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Original article

In *vitro* cytotoxicity and pro-apoptotic activity of phycocyanin nanoparticles from *Ulva lactuca* (Chlorophyta) algae

Abdulrahman L. Al-Malki

Department of Biochemistry, Faculty of Science, King Abdulaziz University (KAU), Jeddah, Saudi Arabia
Bioactive Natural Products Research Group, KAU, Saudi Arabia
Experimental Biochemistry Unit, King Fahd Medical Research Center, KAU, Saudi Arabia

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ABSTRACT

This study investigated the *in vitro* antioxidant, proapoptotic and anti-proliferative activity of phycocyanin extracted from *Ulva lactuca* (Chlorophyta) algae extract loaded on albumin nanoparticle (ULANP). The characterization of ULANP profile was done by using FTIR and its cytotoxicity was investigated by using MTT assay against HepG2 and MCF7 cell lines. The proapoptotic markers caspase 8 & 9 were measured. Analysis of ULANP by FTIR showed the characteristic band (2100 cm^{-1} ~3700 cm^{-1}) that is indicated primarily by —COO, —CO and conjugated double bond. These bonds showed the spectral band at peaks of 2985 cm^{-1} and 2860 cm^{-1}, 2986 cm^{-1} respectively. The antioxidant potential and radical scavenging property of ULANP was also appreciable as compared to the vitamin C and gallic acid. The antiproliferative assay carried out by WST-1 suggests that ULANP was effective against both HepG2 (93.17%) and MCF7 (91.3%). Caspase-8 and −9 were significantly elevated ($p < 0.001$) in both the cell lines of breast and liver cancer. It was concluded that ULANP induced anti-proliferative and pro-apoptotic activities on liver and breast cancer. It is promising as a novel antitumor activity for further investigation the mechanistic pathways mediated this action.

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1. Introduction

Cancer remains a nightmare globally affecting millions of people of varying ages and cultures. Generation of free radicals is a major contributing factor in various chronic diseases, such as cardiac diseases, inflammation, hypertension, diabetes mellitus and neoplasia including cancers (Berber, 2015). Molecular oxygen species can react easily with free radicals to form reactive oxygen species (ROS). The ROS includes hydroxyl radicals and superoxide radicals. These radicals can induce oxidative damage to biomolecules such as membrane lipids, proteins, and nucleic acids. The damage subsequently leads to degenerative diseases (Pietra, 1997). The drug discovery using natural products as medicinal plants or marine organisms remains an important target in current research. Marine microorganisms such as green and blue algae were identified as rich sources of biologically active compounds (Kelecom, 2002). Presently, some countries consume a variety of algae, during food processing, which have high contents of fiber, minerals, vitamins and scavenging activity against free radicals (Parekh and Chanda, 2007). The Saudi coast line in the Red Sea contains different species of marine algae. However, there are a few studies on the biological effects of the marine algae in this region.

Human serum albumin (HSA), the most abundant protein in the body, is preferred for its non-toxicity and ready modifiability (Cohen et al., 2013). Nanoparticles prepared from HSA have many advantages for use in cancer treatment. They are cheap and easily available. They are also non-antigenic, enzymatically degradable, and easy to prepare using promising techniques. It is possible to obtain HSA nanoparticles in a defined size range by manipulating process (Langer et al., 2008).

Nanoparticle technology with much pharmacological applications have been developed recently (Ganesan et al., 2008). Various degradable nanoparticles of natural polymer-origin such as starch and chitosan are widely used as drug delivery systems. In addition, albumin nanoparticles represent a promising novel system with high efficacy. Among these, serum albumin is the most abundant protein in human blood with a molecular weight of 65 kDa with a level of about 50 mg/ml. Albumin nanoparticles offer a new hope

E-mail address: alalmalki@kau.edu.sa
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for the delivery of gemcitabine. The endogenous human serum albumin (HSA) serves as a suitable material for nanoparticles formation as albumin is naturally found in the blood and is thus easily degraded, nontoxic, and non-immunogenic (Kartz, 2008).

For this reason, we are looking for a new source as marine algae extract in nanoparticles from as potent natural antioxidant and antiproliferative activity with lowest side effects. This study was designed to explore in vitro antioxidant and antiproliferative effect of the ***Ulva lactuca* (Chlorophyta) algae** extract loaded on albumin nanoparticles (ULANP) as promising drug for chemotherapy.

2. Materials and methods

2.1. Extraction of Ulva lactuca (Chlorophyta) algae samples

Samples of *Ulva lactuca* were collected at a depth ranging from 25 to 150 cm from the Red Sea at Jeddah, Saudi Arabia. All the collected samples were cleaned sufficiently to remove gastropods and other contaminants. In further, the samples were identified by a marine biologist and compared to the existing herbarium at the faculty of marine sciences at King Abdulaziz University. All the algal samples were stored in −20 °C till analysis.

2.2. Preparation of algal extract

The *Ulva lactuca* samples were air dried at room temperature and ground to a fine powder with a glass homogenizer. Later, 100 g of the sample was extracted by using 500 ml methanol for 4 h at 65 °C and then evaporated by a rotary vacuum pump. The residue obtained was washed with distilled water and stored at −20 °C until further use (Kuda et al., 2005).

2.3. Study in vitro ULANP release

In vitro ULANP release profiles were determined at pH 7.4. The NPs suspension was centrifuged at 20,000 rpm for 45 min and the collected pellet was redispersed in 10 ml of phosphate-buffered saline solution (pH 7.4) at a final concentration of 0.2 mg/ml. The tube was incubated at 37 °C under gentle shaking. At proper time intervals (5 min), an aliquot of eluted drug medium was removed for quantification; this volume must replace with fresh buffer to prevent sink conditions. Drug release was quantified spectrophotometrically.

2.4. Identification of methanol algal extract by GC/MS

The methanol extract of the algae was dissolved in 95% hexane and identified by GC/MS (Agilent 5975; CA, USA) system integrated with gas chromatography (Agilent 7890A; CA, USA). The sample preparation for GC/MS was done by dissolving approximately 1.0 mg of extracted algal sample in 1 ml of hexane and kept at −10 °C until further analysis. During GC/MS, the compound in the sample was identified through both electron impact ionization mass spectrum and modified retention indexes (programmed temperature n-alkane based retention index).

2.5. Preparation and characterization of Ulva lactuca (Chlorophyta) algae extract-loaded albumin nanoparticles (ULANP)

The nanoparticles were prepared by dissolving 10 mg of extract in 4 ml of 10 mM NaCl with continuous stirring. Then methanol was added drop wise until the solution becomes turbid, followed by addition of 100 μl of glutaraldehyde (10%) to enhance particle cross-linking by stirring the suspension for 24 h at 4 °C. The obtained nanoparticles were ultrafiltered by centrifugation (25000g, 10 min; 5 times) and the pellet was dispersed in 10 mM NaCl at a pH of 7.5. Each re-dispersion step was performed in an ultrasonication apparatus (Finkel and Holbrook, 2000). Finally, the Nanoparticles were characterized by Infrared spectra (FTIR/NICOLET–ESPN670).

2.6. Antioxidant activity of ULANP

2.6.1. DPPH assay

The radical scavenging potential of ULANP was determined by a stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) according to the protocol by Matsukawa et al. (1997). The antioxidant activity of ULANP (100 μl) was evaluated by the inhibition percent of scavenging of DPPH reagent in methanol [15]. Inhibition of free radical, scavenging DPPH• (%) was calculated using the formula: I % = $[(A_{blank} - A_{sample})/A\ blank] \times 100$.

2.6.2. Hydroxyl radical (OH^-) scavenging assay

Hydroxyl radical scavenging capacity of ULANP was determined by comparing algal extract degradation of hydroxyl radical (Duan et al., 2006). The inhibition percentage (I%) of algae extracts nanoparticles was measured.

2.7. Antiproliferative effect of ULANP

2.7.1. Cell culture

HepG2, MCF-7 and non-cancerous BHK (Baby hamster kidney) cells were purchased from ATCC (American type culture collection, Manassas, USA). Both the cell lines were cultured in DMEM media supplemented with 10% fetal bovine serum and maintained in a humidified incubator at 5% CO_2 at 37 °C. The cells were grown in monolayer cultures for different experiments.

2.7.2. Cell viability assay

The cell Proliferation assay test is a colorimetric assay that is based on the break of a tetrazolium salt (MTT) by dehydrogenase in mitochondria to form formazan in viable cells. Percentage of cell growth inhibition following exposure to ULNAP was calculated as mentioned (Kelman et al., 2012; Rosaline et al., 2012). The IC_{50} was calculated. The viable cell number count will be proportional to the extent of formazan formed in the assay.

2.7.3 Caspase- 8 and 9 assay

Caspase-8/9 activity was determined by using the Caspase-Glo® 8 Assay and Caspase-Glo® 9. The MCF7 and HepG2 cells were seeded in a 96-well (10^4 cells/well) and treated with 20 μg of ULANP or 10 μg of vincristine. The Caspase-8/9 activities were measured using a microplate reader after 1 h of incubation.

2.8. Statistical analysis

The statistical analysis was done by unpaired Student's*t'* test Using SPSS program version 20. The results were presented as Mean ± SD of triplicates done. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Characterization of Ulva lactuca extract

The FTIR spectrum of the extract of *Ulva lactuca* showed phycocyanins as the most bioactive component with transmittance maxima at 1652 as shown in the Fig. 1 and the GC/MS analysis of the

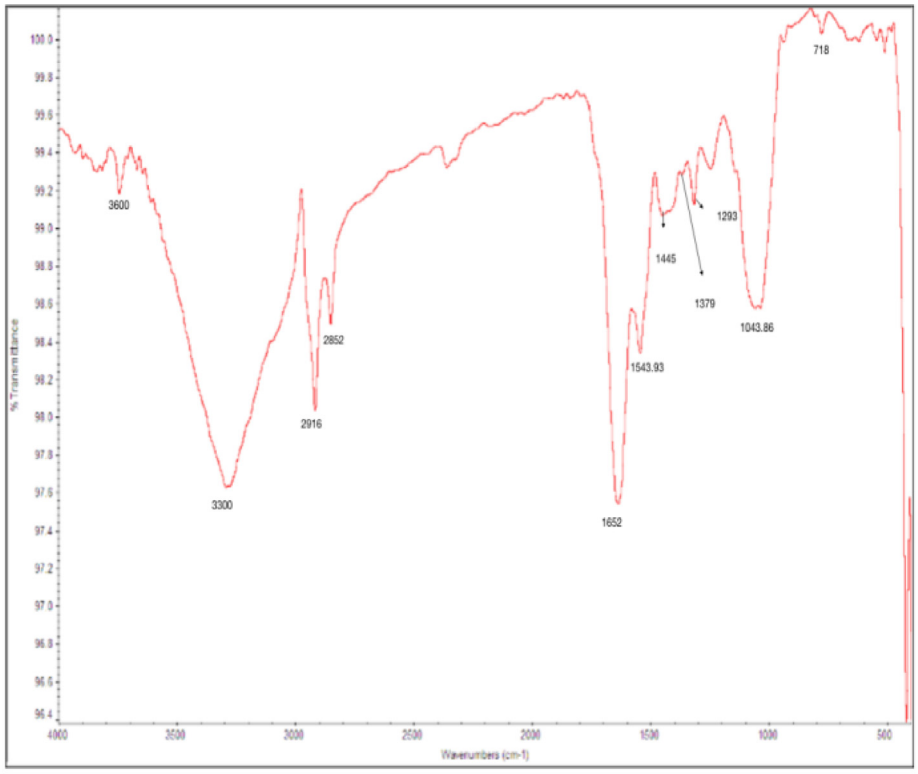

Fig. 1. FTIR spectrum for phycocyanin extract shows a transmittance maxima at 1652.

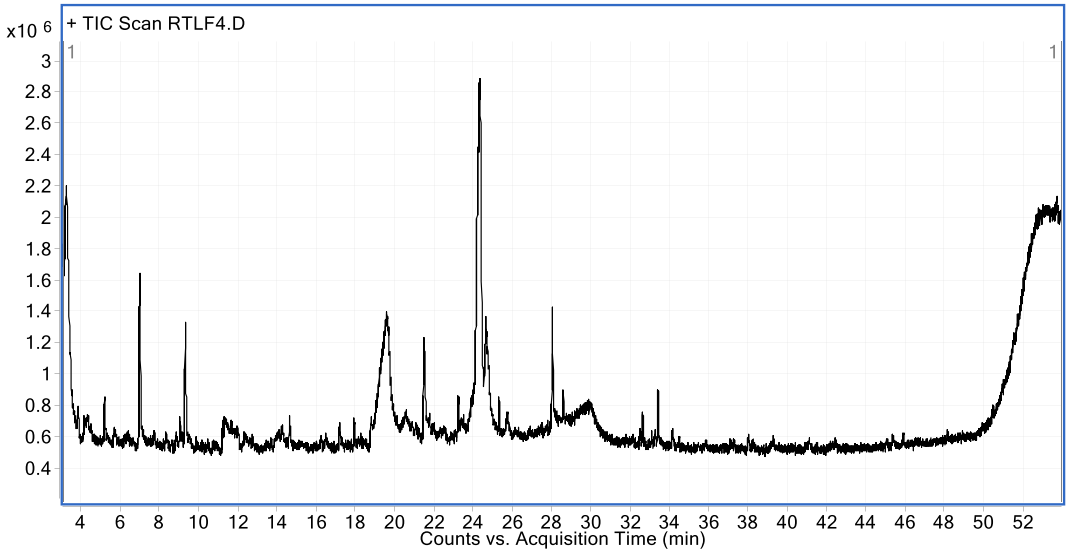

Fig. 2. GC/MS analysis of methanol extract showed the measured RI at 1290 and reference RI at 1287. This signal is specific for phycocyanin.

extract showed the measured RI at 1290 and reference RI at 1287 which is specific for phycocyanin (Fig. 2).

Chemical analysis of methanol extract of by GC–MS reported in Fig. 1. The chromotogram revealed that presence of saturated and unsaturated fatty acids. The major (Phyocyanin). Fig. 2 showed analysis of ULANP by FTIR showed the characteristic band (2100–3700 cm^{-1}) that is indicated mainly from —COO, —CO and conjugated double bond. These bonds showed spectral bands peak 2985 cm^{-1} and 2860 cm^{-1}, 2986 cm^{-1} (see Figs. 3 and 4).

3.2. Antioxidant activity of ULANP

The antioxidant potential of ULANP was assessed by using DPPH assay**.** The comparison of the mean percentage of radical scavenging activity was measured at different concentrations of the extracts of methanol *Ulva lactuca* algae; ULANP and Gallic Acid and Trolox as standard agents. The mean scavenging activity at 100 µg /ml was highest for gallic acid at 91% followed by the methanol extract of *Ulva lactuca* at 83%, ULANP at 70.5% and Trolox at 69% ($p < 0.05$).

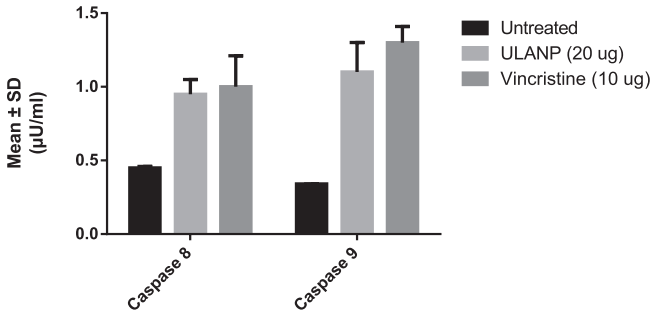

Fig. 3. The levels of Caspases 8 and 9 in untreated and treated MCF7 cell lines.

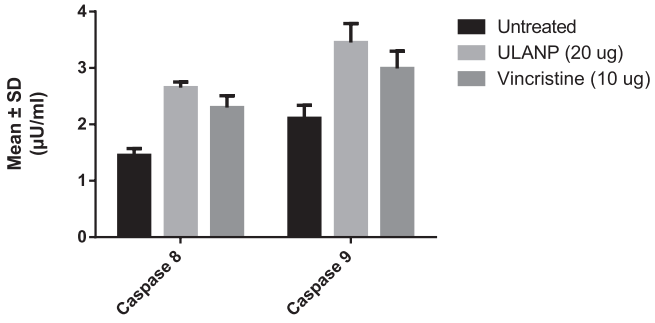

Fig. 4. The levels of Caspases 8 and 9 in untreated and treated HepG2 cell lines.

The mean scavenging activity against hydroxyl radical was measured by using Vitamin C, Vitamin E, the methanol extract of the algae and ULANP. The ULANP showed a scavenging activity at 70% compared to that of the methanol extract which stood at 55% ($p < 0.05$). The reference standards vitamin C and E showed significant activity with values of 90% and 69% ($p < 0.05$) respectively.

3.3. Antiproliferative effect of ULANP

To investigate the impact of ULANP on proliferation of cancer cell lines, we treated HepG2 and MCF7 cells with different doses of ULANP and assessed the antiproliferative potential by using WST-1 assay. Interestingly, there was a significant decrease in the cell viability starting from 0.02 μM (80.9%), which significantly reached to 61.3%, 49.3% and 29.5% at a dose of 2 μM ($P < 0.01$), 4 μM ($p < 0.001$) and 6 μM ($p < 0.001$) respectively. The IC_{50} value of ULANP on HepG2 and MCF7 cells were found to be (Table 1) compared with thymoquinone as control (50 μM). At a concentration of 20 μg of ULANP, the mean values of Caspase 8 were found to be 0.95 ± 0.05 and 2.65 ± 0.09 μU/ml for MCF7 and HepG2 respectively which is a slight increase as compared to the control (MCF7, 0.45 ± 0.06; HepG2, 1.45 ± 0.10 μU/ml). Similarly, Caspase 9 showed a significant increase in the mean values at 0.99 ± 0.07 and 3.45 ± 0.17 μU/ml for MCF7 and HepG2 respectively as compared to the control (MCF7, 0.47 ± 0.04; HepG2, 2.11 ± 0.14 μU/ml). Whereas, the Caspase 8 and Caspase 9 values of positive control (Vincristine) at 10 μg were quite closer in either MCF7 (Caspase 8, 1.00 ± 0.07; Caspase 9, 1.03 ± 0.06) or HepG2 (Caspase 8, 2.10 ± 0.08; Caspase 9, 2.99 ± 0.15) as shown in Table 1.

Table 1
IC_{50} values (μM) of the ULANP against HepG2 and MCF7 are presented as mean ± SD, [a] $p < 0.05$.

Concentration of ULANP (uM)	HepG2	MCF7
10	48.31 + 3.4 [a]	50.39 + 2.4 [a]
20	53.38 + 2.1 [a]	42.4 + 1.9 [a]
40	27.27 + 1.5 [a]	22.20 + 1.0 [a]
Thymoquinone (40 uM)	12.51 + 1.7	12.51 + 1.7

4. Discussion

The present study focused to highlight the utilization of bioactive molecules from natural marine sources that are rich with phycocyanin and can act as a free radical scavenger and antiproliferative agent against tumors. The efficacy of therapeutic effect of some novel drugs is determined by their potential to reach the site of therapeutic action with minimized side effects and off-target toxicities. In most cases, only a small amount of the described dose reaches the target organ, while the major portion of the dose distributes throughout the rest of the body in accordance with its bioavailability. Therefore, designing a drug delivery system optimizes the action of a drug with the lowest toxic side effects *in vivo* is a challenging task (Lordan et al., 2011). The antioxidant and anticancer properties of marine algal exert are still unknown. In this sense, it is of great interest to explore the bioactive components obtained from the algal species loaded with albumin particles to increase the bioavailability. In addition, preparation of nanoparticles will increase the efficacy of this drug delivery system.

The ULANP proved to possess antiproliferative potential with both MCF7 and HepG2 by a demonstrable increase in the levels of caspase-8 and caspase-9 which gives us a hint that the apoptotic mode of cell death could be involved accompanied by the granzyme pathway which needs to be examined further by western blotting and PCR studies. Interestingly, the cyclic stretch of caspase-8 and caspase-9 is playing a significant role in promoting apoptosis. The inhibition of caspase-9 alone or both caspase-8 and caspase-9 significantly inhibited the stretch-induced apoptotic rate and the protein level. These results also suggest the involvement of the intrinsic pathway in the stretch-induced apoptosis (Xu et al., 2011). In the earlier studies, the genes Fas and TNFRSF10B were reported to be involved in the activation of caspase-8 and ultimately leading to the apoptosis (Lin and Richburg, 2014).

Furthermore, the mean scavenging activity of the methanol extract of *Ulva lactuca* and ULANP were compared to that of Gallic acid and Trolox as a reference in the DPPH assay and the highest activity was that of methanol extract of *Ulva lactuca* followed by ULANP. However, ULANP showed better activity against hydroxyl radicals as compared to that of the methanol extract which needs further investigation.

Apoptosis is a physiological programmed cell death to remove damaged or abnormal cells. The steps of apoptosis include chromatin aggregation, cell size shrinking and increased lysosome activity (Rajesh and Natvar, 2011). There are different molecular mechanistic pathways, one of which is increased caspase proteases are involved in the apoptotic mechanism. Caspase assay reveals the significant activity of ULANP in comparison to the standard anticancer drug Vincristine

In conclusion, the ULANP could be a promising lead in the development of a novel chemotherapeutic drug which could target breast and liver cancers if the molecular mechanisms are explored and the specific targets are identified. In the future, we wish to examine the regulation of gene expression which could reflect the mechanism of action and the gene alteration pattern induced by ULANP.

Declaration of Competing Interest

The author declare that they have no financial conflict of interest.

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