EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

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CYTOTOXICITY AND ANTI-CANCER EFFECT OF MANGROVE CRAB (*SCYLLA SERRATA*) SOUP ON HUMAN LEUKEMIC JURKAT CELLS

Maizan Mohamed[1]*, Nani Izreen Mohd Sani[1], Aziana Ismail[2] and Noor Fatmawati Mokhtar[2]

[1]Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, City Campus, Pengkalan Chepa, 16100 Kota Bharu, Kelantan, Malaysia.
[2]Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia.

*Corresponding Author: Maizan Mohamed

Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, City Campus, Pengkalan Chepa, 16100 Kota Bharu, Kelantan, Malaysia.

ABSTRACT

Cancer still remains an aggressive killer worldwide. There are various side effects caused by current cancer therapies especially in infants and pregnant women. Crab is rich in chitin, chitosan and selenium that have been shown to have an anti-cancer activity. This study was aimed to investigate *in vitro* anti-proliferative and anticancer effect of mangrove crab soup on Jurkat leukemic T-cell line. In this study, mangrove crab soup extract was assayed *in-vitro* for their anticancer properties with standard MTT colorimetric procedure and expression of leukemia marker gene in human leukemic jurkat T-cells using semi-quantitative RT-PCR on B2M gene. The results obtained from MTT assay showed that cytotoxicity effect was reduced significantly after 72 hours of exposure at the dose of more than 25% (v/v) and the reduction was in a dose and time-dependent manner with an inhibitory concentration of 50% cell population (IC_{50}) at 35% (v/v). Gene expression study on B2M marker gene for myeloma cancer showed the significant reduction in the level of expression which is similar to the cells treated with cisplatin, the drug used in cancer treatment. The present results revealed that the mangrove crab soup could be a potential alternative agent for leukemia therapy.

KEYWORDS: Cytotoxicity, Anti-cancer, Mangrove crab soup, Human leukemic cancer cells.

INTRODUCTION

It is estimated that there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer in 2012 worldwide. By year 2030, it is projected that there will be 26 million new cancer cases and 17 million cancer deaths per year (Thun *et al*., 2009). The incidence of cancer in Malaysia has increased from 32,000 new cases in 2008 to 37,400 in 2012 and is expected to increase to 56,932 by 2025 with leukemia contributes about 2.7% of all cancer cases worldwide especially in young children http://www.thestar.com.my/News/Community/2014/02/18. Metastasis remains as the main cause of death in most of the cancers (Chaffer & Weinberg 2011).

Despite considerable efforts, cancer still remains an aggressive killer worldwide (Rani *et al.,* 2012). Current cancer treatments are by surgery, chemotherapy and physical therapy such as radiation (Minisini *et al*., 2004, Basta *et al.,* 2015, Vassilakopoulou *et al*., 2016). Current novel synthetic chemotherapeutic agents have not succeeded in fulfilling expectations despite the considerable cost of their development and the emergence of resistance to cancer chemotherapy. These agents also gave lot of side-effects to the patients and cannot be applied on the pregnant women (Basta *et al.,* 2015). Therefore, there is a constant demand to develop new, effective and affordable anticancer drugs. Currently, natural products such as plants and marine products have received increasing attention for their potential as novel cancer preventive and therapeutic agents (Ram & Kumari, 2001, Kinghorn *et al.*, 2009).

Crabs are rich in polysaccharides and those derived polysaccharides possess a wide range of pharmacological activities (Simmons *et al*., 2005, Azuma *et al.,* 2015). It is rich in chitin, chitosan, selenium and natural carotenoids that have been shown to have an anti-cancer activity (Maeda and Kimura, 2004, Wattenberg *et al*., 2010, Al-Shammari *et al*., 2012, Leila *et al*., 2014, Wimardhani *et al*., 2014, Rethna Priya & Ravichandran, 2015). Our previous study on the effect of crab soup on dengue virus, platelet and U937 human monocyte cancer cells has showed 45S pre-ribosomal gene of U937 cancer cells was inhibited in treated cells but not in untreated and normal cells (Maizan *et al*., 2016). Therefore in this study, we are going to investigate on the potential of this crab soup as an anti-proliferative and anti-leukemia agent that could be used as a future treatment for leukemia cancer.

MATERIALS AND METHODS
Preparation of Mangrove Crab Soup Extract
The mangrove crab was obtained from Kg. China market in Kota Bharu, Kelantan, Malaysia. For the cytotoxicity, a stock solution was prepared by boiling 500 g crab with one liter distilled water for 10 min. The solution was centrifuged at 1500g for 15 min and filter-sterilized by using 0.22 μm syringe filter and stored at 4^0C.

Preparation of cells
Jurkat leukemic T-cell line and PCS-800-011 normal PBMC were obtained from American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). Number of cell was determined using haemocytometer and the cells were seeded at a density of 2×10^5/ml cells. The cells were incubated at 37^oC in a humidified 5% CO_2 atmosphere for 24-48 h until 70% confluent for further use.

Anti-proliferative (MTT) assay
To evaluate the cytotoxicity of the crab soup on the Jurkat leukemic T-cell line and normal PBMC cells, MTT assay (Sigma, St. Louis, MO) was used. The cells were seeded onto 96-well microplate at a density of 2×10^4cells/well and treated with neat and 2-fold dilutions of crab soup diluted with RPMI-1640 medium for 24, 48 and 72 hours. MTT solution (1mg/ml) was added and incubated for 2 h and MTT lysis buffer (20% SDS and 50% dimethylformamide) was then added and incubated overnight. Optical density (OD) was measured using a microplate reader (Molecular Devices Co., USA) at 570 nm. Each treatment was performed in triplicates of 3 wells. To calculate the viability of the cancer cells, the percentage of viable cells in the crab soup-treated group versus untreated control was used as the following equation.

The cell viability is calculated as follow:

$$Cells\ viability\ (\%) = \frac{(Average\ absorbance\ value\ of\ treated\ cells - baseline\ value)}{(Average\ absorbance\ value\ of\ untreated\ cells - baseline\ value)} \times 100$$

IC_{50} (Inhibitory concentration at 50% of cell population) value was determined from a graph of percentage of viable cells versus Log2 concentration (mg/ml) of extract.

Cells treatment
Jurkat cells at the density of 1×10^5 cells/ml in RPMI 1640 containing 5% fetal bovine serum were cultured in each well of a 24-well culture dish and incubated in CO_2 incubator for 72 hours. The supernatants were then removed and 0.5 ml of serum-free RPMI 1640 medium containing 25% (v/v) crab soup extract was added to each well. After 72 hours of incubation, the supernatant of each well was removed and further used in gene expression study.

RNA extraction and RT-PCR to determine the expression of *β*-actin and *β*-2 microglobulin (B2M)
Total RNAs were isolated from the supernatant of Jurkat cells treated with crab soup, cysplatin and untreated cells using Trizol reagent (Life Technologies. Inc.). In this study, B2M gene was used as a DNA marker for leukemia as recommended by Massimo *et al.,* 2009. The mRNA expression of the B2M gene was determined using semi quantitative RT-PCR. The primers used in this study were B2M F; 5' CCG TGG CCT TAG CTG TGC TC-3' and B2MR; 5' AGA CAA GTC TGA ATG CTC CAC-3' which were designed based on B2M gene. B2M F; 5' CCG TGG CCT TAG CTG TGC TC-3' and B2MR; 5' AGA CAA GTC TGA ATG CTC CAC-3'. The result was compared with untreated cells as a negative control and cells treated with cysplatin as a positive control. Efficiency of RNA extraction and RT-PCR were checked by RT-PCR amplification on *β*-actin gene using primers β-Actin F; 5'-ATT GCC GAC AGG ATG CAG AAG-3' and R; 5' TAG AAG CAT TTG CGG TGG ACG-3' (Mukhopadhyay et al., 2010). RT-PCR was conducted using AccessQuick one step RT-PCR (Promega, USA) as described in the manufacturer's protocol and 2.5 μL of extracted RNA was added in the amplification reaction.

Statistical analyses
All tests were carried out independently in triplicate (n = 3). Data are expressed as the mean ± standard derivation (SD). The results were processed using Excel 2003 (Microsoft, Redmond, WA, USA).

RESULTS
Cytotoxicity study
In comparison with control untreated cells, crab soup treated cells have minor cytotoxicity effect after 24 and 48 hours post-treatment but the effect was significantly reduced at the concentration of more than 25% (v/v) after 72 hours post-treatment (Fig. 1). The Inhibitory Dose 50 (IC_{50}) of the crab soup was at the concentration of 35% (v/v). No cytotoxicity effect was observed on treated and untreated normal cells (Data not shown).
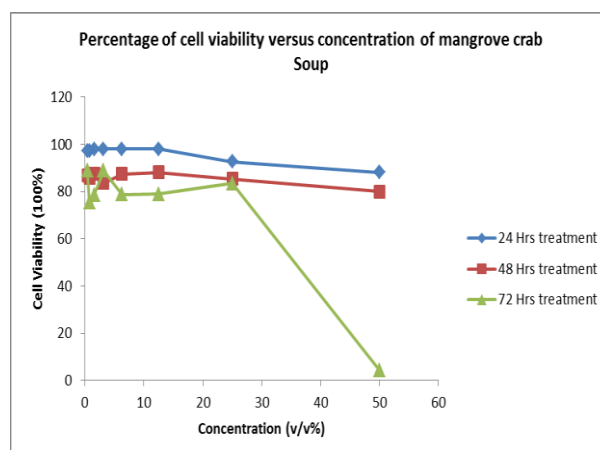

Fig. 1: The percentage of cell viability of Jurkat cell line after 24, 48 and 72 hours post-treatment with

mangrove crab soup. The data are shown as means ± S.D. performed in triplicates with the p value of <0.05

RT-PCR to determine the expression of *β*-actin and *β*-2 microglobulin (B2M)

To determine the RNA extraction efficiency, RT-PCR was conducted on the *β*-actin gene of the untreated, crab soup and cisplatin-treated Jurkat cells. This gene presents in all treated and untreated cells with the same intensity (Fig. 2A), suggesting that all cells were extracted in the same manner for the B2M expression study.

To study the effect of crab soup treatment on human leukemic Jurkat T-cells, the expression of B2M leukemia marker gene was determined using semi-quantitative RT-PCR. The intensity of the PCR band for the Jurkat cells treated with crab soup was significantly reduced compared to the untreated cells and cisplatin-treated cells (Fig. 2B). This result suggested that crab soup has inhibited mRNA expression of B2M gene that is important in the growth on leukemic Jurkat cells.
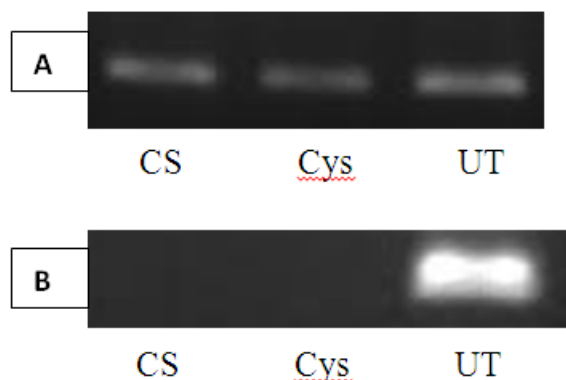

Fig. 2: RT-PCR results of Jurkat cells treated with crab soup (CS) and Cysplatin(Cys) and without treatment (UT) for 72 h using β-actin (A) and B2M (B) primers.

DISCUSSION

The effect of mangrove crab soup on Jurkat leukemic T-cell line was evaluated in this study using MTT cytotoxicity assay and expression of leukemia B2M gene by semi-quantitative RT-PCR. We used Jurkat leukemic T-cell line because this cell line was recommended for studying acute T cells leukemia, T cells signalling and receptors susceptibility of HIV entry (Schneider *et al.,* 1997 and Takeuchi *et al.,*2008). B2M gene was used as it was identified as one of the gene marker to study leukemia (Massimo *et al*., 2009). Cytotoxicity assay of this study have showed no significant cytotoxic effect observed after 24 and 48 hours post-treatment, but the effect started to increase after 72 h post-treatment at the dose of more than 25% (v/v), suggesting that the effects is dose and time-dependent. The result was in agreement with the study conducted by Leila *et al*., (2014), where the extract of crab shell has a significant cytotoxicity effect on breast cancer cell line (MCF7) after 72 hours post-treatment. The IC_{50} of the crab soup was determined

at 35% (v/v) at 72 hours post-treatment, in which at this concentration, 50% of the cancer cell growth was inhibited. This IC_{50} value is important in estimating the minimum non toxic dose (MNTD) of compound on the cells before using in the *in vitro* and *in vivo* treatment.

Expression study on cancer gene based on semi-quantitative RT-PCR has showed a significant inhibition of B2M mRNA expression at 72 hours post-treatment of crab soup which was similar to the result of cells treated with chemotherapy drug, cysplatin, suggesting the potential use of this crab soup as an alternative anticancer agent.

CONCLUSION

Our study demonstrated that mangrove crab soup has growth inhibitory effect on Jurkat leukemic T lymphocyte cells and the effect was in the dose- and time- dependent manner and can be further exploited as a potential anticancer target for leukemia cancer. However, further study on cell apoptosis, use of different form of the extracts and the isolation of the compounds involve in the inhibition of cancer cell growth need to be determined.

ACKNOWLEDGEMENTS

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