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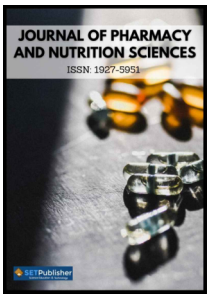

Antibacterial Activity of Bioactive Compounds of Green Coffee Beans on Periodontogenic and Nosocomial Bacteria

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Abstract:

The emergence of antimicrobial resistance and the side effects of synthetic drugs have raised an interest in searching for new antimicrobial compounds. The present study aims to evaluate *in vitro* antibacterial activity of green coffee and its active compounds (chlorogenic acid extract and caffeine extract) against some periodontogenic and nosocomial bacteria. The bioactive compounds, viz. chlorogenic acid and caffeine, were extracted through soxhlet extraction using methanol and water, respectively, and HPLC UV quantified these compounds. The study reported 3 CQA, 4 CQA, and 5 CQA as the significant chlorogenic acids in green coffee beans. Aqueous extract of green coffee beans (AGCB), which is dominant in caffeine, has been found to be the least effective against both periodontal and nosocomial bacteria. The result of our study revealed that the methanol extract of green coffee bean (MGCB), rich in chlorogenic acid, exhibits the highest inhibitory activity against periodontogenic bacteria, followed by the ethanol extract of green coffee bean (EGCB) and AGCB extract. EGCB extract was significantly effective against *Staphylococcus epidermidis* among selected nosocomial pathogens. AGCB extract was least effective against all bacteria. The results highlight that green coffee polyphenols, especially chlorogenic acid, could be used as antimicrobial agents in different biotechnological applications. The antibacterial property of green coffee highlights its potential as a naturally active antibacterial compound.

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INTRODUCTION

The coffee brew is one of the most valuable beverages, known for the old tradition of Ethiopia. Since the early 20th century, coffee has become one of the world's most popular drinks due to its pleasant taste, flavor, and aroma. The International coffee organization reported that during 2019-2020, the average coffee consumption was nearly 167.6 million bags of 60kg, and the everyday coffee intake was probably 500 billion cups. Coffee beans are naturally green beans rich in antioxidants and numerous pharmacologically active compounds such as Caffeine, Chlorogenic acid, diterpenes, and trigonelline [1,2]. Several studies have reported a wide range of therapeutic benefits such as anti-obesity, anti-diabetic, antimicrobial, anti-tumor, and hypocholesterolemic. Recently, coffee has received attention for its ability to inhibit the growth of pathogenic microorganisms [3,4,5]. The incidence and progression of periodontal diseases are related to an increase in pathogenic microbes. *Porphyromonas gingivalis* and *Streptococcus mutant*s are regarded as the primary pathogens in chronic periodontitis etiology [6,7]. Periodontal diseases affect the tissues that support the tooth, and severe periodontal diseases affect nearly 14% of the adult population, representing more than 1 billion cases globally [8].

Nosocomial infections, also called hospital-associated infections, are acquired in search of medical care that did not present at the time of admission. These infections are the most common medical events which affect patient safety; nearly 3.2% of all admitted patients in the US (United States), 6.5% in the European Union, and worldwide prevalence are much higher [9,10]. The responsible bacteria may originate from an endo or exogenous source as a natural flora part; common microorganism includes *Staphylococcus aureus, Staphylococcus epidermidis*, and *Enterococcus faecalis* [11,12]. These nosocomial pathogens are mostly multi-drug-resistant. The increased antimicrobial resistance requires an urgent response as new antimicrobial compounds. Although, developing new antibiotic compounds takes a long time and is expensive. Therefore the preferred way is to optimize already available drugs or a combination of several antibiotic compounds. In addition, society's concern about the safety of synthetic agents has increased interest in naturally occurring compounds. Even though coffee is considered the world's most popular and loved drink, studies on its antimicrobial properties are restricted in the literature. Following an extensive literature search and best of our understanding, this will be the first comparative study to determine green coffee *extract is in vitro* antibacterial effect and its active compounds (chlorogenic acid extract and caffeine extract). Five commonly occurring bacterial species, namely *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Porphyromonas gingivalis*, and *Enterococcus faecalis*, are selected based on available limited literature for the study.

MATERIAL & METHODS

Sample Collection & Preparation

Green coffee beans were purchased from Kannur city in Kerala, India. Beans of similar shape, size, and color were separated manually and crushed through a mortar pestle. Crushed beans were powdered and sieved to get a uniform texture.

Preparation and Extraction

Ethanol Extract from Green Coffee Bean (EGCB)

Green coffee beans extract preparation was accomplished by the Soxhlet extraction method explained by Caballero-Galvan *et al.* [13] with slight modification. 5g powdered sample was disposed of in a porous thimble and placed in a soxhlet extractor with 150 mL of 80 % ethanol at constant reflux for 8 hrs. Finally, the liquid extract was separated through the centrifuge and stored at freeze temperature.

Aqueous Extract of Green Coffee Bean (AGCB)-Caffeine Extraction

Caffeine was extracted from green coffee beans by the method used by Shinde and Shinde [14] with some modifications. 5g Grounded green coffee beans were disposed into a porous thimble, placed into a soxhlet extractor with 150 mL of distilled water, and refluxed for 16 hrs. A constant temperature of 90◦C was maintained to get maximum extraction of caffeine. After the prescribed time, the extract was separated through Whatman filter paper one and stored at freeze temperature.

Methanol Extract of Green Coffee Bean (MGCB)-Chlorogenic Acid Extraction

Chlorogenic acids from the green coffee beans were extracted by the procedure explained by Qiu *et al.* [15] with some changes. 5g Defatted powdered sample was taken into a porous thimble and placed into a Soxhlet extractor with 150 mL of 70% methanol and refluxed for 8 hrs. A constant temperature of 60◦C was maintained

to get maximum extraction. After the prescribed time, the extract was separated through Whatman filter paper one and stored at freeze temperature.

Microorganisms, Antibiotics, and Culture Media

The used bacterial strains were from the ATCC (American Type Culture Collection): *Porphyromonas gingivalis* [ATCC 33277], *Streptococcus mutans* [ATCC 25175], *Staphylococcus aureus* [ATCC 12260], *Staphylococcus epidermidis* [ATCC 14990], *and Enterococcus faecalis* [ATCC 29192]. The antibiotics used in the study were; Streptomycin, Amoxicillin, Tetracycline, Rifampicin, and Cefuroxime. Antibiotics, Mueller Hinton Agar, and Nutrient Agar were purchased from Sigma. Antibacterial activity was performed by Agar well diffusion method, and the zone of inhibition was measured in mm. The stock solution of the antimicrobial agent (Green Coffee bean extract) was prepared by adding 100µL of extract to 1mL thioglycolate broth medium. Mueller Hinton Agar (RANKEM) 1000 mg/L was taken to prepare an antibacterial plate. The Agar plate surface was inoculated by spreading an even volume of the above-prepared stock solution. The well was prepared with a sterile micro tip and filled with antimicrobial agents (extracts). Five wells were made on MH Agar having positive control, negative control, and extract with the consecutively increasing amount (10mg/ml, 15mg/ml, 20 mg/ml).

HPLC Analysis

100µL extract filtrate was mixed with 900 µL Milli-Q water into a 1000 µL volumetric flask. The samples were run in SYSTRONICS [SYS LC-138] High-Performance Liquid Chromatography [HPLC] system. HPLC conditions were: reverse phase column HiQSil C 18-HS and size 4.6 mm × 250 mm × 5µM, isocratic pump type. The injection volume was 20µL, and the flow rate was 1.5 minutes/ mL.

Diluent and Mobile Phase

2% Formic acid and 10% Acetonitrile, prepared by mixing 50mL acetonitrile, 10 mL formic acid, and 440 mL Milli-Q water. Mobile phase A was .1% Trifluoroacetic acid, prepared by adding 1 mL trifluoroacetic acid in 900 mL Milli-Q water in a 1000 mL volumetric flask. The volume was made up to the mark with Milli-Q water. The mobile phase used with CGA extract was methanol and water in 68:32 ratios.

Statistical Analysis

The analysis in this study was carried out in triplicates, and obtained results were expressed as mean ± standard deviation. The obtained data were examined by one-way analysis of variance (ANOVA) at a 95% confidence level, and the mean was equated using Tukey's test of SPSS software version 16.0.

RESULTS

Our research employed the HPLC UV method for qualitative and quantitative evaluation of green coffee beans, which, after authentication, were further screened for their antibacterial properties [16]. The antibacterial activity of EGCB, MGCB and AGCB against *Porphyromonas gingivalis Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis,* and *Enterococcus faecalis* was determined at various concentrations (10mg/ mL, 15mg/ mL, 20mg/ mL).

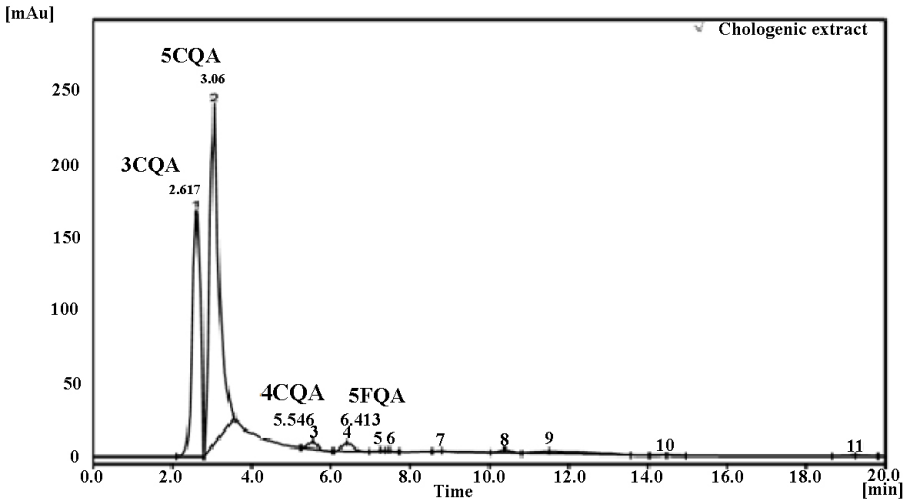

Figure 1: Chromatogram of MGCB.

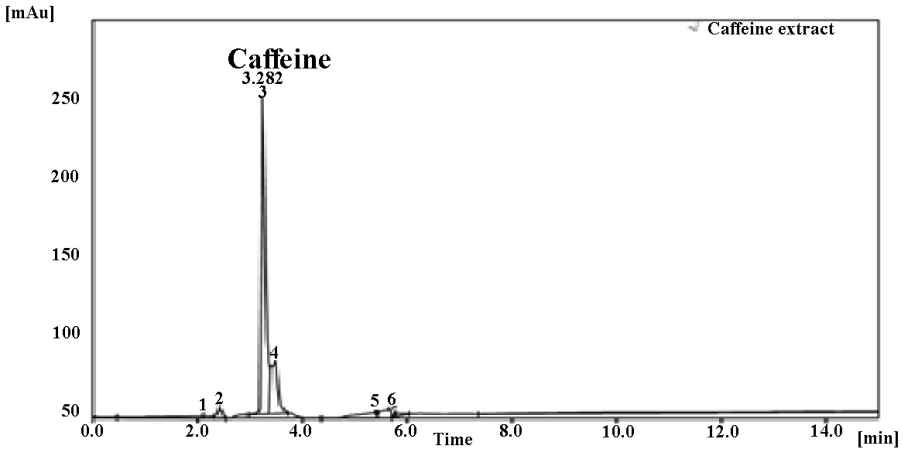

Figure 2: Chromatogram of AGCB.

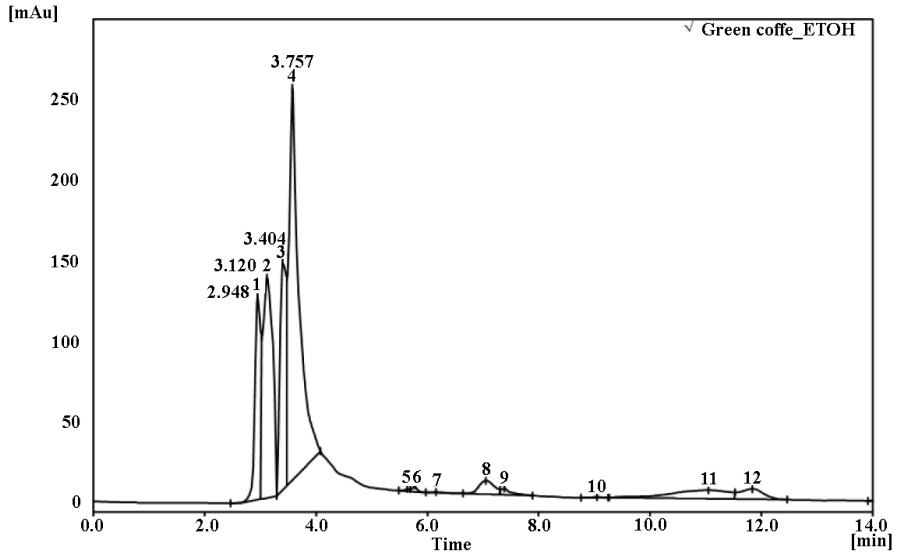

Figure 3: Chromatogram of EGCB.

Characterization of the Extracts

Extract analysis was done by HPLC- UV. Figures **1**, **2**, and **3** show the chromatogram of MGCB, AGCB, and EGCB extract measured at 274nm, 327nm, and 330nm wavelength, respectively.

The chromatogram of MGCB extract is shown in Figure **1**. It can be observed that four CGA isomers have been identified, including 5 Caffeoylquinic acids (5 CQA) peak at 3.063min, 3 Caffeoylquinic acid (3 CQA) at 2.617min, 4 Caffeoylquinic acids (4 CQA) at 5.546 min and 5 Feruloyilquinic acids (5 FQA) at 6.413min [16]**.** The concentration of these isomers can be calculated from their peak area [17]. 5 CQA concentration is .34mg/mL representing 55.86% of total concentration as calculated by total peak areas in Figure **1**. 3 CQA concentration is .21mg/mL, representing 35.85% of total concentrations. 4 CQA and 5 FQA concentration, which is 0.007mg/mL and .01mg/mL representing 1.232% and 1.80% of total concentration.

Figure **2** shows the chromatogram of AGCB extract, and the peak has been identified at 3.232 minutes. The concentration of caffeine is .46mg/mL, calculated by peak area, which represents 47.01% of total concentration as calculated by total peak area in Figure **2**. Figure **3** represents the chromatogram of the EGCB extract. A total of 12 overlapping peaks have been observed, out of which seven are significant. The chromatogram shows the presence of bioactive phytoconstituents in green coffee, including polyphenols, alkaloids, and diterpenes. The highest concentration was seen in peak at 3.575 minutes representing 39.71% of total concentrations of Figure **3**, followed by a peak at 3.120 minutes, representing

21.39%. The peak at 3.404 minutes and 2.948 minutes represents 14.75% and 12.21% concentration, respectively.

Antibacterial Activity against Periodontogenic Bacteria

In the present study, the antibacterial activity of EGCB, MGCB, and AGCB extract against periodontogenic bacteria, including *Porphyromonas gingivalis* (anaerobic gram-negative) and *Streptococcus mutans (anaerobic gram-positive)* at the various concentration (10mg/ mL, 15mg/ mL, 20mg/ mL) were depicted in Table **1**. The results showed that all extracts (10mg/ mL, 15mg/ mL, 20mg/ mL) showed a significant zone of inhibition, while negative control indicated no zone of inhibition. According to Clinical Laboratory Standards Institute [18], the criteria for antibacterial activity are as follows: inhibition zone diameter of 10 mm or less categorized as weak, 10-20 mm categorized as a medium antibacterial activity, and 20 mm inhibition zone or more categorized as strong activity. AGCB extract has been found to be the least effective against both bacteria. The result of our study revealed that MGCB extract exhibits the highest inhibitory activity against periodontogenic bacteria**,** followed by EGCB and AGCB extract. The antibacterial activity of AGCB extract was weak, with a zone of inhibition of 11.2mm at 10mg/ mL. It showed medium activity at 15mg/ mL and 20mg/ mL concentration with a zone of inhibition at 14.96 mm and 18.13 mm, respectively, against *P. gingivalis*. However, extract of EGCB and MGCB exhibit medium antibacterial activity at lower concentrations of 10mg/ mL and 15mg/ mL and was found to have strong activity at 20mg/ mL, which is significantly higher than the positive control.

Similarly, against *S. mutans*, MGCB extract exhibits the highest inhibitory activity (24.73mm), followed by EGCB (23.13mm) at 20mg/ mL concentration. With decreasing the concentration, antibacterial activity was decreased and found to be resistant below 5mg/ mL concentration. The result of our study revealed that the antibacterial activity of AGCB extract was weak with a zone of inhibition of 9.6mm at 10mg/ mL and showed medium activity at 15mg/ mL and 20mg/ mL concentration with a zone of inhibition of 18.86 mm and 19.9 mm respectively.

Antibacterial Activity against Nosocomial Pathogens

The antibacterial activity of EGCB, MGCB, AGCB extracts against Nosocomial bacteria include *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis,* at various concentrations (10 mg/mL, 15 mg/mL, 20 mg/mL) were represented in Table **2**. All extracts (10 mg/mL, 15 mg/mL, 20 mg/mL) showed a significant zone of inhibition against *Enterococcus faecalis*, while these extracts were found less effective against *Staphylococcus aureus* and *Staphylococcus epidermidis*. EGCB extract is found to be most effective against *E. faecalis* and showed a strong inhibition zone at 20 mg/mL (26.06 mm), followed by MGCB (24.93 mm) and AGCB extract (20.23mm). The extracts are found to be least effective against *S. aureus* and *S. epidermidis*. No inhibition zone was observed at 10mg/mL concentration, and medium inhibitory activity was observed at 15mg/mL and 20mg/mL. *S. aureus*, EGCB is most effective against *S. aureus* (19.86mm), followed by MGCB (18.56mm) and AGCB (18.25mm); however, no significant differences were observed between MGCB and AGCB extract activity. EGCB extract showed the

Table 1: Antibacterial Activity of Different Extracts against Periodontogenic Bacteria

Bacteria	Concentration	Inhibition zone [mm]		
		EGCB	MGCB	AGCB
Porphyromonas gingivalis	Streptomycin [20µg/mL]	23.6±.05^{ap}	23.43±.11^{aq}	23.43±.15^{ap}
	10mg/mL	14.93±.11^{br}	17.4±.12as	11.2±.06^{cs}
	15mg/mL	16.96±.06^{bq}	18.76±.25^{ar}	14.96±.57^{cr}
	20mg/mL	24.13±.21^{bp}	24.86±.16^{ap}	18.13±.12^{cq}
Streptococcus mutans	Amoxicillin [20µg/mL]	20.2±.26^{aq}	20.36±.55^{aq}	19.96±.05^{ap}
	10mg/mL	12.4±.34^{bs}	16.03±.05as	9.6±.36^{cr}
	15mg/mL	15.5±.50^{br}	17.93±.12^{ar}	18.86±.11^{aq}
	20mg/mL	23.13±.23^{bp}	24.73±.25^{ap}	19.9±.10^{cp}

Mean values with the similar letter in the same row (a,b,c) or in the same column (p,q,r,s) for a specific extract and concentration do not differ significantly by the Tukey's test at $p<0.05$.

Table 2: Antibacterial Activity of Different Extracts against Nosocomial Bacteria

Bacteria	Concentration	Inhibition zone [mm]		
		EGCB	MGCB	AGCB
Enterococcus faecalis	Rifampicin [20µg/mL]	29.99±.01^{ap}	30±.05^{ap}	29.96±.05^{ap}
	10mg/mL	16.06±.12^{bs}	17.03±.05as	14.66±.28^{cs}
	15mg/mL	20.03±.05^{ar}	19.3±.1^{br}	19.23±.40^{br}
	20mg/mL	26.06±.11^{aq}	24.93±.12^{bq}	20.23±.41^{cq}
Staphylococcus aureus	Tetracycline [20µg/mL]	30.83±.57^{ap}	30.66±.28^{ap}	30.8±.20^{ap}
	10mg/mL	Nil	Nil	Nil
	15mg/mL	20.03±.05^{aq}	19.43±.40^{bq}	19.21±.1^{bq}
	20mg/mL	19.86±.21^{aq}	18.56±.11^{br}	18.25±.50^{br}
Staphylococcus epidermidis	Cefuroxime [20µg/mL]	26.9±.11^{ap}	26.36±.15^{ap}	26.06±.05^{ap}
	10mg/mL	Nil	Nil	Nil
	15mg/mL	17±.21^{ar}	16±.05^{br}	15.96±.05^{br}
	20mg/mL	19.06±.11^{aq}	19±.02^{aq}	18.5±.05^{aq}

Mean values with the similar letter in the same row (a,b,c) or in the same column (p,q,r,s) for a specific extract and concentration do not differ significantly by the Tukey's test at $p<0.05$.

highest inhibition zone, 19.06mm followed by MGCB (18.5mm) and AGCB (19mm) at 20mg/mL against *S. epidermidis*. The inhibition activity of EGCB was non significantly different from the activity of MGCB at a higher concentration of 20mg/mL.

DISCUSSION

Plant extract with therapeutic benefits is an alternative treatment for various diseases or infections. Along with its bitter taste and smell and stimulating effect, coffee is one of the few therapeutic agents that have evidenced its medicinal properties [19]. The antibacterial effect of coffee is one of the medicinal properties evidenced by research, although the studies are limited. Therefore, in the present work antibacterial activity of coffee is evaluated in different extracts, including ethanol (EGCB) methanol and aqueous extract (AGCB). Coffee is the most popular beverage and contains a wide variety of bioactive compounds. It is well-documented that solvents play an essential role in the extraction of bioactive compounds. Previous studies reported several bioactive compounds in coffee, majorly phenolic compounds like chlorogenic acid and its derivatives, methylxanthines such as caffeine, trigonelline, theobromine, and theophylline, and diterpenes including cafestol and kahweol [20].

Further, in the study, extracted compounds are quantified by HPLC and reported 3 CQA, 4 CQA, and 5 CQA as the significant chlorogenic acids in green coffee beans. Our results have the same opinion as Clifford's [21]. The chlorogenic acid content in green coffee beans depends upon genetics, maturation, soil, agricultural practices, and climate [22], and caffeine concentration in coffee beans mainly depends on species [20].

Enterococci have arisen as significant nosocomial microorganisms second to Staphylococci which are the primary source of nosocomial diseases worldwide [23]. The two bacterial species, *S. epidermidis and E. faecalis* have not been tested extensively. Our results showed similarities with Runtime *et al*. [24] in that whole coffee extract showed an antibacterial effect against all three nosocomial pathogens, *i.e*., *S. epidermidis, S. aureus, and E. faecalis. S. epidermidis* is a regular member of the human microbiota, usually found on the skin and mucous layer. It is the most common cause of nosocomial infection and *S. aureus* [25]. Due to its ability to form a biofilm with foreign bodies, *S. epidermidis* emerged as an opportunistic pathogen in patients on medical devices [26, 27]. Among *Enterococci* infections, nearly 60% of infections are caused by *E. faecalis* [23]. The most commonly caused infections are urinary tract infections and intra-abdominal and pelvic infections associated with instruments [28].

Caffeine is not only the active component of coffee responsible for its antibacterial properties, but other components of coffee also contribute to its antibacterial

properties [24]. The bacterial cell wall composition of gram-positive and gram-negative bacteria is different, as the outer membrane of gram-negative bacteria consists of lipopolysaccharide, which makes bacteria more resistant to antibacterial agents [29]. Inouye *et al*.; [30] concluded that gram-positive bacteria are more susceptible to polyphenols as gram-negative bacteria are more resistant to plants' secondary metabolites because their bacterial cell wall is linked to an outer membrane complex, and it decreases the chemical channel. Our findings agree with the observation of Martinez-Tome [31] that the inhibition zone of bacterial growth increases as concentration increases. Our study observed a strong inhibition zone in MGCB extract against gram-negative periodontogenic bacteria *P. gingivalis*. This supports Quiroz *et al.* [32] findings that medium chlorogenic acid concentration has high susceptibility to gram-negative bacteria. The reason behind this could be that the outer membrane of bacteria can be altered by some polyphenols [33, 34]. However, the specific mechanism for its antimicrobial activity is still being determined, as there may be several targets in the cell [35]. The change in cell membrane permeability or modification in intracellular function due to the hydrogen bonding of phenols to enzymes enhances the tendency of phenols to bind with lipids which aids its collaboration with the cell membrane leading to an increase in antimicrobial activity. It can induce irreparable harm to the cytoplasmic membrane, and even thickening in cell content can cause inhibition of intracellular enzymes [36]. AGCB extract showed weak antibacterial activity than MGCB and EGCB extract. The weak antibacterial activity of AGCB could be because caffeine is its dominant component, and caffeine can pass the bacterial cell wall and start inhibiting DNA synthesis; this decreases bacterial cell activity and prevents protein and enzyme synthesis. Nevertheless, before oxidation, bacterial enzyme demethylases convert caffeine to theobromine and paraxanthine, leading to a decrease in antibacterial activity [37].

CONCLUSION

In this study, we reported the antibacterial properties of the green coffee extract against periodontal and nosocomial bacteria. The results are evidence that the antibacterial property of green coffee is mainly due to its polyphenolic compound, especially chlorogenic acid, and caffeine is the least effective antimicrobial agent in coffee The obtained results make green coffee a potential therapeutic agent for dental caries. They can be used as an antimicrobial agent in different biotechnological applications. Effectiveness of green coffee against periodontal bacteria viz. *Porphyromonas gingivalis* and *Streptococcus mutant,* promising its uses as an alternative to mouthwash. Selected nosocomial pathogens viz. *Staphylococcus aureus, Staphylococcus epidermidis*, and *Enterococcus faecalis* are mainly transmitted by hand, considering this green coffee can be used as a component in hand wash preparation.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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