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Original Article

Wound Healing Activity of *Arum Maculatum*

Arum Macalatum Bitkisinin Yara İyileştirici Aktivitesi

Ayse ARZU SAKUL[1], Mehmet Evren OKUR[2], Sule AYLA[3], Benay DAYLAN[4], Ayse Esra KARADAG[5], Sebnem BATUR[6], Ekrem Musa OZDEMIR[7], Ebrar ALTINALAN[1], Mehmet Yalcin GUNAL[8]

[1]Istanbul Medipol University Faculty of Medicine, Department of Medical Pharmacology; Research Institute for Health Sciences and Technologies (SABITA), Istanbul, Turkey
[2]University of Health Sciences Faculty of Pharmacy, Department of Pharmacology, Istanbul, Turkey
[3]Istanbul Medeniyet University Faculty of Medicine, Department of Histology and Embryology, Istanbul, Turkey
[4]Istanbul Medipol University Faculty of Medicine, Department of Histology and Embryology, Istanbul, Turkey
[5]Istanbul Medipol University Faculty of Pharmacy, Department of Pharmacognosy, Istanbul, Turkey
[6]Istanbul University-Cerrahpasa, Cerrahpasa Faculty of Medicine, Department of Pathology, Istanbul, Turkey
[7]Istanbul Medipol University, Research Institute for Health Sciences and Technologies (SABITA); Medical Research Center (MEDITAM), Istanbul, Turkey
[8]Alanya Alaaddin Keykubat University Faculty of Medicine, Department of Physiology, Antalya, Turkey

ABSTRACT

Objective: In this study, the antioxidant properties of *Arum maculatum* plant were evaluated. This study reported for the first time the wound healing activity of the methanol extract of *A. maculatum* fruits. This study aimed to assess and determine the possible pharmacological activities of *A. maculatum* and evaluate its potential to act as a wound care plant.

Methods: The antioxidant and antimicrobial activities of *A. maculatum* were investigated using excisional *in vivo* and *in vitro* wound healing mouse models. A total of 32 Balb-c mice were used, which were equally, divided into four groups: saline control group, control group, *A. maculatum* group, and *Centella asiatica* extract group. Treatment applications were performed topically once per day. Wound area narrowing, wound healing percentage, and epithelialization time were analyzed.

Results: *A. maculatum* application supported the healing process in *in vivo* and *in vitro* wound models. *A. maculatum* contributed to the healing process by promoting granulation tissue formation, epidermal regeneration, and angiogenesis.

Conclusions: Wound healing is a complex and well-organized process that requires communication between cells. The antioxidant and antimicrobial activities of *A. maculatum* extract have been determined by current studies. *A. maculatum* extract may provide significant benefits in promoting the wound healing process.

Keywords: *A. maculatum*, wound healing, mice, antioxidant, extract

ÖZ

Amaç: Bu araştırmada antioksidan özelliklerini değerlendirmek için *Arum maculatum* bitkisi seçilmiştir. Bildiğimiz kadarıyla *A. maculatum* meyvelerinin metanol özünün yara iyileştirici aktivitesi ilk kez bu çalışmada rapor edilmiştir. Bu çalışma, *A. maculatum*'un olası farmakolojik aktivitelerini belirlemek, değerlendirmek ve bir yara tedavi edici bitki olarak etki gösterme potansiyelini değerlendirmek içindi.

Yöntemler: *A. maculatum*'un antioksidan ve antimikrobiyal aktiviteleri, farelerde eksizyonel *in vivo* ve *in vitro* yara iyileşme modelleri kulanılarak araştırılmıştır. Toplamda 32 Balb-c fare kullanılmış olup salin kontrol grubu, kontrol grubu, *A. maculatum uygulanan grup* ve *Centella asiatica* özütü uygulanan grup olmak üzere 4 gruba ayrılmıştır. Tedavi uygulamaları günde bir kez topikal olarak gerçekleştirilmiştir. Skar alanı hacminde gerçekleşen değişim, yara iyileşme yüzdesi ve epitelizasyon süresi analiz edilmiştir.

Bulgular: *A. maculatum* uygulaması *in vivo* ve *in vitro* yara modelinde iyileşme sürecini desteklemiştir. *A. maculatum*, granülasyon dokusunu artırarak iyileşme sürecine katkıda bulunmuş, epidermal rejenerasyonu ve anjiyogenezi artırmıştır.

Sonuçlar: Yara iyileşmesi, hücreler arası iletişimi gerektiren karmaşık ve iyi organize edilmiş bir süreçtir. Mevcut çalışmalar doğrultusunda antioksidan ve antimikrobiyal aktivitesi belirlenmiş olan *A. maculatum* özü, yara iyileşme sürecinin desteklenmesinde önemli bir fayda sağlayabilir.

Anahtar kelimeler: *A. maculatum*, yara iyileşmesi, fareler, antioksidan, ekstrakt

Address for Correspondence: A. Arzu Sakul, Istanbul Medipol University Faculty of Medicine, Department of Medical Pharmacology; Research Institute for Health Sciences and Technologies (SABITA), Istanbul, Turkey
E-mail: aasakul@medipol.edu.tr **ORCID ID:** orcid.org/0000-0002-9354-0000

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INTRODUCTION

As wounds are linked to the increase in loss of life, wound assessment and care are a challenge beyond medical society from ancient times onward[1]. Wounds gradually heal through well-organized processes that require cells to communicate with each other. This process is accompanied by cytokines, growth factors, and extracellular matrix (ECM) elements[2]. ECM proteins should maintain the skin's complex, and their roles are primarily found in connective tissue. Their relationship with skin cells is crucial in maintaining skin homeostasis and regeneration. In the phases of wound healing are as follows: (1) inflammatory phenotype transition that occurs in macrophages during the inflammatory process, (2) new artery development and angiogenesis stimulation by promoting endothelial cell differentiation and cell migration, and (3) granulation tissue, skin-cell, and ECM development, which leads to proliferation and remodeling. The above features can aid treatment plans in treating wounds and skin aging. They also sparked new research and clinical studies[3].

Arum maculatum L. (Arum) belongs to the *Araceae* family and known as "yılan pancarı, yılan yastığı" in Turkey. The plant has many traditional uses such as for cancer, constipation, fungal diseases, hemorrhoids, and rheumatism. It is also used for its diaphoretic, sudorific, and expectorant properties. *A. maculatum* is an acceptable remedy for paralysis[4]. In a previous study about the biological activity of *A. maculatum*, Alencar et al.[5] investigated lectins' pro-inflammatory activity when isolated. In addition, the insecticidal action of lectins obtained from the tuber of the plant was clarified[6]. Previous studies have also investigated the cytogenic, anti-hemorrhoid, antimutagenic, antifungal, antiaflatoxigenic, anticholinesterase, antimicrobial, and antioxidant activities of several plants[7-9]. The analgesic activity and hepatotoxicity of *A. maculatum* methanol extract were examined *in vivo* in rats, and remarkable results were obtained[8,10]. Reactive oxygen species (ROS) can form during the wound healing process, and regular consumption of foods showing antioxidant activity can reduce these harmful effects[11]. Plant extracts have antioxidant effects[12]. In this study, *A. maculatum* was chosen to determine the plant's antioxidant properties; to our knowledge, during the preparation of this manuscript, this was the only study that examined the activity of *A. maculatum* methanol extract in wound healing *in vivo*. This study aimed to identify and establish the potential pharmacological properties of *A. maculatum*, as well as its potential as a wound care product.

MATERIALS and METHODS

Materials

In this study, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid (ABTS)], Trolox, and ascorbic acid were provided by Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Only analytical-grade or higher chemicals, unless specified otherwise, were used.

Preparation of *A. maculatum* Extract

A. maculatum fruits were collected from Silivri province, Istanbul (Turkey), in May 2019. Ayse Esra Karadag described the plant material. Voucher samples IMEF no. 1099 were deposited at the herbarium in the Department of Pharmacognosy of the Istanbul Medipol University Faculty of Pharmacy, Turkey. The plant sample was cut into small pieces and dried under fresh air. The air-dried plant sample was crushed, macerated for 24 h in methanol, and ground into a powder. The methanol extract was used to prepare gel formulations for further analysis after filtration and evaporation.

Antioxidant Activity

DPPH Radical Scavenging Assay

DPPH• was used to determine the total antioxidant capacity[13]. The reaction compound included a total of 100 µM DPPH• in methanol and extract. The absorbance was measured at 517 nm using a UV spectrophotometer (UV-1800, Shimadzu, Japan) at 25 °C±2 °C after 30 min. As a % of radical reduction, the radical scavenging activity (RSA) was determined using the following equation:

$$DPPH{\bullet}RSA\% = [(Absorbance_{control} - Absorbance_{test\_sample}) / Absorbance_{control})] \times 100$$

ABTS Radical Scavenging Assay

The ABTS radical cation decolorization protocol was used to evaluate the antioxidant capacity of *A. maculatum*[14]. To produce ABTS•, a 2.45 mM potassium persulfate and 7 mM aqueous ABTS mixture reacted. Before using it, the mixture was kept at a 25 °C dark room for 16 h. The final mixture was stored in a dark room at a temperature of 25 °C for 16 h. The mixture was added with ethanol, and its absorbance at 25 °C was calculated at 734 nm. The procedure was carried out three times, and Trolox was chosen as the "positive control"[15]. Table 1 presents the results calculated as IC_{50}.

$$ABTS{\bullet}RSA\% = [(Absorbance_{control} - Absorbance_{test\ sample}) / Absorbance_{control})] \times 100$$

Table 1. *A. maculatum* ABTS and DPPH radical scavenging activities (ICD_{50} ± mg/mL).		
	A. maculatum	**Reference compounds**
	IC_{50} ± SEM (mg/mL)	
ABTS•	0.75±0.15	0.013±0.004 (Trolox)
DPPH•	0.52±0.18	0.004±0.001 (Ascorbic acid)
ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), DPPH: 2,2-diphenyl-1-picrylhydrazyl,		

In Vivo Experiments

Research Subjects

Balb-c mice weighing 24-28 g were used. The experimental animals were held inside houses in line with standards at 24 °C, had free access to food and water, and exposed to indoor lighting between 0700 and 1900 hours. Ethical approval was granted by the Istanbul Medipol University Animal Experiments Local Ethics Committee (decision no: 91, date: December 19, 2019) before the experiments.

Protocols of Wound Formation and Experiment Groups

The animals used in the experiment were divided into four (8 animals in each) groups:

Control: Treated with saline (saline control group)

Group 1: Vehicle group (control group)

Group 2: *A. maculatum* group.

Group 3: *Centella asiatica* extract (CAE) group (reference molecule, Madecassolâ cream)

Base gel, which served as the vehicle, was prepared according to a previous study[16]. To prepare the extract gel (5%, w/w), *A. maculatum* extract was added to the base gel and stirred slowly until a homogeneous gel was formed.

Mice were anesthetized with a mixture of ketamine (80-100 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. To create two wound tissues, the back region was shaved, and excisional scar tissue was created using a 5-mm biopsy apparatus on the left side of the midline. Wound tissues were localized 1 cm from each other and 1.5 cm from the midline. Topical treatment application to the wound areas was continued for 10 days.

Macroscopic Assessment

To score wound healing, wound areas were photographed at the beginning (day 0) and end of the application (day 10). Areas of wound surfaces were measured using the Image J software. The wound healing rate was calculated using the formula specified in the literature[17].

Histological Assessment

The scar tissues were removed at the end of day 10 after the animals were euthanized. Then, 10% neutral formalin was used for fixation. Furthermore, ethanol was used to dehydrate the wound samples, and toluene was used to clear them. The samples were then embedded in paraffin. Thereafter, 5 μm-thick sections of the samples were subjected to hematoxylin-eosin (HE) and immunohistochemical staining: vascular endothelial growth factor (VEGF; Santa Cruz sc-7269], collagen (COL1A1; Santa Cruz sc-293182), platelet-derived growth factor (PDGF-A; Santa Cruz sc-9974) on glass slides.

Wound healing was evaluated histologically using the method defined by Galeano et al.[18] and Ayla et al.[16]. In terms of angiogenesis, 1 point indicates one to two vessels/site; 2 points, few neovascularizations (3-4/site); 3 points, newly formed capillary vessels (5-6/site); and 4 points, newly formed and normal looking capillary vessels (>7/site). In the assessment of angiogenesis, four microscopic fields in each subject were evaluated at ×20 magnification.

In terms of epidermal organization, 1 point indicates weak epidermal creation (≥20%); 2 points, incomplete epidermal creation (≥40%); 3 points, moderate epithelial creation (≥60%); and 4 points, complete epidermal organization (≥80%). Granulation tissue thickness was measured as follows: 1 point, weak granulation layer; 2 points, intermediate granulation layer; 3 points, dense granulation layer; and 4 points, very thick layer.

In this study, a quantitative method was used to determine the immunoreactivity of immunohistochemical staining. Five fields were randomly selected, and these fields were evaluated and averaged: 0 points, no staining; 1 point, poor staining; 2 points, moderate staining; and 3 points, strong staining[16,19].

Statistical Analysis

Tests were conducted using IBM SPSS Statistics for Windows version 20 (IBM Corp., Armonk, NY, USA). Intergroup differences were analyzed using analysis of variance and the least significant differences tests. All values were presented as mean ± standard error of the mean, and a p-value <0.05 indicated the significance limit.

RESULTS

Antioxidant Activity

Treatment compounds containing antioxidants have been demonstrated to aid in wound healing and protect them from oxidative damage[16]. Table 1 presents the study results of *A. maculatum* extract's antioxidant activity. According to comparisons with standard substances, *A. maculatum* extract was thought to have effective antioxidant activity. As a result of this study, it has been shown that *A. maculatum* extract has an antioxidant effect. This antioxidant capacity was thought to have an important effect on the wound healing process.

Macroscopic Wound Healing

The wound healing rates and macroscopic wound healing are shown in Figure 1. After day 10, no significant wound contraction difference was found between the *A. maculatum* (Arum) group and the control group (p ≥ 0.05) (Figure 1). However, when compared with day 0, a significant difference in wound contraction was found. Similarly, no significant wound contraction difference was found between the CAE group and the control group after day 10 (p=0.321). However, a significant difference was observed between day 0 and the control group. The outcome was the same for the vehicle group (Figure 1).

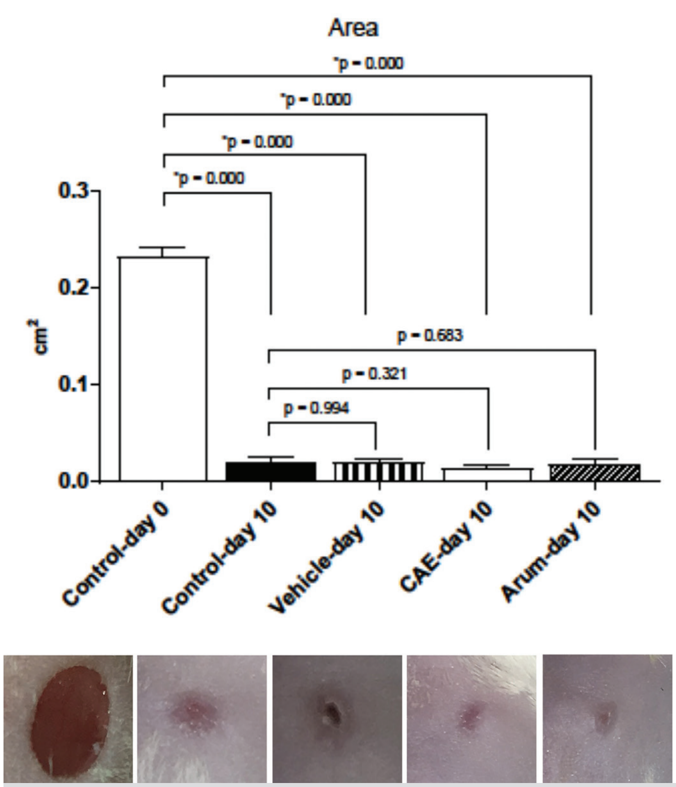

Figure 1. Healing of the wound area and macroscopic wound healing observations for the control group, vehicle group, CAE group, and *A. maculatum* group.

CAE: *Centella asiatica* extract, *A. maculatum*: *Arum maculatum*

Histology of Wound Healing

Histological (H&E) and immunohistochemical (VEGF, PDGF, and collagen) evaluations were appraised separately. The results of the macroscopic evaluation are presented in Figure 1. Histopathologically, histological evaluation (Figure 2) and immunohistochemical staining data, which enable comparison of immunohistochemistry collagen, PDGF, and VEGF wound healing scores between the groups, are shown in Figure 3. Epidermal regeneration, thickness of the granulation tissue, and angiogenesis from HE sections were evaluated and scored separately (Figure 2). Both *A. maculatum* extract (p<0.0001) and CAE (p<0.0001) were more potent than the control and vehicle groups in terms of vascularization, granulation tissue development, and epidermal tissue integrity (Figure 2). PDGF and VEGF immunohistochemical staining performed in the CAE groups was more potent than those in the control and vehicle groups (p≤0.05) (Figure 3). However, only VEGF staining increased significantly for *A. maculatum* (p≤0.02) (Figure 3); however, PDGF (p=0.08) and collagen (p=0.8) staining was not significantly different between the *A. maculatum* group and the control group (Figure 3).

DISCUSSION

Since wound assessment and treatment is a complex process involving various aspects with significant influence on healing, wound healing is an unfulfilled clinical challenge in healthcare[1]. Wounds occur when tissue integrity is disrupted. Wound healing is frequently studied, and some of its mechanisms still need explanation[20]. According to traditional definitions, hemostasis, inflammation, proliferation, and tissue remodeling are four separate stages of wound healing. The arrangement of these stages in wound healing relies on factors such as cytokines, growth factors, proteases, eicosanoids, kinins, and cellular metabolites[21], and these may delay wound healing. Previously, all wounds heal with complications such as scars and infection; however, at present, side effects related to wound healing are reduced. Despite this, chronic wounds still bore doctors[22].

Plants have been traditionally utilized to improve wound healing as topical formulations, and they proved beneficial in wound care and accelerate wound healing without causing distress, pain, and scarring[23].

As a result of phytochemical screening, *A. maculatum* was found to contain glycosides such as flavonoids, saponins, cyanogenes, alkaloids, polyphenols, 2-heptanone, (E)-caryophyllene, indoles, p-cresol, proanthocyanidins, monoterpenes, two sesquiterpenes, and lectins[10].

Because of their capacity to scavenge free radicals, singlet oxygen, and superoxide radicals, phenolic compounds are classified as strong antioxidants. The antioxidant potential related to mature collagen fiber growth and enhanced angiogenesis supported by fibroblasts was determined to be responsible for these effects[24]. According to the above studies, *A. maculatum* fruits have a high phenolic profile. Therefore, the wound healing activity can be considered to result from this phenolic profile.

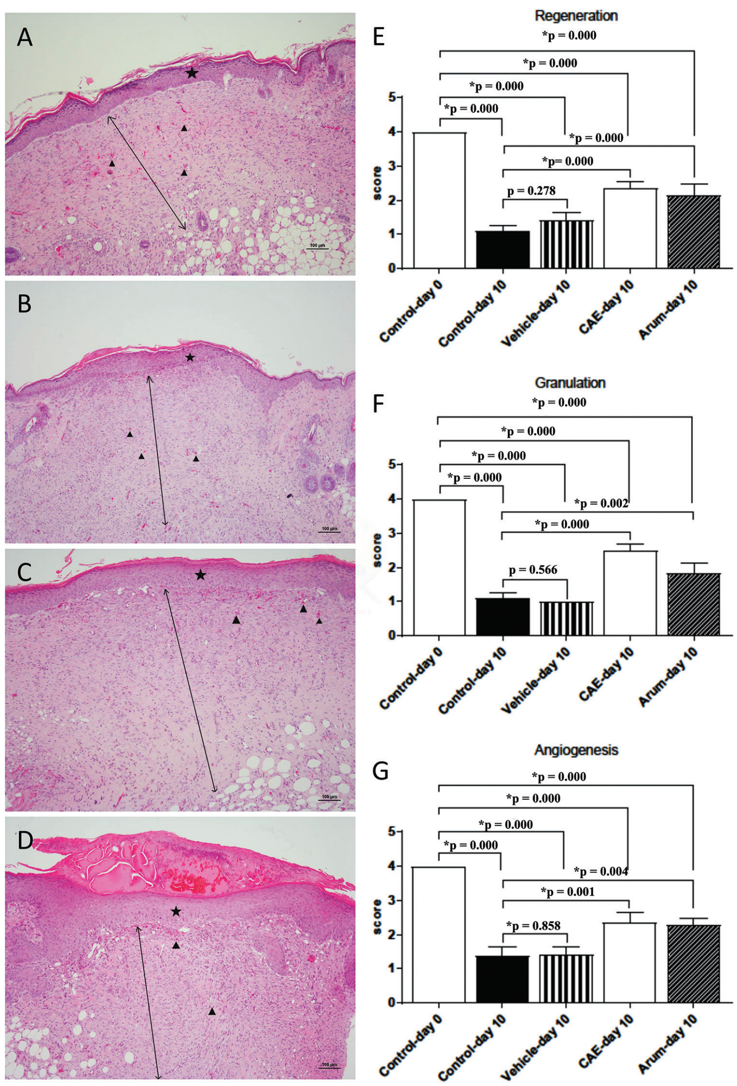

Figure 2. Histopathological view of the injured tissues of the control (A), vehicle gel (B), CAE (C), and *A. maculatum* (Arum) (D) extract gel on day 10 after wound incision (original magnification, ×10). (E-G) Histological scores of epidermal regeneration, granulation tissue thickness, and angiogenesis of the control, vehicle gel, CAE, and *A. maculatum* (Arum) groups. Arrow, granulation tissue thickness; star, epidermal regeneration; triangle, angiogenesis. Statistically significant when compared with the control group; $p<0.05$. Values are presented as the mean ± SEM. The scale bars represent 100 μm.

CAE: *Centella asiatica* extract, *A. maculatum*: *Arum maculatum*

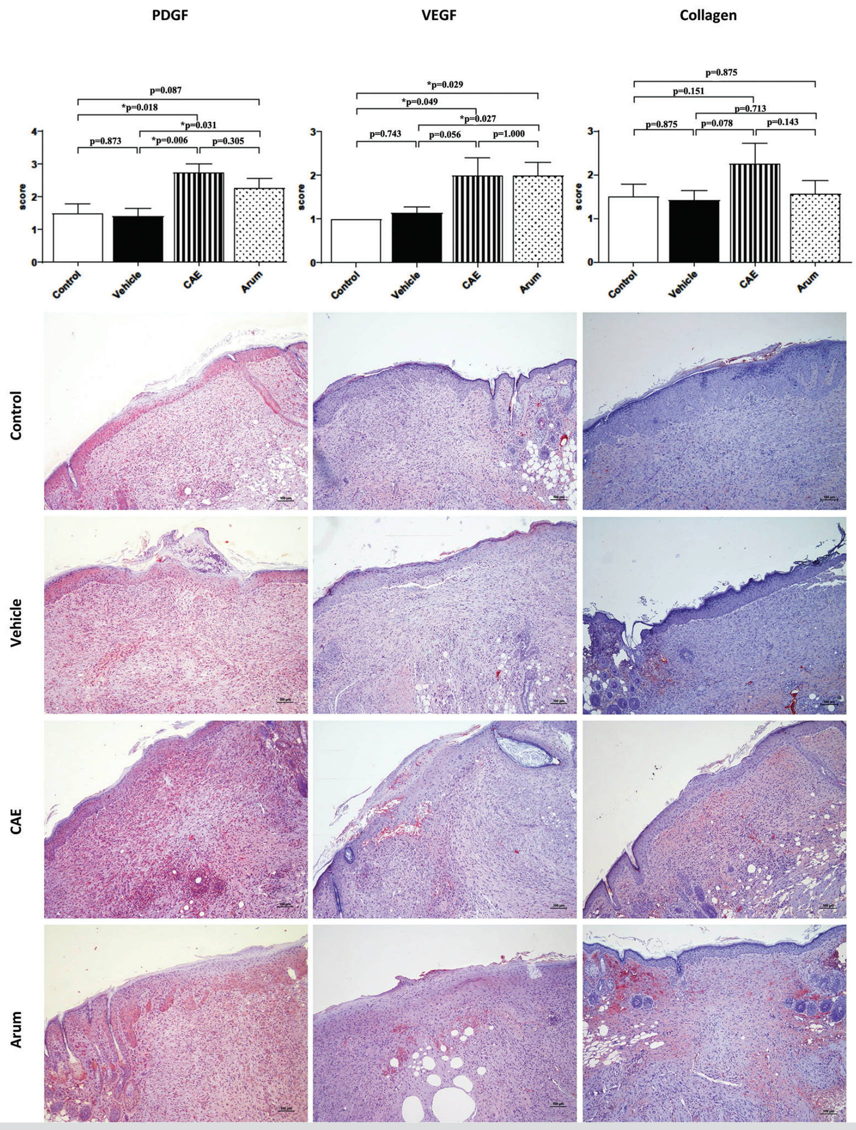

Figure 3. Comparison of immunohistochemical collagen, PDGF, and VEGF wound healing scores among the groups. Statistically significant when compared with the control group; $p<0.05$ (*). Values are presented as the mean ± SEM. Histopathological view of the injured tissues of the untreated (control), vehicle, *A. maculatum* (Arum), and CAE groups onday 10 after wound incision (original magnification, ×10).

CAE: *Centella asiatica* extract, *A. maculatum*: *Arum maculatum*, PDGF: Platelet-derived growth factor, VEGF: Vascular endothelial growth factor

Wounds heal to reduce inflammatory factors and increase tissue formation with a new blood vessel[20]. Studies have used herbal extracts to speed wound healing, and many agents increase cell proliferation, angiogenesis, and collagen production. Therefore, these compounds have been used for these properties[25,26]. Previous studies have also investigated the antimicrobial and antioxidant activities of various plants, such as *A. maculatum*[7]. In fact, *A. maculatum* was used for wound healing purposes. In addition, the use of *A. maculatum* to treat hemorrhoids among the general population may be associated with its wound healing effect[27]. However, no studies have shown its effect on wound healing. In this study, topical application of *A. maculatum* was found to improve skin healing. A statistically significant boost in the re-epithelialization, angiogenesis, and granulation tissue thickness was found in the *A. maculatum* group or the CAE group, compared with the control group. Accordingly, scientific data confirmed the effectiveness of this herbal preparation.

When the hemostatic plug is formed, platelets produce transforming growth factor beta, PDGF, and fibroblast growth factor, inducing angiogenesis. In this study, *A. maculatum* was observed to increase wound healing, primarily by increasing VEGF. Angiogenesis improves fibroblast activity and feeding wound area. The fibroblast activity contributes to the wound healing process by contributing to the formation of granulation tissue. Fibroblast proliferation and migration are decreased by ROS in wounds, which decreased collagen synthesis[28]. We can attribute this to the absence of a difference in collagen staining between the *A. maculatum* group and the control group.

In this study, *A. maculatum* was observed to promote the healing of skin wounds. Epidermal regeneration and granulation tissue were increased in the *A. maculatum* and CAE groups compared with those in the saline control group. Thus, *A. maculatum* extract could be a new alternative in treating wounds, owing to its feature similar to CAE.

CONCLUSION

The results of this show that topical treatment of any wound with *A. maculatum* promotes advanced wound contraction and helps in healing experimental skin wounds in mice. In light of the present results, *A. maculatum* extract could contribute significantly to the wound healing process *in vivo* owing to its antioxidant activity. Owing to the great *in vivo* wound healing behavior of *A. maculatum* extract, further investigation on larger animal models or humans could confirm its possible application for wound healing.

Ethics

Ethics Committee Approval: Ethical approval was granted by the Istanbul Medipol University Animal Experiments Local Ethics Committee (decision no: 91, date: December 19, 2019) before the experiments.

Informed Consent: Animal experiment study.

Peer-review: Externally and internally peer-reviewed.

Author Contributions

Surgical and Medical Practices: A.A.S., M.E.O., S.A., B.D., A.E.K., S.B., E.M.O., E.A., M.Y.G., Concept: A.A.S., Design: A.A.S., M.E.O., Data Collection and/or Processing: A.A.S., M.E.O., S.A., B.D., A.E.K., S.B., E.M.O., Analysis and/or Interpretation: A.A.S., M.E.O., S.A., M.Y.G., Literature Search: A.A.S., M.E.O., A.E.K., E.A., Writing: A.A.S., S.A.

Conflict of Interest: The authors have no conflict of interest to declare.

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