

Article

Cell-Free Coelomic Fluid Extracts of the Sea Urchin *Arbacia lixula* Impair Mitochondrial Potential and Cell Cycle Distribution and Stimulate Reactive Oxygen Species Production and Autophagic Activity in Triple-Negative MDA-MB231 Breast Cancer Cells

Claudio Luparello *, Debora Ragona, Dalia Maria Lucia Asaro, Valentina Lazzara, Federica Affranchi, Vincenzo Arizza and Mirella Vazzana

Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università di Palermo, Viale delle Scienze, 90128 Palermo, Italy; debora0410ale@gmail.com (D.R.); daliaasaro@libero.it (D.M.L.A.); valentina.lazzara@community.unipa.it (V.L.); federica.affranchi@community.unipa.it (F.A.); vincenzo.arizza@unipa.it (V.A.); mirella.vazzana@unipa.it (M.V.)

* Correspondence: claudio.luparello@unipa.it; Tel.: +39-91-238-97405

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Abstract: Triple-negative breast cancer (TNBC) is a highly malignant tumor histotype which lacks effective targeted therapies, thereby being considered as the most aggressive form of breast carcinoma. To identify novel compounds which could counteract TNBC cell growth, we explored the in vitro effects of crude extracts and <10 kDa-filtered fractions of the coelomic fluid obtained from the sea urchin *Arbacia lixula* on TNBC MDA-MB231 cells. We examined cell viability, cycle distribution, apoptotic/autophagic activity, and mitochondrial polarization/cell redox status. Here, we report the first data demonstrating an anti-TNBC effect by *A. lixula*-derived coelomic fluid extracts. Thus, identification of the water-soluble bioactive component(s) contained in the extracts deserve(s) further investigation aimed to devise novel promising prevention and/or treatment agents effective against highly malignant breast tumors.

Keywords: echinoderm; breast cancer; cytotoxicity

1. Introduction

Sea urchins are invertebrates belonging to the class *Echinoidea* (phylum *Echinodermata*), which incorporates more than 700 described extant species worldwide. Recently, they have been the object of growing interest as available sources of new biomolecules which, on the basis of their activity as antibacterial, antiviral, antiprotozoal, antifungal, and anticancer agents, may be potential candidates for drug discovery and development [1–4]. Indeed, these animals have developed complex molecular mechanisms to use in various biological processes, such as communication, defense, reproduction, and antimicrobial resistance. The chemical components of such machineries can be beneficial to drug design due to their interactions with some selected proteins that have been conserved during the evolution and whose human orthologues are likely to be implicated in different pathobiological processes which control disease development, e.g., cell proliferation and death or immune and inflammatory responses [5].

Li et al. [6,7], in their study, reported for the first time the isolation and the chemical characterization of antimicrobial peptides (AMPs), named strongylocins 1, 2, and centrocins 1, 2, derived from the coelomocytes extract of the green sea urchin *Strongylocentrotus droebachiensis*. These peptides showed a significant antibacterial activity against both Gram-positive and Gram-negative bacteria.

Further studies conducted by Björn et al. [8] demonstrated that the synthetic peptides sequentially derived from the previously characterized centrocin 1 (CEN1 HC-Br and CEN1 HC) showed prominent microbicidal and anti-inflammatory activities on in vivo and in vitro mammalian models, representing an interesting resource for the treatment of infections in humans.

Furthermore, a recent study by Katelnikova et al. [9] highlighted that a glycopeptide fraction (GPF) derived from internal organs of the green sea urchins *Strongylocentrotus droebachiensis* possesses relevant anti-inflammatory effects, especially for the treatment of bronchitis. Moreover, the absorption and pharmacokinetics of GPF following single and repeated intranasal administration were evaluated over the course of seven days in rats [10].

Another echinoderm species from which bioactive molecules were studied is *Arbacia lixula*, commonly found in the Mediterranean Sea and in the African Atlantic coast [11]. So far, the biomedical applications of molecules extracted from this marine invertebrate are very limited. Of note, Stabili et al. [12] demonstrated the powerful antioxidant effect exerted by the lysate of *A. lixula*'s coelomocytes, the immune cells present in the coelomic fluid (CF) of the sea urchin, whereas Cirino et al. [13] highlighted that *A. lixula*'s eggs are a rich source of astaxanthin, a radical scavenger that can prevent neurodegenerative diseases. These promising findings prompted an extension of the study on the pharmacological potential of *A. lixula*-derived substances, whose massive sourcing represents an easy task due to the abundance of this marine species in waters surrounding Sicily, also being a thermophilic species which is expanding very quickly due to the increase of the surface temperature of the Mediterranean Sea [14].

Triple-negative breast cancers (TNBC) are categorized among the highly "aggressive" malignant carcinomas. In fact, the lack of expression of estrogen, progesterone, and epidermal growth factor type 2 (HER2) receptors makes them poorly responsive to hormonal therapies and to HER2-targeting drugs, and the TNBC histotype is typically associated with a poorer prognosis than other breast tumors [15]. In light of the paucity of treatment options against this cancer type, multiple approaches have been attempted to discover novel compounds which might interfere with TNBC cell growth and expansion. On the other hand, the development of targeted therapies necessarily requires a detailed examination of the biological aspects of such compounds, aimed to unveil the molecular mechanisms underlying their bioactivity.

In the present paper, the effect of the treatment of the TNBC cell line MDA-MB231 with crude and filtered water extracts of the CF obtained from *A. lixula* was examined. The cell line chosen as the in vitro model system for the present investigation comes from a pleural effusion of a TNBC of the basal subtype and is endowed with properties correlated to a highly malignant phenotype, i.e., the inactivation of p53 owed to a mutation in codon 280 of exon 8, and the capability to form metastases in vivo [16–18]. The aim of our study was to explore the CF extract-mediated effects, if any, on viability cell cycle distribution, apoptosis, autophagic activity, and mitochondrial polarization/cell redox state of cancer cells. Here, we report that both crude extracts and <10 kDa-filtered fractions are cytotoxic against MDA-MB231 cells. Interestingly, their specific cytotoxic activity, when administered at 48 h half maximal inhibitory concentration (IC_{50}), appears to involve different processes among those analyzed. In fact, in the presence of both the crude and filtered extracts, cells underwent cycle perturbation, but a prominent mitochondrial depolarization and a relatively low increase of reactive oxygen species (ROS) was observed in the former case, whereas a higher increase of ROS, the upregulation of the autophagic activity, and the lack of dissipation of transmembrane mitochondrial potential (MMP) was observed in the latter case. In the light of our pilot study, the CF extracts from *A. lixula* appear to merit more detailed investigations, also performed in in vivo models.

2. Materials and Methods

2.1. Catching and Safekeeping of the Animals

A total of 24 individuals of *A. lixula* sea urchins (Figure 1) were caught in the Gulf of Palermo (Sicily, Italy) at a depth of 5–10 m, near a grassland of *Posidonia oceanica*. The species was identified by V. Arizza, coauthor of this work and Full Professor of Zoology at the University of Palermo (Italy). Before starting the experiments, the animals were kept in aquaria for two weeks for acclimation in artificial sea water (9 mM KCl; 0.425 M NaCl; 0.0255 M MgSO₄·7H₂O; 9.3 mM CaCl₂·2H₂O; 0.023 MgCl₂·6H₂O; 2 mM NaHCO₃, pH 8.0) at 15 ± 2 °C with constant oxygenation, and fed with Algamac 3000 (Aqua fauna Bio-Marine Inc., Hawthorne, CA, USA).



Figure 1. A specimen of *Arbacia lixula* sea urchin.

2.2. Bleeding Procedure and Manipulation of the CF

After cutting the peristomial membrane of the sea urchins, the CF was collected separately, on ice, in the presence of ISO-EDTA anticoagulant (20 mM Tris, 0.5 mM NaCl, 70 mM EDTA; pH 7.5). For each animal, an average of 8 mL of CF was obtained. Subsequently, the samples were immediately centrifuged at 1000× *g* for 10 min at 4 °C to discard the coelomocytes. Half of each sample (cell-free CF) was stored at −80 °C and the remaining fluid was filtered by centrifugation with Corning Spin-X UF 6 concentrators, equipped with polyethersulfone membranes (10 K MWCO; Corning Inc., Tewksbury, MA, USA) at maximum speed (4000× *g*) for ~ 3–4 h at 4 °C and then stored at −80 °C. Both the crude and centrifuged CFs, hereafter referred as crude extracts and 10K fraction respectively, were lyophilized in an Alpha 2–4 LD plus freeze-dryer (Martin Christ, Osterode am Harz, D). The powders obtained were then grouped together to have a preparation of total crude extracts and another preparation of total 10K fractions. Both preparations were resuspended in sterile distilled water, with the smallest possible volume obtaining 8.7 mL of crude extract solution and 3.0 mL of 10K fraction solution. Their protein concentrations were evaluated with the Qubit Protein Assay Kit in a Qubit 3.0 fluorometer (ThermoFisher, Waltham, MA, USA), according to the manufacturer's instructions, and the values were 396 and 390 µg/mL, respectively.

2.3. Cell Cultures

MDA-MB231 TNBC cells, taken from laboratory stocks, were cultured in D-MEM medium (Sigma, St. Louis, MO, USA) containing 10% fetal calf serum (FCS; ThermoFisher, Waltham, MA, USA) and antibiotic/antimycotic mixture (100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 mg/L amphotericin B; ThermoFisher, Waltham, MA, USA), at 37 °C in a 5% CO₂ atmosphere.

2.4. Cell Viability Assay

Cell viability was examined with an MTT assay [19]. Essentially, MDA-MB231 cells were seeded at a concentration of 5500 cells/well in 96-well plates, and, after overnight adhesion, exposed to

1, 2.5, 5, or 10 µg/mL of either crude extracts or 10K fractions for 48 h. After addition of MTT, cells were incubated with the solubilization buffer and the absorbance of the dissolved formazan was quantitated in an automated microplate reader ($\lambda = 550$ nm). After calculation of the viability ratio between treated and control cells, the IC₅₀ was evaluated with the online calculator available at <https://www.aatbio.com/tools/ic50-calculator> (accessed on 14th June 2019).

2.5. Flow Cytometry Assays

The following assays were performed as described in References [20,21] using a FACSCanto flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). All the data were analyzed with Flowing Software v.2.5.1 (Turku, SF, Finland). Before each analysis, gating in the FSC versus SSC plot was performed to exclude cell debris displaying low FSC values.

2.6. Cell Cycle

Cell cycle distribution was analyzed after cell fixation with cold 70% ethanol, incubation with 40 µg RNase A/mL, and staining with 20 µg propidium iodide/mL.

2.7. Apoptosis

To individuate the possible onset of apoptosis, the externalization of phosphatidylserine was checked using the Annexin V-FITC kit (Canvax Biotech, Cordoba, Spain), following the manufacturer's instructions.

2.8. MMP

The MMP was monitored by incubating control and treated cells with the dye JC1 (Molecular Probes, Eugene, OR, USA), which produces an intense red fluorescence emission, that is measured in the FL2 channel (585 nm), in case of physiologic MMP, whereas in case of depolarization, it shows a green fluorescence that is measured in the FL1 channel (530 nm). A positive control for MMP loss was made by treating cells with 1 µM valinomycin.

2.9. ROS Production

The extent of ROS accumulation was checked with the ROS Detection Assay Kit (Canvax Biotech, Cordoba, Spain) according to the manufacturer's instructions. A positive control for ROS over-production was made by treating cells with 50 µM H₂O₂.

2.10. Acidic Vesicular Organelles (AVOs)

In order to quantitate the extent of the acidic vesicular organelle (AVO) accumulation, after fixation with cold 70% ethanol, cells were stained with 100 µg acridine orange/mL (Sigma) for 20 min in the dark before the analysis.

2.11. Beclin-1, LC-3-II, and Total LC-3 Expression

In order to quantitate the intracellular amount of the autophagic markers beclin-1 and total LC-3, after fixation with HistoChoice (Amresco, Solon, OH, USA) for 30 min at ambient temperature and wash with phosphate-buffered saline (PBS) + 2% FCS, cells were incubated with the primary antibody dissolved in PBS containing 0.1% Triton X-100, 2% FCS, and 40 µg propidium iodide/mL for 30 min. The primary antibodies were rabbit polyclonal anti-beclin-1 (H-300, sc-11427, Santa Cruz Biotechnology, Dallas, TX, USA; working dilution 1:50, as recommended by He et al. [22]), and rabbit polyclonal anti-LC3 (L8918, Sigma; working dilution 1:250). In the negative controls, the primary antibodies were omitted. The samples were exhaustively washed and incubated with the secondary antibody (FITC-conjugated goat anti-rabbit IgG whole molecule, F0382, Sigma; working dilution 1:80) for 30 min, and again washed three times in PBS and analyzed in the flow cytometer. The evaluation

of the autophagic marker LC3-II expression was made according to Eng et al. [23]. To this purpose, unfixed cells were resuspended in PBS + 0.05% saponin (Sigma, St. Louis, MO, USA) to extract the non-autophagosome-associated soluble LC3-I, and then incubated with the primary and secondary antibodies as in the previous protocol, with the exception that all the dilution buffers contained 0.05% saponin, which also replaced Triton X-100 (Sigma, St. Louis, MO, USA).

2.12. Statistics

Analysis of variance (ANOVA) tests were performed with SigmaPlot 11.0 software (SYSTAT, San Jose, CA, USA), considering a p -value < 0.05 as statistically significant.

3. Results

In a first set of assays, we examined the effect exerted by incubations with either crude extracts or 10K fractions of *A. lixula*'s CF on MDA-MB231 cell survival via an MTT assay. As shown in Figure 2, cell exposure for 48 h to both preparations resulted in a concentration-dependent decrease of cell viability with an average IC_{50} of 9.16 and 8.34 $\mu\text{g/mL}$ for the crude extract and 10K fraction respectively, thereby indicating that the latter preparation is more powerful in exerting its inhibitory effect. These IC_{50} concentrations were used in the following experiments, aimed to gather more detailed data on the molecular aspects of CF extract-induced toxicity on MDA-MB231 cells via a panel of flow cytometry assays.

First, we checked which kind of perturbation was induced on cell cycle by treatment with either crude extracts or 10K fractions. To this purpose, the cells exposed to the preparations for 48 h were stained with propidium iodide and submitted to flow cytometric analysis of the distribution of cell cycle phases. Figure 3 shows that, if compared to controls, in both cases, the result of the exposure was an accumulation of cells in the S phase fraction (average control versus crude extract versus 10K fraction = 5.4% versus 23.5% versus 19.1%), with a reduction of cell percentages in the other two fractions (G_0/G_1 : average control versus crude extract versus 10K fraction = 33.9% versus 24.7% versus 27.6%; G_2/M : average control versus crude extract versus 10K fraction = 59.5% versus 48.2% versus 47.1%). The sub- G_0/G_1 fraction was negligible, accounting for less than 0.6% in all samples.

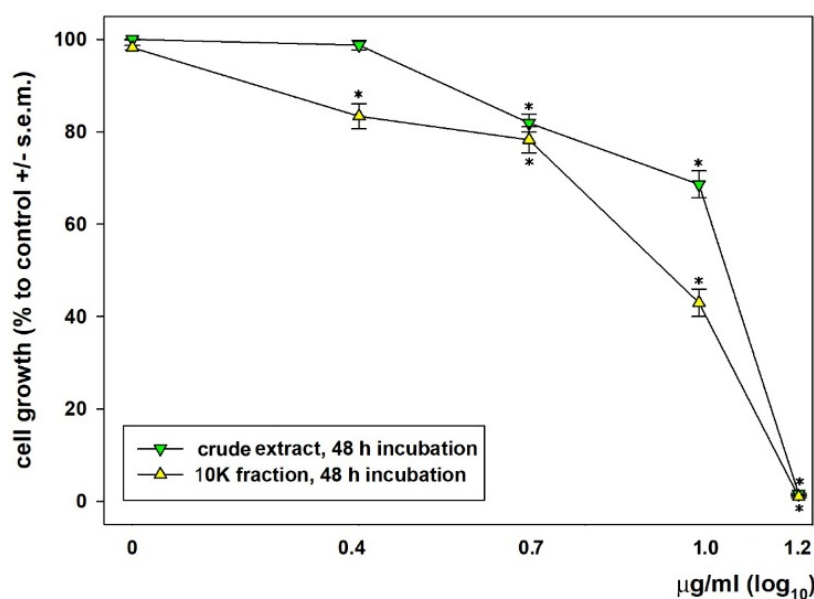


Figure 2. Dose-response effect of crude extracts and 10K fractions of *A. lixula*'s CF on the viability of MDA-MB231 cells after 48 h of treatment. The error bars indicate the standard error of the mean (s.e.m.) of three independent measurements. * $p < 0.05$.

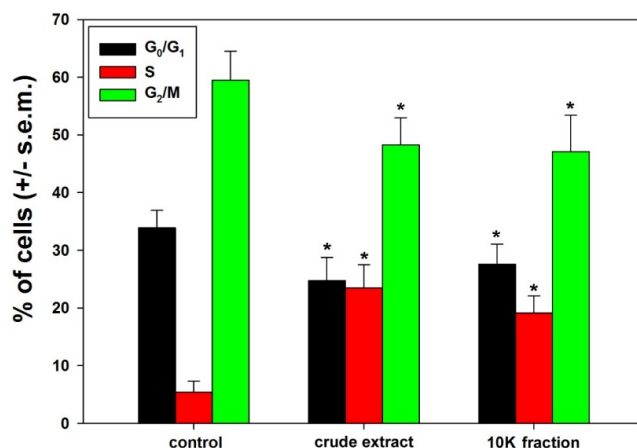


Figure 3. Effect of crude extracts (9.16 µg/mL) and 10K fractions (8.34 µg/mL) of *A. lixula*'s CF on the cell cycle distribution of MDA-MB231 cells after 48 h of treatment, compared to controls. The error bars indicate the standard error of the mean (s.e.m.) of three independent measurements. * $p < 0.05$ versus control.

Then, control and exposed cells were submitted to annexin V-FITC/propidium iodide staining in order to check whether treatment with either crude extracts or 10K fractions could induce phosphatidylserine externalization, which is a hallmark of apoptosis promotion. The data obtained showed no difference between the two experimental conditions with a nearly total absence of annexin V+/propidium iodide cells (about 0.3% of the tested populations; data not shown), thereby demonstrating that there was no involvement of programmed cell death in CF extract-triggered cytotoxic effect. The same result was obtained repeating the assay at earlier times, i.e., after 12 and 24 h of exposure.

Subsequently, we examined whether cell treatment could affect mitochondrial integrity and activity by monitoring mitochondrial polarization status with the membrane-permeable JC1 dye, which is sensitive to changes in MMP. Figure 4 shows that a drastic loss of the MMP can be recorded only in crude extract-treated cells, with the percentage of low red fluorescence-emitting events (bottom quadrant) rising from about 44.9% of the control to 85.5% after exposure, a value close to that of the positive control obtained incubating cells with the ionophore valinomycin (about 95%). On the other hand, the results obtained after cell exposure to the 10K fraction were comparable to those of the untreated controls.

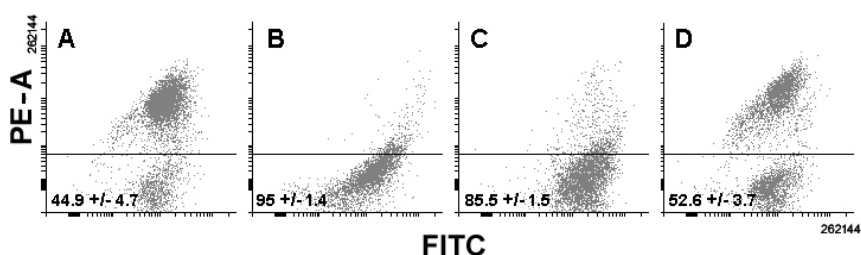


Figure 4. Representative flow cytometric analyses of the MMP in untreated (A), valinomycin- (B); 1 µM), crude extract- (C); 9.16 µg/mL), and 10K fraction-treated (D); 8.34 µg/mL) MDA-MB231 cells for 48 h. The x- and y-axes report the intensities of green and red fluorescence emitted by JC-1 in physiologic or depolarization conditions, respectively. The ionophore valinomycin was used as a positive control to dissipate MMP. The values indicated in the bottom quadrants in each frame correspond to the percentage of low red fluorescence-emitting events attributable to cells that underwent dissipation of MMP. The data are expressed as mean ± s.e.m. of three independent assays.

The possible imbalance of cell redox state was checked through flow cytometric evaluation of intracellular ROS accumulation after gating to exclude from the assay the aliquot of low

fluorescence-emitting dead cells identified for their positivity to propidium iodide staining. As shown in Figure 5, exposure to both preparations determined the upregulation of ROS levels in MDA-MB231 cells but to a lesser extent, i.e., up to $9.3\% \pm 3.1\%$ versus controls, in the presence of the crude extract compared to the rise up to $16.25\% \pm 3.1\%$ versus controls in case of treatment with the 10K fraction.

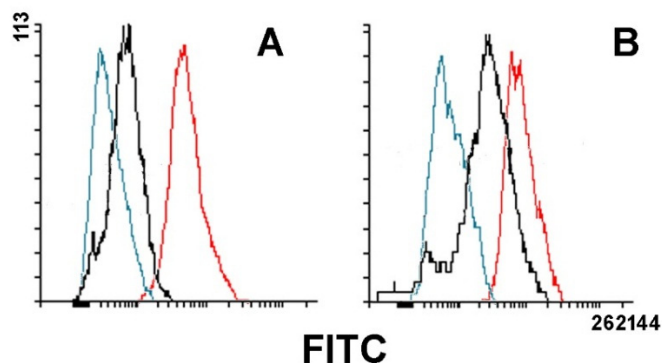


Figure 5. Representative plots showing the accumulation of ROS in gated alive MDA-MB231 cells treated with crude extracts ((A); $9.16 \mu\text{g/mL}$) and 10K fractions ((B); $8.34 \mu\text{g/mL}$) for 48 h (black peaks). The blue peaks correspond to the untreated cell preparations. The orange peaks originate from preparations of cells exposed to H_2O_2 and therefore over-producing ROS. The x-axis reports the intensity of green fluorescence emitted by the dye 2',7'-dichlorodihydrofluorescein, which is proportional to the amount of intracellular ROS.

The last set of analyses was aimed at checking whether the CF-derived preparations could induce a modification of cancer cell autophagic behavior. To this purpose, we first assessed the possible change in the amount of the autophagy characteristic AVOs, which are mainly autophagolysosomes, after staining with acridine orange, a green fluorophore that undergoes a metachromatic shift to red fluorescence when uptaken and concentrated into the organelles. Figure 6 shows that only 10K fraction-treated cells underwent a consistent increase of red fluorescence up to $15.2\% \pm 4\%$ versus controls, with the fluorescence intensities of crude extract-exposed samples being comparable to those of the untreated controls. Therefore, these latter preparations were not subjected to further analyses.

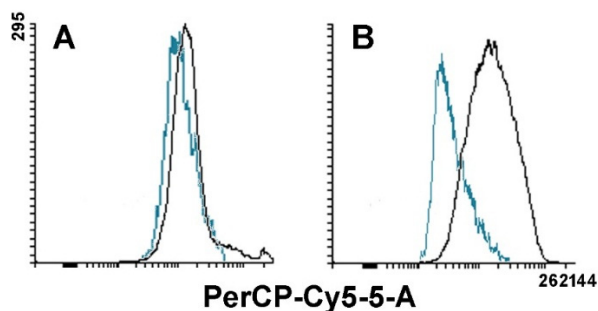


Figure 6. Representative plots showing AVO accumulation in MDA-MB231 cells exposed to crude extracts ((A); $9.16 \mu\text{g/mL}$) and 10K fractions ((B); $8.34 \mu\text{g/mL}$) for 48 h (black peaks). The blue peaks correspond to the untreated cell preparations. The x-axis reports the intensity of red fluorescence emitted by the dye acridine orange, which is proportional to the amount of intracellular AVOs.

It is known that acridine orange can also accumulate in other acidic non-lysosomal compartments [24] and therefore, it represents a reliable marker for monitoring autophagy only when used in conjunction with other molecular markers [25]. Hence, to confirm the acridine orange results of 10K fraction-treated MDA-MB231 cells, we examined the intracellular accumulation levels of beclin-1, a core component of the beclin 1-PI3KC3 complex, which is involved in autophagosome biogenesis and maturation [26], and LC3-II, the autophagosome-linked proteolyzed and lipidated form of the inactive

soluble LC3-I protein, whose concentration increases upon autophagy upregulation [25]. For LC3-II quantification, as reported by Eng et al. [23], cells were not submitted to fixation and the immunoreactive cytosolic LC3-I was extracted and removed by cell resuspension in a saponin-containing buffer before the flow cytometric assays. Moreover, in light of the conflicting evidence about the increment of LC3 transcription levels in various conditions of cell stress-induced autophagy [27–29], we wanted to also study the effect of tumor cell exposure to 10K fractions on the intracellular accumulation of total LC3 through flow cytometric immunoquantitation carried out on fixed cell preparations. The obtained results are shown in Figure 7. Previous observations pointed out the high basal autophagy level of MDA-MB231 cells [20,21,30]. The present results show an accumulation of autophagy markers after cell exposure to the 10K fractions. In fact, under the experimental conditions, the tested amounts of beclin-1, LC3-II, and total LC3 are upregulated by about 1.9-, 1.35-, and 1.7-fold versus controls respectively, thus also substantiating the occurrence of LC3 transcriptional activation in exposed MDA-MB231 cells.

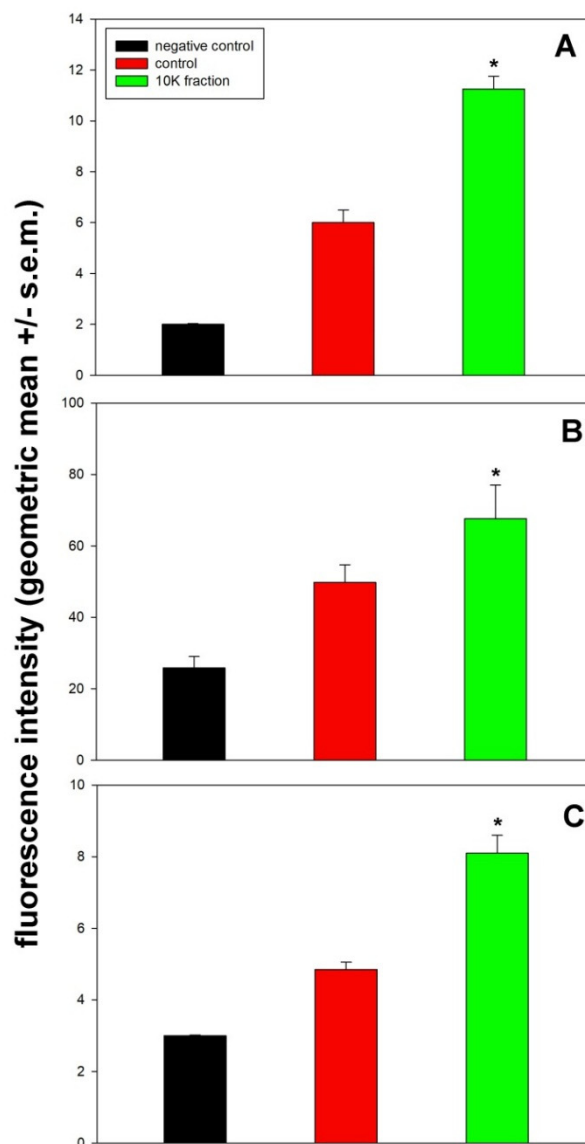


Figure 7. Histograms showing the intensity of the immunofluorescence of beclin-1 (A), LC3-II (B), and total LC3 (C) in MDA-MB-231 cells after 48 h of treatment with 10K fractions from *A. lixula*'s CF, compared to untreated controls. Negative controls lack the incubation with the primary antibody. The error bars indicate the standard error of the mean (s.e.m.) of three independent measurements. * $p < 0.05$.

4. Discussion

Breast cancer is the most common diagnosed tumor among females, being a widely spread neoplasia that accounts for about 12% of all cancers and whose incidence is globally increasing [31]. The TNBC subtype accounts for approximately 15% of breast cancers and it lacks effective targeted therapies, thereby being considered as the most aggressive form of breast tumors to date [32]. In the attempt to identify new compounds endowed with anti-breast cancer action, in the present paper, we have assayed the cytotoxic effect of water extracts of CFs from *A. lixula* on MDA-MB231 TNBC cells, also comparing the activity of the crude samples to that of the centrifuged counterparts retaining peptides and other water-soluble molecules with M.W. < 10 kDa. In preliminary assays (unpublished data), the earlier (i.e., at 24 h) occurrence of cytotoxic effects after MDA-MB231 cell exposure to the preparations was also examined. After identification of the IC₅₀ of the crude extracts and 10K fractions (10.32 and 11.53 µg/mL, respectively) and cell treatment, nonetheless, in this time frame, the sole modification observed was the occurrence of mitochondrial depolarization in the presence of the crude extract down to percentages close to those of the corresponding positive control, whereas there was no difference between treated and control cells in all the other assays. The late occurrence of the phenotypic effects observed, i.e., after 48 h of exposure to CF extracts, is in line with the widely recognized resistance of the MDA-MB231 cell line to treatments and its delayed responses [33,34]. In the light of such preliminary evidence, we decided to examine, in detail, only the effects after 48 h of exposure.

Therefore, to the best of our knowledge, here we report the first data demonstrating an anti-TNBC effect by *A. lixula*-derived extracts. Indeed, our results show that 48 h exposure to either preparation decreases tumor cell number in a dose-response manner. On the other hand, with respect to the biological endpoints chosen, the crude extracts and 10K fractions appeared not to elicit fully overlapping effects. In fact, only the filtered CF samples were able to induce the selective upregulation of autophagy in the absence of the MMP dissipation observed in the presence of the crude extract. Moreover, the upregulation of ROS was about 7-fold more prominent in the 10K fraction-treated cell cultures. Although at present we have no explicative evidence for these different behaviors, it might be assumed that the ultrafiltration to which the 10K fraction is subjected determines the concentration of the responsible compound(s) that, once the threshold level is reached and exceeded, could switch-on a cellular response leading to a more pronounced imbalance of the redox state and an increase in the autophagic activity. Alternatively, ultrafiltration might deplete the 10K fractions of factor(s) counteracting these biological processes, that instead remain(s) available in the crude extract.

One of the most intriguing data reported here is the link between cell exposure to the *A. lixula*-derived 10K fraction and the promotion of the autophagic activity, a biological implication of treatment with extracts from marine organisms which is still poorly investigated in the literature. In particular, our results suggest that autophagy upregulation occurs in parallel with ROS overproduction and S-phase block but in the absence of MMP loss and apoptosis incurrence. Cellular autophagy is a homeostatic autodigestive activity which implies the removal or turnover of cytoplasmic substrates via sequestration in the multi-membrane-bound autophagic vacuoles that eventually fuse with lysosomes generating AVOs or autolysosomes [35]. Autophagy plays a complex and controversial role in breast cancer, operating as either a tumor-suppressor system addressing neoplastic cells to death, or a cancer cell protective system which provides energy and basic elements to allow a fast-proliferative rate in conditions of oxygen and nutrient deficiency [36]. MDA-MB-231 cells are characterized by a constitutively elevated autophagy rate and autophagy restraining has made them vulnerable to cytotoxic treatments in various experimental conditions [20,21,37,38]. On the other hand, it is widely recognized that chemical induction of autophagy can be lethal to MDA-MB231 cells, as reported in some published papers [39,40]. Taking our and literature data into account, we can advance the following hypotheses.

Chen et al. [41] reported that oxidative stress is able to selectively induce autophagic cell death without switch-on of apoptosis in transformed and tumor cells. Thus, it is conceivable that, in the

presence of the 10K fractions, cells undergo metabolic stress and derangement in the antioxidant system in the absence of mitochondrial potential dysfunction with a consequent high rise of the endogenous ROS levels. In turn ROS, acting as redox signaling molecules, may trigger an intracellular transduction pathway leading to autophagy upregulation. Alternatively, as reported by Ling et al. [42] for safinigol-treated MDA-MB231 cells, the rise of autophagic activity could be interpreted as a cell repair mechanism activated to counteract ROS-induced necrotic death but resulting in a wasted effort to maintain cell survival. However, although acridine orange stain and assessment of beclin-1 and LC3-II levels are commonly-used markers for autophagy, in order to get a complete picture of the autophagic flux and activity in our experimental system, as suggested by Klionsky et al. [25], it will be necessary to obtain complementary results with additional methodologies. Therefore, further studies will be addressed to a better clarification of this aspect of CF extract cytotoxicity.

Furthermore, turning to the cytotoxic effect exerted by the total extract on TNBC cells, it may be assumed that cell damage and eventual death is mainly based upon the early and persistent depolarization of mitochondria and consequent loss of function, which can determine an insufficient ability to produce ATP and also interfere with ROS production, thereby accounting for the lower level of ROS accumulation recorded in our experimental system.

Exposure to both crude and filtered CF extracts were also proven to exert a selective effect on cell cycle progression by fostering a noticeable accumulation of MDA-MB231 cells at the S phase. It is conceivable that the induced cell damage also extends to the DNA replication process with a consequent deceleration, if not an arrest, of the specific cycle phase compared to control cells [43]. Taking the cumulative effects exerted by the 10K fraction on TNBC cells into account, it is worth mentioning that molecules which promote autophagy were also proven to induce a preferential impairment of cell cycle progression in the S phase [44,45]. Interestingly, a similar cell cycle-altering property was also found with the water extracts obtained from the sea cucumber *Holothuria polii*, another Mediterranean echinoderm, able to inhibit the S phase progression of MDA-MB231 cells after three days of treatment [46].

Further investigations are needed to get into overall details of the molecular processes through samples under study to accomplish their cytotoxic activity. Nonetheless, our work presents the first data on an additional example of extracts from marine organisms that exhibit antitumoral, in particular, anti-TNBC, activity in pre-clinical studies. Moreover, it gives the second documented evidence of the autophagy-modulating ability owned by extracts obtained by sea urchins, following the paper of Russo et al. [47] about the effect of *Paracentrotus lividus*'s ovothiol on HEP-G2 hepatocarcinoma cells, also providing additional insight into the role played by the autophagic activity in breast cancer cell death. Of note, an in vitro anti-TNBC activity was also described in a previous paper by our group [48], exposing MDA-MB231 cells to the CF extracts of another echinoderm species, i.e., *Holothuria tubulosa*.

This study has a current limitation, i.e., the lack of identification of the component(s) of *A. lixula*'s CF responsible of the effects described here, which has/have not been isolated to date, although separation experiments are in progress. On the other hand, the results shown here strongly suggest that more than just one constituent must be involved in the promotion of the described effects. Our future goal is the characterization and sequencing of the bioactive molecules from *A. lixula*, according to Li et al. [6,7]. To date, we know that these compounds, for the first time extracted and evaluated in cell-free CF, can withstand lyophilization, resuspension, and freeze-thawing cycles, and the massive ROS production- and autophagy-stimulating components must have a M.W. < 10 kDa since these biological activities manifest only after concentration of the crude extract by filtration. On the other hand, substances with a M.W. > 10 kDa promoting a prompt and strong MMP dissipation are surely present in the aqueous extract since this distinctive line is lost upon filtration. Moreover, it would be interesting to evaluate in vitro and in vivo the anti-inflammatory and pharmacokinetic properties of these molecules in mammalian models according to References [8,10] to contribute to the applicability of these compounds in the treatment of infections in humans.

5. Conclusions

In conclusion, although the potential anti-cancer compounds obtained from echinoderms have been widely illustrated in the literature [49], the contribution of sea urchins to this field of research is still very limited and is represented only by a few pilot studies previously published and which focused on steroidal and polysaccharidic constituents [4,50,51]. Here, we have demonstrated the cytotoxicity of extracts from *A. lixula*'s cell-free CF towards TNBC cells in vitro. Hence, this natural material is worth further exploration addressed to the identification of the water-soluble "active ingredient(s)" present to devise novel promising prevention and/or treatment agents effective against highly malignant breast carcinomas.

Author Contributions: D.R., D.M.L.A., V.L., F.A., and V.A. performed the experiments. C.L. and M.V. supervised the work and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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