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# An Analysis on Flavonoids Contents in Mao Luang Fruits of Fifteen Cultivars (*Antidesma bunius*), Grown in Northeast Thailand

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**Abstract:** This investigation was carried out at the Department of Biotechnology, Faculty of Technology, Mahasarakham University, Mahasarakham Province, Northeast Thailand. The study aimed to analyze flavonoids contents in ripe fruits of fifteen Mao Luang cultivars (*Antidesma bunius*) harvested from dipterocarp forest of the mountainous areas of Phupan Valley, Sakon Nakhon Province, Northeast Thailand. The experiment was laid in a Completely Randomised Design (CRD) with five replications. The fifteen cultivars were used as treatments. An amount of 2 kg of ripe fruits of each cultivar was collected and extracted for juice solutions. The analysis was carried out with the use of RP-HPLC laboratory system. The results showed that fruits of the fifteen Mao Luang cultivars contained three different kinds of flavonoids, i.e., catechin, procyanidin B1 and procyanidin B2. These three chemical compounds were the major flavonoids in all analyzed fruit samples of the fifteen cultivars. The highest amount of procyanidin B1 was found with Lompat followed by Maeloogdog with values of 4,122.75 and 3,993.88 mg 100 $g^{-1}$ of fresh weight, respectively and the highest amount of procyanidin B2 was found with Sangkrow 2 followed by Fapratan with values of 5,006.39 and 3,689.42 mg 100 $g^{-1}$ of fresh weight, respectively. Catechin contents in fruits of the fifteen cultivars varied from 73.39 to 316.22 mg 100 $g^{-1}$ of fresh weight for Sangkrow 5 and Fapratan, respectively where Fapratan was the highest among the fifteen cultivars followed by Sangkrow 2 with values of 316.22 and 175.40 mg 100 $g^{-1}$ of fresh weight, respectively. In terms of grand total amounts of flavonoids, Sangkrow 2 was the best followed by Fapratan, Sangkrow 1 and Maeloogdog, whilst the rest were of secondary importance.

**Key words:** *Antidesma bunius*, catechin, mao luang fruits, procyanidin B1, procyanidin B2, RP-HPLC

## INTRODUCTION

Mao Luang (*Antidesma bunius*) is one of many different kinds of medicinal plants in Thailand. The name of Mao Luang is a common Thai name, this crop plant is commonly known to local people in Northeast Thailand as a medicinal plant. The plant is normally found in many dipterocarp forested areas in Thailand and also throughout the region of Southeast Asia (Roger, 2004). In Thailand, it is naturally grown in many forested areas of dipterocarp forests, particularly in Northeast. Its small oval shape of fruit when ripe has an attractive bright red appearance with a mixture taste of sweet and sour where the fruit produces a distinctive flavour. The local people in Thailand, particularly northeastern region traditionally used the fruits when ripe as a medicinal plant for gastric intestinal problems, e.g., diabetes, dysentery, indigestion and constipation. This crop plant normally flowers in the rainy season and able to produce some considerable amounts of ripe fruits in late rainy season (August-September). That is why some plentiful quantities of Mao Luang fruits have been used as a raw material for making tasty jelly jam, drinking juices, juice concentrate and even wine. Nowadays, the products have been used widely among the Thai people. Furthermore, this crop plant has been considered to be a good source of bioactive substances of flavonoids. The flavonoids are commonly known as bioactive substances and it is an important group of secondary plant compounds. The substances play its important role as protective agents against fungus and UV irradiation and also it has some positive effects on human health where the derived compounds in fruits and in beverages possess their nutritive values in man nutrition, particularly the healing of Coronary Heart Disease (CHD) as stated by Hertog *et al.* (1997) and it was found that it could even reduce platelet aggregation, anti-oxidative, anti-carcinogenic protection as found by Knekt *et al.* (1997), Kuulasmaa *et al.* (2000) and Stoclet *et al.* (2004) and also anti-cancer in man as reported by Hollman and

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Katan (1999). The substances of flavonoids are of a large family of over 4,000 ubiquitous secondary plant metabolites, which could be further divided into five subclasses, i.e., flavonols, flavones, anthocyanins, catechins and flavonones as reported by Merken and Beecher (2000). Catechins (flavan-3-ols) and their oligomers are known as procyanidins where two of the major groups of flavonoids possess bioactive compounds, i.e., procyanidin B1 and procyanidin B2. These two substances play an important role in man nutrition as mentioned earlier. Thus the Thai villagers have been using the various products derived from ripe fruits of Mao Luang trees for a number of years. The compounds of procyanidins are of particular interest to man owing to their potent antioxidant activities, ability to scavenge free radicals and nitrogenous compounds (Hagerman *et al*., 1998; Ariga and Hamano, 1990; Arpentine *et al*., 1992; Arteel and Sies, 1999) and it has a protective property against cardiovascular disordered (Teissedre *et al*., 1996; Mangiapane *et al*., 1992; Rein *et al*., 2000). Structurally, procyanidins composed of polyhydroxyl flavan-3-ol units (+)-catechin or (-)-epicatechin and it existed in many kinds of fruits and vegetables as monomers and also in more complex polymeric forms, such as when two monomers condense to form dimers where the derived chemicals include catechin, procyanidins B1 and B2. Oligomeric procyanidins (polymers of the monomeric forms) possess their antioxidant activities in man, which could be increased linearly with the number of reactive catechol and/or pyrogallol groups (Lotito *et al*., 2000).

For industry, flavonoids could be processed by conventional techniques to produce some useful products such as paper, thin-layer and column chromatography, where the process could be time-consuming and rather labourious in carrying out its quantitative analysis. For the past decades, the use of a technique known as Reversed Phase of High Performance Liquid Chromatography (RP-HPLC) has been considered to be a useful technique for the analysis of flavonoids where the methods have been developed to analyze catechins and procyanidins in fruits and other beverages. The UV-visible spectra of catechins and procyanidins provide an absorption range up to a maximum where it ranges between 250 and 400 nm (Revilla *et al*., 1991). Nowadays, it could possibly be inferred that little quantitative information is available on catechin and procyanidins contents derived from the various cultivars of Mao Luang fruits. For this work, RP-HPLC equipped with Diode Array Detector (DAD) was used to simultaneously analyze catechins and procyanidins extracted solutions from Mao Luang fruits where the method could achieve a rapid result with high accuracy and suitable for quantitative assessment in searching for the compounds of flavonoids out of the fruits. Therefore, it is of important value to carry out laboratory work with the use of a Reverse Phase-High Performance Liquid Chromatography equipment (RP-HPLC) in order to obtain information on important chemical compounds in fruits of Mao Luang medicinal plants found in Northeast Thailand so that in the near future some industrial products such as canned juices and other products derived from fruits of this crop could possibly play a significant role in the Thai economy, e.g., juices of Mao Luang crop have been recently served to passengers as a juicy drink by Thai Airways International.

## MATERIALS AND METHODS

This investigation was carried out during the rainy season of the 2006 at the Department of Biotechnology, Faculty of Technology, Mahasarakham University, Northeast Thailand to determine amounts of flavonoids chemical compounds, i.e., Procyanidin B1, Procyanidin B2 and Catechin in ripe Mao Luang fruits. Before the starting of the laboratory analysis, some lab materials required for the analysis had been prepared, they include methanol, acetonitrile and phosphoric acid of HPLC grade (Tedia Company, USA). Deionized water was prepared by a Milli-QWater Purification system (Millipore, MA, USA). Procyanidin B1, Procyanidin B2 and Catechin standards obtained from Sigma Company (USA) were used. Standard stock solutions of these three flavonoids were prepared with methanol at a concentration of 0.50 mg $mL^{-1}$. All of the solution samples were filtered through with a 0.45 μm membrane filter (Millipore) and then they were ready for further analysis. For Mao Luang fruits collection, fruits of fifteen cultivars of the different local names in Thai i.e., Sangkrow 2, Fapratan, Sangkrow 1, Maeloogdog, Phuchong, Sangkrow 4, Sangkrow 3, Sangkrow 5, Nonkloy, Lompat, Kumlhai, Sangkrow 8, Sangkrow 7, Chomphupan and Sawang were collected. The cultivars were used as treatments. Thus the experiment was carried out in a Completely Randomized Design (CRD) with five replications. An amount of 2 kg of ripe fruits of each cultivar was collected from the mountainous areas of dipterocarp forest of Phupan Valley, Sakon Nakhon Province, northeastern region of Thailand during the rainy season of the 2006. Fruits of each cultivar were separately squeezed for fruit juices and then filtered through with the use of a filter paper Millipore mentioned earlier. An amount of 200 g of juices of each cultivar were packed into two plastic bags, i.e., each bag has an amount of 100 g and then the packages of all cultivars were stored in a deep fridge at -20 ±2°C before the samples were used for lab analysis. Juice samples of each cultivar were duplicated five times and used for the analysis.

Laboratory processes of the analysis were carried out with the use of the method described by Hakkinen and Auriola (1998) and Hakkinen *et al.* (1999), i.e., the extracted fruit juice samples of 5 g were hydrolysed into a 50% (v/v) aqueous methanol containing both hydrochloric acid (1.2 M) and antioxidant (ascorbic acid). The mixtures of the samples were refluxed at 85°C for 2 h then filtered through with the use of filter papers of 0.45 μm membrane filter (Millipore) then the samples were ready of a direct testing. Before testing the samples, RP-HPLC (Reverse Phase-High Performance Liquid Chromatography) apparatus consisted of Shimadzu (Shimadzu Cooperation Analytical and Measuring Instruments Division Kyoto, Japan) LC-20AD Series pumping system, SIL-10AD Series Auto injector system and SPD-M20A Series Diode array detector was used to record online UV spectra of the organic acids of the samples. The data were collected and analyzed with a Shimadzu Computing System. The column being used was an Apollo $C_{18}$ (Alltech) (ø 4.6×250 mm, 5 μm) protected with guard column Inertsil ODS-3 (4.0 mm, 10 mm, 5 μm). Twenty-microlitre samples of each sample were analysed with the use of the HPLC system. The mobile phase was carried out with an acetonitrile-deionized water (2:97.8, v/v) containing 0.2% phosphoric acid (solvent A) and acetonitrile-deionized water (97.8:2, v/v) containing 0.2% phosphoric acid (solvent B) at a flow rate of 0.6 mL $min^{-1}$ and the column temperature was maintained at 40°C. The UV-Vis spectra were recorded from 190 to 400 nm and being read at 254 nm. The linear gradient started with 20% solvent B, 50% solvent B for 30 min, 60% solvent B for 35 min, 20% solvent B for 40 min with isocratic elution up to 55 min. Quantification was carried out by an integration of the peak values using an external standard method.

The following parameters for each flavonoid were determined: linearity, precision, accuracy and sensitivity. Calibration graphs being used for linearity determination were established for the flavonoids solutions and each was duplicated five times. The prepared standard samples for each flavonoid include procyanidin B1 (1-100 mg $L^{-1}$), procyanidin B2 (1-100 mg $L^{-1}$) and catechin (0.50-100 mg $L^{-1}$). A precision system was performed, i.e., an injection of five times of the mixture of the same standard was carried out and tested with the use of the RP-HPLC. The Coefficient of Variation (CV) for the reproducibility of the RP-HPLC analysis was calculated for each flavonoid. Accuracy of the results was determined by calibration curves derived from standard solutions of the flavonoids against the attained results. Sensitivity was evaluated by determining the Limit of Detection (LOD) and the Limit of Quantification (LOQ). LOD was performed to provide a peak with a signal-to-noise ratio of 3, whereas LOQ was the lowest amount with a signal-to-noise ratio of 10. The collected data of flavonoids profiles were statistically analysed using SPSS Version 10.0.1 Computer Program for Duncan's Multiple Range Test (SPSS, 2001).

## RESULTS

**RP-HPLC Chromatogram figures of the three flavonoids:** The results derived from RP-HPLC Chromatograme of the three flavonoids are shown in Fig. 1 where the peaks of responses were separately found at a wavelength of 254 nm. The peak result found at 254 nm was higher than the one detected at 360 nm. Thus 254 nm reading wavelength was, more or less, the most suitable wavelength for the determinations of flavonoids of catechin, procyanidin B1 and procyanidin B2 chemical substances.

**Flavonoids laboratory calibrations:** The calibration curve for each flavonoid was established by injecting five different concentrations of the standard mixtures to the RP-HPLC equipment. Other results, such as retention time, Limit of Detection (LOD), linear range and correlation coefficient ($R^2$) were shown in Table 1. Results showed that the LOD of each flavonoid was relatively low (0.50-10 mg $L^{-1}$), where the figure indicates a high accuracy of the method and it was maintained at a high sensitivity level, i.e., correlation coefficient values ranged from 0.9967 to 0.9999 for procyanidin B2 and procyanidin B2, respectively

**Reproducibility of RP-HPLC analysis:** The reproducibility of the RP-HPLC analysis was carried out in two ways, i.e., retention time and peak reading. In this method, the Coefficient of Variation (CV) for the reproducibility of the HPLC analysis was obtained through five injections of the same standard mixture and the results are shown in Table 1. To attain repeatability (intra-day precision) of the RP-HPLC, a hydrolyzed Mao Luang sample of each cultivar was repeatedly analyzed for 5 times. The coefficients of variation (CV) were between 0.11 to 1.05% and 1.09 to 4.07% of retention time and peak reading, respectively. Within-laboratory reproducibility (inter-day precision) of the whole method (including sample extraction and hydrolysis plus HPLC analysis), the readings were carried out by duplications of the hydrolyzed Mao Luang samples within 5 days during a period of 5 weeks of testing. The CV attained ranged between 1.12 to 1.47% of retention time and 3.65 to 5.21% of peak area for flavonoids of ripe Mao Luang fruits were

Table 1: Laboratory calibrations for flavonoids of catechin, procyanidins B1 and B2 being used for the determinations of flavonoids contents in Mao Luang ripe fruits

| Compounds | Retention time (min) | Linear ranges (mg $L^{-1}$) | Calibration values | Correlation coefficients ($R^2$) | Detection limit (mg $L^{-1}$) | Retention time (%CV) | | Peak area (%CV) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Intra-day | Inter-day | Intra-day | Inter-day |
| Catechin | 8.973 | 0.01-100 | Y = 8089.0X - 4882.7 | 0.9998 | 0.50 | 0.11 | 1.27 | 4.07 | 4.94 |
| Procyanidin B1 | 14.139 | 1.00-100 | Y = 2131.6X - 1005.7 | 0.9999 | 10.00 | 0.08 | 1.12 | 1.09 | 3.56 |
| Procyanidin B2 | 16.897 | 1.00-100 | Y = 2195.3X - 989.91 | 0.9967 | 10.00 | 1.05 | 1.47 | 2.15 | 5.21 |

Table 2: Mean values and grand total amounts of flavonoids of catechin, procyanidin B1 and procyanidin B2 contents in ripe fruits of fifteen Mao Luang cultivars, grown on dipterocarp forest of Phupam Valley, Sakon Nakhon Province, Northeast Thailand

| Cultivars | Catechin | Procyanidin B1 | Procyanidin B2 | Grand Total |
|---|---|---|---|---|
| | (mg 100 $g^{-1}$ FW) | | | |
| Sangkrow 2 | 175.40b | 1332.91o | 5006.39a | 6514.70a |
| Fapratan | 316.22a | 2319.71i | 3689.42b | 6325.35a |
| Sangkrow 1 | 147.38c | 2313.51j | 3430.36c | 5891.25b |
| Maeloogdog | 106.74fg | 3993.88b | 1357.52m | 5458.14bc |
| Phuchong | 121.93de | 2372.58h | 2866.09e | 5360.60bcd |
| Sangkrow 4 | 127.94d | 2930.20e | 2215.15i | 5273.29cd |
| Sangkrow 3 | 178.79b | 2403.94g | 2639.41f | 5222.14cd |
| Sangkrow 5 | 73.39h | 3097.65c | 1694.73l | 4865.77de |
| Nonkloy | 104.72fg | 1643.78n | 3100.02d | 4848.52d |
| Lompat | 72.03h | 4122.57a | 329.24o | 4523.84e |
| Kumlhai | 103.64g | 1865.42m | 2585.88g | 4554.94ef |
| Sangkrow 8 | 76.04h | 2505.54f | 1938.02k | 4519.60ef |
| Sangkrow 7 | 112.57efg | 1905.01l | 2243.98h | 4261.56f |
| Chomphupan | 104.66fg | 2930.86d | 1060.52n | 4096.04f |
| Sawang | 115.84ef | 2006.15k | 1939.16j | 4061.15f |
| Maximum | 316.22 | 4122.57 | 5006.39 | 6514.70 |
| Minimum | 72.03 | 1332.91 | 329.24 | 4061.15 |
| Mean | 129.15 | 2516.25 | 2406.39 | 5055.13 |

Letter(s) of the same column indicate least significant differences of Duncan's Multiple Range Test (DMRT) at probability, $p \leq 0.05$

recorded. The CV for the retention time of all peaks of the standards was <2% and the CV for the peak area was <5%, where the readings indicated a good reproducibility, which were reliable and acceptable (Hakkinen and Auriola, 1998).

**Amounts of catechin, procyanidin B1 and procyanidin B2 in ripe Mao Luang fruits:** The results showed that catechin contents of 15 Mao Luang cultivars ranged from 72.03 to 316.22 mg 100 $g^{-1}$ of fresh weight for Lompat and Fapratan cultivars, respectively (Table 2). The differences were large and statistically significant. With procyanidin B1, the content in ripe fruits ranged from 1,332.91 to 4,122.57 mg 100 $g^{-1}$ of fresh weight for Sangkrow 2 and Lompat cultivars, respectively. The differences were large and statistically significant. For procyanidin B2, this chemical compounds found in ripe fruits ranged from 329.24 to 5,006.39 mg 100 $g^{-1}$ of fresh weight for Lompat and Sangkrow 2 cultivars, respectively. The differences were large and statistically significant. Grand total values of the three chemical compounds ranged from 4,061.15 to 6,514.70 mg 100 $g^{-1}$ of fresh weight for Swang and Sangkrow 2, respectively. The differences were large and statistically significant.

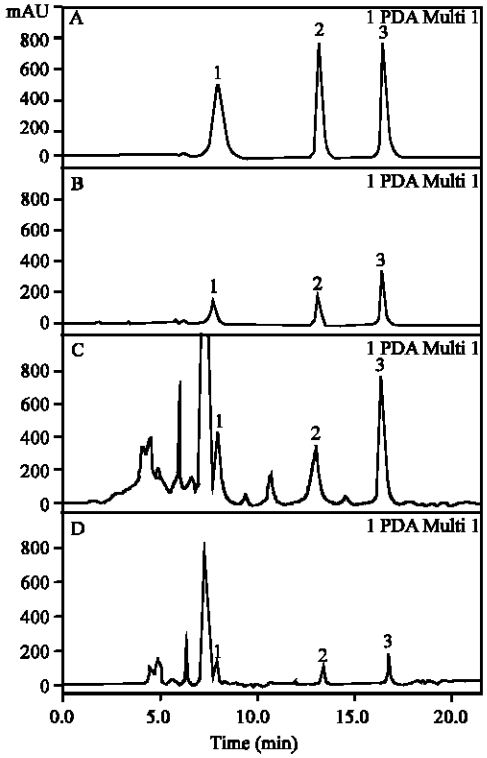


Fig. 1: RP-HPLC chromatogram of three flavonoids standards detected at 254 nm (A), 360 nm (B) and methanol extract chromatogram of Mao Luang fruit sample recorded at 254 nm (C) and 360 nm (D): where 1 = catechin; 2 = procyanidin B1 and 3 = procyanidin B2

## DISCUSSION

Mao Luang (*Antidesma bunius*), a kind of medicinal fruit trees could be found in most of dipterocarp forested areas in Northeast Thailand where the local people have been acquainted with this type of medicinal trees for many decades. The matured trees normally initiate flowers in the rainy season and its fruits reach their ripening stage in late rainy season (August-September). For the past few decades, the local people normally harvest its fruits from most of dipterocarp forests but nowadays some growers paid more attention to this crop plant since its fruits contain some considerable amounts of chemical compounds, e.g., numerous amounts of organic acids and

flavonoids, thus within this decade some growers have sown seeds of this crop for industrial purposes. It has been advocated that juices derived from ripe fruits could be used for healing sickness of man on coronary heart disease as reported by Hertog *et al.* (1997) and it could even reduce platelet aggregation, anti-oxidative, anti-carcinogenic substances as reported by Knekt *et al.* (1997), Kuulasmaa *et al.* (2000) and Stoclet *et al.* (2004) and also anti-cancer in man as reported by Hollman and Katan (1999). Some other workers have stated that Mao Luang ripe fruits contain some certain amounts of phenolics, flavonoids, anthocyanins and carotenoids as reported by Qian *et al.* (2004), Sass-Kiss *et al.* (2005), Trappey *et al.* (2005) and Cieslik *et al.* (2006). From these published information, one may find that this type of medicinal plant could possibly be considered to be an outstanding crop plant useful for man in many ways such as the edible fruits could be consumed (juices) just like some other orchard fruits but it offers some useful bioactive substances to possess its effects as medicinal aids.

For this study, it was found that there were fifteen cultivars of Mao Luang plants scattering on the forested areas of Phupan Mountain, Sakon Nakhon province, Northeast Thailand. The ripe fruits of the fifteen cultivars were collected and used for laboratory analysis for flavonoids contents of catechin, procyanidin B1 and procyanidin B2 chemical compounds. It was found that the fruits of the fifteen cultivars possessed different amounts of flavonoids contents of catechin, procyanidin B1 and procyanidin B2. With catechin, the results showed that Fapratan cultivar gave the highest amount (316.22 mg 100 $g^{-1}$ of fresh weight) and least with Lompat cultivar (72.03 mg 100 $g^{-1}$ of fresh weight). The differences were large and statistically significant. Similarly, the fifteen Mao Luang cultivars did not give similar amounts of both procyanidins (B1 and B2) where B1 was highest with Lompat (4,122.57 mg 100 $g^{-1}$ of fresh weight) and least with Sangkrow 2 (1,332.91 mg 100 $g^{-1}$ of fresh weight) and B2 was highest with Sangkrow 2 (5,006.39 mg 100 $g^{-1}$ of fresh weight) and least with Lompat (329.24 mg 100 $g^{-1}$ of fresh weight). For grand total amounts of flavonoids, the results revealed that Sangkrow 2 gave the highest followed by Fapratan, Sangkrow 1, Maeloogdog and lowest with Swang. Therefore, in terms of grand total amounts of flavonoids, Sangkrow 2 may be considered to be the most appropriate cultivar for crop cultivation followed by Fapratan, Sangkrow 1 and Maeloogdog, whilst the rest may be of secondary importance. Of the attained results, Mao Luang ripe fruits of different cultivars gave different amounts of flavonoids contents. This could possibly be attributable to perhaps the differences in genetic traits of the crop plants and it may be possible that soil conditions provide different available amounts of soil nutrients due to variation in soil pH and nutrients (Mengel and Kirkby, 1987). Suksri (1999) stated that variation in soil pH gave variation in amounts of soil nutrients available to plant roots and the uptake in plants could be related to the available amounts. It has been advocated that most tropical soils tend to possess a high acidity level due to the depletion of soil nutrients, particularly soil calcium. Furthermore, environmental conditions may affect soil pH, e.g., rainfalls could possibly increase intensity of soil acidity, leaching away of soil nutrients due to heavy rainfalls could be another factor apart from respiration of plant roots to form a light carbonic acid in soils and etc. He further stated that macronutrients influence taste of fruits of orchard plants, e.g., more of nitrogen provides sour taste whilst more of potassium increases sweetness in some fruits or otherwise increases tannin content or bitterness taste in some fruits of orchard plants, e.g., with the case of star apple (*Chrysophyllum cainito*). Thus variation in flavonoids contents may be influenced by variation in the amounts of nutrient supply, particularly macronutrients when high intensity of soil acidity is attained. However, genetic traits may also play an important role in the amounts of chemical compounds in fruits of Mao Luang plants (Scalzo *et al.*, 2005). Some other reasons for variation in flavonoids contents may be attributable to stages of ripeness, post-harvest conservation and processing of fruits and even pigment of the fruits (Auger *et al.*, 2004). Colour of fruits has some effects on phenolics, flavonoids and anthocyanins contents in many kinds of fruits as reported by Qian *et al.* (2004), Sass-Kiss *et al.* (2005), Trappey *et al.* (2005) and Cieslik *et al.* (2006).

It is noticeable that the amounts of catechin contents in Mao Luang ripe fruits were much higher than red grape (Pinot-noir) reported by Bourzeix *et al.* (1986) and many other kinds of fruits such as strawberries and vaccinium species (blueberry, wild bilberry and wild bog whortleberry) as reported by De Pascual-Teresa *et al.* (2000) and Hakkinen and Torronen (2000), particularly procyanidin B2.

## CONCLUSION

From this investigation, it was found that the fifteen Mao Luang cultivars possessed different amounts of flavonoids of catechin, proyanidins B1 and B2 in ripe fruits of the crop plants. Based on grand total amounts of flavonoids of catechin, proyanidins B1 and B2, the most outstanding cultivar was found with Sangkrow 2 followed

by Fapratan, Sangkrow 1 and Maeloogdog, whilst the rest were of secondary importance. The differences in flavonoids contents in ripe fruits of Mao Luang trees could be due to genetic traits and soil environments, i.e., soil pH, particularly the release of soil macronutrients available for plant roots.

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