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INFLUENCE OF TRIBULUS TERRESTRIS EXTRACT ON BONE MORPHOLOGY AND BIOMECHANICS OF RAT FEMURS

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Abstract

Introduction: *Tribulus terrestris* (TT) is an herb credited as a food supplement and capable of providing metabolic gains, from the refinement of androgen levels and treatment of various diseases to the improvement of bone mineral content. **Objective**: to evaluate the influence of TT extract on the biomechanical and histomorphologic properties of rat femurs. **Methodology**: An experimental study was performed with Wistar rats, divided into two groups (n=10), Control (CT), treated with 0.5ml of 0.9% SF, orally, and *Tribulus terrestris* (TT), treated with TT extract in a dose of 50 mg/kg in a 0.5 ml solution, orally. After 60 days, the right femurs were dissected and the morphology and biomechanics were analyzed. **Results**: during the experiment, all animals gained weight, being the CT group gain (181±21) higher than the TT one (132±12). Morphometric dimension analyses did not reveal significant differences and interactions between CT and TT groups. The TT group presented significantly inconsistent data in the histomorphometric analysis of the trabecular and cortical area and thickness of the femurs, when compared to the CT group, showing larger dimensions upon consumption of the TT. The biomechanical analyses revealed remarkable differences, as the TT group needed a greater maximum force for rupture, displacement, extrinsic rigidity, elasticity, tension and deformation of the femurs until the fracture was evidenced. **Conclusion**: the consumption of TT had positive effects regarding the biomechanical properties, volume and thickness of the femurs, and in the trabecular area, suggesting a greater bone resistance to the fracture caused by the flexion test.

Keywords: *Tribulus terrestris*; bone biomechanics; androgenic effects; testosterone; bone density, phytotherapy.

1. INTRODUCTION

Over the centuries, folk medicine has been molded by the use of many herbs credited as food supplements capable of providing diverse metabolic gains, from the refinement of androgen levels and treatment of various diseases to the improvement of bone mineral content and athletic performance of the individual. One of these herbs is *Tribulus terrestris*, a creeping herbaceous species of the *Zygophyllaceae* family, which composition, depending of the place of origin and in its internal organs, may has a high active content of sterol saponins, flavonoids, phenolic carboxylic acids, alkaloids, tannins and/or terpenoids (STEFÃNESCU et al, 2020; PARHAM et al, 2020).

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Much of the literature discusses the pharmacokinetic properties of the main compounds of *Tribulus terrestris* in a broad, but often contradictory, way. Even so, as reinforced by MA, GUO and MANG (2017), it is undeniable that steroidal saponins, for example, as main components of the herb, have a permeable membrane thanks to the presence of an amphiphilic molecule in their composition, which, consequently, increases the absorption of other compounds, a fact that should be considered in patients who use *T. terrestris* chronically, since toxic effects may arise (STEFÃNESCU et al, 2020).

Being indicated for the most diverse purposes, the herb discussed in the present study is emphasized, by many authors, as an important antioxidant, vasodilator, antiapoptotic, antibacterial, anthelmintic and larvicidal, postprandial antihyperglycemic (for diabetic patients), anti-inflammatory, anti-caries, anti-urolithic (for the treatment of urinary diseases, such as urolithiasis), anti-aging and neuro-protective, by action on the Central Nervous System, and for the prevention and treatment of cardiovascular and skin diseases and hepatocellular carcinoma, and improves memory, vision and athletic activity. In addition, it acts in sexual disorders, improving sexual behavior, from the activation of libido and the control of menopausal transition symptoms, in women, to the treatment of erectile dysfunction in men, by converting testosterone into dihydrotestosterone, and increasing sperm motility and testosterone levels by the indirect action of steroidal saponins, such as protodioscin (ABDEL-ALL et al, 2021; PARHAM et al, 2020; STEFÃNESCU et al, 2020; VALE et al, 2018; KANG et al, 2017; PARK et al, 2017; SALGADO et al, 2017; ZHU et al, 2017; KIM et al, 2011; GAUTHAMAN and GANESAN, 2008). According to POKRYWKA et al (2014), this effect of rising testosterone levels is due to an association, not yet well known, of *T. terrestris* with other drugs.

Testosterone, as an anabolic androgenic agent that generates long-term anabolic actions, presents some diverging results on clinical studies in animals and in humans, comparing the application of *Tribulus terrestris* alone or in association with other pharmacological substances (MA, GUO and MANG, 2017; QURESHI, NAUGHTON and PETROCZI, 2014). However, in both types of studies, some

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authors have noted that the use of this herb can influence not only the doping tests of athletes, but also the regulation of bone tissue functions, since the increasing sexual steroid levels can evoke periosteal and endocortical growth, as well as in early epiphyseal closure with interruption of bone growth (VAN DER LOOS et al, 2021; LOK, 2015). Proportionally, the decrease in testosterone concentrations, common in aging, can cause periodontal disease and osteoporosis (FIDELIS et al, 2020).

In parallel, prolonged treatment with *Tribulus terrestris* is considered capable of reducing bone loss and stimulating the increase in bone mineral density, promoting a bone protective effect, secondary to its antiarthritic effect, according to MARQUES et al (2019) and BERTOZZO et al (2019). Consequently, this herb may assist other phytotherapeutic drugs in the treatment of osteoporosis, a systemic bone metabolic disease characterized by a higher rate of bone resorption than formation (deficiency in bone strength), and support a decreased risk of fractures. In this sense, the use of medicinal herbs for such a disease associated with hormone replacement therapy, for example, would aim to reduce side effects and the cost of treating fractures and their complications (FIDELIS, 2020).

As highlighted by STEFÃNESCU et al (2020), POKRYWKA et al (2014), NEYCHEY and MITEV (2016) and several other authors, yet more molecular studies are necessary so that the pharmacodynamic experimental analyses of the active components of *Tribulus terrestris* culminate in less diverging results, more effective and safer for humans.

The aim of the present study was to evaluate the influence of *Tribulus terrestris* extract on the biomechanical and morphological properties of Wistar rat femurs.

2. MATERIAL AND METHODS

Ethical aspects - This study was approved by protocol 25A/2014 and all steps were performed in accordance with the Research Ethics Committee of UNIFENAS. The

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research was developed in partnership between the Federal University of Alfenas (UNIFAL-MG) and José do Rosário Vellano University (UNIFENAS).

Animals and experimental protocol - Twenty male albino Wistar rats (*Rattus norvegicus albinus*) weighting 245.6+2.1 g, and supplied by the Central Animal House of the José do Rosário Vellano University- UNIFENAS, Alfenas-MG were used and kept at the Laboratory of Animal Experimentation of UNIFENAS in ventilated shelves (Alesco, Monte Mor, SP, Brazil), under controlled temperature (25±2°C), and 12-h/12-h light/dark cycles.

The animals were randomized into two experimental groups (n=10), the Control Group (CT) with rats treated orally with 0.9% saline solution, in a volume of 0.5 ml, and the *Tribulus terrestris* Group (TT), with of rats treated orally with *Tribulus terrestris* extract, with a dose of 50 mg/kg in a volume of 0.5 ml (solution). All animals received the treatment over 60 days (8 weeks).

All rats were allowed free access to water and a balanced commercial solid diet (Nuvilab CR - Nuvital, Colombo - PR, Brazil). A liquid diet was offered in 500 mL vials for free consumption. Every 48 hours, solid and liquid diets were changed and residual values were recorded; in addition, the animals were weighed weekly.

Euthanasia protocol and biological material collection - After 60 days of treatment the animals were weighed and, later, anesthetized, following the anesthetic protocol with Xylazine Hydrochloride (Rompun® 2g/10mL) - 40mg (0.2ml) in association with Ketamine Hydrochloride (Ketamin®50mg/mL) -10mg (0.2ml), intraperitoneally. The animals' blood was collected via retroorbital and sent for serum and total calcium analysis. Subsequently, the right femurs were removed, cleaned from soft tissues, measured, placed in gauze soaked in saline solution (0.9%) and stored in a freezer (-20°C) until the day before mechanical test (NAKAGAKI et al, 2011; SOARES et al, 2010).

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Morphometric analysis of the femur - The dimensions of the femurs were measured using a digital caliper (Starrett®). Each measurement corresponds to the distance between two referential points of the bone itself (LAMMERS; GERMAN; LIGHTFOOT, 1998). For this purpose, the following were collected: (A) *femur length* (mm) - from the most proximal point of the femoral head to the most distal point of the other end of the bone; (B) *width of the femoral shaft* (mm) - taken from the narrowest portion of the middle of the femur; (C) *width of the proximal femur* (mm) - from the most anterior point of the head of the femur to the tip of the greater trochanter; (D) *width of the distal femur* (mm) - across the condyles (anteroposterior direction), perpendicular to the length of the femur.

For trabecular epiphysis analysis, six histological sections were taken from each femur and two fields were captured in each section. Initially, the samples were fixed in 10% buffered formalin for a period of 72 hours at room temperature, and decalcified in a solution of formic acid, formaldehyde and sodium citrate for 35 days. After this period, they were histologically processed and cross-sections of five micrometers (µm) thick were obtained and stained with hematoxylin-eosin.

Measurements of the trabecular and cortical area and thickness of the femurs were obtained using the software NIS-Elements: Advanced Research 3.0 (USA), with a 20X objective coupled to a Nikon photomicroscope, model Eclipse E400, with a Nikon camera model DXM 1200C (NAKAGAKI et al, 2011).

Mechanical tests - The femurs were submitted to a mechanical resistance test at the Department of Anatomy of UNIFAL-MG, in a three-point flexion module, TA.XTplus (texture analyzer), using a load cell of 50 Kgf at a speed of 1.3 mm/min. The distance between the two bone ends was 50 mm, and, to take the resistance value, a load was applied to the middle third of the bone (SOARES et al, 2010) (diaphysis at the surgical intervention site), through a tip, attached to a universal testing machine. Femurs were tested in the anteroposterior plane (concave facing up), with the anterior side facing up, supporting compression, and with the posterior side facing down, supporting tension. The force was applied on the anterior surface due to the greater stability of

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the bone with the mechanical apparatus, minimizing rotation during the test (HUANG et al, 2003).

The results of the tests were recorded on a computer coupled to the MTS and after obtaining the maximum force and displacement curves, analyses of the mechanical and structural properties of the femurs were performed.

Statistical analysis - For the statistical analyses of the results, the GraphPad Prism 9.1 program was used, and the Analysis of Variance (ANOVA) was applied at 5% of significance, suitable for comparing independent groups.

3. RESULTS AND DISCUSSION

RESULTS

In the present study, it was observed that rats that received *Tribulus terrestris* (TT) gained less body weight when compared to rats in the Control Group (CT). The animals' food and water consumption was satisfactory and statistically equal (Table 1).

Table 1. Weight of animals at the beginning and end of the experiment, weight gain, average daily consumption of water and food of the CT and TT groups rats.

Parameters	CT	TT	p value
Initial weight of rats (g)	247±11[a]	232±4[a]	0,2057
Final weight of rats (g)	401±14 [a]	358±11[b]	0,0144*
Weight gain over 60 days (g)	181±21 [a]	132±12[b]	0,0436*
Average daily feed intake (g)	32,4±0,5 [a]	31,7±0,4 [a]	0,3238
Average daily water consumption (mL)	24±0,4 [a]	24,7±0,5 [a]	0,2813

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Foot note: Means and standard deviation showing the same lowercase letter on the line are statistically equal for p≤0.05.
Source: Authors.

The analysis of the morphometric dimensions of the femurs did not reveal significant differences and interactions in the experimental groups for the femur length (mm) and femur proximal width (mm); however, they showed a remarkable difference for the femoral shaft width (mm) and width of the distal femoral epiphysis (mm) between both groups (Figure 1).

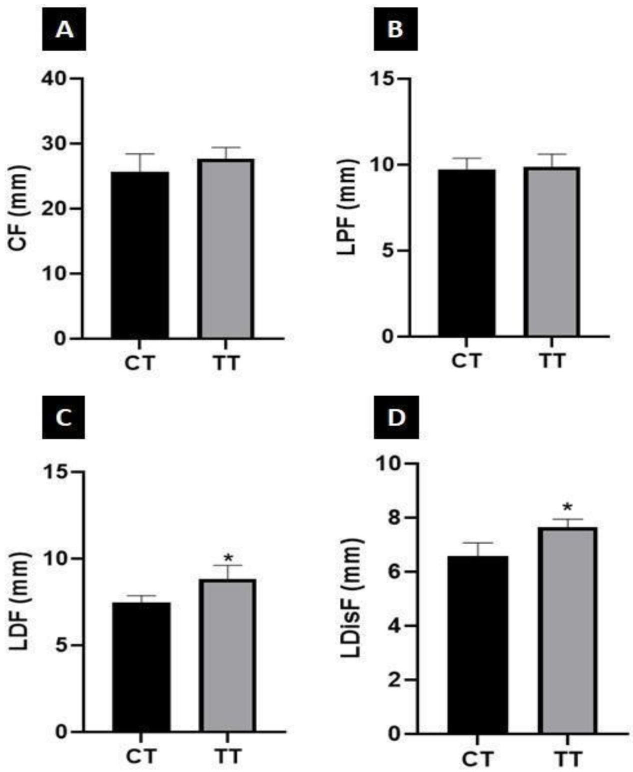

Figure 1. Analysis of the results referring to the dimensions of the right femur of rats in the CT and TT groups:

A) CF – Femoral Length (mm) – The statistical analysis showed that there is no statistically significant difference between CT and TT groups (p = 0.0628).

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B) LPF – Proximal Femoral Epiphysis Width (mm) – The statistical analysis showed that there is no statistically significant difference between CT and TT groups (p = 0.5745).
C) LDF – Femoral Diaphysis Width (mm) – The statistical analysis showed that there is a statistically significant difference of the TT group in relation to CT one (p = 0.0001).
C) LDisF – Distal Femoral Epiphysis Width (mm) – The statistical analysis showed that there is a statistically significant difference of the TT group in relation to CT one (p = 0.0001).
* - Statistically significant difference
Source: Authors.

Histomorphometric analyses of the trabecular and cortical area and thickness of the femurs revealed significant differences and interactions in the larger experimental groups observed in the TT group (Figure 2).

DISCUSSION

The findings regarding bone morphology and biomechanics established a direct relationship to the effects of *Tribulus terrestris* consumption, as the animals in the TT group gained weight throughout the experiment and consumed enough feed and water in order not to characterize malnutrition or dehydration.

It is known that animal experimentation is directly influenced by the ingestion of liquid and solid (feed) in rodents. In this sense, it is important that, as done in the present study, the researchers monitor the consumption of liquids and solids, as well as the weight gain of Wistar rats, so that the solid intake does not exceed the minimum threshold of 25g/day, liquid intake varies from 15 to 80 ml per day and that weight loss shall not be marked throughout the experiment, in order not to characterize malnutrition or dehydration and so compromise the subsequent analysis (WEISBROTH et al., 1977; PALENCIA et al., 1994; HAU and SHAPIRO, 1984).

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Therefore, the results of this study were in agreement with the analysis mentioned above: all Wistar rats consumed an amount of feed greater than the pattern minimum threshold and a volume of liquid in line with the ideal limits. In addition, no significant difference was found between the studied groups, thus ruling out the possibility of malnutrition and dehydration. The results showed a weight gain by all rodents during the experiment, which makes it clear that the morphological and biomechanical findings of the bone structure are directly related to the consequences of the consumption of *Tribulus terrestris*.

According to DALMOLIN et al. (2013), biomechanical tests goals benefits ranging from testing the strength of implants to analyzing the distribution of stresses in the bone, mainly to obtain better answers about the stabilization of fractures. Consonantly, the present study used these tests to evaluate the bone resistance under the use of *Tribulus terrestris*, considering the effects of the consumption of this herb on bone thickness and area and on the following variables: Maximum Strength (N), which is the greater load than the bone tissue is capable of sustaining; Displacement or Distension (mm), which is the amount of deformation that occurs when an external load is applied perpendicularly to the cross-sectional area of the bone tissue and in the opposite direction to it; Extrinsic Rigidity or Hardness (N/mm), which corresponds to the bone's ability to absorb energy without fatigue and breakage; Maximum Tension (Mpa), which reflects the resistance to external load; and Modulus of Elasticity (MPa), which is the bone tissue's ability to return to its original size and shape after the load is released. In the same way, such an evaluation allows the understanding of the way in which all these variables reflect on the deformation (Mpa) of the bone, that is, on the promotion of internal deformations on the structures of the static bone tissue under analysis, as pointed out by DINIZ et al. (2005), in order to obtain a practical-scientific direction for the treatment of long bones fractures (since the tests used the femur as a reference) and cases of osteoporosis, improving prognosis and the quality of daily life of those affected by them.

Bone tissue is constantly submitted to mechanical stimuli that favor its development and architectural structure, arising from the dynamic processes of

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atrophy and hypertrophy, allowing the bones to sustain the loads applied by physiological displacement. Such tissue is composed of collagen fibers (the vast majority being formed by calcium phosphate) and by osteoblasts, osteocytes, osteoclasts and by undifferentiated cells. Such collagen fibers are responsible for absorbing most of the tension overload, that is, of the maximum external force applied in the cortical bone, even though in this area the collagen is anisotropic, that is, it does not have mechanical properties that are equal when is strained in different directions. And it is this anisotropy that corresponds to cortical bone adaptation to loads, whether physiological or not, which is called Wolff's Law (DINIZ et al., 2005).

According to Wolff's Law, the bone has the ability to adapt itself to structural changes, in size and shape, depending on the mechanical stimulus to which it is submitted. When a maximum external force is applied to the bone, tension and stretching or distension stress occurs, as explained by DINIZ et al. (2005). This tension is responsible for strengthening the collagen fibers, allowing them to align in parallel with the tension loads, which consequently increases bone remodeling. Cortical bone tissue, for example, is dense, homogeneous and orients itself more longitudinally than tangentially, since osteons are oriented by the longitudinal axis of the bone (DINIZ et al., 2005).

In the present study, the femurs were tested in the anteroposterior plane, with the concavity and anterior side facing upwards, supporting compression, and with the posterior side facing downwards, supporting tension, according to DINIZ et al. (2005): such bones, when mechanically stretched, develop electrical charges, which tend to be negative in the compressed regions (which should be concave, with a predominance of osteoclastic activity) and positive in the extended regions (which should be convex, with a predominance of osteoblastic activity). In this sense, HUANG et al. (2003) state that it is a matter of bone stability to the mechanical apparatus, and DINIZ et al. (2005) associate the force applied to the anterior surface to the modification of the bone structure by apposition in its concavity and by resorption in its convexity, since the internal bone lesions produced by external mechanical force make the bone reacts with a rapid and massive hypertrophy, which

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this study has shown the contribution of *Tribulus terrestrial* (TT) in a more assertive way with the increase in the thickness and area of the analyzed femurs.

It is known that bone diseases constitute an important public health problem, which affects any age group and gender, and one assumes that in coming years their incidence will continue to rise globally, in view of the increase in life expectancy of the population (FIDELIS, 2020; PARK et al, 2017). In this sense, the life's quality of sick individuals can be affected and, consequently, their daily life activities can be compromised.

In this context, the use of the herb *Tribulus terrestris* as a supplement has been associated with several benefits for human health, including the bone protective effect, which may possibly provide a reversal of the progression and the consequent negative effects of bone diseases, such as osteoporosis.

It is well established in the scientific literature that bone loss accelerates in hypogonadal states, female menopause being a classic example. In a review, GOLDS, HOUDEK, and ARNASON (2017) studied the consequences of adult male hypogonadism on bone metabolism. They concluded that testosterone signaling stimulates osteoblasts to form trabecular bone and helps osteocytes to prevent trabecular bone loss, being a key hormone in bone health by reducing the risk of fractures and potentially contributing to increased bone mineral density.

Regarding this, the androgenicity and potential abilities of *Tribulus terrestris* to increase testosterone levels have been subject of research. In their review of clinical trials with male athletes using *Tribulus terrestris*, PORKRYWKA et al (2014) found that in humans, when used alone, without additional components, *Tribulus terrestris* extract does not improve androgenic status or physical performance. among athletes. Despite this, they found that the combination of the herb with other pharmacological components increases testosterone levels; however, the authors did not reach the conclusion which components of the mixture contributed to this effect, which may serve as a topic for future research. The aforementioned study then pointed out that the Australian Institute of Sport does not recommend the use of *Tribulus terrestris* by athletes and that the published data on the herb do not provide strong evidence of its

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usefulness or safe use in sport, which raises a warning about the need of broader scientific research that addresses the subject to ensure greater safety in recommending the use of the plant.

In the scientific literature review made by SANTOS, HOWELL and TEIXEIRA (2019), the effectiveness of *Tribulus terrestris* and other herbal medicines in increasing serum testosterone levels, improving sperm quality and prostate parameters in adult men was evaluated. They concluded that the use of the herb, when associated with Peruvian Maca (*Lepidium meyenil*), is not able to alter the serum levels of testosterone in men in a scientifically satisfactory way. Despite this, *Tribulus terrestris* may provide beneficial effects on sperm parameters in men with idiopathic infertility.

In the study performed by MARQUES et al (2019), 48 female rats with ovariectomy-induced bone loss were treated with *Tribulus terrestris*, in dosages of 3, 30 and 300mg [kg x day] for 28 days. Bone densitometry, tibial histology, dehydroepiandrosterone (DHEA), testosterone, renal function and estradiol levels were analyzed. Long-term treatment with 300 mg/kg of the herb significantly raised serum DHEA levels by 40%, without significantly altering estradiol or testosterone levels. There was bone mass gain in all rats treated with *Tribulus terrestris*, but those treated with 30g and 300g had bone mass values comparable to normality. In addition, there was lower renal Ca^{++} excretion in this same group. The authors concluded by correlating bone mass gain with the increase in DHEA, that the reduction in DHEA levels is correlated with changes that occur in post-menopause, such as lower muscle and bone mass, greater accumulation of body fat and diabetes mellitus.

Therefore, experimental analyses of the use of *Tribulus terrestris* as a bone protective herbal medicine have shown diverging results which require the performance of new studies, such as this one, to increase the reproducibility of certain data and provide the publication of less contradictory and safer knowledge for use of this herb by society. Furthermore, as DINIZ et al. (2005) emphasizes, the benefits in the treatment of injuries, diseases or bone deformities are only obtained through the

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association with adequate mechanical stimuli, as in controlled physical activities, as a result of the deposition of bone matter, which allows to conclude once more that the use of *T. terrestris* may not be effective if used in a singular way.

4. CONCLUSION

One concludes that the consumption of *Tribulus terrestris* had positive effects regarding morphological and biomechanical properties of the femurs, both in the increase in thickness and trabecular area and in the increase of the maximum force, displacement, extrinsic stiffness, maximum tension, modulus of elasticity and deformation, suggesting greater bone strength in the group of rats that received the herb.

We emphasize that the literature lacks more scientific data about the androgenic potential of *Tribulus terrestris* and associated substances that improve its effect on bone morphology and biomechanics in the context of clinical medical practice, either in the treatment of bone diseases or in the prevention of bone loss, muscle and bone mass, greater accumulation of body fat and the development or progression of diabetes mellitus, common with aging.

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