available at www.sciencedirect.com
ScienceDirect
journal homepage: http://www.elsevier.com/locate/aob

# Inhibitory properties of *Coffea canephora* extract against oral bacteria and its effect on demineralisation of deciduous teeth

A.G. Antonio [a], N.L.P. Iorio [b], V.S.S. Pierro [a], M.S. Candreva [a], A. Farah [c], K.R.N. dos Santos [b], L.C. Maia [a,*]

[a] Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Brazil
[b] Department of Medical Microbiology, Prof. Paulo de Góes Microbiology Institute, Federal University of Rio de Janeiro, Brazil
[c] Food and Nutritional Biochemistry Laboratory, Department of Biochemistry, Chemistry Institute, Federal University of Rio de Janeiro, Brazil

ARTICLE INFO

*Article history:*
Received 7 September 2010
Received in revised form
23 November 2010
Accepted 1 December 2010

*Keywords:*
Coffee
Plaque pH
Antimicrobial activity
Oral bacteria
Acid production
Dental plaque
Microhardness
Microelectrodes
Hydrogen-ion concentration
Tooth
Deciduous

ABSTRACT

*Objectives:* The antibacterial activity of *Coffea canephora* extract was evaluated *in vitro* against *Streptococcus mutans* and *Streptococcus sobrinus*. The viability of planktonic cells was analysed by susceptibility tests (MIC and MBC) and time-kill assays. The effect of the extract on dental demineralisation was also investigated.

*Methods:* Primary 1st molar fragments ($n = 24$) were inoculated with a saliva pool and sustained in a multiple plaque growth system for 10 days to form biofilm. The biofilm was treated with light roasted *C. canephora* extract at 20%, Milli-Q water (negative control) and chlorhexidine (positive control) once a day, during a week. Blank controls comprised fragments without treatment. Biofilm pH was monitored in the last day of treatment. Changes in tooth mineralisation were assessed by cross-sectional microhardness (CSMH) test.

*Results:* MIC and MBC for *S. mutans* were $7 \pm 2$ mg/mL and $160 \pm 0$ mg/mL, respectively, showing no activity for *S. sobrinus*. The extract produced a 4-log reduction in the number of colonies of *S. mutans* after 3-h treatment ($p < 0.05$) with undiluted extract (20%) and MBC concentration (16%). There was no difference among negative/blank controls and coffee plaque pH. Differences between CSMH values of dental fragments subjected to the coffee extract and to chlorhexidine were not significant. At depths up to 30 μm from the enamel surface, coffee extract and chlorhexidine promoted higher CSMH values when compared to blank/negative controls ($p < 0.05$).

*Conclusion:* Our data suggest that light roasted *C. canephora* extract is beneficial as an anticariogenic substance.



## 1. Introduction

Dental biofilm is defined as the diverse microbial community found on tooth surface embedded in a matrix of polymers of bacterial and host origin.[1] From a biochemical point of view, the bacteria in the biofilm are always metabolically active, causing fluctuations in pH. These fluctuations may cause a loss of minerals from the tooth when pH drops, or a gain of minerals when pH increases.[2] The cumulative result of these de- and re-mineralisation processes may be a net loss of minerals, leading to dissolution of the dental hard tissues and the formation of a caries lesion.[1] Therefore, an important strategy for the prevention of dental caries is to reverse or halt the mineral loss.[3]

* *Corresponding author at*: Rua Gastão Gonçalves, 47/501, Santa Rosa, 24240-030 Niterói, Rio de Janeiro, Brazil. Tel.: +55 21 2629 3738; fax: +55 21 2629 3738.
E-mail address: rorefa@terra.com.br (L.C. Maia).
0003–9969/$ – see front matter 
doi:10.1016/j.archoralbio.2010.12.001

Over the past two decades, the anti-caries potential of polyphenols extracted from different types of natural products such as propolis, tea, cocoa, cranberry, has been reported.[4–6] These substances have been proven to be potential agents in the prevention of oral disease, particularly biofilm-related diseases.[7,8]

In the plant kingdom, the sources of polyphenols are numerous.[6] Coffee is not only the most popular and widely consumed beverage throughout the world, but also rich in polyphenols as well.[9] Studies have shown the possibility of dental caries prevention by coffee's antibacterial activity against *Streptococcus mutans*,[10,11] which is a member of the endogenous oral microflora and also a contributor to biofilm formation in the oral cavity.[12]

The antibacterial activity of coffee against *S. mutans* changes according to its chemical composition that is influenced by species and processing such as roasting and decaffeination.[11] Previously, we observed that *Coffea canephora* extracts presented better performance as an antibacterial agent compared to *Coffea arabica*.[11] However, there is no evidence that *C. canephora* extracts exert the same activity against cariogenic bacteria other than *S. mutans*. Another important aspect that has not been investigated is the effect of coffee on the de-remineralisation process of caries disease. Exploring these themes would result in a more direct and realistic picture of coffee anticaries effect.

Therefore, in the present study, we first evaluated, *in vitro*, the antibacterial effect of a *C. canephora* extract against *S. mutans* and *S. sobrinus*. Following, we investigated the demineralisation effect of the extract on deciduous teeth after *ex vivo* treatment of mixed biofilm.

## 2. Materials and methods

### 2.1. *Coffea canephora extract and controls*

Regular *C. canephora* cv. Conillon beans were roasted in a commercial spouted bed roaster (I-Roast, Gumee, IL, USA), operating at a max. temperature of 220 °C, for 6 min, to produce a light roasting degree (SCAA, USA). Roasting coffee beans were ground in a laboratory-scale mill to pass through a 0.46 mm sieve. An aqueous coffee extract at 20% (pH 5.28 ± 0.8) was obtained by a coffee brewing procedure, percolating 100 mL of pre-boiling (95 °C) Milli-Q purified water through 20 g of ground roast coffee. Chlorhexidine at 0.05% and at 0.12% (pH 5.10 ± 0) were used for the Killing kinetics and biofilm assays, respectively, as positive controls, whereas Milli-Q purified water (pH 5.75 ± 0.6) was used as the negative control for both experiments.

### 2.2. *Characterisation of phenolic compounds from Coffea canephora extract*

The contents of caffeic and chlorogenic acids (3-, 4- and 5-caffeoylquinic acids – 3-, 4- and 5-CQA; 3-, 4- and 5-feruloylquinic acids – 3-, 4- and 5-FQA and 3,4-; 3,5- and 4,5-dicaffeoylquinic acids – 3,4-, 3,5- and 4,5-diCQA) were determined by gradient LC–DAD-ESI-MS according to Farah et al.,[9] using a Magic C30 column (150 mm × 2.0 mm, 5 μm, 100 Å, Michrom Bioresources Inc., Auburn, CA). The contents of caffeine were determined by LC–ESI-MS according to Antonio et al.[11]

The liquid chromatograph equipment (Shimadzu, Kyoto, Japan) comprised a LC-10AD*vp* quaternary pump, a CTO-10AS*vp* column oven, a 8125 manual injector (Rheodyne) with a 5 μL loop and a SPD-M10A*vp* diode array detector. This liquid chromatograph system was interfaced with a LC–MS 2010 mass spectrometer (Shimadzu, Kyoto, Japan). For sample clarification prior to the chromatographic analyses of phenolic compounds, 500 μL of each coffee extract was mixed with 500 μL of Carrez solutions and the volume was made up to 50 mL with Milli-Q water. The mixtures were shaken, allowed to rest for 10 min and filtered through a Whatman #1 qualitative filter paper and through a 0.22 μm membrane (Millipore). The filtrates were used directly for chromatography. Chemical analyses were performed in triplicate.

### 2.3. *Bacterial strains and culture to evaluate the Minimum Inhibitory Concentration (MIC)*

The bacterial strains used were from the American Type Culture Collection (ATCC): *S. mutans* ATCC 25175 and *Streptococcus sobrinus* ATCC 33478. Bacteria were kept at −20 °C in Tryptic Soy Broth (Oxoid, Hampshire, England) with 20% glycerol and activated by transfer into blood agar. The plates were incubated at 37 °C during 48 h, in a candle jar. Bacterial cells were suspended in saline solution to produce a suspension of about $1.5 \times 10^8$ CFU/mL. 300 μL of this suspension were mixed with 9.7 mL of Mueller–Hinton bacterial Broth medium (Difco, Sparks, USA), resulting in an inoculum with 4–$5 \times 10^6$ CFU/mL.

### 2.4. *MIC and Minimum Bactericide Concentration (MBC) determination*

MIC was evaluated by the dilution method in Mueller–Hinton Broth medium (Difco, Sparks, USA) according to Antonio et al.,[11] with concentrations ranging from 5 to 160 mg/mL of the 20% aqueous coffee extracts. 100 μL of the inoculum was added to each tube and the MIC value was evaluated after 24 h incubation at 37 °C, as the lowest coffee solution concentration inhibiting observable growth. Samples from tubes where the MIC results showed no bacterial growth were removed with a loop, inoculated onto a blood agar plate, and incubated at 37 °C, for 48 h. MBC was considered to be the lowest concentration at which microorganisms were totally unable to grow. The controls included an inoculated Mueller–Hinton Broth medium (Difco, Sparks, USA) without the test coffee extracts and also tubes with no inoculated Mueller–Hinton Broth medium (Difco, Sparks, USA), but with the test extracts, in all concentrations tested. Experiments were performed three times in duplicate.

### 2.5. *Killing kinetics assays*

The experiment of bacterial killing kinetics of the coffee extract was described by Alviano et al.[13] Only *S. mutans* ATCC 25175 was used in this study because we did not find the MIC and the MBC of the tested coffee extract against *S. sobrinus*.

Please cite this article in press as: Antonio AG, et al. Inhibitory properties of *Coffea canephora* extract against oral bacteria and its effect on demineralisation of deciduous teeth. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.12.001

Accordingly, 0.4 mL aliquots of the bacterial strain culture, after adjustment to 0.5 McFarland scale, which were grown for 10 h in Müeller–Hinton Broth medium (Difco, Sparks, USA) at 37 °C under microaerofilia, were added to 1.6 mL of the extract at 25% to give a final concentration of 20%. Also, coffee extracts at 1% and 16% were tested. After the inoculum addition, 100 μL aliquots of each system were collected at 30 min intervals up to 3 h, to enumerate viable cells by serial dilution ($10^{-3}$ to $10^{-8}$) in sterile saline and spreading of 100 μL of each dilution in blood agar plates. Two more tubes containing 2 mL of Müeller–Hinton Broth medium (Difco, Sparks, USA) were used as controls; one without bacteria for a sterility control and the second with bacteria for a growth control. Chlorhexidine at 0.05% (positive control) and Milli-Q water (negative control) were also tested. The blood agar plates were incubated during 48 h at 37 °C in microaerofilia to determine the CFU/ml. The killing kinetics curve for *S. mutans* strain with the coffee extract and the controls were obtained comparing the bacterial population at the beginning of the experiment (time = 0 min) and at each 30 min interval after the addition of the tested substances.

### 2.6. Tooth selection and sample preparation for biofilm plate assay

The naturally exfoliated primary first molars used in this investigation were collected from children living in Rio de Janeiro, Brazil, in conformity with the rules of the Local Ethics Committee of the Institute of Studies in Public Health, Federal University of Rio de Janeiro, Brazil (Process No. 43/2007). Twelve teeth were inspected using a light microscope (40 X), and none of them was found to have structural alterations. All teeth were stored in physiological solution (changed every week) until the beginning of the experiment. The teeth were sectioned mesiodistally by using a cutting machine (Isomet, Buehler, Lake Bluff, IL, USA), resulting in 24 fragments. Each fragment was coated with an acid-resistant nail varnish leaving a window (4.5 mm × 4.5 mm) of exposed tooth. All fragments were submitted to ethylene oxide sterilisation (Bioxxi, Brazil) prior to the experiment (Fig. 1).

### 2.7. Inoculum to form biofilm on tooth fragments

The inoculum comprised unstimulated whole mixed saliva, collected from three volunteers (1 man and 2 women) aged 25–36 years (mean 29 years), who spit into a graduated collection tube. The subjects were in good general and oral health, and had all natural teeth. None of the subjects was on any medication and they gave their informed consent. Subjects were instructed not to consume food or beverages except water for 1 h before saliva collection. Moreover, none of them consumed coffee habitually. The saliva produced in the first 30 s was discarded and then, it was collected for exactly 5 min. Their mean DMFT (4.6) and mean whole saliva flow rate (0.83 mL/min) were registered.

The saliva (1 mL) from each volunteer was placed into a tube, which was mixed using a vortex, resulting in an inoculum with $2 \times 10^8$ CFU/mL (dilution of 1:200). From this suspension, $0.6 \times 10^3$ CFU/mL of *S. mutans* were identified.

### 2.8. Biofilm plate assay

The biofilm model (Fig. 1) employed in this study was able to form stable and reproducible biofilm with human saliva. It was conducted in polystyrene 24-well tissue-culture plates (TPP, Zellkultur Testplatte 24 F). The tooth fragments were fixed inside the wells with 400 μL of agar at 2% (Noble agar, Difco, USA). First, each fragment was placed inside a well and then the agar was carefully added in its bottom, leaving the teeth surface exposed for treatment. After the agar had become solid in order to fix the referred tooth fragments, the wells were completed with Brain-Heart Infusion growth media (BHI, Difco, 1485 μL/well) already containing the inoculum (15 μL/well). The system was incubated in microaerofilia for 10 days at 37 °C so as to produce biofilm.

After biofilm formation on the teeth, three different types of treatment were performed (six teeth for each treatment): (1) 50 μL of *C. canephora* extract at 20%; (2) 50 μL of Milli-Q purified water (negative control); and (3) 50 μL of chlorhexidine at 0.12% (positive control). Six biofilm-covered teeth, which did not receive any treatment, were considered the blank control. Before substances' application on dental biofilm for 1 min, the medium from each well was removed. After each treatment, dental fragments were rinsed twice with 1500 μL of distilled water (neutral pH) and a new Brain-Heart Infusion growth media (BHI, Difco, Sparks, USA; 1500 μL/well) was inserted into the wells. The treatment procedure was performed once a day, at the same hour, during a week.

### 2.9. Plaque pH measurements

The plaque pH measurements (Fig. 1) were performed with a touch microelectrode (Beetrode MEPH-3L, WPI, New Haven, CT, USA) and with a reference electrode connected to a pH metre (Orion Res Inc., Cambridge, MA, USA). The electrode was calibrated using pH 4.0 and pH 7.0 buffers before the beginning of each test and also after every plaque pH determination. A group of three teeth from the six treated with each substance and also from the six blank controls had their biofilm evaluated. The microelectrode was inserted into the biofilm on each teeth and the plaque pH was measured on 7th day of treatment at time intervals of 0 (immediately after) and 5 min after substances' application. The pH analyses were blind.

### 2.10. Cross-sectional microhardness test of teeth fragments

At the end of the experimental phase, the tooth fragments were removed from the wells, and longitudinally sectioned in the middle of the fragment resulting in two halves (Fig. 1). Each half was included in stubs and the cut surfaces were exposed and polished. The cross-sectional microhardness (CSMH) was performed according to Hara et al.[14] Two sequences of 14 indentations were made at 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, 200 and 300 μm from the outer enamel surface, 150 μm apart. The means were calculated for each distance. CSMH values were measured using a Digital Microhardness Tester (HVS-1000, Time Group Inc., China), with a load of 25 g for 10 s. The data were expressed as Knoop hardness number (kg/mm$^2$). Afterwards, the hardness of the untreated enamel

Please cite this article in press as: Antonio AG, et al. Inhibitory properties of *Coffea canephora* extract against oral bacteria and its effect on demineralisation of deciduous teeth. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.12.001

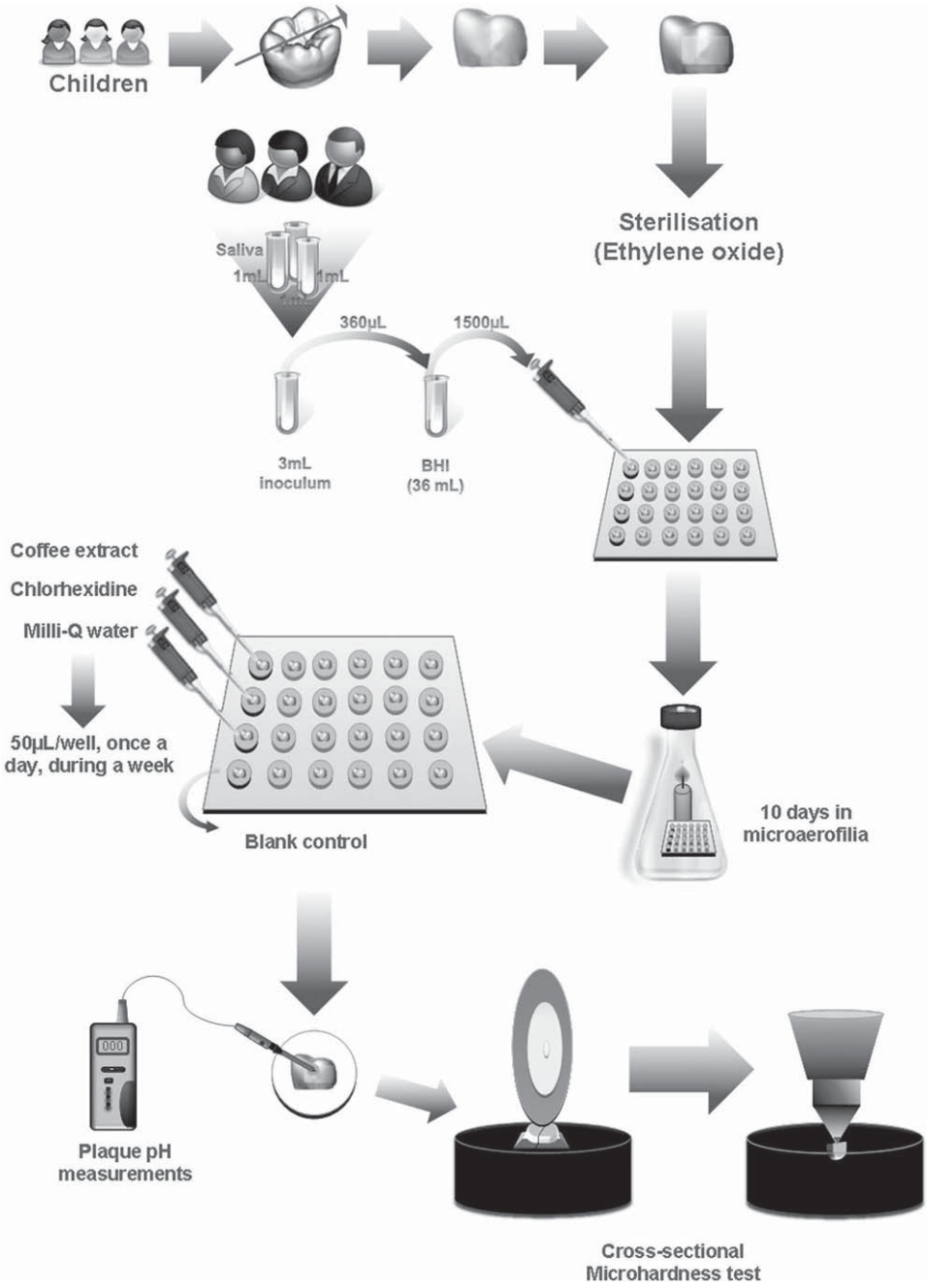


**Fig. 1 – The biofilm model and the tests used to identify the anticariogenic property of *Coffea canephora* extract.**

that was covered with the nail varnish was used as control. The examiner was blind during the experiment.

### 2.11. *Mineral contents of the C. canephora extract*

An aliquot of 3 mL from *C. canephora* extract at 20% and from BHI growth media was sampled and centrifuged (3000 × *g*, 3 min., 4 °C). The supernatant was treated with 250 µL of 65% of $HNO_3$. The solutions were analysed by atomic absorption spectroscopy (Analyst 300 – Perkin Elmer, Germany) in order to calculate the contents of Zinc, Strontium, Silicon, Sulphur, Phosphorus, Sodium, Manganese, Magnesium, Potassium, Iron, Copper, Calcium, Barium, Boron and Aluminium in the coffee and in the media where the teeth were submitted to treatments.

In relation to fluoride contents, it was also analysed in samples of *C. canephora* and BHI, in duplicate, according to Lima and Cury.[15] Fluoride was analysed using an ion specific electrode (Orion – 96-09) after buffering with the same volume of TISAB II (acetate buffer 1.0 M, pH 5.0 containing NaCl 1.0 M and 1,2-cyclohexanediaminetetracetic 0.4%). The electrode was calibrated with five standard solutions ranging from 0.125 to 2.0 µg F/mL. The calibration curve ($r = 0.909$) was tested against fluoride standard solutions (Orion) and blanks.

Please cite this article in press as: Antonio AG, et al. Inhibitory properties of *Coffea canephora* extract against oral bacteria and its effect on demineralisation of deciduous teeth. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.12.001

Analyses were performed in duplicate.

### 2.12. Statistical analysis

The Kruskal–Wallis test was used for statistical comparison of killing kinetics assays results and also for the effect of *C. canephora* extract on acidogenicity of biofilm. For analyses of CSMH values, the assumptions of equality of variances and normal distribution of errors were respectively checked with the Shapiro–Wilk test for all response variables. ANOVA and Tukey test were used to detect differences among treatments. SSPS software, version 17.0 was used for all statistical analyses. Differences between means were considered significantly different when values of $p < 0.05$ were obtained.

## 3. Results

The phenolic compounds, caffeine and minerals concentrations in the *C. canephora* aqueous extract at 20% are presented in Tables 1 and 2, respectively.

### 3.1. Antibacterial and bactericidal activities of coffee extract

The *C. canephora* extract was tested for its antibacterial activity towards *S. mutans* and *S. sobrinus*. MIC and MBC values, $7 \pm 2$ (mg/mL) and $160 \pm 0$ (mg/mL), respectively, showed that the coffee extract is active against *S. mutans*. No antibacterial (MIC) and bactericidal (MBC) activities were found against *S. sobrinus* at tested concentrations.

### 3.2. Killing kinetics results

The different concentrations of coffee extract (1%, 16% and 20%) acted differently on *S. mutans* when compared to the controls and one to another (Fig. 2). *C. canephora* aqueous extract at 16% and 20% ($p > 0.05$) demonstrated a 4-log reduction in the growth of *S. mutans* after 3 h treatment, compared to the untreated control ($p < 0.05$). Chlorhexidine at 0.05% could reduce the CFU count of *S. mutans* ATCC 25175 below the detection limit (50 CFU/ml) from 30 min on ($p < 0.05$). *C. canephora* extract at 1% was not able to reduce the initial bacterial population after 3 h treatment in the same proportion of the extracts with the highest concentrations ($p < 0.05$).

**Table 1 – Contents of cinnamic acid derivatives (phenolic compounds) and caffeine in *Coffea canephora* extract at 20%.[a,b]**

| Chemical compounds | Mean ± SD |
|---|---|
| *Cinnamic acid derivatives* | 3650.78 ± 74.0 |
| 3-CQA | 808.6 ± 16.8 |
| 5-CQA | 1342.2 ± 52.7 |
| 3-FQA | 136.6 ± 7.0 |
| 4-CQA | 863.4 ± 22.3 |
| 5-FQA | 78.7 ± 6.5 |
| 4-FQA | 181.08 ± 7.4 |
| 3,4-diCQA | 125.30 ± 4.4 |
| 3,5-diCQA | 42.75 ± 0.4 |
| 4,5-diCQA | 72.14 ± 1.1 |
| CA | 87.33 ± 35.0 |
| *Caffeine* | 2110 ± 0.4 |

[a] Results are shown as mean of triplicate analysis, expressed in μg/mL.
[b] CA, caffeic acid; CQA, cafeoylquinic acids; FQA, feruloylquinic acids; diCQA, dicaffeoylquinic acids; CGA, chlorogenic acids.

**Table 2 – Mineral contents of *Coffea canephora* extract at 20% and BHI medium.[a]**

| Minerals | *Coffea canephora* extract (20%) (mean ± SD) | BHI medium (mean ± SD) |
|---|---|---|
| Zinc | 0.52 ± 0.03 | 1.57 ± 0.02 |
| Strontium | 1.11 ± 0.05 | 0.04 ± 0.01 |
| Silicon | 6.66 ± 0 | 4.05 ± 0.04 |
| Sulphur | 314.66 ± 9.6 | 243.73 ± 3.2 |
| Phosphorus | 491.67 ± 22.77 | 294.82 ± 10.54 |
| Sodium | 26.66 ± 1.67 | 2714.8 ± 97 |
| Manganese | 1.94 ± 0.03 | 0.04 ± 0.01 |
| Magnesium | 589.69 ± 18.32 | 14.04 ± 2.12 |
| Potassium | 10173.58 ± 182 | 759.35 ± 19.43 |
| Iron | 3.22 ± 0.15 | 0.8 ± 0.01 |
| Copper | 0.46 ± 0.02 | 0.2 ± 0 |
| Calcium | 216.71 ± 11.93 | 10.8 ± 3.2 |
| Barium | 0.14 ± 0 | 0.04 ± 0.01 |
| Boron | 4.23 ± 0.16 | 0.10 ± 0 |
| Aluminium | 0.083 ± 0.08 | 0.3 ± 0 |
| Fluoride | 0.018 ± 0 | 0.044 ± 0.003 |

[a] Results are shown as mean of duplicate analysis, expressed in μg/mL.

### 3.3. Effect of *C. canephora* extract on acidogenicity of biofilm

Although we observed a small raise (from $4.81 \pm 0.10$ to $4.95 \pm 0.05$) of biofilm pH 5 min. after the last treatment (7th day of treatment) with the *C. canephora* aqueous extract at 20%, it was not significant ($p > 0.05$) when compared to the treatment with negative control (from $4.89 \pm 0.11$ to $4.84 \pm 0.13$) and to blank control ($4.83 \pm 0.14$ to $4.85 \pm 0.16$).

It was not possible to evaluate the pH of the biofilm treated with the positive control (chlorhexidine at 0.12%), since these tooth fragments showed no biofilm accumulation after 7 days of treatment.

### 3.4. Cross-sectional microhardness results

For the CMSH analysis (Fig. 3), *C. canephora* aqueous extract significantly ($p < 0.05$) inhibited demineralisation at depths up to 30 μm from the enamel surface when compared to blank and negative controls. Also, at depths up to 20 μm, fragments subjected to chlorhexidine (0.12%) presented less loss of hardness than coffee extract, but the difference between both treatments was not significant for all distances.

The hardness values of the tooth fragments' areas subjected to coffee extract and chlorhexidine (0.12%) did not differ statistically from the adjacent areas that were previously protected with acid-resistant varnish.

Please cite this article in press as: Antonio AG, et al. Inhibitory properties of *Coffea canephora* extract against oral bacteria and its effect on demineralisation of deciduous teeth. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.12.001

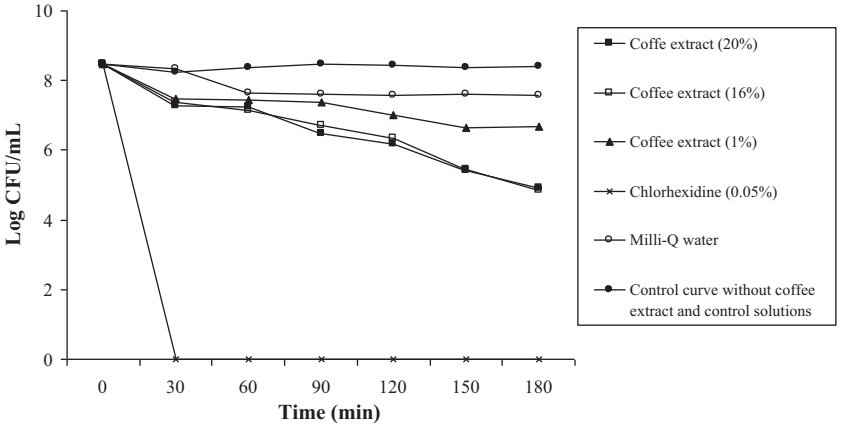


**Fig. 2 – Bacterial killing kinetics of a *C. canephora* extract and controls for 180 min. The *S. mutans* CFU was determined at 30 min intervals after the contact with the following treatments: (1) *C. canephora* aqueous extracts at different concentrations (1%, 16% and 20%), (2) chlorhexidine (0.05%) – positive control and (3) Milli-Q water – negative control. Bacterial growth without the extracts and control solutions was also monitored. Significant differences ($p < 0.05$) were found between controls and coffee extracts at 16% and 20% from 150 min onwards and also between chlorhexidine (0.05%) and all other treatments, in all moments.**

## 4. Discussion

The use of plant extracts with medicinal properties represents a concrete alternative for the treatment of different diseases. This includes the use of natural products as antimicrobial agents,[5,13,16] even though in the absence of scientific basis such practices may generate serious adverse effects.[13]

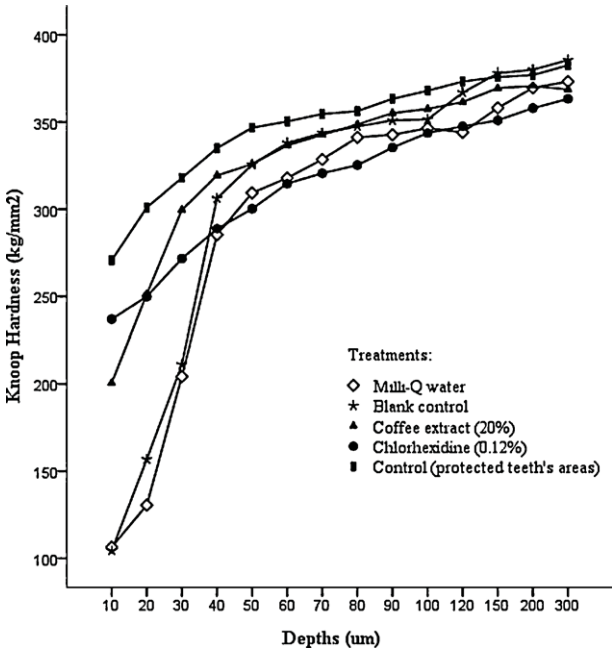


**Fig. 3 – Means of enamel Knoop hardness (kg/mm$^2$) after treatments with coffee extracts and controls, considering the distance (μm) from the enamel surface. A significant difference was found among the coffee extract and blank/negative controls at depths up to 30 μm ($p < 0.05$). No significant difference was observed between this extract and chlorhexidine ($p > 0.05$).**

Regarding the use of coffee extracts, it could not be disapproved, since this beverage is part of the habitual diet for many people, except for those who are very sensitive to caffeine.

There is no doubt that coffee is the most consumed beverage in the world after mineral water. In addition to its pleasant flavour, it has been considered as a potential functional food for its biopharmacological properties demonstrated in clinical and epidemiological researches.[17,18] In the last decade, a series of studies have been performed exploring the anti-infective properties of coffee on different microorganisms,[11,16,19,20] which can generate a significant improvement in managing several kinds of health disorders. The main compounds responsible for such activity in roasted coffee extracts are chlorogenic acids, caffeic acid and caffeine. Other minor compounds described in the literature are trigonelline, α-dycarbonil compounds and protocatechuic acid.[10,11,19]

Anila Namboodiripad and Kori [21] observed that subjects who consumed coffee daily without sugar and milk showed Decayed Missing and Filled Surface (DMFS) scores of 2.9, whereas in subjects who did not consume coffee, the DMFS score was 4.0, demonstrating the anti-caries properties of coffee. The anti-caries effect of a substance has been related to: (1) its physicochemical effects, by inhibiting demineralisation and enhancing remineralisation processes[3]; (2) its antibacterial effects, by inhibiting the critical metabolic processes of mutans streptococci[12]; and (3) by preventing the development of favourable low pH environments for cariogenic bacteria in the biofilm.[12] In the present study, these three effects were evaluated in *C. canephora* extract.

Included in the group of bacteria associated with carious lesions is the group of mutans streptococci, which contains seven species, although two of them, *S. mutans* and *S. sobrinus*, are the main bacteria associated with caries in humans.[22] In the present study, we selected both bacteria to be evaluated. Nevertheless, mutans streptococci species are not considered to be one of the early or primary colonizers of teeth, but rather

Please cite this article in press as: Antonio AG, et al. Inhibitory properties of *Coffea canephora* extract against oral bacteria and its effect on demineralisation of deciduous teeth. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.12.001

colonize at later stages of plaque development, after other species have adhered on the tooth surface. Acquisition of these bacteria, and their final proportions in plaque, may be related in part to the presence of other plaque species and in part to the environmental conditions.[23] Therefore, in the present study, an *in vitro* biofilm/caries model using a saliva pool – where mixed species were controlled by *in vitro* environmental and nutrient conditions – was applied to simulate an *in vivo* situation. According to Fontana et al.[23] a bacterial system is the best *in vitro* caries model to accurately mimic an oral environment.

Still considering the methodology, different concentrations of chlorhexidine were used as positive controls. For time-kill experiment with strains of ATCC, the positive control was chlorhexidine at 0.05%, while for the biofilm assay it was chlorhexidine at 0.12%. Studies[11,24] have been using chlorhexidine at 0.05% as the positive control for biofilm models formed by only one ATCC microorganism strain. According to Filoche et al.,[2] single-species biofilms were more susceptible to treatment with chlorhexidine than a biofilm provided by different species of microorganisms. In the present work, the biofilm formed on teeth surface should be considered as a complex net of different species of bacteria, thus the authors opted for a higher concentration of chlorhexidine (0.12%) as the positive control, just as it is prescribed for the oral cavities.

Regarding the biofilm assay, despite the difficulty to fragment primary teeth due to their small size, such teeth were used as hosts for microorganism biofilm growth because they are physiologically exfoliated, being easier to be obtained than permanent teeth. Additionally, primary teeth are more susceptible to mineral loss than permanent teeth. According to Attin et al.,[25] primary teeth contain higher concentrations of carbonate ion comparing to permanent teeth, being also differently placed in the hydroxyapatite structure of deciduous teeth when compared to permanent teeth. Such characteristic is responsible for a higher susceptibility of primary enamel to loose minerals when exposed to a cariogenic challenge than permanent enamel.[26] Therefore, since primary teeth are more susceptible to caries disease, we found appropriate to use them in our model instead of permanent teeth.

In the current study, *C. canephora* extract did not show bacteriostatic activity against *S. sobrinus* at tested concentrations. Although it might be possible at higher concentrations, this test was not performed because we opted for testing concentrations that still could be normally consumed by people (up to 20%). This concentration has already shown antibacterial properties against enterobacteria.[19]

According to our results, the minimum concentration of the coffee extract that inhibited the growth of *S. mutans* was $7 \pm 2$ (mg/mL). A similar result (MIC = 5 mg/mL) has been previously observed by Antonio et al.,[11] when evaluating the antibacterial activity of *C. arabica* and *C. canephora*. Regarding the minimum bactericidal concentration (160 mg/mL) of this extract against *S. mutans*, it was higher than MIC in from 4 to 5 dilutions. Comparable results were demonstrated by a similar study with natural product.[27]

The outcome of the time-kill assay showed that the *C. canephora* extract exhibited a time- and concentration-dependent killing effect against *S. mutans* ATCC 25175. This means that after 3 h contact between *S. mutans* and the extract, the number of viable cells reduced significantly. Our data suggests that the consumption of coffee can prevent the colonization of *S. mutans*, since coffee has been widely and safely consumed as a beverage for a long period of time. Signoretto et al.[28] observed, in an *ex vivo* study, a positive correlation between the consumption of coffee and oral health in terms of reduction of plaque deposition and lower counts of odontopathogens, such as *S. mutans*.

Considering the results of the cross-sectional microhardness test, it showed that the *C. canephora* extract at 20% inhibited the demineralisation of enamel at depths up to 30 μm from the enamel surface. Despite the similar inhibitory effect of the coffee extract at 16% and at 20% on killing kinetics experiment with planktonic cells of *S. mutans*, we opted to conduct the biofim plate assay testing the extract at 20% since we used mixed biofilm formed by human saliva, which tend to be more resistant than planktonic cells.[11]

The process of enamel demineralisation involves the dissolution of enamel apatite crystals, and the diffusion of ions, such as calcium, phosphate, and hydrogen, into and out of the enamel microstructures. It has been suggested that the ion diffusion pathway in enamel is controlled by the organic matrix network, which occupies the enamel tissue.[16] The changes on enamel organic matrix can affect the demineralisation process through the control of the diffusion pathway in tooth structures.[3] In the present study, the chemical analyses of the coffee extract showed a large amount of calcium and phosphorus in the referred extract. So, the authors supposed that the *C. canephora* extract could interact with the enamel organic matrix through its mineral contents, inhibiting the decomposition of the organic matrix during the acid attack by the microorganisms.

Furthermore, Zhang et al.[16] avowed that interactions between polyphenols and organic matrixes seem to also inhibit the demineralisation process. According to them, the referred interaction involves covalent, ionic, hydrogen bonding or hydrophobic processes, which induces the metamorphism of enamel organic matrix. The metamorphic organic matrix is precipitated in the enamel, resulting in a slow down of the speed of mineral ions loss, and consequently, enamel demineralisation is inhibited. In our study, the *C. canephora* extract was extremely rich in polyphenol compounds, which should have inhibited the demineralisation process of the fragments submitted to the artificial caries/biofilm model. A grape seed extract, another natural substance containing polyphenols, exerted a similar effect on the tooth hard tissue.[24]

Another point should be emphasized. Since BHI medium contained some minerals like calcium and phosphorus, one could say that these minerals could have exerted an effect on teeth remineralisation process. However, all tooth fragments, including blank and negative controls, were equally kept with BHI medium in the wells. A significant difference between the hardness of dental biofilm that received the coffee treatment and the negative and blank controls led us to believe that the amount of the referred chemical compounds in the coffee extract was responsible for such result.

*C. canephora* extract demonstrated a significant inhibitory effect of demineralisation even after applying a small amount of the extract (50 μL) on the biolfim and for a short period of

Please cite this article in press as: Antonio AG, et al. Inhibitory properties of *Coffea canephora* extract against oral bacteria and its effect on demineralisation of deciduous teeth. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.12.001

time. Aspiras et al.[29] stated that the biofilm acts as a storage reservoir for ions such as calcium, fluoride and phosphate, causing a raise in retention and an exchange between these ions and tooth enamel. In addition, dental plaques on enamel tooth have been shown to consist of microbial ''stacks'' surrounded by voids and channels, exhibiting an open and fragmented architecture with a high surface-area in the outermost layers.[16] The void-and-channel system in plaque would serve as a route for the distribution of antimicrobial compounds. This would increase the time needed for remineralisation and thus delaying the caries combat. In the present study, even though the biofilm was washed after treatment, the coffee compounds were probably retained in the biofilm netting, being released over time to the tooth and allowing the constant ion exchange.

We know that small amounts of calcium and phosphorus lost by enamel during the pH drop throughout the de-remineralisation process can be more efficiently recovered if fluoride is present in the oral environment.[30] In a recent study[20] the authors reported that the coffee fraction tested against oral biofilm contained fluoride, which is understood by us that the anticaries action of coffee aqueous extract should be also enhanced by the presence of fluoride in coffee. However, in the present study, the fluoride contents of *C. canephora* and of BHI were lower than the limits needed (0,1 μg F/mL) to raise the pH of biofilm [31] and consequently favour the ionic exchanges during the de-remineralisation process.

In this study, although the coffee extract penetrated through the biofilm net pathways, inhibiting the demineralisation process, it was not able to increase the biofilm pH. The *C. canephora* extract studied was made from a light roasted ground coffee with weak acidic properties (pH $5.28 \pm 0.8$). Although it prevented the rising of dental biofilm pH, it contained a large amount of phenolic compounds and caffeine that favours its antimicrobial action.[11] The authors believe that both the inhibition of demineralisation and the antibacterial properties of this coffee extract against *S. mutans* were more important caries-preventive factors than its influence on the biofilm acidogenecity.

Analysis of data from our CSMH experiment indicates no significant difference between the fragments with biofilm which was treated with the coffee extract and those treated with chlorhexidine (0.12%). However, the hardness values of fragments submitted to chlorhexidine at depths under 20 μm from enamel surface were lower than the same values of fragments/biofilm treated with the coffee extract. It is well documented that chlorhexidine is a classic anticaries agent due to its properties in killing both Gram-negative and Gram-positive bacteria by damaging their cell wall. Its antiplaque action is superior to other antiseptics with greater antibacterial activity.[32] Probably, the effect of this potent antimicrobial agent reduced a great number of viable cells, thus preventing the acid production by the current oral bacteria and consequently the loss of mineral. So, its anticaries action was only due to its antibacterial properties, since it does not contain chemical compounds that are able to interfere with the organic matrix of enamel, enhance the remineralisation of tooth enamel, and consequently elevate the hardness of the present enamel fragments.

In view of the presented data, a light roasted *C. canephora* aqueous extract can be considered as a potential anticariogenic substance due to its capacity of preventing the growth of *S. mutans* and of inhibiting dental demineralisation. However, as much as we have tried to mimic a real situation, these results were obtained in an *in vitro* environment. Further *ex vivo/in vivo* studies should be elaborated with the aim of investigating new approaches for caries management such as the benefits of the retention of this anticariogenic agent into dental biofilms.

## Acknowledgments

We are grateful to Professor Jaime Aparecido Cury for the fluoride analyses performed in the Oral Biochemical Laboratory situated in the Physiologic Science Department of State University of Campinas. The authors would like to thank Mr. Marlei Gomes da Silva for generously providing support for the biofilm assay. The financial support of the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) is also acknowledged.

*Funding*: None.

*Competing interests*: We affirm that we have no conflict of interests among authors or the author's institutions.

Ethical approval: Not required.

## REFERENCES

1. Kidd EAM, Fejerskov O. What constitutes dental caries? Histopathology of carious enamel and dentin related to the action of cariogenic biofilms. *J Dent Res* 2004;**83**(Spec Iss C):C35–8.
2. Filoche S, Wong L, Sissons CH. Oral biofilms: emerging concepts in microbial ecology. *J Dent Res* 2010;**89**:8–18.
3. Featherstone JD. Fluoride, remineralization and root caries. *Am J Dent* 1994;**7**:271–4.
4. Ooshima T, Minami T, Aono W, Izumitani A, Sobue S, Fujiwara T, et al. Oolong tea polyphenols inhibit experimental dental caries in SPF rats infected with mutans streptococci. *Caries Res* 1993;**27**:124–9.
5. Ooshima T, Osaka Y, Sasaki H, Osawa K, Yasuda H, Matsumura M, et al. Caries inhibitory activity of cacao bean husk extract in in vitro and animal experiments. *Arch Oral Biol* 2000;**45**:639–45.
6. Smullen J, Koutsou GA, Foster HA, Zumbé A, Storey DM. The antibacterial activity of plant extracts containing polyphenols against *Streptococcus mutans*. *Caries Res* 2007;**41**:342–9.
7. Koo H, Cury JA, Rosalen PL, Ambrosano GM, Ikegaki M, Park YK. Effect of a mouthrinse containing selected propolis on 3-day dental plaque accumulation and polysaccharide formation. *Caries Res* 2002;**36**:445–8.
8. Yatsuda R, Rosalen PL, Cury JA, Murata RM, Rehder VL, Melo LV, et al. Effects of Mikania genus plants on growth and cell adherence of mutans streptococci. *J Ethnopharmacol* 2005;**97**:183–9.
9. Farah A, de Paulis T, Moreira DP, Trugo LC, Martin PR. Chlorogenic acids and lactones in regular and water-decaffeinated arabica coffees. *J Agric Food Chem* 2006;**54**:374–81.
10. Daglia M, Papetti A, Grisoli P, Aceti C, Spini V, Dacarro C, et al. Isolation, identification, and quantification of roasted

coffee antibacterial compounds. *J Agric Food Chem* 2007;**55**:10208–13.
11. Antonio AG, Moraes RS, Perrone D, Maia LC, Santos KRN, Iorio NLP, et al. Species, roasting degree and decaffeination influence the antibacterial activity of coffee against *Streptococcus mutans*. *Food Chem* 2010;**118**:782–8.
12. Bradshaw DJ, Marsh PD, Hodgson RJ, Visser JM. Effects of glucose and fluoride on competition and metabolism within in vitro dental bacterial communities and biofilms. *Caries Res* 2002;**36**:81–6.
13. Alviano WS, Alviano DS, Diniz CG, Antoniolli AR, Alviano CS, Farias LM, et al. In vitro antioxidant potential of medicinal plant extracts and their activities against oral bacteria based on Brazilian folk medicine. *Arch Oral Biol* 2008;**53**:545–52.
14. Hara AT, Queiroz CS, Paes Leme AF, Serra MC, Cury JA. Caries progression and inhibition in human and bovine root dentine in situ. *Caries Res* 2003;**37**:339–44.
15. Lima YBO, Cury JA. Ingestão de flúor por crianças pela água e dentifrício. *Rev Saude Publ* 2001;**35**:576–81.
16. Zhang L, Xue J, Jiyao L, Zou L, Hao Y, Zhou X, et al. Effects of Galla chinensis on inhibition of demineralization of regular bovine enamel or enamel disposed of organic matrix. *Arch Oral Biol* 2009;**54**:817–22.
17. Ohshima T, Miyakawa Y, Watanabe T, Ohyama K. Effect of amphotericin B dilution with various beverages on the survival of *Candida albicans* cells. *Kansenshogaku Zasshi* 2003;**77**:29–33.
18. Johnston KL, Clifford MN, Morgan LM. Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine. *Am J Clin Nutr* 2003;**78**:728–33.
19. Almeida AAP, Farah A, Silva DAM, Nunan EA, Glória MB. Antibacterial activity of coffee extracts and selected coffee chemical compounds against Enterobacteria. *J Agric Food Chem* 2006;**54**:8738–43.
20. Stauder M, Papeti A, Daglia M, Vezzulli L, Gazzani G, Varaldo PE, et al. Inhibitory activity by barley coffee components towards *Streptococcus mutans* Biofilm. *Cur Microbiol* 2010.
21. Anila Namboodiripad PC, Kori S. Can coffee prevent caries? *J Conserv Dent* 2009;**12**:17–21.
22. Thylstrup A, Fejerskov O. *Cariologia clínica*. 3rd ed. São Paulo: Editora Santos; 2001.
23. Fontana M, Haider A, Gonzáles-Cabezas C. Caries lesion development and biofilm composition responses to varying demineralization times and sucrose exposures. *Biofilms* 2004;**1**:229–37.
24. Xie Q, Bedran-Russo AK, Wu CD. In vitro remineralization effects of grape seed extract on artificial root caries. *J Dent* 2008;**36**:900–6.
25. Attin T, Wegehaupt F, Gries D, Wiegand A. The potential of deciduous and permanent bovine enamel as substitute for deciduous and permanent human enamel: erosion–abrasion experiments. *J Dent* 2007;**35**:773–7.
26. Sønju Clasen AB, Ruyter IE. Quantitative determination of type A and type B carbonate in human deciduous and permanent enamel by means of Fourier transform infrared spectrometry. *Adv Dent Res* 1997;**11**:523–7.
27. Castro ML, Nascimento AM, Ikegaki M, Costa-Neto CM, Alencar SM, Rosalen PL. Identification of a bioactive compound isolated from Brazilian própolis type 6. *Bioorg Med Chem* 2009;**17**:5332–5.
28. Signoretto C, Burlacchini G, Bianchi F, Cavalleri G, Canepari P. Differences in microbiological composition of saliva and dental plaque in subjects with different drinking habits. *New Microbiol* 2006;**29**:293–302.
29. Aspiras M, Stoodley P, Nistico L, Longwell M, de Jager M. Clinical implications of power toothbrushing on fluoride delivery: effects on biofilm plaque metabolism and physiology. *Int J Dent* 2010. [Epub 2010 April 15].
30. Cury JA, Tenuta LMA. Enamel remineralization: controlling the caries disease or treating early caries lesions? *Braz Oral Res* 2009;**23**:23–30.
31. Belli WA, Buckley DH, Marques RE. Weak acid effects and fluoride inhibition of glycolysis by *Streptococcus mutans* GS-5. *Can J Microbiol* 1995;**41**:785–91.
32. Wise MP, Cole JM, Williams DW, Lewis MA, Frost PJ. Efficacy of oral chlorhexidine in critical care. *Crit Care* 2008;**12**:419.

Please cite this article in press as: Antonio AG, et al. Inhibitory properties of *Coffea canephora* extract against oral bacteria and its effect on demineralisation of deciduous teeth. *Archives of Oral Biology* (2010), doi:10.1016/j.archoralbio.2010.12.001