

A micelle mediated extraction as a new method of obtaining the infusion of *Bidens tripartita*

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In this paper, the extracts of three-part beggarticks (*Bidens tripartita*) were prepared using ultrasonic assisted micelle-mediated extraction method (UAMME). Different kind of surfactants and WPC (whey protein concentrate) were applied. In the *B. tripartita* extracts twenty polyphenols were identified by UPLC-DAD-MS. Both aqueous and UAMME extracts mainly contained chlorogenic acid, caftaric acid and its derivatives as well as luteolin 7-O-glucoside. The luteolin was extracted with Rokanol B2 and Triton X-100. Furthermore, antioxidative properties of the extracts were analyzed with two methods: reactions with di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) reagent and Follin's method. The DPPH radical scavenging by micellar extracts was in general comparable with the antioxidant activity of conventional extracts. The most active was sample with Tego Care CG90, reducing about 73% of the radical. Obtained results confirmed that the UAMME might be an alternative method, to the liquid-liquid or solid-liquid extraction, of obtaining specified extracts rich in active compounds. Selecting a suitable surfactant may thus provide the expected composition of the extract.

Key words: ultrasonic assisted micelle-mediated extraction, three-part beggarticks, whey protein concentrate

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INTRODUCTION

Three-part beggarticks (Bur-marigold, *Bidens tripartita*) is a perennial, branched plant from the genus *Bidens*, family *Compositae*, subfamily *Asteroideae*. It grows near the aquatic habitats, periodically flooded or wet. It occurs in Europe, Asia, North Africa and North America. In Poland it is found mainly in the area of Bialogon on the Bobrza River, Cedzyna on the Lubrzanka River and lagoons: Kielce – Szydłówek, Cedro-Mazur, Zagórze (Szafer & Kulczyński, 1953). The herb of three-part beggarticks was used in folk medicine as a diuretic, sudorific and anti-inflammatory agent (for review see Shikov *et al.*, 2014). Now it is used in fever treatment, skin diseases, bladder and kidney troubles, and as an immunological system stimulant (Wolniak *et al.*, 2007). The bur-marigold was also reported to be effective treatment for the allergic itching among children (Sezik *et al.*, 2004). The methylene chloride extract of *B. tripartita* showed high activity in the inhibition of cancer L1210 (mouse leukemia) cells and against thrombin (Goun, 2002). Research by Tomczykowa and coworkers (2008) concerning antimicrobial and antifungal activity

of aerial parts of *B. tripartita* fully explain the use of the flowers and herbs for treatment of *muco-cutaneous candidosis*, as well as skin disease and inflammations caused by microbial and fungal organisms. Moreover, the essential oil obtained from the roots of *B. tripartita* exhibited a strong antifungal activity (Tomczykowa *et al.*, 2011). Tolmacheva and coworkers (2014) examined twenty different herb extracts, from species occurring all over Eastern Europe, using two biological tests: wild-type and reporter *Chromobacterium violaceum*, and came to a conclusion, that only *B. tripartita* herb contains molecules that imitate acyl-homoserine lactone, operating as the agonist for bacteria 'quorum sensing' communication. The investigation on acute toxicity of an alcoholic extract of *B. tripartita* plant in mice suggested that it is relatively safe toxicologically and could be used with some degree of safety (Sandu *et al.*, 2012). The further evaluation of water and alcoholic extracts from this plant indicated good *in vivo* biocompatibility in rats (Sandu *et al.*, 2013).

Phytochemical studies of three-part beggarticks showed that this herb is a rich source of active compounds: flavones, flavanones, chalcones and aurones, coumarins, a small amount of vitamin C, carotenoids and a volatile oil. The green parts of *B. tripartita* contain polyacetylenic compounds, linoleic acid and ocimene, whereas in the flowers mainly thiophene and traces of cosmene as well as eugenol were identified (Wolniak *et al.*, 2007; Pozharitskaya *et al.*, 2010). The main components of the essential oil obtained from the roots of *B. tripartita* by hydrodistillation were α -pinene, β -bisabolene, p-cymene, hexanal, linalool, p-cymene-9-ol, β -elemene, 2-pentylfuran, and silphiperfol-6-ene (Tomczykowa *et al.*, 2011). In turn, α -pinene, p-cymene, β -ocimene, and β -elemene are predominant in extracts prepared by supercritical CO₂ extraction with solid-phase trap (Kaškonienė & Maruška, 2015).

The main flavonoid constituents of the methanol extracts were isookanin-7-O-glucoside, luteolin-7-O-glucoside and luteolin (Wolniak *et al.*, 2007; Lv & Zhang, 2013). The latter is especially important as the *in vivo* antiproliferative activities of luteolin against human breast cancer cell and prostatic cancer were proven (Huo *et al.*, 2013).

Increased demand for safe and natural origin products is clearly visible. Therefore, scientists are elaborating safe methods of obtaining active substances. Un-

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Abbreviations: WPC, whey protein concentrate; UAMME, ultrasonic assisted micelle-mediated extraction method; DPPH, di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium

fortunately, there are still no effective methods of the separation of natural extracts rich in bioflavonoids. As the compounds are very sensitive to temperature and light, the purification deprives them of their valuable properties (Ballesteros-Gomez, 2010). A relatively new method is application of surfactants for the preparation of intermediates, containing the plant origin bioflavonoids in a high concentration.

The micelle-mediated extraction method (MME) in comparison with the liquid-liquid or solid-liquid extraction methods does not require the use of toxic organic solvents, so it offers a convenient alternative to the conventional extraction systems (Szymanowski, 2000). In this method, instead of an organic solvent, an aqueous surfactant solution is used, which solubilizes desired ingredient in the hydrophobic micelles (Paul & Moulik, 2001). Factors, which affect the dissolving capacity of the surfactants solution, are the structure and type of surfactant, the presence of electrolytes and other organic materials and the temperature (Quina & Hinze, 1999; Ferrera *et al.*, 2004). This methodology offers the advantages of safety, low cost, ability to concentrate solutes and low toxicity compared to classical organic solvents (Shi *et al.*, 2007; Zhou *et al.*, Trivedi *et al.*, 2011). The extracted substances can be safely used in foods, pharmaceuticals or cosmetics. The ultrasonic assisted micelle-mediated extraction (UAMME) could be applied to obtain biologically active substances, such as vitamins A, E, K, B1, paracetamol or salicylic acid, from the biological material (Madej, 2009; Tomaszewicz-Potępa *et al.*, 2010), and bioflavonoids from plant material (Tomaszewicz-Potępa *et al.*, 2010; Śliwa *et al.*, 2013).

UAMME method seems to be promising as a pure method to obtain *B. tripartita* extract, due to the rich composition of the actives and interesting raw material for cosmetic and medical application. The aim of this work was to assess four nonionic surfactants (Tego Care CG90, Sucrose Stearate, Triton X-100, Rokanol B2) as well as WPC (whey protein concentrate) in the extraction of three-part beggarticks herb (*B. tripartita*) by UAMME method. The composition and antioxidant properties of UAMME extracts were analyzed in comparison to water. The bioflavonoids composition was determined with UPLC-DAD-MS method. The HLB of studied surfactants as well as the surface tension of 1% solutions of the surfactants were measured, to find out an explanation for the differences in bioflavonoids concentration. Moreover, the hydrophobic properties ($\log P$) of selected flavonoids were compared. In this work, probably for the first time, ultrasonic assisted micelle-mediated extraction (UAMME) method was used to separate bioactive ingredients from *B. tripartita*.

MATERIALS AND METHODS

Chemicals. Aluminum chloride, sodium hydroxide, sodium nitrite were obtained from POCh (Gliwice Poland). The Folin-Ciocalteu's reagent, DPPH, luteolin, gallic acid were supplied by Sigma Aldrich (Poland). Among the used surfactants there were: Rokanol B2 (Alkoxylated (EO/PO) natural alcohol C16-C18) from PCC Rokita (Poland), Triton X-100 (Octylphenol Ethoxylate (EO)) from DOW Chemicals Co. (DOW Poland), TegoCare CG 90 (Cetearyl Glucoside) from Evonik Industries (Germany) and Crodesta F160 (Sucrose Stearate) from Croda Personal Care (Croda, Poland). The 80% WPC was purchased from Bartex

(Pasłek, Poland). *B. tripartita* herb was bought from Dary Natury (Grodzisk, Poland). All the used reagents were of analytical grades.

Extraction procedure. *Aqueous extract.* Dried three-part beggarticks herbs (3 g) were exhaustively extracted with 200 ml of distilled water. The extraction was carried out in an ultrasonic bath at room temperature, for 30 min.

Micelle-mediated extraction. Dried three-part beggarticks herbs (3 g) were exhaustively extracted with 200 ml of aqueous surfactant solution (1%) or solution of 1% WPC. Four non-ionic surfactants were used: Tego Care CG 90 (HLB=11), Sucrose Stearate (HLB=16), Triton X-100 (HLB=13.5), Rokanol B2 (HLB=6.7). Protein solutions were prepared by dissolving WPC in an appropriate amount of distilled water and subsequent mixing with magnetic stirrer until completely dissolved and left for protein swelling for 1 h. In all of the cases, 0.1 g of sodium benzoate and 0.5 g of citric acid were added to the eluent solution. The extraction was carried out in an ultrasonic bath, at room temperature, for 30 min.

Polyphenols content. The content of polyphenols in individual extracts was determined by means of the liquid chromatography (UPLC-DAD-MS) method, applied recently in Oszmiański and coworkers (2015). The identification of the polyphenols of *B. tripartita* extracts was carried out using an ACQUITY Ultra Performance LCTM system with a mass detector G2 QT of Micro-mass spectrometer equipped with an electrospray ionization (ESI) source operating in negative mode (UPLCTM; Waters Corporation, Milford, MA, USA). The separation of individual polyphenols was carried out using a UPLC BEH C18 column (1.7 μm , 2.1 \times 100 mm, Waters Corporation) at 30°C. The elution solvents were aqueous 0.1% formic acid (A) and 100% acetonitrile (B). Samples (10 μl) were eluted according to the linear gradient described by Oszmiański and coworkers (2013). Analysis was carried out using full scan, data-dependent MS scanning from m/z 100 to 1500. The mass tolerance was 0.001 Dalton, and the resolution was 5000. Leucine enkephalin was used as the internal reference compound during ESI-MS accurate mass experiments and was permanently introduced via the LockSpray channel using a Hamilton pump. The lock mass correction was ± 1000 for the mass window. All TOF-MS chromatograms are displayed as base peak intensity (BPI) chromatograms and scaled to 12400 counts per second (cps) (=100%). The effluent was led directly to an electrospray source with a source block temperature of 130°C, a desolvation temperature of 350°C, a capillary voltage of 2.5 kV and a cone voltage of 30 V. Nitrogen was used as the desolvation gas with a flow rate of 300 $\text{L} \cdot \text{h}^{-1}$.

The characterization of the single components was carried out based on their retention time and the accurate molecular masses. Each compound was optimized to its estimated molecular mass $[M-H]^-$ in negative mode before and after fragmentation. The data obtained from LC/MS were subsequently entered into the MassLynx 4.0 ChromaLynx™ Application Manager software. Based on the data, the software allows scanning different samples for the characterized substances.

The runs were monitored at the following wavelengths: (-)-epicatechin and procyanidin at 280 nm, hydroxycinnamates at 320 nm and flavonol glycosides at 360 nm. Retention times (R_t) and spectra were compared with those of pure standards. Calibration curves at concentrations ranging from 0.05 to 5 mg/ml

Table 1. Bioflavonoid composition of the obtained extracts (C – concentration in [mg/l], [%] – part of the total flavonoid content).

	CF-160		TX-100		RB2		TCCG90		WPC		Aq	
	C	[%]	C	[%]	C	[%]	C	[%]	C	[%]	C	[%]
Neochlorogenic acid	2.5	4	1.7	2	2.3	2	6.3	8	18.3	38	2.0	4
Chlorogenic acid	30.1	43	17.8	23	25.9	22	37.0	46	13.8	28	22.4	42
Caftaric acid	8.9	13	5.1	7	7.3	6	10.8	13	3.5	7	6.8	13
Caftaric/coutaric acid derivatives	5.7	8	3.6	5	6.4	6	7.1	9	2.7	5	4.0	8
Cryptochlorogenic acid	1.7	2	1.1	1	1.6	1	2.5	3	4.5	9	1.3	2
Caftaric acid derivatives	2.1	3	1.0	1	1.7	1	3.6	4	0.7	1	1.6	3
Caffeic acid, plus terminal rhamnose	3.6	5	1.9	3	3.1	3	5.8	7	1.3	3	2.8	5
Luteolin-3,7-dihexoside	0.7	1	0.5	1	0.4	0	0.4	0	0.2	0	0.6	1
Quercetin-3-O-hexoside	1.0	2	1.9	2	2.3	2	0.6	1	0.1	0	1.0	2
Quercetin-3-O-rutinoside	0.2	0	0.4	1	0.5	0	0.1	0	0.1	0	0.2	0
Quercetin-3-O-hexoside	0.8	1	1.1	1	1.3	1	0.5	1	0.4	1	0.7	1
Luteolin-7-O-glucoside	6.0	9	13.5	18	16.7	14	2.8	3	1.4	3	5.0	9
Quercetin-3-O-6-acetylglucoside	1.0	1	1.5	2	1.6	1	0.6	1	0.4	1	0.8	2
4,5-di-O-caffeoylquinic acid	0.2	0	1.3	2	1.6	1	0	0	0	0	0.3	0
Luteolin-3-O-hexoside	0.1	0	0.9	1	1.1	1	0	0	0	0	0.2	0
Apigenin-glucoside	1.3	2	4.2	6	5.4	5	0.5	1	0.3	1	1.0	2
3,5-di-O-caffeoylquinic acid	0.5	1	2.4	3	3.6	3	0	0	0	0	0.3	1
Luteolino -3-O -glucosyl-6''-acetate	0	0	1.7	2	2.1	2	0.1	0	0	0	0.3	1
Quercetin-3,4-diglucoside	0.3	0	2.9	4	7.1	6	0.9	1	0.4	1	0.1	0
Luteolin	0	0	4.6	6	12.1	10	0	0	0	0	0	0
Total	69.4		76.0		116.5		81.2		48.7		53.4	

($R^2 \leq 0.9998$) were made from (–)-epicatechin, procyanidin A2, procyanidin B2, neochlorogenic acid, caffeic acid, quercetin-3-O-galactoside, quercetin-3-O-rutinoside and kaempferol-3-O-glucoside as the standards. All incubations were done in triplicate. The results were expressed in milligrams per l of extracts.

In-vitro Antioxidant assay. Flavonoids content was determined using their ability to react with aluminium chloride according to the method specified by Hussain and coworkers (2012). The following procedure was applied: 1 ml of the extract was dissolved in 1.1 ml of distilled water. Next 0.3 ml of NaNO_2 solution (5%) was added and left for 5 minutes. Then, 0.6 ml of AlCl_3 solution (10%) and after 6 min, 2 ml of 1 M NaOH were respectively added. The mixture was diluted with 0.275 ml of distilled water and the absorbance of the sample was measured immediately, at $\lambda=510$ nm. The spectra were recorded with Macherey Nagel Nanocolor UV/Vis spectrophotometer. A standard curve was plotted for luteolin and the result were given by luteolin equivalent, (mg/l). Because of the presence of surfactants, the obtained results were not counted to the dry weight of the extract.

Determination of polyphenols in the extracts was performed with the Folin-Ciocalteu (FC) method described in Grajka (2000) and in Cybul and Nowak (2008). Definite volume (1 ml) of tested extract was transferred to 10 ml volumetric flask. Next, 5 ml of the FC reagent was added and the mixture was incubated for 4 minutes. After that, 4 ml of 7.5% sodium carbonate solution was added. The blank sample was also prepared. The test flasks were placed in the dark and left for 2 hours. After this time, the absorbance was

measured, at wavelength $\lambda = 765$ nm. The results were calculated based on the content of gallic acid, using the calibration curve.

The DPPH scavenging potential was estimated as previously described by Shabir and coworkers (2011) with slight modifications. Definite volume (2 ml) of DPPH methanol solution (0.05%) was added to 4 ml of each extract solution with 4 cm^3 of distilled water and left to react at room temperature, in the dark, for 30 min. The absorbance of the obtained mixtures was measured at $\lambda=517$ nm.

RESULTS AND DISCUSSION

In all prepared extracts the flavonoid compounds were identified. The results of qualitative analysis showed that the final composition of the extracts depended on the type of applied extracting agent (Table 1). The highest content of these compounds was obtained with used of Rokanol B2 (RB2). It should be emphasized that in this case the amount of flavonoids was twice as high as in the aqueous extract. The flavonoid content in the extracts decreased sequentially depending on the applied eluent, as followed: aqueous solution of Rokanol B2 (RB2), Tego Care CG90 (TCCG90), Triton X-100 (TX100), Crodesta F160 (CF160), pure water (Aq) and whey protein concentrate (WPC) solution. As showed in Wolniak and coworkers (2007), the methanol extract of herb contained three main flavonoids: isookanin 7-O-glucoside (0.16%), luteolin 7-O-glucoside (0.18%) and luteolin (0.031%, calculated on dry weight). Using chromatography (UPLC-DAD-MS)

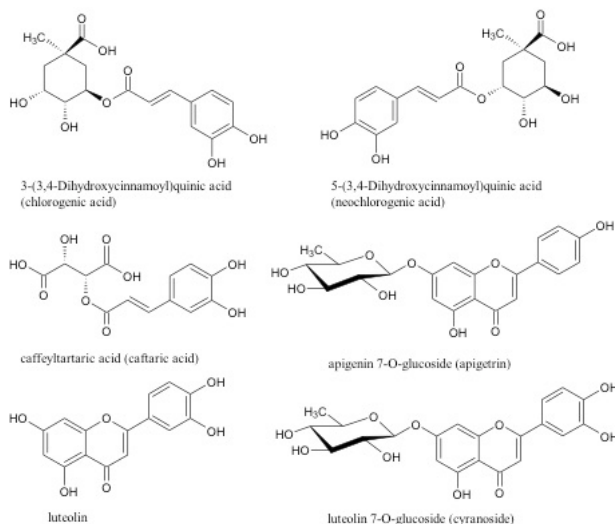


Figure 1. Phenolic compounds identified in the *B. tripartita* extracts.

method twenty polyphenols in *B. tripartita* extracts were identified. Both aqueous and UAMME extracts mainly contained chlorogenic acid, caftaric acid and its derivatives as well as luteolin 7-O-glucoside (Table 1, structures presented in Fig. 1), which was expected due to the polarity of the mentioned compounds. The luteolin was extracted only with Rokanol B2 and Triton X-100, from which the first one gave slightly higher yield. Using these surfactants, instead of water, three-fold more luteolin 7-O-glucoside was obtained which resulted in a significant increase, of the total flavonoids content, compared to other eluents used. The results suggested that in the case of phenolic compounds extraction, RB2 with mixed chain EO/PO (ethoxy/propoxy) is a better agent, although the qualitative composition of Rokanol B2 and Triton X-100 extracts was similar. It could be probably explained by the nature of the surfactants. Both of the compounds belong to a group of polyalkoxylated surface-active agents. The micelles formed by them are characterized by the presence of voids in the palisade layer.

Generally, the main component of all the UAMME extracts was chlorogenic acid. The exception was the WPC extract containing 38% of neochlorogenic acid. The content of neochlorogenic acid was three times higher in comparison to Tego Care CG90 extract and ten times higher compared to the aqueous extraction.

The results indicate, that the differences in the efficiency of phenolic compounds extraction could be the result of different properties of the surfactants. Table 2 shows HLB values and surface tension of used

Table 2. Characteristic of the surfactants.

Surfactant	HLB*	Surface tension** [mN/m]
Rokanol B2	6.7	29.69
Triton X-100	13.5	28.69
Tego Care CG 90	11	33.20
Crodesta F160	14.5	33.58
WPC	-	43.55

*Based on the manufacturer's data sheet; **Average of five measurements

Table 3. The hydrophobic properties ($\log P^*$) of selected flavonoids.

Flavonoid	$\log P$
Neochlorogenic acid	-0.4
Chlorogenic acid	-0.4
Apigenin-glucoside	-0.1
Caftaric acid	0.1
Quercetin-3-O-6-acetylglucoside	0.4
Luteolin-7-O-glucoside	0.5
Luteolin	1.4

*Computed value (XLOGP3-AA)

surfactants. Among the studied compounds, Rokanol B2 was the most hydrophobic (HLB=6.7) and caused higher decrease of the water surface tension. Perhaps this is the main reason for its best efficiency. However, relationship between the HLB value or surface tension and the high content of the particular flavonoids was not found. Finding the correlation requires further studies. There is also no evident relationship between the hydrophobic properties of the flavonoids (Table 3) and their concentration in the obtained extracts.

Table 4 shows the results of the determination of antioxidant properties of the obtained extracts. All of the results are the average of three measurements. Results obtained with qualitative method confirmed the higher content of flavonoids in extracts prepared according to the UAMME method. Incompatibility with chromatographic analyses can be explained by closing of the antioxidant substances inside of the micelles. A similar effect was observed by Guo and coworkers (2007) for Triton X-100. The DPPH radical scavenging by micellar extracts was generally comparable with the activity of conventional extracts. The most active was sample with Tego Care CG90, reducing ca. 73% of the radical. The highest activity of the extract suggests that other active compounds are also present in the mixture, and that antioxidative synergism may occur. This effect was also indicated by Wolniak and coworkers (2007). The advantages of UAMME were revealed by the comparison of the aqueous extract obtained by sonication at room temperature (blank experiment) and treated with boiling water (Table 4). The addition of the surfactant resulted in four- to thirteen-fold increase in the activity of the extracts.

CONCLUSION

In this paper authors performed detailed analysis of the flavonoids content in the extracts from *B. tripartita* obtained by UAMME method. The total flavonoids and polyphenols content in the UAMME-extracts was higher than that obtained by water extract. Moreover, the results proved that WPC could be an effective agent for UAMME extraction, similar to typical surfactants. WPC solution, as the source of micelles, might be convenient method for the preparation of plant extracts rich in natural antioxidants, as well as an alternative for common methods. Additionally, it should also be emphasized that the UAMME extracts do not contain or-

Table 4. Characteristics of UAMME, water, methanol:water and acetone:water extracts of *B. tripartite*.

Surfactant	Concentration of surfactant [%]	Flavonoids content per luteolin [mg/dm ³]	Antioxidant activity [%]**	Polyphenols content per gallic acid [mg/dm ³]
Rokanol B2	1	48.7	32.4	72.7
Triton X-100	1	31.6	26.7	75.4
Tego Care CG 90	1	68.3	72.8	43.1
Crodesta F160	1	33.2	23.0	29.8
WPC	1	64.5	21.8	66.9
water*	–	25.6	5.4 (35***)	34
methanol:water***	–	–	34	–
acetone:water***	–	–	56	–

*Deminerallized water extract was used as a reference sample; **The ability to inhibit the oxidation reaction of DPPH; ***DPPH radical scavenging activities of extracts from *B. tripartite* herb determined by Wolniak *et al.*, 2007

ganic solvents and can be directly used in e.g. cosmetic compositions, also as the source of whey proteins.

The selection of a suitable surfactant may thus provide the expected composition of extract. This will be the subject of further studies.

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