RESEARCH ARTICLE

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Purification and Antitumor Activity of Different Alcohol-Derived Fragment Extracts from Crab Shell

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Abstract

Objective: Crab, a marine animal in the south of Iraq in the Gulf region, has several bioactive ingredients with potential antitumor activity. It is mentioned in Iraqi folklore as medicine with antitumor properties. **Methods:** The study examined the anticancer activity of a pharmaceutically active ingredient derived from crab shells, such as chitosan and purified fractions. Shell material was dried in an oven at 200°C. The baked granular powder was extracted by the Methanolic Extraction method and partially purified as fractions. The study included two parts: in vitro testing on several cancer cell lines and in vivo testing against a mammary adenocarcinoma mouse model. **Results:** Chitosan purified from backed crab shell powder exhibited proliferation inhibition in vitro for two human breast cancer cell lines (AMJ13 and MCF7) and one mouse mammary adenocarcinoma tumor cell line (AMN3). Furthermore, the isolated fractions showed significant cancer cell proliferation inhibition in (Cal51) breast cancer cells and esophageal cancer (SKG). The purified extract showed marked in vivo antitumor activity by significantly reducing tumor growth compared to control untreated groups. Furthermore, It shows no significant toxicity on mice weight by the methanolic extract even when used at a high dose. **Conclusion:** These results suggest that the chitosan purified from backed crab shell powder and the ethanolic fraction have broad antitumor effects that can be used for cancer treatment in clinical trials.

Keywords: Crab Shell Extracts- Chitosan- Broad Spectrum Antitumor Activity- Iraqi Folk Medicine

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Introduction

Marine bioactive compounds are crucial in the study of marine bioactive chemicals with anticancer properties. A specific type of crab, the Leucosiid crab Nursia plicata [1] found in the south of Iraq is a marine species that holds a distinctive evolutionary status, including several bioactive compounds with specialized functions attributed to its primitive nature [2]. Horseshoe crabs' hemocytes and hemolymph plasma include bioactive components such as coagulation factors, protease inhibitors, antibacterial agents, lectins, and more [3, 4].

Tachyplesin has been found in acidic extracts of the hemocyte residue from the horseshoe crab (Tachypleus tridentatus). Tachyplesin, a cationic peptide, inhibits the growth of both Gram-negative and Gram-positive bacteria at low concentrations. Additionally, it possesses anti-cancer characteristics [5, 6]. Tachyplesin may change the malignant morphological and ultrastructural features of the human gastric carcinoma cell line [7]. chemically created preparation of tachyplesin found to inhibit the proliferation of prostate cancer, melanoma, and endothelial cells could all be inhibited in a dose-dependent manner. Tachyplesin inhibits tumor growth in vivo through apoptosis induction in the tumor cells; and may cause tumor cells to die by activating the traditional complement system [8, 9]. Iraqi Leucosiid crab, Nursia plicata and Tachyplesin both have biomedical significance. Leucosiidae crabs, as demonstrated in our study, may possess antitumor compounds. Horseshoe crabs are renowned for their blood, which contains amebocytes used in medical testing.

There are many natural substances in crab shells; one of the essential substances is chitin, which are big polysaccharide molecules connected like beads on a string. Chitin has a strong positive charge, which makes it different from most polysaccharides and enables it to attach to negatively charged surfaces. Antibacterial, antifungal, and antiviral properties are exhibited by chitin and its variants, including chitosan, chitin oligosaccharides, and chitosan oligosaccharides. They are also inert and non-toxic [10]. Chitosan isolated from crab shells exhibited anticoagulant properties [11]. chitosan can boost the therapeutic efficacy of chemotherapy agents against cancer and reduce their adverse consequences, such as myelosuppression, toxicity of the gastrointestinal system, and 5-fluorouracil-induced immunotoxicity [12]. Low molecular weight Chitosan

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can dissolve in culture and can operate as a modulator for the immune system of the intestines in animals. It can also increase the cytotoxic activity of intraepithelial lymphocytes against malignancies [13].

Ancient Iraqi book on Islamic folklore medicine dating back ninety years piqued our interest. It discussed the use of crab shell ash to cure cancer patients [14]. Our group conducted a prior investigation to analyze the anticancer characteristics of four extracts derived from crab shells. (extracts obtained from crab shell powder, both backed and unbacked, using water and methanol as solvents). The aqueous and methanolic extracts of backed crab shell powder demonstrated a dose- and time-dependent proliferation inhibition in vitro for two human carcinoma cell lines; (Hep-2, RD) and one murine tumor cell line (AMN3). These findings suggest that the crab shell powder has anti-cancer properties [15]. The current study is designed to isolate active ingredients from Crab shells and test their antitumor activity, in vitro and in vivo.

Materials and Methods

Crab shell samples

Our team gathered crab shell samples from the southern Iraqi city of Basra and transported them to the laboratory.

Isolation and purification of chitin and chitosan

Shell material was extracted, dried at ambient temperature, and ground for each crab sample. Powder dried in an oven at 200°C for two hours, leaving broiled crab shell material that was directly extracted. The baked granular powder was extracted according to Markham (1982), and Methanolic Extraction was conducted [16]. The extraction method consisted of two stages. In the first, a MeOH: H2O (9:1) solvent was utilized. Next, a 1:1 ratio of the same solvent was used. Extraction lasted seven hours during both phases. Following each extraction procedure, the mixture was filtered. Whatman no. 1 facilitated the filtration process. The two extracts were mixed together and placed in uncovered Petri dishes in an incubator at 37°C until fully dehydrated. This extract was used as a control in the in vivo study.

Through demineralization and deproteinization, chitin was purified. The powdered shell was treated for 24 hours with 2N HCl to eliminate the mineral content and then with 1N NaOH at 80 ˚C for 24 hours to take off the protein content [17]. The deacetylation procedure transformed extracted chitin into chitosan [18]. chitin was deacetylated in a 40% aqueous NaOH solution by heating it under reflux at 110 ˚C for six hours before settling at 22 ˚C. The recovered precipitate was rinsed with distilled water and allowed to stand for 12 hours with constant stirring at 22 ˚C in a 10% acetic acid solution. Using a 40% NaOH solution, the pH was adjusted to 10 using a pH adjuster. The solution was dialyzed against deionized water for a period of 24 hours. The product was centrifuged at 10,000 rpm for 10 minutes to obtain pure chitosan.

Ultra violate visible light spectroscopy scanner

Because the ethanolic extract of the roasted crab shell was unknown to us from the point of its containment of compounds, an ultra violates- visible light spectroscopy scanner (AquaWell Q5000, nanodrop spectrophotometer, England) was used to determine the peaks that may represent the presence of a particular compound. Also, this tool may help characterize these compounds during silica gel fractionation. One mL was used in the test and of the extract, and 70% ethanol was used as a blank.

Fourier-transform infrared spectroscopy (FTIR)

This assay was used to determine the functional groups in the ethanolic extract of the roasted crab shell. Ten microliters of the extract were used to carry out this assay using an FTIR system (Bruker, Tensor 27, Germany) coupled with a thermal unit (Specac, high-temperature golden gate controller) connected to the diamond stage allows using liquid samples with small volumes. The data were collected with OPUS software instilled in a computer system linked to the FTIR system.

Gas chromatography mass-spectroscopy

The active extract was analyzed by Shimadzu QP2010/Japan version 2010 Gas Chromatography/ Mass Spectrometry at the Iraqi Ministry of Science and Technology. The interface between a Gas Chromatograph and a Mass Spectrometer. Column of capillary material (InertCap 1MS, 0.25mm, 30m, 0.25m, Gl Sciences, Japan) Helium (99.999%); constant flow rate of 1 ml/min; Auto-Injector: AOC-20i, Shizuoka. The injection volume was five microliters. Chamber oven Temperature: 100°C. Program for oven temperature: 100 °C for three minutes, 240 °C for nine minutes, 280 °C for five minutes, and 300 °C for two minutes [19].

Silica gel column chromatography

In order to fractionate the component of ethanol extract of the roosted crap shell, a silica gel column was prepared according to the following method. A class column with 30 cm length and 2 cm inner diameter was used. The bottom end of the column consists of a rounded glass mashed disc with 0.5-micrometer pours and a stopcock. The utilized stationary phase (silica gel) was packed in the column after was converted to a slurry. This was carried out by Pouring 150 g of the silica gel into an Erlenmeyer flask. The silica gel-containing Erlenmeyer flask was subsequently added to a 70% ethanol solvent system. The addition of sufficient solvent ensured that all of the silica gel was effectively suspended. Since silica does not dissolve, the solvated mixture must be visually distinct. After adding the solvent, the Erlenmeyer flask was stirred to ensure that all of the silica was thoroughly solvated. A ring stand was used to secure the column in a vertical position. Using a funnel, the prepared silica gel slurry was carefully poured into the column. In order to convey the slurry from the Erlenmeyer flask to the column, an additional solvent was required. Using a pipette, any silica gel that adhered to the column's walls was removed. As the silica gel settled in the column, it was lightly tapped by the sides to ensure that it was densely packed and free of air bubbles. The stopcock was then opened to enable the solvent to drain into a clean Erlenmeyer flask until the

fronts of the silica gel and the 70% ethanol solvent met. The remaining solvent was extracted.

After finishing the column preparation, 500 mg of dried ethanolic extract was dissolved with 1 mL of 99% ethanol. The dissolved extract was placed with a pastor pipet on the top of the silica gel column and was let to enter the stationary phase of the column. Three types of solvent washes were used to fractionate the component of the carb extracts. They were arranged according to their polarity. The First was methanol, the second was ethanol, and the third was acetonitrile. One hundred milliliters of each solvent was used to elute the extract component down the column. Fractions of 10 mL were gathered down the column, and after finishing the liquids, the column was washed with 150 mL of triple distilled water. The absorbance of each fraction was read at 270 nm with the same spectrophotometer to determine the fraction tube that contains the highest active compound concentration. The number of three solvents eluted fractions tubes was reduced by gathering the tubes with similar or the same absorbance values. After that fraction of each solvent that has absorbency was dried in an incubator (Miller, Germany) at 37 ºC, weighed, and dissolved in 2 mL of DMSO to be used in an anti-cancer activity assay.

Cell lines

The AMJ13, MCF7, AMN3, and REF cell lines were cultured in RPMI-1640 media with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C. The human breast cancer cell line CAL51, human esophageal carcinoma cell lines (SKG), and the human epithelial breast tissue cell line HBL were cultured in MEM media supplemented with 10% FBS. The Experimental Therapy Department of the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) provided all the cell lines from the Cell Bank Unit. The cells are regularly assessed for typical growth traits and verified periodically.

In vitro Cytotoxicity assays

AMJ13, MCF7, AMN3, and REF cells were cultured at a density of 7000 cells / well in 96-well microplates and incubated overnight at 37°C. Serial dilutions of pure chitosan were prepared in serum-free media ranging from 1000 to 7.8µg/ml, then incubated for 72 hours to assess growth inhibition. Three distinct fractionations (Acetonitrile, Methanolic, and Ethanolic) were evaluated on the CAL51 breast cancer cell line, the human epithelial cell line (HBL), and SKG, the human esophageal carcinoma cell line. Following the incubation period, cell viability assays were conducted to assess the rate of growth inhibition. Cell viability was assessed 72 hours post-treatment by replacing the medium with 28 µl of a 2 mg/ml MTT solution (Bio-World, USA), and incubated at 37°C for 1.5 hours. The residual crystals in the wells were dissolved by adding 130 µl of Dimethyl Sulphoxide (DMSO) (Santa Cruz Biotechnology, USA), and shaking for 15 minutes. A microplate reader (Optima microplate reader, BMG labtech, Germany) was used to detect absorbance at 492 nm. The assay was performed in triplicate and repeated at least twice. The average viability of the treated cells at each dilution was measured as a percentage compared to the control wells treated with just medium (100% survival) ± SEM. The treated and untreated cells were stained with crystal violet, viewed under an inverted microscope, and photographed for cytopathological examination.

In Vivo Experiments

Laboratory animals

All animals were managed according to the rules of the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) in the animal house unit. The protocols adhered to the International Guiding Principles for Biomedical Research Involving Animals, ensuring humane treatment and care of the animals used. All procedures were reviewed and approved by the Scientific Committee of ICCMGR.

Animal cancer model

The murine mammary adenocarcinoma tumor (AN3) used in the present study has been previously described [20]. AN3 tumor line originated from the mammary tumor of an Albino Swiss mouse. Continuous transplantation of AN3 tumor cells into inbred syngeneic mice maintains the AN3 tumor line. This tumor was supplied by the cell Bank unit and animal house unit of the ICCMGR's Department of Experimental Therapy.

Experiment Design

Tumors were induced by inoculating AN3 cells (106 cells in 100 µl per location) into the right flanks of female Swiss Albino mice aged 6–8 weeks. (ICCMGR, Animal housing unit, Baghdad, Iraq). When tumor nodules reached a diameter of 0.5–1 cm, the animals were randomly divided into three groups of five. One group received intraperitoneal (IP) injections of the extract at a dosage of 0.5g/kg in 100 µl PBS, the second group received 1g/kg IP injections daily for 30 days, and the third group served as the untreated control group. After 30 days, the animals were sedated and euthanized using a lethal dose of diethyl ether.

Antitumor activity assessment

Every third day, tumor diameters were recorded, and tumor size was measured with calipers. Tumor size was measured according to the following equation (0.5 × lengths × width × width) [21] and presented as mean ± SEM for every single group. The animals were euthanized when the tumor volume reached roughly 10% of the total body weight.

In order to measure tumor growth, tumor size was normalized to each tumor's volume at day zero when treatment was initiated. Tumor growth inhibition (TGI) was assessed biweekly using a specific formula [22].

$$GI\% = \frac{\text{tumor volume of untreated group - tumor volume of treated group}}{\text{tumor volume of untreated group}} \text{x}100$$

Tumor growth inhibition >50% is considered meaningful.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., USA). One-way ANOVA was utilized to statistically compare three or more groups. Graphs display data as the mean value ± s.e.m.

Results

Ultra violate visible light spectroscopy scanner

We used UV-Vis scanning spectroscopy to detect λmax of the compounds present in unknown samples. Figure (1) indicates clearly that most of the compound present in the 70% ethanolic extract of roasted crab shell laid in the range of UV spectrum between 200 and 300 nanometers wavelength. This result determined the range of spectroscopy wavelength to be used in the fractionation step for that extract's active compound or ingredient.

Fourier-transform infrared spectroscopy (FTIR)

This analysis revealed that the 70% ethanolic extract of the roasted crab shell contains active compounds with a number of active groups detected in the wavelength ranging from 500 to 2500 nm, as shown in Figure (2).

Silica gel column chromatography

In this step of active compound identification, three types of solvents were used. Since the crab extract was produced using 70% ethanol, a high percentage of its compound consisting of polar properties was expected. Thus, a range of polar solvents was used to elute the active compound from the silica gel column. Building on this strategy, solvents ranging from the highest polarity to the lowest were invested. These solvents were, according to their relative polarity, methanol (with a relative polarity of 0.762), ethanol (with a relative polarity of 0.654), and acetonitrile (a relative polarity of 0.460) [23]. Figure (3) represents the absorbance of the eluted fractions at 270 nm. There were two main peaks in absorbance, indicating the presence of a specific type of compound. As mentioned in the methods, ten tubes were collected from each type of solvent used. After measuring the absorbance of each fraction tube at the indicated wavelength, a similarity in absorbance values was noticed between adjacent tubes. Therefore, the tubes with similar or the same absorbance values were gathered, reducing the number of the collected tubes of each solvent used to 3 instead of 10. The methanolic eluents were reduced to three fractions; the middle one (methanol 2) possesses the peak absorbance at the indicated wavelength. The ethanolic eluents were also reduced to three fractions the last two third of them possess the peak absorbance. The adjacent tubes in the boundaries between the ethanolic and acetonitrile eluents had similar absorbance, so they were gathered and counted with the fractions of acetonitrile because the remaining tubes of acetonitrile did not have any peak absorbance, which indicates that there was no active compound could be detected in them. Table (1) shows the weight of each solvent fraction collected from the silica gel column.

Gas chromatography mass-spectroscopy

The results of GC-MS spectroscopy for the compound contained in the three solvent fractions are presented in the Figures (4 to 6). The methanolic fraction consists mainly of two compounds: Cholesteryl 3-cyclohexyl butyrate, and Piperidinone. The ethanolic fraction consisted of Phenol, 2-[1-(4-hydroxyphenyl)-1-methylethyl]. At the same time, the acetonitrile fraction consists of Cholesterol 3-O-[[2-acetoxy]ethyl]. Each detected compound structure is presented in the GC-MS chromatograms in the Figures mentioned.

In-vitro antitumor activity of purified extract (Chitosan)

To assess the effectiveness of purified crab shell extract (chitosan) and its ability to induce cytotoxicity, cancer cell lines underwent MTT cell viability assay. (AMJ13, MCF7, and AMN3) And normal mice embryonic cells

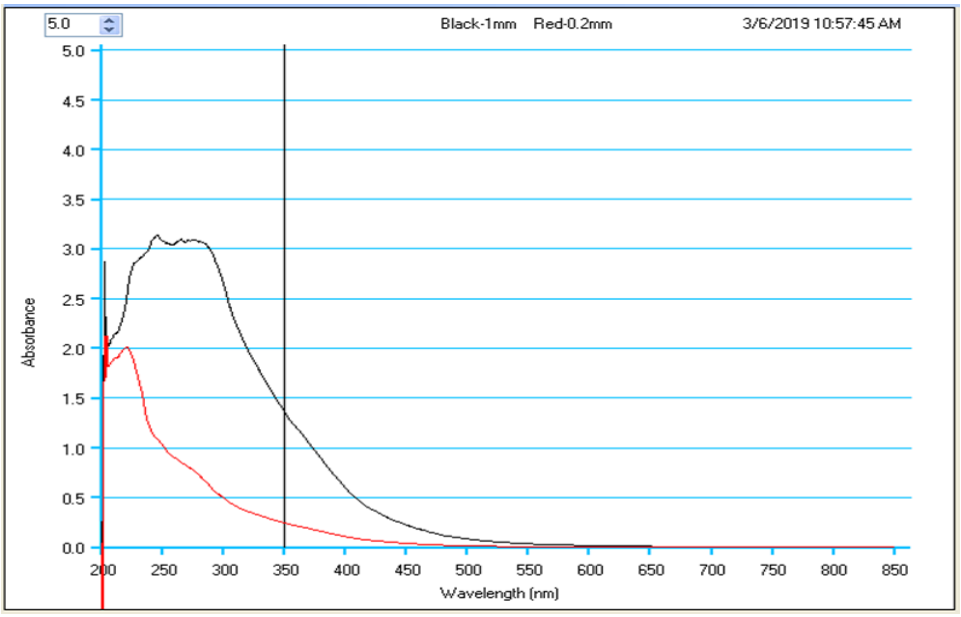

Figure 1. Ultraviolet-Visible Light Scanning for the 70% Ethanolic Extract of the Roasted Crab Shell

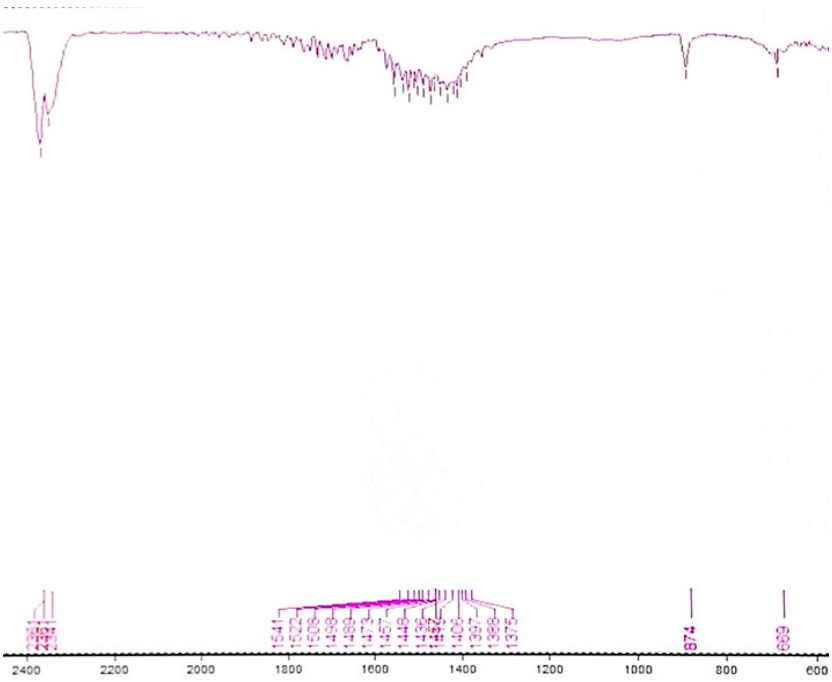

Figure 2. This Analysis Revealed that the 70% Ethanolic Extract of the Roasted Crab Shell Contains Active Compounds with a Number of Active Groups Detected in the Wavelength Ranging from 500 to 2500 nm.

Table 1. The Weight of Each Eluted Fraction Collected after Silica Gel Filtration Chromatography of the Roasted Crab Shell Using Three Different Solvents

The solvent used to elute compound	Collected weight of the fractions eluted
Methanol	153 mg
Ethanol	78 mg
Acetonitrile	22 mg

(REF). The results in Supplementary Figure 7 indicated that most of the concentrations used effectively reduced the viability of cancer cells while having nearly the same cytotoxicity in normal cells. In addition, human breast cancer cells showed more sensitivity to the purified extract. Cytopathological lesions of crystal violet-stained cells showed that treated cancer cells are detached, having cytoplasmic and nuclear shrinkage and cell rounding at a dose of 125 µg/ml compared to untreated cancer cells. While having nearly the less cytotoxic effect in REF

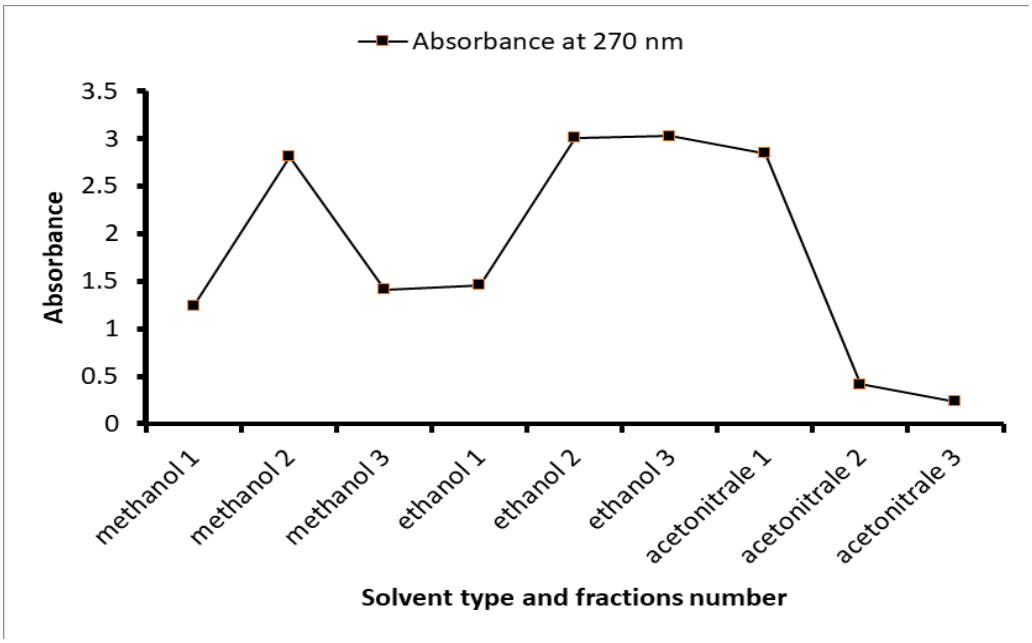

Figure 3. Absorbance of Fractions Eluted from the Silica Gel Column at 270 nm Using Three Different Solvents and Three Concentrated Fractions Gathered from 10 Tubes for each Solvent Used in the Fractionation Process. Numbers with the solvent indicate the overall fractions produced after gathering the tubes with similar or same absorbance values for the solvent used.

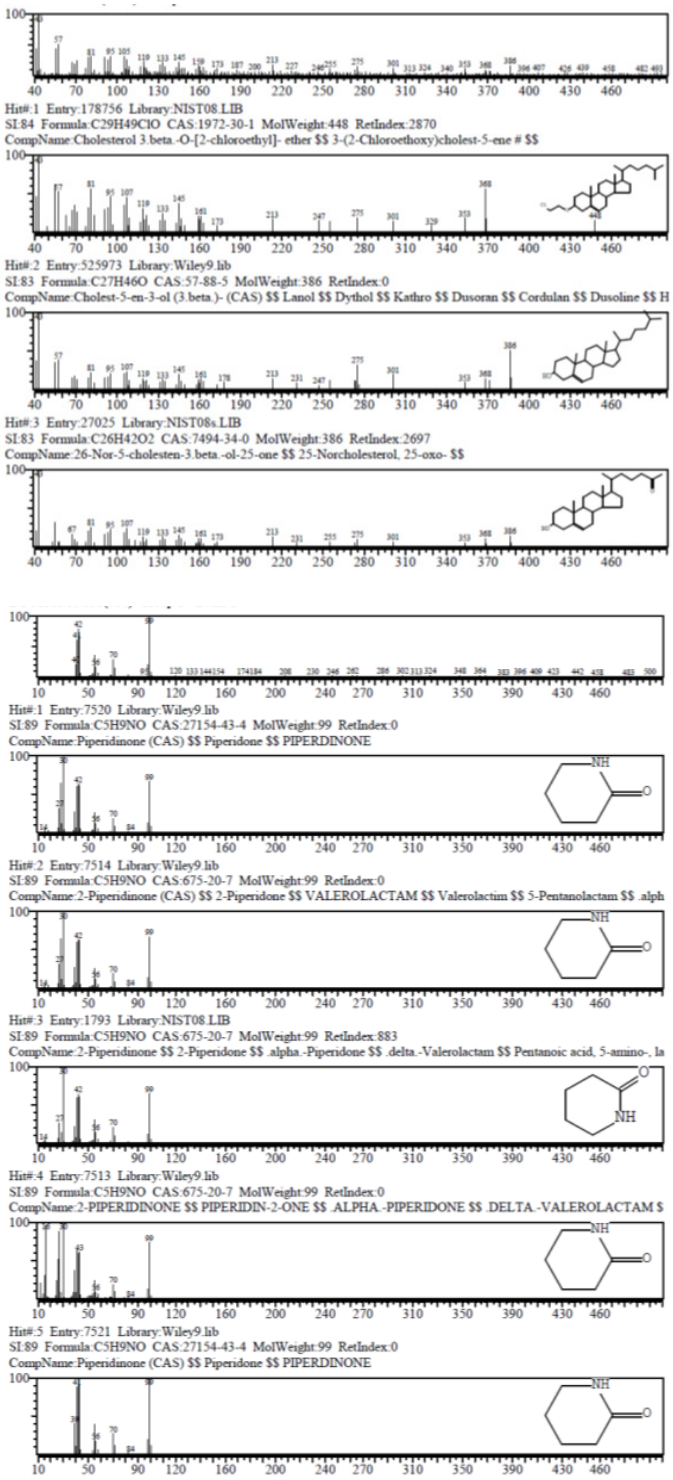

Figure 4. Cholesteryl 3-cyclohexyl Butyrate and Piperidinone was the Identity of the Methanolic Faction Compound Using GC-MS Spectroscopy

normal cells, there are also signs of cell detachment and cell shrinkage but in fewer numbers (Supplementary Figure 8).

In-vitro antitumor activity of purified fractions

Cytotoxicity of the fractionated extract against normal and breast cancer cell lines was done to evaluate the possible anti-cancer. Now, the Acetonitrile fraction IC_{50} was 0.33ug in CAL51 cells (Supplementary Figure-9A). Methanolic fraction IC_{50} was 0.872ug in CAL51 cells (Supplementary Figure-9B). Ethanolic fraction IC_{50} was 1.536ug in CAL51 cells (Supplementary Figure-9C). Acetonitrile fraction IC_{50} was 2.621ug in HBL cells (Supplementary Figure-9D). Methanolic fraction IC_{50} was 13.27ug in HBL cells (Supplementary Figure-9E). Ethanolic fraction IC_{50} was 2.4ug in HBL cells (Supplementary Figure-9F). Comparison between IC_{50} of acetonitrile fraction on cancer and normal cells showing the significant safety margin (Supplementary Figure-9G). Comparison between IC_{50} of the methanolic fraction

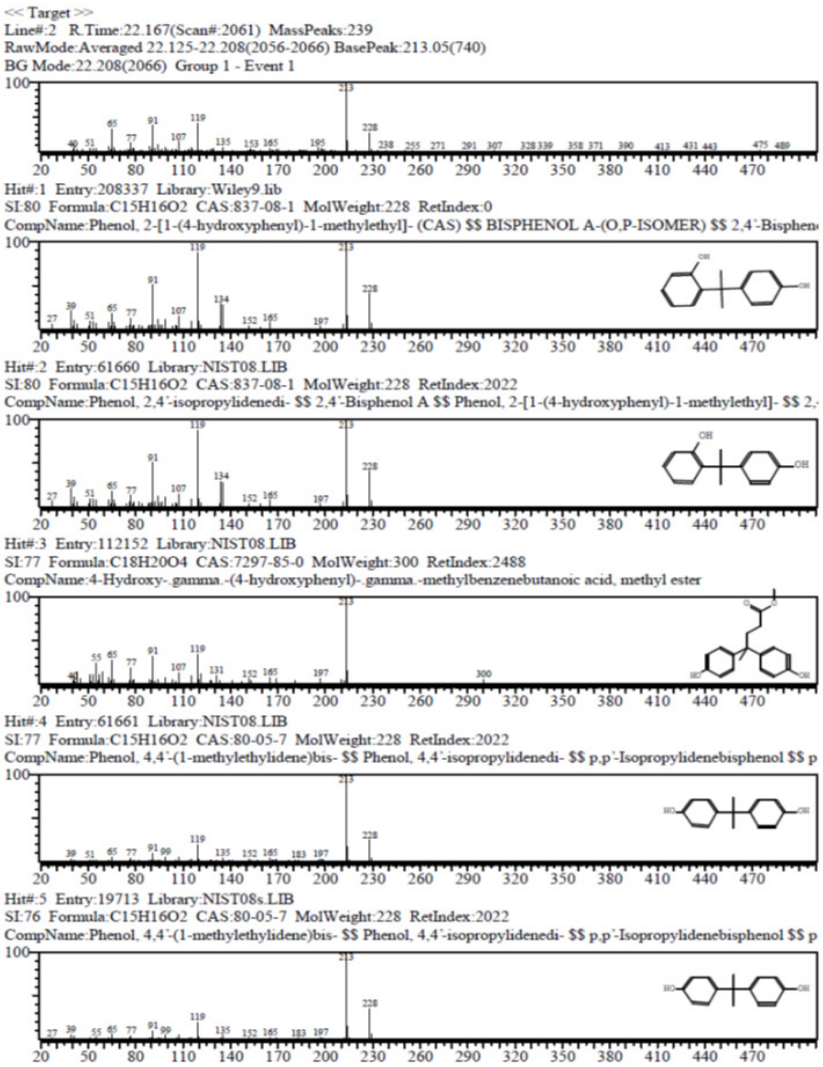

Figure 5. Phenol, 2-[1-(4-hydroxyphenyl)-1-methylethyl] was the Identity of the Ethanolic Fraction Compound Using GC-MS Spectroscopy.

on cancer and normal cells showing the significant (Supplementary Figure-9H). Comparison between IC_{50} of the ethanolic fraction on cancer and normal cells showing the high safety margin (Supplementary Figure-9L). Cytotoxicity of the fractionated extract against human esophageal carcinoma cell lines (SKG) shown in Supplementary Figure-10. Acetonitrile fraction IC_{50} was 38.24ug (Supplementary Figure-10A). Methanolic fraction IC_{50} was 705ug (Supplementary Figure-10B). Ethanolic fraction IC_{50} was 2.357ug (Supplementary Figure-10C). cytopathological effect of the fractionated extracts in normal and cancer cell lines were studied at IC_{50} doses. Acetonitrile fraction treated CAL51 breast cancer cells showed the cell detachment lesion at IC_{50} dose of 0.33ug in compared to other fractions and to untreated cancer cells. For the normal breast epithelial cells HBL, Ethanolic fraction showed the highest killing effect at IC_{50} dose of 2.4ug where it showed mainly cell detachment compared to untreated cells. Cytopathological lesions induced by the Ethanolic fraction at IC_{50} dose of 2.357ug were the highest when used to treat human esophageal carcinoma cell line (SKG) such as cell shrinkage and nuclear condensation in compared to Acetonitrile fraction and Methanolic fraction and untreated cancer cells (Supplementary Figure-11).

Crab extract antitumor activity against AN3 Mouse mammary adenocarcinoma tumor model

To explore the theory that baked crab extract antitumor activity in-vitro will be shown in vivo as well, we transplanted Swiss Albino female mice with mammary adenocarcinoma tumor cells (AN3 cell line). The cells were implanted in the dorsal area through a pincture in the thigh region to avoid tumor cell leakage, this location allows for easy tumor measurement. When the tumors mass reached a diameter of 0.5–1 cm, the animals were sorted into three groups of five in random order. As represented in Supplementary Figure 12, relative tumor volumes were graphed for a period of 30 days. In response to methanolic extracts of baked crab shell therapy, 1g/kg was administered to the first group which showed the highest inhibition in tumor volume ($P < 0.05$), and continued to show a significant tumor growth inhibition rate to the end of the experiment, as shown in Supplementary Figure 13.

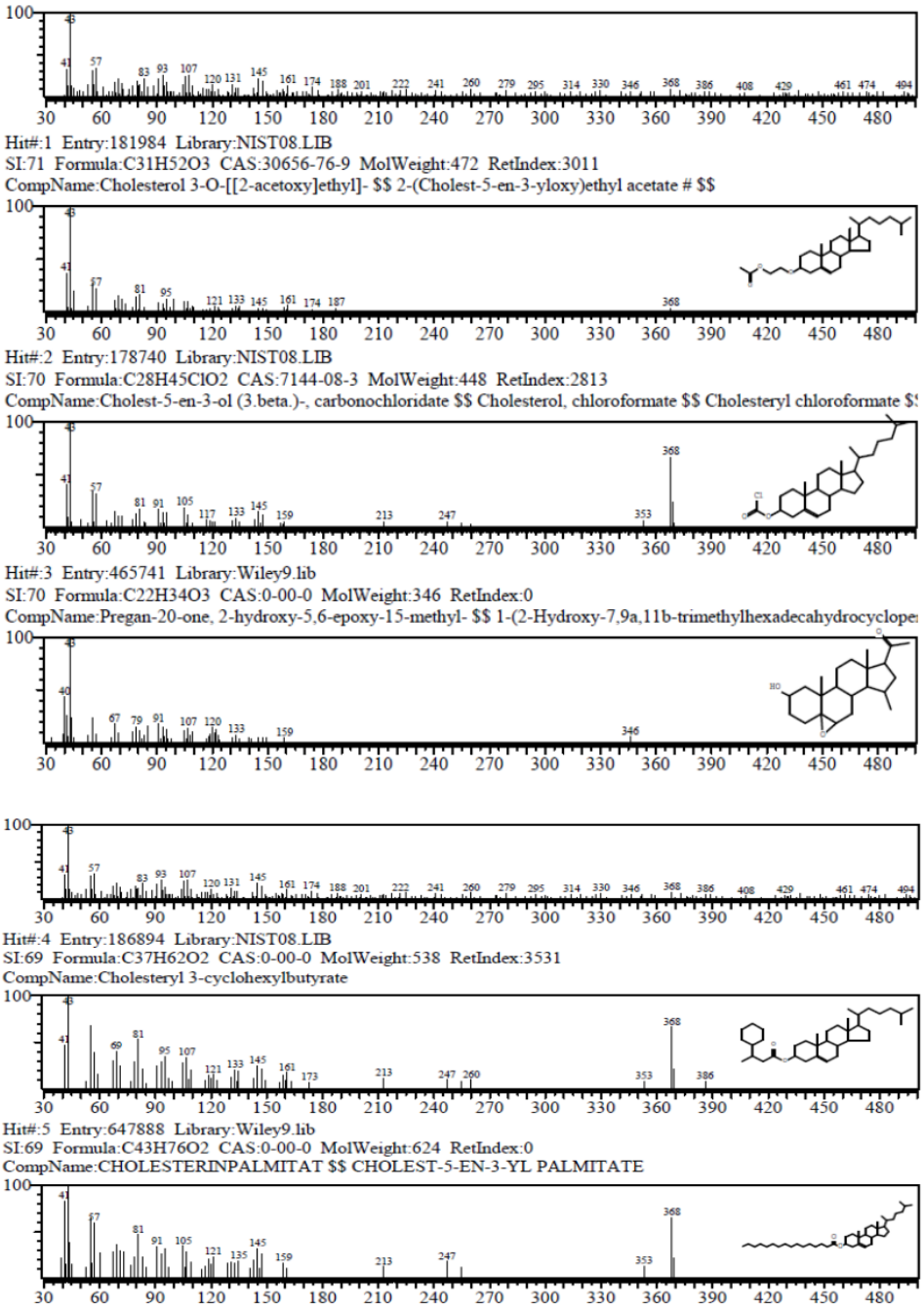

Figure 6. Cholesterol 3-O-[[2-acetoxy]ethyl] was the Identity of the Acetonitrile Fraction Compound Using GC-MS Spectroscopy.

The tumors in the untreated control group continue to grow at all days of the experiment. Figure 14 shows the weight of the treated groups in comparison to the untreated control group which shows no significant toxicity by the methanolic extract on mice weight even when using a high dose of 1 g/kg for 30 days.

Discussion

The current work shows clear evidence for the antitumor effect of the baked crab shell fractions, either purified or not. The purified chitosan showed a cytotoxic effect that needs more investigation and more purification to reduce the cytotoxic effect on normal cells. The in vivo results were very promising and provided encouraging results for future clinical trials. In a prior in vitro investigation, we evaluated extracts from baked and unbaked crab shells. The results showed that extracts from baked crab shells were more effective and had a greater cytotoxic effect compared to extracts from unbaked crab shells, particularly the methanolic extract of baked crab shells [15]. The three isolated fractions showed promising general antitumor activity but the ethanolic fraction was the inducer of cancer cell cytotoxicity.

Earlier research has documented the discovery of anticancer agents in crabs, including tachyplesin found in the leukocytes of the horseshoe crab. Moreover, chitin in crab shells has also been identified as a potential anticancer compound [13].The anticancer action of crab shell extracts is attributed to the presence of chitin and its

derivatives, identified as an antitumor chemical by [12]. One of our research participants in a prior study [24] identified the presence of germanium, selenium, zinc, chromium, magnesium, and copper in crab shell ashes. Hence, we proposed the development of a novel complex by backing it in the oven at 200°C to form minerals, amino acids, vitamins, and polysaccharides found in a crab shell, which requires further research. The backing process may have advantages such as enhanced cytotoxicity, by making it more effective against cancer cells through improved bioavailability. The baking process may help in breaking down the shell, making the active compounds more accessible and bioavailable. However, there are some expected disadvantages of baking, such as potential degradation, over-baking can lead to the degradation of active compounds, reducing the effectiveness of the extract and requiring controlled conditions; maintaining consistent baking conditions (temperature and time) is crucial to ensure reproducibility and effectiveness.

In a study done by Rezakhani [25], he found a decreasing trend in cell viability along with an increase in apoptosis, with significant differences in doses compared with the control group. It appears that the preparation is through a reduction in NO production and the induction of apoptosis. Chitin derivatives, carotenoids, and selenium are important factors to consider in the process of growth inhibition [26]. The same group found that crab shell extract showed an anti-prostate cancer effect by inducing cell apoptosis and decreasing NO production [27]. Moreover, chitosans were found to be antioxidants and possess antitumor activities, which depended on their molecular weight [28]. This is explained by higher Solubility, as lower molecular weight chitosans are more soluble in water, which enhances their interaction with cancer cells and improves their antitumor efficacy. Moreover, increased Bioavailability, smaller chitosan molecules can penetrate tumor tissues more effectively, leading to better bioavailability and stronger antitumor effects. Finally, enhanced Cellular Uptake, lower MW chitosans are more easily taken up by cancer cells, which increases their cytotoxic effects [29]. Our study has some limitations such as a single cancer model for in vivo study which focused solely on a murine mammary adenocarcinoma tumor model (AN3) as in vivo model for human breast cancer. While the in vitro studies included a more other cell line. Moreover, the study primarily focused on demonstrating the antitumor activity of crab shell extracts but did not delve deeply into the underlying mechanisms of action. While the study identified some compounds in the extracts using GC-MS, a more comprehensive characterization is needed. This includes determining the precise composition and concentration of all active compounds, which could contribute to understanding the synergistic effects and optimizing the extraction process.

In conclusion, we present evidence that purified ethanolic fragments from baked crab shells have the broadest spectrum growth inhibitory effect on different types of cancer especially the breast and esophageal cancer cells in vitro, and The backed crab shell had significant in vivo growth inhibition and can be used in clinical trials.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

General

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Funding Statement

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Approval

It is approved by the scientific committee of the Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Declaration

It is approved by the scientific committee of Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq.

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