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ORIGINAL ARTICLE

Garden Cress (*Lepidium sativum* L.) Seeds Enhancing Osteogenesis Postinduced-Bone Fracture

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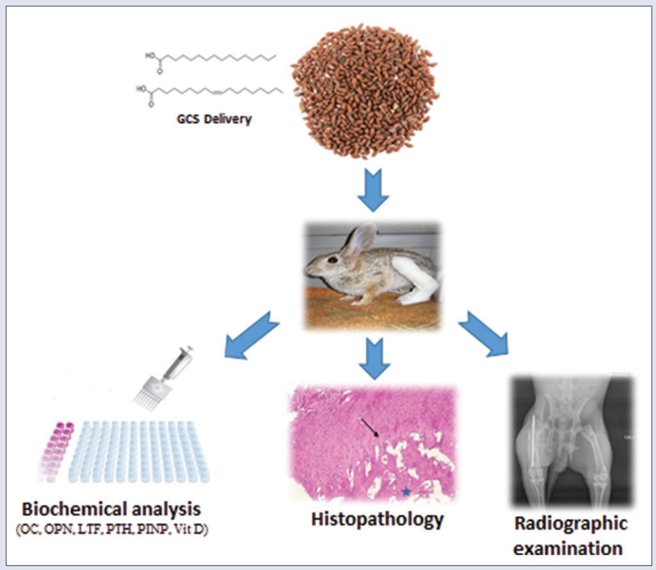
ABSTRACT

Objectives: This study is aimed to evaluate the role of garden cress seeds (GCS) in osteogenic enhancement in bone fractures induced in rabbits. **Materials and Methods:** Thirty New Zealand White rabbits (*Oryctolagus cuniculus*) (n = 30) of 6 months of age and weighing 3–4 kg were included in this study. Rabbits were distributed into two main groups, One served as control and the other were subjected to induced transverse diaphyseal fractures of the left femurs. All rabbits were accommodated in cages and permitted to move freely without external support. Wound care, hygienic conditions, diet and behavior were observed and followed up on daily basis. At the end of the study, five rabbits of each subgroup were sacrificed, followed by dissection of the left femurs. Histomorphometric measurements were performed in all microscopic fields at a ×100 using by Leica microscope DM 2500 connected to a camera (Leica DFC 295) and Leica Q win V3 image analysis software. **Results:** Bone markers analysis revealed that the serum levels of osteopontin and Vitamin D in fractured femur rabbits fed on 6 g GCS showed a significant increase compared to those of untreated fractured femur by the end of the 2nd phase of the study. The serum levels of Osteocalcin in fractured femur rabbits fed on 12 g GCS showed a significant decrease compared to those of untreated fractured femur at the end of the study. The serum levels of Parathormone and Lactoferrin in fractured femur rabbits fed on 12 g GCS showed a significant increase compared to those of untreated fractured femur at the end of the 2nd and 3rd phases of the study accompanied by a significant elevation in liver function test serum levels of fractured femur rabbits fed on 6 g GCS at the end of the 2nd and 3rd phases of the study. The histomorphometric evaluation showed marked improvement of fractured femur rabbits fed on 6 and 12 g of GCS as compared to those of untreated fractured femurs. **Conclusion:** Garden cress seeds could be a promising alternative treatment in bone fracture.

Key words: Fractured femur and *Lepidium sativum*, garden cress seeds, osteogenesis, osteogenic activity

SUMMARY

- In summary, our study aimed to evaluate the role of garden cress seeds (GCS) in osteogenic enhancement in bone fractures induced in rabbits. Thirty New Zealand White rabbits (*Oryctolagus cuniculus*) were included in this study. Rabbits were distributed into two main groups, one served as control and the other were subjected to induced transverse diaphyseal fractures of the left femurs. Bone markers analysis revealed that treatment of GCS through the period of 4, 8, and 12 weeks, has markedly improved the biochemical bone indices and repaired microarchitecture of femurs bones of the GCS treated rats compared to spontaneously healed fractured femur group. The histomorphometric evaluation showed marked improvement of fractured femur rabbits fed on 6 and 12 g of GCS as compared to those of untreated fractured femurs. Garden cress seeds could be a promising alternative treatment in bone fracture.



Abbreviations used: GCS: Garden cress seeds; IDDM: Insulin-dependent diabetes mellitus; GC-MS: Gas chromatography-mass spectroscopy; AMU: Atomic mass units; FAME: Fatty acid methyl esters; H and E: Hematoxylin and eosin; OC: Osteocalcin; OPN: Osteopontin; LTF: Lactoferrin; PTH: Parathormone; PINP: Procollagen I; Ca: Calcium; P: Phosphorus; ALP: Alkaline phosphatase; Vit D: Vitamin D.

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INTRODUCTION

Osteogenesis was the main process that was studied massively experimentally, clinically, and traditionally pointing at simplifying this phenomenon completely and recording it by various methods, such as ultrasound[1] biomechanical measurements,[2] and dual-energy X-ray absorptiometry.[3] The effects of several factors and medicines on bone fracture healing were well-known as well.[4,5]

A fracture is an absence of continuity in the bone substance after severe trauma. Postmenopausal osteoporosis is the main reason for pathological fracture whereby wrist, hip, and vertebral fractures are the most common osteoporotic fractures which take place in the elderly.[6] Bone fractures are classified according to the shape of the fractured fragments and their patterns. Fractures can be divided into oblique, transverse, spiral and comminuted fractures. Further types of bone fractures include crush fracture, compression fracture, gunshot fracture, greenstick fracture, and avulsion fracture. According to the etiology, there are 3three types of fractures including, fatigue, pathological and traumatic. Finally, based on the nature of the fracture, there are open and closed fractures.[7]

Alternative medicines, including traditional folk medicine, have used natural medicinal plants from ancient times till now.[8] This was experienced for the treatment of several ailments in various societies.[9] Traditional and herbal-based medicines play a vital role in the improvement of health.[10] Garden cress seeds (GCS) were involved in the treatment of insulin-dependent diabetes mellitus.[11] These seeds contain mucilage in its dry seed coat that has been isolated using dissimilar solvents and utilized by researchers as an excipient in a variety of pharmaceutical designs for preferred utility.[12] The mucilage of GCS is widely used in several traditional medicinal applications such as cough syrups. GCS possess anti-hyperglycemic properties that add in controlling the blood glucose level in diabetics antiperspirants, stimulants, diuretics, antifungal, and antibacterial properties.[13-15]

Moreover, this plant was used in the community of the Kingdom of Saudi Arabia (KSA) as an important component in Saudi folk medicine for several applications, but mainly in bone healing.[16] Various Arabic names, such as Rashad/Hurf/Thuffa, were given to GCS in the Arabic region, including KSA, which has the plant grown in Hijaz, the Eastern province, and AlQaseem.[17] The plant roots, leaves and their seeds were used in traditional medicine, but the effect of the seeds on osteogenesis was remarkable and famous in folk medicine and has been testified in rats.[16] Good results of bone fractures healing were noticeable over decades by traditional folk medicine practitioners.[16]

This study is aimed to evaluate the role of GCS in osteogenic enhancement in bone fractures induced in New Zealand White rabbits through estimation of bone markers to reach a new natural alternative for acceleration and stimulation of osteogenesis and bone fracture healing to be applied clinically in future.

MATERIALS AND METHODS

Materials

Preparation of garden cress seeds aqueous extract

GCS were obtained locally from the traditional medicine market in Al-Quaseem, KSA and identified by Taxonomist and deposited at the Herbarium of Biological Department, Faculty of Science, King Abdulaziz University. Before extraction, GCS was washed using double distilled water, dried and crushed by pestle and mortar. The seeds were allowed to dry under the sunlight for 2 days then homogenized to a fine powder and stored in free-moisture opaque container until use.

Chromatographic analysis of garden cress seeds using gas chromatography-mass spectroscopy

Chromatographic analysis using gas chromatography-mass spectroscopy (GC-MS), preparation of fatty acid methyl esters, and silylation agent: BSA. N, O-Bis (trimethylsilyl) acetamide were carried out according to Zamzami *et al*.[18]

Experimental design and treatment of rabbits

Thirty New Zealand White rabbits (*Oryctolagus cuniculus*) (n = 30) of 6 months of age and weighing 3–4 kg were included in this study. Rabbits were grouped and housed in a controlled environment including well-ventilated polypropylene cages with husk beds. The photoperiods were regulated at suitable conditions and the temperature was adjusted at 25 ± 1°C as well as 60%–80% relative humidity. Rabbits were fed on standard pellet diets and offered drink water *ad libitum*. Rabbits were acclimatized to laboratory conditions for one week before starting the experiment.[19]

Rabbits were maintained for Care and Use of Laboratory Animals according to the criteria of the US National Institutes of Health (NIH Publication No 8523, revised 1985).[20]

All animals were approved by the Faculty of Medicine-Research Ethics Committee based on the good clinical practice guidelines and followed the National Committee of Bio and Med ethics-King Abdul Aziz City for Science and Technology (HA-02-J-008).

Induction of subperiosteal transverse fractures

Rabbits were distributed into two main groups as follows:

- Group-A (n = 15): Rabbits of this group were divided randomly and equally into three subgroups (A1, A2, and A3) with five rabbits for each: Rabbits of subgroup A1 served as a negative control group and fed on standard pellet diets. Rabbits of subgroups A2 and A3 were fed on standard pellet diets in addition to 6 and 12 g of GCS in their food on daily basis, respectively.
- Group-B (n = 15): Rabbits of this group were subjected to induced transverse diaphyseal fractures of the left femurs. Surgical interference was conducted under intramuscular anesthesia of ketamine HCl 35–40 mg/kg body weight, xylazine 5 mg/kg body weight, and acepromazine (0.75 mg/kg).[21] The mid-shaft of the left femurs was exposed to induce transverse diaphyseal fracture using embryotomy wire, which was reduced and immobilized by intramedullary K-wires.[22]

This group was subdivided into three subgroups (B1, B2, and B3) with five rabbits for each:

Rabbits of subgroup B1 were left for spontaneous femur healing and received standard pellet diets. Rabbits of subgroups B2 and B3 were fed on standard pellet diets in addition to 6 and 12 g of GCS in their food on daily basis respectively and were left for spontaneous femur healing.

All rabbits were accommodated in cages and permitted to move freely without external support. Wound care, hygienic conditions, diet, and behavior were observed and followed up on daily basis and the food was monitored ensuring that rabbits ate all of their food before the addition of extra 6 g or 12 g of the seeds in their new meal.[22] At the end of the 6^{th} and the 12^{th} weeks postoperatively, fracture union was clinically assessed through the absence of pain at the fracture site. Furthermore, blood samples were collected from the ear vein of the rabbits after 4, 8 and 12 weeks and sera were separated to estimate the bone markers using the appropriate method for each; serum osteocalcin (OC), osteopontin (OPN), lactoferrin (LTF), parathormone (PTH), procollagen I (PINP), calcium (Ca), phosphorus (P), alkaline phosphatase (ALP), and Vitamin D (Vit D).

Histopathological evaluation

At the end of the study, five rabbits of each subgroup were sacrificed, followed by dissection of the left femurs which were fixed in 10% buffered neutral formalin solution, decalcified by formic acid embedded in paraffin, sectioned 5 μm and stained by H and E, then examined microscopically for the different stages of repair.

Safranin-O/fast green staining

It was used preferably to refine and enhance the perception of the cartilaginous and the osseous tissue by distinguishing it, by color (bright red for cartilage and green for bone), from a different histologically contiguous tissue.

Histomorphometric quantitation

Histomorphometric measurements were performed in all microscopic fields at a ×100 using by Leica microscope DM 2500 connected to a camera (Leica DFC 295) and Leica Q win V3 image analysis software. The area per cent of both cartilaginous and osseous tissue in Safranin-O/Fast green-stained sections was assessed. The mean values were attained.

RESULTS

The gas chromatography mass spectroscopy analysis of garden cress seeds extract

The pattern of LSS extract for polyphenols and carbohydrates demonstrated by GC-MS analysis showed the presence of 15 active compounds as detailed in our previous study.[18] The mass spectra of these active compounds compared with the spectra of compounds known in the Library Research (NIST 08) were identified, and have unique and critical pharmacological actions.

The effects of garden cress seeds and spontaneous healing on bone markers of induced-femur fracture rabbits 4 weeks postoperatively (Phase-1)

As shown in Table 1, the mean serum levels of OC, LTF, PINP and Ca of 6 g GCS prophylactic rabbits are significantly higher ($P \leq 0.01$) than those of the control. There are insignificant differences ($P \geq 0.05$) of serum levels of OPN, PTH, P, ALP and Vit. D of 6 g GCS prophylactic rabbits compared to those of the control. The mean serum levels of OPN, LTF, PINP and Ca of 12 g GCS prophylactic rabbits are significantly higher ($P \leq 0.01$) than that of the control with a significant decrease ($P \leq 0.01$) in the serum ALP compared to that of the control. Meanwhile, there are insignificant differences ($P \geq 0.05$) of serum levels of OPN, LTF, *P* and Vitamin D of 12 g GCS prophylactic rabbits compared to that of the control. The mean serum levels of PINP and Ca of prophylactic rabbits are significantly higher ($P \leq 0.01$) than that of the control with a significant decrease ($P \leq 0.05$) in the serum OPN compared to that of the control [Figures 1 and 2].

Concomitantly, The mean serum levels of PINP and ALP of 6 and 12 g GCS treated rabbits 4 weeks postoperatively are significantly higher ($P \leq 0.01$) than that of the untreated fractured femur rabbits with significant ($P \leq 0.01$) decrease in the serum levels of Vit. D of 6 and 12 g GCS treated rabbits compared to that of the untreated fractured femur rabbits. However, there are insignificant differences ($P \geq 0.05$) of serum levels of OC, OPN, PTH, LTF, Ca, *P* of 6 and 12 g GCS treated rabbits 4 weeks postoperatively compared to that of the untreated fractured femur rabbits except for serum *P* of 12 g GCS treated rabbits that exhibited significant increase ($P \leq 0.05$) than that of the untreated fractured femur rabbits [Figures 1 and 2].

Table 1: The effects of garden cress seed (6 and 12 g) and spontaneous healing on bone markers of induced-femur fracture rabbits 4 weeks postoperatively (Phase-1)

Groups of phase 1	Calcium (mg/dl)	Phosphorus (mg/dl)	ALP (mg/dl)	Vitamin D (mg/ml)	OC (ng/ml)	OPN (mg/ml)	PTH (mg/ml)	LTF (μg/ml)	PINP (ng/ml)
Control	8.72±0.220	4.5±0.47	166.04±9.45	0.055±0.004	0.27±0.016	0.31±0.01	0.24±0.02	0.1690±0.015	10.02±1.42
GCS (6 g)	12.32±0.43***	5.08±0.47	159.4±5.49	0.046±0.003	0.34±0.02**	0.34±0.03	0.2783±0.02581	0.21±0.021*	21.9±1.6***
GCS (12 g)	11.8±1.4*	4.8±0.35	95.3±8.2**	0.05±0.003	0.29±0.04	0.37±0.02*	0.24±0.02	0.20±0.015*	16.33±1.3**
Untreated fracture	11.5±1.2	3.99±0.5	176±8.7	0.05±0.003	0.28±0.04	0.27±0.01	0.269±0.03	0.17±0.012	11.9±2.2
Fracture+GCS (6 g)	10.1±1.3	4.58±0.62	194.4±6.4##	0.037±0.007##	0.29±0.03	0.31±0.018	0.26±0.01	0.18±0.01	23.8±1.2###
Fracture+GCS (12 g)	10.65±0.01	4.64±0.31#	203.0±6.92###	0.031±0.008#	0.24±0.037	0.29±0.041	0.25±0.03	0.18±0.009	16.7±1.8##

All data were expressed as mean ±SEM. Values were statistically tested using the student's t test and significant differences at $P<0.05$ and $P<0.01$, as indicated by (*) & (**), compared to control and (#) & (##) compared to untreated fracture respectively. SEM: Standard error of mean; GCS: Garden cress seed; ALP: Alkaline phosphatase, OC: Osteocalcin; OPN: Osteopontin; LTF: Lactoferrin; PTH: Parathormone, PINP: Procollagen I, GCS: Garden cress seed

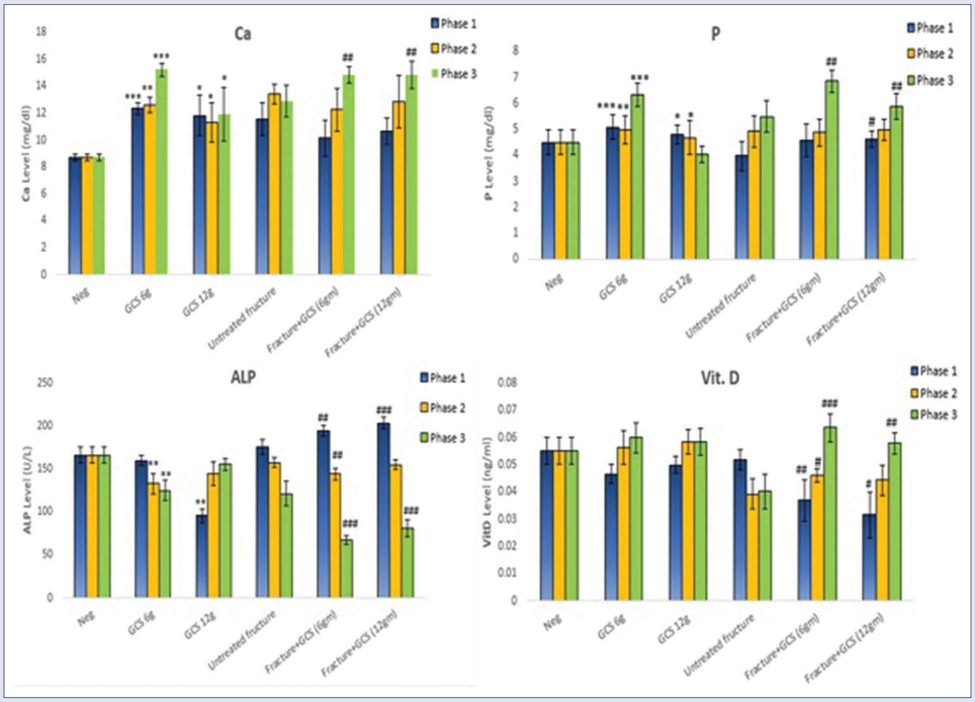

Figure 1: Serum levels of calcium, phosphorus, alkaline phosphatase and Vitamin D of untreated control (Neg.) rabbits garden cress seeds prophylactic and spontaneous healing rabbits. Garden cress seeds treated rabbits 4, 8 and 12 weeks post induced femur-fracture (phase-1, phase-2 and phase-3 respectively) as indicated in the figure. All data were expressed as mean ± standard error of mean values were statistically tested using the Student's *t*-test and significant differences at $P < 0.05$, $P < 0.01$, $P < 0.001$ as indicated by (*), (**), (***) compared to control and (#), (##), (###) compared to untreated fracture respectively

The effects of garden cress seeds (6 and 12 g and spontaneous healing on bone markers of induced-femur fracture rabbits 8 weeks postoperatively (Phase-2)

As shown in Table 2, the mean serum levels of PTH, LTF, PINP, and Ca of 6 g GCS prophylactic rabbits are significantly higher ($P \leq 0.01$) than that of the control accompanying with a significant decrease ($P \leq 0.05$) in the serum levels OC and ALP compared to that of the control. There are insignificant differences ($P \geq 0.05$) of serum levels of OPN, *P* and Vit. D of 6 g GCS prophylactic rabbits compared to that of the control. The mean serum levels of LTF and Ca of 12 g GCS prophylactic rabbits are significantly higher ($P \leq 0.01$) than that of the control accompanying with insignificant differences ($P \geq 0.05$) in the serum OC, OPN, PTH, PINP, P, ALP, and Vitamin D of 12 g GCS prophylactic rabbits compared to the control. Meanwhile, there is a significant decrease ($P \leq 0.05$) in the serum OC compared to that of the control [Figures 1 and 2].

In the meantime, The mean serum levels of OPN and Vit. D of 6 g GCS treated rabbits 8 weeks postoperatively are significantly higher ($P \leq 0.01$) than that of the untreated fractured femur rabbits accompanying with significant ($P \leq 0.01$) decrease in the serum levels of ALP of 6 g GCS treated rabbits compared to that of the untreated fractured femur rabbits. However, there are insignificant differences ($P \geq 0.05$) of serum levels of OC, PTH, LTF, Ca, *P* and Vitamin D of 6 GCS treated rabbits 8 weeks postoperatively compared to that of the untreated fractured femur rabbits [Figures 1 and 2].

The mean serum levels of LTF of 12 g GCS treated rabbits 8 weeks postoperatively are significantly higher ($P \leq 0.01$) than that of the untreated fractured femur rabbits accompanying with insignificant differences ($P \geq 0.05$) of serum levels of OC, OPN, PTH, PINP, Ca, P, ALP, and Vitamin D of 12 g GCS treated rabbits compared to that of the untreated fractured femur rabbits [Figures 1 and 2].

The effects of garden cress seeds (6 and 12 gm) and spontaneous healing on bone markers of induced-femur fracture rabbits 12 weeks postoperatively (Phase-3)

As shown in Table 3, the mean serum levels of OPN, PTH, LTF, PINP, Ca, and *P* of 6 g GCS prophylactic rabbits displayed significant increase ($P \leq 0.05$) than that of the control rabbits accompanying with insignificant increase ($P \geq 0.05$) in the serum levels Vit. D compared to that of the control. While the mean serum levels of ALP exhibited significant decrease ($P \leq 0.05$) than that of the control rabbits accompanying with insignificant decrease ($P \geq 0.05$) in serum OC compared to that of the control. The mean serum levels of LTF, PINP, and Ca of 12 g GCS prophylactic rabbits revealed significant increase ($P \leq 0.05$) than that of the control accompanying with insignificant increase ($P \geq 0.05$) in the serum levels of OPN and Vitamin D of 12 g GCS prophylactic rabbits compared to that of the control. Meanwhile, the mean serum levels of OC, PTH, P, and ALP of 12 g GCS prophylactic rabbits showed insignificant decrease ($P \geq 0.05$) compared to that of the control [Figures 1 and 2].

In the meantime, The mean serum levels of OC, OPN, and ALP of 6 g GCS treated rabbits showed a significant decrease ($P \leq 0.05$) with insignificant decrease ($P \geq 0.05$) in serum PTH of 6 g GCS treated rabbits than that of the untreated fractured femur rabbits 12 weeks postoperatively. Furthermore, the mean serum levels of LTF, Ca, *P* and Vit. D of 6 g GCS treated rabbits showed significant increase ($P \leq 0.05$) with insignificant increase ($P \geq 0.05$) in serum PINP of 6 g GCS treated rabbits than that of the untreated fractured femur rabbits 12 weeks postoperatively [Figures 1 and 2].

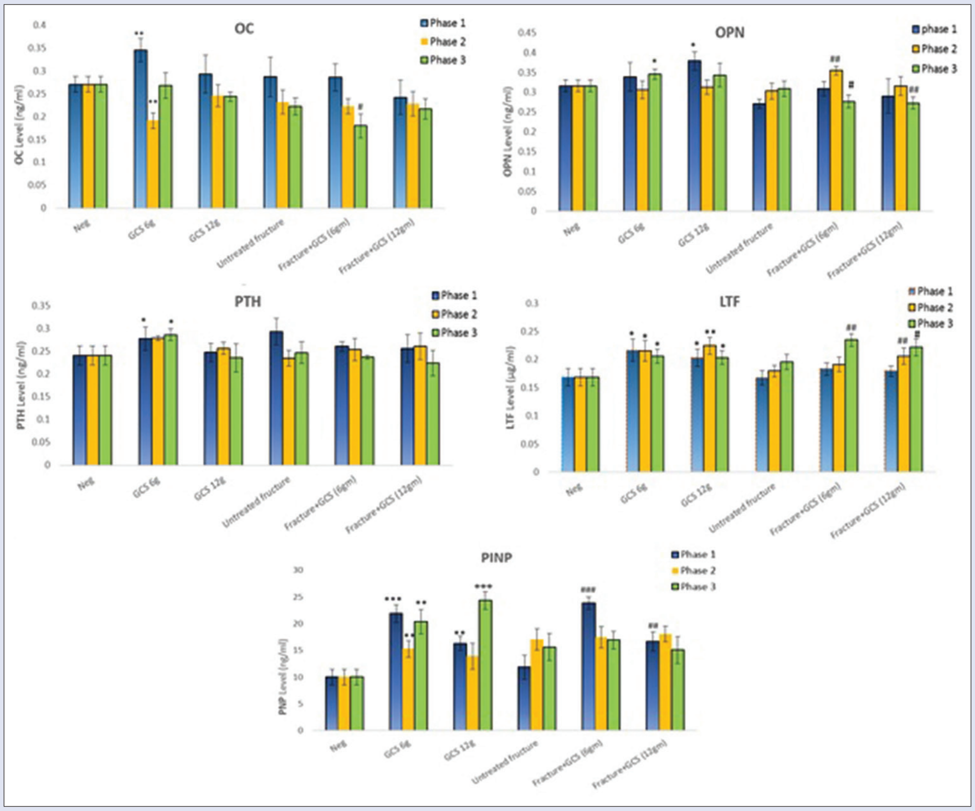

Figure 2: Serum levels of osteocalcin, osteopontin, parathormone, liver function test and procollagen I of untreated control (Neg.) rabbits garden cress seeds prophylactic and spontaneous healing rabbits. Garden cress seeds treated rabbits 4, 8 and 12 weeks post induced femur-fracture (phase-1, phase-2 and phase-3 respectively) as indicated in the figure. All data were expressed as mean abbits 4, 8 and 12 weeks post induced femur-fracture (phas*t*-test and significant differences at $P < 0.05$, $P < 0.01$, $P < 0.001$ as indicated by (*), (**), (***) compared to control and (#), (##), (###) compared to untreated fracture respectively

The mean serum levels of OPN and ALP of 12 g GCS treated rabbits showed a significant decrease ($P \leq 0.01$) with insignificant decrease ($P \geq 0.05$) in serum OC, PTH, and PINP of 12 g GCS treated rabbits than that of the untreated fractured femur rabbits 12 weeks postoperatively. Furthermore, the mean serum levels of LTF, Ca, and Vitamin D of 12 g GCS treated rabbits showed significant increase ($P \leq 0.05$) with insignificant increase ($P \geq 0.05$) in serum *P* of 12 g GCS treated rabbits than that of the untreated fractured femur rabbits 12 weeks postoperatively [Figures 1 and 2].

Histopathological study

The histomorphometric quantitation of the histological sections from the rabbits with untreated fractures showed predominant fibrous and cartilaginous callus tissue compared to the osseous one. The mean area % of the cartilaginous tissue was 60.2 ± 4, while the mean area % of the osseous tissue was 15 ± 7. This subgroup features relatively the highest cellularity level among the studied subgroups. The number of woven bone spicules exceeded the lamellar bone trabeculae [Figures 3a and b].

Histomorphometric evaluation of the histological sections from rabbits received 6 g of GCS were almost showing osseous matrix with the formation of lamellar bone and bone remodeling with resuming the preinjury structure. Scanty cartilaginous matrix of fibrous tissue was seen at the periphery of the osseous tissue. In some cases, complete resorption of the cartilaginous tissue was achieved. The mean of area % of the osseous tissue was 73.9 ± 1, while the mean area % of the cartilaginous tissue was 20 ± 2. Markedly declined cellularity level was noted [Figures 3c and d]. On the other hand, histological sections from rabbits received 12 g of GCS exhibited more cartilaginous tissue undergoing ossification with the formation of woven bone with the mean of area % of the cartilaginous tissue was 50.3 ± 2 compared to 32.5 ± 7in the osseous tissue. Woven bone exceeded the lamellar bone [Figures 3e and f].

DISCUSSION

The current study is aimed to evaluate the potential of GCS on bone healing in rabbits induced fracture. Analysis of GC by GC/MS showed its high content of unsaturated fatty acids which exert its biological activity in improving the osteogenic markers that enhance bone healing in fracture rabbit. In the present study, all fractured femur groups, had insignificant hypocalcemia, accompanied by insignificant higher levels of alkaline phosphatase and inorganic phosphorus at the end of the first 4 weeks of the study compared to those of the untreated fractured femur subgroup at the end of the corresponding period. The values of those parameters were reversed at the end of the study. It is evidenced that the increases in extracellular concentrations of inorganic phosphorous lead to the accumulation of calcium salts, reshaping to crystalline salts (hydroxyapatites) within the organic matrix.[23,24] The serum Ca levels in fractured femur rabbits fed on GCS 4 and 8 weeks postfracture (by the end of 1st and 2nd phases of the study) were insignificantly lower than those of the untreated fractured femur rabbits subgroup. Rabbits with fractured femur received oral administration of GCS showed a high Ca level 12 weeks postfracture (by the end of the 3rd phase of the study) compared to those of the untreated fractured femur subgroup, suggesting that Ca was utilized effectively in the re-mineralization process of bone. These results agreed with those of Soumi and his colleagues who

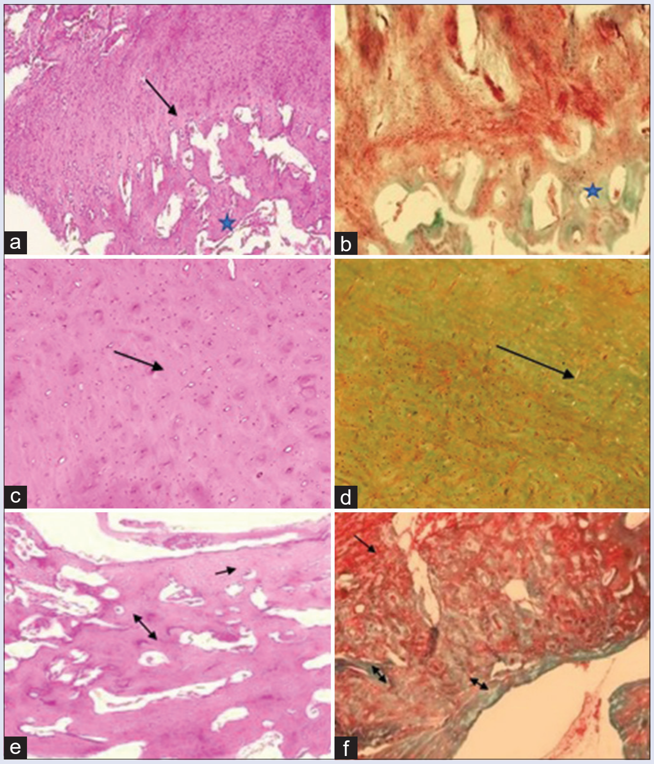

Figure 3: Histological sections changes of femur rabbits at magnification of ×100. (a) and (b) spontaneously healed fractured (femur subgroup B1) showing predominant fibrocartilaginous matrix (arrow) compared to the osseous one which shows woven bony spicules (star) (a and b: H and E, ×100 and Sphranin O, ×100, respectively). (c and d) fractured femur rabbits received 6 g of garden cress seeds (subgroup B2) are almost showing osseous matrix with formation of lamellar bone, restoration of the Haversian system (arrow) and bone remodeling configuration as depicted (c and d: H and E, ×100 and Sphranin O, ×100, respectively). (e) and (f) fractured femur rabbits received 12 g of garden cress seeds (subgroup B3) showing more cartilaginous (arrow) undergoing ossification with formation of woven bone (double arrow). Lamellar bone trabeculae formation is encountered (e and f: H and E, ×100 and Sphranin O, ×100, respectively)

demonstrated that serum calcium reduced to the normal level within one month in fracture normal union and malunion groups.[25]

An increased in inorganic phosphorous level in rabbits fed on 12 g/day of GCS 4 weeks postfracture compare to those of the untreated fractured femur subgroup. While the inorganic phosphorous level in rabbits fed on 6 g/day of GCS 4 weeks postfracture showed significant increase compared to those of the untreated fractured femur subgroup. The serum ALP levels in rabbits fed on 6 and 12 g/day of GCS 4 weeks postfracture were higher than those of the untreated fractured femur subgroup, followed by a decrease at the end of the 2nd and 3rd phases of the study. It was reported that serum ALP concentrations were increased in all subjects after fracture and decreased after treatment in nonunion cases.[25]

Normal fracture healing is accomplished by augmented osteoblastic activity. Osteoblasts, responsible for both bone matrix formation and its mineralization[24,26,27] secrete excessive amounts of ALP, which contribute to the bone healing process.[28-30] Hydroxyapatites accumulated in the organic matrix are composed essentially of calcium and phosphate.[31] It is believed that ALP either intensify the concentration of local, inorganic phosphorus or neutralize inorganic pyrophosphate, an inhibitor of hydroxyapatite crystal formation.[24,30,32]

Table 2: The effects of garden cress seed (6 and 12 g) and spontaneous healing on bone markers of induced-femur fracture rabbits 8 weeks postoperatively (Phase-2)

Groups of phase 2	Calcium (mg/dl)	Phosphorus (mg/dl)	ALP (mg/dl)	Vitamin D (mg/ml)	OC (ng/ml)	OPN (mg/ml)	PTH (mg/ml)	LTF (µg/ml)	PINP (ng/ml)
Control	8.7±0.2	4.5±0.48	166±9.4	0.055±0.004	0.27±0.016	0.32±0.014	0.24±0.02	0.17±0.01	10.02±1.4
GCS (6 g)	12.6±0.58**	5.0±0.5	132.6±11.9*	0.06±0.006	0.19±0.017**	0.31±0.022	0.28±0.005*	0.22±0.018*	15.3±1.5**
GCS (12 g)	11.3±1.4*	4.7±0.65	144.3±13.3	0.06±0.0041	0.25±0.02	0.3±0.01	0.26±0.01	0.23±0.01**	14.0±2.42
Untreated fracture	13.4±0.73	4.9±0.58	157.3±5.6	0.04±0.005	0.23±0.02	0.3±0.02	0.24±0.0170	0.18±0.01	17.1±1.9
Fracture+GCS (6 g)	12.2±1.6	4.9±0.5	144.0±7.0##	0.046±0.002#	0.22±0.016	0.36±0.01##	0.25±0.02	0.19±0.01	17.4±1.9
Fracture+GCS (12 g)	12.8±1.9	5.0±0.4	154.6±5.6	0.04±0.005	0.23±0.02	0.3±0.02	0.26±0.029	0.2±0.01##	17.8±1.4

All data were expressed as mean ±SEM. Values were statistically tested using the student's t test and significant differences at $P<0.05$ and $P<0.01$, as indicated by (*) & (**), compared to control and (#) & (##) compared to untreated fracture respectively. SEM: Standard error of mean; GCS: Garden cress seed; ALP: Alkaline phosphatase, OC: Osteocalcin; OPN: Osteopontin; LTF: Lactoferrin; PTH: Parathormone, PINP: Procollagen I, GCS: Garden cress seed

Table 3: The effects of garden cress seed (6 and 12 g) and spontaneous healing on bone markers of induced-femur fracture rabbits 12 weeks postoperatively (phase-3)

Groups of phase 3	Calcium (mg/dl)	Phosphorus (mg/dl)	ALP (mg/dl)	Vitamin D (mg/ml)	OC (ng/ml)	OPN (mg/ml)	PTH (mg/ml)	LTF (µg/ml)	PINP (ng/ml)
Control	8.7±0.2	4.5±0.48	166±9.4	0.055±0.0053	0.27±0.016	0.31±0.014	0.24±0.02	0.17±0.01	10.0±1.4
GCS (6 g)	15.2±0.49***	6.3±0.4**	124.7±11.68**	0.06±0.005	0.27±0.02	0.34±0.009*	0.29±0.01*	0.2±0.01*	20.4±2.28**
GCS (12 g)	11.9±1.99	4.0±0.3	155.4±6.9	0.059±0.005	0.24±0.01	0.34±0.03	0.24±0.03	0.2±0.01*	24.36±1.6***
Untreated fracture	12.8±1.7	5.5±0.6	121.30±13.9	0.04±0.006	0.22±0.019	0.31±0.019	0.25±0.02	0.19±0.01	15.7±2.5
Fracture+GCS (6 g)	14.8±0.61$^{\#\#}$	6.9±0.4$^{\#\#}$	67.2±4.9$^{\#\#\#}$	0.06±0.005$^{\#\#\#}$	0.18±0.02	0.28±0.015$^{\#}$	0.24±0.004	0.24±0.01$^{\#\#}$	17.0±1.7
Fracture+GCS (12 g)	14.8±1.03$^{\#\#}$	5.9±0.5	81.2±9.8$^{\#\#\#}$	0.06±0.004$^{\#\#}$	0.22±0.022	0.27±0.01$^{\#\#}$	0.22±0.02	0.22±0.01$^{\#}$	15.1±2.5

All data were expressed as mean±SEM. Values were statistically tested using the student's *t*-test and significant differences at $P<0.05$, $P<0.01$, $P<0.001$ as indicated by (*), (**), (***) compared to control and ($^{\#}$), ($^{\#\#}$), ($^{\#\#\#}$) compared to untreated fracture respectively. SEM: Standard error of mean; GCS: Garden cress seed; ALP: Alkaline phosphatase, OC: Osteocalcin; OPN: Osteopontin; LTF: Lactoferrin; PTH: Parathormone, PINP: Procollagen I, GCS: Garden cress seed

In the present study, the serum levels of OPN of rabbits fed on 6 g GCS showed insignificant elevation after 4 weeks post starting of the experiment and significant elevation after 8 weeks post starting of the experiment compared to those of the untreated fractured femur subgroup at the end of the corresponding phases of the study. The role of OPN facilitates osteoclast attachment and directs mineral deposition by influencing crystal size and shape.[33-35] The most remarkable outcome was a significant reduction in fracture toughness owing to OPN deficiency.[36] Fracture toughness is enhanced by many factors including microcracking, porosity, uncracked-ligament bridging, and crack deflection.[37,38]

The levels of serum OC were insignificantly decreased in fractured femur rabbits fed on 6 and 12 g GCS 4 and 8-week post femur fracture compared to those of the untreated fractured femur subgroup at the end of the corresponding phases of the study. This could be due to the repairing process was at the final stage (45 and 60 days after fracture), thus showing decreased OC activity.[39] While, at the end of the last phase of the study, the levels of serum OC were significantly decreased in rabbits fed on 6 g of GCS at the end of the experiment compared to those of the untreated fractured femur subgroup at the end of the corresponding phase of the study.

PTH regulates serum calcium levels by acting mainly on bones and kidneys. Two important functions, for example, are stimulating of renal calcium reabsorption and bone resorption when calcium levels are low.[40] The results of the present study revealed that the levels of serum PTH were insignificantly decreased in fractured femur rabbits fed on 6 and 12 g GCS 4-week post femur fracture compared to those with the untreated fractured femur at the end of the corresponding phase of the study. GCS did not affect PTH levels compared to both the normal control rabbits and rabbits with untreated fractured femur at the end of the 4th phase of the study. On the other hand at the end of both the 2nd and 3rd phases of the study, serum PTH levels showed a significant increase in rabbits fed on 6 g GCS prophylactically and in fractured femur rabbits fed on in 12 g GCS compared to those of the normal control and rabbits with untreated fractured femur, respectively. The increased serum levels of PTH were conforming to the high serum values of Ca at the end of the 3rd phase of the study indicating that renal calcium reabsorption was enhanced by the PTH influence on nephrons.[41]

The results displayed a significant increase in the serum levels of LTF in rabbits fed on 6 and 12 g GCS prophylactically compared to normal control rabbits at the end of the three phases of the study. Moreover, the serum levels of LTF in fractured femur rabbits fed on 6 and 12 g GCS showed a significant increase compared to those with the untreated fractured femur at the end of the 2nd and 3rd phases of the study. This could be due to the impact of GCS to enhance the synthesis of liver function test that accelerate osteogenesis. The serum levels of Vitamin D was insignificantly reduced in rabbits fed on 6 and 12 g GCS prophylactically compared to those of normal control rabbits. Meanwhile, the serum levels of Vitamin D was significantly reduced in fractured femur rabbits fed on 6 and 12 g GCS compared to those with untreated fractured femur at the end of the 1st phase of the study. The values of Vitamin D was insignificantly reduced in rabbits with untreated fractured femur compared to those of normal control rabbits, this agreed with that of Alkalay *et al*. 1989 who stated that studies on animal and human models have demonstrated decreased serum concentrations of Vitamin D following fracture.[42] However, 8 weeks post starting the experiment, the serum levels of Vitamin D was insignificantly increased in rabbits fed on 6 and 12 g GCS prophylactically compared to those of normal control rabbits. While the serum levels of Vitamin D was significantly increased in fractured femur rabbits fed on 6 g and insignificantly increased in fractured femur rabbits fed on 12 g GCS compared to those with untreated fractured femur at the end of the 2nd phase of the study. This could be due to GCS supplementation which might influence the synthesis of Vitamin D. It was reported that Vitamin D supplementation in patients led to significant fracture reduction compared to those not receiving therapy and if Vitamin D appeared to help prevent fracture by affecting the integrity of bone, a conclusion could be drawn that it would help with the bone healing process.[43]

The results of the current study revealed that the serum levels of PINP were significantly increased in rabbits fed on 6 and 12 g GCS prophylactically compared to those of normal control rabbits by the end of the 1st phase of the experiment. Meanwhile, the serum levels of PINP were also significantly increased in fractured femur rabbits fed on 6 and 12 g GCS compared to those with the untreated fractured femur at the end of the 1st phase of the study. This could be due to GCS supplementation which might influence the synthesis of PINP. The sources of PINP other than synthesis at the fracture site should be considered.[44] Furthermore, the serum levels of PINP were also significantly increased in untreated fractured femur rabbits and in fractured femur rabbits fed on 6 and 12 g GCS compared to normal control. However, 8 weeks post starting the experiment, the serum levels of PINP were significantly increased in rabbits fed on 6 g GCS and insignificantly increased in rabbits fed on 12 g GCS compared to those of normal control rabbits. While the serum levels of PINP were insignificantly increased in fractured femur rabbits fed on 6 and 12 g GCS compared to those with untreated fractured femur at the end of the 2nd phase of the study.

At the end of the study, in fractured femur rabbits, the serum levels of PINP were insignificantly increased and insignificantly decreased in rabbits fed on 6 and 12 respectively compared to those with the untreated fractured femur at the end of the 3rd phase of the study.

Meanwhile, the serum levels of PINP were significantly increased in rabbits fed on 6 and 12 g GCS compared to those of normal control rabbits.

The histomorphometric evaluation showed marked improvement of fractured femur rabbits fed on 6 and 12 g of GCS as compared to those of untreated fractured femurs that conforming to the previous work.[45] In some cases, complete resorption of the cartilaginous tissue was achieved. The mean area of the osseous tissue was 73.9 ± 1%, while that of the cartilaginous tissue was 20 ± 2%. Markedly declined cellularity level was noted. Meanwhile, histological sections from rabbits received 12 g of GCS showed more cartilaginous tissue undergoing ossification with the formation of woven bone with the mean area of the cartilaginous tissue was 50.3 ± 2% compared to 32.5 ± 7% in the osseous tissue. In addition, woven bone exceeded the lamellar bone.

The affirmative effect on bone density of GCS could be owing to its high content of Ca and on its capability to elevate serum and liver docosahexaenoic acid, alpha-linolenic acid and eicosapentaenoic acid[46-48] that have been revealed to have advantageous effects on bone. These results are in agreement with formerly described benefits of GCS that enhanced a marked impact on fracture healing in rabbits.[22]

CONCLUSION

It was concluded that GCS enhances bone healing by improving biochemical markers related to osteogenesis and calcification. It is promising as complementary or alternative therapeutic osteogenic agent in bone fracture. Further study needed to explore the mechanism of action of these ingredients in bone healing.

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Conflicts of interest

There are no conflicts of interest.

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