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Hancornia speciosa Gomes Latex Increases Bone Mineralization in Rats: A Preclinical Study*

Látex de Hancornia speciosa Gomes aumenta a mineralização óssea em ratos: Um estudo pré-clínico

Francielly Andressa Felipetti[1] Victor Seabra Lima Prado Costa[2] Juliana dos Santos Neves[1] Ingrid Grazielle Sousa[1] Sônia Maria De Stefano Piedade[3] Pedro Duarte Novaes[1]

[1] Departament of Morphology, Faculdade de Odontologia de Piracicaba (FOP), Universidade Estadual de Campinas (UNICAMP), Piracicaba, SP, Brazil
[2] Departament of Health Sciences, Universidade Federal de Santa Catarina (UFSC), Araranguá, SC, Brazil
[3] Departament of Exact Sciences, Escola Superior de Agricultura Luiz de Queiroz (ESALQ), Universidade de São Paulo (USP), Piracicaba, SP, Brazil

Address for correspondence Francielly Andressa Felipetti, PhD, Departamento de Ciências da Saúde, Universidade Federal de Santa Catarina (UFSC), Rua Governador Jorge Lacerda, 3.201, Jardim das Avenidas, Araranguá, 88900-000, SC, Brazil (e-mail: fafelipetti@gmail.com).

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Abstract

Objective To evaluate the systemic effect of *Hancornia speciosa* latex on bone neoformation and mineralization in rats.

Methods For that, the latex was first collected, and its composition was analyzed. A total of 30 male Wistar rats were used, which were simultaneously submitted to two surgical procedures: extraction of an incisor and creation of a defect with 2 mm in diameter in the parietal bone. The rats were divided into two groups: systemic control (SC) systemic latex (SX) which were administered, orally and daily, 1.5 mL of water or a solution containing 50% of water and 50% of latex by gavage, respectively. After 15 days of the treatment, the animals were euthanized and their samples were collected.

Results The results were statistically analyzed, and the level of significance was set at 0.05. We showed that *H. speciosa* latex contained calcium. The oral and daily administration of the latex for 15 days increased the contents of calcium and phosphorus in the basal bone and newly-formed bone in the mandibular alveolus of rats.

Conclusion The present was a pioneer study demonstrating the potential of *H. speciosa* latex in increasing bone mineralization. Our results may aid in the conception and development of a natural drug.

Keywords
- apocynaceae
- complementary therapies
- histology
- hydroxyapatites
- scanning electron microscopy

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Resumo

Objetivo Avaliar o efeito sistêmico do látex de *Hancornia especiosa* na neoformação óssea e mineralização em ratos.

Métodos Para isso, primeiro o látex foi coletado, e sua composição foi analisada. No estudo, foram utilizados 30 ratos Wistar machos submetidos simultaneamente a dois procedimentos cirúrgicos: extração de incisivo e criação de um defeito de 2 mm de diâmetro no osso parietal. Os ratos foram divididos em dois grupos: controle sistêmico (CS) e látex sistêmico (XS), aos quais foi administrado, oral e diariamente, 1,5 mL de água ou uma solução contendo 50% de água e 50% de látex por gavagem, respectivamente. Após 15 dias do tratamento, os animais foram eutanizados, e suas amostras, coletadas.

Resultados Os resultados foram analisados estatisticamente, e o nível de significância foi fixado em 0,05. Mostramos que o látex de *H. speciosa* continha cálcio. A administração oral e diária deste látex por 15 dias aumentou o conteúdo de cálcio e fósforo de osso basal e de osso recém-formado no alvéolo mandibular de ratos.

Conclusão Este foi um estudo pioneiro, que demonstrou o potencial do látex de *H. speciosa* no aumento da mineralização óssea. Nossos resultados podem ajudar na concepção e no desenvolvimento de uma droga natural.

Palavras-chave

- apocynaceae
- terapias complementares
- histologia
- hidroxiapatitas
- microscopia eletrônica de varredura

Introduction

Bone is a mineralized connective tissue mainly composed of osteoblasts, osteoclasts, bone lining cells, and osteocytes. These cells are essential for the bone regeneration process, and play an important role after the development of bone defects.[1–5] However, in extensive bone defects, the repair must be supported by other biological products.[6,7]

Some biological products have an important osteogenic potential, such as the latex extracted from the trunk of *Hevea brasiliensis* (rubber tree)[6–11] and *Hancornia speciosa* Gomes (*mangaba* tree, or *mangabeira*, in Portuguese).[12–14]

A study[12] conducted with *H. speciosa* latex has shown that its topical application increased the area of newly-formed bone on the calvarial defect of rats. Besides that, there are also popular beliefs in Brazil that support the benefits of this product. In Northeastern Brazil, some communities collect the *H. speciosa* latex and mix it with water to obtain a 50% latex solution called"leite da mangaba" (*mangaba* milk), which is used in the treatment of bone fractures.[13] However, to date, no studies have been conducted to confirm this effect.

These findings motivated us to study the regenerative potential of the *H. speciosa* latex. Therefore, the present study aims to assess the effect of the oral administration of *H. speciosa* latex on bone neoformation and mineralization in Wistar rats.

Materials and Methods

Ethical Statement

The procedures were performed according to the guidelines of the Brazilian National Council for Animal Experimentation Control (Conselho Nacional de Controle de Experimentação Animal, CONCEA, in Portuguese), and were approved by the institutional Ethics Committee on the Use of Animals (under protocol 37901). The study data were developed based on a PhD thesis, and are available in the university repository at the following link: http://acervus.unicamp.br/index.asp?codigo_sophia=987041.

Study Design and Experimental Procedures

The present study was designed according to the Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines.[15] The sample size was calculated using the data obtained from the pilot project (protocol: 34271) and the following formula: $n = (N[S]^2\ [t]^2) / (N(Ex\text{-})^2 + [S]^2\ [t]^2$. A total of 30 healthy male Wistar rats of the HanUnib strain, with an average weight od 390 g, and age of 10 weeks, were obtained from a center for biological research at the university and accommodated at its licensed bioterium. The rats were housed in individual plastic cages with bedding materials, and maintained under standard conditions of temperature and light (12:12h light-dark cycle). To minimize potential confounders regarding the order of treatment of each rat, the cages were numbered individually. They received distilled water and rodent feed ad libitum. The rats were anesthetized with an intraperitoneal injection of 80 mg/kg ketamine (Dopalen, Sespo Indústria e Comércio Ltda., Paulínia, SP, Brazil) and 8 mg/kg xylazine (Rompun, Bayer SA, São Paulo, SP, Brazil). Then, two procedures were simultaneously performed: extraction of the lower-left incisor and creation of a defect in the left parietal bone (diameter: 2 mm),[12] using an adapted diamond drill bit number 4142 (KG Sorensen, Cotia, SP, Brazil) on a low-speed handpiece (KAVO, Kaltenback & Voigt, São Paulo, SP, Brazil) with mounted irrigation with physiological saline solution. These procedures developed a bone injury to verify the potential of the latex regarding bone regeneration and mineralization. After surgery, the rats were administered 2 mg/mL of tramadol hydrochloride intramuscularly (Tramal, Grunenthal

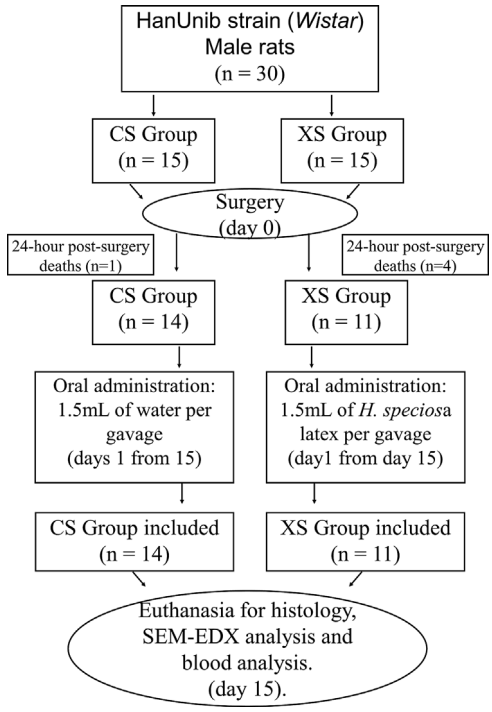

Fig. 1 Flowchart of the experimental protocol with the number of animals used, those that died, and those that were included in the study.

do Brasil Farmacêutica Ltda, São Paulo, SP, Brazil) and were monitored for 24 hours. To minimize the effects of subjective bias during the allocation to treatment, the rats were randomly divided into 2 groups: systemic control (SC, $n = 15$) and systemic latex (SX, $n = 15$). The rats were numbered, and randomization was performed using sealed opaque envelopes. The day after the surgery, we noticed that five rats had died, and these animals were excluded from the study. Therefore, the SC group was then composed of 14 animals, and the SX group, of 11 (►**Fig. 1**). The SX was submitted to a regimen of 5 hours of fasting, followed by the administration by gavage of 1.5 mL of a solution containing 50% of water and 50% of latex. The SC group was treated the same way, but with the administration of 1.5 mL of distilled water. As recommended by CONCEA, 15 days after the surgery, all rats were euthanized by cervical dislocation, and their calvaria, hemimandible, stomach, and blood were collected.

Experimental Outcomes Assessed

All of the analyses (of the latex, of the blood, of histology, and through scanning electron microscopy [SEM] with energy dispersive X-ray [EDX] spectroscopy) were performed by a single-blind examiner, previously trained on animal care, laboratory, and histological techniques.

Latex Collection and Analysis

H. speciosa latex was collected in the town of Mata de São João, state of Bahia, Brazil (12°27′42″S 37°56′38″O 69NE),[12] and mixed with distilled water (ratio: 1:1) to obtain a 50% latex solution. The solution was stored in syringes at 4°C. Then, the *H. speciosa* latex was diluted eleven times and subjected to colorimetry with arsenazo III and molybdic acid to assess the presence of calcium (Ca) and phosphorus (P).

Blood Analysis

From each rat, a total of 1 mL of blood was collected and centrifuged at 3,000 g-force (5,000 rpm) for 10min at 4°C. After centrifugation, 450 µL of the supernatant containing plasma was collected and examined by the colorimetric method using a calcium arsenazo III Kit and inorganic phosphorus by ultraviolet photometry (phosphorus UV) with the BS 120-Mindray/Bioclin (Bioclin, Belo Horizonte, MG, Brazil) automation equipment. The concentrations of Ca and P were recorded in mg/dL and compared between the groups.

Histological Processing

The stomach, hemimandible, and calvaria were fixed in Karnovsky solution and subjected to conventional histological processing.[16] First, the stomach was cut in halves, and a ring-shaped portion was collected. Second, the hemimandible was sectioned at the level of the mesial surface of the first molar, and two (one anterior and one posterior) fragments were obtained. Third, the calvaria was transversely sectioned, and only the bone portion was obtained. Subsequently, the anterior fragment of the hemimandible and the calvaria were decalcified with an ethylene diamine tetraacetic acid (EDTA) solution at 4%, and pH of 7.4, for 1 month. The ring-shaped portion of the stomach, the clavaria defect, and the anterior fragment of the hemimandible were dehydrated, diaphonized, and embedded in paraffin.[16] Sections were collected, the slides were prepared (stained with hematoxylin/eosin) and observed under a light microscope.

Descriptive and Histomorphometric Analysis

The slides were photographed using the OpticaView7 software. The images recorded were analyzed by a trained single examiner using the Image J software. The stomach, mandibular alveolus, and calvarial morphology were demonstrated using descriptive analysis. The are of newly-formed bone within the entire mandibular alveolus and calvarial bone defect were examined using histomorphometric analysis. The quantified values were compared between the groups.

SEM-EDX Analysis

The posterior fragment of the hemimandible was washed with phosphate-buffered saline (PBS), dehydrated with an increasing ethanol series, exposed to room temperature for drying, and attached on aluminum stubs. After conductive carbon coverage, the mandibular alveolar region was analyzed using SEM-EDX.[17] Once the sample image was acquired by SEM, the following areas of interest were selected: vestibular and proximal regions of the newly-formed bone and the basal bone. These regions were examined by EDX (acceleration voltage of 15 kV; working distance of 20 mm; acquisition time of 100 s). The atomic compositions were recorded, and the Ca/P ratio was calculated. The averages of the atomic content and proportions were compared between the groups.

Table 1 Plasma concentration of calcium and phosphorus

Groups	Total calcium (mg/dl)	Total phosphorus (mg/dl)
Systemic control	8.79 ± 0.83 A	5.37 ± 1.32 A
Systemic latex	8.89 ± 0.52 A	5.05 ± 1.19 A

Note: Values are expressed as means ± standard deviations. Equivalent letters indicate that there is no statistically significant difference between the groups, as calculated using the *t*-test with $p < 0.05$.

Statistical Analysis

Data were analyzed using the R (R Foundation for Statistical Computing, Vienna, Austria) software. Once the homogeneity of variance and normal distribution was confirmed with the Bartlett and Shapiro-Wilk tests, either the *t*-test or Welch-test was used to compare the SC and SX groups. Significance was set at 5% ($p < 0.05$).

Results

Latex Analysis

The *H. speciosa* latex diluted 11 times contained 0.1780 mg/mL of Ca. The concentration of P was not significant.

Analysis of Plasma Ca and P

►**Table 1** shows that both groups had an equal amount (mg/dl) of total Ca and P in blood plasma.

SEM-EDX Analysis

The results revealed that the main elements of all the evaluated samples and bone regions were sodium (Na), magnesium (Mg), P, and Ca.

Mineralization of the Basal Bone in the Mandibular Alveolus

We observed a similar Na content (%) in both groups. However, the content of Mg (%) was reduced by ∼ 50%, whereas those of Ca (%) and P (%) increased, respectively, ∼ 20% and 15% in the basal bone in the SX group when compared with the SC group (►**Table 2**). The Ca/P ratio in the SX group was elevated because the increase in Ca content was higher than that of the P content. An increase in the Ca and P contents indicates a higher degree of mineralization.

Mineralization of the Newly-Formed Bone in the Mandibular Alveolus

On the newly-formed bone in the mandibular alveolus, we observed a similar Na content in both groups. However, the content of Mg was reduced by ∼ 40%, whereas those of Ca and P increased by ∼ 19% each in the newly-formed bone in the SX group when compared with the SC group (►**Table 3**). The Ca/P ratio was similar for both groups because of an equal increase in the Ca and P contents. Nevertheless, the newly-formed bone in the SX group was more mineralized due to its higher mineral content.

Histological Analysis of Mandibular Alveolus and Calvarial Defect

We used these analyses to evaluate the newly-formed bone in the samples. A similar bone repair stage between both groups was observed. The trabeculae of the newly-formed bone extended from the edges of the basal bone toward the center of the defect (►**Fig. 2**) and the mandibular alveolus (►**Fig. 3**). The amount of new bone in these structures was also similar in both groups (►**Fig. 4**).

Histological Analysis of Stomach Morphology

We evaluated the morphology of the body region of the stomach, and we observed that the stomachs of the rats on the SX group presented normality in its four layers (►**Fig. 5**). The mucosal layer exhibited regularity throughout the entire extension of the epithelium. No damage was observed in the gastric pit and gastric glands. The submucosal layer tipically exhibits large blood vessels and nerves

Table 2 Semi-quantitative chemical analysis of the basal alveolus (SEM-EDX)

Groups	Sodium (%)	Magnesium (%)	Calcium (%)	Phosphorus (%)	Calcium/Phosphorus ratio (%)
Systemic control	1.09 ± 0.09 A	0.82 ± 0.05 A	50.54 ± 3.52 A	23.36 ± 1.32 A	2.14 ± 0.03 A
Systemic latex	0.82 ± 0.09 A	0.42 ± 0.12 B	63.1 ± 1.93 B	27.33 ± 0.53 B	2.30 ± 0.03 B

Abbreviation: SEM-EDX, scanning electron microscopy with energy dispersive X-ray.
Notes: Values are expressed as means ± standard errors. The percentages of sodium, calcium, and phosphorus were evaluated using the *t*-test, and that of magnesium, using the Welch test. Different letters indicate a statistically significant difference in the results, with $p < 0.05$.

Table 3 Semiquantitative chemical analysis of the newly-formed bone in the alveolus (SEM-EDX)

Groups	Sodium (%)	Magnesium (%)	Calcium (%)	Phosphorus (%)	Calcium/Phosphorus ratio (%)
Systemic control	0.98 ± 0.07 A	0.80 ± 0.02 A	51.98 ± 3.79 A	21.82 ± 1.37 A	2.36 ± 0.04 A
Systemic latex	0.75 ± 0.09 A	0.47 ± 0.12 B	63.86 ± 1.90 B	26.75 ± 0.53 B	2.38 ± 0.04 A

Abbreviation: SEM-EDX, scanning electron microscopy with energy dispersive X-ray.
Notes: Values are expressed as means ± standard errors. The percentages of sodium, calcium, and phosphorus were evaluated using the *t*-test, and that of magnesium, using the Welch test. Different letters indicate a statistically significant difference in the results, with $p < 0.05$.

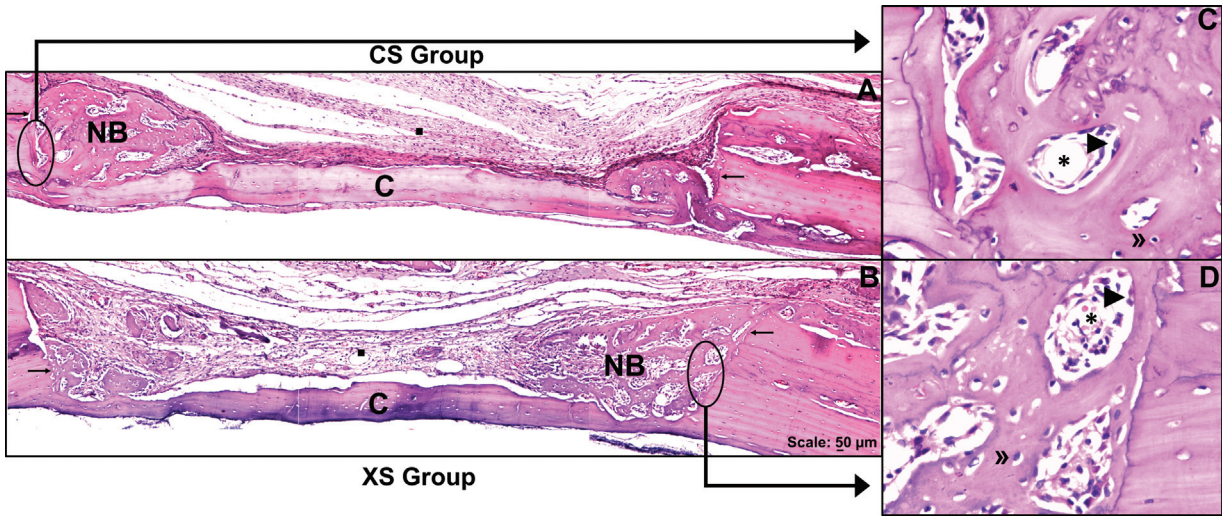

Fig. 2 Photomicrography of the calvarial defect in the SC and SX groups. Note: This is a representative image of the SC and SX groups. Coronal section. Notice the newly-formed bone extending from the edge of the defect toward the center. Abbreviations: C, calvaria; NB, newly-formed bone; →, edge of the defect; ■, connective tissue; *, marrow spaces; ►, osteoblasts; «, osteocytes (hematoxylin and eosin; A and B, 100x; C and D, 200x).

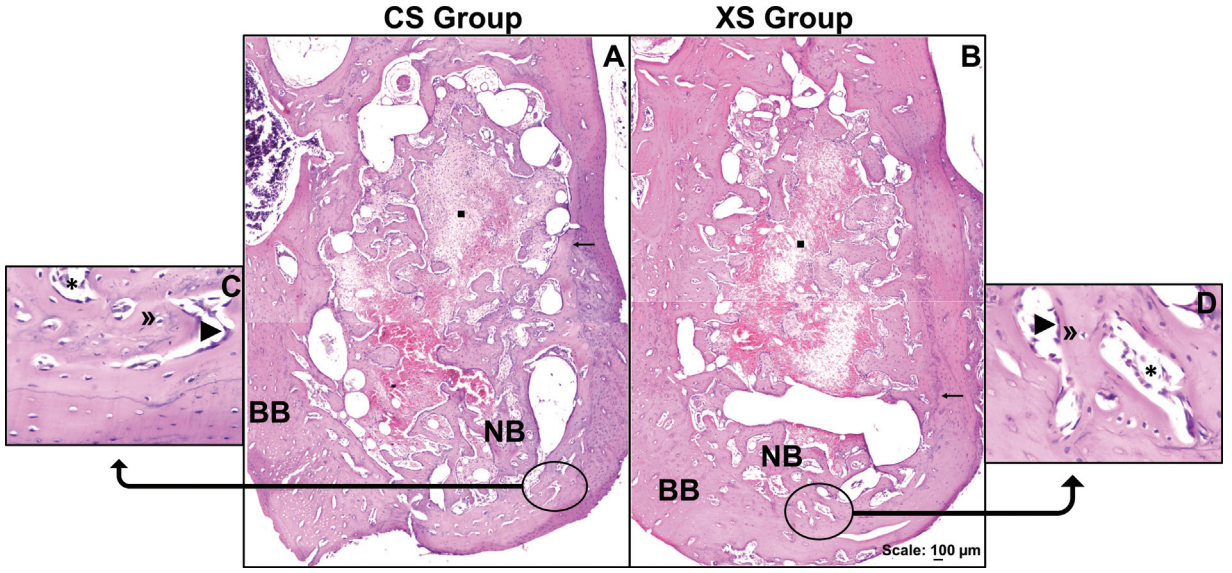

Fig. 3 Photomicrography of the mandibular alveolus in rats (SC and SX groups). Note: This is a representative image of the SC and SX groups. Cross-section. Notice the newly-formed bone extending from the periphery of the basal bone toward the center of the mandibular alveolus. Abbreviations: BB, basal bone; NB, newly-formed bone; →, periphery of the basal bone; ■, connective tissue; *, marrow spaces; ►, osteoblasts; «, osteocytes (hematoxylin and eosin; A and B, 50x; C and D, 400x).

intertwining with the dense connective tissue. No lesion was visualized along the muscularis externa and serous layer. In addition, there was no infiltration of leukocytes, ulcers, erosions, perforations, or gastric bleeding. Therefore, the latex administered systemically did not cause any damage to the stomach.

In 2011, Marinho et al.[18] demonstrated the absence of toxic effects of the *H. speciosa* latex. In the present research, we state that the rats demonstrated no noticeable adverse effects, and to reduce any side effects of the latex in the stomach, we diluted it in distilled water.

Discussion

The present research evaluated the systemic effect of *H. speciosa* latex on bone neoformation and mineralization in Wistar rats.

We found that Ca is one of the components of *H. speciosa* latex. This element was also found in the fruit of *H. speciosa*,[19,20] and in the *Hevea brasiliensis* latex.[21] Possibly, after the ingestion of the latex, the blood Ca concentration increased in the SX group. However, we did not detect this change, possibly because of the rapid action of calcitonin and

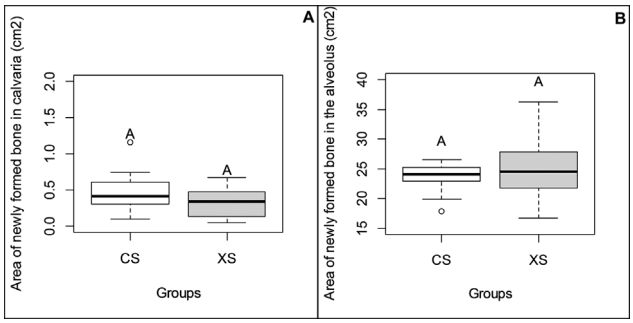

Fig. 4 Area of newly-formed bone in the calvaria defect and the mandibular alveolus (cm^2) of rats in the SC and SX groups. Note: Equivalent letters indicate that there is no statistically significant difference between the groups, as calculated using a *t*-test with $p < 0.05$.

the deposition process of exchangeable salts. These actions may have culminated in blood homeostasis and deposition of Ca and P in the bones. Therefore, we observed an increase in the relative Ca and P contents in the basal bone of the mandibular alveolus in the SX group compared with the SC group.

Our results showed that, in addition to the increasing Ca and P contents, the latex treatment also resulted in the reduction of the Mg content. Therefore, we hypothesize that the amorphous crystals present in the bones were converted into hydroxyapatite crystals by the replacement/addition of atoms. Thus, the basal bone of the mandibular alveolus in the SX group became more mineralized.

The new mineralized bone can be analyzed by SEM-EDX to calculate the Ca/P ratio and to identify the degree of bone mineralization.[22,23] The present pioneering study demonstrated that *H. speciosa* latex increased the content of Ca and P, thus leading to an increase in the mineralization of the newly-formed bone in the mandibular alveolus of Wistar rats after 15 days of treatment. This effect can be attributed not only to the presence of Ca in the latex, but also to the presence of some phytochemicals (chlorogenic acid and naringenin-7-O-glucoside).[12] Studies[24,25] have shown that these phytochemicals can stimulate osteoblastic activitiy. Active osteoblasts secrete essential molecules for bone mineralization.[26] Thus, these compounds may improve bone mineral density and microarchitecture.[24,25]

The histological analysis showed that the oral administration of *H. speciosa* latex at 50% did not increase the newly-formed bone in the mandibular alveolus or the calvarial defect. These data corroborate other results previously

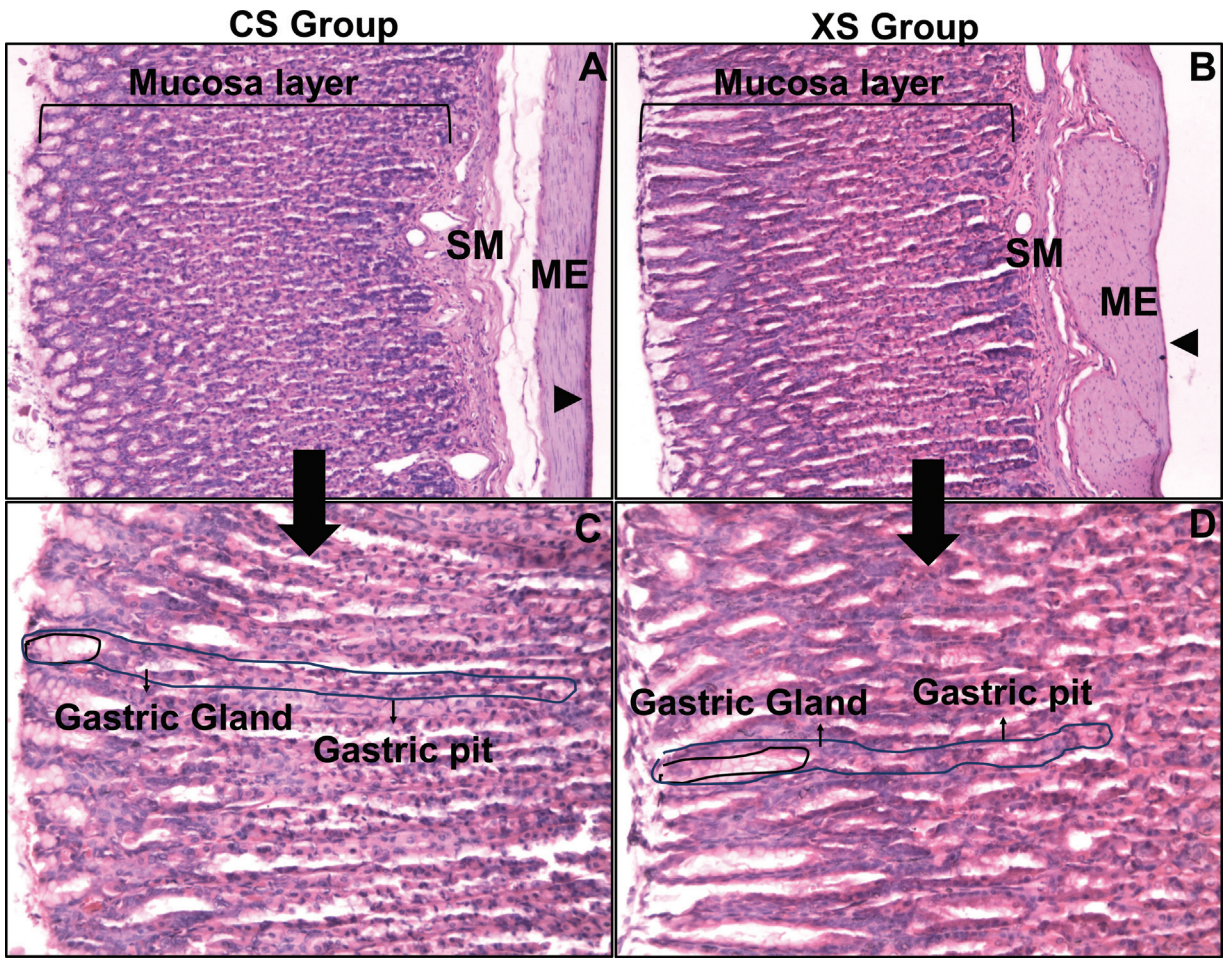

Fig. 5 Photomicrography of the body region of the stomach in rats (SC and SX groups). Note: This is a representative image of the SC and SX groups. Cross-section. Notice the aspect of normality in all the stomach layers of both groups. Abbreviations: SM, submucosa; ME, muscularis externa; ► serosa (hematoxylin and eosin; A and B, 100x, C and D, 200x).

obtained by our team[27] with the oral administration of the latex in another concentration (of 50% and 3%).

On the other hand, it has been demonstrated that, in rats treated with topical applications of natural latex, there was an increase in the area of newly-formed bone in the calvaria[12] and mandibular alveolus.[8] This difference occurred due to the administration by distinct routes.

Although the oral treatment with 50% latex did not influence the amount of newly-formed bone, the main finding of the present paper is that the solution containing 50% latex increased bone mineralization.

We also demonstrated that the oral administration of the latex did not cause any stomach injury. Marinho et al.[18] administered different doses of *H. speciosa* latex by gavage, and observed that the product did not lead to the development of any lesions in the stomach. These results suggest that the latex has beneficial effects in the body, without damaging the gastric layers. Furthermore, Marinho et al.[18] also demonstrated the absence of toxic effects of this product. During the conduction of the present study, no behavioral alteration neither signs of intoxication were observed in the rats in the SX group.

Despite the advances in alternative methods, animal models still have the main advantage of providing information about the organism as a whole.[28] Rats are one of the most used vertebrates in research due to their genetic similarities to the human species.[28] Thus, the results of these studies can be extrapolated to human biology.

In addition, the authors valued the principles of the 3Rs (reduction, refinement, and replacement) for the use of animals.[29]

Finally, the authors are aware that the present study has some limitations, such as the non-identification of significant concentrations of P by the colorimetric method, as well as the death of some animals after the surgical procedure, which reduced the number of samples. Nevertheless, these unforeseen events were not able to generate losses to the statistical analysis and the reliability of the data obtained with the research.

Conclusion

We found that *H. speciosa* latex contains Ca in its composition, and we demonstrated that the daily oral administration of the product for 15 days increases the Ca and P contents and decreases the Mg content of the basal and newly-formed bones in the mandibular alveolus. An increase in the Ca and P contents indicates higher Ca phosphate deposition in the bone; a decrease in the Mg content indicates that amorphous Ca phosphate present in the bones of the latex-treated rats was converted into hydroxyapatite crystals by the replacement/addition of atoms. Therefore, the basal and newly-formed bone in the mandibular alveolus became more mineralized after the latex treatment. Besides that, the oral treatment with latex did not change stomach morphology and plasma Ca and P concentrations. On the other hand, we showed that *H. speciosa* latex did not contribute to increasing the area of newly-formed bone in the calvarial defect and mandibular alveolus.

In conclusion, we may state that these results support the popular belief regarding the benefit of consuming *mangaba* milk daily for the treatment of fractures. Furthermore, our results may aid in the conception and development of a natural drug and favor the entire population that ingests the product.

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Conflict of Interests
The authors have no conflict of interests to declare.

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