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The Effects of Rosemary (*Rosemarinus officinalis*) Extract on Wound Healing in Rabbits*

In the present study, we examined the effects of 5% *Rosemarinus officinalis* (rosemary) extract on wound healing as compared to those of povidone-iodine and isotonic saline solution by daily visual observation, histopathological and statistical methods. Six male and six female New Zealand rabbits were used in the study. On dorsal aspect of each rabbits, experimentally 3.14 cm in diameter, three full-thickness wounds was constituted. One of these wounds received daily 5% rosemary extract, another one received 10% povidone-iodine solution and the last one received 0.9% isotonic saline solution containing the gauze tampon for control purposes. Size of each wound was measured on days 4, 7, 10 and 14 using a caliper and biopsies were taken from different regions in clockwise direction proceedingly. Wound healings were assessed histopathologically in terms of neutrophil and macrophage infiltration with fibrocyte and fibroblast proliferation, collagen deposition, epithelialization, and vascularization. The aim of this study was to investigate the effect of rosemary extract on wound healing compared to povidone-iodine and isotonic saline solution.

Key Words: Histopathology, rabbit, *Rosemarinus officinalis*, wound healing.

Tavşanlarda Biberiye (*Rosemarinus officinalis*) Ekstraktının Yara İyileşmesi Üzerine Etkileri

Bu araştırmada, %5'lik *Rosemarinus officinalis* (biberiye) ekstraktının yara iyileşmesi üzerine etkileri iyot çözeltisi ve serum fizyolojik ile karşılaştırılmalı olarak günlük gözlem, histopatolojik ve istatistiksel yöntemlerle incelendi. Çalışmada 6 erkek, 6 dişi Yeni Zellanda tavşanı kullanıldı. Her tavşanın sırt bölgesine deneysel olarak 3.14 cm çapında üçer adet, tam katlı yara oluşturuldu. Bu yaralardan birine günlük olarak %5'lik biberiye ekstraktı, diğerine %10'luk povidone-iyot solüsyonu ve sonuncusuna ise kontrol amaçlı %0.9'luk serum fizyolojik içeren gazlı bez tamponu uygulandı. Tüm hayvanların yara çapları 4, 7, 10 ve 14. günlerde kumpasla ölçüldü ve her defasında saat yönünde ilerleyerek farklı bölgelerden biyopsi örnekleri alındı. Yara iyileşmesi, histopatolojik olarak nötrofil ve makrofaj infiltrasyonları ile fibrosit, fibroblast proliferasyonu, kollagen birikimi, epitelizasyon ve damarlaşma yönünden değerlendirildi. Bu çalışmanın amacı, biberiye ekstraktının yara iyileşmesi üzerindeki etkisinin iyot ve serum fizyolojik solüsyonları ile karşılaştırılmalı olarak incelenmesidir.

Anahtar Kelimeler: Histopatoloji, *Rosemarinus officinalis*, tavşan, yara iyileşmesi.

Introduction

Wound healing is process that starts following injury of the skin and soft tissues. Primary objective in treatment is prevention of secondary infection using medications and bandages, rapid restoration, contraction and epithelialization of the wound. Healing of skin wounds occur via a cascade of cellular and molecular events including cell proliferation, differentiation, migration and increased biosynthetic activity (1, 2). In full-thickness skin wounds, there is an injury that disrupts the integrity of the vessels, mesenchymal tissue and epithelium. In the initial stage, wound site is filled with blood clot and fibrin clusters. Fibrin comprises the first line of defense against infection. Granulocytes migrate to the wound site to demarcate the necrotic tissue. In the second stage of healing, granulation tissue, characterized by an increase in several cells develops. Scar tissue forms depending on mature fibrocyte and getting dense collagen in the third stage (3).

Rosemary is an evergreen, fragrant, decorative, perennial plant that may grow up to 2 m in many parts of the world. It is mainly native to the Mediterranean Region (4, 5, 6). The Rosemary extracts, ursolic acid, oleanolic acid, and micromeric acid are considered to have the most effective anti-inflammatory (7, 8) and anti-tumor activities (8). Rosemary has an antibacterial (9) and antioxidant effect in vivo (10, 11). It increases the synthesis of prostaglandin E₂, decreases production of leucotrien B₄ in the polymorph leucocytes and prevents complement system in humans (10). It decreases plasma

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glucose level (12) and has antitumorigenic activity (13, 14). Sprouts of the plant have been known to have hepatoprotective and increasing bile secretion activity in rats (13, 15) Rosemary powder is used as anti-inflammatory agent in horses, pigs and cattle while rosemary ointment can be used for breast disinfection and its solution is used locally for wound treatment (16).

The aim of this study was to investigate the effects of *Rosemarinus officinalis* extract on wound healing compared to povidone-iodine and isotonic saline solution.

Material and Methods

Preparation of the extract

To prepare 5% *Rosemarinus officinalis* (rosemary) extract, the leaves of rosemary were cleaned with water. Then 5 g of cleaned and crashed leaves was heated until boiling in a container of 100 mL distilled water and then filtered through a piece of double layered muslin cloth (17). Fresh extract was prepared for daily use.

Animals

Twelve healthy, adult New Zealand rabbits (6 male and 6 female), weighing about 2500±200 g were used for the study. They were individually housed and fed in normal diet and water ad libitum in a room with natural light cycle and constant temperature (24±2 °C). The Harran University Animal Care and Use Committee has approved the study protocol.

Instrumentation and surgical procedure

All rabbits were anaesthetized with intramuscular administrations of 10 mg/kg xylazine hydrochloride and 50 mg/kg ketamine hydrochloride. The dorsal aspects of 12 rabbits were clipped and prepared for aseptic surgery. On dorsal aspect of each animal, one cranially and two caudally located full-thickness skin wounds in 3.14 cm diameter were created using a template prepared from a used X-ray film (Figure 1.A).

Treatment protocol

Each rabbit was treated once daily, before bandaging, with topical 5% *Rosemarinus officinalis* (rosemary) extract and 10% povidone-iodine and 0,9% isotonic saline solution respectively.

Evaluations of wound healing

Observations during daily wound care: Each wound was evaluated at the time of the daily bandage changes for the presence of exudate and wound healing until day 14.

Measurement of wound diameter: The wound diameters was measured by caliper both horizontally and vertically and the average was calculated postoperative on the 4th, 7th, 10th and 14th days.

Histopathological evaluation: Biopsy specimens, which were collected with clockwise rotation on the 4th,

7th, 10th and 14th postoperative days (POD), wounds' diameters were measured with calipers in millimeters. Skin specimens were fixed in 10% neutral buffered formalin and processed routinely for histopathological examination. Five micrometers sections were stained with haematoxylin and eosin (H&E). Progressive decrease in neutrophil numbers, progressive increase in angiogenesis, density of macrophages, the ratio of fibroblast, fibrocyte and collagen deposition etc. are the parameters used to assess the progression of healing from the inflammatory into the repair stage. In every skin section, an area just beneath the epidermis or crust formation was randomly selected. Thereafter, four consecutive areas moving towards the deep dermis were selected. The five selected areas were examined under ×400 magnification. The number of neutrophils, fibroblast, fibrocyte was scored as 0–25 = 1, 26–50 = 2, 51–75 = 3, and > 75 = 4 (18). The same areas were also examined for the number of vessels and the actual count was noted. Masson's trichrom staining was made for the purpose of find out connective tissue proliferation in wound areas (19).

Statistical analysis

Statistical analyses were carried out using statistics software (SPSS). Kruskal Wallis and Jonkheere-Terpstra methods were used to test the significances of intergroup differences in quantitative (wound size) and qualitative variables, respectively.

Results

Day 4: When measured by caliper, wounds dressed with gauze soaked in povidone-iodine solution and rosemary extract were smaller in size than those dressed with gauze soaked in the isotonic saline solution. However, differences between groups were not statistically significant. On microscopical examination hemorrhage, intensive edema and neutrophil infiltration and moderate macrophage infiltration were distinctive findings (Figure 2.A).

Day 7: Reduction in the sizes of wounds treated with rosemary extract and povidone-iodine solution, compared to isotonic saline solution, was statistically significant. Even though reduction in wound sizes of rosemary extract group smaller than the povidone-iodine solution group, the difference was not statistically significant. Microscopically, as opposed to other groups, re-epithelialization has been found to have started in the rosemary extract group. The number of neutrophils was lower than that on day 4 in the povidone-iodine solution and rosemary extract groups while neutrophils were still abundant in the isotonic saline group. The differences in wound size measured on day 7 between groups were statistically significant ($P < 0.01$) while those measured on other days were not ($P > 0.05$) (Table 1).

Day 10: Though scarce, moderate macrophage infiltrations were still present in the inflamed regions of the rosemary extract and povidone-iodine solution groups on day 10 of healing. Furthermore, fibroblasts

were abundant and formation of numerous vessels and increase in the amount of collagen were noted. Re-epithelialization was ongoing. The moderate neutrophil and macrophage infiltrations and infrequent fibroblasts were still present in the isotonic saline group.

Day 14: On day 14 of healing process, microscopical examination showed that the number of fibroblasts was lower, the number of fibrocytes was higher and the amount of collagen was increased in povidone-iodine solution and rosemary extract groups (Figure 2.B). Re-epithelialization process was found to be complete (Figure 1.B).

Presence of infrequent macrophages and neutrophils, as well as fibroblasts and fibrocytes were observed with isotonic saline solution dressing. This was statistically significant. Numbers of neutrophils counted on day 14 were significantly different between groups

($P < 0.01$) (Table 2) but the differences in other days were not significant ($P > 0.05$).

Measurements of wound size revealed that wounds healed faster with rosemary extract than povidone-iodine or isotonic saline solution. Neutrophil infiltration significantly regressed on day 7 with rosemary extract and on day 10 with povidone-iodine solution. On the other hand, neutrophil infiltration in the wound region was still present on day 14 with isotonic saline solution. In conclusion, rosemary extract was found to be more effective on wound healing than povidone-iodine or isotonic saline solution. In the wound area, the connective tissue proliferation was confirmed by Masson's trichrom staining. The connective tissue proliferation was defined as the least on day 4th, getting increased on days 7th, 10th and 14th in the wound area.

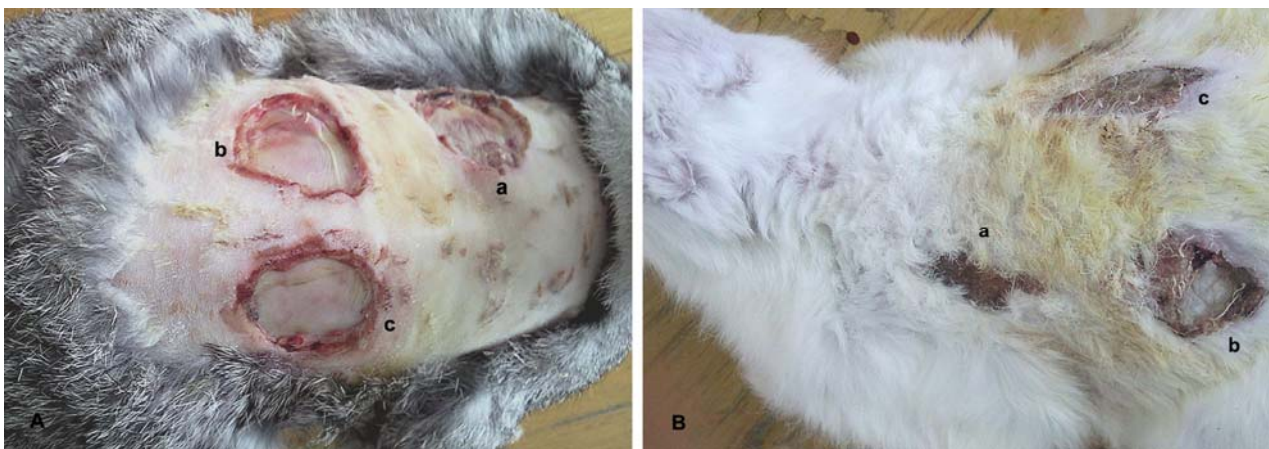


Figure 1: **A.** Representative open wounds of rabbits on day 0: a. *Rosmarinus officinalis* group, b. Iodine group, c. Isotonic saline group. **B.** Representative open wounds of rabbits on day 14: a. *Rosmarinus officinalis* group, b. Iodine group c. Isotonic saline group.

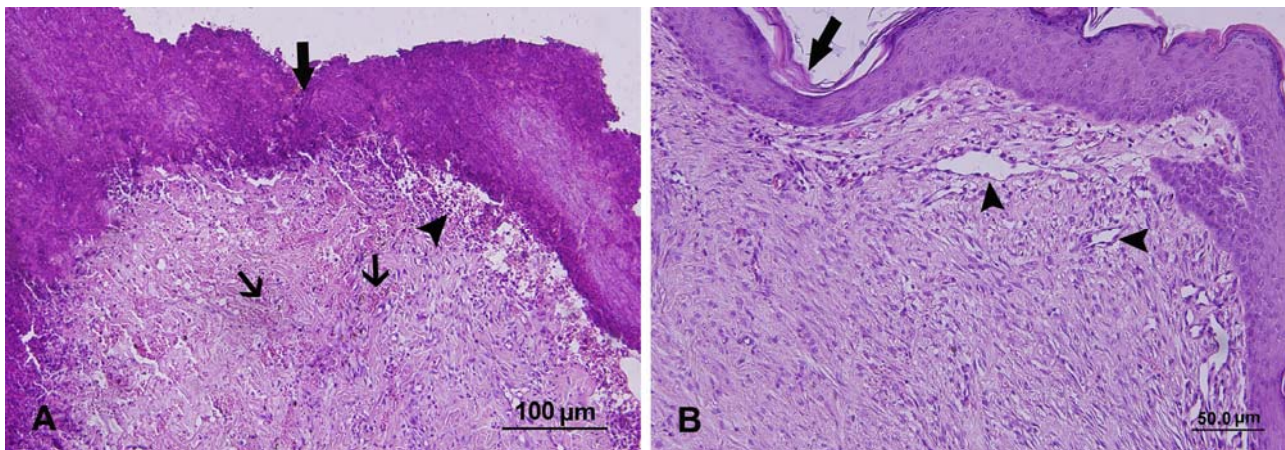


Figure 2: **A.** General histological aspect of the wounds on day 4th *Rosmarinus officinalis* group. Crust (bold arrow), infiltration of numerous inflammatory cells (mainly neutrophils) in the dermis (head arrow), haemorrhage (thin arrows). H&E., Bar: 100 µm. **B.** General histological aspect of the wounds on day 14th *Rosmarinus officinalis* group. Completed re-epithelialization (bold arrow), neovascularization in the dermis (head arrows). H&E., Bar: 50 µm.

Table 1. Measurements of the wound diameter in the groups.

APPLICATION	DAYS			
	4	7	10	14
	X±S _x	X±S _x	X±S _x	X±S _x
ROSEMARY	2.3±0.0	2.27 ^b ±0.38	1.97±0.43	1.63±0.18
POVIDONE-IODINE	2.9±0.21	2.57 ^b ±0.23	1.90±0.15	2.07±0.17
ISOTONIC SALINE	2.53±0.17	3.07 ^a ±0.89	2.23±0.09	1.97±0.22
GENERAL	2.57±0.12	2.63±0.18	2.03±0.14	1.89±0.11
SIGNIFICANCE (P)	–	*	–	–

X±S_x: Average;± Standart error.

*: Statistically important difference between the averages (P<0.05).

a, b: Difference between the averages shown in the same column with different symbols in terms of statistical significant (P <0.05).

Table 2. Neutrophil count in the groups.

APPLICATION	DAYS			
	4	7	10	14
	X±S _x	X±S _x	X±S _x	X±S _x
ROSEMARY	2.0±0.58	1.0±0.0	1.0±0.0	1.0 ^b ±0.0
POVIDONE-IODINE	2.0±0.58	2.67±0.33	1.0±0.0	1.0 ^b ±0.0
ISOTONIC SALINE	3.0±0.0	2.33±0.67	2.33±0.33	2.67 ^a ±0.33
GENERAL	2.3±0.29	2.0±0.33	1.44±0.24	1.56±0.30
SIGNIFICANCE (P)	–	–	–	*

X±S_x: Average; ± Standart error.

*: Statistically significant difference between the averages (P<0.05).

a, b: Difference between the averages shown in the same column with different symbols in terms of statistical significance (P <0.05).

Discussion

The aim of this preclinical study was to evaluate the wound healing activity of the extract of *Rosemarinus officinalis* (rosemary) extract as compared to povidone-iodine and isotonic saline solution in the incisional cutaneous wounds in rabbits, an appropriate model for surgical cutaneous wounds.

The wound healing process involves many complex factors. These may be classified as local and systemic factors, organ and species variability in response to injury. In this study, we adopted an experimental design including daily observation, macroscopical and histological evaluations of the wound healing process and statistical methods.

Re-epithelialization, collagen deposition and neovascularization are all among the parameters used for the analysis of healing wounds (20-22). The immature vessels are originated from mature vessels of deeper tissues. Anastomoses also occur among these vessels to

provide the wound area with sufficient blood supply (23). In the present study, increase in neovascularization was observed in the rosemary extract and povidone-iodine solution groups on day 10 significantly.

Rosemary extract has long been used in medicine and food industry, thanks to its antibacterial (7, 8), antiinflammatory and antioxidant (10, 24, 25) activities. Many studies have been carried out on Rosemary's bactericidal and bacteriostatic effects (9). The present study is important since it demonstrated the antiseptic, antimicrobial and epithelializing effects of rosemary extract on wound healing.

As a result, we have noted that open wounds in rabbits treated with rosemary extract tend to heal faster than similar wounds treated with povidone-iodine and isotonic saline solution. These results indicate that rosemary extract provide a good alternative for the treatment of open wounds in rabbits.

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