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Research Article

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Determination of the Wound Healing Effect of Walnut (*Juglans regia*) Hull Waste in Rats

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Abstract: Today, many problems such as skin disorders, dermatological diseases, especially cuts and injuries due to bacterial, fungal and viral factors are increasing day by day. Due to the side effects, cost and resistance development potential of drugs, creams and ointments used in the treatment of these diseases, alternative treatment methods are being investigated. In this context, walnut plant (Juglans regia L.) plays an important role in the treatment of eczema, wounds and skin diseases due to its antibacterial, antifungal and antiviral, wound healing and tissue repairing effects. The aim of this study is to determine the wound healing effect of Juglans regia L. hull in topical application on rats. In the study, the wound healing effect of cream formulations prepared by adding walnut hull extract (0%, 25%, 50%) was examined in rats. Wound size and tissue examination were performed to evaluate wound healing. Statistically significant differences were found between the groups in terms of histopathologic and histochemical parameters ($p < 0.01$). Wound diameter in mice was reduced to 420.28±37.46 mm and 336.28±21.66 mm with creams containing 25% and 50% extracts, respectively. Compared to the control group (578.85±27.23 mm), these values show that the extract increased the wound closure feature in a dose-dependent manner. Therefore, the addition of 50% extract was determined as the most effective application for wound healing. This study is of great importance in terms of both the utilization of waste and the scientific characterization of the knowledge in traditional medicine.

Keywords: Juglans regia, walnut hull, wound healing, natural cream, waste utilization.

1. Introduction

Plants, which have served various purposes such as food, beverage, medicine, ointment, spice, spice, dye, cosmetics, phytotherapy, aromatherapy from the past to the present, continue to increase their importance and usage areas by taking advantage of the gains obtained with the developing science and technology [2].

As in many fields, healing plants are very popular in the medical field, especially in folk medicine. In this context, despite the rapidly growing developments in modern medicine, pharmaceuticals and chemical industry, alternative treatment methods and treatment with plants still maintain their popularity and the demand for plants has been increasing in developed countries in recent years [1].

Disruption of the integrity and functioning of tissues is called a wound. The restoration of this functioning and the regaining of tissue integrity is called wound healing. Wound healing generally occurs in four stages: haemostasis, inflammation, cell proliferation and remodelling. This process represents the desire of the tissue to restore its normal anatomical structure and function [2,4]. Many in the scientific and medical communities continue to conduct research in search of improved wound care [3]

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The leaves and fruits of the walnut plant, which is the plant used in both natural dye and alternative treatment methods, serve this purpose. In recent years, natural compounds have become an attractive research topic due to their potential to promote wound healing. In this context, walnut (Juglans regia) walnut hull is an important focus for its potential health benefits. [5,6].

Walnut plant is a plant that supports wound healing and accelerates the healing process thanks to bioactive components such as phenolics, flavonoids, organic acids, terpenoids, juglon, which have antibacterial, antifungal, antioxidant properties especially in its leaves and fruits. Walnut hull is a rich source of such biological molecules. In addition, thanks to juglon, a phenolic type that it contains a lot of, it prevents cell damage by reducing oxidative stress and can be effective in wounds and cancerous cells. In addition, the minerals contained in walnut hull, especially zinc and magnesium, help cellular regeneration and healing processes [9]. Factors such as industrial wastes, waste of natural resources and consumption imbalances lead to the deterioration of the natural balance and endanger human health. Walnut hull from walnut shelling machine, which constitutes an important part of these wastes, is harmful to the environment if it is not utilised. Considering that the average shell rate of walnuts produced in Turkey is 48.422% (85.847 tonnes of shells), it is clearly seen that these shells are wasted [10].

The aim of this study is to systematically evaluate the potential effects of walnut hull on wound healing. The biochemical content of walnut hull and its contribution to wound healing processes will be discussed in the light of literature review and existing research. In addition, the clinical application potential of walnut hull and future research directions in this field will also be discussed.

2. Material and Methods

Plant Material

Pulp of walnut (Juglans regia) hull were obtained from walnut production factories in Bursa. The liquid pulps obtained from the walnut shelling machine were taken into storage containers and kept at +4 degrees during the analysis period in the laboratory.

Obtaining Walnut Hull Pulp Extract

Walnut pulp was boiled at 100 C for 1 hour in a similar way to the method prepared for natural dyeing, then the pulps were filtered and stored at +4 C to be added to the cream formulation.

Preparation of Cream

Table 1: Cream formulation containing walnut pulp extract

			25%	50%	blank krem
A	Emulgade A 165	Glyceryl Stearate and PEG-100 Stearate	6,5	6,5	6,5
	cutina pes	Pentaerythrityl Distearate	2,5	2,5	2,5
	cetiol mm	Myristyl Myristate	2	2	2
	cutina hvg	Hydrogenated Vegetable Glycerides	3	3	3
	cetiol c5	Coco caprylate	2	2	2
	cetiol pgl	Hexyldecanol and hexyldecyllaurate	3	3	3
	lanetteo 0	Cetearyl Alcohol	2	2	2
	stearic acid	Stearic acid	1	1	1
B		AQUA	47,8	22,8	72,8
		Glycerin	5	5	5
		Extract of pulp of walnut hull	25	50	0
C		Dehydroacetic acid and benzyl alcohol	0,2	0,2	0,2

Firstly, phase A was prepared, then walnut pulp was added in phase B and the solid liquid mixtures were thoroughly mixed in phase C in a homogeniser (Table 1). Finally, the pulp cream was prepared for animal experiments.

Determination of the efficacy of the products obtained on experimental animals

All experiments were performed in accordance with the principles of National Institutes of Health Animal Research. The study was approved by Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee walnuts produced in Turkey is 48.422% (85.847 tonnes of shells), it is clearly seen that these shells are wasted [10].

The aim of this study is to systematically evaluate the potential effects of walnut hull on wound healing. The biochemical content of walnut hull and its contribution to wound healing processes will be discussed in the light of literature review and existing research. In addition, the clinical application potential of walnut hull and future research directions in this field will also be discussed. (Ethics No: 669). Wistar Albino (250-300 g) rats were used in the study. Animals were kept under standard conditions (temperature; 22±3 0C, humidity; 55-60% and 12 hours light/12 hours dark environment). Animals were kept in plastic cages without any restriction. All rats were fed with standard feed (Korkuteli feed) and tap water. Animals were randomly allocated for the study. The animals were randomly assigned for the study. Under ketamine-xylazine anesthesia, a 7x7 mm skin defect was created on the shaved back area of the rats. The products were applied to the wound area for 15 days to evaluate wound healing. At the end of this period, euthanasia was performed, and wound healing was assessed.

Walnut pulp extract added extracts were applied to the shaved skin and the skin reaction was examined every day. After 15 days of application, the rats were euthanised and the reaction in the contact area was examined histopathologically.

Creating a skin defect

Before starting the study, approval was obtained from Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (MAKÜ-HADYEK) and all procedures on animals were performed according to ARRIVE (Animal Research: Reporting in-vivo Experiments) 2.0 rules. The mice used for the study were carried out at Burdur Mehmet Akif Ersoy University Experimental Animal Production and Experimental Research Centre. In the experimental phase of the study, a total of 21 animals were used in 3 groups of 7 CD-1 mice each. To create defects, all mice were anesthetized with Xylazine HCl (Alfazin, Alfasan IBV) and Ketalar HCl (Alfamin, Alfasan IB) and their backs were shaved. Then, skin defects were created with a 4 mm diameter biopsy punch (Figure 1). Empty cream material, 25% walnut pulp added cream and 50% walnut pulp added cream (Figure 2) were applied to the defect area every day during the study (Figure 3). On the 15th day of the study, the mice were again anesthetized and euthanized. Skin samples were collected for histopathologic and immunohistochemical examinations and fixed in 10% buffered formaldehyde solution.

Figure 1: Appearance after creating a defect in the dorsal

Figure 2: Appearance of the creams used for the experiment.

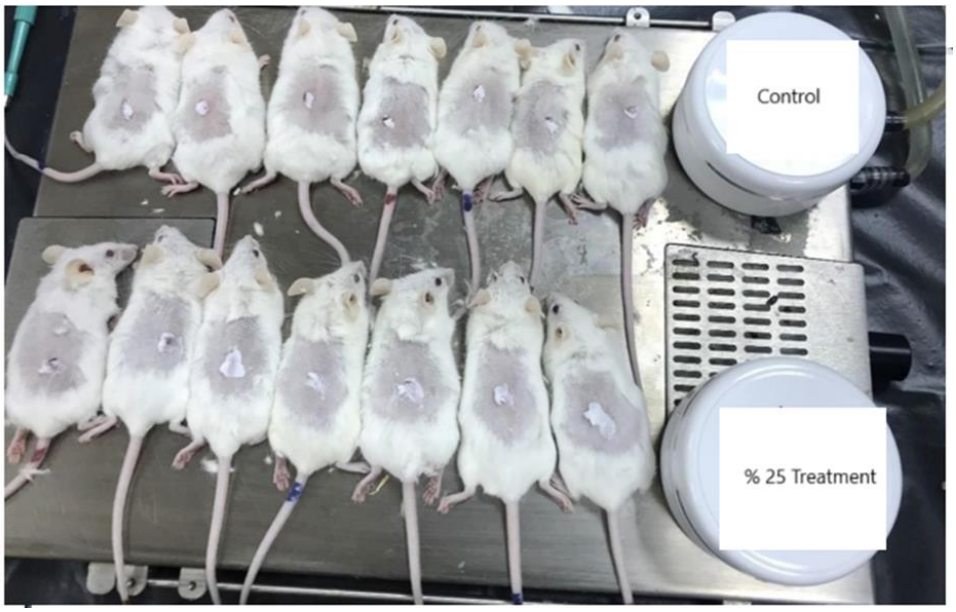

Figure 3: Application of creams to the defect area.

Histopathological Method

Skin samples taken from the rats during necropsy including the defect area were fixed in 10% neutral formaldehyde solution. After two days of formaldehyde fixation, the samples were placed in tissue tracking cassettes. Group and animal number were written on each cassette and placed in formaldehyde again. Skin samples kept in formaldehyde overnight were placed in the basket of a fully automatic tissue tracking device (Leica ASP300S; Leica Microsystem, Nussloch, Germany) for routine histopathological tissue tracking. Tissue tracking was performed by setting the daily tissue tracking programme. For this purpose, the cassettes were passed from low-grade alcohols to high-grade alcohols (from 70% to 100%) overnight in the device to remove the water in the tissue, to remove the fat in the organs by passing through two xylol, and to allow the paraffin to settle in the tissue cavities by placing them in hot paraffin. The following morning, the tissue samples were blockaged. For this purpose, the samples were taken from the autotechnicon device and taken to the hot section of the tissue embedding device (Leica Histocore Arcadia H) for blocking and blocked in paraffin in vertical figure. The blocks were then placed on the cold table section of the paraffin device and kept there for one hour to freeze the paraffin. After cooling the blocks for 4-5 hours, 5 micron thick serial sections were taken on a Leica 2155 model fully

automatic rotary microtome (Leica Microsystem, Nussloch, Germany). The sections were kept at room temperature overnight for drying.

The next day, the sections were placed on bridges for haematoxylin-eosin (HE) staining and the staining procedure was started. Firstly, the sections were kept in an oven at 60ºC for 2 hours to melt the paraffin on the sections and for better adhesion of the tissues on the slide. Then they were passed through 3 different xylol series for 30 minutes each to remove the remaining paraffin. Then, the tissues were quenched by passing through 100%, 96, 90, 80 and 70 alcohols, respectively. Then they were stained with haematoxylin for 15 minutes, washed with distilled water and stained with eosin for 3 minutes. The tissues were then passed through 70, 80, 90, 96, 90, 96 and 100 per cent alcohol, respectively, and the water was removed. After the tissues were polished in xylol, entellan was dripped onto the tissues and coverslips were attached. After staining, the sections were dried for one day and prepared for examination under a microscope. The preparations were examined under an Olympus CX21 model light microscope and microscopic digital photographs were taken with an Olympus DP26 model camera and transferred to a computer. Defect lengths were measured and statistical analyses were performed. Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan) was used for microphotography.

Immunohistochemical Method

While sections were taken for histopathological examination, two serial sections were taken for immunohistochemical analysis and mounted on Poly-L-lysine slides. The sections were immunohistochemically stained with collagen-4 [collagen 4 polyclonal antibody (bs-4595R), Bioss Antibodies Inc., Massachusetts USA1/100 dilution] and cytokeratin-1 [cytokeratin 1 (4D12B3): sc-65999), 1/100 dilution] by streptavidin-biotin complex peroxidase method according to the manufacturer's instructions. For this purpose, the sections were first kept in an oven at 45 ºC overnight to ensure better adhesion of the sections on the slide. Then, the paraffin was removed from the sections passed through xylol and the tissues were rehydrated by passing through graded alcohols (from 100% to 70% alcohol). After removing the slides from the last alcohol, they were kept in distilled water for 10 minutes. After this step, the tissue sections were placed in a humid chamber and all steps except boiling in a microwave oven with citrate buffer solution were performed in a humid chamber. For immunohistochemical staining, the sections were first kept in 3% hydrogen peroxide solution prepared in methanol for 20 minutes to remove endogenous peroxidase activity. Then the tissue sections were placed in citrate buffer solution and boiled in the microwave oven twice for five minutes each.

Then, they were washed twice in phosphate buffer solution (PBS). Normal goat serum was placed on the sections and incubated for 45 minutes in order to remove nonspecific bindings that may be figured in the tissues. After the incubation was completed, the excess protein block was discarded without washing and the primary antibody was dropped on the sections and kept at +4 °C overnight. Then the sections were washed again with PBS and streptavidin was added and incubated for 30 minutes. At the end of this period, the sections were washed twice with PBS. Then, biotinised serum was dripped on the sections and treated for 30 minutes and washed twice more in PBS. Then, 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen was dripped on the tissue sections to make the figure reaction visible. Then, the process was terminated by counterstaining with Harris haematoxylin stain. After all these steps, the sections were covered with coverslips and made ready for examination under light microscope. Immunohistochemical expressions were scored semiquantitatively between 0 and 3. In this scoring, 0: negative, 1: mild, 2: moderate and 3: severe positive. The scores were statistically analysed and the differences between the groups were determined.

Picrosirius Red Method

A series of sections taken from the defect area were stained with Picro Sirius Red method using a ready-made kit (ab150681, Abcam, UK) to evaluate collagen development. After staining, the sections were passed through 70, 80, 90, 96, 90, 96 and 100 % alcohol respectively to remove the water from the tissues. For polishing, the tissues were passed through xylol, dripped with entellan, coverslipped and examined under a light microscope.

Total Phenolic Content

Total phenolic analysis is measured in alkaline medium with folin solution based on the formation of a blue color at 765 nm. The total increase in blue color is directly proportional to the increase in total phenolic compound. The amount of total phenolic compound is calculated as mg Gallic Acid Equivalent (GAE)/g extract using the gallic acid standard curve (Singleton and Rossi, 1965).

Statistical analysis

SPSS package programme was used for all analyses, variables were presented as mean ± standard deviations and ANOVA (post hoc Duncan test) tests were used to compare immunohistochemical scores between groups ($p<0.05$).

3. Results & Discussion

Clinical

During the experiment, no mortality was observed in any of the groups of mice. Throughout the study, it was noted that defects in the groups treated with cream containing walnut pulp began to close more rapidly. The rate of defect closure was particularly notable in the group receiving 50% walnut pulp.

Necropsy

Pre-necropsy examinations revealed that the defects in the control cream group still exhibited extensive tissue loss, whereas the defects in the groups treated with walnut paste showed significant reduction. (Figure 4-6).

Figure 4: Appearance of skin defects of mice in the control group

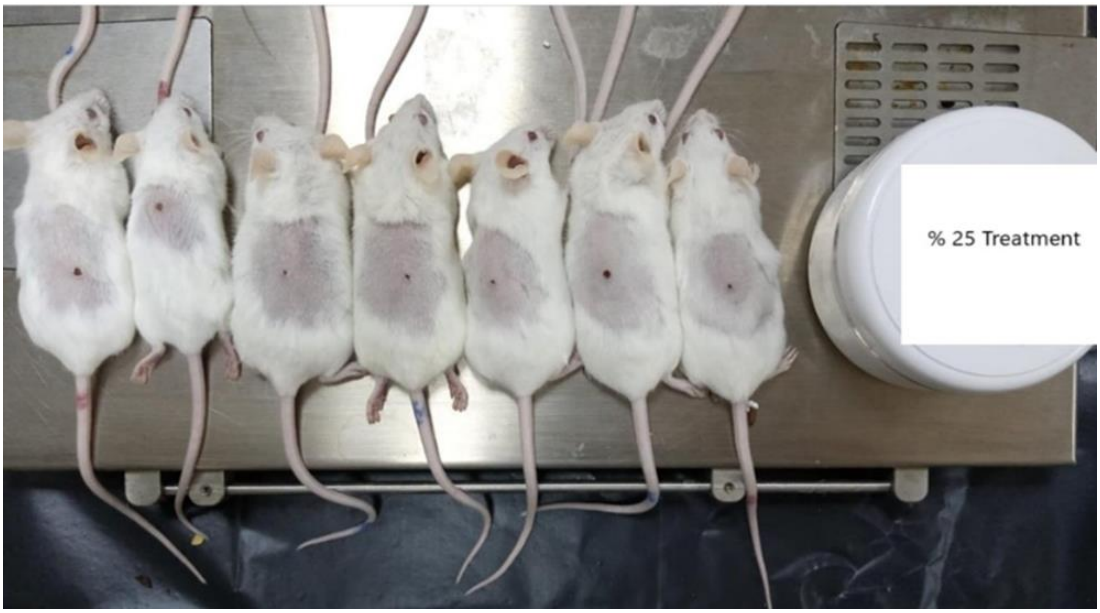

Figure 5: The appearance of the skin defects of the mice in the group treated with 25% walnut pulp cream at the end of the study.

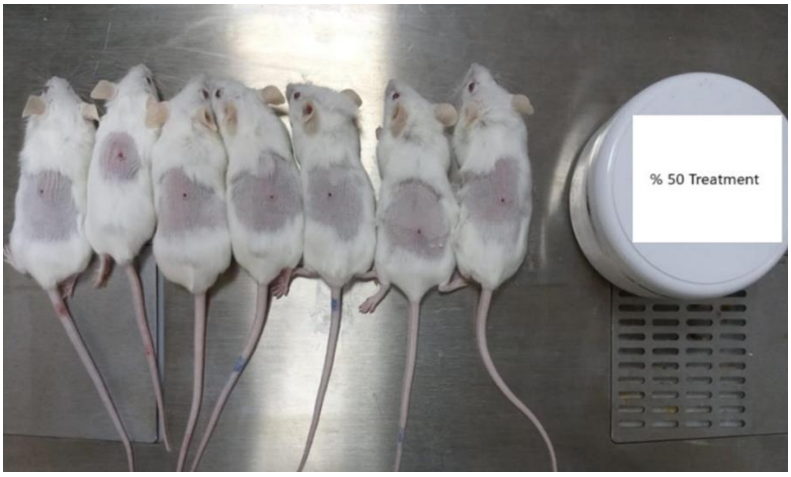

Figure 6: The appearance of the skin defects of the mice in the group treated with 50 % walnut pulp cream at the end of the study

Histopathological

Histopathological examination revealed that the defect size was significantly reduced in the walnut pulp cream group compared to the control group in parallel with the necropsy findings. In addition, epithelialisation was significantly increased in the walnut pulp groups. Complete closure was not observed in any group (Figure 7).

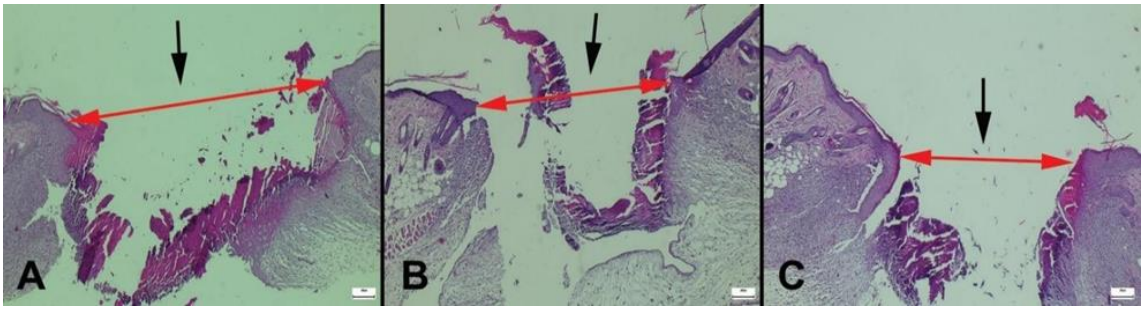

Figure 7: Histopathological appearance of skin defects according to the groups, (A) large defect area (arrow) in the control group, (B) decreased defect size in the group treated with 25% walnut pulp added cream, (C) decreased defect size in the group treated with 50% walnut pulp added cream, red double headed arrow shows defect size, HE, Bars= 100µm.

Picrosirius Red

In the picrosirius red staining performed for the determination of connective tissue development and collagen density, it was observed that the amount of collagen increased in the groups to which walnut pulp was added, while slight collagen formation was observed in the control group. The increase was observed to be more pronounced in the group to which 50% walnut pulp was added (Figure 8).

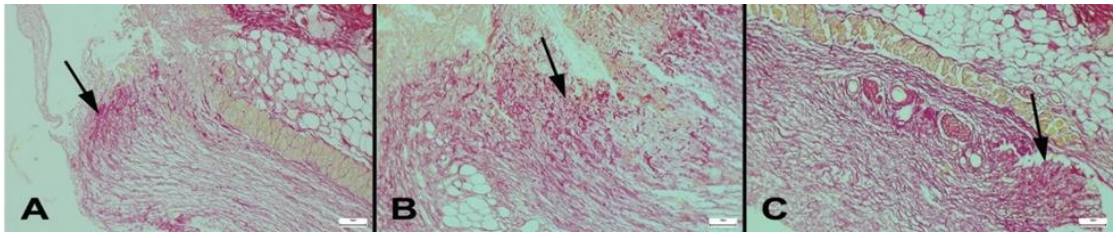

Figure 8: The appearance of collagen amounts according to the groups (A) slightly in the control group, (B) increased in the group treated with 25% walnut pulp added cream, (C) markedly in the group treated with 50% walnut pulp added cream Increased collagen amount in the figure (arrows), Picrosirius red staining, Bars= 50µm.

Immunohistochemical

Cytokeratin and collagen 1 expressions increased with the addition of walnut pulp. The intensity of the expressions increased with the walnut pulp ratio. Statistical analysis results of immunohistochemical expression scores are shown in Table 1.

Cytokeratin immunohistochemistry

When the cytokeratin expressions in the newly formed epithelial layer in the defect area were analysed according to the groups, it was observed that the expressions increased in the groups to which walnut pulp was added, while a slight expression was detected in the control group, and the most significant increase was observed in the group to which 50% walnut pulp was added (Figure 9).

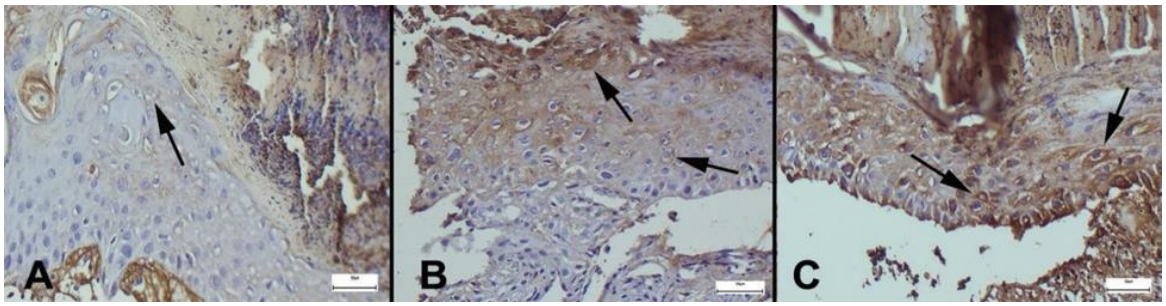

Figure 9: Appearance of cytokeratin expressions in the newly formed epithelial layer at the defect site, (A) mild expression in the control group (arrow), (B) increased expression in the group with 25% walnut pulp (arrows), (C) markedly increased expression in the group with 50% walnut pulp (arrows), Streptavidin biotin peroxidase technique, Bars= 50µm.

Collagen-1 Immunohistochemistry Findings

When the appearance of collagen 1 expression was evaluated according to the groups, it was observed that the expression increased in the group given walnut pulp compared to the control group. The most significant increase was observed in the group given 50% walnut pulp (Figure 10).

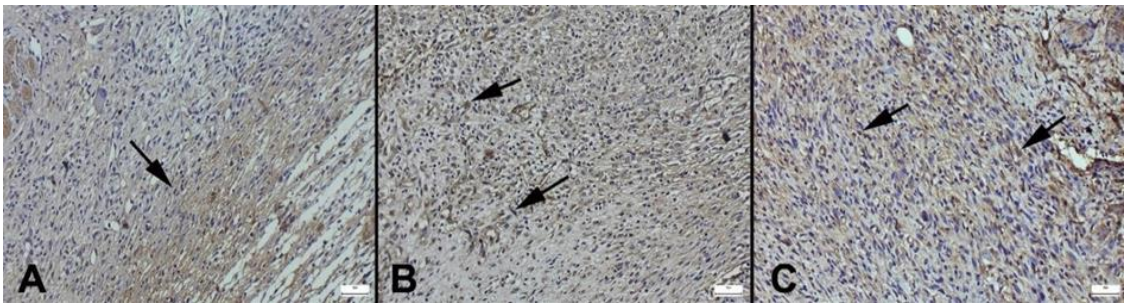

Figure 10: Expression of collagen 1 in the defetk region, (A) very mild expression in the control group (arrow), (B) increased expression in the group with 25% walnut pulp added (arrows), (C) significantly increased expression in the group with 50% walnut pulp removed (arrows), Streptavidin biotin peroxidase technique, Bars= 50µm.

Table 2: Statistical analysis results of defect size and immunohistochemical scores

	Defect size	Cytokeratin	Collagen 1
Control	578.85±27.23^{a}	0.57±0.53^{a}	1.14±0.37^{a}
%25 walnut hull	420.28±37.46^{b}	1.71±0.48^{b}	2.28±0.48^{b}
%50 walnut hull	336.28±21.66^{c}	2.28±0.48^{c}	2.42±0.53^{b}
P	<0.001	<0.001	<0.001

Data are given as mean ± standard deviation (mean ±SD). Differences between groups with different superscripts in the same column are statistically significant.

Total Phenolic Content (mg/g)

The total phenolic content of the liquid pulp in the walnut hull was determined as 187.73 mg/g. In the study conducted by Labuckas et al., 2008, it was reported that this value changed in different varieties and with different solvents and this range was determined as 95-490 mg/g.

4. Conclusion

Based on the obtained data, in general, the application of cream with 50% plant extract showed more positive effects compared to the control. It was determined that wounds in the groups treated with walnut pulp cream healed more rapidly. Pre-necropsy examinations revealed that the control cream group still exhibited extensive tissue loss, whereas the groups treated with walnut pulp showed significant reduction. Histopathological examination results indicated that the wound size in the walnut pulp cream group significantly decreased compared to the control group, and epithelialization (new tissue formation) increased markedly. However, complete closure was not achieved in any group. Picrosirius red staining showed an increase in collagen amount in the groups with added walnut pulp, while slight collagen formation was observed in the control group; this increase was more pronounced in the group with 50% walnut pulp. Immunohistochemical findings demonstrated that cytokeratin and collagen 1 expressions increased in the groups with added walnut pulp; the highest increase was seen in the 50% walnut pulp group. The total phenolic content of the liquid pulp in the walnut hull was determined to be 187.73 mg/g. These findings indicate that walnut pulp has a significant clinical and histopathological impact on the wound healing process.

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