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The Effect of Aqueous Extract of Cinnamon on Anaerobic Peri-Implantitis Bacteria

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ABSTRACT

Background and Objective: Even though dental implants have a high success rate, the most frequent implant dentistry problem is peri-implantitis. With increased bacterial resistance to antibiotic, the herbal products are more preferred. This study aimed to find a concentration of aqueous cinnamon extract that prevents the patient's anaerobic bacterial flora from growing with peri-implantitis and to evaluate the phytochemical composition of Aqueous Extract of cinnamon.

Methods: This experimental in vitro study was conducted using total anaerobic bacterial samples isolated from the implant pockets of 10 patients (male and female) aged 40-60 years suffering from chronic peri-implantation requiring the presence of a probing pocket depth≥10 mm and clinical attachment loss of (1-2) mm or more. Antibacterial assay was performed using well diffusion agar at an extract concentration of 5, 10, 12, 15, 20, and 25 mg/mL, which were chosen randomly according to the pilot study with a positive control as CH2% and a negative one as distilled water to measure the inhibition zone through the center from edge to edge of inhibition zone. Final concentrations (1, 3, 6, 12, 24, 48) mg of the extracts were used to find the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Secondary metabolites of the prepared cinnamon aqueous extract were detected using Gas Chromatography-Mass Spectrometry (GC-MS) and phytochemical test.

Findings: The present study showed that Cinnamon contains alkaloid, flavonoids, saponins, and glycoside, and it significantly inhibited the isolated bacteria with MBC at 24 mg/mL and Minimum Inhibitory Concentration (MIC) at 12 mg/mL. Well-diffusion assay revealed that the mean values of inhibition zone diameter were 1.15±0.118, 1.27±0.142, 1.38±0.132, 1.51±0.13 and 1.46±0.084 cm for concentrations of 12.5, 15, 20, 25 and Chlorhexidine (CHX) 2% mg/mL, respectively ($p<0.05$). The increase in inhibition zone diameters was associated with the increase in cinnamon extract concentration, while concentrations of (5.10) mg/mL revealed no zone of inhibition.

Conclusion: According to the results, this study determined that cinnamon was a potent antimicrobial agent, and that it can be used as anti-infective therapy against peri-implantitis to overcome implant failure.

Keywords: *Herbal Extract, Cinnamomum Aromaticum, MBC, MIC, Phytochemical, Dental Implant*.

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Introduction

Peri-implantitis is a multifactorial disease and a local inflammatory response associated with the loss of supporting bone in the tissues surrounding the implant. The main etiological agent of this condition is bacterial colonization of the pocket around the implant (1).

Although antibiotics are commonly used and are among agents effective in dealing with this bacterial infection, researchers are more interested in using herbal derivatives for treating it due to the resistance of human pathogenic microorganisms to these antibacterial agents as well as the side effects and ineffectiveness of these drugs (2). In addition, medicinal plants are generally better tolerated by the body and usually have no side effects. Therefore, these drugs are more suitable for chronic diseases, particularly when used for a long period of time. These drugs are also inexpensive and readily available (3).

Cinnamomum cassia (also known as Cinnamomum aromatic, Chinese cinnamon, or Chinese cassia) is a member of the laurel family (Lauraceae). Various pharmacological properties of Cinnamomum cassia, with an antioxidant, a cardioprotective, and an anti-inflammatory and antimicrobial properties have been also documented (3). It contains many antibacterial elements such as saponins, flavonoids and phenol (4).

For instance, EO cinnamon, cinnamon extracts, and pure compounds can be potentially used in mouthwashes and toothpaste or as a root canal irrigant due to their antifungal, antibacterial, and other properties, showing great promise as antimicrobial agents for the future of dentistry (5).

The antibacterial activity of cinnamaldehyde extracts of Cinnamon against the periodontal pathogens such as A. actinomycetemcomitans, Treponema denticola and Tannerella forsythia was also investigated. The results showed high efficacy of cinnamon against all pathogens tested. MBCs ranged from 6.25 to 75 mg/mL and MICs ranged from 1.56 to 12.5 mg/mL against various bacteria (6) Cinnamon's antimicrobial properties on both Gram-positive and Gram-negative bacteria have been the subject of numerous studies, suggesting that it could serve as an alternative treatment for periodontal diseases (7), but few have explored its effect on the total anaerobic bacteria associated with peri-implantitis that are responsible for most implant failures.

Methods

Collection and preparation of plant material: Cinnamon (Cinnamomum cassia) bark was purchased from the local herbal medicine market (Baghdad, Iraq). The purchased cinnamon was identified and approved by the Ministry of Industry and Minerals, the Institute for Research and Industry Development, and the Ibn Al Betar Research Center. Then the cinnamon bark was transferred to the laboratory and thoroughly washed in distilled water to remove unwanted residue and dust. Dust-free parts were dried in the shade at room temperature for seven days until they were dry enough for sanding, then stored at room temperature in a dry, sterile container until use (8).

Plant extracts preparation: Preparation of the cinnamon extract: The cinnamon bark was ground into a fine powder in a milling machine and sieved with a mesh sieve to obtain a very fine powder. After that, one liter of sterile water was mixed with 100 grams of cinnamon powder, left in a water bath at 60 degrees Celsius for five hours, and then filtered through sterile filter paper No. 1 (Whatman, UK) (9). A rotary vacuum evaporator was used to collect and concentrate the filtrate at a lower pressure until a semi-solid material was obtained. This material was then dried in a crucible at a controlled temperature (45^{o}C) in a convection oven to evaporate water and produce a solid powder (10). The extract was stored at 4°C in the refrigerator and had a viscous brown color until use (11).

Chemical analysis of the cinnamon extract:

A: Phytochemical tests: Tests for alkaloids, glycosides, saponins, tannins, flavonoids, steroids, and triterpenoids were performed on the extract. A form of investigation where a color change or reaction took place in accordance with the findings following treatment of the sample or cinnamon extract with the reagent yielded positive qualitative results (+).

B: Chromatography-mass spectrometry (GC-MS):

GC-MS conditions: Cinnamon extract was diluted in methanol and analyzed using an Agilent 7820A USA GC mass spectrometer. Chromatograms were screened in scan modes from 25 to 1000 m/z. Injections were performed in splitless mode. Component identification was performed by comparing the mass spectra with standards from the C:\GCMS\NIST11.L. Quantitative analysis of the components of interest, expressed as area percent, was performed by normalized measurements of the peak areas.

Microbiological sample collection:

Study design, individuals and clinical parameters: In this experimental in vitro study, the sample design was done according to previous studies (12, 13); ten patients with at least one implant with peri-implantitis. All individuals showed good general health and were non-smokers. They had not received any antibiotic therapy or anti-inflammatory drugs within the last 6 months, nor had they used antimicrobial mouth washes within the last 2 weeks before the onset of the study. No peri-implantitis therapy had occurred within the last 2 years. The age of the individuals was between 40 and 60 years. The following clinical examinations of the implants with peri-implantitis were conducted and registered: 1) bleeding on probing (BOP), 2) probing depth (PD) 10 mm, and clinical attachment loss of 1-2 mm or more, 3) recession or gingival hyperplasia Intraoral radiograph (14).

The study design and all study protocols were examined and approved by the research Ethics Committee of the University of Baghdad College of Dentistry with Ref. number: 638 at 4-8-2022 and with Project No. 638222.

Sterilizations: The glassware and steel equipment were sterilized in a ventilated oven at 160°C for two hours, while the culture media were autoclaved at 249.8°F at 15 pounds/square inch for 15 minutes. The benches and the floor of the laboratory were disinfected with Dettol antiseptic solution. All laboratory work was performed under the UV sterilization cabinet-biological safety cabinet (13).

Selective media for bacterial growth: Brain Heart Infusion Agar BHI.A, Brain Heart Infusion-Blood Agar BHIB and Hinton agar MHA were prepared from Oxoid Laboratories Company. Brain heart infusion broth BHI was prepared from Mast Group Ltd. All media were prepared and sterilized according to the instructions of the manufacturers (15).

Culture and microbiological analysis: Samples were taken from the deepest part of the peri-implant pocket using sterile point F1 paper (Dentsply Millefer paper) inserted in phosphate-buffered saline with sterile scissors for five minutes and immediately transported to the microbiology laboratory for bacterial culture. Samples were vortexed with buffer saline and then inoculated onto the blood agar medium under anaerobic conditions using an anaerobic gas pack and anaerobic vessel at 37°C for 48 hours (16).

Maintenance of bacterial isolate: A single colony of the bacterial isolate was picked from the bacterial agar medium, transferred to 10 mL of sterile BHI broth, and incubated anaerobically for anaerobic bacteria at 37°C for 24 hours. These broths were stored in the refrigerator until use (16).

Activation of isolates: The bacterial strain inoculum was sterilized and activated by adding, approximately 0.1 mL of bacterial isolates to 10 mL pure Brain-heart infusion broth (pH 7.0) which was incubated anaerobically for 24 hours at 37°C before each experiment (17).

Gram stain: After 48 hours of anaerobic incubation in the incubator, the bacterial colony with Gram stain was studied. This was used for identifying the Gram-positive and Gram-negative microorganisms. A stain of the bacterial isolate was made on a clean slide stained with the Gram stain reagents. Their responses in grams and their morphology were observed under the microscope using an x100 objective lens.

Microbiological Assay:

1. Sensitivity of totally anaerobic bacteria to different concentrations of cinnamon extract in water in vitro: The antimicrobial activities of cinnamon extract were assessed by adopting the agar well-diffusion method (18). Ten plates were prepared. 25 mL Mueller Agar was poured into each sterile glass. Petri dishes were then kept at room temperature for 24 hours. Then, 0.1 mL of activated standardized inoculum of anaerobic bacteria was added to each plate that was adjusted to (10 CFU/mL; 0.5 MacFarland) and spread using sterile spreader onto sterile Muller-Hinton Agar (MHA) to achieve confluent growth. The plates could dry and a sterile cork borer (6 mm diameter) was used to bore wells in each plate. Each well was filled with 0.2 mL of five concentrations of water cinnamon extract (5, 10, 12, 20, 25 mg/mL), which were selected according to pilot study using sterile deionized water which served as a negative control. Positive control in the form of chlorhexidine 2% was also included in the study. Plates were left at room temperature for 10 minutes for diffusion and, then, incubated anaerobically in an anaerobic jar using a gas pack for 24 hrs at 37°C. Some of inhibition were located across the diameter of each well. The inhibition diameter around the wells containing the test materials was measured and recorded after incubation under aseptic conditions. The zone of inhibition was assumed to be measured in cm with calipers. None of incubation zones indicated complete resistance of bacteria to the test agent (19).

2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination of aqueous cinnamon extract: Final concentrations (1, 3, 6, 12, 24, 48 mg) of the extracts were mixed separately with Brain Heart Infusion (BHI) agar to obtain 10 mL of agar, then poured into Petri dishes and allowed to harden, and then inoculated with 0.1 mL of activated anaerobic isolates bacteria. All these Petri dishes were incubated for 24 hours at 37°C, including the control plates (i.e., negative control plates containing Brain Heart Infusion (BHI) agar with microbial inoculation without the addition of the extracts, as well as the positive control plates containing Brain Heart Infusion (BHI) agar and different concentrations of extracts without microbial inoculation). Each petri dish was checked and examined for microbial growth. The minimal bactericidal concentration (MBC) was determined as the lowest concentration of the extract that killed the microorganisms (15).

Statistical analysis: Statistical analysis was performed using mean (mm), standard deviation, The Kolmogorov-Smirnov test was used to determine the normality of the collected data. A one-way analysis of variance statistical test was used to examine the differences between each group, with a significance threshold of $p \leq 0.05$. Significance of all statistical tests was determined using SPSS (Statistics Package for Social Sciences).

Results

Identification of bacterial colonies: The appearance of bacterial colonies was whitish accumulation with small and large size with smooth surface as show with Figure 1A. The gram stain test indicated collected bacterial samples from negative and positive bacteria as show in figure 1B.

Qualitative Phytochemical Test Methods: Chemical analysis of the studied extracts from cinnamon bark showed that they contained the active compounds as a secondary metabolism as shown in (Table 1).

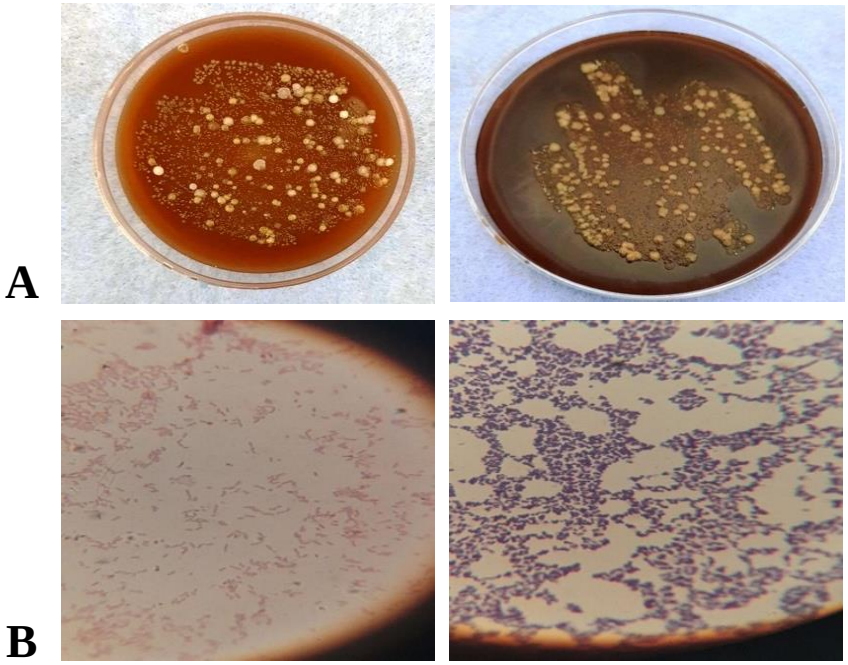

Figure 1. A: Colonies of Anaerobic Bacteria, B: Colonies of Anaerobic Bacteria under Light Microscope Gram-positive and Negative

Table 1. Phytochemical Test Results of the Cinnamon Extract (Qualitative) Note: (+ve) Detected; (-ve) Not Detected

No.	Testing type	Testing result
1	Flavonoid	+ve
2	Alkaloid	+ve
3	Saponin	+ve
4	Tannin	+ve
5	Quinone	+ve
6	Terpenoid	-ve
7	Coumarin	+ve
8	Resin	-ve
9	Phenols	+ve
10	Glycosides	+ve
11	Protein	-ve
12	Carbohydrate	-ve

Chromatography-mass spectrometry (GC-MS): 25 chemical peaks were identified in cassia extract using the GC-MS method. The compounds included alcohols, fatty acid aldehydes, esters, organic acids, alkanes, and ketones, Ester, 9-Octadecenoic acid (Z)-, 2,3-dihydroxy propyl ester, Octadecadienoic acid (Z, Z)-, methyl ester Alcohols: glycerin; 1,2,3-Butanetriol Aldehyde: Octadecenal, (Z)-cis-11-Hexadecenal Ketones3- Methoxy acetophenone Acid: cinnamic acid, and Oleic acid as observed in (figure 2).

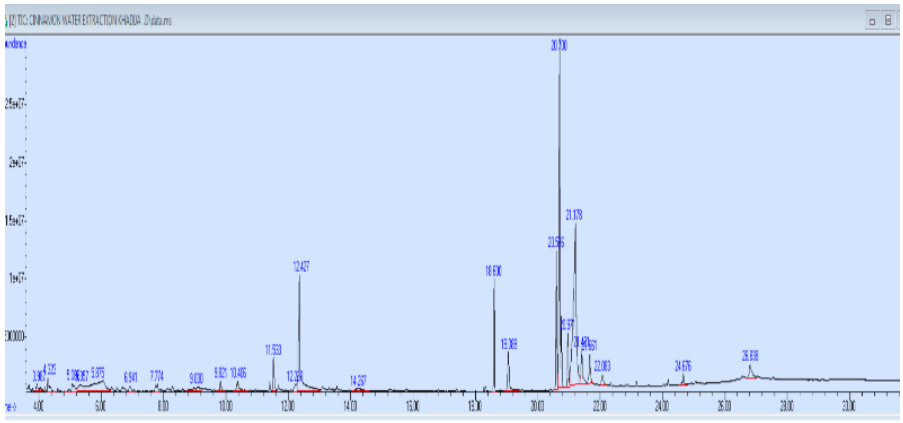

Figure 2. GC-MS Profile of Cinnamon Aqueous Extract

Sensitivity of totally anaerobic bacteria to different concentrations of cinnamon extract in water in vitro: The mean zones of inhibition of growth against total anaerobic bacteria tested in response to different concentrations of CN.A.E (5, 10, 12.5, 15, 25) mg/ml were (0, 0, 1. 15±0.12, 1.27±0.14, 1.51±0.14,) cm respectively and for chlorhexidine was 1.46±0-08 as shown in (Figures 3) and (Table 2). Statistical analysis of the normal distributed data was showed a p value of 0.000, indicating a significant difference across the whole data as observed in (Table 2).

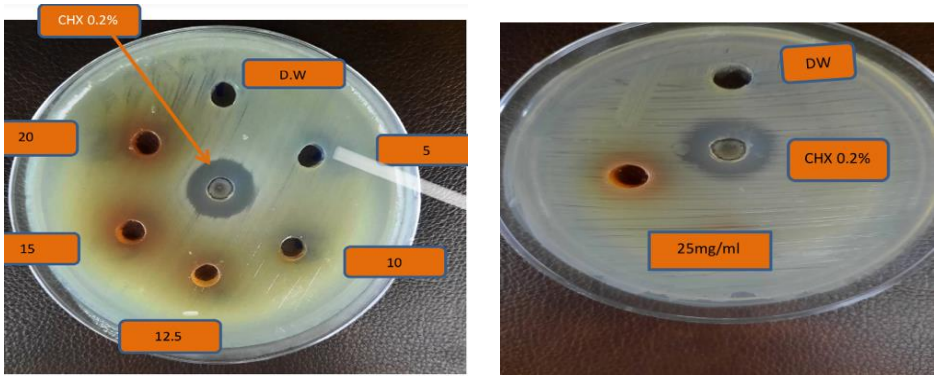

Figure 3. Sensitivity of Total Anaerobic Bacteria to Different Concentrations of Cinnamon Extract, CHX, and D.W by Agar Well-diffusion Method

Table 2. Antibacterial Activity of the Extracts was given as the mean diameter of the Inhibition Zone and ANOVA

Concentration	N	Mean±SD	ANOVA Table	
			F	Sig.
5.00	10	0.000	521.299	0.0001
10.00	10	0.000	521.299	0.0001
12.50	10	1.15±.118	521.299	0.0001
15.00	10	1.270±0.142	521.299	0.0001
20.00	10	1.380±0.132	521.299	0.0001
25.00	10	1.510±0.137	521.299	0.0001
CHX 0.2%	10	1.460±.084	521.299	0.0001
DW	10	0.000	521.299	0.0001

Along with a greater extract concentration, the inhibitory zone's mean diameter increased, as shown in figure 4.

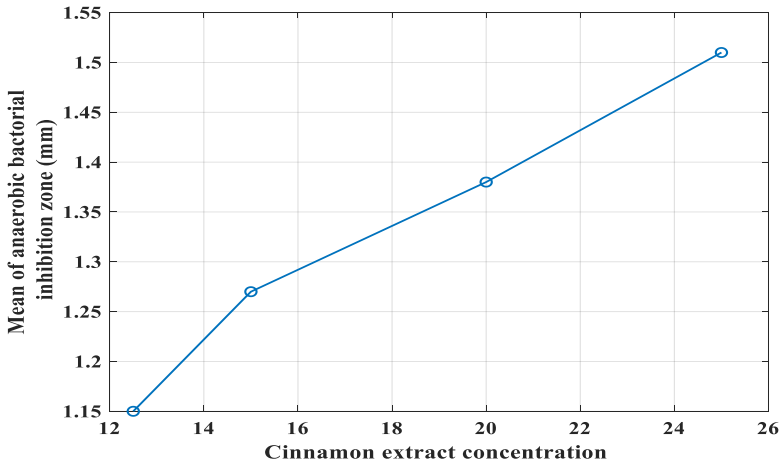

Figure 4. Mean Plots inhibition zone in relation to cinnamon extract concentration

Comparison in growth inhibition zone of total anaerobic bacteria between chlorhexidine and different concentrations of cinnamon extract: The mean of growth inhibition zone was significantly higher in chlorhexidine than 12.5 and 15 mg/mL concentrations of cinnamon ($p<0.001$). No statistically significant difference ($p\geq0.05$) was detected in mean of growth inhibition zone of test bacteria when we used 20 and 25 mg/mL concentrations of cinnamon and chlorhexidine, as shown in (Table 3).

Table 3. Comparison in means of growth Inhibition Zone of total anaerobic bacteria for Different Concentrations of Watery Extract of Cinnamon and Chlorhexidine by LSD test

Concentration	Concentration of CN.A.E	Mean Difference	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
CHX 0.2%	5.00	1.46000*	0.000	1.3724	1.547
	10.00	1.46000*	0.000	1.3724	1.547
	12.50	0.31000*	0.000	0.2224	0.397
	15.00	0.19000*	0.000	0.1024	0.277
	20.00	0.08000	0.073	-0.0076	0.167
	25.00	-0.05000	0.259	-0.1376	0.037

***This is a lower bound of the true significance, highly significant difference (p<0.01).**

Determination of MBC for cinnamon extract against total anerobic bacteria: The results of these experiments showed that the minimum bactericidal concentration of the aqueous cinnamon extracts for total anaerobic bacteria was 24 mg/mL. This concentration showed no growth after re-culture on pure BHI-A medium; in other words, the concentration of 24 mg/mL had a bactericidal effect and killed the bacteria with 12 mg/mL representing the MIC; the minimum concentration of cinnamon extract that inhibited bacterial growth as observed (Figure 5) and (Table 4).

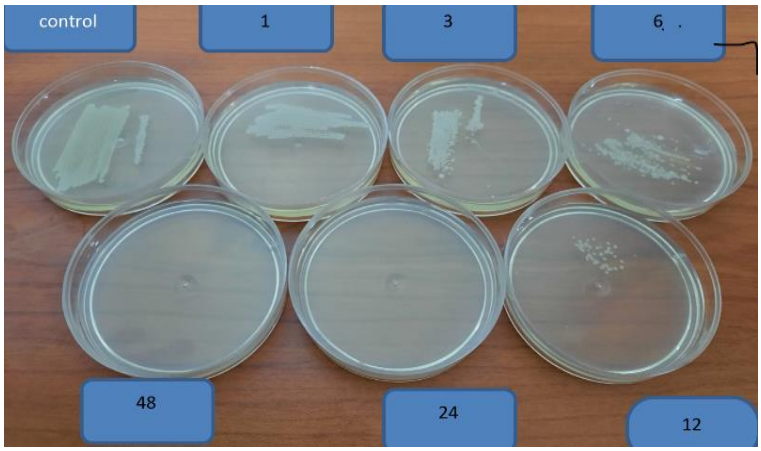

Figure 5. MIC and MBC Determination of Cinnamon Aqueous Extract

Minimum Inhibitory Concentration (MIC)= 12mg/ml. Minimum Bactericidal Concentration (MBC)= 24 mg/mL.

Table 4. MIC and MBC for Cinnamon Watery Extract

Conc. of cinnamon extract (mg/mL)	
Control	Growth
1	Growth
3	Growth
6	Growth
12	Growth
24	Non
48	Non

Discussion

This research indicated that cinnamon is effective on inhibition zone diameter of total anerobic bacteria under in vitro condition and acts as a broad-spectrum antibiotic. Many studies reported the antibacterial effects of cinnamons on both Gram-positive and Gram-negative bacteria (20). A zone of inhibition greater than 5 mm in diameter was considered as a positive result of cinnamon extract effect on gram negative and gram positive bacteria (21). In present study, the minor inhibition zone was 11.5 mm (1.1 cm) with concentration 12.5+0.12 mg/mL, which was indicative of the activity of tasted extract against tasted bacteria. Moreover, the anti-microbial effect of cinnamon was in agreement with the findings from other studies demonstrating the antimicrobial activity of cinnamomum cassia against various bacterial flora (22, 23), as well as with the findings from-numerous research reporting the vast range of antibacterial benefits of cinnamomum cassia (24).

The (MIC) and MBC of E CN. A. in this study were 12 mg/mL and 24 mg/ml respectively and this finding are consistent with those of a study by Saquib et al., which demonstrated cinnamon's high efficacy against all of the periodontal pathogens tested. MICs vary from 1.56 to 12.5 mg/mL and MBCs vary from 6.25 to 75 mg/mL against different microorganisms (6). This is in agreement with Aneja et al., who found that C. Cinnamon extracts in acetone, ethanol, and methanol had substantial antibacterial activity against yeast and bacteria in the mouth (25).

This study showed that CN.A.E. was as effective as chlorhexidine 2% at 20 and 25 mg/mL. However, this results were consistent with the findings from earlier studies by Zhou et al and Sethi et al showing that cinnamon extract was as effective as chlorhexidine, and explaining cinnamon's activity against plaque and gingivitis due to its active ingredients including Cinnamaldehyde, aromatic aldehydes, and eugenol (26, 27). Photochemical examination of CN.A.E. showed that extract contained secondary metabolite bioactive compounds such as alkaloids, flavonoids, tannins, phenol, and saponins, which serve as defensive mechanisms against microbes (28). Our study result in this regard was in agreement with the findings from other studies documenting that cinnamon contained secondary metabolite compounds such as alkaloid, saponin, flavonoid, and glycoside groups (29).

Several studies reported that cinnamon bark contained cinnamic aldehyde as the main component which is the main antimicrobial compound (30, 31); however, this study found that the concentration of cinnamic aldehyde was very low because of the extraction method and polarity of solvent. Liang et al showed that the extraction method affected the composition of the extract from Cinnamomum species (32).

The GC mass analysis was performed. Photochemical findings represented the friction of fatty acids, esters, ketones, alcohols, aldehydes, and organic acids, were responsible for the antibacterial effects of cinnamon extract. Our study result in this regard was also in line with the findings from other studies suggesting that most aromatic plants contained dozens of these compounds (21). However, our study results were not consistent with the findings from other studies indicating that chemical composition and quantity of cinnamon plants were strongly influenced by the variety and age of the plant, drying methods, geographical conditions, time and treatment of harvest (33), market storage condition (31), as well as the methods of extraction and polarity of solvent (34).

The antibacterial activity of extract is belonging to the Phytochemicals function in a variety of bioactivities through several mechanisms. They may prevent the growth of microorganisms, obstruct various biological metabolic processes, or modify the routes for signal transduction and gene expression. When bacterial cell walls and membranes collapse under the influence of phytochemicals, cell components leak out, the proton motive force is disrupted, the efflux pump and enzyme are dysfunctional, and cytosis results (32, 35).

In conclusion, the results indicated that aqueous cinnamon extract had a good antimicrobial effect on total anaerobic bacteria, and that the application of higher concentrations produced more favorable results as the same effect as chlorhexidine while the application of lower concentrations of 5 and 10 mg/mL did not affect the growth of anaerobic bacteria. The MIC was 12.5 mg/mL and MBC was 24 mg/mL. The contribution of this study lies in the facts that it added to our knowledge regarding the antibacterial activity of cinnamomum cassia. Furthermore, it was found that Chromatography-mass spectrometry (GC-MS) was widely used to identify various bioactive compounds present in plants, like Alkaloids, flavonoids, organic acids, amino acids, etc. It was recommended that the computational tools emerged as sophisticated drug discovery tools should be employed in order to screen the drugs for bioactive compounds present in medicinal plants. Since cinnamon is readily available, inexpensive, safe and cost-effective source of natural antibacterial compounds, it can be introduced as a definite treatment in near future to reduce infection that cause implant failure.

Conflict of interest: The authors have no conflicts of interests relevant to this article.

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