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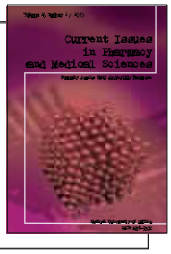

Determination of vitamin C and selected low molecular weight organic acids in aqueous extract of mulberry leaves used as dietary supplements

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ABSTRACT

Some scientific reports indicate the possibility of using different parts of mulberry (*Morus alba* L.) as the source of bioactive compounds to prevent type 2 diabetes. The aim of the present study was to determine the content of vit. C (ascorbic acid) and four different low molecular weight organic acids (citric, malic, oxalic, tartaric) in aqueous extracts made from the two kinds of dietary supplements: 1) containing 100% of *M. alba* leaf (coded as SICOM) and 2) containing admixture of mulberry leaves and other medicinal herbs (MUCOM). The content of vitamin C was determined by Tillmans method, oxalic acid - by manganometric titration, tartaric acid - by colorimetric procedure, citric and malic acid by enzymatic assays. Total acidity (expressed as content of citric acid) was determined by potentiometric titration. The average content of vitamin C in aqueous extracts of SICOM was found as 0.30 mg/100 mL, while the concentration of individual acids were found to be in range: oxalic 12.66-32.34 mg/100 mL, tartaric 14.18-26.29 mg/100 mL, citric 9.74-19.99 mg/100 mL, malic 4.52-5.23 mg/100 mL. Aqueous extracts prepared from SICOM containing coarse powder of mulberry leaves indicated the highest content of vitamin C and tartaric acid, respectively, 0.34 and 26.29 mg/100 mL, while the content of citric acid was the lowest (9.74 mg/100 mL). The results of chemometric analysis with PCA method showed that measured profile of vitamin C and the four low molecular weight organic acids could be used for superior differentiation of aqueous extracts obtained from SICOM and MUCOM dietary supplements, as well as enabling to distinguish such extracts prepared from the fine and coarse powdered mulberry leaves in these supplements.

Keywords: dietary supplements, micronutrients, enzymatic methods, dicarboxylic acids

INTRODUCTION

Numerous scientific reports point to the possibility of using white mulberry (*Morus alba* L.) leaves as a source of compounds to prevent hepatitis B virus infection [5], human breast and colon cancer [3], atherosclerosis [2], hypertension [10], and, especially, type 2 diabetes [2,7] which has become one of the major causes of premature illnesses and death through the increased risk of cardiovascular diseases [7]. Recent investigations have revealed that beneficial hypoglycaemic effect is caused in diabetic humans by polyhydroxylated piperidine alkaloids, as the most potent α-glycosidase inhibitors, present in leaves and bark of *M. alba* [7].

It is well known that low-molecular-weight organic acids, as common micronutrients in vegetable rich diet, regulate the state of acid-base homeostasis and course of intestine absorption of many microelements and bioactive phytochemicals in humans [6]. However, recently Bryland et al. [1] have reported that citric acid might be responsible for significant reduction of oxidative stress and inflammation caused by hyperglycaemia induced damage effects leading to vascular endothelial disfunction in human umbilicial vein cells. Plant derived oxalic acid (e.g. from *Eugenia jambolana* Lam.) or ascorbic acid (e.g. from *Limonia acidissima* L.) in mixture with tri-terpenoids, tannins and gallic acid indicated a synergistic effect in lowering blood sugar in diabetic rats [14, 15], while tartaric acid diesters isolated from *Equisetum arvense* showed endothelium-dependent vasodilation activity in rat aorta cells lines [13]. The content of low-molecular-weight organic acids in the fruits of different genotypes of *M. alba*, *M. nigra* and *M. rubra* is rather well described [6,9]. However there is no satisfactory data on their amount in the *M. alba* leaves which are commonly used as the components of various dietary supplements

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offered recently on the functional food market in Poland and EU. The aim of present study was to determine the content of ascorbic acid (vit. C) and four different low molecular weight organic acids (citric, malic, oxalic, tartaric) in the freshly prepared aqueous extracts made from the six various dietary supplements containing different amounts of *M. alba leaf.*

MATERIALS AND METHODS

Reagents, chemicals, plant material and aqueous extracts. L-(+)-Ascorbic acid, dihydrate oxalic acid, acetone, sodium bicarbonate and 25% calcium chloride were purchased from POCH (Gliwice, Poland) while 2,6-dichloroindophenol sodium salt hydrate (Tillmans reagent), L-malic and tartaric acid were obtained from Sigma Aldrich (Poznań, Poland). All chemicals were of analytically pure. The deionized water with conductivity 0.06 μS/mL was prepared by the water purification system HLP Smart 2000 (Hydrolab, Gdańsk, Poland). The studied here dietary supplement samples included 6 products (5 packed in sachets and 1 as the loose leaves) supplied from the 4 different manufactures in Poland. Four examined dietary supplements (coded as SICOM) contained only leaves of *M. alba*. While two other (coded as MUCOM), except leaves of *M. alba*, contained addition of different medicinal plant species: one contained 0.05% addition on cinnamon, the next one – 70% addition of mixed composition of *Phaseoli pericarpium*, *Urticae folium, Foeniculi fructus* and *Aronia fructus.* These six dietary supplements were characterized by different degree of leaves fragmentation (see Table 1 and 2). Four tested dietary supplements contained fine powdered *M. alba* leaves, one – very fine powdered, and one – coarse powdered. The aqueous extracts were prepared for 5 minutes from 1.0 g of tested dietary supplement and using 50.0 mL deionized water at 35°C. These extracts were purified by using the syringe filter Chromafil PES 45/25 (45 μm, Macherey-Nagel, Germany). Then the extracts were cooled to the room temperature 20°C and analyzed.

Determination of citric, L-malic and tartaric acid. Citric and L-malic acids were determined with enzymatic procedures using the diagnostic kit supplied from Megazyme (Brey, Ireland) according to the manufacturer's instructions. Then the amount of tartaric acid was analyzed by colorimetric method with ammonium metavanadate procedure (Megazyme). The absorbance of analyzed solutions was measured by using UV-VIS spectrophotometer type 1300 (Zeiss, Jena, Germany).

Determination of ascorbic acid, oxalic acid and total acidity. After filtration and cooling to the room temp., 5 mL of the studied aqueous extract was dissolved in 45.0 mL of the 3.0% oxalic acid. The solution was mixed and collected three times with 10.0 mL for final titration. Vitamin C was determined by the Tillmans method. The titrant was Tillmans reagent at concetrations 0.005 mg/mL. Oxalic acid was determined by manganometric titration using 0.002 M $KMnO_4$ solution as titrant. Total acidity was determined by potentiometric titration using a semi-automatic analyzer DL-22 (Mettler Toledo, Greifensee, Switzerland) in the solution prepared by dissolving 5.0 mL of aqueous extract in 50.0 mL of deionized water. The results of measurements were expressed as equivalents of citric acid content (mg/100 mL).

Statistical analysis. Three randomly selected lots of each dietary supplement differing in production date were collected in 2012 year. Next, all chemical analyses were performed for three samples taken from individual lot of the each supplement in three replications. Results of analyses for each studied here supplement were expressed as mean of 27 independent experiments (3 lots × 3 samples × 3 replications = 27) and standard deviation (SD). Basic (analysis of variance followed by Tuckey and Kruskal-Wallis post hoc tests) and multivariate statistical (principal component analysis PCA) calculations were carried out using the procedures available in Statistica v.8.0 for Windows software (StatSoft, Tulsa, USA). The level of significance was uniformly set at $p<0.05$.

RESULTS AND DISCUSSION

To our best knowledge, our work is the first report on the profile of the low-molecular-weight organic acids in the aqueous extracts from dietary supplements containing *M. alba* leaves. Nevertheless their content was primary determined in the *M. alba* and *M. nigra* fruits by using variety of HPLC and spectrophotometric methods [4,6, 9,11]. The content of the four low molecular weight organic acids and vitamin C in the aqueous extracts prepared from studied here SICOM- and MUCOM-type dietary supplements were presented in Table 1 and 2.

The total contents of four determined here organic acids in the SICOM-type supplements were in the range of 51.79 to 72.86 mg/100 mL. As seen from data in Table 1, Table 2 and Fig. 1, the oxalic acid was predominant acid in studied extracts of SICOM and MUCOM type supplements. The average content of oxalic acid in the SICOM extracts was 25.18 mg/100 mL, which is equivalent of 1185.02 mg/100 g dry mass of mulberry leaves (d.m.), and followed by tartaric 20.39 mg/100 mL (806.25 mg/100 g d.m.), citric 14.18 mg/100 mL (684.96 mg/100 g d.m.) and malic acid 4.85 mg/100 mL (226.27 mg/100 g d.m.). By comparison, the significantly lower average content of oxalic acid in *M. alba* leaves (183.02 mg/100 g d.m.) and *M. nigra* fruits (49.30 mg/100 g d.m.) was reported previously by Butt et al. [2] and Koyuncu [9], respectively. Considerable difference between previously reported [2] and observed here oxalic acid content in the SICOM-type mulberly leaf products is caused mainly by

Table 1. Content (± SD) of vitamin C (ascorbic acid) and selected low molecular weight organic acids in the examined aqueous extracts of single-component dietary supplements SICOM containing 100% of *M. alba* leaf (n = 27, p < 0.05)

Code	Dietary supplement SICOM	Trade form	pH [z] Color [z]	Organic acid [mg/100mL]						
				Ascorbic	Citric	Malic	Oxalic	Tartaric	Total content [a]	Total acidity [c]
M4	*Morus alba* leaf (100%)	sachets (2g) fine powder	6.0 light yellow	0.33 ± 0.02	19.99 ± 0.48	4.96 ± 0.15	12.66 ± 0.28	14.18 ± 0.68	51.79	86.16 ± 21.15
M7	*Morus alba* leaf (100%)	sachets (2g) fine powder	6.0 light yellow	0.24 ± 0.02	15.80 ± 0.09	5.23 ± 0.08	30.60 ± 0.18	16.26 ± 0.71	67.89	74.22 ± 4.30
M8	*Morus alba* leaf (100%)	loose leaf coarse powder	6.0 yellow	0.34 ± 0.03	9.74 ± 0.05	4.70 ± 0.11	25.14 ± 0.45	26.29 ± 0.75	65.87	68.00 ± 2.45
M10	*Morus alba* leaf (100%)	sachets (2g) fine powder	6.0 yellow	0.30 ± 0.02	11.18 ± 0.05	4.52 ± 0.10	32.34 ± 0.45	24.82 ± 0.73	72.86	68.80 ± 1.60
			Mean	0.30	14.18	4.85	25.18	20.39	64.60	74.29

[z] fresh aqueous extract; [a] sum of four organic acids without content of vit. C (ascorbic acid); [c] expressed as the mg of citric acid per 100 mL of fresh aqueous extract

Table 2. Content (± SD) of vitamin C (ascorbic acid) and selected low molecular weight organic acids in the examined aqueous extracts of multiherbal dietary supplements MUCOM containing *M. alba* leaf together with other medicinal herbs (n = 27, p < 0.05)

Code	Dietary supplement MUCOM	Trade form	pH [z] Color [z]	Organic acid [mg/100mL]						
				Ascorbic	Citric	Malic	Oxalic	Tartaric	Total content [a]	Total acidity [c]
M5[b]	Tea for diabetics[b]	sachets (2g) fine powder	6.0 yellow	0.21 ± 0.04	19.89 ± 0.18	4.24 ± 0.11	23.34 ± 0.27	20.86 ± 0.67	68.33	86.38 ± 14.56
M12	*M. alba* leaves and 0.05% cinnamon	sachets (2g) very fine powder	6.0 yellow	0.24 ± 0.06	19.95 ± 0.05	4.14 ± 0.10	30.60 ± 0.54	13.31 ± 1.01	68.00	71.50 ± 1.54
			Mean	0.22	19.92	4.19	26.97	17.08	68.16	78.94

[z] fresh aqueous extract; [a] sum of four organic acids without content of vit. C (ascorbic acid); [b] multiherbal dietary supplement MUCOM (30% of *Morus alba* leaf and 70 % of mixture containing *Phaseoli pericarpium, Urticae folium, Foeniculi fructus, Aronia fructus*); [c] expressed as the mg of citric acid per 100 mL of fresh aqueous extract

the inaccuracy of the manganometric method, different type of studied *M. alba* materials, its genetic differences and environmental conditions of cultivation.

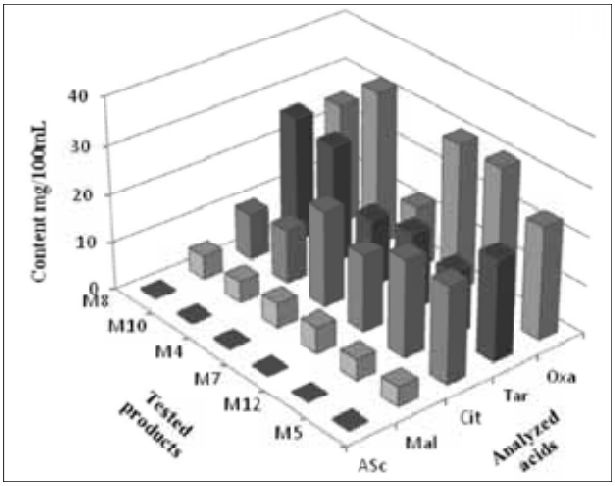

Fig. 1. Profile of vitamin C (Asc) and organic acids (Mal, Cit, Tar, Oxa) content in the analyzed aqueous extracts of dietary SICOM- and MUCOM-type dietary supplements containing *M. alba* leaf

The present work expresses a lower average amount of malic acid in the SICOM-type mulberly leaf products as compared with previously published results by Gundogdu et al. [6] and Koyuncu [9], respectively, in Turkish *M. alba* fruit (3095.01 mg/100 g d.m.) and *M. nigra* fruit (5747.50 mg/100 g d.m.). However, the average amount of malic acid was determined at much lower concentration in Pakistani *M. alba* fruits (4.10 mg/100 g d.m.) and *M. nigra* fruits (2.10 mg/100 g d.m.) [12]. The average concentration of the citric acid (684.96 mg/100g d.m.) determined in this work in the the SICOM-type mulberly leaf products is quite comparable with the results published earlier by Mahmood et al. for the *M. nigra* fruit from Pakistan (773.80 mg/100 g d.m.) [12]. However, the contents of citric acid observed in our study of the SICOM-type mulberly leaf products were higher than average results of study by Gundogdu et al. for the *M. alba* fruit from Turkey (393.00 mg/100g d.m.) [6]. The determined in our study average content of tartaric acid in the SICOM-type mulberly leaf products (806.25 mg/100g d.m.) is near four times higher than in the *M. alba* fruit (223.00 mg/100g d.m.) [6] and twice higher than in *M. nigra* fruit (445.00 mg/100 g d.m.) [9]. The average content of vit. C in the SICOM-type mulberly leaf products was found to be 0.30 mg/100 mL (14.38 mg/100 g d.m.). A higher average level of vit. C was found in *M. alba* fruit, which was determined earlier by HPLC method (in range from 24.20 to 32.20 mg/100 g d.m.) [6,11]. On the other hand the average content of ascorbic acid determined in the *M. alba* fruits by spectrophotometric method was on the same range (15.20 mg/100 g d.m.) [8] as in our studies. In the aqueous extracts obtained here from the SICOM supplements containing coarse powder of the *M. alba* leaves (M8, see Tab. 1) the increased content on ascorbic acid 0.34 mg/100 mL (16.36 mg/100 g d.m.) and tartaric acid (26.29 mg/100 mL) and decreased amount of citric acid (9.74 mg/100 mL) have been found. In the aqueous extracts prepared from the MUCOM-type supplement containing a very fine powder of *M. alba* leaves and addition of 0.05% cinnamon (M12), the lowest amount of malic acid (4.14 mg/100 mL) was observed. While in the aqueous extracts prepared from the MUCOM-type supplement M5 containing 70% admixture of other plant

materials the lowest content of vit. C (0.21 mg/100 mL) was recorded and the increased content of citric acid (19.89 mg/100 mL). It was also found that in the aqueous extracts obtained from the SICOM-type supplements M4 and M10 with fine powder *M. alba* leaves the increasing amount of oxalic acid (from 12.66 to 32.34 mg/100 mL) is accompanied by the increased total content of the all four tested acids (from 51.79 to 72.86 mg/100 mL).

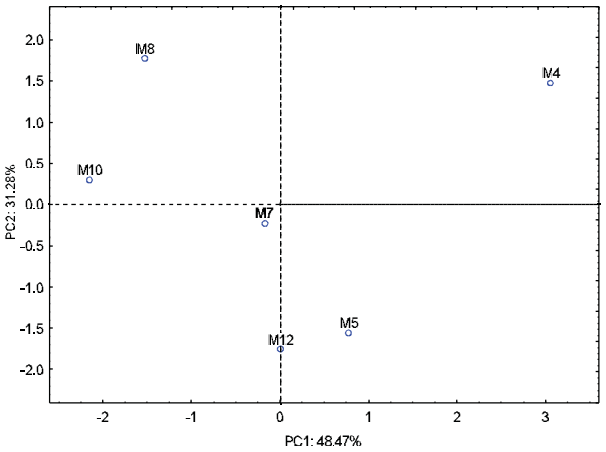

Fig. 2. Loadings plot of the calculated first two principal components, PC1 and PC2, by the variables of individual aqueous extracts made from SICOM- and MUCOM-type dietary supplements with *M. alba* leaf. The symbols on the plane denote the code of examined dietary supplement as in Tab. 1 and 2

To elucidate similarities and dissimilarities in the tested acids profiles of the 6 studied here *M. alba* leaf based SICOM- and MUCOM-type supplements, principal component analysis (PCA) was performed with result presented in Fig. 2. The first PC1 and second PC2 principal component explained together 79.75% of the total variance of analyzed data from Table 1 and 2. Calculated PC1 component expresses the changes in the oxalic acid and in the total four organic acids content in analyzed aqueous extracts, while PC2 component can be related with decreasing citric acid content in these extracts. In Fig. 2 one can observe the superior differentiation of the aqueous extracts obtained from the studied here SICOM- (M4, M7, M8, M10) and MUCOM-type (M5, M12) mulberry dietary supplements. In addition, the aqueous extracts prepared from the fine (M4, M5, M7, M10, M12) and coarse (M8) powdered plant materials in these supplements have been effectively distinguished by used PCA procedure.

CONCLUSIONS

Increasing content of vitamin C and tartaric acid in the aqueous extracts prepared from the SICOM-type supplements with coarse powdered *M. alba* leaves was observed in this study, while the total content of the four studied here organic acids was highest in the aqueous extracts prepared from the supplements with a fine powdered *M. alba* leaves. This study reveals that PCA procedure on the determined here organic acids profile appears a useful tool for reliable differentiation of aqueous extracts obtained from supplements with the *M. alba* leaves, especially in view of its micronutrients quality, plant authenticity and manufacturing process. It could be hypotesized that a high content of citric acid in the SICOM-type mulberly leaf supplements may have beneficial therapeutic potential enabling to abolish hyperglycaemia-induced vascular endothelial dysfunction in diabetic subjects.

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