ORIGINAL ARTICLE

Nr 2023;13 (4):558-563

# Healing Effect of *Morus nigra* Leaf Extract over Tendon Injuries on Proliferative Phase

**Luiza Oliveira Borges[1], Rafael Junio Ribeiro[1], Teresa Maria Da Ré Silva[2], Flávia Da Ré Guerra[3], Petrus Pires Marques[1]**

[1] *School of Medicine, Universidade Edson Antônio Velano (UNIFENAS), Alfenas, Brazil*
[2] *Department of Functional and Structural Biology, Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil*
[3] *Department of Anatomy, Institute of Biomedical Science, Federal University of Alfenas – UNIFAL-MG, Alfenas, Brazil*

**CORRESPONDING AUTHOR:**
Petrus Pires Marques
School of Medicine
Universidade Edson Antônio Velano (UNIFENAS)
Rua Dr. Geraldo Cardoso 54
Residencial São Lucas, Alfenas (Minas Gerais)
37131590 Brazil
E-mail: petrus.marques@unifenas.br

**DOI:**
10.32098/mltj.04.2023.06

**LEVEL OF EVIDENCE: 3**

**SUMMARY**

**Background.** Tendon injuries are difficult to heal properly, and many researchers have attempted to overcome this problem using various approaches.
**Materials and methods.** The authors evaluated the capabilities of *Morus nigra*, a widely known plant with many verified therapeutic properties, on injured tendons in the early stages of recovery.
**Results.** The extract did not demonstrate many benefits regarding tendon resistance to applied forces during either phase; however, an increase in the tendon capacity to stretch without rupture was observed in the early stages of repair. The leaf extract visibly accelerated the recovery process after 15 days of treatment, altering the profile of collagen/GAG deposition in the tendons, reducing inflammatory infiltrate and cellularity, with an increase in all MMP-2 forms expression.
**Conclusions.** The leaf extract accelerated the recovery of tendon morphology. Further studies to identify molecule-specific mechanisms could prove beneficial for tendon healing.

**KEY WORDS**
*Biomechanics; healing; Morus nigra; tendon injury; tendinopathy; phytotherapy.*

## INTRODUCTION

The tendon consists of a firm structure formed by bundles of collagen grouped in the same direction. It connects muscles to bones and transmits contraction forces to the skeletal system to maintain posture and execute movement (1, 2). Its arrangement, formed by fibers organized in an orderly and parallel manner, allows the tendon to endure high loads of force during work (2). However, it is not uncommon for tendon ruptures to occur during common daily and physical activities. This type of injury concerns all age groups, affecting an individual's quality of life since its treatment tends to be complex (3). In general, there are some factors such as sex, age, and chronic diseases – rheumatoid arthritis and diabetes –, that suggest a relation with tendon ruptures (4).
In this context, the calcaneus tendon is the most likely to rupture spontaneously, especially in athletes, as it is subjected to a large amount of static and dynamic loads (2). Its rupture occurs due to forced back flexion, making it impossible for the individual to place the foot in a plantar position (2, 5). The healing is a longstanding process, due to the extracellular matrix (ECM) complexity and is hindered by low vascularization, which characterizes this type of injury as severe. The process results in tissue without its original properties and, therefore, more susceptible to further ruptures (3).
The tendon healing process starts immediately after injury and is divided into three phases: inflammatory, proliferative, and remodeling. Tissue revascularization occurs in the inflammatory phase, which lasts until the seventh day after injury. In addition, signaling factors stimulate the proliferation of tenocytes, recruiting more inflammatory cells and fibroblasts to initiate the synthesis of type III collagen and fibril formation. Then, in the proliferative phase, until the twentieth day of injury, there is a high synthesis of collagen, especially type III and other components of the ECM. At this point, the ECM is much more disorganized; therefore, the probability of a new rupture occurring is high. Remodeling occurs on the twen-

ty-first day and has no maximum deadline for completion. A decrease in angiogenesis, collagen, and glycosaminoglycans (GAG) synthesis is observed. Thus, type III collagen is replaced by type I collagen, which is then replaced by fibrous tissue and soon replaced by scar tissue (6, 7).

Studies in rats have shown that mechanical strength during the repair process stimulates tissue healing, whereas immobilization is detrimental (2, 3). In addition, the extent and nature of the lesion usually determine the success of healing (8). Thus, the most appropriate treatment for these injuries remains unclear. However, if the healing period is reduced, complications can be minimized (9). Hence, the search for new therapies capable of improving the healing process is necessary.

A possible treatment approach can be based on phytotherapy and cultural and ancient practices, which have been scientifically explored. This study sought to analyze the properties of *Morus nigra*, popularly known as the blackberry. It is a plant native to southwest Asia and is used as a traditional herbal medicine in animals and humans. It has important therapeutic properties, such as anti-inflammatory, antimicrobial, antidiabetic, analgesic, anti-obesity, anticancer, and organ-protective properties (10-12).

Phytochemical analyses have revealed the presence of flavonoids, alkaloids, and tannins in *M. nigra* leaves (13). The presence of alkaloids, depsides, depsidones, foamy saponins, and tannins in the aqueous extract of *M. nigra* has also been demonstrated (14). In addition to their antioxidant properties, tannins have healing functions, as they help to form a protective layer on injured tissues, and the healing process may normally occur below this layer. These secondary metabolites of plant origin are pharmacologically active substances that are used as raw materials in the manufacture of cosmetics and herbal medicines. Their use is interesting because of their high availability, low toxicity, minimal risk of side effects, and especially low cost when compared to allopathic medicines.

Most of the properties of *Morus nigra* are due to its antioxidant capacity, which results from abundant phytochemical constituents, such as polyphenols, flavonoids, and anthocyanins. Therefore, this plant can be an important nutraceutical for the control and prevention of chronic diseases and injuries (12, 15). Therefore, this study sought to determine whether the substances present in *Morus nigra* can induce better healing conditions in tendons, reducing the problems arising from the long repair time normally present in this tissue.

## MATERIALS AND METHODS

### Use of animals

32 male Wistar rats were used in the present study. The animals were provided by the Bioterium of José do Rosário Vellano University (UNIFENAS). The rats were housed in two or three per cage under a 12-hour light-dark cycle and had free access to commercial food and liquid (water or tea). The contralateral paws of each animal were used as the control group to avoid the use of more animals.

The use of animals was approved by the CEUA (animal ethics use committee of the institution): protocol number: 47:48 A/2017 – Date of approval: April 17, 2018.

### Experimental groups

Two trials were conducted for this study. In the first trial, 16 animals were used, which, after seven days of treatment, were euthanized for the proposed biochemical, biomechanical, and morphological analyzes. The animals were arranged as follows:

- GW7: eight animals with partially transected tendons and access to food and water.
- GT7: eight animals with partially transected tendons and access to food and *Morus nigra* tea.
- GC7: control group. Intact contralateral paws of previous animals.

For the second trial, 16 more animals were used, which, after 15 days of treatment, were also euthanized, and the analyses were completed. The animals were distributed as follows:

- GW15: eight animals with partially transected tendons and access to food and water.
- GT15: eight animals with partially transected tendons and access to food and *Morus nigra* tea.
- GC15: control group. Intact contralateral paws of previous animals.

Researchers responsible for the animal care were aware of how animals were been treated, given the nature of the treatment (they had to offer the tea in the cages). Since the cages were labeled using numeric code. the researchers responsible for euthanasia and animal surgery were not aware of group distribution. Any animals showing signs of starvation, dehydration, or behavioral changes were excluded from the experiment.

### Procedure for partial transection of the tendons

Animals were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (12 mg/kg). After removing the skin, transverse partial transection was performed in the tension region of the calcaneal tendon, located at an approximate distance of 3 mm from the tendon insertion into the calcaneus bone. Subsequently, the tissues were repositioned, and the region was sutured (16).

### Biomechanical and microscopy analysis

Tendon biomechanics were analyzed using a texturometer TA.XT plus, from Stable Micro Systems. The tendons were maintained in physiological solution until testing. Before the test, the length, width, and thickness of the tendons were measured using a digital caliper. The last two were determined at the midpoint of the distal region of each tendon and the cross-sectional area was calculated using these measurements. During the test, both ends of the tendons were fixed in the machine, which applied an increasing load at a constant rate of 20 mm/min until tendon rupture. The force necessary to rupture the tendon was designated as the failure load (F). Using this parameter and tendon displacement, it was possible to calculate the stress in MPa ($\sigma = F/A$), strain in % ($\varepsilon$ = displacement/initial length), and Young's modulus ($E = \sigma/\varepsilon$) (17).

## Morphological analysis

The tendons were fixed using a 4% formaldehyde solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH– pH 7.4) for 18 h at room temperature and washed in water, ethanol dehydrated, diaphanized with xylene and paraffin-embedded. Longitudinal serial sections of 7 µm were stained with hematoxylin and eosin (HE) and analyzed under an Olympus BX 60 light microscope.

## Biochemical analysis

### *Extraction procedure*

The calcaneal tendons were removed and incubated in extraction buffer (50 mM Tris-HCl pH 7.4, 0.2 M NaCl, 0.1% Triton X-100, 10 mM $CaCl^2$, and protease inhibitor 100 µL/10 mL) at 4 °C for 3 h. Subsequently, the material was centrifuged (13,000 × g, 20 min, 4 °C) (16).

### *Quantification of proteins*

Non-collagenous proteins were quantified according to the Bradford method, using bovine serum albumin as the standard. Absorbance was measured at 595 nm (18).

### *Zymography*

Metalloproteinase (MMP) analyses were performed using zymography methods (19). The samples were incubated in extraction buffer (50 mM Tris-HCl pH 7.4, 0.2 M NaCl, 0.1% Triton X-100, 10 mM $CaCl^2$, and protease inhibitor 100 µL/10 mL) at 4 °C for 24 h. In total, 20 µg protein was loaded into each well. Polyacrylamide gels were prepared using 2 mg/mL of gelatin. The gels were incubated in incubation buffer (50 mM Tris-HCl pH 8.4, 5 mM $CaCl^2$, and 1 µM $ZnCl^2$) overnight at 37 °C. The gels were stained with Coomassie Brilliant Blue R-250 and bleached (30% methanol and 10% acetic acid in water). Finally, the gels were placed in a shrinking solution (30% methanol and 3% glycerol).

## Statistical analysis

All results are expressed as the mean ± standard deviation. The Mann-Whitney test was used to analyze birefringence measures. Other quantitative data were compared between groups using the Student's t-test. The level of significance was set at $p < 0.05$. Analysis was performed using GraphPad Prism® (GraphPad Software, La Jolla, CA, USA).

# RESULTS

After the respective treatment periods, 7 and 15 days, receiving water or blackberry tea, the animals were euthanized, and the tendons were subjected to the following analyses.

## Biochemical analysis

Through comparative graphical analysis of the densitometry of zymography bands (**figure 1**), metalloproteinases (MMP) were quantified: MMP-2 of latent, intermediate, and active isoforms. In the groups treated for seven days, less MMP-2 was observed in the tendons of rats that consumed blackberry tea (GT7). Thus, this group came closer to the concentration of the contralateral control tendons (GC7) compared to the tendons of animals that consumed only water (GW7). The results were different in the groups treated for 15 days. In this case, MMPs-2 were statistically higher in animals that received blackberry tea (GT15), when compared to the group that received only water (GW15), and to the control group (GC15).

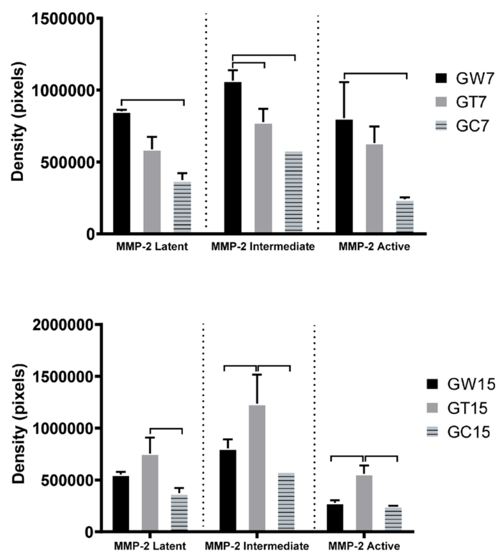


**Figure 1.** Band densitometry of zymography for the different isoforms of MMP-2 in the inflammatory (above) and proliferative phases (below).

The groups connected in brackets indicate statistically significant differences ($p < 0.05$).

### Morphological analysis

Inflammatory changes (vascular changes and inflammatory infiltrates) and reparative processes were analyzed. The morphological analyses of the groups differed in terms of cellularity and inflammatory processes. Image samples can be viewed in **figure 2**.

Microscopy could not demonstrate evident differences between groups treated with water or tea for only seven days (GW7 and GT7). The lesion area is well marked with inflammatory infiltrate and a great number of fibroblasts, with basophilic ECM. When treating the animals for 15 days, differences arose in the images (compare GW15 and GT15). The tendons of GT15 are clearly more eosinophilic with reduced cellularization in the lesion area.

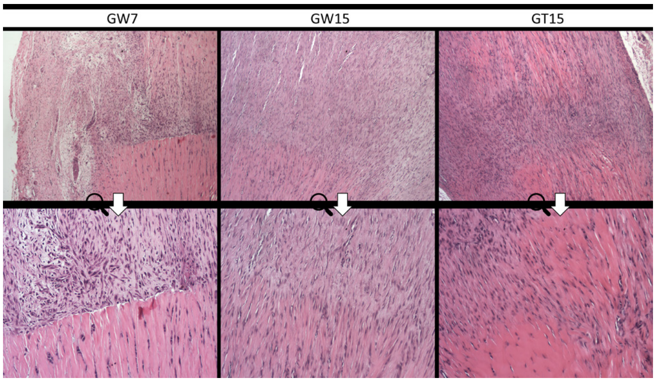


**Figure 2.** Image samples from histological slides obtained from the experimental groups.

First row, images at 40x magnification; second row, 100x magnification.

### Biomechanical analysis

The biomechanical properties were evaluated by analyzing the following variables: strain (%) or stretching, stress (Mpa) or tension, and Young's modulus (Mpa), which refers to elasticity. All the results were summarized in **figure 3**.

Analyzing the animals submitted to seven days of treatment, the statistical analysis showed, with $p < 0.05$, that the strain (elongation of the tendon) presented no differences between the control (GC7) and animals treated with water (GW7). Nevertheless, the strain was considerably higher in the group treated with tea (GT7). In the fifteen-day treatment, however, there was no statistical difference between the groups.

Stress demonstrated that in both treatment periods, the transected groups had a reduction in their resistance to tension, with no statistically significant difference between the treated and control groups. Meaning the force required to rupture the control tendons was considerably higher than that required to break the transected tendons that received tea or just water.

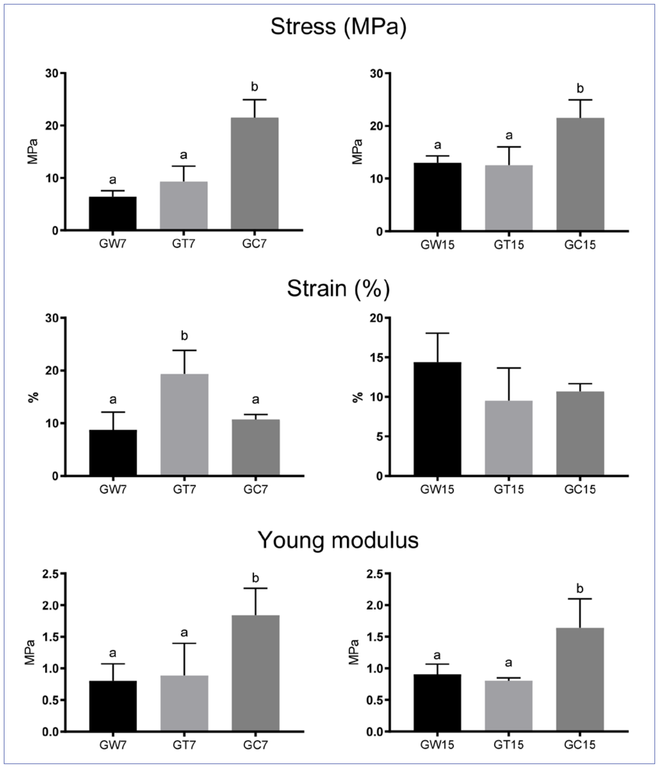


**Figure 3.** Summary of biomechanical results for all experimental groups.

Different letters indicate statistically significant differences ($p < 0.05$).

From Young's Modulus, it was observed, also in both treatment periods, that there were no statistical differences between the transected groups – treated with tea or water. Both presented lower values than the control. In this way, the control tendons, with greater stiffness, endured greater strength but were less deformed and could suffer ruptures.

## DISCUSSION

This study aimed to evaluate the effects of blackberry tea (*Morus nigra*) on the healing process of the calcaneus tendon in rats during the inflammatory and mid-proliferative phases after partial transection. The data obtained can assist in further studies to seek alternatives for the treatment of tendon injuries.

Remodeling of the ECM of the tendon during the healing process depends on the action of MMPs, which are proteins that play an important role in the degradation of collagen, proteoglycans, and elastin (20). MMPs play a role in many biological processes, including tissue remodeling and growth, and are also involved in defense mechanisms and immune responses of tissues. Physiological processes, such as growth and wound healing, involve increased expression of MMPs (21).

The MMP-2 is a constitutively expressed gelatinase, involved in the natural collagen turnover. The physical lesion naturally brought an increase to the expression of the protease in the tendons, but our results demonstrated that the animals treated with tea kept the values closer to the uninjured animals during the inflammatory phase (**figure 1**). Although necessary for the tendon recovery, large amounts of MMPs results in excessive collagen degradation, making the tendon more susceptible to ruptures (22).

Interesting results were observed after 15 days of treatment with *Morus nigra*. The MMP-2 expression of the control animals was similar to the ones treated with water, but there was a significant increase in the tea-treated animals (GT15). At the same time, **figure 2** shows the difference in morphology between GW15 and GT15, the last having an ECM much more eosinophilic around the lesion, and visible reduction in the inflammatory infiltrate. This eosinophilia is evidence of a shift in the concentrations of GAGs (very anionic) and collagen fibers deposition by fibroblasts, approximating the tendon of a healthy one. Reduced cellularization in GT15 also evidences a more organized tendon and an evolution in the recovery of the structure.

The anti-inflammatory capabilities of the Morus genus were demonstrated by other researchers. Substances isolated from the root bark of *M. nigra* were able to decrease the secretion of the pro-inflammatory cytokines TNF-α and IL-1β in macrophages stimulated by LPS, thus reducing inflammation (23). It was also demonstrated that treatment with blackberry leaf extracts and pulps reduced the number of total leukocytes in bronchoalcoholic lavage fluid in septic mouse models, indicating a reduction in the inflammatory infiltrate in the lung (24).

The parameters analyzed regarding the biomechanical properties were stress, which corresponds to the strength needed to cause the tendon rupture, strain refers to how much the tendon could be stretched before the rupture, and Young's modulus, whether the tendon is elastic, or if its structure is rigid.

Analysis of the biomechanical properties revealed that the transected tendons were less resistant to the applied pressure (stress). During the inflammatory process, the ECM changes, and the complex arrangement of collagen fibrils responsible for tendon resistance becomes disorganized. Therefore, during that period, the tendon became more susceptible to further ruptures (22). The use of anti-inflammatory substances, by reducing the duration of inflammation, can prevent new injuries. In GT7, greater elasticity was observed, which resulted in a greater elongation capacity; however, less load was supported. Other parameters tested could not assert differences between the groups for the treatment times chosen.

The disorganization of collagen fibers, a characteristic of an injured tendon, is related to the increased performance of MMPs due to inflammation. During the tendon healing process, the ECM has a disorganized appearance, and even with treatment, the original biomechanical properties are never recovered (25).

## CONCLUSIONS

Studies that specifically relate the effects of *Morus nigra* tea on tendon recovery are scarce. However, its properties can be explored and made suitable for the treatment of these lesions, especially anti-inflammatory substances. This paper demonstrates the value of the extract in accelerating the tendon recovery, but the mechanisms by which it is achieved need to be elucidated. Further studies could provide more molecule-specific answers to increase even more the desired recovery responses.

## FUNDINGS

This study was funded by FAPEMIG and received support of all the institutions involved both in the form of financial funding and facility use.

## DATA AVAILABILITY

The data supporting the findings of this study are available within the article itself. Any request for additional content can be requested to the corresponding author.

## CONTRIBUTIONS

LB, RR, TS: investigation, writing – original draft, formal analysis. FG: formal analysis, editing, writing – review & editing. PM: funding acquisition, project administration, resources, formal analysis, translating, writing – review & editing.

## CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

## REFERENCES


1. Voleti PB, Buckley MR, Soslowsky LJ. Tendon healing: repair and regeneration. Annu Rev Biomed Eng. 2012;14:47-71. doi: 10.1146/annurev-bioeng-071811-150122.
2. Guerra F, Vieira C, Almeida M, et al. Pulsed LLLT improves tendon healing in rats: a biochemical, organizational, and functional evaluation. Lasers Med Sci. 2014;29(2):805-11. doi: 10.1007/s10103-013-1406-2.
3. Nourissat G, Berenbaum F, Duprez D. Tendon injury: from biology to tendon repair. Nat Rev Rheumatol. 2015;11(4):223-33. doi: 10.1038/nrrheum.2015.26.
4. Szaro P, Witkowski G, Śmigielski R, Krajewski P, Ciszek B. Fascicles of the adult human Achilles tendon – An anatomical study. Ann. Anat. 2009;191(6):586-93. doi: 10.1016/j.aanat.2009.07.006.
5. Kannus P, Natri A. Etiology and pathophysiology of tendon ruptures in sports. Scand J Med Sci Sports. 1997;7(2):107-12. doi: 10.1111/j.1600-0838.1997.tb00126.x.
6. Sharma P, Maffulli N. Biology of tendon injury: Healing, modeling and remodeling. J Musculoskelet Neuronal Interact. 2005;6(2):181-90. Available at: https://www.ismni.org/jmni/pdf/24/14MAFFULLI.pdf.
7. Abate M, Salini V, Schiavone C. Achilles tendinopathy in elderly subjects with type II diabetes: the role of sport activities. Aging Clin Exp Res. 2016;28(2):355-8. doi: 10.1007/s40520-015-0391-7.
8. Ferrari R, Picchi L, Botelho A, Minamoto V. Processo de regeneração na lesão muscular: uma revisão. Fisioter Mov. 2005;18(2):63-71.
9. Guerra FDR, Vieira CP, Almeida MS, Oliveira LP, de Aro AA, Pimentel ER. LLLT improves tendon healing through increase of MMP activity and collagen synthesis. Lasers Med Sci. 2013;28(5):1281-8. doi: 10.1007/s10103-012-1236-7.
10. Wei H, Zhu J, Liu X-Q, et al. Review of bioactive compounds from root barks of Morus plants (Sang-Bai-Pi) and their pharmacological effects. Cogent Chem. 2016;2(1). doi: 10.1080/23312009.2016.1212320.
11. Chen H, Pu J, Liu D, et al. Anti-Inflammatory and Antinociceptive Properties of Flavonoids from the Fruits of Black Mulberry (Morus nigra L.). PLoS One. 2016;11(4):e0153080. doi: 10.1371/journal.pone.0153080.
12. Lim SH, Choi C-I. Pharmacological Properties of Morus nigra L. (Black Mulberry) as A Promising Nutraceutical Resource. Nutrients. 2019;11(2):437. doi: 10.3390/nu11020437.
13. Guizzo P, Bredda T, Scarpa M, Navarro F. Controle de Qualidade e triagem fitoquímica da droga vegetal das folhas de Morus nigra L. (MORACEAE). Revista de Ciências Farmacêuticas Básica e Aplicada. 2015;36(2):259-65.
14. Rosa R, Ribeiro L, Souza A, Fonseca T. Triagem fitoquímica dos extratos aquosos de Bauhinia candicans, Foeniculum vulgare, Mentha pulegium e Morus nigra. Conexão Ciência. 2016;11(1):44-51. doi: 10.24862/cco.v11i1.381.
15. Wang L, Yang Y, Liu C, Chen R-Y. Three new compounds from Morus nigra L. J Asian Nat Prod Res. 2010;12(6):431-7. doi: 10.1080/10286020.2010.489824.
16. da Ré Guerra F, Vieira C, Oliveira L, Marques P, Almeida M, Pimentel ER. Low-level laser therapy modulates pro-inflammatory cytokines after partial tenotomy. Lasers Med Sci. 2016;31(4):759-66. doi: 10.1007/s10103-016-1918-7.
17. Vieira C, Oliveira L, Guerra F, Almeida M, Marcondes M, Pimentel E. Glycine improves biochemical and biomechanical properties following inflammation of the achilles tendon. Anat Rec (Hoboken). 2015;298(3):538-45. doi: 10.1002/ar.23041.
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54. doi: 10.1006/abio.1976.9999.
19. Marqueti R, Parizotto N, Chriguer R, Perez S, Selistre-de-Araujo H. Androgenic-anabolic steroids associated with mechanical loading inhibit matrix metallopeptidase activity and affect the remodeling of the achilles tendon in rats. Am J Sports Med. 2006;34(8):1274-80. doi: 10.1177/0363546506286867.
20. Lu P, Takai K, Weaver V, Werb Z. Extracellular Matrix Degradation and Remodeling in Development and Disease. Cold Spring Harb Perspect Biol. 2011;3(12):a005058. doi: 10.1101/cshperspect.a005058.
21. Benjamin MM, Khalil RA. Matrix metalloproteinase inhibitors as investigative tools in the pathogenesis and management of vascular disease. Exp Suppl. 2012;103:209-79. doi: 10.1007/978-3-0348-0364-9_7.
22. Marques P, Porto N, Vieira C, et al. Effects of supraphysiological doses of nandrolone decanoate in Achilles tendon of rats. Muscles Ligaments Tendons J. 2019;9(2):255-61. doi: 10.32098/mltj.02.2019.15.
23. Zelová H, Hanáková Z, Čermáková Z, et al. Evaluation of Anti-Inflammatory Activity of Prenylated Substances Isolated from Morus alba and Morus nigra. J Nat Prod. 2014;77(6):1297-303. doi: 10.1021/np401025f.
24. Lúcio K, Rabelo A, Araújo C, et al. Anti-Inflammatory and Antioxidant Properties of Black Mulberry (Morus nigra L.) in a Model of LPS-Induced Sepsis. Oxid Med Cell Longev. 2018;2018:5048031. doi: 10.1155/2018/5048031.
25. Oliveira L, Vieira C, Guerra F, Almeida M, Pimentel E. Structural and biomechanical changes in the Achilles tendon after chronic treatment with statins. Food and Chemical Toxicology. 2015;77:50-7. doi: 10.1016/j.fct.2014.12.014.