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

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Abstract

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

Keywords: Betulin diacetate; Arabinogalactan; Complexes; Microwave synthesis; Films; Antitumor activity.

Introduction

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

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

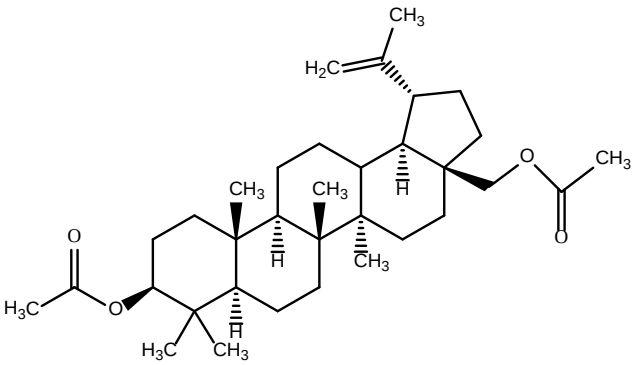


Fig. 1 Molecular structure of betulin diacetate

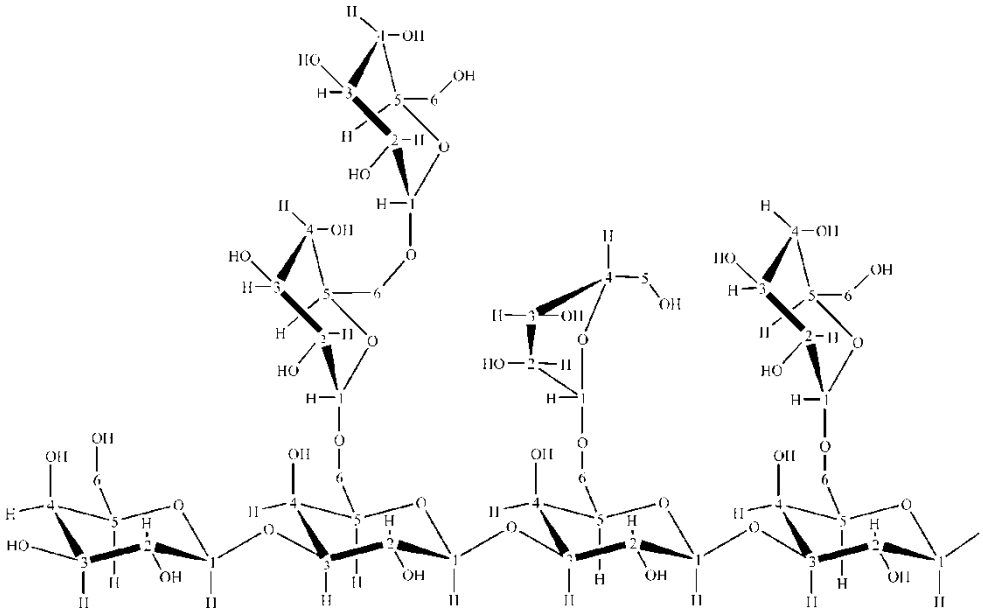


Fig. 2 Fragment of arabinogalactan molecular structure

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

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

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

Experimental

BDA was obtained in a one-step synthesis directly from the birch bark, without a separate stage of betulin isolation (Kuznetsova et al. 2008). The product was purified, leaving final impurities below 1.4 wt %. Arabinogalactan (Mw ~16,000) was isolated from larch (Larix sibirica Ledeb.) wood using an established method (Kuznetsova et al. 2006). High-purity dimethylsulfoxide (Soyuzkhimprom Ltd, Russia) was dried with calcined Na_2SO_4.

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

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

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

The content of BDA in the films was determined by means of high-performance liquid chromatography (HPLC). Firstly, BDA was extracted by chloroform; then the $CHCl_3$ extracts were evaporated, and the solid residuals were dissolved in ethanol. The HPLC analysis of

ethanol solutions was performed using a Millichrom A-02 chromatograph (Econova, Russia) (35 ^{o}C, ProntoSIL 120-5-C18 AQ, 2.0 x 75 mm, H_2O (A) – CH_3CN (B), 80-100-100 % B, 100 μL/min).

Molecular weight distribution of the polymer was determined by gel-permeation chromatography (GPC) on an Agilent 1200 chromatograph with a 1260 Infinity refractive-index detector (30 ^{o}C, PL aquagel-OH 40, 300 x 7.5 mm, 0.1 M $LiNO_3$, 1 mL/min).

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

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

The $^{13}C\{H\}$ NMR spectra were recorded with a Bruker Avance III 500 spectrometer (working frequencies 500.13 (^{1}H) and 125.76 MHz (^{13}C)). The samples were dissolved in deuterated water. An external sample of acetone/D_2O was used as a standard for $^{13}C\{H\}$ data.

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

Antitumor activity of the BDA-AG composite films was determined by estimating the viability of Ehrlich carcinoma cells after 24 hours of incubation at 37 ^{o}C, 5% CO_2 with BDA-AG complex at the final concentration of 0.5 mg·mL^{-1}. Viability was estimated using trypan blue in accordance with the standard manufactures protocol. Each sample was examined in 5 experiments, and statistical processing was performed.

Results and discussion

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

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

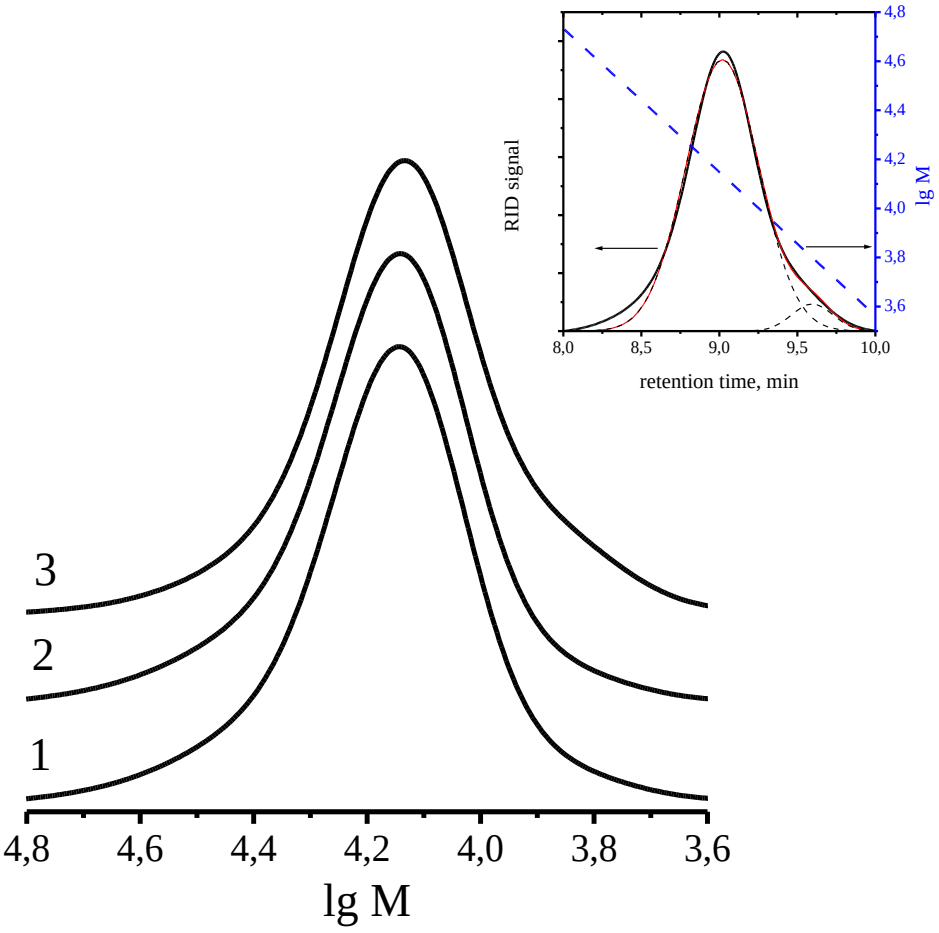


Fig. 3 Gel-permeation chromatograms of the starting AG sample (1) and after microwave heating: AG1 (2) and AG2 (3) samples. At the inset, the decomposition of the curve 3 into the components is presented.

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

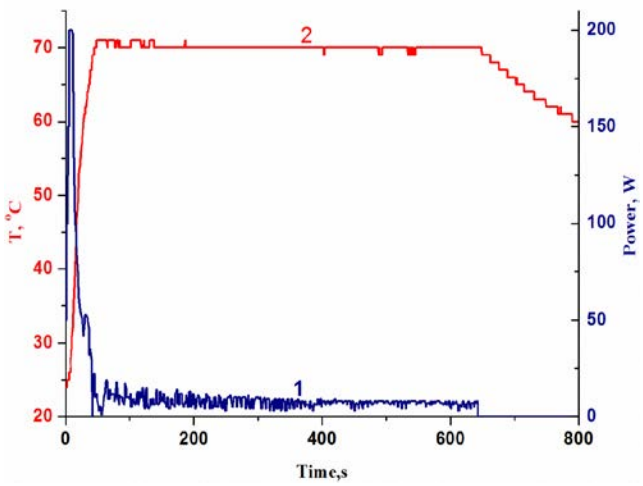
Sample	Number average molecular weight, M_n	Weight average molecular weight, M_w	M_w/ M_n	MW irradiation conditions			
				$T_{initial}$, °C	T_{max}, °C	Time, s	Total energy, J
Starting AG	13397	16006	1,19				
MW heated AG (Sample AG1)	13311	15883	1,19	70	89	600	3610
MW heated AG (Sample AG2)	12189	14528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

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

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

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

Sample No.	Microwave power, W	Reaction temperature, °C	Time of treatment, min	BDA content, wt %
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



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

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

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

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

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

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

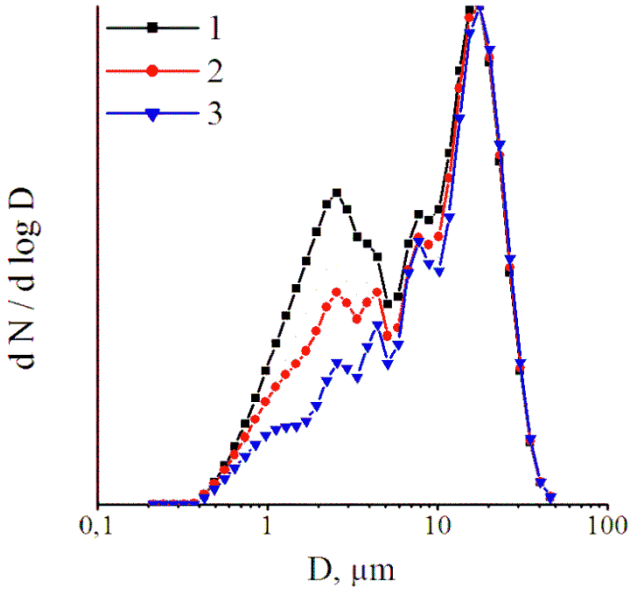
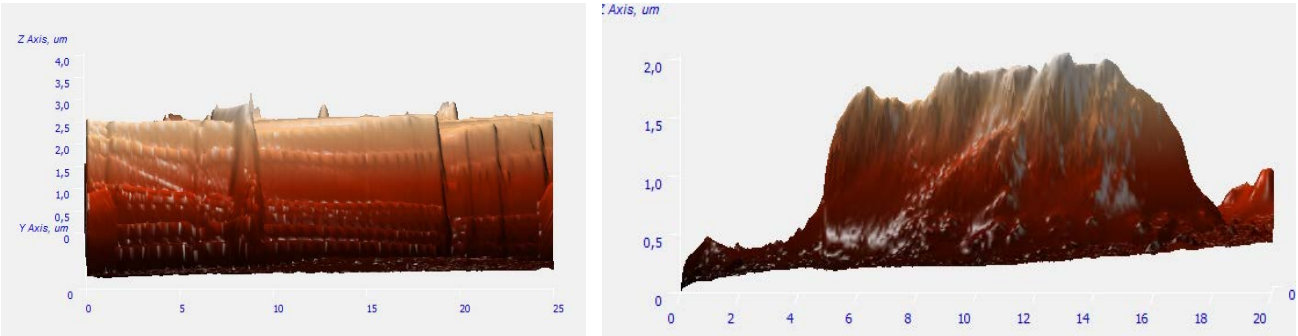


Fig. 4 Size distributions of BDA particles in water suspensions before (1) and after (2, 3) microwave irradiation under different conditions: 2 – 70 °C, 10 min, 3 – 100 °C, 20 min (input MW power, 200 W). Curves are normalized to the maximal number of particles.

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

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



a b

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

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

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

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

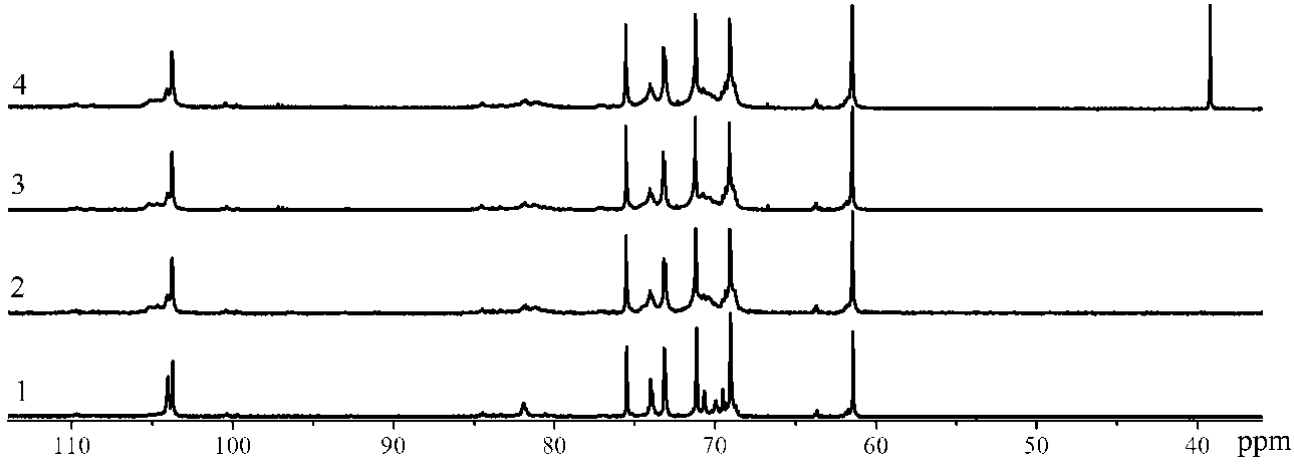


Fig. 6 Fragments of the $^{13}C\{H\}$ NMR spectra of the D_2O solutions of initial AG (1) and BDA-AG complex prepared as a film after conventional heating (2) and microwave heating of water suspension (3) or DMSO solution (4).

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

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

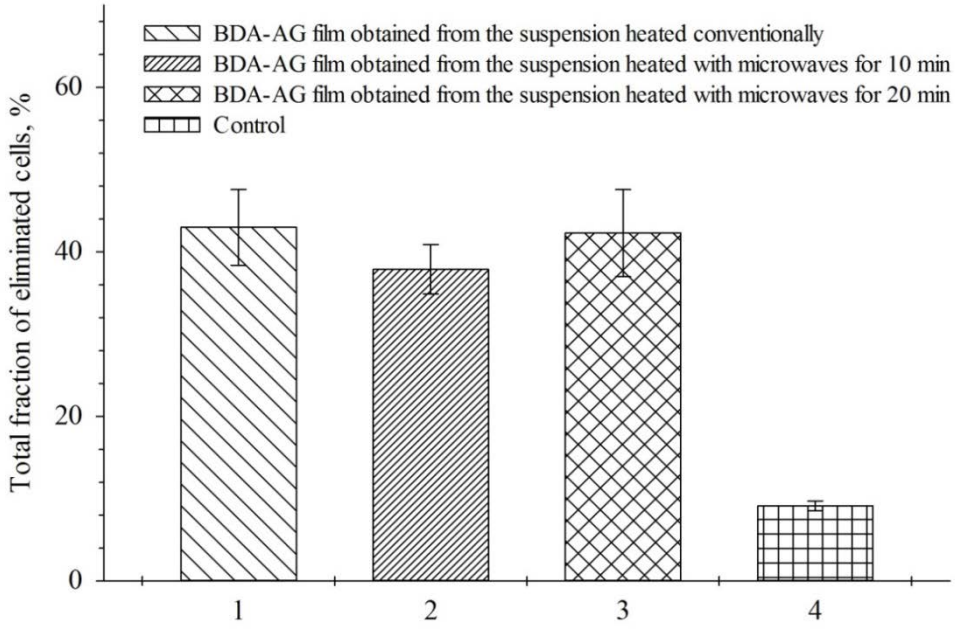


Fig. 7 Antitumor activity (against EAC cells) of the BDA-AG complexes prepared as the films from the suspensions heated conventionally (1) and heated with microwaves for 10 (2) and 20 (3) min (input MW power, 200 W) in comparison with control (4).

Conclusions

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

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