Determination of the Antioxidant Activity and Bioactive Compounds of Mulberry Fruit Extracts

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Abstract. White mulberry (Morus alba L.) is a berry native to China which fruit is usually eaten fresh or after processed. Its bioactive compounds vary depending on species, cultivation, location and others. In this study, the total flavonoids content, total phenolic content, total anthocyanins content and antioxidant activity of Morus alba L. fruit grown in Tuaran, Sabah were analyzed. Fruit was extracted with 60⁰C hot water and 80% ethanol, while the total bioactive compounds analysis utilized aluminum chloride, Folin-Ciocalteu and pH differential method in determining the flavonoids, phenolic, and anthocyanins content. Their antioxidant activity was determined using Free Radical Scavenging 2,2-Diaryl-1-Pikrilhidrazil (DPPH), Ferric Reduction Antioxidant Power (FRAP) and Radical Cation 2,2'-Aznio-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay. As a result, significantly higher (p < 0.05) content of total flavonoids (104.34 mg QE mg-1), phenolic (1.21 mg GAE mg-1) and anthocyanins (0.74 mg c-3-gE mg -1) were obtained from ethanolic extract than the hot water extract. Also, significantly higher (p < 0.05) antioxidant activity was observed in ethanolic extract for DPPH (0.50 mg mL-1), FRAP (3.74 mM Fe (II) g-1) and ABTS (6.05 mg AEAC g-1). Data showed that ethanol solvent (80%) is a better solvent for Morus alba L. fruit’s extraction.

Keywords. mulberry, total phenolic, total flavonoids, total anthocyanins, antioxidants activity

1. Introduction
Morus alba L. is a mulberry belonging to genus Morus L. from Moraceae family that can adapt to a variety of climates, topography, and soil conditions [1]. White mulberry is native to China and the fruit have been regarded as edible traditional medicine by the Chinese [2]. Nowadays, white mulberry fruits are eaten fresh or after processed. Among the products that have been produced from mulberry fruit are sweets, frozen desserts, juices, pastes, ice cream, wine and mulberry jelly [3][4]. The fruit is rich in various bioactive components such as anthocyanins, flavonoids, phenolic acids, vitamins, fats, sugars and minerals [5][6]. Bioactive compounds are components capable of modulating metabolic processes and benefit health through antioxidant activity, inhibition or induction of enzymes, inhibition of receptor activity, as well as induction and inhibition of gene expression [7]. Meanwhile, antioxidative compounds are capable of inhibiting the oxidation of lipids, proteins, carbohydrates and Deoxyribonucleic acid (DNA) as well as removing free radicals in the human body [8]. High level of free radicals might cause oxidative stress which leads to variety...
of chronic diseases including atherosclerosis, cardiovascular disease, cancer, diabetes, rheumatoid arthritis, chronic inflammation, premature aging, stroke, septic shock and other degenerative diseases in human [9][10]. Therefore, adoption of natural antioxidants-rich diets including fruits, vegetables, natural juices, teas and cereals will aid in protecting the body from free radicals and its negative oxidative stress impacts.

Studies on the activity of antioxidants and bioactive compounds have been extensively done on fruits and plants. Several studies have found that phenolics compounds in mulberry possess numerous positive effects on human health including antioxidative, antitumor, hypolipidemic effect, neuroprotective activity and macrophage activation effect [11]. However, the chemical composition of mulberries such as the content of polyphenols, flavonoids, anthocyanins and carotenoids vary depending on mulberry species, cultivars, variations, location, cultivation treatment, plant nutrition, maturity, harvest, storage conditions and extraction processes [12][13]. Most studies on white mulberry fruit (Morus alba L.) were conducted abroad such as in China, Serbia, Spain, Iran and others with various extraction solvents; methanol, acetone, ethanol and ethyl acetate [3][14][15][16][17][18]. Besides, a three hectares white mulberry plantation with about 1,000 trees has existed in Kampung Tudan, Tuaran, Sabah, indicating the state’s ability to cultivate this plant and their economy potentiality.

To date, no study have been conducted on the chemical composition and antioxidant activity of Morus alba L. fruit grown in Sabah, Malaysia. Therefore, to fill in this knowledge gap, the total phenolic, flavonoids, anthocyanins and antioxidants activity of mulberries were analyzed, as well as finding out a better extraction solvent for the fruit. It is hoped that this information can be of used as preliminary knowledge about mulberry cultivated in Sabah and also for mulberry-based functional foods and products production. Also, as the mulberry-based product would fit in the thriving demand and production of healthy beverages, snacks and health supplements, this would give the country a good marketing and economic growth potential.

2. Materials and methods

2.1. Sample Collection and preparation

White mulberry fruit (Morus alba L.) in blackish red colour (maturity index of four) was obtained from Kampung Tudan, Tuaran, Sabah. The fruit was washed, air-dried and chopped. Then, fruit was froze (New Brunswick Scientific U410, Germany) at -80°C for 24 hours before being freeze-dried (Labconco, USA) for 48 hours. Afterward, the fruit was ground to powder, kept in an airtight container and stored at -20°C until further analysis.

2.2. Sample Extraction

Extraction method was carried out using 80% ethanol (Merck, Germany) and 60°C hot water. A 0.1 g of frozen samples was extracted with 30 mL of 80% ethanol (v/v). The mixture was shaken for 2 hours using an incubator shaker (Thermo Fisher, USA) set at 200 rpm at 25°C. Meanwhile, 2 gram of frozen samples were extracted with 100 ml of 60°C distilled water for 2 hours in water bath (Memmert WMB 22, Germany). The mixtures were filtered using Whatman No. 1 filter paper. The supernatant was collected and the residues were re-extracted under the same conditions.

2.3. Bioactive compounds analysis

2.3.1. Determination of total flavonoids content. Aluminum chloride assay described by Izzreen & Fadzelly [19] was used. First, 0.3 mL of 10% aluminum chloride was added to 5 mL diluted sample. After 6 minutes, 2 ml of sodium hydroxide (1M) and 2.4 mL of distilled water were added to the mixture. The mixture was left for 15 minutes before measured at 510 nm wavelength using a UV-Vis spectrophotometer (Perkin
Elmer, USA). Quercetin was used as standard (0 - 0.1 mg mL⁻¹). Result was expressed as mg equivalent to quercetin (mg QE g⁻¹) from the dry sample.

2.3.2. Determination of total phenolic content. Folin-Ciocalteu method was used as described by Singleton & Ross [20] with slight modifications. First, 7.5% sodium carbonate solution and Folin-Ciocalteu solution (Sigma-Aldrich, USA) were prepared by mixing Folin-Ciocalteu reagent and distilled water (1:10). Then, 1.0 mL of the Folin-Ciocalteu reagent solution was mixed with 0.2 mL of sample extract. After 10 minutes, 0.8 mL of sodium carbonate solution was added and left at 22°C for 30 minutes before measured at 743 nm using UV-Vis spectrophotometer. Gallic acid (Sigma-Aldrich, USA) was used as a standard (0.1 to 0.5 mg mL⁻¹) and result was expressed as mg of the equivalent gallic acid (mg GAE g⁻¹) of dry weight.

2.3.3. Determination of total anthocyanins content. A pH difference-spectrophotometric method described by Giusti & Wrolstad [21] was used, where 0.025 M of potassium chloride buffer (Merck, Germany) (pH 1.0) and 0.4 M of sodium acetate buffer (Sigma-Aldrich, USA) (pH 4.5), adjusted with 0.1 M hydrochloric acid (Sigma-Aldrich, USA) were used. A total of 3.5 ml 0.025 M of potassium chloride buffer, 3.5 ml of 0.4 M of sodium acetate buffer and 0.5 ml of extract sample were mixed. The absorption values were measured at 515 nm and 700 nm wavelengths. Absorption readings were taken based on plotted graphs against distilled water. Cyanidin-3-glucoside was used as standard. Result was expressed as mg cyanidin-3-glucoside equivalent (c-3-gE) g⁻¹ from the dry sample. Total anthocyanins content is measured using the formula below:

\[
\text{c-3-gE} = \frac{(A x Mw x DF x 1000) / \varepsilon x 1)}{	ext{(1)}}
\]

\[A = \text{absorption of } (A_{515} - A_{700}) \text{ of pH 1.0 - (A}_{515} - A_{700}) \text{ of pH 4.5}
\]

\[Mw = \text{weight for cyanidin-3-glucoside molecule = 449.2}
\]

\[DF = \text{sample dilution factor}
\]

\[\varepsilon = \text{molar absorption of cyanidin-3-glucoside = 26,900}
\]

2.4. Antioxidant activity analysis

2.4.1. Free Radical Scavenging 2.2-Dyphenyl-1-Pikrilhidrazil (DPPH) Assay. According to Mensor et al. [22], a total of 1 mL of 0.3 mM DPPH solution was mixed with 2.5 ml of extract sample and left for 30 minutes in the dark at room temperature before measured at 518 nm using a UV-Vis spectrophotometer. Free radical scavenging activity was calculated using the formula below in which 1 ml of solvent + 2.5 ml of extract were used as blank, while 1 ml of 0.3 mM DPPH + 2.5 ml of solvent were used as control. The result was reported in IC₅₀.

\[IC₅₀ = 100 - [(\text{Abs sample} - \text{Abs blank})/ \text{Abs control}] \times 100 \quad (2)
\]

2.4.2. Ferric Reduction Antioxidant Power (FRAP). According to Bakar et al. [23], FRAP reagent was prepared by mixing 25.0 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 10 mM 2,4,6-trpyridyl-s-triazine (TPTZ) and 2.5 ml of 20 mM ferric chloride (10: 1: 1). The fresh FRAP reagent was heated in water bath at 37°C. Next, 300 μl of FRAP reagent, 100 μl of sample extract and 300 μl of distilled water were mixed. Mixture was left for 4 minutes before measured at 593 nm wavelength using a UV-Vis spectrophotometer. Fe (II) (Sigma-Aldrich, USA) was used as a standard (0.01 - 0.1 mg mL⁻¹) and result obtained was expressed as the concentration of antioxidants has the ability to reduce ferric in 1 gram of sample (mM /g).

2.4.3. Radical Cation 2,2’-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) Assay. According to Bakar et al. [23], ABTS radical solution was first prepared by mixing 2.5 mL of potassium persulfate solution (2.45 mM) with 5 mL of ABTS solution (7 mM). The mixture was incubated at room temperature
for 16 hours. Then, the ABTS solution was diluted with 80% methanol until obtaining 0.700 ± 0.02 at a 734 nm. Next, 200 μL of sample was mixed with 2.0 ml of ABTS solution and measured at 734 nm using a UV-Vis spectrophotometer. Ascorbic acid (Merck, Germany) was used as the standard and the result was expressed as mg equivalent to the antioxidant capacity of ascorbic acid in 1 g sample (mg AEAC g⁻¹).

2.5. Statistical Analysis.
All tests were conducted in duplicate and reported in the form of mean ± standard deviation (n=2). Data were analyzed using T-test in SPSS version 26.0 with significant difference of p < 0.05.

3. Results and discussion

3.1. Bioactive compound of Morus alba L. fruit.

Table 1. Bioactive compounds in Morus alba L. fruit.

| Analysis                              | Hot Water Extract (60°C) | Ethanol Extract (80%) |
|---------------------------------------|--------------------------|-----------------------|
| ³Total flavonoids content             | 4.58 ± 0.05a             | 104.34 ± 1.53a        |
| ²Total phenolic content               | 0.09 ± 0.00b             | 1.21 ± 0.03b          |
| ³Total anthocyanins content           | 0.03 ± 0.00c             | 0.74 ± 0.00c          |

¹Result was expressed as mg of quercetin equivalent in 1 mg (mg QE mg⁻¹)
²Result was expressed as mg of gallic acid equivalent in 1 mg (mg GAE mg⁻¹)
³Result was expressed as mg of cyanidin-3-glucoside equivalent in 1 mg (mg c-3-gE mg⁻¹)

The data were expressed in mean ± standard deviation where (n = 2). From Table 1, significant difference (p < 0.05) between the two solvents could be seen with 80% ethanol extract containing higher flavonoids (104.34 mg QE mg⁻¹), phenolic (1.21 mg GAE mg⁻¹) and anthocyanins (0.74 mg c-3-gE mg⁻¹) content than the 60°C hot water extract. The difference in compounds values could be associated with the different polarity of the used solvents. This was because of solvents “like-dissolves-like” general principle which indicates solvents preference to only extract the same polarity phytochemicals [24]. The results showed that the bioactive compounds found in the Morus alba L. fruit have similar polarity as ethanol, hence revealing higher flavonoids, phenols and anthocyanins readings. Besides, it was previously found that polyphenolic compounds are more soluble in organic solvents with lower polarity than water [33]. This justifies the higher results of ethanolic extract as ethanol is a less polar solvent than water. The effect of extraction solvents was also reported by Kostić et al. [25] in their study using 4 different solvents which reported the highest flavonoids reading in methanol extract (1.38 mg CE g⁻¹), and highest phenolic content in ethanol extract (4.33 mg GAE g⁻¹), however anthocyanins content was not detected in any of the solvent. The result was also supported by Cui et al. [14] which obtained the highest flavonoids content in EtOH extract (50.52 mg RE g⁻¹) followed by ethyl acetate, n-butanol and petroleum ether. These showed that polarity of a solvent is indeed capable of influencing the bioactive compounds’ value. In this study, 80% ethanol was found to be a more suitable solvent in extracting flavonoids, phenolic and anthocyanins compounds from Morus alba L. fruit than 60°C hot water.

The effect of solvent aside, the total content of bioactive compounds in white mulberry fruit can also be influenced by location factors and environmental conditions of cultivation. Natić et al. [17] used 11 genotypes with different morpho-anatomical features represented by individual trees Morus alba L. collected from five locations around Northern Serbia. The results of the study found that the total content of phenolic and anthocyanins in the samples taken from the five locations gave different readings. In this study, white mulberry from Sabah revealed lower total flavonoids content (104.34 mg QE mg⁻¹) than mulberry from China (20.4 mg QUE g⁻¹) extracted with ethanol [26]. This showed that the bioactive
compounds in mulberries can be influenced by different geographical coordinates at each location and environmental conditions of cultivation. In conclusion, the solvent effect used in the extraction method influences the results obtained in this study and ethanol solvent is more suitable for use in extracting flavonoids, phenolic and anthocyanins compounds in Morus alba L. fruit. In comparison with previous studies, the total content of bioactive compounds can also be contributed by several factors such as the type of species as well as the location and conditions of the planting environment.

3.2. Antioxidant activity of Morus alba L. fruit.

Table 2. Antioxidant activity in Morus alba L. fruit.

| Analysis       | Hot Water Extract (60°C) | Ethanol Extract (80%) |
|----------------|--------------------------|-----------------------|
| 1DPPH assay    | 0.58 ± 0.00a             | 0.50 ± 0.00a          |
| 2FRAP assay    | 0.06 ± 0.00b             | 3.74 ± 0.00b          |
| 3ABTS assay    | 0.59 ± 0.00c             | 6.15 ± 0.02c          |

1Result was expressed in IC$_{50}$ (mg mL$^{-1}$).
2Result was expressed in mM of ferric to ferrous reduction in 1 g of dry sample.
3Result was expressed as mg of ascorbic acid equivalent (AEAC) in 1 g of dry sample.
a, b, c Difference between values were statistically significant (p < 0.05).

The data were expressed in mean ± standard deviation where (n = 2). The IC$_{50}$ reading of DPPH was statistically higher (p < 0.05) in 60°C hot water extract (0.58 mg mL$^{-1}$) compared to 80% ethanol extract (0.50 mg mL$^{-1}$), indicating the higher antioxidant activity of ethanol extract (Table 2). Higher FRAP and ABTS values were also observed in 80% ethanol extract than the hot water. Solvent effect is an important parameter which not only would influence the amount of dissolve compounds but also the chemical behavior of antioxidative compounds [27]. According to Çelik et al. [28] and Boeing et al. [29], different polarities of extraction solvents could significantly affected antioxidant performance based on single electron transfer (SET) and hydrogen atom transfer (HAT) due to the antioxidant compounds' different solubility in different solvents. For example, hydrogen bonding in polar solvents could dramatically change the donor activity of phenolic antioxidant H-atoms which in turn affected or reduced the measured antioxidant ability [28]. This might be the reason for the low activity in water extract. On the other hand, studies found that the usage of organic solvents-aqueous mixtures as extraction solvents generally exhibited higher antioxidant activities as compared to those of absolute organic solvents or water, which justified the higher result of our 80% ethanol extract [30][32]. Also, the difference in antioxidant activity could be linked to the higher content of phenolic (1.21 mg GAE mg$^{-1}$), flavonoids (104.34 mg QE mg$^{-1}$) and anthocyanins (0.74 mg e-3-gE mg$^{-1}$) in 80% ethanol extract than the 60°C hot water extract, as these compounds are known to possessed potent antioxidant ability [18]. Our data was similar with previous study conducted by Li et al [30] which obtained higher total phenolic and anthocyanins content in 70% ethanol extract, thus leading to its higher DPPH and ORAC activities (3.21 g VCE/ kg and 940.56 μmol TE/ g DW, respectively), as compared to their water extract (3.00 g VCE/ kg and 499.35 μmol TE/ g DW, respectively).

In addition, different geographical factors and environmental conditions of cultivation were also contributing factors to mulberry fruit’s antioxidant activity. Study of Morus alba L. fruit grown in five different locations around Vojvodina, Northern Serbia revealed different DPPH radical scavenging activity [17]. Moreover, the result of this study exhibited a more potent antioxidant activity of DPPH (IC$_{50}$ = 0.50 mg mL$^{-1}$) compared to China mulberry (IC$_{50}$ = 0.518 mg mL$^{-1}$) in Raman et al. [26]. This clearly shows that factors of location and environmental conditions of cultivation can influence the results of antioxidant activity in the fruit of mulberry. Overall, the type of solvent used in this study could affected the results of
antioxidant activity obtained and that 80% ethanol was more effective in extracting antioxidant compounds in *Morus alba* L. fruit grown in Sabah, Malaysia.

4. Conclusion

It could be concluded that *Morus alba* L. fruit grown in Sabah, Malaysia contained high flavonoids compounds compared to the phenolic and anthocyanins compounds. Ethanol (80%) is a better solvent than hot water (60°C) in extracting bioactive compounds and high antioxidant activity from *Morus alba* L. fruit. Through this study, it was shown that factors such as solvents, species and cultivars as well as geographical location could influenced the activity of antioxidants and bioactive compounds in *Morus alba* L. It is hoped that this data can provide useful preliminary information on bioactive compounds and antioxidants activity of *Morus alba* L. fruit grown in Sabah, Malaysia. Nevertheless, further studies using other solvents should be conducted to obtain best extraction solvents data for *Morus alba* L. in Sabah, Malaysia.

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