# CHAPTER IV

# RESULTS

## 1. Tyrosinase inhibitory activity of the crude extracts from *Mallotus spodocarpus* and *Excoecaria bicolor*.

Tyrosinase is a multifunctional enzyme that is found in fungi, plant materials and animal tissues and responsible for melanin biosynthesis. Because tyrosinase is the rate-limiting step in melanin synthesis, tyrosinase inhibitors have become increasing important as cosmetic and medical products. The objective of this experiment was to examine and compare tyrosinase inhibitory activity of the two euphorbiaceous plant extracts using mushroom tyrosinase assay. Positive control used for this assay was kojic acid. Its serial dilutions (0.58 μg/ml, 0.29 μg/ml, 0.145 μg/ml, 0.0725 μg/ml, 0.03625 μg/ml) were examined, as previously described. [76] The results showed that kojic acid inhibited muchroom tyrosinase activity in a dose-dependent manner (Figure 6). Then, various doses of the extracts from *Mallotus spodocarpus* and *Excoecaria bicolor* were screened for tyrosinase inhibitory activity. As shown in figure 7, the extract from *Mallotus spodocarpus* exhibited about 20% tyrosinase inhibitory activity at a minimum dose (0.125 mg/ml) and about 28% inhibition at the highest dose examined (2 mg/ml). On the other hand, *Excoecaria bicolor* extract at dose of 0.125 mg/ml exhibited about 40% inhibition and showed a dose-dependent pattern with approximately 80% inhibition at a dose of 2 mg/ml (Figure 8). Therefore, these results demonstrated that the extract from *Excoecaria bicolor* exhibited more potent inhibitory activity than that of *Mallotus spodocarpus*.

**2. Cytotoxic effect of the crude extracts in melanocyte cell-based assay.**

The objective of this experiment was to examine the cytotoxicity of the crude extracts using MTT assay in melanocyte cell line (CRL-1676). Melanocytes were exposed for 24 h to *Mallotus spodocarpus* extract or *Excoecaria bicolor* extract at various concentrations ranging from 0.125 mg/ml- 2 mg/ml. The results demonstrated that *Mallotus spodocarpus* extract was extremely toxic to melanocytes. As shown in figure 9, over 95% cell death was observed at a minimum concentration of 0.125 mg/ml. However, as shown in figure 10, *Excoecaria bicolor* extract at concentration of 0.125 mg/ml exhibited about 14% cytotoxicity and approximately 44 % cytotoxicity at a dose of 2 mg/ml.

**3. The crude extracts from from *Mallotus spodocarpus* and *Excoecaria bicolor* inhibit the expression of the tyrosinase and MITF RNAs.**

Although *Mallotus spodocarpus* extract appeared to be very toxic to melamocytes as shown in the previous experiments, fur ther to examine its effect together with that of *Excoecaria bicolor* extract on the expression of the tyrosinase and MITF RNAs. Melanocytes were exposed to *Mallotus spodocarpus* extract or *Excoecaria bicolor* extract at the concentrations of 10 μg/ml or 100 μg/ml for 24 h. As shown in figure 11 A and B, the crude extracts from *Mal lotus spodocarpus* and *Excoecaria bicolor* down-regulated the expression of the tyrosinase and MITF RNAs in a dose-dependent manner. However, *Mallotus spodocarpus* extract appeared to be more effective in lowering the expression of tyrosinase and MITF than that of *Excoecaria bicolor* extract (A, lanes 2, 3 vs lanes 4, 5; B, lanes 2, 3 vs lanes 4,5).

**4. *Excoecaria bicolor* crude extract increases the phosphorylation of ERK in a dose-dependent manner.**

An inhibitory effect of *Excoecaria bicolor* crude extract on signaling cascade leading to the expression of the tyrosinase and MITF RNAs was further examined by means of western blot analysis. Melanocytes were exposed to 10 μg/ml or 100 μg/ml concentration of *Excoecaria bicolor* crude extract for 24 h. Total cell lysates from untreated and treated cells were collected for SDS-PAGE. Equal amount of protein samples (100 μg/lane indicated by the expression of beta actin in each lane; figure 12B) was separated on acrylamine gel, transferred to PVDF membrane and probed with anti-phosphorylated form of ERK (at Thr202/Tyr204). Activation of ERK signaling cascade is involved in the degradation of MITF leading a decrease in the expression of tyrosinase RNA. As shown in figure 12A, the results demonstrated that *Excoecaria bicolor* crude extract increased the expression of phosphorylated ERK in a dose dependent manner. These findings suggest that *Excoecaria bicolor* crude extract may down regulate tyrosinase expression through the mechanism involved ERK phosphorylation.

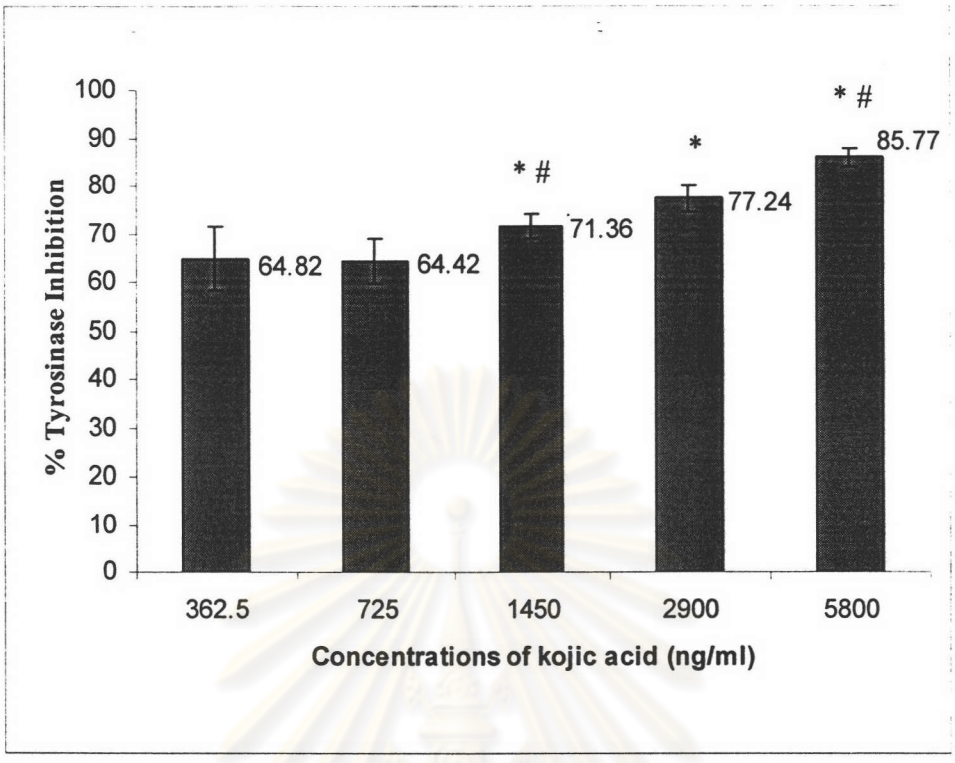


**Figure 6. The mushroom tyrosinase inhibitory activity of kojic acid.** evaluating % tyrosinase inhibition (described in material and method) of kojic acid that uses as positive control to various concentration. Represent the mean (±SEM) of five experiments.

* Significant of difference with 0.125 mg/ml by one way-ANOVA at $p<0.05$ (n=5)

# Significant of difference with 0.25 mg/ml by one way-ANOVA at $p<0.05$ (n=5)

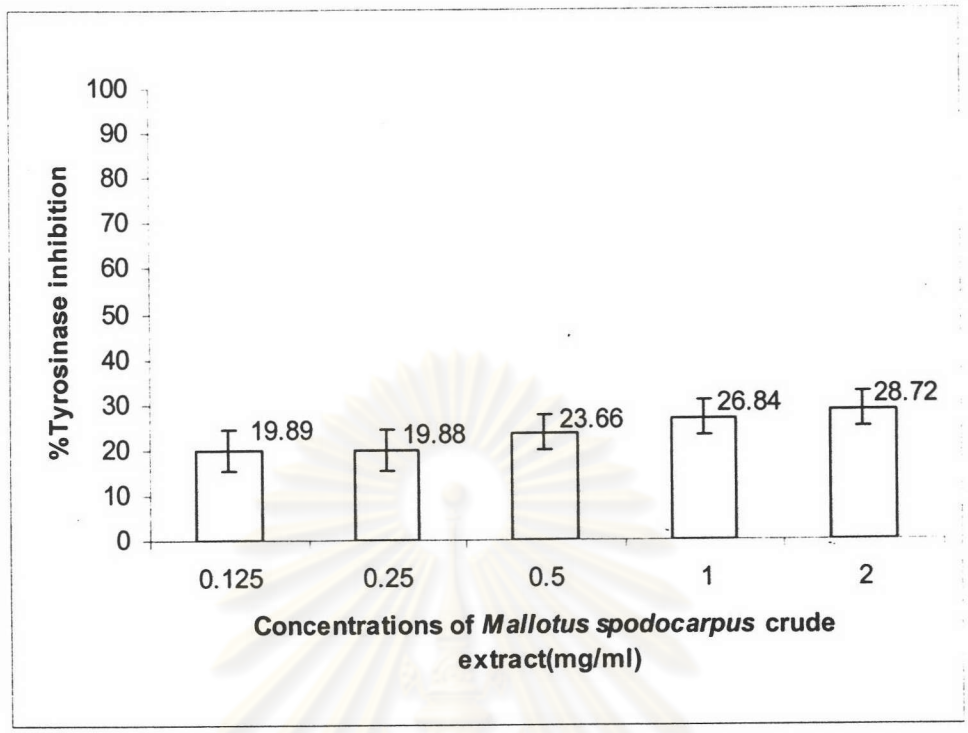


**Figure 7. The mushroom tyrosinase inhibitory activity of *Mallotus spodocarpus* crude extract.** evaluating % tyrosinase inhibition (described in material and method) of *Mallotus spodocarpus* crude extract to various concentration. Represent the mean (±SEM) of five experiments. There was no significant of difference among this experiment.

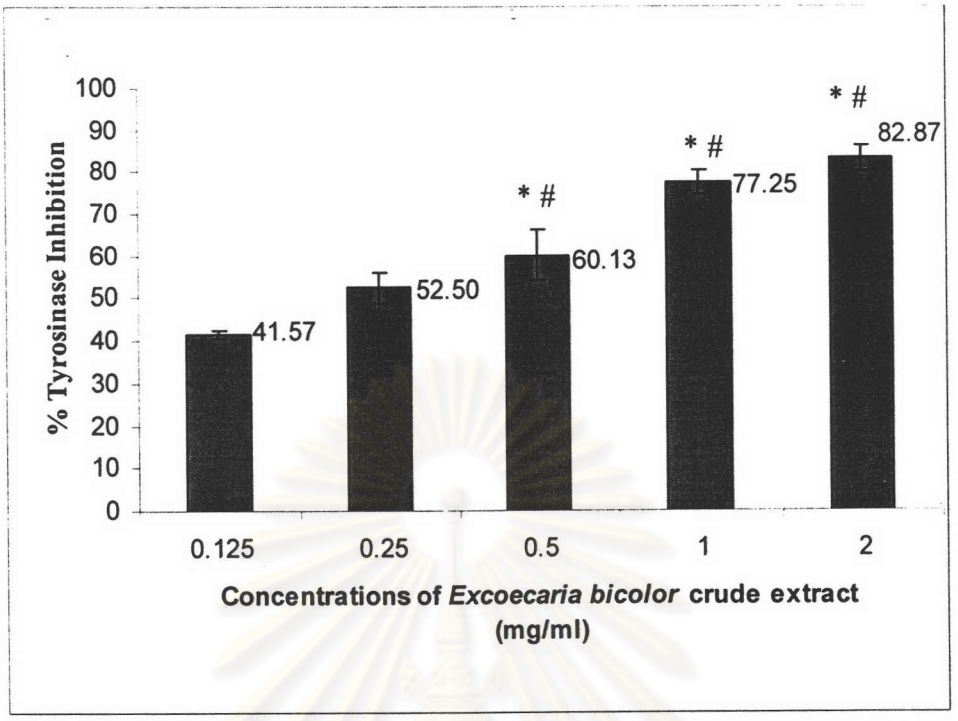


**Figure 8. The mushroom tyrosinase inhibitory activity of *Excoecaria bicolor* crude extract.** evaluating % tyrosinase inhibition (described in material and method) of *Excoecaria bicolor* crude extract to various concentration. Represent the mean (±SEM) of five experiments.

* Significant of difference with 0.125 mg/ml by one way-ANOVA at $p<0.05$ (n=5)

# Significant of difference with 0.25 mg/ml by one way-ANOVA at $p<0.05$ (n=5)

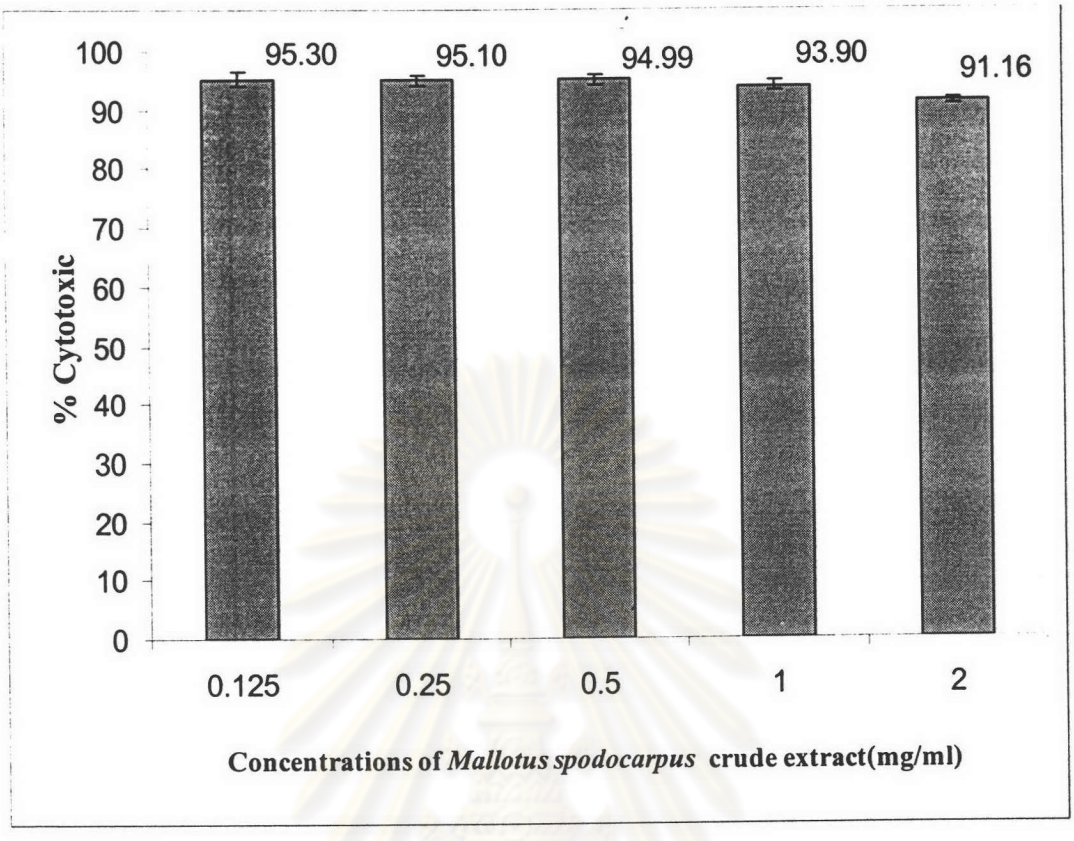


**Figure 9. Cytotoxicity of *Mallotus spodocarpus* crude extract in melanocyte cell line.** CRL-1676 cells was treated with *Mallotus spodocarpus* crude extract to various concentration at 24 h. Compared with the untreated control and represent the mean (±SEM) of four experiments. There was no significant of difference among this experiment.

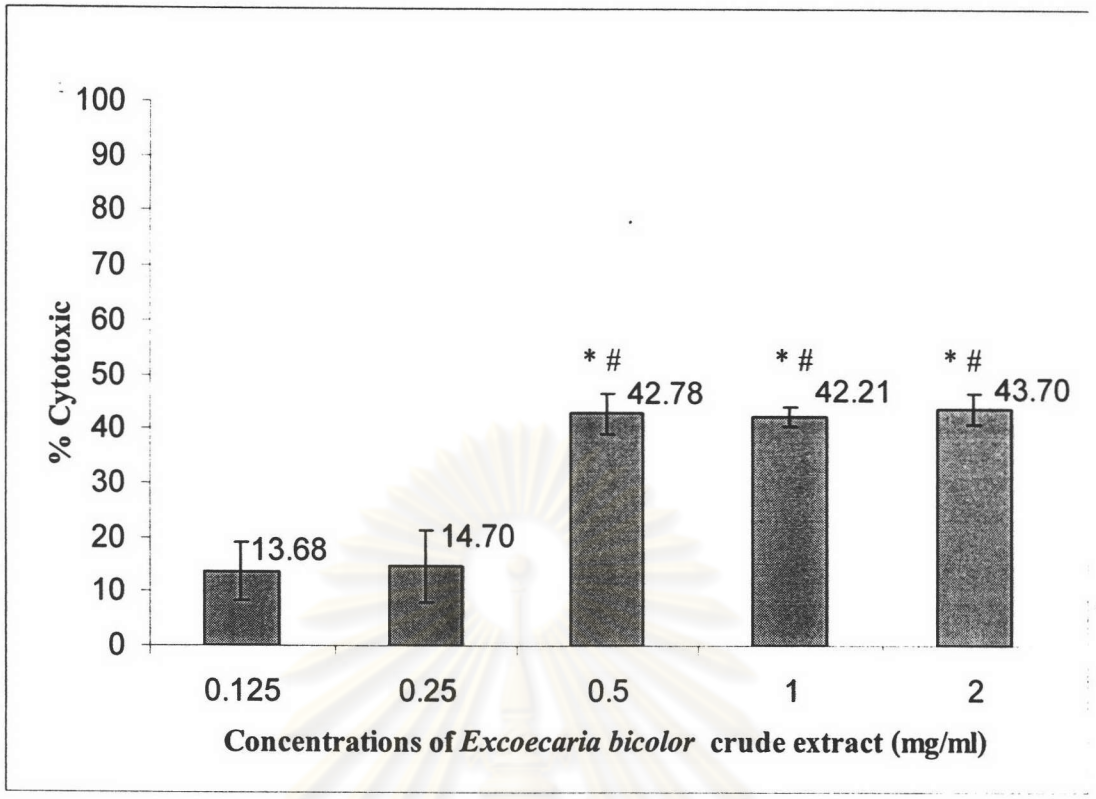


**Figure 10. Cytotoxicity of *Excoecaria bicolor* crude extract in melanocyte cell line.** CRL-1676 cells was treated with *Excoecaria bicolor* crude extract to various concentration at 24 h. Compared with the untreated control and represent the mean (±SEM) of four experiment.

* Significant of difference with 0.125 mg/ml by one way-ANOVA at $p<0.05$ (n=5)

# Significant of difference with 0.25 mg/ml by one way-ANOVA at $p<0.05$ (n=5)

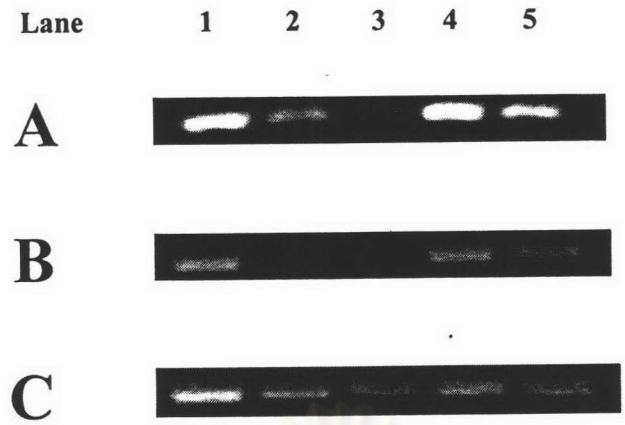


**Figure 11.** ***Mallotus spodocarpus*** **and** ***Excoecaria bicolor*** **crude extract induced gene expressions of tyrosinase and MITF in melanocyte cells.** CRL-1676 cells was treated with *Mallotus spodocarpus* and *Excoecaria bicolor* crude extract at concentrations of 10 μg/ml and 100 μg/ml. Cells exposed to the medium without crude extract were used as control. The representative bands were shown. (A) represents for result of tyrosinase gene expression, (B) represents for MITF gene expression, (C) represents for internal control β-actin. Bands in lane 1 indicated basal expression of tyrosinase and MITF in untreated control. Bands in lane 2, 3 that treated with *Mallotus spodocarpus* crude extract at 10 μg/ml and 100 μg/ml have shown an decreased expression of tyrosinase and MITF. Band in lane 4, 5 that treated with *Excoecaria bicolor* crude extract at 10 μg/ml and 100 μg/ml have shown decreased expression of tyrosinase and MITF.

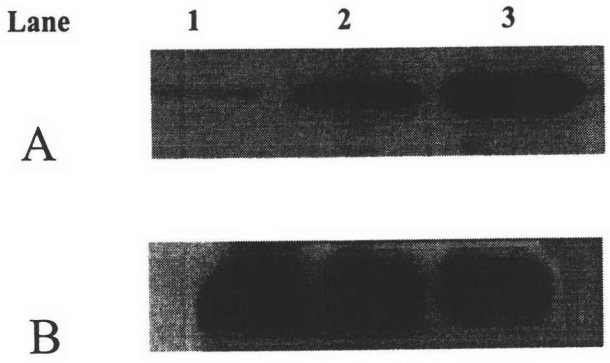


**Figure 12. *Excoecaria Bicolor* crude extract induced phospho-ERK protein expression in melanocyte cell line.** CRL-1676 cells was treated with *Excoecaria bicolor* crude extract at concentrations of 10 μg/ml and 100 μg/ml. Cells exposed to the medium without crude extract were used as control. The representative bands were shown. (A) represents for result of *Excoecaria bicolor* crude extract treated phospho-ERK protein expression, (B) represents for β-actin. Bands in lane 1 indicated basal protein expression of phospho-ERK in untreated control cells. Bands in lane 2, 3 showed an increased expression of phospho-ERK induced by crude extract treatment in a dose-dependent manner. The expression of β-actin in each sample was used as an internal control for loading.