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RESEARCH

The protective effect of tangeretin and pomegranate separately and in combination on ethanol-induced acute gastric ulcer model

Etanol ile indüklenmiş akut gastrik ülser modelinde tangeretin ve pomegrenatın ayrı ayrı ve kombinasyon halinde koruyucu etkisi

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Abstract

Purpose: This study was designed to find an answer to the question, "Is it beneficial to use pomegranate (POM) and tangeretin (TAN) separately or in combination, for the prevention of acute gastric ulcer?".

Materials and Methods: The gastroprotective effect of tangeretin and pomegranate was determined by measuring the levels of the selected inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β and IL-10], lipid peroxides, and enzymatic activities of antioxidants in gastric tissue samples.

Results: When all groups are written as control, gastric ulcer, POM+EtOH, TAN+EtOH and POM+TAN+EtOH, respectively; IL-1β cytokine levels were measured as 0.147, 0.24, 0.228, 0.195 and 0.182 pg/g protein. IL-6 levels; 16,857, 25,923, 19,797, 18,838 and 17,896 pg/mg protein. TNF-α levels were 39,916, 49.97, 44,678, 41,673 and 40,844 pg/mg protein. Finally, IL-10 levels were measured as 33,496, 28,071, 29,693, 30,073 and 30,008 pg/mg protein. SOD activities were determined as 18,038, 13,731, 15,506, 14,439, and 15,943. CAT activities were 674,638, 639,964, 673,382, 664,691, and 671,203. Protein carbonyl levels were measured as 26,799, 40,30, 33,052, 34,579 and 32,79. Finally, MDA levels were found as 5,239, 9,814, 6,695, 5,771 and 5,836. Briefly, POM and TAN showed their antioxidant functions by decreasing the levels of malondialdehyde (MDA), and protein carbonyl and increasing the activity of superoxide dismutase (SOD) and catalase (CAT). And also, these protective agents exhibited their anti-inflammatory functions by decreasing the content of TNF-α, IL-6, and IL-1β, and increasing the IL-10 levels.

Conclusion: Tangeretin and pomegrenate have a potential gastroprotective effect against ethanol-induced acute gastric ulcer and that the combined treatment is more

Öz

Amaç: Bu çalışma, "Akut gastrik ülserinden korunma konusunda pomegrenat ve tangeretinin ayrı ayrı veya birlikte kullanılması yararlı mıdır?" sorusuna cevap bulmak amacıyla tasarlanmıştır.

Gereç ve Yöntem: Mide dokusunda tangeretin and pomegranatın gastroprotektif etkisi, seçili inflamatuar sitokinler [tümör nekroz faktörü-α (TNF-α), interlökin-6 (IL-6), IL-1β ve IL-10)], lipid peroksit düzeyleri ve enzimatik antioksidan aktiviteleri ölçülerek değerlendirildi.

Bulgular: Tüm gruplar kontrol, gastrik ülser, POM+EtOH, TAN+EtOH ve POM+TAN+EtOH şeklinde sırasıyla yazıldığında; IL-1β sitokin düzeyleri: 0,147, 0,24, 0,228, 0,195 ve 0,182 pg/g protein olarak ölçüldü. IL-6 düzeyleri; 16,857, 25,923, 19,797, 18,838 ve 17,896 pg/ mg protein idi. TNF-α düzeyleri 39,916, 49,97, 44,678, 41,673 ve 40,844 pg/mg protein idi. Son olarak IL-10 düzeyleri ise 33,496, 28,071, 29,693, 30,073 ve 30,008 pg/mg protein olarak ölçüldü. SOD aktiviteleri 18,038, 13,731, 15,506, 14,439, 15,943 olarak tayin edildi. CAT aktiviteleri ise 674,638, 639,964, 673,382, 664,691, 671,203 idi. Protein karbonil düzeyleri 26,799, 40,30, 33,052, 34,579 ve 32,79 olarak ölçüldü. Son olarak MDA düzeyleri ise 5,239, 9,814, 6,695, 5,771 ve 5,836 olarak bulundu. Kısaca; POM ve TAN, antioksidan fonksiyonlarını malondialdehit (MDA) ve protein karbonil düzeylerini düşürerek ve süperoksit dismutaz (SOD) ve katalaz (CAT) aktivitesini artırarak gösterdiler. Ayrıca bu ajanlar anti-inflamatuar etkilerini TNF-α, IL-6 ve IL-1β düzeylerini azaltarak ve IL-10 düzeylerini yükselterek sergilemişlerdir.

Sonuç: Tangeretin ve pomegrenatın etanol kaynaklı akut gastrik ülserine karşı potansiyel bir gastroprotektif etkiye sahip olduğu ve kombine tedavinin tek başına POM veya TAN'ın etkisinden daha faydalı olduğunu göstermektedir. Ayrıca bu etkinin, seçilen her iki flavonoidin sinerjistik etki

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beneficial than the effect of POM or TAN alone. In addition, this effect is thought to be due to the fact that both selected flavonoids can show a synergistic effect, reducing the levels of inflammation parameters and increasing antioxidant levels.
Keywords: Gastric ulcer, pomegranate, tangeretin, ethanol; rat

göstererek inflamasyon parametrelerinin düzeylerini azaltabilmesi ve antioksidan düzeylerini yükseltebilmesinden kaynaklandığı düşünülmektedir.

Anahtar kelimeler: Mide ülseri, pomegranate, tangeretin, etanol; rat

INTRODUCTION

Gastric ulcer is the most widespread type of gastrointestinal inflammatory disorder and approximately 10% of the world population suffers from this disease[1]. Although there are many factors that cause gastric ulcer, high alcohol consumption is known as one of the biggest reasons of damage to the gastric mucosa. When ethanol (EtOH) comes into contact with the gastric mucosa, it causes an oxidative stress environment that causes gastric cell necrosis[2]. The use of absolute ethanol is often preferred to induce gastric ulcers in animal models designed *in vivo*. For example, in a study, the protective efficacy of Spirulina platensis, Golden Kiwifruit Flesh, and Golden Kiwifruit Peel extracts, separately and in combination, against experimentally induced gastric ulcer was investigated[3]. In another study, the potential protective effect of Reichardia picroides extract on gastric ulcer model induced using ethanol and hydrochloric acid was investigated[4]. Ethanol directly damages the gastric mucosa and triggers the production of excessive oxidative stress, which leads to an inflammatory response. Damage to the stomach increases the levels of various proinflammatory cytokines, triggering the recruitment of leukocytes that stimulate inflammatory responses[5]. Tumor necrosis factor-α (TNF-α) stimulates the transcription of inflammatory mediator cytokines such as interleukin-6 (IL-6) and IL-1β[6,7].

Although existing antiulcer drugs cause a decrease in the incidence of gastric ulcers, there is a need to develop new drugs that are natural, have no side effects, and are readily available, as tolerance and undesirable side effects may develop with many of these drugs[8]. For this reason, natural products of plant origin have attracted the attention of many researchers[9,10]. Flavonoids are natural compounds that consist of variable phenolic composition found in fruits, vegetables, grains, and bark, as well as in the roots, stems, and flowers of plants. These natural compounds have a range of therapeutic and pharmacological effects[11]. Among these flavonoids, tangeretin (TAN), a phytochemical found in citrus peels, and due to the lipophilic structure of multiple methoxy groups in its structure, its bioavailability is high and it is easily absorbed from the intestines because it lacks glycoside structure[12,13]. This flavonoid has a number of valuable biological activities[14-16]. For instance, in a study in which hepatocyte damage was created with bisphenol, it was reported that tangeretin had a healing effect by inhibiting inflammation and oxidative damage[17]. In another study, it was reported that tangeretin protected renal tubular epithelial cells against experimental cisplatin toxicity[18]. Pomegranate (POM) fruit belonging to the *Punicaceae* family contains plenty of natural antioxidants and anticarcinogenic phytochemical components[19]. For example, it has been reported that the use of pomegranate peel extract as an antioxidant agent in a rat model of diabetes reduces the complications of the disease[20]. Moreover, pomegranate exhibits a role in the protection against oxidative stress, reducing of risks of chronic diseases, and also preventing their progression[21].

Taking the aforementioned information into consideration, it becomes evident that tangeretin and pomegranate exert significant pharmacological effects across various types of diseases. Consequently, the inquiry of whether the specific agents chosen for this study could potentially play a role in preventing gastric ulcers emerged as an area of interest. As a result, the present study was designed to investigate the gastroprotective potential of TAN and POM, both individually and in combination, within a rat model of acute gastric ulcers induced by ethanol exposure.

MATERIALS AND METHODS

Chemicals

The chemicals and solvents used in all experiments were of analytical grade. EtOH and Dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich. The powder form of pomegranate extract

(Pomella, Verdure Sciences, Noblesville, USA) was >95 (by HPLC). The powder form of tangeretin (AvaChem Scientific, San Antonio, USA) was 98% (by HPLC).

Animals

A total of 35 Wistar Albino female rats, 8-10 weeks old, weighing 180±20 gram, were purchased from Firat University Experimental Research Center and in accordance with the ethical rules of standard experimental practices. Our study was approved by the Firat University animal experiments local ethics committee (protocol ID: 2019/92; date: 03.07.2019). The care and housing of the experimental animals were carried out also Firat University Experimental Research Center. The study was performed in the Medical Biochemistry Laboratory, Faculty of Medicine, Firat University. Animals were fasted 24 hours before the start of the experiment, but no water restriction was made. The animals were allowed to drink water ad libitum. Before and during the experiment, all animals were housed in clean cages prepared for animals in automatically air-conditioned rooms with a constant temperature of 21±1°C.

Experimental design

The animals were arbitrarily divided into five groups (seven rats in each group). Group 1 was designed as a control group and the rats in this group were administered a single dose of 1 mL of DMSO via oral gavage. Group 2 represented the study group where gastric ulcers were induced using absolute ethanol (EtOH). To establish the ulcer model, 5 mL/kg BW absolute EtOH was applied once by gavage[22]. Groups 3, 4 and 5 were designed as gastro protective groups. 100 mg/kg BW POM for Group 3, 100 mg/kg BW TAN for Group 4, 100 mg/kg BW POM+TAN for Group 5 were administered by oral gavage, respectively. POM and TAN were dissolved in DMSO. *Two* hours after POM and TAN applications, 5 mL/kg BW absolute EtOH was administered by gavage to form gastric ulcer. Sacrifice of the rats occurred 90 minutes after EtOH administration, performed under anesthesia induced by Xylazine (10 mg/kg) and Ketamine (60 mg/kg). Table 1 provides a summary of the dosages and experimental groups.

Following the sacrification process, the stomach tissues of the animals were dissected for macroscopic evaluation. The stomach tissue was cut along the greater curvature and its contents were empited. Then, stomach tissues were washed with 0.9% cold (+4ºC) sodium chloride and dried with blotting paper. Afterwards, the tissues were homogenized with a homogenizer in 0.01M phosphate buffered saline solution (1:10, pH:7.0) at 16000 rpm for 4 minutes.

Table 1. Design of Study Groups

<table>
<tr><th colspan="5">Groups</th></tr>
<tr><th>Group 1 (n=7)</th><th>Group 2 (n=7)</th><th>Group 3 (n=7)</th><th>Group 4 (n=7)</th><th>Group 5 (n=7)</th></tr>
<tr><td colspan="5">1 week adaptation</td></tr>
<tr><td colspan="5">After a 24-hour fasting period without water restriction</td></tr>
<tr><td rowspan="3">Control Group
1 mL of DMSO was administered by oral gavage.</td><td>Gastric ulcer group
Gastric ulcer was formed by administering 5 mL/kg absolute Ethanol by gavage.</td><td>Pomegranate+Ethanol
100 mg/kg of Pomegranate was dissolved in DMSO and administered by oral gavage.</td><td>Tangeretin+ Ethanol
100 mg/kg of Tangeretin was dissolved in DMSO and administered by oral gavage.</td><td>Pomegranate+ Tangeretin+Ethanol
100 mg/kg Pomegranate + 100 mg/kg Tangeretin were dissolved in DMSO and administered by oral gavage.</td></tr>
<tr><td></td><td colspan="3">After 2 hours, 5 mL/kg of absolute Ethanol was administered by oral gavage.</td></tr>
<tr><td colspan="4">After 90 minutes of waiting period, the rats were sacrificed and the study was terminated.</td></tr>
</table>

Biochemical measurements

Protein Measurement with the LOWRY Method

The amount of protein in the supernatant samples was determined by using the Folin-Lowry method[23].

the Cytokine and MDA Levels

Levels of IL-6 (pg/mg prot), IL-1β (pg/gr.prot), TNF-α (pg/mg prot), IL-10 (pg/mg prot) and MDA (nmol/mg prot) were assessed using enzyme-linked immunosorbent assay according to the manufacturer's instruction. All ELISA kits were sourced from SunRed (Sunred Biological Tech. Company, Shangai, China).

the Tissue SOD Activity

For the measuring of SOD activity, the method described by Sun et al. was used[24].

Tissue the CAT Activity

For the measuring of CAT activity, the method developed by Aebi was used[25]. In principle, the method is based on spectrophotometric monitoring of the decrease in hydrogen peroxide concentration per unit time at 240 nm.

Protein Carbonyl

Protein carbonylation was determined by Levin's 2,4-dinitrophenyl hydrazine method[26]. Keto or aldehyde groups react with one molecule of phenyl hydrazine. The molecule is then rearranged to form the keto group, and a second phenyl hydrazine molecule binds. The resulting yellow-orange colored product is measured spectrophotometrically.

Statistical analysis

The software developed by Arslan et al. was employed to determine the sample size[27]. Accordingly, with a type 1 error (alpha) of 0.05 and an effect size of 1.32, the number of animals in each group was set at 7. Conformity of continuous variables to normal distribution was evaluated using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was conducted for IL-1β, IL-6, TNF-α, IL-10, protein carbonyl, MDA, and SOD and CAT activities. p value < 0.05 was accepted as statistically significant.

RESULTS

Figure 1 illustrates the impact of pomegranate and tangeretin on inflammatory signals in response to ethanol-induced gastric lesions in rats. Within the gastric ulcer group, proinflammatory factors such as TNF-α, IL-1β, and IL-6 exhibited markedly higher levels compared to the control group ($p < 0.001$). A significant reduction was observed in serum levels of TNF-α, IL-1β, and IL-6 across all groups treated with gastroprotective agents, with the most notable decrease seen in the combined treatment group. In contrast, the level of IL-10—an anti-inflammatory factor—was found to be higher in the control group than in the gastric ulcer group. Pretreatment with the selected gastroprotective agents resulted in a marginal, though statistically insignificant, increase in IL-10 content ($p > 0.05$).

Ethanol-treated rats exhibited significantly decreased SOD and CAT activities in stomach tissue when compared to the control group ($p < 0.001$ for SOD; $p < 0.05$ for CAT activities). Treatment with the selected gastroprotective agents led to a significant increase in the activities of both SOD and CAT in comparison to animals exposed only to ethanol.

The gastric ulcer group displayed notable oxidative changes, evident through elevated MDA and protein carbonyl levels in comparison to the control group. Interestingly, both POM and TAN, either alone or in combination, exhibited protective effects against lipid and protein oxidation in ulcerated tissues. Notably, the combined treatment group demonstrated the most effective protection against oxidative damage (Figure 2).

DISCUSSION

The present study aimed to investigate the gastric protective effects of tangeretin alone and in combination with pomegranate. The ethanol-induced gastric ulcer model was chosen due to its resemblance to ethanol-related gastric ulceration observed in humans[28]. With the advancement of technology, new treatment methods are being developed in addition to the existing treatments for gastric ulcers. However, beyond the treatment of gastric ulcers, it is of great importance to identify the factors causing the ulcers and to prevent their occurrence beforehand[29]. In contemporary times, the utilization of natural components in diverse experimental and clinical studies is on the rise[30,31].

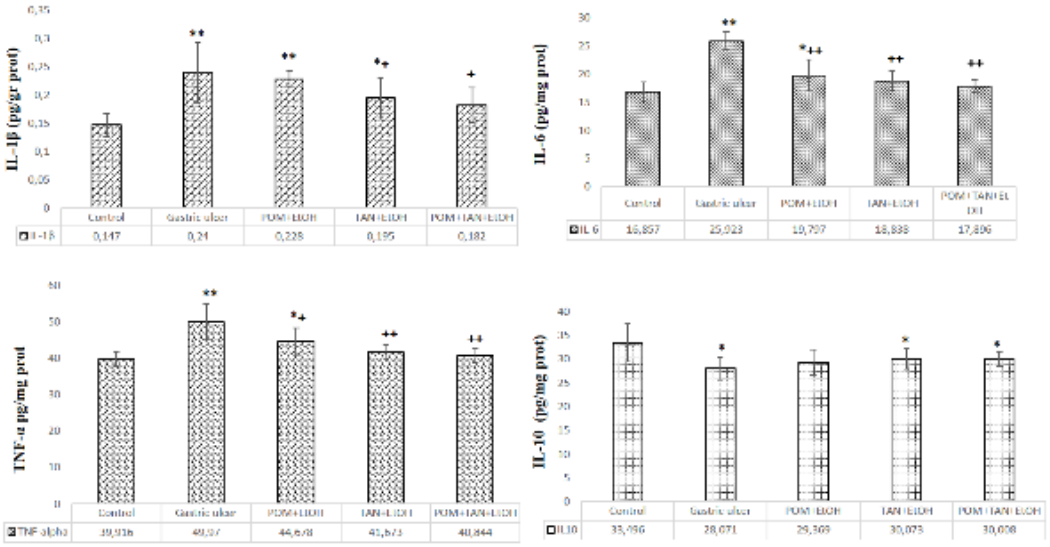

Figure 1. Effect of the POM, TAN and combined (POM+TAN) on the levels of IL-1β, IL-6, TNF-α and IL-10 in supernatant of gastric tissues.

*p < 0.05, **p < 0.001 vs. control group; +p < 0.05, ++p < 0.001 vs. gastric ulcer group.

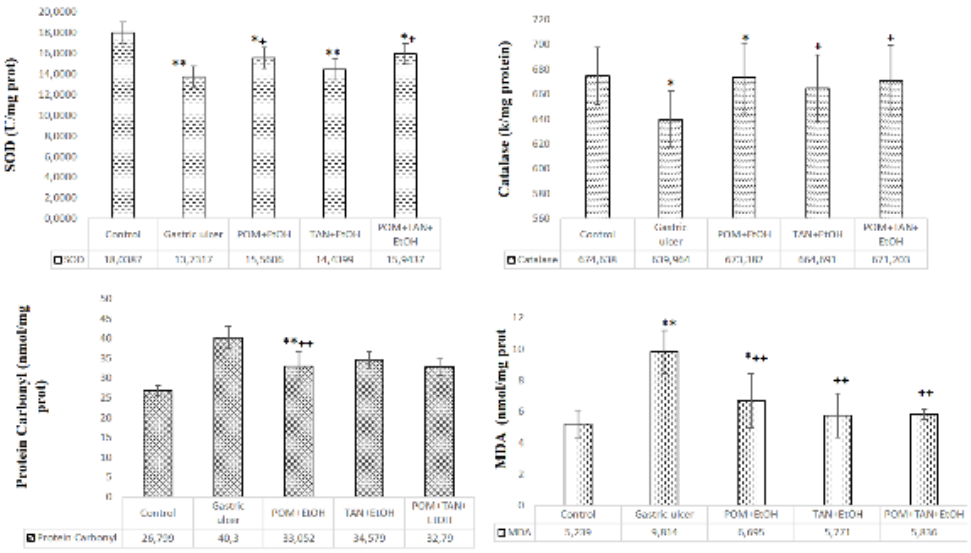

Figure 2. Effect of POM, TAN and combined therapies (POM+TAN) on SOD and CAT activities, protein carbonyl and MDA levels in supernatant of gastric tissues.

*p < 0.05, **p < 0.001 vs. control group; +p < 0.05, ++p < 0.001, vs. gastric ulcer group.

Ethanol (EtOH) can lead to an excessive release of inflammatory cytokines and the production of free radicals, which in turn causes damage to the gastric mucosa. TNF-α, a significant proinflammatory cytokine, stimulates neutrophil infiltration in areas of gastric inflammation and hinders the healing of gastric ulcers[28]. In our study, it was observed that the administration of EtOH induced an inflammatory response by elevating TNF-α levels compared to the control group. This finding is consistent with the study conducted by Ercan et al., where they reported that TNF-α, interleukin (IL)-6, and IL-8 levels increased as indicators of inflammation[32]. This outcome also aligns with the results of a previous study that confirmed elevated TNF-α levels in gastric ulcers induced by indomethacin[33]. In other studies, it has been noted that the levels of gastric TNF-α and other proinflammatory cytokines increase in animals exposed to substances like NSAIDs, ethanol, and acetic acid. Furthermore, TNF-α has a direct association with the escalation of gastric lesions. It has been suggested that anti-TNF-α agents might possess ulcer-healing effects[34,35]. IL-1β and IL-6 are other secreted proinflammatory cytokines in addition to TNF-α in almost all inflammatory responses to gastric tissue may be used as biomarkers of gastric damage[36,37]. In a separate study, it was indicated that levels of IL-6 and IL-1β in gastric tissue are correlated with the severity of gastric ulceration[38]. In a study conducted by Wang et al., it was reported that the cytokines of IL-1β and IL-6 in serum were elevated in the gastric ulcer mice model which was induced by absolute ethanol[39]. Our findings were also consistent with the study examining the effect of gallic acid in an ethanol-induced gastric ulcer model[40]. In also our study, it was observed that IL-1β and IL-6 levels in gastric supernatant increased according to the control. On the other hand, results here showed, that tangeretin alone or in combination with pomegranate significantly decreased TNF-α relative to gastric ulcer group as indicated in Figure 1. The results of this study suggested that pretreatment with the selected gastroprotective agents (POM and TAN) decreased TNF-α, IL-6 and IL-1β levels in gastric supernatant, which could possibly contribute to alleviating the inflammatory injury of gastric ulcer.

Apart from inflammation, the formation of free radicals and heightened oxidative stress are additional factors contributing to the development of gastric ulcers. The disruption in the balance between the generation of free radicals and their scavenging capacity leads to oxidative stress, which, in turn, gives rise to various pathological conditions[41]. The end product of lipid peroxidation, known as MDA (malondialdehyde), is also recognized as an indicator of damage to the gastrointestinal mucosa. Furthermore, it can serve as an assessment tool for ulcerative and inflammatory conditions of the gastrointestinal tract[42,43]. It has been reported that animal models of ethanol-induced gastric ulcers exhibit elevated MDA levels in comparison to control groups[28,44]. In our study, a significant increase in tissue levels of MDA was also observed. This elevation could potentially be attributed to the escalated production of reactive oxygen species resulting from the EtOH-induced suppression of the mitochondrial respiratory chain[45].

Protein carbonyl levels are commonly utilized as markers of oxidative damage to proteins, reflecting cellular damage caused by reactive oxygen species[46]. In a study involving the creation of aspirin-induced gastric injuries in rats, it was reported that the group administered with aspirin exhibited increased protein carbonyl levels in comparison to the control group[47]. Similarly, in another study, it was observed that the administration of Ethanol/HCl significantly raised protein carbonyl levels when compared to the control group[46]. Consistent with these conducted studies, our investigation also indicated an elevation in protein carbonyl levels within the ulcer group, serving as an oxidative stress marker, as opposed to the control group.

The enzyme SOD, responsible for diminishing the levels of intracellular superoxide radicals, converts oxygen radicals into hydrogen peroxide molecules, thereby creating a more stable structure[48]. However, the hydrogen peroxide molecule still possesses the potential to present an oxidative threat to the organism as it can be reduced to its more reactive metabolites, namely the hydroxyl radical and/or the superoxide radical[49]. To counter this, the enzymes CAT and glutathione peroxidase (GPx) come into action. CAT and GPx exhibit antioxidant effects by breaking down the hydrogen peroxide molecule into water molecules[50]. In our study, we observed a clear reduction in the activity of both SOD and CAT in the group administered with ethanol, in comparison to the control group. These findings are consistent with other reports[28,51]. Antioxidant enzymes function as defense mechanisms against deleterious free radicals that manifest due to oxidative stress. While these

enzymes counteract the damage induced by free radicals, their own concentrations tend to decrease[40].

In conclusion, the findings suggest that tangeretin and pomegranate exhibit potential gastroprotective effects against acute gastric ulcers induced by ethanol. Furthermore, the combined treatment appears to offer greater benefits compared to the individual effects of POM or TAN alone. This enhanced effectiveness is believed to stem from the fact that both selected flavonoids can collectively reduce inflammation parameters while simultaneously increasing antioxidant levels through a synergistic mechanism. However, it is important to note that large-scale randomized and controlled clinical trials are necessary to uncover previously unknown details concerning the ameliorative effects of TAN and POM on human organs.

One limitation of our study is that, while animal models play crucial roles in comprehending human diseases, the progression of the disease and the mechanisms of action of therapeutic agents employed may not perfectly align with those observed in humans.

Author Contributions: Concept/Design : FT, NI, BB; Data acquisition: NI; Data analysis and interpretation: SS, FT, NI, BB; Drafting manuscript: SS; Critical revision of manuscript: ; Final approval and accountability: SS, NI, FT, BB, AŞİ; Technical or material support: -; Supervision: -; Securing funding (if available): n/a.
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Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
Financial Disclosure: Authors declared no financial support

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