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ORIGINAL PAPER

Anti-proliferative and Pro-apoptotic Effect of Dichloromethane Extract of *Octopus vulgaris* By-Products on Human Breast Cancer Cell Lines

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Abstract
Purpose Octopus by-products are considered as a waste and no data of the activity of their lipid fractions have ever been reported. The aim of this study was to valorize *Octopus vulgaris* by-products by investigating their dichloromethane extract effect on proliferation and apoptosis of human breast cancer cell lines.
Methods Two lipidics subfractions, F3336 and F3740, of Octopus by-products were obtained by silica gel chromatography after total lipid extraction. Their effects on proliferation, migration and apoptosis were examined on MCF-7 and MDA-MB-231 human breast cancer cell lines. The 3-(4,5-dimethyl-2-thiazol)-2,5 diphenyltetrazolium bromides (MTT) assay was used for the cell viability. Cell death was determined by flow cytometric analysis after 7-aminoactinomycin D (7-AAD) staining. MDA-MB-231 invasion and migration were analyzed using the Boyden chamber and wound healing assay.
Results Treatment with the two lipidics subfractions showed a reduction in the proliferation of MCF-7 and MDA-MB-231 cell lines in a dose dependent manner. Treatments significantly inhibited MCF-7 cells growth than MDA-MB 231 cells. Moreover, the two lipidics subfractions induced apoptosis of MCF-7 cells and reduced invasion and migration of MDA-MB 231 cells.
Conclusion This study shows for the first time that *O. vulgaris* lipid extracts have an antiproliferative and apoptotic effects on human breast cancer cell lines.

Keywords *Octopus vulgaris* · By-products · Lipids · Breast cancer

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Introduction

The marine environment is actually overfishing [1] and captures of cephalopods do not cease increasing, at least 35 % of the world capture during the five last years. Before exporting, the fish products generate an enormous quantity of by-products which are considered as a waste and have never been valorized.

It is generally known that marine fishes synthesize and are a source of lipids that are used in dietary fish oils for humans. On the other hand, several studies described beneficial effects of marine lipids (n-3) poly-unsaturated fatty acids [2], Phospholipids [3] and Sterols [4] in tumor and metastasis inhibition.

In the past, anti-proliferative effects of sterols have been observed in various types of human cancer such as lung [5], stomach [6], ovary [7] and breast [8], [9]. Moreover, it has been proposed that sterols inhibit migration and invasion and promote apoptosis of cancerous cells [10, 11].

The present study was aimed for the isolation of lipids fractions from octopus by-products obtained from Copefrito company (Toliara, Madagascar) and for the evaluation of the two lipidics subfractions on proliferation, migration and apoptosis of breast cancer cells.

Materials and Methods

Materials

The human breast cancer MCF-7 and MDA-MB-231 cells lines were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury). The 3-(4,5-dimethyl-2-thiazol)-2,5 diphényltétrazolium bromides (MTT) and 7-aminoactinomycin D (7-AAD) were from Sigma-Aldrich. 8 µm filter inserts were purchased from nunc company (Denmark).

Cell Culture

MCF-7 and MDA-MB-231 cells lines were cultured at 37 °C in a humidified incubator with 5 % CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 1 % glutamine and 1 % penicillin–streptomycin.

Obtention of Lipids Fractions and Subfractions

Octopus by-products were obtained from Copefrito company, Toliara, Madagascar. They were parts of octopus (viscera, pocket of ink, eyes and beak) not intend for human consummation.

Octopus by-products from several lots were maintained at 4 °C until treatment. Lipid extraction was carried out by Folch method [12]. Briefly, 100 g of Octopus by-products were macerated in chloroform–methanol (2:1 v/v) during 24 h. They were homogenized by magnetic stirring for 30 min and then filtered. Two distinct phases were appearing, an organic phase which contains lipids and an aqueous phase. The organic phase was collected; its water content was discarded using anhydrous sodium sulfate (Na_2SO_4) and was evaporated to obtain the lipid crude extract (0.48 %). It was then dissolved with chloroform–methanol (2:1 v/v) and chromatographed over silica-gel column. Two different solvents were added according to their polarity (cyclohexane and dichloromethane). Cyclohexane fraction was discarded.

Thin-layer chromatographic of all dichloromethane fractions were carried out on Kisselgel plates with dichloromethane. The chromatograms were visualized by spraying with ethanol/sulfuric acid (95/5, v/v) and heating at 95 °C for 2 min. Following thin-layer chromatography analysis, eluates with similar profiles were combined to produce five fractions: F9, F1020, F3336, F3740 and F4160 (Fig. 1).

Cell Viability Test

MCF-7 and MDA-MB-231 Cells were plated in 96-wells at a density of 10^4 cells per well in 200 µl of culture medium and allowed to adhere overnight. After 24 h, the culture medium was removed and cells were treated with different concentrations of F3336 and F3740 fractions diluted in 0.1 % BSA containing medium for 24 h. For the MTT assay, 100 µl of culture medium was removed and 50 µl of MTT at concentration of 2,5 mg/ml were added to each well. The mixture was further incubated for 4 h at 37 °C, and the liquid in the well was removed thereafter. Dimethylsulfoxide (DMSO; 200 µl) was then added to each well to solubilize the formazan product and absorbance was measured at 540 nm. Relative cell viability was expressed as a percentage of the untreated cells.

Flow Cytometric Quantification of Cell Death

Cell death was determined with a fluorescent DNA-binding agent, 7-AAD. MCF-7 cells were cultured in 12-wells plate at 75.10^3 cells per well in 1 ml of culture medium for 24 h. Cells were treated with 100 ng/ml of F3336 and F3740 fractions. After treatment, cells were trypsinized and harvested in another 96-wells plate. After centrifugation at 1,600 rpm for 1 min, supernatant was removed. Then, 100 µl of culture medium were added with 5 µl 7-AAD (diluted in ¼ PBS) and 95 µl PBS/EDTA.

The apoptotic cells were analyzed on fluorescence-activated cell sorting (FACS) by using BD Accury C6 flow cytometer.

Cell Migration Assay (Cell Invasion Assay)

MDA-MB-231 cell line was used for the cell migration assay for its significant migration ability [13]. The ability of these cells to migrate was measured using Boyden Chamber assay.

Cells were plated in 24-microwells at 10.10^4 cells in 200 µl culture medium into the inserts and incubated 24 h at 37 °C. Cells were then treated with 100 ng/ml of F3336 and F3740 and incubated at 37 °C for another 24 h in a humidified incubator with 5 % CO_2. At the end of incubation, the cells in the upper surface of the inserts were carefully removed with a cotton swab. The inserts was washed with PBS and placed in 500 µl glutaraldehyde 1 % for 30 min. After washing with PBS, 100 µl of crystal violet were added in the insert for 10 min before cleaning with PBS.

The results were obtained by analysis of the cells invasion to the lower surface of the membrane filter with a light microscope.

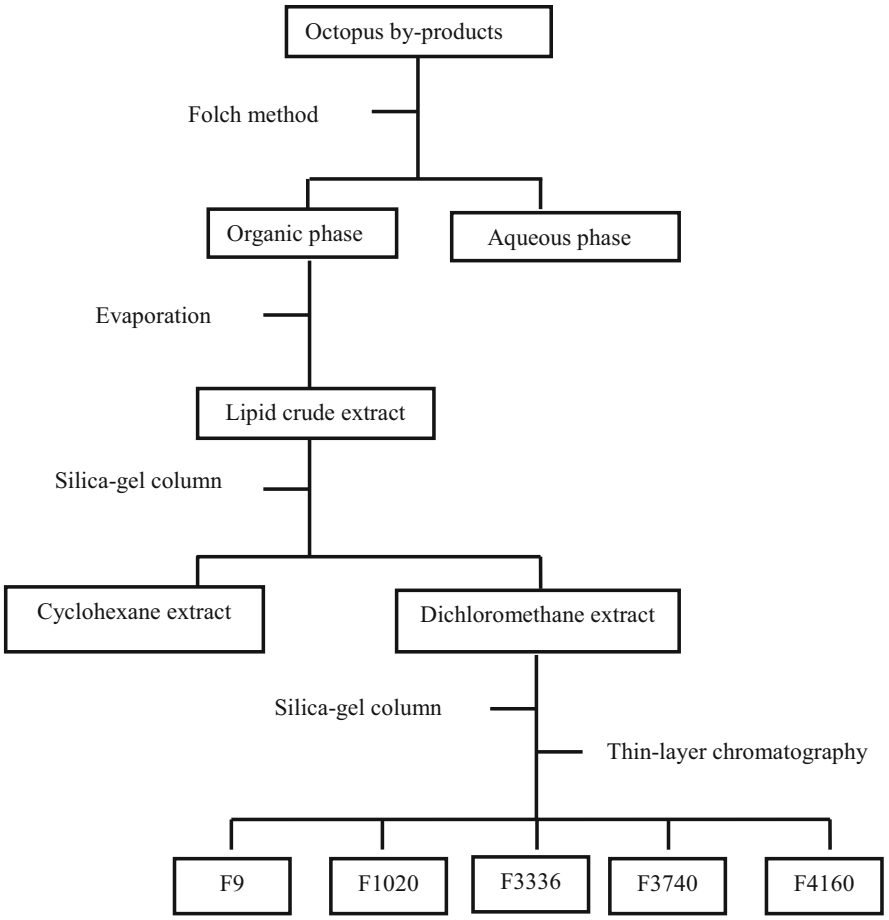

Fig. 1 Extraction procedure to obtain the two lipidics subfractions F3336 and F3740 of octopus by-products. Octopus by-products were macerated with chloroform–methanol (2:1 v/v) according to Folch method. Lipids extract was fractionated by column chromatography using different solvent. Dichloromethane was then fractioned and purified to obtain different subfractions

Wound-Healing Assay

MDA-MB 231 cell line was seeded in a 12 well plate at concentration 3.10^5 cells per well. After overnight incubation, culture media were removed and a scratch wound across the well was made using fine tips. The wells were then washed with PBS and cells were incubated with F3336 and F3740. After 24 h wound healing pictures were taken under a microscope.

Statistical Analysis

Statistical analyses were made using student's *t* test.

Results

Effect of Lipidics Subfractions on Cell Viability

The inhibitory effect of lipidic subfractions on cancerous cells viability was performed with MTT assay. Cells were treated with the two lipidics subfractions, F3336 and F3740, with different concentrations for 24 h. The results demonstrated that F3336 and F3740 reduced MCF-7 and MDA-MB 231 cells proliferation in a dose dependent manner. They were very active in MCF-7 than in MDA-MB 231. At lowest concentration (46 ng/ml) F3336 and F3740 inhibited MCF-7 proliferation by 40 and 70 % respectively (Fig. 2).

Effect of Lipidics Subfractions on MCF-7 Cell Apoptosis

MCF-7 cell apoptosis was analyzed by FACS flow cytometer. Dead cells were stained with 7-AAD. The results are shown in Fig. 3. The two lipidics subfractions induced apoptosis of MCF-7. In fact, F3336 and F3740 resulted in an increase of 7-AAD positive cells by 45 and 51.6 % respectively versus untreated cells.

Effect of Lipidics Subfractions on Migration and Invasion of MDA-MB231 Cells

In order to determine the effect of the two lipidics subfractions on invasion ability of MDA-MB 231 cells, a Boyden Chamber assay was realized. The results show a significant reduction of cells invasion treated with F3336 and F3740 compared with untreated cells (Fig. 4). To

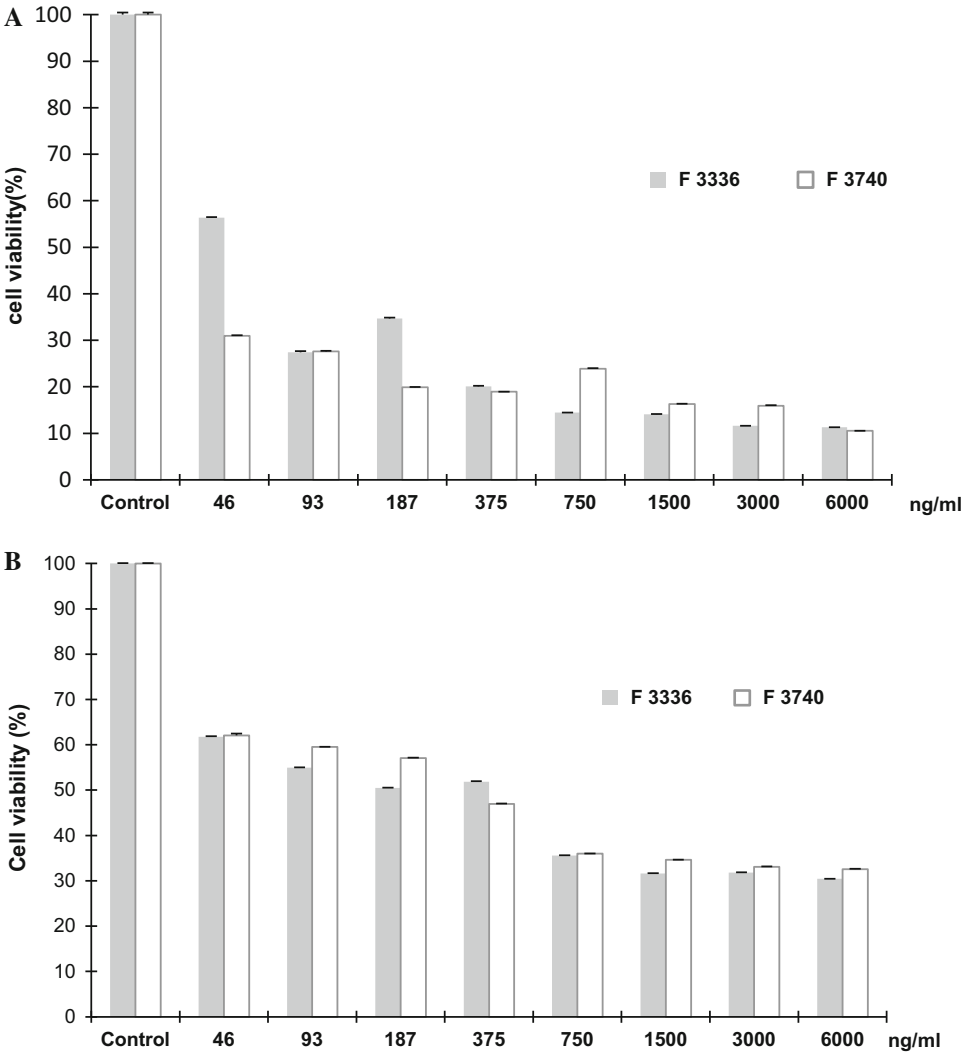

Fig. 2 Effect of lipidics subfractions, F3336 and F3740, in MCF-7 and MDA-MB 231 cells proliferation. **a** Effect of F3336 and F3740 on MCF-7 cells proliferation. **b** Effect of F3336 and F3740 on MDA-MB 231 cells proliferation. Data are the mean ± SD of three different experiments. Each experimental condition was carried out in triplicate. Treated cells versus control ($p < 0.01$)

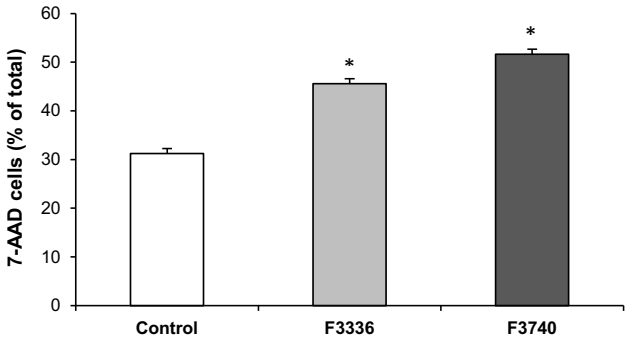

Fig. 3 Effect of lipidics subfractions F3336 and F3740 on apoptosis of MCF-7 cells. Cells were treated with the subfractions (100 ng/ml). Cell death was determined by flow cytometer analysis after 7-AAD staining. Data are the mean ± SD of three different experiments. Each experimental condition was carried out in triplicate. * $p < 0.05$ versus control

investigate the effect of the two lipidics subfractions on cancerous cells migration, we performed the wound-healing assay. After 24 h incubation period at 37 °C and 5 % CO_2, cells were scratched by a sterile fine tip and the cells were treated with 100 ng/ml concentration of lipidics subfractions. As demonstrated in Fig. 5, F3336 and F3740, decreased the migration capacity of MDA-MB231 as compared with untreated cells.

Discussion

Octopus by-products were considered as a waste and no data on the anticancer activity of their lipid fractions have been reported. In this study, they were valorized by analyzing the lipid fractions on breast cancer cells growth and apoptosis. Two lipidics subfractions F3336 and F3740 were obtained after total lipid extract and silicate gel chromatography of dichloromethane fraction.

The results suggested that the antiproliferative capacity of the two lipidics subfractions are variables for the two types of breast cancer cells screened MCF-7 and MDA-

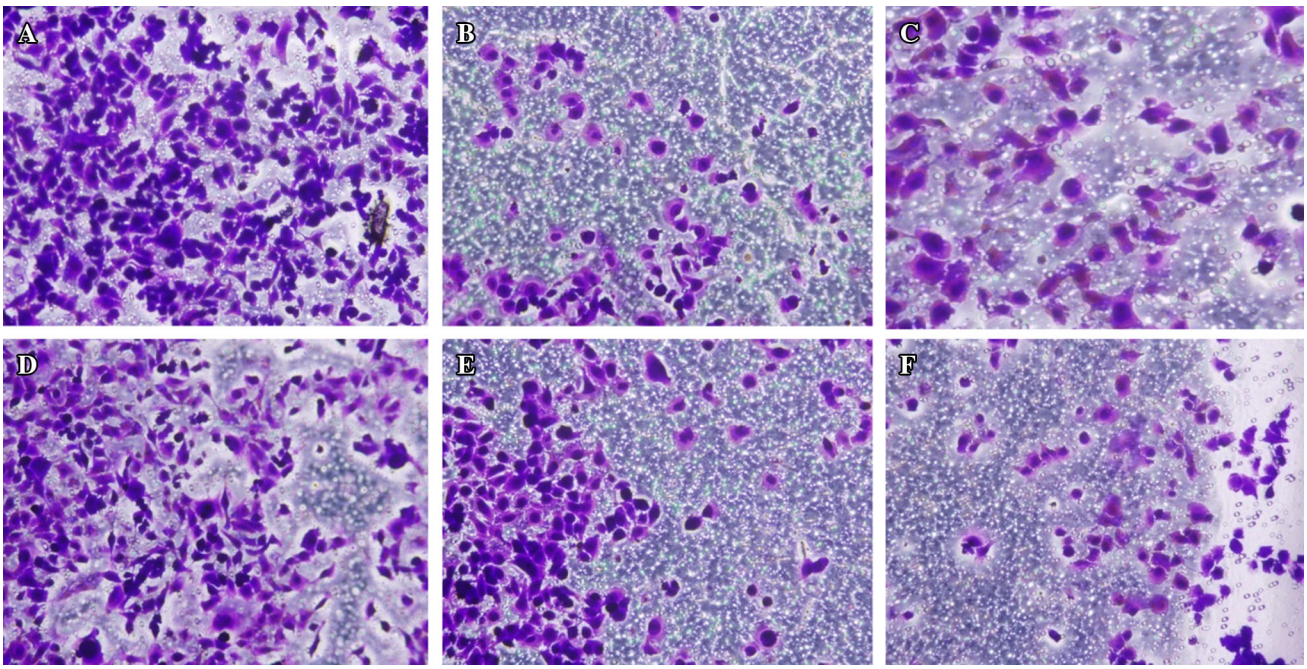

Fig. 4 Effect of lipidics subfractions on invasion of MDA-MB 231 cells. Cells were grown on filter inserts and were treated for 24 h with 100 ng/ml concentrations of F3336 and F3740. **a** Untreated cells. **b** F3336. **c** F3740. Data are representative of three separate experiments realized in triplicates

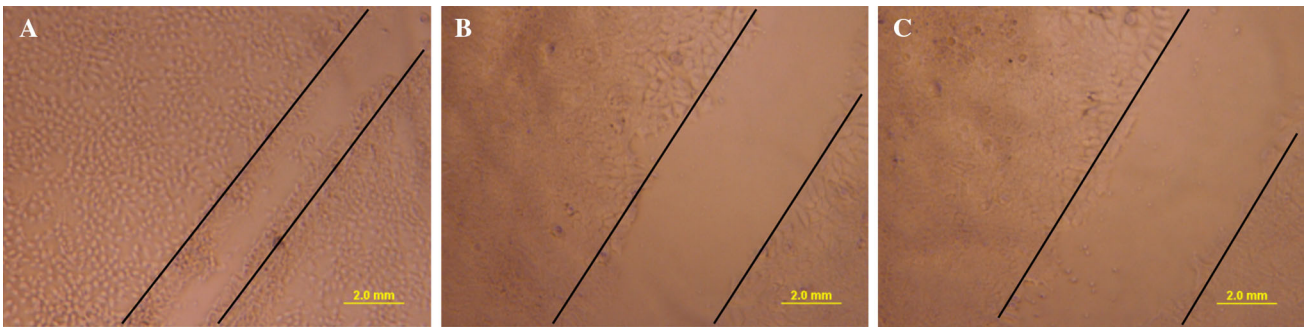

Fig. 5 Effect of lipidics subfractions, F3336 and F3740, on MDA-MB231 cells migration. Confluent cells were scratched by a sterile fine tip and treated with 100 ng/ml concentration of lipidics subfractions. **a** Untreated cells, **b** F3336, **c** F3740. Data are representative of three separate experiments realized in triplicates

MB231. There are apparent decreases of cells proliferation in a dose dependent manner in both cell lines. F3336 and F3740 fractions exhibited significant anti-proliferative effect on MCF-7 more than in MDA-MB231. This may be due to the phenotypes of these two cancer cells. In fact MCF-7 is ER^+ and MDA-MB231 is ER^-. This difference of phenotypes is generally considered as a relevant parameter to their degree of malignancy, and as a consequence, to the response that could be expected from a chemopreventive treatment [14, 15]. Many studies have demonstrated the effect of dichloromethane extracts from plant (*Dunaliella tertiolecta*) [17], algae (*Croton macrobothrys*) [16] and aquatic crustaceans (*Artemia ciniformis*) [18] on MCF-7 cells proliferation.

Moreover, our data show a reciprocal effect of these two lipids subfractions on proliferation and apoptosis. In fact, the two lipids subfractions, F3336 and F3740 also stimulated MCF-7 apoptosis quantified by Flow cytometric analysis after 7-AAD staining.

In addition to proliferation and apoptosis, traits of cancerous cells include migration and invasion which are essential for them to leave the primary tumor and disseminate throughout the body. Our results indicated that lipidics subfractions, F3336 and F3740, inhibited MDA-MB 231 cells migration and invasion.

Various compounds which have biological functions were isolated by Dichloromethane. Among them, sterols and terpenes have been reported to inhibit proliferation,

induce apoptosis and reduce migration [19–21]. These findings, coupled with the fact of dichloromethane extract from *Octopus vulgaris* have the same activity, indicate that sterols or other compounds (e.g. terpenes) may be responsible for these biological observations.

Our study mainly utilized two subfractions of dichloromethane which we consider contain many components raising the question as to which compounds within F3336 and F3740 subfractions are responsible for generating the observed anti-cancer effects. The identification of the active components of Octopus by-products and confirmation of their properties should elucidate the precise mechanisms of their anti-cancer effect and may provide a basis for the chemopreventive and chemotherapeutic strategies in breast cancer.

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