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Brief Report

Evaluation of Anti-Inflammatory Activity in Methanolic Seed Extracts of International *Sorghum bicolor* L. Resources

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Abstract: Sorghum is an important cereal with high value as a health food ingredient because it contains various phenolic compounds. Anti-inflammatory activity was assessed using 12 sorghum resources collected from various countries to explore their potential as medicinal resources. The findings revealed that, at extract concentrations of 25 μg/mL and 50 μg/mL, cell survival rates were observed to be between 70 and 80% for most varieties, with the exception of K159081. In the analysis of anti-inflammatory activity, measured by the rate of nitric oxide (NO) production, sorghum varieties K159041 and K159081 exhibited NO production rates of 0.46 ± 0.38% and 2.58 ± 0.20%, respectively, indicating significant anti-inflammatory properties. The investigation into anti-inflammatory effects also included examining the expression of the inducible nitric oxide synthase (iNOS) gene, which is related to the inflammatory response triggered by LPS in macrophages. Varieties K159041, K159048, K159077, K159078, K159081, K159089, and K159096 were analyzed for this purpose. Further, an expression test of the cyclooxygenase 2 (COX-2) gene revealed values less than 0.4 in K159077, K159081, and K159089, suggesting these sorghum lines possess higher anti-inflammatory activity compared to others. Additionally, the expression analysis of tumor necrosis factor alpha (TNF-α), a gene identified as an inflammatory cytokine, showed that the mRNA levels in the lines K159048, K159077, K159078, K159088, K159089, K159093, and K159096 were expressed at lower levels relative to other sorghum resources, categorizing them as having high anti-inflammatory activity. Notably, the K159081 line exhibited the lowest expression level of all genes associated with inflammation, marking it as a valuable medicinal resource with potential development as an anti-inflammatory agent.

Keywords: anti-inflammatory activity; COX-2; iNOS; NO production; sorghum resources; TNF-α

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1. Introduction

Sorghum, a staple crop in Africa, Asia, and South America, has seen an increase in value due to its rich nutrient and energy content [1]. Amidst concerns over the potential impact of climate change on global crop production, researchers are exploring alternative food sources to sustain the growing population and enhance agricultural yields. Sorghum stands out as a viable option for meeting these food needs [2]. Studies have focused on developing sorghum into a tailored biomaterial for creating functional foods, owing to its content of essential nutrients like proteins, vitamins, minerals, lipids, and fiber, as well as significant quantities of secondary metabolites such as flavonoids, tannins, and anthocyanins. This underscores the importance of interdisciplinary research in this area [3,4]. These secondary metabolites in sorghum seeds are recognized for their potential

health benefits, including their roles in disease prevention and the promotion of health through their antioxidant, anti-inflammatory, and anti-cancer properties [5,6].

Oxidative stress, characterized by an imbalance between free radicals and antioxidants, leads to various inflammatory and cancer-related diseases [7,8]. Inflammation can damage cells, making them prone to infections and chronic diseases, with cytokines such as interleukin 1, β (IL-1β), tumor necrosis factor (TNF-α), and interleukin 6 (IL-6) playing critical roles in its development [9]. In research models, macrophages involved in inflammation release nitric oxide and inflammatory molecules upon LPS stimulation; excessive NO production can lead to cancer metastasis and DNA damage, causing mutagenesis [10,11]. Phytochemicals have been identified as agents capable of mitigating inflammation by inhibiting inflammation-promoting enzymes [12,13]. Therefore, identifying plant-based substances that can suppress inflammatory markers is crucial for preventing chronic diseases.

There is a report in activity research that studied the correlation between cultivation environment and content, such as phenol, according to the sorghum genotype. Recently, as research on the biological functionality of sorghum has become more active, there have been reports on active components such as flavonoids and various phenolic components, including tannin [14]. The polyphenol extract of sorghum is known to exhibit antioxidant activity and inhibit the activity of enzymes related to cholesterol biosynthesis, making it a valuable crop as a food [15]. Although numerous studies have highlighted the anti-inflammatory effects of sorghum extracts, the underlying mechanisms remain partially understood [12,16,17]. Research continues to delve into the anti-inflammatory potential of sorghum's phenolic extracts, emphasizing the need to elucidate the mechanisms connecting sorghum extracts to their anti-inflammatory activity.

In a recent study involving 12 sorghum resources collected internationally, comprehensive analyses were conducted on antioxidant activity, total phenol and flavonoid contents, phenolic compound analysis, and intracellular antioxidant gene expression [18]. Extracts of sorghum seeds collected from overseas showed different differences in antioxidant activity, depending on the genetic characteristics of the resources. Building on this foundation, the current study further examines the cytotoxicity and anti-inflammatory effects on LPS-induced macrophages, as well as the expression of inflammation-related genes using real-time polymerase chain reaction (PCR). The findings underscore sorghum's efficacy in alleviating and improving inflammation, reinforcing its potential as a valuable material for health improvement.

2. Materials and Methods

2.1. Extract Preparation

Sorghum seeds from 12 genetic resources (K159041, K159042, K159078, K159081, K159088, K159089, K159093, K159097, K159100, K159096, K159048, and K159077), originating from Australia, the former Soviet Union, the USA, Sudan, and Guadeloupe, were acquired through the National Agrobiodiversity Center at the National Institute of Agricultural Sciences, Rural Development Administration, Republic of Korea. Three grams of seeds per resource were ground using a grinder (HG-7113, Haeger, Barcelona, Spain), then extracted in 100% methanol at room temperature for 48 h. The extracts were filtered using filter paper (Whatman No. 42). The filtrate was then concentrated to a final concentration of 10,000 μg/mL using a rotary vacuum concentrator (EYELA N-1110, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) for experimental use.

2.2. MTT Assay

Raw 264.7 cells were seeded at a density of 1×10^5 cells/well in 100 μL per well in a 96-well plate (SPL Life Science Korea, Pocheon, Republic of Korea) and incubated in a CO_2 incubator for 24 h [19]. Following this incubation, the medium was discarded, and the cells were treated with sorghum seed extract at concentrations of 25, 50, and 100 μg/mL, diluted in DMEM/High Modified medium (Hyclone Laboratories Inc., South Logan, UT, USA), for 24 h. After the treatment period, the extract was removed, and 100 μL of 500 μg/mL MTT

reagent was added to each well. The cells were then incubated in a CO_2 incubator for 4 h, followed by the addition of 100 µL of DMSO to dissolve the formazan crystals. The plates were incubated at room temperature for 20 min to facilitate dissolution. The absorbance was measured at 519 nm using a UV-spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cytotoxicity of the sorghum seed extract in Raw 264.7 cells was calculated using the formula: Cytotoxicity % = [(Abs(sample) − Abs(blank)/Abs(control)] × 100.

2.3. Analysis of Anti-Inflammatory Activity Using LPS-Induced Macrophages

To evaluate the rate of NO production in LPS-induced macrophages, 100 µL of RAW 264.7 cells, at a density of 1×10^5 cells/well, were seeded into a 96-well plate and incubated in a CO_2 incubator for 24 h [19]. Subsequently, 50 µL of 4 µg/mL LPS and 50 µL of sorghum seed extract, diluted to concentrations of 25, 50, and 75 µg/mL using DMEM/High Modified, were added and incubated for another 24 h in a CO_2 incubator (Sanyo Co., Ltd., MCO-19AIC, Osaka, Japan). After incubation, 50 µL of the supernatant was collected for analysis. Griess reagent A (1% sulfanilamide, Sigma-Aldrich Co., Ltd., St. Louis, MO, USA) and Griess reagent B (0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, Thermo Fisher Scientific Inc., Waltham, MA, USA) were mixed in a 1:1 ratio, and 50 µL was added to the supernatant. The mixture was then incubated at room temperature for 20 min, and the absorbance was measured at 519 nm using a UV–vis spectrophotometer. The anti-inflammatory activity in Raw 264.7 cells was calculated as: Rate of NO production % = [(Abs(sample) − Abs(blank))/Abs(control)] × 100.

2.4. Analysis of Inflammation-Causing Genes Using Real-Time PCR

cDNA synthesis was performed using PrimeScript™ RT Master Mix (Perfect Real Time, Takara Korea Biomedical Inc., Seoul, Republic of Korea). For real-time PCR, a 25 µL reaction mixture was prepared using TB Green® Premix Ex Taq™ (Tli RNaseH Plus, Takara Korea Biomedical Inc.), and the analysis was carried out on a CronoSTAR™ 96 Real-Time PCR System (Takara Korea Biomedical Inc., Seoul, Republic of Korea) [18]. The PCR conditions included an initial denaturation at 95 °C for 30 s, followed by a two-step amplification process (denaturation at 95 °C for 5 s and annealing at 60 °C for 30 s) for 40 cycles, and a melting curve analysis (95 °C for 1 min, 60 °C for 15 s, and 98 °C for 5 s). The nucleotide sequences of the primers used are detailed in Table 1.

Table 1. Nucleotide sequences of primers used in real-time PCR.

Primer	Orientation	Sequence (5′ to 3′)
β-actin	Forward Reverse	AGAGGGAAATCGTGCGTGAC CGATAGTGATGACCTGACCGT
TNF-α	Forward Reverse	AGGGGATTATGGCTCAGGGT GAGTCCTTGATGGTGGTGCA
COX-2	Forward Reverse	CCCTCCTCACATCCCTGAGA ACTCTGTTGTGCTCCCGAAG
iNOS	Forward Reverse	CTATGGCCGCTTTGATGTGC TTGGGATGCTCCATGGTCAC

2.5. Statistical Analysis

The statistical analysis was based on the results of three independent experiments, presented as mean ± standard deviation. Data were analyzed using IBM SPSS Statistics 26 software. Duncan's multiple range test was applied to determine statistical significance through analysis of variance, with a significance threshold set at $p < 0.05$.

3. Results and Discussion

3.1. Toxicity Evaluation of RAW 264.7 Cells to Measure Anti-Inflammatory Activity

The experiment utilized sample concentrations of 25, 50, and 100 μg/mL to assess cytotoxicity towards normal cells. Figure 1 illustrates the cytotoxicity in RAW 264.7 cells. All tested extracts from the collected resources demonstrated low cytotoxicity at a concentration of 100 μg/mL. Notably, the extract from the K159078 sorghum resource exhibited the lowest cytotoxicity, at 5.89 ± 0.17%, while the K159081 resource extract displayed similar low cytotoxicity levels, with a statistical value of 14.20 ± 0.40%. The concentrations that maintained cell viability above 70–80% were identified as 25 μg/mL and 50 μg/mL; however, K159078 was the only variant showing a cell viability lower than this range.

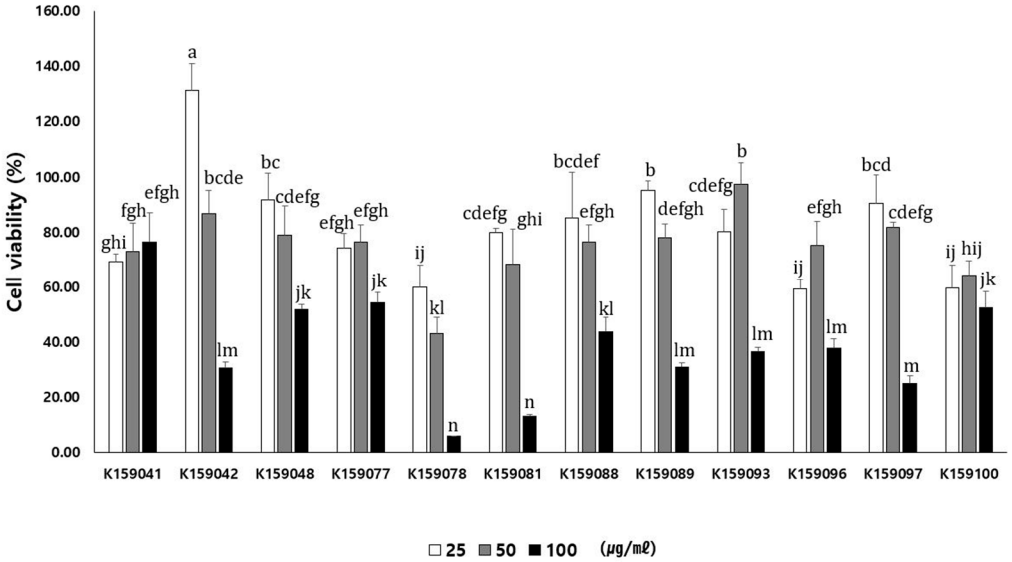

Figure 1. Cytotoxicity comparison with 12 sorghum resources collected from foreign region using RAW 264.7 cells. Significance was indicated with different letters according to statistical analysis.

Cell viability is commonly assessed using the MTT assay, which involves the conversion of the yellow tetrazolium MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan by active mitochondrial dehydrogenase in viable cells. The absorbance of this conversion is measured at 490 nm using a spectrophotometer [20]. When treated with various concentrations of sorghum extract (50, 100, 200, 400, 500 μg/mL), no cytotoxicity was observed in either the hull or grain even at the highest concentration of 500 μg/mL. However, the bran extract maintained a survival rate above 90% at concentrations up to 200 μg/mL [21]. Another study that applied sorghum seed extract at 50, 100, and 200 μg/mL to RAW 264.7 cells found that treatment with 100 μg/mL of extract resulted in cell viability exceeding 89.28 ± 15.30%, indicating robust cell vitality across all tested seed extracts [22]. Our results showed a 70–80% cell survival rate at a lower concentration than those reported, which may be due to differences in the type of extraction solvent and extraction method.

These findings highlight that MTT assay results can vary significantly based on the plant species used, as well as the differences in extraction solvent, site, and method. This underlines the importance of considering these variables when evaluating the cytotoxicity and therapeutic potential of plant extracts.

3.2. Anti-Inflammatory Activity Using Sorghum Extract

To assess the anti-inflammatory effects of sorghum extract on LPS-induced inflammation in RAW264.7 cells, the reduction in NO production was quantified using Griess reagent. The extracts from various sorghum resources were prepared and cultured to achieve final concentrations ranging from 25 to 75 μg/mL in the culture medium. The results, depicted

in Figure 2, highlight the relationship between NO production rates and inflammation. At a concentration of 75 μg/mL, NO production was significantly reduced, with sorghum resources K159041 and K159081 demonstrating the most pronounced decrease in NO production rates at 0.46 ± 0.38% and 2.58 ± 0.20%, respectively. These findings indicate their superior anti-inflammatory activity. The relative effectiveness of the sorghum resources, in descending order of anti-inflammatory potential at this concentration, are as follows: K159097, K159077, K159078, K159042, K159088, K159100, K159093, K159089, K159096, and K159048.

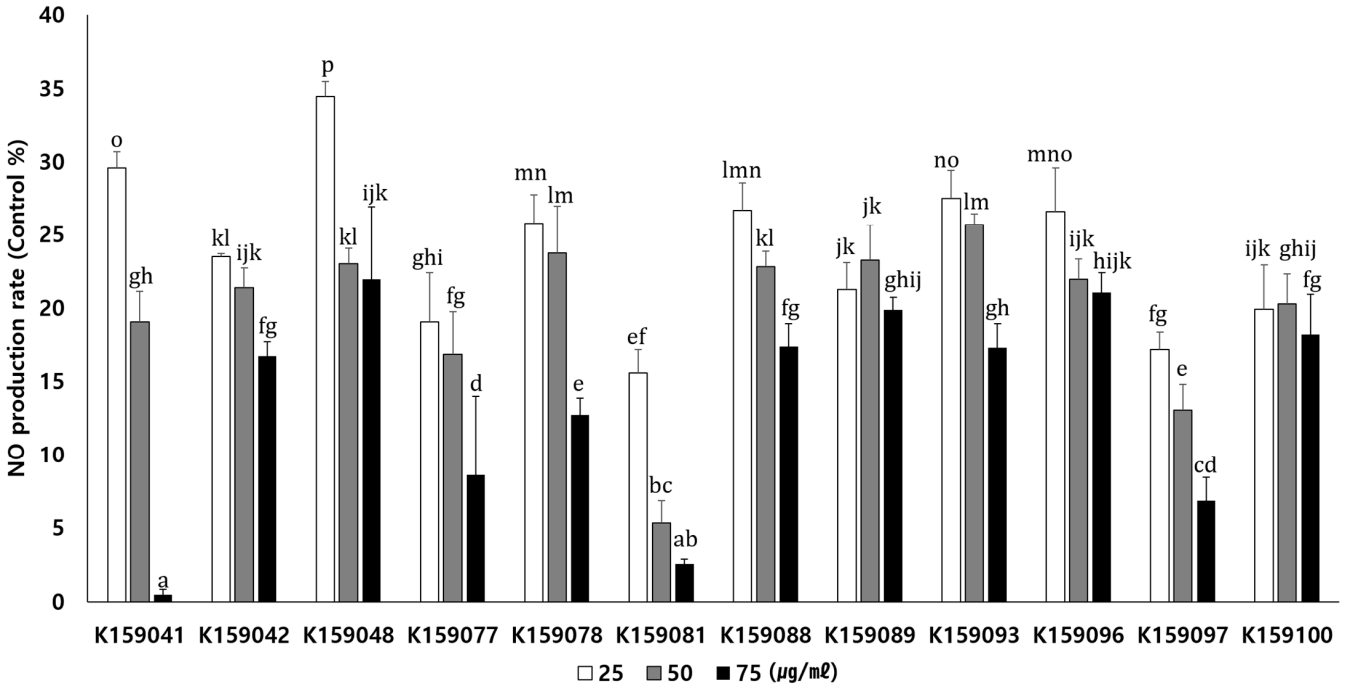

Figure 2. Ratio of NO production 12 sorghum resources collected from foreign region using RAW 264.7 cells. Significance was indicated with different letters according to statistical analysis.

A study on the NO inhibitory activity of sorghum seed extract from Uzbekistan treated on LPS-induced RAW264.7 cells at concentrations of 10, 50, and 100 μg/mL reported a significant inhibitory effect, with an inhibition rate of 144.35 ± 4.55% at 100 μg/mL [18]. Furthermore, the use of extruded sorghum bran extract resulted in a more than 14% increase in NO production suppression compared to non-extruded extracts [23]. These variations in NO production inhibition are attributed to differences in the sorghum extract and the method of sample preparation. Notably, K159041 and K159081 demonstrated nearly zero NO production rates, indicating their potent anti-inflammatory effects. This underscores the necessity for further investigation into their potential medicinal applications as anti-inflammatory agents.

3.3. Inflammatory Gene Expression Analysis

To evaluate the impact of sorghum extract treatment on the expression of inflammation-related genes in LPS-induced RAW264.7 cells, primers were designed for iNOS, TNF-α, and COX-2 genes, and qPCR analyses were performed. The expression level of the iNOS gene was found to be 0.4 or lower in seven sorghum resources: K159041, K159048, K159077, K159078, K159081, K159089, and K159096. Notably, K159048 and K159081 exhibited significantly reduced expression levels, nearing zero (Figure 3). For the COX-2 gene, three out of the twelve sorghum resources—K159077, K159081, and K159089—demonstrated low expression levels (<0.4), with K159081 showing almost no expression (Figure 4). TNF-α gene expression analysis revealed that K159048, K159077, K159078, K159088, K159089, K159093, and K159096 had expression levels below 0.4 (Figure 5).

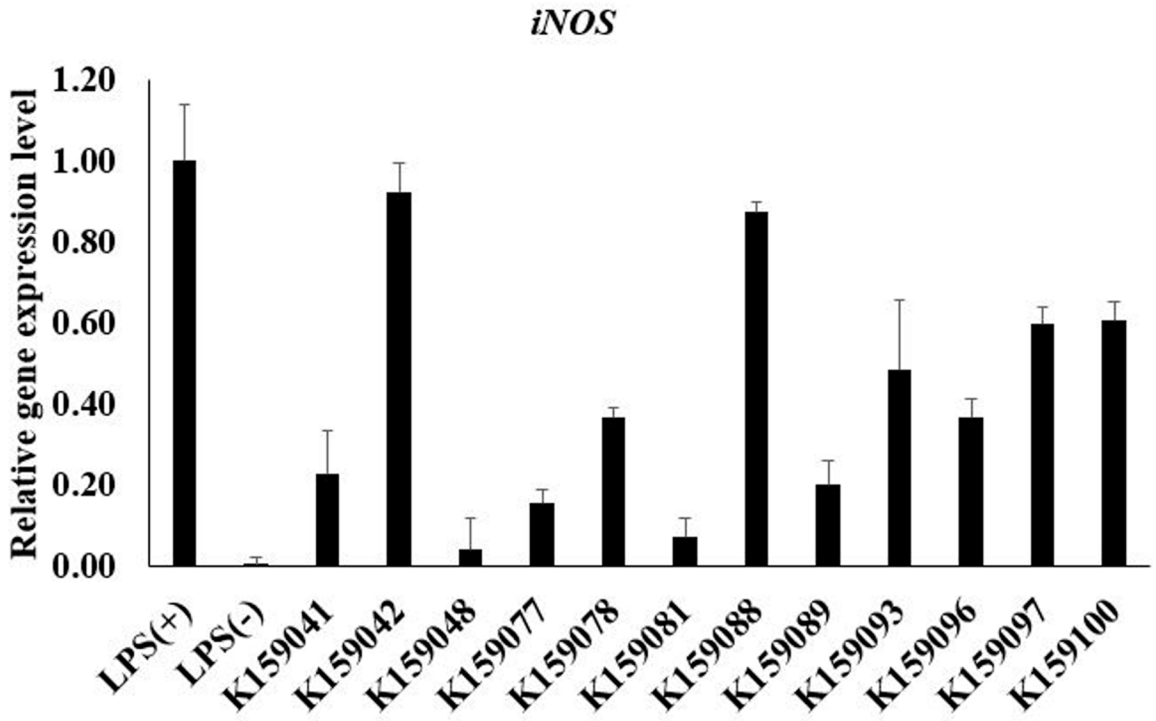

Figure 3. Expression comparison of *iNOS* gene in 12 sorghum resources collected from foreign region using RAW 264.7 cells.

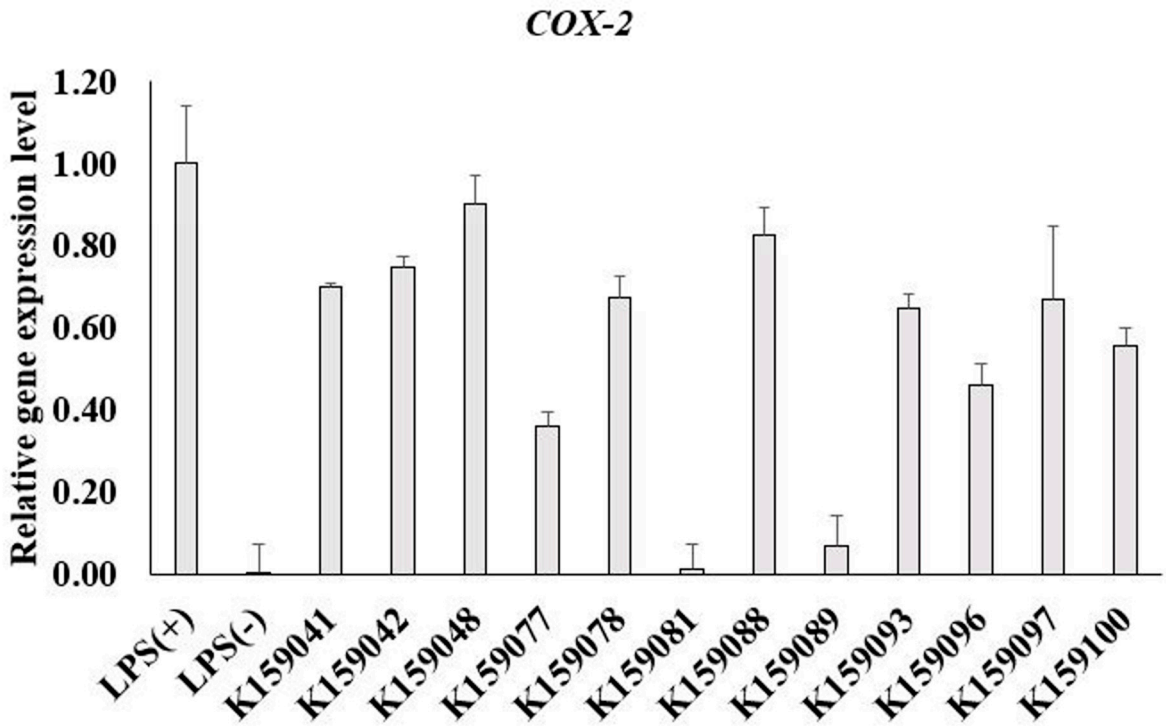

Figure 4. Expression comparison of *COX-2* gene in 12 sorghum resources collected from foreign region using RAW 264.7 cells.

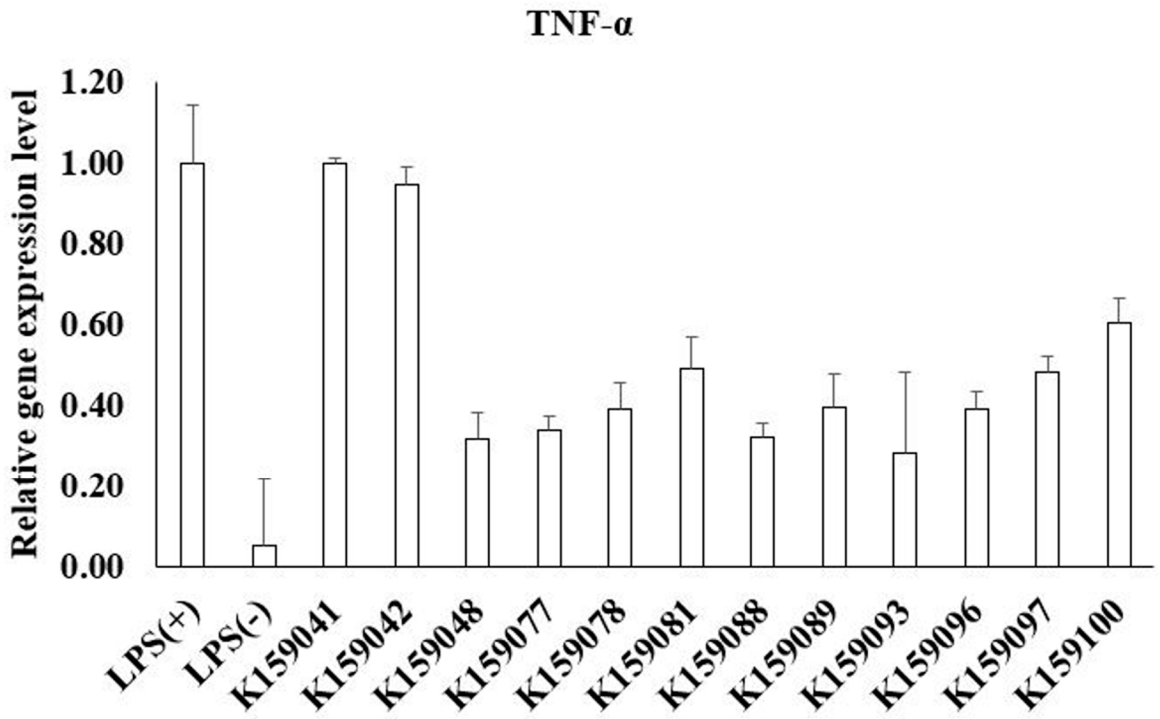

Figure 5. Expression comparison of *TNF-α* gene in 12 sorghum resources collected from foreign region using RAW 264.7 cells.

Inflammation is a localized response to infection and injury, arising from immune system reactions to internal and external stimuli [15]. The inflammatory response in macrophages, particularly LPS-induced inflammation, is known to be regulated by iNOS and COX-2 enzymes [24]. Methylene chloride extract of 'Hwanggeumchal' sorghum (25–100 μg/mL) was shown to decrease mRNA expression levels of iNOS and COX-2, as well as the protein levels, in a dose-dependent manner in LPS-induced RAW 264.7 cells. This was accompanied by a reduction in mRNA expression levels of the inflammatory cytokine TNF-α [25]. Various in vitro studies utilizing sorghum extract have reported its efficacy in suppressing inflammation by reducing the expression of inflammatory molecules. For instance, acetone extract of sorghum significantly inhibited COX-2 mRNA expression in LPS-induced RAW 264.7 cells [26].

4. Conclusions

The sorghum genetic resources used in this study have already been published on changes in antioxidant activity [Seo Ji-won Paper]. The results of this study, conducted to study another anti-inflammatory activity, are consistent with several other studies highlighting the anti-inflammatory potential of sorghum extract. Specifically, in this study, comprehensive gene expression analysis of iNOS, TNF-α, and COX-2 confirmed that K159081 sorghum resource had significantly reduced expression of all genes investigated compared to the control and other sorghum cultivars. K159081 sorghum resource is the resource that showed the highest total flavonoid activity in previous studies [Seo Ji-won Paper]. Therefore, we suggest that the relationship between total flavonoid content and anti-inflammatory activity should be further studied in the future. Resources selected for their outstanding anti-inflammatory activity can be used not only for the development of functional foods, but also as pharmaceuticals to relieve and improve inflammation.

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Data Availability Statement: The data presented in this study are contained within the article.

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Conflicts of Interest: The authors declare no conflicts of interest.

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