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Original Research Article

POLYSACCHARIDE EXTRACTS FROM *SARGASSUM SILIQUOSUM* J.G. AGARDH MODULATES PRODUCTION OF PRO-INFLAMMATORY CYTOKINES IN LPS-INDUCED PBMC AND DELAYS COAGULATION TIME IN-VITRO

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Abstract

Sulfated polysaccharides from brown seaweeds exhibit various biological activities, structural diversity, and are potential reagents for the development of therapeutic drugs. This study aimed to determine the effect of aqueous and fucoidan extracts from *Sargassum siliquosum* J. G. Agardh on viability of peripheral blood mononuclear cells, production of pro-inflammatory cytokines and plasma coagulation using *in vitro* assays. Sulfate contents of the polysaccharides were quantified using Acid-Ashing Digestion Ion chromatography. Effect on viability of the extracts on peripheral blood mononuclear cells was determined by MTT Assay. Estimation of pro-inflammatory cytokines concentrations was done through Enzyme-Linked Immunosorbent Assay, while anticoagulant activity was measured by Prothrombin Time and Activated Partial Thromboplastin Time. Results revealed that both extracts were non-cytotoxic to PBMCs, reduced significantly the production of IL-1, IL-6, TNF-α and exhibited normal anticoagulant activity in PT assays and prolonged APTT remarkably in dose-dependent manner. In conclusion, extracts of the *Sargassum siliquosum* J.G. Agardh is a potential alternative source in producing anti-inflammatory and anticoagulant substances in the future.

Keywords: *Sargassum siliquosum*, sulfated polysaccharides, ashing-acid digestion, peripheral blood mononuclear cells, cytokines, inflammation, coagulation

Introduction

Sulfated polysaccharides from brown seaweeds exhibits biological activities including vasodilation (Park *et a*l., 2008); anticoagulant (Arivulsevan *et al*., 2011); antivirus (Sinha *et a*l., 2010); antitumor (Khanavi *et al*., 2010); immunomodulatory (Khanavi *et al*., 2010); anti-inflammatory (Yoon *et al*., 2009); antivasculogenic (Dias *et al*., 2008); antioxidant (Kim *et al*., 2010); antiherpetic (Lee *et al*., 2010); and hepatoprotective (Josephine *et al*., 2008). This compound is unique, displays varying degree of sulfation and structurally different in each

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algal species. Generally, the biological activity of polysaccharides from marine algae is related to the molecular size, type of sugar, sulphate content, type of linkage and molecular geometry are also known to have a role in their activities (Vischuk *et al.*, 2011). The unique distributed sulfation pattern of high sulfated polysaccharide is believed to regulate its functional specificity (Karmakar *et al*., 2009).

Sargassum siliquosum J. G. Agardh is widely distributed in the Philippines and has been reported for its ethnobiological uses such as food, fertilizer, insecticidal, sea-urchin and abalone culture and also in health drinks (Tupas and Montano, 1987). Despite the wide variety of application of *Sargassum siliquosum* J. G. Agardh, its effects on production of pro-inflammatory cytokine and coagulation have never been reported.

Thrombosis has been recognized as a protective mechanism critical for staunching blood loss after injury in simple pathological states. However, in diseases such as atherosclerosis, the pictures differ substantially from this simplistic method. In the natural history of atherosclerosis, thrombosis involves an inciting injury more subtle than a wound. In such pathological states, the importance of an intricate interface between inflammation and thrombosis becomes apparent (Libby and Simon, 2001).

The study focused on the effects of sulfated polysaccharides from *S. siliquosum* on production of pro-inflammatory cytokines in LPS-induced PBMCs and plasma coagulation in vitro. The results showed that *S. siliquosum* extracts are potentially useful for the development of a natural drug against inflammation and thrombosis whose interface results to vascular pathology.

Materials and Methods

Extraction of polysaccharides from *S. siliquosum* J.G Agardh

S. siliquosum was collected from Balibago, Calatagan Batangas, Philippines. The algal species was authenticated by Dr. Gavino C. Trono of the Marine Science Institute College of Science, University of the Philippines Diliman. About 1000g fresh weight biomass of *S. siliquosum* were cleaned and washed with distilled water. The samples were air-dried at room temperature, grounded into fine particles using Wiley mill, and sieved in a 2mm mesh. Polysaccharides were extracted from *S. siliquosum* according to Michailovna *et al*.(2009) and Gamal-Eldeen *et al.(*2009) with some modifications *(Fig. 1).* The powdered algal sample was extracted repeatedly with 80% ethanol at 22°C for 22 h to remove the low molecular weight components. The resulting residual materials were extracted with boiling distilled water for 5 h repeatedly for three times. The filtrate of the water extract was concentrated by rotary evaporation to give **(AQ).** The dried residue was subjected to further extraction using 0.1 M HCl at 20-25°C for 12 h. The filtrate was adjusted to pH 6.0 by 1M NaOH and was concentrated to 1/5 of its original volume, precipitated with 96% ethanol, and then centrifuged for 20 min at 3000g to give **(F1).**The prepared extracts (AQ and F1) were lyophilized, analyzed, and used for cytotoxicity, ELISA and anticoagulant assays.

Ashing-Acid Digestion Ion Chromatography for sulfate content

The percentage of sulfate composition was quantified using Dionex DX-120 Ion Chromatography with IonPac AS4A-SC 2-mm column. Briefly, 5.0 g of lyophilized sample were ashed, digested with 1M HCl and filtered through 0.45µm membrane filter. Five microliters (5µl) were used as the injection volume. A solution of 1.8 mM$NaHCO_3$ + 1.7 mM Na_2CO_3 was used as the eluent, and electrical conductivity was used as the mode of detection. The degree of sulfation was computed from standard curve prepared from the peak area readings of standard sulfates.

Preparation of the extracts for stimulation of Peripheral Blood Mononuclear Cells (PBMC)

The individual lyophilized extract was dissolved in RPMI 1640 and filter-sterilized with 0.20 µm membrane filter (Minisart, Sartoriusstedim biotec) to prepare the desired concentration.

Blood collection

Twenty milliliters (20 mL) of blood were collected from healthy individuals who were screened using the guidelines by the Philippine National Voluntary Blood Services Program. Blood was collected through aseptic technique in heparinized and citrated tubes with written informed consent following an approved protocol by the Human Ethics Committee of the Philippine Orthopedic Center Blood Bank.

PBMC separation and cell proliferation assay

PBMC were isolated by density-centrifugation using Lympoprep gradient media. Cell viability was assessed using Trypan Blue exclusion assay. The cells were washed in phenol free RPMI 1640 and 1 X 10^6 cells/mL were resuspended in RPMI 1640 supplemented with 5% FBS, 1% sodium pyruvate, 100 U/ml penicillin and 100µg/mL streptomycin (pen-strep), L-glutamine (4mM) with or without *S. siliquosum* extracts (0-200 µg/ml), 10µg/ml of bacterial lipopolysaccharides LPS (Sigma-Aldrich) in a 96 well/plates (BD Falcon). The cells were incubated at 37ºC in 5% CO_2. After 72 h, the cell supernatant was collected and frozen at -80ºC until cytokine assay. MTT assay kit (Invitrogen, USA) was used to measure viability. Mitomycin was used as a reference standard drug. The absorbance was read at 550nm using a microplate reader (Tecan Microplate GENios). The experiment was performed in triplicate.

Cytokine assay

The tumor necrosis factor-α (TNF-α), interleukin- 1, and interleukin-6 concentrations in the supernatants were measured with ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's protocols. The absorbance was read at 550nm and corrected at 620nm using a microplate reader (Tecan Microplate GENios). The experiment was performed in triplicate.

Plasma coagulation assays

Varying concentrations of F1 and AQ extracts were prepared using a two-fold serial dilution. Plasma was separated from citrated whole blood samples. Plasma (90µL) and extracts (10µL) of varying dilutions were mixed. Standardized coagulation assays, Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) (STA-Neoplastine CI Plus), were performed in triplicate for each concentration against the control sample (100µL plasma).

Statistical Analysis

The data were expressed as MEAN ± SEM and analyzed by Multivariate Analysis and ANOVA. A p value of <0.05 was considered statistically significant.

Results

Extraction and Ashing-Acid Digestion Ion Chromatography

Extracts from *S. siliquosum* are brownish, powdery and with characteristic phosphine odor. Both are soluble in water and culture media. Sulfate was successfully separated from polysaccharides after acid treatment and was eluded between 6.35-6.52 minutes. The average retention time, peak areas, amount of sulfate in extracts per 5 mg ashed sample were summarized in *Table 1* and *Fig. 2*.

MTT Assays

As shown in *Fig. 3*, there was a significant ($p<0.05$) increase of PBMC proliferation with increasing concentrations. At 175 µg/ml, AQ induced proliferation by 83% and F1 (59%). It can be seen that proliferation correlated with the degree of sulfation. The standard drug Mitomycin showed cytotoxicity starting at the lowest concentration.

The observed mitogenic activity of *S. siliquosum* extracts is the same with low molecular weight fractions from *Sargassum chordalis'* carrageenans which induced polyclonal proliferation of T- and B-lymphocytes in vitro (Stephanie *et al*., 2010). The stimulation was mediated by the binding of the polysaccharides to their corresponding receptors including TLRs, which triggers cell activation by mitogen-activated protein kinases (Han *et al*., 2003).

ELISA Assays for pro-inflammatory cytokines

Bacterial lipopolysaccharides induced excessive production of IL-1 as high as 290 (±1.81) pg/mL and 217.9(±62.93) pg/mL after 48 and 72 hours respectively. This excessive production was thirty-fold times higher than the basal concentration of IL-1 in unstimulated PBMCs (9.7 pg/mL). The stimulation of IL-1 production by LPS was significantly ($p<0.05$) reduced by co-incubation of PBMCs with sulfated polysaccharides (0-200 µg/mL). The observed reduction was dose-dependent**.** LPS-induced IL-1 production was reduced by 82% (F1) and 81% (AQ) and 63% after 48 hours of co-incubation at 200 µg/mL. The reduction in LPS-stimulated IL-1 production was still evident after 72 hours *(Fig. 4).* Multivariate analysis shows no significant interaction between extracts, concentration and time ($p<0.05$).

LPS also induced excessive production of IL-6 as high as 612 (±43.75) pg/mL and 615 (±36) pg/mL after 48 and 72 hours of stimulation. This excessive production was 153 times fold higher than the basal concentration of unstimulated PBMCs (4±.81 pg/mL). This LPS-stimulated IL-6 production was significantly reduced by co-incubation with *S. siliquosum* extracts. As shown in *Fig. 5,* PBMC treated with *S. siliquosum* extracts exhibited significantly lower IL-6 production when compared to LPS-stimulated IL-6 production ($p<0.05$)**.** The optimum inhibition was seen at 200µg/mL where all extracts reduced LPS-induced production by 97% after 48 hours of co-incubation and significant inhibition was maintained until 72 hours ($p<0.05$). There were no differences in IL-6 production between *S. siliquosum* extracts. Further, treatment with100µg/mL to 200µg/ml produced significantly lower IL-6 than treatment with 50µg/mL and 25µg/mL.

Addition of LPS resulted also to significant increase in the production of TNF-α as high as 652 (±66) pg/mL and 427 (±108) pg/mL after 48 and 72 hours respectively. This over-expression was 54 times and 35 times fold higher than the concentration of TNF-α in unstimulated PBMCs at 12 pg/mL. The optimum effect was seen at 200 µg/mL and reduction was higher in AQ (96%) than F1 (79%) *(Fig.6)*. However, the effect was not dose-dependent.

PT And APTT Coagulation Assays

PT is a coagulation assay which measures the activity of extrinsic and common coagulation factors. The mean PT (in seconds) of the control and all the varying concentrations of both AQ and F1 extracts did not exceed the reference range (11.4 to 15.8 seconds). ANOVA revealed that the anticoagulant activity of AQ extract did not differ significantly with F1 in PT assay.

APTT is clot-based screening test for intrinsic and common coagulation pathways. The mean APTT (in seconds) of the control was within the reference range (24.5-36.5 seconds). However, both F1 and AQ extracts prolonged the APTT remarkably than the control. ANOVA showed that F1 extract had significantly higher anticoagulant activity than AQ extract in APTT assay. The most potent concentration that delayed APTT for up to 252 seconds and 304 seconds was noted at 100 mg/dl for both AQ and F1 extracts, respectively. *(Fig 7)*

Discussion

The cytokines are a very diverse group of soluble glycoproteins and low-molecular weight peptide. They directly mediate interactions between cells, regulate cell and tissue functions, coordinate embryonic development, cell growth and maturation, wound repair and healing. They also mediate immune responses including acute phase reactions and septic shock, and new blood vessel formation. The biologic effects and function of pro-inflammatory cytokines involve systemic and local effects that have influence on immunologic properties including T-cell and B-cell growth activation (Dunlop and Campbell, 2000). Clinical and experimental evidence suggests that shock, arthritis, osteoporosis, colitis, leukemia, diabetes, atherosclerosis and cancers are mediated, in part by excessive production of these cytokines. Over-expression of these

cytokines has been implicated in cancer-related inflammation and in the tumourigenesis of breast cancer, ovarian cancer, prostate cancer, lung cancer, cervical cancer, multiple myeloma, renal cell carcinoma, and melanoma (Chang *et al*., 2005(b); Gerlo *et al*., 2008; Grivenikov *et al*., 2009; Matzaraki *et al*., 2007; Li *et al*., 2009).

The production of IL-1, IL-6 and TNF-α was upregulated by nanomolecular dose of LPS as a result of its binding to the monocyte surface and the subsequent intracellular signalling leading to proinflammatory cytokine gene transcription (Baldwin, 1996; Gori *et al*., 2004). The addition of LPS to PBMC cells result in an activation of the transcription factor NF-κB, which plays a crucial roles in regulating the expression of many pro-inflammatory cytokine genes involved in the inflammatory responses, such as those encoding for TNF-α, IL-6, IL-1β (Hochart *et al*., 2006). The exact mechanisms of the inhibitory effect of sulfated polysaccharides on the secretion of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 by LPS-stimulated PBMC cells are unknown (Matsumoto *et al*., 2004).

Blood coagulation comprises a series of enzymatic reactions whereby an inactive proenzyme is converted to the active enzymatic form, which, in turn, promotes activation of other proenzymes by cascade mechanisms. The next step is the conversion of fibrinogen to fibrin, and its polymerization to cross-linked form and therefore the clot formation (De Lara-Issasi, *et al. 2004).* It was observed that AQ and F1 extracts have stronger effect on APTT than PT. It was also noted that F1 extract delayed APTT longer than AQ. Studies have shown that most widely recognized bioactivity of marine sulfated polysaccharides is the heparin-like anticoagulant activity exhibited by fucoidans and other fucans of brown seaweeds (Jiao *et al*, 2011, Irhimeh *et al*. 2010). In this case, the exact mechanisms of the delaying effect of sulfated polysaccharides on the coagulation time have to be investigated further.

In conclusion, extracts of the *S. siliquosum* J.G. Agardh is a potential alternative source in producing anti-inflammatory and anticoagulant substances in the future. Its effect in the reduction of cytokines and inhibition of the intrinsic coagulation cascade is dose-dependent.

Acknowledgement

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FIGURE LEGENDS

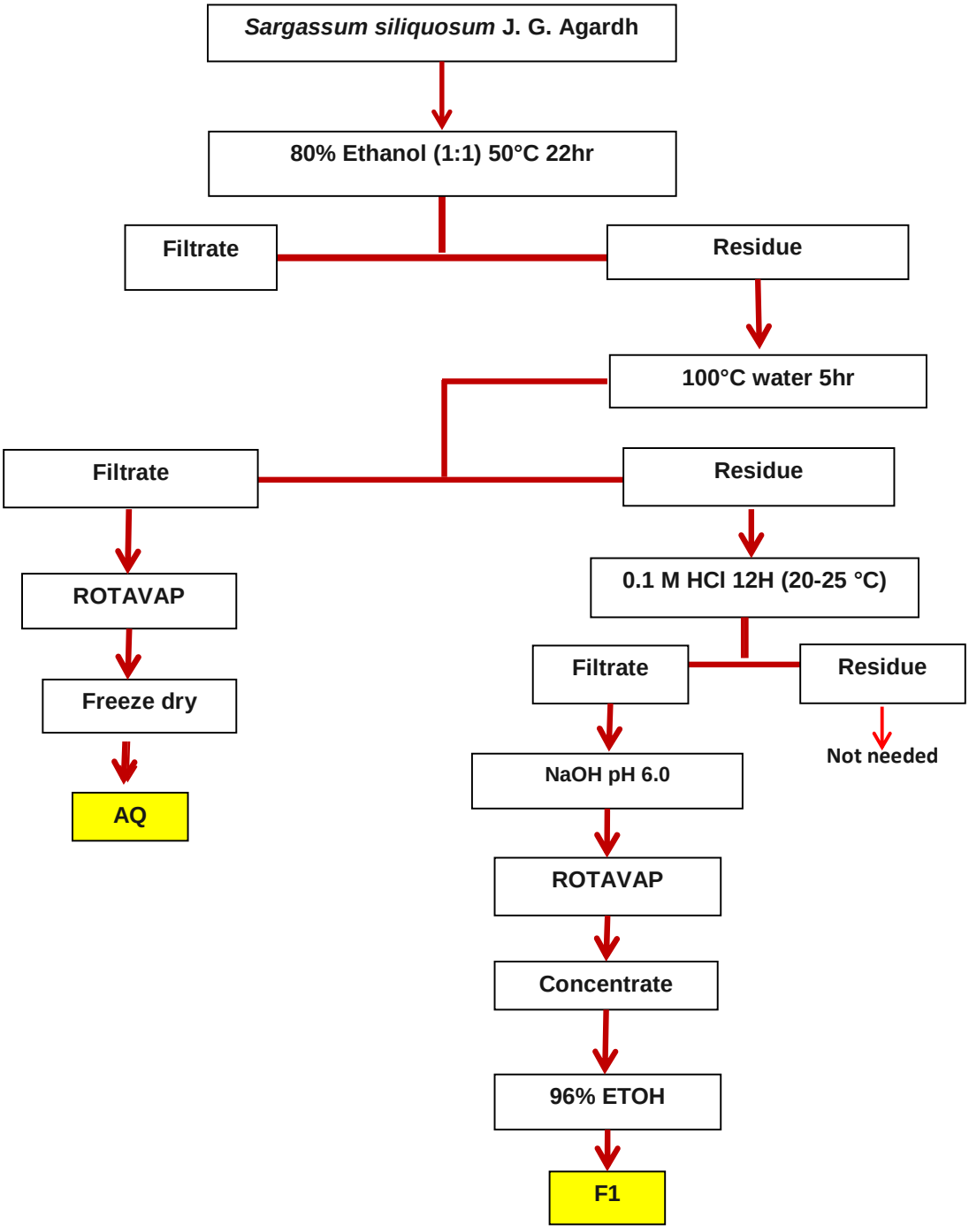

Fig 1.Scheme of the extraction of polysaccharides from *S. siliquosum* J.G. Agardh. Lyophilized fractions (AQ, F1) were used for the analysis of cytokines production and anticoagulant activity.

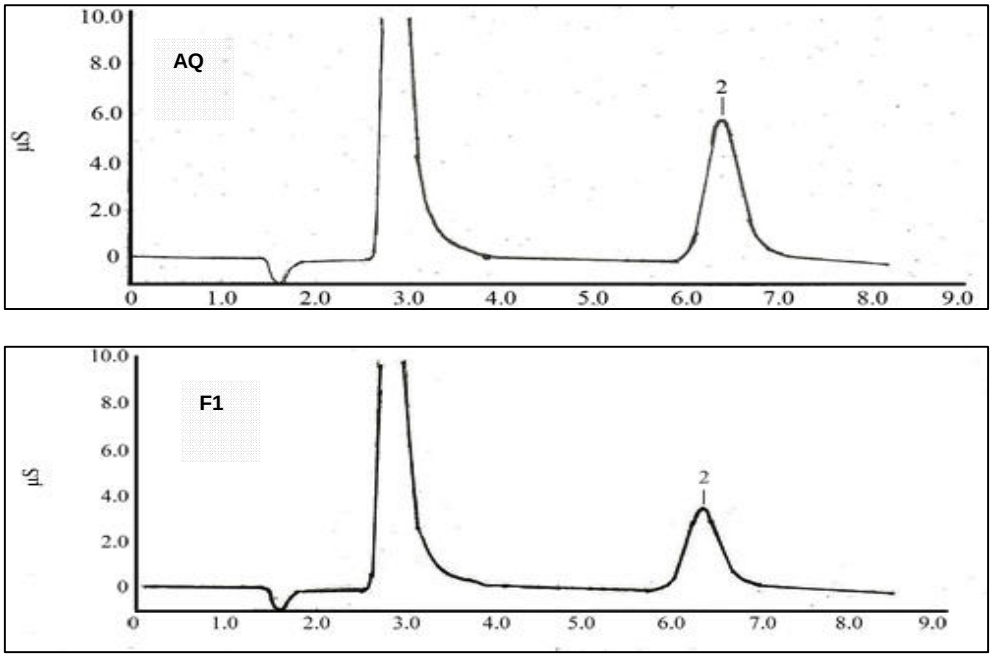

Fig 2.The sample chromatograms of 2 polysaccharides from *S. siliquosum*including aqueous (AQ) and fucoidan (F1) extracts. The sulfate analysis was carried out as described in the experimental section. Sulfate was successfully eluted.

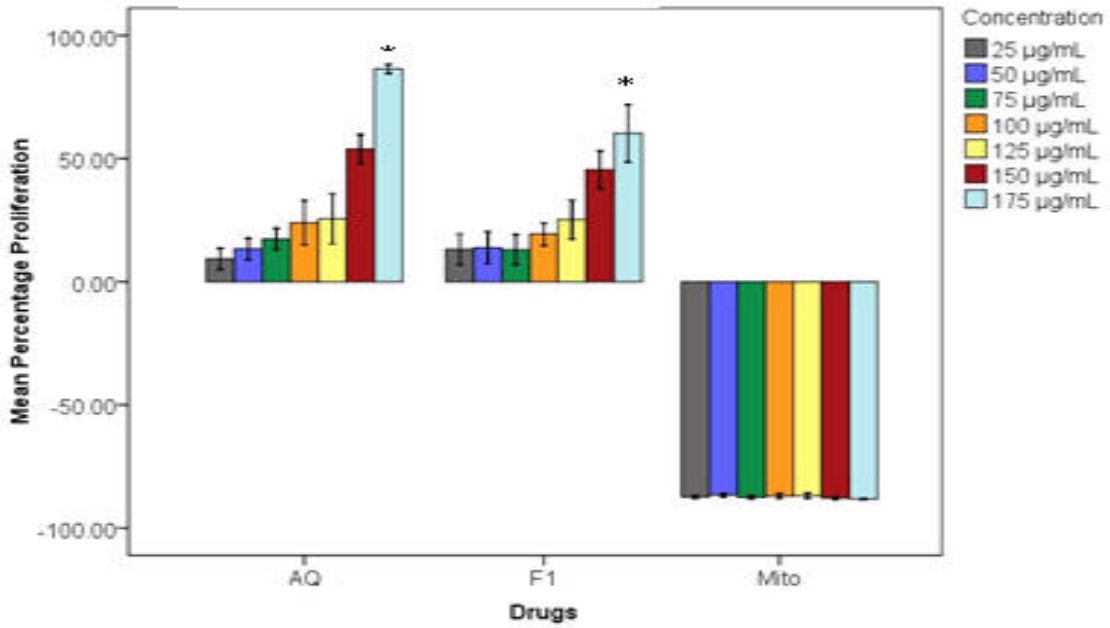

Fig. 3. Effect of the sulfated polysaccharides AQ and F1 on cell proliferation of PBMCs after 72 hours. Cells were seeded (1.6 x 10^6cells/mL in 96-well plates in 100µl of RPMI 1640 medium followed by treatment with extracts (25-175 µg/mL or Mitomycin (25-175 µg/mL). The cell viability was assessed using the MTT assay. Data representas the means ± SEM of 3 trials (n=3), * significantly different ($p<0.05$).

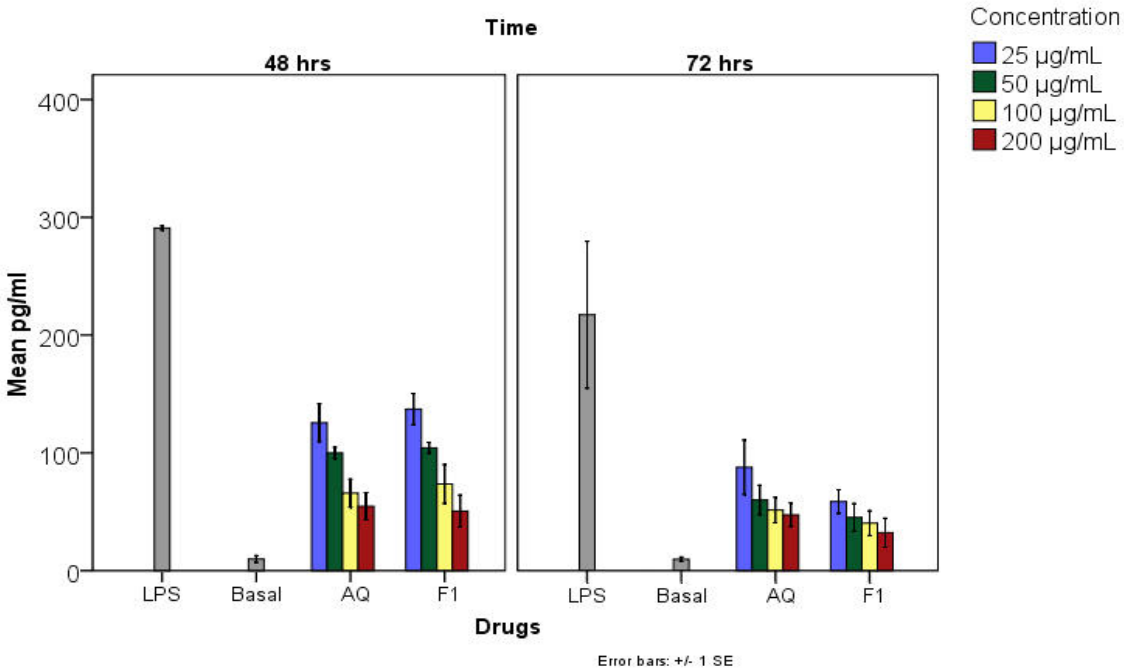

Fig. 4 Effects of *S. siliquosum* extracts on the production of IL-1. PBMC cells (1.0 X 10^6) cells/mL were stimulated with LPS (10µg/mL) alone and with the indicated doses of the extracts for 48 and 72 hours. The level of the cytokine in the supernatants of the culture was determined by ELISA (eBioscience). Data represent as the means ±SEM of 3 trials (n=3).

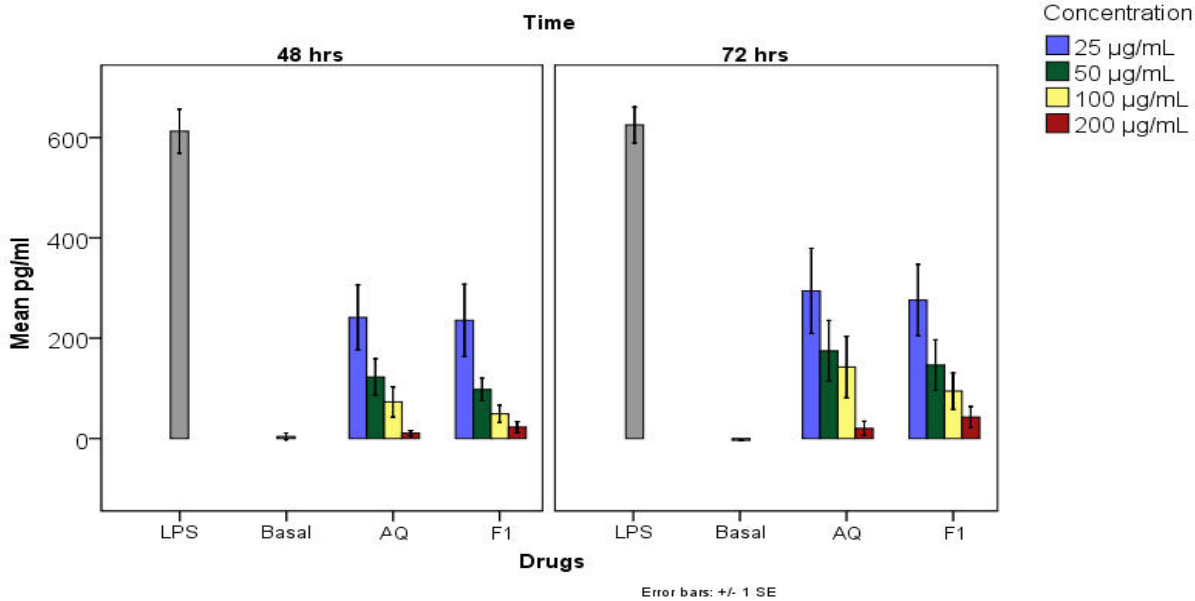

Fig. 5 Effects of *S. siliquosum* extracts on the production of IL-6. PBMC cells (1.0 X 10^6) cells/mL were stimulated with LPS (10µg/mL) alone and with the indicated doses of the extracts for 48 and 72 hours. The level of the cytokine in the supernatants of the culture was determined by ELISA (eBioscience). Data represent as the means ±SEM of 3 trials (n=3).

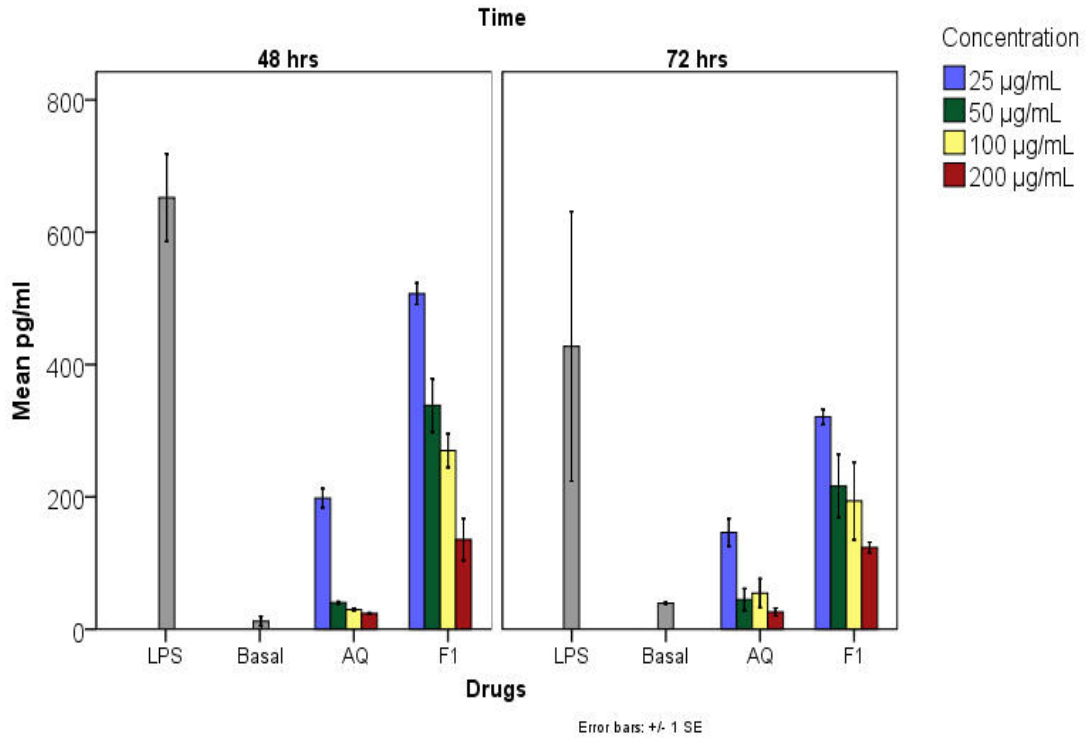

Fig. 6. Effects of *S. siliquosum* extracts on the production of TNF-α. PBMC cells (1.0 X 10^6) cells/mL were stimulated with LPS (10µg/ml) alone and with the indicated doses of the extracts for 48 and 72 hours. The level of the cytokine in the supernatants of the culture was determined by ELISA (eBioscience). Data represent as the means ±SEM of 3 trials (n=3).

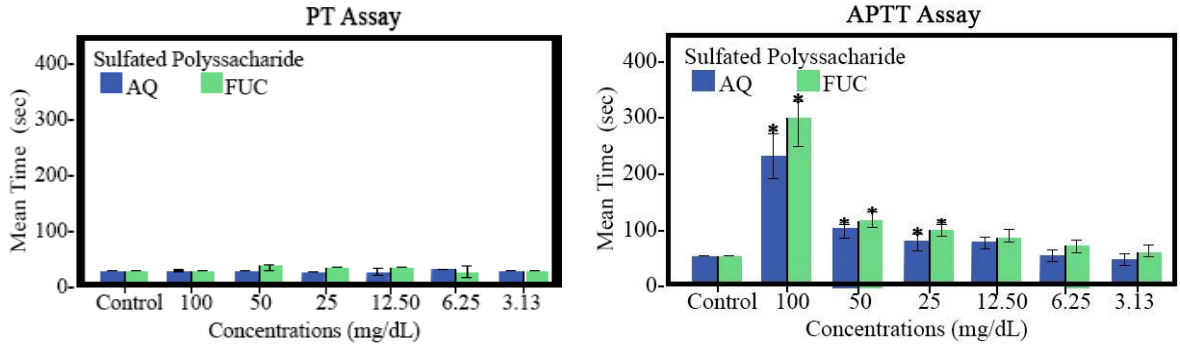

Fig. 7 Effects *of S. siliquosum* extracts in coagulation time. Citrated plasma (90µl) was mixed with varying concentrations of AQ and F1 extracts (10 µl) which were prepared by 2-fold serial dilutions. The clotting time was determined by PT and APTT assays (STA-Neoplastine CI Plus). Data represent as the means ±SEM of 3 trials (n=3).
* significantly different ($p<0.05$).

Table 1: Retention time, peak areas and percent sulfate in the two extracts from *S. siliquosum.* Values are means of duplicate experiments.

Extracts	**Retention time**	**Peak areas**	**Sulfate, %**
AQ	6.40	940956	11
F1	6.52	834406	3.5