Biomimetic effect of a natural leaf extract in clavarial defects (Experimental study)

Original Article

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ABSTRACT

Defects in Oral and Maxillofacial region constitutes a challenge for surgeons to reconstruct. Invention of different strategies involving new biomaterials that hasten the natural regenerative steps is a persistent need. Natural sources gained attention in the past few years.

Objective: Evaluate the effectiveness of Moringa Oleifera leaf (MOL) powder and extract mixture locally and MO leaf extract repeated injections in critical size clavarial defects in rabbits.

Materials and Methods: Moringa oleifera fresh leaf powder and aqueous extract were prepared. Eight new zealand rabbits were included in this study; two animals were used as control. The rest six were used as intervention groups. All animals were subjected to creation of bilateral clavarial defect in the parietal bone, both sides of the test animals were treated with a mixture of Moringa Oleifera leaf powder (0.35 gm) and water extract (½ml). The right side was additionally injected with Moringa Oleifera leaf water extract (¼ ml) twice (3 days a part postoperatively). The specimens were sent for histopathologic examination by H&E and Masson Trichrome stain to quantify new bone formation, defect narrowness.

Results: UMoringa Oleifera leaf (MOL) extract repeated injections sites showed more newly formed bone, fewer inflammatory cells and narrower bone defects.

Conclusion: Moringa Oleifera leave Polyphenolic extracts: Gallic, Chlorogenic acids, and Rutin affected bone healing parameters positively.

Key Words: Natural biomimetic, bone regeneration, Green synthesis, Moringa oleifera lam leaves, natural hydroxyl-appatites, local delivery, rabbit clavarial defects, critical size defect.

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INTRODUCTION

Bone is a highly dynamic tissue. As a load carrier, it is in a state of continuous remodeling including both processes of bone formation and resorption. Defects in Oral and Maxillofacial region constitutes a challenge for surgeons to reconstruct. Defects such as bone fractures, pathological lesions, infections, and congenital malformations, retard the regenerative steps which subsequently affect the quality of life, the patient psychology and represent a financial burden. Hence came the need of different techniques and strategies involving new biomaterials that stimulate the natural regenerative process constitutes the field of "regenerative medicine" [1,2].

Numerous biomaterials have been tried as bone substitutes such as allografts, xenografts, and synthetic biomaterials. While the greatest success in bone grafting has been achieved with autogenous bone. However, it was faced by its limited supply, liability of resorption and donor site morbidity. Synthetic hydroxyapatites were preferable than other bone grafts due to their availability, reasonable price, the absence of risk for disease transmission and decreased allergic potential. Natural hydroxyapatite substitutes, such as marine based and plant based preparations (Green synthesis of hydroxyapatite) came to site recently [3-.6]

The choice of surgical technique for removing maxillofacial Plant sources of potential role for bone regeneration are numerous uncountable for example Eugenol, Gum Arabic, Aloe vera, Nigella sativa, Dalbergia sissoo, Cissus quadrangularis (CQ), Withania somnifera (WS) and Tinospora cordifolia (TC), Curcuma, Danggui buxue, Morinda citrifolia Leaf, Mate tea, Danggui buxue, Broussoneta kazinoki, Soy bean, grape seeds and Moringa oleifera (MO) [6- 12] . In return, marsupialization allows a gradual reduction in Different parts of Moringa Oleifera plant (stem, roots, flowers and leaves) contains various nutrients such as vitamins, minerals, amino acids, and phytochemicals (polyphenolic compounds). Most studies have focused on the leaves that have been confirmed for being antioxidant, anti-inflammatory, anticancer, and antimicrobial properties [13- 18].

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Herbal preparations gain repetition for bone healing improvements upon its local application besides its oral intake with special concern to phytochemical (Polyphenol) compounds as a specific new drug era. Many researches were interested in investigating the drying method, suitable dose, solvent extraction and local application delivery systems to meet the regenerative process requirements. Therefore, there is no universal extraction method to extract plant phenols. In near future the local use of phenolic compounds will become a reality in clinical practice. The suggested mechanism of Polyphenols action is to suppress osteoclast function and differentiation. Moreover, to stimulate osteoblast proliferation with reduction of its apoptosis rate [13- 19]

Different types of solvents were tried when preparing plant extracts. However, water extract has been confirmed with its high antibacterial activity against main pathologic flora (Gram positive and negative) besides anti-inflammatory properties[20- 22] A study confirmed cytotoxicity for ethanolic extracts over the water extract [14,19] Diverse opinions on difference between levels of phytochemicals in different solvents (Ethanolic, Methanolic and Water extracts). Most experiments confirmed ethanol upper hand. While, Kwon research confirmed superior results for water extract of moringa over ethanolic extract (Table 2) [23]

The rabbit calvaria model has grabbed attention when evaluating different biomaterials due to its easy surgical access compared with small animal models (rodents) and faster data results compared to large animal models (dogs). Moreover, rabbit calvaria are close to the anatomical and physiological characteristics of humans [24- 31] Among the polyphenol extracts of moringa oleifera leave, Gallic acid, Rutin and Cholinergic acid were found to exhibit antioxidant, anti-inflammatory properties, and antimicrobial action Although, decreased inflammation is not enough alone to promote bone formation. Various Studies showed improvements of bone regeneration through stimulating angiogenesis, mesenchymal stem cells and osteoblastic activity. The healing process showed increased expression of osteoprotegerin, osteopontin, osteocalcin and bone morphogenetic protein-2 bone mediators. In addition, an in vitro study reported decreased demineralization rate of enamel.

when polyphenol composite containing chlorogenic acid was applied on the enamel surface [32- 43]

MATERIAL AND METHODS

Preparation of the implant

The fresh leaves of Moringa oleifera (MO) were harvested from Agriculture research center, Egypt. The leaves were soaked for 15 min.in water to remove dirt and dried (to low moisture content of 67%-) for seven days under shade, at room temperature, to avoid loss of active compounds. The dried leaves were stored in air tight container at 7°C. The stored leaves were ground to powder using home grinder at the same day of use. For preparing aqueous extracts, macerate the dried leaf powder 40 gm in 100 ml boiled water with stirring and then left for 24 h at room temperature. The extract was obtained by filtration. Filtrate was concentrated using an evaporator to 11.7% of their initial volume. High performance liquid chromatography (HPLC) was used to estimate polyphenols in the mixture and extract at the center of excellence, National research center, Egypt.

The surgical steps

The experimental study was performed at animal house, Cairo University. Eight New Zealand white male rabbits weighing 2 to 2.5 kg were used. All surgical procedures were performed using injectable general anesthesia. Each animal was premedicated according to weight with an intramuscular injection of Ketamine* (0.5 mg/kg body weight) IV TEKAM®50, HIKMA, Pharmaceutical Co..Amman-Jourdan and Xylazine**IM (5 mg/kg body weight) ROBMPUN®,Bayer, Leverkusen,Germany

The surgical site was shaved and disinfected with betadine, local anesthesia was provided using a 2% Mepecaine solution. An incision was made along the midline of the scalp from a point midway between the bases of the ears to approximately 5 cm anteriorly through the full thickness of the skin. Sharp subperiosteal dissection reflected the pericranium from the outer table of the cranial vault exposing the parietal bones (Figure 1A).

Bilateral full thickness calvarial defects were prepared of each rabbit, using a fine round bur which is mounted on a low-speed handpiece, under copious irrigation with saline solution (Figure 1B). The defect size was about 10 mm. Two rabbits were left without intervention as a control group to exclude presence of any environmental factor affecting the wound healing.

A mixture of Moringa oleveira exctract ½ ml mixed with 0.35 gm MOL powder (Figure 2). In the remaining 6 rabbits, both defects were filled with the MOL (powder extract mixture) (Figure 3A&B) . Cannulation and injection of prophylactic antibiotic were carried out using Flumox 250 mg*** (Egyptian International Pharmaceutical Industries co (EIPICO) 1O TH of Ramadan city, EGYPT.) , in divided dose intramuscular injection every 24 hours for 3 days, for each animal. A topical antibiotic was applied after suturing once Bivatracin spray*** ACDIMA International The right side defect was repeatedly injected by the moringa oleifera extract ¼ ml twice (3 days apart) at the defect center (Figuer 3B). Animals were kept in individual cages and received food. They were observed every now and then by a specialist. All animals were sacrificed at end of second week. The implanted bony defects were dissected and fixed in neutral formalin solution 10%.

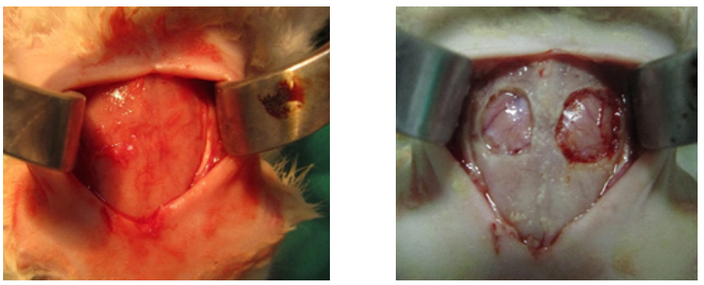


Figure 1: A: Incision, dissection and reflection exposing the parietal bone
B: Showing bilateral calvarial defects almost 10 mm in size.

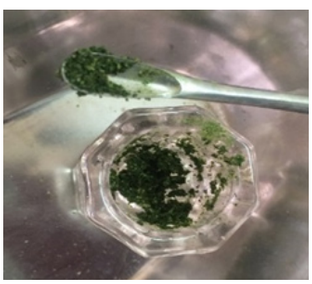


Figure 2: Showing moringa oleveira leaf and extract mixture

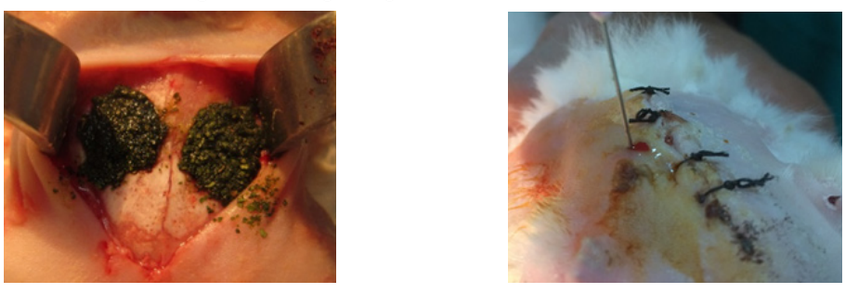


Figure 3: A: Showing application of the moringa olefeira leaf and extract mixture
B: Showing injection of MO extract at the defect center (white arrow)

Histomorphometric analysis :

The samples were prepared and analyzed at the histopathology Unit, Pathology department, Faculty of Oral and Dental Medicine, cairo University. The specimens were decalcified in EDTA for four weeks, processed and embedded in paraffin. A 4µm tissue section was cut from each specimen, deparaffinized, hydrated and stained with hematoxylin and eosin (H&E) for microscopic examination. Another tissue section was prepared and stained by Masson trichrome (MTC) stain for morphometric analysis. A blinded histological assessment was carried out (the pathologist didn't know which side received the repeated injection) *.

Assessment of wound healing:

Using Leica Qwin 500 computer image analyzer, the MTC-stained sections were examined to detect mineralized bone tissue (stained red) and collagen and osteoid tissue (stained green). Then, area percent (%) of the newly-formed bone in the center of the wound was measured under magnification power of ×200. In addition, the distance between the wound margins were measured in microns under magnification power of ×40 to assess the amount of bone that is formed and fused to the adjacent normal bone (Defect narrowness).

Statistical analysis:

The data obtained from computer image analyzer was presented as mean and standard deviations (SD). The paired t-test was used to compare between right and left sides. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Microscopic examination of H&E stained sections:

In the control group, bone defect was filled with granulation tissue which was heavily infiltrated by inflammatory cells and covered by fibrous band. No bone trabeculae could be seen within the bony defects.(Figure 4)

In the intervention group, there was an evidence of healing but with different degrees. The wound was filled by granulation tissue infiltrated by chronic inflammatory cells and covered by dense fibrous band. A newly formed bone was detected in the center of the lesion and at the periphery where it fused with marginal normal bone. Many reversal lines were also seen demarcating the fusion between old and new bone.(Figure 5,6)

More bone trabeculae, fewer inflammatory cells and narrowing of bone defect were observed in the Right-side group (repeated injection side). Table 1

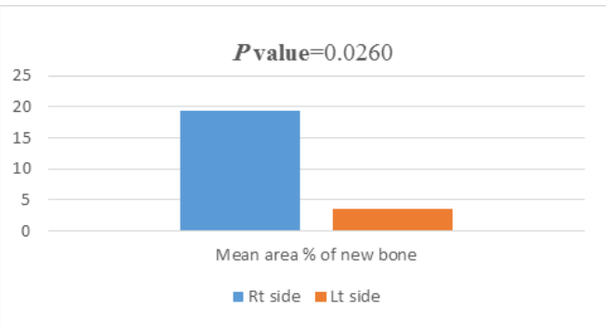
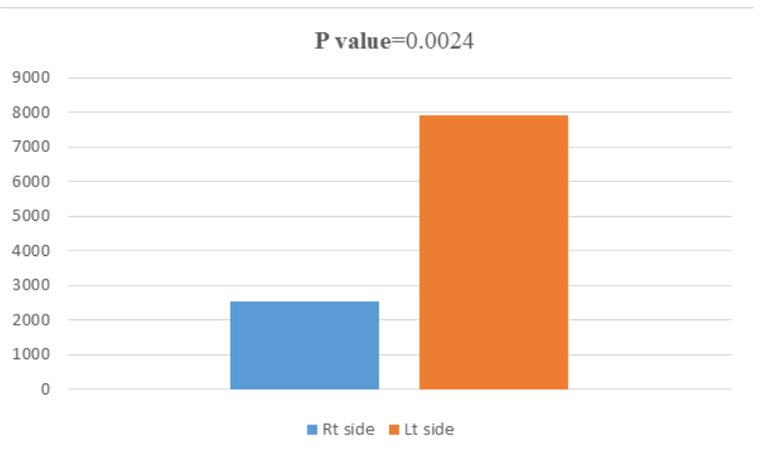


Figure. 8: Histogram comparing bone area % between two groups



Figuer 9: Histogram comparing defect narrowness between two groups

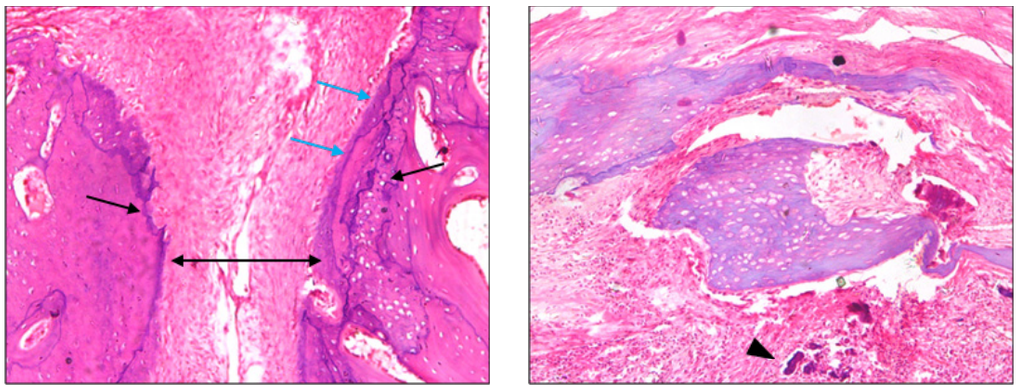


Figure 5 : Photomicrographs of the right-side group showing (a) approximation of defect margins, osteoblastic rimming (blue arrows) and many reversal lines (black arrows) separating old and new bone, (b) bone trabeculae and areas of calcification (arrow head) within the granulation tissue filling the defect (H&E x100).

Table 1: Microscopic examination of H&E stained sections

Study Groups	Control group	Intervention group	
		Right side	**Left side**
Wound surface	Thin fibrous band	Thick and dense fibrous band	Thick fibrous band
Defect margin	Osteoblastic rimming with no bone formation	Bone formation and many reversal lines. Defect narrowing.	Bone formation and reversal lines.
Defect center	Granulation tissue heavily infiltrated by chronic inflammatory cells	Granulation tissue infiltrated by mild chronic inflammatory cells. More bone trabeculae	Granulation tissue infiltrated by mild chronic inflammatory cells. Some bone trabeculae

Assessment of mineralized bone tissue:

In the center of the defect, a greater area percent of mineralized bone, stained red with MTC(Figure 7) , was detected in the right-side group (19.4175±5.7832), compared to the left-side (3.5850±2.4730) the difference was statistically significant (p= 0.026). Table 2(Figure 8)

To assess bone formation at the periphery, Narrowness of the defect margins was measured and the narrower defect was recorded in the right-side group (2536.685±1286.6508), compared to the left-side one (7930.6150±575.8805) the difference was statistically significant (p= 0.0024). Table 3(Figure 9)

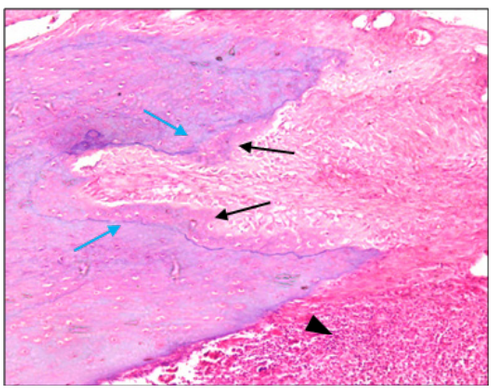


Figure 6 : Photomicrograph of the left-side group showing reversal line separating old (blue arrows) and new bone (black arrows) and chronic inflammatory cell infiltrate (arrow head) (H&E x100).

Table 2: Area percent of mineralized bone

The studied groups	Right-side(repeated injections)	Left-side (single application)
Mean	19.4175%	3.5850%
St.d deviation	5.7832	2.4730
SEM	2.8916	1.2365
P value	0.0260	
95% confidence interval of this difference	3.5915 to 28.0735	

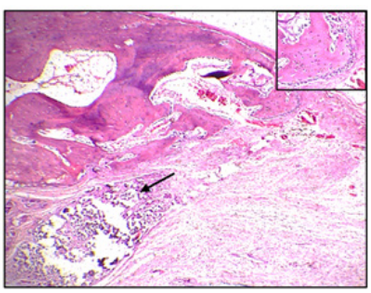


Figure 4 : Photomicrograph of the control group showing loose granulation tissue infiltrated by chronic inflammatory cells (black arrows) and osteoblastic rimming (inset x200). New bone formation could not be observed (H&E x40).

Table 3: Narrowness of the defect margins

The studied groups	Right-side(repeated injections)	Left-side (single application)
Mean	2536.6850 microns	7930.6150 microns
St.d deviation	1286.6508	575.8805
SEM	643.3254	287.9403
P value	0.0024	
95% confidence interval of this difference	-7173.8175 to -3614.0425	

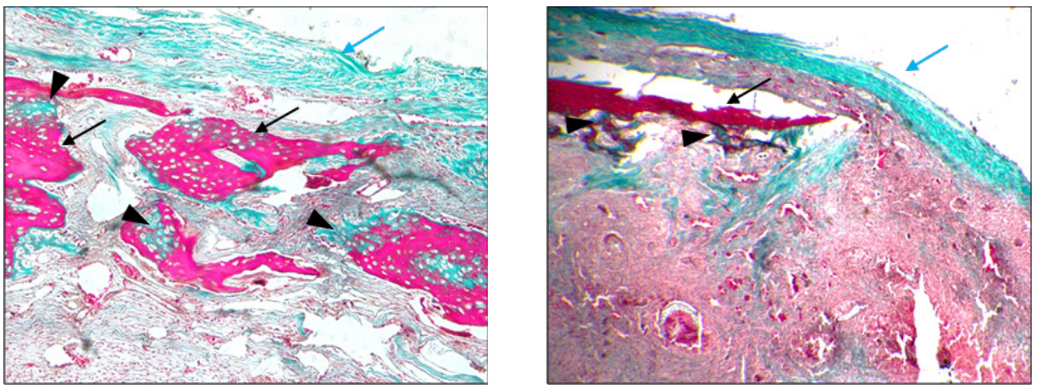


Figure 7 : Photomicrographs of MTC stained sections of right-side (a) and left-side (b) groups showing fibrous band covering the defects (blue arrows) and new bone formation in both groups. More mineralized bone trabeculae (stained red) with peripheral osteoid tissue (stained green) (arrow heads) could be seen in the right-side group (MTC x100).

DISCUSSION

Biomimetic materials extracted from plant sources gained repetition in the past few years. The miracle tree Moringa oleifera as one of the plant sources, has multiple uses in the field of biomedicine. The leaves have the greatest concern due to multi-beneficial contents (various nutrients, and phytochemicals). Polyphenols are examples of phytochemicals, with known antioxidant, anti-inflammatory, anticancer, and antimicrobial properties. Besides its bone regeneration suggestions, they have a documented role in re-mineralization processes. Moreover, it improves the adhesion between adhesives and dentin or enamel.

Table 4: Polyphenols contents in both water extract & powder and water extract mixture. High performance liquid chromatography (HPLC) was used to estimate polyphenols in the mixture and extract.

Polyphenols contents	Injection liquid) Water extract Conc. (µg / ml)	Scaffold Mixture: (Powder and water extract) Conc. (µg / ml)	Percent of biding to HA (Hydroxy appatite)Reference
Gallic acid	108.02	594.08	6
Rutin	153.10	479.11	52
Chlorogenic acid	189.41	458.22	99
Catechin	100.47	383.74	9
Naringenin	123.34	206.10	0
Ferulic acid	161.02	176.73	17
Querectin	56.08	134.64	1
Coumaric acid	3.53	92.09	58
Vanillin	60.14	77.13	2
Syringic acid	16.01	24.41	33
Coffeic acid	13.43	18.91	23
Cinnamic acid	2.46	9.38	-

High power liquid chromatography (HPLC), was used to estimate polyphenol compounds actually present in the study mixture and extract. Locally administered polyphenols scaffolds extracted from Moringa oleifera leaves (Contain mainly Gallic acid Rutin and Cholinergic acid in high concentrations) as illustrated in table 4. While polyphenols extracts (Contain mainly chlorogenic, ferulic, rutin acids) in high concentrations. Gallic acid was the most abundant in the mix (594 µg / ml) followed by Rutin (479 µg / ml) and Chlorogenic acid (458 µg / ml) table 4. Information about percentage binding of these polyphenols to hydroxyapatite (HAP) are surprising showing that Chlorogenic acid is 99 % bound to bone, followed by Rutin 52% where the mix and the extract are rich in them. [36]

High affinity of Gallic acid and Rutin to hydroxylappetite on oral intake was documented by Alldritt et al research 2019. [36] Upon local administration of these polyphenols, concept is also applicable. Further injections of the liquid extract at different time interval allow the concept of sustain release of these polyphenols (not just one shot in the primary scaffold) which explains the results.

Locally administered scaffolds were stable enough, besides the extract repeated injections was easy and applicable. The significant results could be due suspected high bone binding of chlorogenic acid and rutin on local administration by repeated injection and to the potential local sustain release of them. Previous studies on rabbit calvarial defect models used 4 weeks, 6 weeks and 8 weeks as test periods. Lapplainem 2014 concluded that 2 weeks would be an appropriate period for observing bone healing in this defect model. Although the critical-size aspect of this area is debated, many studies recommended 10 mm diameter, bilateral calvarial defects as critical size. The poor blood supply and deficient bone marrow of calvarial defects, make it a good test variant.[2,12,24-27,35]

A study 2020 on in critical-sized defects in Wister rats used Gallic acid (an example of polyphenols) in conjugation with scaffolds demonstrated anti-inflammatory and significant bone regeneration 26. Moreover, it was used in socket healing after extraction of central incisors, sockets treated with 3 ml of Gallic acid single applications howed higher osteocalcin expression and lower trabecular separation [35]

Gallic acid also increases osteoblastic cell adhesion to implants, osteoblast proliferation and preventing fibroblast proliferation which resulted in a better osteointegration and biocompatibility [26].

Chauhan et al 2018 also reported reduced fracture risk and bone fragility due Gallic acid inclusion in his treatment. Hou et al. 2019 reported also success of Gallic acid in rat calvarial model that showed increased defect fill, osteoblast proliferation, and activity.[26,35,49] According to High power liquid chromatography (HPLC), the chlorogenic acid was the most abundant in extract used for injection (189 µg / ml) followed by Ferulic acid (161 µg / ml) and Rutin (153 µg / ml). Rutin demonstrated increase in the fibroblast proliferation and collagen production as reported by Sharma et al 2013[47].While, Februic acid (FA) had positive effects on stem cells signaling pathways documented by Liang JW 2021. From all above, repeated injection demonstrated increase in the fibroblast proliferation and collagen production forming the bone matrix besides, the role of februic acid on stem cells to stimulate bone forming cells. [40,44,45,46,48]

The present results revealed significant mineralized bone area % in the calvarial defects treated with repeated injection sites over single application side, Moreover, the defect narrowness was superior.

In conclusion, Within the limitation of this study, Moringa mixtures and extracts containing Gallic, Chlorogenic acids, and Rutin affected bone healing parameters positively, taking into consideration the differences between the animal and human biology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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