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Exposure to fluoride of children during the critical age for dental fluorosis, in the semiarid region of Brazil

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Abstract *There is a concern about the increasing prevalence of dental fluorosis, which depends on the dose of fluoride (F) to which children are subjected during tooth formation. Environmental temperature affects water intake and therefore it would be important to assess whether children living in the semiarid region are exposed to an excessive dose of F. Therefore, the objective of this study was to determine the total dose of F to which children are exposed during the critical age for dental fluorosis, with diet (water and food) and toothpaste as F sources, in the semiarid region of Brazil. Methodology: 26 children aged 25.2 ± 9.1 months, residents in Feira de Santana, State of Bahia (with F in the public water supply) were selected. Duplicate-diet, water, products from toothbrushing and toothpaste samples were collected. F concentration was determined using an ion-specific electrode, after proper sample preparation. Results: the mean and standard deviation of dose (mg F/kg/day) from diet, toothpaste and total were respectively: 0.016 ± 0.010; 0.030 ± 0.039 and 0.047 ± 0.043. Conclusions: the children evaluated living in the semiarid region are not exposed to a risk dose for dental fluorosis.*

Key words *Dental fluorosis, Fluorides, Food, Water intake, Toothpastes*

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Introduction

The widespread use of fluoride (F) was a fundamental factor in the decline of caries reported worldwide[1], however, it has been accompanied by concern about the increase in the prevalence of dental fluorosis[2-4].

Dental fluorosis is a defect in the mineralization of teeth in formation, due to the presence of F, and is related to chronic ingestion of this element, with its severity being dose-dependent. Clinically acceptable dental fluorosis is characterized by small white and opaque striae on the tooth surface, but it does not make the affected tooth more, or less susceptible to caries. From the esthetic aspect, it is of major relevance when it occurs in permanent maxillary incisors[5]. The critical age range for the development of dental fluorosis is around 15 to 30 months of life[6], when the permanent maxillary incisors are in the stage of transition between the enamel secretory and maturation phase. Associated with this, children at this age swallow a large part of the toothpaste during tooth brushing, increasing the ingestion of F, if the toothpaste is fluoridated[7-9].

The prevalence and severity of fluorosis are directly related to the quantity of F ingested; According to Fejerskov et al.[5], for each increase in the dose of 0.01 mg F/ kg, an increase of 0.2 in the community fluorosis index (CFI) is expected. Therefore, since past times, limits were established with a view to minimizing the risk (fluorosis) and maximizing the benefit (prevention and control of dental caries). Burt[10] estimated that if systemic F is important, a maximum dose of 0.05 to 0.07mg F/ kg must be obeyed for safety in terms of clinically acceptable dental fluorosis (without compromising esthetics).

Determining the dose of exposure to fluoride in the critical age range for dental fluorosis has been the aim of some studies conducted in different countries. Generally speaking, diet and toothpastes are considered the most representative sources of F. The study of Guha-Chowdhury et al.[11], conducted in New Zealand, demonstrated that children who consumed optimally fluoridated water were exposed to a daily dose of 0.019 mg F/kg from their diet, and 0.036 mg F/kg when diet and toothpastes were considered. In Brazil, the first studies were conducted in the southeastern region, in cities with optimally fluoridated water (Piracicaba/SP, subtropical climate[7]; and Ibiá/MG, tropical climate[8]). Both studies indicated a high total dose, above that of the parameter 0.07 mg F kg/day. However, this high dose was not reflected in high prevalence of dental fluorosis in the same volunteers later re-evaluated[12], so that the relationship between fluoride ingestion and dental fluorosis continued to be undefined[13]. Although this relationship still needs to be established, determining the dose of exposure continues to be important.

Estimates have indicated that fluoridated water supplies contribute to over 50% of the dose in diet, and that the ingestion of liquids is directly proportional to the ambient temperature. Therefore, the formula of Galagan and Vermillion[14] is used to estimate the ideal concentration of F in water supplies considering the mean value of maximum temperatures in each city. As all the states of Brazil, with the exception of RS and SC, have a mean monthly maximum temperature in the range between 26.4 and 32.5ºC, the ideal concentration of F in the water supply must be between 0.6 and 0.8 µg F/ml. However, this temperature range is extensive; it does not take into account the variations throughout the seasons of the year, and the relative humidity of the air is not considered in these estimates. Lima and Cury[15] verified that in a city with a sub-tropical climate (Piracicaba, SP), there is an increase in the volume of liquids consumed during the hotter seasons of the year. As this liquid was fluoridated water, a dose of F higher than 19% would be expected in the hotter periods. This estimate was also reported for some regions of the United States[16].

As a result of this concern, some studies were conducted in Brazilian regions with higher ambient temperatures. In the city of Penedo (AL), with a coastal tropical climate a low dose from water ingestion (0.021 mg F/kg), was reported, although this water contained an F concentration above the recommended level (0.94 µg/ml). Whereas, in the city of Brejo dos Santos (PB), which has a semi-arid climate and water naturally containing 0.6 to 0.9 µg F/ml in the water, an almost three times higher dose from diet (0.06 mg F/kg) was reported[17].

In addition, this higher level of F ingestion was expected in the city of Feira de Santana, BA, considering the data of an oral health survey conducted in the municipality, in which a 45.6% prevalence of dental fluorosis was verified in the State schools; 36.5% in the Municipal, and 34% in Private schools[18]. These values were much higher than those found in the Northeastern region (14.5%), or that of the national mean value (16.7%)[19].

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Methodology

Ethics

This study was approved by the Ethics Committee on Research involving Human Beings of UEFS. The volunteers were selected after their parents/guardians had read and signed a Term of Free and Informed Consent, in which all the necessary information about the research was stated. In addition, the researchers personally provided them with explanations.

Sample

A convenience sample was selected, with the size based on those of similar studies[8,11], composed of 40 volunteers, of both genders, with a mean age of 25.2 ± 9.0 months, resident in the city of Feira de Santana, BA. Five volunteers frequented the daycare center of the State University of Feira de Santana on a daily basis, and 35 remained at home with their family members. During the study, some volunteers withdrew from the study, so that the necessary samples (duplicated diet, water and tooth brushing products), were collected from only 26 volunteers (three who frequented the daycare center and 23 who did not), who thus composed the study casuistic. Their age was 25.2 ± 9.1 months, and their weight equal to 12.5 ± 2.5 kg.

Water collection

A sample of the water used for drinking by each volunteer was collected in a flask provided by the researchers. The origin of the water (public water supply, mineral, well) was questioned by the researchers.

Duplicated diet collection

For the meals consumed, this procedure was performed by the parents/guardians at home; or by one of the researchers, in the case of children who frequented the daycare center. On two days, either consecutively or not, everything the child ate or drank (including water) was collected in a receptacle provided by the researchers. The method was based on the study of Guha-Chowdhury et al.[11]. In this method, the same quantity of all the solid and liquid components of the diet (including water) was collected, as the quantity consumed and ingested in a certain period of time. For this purpose, the person responsible for the collection was given detailed instructions, both verbally and in writing. In these instructions the recommendation was to prepare two portions of the meal: one for the child, and the other for the research (for example, two equal plates of food). After the child had finished eating, if anything was left over, the same quantity had to be removed from the portion destined for research. Thus, the quantity collected had to be similar to the quantity ingested. The parts of foods that were not ingested, such as skin and bone, were not to be collected. The diet sample collected was homogenized in a liquidizer, the volume was measured, and a sample was frozen for later analysis. The concentration of F in the diet was multiplied by its daily volume, and divided by the child's weight, thereby obtaining a dose value (mg F/ kg/ day). The mean value of two days was calculated. To reimburse the expenses incurred by volunteers with the duplicated diet collection, they were provided with kits containing non-perishable food, a tube of toothpaste and a tooth brush.

Collection of tooth brushing products

This procedure was always followed-up by one or more researchers. For this procedure, the volunteers were asked to bring the toothpaste habitually used by the children, so that a sample could be taken (approximately 1 g) and its total soluble fluoride content could be determined. When the volunteers did not bring the toothpaste, the procedure was performed with the toothpaste provided by the researchers (Strawberry flavored Tandy), if the volunteers reported that the child used fluoridated toothpaste for tooth brushing. For the children who did not yet brush their teeth, or those who used non-fluoridated toothpaste, the collection of tooth brushing products was omitted.

According to the methodology described by Rojas-Sanchez et al.[20], an appropriate chart was used for taking notes of: the daily tooth brushing frequency (d.tb.f), the person responsible for this, and which toothpaste was used. The tooth brushing performed by the teacher at the daycare center (in the case of children who frequented the UEFS daycare center) and that performed by the person responsible for each child, which occurred every day, was followed up.

The tooth brush was weighed; the scale (digital, with 2 decimal places) was zeroed, and the person responsible for tooth brushing put the toothpaste on the brush, and it was weighed again. The weight of toothpaste was registered, and tooth brushing was performed in the habitual manner; the child expectorated (if it did so) the foam and saliva into a plastic cup; as was done with rinsing the mouth (with deionized distilled water; the brush was vigorously washed with deionized distilled water, which was also collected in the plastic cup. This suspension, denominated *Product of Tooth Brushing*, was homogenized; its final volume was measured, and a sample (approximately 10 ml) was frozen for later analysis.

F dosing in samples

All the analyses were performed with the use of an F-specific electrode (Orion 96-09) coupled to a potentiometer (Orion Star A214). On every day of analysis, this electrode was previously calibrated with samples containing known concentrations of F (prepared with standards of F, in the same conditions as those of the samples). The electrode provided results in millivolts (mV). As there is a ratio between mV and log[F] (mV = a - b * log [F]), the values in mV were used to reach the F concentration values. For the calculations, Microsoft Office Excel software was used.

To the water samples, a specific buffer was added (TISAB II, total ionic strength and pH adjustment buffer), in the ratio of 1:1, and the electrode was calibrated with standards containing between 0.2 and 3.2 µg F/ml.

To extract F from the diet samples, the hexamethyldisiloxane (HMDS-facilitated) microdiffusion technique was used[21]. The technique consists of adding the sample to a petri dish containing a strong acid (6N hydrochloric acid) saturated with hexamethyldisiloxane. In the center of the dish, there must be a deposit containing a strong base (1.65N sodium hydroxide). The plate containing the reagents remains under agitation for a period of 12 hours, during which the F is extracted from the sample, volatilized (fluorsilane) and attracted to the deposit containing the strong base. Then this deposit was removed from the plate, dried in an oven, and the solid fluoride crystals formed were dissolved in a weak acid (0.66N acetic acid), for the readout to be taken with an electrode calibrated with standards containing between 0.2 and 3.2 µg F/ml. To calculate the dose of F from diet, the F concentration in the samples was multiplied by the volume of diet, and divided by the child's weight.

In the samples of toothpaste and tooth brushing products, the Total Soluble Fluoride concentration was determined, according to the methodology described by Cury[22]. For this purpose, the samples were centrifuged at (3000 x g 10 min) and 0,25 ml of 2M hydrochloric acid was added to a volume of 0.25 ml of the supernatant. This solution was maintained at 45°C for 1h, and then buffered with 0.5 ml of 1M sodium hydroxide and 1 ml of TISAB II. An initial dilution of 100 times was made of each toothpaste; the electrode was calibrated with standards containing between 4 and 64 µg F/ml. Subtracting the amount of F that was recovered (TSF in the tooth brushing product) from the quantity initially used (weight of toothpaste times its TSF concentration), the quantity of F ingested during tooth brushing was determined. This value was multiplied by the daily tooth brushing frequency (d.tb.f) and divided by the child's weight to determine the dose of F to which the child was being submitted daily by brushing with fluoridate toothpaste.

Statistical data analysis

Initially, descriptive statistical analysis of the results was performed, with the purpose of determining the mean, standard deviation and variance values for each variable of the determined response. The t-test (bicaudal) was applied for comparison of the dose of F from diet, between children who drank from the water supply and those who drank mineral water. The limit of significance was established at 5%. Microsoft Office Excel software was used.

Results

Table 1 presents the results of the daily dose of exposure to F, considering the diet and tooth brushing with fluoridated toothpastes as sources of F. On an average, the volunteers were found to be exposed to a safe dose, in terms of dental fluorosis (0.057 mg F/kg), however 34.6% of them (9 volunteers) were exposed to a total dose equal to or higher than the limit of 0.07 mg F/kg. Considering only the dose due to toothpastes, 19.2% (5 of the volunteers) were exposed to a dose equal to or higher than this limit. The fluoridated toothpastes contributed to the larger portion of the total dose (71%). When the volunteers who did not brush their teeth (n = 2), or those who brushed with toothpaste without F (n = 2) were excluded, the values of F dose from diet, toothpaste, and total dose were: 0.017 ± 0.010; 0.048 ± 0.038 and 0.064 ± 0.043 mg F/kg, respectively.

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In Table 2, the values of F concentration in the samples of drinking water used by the volunteers are demonstrated. On an average, the water supply samples were verified to present adequate F content (between 0.6 and 0.8 μg F/ml), and the mineral water samples presented low F concentration.

Table 3 shows a comparison of the dose of F from diet between children who drank water from the fluoridated water supply (n = 9), and children who drank mineral water with low concentrations of F (n = 17). There was no statistically significant difference between the groups ($p > 0.05$).

Table 4 presents the results with reference to the dose of F considering tooth brushing with fluoridated toothpastes. Worth pointing out is that two children used non-fluoridated toothpaste at home, and two did not yet brush their teeth (d.tb.f. = 0). At the daycare center, tooth brushing was performed with non-fluoridated toothpaste. For the children who frequented the daycare center, the results for both brushing performed by the teachers at the daycare center and that performed by the parents were considered.

Discussion

As the semi-arid climate is characterized by high temperatures, low relative humidity of the air, and low volume of rainfall, there may be a higher level of liquid ingestion. Thus, a higher total dose of exposure to F could be suspected than that determined in Brazilian regions with a subtropical or tropical climate[8]. The results of Rodrigues et al.[17] confirmed this. Their study was conducted in five different localities, with one of them (Brejo dos Santos-PB) having a semi-arid climate and water that naturally contained from 0.6 to 0.9 μg

Table 1. Dose of F (mg F/ kg/ day) to which the volunteers were submitted (n = 26).

	Mean	(SD)	Minimum	Maximum
Diet	0.016	0.010	0.003	0.033
Toothpaste	0.030	0.039	0.000	0.148
Total	0.047	0.043	0.004	0.181

Table 2. F concentration in drinking water samples (μg/ml).

	Mean	(SD)	Minimum	Maximum
Water Supply (n = 9)	0.782	0.157	0.538	1.052
Mineral Water (n = 17)	0.070	0.037	0.004	0.148

Table 3. Dose of F from diet, for children who drank water from water supply or mineral water (mg F/ kg/ day).

		Mean	(SD)	Minimum	Maximum
Dietary F Dose (mg F/ kg/ day)	**Water Supply**	0.021a	0.010	0.003	0.033
	Mineral Water	0.014a	0.010	0.003	0.030

p = 0.1578; t-test.

Table 4. Data relative to tooth brushing: daily tooth brushing frequency (d.tb.f.), percentage of toothpaste ingested, and weight of toothpaste placed on brush (g).

	Mean	(SD)	Minimum	Maximum
d.tb.f.	1.6	1.0	0.0	4.0
% toothpaste ingested	70.5	24.0	37.0	100.0
g toothpaste	0.47	0.31	0.14	1.10

F/ml. The dose of F coming from diet, also obtained by the duplicated-diet method, was 0.06 mg F/kg, close to the established limit of 0.07 Mg F/kg. Tooth brushing with fluoridated toothpastes would certainly elevate the daily dose to above this limit.

In the present study the mean dose of F coming from diet was 0.016 g F/kg, well below that expected for a region with a semi-arid climate. The F dose value was also lower in studies conducted in regions of Brazil with more amenable temperatures, which also had fluoridated water supplies: Piracicaba, SP (0.040 mg F/kg) and Ibiá, MG (0.027 mg F/kg)[8]; Bauru, SP (0.06 mg F/kg)[17].

In view of this dose below the expected value, the first aspect to be considered was the fact that 65% of the volunteers drank mineral water with very low quantities of F (Table 2). However, it is important to point out that even when drinking water with negligible quantities of F, the children consumed food prepared with water from the fluoridated water supply, as reported by the parents/guardians. When food is prepared with fluoridated water, F is known to be incorporated into the food, and thus the concentration of F in these foods reflects the concentration of F in the liquid used to prepare it[23-26]. This may explain the fact that there was no significant difference in the dose of F from diet between the children who drank mineral water (0.014 mg F/kg/day) and those that drank water from the fluoridated water supply (0.021mg F/kg/day).

The second aspect to be considered for the dose from diet being below the expected value, may have been imprecision in the duplicate-diet collection made by the persons responsible for the children, in spite of the fact that they had been duly instructed by the researchers. A relatively low total daily volume was verified (685.4 ± 269.8 ml). Only one of the food collections performed at the daycare center by three volunteers was made by one of the researchers. Worth pointing out is the fact that in the study of Lima and Cury[7], the children frequented a daycare center throughout the entire day, and the food was collected by one of the researchers; in the study of Paiva et al.[8] the researcher personally accompanied the collections made in the volunteers' homes. This care is not always possible, and is a risk inherent to the methodology; therefore it was a limitation of the present study. Although subject to this type of imprecision, the duplicated-diet technique appears to result in more realistic values, when compared with the diet diary, which resulted in much higher dose values[11,27].

With regard to fluoridated toothpastes, their early and inadvertent use by children in early childhood is a risk factor for dental fluorosis[28], seeing that they swallow a large portion of this product during tooth brushing, because they are unable to expectorate adequately[7,8]. The quantity of toothpaste used[8,27] and frequency of brushing[8] are directly related to the dose of F to which children are submitted by tooth brushing, although the relationship of these factors with the occurrence of dental fluorosis has not been related[28].

The mean dose of F considering the ingestion of fluoridated toothpastes during tooth brushing was 0,030 mg F/kg; that is, below the critical values. However, when the volunteers were evaluated individually, the authors verified that five of them (19.2%) were exposed to a dose equal to or over the limit of 0.07 mg F/kg only from the F in the toothpaste.

In addition, the mean quantity of toothpaste placed on the brush was 0.47g, almost five times more than the amount recommended for the age group (0.1 g, a size similar to that of a grain of rice)[29]. Worth pointing out is that for all the volunteers, the weight of toothpaste was higher than 0.1g, and the three volunteers with the dose above the limit used a quantity of toothpaste that was well over the limit (between 0.9 and 1.1 g). Whereas, the daily tooth brushing frequency (d.tb.f) was 1.6 on an average, ranging from 0 to 4. In the literature, higher values were verified, as in the study of Omena et al.[30], in which 69% of the volunteers brushed their teeth three or more times a day, resulting in a mean dose of 0.107 mg F/kg from toothpastes. A fact to be observed is that two of the children in the present study did not yet brush their teeth, which does not normally occur with children who frequent daycare centers, and this may explain the higher doses related by Lima and Cury[7] (0.052 mg F/kg; d.tb.f. = 2.2). On an average, 70.5% of the toothpaste put onto the brush was ingested, a value higher than that reported in the literature for the same age range (57.4%[7]; 64.6%[8]; 49 to 64%[9]). According to Nascimento et al.[9], there is an inverse relationship between the age of children and ingestion of toothpastes during tooth brushing. Furthermore, here we point out another aspect: the influence of toothpaste flavor on its ingestion. According to Oliveira et al.[31], when the toothpaste has a sweet flavor (such as Tandy and similar types), there may be a higher percentage of ingestion by children. The fact that we provided Tandy toothpaste when the volunteers forgot to bring the toothpaste they habitually used at home (85% of them did not bring any) could have overestimated the dose found, however, we found that this was the toothpaste most used by the volunteers (50%), followed by the toothpaste without F (23.1%), any toothpaste (11.5%), volunteer did not use toothpaste (7.7%), toothpaste with low F concentration (3.9%) and only one volunteer (3.9%) reported that he/she used toothpaste specifically indicated for adults (without sweetened flavor).

Thus, in view of the large quantity of toothpaste put onto the toothbrush, allied to a high percentage of its ingestion during tooth brushing, we justified the fact that toothpastes contributed the major portion of the total dose of exposure to F, in spite of the present study having been conducted in a region with a semi-arid climate, corroborating the findings of Omena et al.[30]. This is further evidence that in regions with water supplies that are fluoridated at adequate concentrations, the problem with regard to F ingestion is the use of large quantities of toothpaste. Considering the data of high F ingestion due to the use of toothpastes, there is clearly a need to educate the parents/guardians to place small quantities of toothpaste on the children's toothbrushes.

An important point to mention was the use of non-fluoridated toothpaste by six volunteers (23.1%) at home, and by the three at the daycare center they frequented. In all the situations, both the parents and Dentists responsible for the daycare center used the above-mentioned type of toothpaste to prevent dental fluorosis. Nevertheless, the international literature has shown us that this attitude is incorrect[32], because the use of toothpastes with reduce concentrations of fluoride has been shown to be less effective in the control of caries in deciduous teeth[33,34], and this could be aggravated with the use of non-fluoridated toothpastes.

Considering the diet and toothpastes as sources of F, a total dose of 0.047 mg F/kg was verified, below the limit established as being safe (0.07), and incompatible with the high prevalence of fluorosis in the city (mean of 38.7%). Six volunteers (23.1%) were exposed to a dose higher than 0.07 mg F/kg, due to the use of fluoridated toothpaste.

In this sense, discussions are necessary about: the validity of the parameter of 0.07 mg F/kg established in the literature[10]; and the significance of the high prevalence of dental fluorosis reported[18].

Relative to the parameter recommended by Burt[10], it is known that this was not the result of an experiment about the dose-response to F ingestion and dental fluorosis. Therefore, care must be taken when comparing experimental data with this limit, since reports have indicated that exposure to high doses do not always result in high prevalence of dental fluorosis[12]. The fact that not all the F ingested is absorbed, is not considered in dose determination studies. However, *in vivo* studies have demonstrated that the presence of food in the stomach diminishes the absorption of fluoride (F) coming from the toothpaste ingested after meals[35]. Thus, the dose values may have been overestimated.

Whereas, relative to the significance of high prevalence of dental fluorosis, it is relevant to question what would be acceptable in terms of the prevalence of dental fluorosis. Discussions have indicated that more important than prevalence, would be the severity of dental fluorosis. Except in regions that have water that naturally contains excessive quantities of F, the majority of case reported have been of very light and light degrees of fluorosis. Moreover, Menezes et al.[36] have demonstrated that in the majority of instance, fluorosis at this degree of severity is not perceived by the population. Narvai[37] considered this dental fluorosis that affects a good portion of the population, without however leading to esthetic or functional compromise of the affected teeth, to be "endemic and iatrogenic".

The prevalence of dental fluorosis in Feira de Santana was high when compared with the data presented by the National Oral Health Survey in 2011, with reference to the "Projeto SB Brasil 2010" (national mean = 16.7%). However, it is similar to that of the city of São Paulo, where four surveys have been conducted over the course of twelve years, which revealed prevalence values of 43.8; 33.7; 40.3, and 38.1% in the years 1998, 2002, 2008 and 2010, respectively, without demonstrating a trend towards increasing with the passage of time[38]. In Salvador, BA, it was also verified that there was a tendency towards increasing prevalence of fluorosis in the period between 2001 and 2004[39].

In addition, we point out the difficulties existent for the diagnosis of dental fluorosis: it is difficult to adequately calibrate many researchers; the existent indexes are subjective[40]; the differential diagnosis may be confused (for example, with enamel hypoplasia), and there is also the question of bias when the examiner "wants to find" the problem; thus there are reports of many false-positive cases[38]. Important technical questions also interfere in the results, especially in the milder forms of dental fluorosis: the visual acuity of the examiner; the lighting conditions and hydration of the tooth; presence of bacterial dental biofilm, and the angle of observation[41]. Thus, care is required when comparing the values of dental fluorosis prevalence among different studies.

Finally, it could be concluded that the children in the critical age range evaluated, for the development of dental fluorosis, and residents of a region with a semi-arid climate were not exposed to an excessive dose of fluoride, considering the limit established between 0.05 and 0.07 mg F/kg/day. Nevertheless, in view of the results with reference to the dose coming from toothpaste, it is important to point out the need for promoting educational actions about its correct use, with the aim of gaining the maximum benefit in the control of dental caries, and minimizing the risks of dental fluorosis: use of a toothpaste with a conventional concentration of F (between 1000 and 1500 µg/g) in a small quantity (0,1 to 0.3g)[29,32].

Collaborations

YBO Lima-Arsati worked on the experimental design of the study, selection of volunteers, supervision of sample collections and their laboratory analysis, in addition to writing the article. ARLF Gomes worked on the selection of volunteers, supervision of sample collections and their laboratory analysis, in addition to writing the article. HKA Santos worked on the selection of volunteers, collection and analysis of products of tooth brushing samples. F Arsati helped with sample collection and writing the scientific article. MC Oliveira worked on the experimental design of the study and writing of the article. VS Freitas worked on the experimental design of the study, selection of volunteers, and statistical analysis of the data.

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