

Anti-inflammatory properties of saponin fraction from (Spiny brittle star) *Ophiocoma erinaceus* (Müller and Troschel, 1842)

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Received: February 2016

Accepted: December 2018

Abstract

Marine saponins are naturally glycosides compounds which possess important biomedical properties such as anti-cancer, antioxidant and anti-inflammatory. Nevertheless, no systematic studies on the anti-inflammatory potential of brittle star saponin and its underlying mechanisms have been reported. In this study, *Ophiocoma erinaceus* extracted saponin was investigated for its in vitro anti-inflammatory capacity. The antioxidant capacity of extracted saponin has evaluated using anion superoxide scavenging assay. Further, the anti-inflammatory effect of brittle star saponin on the production of inflammatory cytokines in THP-1/M cells has evaluated. Molecular mechanisms inhibitory effects of brittle star saponin were assessed by analyzing the expression of inflammatory mediators at transcriptional level. The results showed scavenging properties of extracted saponin against superoxide anion radical and demonstrated that brittle star saponin dose-dependently attenuated the release of inflammatory mediators such as NO, TNF- α , and COX-2, Real Time-PCR analysis elucidated that the anti-inflammatory action of brittle star saponin is correlated with its inhibitory effect on lipopolysaccharide (LPS)-induced TNF- α and COX-2 mRNA expression in THP-1/M cells. The results have been showed, intensely afford the anti-inflammatory combined with its antioxidant potential of marine saponin extracted from brittle star.

Keywords: Inflammation, Saponin, Cytokines, Brittle star, Natural product

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Introduction

Inflammation is one of the most critical natural host defense systems which is known as a localized protective response of vascular tissue to pathogen, irritation, injury and damaged cells (Lu *et al.*, 2006). This disorder characterized by redness, swelling and disturbed physiological functions to restore homeostasis at damaged sites which can stimulate the activation of various immune cells such as macrophages, monocytes and neutrophils (Mittal *et al.*, 2014).

Acute and chronic inflammatory barriers have a critical function in protecting our bodies to maintain immune homeostasis (Hossen *et al.*, 2015). Macrophages are important cells of the inflammatory immune responses through secrete of several pro-inflammatory mediators and cytokines (Yayeh *et al.*, 2012). Cytokines are a group of small nonstructural proteins of the immune system secreted by various cells and communicating in paracrine, autocrine and endocrine manners (Tanekhy and Sakai, 2019).

There is an increase of inflammatory mediators including reactive oxygen species (ROS), reactive nitrogen species (RNS), nitric oxide (NO), prostaglandin E₂ (PGE₂), tumor necrosis factor- α (TNF- α), interleukin (IL)-1, IL-6, interleukin-1 β (IL-1 β) and cyclooxygenase (COX)-2 during inflammation (Sanjabi and Zenewicz, 2009; Arranz *et al.*, 2015). The cytokines such as IL-1, TNF, and IL-6 promote inflammation are known as pro-inflammatory cytokines because they initiate local inflammation of

injured tissues (Liu and Lin, 2013). All of the immune responses lead to protect the body against infection though exaggerated responses of inflammatory mediators which can cause sustained or chronic inflammation, lead to various diseases such as atherosclerosis, arthritis, diabetes and Alzheimer's disease (Yang *et al.*, 2012).

Recent studies have been shown that chronic inflammation can be associated with a variety of cancers (Jeong *et al.*, 2013). The progression of cancer might be a process driven by inflammatory modulators and their multiple signaling pathways which result in a higher cell proliferation, mutagenesis, oncogenesis and angiogenesis (Suresh *et al.*, 2011; Frontela-Saseta *et al.*, 2013).

Over the last few decades, Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for management of inflammatory conditions which are used to relieve or alleviate signs of inflammation (Apetz *et al.*, 2014). Nonetheless, anti-inflammatory drugs are also associated with some undesirable side effects, these complications recruit a great deal of interest to development of safe and effective anti-inflammatory medicines with the capacity to modulate the inflammatory response and prevent or cure chronic inflammatory conditions (Meek *et al.*, 2010). Meanwhile, potential natural products with minimizing side effects to avoid immune disorder have been paid more attention to overcome this issue (Yayeh *et al.*, 2012).

Brittle star Muller and Troschel (Ophiocomidae) are one of the marine

invertebrates habitually found on the sea floor worldwide, ophiroids are diverse group of echinoderm with worm-like arms and various colors (Stohr *et al.*, 2012). Biological properties of brittle stars can be linked to the presence of a wide array of bioactive compounds especially steroids, naphthaquinone, terpens, phenylpropanoid, cerebrosides and glycosides (Soltani *et al.*, 2014). Furthermore, previous researchers have been demonstrated that extracted saponin fraction from brittle star possess biological and pharmacological activities including antioxidant, anticancer, and fungicidal activities (Esmat *et al.*, 2013).

In this study, the main aim was the assessment of anti-inflammatory potential of saponin fraction extracted from *Ophiocoma erinaceus* and their molecular mechanisms underlying the anti-inflammatory properties.

Materials and methods

Inhibition of albumin denaturation

Inhibition of protein denaturation was evaluated according to the below method with minor modifications (Hossain *et al.*, 2012). The reaction mixture was consisting of saponin fraction at the different concentrations (0-200 $\mu\text{g ml}^{-1}$) and 100 $\mu\text{g/ml}$ diclofenac sodium (Standard anti-inflammatory drug) with 1% aqueous solution of bovine albumin fraction. The samples were the mixture was incubated at 37 °C for 20 min and then heated kept at 57 °C for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at

660 nm. The experiment has performed in triplicate. Percent inhibition of the percentage inhibition of protein denaturation was calculated as follows: Percentage inhibition= (Abs control– Abs sample)×100/Abs control

Protection of red blood cells (RBCs) membrane

Fresh whole human blood (10 ml) was collected and transferred to the heparinized centrifuged tubes. The tubes were centrifuged at 3000 rpm for 10 min and washed up three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline. Briefly, the control group composed of saline, erythrocytes and aspirin, the experimental groups consist of various concentrations of brittle star saponin fraction (0-200 $\mu\text{g ml}^{-1}$) plus erythrocyte solution. All groups were incubated at 56 °C for 30 min, centrifuged at 2500 rpm and measured the supernatant optical density at 560 nm.

Proteinase inhibitory effect

The test has been performed according to the to the below method with slight modifications (Sakat *et al.*, 2010). In brief, the reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml different concentrations of saponin fraction. The mixture has been incubated at 37 °C for 5 min and added 1 ml of 0.8% (w/v) casein. The mixture has been incubated for an additional 20 min. Then, 2 ml 70% perchloric acid was added to terminate the reaction.

Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank.

Cell culture

The human THP-1 monocytes (NCBI, Iran) were cultured in RPMI-1640 medium containing 10% FBS supplemented with 1% penicillin-streptomycin, 2 mM l-glutamine and 0.05 mM-mercaptoethanol at 37 °C, 5% CO₂ incubator. The cells (4×10⁵) have been cultured in 24 wells plates. Differentiation to macrophages was induced by incubation of THP-1 cells for 48 h in presence of 100 ng ml phorbol 12-myristate 13-acetate (PMA) (Sigma, USA). After differentiation to macrophages, the cells having been rinsed with PBS and exposed with 0.05 µg ml⁻¹ LPS in presence of different concentrations of brittle star saponin fraction for 6, 12 or 24 h in FBS free medium. At next, the supernatant was frozen at -80 °C to conduct later experiments. All experiments have been repeated for at least three times.

Cytotoxicity assay

For evaluation cytotoxicity of brittle star saponin on THP-1/M cells, MTT assay was conducted. Briefly, the cells have cultured in 96 well plates overnight, and were then exposed to various concentration of brittle star saponin. After 24 h, MTT solution (1 mg ml⁻¹) has added to each well in darkness and plate were incubated 4 h at CO₂ incubator. Then the supernatant has discarded gently and formazan crystals dissolved in 80 µl of dimethyl sulfoxide. The plates have then shaken

for 2 h and the optical density was recorded at 560 nm by plate reader (Epoch, USA).

Determination of antioxidant activity by superoxide anion assay

The antioxidant activity has evaluated by reaction of 1 ml nitro blue tetrazolium (NBT) solution, 1ml NADH solution, 100 µl phenazine methosulphate and 0.1 from different concentrations of brittle star saponin. The reaction has carried out at 25 °C for 5 min. The absorbance was measured at 560 nm. The reduced absorbance reveals the induced superoxide anion scavenging effect. The percentage of superoxide anion scavenging was calculated as follows: scavenging rate % (1-Ai/Ao)×100. Ao: absorbance without sample, Ai: absorbance with sample.

The determination of NO, COX-2 and TNF-α levels

The release of NO, COX-2 and TNF-α was measured by ELISA method. Briefly, after the pre-incubation of THP-1/M cells for 18 h, the cells were pre-treated with brittle star saponin fraction (0–200 µg ml⁻¹) for 30 min and were further exposed with LPS (2 µg ml⁻¹) for 24 h. The inhibitory effect of extracted saponin on NO, COX-2, and TNF-α level was evaluated by an ELISA kit (BD biosciences, Madrid, Spain) according to manufacturer's protocol by measuring the OD at 450 nm using an Epoch plate reader.

Evaluating the expression of TNF- α by flow cytometry

For evaluating TNF- α expression in cells, flow cytometry analysis has conducted. In this assay, the treated and untreated THP-1/M cells having been trypsinized and transferred to centrifuge in 2000 rpm at 4 °C. Then the cells, rinsed with PBS, centrifuged and have added TNF- α primary antibody (Abcam, UK) (1:100) overnight. On the next day, the washing was performed and centrifugation has exerted, then conjugated secondary antibody (1:20) has added for 45 min. Finally, 300 μ l 0.1 % formalin has added and flow cytometry has conducted using FACS caliber flow cytometry (Becton Dickinson, USA) (Mandal *et al.*, 2010).

PCR amplification and quantification (RT-PCR)

The changes of TGF- β mRNA expression were analyzed by RT-PCR. The study has conducted in 6 well plates and groups were divided into control and experimental. The normal group has kept untreated and the extract treatment groups were treated with respective doses of saponin fraction. Total cellular RNAs of THP-1 treated cells and untreated cells were isolated by the High Pure RNA Isolation kit (Roche, Germany). The RNA was quantified using a spectrophotometer (Epoch, USA) at 260 nm. cDNA was prepared from the total RNA (2 g) using an oligo (dT) as a primer and M-MLV reverse transcriptase. The expression levels of COX-2, TNF- α and GAPDH (internal control) were analyzed by PCR amplification of these

genes by using specific primers at specific annealing temperatures in a thermal cycler (Bio rad, USA). The PCR reactions were carried out in 2mM MgCl₂, 10mM dNTPs, PCR buffer and 2 units of Taq DNA polymerase in the thermal cycler. The amplicons were electrophoresed in 2% agarose gel. The bands were quantified by densitometry on Gel documentation System using image J software (Biorad, USA). Values were normalized by calculating densitometry ratio of internal control GAPDH as base line.

Statistical analysis

The results are presented as the mean \pm SEM. The experiments were carried out in triplicate. The significant differences among the means were analyzed by one-way ANOVA followed by the Tukey test. The level of $p \leq 0.05$ was considered to be significant.

Results

In vitro Anti-inflammatory bio assays

The *in vitro* anti-inflammatory effect of brittle star saponin fraction was investigated against the denaturation of egg albumin, hemolysis of red blood cells and proteinase activities. The results of protein denaturation assay showed a dose dependent suppression of albumin denaturation by extracted saponin in the concentration ranging from 6.25 to 100 μ g ml⁻¹, so that the most inhibitory effect were indicated at 100 μ g ml⁻¹ ($p \leq 0.001$). Diclofenac sodium was used as reference anti-inflammatory drug (Fig. 1A).

The findings of protective effect against erythrocyte membrane showed a

dose dependent protective effect by brittle star saponin in the concentration ranging from 6.25 to 50 $\mu\text{g ml}^{-1}$, so that maximum protective effect were demonstrated at 50 $\mu\text{g ml}^{-1}$ ($p \leq 0.001$) (Fig. 1B).

Further, the Anti-inflammatory capacity was calculated by measuring the absorbance of saponin fraction treated groups and converting it into total suppression of proteinase activity. The present data indicated

concentration dependent of proteinase inhibition by brittle saponin fraction so that the high inhibitory effect of proteinase suppression was determined as 50 $\mu\text{g ml}^{-1}$ ($p \leq 0.01$). Diclofenac sodium has been used as reference anti-inflammatory drug with 32.8 % proteinase suppression in 300 $\mu\text{g ml}^{-1}$ concentration (Fig. 1C).

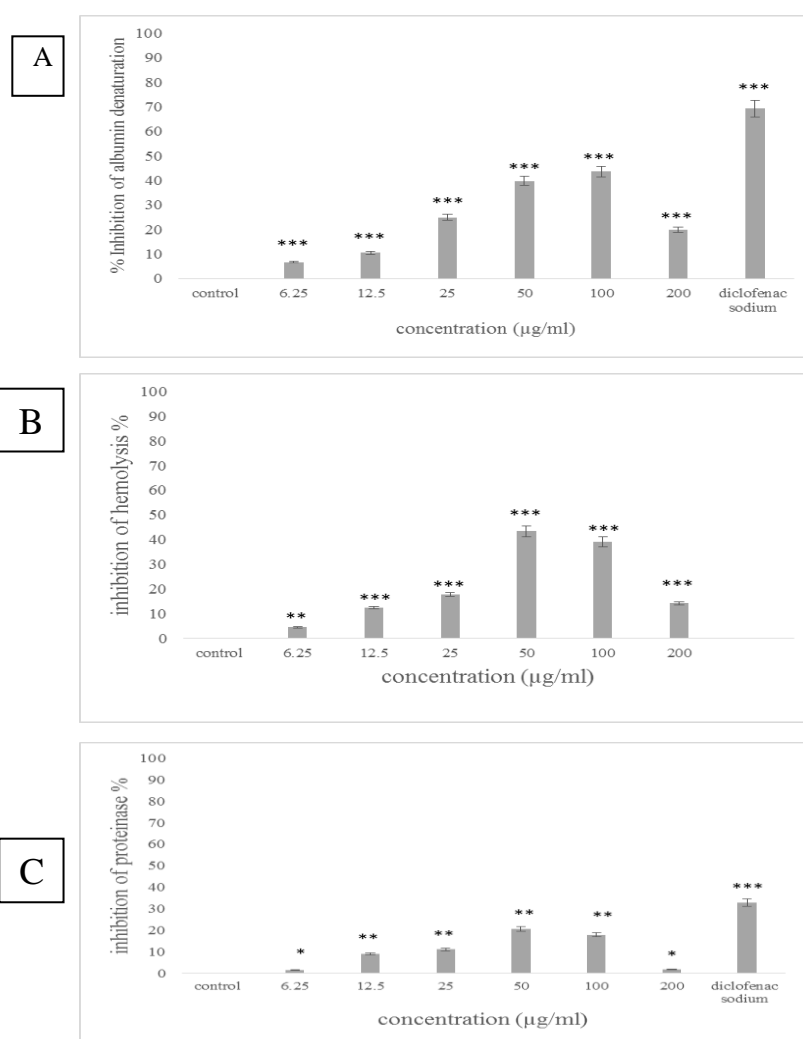


Figure 1: A) Influence of different concentrations of brittle star saponin fraction against protein denaturation. B) The effect of different concentrations of brittle star saponin fraction against suppression of hemolysis. C) The effect of various concentrations of brittle star saponin fraction against inhibition of proteinase activity. The data were represented as Mean \pm SD. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$ were considered significant.

Assessment the cytotoxicity of brittle star saponin in THP-1/M cell

For investigating the mechanism underlying the anti-inflammatory capacity of brittle star saponin, we selected the appropriate concentration of the saponin fraction that would not affect the cell viability in THP-1/M cells. For this purpose, THP-1/M cells

were treated with different concentrations of brittle star saponin ($0\text{--}800\ \mu\text{g ml}^{-1}$) and MTT assay was performed. Interestingly, THP-1/M cells have been found to indicate $100\text{--}89\%$ viability below to $200\ \mu\text{g ml}^{-1}$ concentration of saponin fraction (Fig. 2).

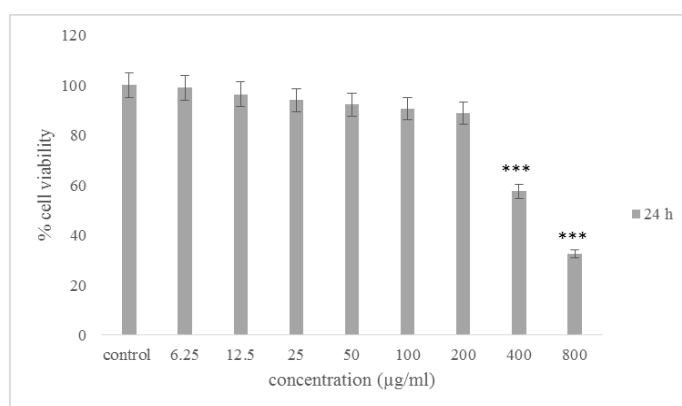


Figure 2: Cytotoxicity of saponin fractions in THP-1/M cells.

The cells were treated with brittle star saponin fraction in the range of $0\text{--}800\ \mu\text{g ml}^{-1}$ for 24 h. As indicated, extracted saponin didn't have any effects on cell survival in the range of $0\text{--}200\ \mu\text{g ml}^{-1}$. The data were represented as Mean \pm SD of three independent experiments, each performed in triplicates. * $p<0.5$, ** $p<0.01$, *** $p<0.001$ were considered significant.

Superoxide anion scavenging assay

Superoxide anion radical scavenging activity was conducted to assess antioxidant capacity of brittle star saponin. Vitamin C was used as an approved antioxidant compound. The results showed that saponin fraction scavenges the superoxide anion up to

38 % at $2000\ \mu\text{g ml}^{-1}$ concentration, whereas Vitamin C scavenged superoxide anion radical as 82 %. The results are represented in Fig. 3 and showed that the brittle star saponin fraction possess antioxidant activity due to scavenging superoxide anion free radical.

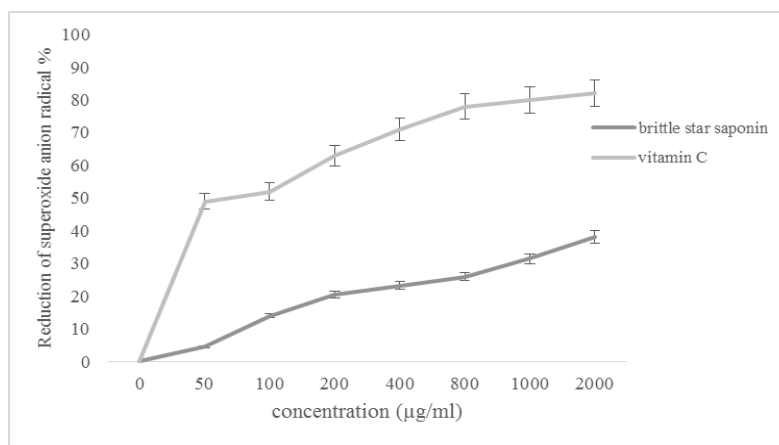


Figure 3: Scavenging activity of brittle star saponin fraction on superoxide anion radical. The values are significantly different ($p < 0.05$) when compared with the control (vitamin C) at the same concentrations.

Effect of brittle star saponin on the cytokine release in LPS - activated THP-1/M cells

The suppressive effect of brittle star saponin fraction on the level of inflammatory cytokines such as NO, COX-2 and TNF- α has measured using ELISA kit. As shown in Fig. 4, the THP-1/M cells released detectable basal levels of NO, COX-2 and TNF- α as compared with non-activated cells. The

pre-treatment of 100 $\mu\text{g ml}^{-1}$ of brittle star saponin fraction attenuated TNF- α release. Further, the secretion of NO and COX-2 has also significantly reduced with 100 $\mu\text{g ml}^{-1}$ of brittle star saponin fraction. These results have indicated that the anti-inflammatory activity of brittle star saponin was exerted by inhibition of the inflammatory mediator's release.

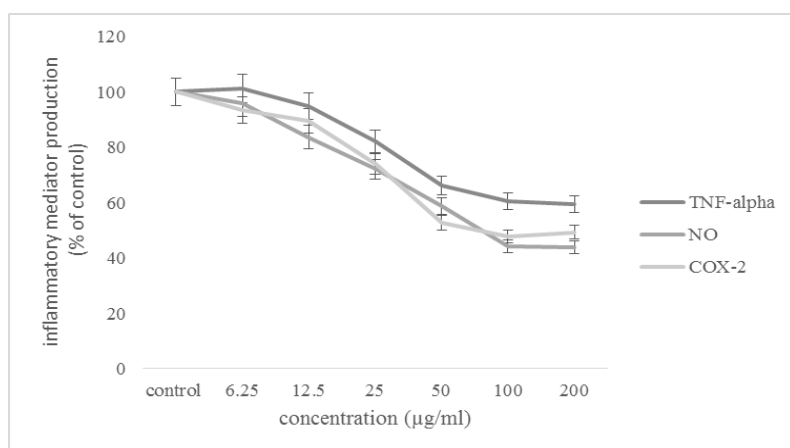


Figure 4: Effect of brittle star saponin on the production of inflammatory cytokines. Levels of NO, COX-2, and TNF- α were measured by ELISA from supernatants of THP-1/M cells with different concentrations of extracted saponin and LPS ($1 \mu\text{g ml}^{-1}$) for 24 h.

Effect of brittle star saponin on cytokine gene expression in THP-1/M cells with LPS

As previously mentioned, TNF- α is well-known inflammatory cytokine that modulate inflammatory process, COX-2 is a key enzyme in the biosynthesis of prostaglandins which activated by extracellular stimuli such as and its expression promoted during inflammation. In order to assess the presence of relationship between

cytokine production and gene expression, RT-PCR was performed. As exhibited in the Fig. 5, the mRNA levels of pro-inflammatory mediators such as TNF- α and COX-2 has significantly decreased in presence of the brittle star saponin fraction. The comprehensive analysis of these results confirmed that the brittle star saponin exerted anti-inflammatory effects by reducing the expression of inflammatory cytokines.

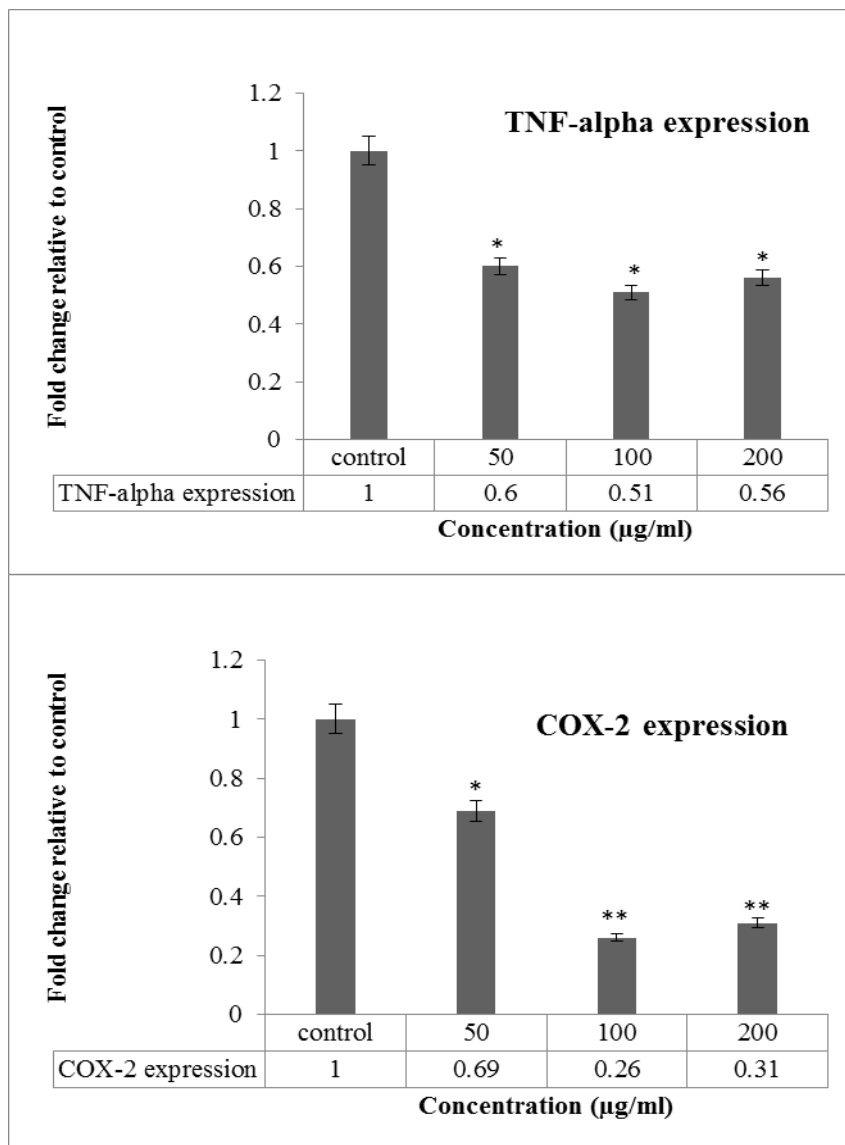


Figure 5: The Effect of brittle star saponin fraction on the mRNA level of pro-inflammatory genes. A) TNF- α gene expression and B) COX-2 gene expression were analyzed at transcriptional level by RT-PCR. GAPDH was used as a loading control.

Discussion

During inflammation, ROS level partly were increased to administrate functional defense role against pathogens (Khansari *et al.*, 2009). ROS generation can elicit caspase activation which indicates that apoptosis induction mediated by reactive oxygen species directly or indirectly (Liou and Storz, 2010). In the previous study we indicated that *O. erinaceus* extracted saponin induced apoptosis in cervical cancer cells through ROS mediated pathway (Amini *et al.*, 2017). It has been reported that persistent inflammatory stimuli are currently contributed with an increased risk of chronic conditions such as cancer and cancer cells express elevated levels of antioxidant to detoxify from ROS. Therefore, the inhibition of inflammation and oxidative stress by natural products is considered crucial in prevention of inflammatory related disease (Yang *et al.*, 2015).

Saponins have been demonstrated to act as antioxidants for protecting tissues from induced injuries by oxidative stress. Moreover, the previous literature was indicated that saponins possess anti-inflammatory capacities (Li *et al.*, 2014). Therefore, the aim of this paper for first time was to evaluate the anti-inflammatory potential of saponin fraction obtained from *O. erinaceus* brittle star.

The present study clearly demonstrates that the brittle star saponin fraction possesses significant anti-inflammatory and antioxidant activity *in vitro*. Moreover, it was demonstrated that extracted saponin

fraction was able to attenuate the expression of inflammatory markers in THP-1 human macrophages activated with lipopolysaccharide (LPS).

We initially evaluated *in vitro* inflammatory capacity of brittle saponin fraction using three assays. Protein denaturation bioassays showed a concentration dependent inhibition of albumin denaturation of brittle star saponin fraction which was supported by erythrocyte hemolysis and proteinase inhibitory assays.

Furthermore, we investigated the cytotoxicity of saponin fraction in THP-1 cells. Surprisingly, it has found that the examined brittle star saponin fraction didn't reduce cell viability. Therefore, the lowest toxic concentration of saponin fraction (400 $\mu\text{g ml}^{-1}$) was selected for further experiments in order to assess its anti-inflammatory activity.

Inflammation is commonly associated with an over expression of a number of pro-inflammatory cytokines and chemokines including IL-1 β , IL-6, IL-1, COX-2 and TNF- α (Fangkrathok *et al.*, 2013). It must be pointed out that our results exhibited that brittle star saponin fraction were able to reduce the pro-inflammatory cytokine secretion and gene expression in LPS activated macrophages.

The TNF- α , as a master regulator of inflammatory cytokine production confers critical role in inflammation and cancer development (Teiten *et al.*, 2009). During triggering the inflammatory response, the pro-inflammatory cytokines TNF- α and IL-1 β initially secreted by macrophages

which exert an appreciable role in the initial development of the reaction (Nalbantsoy *et al.*, 2012).

The results have presented in this paper showed that extracted saponin fraction remarkably inhibited the production of TNF- α in LPS-activated THP-1 cells without affecting on cell viability.

Macrophages have an essential role in the inflammatory cascade and stimulation of these cells with LPS induces pro-inflammatory enzyme of COX-2 and inducible enzyme iNOS production (Apetz *et al.*, 2014). Up-regulation of COX-2 has been proven to play a vital role in the pathogenesis of many inflammatory related diseases, so that diversity of anti-inflammatory bioactive compounds has been indicated to inhibit COX-2 expression (Fangkrathok *et al.*, 2013).

Our results revealed that saponin fraction significantly suppressed COX-2 expression elicited by LPS in macrophages which proves potent anti-inflammatory capacity of brittle star saponin fraction. Additionally, it is demonstrated that suppression of TNF- α and COX-2 at mRNA levels associated with the anti-inflammatory potential of brittle star saponin fraction may be occurred at the transcriptional level.

The results of present study were in agreement with other investigations presented by other researchers based on inflammation inhibitory effects of saponin compounds by down-regulation of pro-inflammatory cytokine.

Numerous studies have been reported that saponins have hemolytic,

antimicrobial, antitumor and anti-inflammatory activities (Hassan *et al.*, 2012). *Polygala japonica* has been widely used in traditional Chinese medicine. Wang *et al.* (2008) evaluated the anti-inflammatory effects of isolated saponins from *Polygala japonica*, and explored the potential therapeutic efficacy of bioactive saponins from *P. japonica* to inhibit acute inflammation (Wang *et al.*, 2008).

The inflammation inhibitory potential of flavonoid and saponin fractions from the leaves and root bark of *Zizyphus lotus* has been evaluated and it indicated that *Z. lotus* exhibits anti-inflammatory activity via inhibition of NO production (Borgi *et al.*, 2008). Borges *et al.* evaluated the anti-inflammatory activity of *Chiococca alba* (L.) Hitchc. (Rubiaceae) of saponin fractions against in vitro LPS-induced inflammation and displayed anti-inflammatory properties of saponin fraction (Borges *et al.*, 2013).

In 2015 a study was designed to evaluate the cytotoxicity and anti-inflammatory activities of steroidal saponins from the ethanol extract of the roots of *Bletilla striata* and reported that some isolated saponin exert selective suppression of Cox-2 as compare with the anti-inflammatory drug. It implied that steroidal saponins may be applicable as novel anti-inflammatory therapeutics (Wang and Meng, 2015). Yang *et al.* (2013) performed a study to assess anti-inflammatory effect of extracted saponins from tetraploid *G. pentaphyllum* and indicated the anti-

inflammatory effect of saponin compounds by suppression the expression of IL-1b, IL-6 and COX-2 mRNA in LPS-activated RAW 264.7 macrophage cells.

Korean Red Ginseng (KRG) is a representative traditional herbal medicine strongly proposes that the anti-inflammatory effect mediated by a reduction in the activation of p38-, JNK2-, pathways and their corresponding transcription factors (ATF2 and IRF3). Yayeh *et al* investigated the inflammatory inhibitory effect of red ginseng saponin fraction and exhibited that the saponin fraction suppresses the expression of pro-inflammatory mediators in vitro (Yayeh *et al.*, 2012).

Considering the results presented in this paper, brittle star saponin fraction revealed antioxidant potential and exhibited an anti-inflammatory capacity, through the inhibition of albumin denaturation, erythrocyte hemolysis, proteinase activity, suppression of pro-inflammatory cytokines production and gene expression in THP-1/M cells. These data suggested the initial evidence that brittle star saponin fraction possesses significant anti-inflammatory properties which may be utilized in the future.

Acknowledgements

The authors would like to thank Research Center for Animal Development Applied Biology, Islamic Azad University, Mashhad, Iran

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