ATIVIDADE ESPECÍFICA DO COMPLEXO BIOLÓGICO-ATIVO EM EXTRATOS DE DROGAS LÍQUIDOS DE ERVAS, ESTUDADAS EM DIFERENTES CONDIÇÕES DE SECAGEM

SPECIFIC ACTIVITY OF BIOLOGICALLY ACTIVE COMPLEX IN LIQUID HERBAL DRUG EXTRACTS, STUDIED UNDER DIFFERENT DRYING CONDITIONS

СПЕЦИФИЧЕСКАЯ АКТИВНОСТЬ КОМПЛЕКСА БИОЛОГИЧЕСКИ АКТИВНЫХ ВЕЩЕСТВ ЖИДКОГО РАСТИТЕЛЬНОГО ЭКСТРАКТА ПРИ РАЗНЫХ РЕЖИМАХ ЕГО СУШКИ

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RESUMO

O objetivo deste trabalho foi avaliar a influência de diferentes métodos de remoção de solventes de extração de extratos líquidos de fitoterápicos sobre sua atividade farmacológica (imunomodulação, adaptogene, antioxidante e antibacteriana). Os autores utilizaram sistemas enzimáticos biotestes *in vitro* baseados em enzimas-chave do sistema de proteção antioxidante - glutathione redutase e catalase, bem como na enzima limitante da fase terminal da fagocitose - NADPH-oxidase, que permitem identificar adaptogênicos, antioxidantes, antibacterianos e antimicrobianos. atividade imunomoduladora da BAS e estimar possíveis alterações na bioatividade dos objetos estudados. Os resultados obtidos mostraram que a temperatura de secagem dos extratos de fitoterápicos combinados líquidos não influencia significativamente na bioatividade do complexo BAS. Amostras extraídas, secas a diferentes temperaturas, possuem atividade imunomoduladora e antibacteriana. Os melhores resultados foram mostrados pelas duas amostras: 1) secas em $t = 60 \pm 1^\circ\text{C}$ sem aplicação de vácuo, e 2) adsorvidas preliminarmente na mistura de pó como isomalte GalenIQ® e Aeroperl® 300 Pharma, e então secas à temperatura ambiente. Os materiais deste artigo podem ser úteis para especialistas em tecnologia farmacêutica, bioquímica e farmacologia. Os resultados obtidos contribuem para o desenvolvimento de novas formulações avançadas de fármacos baseadas em extratos de fitoterápicos da BAS.

Palavras-chave: extrato de drogas fitoterápicas, sistemas biotestes *in vitro*, atividade farmacológica, substâncias biologicamente ativas, enzimas limitantes.

ABSTRACT

The aim of this study was to evaluate the influence of different methods of extraction solvents removal from liquid herbal drugs extracts on their pharmacological activity (immunomodulating, adaptogene, antioxidant and antibacterial). The authors used specific enzyme biotest systems *in vitro* based on key enzymes of antioxidant protection system - glutathione reductase and catalase, as well as on limiting enzyme of phagocytosis terminal phase - NADPH-oxidase, that allow the researchers to identify adaptogene, antioxidant, antibacterial and immunomodulating BAS activity and to estimate possible alterations in bioactivity of the studied objects. The obtained results showed that the drying temperature of liquid combined herbal drugs extracts does not influence significantly on its BAS complex bioactivity. Extract samples, dried at different temperatures, have immunomodulating and antibacterial activity. Best results were shown by the two samples: 1) dried at $t=60 \pm 1^\circ\text{C}$ without vacuum application, and 2) preliminary adsorbed on the mix of powder like excipients isomalt GalenIQ® and Aeroperl® 300 Pharma, and then dried at room temperature. The materials of this article can be useful for specialists in pharmaceutical technology, biochemistry, and pharmacology. The obtained results contribute to the development of new advanced drug formulations based on BAS of herbal

drugs extracts.

Keywords: herbal drugs extract, biotest-systems in vitro, pharmacological activity, biologically active substances, limiting enzymes.

АННОТАЦИЯ

Целью настоящей работы является исследование влияния разных способов удаления экстрагента из жидкого экстракта на его специфическую активность (иммуномодулирующую, адаптогенную, антиоксидантную и противомикробную). Авторы использовали специфические биотест-системы ферментов в опытах in vitro на основе ключевых ферментов антиоксидантной защитной системы – глутатионредуктазы и каталазы, а также для ограничения фермента фагоцитозной терминальной фазы - НАДФ-оксидазы, которые позволяют установить адаптогенные, антиоксидантные, антибактериальные и иммуномодулирующие активности БАВ, а также оценить изменения биоактивности исследуемых объектов. Полученные результаты показали, что температура сушки жидких комбинированных экстрактов лекарственных растений существенно не влияет на биологическую активность комплекса БАВ. Экстракты, высушенные при разных температурах, обладают иммуномодулирующей и антибактериальной активностью. Наилучшие показатели были у следующих образцов: 1) экстракт, высушенный при $t = 60 \pm 1^\circ\text{C}$ без вакуума; и 2) адсорбированный на смеси изомальта GalenIQ® и Aeroperl® 300 Pharma экстракт, высушенный при комнатной температуре. Материалы этой статьи могут быть полезны специалистам в области фармацевтической технологии, биохимии и фармакологии. Полученные результаты способствуют разработке инновационных лекарственных препаратов на основе БАВ экстрактов лекарственных растений.

Ключевые слова: биологически активные вещества, растительный экстракт, лимитирующие ферменты, биотест-системы in vitro, фармакофоры.

INTRODUCTION

Liquid extracts are concentrated preparations of herbal drugs, which can be used as a part of formulation for drugs production and as separate drugs. They can be produced from one or several species of medical plants (National Pharmacopoeia, n.d.; National register, 2015).

An example of such preparation is combined liquid extract obtained from *Matricaria chamomilla* L. flowers, *Calendula officinalis* L. flowers, and *Achillea millefolium* L. herb (FSP..., n.d.). 40% ethanol solution was used as an extraction solvent. This extract is used as an anti-inflammatory and wound healing drug (Baginskaya *et al.*, 2000). Therapeutic effect of the drug is associated with the contained BAS complexes: flavonoids, terpenoids, polyolefinic compounds, polysaccharides, basic character substances, etc. (Vichkanova *et al.*, 2009).

The interest and attention of specialists in herbal drugs extracts are still high, which is explained by a number of their advantages,

proved the efficiency and complex impact on the organism (Ivanichkin, 2011; Mironov and Fetisova, 2013; Radtsig, 2011; Vishnyakov and Sinkov, 2013). However, it is known that liquid extracts have some weaknesses, they are badly shippable, they can form sediment at moderate temperature decrease or at partial alcohol evaporation, the temperature range at shelf-life should be $15\text{--}20^\circ\text{C}$ (Chueshov, 2002; Menshutina, 2013) and they are not always convenient to use. Since they are commonly indicated for mouth rinsing, they are not suitable for some categories of patients.

Due to these facts, improvement of the pharmaceutical form "Liquid extracts" is an acute issue. For liquid extracts, this can be extraction solvent removal, the inclusion of their BAS into pharmaceuticals forms that are more convenient to use, etc. However, extraction solvent removal is associated with temperature exposure, which may result in structure and features alterations of herbal drugs BAS complexes.

The scientists in "All-Russian Scientific Research Institute of Medicinal and Aromatic

Plants" (ASRIMAP) developed a biochemical method, based on specific enzyme biotest-systems, for bioactivity evaluation of BAS in vitro. Their development is based on fundamental knowledge in pharmacology and biochemistry, primarily, on the understanding of specific pharmacophores, that are related to each BAS pharmacologic group, complementary to a respective receptor, and define the type of pharmacological activity (Mineeva, 1995; Bykov *et al.*, 1997). BAS pharmacophores selectively interact with the respective targets, primarily with limiting enzymes that play an important role in biochemical processes. Limiting enzymes quickly respond to environmental changes by alterations of their activity. For the present study, the authors chose the following enzyme biotest systems in vitro: glutathione reductase (GR) in combination with catalase (CAT) were used for identification of adaptogene, antioxidant and antibacterial activity, and NADPH-oxidase was used for identification of immunomodulating activity of the studied objects.

The purpose of the present study is to evaluate the influence of different methods of extraction solvent removal from liquid herbal drug extract on their bioactivity (immunomodulating, adaptogene, antioxidant and antibacterial), using NADPH-oxidase, glutathione reductase and catalase specific enzyme biotest systems in vitro for further use of dry extract of BAS complex as a part of new drug formulations that will allow to expand their therapeutic indications.

MATERIALS

The following objects of the study were chosen:

- liquid aqueous alcoholic extract from *Matricaria chamomilla* L. flowers, *Calendula officinalis* L. flowers, and *Achillea millefolium* L. herb (further in the text called liquid extract, No. O5), produced according to the regulatory document ND 42-0171-1463-01 (FSP..., n.d.);
- samples of dried liquid extract under different drying temperatures:
 - O1 – at room temperature;
 - O2 – 30±1°C;
 - O3 – 45±1°C;
 - O4 – 60±1°C;
 - O6 – dried at room temperature on the

excipients mix of SmartEX® and Aeroperl® 300 Pharma*;

- O7 – dried at room temperature on the excipients mix of isomalt GalenIQ® and Aeroperl® 300 Pharma;
- specific enzyme biotest systems in vitro, developed in ASRIMAP (Bykov *et al.*, 2001a, 2001b, 2002a, 2002b), which are a part of ASRIMAP Biologic collection of specific enzyme biotest systems in vitro (BC-SEBTS);
- prothymosin-α – thymic hormone, natural immune activator, manufactured by Merck (Germany).

NADPH-oxidase was received from quiet polymorphonucleocytes from peripheral rabbit blood, obtained during the experiment (Lupanova, 2011).

The following reagents were used:

- NADPH, oxidized glutathione, nicotinamide adenine dinucleotide phosphate (NADPH) and highly purified enzymes glutathione reductase and catalase, produced by Sigma-Aldrich (USA);
- hydrochloric acid marked ACS, Sodium phosphate and Potassium Phosphate, Potassium chloride and Magnesium chloride, Hydrogen peroxide – (Reachim).

METHODS

BAS immune modulating activity was studied by the influence on the limiting enzyme activity in the terminal stage of phagocytosis – NADPH oxidase (Popova *et al.*, 1999) by the change of optical density of the tested solutions at the wavelength of 340 nm and at 24°C by means of biochemical analyzer Clima MC-15 (Spain).

Adaptogene, antioxidant and antibacterial activity was studied by means of biochemical analyzer Clima MC-15 (Spain) using complex specific biotest system in vitro, based on key enzymes of antioxidant protection system - glutathione reductase and catalase, that allow the researchers to obtain objective results (Brodova, 2003; Bushueva *et al.*, 2016; Daniels *et al.*, 1994; Dubinskaya *et al.*, 2013; Kolokhir *et al.*, 2012; Popova, 2000).

Glutathione reductase reaction rate was measured by the decrease of optic density at 340 nm wavelength due to NADPH oxidation by an equimolar amount of reduced glutathione. To

measure the catalase reaction rate, the authors used the method, based on hydrogen peroxide capability to form a stable colored complex compound with Molybdenum salts, to evaluate hydrogen peroxide concentration decrease during the reaction in the tested solution by means of biochemical analyzer Clima MC-15 (Spain) at the respective optical filter.

Statistical analysis of the obtained data was conducted at normal distribution of values in the samplings (in 5 replications), arithmetic mean and error of the mean ($M \pm m$) was calculated. The significance of differences between the samplings was estimated by Student's t-test (Bushueva *et al.*, 2016). The differences between the samplings were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The study of immunomodulating activity in vitro

For comparative quantitative estimation of the direct activating influence of the studied compounds on NADPH-oxidase reaction rate in vitro, the authors compared reactions rates, obtained in the mentioned samples, with the NADPH-oxidase reaction rate in the sample, that contained natural immunostimulator prothymosin α , taking it as 100% (Khabriev, 2005).

The results of the estimation of the direct influence of the studied samples on the rate of NADPH-oxidase reaction in vitro are shown in Table 1.

As it is seen from Table 1, the control sample did not contain BAS, and its NADPH-oxidase reaction rate was equal to 0. When prothymosin- α was added into inactive leukocytes homogenate, NADPH-oxidase reaction was activated. NADPH was oxidized at 44.2 nmol/min for 10 μ l of homogenate, which was taken as 100%.

When extract samples that were dried under different conditions (No. O1 - O4, O6, O7) were added, they expressed mild activating influence on NADPH-oxidase, which proved immunostimulating properties of these objects. The maximal immunostimulating effect was shown by the sample No. O4, that was dried at $t = 60 \pm 1^\circ\text{C}$ in both concentrations, and the samples No. O6 and O7 dried in 6.6 μ l/ml concentration.

The study of adaptogene, antioxidant and antibacterial activity in vitro

The study is based on the fact that GR is activated when adaptogens and antioxidants are added. Adaptogens, unlike antioxidant BAS, express antibacterial effect. Thus, testing of substances in vitro with GR in combination with CAT reaction allows the researchers to identify adaptogens selectively that activate GR and inhibit CAT. Antioxidants increase GR reaction rate but do not inhibit CAT reaction.

The results of the evaluation of the direct influence of test samples on the rate of glutathione reductase and catalase reactions in vitro are shown in Table 2.

The measurement of GR and CAT reactions rate at the presence of extract samples (Table 2) showed that the sample, dried at $t = 60 \pm 1^\circ\text{C}$, liquid extract and 2 samples of extracts, dried with excipients, barely reduced the GR reactions rates and inhibited CAT reactions in vitro, expressing moderate antibacterial effect. The sample No. O7, dried on the mix of excipients isomalt GalenIQ[®] and Aeroperl[®] 300 Pharma, showed dose-dependent effect.

CONCLUSIONS:

1. The temperature of drying of a liquid extract made from *Matricaria chamomilla* L. flowers, *Calendula officinalis* L. flowers, and *Achillea millefolium* L. herb has no significant influence on the bioactivity of its BAS complex.
2. Extract test samples, dried at different temperatures (7 samples), showed moderate immunomodulating and antibacterial activity.
3. The best results on specific activity were shown by test extract samples, dried at $60 \pm 1^\circ\text{C}$ (No. O4), and test samples No. O7 (extract, dried on the mix of excipients isomalt GalenIQ[®] and Aeroperl[®] 300 Pharma).
4. The obtained results contribute to the implementation of herbal drugs BAS complexes from dry extracts into the development of solid pharmaceutical forms of new advanced drugs, based on the BAS of herbal drugs extracts, for expansion of therapeutic indications.

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Table 1. The influence of test samples on the NADPH-oxidase reaction rate in vitro

Test sample	NADPH-oxidase reaction rate, M±m	
	nmol/min per 10 µl of homogenate	%
Control	0	0
Prothymosin-α	44.2±2.08	100.0
O1 3.3 µg/ml	7.6±0.33*	17.3
O1 6.6 µg/ml	7.87±0.32*	17.8
O2 3.3 µg/ml	7.29±0.28*	16.5
O2 6.6 µg/ml	7.07±0.30*	16.0
O3 3.3µg/ml	7.51±0.35*	17.0
O3 6.6µg/ml	7.43±0.36*	16.8
O4 3.3µg/ml	8.18±0.34*	18.5
O4 6.6µg/ml	7.91±0.34*	17.9
O5(10 µl)	7.78±0.37*	17.6
O5(20 µl)	6.94±0.33*	15.7
O6 3.3 µg/ml	7.47±0.33*	16.9
O6 6.6 µg/ml	7.96±0.39*	18.3
O7 3.3 µg/ml	7.69±0.35*	17.4
O7 6.6 µg/ml	8.27±0.40*	18.7

Table 2. The influence of the test samples on the glutathione reductase and catalase reactions *in vitro*

Test sample	Reaction rate, M±m			
	Glutathione reductase		Catalase	
	μmol/ (min/mg of protein)	%	μmol/ (min/mg of protein)	%
Control	2.98±0.136	100	0.140±0.0068	100
O1 3.3 μg/ml	2.96±0.135	99.3	0.144±0.0065	102.9
O1 6.6 μg/ml	2.98±0.146	100.1	0.148±0.0071	105.7
O2 3.3 μg/ml	2.87±0.121	96.4	0.135±0.0066	96.2
O2 6.6 μg/ml	2.85±0.130	95.8	0.138±0.0059	98.8
O3 3.3μg/ml	2.81±0.135*	94.3*	0.137±0.0065	98.4
O3 6.6μg/ml	2.78±0.136*	93.2*	0.107±0.0053*	76.6*
O4 3.3μg/ml	2.62±0.118*	87.8*	0.106±0.0049*	75.7*
O4 6.6μg/ml	2.58±0.121*	86.4*	0.102±0.0043*	73.2*
O5(10μl)	2.63±0.118*	88.3*	0.108±0.0047*	77.3*
O5(20μl)	2.61±0.117*	87.5*	0.107±0.0052*	76.5*
O6 3.3 μg/ml	2.77±0.130*	93.0*	0.110±0.0052*	79.0*
O6 6.6 μg/ml	2.68±0.115*	90.1*	0.110±0.0051*	78.8*
O7 3.3 μg/ml	2.73±0.126*	91.7*	0.106±0.0050*	75.4*
O7 6.6 μg/ml	2.60±0.108*	87.3*	0.102±0.0049*	73.1*

Note: here and further * - statistically significant difference from the control sample at $p<0.05$.