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Original Article

The antibacterial effects of coffee extract, chlorhexidine, and fluoride against *Streptococcus mutans* and *Lactobacillus plantarum*: An *in vitro* study

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ABSTRACT

Background: The aim of the present study was to compare the antibacterial effects of coffee extract with those of 0.2% sodium fluoride and chlorhexidine (CHX) mouthrinses on *Streptococcus mutans* and *Lactobacillus plantarum in vitro*.

Materials and Methods: In this experimental *in vitro* study, the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and disk diffusion method were determined for different concentrations of coffee extract, 0.2% CHX, and 0.2% fluoride against *S. mutans* and *L. plantrum*. Data were analyzed using Kruskal–Wallis analysis. Statistical significance level was established at $P < 0.05$.

Results: The MIC of coffee was achieved at 62.5 and 500 mg/mL against *S. mutans* and *L. plantarum*, respectively. The MBC against *S. mutans* was 125 mg/mL. The diameter of the zone of inhibition around *S. mutans* for pure coffee extract (100%), CHX (0.2%), and fluoride was 19.8 mm, 9.92 mm, and 0, respectively. At a concentration of 6.25%–100%, coffee had a significantly larger zone of inhibition compared to CHX and fluoride) $P = 0.01$). The MBC of coffee and fluoride was 0 against *L. plantarum*. The lowest inhibitory concentration belongs to CHX (MIC: 0.624 mg/ml for *L. plantarum* and 0.125 mg/ml for *S. mutans*).

Conclusion: The coffee had an antibacterial effect against *S. mutans* on 62.5–1000 mg/ml concentrations. The zone of inhibition around *S. mutans* for higher concentrations of coffee (6.25%–100%) was significantly higher than that of CHX and fluoride 0.2%. Bacteriostatic effect of coffee against *L. plantarum* was obtained at 500–1000 mg/ml. However, coffee and fluoride did not show any bactericidal effects against *L. plantarum*.

Key Words: Chlorhexidine, coffee, sodium fluoride, *Streptococcus mutans*

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INTRODUCTION

Dental caries is a multifactorial disease which mainly results from reactions among oral flora, susceptible host, and diet. *Streptococcus mutans* and *Lactobacillus* are the two main cariogenic bacteria in human beings.[1] There are various species of *Lactobacillus* in oral cavity; however, one of the most powerful

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acid-producing bacteria in oral environment which might play an important role in caries development is *Lactobacillus plantarum*.[2,3]

One of the most common chemical methods of caries prevention is topical mouthwash use including fluoride or chlorhexidine (CHX).[4] Swallowing a large amount of fluoride among children younger than 8 years might lead to fluorosis.[5] In addition, CHX is one of the most effective antiplaque agents, which has some side effects including changes in taste and stained teeth.[4,6] To overcome such side effects, the WHO has recommended investigating the possibility of using natural substances such as herbal extracts.[7]

Several studies have suggested that many natural foods and beverages might lead to inhibition of adhesion and activity of oral pathogenic bacteria,[8-15] and this biologic activity is principally due to their polyphenol. Polyphenols are the representative of the significant photochemical material in plants, particularly in fruits, seeds, and leaves.[8] The highest component of polyphenols is in particular flavonoids, such as catechin, catechin gallate, and proanthocyanidin. Thousands of plant polyphenols have been recognized; however, coffee is one of the most popular drinks around the world and one of the main sources of phenolic compounds.[9,11,15]

Anila Namboodiripad and Kori[14] observed that individuals who consumed coffee daily without sugar and milk showed a reduction of dental caries compared to others without the same custom, demonstrating the anticaries properties of coffee. Signoretto *et al*.[16] suggested a significant reduction of *S. mutans* and *Lactobacillus* in plaque and saliva and a decrease in microbial plaque index of those who used to drink coffee.

Studies have indicated that, due to interference with proteins of microbial membrane, coffee inhibits *S. mutans* adhesion and leads to reduction in oral acid production by the inhibition of glucosyltransferase and amylase.[7,17,18]

It has been found that, in addition to polyphenol, coffee shows antibacterial activity against Gram-positive and Gram-negative bacteria due to having trigonelline, caffeine, and α-dicarbonyl compounds.[9,11] Antibacterial activity of coffee is different based on coffee's chemical composition, type, and processing including roasting and decaffeination.[11,13] Coffee can even have anti-demineralization effect on teeth surfaces; therefore, it is a promising way against dental caries, as it is quite safe with pleasant odor and taste and popular almost all around the world.[13] However, there is no evidence that coffee extract exerts the same activity against cariogenic bacteria other than *S. mutans*,[13] and studies clarifying the role of *Coffea canephora* extract on lactobacilli are still needed. The purpose of the present study was to evaluate and compare the antibacterial effects of *Coffea arabica* extract with 0.2% CHX and fluoride on *S. mutans* and *L. plantarum in vitro*.

MATERIALS AND METHODS

In this experimental *in vitro* study, 0.2% sodium fluoride (Behsa, Arak, Iran) and 0.2% CHX (Nazhou, Behsa, Tehran, Iran) mouth rinses were used. Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, USA) was used for serial dilutions of coffee extract and CHX and fluoride mouthrinses to determine the minimum inhibitory concentration (MIC).[10] Disk diffusion method[9] was used to determine the diameters of the zones of inhibition, and solid blood agar culture medium (Blood Agar, HIMEDIA Company, Hyderabad, Telangana India) was used to determine the minimum bactericidal concentration (MBC).[10]

This experimental design, *in vitro* laboratory setting study was approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran (Project No. 394122). It was performed in the School of Pharmacy and Dental Research Center of Dentistry Faculty. Pure cultures of *S. mutans* (PTCC 1683) and *L. plantarum* (ATCC 1491T, America) were achieved from the department of microbiology.

Bacteria were kept at −20°C in TSB (Difco Laboratories, Detroit, MI, USA) with 20% glycerol and activated by transfer into blood agar (Blood Agar, HIMEDIA Company, Hyderabad, Telangana India). Incubation were performed at 37°C for 48 h, with 5% CO_2 for cariogenic bacteria, and anaerobic condition for *L. plantrum*.

Preparation of coffee extract

Coffee beans (*C. canephora*) procured from the local market in Isfahan, Iran. The beans were roasted in a commercial spouted bed roaster (I-Roast-2, Gumee, IL, USA), operating at a maximum temperature of 220°C, for 6 min, to produce a moderately light roasting degree (SCAA, USA).[9,10]

The roasted coffee beans were ground in a laboratory-scale mill to pass through a 0.46-mm

sieve. The aqueous coffee extract was obtained by a coffee brewing procedure, percolating 100 mL of preboiling (95°C) Milli-Q purified water (Millipore Corporation) through 100 g of ground roasted coffee. The extracts were filtered through Whatman #1 qualitative filter paper (Whatman). The specimens were identified by a botanist and a pharmacognosist for their authenticity. Further dilution was done using sterile water to obtain the concentrations of 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, and 0.75%. All the extracts were stored separately in sterile containers and were labeled accordingly. The samples were stored in a refrigerator at 4ºC and transported to the department of microbiology.

Determination of phenolic constituents in the extract

The total phenolic content (TPC) of coffee extract was determined spectrophotometrically according to a modified method of Lachman *et al*.[19] To determine the total flavonoid content (TFC), these compounds were precipitated using formaldehyde, which reacts with C-6 or C-8 atoms of 5,7-dihydroxyflavonoids to form methyl derivate that further react with other flavonoid compounds and also at C-6 and C-8 positions. The condensed products of these reactions were removed by filtration, and the remaining nonflavonoid phenols were determined as previously described. Flavonoid content was calculated as the difference between total phenol and nonflavonoid phenol content. Gallic acid was used as the standard, and the results were expressed as mg gallic acid equivalents (GAEs)/g of coffee.[20] All measurements were performed in triplicate.

The TPC of plain coffee extract was obtained as 198.75 mg GAE/g, whereas the TFC was determined as 77.1 mg GAE/g. Flavonoids constitute an average 45% of total polyphenols, which designated that a larger portion of total polyphenols was attributed to nonflavonoid compounds, represented by phenolic acids.

Disk diffusion method to determine the diameters of the zones of inhibition

Spread plate method was used to prepare a culture from the bacterial suspension at 0.5 McFarland concentrations on blood agar.[9] After 5–10 min, wells measuring 6 mm in diameter were prepared in the culture and the bottoms of the wells were sealed with blood agar medium; the distance between the wells and the edge of the plate was 1.5 cm, and the wells were 2–2.5 cm apart. Then, 50–100 μL of the pure concentrations of the extracts and 0.2% fluoride and CHX were transferred into the wells. The negative and positive control wells were filled with physiologic serum and 10 μg of penicillin, respectively. The plates were placed in a refrigerator for 1–2 h to provide the antimicrobial agent with the opportunity to diffuse into the environment. The plates were incubated at 37°C for 18–24 h. Then, Vernier calipers were used to determine the diameters of the zones of inhibition. This test was repeated separately seven times for each material by one microbiologist who also examined the MIC and MBC.

Determination of minimum inhibitory concentration and minimum bactericidal concentration using the agar dilution method

The agar dilution method recommended by the National Committee for Clinical Laboratory Standards was used.[21]

100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, and 0.75% concentrations of each extract were prepared. These concentrations are equal to 1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.6 mg/mL, respectively.

1 mL of the microbial suspension containing 1.5×10^8 CFU/mL (0.5 McFarland turbidity standards) of bacterial sample was added to each test tube.

The test tubes were incubated at 37°C for 24 h, and the results were evaluated in terms of turbidity of the test tubes compared to the time before incubation and the positive and negative controls. After incubation, the minimum concentration of each extract that resulted in the inhibition of bacterial growth was considered as MIC.[10] The turbidity in each test tube indicated the inefficacy of that concentration in inhibiting bacterial growth. Subsequent to determination of MIC, approximately 50 μL of the solutions from the test tubes with no turbidity was transferred onto the solid blood agar culture plates (HIME DIA., India) and cultured with a swab using the spread plate method, followed by incubation at 37°C for 48 h to determine the presence or absence of bacterial growth macroscopically (observing the colonies by a microbiologist). The minimum concentration in which no bacterial proliferation was detected (no colonies) was reported as MBC.[10] Each examination was tested seven times by one microbiologist. In relation to the determination of MIC, it should be pointed out that, due to the turbid nature of the extracts used in the present study and the inability to determine the

accuracy of turbidity or clarity of the test tubes after incubation, microbial plates were prepared from the turbid test tubes and evaluated under a microscope to evaluate the growth or inhibition of growth of bacteria.

The culture medium along with the strain was considered the negative control, and the tube with SM and penicillin was the positive control.

Statistical analysis

Statistical analysis was performed using the software SPSS (version 20, Chicago, IL, USA). Descriptive statistics were retrieved, and the data of the zones of inhibition were compared using Kruskal–Wallis test. Statistical significance level was established at $P < 0.05$.

RESULTS

Table 1 summarizes MIC and MBC which were the same in all seven times of examinations.

Minimum inhibitory concentration and minimum bactericidal concentration

The coffee had an antibacterial effect against *S. mutans* on 62.5–1000 mg/ml concentrations. MIC achieved at 62.5 mg/mL and 500 mg/mL against *S. mutans* and *L. plantarum*, respectively. It could be concluded that the coffee inhibited *S. mutans* growth more than *L. plantarum*.

The MBC against *S. mutans* for coffee extract was determined at 125 mg/mL, at which no bacterial colonies were observed. Coffee and fluoride did not show any bactericide activity against *L. plantarum* (MBC = 0).

However, the MIC and MBC of coffee extract against *S. mutans* were higher than CHX (0.125 mg/mL) and fluoride (2 mg/mL). In fact, antibacterial effects of CHX were revealed in lower concentrations (<0.2%) compared to fluoride and coffee. The bactericide activity of all materials tested and the inhibitory activity of coffee and CHX were lower against *L. plantarum* compared to *S. mutans*. Fluoride showed a similar inhibitory activity against *S. mutans* and *L. plantarum*.

Inhibition effect of coffee was lower than 0.2% fluoride. The lowest inhibitory concentration belongs to CHX (0.624 mg/ml for *L. plantarum* and 0.125 mg/ml for *S. mutans*). In other words, CHX and fluoride mouthwashes had a higher bacteriostatic effect on both the above-mentioned bacteria, compared to polyphenolic extract of coffee.

Considering seven measurements made for MIC and MBC and the similarity of all the values in each sample (with a variance of 0 for the values), there were no significant differences between MIC and MBC of each sample ($P = 1$). The correlation coefficient for MIC and MBC values in all the materials was the same ($r = 1$, $P < 0.01$).

Results of the disk diffusion method

Against Lactobacillus plantarum

Bacterial growth was not inhibited around the coffee extract and fluoride mouthrinse, and the diameter of the zone of inhibition was 0. The diameter of the zone of inhibition (mean) for Nazhou and Behsa 0.2% CHX mouthrinses was 11.94 mm and 8.96 mm, respectively. The difference between various concentrations of coffee and both CHX mouthrinses was statistically significant) $P < 0.05$). However, the difference between various concentrations of coffee and fluoride was not statistically significant) $P > 0.05$). The difference between each concentration of CHX and fluoride was statistically significant) $P = 0.03$). There was no significant difference between the zones of inhibition of the two CHX mouthrinses (Nazhou and Behsa) as well ($P = 0.18$). It can be concluded that higher concentrations of coffee extract (6.25%–100%) showed a significantly higher antibacterial effect on *S. mutans*, compared to CHX and fluoride mouthwash (0.02% or lower); however, only CHX was significantly able to inhibit *L. plantarum*.

Table 1: Minimum inhibitory concentration and minimum bactericidal concentration of coffee extract, 0.2%. chlorhexidine, and 0.2% sodium fluoride

Bacteria	*Streptococcus mutans*		*Lactobacillus plantarum*	
MIC/MBC	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Coffee	62.5	125	500	0
Chlorhexidine 0.2% (Nazhou)	0.125	0.125	0.624	1.25
Chlorhexidine 0.2% (Behsa)	0.125	0.125	0.624	1.25
Sodium fluoride 0.2%	2	2	2	0

MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration

Table 2 represents a comparison of various concentrations of each substance and indicates a comparison of different materials in each concentration against *L. plantarum*.

Against Streptococcus mutans

The diameter of the zone of inhibition (mean) for pure coffee (100%), Nazhou, and Behsa CHX mouthrinses (0.2%) was 19.8 mm, 11.8 mm, and 9.92 mm, respectively. Bacterial growth was not inhibited around the fluoride mouthrinse, and the diameter of the zone of inhibition was 0. The comparison of the diameter of the zone of inhibition in each concentration (62.5–1000 mg/ml) of coffee was significantly larger compared to CHX And fluoride ($P = 0.01$) [Table 3]. Both CHX mouthrinses revealed a larger zone of inhibition significantly compared to fluoride. There was no significant difference between the zones of inhibition of the two CHX mouthrinses (Nazhou and Behsa) as well ($P = 0.08$).

Table 3 represents a comparison of various concentrations of each substance and indicates a comparison of different materials in each concentration against *S. mutans*.

DISCUSSION

It seems that using natural products as a part of common daily diet might be a better and safer alternative for caries prevention if they are supported by scientific-based evidence.[16] In this study, antibacterial effect of coffee extract was compared to CHX and fluoride. The two most commercialized coffee species are *C. arabica* and *C. canephora*,[22] and a study[23] indicated higher efficacy of the latter in relation to the inhibition of S. *mutans*. In this regard, *C. canephora* was used in the present study.

The results indicated that coffee has a higher bacteriostatic effect on *S. mutans* (MIC = 62.5 mg/ml) than on *L. plantarum* (MIC = 500 mg/ml). Nevertheless, bacteriostatic effect of coffee on *S. mutans* was obtained in higher concentrations than CHX and fluoride 0.2% (2 mg/ml). Bacteriostatic effect of both the CHX types was higher than other materials (0.624 mg/ml for *L. plantarum* and 0.125 mg/ml for *S. mutans*).

Coffee, with a concentration of 125 mg/ml, showed a bactericidal effect on *S. mutans*. Among the tested materials, only CHX showed a bactericidal effect on *L. plantarum*. Compared to coffee and fluoride, CHX mouthwash showed higher bacteriostatic and bactericidal effects on both bacteria in lower concentrations.

Based on the obtained results from the diameter of the zone of growth inhibition, it can be said that antibacterial effect of coffee against *S. mutans* in 62.5–1000 mg/ml was significantly higher than that of CHX and fluoride with the concentration of 0.2% or lower.

Table 2: Zone of inhibition (mm) analysis in different concentrations of coffee, fluoride, and chlorhexidine against *Lactobacillus plantarum*

Concentration (%)	Coffee	Chlorhexidine 0.2% (Nazhou)	Chlorhexidine 0.2% (Behsa)	Fluoride 0.2%	Penicillin (positive control)
6.25	$0^{A,a}$	$4.98^{B,a}$	$3.98^{B,a}$	$0^{A,a}$	15
12.5	$0^{A,a}$	$5.94^{B,b}$	$5^{B,b}$	$0^{A,a}$	15
25	$0^{A,a}$	$7.94^{B,c}$	$6.06^{B,c}$	$0^{A,a}$	15
50	$0^{A,a}$	$8.94^{B,d}$	$8.04^{B,d}$	$0^{A,a}$	15
100	$0^{A,a}$	$11.94^{B,e}$	$8.96^{B,e}$	$0^{A,a}$	15

Small letters represent a comparison of various concentrations of each substance (vertical comparison). Large letters indicate comparison of different materials in each concentration (horizontal comparison)

Table 3: Zone of inhibition analysis (mm) in different concentrations of coffee, fluoride, and chlorhexidine against *Streptococcus mutans*

Concentration (%)	Coffee	Chlorhexidine 0.2% (Nazhou)	Chlorhexidine 0.2% (Behsa)	Fluoride 0.2%	Penicillin (positive control)
6.25	$11.7^{A,a}$	$4.05^{B,a}$	$1.92^{B,a}$	$0^{C,a}$	15
12.5	$14^{A,a}$	$5.87^{B,a}$	$3.96^{B,a}$	$0^{C,a}$	15
25	$15.9^{A,b}$	$8.02^{B,a}$	$5.96^{B,a}$	$0^{C,a}$	15
50	$17.8^{A,b}$	$10.1^{B,b}$	$7.95^{B,b}$	$0^{C,a}$	15
100	$19.8^{A,b}$	$11.8^{B,b}$	$9.92^{B,b}$	$0^{C,a}$	15

Small letters represent a comparison of various concentrations of each substance (vertical comparison). Large letters indicate comparison of different materials in each concentration (horizontal comparison)

This is consistent with the findings of Mehta *et al*.'s[13] study which reported that the antibacterial efficacy of coffee extract increases with an increase in the extract concentration.

However, for *L. plantarum*, even higher concentration of coffee did not show any antibacterial effect and just showed bacteriostatic effect at 500–1000 mg/ml. To confirm the results of MBC and MCI, the zone of growth inhibition was not observed around coffee and fluoride in *L. plantarum* culture medium, at any concentration. Assuming that the diameter of the zone might increase with an increase in the exposure time of the substance and bacteria, the samples were observed for more 48 h. However, no diameter of the zone of growth inhibition was observed. Only CHX showed an antibacterial effect against *L. plantarum*.

Direct comparison of the results by various studies is difficult due to different analysis methods, types of bacteria strain, the presence of sucrose in culture media, available sources, substance type, manufacturers, and extracting method.[17] In addition, antibacterial activity of coffee is different according to coffee's chemical composition, type, and processing, including roasting and decaffeination.[11,13]

MIC and MBC of coffee against SM were obtained to be 7 and 160, respectively, in a study by Antonio *et al*.;[11] in particular, MIC of coffee was higher than our study; however, MBC was almost similar to the present study. *C. canephora* extract did not show bacteriostatic activity against *Streptococcus sobrinus*.

Almeida *et al*.[9] did not investigate MIC and MBC, but reported the diameter of the zone of growth inhibition around Arabica coffee as 6.9 mm, which was much smaller than the value obtained by the present study (19.8). In fact, antibacterial effect of coffee extract in the present study was higher, and it can be attributed to the issue that antibacterial effect of *C. canephora* is probably higher than that of Arabica coffee.[23]

The results by Mehta *et al*.'s[13] study were closer to the present study, and the zone of growth inhibition around *S. mutans* has been reported to be 23 mm.

The main compounds responsible for antimicrobial action of coffee extracts are phenolic compounds including chlorogenic acids, caffeic acid, and caffeine and minor compounds such as trigonelline, a-dicarbonyl compounds, and protocatechuic acid.[11] Phenolic compounds inactivate the bacterial cellular enzymes which depend on the rate of penetration of the substance into the cell or caused by membrane permeability changes.[12,13]

It should be noted that we investigate the pure *C. canephora* extract and diluted concentrations 50%, 25%, 12.5%, and 6.25%. However, the coffee concentration which could be habitually consumed by people is up to 20%.

Theoretically, a cup of 100 mL usually contains 39.75 mg of polyphenols.[16] In the present study, 62.5 mg/mL concentration of coffee contained 12.42 mg/mL of polyphenols. Therefore, the concentration of polyphenols in one cup of coffee is much higher than that reported as MIC in the present study, and as a result, the probability of *S. mutans* inhibition by drinking coffee is high. However, 500 mg/ml concentration (MIC for *L. plantarum* = 50%) contained 99.37 mg/mL of polyphenols. Consequently, the inhibition of *L. plantarum* might not obtained by drinking coffee. It could be suggested that high-concentration mouthrinses prepared from coffee can be effective against lactobacilli, while daily drinking coffee can inhibit *S. mutans* growth.

Several studies have revealed the potential of coffee against *S. mutans*.[8,9,22,23] However, its potential against lactobacilli is not determined.

One of the strengths of the present study was that the diameter of the zone of growth inhibition around *1ntarum* and *S. mutans* was investigated at various concentrations of coffee, CHX, and fluoride.

Da Silva *et al*.[10] reported that MIC of *C. canephora* against *Lactobacillus rhamnosus* was 10 mg/ml. Similar to the present study, *C. canephora* did not show any bactericidal effect against *L. rhamnosus*. They did not investigate the diameter of the zone of growth inhibition.

In the present study, it has been indicated that 500–1000 mg/ml concentration of coffee shows bacteriostatic effect against *L. plantarum* (MIC = 500 mg/ml); however, no bactericidal effect was observed.

In the present study, only *C. canephora* of the commercially available coffee in the Iranian market was investigated. It is recommended, for further studies, to investigate antibacterial effect of various types of coffee and its different components on other bacterial species including *Lactobacillus*, and the

bacteria related to periodontal disease and clinical trials should also be conducted.

CONCLUSION

The coffee had an antibacterial effect against *S. mutans* on 62.5–1000 mg/ml concentrations. Polyphenolic extract of coffee has inhibitory effect (bacteriostatic) on growth of *S. mutans* and *L. plantarum*. However, this effect is lower than that of CHX and fluoride. Coffee only shows a bactericidal effect on *S. mutans*; this effect for higher concentrations of coffee (6.25%–100%) is significantly higher than that of CHX and fluoride 0.2%. Coffee, at 500–1000 mg/ml, showed a bacteriostatic effect against *L. plantarum* (MIC = 500 mg/ml). However, no bactericidal effect was observed. CHX with lower concentration showed higher bactericidal and bacteriostatic effects, particularly against *S. mutans*, compared to other tested materials. Bacteriostatic effect of fluoride was similar on both bacterial strain; however, it showed bactericidal effect only against *S. mutans*.

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Conflicts of interest

The authors of this manuscript declared that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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