Full Length

EFFECTIVENESS OF *CISSUS QUADRANGULARIS* IN ENHANCING THE BONE HEALING PROCESS IN WISTAR RATS

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ABSTRACT

Bone fractures pose significant health concerns in both humans and animals, necessitating effective strategies for accelerated healing. This study is aimed to evaluate the bone healing potential of Cissus quadrangularis (CQ) in Wistar rats. A total of 24 rats were randomly assigned into four groups (n=6 each). Group I served as healthy control, group II as fractured untreated control, group III received oral CQ treatment (400 mg/kg body weight daily for 30 days), and group IV received topical CQ application at the fracture site for the same duration. Biochemical and radiological assessments were conducted to monitor healing progress. Results demonstrated significant ($p<0.05$) improvements in biochemical markers of bone healing in treated groups compared to the untreated control. Notably, oral administration of CQ in group III showed the most rapid and effective fracture healing, while topical application in group IV also yielded beneficial effects, albeit slightly delayed. In conclusion, Cissus quadrangularis exhibits promising bone-healing properties in Wistar rats, with oral administration proving more effective than topical application. These findings support further investigation into CQ as a natural therapeutic agent for fracture management.

Key words: *Cissus quadrangularis*, fracture healing,Wistar rats & bone regeneration

Received :26.06.2025 Revised : 11.09.2025 Accepted : 12.09.2025

INTRODUCTION

The rat (*Rattus norvegicus*) is a widely used model organism in biomedical research due to its physiological, genetic, and anatomical similarities to humans. Rats have been instrumental in research areas such as toxicology, pharmacology, neuroscience, and more recently, regenerative medicine and orthopedics (Chen *et al*., 2019). *Cissus quadrangularis*, also known as 'Hadjod' in the local language meaning 'fractured bone healer', is one of the most important species of plants scattered all over India, especially in tropical regions (Bakshi *et al*., 2001). *Cissus quadrangularis* belongs to the family Vitaceae, which is a perennial plant commonly known as 'Veldgrap' or 'Devil's

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backbone' (Kumbhojkar *et al*., 1991). It has healing properties in tendons and ligaments (Justin and Joseph, 2011). Phytochemical studies have revealed the presence of carotene, phytosterol, terpenoids, β-sitosterol, δ-amyrin, δ-amyrone, and calcium in *Cissus quadrangularis* (Mishra *et al*., 2011). The stem of *Cissus quadrangularis* is an important medicinal plant in Ayurveda and is used as an alternative to anthelmintics, dyspeptic, digestive tonic, analgesic in eye and ear diseases, treatment for irregular menstruation and asthma, and in complaints of the back and spine (Sen *et al*., 1966). The plant is also used for treatment as a carminative, anthelmintic, and poultice for bone setting (Kumar *et al*., 2005). The paste of stem is used in the treatment of asthma, burns and wounds, bites of poisonous insects, and for saddle sores of horses and camels (Paulsen *et al*., 2007). The World Health Organization (WHO) has recommended member countries to actively promote native medicines and take steps to conserve and cultivate medicinal plants. Presently, focus on plant research has increased globally and a large body of evidence supports its immense potential in traditional medicine. Medicinal herbs are in high demand due to their widespread use and less adverse effects (Mate *et al*., 2008). Ancient Ayurvedic texts reported *Cissus quadrangularis* as a general tonic with analgesic and specific bone fracture healing properties. Anabolic and androgenic compounds are well known to antagonize glucocorticoid receptors and promote bone growth and fracture healing (Siddiqua and Mittapally, 2017).*Cissus quadrangularis* accelerates bone remodeling and increases bone tensile strength. Vitamins and steroids found in this plant influence bone fracture healing, promoting early regeneration of connective tissues and quicker callus mineralization (Mishra *et al*., 2010). Paste of crushed stem or alcoholic extracts applied locally or administered intramuscularly facilitate rapid healing of fractured bones in albino rats, mice, and dogs (Udupa and Prasad, 1964). Despite these promising reports,systematic in vivo validation of *Cissus quadrangularis* in standard bone fracture model remains limited. The precise mechanisms through which its phytoconstituents influence bone remodeling and mineralization are not fully understood and there is insufficient comparative evidence regarding different forms of administration. In light of the World Health Organization's recommendation to promote and scientifically evaluate medicinal plants, further research is required to substantiate traditional claims with modern biomedical evidence. Therefore, keeping in view of the above facts, the studies on healing of long bonefractures in Wistar rats with the use of osteoinducers was undertaken with theobjectives to study the efficacy of *Cissus quandrangularis* extract as an osteoinducer in the process of bone healing (Subhashri *et al*., 2013).

MATERIALS AND METHODS

Animal model and housing conditions

A total of 24 healthy female Wistar rats were selected and randomly allocated into four experimental groups, with six animals in each group (n = 6). The rats were maintained under standard laboratory

conditions temperature (22 ± 2°C), relative humidity (50–60%) and a 12h light/dark cycle. Standard rodent chow and water were provided ad -libitum throughout the study.

The group size (n = 6) was determined using the resource equation method where, E = Total number of animals − Total number of groups. In the present study, with 4 groups × 6 rats = 24 animals and 4 groups. which falls within the recommended range of 10–20 for exploratory animal experiments. This group size is consistent with similar experimental designs and was selected in accordance with ethical guidelines to minimize animal use while ensuring statistical validity.

Approval of animals from IAEC

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) (vide No. IAEC/VCA/Durg dated 06.03.2019) of College of Veterinary Science & Animal Husbandry, Anjora, Durg, India. Experiments followed the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India. Proper care and management of animals were ensured throughout the study.

Extraction procedure for *Cissus quadrangularis*

Fresh stems of *Cissus quadrangularis* were procured from the local market (Fig.1) and cleaned to remove any extraneous materials and cut into small pieces. The pieces were then shade-dried and ground into a fine powder using an electric mixer. (Fig.2). A 100 g portion of the powdered material was placed in a thimble and loaded into each Soxhlet apparatus. A methanol-water solution in a 7:3 ratio was added in the required quantity. The heater plate was connected to an electrical supply, and the extraction process was monitored (Fig.3). After 24-48 hours, the final filtrate was collected and poured into a tray for methanol evaporation. The tray was placed on a hot water bath at 40°C to facilitate evaporation. Finally, the tray containing the semi-solid extract was weighed to determine the exact yield.

Preparation of working solution

The required amount of extract of Cissus quadrangularis was measured as per the dose/ kg body weight (400mg/kg b.wt) in rats and daily fresh solution was prepared using distilled water.

Determination of LD50 toxicity

The acute toxicity of Cissus quadrangularis was evaluated by determining the median lethal dose (LD_{50}), as per OECD guideline 423 (Jonsson *et al.*, 2013) which represents the dose expected to be fatal in 50% of experimental animals. The study was performed in Wistar rats following standard guidelines. A limit dose of 2000 mg/kg body weight of the methanolic extract of *Cissus quadrangularis* was administered orally to five rats, and the animals were observed for behavioral changes, signs of toxicity, and mortality for a period of five days. If 50% of rats do not die, then 1/5th of limit dose would be used as therapeutic dose. In the

present study, no rats were affected as per the trial dose and its 1/5th limit dose was used for treatment purpose.

Preparation of ointment

The simple ointment base was prepared in accordance with the method prescribed in the Indian Pharmacopoeia using wool fat (0.5 g), hard paraffin (1 g), and white/yellow soft paraffin (8.5 g) to obtain 10 g of base. For the formulation of *Cissus quadrangularis* ointment, 50 g of preparation was obtained by incorporating 5 g of *Cissus quadrangularis* extract into 45 g of the simple ointment base. The extract was accurately weighed and transferred onto an ointment slab, where it was triturated with the base using an ointment spatula until a uniform and homogeneous mixture was achieved.

Preparation of animals and tibial fracture procedure

Twenty-four female Wistar rats (150–200 g) were acclimatized, clinically evaluated, and underwent baseline tibial radiography. Anesthesia was induced via intramuscular injection of Xylazine (20 mg/kgb.wt.) and Ketamine (50 mg/kgb.wt.) for a 200 g rat, this corresponded to 4 mg Xylazine (0.2 mL of 20 mg/mL stock) and 10 mg Ketamine (0.1 mL of 100 mg/mL stock), giving a total volume of 0.3 mL, which was split between both quadriceps to avoid exceeding the recommended site volume (0.2 mL) and prevent sciatic nerve injury. Anesthesia allowed proper positioning for radiography and surgical procedures. The operative site was shaved and disinfected with Betadine, and a controlled tibial fracture was created using a 2.7 mm drill bit (Song *et al.*, 2014). The drill was guided carefully along the tibial shaft to produce a standardized transverse fracture with minimal soft tissue disruption the small incision and stability of the fracture allowed the procedure to be completed without suturing. Fracture creation was confirmed radiographically using mediolateral and craniocaudal views with a digital radiography system (small focal spot 0.6 mm, pixel size 100–150 µm, 40–50 kVp, 2–4 mAs, exposure ≤50 ms, source-to-image distance 90–110 cm), while the limb was positioned on foam pads to minimize rotation and motion. Humane endpoints were strictly observed, with daily monitoring for pain, distress, or abnormal behavior; analgesics and supportive care were provided as needed, and any severely affected animal would have been humanely euthanized in accordance with CCSEA guidelines. Feed and water were provided ad-libitum at cage floor level to accommodate mobility limitations, and body weight and feed intake were monitored to ensure general well-being, with no significant changes observed during the study period.

Experimental design and treatment protocol

The study was conducted to evaluate the effects of *Cissus quadrangularis* on bone healing in a bone fracture model. The treatment protocol for each group was as follows:

Group I (healthy control, n=6):

This group includes healthy, unfractured animals that did not receive any treatment. They served as baseline control for comparative analysis.

Group II (untreated control, n=6):

Animals in this group underwent standardized fracture induction, after which the fractured bone was immobilized using a splint. Additionally, daily antiseptic wound dressing was performed for up to 30 days, but no pharmacological treatment was administered.

Group III (oral *Cissus quadrangularis* Treatment, n=6):

The animals in this group underwent fracture induction, followed by fracture immobilization using a splint and daily antiseptic wound dressing. In addition, *Cissus quadrangularis* was administered orally at a dose of 400 mg/kg body weight per day for 30 days to assess its systemic effect on bone healing.

Group IV (topical *Cissus quadrangularis* Treatment, n=6):

Similar to Group III, the animals in this group underwent fracture induction, followed by fracture immobilization using a splint and daily antiseptic wound dressing. However, instead of oral administration, *Cissus quadrangularis* was applied topically at the wound site once daily for 30 days to assess its localized effect on bone healing. After each application, the ointment-covered fracture site with sterile gauze to maintain contact of the ointment with the periosteal and surrounding tissues, prevent contamination, and enhance localized absorption. Daily antiseptic wound care was performed prior to each application to maintain hygiene. The gauze covering was well tolerated, and no adverse effects such as wound irritation or infection were observed during the treatment period.

Parameters Studied

A) Biochemical analysis and statistical evaluation:

Rats were anesthetized by placing them in a transparent induction chamber for light anesthesiawith isoflurane anesthetic for approximately one minute. Blood samples (1 mL) were collected from the retro orbital site of ratson days 0, 7, 14, and 28, and serum was separated for the estimation of biochemical parameters: alkaline phosphatase, serum calcium, and serum phosphorus. These parameters were measured using standard methods with a semi-automated biochemistry analyzer (Diasil-100, Systronics) The collected data were expressed as mean ± standard error and subjected to statistical analysis using analysis of variance (ANOVA) to determine significant differences among groups, following the standard procedures outlined by Snedecor and Cochran (1994).

B) Radiological findings:

Medio-lateral radiograph of the tibia was taken using 100 mA X-ray machine on 0, 14th, and 28th days postoperatively to evaluate the healing process of fractured

bone such as callus formation, filling of the created defect, organization of callus and response of bone to the plant extract *Cissus quandrangularis*.

RESULT AND DISCUSSION

A) Biochemical Analysis and Statistical Evaluation

1) Alkaline Phosphatase (ALP):

Table 1 presents the mean ± standard error (SE) values of alkaline phosphatase (ALP) activity (U/L) at various time intervals across different experimental groups, with a graphical representation in (Fig 5). No significant differences in ALP levels were observed in group I, whereas groups II, III, and IV exhibited a significant increase on day 14 and day 28. The ALP values ranged from 178.81 ± 7.11 to 295.4 ± 3.69 across all four groups at different time points. Alkaline phosphatase plays a crucial role in bone formation and fracture healing. Secreted by osteoblasts, the enzyme enhances mineralization by increasing the local concentration of inorganic phosphate or by activating collagen fibers to promote calcium salt deposition at the fracture site. *Cissus quadrangularis* has been reported to elevate ALP levels during fracture healing in dogs (Mishra *et al*., 2010). In the present study, a significant rise in ALP during the early postoperative period could be attributed to adrenal hyperfunction caused by stress, trauma to skin and muscle, and increased osteogenic activity and calcium salt deposition at the fracture site, as noted by Pardeshi and Ranganath (2009). Similarly, (Mahendra *et al*., 2007) observed a significant increase in serum ALP activity throughout the observation period in dogs with femoral fractures repaired using polymethyl methacrylate.

2) Serum calcium:

Table 1 presents the mean ± standard error (SE) values of serum calcium (mg/dL) at various time intervals across different experimental groups, with a graphical representation in (Fig 6). There was no significant difference in serum calcium levels between groups I and II, while significant differences were observed between groups III and IV at various time points. The values ranged from 8.66 ± 0.19 to 12.09 ± 0.78 across all four groups over the time. In this study, non-significant fluctuations in mean serum calcium levels were observed on days 7, 14, and 28 in group II. In contrast, group III and IV showed a significant increase in serum calcium on the 7th post-operative day, followed by a non-significant decrease on days 14 and 28. The initial rise in serum calcium levels, followed by a decline in groups III and IV, treated with *Cissus quadrangularis*, may be attributed to faster fracture healing with increased calcium mobilization for the formation of bridging callus. Similar results were reported by Maiti *et al.* (1999), who found that *Cissus quadrangularis* accelerated the healing process in experimentally fractured radius-ulna in dogs.

3) Serum phosphorus:

Table 1 presents the mean ± standard error (SE) values of serum phosphorus (mg/dL) at various time intervals across

different experimental groups, with a graphical representation in (Fig.7). There was no significant difference in serum phosphorus levels between groups I and II, whereas groups III and IV showed significant differences at various time points. The values ranged from 12.72 ± 0.29 to 15.31 ± 0.58 across all four groups. In this study, group II exhibited a non-significant increase in serum phosphorus on days 7 and 14, followed by a decline on day 28 post-treatment. In contrast, groups III and IV showed a significant increase in serum phosphorus on days 7, 14, and 28 post treatments. This rise in serum phosphorus could be attributed to osteoclastic activity, leading to the reabsorption of dead bones which in turn resulted in elevated serum phosphorus levels. These findings are consistent with those reported by Pandey and Udapa (1981) in dogs, Rani and Ganesh (2003) in goats who has also observed higher serum phosphorus levels in the early stages of fracture healing.

B) Radiological findings

Radiological examination of the fractured bone in both control and treatment group animals was conducted to assess the rate and extent of healing. Radiographs were obtained at designated intervals of 0, 14, and 28 days throughout the observation period in control (Fig.8) as well as treatment groups (Fig 9, 10 and 11). The rats treated with *Cissus quandrangularis* presented a remarkable increase in the rate of healing in bone fracture with decrease in pain and swelling as compared to group II (untreated control). However, in group II (untreated control), the radiograph obtained on 28th day showed persistence of slight fracture (radiolucent area) i.e. incomplete bridging of fracture gap. In group II, radiographs obtained immediately after creating fracture showed a 2 mm radiolucent area at the mid tibial region. There was also very mild degree of osteogenesis, and obliteration of fracture gaps by distinct callus formation at 14days of radiological examination as seen in (Fig.9), whereas, in group III (oral administration), the fracture gap was completely reduced suggesting quick and early healing as seen in (Fig.10). Similarly, in rats of group IV (topical application), small radiolucent area was still evident, i.e. incomplete bridging of fracture gap at 14 days of radiograph which was completely healed by 28th day of observation as seen in (Fig.11). In the entire fracture groups, i.e. group II, III and IV, the fracture was supported with small rectangular piece of x-ray film which served as splint. Nutrition supply is an essential aspect for bone healing, and the mineral calcium helps in healing of bone. The rate of new bone formation does not improve by increased intake of calcium alone. The ability for absorption and utilization of calcium should be improved for hastening fracture healing. Phytochemical studies of *Cissus quandrangularis* have shown the presence of various versatile constituents such as flavanoids, triterpenoids, Vitamin C, stilbene derivatives and phytosterols (Jainu and Devi, 2003).

Cissus quandrangularis acts by the stimulation of metabolism and increased uptake of minerals, calcium, sulphur and strontium by the osteoblast in fracture healing (Mishra *et al.*, 2010). It increases

the rate of bone regeneration and improves blood circulation and nutrient supply to the bones. It preserves bone tissue anabolism and regeneration and promotes osteoblastic proliferation and differentiation (Muthusami *et al*., 2011). In the present study, radiological examination of the treated rats showed good response in terms of healing in group III (oral administration) which was completed at the end of the 14th day. The bone healing in the animals of group IV (topical application) was less effective, although better than the untreated group II. Similar findings were also observed by Singh and Udupa (1962) as they observed that *Cissus quadrangularis* extract initiated the healing process by the 11th day. This may be because of accumulation of a larger quantity of mucopolysaccharide in the first week followed by more rapid fall and its earlier disappearance from the fractured area, and both actions have a beneficial effect on healing of the fractures.

CONCLUSION

The study demonstrated that oral administration of *Cissus quadrangularis* at 400 mg/kg body weight significantly accelerated fracture healing, resulting in complete bridging of the fracture gap as confirmed by radiographic analysis. In contrast, its topical application produced comparatively slower healing. Furthermore, biochemical parameters revealed no significant alterations following oral administration, indicating safety at the tested dose. These findings suggest that *Cissus quadrangularis* possesses potent osteoinductive and anti-inflammatory properties, making it a promising natural therapeutic agent for enhancing bone regeneration

ACKNOWLEDGEMENT

The authors express their sincere gratitude to the Department of Veterinary Surgery & Radiology and Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Anjora, Durg (Chhattisgarh) for providing the necessary facilities and support to carry out this research. We also extend our heartfelt thanks to the technical staff and animal attendants for their assistance in animal handling and care throughout the study. The guidance and support received from the Institutional Animal Ethics Committee (IAEC) are deeply appreciated.

Fig.1. Stems of CQ plant

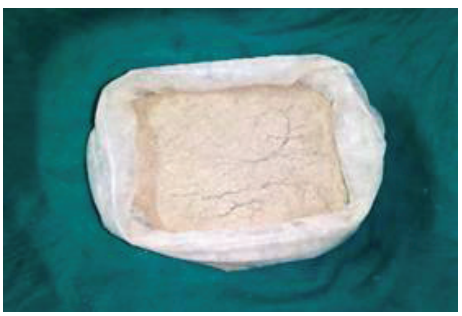


Fig.2. Powder of CQ plant

Fig.3. Soxhlet extraction and powder preparation of CQ

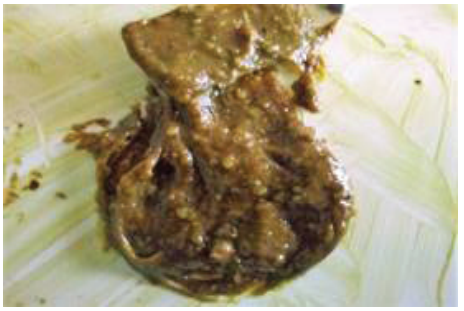


Fig.4. Preparation of CQ ointment

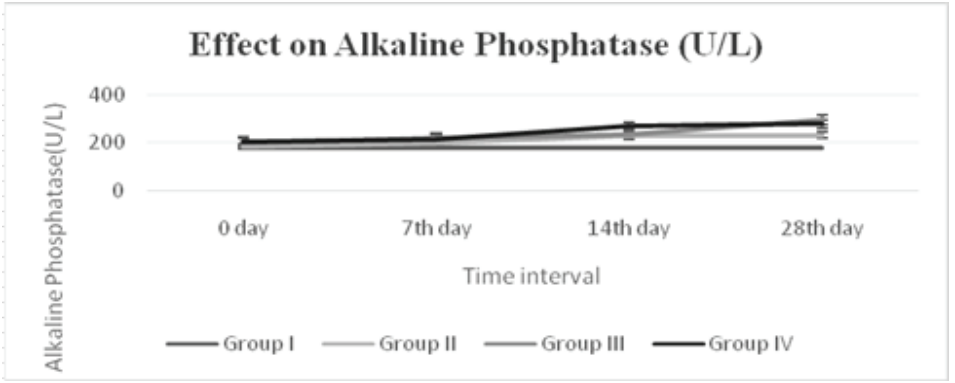


Fig.5. Mean values of Alkaline phosphatase (U/L) at various time interval in different group.

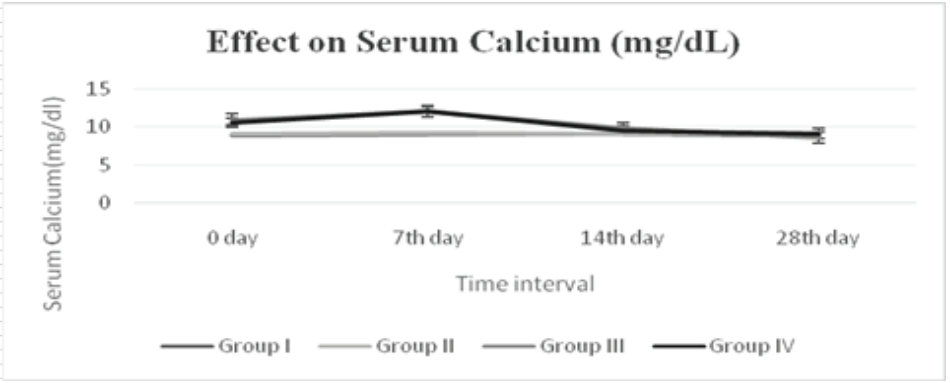


Fig.6. Mean values of Serum calcium (mg/dL) at various time interval in different groups.

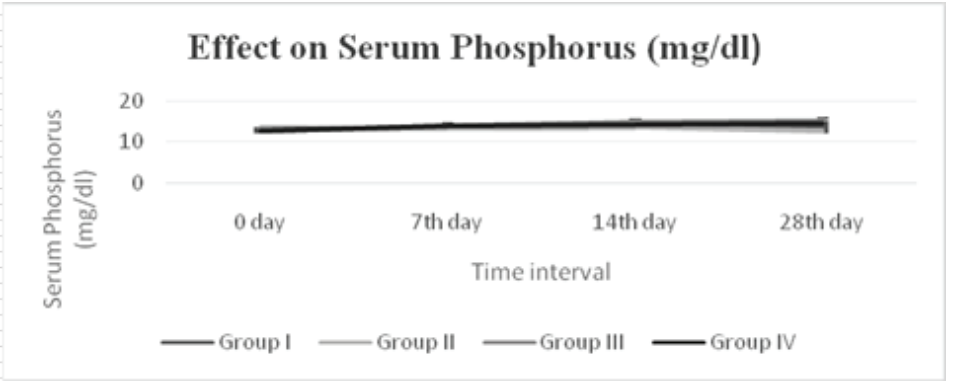


Fig.7. Mean values of Serum phosphorus (mg/dL) at various time interval in different groups.

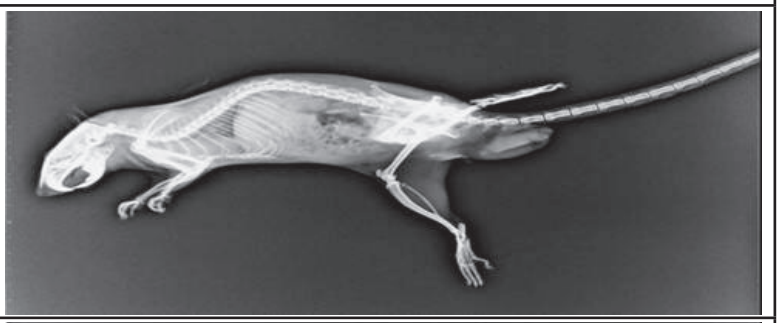
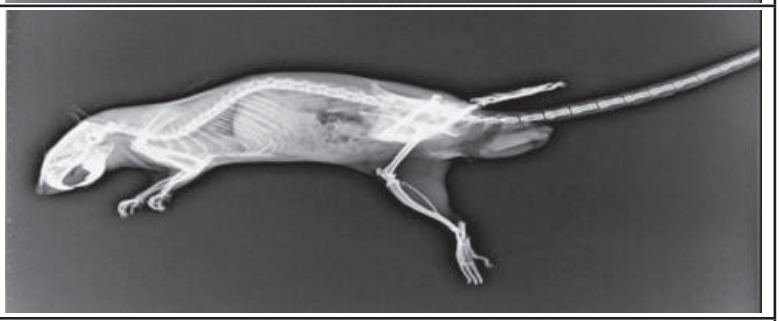
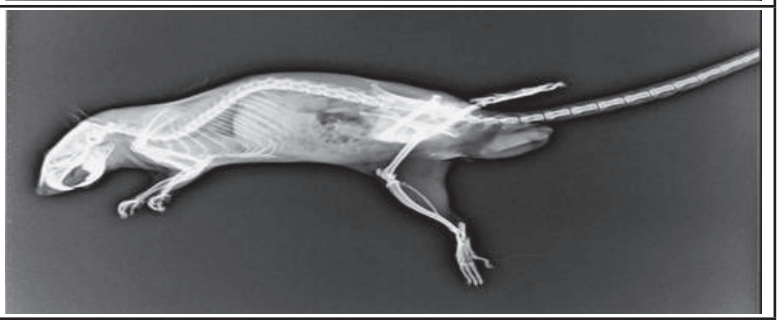
Time Intervals	Radiographs
0 day	
14th day	
28th day	

Fig.8. Radiographical observation of normal bone at various time intervals in group- I.

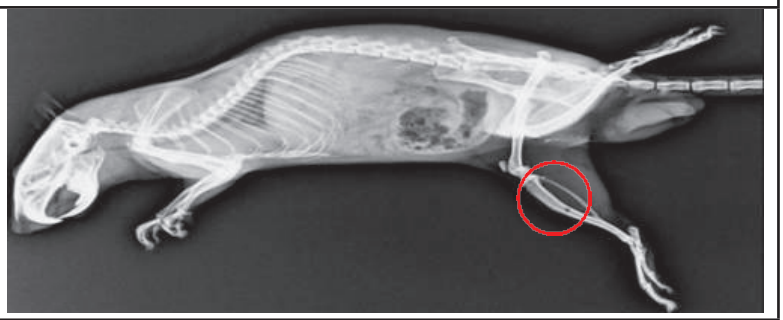
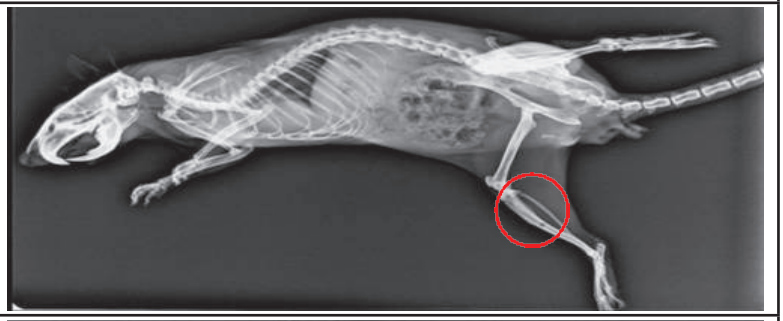
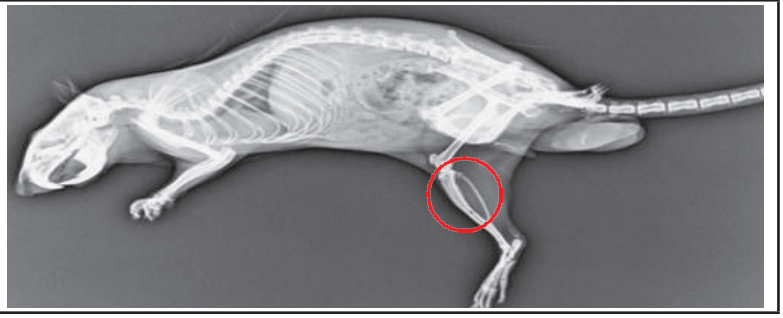
Time Intervals	Radiographs
0 day	
14th day	
28th day	

Fig.9. Radiographical observation (red circle) of fracture healing at various time intervals in group-II.

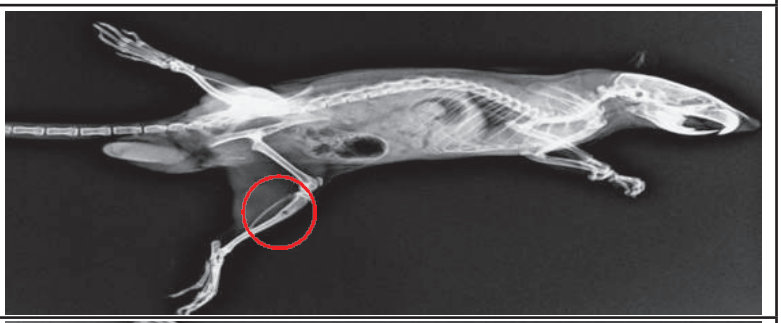
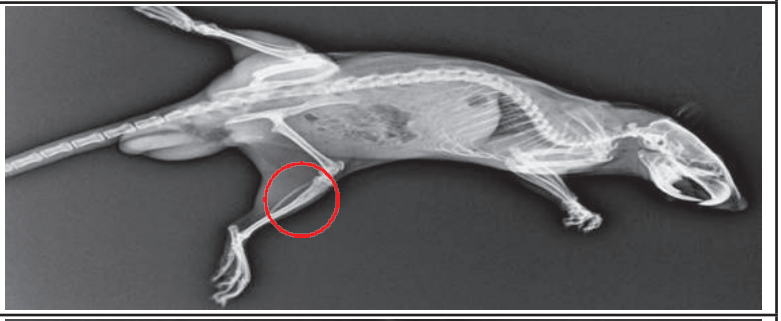
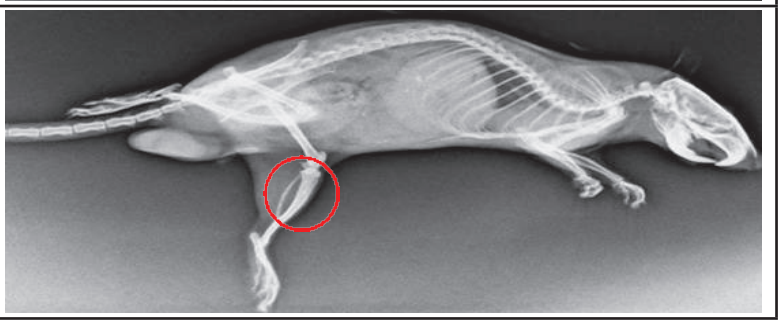
Time Intervals	Radiographs
0 day	
14th day	
28th day	

Fig.10. Radiographical observation (red circle) of fracture healing at various time interval in group-III

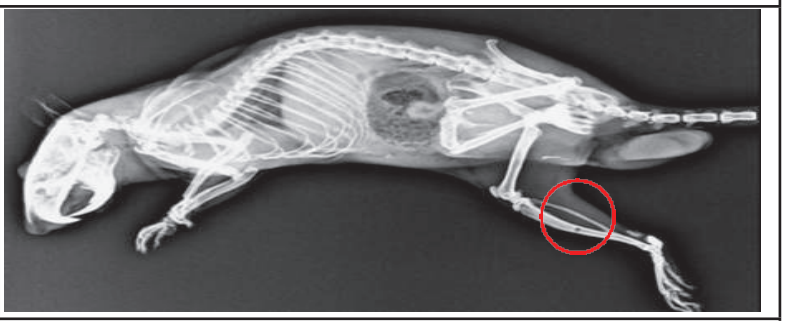
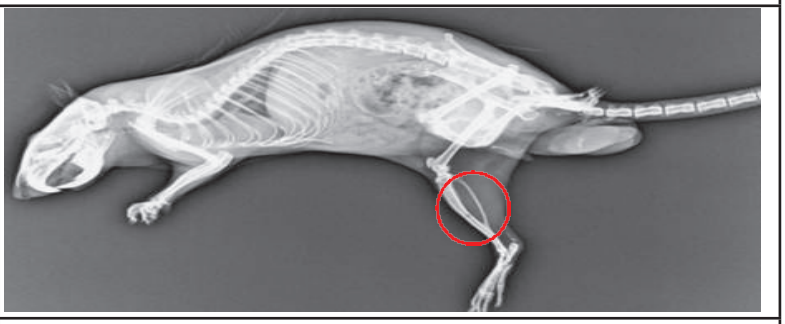
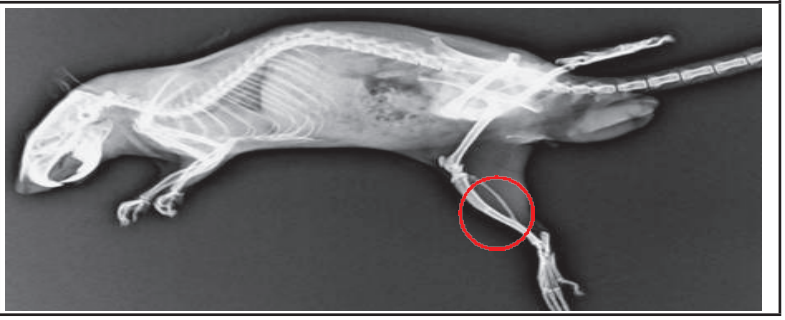
Time Intervals	Radiographs
0 day	
14th day	
28th day	

Fig.11. Radiographical observation (red circle) of fracture healing at various time intervals in group -IV.

Table.1. Summary of Biochemical parameters at various time interval in different groups.

Parameters	Groups	Time interval (days)			
		0 day	7 day	14 day	28day
ALP (U/L)	I	178.81 ± 7.11^{aA}	179.13 ± 9.33^{aA}	180.21 ± 12.20^{aA}	179.36 ± 11.76^{aA}
	II	185.13 ± 17.42^{aA}	198.56 ± 8.17^{aA}	229.1 ± 11.11^{bB}	231.46 ± 9.61^{bB}
	III	201.35 ± 12.59^{aA}	221.46 ± 9.32^{abA}	234.98 ± 8.75^{bB}	295.4 ± 3.69^{cC}
	IV	204.85 ± 13.25^{aA}	213.48 ± 7.42^{aA}	267.65 ± 5.95^{bC}	278.58 ± 6.63^{bC}
Ca (mg/dl)	I	8.90 ± 0.14^{aAB}	8.98 ± 0.11^{aA}	9.01 ± 0.19^{aA}	8.92 ± 0.54^{aA}
	II	9.04 ± 0.19^{aAB}	9.13 ± 0.37^{aA}	9.03 ± 0.10^{aA}	9.18 ± 0.54^{aA}
	III	10.95 ± 0.39^{bB}	12.09 ± 0.78^{dB}	9.89 ± 0.13^{cB}	8.66 ± 0.19^{aA}
	IV	10.53 ± 0.25^{cA}	11.98 ± 0.43^{aB}	9.52 ± 0.13^{bB}	9.13 ± 0.86^{aA}
P (mg/dl)	I	13.29 ± 0.44^{aA}	13.18 ± 0.37^{aA}	13.67 ± 0.55^{bA}	13.52 ± 0.40^{bAB}
	II	13.09 ± 0.29^{aA}	13.33 ± 0.29^{aA}	13.49 ± 0.38^{aA}	12.72 ± 0.29^{aA}
	III	12.84 ± 0.33^{aA}	14.17 ± 0.42^{bB}	14.88 ± 0.23^{bB}	15.31 ± 0.58^{bC}
	IV	12.83 ± 0.29^{aA}	14.00 ± 0.58^{bB}	14.12 ± 0.27^{bAB}	14.46 ± 0.38^{bBC}

Superscripts 'a', 'b', and 'c' indicate the level of significance of values within groups when compared to their respective baseline values. Specifically, mean values carrying the superscript 'a' denote no significant difference from those in Group I and Group II. Mean values with the superscript 'b' indicate no significant difference from those in Group III and Group IV. In contrast, mean values marked with the superscript 'c' reflect a statistically significant difference ($p<0.05$) within the groups. Additionally, superscripts 'A'and 'B' are used to denote values across different time intervals. Superscript 'A' indicates that there is no statistically significant difference across the time intervals in a given group, while superscript 'B' suggests statistically significant difference ($p<0.05$) across the time intervals in a given group.

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