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Original Article

# Increased Osteoblastic and Osteocytic *in Vitro* Cell Viability by Yerba Mate (*Ilex paraguariensis*)

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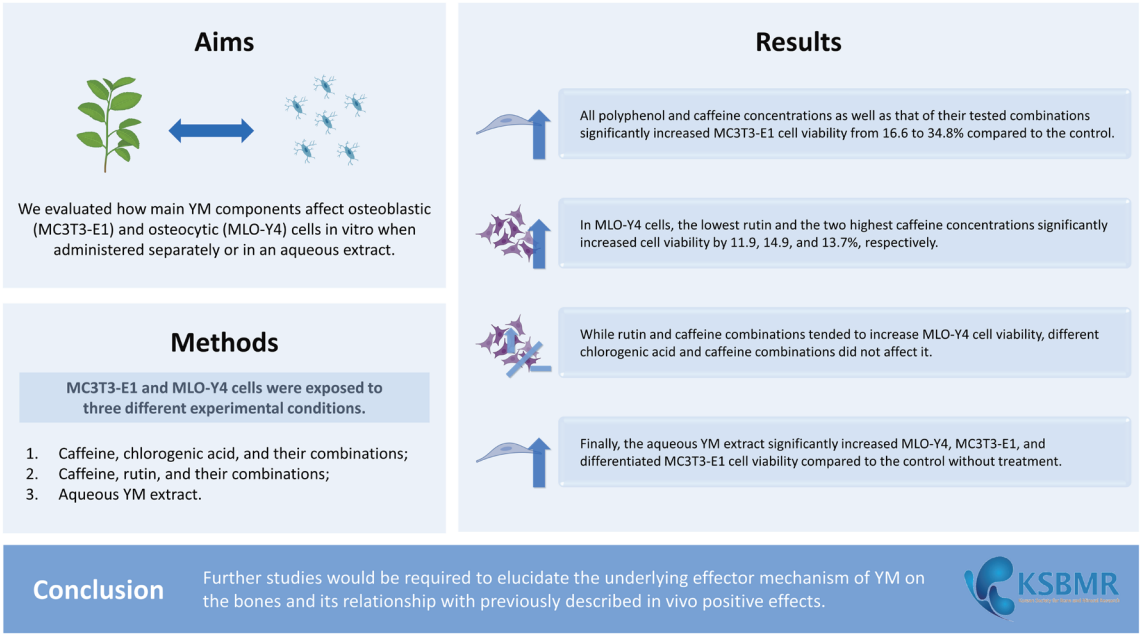
**Background:** Yerba mate (YM, *Ilex paraguariensis*) consumption beneficially affects the bones. However, whether YM components exert their effect on bone cells directly remains elusive. **Methods:** We evaluated how main YM components affect osteoblastic (MC3T3-E1) and osteocytic (MLO-Y4) cells *in vitro* when administered separately or in an aqueous extract. MC3T3-E1 and MLO-Y4 cells were exposed to three different experimental conditions: (1) Caffeine, chlorogenic acid, and their combinations; (2) Caffeine, rutin, and their combinations; (3) Aqueous YM extract. **Results:** All polyphenol and caffeine concentrations as well as that of their tested combinations significantly increased MC3T3-E1 cell viability from 16.6% to 34.8% compared to the control. In MLO-Y4 cells, the lowest rutin and the two highest caffeine concentrations significantly increased cell viability by 11.9, 14.9, and 13.7%, respectively. While rutin and caffeine combinations tended to increase MLO-Y4 cell viability, different chlorogenic acid and caffeine combinations did not affect it. Finally, the aqueous YM extract significantly increased MLO-Y4, MC3T3-E1, and differentiated MC3T3-E1 cell viability compared to the control without treatment. **Conclusions:** YM components (rutin, chlorogenic acid, and caffeine) positively affected bone cells, mainly pre-osteoblast cells. Moreover, the aqueous YM extract significantly increased MLO-Y4, MC3T3-E1, and differentiated MC3T3-E1 cell viabilities indicating an additional relevant nutritional property of YM infusion. Further studies would be required to elucidate the underlying effector mechanism of YM on the bones and its relationship with previously described *in vivo* positive effects.

**Key Words:** Yerba mate · *Ilex paraguariensis* · Osteoblast · Osteocyte · Bone

## INTRODUCTION

Yerba mate (YM) infusion, prepared with dried leaves of *Ilex paraguariensis A.St.-Hil*, is a highly consumed beverage in Latin America, mainly in Argentina, Brazil, Paraguay, and Uruguay, as tea or coffee. The highest YM consumption occurs in Uruguay (~8 kg/person/year) followed by Argentina (~6.5 kg/person/year) and it is exported from Argentina to more than 50 countries in the world.[1] Several active phytochemicals have been identified in aqueous extracts of *Ilex paraguariensis* such as xanthines, polyphenols, and saponins which would be responsible for

## Graphical Abstract




beneficial actions of YM on health.[1-7] It has been previously reported that YM could decrease triglycerides and cholesterol in hypercholesterolemic rats [8] and could also result in an improvement in the lipid profile in patients with dyslipidemia.[9] Additional studies showed that YM has anti-obesity, anti-inflammatory, antibacterial and immunomodulatory effects.[10-14]

A positive effect of YM has been previously observed on bone, both in experimental animals and in postmenopausal women. A higher femoral neck and lumbar bone mineral density (BMD) was found in postmenopausal women who drank more than 1 liter of YM per day for at least 5 years compared to controls who did not drink YM infusion.[15] In addition, YM administration had a positive effect on BMD and trabecular bone volume in rats, by partially reversing bone loss due to low Ca intake.[16] However, in a case-control study carried out in South Brazil there was no significant difference between the frequency of fractures in women who drank YM infusion and women who did not.[17]

Similarly to YM, consumption of black tea and green tea (*Camellia sinensis*), which are also rich in polyphenols and xhantines, result in a protective effect on bone, lowering fracture risk.[18-20] The presence of polyphenols with antioxidant effect could explain this favorable effect on bone tissue,[21] considering that reactive oxygen species induce the apoptosis of osteoblasts and osteocytes and increase osteoclastogenesis leading to bone loss.[22] A relationship between bone loss with age and oxidative stress was found by the assessment of advanced protein oxidation products such as malondialdehyde (MDA) and superoxide dismutase (SOD) in femur samples of young, adult, and elderly rats. Increased MDA levels and decreased SOD activity with aging were found.[23] Additionally, it has been suggested that foods rich in antioxidants may represent a strategy to decrease age-related bone loss, while foods rich in polyphenols have been associated with better bone health attributable to their antioxidant capacity.[20,24] However, although there are few *in vitro* studies reporting the effects of YM on osteoblastic cells,[25,26] whether particular components of the infusion have effects on cell survival or not remains unknown.

Therefore, we aimed to assess the content of components with possible effects on bone tissue of commercial brands of *Ilex paraguariensis* and to evaluate the *in vitro* effect of the most relevant YM components (chlorogenic acid, rutin, and caffeine) on pre-osteoblastic and osteocytic cells. Considering that the YM is a complex mixture with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its corresponding matrix, we analyzed the effects of an aqueous YM extract on pre-osteoblastic cells, mature osteoblasts, and osteocytic cells.

## METHODS

### 1. Preparation of YM infusion

YM infusions were prepared with 50 g of dried *Ilex paraguariensis* leaves in 500 mL of tap water (phosphate, 0.37±0.40 ppm; calcium, 12.48±3.83 ppm; fluoride, 0.11±0.04 ppm) at 70 or 90°C, under constant stirring for 5 min. The lowest temperature (70°C) is the condition used by the manufacturer companies for the analysis of YM composition informed in the package and the highest temperature (90°C) was selected to analyze the maximum conditions in which YM is consumed. Samples were filtered (pore 10 μm) and stored at -20°C. The analyses were performed in three replicates. Twelve commercial brands of YM infusions (Taragüí, Taragüí Energía y Unión [Las Marías], Rosamonte [Hreñuk], Amanda [La Cachuera], Cruz de Malta y Nobleza Gaucha [Molinos Río de la Plata], La Tranquera [Llorente], Playadito [Coop. Liebig], Piporé [Coop. Santo Pipo], Aguantadora [Coop. Montecarlo], Andresito [Coop. Andresito]) were analyzed. These brands comprise more than 80% of the *Ilex paraguariensis* brands sold in Argentina.

### 2. YM components with possible effects on bone tissue

To determine the concentration of components with potential activity bone-active YM components, the following procedures were followed: *Caffeine*: the content was determined by reversed-phase high-performance liquid chromatography on a C18 column (Ultrasphere, Beckman, USA; 250 mm×4.6 mm) with mobile phase 0.1% acetonitrile-water (20:80 v/v) and read at 273 nm.[27] *Polyphenol*: total polyphenol content (TPC) was determined spectrophotometrically at 765 nm using the Folin-Ciocalteu method (ISO 14502-1, 2005).[28] A standard curve for different concentrations of gallic acid (0, 10, 20, 30, 40, and 50 mg/L) ($R^2$=0.9995) was plotted. The TPC was expressed as grams equivalent of gallic acid/100 g dry YM.[15] *Calcium*: its concentration was measured by atomic absorption spectroscopy (Arolab MK II, Buenos Aires, Argentina). *Inorganic phosphorus*: it was measured spectrophotometrically at 690 nm (Rayto RT 6000) by inducing a phosphorus reaction with molybdate in an acid medium (Wiener Lab, Rosario, Argentina).[15] *Fluoride*: its concentration was measured with ion selective electrode ORION 94-09 with a reference electrode Ag/AgCl. The measurement is based on the linear relation between the mV developed by the electrode and the logarithm of the fluoride concentration of the standards: $10^{-3}$-$10^{-6}$ M of NaF.[29]

### 3. Antioxidant activity

The free radical-scavenging activity of YM infusions was evaluated by measuring the absorbance at 517 nm of the samples incubated with 2,2-difenil-1-pricryl-hidrazil (DPPH) radical.[30] Butylated hydroxytoluene was used as a positive control. The DPPH scavenging effect (%) of the infusions was calculated using the formula: $([A_0–A_1]/A_0)\times100$, where $A_0$ is the absorbance of the control and $A_1$ is the absorbance of the sample. The mean inhibitory concentration at 50% ($IC_{50}$) was calculated with the TPC that could scavenge 50% of the DPPH. A lower $IC_{50}$ value corresponds to a higher antioxidant capacity of the YM infusion. In addition, the DPPH inhibition (%) was calculated considering a TPC of 30 μg/mL.

### 4. Cell culture

The murine pre-osteoblast cell line was generously provided by Dr. McCarthy (LIOMM, La Plata, Argentina). MC3T3-E1 cells were cultured in complete Dulbecco's modified Eagle's Medium (DMEM; Gibco; Life Technologies, Carlsbad, CA, USA; supplemented with 10% fetal bovine serum [FBS], 1% penicillin and streptomycin, 1% L-glutamine) in a humidified 5% $CO_2$ atmosphere at 37°C ($CO_2$ incubator; Thermo Fisher Scientific, Waltham, MA, USA).[31,32]

The murine osteocyte MLO-Y4 cell line was obtained from Dr. Delpino (INIGEM, Buenos Aires, Argentina), with Dr. Bonewald´s permission (Indiana Center for Musculoskeletal Health, Indianapolis, IN, USA).[33] MLO-Y4 cells were cultured in complete α-MEM (α-Minimum Essential Medium; Gibco-BRL, Carlsbad, CA, USA; supplemented with 10% FBS, 1% penicillin, and streptomycin), at 37°C in a 5% $CO_2$ incubator (Thermo Fisher Scientific, Waltham, MA, USA) on 0.1% type I collagen (Sigma-Aldrich, St. Louis, MO, USA) coated bottles or multi-well plates. The medium was refreshed every 2 to 3 days. Cell morphology was analyzed qualitatively through a phase contrast inverted microscope (Zeiss, Oberkochen, Germany).

## 5. Differentiation of pre-osteoblast MC3T3-E1 cells

Pre-osteoblast MC3T3-E1 cells were cultured in an osteogenic medium (complete DMEM supplemented with 50 μg/mL ascorbic acid and 5 mM β-glycerophosphate).[34] To evaluate the model of differentiated MC3T3-E1 cells, total alkaline phosphatase activity and calcified nodules were determined at days 1, 7, and 14 of culture. Enzymatic activity was determined by spectrophotometry (405 nm) using a commercial kit (ALP 405 AA Wiener lab) and expressed concerning the total protein content assessed by spectrophotometry (540 nm; commercial kit Proti U/LCR Wiener Lab). Protein assessment was performed in a homogenization obtained by washing the cells with phosphate-buffered saline, adding radioimmunoprecipitation assay buffer and using a scrapper and sonication to break the cell walls. The mineralization assay was performed by fixing the cells with 4% formaldehyde for 10 min and staining them with a 2% alizarin red (Sigma-Aldrich) solution for 30 min at room temperature, allowing the visualization of calcified nodules under a microscope.[27]

## 6. Aqueous YM extract for cell culture

The aqueous YM extract was prepared from 0.1 g of a lyophilized sample (provided by Dr. Juan Ferrario; Faculty of Exact and Natural Sciences, Buenos Aires, Argentina),[35,36] which was diluted with sterile distilled water to obtain a final concentration of 0.3 mg/mL of chlorogenic acid, one of the main YM components. Culture cells were exposed to 1/500, 1/1,000, and 1/2,000 dilutions of stock solution, corresponding to a final concentration of chlorogenic acid of 0.15, 0.3, and 0.6 μg/mL, respectively.

## 7. Cell viability exposed to YM components

MC3T3-E1 and MLO-Y4 cells were seeded on 96 well plates and cultured at 37°C until they reached 70% confluence. Subsequently, cells were exposed for 48 hr to different caffeine concentrations and polyphenol (rutin or chlorogenic acid) in two different experiments (3 repetitions each): (1) Caffeine (C 0.66, 1.66 y 3.33 μg/mL), chlorogenic acid (1, 5 y 10 μg/mL) and their respective combinations (N=6/group). (2) Caffeine (C 0.66, 1.66 y 3.33 μg/mL), rutin (R 1, 5 y 10 μg/mL) and their respective combinations (N=6/group). For both experiments, cells incubated with a complete medium without treatment were used as control group. Caffeine, rutin, and chlorogenic acid were purchased from Sigma Aldrich.

After incubation at 37°C for 48 hr, 10 μL of WST-1 (Cell Proliferation Reagent; Roche Diagnostics, Basel, Switzerland) were added to each well for 120 (MC3T3-E1) or 90 min (MLO-Y4). The absorbance of each well was measured at 450 nm using a microplate reader (Rayto RT-2100C).

The effect of the compounds on cell viability was calculated based on the optical density for each condition (ODt), considering the control group (ODc) as 100% (100*ODt/ODc).

## 8. Cell viability after exposition to aqueous YM extract

Pre-osteoblast MC3T3-E1 and MLO-Y4 cells were seeded on 96 well plates and cultured at 37°C until they reached 70% confluence. In differentiated MC3T3-E1, the aqueous YM extract was added on day 14 of differentiation. Subsequently, cells were exposed for 48 hr to different dilutions of the YM extract. MLO-Y4 cells were exposed to dilutions of 1/1,000 and 1/2,000 and both MC3T3-E1 cells were exposed to dilutions of 1/500 and 1/1,000. In the same plates, cells were cultured without any additional treatments used as control cells.

During following incubation at 37°C for 48 hr, 10 μL of WST-1 (Roche) were added to each well and the absorbance at 450 nm was measured over a period of 60 min using a microplate reader (Rayto RT-2100C). Absorbance versus time was plotted to find the time when the groups showed significant differences. The effect of the compounds on cell viability was calculated based on the optical density for each condition (ODt), considering the control group (ODc) as 100% (100*ODt/ODc).

## 9. Data analysis

Shapiro-Wilk and Bartlett tests were used to assess normality and equal variances respectively and parametric or non-parametric tests were used, as appropriate. Continuous variables were expressed as mean±standard error or median (interquartile range [IQR]), according to data distribution. Cell viability data were analyzed by one-way ANOVA and Dunnett's multiple comparison test or Brown-Forsythe and Welch's ANOVA test and Holm-Sidak's multiple comparison test. Differences were considered significant if *P* value less than 0.05. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software


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Inc., San Diego, CA, USA).

## RESULTS

### 1. YM components with possible effect on bone tissue

No differences in calcium, phosphate, fluoride, total polyphenol, and caffeine content were found among the different commercial *Ilex paraguariensis* brands evaluated (data not shown, One-way ANOVA and Kruskal-Wallis test). Consistent with those results, no differences among commercial brands were observed in antioxidant activity: $IC_{50}$, mean 70.1 μg/mL; range, 47.2–90.4; IQR, 61.9–73.9; DPPH inhibition 21.8%; range, 13.4–36.2; IQR, 19.4–24.7.

The concentration of YM infusion components obtained with different temperatures of the water did not show significant differences in calcium, phosphate, or fluoride concentration. On the contrary, significantly higher TPC and caffeine levels were observed at 90°C compared to 70°C (Table 1).

### 2. YM components effects on osteocytic cultured cells (MLO-Y4)

MLO-Y4 cells did not show evident changes in morphology after exposure to treatments. After 3 hr of seeding, cells were small, stretched and stellated in shape, with many short processes. After 48 hr, the MLO-Y4 cells were more confluent and dendritic morphology, a characteristic morphologic feature of osteocytes, was evident.

Rutin (R, 1 μg/mL) and caffeine (C, 1.66 and 3.33 μg/mL) significantly increased the MLO-Y4 cells viability compared to the control without treatment by 11.9%, 14.9%, and 13.7%, respectively (Fig. 1A). The remaining concentrations did not show significant effects on cell viability.

All the combinations of rutin and caffeine showed a tendency to increase MLO-Y4 cell viability, reaching statistical significance for several of them (Fig. 1B). We highlighted the R 10 μg/mL+C 0.66 μg/mL combination, which represents approximately, the TPC/caffeine ratio found in YM infusions, which showed a significant increase of 8.4% compared to the control. No additive or synergistic effect of the combination was observed.

On the other hand, the different combinations of caffeine and chlorogenic acid did not show an effect on cell viability (Fig. 1C). Moreover, the highest chlorogenic acid concentration employed in combination with caffeine showed a tendency to decrease MLO-Y4 cell viability, compared to controls.

### 3. Effect of YM components on pre-osteoblastic cultured cells (MC3T3-E1)

MC3T3-E1 cells did not show evident morphological changes after exposure to rutin, chlorogenic acid, or caffeine or combinations at their different concentrations, compared to controls. The cells presented rounded morphology after 3 hr of seeding and began forming a monolayer with a fibroblastoid shape after 1 day of culture. After 48 hr, the cells were semi-confluent.

Each polyphenol (rutin or chlorogenic acid) and caffeine concentration significantly increased from 16.6% to 34.8% MC3T3-E1 viability cells compared to control, considered as 100% of viability (Fig. 2A). Furthermore, all combinations between chlorogenic acid and caffeine (Fig. 2B), and rutin and caffeine (Fig. 2C) showed a significant increase in MC3T3-E1 cell viability. We highlighted two particular combinations (AC 10 μg/mL+C 0.66 μg/mL and R 10 μg/mL+C 0.66 μg/mL), which had, approximately, the TPC/caffeine ratio found in YM infusions, with an increase of 17.7% and 25.5% in viability, respectively.

**Table 1.** Concentration of yerba mate components in infusions prepared at different temperatures in 12 commercial brands

| | 70°C | 90°C | *P*-value |
|---|---|---|---|
| Calcium (mg/L) | 14.6 (12.2–20.2) | 14.6 (10.0–19.0) | NS |
| Phosphate (mg/L) | 102.8 (73.2–134.0) | 109 (83.6–150.8) | NS |
| Fluoride (mg/L) | 0.18 (0.08–0.33) | 0.17 (0.07–0.43) | NS |
| Total polyphenol content (g GAE/100 g YM) | 14.3 (13.6–14.8) | 17.1 (15.9–18.6) | <0.0001 |
| Caffeine (g/L) | 0.64 (0.61–0.83) | 0.81 (0.73–0.87) | 0.0161 |

The data is presented as median (interquartile range).
GAE, gallic acid equivalent; YM, yerba mate; NS, not significant.

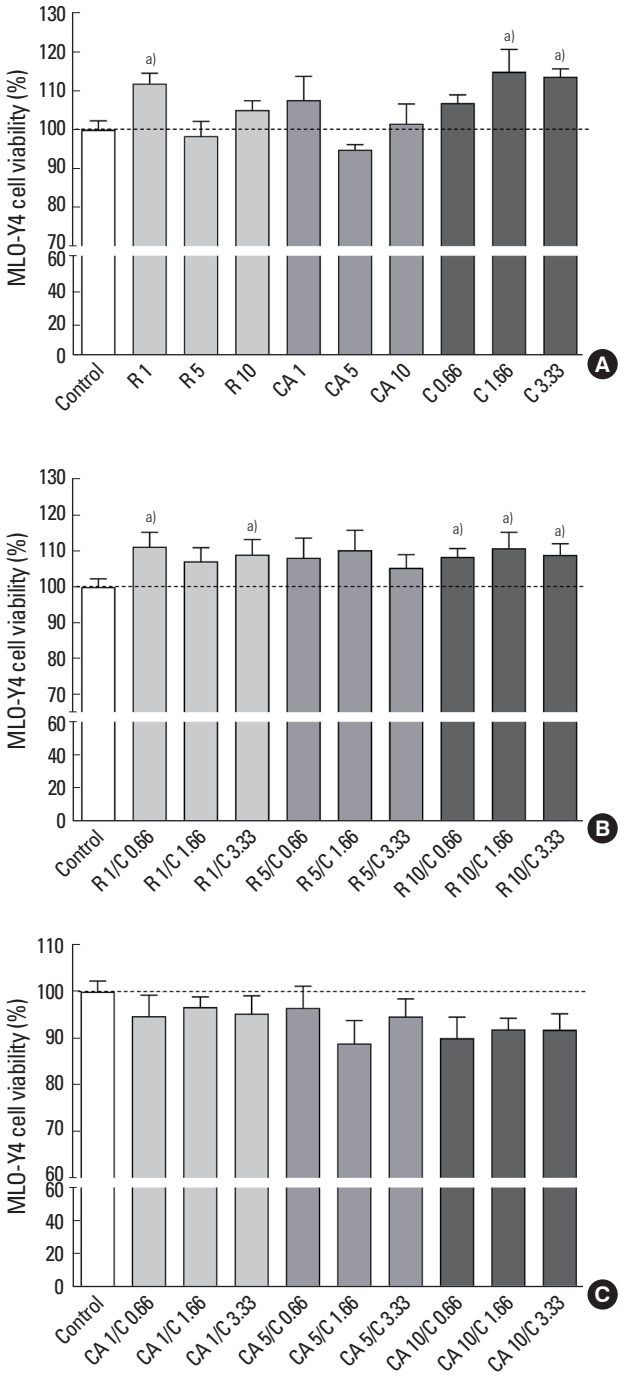


**Fig. 1.** Yerba mate (YM) components effects on MLO-Y4 cell viability. Data are expressed as mean±standard error (%) compared to controls (white bar, 100%). The x-axis indicates the concentrations of YM components in μg/mL. (A) Effects of individual YM components. (B) Effects of rutin and caffeine combinations. (C) Effects of chlorogenic acid and caffeine combinations. [a]$P<0.05$ by one-way ANOVA and Dunnett's multiple comparison test. R, rutin; CA, chlorogenic acid; C, caffeine.

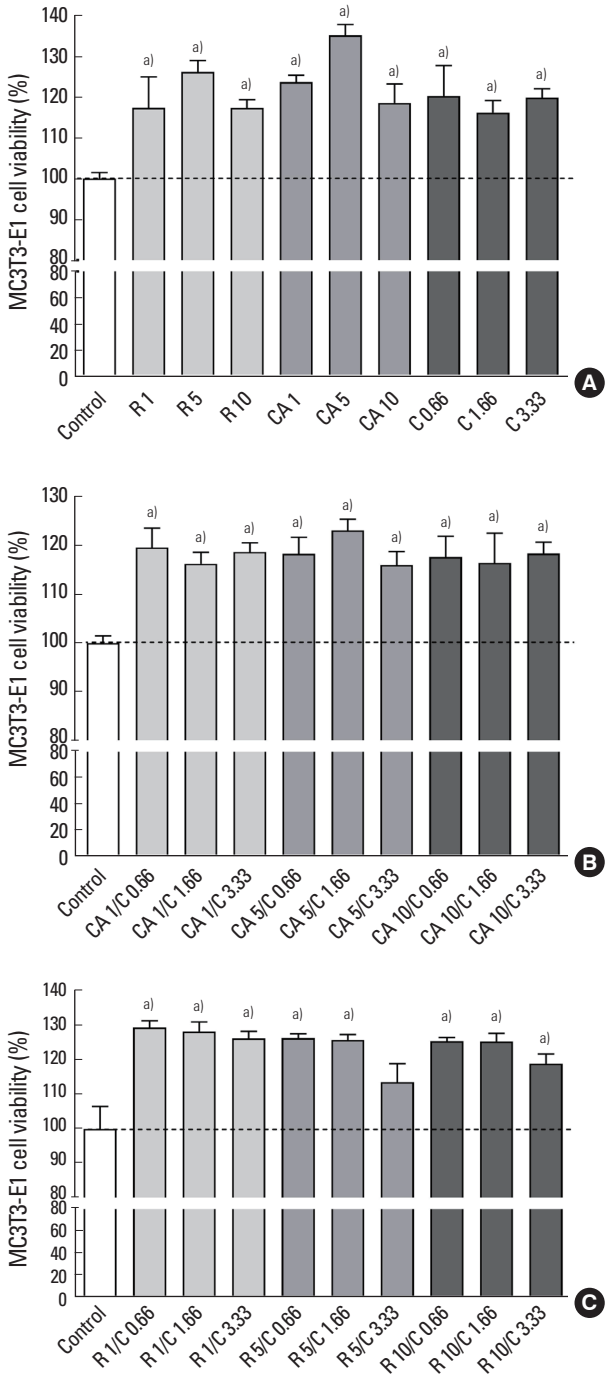


**Fig. 2.** Yerba mate (YM) components effects on MC3T3-E1 cells viability. Data are expressed as mean±standard error (%) compared to controls (white bar, 100%). The x-axis indicates the concentrations of YM components in μg/mL. (A) Effects of individual YM components. (B) Effects of rutin and caffeine combinations. (C) Effects of chlorogenic acid and caffeine combinations. [a]$P<0.05$ by one-way ANOVA and Dunnett's multiple comparison test. R, rutin; CA, chlorogenic acid; C, caffeine.



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### 4. Aqueous YM extract effects on bone cultured cells

The aqueous YM extract significantly increased MLO-Y4, MC3T3-E1 and differentiated MC3T3-E1 cell viability compared to controls after 48 hr of treatment (Fig. 3, 4). Considering the same YM dilution (1/1,000) the cell viability was increased by 23.6% in MLO-Y4 cells, 15.4% in MC3T3-E1 cells and 105.8% in differentiated MC3T3-E1 cells. Despite there being no significant differences between both aqueous YM extracts, there is a trend to greater viability in

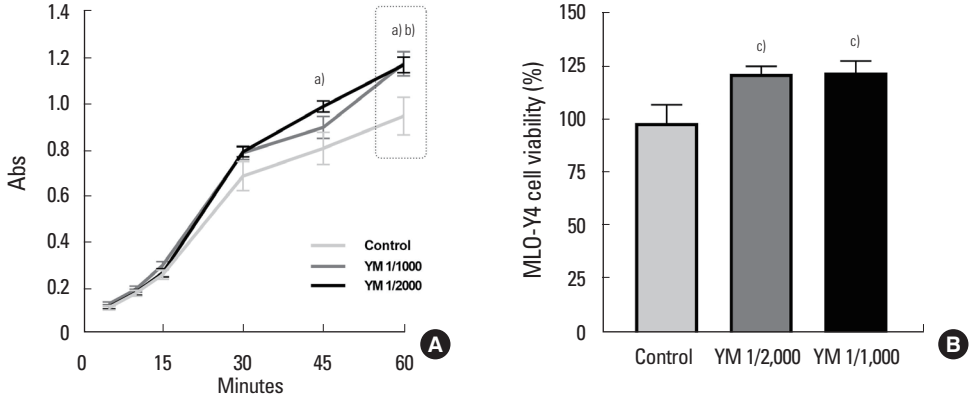


**Fig. 3.** Aqueous yerba mate (YM) extract effects on MLO-Y4 cell viability. (A) Absorbance (Abs) versus time graphs. Data are expressed as mean±standard error (SE). Data into the segmented line is shown in (B) after calculating cell viability. (B) MLO-Y4 cell viability expressed as mean±SE (%) compared to controls (light gray bar, 100%) at 60 min. [a]Indicates significant differences between YM 1/2,000 and control. [b]Indicates significant differences between YM 1/1,000 and control. [c]$P<0.05$ by Brown-Forsythe and Welch's ANOVA test and Holm-Sidak's multiple comparison test.

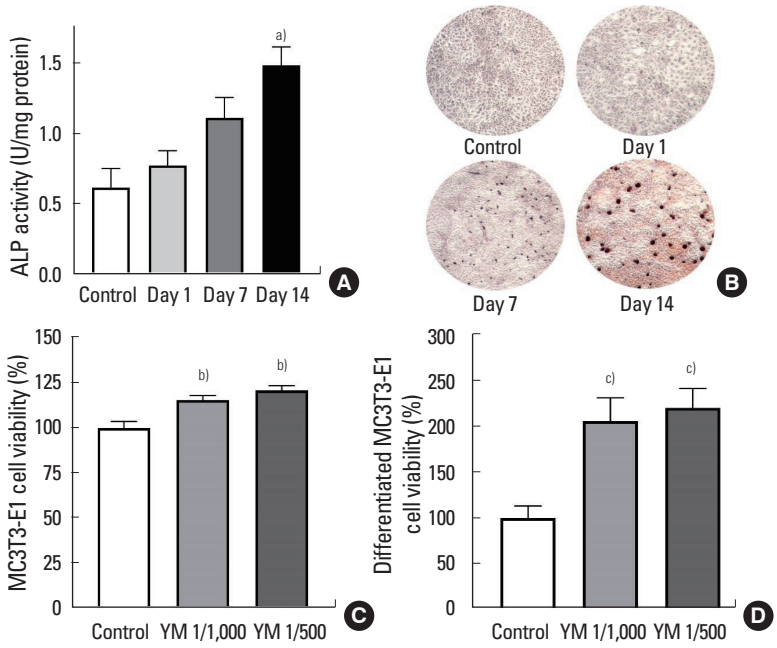


**Fig. 4.** Aqueous yerba mate (YM) extract effects on MC3T3-E1 cultured cells. (A, B) Assessment of differentiation by total alkaline phosphatase (ALP) activity (C) and calcified nodules (D) increase. (C, D) The aqueous YM extract significantly increased MC3T3-E1 and differentiated MC3T3-E1 cells viability. Data are expressed as mean±standard error (%) compared to controls (white bar, 100%) at 30 and 15 min respectively. [a]$P<0.05$ vs. control and day 1 by Brown-Forsythe and Welch's ANOVA test (Holm-Sidak's multiple comparison test). [b]$P<0.05$ by one-way ANOVA (Dunnett's multiple comparison test) for MC3T3-E1. [c]$P<0.05$ by Brown-Forsythe and Welch's ANOVA test (Holm-Sidak's multiple comparison test) for differentiated MC3T3-E1 cells.

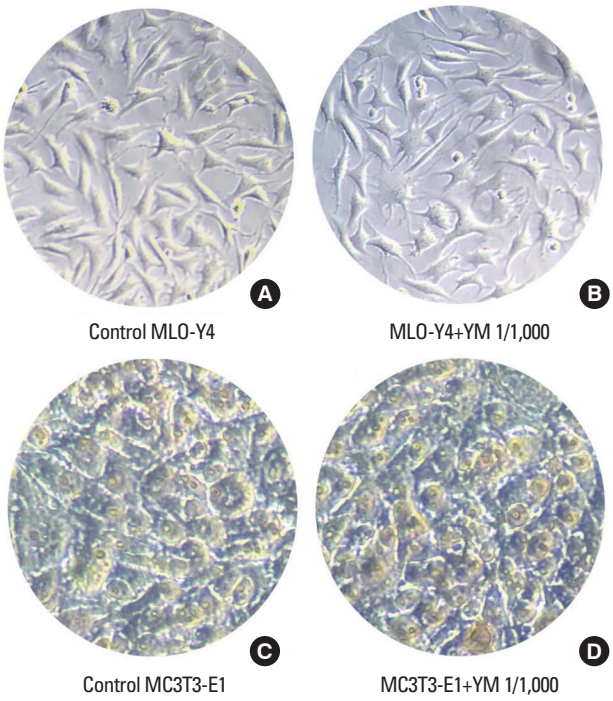


**Fig. 5.** Cell morphology on a contrast phase microscope. (A) MLO-Y4 cells without treatment. (B) MLO-Y4 cells after 48 hr treated with a 1/1,000 dilution of aqueous yerba mate (YM) extract. (C) Differentiated MC3T3-E1 cells without treatment. (D) Differentiated MC3T3-E1 cells after 48 hr treated with a 1/1,000 dilution of aqueous YM extract.

the more concentrated extract.

Representative pictures of MLO-Y4 and differentiated MC3T3-E1 cells show no evident changes in morphology after exposure to YM treatment (Fig. 5).

## DISCUSSION

Elemental composition analysis of *Ilex paraguariensis* revealed the presence of many macro- and microelements. Amino acids, minerals (aluminum, chromium, copper, iron, manganese, nickel, potassium, and zinc, among others) and vitamins have been described in variable concentrations due to factors such as the characteristics of the soil and the seasons of the year.[37,38] The average calcium concentration found in this study in 12 commercial brands of YM was 15 mg/L without differences between them and both water temperatures evaluated. Despite the calcium content reported here being lower than the one informed previously (28.5 mg/L at 80°C and 28.7 mg/L at 90°C),[15] the values in both studies are low and only contribute to a 1.5% to 3% of the Recommended Dietary Allowances (RDA; 1,000 mg/day) considering 1 L of YM infusion per day. Phosphate represents 16.9% of the RDA (700 mg/day) and fluoride content represents a small amount below the recommended upper limit (6 mg/day). A previous study assessed the content of selected elements (copper, zinc, iron, manganese, but not calcium),[6] the authors estimated that the consumption of one cup (200 mL) of YM infusion can cover 57.6% to 72.4% of RDA for manganese, 2.0% to 2.4% for copper, 0.42% to 1.43% for iron, and 0.56% to 0.84% for zinc. The bone effect of YM could not be attributed to calcium, phosphate, or fluoride content. In addition, we did not find differences in the levels of these extracted compounds when the temperatures used were above 70°C.

On the other hand, caffeine concentration and total polyphenol increased at higher extraction temperatures. Caffeine consumption has a negative impact on BMD with accelerated bone loss [39] and increased risk of fractures,[40] mainly associated with low calcium diets.[41,42] This negative effect was also observed in experimental animals.[43,44] Caffeine administration enhanced osteoclastogenesis from bone marrow hematopoietic cells and bone resorption activity *in vivo*.[45] Moreover, caffeine enhanced the expression of the receptor activator of nuclear factor-κB ligand (RANKL) and reduced osteoprotegerin protein levels in MC3T3-E1 pre-osteoblastic cells.[42] Caffeine (10 mM=1,942 μg/mL) also showed a negative effect on viability of the osteoblasts, the formation of ALP-positive staining colonies and mineralization nodules.[46] However, in the current study, we found an increase in bone cell viability, mainly for pre-osteoblast (MC3T3-E1) cells following low caffeine concentrations treatment (0.66–3.33 μg/mL). A systematic review showed both effects, caffeine can negatively interfere with bone metabolism by accelerating bone loss and delaying bone repair, or positive effect by activating osteogenesis and bone neoformation.[47]

According to recommendations, caffeine intake should be below 400 mg/day [48] because a negative association between caffeine (>200–300 mg/day=~400–500 mL of coffee) and BMD has been reported, an effect which was attenuated with Ca intake >750 mg/day.[38,43] Consequently, the caffeine concentration found in our study (800 mg/L; 95% confidence interval, 610–860) for an estimated intake of 1 liter of YM per day would be above the daily recommendation. Therefore, it could be expected that YM


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consumption was deleterious for bone tissue. However, previous studies have found a positive effect of YM infusion on BMD in postmenopausal women and experimental animals.[14,15]

These positive effects could be explained by the antioxidant action of polyphenols which have shown a positive impact on bone metabolism.[20,23] Polyphenols can preserve bone health potentially by different mechanisms: the antioxidant effect, which could be lower and be shared with other infusions or foods such as tea, wine, and blueberries, among others. YM was able to decrease bone resorption in rats by inhibiting osteoclastogenesis in a RANKL-dependent signaling pathway activated by oxidative stress.[49] Moreover, polyphenols have proven osteoblastogenesis improvement and osteoclastogenesis reduction.[50,51] Among the polyphenols known to have bone effects, dietary soy isoflavones suppress bone depletion in rodents and post-menopausal women, icariin has been reported to have osteogenic properties both *in vitro* and *in vivo* and fisetin promotes osteoblasts differentiation through Runx2 transcriptional activity.[45,52]

In this study, we found that the main polyphenols present in YM significantly increased pre-osteoblast (MC3T3-E1) cell viability at all concentrations evaluated, from 1 to 10 μg/mL. On osteocytes, the individual effect was lower and only the lowest rutin concentration (1 μg/mL) showed a significant increase in cell viability. In agreement with our results, chlorogenic acid prevented RANKL-induced osteoclastogenesis,[53] promoted osteogenic differentiation of human dental pulp stem cells through Wnt signaling [54] and prevented osteoporosis in ovariectomized rats through the Shp2/phosphoinositide 3-kinase/Akt pathway.[55] Moreover, rutin from *Chrozophora tinctoria* increased osteocyte and osteoblast-related gene expression and decreased the expression of members of the Runx2 suppressor family and of osteoclastogenic genes in the SAOS-2 cell line.[56]

We also evaluated the combinations of both polyphenols (chlorogenic acid or rutin) plus caffeine. While all combinations showed a significant increase in pre-osteoblast (MC3T3-E1) cell viability, the effect in osteocyte cells (MLO-Y4) was less clear. Furthermore, it would appear that there is a competition between chlorogenic acid and caffeine in osteocytic cells because chlorogenic acid inhibits the caffeine-induced increase in osteocyte viability, an effect which was not observed in MC3T3-E1 cells.

Despite the value of the evaluated individual YM component and its combinations on bone cells, we considered the importance of assessing the YM extract effect because of its matrix and possible interactions. Here we found that the aqueous YM extract significantly increased the viability of MLO-Y4 (~23%), MC3T3-E1 (~15%) and differentiated MC3T3-E1 (~100%) cells compared to the control without treatments. In accordance with YM components effects results, the aqueous YM extract increased cell viability mainly on MC3T3-E1 differentiation cells. Furthermore, the same YM extract showed a positive effect on survival and growth of dopaminergic neurons in culture.[36] It was also recently demonstrated that pre-administration of YM extract may prevent deleterious effects in cell morphology, increasing cell adhesion and proliferation rate in MC3T3-E1 cells exposed to $H_2O_2$, which could enable the maintenance of extracellular matrix in the presence of oxidative stress.[25] Moreover, a positive effect of low concentration of soluble YM on osteoblast of bone marrow-derived mesenchymal stromal cells differentiation was found, with increased alkaline phosphatase activity, mineralization and gene expression of transcription factors (Runx2, Osterix, and β-catenin) and bone matrix proteins (osteopontin, bone sialoprotein, osteocalcin, and bone morphogenetic protein-2).[26] However, the same study showed that a higher YM concentration (≥50 μg/mL) had deleterious effects, including cytotoxicity.[26]

Some limitations of the study must be pointed out: it is an *in vitro* study with a limited range of concentrations evaluated and without considering the bioavailability of the components included in a complex matrix of YM. In addition, the viability assay (WST-1) does not allow complete discrimination between cell survival and cell proliferation, because both situations could increase the overall activity of succinate-tetrazolium reductase (EC 1.3.99.1), only active in metabolically intact cells.

In conclusion, main YM components (rutin, chlorogenic acid, and caffeine) have shown positive effect on bone cells, mainly pre-osteoblast cells (MC3T3-E1). Moreover, the aqueous YM extract significantly increased the viability of osteocytic (MLO-Y4), pre-osteoblast cells (MC3T3-E1), and differentiated MC3T3-E1 cells indicating an additional relevant nutritional property to YM infusion. Latin America, mainly Argentina and Brazil, are the main producers of YM in the world. According to the National Institute of YM (INYM) from Argentina, in 2019 they exported almost 80 million kilos

mainly to Syria, Chile, Lebanon, USA, and Spain.

However, further studies are necessary to elucidate the mechanism of action of YM on bone and its relationship with previously positive YM effects on the bone described *in vivo*.

## DECLARATIONS

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### Ethics approval and consent to participate

This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the School of Medical Sciences, Rosario National University, Argentina (1MED474).

### Conflict of interest

No potential conflict of interest relevant to this article was reported.

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