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Influence of different extraction solvents on antioxidant and antityrosinase activities of *Morus alba* Linn. leaf extract.

Wachira Yodthong[1], Suraphon Chaiwongsar[1], Penpicha Wanachantararak[2], Yanee Keereeta[1], Watee Panthuwat[1], Benjaporn Saovapha[3], Tanongsak Sassa-deepaeng[1]*

[1]Agricultural Biochemistry Research Unit, Faculty of Science and Agricultural Technology, Rajamangala University of Technology Lanna, Lampang, Thailand
[2] Dentistry Research, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand
[3] Boromrajonani College of Nursing Nakorn Lampang, Lampang, Thailand

***Corresponding author**: nai.rmutl@gmail.com
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Abstract

The objective of this study was to investigate the impact of different extraction solvents such as hexane, benzene, ethyl acetate, acetone, ethanol, and water on antioxidant and antityrosinase efficiency of mulberry leaf extract. The sample was extracted from leaves by maceration method. The obtained extract was filtered and evaporated to dryness by using the rotary evaporator prior to measuring the total phenolic content, flavonoid content, antioxidant activity, antityrosinase activity, and morin content in triplicate. The results indicated that the highest antioxidant activity was found in ethyl acetate (EA) fraction of CM60 with ABTS value of 57.8 ± 7.7 % inhibition and DPPH value of 58.2 ± 0.7 % inhibition that were positively related to its phenolic content (36.9 ± 1.3 μg GAE/mg DW). Interestingly, The highest antityrosinase activity was found in acetone fraction of CM60 which inhibited tyrosinase activity by 3.0 ± 0.1 μg KE/mg DW that was positively related to its flavonoid content (32.71 ± 0.1 μg QE/mg DW) and was related to its highest morin content (0.71 ± 0.01 μg/mg DW) measured using HPLC. The present results indicated that the EA fraction of CM60 possessed the highest antioxidant property related to its phenolic content, and the acetone fraction showed the highest antityrosinase activity related to its flavonoid content especially morin which is tyrosinase inhibitor. Both fractions of *M.* alba were potential candidates for skin protector from oxidative damage and skin-whitening agent development. Further studies are necessary to formulate the compounds responsible for antioxidant and antityrosinase properties and to investigate antityrosinase properties *in vivo* prior to transferring technology to communities.

Keywords: mulberry, antioxidant, antityrosinase, solvent extraction

Introduction

Melanins are multifuctional polymers that are found in animals, plants, arthropods, bacteria, and fungi (Solano, 2014). They are classified into 3 forms, including eumelanin, pheomelanin, and neuromelanin. Eumelanin is the dominant form of melanin found in human melanocytes which plays important role in camouflage, thermoregulation and photoprotection of the skin against ultraviolets (Nasti and Timares, 2015; Ulbing et al., 2019). However, the over-exposure in sunlight lead to promoting synthesis of melanin resulted presenting of unpleasant skin color (Yun et al., 2015; Ribeiro et al., 2015). In addition, this excessive melanin synthesis and deposition in skin may cause melasma, freckles, lentigo and geriatric pigment spot lead to may not pursuit of most Thai women (Yun et al., 2015; Ya et al., 2015). Although numerous synthetic chemicals, such as hydroquinone and some derivatives, revealed high effective in skin lightening and in treatment of pigmentation disorders through inhibit melanin production and through decreasing the number of melanocytes, these can also lead to skin irritation and has been reported as carcinogen and mutagen (Agorku et al., 2016; McGregor, 2007; Parvez et al., 2006). It is important to find novel plant-based compounds which are safe and effective to overcome this side effects. Additionally, some active compounds from plants also showed as high potential antioxidants which are important in prevention of oxidative process in melanin production.

Mulberry (*Morus alba* L.), belonging to the Moraceae family, is a deciduous plant which is widely distributed in Thailand regarded as nutritious fruit for human, forage and silkworms. The leaves, twigs, and root barks have been used in medicine recipes to treat diabetes (Ranjan et al., 2017; Riche et al., 2017), diabetic nephropathy (Sheng et al., 2018), obesity (Kim, 2017; Chang et al., 2016), antioxidant (Kim and Lee, 2017; Bae and Suh, 2007), anti-dopaminergic (Hussain et al., 2017; Yadav and Nade,

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2008), atherosclerosis, and cancer (Hashemi and Khadivi, 2020; Chon et al., 2009). It was also found that numerous flavonoids, the most water-insoluble compounds, were the main active constituents of mulberry, including morin, resveratrol, quercetin, and quercetin derivatives (Chauhan et al., 2013; Sun et al., 2011). To achieve specific active compounds, various solvents with different polarities were employed in mulberry leaf extraction, including hexane, benzene, ethyl acetate, acetone, ethanol and water.

In this study, to obtain the most potent tyrosinase and oxidative inhibitory fraction, the impact of different polarity of solvents on antioxidant and antityrosinase activities of *Morus alba* Linn. leaf extract were investigated.

Materials and methods

Plant materials

The mature leaves of mulberry var. Chiangmai 60 (CM60) were collected randomly from 10 plants on May 7th, 2018 as recommended by Zou et al. (2012) in Rajamangala University of Technology Lanna Lampang (18.367053, 99.596299), Mueang Lampang District, Lampang, Thailand and then authenticated by an herbalist from Rajamangala University of Technology Lanna (RMUTL) whereas var. Buriram 60 (BR60) and Sakhonnakhon 75 (SN75) were available in the same manner on May 9th, 2018 as the voucher specimen from Queen Sirikit Sericulture Center Chiang Mai (18.903564, 99.013597) San Sai District, Chiang Mai, Thailand.

Sample preparation

Upon arrival, 500 mg of fresh sample was washed with tap water followed by distilled water prior to drying in hot air oven at 55 °C until constant weight. The dried sample was ground by using electric blender is obtained. Fine powder was collected by using 20-mesh sieves before stored at -20 °C for further analyses.

Sample Extraction

Extraction was conducted according to the sequential extraction method using different solvents including hexane, benzene, ethyl acetate, acetone (from RCI Labscan, Bangkok, Thailand), ethanol (from Liquor Distillery Excise Department, Bangkhla, Thailand) and water. Each solvent extraction was performed in triplicate as described by Thavamoney et al. (2018) with some modifications. The 10 g of mulberry powder was immersed in 250 mL of solvent at ambient temperature and was agitated at 180 rpm assisted by an orbital shaker for 180 min twice a day and then filtered. The remaining residue was subjected to re-extraction. The filtrates of each solvent were pooled and concentrated by using a rotary evaporator, while water filtrate was dried by using a freeze dryer.

ABTS assay

The ABTS free radical-scavenging activity was examined according to the modified method described by Sassa-deepaeng et al. (2017). The 7.0 mM ABTS (Sigma–Aldrich, Steinheim, Germany) aqueous solution and 2.45 mM Potassium persulfate (VolChem, Shantou Xilong Chemical Plant Co Ltd., China) was mixed and incubated at the ambient temperature for 16 hours in the dark to generate pre-formed radical cation $ABTS^{+}$. The working solution was diluted with DI water to absorbance values between 0.8±0.1 at 734 nm. The reaction was started by adding 20 µL of various concentrations (125, 250, 500, 1000 µg/mL) of samples to 980 µL of the working solution and then incubated at the ambient temperature for 30 min in the dark. The unpaired electrons were sequestered by antioxidants in the sample resulted the test solution turned into colorless. The measurement was performed against the bank at 734 nm using V-1200 spectrophotometer (Dshing Instrument Co., Ltd., China) with UV-Professional analysis software. The percentage of inhibition of antioxidant was calculated using the equation:

$$\% \text{ inhibition} = [(A_c - A_s)/A_c] \times 100$$

Where A_c was the absorbance of the control and A_s was the absorbance of the reaction mixture.

The linear curves were constructed by plotting the percentage of inhibition against the concentration in µg/mL ($R^2 = 0.99$).

DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical-scavenging activity was examined using modified method described by Sassa-deepaeng et al (2019). DPPH (Sigma–Aldrich, Steinheim, Germany) solution (oxidized form) was prepared in absolute ethanol to get final absorbance of 0.8 ± 0.1 at 517 nm. One hundred µL of various concentrations (125, 250, 500, 1000 µg/mL) of samples were mixed with 900 µL of DPPH radical solution and then incubated at the ambient temperature for 30 min in the dark. The antioxidant of the sample, which can donate an electron to DPPH radical contributed in solution color develop into yellow. The solution was measured against the bank at 517 nm using V-1200 spectrophotometer (Dshing Instrument Co., Ltd., China) with UV-Professional analysis software.

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The percentage of inhibition of antioxidant was calculated using the equation:

$$\% \text{ inhibition} = [(A_c-A_s)/A_c] \times 100$$

Where A_c was the absorbance of the control and A_s was the absorbance of the reaction mixture.

The linear curves were constructed by plotting the percentage of inhibition against the concentration in µg/mL ($R^2 = 0.99$).

Total phenolic content

The total phenolic content (TPC) was examined using the Folin-Ciocalteu (FC) colorimetric method. Twenty µL of various concentrations (125, 250, 500, 1000 µg/mL) of the extract was added to 100 µL of FC (Merck, Damstadt, Germany) reagent in 1,980 µL of DI water and then was incubated for 5 min at the ambient temperature. Afterward, 300 µL of 7% of Na_2CO_3 (Univar, Ajax Finechem, Australia) was added and incubated again for 60 min at the ambient temperature in the dark, the absorbance of the solution was measured at 765 nm using V-1200 spectrophotometer with UV-Professional analysis software. TPC quantitation was performed in triplicate. The gallic acid at different concentrations was used to generate the calibration curve ($R^2 = 0.99$). The TPC was expressed as microgram gallic acid (Bio Basic Inc., Ontario, Canada) equivalent (GAE) per milligram dry weight (Sassa-deepaeng et al., 2017).

Flavonoid content

The flavonoid content (FC) was examined using aluminum trichloride ($AlCl_3$) colorimetric method with some modified by Sassa-deepaeng et al. (2019). Twenty µL of various concentrations (125, 250, 500, 1000 µg/mL) of the extract was added to 380 µL of DI water followed 100 µL of 5% $NaNO_2$ (Univar, Ajax Finechem, Australia) and incubated for 5 min at the ambient temperature. Afterward, 100 µL of 10% $AlCl_3$ (Lobachemie, Mumbai, India) was added and was incubated again for 6 min at the ambient temperature. At the final step, 400 µL of 1M NaOH (Merck KGaA, Darmstadt, Germany) was added and then incubated for 15 min in the dark. The absorbance was measured at the wavelength of 415 nm using V-1200 spectrophotometer with UV-Professional analysis software. The flavonoid quantitation was carried out in triplicate and the FC was expressed as microgram quercetin (Sigma–Aldrich, Steinheim, Germany) equivalent (QE) per milligram dry weight.

Antityrosinase activity

The antityrosinase activity was performed according to the method described by Momtaz et al. (2008). The standard inhibitor and extracts (400 µg/mL) were dissolved in ethanol (Liquor Distillery Excise Department, Bangkhla, Thailand) and then diluted in 50 mM dibasic sodium phosphate buffer, pH 6.6. The extracts were firstly pre-tested at one concentration of 400 µg/mL for their inhibitory effect on tyrosinase. Afterwards, the samples which possessed the inhibitory effect were selected for further study in various dilutions ranged from 3.125 to 800 µg/mL. Kojic acid was used as positive control. The 70 µL of each extract was mixed with 30 µL of tyrosinase (333 U/mL) in 96-well microplate (Sterilin, Sterilin Limited, U.K.) and incubated for 5 min at the ambient temperature. Afterward, 110 µL of substrates (4 mM L-DOPA) was added to each well. The mixtures were then incubated for 30 min at the ambient temperature in the dark. The amount of dopachrome was measured at the wavelength of 492 nm using microplate reader (BIOBASE-EL10, Biobase Biodustry (Shandong) Co., Ltd., China). The percent inhibition of tyrosinase was calculated as the following equation:

$$\%\text{Tyrosinase inhibition} = [(A-B)-(C-D)/(A-B)] \times 100$$

Whereas,
A = absorbance of blank solution with tyrosinase
B = absorbance of blank solution without tyrosinase
C = absorbance of sample solution with tyrosinase
D = absorbance of sample solution without tyrosinase

HPLC apparatus and conditions

The morin content was determined by and in-house validated HPLC method on Hitachi CM-5000 Chromaster series (Chromaster, Hitachi, Japan) equipped with a 5110 pump, 5260 autosampler, 5310 column oven and 5430 diode array detector. The separation was performed on a Purospher® STAR RP-18 endcapped (250 mm x 4.6 mm i.d., 5 µm) column (Merck KGaA, Damstadt, Germany). The elution was conducted on isocratic solvent system using 0.1% phosphoric acid in acetonitrile (1:9) as mobile phase, filtered through a 0.45 µm Nylon membrane filter (Merck KGaA, Darmstadt, Germany) and sonicated for 10 min before use; flow rate of 1.0 mL/min for 10 min with controlled temperature at 25°C. DAD detector was set at the wavelength of 252 nm. The injection volume of samples and standards was 10 µL.

Statistical analysis

The data were analyzed using tool pack of the Microsoft Excel 2016 software. (Microsoft Corporation., CA, USA). One-way analysis of

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variance and the LSD range test were used to determine significant differences and the differences at $p < 0.05$ were considered significant.

Results and discussion

Sequential extraction procedures and solvents are the critical step which is responsible for dissolving the endogenous active compounds from mulberry leaves. To achieve the highest yield of active compound from sample, the different polarity of solvents (Reichardt, 2003) including hexane, benzene, ethyl acetate, acetone, ethanol and water with the polarity of 0.009, 0.111, 0.228, 0.355, 0.654, and 1.00 were used. Thus, due to the fundamental principle of solubility, active compounds in samples can be extracted by using appropriate solvents. The yield obtained from the extraction is shown in Figure 1.

The percent extraction yield in each of the mulberry variety up to 100% as indicated in Figure 1. The maximum yield was ethanolic extract of BR60 (47.5 ± 8.8), following of SN75 and of CM60, respectively, while the minimum extraction yield was found in acetonic extracts of BR60 (4.5 ± 1.5), following that of CM60 and SN75, respectively. It can be suggested that the major phytochemicals in mulberry leaves were mostly high in polarity as indicated by Andallu et al. (2014), and Zhang et al. (2010). Interestingly, the color of hexane extract was yellow with oily odor, while of the others were dark-greenish without oily smell. The possible explanation for this observation was the presence of lipid or lipophilic compound in hexane fraction. However, the result was not indicated which fractions possessed the active gradient. Therefore, ABTS assay for assessing the antioxidant activity of each extract was firstly conducted.

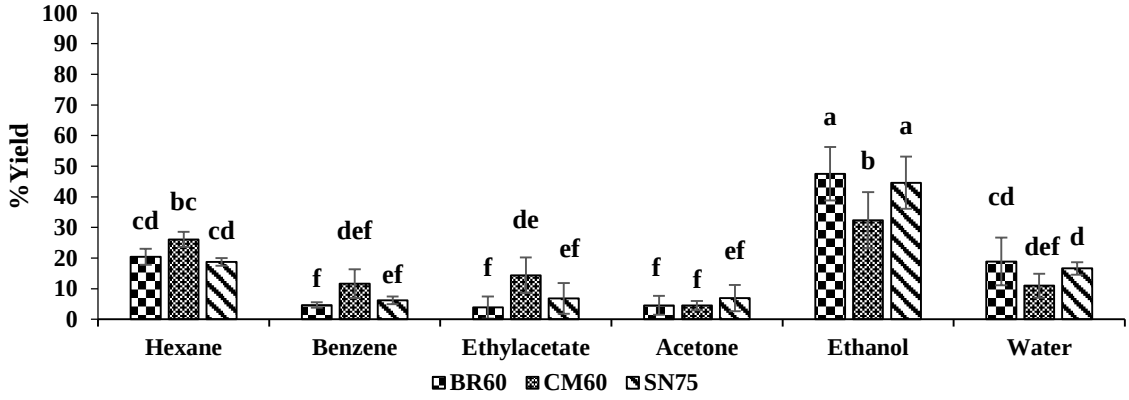

Figure 1. Yield of various extract of mulberry leaves. The error bars represent the standard deviation of mean. The different letters indicate significant difference ($p < 0.05$).

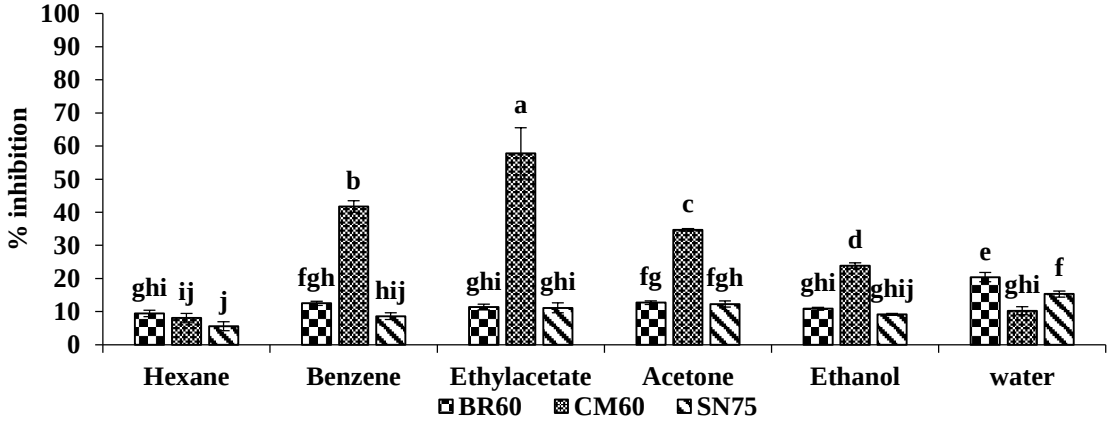

Figure 2. ABTS assay for assessing the antioxidant activity of *M. alba* (CM60, BR60 and SN75) leaf extracts in different solvents. The error bars represent the standard deviation of mean. The different letters indicate significant difference ($p < 0.05$).

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The free radical scavenging activity was screened in terms of the percentage inhibition of the ABTS radical cation by the antioxidants in sample. It was found that there was a significant variation in the percentage inhibition of the different solvent extracts (8.1 to 57.8 % inhibition) revealed in CM60 samples which were possessed the scavenging activity greater than BR60 and SN75 samples. The ranging inhibitory effect in the order of CM60 was ethyl acetate > benzene > acetone > ethanol as indicated in Figure 2. Concurrently, hexane extract had proved the most inefficient scavenger of the ABTS radical. It was confirmed that mainly antioxidant compounds were deposited in polar solvent extracts or the high-polarity components contributed to reducing power as documented by Li et al. (2017). Therefore, The DPPH scavenging activity was performed to insist upon the finding.

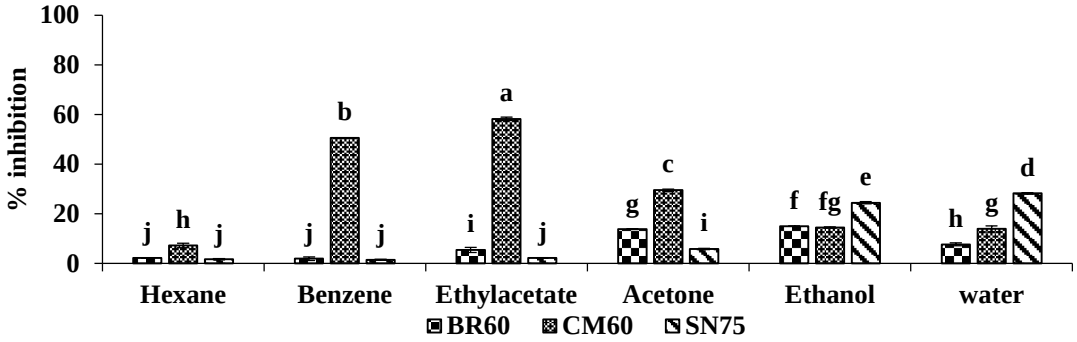

Figure 3. DPPH assay for assessing the antioxidant activity of *M. Alba* (CM60, BR60 and SN75) leaf extracts in different solvents. The error bars represent the standard deviation of mean. The different letters indicate significant difference ($p < 0.05$).

The DPPH scavenging activity was quantified in terms of the percentage inhibition of a pre-formed free radical by the antioxidants in sample. The similar pattern of the antioxidant inhibitory properties as mentioned in ABTS assay was obtained, but the values were slightly lower than ABTS assay as indicated by Saravanakumar et al. (2019) due to ABTS is more sensitive than DPPH when the sample consist of a large quantity of hydrophilic compounds. It was also found that the mostly DPPH radical scavenging activities of the CM60 extracts were greater than BR60 and SN75, and ranging in the order: ethyl acetate > benzene > acetone > water > ethanol > hexane as indicated in Figure 3. There was a significant variation in the percentage inhibition of the DPPH radical by the solvents (7.2 to 50.6 % inhibition). Again, ethyl acetate extract of CM60 showed the highest antioxidant capacity. Concurrently, all hexane extracts also had a considerably the lowest DPPH scavenging capacity. Interestingly, there was to be trend for the radical scavenging capacity of sample next to ethyl acetate seem to be slightly decreased following the increasing of solvent polarity. It was also found these characteristics in using less polarity solvents. Overall, the greatest DPPH radical scavenging activity was found in ethyl acetate extract of CM60. Therefore, the TPC assay was conducted to ascertain whether it was a factor which was related to the free radical scavenging activity.

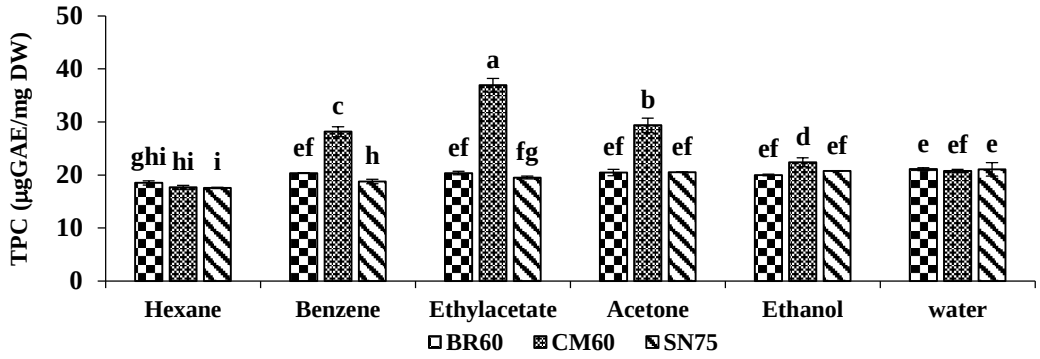

Figure 4. The total phenolic content of *M. alba* (CM60, BR60 and SN75) leaf extracts in different solvents. The error bars represent the standard deviation of mean. The different letters indicate significant difference ($p < 0.05$).

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Mulberry leaves were harvested in May as recommended by Zou et al. (2012) due to the highest total phenolic content was obtained. The TPC value was quantified by using the Folin–Ciocalteu reagent assay. It was found that the TPC values in three mulberry cultivars ranged from 17.55 to 36.92 µg GAE/mg DW, with the highest TPC content in ethyl acetate extract of CM60 and the lowest TPC content in hexanic extract of BR60 as shown in Figure 4. It can be suggested that the TPC was extracted at the appropriate polar as indicated by Abarca-Vargas et al. (2016). These findings were similar to report by Hao et al. (2018) who found a variation of the TPC of 24 Chinese mulberry leaves, ranging from 23.4 to 39.4 µg GAE /mg DW and similar the finding of Zou et al. (2012) who found a variation of the TPC of 6 Chinese mulberry leaves, ranging from 30.4 to 44.7 µg GAE/mg DW. In addition, acetonic extract and benzenic extract of CM60 were also revealed high TPC content. The values of phenolic content in this current study varied slightly compared to those literatures might be due to the geographical variation and flavonoid content. To investigate the influence of solvents on flavonoid content, the aluminum chloride was used for further study and presented in Figure 5.

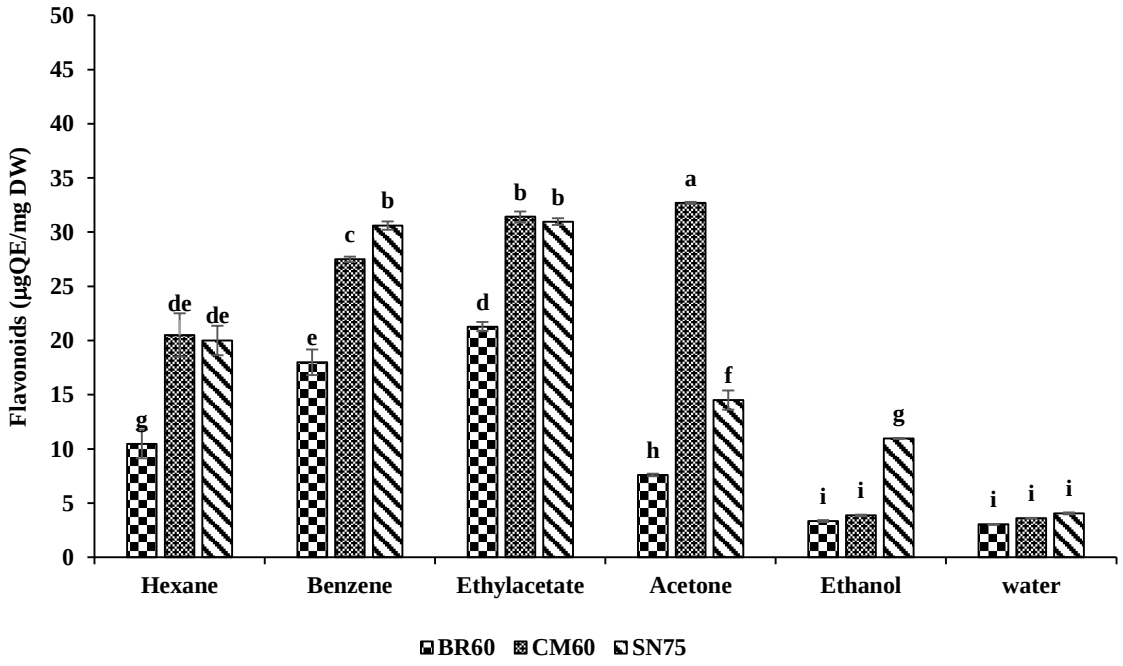

Figure 5. The flavonoid content of *M. alba* (CM60, BR60 and SN75) leaf extracts in different solvents. The error bars represent the standard deviation of mean. The different letters indicate significant difference ($p < 0.05$).

The flavonoid content in mulberry extracts was determined using aluminum chloride in a colorimetric method. It was found that the flavonoid values in three mulberry cultivars ranged from 3.0 to 31.4 µg QE/mg DW, with the highest flavonoid content in acetonic extract of CM60 and the lowest flavonoid content in all water extracts as shown in Figure 5. The flavonoid content was similar to report by Chang et al. (2011) who found the value of Taiwan mulberry leaf was 23.5 µg Rutin Equivalent /mg DW, but was slightly lower than the finding of Chauhan et al. (2013) who reported that flavonoid contents of mulberry varieties ranged from 40.7 to 165.6 µg GAE/mg DW. As indicated by Aryal et al. (2019), the genetic diversity, geographical variation, environmental factors, and season were also significantly affected the flavonoid content. To explore correlations of flavonoid and total phenolic content with their antioxidant activities, the correlation curve was generated and presented in Figure 6.

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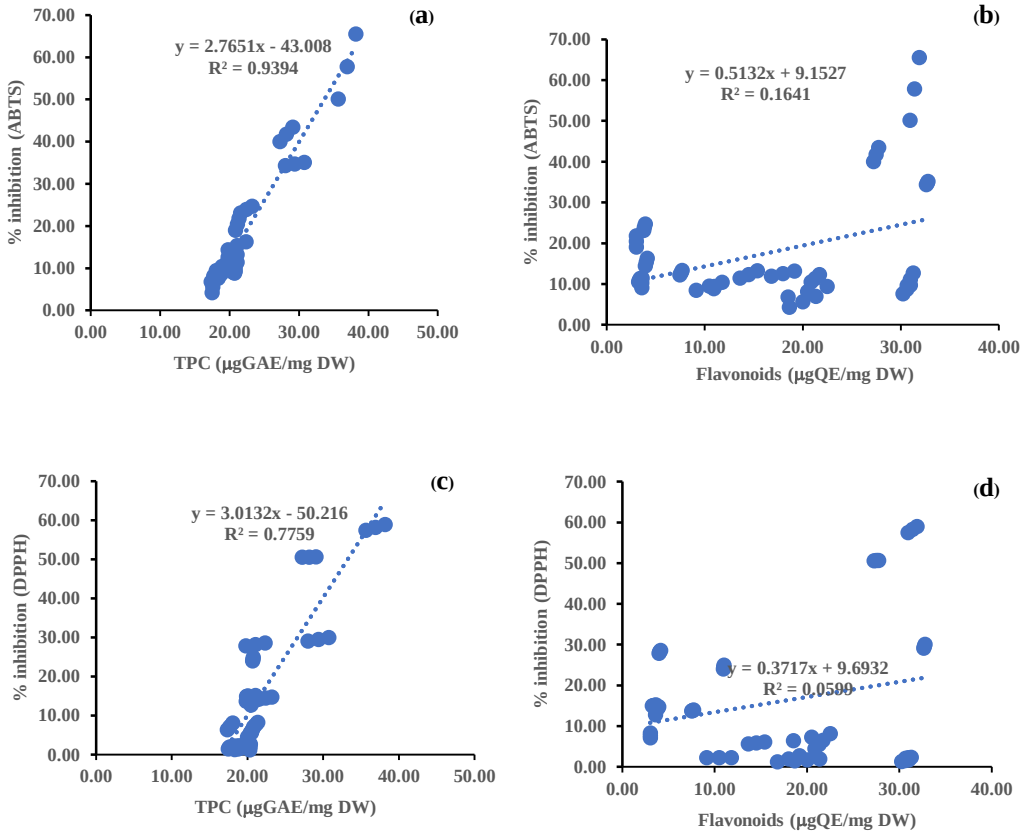

Figure 6. The correlation between (a) TPC content and % inhibition of ABTS, (b) Flavonoid content and % inhibition of ABTS, (c) TPC content and % inhibition of DPPH, and (d) Flavonoid content and % inhibition of DPPH.

It was found that the TPC content value was significantly correlated with percentage inhibition of ABTS ($R^2 = 0.9394$) and DPPH ($R^2 = 0.7759$) assays as presented in Figure 6, whereas the flavonoid content was not correlated with any antioxidant assay. The result was similar to the finding of Andarwulan et al. (2010) who found that the TPC value was highly correlated with DPPH and ABTS radical scavenging assays, but the flavonoid content was not correlated with those antioxidant activity. It could be associated with other pro-oxidant constituents or consisting of flavonoids which possessed low potential antioxidant property in the extracts. Interestingly, only the extracts of CM60 showed the correlation significantly with ABTS and DPPH assay. Thus, it can be suggested that CM60 extracts were trend to be high potential for cosmetics development. However, the results from antityrosinase assay were also need to be used for that purpose.

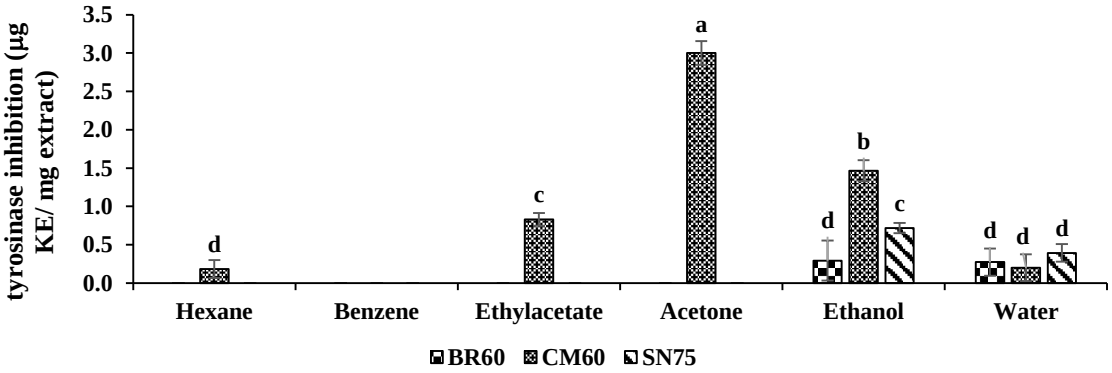

Figure 7. The antityrosinase inhibitory activity of *M. alba* (CM60, BR60 and SN75) leaf extracts in different solvents. The error bars represent the standard deviation of mean. The different letters indicate significant difference ($p < 0.05$).

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The antityrosinase inhibitory activity of different solvent extracts was determined in terms of the percentage inhibition of tyrosinase by various inhibitors in sample. It was found that the mostly inhibitory activities of CM60 extracts were greater than BR60 and SN75, and ranging in the order: acetone > ethanol > ethyl acetate as indicated in Figure 7. Unfortunately, the inhibitory activity was undetectable in any benzenic extracts and found only a small amount of activity in hexanic and water extracts. It was also found that there was a significant variation in the percentage inhibition of tyrosinase of CM60 extracts by the solvents (0.0 to 3.0 μg KE/mg extract). Interestingly, acetonic extract of CM60 showed the highest antityrosinase capacity. It was confirmed that mulberry leaf extract also possessed antityrosinase compound as it was found in twig and root (Chang et al., 2011). The antityrosinase activity might be involved the flavonoid composition such as morin as reports of Chang et al. (2011), and Zhang et al. (2016). Therefore, the HPLC method was conducted to further investigate the morin content in CM60 extracts and presented in Figure 8.

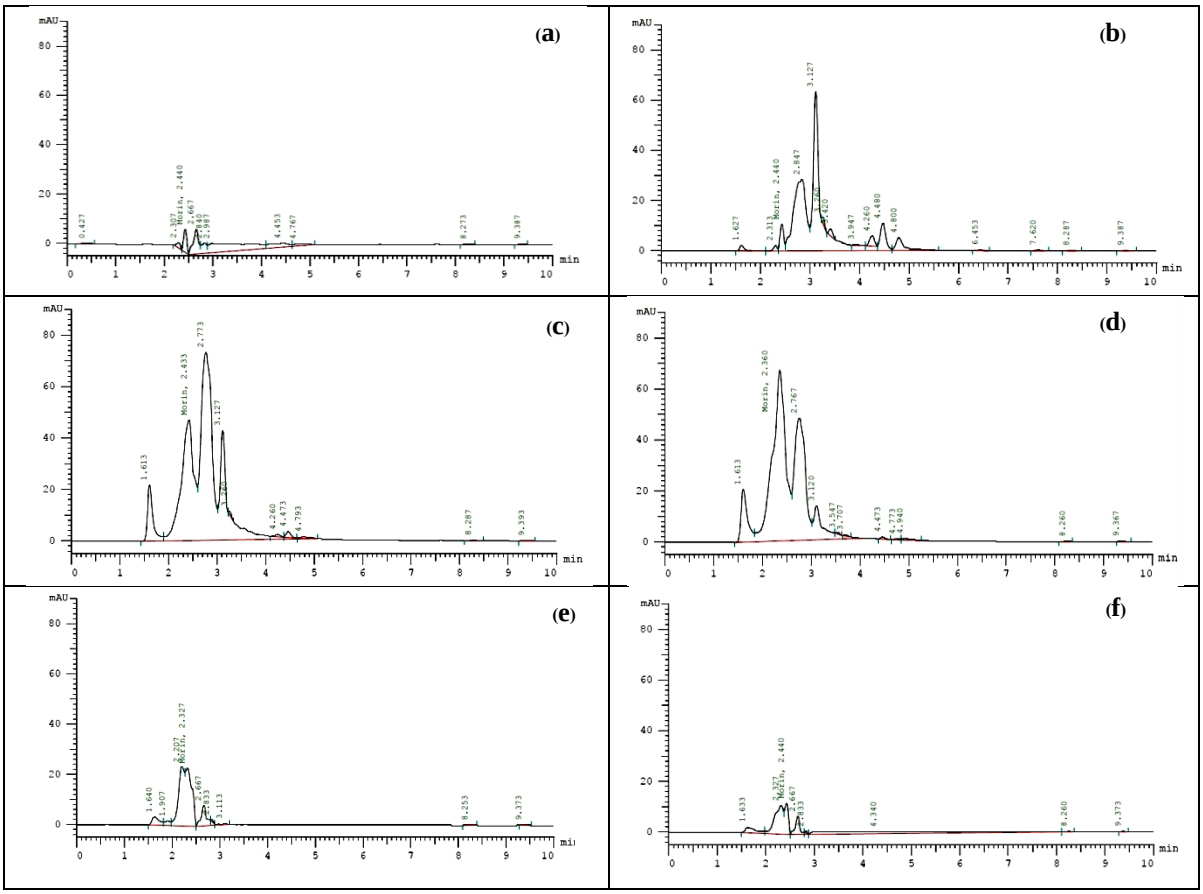

Figure 8. HPLC chromatograms of *M. alba* (CM60) leaf extracts in different solvents (a) hexane, (b) benzene, (c) ethyl acetate, (d) acetone, (e) ethanol, and (f) water

The HPLC method was validated for the analysis of morin content in the different solvent extracts of CM60. The linear curve of standard was generated by using morin hydrate (Sigma-Aldrich, Germany) dissolved in mobile phase at the concentration range of 1.25-10.0 μg/mL. The good linearity was exhibited by the 4-point calibration curve performed in triplicate with correlation coefficients (R^2) within the range of 0.9996-0.9999, which indicated that the method was effective for the quantification of morin. The HPLC chromatogram of morin in each extract of CM60 is presented in Figure 9.

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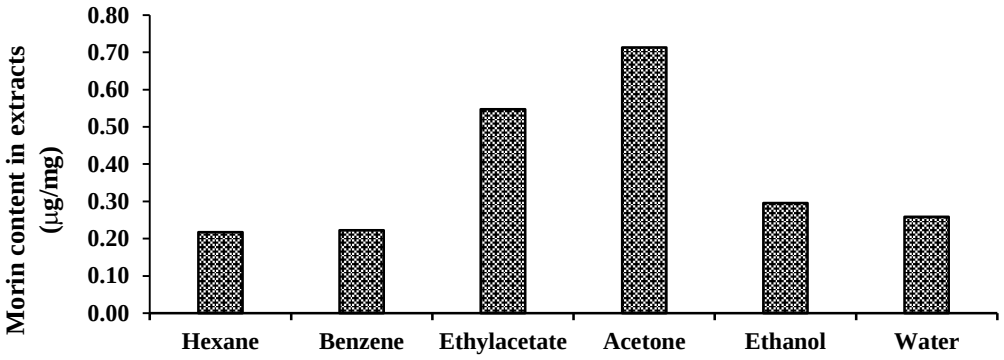

Figure 9. The morin content of *M. alba* (CM60) leaf extracts in different solvents.

The in-house validated HPLC method was applied to determine the contents of morin in the six different polar extracts of CM60. The results in Figure 9 showed that acetonic extract of CM60 possessed the greatest amount of morin, ranging in the order: that of ethyl acetate, ethanol, water, hexane = benzene. Interestingly, the acetonic extract of CM60 was revealed the greatest inhibitory effect of tyrosinase and related to the highest contents of flavonoid and morin. Therefore, it can be assumed that morin possessed inhibitory effect of tyrosinase accompany with other flavonoids such as rutin, maclurin, resveratrol, isoquercitrin and the others in the category of flavonols as the reports of Chang et al. (2011) and Yang et al. (2017).

Conclusions

The ethyl acetate extract of CM60 showed the greatest antioxidant inhibitory activity evaluated by using ABTS and DPPH assays and showed positively related to TPC content. Meanwhile, the acetonic extract from CM60 exhibited better tyrosinase inhibitory activity than other varieties and revealed positively related to the flavonoid content. Additionally, morin had a high content in the mulberry leaf extract from CM60 especially in acetonic extract, thus CM60 leaf extracted using acetone and ethyl acetate was the most suitable as potential resources for skin-whitening agent development.

Acknowledgements

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