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ANTI-INFLAMMATORY AND ANTIOXIDANT EFFECT OF *ALLIUM SCORODOPRASUM L.* ETHANOLIC EXTRACT (ASE) IN MICE

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ABSTRACT

The antioxidant and anti-inflammatory properties of Allium species plants have been reported in studies. However, no study was found showing the antioxidant and anti-inflammatory properties of *Allium scorodoprasum L.* ethanolic extract (ASE) in the experimental carrageenan (Carr) induced paw edema mice model. This study aimed to investigate the antioxidant and anti-inflammatory effects of ASE in Carr-induced paw edema mice model. The polyphenolic compounds of ASE were analyzed with HPLC. A total of 30 male Balb/C mice were randomly divided into Control, Carr, Carr + Diclofenac Sodium, Carr + ASE200, Carr + ASE400, and Carr + ASE600 groups (n=5/group). The mice paw edema was induced by 0.05 ml subplantar injection of 1% Carr. Different groups of mice with Carr-induced paw edema were administered by oral gavage with 10 mg/kg Diclofenac Sodium and 200, 400, and 600 mg/kg ASE, respectively. Orally administered ASE at 400 and 600 mg/kg significantly decreased paw swelling, and serum levels of TNF-α, IL-1β, IL-6, AST, ALT, and ALP. ASE also decreased MDA levels and total oxidant status (TOS) but increased total antioxidant status (TAS) in the liver. In addition, ASE decreased paw and liver histopathologic values and neutrophil counts in paw tissues. The results of this study showed that ASE has an anti-inflammatory and antioxidant effect through strong polyphenolic compounds.

Key words: *Allium scorodoprasum L.,* antioxidant, anti-inflammatory, carrageenan, paw edema, mice.

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INTRODUCTION

The inflammatory response, under physiological conditions, allows the body to eliminate detrimental stimuli and promote homeostasis, thereby allowing weakened tissues to regenerate and recover (Cui *et al*., 2020). Immune cells such as macrophages, dendritic cells, lymphocytes and neutrophils play an important role in the production of inflammatory responses (Akira *et al*., 2006). Inflammatory reactions are strongly associated with the formation of free radicals and the development of oxidative stress. During the inflammatory process, neutrophils and macrophages release reactive oxygen species (ROS) and have a significant role in host defense (Deng *et al*., 2011). The disruption of the balance between oxidant-antioxidant and inflammation can cause tissue damage, membrane lipid peroxidation and the development of various diseases (Liguori *et al*., 2018).

The progression of acute inflammatory responses is caused by conditions such as excessive production of free radicals, activation of complex enzymes, and release of inflammatory and pro-inflammatory mediators. The release of different inflammatory vasoactive components such as histamine, bradykinin, prostaglandin and serotonin occur following mast cell activation at the onset of acute inflammation (Kulinsky, 2007). Through inducing the release of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and interferon-gamma (IFN-ɣ), reactive oxygen species (ROS) enhances inflammation. Therefore, free radicals are significant mediators that induce or maintain inflammatory processes. These reactive oxygen species must be neutralized and degraded by the cells and tissues in the form of an antioxidant enzyme, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Deng *et al*., 2011). As the basis of their action, many anti-inflammatories, anti-necrotic, neuroprotective, and hepatoprotective medications have an antioxidant and radical scavenging function (Franzotti *et al*., 2000; Huang *et al*., 2012). Free radicals are the critical mediators that activate inflammatory processes and thus neutralize inflammation by antioxidants and radical scavengers (Geronikaki and Gavalas, 2006). To suppress inflammation, steroidal and non-steroidal anti-inflammatory medications (NSAIDs) are commonly used. However, severe side-effects including dyspepsia, gastrointestinal and cardiovascular complications limit its

use (Moghadam-Kia and Werth, 2010; Al-Saeed, 2011; Varga *et al.*, 2017). Therefore, the study of therapeutically active natural products has recently become increasingly relevant.

Recently, antioxidant and anti-inflammatory components in the structure of plants have become a new focus of interest to examine and evaluate potential health functions against various pathological processes (Huang *et al*., 2012; Ou *et al*., 2019). In the therapeutic anti-inflammatory treatment, the use of plant products with known anti-inflammatory and antioxidant properties can be very important. Many substances such as phenolic compounds, terpenes and polyphenols present in the structure of plants are biologically active in a wide range of ways, including anti-cancer, antibacterial, antioxidant, anti-diabetes and anti-inflammatory effects (Demir *et al.*, 2020). Therefore, for the assessment of traditional medicine, the study of the biological processes of plant extracts is quite important. Allium species was recorded as one of the world's oldest cultivated plants (Oosthuizen *et al.*, 2018). Organs of Allium plants have powerful antioxidant properties as they have high levels of flavonoid, carotenoid, and chlorophyll as well as low levels of toxic oxygen radicals (Stajner *et al.*, 2006). *Allium scorodoprasum L. subsp. rotundum (L.)* is also known as wild garlic or wild leek. *Allium scorodoprasum*'s leave and other parts can be consumed raw or cooked (fried) and used as a flavouring. On the other hand, *Allium scorodoprasum* has been used in folk medicine for diabetes control and vision enhancement. The *Allium scorodoprasum* root tubers have been reported to have antibacterial, antifungal, antioxidant and antiviral properties. According to pharmacological research, *Allium scorodoprasum L.* has diuretic, antibacterial, antifungal, antihypertensive, hepatoprotective, anti-obesity, and antitumor properties (Tasci and Koca, 2016). The antioxidant and anti-inflammatory properties of Allium species plants have been reported in studies. However, no study was found showing the antioxidant and anti-inflammatory properties of *Allium scorodoprasum L.* ethanolic extract (ASE) in the experimental carrageenan (Carr) induced paw edema mice model.

The aim of this study was to investigate the protective effect of ASE in the inflammatory model of the paw caused by carrageenan in mice on anti-inflammatory and antioxidant pathways.

MATERIALS AND METHODS

Chemicals and Medications: Carrageenan was acquired from Boston USA Chemistry (Boston, Massachusetts, United States). The standard anti-inflammatory drug Diclofenac Sodium was purchased from Abdi Ibrahim Pharmaceuticals (Istanbul, Turkey). 2-Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Formalin solution and Hydrochloric acid (HCl) were purchased from Merck (Merck, Darmstadt, Germany). From SunRed Biological Technology (Shanghai, China) TNF-α, IL-6, IL-1β, TAS and TOS were purchased.

Plant source: *Allium scorodoprasum L.* was collected from the Kösedağ area in April-May in Sivas province of Turkey. The voucher specimen number is 1400–1500 m, AA 4761.

Preparation of Plant Extract and HPLC analysis: *Allium scorodoprasum* leaf was slim cut, then dried in the oven at 40 °C. The method optimized by Cai *et al.* (2004) was applied for the drying process of the plant. The extraction of *Allium scorodoprasum L.* was conducted in amber glass bottles (0.1 L) within a shaker water bath (Memmert-WBN 14, Germany). A 10 g of *Allium scorodoprasum L.* was put in the bottle and waited at experimental extraction temperature of 100 ml extraction solvent (different proportions of 28% EtOH: H_2O). All extraction protocols were performed at 79 °C with shaking at 200 rpm for 118 min. The samples were filtered by the Whatman No: 1 filter paper after extraction. The filtrates were combined and dried at 79 °C using a rotary evaporator (Rotavapor® R-100, Buchi, Switzerland) (Demir *et al*., 2022). ASE extraction contents were determined by HPLC (Agilent Technologies, USA). Acetonitrile (solvent A) and 0.1% formic acid solution in water (solvent B) were used as the mobile phase. The dilution was performed on Zorbax SB-C18 chromatographic column (150-mm x 4.6 mm x 3.5 μm). Chromatographic mode is the flow rate of the mobile phase through column 0.5 ml/min and injection volume 3 μL. The elucidation of the compounds was done at 25 ° C from the column by adjusting the ratio of solvent A from 100% to 52% (0–10 min 100% A, 10 min 100% A, 10 min 100%–90% A, 15 min 90%–85% A, 15 min 85%–65% A, 10 min 65%–52% A).

Analysis of toxicity of ASE: To determine whether the ASE exerts any signs of toxicity on the mice model, the acute toxicity analysis was performed according to the Organization for Economic Cooperation and Development (OECD, guideline 423).

Animal Experiments: Thirty male Balb/C mice having 25-35 g body weight were purchased from the Sivas Cumhuriyet University Faculty of Medicine's Experimental Animals Laboratory. The animal experiment was compliant with EU Directive 2010/63/EU and approved by Sivas Cumhuriyet University Ethical Committee for Animal Experiments (CÜHADYEK) (Number: 65202830-050.04.04-317). In an environmentally controlled testing room (temperature 22 ± 1 °C, 50%-60% relative humidity, 12/12 h light/dark cycle), the mice were housed. *Ad-libitum* food and water were given to the mice. After a one-week acclimation period, the mice were randomly divided into six groups

(n=5/group). The groups were designed as follows: Control group, Carrageenan group (Carr), Carrageenan + Diclofenac Sodium (10 mg/kg) group, and Carrageenan + *Allium scorodoprasum L.* extract 200 mg/kg (Carr + ASE200), 400 mg/kg (Carr + ASE400), 600 mg/kg (Carr + ASE600) groups, respectively. The mice in the treatment groups were given different doses of ASE orally, while the mice in the Control and Carr groups were given 0.5 ml of distilled water. On the other hand, 10 mg/kg doses of Diclofenac Sodium were applied by oral gavage in Carr + Diclofenac Sodium group. To form a paw edema model, 0.05 ml subplantar injection of 1% carrageenan was given to mice at 60 minutes after administration of various doses of ASE, Diclofenac Sodium and the Control group received an equivalent amount of sterile saline. The developed swelling of the right hind paw was continuously monitored for 5 h. Blood sample was collected by cardiac puncture under ketamine (100 mg/kg) / Xylazine (10 mg/kg) anesthesia. The collected blood was centrifuged at 3000 x rpm 15 min +4℃ and IL-1β, IL-6, and TNF-α proinflammatory cytokine levels in serum were determined by ELISA kit analysis. Later, mice were killed with cervical dislocation and the right hind paw and liver were dissected. A part of livers tissue was homogenized (Bead Blaster™ 24, Edison, USA) in 1 g tissue/10ml PBS (pH:7.4) and 1.15% KCl, and then centrifuged at 4000 rpm for 10 min at +4℃. Total oxidative and antioxidative status was analyzed of obtained supernatant. Liver function test was evaluated from serum. The other part of the liver tissue and the right hind paw was fixed in 10% formalin for histopathological examination.

Evaluation of paw edema swelling: The right hind paw edema swelling of mice was measured by using a digital Vernier caliper at 0, 1, 2, 3, 4, and 5 h before and after subplantar injection of carrageenan and was expressed millimeters (mm). The percentage inhibition of edema is determined by the formula (Hisamuddin *et al.*, 2019):

$$\%\text{Inhibisyon edema} = \frac{[(\text{Ct} - \text{Co})\text{in control mice} - (\text{Ct} - \text{Co})\text{ intreated mice}]}{(\text{Ct} - \text{Co})\text{in control mice}} \text{x}100$$

Evaluation of liver lipid peroxidation, TAS and TOS levels: Lipid peroxidation of the liver tissue was identified as malondialdehyde (MDA) according to the previous method (Ohkawa *et al.*, 1979). To generate a red complex TBARS, MDA reacts with thiobarbituric acid at high temperatures in an acid medium. TBARS absorbance at 532 nm was measured. Total antioxidant status (TAS) and total oxidant status (TOS) levels in the liver tissue were analyzed with a commercial ELISA kit (Sun Red Biotechnology, Shanghai, China). The total protein of liver supernatants was analyzed by an autoanalyzer (BS 200 Mindray, China).

Assessment of pro-inflammatory cytokines serum levels in mice: Commercial ELISA kits (Sun Red Biotechnology, Shanghai, China) were used to detect tumor necrosis factor (TNF)-α, interleukin (IL)-1β and serum interleukin (IL)-6 levels according to manufacturer's protocol and using Microplate Photometer (MultiSkan FC, Thermo Fisher Scientific K.K). The level of TNF-α was expressed in nanograms per milliliter of serum. IL-1β serum level was expressed in picogram per milliliter and IL-6 serum level was expressed in nanogram per liter.

Evaluation of serum liver function associated parameters in mice: An autoanalyzer (BS 200 Mindray, China) was used to measure serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). The level of these parameters is expressed in units per liter.

Evaluation of right hind paw and liver tissue of mice histopathologic morphology: The right hind paw and liver tissue were collected following cervical dislocation under high-dose anesthesia. These were fixed in a 10% formalin solution for 24 h. Decalcification was performed on paw tissue in 5% nitric acid solution for 6 h. All tissues were processed and embedded in paraffin according to standard procedures. With the paraffin blocks obtained, sections with a thickness of 5 µm were obtained in the microtome (Leica RM 2125RT). The sections were deparaffinized with xylene and Hematoxylin-Eosin (H&E) stained. Histopathological evaluations were made under a light microscope (Olympus BX51, Tokyo, Japan) and photographs of characteristic findings (Olympus, EP50) were taken. Scoring 0–5 was made based on the prevalence and severity of edema and inflammatory cell infiltrates in the epidermis, dermis and hypodermis layers in the evaluation of lesions in paw tissues. It was scored as; 0 = no lesion; 1 = light; 2 = light/medium; 3 = medium; 4 = moderate/severe; 5 = severe (Coura *et al.*, 2015). In addition, neutrophil granulocyte count was performed in five areas at 40X magnification in each case. Liver lesions were scored as previously reported (Ates and Ortatatli, 2021); 0 = no histopathological change; 1 = (mild) hepatocellular swelling due to hydropic degeneration in the centrilobular region; 2 = (moderate) moderate degeneration and hepatocellular swelling in the centrilobular and intermediate region; 3 = (severe) diffuse, severe hepatocellular swelling and disintegration. All evaluations were performed blindly.

Statistical analysis: The mean ± standard error (SEM) was expressed for all data. Biochemical parameters, paw edema-swelling measurement and paw tissue neutrophil counts were statistically evaluated by one-way ANOVA *post hoc* Tukey multiple comparison tests. The histopathological scores were evaluated with Kruskal-

Wallis with the Bonferroni correction test. Values of less than 0.05 were assumed to suggest significance. The statistical analyses were conducted using version 26 of the SPSS program.

RESULTS

Quantification of phenolic composition in an ethanolic ASE by HPLC: Component profiles of ethanolic extracts of *Allium scorodoprasum L.* were analyzed by HPLC. HPLC chromatogram (**Figure 1**) of the extract showed sharp peaks for vanillic acid, protocatechuic acid, p-hydroxybenzoic acid, gallic acid, protocatechuic aldehyde, rutin, m-coumaric acid, p-coumaric acid, caffeic acid, quercetin, catechin, ferulic acid, naringenin, kaempferol, chlorogenic acid and rosmarinic acid. The estimated number of detected constituents in the extract are 5.56 mg/g caffeic acid, 2.09 mg/g catechin, 8.26 mg/g chlorogenic acid, 2.65 mg/g ferulic acid, 4.73 mg/g gallic acid, 8.11 mg/g kaempferol, 5.84 mg/g m-coumaric acid, 6.65 mg/g naringenin, 5.31 mg/g p-coumaric acid, 9.17 mg/g p-hydroxybenzoic acid, 5.09 mg/g protocatechuic acid, 6.36 mg/g protocatechuic aldehyde, 10.93 mg/g quercetin, 4.76 mg/g rosmarinic acid, 3.09 mg/g rutin, 6.73 mg/g vanillic acid, 8.1 mg/g Σ HBA (sum of benzoic acid derivatives), 6.9 mg/g Σ HCA (sum of cinnamic acid derivatives) and 10.7 mg/g Σ PHA (sum of phenolic acids) respectively.

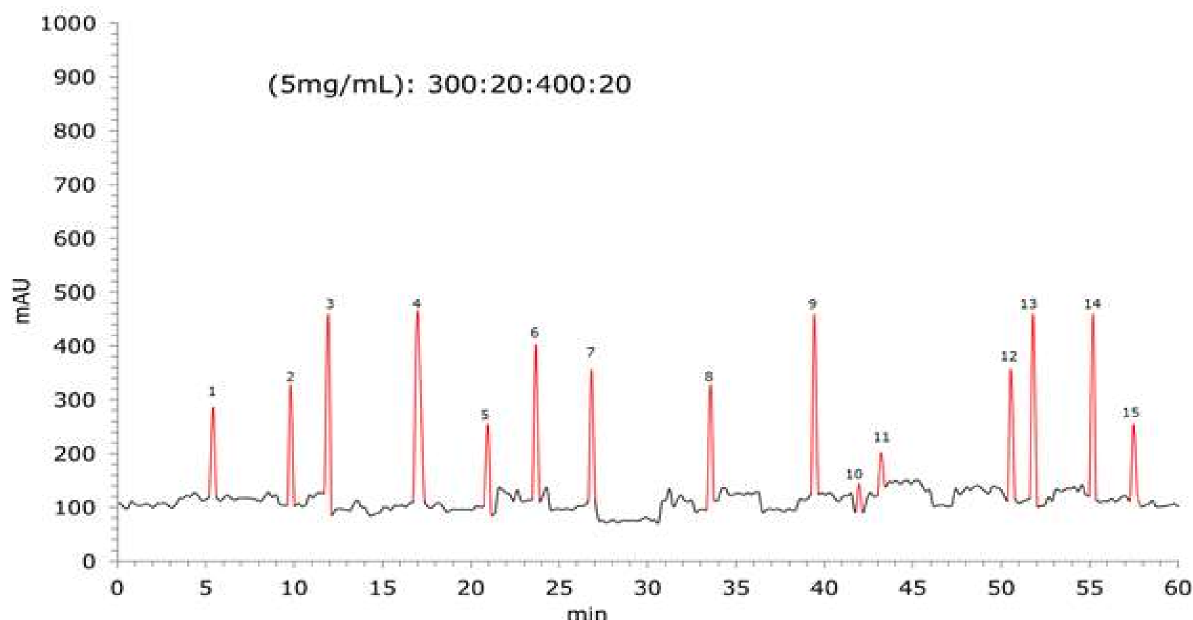

Figure 1: The phenolic compounds of ethanolic extract of *Allium scorodoprasum L*. were determined by HPLC. These are shown in HPLC chromatograms: 1; Gallic acid, 2; Protocatechuic acid, 3; Protocatechuic aldehyde, 4; p-Hydroxybenzoic acid, 5; Rutin, 6; Vanillic acid, 7; m-Coumaric acid, 8; p-Coumaric acid, 9; Caffeic acid, 10; Quercetin, 11; Catechin, 12; Ferulic acid, 13; Naringenin, 14; Kaempferol, 15; Chlorogenic acid, 16; Rosmarinic acid.

Analysis of ASE Toxicity*:* For an experimental period of 14 days, mice who received ASE with a dose of 2000 mg/kg did not present any type of illness effect or mortality. No major health symptoms have been observed. Compared to normal untreated mice, there were no obvious changes in body weight, food and water consumption rates.

Effect of ASE on carrageenan-induced mice acute paw edema: The carrageenan-induced mice paw edema assay was used to measure the anti-inflammatory activity (Sarkhel, 2015). The maximum edematous inflammation was seen at 4 h. There was no significant difference in edema inhibition between the Carr + ASE200 and Carr groups ($p>0.05$). The Carr-induced mice paw edema was notably suppressed by ASE400 and ASE600. Doses of ASE 400 mg/kg following the application of carrageenan 50% in 2 h, 60% in 3 h, 81.48% in 4 h and 73.17% in 5 h inhibited respectively ($p\leq0.01$). Doses of ASE 600 mg/kg following the application of carrageenan 42.59% in 2 h, 38% in 3 h, 44.44% in 4 h, and 60.98% in 5 h inhibited respectively ($p\leq0.05$). The rate of inhibition exerted by the standard nonsteroidal anti-inflammatory drug Diclofenac Sodium was found to be 27.78% in 2 h, 38% in 3 h, 74.07% in 4 h and 56.10% in 5 h ($p\leq0.01$) (**Table 1**). The ASE400 and ASE600 showed effectiveness in reducing carrageenan-induced inflammation.

Table 1. The effect of ethanolic extract of *Allium scorodoprasum L.* on carrageenan-induced paw edema model in mice.

Groups n=5 (in mm) (%inhibition)	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr
Control	2,45 ± 0,09	2,80 ± 0,06	2,76 ± 0,06	2,68 ± 0,04	2,57 ± 0,04	2,53 ± 0,04
Carr + Diclofenac Sodium	2.50±0.03	2.89±0.02 (18.75%)	2.89±0.02** (27.78%)	2.79±0.02** (38.00%)	2.77±0.03** (74.07%)	2.68±0.04** (56.10%)
Carr	2.55±0.06	3.04±0.04	3.09±0.03	3.05±0.02	3.07±0.01	2.96±0.01
Carr + ASE200	2.48±0.04	2.96±0.09	2.94±0.04* (14.81%)	2.95±0.04 (6%)	2.96±0.07 (12.96%)	2.86±0.05 (7.32%)
Carr + ASE400	2.55±0.02	2.86±0.02 (35.42%)	2.83±0.02*** (50.00%)	2.75±0.03*** (60.00%)	2.68±0.04*** (81.48%)	2.66±0.03** (73.17%)
Carr + ASE600	2.53±0.01	2.84±0.04* (35.42%)	2.84±0.02*** (42.59%)	2.84±0.07** (38.00%)	2.83±0.06* (44.44%)	2.69±0.07** (60.98%)

Values were expressed as mean ± SEM. The significances were expressed by *p≤0.05, **p≤0.01, ***p≤0,001 compared to Carr group (one-way ANOVA *post hoc* Tukey test).

Effect of ASE on liver lipid peroxidation, TAS and TOS in mice induced by Carrageenan: In **Table 2**, the levels of MDA, TAS, and TOS in liver tissue are present. In the Carr group, the MDA and TOS levels were significantly higher and the TAS levels were significantly lower than in the Control and Carr + Diclofenac Sodium groups (p≤0.05, p≤0.05). However, MDA and TOS levels in Carr + ASE400 and Carr + ASE600 groups notably decreased, and TAS levels significantly increased compared to the Carr group (p≤0.05). There was no significant difference between the Carr + ASE200 and Carr groups in MDA and TAS levels (p>0.05). However, the TOS levels were notably lower in the Carr + ASE200 group than in the Carr group (p≤0.05).

Table 2. The effect of ethanolic extract of *Allium scorodoprasum L.* on MDA, TAS, TOS, TNF-α, IL-1β and IL-6 levels in carrageenan-induced paw edema model in mice.

	Control	Carr + Diclofenac Sodium	Carr	Carr + ASE200	Carr + ASE400	Carr + ASE600
MDA (nmol/mg protein)	1.17 ± 0.12	1.74 ± 0.17*	2.46 ± 0.09###	2.44 ± 0.13###	1.83 ± 0.18*	1.85 ± 0.11*
TAS (U/mg protein)	0.21 ± 0.01	0.19 ± 0.01	0.16 ± 0.01#	0.19 ± 0.02	0.21 ± 0.01*	0.21 ± 0.002*
TOS (nmol/mg protein)	0.0478 ± 0.003	0.0477 ± 0.003*	0.0617 ± 0.003#	0.0491 ± 0.002*	0.0437 ± 0.002**	0.0498 ± 0.001*
TNF-α (ng/ml)	275.70 ± 24.17	283.74 ± 17.26**	380.80 ± 8.90 ###	307.47 ± 13.81*	307.18 ± 8.52*	307.42 ± 5.41*
IL-1β (pg/ml)	13.69 ± 1.15	15.11 ± 0.93*	19.60 ± 0.91##	18.64 ± 0.61#	14.20 ± 0.43**	14.47 ± 1.36*
IL-6 (ng/L)	126.41 ± 4.43	125.56 ± 6.77**	157.73 ± 6.23##	138.24 ± 5.04	126.63 ± 2.60**	132.83 ± 3.42*

Values were expressed as mean ± SEM. The significances were expressed by #p≤0.05, ##p≤0.01, ###p≤0.001 compared with the Control group, *p≤0.05, **p≤0.01, ***p≤0.001 compared with the Carr group using one-way ANOVA *post hoc* Tukey HSD.

Effects of ASE on proinflammatory cytokine serum levels in Carrageenan-induced mice: Table 2 demonstrates the effect of ASE on TNF-α, IL-1β and IL-6 levels. In Carrageenan-induced paw edema, TNF-α, IL-1β and IL-6 were significantly increased in contrast to the Control and Carr + Diclofenac Sodium groups (p≤0.01, p≤0.05). Increased levels of TNF-α decreased with ASE treatment at doses of 200, 400 and 600 mg/kg (p≤0.05), as well as Diclofenac Sodium at doses of 10 mg/kg (p≤0.01). The IL-1β levels decreased by treatment with ASE 400 mg/kg (p≤0.01), ASE 600 mg/kg (p≤0.05) and Diclofenac Sodium 10 mg/kg (p≤0.05). The IL-6 levels decreased by treatment with ASE 400 mg/kg (p≤0.01), ASE 600 mg/kg (p≤0.05) and Diclofenac Sodium 10 mg/kg (p≤0.01). There was no change in IL-β and IL-6 levels in the treatment ASE 200 mg/kg group compared to the Carr group (p>0.05).

Effect of ASE serum liver function associated parameters Carrageenan-induced mice: The effects of ASE on serum levels in AST, ALT and ALP in experimental mice is shown in **Figure 2**. Carrageenan-induced mice displayed a significant increase in AST, ALT and ALP compared to the Control and Carr + Diclofenac Sodium groups, respectively ($p \le 0.05$, $p \le 0.05$). The increased levels of serum AST significantly decreased with ASE 200 mg/kg ($p \le 0.01$), ASE 400 mg/kg ($p \le 0.001$), and ASE 600 mg/kg ($p \le 0.01$). The ALT and ALP levels decreased by treatment with ASE 400 mg/kg ($p \le 0.05$), ASE 600 mg/kg ($p \le 0.05$).

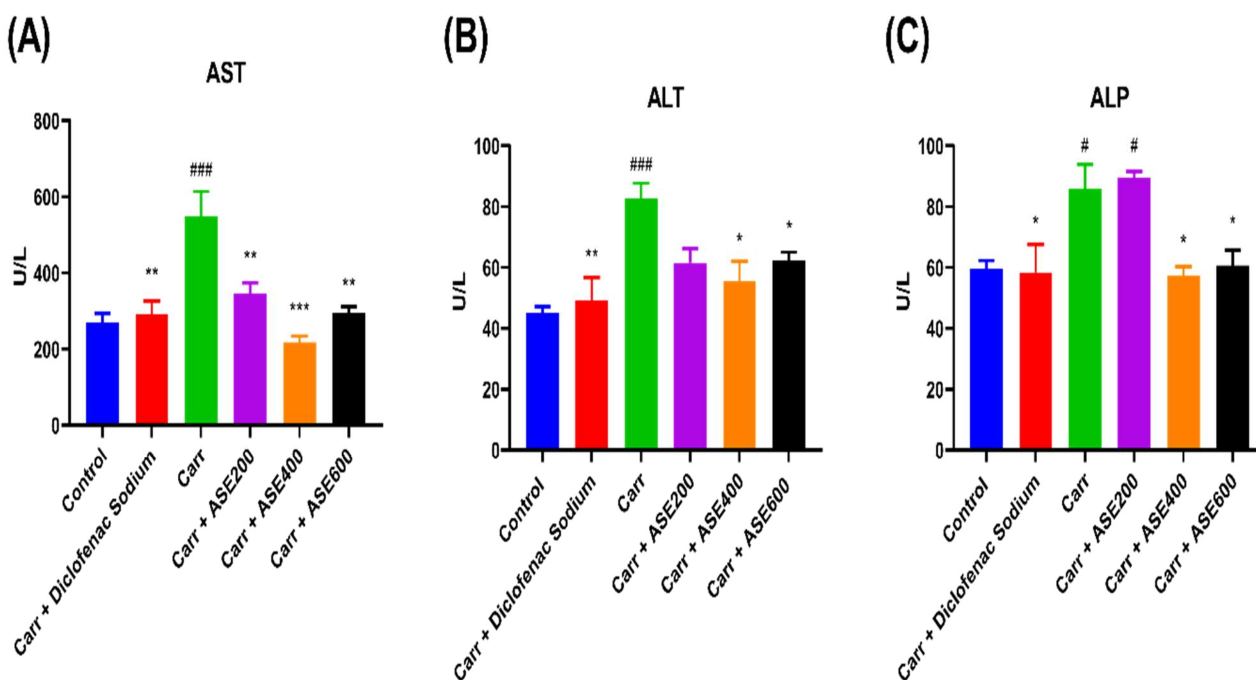

Figure 2: (A), (B), (C) The effect of ethanolic extract of *Allium scorodoprasum L.* on serum AST, ALT and ALP levels in carrageenan-induced paw edema model in mice. Values were expressed as mean ± SEM. The significance values were $^{\#}p \le 0.05$, $^{\#\#}p \le 0.01$, $^{\#\#\#}p \le 0.001$ in comparison with the Control group; $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$ in comparison with the Carr group using one-way ANOVA *post hoc* Tukey HSD.

Effect of histopathological analysis of right hind paw and liver tissue: In the histopathological examinations performed semi-quantitatively, no lesion was observed in the paw tissue epidermis in all groups. In the Carr group, edema, hyperemia, hemorrhage and inflammatory cell infiltrates, the greater part of neutrophil granulocytes was observed in the dermis of paw tissues (**Figure 3A**). In this group, it was determined that inflammatory cells concentrated around the vessel, especially in the hypodermis, spread to the muscle tissue. It was determined that these findings decreased statistically in the experimental groups except the Carr + ASE200 group ($p \le 0.05$; **Figure 3C**). It was determined that neutrophil granulocyte counts in paw tissue decreased in Carr + Diclofenac Sodium, Carr + ASE400 and Carr + ASE600 groups compared to Carr and Carr + ASE200 groups ($p \le 0.05$; **Figure 3B**). In the histopathological examination of the liver, there was no lesion in the control group. Diffuse hepatocellular swelling/hydropic degeneration findings were observed in the Carr group. Some hepatocytes were observed to be disintegrated. Although there was a decrease in liver lesions at various levels in the experimental groups, these were statistically significant in the Carr + Diclofenac Sodium, Carr + ASE400 and Carr + ASE600 groups ($p \le 0.05$; **Figure 3D**).

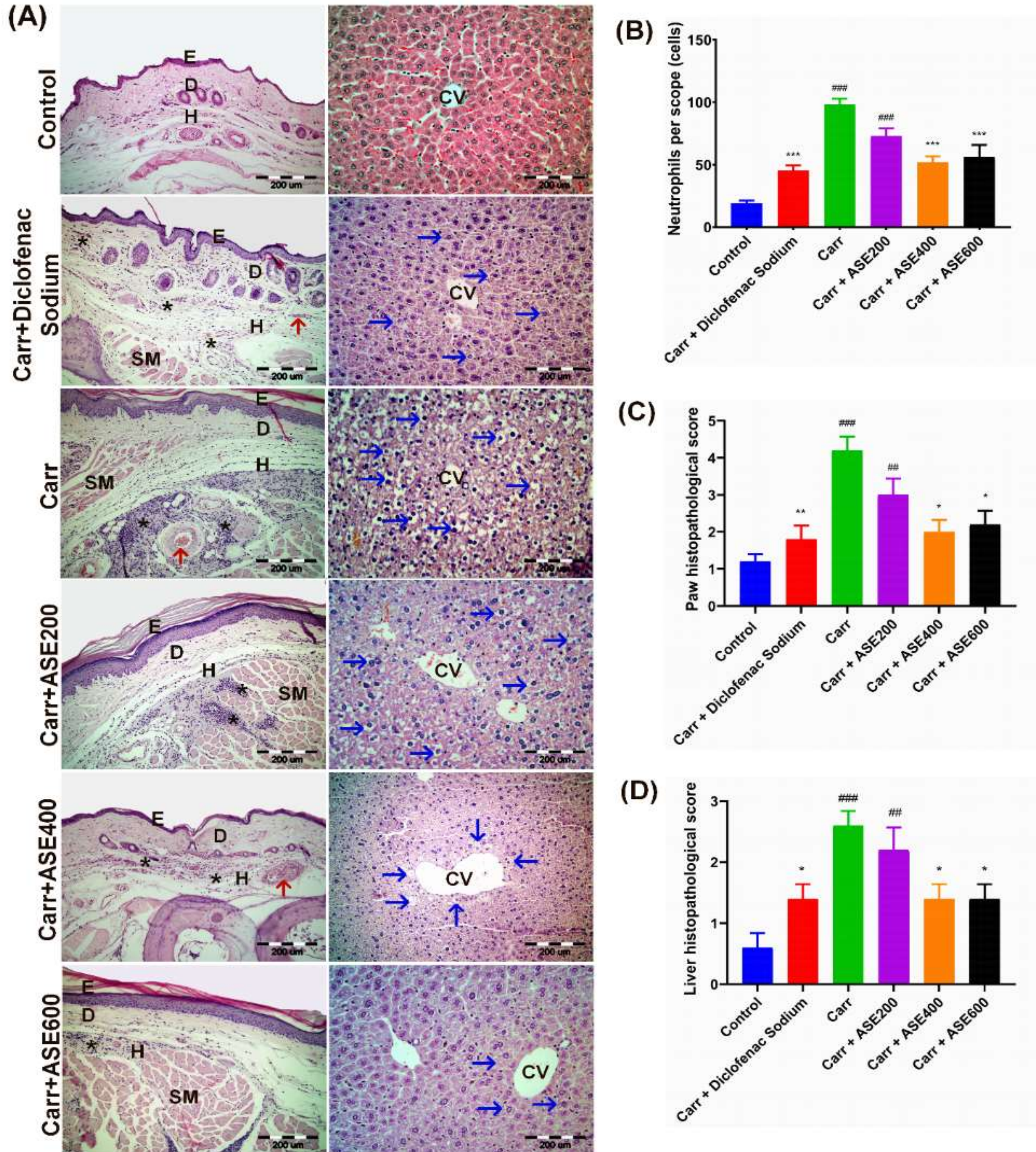

Figure 3: The effect of ethanolic extract of *Allium scorodoprasum L.* on histopathological findings in carrageenan-induced paw edema model in mice. (A) Representative photomicrographs of the protective efficacy of ASE against carrageenan-induced paw inflammation and liver damage, HE, 20X. E: Epidermis; D: Dermis; H: Hypodermis; SM: Skeletal muscle fiber, Asterisks: Inflammatory cell infiltration; Red arrow: Hyperemia; CV: Central vein; Blue arrow: Degenerated hepatocyte. (B) Number of neutrophils in scope (40X); significance values were $^{\#}p\leq0.05$, $^{\#\#}p\leq0.01$, $^{\#\#\#}p\leq0.001$ in comparison with the Control group, $^{*}p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$ compared to the Carr group using one-way ANOVA *post hoc* Tukey HSD. (C) Paw histopathological score (40X) and (D) Liver histopathological score (40X); the significance values are $^{\#}p\leq0.05$, $^{\#\#}p\leq0.01$, $^{\#\#\#}p\leq0.001$ in comparison with the Control group, $^{*}p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$ in comparison the Carr group using Kruskal-Wallis with Bonferroni correction.

DISCUSSION

The repeated use of current anti-inflammatory drugs could lead to severe side effects. Thus, alternative therapeutic agents are required (Gurib-Fakim, 2006). Recently, reliance on herbal compounds for primary health care has increased, especially in developing countries, due to their better tolerable for the human body and fewer side effects during the treatment of many diseases in the world (Banerjee *et al.*, 2018). However, improper use of plant compounds has been reported to have severe adverse effects such as toxicity and death (Kao *et al.*, 1992; Şeremet *et al.*, 2016). It has been a worldwide trend to explore, research, and analyze plants in the local flora that have anti-inflammatory and antioxidant effects. This research has focused for the first time on the anti-inflammatory and antioxidant activity of *Allium scorodoprasum L.* ethanol extract in Carrageenan-induced paw edema in mice.

Changes in behavioral patterns and mortality in mice are signs of toxicity. In the present study, the ASE acute toxicity testing on mice showed that no toxicity-related physiopathological changes were observed in mice. The most common toxicity symptoms such as weight loss, lethargy, convulsion, coma, and tremors were not observed. Moreover, no changes were observed in the skin, hair or the mucous membranes for 2000 mg/kg doses of ASE.

The paw edema model caused by carrageenan is widely used in laboratory rodent models to study the anti-inflammatory activity of natural products. The inflammatory response caused by carrageenan consists of two stages. The early phase (first 2 hours after carrageenan injection) activates pro-inflammatory mediators such as serotonin and histamine; mainly quinines, cytokines, prostaglandin, cyclooxygenase, nitric oxide and neutrophil mediated free radicals are released mostly in the second phase (3-5 hours after carrageenan injection) (Moon *et al.*, 2018). In our study, ASE 400 and 600 mg/kg doses decreased the edema thickness after carrageenan injection from the 2nd hour to the 5th. The maximum edema inhibition was seen at the ASE dose of 400 mg/kg in the 4th hour. It was found that the ASE400 group (81.48%) was more effective in decreasing the carrageenan-induced paw edema than the standard anti-inflammatory drug Diclofenac Sodium group (74.07%). These findings suggest that ASE reduces edema formation on different inflammation pathways by acting as an anti-inflammatory agent.

Inflammation is a complex mechanism that includes the recruitment drive of inflammatory cells, the release of pro-inflammatory cytokines and various inflammatory mediators. By stimulating T cells and macrophages and releasing kinins and leukotrienes and further stimulating the development of additional inflammatory cytokines, TNF-α is the most important player in inflammatory reactions, forming natural defensive responses (Zelová and Hošek, 2013). IL-1β is also a key pro-inflammatory cytokine to initiate and strengthen the inflammatory reaction (Lopez-Castejon and Brough, 2011). Produced in the inflammatory region, IL-6 can promote differentiation of T and B lymphocytes and release most acute phase proteins and some inflammatory cytokines (Del Giudice and Gangestad, 2018). Available information in the literature suggests that many cytokines including TNF-α, IL-1β, and IL-6 play a major role in carrageenan-induced inflammation (Loram *et al.*, 2007). To regulate the inflammatory reaction, the inhibition of proinflammatory cytokines and mediators is essential. Carrageenan applications in our study increased serum TNF-α, IL-1β, and IL-6 pro-inflammatory cytokines levels. Increased ASE 400 and 600 mg/kg dose improved serum TNF-α, IL-1β, and IL-6 levels. These findings showed that the anti-inflammatory activity of ASE was identified by suppressing the secretion of pro-inflammatory cytokines and mediators in the carrageenan-induced mice paw edema model.

Inflammatory reactions induced by carrageenan are associated with neutrophil penetration and release of reactive oxygen species such as hydrogen peroxide, superoxide and hydroxyl radicals produced from neutrophils and other neutrophil-mediated mediators and decreased activity of antioxidant enzymes in the liver (Lu *et al.*, 2007; Lai *et al.*, 2009). Lipid peroxidation is known to induce inflammatory processes. MDA is a lipid peroxidation marker caused by free radical activity, tissue damage and oxidative stress. For cellular defense against reactive oxygen species and free radicals, antioxidant capacity and antioxidant enzymes play a primary function (Cuzzocrea *et al.*, 1999). In the previous study, carrageenan increased MDA level, serum AST and ALT levels in rat liver (Mansouri *et al.*, 2015). Carrageenan-induced inflammatory reaction increased levels of MDA and TOS in liver tissue and decreased levels of TAS in this research. Carrageenan application also increased serum AST, ALT and ALP in mice. The application of ASE decreased MDA and TOS levels and increased TAS levels as well as serum AST, ALT and ALP levels in carrageenan-induced inflammatory condition. The possible reason why ASE application improves the increase in AST, ALT and ALP levels is the inhibition of lipid peroxidation and increased TAS levels. Therefore, it can be suggested that ASE has *in vivo* antioxidant properties with its strong herbal phenolic content.

Acute inflammatory response is associated with an increase in vascular permeability and cellular infiltration that contributes to the forming of edema due to the accumulation in the inflammatory zone of leukocytes, proteins and interstitial fluids (Scallan *et al.*, 2010). Moreover, neutrophil infiltration has been well characterized in carrageenan-induced acute inflammation in paw tissue (Chang *et al.*, 2012). The histopathological

evaluation of mice paw tissue showed notably reduction in neutrophil count, edema and inflammatory states such as inflammatory cell infiltration in Carr + ASE400 and Carr + ASE600 groups. These findings were similar in the Carr + Diclofenac Sodium group. In addition, the mice liver tissue histopathological assessment indicated that doses of ASE 400 mg/kg and 600 mg/kg alleviated liver lesions. This evidence shows that ASE has potential anti-inflammatory efficacy through the possess strong polyphenolic compounds.

The results of this research showed the ethanolic extract of *Allium scorodoprasum L.* possessed an anti-inflammatory and antioxidant activity in carrageenan-induced paw mice model through inhibition of proinflammatory cytokines (TNF-α, IL-1β, and IL-6), decreased lipid peroxidation and TOS levels, but increased TAS level. These effects of ethanolic extract of *Allium scorodoprasum L.* was confirmed by inhibition of edema, and reduced inflammatory cell infiltration in paw tissue, and improved liver lesions. In conclusion, the fact that this plant has anti-inflammatory and antioxidant potential may have a guide for new therapeutic strategies.

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