Trop J Nat Prod Res, April 2019; 3(4):113-123 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Crab Ash Extract has Anti-Proliferative Effects on SK-MEL-28 Melanoma Cells and Induces a Cellular Stress Response and Metabolic Changes

Hedeel M. Katran*, Brian Brestovac, Danielle E. Dye

School of Pharmacy and Biomedical Science, Curtin Health Innovation Research Institute, Faculty of Health Science, Curtin University, Bentley WA, Australia

ARTICLE INFO

Article history:
Received 27 March 2019
Revised 30 April 2019
Accepted 05 May 2019
Published online 07 May 2019

ABSTRACT

The incidence of metastatic melanoma continues to rise worldwide and although there has been recent advances in treatment, outcomes remain poor for many patients. Therefore, there remains a need for novel treatments. Recently, *in vitro* studies have shown that some compounds derived from crab shell or hemolymph may have anticancer properties. Furthermore, whole crab ash has been recorded as a traditional folk medication used to treat solid tumours. This study examined the anticancer properties of extracts derived from the crab genus *Portunus*. The SK-MEL-28 melanoma cell line was treated with ethanol and aqueous extracts derived from whole crab ash (CA), shell, or muscle fibres. Concentrations tested were from 312.5 µg/mL to 5000 µg/mL. CA ethanol extract showed the highest amount of cell death, with 100% cell death observed at 2500 µg/mL and 50% cell death at 1250 µg/mL. Cells that survived CA treatment showed altered morphology and intracellular granulation. Next, lysates of melanoma cells treated with a sub-lethal concentration (750 µg/mL) of ethanolic CA were analysed by semi-quantitative mass spectrometry. This revealed upregulation of proteins associated with protein synthesis, folding and degradation, ER chaperones, carbohydrate and lipid metabolism, cytoskeletal and some nuclear proteins. Taken together, the proteomic data suggest activation of cellular stress pathways and changes in metabolism.

Keywords: Melanoma, crab extract, apoptosis, mass spectrometry, cellular stress.

Introduction

Brachyuran whole crab ash is an ancient folk medication used to treat solid tumors in the Middle East.[1] Avicenna, a medical scientist,[2] in his 11th-century records precisely describes solid tumors and how whole freshwater crab ash (CA) was used to treat the disease.[3] Several recent studies have demonstrated that the crab compounds derived from shell or hemolymph have anticancer effects *in vitro*.[1,4-6] For example, both MCF7 breast carcinoma and LNCaP prostate carcinoma cells showed reduced proliferation, increased apoptosis and decreased nitric oxide (NO) production when treated with the ethanol extract of the shell of the freshwater crab *Potamon persicum*.[7,8] In addition, hemolymph of brachyuran crab (*Dromia dehaani*) was reported to show cytotoxicity against the hepatocellular carcinoma cell line, HepG2.[9] Although the active compound(s) leading to these anti-cancer effects is not known, although it has been suggested that chito-oligosaccarides, chitosan, carotenoids and selenium may play a role.[1, 7]

These data have led to increased interest in the anti-tumor activity of novel crab extracts, including those derived from species of the genus Portunus. Portunus is an abundant crab genus, comprising over 90 species, and is found in diverse regions. It contains several important species for fisheries and is cultured in farms for human consumption.[10]

There is very limited published data describing medicinal uses of Portunus extract. A recent report describes the identification of a glycosaminoglycan extracted from *Portunus pelagicus* as an inhibitor of *β*-secretase 1 (BACE), which plays a role in the pathogenesis of Alzheimer's Disease.[11] However, to our knowledge, there are no publications describing anti-cancer properties of Portunus extract.

Data from the International Agency for Research on Cancer indicates there were 17 million new cases of cancer worldwide in 2018, with this projected to increase to 27.5 million new cases per year by 2040, if recent trends continue.[12] The most common cancers in 2018 were breast, prostate, lung and colorectal.

Cutaneous melanoma was the 19th most common cancer worldwide in 2018 but shows significant variation in incidence rates across ethnic groups, with fair skinned people the most affected.[13] Melanoma is also one of the most common cancers in young adults and as such, is responsible for many years of life lost per death. Australia and New Zealand have the highest incidence of melanoma with 15,229 new diagnosed cases predicted in 2019.[14]

Until 2011, the main treatment of metastatic melanoma was dacarbazine, which showed limited efficacy.[15] Since then, the Food and Drug administration (FDA) has approved immunotherapies, targeted therapies and an oncolytic virus for treatment of melanoma.[16] However, these therapies can have significant side effects, and in the case of targeted therapies (such as BRAF inhibitors), patients often acquire resistance within months, limiting their clinical use.[16] Thus, the identification and development of new therapeutic options for melanoma remains a priority.

In this study, the melanoma cell line SK-MEL-28 cell line was treated with Portunus extracts, derived from whole crab ash (CA), shell, and muscle fibers, at different concentrations. CA ethanol extract had the greatest effect on cell proliferation, with 50% cell death after 24 hours at 1250 µg/mL. SK-MEL-28 cells were then treated with a sub-lethal concentration of CA ethanol extract (750 µg/mL, 24 hours) and cell lysates subjected to proteome analysis. Compared to control cells, cells

*Corresponding author. E-mail: hedeel.katran@postgrad.curtin.edu.au
Tel: +61435095020

Citation: Katran HM, Brestovac B, Dye DE. Crab Ash Extract has Anti-Proliferative Effects on SK-MEL-28 Melanoma Cells and Induces a Cellular Stress Response and Metabolic Changes. Trop J Nat Prod Res. 2019; 3(4):113-123. **doi.org/10.26538/tjnpr/v3i4.3**

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

treated with CA ethanol extract showed significant upregulation of 54 proteins, many of which were involved in protein folding, metabolic dysregulation and the cellular cytoskeleton.

Materials and Methods

Crab taxonomic classification

The crab was purchased fresh from a food market. According to the classification keys and identification criteria in published resources,[10, 17-25] the crab used in this study belongs to the family, Portunidae; subfamily, Portuninae; genus, *Portunus.* Figure 1 shows some morphological features of this genus. The following features were used to identify the crab as a member of the Portunidae family: 1) transversal hexagon shaped carapace, slightly convex dorsal surface, broad front and dental margin with several teeth or spines; and 2) lateral and last flattened legs adapted for swimming, the later have paddle-like dactyl. Features of the subfamily Portuninae were: 1) eyestalks are not long, 2) chelipeds are the longer limbs. Genus *Portunus* determinant criteria were: 1) carapace width is more than its length, 2) margin has nine teeth (spines) with the last one enlarged, 3) propodus granular and ridged, and basal antennal joint is wide, 4) outer maxillipeds contain semi-cylindrical segments of palp.[10,17-25] This genus can be distinguished from the genus *Callinectes* by its triangular male abdomen, which is an inverted T shapee in *Callinectes* and the presence of inner spine on the chelipeds.

Crab extract preparation

Six extracts were prepared including ethanol and aqueous extracts of whole crab ash (CA), shell extract and protein extract. Whole CA was obtained by heating the entire crab at 150^{o}C for 2 hours before grinding the crab into a powder. Shell extract was prepared as for CA, except using only the shell. Protein extract was prepared by freezing and lyophilizing the muscle fibres (Freeze Dryer, SCANVAC®, Germany), prior to grinding into a powder.

For the ethanol extraction, each of the three powders were extracted according to method described by Rezakhani *et al.*,[7] as follows: for each 1 g crab derived powder, 30 mL 70% ethanol was added and the mixture left in the dark at room temperature (RT) for 48 hours before being filtered with filter paper. The filtrate was allowed to dry for 48 hours before being lyophilised at -48°C, 0.563 Mbar for 24 hours. Following this, extracts were resuspended in cell culture media without fetal bovine serum (described below) and filter sterilized through a 0.22 µm filter.

The aqueous extracts were prepared for CA, shell, and muscle fibres, as for the ethanol extraction, except that distilled water was used as the initial solvent instead of 70% ethanol.

Cell Culture

SK-MEL-28 media included 90% v/v Roswell Park Memorial Institute (RPMI) 1640 Medium, 1 mM sodium pyruvate, 2 mM glutamine, and 10 mM HEPES buffer (all from Gibco®, Life Technologies, Carlsbad, CA) and 10% fetal bovine serum (FBS) (Serana, Bunbury, Australia). Cells were thawed, seeded into 25 mL flasks and incubated in a humidified incubator at 37°C and 5% CO_2. Cells were grown to 70 - 85% confluence before passaging (every 2-3 days). After several passages, cells were seeded into 24 well plates (5×10^4 cell/well) for treatment with crab extract.

Crab extract treatment

Each of the extracts were added to the cell line in the concentrations of 5000, 2500, 1250, 625, 312.5, and 156.25 µg/mL in normal growth media, in triplicates in a 24 well plate. The results were observed after 24, 48, and 72 hours and the concentrations then narrowed to 2500, 1250, 950, 625, 312.5 µg/mL. Combinations of shell and protein extracts (50/50) were also prepared at total concentrations of 1200, 1000 and 800 µg/mL and cells observed over 72 hours. Control cells were grown in culture media alone.

Cytopathic effect (CPE)

Cells were observed for rounding up[26] and blebbing,[27] which are morphological changes associated with apoptosis, known as cytopathic effect (CPE). These changes were observed via inverted light microscopy (Olympus, Tokyo, Japan) at 400x magnification and rounded cells counted manually in non-overlapping fields of view.

DAPI staining

4',6-Diamidino-2-Phenylindole (DAPI) staining was used to visualize the nucleus of control and treated cells, to identify if nuclear damage such as shrinkage or fragmentation, had occurred.[26, 28] Control cells and cells treated with 1250 and 950 µg/mL CA ethanol extract were washed with phosphate buffered saline (PBS) (Sigma-Aldrich, St Louis, MI) twice, and 0.3 µM DAPI (Thermo Fisher Scientific, Waltham, MA) in PBS was added and incubated in the dark for 5 min. Cells were rinsed with PBS twice before being fixed in 4% paraformaldehyde (PFA) in PBS.

Preparation of cell lysate from SK-MEL-28 for mass spectrometry

SK-MEL-28 cells were grown in two 25 cm^2 flasks, and when near confluence, one flask treated with 750 µg/mL CA (treated), and the other allowed to grow in normal medium (control) for 24 hours. The media was removed, cells washed in PBS and detached by incubating with 2.5 mM Ethylenediaminetetraacetic acid (EDTA) at 37℃ for 2 min, followed by mechanical disruption using a plastic cell scraper. Cells were collected in 5 mL PBS and centrifuged at 1200 rpm for 5 min at 20℃. The cells were then washed in 5 mL PBS and re-centrifuged. The cell pellet was resuspended in 200 µL lysis buffer (PBS, 1% NP40, 1x cOmplete™ protease inhibitor; all from Sigma Aldrich), incubated on ice for 30 min and centrifuged at 12000 rpm at 4℃ for 10 min. The supernatant was harvested, and protein concentration measured before being stored at -20℃.

BCA Protein Assays

The Bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific) was used to estimate the protein concentration in SK-MEL-28 cell lysates. Briefly, a standard curve was constructed by serial dilution of 1 mg/mL bovine serum albumin in the wells of a 96-well plate, and a range of cell lysate volumes were tested. Following the addition of the BCA reagent, the assay was incubated at 37^{o}C for 30 min before the absorbance measured at 562 nm (EnSpire Multimode Plate Reader, PerkinElmer, Waltham, MA). Standard curves and concentrations were calculated using Microsoft Excel.

Acetone Precipitation

900 µL of -20°C cooled acetone was added to the treated and control cell lysates, vortexed, incubated in -20°C for 1 hour, and then centrifuged at 14 000 x g for 10 min. The supernatant was removed, and the precipitated proteins were air dried at 20^{o}C for 30 min.

Mass Spectrometry

The protein samples of treated and control cells were processed by Proteomics International (PI) laboratories, NATA ltd (Nedlands, Australia). Samples were reduced, alkylated and trypsin digested according to the iTRAQ protocol (Sciex). Samples were analysed by electrospray ionisation mass spectrometry using the Shimadzu Prominence nano HPLC system (Shimadzu) coupled to a 5600 TripleTOF mass spectrometer (Sciex). Peptides were loaded onto a Zorbax 300SB-C18, 3.5 µm separation column (Agilent Technologies) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). ProteinPilot™ 5.0 (SCIEX) was applied against Swissport database to identify the peptides.

The total number of distinct peptides identified was 2074 for treated and 1347 for control (>95% confidence level). This was reduced to 366 for treated and 261 for control, when only proteins with two unique peptides (>95% confidence level) were considered. When these two protein data sets were compared. This smaller number of unique proteins underwent analyses. The global and local False Discovery Rate (FDR) was <0.1%.

Functional Annotation

In addition to text mining, Visualization and Integrated Discovery (DAVID) v6.8 [29], as well as Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) protein interactions tool version 10.5 [30] were used.

Statistical analysis
Fisher's exact test applied to identify significant changes in unique peptide quantity between control and treated cell lysates, with $p<0.10$ considered significant.

Results and Discussion

Treatment of SK-MEL-28 melanoma cells with ethanol and aqueous crab extracts
SK-MEL-28 cells showed higher levels of CPE (e.g. cell rounding) and cell death when treated with ethanol extracts compared to aqueous extracts (Figures 2 - 4, Table 1). Aqueous extracts of whole crab ash (CA), shell and muscle protein were associated with minimal cell death or morphological changes at all concentrations up to 2500 µg/mL (CA) or 5000 µg/mL (Figure 2, Table 1). This suggests that the aqueous extraction method was a poor method of extracting potentially cytotoxic compounds from the Portunus sp. crab. This is consistent with Al-Shammari *et al*, who reported that burned crab shell extracted in methanol was more effective at inhibiting cellular proliferation than an aqueous extract.[1]
In contrast, SK-MEL-28 cells treated with ethanol extracts displayed CPE and cell death at concentrations greater than 1250 µg/mL (Figures 3, 4; Table 1). In particular, ethanol whole crab ash (CA) extract was associated with cell death at lower concentrations than ethanol shell or protein only extracts. Ethanol CA extract at 2500 µg/mL was associated with 100% cell death at 24 hours and clear morphological changes at 1250 µg/mL (48-58% cell rounding) (Figure 3, Table 1). In contrast, ethanol shell and protein extracts showed minimal morphological changes at 1250 µg /mL (6 – 10% rounding) and modest changes at 5000 µg/mL (20-24% rounding) (Figure 4, Table 1).
Interestingly, combining ethanol shell and protein extracts was associated with increased cell death and rounding (22-28% at 1200 µg/mL) compared to when the ethanol shell and protein extract were applied separately (5 – 8% at 1250 µg/mL) (Figure 3). This suggests there may be a combination of molecules when these two were applied together than stimulated increased CPE and cell death in the SK-MEL-28 cells. However, extracting the shell and protein separately and then combining them did not cause the same level of morphological changes or cell death as when similar concentrations of whole crab ash, extracted in a single process, were applied to the cells. For example, at 1200-1250 µg/mL there was 48 - 57% cell rounding in CA extract compared to 22 - 28% in the combined shell/protein extract. This may be because the ethanol extraction of bioactive molecules from whole crab was more efficient than separate extractions. In addition, the CA extract and protein extract were prepared differently. CA extract was subjected to 150^{o}C for 2 hours, which would have denatured all proteins in the crab muscle, whereas the ethanol protein extract may have contained active peptides. Al-Shammari also found burnt crab extract to be more effective than non-heated crab shell extract and hypothesized that the heating process may lead to the formation of novel complexes in the crab shell not seen in the native (non-burned) state.[1] It is possible that the heating process of our whole crab extract, which would lead to denatured protein, has produced a novel compound or conformation with increased cytotoxicity, which is not contained in non-heated protein material. As the ethanol CA extract was found to be the most effective, further microscopic analyses was performed on SK-MEL-28 cells. Cells treated with 1250 µg/mL ethanol CA extract showed signs of apoptosis such as membrane blebbing and cell rounding up,[26,27] as well as a granulated cytoplasm; when compared to control cells (Figure 5). Melanoma cells treated with a lower concentration of 625 µg/mL also showed clear areas near the nucleus (Figure 5). Further studies using DAPI staining also revealed the presence of pyknotic nuclei in cells treated with ethanol CA extract, with increasing nuclear changes seen with increasing concentration, as well as fewer cells per field of view (Figure 6). These data are consistent with that of Rezakhani *et al*, who found a similar concentration (1000 µg/mL) of *Potamon Persicum* ethanol extract was associated with cell viability of approximately 75% in LnCap prostate and MCF7 breast cancer cells at 24 hours exposure, which fell to approximately 50% at 72 hours. In both cell lines, TUNEL assays confirmed a dose-dependent relationship between the extract and levels of apoptosis.[7,8] Cell viability and signs of apoptosis (e.g. membrane blebbing) also increased at 48 and 72 hours in our assays (data not shown).

Mass Spectrometry (MS) and Functional Annotation
Approximately 40-50% of SK-MEL-28 cells survived treatment with 1250 µg/mL of ethanol CA extract over 24 hours, which suggests these cells may be able to tolerate the cytotoxic compound(s) contained in the extract. Understanding the mechanisms that enable cell survival may provide information about the pathways stimulated by this extract and the nature of the chemoresistance seen in these cells.
SK-MEL-28 cells were treated with a sublethal dose (750 µg/mL) of CA ethanol extract, or medium only (control) for 24 hours. Protein lysates of treated and control cells were analysed by mass spectrometry (MS), followed by functional analysis applying bioinformatics online tools and text mining. There were 54 proteins that were significantly upregulated, based on the number of unique peptides identified, in treated cells compared to untreated cells (Fishers exact test, $p < 0.1$) (Table 2). However, it must be noted that this MS analyses was semi-quantitative. As such, these data provide some clues about the cellular response to the crab extract applied, which require validation and further investigation.
The proteins with altered levels in treated versus control cells were grouped into categories based on their primary roles and/or locations within the cell. As many proteins have multiple functions, the grouping, discussion and interpretation is not exhaustive (Table 2).

Protein synthesis, folding, degradation; endoplasmic reticulum (ER) chaperone proteins
The largest group of proteins that were upregulated in treated cells were those involved in protein synthesis, folding and ER chaperones, suggesting cellular stress was induced in the SK-MEL-28 cells by the crab extract. Following cellular stress, cells have to cope with protein damage in order to survive. To do this they have developed a dual response – damaged proteins are either repaired or eliminated, via molecular chaperones and proteolysis, respectively.[31]
A number of members of the cytoplasmic heat shock protein (HSP) family were upregulated, including HSP90α, HSP90β, HSP70 members 2/8, and protein deglycase; in addition to the delta, epsilon and zeta subunits of the chaperonin, T-complex protein (TCP) (CCT4, CCT5, CCT6a). A number of other studies have found upregulation of cytoplasmic molecular chaperones in chemo-resistant cancer cells, suggesting HSP upregulation is involved in chemo-resistance. For example, cisplatin-resistant cervical cancer cells[32] and paclitaxel-resistant ovarian cells[33] both showed upregulation of HSPs and other molecular chaperones/chaperonins, including the TCP subunits. Misfolded proteins in the endoplasmic reticulum (ER) are processed via ER resident chaperones, a number of which were also upregulated in treated SK-MEL-28 melanoma cells. A role for ER chaperone proteins in chemo-resistance has been previously described. For example, prolyl-4-hydroxylase subunit β has been implicated in temozolomide resistance in glioma[34] and peptidyl-prolyl cis-trans isomerase A has been implicated in paclitaxel resistant breast cancer cells.[35]
Several proteins involved in degradation (transitional ER ATPase, and ubiquitin-like modifying activating enzyme) were also upregulated in treated cells, along with molecules associated with the redox level of the cell, e.g. peroxiredoxin-1 and glutathione S-transferase P. This is consistent with Di Michele *et al.*[33] who describe increased levels of peroxiredoxin-2, 3 and 6 in cisplatin-treated ovarian cancer cells, and Castagna *et al.*[32] who described upregulation of peroxiredoxins-2 and 6 and glutathione-S-transferase in cisplatin treated cervical cancer cells.
There was also upregulation of a number of proteins involved in protein synthesis/translation, namely elongation factors and the components of the small and large ribosomal subunits. mRNA translation elongation has been studied in melanoma, with eukaryotic translation initiation factor 5A (eIF5a) associated with tumour thickness and poor prognosis[36]

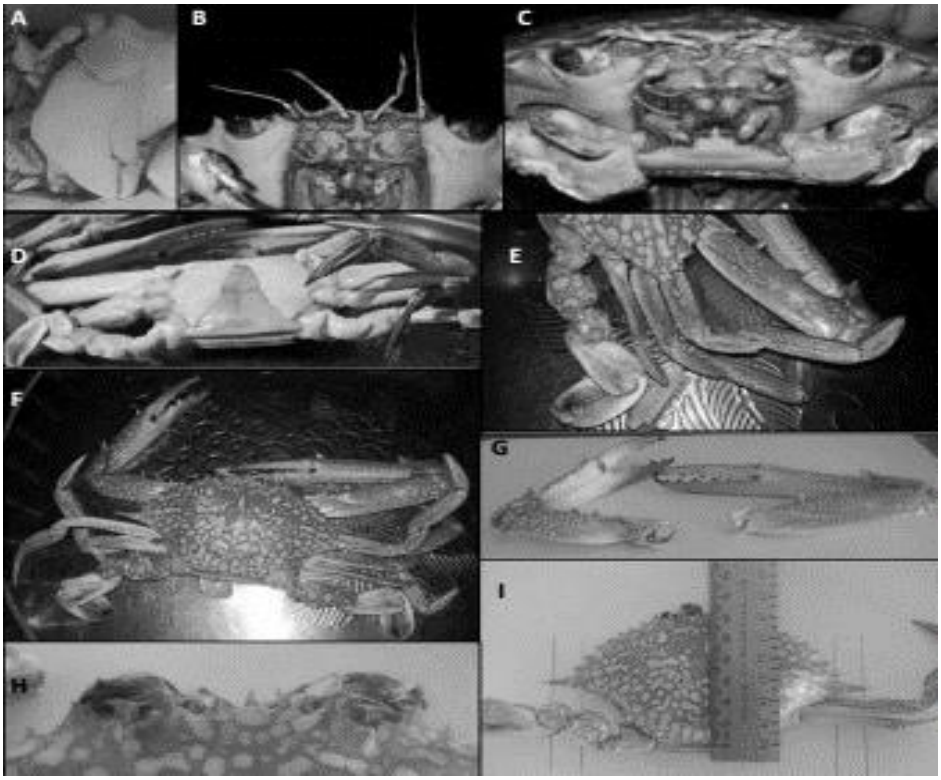


Figure 1: *Portunus* sp morphological features. A, maxilliped; B, antenna; C, front view; D, abdominal view; E, legs; F, dorsal whole view; G and L, chelipeds; H, front board; and I, carapace.

and eukaryotic elongation factor 2 (eEF2) identified as a potential serum biomarker in melanoma.[37] Interestingly, elongation factor 1 delta (EF1-delta) was described as one of four proteins upregulated in melanoma cells treated with cisplatin, vindesine, fotemustine and etoposide to produce drug resistant sub-lines,[38] but there are no recent publications describing the role of this protein in melanoma. It is possible that elongation factors may contribute to chemoresistance in melanoma and/or provide options for therapeutic targeting, but current data is limited and this area requires further investigation. [39]

Metabolic changes (glycolysis, lipid metabolism, mitochondrial proteins)
A number of proteins involved in glycolysis were upregulated in treated SK-MEL-28 cells including alpha enolase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate isomerase, hexokinase-2 and L-lactate dehydrogenase A chain (Table 6). In addition, a key regulator of the citric acid cycle, isocitrate dehydrogenase, was upregulated, along with fatty acid synthase. Interestingly, two components of the ATP synthase complex involved in oxidative phosphorylation (OXPHOS) in the mitochondria showed increased expression in treated cells, suggesting that both glycolysis and OXPHOS are active in treated cells. Moreover, the increase in fatty acid synthase may suggest that some of the pyruvate produced in glycolysis is converted into fatty acids. Aerobic glycolysis, or the Warburg effect, is a hallmark of cancer.[40] The Warburg effect describes the increased use of glycolysis rather than OXPHOS by tumor cells in physiological oxygen conditions (reviewed in Bhattacharya *et al*).[41] Although OXPHOS produces larger amounts of ATP, it is slower to respond to changing conditions, whereas glycolysis can respond quickly. It has been proposed that tumor cells can optimize energy production by maintaining a mix of glycolysis and OXPHOS, where glycolysis may be used to meet short term increases in energy demands.[42] Interestingly, upregulation of alpha-enolase (ENO1), with associated increased glycolysis, was described in cisplatin-resistant cells in gastric cancer, and associated with poor patient outcomes.[43] Moreover, *ENO1* knockdown was found to reduce glycolysis, increase OXPHOS, and cause growth arrest.[44,45] Other studies have found increased aerobic glycolysis is important in maintaining chemo-resistance, and it has been hypothesised that glycolytically-derived ATP contributes to, among other things, drug inactivation and increased intracellular survival signalling.[46] The upregulation of the mitochondrial MICOS complex subunit MIC60 (IMMT) and mitochondrial 60kDa HSP suggests there may have been some mitochondrial damage/stress associated with the treatment – MIC60 is a core unit of MICOS, which regulates cristae morphology and protein transport;[47] while mitochondrial HSP60 maintains proteins in an unfolded state to enable transport across the inner mitochondrial membrane.[48]

Nuclear proteins
Poly (ADP-ribose) polymerase 1 (PARP-1) and X-ray repair cross-complementing protein 5 (XRCC5) are involved in single stranded and double stranded DNA repair, respectively. Intuitively, increased DNA repair mechanisms could be associated with chemo-resistance, and indeed with PARP-1, this seems to be the case. Increased PARP activity is believe to be associated with resistance to DNA damaging agents.[49] However, for XRCC5 in melanoma, there have been conflicting reports - in some studies increased levels of XRCC5 have been associated with worse patient outcomes,[50] whereas in others the reverse was true.[51] There is no data regarding XRCC5 and chemo-resistance. Nucleophosmin (NPM1) is known to be involved in a number of pathways including mRNA transport, chromatin remodelling and apoptosis, but has also recently been implicated in genome stability and DNA repair (homologous recombination).[52] In addition, recent data has suggested reduced levels of NPM1 are associated with chemotherapeutic sensitivity[53] whereas elevated levels confer multiple drug resistance in breast cancer cells.[54] Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and polypyrimidine tract-binding protein 1 (PTBP1) are both members of the heterogeneous nuclear ribonucleoprotein family that bind to pre-mRNAs in the nucleus and affect pre-mRNA processing and transport. PTBP1 has been implicated in melanoma via its role in splicing CD44,[55] a cell surface molecule involved in migration metastasis. Conversely, in breast cancer PTBP1 activity is believed to regulate splicing of pyruvate kinase and contribute to altered glucose metabolism.[56]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

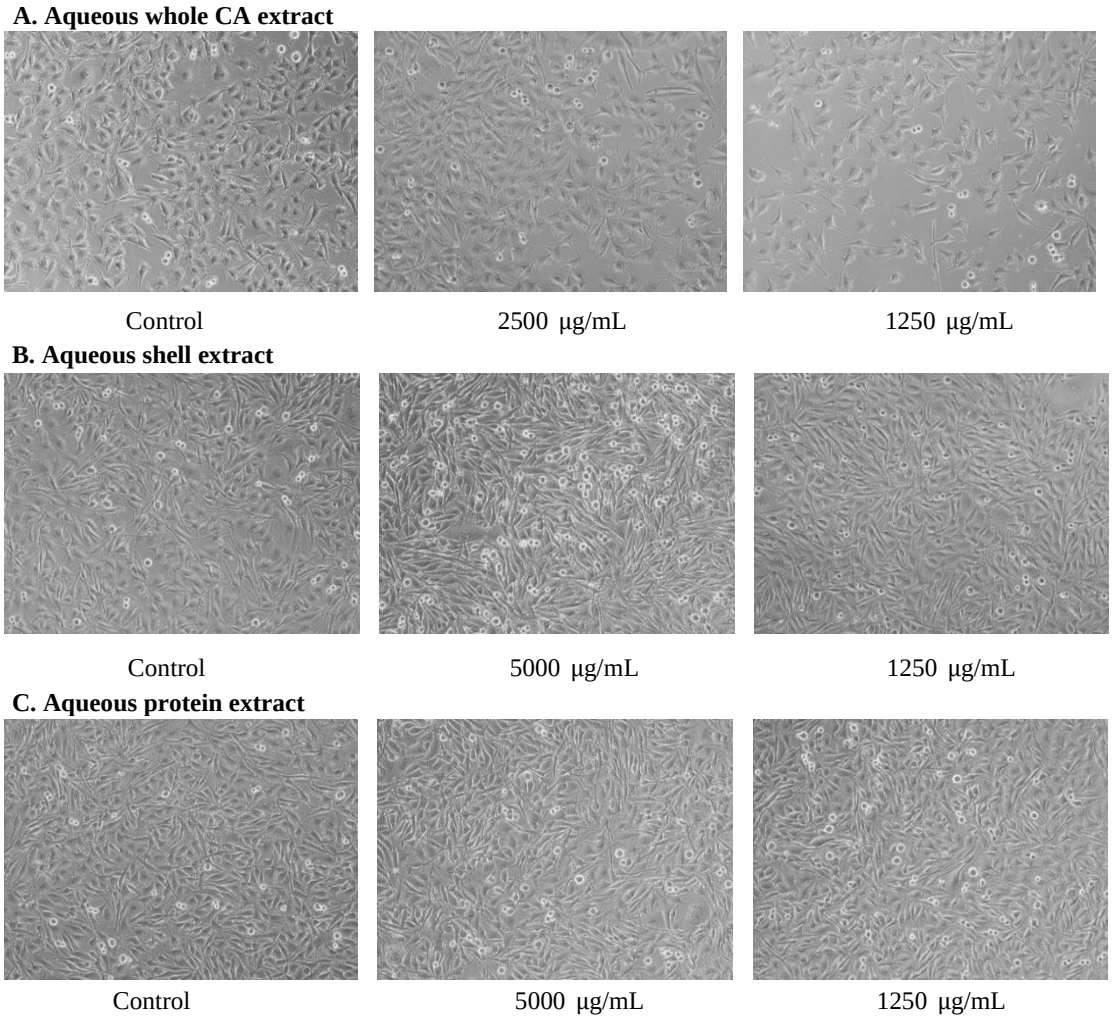


Figure 2: SK-MEL-28 cells treated with aqueous extracts of A) whole crab ash, B) shell and C) protein, for 24 hours. All images were taken at 100x magnification on an inverted phase contrast microscope and captured using the Olympus cellSens platform.

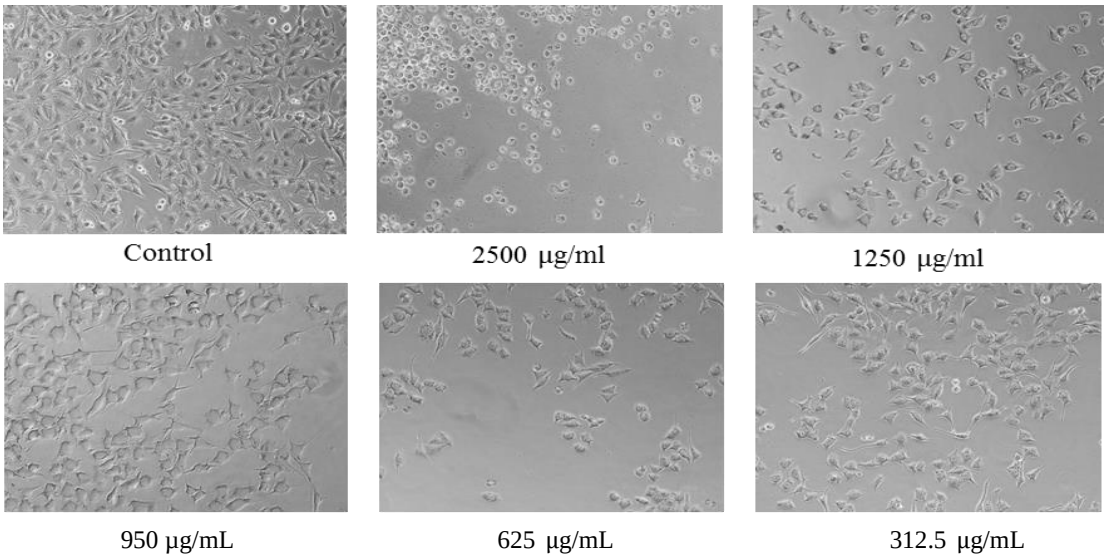
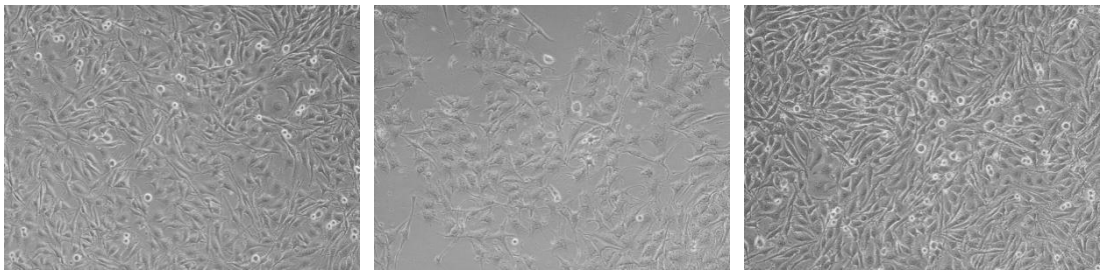


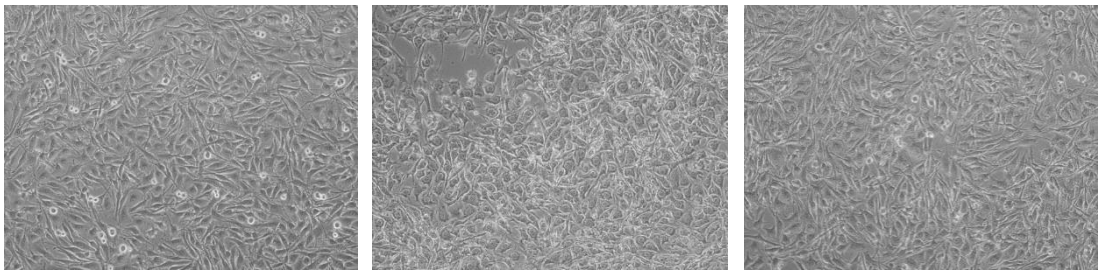
Figure 3: SK-MEL-28 cells treated with ethanolic whole crab ash (CA) extract for 24 hours. All images were taken at 100x magnification on an inverted phase contrast microscope and captured using the Olympus cellSens platform.

A. Ethanolic shell extract



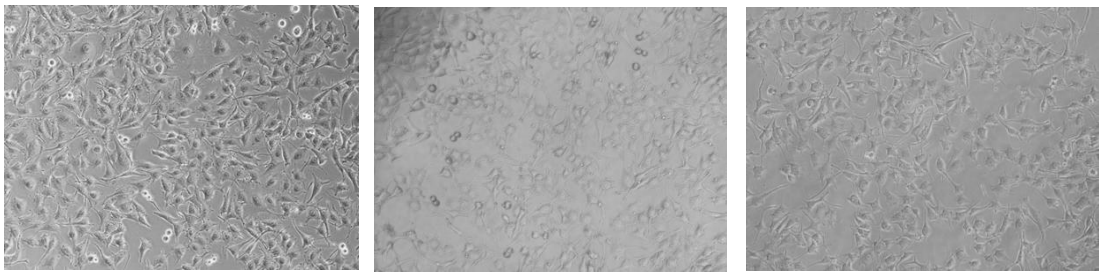
Control 5000 µg/mL 1250 µg/mL

B. Ethanolic protein



Control 5000 µg/mL 1250 µg/mL

C. Ethanolic shell and protein extract



Control 1200 µg/mL 800 µg/mL

Figure 4: SK-MEL-28 cells treated with ethanolic extracts of A) shell, B) protein, and C) combined shell and protein for 24 hours. All images were taken at 100x magnification on an inverted phase contrast microscope and captured using the Olympus cellSens platform.

Table 1: Percentage of cell rounding in SK-MEL-28 cells treated with ethanolic and aqueous *Portunus* extracts for 24 hours.

	Percentage of rounded up cells						
Extract Conc. (µg/mL)	**Ethanolic CA**	**Ethanol shell**	**Ethanol protein**	**Ethanol shell + protein**	**Aqueous CA**	**Aqueous shell**	**Aqueous protein**
0	5%	5%	5%	5%	5%	5%	5%
312.5	6-8%	5-7%	5-7%		5-7%	5-7%	5-7%
625	14-18%	5-7%	5-7%		5-7%	5-7%	5-7%
950	27-32%						
1250	48-57%	6-8%	5-7%	22-28%[1]	5-7%	6-8%	5-7%
2500	100%	6-10%	8-10%		18-24%	8-12%	5-7%
5000		20-24%	22-24%			22-25%	5-7%

[1]1200 µg/mL (600 µg/mL shell + 600 µg/mL protein extract)

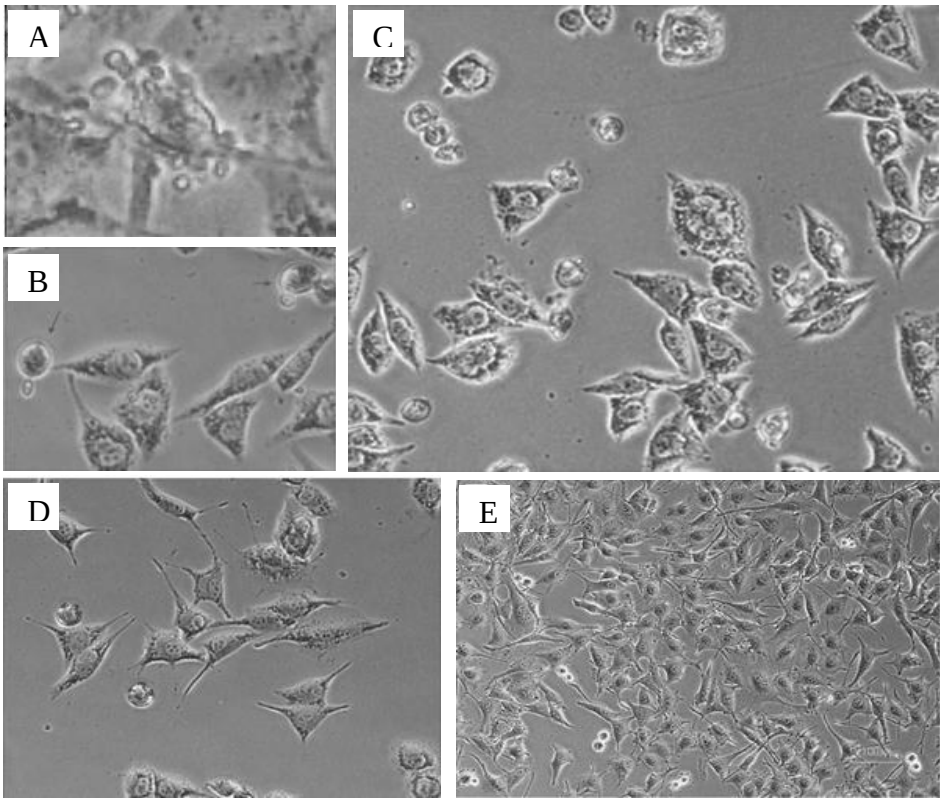


Figure 5: Apoptosis-related cytopathic effect (CPE) in SK-MEL-28 cells treated with 1250 ug/mL ethanolic CA extract. A, blebbing (400x, zoom); B, cell roundup (200x, zoom); C, Dark granules in the cytoplasm (200x, zoom); D, Clear area around the nuclei (this is a 625 ug/mL CA ethanolic extract treated cells)(200x); E, control (100x). Images were taken at on an inverted phase contrast microscope and captured using the Olympus cellSens platform.

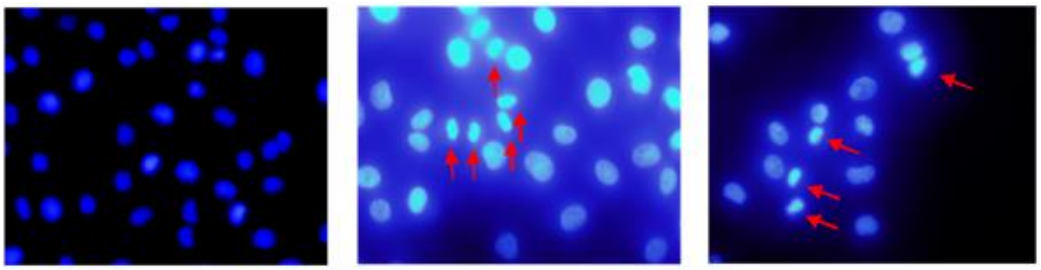


Figure 6: DAPI staining after 24 hours of treatment with ethanolic CA extract. Nuclear pyknosis is indicated. Top images are taken at 400x magnification, bottom images at 100 x magnification. Images were taken at on an inverted fluorescent microscope and captured using the Olympus cellSens platform.

Interestingly HNRNPA1 has also been shown to regulate CD44 splicing in hepatocellular carcinoma, with expression associated with poor prognosis.[57] However, there is no data available regarding the contribution of HNRNP family members to chemo-resistance.

Cytoskeletal proteins

The contractive proteins, myosin-9 and β-actin were upregulated in treated cells, as was the actin binding protein α-actinin-4 and two tubulin isoforms (α-1B chain and β-4B chain). Di Michele *et al.*[33] also describe changes in a range of cytoskeletal proteins in paclitaxel treated cells, but in contrast to our study, found decreased β-actin. The actin cytoskeleton provides a scaffold for the cell and is essential for cellular functions including cell migration, endocytosis and vesicular trafficking, cytokinesis and apoptosis.[58] Actin interacts with actin binding proteins (ABPs) such as cofilin and α-actinin and is regulated by Rho GTPases, which control ABP function, leading to changes in actin structures such as the formation of stress fibres and lamellipodia.[58] Several reports have reported that changes in actin cytoskeleton organisation in osteosarcoma and leukaemic cells are associated with drug resistance and worse prognosis (reviewed in Desouza *et al.*[58]). In addition, increased actin/myosin expression may also represent a more motile/metastatic phenotype of surviving cells. Tubulins are components of the microtubule cytoskeleton. During interphase, microtubules are involved in maintenance of cell shape and trafficking intracellular proteins and organelles, whereas during mitosis microtubules form the mitotic spindle integral to chromosome segregation.[59] Some chemotherapeutics directly target microtubules and suppress microtubule dynamics, leading to failure of mitosis.[60]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 2: Upregulated proteins in SK-MEL-28 melanoma cells after treatment with 750 µg/mL ethanolic CA for 24 hours.

Uniprot Accession #	Gene Name	# Peptides C[1]	# Peptides T[2]
Protein Synthesis			
P07237	Elongation factor 1-alpha 1 (EEF1A1)	15	24
P13639	Elongation factor 2 (EEF2)	11	22
P05387	60S acidic ribosomal protein P2 (RPLP2)	0	8
P24534	Elongation factor 1-beta (EEF1B2)	0	5
P15880	40S ribosomal protein (RPS2)	0	4
P55884	Eukaryotic translation initiation factor 3 subunit B (EIF3B)	0	4
Protein folding, degradation and oxidative stress			
P11142	Heat Shock Protein Family A (Hsp70) Member 8 (HSPA8)	16	22
P55072	Transitional endoplasmic reticulum ATPase (VCP)	13	21
P08238	Heat shock protein HSP 90-beta (HSP90AB1)	11	23
P07900	Heat shock protein HSP 90-alpha (HSP90AA1)	10	24
Q99497	Protein deglycase/Parkinson disease protein 7 (PARK7)	4	11
P22314	Ubiquitin-like modifier-activating enzyme 1 (UBA1)	2	11
P48643	T-complex protein 1 subunit epsilon (CCT5)	2	9
Q06830	Peroxiredoxin-1 (PRDX1)	2	8
P40227	T-complex protein 1 subunit zeta (CCT6A)	1	7
P54652	Heat shock-related 70 kDa protein 2 (HSPA2)	0	7
P09211	Glutathione S-transferase P (GSTP1)	0	6
P50991	T-complex protein 1 subunit delta (CCT4)	0	5
ER chaperones			
P07237	Prolyl 4-hydroxylase subunit beta (P4HB)	9	17
P14625	Heat shock protein 90 kDa beta member 1 (HSP90B1)	6	13
P27824	Calnexin (CANX)	3	11
P23284	Peptidyl-prolyl cis-trans isomerase B (PPIB)	1	7
P13667	Protein disulfide-isomerase A4 (PDIA4)	0	7
Glycolysis and Metabolism			
P06733	Alpha-enolase (ENO1)	21	44
P14618	Pyruvate kinase PKM (PKM)	19	34
P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	13	38
P00338	L-lactate dehydrogenase A chain (LDHA)	6	22
O75874	Isocitrate dehydrogenase [NADP] cytoplasmic (IDH1)	1	6
P06744	Glucose-6-phosphate isomerase (GPI)	1	6
P52789	Hexokinase-2 (HK2)	0	6
Lipid metabolism and lipid binding proteins			
P49327	Fatty acid synthase (FASN)	9	20
P08758	Annexin A5 (ANXA5)	6	17
P04083	Annexin A1 (ANXA1)	5	12
Q00341	Vigilin (HDLBP)	0	6
Mitochondrial proteins			
P10809	60 kDa heat shock protein, mitochondrial (HSPD1)	16	37
P06576	ATP synthase subunit beta, mitochondrial (ATP5B)	8	19
P25705	ATP synthase, mitochondrial alpha subunit 1, (ATP5F1A)	6	13
Q16891	MICOS complex subunit MIC60 (IMMT)	0	6
Nuclear proteins			
P13010	X-ray repair cross-complementing protein 5 (XRCC5)	5	12
P09874	Poly [ADP-ribose] polymerase 1 (PARP1)	1	6
P09651	Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1)	0	7
P26599	Polypyrimidine tract-binding protein 1 (PTBP1)	0	6
P06748	Nucleophosmin (NPM1)	0	6
Q96AG4	Leucine-rich repeat-containing protein 59 (LRRC59)	0	5
Cytoskeleton and cytoskeletal binding/regulating proteins			
P35579	Myosin-9 (MYH9)	16	26
P60709	Actin, cytoplasmic 1 (ACTB)	15	26
P68363	Tubulin alpha-1B chain (TUBA1B)	13	29
O43707	Alpha-actinin-4 (ACTN4)	7	19
P68371	Tubulin beta-4B chain (TUBB4B)	0	20

Q14204	Cytoplasmic dynein 1 heavy chain 1 (DYNC1H1)	0	9
Other proteins			
Q09666	Neuroblast differentiation-associated protein (AHNAK)	18	40
P46940	Ras GTPase-activating-like protein IQGAP1 (IQGAP1)	5	15
P05023	Sodium/potassium transporting ATPase subunit alpha (ATP1A1)	2	7
P31949	Protein S100-A11 (S100A11)	0	4

Recently Wang *et al.*[61] described a novel inhibitor of tubule polymerisation that reduced lung metastasis in a murine melanoma model, demonstrating tubulin is a potential target in melanoma treatment. Thus, upregulation of tubulin may also be involved in chemo-resistance.

Other proteins

Neuroblast differentiation-associated protein (AHNAK), Ras GTPase-activating-like protein (IQGAP1), sodium/potassium transporting ATPase subunit alpha (ATP1A1), and S100 calcium-binding protein (S100A11) did not fit well into any of the previous categories and were listed separately. AHNAK is described as a large scaffolding protein and is believed to contribute to actin cytoskeletal organisation and cell-cell contacts. A recent study has described reduced AHNAK expression in melanoma compared to melanocytes,[62] whereas we observed an increase in our treated cell line. IQGAP1 belongs to a family of scaffold proteins that contribute to that cytoskeletal dynamics, and intracellular signalling, and is often over expressed in cancer.[63] Recently, Pan *et al.*[64] identified the O- class forkhead factor, FOXO1, as a binding partner of IQGAP1. These authors describe that phosphorylated FOXO1 binds to IQGAP1 in the cytoplasm to decrease IQGAP1-dependent activation of the ERK/MAPK pathway. This interaction is blocked by taxane based chemotherapeutics, leading to IQGAP1 activation of the extracellular signal-regulated kinase/ mitogen-activated protein kinase (ERK/MAPK) pathway and chemo-resistance.[64] Thus, IQGAP1 upregulation may be contributing to resistance in our treated melanoma cells.

Conclusion

This study reveals that an ethanol extract of whole crab ash, from the crab of the genus *Portunus*, resulted in reduced proliferation and apoptosis in the SK-MEL-28 melanoma cell line. Ethanol extracts of shell alone or protein alone, and aqueous extracts, were much less cytotoxic compared to the ethanol whole crab ash extract. This suggests that the cytotoxic agent/s were extracted more efficiently with ethanol. In addition, the heating involved in preparation of the whole crab ash; and the combination of shell plus protein, appear to produce a more cytotoxic mixture.

A sub-lethal concentration of whole crab ash caused apoptosis in some cells within melanoma SK-MEL-28 cell line, and at the same time, may have induced resistance mechanisms in cells that survived treatment. In broad terms, there were changes in expression of cytoskeletal and ER proteins involved in protein folding, and some indications of increased protein synthesis. A number of enzymes in the glycolytic pathway were upregulated, suggesting a role for aerobic glycolysis, and expression of some mitochondrial proteins was increased. Cytoskeletal changes included increased expression of actin, myosin and tubulin; and nuclear changes involved proteins involved in mRNA splicing.

These data are preliminary and based on semi-quantitative proteomic analyses. Further investigation and validation of this data should be performed using increased sample sizes, and quantitative mass spectrometry.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors would like to thank Professor Deirdre Coombe for supplying the SK-MEL-28 melanoma cells and tissue culture reagents. The authors would like also to thank Dr Carl Mousley for his support in the protein functional analysis. The authors acknowledge the Higher Committee of Education of Iraq (HCED), The School of Pharmacy and Biomedical Science at Curtin University, and the Curtin Health Innovation Research Institute for supporting this study.

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