CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

The dried powdered berries of *C. nervosum* var. *paniala* showed capacity of ethanolic extract more than water extract because the ethanol soluble extractive value was more than water soluble extractive value (5.94 and 1.92%). Its ash showed high soluble in acid and total ash less than < 1%. Its meaning crude drug from this plant showed less inorganic matter. From the result of phytochemical screening found that most type of the chemical constituent were phenolic content in form of tannin and flavonoid, less alkaloid content and absent of saponin, anthraquinone and coumarin. The results of phytochemical screening related with TLC.

The fresh ripe berries of *C. nervosum* var. *paniala* were preliminary extracted with several different methods such as squeezed to give juice, macerated with 50% ethanol, 95% ethanol or decocted with water, and dried by freeze dry, vacuum dry and spray dry methods. It was found that the ethanolic extract and dry by freeze dry (E95FD) and water extract and dry by freeze dry (WSD) showed the highest percentage of yield (3.22%, 3.13%) and also showed the highest antioxidant activity on both assay. This extract (E95FD) also exhibited the highest active compound such as total monomeric anthocyanin (5.36 mg/g), total phenolic content (111.28 mg GAE/g), catechin (0.26 mg/g) and quercetin (1.06 mg/g). However the residue of this extract still continued extract and found that this extract showed the highest quercetin (1.27 mg/g). The water extract and dry by freeze dry also showed the highest active compounds such as ascorbic acid (0.51 mg/g) and gallic acid (2.26 mg/g). Ascorbic acid solute in water and stable in low temperature so this compound was found in the water extract and dry by freeze dry. This result related with the previous report which found that the water extract found ascorbic acid and it stable in low temperature (Sirimerngmoon et al., 2006).

The content of ascorbic acid, catechin, cyanidin 3-glucoside, gallic acid, kaempferol and quercetin in the crude extracts of *C. nervosum* var. *paniala* from different extraction methods were evaluated by HPLC. HPLC analysis of each reference standard showed single peaks at retention times of ascorbic acid, catechin,

cyanidin 3-glucoside, gallic acid, kaempferol and quercetin were 4.70, 13.4, 22.8, 9.3, 83.4 and 80.4 min, respectively.

The major compound of all samples was cyanidin 3-glucoside. The contents of cyanidin 3-glucoside in all extracts were found as 0.29 to 2.19 mg/g dry weight. The highest average contents of cyanidin 3-glucoside (2.19 mg/g dry weight) were found in the E50FD extract follow by DWFD extract (1.78 mg/g dry weight) and E95FD extract (1.72 mg/g dry weight). This compound is polar compound so it was found by extraction with polar solvent such as water or 50% or 95% ethanol. The highest average contents of ascorbic acid (0.51 mg/g dry weight) were found in the WFD extract follow by WSD extract (0.27 mg/g dry weight). The highest average contents of catechin (0.26 mg/g dry weight) were found in the E95FD extract follow by REFD extract (0.21 mg/g dry weight). The highest average contents of gallic acid (2.26 mg/g dry weight) were found in the WFD extract follow by REFD extract (1.00 mg/g dry weight). The highest average contents of kaempferol (0.11 mg/g dry weight) were found in the E50FD extract and the others were less content of kaempferol. The highest average contents of quercetin (1.27 mg/g dry weight) were found in the REFD extract follow by E95FD extract (1.06 mg/g dry weight) and WFD extract (0.51 mg/g dry weight). These results are the first report of discovery these ingredients for health from its ripe fruits especially cyanidin 3-glucoside as major substance which make it be color.

Cyanidin 3-glucoside exhibited the highest scavenging activity, followed by quercetin, ascorbic acid and BHT by DPPH; EC_{50} were 1.23, 1.60, 2.26 and 12.26 μg/ml, respectively. E95FD extract showed the highest scavenging activity follow by E50FD extract, WFD extract) and REFD extract (EC_{50}= 15.08, 17.70, 18.68 and 19.36 μg/ml, respectively). The lowest scavenging activity were JuFD extract (EC_{50}= 182.22 μg/ml).

The inhibition of lipid peroxidation on liposome assay of the crude extracts of *C. nervosum* var. *paniala* from different extraction methods. REFD extract showed the highest inhibition of lipid peroxidation follow by E95FD extract, E50FD extract, WFD extract (IC_{50}= 39.45, 50.85, 101.95 and 186.20 μg/ml, respectively). The lowest inhibition of lipid peroxidation were DWFD extract (IC_{50}= 880.20 μg/ml).

The DPPH radical-scavenging activity was correlated with the content of phenolics compound. This study was strong correlated with the findings of previous studies (Mahor *et al*, 2006). The inhibition of lipid peroxidation in liposome assay also gave a similar result. Therefore, these activities seemed to be due to polyphenols. The ethanolic extracts of *C. nervosum* var. *paniala* show the higher inhibition of lipid peroxidation than the water extract. Meanwhile the extracts from dried berries of *C. nervosum* var. *paniala* did not demonstrate effective antioxidant activity.

The crude extracts of *C. nervosum* var. *paniala* from different extraction methods were tested with two different types of human carcinoma cell lines (HeLa and COR L23) and one type of human normal cell lines (MRC-5, lung fibroblast) in this study. The REFD extract of was the highest percentage of inhibition at concentration 100 µg/ml of HeLa, COR L23 and MRC-5 as 94.78, 42.88 and 93.54%, respectively. The E50FD extract showed the second effective activity against to all cells line, Hela, CORL-23 and MRC-5 as 32.38, 35.90 and 33.73 ± 3.16%, respectively. The E95FD extract showed the third cytotoxic activity. The JuFD, WFD, WSD, DWFD and DEFD were less effective to all cells.

The data showed that ethanolic extracts of *C. nervosum* var. *paniala* had the higher cytotoxic activity than the water extracts. Although ethanolic extracts of *C. nervosum* var. *paniala* exhibited the highest cytotoxic activity against HeLa and lung cancer, but also showed cytotoxic against normal. This indicates that they have nonspecific cytotoxic activity because they killed all types of cancer and normal cells. However these extract showed less cytotoxic activity because IC_{50} value were more than 50 µg/ml. According to National Cancer Institute guidelines, IC_{50} values of the extracts should be less than 20 µg/ml, it will be considered as “active”. Thus, this result was concluded that the ethanolic and water extracts from the riped berries of *C. nervosum* var. *paniala* had no cytotoxic activity against cancer cell line.

From these data could be concluded that the method of extraction which made values for health was 95% ethanolic extract and dried by freeze dry (E95FD) because it showed the highest antioxidant activity on DPPH assay (EC_{50}=15.08 µg/ml) showed high activity on lipid peroxidation (IC_{50}=50.85 µg/ml) but there was no cytotoxic activity. However the residue from this extract still had this activity and also

showed the highest activity for lipid peroxidation (IC_{50}= 39.45 μg/ml) and high antioxidant for DPPH (EC_{50}= 19.36 μg/ml). The major compound for antioxidant were cyanidin 3-glucoside because it was found highest amount and also exhibit the highest antioxidant activity by DPPH assay (EC_{50}= 1.23μg/ml). The active ingredient for health still found such as quercetin in this extract and its residue. The water extract by boiling method showed less activity and less active ingredients for health than ethanolic extract. Its juice and freeze dry showed low antioxidant activity by both assay and also showed less active antioxidant compound such as cyanidin 3-glucoside. From the extraction method was concluded that the extraction for health product should used 95% ethanolic extract because it showed highest activity and active component for health such as quercetin, cyanidin 3-glucoside and catechin. The water extract from fresh ripe fruit gave the highest gallic acid and vitamin C content. However water extraction method should be boiled and dried by freeze dry better than spray dry because this extract showed health compounds content and high antioxidant activity more than dried by spray dry. The comparison between ripe fruits and dry fruit found that the fresh fruit showed higher antioxidant activity than dry fruit. The preparation for health product for health should used ethanolic extract from fresh ripe fruit. For food product should be wine because used ethanolic and water extract which showed high antioxidant activity on both assay and the highest anthocyanin in for cyanin 3-glucoside. For cosmetic, 95% ethanolic extract and dried by freeze dry would be the best method for fruit extraction because this method gave high antioxidant ingredients such as quercetin, catechin. These ingredients were used mostly in cosmetic and made in liposome for nano product.

The nutrition value was determine and found that E95FD extract and WFD extracts composed with the major compound as carbohydrate (89.6 - 92.7 %), and rich of mineral such as, sodium, potassium, calcium and magnesium (9.1 - 23.7, 10.5 - 15.2, 1.2 - 2.2 and 0.8 - 2.5 mg/kg, respectively). The crude fiber and heavy metals (lead and iron) were not found in the extracts.

E95FD extract which exhibited the best antioxidant activity on DPPH assay was also isolated pure compound by bioassay guild fractionation of VLC and HPLC method and elucidation by proton NMR and EIMS. A pure compound was identified

as cyanidin 3-glucoside by comparison spectrum data from previous publication (Robert *et al*, 2007) and confirmed with authentic standard (Fluka product) by HPLC for 3 solvent systems.

The stability of E95FD and WFD extracts was also studied by kept at accelerated condition (45 °C with 75% relative humidity for 120 days). Results showed that the physical properties of the extracts changed according to time especially stickiness and color. The color of both of extracts kept in this condition changed rapidly. The color changed (ΔE) of the E95FD extract was more than 2 at days 22 and color changed (ΔE) of the WFD extract was more than 2 at days 8. The characteristic of both extracts changed from purplish-red powder to purplish-black stickiness. The color changed (ΔE) of the E95FD extract and WFD at the end of this condition (120 days) were 4.51 and 5.28, respectively.

The amount of active ingredients measured as total phenolics compound, cyanidin-3-glucoside, and quercetin content in the extracts were also quickly decreased. In this condition, the total phenolics compound content of E95FD and WFD extracts were 43.9 and 43.2%, respectively after 120 days. Quercetin content of E95FD and WFD extracts were 43.9 and 43.2%, respectively. While cyanidin 3-glucoside content was 0% after 30 days of both E95FD and WFD extracts. The effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) of both E95FD and WFD extracts were more than 2 times after 22 days (compared with 0 day). After 120 days the EC_{50} of both E95FD and WFD extracts were more than 200 μg/ml.

The pH of both E95FD and WFD extracts rather stable after 120 days, however the color of the extracts were changed. It might concern with the monomeric anthocyanin, the color profile of the extract. The content of total monomeric anthocyanin was 0 % after 120 days, in contrast the polymeric anthocyanins were increased from 51.9 to 84.7% and 58.4 to 83.3% of E95FD and WFD extracts, respectively. The results indicated that the monomeric anthocyanin transformed into polymeric anthocyanin. However, the antioxidant activities of the extracts were low.

The stability of E95FD and WFD extracts were also kept at ambient condition (25 - 32 ^{o}C with 55 - 60% relative humidity for 180 days). Results showed the physical properties of the extracts slowly changed according to time especially

stickiness. The color of both extracts kept in this condition was slowly decreased. The color changed (ΔE) of the E95FD and WFD extract less than 2 after 180 days. The characteristic of both of the extracts was few changed from purplish-red to deep purplish-red, but the extracts changed from powder to stickiness at the end of this condition.

The amount of active ingredients measured as total phenolics compound, cyanidin-3-glucoside, and quercetin content in the extracts were also slowly reduced. In this condition, the total phenolics compound content of E95FD and WFD extracts were 70.3 and 66.6%, respectively after 180 days. Quercetin content of E95FD and WFD extracts were 60.4 and 29.7%, respectively. While cyanidin 3-glucoside content of E95FD and WFD extracts were 31.5 and 23.0%, respectively. The effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) of both E95FD and WFD extracts were more than 2 times (compared with 0 day) after 45 days. After 180 days the EC_{50} of E95FD and WFD extracts were 83.8 and 88.6 µg/ml, respectively.

The pH of both E95FD and WFD extracts rather stable after 180 days, however the color of the extracts were also few changed. The content of total monomeric anthocyanin content in E95FD and WFD extracts were 60.4 and 50.2 %, respectively after 180 days, meanwhile the polymeric anthocyanin were increased from 51.9 ± 0.57 to 62.2 ± 0.84% and 58.4 ± 1.32 to 62.9 ± 0.48% of E95FD and WFD extracts, respectively.

From the stability results concluded that Ma-kiang extract by water or ethanol were neither stable at room temperature nor higher than room temperature so the storage crude extract should be keep in freezer. This results related with previous result of Sirimerngmoon *et al* (2006) which found the anthocyanin, total phenolic content, ascorbic acid and antioxidant activity were decreased at high temperature. The storage of the extract from Ma-kiang should be kept in freezer or 0 ^{o}C which Khemsawat *et al (*2006) reported. Development of its extract should be added some adsorbent for adsorption moisture such as lactose because it would make extract dry. If the extracts dry, the component would be more stable than no adsorbent. Moisture or water made the antioxidant activity change because the oxygen from moisture would make oxidation reaction which some ingredients especially anthocyanin.

From this study it could be concluded that the method of extraction of Ma-kiang ripe fruits should be 95% ethanol because showed high active ingredients for health. The temperature of extract should avoid high temperature and fresh fruit extract would be better than dry extract. The health product from this plant should be wine, extract in tablet or capsules because the taste of the ethanolic extract was nearly bitter and little astringent. The ethanolic extract should also used for nano-cosmetic such as Ma-kiang liposome for anti-aging because the process of trapping extract in liposome should used ethanolic extract which liposome could dissolve in ethanol so the ethanolic extract prepared in liposome was better than water extract. The residue from ethanolic extract should also be used in health product because it showed high health ingredients and high antioxidant activity. It should continue studied for fiber content for apply to use for obesity product. However the water extract could make good health product but the dry method should used vacuum freeze dryer method because the ripe berries were not stable in high temperature and adsorbent should also be added in this extract for moisture protection and prolong the extract. Thus, the storage the extract from both solvent extracts should be kept in the freezer. The marker or active ingredients of analysis for extract from Ma-kiang on both solvent extractions should be cyanidin 3-glucoside because it was found high amount and it was also showed the highest antioxidant activity.