RESEARCH ARTICLE

Editorial Process: Submission:12/17/2017 Acceptance:07/12/2018

Selective Cytotoxicity of α-Santonin from the Persian Gulf Sponge *Dysidea Avara* on Pediatric ALL B-lymphocytes via Mitochondrial Targeting

Marjan Aghvami[1], Arghavan Keshavarz[1], Melika Nazemi[2], Mohammad Hadi Zarei[1], Jalal Pourahmad[1]*

Abstract

Background: Acute lymphoblastic leukemia (ALL) is one of the most dominant malignancies among children, characterized by production of immature and dysfunctional blasts which are resistant to cytotoxic chemotherapeutic agents. Therefore, research protocols are currently focusing on discovery of novel anti-cancer agents to enhance survival rates and decrease unwanted side effects. Approximately two-thirds of the planet is covered by oceans with a massive range of marine organisms of interest to scientists in pharmaceutical fields. **Methods:** Among marine resources, sponges are known to have beneficial effects in the treatment of numerous malignancies. One fraction of crude extracts containing α-Santonin was made from the Persian Gulf marine sponge, *Dysidea avara*, and investigated for anticancer effects. **Results:** Treatment of ALL B-lymphocytes with the *Dysidea avara* extract caused augmentation in ROS generation, decline in mitochondrial membrane potential, mitochondrial swelling, release of cytochrome c from mitochondria and activation of caspase-3 only in mitochondria isolated from B-ALL lymphocytes. **Conclusion:** In brief, our results suggest that *Dysidea avara* extracts may selectively induce apoptosis in malignant pediatric lymphocytes.

Keywords: B-ALL lymphocyte- *Dysidea avara*- mitochondria- apoptosis, Persian Gulf

Asian Pac J Cancer Prev, **19 (8)**, 2149-2154

Introduction

Cancer is one of the main causes of death around the worldand according to WHO estimation in 2020 almost 10.3 million people would die because of this incurable malignancy.

Pediatric Acute lymphoblastic leukemia (ALL) is one of the life-threatening malignancieswhich affects children with a high incidence below the age 6 years old (Schwab and Harrison, 2011; Roganovic, 2013). The complete eradication of the injured and dysfunctional lymphoid cells without alteration in normal precursors is desirable and the main objective of each therapeutic protocol. Chemotherapy is the first line treatment strategy, however, the standardized chemotherapy regimen have a serious or permanent side effects. Due to the undesired toxic effects of chemical compounds,many new naturally occurring compounds are introduced as a replacement (Pui et al., 2004; Schwab and Harrison, 2011).

Marine organisms are an abundant source of bioactive molecules, and the Persian Gulf is the rich environment which contains a huge number of various species.Among marine resources, more than 15,000 species of marine sponges are known worldwide (Thomas et al., 2010). and they considered as a beneficial natural sourcein pharmaceutical studies for many years (Simmons et al., 2005). A lot of studies on marinesponges indicated that sponge *Dysidea avara*, have cytotoxic effects on cancer cell lines; thus it is suggested that marine drugs can potentially be used as a beneficialmedicine in cancer therapy.These studies have just concern about clinical consequences. However, in this study. Fractions containing α-Santonin was investigated for cytotoxic activity in human B-ALL lymphocytes comparing with healthy normal cells. Here in, we emphasis on the mechanism of *Dysidea avara* fractions containing α-Santonin to onset the intrinsic pathway of apoptosis.

By consideration of mitochondrial parameters such as reactive oxygen species (ROS) generation, mitochondrial membrane potential (MMP) decline, mitochondrial swelling, release of cytochrome c, and caspase-3 activity, we demonstrated that Dysideaa vara. Fractions containing α-Santonin selectively activate apoptosis through mitochondrial pathways in human leukemic cells.

*[1]Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, [2]Persian Gulf and Oman Sea Ecological Center, Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Bandar Abbas, Iran. *For Correspondence: j.pourahmadjaktaji@utoronto.ca*

Materials and Methods

Chemicals

Trypan blue, 2′,7′-dichlorofuorescin diacetate (DCFH-DA), Rhodamine123, bovine serum albumin (BSA), N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma-Aldrich Co., RPMI1640 and FBS (Fetal Bovine serum) were purchased from Gibco, Life Technologies, Grand Island, NY. Ficoll-paque PLUS was obtained from Ge Healthcare Bio-Science Company. Marine sponge extract and fractions containing α-Santonin were prepared in Persian Gulf and Oman Sea ecological research organization, Bandar Abbas, Iran.

Sampling and identification

Dysidea avara was collected by scuba diving in June 2016; from reef those habitats at depths of 20-25 m in Hengam Island on the Persian Gulf. After identification of species the samples were lyophilized and dried samples used for extraction.

Extraction

The sponge dried sample powder extracted with acetone solvent. After 72 hours of soaking in acetone the solvent filtered and acetone evaporated to dryness, at low pressure at 35- 40°C by using Rota vapor (Çitoğlu and Acıkara, 2012).

Isolation and Identification of α-Santonin compound

Silica gel column chromatography with 70 cm height and 2 cm diameter used for purification of sponge acetone extract (6.2 gram). The packed column washed by different combination (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100) of hexane: ethyl acetate solvent and all fraction collected in 10 ml tube (147 fractions) (Çitoğlu and Acıkara, 2012). Obtained fractions from the column chromatography were performed by thin layer chromatography with a mobile phase including methanol-chloroform-ethanol solvents with ratios of 70:20:10. To identify the compound belong to triterpenoid, vanillin-sulphuric acid reagent was used as a 1% solution of vanillin in ethanol and 5% sulphuric acid solution in ethanol by spraying on a thin-layer chromatography plate. After spraying, thin-layer chromatography plates were placed in the oven at 110 °C for 10 minutes; changes in visible light were observed; the portions of the teriterpenoids were changed to pale purple-full purple colour (Attaway et al., 1965). The spots with purple colour were injected into a gas chromatography (Agilent7000 Series Triple Quad GC / MS Main Frame) to identify the α-Santonin compound.

Selection of healthy donors and patients with ALL

Five ALL patients, and 5 healthy donors (aged 2-9 years) were enrolled in this study. ALL was diagnosed and confirmed according to the definition of the World Health Organization (WHO) classification by oncologist. Only patients with no previous treatments within the last six months were included in this study. All 5 patients have sampled prior any medication. This study was approved by the Shahid Beheshti University of Medical Science's ethics committee, and all the patients signed an informed consent form.

Isolation of Mitochondria

Mitochondria were isolated from the ALL B-lymphocytes by mechanical lysis and multiple centrifugations. Briefly, B-lymphocytes were washed with cold PBS and centrifuged at 450× g. The pellet was re-suspended in cold isolation buffer. Non- lysed cells and nuclei were spun down by centrifugation at 1,000 ×g for 10 minutes. The supernatant was further centrifuged at 20,000 × g for 25 minutes. The pellet, designated as the mitochondrial fraction, was suspended in assay buffer. The accuracyof mitochondrial isolation was determined by succinate dehydrogenase measurement (Rotem et al., 2005).

Succinate Dehydrogenases Activity Assay

The activity of succinate dehydrogenases was examined by estimation of MTT reduction (Berridge and Tan, 1993). The colorimetric assay was performed by ELISA reader (Tecan, Rainbow hermo, Austria).

In this case we used an internationally accepted protocol using at least three different concentrations as $IC_{50}/2$ (sub-toxic concentration), IC_{50} (toxicity threshold concentration) and $2\times IC_{50}$ (very toxic concentration). In our research the IC_{50} determined for sponge fractions containing α-Santonin on isolated B-ALL lymphocyte mitochondria was approximately 300µg/ml and therefore we used 150µg/ml as sub-toxic and 600µg/ml as very toxic concentrations. To determine the IC_{50} of sponge fractions containing α-Santonin which defined as a concentration which decreased the activity of mitochondrial succinate dehydrogenase down to 50% and considered as toxicity threshold in accelerated cytotoxicity mechanisms screening (ACMS) technique, we applied 7 different concentrations obtained from literature review (shown in Figure 3) on isolated B-ALL lymphocyte mitochondria using MTT assay. In order to determine IC_{50} value for our investigated compound, dose–response curves were plotted and the IC_{50} determination was based on a regression plot of 7 different concentrations (data and curves not shown).

Determination of ROS formation

The mitochondrial ROS formation was performed using the fluorescent dye DCFH-DA. Briefly, isolated mitochondria were placed in respiration buffer, and then added different concentrations of sponge fractions containing α-Santonin at 37 °C for 60 minutes. Finally, the fluorescence intensity was measured by fluorescence spectrophotometer at an excitation and emission wavelength 488 nm and 527 nm respectively (Rezaei et al., 2014).

Determination of Mitochondrial Membrane Potential (MMP)

MMP assayed by a fluorescent dye, rhodamine 123. The mitochondrial fractions were incubated with 10 µM of Rhodamine 123 in MMP assay buffer and then various concentrations of sponge fractions containing α-Santonin at 37 °C were added to this suspension.

The fluorescence intensity was monitored using fluorescence spectrophotometer at the excitation and emission wavelength of 490 nm and 535 nm, respectively (Hosseini et al., 2013).

Determination of Mitochondrial Swelling

Mitochondria suspensions were incubated in in swelling buffer supplemented with 1 mg/mL rotenone and 10 mmol/L succinate at 37 °C.

After 10 minutes of pre-incubation, various concentrations of sponge fractions containing α-Santonin were added. Mitochondrial swelling was measured spectrophotometrically (at 540 nm) within 60 minutes (Rotem et al., 2005).

Determination of caspase-3 activity

For determination of caspase-3 activity in cell lysate of lymphocytes, the colorimetric assay was performed based on the Sakahira protocol (Sakahira et al., 1998) using Sigma's caspase-3 assay kit (CASP-3-C).

Determination of Cytochrome c Release

The concentration of cytochrome c was determined in the isolated mitochondria using the Quantikine Human Cytochrome c Immunoassay kit (Eskandari et al., 2012).

Statistical analysis

Results are presented as mean ± SD. Assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test when appropriate. Statistical significance was set at $P < 0.05$ and the parameters of mitochondrial dysfunction were analyzed by two way ANOVA and Bonferroni post-hoc test. In all graphs were expressed as mean ± SD and $P < 0.001$ was considered statistically significant.

Results

Isolation of fractions containing triterpenoid

Thin layer chromatography used to separate the column chromatography fractions (147 fractions) by vanillin reagent based on colure change of terpenoid content from pink to purple (Figure 1).

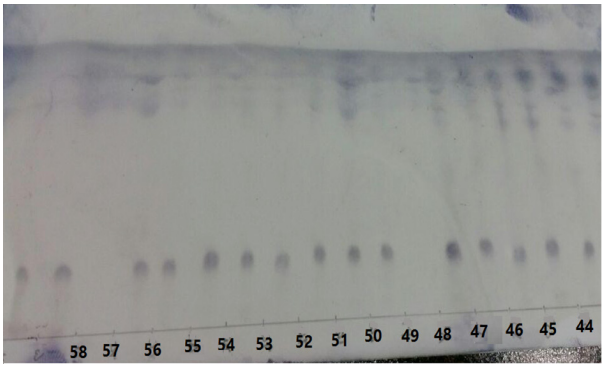

Figure 1. Thin Layer Chromatography of Acetone Fractions of Sponges

Identification of α-Santonin Compounds

The α-Santonin with the chemical formula $C_20H_{30}O_3$ (Figure 2), belonging to the teriterpenoid group identified with 92% purity in fraction 50-55.

Succinate Dehydrogenase Activity Assay

In this investigation, MTT assay was used to evaluate the impact of sponge fractions containing α-Santonin on mitochondrial succinate dehydrogenase. As shown in Figure 3 this extract can effectively inhibit succinate dehydrogenase activity in isolated ALL mitochondria without any significant effect on normal mitochondria.

ROS formation assay

As a consequence of the fact that Radical Oxygen Species (ROS) formation is a critical point in apoptosis initiation, we tested if sponge fractions containing α-Santonin could change these species level in ALL and healthy mitochondria. As shown in Figure 4, treatment with sponge fractions containing α-Santonin at 150, 300, and 600µg/ml (1/2 IC_{50}, IC_{50}, 2 IC_{50}) for 60 minutes, significantly increased ROS generation ($p < 0.0001$). In contrast, any significant effects on healthy mitochondria were not seen after treatment with IC_{50} (300 µg/ml) of sponge fractions containing α-Santonin.

Mitochondrial Membrane Potential (MMP) assay

We evaluated the impact of sponge fractions containing α-Santonin on mitochondrial membrane potential on both healthy and ALL samples. Treatment with different concentrations (150, 300, and 600µg /ml for

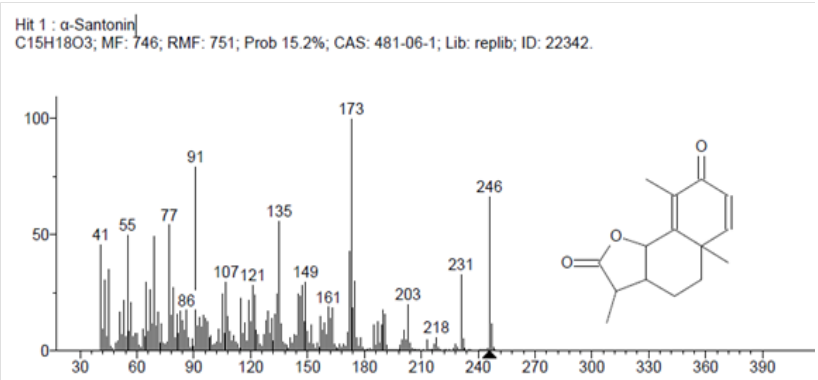

Figure 2. Gas Chromatography Chromatogram of Fraction no. 50 of Column Chromatography Containing α-Santonin Compound

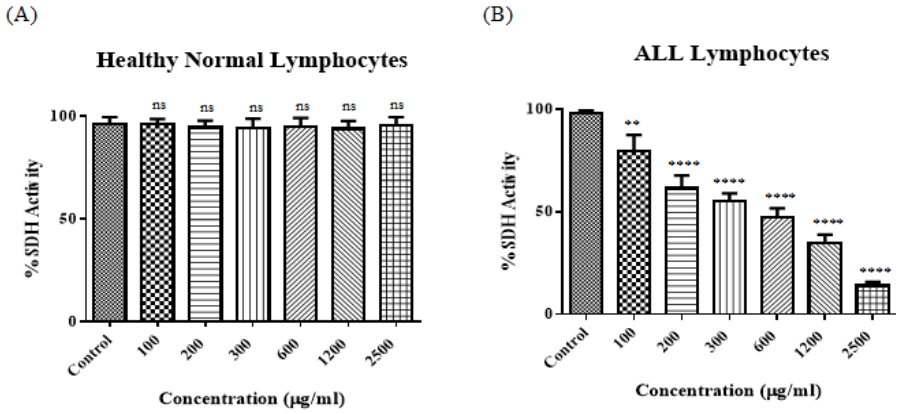

Figure 3. The Effect of Sponge Fractions Containing α-Santonin on Succinate Dehydrogenase Activity of Healthy (A) and B-ALL (B) Mitochondria Obtained from Lymphocytes. The effect of sponge fractions containing α-Santonin) on succinate dehydrogenase activity were evaluated by MTT assay following 1 h of treatment. Values (mean ± S.D.) are from three independent experiments (n = 3). **p< 0.01, **** p < 0.0001.

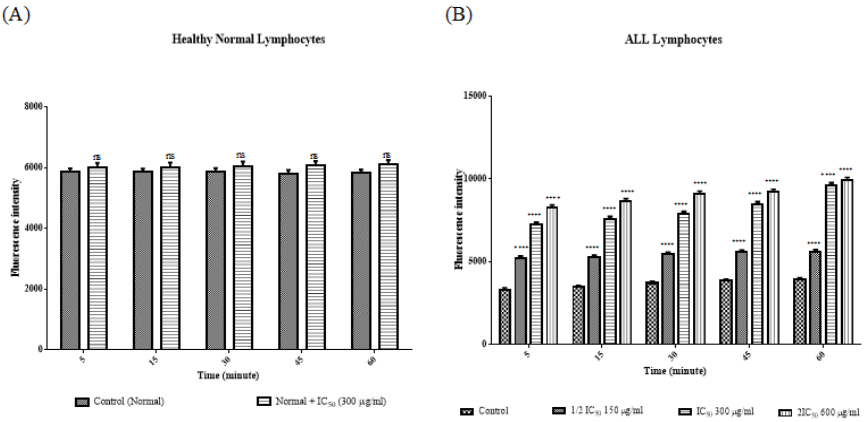

Figure 4. The Effect of Sponge Fractions Containing α-Santonin on ROS Formation in Mitochondria Obtained from Healthy (A) and B-ALL (B) Lymphocytes. Isolated mitochondria were obtained from healthy (A) and ALL (B) donors incubated with of sponge fractions containing α-Santonin for 1 hour. Columns represent mean of DCF fluorescence intensity in each group treated with sponge fractions containing α-Santonin for 5–60 minutes. Values (mean ± S.D.) are from three independent experiments (n = 3). ****p< 0.0001.

60 minutes) significantly (p< 0.0001) reduced MMP in B-ALL mitochondria. Conversely, the IC_{50} concentration (300 µg/ml) did not cause anysignificant effects on mitochondrial potential in healthy samples (Figure 5).

Mitochondrial swelling assay

Mitochondrial swelling followed up by detecting the absorbance of mitochondria within 60 minutes at 540 nm. As demonstrated in Figure 6, following addition of different concentrations (150, 300, and 600 µg/ml) of

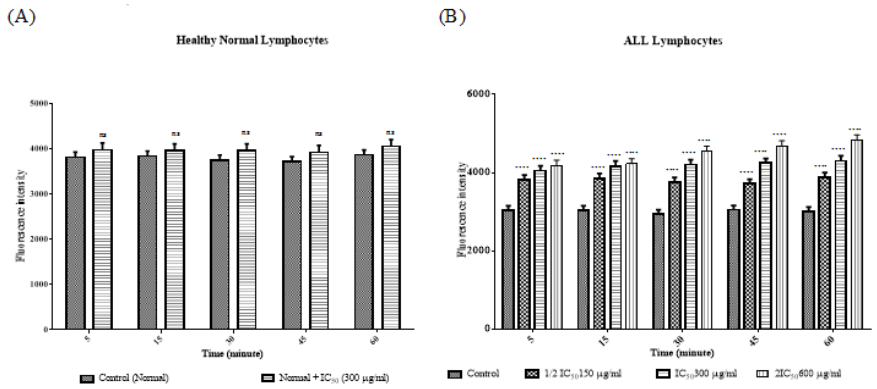

Figure 5. The Effect of Sponge Fractions Containing α-Santonin on MMP Decline in Mitochondria Obtained from Healthy (A) and B-ALL (B) Lymphocytes. Isolated mitochondria were obtained from healthy (A) and ALL (B) donors incubated with of sponge fractions containing α-Santonin for 1 hour. Columns represent mean of Rhodamine123 fluorescence intensity in each group treated with sponge fractions containing α-Santonin for 5–60 minutes. Values (mean ± S.D.) are from three independent experiments (n = 3). ****p< 0.0001.

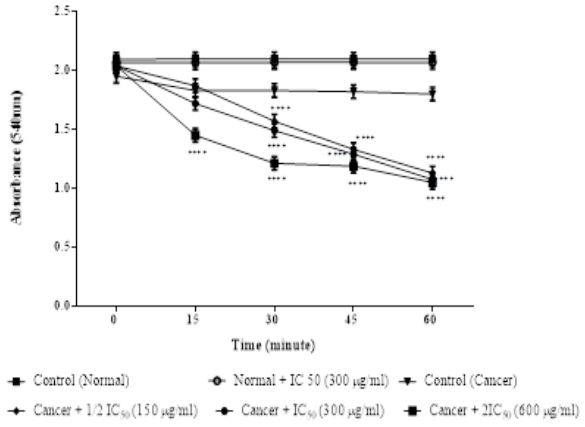

Figure 6. Effects of Sponge Fractions Containing α-Santonin on Mmitochondrial Swelling in Mitochondria Obtained from Healthy and B-ALL Lymphocytes. Addition of sponge fractions containing α-Santonin (150,300, and 600µg/ml) induces mitochondrial swelling in ALL B-lymphocyte mitochondria in a concentration depending manner. Mitochondria which obtained from healthy lymphocytes endure no changes when treated by IC_{50} of sponge fractions containing α-Santonin during 60 minutes. Values (mean ± S.D.) are from three independent experiments (n = 3). ****p< 0.0001.

sponge fractions containing α-Santonin, a great swelling in mitochondria obtained from B-ALL lymphocytes was observed. However, sponge fractions containing α-Santonin at IC_{50} concentration did not cause mitochondrial swelling in healthy samples.

Determination of Cytochrome c Release

As shown in Figure 7 sponge fractions containing α-Santonin at IC_{50} (300µg/ml) significantly ($p < 0.01$) caused release of cytochrome c into incubation buffer. Our results revealed that sponge fractions containing α-Santonin can cause mitochondrial permeability

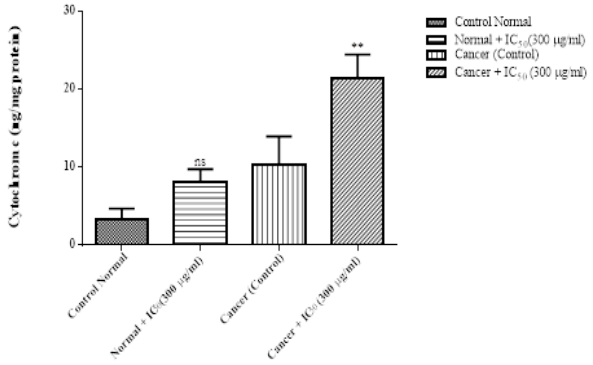

Figure 7. The Effect of Sponge Fractions Containing α-Santonin on Cytochrome c Release from Mitochondria which Obtained from Healthy and B-ALL Lymphocytes. Sponge fractions containing α-Santonin at IC_{50} (300µg/ml) cause cytochrome c release in the ALL mitochondria but not in healthy one. The amount of expelled cytochrome c from mitochondrial fraction into the suspension buffer was determined using human Cytochrome c ELISA kit as described in above. Values (mean ± S.D.) are from three independent experiments (n = 3). **p< 0.01.

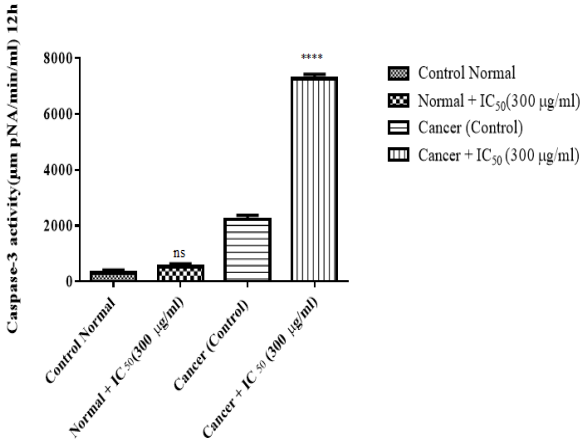

Figure 8. The Effect of Sponge Fractions Containing α-Santonin on Caspase-3 Activity in B-ALL and Healthy Lymphocytes. Caspase-3 activity was determined by Sigma-Aldrich kit. Columns represent caspse-3 activity (µM pNA/min/ml) in both healthy and B-ALL lymphocytes treated with IC_{50} (300µg/ml) of sponge fractions containing α-Santonin for 3 h. Values (mean ± S.D.) are from three independent experiments (n = 3). **** p <0.0001

transition (MPT) pore opening and cause cytochrome c expulsion from mitochondria.

Determination of Caspase-3 Activity

As shown in Figure 8, sponge fractions containing α-Santonin at IC_{50} (300 mg/ml) significantly ($p < 0.0001$) enhanced the activity of caspae-3, the critical apoptosis mediator, in B-ALL but not in healthy lymphocytes.

Discussion

Cancer is classified inthe category of complicated diseases with involvement of multiple factors such as genetic, lifestyle, and age (Schnekenburger et al., 2014). Traditional chemotherapeutic agents applied their anticancer effects by the direct cytotoxic mechanism. Nevertheless, thesecytotoxic effects are not always restrictedto malignant cells and are usuallyaccompanied by several side-effects. Therefore, the search for more selective anticancer compounds is the main goal of medicine (Rady, 2014). Literature documents reveal that somenaturally occurring molecules which isolated from marine sponges have exhibited antineoplastic properties (Müller et al., 1985; Sawadogo et al., 2015).

Natural compounds which are recently in consideration as an alternative medicine for many types of cancers including leukemia have demonstrated low toxicities against normal cells (Zhang et al., 2001; Nobili et al., 2009). It has proven that marine organisms areamongst the most valuable resources for bioactive natural compounds, especiallyin the domain of novel anticancer discovery (Mayer and Gustafson, 2008; Gordaliza, 2010; Trianto et al., 2017). α-Santonin, is one of these natural compounds. Recent studies have been demonstrated antitumor properties of marine sponge extract on many types of cancers (Müller et al., 1985; Schwartsmann et al., 2001).

In this study, we investigated that marine sponge

extract can trigger apoptosis through mitochondrial pathway.

Our result showed that incubation of mitochondrial suspension with sponge fractions containing α-Santonin in IC_{50} concentration significantly increased ROS formation. These reactive species may promote MPT (mitochondrial permeability transition) pore by oxidation of thiol groups which induced ΔΨm collapse, mitochondrial swelling and finally release of the mitochondrial apoptogenic factor. Our findings also revealed that sponge fractions containing α-Santonin can induce mitochondrial swelling in a time and concentration-dependent manner only in ALL B-lymphocyte. We, therefore, concluded that sponge fractions containing α-Santonin has a considerable role in initiating apoptosis on mitochondria isolated from ALL B-lymphocytes without significant effect on normal healthy mitochondria.

In conclusion, the anticancer activity of sponge fractions containing α-Santonin could associate with its apoptosis induction of ALL cells through mitochondrial pathway. Based on our findings, sponge fractions containing α-Santonin may be an efficacious candidate as a selective naturally occurring chemotherapeutic agent against pediatric lymphoblastic ALL.

References

Attaway J, Barabas L, Wolford R (1965). Analysis of terpene hydrocarbons by thin layer chromatography. *Anal Chem*, **37**, 1289-90.

Berridge MV, Tan AS (1993). Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys*, **303**, 474-82.

Çitoğlu GS, Acıkara ÖB (2012). Column chromatography for terpenoids and flavonoids. In 'chromatography and its applications', Eds Dhanarasu S. InTech, Rijeka, pp13-49.

Eskandari MR, Fard JK, Hosseini MJ, et al (2012). Glutathione mediated reductive activation and mitochondrial dysfunction play key roles in lithium induced oxidative stress and cytotoxicity in liver. *Biometals*, **25**, 863-73.

Gordaliza M (2010). Cytotoxic terpene quinones from marine sponges. *Mar Drugs*, **8**, 2849-70.

Hosseini MJ, Shaki F, Ghazi-Khansari M, Pourahmad J (2013). Toxicity of Vanadium on isolated rat liver mitochondria: A new mechanistic approach. *Metallomics*, **5**, 152-66.

Mayer AM, Gustafson KR (2008). Marine pharmacology in 2005–2006: Antitumour and cytotoxic compounds. *Eur J Cancer*, **44**, 2357-87.

Müller WE, Maidhof A, Zahn RK, et al (1985). Potent antileukemic activity of the novel cytostatic agent avarone and its analogues in vitro and in vivo. *Cancer Res*, **45**, 4822-6.

Nobili S, Lippi D, Witort E, et al (2009). Natural compounds for cancer treatment and prevention. *Pharmacol Res*, **59**, 365-78.

Pui CH, Relling MV, Downing JR (2004). Acute lymphoblastic leukemia. *N Engl J Med*, **350**, 1535-48.

Rady H (2014). Sponge mesohyl induces anti-proliferation activity and cell cycle arrest in colon cancer in-vitro. *Res J Pharm Biol Chem Sci*, **5**, 1070-4.

Rezaei M, Salimi A, Taghidust M, et al (2014). A comparison of toxicity mechanisms of dust storm particles collected in the southwest of Iran on lung and skin using isolated mitochondria. T*oxicol Environ Chem*, **96**, 814-830.

Roganovic J (2013). Acute lymphoblastic leukemia in children. In 'Leukemia', Eds INTECH, pp 39-74.

Rotem R, Heyfets A, Fingrut O, et al (2005). novel anticancer agents acting directly and selectively on human cancer cell mitochondria. *Cancer Res*, **65**, 1984-93.

Sakahira H1, Enari M, Nagata S (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*, **391**, 96-9.

Sawadogo WR, Boly R, Cerella C, et al (2015). A survey of marine natural compounds and their derivatives with anti-cancer activity reported in 2012. *Molecules*, **20**, 7097-142.

Schnekenburger M, Dicato M, Diederich M (2014). Epigenetic modulators from "The Big Blue": A treasure to fight against cancer. *Cancer Lett*, **351**, 182-97.

Schwab C, Harrison CJ (2011). Acute lymphoblastic leukaemia. *Methods Mol Biol*, **730**, 99-117.

Schwartsmann G, da Rocha AB, Berlinck RG, et al (2001). Marine organisms as a source of new anticancer agents. *Lancet Oncol*, **2**, 221-5.

Simmons TL, Andrianasolo E, McPhail K, et al (2005). Marine natural products as anticancer drugs. *Mol Cancer Ther*, **4**, 333-42.

Thomas TRA, Kavlekar DP, LokaBharathi PA (2010). Marine drugs from sponge-microbe association-A review. *Mar Drugs*, **8**, 1417-68.

Trianto A, Ridhlo A, Triningsih D, et al (2017). Study on anticancer activity of extracts of sponges collected from biak water, indonesia. *IOP Conf Ser: Earth Environ Sci*, **55**, 012069.

Zhang-LP JJ, Tam JW, Zhang Y, et al (2001). Effects of matrine on proliferation and differentiation in K-562 cells. *Leuk Res*, **25**, 793-800.