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Effect of Supercritical and Aqueous–Alcoholic Extracts from *Halocynthia Aurantium* on Gametes, Zygotes, and Embryos of Sea Urchin

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Abstract—The influence of supercritical (SC) and aqueous–alcoholic extracts of *Halocynthia Aurantium* (Ascidiaceae, Tunicata) on gametes, embryos, and larvae of sea urchin *Strongylocentrotus intermedius* was studied. It was demonstrated that aqueous–alcoholic extract produce no appreciable effect on embryos and larvae of sea urchin. When supercritical extract is added at the stage of gamete fusion, fission is not affected, but gastrulation process changes, producing exogastrulae. In addition, the development of infusoria was suppressed in the presence of supercritical extract. The distinctions in the biological activity of aqueous–alcoholic and SC–CO_2 extracts of *Halocynthia Aurantium* tunic are evidently associated not with the lipid fraction but with the fractions of peptides and secondary metabolites, since the lipid fractions were virtually identical.

Key words: supercritical and aqueous–alcoholic extracts, ascidia, *Halocynthia Aurantium*, biotesting, sea urchins, embryos, exogastrulation, infusoria.

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INTRODUCTION

At present, various substances extracted from herbal raw materials with liquefied and supercritical carbon dioxide (SC-CO_2) are finding ever-new applications. Note, however, that, in Russia, the extraction technology, raw material assortment, and areas of commercial use of CO_2-extracts are limited to two or three branches of industry, being so far ignored in pharmacology and the foodstuff industry for manufacturing gerodietetic products. Therefore, the development of the technology of preparation of CO_2-extracts from various raw materials for use in manufacturing functional products is a topical problem. Liquid and supercritical CO_2 is an effective solvent of hydrophobic substances. Normally, substances extracted using SC-CO_2 technology have a more complex composition than those obtained by means of aqueous–alcoholic extraction. The SC-CO_2 technology makes it possible to extract almost completely both saturated and unsaturated fatty acids, as well as fat-soluble vitamins, waxes, terpenes, terpenoids, phenol-containing compounds, pigments, alkaloids, and phytosterols.

Ascidia are considered as a promising source of antitumor drugs with a unique mechanism of therapeutic action [1–3]. A number of polycyclic aromatic alkaloids with a well-pronounced antitumor activity were extracted from various ascidian species [4–10]. The mechanisms of action of these compounds remain unclear, but there are reasons to believe that they differ from those of the known antitumor drugs. It was revealed that asciddemine, belonging to the class of pyridoacridines, not only disrupts the transport of Ca^{2+} ions from the intracellular depots (mitochondria and sarcoplasmic reticulum) into the cytoplasm, but also affects the DNA of cells, causing its fragmentation, and, as a result, the apoptosis of cells. Ecteinascidins, specifically binding to promoter DNA, are likely to inhibit transcription [7, 9].

Ascidia also contain antibiotics. For example, halocidin, an antimicrobial peptide, was extracted from *Halocynthia Aurantium* hemocytes [12].

In 1970–1990, researchers from different countries developed a method of biotesting with the use of gametes, embryo, and larvae of sea urchin, known as the Sea Urchin Test System [13–15]. The Sea Urchin Embryo Test, a method based on an analysis of embryonic and earlier larval development of sea urchin has

become most popular. It is frequently used to test various bioactive substances [16, 17].

The present work concerns the preparation of new biologically active substances with antiteratogenic, sorption, antitumor, immunomodulating properties. Sea hydrobionts, including ascidia, offer a wide variety of biologically active substances. Among these substances, some were shown to possess the indicated biological properties. At present, Far Eastern research institutes intensely study preparations obtained from trepang, cucumaria, squid ganglia, muscular tissue of mollusks, and other hydrobionts. *Halocynthia Aurantium* is one of such objects, which was selected during screening of widespread invertebrates from the Peter the Great Gulf for stress-modulating effect of alcohol extracts. These filtering animals belong to Chordata type, Tunicata subtype. They are widespread in Far Eastern and Arctic seas at depths from 4 to 400 m. Pharmacologically, an alcohol extract from *Halocynthia Aurantium* tunic, known as Haurantin, has been most extensively studied [17–19].

Haurantin is a aqueous–alcoholic extract (1 : 1) prepared from dry *Halocynthia Aurantium* tunic. This preparation was demonstrated to contain 15 amino acids, 7 of which are unique. It contains phospholipids, fatty acids, and neutral lipids. The lipids are characterized by a high degree of unsaturation due to the presence of polyene fatty acids. The extract was shown to contain prostaglandins, carotenoids, and vitamin C. Haurantin is prepared using the standard technology for producing galenical preparations, by means of aqueous–alcoholic extraction. Since this method does not ensure a complete extraction of active substances, search for new methods for extracting biologically active substances from ascidia presents a pressing problem. We proposed a new method for extracting active substances from dry *Halocynthia Aurantium* tunic, by means of SCF extraction.

In the present work, we report the results of studies on preparation of extracts from ascidia of the Sea of Japan with the help of supercritical carbon dioxide and on testing their biological activity in comparison with the aqueous–alcoholic extract.

EXPERIMENTAL

Halocynthia Aurantium (Tunicata, Ascidiaceae, Pleurobranchiata, Pyuridae) was collected in the Peter the Great gulf of the Sea of Japan at depths of 5 to 20 m. The test object was sea urchin *Strongylocentrotus intermedius*. Sea urchins from the Sea of Japan were kept in the Aquarium Laboratory of the Institute of Marine Biology of the Far East Branch of the Russian Academy of Sciences. Sexually mature specimens of approximately the same size at the stage of gonad fluidity were selected. The experiments were performed with ovums, spermatozoids, embryos, and just-fertilized ovums. A suspension of mobile spermatozoids was added to mature ovums, and then an aqueous–alcoholic or SCF extract of *Halocynthia Aurantium* tunic was added. The experiments were performed with zygotes during fertilization (200–250 ovums) and larvae (50–100 larvae in each experiment): early and intermediate blastulae, early, intermediate, and late gastrulae and pluteuses. Gametes, embryos, and larvae were transferred to Petri dishes filled with seawater at a temperature of 19 to 20°C. We used embryo cultures with a fertilization percentage of ~98%. In different experiments, the seawater was doped with 0.1–0.4 mg/ml of the test substances.

The reference samples were unaffected embryos and larvae at the corresponding stage of development. The experiments were conducted in three repeatabilities. The experimental procedure was the same for all the development stages studied. As the aqueous–alcoholic extract of *Halocynthia Aurantium* tunic, we used Haurantin, which was prepared from dry ascidia tunic and 70% alcohol. Before biotesting, the extract was evaporated in a vacuum evaporator.

Extraction from *Halocynthia Aurantium* tunic with SC-CO_2 was conducted on a SFE-1000 extractor (Thar Technologies Inc., USA). This device makes it possible to perform extraction in the flow regime with the use of various modifiers of polarity at a fluid mass flow rate of up to 200 g/min, pressure of up to 650 bar, and temperature of up to 120°C. A 160-g portion of minced tunic was placed into a 200-ml vessel. The vessel was sealed and thermostated, after which the setup was started. Extraction was considered to begin when all the working parameters of the system stabilized. The experiments were performed using 99.8% pure CO_2 (State standard GOST 8050-85, OAO "Linde Gas Rus") without polarity modifiers under the following conditions: pressure, 300 bar; extractor temperature, 40°C; fraction collector temperature, 35°C; mass flow rate, 30 g/min; time of extraction, 120 min. After the end of extraction, the pressure in the system was released, and the Teflon vessel was removed from the fraction collector and degassed for 3 h.

Extraction of lipids from the extracts. The lipid component was isolated from all extracts by using the Bligh–Dyer method [21]. The powdery mass was poured over with an 1 : 1 methanol–chloroform mixture (3 ml of solvent per 1 g of extract) and homogenized in an Waring blender with ultrasonic amplification for 2 min. The procedure was repeated twice; next, the extracts were blended and diluted with a small amount of water to initiate phase stratification. The extract was filtered to remove insoluble particle and then centrifuged at 3000 rpm for 15 min, until a complete separation of the organic and aqueous–alcoholic phases was achieved. The lower layer of the organic phase was sucked out with a pipette transferred into a flask, where the major part of the solvent was removed by evaporation. Concentrated extract was poured into 2-ml viols, where the residuals of solvent were removed with a flow of an inert gas.

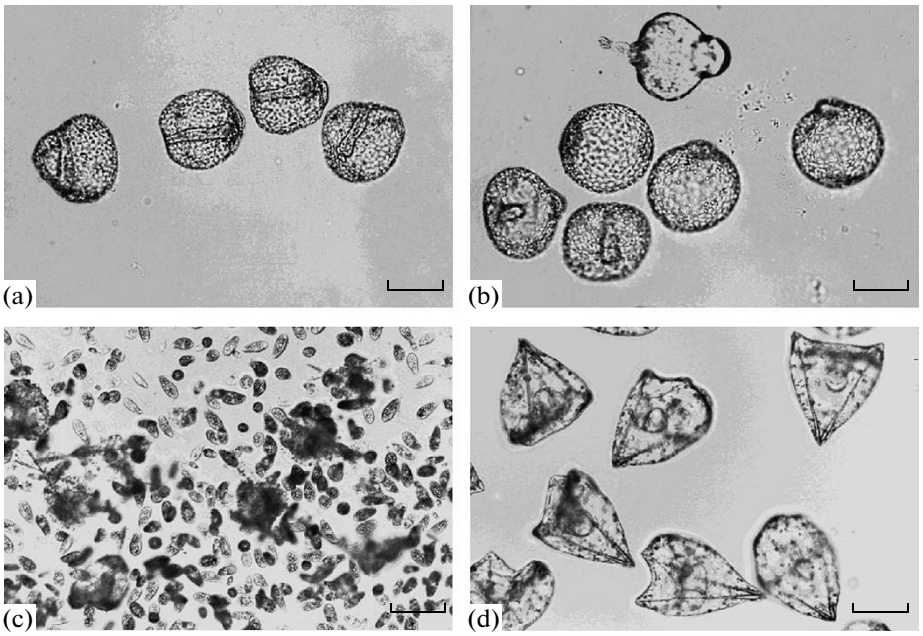

Fig. 1. Embryos and larvae of sea urchin (50-μm scale): (a) reference embryos of sea urchin 24 h after fertilization (normal gastrula) (b) test embryos 24 h after fertilization in the presence of the supercritical extract (gastrulation disrupted, exogastrulae are formed); (c) reference embryos 73 h after fertilization (numerous infusoria appeared in the cultural media, lysing sea urchin embryos); and (d) sea urchin larvae at the stage of early pluteus, 73 h after fertilization (supercritical extract was added to the embryos 24 h after fertilization, at the stage of late gastrula; normal development takes place in the complete absence of infusoria in the cultural medium).

GC-MS analysis of methyl esters of fatty acids. Hydrolysis of fatty acids with subsequent methylation was conducted as described in [21]. Analysis was performed on HPGC/MSD 5393 chromatograph equipped with a HP-5-MS capillary column during heating the column from 100 to 230°C at a rate of 5 K/min. The spectra were deciphered using the US NBS mass spectra database.

RESULTS

When aqueous–alcoholic extracts or minced tunic subjected to extraction in SC-CO_2 were added to Petri dishes containing spermatozoids, ovums experiencing fertilization, dividing embryos, embryos at the stages of early and intermediate gastrula, and larvae at the stage of early pluteus, no visible distinctions from the reference were observed: spermatozoids remained mobile, normal fertilization percentage (98%) was in place, fission occurred without deviations, and normal gastrula and early pluteuses were formed. Only upon addition of the aqueous–alcoholic extract, a small decrease in the fractionation rate was observed, which disappeared at the stage of late blastula.

When the same samples (spermia, embryos, and larvae) were treated with supercritical extracts from *Halocynthia Aurantium* tunic also no deviations from the reference samples were found. However, if a supercritical extract was added when the gametes fuse together, the development was disrupted. The formation of the fertilization membrane and fission occurred as usual, but the process of gastrulation was perturbed. Instead of the formation of a normal archenteron through the bulging of the blastomeres of the vegetative pole of the blastula, as it occurred in the reference sample (Fig. 1a), exogastrulae were formed (Fig. 1b). During exogastrulation, the blastomeres of the vegetative pole do not invaginate into the blastocoel, but, on the contrary, bulge outward. Later, such exogastrulae do not develop, but continue to be alive for several days, remaining mobile. They can somewhat increase in size compared to the reference late blastulae and become more transparent because of a depletion of yolk granules.

Embryos and larvae were cultivated in seawater from the aquarium of the Institute of Marine Biology of the Far East Branch of the Russian Academy of Sciences, which, in turn, was supplied from the Amur Gulf. This water contains a large number of bacteria and single-cell eukaryotes, which intensely replicate in Petri dishes with sea urchin embryos, especially infusoria. After four or five days, bacteria became so numerous that they cause lysis of sea urchin embryos and larvae (Fig. 1c). A similar picture was observed when the culture was treated with the aqueous–alcoholic extract or with minced tunic after extraction. Note, however, that addition of the SC-extract to a medium with embryos after gastrulation suppressed the development of infusoria, allowing larvae to

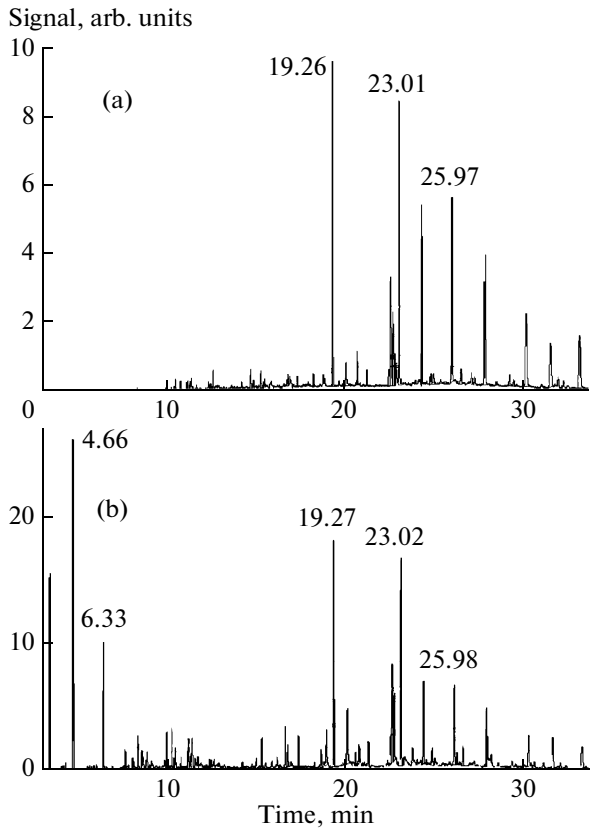

Fig. 2. Gas chromatograms of extracts of *Halocynthia Aurantium* tunic: (a) supercritical and (b) aqueous–alcoholic.

develop in a clean medium with a number of single-cell eukaryotes too small to interfere (Fig. 1d).

Examination of extracts by gas chromatography mass spectrometry involved a special preparation of probes for analyzing nonpolar compounds. Figure 2 shows gas chromatograms of the supercritical (Fig. 2a) and aqueous–alcoholic (Fig. 2b) extracts of *Halocynthia Aurantium* tunic. Both chromatograms show numerous peaks belonging to fatty acids and hydrocarbons.

DISCUSSION AND CONCLUSIONS

Fertilization is the most delicate period in the development of sea urchin. That the fusion of gametes in the presence of the SC extract occurs as usual suggests that the extract components do not affect the ability of a spermatozoid to penetrate through the ovum membranes—they act at later stages. Since, the extract does not markedly affect spermatozoids, ovums, embryos, or larvae, it is reasonable to conclude that it acts only for a first few minutes after fertilization, influencing the fusion of the ovum with the spermatozoid.

What happens in the zygote cytoplasm immediately after the gametes were brought in contact? In the ovum and in the spermatozoid, calcium ions are released, a process accompanied by acrosome and cortical reactions. In the presence of the SC extract, the transport of calcium ions can be disrupted. The depolarization of the ovum membrane gives rise to an activation potential that initiates the formation of a network of bundles of helical actin microfilaments in the zygote cortex, ensures the release of the content of cortical granules and the stabilization of morphogenetic determinants [22–24]. This period of embryogenesis is sensitive to the action of alterating factors [25–28]. Within first minutes after fusion of the gametes, embryos are sensitive to the action of the SC extract. The mechanism underlying this effect encompasses the processes occurring from the instant the spermium contacts the ovum to the moment of formation of a first cleavage furrow, processes that result in the fusion of the gametes.

Thus, we believe that, within first minutes of fertilization, the SC extract disrupts the transport of calcium ions and impedes the regular assembly of bundles of actin filaments, an action that later manifests itself through the disruption of gastrulation: instead of a normal invagination, exogastrulation takes place. Exogastrulation is a nonspecific reaction of sea urchin embryos in response to the action of various alterating factors (for example, a deficiency of calcium ions in seawater [29, 20]).

An important result of the study is that the SC extract inhibits the growth of infusoria, abundant in the seawater in which embryos are cultivated, an observation that signifies that SC extracts contain substances toxic for infusoria. In aqueous–alcoholic extracts, these compounds are either absent or inactive.

Gas chromatography revealed a number of distinctions in the composition of aqueous–alcoholic and SC extracts (Fig. 2). These fractions are qualitatively similar in the composition of fatty acids (Figs. 2a, 2b). Both extracts contain C_{14}–C_{22} acids, branched carboxylic acids, and C_{11}–C_{26} hydrocarbons. At the same time, significant quantitative distinctions were revealed, which can be accounted for by the fact that SC extraction provided a complete removal of the lipid fraction from tunic. That some of the components present in the aqueous–alcoholic extract are absent in the SC extract suggests that the conditions of flow extraction with supercritical CO_2 are far from optimal.

The distinctions in the biological activity of aqueous–alcoholic and SC-CO_2 extracts of *Halocynthia Aurantium* tunic are evidently associated not with the lipid fraction but with the fractions of peptides and secondary metabolites. This conclusion is supported by the fact that halocidin, a peptide with antimicrobial action, was extracted from *Halocynthia Aurantium*, [12].

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