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IN VITRO EVALUATION OF THE IMMUNOMODULATORY AND ANTICARCINOGENIC ACTIVITY OF THE FRESHWATER BRYOZOAN *HYALINELLA PUNCTATA* METHANOLIC EXTRACT

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The immunomodulatory and anticarcinogenic activity of the freshwater bryozoan *Hyalinella punctata* methanolic extract (MEx) was evaluated *in vitro* on selected biosystems for the first time. Murine lymphocytes and macrophages were used for testing of MEx effects on cell proliferation and nitric oxide (NO) production, respectively, while human cancer cell lines were utilized for measuring its activity against cancer cells. The results suggest a strong and tissue-specific immunomodifying activity (IC_{50} values for inhibition of proliferation of lymph node and spleen-derived lymphocytes were 4.1 µg/ml and 9.7 µg/ml, respectively) and moderate anticancer activity of MEx (IC_{50} value for the MCF-7 cell line was 24.13 µg/ml). Down-regulation of macrophage NO production was also obtained. The potential use of *H. punctata*-derived natural products for the treatment of human chronic inflammatory diseases and cancer is worthy of further investigation.

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1. Introduction

Bryozoa (moss animals) are a phylum which many people, both scientist and layperson alike, have little if any familiarity, despite the fact they are widely distributed throughout the world's marine and freshwater environments [1]. Bryozoans are sessile, colonial, filter-feeding organisms with about 4.000 extant species most of which are marine [2]. *Hyalinella punctata* (Hancock, 1850) is not a very frequent bryozoan species of freshwater origin, but it has been noticed in Serbia [3,4].

An intricate network of secondary metabolites is present in the extracts of bryozoans; with a few exceptions, bioactive natural products identified from their marine representatives so far are either alkaloids, sterols, bryostatins or heteroatom-containing compounds [5]. Like many invertebrates, bryozoans lack an immune system and are vulnerable to predation, overgrowth or infection, so these compounds might have a defensive role. However, most of the studies have focused strictly on the marine species.

Inflammation is a complex physiological process which can be defined as a response to cellular and tissue injures caused by infections or physical and chemical stimuli. When the antigen stimulus persists, or when the anti-inflammatory network does not operate efficiently,

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inflammatory cell survival is prolonged, and the acute inflammatory response becomes chronic, facilitating the development of pathological conditions [6]. Many diseases, such as cancer, diabetes, and cardiovascular disorders, have a component of inflammation, or the disease is exacerbated by inflammatory mediators. Concomitant with inflammation is the generation of reactive oxygen species (ROS), which increase oxidation of proteins and lipids, resulting in signals that trigger more inflammation. In addition to ROS, there are analogous reactive nitrogen species (RNS), including the radical nitric oxide (NO·) which is also implicated in inflammation, cancer and other pathological conditions when it is produced in excess [7]. Therefore, much attention has been focused on the use of antioxidants, especially of natural origin, to inhibit lipid peroxidation or to protect the damage of free radicals [8].

In this work, which is in the course of previous study of Pejin *et al.* on natural products of invertebrates [9], we present *in vitro* obtained data suggesting "non-food organism" *H. punctata* as a promising resource of novel substances for the modulation of inflammation and immune responses, as well as those with anticarcinogenic effects. We screened its crude methanolic extract (MEx) to identify putative new drugs with the aforementioned efficacy focusing our research on the influence on murine immune cells including lymphocytes and macrophages, and three selected human cell lines, two of which were cancer cell lines. To the best of our knowledge, this is the first report of immunomodulatory and antineoplastic activity of *H. punctata.*

2. Experimental

2.1. Animal material and extract preparation

The sample of *Hyalinella punctata* (Hancock, 1850) was collected in Belgrade (the river Danube, Serbia, November 2011). Voucher specimen has been deposited in the Zoology Collection of the Department of Biology and Ecology of the University of Novi Sad, Serbia (BRY 003). After carefully cleaning from contaminants, the bryozoan sample was lyophilized. The dried parts of *H. punctata* were ground (2 g) and extracted thrice with methanol for 30 min at room temperature, respectively. The extract was evaporated to dryness and stored at - 20 °C until further use. Stock solution for dry methanol extract (1 mg/ml) was prepared in dimethyl sulfoxide or methanol, as specified in the Results. The final concentration of diluents did not exceed 1%, which alone had no effect on cell response.

2.2. Immunomodulatory activity

2.2.1. Animals and materials

For isolation of lymph node cells (LNC), splenocytes (SPC) and resident peritoneal cells (PC), normal adult inbred C57BL/6 mice, and Dark Agouti (DA) rats were used. The animals were housed at the animal facility of the Institute for Biological Research “Sinisa Stankovic” (Belgrade, Serbia) under standard laboratory conditions (non-specific pathogen-free) with free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Institute for Biological Research “Sinisa Stankovic”, University of Belgrade and were carried out in accordance with established International Guiding Principles for Animal Research. Sex and age-matched (10-12 weeks old) animals were killed by cervical dislocation and tissues were removed aseptically. Fetal calf serum (FCS), RPMI-1640 medium, phosphate-buffered saline (PBS), lipopolysaccharide (LPS, *E. coli* 055:B5), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and concanavalin A (ConA) were purchased from Sigma (St, Louis, MO, USA). All dishes for culturing cells were from Sarstedt (Numbrecht, Germany). Recombinant mouse IFN-γ was from R&D (Minneapolis, MN, USA).

2.2.2. Cell isolation and treatment

Lymph node cells (LNC) were prepared from cervical lymph nodes, and total splenocytes (SPC) were isolated from spleens, both from age- and sex-matched healthy animals. To obtain single cell suspension, organs were mechanically disrupted by gentle teasing tissue through a cell strainer (BD Falcon, BD Bioscience, Bedford, USA), and cells were collected by centrifugation. Resident peritoneal cells (PC) were collected by peritoneal lavage with cold PBS. Erythrocytes from cell suspensions were lysed using erythrocytes lysis buffer (eBioscience, San Diego, CA, USA). Cells were washed and suspended in RPMI-1640 medium supplemented with 2mM-glutamine, 0.01% sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, antibiotics and 5% (v/v) heat-inactivated FCS (culture medium). The capacity of SPC and LNC to proliferate was determined by culturing 3×10^5 and 4×10^5 cells/well (96-well plate), respectively, for 48 h in the presence or absence of ConA and methanol extract (range 0.01 - 100 μg/ml) or the vehicle, as specified in the results. PC were stimulated for 48 h with LPS (5 μg/ml) + recombinant IFN-γ (10 ng/ml), or left non-stimulated, both in the absence or presence of methanol extract (range 0.01 - 100 μg/ml). All cultures were set up in triplicate in a final volume of 200 μl and incubated in a humidified (5% CO2, 95% air) atmosphere at 37°C. Cell viability was assessed on freshly obtained cells immediately before plating and after 48 h of cultivation by conventional trypan-blue or MTT assay, while cell-free culture supernatants were collected after 24 h and 48 h for determination of NO production by Griess reaction.

2.2.3. Determination of cell viability by trypan-blue assay

In the trypan-blue (TB) assay, the ability of viable cells to exclude the stain was estimated. Cells were collected and counted in 0.2% TB solution using a hemocytometer. The relative numbers of dead cells were calculated as percentages of the number of stained cells [10].

2.2.4. Evaluation of cell mitochondrial activity by MTT-assay

In the MTT assay, cell respiration, and thus cell viability and/or proliferation, was assessed based on the mitochondrial-dependent reduction of MTT to the colored formazan product [11]. The assay is based on the cleavage of the tetrazolium salt MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), to formazan by mitochondrial dehydrogenases in viable cells. Briefly, at the end of appropriate LNC, SC or PC treatments, cell culture supernatants were removed from the plates and MTT solution (1 mg/ml) was applied. Incubation with MTT lasted for 30 min at 37°C. DMSO was added to the cells to dissolve the formazan crystals. The absorbance was measured at 570 nm, with a correction at 690 nm, using an automated microplate reader (LKB 5060-006, LKB, Vienna, Austria).

2.2.5. Assay of NO release

Nitrite accumulation, as an indirect measure of NO release, was determined in cell culture supernatants using the Griess reaction. Briefly, 50 μl of cell culture supernatant was removed and combined an equal volume of Griess reagent (a 1:1 mixture of 0.1% naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% H_3PO_4) in a 96-well plate. The absorbance at 570 nm was determined in a microplate reader (LKB 5060-006; LKB, Vienna, Austria). The nitrite concentration was determined through a comparison with a sodium nitrite standard curve. The data obtained from triplicates are presented as μM of nitrite.

2.2.6. Statistical analysis

Results are presented as means ± SD obtained in independent experiments. Each experiment was performed in triplicate and repeated at least three times. The significance of the changes was evaluated by Student's *t*-test, using the Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA) package program and p values < 0.05 were considered to be significant.

2.3. Anticarcinogenic activity

2.3.1. Cell lines

The cell lines used in the study were MCF-7 (human breast adenocarcinoma, ATCC HTB22), HT-29 (human colon adenocarcinoma, ATCC HTB38) and MRC-5 (human foetal lung fibroblasts, ATCC CCL 171). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose, supplemented with 10% of fetal calf serum (FCS, NIVNS) and antibiotics: 100 IU/cm^3 of penicillin and 100 μg/cm^3 of streptomycin (ICN Galenika). The cells were sub-cultured twice a week and a single cell suspension was obtained using 0.25% trypsin in EDTA (Serva). All cell lines were cultured in flasks (Sarstedt, 25 cm^2) at 37^0C in the 100% humidity atmosphere and 5% of CO_2. Exponentially growing cells were used throughout the assays. The cell density (number of cells per unit volume) and percentage of viable cells were performed as previously described [12]. Viability of cells used in the assay was over 95%.

2.3.2. MTT assay

Cytotoxicity was evaluated by tetrazolium colorimetric MTT assay (SIGMA). Cells were plated into 96-well microtitar plates (Costar) in a volume of 90 μl per well, in the complete medium at optimal seeding density of 5×10^3 cells per well to assure logarithmic growth rate throughout the assay period. Tested substances at concentration ranging from 10^{-8} μg to 10^{-4} μg were added to all wells except to the control ones. Plates were incubated at 37°C for 48 h. Three hours before the end of incubation period 10 μl of MTT solution (5 mg/ml) was added to all wells and plates were incubated for 3 h at 37^0C, after which medium and MTT were removed by suction. The formazan product was then solubilised in 100 μl 0.04 M HCl of isopropanol. After a few minutes at room temperature, the plates were read on a spectrophotometer plate reader (Multiscan MCC340, Labsystems) at 540/690 nm. The wells without cells containing complete medium and MTT only acted as blank. Cytotoxicity was expressed as a percent and calculated according the formula: $\% C = 1 - (OD_{test}/OD_{control}) \times 100$.

2.3.3. Data analysis

Two independent experiments were set out with quadruplicate wells for each concentration of the compound. IC_{50} value defines the dose of compound that inhibits cell growth by 50%. The IC_{50} of the extract was determined by Median effect analysis [11].

3. Results

3.1. The effect of MEx on lymphocyte proliferation

To define the influence of MEx on lymphocyte proliferation, LNC were isolated from C57BL/6 mice, and were stimulated with polyclonal mitogen ConA (1 μg/ml) in the absence or presence of wide range of ten-fold concentrations of MEx (ConA-stimulated proliferation), or the cells were cultured with MEx but left without mitogen (spontaneous proliferation). Since lymphocyte blastic transformation [13] and associated increased mitochondrial activity [11] are among the earliest cellular changes attributed to mitogen or antigen-stimulated lymphocyte proliferation, we determined mitochondrial activity of the cells using MTT assay. The results obtained clearly showed that MEx significantly inhibited spontaneous, as well as ConA-induced LNC proliferation only in the highest extract doses tested, i.e. 10 and 100 μg/ml of MEx, respectively (Figure 1); the corresponding concentrations of vehicle had no effect on cell responses even at the highest dose present (1% v/v). Interestingly, while 100 μg/ml of MEx was toxic to the cells, the dose of 10 μg/ml produced cytostatic effects, as revealed by cell morphology and TB exclusion assay (not shown). In the next set of experiments we examined the effect of MEx on ConA-induced proliferation of lymphocyte populations present within two different immune organs, i.e. lymph nodes and spleen,

both derived from Dark Agouti rats. As monitored by MTT assay, mitochondrial activity of stimulated lymphocytes was compromised by MEx in a dose-dependent way (Figure 2). However, the inhibitory effect was tissue-specific, since the IC_{50} values, which stand for inhibition of LNC and SPC growth by 50%, were 4.1 µg/ml and 9.7 µg/ml, respectively. Moreover, the dose-dependent inhibitory effect of MEx on ConA-stimulated lymphocytes was further evaluated by light microscopy of the cell cultures. In the presence of MEx there was a dose-dependent decrease of cell aggregates (the sign of intensive cell-cell interactions), as well as the reduction of enlarged "blast cell" appearance (cells in the phase of proliferation) as the MEx concentration increased (from 1.6 - 50 µg/ml) (not shown). As revealed by cell morphology and TB exclusion assay (not shown), even the highest tested dose of 50 µg/ml MEx was not toxic to LNC or SPC, but only the morphology of cells was different, i.e. ConA did not induce blasts in the presence of extract.

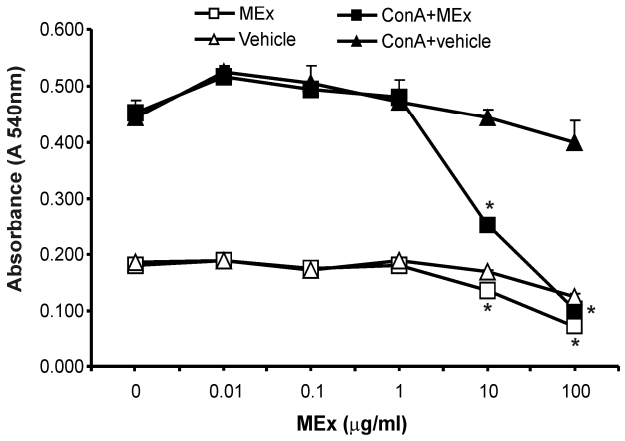

Fig. 1. The effect of MEx on mouse lymphocyte proliferation. Mouse LNC (4×10^5/well) were stimulated with Con A (1 µg/ml), or were without mitogen (spontaneous proliferation) and were cultured for 48 h in the presence of varying concentrations of MEx, or corresponding DMSO concentrations, or in medium alone. Cell proliferation was measured using colorimetric MTT assay. %.$p<0.05$ (Student's t test) refers to values obtained in control.*

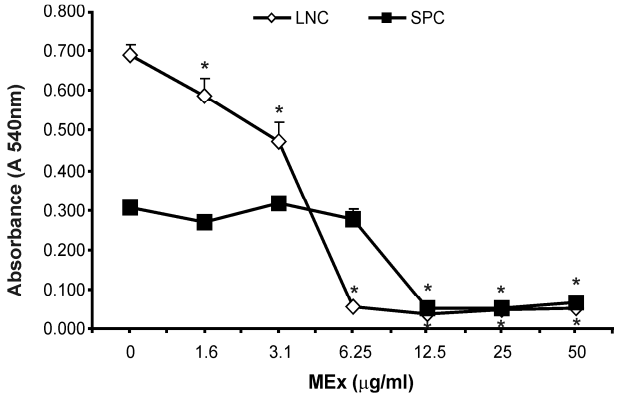

Fig. 2. The effect of MEx on ConA-induced proliferation of rat lymphocytes. Rat LNC (3×10^5/well) and SPC (3×10^5/well) were stimulated with Con A (2.5 µg/ml) in the presence of varying concentrations of MEx and cell proliferation was measured using colorimetric MTT assay. %.$p<0.05$ (Student's t test) refers to values obtained in control.*

3.2. The effects of MEx on macrophage NO production

The next experiments has been undertaken to assess the effects of MEx on the release of NO as part of the macrophagic response. To that end, we employed cells that are known to respond to proper stimuli with enhanced NO production: the primary cultures of mouse PC, as well as resident rat spleen macrophages. NO is generated by the enzyme nitric oxide synthase (NOS) that can be induced in murine macrophages by lipopolysaccharide (LPS) and cytokine IFN-γ. In order to test the effect of MEx on PC, cells were treated with different doses of MEx (0.01-100 μg/ml) in the presence of LPS + IFN-γ as murine macrophage stimulators, or were left nonstimulated. Since macrophages usually do not proliferate, MTT assay was used to control PC survival. Obtained results revealed that only the highest dose of MEx (100 μg/ml) impaired PC viability (Figure 3a). As evaluated by Griess reaction, activation of cells with LPS + IFN-γ induced significant NO production, while NO release by resident nonstimulated PC was at low level (Fig. 3b). However, in the presence of MEx, NO release into cell culture of these cells was significantly reduced. Importantly, down-regulation of NO was obtained even with the MEx doses that did not affect cell survival (1 and 10 μg/ml) (Figure 3a *versus* Figure 3b). In addition, MEx *per se* did not trigger nitrite accumulation, suggesting that it does not interfere with the basal processes involved in NO generation, nor it contains NO stimulatory components. Since rat spleens are rich in macrophages (approximately 10% of total SPC, our unpublished observation), we also tested the effect of MEx on NO production of SPC. In order to stimulate spleen macrophages indirectly, through endogenous lymphocyte products, we used T lymphocyte mitogen ConA. Examination of culture media from ConA-stimulated SPC revealed that spleen macrophages produced readily detectable NO (Figure 4). Addition of the MEx caused a reduction in measured amounts of nitrite and the observed scavenging of NO by the extract was concentration-dependent (Figure 4). Together, these results indicate that MEx is negative regulator of macrophage NO response to proper stimuli.

3.3. The influence of MEx on viability of three human cell lines

The anticarcinogenic activity of MEx was evaluated by MTT assay against two human cancer cell lines (MCF-7 and HT-29) and one human normal cell line (MRC-5). As depicted in Figure 5, MCF-7 human breast adenocarcinoma cells were only found to be sensitive to the extract (IC_{50} value 24.13 μg/ml).

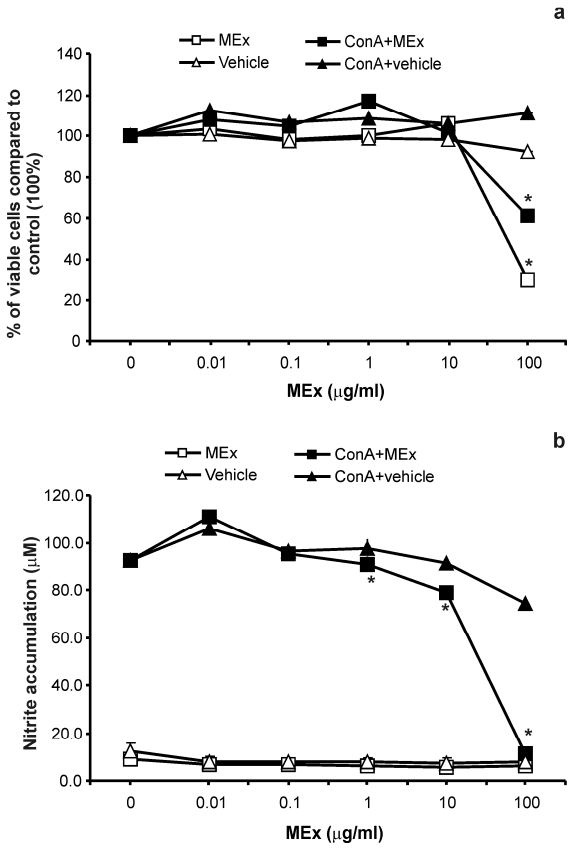

*Fig.3. The effect of MEx on PC viability and NO production. Nonstimulated or LPS (5 μg/ml) + IFN-γ (10 ng/ml) stimulated mouse PC (2×10^5 cell/well) were cultured for 48 h in the absence or presence of different doses of MEx as indicated. (a) Assessment of viability was performed by MTT test. Data are presented as % of viable cells compared to the values of matching untreated or LPS+IFN-γ-treated cell cultures which was taken as 100%. (b) Cell-free supernatants were collected from the cell cultures and analyzed for levels of nitrites by Gries reaction. Data from one representative experiment are presented as mean nitrite concentration (triplicate cultures) ± SD. *p<0.05 (Student's t test) refers to values obtained in control.*

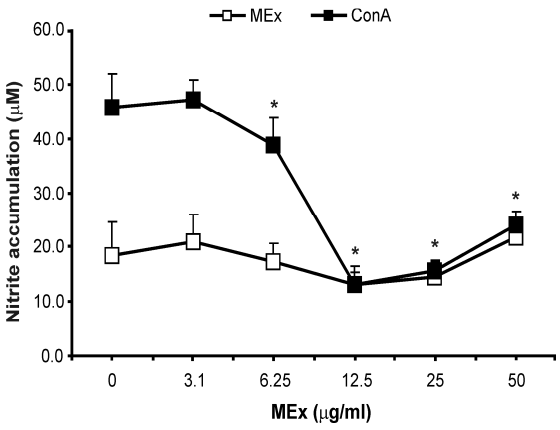

*Fig. 4. The effect of MEx on NO production by SPC. Nonstimulated or ConA (2.5 μg/ml) stimulated rat SPC (3×10^5 cell/well) were cultured for 48 h in the absence or presence of different doses of MEx as indicated. Subsequently, cell-free supernatants were collected from the cell cultures and analyzed for levels of nitrites by Gries reaction. Data from one representative experiment are presented as mean nitrite concentration (triplicate cultures) ± SD. *p<0.05 (Student's t test) refers to values obtained in control.*

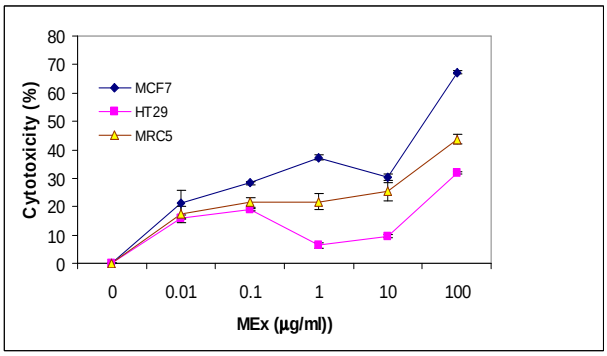

Fig. 5. The effect of MEx on the growth of human cell lines. Breast adenocarcinoma MCF-7, human colon adenocarcinoma HT-29, or human normal fetal lung fibroblast MRC-5 were cultured for 48 h in the absence or presence of different doses of MEx as indicated and cell growth was measured using colorimetric MTT assay. Results are presented as % of death cells compared to untreated cells (100% viable).

4. Discussion

In the present paper we have showed that MEx potently affects proliferation of polyclonally-stimulated mixed populations of immune cells (SPC and LNC), whereas in the macrophage model system it exhibits inhibitory effects on NO generation. The MEx effect was most probably an indirect, mediated through inhibition of lymphocyte-derived macrophage-stimulating cytokines, such as IFN-γ. Moreover, MEx expresses a potent cytotoxic activity against the tumor cell line MCF-7. These results imply a significant role of the investigated extract in the control of pro-inflammatory responses in macrophages and lymphocytes and human breast cancer proliferation. It is well documented that prolonged or chronic inflammation, associated with ROS and RNS generation, is detrimental and has an important role in the development of diseases such as arthritis, diabetes, cardiovascular disorders, neurodegenerative diseases, and cancer [14-17]. Although immune surveillance and cytotoxic actions of NK cells and $CD8^+$ T cells are considered essential for prevention of tumor development and restraint of its progression [18], it is widely accepted that persistent inflammation in tumor (micro)environment contributes to tumor genesis, progression and metastasis [19]. Recently, researchers have focused on designing effective therapeutics for such inflammatory diseases by modulating inflammation. Phagocytic cells, especially macrophages, are sensitive to changes in the oxidant-antioxidant balance because of the production of ROS and RNS as part of their normal function. Because of this, macrophages offer an excellent model system to study the anti-oxidant and NO inhibitory activities of natural materials. We have therefore evaluated the potential of active components from *H. punctata* to scavenge RNS using macrophage model system. Our results provide evidence that these components might contribute to protection against damage by RNS thought to be involved in the pathology of chronic diseases. In addition, the direct anti-cancer effect of MEx *in vitro* against human breast adenocarcinoma was also demonstrated. Moreover, the results regarding immunomodulatory effects of MEx on lymphocytes are of importance for the potential anticarcinogenic application of *H. punctata* active constituents, as they might affect tumors not only directly, but also through modulation of immune response thus favoring anti-tumor *milieu*.

According to National Cancer Institute guidelines the crude extract with an $IC_{50} < 30$ µg/ml is considered active [20], so MEx may represent a good natural resource of new anticarcinogenics. In comparison, the alkaloids pterocellins A and B, isolated from the marine bryozoan *Pterocella vesiculosa* and considered to be promising new leads for antitumor drugs, have not affected MCF-7 [21]. On the other hand, MEx has shown a low cytotoxicity on the nonmalignant cell line MRC-5.

An important issue that requires further investigation is whether *H. punctata*-derived natural products are absorbed and retain their properties *in vivo*. For example, the MEx components might exert protective actions within the gastrointestinal tract on radical-driven pathologies, as well as after absorption into the systemic circulation [22]. In addition, in order to find most effective approaches, which at the same time have fewer side effects, it is of primary interest to investigate the effects of combined treatment of MEx with conventional chemotherapeutics. Future availability of *H. punctata*-derived pure compounds and appropriate methodologies will help to clarify these points.

5. Conclusion

Together, our results suggest that MEx appears to confer protection against a variety of important chronic debilitating conditions. Therefore, the potential use of freshwater bryozoan *H. punctata*-derived components for the treatment of human chronic inflammatory diseases, cardiovascular disorders and cancer is worthy of further investigation.

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