**DEVELOPMENT OF THE BLACK SEA HYDROBIONT RESOURCE STUDY METHOD AS A NEW ECONOMICALLY FEASIBLE DIRECTION OF PHARMACOLOGY AIMING TOWARD SAVE HUMAN HEALTH AND ECOLOGY**

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**Abstract.** The selection of the Black Sea aquatic area as a hydrobiont biologically active compound source allows resolving several environmental problems including development of an integrated hydrobiological resource management system for the Black Sea as a new sector of modern pharmacology. Aquatic organisms produce unique secondary metabolites. This paper presents the results of studies on the development of the biologically active substance extraction technology from non-commercial aquatic organisms as well as a preliminary assessment of the biochemical activity of the substrates obtained. Biologically active substances were extracted using the of two-phase extraction method in conjunction with ultrasound. For the substrates, the antioxidant activity was determined utilising the method that allow conducting screening of pharmaceutical raw materials and biologically active substances with high antioxidant activity. The protective activity evaluation was carried out during the study of the enzymatic alcoholic fermentation kinetics within a self-contained system. The evaluation of lymphocytes proliferative activities influenced by the obtained substrates was carried out using the cultivation method. The acquired data allows recommending the two-phase extraction method combined with ultrasonic voiceover as the effective one when processing analogical marine raw materials. The isolated substrates are characterised by a pronounced biochemical activity in relation to the living systems cells, which suggests a significant synergistic effect with derivatives of the microalgae Chlorella Vulgaris.

1. **Introduction**

The rational and integrated consumption of the World Ocean’s biological resources provides for maximum involvement of non-commercial waste and inedible hydrobionts within the national economy as new sources of dietary, fodder, and technical products. At the same time, within the current stage of the ecological and biological state of the environment, some serious breakthroughs are required in the traditional attitude towards the World Ocean’s marine and freshwater aquatic area’s biological resources, which are mainly used within the nutrition and forage production sphere. The exacerbation of the "raw material" shortage in the pharmaceutical industry, an acute deficiency of biologically active substances, a narrowing of the land sources base and the outlined revision of customs in the exploitation of the World Ocean’s valuables in general, also serves as a basis for the
development of new principles for the marine aquatic organisms utilization as producers of chemical compounds relevant for pharmaceutical science and the development of a specialised medicinal science direction - marine pharmacy [1-4].

The selection of the Black Sea water area as sources of aquatic organisms’ biologically active compounds provides the possibility for resolving several ecological problems in the region. Firstly, the problem of sharp depletion of traditional sources for obtaining new medicinal products which are based on biologically active substances of terrestrial organisms (plants and animals), and the constantly increasing costs of obtaining them. Secondly, it allows avoiding significant losses during the processing of commercial aquatic organisms (up to 60% of the catch weight), which is a very large proportion of the resource reserve, as well as effective utilization of the rational and integrated aquatic biological resource consumption principle, it also allows the maximum involvement of substantial concentrations of non-commercial hydrobionts in the territorial waters, in particular, marine invertebrates, in combination with the development of relevant resource-saving and low-waste technologies for their utilization, which, in turn, reduces additional pressure to the ecosphere. Thirdly, it provides a start to the formation of the Black Sea marine organisms integrated resource management system principle as a new direction of modern pharmaceutical science, requiring significant efforts in the field of geographical distribution and reserve determination [5,6].

In addition to all above, aquatic animals and plants’ biologically active substances possess the unique properties associated with water based organisms’ living conditions within the marine environment characterized by high salt content, low or no illumination, elevated pressure and unusually high or low temperature, which forces marine organisms and their metabolites to differ from organisms with terrestrial biology.

The organisms within aquatic environments produce structurally unique and diverse secondary metabolites that are not typical for terrestrial organisms, and many of them are characterized by extremely high biological and pharmacological activity. The aquatic organisms’ metabolites are represented by functional compounds such as carotenoids, phospholipids, saponins, polyunsaturated fatty acids of the omega three and omega six classes, with high antioxidant, immunomodulatory, radioprotective and antitumor activity when used in doses required for the treatment and prevention of numerous diseases, which supposed to be extremely low due to their exceptionally high activity [7-14].

Another advantage of the marine origin biologically active substance utilization is the economic benefit due to the relative availability, large variety of structures, remarkable volumetric quantity and successful reproducibility of the raw material base [4-6].

2. Research objective
The paper presents the results of studies on the development of low-waste and resource-saving technologies for the extraction of biologically active substances from non-commercial hydrobionts of the coastal zones of the Black Sea, as well as a preliminary assessment of the obtained substrate biochemical activity.

3. Materials and methods
Marine invertebrates (rapana - Rapana Venosa, mussels – Mytilus Galloprovinciales) and concentrated culture of Chlorella Vulgaris in its media were used as study objects. Substrates of biologically active substances from invertebrate tissues were extracted using the two-phase extraction method in combination with ultrasound. The two-phase extraction method with specific utilization of the two-phase solvent system (alcohol-water mixture/oil) as an extractor, allows extraction of hydrophilic and hydrophobic substances simultaneously from raw materials when preparing extracts and compounds based on marine hydrobionts, while the hydrophilic substances predominantly remain in the alcohol-water phase and lipophilic substances pass into the oil. The combination of two-phase extraction and ultrasound provides a deeper penetration of the solvent into material with a cellular structure, reduces
the processing time, provides an efficient productivity and reproducibility, reduces solvent consumption, increases the process speed, and allows the thermolabile substance extraction [15-17].

For the resultant substrates, antioxidant activity (AOA) was determined using a method that allows conducting screening of pharmaceutical raw materials, phyto-medications and biologically active substances with high AOA including the interaction of the analyzed sample with potassium permanganate up to the discoloration of the latter in an aqueous sulfuric acid medium at room temperature [15,16]. The antimicrobial activity was determined using a method based on germination in a nutrient medium.

A preliminary assessment of the protective activity of the obtained medicinal forms was carried out in a detailed study of the total process of enzymatic reactions of alcoholic fermentation kinetics in a self-contained system, and the subsequent comparison of the individual pattern parameters of reaction course of the cases when medicinal substances were absent with their various concentrations in solution. In these experimental series, the microscopic fungi belonging to the biological species Saccharomyces Cerevisiae (top-fermented yeast) was selected as a model organism due to it being the one of the most studied model organisms. Heavy metal salt was used as an exogenous toxicant.

Evaluation of the proliferative activity (growth and mitogenic activity) of lymphocytes in the “in vitro” culture under influence of substrates of invertebrate’s biologically active substances was carried out by the standard semi-micro method of culturing peripheral blood lymphocytes of inferior primates, the monkeys, with some modifications, followed by cytogenetic analysis of the studied karyotypes (preparation of metaphase chromosomes specimens, chromosome staining and the quantitative analysis of metaphase plates) [18].

The effect of antiulcerogenic activity of Chlorella concentrated culture in its media was studied using an experimental model of the gastric mucosa damage on 10 mice of both sexes. Experimental damage to the gastric mucosa was modelled by a single intragastric administration of ethanol (1 ml 96° / 200 g animal weight). The assessment of ulcerogenic effect was carried out 1 hour after the administering of alcohol. The damage degree to the gastric mucosa and its severity were characterized on the basis of a total length of ulcerative lesions (in mm) calculation in each mouse.

4. The results and discussion

The combination of two-phase extraction with ultrasonic impact provided possibility to achieve AOA values equal to the AOA of substrates obtained using classical extraction with stirring after 96 hours, during 60-180 minutes, depending on the properties of the initial raw material. Analysis of the research results on biologically active substance substrates antioxidant activity allowed the possibility to draw the following conclusions. AOA increases in accordance with the duration of ultrasound exposure. The AOA of substrates in the oil phase is 2-3 times higher than the AOA of substrates obtained under similar conditions in the aqueous-alcoholic phase. The AOA of substrates of biologically active substances extracted from the tissues of rapanas is higher than the AOA of the substrates obtained from mussel tissues, in condition of an ultrasound exposure time from 6 to 12 hours. The ultrasound exposure time for obtaining the extracts with optimal AOA values in the oil and water-alcohol phases is 3 hours. The research results are presented in Figs. 1, 2.
The study of antimicrobial activity was carried out using the method of substrate germination within a nutrient medium with optimal values of AOA in oil and water-alcohol phases (with ultrasonic treatment time 3 hours) in the ratio 20 μl of extract per 1 ml of medium. The samples obtained were placed in the thermostat and visually observed for a considerable time. Germination of the nutrient medium was not observed within 30 days from initial of sowing, which may be an indirect confirmation of the pronounced antimicrobial activity of the studied samples.

Assessment of the protective properties of the invertebrate’s substrates indicated the following results. The influence of the biologically active substance substrates on the maximum possible rate of enzymatic fermentation of yeast in the presence of heavy metal salts is generally positive, i.e. they increase its change. In general, all substrates significantly slow down the degeneration stage of the yeast’s enzymatic fermentation in the presence of heavy metal salts within a self-contained system. The protective properties of alcohol extracts are more pronounced at a higher content in a self-contained system and are almost identical at all heavy metal (lead) salt concentrations. The protective activity of oil extract is twice as high as that of alcohol extracts. At the same time, the protective properties of these samples are much higher within the ranges of high and low heavy metal (lead) salt.
concentrations. If the content of oil extract increases, the protector activity decreases. The research results are presented in Fig. 3.4.

**Fig. 3.** Experimental dependence of the total process of yeast’s enzymatic fermentation rate (the volume of CO$_2$ (cm$^3$) on the reaction time) in a self contained system in the presence of a mussel biologically active substances alcohol (1.0 ml) and various concentrations of a heavy metal salt (1 - 0.1 M; 2 - 0.002 M; 3 - 0.004 M; 4 - 0.0008 M; 5 - 0.00016 M; 6 - 0.000032 M).

**Fig. 4.** Experimental dependence of the yeast’s total process of enzymatic fermentation rate (the volume of CO$_2$ (cm$^3$) on the reaction time in a self contained system in the presence of a mussel biologically active substances of oil extract (1.0 ml) and various concentrations of heavy metal salt (1 - 0.1 M; 2 - 0.002 M; 3 - 0.004 M; and 4 - 0.0008 M; 5 - 0.00016 M; 6 - 0.000032 M).

The effect of biologically active substances extracts derived from the mussel tissues on living cells is multifactorial: firstly, the components of the extracts can be transported inside the cell for subsequent utilisation as a source of missing macro elements (N, P, S, etc.) - thus, demonstrating the activity as a substrate; secondly, the components of the extracts, being adsorbed on the surfaces of biological membranes, affect the regulation of metabolic processes, contributing to the cells protection from adverse concentrations of metabolic products or blocking the membrane penetrability capacity in the case of high concentrations and demonstrating protective activity (in more complex systems that include heavy metals ions, the protective function will probably be expressed as an adsorption of these ions).

The growth and mitogenic activity of peripheral blood lymphocytes of lower monkey were evaluated under the influence of the biologically active substance extracts derived from mussel tissues in the oil and water-alcohol phase at an ultrasonic treatment time of 3 hours. The level of mitogenic
activity for the control sample was 18 mitoses per 1000 cells; for the sample with the introduced complex of biologically active substances in the water-alcohol phase accounted for 22 mitoses; for the sample with the introduced complex of biologically active substances in the oil phase it was 48 mitoses. When an endogenous toxicant (a heavy metal salt) was introduced into the medium, the level of mitogenic activity decreased, while in the presence of an alcohol extract it remained at the level of the control sample, and in the presence of the oil extract, a pronounced increase was maintained.

The results of the previously studied effect of the antiulcerogenic activity of concentrated culture of Chlorella Vulgaris in its media on the model of experimental damage to gastric mucosa demonstrated a positive dynamic of mucosal healing. The utilised concentrate reduced the average number of ulcerations by 27% (p <0.001) 3 days after an application. This allows considering the agent as active and capable to sufficiently rapid reparative regeneration capabilities, possibly due to the anti-edema, antioxidant and capillary-strengthening effect.

5. Conclusions

The obtained data allows using the two-phase extraction method in combination with ultrasonic voicing, as sufficiently effective in processing raw materials of this type and to recommend it within the frame of actual resource-saving and low-waste technologies. The isolated substrates containing biologically active substances of aquatic organisms possess a significantly pronounced biochemical, protective, regenerating, growth and pharmacological activity in relation to living systems cells which allows an assumption of their high synergistic effect in combination with the Chlorella Vulgaris derivatives.

Taking into account the abovementioned emphasis on the possibilities of creating universal bio substrates, consideration should be given that it is appropriate to continue an experimental research on the development of technologies for deriving biologically active substances of aquatic organisms in combination with methods based on the microalgae Chlorella Vulgaris in order to develop highly effective pharmaceuticals preparations of various somatic orientations.

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