Islamic Azad University
Urmia Branch

Journal of Basic and Clinical Veterinary Medicine

2025; 6(1): 35-45

Official Journal of Veterinary Faculty of Islamic Azad University
Urmia Branch

Journal Homepage: https://sanad.iau.ir/journal/jbcvm/

Original Article

Radiological and histopathological study of the effects of Kakuti plant (*Ziziphora tenuior*) on bone defect repair in rabbit animal model

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ARTICLE INFO

Received: 13 October 2024

Accepted: 15 February 2025

DOI: 10.82561/JBCVM.2025.1186687

KEYWORDS:

Bone defect
Histopathology
Kakuti plant (*Ziziphora tenuior*)
Rabbit
Radiology

ABSTRACT

This study aimed to assess the radiological and histopathological effects of the hydroalcoholic extract of the Kakuti (*Ziziphora tenuior*) plant on bone healing in a rabbit model. The current study involved 10 male New Zealand white rabbits, aged 12–18 months with a mean weight of 1.1-1.7 kg. The rabbits were randomly divided into two groups: a control group and a treatment group. The rabbits were anesthetized via intramuscular injection of a mixture containing xylazine and ketamine; then, a 3 mm diameter and depth defect was created in the radius bone. In the sample group (n=5), 1 g of Kakuti extract was placed inside the defect cavity, and the skin and muscles were sutured as usual. Both groups were evaluated radiologically and histopathologically at two- and seven-weeks post-surgery. Results showed no significant difference between the two groups in callus formation, healing, or remodeling on radiographs at these time points ($p> 0.05$). Histopathological findings indicated no significant difference in connective tissue formation between the control and treatment groups at either time point, while bone tissue formation showed a significant difference ($p> 0.05$). These findings suggest that *Ziziphora tenuior* extract accelerates bone healing in the rabbit radius but does not significantly influence cartilage healing.

مطالعه رادیولوژیکی و هیستوپاتولوژیکی اثرات گیاه کاکوتی (*Ziziphora tenuior*) بر ترمیم نقیصه استخوانی در مدل حیوانی خرگوش

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چکیده

هدف از این مطالعه بررسی رادیولوژی و هیستوپاتولوژی اثرات گیاه کاکوتی (*Ziziphora tenuior*) بر ترمیم نقیصه استخوانی در مدل حیوانی خرگوش بود. در این تحقیق ۱۰ سر خرگوش نر سفید نیوزیلندی با میانگین سنی ۱۸-۱۲ ماه و محدوده وزنی ۱/۷-۱/۱ کیلوگرم بطور تصادفی در دو گروه شاهد و تیمار قرار داده شدند. هر یک از خرگوش‌ها با تزریق داخل عضلانی کوکتل زایلازین و کتامین بی هوش شده و سپس نقیصه‌ای به قطر و عمق ۳ میلی‌متر در استخوان زند زبرین آن ها ایجاد گردید. در گروه تیمار (n=۵)، به مقدار ۱ گرم از عصاره کاکوتی در داخل حفره نقیصه قرار داده و پوست و عضلات این قسمت به صورت معمول بخیه زده شد. هر دو گروه در دو و هفت هفته پس از عمل جراحی از نظر رادیولوژیکی و هیستوپاتولوژیکی ارزیابی شدند. نتایج نشان داد که هیچ تفاوت معنی‌داری بین دو گروه در تشکیل کالوس، بهبودی یا بازسازی در رادیوگرافی‌ها در این مقاطع زمانی وجود نداشت ($p> ۰/۰۵$). اما در رابطه با تشکیل بافت استخوره خرگوانی این اختلاف معناداری بود در هفته ششم از نظر میزان تشکیل بافت همبند بین گروه کنترل و تیمار اختلاف معناداری وجود داشت اما در رابطه با میزان تشکیل بافت غضروفی اختلاف معناداری مشاهده نشد ($p< ۰/۰۵$). نتایج این مطالعه نشان می‌دهد که عصاره گیاه کاکوتی (*Ziziphora tenuior*) باعث تسریع در روند ترمیم نقیصه تجربی استخوان زند زبرین خرگوش می شود اما این تسریع روند ترمیمی استخوان چندان معنی‌دار نمی‌باشد.

واژه های کلیدی: نقیصه استخوان، هیستوپاتولوژی، گیاه کاکوتی(*Ziziphora tenuior*)، خرگوش، رادیولوژی

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INTRODUCTION

One important reason for radiographic evaluation of a fracture is to assess its healing [1]. Bone repair occurs primarily (intramembranous) and secondary (endochondral) [2]. In primary repair, undifferentiated mesenchymal cells produced in the periosteum and endosteum transform into bone cells. As ossification matures, callus formation will not occur [3]. Bone fractures usually heal secondarily, in which undifferentiated mesenchymal cells first form cartilage and then bone [4]. In this process, a hemorrhage first occurs at the fracture site, which then turns into a hematoma. The undifferentiated mesenchymal cells are produced from the periosteum and endosteum, and then chondroblasts and chondrocytes are formed. Callus is formed by the differentiation of chondrocytes [5]. Most causes, such as accidents, falls from a height, getting stuck between appliances, and conflicts with other animals, can cause bone fractures in pets [6]. Some diseases, such as osteomyelitis, bone infections, and bone marrow cancer, may predispose the animal to bone fractures [7]. In the normal state, bone healing includes the stages of hematoma, subperiosteal and endosteal cellular proliferation, callus formation, and consolidation [8]. Non-union or malunion is very important because, if the healing process is not properly managed, the bone's shape can become abnormal, and in the future, this can lead to severe consequences such as movement disorders, recurrent fractures, or even failure of bone function in the target area. The process and speed of fracture healing are important because animals quickly recover from pain after surgery and make unexpected movements. They may neglect or overuse their injured limb, which can disrupt fracture healing. Therefore, stimulating osteogenesis with compatible biomaterials to accelerate healing is highly important [9]. Bone fractures in animals are among the most important challenges because their healing takes a long time due to the tissue's hardness, leading to many side effects in the affected organ [10]. One promising treatment approach involves using mineral supplements and vitamins that are not sensitive or toxic to living tissues. Various biomaterials are being investigated to support bone tissue regeneration and promote its recovery. Synthetic or nature-derived materials that provide a foundation for bone regeneration have become valuable and practical. In some studies, reports have been provided on the level of effects of various biomaterials on bone fracture healing. In fact, these biomaterials are biocompatible substances that elicit minimal inflammatory cell responses in the affected area [11]. In the past, medicinal plants were used to treat bone fractures quickly. Nowadays, with advances in science and access to new methods for extracting plant-based active ingredients, a new avenue has been opened for researching and investigating plants and their constituent substances [12]. The Kakuti plant (*Ziziphora tenuior*) belongs to the *Lamiaceae* family and has analgesic and anti-inflammatory medicinal properties [13]. One of the main active chemical compounds in this plant is Pulegone, whose analgesic and anti-inflammatory effects are well-known [14]. Some reports also indicate analgesic effects of *Lamiaceae* plants, attributed to compounds such as carvacrol, flavonoids, and steroids [15]. Also, several reports have documented the involvement of opioidergic and serotonergic systems in the analgesic effects of extracts from these plants [16, 17]. The Kakuti plant shows a wide range of polysaccharide structures. It contains mineral elements such as calcium, potassium, and magnesium, as well as galacturonic acid, lactose, arabinose, and rhamnose [18]. A 2016 study by Mohammadi and colleagues reported that the Kakuti plant was highly effective in skin repair, an effect attributed to its ability to stimulate

myofibroblast contraction in the wound bed [19]. In 2018, Deng & Sun examined the effects of five-finger extract on osteogenic and angiogenic factors and long-bone fracture repair. It has been reported that 2 months of treatment can significantly increase levels of angiogenic factors in treated groups [20]. In another study, Kraujalienė and colleagues (2017) investigated the protective effects of goldenrod plant extract on bone following administration in streptozotocin-induced diabetic rats. The results of this study indicated that a low dose of goldenrod alcohol extract has protective effects on trabecular bones in diabetic rats [21]. Huang and colleagues in 2023 investigated the effect of tea consumption on the risk of osteoporotic fractures in elderly individuals. The results of their study showed that tea, due to its high levels of flavonoids and polyphenols and its abundant antioxidant properties, can reduce the risk of osteoporotic fractures [22]. Upon reviewing previous studies, we found that no research has been conducted on the effects of the plant Kakuti on bone fracture healing. Given the therapeutic effects of the Kakuti plant and its use in traditional medicine to treat bone pain, this study aimed to investigate the radiological and histopathological effects of its hydroalcoholic extract on bone defect healing in a rabbit model.

MATERIALS AND METHODS

Study design and animals

The current study involved 10 adult male New Zealand white rabbits with an average age of 12–18 months and a mean weight of 1.1-1.7 kg. The rabbits were randomly divided into two groups: a control group and a treatment group. Preparation of Kakuti (Ziziphora tenuior) extracts

In July 2023, when *Ziziphora tenuior* was in full bloom, aerial parts were harvested from wild plants growing near Urmia, Iran. Herbaria and voucher samples were created and submitted to the Department of Pharmacognosy at Urmia University of Medical Sciences, Faculty of Pharmacy. Subsequently, 350 mL of 80% aqueous ethanol was used to extract 150 g of air-dried and ground aerial parts of the plant for 24 hours at room temperature (21˚C). The extract was then filtered through Whatman No. 3 paper and concentrated using a rotary evaporator IGA TQ-04 AT (IGA®-Werttke GmbH & Co. KAAG, Germany) to a final weight of 13.5 g. Before use, the samples were kept at 50˚C. Following a pilot study, the range of 30-150 mg/kg was utilized to select extract doses for further investigations. Fractionation was employed to ascertain the composition in grams of the ethanolic extract [18].

Anesthesia and surgery

At the beginning of the study, each rabbit was administered 1 mg/kg of body weight of acepromazine intramuscularly, and then the left forelimb motor function was immobilized from the mid-humerus to the wrist joint. After inducing anesthesia with an intramuscular injection plus of xylazine (8 mg/kg bw) and ketamine (70 mg/kg bw) [23], a skin incision was made on the inner aspect of the left forearm and then soft tissues were dissected and periosteum was separated to create a defect with a diameter and depth of 3 mm in the radius bone using a surgical motor and trephine [24]. In the sample group (n=5), 1 gram of Kakuti extract was placed inside the defect cavity [25] and in the control group, physiological saline with the same dose volume as the Kakuti extract was applied to the defect site, then the soft tissue was closed over the defect and the skin was sutured with a simple interrupted pattern with

Nylon 4-0 (Figure 1). Following the surgery, sulfazolin antibiotic (25 mg/kg bw) was injected intramuscularly for three days. Both groups of rabbits were evaluated radiologically and histopathologically in the second and seventh weeks after surgery.

Radiographic study

Radiographic evaluations were conducted on days 0, 14, and 49. The score is as follows: 0= observing a complete defect and a lack of radiopacity in the defect region, 1= a slight increase in radiopacity in the defect region and initiating the filling of the defect, 2= the defect region is not clear and an increase in radiopacity, and 3= the defect is completely filled and/or the defect region is similar to adjacent bones. On day 0, and at weeks two and seven after surgery, lateral and craniocaudal radiographs were taken from the left forelimb area of the rabbits (Figure 2). The radiology device used for this purpose was a digital model GXR-SD 152 DDR (Varian N.V. Co., made in the USA). The focus-film distance was set at 100 cm, and the peak kilovoltage and milliamperes per second were set to 42 and 5, respectively. The detector used was a flat panel SCI with dimensions of 24 × 30 centimeters, and the software used for image processing and tissue structure measurement was Varian and DRGEM. Grading was performed based on callus formation, degree of union, and bone remodeling. The modified Lane and Sandhu grading system was used to evaluate and grade the radiographs (Table 1).

Histopathological study

At weeks two and seven post-surgery, three rabbits from each group were euthanized humanely for bone sampling. For histopathological investigation, sections were placed in formalin 10% and then placed in formic acid (as a decalcifying agent) and transferred to the histology lab, where the samples were cut. They were embedded in paraffin and sectioned. They were prepared at a thickness of 5 µm, deparaffinized, and stained with hematoxylin–eosin. The sections were investigated by a light microscope.

Statistical analysis

A confidence interval (CI) was used to estimate the size of the callus. Data were analyzed using a t-test in SPSS version 21. All values were expressed as the mean and standard deviation (Mean ± SD), and the value of $p \leq 0.05$ was used as statistical significance. All data presented are three replicates.

RESULTS

Radiography

Radiographic evaluation of the radius bone-healing process in the study rabbits was performed on day 0 and at weeks 2 and 7 after surgery. According to Table 2, there was a significant difference between the control and treatment groups in terms of callus formation, fusion, and remodeling in the second and seventh weeks ($p > 0.05$) (Figures 3 and 4).

Histopathology

According to Table 3, there was no significant difference in connective tissue formation between the control and treatment groups in the second week, whereas bone tissue formation showed a significant difference (p> 0.05). In the seventh week, there was a significant difference between the control and treatment groups in connective tissue formation, but no significant difference in cartilage formation (p> 0.05). This week, bone tissue was formed in the samples, and bone plates were also visible in the tissue (Table 3) (Figure 5). According to Table 3, there was no significant difference in connective tissue formation between the control and treatment groups in the second week, whereas bone tissue formation showed a significant difference (p> 0.05). In the seventh week, there was a significant difference between the control and treatment groups in connective tissue formation, but no significant difference in cartilage formation (p>0.05). This week, bone tissue was formed in the samples, and bone plates were also visible in the tissue (Table 3) (Figure 5).

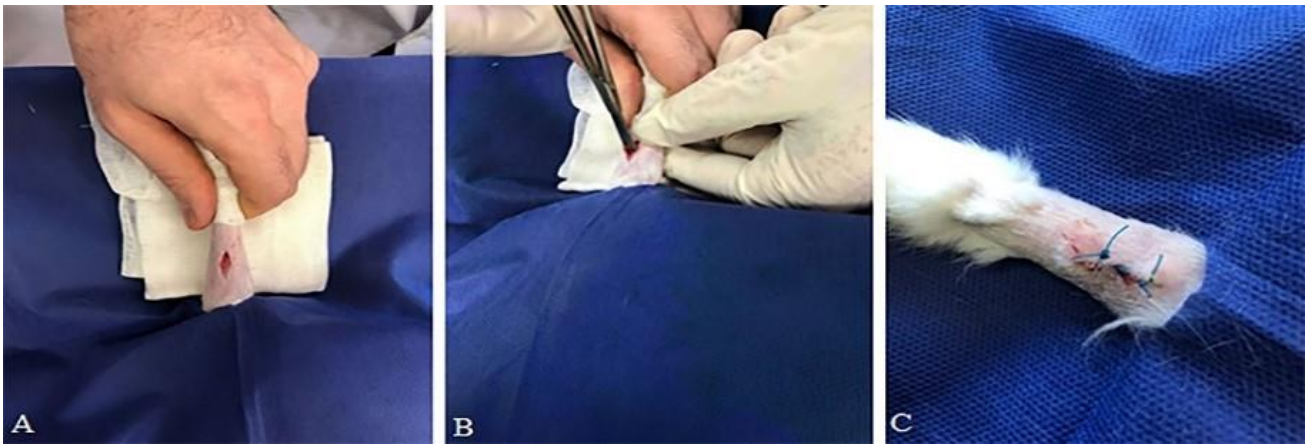


Figure 1: Creation of an experimental defect in the rabbit radius bone. A) Cutting the skin and muscles. B) Creating a defect in the radius bone. C. Suturing.

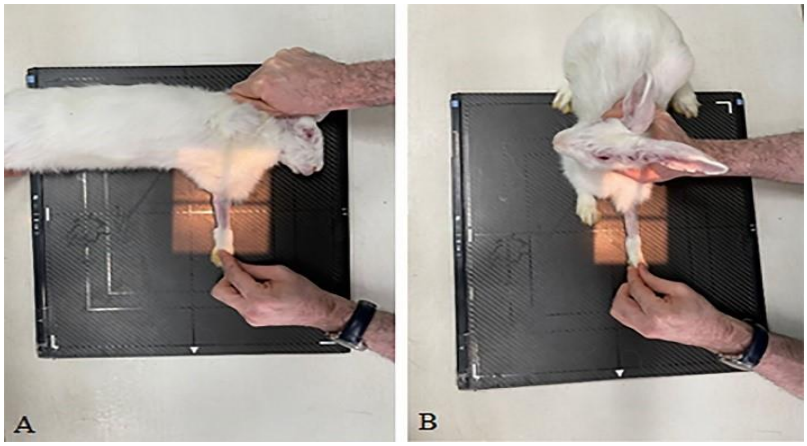


Figure 2: Lateral (A) and Craniocaudal (B) radiographs of the left forearm of a rabbit.

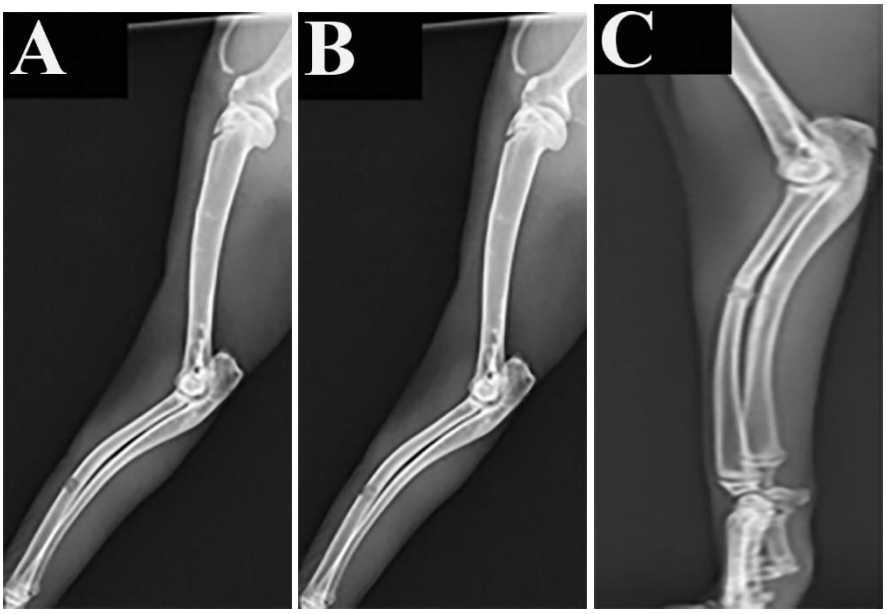
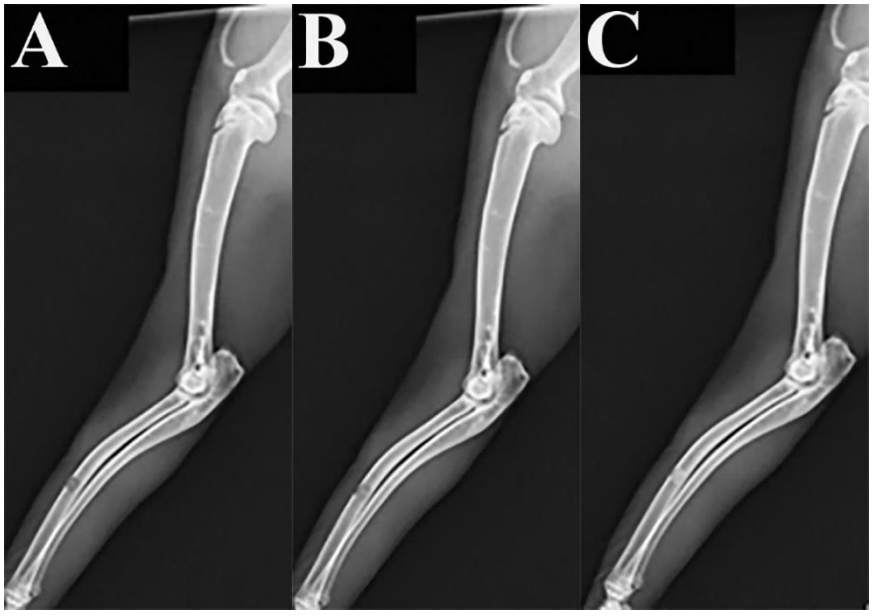


Figure 3: Radiograph of the control group. A) 0 day, B) second week, and C) seventh week.



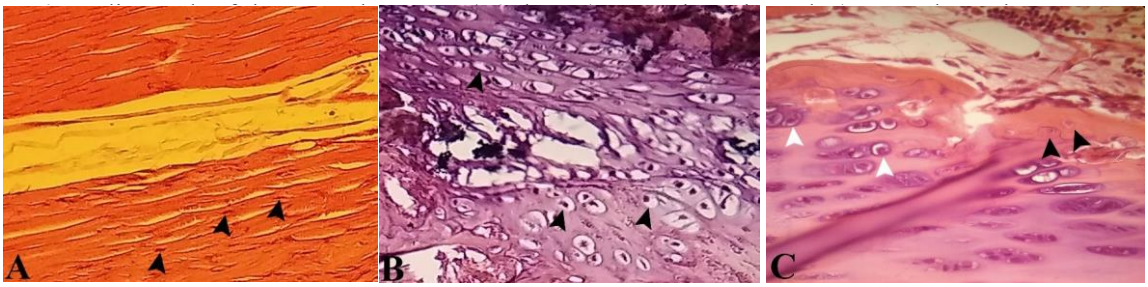


Figure 5: Histopathology of bone healing. A) Transverse section of the bone defect in the first week; Fibrous tissue (collagen material) is visible. Arrows indicate fibroblasts. B) Second week; Cartilage tissue has filled the entire lesion. Arrows indicate cartilage (chondrocytes). C) Seventh week; There is a lot of cartilage tissue and a small amount of bone tissue. Black arrows indicate bone tissue (osteocytes) and white arrows indicate cartilage tissue (chondrocytes). (hematoxylin-eosin staining, magnification x100).

Table 1: Scoring for radiographs obtained using the Lane and Sandhu method

Grade	Bone formation
0	There is no sign of bone formation
1	Bone formation and filling of 25% defect
2	Bone formation and filling of 50% defect
3	Bone formation and filling of 75% defect
4	Bone formation and filling of 100% defect
	Union
0	Non union
1	Possibility of union
2	Complete union
	Remodeling
0	There is no sign of bone remodeling
1	There is weak sign of bone remodeling
2	Complete remodeling

Table 2: Comparisons of radiographic evaluation of the variables studied between groups on different days

Qualitative variables	New bone formation			Union			Remodeling		
	Control	Treatment	P value	Control	Treatment	P value	Control	Treatment	P value
Second week	0	1	0. 001	0	0	1.000	0	0	0.001
Seventh week	3	4	0.001	1	2	1.000	1	2	0.001

p< 0.05 is significant.

Table 3: Comparisons of histopathological evaluation of the studied variables between groups in the second and seventh weeks

Qualitative variables	Fibroplasia			Chondroplasia			Osteoplasia		
	Control	Treatment	P value	Control	Treatment	P value	Control	Treatment	P value
Second week	2(2-1)	3(3-2)	0. 990	2(2-1)	2(2-1)	1.000	0(0-0)	2(2-1)	0.430

p< 0.05 is significant.

DISCUSSION

This study aimed to evaluate the radiographic and histological effects of Kakuti (*Ziziphora tenuior*) plant on the repair of radial bone defects in a rabbit model. Results showed that Kakuti administration significantly accelerated bone healing compared with the control group. Extensive research has been conducted on bone grafts in order to reduce the issues associated with auto grafts, such as postoperative pain and high surgical costs, and to reduce the risk of bleeding and ultimately patient mortality [26]. The aim of this study was to investigate the histopathology and radiography of new bone formation in a defect created in the rabbit radius bone. The lack of clinical reactions and no infections at the site indicated good biocompatibility of Kakuti bone healing. Availability is also an important factor in selecting a substitute material for bone tissue since Kakuti is abundantly found in nature. Another advantage was its compatibility with imaging systems; its lack of systemic effects, such as allergic reactions during surgery and maintenance, was another. From the researchers' perspective, bone graft substitutes should possess certain characteristics, such as predictable absorption time, good efficacy for surgeons, no interference with new imaging systems, an affordable price, resistance to internal and external heat, and the ability to induce osteogenesis [27]. Elbahnasawy and colleagues in 2019 investigated the effect of thyme plant extract on osteogenic factor I and angiogenic factor I, as well as bone fracture healing in rat tibia. The results showed that receiving this extract for three months had a positive effect on the healing process [28]. In

another study, Pouille and colleagues reported in 2020 that dandelion root extract improved bone strength in mice. *Dandelion* root extract at a dose of 5 milligrams per kilogram of body weight in treated mice had protective effects on the diaphysis and epiphysis of long bones and may increase angiogenic factor 1 in the treatment group. In fact, this study's detailed results indicate intramembranous bone formation within the defect created in the compact tibial bone of laboratory mice [29]. Rashidi and colleagues in 2024 examined the effects of ethanol extract from barberry roots on the proliferation and differentiation of human mesenchymal stem cells into osteoblasts. The results showed positive effects on proliferation and bone differentiation [30]. In another study, Jiang and colleagues in 2015 investigated the effect of calcitonin hormone on bone healing in Indian Hedgehogs. They reported a significant difference between the control group and the experimental group receiving calcitonin, and that calcitonin hormone was helpful in the formation of callus during the initial stages of bone healing [31]. Gao and colleagues (2021) examined the effect of Shahi on tibial bone fractures in desert rats, and their results after 8 weeks indicated that this plant had beneficial effects on fractures. A study was also conducted by Bernela and colleagues in 2016, comparing the analgesic effects of katira gum with those of morphine and diclofenac in laboratory white mice using the tail-flick and hot-plate tests. The results of this study showed that in the tail-flick test, doses of 125, 250, and 500 µg/kg of katira gum decreased the number of abdominal contractions and increased the pain inhibition percentage compared to the control group [32]. In the hot plate test, only at the dosage levels of 125 µg/kg and 500 µg/kg, katira gum reduced pain after injection within the first 15 minutes. At this time point post-injection, katira gum at a dosage level of 125 µg/kg had a significant difference compared to control groups as well as diclofenac at a dosage level 30 mg/kg and morphine at a dosage level 2 mg/kg Gao and colleagues in 2021 examined the effect of Shahi on tibial bone fracture in desert rats, and their research results after 8 weeks indicated beneficial and positive effects of this plant on fracture [33]. A study was also conducted by Bernela and colleagues in 2016, comparing the analgesic effects of katira gum with those of morphine and diclofenac in laboratory white mice using the tail-flick and hot-plate tests. The results of this study showed that in the tail-flick test, doses of 125, 250, and 500 µg/kg of katira gum decreased the number of abdominal contractions and increased the pain inhibition percentage compared to the control group. In the hot plate test, only at the dosage levels of 125 µg/kg and 500 µg/kg, katira gum reduced pain after injection within the first 15 minutes. At this time point post-injection, katira gum at a dosage level of 125 µg/kg showed a significant difference compared to the control groups, as well as diclofenac at a dosage level of mg/kg30 and morphine at a dosage level of mg/kg2 ($p > 0.01$) (24). A study was also conducted by Bernela and colleagues in 2016, comparing the analgesic effects of katira gum with those of morphine and diclofenac in laboratory white mice using the tail-flick and hot-plate tests. The results of this study showed that in the tail-flick test, doses of 125, 250, and 500 µg/kg of katira gum decreased the number of abdominal contractions and increased the pain inhibition percentage compared to the control group [32]. In the hot plate test, only at the dosage levels of 125 µg/kg and 500 µg/kg, katira gum reduced pain after injection within the first 15 minutes. At this time point post-injection, katira gum at a dosage level of 125 µg/kg had a significant difference compared to control groups as well as diclofenac at a dosage level 30 mg/kg and morphine at a dosage level 2 mg/kg Gao and colleagues in 2021 examined the effect of Shahi on tibial bone fracture in desert rats, and their

research results after 8 weeks indicated beneficial and positive effects of this plant on fracture [33]. A study was also conducted by Bernela and colleagues in 2016, comparing the analgesic effects of katira gum with those of morphine and diclofenac in laboratory white mice using the tail-flick and hot-plate tests. The results of this study showed that in the tail-flick test, doses of 125, 250, and 500 µg/kg of katira gum decreased the number of abdominal contractions and increased the pain inhibition percentage compared to the control group. In the hot plate test, only at the dosage levels of 125 µg/kg and 500 µg/kg, katira gum reduced pain after injection within the first 15 minutes. At this time point post-injection, katira gum at a dosage level of 125 µg/kg showed a significant difference compared to the control groups, as well as diclofenac at a dosage level of 30 mg/kg and morphine at a dosage level of 2 mg/kg ($p > 0.01$) [24]. In our study, in the Kakuti extract treatment group, after 6 weeks, mature bone trabeculae filled the created defect. Comparative evaluation of radiographic variables between groups at the second and seventh weeks showed no significant differences in bone remodeling, fusion, or post-surgical remodeling between the control and treatment groups. However, fusion and bone formation were higher compared to the second week.

CONCLUSION

The results of our study indicate that Kakuti plant extract (*Ziziphora tenuior*) accelerates the healing of an experimental radial bone defect in rabbits, but this acceleration is not statistically significant.

ETHICS

Approved.

CONFLICT OF INTEREST

None.

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