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Research Article

IMMUNOGENICITY ANALYSIS OF TRITERPENE GLYCOSIDE FROM HOLOTHURIA ATRA TO DETECTING FAS AND BCL-2 PROTEIN ON THE SP-C1 CELL OF TONGUE CARCINOMA

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ABSTRACT

Objective: The objective of this study is to assess the role of triterpene glycoside of *Holothuria atra* to induce the Fas and Bcl-2-regulated apoptosis in *Supri's Clone 1* (Sp-C1) cell of tongue carcinoma.

Methods: The triterpene glycoside of *H. atra* was isolated by high-performance liquid chromatography. The Sp-C1 cell of tongue carcinoma was cloned by Dulbecco's Modified Eagle Medium and cytotoxicity assay by 3-4-5-dimethylthiazol-2yl 2,5-diphenyltetrazolium bromide assay. Expression Fas and Bcl-2 protein were analysed by immunocytochemistry also apoptosis detected by double staining ethidium bromide acridine. The datum of studied was analyzed by one-way analysis of variance (ANOVA), significance ($p<0.05$), and strength correlation ($p<0.001$) with R=1.

Results: The *H. atra* has triterpene glycoside, and in the dose of 4 mg/ml, it has been cytotoxic activities on the Sp-C1 ($p<0.05$), mortality 80%; inhibitory concentration 50 (IC_{50})=0.6 and anti-logarithm = 4. In general, the concentration of 2.5 mg/ml of triterpene glycoside has triggered the expression of Fas protein (active, 71%; moderate, 10%; and no-active, 27%), whereas the Bcl-2 protein (active, 59%; moderate, 14%; and no-active 27%). Statistically, both expressions of protein were significant ($p<0.05$). Triterpene glycoside caused the apoptosis of Sp-C1 cell (strong, 87%, and moderate, 13%).

Conclusion: The triterpene glycoside has the properties of cytotoxicity, and apoptosis in the SP-C1 cell also could be triggering the expression of Fas and Bcl-2 proteins.

Keywords: *Holothuria atra*, Cytotoxic-apoptosis, Bcl-2 and Fas proteins, *Supri's Clone 1* cell, Triterpene glycoside.

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INTRODUCTION

Ninety-five percent of head, neck, and mouth cancers are squamous cell carcinomas (SCC) [1]. The frequency of the oral cavity SCC reached the sixth of the 10 most advanced cancers around the world and tended to increase [2]. The tongue is an area of concern for the incidence of oral cavity occurrence. SCC of the tongue represents 25%–50% of the total number of oral cavity SCC [3].

SCC is treated with surgery, radiotherapy, and chemotherapy or in combination, but the 5-year survival rate is poor, about 50% [4], and according to Zhang *et al.* [5], even with 30% local and regional recurrence combination therapy, 25% metastasis, and 5% survival by 40%. Therefore, the target of developing anticancer drugs is directed to the induction of apoptosis [6], derived from natural materials, and one of them is a sea cucumber [7].

The tumor was called a disorder physiologically of cell growth in the body [8]. This is as a result of an apoptotic failure that caused by unsuccess checkpoint in G0 phase of cell cycle [9,10]. Theoretically, Bcl-2 and Fas proteins on *Supri's Clone* 1 (SP-C1) are being the target of chemotherapy of anti-tumor [11]. Bcl-2 engaged in intrinsic pathway, whereas the Fas protein in the extrinsic pathway [12].

The natural products have an important role in cancer therapy, and a substantial number of clinically-used chemicals are derived from plants or animal [13]. A number of active component of plant reported to adherence the cancer cell metastatic such as *Arctium lappa* L. [14]. Liu *et al.* [15] reported that the triterpene glycoside of monk fruit was inhibited cancer into the body. It has to suppress P53 protein and decreasing regulation of matrix metallopeptidases and phosphorylated extracellular signal-regulated kinases.

Seas cucumbers are marine invertebrates that produce the secondary metabolites which have unique structures and useful biological activities [16]. In Indonesia, there are many sea cucumbers, one of them is *Holothuria atra*, originating from the of Mentawai (West Sumatra) [17]. The isolation of sea cucumber is triterpene glycoside which is the main bioactive compound of sea cucumber, with the wide structure of biological activity such as antifungal, cytotoxic, hemolytic, immune-modulatory effect, and antitumor [18].

The research of triterpene glycoside *H. atra* as the anticancer was reported by Aminin *et al.* [19] as the anti-malignant tumor of animal model, such as the cytotoxicity assay and apoptotic assay. Therefore, the development of anticancer by inducing apoptosis is importantly a

targeted therapy. The study of triterpene glycoside *H. atra* to detect the Bcl-2 and Fas protein has been not reported this fac In consequence, this research was reposted the role of triterpene glycoside *H. atra* to induce the Bcl-2 and Fas protein-regulated apoptosis in Sp-C1 cell of tongue carcinoma.

METHODS

Materials

The research has approved with the ethical clearance No.730/TGL/KEPK/FK USU-RSUP HAM/2016 issued by Faculty of Medicine, University of Sumatera Utara, Medan, Indonesia. The eight concentrations of triterpene glycoside of *H. atra* assayed on the Sp-C1 cell related the immune-expression of Bcl-2 and Fas proteins, cytotoxicity assay, and apoptosis assay.

Purification of triterpene glycoside of *H. atra*

Isolation and purification of triterpene glycoside were used the column chromatography method with silica gel phase G60 and thin-layer chromatography with silica gel GF256 and Sephadex LH20 column method with methanol solvent to obtain one spot. Qualitatively of triterpene glycoside was analyzed by high-performance liquid chromatography (also used to triterpenoid assay with added 50–100 mg/ml of triterpene glycoside in 0.5 ml of glacial acetate acid and incubated 15 min in tubes, and then added strong sulfate acid 0.5 ml). Positive has triterpene glycoside emerged brownish-red and purple colors [20,21].

Cytotoxicity assay

Cytotoxicity assay of Sp-C1 cell of tongue carcinoma had been analyzed by 3-4-5-dimethylthiazol-2yl 2,5-diphenyltetrazolium bromide (MTT) assay [22]. Triterpene glycoside concentrations used to inhibitory concentration 50 (IC_{50}) as the standard referred of the research model. 100 µl triterpene glycoside in various concentration entered into the well-microplate 96 as well as Sp-C1 cancer cells. Microplates were incubated in CO_2 incubators 24 h (5% CO_2, 37°C, 98% moisture) and added each well plate 20 µl 5 mg/ml MTT solution, a solution in plate + 100 µg dimethyl sulfoxide to dissolve formazan crystals. Absorbance read by ELISA reader 520 nm. The frequency of the percentage of Sp-C1 mortality was adopted by Li *et al.* [23].

Immunocytochemistry assay

In this performance research methods, we were used the product of Abcam, Cambridge, MA, USA. The 80% confluent Sp-C1 cells did cell harvest and centrifuged at 1500 rpm 5 min, then the supernatant was removed, washed with phosphate-buffered saline (PBS), and fixed by methanol 5 min. Immunocytochemistry assay was adopted by Yuliani et al. [24]. The sample was blocked by hydrogen peroxidase during 15 min and washed with Phosftae buffer saline (PBS) 2 times, then added ultra V block and incubated 5 min at room temperature, washed with PBS. Identify Fas and Bcl-2 protein with adding the primary antibody (1:100), incubated 30 min, rinsed with PBS 4 times, futhermore added biotinylated goat anti polyvalent and incubated 10 min at room temperature, washed with PBS 4 times, then, added streptavidin-peroxidase also incubated 10 min at room temperature, rinsed with PBS respectively 4 times and added diaminobenzidine (DAB) plus chromogenic 10 µl + 500 ul DAB and substrate, incubated 15 min and washed with PBS. The last was colored by Mayer's Hematoxylin 10 min and flushed by aquadest and dry, and the result was observed under a light microscope ×40 [25].

Apoptosis assay

In this apoptosis assay, we used the product of Invitrogen Life Science Technologies, Foster City, CA, USA. Influence of triterpene glycoside of *H. atra* on the apoptosis activity of Sp-C1 was assayed by double acting ethidium bromide acridine orange method. The 80% confluent Sp-C1 cells harvested and centrifuged at 1500 rpm 5 min, then the supernatant was discarded. Pellet/precipitate added 1 ml complete medium and reset it after doing the calculation with the counting chamber. Perform starvation with a media concentration containing 0.5% fetal bovine serum (FBS). The cell is grown on 24 well-supplied slipover plates, as many as 500,000 per 500 µl at each well and 24-h incubated in the CO_2 incubator. The cell is grown on 24 well-supplied slipover plates, as many as 500,000 per 500 µl at each well and 24-h incubated in the CO2 incubator, then substituted 10% FBS medium and treated cells with various concentrations, incubated 24 h in the CO2 incubator and the next day disposes of media and washed with PBS 2 times, covered by ethidium bromide acridine orange, observed in fluorescein microscope 40X [26,27].

Statistical analyses

The cytotoxicity, immunoexpressions Bcl-2, Fas proteins, and apoptosis of the Sp-C1 cell of tongue carcinoma were analyzed by one-way analysis of variance (ANOVA) with a $p<0.05$ and a correlation value of $p<0.01$.

RESULTS AND DISCUSSION

Triterpenoid analyses of *H. atra*

The extracted total of H. atra tested the compound of triterpene by the triterpenoid assay. Furthermore uses to evaluating the cytotoxicity, apoptosis, and expression of Bcl-2 and Fas protein (Fig. 1) [28]. Triterpene has the natural bioactive plant, animal, and fungus. It has anticancer, inflammatory effect, antioxidative, antiviral, antibacterial, and anti-fungal and also has the cytotoxically and chemoprotective activities on the neoplasm therapy [29]. Bishayee *et al.* [30] reported that triterpene takes a role in the apoptotic response of chemoprevention and tumor mammary cases.

Cytotoxicity of triterpene glycoside

The IC_{50} be a standard to measure the inhibition effect of triterpene glycoside with various doses (mg/ml). The minimum concentration (4 µg/ml) has a strong effect on cytotoxicity to Sp-C1 cell with mortality scores of 80%, IC50=0.6, and anti-logarithm 4, as well as mortality, is significant ($p<0.05$). These data are referenced to prescribe a concentration of the cytotoxicity assay of triterpene glycoside against Sp-C1 cell of tongue carcinoma (Table 1). The American National Cancer Institute suggested that the plant extract has the potential cytotoxic effect if they have IC_{50}<20 µg/ml [31]. Molyneux [32] declared that IC_{50} is the antioxidant concentration related to obstruct of 50% free radical activity and be avowed active in cytotoxic if the mortality of cell is achieved 80–100% (active), 50–79% (moderate), and 49% down is non-active [4].

The evaluation triterpene glycoside doses 0.5–4 µg/ml were assayed with minimal cytotoxic elicited an expression of Bcl-2 and Fas proteins also apoptosis on the Sp-C1 cell. Based on the cytotoxic assay, the doses of 2.5 (µg/ml) is the best standard evaluated of cytotoxic with anti-logarithm (2.197) and antioxidant (81%). The scale of cytotoxicity are strong (0.049– 0.199), moderate (0.222–0.699) and non-active

Fig. 1: The triterpene glycoside of *Holothuria atra* (circle of color)

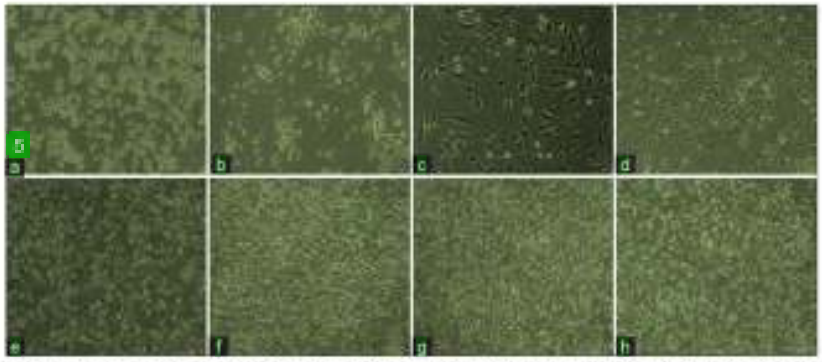

Fig. 2: Cytotoxicity of *Suprt's Clone 1* cells after 24 h treatment by triterpene glycoside of *Holothuria atra* in various doses (a) 0.5 µg/ml, (b) 1 µg/ml, (c) 1.5 µg/ml, (d) 2 µg/ml, (e) 2.5 µg/ml, (f) 3 µg/ml, (g) 3.5 µg/ml, and (h) 4 µg/ml. All images are magnified at ×40 (a-d) and ×10 (e-h). The images are representative of at least five such fields of view per sample and three independent trials

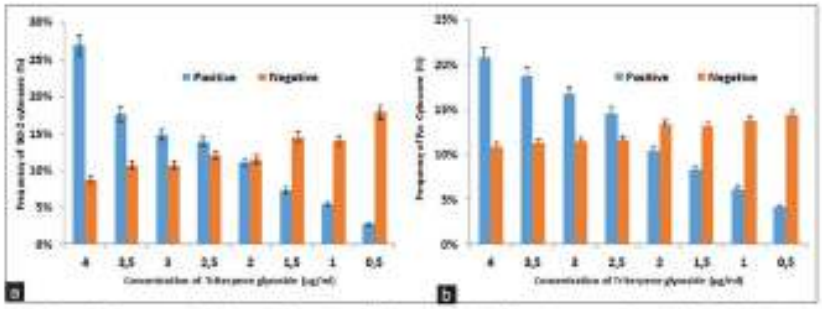

Fig. 3: Expression of Bcl-2 and Fas protein of *Suprt's-Clone 1* cell after sensitized by triterpene glycoside *Holothuria atra* (a) expression of the Bcl-2 protein and (b) expression of Fas protein

(0.745>). Fajarningsih *et al.* [33] explained the IC_{50} triterpene glycoside 0.239 µg/ml be included strong to adherent the development of cancer cells. The cytotoxic effect of triterpene glycoside on the Sp-C1 cell is shown in Fig. 2.

Our research has been in line with the study of Jangwan and Singh [34], and the triterpene extracted from *Randia dumetorum* Lamk was shown over cytotoxic effect (IC_{50} = antilog 2.55 = 354.8 µg/mL). Those findings are clarified again by Han *et al.* [35], triterpene glycosides (glycosides 1–3) isolated by sea cucumber showed the cytotoxicity activities on the tumor cell of P-388, A549, MKN-28, HCT116, and MCF-7 with concentration IC_{50} 0.93–2.60 µmol/L. In our research used to concentration 0.5–4 µg/mL. Based on the data obtained from this study, the cytotoxic activity of the glycosides of *H. atra* is highly sensitive to the Sp-C1 cell of tongue carcinoma.

Expression of Bcl-2 and Fas proteins

In general, the triterpene glycoside of *H. atra* has better than the potential for inducing the expression of Fas protein (active 71%; moderate 10%; and non-active 27%). Meanwhile, Bcl-2 protein has active 59%, moderate 14%, and non-active 27%. Both Fas and Bcl-2 are statistically significant ($p<0.05$) (Fig. 3). These results were shown that the triterpene glycoside of *H. atra* has immunogenically better that to Fas protein compared to Bcl-2 (Fig. 4). Zhao *et al.* [2012] [31] reported in his research that the triterpene glycosides could be causing to decrease the expression of the Bcl-2 protein and Mcl-1 and also to increase the sub-G0/G1 population of apoptotic cells and expression of Bax protein. These are a role in the expression of inhibitor cyclin-dependent kinase, p21, and the last to activated caspases 3, 7, and 9 [27].

The result of the study identified that triterpene glycosides of *H. atra* can trigger the expression of Bcl-2 and Fas protein. Its active component

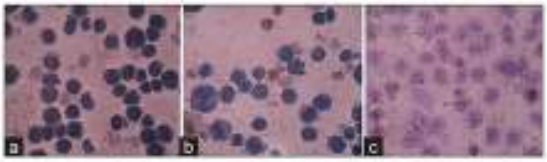

Fig. 4: Profile immunocytochemistry of Bcl-2 (a), Fas (b), and negative control (c)

possibility can be used to early detect the tumor of tongue carcinoma. Aminin *et al.* [19] give expression which the holothurian triterpene glycosides be a biology agent for cytostatics therapy.

Bcl-2 and Fas are the protein that mixed up with activated the tumor cell. Commonly, both expression Bcl-2 and Fas proteins were facilitated by ligand (FasL) contained in the cell surface that roles to improve the cell cycle and to prevent the apoptosis. Fas protein resistance-pathway also to contribute the Bcl-2 protein through linked-phosphatase-1, and soluble Fas (sFas) mRNA [37]. Expression of Fas protein and its ligand FasL was detected on the 44 subjects (88%) of the mount 50 subjects (100%) [38].

Apoptosis analyses

Build on the study, triterpene glycoside has apoptosis effect on the Sp-C1 cell of tongue carcinoma 87% (strong) and 13% (moderate) (Fig. 5), with indicator scale 0–5% (weak), 5–25% (moderate), and 25–100% (strong), significant ($p<0.05$) and strong correlation ($r=0.92$). Yun *et al.* [39] suggested that this research has been the strongly correlated effect of triterpene glycoside to induce the apoptosis in a way inactivated the Fas protein and caspase-8, cleavage of Bid, mitochondrial damage, and caspase-3 activation [40]. Piati *et al.* [41] adduced that the apoptosis

Table 1: Cytotoxicity assay of triterpene glycoside *H. atra* on the Sp-C1 cell

Doses (µg/ml)	Average (OD)	SDV	Mortality (%)	Scale	Log10-Concen	Anti-Log	p	R
4	0.049	0.058	88	Strong	0.602	0.274	0.014 (p<0.05)	0.939
3.5	0.096	0.076	83	Strong	0.544	0.248		
3	0.082	0.069	85	Strong	0.477	0.217		
2.5	0.118	0.021	81	Strong	0.398	0.181		
2	0.326	0.090	57	Moderate	0.301	0.137		
1.5	0.356	0.072	54	Moderate	0.176	0.080		
1	0.515	0.006	36	Non-active	0.000	0.000		
0.5	0.745	0.118	10	Non-active	−0.301	−0.137		

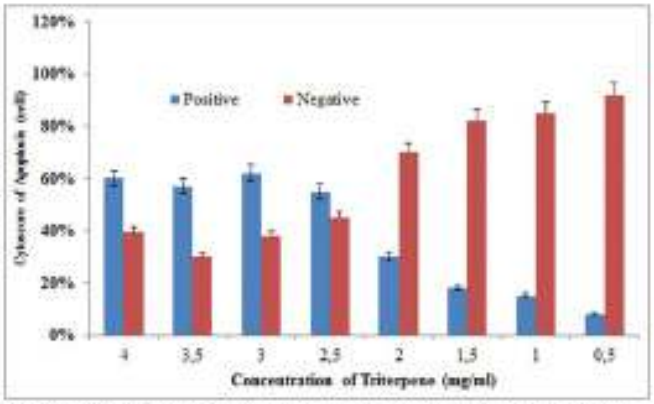

Fig. 5: Apoptosis frequency of *Supri's-Clone 1* cell of tongue carcinoma after administrated by triterpene glycoside of *Holothuria atra*

is inducible undergo FasL, tumor necrosis factor, and TRAIL bind on the target receptor. Meanwhile, caspases, family protein Bcl-2 will be the programming of death naturally. Meanwhile, caspases, family protein Bcl-2 will be the programming of death naturally in the regulation of immune response [36]. Furthermore, the flavonoid of plant herbal has to the prevention of cancer by inhibiting signal transduction enzymes, protein tyrosine kinase, protein kinase C, and phosphoinositide 3-kinases. The signals are involved in the regulation of cell proliferation [42].

Sp-C1 cell that experienced apoptotic to expressed the Fas and its ligand on the tumor cell surface [Fig.6]. In the case of hepatocellular carcinoma, changing the structure of Fas protein was related to the expression of the Bcl-2 protein and reported to inhibit the apoptosis [12]. In this guided that shown triterpene glycoside can interfere with Bcl-2 and fast protein expressions, so as the tumor cell is not developed and the checkpoint phase will be back operated in the apoptosis occurrence [43].

CONCLUSION

Triterpene glycoside of *H. atra* has been cytotoxicity effect on the Sp-C1 cell of tongue carcinoma, also inducible to expression the Bcl-2 dan Fas protein, at once to regulated the apoptosis of the Sp-C1 cell. Based on the result, triterpene glycoside of *H. atra* be possibility will be used as the active biology material to prevent the tumor metastatic of the tongue and applicable on the cancer whole body.

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Laboratory of integrated research, Universitas Gadjah Mada, Yogyakarta, Indonesia, was given Sp-C1 cell. The best thank to the Sumatera Biota Laboratory, Andalas University, Padang, Indonesia had been facilitated preparation triterpene glycoside of *H. atra*.

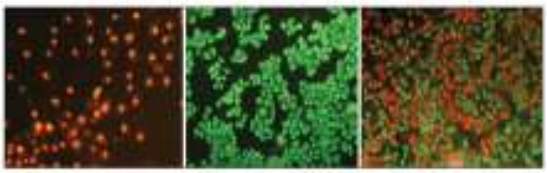

Fig. 6: Analyzed by double staining ethidium bromide acridine orange on the culture of the *Supri's-Clone 1* cell, apoptosis cell (red), and non-apoptosis (green), magnified at ×40. (a) Control positive, (b) control negative, (c) treatment

AUTHOR'S CONTRIBUTIONS

UA carried out the conception, cytotoxicity assay, immunocytochemistry assay, and apoptosis assay also drafted the manuscript with BAG and MHS. Whereas, SI, DH, AP, and NK have been given the research ideas and design of research and include the preparation of triterpene glycoside of *Holothuria atra*. Specifically, BAG has been arranged the manuscript, statistical analysis, and corresponding author. All of the authors were read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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