Anti-inflammatory effects and mechanisms of *Hizikia fusiformis* via multicellular signaling pathways in lipopolysaccharide-induced RAW 264.7 cells

Bhesh Raj Sharma[1], Chan Seon Park[2], Seung Jin Ma[3] and Dong Young Rhyu[1,4*]

[1]Department of Oriental Medicine Resources and Institute of Korean Medicine Industry, Mokpo National University, Jeonnam, Republic of Korea

[2]Department of Marine and Fisheries Resources, Mokpo National University, Jeonnam, Republic of Korea

[3]Department of Food Engineering, Mokpo National University, Jeonnam, Republic of Korea

[4]Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, USA

Abstract: In this study we investigated the anti-inflammatory effects and mechanisms of *Hizikia fusiformis* (HF) extracts in lipopolysaccharide (LPS)-induced RAW 264.7 cells. We extracted HF using solvent and sub-critical water techniques. In results, HF extracts inhibited nitric oxide (NO) production in cell-free and LPS-stimulated RAW 264.7 cells. HF210 (extract prepared with sub critical water at 210°C) was most effective. The HF210 extract dose-dependently inhibited inducible nitric oxide synthase expression (iNOS) and nuclear factor kappa (NF-κB) p65 translocation from cytosol to the nucleus. Furthermore, HF210 extract dose-dependently inhibited the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), Jun N-terminal kinase (JNK), and signal transducers and activators of transcription (STAT)-1in LPS-induced RAW 264.7 cells. Thus, our results suggest that anti-inflammatory effects of HF210 extract showed a noticeable distinction by regulation of multiple signaling pathways in LPS-induced RAW 264.7 cells.

Keywords: Sub critical water extract, inflammation, nuclear factor kappa B, mitogen-activated protein kinase.

INTRODUCTION

Inflammation is a complex defense mechanism against microbial and endotoxin infections, wounds and irritants, which seeks to restore the normal cell structure and function (Buckley *et al.,* 2013). In response to inflammation, macrophages play key roles for important homeostatic function through the production of various cytokines and growth factors (Fujiwara and Kobayashi, 2005). However, when macrophages activate by lipopolysacchride (LPS) in cell culture system, it stimulate the excessive production of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, and pro-inflammatory mediators, such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 (Kim and Kim, 2011). The release of various inflammatory mediators is associated with the progress of many inflammatory diseases, such as asthma, rheumatoid arthritis, cancer, diabetes, obesity, and atherosclerosis (Caruso *et al.,* 2004, Pan *et al.,* 2008). Nuclear factor kappa B (NF-κB) and Janus kinase/signal transducers and activators of transcription (JAK/STAT) are transcriptional regulators that are crucial in responses to inflammatory signaling, and can lead to the over expression of inflammatory mediators and cytokines during inflammation (Walker *et al.,* 2006; Lawrence, 2009). Mitogen-activated protein kinases (MAPKs) are critical signaling pathway modulating the activation of inflammatory transcription factors in inflammatory cells (Johnson and Lapadat, 2002). Blockage of signaling pathways to inhibit the release of inflammatory molecules would be a novel strategy for the treatment of inflammatory diseases. However, currently available anti-inflammatory drugs, including glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs), and other immune-suppressants are associated with side effects which limits their long-term use (Higuchi, 2006). Natural products that can block these inflammatory pathways may be developed as potential anti-inflammatory agents with safety and utility (Maroon *et al.,* 2010).

Hizikia fusiformis (HF), an edible brown seaweed that grows largely in temperate seaside areas of Korea, Japan, and China, has been used as a healthy food complement for millennia in East Asian Countries (Wu *et al.,* 2013). To date, immune-modulatory (Shan *et al.,* 1999), anti-cancer (Huh *et al.,* 2012), and anti-oxidative (Wu *et al.,* 2013) activities of HF have been reported. However, the other potential therapeutic effects of HF have not been studied, yet. In this regard, we evaluated NO inhibitory effects of different HF extracts, which were prepared by conventional solvent and sub critical water extraction techniques. Then, we measured anti-inflammatory mechanisms of the HF extract, using LPS-induced RAW 264.7 cells.

**Corresponding author:* e-mail: rhyudy@mokpo.ac.kr

MATERIALS AND METHODS

Reagents

LPS, sodiumnitropruside (SNP), thiazolyl blue tetrazolium bromide (MTT), N-(1-naphthyl) ethylene-diaminedihydrochloride, sulfonamide, phosphatase inhibitor cocktail 2 and β-actin (A5441) antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). iNOS (SC-650) and NF-κB p65 (SC-372) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). JNK (9258), p-JNK (4671), p38 MAPK (9212), p-p38 MAPK (9211), STAT1 (9172) and p-STAT1 (9171) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Nuclear and cytoplasmic extraction reagent (78833) was obtained from Thermo Scientific (Rockford, IL, USA). All chemicals used in the experients were of analytical grade and obtained from Sigma-Aldrich unless otherwise specified.

Preparation of extracts

HF was collected from the coastal area of Jangheung-gun, Korea. The material for extraction was cleaned up by the removal of epiphytes and sand using tap water, and dried at RT for about 24h. Then, dried HF was ground into a fine powder and was extracted by solvent extraction and sub critical water extraction. Solvent extracts were prepared by extracting the fine powder with 80% ethanol, 80% methanol, and distilled water at room temperature for 24h. Sub critical water extracts were prepared using 3-mega-pascal pressure and hot water for 1min at different temperatures; 90, 150 and 210°C.

Measurement of NO scavenging activity in cell-free system

SNP (10mM) and different concentrations of the sample were mixed for 150min at 25°C. Griess Reagent (5% phosphoric acid containing 0.1% NEDD and 1% sulfanilamide) was added to the reaction mixture and absorbance was measured at 540nm using an Immuno Mini NJ-2300 spectrophotometer (Inter-Med, Tokyo, Japan).

Cell culture and cell viability assay

RAW 264.7 cells (KCLB 40071), purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea), were cultured in RPMI 1640 medium which contained 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2. MTT assay was used to measure the cell viability. Briefly, RAW 264.7 cells (2x10^4 cells/well) were seeded in a 96-well plate. After 4 h, the different concentrations of HF extracts were added to each wells and then incubated for 24h. Then, MTT solution (final concentration 0.2mg/ml) was added to the wells and incubated for 4h at 37°C. Finally, dimethylsulfoxide was added to dissolve the formazan crystals, and absorbance was measured at 540nm using the spectrophotometer.

Measurement of NO production in cell culture system

Briefly, after treatment of the sample and LPS (1μg/ml) for 24h in RAW 264.7 cells, the culture supernatant was incubated with Griess reagent for 5min and absorbance was measured as described above. The concentration of NO was calculated by a linear standard curve generated from serial dilutions of sodium nitrite in working medium (Sharma *et al.,* 2014).

Western blot analysis

Western blot analysis was performed as described previously (Sharma and Rhyu, 2014)

STATISTICAL ANALYSIS

SPSS (SPSS, Chicago, IL, USA) was used for statistical data analysis. All data are presented as mean ±SE. Data were analyzed by one-way ANOVA and Duncan's *post hoc* test of multiple comparisons. Bars not mentioning the same letters are significantly different at p<0.05 by Duncan's multiple comparison test.

RESULTS

Effect of different HF extracts on NO scavenging activity in cell-free system

Solvent and sub critical water extracts of HF were initially tested for their NO scavenging activity in a cell-free system (table 1). The sub critical water extracts of HF were more effective than solvent extracts of HF. We found that HF210 extract inhibited NO production by 51%, which is stronger inhibition than other extracts.

Effect of different HF extracts on NO production and iNOS expression in LPS-induced RAW 264.7 cells

RAW 264.7 cells were exposed to the various concentrations of HF extracts for 24h to test their cytotoxicity. MTT assay revealed no evidence of cytotoxicity (data not shown). LPS treatment significantly increased NO production (from 11.6 to 49.3μM) in RAW 264.7 cells. However, the addition of different HF extracts effectively inhibited NO production in LPS-induced RAW 264.7 cells (fig. 1). HF210 extract showed strong inhibition in a dose-dependent way (fig.1 and 2). Further, 125 and 250μg/ml of HF210 extract dose-dependently decreased iNOS expression in LPS-induced RAW 264.7 cells (fig. 2).

Effect of HF210 extract on NF-κB p65 nuclear translocation in LPS-induced RAW 264.7 cells

LPS stimulated the translocation of NF-κB p65 from cytosol to the nucleus in RAW 264.7 cells (fig. 3). NF-κB p65 was over-expressed in the nucleus and is under-expressed in the cytosol by exposure to LPS. However, the addition of HF210 extract inhibited NF-κB p65 translocation from cytosol to the nucleus in a concentration-dependent way, by decreasing and

increasing its expression to the nucleus and cytosol, respectively.

Effect of HF210 extract on MAPKs expression in LPS-induced RAW 264.7 cells

The expression of p-JNK and p-p38 were markedly increased by the treatment of LPS in RAW 264.7 cells (fig. 4). However, the addition of 125 and 250µg/ml of HF210 extract significantly decreased their expression in a dose-dependent way.

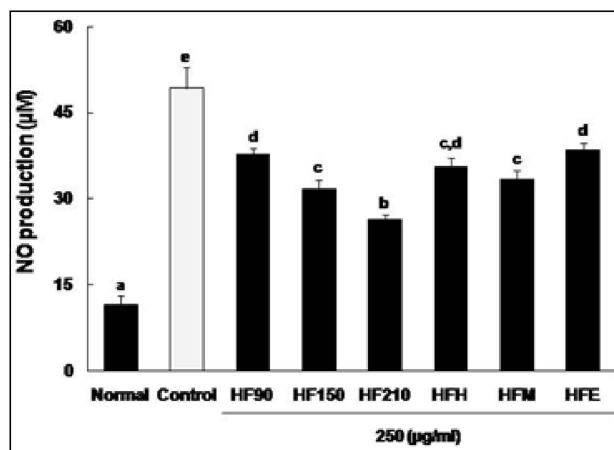

Fig. 1: Effect of different HF extracts on NO production in RAW 264.7 cells. Cells were pretreated with 250µg/ml of HF for 1 h and then treated with LPS (1µg/ml). After 24h, medium was collected, and the released NO was measured using Griess reagent. Normal, LPS untreated; Control, LPS treated; HF90, sub critical water extract at 90°C and 3MPa pressure; HF150, sub critical water extract at 150°C and 3MPa pressure; HF210, sub critical water extract at 210°C and 3MPa pressure; HFH, water extract; HFM, methanol extract; HFE, ethanol extract. Each value is the mean±SE of three independent experiments. Bars with different letters are significantly different at p<0.05 by Duncan's multiple comparison test.

Effect of HF210 extract on STAT1 expression in LPS-induced RAW 264.7 cells

As shown in fig. 5, STAT1 is highly phosphorylated by the treatment of LPS in RAW 264.7 cells. However, the addition of 125 and 250µg/ml of HF210 extract significantly decreased p-STAT1 expression in a dose dependent manner.

DISCUSSION

Plant-derived natural products have been used as an alternative therapy for the treatment of various biological disorders for centuries. Recently, several natural products have been reported to have anti-inflammatory activities (Ali *et al.,* 2011; Sharma *et al.,* 2015). We found that, among the different HF extracts, HF210 extract strongly inhibited NO production in both cell-free system and cell-based system, when compared with other extracts. This was further confirmed from its potency to decrease iNOS expression in LPS-induced RAW 264.7 cells. Moreover, HF210 extract effectively inhibited NF-κB translocation from the cytosol to the nucleus, MAPKs activation, and STAT1 phosphorylation, which are the main targets of novel anti-inflammatory drugs.

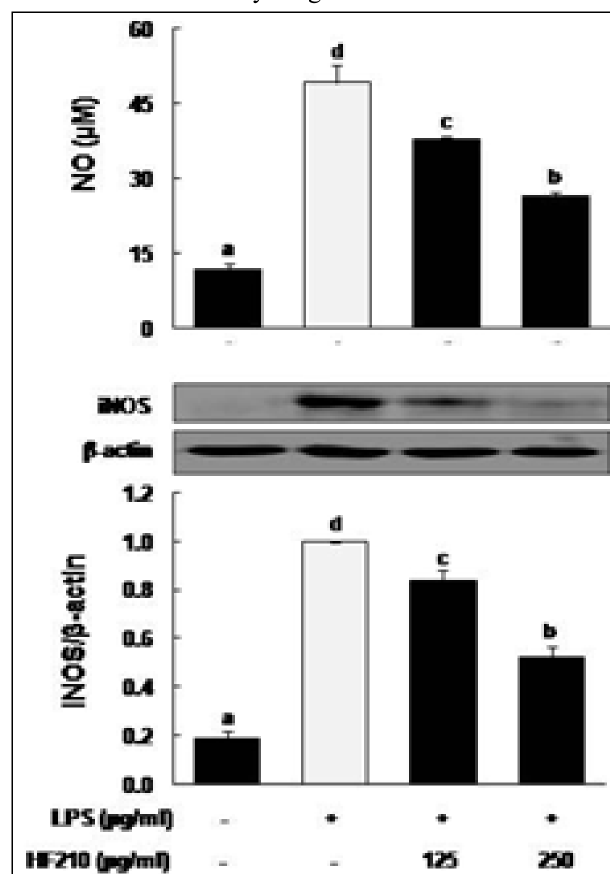

Fig. 2: Effect of HF210 extract on NO production and iNOS expression in LPS-induced RAW 264.7 cells. Cells were pretreated with the indicated concentration of HF210 extract for 1h and then treated with LPS (1µg/ml). After 24h, the cells were lysed and the level of protein in the lysate was examined by western blot. Each value is the mean±SE of three independent experiments. Bars with different letters are significantly different at p<0.05 by Duncan's multiple comparison test.

Numerous anti-inflammatory plant extracts are reported to have NO scavenging activities in a cell free system (Karki *et al.,* 2013). Therefore, sub critical water and solvent extracts of HF were initially tested for their NO scavenging activity in the cell-free system (table 1). The sub critical water extracts of HF were more effective than solvent extracts of HF. Conventional solvent extraction needs long extraction time, and results in the low yield of extracts; however, sub critical water extraction takes a short time with high yield of bioactive compounds, including polyphenols and flavonoids (Lianx and Fan,

2013). Therefore, we can speculate that different bio-active compounds formed during sub critical water extraction could scavenge NO formation.

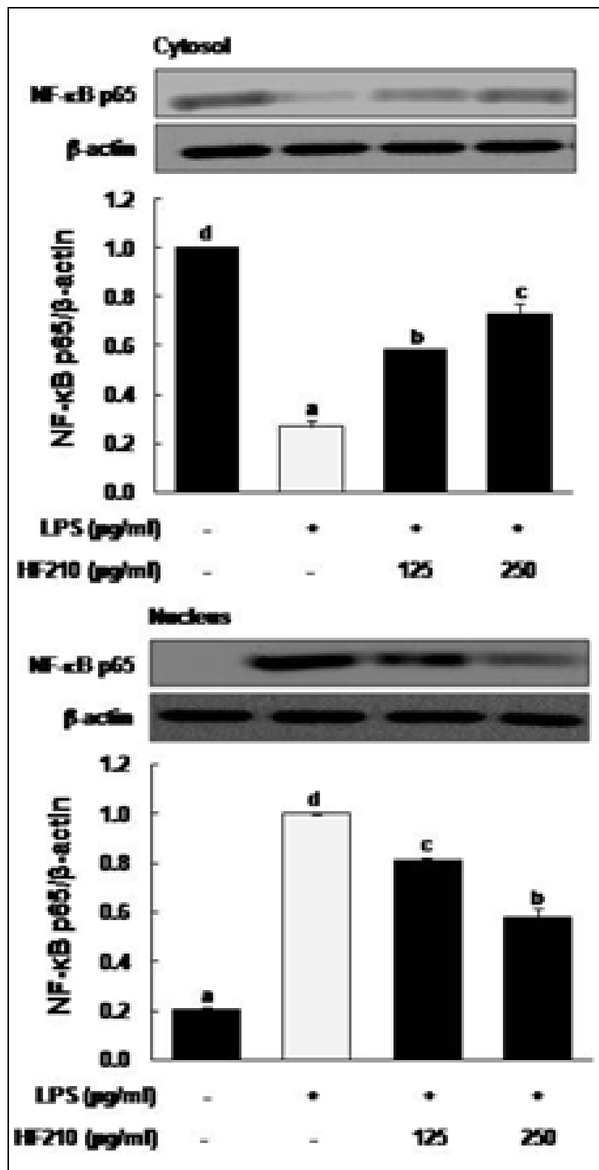

Fig. 3: Effect of HF210 extract on NF-κB p65 translocation in LPS induced RAW 264.7 cells. Cells were pretreated with the indicated concentration of HF extract for 1h and then treated with LPS (1μg/ml) for 20 min, and cytosolic and nuclear protein levels were prepared by cell lysis. Then, the level of protein was examined by western blot. Each value is the mean±SE of three independent experiments. Bars with different letters are significantly different at $p<0.05$ by Duncan's multiple comparison test.

Macrophages, which are widely distributed throughout the body, has an important role for the initiation and amplification of the various inflammatory diseases. They secrete excessive pro-inflammatory molecules, including NO, which are up-regulated by iNOS and COX-2 over-expression during inflammation (Verma *et al.,* 2012). Therefore, we measured the anti-inflammatory effects of different HF extracts using established murine macrophage RAW 264.7 cell line. HF210 extract exhibited stronger NO inhibitory activity than other extracts. High yield of bioactive compounds, such as flavonoids, tannins, polysaccharides, lignin etc., formed during sub critical water extraction (Lianx and Fan, 2013) may have exhibited strong NO inhibitory activities. In fact, several natural products have been reported to have NO inhibitory activity by preventing iNOS expression in RAW 264.7 cells (Lauren *et al.,* 2009). In our results, we also found that HF210 extract strongly inhibited iNOS expression. Therefore, anti-inflammatory effects of HF210 extract could be through the regulation of iNOS expression.

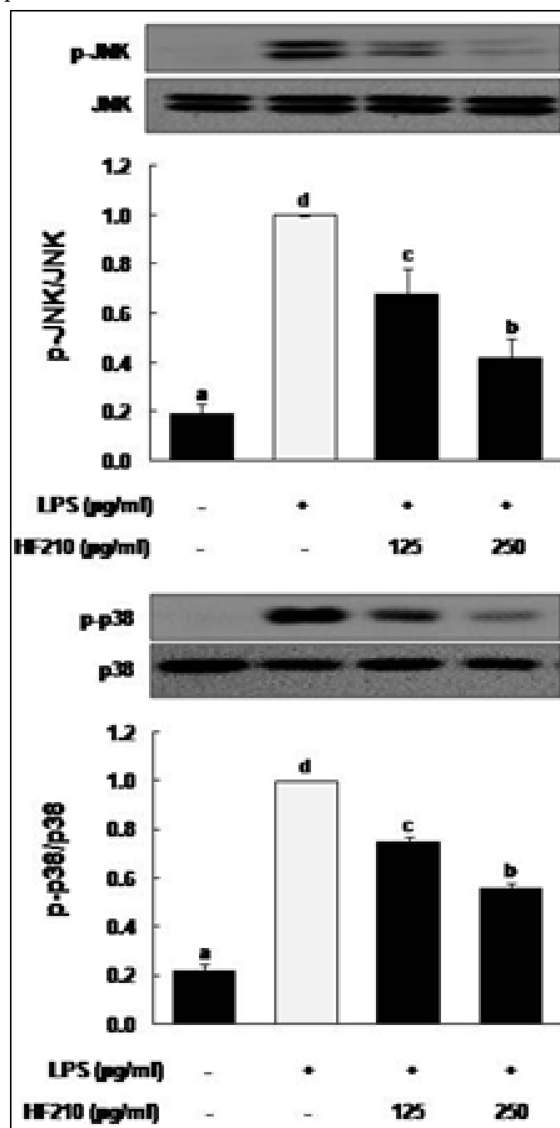

Fig. 4: Effect of HF210 extract on MAPKs (JNK and p38) expression in LPS induced RAW 264.7 cells. Cells were pretreated with the indicated concentration of HF

extract for 1 h, and then treated with LPS (1µg/ml) for 25 min, then the cells were lysed and the levels of proteins in the lysates were examined by western blot. Each value is the mean±SE of three independent experiments. Bars with different letters are significantly different at $p<0.05$ by Duncan's multiple comparison test.

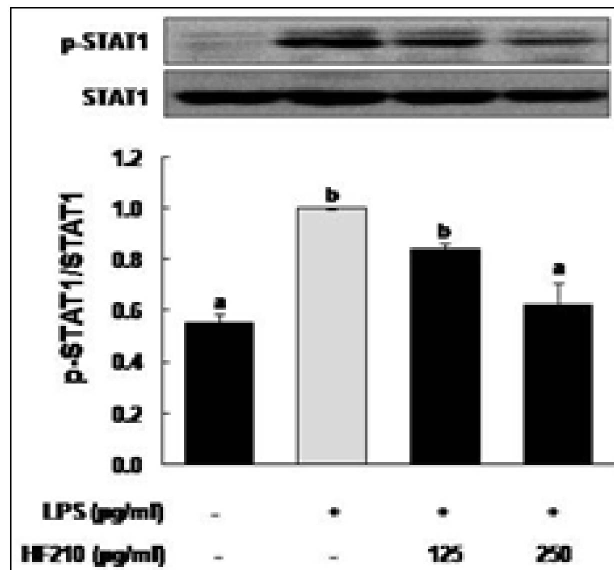

Fig. 5: Effect of HF210 extract on p-STAT1 expression in LPS induced RAW 264.7 cells. Cells were pretreated with the indicated concentration of HF210 extract for 1h and then treated with LPS (1µg/ml) for 30min, then the cells were lysed, and the level of protein in the lysate was examined by western blot. Each value is the mean±SE of three independent experiments. Bars with different letters are significantly different at $p<0.05$ by Duncan's multiple comparison test.

NF-κB is a major transcription factor that regulates the expression of genes encoding inflammatory cytokines and modulators during inflammation. It forms a cytoplasmic complex under normal physiological conditions, but when activated by LPS, it is released from the inactive complex and trans locates into the nucleus to activate different pro-inflammatory target genes, such as iNOS and COX-2 (Israf *et al.,* 2007). Determination of NF-κB translocation by western blotting shows that HF210 extract strongly inhibited its translocation from cytosol to the nucleus. Since MAPKs regulate NF-κB transcriptional activity by phosphorylation of its IκBα subunit and mediate cell signal transduction from the cell surface to the nucleus (Kaminska, 2005; Hoesel and Schmid, 2013), we measured its effect on MAPKs, such as JNK and p38 activation in RAW cells. Interestingly, HF210 extract strongly inhibited both JNK and p38 activation in RAW 264.7 cells. Therefore, the anti-inflammatory mechanism of HF210 extract is closely linked with the modulation of NF-κB activation through JNK and p38 MAPK signaling pathway in LPS-mediated RAW 264.7 cells.

Table 1: Effect of different HF extracts on NO scavenging activity in cell-free system.

HF extracts	NO scavenging activity (%)		
	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)
HF90	14.6±0.5	21.3±0.4	43.0±1.2
HF150	19.1±0.4	29.7±0.2	46.1±0.1
HF210	47.2±0.2	49.9±0.3	50.9±0.2
HFH	12.9±0.5	21.2±0.3	34.3±0.3
HFM	4.8±0.2	4.1±3.3	5.9±4.4
HFE	0.5±2.8	1.1±0.9	9.9±6.0

NO scavenging activities of different HF extracts in cell free system, using sodium nitroprusside as a NO donor. HF90, sub critical water extract at 90°C temperature and 3 MPa pressure; HF150, sub critical water extract at 150°C temperature and 3 MPa pressure; HF210, sub critical water extract at 210°C temperature and 3 MPa pressure; HFH, water extract; HFM, methanol extract; HFE, ethanol extract. Each value is the mean±SE of 3independent experiments.

The JAK/STAT pathway is activated by a large number of cytokines during the inflammatory response. The STAT proteins are basically involved in the regulation of growth, survival, and differentiation in various cells, and these transcription factors are activated by phosphorylation of JAK (Aaronson and Horvath, 2002). The control of cytokine-mediated JAK/STAT signaling pathway has been recently identified as a therapeutic target for inflammatory diseases (Coskun *et al.,* 2013). Recently, Jin *et al*., reported the anti-inflammatory effects of isoegomaketone by inhibiting p-STAT1 expression in LPS-induced RAW264.7 cells (Jin *et al.,* 2010). Similarly, our results showed that the treatment of 125 and 250µg/ml of HF210 extract significantly decreased p-STAT1 expression in a dose dependent manner in LPS-induced RAW 264.7 cells (fig. 5).

CONCLUSION

Taken together, we found the anti-inflammatory effects of HF210 extract, using LPS-stimulated RAW 264.7 cells. Moreover, the anti-inflammatory effects of HF210 extract are mediated by blocking NF-κB translocation, MAPKs activation, and STAT1 phosphorylation in LPS-treated cells. Based on all these evidence, HF210 extract is proposed as a promising natural product for the prevention and therapy of inflammation related diseases. However, further studies are needed to standardize HF210 extract by characterizing their chemical components.

ACKNOWLEDGEMENTS

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