

The effect of daily intake of green coffee bean extract as compared to Agiolax® on the alveolar bone of albino rats

Wpływ codziennego spożycia wyciągu z ziaren zielonej kawy w odniesieniu do preparatu Agiolax® na kość wyrostka zębodołowego szczurów albinosów

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Abstract

Background. Obesity is a worldwide medical problem in which excess body fat is accumulated in the body. The use of weight loss supplements such as green coffee bean extract and Agiolax has become a common trend among people who want to lose weight in a fast and non-tiring way. As a result of their effect on fluid excretion, both of these products may be expected to have a damaging effect on the alveolar bone.

Objectives. The aim of the present study was to evaluate the histopathological effect of green coffee bean extract as compared to Agiolax on the alveolar bone of albino rats.

Material and methods. Twenty-seven adult male albino rats were randomly assigned to 3 groups. Nine received distilled water daily for 2 months by oral gavage (the control group); the other 2 groups received 1 mg/100 g. body weight green coffee bean extract or Agiolax 8 mg/100 g. body weight daily for 2 months by oral gavage (the GC and Ag groups, respectively). The alveolar bones were dissected and examined histologically, histomorphometrically and by western blotting.

Results. The bone area percentage and the calcium level in serum were significantly decreased in the GC and Ag groups, while the calcium level in urine was significantly increased in both the experimental groups as compared to the control group. On the other hand, RANKL expression was significantly increased only in the GC group, and the tissue calcium (Ca) level was significantly decreased only in the GC group as compared to the control group.

Conclusions. Long-term oral administration of green coffee bean extract and Agiolax might lead to alveolar bone loss. A greater deleterious effect was caused by green coffee bean extract, as it caused more RANKL expression, significantly reduced Ca level in the tissue and consequently decreased the bone area percentage.

Key words: obesity, RANKL, alveolar bone, Agiolax, green coffee bean extract

Słowa kluczowe: otyłość, RANKL, kość wyrostka zębodołowego, Agiolax, ekstrakt z ziaren zielonej kawy

Obesity, defined as an abnormal or extensive fat accumulation, is an important health issue. It is a chronic medical condition requiring long-term therapy. It increases the likelihood of various diseases, particularly heart disease, type 2 diabetes, obstructive sleep apnea, certain types of cancer, and osteoarthritis. In addition, obesity can diminish one's quality of life.^{1,2} Obesity is the 5th leading risk for global deaths, killing more people than underweight; at least 2.8 million adults die as a result of obesity each year.³

Weight loss reduces susceptibility to weight-related diseases, decreases pain, increases physical functions and improves the overall quality of life. Several options for weight loss exist, ranging from medications to exercise, dietary modification, and surgery.⁴ Dietary supplements, which include vitamins, minerals, herbs, and amino acids, are widely used for weight loss, muscle building and sexual function.⁵

Green coffee bean extract (GCBE) is widely marketed as a dietary supplement aiding in weight control. It functions by blocking starch absorption by means of an α amylase inhibitor. Biologically, 3 major components of coffee are considered to contribute to its beneficial effects: chlorogenic acids, caffeine and the coffee diterpenes (cafestol and kahweol).^{6,7}

Shimoda et al. reported that oral administration of GCBE for 13 days reduced visceral fat and body weight by inhibiting fat absorption and activating fat metabolism in the liver.⁸ Vinson et al. also demonstrated the efficacy of GCBE in reducing body weight, body mass index and body fat percentage.⁹

Laxatives are usually used to speed up the digestion process, to avoid chronic constipation and also to regulate bowel movements.¹⁰ Lately, laxatives are among the drugs that are frequently used among those looking to lose weight quickly. It has been estimated that more than 4% of the general population engages in laxative abuse.¹¹

Agiolax is a combination of bulk and stimulant laxatives, as it contains natural dietary fiber from the seeds and husks of *Plantago ovata* (PO) combined with the herb senna.¹² Each sachet of 5 g contains 2.60 g seeds of *Plantago ovata* (PO), 0.62 g of tinnevelly senna pods and 0.11 g of ispaghula husk. The oral administration of senna to Dawley rats resulted in a slight reduction in the rats' body weight, increased water consumption and caused notable changes in electrolyte levels in serum and urine.¹³ Romero et al. demonstrated the hypolipidemic effect of PO on male guinea pigs, whose plasma triglycerides and cholesterol LDL were significantly lowered, while the activity of cholesterol hydrolase was significantly increased.¹⁴

Objectives

Since green coffee bean extract and Agiolax are commonly used worldwide as weight loss and laxative dietary supplements, the aim of the present study was to investigate their histopathological effects on the alveolar bone of albino rats upon daily administration.

Material and methods

Experimental procedures

Twenty-seven adult male inbred Wistar albino rats, with an age range from 3 to 4 months and weight range from 150 to 200 g, were used in the study. The animals were housed in a sterile, controlled environment (temperature $25 \pm 2^\circ\text{C}$ and 12 h dark/light cycles) and fed a standard pellet diet and tap water. All the experiment procedures were conducted in the animal house of the Faculty of Medicine, Cairo University (Egypt), in accordance with the recommendations and approval of the Institutional Animal Care and Use Committee (IACUC), Cairo University, Egypt (approval No: CU/III/F/18/18).

The rats were randomly distributed into 3 groups of 9 rats each. The control group received 5 mL of distilled water via oral gavage a daily for 60 days. The green coffee bean extract (GC) group received 1 mg/100 g. body weight of green coffee bean extract (standardized to contain 45% chlorogenic acid; Puritan's Pride, Oakdale, USA) in 5 mL of distilled water via oral gavage daily for 60 days. The Agiolax (Ag) group received 8 mg/100 g. body weight of Agiolax (granulated mixture of *Plantago ovata* seeds, tinnevelly senna pods and ispaghula husk; Madaus/CID, Giza, Egypt) in 5 mL of distilled water via oral gavage daily for 60 days.

The animals were sacrificed by ketamine overdose and the mandibles were dissected. The right side of each mandible was used for light microscopic examination and histomorphometric analysis. The left sides of the mandibles were used for detection of the calcium (Ca) level in the bone tissue and for assessment of RANKL expression using western blotting.

Light microscopic examination

The specimens were fixed in 10% neutral formalin for 48 h, washed, soaked in 10% ethylene diamine tetra-acetic acid (EDTA) for decalcification for 6 weeks and then rinsed in distilled water. The specimens were dehydrated in ascending grades of alcohol and embedded in paraffin.

Mesiodistal sectioning of the right side of the jaws was carried out. Histological sections (5 μm thick) were prepared. The sections were subjected to hematoxylin and eosin (H&E) stain according to the conventional method. Histopathological examination was performed using a light microscope.

Histomorphometric analysis

A Leica Qwin 500 image analyzer computer system (Leica Micro Systems Ltd., Milton Keynes, UK) was used for the analysis. In the hematoxylin and eosin-stained sections, the bone volume, defined as the percentage of trabecular bone volume to tissue volume, was measured.¹⁵

The measurements were taken using an objective lens of magnification 10, that is, a total magnification of 100. Ten fields were measured from each group and the mean values were calculated.

Western blotting

The antibody used was the antigen affinity purified receptor activator of nuclear factor- κ B ligand (RANKL) monoclonal antibody (Santa Cruz Biotechnology Inc., Dallas, USA). The Ready Prep™ protein extraction kit (Bio-Rad Inc., Hercules, USA) was used for protein extraction from the tissues. The Bradford Protein Assay Kit (Bio Basic Inc., Markham, Canada) was used for the quantitative protein analysis. Sample proteins were then separated on polyacrylamide gel (TGX Stain-Free FastCast Acrylamide Kit, Bio-Rad Inc.), loading 20 μ g of total protein per each mini-gel well. The gel was then assembled in transfer sandwiches with polyvinylidene difluoride (PVDF) membranes. The blot was then run to allow protein bands to transfer from gel to membrane using BioRad Trans-Blot Turbo. The PVDF blots were incubated in 5% non-fat dry milk, Tris-HCL, 0.1% Tween 20 for 1 h. RANKL antibody was then added to the membrane containing the specimen samples and incubated at 4°C overnight. An appropriate secondary antibody was incubated for 2 h at room temperature. After the specimens were washed twice in 1 \times TBS-T, a densitometric analysis of the immunoblots was performed to quantify the amounts of RANKL in all the samples against control sample beta actin (housekeeping protein) by protein normalization using the Chemi-Doc MP imaging system.

Biochemical analysis

After the completion of the experiment and before the animals were sacrificed, urine and blood (serum) samples were obtained from each group. Following the sacrifice of the animals, tissue samples from left mandibles were obtained from each group. The level of calcium was measured in all these samples.

Statistical analysis

Values were presented as mean and standard deviation (SD) values. The data was checked for normality using the Kolmogorov-Smirnov test. The results of the Kolmogorov-Smirnov test indicated that most of the data was normally distributed (parametric data), therefore a one-way analysis of variance (ANOVA) test was used between groups. When the ANOVA yielded a significant difference, it was followed by Tukey's post hoc test. The significance level was set at $p < 0.05$. The statistical analysis was performed using SPSS for Windows v. 18.0 (SPSS, Inc., Chicago, USA).

Results

Histological results

The alveolar bone of the control group showed regular shape and orientation. It consisted of interconnected bone trabeculae containing osteocytes in their lacunae with intervening red bone marrow spaces (Fig. 1A, B).

The histological sections of the mandibles from the green coffee bean extract group showed marked changes in the alveolar bone. The marrow spaces were extremely widened and interconnected, containing red marrow and multinucleated large cells (Fig. 2A). Some marrow spaces showed fatty bone marrow (Fig. 2B). Wide white spaces with irregular scalloped surfaces (reversal lines) representing resorbed areas of bone were observed (Fig. 2A–C). Osteocytes appeared with widened lacunae and shrunken nuclei. Few lacunae appeared empty (Fig. 2C).

The alveolar bone of Agiolax group revealed multiple medium sized irregular bone marrow spaces (Fig. 3A). A marked increase in lines of demarcation, cement lines and reversal lines gave the alveolar bone a mosaic pattern (Fig. 3B). The lines of demarcation separated newly formed bone surrounding the marrow spaces with uniform staining patterns, bone with exposed collagen fibers, extensive vacuolation and empty osteocyte lacunae (Fig. 3C).

Histomorphometric analyses

Significant differences in the area percentage occupied by alveolar bone trabeculae between specimens of all the study groups were noted. A significant decrease in bone area percentage was observed in the jaws of the 2 experimental groups (GC and Ag) as compared to the control group ($p = 0.000$). Moreover, a significant decrease in bone trabeculae area percentage in the GC group as compared to the Ag group was revealed ($p = 0.002$; Table 1).

RANKL expression

Significant differences in RANKL expression were found between all the groups. The highest mean value for RANKL expression was detected in the GC group, whereas the lowest mean value was recorded in the control group, with a significant difference between the 2 ($p = 0.000$). Moreover, a significant increase in RANKL expression was detected in the GC group as compared to the Ag group ($p = 0.001$), while a nonsignificant difference was revealed between the Ag group and the control group ($p = 0.534$; Table 1).

Biochemical results

Differences were noted in Ca levels between all the groups in serum, urine and bone tissue.

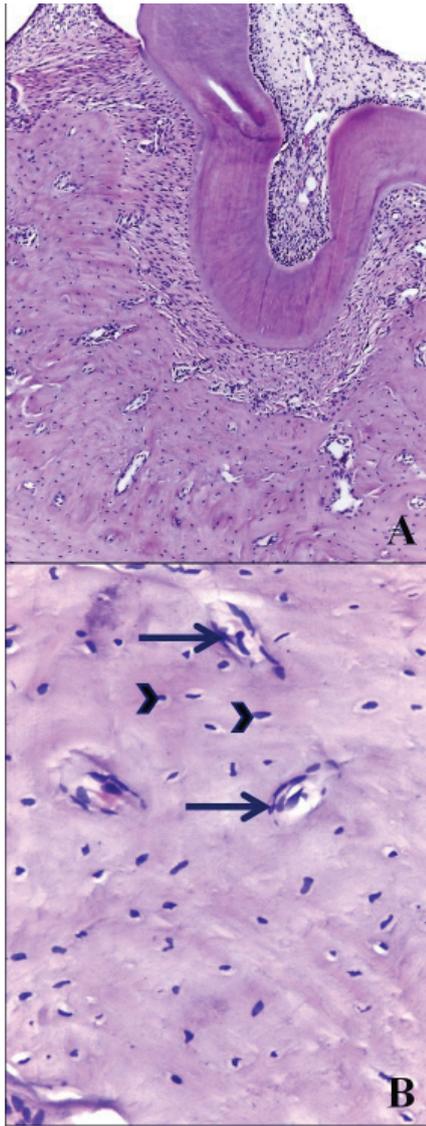


Fig. 1. A photomicrograph of an alveolar process from the control group

A – the normal structure of the alveolar bone; B – bone marrow spaces (arrows), bone trabeculae and osteocytes in their lacunae (arrowheads) (H&E, orig. mag. A: $\times 10$, B: $\times 40$)

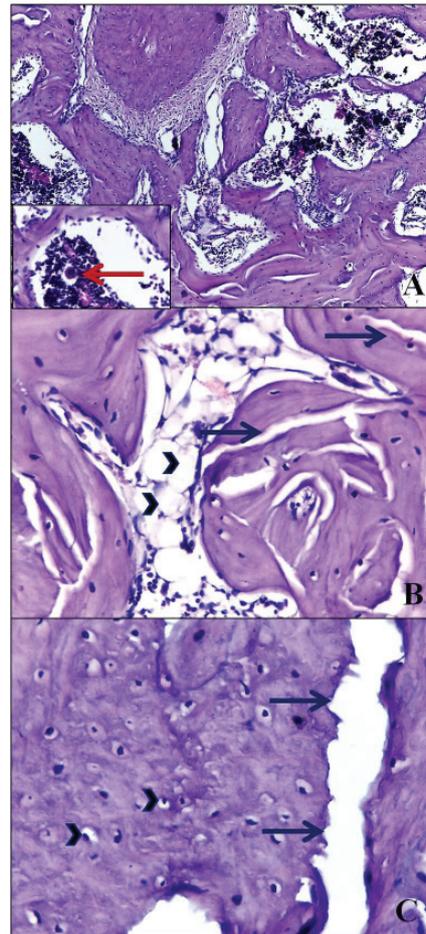


Fig. 2. A photomicrograph of an alveolar process from the GC group

A – wide interconnected marrow spaces enclosing bone trabeculae; inset showing a large multinucleated cell in a marrow space (arrow); B – multiple sequestered bone trabeculae with scalloped borders (arrows) surrounded with fatty bone marrow (arrowheads); C – bone trabeculae with scalloped borders (arrows) as well as osteocytes with widened lacunae and shrunken nuclei (arrowheads). Few lacunae were empty (H&E, orig. mag. A: $\times 10$, inset, B and C: $\times 40$)

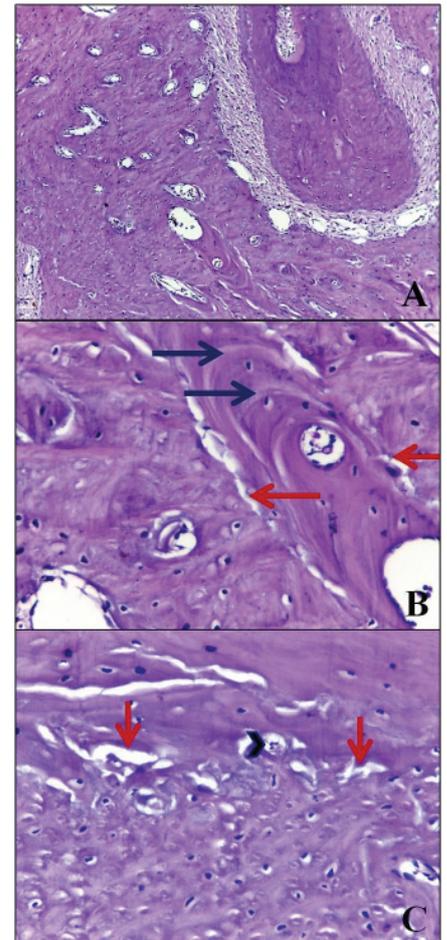


Fig. 3. A photomicrograph of a rat alveolar bone from the Ag group

A – multiple relatively wide marrow spaces; B – a higher magnification showing cone-shaped rest lines (arrows) surrounding marrow spaces, scalloped lines of demarcation (red arrows) giving the bone a mosaic pattern appearance; C – lines of demarcation (red arrows) that separate newly formed bone with uniform staining, osteocytes occupying their lacunae and bone with multiple vacuoles that represent empty, widened lacunae and exposed collagen fibers. Osteoclasts (arrowhead) were evident on this demarcation line (H&E, orig. mag. A: $\times 10$, B and C: $\times 40$)

In serum, a significant decrease in Ca level in the Ag group and the GC group as compared to the control group was revealed ($p = 0.000$). Despite the calcium level being lower in the Ag group than the GC group, the difference between them was not significant ($p = 0.977$; Table 1).

On the other hand, the calcium level in urine was significantly increased in the Ag group and the GC group as compared to the control group ($p = 0.000$). Although the calcium level in the urine of the Ag group was higher than in the GC group, there was no significant difference between them ($p = 0.275$; Table 1).

Regarding Ca levels in the mandible, the highest mean value was detected in the control group, whereas the lowest mean value was recorded in the GC group, with a significant difference between the 2 groups ($p = 0.006$). No

significant differences in tissue Ca levels were revealed between the GC group and the Ag group ($p = 0.413$) or between the Ag group and the control group ($p = 0.102$; Table 1).

Discussion

Over the last 20 years, obesity has become the most prevalent nutritional problem in the world, affecting not only adults but also children and adolescents. It is the key risk factor for many chronic and noncommunicable diseases.¹⁶ Dietary supplements for weight loss encompass a wide variety of products. Herbs, dietary fiber, caffeine and minerals are common ingredients in these supplements.¹⁷

Table 1. Comparison of values of different parameters between groups (ANOVA test)

Measured items	Groups	Mean \pm SD	95% confidence interval for mean		p-value
			lower	upper	
Bone area [%]	control	40.91 ^a \pm 4.37	37.26	44.57	0.000*
	green coffee bean extract	22.01 ^c \pm 2.23	20.15	23.87	
	Agiolax	30.18 ^b \pm 4.98	26.01	34.34	
RANKL	control	2.95 ^b \pm 0.20	2.78	3.11	0.000*
	green coffee bean extract	3.76 ^a \pm 0.22	3.58	3.94	
	Agiolax	3.11 ^b \pm 0.42	2.75	3.46	
Ca in serum [mg/dL]	control	13.16 ^a \pm 1.97	11.5	14.8	0.000*
	green coffee bean extract	7.88 ^b \pm 1.82	6.36	9.40	
	Agiolax	7.66 ^b \pm 2.64	5.46	9.87	
Ca in urine [mg/mL]	control	119.65 ^b \pm 9.14	112.0	127.3	0.000*
	green coffee bean extract	263.14 ^a \pm 83.6	168.1	358.2	
	Agiolax	332.81 ^a \pm 101.3	248.2	417.5	
Ca in tissue [mg/mg]	control	11.13 ^a \pm 2.03	9.43	12.82	0.000*
	green coffee bean extract	8.13 ^b \pm 1.25	7.08	9.17	
	Agiolax	9.25 ^{ab} \pm 1.83	7.72	10.78	

Significance level – $p < 0.05$; SD – standard deviation; * significant; values sharing the same superscript letter within the same comparison are not significantly different in Tukey's post-hoc test.

In the current study, oral administration of green coffee bean extract caused obvious changes in the alveolar bone of rats' mandibles. There were multiple huge marrow spaces and wide white spaces with irregular scalloped surfaces (reversal lines), representing resorbed areas of bone. These results coincide with Liu et al., who reported that caffeine effectively increased bone resorption activity via enhancement of the receptor activator of NF- κ B ligand (RANKL).¹⁸ Moreover, Tsuang et al. demonstrated that adding caffeine to the culture medium of osteoblasts significantly increased the intracellular prostaglandin PGE₂ content and PGE₂ secreted into the medium.¹⁹ Prostaglandins (PGs) are local mediators that stimulate osteolysis in bone organ cultures, and when administrated systemically or locally in vivo, result in increased bone loss. The authors added that caffeine may induce osteoblast apoptosis, which then led to decreased bone cell viability.

Moreover, the histological results of the present study demonstrated osteocytes with shrunken nuclei and widened lacunae in the green coffee group. The latter finding is in accordance with Wysolmerski, who reported that osteocytes appeared with large sized lacunae due to perilacunar and pericanalicular matrix remodeling that occurred as a result of high levels of parathyroid hormone (PTH) in response to an increased systemic demand for calcium.²⁰

In the current study, large multinucleated cells were observed in the bone marrow spaces in the GC group. These cells might be osteoclast precursor cells and their presence in large amounts could be attributed to the production of osteoclast-inducing cytokines such as RANKL, which play a major role in tissue destruction.²¹

Interestingly, both the biochemical results and the histomorphometric data of our study supported the histopathological results, as the measured Ca level in serum and in the alveolar bone tissue in the GC group was significantly decreased in comparison to the control group, but in the urine it was markedly increased. Furthermore, a decrease in the bone area percentage was found in the GC group as compared to the control group ($p = 0.000$). Moreover, the highest level of RANKL expression was reported in the GC group, indicating increased bone resorption activity. These findings are supported by Rapuri et al., who detected significant increased bone loss in the spine in a high caffeine consumption group compared with subjects with lower caffeine consumption.²²

Lacerda et al. reported similar results when they investigated the effects of coffee on bone metabolism in male rats born of females treated daily with coffee and with coffee intake since birth.²³ The results showed significantly greater amounts of calcium in the plasma and urine and significantly less calcium in bone. Moreover, the significant decrease in the Ca level in the mandibular bone tissue in the GC group in the current work is consistent with Shapses and Riedt, who reported that decreased calcium absorption during caloric restriction for weight reduction led to high serum levels of PTH.²⁴ Parathyroid hormone is secreted in response to low blood Ca levels, and it indirectly stimulates osteoclast activity to release more calcium into the blood through RANKL expression.²⁵

With regard to the Agiolax group, the results of the present study demonstrated a mosaic pattern of the alveolar bone with several demarcation and reversal lines, denoting bone remodeling. Irregular white spaces surrounded by reversal lines were seen, representing resorbed areas

of bone. Newly deposited bone was observed around the marrow spaces. The lines of demarcations separated newly formed bone (surrounding the marrow spaces with uniform staining patterns and many rest lines) and bone (with exposed collagen fibers, extensive vacuolation and empty osteocytes lacunae).

The histomorphometric results of the study confirmed the histological results, which showed a significant decrease in bone area percentage in the Ag group compared to that of the control group, and a significant increase in the bone area percentage in the Ag group compared to the GC group. However, the RANKL level was slightly increased in the Ag group compared to that of the control group; this difference did not reach the level of statistical significance. The biochemical findings denoted a significant increase in the Ca level in urine, a significant decrease in the Ca level in serum and a nonsignificant decrease in the Ca level in the mandible bone tissue in the Agiolax group as compared to the control group. Therefore, the exposed collagen fibers observed in the Ag group could be related to the negative effect of Agiolax on calcium retention, as long-term Agiolax abuse leads to severe electrolyte and water losses.²⁶

The results for the Ag group in the present study are supported by Lin et al., who reported that the administration of senna (one of the main components of Agiolax) and rheum polysaccharides led to a significant decrease in free Ca^{+2} levels in rats' liver cells as compared to a control group ($p < 0.01$).²⁷ Moreover, Mitchell et al. reported that the oral administration of different doses of senna to Dawley rats increased potassium and chloride in serum and decreased sodium, potassium and chloride in urine.¹³

By comparing bone area percentage results, RANKL expression and the biochemical results of the green coffee group with the Agiolax group, we found that the bone area percentage was significantly decreased in the Agiolax group, while RANKL expression was significantly increased. No significant differences in Ca levels were reported between the 2 experimental groups. These outcomes support that green coffee (caffeine) has a direct effect on the viability of bone cells, as it induces osteoblast apoptosis and enhances osteoclast activity, which means bone repair in the GC group is reduced compared with that in the Ag group.

He et al. reported that apoptosis was induced by caffeine at a relatively low concentration in JB6 Cl41 cells, and that the percentage of apoptotic cells gradually increased as the caffeine concentration was increased.²⁸ Bode et al. investigated the effect of caffeine on cell cycle function.²⁹ At concentrations $< 1\mu\text{M}$, caffeine has been reported to induce p53-dependent apoptosis associated with increased expression of pro-apoptotic Bax and caspase-3. At concentrations of $1\text{--}2\mu\text{M}$, caffeine induced G1 arrest, whereas concentrations of $2\text{--}4\mu\text{M}$ appeared to block G1 arrest and induced apoptosis.

The bone repair that was observed in most of the specimens from the Ag group denoted that Ag acts as a diuretic, causing change in body electrolytes without affecting bone cell viability; the newly formed bone trabeculae surrounding the marrow spaces had uniform staining, while old bone stained differently and revealed exposed collagen fibers and vacuoles. This denoted that old bone became poorly calcified (less mineralized), which was supported by the significant increase of Ca in urine.

The empty and widened osteocyte lacunae detected in the Ag group in the current research could be explained by Sohn et al., who reported that the products of PO fermentation induced apoptosis in colorectal cancer cells by increasing expression of caspase activation, up-regulation of B-cell lymphoma protein 2 homologous antagonist killer (BAK) and death receptor (DR5).³⁰

Conclusions

The overall results of the present study clearly evidenced that long-term oral administration of green coffee bean extract and Agiolax led to alveolar bone loss, which was demonstrated in the rats' mandibles using histopathological, histomorphometric, western blotting and biochemical analyses. A greater deleterious effect was caused by green coffee bean extract, as it caused more RANKL expression, lower Ca levels in the tissue and subsequently less bone area percentage. Further investigations are needed to clarify the mechanism of the action of Agiolax on bone cells.

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