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Effects of black mulberry, chokeberry, and elderberry extracts on the healing of burn wounds

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ABSTRACT

Background: In the burn affected area of the skin, the progression or deepening of wounds is related to oxidative stress. Especially in the highly susceptible stasis zone, tissues survive to the extent that they can cope with oxidative stress.
Objective: This study investigated the potential of extracts (E) derived from the fruits (F) and leaves (L) of elderberry (E), chokeberry (C), and black mulberry (M), which are rich in antioxidant properties, to enhance the recovery of the stasis zone in burn wounds.
Methods: The study employed a sample size of 56 healthy rats. The comb burn model was used. The rats were administered the extracts via gastric gavage for a period of 21 days. Histological examination and biochemical analyses were conducted on biopsies taken from the stasis zone on the 3rd, 7th, and 21st days of the study. Photography was performed for macroscopic evaluations. The histological assays included the evaluation of inflammatory cell infiltration, reepithelialization, and collagenization, as well as immunohistochemical analysis of vascular endothelial growth factor (VEGF). Bioassays pertaining to the enzymatic activities of catalase, superoxide dismutase, glutathione peroxidase, and malondialdehyde (MDA) levels were performed.
Results: In macroscopic evaluation, a significant difference was found between the groups in terms of stasis area ($F=3.58$, $p_2< 0.001$). Post-hoc analyses showed that there was a significant difference between CFE-ELE, EFE-Burn, ELE-Burn and MLE-Burn groups in terms of stasis zones ($p < 0.05$). However, the difference between the groups according to time was not significant ($F=1.36$, $p = 0.16$). At the end of the 21-day experiment, inflammatory cell infiltration was higher in the burn group compared to the other groups, but similar to the CFE group. Re-epithelialization was similar in the burn group compared to the fruit extract groups and significantly lower compared to the leaf extract groups. Furthermore, a significant increase in collagenization and VEGF immunoreactivity was observed in all treatment groups compared to the burn group ($p < 0.05$). However, no significant difference was detected between the treatment groups. The treatment groups presented a notable reduction in MDA levels in comparison to the burn group ($p < 0.001$).
Conclusion: This study demonstrated the efficacy of fruit and leaf extracts in burn healing. Histological examination revealed that leaf extracts exhibited superior healing effects compared to fruit extracts. These results suggest that bioactive components in fruits and leaves may have different biological effects.

1. Introduction

The diversity of plant species and research on their use as herbal remedies constitute fundamental elements in the continuity of traditional medicine. In general, due to their richness in chemical composition, plant resources have provided an alternative approach not only in

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the treatment of numerous health issues but also in the healing of wounds [1]. The use of medicinal plants for the treatment of skin diseases is not a novel phenomenon. In fact, the use of such plants extracts to treatment wounds, cuts, and burns dates back to ancient times. [2]. Heteroglycans, flavonoids, tannins, volatile oils, and plant sources rich in vitamins and minerals contribute to the acceleration of the healing a wound process due to their emollient, antiinflammatory, astringent and antioxidant effects [3,4]. The primary effects of a formulation designed for skin wounds or burns treatments are to the skin protection from infection, reduce inflammation, and stimulate cell proliferation for the healing of cellular damage [4].

Elderberries (Sambucus nigra L.) are widely utilized in herbal medicine due to their health benefits [5,6]. Published research indicates that Sambucus sp. are used in Turkish folk medicine to relieve abdominal discomfort, hemorrhoids, rheumatic pain, respiratory illnesses, elevated fever, snake bites and injuries. Furthermore, the same treatments have been applied to toothaches and burns [7–10]. In traditional medicine, elderberry leaves are used to cure burns, boils, and skin inflammation as well as for the cure of chronic and infected wounds [11–13]. In humans and animals, freshly crushed leaves are applied to the wound in order to exploit their wound healing properties [14]. Aronia melanocarpa (Michx.) Elliot, commonly known as chokeberry, is prominent for its high procyanidin, anthocyanin and flavonoid contents [15]. It has been a popular ingredient in the field of health and wellness, with its fruits and products being used for a multitude of purposes. These include the treatment of cardiovascular diseases and other heart related illnesses such as the reduction of hypertension; the defence against bacteria and viruses; and reducing cholesterol, diabetes and obesity [15–18]. Chokeberry leaves are used in traditional medicine as antiviral, antiinflammatory, antimicrobial and antiproliferative agents. Black mulberry (Morus nigra L.) is a good source of various bioactive flavonoids including quercetin, catechinand rutin, as well as other components. However, the most abundant anthocyanins consist of cyanidin-3-O-glycoside and cyanidin-3-O-rutinoside [19,20]. Black mulberry leaves have been idenitified to contain four major phenolic compounds: rutin, caffeolquinic acid, ferulic acid, and chlorogenic acid. Considering the number of identified phenolic compounds, flavonoid compounds appear to be the dominant class [21].

Burns, which are difficult and complex to heal, are a prevalent and destructive form of wounds [22]. A number of pathophysiological processes take place in the burn wound area, resulting in the generation of numerous agents and reactive oxygen species (ROS). Those ROS consist of superoxide radical monoanion (O^{2-}), peroxide dianion (O_2^{2-}) and hydrogen peroxide (H_2O_2) [23]. Due to their extreme reactivity, ROS can interact with a wide range of biomolecules present in their environment, most notably lipids being their prime target. This complex process is commonly known as “oxidative stress” [24]. Their action as oxidizing agents result in the damage of cell membranes and assemblage of nuclear structures in the stasis zone. Additionally, these radicals contribute to deeper damage of affected tissue [25,26]. Research into the maintenance of the stasis zone has consistently demonstrated that agents with potent antioxidant and anti-inflammatory properties are a primary focus of attention. In a series of experiments conducted on animals, it was demonstrated that antioxidant treatments gave rise to a significant improvement across a range of measured outcomes. These improvements are thought to be the output of reduced oxidative stress. Antioxidants achieve this by neutralizing reactive oxygen species (ROS), making them a promising area of research for developing new treatments for wound healing [27].

Antioxidants, which are molecules that act to counteract free radicals, play a role in various healing processes, including preventing the formation of radicals, scavenging existing radicals, and repairing damage caused by radicals. Given the diversity of defense dismutases (SOD), the initial (first-line) category of these enzymes includes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [28]. The action of such antioxidants offers the potential to attenuate or inhibit radicals’ formation that would otherwise damage the healthy tissue’s biomolecules and, subsequently, the entire body [29]. One of the significant indicators of oxidative stress (lipid peroxidation) is malondialdehyde (MDA).

The current study intended to evaluate the effectiveness of elderberry, chokeberry, and black mulberry fruit and leaf extracts on burn healing in rats. The experimental process entailed the photographic documentation of burn areas and the collection of biopsies from the stasis zone with the objective of assessing the effects in terms of histopathological and biochemical parameters. The hypothesis is that the antioxidant effects of these species can be used to reduce oxidative stress in burn-injured tissue and promote healing in the stasis zone.

2. Materials and methods

In the study, fruits and leaves of elderberry, chokeberry, and black mulberry species were used as plant materials. Samples were collected from the Apricot Research Institute, Malatya province (Türkiye). Leaf samples were collected during the flowering period (in May), while fruit samples were harvested at the fully ripe stage (elderberry and chokeberry in September, black mulberry in July). The collected fruit and leaf samples were lyophilized and turned into powder for use in the study. The İnönü University ethics committee approved this study with decision numbered 2020/17–7. Experimentation took place in the İnönü University/ the Experimental Animals Production and Research Center, where test animals were reared and obtained. The experiment employed 16 weeks old rats, Wistar albino (n:7), homogeniouslyweighing 260 ± 20 g. Rats were kept in a conditioned room at 22 ± 2 °C of temperature, 12 h light/dark cycle. The rats were fed with standard feed and water throughout the study.

2.1. Preparation of extracts and treatment protocol

Fruit and leaf extracts to be applied were separately extracted in a hydrochloric acid: water:methanol (0.1:29.9:70, v/v/v) solvent mixture [30]. Maceration method was used for extracting active compounds from fruits and leaves. After the extraction processes, the individual supernatant fractions from each sample were combined and solvents were subsequently eliminated using an evaporator. The remaining part was completely dried in a lyophilizer for the complete removal of water. Finally fruit and leaf extracts in powder form were obtained, and the extracts were stored at −20 ºC in sealed tubes. The applied extracts were made daily in distilled water (vehicle solvent) at the predetermined concentrations. The administration of extracts and vehicle solvent was carried out via gastric gavage at a dose of 1 mL on a single daily occasion for 21 consecutive days. All daily administrations were conducted in accordance with the following protocol:Group C received a vehicle solvent (control), group B received a vehicle solvent (burn), group EFE received an elderberry fruit extract at 15 mg/kg, group ELE received an elderberry leaf extract at 1 mg/kg, group CFE received a chokeberry fruit extract at 100 mg/kg, group CLE received a chokeberry leaf extract at 200 mg/kg, group MFE received a black mulberry fruit extract at 100 mg/kg, and group MLE received a black mulberry leaf extract at 250 mg/kg [31–33].

2.2. Experimental protocol and burn model

Our sample size was allocated to 8 experimental groups. The burn group (B group, n = 7), control group (C group, n = 7), elderberry fruit extract group (EFE group, n = 7), elderberry leaf extract ELE group (ELE group, n = 7), chokeberry fruit extract group (CFE group, n = 7), chokeberry leaf extract group (CLE group, n = 7), mulberry fruit extract group (MFE group, n = 7) and mulberry leaf extract group (MLE group, n = 7) were determined.

By employing comb-brass protocol, which was initially proposed by Regas (1992), burn wounds were generated [34]. The protocol is a

frequently utilized and standardized methodology for investigating the stasis zone [35]. This model was made using a metal stamp that contained four rows of rectangular protrusions (1 cm×2 cm) and three spaces (0.5 cm×1 cm) and weighed approximately 290.0 g (Fig. 1). General anaesthesia was induced with intraperitoneal 75 mg/kg ketamin (Ketasol 10 % w/v; Richter Pharma Ag, Wels, Austria) and 8 mg/kg xylazine hydrochloride (XylazinBio 2 % injectable solution Solvent, Bioveta PLC, Ivanovice na Hané, Czech Republic) to the rats. Following the anesthesia; except for group control, the entire dorsum was entirely shaved in all rats. The metal stamp, which was maintained at a boil in water for a period of five minutes, was applied to the rats' skin for a duration of ten seconds without exerting any pressure. The identical methodology was employed on the rats' contralateral side. The coagulation zones were identified in four discrete locations where contact between the metal stamp and the rats was established. These zones were observed to exist in three separate interspaces. This suggests the existence of three distinct zones of stasis. In order to prevent the rats from becoming dehydrated, an intraperitoneal injection of isotonic saline (sodium chloride) solution (0.9 % NaCl) was administered at a dose of 10 mL/kg following the completion of the burn process. The control group remained devoid of any burn procedure. Tissue samples obtained from this group were used for comparative histological and biochemical assessments conducted at the conclusion of the experiment.

2.3. Macroscopic analyses

Macroscopic examination of the rat subjects was conducted using digital images taken under general anaesthesia on the 3, 7 and 21 days in other groups except for group control. Photographs were taken from a standard distance. (25 cm). Burn areas were calculated in cm^2, using a ruler placed in the image taken from the same distance. The zones of stasis were calculated using the Photoshop CS6 program (Fig. 1).

2.4. Histopathological analyses

Samples of the skin tissues were taken from the zones of stasis on 3rd, 7th, and 21th after general anesthesia, excluding the control group. The tissues were fixed in 10 % formaldehyde to preserve their structure. After fixation, the samples were processed for histological analysis, including dehydration and clearing, followed by embedding in paraffin. The paraffin-embedded blocks were cut at a thickness of 4 μm for the assessment of inflammatory cell infiltration (ICI), which was conducted via hematoxylin and eosin staining. Additionally, the extent of reepithelialization was evaluated. Furthermore, Gomori trichrome staining was utilized to assess the density of collagen in the samples. For the purpose of microscopic examination, a Leica DFC-280 microscope and Leica Q Win Image Analysis System were utilised (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

In order to assess the ICI, a semi-quantitative scoring system was performed. This entailed evaluating 10 random areas at a magnification of 40x. The extent of infiltration was evaluated on a scale of 0–3. The scoring system employed was as follows: 0 (normal), 1 (minimal), 2 (moderate), and 3 (severe).

In the evaluation for reepithelialization, a scale ranging from 0 to 3 was used, where the entire surface of each region was examined. Accordingly, 0: absence of epithelial tissue in the stasis zone, 1: epithelial tissue not completely covering the stasis zone, 2: epithelial tissue covering the stasis zone thin-thickness, and 3: epithelial tissue covering the stasis zone medium-thickness.

In a similar fashion, collagenization was assessed via a scale of 0–3, with scoring based on comparison to normal tissues. A total of ten areas were randomly selected and examined at 40x magnification. Each specimen was scored according to the following criteria: 0 (absence of staining compared to normal tissue), 1 (slightly reduced staining intensity), or 3 (similar or slightly reduced staining intensity) [36].

2.5. Immunohistochemical analyses

To determine angiogenesis density, an immunohistochemical staining method was applied for the VEGF protein. The angiogenesis score was assigned semi-quantitatively based on the intensity of VEGF immunoreactivity as observed through light microscopy at 40x magnification. This intensity was scored on a scale from 0 (no staining) to 3 (severe staining). Ten randomly chosen fields of view were analyzed per section.

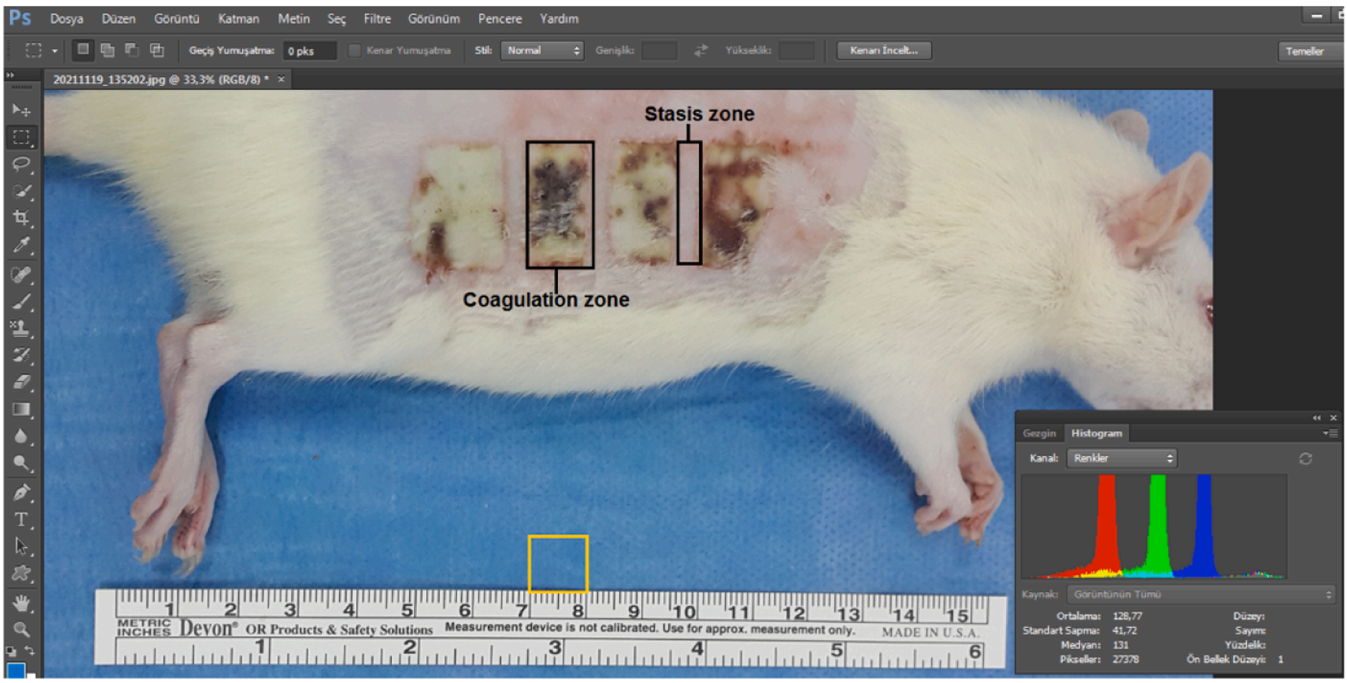

Fig. 1. Calibration process performed on the computer and the calculation of zone of stasis on the 3rd, 7th and 21st days.

2.6. Biochemical analysis

The tissues taken from the control and other treatment groupson the 3rd, 7th and 21st days were stored at a temperature −80 °C. In the biochemical analysis, MDA and CAT, SOD and GPx enzyme activity were determined in samples.

2.6.1. Homogenization of tissues

The skin samples from all groups (approximately, 0.1 g) were put into tubes and 1 milliliter of the pH 7.4 phosphate buffer was added. Using a homogenizer (Ultra Turrax T2, IKA Werke GmbH), the samples were homogenized until they were entirely disintegrated (homojenization at 6000 rpm for total of 5 min). A refrigerated centrifuge (Centrifuge 5415 R, Eppendorf AG) operating at 13,500 rpm at the temperature of + 4°C for 10 min, was utilized for the centrifugation [27]. The Bradford method was employed for the assessment of protein concentrations across all experimental groups [37].

2.6.2. Catalase (CAT)

The determination of CAT activity was conducted in accordance with the method originally described by Lück (1965) [38]. The activity of CAT was quantified via the spectrophotometric monitoring of hydrogen peroxide (H_2O_2) decomposition into water and molecular oxygen at a wavelength of 240 nm. The specific activity results were expressed as units per milligram of protein (U/mg protein)

2.6.3. Superoxide dismutase (SOD)

The total superoxide dismutase (SOD) activity was determined in accordance with the methodology described by Sun et al. [39]. This method is based on the principle of preventing the reduction of nitro blue tetrazolium (NBT) by the xanthine oxidase system, which generates superoxide. The quantity of SOD required to impede the reduction of NBT by 50 % is considered to be a single unit of the enzyme. The activity of SOD was expressed in units per milligram of protein (U/mg of protein).

2.6.4. Glutathione peroxidase (GPx)

The activity GPx was quantified using the methodology described by Lawrence and Burk [40]. The method entails determining the rate of oxidation of NADPH at a wavelength of 340 nm for a period of 3 min at a temperature of 25°C, with the inclusion of glutathione reductase (GR) and glutathione (GSH) in the reaction milieu. Glutathione peroxidase activity was quantified as units per milligram of protein (U/mg protein).

2.6.5. Malondialdehyde (MDA)

MDA levels were quantified through the use of a TBARs (thiobarbituric acid-reactive substances) assay, as originally described by Mihara and Uchiyama [41]. The results were presented as nanomoles per milligram of protein (nmol/mg protein).

2.7. Statistical analyses

The resulting data were summarized employing a median (minimum-maximum) value approach. In order to analyze the zone of stasis measurements, a two-way permutation analysis of variance (PERMANOVA) was performed with factors including group (7 levels: CFE, CLE, MFE, MLE, EFE, ELE, Burn) and measurement time (3 levels: at the day 3, 7, and 21). The design that was used was a two-way crossover design. According to normality tests, the data did not exhibit a normally distributed pattern ($p > 0.05$) for the histologic results. Therefore, nonparametric tests were employed for the comparison of the groups. Kruskal-Wallis analysis of variance was used for group comparisons for all variables, and Mann-Whitney *U* test was used for pairwise group comparisons with Bonferroni correction. Analysis was conducted with Python 3.9 and IBM SPSS Statistics for Windows version 26.0 (NewYork, NY, USA).

3. Results

3.1. Macroscopic analyses

The changes in the zones of stasis measurements of rats on a daily basis (3rd, 7th and 21st day) are presented in the Table 1 and Figs. 2 and 3. Statistical analysis revealed a significant difference in the zone of stasis measurements ($F = 138.83$, $p < 0.001$). Post-hoc comparisons showed significant differences between all measurement pairs (day 3 to day 7, day 3 to day 21, day 7 to day 21) ($p_3<0.001$). Statistically significant differences were found of zones of stasis values among the groups (CFE, CLE, MFE, MLE, EFE, ELE, Burn) ($F=3.58$, $p_2<0.001$). In post-hoc analyses, there were significant statistical differences between

Table 1
The results of statistical analysis on zones of stasis (Median (Min-Max), cm^2).

Groups and Days	Stasis Area Median (Min-Max)	Between measurements F value p value	Between Groups F value p value	Interaction (Group*Measure)
Burn−3rd	0.600 (0.421–0.884)	F= 138.83 $p_1< 0.001$	F= 3.58 $p_2< 0.001$	F= 1.36 p = 0.16
Burn−7th	0.662 (0.412–0.909)	3th day−7th day $p_3< 0.001$	CFE-CLE $p_4= 0.386$	
Burn−21st	1.149 (0.764–1.847)	3thday−21th day	CFE-EFE $p_4= 0.212$	
CFE−3rd	0.624 (0.534–0.768)	$p_3< 0.001$ 7th day−21th day	CFE-ELE $p_4= 0.039$	
CFE−7th	0.819 (0.662–0.914)	$p_3< 0.001$	CFE-MFE $p_4= 0.640$	
CFE−21st	1.158 (0.858–1.986)		CFE-MLE $p_4= 0.267$	
CLE−3rd	0.683 (0.531–0.951)		CFE-Burn $p_4= 0.368$	
CLE−7th	0.838 (0.481–1.053)		CLE-EFE $p_4= 0.700$	
CLE−21st	1.373 (1.035–2.376)		CLE-ELE $p_4= 0.234$	
MFE−3rd	0.663 (0.322–0.743)		CLE-MFE $p_4= 0.684$	
MFE−7th	0.833 (0.645–1.172)		CLE-MLE $p_4= 0.800$	
MFE−21st	1.298 (0.869–4.336)		CLE–Burn $p_4= 0.070$	
MLE−3rd	0.742 (0.433–0.95)		EFE-ELE $p_4= 0.410$	
MLE−7th	0.848 (0.702–1.097)		EFE-MFE $p_4= 0.430$	
MLE−21st	1.202 (0.901–1.711)		EFE-MLE $p_4= 0.890$	
EFE−3rd	0.731 (0.417–0.889)		EFE-Burn $p_4= 0.03$	
EFE−7th	0.847 (0.632–1.153)		ELE-MFE $p_4= 0.11$	
EFE−21st	1.390 (0.872–1.943)		ELE-MLE $p_4= 0.34$	
			ELE-Burn $p_4= 0.003$	
			MFE-MLE $p_4= 0.510$	
			MFE-Burn $p_4= 0.17$	
			MLE-Burn $p_4= 0.044$	
ELE−3rd	0.769 (0.628–0.973)			
ELE−7th	0.996 (0.718–1.273)			
ELE−21st	1.223 (0.838–1.920)			

p_1 value: The significance test result among measurements, p_2 value: The significance test result of inter-group PERMANOVA, p_3 value: Results of post-hoc comparison significance test among measurements, p_4 value: Results of post-hoc comparison significance test between groups.

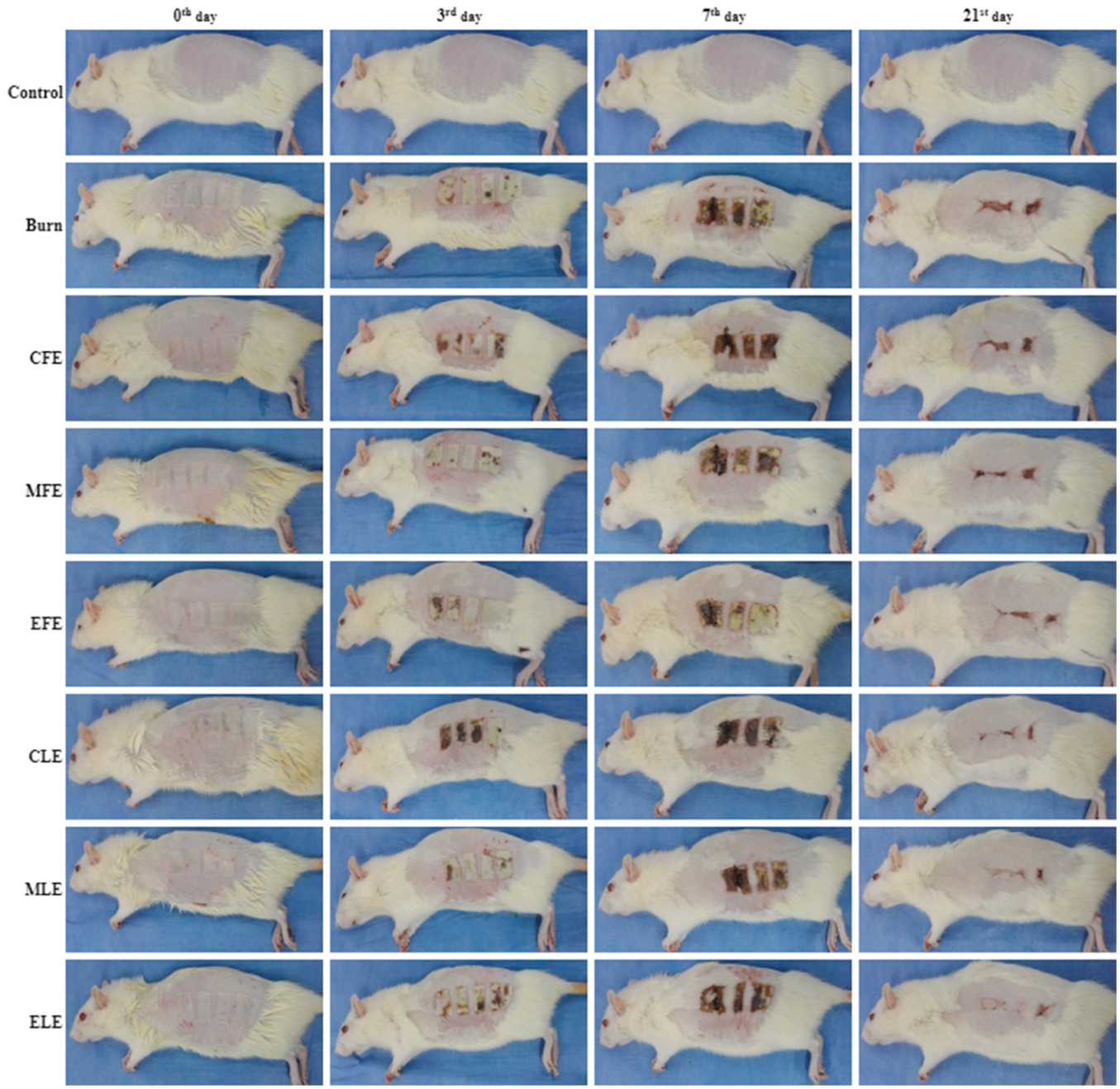

Fig. 2. Macroscopic views of the burned areas in rats at distinct time of experiment. CFE: Chokeberry fruit extract, CLE: Chokeberry leaf extract, EFE: Elderberry fruit extract, ELE: Elderberry leaf extract, MFE: Black mulberry fruit extract, MLE: Black mulberry leaf extract.* : Different for EFE, ELE, and MLE groups.* *: Different for CFE group.

the CFE-ELE, EFE-Burn, ELE-Burn, and MLE-Burn groups in terms of stasis zones ($p_4<0.05$). Additionally, the analysis revealed that the observed difference among the groups over time was not statistically significant ($F=1.36$, $p = 0.16$). Furthermore, the interaction effect was not found to be statistically significant ($p > 0.05$).

3.2. Histopathological analyses

The histological structures of the skin in the control group, labelled A and B, exhibited the typical characteristics of the epidermis and dermis, respectively. Upon closer examination, VEGF immunoreactivity was observed in the endothelial cells (indicated by thin arrow) and fibroblasts (indicated by thick arrow) that lined the blood vessels in the dermis. The control group exhibited typical histological characteristics, with no abnormalities noted (Fig. 4). In addition, VEGF immunoreactivity was clearly apparent in the endothelial cells and fibroblasts of the vessel walls.

3.2.1. 3rd day histopathological analyses

Histological evaluation of tissue samples focused on ICI. The burn group demonstrated the highest ICI density among all groups ($p < 0.001$). A comparative analysis was conducted on the treatment groups, it was seen that the infiltration density of the MLE, ELE and CLE groups was statistically lower than the MFE, EFE and CFE groups. VEGF was no detected in all of the groups (Fig. 5).

3.2.2. 7th day histopathological analyses

Samples of tissue were evaluated histologically in terms of ICI,

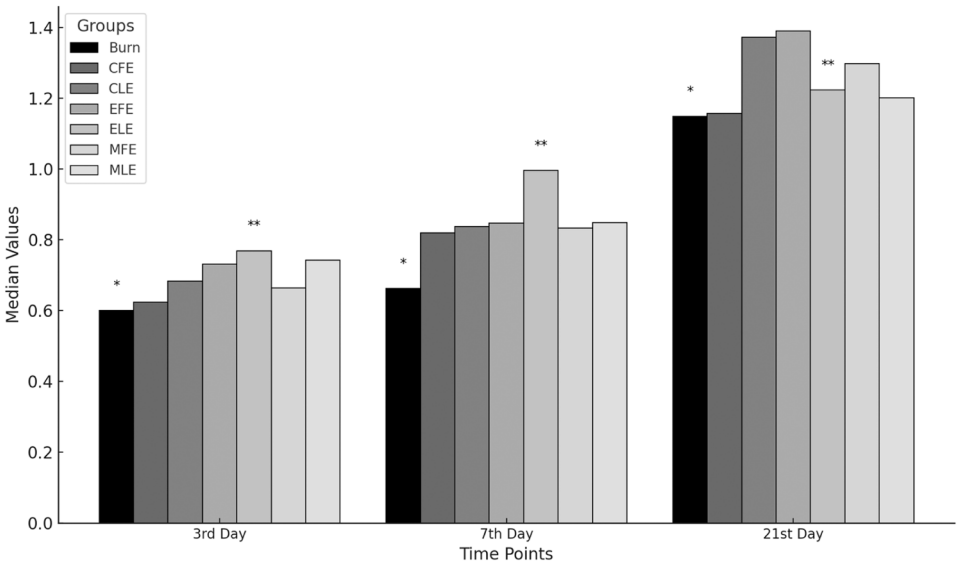

Fig. 3. Comparison of median stasis area across different treatment groups and days post-burn.

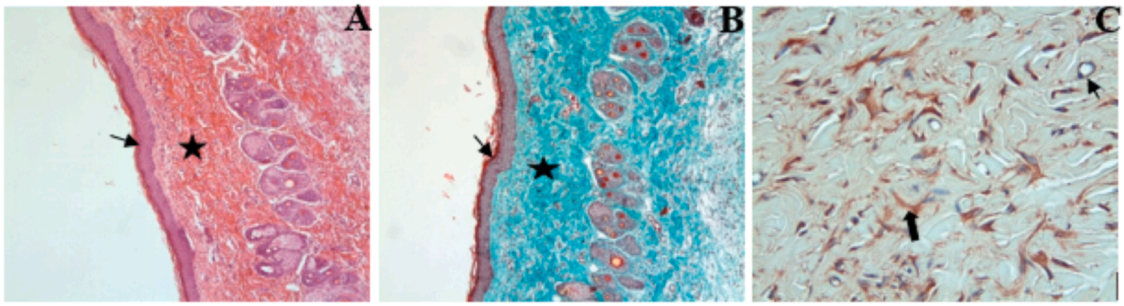

Fig. 4. In the control group, the epidermis (A, arrow) and dermis (B, star) exhibited a normal histological appearance; VEGF immunoreactivity (C) was observed in the endothelial cells (thin arrow) and fibroblasts (thick arrow) lining the walls of the blood vessels in the dermis. A: Hematoxylin-Eosin x10, B: Gomori trichrome x10, C: VEGF immunohistochemical staining x40.

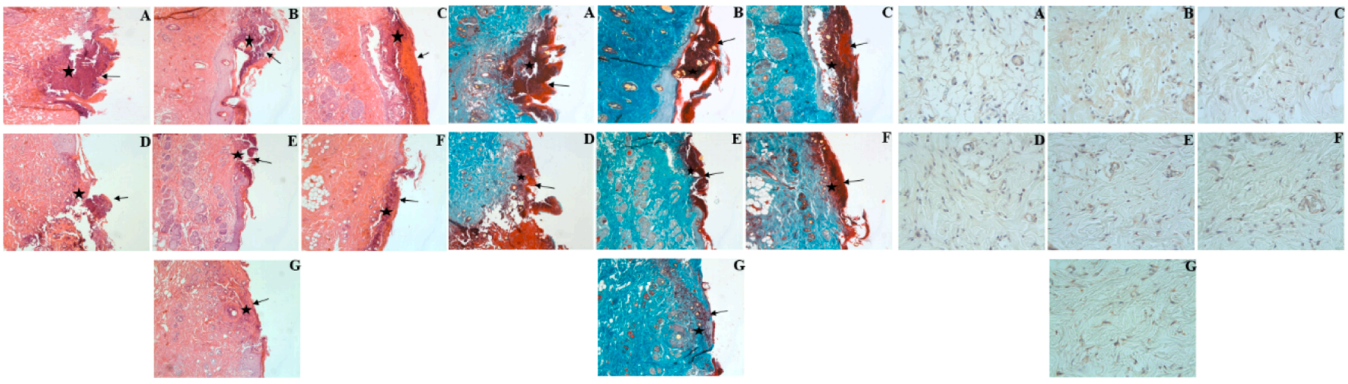

Fig. 5. Histological appearance of the 3rd day zone of stasis in the all groups. In all groups, it is seen that the epidermis is shed and the necrotic of the dermis. The arrows indicate epidermal exfoliation, and the stars point to necrotic dermis. A: Burn, B: MFE, C: MLE, D: EFE, E: ELE, F: CFE, G: CLE.

reepithelialization collagenization and angiogenesis. It was determined that 7 days after the burn application, the infiltration density was higher in the burn group and the observed difference was found to have statistical significance when compared to the other groups ($p < 0.05$). The ICI was significantly lower in groups MLE, ELE and CLE than in the groups MFE, EFE and CFE ($p < 0.05$) (Fig. 6).

3.2.3. 21st day histopathological analyses

Samples of tissue were examined histologically for ICI, reepithelialization collagenization and angiogenesis (Table 2). ICI was considerably higher in burn group in comparison to the treatment groups and was similar to the CFE group. While reepithelialization was found to be similar in the burn group to the MFE, EFE and CFE groups, it was found

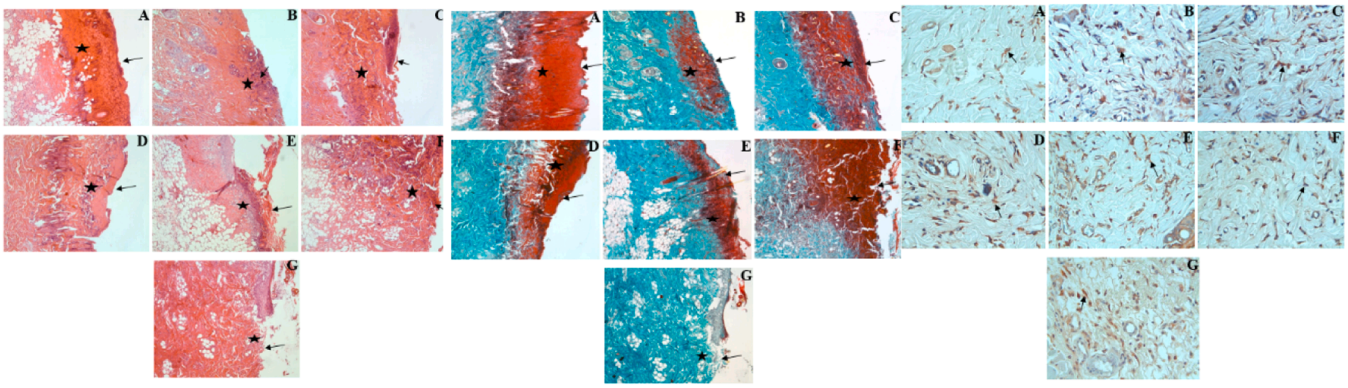

Fig. 6. Histological appearance of the 7th day zone of stasis in the all groups. In all groups, it is seen that the epidermis is shed and the necrotic of the dermis. The arrows indicate epidermal exfoliation, and the stars point to necrotic dermis. A: Burn, B: MFE, C: MLE, D: EFE, E: ELE, F: CFE, G: CLE.

Table 2
Histopathological and VEGF evaluation results of tissue samples (Median (Min-Max)).

Groups	Days	İnflammatory cell infiltration	Epithelialization	Collagenization	VEGF
Control	3rd	-	-	-	-
Burn		2 (0–3)	0 (0–0)	0 (0–0)	0 (0–0)
EFE		2 (0–3)[a]	0 (0–0)	0 (0–0)	0 (0–0)
ELE		1 (0–3)[a,b]	0 (0–0)	0 (0–0)	0 (0–0)
CFE		2 (0–3)[a]	0 (0–0)	0 (0–0)	0 (0–0)
CLE		1 (0–3)[a,b]	0 (0–0)	0 (0–0)	0 (0–0)
MFE		2 (0–3)[a]	0 (0–0)	0 (0–0)	0 (0–0)
MLE		1 (0–3)[a,b]	0 (0–0)	0 (0–0)	0 (0–0)
[a]Statistically significantly decreased in comparison to the burn group ($p < 0.05$)					
[b]Statistically significantly increase in comparison to the group MFE, EFE, CFE ($p < 0.05$)					
Control		-	-	-	-
Burn		2 (0–3)	0 (0–0)	0 (0–2)	1 (1–2)
EFE		2 (0–3)[a]	0 (0–0)	1 (0–3)[c]	1 (1–2)[c]
ELE		1 (0–3)[a,b]	0 (0–0)	1 (0–3)[c]	2 (1–2)[c]
CFE	7th	2 (0–3)[a]	0 (0–0)	1 (0–3)[c]	2 (1–2)[c]
CLE		1 (0–3)[a,b]	0 (0–0)	1 (0–3)[c]	2 (1–2)[c]
MFE		2 (0–3)[a]	0 (0–0)	1 (0–3)[c]	2 (1–3)[c]
MLE		1 (0–3)[a,b]	0 (0–0)	1 (0–3)[c]	2 (1–3)[c]
[a]Statistically significantly decreased in comparison to the burn group ($p < 0.05$)					
[b]Statistically significantly increase in comparison to the group MFE, EFE, CFE ($p < 0.05$)					
[c]Statistically significantly increase in comparison to the burn group ($p < 0.05$)					
Control	21st	-	-	-	-
Burn		1 (0–2)	1 (0–2)	0 (0–2)	1 (1–2)
EFE		0 (0–1)[a]	1 (0–3)[b]	2 (0–3)[c]	2 (1–3)[c]
ELE		0 (0–1)[a]	2 (0–3)[c]	2 (0–3)[c]	2 (1–3)[c]
CFE		1 (0–2)[b]	1 (0–3)[b]	2 (0–3)[c]	2 (1–3)[c]
CLE		0 (0–1)[a]	2 (0–3)[c]	2 (0–3)[c]	2 (1–3)[c]
MFE		0 (0–1)[a]	1 (0–2)[b]	1 (0–3)[c]	2 (1–3)[c]
MLE		0 (0–1)[a]	2 (0–3)[c]	2 (0–3)[c]	2 (1–3)[c]

[a] Statistically significantly decreased in comparison to the burn group ($p < 0.05$).
[b] Statistically significantly was no difference in comparison to the burn group ($p > 0.05$).
[c] Statistically significantly increase in comparison to the burn group ($p < 0.05$).

to be lower compared to the MLE, ELE and CLE groups. Collagenization was found to be markedly elevated in the treated groups in comparison to the burn group. Nevertheless, no statistically difference among the treatment groups (Fig. 7).

3.3. Biochemical analyses

The CAT, SOD, GPx, and MDA parameters differed significantly between the groups (Table 3). On day 7; SOD and CAT levels were found to be elevated in the treatment groups in comparison to the burn group ($p < 0.05$). At the 21st day of the study, no statistically significant difference found between the parameter of GPx. On day 21, the treatment groups had lower MDA levels than the burn group. (Table 3) ($p < 0.001$). On day 21, mean MDA levels were significantly reduced in both the EFE and MFE groups compared to the ELE and MLE groups. In the proliferation stage of healing the wound, CLE demonstrated efficacy in reducing tissue lipid peroxidation, with a more pronounced effect than that observed with CFE on day 21. On the seventh day, except for the EFE group, the MDA levels of other treatment groups were found to be lower compared to the burn group. On day 3, the group that received the CLE treatment exhibited lower levels of MDA than the burn group.

4. Discussion

Wound repair, particularly in burn healing, is a complex process involving three interdependent metabolic stages: inflammation, proliferation, and tissue remodeling [42]. Upon the skin is exposed to elevated temperatures, an outward radiating response is initiated at the site of initial contact, resulting in the formation of three distinct zones in all directions. In 1953, Jackson outlined the anatomical classification of burn wounds into three distinct zones: the initial zone of coagulation, the subsequent zone of stasis, and finally, the zone of hyperemia [43].

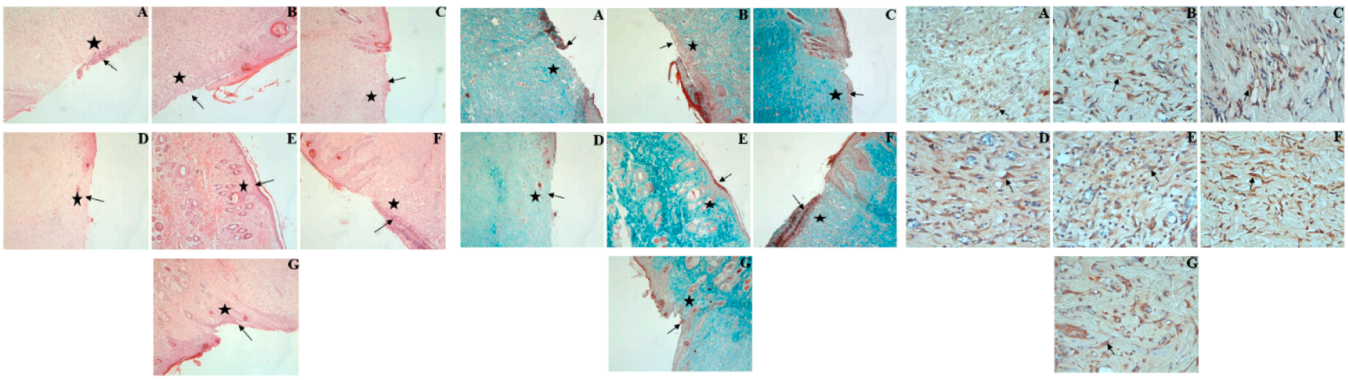

Fig. 7. Histological appearance of the 21st day zone of stasis in the all groups. The arrows indicate epidermal exfoliation, and the stars point to necrotic dermis. A: Burn, B: MFE, C: MLE, D: EFE, E: ELE, F: CFE, G: CLE.

Table 3
Result of SOD, CAT, GPx and MDA analysis in tissue samples from all groups (Median (Min-Max: Minimum-Maximum)).

Days	Groups*	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	MDA (nmol/mg protein)
3rd	Control	0.27^{d} (0.2–0.38)	120.54^{c} (94.59–149.52)	0.01^{c} (0.01–0.02)	91.94^{b} (54.64–123.73)
	Burn	0.19^{a} (0.11–0.23)	86.00^{a} (83.35–111.52)	0.01^{c} (0.01–0.01)	121.04^{bcd} (72.82–175.55)
	EFE	0.14^{b} (0.1–0.28)	78.77^{b} (71.55–93.88)	0.02^{b} (0.02–0.02)	230.16^{a} (158.27–299.46)
	ELE	0.16^{ab} (0.12–0.22)	138.3^{c} (96.37–182.62)	0.02^{ab} (0.01–0.03)	149.71 ^{cd} (76.45–175.09)
	CFE	0.19^{a} (0.17–0.20)	93.08^{ab} (62.68–140.15)	0.02^{ab} (0.01–0.04)	260.45^{a} (218.27–272.82)
	CLE	0.12^{b} (0.1–0.19)	90.40^{ab} (70.95–108.62)	0.01^{c} (0.01–0.01)	112.09^{bc} (60.09–197.36)
	MFE	0.06^{c} (0.02–0.14)	84.36^{a} (78.93–124.2)	0.01^{c} (0.01–0.02)	145.55^{d} (103.73–169.18)
	MLE	0.04^{c} (0.01–0.13)	77.59^{ab} (65.20–95.00)	0.01^{ac} (0.01–0.02)	222.02^{a} (174.64–290.09)
	p	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
7th	Control	0.27^{d} (0.2–0.38)	120.54^{c} (94.59–149.52)	0.01^{c} (0.01–0.02)	91.94^{b} (54.64–123.73)
	Burn	0.09^{b} (0.05–0.13)	78.09^{c} (54.81–86.53)	0.01 ^{cd} (0.01–0.02)	123.36^{d} (89.18–184.64)
	EFE	0.13^{ab} (0.06–0.20)	143.57^{b} (116.93–169.69)	0.02^{acd} (0.01–0.05)	250.93^{e} (211.91–337.13)
	ELE	0.14^{ab} (0.06–0.20)	106.80^{a} (68.12–146.14)	0.05^{b} (0.01–0.08)	42.36^{b} (16.45–75.09)
	CFE	0.16^{a} (0.04–0.22)	100.83^{a} (36.95–178.19)	0.03^{ab} (0.01–0.04)	55.54^{ab} (19.18–113.73)
	CLE	0.11^{ab} (0.05–0.22)	108.15^{a} (74.44–149.67)	0.02^{acd} (0.01–0.03)	73.54^{ac} (39.18–105.55)
	MFE	0.14^{ab} (0.09–0.22)	108.6^{a} (54.32–122.26)	0.02^{ac} (0.01–0.05)	78.27 ^{cd} (42.82–223.27)
	MLE	0.14^{a} (0.10–0.17)	106.03^{a} (84.89–112.73)	0.02^{acd} (0.01–0.02)	104.2 ^{cd} (69.91–158.09)
	p	**0.0023**	**0.0033**	**0.0059**	**< 0.001**
21st	Control	0.27^{d} (0.2–0.38)	120.54^{c} (94.59–149.52)	0.01 (0.01–0.02)	91.94^{b} (54.64–123.73)
	Burn	0.10^{a} (0.03–0.20)	137.77^{bc} (96.07–149.95)	0.02 (0.01–0.02)	131.93^{e} (103.73–164.64)
	EFE	0.13^{a} (0.04–0.21)	144.86^{c} (135.05–187.69)	0.02 (0.01–0.02)	82.82^{bc} (43.73–95.55)
	ELE	0.126^{a} (0.10–0.13)	142.52^{c} (127.65–169.43)	0.02 (0.01–0.03)	102.82^{a} (70.09–142.81)
	CFE	0.09^{a} (0.06–0.20)	101.63^{a} (64.91–150.58)	0.01 (0.01–0.01)	86.72^{ab} (72.82–102.82)
	CLE	0.12^{a} (0.08–0.18)	132.41^{bc} (110.45–150.68)	0.02 (0.01–0.02)	61.72^{c} (43.73–92.82)
	MFE	0.10^{a} (0.07–0.16)	125.75^{ab} (114.17–141.22)	0.02 (0.01–0.02)	27.36^{d} (25.54–41.91)
	MLE	0.12^{a} (0.09–0.14)	126.37^{bc} (113.49–154.3)	0.01 (0.01–0.07)	90.09^{ab} (61.91–122.82)
	p	**0.0052**	**0.0093**	**0.0602**	**< 0.001**

The variables are presented as 'Median (Min.-Max.)'
* :There is a statistically significant difference in categories containing different letters.

These zones have been defined in accordance with on the severity of damage to tissue and the blood circulation [44]. The initial zone of coagulation at the main region of injury is usually assumed to consisting of damaged tissue. The most peripheral zone is recognized as hyperaemia, which is delineated with vascular dilation and inflammatory changes, but no discernible structural impairment. Between these two areas, an intermediate region of unidentified prognosis occurs, which is referred to as the stasis zone [45]. The stasis zone is defined as a critical region in burn wounds, where tissues remain viable but are at significant risk of ischemic necrosis without timely intervention. [46].

The basis of burn treatment research is the recovery process of the burn's zone of stasis [47,48]. In the absence of appropriate intervention to the healing potential of the stasis zone, necrosis eventually sets in and blood circulation ceases entirely. Preventing the inflammation occurring in the zone of stasis and controlling the release of ROS in the acute phase are vital for the rescue of the zone of stasis. Recently, several studies have reported the efficacy of various agents (N-acetylcysteine, ozone, β-glucan, caffeic acid, etc.) in rescuing this zone.When examining the literature on the salvage the zone of stasis, it is usually observed that agents with high antioxidant properties and anti-inflammatory characteristics are preferred [49,50]. Furthermore, in serious burn patients, Reactive Nitrogen Species (RNS) and ROS levels rise in damaged tissue. The use of antioxidants in treatment has been shown to be an efficacious approach to reducing oxidative stress [51]. A diet rich in antioxidant content is a vital component of burn treatment, and it should be considered a necessary part of the healing process for burn patients [52].

Nutritional therapy represents a crucial component of the healing of burn wounds protocol [53]. The present findings demonstrate that components with antioxidant properties contribute to various parameters involved in the healing of burns and help to reducecell death resulting from injuries, improving situations conducive to maintaining the zone of stasis [53–55]. In our study, we used the extract of elderberry, chokeberry and black mulberry fruits and leaves and evaluated their effect on the burn wound healing estimated macroscopic, histopathologically and biochemically. Our main aim was to inhibit the progression of necrotic tissue in burns by preserving the zone of stasis.

A number of experiments have been conducted on animals to ascertain the impact of elderberry fruit, leaves, and flowers on burn wounds. In their investigation, Mogoşanu et al. [4] examined the impact of an extract derived from the flowers and leaves of the elderberry tree (S. nigra L.) on the wound healing process. The findings of this study indicate that elderberry extracts derived from flowers and leaves have a notable impact on burn healing in rats. The primary constituents responsible for these effects are reported to be flavonoids and tannins present in the extracts. In many studies, it has been reported that, overall, flavonoids and tannins contribute to the observed effects with their astringent, antiseptic, anti-inflammatory, and burn healing properties [25,56]. Elderberry leaf extracts (aqueous and ethanolic) have been investigated for their effects on the inflammatory phase in tissues resulting from injury, and it has been determined that extracts from elderberry leaves possess significant anti-inflammatory properties because of their capacity to neutralize reactive oxygen species [13].

In another study, fractions derived from elderberry leaves (S. ebulus L.) were investigated for their potential wound healing properties in rats' samples, with a comparison made to the reference drug 'Madecassol'. Through macroscopic and histological evaluations, the portion of the mixture exhibiting the most pronounced therapeutic impact was isolated, and the quercetin-3-O-glucoside compound was identified within it [54]. It has been demonstrated that the extract of mulberry leaves (Morus nigra L.) accelerate the healing of thermal burns, resulting in less tissue damage than in control groups of animals [57]. Another investigation, the healing effects of the fruit and leaves of mulberry (Morus nigra L.) have been investigated through an in vivo study, and the fruit extract has been fractionated to reveal the structures of quercetin-3-O-rutinoside and kaempferol-3-O-rutinoside compounds, which have been indicated to be effective in wound healing [58].

In our study, the macroscopic evaluations conducted on the 3 day post-burn, the wound was covered by a scab. On the 7th day, the burn areas of all rats decreased while the zone of stasis increased. Additionally, on the 7th day, the burn areas of all rats crusted (Fig. 4). On the 21st day, it was determined that the mean zone of stasis values of the treatment groups were higher mean for these than for the burn group. Additionally, the scabs reduced to the center, some of them fell off and the wounds of each group had almost healed. The process of wound healing involves the occurrence of an inflammatory phase. Indeed, an extended inflammatory phase has a significantly inhibitory effect on the healing process [54]. In burn wounds, chemical substances produced by damaged tissues are activated in a two-phase proinflammatory and antiinflammatory response. After a severe burn injury, nuclear factor κB, a transcriptional activator (protein), is activated to regulate the expression of proinflammatory mediators such as tumor necrosis factor alpha (TNF-α) and intercellular adhesion molecule-1 (ICAM-1). These mediators start antimicrobial activity by triggering monocytes and neutrophils. TNF-α is also in responsible for inducing apoptosis in a variety of cells around the wound and secreting other proinflammatory mediators like interleukins 1 and 6 (IL-1, IL-6) [27]. Determined the possible additive role of anti-inflammatory components in fruits and leaves extracts on inflammatory cell infiltration, which may be associated with wound healing.

The results of the current study indicated that, on the third day after the burn, the process of epithelialization and collagenization had not yet begun. Additionally, the presence of VEGF was not detected in all experimental groups. On the third day after the burn, cross-sections from all experimental groups were notable for the presence of necrotic dermis and sloughed epidermis. In addition, ICI was observed in numerous samples. When the obtained data were examined, it was found that the highest ICI density on day 3 was in the burn group ($p < 0.001$).

In the study, biopsies taken 7 days after the burn injury showed that necrotic dermis and sloughed epidermis persisted in the groups exposed to the burn. Meanwhile, epithelialization and collagen formation began in all treatment groups, with the burn group exhibiting the slowest progress. Despite the elevated collagen density observed in treatment groups compared to the burn group, no statistically significant differences were identified among the treatment groups.

Angiogenesis (the formation of new blood vessels) is a critical component of the wound healing process, as it facilitates the delivery of essential oxygen and nutrients to the affected area [59]. However, disruptions to this process can delay healing. VEGF is a key mediator of angiogenesis, which is regulated by multiple other factors during the wound healing process [47]. VEGF was found to be significantly higher in the treatment groups in comparison to the burn group. Nevertheless, no statistically significant differences were observed between the treatment groups. On the 21st day, the histological examination revealed that the ICI was considerably higher in burn group in comparison to the treatment groups, and exhibited a profile comparable to that of the CFE group.

Reepithelialization was observed in all groups 21 days after the burn treatment. Reepithelialization was found to be similar in the burn group to the EFE, CFE, and MFE groups, while it was observed to be lower when compared to the ELE, CLE, and MLE groups. Assessment of collagenization indicated a notable increase in this process in the treatment groups when compared to the burn group. However, when comparing the treatment groups, no significant difference could be identified ($p < 0.05$). It was observed that there was a statistically significant increase in VEGF levels in the treatment groups in comparison to the burn group. However, no significant intergroup difference was identified amongst the treatment groups themselves.

During the inflammatory response, immune cells chiefly neutrophils and macrophages are observed to accumulate at burn lesions. This phenomenon is accompanied by a process designated as respiratory explosion, resulting in the production of ROS. Consequently, the amount of ROS rises to an extent that surpasses the antioxidant system's capacity to neutralize and eliminate them. Antioxidant enzymes, including CAT, SOD, and GPx are synthesized with the objective of defending the organism from oxidative stress-related damage [29]. These are antioxidant enzymes that function to either reduce the formation of free radicals or inhibit their activity within cells. They can neutralize any molecule that has the potential to become a free radical or that has the capacity to cause other radicals to be produced very quickly [28].

These enzymes have been previously researched and it is well established how important they are to the process of skin repair [29]. SOD plays a pivotal role in antioxidant defense mechanisms, serving to mitigate the effects of oxidative stress in the wound area. According to reports, the level of SOD enzyme within acute wounds demonstrates a decline until the seventh day post-injury after which they exhibit a gradual return to normally [60]. At day 21, no statistically significant difference found between the treatment groups and the burn group. However, by day 7, the antioxidant enzyme SOD exhibited significantly elevated values in all treatment groups relative to the burn group ($p < 0.05$). On the seventh day, CAT levels increased in all treatment groups. On day 21, the EFE, ELE, and CLE groups exhibited significantly higher CAT levels compared to the burn group (Table 3). It is noteworthy that no significant difference was observed in the GPx parameter in the course of this study.

MDA is generated as an intermediate product of the lipid peroxide reaction, which is triggered in the wound area by free radicals [60]. The increase in this substance generally denotes destruction to the cell membrane, which often leads to a regional rise in oxidative stress [61]. Treatment groups reduced MDA levels on day 21st in comparison to burn group ($p < 0.001$). This result shown that the use of the fruit and leaf extracts reduces the lipid peroxidation. However, we could not significantly interpret the results of the treatment and control groups on days 3 and 7 of the study. In previous studies, it has been noted that MDA analysis may not give a highly sensitive results [27,62]. Although MDA levels are often measured in studies, it is recommended that other markers of lipid peroxidation are also assessed [63] In particular, the position of the tissue to be investigated should be standardised so as not to influence the results and normal skin tissue should not include [27].

5. Conclusion

Recent research has focused on identifying compounds that enhance the skin repair process. A variety of plants with strong antioxidant properties have been traditionally used in the treatment of wounds, and have also been demonstrated to have wound-healing properties.

The use of new antioxidant approaches in the treatment to reduce the effects of oxidative stress associated with burns is considered a promising step in burn management. In sum, this study found that daily oral treatment with elderberry, chokeberry and black mulberry extracts supported burn healing compared to the burn group within a rat comb burn model. Upon the fruits and leaves groups were compared, it was found that the findings of the histopathological healing process were better in the leaves groups. In the in vitro tests we conducted, we found that leaf samples contained significantly higher amounts of flavonoid compounds compared to fruits and had a higher antioxidant capacity. This effectiveness is thought to be related to their higher content of flavonoid components compared to fruit samples. Our findings demonstrated the therapeutic potential of medicinal plants and polyphenolic compounds in promoting wound healing.In this respect, we believe that our study will contribute to the literature investigating new drug treatments for burn management.

Ethical approval

This study was approved by the İnönü University Local Ethics Committee (decision number 2020/17–7) and conducted in accordance with ethical guidelines.

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CRediT authorship contribution statement

Rukiye Zengin: Resources, Investigation, Writing - Original Draft. **Selim Erdoğan:** Project administration, Resources. **Onural Özhan:** Methodology, Resources. **Elif Taşlidere Karaca:** Investigation, Formal analysis. **Semih Özçinar:** Methodology. **Yakup Yilmaztekin:** Investigation. **Fatma Hilal Yağin:** Formal analysis. **Yılmaz Uğur:** Investigation. **Cemal Firat:** Methodology, Writing - Review & Editing. **Hakan Parlakpinar:** Writing - Review & Editing. **Ayşe Burçin Uyumlu:** Writing - Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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