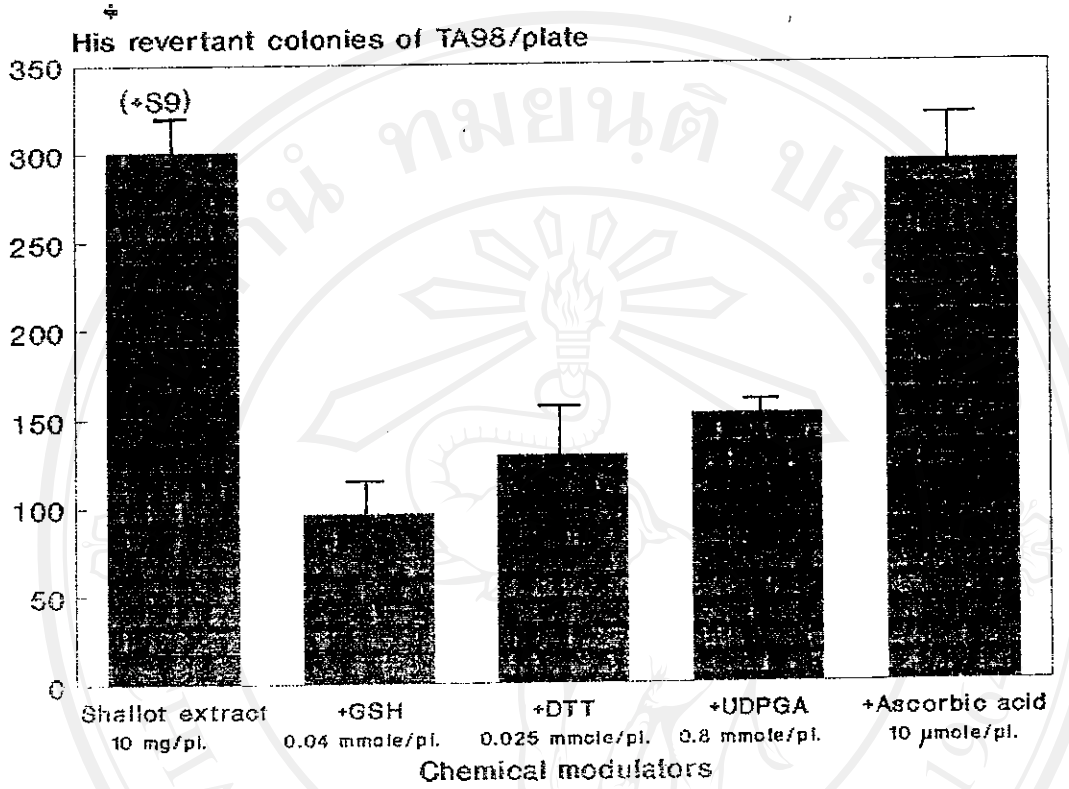
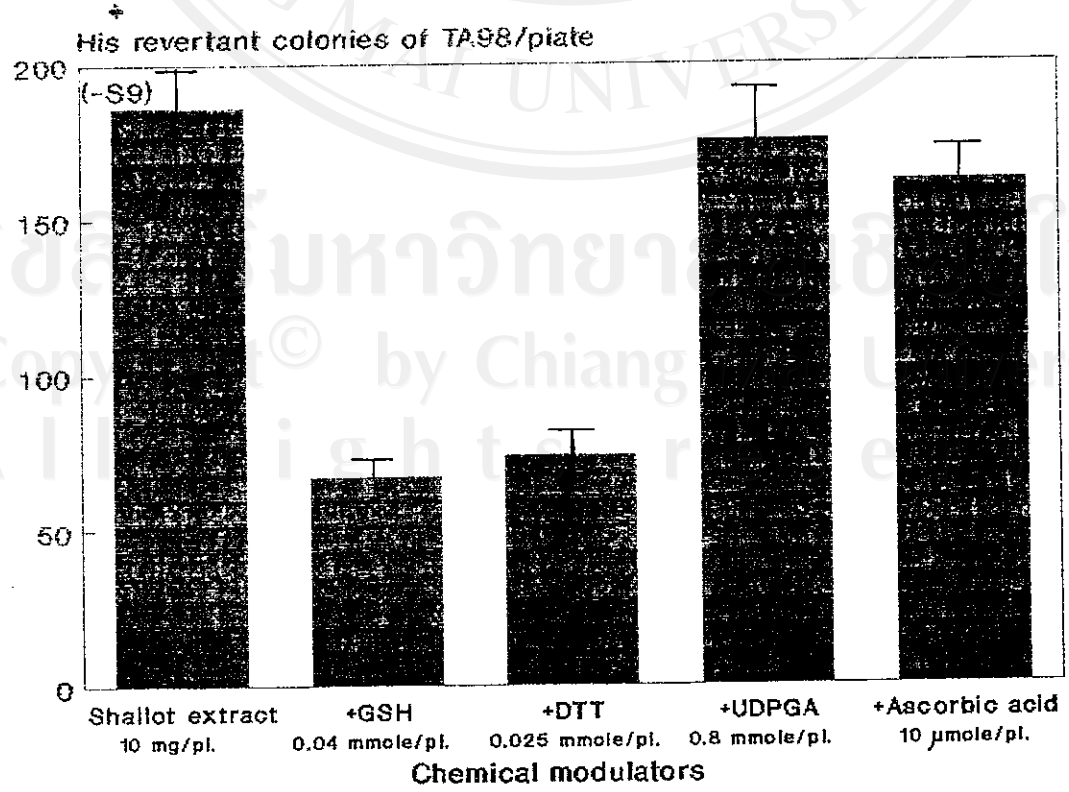



Fig.19 Effect of chemical modulators on shallot mutagenesis.



3.4 Effect of incubating GSH with shallot extract prior to mutation assay.

The mutagenicity of shallot was reduced after treatment with GSH in the absence of metabolic activation. It is possible that a chemical reaction may have occurred between GSH and mutagenic substances in shallot extract. The result indicated that incubation of GSH with shallot extract prior to mutagenicity caused a reduction of mutagenesis of approximately 49 % , compared with 43 % inhibition when GSH was mixed during the mutation assay (Fig.20). It was suggested that GSH may inhibit shallot mutagenesis by a direct reaction between the SH group in GSH and OH group of active mutagenic components. In the presence of microsomal enzymes (S9 fraction), conjugation was noted to enhance the inhibitory effect of GSH.

No difference of the inhibition percentage of quercetin was observed when GSH was added either during mutation assay or prior to the mutation assay ( Fig.21).

Fig.20 Effect of GSH on shallot mutagenesis.(A) GSH addition during mutation assay.(B) GSH incubation prior to mutation assay.

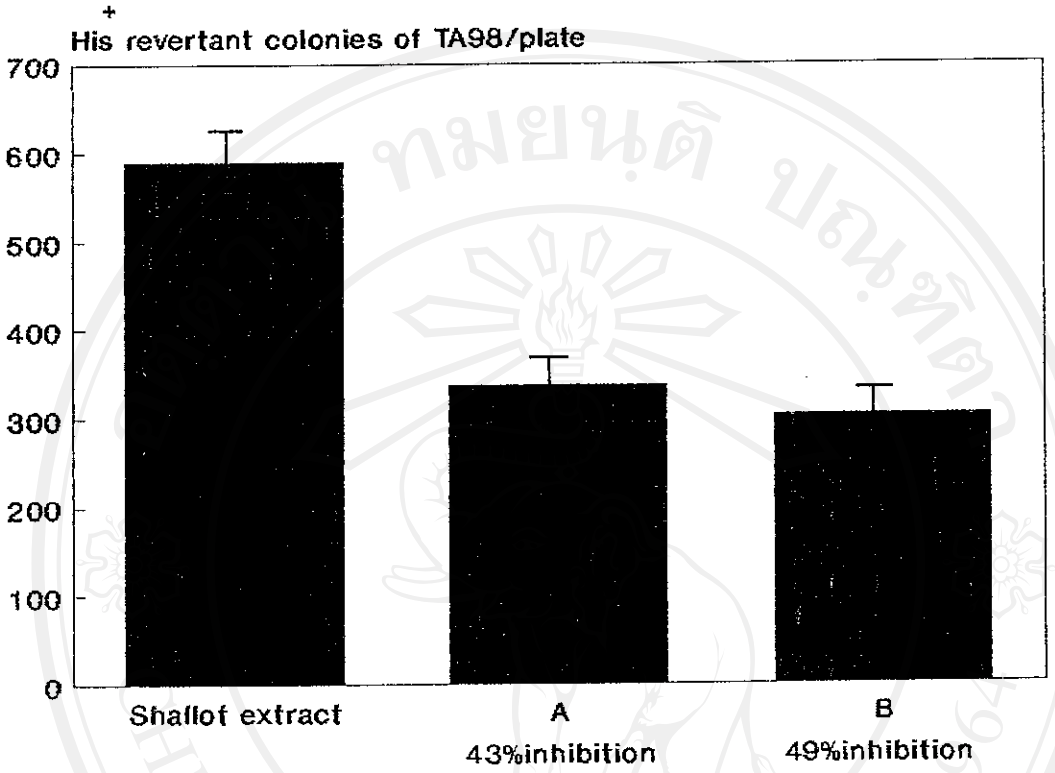
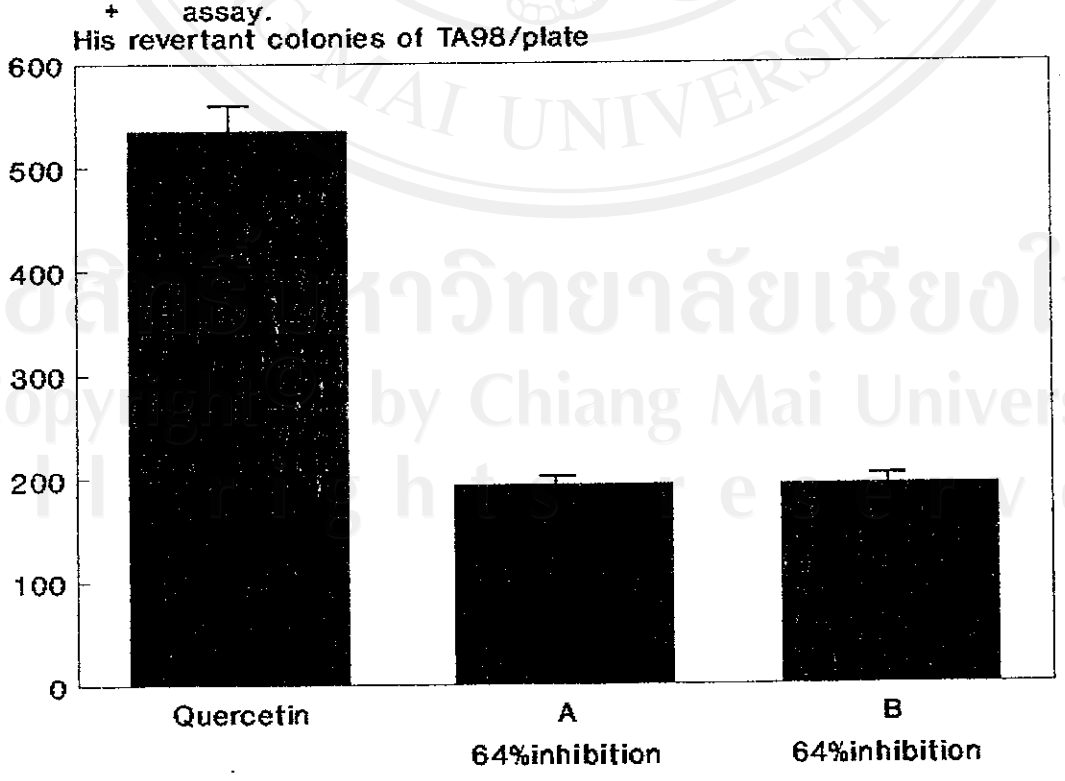



Fig.21 Effect of GSH on quercetin mutagenesis. (A) GSH addition during mutation assay.(B) GSH incubation prior to mutation assay.



3.5 Partial purification of mutagenic substances in shallot by column chromatogram

3.5.1 Partial purification of shallot extract by Bio-gel P-10 column

The Bio-gel column chromatogram of methanol extract of shallot is shown in Fig.22. At least five peaks (a,b,c,d and e) were separated. The mutagenic peak is shown in fraction number 37 to 42 (peak b), which were eluted with distilled water. However, the mutagenic recovery by this separation was very low, only 2 %. Therefore, it was suggested that Bio-gel p-10 may not be suitable for further separation of the mutagenic substances in shallot.

3.5.2 Chromatographic fractionation by SEP-PAK cartridges

SEP-PAK cartridges , is a commercial column supplied by Waters Co. It is widely use to separate many compounds of a mixture and was the first step using for selecting the most suitable eluent for the compound studied. uBonda Pak $C_{18}$ cartridge was used in this investigation. It is a type of octadecylsilane bonded $C_{18}$ phase.

Table 3 indicates that 50% MeOH and 100% MeOH were suitable to elute mutagenic substances present in methanol extract of shallot, from SEP-PAK column.

Table 4 $His^+$ revertant colonies of SEP-PAK cartridge's fraction with different eluents

| Fraction | Eluent | $His^+$ revertant colonies of TA98 / plate | |
|---|---|---|---|
| | | +S9 | -S9 |
| 0 | Non-eluted | 17 | 6 |
| $1^{st}$ | water | 11 | 8 |
| $2^{nd}$ | 15% MeOH | 0 | 0 |
| $3^{rd}$ | 30% MeOH | 15 | 0 |
| $4^{th}$ | 50% MeOH | 322 | 125 |
| $5^{th}$ | 100% MeOH | 682 | 281 |
| $6^{th}$ | Acetone | 24 | 0 |
| $7^{th}$ | Hexane | 9 | 0 |

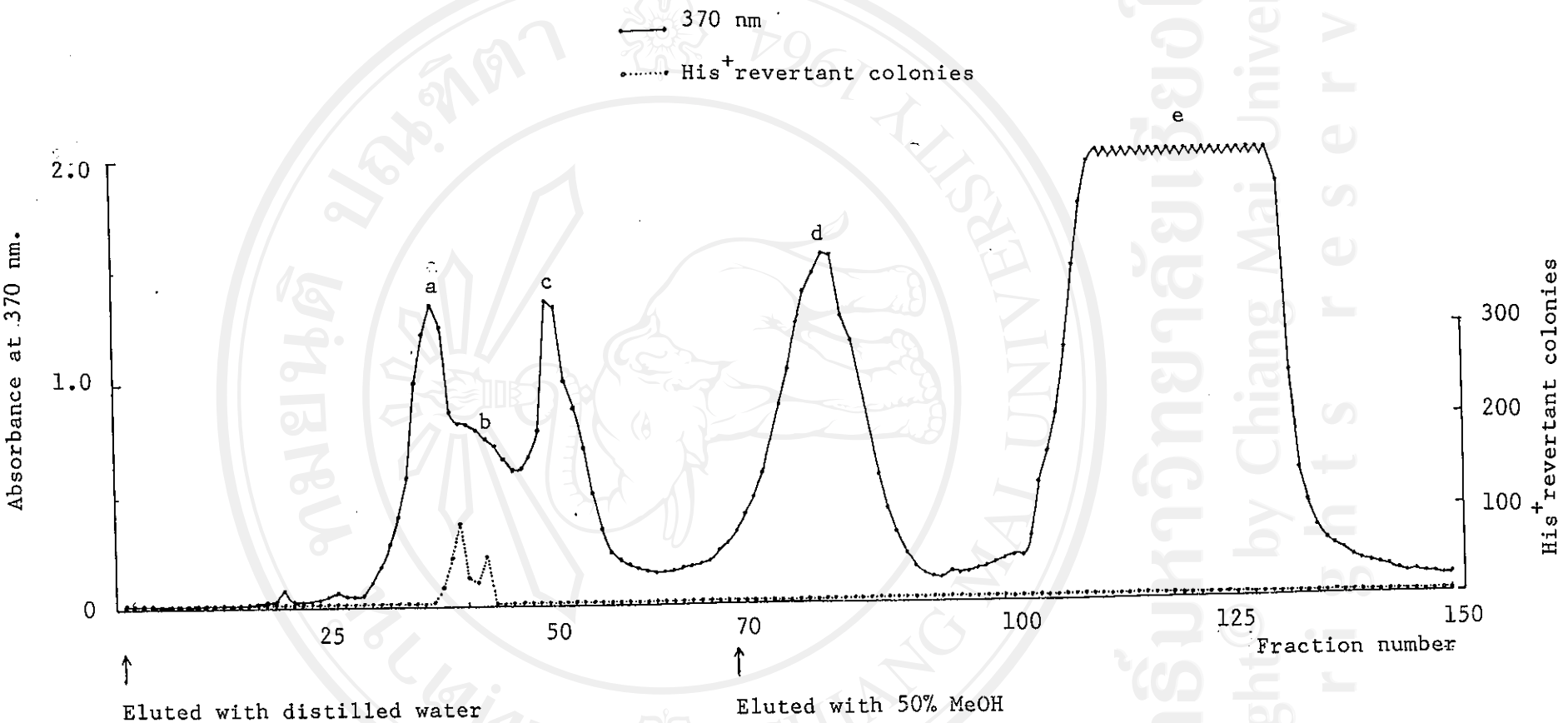



Fig. 22 The partial purification profile of shallot extract on Bio-gel P-10 column.

3.5.2.1 Partial purification of mutagenic substances in 50% methanol eluent by Sephadex LH-20 column

The 50% methanol eluate from SEP-PAK cartridge was further purified by Sephadex LH-20 column (2 x 45 cm) which was eluted with absolute methanol. Four $OD_{370}$ peaks (I,II, III,IV) were obtained. Each peak was pooled and dried by vacuum distillation. The remained residue was assayed for mutagenicity. The specific mutagenic activity was calculated as the number of $His^+$ revertant colonies per mg residue used. The main mutagenic peaks as detected in the last peak (peak IV), were fractionated in volume 220 ml to 280 ml (Fig. 23). This main mutagenicity peak was repurified by the same column and eluted with absolute methanol. The main peak ($IV_{a1}$) of major mutagenicity was contained in volume 240 ml to 320 ml (Fig. 24). The main mutagenic peak was finally purified by Sephadex LH - 20 column (1 x 18 cm.) by using methanol as an eluant. The single peak ($IV_{a2}$) was in the volume of 27.5 ml to 40 ml (Fig. 25).

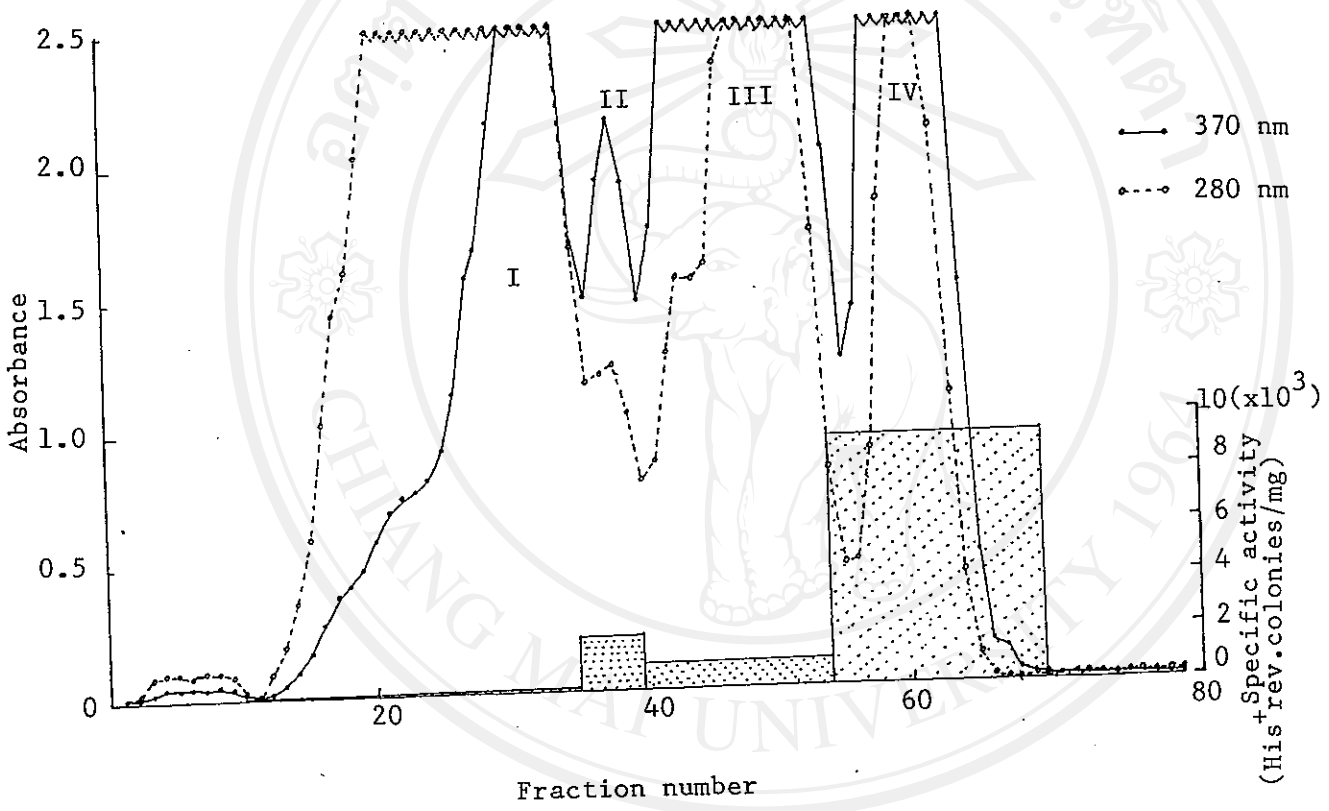



Fig. 23 Sephadex LH-20 column chromatographic profile of 50% MeOH eluate and their mutagenicity.

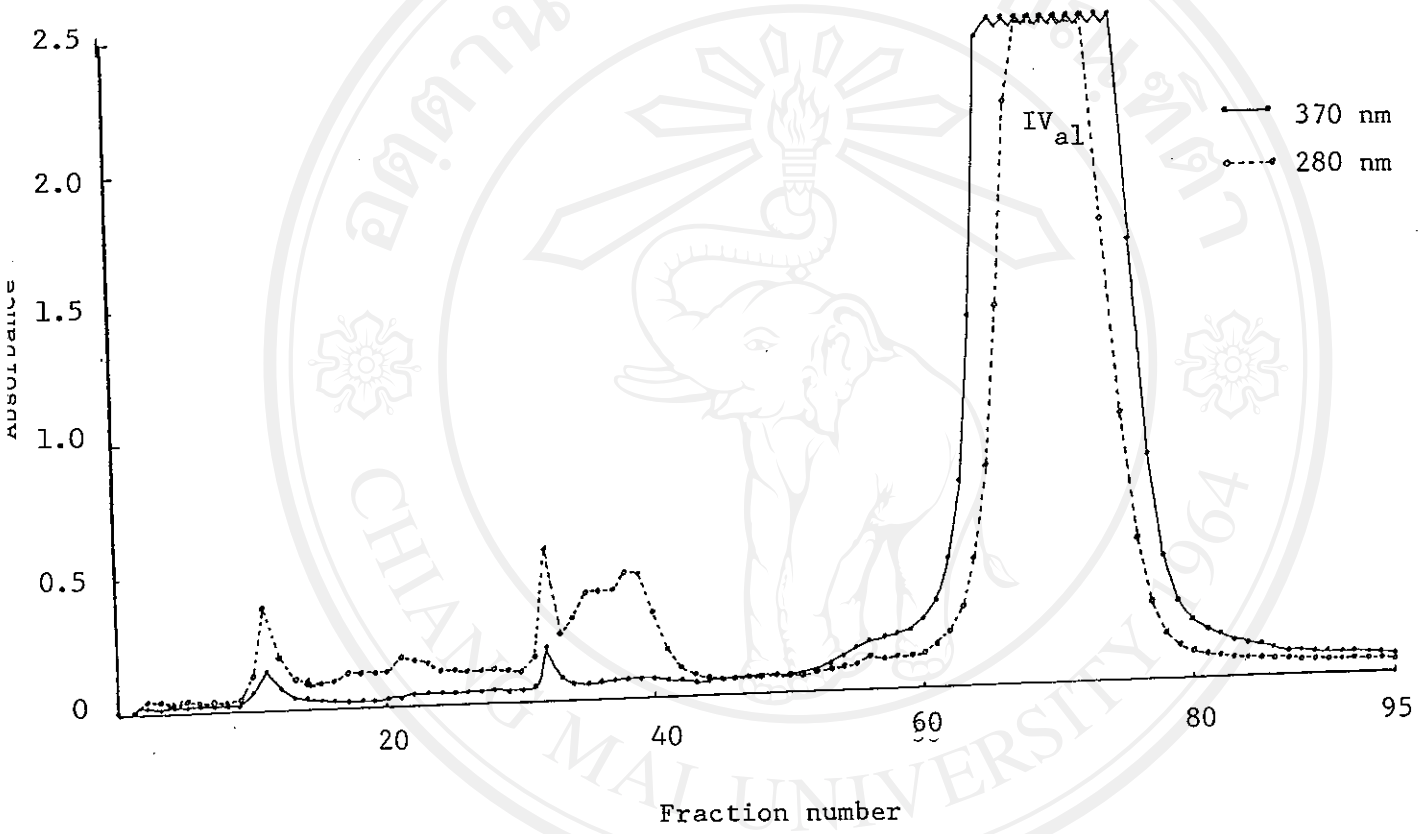



Fig. 24 The chromatographic profile of 2nd Sephadex LH-20 column.

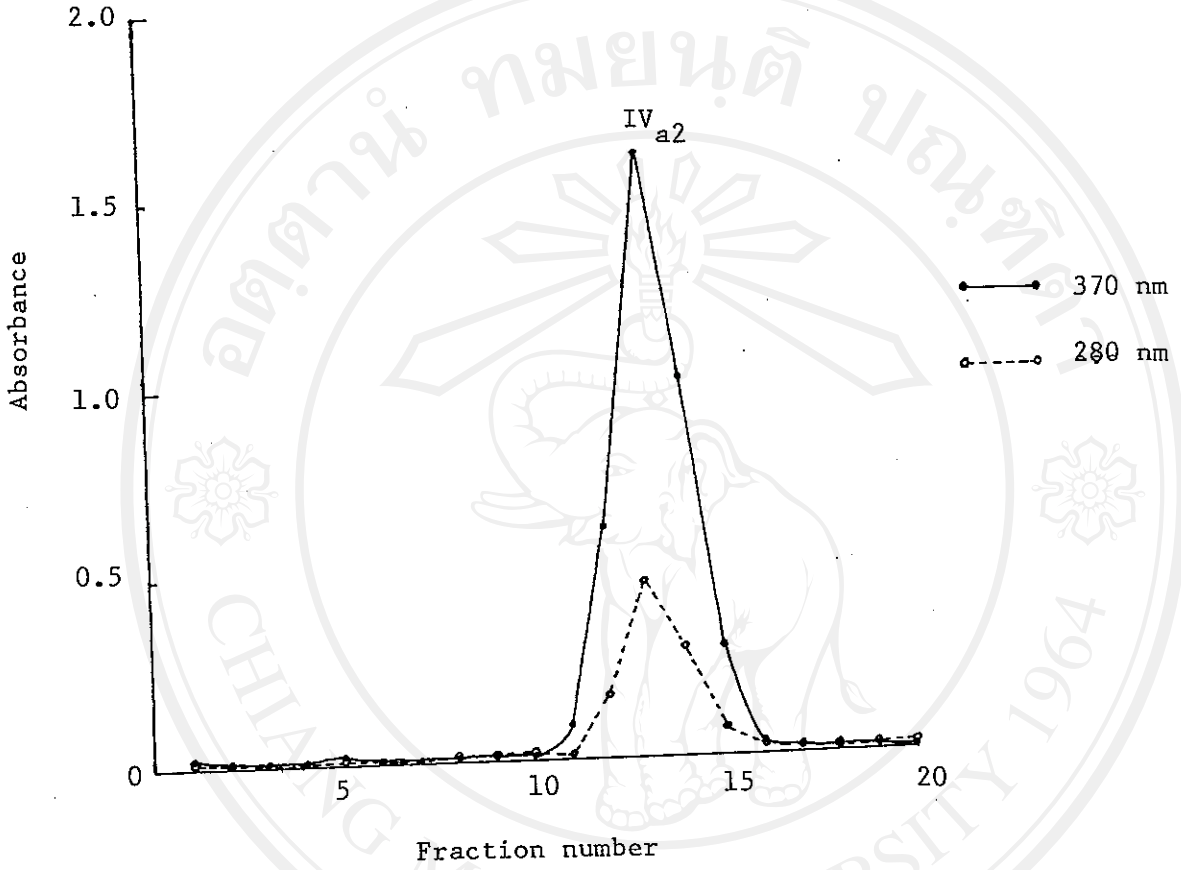



Fig. 25 The chromatographic profile of the third purification by Sephadex LH-20 column.

3.5.2.2 Partial purification of mutagenic substances in 100% methanol eluent by Sephadex LH-20 column

The 100% methanol eluted from SEP-PAK cartridge was also further purified by the Sephadex LH-20 column(2 x 45 cm) which was eluted with absolute methanol. Each peak was pooled and dried by vacuum distillation. The remained residue was assayed for mutagenicity. The specific mutagenic activity was calculated as the number of $His^+$ revertant colonies per mg residue used. The main mutagenicity was also detected in the last peak (M-6) as shown in Fig. 26. The amount of this main mutagenic peak was too small and its specific mutagenicity was too low for further purification at the present. By thin-layer chromatography, this peak was not identical with the last peak(peak IV) separated from the 50% MeOH eluent. Their $R_f$ values were also different from each other as shown in Table 5.

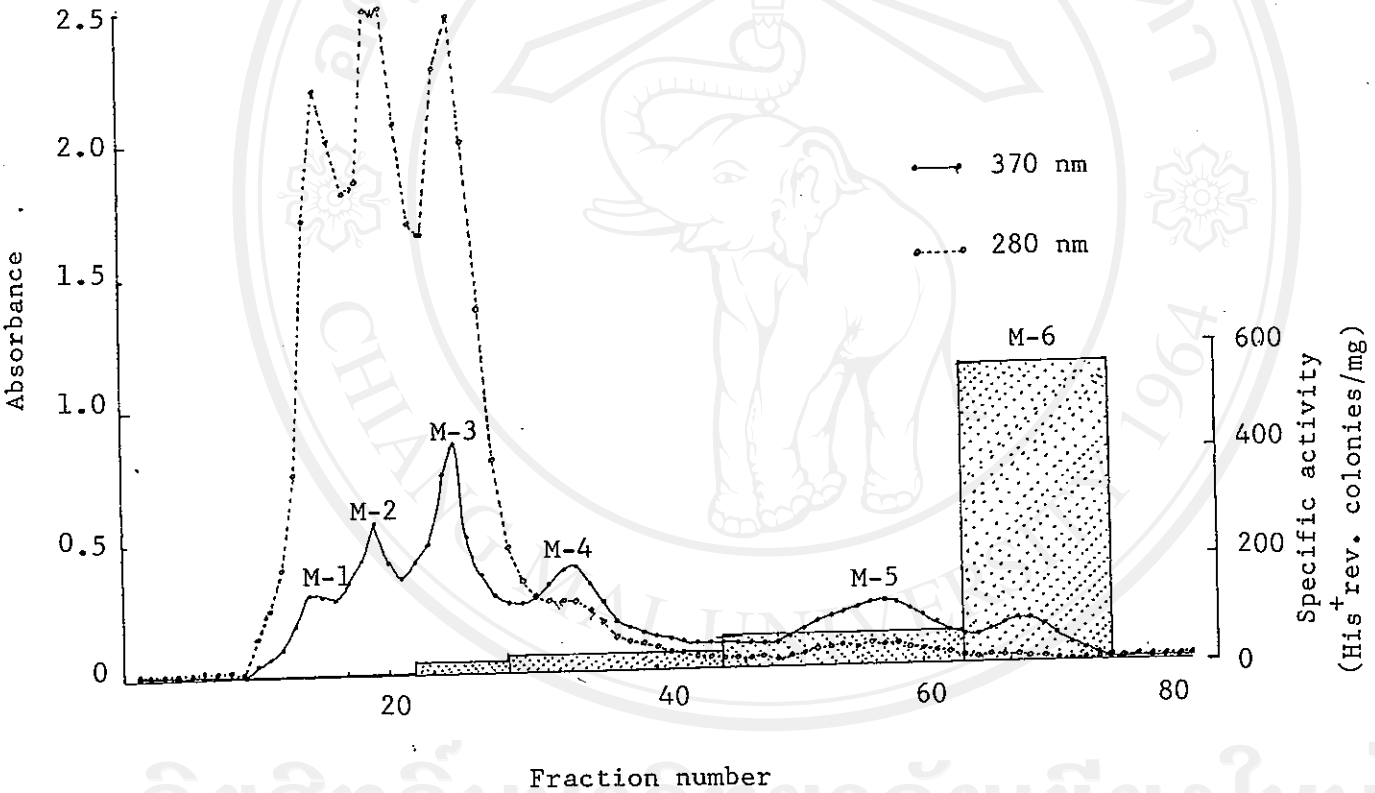



Fig. 26 Sephadex LH-20 column chromatographic profile of 100% MeOH eluate and their mutagenicity.

3.6 Characterization of the partially purified mutagenic substance (peak IV) in 50% methanol.

3.6.1 $R_f$ value on Silica gel 60 G

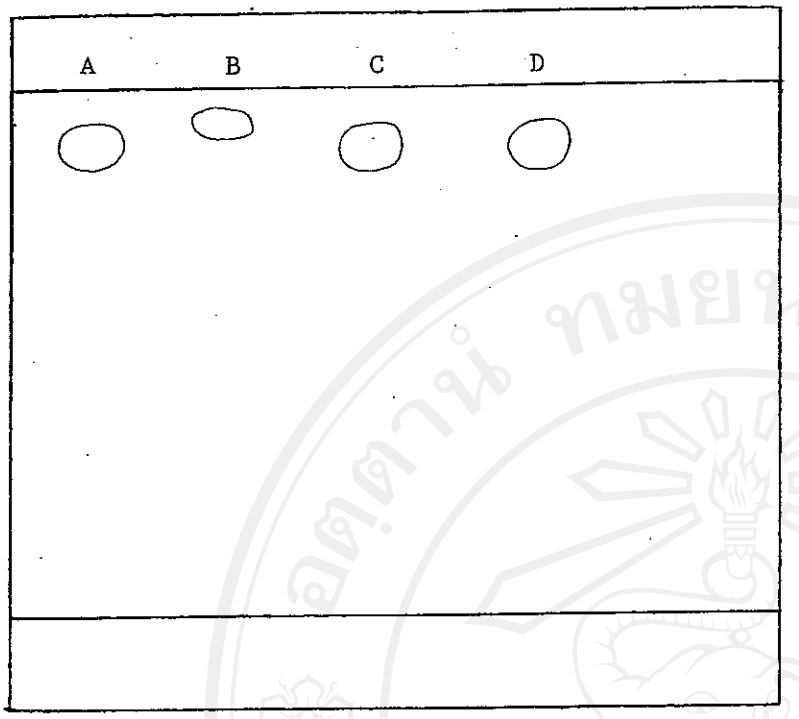
The partial purified mutagenic substance (peak $IV_{a2}$) showed the same $R_f$ value as authentic sample of quercetin in solvent system No. 1, but in solvent system No.2 its $R_f$ value was different from the standard flavonoid as shown in Table 5 and Fig. 27. Co-chromatography of this substance with standard quercetin using solvent system No.1, only one spot was detected, but by solvent system No.2, the peak $IV_{a2}$ substance showed $R_f$ value different from that of the mixture (quercetin + peak $IV_{a2}$).

Table 5 $R_f$ value of partially purified extract, authentic quercetin and kaempferol

| Substances | $R_f$ value | |
|---|---|---|
| | Solvent system No.1 | Solvent system No.2 |
| Peak $IV_{a2}$ | 0.892 | 0.864 |
| Peak $IV_{a2}$ + Quercetin | 0.892 | 0.871 |
| Peak M-6 | 0.914 | 0.850 |
| Quercetin | 0.892 | 0.875 |
| Kaempferol | 0.928 | 0.885 |

System No.1=Chloroform:Ethanol:Butanone:Acetyl acetone (16:10:5:1)

System No.2=Chloroform:Methanol:Water (65:45:12)



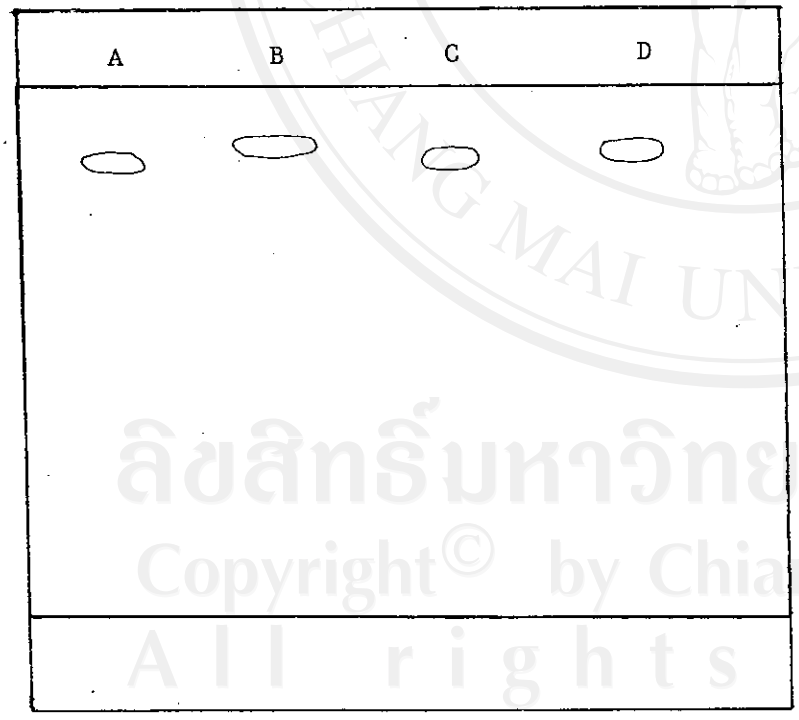

A : Quercetin

B : Kaempferol

C : Partially purified extract

D : Mixtures of quercetin and partially purified extract

Solvent system No.1 ; Chloroform:Ethanol:Butanone: Acetylacetone (16:10:5:1)

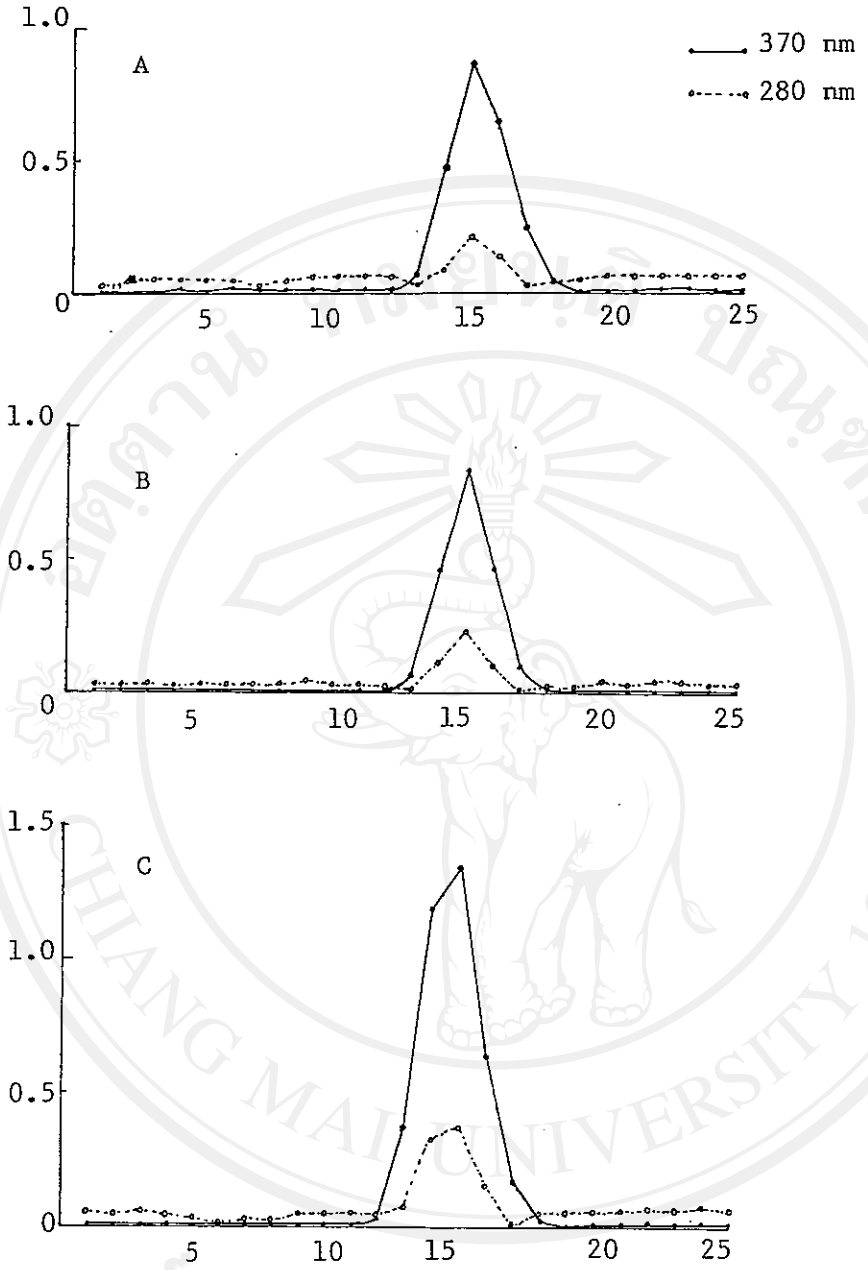



Solvent system No.2 ; Chloroform:Methanol:Water (65:45:12)

Fig. 27 TLC chromatogram of partially purified extract and standard samples.

3.6.2 Co-chromatography of partially purified mutagenic substance (peak $IV_{a2}$) with quercetin on Sephadex LH-20 column.

By Sephadex LH-20 column chromatography, The partially purified substance (peak $IV_{a2}$) showed similar peak as that of standard quercetin. However, Its co-chromatography with authentic quercetin exhibited the enlarged peak with a small shoulder as shown in Fig. 28 (C).




A : Partially purified substance (220 µg)

B : Standard quercetin (150 µg)

C : The mixtures of partially purified substance (220 µg) and authentic quercetin (100 µg)

Fig. 28 Chromatographic profiles of partially purified substance and authentic quercetin.

3.6.3 UV - spectra pattern of peak $IV_{a2}$ and known compounds.

The UV spectra of the peak $IV_{a2}$ was similar to that of standard quercetin (Fig. 30 a, and b). It was composed of 2 absorption peaks, the major peak was at the wavelength of 360-370 nm and another was at 250-260 nm.

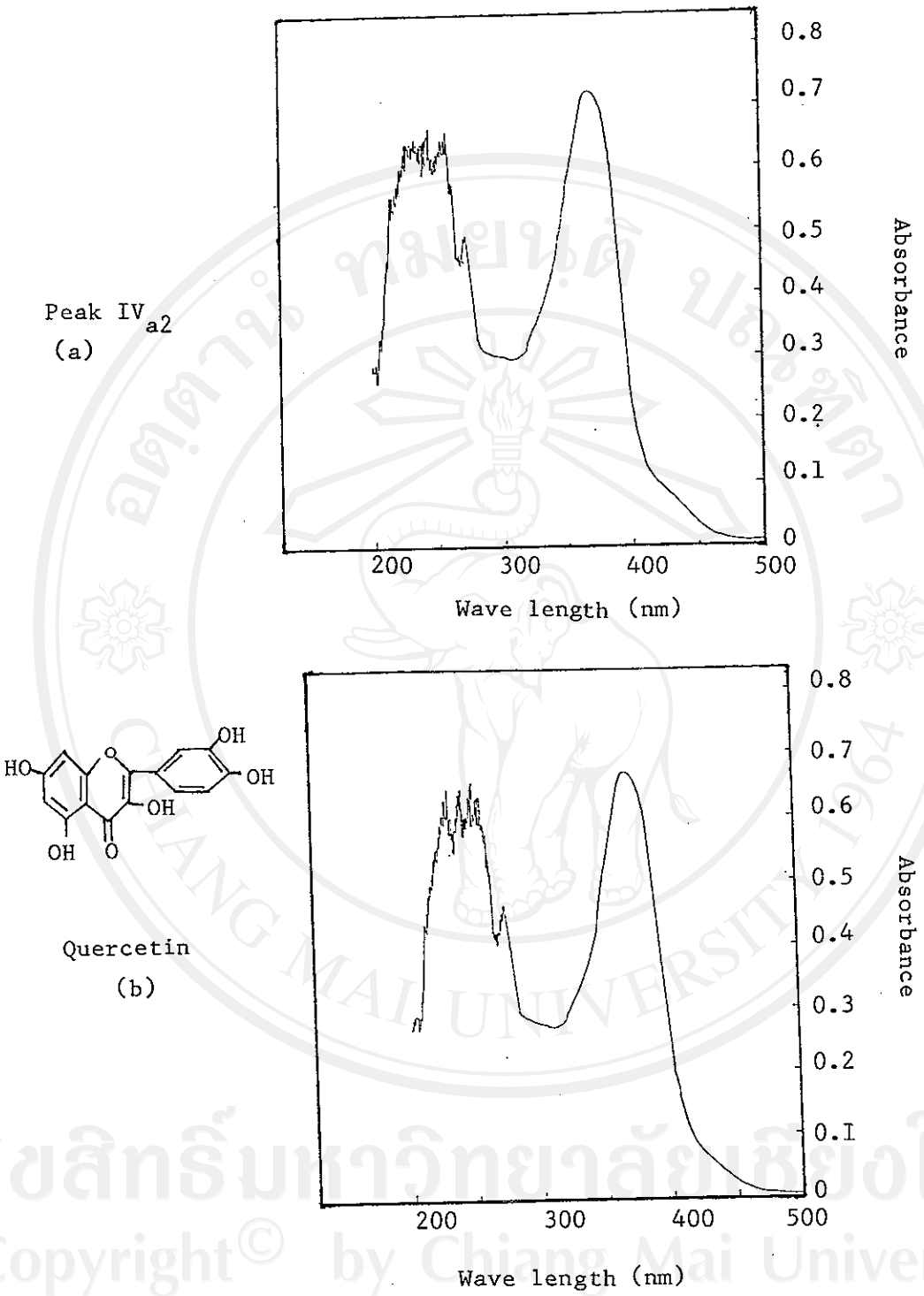
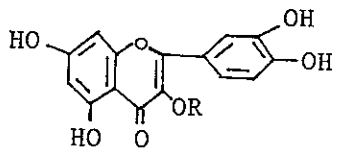


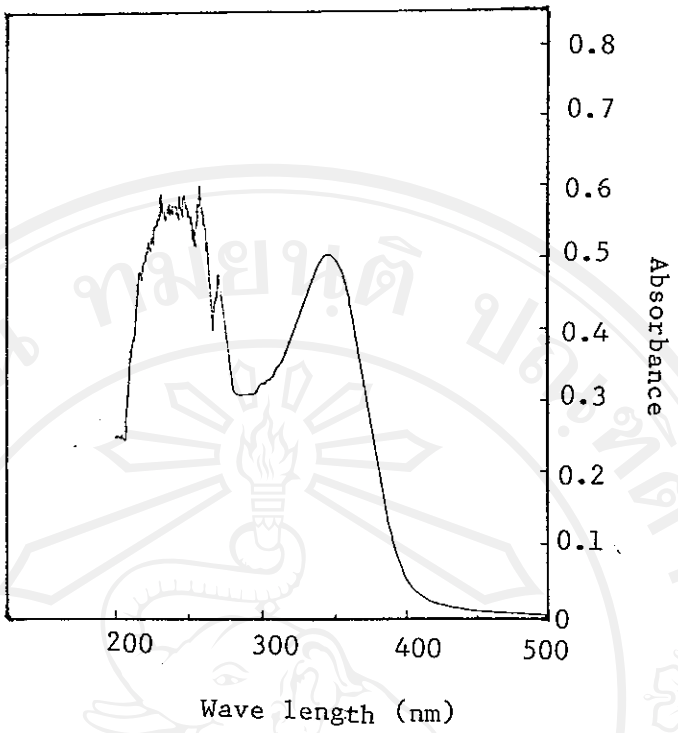

Fig. 29 UV-spectra pattern of peak $IV_{a2}$(a) and various standard flavonoids (b) quercetin (c) quercetrin and (d) flavanone.

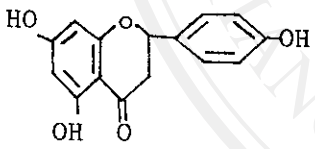



R = Rhamnose

Quercetrin

(c)




Flavanone

(d)

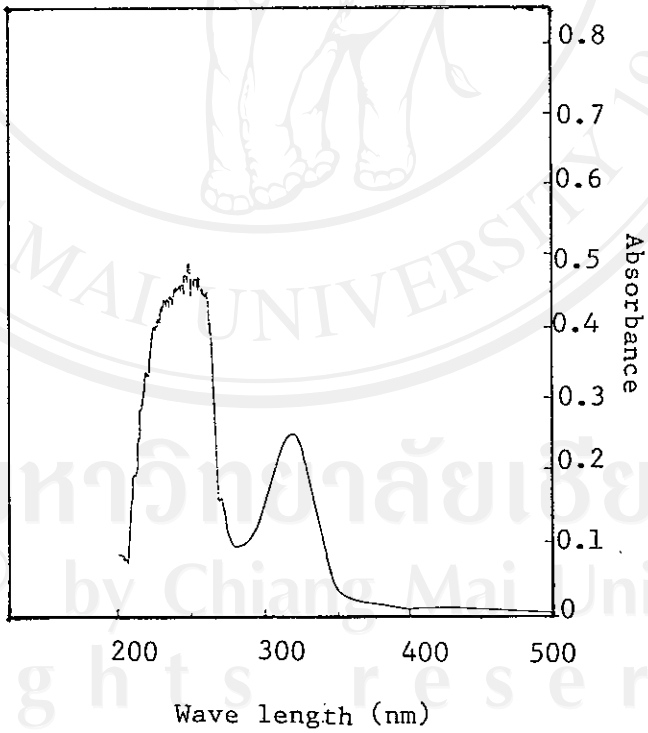



Fig. 29 (continued)