6-1-2018

Cytotoxic activity of the Red Sea anemone entacmaea quadricolor on liver cancer cells

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Cytotoxic Activity of the Red Sea Anemone *Entacmaea quadricolor* on Liver Cancer Cells

A Thesis Submitted to

The Biotechnology Master's Program

In partial fulfillment of the requirements for the degree of Master of Science

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February 2018

DEDICATION

To Farida, the beautiful little angel who left our world, your courage was my true inspiration to continue.

To every cancer patient who suffered and is suffering from pain.

To every family that suffered from the loss of their beloved child, father, mother or sibling

I dedicate this work.

May ALLAH Bless their souls and accept this work as a good deed.

To my family; my Husband, Father, Mother, Brother and Sisters without your help nothing of this work could be done.

To Omar, the precious gift from ALLAH

Thank you is never enough.

ACKNOWLEDGMENTS

Praise is to ALLAH by Whose grace good deeds are completed

I would like to show the deepest gratitude to my advisors; **Dr Arthur Bos** and **Dr Asma Amleh** for their continuous guidance and support. And to extend my sincere graduate to **Dr. Ahmed Moustafa** for offering his help when needed. In addition, I would like to express my gratitude to **Dr Hamza El Dorry** for his valuable advice and to **Dr Michail kontominas** at chemistry department AUC as well.

Special thanks to Professor **Mehmet Ozturk** from the Department of Molecular Biology and Genetics, Bilkent University for kindly providing SNU-449 cell line as a gift from him.

I highly appreciate **Mona El Rady** and **Mohammed Maged** for their valuable advices that helped me a lot in the practical work and thesis writing. I extremely thank **Amgad Ouf** and **Aya Youssef** for their continued support.

To all my friends and colleagues, I would like to express my appreciation especially to, **Menna Ghouraba, Nouran Adly, Youssef Abdo, Laila Ziko, Menna El Far, Nancy Youssef, Noha Saad, Nahla Hussien, Sara Sonbol and Myreit Ghabrial.**

I specially thank the most helpful lab aids**; Mr Zien and Mr Mohammed.**

Finally, I would like to express much gratitude to the AUC for providing the grant that funded this research and for partially funding my studies at the AUC.

ABSTRACT

*Entacmaea quadricolor (*Phylum *Cnidaria,* class *Anthozoa)* is a marine anemone found in *Coral Reefs* in the Red Sea. Its venom is reported to be a potential anticancer agent when tested on several cancer cell lines; such as skin cancer, and lung cancer cell lines. Yet, none of the earlier studies have characterized the extracted venom, nor tested its activity on hepatocellular carcinoma (HCC).

The purpose of this study was to determine for the first time the potential cytotoxic activity of the Red Sea anemone *E. quadricolor* on HCC cell line. In addition to the effects of bleaching, seasonality and light exposure during storage on the venom were studied. Moreover, it was aimed to determine whether the cytotoxic activity was apoptotic or necrotic.

In this study, the venom of the anemone *E. quadricolor* was extracted and stored under three different conditions; winter vs. summer, light vs. dark and bleached vs. unbleached. The cytotoxic activity of each venom was tested on SNU-449 HCC cells using the MTT assay. SDS-PAGE was used to differentiate between venoms based on protein composition. Moreover, Annexin-V/PI assay was used to determine the type of cytotoxic activity.

The results revealed that *E. quadricolor* had potent cytotoxic activity against SNU-449 cells that was mediated by a necrotic pathway. The maximum activity was found during summer at the half-inhibitory concentration (IC_{50}) of 20 µg/ ml. However, this cytotoxic activity was neutralized when the venom had been exposed to light when stored. Furthermore, cytotoxic activity was significantly decreased upon Bleaching of the anemone. A protein of 28 kDa was found in the composition of the venoms of bleached and unbleached organisms possibly identifying the cytotoxic active protein.

The present results underline the findings of previous studies showing the cytotoxic activity of the sea anemone venom on cancer cell lines extending this to HCC. Furthermore, the findings are unique in showing that a bleached organism still produces toxic proteins and the venom loses its toxicity when exposed to light.

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List of abbreviations:

A.U.C: Area Under the Curve.

APs: Actinoporines

B: *E. quadricolor* venom; extracted after bleaching of anemone and stored in dark conditions

BCA: Bicinchoninic acid

E. quadricolor: *Entacmaea quadricolor*

ED_{50}: median effective dose

EqTx-II: equinatoxin-II

FBS: fetal bovine serum

FDA: food and drug administration

FITC: Fluorescein isothiocyanate

HCC: Hepatocellular Carcinoma

HCV: Hepatitis C virus.

IC_{50}: half-inhibitory concentration

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide

PBS: phosphate buffered saline

PI: propidium iodide

PS: phosphatidyl serine

RFPs: Red fluorescent proteins

RPMI: Roswell Park Memorial Institute medium

SD: *E. quadricolor* venom; extracted in summer and stored in dark conditions

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNU: Seoul National University

WD: *E. quadricolor* venom; extracted in winter and stored in dark conditions

WL: *E. quadricolor* venom; extracted in winter and stored in light conditions

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Chapter 1: Literature review and study objectives

1.1. Marine Biotechnology: The Future Era of Cancer Therapy.

Nowadays the era of cancer therapy is witnessing a huge development. Even though, the search for new anticancer agents has to be continued to cope with the new challenges and to tackle the new problems that emerge in cancer therapy field, the eyes of researchers were turned towards the marine environment as a source of anticancer drugs.

The last century had witnessed great discoveries of marine bioactive products showing anticancer activity. For example, Cytarabine (cytosine arabinoside); which was the first anticancer drug of marine origin that was approved by Food and Drug Administration (FDA).Cytosine arabinoside was isolated from the Caribbean sponge *Cryptotheca crypta* and it is currently used as an anticancer drug for leukemia and lymphoma (Sarfaraj et al., 2012). Recently, FDA has proved two more drugs of marine origin for cancer therapy, Adcetris™ and YONDELIS®. Adcetris™ (brentuximab vedotin) is currently indicated for cases of resistant Hodgkin lymphoma and systemic anaplastic large cell lymphoma. The active cytotoxic peptide of brentuximab vedotin is monomethyl auristatin E that is a synthetic analog of a peptide isolated from the marine shell-less mollusk *Dolabella auricularia* (Bai et al., 1990, Cheung et al., 2015). The second Drug, YONDELIS® (Trabectedin) is derived alkaloid isolated from the tunicate *Ecteinascidia turbinata*, it is currently indicated for cases suffering from metastatic soft tissue sarcomas; liposarcoma and leiomyosarcoma. (Allavena et al., 2005, "Press Announcements - FDA approves new…" 2015).

Currently, many marine bioactive products have shown promising results as anti-cancer agents and are in the clinical pipeline waiting for their release. (Cheung et al., 2015). All these studies might allow us to predict that this century could be the golden era for new effective cancer therapies of marine origin.

1.2. Sea Anemone Toxins and Cancer Therapy

For many years, *Cnidaria*ns and especially Sea anemones (class: *Anthozoa*) had captured the researchers' attention. With their simple structure, catchy colors and

peaceful appearance, yet they revealed to have a potent defense mechanism, which is venom production. Sea anemones contain highly specialized cells called nematocysts. Nematocysts are responsible for the production of a mixture of toxic polypeptides in order to defend the anemone against predators and to capture prey (Samejima et al. 2000, Mariottini and Pane, 2013).

Extensive studies have been conducted on multiple sea anemones in order to analyze and characterize their peptide toxins. Most of the analyzed toxins were classified according to their action into cytolytic and cytotoxic peptides. (Mariottini and Pane, 2013)

1.2.1 Cytolytic peptides:

Cytolytic peptides were classified by Anderluh and Maček (2002) based on their molecular size into 4 groups; Group I which consists of small peptides ranging from 5 to 8 kDa, due to their small size they can penetrate through the cell membrane that contains phosphatidylcholine. Group II which consists of pore-forming peptides called Actinoporins (APs) of a molecular size around 20 kDa. APs were found to have a specific structure of β-sandwich flanked by two α-helices; where the N-terminal of the α-helix consists of a cluster of aromatic amino acid residues that recognize and bind to the sphingomyelin lipids in cell membrane forming a pore that allows water and cations Ca^{2+} to penetrate into the cells leading to its lysis. (Samejima et al., 2000, Rojko et al., 2016). Group III; includes lethal phospholipases A2 of molecular size range from 30 to 40 kDa. Group IV; includes one large sized thiol-activated cytolysin of 80 kDa isolated from Metridium senile. (Mariottini and Pane, 2013)

1.2.2 Cytotoxic peptides

Multiple studies have been conducted to assess the cytotoxic activity of multiple sea anemones' venoms on different cancer cell types. For example, the venom of the anemone *Cryptodendrum adhaesivum* was shown to have cytotoxic activity against A549 (lung cancer), T47D (breast cancer) and A431 (Skin cancer). In other studies, the researchers succeeded in the isolation and identification of the protein which is responsible for the cytotoxic of the venom such as equinatoxin-II (EqTx-II) which is a cytolytic protein isolated from the anemone *Actinia equina*.

EqTx-II was shown to have cytotoxic activity against two human glioblastoma cell lines; U87 and A172. It is worth mentioning, the study revealed that EqTx-II at non-cytotoxic concentration showed to have a synergistic action when combined with low doses of three classical anticancer agents; cytosine arabinoside, doxorubicin, and vincristine. (Soletti et al., 2008, Mariottini and Pane, 2013). Other examples of cytotoxic extracts of sea anemones on human cancer cell lines are listed in Table.1. All studies showed the high potential of cytotoxic peptides extracted from Sea anemones to be effective cancer therapeutic drugs for different types of cancer as monotherapy or in combination with other classical cancer drugs where they enhance their activity and reduce their side effects. (Monroy-Estrada et al., 2013, Mariottini and Pane, 2013).

Table 1 Cytotoxic activity of sea anemones' extract on different human cancer cell lines (Mariottini and Pane, 2013)

Anemone	Compound or material	Cell line	Tissue/organ/ histology	IC50-ED50	Reference
Actinia equina	EquinatoxinII -I18Cmutant	MCF 7 ZR 751 HT1080	Breast adenocarcinoma Breast carcinoma Fibrosarcoma	0.2–0.3 µg/mL 5.8 µg/mL 14.2µg/mL	Potrich et al.,2005
	EqTx-II	U87 A172	Glioblastoma Glioblastoma	Not indicated	Soletti et al.,2008
Aiptasia mutabilis	Crude venom	Hep-2	Epithelial carcinoma	Not indicated	Marino et al.,2004
Bunodosoma Caissarum	Bc2	U87 A172	Glioblastoma Glioblastoma	Not indicated	Soletti et al.,2008
Cryptodendrum adhaesivum	Venom	A549 T47D A431	Non-small cell lung carcinoma Breast cancer Skin cancer	Not indicated	Ramezanpour et al.,2012

Entacmaea quadricolor	Venom	A549 A431	Non-small cell lung carcinoma Skin cancer	Not indicated	Ramezanpour et al.,2012
Heteractis crispa	Actinoporin RTX-A Venom A549	HL-60 HeLa THP-1 MDA-MB-231 SNU-C4 lung carcinoma	Promyelocytic leukemia Cervix carcinoma Monocytic leukemia Breast cancer Colon cancer lung carcinoma	1.06 nM 2.26 nM 1.11 nM 4.64 nM 4.66 nM Not indicated	Fedorov et al.,2010 Ramezanpour et al.,2012
Heteractis malu	Venom	A549 T47D A431	lung carcinoma Breast cancer Skin cancer	Not indicated	Ramezanpour et al.,2012
Heteractis magnifica	Venom	A549	lung carcinoma	Not indicated	Ramezanpour et al.,2014
Sagartia rosea	Acidic actinoporin Src I	U251 NSCLC BEL-7402 BGC-823	Glioblastoma lung carcinoma Liver carcinoma Stomach adenocarcinoma	3.5µg/mL 2.8µg/mL 3.6 µg/mL 7.4µg/mL	Jiang et al.,2003
Urticina piscivora	Venom UpI	HEL299	Embryonic lung	10.07µg/mL 29.99 µg/mL	Cline et al.,1995

1.3. *Entacmaea quadricolor*

1.3.1 Distribution and structure

Entacmaea quadricolor (Phylum: *Cnidaria*, Class *Anthozoa*, Order: *Actinaria)* is the target anemone in this study. It is widely distributed throughout the Indo-Pacific tropical waters, including the Gulf of Aden, and the Red Sea (Nanninga et al., 2014). The holotype was described in 1827 and it was collected near Suez along the Egyptian coast of the Red Sea (Rueppell and

Leuckart, 1828). *E. quadricolor* gained its nomenclature as it has multiple morphs based on the color of its tentacles; green, pink to purple, orange to red and brown (Scott and Harrison, 2009).

Its structure consists of a well-developed base that adheres to rocks and sea stones, a smooth contractile column, and a flat oral disc that contains a small central mouth in addition to long bulbous-ended tentacles (Figure1). The common name is bubble-tip anemone. (Dunn, 1981, Fautin and Allen, 1997).

Figure 1. Bubble tip sea anemone *Entacmaea quadricolor*

Source: https://thereeftankblog.com/my-livestock/rainbow-bubble-tip-anemone/

1.3.2. Symbiosis

E. quadricolor has two types of symbiosis ectosymbiosis and endosymbiosis. These symbionts are vital for the survival of the anemone.

The ectosymbionts such as anemonefish including 7 fishes of the *Amphiprion* genus, *Dascyllus trimaculatus*, and *Premnas biaculeatus*, besides several forms of anemone shrimp such as *Periclimenes brevicarpalis*. It is worth mentioning that *Amphiprion bicinctus*was only found in the Red Sea; that is why it is known by the Red Sea clownfish. (Dunn, 1981).

This mutualistic relationship provides protection to both the anemonefish and the host anemone; where anemone provides protection to the

anemonefish and their laid eggs from predators thus elongates their lifespan (Nedosyko et al., 2014). Interestingly, each anemone acts as a selective host for the symbiotic anemonefish, where it was believed that a certain type of mucus secreted by the anemonefish that makes it resistant against the venom of the hosted anemone whereas other fish are susceptible to this venom. (Mebs, 2009; Nedosyko et al., 2014). Also, the anemonefish protects the anemone from predators due to their aggressive behavior toward intruders. In addition to the ammonia excreted by anemonefish enhances the growth of the anemone (Porat and Chadwick-Furman, 2005).

The endosymbiosis include a dinoflagellate called zooxanthellae; which are unicellular algae belonging to genus *Symbiodinium* sp. They are capable of photosynthesis as they contain chlorophyll, and thus they provide fixed carbon that is an essential food source for anemone (Muscatine and Porter, 1977, Onodera et al., 2004).

1.3.3. Anemone bleaching

The loss of the endosymbiotic zooxanthellae leads to the loss of the anemone color in a phenomenon called "bleaching" (figure 2). This phenomenon occurs if the anemone was subjected to harsh conditions such as metal toxicity, increase in water salinity, rapid decrease in temperature, increased light intensity, bacterial infections, but the most significant factor is the increase in water temperatures (Fujise et al., 2014). Normally, sea anemones have low mortality rates. However, due to the continuous increase in seawater temperature the mortality rates are increasing. This is an environmental alert where global warming is increasing leading to the elevation in seawater temperature putting the life of anemones and all the marine life in great danger (Hobbs et al., 2013).

Figure 2. Bleached *E. quadricolor*

Source: https://www.flickr.com/photos/wildsingapore/30027134601/

1.3.4. Dinoflagellate toxins

It is worth mentioning that some species of dinoflagellates (to which zooxanthellae belong) are toxic to fish and human. Multiple studies were done to isolate these toxins and to determine the type of their toxicity. Dinoflagellate toxins include; neurotoxin as paralytic shellfish poison and hemolytic toxins as the G.breve toxin (Schever, 2012). Interestingly, it was found out that some of the dinoflagellates uniquely contain a carotenoid compound called Peridinin that was proved to have cytotoxic activity when tested on human colorectal cancer cells (Sugawara et al., 2007).

1.3.5. *E. quadricolor* bioproducts:

Bio Fluorescence:

E. quadricolor showed to emit fluorescence similar to most of *Cnidaria*ns. It expresses both green fluorescent protein (GFP) and Red Fluorescent protein (RFP) as shown in figure 3. RFP is much more important due to its vast application in tissue and whole body imaging. *E. quadricolor* expresses two types of RFP; eqFP578 and eqFP611. eqFP611 is around 26 kDa in size and it is considered to be a unique far-red shifted RFP as it has maximum emission at 611nm, and that is the highest recorded natural emission (Peterson et al, 2003, Vallone et al.,2005).

It is worth mentioning that some of RFPs as DsRed which is isolated from *Discosoma* sp. (Phylum *Cnidaria*), was reported to have cytotoxic activity against cervical cancer cells (Hela) (Zhou et al., 2011). On the other hand, no studies were conducted to address the cytotoxic activity of wild *E. quadricolor* RFPs. (Piatkevich et al, 2011)

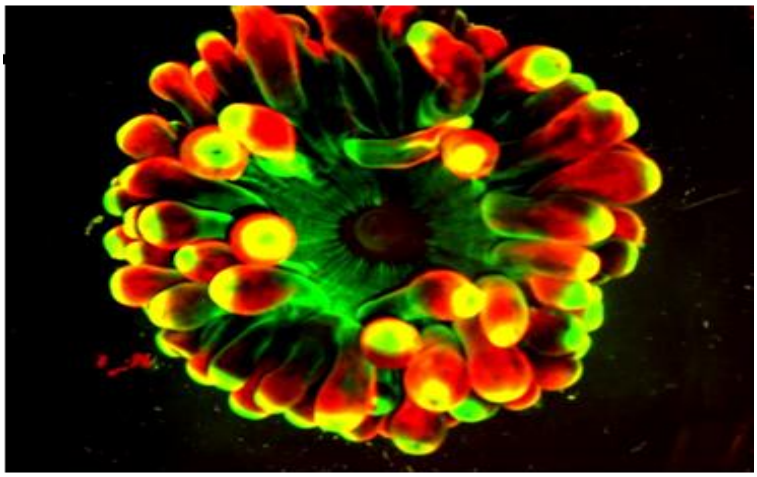

Figure 3 Green and red fluorescence of *E. quadricolor*

Source:http://www.gbri.org.au/SpeciesList/Entacmaeaquadricolor%7CNicolaWood.aspx?PageContentID=4474

Cytolytic activity:

Like most of the *Cnidaria*ns, *E. quadricolor* was reported to have a cytolytic activity. A cytolytic peptide was isolated from *E. quadricolor* named cytolysin EnT. Samejima et al., 2000determinedthe N-terminal sequence of the cytolysin. The 40 amino acid residue shown to have a high degree of similarity to other isolated cytolysins as HmT; a highly active cytolysin that was isolated from the sea anemone *Heteractis magnifica* (Mebs et al., 1992; Mebs, 1994).

Cytotoxic activity:

Multiple studies have been recently conducted on sea anemones revealed that their venom had cytotoxic activity against multiple human cancer cell lines. Only one study was done on *E. quadricolor* addressing its potential cytotoxic activity. The study tested the cytotoxic activity of the venom of *E. quadricolor* (besides four other sea anemones) on three different cancer cell lines; A549 lung cancer, T47D breast cancer, and A431 skin cancer. The study assessed the cytotoxic activity by using the MTT assay and the crystal violet assay. The results showed a significant inhibitory effect on both A431 and A549 cancer cell lines at 40µg/ml protein concentration of the venom. (Ramezanpour et al., 2012)

1.4 Hepatocellular carcinoma, the heavy burden in Egypt

Hepatocellular carcinoma (HCC) is one of the most notorious types of cancer; it was recorded to be the second leading cause of cancer-related mortality worldwide (GLOBOCAN 2012 database). In Egypt, it is considered to be a major health problem that is attributed to the high incidence of HCV (Hepatitis C virus) infection. Throughout the last century, Egypt had suffered from increasing incidences of HCV, majorly due to the parenteral treatment of Bilharzia, in addition to the intra-familial transmission. (Shaker et al., 2013, Saleh et al., 2015). Since then, the prevalence of HCC in Egypt is highly increasing in a way that is affecting the Egyptian lifestyle in multiple aspects. This fact makes searching for an effective anticancer agent is the highest priority.

HCC has been considered one of the most notorious cancers due to its poor prognosis; where the early stages of the disease are mostly asymptomatic and in most cases, the disease is only diagnosed in late incurable stages. Although multiple studies have been conducted in order to find an effective therapy, the treatment of HCC is still a great challenge. Besides surgical intervention, Sorafenib was considered the only systemic treatment for advanced HCC that is currently approved by FDA. Unfortunately, Sorafenib showed to be less effective and extended the patient's life by only a few months. (Daher et al., 2017).

1.5 Study Objectives:

E. quadricolor like most of sea anemones had shown to have cytotoxic activity against human cancer. However, the biotoxin of *E. quadricolor* has been fully studied neither on molecular level nor its cytotoxic activity on different cancer cell types. In this study, I aim to examine for the first time the cytotoxic activity of the Red Sea anemone *E. quadricolor* on HCC cell line. SNU-449 cells were the cell line of choice as it is characterized by multiple drug resistance against anticancer drugs (Boulin et al.,2011 and Park et al.,1994) and it demonstrates grade II-III/IV which is the most demonstrated grade of HCC (Paradis,2013). Moreover, I aim to characterize the venom in terms of

1st Addressing the effect of anemone bleaching on the cytotoxic activity of *E. quadricolor*.

2nd Identifying the optimum storage conditions by assessing the effect of light on the venom.

3rd Investigating the effect of seasonal variation on *E. quadricolor* cytotoxic activity.

4th Determining the type of cytotoxic activity whether it's necrotic or apoptotic.

Chapter 2: Materials and Methods

2.1 Sample collection:

Two *E. quadricolor* anemones were purchased from a local aquarium shop in Cairo, Egypt. Both anemones have been collected from the Egyptian coast of the Red Sea. Both individual anemones looked healthy, had the same color (brown) and weighed about 80g. Their sex was not identified. The first anemone had been collected in the winter (February 2015) and the other at the end of the summer (September 2015). The anemones were kept in a marine aquarium in a laboratory at the American University in Cairo. Water salinity (1.024 - 1.026 g/ml), pH (8.1-8.3) and temperature were adjusted to mimic the Red sea water (20-22^{o}C in winter and 22-25^{o}C in summer). The animals were fed twice weekly with chopped shrimps and fasted one week before each venom extraction. (Sencic and Macek, 1990, Ramezanpour et al., 2012). After the first extraction in March 2015, the anemone was subjected to elevated water temperature (28-29 ^{o}C) causing the anemone to lose its color (hence zooxanthellae) (Hill and Scott 2012). Venom extraction was conducted when the animal was fully bleached.

2.2 Venom extraction

The crude venom was extracted using the milking technique (Sencic and Macek, 1990). Each anemone was placed in a sterile tray to allow the anemone to release excess water and to remove attached rubble. After placing each anemone in a plastic bag, the tentacles were gently massaged until no more fluid was produced. It was observed that after extraction the anemones were able to regain their weight and health. In contrary, other methods of extraction cause severe harm or death to the anemone (Nedosyko et al., 2014). The collected venom was then lyophilized using a TOPT-10C freeze dryer and kept in -80^{o}C. (Sencic and Macek, 1990, Ramezanpour et al., 2012).

The date of anemone collection, date of sample extraction, anemone condition, and sample storage conditions are listed in Table.2.

Table 2 Crude protein venoms collected from *E. quadricolor*

Venom	Date of Anemone collection	Anemone condition	Venom Storage	Volume of venom
Winter dark(WD)	Feb 2015	Kept in Temp. (20-22^{o}C)	Stored in Dark	100ml
Winter light (WL)	Feb 2015	Kept in Temp. (20-22^{o}C)	Stored in light	100ml
Winter Bleached (B)	Feb 2015	Kept in Temp. (28-29^{o}C)	Stored in Dark	20ml
Summer Dark(SD)	Sep 2015	Kept in Temp. (22-25^{o}C)	Stored in Dark	60ml

2.3 Protein determination

The Lyophilized powder of each venom was dissolved in MilliQ water at 100mg/ml (Ramezanpour et al., 2012) and filtered using a CHM 0.2 µm Syringe filter to eliminate undissolved particles. The protein level was quantified using Bicinchoninic acid (BCA) protein assay (Serva *BCA Protein Macro Assay Kit Cat. No. 39228*); which is a colorimetric technique based on the formation of a purple colored product. (Equation 1 and 2) ("BCA Protein Assay Macro Kit", 2011)

1. Protein (peptide bonds) + $Cu^{2+} \longrightarrow Cu^{1+}$ Biuret complex
2. Cu^{1+} + 2 Bicinchoninic Acid $\longrightarrow$ BCA-Cu^{1+} complex (purple colored detected at 562 nm)

A 2.0 mg/ml standard Bovine serum albumin (BSA) stock solution was prepared and serially diluted with MilliQ water to the following concentrations;1000, 750, 500, 250, 100, 50, 25 and 0 µg/ml. The working reagent was prepared by mixing Reagent A and Reagent B at 50:1 ratio according to the manufacturer's instructions.

Hundred micro liters of each standard and sample were mixed with 2 ml of working reagent induplicate. Each mixture was transferred to sterile cuvette and incubated for 30 minutes in 37°C incubator, then left at room temperature for 15 minutes. Subsequently, the optical density (OD) was detected at 562 (nm) using a spectrophotometer (GE Healthcare / Amersham Biosciences Ultrospec 3100 Pro UV/Visible Spectrophotometer). The protein concentration of each venom (µg/ml) was determined relative to BSA standard curve (Table.3). (Smith et al., 1985, "BCA Protein Assay Macro Kit", 2011).

Table 3 Total protein level of each venom (µg/ml) using BCA protein assay

Type of venom	Protein concentration (µg/ml)
WD	1500 µg/ml
WL	1500 µg/ml
B	650 µg/ml
SD	600 µg/ml

2.4 Cell culture

SNU-449 cells were used (ATCC no. CRL-2234). It was derived from a 52-year-old Asian male suffering from grade II-III/IV hepatocellular carcinoma (Park et al., 1995). The cells were kept in RPMI-1640 medium (Roswell Park Memorial Institute medium) (Lonza, USA) supplemented with 10% FBS (Fetal bovine serum) (Lonza, USA) and 5% Penicillin-streptomycin antibiotic (Lonza, USA). The cells were seeded in sterile T-75 cm^2 tissue culture flasks at seeding density = 1.5×10^6 cells/ml and incubated at 37°C temperature and 5% carbon dioxide fully humidified incubator (SHEL LAB, USA). The cells were checked each 2-3 days under an inverted microscope (ZEISS AXIO Vert.A1) and subcultured at 90% confluency.

2.5 Cell viability

The cytotoxic activity of each venom on SNU449 cell line was tested using the MTT assay. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay is a colorimetric assay based on measuring the ability of viable cells to convert the yellow tetrazolium salt MTT into violet formazan crystal by the action of mitochondrial NADPH-dependent cellular oxidoreductase enzymes. A solubilizing agent is used to solubilize the formazan crystals and the cell viability can be detected by measuring the optical density (OD) where the increase in OD is linear to the number of viable cells. (Berridge et al., 2005, Meerloo et al., 2011)

In this study, SNU449 cells were seeded in sterile flat-bottom 96-well plates at a density of 10^4 cells/well with 100µl complete RPMI media/well. The plates were incubated at 37°C temperature and 5% carbon dioxide fully humidified incubator (SHEL LAB, USA) for 24 hours. Each lyophilized venom was dissolved in MilliQ water at 100 mg/ml of mass concentration and serially diluted with complete RPMI media to 0.1, 1, 10, 40 µg/ml final protein concentrations based on the BCA assay results. The plates were incubated for 24, 48 and 72 hours' intervals. After each time interval, the media and added venom were removed and 20 µL of MTT (5mg/mL dissolved in sterile PBS) (Serva, Germany) added to 100 µL of complete RPMI media. The mixture was added to each well and incubated at 37°C in complete darkness for 3 hours. After 3 hours, 100 µL/well of DMSO (Dimethyl sulfoxide, SERVA, Germany) was added to solubilize the formazan crystal. Absorbance was measured at 570 nm (Ramezanpour et al., 2012) using SPECTROstar Nano (BMG LABTECH) plate reader.

All the experiments were conducted under dark conditions. The control groups included; 1st, negative control where only media was added to SNU449 cells and the percentage cell viability was considered 100%. 2nd, solvent control where MilliQ water was added to cells to detect if the solvent used had any effect on cell viability. 3rd, positive control; Cisplatin (cis-diammineplatinum(II)dichloride), a drug commonly used in cancer treatment was used to compare its cytotoxic activity to *E. quadricolor* venom on SNU449 cell line. Cisplatin (Sigma-Aldrich, USA) was prepared at 1mg/mL stock solution in sterile-filtered 0.9%NaCl (Ziko et al., 2015) and

serially diluted with complete RPMI media to 0.1, 1, 10, 20, 40 and 80 µg/ml final concentrations.

2.6 Statistical analyses

All MTT assays were conducted in triplicates (three independent experiments). The data was analyzed using GraphPad prism (version 5); a non-parametric test was used to analyze the data as Bartlett's test showed that the variances were significantly different. Kruskal-Wallis test was used to analyze the effect of each venom on SNU-449cell viability. Dunn's multiple comparison test was used to compare the cell viability of each condition to that of any other condition. Differences in cell viability were considered significant at p-value < 0.05.

2.7 Identification of the protein size

In order to compare the protein composition of the unbleached with the bleached venom; 20 µg of protein of each venom were mixed with 5x Laemmli dissociation buffer, the mixture was boiled in thermal block at 99°C for 10 minutes, and then each sample was loaded on12% SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis). ProSieve™ QuadColor™ of range 4.6 to 300 kDa was used as Protein Marker. The gel was run at 130V using 1x SDS running buffer. After the run, the gel was stained with Coomassie Blue stain and imaged by Gel Doc EZ System (Bio-Rad, USA). The intensity of the observed bands was analyzed using ImageJ; histograms obtained showed the differential expression of each protein in the bleached and unbleached *E. quadricolor*.

2.8 Determination of the type of cell death

In order to determine the type of the cell death caused due to *E. quadricolor* venom on SNU449 cells, Annexin –V/ PI (propidium iodide) assay was used to differentiate between apoptotic and necrotic cells. The Annexin –V/ PI assay is based on the detection of morphological changes that occur in the cell membrane of the apoptotic and necrotic cells. In healthy cells, the cell membrane is intact and phosphatidyl serine (PS) is exclusively found in the inner surface of the membrane. In early apoptosis, PS is exposed to the outer surface of the cell membrane. Annexin V;

which is a phospholipid-binding protein conjugated to FITC (Fluorescein isothiocyanate) has high affinity and specifically bind to PS staining the apoptotic cells with green fluorescence. On the other hand, in necrosis; the cell membrane loses its integrity and becomes permeable to PI that binds to nucleic acid staining necrotic cells with red-fluorescence that can be detected by fluorescent microscope. (Van et al., 1998).

In this study, SNU449 cells were seeded in sterile flat-bottom 6-well plate at seeding density = 0.3x 10^6cells/ml with 3ml of complete RPMI media/well and incubated for 24 hours at 37°C temperature and 5% CO_2 incubator (SHEL LAB, USA). In this assay two concentrations of venom SD were tested; IC_{50}= 20 µg/ml (calculated by GraphPad Prism (version 5) based on MTT assay) and 40 µg/ml, complete RPMI media was only added for the negative control. After venom addition the plate was incubated at 37°C temperature and 5% CO2 incubator for 24 hours. After that, the cells of each treatment were washed twice with PBS and trypsinized using Trypsin-EDTA (Lonza, USA). The cells were then collected and centrifuged, the formed pellet was re-suspended and the cell count was determined using the trypan blue assay. After that, the cell suspension of each treatment was re-centrifuged and the pellet was re-suspended in Annexin -binding buffer at 10^6 cells/ml. 10 µl of each cell suspension were mixed with 5µl of the Annexin V conjugate and 1 µl PI working solution (Annexin V Alexa Fluor™ 488 &PI (Thermo Scientific, USA). The cells were incubated for 15 minutes in darkness at room temperature. Subsequently, cells were washed with Annexin-Binding buffer and deposited on glass slides. Fluorescence emitted by cells was detected using an Olympus 1x70, USA fluorescence microscope.

Chapter 3: Results

3.2 Cell viability

3.2.1 Cytotoxicity assay of *E. quadricolor* venoms

After 24 hours (Figure 4.A), no cytotoxicity was observed at 0.1, 1 and 10 µg/ml, but the percentage cell viability in all the venoms was slightly higher than the control, and the maximum increase was observed in WL venom. On the other hand, at 40 µg/ml, the percentage cell viability was significantly decreased to 23% and 8.5% in WD and SD venom respectively, a slight decrease was observed in B venom to 85%. However, in WL venom the percentage cell viability continued to increase up to 160%.

After 48 hours (Figure 4.B); similar to the 24-hour interval, the decrease of the cell viability was only observed at 40µg/ml for all the venoms except for WL venom. For WD and SD venom, the percentage cell viability was decreased to 18% and 0.4% in WD and SD venom respectively. In B venom, the percentage cell viability was decreased to 75%. While in WL venom, the percentage cell viability continued to increase up to 200%.

After 72 hours (figure 4.C), the maximum decrease in the percentage cell viability was observed at 40µg/ml in WD and SD venoms to 17.5% and 0.1% respectively. In venom B; the percentage cell viability was nearly unchanged from its value at 48-hour interval.

In comparing the percentage cell viability of venoms to the control, at all-time intervals, both WD and SD at 40 µg/ml were significantly different (Table 4.). Interestingly, SD and WD venoms only showed a significant difference at 40 µg/ml at 72-hour interval. (Significant differences between the venoms were listed in Table.4)

The solvent control showed almost equal cell viability to the control; which means that the MilliQ water used to dissolve the venom had no effect on the viability of the cells.

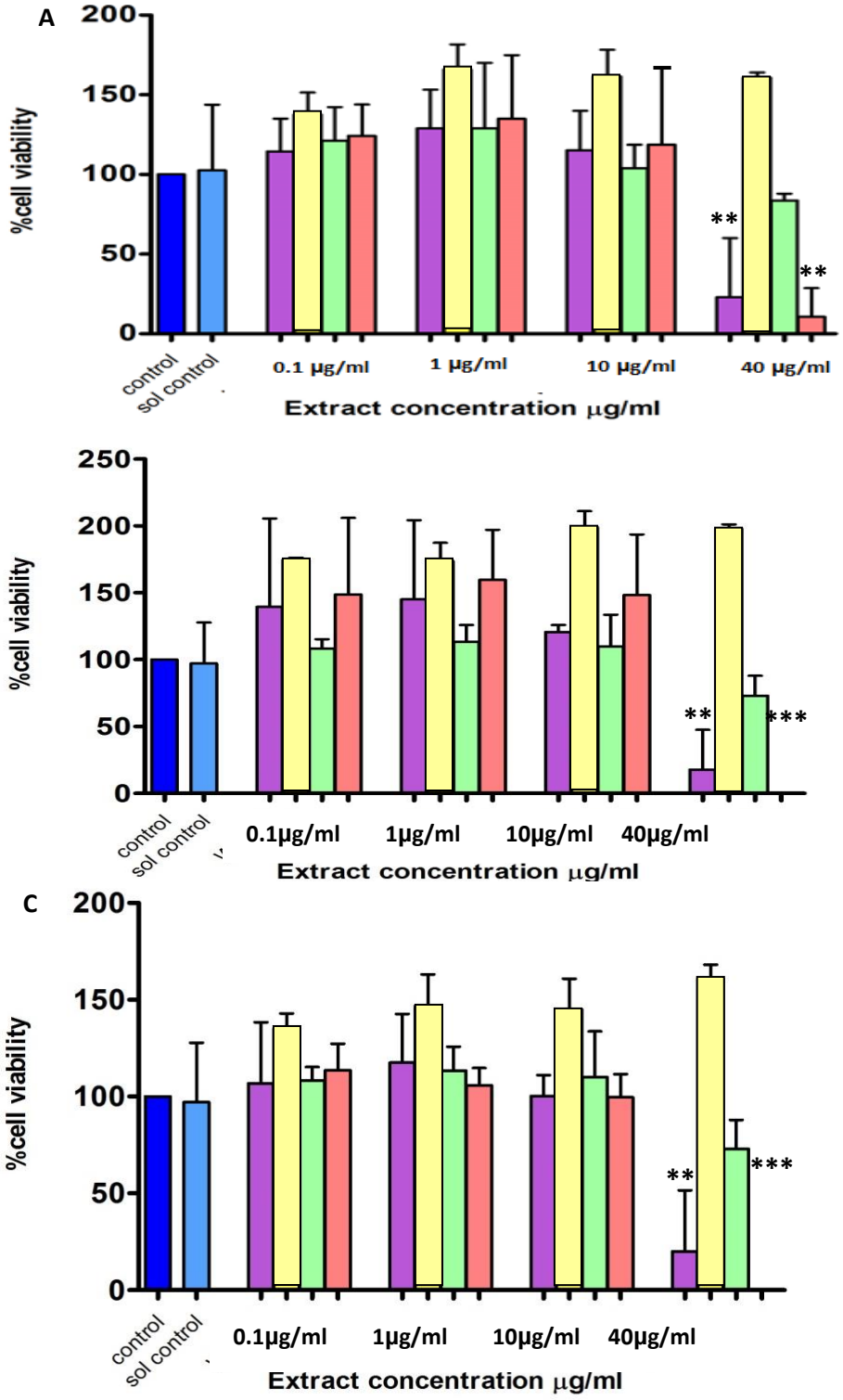

Figure 4. The effect of *E. quadricolor* venoms on SNU-449 cell viabilityafter 24, 48 and 72 hours; A, B and C respectively.

Data shown as mean ±SD, n = 3.Significant difference was calculated compared to control (**p value <0.01, *** p value < 0.01). Each venom was tested at 4 concentrations; 0.1, 1, 10 and 40 µg/ml. Dark blue bar represents the control, light blue bar represents the solvent control, purple; winter dark venom, yellow; winter light venom, green; bleached venom and pink; summer dark venom.

Table 4 P-values and significant differences between the means of SNU-449 cell viability after exposure to *E. quadricolor* venoms at 24, 48 and 72 time intervals. C; control, WD40; winter dark venom at 40 µg/ml, SD40; summer dark venom at 40 µg/ml, B; bleached venom at 40 µg/ml and WL; winter light venom at 40 µg/ml. (* P < 0.05, **P < 0.01 & *** P < 0.001)

Time interval	sample	p-value	significance
24 hour	C-WD40	<0.01	**
	C- SD 40	<0.01	**
	WD40-B40	<0.05	*
	WD40-WL40	<0.001	***
	SD40-B40	<0.01	**
	SD40-WL40	<0.001	***
48 hour	C-WD40	<0.01	**
	C- SD 40	<0.001	***
	SD40-B40	<0.01	**
	WL40-WD40	<0.001	***
	WL40-SD40	<0.001	***
72 hour	C-WD40	<0.01	**
	C- SD 40	<0.001	***
	SD40-WD40	<0.01	**
	B40-SD40	<0.001	***
	WL40-SD40	<0.001	***

3.2.2 Cytotoxicity assay of Cisplatin

The results showed that the cell viability was slightly higher than control at 0.1 and 1 µg/ml at all-time intervals (Figure 5). However, the decrease of the percentage of cell viability was observed after 72 hours to 62%, 45.5% and 32.5% at 10, 20 and 40 µg/ml respectively. The maximum decrease was observed at 80 µg/ml, where the percentage of cell viability reached 6.4% after 72 hours.

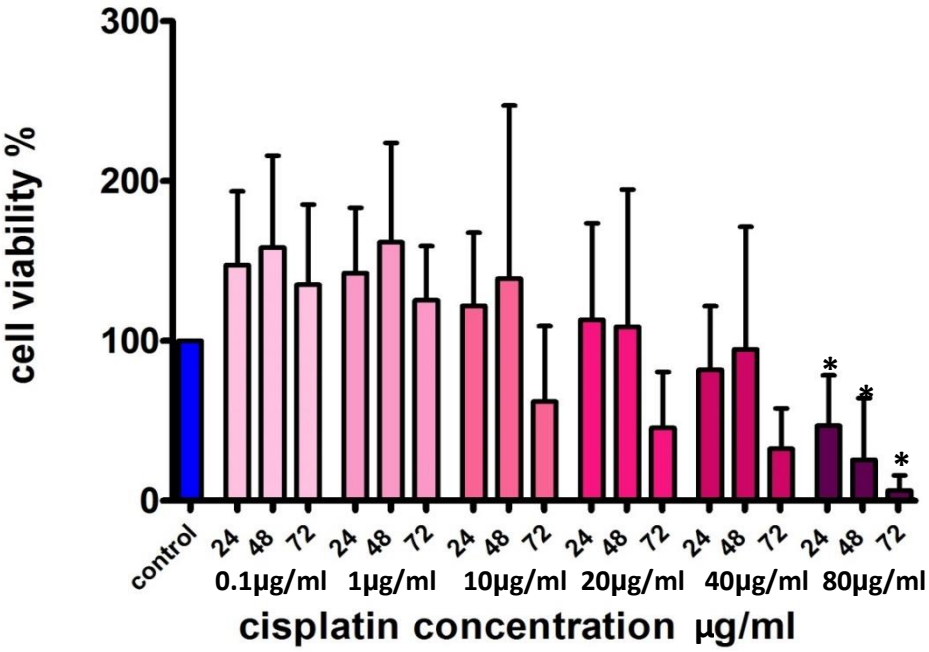

Figure 5The effect of Cisplatin on SNU449 cell viability after 24, 48 and 72 hours. Data shown as mean ± SD, n = 3. The maximum decrease in %cell viability was observed at 80 µg/ml after 72 hours. Significant difference was calculated compared to control (* P <0.05)**.**

3.3 Identification of protein size

The unbleached venom (Figure 6. A) Lane 1 and 2 showed 2 strong bands around 28 kDa and 15 kDa in addition to other multiple faint bands. On the other hand, multiple bands were observed in lane 5 and 6 (bleached venom) at 300, 250, 70, 55, 46, 45, 28, 15 and 9 kDa. Both venoms showed bands at almost the same size. however, the bleached venom showed multiple detectable bands but a very faint band at 28 kDa. In contrary to the unbleached one which showed a strong band at 28 kDa but the other bands were hardly detectable.The ImageJ obtained histogrames supported the observed defferentail expression of proteins in the bleached and unbleached venoms (Figure.6.B). The 15 kDa band was overlapped with the frontal dye. So in order to calculate the band intesity in each lane, the intensity of the dye alone was determined using ImageJ and subtracetd from the overlaped protein band.

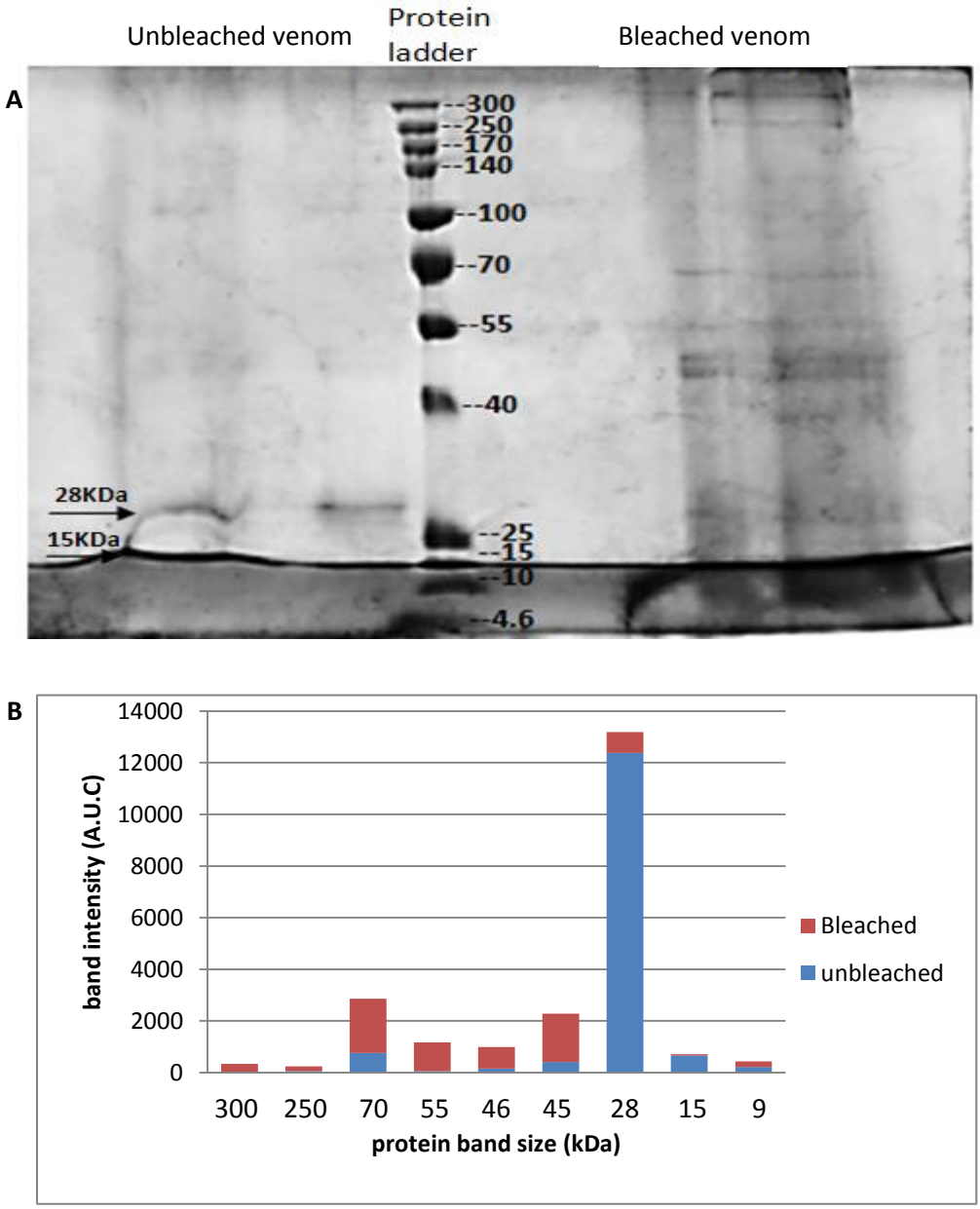

Figure 6 Protein size identification of bleached and unbleached venoms of *E. quadricolor*. A. Image of stained SDS-PAGE of unbleached and bleached venoms: lane 1 and 2; unbleached venom, lane 3; protein marker, lane 5 and 6; bleached venom. At around 28 kDa, a strong band was detected in lane 1 and 2, while it was very faint in lane 5 and 6. Another band was observed at 15kDa in the 2 venoms (marked with black arrow). B. Histograms obtained by ImageJ showed the differential expreesion of proteins in the bleached and unbleached venoms;the bleached venom showed higher protein expression in all protein sizes except for 28 kDa and 15 kDa. (A.U.C); Area under the curve.

3.4 Determination of the type of cell death

Based on the trypan blue exclusion assay, the percentage cell viability of the untreated cells (control) was calculated to 83.33%. While no viable cells were found in the examined field of cells treated with 20 µg/ml and 40 µg/ml of SD venom.

Based on the Annexin-V/PI assay, the bright field of the control showed multiple cells of normal rounded shape and well defined intact cell membrane in addition to three enlarged cells. In the PI staining; around three strong red signals were observed. In Annexin-v + PI staining, three identified cells emitted both green and red fluorescence (orange when merged) along with weak green signal emitted by viable cells. These results indicated the presence of three necrotic cells in the examined field (Figure 7.A). In the bright field in figure 7.B the number of well-defined rounded cells was decreased when adding SD venom at 20 µg/ ml, in the PI staining as well as the Annexin –V staining around 7 cells emit both red and green fluorescence in the examined field. Moreover, in figure 7.C upon adding 40 µg/ml of SD venomcells showed to be enlarged in size and some of them lost the integrity of the cell membrane and expelled their cytoplasmic content outside the cells (white arrow). In PI and Annexin-V staining; almost all the cells emitted both red and green fluorescence with the exception of one cell that emitted only green fluorescence in the examined field.

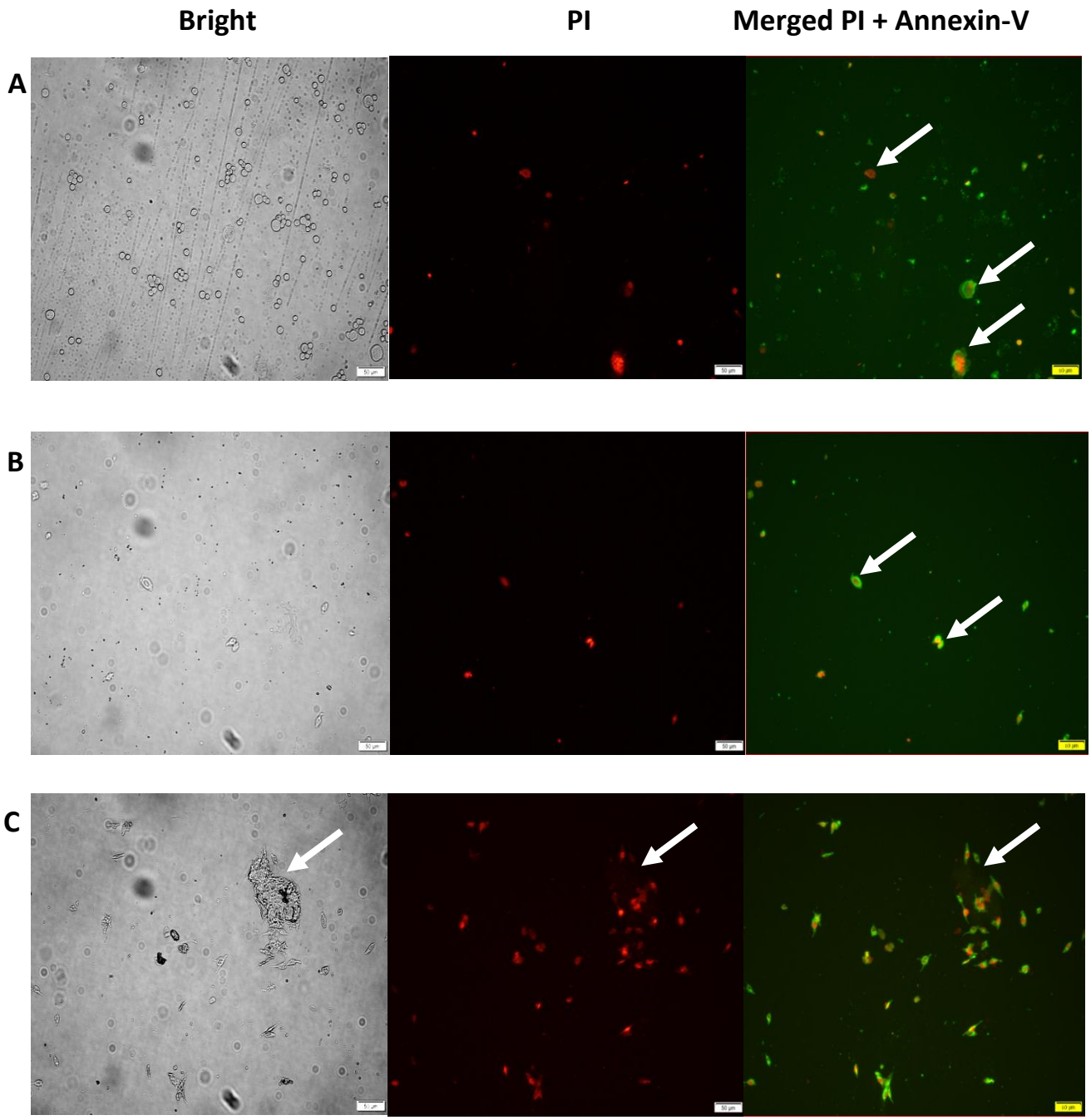

Figure 6. Annexin-V/PI assay of SNU-449 cells under fluorescence microscope

A; represents untreated cells (control), B; cells+ SD venom at 20 µg/ml and C; cells+ SD venom at 40µg/ml. Bar scale = 50 µm. A; multiple intact rounded cells were observed in bright field but only three cells emitted strong red and green fluorescence in merged field. B and C; the number of viable cells was decreased and cells were enlarged in size in the bright field, in PI and Annexin-V staining; almost all the cells emitted both red and green fluorescence. Some of the observed necrotic cells was marked by white arrows.

Chapter 4: Discussion

Multiple studies were conducted by researchers in order to identify and characterize the cytotoxic activity of sea anemones on different cancer cell lines. However, there was only one study that assessed the cytotoxic activity of *E. quadricolor* on lung, breast and skin cancer cell lines (Ramezanpour et al., 2012). To the best of our knowledge; this is the first study to examine a venom from the sea anemone *E. quadricolor* on a liver cell line. Moreover, it is the first time to address the effect of seasonal differences and venom storage on the cytotoxic activity of a sea anemone venom.

4.1 *E. quadricolor* cytotoxic activity among different cancer cell lines:

In the present study, the venom of *E. quadricolor* was tested on HCC cell line SNU-449. Based on the conducted MTT assay, the study revealed that the venom showed high cytotoxic activity reached to 0.1 % cell viability at 40µg/ml concentration after 72 hours' incubation period. This is the highest percentage of cytotoxic activity recorded compared to other tested cell lines. Ramezanpour et al., 2012 had showed that *E. quadricolor* venom had a significant cytotoxic activity against lung and skin cancer cells (A549 and A431 respectively) at 40µg/ml, while on T47D (breast cancer cells) no significant cytotoxicity was observed (Table 5).

Table 5. Percentage cell viability of *E. quadricolor* venom at 40 µg/ml among different cancer cell lines

Sample	Cell line	%cell viability after 24hours	%cell viability after 48 hours	%cell viability after 72 hours
SD venom	SNU449 (Liver cancer)	8.5%	0.4%	0.1%
Ramezanpour et al., 2012	A549 (lung cancer)	39%	30%	19%
Ramezanpour et al., 2012	T47D (breast cancer)	100%	100%	60%
Ramezanpour et al., 2012	A431 (skin cancer)	50%	30%	20%

4.2 Characterizing the effect of light on the cytotoxic activity of *E. quadricolor*

In order to characterize the effect of light on the cytotoxic activity of *E. quadricolor*, the winter venom was stored under two different conditions; one was stored in dark (WD) and the other was stored in light (WL). Based on the MTT assay, WD venom showed to have high cytotoxic activity at 40µg/ml where the percentage cell viability was decreased to 17.5%. On the other hand, WL venom showed no cytotoxic activity at all concentrations at all-time intervals but in fact, the cell viability was increased up to 200% as if the venom is nourishing the cells. This increase in the cell viability was not discussed by literature.

This is the first study reporting the effect of light on the cytotoxic activity of extracted venom from sea anemone in specific and *Cnidaria*ns in general. Other studies had addressed other stability conditions such as temperature, pH, and storage condition. For instance, a study was done on jellyfish *Cyanea nozakii* (Phylum: *Cnidaria*) where the extracted venom showed to have cytotoxic activity against liver and colon cancer cells (Cuiping et al., 2012). Upon subjecting the venom to different temperatures and pH levels, the cytotoxicity was changed. At temperatures above 60^{o}C the cytotoxic activity was diminished. In addition, the pH range where the venom showed maximum activity was between 4.5 and 8.5 above that range or below the cytotoxicity was distinctly decreased. Moreover, the study had tested the effect of several storage conditions on the cytotoxic activity of the venom; either in solubilized or lyophilized form at different storage temperatures. The most stable storage condition was in lyophilized form at -80 ^{o}C. (Cuiping et al., 2012)

In the present the study, all the cell viability assays were conducted at 37 ^{o}C, the pH was around 7.5-8, the venoms were stored in solubilized form at -20 ^{o}C and no distinctive change was observed on the cytotoxic activity of the venoms by time of storage that was 6 months.

4.3 Bleaching effect on *E. quadricolor* cytotoxic activity

"Bleaching" is a phenomenon that occurs when a sea anemone is subjected to harsh conditions as increased water temperature (refer to chapter one section 1.3.3). The main reason of bleaching is the expulsion of the endosymbiotic zooxanthellae. (Fujise et al., 2014).

It was previously reported that types of dinoflagellates (to which zooxanthellae belong) isolated from marine organisms had shown to have cytotoxic activity against cancer cells. Peridinin, which is a carotenoid compound uniquely isolated from dinoflagellate *Heterocapsa triquetra* was reported to have cytotoxic activity against human colorectal cancer cells (Sugawara et al.,2007). Moreover, a derived fatty acid called zooxanthellactone was isolated from dinoflagellates had shown to have cytotoxic activity when tested on human squamous cell carcinoma (Onodera et al., 2004). Another study was done on dinoflagellates isolated from soft coral *Clavularia viridis*. The study investigated the cytotoxic activity of two compounds; a peridinin-related Norcarotenoids compound isolated from the symbiotic dinoflagellate and a prostanoid compound isolated from the host coral. Interestingly, both compounds were shown to have cytotoxic activity against tested cancer cells. (Suzuki et al., 2003)

Moreover, several peptides isolated from different sea anemones have been proven to have cytotoxic activity against multiple cancer cells. Such as EqTx-II, SrcI and UpI (Table.1). (Soletti et al., 2008, Jiang et al., 2003 and Cline et al., 1995, respectively)

Here, the effect of bleaching on the cytotoxic activity of sea anemones is addressed for the first time. In this study, *E. quadricolor* was subjected to elevated temperature (28-29^{o}C) until it was totally bleached. The first observation recorded when comparing the bleached to unbleached venom, was the remark decrease in the amount of the milked venom of venom B (20 ml). In Addition, the protein concentration of venom B was calculated based on the BCA assay to be 650 µg/ml. On the other hand the unbleached venom had much higher venom yield (100 ml) and much higher protein concentration = 1,500 µg/ml. (Table .3).

The MTT assay showed that the bleached venom lost its significant cytotoxic activity compared to unbleached venoms; where at 40 µg/ml concentration after 72 hours' incubation the cytotoxic activity was significantly decreased from 0.1% cell viability to 75% cell viability for SD and B respectively. (Figure 4.C)

In order to compare between the unbleached and bleached venom on bases of protein composition, both venoms were run on 12% SDS-PAGE. The stained gel showed differences between the fractionated bands in the tested venoms (Figure 6). The unbleached venom showed a strong band at around 28 kDa, while the bleached

one showed a much fainter band of a similarly-sized protein. This finding might suggest that the 28 kDa protein fraction might be the fraction responsible for the cytotoxic activity of the unbleached venom.

These findings might provoke a question; whether the cytotoxic activity of *E. quadricolor* venom is due to the anemone itself or the endosymbiotic Zooxanthellae.

Based on the previous findings; in the present study three assumptions could be made;

First, only one organism is responsible for the cytotoxic activity of *E. quadricolor* venom either the symbiotic zooxanthellae or the host anemone.

Second, both the anemone and zooxanthellae contain cytotoxic compounds and these compounds act in synergy producing the cytotoxic activity of the venom.

Third, the cytotoxic activity is due to the host anemone itself, but the presence of the endosymbiotic zooxanthellae is essential for the production of the cytotoxic peptide.

4.4 *E. quadricolor* venom is more potent than Cisplatin on SNU-449

HCC cell lines especially SNU-449 had shown multiple drug resistance against different chemotherapeutic agents. (Boulin et al., 2011 and Park et al., 1994). Cisplatin is one of the drugs that were tested on SNU-449 cells, where the IC_{50} was calculated based on cytotoxicity assay ≈166 µg/ml after 30 minutes incubation period. (Boulin et al., 2011)

In this study, Cisplatin was used as a positive control. Based on the MTT assay done at 6 concentrations of Cisplatin the maximum decrease in cell viability was observed at 80µg/ml concentration where the percentage cell viability decreased to 6.4 %. On the other hand, the cell viability of SNU-449 cells was decreased to 0.1% at 40µg/ml concentration of SD venom. Moreover, when comparing the cytotoxic activity in terms of the IC_{50} calculated based on the MTT assay, Cisplatin IC_{50} ≈ 66 µg/ml. While the IC_{50} of SD venom ≈ 20 µg/ml after 72 hours incubation period. Based on the calculated IC_{50}, SD venom showed to be 3-folds more potent than Cisplatin.

This finding suggests that *E. quadricolor* could be a potential chemotherapeutic agent in treating grade II/III hepatocellular carcinoma either as monotherapy or in combination with other classical anticancer agents. This result is consistent with a previous study that proved the cytotoxic effect of sea anemone

Sagartia rosea venom on HCC. SrcI, which is an acidic actinoporin of size 19.6 kDa was isolated from *Sagartia rosea*. SrcI was found to have cytotoxic activity against multiple cancer cell lines including HCC cells (BEL-7402) with $IC_{50}\approx$ 3.6 µg/ml (Table.1) (Jiang et al., 2003). Furthermore, toxins extracted from marine organisms other than sea anemones had shown to have cytotoxic activity against different HCC cells. For example, the venom extracted from jellyfish *Cyanea nozakii* (Phylum: *Cnidaria*) was reported to have cytotoxic activity against 2 human hepatoma cells; Bel-7402 and SMMC-7721 with $IC_{50} \approx$ 17.9 and 24.3 µg/ml, respectively. (Cuiping et al., 2012)

4.5 Seasonal effects on *E. quadricolor* cytotoxic activity

For decades, scientists had questioned the effect of seasonal variation on the pharmacological activity and biochemical composition of marine organisms. For example, a study was conducted on the toxic sponge *Crambe crambe* (Phylum: *Porifera*), which was reported to have antiviral activity and cytotoxic activity against L1210 (murine leukemia cells) (Jares-Erijman et al.,1991). It was reported that the toxin production was changed according to the season, where the maximum toxic activity was observed in summer and autumn, whereas the minimum activity was observed in winter and spring seasons. (Turon et al. 1996).

Another study was done on multiple species of British marine algae that were proved to have antimicrobial activity. Some of the tested species showed no variation of the antibiotic activity through different seasons, while others showed seasonal pattern related antibiotic activity. (Hornsey and Hide, 1976)

In the present study, the effect of seasonal variation on the cytotoxic activity of a sea anemone was addressed for the first time. The seasonal effect was addressed in terms of the change in water temperature, where the winter anemone was kept at 20-22^{o}C while the summer anemone was kept at 22-25 ^{o}C (Table 2). Unfortunately, the adjustment of other changes related to nutrient levels such as nitrogen and phosphorous to mimic their level in the Red sea during different seasons was not applicable in this study (Fahmy, 2003).

The cytotoxic activity of winter and summer *E. quadricolor* venoms was tested (WD and SD venom respectively). Both venoms showed significant reduction of cell viability at 40 µg/ml concentration. Nevertheless, the SD venom showed to

have a time-depended cytotoxic activity where the cell viability was decreased to 0.1% after 72 hours. On the other hand, the cytotoxic activity of WD venom showed no significant time –dependence. Interestingly, in comparing the cell viability of both venoms no significant differences were observed except after 72 hours (Figure 4.A, B, and C). In comparing both venoms on the term of IC_{50}; SD venom showed to have lower $IC_{50} \approx 20$ µg/ml, while the IC_{50} of WD venom ≈ 28.5 µg/ml.

Based on this finding, we cannot claim that seasonal differences have a major effect on the cytotoxic activity of *E. quadricolor*. However, we might assume that its cytotoxic activity reaches its maximum in the summer season.

4.6 *E. quadricolor* has a necrotic effect on SNU-449

The type of cell death either apoptotic or necrotic was investigated on different cytotoxic anemone venoms. Some of these venoms had shown to have apoptotic mediated cell death. For example, RTX-A which is an actinoporin isolated from the sea anemone *Heteractis crispa* was proven to have cytotoxic activity against different cancer cells (Table.1). Based on Annexin-V/PI double staining assay, RTX-A showed to induce apoptosis to tested cells. (Fedorov et al., 2010). While other venoms had shown to have a necrotic effect on tested cells such as the crude venom extracted from the sea anemone *Aiptasia mutabilis* which had a necrotic mediated cell death on human epithelial cells (HEp-2). (Marino et al., 2004).

In the present study, based on the conducted Annexin-V /PI assay (Figure.7 B and C) the venom of *E. quadricolor* was shown to have a necrotic-mediated cytotoxic effect on SNU-449 cells. Moreover, this finding might suggest that the active protein is probably a membrane-damaging peptide.

Chapter 5: Conclusions:

In the sight of the reported findings in this study, the following was concluded;

- The venom of *E. quadricolor* was found to have cytotoxic activity against grade II/III HCC cell line with IC_{50} = 20µg/ml.
- On characterizing this cytotoxic activity in terms of the optimum storage condition, it was found that this cytotoxic activity was completely neutralized when storing the venom in light conditions. This suggests that the active protein might be light sensitive.
- On addressing the seasonal effect on the cytotoxic activity of *E. quadricolor*, both winter and summer venoms showed significant cytotoxic activity.
- The effect of bleaching on the cytotoxic activity of the sea anemone was addressed for the first time. It was reported that the venom taken from bleached *E. quadricolor* had lost its cytotoxic activity.
- Moreover, the study reported that *E. quadricolor* cytotoxicity on SNU-449 cells is most probably mediated by a necrotic pathway.

Future perspectives;

Despite of the new findings reported in the present study regarding the characterization of *E. quadricolor* cytotoxic activity on HCC cell line. Further studies are recommended to understand more about the cytotoxic activity of *E. quadricolor* on the pharmacological and biochemical level.

Hereby, I highly recommend;

- The expansion of the spectrum of the tested cancer cell lines to include more HCC cell lines other than SNU449 in order to get more understanding regarding the mechanism of action of *E. quadricolor* venom on HCC.
- The isolation of the symbiotic zooxanthellae and testing its cytotoxic activity alone and in combination with *E. quadricolor* venom in order to understand the relationship between both venoms.
- The purification of the venom and the fractionation of the protein and testing the cytotoxic activity of each fraction in order to determine the active protein fraction.
- The determination of the effect of *E. quadricolor* venom in combination with other cancer therapies.
- The assessment of the physiological status of the unbleached versus the bleached anemone using Fluorescence spectrum.

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