

1 **Microwave-assisted synthesis and antitumor activity of the supramolecular**
2 **complexes of betulin diacetate with arabinogalactan**

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Abstract

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29 In this work, a water-soluble supramolecular complex was synthesized in an aqueous
30 suspension of betulin diacetate (BDA) and arabinogalactan (AG) upon microwave heating.
31 Microwave heating allows reducing the time required for the complex formation, compared
32 with conventional heating in a water bath. The specific effect of microwave irradiation on the
33 initial reagents and preparation of a supramolecular complex was studied. In contrast to
34 conventional heating, under microwave heating AG macromolecules may break into roughly
35 equal fragments when the temperature increases up to 100 °C. A change in the surface
36 morphology of BDA crystals under microwave heating of the suspension suggests that
37 microwave irradiation facilitates the dissolution of BDA in water. It has been shown that the
38 use of dimethylsulfoxide as a reaction medium for microwave heating led to a decrease in
39 BDA content in the product due to the inclusion of DMSO into AG macromolecules. The
40 BDA-AG complex was isolated from the microwave-heated aqueous solution, after water
41 evaporation, as a thin amorphous film, which exhibited antitumor activity against Ehrlich
42 ascites carcinoma cells and can be a promising material for pharmacological applications.

43
44 **Keywords:** Betulin diacetate; Arabinogalactan; Complexes; Microwave synthesis; Films;
45 Antitumor activity.

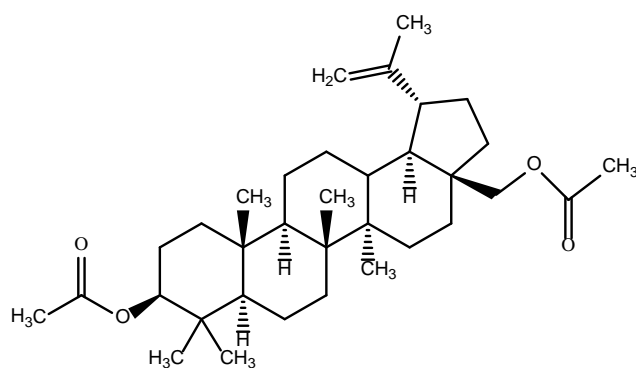
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Introduction

49 Betulin diacetate (BDA), 3 β ,28-diacetoxylup-20(29)-ene, (Fig. 1), an ester of acetic
50 acid and betulin, exhibits versatile biological activities (Lu 2013). Nevertheless, the poor
51 solubility of BDA in water greatly hampers its bioavailability and limits its application.

52 Previously (Shakhtshneider et al. 2013), we reported on the mechanochemical
53 preparation of the composites of BDA with water-soluble polysaccharide arabinogalactan
54 (AG) (Fig. 2) possessing a higher solubility due to complex formation. The BDA-AG
55 complex was prepared also as a thin film isolated from an aqueous solution by water
56 evaporation. The BDA-AG composite films exhibited anti-cancer activity against lung
57 adenocarcinoma A549 cells, which was significantly higher than the activity of both pure
58 BDA and its physical and ball-milled mixtures with AG (Shakhtshneider et al. 2016).

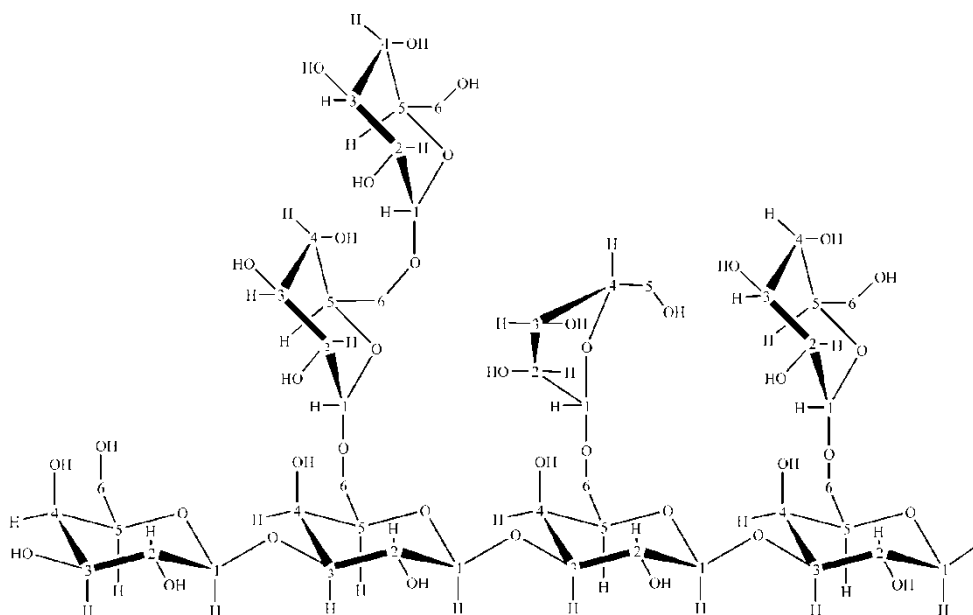
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61 **Fig. 1** Molecular structure of betulin diacetate

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63

64 **Fig. 2** Fragment of arabinogalactan molecular structure

65

66 To prepare the BDA-AG complex in solution, heating in a water bath for some hours
 67 was required (Shakhtshneider et al. 2016). The purpose of this work was to improve the
 68 method of BDA-AG complex preparation, by decreasing the process time and enhancing the
 69 yield of the product.

70 In the recent decades, high-speed synthesis with microwaves has attracted a
 71 considerable amount of attention particularly in organic synthesis, drug discovery,
 72 supramolecular chemistry, and carbohydrate chemistry (Singh et al. 2015; Kappe 2004;
 73 Bandyopadhyay et al. 2015; Alexandre et al. 2003; Doehler et al. 2015; Pistarà et al. 2014). In
 74 contrast to conductive heating, microwave irradiation produces efficient internal heating by
 75 the direct coupling of microwave energy with the molecules in the reaction mixture, leading
 76 to shorter reaction time, higher product yield, cleaner reaction profiles.

77 In this study, the possibility to synthesize the BDA-AG supramolecular complex
78 through controlled microwave heating was evaluated. Various regimes of the microwave
79 treatment of reactive mixtures in the presence of a solvent were used to increase the yield of
80 the complex.

81 Experimental

82
83 BDA was obtained in a one-step synthesis directly from the birch bark, without a
84 separate stage of betulin isolation (Kuznetsova et al. 2008). The product was purified, leaving
85 final impurities below 1.4 wt %. Arabinogalactan (Mw ~16,000) was isolated from larch
86 (*Larix sibirica* Ledeb.) wood using an established method (Kuznetsova et al. 2006). High-
87 purity dimethylsulfoxide (Soyuzkhimprom Ltd, Russia) was dried with calcined Na₂SO₄.

88 Microwave irradiation experiments were performed using a dedicated single-mode
89 microwave reactor (Discover-S-Class, CEM, USA) with 300 W maximum magnetron output
90 power allowing sealed vessel processing up to 300 °C and 20 bar of pressure in combination
91 with an efficient magnetic stirring system. The temperature and the excess pressure in the
92 microwave vessel were monitored during the experiment. Sealed-vessel microwave
93 technology was employed; water and dimethylsulfoxide (DMSO) were used as the solvents.
94 The following parameters of the microwave-assisted reaction were varied: input power,
95 reaction temperature, and time of microwave treatment. Each experiment was repeated
96 triplicate.

97 For BDA-AG complex preparation, a mixture (0.5 g) of dry initial substances with
98 BDA : AG ratio of 1 : 9 (w/w) was put into a microwave vessel, and then 4 mL of distilled
99 water (or DMSO) was added. The suspension was subjected to microwave irradiation with
100 simultaneous stirring by the magnetic stirring system. After cooling, the suspension was
101 filtered through a 0.22 µm filter to remove undissolved BDA precipitate, and the filtrates were
102 evaporated under reduced pressure at 35-40 °C. A thin flexible film remained at the bottom of
103 the flask after evaporation.

104 To compare with the microwave-assisted synthesis, a mixture (0.5 g) of BDA and AG
105 (1 : 9, w/w) was placed into the microwave vessel with water (4 mL) and stirred at 70 °C
106 using a glycerin bath. Stirring time was 20 minutes. After that, the solution was filtered, and
107 the filtrate was subjected to solvent evaporation under vacuum to obtain the film.

108 The content of BDA in the films was determined by means of high-performance liquid
109 chromatography (HPLC). Firstly, BDA was extracted by chloroform; then the CHCl₃ extracts
110 were evaporated, and the solid residuals were dissolved in ethanol. The HPLC analysis of

111 ethanol solutions was performed using a Millichrom A-02 chromatograph (Econova, Russia)
112 (35 °C, ProntoSIL 120-5-C18 AQ, 2.0 x 75 mm, H₂O (A) – CH₃CN (B), 80-100-100 % B,
113 100 µL/min).

114 Molecular weight distribution of the polymer was determined by gel-permeation
115 chromatography (GPC) on an Agilent 1200 chromatograph with a 1260 Infinity refractive-
116 index detector (30 °C, PL aquagel-OH 40, 300 x 7.5 mm, 0.1 M LiNO₃, 1 mL/min).

117 To prepare the water suspension containing BDA crystals of rather good quality, a
118 saturated solution of BDA in ethanol was added dropwise into water. After BDA
119 crystallization, ethanol was removed by evaporation under low pressure at a temperature of
120 35 °C. The particle size distribution in the suspension was measured with a Microsizer 201A
121 (VA Instalt Company Ltd, Russia) laser particle analyzer.

122 Atomic force microscopy (AFM) studies were carried out in the tapping mode using
123 an INTEGRA scanning probe nanolaboratory (NT-MDT, Russia). To study the particle
124 surface morphology by means of AFM, the suspension diluted with water was deposited on a
125 freshly cleaved mica surface (3 × 3 mm). The measurements were carried out in a semi-
126 contact regime using NSG01_DLC cantilevers. The scanning area was 20 × 20 µm.

127 The ¹³C{H} NMR spectra were recorded with a Bruker Avance III 500 spectrometer
128 (working frequencies 500.13 (¹H) and 125.76 MHz (¹³C)). The samples were dissolved in
129 deuterated water. An external sample of acetone/D₂O was used as a standard for ¹³C{H} data.

130 Ball-milling was carried out in a SPEX 8000 mixer mill (CertiPrep Inc., USA) in a
131 stainless steel vial (60 mL) with steel balls (6 mm in diameter, total 30 g) for 15 min.

132 Antitumor activity of the BDA-AG composite films was determined by estimating the
133 viability of Ehrlich carcinoma cells after 24 hours of incubation at 37 °C, 5% CO₂ with BDA-
134 AG complex at the final concentration of 0.5 mg·mL⁻¹. Viability was estimated using trypan
135 blue in accordance with the standard manufactures protocol. Each sample was examined in 5
136 experiments, and statistical processing was performed.

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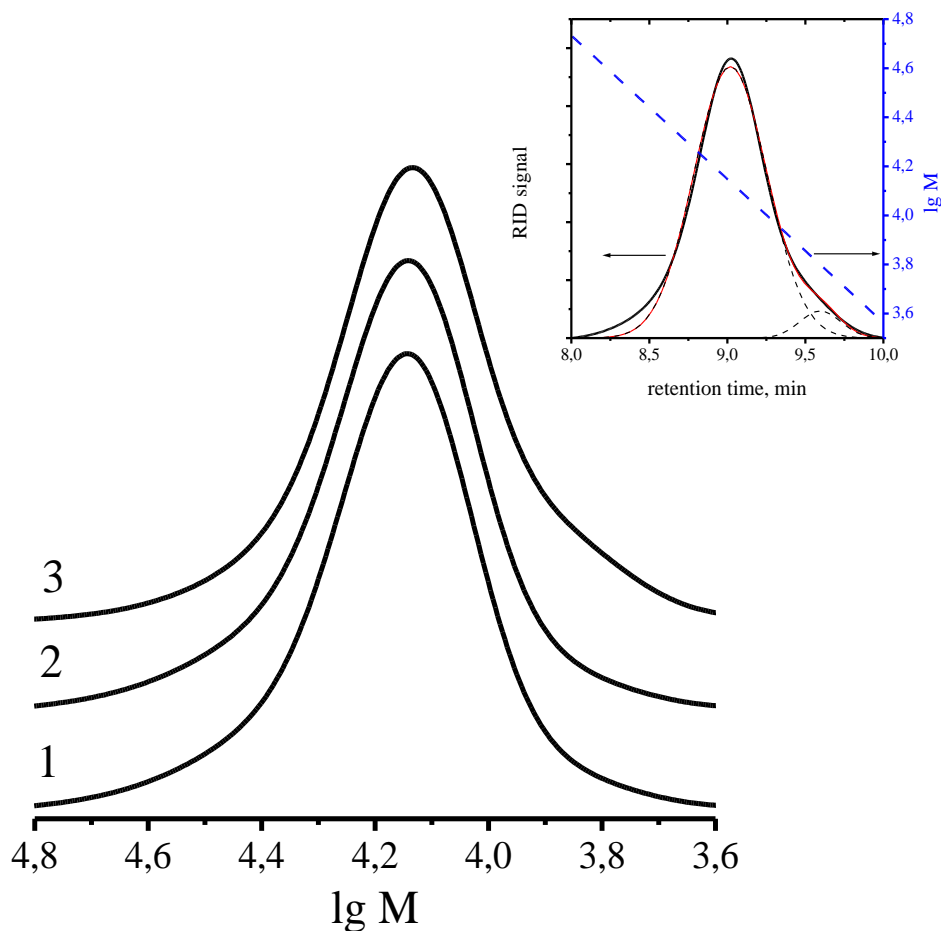
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138 Results and discussion

139

140 Initially we studied the effect of microwave irradiation on the structure and properties
141 of AG alone. GPC investigations of AG after microwave heating revealed that under
142 microwave irradiation at 70 °C (AG1 sample), the chains of AG were practically unbroken,
143 and the molecular weights, M_w and M_n, as well as polydispersity were approximately equal
144 (Fig. 3 and Online Resource 1). Nevertheless, microwave heating at 100 °C, with the total

145 energy being more than 7 KJ (AG2 sample), led to a slight shift of the $\lg M$ 4.2 ($M_w \sim 16,000$)
 146 peak and to the appearance of a new peak corresponding to a lower molecular mass
 147 ($M_w \sim 8,000$). It can be suggested that under these conditions, partial destruction of the
 148 polysaccharide macromolecules occurs. It should be noted that under heating AG aqueous
 149 solution at 100 °C in an oil bath, there were no changes in the molecular weight of the
 150 polymer. The breaking of AG macromolecules into roughly equal fragments was observed
 151 earlier during the mechanical treatment of AG in a planetary mill (Dushkin et al. 2012) and is
 152 probably connected with the structure of polymer molecules and chain breakdown mechanism
 153 (Grassie et al. 1985). In this case, it can be concluded that the microwave heating experiments
 154 should be conducted at temperatures below 100 °C to avoid the destruction of the AG
 155 macromolecules.



156

157 **Fig. 3** Gel-permeation chromatograms of the starting AG sample (1) and after microwave
 158 heating: AG1 (2) and AG2 (3) samples. At the inset, the decomposition of the curve 3 into the
 159 components is presented.

160

161 **Suppl. Table 1** Molecular weights of AG before and after microwave (MW) heating (input
 162 MW power, 200 W) AG aqueous solution.

| Sample | Number average molecular weight, M_n | Weight average molecular weight, M_w | M_w/M_n | MW irradiation conditions | | | |
|------------------------------|--|--|-----------|---------------------------|---------------------|---------|-----------------------|
| | | | | $T_{initial}, ^\circ C$ | $T_{max}, ^\circ C$ | Time, s | Total energy, J |
| Starting AG | 13397 | 16006 | 1,19 | | | | |
| MW heated AG (Sample AG1) | 13311 | 15883 | 1,19 | 70 | 89 | 600 | 3610 |
| MW heated AG (Sample AG2) | 12189 | 14528 | 1,19 | 100 | 102 | 1200 | 7881 |

163

164 As for the microwave heating of BDA alone, the HPLC study did not show any
 165 changes in BDA structure after microwave heating.

166 In Table 1, BDA content in the obtained BDA-AG composite films is presented. One
 167 can see that an increase in the duration of MW treatment did not lead to an increase in the
 168 product yield (samples Nos. 1 and 2, and Nos. 3 and 4). At the same time, as the input
 169 microwave power increased up to 200 W (sample No. 3), BDA content has increased.
 170 Nevertheless, an increase in the temperature up to 100 °C (sample No. 5) resulted in a
 171 decrease in BDA content.

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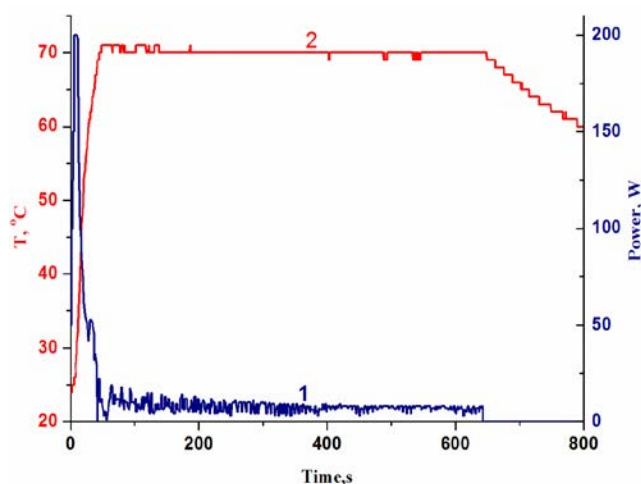
173 **Table 1** BDA content in the films prepared from the microwave heated BDA-AG (1 : 9, w/w)
 174 suspensions depending on the microwave irradiation conditions

| Sample No. | Microwave power, | Reaction | Time of treatment, | BDA content, wt % |
|------------|------------------|-----------------|--------------------|----------------------|
| | W | temperature, °C | min | |
| 1 | 70 | 70 | 10 | 1.9 ± 0.1 |
| 2 | 70 | 70 | 30 | 2.1 ± 0.1 |
| 3 | 200 | 70 | 10 | 2.6 ± 0.1 |
| 4 | 200 | 70 | 20 | 2.2 ± 0.1 |
| 5 | 200 | 100 | 20 | 1.9 ± 0.2 |
| 6 | 200 | 70 | 10 | 1.6 ± 0.2 |

175

176

177



178

179 **Suppl. Fig. 1** Power profile (1) and temperature (2) of the microwave-assisted synthesis of
180 BDA-AG complex (sample No. 3).

181

182 The temperature and power curves of MW synthesis are depicted in Online
183 Resource 2. Microwave experiments were carried out in the "dynamic" mode, in which the
184 microwave energy level varied depending on the achievement and maintenance of the set
185 temperature. The time necessary to reach the required temperature depends on the dielectric
186 properties of the substances in the reaction mixture and can be varied, in part, due to the
187 heterogeneity of the mixture. In our experiments, the temperature was risen quickly (for 20-50
188 seconds) to the set value. It can be suggested that the synthesis of the BDA-AG complex
189 appears to occur during relatively short exposure times (maybe even less than 10 min), and
190 continuous MW heating, especially under severe process conditions, can lead to partial
191 degradation of the complex.

192

193 It could be expected that mechanical activation will cause an increase in the reactivity
194 of the reactive mixture in the microwave-assisted synthesis. To test this assumption, a ball-
195 milled 1 : 9 (w/w) BDA-AG mixture was subjected to microwave heating (sample No. 6).
196 Nevertheless, proceeding ball-milling of BDA-AG mixtures had an adverse effect on the
197 formation of the BDA-AG complex, resulting in a decrease in the product yield. It is likely
198 that under milling, BDA was dispersed, and it covered the surface of AG particles preventing
199 their subsequent dissolution. The presence of AG in the precipitate after the filtration of the
200 microwave-irradiated suspension confirmed this hypothesis.

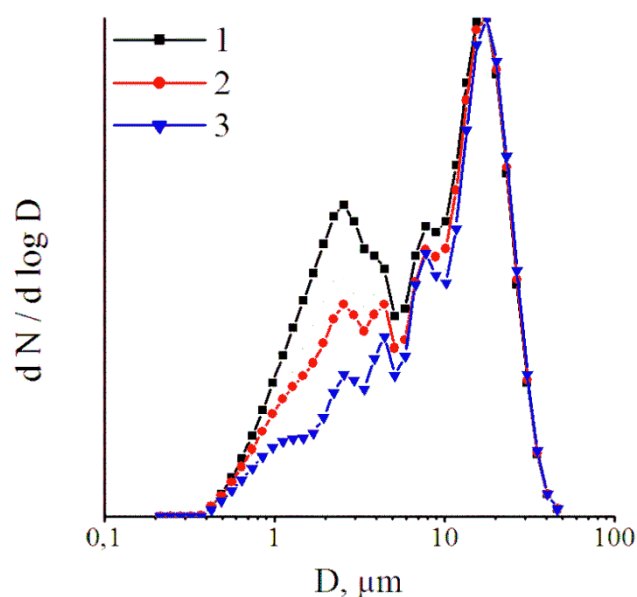
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202 To elucidate the specific role of microwave irradiation in the preparation of a
supramolecular complex, the BDA-AG complex was obtained by a conventional method,
applying (as far as possible) identical conditions as those for the microwave-assisted

203 synthesis. The BDA content in these films was equal to about 0.7 wt %, which is significantly
 204 less than for the samples prepared under microwave heating.

205 It is known that the size and morphology of particles play an important role in solid-
 206 phase synthesis (Butyagin 2000). The same is true for slurry processes when a liquid not
 207 dissolving at least one of the reactants is used. On the other hand, it is known that microwave
 208 heating can affect the dissolution behavior of the substances (Olubambi et al. 2007; Wang et
 209 al. 2013). Therefore, we studied the change of BDA particle size distribution and their
 210 morphology under microwave irradiation conditions. For these experiments, the suspension of
 211 BDA in water containing BDA microcrystals of rather good quality was prepared. In Figure 4,
 212 the size distributions of BDA particles in water suspensions before and after microwave
 213 heating are presented. In the initial suspension, the size distribution is bimodal with the
 214 maximums near $\sim 3 \mu\text{m}$ and $\sim 20 \mu\text{m}$. After microwave irradiation, the intensity of the first
 215 peak decreased, and the second one increased. This suggested that small BDA particles were
 216 dissolved under the conditions of microwave irradiation, and more stable aggregates of the
 217 particles were formed.

218



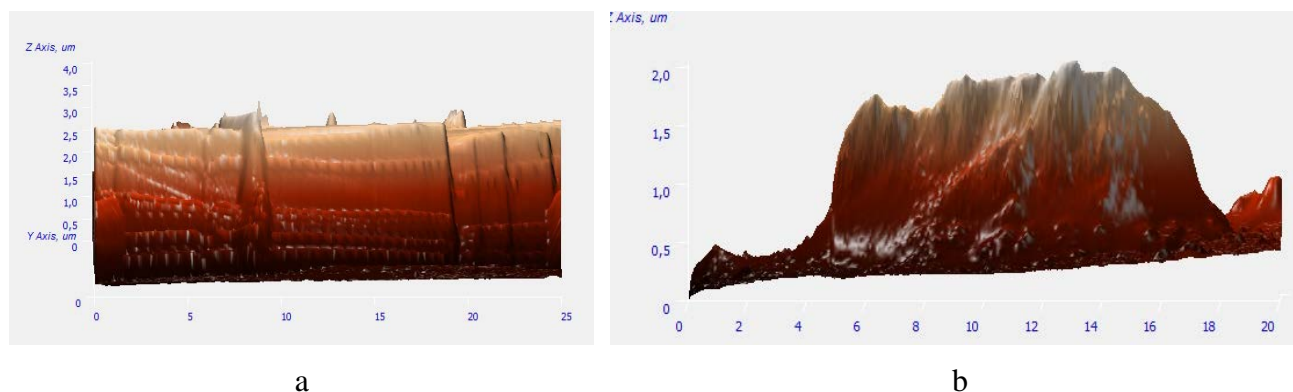
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220 **Fig. 4** Size distributions of BDA particles in water suspensions before (1) and after (2, 3)
 221 microwave irradiation under different conditions: 2 – 70 °C, 10 min, 3 – 100 °C, 20 min (input
 222 MW power, 200 W). Curves are normalized to the maximal number of particles.

223

224 Figure 5 shows the AFM images of BDA particles in water suspensions before and
 225 after microwave irradiation. In the initial suspension, the particles looked as the rod-shaped
 226 crystals combined in aggregates. One can see the well-defined edges and the steps at the

227 surface of the crystals. The surface morphology did not change even after aging the
 228 suspension for one day. Nevertheless, after microwave irradiation for 10 min, the particles
 229 acquired an irregular shape. In contrast to the initial suspension, there were no flat surface
 230 edges and growth steps at the surface of the particles after microwave irradiation. It seems
 231 that under microwave irradiation, BDA particles were dissolved in water rather rapidly, which
 232 led to particle surface smoothing.



233

234 **Fig. 5** Tapping-mode AFM images of BDA particles in water suspensions displaying the
 235 height of AFM signal: a – before MW irradiation, b – after MW irradiation.

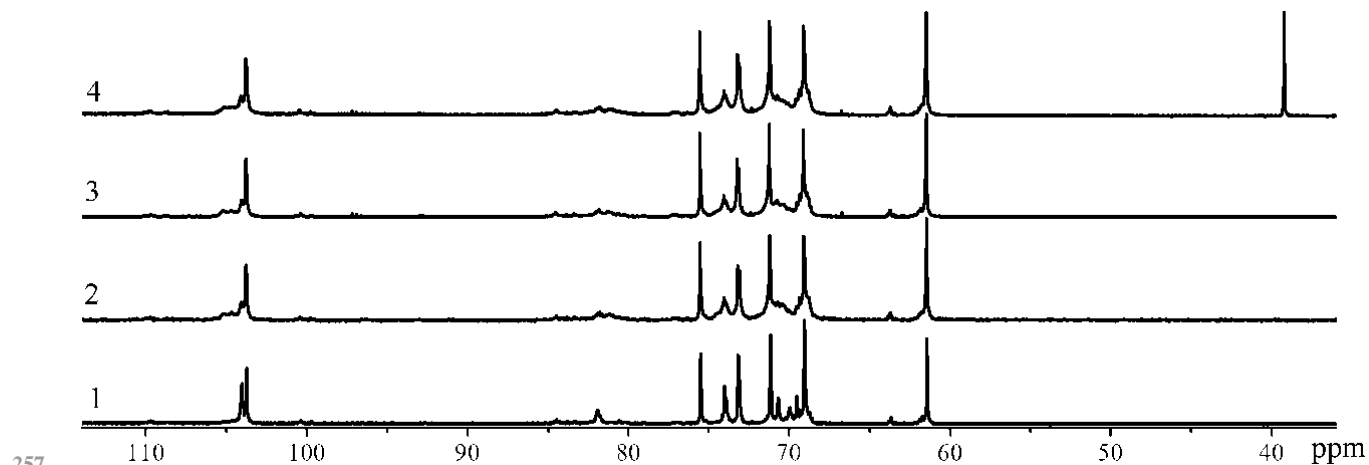
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237 It is known that a reaction medium with a high loss factor ($\tan\delta$) is required for
 238 efficient absorption and, consequently, for rapid heating. With its comparatively high $\tan\delta$
 239 value of 0.123 (Kappe 2004), water is a very useful solvent for microwave-mediated
 240 synthesis. Besides, water as a readily available, nontoxic, and nonflammable substance has
 241 clear advantages as a solvent for use in organic synthesis. Nevertheless, it was interesting to
 242 test other reaction media for the synthesis. DMSO ($\tan\delta$ 0.825) is one of the solvents that can
 243 be classified as high microwave absorbing (Kappe 2004). Moreover, both reactants, BDA and
 244 AG, are soluble in this solvent. It can be suggested that the reaction in the solution will
 245 proceed more easily and without overheating. Under the same conditions as those used for
 246 experiments in water (200 W, 70 °C, 10 min), the product was obtained with BDA content
 247 equal to 1.6 %, which was significantly less than in the experiments in water.

248 Figure 6 shows $^{13}\text{C}\{\text{H}\}$ NMR spectra of the aqueous solutions of initial AG and BDA-
 249 AG complex prepared as a film after conventional and microwave heating in water and
 250 DMSO. One can see that in the case of MW heating of DMSO solutions, the obtained
 251 complex contained a lot of DMSO molecules in the structure. Besides, a slight broadening
 252 and shift of the AG C6 signal was observed. The ratio of the areas of DMSO (39 ppm) and C6

253 (61.3 ppm) signals was estimated as 1.2. This means that the composite film may contain up
254 to 20 wt % of DMSO. This can be the possible reason of a decrease in BDA content in the
255 product.

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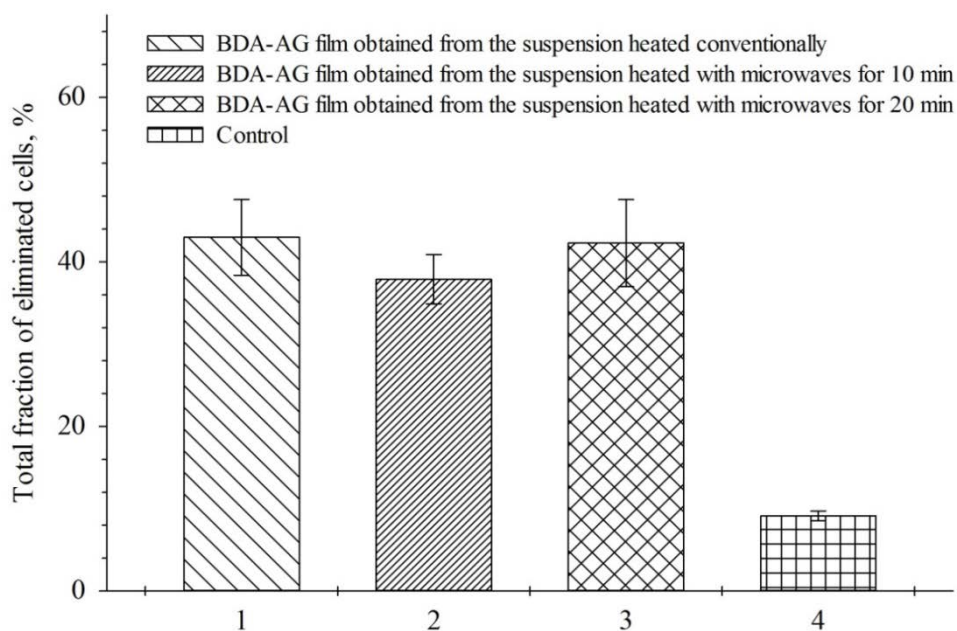
259 **Fig. 6** Fragments of the $^{13}\text{C}\{\text{H}\}$ NMR spectra of the D_2O solutions of initial AG (1) and
260 BDA-AG complex prepared as a film after conventional heating (2) and microwave heating of
261 water suspension (3) or DMSO solution (4).

262

263 In the case of the films obtained by evaporation of the microwave heated water
264 filtrates, in comparison with AG, similar changes in NMR spectra were observed as for
265 conventional heating (Mikhailenko et al. 2016) suggesting that the same complex was formed
266 under microwave irradiation conditions. This gave us the reason to believe that the complex
267 obtained by microwave treatment will also possess pharmacological activity, similarly to the
268 complex obtained by traditional way.

269 We studied the antitumor activity of the BDA-AG complex, prepared as the films
270 using conventional and microwave heating in water, against Ehrlich ascites carcinoma (EAC)
271 cells. *In vitro* experiments showed (Fig. 7) that the composite films obtained from
272 suspensions heated with microwaves exhibited antitumor activity against EAC cells, which
273 was not less than that of the films obtained by the conventional procedure.

274



275

276 **Fig. 7** Antitumor activity (against EAC cells) of the BDA-AG complexes prepared as the
 277 films from the suspensions heated conventionally (1) and heated with microwaves for 10 (2)
 278 and 20 (3) min (input MW power, 200 W) in comparison with control (4).

279

280

Conclusions

281

282 The obtained results demonstrate that microwave heating is a highly efficient
 283 technique to prepare the supramolecular complex of betulin diacetate (isolated from birch
 284 bark) and natural polysaccharide arabinogalactan. Under microwave irradiation conditions,
 285 the reaction time was reduced from several hours to a few minutes in comparison with
 286 traditional procedure. The change in the size and surface morphology of BDA crystals under
 287 microwave heating was observed, suggesting that microwave impact facilitate BDA
 288 dissolution in water, that could contribute to high-speed synthesis of the supramolecular
 289 complex. The BDA-AG complex isolated from the microwave heated aqueous solution as a
 290 thin film exhibited antitumor activity against Ehrlich ascites carcinoma cells, which was not
 291 less than that of the films obtained by the conventional way.

292

293

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