

Effect of Topical Application of Flavanoids Extract of Hibiscus Sabdariffa on Experimentally Induced Bone Defect

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Abstract

Background: The restoration of bone continuity and bone union are complex processes, and their success is determined by the effectiveness of osteosynthesis. The use of plants for healing purposes predates human history and forms the source of current modern medicine

The aim of this study: It was histological evaluation of effect of topical application of flavonoid on healing of induced bone defect in rabbit tibia.

Material and method: Twenty-four Adult New Zealand rabbits used in this study, they were divided into four groups for the healing periods (3days, 1,2, and 4weeks) Two holes were induced in rabbit tibia one on the left side and has been left to heal normally as control. While, the other hole made on the right side filled with flavonoid extract regarded as experimental. Sacrificing of the animals were done according to the healing periods. Routine processing and sectioning technique was performed for histological and histomorphometric evaluation.

Results: Histological findings revealed that progression of bone healing process was accelerated after flavonoid application as shown by examination of histological sections and results of statistical analysis that indicated increase of mean count difference of cells between control and experimental groups obviously noticed at early healing durations. Histomorphometric analysis for all bone parameters examined in this study, showed increase in mean count difference while bone marrow space decreased.

Conclusion: The study revealed that topical application of flavonoid was effective in promoting bone healing process

Key words: Bone defect, flavonoid extract of Hibiscus Sabdariffa. (J Bagh Coll Dentistry 2018; 30(1): 33-38)

INTRODUCTION

Bone defects are a serious illness that can result after a pathological process has destroyed vital components of the bone. Most commonly the causative event is extensive trauma and subsequent infection. This damage to the bone and soft tissues heals slowly and restitution can be only expected after some time of rest and procedures of debridement⁽¹⁾.

Bone defect healing is a process of reconstruction of the bone tissue, which generally undergoes a multidimensional procedure with an overlapping timeline. The vast majority of bone defects can heal spontaneously under suitable physiological environmental conditions due to the regeneration ability of bone. However, the healing process of bone defect is time consuming, and new bone generation takes place slowly due to the size of defects or unstable biomechanical properties, unfavorable wound environment, suboptimal surgical technique, metabolic factors, hormones, nutrition, and applied stress⁽²⁾.

Flavonoids may protect against bone loss by up regulating signaling pathways that promote osteoblast function, by reducing the effects of oxidative stress or chronic low-grade inflammation⁽³⁾.

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MATERIALS AND METHODS

The materials used in the present study were flavonoid extract 2 µml, anesthetic solution (Ketamine hydrochloride 10% Woerden Xylazine 20 mg), formalin 10%, ethanol alcohol 96%, xylol, paraffin wax, and Hematoxylin and Eosin (H&E) stain.

Experimental design:

The total animals (24) were divided into four groups according to each healing interval (3 day, 1, 2, and 4 weeks) six animals for each. A hole induced in left tibia as control was left to heal spontaneously, a second hole induced in right tibia where topical application of flavonoid extract was done as experimental side, the flavonoid extract was applied by pipette.

Surgical procedure:

The surgical procedures were done under general anesthesia drugs by a mixture of ketamine hydrochloride 10% (50 mg/kg B.W) and xylazine 2% at a dose (5 mg/kg body weight) The surgery was performed in a well sterilized conditions and with gentle surgical technique. Sharp blade was used to make a skin incision and tibia was exposed, initial intermittent drilling was done to diameter of about 3mm and depth 3mm, vernier was used to check the depth and diameter of the holes⁽⁴⁾. The animals were sacrificed by an overdose of anesthetic solution at (3 days, 1, 2 and 4 weeks) healing intervals. Removal of skin, fascia and muscles at the operation site was performed; afterwards bone

specimens were prepared by cutting the bone about 5 mm away from operation site with continuous irrigation with saline to avoid bone damage.

The specimens were fixed in 10% formalin for 48h, decalcified with solution of formic acid, then bone tissue dehydrated with alcohol and embedded in paraffin. Sections of 5µm were prepared in the usual fashion, and stained with hematoxylin and Eosin. Histological examination was performed using light microscope. Histomorphometric assessment of bone cells (osteoblasts, osteocytes, and osteoclasts), trabecular area (mm²), trabecular number and bone marrow area (mm²) was performed by image processing software program (ImageJ.exe). Two microphotographs were taken by a camera attached to the microscope at power X4, one in upper part and other picture in lower part to cover approximately all defect area ⁽⁵⁾.

Results

Histological findings

Three days duration

The histological section of the defect area of the control group shows granulation tissue, inflammatory cells infiltration and fat cells (Fig. 1). While histological examination of defect area of 3days duration after flavonoids application shows bone marrow, inflammatory cells and fat cells (Fig. 2 and 3).

One-week duration

Histological examination of defect area after 1 week in control group shows fibroblasts, blood vessel with adjacent inflammatory cells, differentiated osteoblasts at rims of bone matrix enclosing osteocytes (Fig. 4). While, histological examination of defect area after 1 week of flavonoids application shows deposition of bone matrix within marrow tissue rimmed by formative osteoblasts, and enclosing large number of osteocytes (Fig. 5 and 6).

Two weeks duration

Microphotograph view of control group after 2weeks shows new immature bone trabeculae filled with irregularly arranged osteocytes and osteoblasts seen at peripheries of trabeculae, reversal line separating old and new bone, osteoclast resides howship lacuna also illustrated (Fig. 7). While, histological section of flavonoid group after 2weeks shows newly formed bone trabeculae enclosing areas of bone marrow, and including numerous irregularly arranged osteoblast, osteocyte, osteoclast and reversal line between old and new bone (Fig. 8).

Four weeks duration

Histological section of control group after 4weeks shows dense bone trabeculae almost filling defect site with osteocytes arranged around haversian

system, also reversal lines separating old and new bone are detected (Fig. 9 and 10). While, histological section of experimental group after 4weeks shows mature dense bone, osteocytes that are surrounding haversian system lined by osteoblasts, also reversal line is shown (Fig. 11 and 12).

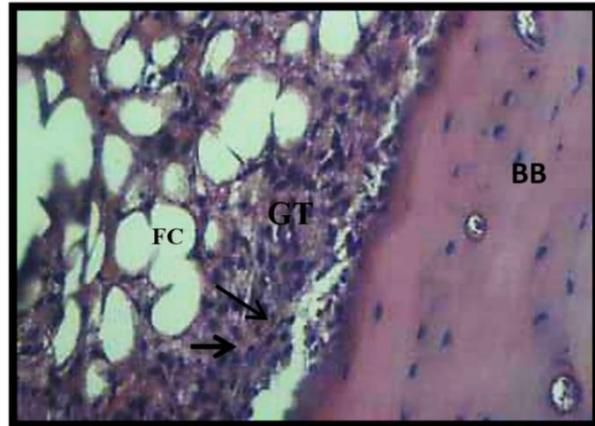


Figure 1: Microphotograph view after 3days of control group shows basal bone(BB), area in filtrate by inflammatory cells (arrows) and fatty cell(FC) H&Ex40

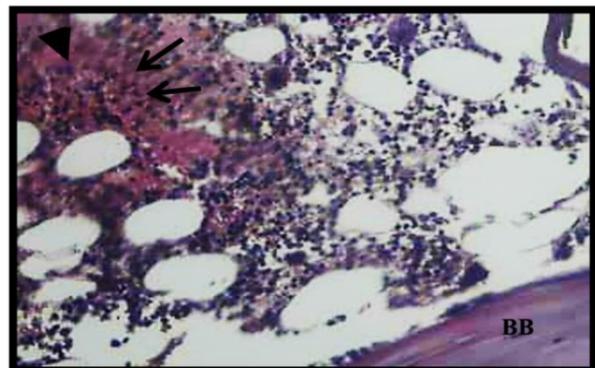


Figure 2: Microphotograph view after 3 days of flavonoid group shows areas of blood congestion (arrow head) inflammatory cells (arrows) and basal bone(BB). (H&E X20).

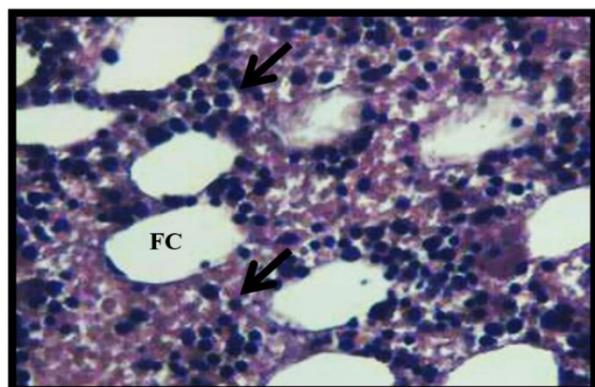


Figure 3: Magnified view of flavonoid group after 3 days shows, inflammatory cells (arrows) and fat cells(FC) are seen. H&E X40.

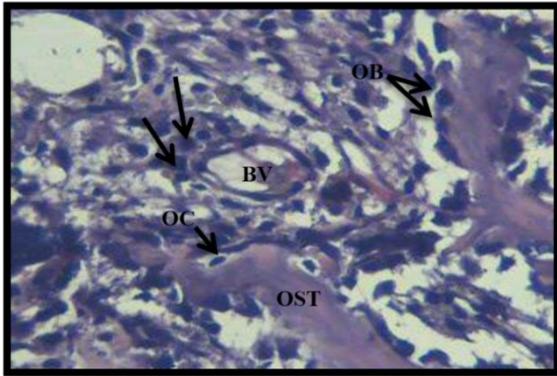


Figure 4: View of defect site of control group after 1 week, shows osteoid tissue, osteoblasts, osteocytes and inflammatory cells (arrow). H&E X40.

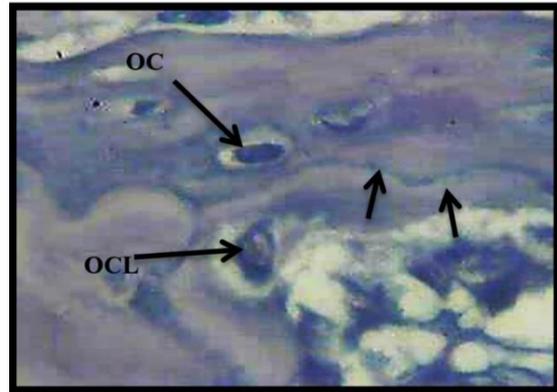


Figure 7: View shows osteocytes (OC), reversal line (arrow), osteoclast (OCL). H&E x100.

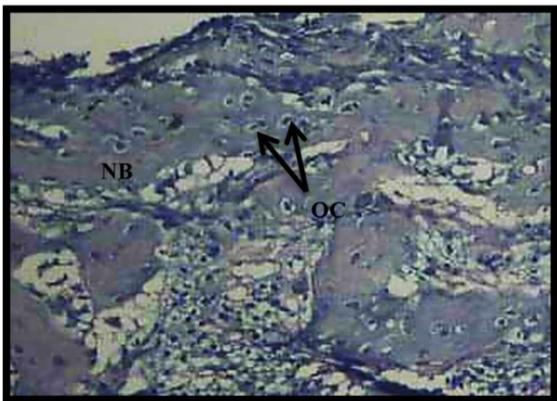


Figure 5: View after 1 week of flavonoid application shows new bone matrix enclosing osteocytes and rimmed by osteoblasts (OB). H&E X20.

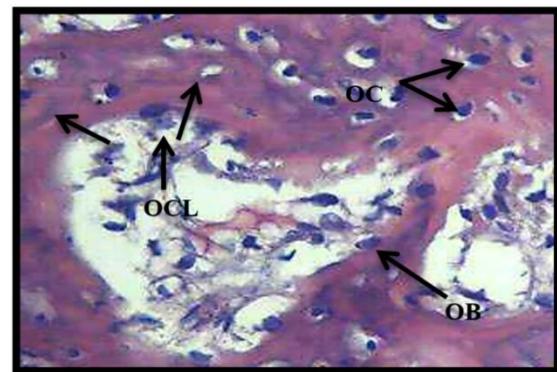


Figure 8: View of 2 weeks experimental group shows bone trabeculae with numerous osteocytes (OC), osteoblast (OB), osteoclast (OCL), reversal line (arrows). H&E x40.

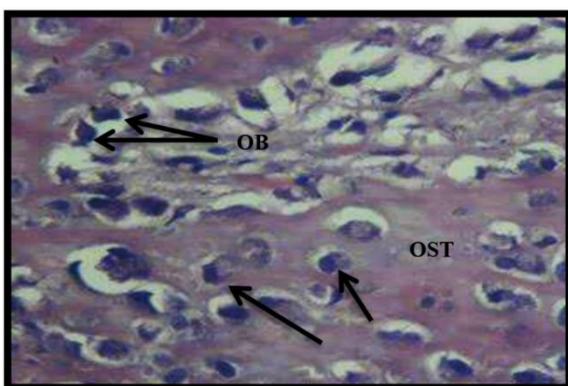


Figure 6: View after 1 week of flavonoid application shows osteoid bone enclosing osteocytes (arrows) and rimmed by osteoblasts (OB). H&E X40.

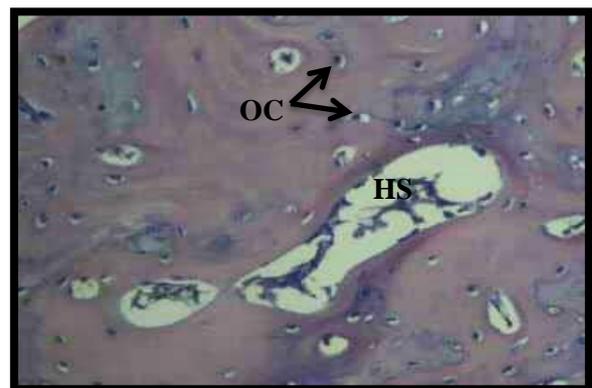


Figure 9: View of control group after 4 weeks shows Haversian system (HS) surrounded by osteocytes (OC). H&E x20

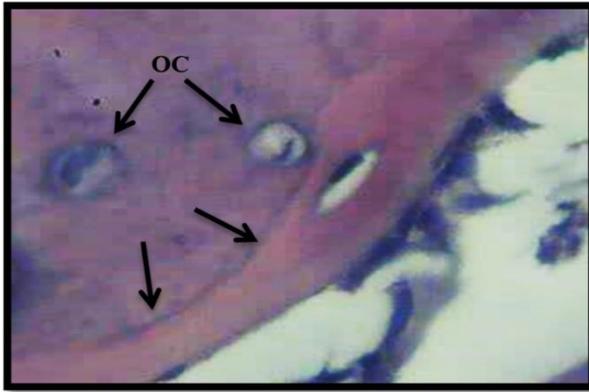


Figure10: View of control group of 4weeks duration shows osteocytes (OC) and reversal line (arrows). H&E x100.

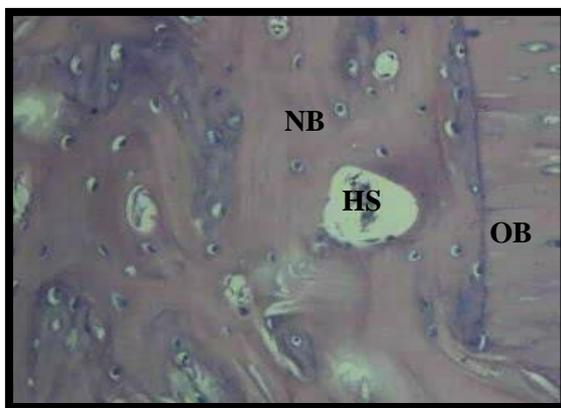


Figure 11: View of flavonoid group after 4 weeks shows new bone (NB) and old bone(OB) with osteocytes arranged around haversian system(HS). H&E x20.

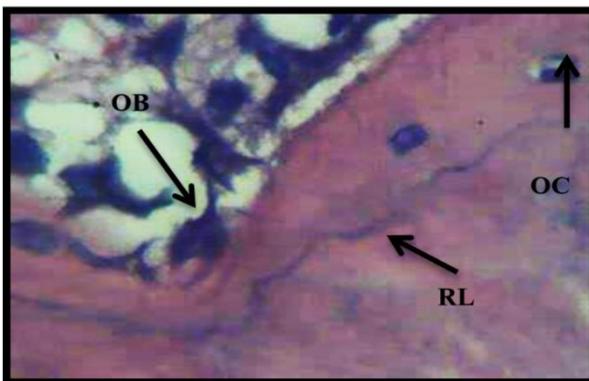


Figure 12: Magnified view of flavonoid group after4 weeks shows osteoblasts (OB), osteocytes (OC), reversal line(RL). H&E x100

Inflammatory cell parameter:

As illustrated in table 1, the results of the present study show obvious increase in median value measured as mean count difference of cell number between control and experimental groups at 3days and 1week durations, whereas almost little or no difference was recorded at 2 and 4 weeks durations

Non significant difference in mean values of inflammatory cells regarding both groups throughout the healing periods was detected.

Table 2 shows very strong linear correlation in both control and experimental side during the healing intervals.

Histomorphoetric analysis of studied groups for bone architecture parameters:

The statistics for the trabecular number, trabecular area, bone marrow area and bone cells count (osteoblasts, osteocytes and osteoclasts) were estimated in the studied groups in duration 1,2, and 4 weeks as illustrated in (Figure 13)

Bone cells

For the studied groups (control and experimental) the mean count difference of bone cell (osteoblast) increased between experimental and control groups after 1week duration and decrease or no difference in 2, and 4 weeks duration but this change was statically significant in 1 and 2 week duration and not significant in 4 week duration. Regarding bone cells (osteocytes and osteoclast) there is slight difference in median value of the mean count difference at 1, 2 and 4 weeks, and it was non-significant statistics

Trabecular number, trabecular area, and bone marrow area

At 2weeks there was increase in mean count difference of trabeculae number while bone marrow area showed slight decrease in mean values but no significant statistics were recorded. Trabecular area showed increase in mean count difference with significant statistics.

After 4 weeks, trabeculae number, and trabeculae area showed increase in mean count difference between experimental and control groups but this change was statistically not significant while bone marrow area showed slight decrease in mean values and was statically significant.

DISCUSSION

This study was designed to evaluate the effect of topical application of flavonoid extract on experimentally induced bone defects in tibiae of rabbits.

Adult rabbits were selected because they provide many desired characteristics to fulfill the requirement of the study. The rabbit tibia model has been successfully used as operating site in many previous investigations. New Zealand white strains of rabbits are commonly being used for research activities ^(6,7).

Flavonoids, found in a wide diversity of foods fruits and vegetables, herbs and spices, essential oils, and beverages, have the most potential of dietary components for promotion of bone health beyond calcium and vitamin ⁽⁸⁾.

Table 1: Control vs experimental groups comparison in median values for inflammatory cells indices measured as mean count of cells per HPF

Duration	Variances	No.	Range	Median	Mean Rank	R(effect size)	P value
3 days	Experimental group	6	107to211	161	21.5	0.38	0.35[NS]
	Control group	6	96to243	131.5	21.5		
	Mean count difference between experimental and control groups	6	-136to89	14.5			
1 week	Experimental group	6	48to101	68	15.5	0.64	0.12[NS]
	Control group	6	13to88	41.5	13.3		
	Mean count difference between experimental and control groups	6	-3to50	33			
2 week	Experimental group	6	13to29	26	9.5	0.3	0.46[NS]
	Control group	6	17to30	22	11.7		
	Mean count difference between experimental and control groups	6	-4to7	1			
4 week	Experimental group	6	4to11	10	3.5	-0.28	0.49[NS]
	Control group	6	4to12	9	3.5		
	Mean count difference between experimental and control groups	6	-2to5	-1			

Table 2: Linear correlation and p value for (Kruskall-Wallis) and (Mann-Whitney) for difference in median between all the 4 follow up intervals for inflammatory cells

	Control groups	Experimental groups
Linear correlation with duration of follow ups	r=-0.9 P<0.001	r=-0.971 P<0.001
Kruskall-Wallis for difference in median between all the 4 follow up intervals	P<0.001	P<0.001
Mann-Whitney for difference in median between:		
3 days X 1week	P<0.001	P<0.001
3 days X 2 weeks	P=0.004	P=0.004
3 days X 4weeks	P=0.004	P=0.004
1week X 2weeks	P=0.004	P=0.004
1 week X 4weeks	P=0.42[NS]	P=0.004
2weeks X 4weeks	P=0.004	P=0.004

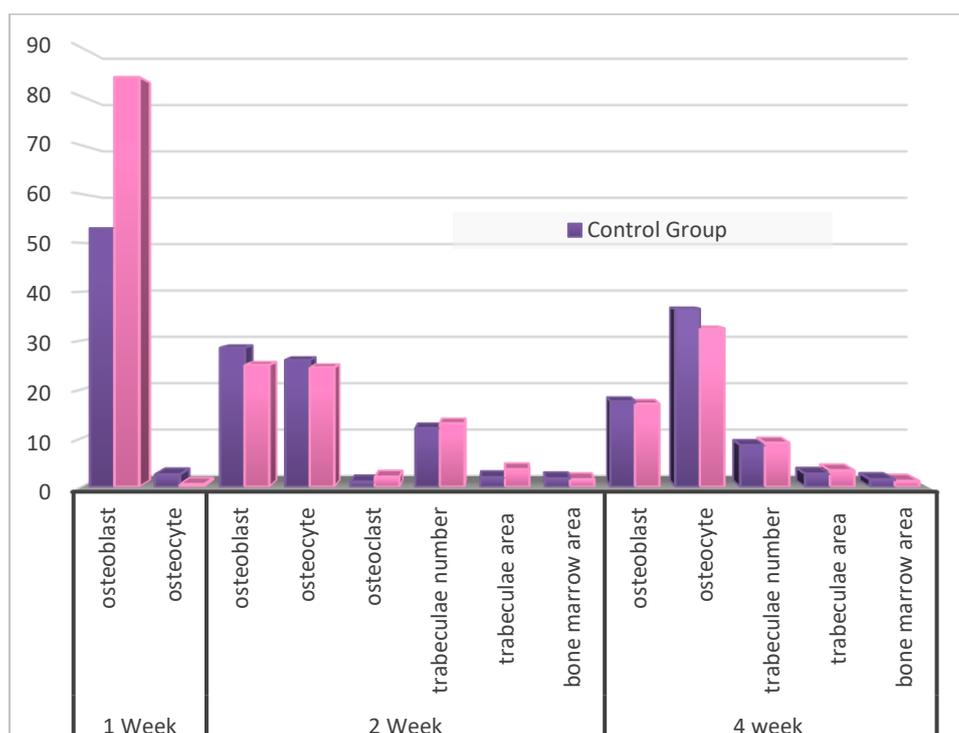


Figure 13: Comparison of median value for control and experimental groups in different durations.

Results obtained in this study showed increase in median value measured as mean count difference of cell number between control and experimental groups at early days of healing (3 days and 1 week durations), which indicates activity of FLV in accelerating healing process in agreement with Bhaskar and Nithya ⁽⁹⁾ who investigated wound-healing potency of the ethanolic extract of the flowers of *Hibiscus rosa sinensis* on Wistar albino rats and found that the extract increased cellular proliferation and collagen synthesis at the wound site attributed to its high flavonoid content with antimicrobial property. Mountziaris and Mikos ⁽¹⁰⁾ stated that at the periphery of fracture site, stem cells differentiate into osteoblasts, as a result bone forms 7–10 days after injury which agrees with the findings of this study where deposition of bone matrix by differentiating osteoblasts was detected at 1 week duration in both groups. The progression of 2 weeks bone healing as indicated by matrix deposition by differentiating osteoblasts during the first week and bone trabeculae formation within newly formed bone as shown in histological sections that increased in number and width throughout the 2nd and 4th weeks intervals. The presence of osteoclasts residing Howship lacunae indicates remodeling process, there was decrease in marrow space area with time which was more obvious in experimental groups as it was detected by histomorphometric analysis. These findings could be explained according to a research conducted by Weaver et al. ⁽¹¹⁾ who reported that some flavonoids appear to have bone anabolic activity, which has exciting implications beyond merely inhibiting bone resorption through suppressing osteoclast activation. Histomorphometrical analysis have shown that the equivalence of median of mean difference count of all parameters tested for micro architecture records between control and experimental groups clarified a high value in experimental groups than those of control groups, this result can be explained on a fact of the effect of flavonoid on the progenitor cells to be differentiated into osteoblasts (bone formative cells) and enhancement of osteoid tissue formation which need more nutrient supplements. The results also showed that there was a significant difference in bone architecture parameters in different intervals time. Increase in trabecular width cortical width and trabecular number as

shown at 2 and 4 weeks which could be attributed to time consuming for apposition of bone and its maturation, while the decrease in number of osteoblasts with time could be explained on the fact that more formative cells, more supplements and more nourishment is needed for any new tissue formation and when the formation of the bone settled and reached to its final measurement no more osteoblasts and blood vessels needed except for preservation of the biological activity.

CONCLUSION

Results obtained in this study have shown that flavonoid extract from *hibiscus sabdariffa* had potential activity in promoting healing process of bone defects.

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