Journal of Fisheries
Volume 9 Issue 1 April 2021
Peer Reviewed | Open Access | Online First

eISSN 2311-3111
pISSN 2311-729X

DOI: https://doi.org/10.17017/j.fish.218

Original Article

Investigation on the effects of *Inula viscosa* L. on rainbow trout gonad cells induced by lipopolysaccharide

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Manuscript history
Received 2 March 2020 | Revised 13 December 2020 | Accepted 27 February 2021 | Published online 13 April 2021

Citation
Küçükgül A, Küçükgül A, Metin Korkmaz T (2021) Investigation on the effects of *Inula viscosa* L. on rainbow trout gonad cells induced by lipopolysaccharide. Journal of Fisheries 9(1): 91203. DOI: 10.17017/j.fish.218

Abstract
Fish cells have been accepted as an alternative to in vivo assay for inflammatory effects of therapeutic experimental systems. To reveal the anti-inflammatory effects of *Inula viscosa* (IV) fish cell line RTG-2, derived from the gonadal tissue of rainbow trout, was infected with lipopolysaccharide (LPS). Effective concentrations for different concentrations of LPS (1, 5, 10, 20 µM) and IN (1, 5, 10, 20 µg ml^{-1}) were determined. While the 20 µM concentration of LPS, which was effectively selected from preliminary tests, caused 27% cell loss, the effective 1 µg ml^{-1} concentration of IN caused 1.1% proliferation in the cells when compared to the control group. All pro-inflammatory parameters investigated in LPS-induced RTG-2 cells showed up-regulation, with the highest increase in TNF-α gene expression level (11.3 fold changes). Down-regulation was determined in the IN together with LPS administered group and IL-1β had the highest effect with 96%. IL-6 protein level decreased by LPS at a rate of 4% and IN together with LPS increased by 9%. The IN exhibited significant efficacy against inflammation caused by LPS. However, further studies are needed to determine pharmacological activity of *I. viscosa* in details.

Keywords: Endotoxin; *Inula viscosa*; inflammation; rainbow trout gonad cell line

1 | INTRODUCTION

Infectious bacterial diseases in fish have attracted researchers' attention and of interest for many years because of their impacts on fish health. Mortality and economic losses associated with these diseases make up almost 10% of total production (Jarp *et al.* 1994). Bacterial infections that cause diseases in fish are mostly originated from gram negative (Gr–) bacteria (Akaylı *et al*. 2015; Olesen and Vendramin 2015; Küçükgül et al 2019a, 2019b). The lipopolysaccharide (LPS), which plays a key role in the pathogenesis of Gr– bacterial infections, is a central component of the outer membrane of these bacteria (Whitfield and Trent 2014). Bacterial LPS, also called endotoxins which are considered to be the main virulence factor, are responsible for the lethal effects and clinical manifestations of diseases in humans and animals (Morrison *et al.* 1985; Brandtzaeg *et al.* 2001; Küçükgül *et al.* 2019a). High vertebrate animals are susceptible to endotoxin even at very low doses, while lower vertebrates such as fish are often resistant to endotoxic shock. However, LPS has the ability to express cytokines and acute phase proteins and also exhibits immunological, pathological, physiological, immuno-endocrinological and neuroimmunological effects in various fish species (Pepels *et al.* 2004; Küçükgül *et al.* 2019a). During infection, bacterial LPS is exposed to the release of cytokines by inflammatory cells that play an indispensable role in the host's fight against microorganisms. However, excessive cytokine

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production (TNF-α: tumor necrosis factor-alpha, IL-1ß: Interleukin-1beta and IL-6: Interleukin-6, etc.) causes cell damage and inflammatory diseases (Jin *et al.* 2011). During cytokine stimulation following bacterial infection, IL-6, an important interleukin and proinflammatory cytokines such as TNF-α and IL-β, are released and they have a key role in antibacterial, antiparasitic and antiviral immunity (Nam *et al.* 2007; Mladineo and Block 2010; Verriera *et al.* 2011; Küçükgül *et al.* 2019a).

Cell culture applications have many advantages such as compatibility with the results obtained in in vivo studies and the possibility of examination at the cellular level. Fish cell lines play an important role in the studies of aquatic virology, developmental biology, genetics, immunology, physiology, toxicology and pharmacology (Baksi and Frazier 1990; Bols 1991; Kohlpoth *et al.* 1999). RTG-2 is a well-characterized fibroblastic rainbow trout cell line derived from both male and female gonadal tissue (Wolf and Quimby 1962). Many in vitro rainbow trout studies have been performed using this cell line (Bols 1991; Kohlpoth *et al.* 1999).

Antibiotic treatments, which were used unconsciously in aquaculture in the past, have been chosen as an effective way of combating fish diseases. However, many handicaps of antibiotics (such as resistance development and residues) caused more harm than good (Schmidt *et al.* 2000; Miranda and Zemelman 2002; Sorum 2006). In recent years, scientists working on the subject have looked for alternative methods which are more useful and have less risk factor and herbal products used for medical purposes have been considered an alternative treatment strategy (Harikrishnan and Balasundram 2008; Citarasu 2010; Harikrishnan *et al.* 2011; Kucukgul *et al.* 2019a).

Inula viscosa L., a member of the Asteraceae family, is popularly known as sticky stalk. This species, which is considered an alternative to antibiotics in the treatment of diseases, plays a role in many biological activities (antifungal, anti-inflammatory, antibacterial, anticancer, etc.) with sesquiterpene and monoterpenes (Danino *et al.* 2009; Lee *et al.* 2010). This species has been reported to have an active role in the immune mechanism of fish due to its antibacterial and antioxidant effects (Gökbulut *et al.* 2013; Sırakov *et al.* 2018). In this study, antiinflammatory effects of *I. viscosa* on rainbow trout gonad cells (RTG-2) after infection with LPS were investigated by detecting changes in IL-1β, TNF-α and IL-8 gene expressions and IL-6 protein levels.

2 | METHODOLOGY

In the present study conducted in vitro, Lipopolysaccharide (LPS; *Escherichia coli* O127: B8) (Sigma-Aldrich, St, Louis, CO) was used to induce experimental infection; *I. viscosa* essential oil was used to determine anti-inflammatory activities on LPS-infected rainbow trout gonad cell / RTG-2 (ATCC® CCL-55 ™).

2.1 *Inula viscosa* essential oil

Inula viscosa was collected from the vicinity of Hatay during the period August – September 2018 and dried in moisture free environment. Essential oil of *I. viscosa* was extracted by water distillation technique. For this process, the leaves and flowers were separated into small pieces, boiled under constant pressure and the final extract was prepared. Subsequently, the extracts were taken into sterile 15 ml falcon tube and the mouths of the tubes were covered with paraffin strips and stored at 4°C in a refrigerator until the working process.

2.2 Cell culture

Rainbow trout gonad cells / RTG-2 (ATCC® CCL-55™) were maintained in Lebovitz L-15 supplemented with 10% FBS and 1% penicillin / streptomycin / amphotericin solution in a 23°C incubator under the atmosphere of 0% CO_2. On attaining 75 – 80% confluency, the cells were sub cultured by trypsinization (0.025% Trypsin / EDTA) and then the experimental groups were formed. All experiments took place for 24 h after seeding.

2.3 Cytotoxicity assay

At the preliminary studies, we determined the cytotoxic effects of LPS and *I. viscosa* (IN) on gonad cells by using MTT test. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Merck (Fluka, USA) and followed the method of Papachristou *et al.* (2013). The toxic and effective doses were determined by adding the concentrations of LPS (1, 5, 10 and 20 µM) and IV (1, 5, 10 and 20 µg ml^{-1}) in the incubator conditions for 24 h (24-well cell dish, 4×10^4 cells $well^{-1}$) and then the adherent cells were removed from the medium. Each well received 200 µL of fresh medium containing 20 µL of MTT solution (5 mg ml^{-1} in phosphate buffered saline) for 4 h. MTT crystals were dissolved by adding 100 µL 0.04 M HCL / isopropanol, for 15 minutes at 23°C. The supernatants were then transferred to separate tubes and centrifuged at 12×10^3 rpm and 4°C. Absorbance was determined at 570 nm by an ELISA reader (µQuant-SK).

2.4 Experimental design

Effective LPS and IV concentrations determined by MTT analysis were used in the experimental study. The effective dose of LPS was 20 µM while that of the plant was 1 µg ml^{-1}. According to the cytotoxicity results, the first group was control (cont). The second group, infection group, was formed by LPS (LPS). For this, the cells were exposed to *E. coli* LPS (20 µM) to cause inflammation. The third group which only *I. viscosa* essential oil (1 µg ml^{-1}) was applied to the cell). The last group was evaluated as the treatment group of *I. viscosa* (1 µg ml^{-1}) on RTG-2 cells stimulated with 20 µM LPS (IN1 / LPS20). The animal

experiment was repeated three times.

2.5 Lysate preparation and investigation of protein levels

After the application of LPS and *I. viscosa* to the cells, the plate (six wells and 1.6×10^4 cells well^{-1}) was incubated with ice and the media on the cells was discarded and washed three times with ice-cold PBS. Lysates of the cells in the experimental groups were obtained using the M-PER (Thermo, EU) solution kit. Then, protease / phosphatase inhibitor cocktail was added in the ratio of 1 / 100 and rinsed gently for 5 min. Next, supernatants were obtained by centrifugation of tubes at 14000 rpm, for 10 min. Protein level of IL-6 was determined using commercially available specific ELISA kit (Bioassay Technology Laboratory, PRC).

2.6 RNA isolation and gene analyses

Isolation of RNAs in samples was performed following the protocol of RiboEx reagent (GeneAll-SK) kit. Polymerase chain reactions were performed in a real-time thermal cycler (Bio-RAD, Life-cycle, CFX96, SK) using Syber green mastermix (iTaq™ Universal SYBR® Green Supermix, EU). The general temperature cycle protocol was performed as 15 min at 95°C (cDNA synthesis—1 cycle) and 15 min at 95°C (first denaturation—1 cycle). Afterwards, 15 s at 95°C (denaturation), 30 s at 56 – 60°C (annealing), and 30 s at 72°C (final extension) were performed for 40 cycles. Specific primer sequences of the genes studied are given in the table (Table 1). Net values (with 2−DDCt formulation) were calculated as relative ratios by optimizing the expression levels of the target genes with β actin expression, as a housekeeping gene.

2.7 Statistical analyses

Data were analyzed by SPSS 21.0 program, Descriptive and One-way ANOVA analysis and shown as mean standard errors, unless otherwise specified. Differences between groups were analyzed by Duncan test. Statistical significance was defined as $p < 0.05$.

TABLE 1 Target gene specific primer sequences.

Gen	Reverse	Forward
IL-1β (Sentegen, TR)	5′–TTG AGC AGG TCC TTG TCC TTG- 3′	5′–ACA TTG CCA ACC TCA TCG-3′
IL-8 (Sentegen, TR)	5′ –TCT CAG ACT CAT CCC CTC AGT- 3′	5′–AGA ATG TCA GCC AGC CTT GT-3′
TNF-α (Sentegen, TR)	5′ –TGA GGC CTT TCT CTC AGC GAC AGC-3′	5′–TGG AGG GGT ATG CGA TGA CAC CTG - 3′
β-Act (Sentegen, TR)	5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′	5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′

3 | RESULTS

In the present study, 2% proliferation was detected in the group treated with LPS at a concentration of 5 µM (0.768 ± 0.0045) compared to the control group (0.7484 ± 0.0015). In other doses of LPS, cell viability was significantly decreased by 4% and 27% in the groups treated with 10 µM (0.7172 ± 0.0042) and 20 µM (0.5494 ± 0.0040) of LPS respectively ($p < 0.05$). LPS of 20 µM was chosen as the effective concentration to produce inflammation in subsequent studies (Figure 1A). In addition, the solution containing IN obtained by water distillation method was applied to the cells at different concentrations (1, 5, 10 and 20 µg ml^{-1}). At the end of the incubation time, 1.1% cell proliferation was detected in the group treated with 1 µg ml^{-1} of IN (0.775 ± 0.0035) compared to the control group (0.7484 ± 0.0015). However, increasing concentrations (5 µg ml^{-1}, 0.7364 ± 0.0044; 10 µg ml^{-1}, 0.603 ± 0.0070; 20 µg ml^{-1}, 0.481 ± 0.0020) of IN were found to have a reducing effect on the cell viabilities by 1.7%, 19% and 36% respectively (Figure 1B). Based on the data obtained, it was determined that IN may have anti-cytotoxic activity at a concentration of 1 µg ml^{-1} and was selected as the effective concentration and this dose was used as a reference during the study ($p < 0.05$). In this study, the efficacy of IN against the inflammatory cytotoxic activity of LPS was investigated. According to the data obtained, cell loss caused by 20 µM LPS (0.5494 ± 0.0040) was improved in 1 µg ml^{-1} IN group (0.613 ± 0.0040) by 12% ($p < 0.05$; Figure 2). Cell images are shown in Figure 3. The effective concentration of *I. viscosa* essential oil which applied to the cell at different concentrations (1, 5, 10 and 20 µg ml^{-1}) was detected as 1 µg ml^{-1} due to 1.1% (0.775 ± 0.0035) cell proliferation when it was compared with the control group (0.7484 ± 0.0015).

3.1 Gene expression

The expression level of IL-1β with LPS insignificantly up regulated when compared with the control group. When *I. viscosa* treatment following LPS infection (IN1 / LPS20) compared with the LPS group, a significant down-regulation of 51% was determined ($p < 0.05$). In LPS application, the expression level of IL-8 was up-regulated with 4.69-fold relative changes while 96% down-regulation was detected in IN1 / LPS20 group. The expression level of TNF-α compared with the control group showed up-regulation (11.3 fold) in the LPS group. For the other group (IN1 / LPS20), 92% down-regulation was observed when compared with the LPS group (Figure 4).

3.2 Protein level

According to the results, intracellular IL-6 protein level decreased by 4% in the LPS added group (3.9672 ± 0.105) when compared to control group (4.0961 ± 0.074). In the group that added IN together with LPS (4.3252 ± 0.133), it

was found that this level increased by about 9% (p = 0.059; Figure 5).

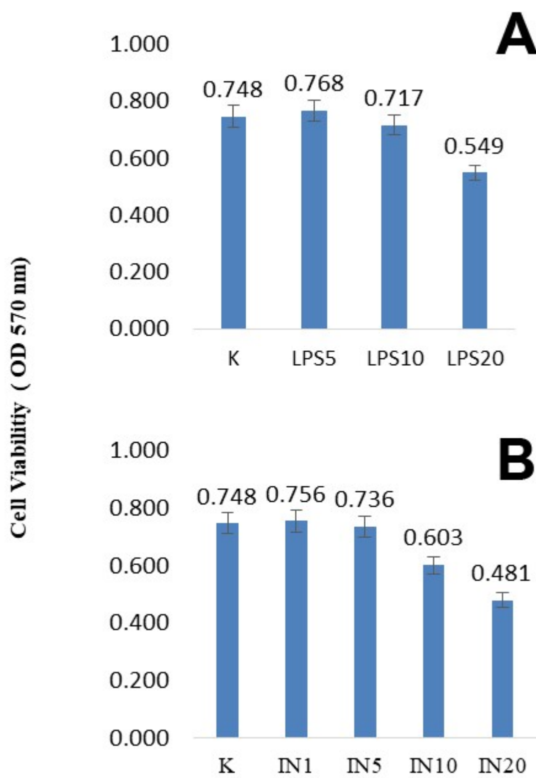

FIGURE 1 Efficiency of LPS (A) and IN (B) on the viability of RTG-2 cells.

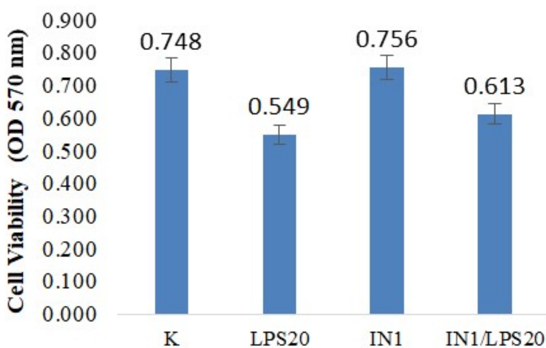

FIGURE 2 Efficiency of *Inula viscosa* treatment (1 µg ml^{-1}) after LPS-induced infection (20 µM) on the viability of RTG-2 cells. Lipopolisakkarit (LPS); *I. viscosa* essential oil (IN); IN1: 1 µg ml^{-1} is the effective concentration for IN; LPS20: 20 µM is the effective concentration for LPS; Data were expressed as means ± standard deviation (SD), n = 3.

4 | DISCUSSION

Diseases are among the most important problems in aquaculture as they cause high economic loses (Mohsin *et al.* 2012). Therefore, antibiotic treatment has been used since the past to prevent such losses in the aquaculture sector. Due to many handicaps of antibiotics (residual, development of resistant strains etc.), scientists have focus on new alternative treatment methods for a long time. Recent studies showed that herbal-derived treatments do not have any side effects. In particular, bioactive structures and natural compounds of plant extract having many antimicrobial, antioxidant and anti-inflammatory activities have been another preferred reason. In this study, gonad cell line (RTG-2) isolated from rainbow trout was infected with lipopolysaccharide (LPS) and the anti-inflammatory effects of *I. viscosa* were investigated.

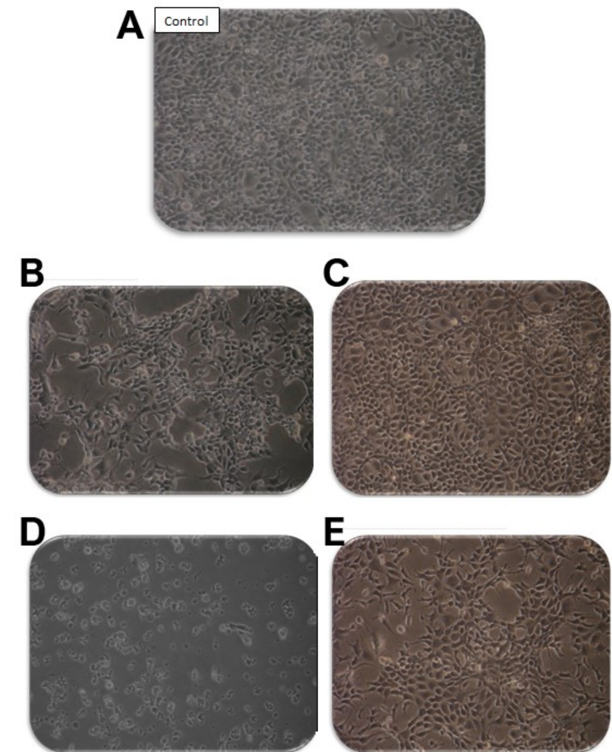

FIGURE 3 Cell images. A, control; B, LPS 20 µM ml^{-1}; C, IN 1 µM ml^{-1}; D, IN 20 µM ml^{-1}; E, IN 1 µM ml^{-1} + LPS 20 µM ml^{-1}. Cell densities were monitored by invert microscope (Olympus CK40, JP). LPS showed significant cell losses. IN was observed to prevent significant losses of LPS, (IN1, 1 µg ml^{-1} is the effective concentration for IN; LPS20, 20 µM is the effective concentration for LPS).

Talib *et al.* (2012) examined the antiproliferative and antimicrobial effects of the chemical components of *I. viscosa* and determined an antiproliferative activity by MTT on three cell lines (MCF-7, Hep-2 and Vero cell line). Four flavonoid MCF-7 present in the chemical components of *I. viscosa* showed high antiproliferative activity against Hep-2 cell lines, but showed limited activity in Vero cell line. The antiproliferative activity of methanol and water extracts obtained from *I. viscosa* plant was evaluated on MCF-7 and T-98 cell lines (Özkan *et al.* 2019). It was reported that methanol extract of *I. viscosa* had an antiproliferative effect on MCF-7 and T-98 cell lines better than those of water extract with IC50 values of 179.5 ± 2 µg ml^{-1} and 121 ± 3 µg ml^{-1} respectively.

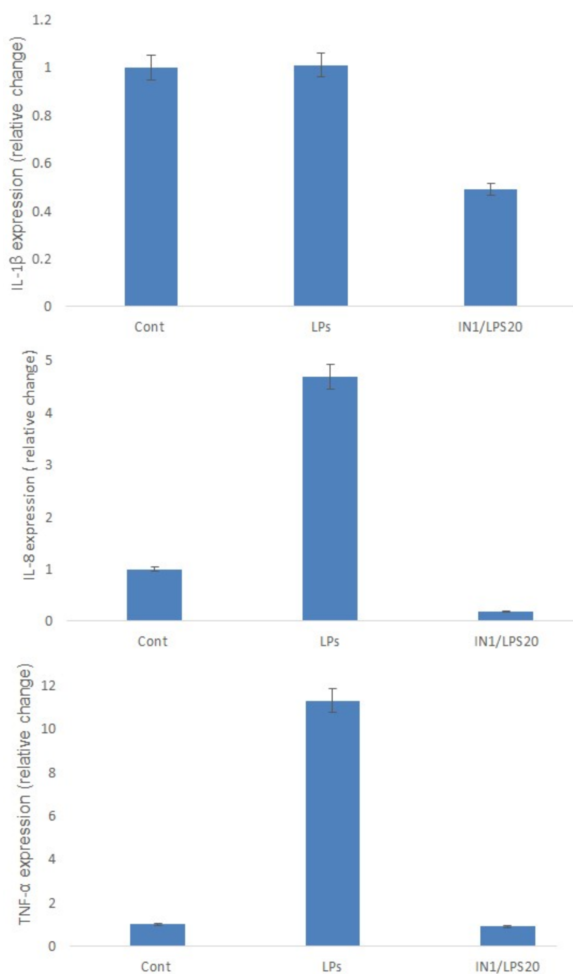

FIGURE 4 Gene expression levels of pro-inflammatory cytokines (IL-1β, IL-8, TNF-α).

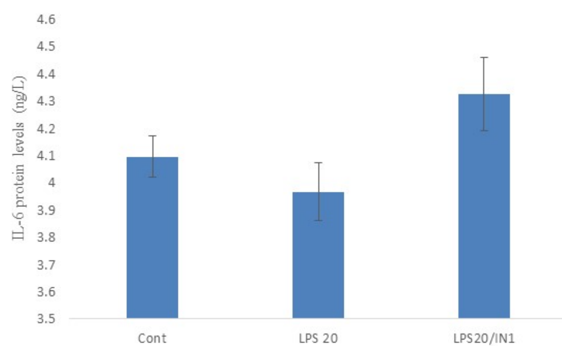

FIGURE 5 IL-6 protein levels. IN1, 1μg/ml is the effective concentration for IN; LPS20, 20 μM is the effective concentration for LPS; Data were expressed as means ± standard deviation (SD), *n* = 3.

Abu-Dahab and Afifi (2007) reported that *I. viscosa* extract had no toxic effect on A549 and HL60 cell lines, but showed a strong antiproliferative and cytotoxic activity in the MCF-7 cell line. In our study, the effective concentration of *I. viscosa* essential oil which applied to the cell at different concentrations was detected as 1 μg ml^{-1}. Because 1.1% cell proliferation was detected in the group treated with 1μg ml^{-1} of IN (0.775 ± 0.0035) compared to the control group (0.7484 ± 0.0015). Since the biological effects of *I. viscosa* are mainly dependent on their chemical structure and relative orientation of the different moieties in the molecule, it is believed that the differences between studies may be resulted from this reason.

Bacterial infections causing fish diseases are mostly gram negative origin (Akaylı *et al.* 2015; Olesen and Vendramin 2015; Küçükgül *et al*. 2019b) and it is associated with LPS which plays a key role in the pathogenesis of these infections (Whitfield and Trent 2014). LPS derived from *E. coli* is a well-characterized inducer of inflammatory response in vivo (Kruzel *et al.* 2002; Zhang *et al.* 2008). LPS which is responsible for the lethal effects and clinical manifestations of diseases leads to the release of cytokines that are effective in the host's fight against microorganisms. However, excessive cytokine production (TNF-α, IL-1ß and IL-6, etc.) leads cell damaging and inflammatory diseases (Jin *et al.* 2011). Following bacterial infection, IL-6, TNF-α and IL-β are released during cytokine stimulation, which plays a key role in antibacterial, antiparasitic and antiviral immunity in fish (Nam *et al.* 2007; Mladineo and Block 2010; Verriera *et al.* 2011). Costa *et al.* (2011) reported that IL-6 and IL-1ß expressions were induced by LPS during inflammation in rainbow trout (RTS-11) cells, and that IL-6 was significantly down-regulated. It was shown that pro-inflammatory cytokines (IL-6, etc.) and anti-inflammatory cytokines (IL-10, etc.) increased during infection (Van der Poll *et al.* 1996; Peters and Nover 2013). Mueller *et al.* (2010) used LPS-stimulated macrophage cells to determine the proliferative or antiinflammatory activity of plant extracts and they reported that at a concentration of 0.5 mg ml^{-1} thyme (*Origanum onites*) reduced IL-6 expression levels, while 0.2 mg ml^{-1} thyme increased IL-10 expression levels. For *Staphylococcus aureus* infections, IL-6 expression has been reported to reach high levels (Gómez *et al.* 2005). Schildberger *et al.* (2011) expressed that *S. aureus* and *Pseudomonas aeruginosa* strongly activate IL-6 expression from pro-inflammatory cytokines. Sun *et al.* (2009) showed that 1,000 ng ml^{-1} of LPS from *P. aeruginosa* leaded to significant secretion of TNF-α, IL-1, IL-6, IL-8 and IL-10 in whole blood.

Our result of IL-1β and TNF-α expressions with LPS was up regulated. In groups of *I. viscosa* treatment following LPS infection (IN1 / LPS20) were significantly down-regulated with 51% and 92% respectively. IL-1β had the highest effect with 96% in IN1 / LPS20 group. IL-6 protein level decreased 4% in LPS. In other group (IN1 / LPS20), IL-6 was found that increased by about 9%. According to our findings, up-regulation of all pro-inflammatory gene expression levels by LPS stimulation was similar to those of Costa *et al.* (2011) in respect to our IL-6 expression results. Up-down regulation levels at pro-inflammatory gene expression levels are affected by many factors such

as LPS concentration differences, application time, cell lines and therefore, the differences in results in different studies may be resulted from above mentioned factors.

5 | CONCLUSIONS

In the present study the efficacy of *I. viscosa* on pro-inflammatory cytokines was determined and it was shown that IN exhibited significant efficacy against inflammation caused by LPS. However, further studies are needed to determine pharmacological activity of *I. viscosa* in detail.

ACKNOWLEDGEMENTS

Thanks are due to the University of Munzur, Turkey for funding this research through the Scientific Research Projects Coordination Unit (Project Number- YLMUB017-22).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

AK & **AK** designed the experiment, analysed the data and prepared the manuscript. **TMK** conducted sampling and also prepared the manuscript. All authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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