# The Analysis of Arbutin in Mao (*Antidesma thwaitesianum* Muell. Arg.) Extracts

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## ABSTRACT

Arbutin is a skin-lightening agent that was discovered in berry fruit plants such as bearberry. The Mao tree produces the Mao fruit, which is quite similar to berries. The hypothesis of this research is based on the possibility of the discovery of arbutin in different parts of the Mao tree (*Antidesma thwaitesianum* Muell. Arg.). The purpose of this research was to determine the presence of arbutin in the unripe fruits (green Mao fruits, GMF), ripe fruits (red Mao fruits, RMF), mature fruits (black Mao fruits, BMF), young Mao leaves (YML) and mature Mao leaves (MML). The arbutin in the samples was isolated by thin layer chromatography (TLC) and quantified using high performance liquid chromatography (HPLC). The results showed that MML contained the greatest amount of arbutin (10.6 mg/100 g of raw material). The arbutin in MML was isolated using solid phase extraction (SPE) and preparative thin layer chromatography (PTLC) and was characterised via ESI-MS. The results of MS confirmed the presence of arbutin in the PTLC extract of MML. The crude and PTLC extracts of MML were tested for inhibitory activity against tyrosinase compared with the arbutin standard. The tyrosinase inhibition activities suggested that $IC_{50}$ of the crude extract of MML ($IC_{50}$ = 7.703 mg/l) and PTLC extract of MML ($IC_{50}$ =9.428 mg/l) were more effective than the arbutin standard ($IC_{50}$ = 14.012 mg/l).

*Keywords: Antidesma thwaitesianum* Muell Arg., arbutin, Mao fruit analysis, Mao leaf analysis, tyrosinase inhibitor

## INTRODUCTION

Mao or Maoluang, whose scientific name is *Antidesma thwaitesianum* Muell., is a wild plant in the *Euphorbiaceae* family. It is mostly grown in northeastern Thailand. The flowers of the Mao tree bloom from

May to July and the fruit matures from August to October (Morton, 1987).

Mao fruits form in a cluster similar to grape fruits and turn red when they ripen. The ripened (red) and mature (black) fruits are consumed fresh and processed as juice or local wine products. Mao wine was reported to contain high amounts of caffeic, a beneficial phenolic compound (Nuengchamnong & Ingkaninan, 2010). Mao fruits have been found to be rich in polyphenolic compounds such as gallic acid, epicatechin, catechin and cyanidin-3-*O*-glucoside (Jorjong, Butkhup, & Samappito, 2015). Besides the fruits, other parts of the Mao tree have been revealed as having anti-apoptotic and anti-inflammatory effects (Puangpronpitag et al., 2011).

Arbutin, p-hydroxyphenyl β-D – glucopyranoside (Figure 1), is a tyrosinase-based inhibitor, well known as a whitening agent popularly used in cosmetics for its effectiveness in the treatment of skin hyperpigmentation. Arbutin is noncytotoxic to humans. Its chemical structure is similar to that of hydroquinone, consisting of a phenol molecule with a glucose connecting to para position. Arbutin can be used as a substitute to hydroquinone, as the latter is cytotoxic to melanocytes in the human body (Hu et al., 2009). The presence of arbutin has been detected in various plants, including bearberry leaves (*Arctostaphylos uva – ursi* Ericaceae), (Lin, Yang, & Wu*,* 2007), pear trees (*Pyrus communis L.*, Rosaceae) (Cui et al., 2005), cowberry (*Vaccinium vitis-idaea L.*, Ericaceae, *Bergenia crassifolia* (Saxifragaceae) (Nycz et al*.,* 2010), *Origanum majoana L.*(Lamiaceae) (Assaf, Ali, & Makboul, 1987; Lukas et al., 2010), *Myrothamnus flabellifolia* Welw. (Myrothanmnaceae) (Suau et al., 1991) and some other plant families. Arbutin can be classified into α-arbutin (alpha arbutin) and β-arbutin (beta arbutin). Even though the inhibitory activity against tyrosinase of α-arbutin is 10 times higher than that of β-arbutin, β-arbutin is obtained from natural products of various plant extracts, while α-arbutin is only produced by enzymatic synthesis of amylase through *Bacillus subtilis* (Liu et al*.,* 2013). Arbutin, thus, generally refers to β-arbutin. (Figure 1) Arbutin has also been reported to have anti-bacterial properties useful for the treatment of urogenital tract infection (Rychlinska & Nowak, 2012).
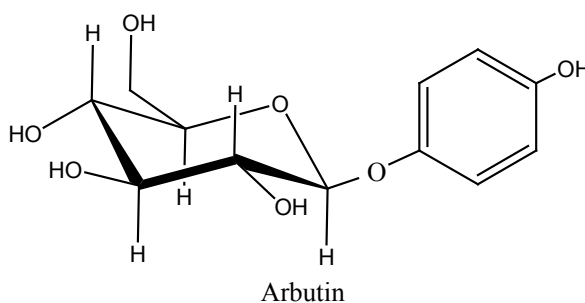


Arbutin

*Figure 1.* A chemical structure of arbutin

Tyrosinase is a polyphenol oxidase enzyme with a code of EC 1.14.18.1 (Jeon et al., 2005), which is a copper-containing enzyme in animals, higher plants and fungi. It is functionally related to melanogenesis, the process which produces the brown colour of the human skin or fruits. Tyrosinase is involved in two steps of melanogenesis; the first step is to convert tyrosine to 3, 4-dihydroxy phenylalanine (L-DOPA), and the second is the oxidation of L-dopa to dopaquinone. Dopaquinone converts to dopachrome and finally to eumelanins, manifesting as a brown pigment on human skin and hair (Ito, 2003). Tyrosinase-inhibiting activity will block the emergence of brown colouration. There are various types of tyrosinase inhibitors, and arbutin is one of these inhibitors, and is also well known as a skin depigmentation agent (Hori, Nihei, & Kubo, 2004). Both forms of α- and β-arbutin and also arbutin derivatives have been investigated for their depigmentation ability. The synthetic arbutin derivative in the form of acyl arbutin has been reported to be more effective in the inhibitory function of melanin production than arbutin (Tokiwa et al., 2007).

In this paper, arbutin was determined in the five samples from different parts of the Mao tree. The objectives of this study were to determine the presence of arbutin both qualitatively and quantitatively. The characterisation of arbutin was analysed to determine which of the samples contained the greater amount of arbutin, as well as to test the levels of inhibitory activity against tyrosinase produced by the compounds of these samples compared with the arbutin standard.

## MATERIALS AND METHODS

### Materials and Chemicals

Samples of different parts of Mao trees, comprising unripe Mao fruits (green Mao fruits, GMF), ripe fruits (red Mao fruits, RMF), mature fruits (black Mao fruits, BMF), young Mao leaves (YML) and mature Mao leaves (MML) (Figure 2-Figure 6) were collected from Sakon Nakhon province in Northeastern Thailand. Analytical agents were obtained from various suppliers in Thailand, Europe and America: arbutin, mushroom tyrosinase (EC 1.14.18.1, 500 U/mg) and L-dihydroxyphenylalanine (L-DOPA) standards were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA); methanol, ethyl acetate and acetonitrile were obtained from Lab Scan (Bangkok, Thailand); silica gel 60 G for thin layer chromatography was bought from Merck (Darmstadt, Germany); and dichloromethane from Fluka. Disodium hydrogen phosphate and sodium hydrogen phosphate were obtained from Univar (NSW, Australia). Milli-Q water (Millipore, Befford, MA, USA) was used in the HPLC method.

*Figure 2.* Mature Mao leaves (MML)



*Figure 5*. Red Mao fruits (RMF)



*Figure 3.* Young Mao leaves (YML)



*Figure 6.* Black Mao fruits (BML)



*Figure 4.* Green Mao fruits (GMF)

## Instruments

The analytical process was conducted using High Performance Liquid Chromatography (HPLC) and a mass spectrometer. The model of the HPLC that was used in this experiment was the Shimazu 10-VP series, utilising Inertsil ODS-3 with a diode array detector of dimensions 4.6 × 250 mm, 5 μm column. The chromatograms were monitored at 224 nm. The model of the mass spectrometer was Quattro micro API with Electrospray (ESI).

## Qualitative and Quantitative Analysis of Arbutin

**Crude extraction.** Fresh samples of unripe Mao fruits (green Mao fruits, GMF), ripe fruits (red Mao fruits, RMF), mature fruits (black Mao fruits, BMF), young Mao leaves (YML) and mature Mao leaves (MML) were weighed at 500-1500 g and dried at a temperature of 50ºC for 6 h before being ground into small pieces. Each sample was soaked in 1500 ml of methanol for five days at room temperature. Then the extracting solvent was decanted and removed via the evaporation process. The five crude extracts were weighed and kept for further analysis.

**Thin layer chromatography (TLC).** Each crude extract of five samples, weighing about 1.0 g was spotted on 5 x 10 cm of TLC plates (about 10 plates) in which the arbutin standard level was marked as a reference. The plates were placed into a vessel with mobile phase system of 100 % of acetonitrile and left on the plates until the solvent permeated the samples to separate the arbutin. All the spots were detected at 254 nm of UV light source to compare the level of samples moving up to the level of the arbutin standard. The spots from the extracts that were of the same level as the arbutin standard were scraped from all the TLC plates and collected. The isolated arbutin was extracted from the silica particles using methanol. The extracting solvent was then separated from the silica particle via centrifugation.

**High performance liquid chromatography (HPLC).** After centrifugation, the separated extracting solvent was removed. Then 0.01 g of the isolated arbutin was weighed and dissolved in 10.0 ml of ultrapure water (Milli-Q) for chromatographic analysis. A reverse phase HPLC system was equipped with Inertsil ODS-3 4.6 × 250 mm, 5 µm column and a diode array detector at the wavelength 224 nm. The column temperature was set at 35°C. The injection volume of 20 µl was used and the elution was performed at a flow rate at 1 mL/min under the isocratic system of acetonitrile: water, 80 : 20 v/v.

***The qualitative and quantification of arbutin.*** The quantification of arbutin in the five crude extracts was performed as described by Lukas et al. (2010) with modifications. Briefly, 1.0 g of the weighed methanolic crude extract was isolated by TLC method. The isolated arbutin samples were dissolved in 10.0 ml methanol. The samples were then filtered through a 0.45-µm Nylon membrane. An aliquot of 20 µl of each sample, with or without co-elution with the purified arbutin, was then subjected to analytical HPLC analysis as described earlier to determine the presence of arbutin. A standard calibration curve for arbutin was prepared with solutions ranging from 10 to 100 mg/l. A concentration curve was constructed using the average area calculated by the software, Shimadzu Class VP. An aliquot of 20 µl of the isolated arbutin extract was injected onto the HPLC column and eluted as described earlier. The calculated concentration of arbutin was expressed in mg/l.

## Isolation and Identification of MML

**Solid phase extraction (SPE).** Crude extract of MML was weighed at 100.0 g and 20 ml of methanol was added, after which the crude MML was separated by centrifuging and the sample solution was kept for SPE loading. The $C_{18}$ Discovery cartridge was pre-eluted with 3 ml of methanol and followed with 3 ml of water. Then, the sample solution was loaded into the cartridge at a flow rate of 3 ml/min with 10-times reloading. The sample was extracted with 50% dichloromethane and 50% methanol. The solution of extract sample was kept for the next PTLC method.

**Preparative thin layer chromatography (PTLC).** The solution from the SPE method was spotted on the PTLC plates prepared from Silica gel 60 G (from Merck). The arbutin standard was marked at the first spot for a reference. Several solvent systems were experimented with to optimise the separation of arbutin on the TLC plate and it was found that dichloromethane:methanol (50:50, v/v) was the most suitable solvent system for separation of arbutin. The compound was separately removed by scraping off the silica at the same level of the arbutin standard. The isolated compound was dissolved in methanol. The solution was centrifuged to remove silica, and kept for the next mass spectrometry method and tyrosinase inhibition analysis.

**Mass spectrometry**. The solution that was extracted from the PTLC was analysed using an ESI-MS (quattro micro API, micromass) process.

## Tyrosinase Inhibition Analysis

The method was adapted from previous research for a suitable condition for storage of arbutin to be used as a positive control (Wu et al., 2012). In this stage the method suggested by Wu et al. (2012) i.e. to use arbutin as a positive control was adapted. The crude extract of MML and the PTLC extract of MML were analysed for enzyme tyrosinase inhibition by monitoring for dopachrome occurrence. L-dopa was used as a tyrosinase substrate in comparative conditions with samples and without samples. The samples were prepared at different concentrations, 20, 30, 50, 100 and 150 mg/l. The method was designed in four test tubes: labelled for convenience as A, B, C and D. Test tube A contained 0.02 M phosphate buffer pH 6.8 (800 µl), tyrosinase (400 µl) and 20 % ethanol (400 µl); B contained 0.02 M phosphate buffer pH 6.8 (1200 µl) and 20% ethanol (400 µl); (blank for A); C contained tyrosinase (400 µl), 0.02 M phosphate buffer (800 µl) and the samples of crude extract of MML or PTLC extract of MML; and D contained a phosphate buffer and ethanol (blank for C). All the test tubes were incubated at 25°C for 10 min. Then, the substrate L-dopa was mixed into all the test tubes. The spectrophotometric analysis at wavelength 492 nm in all the test tubes

was processed immediately. Subsequently, all the test tubes were incubated at 25°C for 2 min before measuring again with a spectrophotometric method. The values of spectrophotometric absorbance of each test tube before a 2-min incubation were deducted by the absorbance value after incubation. The difference in values of deduction absorbance were used for calculation in the percentage of tyrosinase inhibition values as shown in Equation 1, where A, B, C, D represented the difference values of absorbance of each test tube.

Percentage of tyrosinase inhibition =

$$\frac{(A-B)-(C-D)}{(A-B)} \times 100 \qquad (1)$$

The $IC_{50}$ was calculated from the plot between concentration and percentage of tyrosinase inhibition at the half-way mark of the experiment.

## RESULTS AND DISCUSSION

### Qualitative and Quantitative Analysis of Arbutin

The methanolic crude extract of the five samples, GMF, RMF, BMF, YML and MML that were spotted on the TLC plates was compared with the arbutin standard as a reference. The spots of all the samples and arbutin were shown in brown under a UV-visible spectrophotometer. The spots of the five samples were removed at the $R_f$ value 0.63 of arbutin in a mobile phase of acetonitrile. In the chromatographic analysis, the retention time of arbutin standard appeared at 2.78 min (Figure 7a). In Figure 7b, the MML, GMF and RMF extract is shown as a distinctive peak at the retention time 2.784 min, and co-elution with the arbutin standard showed an increase in peak height; thus, the peak at 2.784 min showed the presence of arbutin in these extracts. The YML and BMF extract, however, only showed the presence of arbutin after co-elution with the standard at 2.773 min and 2.763 min, respectively. The concentration of arbutin in all the samples was calculated using the linear equation from the calibration curve that showed the correlation between the peak area and concentration (Figure 9). From the standard calibration curve, the amount of arbutin in each sample was calculated apart from the weight of the samples. The weight of five samples in each step was comparatively calculated, TLC extracts (0.01-0.3 g), methanolic crudes (1.0-11.0 g) and fresh samples (500-1500 g). The graph (Figure 10) demonstrated the comparative amounts of arbutin in five samples, MML, YML, GMF, BMF and RMF, 10.6, 9.9, 1.2, 0.05 and 0.013 mg/100 g of raw material, respectively. Therefore, the results showed that MML contained the most amount of arbutin among the five samples. The results displaying the amount of arbutin in the Mao leaves correlated with reports on arbutin found in leaves of various plants such as bearberry (*Arctostaphylos uva-ursi* Ericaceae) (Lin et al., 2007), cranberry, blueberry and pears (Kenndler et al., 1990; Nihei & Kubo, 2002). Consequently, only mature Mao leaves (MML) were further studied for inhibitory activity against tyrosinase and also isolated for arbutin identification.
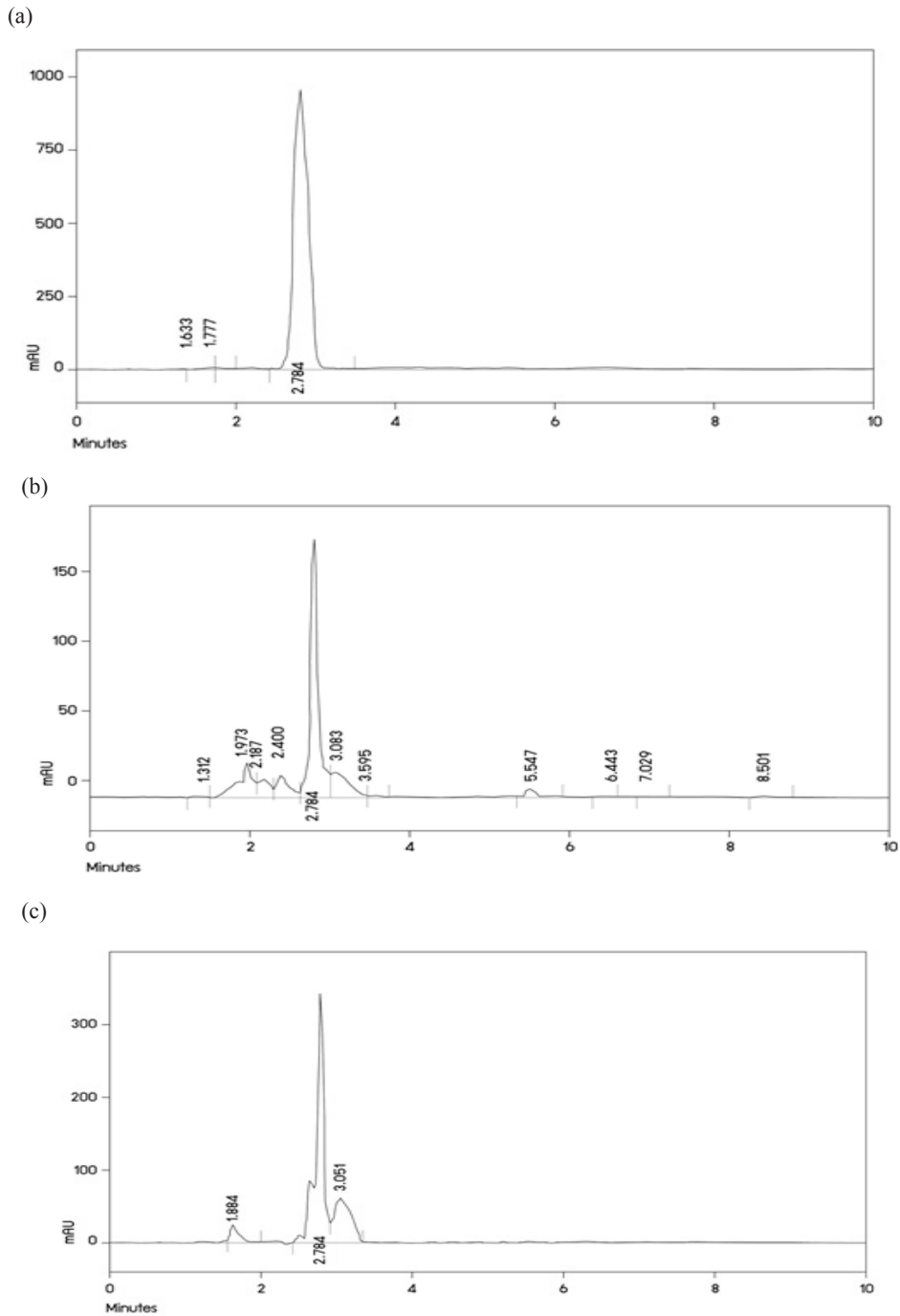
(a)

(b)

(c)

*Figure 7.* Chromatograms of (a) an arbutin standard  (b) mature Mao leave (MML) extract and (c) mature Mao leaf (MML) extract spiked with arbutin standard shown at the peaks at retention time 2.784 min under the condition of mobile phase acetonitrile:water, 80:20, v/v, Inertsil ODS-3, 4.6 × 250 mm, 5 μm, column at 35°C and DAD 224 nm
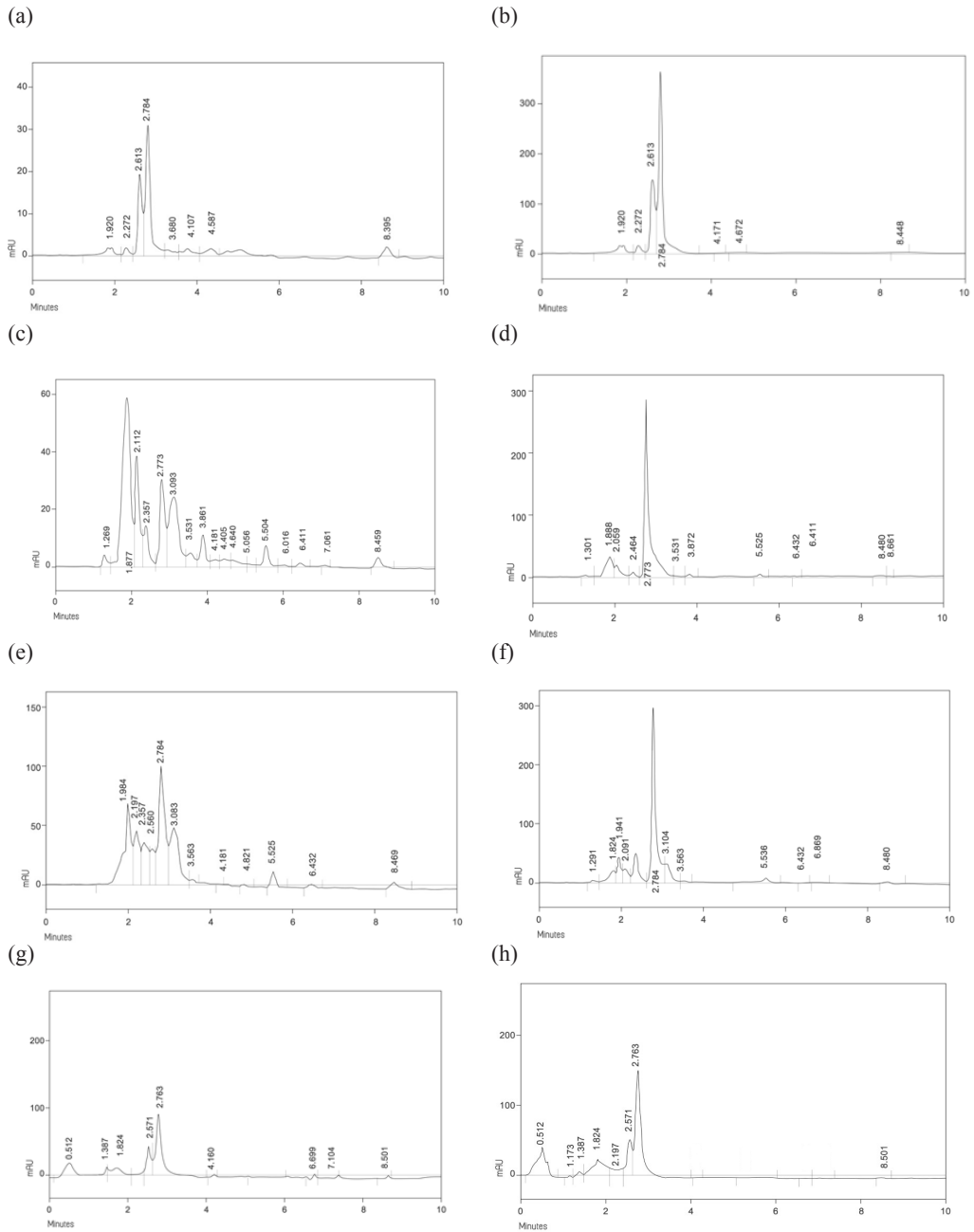
*Figure 8.* Chromatograms of (a) red Mao fruits (RMF) (b) RMF with arbutin spiking, (c) black Mao fruits (BMF), (d) BMF with arbutin spiking, (e) green Mao fruits (GMF), (f) GMF with arbutin spiking, (g) young Mao leaves (YML) and (h) YML with arbutin spiking shown at the peaks under the condition of mobile phase acetonitrile:water, 80:20, v/v, Inertsil ODS-3, 4.6 × 250 mm, 5 µm, column at 35°C and DAD 224 nm
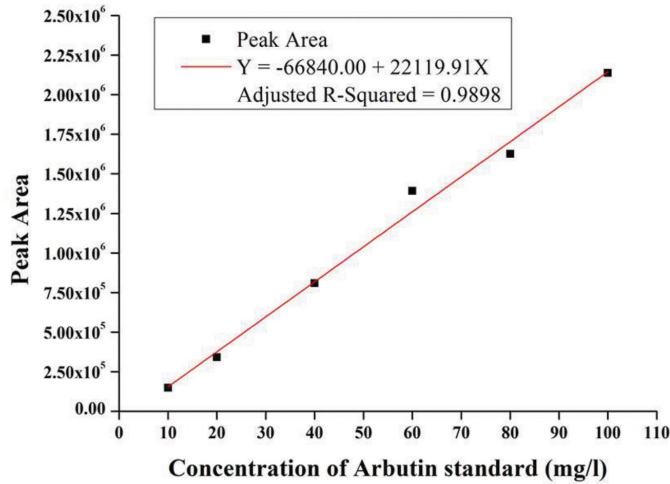
*Figure 9.* The standardisation of the arbutin graph at various concentrations vs. peak area
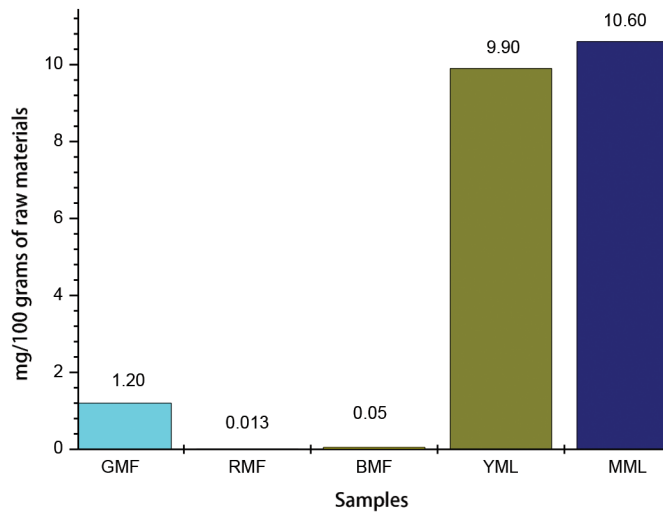


*Figure 10.* The content of arbutin in each part of the Mao tree

**Tyrosinase Inhibitory Activity of Mature Mao Leaf (MML) Crude Extract**

Melanogenesis is the pathway for producing melanin granules from cells named melanocytes located at the basal layer of the epidermis that is controlled by tyrosinase.

There are two pathways for melanogenesis to produce eumelanins and pheomelanins. Tyrosinase has a role in skin pigmentation, which is related to eumelanins produced to make skin darker (Ito, 2003). The systems, which retard tyrosinase from working, have been studied for years. The dopachrome

method was used to determine $IC_{50}$, the concentration of samples at which half the original tyrosinase activity is inhibited. The lower the rate of spectroscopic absorption of dopachrome, the better the inhibition against tyrosinase is. The inhibitory activity against tyrosinase of crude, isolated (PTLC) extract of MML and the standard arbutin was comparatively tested. The results showed that the tyrosinase inhibitory activity of MML crude extract is potentially the greatest among the PTLC extract of MML and the arbutin standard, as shown in Figure 11 and Figure 12. In $IC_{50}$ values, the crude and PTLC extract of MML reduced melanin production more efficiently when compared with arbutin. From the results it can be hypothesised that there were some other natural active compounds in the crude MML extract that were co-working with arbutin to exert

the inhibitory effect against tyrosinase. The isolated compound from PTLC also presented better inhibition than arbutin, and the pathway can be explained in the same way as for the crude extract. There is a possibility that the presence of other phenolic compounds in the MML crude extract was working in synergy with arbutin to decrease the production of melanin. Phenolic compounds can be tyrosinase inhibitors as they are examined as substrates (Ito & Wakamatsu, 2015). Polyphenolics such as gallic acid, catechin and cyanidin-3-*O*-glucoside, which were reported in *Antidesma bunius* Linn. cultivars (Jorjong, Butkhup, & Samappito, 2015), may also be found in the MML crude extract and may have contributed to tyrosinase's inhibitory activity. The polyphenolic content should be considered for further analysis of *Antidesma thwaitesianum* Muell Arg.
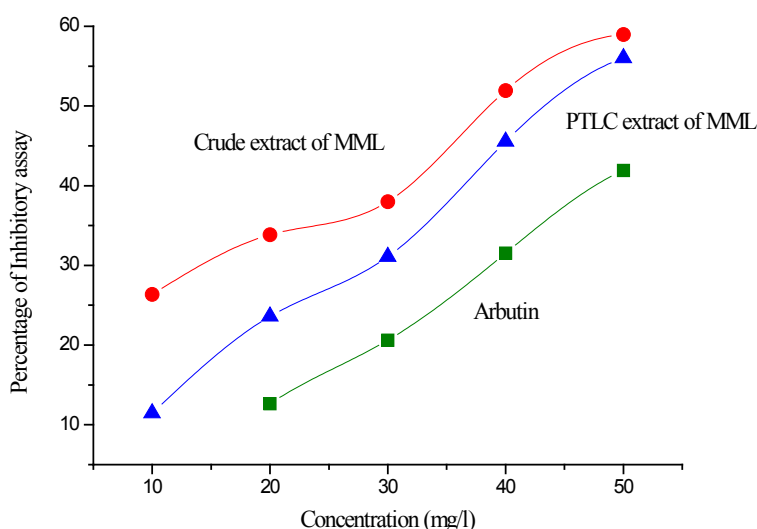


*Figure 11.* Percentage of inhibitory activity against tyrosinase of arbutin standard, crude extract of MML and PTLC extract of MML
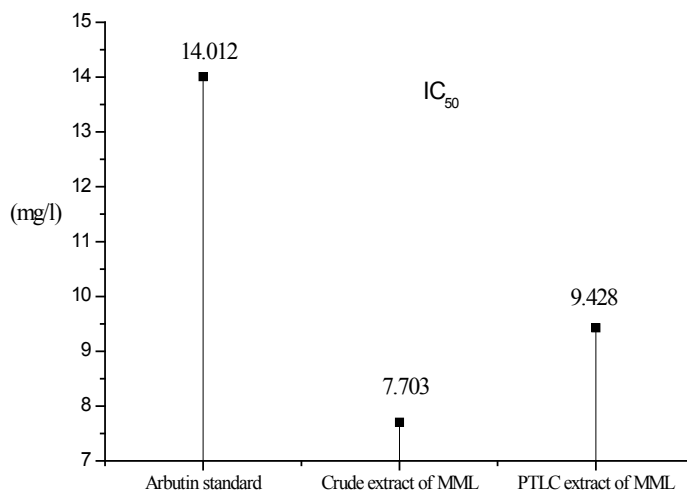
*Figure 12.* The inhibitory concentration against tyrosinase at 50% of arbutin standard, crude extract of MML and PTLC extract of MML

## Identification of Arbutin in MML

MML crude extract was isolated by SPE and PTLC extraction. The clear solution, which was obtained from SPE extraction using a $C_{18}$ cartridge, showed some impurities. In the PTLC extraction, the compound was removed from the plate at the $R_f$ value of 0.81 with reference to arbutin. The isolated compound from MML was characterised in mass spectroscopy, which was recorded using the Micromass Quattromicro (Quattro micro API, micromass) in methanol solvent using the positive mode. The mass spectrum of the PTLC extract of MML is shown in Figure 13. There was a significant peak that indicated the presence of an arbutin compound. It showed the ion number, m/z = 305, indicating a possibility that arbutin molecular ions (m/z=272.08) were represented, signalling the possibility of arbutin molecular ions attaching to methanol ($CH_3OH$) and 1 atom of

hydrogen ion corresponding to the number 305.14. The possibility of the presence of methanol, which is used as a solvent can be attributed to the molecular ions of arbutin. The molecular ion of arbutin (272.08) was not shown in the mass spectrum according to the low resolution of the machine. In Figure 14, the mass spectrum of the standard arbutin did not show the molecular ions at 272.08 either, but the ion number in the arbutin standard showed the same number of the peak in the extract of MML at 305, with methanol and 1 atom of hydrogen ion attached to the arbutin molecular ions, showing the ion number, 305.11. Therefore, the isolated compound of MML was believed to identify as arbutin. Further identification was made possible through analysis of ^{1}H-NMR (500 MHz, MeOD), as seen in Figure 15(a), MML extract and Figure 15(b), the arbutin standard. The comparative spectrum presented similar

results for the aromatic ring in both (δ 6.66-7.02). At the upfield (δ 3.29-3.90), even though the peaks of the MML extract showed some difference from the arbutin standard, all the peaks of the arbutin standard appeared in the spectrum of the MML extract. The significantly distinct peaks between the MML extract (δ 5.27-5.29) and the standard spectrum (δ 4.71-4.74) can be described by the difference between α-arbutin and β-arbutin (Cepanec & Litvic, 2008) i.e. the MML extract was α-arbutin, while the arbutin standard was β-arbutin.
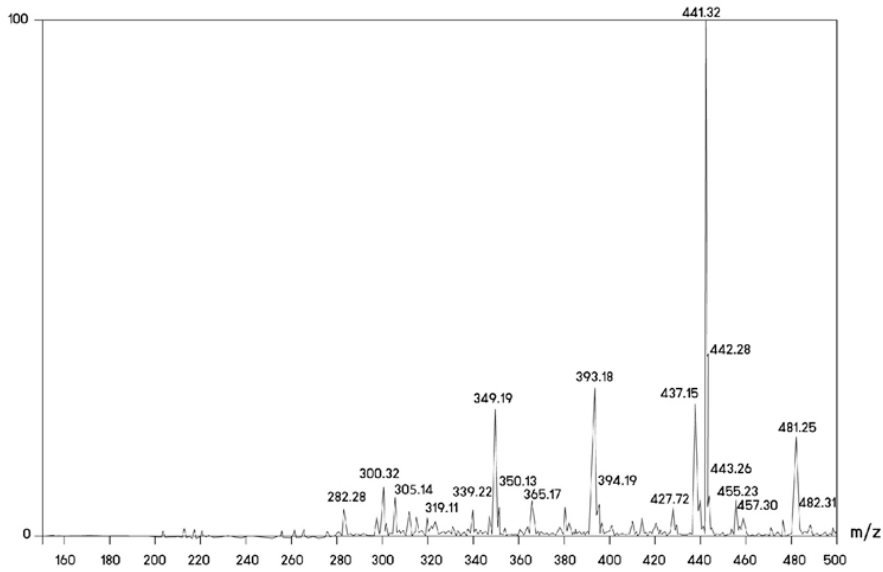


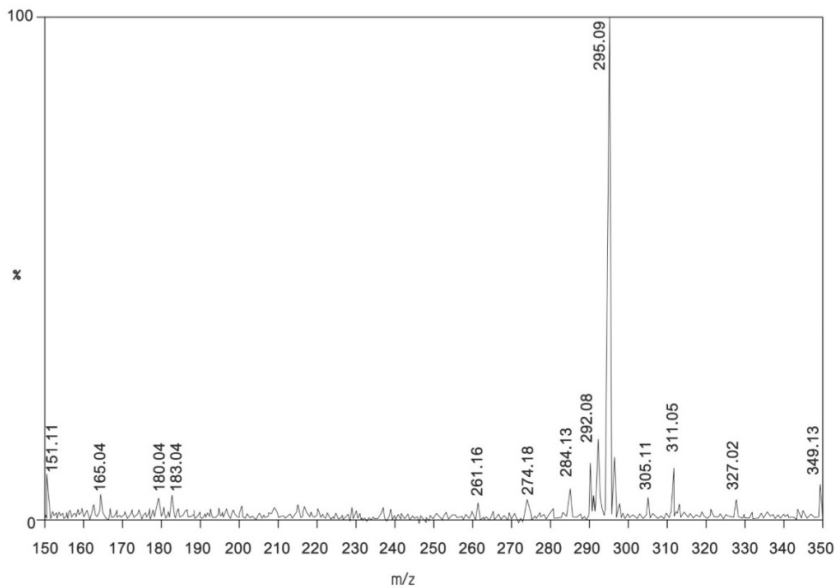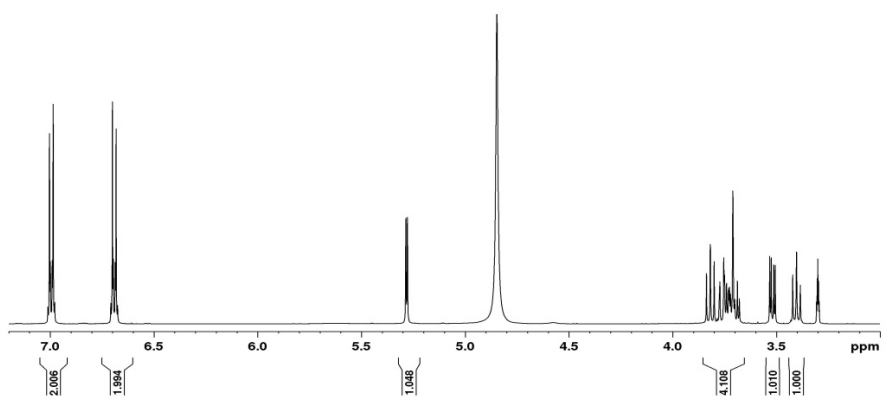*Figure 13.* A mass spectrum of mature Mao leaf (MML) extract



*Figure 14.* A mass spectrum of the arbutin standard
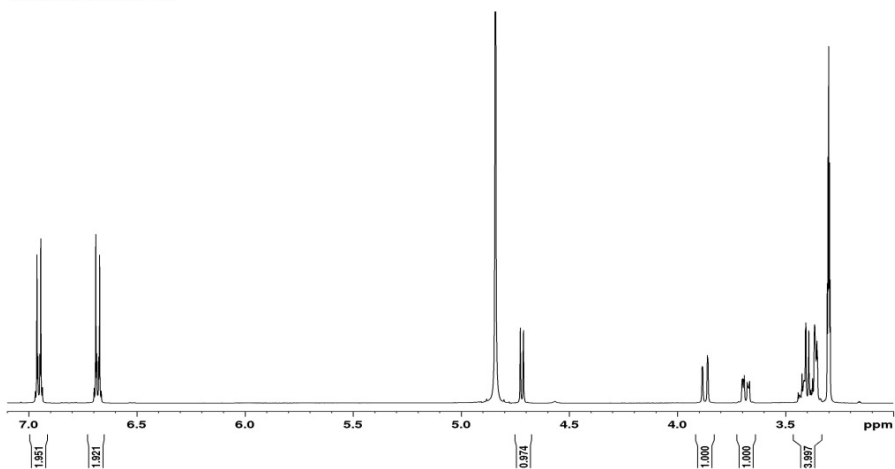
(a)

arbutin-1H



(b)

standard arbutin-1H



*Figure 15.* The ¹H-NMR spectrum of (a) mature Mao leaf (MML) extract and (b) the arbutin standard

## CONCLUSION

The results showed that arbutin was found in green Mao fruits (GMF), ripe red Mao fruits (RMF), mature fruits (black Mao fruits, BMF), young Mao leaves (YML) and mature Mao leaves (MML). Among these samples, MML presented the greatest amounts of arbutin (10.6 mg/100 g of raw material).

The results of inhibitory activity against tyrosinase showed that the $IC_{50}$ of the crude extract of MML ($IC_{50}$=7.703 mg/l) and the isolated extract from PTLC of MML ($IC_{50}$=9.428 mg/l) were more effective than the arbutin standard ($IC_{50}$=14.012 mg/l). It is reasonable to assume that there were some active compounds in the Mao extracts that decreased melanin production efficiently compared with arbutin. The active chemicals in the MML extract appears to have been polyphenols. Polyphenols can be accepted as substrates by tyrosinase, which depend on the position and presence of their structure for inhibition. The polyphenolic compounds represented in natural products are mostly flavonoids, which are reported to be widely distributed in the leaves, seeds, bark and flowers of plants (Chang, 2009). It is suggested that further research might be undertaken relative to the polyphenolic compounds that occur in Mao leaf extracts. The significant results of this research were the discovery that the greatest amount of arbutin was found in the leaves of the Mao tree (*Antidesma thwaitesianum* Muell. Arg.) and that Mao leaf extract imposes inhibitory activity on tyrosinase. The Mao tree is a local plant cultivated in Northeastern Thailand, where the Mao fruit is consumed as a nutritious and medicinal food. In addition, the leaves of this tree can be used in skin-whitening cosmetic products. It is recommended that future studies on this plant focus on the antibacterial properties of its leaves.

## ACKNOWLEDGEMENT

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