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ORIGINAL ARTICLE

The effects of Tendoflex® (polytendon complex) and *Hypericum perforatum* (St. John's wort oil) on repaired Achilles tendon healing in rats

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ABSTRACT

Objectives: This experimental study aims to examine the effects of Tendoflex® and *Hypericum perforatum* on tendon healing in rat models undergoing iatrogenic Achilles tendon rupture and similar surgical treatments.

Materials and methods: Eighty Wistar albino rats weighing 250 to 350 g were randomly divided into four groups. Group A: Tendoflex® was administered orally as 1 capsule/2.5 kg daily by gavage. Group B: *Hypericum perforatum* was administered orally as 300 mg/kg daily by gavage. Group C: Tendoflex® and *Hypericum perforatum* were co-administered orally by gavage at the prespecified doses. Group D: No medication was given to the control group. Five rats from each group were sacrificed weekly, and the tissue samples were examined histologically, followed by the biomechanical tests of the Achilles tendon.

Results: In the mechanical testing, pulling forces were superior in all intervention groups and in all weeks over the control group. In particular, in the early periods (Weeks 1, 2, and 3), the mixed group showed the most favorable results, followed by the *Hypericum perforatum* group (p=0.010, p=0.591, and p=0.130, respectively). The most favorable collagen type I and type III expression values were found in the mixed and *Hypericum perforatum* groups at Weeks 2 and 3, respectively (p=0.025 and p=0.018). In the immunohistochemical and Western Blot examinations, extreme collagen type I and type III expression were detected in the mixed and *Hypericum perforatum* groups at Weeks 2, 3, and 4.

Conclusion: Tensile strength of the Achilles tendon increased by using *Hypericum perforatum* and Tendoflex® following rupture and repair of the Achilles tendon in rats. The combined use of these two agents yielded the most favorable mechanical and histological results, particularly in the early period. This result may be related to the higher level of collagen type I and type III immunity in all groups, compared to the control group.

Keywords: Achilles tendon, centaury, *hypericum perforatum*, St. John's wort oil, tendoflex.

The Achilles tendon is the body's largest and strongest ligament, connecting the soleus and gastrocnemius muscles to the calcaneus. Its role is essential for flexion of the ankle and knee and stabilization of the ankle.[1] The Achilles tendon is frequently injured, and open surgical repair is the first choice of treatment for acute full-thickness injury of the Achilles tendon, particularly in young adults.[2]

After the open repair of an Achilles tendon's full-thickness rupture, there is a long recovery time and high rates of re-rupture, resulting in severe disability and long-term workforce loss.[3] Although

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many studies have been conducted on tendon healing in recent years and many medical options such as pulsed electromagnetic field therapy, glucosamine chondroitin sulfate, platelet-rich plasma, intermittent pneumatic compression, low-molecular-weight heparin and rivaroxaban have been investigated, it is still unclear in the literature how to treat Achilles tendon rupture.[1-6]

The fibroblasts from the extrinsic area in the inflammatory space induce collagen synthesis during healing of the tendon. The tenocytes are, then, produce a more intense collagen synthesis. This intensified collagen synthesis matures with the normal physiological movements of the tendon and are arranged in parallel to each other along the force applied in the remodeling period.[7]

The Tendoflex® (Mega-Farma Ilaç & Kozmetik San. Tic. Paz. Ltd. Şti. Istanbul, Turkey) (methyl sulfonyl methane, L-arginine, bromelain, vitamin C, rutin, collagen type I) is a polytendon complex and has properties that accelerate the tendon healing process and causes the repaired tissues to be rich in collagen. The ingredients of the Tendoflex® such as vitamin C and collagen have been shown to increase tendon healing.[7,8] *Hypericum perforatum* (St. John's wort oil), popularly known as centaury oil, is known to increase the accumulation of collagen and reduce the duration of inflammation.[9] In the present study, we aimed to examine the effects of Tendoflex® and *hypericum perforatum* on tendon healing in a rat model created by iatrogenic Achilles tendon rupture and similar surgical treatments.

MATERIALS AND METHODS

This experimental study was approved by the Atatürk University Animal Experiment Local Ethics Committee (Date: 29/05/2019, No: 75296309-050.01.04-E.1900162998). The study was conducted in accordance with the principles of National Research Council. Guide for the Care and Use of Laboratory Animals.[10] Eighty 250 to 350 g Wistar-Albino rats were randomly divided into four groups of five rats, after the Achilles tendons of the animals were iatrogenically ruptured and repaired. Group A: Tendoflex® was given by oral gavage as 1 capsule/2.5 kg daily. Group B: *Hypericum perforatum* was administered orally as 300 mg/kg daily by gavage. Group C: Tendoflex® and *Hypericum perforatum* were administered together at the prespecified daily doses orally via gavage. Group D: This group was the control group and no drug was given.

All surgical techniques were performed in bilateral lower extremities of the rats in the prone position under sterile conditions. The rats were anesthetized with ketamine hydrochloride (50 to 75 mg/kg, intramuscular, Ketalar®; Pfizer, Istanbul, Turkey) and xylazine (5 to 10 mg/kg, intramuscular, Rompun®, Bayer Türk Kimya San. Ltd. Şti., Istanbul, Turkey). Preoperative antibiotic prophylaxis was provided using cefazolin sodium (10 mg/kg, subcutaneous, Sefazol®, Mustafa Nevzat Ilac San., Istanbul, Turkey). Both legs of the anesthetized animals were shaved. The rats were placed on a heated operating table, disinfected, and covered to expose both legs. In the surgical procedure, the deep fascia was passed through a 10 mm skin-subcutaneous skin incision and the Achilles tendon was reached. A 2-mm transverse full layer incision was made from 5 mm proximal to the adhesion site of the calcaneus. The Achilles tendons of the rats in all groups were repaired with the modified Kessler method using 5.0 nylon sutures. Subsequently, the skin was sutured with 4.0 nylon monofilament. After surgical treatment, the animals were kept in cages without restriction of movement or immobilization. The rats were monitored for 2 h after anesthesia. Vital signs of all rats and their mobility were maintained.

To assess the weekly effects of the active ingredients, tissue samples were taken from the repair area after performing the biomechanical tests, and five rats from each group were sacrificed weekly to be investigated by histopathological and Western Blot methods. Thus, the active ingredients were given daily for one week to the first subgroup, two weeks to the second subgroup, three weeks to the third subgroup, and four weeks to the fourth subgroup.

Weekly sacrificed Achilles tendons were measured in a mechanical test device (Shimadzu AG-IS 100, pulling speed 5 mm/min working speed, loading speed 5 mm/min, Atatürk University Engineering Faculty, Mechanical Engineering Department R&D Laboratory) regarding their endurance (Figure 1). The load values measured by the load cell of the device, with 0.1% accuracy, were entered into the software, and force values (F) were obtained. The tests were terminated after a force reduction and specimen/sample rupture was detected. The highest force value among the obtained data was recorded.

Tendon tissues taken by necropsies of the rats were fixated in a 10% neutral formalin solution. Tissues were, then, taken into paraffin blocks after

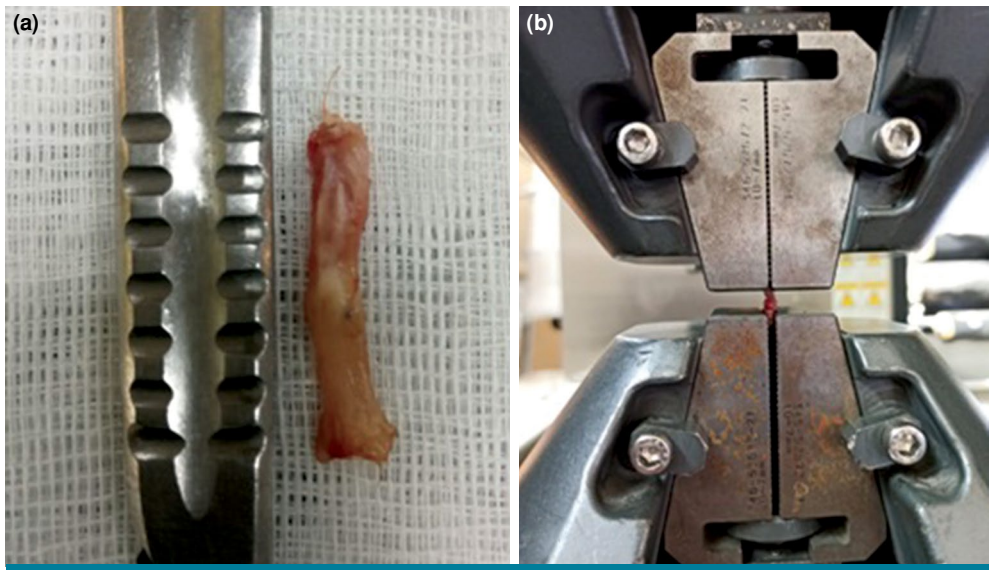


FIGURE 1. (a) Macroscopic image of the sacrificed Achilles tendon, **(b)** measurement of tendon strength in a mechanical measurement device.

routine alcohol-xylol follow-up. Four-μm sections taken on the poly-L-lysine slides were stained by the immunohistochemical method. Three sections taken on slides were passed through xylol and alcohol series and washed with phosphate-buffer saline (PBS) and, then, endogenous peroxidase inactivation was achieved by keeping them in 3% hydrogen peroxide (H_2O_2) for 10 min. The antigen was treated with a retrieval solution at 500 Watts for 2×5 min to reveal the antigen in the tissues. After the protein blocking, the tissues washed with PBS were incubated with Monoclonal collagen type I (Abcam, Catalog No. ab90395, 1/200 dilution ratio) and Monoclonal collagen type III (Abcam, Catalog No. ab6310, 1/200 dilution ratio) primary antibodies at room temperature for 20 min. As the second step, the Large Volume Detection System: anti-Polyvalent, Horseradish Peroxidase (HRP) (Thermo Fisher, Catalog number: TP-125-HL) was used as recommended by the manufacturer. The DAB (3,3'-diaminobenzidine) was used as the chromogen. After contrast staining with Mayer's hematoxylin, it was covered with Entellan™ (Merck Millipore Corp., MA, USA) and examined under a light microscope. Immunopositivity in the tendon tissues was categorized as none (-), mild (+), moderate (++), extreme (+++), and very extreme (++++).

Tendon samples taken were stored at -80°C until analysis. Before starting the Western Blot analysis, the tendon tissues were dissected with a tissue homogenizer at the required level. Protein concentration was prepared by using the Bradford (Biorad) method. For each tissue, 10 μg of protein 4 to 5% sodium dodecyl sulfate (SDS) was loaded on a polyacrylamide gel. Proteins were transferred to the nitrocellulose membrane (Merck Millipore Corp., MA, USA) and blocked in a blocking solution for 1 h. The membranes were, then, incubated at 4°C overnight with the collagen type I and type III primary antibodies used in the immunohistochemical method. Subsequently, they were incubated with secondary antibodies conjugated with HRP. Protein bands were visualized using the chemiluminescence detection kit (ECL™; Amersham BioSciences, Buckinghamshire, UK).

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD) or median (min-max). The difference between the groups was analyzed using the Kruskal-Wallis test, a nonparametric test, and the group that made the difference was assessed using the Mann-Whitney U test. The density of the bands was determined using the Quantity One 1-D analysis software version 4.6.6. (Bio-Rad Laboratories, CA, USA) for Western Blot analysis. A p value of <0.05 was considered statistically significant.

RESULTS

A total of 10 rats were lost from the beginning to the end of the study (Table I). Data of these rats were excluded from the analysis.

TABLE I
Distribution of the mechanical endurance strengths between groups by weeks

	Week	n	Mean±SD	Min-Max
1st Week	*Hypericum perforatum*	4	35.9±7.4	28.13-43.75
	Control	4	31.3±8.1	21.88-40.63
	Mixed	4	50.8±8.2	43.75-62.50
	Tendoflex	4	36.7±1.6	34.38-37.50
	p		0.010	
2nd Week	*Hypericum perforatum*	5	43.8±11.9	28.13-59.38
	Control	4	38.3±13.4	21.88-53.13
	Mixed	4	49.2±9.3	37.50-59.38
	Tendoflex	4	41.4±10.3	28.13-53.13
	p		0.591	
3rd Week	*Hypericum perforatum*	5	58.8±17.5	34.38-75.00
	Control	4	39.8±7.4	34.38-50.00
	Mixed	4	64.1±18.5	50.00-90.63
	Tendoflex	4	52.3±6.9	43.75-59.38
	p			0.130
4th Week	*Hypericum perforatum*	5	77.6±34.9	40.63-125.00
	Control	5	35.2±11.2	25.00-50.00
	Mixed	5	62.5±14.0	46.88-81.25
	Tendoflex	5	51.9±18.3	28.13-71.88
	p		0.067	

SD: Standard deviation.

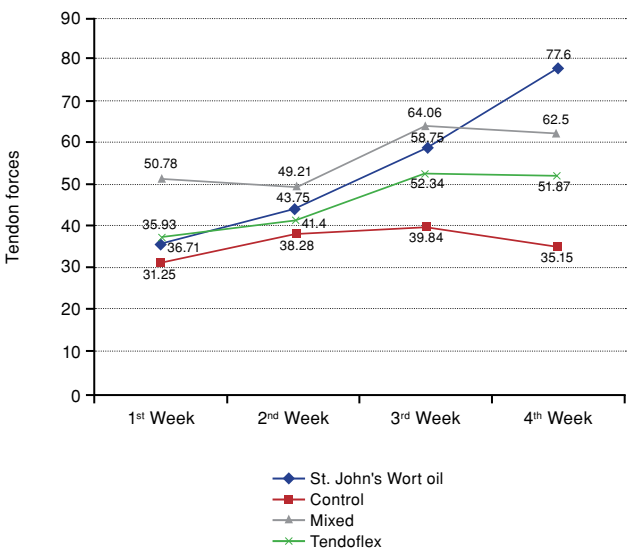


FIGURE 2. Biomechanical strength of tendons according to weeks.

In the examinations during the sacrifice of the rats, no macroscopic defect was found in the integrity of the tendons in any rat. When the endurances of Achilles' tendons at Week 1 were examined, the most favorable results were observed in the mixed group with a significant difference followed by the Tendoflex® and *Hypericum perforatum* groups, respectively. The lowest tendon strengths were seen in the control group (Table I, Figure 2).

Similarly, when the endurance strengths at Weeks 2 and 3 were examined, the most favorable results were obtained in the mixed group, followed by the *Hypericum perforatum* and Tendoflex® groups, respectively. However, these two groups switched in the second week. The lowest tendon strengths were seen in the control group (Table I, Figure 2).

According to the ultimate strength at Week 4, the *Hypericum perforatum* group had the best stamina, followed by the mixed and the Tendoflex® groups, respectively. The lowest tendon strengths in the last

TABLE II
Collagen type I expression in groups by week

	Control	Tendoflex	*Hypericum perforatum*	Mixed	
Collagen I	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p* value
1st week	0.2±0.4^{Aa}	1.2±0.4^{Ab}	1.3±0.5^{Ab}	2.2±0.4^{Ac}	0.030
2nd week	0.3±0.5^{Aa}	1.2±0.4^{Ab}	2.2±0.4^{Bc}	2.8±0.4^{Ad}	0.025
3rd week	1.2±0.4^{Ba}	1.3±0,5^{Aa}	2.2±0.4^{Bb}	2.8±0.4^{Ac}	0.018
4th week	1.2±0.4^{Ba}	1.3±0.5^{Aa}	2.8±0.4^{Cb}	2.2±0.4^{Ac}	0.028
p value	0.013	0.07	0.028	0.085	

A,B,a,b,c designate differences between the groups (p<0.05).

TABLE III
Collagen type III expression in groups by week

	Control	Tendoflex	*Hypericum perforatum*	Mixed	
Collagen III	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p* value
1st week	0.2±0.4^{Aa}	1.2±0.4^{Ab}	1.3±0.5^{Ab}	2.2±0.4^{Ac}	0.030
2nd week	0.3±0.5^{Aa}	1.2±0.4^{Ab}	2.2±0.4^{Bc}	2.8±0.4^{Ad}	0.025
3rd week	1.2±0.4^{Ba}	1.3±0.5^{Aa}	2.2±0.4^{Bb}	2.8±0.4^{Ac}	0.018
4th week	1.2±0.4^{Ba}	1.3±0.5^{Aa}	2.8±0.4^{Cb}	2.2±0.4^{Ac}	0.028
p value	0.013	0.070	0.028	0.085	

A,B,a,b,c designate differences between the groups (p<0.05).

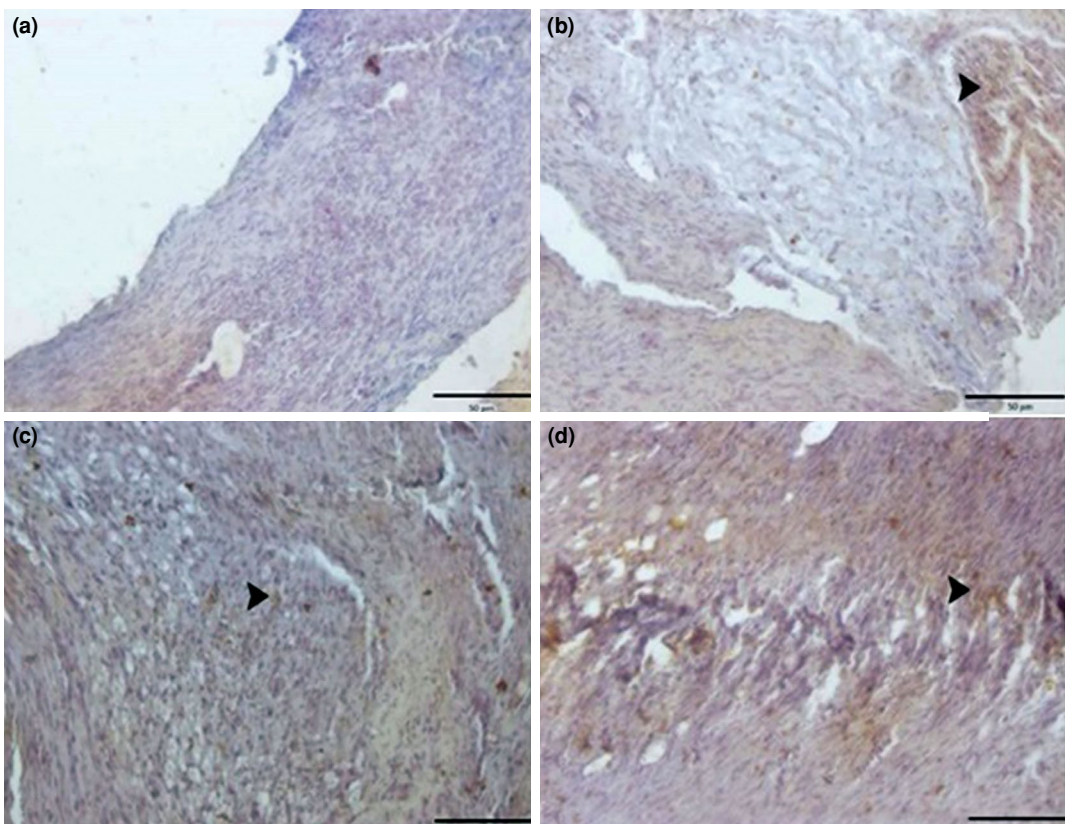


FIGURE 3. Week 1. **(a)** Control group, **(b)** Tendoflex® group. Mild level, **(c)** *Hypericum perforatum* group. Mild level, **(d)** Mixed group. Moderate expression of collagen type I (arrowhead) (×40-immunohistochemistry).

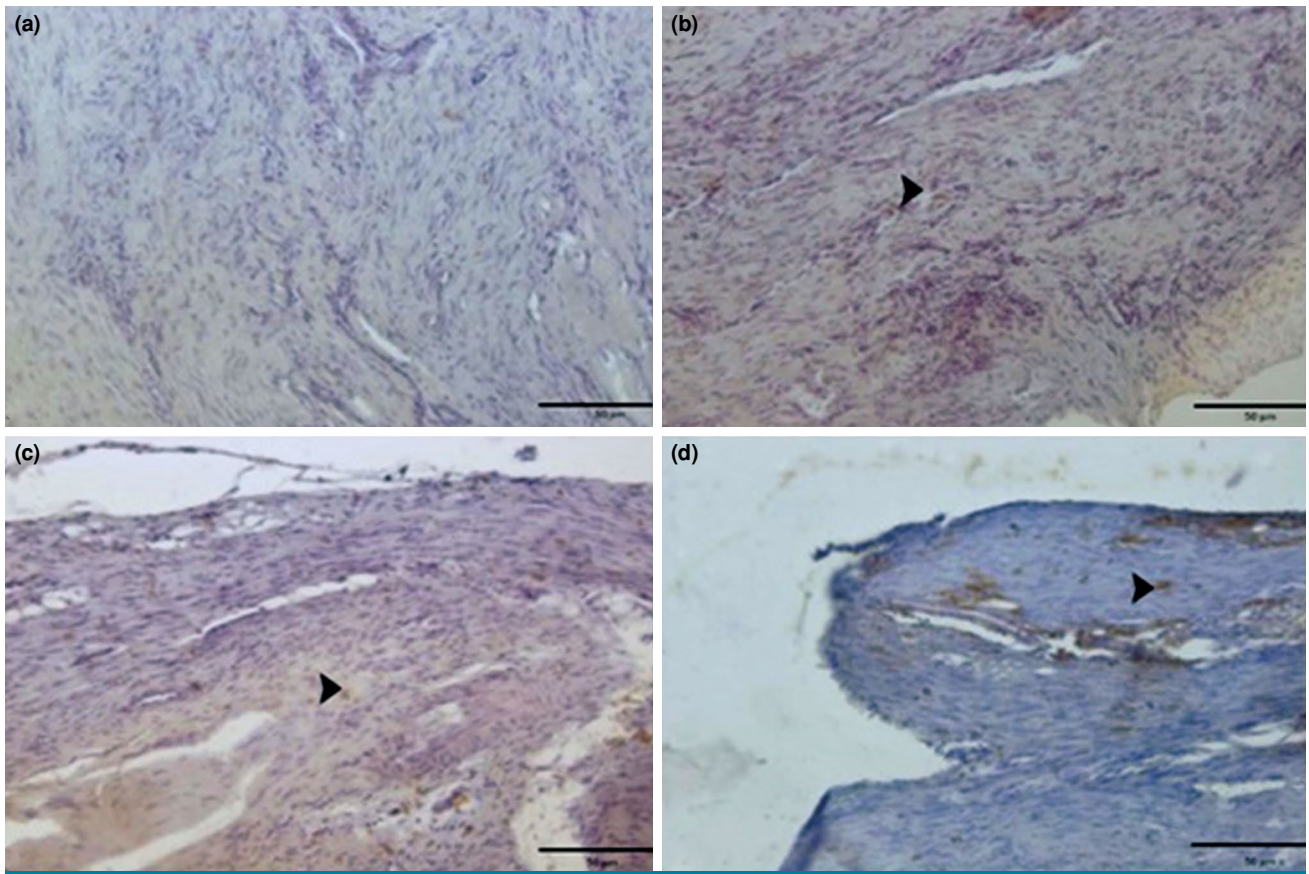


FIGURE 4. Week 2. **(a)** Control group, **(b)** Tendoflex® group. Mild level, **(c)** *Hypericum perforatum* group. Mild level, **(d)** Mixed group. Moderate expression of collagen type I (arrowhead) (×40-immunohistochemistry).

week were observed in the control group, as was the case in all other weeks (Table I, Figure 2).

There was a significant difference in the collagen type I and type III immunity in the tendon tissues among the groups (Tables II and III). Collagen type I expression was not seen in the control group at Week 1, while it was observed at a mild level in the Tendoflex® and *Hypericum perforatum* groups and moderately in the mixed group. The expression level started to increase in the treatment groups, while no expression was observed in the control group at Week 2, which was mild in the Tendoflex® group, medium in the *Hypericum perforatum* group, and extreme in the mixed group. Collagen type I expression, which was observed in the control and Tendoflex® groups at Week 3, was moderate in the *Hypericum perforatum* group and extreme in the mixed group. At Week 4 and the last week of the study, collagen type I expression was mild in the control and Tendoflex® groups, extreme in the *Hypericum perforatum* group (p=0.028), and moderate in the mixed group (p=0.085) (Figures 3-6).

According to the collagen type III expression, immunity was not observed in the control and Tendoflex® groups at Week 1, while mild immunity was observed in the *Hypericum perforatum* group and moderate immunity in the mixed group. Collagen type III expression was not found in the control groups at Weeks 2 and 3, while it was found to be moderate in the Tendoflex® groups at these time points. Collagen type III expression was moderate in the *Hypericum perforatum* group at Week 2 and increased to extreme levels at Week 3. In the mixed group, it was extreme at Weeks 2 and 3. Collagen type III expression was mild in the control and Tendoflex® groups in the last week of the study, while it was extremely high in the *Hypericum perforatum* group (p=0.028) and high in the mixed group (p=0.085) (Figures 7-10).

There was a difference between collagen type I and type III levels in the groups in the Western Blot findings. Similar to the immunohistochemical findings, collagen type I and type III were extremely

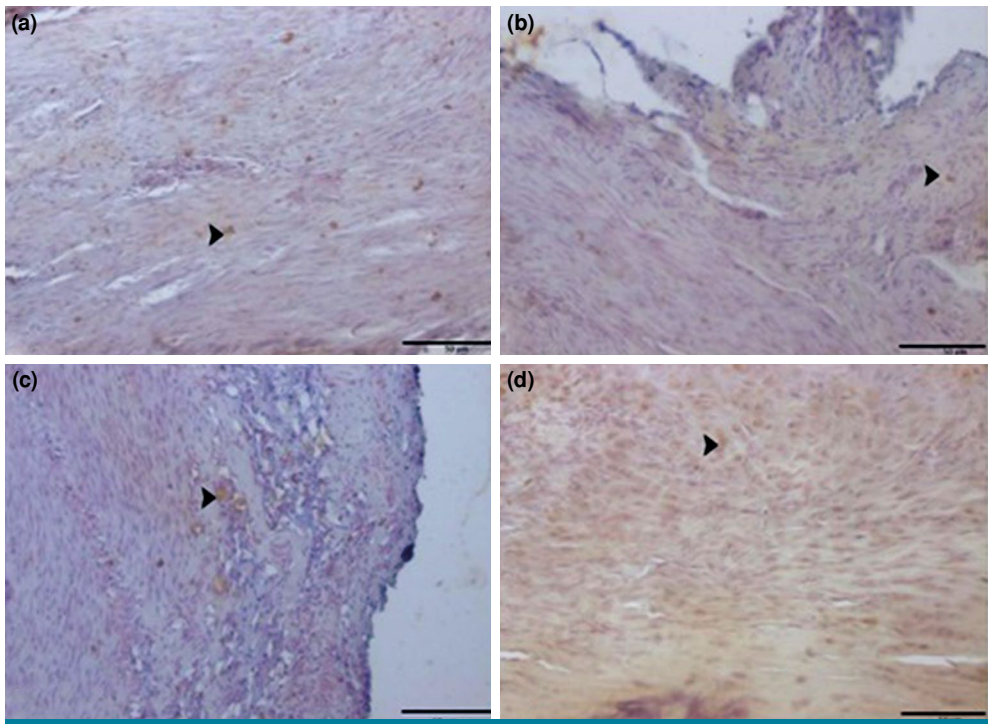


FIGURE 5. Week 3. **(a)** Control group. Mild level, **(b)** Tendoflex® group. Mild level, **(c)** *Hypericum perforatum* group. Medium level, **(d)** Mixed group. Extreme expression of collagen type I (arrowhead) (×40-immunohistochemistry).

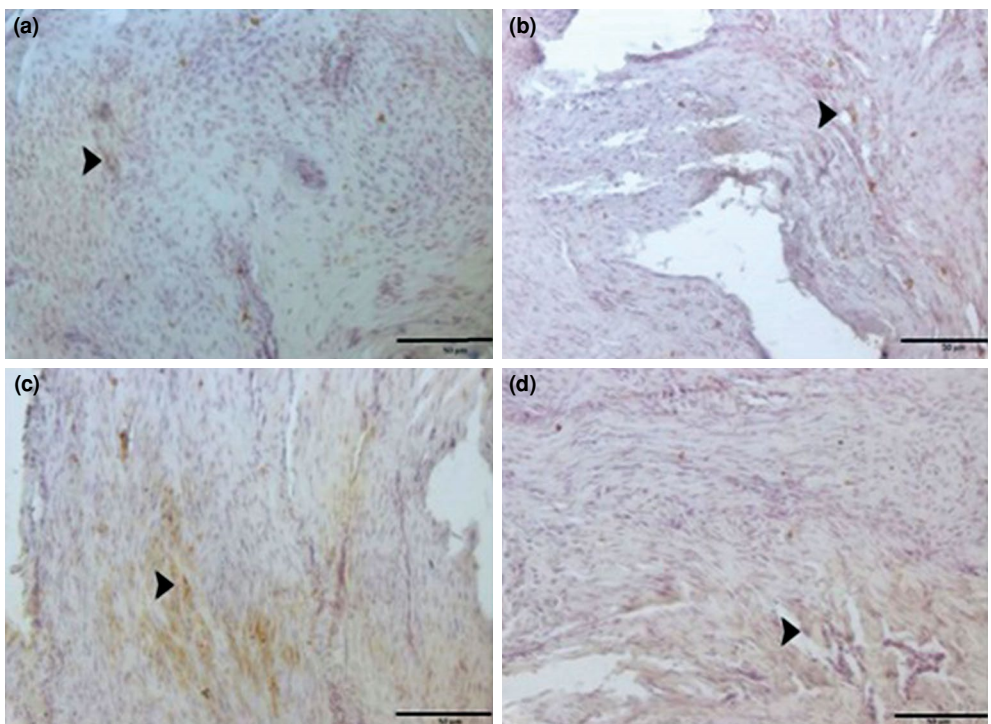


FIGURE 6. Week 4. **(a)** Control group. Mild level, **(b)** Tendoflex® group. Mild level, **(c)** *Hypericum perforatum* group. Extreme level, **(d)** Mixed group. Moderate collagen type I expression (arrowhead) (×40-immunohistochemistry).

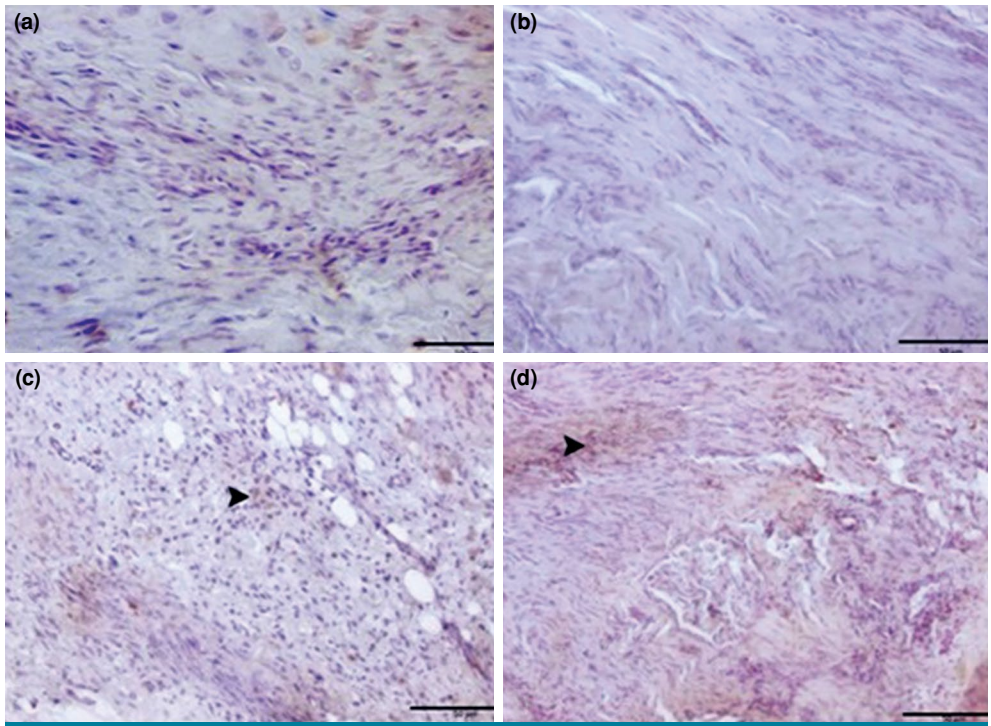


FIGURE 7. Week 1. **(a)** Control group, **(b)** Tendoflex® group, **(c)** *Hypericum perforatum* group. Mild level, **(d)** Mixed group. Moderate expression of collagen type III (arrowhead) (×40-immunohistochemistry).

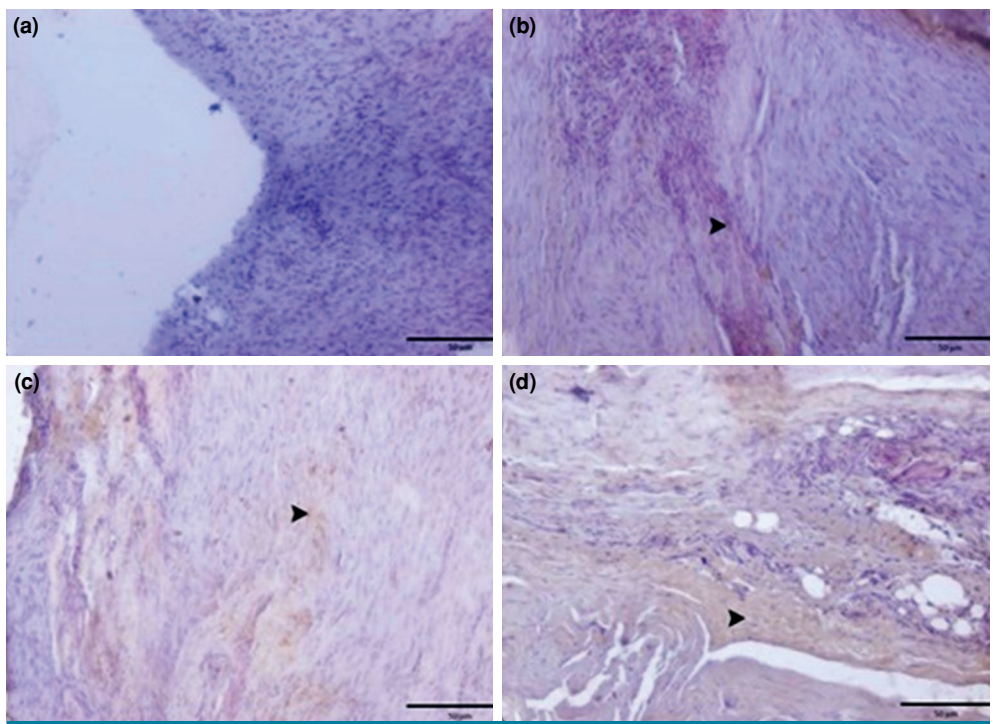


FIGURE 8. Week 2. **(a)** Control group, **(b)** Tendoflex® group. Mild level, **(c)** *Hypericum perforatum* group. Medium level, **(d)** Mixed group. Extreme expression of collagen type III (arrowhead) (×40-immunohistochemistry).

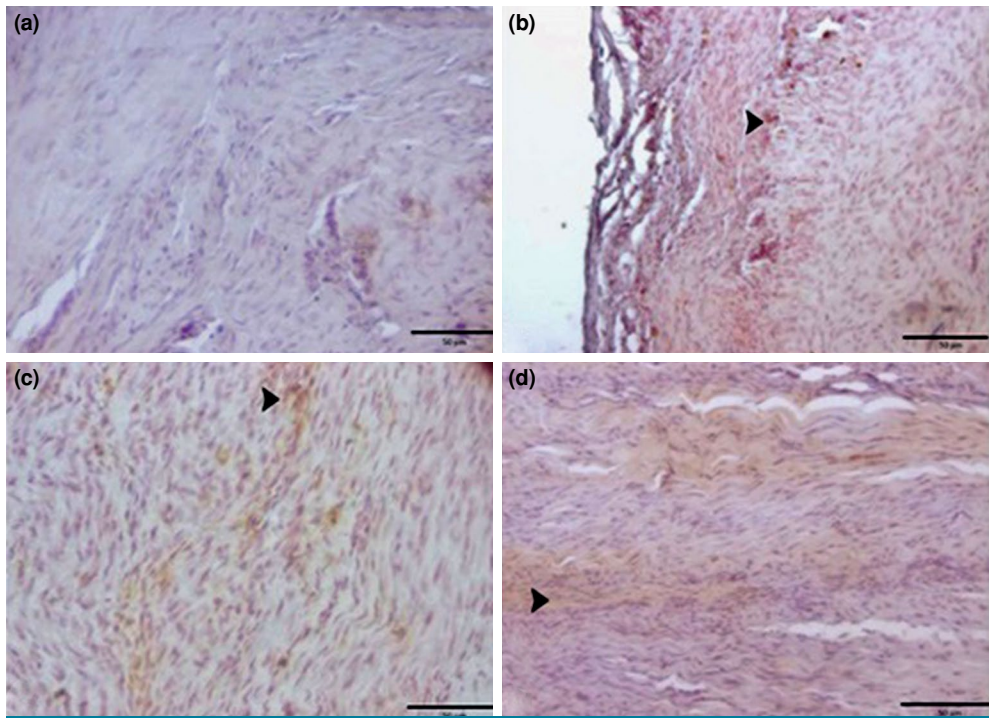


FIGURE 9. Week 3. **(a)** Control group, **(b)** Tendoflex® group. Mild level, **(c)** *Hypericum perforatum* group. Extreme level, **(d)** Mixed group. Extreme expression of collagen type III (arrowhead) (×40-immunohistochemistry).

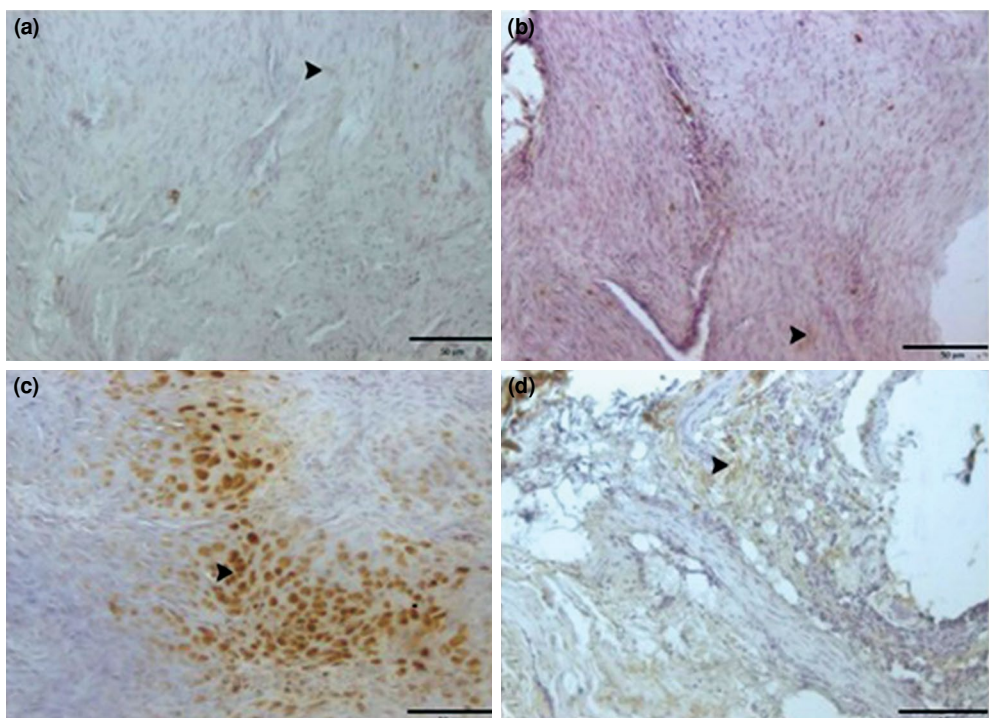


FIGURE 10. Week 4. **(a)** Control group. Mild level, **(b)** Tendoflex® group. Mild level, **(c)** *Hypericum perforatum* group. Very extreme, **(d)** Mixed group. Moderate expression of collagen type III (arrowhead) (×40-immunohistochemistry).

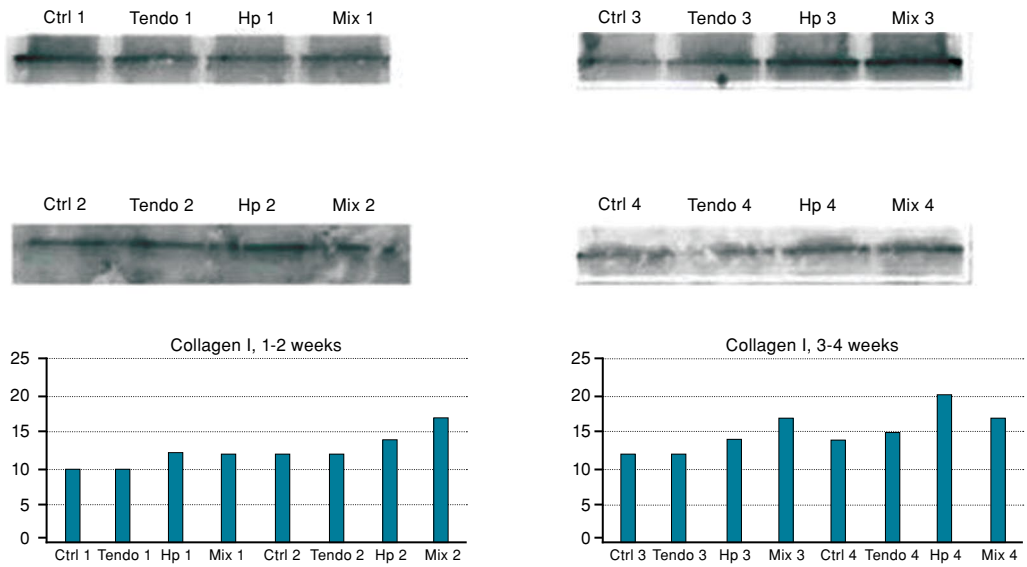


FIGURE 11. Collagen type I. Western Blot analysis.
Ctrl: Control group; Tendo: Tendoflex group; HP: Hypericum perforatum group; Mix: Mixed group.

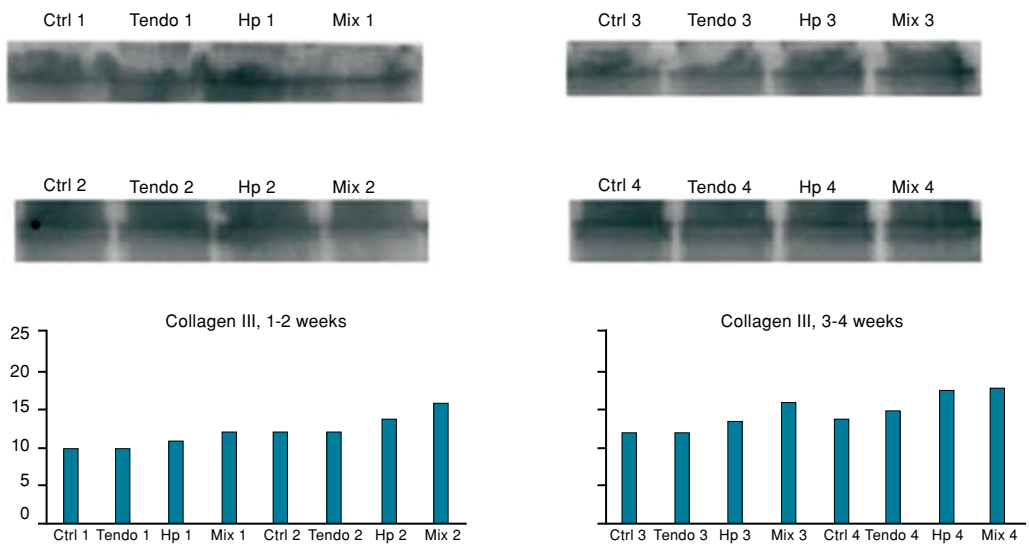


FIGURE 12. Collagen type III. Western Blot analysis.
Ctrl: Control group; Tendo: Tendoflex group; HP: Hypericum perforatum group; Mix: Mixed group.

elevated in the *Hypericum perforatum* group and the mixed group at Week 2. Similarly, at Weeks 3 and 4, the expression of collagen type I and collagen type III increased in the mixed and *Hypericum perforatum* groups (Figures 11 and 12).

DISCUSSION

The Achilles tendon is often injured and data supporting specific treatment strategies for partial and complete ruptures are still insufficient. Regardless of the treatment, patients are at risk of re-rupture and have typically long-term functional deficits. In a recent experimental study, the effects of Tendoflex® and *Hypericum perforatum* on healthy Achilles tendons of the rats were investigated.[11] These active ingredients were reported to increase tensile strength of the tendon in the mechanical investigations and to increase the amount of collagen type I and

type III in the immunohistochemical analysis. In the present study, we examined the effect of these two active ingredients on the repaired Achilles tendon ruptures. We found that the combined use of these two ingredients increased the tendon strength at the most optimal rate, particularly within the first three weeks. Overall, better results were achieved with both individual and combined use of these agents in all weeks, compared to the control group.

In the present study, the mixed group had the most favorable results within the first three weeks, followed by *Hypericum perforatum* and Tendoflex® groups. The *Hypericum perforatum* yielded the most favorable results at Week 4. These results indicate that both active ingredients are beneficial in the healing process of the tendon. These ingredients were highly effective in the early period, which is a very important part of the tendon's healing process, showing that these agents can be recommended following the repair of the Achilles tendon. At Week 4, *Hypericum perforatum* was seen to obtain better results than the mixed group. Mechanism of action differs between these two ingredients and, thus, the two ingredients may eliminate the effect of each other.[2,8,9] Further large-scale clinical studies are needed to understand the underlying reason for this condition.

In our study, three groups, except for the control group, were given the appropriate doses, as previously described, using the active substance. Tendoflex® preparation contains 125 mg of methyl sulfonyl methane, 100 mg of L-arginine, 75 mg of bromelain, 60 mg of vitamin C (L-ascorbic acid), 50 mg of rutin, and 40 mg of collagen type I. Since Tendoflex® contains more than one active ingredient, the amount to be given to the rats was decided by checking these active ingredients separately.[12-16] Bromelain, L-arginine, and methyl sulfonyl methane were used as reference for the dose calibration according to the whole capsule. In a study on bromelain, a dose of 30 mg/kg was shown to be beneficial on tendon damage.[12] One capsule containing 75 mg bromelain was used for each rat to use 30 mg/kg. Again, in another study, L-arginine was used in rats at a dose of 30 to 60 mg/kg.[14] In another study, methyl sulfonyl methane was used at a dose of 50 mg/kg.[13] One tablet containing 125 mg of methyl sulfonyl methane was used for each 2.5 kg of a rat to use 50 mg of methyl sulfonyl methane per kg. In the light of all these studies, one capsule of Tendoflex® was weighed to correspond to 2.5-kg weight of a rat and given by gavage daily according to the weight of the animal. The *Hypericum perforatum* extract was administered orally at a daily dose of 300 mg/kg via gavage based on the previous studies.[17,18]

Many experimental studies focusing on the tendon healing process after different treatment protocols have shown variable outcome criteria.[8] As the main building blocks of the Achilles tendon are collagen type I and type III, in this study, we chose the expression degree of collagen type I and type III as the outcome measure in histopathological examinations, as well as mechanical measurements, and compared these values with the control group. In the tendon healing process, collagen type III is the unorganized scar collagen produced in the first stage. Collagen type I produced later is strong, high-quality, and organized.[4] In the current study, the increased rates of collagen type I and type III were parallel to the mechanical strength of the tendons and superior to the control group in all intervention groups.

Hypericum perforatum is a plant that is widely found in the nature. Some of its positive effects, which have been proven by *in vivo* and *in vitro* experimental studies, are shortening the inflammation time, increasing fibroblast migration, and collagen storage.[19] Bromelain, an enzyme derived from the stem of the pineapple plant, provides a therapeutic effect in the tendon healing process by promoting tenocyte proliferation and reducing malondialdehyde levels in acute tendon injuries.[12] L-arginine is a nitric oxide (NO) precursor.[20] It increases collagen content during tendon healing and is widely used as a dietary supplement to improve repair integrity after tendon repair.[20,21] Murrell et al.[22] found a significant regression in the Achilles tendon cross-sectional area and load during the Achilles tendon healing process in mice using oral N-nitro-L-arginine methyl ester (L-NAME), which inhibits NO synthase activity. Vitamin C plays a role in collagen type I and type III synthesis.[8] In the current study, collagen levels in both Tendoflex® and *Hypericum perforatum* groups were higher than the control group.

Due to the prolonged postoperative recovery time and complications such as rupture, Achilles tendon injury is a significant health problem for middle-aged adults and athletes. In the literature, there are many studies investigating medical treatments in the Achilles tendon healing process. In their study examining the effects of rivaroxaban and low-molecular-weight heparin, Eren et al.[6] reported that these factors contributed to healing in histological examinations, but they could not show the same effect in the biomechanical tests. In another study examining the effect of oral glucosamine, Özer et al.[2] showed its effect on tendon healing in both histological and mechanical examinations. However, the authors concluded that the positive

results in mechanical tests were not statistically significant. In the current study, mechanical tests were superior to the control group, similar to the histological examinations. The most favorable results were obtained in the mixed and *Hypericum perforatum* groups. However, in the current study, the superiority in biomechanical tests was not statistically significant.

Nonetheless, this study has certain limitations. First, clinical and functional results could not be obtained due to the experimental nature of the research. Second, only the short-term effects of active ingredients were able to be observed. Further studies, including clinical and functional outcomes and long-term results with a higher number of subjects are needed.

In conclusion, tensile strength of the Achilles tendon increased by using *Hypericum perforatum* and Tendoflex® following iatrogenic rupture and repair of the Achilles tendon in rats. The combined use of these two agents yielded the most favorable mechanical and histological results, particularly in the early period. These most favorable results were followed by the isolated use of *Hypericum perforatum* and Tendoflex®. This result may be related to the higher level of collagen type I and type III immunity in all groups compared to the control group. These two agents can provide faster and stronger recovery in patients undergoing surgical tendon repair.

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