Marine Oomycetes (*Halophytophthora* and *Salispina*): A Potential Source of Fatty Acids with Cytotoxic Activity Against Breast Adenocarcinoma Cells (MCF7)

Mark Kevin P. Devanadera¹, ², ³, ⁷*, Reuel M. Bennett⁴, Kenshi Watanabe⁶, Myla R. Santiago¹, ³, ⁷, Maria Cristina Ramos¹, ⁵, Tsunehiro Aki⁶, and Gina R. Dedeles¹, ², ⁴**

1 The Graduate School, University of Santo Tomas, Manila, PHILIPPINES
2 Laboratory of Pure and Applied Microbiology, Research Center for the Natural and Applied Sciences, Thomas Aquinas Research Complex, University of Santo Tomas, Manila, PHILIPPINES
3 Department of Biochemistry, Faculty of Pharmacy, University of Santo Tomas, Manila, PHILIPPINES
4 Department of Biological Sciences, College of Science, University of Santo Tomas, Manila, PHILIPPINES
5 Department of Chemistry, College of Science, University of Santo Tomas, Manila, Philippines
6 Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Hiroshima, JAPAN
7 Mammalian Tissue Culture Laboratory, Research Center for the Natural and Applied Sciences, Thomas Aquinas Research Complex, University of Santo Tomas, Manila, PHILIPPINES

Abstract: Marine oomycetes are ubiquitous, fungus-like eukaryotes known to produce fatty acids with potential anticancer activity. The long chain omega-3 and omega-6 fatty acids are currently popular and considered as safe when used as nutraceuticals in cancer treatment. In this study, crude fatty acids from three marine oomycetes, *Halophytophthora* spp. (T12GP1 and T12YBP2) and *Salispina hoi* (USTCMS 1611), were explored for their cytotoxic and apoptotic potentials against human adenocarcinoma/breast cancer cells (MCF7) and normal human dermal fibroblasts (HDFn). Extracts from mycelia mats consisted of diverse saturated, monounsaturated, and polyunsaturated fatty acids such as linoleic, α-linolenic, γ-linolenic, eicosatrienoic and eicosapentaenoic acids. The crude fatty acids from all three oomycetes in *in vitro* assays for cytotoxicity showed no toxicity (30% toxicity values) on HDFn cells. On MCF7 cells, however, IC₅₀ values of 23.44, 15.63, and 26.15 µg/mL were obtained with extracts from *Halophytophthora* T12GP1 and T12YBP2 and S. hoi, respectively. Treated MCF7 cells exhibited deformed cell membrane in MTT assay and also aggregation of DNA and disruption of nuclear membrane aggregation in nuclear staining; further, green signals indicative of apoptosis was recorded in caspase 3/7 assay.

Key words: apoptosis, breast cancer, cancer cells, oomycetes, PUFA

1 Introduction

Marine oomycetes are a group of fungal-like eukaryotes of the kingdom Straminipila¹. Members of this group are considered as initial colonizers of fallen senescent mangrove leaves¹ and are seen as a potentially good source of fatty acids (e.g. polyunsaturated and monounsaturated) that are of industrial and medical importance²–⁴. Polyunsaturated fatty acids (PUFAs) are biological lipid derivatives which are essential in human metabolism and other biological activities⁵. One application of PUFAs in the medical field is their cytotoxic and apoptotic activity against cancer cells. Cancer is one of the major burdens of human diseases worldwide; it ranks as the second most common cause of mortality. Treatments administered to cancer patients are classified as either local (i.e. surgery and radiation) or systemic (i.e. hormone, chemotherapy, targeted therapy). However, despite of the progress in cancer research such as the chemical- and radiation-based therapies, there are still limitations that lead to treatment failure and metastasis. Relative to this, scientists and researchers are currently exploring combinational therapies like site-directed administration, targeted drug release, pathway-specific drugs,

*Correspondence to: Gina R. Dedeles, Laboratory of Pure and Applied Microbiology, Research Center for the Natural and Applied Sciences, University of Santo Tomas, Manila, PHILIPPINES
E-mail: grdedeles@ust.edu.ph, grdeddeles@yahoo.com
Accepted September 5, 2019 (received for review January 31, 2019)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/     http://mc.manuscriptcentral.com/jjocs
use of bio-macromolecules toxic-specific to cancer cells but not to normal cells and most importantly, the use of alternative treatment compounds such as PUFAs. The attachment of PUFAs in the membrane of cancer cells may affect the function and physical properties of the cell (e.g., membrane integrity and signalling pathways), thereby inhibiting cell cycle, and even induce cell death or apoptosis. Further, other factors that promote cancer growth like the free radical formation and promoter signals may also be affected by the interference of PUFAs.

The PUFAs from thraustochytrids (e.g., Aurantiochytrium, Thraustochytrium and Schizochytrium, Pythium and other microorganisms have been extensively characterized and identified but published literature on the fatty acid profile of Halophytophthora species is scarce. Further, as to the application of PUFAs as anticancer agents, studies are often centered on docosahexaenoic acids (DHA) and eicosapentaenoic acids (EPA) whereas other fatty acids remain unexplored. Hence, this study presents the diverse fatty acids from three marine oomycetes from the Philippines and their anticancer activity against MCF7 cancer cell line.

2 Materials and Methods

2.1 Marine oomycete isolates

Three marine oomycete isolates, Halophytophthora T12GP1, Halophytophthora T12YBP2, and Salisipina hoi USTCMS 1611, obtained from the University of Santo Tomas Collection of Microbial Strains (USTCMS) were cultivated in vegetable juice agar following the protocol of Pang et al. with few modifications. Isolates were incubated at room temperature for 7 days and allowed to sporulate in 5 ppt marine water. The morphology of the organism was observed using a light microscope (EvosFL, Life Technologies, USA) and scanning electron microscope (SEM) (Hitachi Table Top SEM, TM3030, Japan). Further, published morphological descriptions of marine oomycetes elsewhere were used.

2.2 Mycelial mass production, fatty acid extraction, and fatty acid analysis

The medium for fatty acid production used in this study was adapted from Pang et al. and Say et al. The cultivation medium was composed of 5% (v/v) clarified vegetable juice with 4 g/L peptone, 4 g/L yeast extract, 4 g/L glucose, and 10 g/L marine salt. Eight 7-mm mycelial discs were placed in 100 mL production medium, and cultures were incubated statically at room temperature for 14 d. The mycelial mass was collected through centrifugation and filtration and then washed three times with sterile distilled water.

The isolation of fatty acids was adapted from Tilay and Annapure and Pote and Bhadekar. Mycelial mass was freeze-dried, homogenized, and soaked in ethyl acetate/methanol solution (2:1 v/v) for 24 h. The suspension was collected, concentrated using a rotary evaporator at 40°C, saponified using 0.5 N sodium hydroxide or was esterified using 0.5 N methanolic sodium hydroxide solution and heated at 90°C for 15 min. The solution for esterification was cooled and mixed with 0.7 N hydrochloric acid-methanol and 14% boron trifluoride in methanol followed by heating at 90°C for 15 min. After which, saturated sodium chloride solution and hexane were added and thoroughly mixed. The upper liquid layer was collected, transferred in an amber bottle, and dried at ~40°C. The hydrolysate was used for toxicity analysis and the esterified sample was used for the fatty acid composition analysis using gas chromatography.

Esterified or FAME (Fatty Acid Methyl Esters) samples were analyzed using gas chromatography (GC) following several published protocols. Samples were filtered into a sample vial using Whatman No.1 and filtrates were placed in an autosampler injector. The injector was set at 250°C and the interface temperature at 270°C. The column was set at 50–150°C with 15°C/min temperature increments. The gas pump was noted at 38.0 cm/min. The capillary was used in a gas chromatography-mass spectrophotometry (GC-MS) to determine the other class of lipids present. The sample was injected into a gas chromatography system (Agilent CG7890A GC System) with a front inlet temperature of 300°C. The gas was used as a carrier gas with a split ratio of 1:20 and a flow rate of 1.19 mL/min. Column used in the analysis was DB-5HT (Agilent) and the temperature was set at 100°C–380°C with increments of 10°C/min and run for 7 mins. Detector used in the analysis was mass spectrophotometer (Jeol JMS-T100GCx) with electron ionization (EI) as a mode of ionization. The detector has an ionization voltage of 70V and detector voltage of 190V, it also has an ion chamber temperature of 280°C and a mass range of 29.0–800.0 m/z.

2.3 Cell proliferation and morphology analyses

Cell lines (human dermal fibroblasts, HDFn, Invitrogen, USA and human breast adenocarcinoma, MCF7, University of the Philippines Mammalian Cell Culture Laboratory) were cultured in Dulbecco’s Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 U/mL), and penicillin (100 U/mL) (Invitrogen, USA). Cell lines (MCF7 and HDFn cells, density at 1.0 × 10⁵) were seeded into 96-well plate with DMEM medium and incubated for 24 h in a humidified incubator at 37°C and 5% CO₂. After 24 h incubation, cells
were treated with extracted fatty acid samples and standard drug (doxorubicin) and incubated for another 24 h. After treatment, media with samples and standards were removed and cells were washed with phosphate buffer saline (PBS, pH 7.2)\(^{20}\). The PBS solution was decanted and replaced with 20 \(\mu\)L of 1% MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Dimethyl sulfoxide (DMSO) (200 \(\mu\)L) was added into each well and the absorbance was read at 570 nm\(^{24,25}\). Cellular morphology was analyzed and documented using an inverted light microscope (Evos FL, Life Technologies, USA).

2.4 Cellular apoptosis by Caspase 3/7 assay

Caspase activity was determined by luminescence method using the Caspase-Glo 3/7 (Promega) protocol. The Caspase-Glo substrate and Caspase-Glo buffer were mixed according to the manufacturer’s instructions. Caspase-Glo mixture (100 \(\mu\)L) was added into each well of untreated and treated cells (MCF7 and HDFn cells) and incubated for 3 h at 37°C. The luminescence reaction was read using Glomax Multiplate reader (Promega) at 425 SP emission filter. Cellular apoptotic activity was conducted by adding Caspase 3/7 fluorescent dye (Life Technologies, USA) and Hoechst 33342 fluorescent dye (Thermofisher Scientific, USA) to MCF7 cells and incubated at 37°C for 1 h. After incubation, MCF7 cells were viewed under blue and green filtered fluorescent microscope (Evos FL, Life Technologies, USA).

2.5 Statistical analysis

Quantitative data generated from the analyses were treated with one-way analysis of variance (ANOVA) using PHStat application in Microsoft Excel. ANOVA was applied on the average values of every sample treatment across the different concentrations at 95% level of significance.

3 Results

3.1 Marine oomycetes from Philippine mangrove leaves

Both Halophytophthora T12GP1 and T12YBP2 (Fig. S1) exhibit similar sporangium morphology to H. batemanaensis\(^{20}\). However, these isolates were neither assigned to this species or other congener pending multigene phylogeny. Salispina hoi USTCMS 1611 was a recently proposed taxon\(^{20}\) based on its morphology, physiology, and phylogenetic placement. This taxon is closely related to S. spinosa and S. lobata. The sporangium and colony morphologies of the three isolates are presented in Fig. S1.

3.2 Fatty acid and lipid profile of marine oomycetes

The weights of mycelia per production and crude fatty acid produced by the three isolates are presented in Table 1. Saturated and unsaturated fatty acids (i.e. monounsaturated and polyunsaturated) were present in the three isolates. The saturated fatty acids were lauric, pentadecanoic, palmitic, heptadecanoic, stearic, arachidic, behenic, tricosanoic, and lignoceric acids (Table 2). Monounsaturated fatty acids identified from the isolates were palmitoleic, oleic, and erucic acids (Table 2), while linoleic, \(\gamma\)-linolenic, \(\alpha\)-linolenic, eicosatrienoic, and eicosapentaenoic acids were the polyunsaturated fatty acids (Table 2).

The lipid component of the three species showed the presence of free fatty acids (Fig. 1). Other classes of lipids, like the triglycerides, sterols, phospholipids, were not found in the crude extract sample. Three fatty acids (palmitic, oleic and linoleic acids) were of high concentrations and common among the three isolates. Palmitic acid was present in Halophytophthora T12GP1 and T12YBP2, and S. hoi at 15.2, 12.13, and 21.25%, respectively. Oleic acid constituted 8.2% for T12GP1, 6.3% for T12YBP2, and 17.70% for S. hoi. Linoleic acid constituted 6.5% for both Halophytophthora T12GP1 and T12YBP2, and 10.24% for S. hoi.

3.3 Cytotoxicity assessment of crude fatty acids from Halophytophthora spp. T12GP1 and T12YBP2, and Salispina hoi

Analysis of the cytotoxic effect of crude fatty acids of Halophytophthora T12GP1 and T12YBP2 and S. hoi against MCF7 cells using MTT assay showed 62–67% toxicity against the cancer cell line at 200 \(\mu\)g/mL for each isolate (Fig. 2). The computed IC\(_{50}\) values were 23.44 \(\mu\)g/mL for Halophytophthora T12GP1, 15.63 \(\mu\)g/mL for Halophytophthora T12YBP2, and 26.15 \(\mu\)g/mL for S. hoi. These IC\(_{50}\) values of the crude fatty acids are close to the IC\(_{50}\) value of 20 \(\mu\)g/mL for cytotoxic agents\(^{14,24,35}\). Crude fatty

| Organism                        | Weight of mycelia per production (g/L) | Weight of crude fatty acid extracted (g) | Weight of extract per mycelial mass (mg/g sample) |
|--------------------------------|----------------------------------------|-----------------------------------------|-----------------------------------------------|
| Halophytophthora sp. T12GP1     | 1.31                                   | 0.61                                    | 466.88                                        |
| Halophytophthora sp. T12YBP2    | 1.73                                   | 0.70                                    | 404.65                                        |
| Salispina hoi USTCMS 1611       | 1.89                                   | 1.04                                    | 551.10                                        |
acids used in the cytotoxic assessment are referred to as fatty acids isolated and saponified from the crude extract of the marine oomycetes in ethyl acetate-methanol solvent system. The crude fatty acids used in the experiment did not contain polyphenols because all were extracted from the semi-polar extraction, saponification, and solvent partitioning using hexane.

Comparing the morphology of untreated cells from cells treated with the crude fatty acid of *Halophytophthora* T12GP1 and T12YBP2 and *S. hoi*, the latter treatment confirmed the toxic effect against MCF7 cells (Figs. S2–S4).

MCF7 cells treated with crude fatty acids of *Halophytophthora* T12GP1, in dose-dependent manner, showed cell detachment, irregular shape formation, cell membrane shrinkage, and even blackening of cells (Fig. S2). Shrinkage of the cell membrane and darkening of the whole cell were observed for MCF7 cells treated with crude fatty acids of *Halophytophthora* T12GP1, in dose-dependent manner, showed cell detachment, irregular shape formation, cell membrane shrinkage, and even blackening of cells. Comparing the morphology of untreated cells from cells treated with the crude fatty acid of *Halophytophthora* T12GP1 and T12YBP2 and *S. hoi*, the latter treatment confirmed the toxic effect against MCF7 cells (Figs. S2–S4). MCF7 cells treated with crude fatty acids of *Halophytophthora* T12GP1, in dose-dependent manner, showed cell detachment, irregular shape formation, cell membrane shrinkage, and even blackening of cells (Fig. S2). Shrinkage of the cell membrane and darkening of the whole cell were observed for MCF7 cells treated with crude fatty acids of *Halophytophthora* T12GP1, in dose-dependent manner, showed cell detachment, irregular shape formation, cell membrane shrinkage, and even blackening of cells. MCF7 cells treated with crude fatty acids of *Halophytophthora* T12GP1, in dose-dependent manner, showed cell detachment, irregular shape formation, cell membrane shrinkage, and even blackening of cells. MCF7 cells treated with crude fatty acids of *Halophytophthora* T12GP1, in dose-dependent manner, showed cell detachment, irregular shape formation, cell membrane shrinkage, and even blackening of cells.

High luminescent signals were detected from MCF7 cells treated with 31.25–250 μg/mL crude fatty acids in comparison to untreated cells. Emitted luminescent signals by cells corresponded to the detection of caspase 3/7 proteins present during apoptosis (Fig. 3). Luminescent signals were directly proportional to increasing fatty acid concentrations. Further, MCF7 cells treated with crude fatty acids emitted blue signals (Fig. 4) indicating nuclear disruption, and DNA aggregation. Green fluorescent signals, generated upon activation of caspase 3/7, were observed on treated MCF7 cells. Interestingly, crude fatty acids were non-toxic to HDFn cells based on MTT assay (Fig. 5). The nucleus of treated HDFn remained intact and showed no aggregations (Fig. 6). Further, no apoptotic green signals were detected on the caspase 3/7 fluorescent dye treatment (Fig. 6).

Fatty acid standards of γ-linolenic, α-linoleic, linolenic, eicosatrienoic and oleic acids at 50 μg/mL showed high toxicity towards MCF7 cells (Fig. 7). γ-linolenic acid inhibited MCF7 cells at 68.51 % ± 4.54, while linoleic acid and

---

**Table 2** Fatty acid profile of *Halophytophthora* spp. T12GP1 and T12YBP2, and *S. hoi* USTCMS 1611.

| Fatty acids                        | *Halophytophthora* sp. T12GP1 (%) w/w | *Halophytophthora* sp. T12YBP2 (%) w/w | *S. hoi* (%) w/w |
|-----------------------------------|--------------------------------------|---------------------------------------|-----------------|
| Decanoic acid (C10:0)             | –                                    | –                                     | 0.29            |
| Lauric acid (C12:0)               | 2.13                                 | 0.81                                  | 0.69            |
| Myristic acid (C14:0)             | 8.64                                 | 5.90                                  | 1.32            |
| Pentadecanoic acid (C15:0)        | 0.79                                 | 0.59                                  | –               |
| Palmitic acid (C16:0)             | 32.62                                | 30.00                                 | 38.56           |
| Palmitoleic acid (C16:1)          | 2.71                                 | 2.50                                  | 0.90            |
| Heptadecanoic acid (C17:0)        | 0.82                                 | 0.56                                  | –               |
| Stearic acid (C18:0)              | 6.82                                 | 8.08                                  | 4.93            |
| Oleic acid (C18:1n9c)             | 17.43                                | 15.56                                 | 32.12           |
| Linoleic acid (C18:2n6c)**         | 13.86                                | 16.12                                 | 18.59           |
| Arachidic acid (C20:0)            | 1.46                                 | 1.87                                  | –               |
| γ-linolenic acid (C18:3n6)**       | 1.13                                 | 1.98                                  | –               |
| α-linolenic acid (C18:3n3)*        | –                                    | 0.93                                  | 1.38            |
| Behenic acid (C22:0)              | 1.77                                 | 2.20                                  | 0.57            |
| Cis-8, cis-11, cis-14-Eicosatrienoic acid (C20:3n6) ** | 2.11 | 3.25 | – |
| Eruccc acid (C22:1n9)             | 3.23                                 | 2.09                                  |                 |
| Tricosanoic acid (C23:0)          | 3.08                                 | 4.61                                  | 0.41            |
| Lignoceric acid (C24:0)           | –                                    | 0.89                                  | 0.25            |
| Cis-5, cis-8, cis-11, cis-14, cis-17-Eicosapentanoic acid (C20:5n3)* | 1.40 | 2.07 | – |

* omega-3 fatty acids; ** omega-6 fatty acids
Fig. 1 Gas Chromatography-Mass Spectrophotometry analysis of lipids from the crude fatty acid sample isolated from Halophytophthora sp. T12GP1, Halophytophthora sp. T12YBP2, and S. hoi. The identified fatty acids for Halophytophthora sp. T12GP1 (8.20: Tetradecanoic acid, 9.95: Hexadecenoic acid, 10.15: Hexadecanoic acid, 11.72: 9,12-Octadecadienoic acid, 11.77: cis-9-Octadecenoic acid, 11.95: Octadecanoic acid 13.14: 5,8,11,14-Icosatetraenoic acid, 13.27: 8,11,14-Eicosatrienoic acid; Halophytophthora sp. T12YBP2 (10.15: Hexadecanoic acid, 11.75: cis-9-Octadecenoic acid, 11.94: Octadecanoic acid); and Salispina hoi (10.14: Hexadecanoic acid, 11.69: 9,12-Octadecadienoic acid, 11.74: cis-9-Octadecenoic acid, 11.95: Octadecanoic acid).

Fig. 2 Cytotoxicity assessment of crude fatty acids from Halophytophthora spp. T12GP1, T12YBP2, and S. hoi using MTT assay. Note: (*) statistically insignificant ($p \geq 0.05$) values between different concentrations for Halophytophthora sp. T12GP1; while (**) statistically significant ($p < 0.05$) values between different concentrations for Halophytophthora sp. T12YBP2 and S. hoi.
eicosatrienoic acid were at 69.11 ± 4.91 and 71.01 ± 1.16. With regard to the result of their MTT assay, the toxicity exhibited by the crude fatty acids can somehow correlate with the effect of PUFAs on the cells as shown in the toxicity values of the standard fatty acids. In relation to the toxic effect of standard fatty acids against MCF7, assessment on the morphology of the cells revealed that all of these fatty acids produced an apoptotic-like cell death on MCF7 cells wherein detachment of cells from the surface of wells and formation of blebbing-like structure were observed. On the other hand, MCF7 treated with linolenic acid showed darkening of the whole cell and bursting of cell membrane which are some of the characteristics of necrotic cell.

4 Discussion

Fatty acids are abundant in marine oomycetes such as *Aurantiochytrium limacinum* and *Halophytophthora* spp. In the recent work of Pang et al.2, a total yield of 0.004 to 0.052 g/L of arachidonic acid (ARA) from *Halophytophthora* spp. *H. spinosa* var. *spinosa* IMBI62 obtained the highest yield, but it showed no production of eicosapentaenoic acid (EPA). On the production of EPA, a yield of 0 to 0.047 g/L was observed from their isolates of *Halophytophthora*. A total percentage of 7.16 ′′ to 25.02 ′′ of ARA was determined for the total fatty acids. The *Halophytophthora* species used by Pang et al.2 were isolated from leaf litters of mangrove trees in Taiwan. In the study of Say et al.4, significant amounts of EPA, ARA, and DHA were also detected from *Halophytophthora* sp. isolated from yellow mangrove leaves collected from Samal Island, Philippines. Here in the present study, three fatty acids (palmitic, oleic and linoleic acids) were found present in all these oomycetes. Currently, monounsaturated and polyunsaturated fatty acids are being explored for their potential as anticancer agents against known cancer cells lines (e.g. MCF7) by exploring several pathways such as caspase activity, DNA damage, and cell membrane integrity.

Crude fatty acids from the three species of marine oomycetes showed toxic effects on MCF7 cells but there were no significant differences between the three samples. However, toxic effects of three crude fatty acid samples

---

**Fig. 3** Caspase 3/7 luminescence signal detection on the crude fatty acid of *Halophytophthora* spp. T12GP1, T12YBP2 and *S. hoi* treated on MCF7 cells. Note: (* * *) statistically significant values between different concentrations ($p < 0.05$).
Cytotoxic Activity of Fatty Acids Sourced from Marine Oomycetes: Halophytophthora and Salispina spp.

were due to the presence of monounsaturated fatty acid (oleic acid) and polyunsaturated fatty acids (linoleic, γ-linolenic, α-linolenic and eicosatrienoic acids) present in the samples. Presence of omega-3 and omega-6 (MUFA and PUFA) with respect to its ratio was observed to have cytotoxic effect on cancer cells and affect different cellular pathways to attain toxicity, mitochondrial impairments, and membrane integrity.

Consequently, the mixture of these SFA, MUFA, and PUFA in crude fatty acid samples exhibited high cytotoxic activity on MCF7 cells (Fig. 2). This could be attributed to the high amount of palmitic acid (a SFA), oleic acid (a MUFA), linoleic acid (a PUFA) present in the sample. In the study of Zhang et al., supplementation of a mixture of PUFAs that include linoleic, gamma linoleic, arachidonic, alpha linoleic, and eicosapentaenoic acid was able to inhibit the growth of human colon cancer cells (LoVo and RKO cells) and exhibit pro-apoptotic reactions.

On the other hand, Pacheco et al. used a mixture of fatty acids (9 SFA, 4 MUFA, 9 PUFA) isolated from antarctic macroalgae (Adenocystis utricularis, Curdiea racovitzae, and Georgiella confluens) to treat breast cancer cells (MCF7 and MDA-MB-231). They hypothesized that the abundance of palmitic, oleic, linoleic, and arachidonic acids on their extracts resulted to cytotoxicity for both breast cancer cell lines yet low toxicity on normal cells (CHO cells) indicating a selective tumorigenic activity of fatty acids.

Fig. 4 Comparative analysis of cellular morphology, nuclear integrity (Hoechst dye), and apoptotic signals (Caspase 3/7) of MCF7 cells treated with crude fatty acids from Halophytophthora spp. T12GP1, T12YBP2, and S. hoi. Cells viewed under fluorescent microscope at 100 x magnification.
SFA and MUFA are commonly associated to pathways related to an increased risk of cancer while PUFAs are known to possess cytotoxic activity on many cancer types. One of the ways that SFA play a role in carcinogenesis is through the activation of toll-like receptors (TLRs) linked to pro-inflammatory activities and tumor activation, whereas PUFAs have an inverse activity\(^{29}\). Thus, the mixture of fatty acids present in three oomycete strains may have the same activity as that of the fatty acids from macroalgae. The mixture of SFA, MUFA, PUFAs may possess any sort of activity towards tumorigenic activity of breast cancer cells.

Caspase activation in treated MCF7 cells (Fig. 3) was observed using the luminescence method where low relative light unit (RLU) was detected in the untreated cells and high RLU for cells treated with crude fatty acids. It, therefore, indicates that the caspase activation pathway, upon binding of the fluorescent dye to the caspase protein, was the putative explanation for the toxicity of crude fatty acids against MCF7 cancer cells. Apoptosis is the controlled cell death conserved for each cell type and the deregulation of apoptosis is a known hallmark of all cancer cell types. There are two mechanisms that affect cellular apoptosis; first is the intrinsic pathway, and the second is the extrinsic pathway.\(^{13}\) The former, specifically, is influenced by the mitochondrial membrane disruption followed by the liberation of cytochrome c in the cytoplasm. It is followed by the binding of cytochrome c to apoptotic protease factor complex which triggers the activation of initiator caspase-9 and the executor caspase-3, caspase-6, and caspase-7.\(^{6,13}\) The activation of caspase-8 and caspase-10 and other effector molecules follow, thereby inducing the actual cell death.\(^{13}\) The caspase cascade pathway activates when a foreign substance attaches to the cell membrane that switches the signalling caspase protein on. In some cellular systems, apoptosis activates the Fas/TNF receptors which, in turn, activates cysteine proteases (or caspases) that send signal to the mitochondria and nucleus to proceed with apoptosis.\(^{5,28,30}\)

---

**Fig. 5** Cytotoxicity assessment of crude fatty acids from *Halophytophthora* spp. T12GP1, T12YBP2, and *S. hoi* using MTT assay against HDFn cells. Note: (**) statistically significant values between different concentrations (\(p < 0.05\)).

**Fig. 6** Comparative analysis of cellular morphology, nuclear integrity (Hoechst dye), and apoptotic signals (Caspase 3/7) of HDFn cells treated with crude fatty acids from *Halophytophthora* spp. T12GP1, T12YBP2, and *S. hoi*. Cells viewed at 100 × magnification.
Polysaturated fatty acids, especially those that belong to omega-3 and omega-6 fatty acids (Table 2), are required for human health\(^{23}\). They are also known to have effects on different pathological processes such as cardiovascular diseases, immune diseases, neurological diseases, and even cancer\(^{23}\). Studies have shown that PUFAs induce apoptosis and affect cell proliferation due to the sensitivity of cancer cells to different PUFAs\(^{14, 23-25, 31}\). There is also variability on the effect of PUFAs depending on their concentration and the type of cancer cells being treated. Omega-3 fatty acids arrest cancer cell growth, while the effects of omega-6 fatty acids are attributed to the production of eicosanoids that are pro-inflammatory in nature\(^{23}\). Omega-3 and omega-6 fatty acids are responsible in releasing protons in the mitochondrial matrix of NT2 human teratocarcinoma cells\(^{33}\), asymmetry. Both omega-3 and omega-6 fatty acids are known to have effects in the membrane which could influence transbilayer lipid asymmetry. Both omega-3 and omega-6 fatty acids are also able to inhibit the proliferation of human prostate cancer (PC-3) and human prostate epithelial (RWPE-1) cells while affecting the production of interleukin-6 (IL-6), lipoxin A4, and tumour necrosis factor-α (TNF-α)\(^{34}\). According to the study of Meng et al.\(^{9}\), introduction of PUFAs into the cell enhances the fatty acid content in the membrane. Further, introduction of excess PUFAs in the cellular environment of human prostate cancer (PC-3) decreased cell proliferation, but that the results of the correlation between direct cell proliferation and free radical generation within the cell was premature to conclude\(^{9}\). The mechanism through which these PUFA affects cellular suppression on various cancer cell types remains unknown and may be tissue-specific on the way cells interact with certain fatty acids\(^{34-36}\).

Corsetto et al.\(^{28}\) showed that EPA and DHA promote cellular toxicity, apoptosis, and up-regulate the expression of Bcl2 and procaspase-8 of ER receptor expressing MCF7 cells, and EGFR receptor expressing MDA-MB 231 cells. Omega-3 fatty acids (i.e. EPA and DHA) influence the prevalence of omega-6 fatty acids in the cytosolic environment and cellular membrane\(^{28}\). In the study of Achenef and Arifah\(^{32}\), cis-9, trans-11 and trans-10, cis-12 conjugated linoleic acids, exhibit cytotoxic activity, apoptosis and cell cycle arrest in G0/G1 phases of MCF7 cells.

Passage of free fatty acids present from the outer environment to the intracellular environment is affected by slow bilayer diffusion, protein-lipid interactions, and protein channel-mediated transport. Excessive concentration of fatty acids affects the viability and function of cancer cells through alteration of membrane ion channels and transporters (i.e. calcium channels). Polysaturated fatty acids are responsible in releasing protons in the mitochondrial matrix of NT2 human teratocarcinoma cells\(^{23}\). Djemli-

Shikoye et al.\(^{33}\) suggested that membrane modification after fatty acid treatments is correlated to ATPase activity in the membrane which could influence transbilayer lipid asymmetry. Both omega-3 and omega-6 fatty acids (i.e. arachidonic, γ-linolenic, linoleic, α-linolenic, eicosapentaenoic and docosahexaenoic acids) were also able to inhibit the proliferation of human prostate cancer (PC-3) and human prostate epithelial (RWPE-1) cells while affecting the production of interleukin-6 (IL-6), lipoxin A4, and tumour necrosis factor-α (TNF-α)\(^{34}\). According to the study of Meng et al.\(^{9}\), introduction of PUFAs into the cell enhances the fatty acid content in the membrane. Further, introduction of excess PUFAs in the cellular environment of human prostate cancer (PC-3) decreased cell proliferation, but that the results of the correlation between direct cell proliferation and free radical generation within the cell was premature to conclude\(^{9}\). The mechanism through which these PUFA affects cellular suppression on various cancer cell types remains unknown and may be tissue-specific on the way cells interact with certain fatty acids\(^{34-36}\).

On the other hand, oxidative stress has a big role in

**Fig. 7** Cytotoxicity assessment of standard fatty acids (γ-linolenic acid, linolenic acid, linoleic acid, eicosatrienoic acid, and oleic acid) using MTT assay against human breast cancer cells.
apoptosis induction generated by reactive oxygen species (ROS) which leads to cell cycle arrest and apoptosis. Fatty acids may exhibit both ROS and non-ROS mediated apoptosis. The ROS-mediated apoptosis involves lipid peroxidation reaction and free radical formation such as peroxyl and alkoxyl radicals. Formation of malondialdehyde and 4-hydroxyalkenals from the lipid peroxidation can remain inside the system for a long period and can attack various targets on the cellular system of cancer cells. The non-ROS mediated apoptosis occurs when cell growth is inhibited in the absence of cellular oxidative stress and eicosanoid production. Through the introduction of PUFAs in the cellular system, the fatty acid suppresses the process of eicosanoid synthesis. A characteristic of PUFA is susceptibility to oxidation reaction where the methylene group between two double bonds is prone to radical attack of reactive species in the cell system. Cancer cells produce high amounts of reactive oxygen species compared to normal cells, and PUFAs easily react with free radicals and undergo isomerization to arrange the double bonds that lead to the formation of dienes and lipid hydroperoxides. This process leads to the formation of alkoxyl radicals such as malondialdehyde, which are highly toxic to cells.

In summary, Dai et al. mentioned that PUFAs are cytotoxic to cancer cells that can elicit increased ROS level, increased lipid peroxidation reaction that produces toxic lipid peroxides, caspase activation for apoptosis activity, modulation of anti-oncogene, activation of peroxisome proliferator-activated receptor, and DNA/chromosome damage.

5 Conclusion

The three marine oomycetes isolates, Halophytophthora T12GP1 and T12YBP2 and S. hoi, produced saturated, monounsaturated, and polyunsaturated fatty acids. These fatty acids were toxic to MCF7 cancer cells and are non-toxic to normal HDFn cells. Further, these exhibited apoptotic activity through caspase activation. This study confirmed that these marine oomycetes are good sources of fatty acids that influence the viability of human breast cancer cells and can be a potential drug, drug additive, or dietary supplement. It also leads to more avenues in exploring medically important fatty acids from marine oomycetes in cancer research.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

This research was funded by the National Research Council of the Philippines (NRCP), and the Philippine Council for Health Research and Development (PCHR&D), Department of Science and Technology (DOST). The Department of Environment and Natural Resources (DENR) is acknowledged for providing sampling permits and UP Mammalian Cell Culture Laboratory, Institute of Biology, University of the Philippines Diliman is acknowledged for the breast cancer cell line (MCF7) used in this study.

Supporting Information

This material is available free of charge via the Internet at http://dx.doi.org/jos.68.10.5650/jos.ess19033

References

1) Leano, E.M. Straminipilous organisms from fallen mangrove leaves from Panay Island, Philippines. Fungal Div. 6, 75-81 (2001).
2) Pang, K.L.; Lin, H.J.; Lin, H.Y.; Huang, Y.F.; Chen, Y.M. Production of arachidonic and eicosapentaenoic acids by marine oomycete Halophytophthora. Mar. Biotechn. 17, 121-129 (2015).
3) Deelal, S.; Suetrong, S.; Damrianant, S.; Unagul, P.; Sakkayawong, N. Isolation and identification of native lower fungi for polyunsaturated fatty acid (PUFA) production in Thailand, and the effect of carbon and nitrogen sources on the growth and production. African J. Biotech. 14, 1449-1460 (2015).
4) Say, E.K.P.; Yabut, A.T.V.; Cinco, N.E.T.; Caguimbal, N.A.L.E.; Devanadera, M.K.P.; Bennett, R.M.; Arafiles, K.H.V.; Aki, T.; Dedelles, G.R. Growth and fatty acid production of Halophytophthora S13005YL1-3.1 under different salinity and pH levels. Phil. Agric. Sci. 100, S6-S11 (2017).
5) Ahangar, P.; Sam, M.R.; Nejati, V.; Habibian, R. Treatment of undifferentiated colorectal cancer cells with fish-oil derived docosahexaenoic acid triggers caspase-3 activation and apoptosis. J. Cancer Res. Ther. 12, 798-804 (2016).
6) D’Eliseo, D.; Velotti, F. Omega-3 fatty acids and cancer cell cytotoxicity: Implications for multi-targeted cancer therapy. J. Clin. Med. 5, 15 (2016).
7) Conklin, K.A. Dietary polyunsaturated fatty acids: impact on cancer chemotherapy and radiation. Alternative Med. Rev. 7, 4-21 (2002).
8) Dai, J.; Shen, J.; Pan, W.; Shen, S.; Das, U.N. Effects of polyunsaturated fatty acids on the growth of gastric cancer cells in vitro. Lipids Health Dis. 12, 71 (2013).
9) Meng, H.; Shen, Y.; Shen, J.; Zhou, F.; Shen, S.; Das,
Cytotoxic Activity of Fatty Acids Sourced from Marine Oomycetes: Halophytophthora and Salispina spp.

J. Oleo Sci.

U.N. Effect of n-3 and n-6 unsaturated fatty acids on prostate cancer (PC-3) and prostrate epithelial (RPWE-1) cells in vitro. Lipids Health Dis. 12, 160 (2013).

10 Arafiles, K.H.V.; Alcantara, J.C.O.; Batool, J.A.L.; Galura, F.S.; Cordero, P.R.F.; Leaño, E.M.; Dedeles, G.R. Cultural optimization of thraustochytrids for biomass and fatty acid production. Mycosphere 2, 521-531 (2011).

11 Leaño, E.M.; Gapasin, R.S.J.; Polohan, B.; Vrijmoed, L.L. Growth and fatty acid production of thraustochytrids from Panay mangroves, Philippines. Fungal Div. 12, 111-122 (2003).

12 Marchan, L.S.; Chang, K.J.L.; Nichols, P.D.; Mitchell, W.J.; Polglase, J.L.; Gutierrez, T. Taxonomy, ecology and biotechnological applications of thraustochytrids: A review. Biotech. Adv. 36, 26-46 (2017).

13 Merendino, N.; Costantini, L.; Manzi, L.; Molinari, R.; D’Eliseo, D.; Velotii, F. Dietary ω-3 polyunsaturated fatty acid DHA: A potential adjuvant in the treatment of cancer. Biomed Res. Int. ID310186 (2013).

14 Zhang, C.; Yu, H.; Ni, X.; Shen, S.; Das, U.N. Growth inhibitory effect of polyunsaturated fatty acids (PUFAs) on colon cancer cells via growth inhibitory metabolites and fatty acid composition changes. PLoS One 10, e0123256 (2015).

15 Ho, H.H.; Joung, S. Halophytophthora gen. nov., a new member of the family Phytaecae. Mycotaxon 36, 377-382 (1990).

16 Fell, J.W.; Master, I.M. Phycomycetes (Phytophthora spp. nov. and Pythium sp. nov.) associated with degrading mangrove (Rhizophora mangle) leaves. Can. J. Bot. 53, 2908-2922 (1975).

17 Nakagiri, A. Halophytophthora species from tropical and subtropical mangroves: a review of their characteristics. Fungal Div. 7, 1-14 (2002).

18 Ho, H.H.; Chang, H.S.; Huang, S.H. Halophytophthora elongata, a new marine species from Taiwan. Mycotaxon 85, 417-422 (2003).

19 Bennett, R.M.; Devanadera, M.K.; Dedeles, G.R.; Thines, M. A revision of Salispina, its placement in a new family, Salispinaceae (Rhipidiales), and description of a fourth species, S. hoi sp. nov. IMA Fungus 9, 259-269 (2018).

20 Bennett, R.M.; Nam, B.; Dedeles, G.R.; Thines, M. Phytopythium leanoii sp. nov., and Phytopythium dogmae sp. nov., Phytopythium species associated with mangrove leaf litter from the Philippines. Acta Mycol. 52, 1103 (2017).

21 Tilay, A.; Annapure, U. Novel simplified and rapid method for screening and isolation of polyunsaturated fatty acids producing marine bacteria. Biotechnol. Res. Int. ID 542721 (2012).

22 Pote, S.; Bhadkar, R. Statistical approach for production of PUFA from Kocuria sp. BRI 35 isolated from marine water sample. BioMed Research International ID 570925 (2014).

23 Notarnicola, M.; Messa, C.; Refolo, M.G.; Tutino, V.; Micolis, A.; Caruso, M.G. Polyunsaturated fatty acids reduce fatty acid synthase and hydroxyl-methyl-glutaryl CoA-reductase gene expression and promote apoptosis in HepG2 cell line. Lipids Health Dis. 10, 1-7 (2011).

24 Dos Santos-Dias, A.C.; Ruiz, N.; Couzinnet-Mossion, A.; Bertrand, S.; Duflos, M.; Pouchus, Y.F.; Barnathan, G.; Nazih, H.; Weilgosz-Collin, G. The marine-derived fungus, Clonostachys rosea, source of a rare conjugated 4-ME-6E,8E-decadienoic acid reducing viability of MCF-7 breast cancer cells and gene expression of lipogenic enzymes. Mar Drugs 13, 4934-4948 (2015).

25 Zhang, B.X.; Ma, X.; Zhang, W.; Yeh, C.K.; Lin, A.; Luo, J.; Sprague, E.A.; Swerdlow, R.H.; Katz, M.S. Polyunsaturated fatty acids mobilize intracellular Ca²⁺ in NT2 human teratocarcinoma cells by causing release of Ca²⁺ from mitochondria. Am. J. Physiol. Cell Physiol. 290, 1321-1333 (2006).

26 Agostoni, C.; Bresson, J.L.; Fairweeht-Tait, S.; Flynn, A.; Golly, I.; Korhonen, H.; Lagiou, P.; Lovik, M.; Marchelli, R.; Martin, A.; Moseley, B.; Neuhauser-Berthold, M.; Przyrembel, H.; Salminen, S.; Sanz, Y.; Strain, S.J.J.; Strobel, S.; Tetens, I.; Tone, D.; van Loveren, H.; Verhagen, H. Scientific opinion on dietary reference values for fats, including saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, trans fatty acids, and cholesterol. EFSA J. 8, 1461 (2010).

27 Nagano, N.; Taoka, Y.; Honda, D.; Hayashi, M. Optimization of culture conditions for growth and docosahexaenoic acid production by a marine thraustochytrid, Aurantiochytrium timacum MH0186. J. Oleo Sci. 58, 623-628 (2009).

28 Corsetto, P.A.; Montorfano, G.; Zava, S.; Jovenitti, I.E.; Cremona, A.; Berra, B.; Rizzo, A.M. Effects of n-3 PUFAs on breast cancer cells through their incorporation in plasma membrane. Lipids Health Dis. 10, 73 (2011).

29 Pacheco, B.S.; Dos Santos, M.A.Z.; Schultz, E.; Martins, R.M.; Lund, R.G; Seixa, F.K.; Colepicolo, P.; Collares, T.; Paula, F.R.; De Pereira, C.M.P. Cytotoxic activity of fatty acids from antarctic macroalgae on the growth of human breast cancer cells. Front Bioeng. Biotechnol. 6, 185 (2018).

30 Zajdel, A.; Wileczek, A.; Latocha, M.; Tarkowski, M.; Kokocinska, M.; Dzierzewicz, Z. Polyunsaturated fatty acids potentiate cytotoxicity of cisplatin in A549 cells. Acta Pol. Pharm.-Drug Res. 71, 1060-1065 (2014).

31 Lu, X.; Yu, H.; Ma, O.; Shen, S.; Das, U.N. Linoleic acid suppresses colorectal cancer cells growth by inducing oxidant stress and mitochondrial dysfunction. Lipids

J. Oleo Sci.
32) AcheneF, M.B.; Arifah, A.K. Cytotoxic effect of conjugated linoleic acids on human breast cancer cells (MCF7). *Acad. J. of Cancer Res.* 5, 11-16 (2012).

33) Djemli-Shipkoyle, A.; Raccah, D.; Pieroni, G.; Vague, P.; Coste, T.C.; Gerbi, A. Differential effect of omega-3 PUFA supplementations on Na, K-ATPase and Mg-ATPase activities: possible role of the membrane omega6/omega3 ratio. *J. Mem. Biol.* 191, 37-47 (2003).

34) Das, U.N. Essential fatty acids enhance free radical generation and lipid peroxidation to induce apoptosis of tumor cells. *Clin. Lipidol.* 6, 463-489 (2011).

35) Santos, C.R.; Schulze, A. Lipid metabolism in cancer. *FEBS J.* 279, 2610-2623 (2012).

36) Ayala, A.; Muñoz, M.F.; Arguelles, S. Lipid peroxidation: production, metabolism, signalling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid. Med. Cell. Longev.* ID360438 (2014).