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A comparative evaluation of antimicrobial activity of the ethanolic extract of *Cinnamomum* zeylanicum and NaOCl against oral pathogens and against swabs taken from nonvital teeth - An in vitro study

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Abstract : Background: The aim of this study is to evaluate the antimicrobial activity of Cinnamon Ethanolic Extract (CEE) against clinical isolates of oral pathogens (*Enterococcus Faecalis, Candida Albicans, Staphylococcus aureus, Pseudomonas aeruginosa, and Streptococcus mutans*),and against swabs taken from nonvital teeth, with comparison of NaOCl.
Material and method: Ethanolic extract of *Cinnamomum zeylanicum* was prepared using Soxhlet apparatus. Agar disk diffusion method was used to determine the zone of inhibition of 25% CEE and 5.25% NaOCl. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured using broth dilution method.
Results: The 25% CEE was effective against all microorganisms with MIC values of 390 µg/ml against *Staph. aureus*, 781 µg/ml against each of *E. faecalis*,*C. albicans and Strep. mutans*, and 1562 µg/ml against *P. aeruginosa*, while the MBC values were 781 µg/ml against *Staph. aureus*, 1562 µg/ml against each of *E.faecalis, C. albicans and Strep. mutans*, and1562 µg/ml against *P. aeruginosa*. CEE produced wider zones of inhibition than NaOCl against *C. albicans* and anaerobic swabs, while NaOCl produced wider zones against the other microorganisms.
Conclusion: The antimicrobial activity of 25% CEE is comparable to 5.25% NaOCl against oral and endodontic pathogens.
Key words : Cinnamon, antimicrobial, *Enterococcus Faecalis.*

Introduction

It is well accepted that microorganisms constitute the major etiologic agent of pulpal and periradicular diseases[1,2]. A wide variety of synthetic antimicrobial agents have been used over the years as endodontic irrigants. Sodium hypochlorite (NaOCl) is the most commonly used antimicrobial agent in root canal treatment[3]. Despite its excellent antimicrobial properties, NaOCl is very irritant and can cause serious consequences if extrude to the periradicular tissues[4-6].The undesirable side effects caused by synthetic irrigants had shifted the research towards developing more safe herbal alternatives.

It has been found that natural plant extracts have potent antimicrobial activity[7].Cinnamon (*Cinnamomum zeylanicum* L.) which is a member of family Lauraceae, is one of the oldest and highest quality

spices that was considered by some ancient cultures a holy spice used in religious ceremonies and to expel evil spirits[8].Besides its use as a spice, it has a long history of use as traditional medicine for its antimicrobial, anti-inflammatory, and analgesic properties[9]. Cinnamon origin is from Sri Lanka and India, but now it is produced commercially in India, Africa, South America, the West Indies, Indonesia, and the Seychelles[10].

This study was designed and performed to define the chemical composition of CEE to provide a better understanding of its bioactivity, and to evaluate its antimicrobial activity in comparison with 5.25% NaOCl. The test was done against selected oral pathogens and against swabs taken from nonvital teeth with chronic periapical lesion. These swabs were incubated under aerobic and anaerobic conditions.

In this study, five species of microorganisms were selected, two of which are often used as quality control microorganisms in the antimicrobial sensitivity tests since they are of known susceptibility to many antimicrobial agents, those were: *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The other three microorganisms were: *Enterococcus faecalis, Streptococcus mutans* and *Candida albicans*, which were selected due to their relevance to both primary and persistent endodontic infections[11-13].

Materials and Methods

Preparation of Cinnamon Ethanolic Extract

The cinnamon bark was crushed into smaller pieces and placed in a thimble made of filter paper and then placed into the extraction chamber of the Soxhlet apparatus. About 400 ml of 90% Ethanol was used for the extraction, which was heated to boiling (78.37°C). The extraction process continued for 8 hours. At the end, the extract was accumulated at the bottom of the boiling flask. Next the extract was concentrated by evaporation to dryness (under vacuum), grounded to fine powder and finally stored in refrigerator at 4 °C to decrease the possibility of degradation of bioactive constituents.

Preparation of the working solution

The concentration of CEE (25%) used in this research was settled in a pilot study, where it was found to give best results. Increasing the concentration of the extract more than 25% did not promote its antimicrobial activity. It was prepared by placing 2.5 gram of CEE powder in in a volumetric flask (10 ml), and 5 ml of 10% Dimethyl Sulfoxide (DMSO) was added slowly with shaking to dissolve the powder. Then the volume was completed with distilled water to 10 ml to obtain concentration of 25%.

Preparation of microbial inocula

Microbial inocula of the species mentioned above, were prepared by direct colony suspension method. Three or four colonies from an overnight culture were taken with a sterile loop and inoculated into a sterile test tube containing 5 ml of sterile nutrient broth. The turbidity of this bacterial suspension was adjusted to reach 0.5 McFarland that is equal to 1×10^8 bacterial cell/ml[14].

Measurement of Antimicrobial Activity

Determination of MIC and MBC

Determination of minimum inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) were performed by broth microdilution method according to clinical and laboratory standards institute[15] with some modifications. MICs were obtained from the lowest concentration of CEE that inhibited microbialgrowth. In the first well of the microtiter (96-wells plate), the stock of CEE was added and mixed with Mueller-Hinton broth (MHB) to achieve a final volume of 100 µl and concentration of 100 mg/ml. Then, we did serial two-fold dilution to obtain final concentrations ranging from 50-0.0976 mg/ml. Finally, 100 µl of bacterial inoculum was added to each well, then plate was sealed with parafilm and incubated at 37° C for 16-20 hours. After incubation, the wells were examined for bacterial growth (turbidity) using spectrophotometer. The least concentration that lacks turbidity matching the (-ve) control was considered as MIC.

MBC was measured by subculture of 50 µl from each well with no visible microbial growth on a Mueller-Hinton agar (MHA) plate followed by incubation at 37°C for 24 h.

The disk-diffusion method

The disk-diffusion method was used to assess the antimicrobial effect of CEE extract by direct contact against the same microorganisms and against swabs taken from necrotic, open-canal, single rooted teeth, and comparing it with NaOCl 2.5%.

Sampling procedure.

Swabs were taken from ten patients having teeth diagnosed to be with pulp necrosis and periapical changes, and indicated for endodontic treatment. All the selected teeth were asymptomatic, single rooted, with open pulp and without periapical swelling or fistula.

Each tooth to be sampled was cleaned with pumice and isolated with a rubber dam. The tooth and surrounding field was disinfected with 35% hydrogen peroxide (H_2O_2) and decontaminated with a 5.25% NaOCl solution. New large round carbide bur mounted on slow speed contra-angle handpiece was used to remove the caries. An access opening was done with a new diamond fissure bur mounted on a high speed turbine handpiece with water cooling. Care was taken not to enter the bur deep inside the pulp chamber in order not to disturb the microflora. After completion of the access cavity, the tooth, clamp, and adjacent rubber dam were once again disinfected with 5.25% NaOC[14,16].

A sterile K-file (size 20) was used for sample collection. The handle of the file was cut off with a sterile diamond bur; the file was then inserted into the canal approximately 2-3 mm short of the apex (estimated radiographically), and a separate filing action was used to obtain the sample. If the root canal was dry, few drops of sterile saline solution were introduced into the canal[17]. Then the file was transferred with the aid of a sterile dental tweezer into a sterile screw-capped tube containing 2 ml of Amies transport media. Next, two sterile paper points (size 20) were introduced into the canal separately and each one left in position for 1 min to absorb all the fluids inside the canal. The paper points were then transferred to the tube with the K-file. Afterward, the samples were transported in an anaerobic jar to the laboratory within two hours for testing[14,16].

Inoculation and culturing.

Microbial inocula of 0.5 McFarland standard of the microorganisms and of the swabs taken from the patients were prepared. Then, 100 µl of each microorganism and of the swabs were spread onto MHA petri dishes. The inocula were spread on the MHA in all directions by mean of sterilized cotton swab. For the swabs taken from the patients, two plates were prepared for each patient; one for aerobic culturing and the other for anaerobic.

Sterilized filter paper disks (5 mm diameter) were impregnated with 15 µl of CEE (25%) or NaOCl (5.25%) placed directly on the MHA, incubated for 24 hours at 37° C as follows[14,16]:

- The dishes inoculated with standard microorganisms incubated aerobically.
- The dishes inoculated with patient swabs were divided into 2 groups for each sample; one group incubated aerobically and the other anaerobically. Anaerobic conditions were applied using anaerobic jar and gas packs.

After 24hrs, the diameters of zones of inhibition were measured in mm and results were recorded.

HPLC and phytochemical analysis.

Sample from CEE was analyzed by HPLC. The main compounds were separated on FLC (Fast Liquid Chromatographic) column under optimum conditions, using Phenomenex C-18, 3 µm particle size (50x2.0 mm ID) column. The mobile phase composed of linear gradient of; solvent A (0.1% acetic acid) and solvent B (6:3:1 v/v of acetonitrile: methanol: 0.1% acetic acid). Gradient program from 0% B to 100% B for 12 minutes. Flow rate was 1.2 ml/min. The results were compared with standard figure.

The separation occurred on liquid chromatography Shimadzu 10AV-LC equipped with binary delivery pump model LC-10A Shimadzu, the eluted peaks were monitored by UV-Vis 10 A-SPD spectrophotometer.

Results

The CEE showed good antimicrobial activity against all the test microorganisms. The antimicrobial activity was assessed qualitatively and quantitatively by calculating the values of MIC, MBC, and by measuring the zone of inhibition.

Tested by broth microdilution method (figure 1), CEE demonstrated MIC values of 390 µg/ml against *Staphylococcus aureus*, 781 µg/ml against each of *Streptococcus mutans*, *Enterococcus faecalis* and *Candida albicans*, while against *Pseudomonas aeruginosa* it was 1562 µg/ml.The MBC values were 781 µg/ml against *Staphylococcus aureus*, 1562 µg/ml against each of *Streptococcus mutans*, *Enterococcus faecalis* and *Candida albicans*, while against *Pseudomonas aeruginosa* it was 1562 µg/ml.

The mean values and the standard deviation of the inhibition zones (measured in millimeters) formed around the disks for both NaOCl 5.25%, and CEE 25% against each of test microorganisms are listed in table (1) and represented in figure (2). By observing the table and the bar chart, we can notice that NaOCl 5.25% produced wider zone of inhibition than CEE 25% against *Staph. aureus, E. faecalis, Strept. mutans, Pseudo. aeruginosa*, and for swabs incubated under aerobic conditions. While CEE 25% produced wider zones for *C. albicans* and swabs incubated under anaerobic conditions.

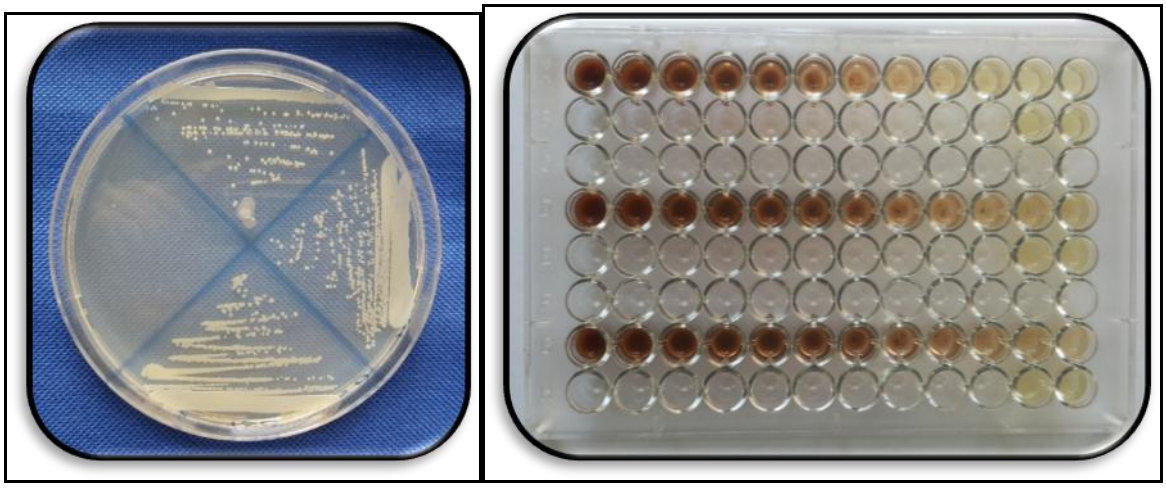

Figure 1: Finding MBC by subculturing samples with no turbidity.

Table 1: Mean and standard deviation of the inhibition zone (in mm) for cinnamon ethanolic extract (CEE) and sodium hypochlorite (NaOCl).

Antimicrobial agent / Microorganism	CEE 25%		NaOCl 5.25%	
	Mean	**SD**	**Mean**	**SD**
Pseudomonas aeruginosa	16.8	0.2	17.3	0.434
Enterococcus faecalis	22.5	0.182	23	0.641
Streptococcus mutans	22.3	0.216	23.5	0.452
Staphylococcus aureus	29.4	0.416	33	0.959
Candida albicans	23.7	0.226	23	0.489
Swabs from patients (aerobic)	23	3.579	26	3.397
Swabs from patients (anaerobic)	23	3.414	22.2	3.269

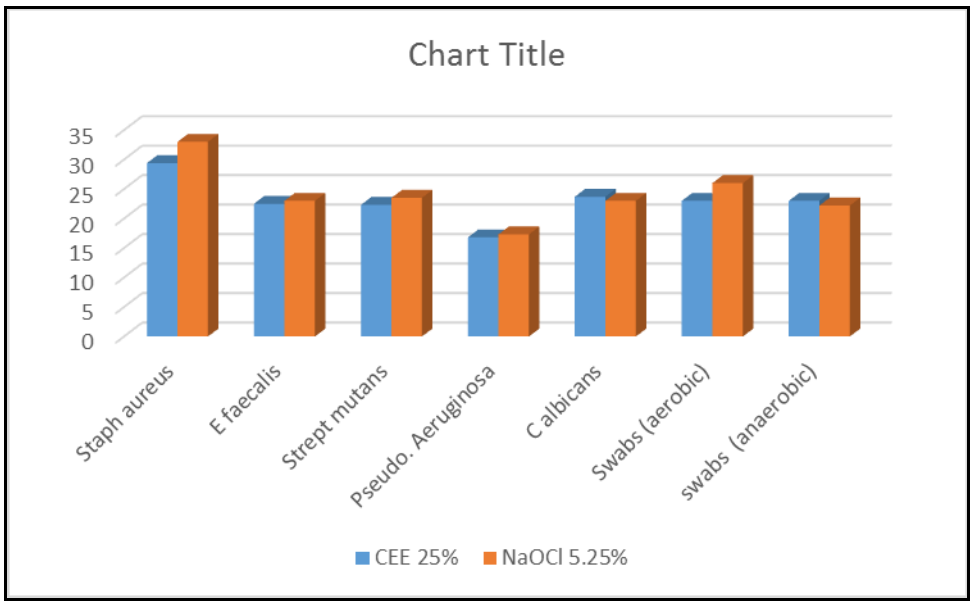

Figure 2: Bar chart showing the mean values of the zones of inhibition (in mm) produced by CEE 25% and NaOCl 5.25%.

We can also notice that the antimicrobial activity of both NaOCl 5.25% and CEE 25% was highest against *Staph. aureus*, and least against *Pseudo. aeruginosa*.

In order to compare the antimicrobial activity of CEE 25% and NaOCl 5.25% against the selected microorganisms and swabs, student t-test was done to see if their results are significantly different from each other. These results showed that there were statistically non-significant differences between the CEE 25% and NaOCl 5.25% regarding the zones of inhibition against all tested microorganisms except *Staphylococcus aureus*, and the swabs incubated under aerobic conditions. For these two microorganism samples, there were statistically significant differences, with NaOCl 5.25% produced wider zone of inhibition than CEE 25%.

Chemical Composition of the CEE

Depending on the HPLC analysis of the CEE,ten components were recognized, which are listed in table 2.

Table 2: Composition of CEE.

No	Component	Rt.	Area	Con. %
1	Benzaldehyde	1.35	193879	14.25
2	Cinnamaldehyde	2.50	90465	6.65
3	Cuminaldehyde	3.42	59688	4.38
4	Eugenol	4.57	24918	1.83
5	Cymene	5.27	262261	19.28
6	Methyl eugenol	6.19	2833231	20.83
7	Cinnamylacetate	7.23	71388	5.25
8	Caryophyllene	8.07	54153	3.98
9	Phellandrene	9.17	49918	3.67
10	Benzylbenzoate	10.90	253468	18.64
Total				100%

Discussion

None of the antimicrobial agents available today in the dental market can perform without any cytotoxicity concerns[18-20].

The antimicrobial potential of alcoholic extract of cinnamon is superior to its aqueous extract[21,22], because the antimicrobial components of the cinnamon bark are more soluble in alcohol as compared to water. Furthermore, the ethanolic extract of cinnamon was found to be more effective than other alcoholic extracts[23,24]. Based on these findings, the ethanolic extract of cinnamon was used in this research.

The antimicrobial activity of CEE is attributed to its bioactive components listed in table 2. Although, the concentration of cinnamaldehyde was low in our extract, this might explain the relatively high concentration needed to achieve antimicrobial effect comparable to 5.25% NaOCl.

Measuring the MIC represents the starting point for wider preclinical assessments of new antimicrobial agents.Both MIC and MBC provide useful information that allows us to optimize the safest concentration which gives best antimicrobial results.

The MIC and MBC values for herbal extracts can vary significantly depending on factors such as: chemical compositions differences between herbs collected in different countries (differences in the climate, soil composition, age and vegetative cycle stage)[25], different botanical parts used for extraction[26], method of extraction, as well as differences in strains of microorganisms used (standardized or clinical isolates)[27].

The MIC values of CEE against different microorganisms used in this study ranged from 390-1562 µg/ml, while the MBC ranged from 781-3125 µg/ml. These results are in accordance with previous literatures[28-31], that studied the antimicrobial properties of different Cinnamon extracts, as well as its essential oils against different microorganisms (including those in our study), and had reported comparable results.

The greatest zone of inhibition was against *Staph. aureus* (29.4 ± 0.416 mm), followed by *C. albicans* (23.7 ± 0.226 mm), swabs from patients (aerobic and anaerobic) (23 ± 3.5 mm), *E. faecalis* (22.5 ± 0.182 mm), *Strep. mutans* (22.3 ± 0.216 mm),and the smallest zone of inhibition was against *P. aeruginosa* (16.8 ± 0.2mm).

These results of this study coincide with the findings of previous studies that measured the zones of inhibition for cinnamon essential oils and extracts against the same microorganisms [32-35].

However,Bardajı´ et al[36]mentioned that the essential oil of cinnamon didn't inhibit the growth of *E. faecalis*.He also stated that cinnamaldehyde was not detected in his specimen, which is one of the major constituents of cinnamon. This difference in the chemical composition might explain this disagreement with our findings. Other researcher,Bayoub et al[37], also reported similar findings to Bardajı´ et al regarding *E. faecalis*. Their findings could be attributed to different method of extraction, and to the presence of trans-cinnamaldehyde in their specimens.

The results of this study had shown that gram negative bacteria *(P. aeruginosa)* are more resistant to both antimicrobial agents (CEE and NaOCl) than gram positive bacteria (*Staph. aureus, E. faecalis* and *Strep. mutans*). This is probably because of the structural differences in the outer membrane of these bacteria. Gram negative bacteria have a thicker layer of lipopolysaccharide outer membrane, that covers the cell wall, which block the penetration of antimicrobial compounds, making them more resistant compared to the gram-positive bacteria[38,39].

In this study 25% CEE produced, insignificantly, wider zone of inhibition than 5.25% NaOCl against the anaerobic swabs. These findings coincide with the results of Jahromi et al[40], who reported that under anaerobic conditions, 25% NaOCl was less effective than propolis, insignificantly. Another research by Canga and Subashi[41], found that 2% CHX was more effective against anaerobic bacteria than 5.25% NaOCl, however the differences were not statistically significant. These findings could probably be rationalized through the fact that one of the mechanisms of action of NaOCl to destroy microbial cells is through oxidation of sulphydryl group of essential enzymes[42,43], hence this mechanism is impaired in the absence of oxygen.

Conclusion

Under the conditions of this study, 25% CEE exhibited a strong antimicrobial efficiency,almost similar to 5.25 NaOCl, against all the test microorganisms and against swabs taken from nonvital teeth.

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