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Evaluation of the antibacterial effects of aqueous and ethanolic leaf extracts of *Aloysia Citriodora* (*Lemon verbena*) on Streptococcus mutans and Streptococcus sobrinus

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Abstract

Introduction: The *Aloysia citriodora* plant from the family of *Verbenaceae* has many uses in traditional medicine. The aim of the current study was to determine the effects of the aqueous and ethanolic extracts of *A. citriodora* on *Streptococcus mutans* and *Streptococcus sobrinus*, which cause tooth decay.

Methods: This 2016 study was performed on standardized strains of *S. mutans* PTCC1683 and *S. sobrinus* PTCC1601 and clinical isolates. Twenty clinical samples were obtained from the dental caries of children admitted to the pediatric ward at the Faculty of Dentistry of Babol University of Medical Sciences (Babol, Iran). The aqueous and ethanolic extracts of *A. citriodora* leaves were prepared in several concentrations ranging from 625–20,000 µg/ml. These concentrations of the extracts were applied to the bacteria by disk diffusion, agar well diffusion, and macrotube dilution. The antibacterial effects of amoxicillin and chlorhexidine digluconate 0.2% (CHX) were also carried out. Data were analyzed by SPSS version 18 software using independent-samples t-test.

Results: *Streptococcus spp.* was successfully isolated from nine out of 20 (45%) specimens. Of the 9 positive samples cultured, 8 (88.8%) were S. mutans and 1 was S. sobrinus (11.2%). No inhibitory zone was observed around the disks and wells containing all concentrations of *A. citriodora* extracts. The minimum concentrations for inhibition of growth (MIC) resulted in turbidity in all tubes and were negative except for the control tubes. Inhibition zones were observed for amoxicillin and CHX disks ($p < 0.001$).

Conclusion: This study found that all studied bacteria were resistant to both types of the extracts; therefore, they are not a suggested replacement for chemical agents in mouthwash. It also shown that CHX is less effective than amoxicillin.

Keywords: *A. citriodora,* Amoxicillin, Chlorhexidine digluconate (CHX), *S. mutans, S. sobrinus*

1. Introduction

Dental caries is the most common chronic disease worldwide (1). Dental caries is defined as infectious bacterial disease that results in destruction of the calcified tissue of the teeth (1). One of the bacteria attributed to dental caries is *Streptococcus mutans*, with eight serotypes that are part of the normal flora in oral cavity. The two species responsible for the initiation of dental caries in man are *Streptococcus mutans* and *Streptococcus sobrinus* (2). A caries prevention method is a complex process comprised of multiple aspects (1). Its primary aim is to reduce the numbers of cariogenic bacteria. To reach this goal, limiting substrate, disrupting of plaque formation with brushing and flossing, modifying tooth surface with different topical treatment such as CHX or antibiotic treatment can be applied (1). The golden standard for mouth rinses is CHX. Reversible side effects of CHX use are brown staining of

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the teeth, sores, sloughing, and dry mouth. Furthermore, the development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for a new antimicrobial substance from other sources, including plants (3). The therapeutic benefits of herbal antimicrobial agents as an alternative to chemical antimicrobial agents has been considered in medicine. Their antimicrobial characteristics, which do not affect the normal flora of the oral cavity, make them a suitable replacement for chemical substances. This replacement could result in overcoming the chemical side effects, which is a positive step in improving health and oral hygiene (4). *Aloysia citriodora* is an herb from the family of *Verbenaceae*, with many medicinal properties. *A. citriodora* is listed as a safe substance by the American Food and Drug Administration (FDA), and the consumption of its alcoholic and tea forms have been deemed safe for use (5). The leaves of this plant contain aromatic compounds and are used to make herbal tea. Phenolic compounds are part of the aromatic substances identified in the aqueous and ethanolic extracts of *A. citriodora*, which include dihydrocaffeic acid, 4-hydroxycinnamic acid, luteolin-7-o-glycoside. These substances are responsible for the antimicrobial, anti-inflammatory, and antioxidant activities (6, 7). The objective research was to determine the effects of the aqueous and ethanolic extracts of *A. citriodora* on clinical isolates and standardized strains of *S. mutans* and *S. sobrinus*.

2. Material and Methods

2.1. Collection of plant sample and preparation of aqueous and ethanolic extracts of A. citriodora

A. citriodora leaves were collected from the campus at Babol University of Medical Sciences and washed and dried in the shade at room temperature (RT) for 72 hours. Then, they were grinded to make powdered. To prepare the aqueous extract, 48 g of the powdered herb was mixed with 100 ml boiled water cooled to 70 °C–80 °C and placed at RT for 24 hours. Then, the mixture was passed through filter paper (Whatman filter paper number 1) and left to dry in an oven at 40 °C–50 °C. The dried remains were weighted and dissolved in 100 ml sterilized distilled water. This extract was considered an aqueous extract of *A. citirodora.* For the ethanolic extract, 82 g of powdered plant was soaked in 250 ml of 95% ethanol in a beaker. The mixture was filtered after 48 hours, as described above. The mixture was then dried by a vacuum device. The weight of the ethanolic extract was measured after drying and diluted in 100 ml sterilized distilled water. The mixture was considered an ethanolic extract of *A. citriodora.* These stock extracts were stored at 4 ºC and used to make several concentrations (625–20,000 µg/ml).

2.2. Sample collection and tested bacteria

In this 2016 *in vitro* study, 20 clinical samples were obtained from the oral caries of children admitted to the children's ward at the Faculty of Dentistry, Babol University of Medical Sciences, after obtaining a signed agreement document from their parents. Exclusion criteria were no history of systemic diseases, no medication, no usage of topical fluoride and anti-septic mouthwash during the past month, and a caries index ≤ 5. Dental plaque from decayed teeth was taken with a sterile swab and placed in a capped tube containing 5ml of brain heart infusion broth (BHI) (Himedia) and sent to the microbiology laboratory in a cool box with ice (8). Standardized strains of *S. mutans* PTCC1683 and *S. sobrinus* PTCC1601 were also obtained. The dental swabs were cultured on Columbia sheep blood agar (SBA); then a streak culture method was used to separate bacterial colonies, incubated for 48 hours at 35°C and 5% CO2 (9, 10). A gram stain was carried out for colonies suspected as *Streptococcus spp.* Then, the catalase test and hemolysis reaction were assessed. Furthermore, biochemical tests such as bile tolerance, susceptibility to optochin disks, and hydrolysis of Pyrrolidonyl-α-naphthylamide (PYR) (Himedia) were preformed (11). In order to differentiate *S. mutans* and *S. sobrinus,* other biochemical tests such as esculin hydrolysis, production of urease, fermentation of sorbitol, melibiose, salicin, mannitol, and raffinose were carried out (10).

2.3. Disk diffusion

Bacterial suspensions with a concentration equal to 0.5 McFarland were made for the standardized strains and the isolated bacteria from the clinical samples. These bacteria were cultured on Muller-Hinton agar (Himedia) containing 5% sheep's blood using the spread plate method (8, 9). Then, sterile paper disks containing concentrations ranging from 625–20,000 µg/ml of the aqueous and ethanolic extracts, 25µg amoxicillin disks (A25C MAST), CHX mouthwash 0.2% (Mehban) were placed on the medium. Disks were placed 15mm from the edge of the plate and 24mm from the center of adjacent disks. The plates were incubated at 35°C and 5% CO2 for 24 hours, and then the diameters of the growth inhibition zone were measured (8, 9). Disks containing only sterilized distilled water and ethanol (extract solvent) were used as negative controls (11, 16).

2.4. Agar well diffusion

Bacterial suspensions were prepared as the disk diffusion method and cultured on BHI agar by the spread plate technique. Then, wells were made on the plates by a sterilized Pasteur pipette and 50–100 µl of varying

concentrations of extracts and mouthwash were added to the wells. Mouthwash was used as a positive and sterilized distilled water and ethanol (extract solvent) were used as a negative control. The plates were kept in RT for 30 min and then incubated at 35°C for 24 hours. The diameters of the inhibition zones were measured (13, 14).

2.5. Determination of MIC values

In order to calculate MIC, cation-adjusted Muller-Hinton broth (CAMHB) (Himedia) medium containing lysed horse blood (LHB) was used in accordance with Clinical & Laboratory Standards Institute (CLSI guidelines) (12). In brief, different concentrations (1–32 µg/ml) of amoxicillin solution were made using phosphate buffer saline (PBS) and then added to tubes containing CAMHB medium (12). Microbial suspensions with a concentration equal to 0.5 McFarland were also added to the tubes resulting in 5×10^5 cfu/ml bacteria. The tubes were incubated at 35°C for 24 hours, and turbidity was measured by a spectrophotometer at 625 nm (9). To confirm MIC results, a loop of each tube was cultured on Columbia blood agar medium at 35°C in the presence of 5% CO2 for 48 hours for colony counting. Each experiment had a positive (medium with bacteria) and negative controls (medium without bacteria). The same experiments as described above were carried out for different concentrations (625–20,000 µg/ml) of the *A. citriodora* extracts.

2.6. Statistical analysis and ethical consideration

The data were processed by SPSS version 18.0 and an independent-samples t-test was used to compare the results of disks of amoxicillin and CHX groups, and a $p < 0.05$ was considered significant. This study was certified by the Ethics Committee (grant number 9441012) of the Research Council of Babol University of Medical Sciences, Babol, Iran.

3. Results

Twenty children, aged 6–12 years old, including nine girls and 11 boys, participated in this study. The mean of caries indices were DMFT = 3.8 and dmft = 5.3. *Streptococcus spp.* was successfully isolated from nine out of 20 (45%) specimens. Of the 9 positive samples cultured, 8 (88.8%) were *S. mutans* and 1 (11.2%) was *S. sobrinus.* All bacteria grew in the presence of all concentrations of the aqueous and ethanolic leaf extracts of *A. citrodora* (625–20,000 µg/ml), and no inhibition zones were observed around the disks and wells using the disk diffusion and agar well diffusion methods. This study demonstrated that all the studied bacteria with inhibition zones of more than 30 mm and 12 mm grew around the amoxicillin and CHX disks ($p < 0.001$) and wells, respectively, using the aforementioned methods (Tables 1, 2). In the third test, which was used to determine MIC, turbidity was seen in all the tubes excluding the negative control tubes. This shows the significant growth of bacteria and the ineffectiveness of the extracts. The MIC results for amoxicillin is shown in Table 3.

Table 1. Mean of inhibition zones diameters (mm) of amoxicillin, CHX, aqueous and ethanolic extracts of *A. citriodora* determined using disk diffusion method

Bacteria		Antibacterial agents			
		Amoxicillin	CHX 0.2%	Aqueous extract	Ethanolic extract
Standardized strains	*S. mutans(n=1)*	46	20	0	0
	S. sobrinus(n=1)	48	16	0	0
Clinical isolates	*S.mutans (n=8)*	38.25 ± 3.8*	16.00 ± 1.77*	0	0
	S. sobrinus (n=1)	32	12	0	0

0 means no growth inhibition zones, * Mean ± SD ($p<0.001$), Amioxicillin 25 µg/disk

Table 2. Mean of inhibition zones diameters (mm) of CHX, aqueous and ethanolic extracts of *A. citriodora* determined by well diffusion method.

Bacteria		Antibacterial agents		
		CHX	Aqueous extract	Ethanolic extract
Standardized strains	*S. mutans (n=1)*	25	0	0
	S. sobrinus(n=1)	22	0	0
Clinical isolates	*S. mutans (n=8)*	20.25 ± 2.49*	0	0
	S. sobrinus(n=1)	15	0	0

0 means no growth inhibition zones, * Mean±SD

Table 3. MIC values of amoxicillin, aqueous and ethanolic extracts of *A. citriodora*

Bacteria		Antibacterial agents		
		Amoxicillin	Aqueous extract	Ethanolic extract
Standardized strains	*S. mutans (n=1)*	16	0	0
	S. sobrinus (n=1)	14	0	0
Clinical isolates	*S. mutans (n=8)*	12.13 ± 1.52*	0	0
	S. sobrinus (n=1)	32	0	0

0 means no growth inhibition zones, Amoxicilline concentrations 1–32 µg/ml, aqueous and ethanolic extracts concentration 625–20,000 µg/ml, * Mean±SD

4. Discussion

A. citrodora leaves have many medicinal properties, which may make their extract a suitable replacement for chemical substances in mouthwash. However, no studies have been performed on their effects on tooth decay causing bacteria until now. The results obtained from our study showed that both *S. mutans* and *S. sobrinus* were resistant to the aqueous and ethanolic extracts of *A. citriodora*, as no inhibition of growth of the studied bacteria was observed. A 2010 study by Ramzi showed that *Staphylococcus aureus, Bacillus subtilis*, and *Micrococcus flavus* were susceptible to 4000 µg/ml of methanolic extract of *A. citriodora* while *Escherichia coli, Pseudomonas aeruginosa*, and the fungal specie of *Candida maltosa* were resistant (16). A 2005 study by Oskay determined the antimicrobial effects of ethanolic extracts of *A. citriodora* in concentrations ranging from 4000–20,000 µg/ ml on nine species of pathogenic bacteria and four species of fungi. The authors found that the most inhibition of growth was observed for *S. aureus* at 4000 µg/ml. *Bacillus cereus* and *Micrococcus luteus* had low susceptibility. *Pseudomonas fluorescens* and *Saccharomyces cerevisiae* were completely resistant (23). However, one possible explanation for our results is the production of biofilm by *S. mutans*, as it is a planktonic bacteria that can produce intra and extracellular polysaccharides by producing the glycosyl-transferase enzyme (27, 28). This is an important factor in the formation and cohesion of biofilm. Bacteria, which produce biofilm, are generally more resistant to antimicrobial agents (24, 25). Another reason may be due to the chemical compounds in the extract. Studies have reported that extracts are rich in polyphenols, which have anti-*Streptococcus spp.* activities via the inhibition of cell adhesion and formation of biofilm and the reduction of cell surface hydrophobicity. Literature has stated that antibacterial activity may be related to the number of oligomeric epicatechin units and interflavonoid bonds in *A. citrodora* extract, while monophenolic structures and compounds such as phenolic acid do not affect the bacteria (25). It should be mentioned that the chemical components of a plant extract may be different from region to region based on climatic conditions and different varieties of the plant (26).

5. Conclusions

The current study demonstrated that aqueous and ethanolic extracts of *A. citriodora* have no bactericidal or bacteriostatic effects on *S. mutans* and *S. sobrinus*. Because the aim of this study was not to fractionize the components present in the extract and assess their roles, we cannot give a definite opinion based on the results. Taking into account the medicinal uses in other circumstances, future studies, particularly on the essence, may clarify the possible mechanism involved.

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Conflict of Interest:
There is no conflict of interest to be declared.

Authors' contributions:
All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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