Effect of nutraceutical from mulberry on anthropometric measures in overweight and obese adults: a randomized, double-blinded, and placebo-controlled trial

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Abstract

Mulberry fruit extract (MFE) has been reported to show remarkable anti-obesity properties in vitro. While the trend for nutraceuticals is increasing, the study on the anti-obesity potential of MFE in humans is limited. This study aimed to develop and evaluate the nutraceutical from MFE and examine the effects of the MFE capsule on anthropometric measures in overweight and obese adults. The nutraceutical was developed, and quality control was carried out. Cyanidin-3-O-glucoside, the major bioactive compound in MFE, was found at 21.50±0.54 mg in each MFE capsule when analyzed by using the validated HPLC method. The weight variation, disintegration, and dissolution profile of MFE capsules were acceptable within the USP 42 requirement for dietary supplements. A total of thirty-two overweight participants were assigned to consume a capsule containing MFE or a placebo capsule daily for 8 weeks in a randomized, double-blinded, and placebo-controlled trial (n = 16 per group). At the end of the study, MFE intake significantly caused a reduction in the visceral fat level when compared to the placebo group (p < 0.05). Moreover, in the MFE group, body weight, BMI, waist circumference, subcutaneous fat of the whole body, trunk, arms, and legs, total body fat, and visceral fat were significantly decreased (p < 0.001). In conclusion, MFE was successfully developed into capsules. MFE capsules showed a favourable effect in reducing anthropometric measurements in overweight and obese adults. This is the first report on the promising anti-obesity potential of MFE capsules in humans.

1. Introduction

Obesity is defined as excessive fat accumulation altering immoderate body weight (WHO, 2021). World Health Organization reported that the number of overweight and obese populations were 39 and 13% globally (WHO, 2021). In Thailand, the report showed approximately 38% of the population was overweight and obese (Health Data Center, 2018). WHO states the definition of overweight and obese based on the data analysis of Body Mass Index (BMI) for Asian people, a BMI of 23 to 29.9, or 30 and greater are considered overweight and obese, respectively (WHO, 2021). Obesity can cause many non-communicable diseases such as diabetes mellitus, hypertension, dyslipidemia, and metabolic syndromes. However, it can be managed by lifestyle modification, diet control, physical exercise, and medications (WHO, 2021). There are currently six medications for the treatment of obesity approved by the U.S. Food and Drug Administration: phentermine, phentermine/topiramate, liraglutide, lorcaserin, naltrexone/bupropion, and orlistat, however, they can cause severe side effects such as dry mouth, constipation, abdominal pain, faster pulse, cough, dizziness, leakage of oily stools, and nausea (National Institute of Diabetes and Digestive and Kidney Diseases, 2020). In Thailand, the Thai FDA approved only orlistat for weight loss purposes (National Drug Information, 2020). In addition, natural products were reported to be alternative choices to counteract obesity (Helal et al., 2020).

Many natural products are available as dietary supplements or nutraceuticals (Helal et al., 2020). Nutraceutical is explained as a food or part of a food that...
provides benefits in addition to its nutritional content. A suitable nutraceutical form can be used for the prevention of some pathogenic conditions (Santini et al., 2017). Most of them are unlikely to provide the intended pharmacological activities without appropriate formulations, which contribute to the instability during processing, storage, or absorption in the GI tract (Bone and Mills, 2013). The United States Pharmacopeia 42 (USP 42), the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), and the Association of Official Agricultural Chemists (AOAC) have provided guidelines for method validation and quality control of pharmaceutical products (European Medicines Agency, 2006; AOAC INTERNATIONAL, 2014; The United States Pharmacopeial Convention, 2019). Among various dosage forms, the capsule is the most common dosage form since it conceals unpleasant tastes or textures and is convenient to be administered (Bone and Mills, 2013).

*Morus alba* L. (white mulberry) is widely cultivated in China, South Korea, and Thailand. Its fruit has been used as a traditional Chinese medicine over decades for anti-inflammation, anti-oxidative stress, and improvement of cardiac function (Chan et al., 2016). The mulberry fruit extract (MFE) contains various bioactive compounds including flavonoids, polysaccharides, and phenolic acids, of which the major constituent is anthocyanin (Bao et al., 2016). Anthocyanin extracts were reported to have anti-obesity effects, including preventing body weight gain, inhibiting lipid accumulation, and exhibiting anti-obesity effects (Prior et al., 2010; Cardile et al., 2015). Cyanidin-3-O-glucoside (C3G), the major anthocyanin in mulberry, has been addressed that it could attenuate obesity by increasing the number of mitochondria during brown adipose tissue differentiation which leads to higher energy expenditure (You et al., 2017). In animal models, MFE reduced total body weights and hepatic lipids (Peng et al., 2011). Freeze-dried MFE was found to suppress weight gain in mice and rats by deflecting lipid accumulation and decreasing the secretion of leptin (Yang et al., 2010; Wu et al., 2013). Encapsulated MFE decreased metabolic syndrome parameters in rats (Wattanathorn et al., 2019). Apart from that, the evaluation of the toxicity of MFE revealed no abnormalities at a single dose in the acute toxicity test and the subchronic regimen in mice. And there was no genotoxicity when tested in*Salmonella* (Wattanathorn et al., 2012; Chang et al., 2016).

Despite the fact, MFE exhibited potential anti-obesity properties in vitro and in vivo, its effect in human subjects has not been examined. This study aimed to develop and evaluate the nutraceutical from MFE and examine the effect of the MFE capsule on anthropometric measures in overweight and obese adults.

2. Materials and methods

2.1 Materials and reagents

Spray-dried ethanolic extract of MFE was purchased from Baoji Oasier Nutri-tech Co., Ltd. (Baoji, China). Analytical-grade acetonitrile (J.T. Baker, USA), methanol (Burdick and Jackson, USA), and phosphoric acid (Sigma-Aldrich, USA) were obtained. Hydrochloric acid and sodium acetate trihydrate were purchased from Merck KGaA (Merck, Germany). C3G chloride (≥98%) was used (ChemFaces, China).

2.2 Determination of C3G in mulberry fruit extract powder by HPLC

Sample extraction was performed by following the method described by Chen et al. (2017) with minor modifications. Five hundred micrograms of MFE sample were extracted in 0.1% hydrochloric acid in water and methanol (50:50, v/v) at 30°C by using an ultrasonic bath to obtain the concentration of 100 µg/mL. The extract was then filtered through a 0.20-µm nylon filter. C3G was used as a standard reference in this analysis. A stock solution of C3G was prepared in 0.1% phosphoric acid in water and acetonitrile (50:50, v/v) at 30°C by using an ultrasonic bath to obtain the concentration of 100 µg/mL. The stock solution was filtered through a 0.20-µm nylon filter for further analysis (Fang et al., 2020). An HPLC system used in this study was a Shimadzu Prominance equipped with a PDA detector (Shimadzu, USA). A Purospher® STAR RP-18 column (150 × 4.6 mm, 5 µm, Merck, Germany) was fitted with a guard column Purospher® STAR (5 µm, Merck, Germany). The mobile phase consists of acetonitrile as solvent A and 0.1% phosphoric acid in water as solvent B. The flow rate was 0.8 mL/min. The column temperature was maintained at 40°C. The injection volume was 10 µL for all prepared samples. A gradient elution program was performed as follows: 0 min, 95-80% A, 5-25 min, 80% A, 25-30 min, 80-70% A, and 30-35 min, 70-95%. The detection wavelength was set at 520 nm. Each sample was analyzed in triplicate (Fang et al., 2020). The HPLC method was validated according to ICH and AOAC guidelines for linearity, accuracy, precision, and specificity (European Medicines Agency, 2006; AOAC INTERNATIONAL, 2014; The United States Pharmacopeial Convention, 2019). The calibration curve of the reference standard, C3G, was obtained from five different concentrations in the range of 20-60 µg/mL.

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2.3 Preformulation study of mulberry fruit extract powder

The moisture content of MFE powder was determined by the gravimetric method by using a moisture analyser (RADWAG, USA), and the percentage of moisture content was calculated by dividing weight loss by weight before drying (%). Determination of bulk and tapped density were performed by compression volume apparatus (ERWEKA SVM, Germany) according to the USP 42 guideline, wherewith the bulk density was calculated by dividing the MFE weight (g) by unsettled apparent volume (mL) and the tapped density was calculated by dividing MFE weight (g) by a tapped volume (mL). Flowability was measured by using the compressibility index \(100 \times \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}}\) and Hausner’s ratio \(\frac{\text{Tapped density}}{\text{Bulk density}}\) (The United States Pharmacopeial Convention, 2019). All preformulation study was done in triplicate. According to a previous study, 200 mg of MFE containing 293.62±4.90 µg C3G alleviated changes in the metabolic animal model at the dose of 250 mg/kg BW for 8 weeks, the effective dose for anti-obesity effect in rats was converted to that in healthy volunteers. Human equivalent dose (HED) was calculated \(\text{HED (mg/kg)} = \frac{\text{Animal dose (mg/kg)}}{\text{Reference body weight (kg)}} \times \frac{\text{Animal } K_m}{\text{Human } K_m}\), where \(K_m\) is the correction factor derived from the division of the reference body weight (kg) of species to its body surface area (m²) (U.S. Department of Health and Human Services, 2005; Wattanathorn et al., 2019).

2.4 Quality control of nutraceutical product

A nutraceutical containing MFE powder was developed. MFE was mixed with lactose monohydrate (Meggle, Germany), magnesium stearate (Tablube, India) and sodium starch glycolate (Primojel, USA), and the placebo was mixed without the MFE powder as described in Table 1. Each formulated powder was weighed for a batch filling, then filled in capsule shells no.0 (Thai Meochems, Thailand) by a semiautomatic capsule filling machine (Model Panviv A01 No.310, The Union Chemical and Surgical, Thailand). Formulated capsules were stored in a light-protected amber plastic bottle with silica gel in a sachet prior to quality control analysis and clinical trial.

MFE and placebo capsules were subjected to quality control as follows.

2.4.1 Weight variation test

Twenty capsules were weighed individually, and the average weight was determined following the method of USP 42, NF37 in \(<2091>\) weight variation of dietary supplements (The United States Pharmacopeial Convention, 2019).

2.4.2 Disintegration test

The disintegration time of both formulae was evaluated according to the method of USP 42, NF37 in \(<2040>\) disintegration and dissolution of dietary supplements. A basket-rack assembly with fluted plastic disks was connected with disintegration apparatus. A 0.05 M acetate buffer was used as immersion fluid, maintained at 37±2°C. All 6 capsules were observed visually within 30 mins (The United States Pharmacopeial Convention, 2019).

2.4.3 In vitro dissolution test

The dissolution test was performed according to the USP 42, NF37 guidance. The basket-type dissolution apparatus (VISION® G2 ELITE™, USA) containing 500 mL of the simulated gastric fluid with pH 1.2 as a dissolution medium was used and maintained at 37±0.5°C. The basket was set to rotate at 100 rpm for 60 mins. The dissolution medium (8 mL) was withdrawn at 5, 10, 15, 30, 45, and 60 mins, then each withdrawn sample was prepared as described previously for HPLC analysis (The United States Pharmacopeial Convention, 2019).

2.4.4 Assay of anthocyanin content

Three capsules were randomly selected for the determination of C3G content individually by using validated HPLC analysis (The United States Pharmacopeial Convention, 2019). Each capsule was prepared as described previously for HPLC analysis. The sample was analysed in triplicate.

2.5 Clinical trial

The anti-obesity effect of the MFE capsule was evaluated in healthy overweight and obese adults both males and females. The inclusion and exclusion criteria were described in Figure 1. The sample size was

<table>
<thead>
<tr>
<th>Ingredients (mg)</th>
<th>Use</th>
<th>MFE capsule</th>
<th>Placebo capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mulberry fruit extract</td>
<td>Active compound</td>
<td>14.60</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>Diluent</td>
<td>475.00</td>
<td>507.60</td>
</tr>
<tr>
<td>Magnesium stearate</td>
<td>Lubricant and glidant</td>
<td>2.40</td>
<td>2.40</td>
</tr>
<tr>
<td>Sodium starch glycolate</td>
<td>Super disintegrant</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Total weight</td>
<td></td>
<td>502.00</td>
<td>520.00</td>
</tr>
</tbody>
</table>

Table 1. The composition of MFE and placebo capsules
calculated based on the independent samples t-test \( (n = 16 \text{ per group}) \) (Sakpal, 2010). This trial was conducted at Bamrasnaradura Infectious Diseases Institute (BIDI) for 8 weeks. All subjects have undergone a thorough medical history review by a physician to confirm their healthiness, then were randomly assigned to receive an MFE or placebo capsule once a day after a meal at dinner. The daily food intake was recorded by dietitians via the 24-hour dietary recall method at the baseline and the end of the study. The averages of micronutrient and macronutrient intakes in the first and final weeks were analyzed using INMU-CAL software (version 3.2, Thailand). Participants, a physician, and dietitians were blinded to the treatment allocation generated by using the Website Randomization.com (http://www.randomization.com) (Dallal, 2018). Participants were designed to visit at weeks 0, 4, and 8. The trial was approved by the Institutional Review Board of the Faculty of Pharmacy, Mahidol University (MU-DT/PY-IRB 2019/PY042) and BIDI for clinical study (IRB/BIDI R027h/62) with the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines, and the ICH-GCP.

2.5.1 Anthropometric measures

Anthropometric measures including height, waist circumference (WC), and hip circumference (HC) were evaluated using standardized techniques and equipment by dietitians. A body composition analyser was used for the measurement of weight, body composition (subcutaneous fat and skeletal muscle) at the whole body, trunk, arms, and legs, total body fat, and visceral fat level (Omron HBF 375, Japan).

2.5.2 Statistical analysis

Statistical analysis for the preformulation study and quality control was carried out by mean-standard deviation (SD) and percentage of relative standard deviation (%RSD). Determination of the significance of anthropometric measures changed from baseline within each group was carried out by paired samples t-test. Inter-group comparison was performed by independent samples t-test. Mean±SD was represented for all data. A \( p \)-value of \( \leq 0.05 \) was considered significant. SPSS statistic version 21 was used for analyzing all the data.

3. Results

3.1 Determination of C3G in mulberry fruit extract powder by HPLC

The HPLC method was successfully validated according to ICH and AOAC guidelines (European Medicines Agency, 2006; AOAC INTERNATIONAL, 2014). The content of C3G in MFE was quantified by using the developed HPLC method. The HPLC chromatograms of MFE and C3G standards were shown in Figure 2. The peak corresponding to C3G in the extract was confirmed by comparing the UV spectrum with that of the reference standard. The extract was spiked with the standard C3G to confirm the presence of C3G.

Linearity was done by plotting the calibration curve of C3G. The linear regression equation was \( y = 1499.7x - 8057.6 \) with a correlation coefficient \( (R^2) = 0.9951 \). The accuracy of the HPLC method was studied via recovery study at 50, 100, and 150% of the expected concentration from the analyzed value of the extract. The average percentage for C3G from the recovery study was found
to be 112.06±1.64 with a %RSD of 1.47. The repeatability and the intermediate precision of C3G obtained %RSD at 1.85 and 1.38. The specificity for C3G in MFE was confirmed by comparing the retention time and UV spectrum of the corresponding peak in the extract with those of C3G. MFE was found to contain C3G at 1288.91±3.02 mg/g extract (128.8±0.30% w/w).

3.2 Preformulation study of mulberry fruit extract powder

The MFE has a dark purple colour and a bitter sour taste with a typical mulberry odour. The MFE powder seemed to be very bulky and slightly hygroscopic. The percentage of moisture content of MFE powder was 4.08±0.00. The average bulk density of MFE powder was 0.52±0.00 g/mL and the average tapped density was 0.87±0.01 g/mL. Carr’s compressibility index was 40.22±0.82 and Hausner’s ratio was 1.67±0.02. Its compressibility index and Hausner’s ratio indicated that the powder had a very very poor flow character (The United States Pharmacopeial Convention, 2019).

3.3 Quality control of nutraceutical product

MFE was successfully formulated into a capsule. Quality control of nutraceutical products was achieved. The results of weight variation and disintegration tests were shown in Table 2. The in vitro dissolution profile of the MFE capsule indicated that the amount of anthocyanin which can be dissolved from the unit tested in 60 mins was demonstrated in Figure 3. The assay of anthocyanin content found that each capsule contained 21.50±0.54 mg of C3G (114.36%) as shown in Table 2.

3.4 Clinical trial

3.4.1 Participants’ characteristics at baseline and week 8

The average mean age of the MFE group was 44.4±10.6 years old and that of the placebo group was 44.4±10.6 years old. The gender ratio of participants (male: female) was 2:14 in both groups. The mean dietary intake was presented in Table 3. Baseline characteristics such as age, gender, and dietary intake record, did not differ between the MFE and placebo groups. There was no statistically significant difference of ages (p = 0.11), genders (p = 0.10), and dietary intake record at week 0 (p = 0.23) and week 8 (p = 0.66) in the two groups. The average BMI at the baseline in the MFE group was 28.15±2.75 and that of the placebo group was 27.89±4.13, which is classified as obese class I according to the BMI classification of Asian adults (WHO, 2021). Therefore, there was no statistically significant difference in all anthropometric measures in the two groups including body weight, waist circumference, hip circumference, waist-to-hip ratio, subcutaneous whole-body fat, subcutaneous trunk fat, subcutaneous arms fat, subcutaneous legs fat, skeletal whole-body muscle, skeletal trunk muscle, skeletal arms muscle, skeletal legs muscle, total body fat, and visceral fat level. The data of anthropometric measures at the baseline was shown in Figure 4.

3.4.2 Effect of mulberry fruit extract capsules on anthropometric measures

Table 3. Baseline and final comparison of dietary intake in MFE and placebo groups

<table>
<thead>
<tr>
<th>Dietary intake</th>
<th>Baseline Mean±SD</th>
<th>Placebo Mean±SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy (kcal)</td>
<td>1474.22±126.33</td>
<td>1490.96±159.33</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>46.84±3.74</td>
<td>47.72±4.49</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>19.23±1.70</td>
<td>18.76±2.09</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>33.93±4.03</td>
<td>33.50±5.01</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

No statistical significant difference was found when compared with baseline within group and placebo group (p>0.05).
Results obtained on anthropometric measures and body compositions among overweight and obese participants were reported in Figure 4. From baseline to week 4 of the treatment, it showed that significant reductions of body weight (-0.69±0.51 kg), BMI (-0.28±0.20 kg/m²), WC (-0.36±0.48 cm), subcutaneous fat of whole-body (-0.60±0.64%), trunk (-0.58±0.59%), and arms (-0.50±0.49%), total body fat (-0.53±0.57%) and visceral fat (-0.47±0.46%) were observed among group treated with MFE (*p < 0.001 in all parameters). From baseline to week 8 of the study, significant reductions of body weight (-1.45±0.61 kg), BMI (-0.57±0.23 kg/m²), WC (-0.81±0.52 cm), subcutaneous fat of whole-body (-1.56±0.77%), trunk (-1.39±0.61%), arms (-1.75±0.69%), and legs (-1.97±1.01%), total body fat (-1.64±0.63%), and visceral fat level (-0.97±0.49) were observed among the MFE group (*p < 0.001 in all parameters), whereas no significant alleviation in any anthropometric measures in the placebo group at all time points (*p > 0.05). In addition, there was a significant increment in skeletal muscle in the legs (0.61±0.57%) and arms (0.60±0.67%) in the MFE group at the end of the study (both *p < 0.001), whereas there was a significant increment in WC (0.29±0.46 cm) and visceral fat level (0.19±0.30) in the placebo group at week 8 (*p = 0.02 and 0.03, respectively). Moreover, the MFE group had a significant reduction in visceral fat levels in week 4 (-2.65±1.32) and week 8 (-3.46±1.33) in comparison to the placebo group (*p = 0.05 and 0.01, respectively). There were no side effects reported in both group during the study.

Figure 4. Effect of MFE and placebo capsules on anthropometric measures among overweight and obese participants: (a) body weight; (b) BMI; (c) waist circumference; (d) hip circumference; (e) waist to hip ratio; (f) body fat; (g) visceral fat; (h) subcutaneous fat (whole body, arms, legs, and trunk); (i) skeletal muscle (whole body, arms, legs, and trunk)

* *p < 0.05 was significantly different when compared with the baseline within the group
** *p < 0.05 was significantly different versus the placebo group
4. Discussion

Nutraceuticals from MFE met the requirement of USP 42. The preformulation study indicated that the flowability was very very poor. A formulation of nutraceutical was developed using the appropriate excipients by adding magnesium stearate as a lubricant to improve its flowing property and sodium starch glycolate to enhance disintegration time and dissolution rate (The United States Pharmacopeial Convention, 2019). The experimented HPLC method, was successfully validated according to the ICH and AOAC guidelines (European Medicines Agency, 2006; AOAC INTERNATIONAL, 2014), and was used to indicate the amount of C3G in the MFE powder. The C3G amount in the powder was used for dose calculation prior to the production of the capsule. MFE and placebo capsules passed the weight variation test and disintegrated within 30 mins. The dissolution profile indicated that C3G was dissolved in 45 mins more than 80% which was a greater amount when compared to the previous dissolution test of mixed berries capsule (mulberry, bilberry, and blueberry) where C3G was dissolved approximately 56% in 46 mins (Makato, 2017). Therefore, these tests met the requirement of USP 42 (The United States Pharmacopeial Convention, 2019). An assay of anthocyanin content showed that the average amount of C3G was 21.50±0.54 mg and was in the range of 80 to 120% of the labelled amount (15.05 - 22.58 mg) which met the requirement of ICH guideline (European Medicines Agency, 2006).

Previous in vitro and in vivo studies have proven that consumption of MFE is able to reduce fat accumulation (Yang et al., 2010; Peng et al., 2011; Wu et al., 2013; You et al., 2017; Wattanathorn et al., 2019). A recent study reported that MFE could increase the number of mitochondria which suggested that it could regulate lipid metabolism (You et al., 2017). MFE intake in hamsters fed with a high-fat diet for 10 weeks decreased weight, hepatic lipids, and fatty acid synthesis (Peng et al., 2011). Freeze-dried powder of MFE suppressed weight gain in mice and rats by deflating lipid accumulation, decreasing the secretion of leptin, and reducing metabolic syndrome parameters (Yang et al., 2010; Wu et al., 2013; Wattanathorn et al., 2019). This study further confirmed the anti-obesity effect of MFE in which MFE administration was able to decrease body weight, subcutaneous fats, body fat, and visceral fat in humans. Further studies on stability testing of developed products and evaluation of biochemical profiles and serum biomarkers are strongly recommended to confirm its anti-obesity effect.

5. Conclusion

MFE has been acknowledged to contain a potential anti-obesity property. C3G is a major anthocyanin found in MFE. In this study, the validated HPLC method was used to identify the C3G amount in the MFE powder and was applied in the anthocyanin content assay to evaluate C3G content in a developed nutraceutical product. Preformulation study of MFE powder indicated typical mulberry characteristics and its poor flow character. The developed formulations have improved flowability, weight variation, disintegration time, and dissolution rate. Thus, both MFE and placebo products passed the quality control criteria. Additionally, this clinical trial is the first report to demonstrate the ability of MFE consumption to decrease body weight and fat accumulation in overweight healthy participants.

Conflict of interest

The authors declare no conflict of interest.

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