Original Article

Comparison Between Antibacterial Effect of Chlorhexidine 0.2% and Different Concentrations of *Cyperus rotundus* Extract: An *In vitro* Study

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Abstract

Aims and Objectives: Modern methods of caries prevention concentrated on natural ingredients usage such as probiotics and polyphenols that are safer for young children with *Streptococcus mutans* inhibitory properties. The purpose of this study was to compare antibacterial effects of different concentration of *Cyperus rotundus* extract and chlorhexidine (CHX) 0.2% mouthwash on *S. mutans* and *Lactobacillus acidophilus*.

Materials and Methods: In this *in vitro* study, the antibacterial effectiveness of the *C. rotundus* extract and CHX was compared with minimum inhibitory concentration (MIC) test in tube, minimum bactericidal concentration (MBC) test in solid medium, and disc diffusion for measurement of inhibition zone. Data were analyzed using one-way ANOVA, one sample *t*-test, and independent sample *t*-test statistical methods by SPSS. 24 software (SPSS Inc., Chicago, USA).

Results: MIC and MBC values of the *C. rotundus* extract were obtained 225 and 450 mg/ml, respectively, for *S. mutans* and 108 and 225 mg/ml for *L. acidophilus*, which are more than CHX (0.5, 1 res.). The inhibition zone increased in a dose-dependent manner but lower than CHX.

Conclusion: The *C. rotundus* extract had antibacterial effects (bactericide and bacteriostatic) on *S. mutans* and *L. acidophillus*. Although this effect was lower than CHX. With regard to adverse effect of CHX, this extract can be a potential antibacterial agent.

Keywords: *Chlorhexidine, Cyperus rotundus, Lactobacillus acidophilus, polyphenol, Streptococcus mutans*

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Introduction

Dental caries is the most common disease in children[1] which is the result of factors such as oral flora, anatomic characteristics, and food diet. *Streptococcus mutans* and *Lactobacillus acidophilus* are two of the most important factors in dental caries.[2] Cariogenic properties of *S. mutans* consist of adhesion to dental surfaces, acid production, and ability of living in acidic environment.[1,3] *Lactobacilli* are more important in dentin caries and caries progression.[2] As dental caries can impose high expenses on societies,[4] different methods have been introduced to prevent it. Due to the plaque-related diseases tend to remain localized than offensive more likely, local application of antibacterial agents is more effective than systemic prescription.[5] Among antibacterial agents, chlorhexidine (CHX) mouth rinse has limited indication in children under 7 years old due to the probability of swallowing and tooth staining.[1] Hence, modern methods for decay prevention are utilization of natural safe materials with bacterial inhibition such as probiotics and polyphenols for children.

Polyphenols are one of the main compounds presented in plants[6] with various biological activities such as antioxidant[7] and anti-inflammation properties.[8] Some studies showed that plant-derived compounds have

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antibacterial and antiplaque formation properties which are the results of their effects on *S. mutans*. These effects divided into three main groups: (1) direct effects with antiproliferative properties, (2) interfering with cell membrane proteins caused to low adherence of bacteria to surfaces, and (3) inhibiting enzymatic system of bacteria through inhibiting glycosyltransferase and amylase.[9]

One of the important plants in oral treatment in traditional medicine of Iran and other countries such as India and China are *Cyperaceae*.[10-12] In modern medicine, various investigations represent antibacterial, antitumoral, antimutagen, antitoxic, anti-inflammation, analgesic, and antioxidant effect of this plant. Furthermore, phytochemical analysis of this plant shows some phenolic components such as flavonoids, coumarins, tannins, and phenolic acids. These compounds are responsible for pharmacologic properties of many herbal species.[13]

Antimicrobial effect of ethanol extract of *Cyperus rotundus* was shown in several studies. In addition, some researches showed the effectiveness of this extract on Gram-positive bacteria.[11-14] Furthermore, *C. rotundus* exerted virucidal effect on HSV. Anti-HBV active constituents were isolated from the rhizomes of *C. rotundus*.[15] Only one research investigated *C. rotundus* anticariogenic properties of the *C. rotundus* extract.[16] They founded that the tuber extract of *C. rotundus* had growth reduction effect on *S. mutans*, but they did not compare with positive control.

Since *S. mutans* and *Lactobacillus,* as major cause of dental caries, are Gram-positive bacteria and due to antibacterial effect of *C. rotundus* on these bacteria and its antibacterial component (polyphenol), this research focused on comparison of antibacterial effect of various concentration of ethanol extract of *C. rotundus* on *S. mutans* and *L. acidophilus* with CHX and penicillin.

Materials and Methods

This laboratory study was done in Kerman University microbiology laboratory with 8-week period. The Institutional Ethical Committee of Shahed University approved the study (IR Shahed REC.1395.35). The rhizome of *C. rotundus* extract preparation was gathered from Tehran suburban gardens. The plant extract was prepared according to percolation method. Before interance in percolator, the plant was moistened with ethanol 70% as solvent (about 30 min). After the extraction, excessive solvent was vaporized with rotary device and then was sterilized with ultraviolet. Finally, the extract was obtained with concentration of 900 mg/ml.

Determination of total phenolic contents

Amount of total phenolic content (TPC) was assessed using Folin-Ciocalteu reagent. Briefly, 50 mg of dry mass of *C. rotundus* extract was mixed with 0.5 mL of Folin-Ciocalteu reagent and 7.5 mL deionized water. The mixture was kept at room temperature for 10 min, and then, 1.5 mL of 20% NaCO (w/v) was added. The mixture was heated on a water bath at 40°C for 20 min and then was cooled in an ice bath. The absorbance was measured at 755 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). Amounts of TPC were calculated using gallic acid calibration curve within range of 10–50 ppm. The results were expressed as gallic acid equivalents mg/100 g of dry plant matter. The results were reported on dry weight basis.

Test microorganism

The bacteria strains *S. mutans* (PTTC 1638) and *L. acidophilus* (PTCC 1683) were purchased from Iranian Research Organization for Science and Technology. The lyophilized bacteria were cultured on Mueller-Hinton blood agar (Merck co, Germany) medium and were incubated for 48 h at 37°C.

Serial dilution method

First, 10 sterilized test tubes were prepared and 2 ml culture medium (Mueller-Hinton broth [Merck, Germany]) was added to each tube. In this study, the extract used at 27, 54, 108, 225, 450, 675, and 900 mg/ml. One tube was considered as positive control without the extract (include culture medium) and one tube as negative control without m0069croorganism. Then, 2 ml bacterial suspension with concentration of 0.5 McFarland was added to each tubes and tubes were incubated at 37°C for 24 h. The lowest concentration of the extract inhibited growth of bacteria was selected as minimum inhibitory concentration (MIC).

Minimum bactericidal concentration determination

Fifty microliter suspensions from tubes that showed no bacterial growth was cultured on the plates containing solid medium (blood agar) and were incubated for another 24 h at 37°C.

The lowest concentration that inhibited 99% of organisms was considered as minimum bactericidal concentration (MBC). MIC and MBC of CHX and penicillin were also measured.

Disc diffusion assay

Discs were soaked in different concentrations of the extract, CHX 0.2% and penicillin 500 mg (positive controls). Different concentrations were prepared with dimethyl sulfoxide as solvent and negative control. Twenty-four hours after complete absorption of the extract, discs were

placed in incubator at 37°C for 1 h. Next, 50 µl suspension of bacteria with concentration of 0.5 McFarland was cultured to the plates containing blood agar medium.

Then, disks were placed on the medium in proper distances (15 mm from center of disc) and were incubated in 37°C, and every 24 h for 3 days, diameter of inhibition zone was measured.

Results

Total phenolic content

TPC of the extract according to standard curve of gallic acid absorbance was obtained 10%.

Minimum inhibitory concentration and minimum bactericidal concentration assessments

The value of MIC and MBC of the extract on *S. mutans* was 225 and 450 mg/ml, respectively. Furthermore, MIC and MBC of the extract for *L. acidophilus* were 108 and 225 mg/ml consequently. The value of MIC and MBC of CHX 0.2% were 0.5 and 1 mg/ml and were 60 and 125 mg/ml for penicilin, respectively [Table 1].

The MIC and MBC of the *C. rotundus* extract on *S. mutans* and *L. acidophilus* had a significant difference with MIC and MBC of penicillin and CHX 0.2% as shown in Table 1. Although MIC and MBC of the extract on *L. acidophilus* were lower than *S. mutans*.

Disc diffusion assessment

Three repeat of the test and effect of time on inhibition zone were not assessed with one-way ANOVA. Differences between three replicates of disc diffusion method were not significant ($F_{(2, 60)} = 0.929$, $P < 0.400$ for *S. mutans*) ($F_{(2, 60)} = 0.096$, $P < 0.934$ for *L. acidophilus*). Furthermore, time elapsed from 24 h to 72 h did not change the inhibition zone significantly ($F_{(2, 18)} = 0.375$, $P < 0.0.693$ for *S. mutans*) ($F_{(2, 18)} = 0.305$, $P < 0.741$ for *L. acidophilus*).

Comparison between inhibition zones of different concentrations

The result of inhibition zone was shown in Figures 1 and 2. There is a significant difference between the extract concentrations with one-way ANOVA ($F_{(6, 14)} = 12.53$, $P < 0.001$ for *S. mutans*) ($F_{(6, 14)} = 15.28$, $P < 0.001$ for *L. acidophilus*). Since the result of ANOVA is significant, the least significant difference *post hoc* test must be reported [Tables 2 and 3].

As shown in these tables, the extract demonstrated antibacterial activity against *S. mutans* and *L. acidophilus* in a dose-dependent manner significantly.

Comparison between the controls and extract

Amount of inhibition zone for *S. mutans* with penicillin was 16.85 mm and with CHX was 13.85 mm.

Table 1: Minimum inhibitory concentration and minimum bactericidal concentration values (mg/mL)

Agent	MIC	MBC
The extract on *Streptococcus mutans*	225	450
The extract on *Lactobacillus acidophilus*	108	225
CHX	0.5	1
Penicillin	60	125

MBC=Minimum bactericidal concentration, MIC=Minimum inhibitory concentration, CHX=Chlorhexidine

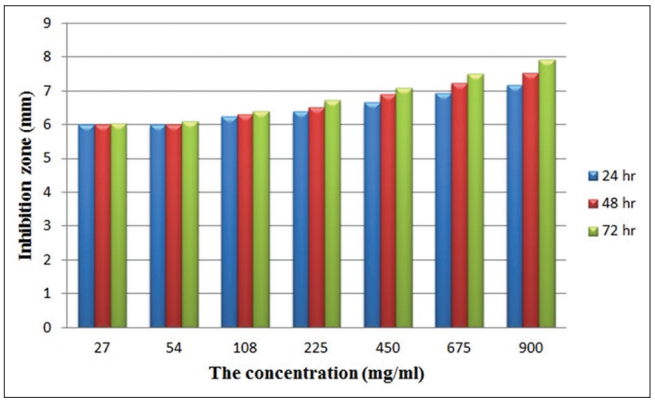

Figure 1: The mean of inhibition zone of *Streptococcus mutans* in different concentrations of *Cyperus rotundus* extract

Furthermore, the inhibition zone for *L. acidophilus* with penicillin was 17.35 mm and with CHX was 14.23 mm. There is a significant difference between all concentrations and positive controls in two tested bacteria with one sample *t*-test (df = 8, $P < 0.001$). All inhibition zones were lower than positive controls but higher than negative control (df = 8, $P < 0.001$).

Comparison between the same extract concentrations on *Streptococcus mutans* and *Lactobacillus acidophilus* inhibition zone

There is a significant difference between two tested bacteria with independent *t*-test ($t = 1.97$, df = 124, $P < 0.05$) [Table 4].

As shown above, there is a significant difference only in 900 mg/ml.

Discussion

With regard to antibacterial properties of *C. rotundus* on Gram-positive and lack of investigation in this area, this research focused on assessment of antibacterial effect of different percentages of ethanol extract of *C. rotundus* and comparison with CHX.

As mentioned above, TPC obtained 10% of the extract that had antibacterial effect on *S. mutans* and *L. acidophilus* in a dose-dependent manner. Although the extract inhibition zone was higher than negative control, comparison of inhibition zone between two tested

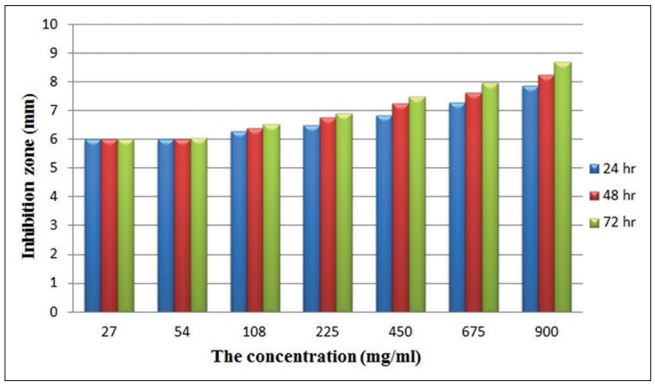

Figure 2: The mean of inhibition zone of *Lactobacillus acidophilus* different concentrations of *Cyperus rotundus* extract

bacteria showed only 100% of the extract (900 mg/ml) was significantly more in *L. acidophilus* than *S. mutans*. In other percentages, there was no difference between two bacteria. MIC and MBC of the extract were more than positive controls.

Direct comparison of different studies is so difficult because of variety in microbial surveys, microbial species, sucrose existence in plates, herb culture area, extraction method, and other interventional parameters.[17] Our TPCs of *C. rotundus* extract were higher than Bashir *et al.*[18] This diversity was related to different cultivation area of the plant.

The present study was concordant with Yu *et al.* who found *C. rotundus* extract reduced *S. mutans* growth in a dose-dependent manner. Although they used tuber extract and had different microbial test (optical density). Furthermore, they did not compare the extract results with NaF 1% as positive control.[17]

MIC values of Sharma *et al.* were similar to ours. They found 250 mg/ml as minimum inhibition concentration of Gram-positive-tested bacteria.[14]

Several researches represented that ethanol extract of *C. rotundus* was more effective on Gram-positive bacteria.[11,15,16,19-21]

Furthermore, Prasad showed that ethanol extract of *C. rotundus* had the most effect on *Streptococcus*.[12]

Various researchers have already shown that Gram-positive bacteria are more susceptible toward plants extracts as compared to Gram-negative bacteria. These differences may be attributed to the fact that the cell wall in Gram-positive bacteria is of a single layer, whereas the Gram-negative cell wall is multilayered structure.[12]

Traditional medicine practitioners make use of water primarily as a solvent, but studies have shown that alcohol extracts of plants are much better and powerful.

Table 2: The result of least significant difference test for comparison of *Streptococcus mutans* inhibition zone of different concentrations

I	J	MD	*P*
27	54	0.00	1
	108	−0.286	0.25
	225	−0.516	0.04
	450	−0.853	0.003
	675	−0.820	0.004
	900	−1.94	0.001
54	108	−0.286	0.250
	225	−0.516	0.049
	450	−0.853	0.003
	675	−0.820	0.004
	900	−1.94	0.001
108	225	−0.230	0.351
	450	−0.566	0.033
	675	−0.533	0.043
	900	−1.65	0.001
225	450	−0.336	0.180
	675	−0.303	0.224
	900	−1.42	0.001
450	675	−0.033	0.891
	900	−1.08	0.001
675	900	−1.12	0.001

MD=Mean difference

Table 3: The result of least significant difference test for comparison of *Lactobacillus acidophilus* inhibition zone of different concentrations

I	J	MD	*P*
27	54	0.00	1
	108	−0.286	0.240
	225	−0.516	0.044
	450	−0.853	0.003
	675	−0.820	0.003
	900	−1.853	0.001
52	108	−0.286	0.240
	225	−0.516	0.044
	450	−0.853	0.003
	675	−0.820	0.003
	900	−1.853	0.001
108	225	−0.230	0.342
	450	−0.566	0.030
	675	−0.533	0.039
	900	−1.56	0.001
225	450	−0.336	0.172
	675	−0.303	0.215
	900	−1.33	0.001
450	675	−0.033	0.889
	900	−1.00	0.001
675	900	−1.033	0.001

MD=Mean difference

This may be due to the better solubility of the active components in organic solvent.[22]

Table 4: Independent *t*-test results of the two bacteria inhibition zone of the same extract concentration

Concentrations	MD	df	*t*	*P*
27	0	-	-	-
54	0.020	16	0.923	0.370
108	0.091	16	1.442	0.169
225	0.195	16	1.93	0.071
450	0.317	16	2.092	0.053
675	0.456	16	1.92	0.072
900	0.737	16	2.11	0.050

MD=Mean difference, df=Degree of freedom

The present study was similar to Mehta *et al*.[23] because of the effectiveness of alcoholic extract of *C. rotundus* on *Streptococcus* but apposing with them for durability and substansivity. Their results showed reduction of inhibition zone (from 24 h and 72 h); but here, despite of increase in most cases, there was no significant difference between different times.

In the present study, all of the agents had good antibacterial effects. However, potency of the extract was lower than control agents, but this point is less important than safety of the herb. Several studies confirmed safety of the extract.[13,15,22] Hence, it is compatible and safe therapy for long-term use in human subjects.[24] Moreover, the extract had more potency on tested bacteria particular on *Lactobacillus* than *S. mutans* that can be a base for further investigations.

It is suggested assessment of anticariogenic effect of the *C. roturdus* extract on other cariogenic bacteria. Hence, extract of other cultural areas selects for extraction and compare with other mouth rinses. Finally, clinical researches should assess the *in vivo* effect of mouth rinses of this beneficial herb.

Conclusion

With regard to remarkable antibacterial effect of the ethanol extract of *C. rotundus* on *S. mutans* and *L. acidophilus* and safety of this herb, it can be a new horizon for production of new preventive mouth rinse for tooth diseases.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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