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Cytotoxic and Apoptotic Potential of Scylla serrate (Mud Crab) Shell Extract Silver Nanoparticles Biosynthesized against Breast Cancer (MCF-7) Cell Lines

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Abstract

Breast cancer is the most common type of cancer in women. Despite various pharmacological developments, the identification of new therapies is still required for treating breast cancer. Crab is often recommended as a traditional medicine for cancer. This study aimed to determine the in vitro effect of a hydroalcoholic crab shell extract on a breast cancer cell line. In this experimental study, MCF7 breast cancer cell line was used. Crab shell was powdered and a hydroalcoholic (70° ethanol) extract was prepared. Five concentrations (100, 200, 400, 800, and 1,000 µg/mL) were added to the cells for three periods, 24, 48, and 72 hours. Increasing the dose significantly increased apoptosis ($p<0.001$). NO secretion from MCF7 cells significantly decreased in response to different concentrations of the extract in a dose- and time-dependent manner ($p<0.050$). The crab shell extract inhibited the proliferation of MCF7 cells by increasing apoptosis, MTT assay.

Keywords: Apoptosis, Breast neoplasms, Cell survival, MTT assay, MCF-7

Introduction

Cancer is a major health problem and one of the main causes of mortality worldwide [1]. At present, breast cancer is the most common cancer in women around the world [2]. The common therapies for cancer are surgery and chemotherapy. Chemotherapeutical agents greatly damage cell DNA, affecting normal cells as well as cancer cells, and cause side effects such as digestive problems, leukopenia, and hair loss. Furthermore, drug resistance limits the effectiveness of chemotherapy [3].

Approximately 60% of the drugs approved for cancer treatment are derived from natural sources. Vincristine, etoposide, irinotecan, taxane, and camptothecin are examples of plant-derived compounds [4]. One of the important compounds in cancer treatment is chitin, $(C8H13O5N)n$. It was first derived from a fungus and is the first known polysaccharide [5]. Potent acids can dissociate chitin, a water-insoluble polymer, into acetic acid and chitosan. Chitosan is a nontoxic polymer produced by the deacetylation of chitin. Chitin is the main component of arthropod exoskeletons, tendons, and respiratory tract lining, and cell walls of the majority of fungi. Crustacean shells are a source of chitin (20%–30%). Crab shell also contains lipidic pigments such as carotenoids (astaxanthin, astatine, and canthaxanthin), lutein, and β-carotene [6].

Current therapeutic modalities for cervical cancer encompass a combination of surgical resection, radiation therapy, and systemic chemotherapy. Platinum-based agents such as cisplatin have served as the cornerstone of chemotherapy regimens for decades; however, their clinical utility is frequently compromised by dose-limiting toxicities, including nephrotoxicity, neurotoxicity, and myelosuppression. Furthermore, the emergence of acquired drug resistance significantly diminishes treatment efficacy over time, leading to disease recurrence and poor patient outcomes. The development of resistance mechanisms, coupled with the severe off-target effects of conventional chemotherapeutic agents, underscores an urgent and unmet need for the discovery and development of novel anticancer agents that demonstrate enhanced selectivity toward malignant cells while sparing normal tissues. This imperative has catalysed a paradigm shift in oncological research, directing attention toward the vast and relatively unexplored reservoir of bioactive compounds derived from natural sources.

The marine environment represents an extraordinary repository of biological and chemical diversity, harbouring organisms that have evolved unique metabolic pathways to thrive under extreme conditions of pressure, temperature, and salinity. This evolutionary pressure has resulted in the biosynthesis of structurally distinctive secondary metabolites with remarkable pharmacological properties, including potent anticancer activities. Marine crustaceans, in particular, have emerged as promising sources of bioactive compounds, with their exoskeletons containing substantial quantities of chitin—a natural polysaccharide that can be deacetylated to produce chitosan. These biopolymers, along with other constituent molecules, have demonstrated diverse biological properties, including antimicrobial, antioxidant, and anticancer activities. The therapeutic potential of crustacean-derived compounds is further substantiated by their historical utilization in traditional medicinal practices across various cultures, where crab shells and ash have been employed for the treatment of tumours and inflammatory conditions, providing ethnopharmacological validation for contemporary scientific investigation.[7]

Jeon and Kim [8] found that oligomers of chitosan exhibit antitumor activities in vitro and in vivo. Low-molecular-weight chitosan has been shown to have remarkable antimetastatic effects against lung cancer in mice [9]. Maeda and Kimura [10] investigated the antitumor effects of low-molecular-weight chitosan and reported a decline in tumor growth and final tumor weight in mice with sarcomas. In recent years, the viability of cancer cells has remarkably decreased in some cell lines, regardless of whether the chito-oligosaccharide (COS) derivatives were positively or negatively charged [11].

Carotenoids are yellow-orange pigments that are found in all higher plants and some animals. A diet containing carotenoids can decrease the risk of cancer in various tissues. Clinical studies have shown that some carotenoids have powerful antitumor activities both in vitro and in vivo [12]. The anticancer activities of different natural carotenoids as well as β-carotene have been documented. Carotenoids (α-carotene and lutein), zeaxanthin, lycopene, astaxanthin, crocetin, and β-carotene can

prevent cancer [13]. Sachindra et al. [14] examined the quantitative and qualitative distribution of carotenoids in the meat and shell of the major marine crab (Charybdis cruciata) and fresh-water crab (Potamon). The total carotenoid content in both species was low. Astaxanthin and its esters, and zeaxanthin were the major carotenoids found in the fresh-water crab extract. Selenium is an essential part of the diet of organisms, including humans, and acts as a protective factor against cancer. Although the anticancer activity of selenium is not completely known, it has been suggested that various mechanisms, such as antioxidant protection by selenoenzymes, inhibit the development of tumor cells, regulate and modulate the cell cycle, inhibit apoptosis, and repair DNA. Recent studies have indicated that selenium not only has the potential to prevent cancer but could also be used to treat cancer and enhance the effectiveness of cancer therapy in combination with other anticancer drugs or radiation [15]. In cell culture models, selenium compounds have been shown to inhibit the development of cancer cells by reducing cell proliferation via cell cycle control or an increase in apoptosis [16].
Considering the current prevalence of breast cancer and the necessity to identify and develop new pharmaceutical compounds, especially those with a natural origin, as well as the primary information obtained from traditional medicine, this study was aimed to determining the effect of hydroalcoholic crab shell extract on the viability, apoptosis, MCF7 cell line.

MATERIALS AND METHODS

Crab Shell Collection

A local fish market in Thiruvananthapuram, Kerala, was used to purchase sea crabs' shells. The shells were then rinsed abundantly in distilled water to wash away the clinging impurities, and allowed to dry in air at room temperature. After drying the shells were ground and powdered with a mechanical grinder. The shell material was crushed into powder and kept in an airtight container until it was to be further processed. Recovery of Chitosan from Sea Crab Shells The sequence of chemical steps in the extraction of chitosan was done in three broad procedures, namely: deproteinization, demineralization and de-acetylation of sea crab shells [17]. First, 5 g of finely ground crab shells were de-proteinized through the use of 1.25 M sodium hydroxide (NaOH). This mixture was stirred continuously at room temperature, 3 hours to successfully remove the protein content. Upon completion of the reaction, the material was filtered, washed continually with distilled water until a neutral pH was achieved and then airdried at 100oC in an oven. This dried and de-proteinized sample was then demineralized by incubation at 80oC in 1.25 M hydrochloric acid (HCl) over 5 hours. This enabled the elimination of calcium carbonate and other minerals present in the shells. The chitin obtained was re-filtered, cleaned using distilled water to eliminate any remaining acid, and then dried in a hot air oven. The last process involved the de-acetylation of the purified chitin in order to obtain chitosan. This was done through the treatment of the chitin with 0.5 M NaOH at 100oC over 2 hours. After the treatment, the mixture was filtered and washed with distilled water until the pH was neutral and the mixture was dried. White to off-white chitosan powder was collected as final product that was kept in an airtight container to be used in further analysis.



Fig.1: Crab Shell extract

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The basis of this test is that the mitochondrial enzyme succinate dehydrogenase in viable cells is capable of reducing the tetrazolium MTT salt to insoluble formazan, which is purple in color; the greater the number of viable cells, the greater the intensity of the stain and vice versa. To perform the test, 15×103 MCF7 cells were loaded into a 96-well plate and 200 µL of RPMI medium containing 7% serum was added. After 24-hour incubation, 200 µL of various concentrations of the extract (0, 100, 200, 400, 800, and 1,000 µg/mL) dissolved in serum-free RPMI 1640 medium was added to the wells. The cells were separately incubated with different doses for 24, 48, and 72 hours.
After incubation, the MTT test was performed in which the supernatant from each well was removed and 500 µL of MTT solution (5 mg/mL) was added to each well and incubated for 3 hours. The supernatant from each well was then removed and 100 µL of dimethyl sulfoxide was added to dissolve the formazan crystals at room temperature for 30 minutes. The optical density of each well was measured using an enzyme-linked immunosorbent assay (ELISA) reader (STAT FAX 2100; Awareness Technology, Westport, USA) at 570 and 630 nm. The viability of the cells for each concentration was calculated using the following formula [16]:
Cell viability (%)=[A570, 630(sample)/A (control)]×10MIC

Morphological Assessment of Apoptosis

Phase Contrast Microscopy

Cells will be seeded in 6-well plates at a density of 2×10^5 cells/well and treated with IC_{50} concentrations of the extract for 24 and 48 hours. Morphological changes characteristic of apoptosis, including cell shrinkage, membrane blebbing, and detachment from the substratum, will be observed and photographed using an inverted phase-contrast microscope equipped with a digital camera.

Fluorescence Microscopy Using Hoechst 33342/PI Dual Staining
Nuclear morphological changes associated with apoptosis will be assessed using Hoechst 33342 and propidium iodide (PI) dual staining.
Procedure:
1. Cells cultured in chamber slides will be treated with IC_{50} concentrations of extract for 24 and 48 hours.
2. After treatment, cells will be washed twice with PBS and stained with Hoechst 33342 (10 µg/mL) and PI (5 µg/mL) for 15 minutes at 37°C in the dark.
3. Stained cells will be observed under a fluorescence microscope using appropriate filters: Hoechst 33342: excitation 350 nm, emission 461 nm (blue)
Propidium iodide: excitation 535 nm, emission 617 nm (red)

4. Apoptotic cells will be identified by condensed and fragmented nuclei (bright blue fluorescence), while necrotic cells will show red fluorescence due to PI uptake [19].

Apoptosis Quantification by Flow Cytometry
Annexin V-FITC/PI Double Staining
Quantitative analysis of apoptotic and necrotic cell populations will be performed using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocol.
Procedure:
Cells (5×10^5 cells/well) will be seeded in 6-well plates and treated with IC_{50} and $2\times$ IC_{50} concentrations of the extract for 24 and 48 hours.
Both floating and adherent cells will be collected, washed twice with cold PBS, and resuspended in 100 µL of 1× binding buffer.
Cells will be incubated with 5 µL of Annexin V-FITC and 5 µL of PI for 15 minutes at room temperature in the dark.
After incubation, 400 µL of binding buffer will be added to each tube.
Samples will be analyzed within 1 hour using a flow cytometer (BD FACS Canto II, USA) with excitation at 488 nm and emission filters at 530 nm (FITC) and 650 nm (PI). A minimum of 10,000 events will be acquired per sample.
Data analysis will be performed using BD FACS Diva software. Cells will be categorized into four populations: Q1 (Annexin V-/PI+): Necrotic cells
Q2 (Annexin V+/PI+): Late apoptotic cells
Q3 (Annexin V+/PI-): Early apoptotic cells
Q4 (Annexin V-/PI-): Viable cells

Data analysis
Data were analyzed using one-way analysis of variance with SPSS version 16.0 (SPSS Inc., Chicago, USA) software, and $p<0.05$ was considered significant.

RESULTS AND DISCUSSION
MTT ASSAY OF MUDCRAB MCF7 viability
Comparison of the mean MCF7 cell viabilities analyzed using the MTT assay after 24, 48, and 72 hours revealed a significant difference between the groups treated with crab shell extract (400, 800, and 1,000 µg/mL) in comparison with the control group. Cell viability was again significantly decreased by increasing the dose (Figure 2 (A,B)).

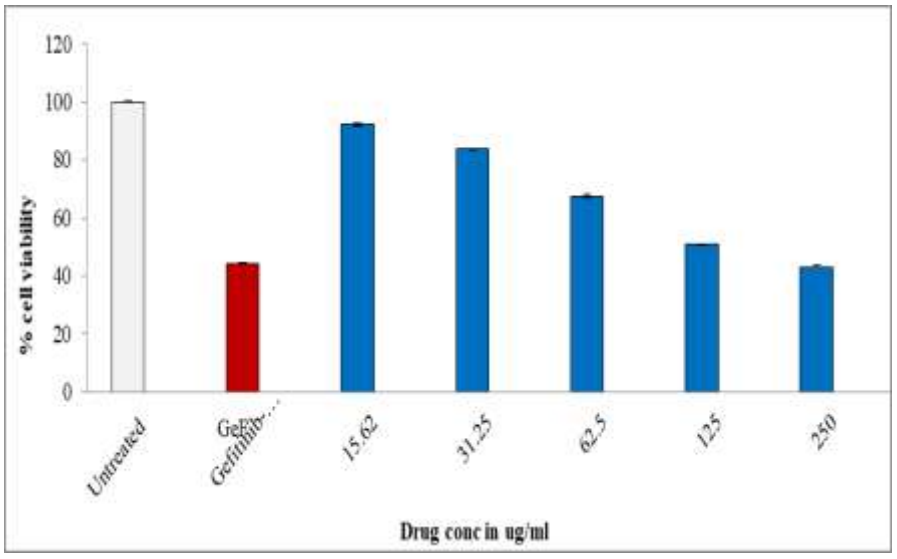


Figure 2.(A) MCF7 cell line viability (%) in different crab shell extracts and different culture periods. There is significant difference between the groups.
**p<0.05; †p<0.001.*

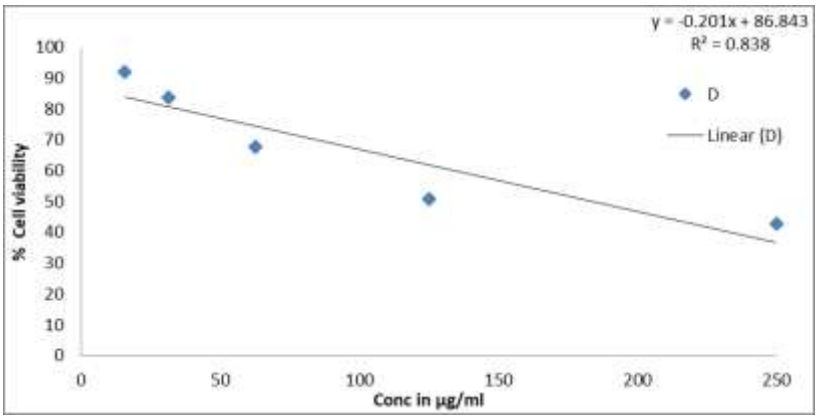


Figure 2. (B) MCF7 viability (%) after 24, 48, and 72-hour exposed to different doses of crab shell extract.
Significant difference between the experiments and control groups were seen.
*p<0.05; †p<0.001. (B) MCF7 cells in control and different doses of the extract at 72 hours by invert microscope (×100).

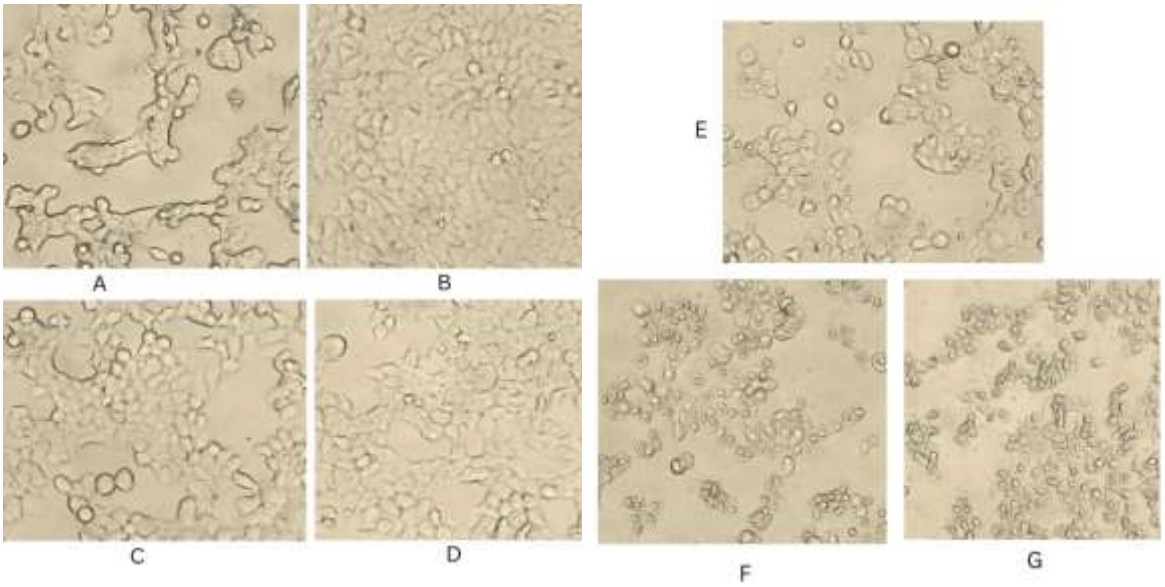


Figure 3. MCF-7 crab shell cell line.

MCF7 apoptosis

The apoptosis index of MCF7 cells treated with crab shell extract showed an increase from control to experimental groups in order of concentration, exhibiting a significant difference with 400 (p<0.050), and 800 and 1,000 µg/mL (p< 0.001) doses (Figure 4).

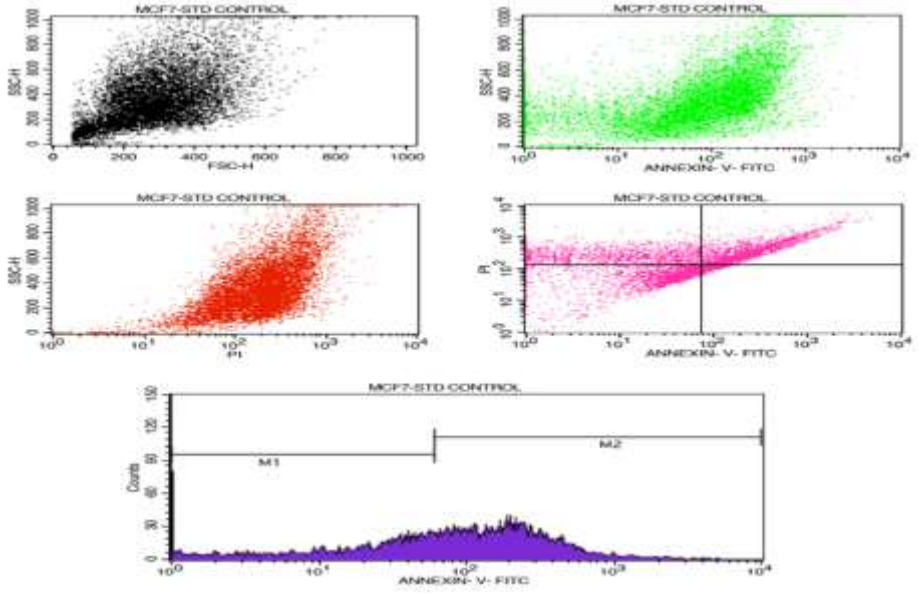


Figure 4. MCF-7 apoptosis Std Control

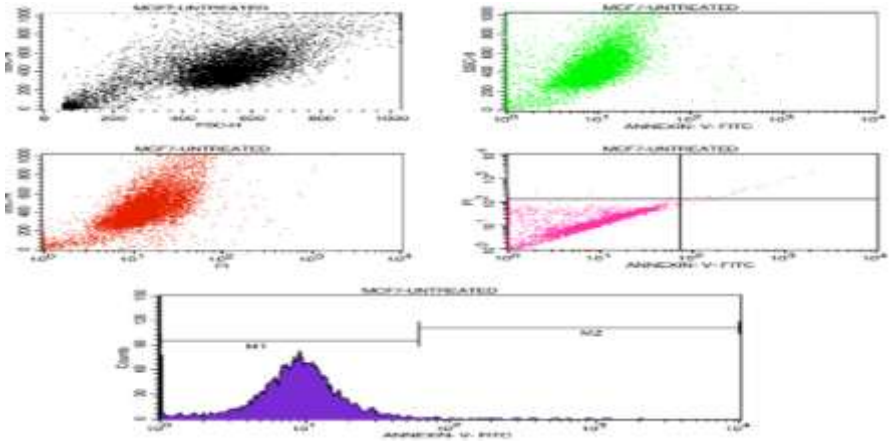


Figure 5. MCF-7 apoptosis untreated

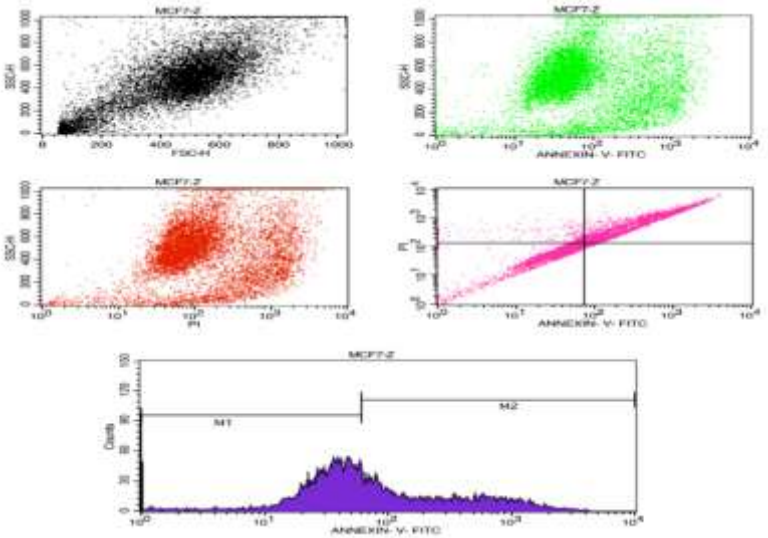


Figure 6. MCF-7 apoptosis Treated

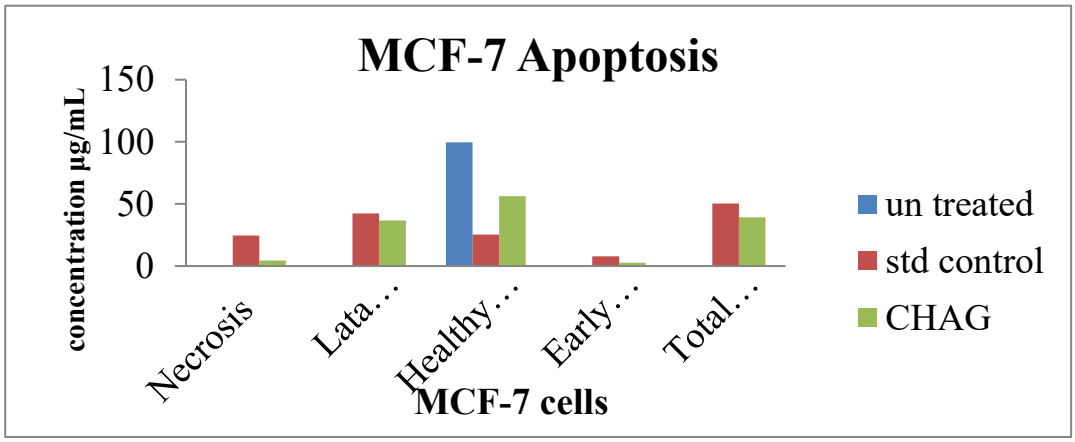


Figure 7. MCF-7 apoptosis Overall

Discussion

In this study, the effect of crab shell hydroalcoholic extract on a breast cancer cell line (MCF7) was evaluated. Five doses (100, 200, 400, 800, and 1,000 µg/mL) of the extract were prepared and their effects on the cells were analyzed over three time periods of 24, 48, and 72 hours. MTT assay used to determine cell viability. A decreasing trend was observed in cell viability along with a rising trend in apoptosis, with significant differences with 400, 800, and 1,000 µg/mL doses compared with the control group. Chitin is the second natural polymer frequently found in the exoskeleton of crustaceans and cell wall of fungi. Chitosan and COS are its main derivatives, for which the antiproliferative properties have been reported in different cancer cell lines in various studies. COS extracted from fungi has also been reported to inhibit the development of a liver carcinoma cell line (HepG2) at five concentrations and over three periods [20], which is similar to the findings of the present study in terms of inhibition of MCF7 cell proliferation at different doses and times.

The effect of raw and cooked crab shell extract and its derivative carotenoids on a liver-tumor cell line (AH109A) has previously been evaluated and the inhibitory effect observed has been attributed to the antioxidant effect of the extract [21]. However, in the present study, the inhibitory effect of crab shell extract on a breast cancer cell line was associated with NO

decrease and induction of apoptosis. The inhibitory effect on MCF7 cells was due to decreasing NO secretion, as reported by Yin et al. [22], although decreasing cyclooxygenase 2 and cytokine expression are additional factors to consider.

A decrease in NO secretion and dose- and time-dependent decrease in the viability of a liver carcinoma cell line (HepG2) and subsequent larynx carcinoma (Hep2) have been attributed to saffron extract and its carotenoids [23]; this finding is in line with the results of the present study. The effect of one of the components of selenium, methyl selenic acid, on the viability of MCF7 cancer cells assessed using the MTT assay showed inhibition of cell development by 50% over three periods of 24, 48, and 72 hours and an increase in cell apoptosis after 48 hours [19]. As in the present study, increasing the dose and time caused cell growth to decline but viability did not fall below 50%, which could be because raw extract was used in the present study unlike the previous study that used distinct and pure compounds. Furthermore, similar apoptotic effects of selenocysteine on MCF7 cells and of sodium selenite on a prostate cancer cell line (JCA1, DU145) have been reported in the studies of Chen and Wong [18] and Gasparian et al. [24], respectively.

Moreover, an antioxidant and antiproliferative effect on a human bladder cancer cell line (T24) has been reported after 48 hours, depending on chitosan concentration [25]. The effect of one of the compounds derived from chitin, astaxanthin, on a human esophagus cancer cell line (TE-4) over 24, 48, and 72 hours was investigated, and dose-dependent inhibition was observed [26]. Furthermore, astaxanthin derived from the green alga Haematococcus pluvialis exerted an inhibitory effect on a large intestine cancer cell line (HCT-116) via apoptosis induction [27]. The findings obtained in the present study are similar to those obtained previously, and confirm the findings regarding different cancer cell lines. Our study had limitations: we did not evaluate the effect of crab sell extract on normal breast epithelial cells. Moreover, using MCF-7 cell as normal breast epithelial cell line, can't substitute normal cell characteristic completely. MCF-7 cell line has a near-diploid karyotype with modest genetic modifications [28].

Silver nanoparticles AgNPs synthesis is an eco-friendly technology widely used in biosynthetic methods which utilize medicinal plants or biological microorganisms. Research lacks in the study of marine crabs and other aquatic animals capable of taking on the role as reducing or capping agents. Marine animals have the benefits of being easily available, safe to handle and posses' variable metabolites that may be considered. In this study, biological synthesis of AgNPs from whole-body marine crab, Portunus pelagicus, extract revealed great antibacterial activity against various species of pathogenic fish bacteria including; Streptococcus iniae, Streptococcus agalactiae, Encherichia coli, Edwardsiella tarda and Vibrio parahaemolyticus. The AgNPs have potential to be a new antimicrobial drug, owing to the high ability to inhibit bacterial growth. [29] study reported silver nanoparticles exhibiting a broad bactericidal activity against both gram-positive and gram-negative bacteria. [30] effectively used silver nanoparticles biosynthesized using plant extract against Staphylococcus aureus. A related result was observed with silver nanoparticles synthesise from the haemolymph of two marine crabs, Carcinus maenas and Ocypode quadrata, that determined AgNPs possess antimicrobial activity on human and fish pathogens [31]. In another reported study, scientists conducting experiments on silver nanoparticles (AgNPs) synthesized from Streptomyces rochei proved successful in inhibiting the growth of medically important pathogenic bacteria Escherichia coli, Pseudomonas aeruginosa, , Vibrio damsela, Staphylococcus aureus, Vibrio fluvialis, Bacillus cereus, Bacillus subtilis and Salmonella typhimurium[32]. The mechanism of AgNPs against bacterial isolates may be due to the interaction of positively charged Ag+ ions on the negative charge of the microorganism cell membrane causing bacterial cell death. [33] reports that adherence to bacterial cells of the cell wall is effortlessly made by smaller particles, obstructing the cells permeability, respiration and other normal behaviour with the release of Ag ions from the AgNPs particle. In agreement, [34] have also observed AgNPs adhered to the cell wall of the gram-negative bacteria (E. coli), causing destruction of the bacterial cell, while the study of agrees on silver nanoparticles mechanism of action as a bactericidal agent by disturbing the integrity and functionality of the bacterial cell membrane [35]. In addition, the Scylla serrata (mud crab) crab shell extract inhibited the proliferation of MCF7 cells by increasing apoptosis, MTT assay.

Conclusion

The present study systematically investigated the cytotoxic and apoptotic potential of Scylla serrata (mud crab) shell extract breast cancer (MCF7) cell line in a dose- and time-dependent manner. It seems that the extract exerts its effect through a reduction in NO production and induction of apoptosis.

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