

Berberis Vulgaris Extract/ β Cyclodextrin Nanoparticles Synthesis and Characterization

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This paper presents a study regarding the evaluation of the main alkaloid (berberine) from different parts of *Berberis vulgaris* L. from the West side of Romania (Arad), the antioxidant activity of extracts, and the obtaining and characterization of *B. vulgaris* extract/ β cyclodextrin complexes. The maximum concentration of berberine was found in bark (~6%), followed by root (3.8%), and the least was found in leaves and fruits (by using the high performance liquid chromatography analysis). Leaves and fruits have the best antioxidant activity (revealed by DPPH free radical scavenging assay). *B. vulgaris* extract/ β cyclodextrin complexes were obtained with good yields (>69%) by using the ethanol-water solution method, the particles obtained having dimensions between hundreds of nanometers to micrometers (by SEM analysis), and the complex formation was confirmed by thermogravimetry (~2% mass loss in the 100-275°C temperature interval) and differential scanning calorimetry (endothermic peak with an energy consumption of ~116 J/g, corresponding to the dissociation of the biocompounds/cyclodextrin complex).

Keywords: cyclodextrins, *Berberis vulgaris*, berberine, nanoparticles, high performance liquid chromatography, scanning electron microscopy, thermogravimetry, differential scanning calorimetry

Berberis vulgaris L. (barberry) belongs to the Berberidaceae family and it is a small shrub which grows in Europe, but also in Africa and Asia, especially to the forest edge, in shining places. It is cultivated for its valuable biological properties. Leaves have ovoidal shapes and are grouped in rosettes. It has spiny stems, yellow flowers, and fruits are ellipsoidal, with sour, astringent taste, and red color [1-3]. The main bioactive compounds from *B. vulgaris* are alkaloids (berberine, berbamine, jatrorrhizine, columbamine, berberubine, oxicanthine, palmatine; figure 1), vitamin C, resin, and tannins, but also flavonoids like quercetin and kaempferol [1,2,4-7]. The most used plant parts in phytotherapy are bark (from stem and root); also leaves and fruits are used. The most important pharmacological uses of *B. vulgaris* are in kidney, bile, and liver diseases (hepatoprotective), and also in ocular and mouth problems. *B. vulgaris* have tonic, antimicrobial, antiemetic, antipyretic, antipruritic, and cholagogue properties, and has also been used in some cases in cholecystitis, cholelithiasis, jaundice, dysentery, leishmaniasis, malaria, and gall stones [4-14]. Most of these properties are due to the presence of some alkaloids: berberine and palmatine have the capacity to inhibit MAO enzyme, jatrorrhizine has the same pharmacologic properties like berberine, but it has lower toxicity; oxicanthine acts as vasodilator and hypotensive, as well as berbamine; the latest has also anti-inflammatory and antioxidative properties [5]. Most of bioactive properties of *B. vulgaris* are due to the overall antioxidant activity generated by the presence of some flavonoid compounds, alkaloids or other compounds with phenolic hydroxyl groups.

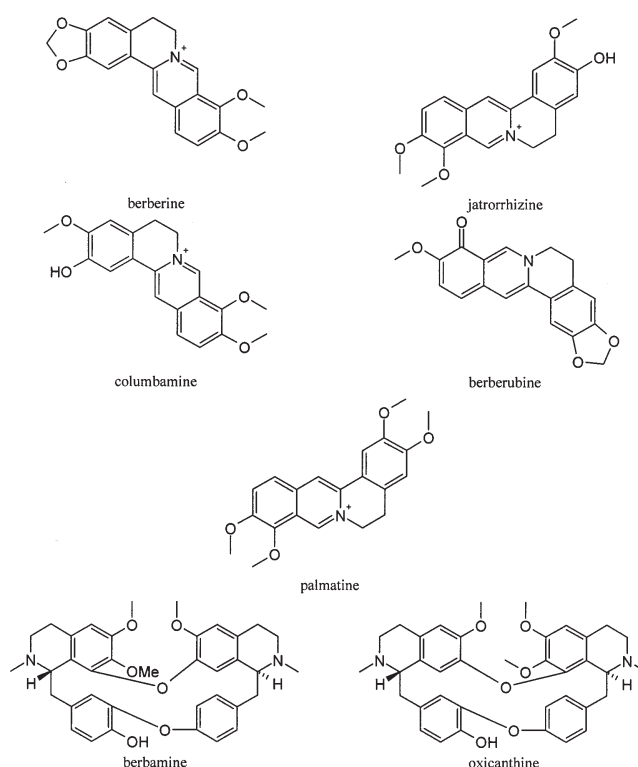


Fig. 1. Structures of the main alkaloids from *Berberis vulgaris* L.

In order to enhance the bioactive properties of compounds, nanoencapsulation methods are widely used. From the wide range of encapsulation matrices, naturally or chemically modified cyclodextrins are extensively used

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in medicine and food fields. Naturally occurring cyclodextrins are α -, β -, and γ -cyclodextrin, which are cyclic oligosaccharides with 6, 7, and 8 glucopyranose moieties; they are obtained from starch by using various *Bacillus* species (like *B. macerans*). The specific architecture of the cyclodextrin structure (truncated cone with exterior hydroxyl groups and hydrophobic inner cavity) determine to use them for nanoencapsulation of hydrophobic and geometrically compatible bioactive molecules (like drugs, food additives, odorant, and flavoring compounds etc.) in order to obtain powdery formulations with higher water solubility, protecting capacity (against air, light, humidity), and controlled release properties [15-32].

In the case of *Berberis* alkaloids, cyclodextrins were used in order to obtain some optical sensors. Thus, berberine was encapsulated in butylated β -cyclodextrin (HDB- β -CD), which was immobilized in PVC [33-35], and also in a β -cyclodextrin dimer [36]. More recently, *Berberis* extract/cyclodextrins are studied in order to obtain formulations which can be used for the treatment of some cancer types [37].

In this paper we try to evaluate the berberine concentrations from different parts of *Berberis vulgaris* L. collected from the West side of Romania, the antioxidant activity of the corresponding extracts (which can be correlated with the chemical composition of extracts), obtaining and characterization of *B. vulgaris* extract/ β cyclodextrin complexes, which can be used in hepatoprotective formulations with enhanced bioavailability.

Materials and methods

Samples of *Berberis vulgaris* L. from the West side of Romania (Arad, Macea Botanical Garden) were collected in the autumn of 2008 (table 1); the main parts of the plant (bark, root, fruit, and leaf) were separated and frozen until extraction. Berberine (>99%, Sigma) was used as the main standard alkaloid from *Berberis*. For the extraction of bioactive compounds from samples ethanol 96% (v/v; Chimopar, Bucuresti) was used. For HPLC (high performance liquid chromatography) analysis of *Berberis* extracts HPLC grade solvents were used (acetonitrile and methanol, Fluka). DPPH (2,2 diphenyl 2 pycrylhydrazyl, >99%, Sigma) was used in order to evaluate the antioxidant activity of extracts.

Solid-liquid extraction and concentration

Bioactive compounds (especially alkaloids and some flavonoids) from *Berberis vulgaris* samples were separated by solid-liquid extraction in a 100 mL discontinuous extractor equipped with reflux condenser. Approximately 5 g finely grounded sample was moistening with 5 mL 10% Na_2CO_3 for alkalization, and then 25-50 mL 96% ethanol was added. The extraction was realized on a water bath at reflux for 0.5 h. After cooling the extraction mass was filtered in vacuum and the residue was extracted again in the same manner. The filtered extract was concentrated (five to six times) by vacuum distillation and the concentrate was filtered again. The final extract was subjected to the HPLC analysis, to the antioxidant activity evaluation and for obtaining cyclodextrin nanoparticles.

HPLC analysis

Identification and quantification of the main bioactive compound (berberine, standard, >99%) from *Berberis vulgaris* extracts were realized by reversed phase high performance liquid chromatography (RP-HPLC). *Berberis vulgaris* extracts and berberine standard solutions were analyzed by using a HPLC Jasco chromatograph, equipped with a quaternary pump (PU-2080 Plus), mixing unit (LG-2080-04 Quaternary gradient), degassing unit (DG-2080-54 4), spectrophotometric detector (UV-2070 Plus Intelligent UV/VIS Detector), with the possibility of acquisition and data handling by using the Jasco ChromPass Chromatography Data System, ver. 1.7.403.1, LC-Net II / ADC Interface. The HPLC conditions were: Nucleosil 100 C18, 250 x 4.6 mm x mm column, 5 μm particle diameter, UV wavelength 254 nm, eluent Citric acid - disodium phosphate buffer solution (pH 4.6) : Acetonitrile 85 : 15, temperature 25°C, flow 1.0 mL/min, volume injected 20 μL .

Spectrophotometric analysis and antioxidant activity evaluation

The presence of some alkaloids containing phenolic OH groups (like columbamine, jatrorrhizine, berbamine) and/or some flavonoids (like quercetin and kaempferol) confer to the *Berberis vulgaris* extracts antioxidant character. The antioxidant activity of *B. vulgaris* extracts was evaluated by using DPPH spectrophotometric method, according to our previous work [38-41].

DPPH is a relatively stable free radical which can be scavenged by antioxidants such as aromatic and enolic

Table 1
CODES AND CHARACTERISTICS OF THE *BERBERIS VULGARIS* SAMPLES

No	Code	Species	Part of the plant	Source and extraction method
1	29_BFzM1	<i>Berberis vulgaris</i> L.	Leaf	Cultivated (Arad), EtOH 96% solid-liquid extraction, vacuum concentration (filtration)
2	30_BFcM1		Fruit	Cultivated (Arad), EtOH 96% solid-liquid extraction, vacuum concentration (filtration)
3	31_BRdM1		Root	Cultivated (Arad), EtOH 96% solid-liquid extraction, vacuum concentration (filtration)
4	32_BScM1		Bark	Cultivated (Arad), EtOH 96% solid-liquid extraction, vacuum concentration (filtration)

Table 2
SEPARATION/CONCENTRATION CONDITIONS AND RESULTS FOR THE OBTAINING *BERBERIS VULGARIS* EXTRACTS

No	Code	m _{sample} (g)	V _{Na2CO3 10%} (mL)	V _{EtOH 96%} (mL)	V _{extract} (mL)	V _{concentrate} (mL)
1	29_BFzM1	5.001			21.5	4
2	30_BFcM1	5.006	5	25	25	4
3	31_BRdM1	5.001			26	4
4	32_BScM1	5.003			21.5	3.2

hydroxyl-containing compounds (*i.e.* flavonoids and some alkaloids from *B. vulgaris* extracts). The violet colour of DPPH becomes light yellow after the reaction and the consumption of DPPH can be measured spectrophotometrically at the wavelength corresponding to the maximum absorption of DPPH (517 nm) [42-45]. In order to evaluate the antioxidant activity, the behavior of DPPH in the presence of *B. vulgaris* extract was compared with standard natural compounds with such activity (quercetin and vitamin C) [45].

An UV-VIS CamSpec 501 spectrophotometer was used for antioxidant activity evaluation; the acquisition and handling of the data were realized with the UV-Vis Analyst program, ver. 4.67. Thus, the sample cuvette contain 2 mL ethanol (96%, v/v), 0.5 mL *B. vulgaris* extract (undiluted or diluted) or positive standard quercetin and vitamin C solution (0.01-1.5 mM), and 0.5 mL 1mM DPPH ethanolic solution. The spectrophotometric analysis was realized at 517 nm for maximum 30 min. Ethanol was used as reference solvent.

Obtaining of *B. vulgaris* extract/ β cyclodextrin nanoparticles

Protecting and controlled release of the bioactive compounds from *B. vulgaris* were realized by β cyclodextrin micro/nanoencapsulation. The cyclodextrin was dissolved in 4 mL distilled water in a thermally controlled minireactor, equipped with reflux condenser, dropping funnel, and magnetic stirrer; the cyclodextrin solution/suspension was heated to 50°C and the *B. vulgaris* extract (a volume corresponding to the main biocompound : cyclodextrin ratio of 1 : 1) was added to the cyclodextrin solution for 15 min. The complex solution/suspension was stirred for another 15 min. The complexation mass was slowly cooled, the complex crystallized and the suspension was put in a refrigerator for completing the crystallization. The *B. vulgaris* biocompounds:cyclodextrin nanoparticles were filtered, washed with ethanol and dried at environmental

temperature. The complexation conditions and yields are presented in table 3. The obtained micro/nanoparticles were analyzed by scanning electron microscopy (SEM), thermogravimetry (TG), and differential scanning calorimetry (DSC).

Scanning electron microscopy (SEM) analysis. Morphological and dimensional analysis of the *B. vulgaris* extract/ β cyclodextrin particles were performed by using an INSPECT S SEM apparatus, voltage of 25 kV, 3000-12000⁷ magnitude level, focusing 10-14.1 mm.

Thermogravimetry (TG).

Thermogravimetric analysis of the *B. vulgaris* extract/ β cyclodextrin nanoparticles was realized by using a TG 209 Netzsch apparatus, a program temperature of 20-550°C with a heating rate of 4°C/min. All determinations were conducted under nitrogen atmosphere. Data acquisition was performed with the TG Netzsch 209-Acquisition Soft/2000 and the data analysis was realized with the Netzsch Proteus-Thermal Analysis ver. 4.0/2000 soft.

Differential scanning calorimetry (DSC)

The DSC analysis of the nanoparticles was performed by using a DSC Netzsch 204 apparatus. Aluminum oxide dishes were used for weighting and analysis of the samples (7 \pm 2 mg sample). The DSC conditions were: temperature program 20-400°C, with a heating rate of 4°C/min, cooling of the sample was achieved with liquid nitrogen. Data acquisition was performed by using the DSC Netzsch 204-Acquisition soft/2000 and data handling was realized with the same program as for TG analysis (Netzsch Proteus-Thermal Analysis ver. 4.0 / 2000).

Results and discussion

In order to identify and quantify the main alkaloid from *Berberis vulgaris* extracts by RP-HPLC analysis, standard

No	Code	Species	Part of the plant	V _{extract} (mL)	m _{p-CD} (g)	m _{complex} (g)	Yield (%)
1	29_BFzM1	<i>Berberis vulgaris</i> L.	Leaf	1	0.67	0.5019	74.91
2	31_BRdM1		Root				
3	32_BScM1		Bark				

No	Code	Part of the plant	V _{extract} (mL)	Dilution factor	Peak area (mV·min)	C _{berberine} (mg/mL extr)	C _{berberine} (% in the sample)
1	29_BFzM1	Leaf	4	30	24.5	0.54	1.29
2	30_BFcM1	Fruit	4	30	22.4	0.49	1.18
3	31_BRdM1	Root	4	30	71.5	1.57	3.76
4	32_BScM1	Bark	3.2	50	85.5	1.88	6.00

Table 3
COMPLEXATION CONDITIONS AND YIELDS FOR OBTAINING *B. VULGARIS* EXTRACT/ β CYCLODEXTRIN NANOPARTICLES

Table 4
RESULTS OF THE HPLC ANALYSIS OF *B. VULGARIS* SAMPLE

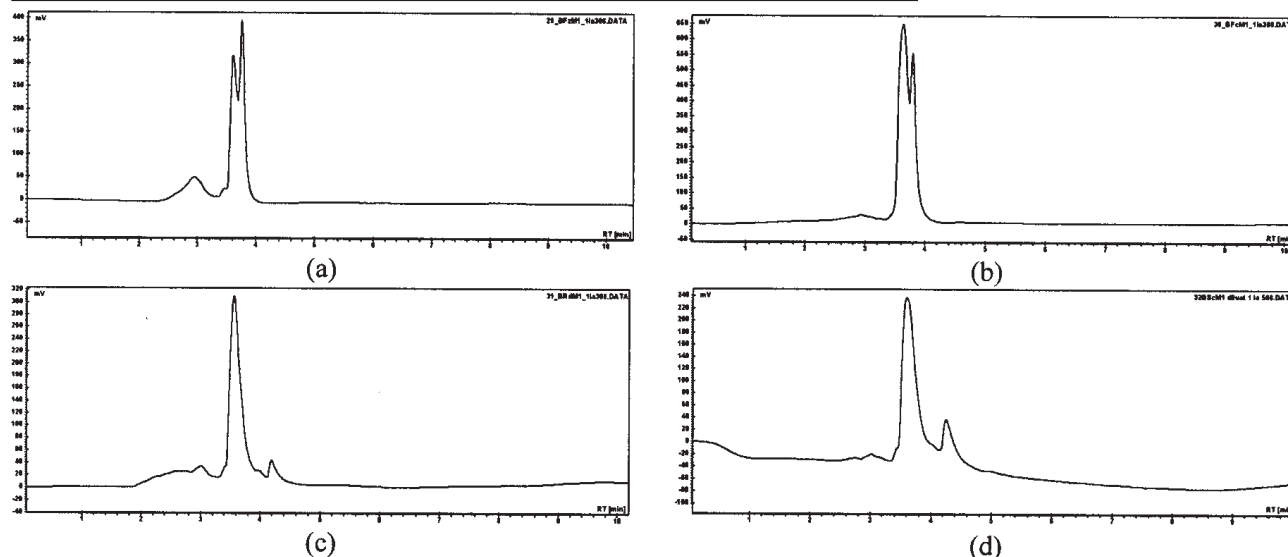


Fig. 2. HPLC chromatograms for *B. vulgaris* leaf (a), fruit (b), root (c), and bark (d) extracts

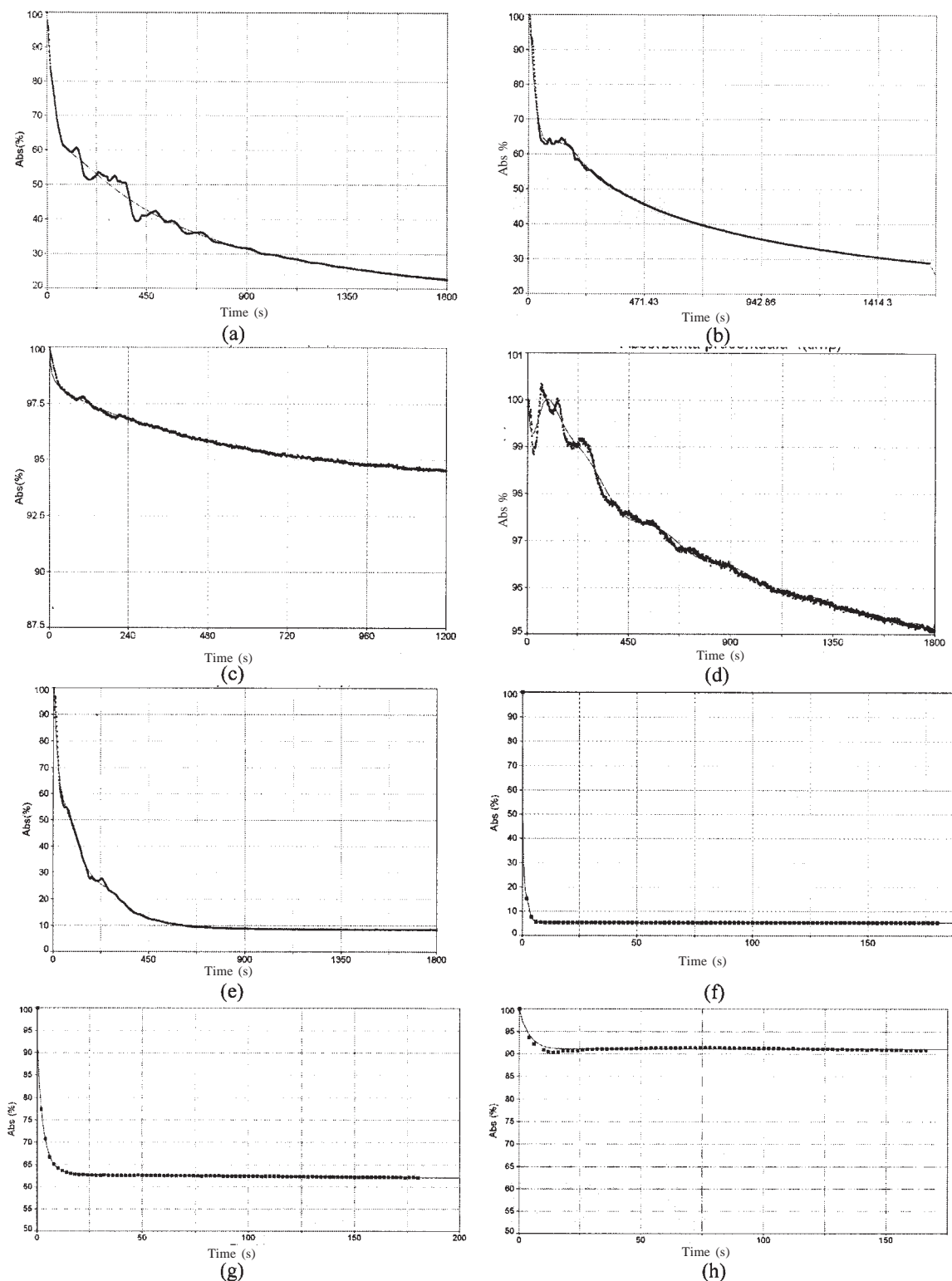


Fig. 3. Antioxidant activity (expressed as residual absorbance percent, %Abs) of *B. vulgaris* leaf (a), fruit (b), root (c), bark (d) extracts, and of positive control quercetin (1.5 mM, (e)) and vitamin C solutions (0.01-1 mM, (f-h))

berberine was used. The calibration curve obtained for berberine is: $Area(mV \cdot min) = 45.34 \cdot c^{0.14}$, where $Area(mV \cdot min)$ is the corresponding berberine peak area (retention time of 3.3-3.6 min) and c is the berberine concentration (mg/mL). By using this calibration curve the concentration of berberine in extracts and forward in the raw samples were in the range of 1.2-6%, more concentrated in bark (table 4, fig. 2).

For the evaluation of antioxidant activity of *B. vulgaris* extracts the DPPH method was used. The antioxidant activity was calculated as the residual absorbance percentage (%Abs) (the ratio of DPPH solution - *B. vulgaris* extract/standard solution mixture and blank sample

absorbances, at 517 nm, calculated as percent); this indicate the reaction capacity of the extract in the presence of DPPH and a lower value of the %Abs suggest a high antioxidant activity.

Thus, the leaf and fruit extracts have the higher antioxidant activity (%Abs of 22% and 30%, respectively), close to the antioxidant activity of quercetin (1.5 mM) and vitamin C (1 mM) standard solutions, while the root and bark extracts have lower activity (>94% in all cases, close to the value corresponding to diluted vitamin C standard solution, 0.01 mM, fig. 3).

Table 5
DPPH MEAN RATE ($\mu\text{M/s}$) FOR DIFFERENT TIME RANGES ($i1$: 400-1200s, $i2$: 1200-1800s) FOR
B. VULGARIS EXTRACTS AND FOR THE QUERCETIN AND VITAMIN C POSITIVE CONTROL SOLUTIONS

No	Code	Description	\bar{v}_{i1} ($\mu\text{M/s}$)	\bar{v}_{i2} ($\mu\text{M/s}$)
1	29_BFzM1	<i>Berberis vulgaris</i> hot extract – leaf, undiluted	0.0056	0.0056
2	30_BFcM1	<i>Berberis vulgaris</i> hot extract – fruit, undiluted	0.00058	0.00058
3	31_BRdM1	<i>Berberis vulgaris</i> hot extract – root, undiluted	0.061	-
4	32_BScM1	<i>Berberis vulgaris</i> hot extract – bark, undiluted	0.025	-
5	Q	Quercetin solution (1.5 mM in ethanol)*	0.053	0.00017
6	VitC1	Vitamin C solution (1 mM in water)**	-	0.00009
7	VitC2	Vitamin C solution (0.1 mM in water)**	-	0.0045
8	VitC3	Vitamin C solution (0.01 mM in water)**	-	0.0061

* 1.1 $\mu\text{M/s}$ for the range of 0-50s and 0.1 $\mu\text{M/s}$ for the range of 50-1200s
** >1 $\mu\text{M/s}$ for the range of 0-10s

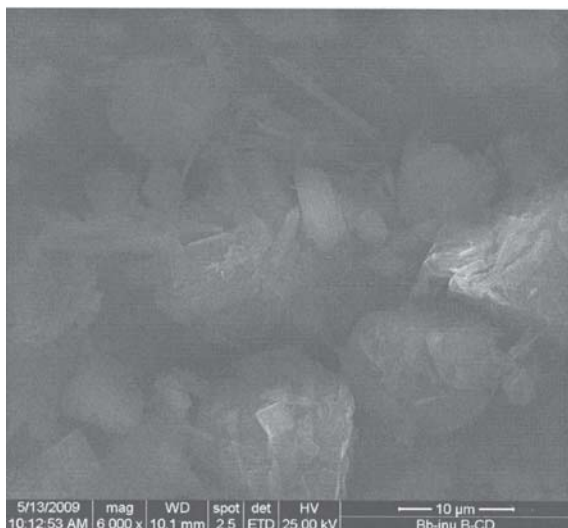


Fig. 4. SEM micrograph of the *B. vulgaris* bark extract/ β -cyclodextrin micro/nanoparticles (code 32_BScM1_bCD; 6000 x magnitude)

Not only the overall antioxidant activity is important for the bioactivity of extracts but also the rate of consumption of biocompounds in the presence of free radicals, which can be evaluated from the DPPH model system above mentioned. The rate of DPPH consumption can indicate the long time antioxidant activity of the *B. vulgaris* extracts and can be evaluated from the DPPH concentration (μM) vs. Time (s) dependence:

$$\bar{v} = -\frac{\Delta c_{\text{DPPH}}}{\Delta t} \text{ - mean rate } (\mu\text{M/s})$$

where:

v is the DPPH reaction mean rate ($\mu\text{M/s}$),
 $\Delta c_{\text{DPPH}}/\Delta t$ is the variation of the DPPH concentration (μM) on the specified time range.

The DPPH rate is therefore the drift of the pseudolinear equation for this time interval. In order to evaluate the momentan DPPH concentration an Absorbance vs. concentration (μM) calibration curve, with a linear dependence (Absorbance = $0.025 + 10960 \cdot \text{concentration}$, $r^2 = 0.997$) is obtained. The rate of DPPH consumption (table 5) on two significant interval ($i1$: 400-1200s and $i2$: 1200-1800s) are small ($<0.006 \mu\text{M/s}$), especially for *B. vulgaris* leaf and fruit extracts (more probable the concentration of flavonoid compounds are higher than for the alkaloid compounds, the first having higher rate of consumption; in this case the maximum rate was up to 50s, but it is hard to evaluate). For the bark and root extracts (table 5), where the total alkaloid concentration (and also the alkaloids with antioxidant activity) is higher, the rate of DPPH consumption is higher on the $i1$ and $i2$ intervals ($0.02\text{--}0.06 \mu\text{M/s}$). These results are close to the positive controls: the behavior of *B. vulgaris* extracts is similar to the quercetin standard solution; the vitamin C is more reactive in the presence of DPPH and after $\sim 10\text{s}$ the rate of DPPH consumption is very small (table 5).

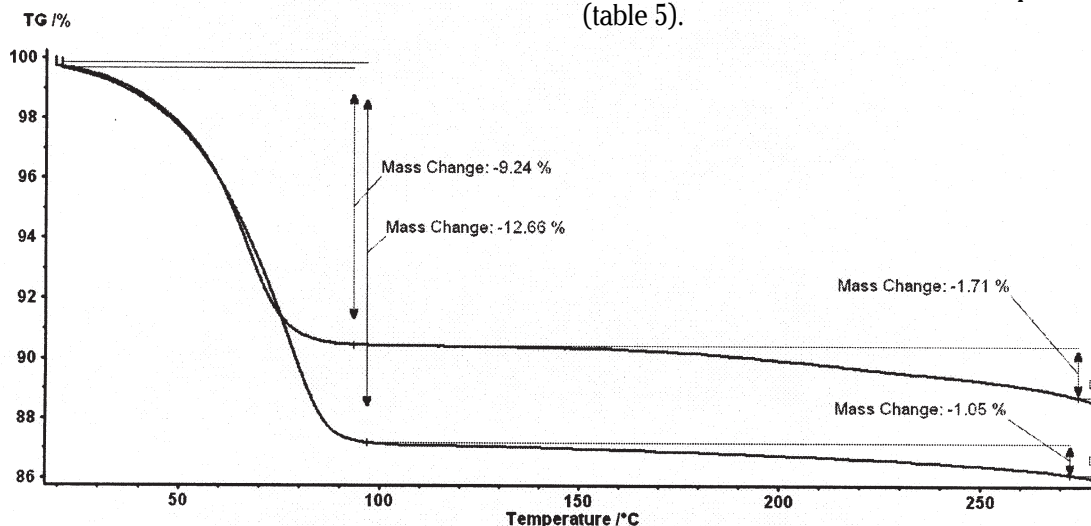


Fig. 5. Superimposed thermograms from the TG analysis for β -cyclodextrin (red; lower) and for the *B. vulgaris* leaf extract/ β -cyclodextrin complex (code 29_BFzM1_bCD; green; upper), for 20-275°C temperature interval

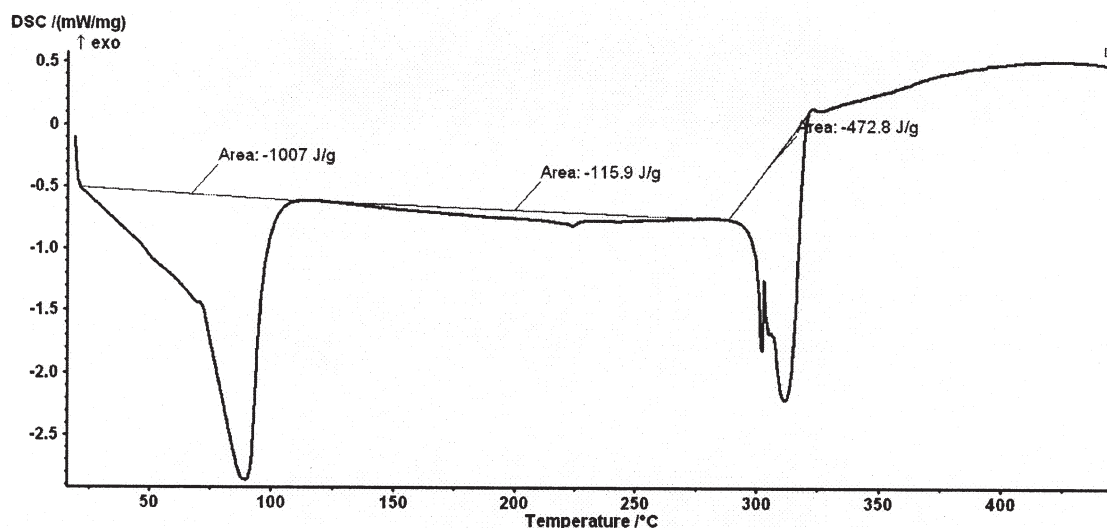


Fig. 6. DSC analysis of the *B. vulgaris* leaf extract/ β -cyclodextrin complex (code 29_BFzM1_bCD)

In order to protect (e.g. against oxidation) and controlled release of the bioactive compounds from *B. vulgaris* (alkaloids and/or other bioactive compounds like flavonoids), the β cyclodextrin encapsulation was used. The best complexation yields (table 3) were obtained for leaf and fruit *B. vulgaris* extract samples (75-77%), and the lower was obtained for bark sample (~69.5%).

The SEM analysis of the *B. vulgaris* extract/ β -cyclodextrin complex indicates a prismatic shape of the crystals (more of them agglomerated), with dimensions of hundreds of nanometers to micrometers (fig. 4).

The TG and DSC analyses clearly indicate the formation of the bioactive compounds/ β -cyclodextrin complex. Thus, the TG analysis of the reagent grade β cyclodextrin indicate a mass loss of ~13% up to 100°C (corresponding to the water dissociation) and lower than 1% up to 275°C (after that the decomposition of cyclodextrin appear), while in the case of *B. vulgaris* leaf extract/ β -cyclodextrin complex (code 29_BFz/bCD) the mass loss corresponding to the dissociation of the bioactive compounds is ~2% (in the range of 100-275°C) and the mass loss corresponding to water (and probably ethanol) release is lower (9.2%) (fig. 5).

The DSC analysis for these complexes is well correlated with the TG analysis: a large endothermic peak appear on the 100-275°C temperature range (116 J/g), corresponding to the bioactive compound release, and no peak appear in the case of β -cyclodextrin for this interval; but the peak corresponding to the water release exists in both cases (1007 J/g for the *B. vulgaris* extract/ β -cyclodextrin complex, figure 6, and 1326 J/g for β -cyclodextrin only).

Conclusion

The following conclusion on the obtaining, analysis, antioxidant activity evaluation of the *Berberis vulgaris* extracts (leaf, fruit, root, and bark) and their β -cyclodextrin micro/nanoparticles can be drawn: (1) the maximum concentration of the main alkaloid, berberine, was found in bark and also in root; (2) the best antioxidant activity have been obtained in the case of leaf and fruit extracts, probable due to the higher flavonoid concentration. This observation is sustained by the DPPH consumption rate on different time interval: higher rate in the time interval up to 50s for the case of leaf and fruit extracts, reduced rate in the next interval, and higher rate on the last interval

in the case of root and bark extracts; (3) the β -cyclodextrin nanoencapsulation of the biocompounds from *Berberis vulgaris* extracts was achieved with moderate yields (69.5-77%), the best being obtained in the case of leaf and root extracts, and the specific physico-chemical analyses (SEM, TG, and DSC) indicate the formation of biocompounds/ β -cyclodextrin complex.

Acknowledgements

The authors want to thanks to Dr. Ioan Grozescu (INCDEMC Timișoara, Romania) for the permission to evaluate the particle dimensions by scanning electron microscopy. This work was supported by Ministry of Education and Research from Romania, PN2 62072/2008.

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Manuscript received: 6.10.2009