Immobilization of Chaga extract in alginate beads for modified release: simplicity meets efficiency

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Abstract

Chaga (*Inonotus obliquus*) is a parasitic fungus, which has been used in traditional medicine in Russia and other northern European countries in the treatment of cancer, gastrointestinal andliver diseases. It has been a subject of intensive researchrecently, confirming many of its health-beneficial effects. In order to obtain a product that would allow modified and prolonged release of the Chaga's active metabolites, hot water Chaga extract was immobilized using calcium - alginate. The extract, which was predominantly composed of carbohydrates (57 %), also contained a relatively high amount of antioxidants/phenolic compounds (130 mg gallic acid equivalents per g of dry extract) and exhibited pronounced radical scavenging activity. It showed significant antibacterial activity as well, inhibiting growth of tested bacterial strains at concentrations of 1.25-20 mg/mL. Entrapment efficiency was about 80 %, and the extract-alginate system showed pH-dependant extract release; there was negligible release at pH 1.75 (gastric pH), and the release gradually increased with the increase in pH, reaching ~43 % of immobilized extract at pH 8.5 after 90 min. Such a product could be used as a dietary supplement, adjuvant in therapy of gastrointestinal diseases or as a food additive.

Keywords: mushrooms, natural product, antioxidant, antimicrobial, encapsulation

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1. INTRODUCTION

Chronic diseases like cardiovascular diseases and diabetes, as well as cancer are among leading causes of death worldwide [1]. Apart from that, the growing microbial resistance to antibiotics is causing a comeback of life-threatening infections [2]. Natural products are still among the most important sources of therapeutic agents in medicine, especially in development of effective therapeutic protocols for therapy of cancer, neurological, cardiovascular, autoimmune, bacterial and fungal diseases [3]. Functional foods, rich in nutraceuticals (vitamins, ω-3 fatty acids, antioxidants, fibers…) are recognized as an important factor in prevention, but also in treatment of chronic diseases [4]. Chaga (*Inonotus obliquus*) is a parasitic fungus that grows predominantly on birch trees (*Betula* spp.) and is gaining popularity as a promising natural source of all kinds of physiologically active metabolites. The fungus produces dark sclerotium, which has been used in traditional medicine in Russia and other northern European countries, in form of decoctions ("tea"), for treatment of stomach diseases, intestinal worms, liver and heart ailments, as well as different kinds of cancer [5-7]. Recent studies revealed that Chaga has a huge medical potential, showing antioxidant, anti-inflammatory, immunomodulatory, antitumor, hypolipemic, hypoglycaemic and antimicrobial activity [5]. The identified active

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principles of Chaga extracts include polysaccharides, terpenoids and various phenolic structures, including water-soluble polymeric pigments [5,7-10]. Although the use of bioactive extracts of natural origin is a growing area of interest in both developing and developed countries, potential issues with such products may be long-term instability, low bioavailability and short-term effects [11]. Moreover, specific conditions in gastrointestinal tract (different pH values and the presence of various enzymes and nutrients) cause degradation of active compounds such as polyphenols [12]. Often bitter, astringent and unpleasant taste of such products may limit their oral application as well [13-14]. Some of these problems may be overcome by using biocompatible polymers as carriers of active ingredients [11]; encapsulation/entrapment of an extract may result in extended shelf-life by ensuring better product stability *via* isolation and protection of active compounds from detrimental effects of oxygen or moisture. This approach could allow controlled delivery and enhanced bioavailability of an active compound by customizing the release mechanism, or release rate in gastrointestinal tract (GIT) [15]. Hence, in order to improve stability and bioavailability of extracted principles, as well as to protect their health-beneficial properties and provide modified delivery of active substances, numerous entrapment techniques have been established [12-13,16-18]. Microencapsulation is considered to be an appropriate method for protection and prolonged or controlled release of extracted antioxidant components (as constituents of food and pharmaceutical products) in relation to specific targeted parts of the human gastrointestinal tract [19]. Encapsulation/entrapment matrix should meet some requirements: to possess good rheological properties, to be inexpensive, food-grade, legally approved and inert to the encapsulated/entrapped material, as well as to provide maximal protection from environmental factors. Such a low-cost and biodegradable polymer is alginate [20].

The aim of this study was to investigate the possibility of immobilization of hot water Chaga extract in alginate beads in order to obtain modified, pH-dependant and prolonged release of active principles, with the accent on compounds with radical scavenging activity. Total carbohydrates, proteins and antioxidant/phenolic compounds were determined, and the extract was tested for radical-scavenging and antimicrobial activity. There are several commercial products that contain Chaga extracts (Befungin is certified in EU), but none of these offers modified release of the extract, which could lead to better bioavailability of the active compounds.

2. MATERIAL AND METHODS

2. 1. Materials

Gallic acid, phenol, Brilliant Blue G, triphenyltetrazoliumchloride (TTC), Folin-Ciocalteu reagent, albumin, medium viscosity sodium alginate (A2033),2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and tris(hydroxy-methyl)aminomethane (Tris) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Malt broth, malt agar, Müller-Hinton broth and Müller-Hinton agar were obtained from Biolife (Milan, Italy). Orthophosphoric acid (H_3PO_4), sodium citrate dehydrate, potassium persulfate ($K_2S_2O_8$) and sulfuric acid (H_2SO_4) were obtained from Centrohem (Stara Pazova, Serbia). D-(+)-glucose, dimethylsulfoxide (DMSO) and ethanol were obtained from Fisher Scientific, (Loughborough, UK). Potassium hydroxide (KOH),potassium dihydrogen phosphate (KH_2PO_4) and sodium carbonate (Na_2CO_3) were purchased from Fagron B.V. (Capelle aan den Ijssel, Netherlands). Calcium chloride ($CaCl_2$) was obtained from Analytika (Prague, Czech Republic). Hydrochloric acid (HCl) was purchased from Zorka Pharma (Šabac, Serbia). Sodium chloride (NaCl) was purchased from Alkaloid (Skopje, North Macedonia). Pronase from *Streptomyces griseus* was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Deionized water was used in all experiments, unless stated otherwise. All chemical reagents used were of analytical grade.

2. 2. Extract preparation and isolation of the macromolecular fraction

Chaga was collected on Vlasina Lake, Serbia, in 2016. Dry sclerotium was powdered and mixed with water (100 g/L). The mixture was subjected to hot water extraction in an autoclave (120 °C, 1.2 bar, 45 min), centrifuged (2750 g, 10 min) and filtered through a Whatman® No. 5 filter paper (Sigma-Aldrich, St. Louis, MO, USA). Part of the filtrate (hot water extract) was collected and lyophilized (-60 °C, 0.011 mbar, 24 h) in a Christ BETA 2-8 LD plus freeze dryer (Osterode, Germany), and as such was used for further investigation. The other part was used to estimate the non-protein

macromolecular content in the extract; the volume of the filtrate was reduced to 10% by boiling, the concentrate was cooled down to room temperature and three volumes of ethanol were added. The mixture was left overnight at 4 °C and then centrifuged (2750 g, 10 min). The precipitate was collected and lyophilized (-60 °C, 0.011 mbar, 24 h). The dry sample was then resuspended in water (10 mg/mL) and subjected to dialysis, against water, using cellulose membrane tubes (SERVAPORE, MWCO 6000-8000 Da, SERVA Electrophoresis GmbH, Heidelberg, Germany), for 24h, to remove salts and small molecules from the extract. Tube content was collected and lyophilized. Dry dialyzed extract was subjected to enzymatic deproteinization; the extract was resuspended in Tris/HCl buffer (0.1 M, pH 8.2), with addition of $CaCl_2$ (10 mM). Pronase, dissolved in the same buffer (10 mg/mL) was added to the sample solution (1 mg of pronase per 100 mg of the extract). Deproteinization was conducted for 24 h, at 30 °C. The reaction was stopped by bringing the mixture to boiling, after which it was subjected to dialysis and lyophiilization, as described above. The obtained fraction represented the deproteinized macromolecular part of the hot water extract.

2. 3. Chemical analysis

2. 3. 1. Carbohydrate content

Total carbohydrates were determined using a method described by DeBois and coworkers [21]. Extract aqueous solution (0.2 mg/mL, 200 µL) was mixed with water (200 µL), phenol aqueous solution (5 % w/v, 400 µL) and concentrated H_2SO_4 (98 %, 2 mL). The mixture was mixed well and the absorbance was read after 40 min at 490 nm on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). D-(+)-glucose was used to construct the standard curve (0.025-0.3 mg/mL; y=5.1114x+0.0851, R^2=0.9985) and the results were expressed as sugar percentage. The experiment was done in triplicate.

2. 3. 2. Protein content

Total proteins were determined using the Bradford protein assay [22]. Bradford reagent was prepared by dissolving Coomassie Brilliant Blue G250 (100 mg) in ethanol (96 %, 50 mL), adding H_3PO_4 (85 %, 100 mL), and adjusting solution to 1 L with water. Aqueous solution of the extract (20 mg/mL, 100 µL) was mixed with the Bradford reagent (5 mL) and absorbance of the mixture was read after 10 min at 595 nm, on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Albumin was used for standard curve construction (0.125-2 mg/mL; y=0.5817x+0.0293, R^2=0.9985) and the results were expressed as protein percentage. The experiment was done in triplicate.

2. 3. 3. Total phenol content

Total phenol content was estimated using a procedure proposed by Jovanović and coworkers [23]. Extract aqueous solution (100 µL, 10 mg/mL) was mixed with aqueous solution of Folin-Ciocalteu reagent (1:3 v:v, 500 µL) and water (6 mL). After 6 min, aqueous solution of Na_2CO_3 (20 % w/v) was added to the mixture and volume was adjusted to 10 mL with water. The absorbance was read after 2 h at 765 nm on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Gallic acid was used for standard curve construction (0.125-2 mg/mL; y=0.9104x+0.0217, R^2=0.9998) and the results were expressed as mg of gallic acid equivalents per gram of the extract (mg GAE/g). The experiment was done in triplicate.

2. 4. *In vitro* antioxidant analysis – ABTS assay

The ability of Chaga extract to scavenge free radicals was estimated by the 2,2'-azino-bis(3-ethylbenzothiazoline-6--sulphonic acid) (ABTS) assay [24] with some modifications. Aqueous solutions of $K_2S_2O_8$ (3.8 %, 88 µL) and ABTS (0.38 %, 5 mL) were mixed and left for 16 h to allow formation of ABTS radicals; the solution was diluted with water (100 fold) to obtain the working solution (0.0037 %). Serial aqueous solutions of the sample (0.313-5 mg/mL, 200 µL) were mixed with the ABTS radical solution (2.8 mL) and the absorbance of the mixtures was read after 20 min at 735 nm on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Mixture of water (200 µL) and the ABTS solution (2.8 mL) was used as a control. For each sample concentration, percentage of the ABTS radical neutralization was calculated according to the formula:

$$((A_c\text{-}A_s) / A_c) 100 \quad (1)$$

where A_c and A_s are absorbance values of control and sample, respectively.

The results were expressed as a sample concentration, which neutralized 50 % of ABTS radicals (EC_{50}, mg/mL). Ascorbic acid was used as standard. The experiment was done in triplicate.

2. 5. Broth microdilution antimicrobial assay

Antimicrobial activity of the Chaga extract was evaluated by using a broth microdilution method [25-26]. The extract was tested against 8 microbial strains of the American Type Culture Collection (ATCC): *Staphylococcus aureus* 25923, *Enterococcus faecalis* 29212, *Bacillus cereus* 10876, *Listeria monocytogenes* 19115, *Salmonella enteritidis* 13076, *Yersinia enterocolitica* 27729, *Escherichia coli* O157:H7 35150, *Candida albicans* 10259 and a clinical strain of methicillin-resistant *Staphylococcus aureus* (MRSA), obtained from the City Institute of Public Health (Belgrade, Serbia). The antimicrobial assay was performed in 96-well microplates (Sarstedt, Germany). The extract was dissolved in the aqueous solution of DMSO (5 % v/v), covering the concentration range from 0.01 to 20 mg/mL. Density of microbial suspensions was set at 10^5 colony forming units (CFU) and TTC (0.0075 %) was added as a growth indicator. Suspensions (50 µL) were added to wells containing serial sample dilutions (50 µL). The growth control was prepared by mixing the microbial suspension (50 µL) with DMSO water solution (5 % v/v, 50 µL). All experiments were performed in duplicates. Bacterial strains were incubated for 24 h at 37 °C, while the yeast strain was incubated for 48 h at 30 °C. The lowest concentration of the sample at which microbial growth was not visible in neither of the replicates (absence of red color for bacterial strains and absence of visible colonies for *C. albicans*) was adopted as a minimal inhibitory concentration (MIC). The minimal bactericidal/fungicidal concentration (MBC/MFC) was determined by serial sub-cultivations of the samples taken from each well on a solid medium (Müller Hinton agar for bacterial strains and malt agar for *C. albicans*); the lowest concentration of the sample without any visible growth after repeated incubations was considered as a MBC. Amoxicillin was used as a standard for bacterial strains (0.05-50 µg/mL) and fluconazole for *C. albicans* (0.1-100 µg/mL).

2. 6. Beads preparation and analysis

2. 6. 1. Beads preparation

Colloid solution of sodium alginate (2 % w/v) and Chaga extract (2 % w/v) was extruded through a blunt stainless still needle (18 G) using a Razel Scientific Instruments R-99 syringe pump (Fairfax, VT, USA) under a constant flow rate of 70 mL/h, into a $CaCl_2$ solution (2 % w/v). The distance between the needle tip and the collecting solution was 3 cm. After extrusion, the beads were left in the collecting solution for 30 min, under constant stirring, then rinsed in distilled water several times and dried at 25°C to the constant weight.

2. 6. 2. Bead size determination

Dry beads were measured using a Motic light microscope (BA 210, Xiamen, China), equipped with a Moticam digital camera (1SP, 1.3 MP) and a Motic Images Plus 2.0 software. Maximal diameters of the beads were measured; the average diameter of the beads and standard deviation were calculated based on measurements of 20 different beads.

2. 6. 3. Entrapment efficiency determination

Entrapment efficiency was estimated by the difference of the total mass of starting materials (alginate + extract) and dry alginate beads, with the assumption that the complete alginate content underwent gelation.

2. 6. 4. Extract releasing study

The pH-dependant release of the extract from alginate beads was investigated by using three solutions with different pH values, approximately covering the physiological GIT pH range (pH 1.75, 5 and 8.5); HCl was diluted in water to prepare the solution of pH 1.75 while KH_2PO_4 and KOH were used to prepare buffer solutions of pH 5 and 8.5 (0.1 M). Beads (350 mg) were suspended in the HCl/buffer solutions (30 mL) and stirred constantly for 90 min. Medium aliquots

(0.5 mL) were sampled after 1, 10, 20, 30, 45, 60 and 90 min, returning the same volume of the medium to the system. Samples were centrifuged and the supernatant was used for total phenol determination (described in the section 2.3.3). The percentage of released extract at each time point was estimated based on the phenol content, which was calculated according to the following formula:

$$(c_s / c_{max}) 100 \quad (2)$$

where c_s is the concentration of phenols determined in the medium and $c_{max}$is the maximal calculated concentration of phenols that could be released. The measurements were done in triplicate.

2. 7. Statistical analysis

Data were expressed as a mean ± standard deviation, if not stated differently. The experimental data were subjected to one-way analysis of variance (ANOVA), whereas Tuckey's HSD test was used to detect significant difference (p=0.05). OriginPro 8 (OriginLab, Northampton, MA, USA) was used for statistical analyses.

3. RESULTS AND DISCUSSION

3. 1. Chemical characterization of the extract and antioxidant activity

The yield of hot water extraction in the present study was 19.8 wt% (Table 1), although some sources state that water extractable compounds make up to 40 wt% of Chaga sclerotium [7]. Carbohydrates made up the greatest part of the extract, ~57 wt%, while proteins accounted for ~7 wt% of the extract. Carbohydrate content of Chaga is known to include various polysaccharides, both heteroglycans and β-glucans, latter being more important as they are responsible for immunomodulatory activity [27]. The extract contained a very high amount of antioxidant/phenolic compounds, ~130 mgGAE/g; the nature of phenolic fraction of Chaga is yet to be fully understood. Chaga contains simple, ubiquitous phenolic acids, such as protocatechuic, caffeic, syringic and *p*-hydroxybenzoic acids [28-29]. Chaga also contains styrylpyrone compounds (hispidin and its analogues), yellow pigments found in some lignicolous fungi, which are thought to have a similar role as plant flavonoids, protecting the fungi from oxidative stress, and thus exhibiting prominent antioxidant activity [5,30-31]. Also, Chaga is known to contain water soluble macromolecular pigments, interpreted by various authors as melanins ("allomelanins"), a "chromogenic complex"(ChC) or a "polyphenol hydroxycarboxylic complex", although their chemical structure is still not understood well [7-8,32-34]. The dark color of the extract suggests that a relatively high amount of such structures is present. Some authors characterized water-soluble "lignin-like structures" and "polysaccharide-lignin complexes", isolated from Chaga [35-36]. Indeed, there is evidence that at least part of Chaga phenolic compounds/pigments represent polymeric, humic acid-like, water soluble lignin derivatives, which some authors hypothesized are derived by fungi mycelium, after partial digestion of birch lignin. Macromolecular pigment complexes, accounting for 50-60 % of the water extractable matter, are precipitated upon acidification of the water extract solution [7]. Chromogens are recognized by Russian researchers as carriers of therapeutic properties of Chaga, and they are even used to standardize products such is Befungin (Tatkhimpharmpreparaty, Russia) [34]. The water-soluble macromolecular fraction of Chaga obtained in this study was dark colored and accounted for 48 % of the extract; the enzymatic deproteinization led to a loss of about 15 % of this fraction, with deproteinized macromolecular fraction representing about 41 % of the extract, correlating well with total proteins determined by the Bradford assay. The fraction obtained after ethanol precipitation, deproteinization and dialysis, was shown to have a high phenolic content, ~90 mg GAE/g, thus confirming that a great part of antioxidant compounds present in Chaga are "bound" to the macromolecular fraction. It is important to note that the chemical structure of Chaga may differ significantly, in relation with its geographical origin [28,37].

Table 1. Chemical analysis of the hot water Chaga extract and ABTS radical scavenging ability, expressed as EC_{50} – the concentration of the extract that neutralizes 50 % of radicals.

Extract yield, %	Carbohydrate content, %	Protein content, %	Phenol content, mgGAE/g	EC_{50}, mg/mL
19.8	57.13±1.00	7.2±0.36	13.11±0.82	1.19±0.02

The Chaga extract expressed a very pronounced radical scavenging activity (EC_{50} = 1.2 mg/mL), which was almost certainly due to the high phenolic content (Table 1); positive correlations between the phenolic content and radical scavenging activity were found by other authors [23,38]. The plateau was reached at 2.5 mg/mL already, with almost complete neutralization of ABTS radicals (Figure 1). Free radicals, which have an important role in various cell functions, as part of ROS – reactive oxygen species, also play one of the crucial roles in the development of diabetes, cancer, cardiovascular and neurodegenerative diseases. This happens when the balance between free radical production and antioxidant defenses is disturbed, leading to oxidative stress and damage of lipids, proteins, and nucleic acids [39]. Beside endogenous free radicals, there are numerous exogenous sources of free radicals, like tobacco, alcohol, processed food, as well as food and water polluted with heavy metals and pesticides.GIT is directly affected by both endogenous and exogenous free radicals. In 2018 there were more than a 1 million registered new cases of colon and 700,000 of rectal cancer worldwide with more than 860,000 deaths combined [40]. Dietary intake of polyphenols was found to decrease the incidence of colorectal cancer [41-42]; it is even suggested that polyphenols may be useful in the treatment of this cancer type, in combination with cytostatics [43]. In addition to being rich in antioxidants and having radical scavenging activity, Chaga possesses a well documented immunostimulatory-mediated antitumor activity, which is associated with its carbohydrates, and also direct cytotoxic activity towards malignant cells, through secondary metabolites such is betulinic acid. Chaga water extract was reported to exhibit cytotoxic activity on colon cancer cell lines, and certain polyphenol compounds were designated as potentially responsible [44].

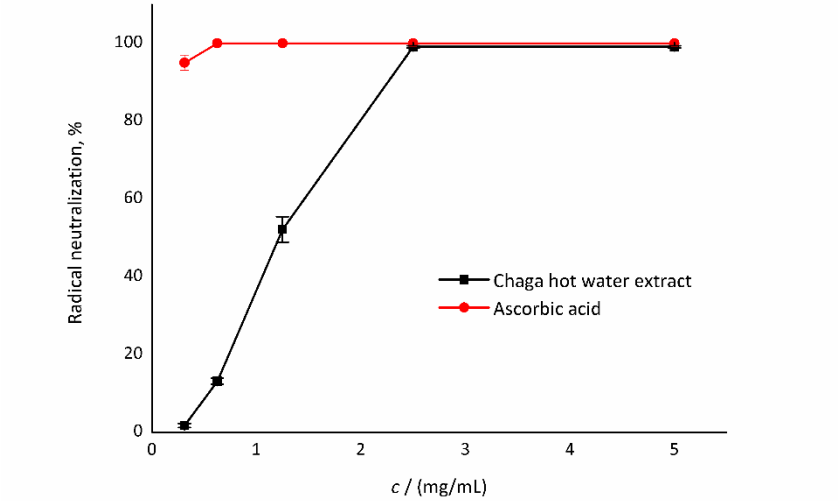

Figure 1. ABTS radical scavenging activity of the hot water Chaga extract

3. 2. Antimicrobial activity

The Chaga extract exhibited inhibitory activity on growth of all tested microbial strains and bactericidal activity against all tested bacterial strains (Table 2). Preferences towards Gram(+) or Gram(-) bacteria were not observed with the greatest growth-inhibition effect achieved on *Y. enterocolitica* (MIC = 1.25 mg/mL) and the least activity against *E. coli* O157 H7 (MIC= 20 mg/mL). Both of these Gram(-) bacteria cause gastrointestinal infections. The extract also showed prominent activity against two bacteria that cause foodborne illnesses, *L. monocytogenes* (MIC = 2.5 mg/mL) and *B. cereus* (MIC = 5 mg/mL), implying that it could act as a natural conservative in food products. The activity was similar against the two other Gram(+) strains, which cause serious intrahospital infections, *S. aureus* (MIC = 2.5 mg/mL) and *E. faecalis* (MIC = 5 mg/mL). The extract was least active against the yeast, *C. albicans*, with inhibitory activity reached at the highest concentration used (20 mg/mL) without fungicidal activity observed. Bactericidal activity was achieved at the highest extract concentration (20 mg/mL) against all the bacterial strains, except in the case of *Y. eneterocolitica*, which was shown to be more susceptible to active compounds of the extract (MBC=5 mg/mL). Glamočlija and coworkers investigated antimicrobial activity of three different commercial Chaga products from Russia, Finland and Thailand and found similar inhibitory activity on *L. monocytogenes* (1.1-3.75 mg/mL) but somewhat better activity against both *B. cereus* (0.75-1.1 mg/mL) and *S. aureus* (0.4-0.75 mg/mL). Preferences towards Gram(+) or

Gram(-) were not found either [28]. Thus, hot water extract of Chaga could be used either as a food additive, to prevent growth of bacteria that cause food spoilage, or as an adjuvant in treatment of some gastrointestinal infections caused by Gram(-) bacteria. Since it is not yet known if the active metabolites could be absorbed from GIT, potential applications of the extract might be even broader.

Table 2. Antimicrobial activity of the hot water Chaga extract, expressed as the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC). Amoxicillin and fluconazole were used as standards for bacterial and fungal strains, respectively

Microbial strain	Chaga extract		Amoxicillin/Fluconazole	
	MIC, mg/mL	MBC/MFC, mg/mL	MIC, mg/mL	MBC/MFC, mg/mL
Bacillus cereus	5	20	0.0014	0.0054
Listeria monocytogenes	2.5	20	0.0003	0.0014
Staphylococcus aureus	2.5	20	0.0002	0.0014
Enterococcus faecalis	5	20	0.0003	0.0027
Escherichia coli O157 H7	20	20	0.0054	0.0217
Salmonella enteritidis	10	20	0.0027	0.0217
Yersinia enterocolitica	1.25	5	0.0217	0.0434
Candida albicans	20	-[1]	0.0125	0.0500

[1]not achieved

3. 3. Beads analysis

The obtained dry beads were irregularly spherically to elliptically shaped, with the diameter of 1.5±0.2 mm. They were almost black in color, reflecting the color of the Chaga extract itself (Figure 2).

Figure 2. The Chaga hot water extract solution (10 mg/mL) next to dry alginate particles containing the extract (left) and the optical micrograph of dry alginate beads (scale bar = 1 mm)

The entrapment efficiency of Chaga extract in alginate beads was relatively high, about 80 %. According to the literature, the percentage of entrapped plant polyphenol extracts into alginate particles was significantly lower in comparison to the value of the entrapment efficiency of the Chaga extract obtained in the present study. It was reported that the entrapment efficiency of *Pterospartum tridentatum* extract was 49 % [16], whereas it was 51 % for*Thymus serpyllum* polyphenols [45]. Somewhat better entrapment, for example, was achieved in the case of polyphenol compounds from *Olea europea* leaves, with the entrapment efficiency of approximately 61 % [46] but still significantly lower than in the case of Chaga. However, the entrapment efficiency of pure resveratrol (polyphenol commonly found in red grape) in alginate particles was only 24.5 % [46]. Presence of different phenolic structures in the Chaga extract, which interact better with alginate than those found in plant extracts, may be the reason why the entrapment was more successful.

Release profiles of Chaga phenolic/antioxidant compounds from the alginate beads in different buffers (pH 1.75, 5 and 8.5) during 90 min are shown in Figure 3. It could be seenthat phenols from alginate beads were not released at the acidic pH (<1 % of released phenols), whereas the increase in the pH led to alginate swelling and higher phenol release. At pH 5, the release started after 30 min, and the total amount of released phenolics was only 13.6 % after 90 min. On the other hand, at alkaline pH (8.5), the release started after 20 min and a significantly higher content of the extract in the medium was achieved after 90 min (approximately 43 %).

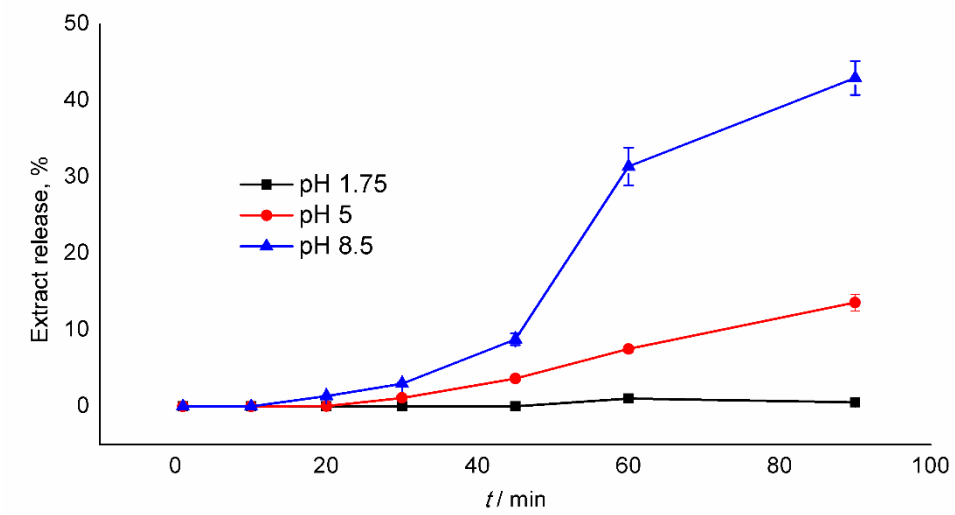

Figure 3. Release profiles of the hot water Chaga extract at different pH values; the percentage of the released extract was estimated by the total phenol analysis

According to literature data, release of plant polyphenols from alginate microbeads is often very rapid, reaching plateau after 10 min in cases of *Aronia melanocarpa* and *T. serpyllum* [45,47] and after 20 min in the case of *P. tridentatum* [16]. As can be seen from the presented results, the entrapment of Chaga extracts into alginate matrix has provided a modified, pH-dependent, prolonged delivery of phenolic/antioxidant compounds. The pH-dependant release kinetics imply that the beads could pass through stomach unchanged and would start liberating active principles after the gastric acid is neutralized. Even then, the release would be prolonged, and the antioxidant compounds could reach distant parts of GIT, without being "spent" in the process. The active compounds could thus act over a large portion of GIT, as radical scavengers, antimicrobial agents or may exhibit other activities that Chaga water extracts are known to possess. The protective effect of alginate would also prevent precipitation of the extract in gastric acidic conditions. Slow release of Chaga phenolics from alginate beads observed at higher pH values implies that these compounds are more complex, larger molecules than those found in plant materials, as mentioned above. Dosage of the beads would be optimized, depending on the purpose of the extract use – whether it is for its antioxidant or antimicrobial activity.

4. CONCLUSION

In the present study, a hot water Chaga extract was obtained, which is regarded safe for humans, as preparations of Chaga have well documented traditional use. The extract was shown to be extremely rich in antioxidant/phenolic compounds and to possess prominent radical scavenging and antibacterial activities. It could be thus potentially used as a dietary supplement, natural antioxidant and/or conservative in food products or even as an adjuvant in therapy of some forms of bacterial infections. Immobilization of the extract in alginate beads, using a cheap, simple method was successful, with about 80 % of the extract being incorporated into the beads. Additionally, pH-dependant and prolonged modified release was achieved, with extract being retained in beads in gastric pH (1.75), and only a small portion of the extract released at pH 5, after 90 min (~13%). The extract release was faster in a weak alkaline medium (pH 8.5), but

sufficiently prolonged so that it was slightly over 40 % after 90 min. Overall, calcium-alginate as a carrier of Chaga extract was shown to be very effective, providing several potential applications of a final product.

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REFERENCES

[1] Grover A, Joshi, A. An Overview of Chronic Disease Models: A Systematic Literature Review. *Glob J Health Sci*. 2014; 7(2): 210-227.

[2] Zaman SB, Hussain MA, Nye R, Mehta V, Mamun KT, Hossain N. A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Cureus*. 2017; 9(6): e1403

[3] Bills GF, Gloer JB. Biologically Active Secondary Metabolites from the Fungi. *Microbiol. Spectr.* 2016; 4(6): 1-32.

[4] Padmavathi M. Chronic Disease Management with Nutraceuticals. *Int J Pharm Sci Invent.* 2013; 2(4): 1-11

[5] Patel S. Chaga (Inonotus Obliquus) Mushroom: Nutraceutical Assesement Based on Latest Findings. In: Patel S, ed. *Emerging Bioresources with Nutraceutical and Pharmaceutical Prospects.* Basingstoke, England: Springer; 2015: 115-126

[6] Lemieszek MK, Langner E, Kaczor J, Kandefer-Szerszen M, Sanecka B, Mazurkiewicz W, & Rzeski W. Anticancer Effects of Fraction Isolated from Fruiting Bodies of Chaga Medicinal Mushroom, *Inonotus obliquus* (Pers.:Fr.) Pilát (Aphyllophoromycetideae): *In Vitro* Studies. *Int. J. Med. Mushrooms*. 2011; *13*(2): 131-143.

[7] Shashkina MY, Shashkin PN, Sergeev, AV. Chemical and medicobiological properties of chaga (review). *Pharm Chem J.* 2006; 40(10): 560-568.

[8] Lee J, Hyun C. Insulin-Sensitizing and Beneficial Lipid-Metabolic Effects of the Water-Soluble Melanin Complex Extracted from *Inonotus obliquus*. *Phytother. Res*. 2014; 28(9): 1320-1328.

[9] Liu C, Zhao C, Pan H, Kang J, Yu X, Wang, H, Li B, Xie Y, Chen, R. Chemical Constituents from *Inonotus obliquus* and Their Biological Activities. *J. Nat. Prod.* 2013; 77(1): 35-41.

[10] Hwang BS, Lee I, Yun B. Phenolic compounds from the fungus *Inonotus obliquus* and their antioxidant properties. *J Antibiot.* 2015; 69(2): 108-110.

[11] Rijo P, Matias D, Fernandes A, Simões M, Nicolai M, Reis C. Antimicrobial Plant Extracts Encapsulated into Polymeric Beads for Potential Application on the Skin. *Polymers*. 2014; 6(2): 479-490.

[12] Pasukamonset P, Kwon O, Adisakwattan S. Alginate-based encapsulation of polyphenols from *Clitoria ternatea* petal flower extract enhances stability and biological activity under simulated gastrointestinal conditions. *Food Hydrocolloid*. 2016; 61: 772–779.

[13] Fang Z, Bhandari B. Encapsulation of polyphenols - a review. *Trends Food Sci. Technol*. 2010; 21: 510–523.

[14] Amatrejo S, Hr T. Tinospora extract (*Tinospora crispa* (L.) Miers.) encapsulation with sodium alginate isolated from brown algae (*Sargassum ilicifolium*). *Asian J Pharm Clin Res.* 2017; 10(11): 267-269.

[15] Chan E, Yim Z, Phan S, Mansa RF, Ravindra P. Encapsulation of herbal aqueous extract through absorption with ca-alginate hydrogel beads. *Food Bioprod Process.* 2010; 88(2-3): 195-201.

[16] Balanč B, Kalušević A, Drvenica I, Coelho MT, Đorđević V, D Alves V, Sousa I, Moldao-Martins M, Rakić V, Nedović V, Bugarski B. Calcium-alginate-inulin microbeads as carriers for aqueous carqueja extract. J. Food Sci. 2016; 81: E65–E75.

[17] Jovanovic A, Balanč B, Djordjević V, Ota A, Skrt M, Savikin K, Bugarski B, Nedović V, Ulrih Poklar N. Effect of gentisic acid on the structural-functional properties of liposomes incorporating β-sitosterol. *Colloid Surface B*. 2019; https://doi.org/10.1016/j.colsurfb.2019.110422

[18] Đorđević V, Balanč B, Belščak-Cvitanović A, Lević S, Trifković K, Kalušević A, Kostić I, Komes D, Bugarski B, Nedović V. Trends in encapsulation technologies for delivery of food bioactive compounds. *Food Eng Rev.* 2015; 7: 452–490.

[19] Gorbunova N, Bannikova A, Evteev A, Evdokimov I, Kasapis S. Alginate-based encapsulation of extracts from beta Vulgaris cv. beet greens: Stability and controlled release under simulated gastrointestinal conditions. *LWT*. 2018; 93: 442-449.

[20] Desai KG, Jin Park H. Recent Developments in Microencapsulation of Food Ingredients. *Dry Technol*. 2005; 23(7): 1361-1394.

[21] DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal Chem*. 1956; 28(3): 350-356.

[22] Bradford M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem.* 1976; 72(1-2): 248-254.

[23] Jovanović A, Đorđević V, Zdunić G, Šavikin K, Pljevljakušić D, Bugarski B. Ultrasound-assisted extraction of polyphenols from *Thymus serpyllum* and its antioxidant activity. *Hem Ind*. 2016; 70: 391–398.

[24] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free *Radic Biol Med.* 1999; 26(9-10): 1231-1237.

[25] Petrović P, Vunduk J, Klaus A, Carević M, Petković M, Vuković N, Cvetković A, Žižak Ž, Bugarski B. (2019). From mycelium to spores: A whole circle of biological potency of mosaic puffball. *S. Afr. J. Bot.* 2019; 123: 152-160.

[26] CLSI. Performance standards for antimicrobial susceptibility testing: 15th informational supplement. CLSI document M100-S15PA, USA: Wayne. 2005.

[27] Wold CW, Kjeldsen C, Corthay A, Rise F, Christensen BE, Duus JØ, Inngjerdingen KT. Structural characterization of bioactive heteropolysaccharides from the medicinal fungus *Inonotus obliquus* (Chaga). *Carbohyd Polym*. 2018; *185*: 27-40.

[28] Glamočlija J, Ćirić A, Nikolić M, Fernandes Â, Barros L, Calhelha RC, Ferreira I, Soković M, Van Griensven, L. (2015). Chemical characterization and biological activity of Chaga (*Inonotus obliquus*), a medicinal "mushroom". *J Ethnopharmacol*. 2015; 162: 323-332.

[29] Nakajima Y, Sato Y, Konishi T. Antioxidant Small Phenolic Ingredients in *Inonotus obliquus* (persoon) Pilat (Chaga). *Chem. Pharm. Bull.* 2007; 55(8): 1222-1226.

[30] Lee I, Yun B. Styrylpyrone-class compounds from medicinal fungi *Phellinus* and *Inonotus* spp., and their medicinal importance. *J Antibiot*. 2011; 64(5): 349-359.

[31] Zheng W, Miao K, Liu Y, Zhao Y, Zhang M, Pan S, Dai Y. Chemical diversity of biologically active metabolites in the sclerotia of Inonotus obliquus and submerged culture strategies for up-regulating their production. *Appl. Microbiol. Biotechnol*. 2010; 87(4): 1237-1254.

[32] Burmasova MA, Utebaeva AA, Sysoeva EV, Sysoeva MA. Melanins of *Inonotus obliquus*: Bifidogenic and Antioxidant Properties. *Biomolecules*. 2019; *9*(6): 248.

[33] Kukulyanskaya TA, Kurchenko NV, Kurchenko VP, Babitskaya VG. Physicochemical Properties of Melanins Produced by the Sterile Form of *Inonotus obliquus* ("Chagi") in Natural and Cultivated Fungus. *Appl Biochem Micro+.* 2002; 38(1): 58-61

[34] Sysoeva MA, Yumaeva LR, Kuznetsova OY, Ziyatdinova GK, Budnikov GK, Melnikova, NB. Study of the composition of biologically active compounds in chaga meal. Perspectives of application of chaga meal in pharmaceutical industry. *Russ. J. Gen. Chem*. 2012; 82(3): 586-594.

[35] Niu H, Song D, Mu H, Zhang W, Sun F, Duan J. Investigation of three lignin complexes with antioxidant and immunological capacities from *Inonotus obliquus*. *Int. J. Biol. Macromol.* 2016; 86: 587-593.

[36] Ichimura T, Watanabe O, Maruyama S. Inhibition of HIV-1 Protease by Water-Soluble Lignin-Like Substance from an Edible Mushroom, *Fuscoporia obliqua*. *Biosci. Biotechnol. Biochem*. 1998; 62(3): 575-577.

[37] Géry A, Dubreule C, André V, Rioult J, Bouchart V, Heutte N, De Pécoulas P, Krivomaz T, Garon D. Chaga (*Inonotus obliquus*), a Future Potential Medicinal Fungus in Oncology? A Chemical Study and a Comparison of the Cytotoxicity Against Human Lung Adenocarcinoma Cells (A549) and Human Bronchial Epithelial Cells (BEAS-2B). *Integr. Cancer Ther*. 2018; 17(3): 832-843.

[38] Islam T, Yu X, Xu B. Phenolic profiles, antioxidant capacities and metal chelating ability of edible mushrooms commonly consumed in China. *LWT*. 2016; 72: 423-431.

[39] Phaniendra A, Jestadi DB, Periyasamy L. Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. *Indian J Clin Biochem.* 2014; 30(1): 11-26.

[40] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018; *68*(6): 394-424.

[41] Wang Z. Dietary polyphenols and colorectal cancer risk: The Fukuoka colorectal cancer study. *World J Gastroenterol*. 2013: 19(17): 2683-2690

[42] Bahrami A, Jafari S, Rafiei P, Beigrezaei S, Sadeghi A, Hekmatdoost A, Rashidkhani B, Hejazi E. Dietary intake of polyphenols and risk of colorectal cancer and adenoma–A case-control study from Iran. *Complement Ther Med*. 2019; 45: 269-274.

[43] Alam MN, Almoyad M, Huq F. Polyphenols in Colorectal Cancer: Current State of Knowledge including Clinical Trials and Molecular Mechanism of Action. *Biomed Res Int*. 2018; *2018:* 1-29.

[44] Blagodatski A, Yatsunskaya M, Mikhailova V, Tiasto V, Kagansky A, Katanaev VL. Medicinal mushrooms as an attractive new source of natural compounds for future cancer therapy. Oncotarget. 2018; 9(49): 29259-29274

[45] Stojanović R, Belščak-Cvitanović A, Manojlović V, Komes D, Nedović V, Bugarski B. Encapsulation of thyme (*Thymus serpyllum* L.) aqueous extract in calcium-alginate beads. *J Sci Food Agr*. 2012; 92: 685–696.

[46] González E, Gómez-Caravaca AM, Giménez B, Cebrián R, Maqueda M, Martínez-Férez A, Segura-Carretero A, Robert P. Evolution of the phenolic compounds profile of olive leaf extract encapsulated by spray-drying during in vitro gastrointestinal digestion. *Food Chem*. 2019; 279: 40–48.

[47] Istenič K, Balanč B, Djordjević V, Bele M, Nedović V, Bugarski B, Poklar-Urlih N. Encapsulation of resveratrol into calcium-alginate submicron particles. *J. Food Eng.* 2015; 167: 196–203.

[48] Ćujić N, Trifković K, Bugarski B, Ibrić S, Pljevljakušić D, Šavikin K. Chokeberry (*Aronia melanocarpa* L.) extract loaded in alginate and alginate/inulin system. *Ind Crop Prod.* 2016; 86: 120–131.

SAŽETAK

Imobilizacija ekstrakta čage u alginatne čestice u cilju modifikovanog oslobađanja: spoj jednostavnosti i efikasnosti

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(Naučni rad)

Čaga (*Inonotus obliquus*) je parazitska gljiva koja se tradicionalno koristi u Rusiji i drugim severnoevropskim zemljama u tretmanu raka, bolesti gastrointestinalnog trakta i jetre, kao i drugih poremećaja. Poslednjih godina je predmet intenzivnog proučavanja, koja su potvrdila mnoga blagotvorna dejstva čage na ljudski organizam. Da bi se dobio proizvod koji bi omogućio modifikovano i produženo oslobađanje aktivnih sastojaka čage, vreli vodeni ekstrakt ove gljive imobilisan je u alginat kao nosač. Ekstrakt, koji se sastojao najviše od ugljenih hidrata (~57%), sadržao je i veliku količinu antioksidantnih/fenolnih komponenti (130 mg ekvivalenata galne kiseline po g suvog ekstrakta) i pokazao značajnu antiradikalsku aktivnost. Ekstrakt je delovao i antibakterijski, inhibirajući rast testiranih sojeva pri koncentracijama od 1.25-20 mg/mL. Efikasnost imobilizacijebila je oko 80%, a sistem je pokazao pH-zavisno oslobađanje ekstrakta. Skoro da i nije bilo otpuštanja komponenti ekstrakta pri pH 1.75 (želudačna pH), dok je s porastom pH došlo i do boljeg otpuštanja, koje je dostiglo maksimum od oko 43% oslobođenog ekstrakta pri pH 8.5, nakon 90 min. Ovakav proizvod mogao bi da nađe primenu kao dijetetski suplement, adjuvans u terapji gastrointestinalnih bolesti ili kao aditiv u hrani.

Ključne reči: gljive, prirodni proizvod, antioksidativno dejstvo, antimikrobno dejstvo, inkapsulacija