Research Article

Determining the antibacterial efficacy of Amoxicillin-Clavulanic acid (Augmentin®), Metronidazole and Ethanolic extract of *Moringa oleifera L.* against clinically isolated *Porphyromonas gingivalis* (in vitro study)

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Abstract: Background: Amoxicillin-clavulanic acid (Augmentin®), Metronidazole, Amoxil/Metro combination, and Azithromycin are commonly utilized as adjunctive treatment of periodontitis. Moringa oleifera L (Mo) leaves extracts have demonstrated the ability to inhibit the growth of facultative anaerobic bacteria including *Porphyromonas. gingivalis (P. gingivalis), Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Shigella sonnei, Staphylococcus aureus, Bacillus subtilis, Beta-hemolytic streptococcus, Bacillus megaterium* as well as dental biofilm development. Objectives: The present study was set out to determine the antibacterial efficacy of Amoxicillin-clavulanic acid (Augmentin®), Metronidazole and Ethanolic extract of Mo leaves against *P. gingivalis* clinical isolate. Materials and Methods: The minimum inhibition concentrations (MIC), minimum bactericidal concentrations (MBC), and sensitivity of the clinical isolated *P. gingivalis* against Mo ethanolic leaves extracts, Augmentin, and Metronidazole were determined using two-fold serial dilution, agar well diffusion, and disk diffusion, respectively. The gas chromatography–mass spectrometry (GC-MS) was used for phytochemical analysis of the Mo leaves extract using ethanol solvent. Results: *P. gingivalis* exhibited inhibition to Mo leaves extract, while it demonstrated resistance to Augmentin and Metronidazole. MIC and MBC of Mo leaves extract were found to be 0.78 mg/ml and 1.56 mg/ml against *P. gingivalis,* respectively. The extract had highest anti-biofilm effect at the minimum concentrations of 1.55 mg/ml and 0.78 mg/ml equivalent to 0.06% Chlorhexidine compared to its lowest conc at 0.34 mg/ml, and this activity was inversely proportional to the extract's concentration. Conclusion: While Augmentin and Metronidazole showed no sensitivity against *P. gingivalis*, the promising antibacterial effect of Mo leaves extract against this bacterium may indicates its potential for use in oral health promotion, suggesting further *in vivo* studies to validate Mo extracts as alternative adjuncts in periodontitis therapy.

Keywords: Augmentin, Metronidazole, *Moringa oleifera L, P. gingivalis.*

Introduction

Periodontal disease is a multifactorial infection with multiple contributing factors and a diverse array of microbes causing devastating influence on the teeth-supporting tissues [(1)]. It begins as reversible inflammation of the gingiva, and if left untreated, it can result in irreversible deterioration of the supportive tooth-related tissues and eventually tooth loss. Extensive research over the years have revealed that only a small proportion of microorganisms in the sub-gingival environment play a fundamental role to initiate and advancing the deterioration of the periodontal health [(1)].

Porphyromonas gingivalis (P. gingivalis), a major virulent dysbiotic pathogen, is a key bacterium that significantly contributes to disruption of the entire micro-environment of periodontal ecosystem through switching the bacterial profile from symbiotic to dysbiotic one [1]. Possessing an extensive arsenal of virulence factors empower this bacterium to invade and destroy the gingival tissues through both direct bacterial engagement and indirect host modulation at the target sites. Subverting innate immune response including manipulating complement-Toll-like receptors (TLRs) crosstalk represents one of the important mechanisms that *P. gingivalis* induce a destructive influence in the homeostatic equilibrium with the host through altering the entire microbial growth and development [1]. In addition, *P. gingivalis* has shown to manipulate T cell development by promoting Th17 cells- mediated inflammatory response while downregulating Th1-dependent cell mediated effect, that enhance immune-mediated elimination of *P. gingivalis* [2]. However, the disruption of this host-microbial homeostasis can also be resulted from several congenital, epigentic modification to environmental triggers, environmental factors such as stress, dietary intake, and smoking, immunoregulatory/deficiency defects, and systemic diseases including diabetes, which shown as alone or in combination, can enhance the breakdown of homeostatic control [3].

The treatment of periodontal disease has traditionally involved professional mechanical plaque removal, along with, in certain situations, using adjunctive antibiotic medications and antiseptic mouthwashes [4]. However, an effective complementary systemic antimicrobial therapy has the potential to significantly suppress or eliminate persistent subgingival-associated periodontal pathogens [5]. Nevertheless, if the targeted pathogens exhibit resistance to these antibiotics, there is a risk of continuous positive feedback loop of growing pathogen-dysbiotic environment, developing more severe forms of periodontitis. This could be explained through the survival of antibiotic-resistant bacteria if exist by expression of several antibiotic-resistant genes from horizontal transfer and phenotypic expression of these genes from other oral microorganisms [6], leading to continuous induction of pathogen-environmental crosstalk [2]. The administration of metronidazole and/or amoxicillin/clavulanic acid has demonstrated suppression of *P. gingivalis* and reduction in the numbers of other potential periodontal pathogens [7]. However, the frequent use of these medications can lead to undesirable side influences, including bacterial-drug resistance. Elevated antibiotic resistance of periodontitis-associated bacteria including *P. gingivalis* [8], makes the use of synthetic antimicrobial agents as effective adjuncts in the treatment of periodontal diseases questionable for their undesirable adverse effects including bacterial resistance. Thus, the concept of using alternative medicinal-based plants becomes extremely important as adjunctive treatment of periodontal diseases.

The use of plants for therapeutic properties is an old concept which garnered significant interest recently. Plant-based therapies have been shown to deliver a variety of biological actions alongside safe application favored their use over traditional synthetic medications. Owing to their extensive antibacterial, anti-inflammatory, and antioxidant characteristics, plant-derived alternatives may serve as a stable, safe, and enriched bioactive substitute to synthetic medications [9]. Moreover, plant-based therapies offer advantages in terms of availability, easily accessible and more economical compared to conventional antibiotics since they do not necessitate complex or costly chemical and pharmaceutical processes. Conversely, the development of new antibiotics involves a highly intricate and prolonged process that is susceptible to challenges and substantial expenses, for example, developing new antibiotics is resource-intensive, often requiring 10–15 years and over a billion dollars [10].

An example of such medicinal herbs is the leaves of Moringa oleifera L (Mo), a member of the Moringaceae family. These leaves have been utilized due to their antioxidant, anti-inflammatory, and

antimicrobial properties. Initial epidemiological studies indicate that it exhibits antimicrobial effects against primary colonizers of dental biofilm, including *Streptococcus mutans, Streptococcus salivarius, Streptococcus mitis, and Streptococcus anginosus* [11]. The antimicrobial efficacy is attributed to the existence of tannins, fatty acids, alkaloids, polyphenols and other active ingredients.

Despite numerous investigations reported the antibacterial effects of Mo against both primary and secondary colonizers, the antimicrobial impact of Mo leaves ethanolic extract compared to the use of metronidazole and amoxicillin/clavulanic acid against specific key anaerobic bacterium such as *P. gingivalis* has not been well demonstrated. Thus, we hypothesized that M. oleifera ethanolic extract would demonstrate superior antibacterial activity against *P. gingivalis* compared to conventional antibiotics.

Materials and Methods

Study design and settings

This research was conducted under the approval of the ethics committee in the College of Dentistry, University of Baghdad (Reference: 256, dated 20/03/2021) (Supplementary 1). All participants were informed about the study's objectives, and their participation was voluntary, requiring the signing of a consent form (Supplementary 2).

Participants who had used antibiotics and received periodontal treatment in the past 3 months were excluded. Twenty patients diagnosed with stage 3 and 4 periodontitis [12], with clinical attachment loss > 5 mm and probing depth >6 mm, and willing to participate in the study were included for the collection of subgingival plaque samples for clinical isolation of *P. gingivalis*.

Subgingival biofilm sampling

The sampling procedure was conducted following supragingival biofilm removal using wiping with cotton wool pledgets to have access to the subgingival area for pocket measurement and subgingival plaque sampling. The subgingival pockets of the buccal surfaces of the molar teeth with depths exceeding 6 mm were sampled following isolation using cotton rolls. The identified periodontal pocket was then sampled using sterile paper point (F2 Dia-ProTTM), which was inserted till resistance was felt and maintained for 60 seconds [13]. The paper point was gently withdrawn and promptly inoculated onto *P. gingivalis* agar (ANAEROBE SYSTEMS, USA). The inoculated plates were incubated for 4-6 days at 36.5 C in an anaerobic environment (Thermo Scientific) in an anaerobic jar (Oxoid™ AnaeroJar).

Porphyromonas gingivalis clinical isolates verification

After conducting several sub-culturing of *P. gingivalis* isolates on the agar plates, validation was confirmed by colony morphology i.e. black pigmented colonies, Gram-staining, morphological identification by microscopic examination, and DNA analysis using polymerase chain reaction (PCR).

DNA Isolation: An Eppendorf tube having 50 µl of disinfected hyper deionized-distilled water (DDW) was mixed with an appropriate and confirmed colony of each sample, homogenized using vortex mixer, incubated in a heat block for 10 minutes at 96 C (Thermo Fisher Scientific), and centrifuged for 5 minutes at 1000 rpm. The DNA template was collected as a precipitate following discarding the supernatant layer [14].

PCR Analysis: A 16S rRNA gene of *P. gingivalis* was analyzed using *P. gingivalis*-specific primers by conventional PCR [15]. The primer sequences were as follows: Forward primer 5′-AGTCAGCTTGCCATACTGGC-3′ and reverse primer 5′-ACCGTTAGCAACTACCGATTG-3′. The amplification process was conducted in triplicate using the VeritiTM 96 well Thermocycler PCR. The final volume of 20 µl of reaction mixture was prepared, comprising: 1 µl of DDW, 2 µl (10 pmol/L)/each *P. gingivalis* primer, 5 µl of DNA template, 10 µl of 2X Prime Taq Premix (GeNet BioG-2000). The PCR cycleis illustrated as follows: an initial denaturation at 96 C for 5 minutes; amplification sequences as DNA denaturation at 95 C for 30 seconds, annealing at 61 C for 30 seconds, extension at 71 C for 30 seconds; and final extension at 71°C for 5 minutes. The PCR product was subsequently subjected to analysis via 2% agarose-gel electrophoresis, running for 36 minutes at 81 V. For gel staining, 3 µl of ethidium bromide was applied. To mark the molecular mass, a 100 bp + DNA ladder was applied. Purification of the bands present on the gel was carried out using the genomic JETTM Gel extraction kit (Fermentas, UK). Ultimately, the standard PCR template was sequenced at Macrogen in South Korea

Moringa oleifera L. Extraction

Ethanolic extracts of Mo leaves (Batch Number: 850703004221, PVT LTD, Coimbatore-641108, India) were obtained through a maceration process [16]. The dried leaves underwent crushing and blending, resulting in 100 grams of the extract disolved in 1 liter of distilled water, yielding a mixture with a proportion of 100 mg/ml. Filtering was employed to remove coarse plant material, utilizing muslin cloth to extract additional solvent from the exhausted plant matter. The ethanolic extract underwent centrifugation (K Centrifuge, PLC series) at 8000 rpm for 10 minutes, filtered using Whatman No. 1 filter paper (England). The refined extract was subsequently dried with a spray drier (Yenchen Machinery) to obtain powdered extract, which was stored in sterile containers in a refrigerator, ensuring its readiness for subsequent experiments.

Analysis of compounds of Mo Leaves extract

The constituents of the extracts was characterized using Gas Chromatography–Mass Spectrometry (GC/MS) analysis [17], (Agilent Technologies 7820A GC System, USA). Each extract was loaded as a 1 µl sample solution into the GC-MS system. A standard capillary column (30 mm x 0.25 mm x 0.25 µm film thickness) was utilized within the mass spectrometer. The column temperature initially held at 100°C for 6 minutes was gradually increased to 255 C at a rate of 10 C/min and maintained at this temperature for 10 minutes. Helium, at a continuous flow rate of 1 ml/min, was served as the carrier gas. The mass spectrometer operated in the electron impact (EI) ionization mode with parameters set at 1500 V for the ion source temperature 245 C and 150 C for the MS quad temperature. Mass spectral data were collected using the GC-MSD Agilent Chem Station Software. Identification of the extracted components was based on the comparison of the obtained MS values with the collection of the National Institute of Standards and Technology (NIST).

Agar well and disk diffusion test

The agar well diffusion method was employed to assess the antimicrobial sensitivity of *P. gingivalis* to the ethanolic extract of Mo leaves [18]. The inoculation of 50 µl of *P. gingivalis* suspension was inoculated using L-shaped loop on Mueller Hinton agar (MHA) plates and left for dryness. A sterile micropipette tip was punctured the MHA plate used to make holes (6 mm in diamenter). Subsequently, 100 µl of Mo ethanolic extract at concentrations 25, 50, 75, and 100 mg/ml was applied to the well holes [19]. The agar disk

diffusion was employed to assess the antibacterial efficacy of Metronidazole and Augmentin against *P. gingivalis*. Two antibiotic discs (HiMedia, India) of Augmentin (30 µg) [20] and metronidazole (5 µg) [21] were placed onto the surface of agar plates within 15 min of inoculation. 100 µl of sterilized distilled water (DW) and alcohol free 0.12% chlorhexidine (CHX, periokin) were used as negative and positive controls, respectively. All plates were then anaerobically incubated for 48 hours at 36.5°C. The diameter of the inhibition zones was assessed using vernier caliper and recorded as the average width of the inhibition zone surrounding each well containing the extracts solutions. An inhibition zone (IZ) measuring more than 8mm considers *P. gingivalis* as susceptible against used antibiotics, and subjected for further MIC and MBC testings, other wise, it considered resistant if it was less than 8mm IZ, and not underwent MIC and MBC analysis [22].

Determination of the minimum inhibitory concentration and the minimum bactericidal concentration of Mo leave extract

The Minimum Inhibitory Concentration (MIC) for ethanolic extract of Mo leaves was determined using a macro two-fold serial dilution method. In this process, each of the 10 labeled test tubes (T1-T10) was loaded with 900 µl of Mueller-Hinton broth (MHB). The first tube received 900 µl of the extract at a concentration of 50 mg/ml, and subsequent serial dilutions were made from the 1st to the 8th tube, leading to concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 mg/ml. The negative and positive controls consisted of MHB in T9, while T10 contained 900 µl of a 0.12% Chlorhexidine-MHB mixture, respectively. The *P. gingivalis* suspension was made by the following steps: The well-determined colonies of *P. gingivlais* growing on the agar were inoculated in 3 ml of MHB, containing 1 µg/ml of menadione and 5 µg/ml of hemin using sterile loops. The *P. gingivalis* suspension then adjusted to 5×10^5 CFU/ml concentration at 660 nm optical density adjusted to the 0.5 McFarland's turbidity standard [23]. To each dilution, the addition of 100 µl *P. gingivalis* suspension was conducted and ensured that the total volume per tube reached 1000 µl. The tubes were then incubated anaerobically for 48 hours at 36.5 C. Then, bacterial growth (turbidity) was assessed visually using a spectrophotometer (EMC LAB, Germany, v-1100 DIGITAL SPECTROPHOTOMETER) at 620 nm optical density. The MIC was defined as the lowest concentration of the extract showing no turbidity. For result validation, all antimicrobial evaluations of were conducted in triplicate at three different instances. Following MIC determination, 50 µl from tubes devoid of visible bacterial growth were cultured on MHA plates and incubated for 48 hours at 36.5 C within an anaerobic environment using an anaerobic gas pack within the anaerobic jar. For minimum bactericidal concentration (MBC) value, it was computed as the lowest dosage of ethanolic leaves extract at which either fewer than three colonies or no growth were observed. This outcome indicated a bactericidal activity ranging from 99 to 99.5%, signifying a substantial level of bacterial elimination [24].

Anti-Biofilm Assay of Moringa oleifera Leaf ethanolic Extract

The extract's anti-biofilm effect was detected using the tube adhesion method [25]. The serial dilutions of the test tubes were poured and rinsed for 1 min with phosphate-buffered solution at pH 7.3, and left to dry. Staining with 0.1% crystal violet were conducted with all tubes for 1 min and rinsing with distilled water to remove the remaining stain. All tubes were then kept for dryness in upside down position for 24 h. The biofilm formation was viewed visually as positive when an observable stain was lined the tube's wall and bottom. This stain was graded as none or weak (score=1 with strong anti-biofilm activity), moderate (score=2 with moderate anti-biofilm activity), and strong (score=3 with weak anti-biofilm

activity). All meaurments were conducted with inter-examiner callibartion at three different times in triplicate.

Statistical analysis

The antimicrobial sensitivity test results were initially assessed at a 5% significance level utilizing the Kruskal-Wallis test. Subsequently, at a significance level of 0.05, the Mann-Whitney U test was employed to assess differences between the groups. All these tests were conducted following checking the normality of the sample using Shapiro-Wilk test. GraphPad Prism 10.5.0 software was used for statistical analysis of all presented data

Results

Porphyromonas. gingivalis Verification

Black-pigmented bacterial isolates were detected in 60% (n=12) of the samples examined. After a 48-hour incubation period, the colonies exhibited a round, small, convex growth, and an opaque appearance. Following 5-7 days on lysed blood, black-pigmented colonies became visible, as illustrated in Figure 1A. Following DNA sequencing, it was determined that all examined colonies bore genetic resemblance to *P. gingivalis* strain ATCC 33277 as shown in Figure 1.B.

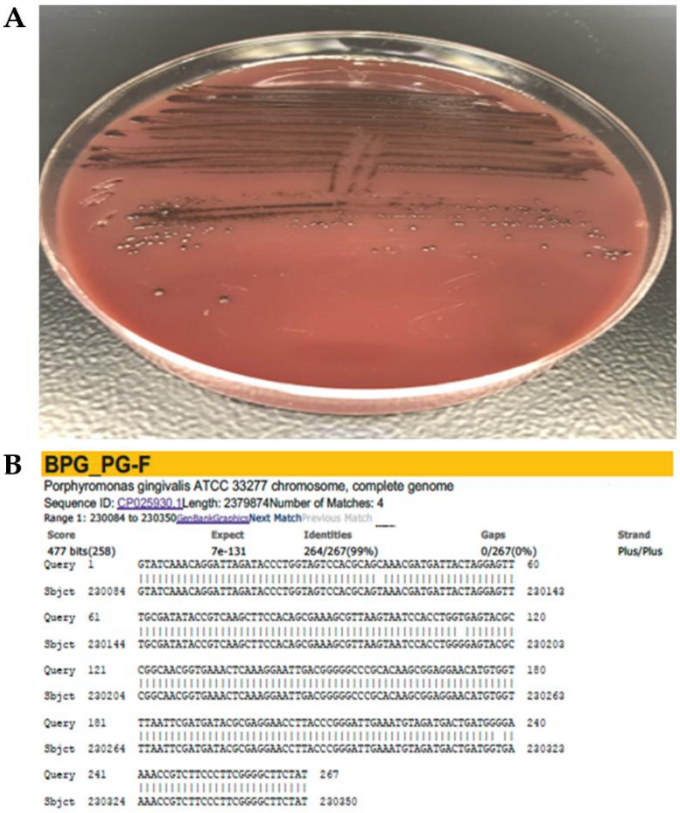

Figure 1: *P. gingivalis* verification: (A) Following a 5-7-day incubation period at 36.5 C in an anaerobic gas pack in an anaerobic jar. The colonies of *P. gingivalis* exhibited a black pigment, spherical shape, and a smooth, shiny appearance. (B) 16s RNA Sequnceing graph of *P. gingivalis.*

GC-MS analysis of Mo ethanolic leaves extracts

In the GC-MS analysis of Mo leaves, the primary constituents identified were Trans-11 octadecenoic acid and Oleic Acid (18.15%), with 1H,3H-Quinoline-2,5-dione, 1-4-fluorophenyl (16.28%) being the next major alkaloid components, as shown in Table 1 and Figure 2. However, the rest components following

GC-MS analysis showed minor components ranged from 0.79% of Oxirane, trimethyl-1,3-Dioxane, 4,5-dimethyl- to 4.75% of Ethanol, 2-bromo-N-(2- Methoxyethyl) alanine Triethyl borate.

Table 1: GC-MS profile of the Mo ethanolic Leaves extracts.

Peck	Retention time	Area %	Name
1	4.734	1.57	Adenosine, 4'-de(hydroxymethyl)-4-[N-ethylaminoformyl] - Acetamide, 2-(2-hydroxyethoxy)-m-Dioxan-4-ol, 2,6-dimethyl-
2	7.155	1.62	Adenosine, 4'-de(hydroxymethyl)-4' 2H-Pyran-2-one, tetrahydro-6,6-dimethyl-
3	9.143	0.79	Oxirane, trimethyl-1,3-Dioxane, 4,5-dimethyl-
4	11.207	16.28	2-Hexanol, 3-methyl- Ethanol, 2-[2-(2-methoxyethoxy) ethoxy]-, acetate Pentane, 1-methoxy-
5	11.938	2.20	1,3-Butanediol 1-Butanol, 4-methoxy-1,4,7,10,13,16-Hexaoxacyclooctadecane
6	12.337	1.36	Acetaldehyde, di-sec-butyl acetal Butyric acid, 2,3-dioxo-, 2-methyl oxime, ethyl ester
7	13.951	16.28	1H,3H-Quinoline-2,5-dione, 1-4-fluorophenyl -4H-Pyran-4-one, 2,3-dihydro-3,5-di hydroxy-6-methyl-
8	15.039	18.15	Imidazole, 2-fluoro-5-hydroxy-1-ri bofuranosyl- Trans-11-octadecenoic acid, Oleic Acid
9	18.615	0.93	(2,5-Dimethyl- [1,3] dioxan-4-yl)-methanol Acetic acid, hydroxy-, ethyl ester, Hydroperoxide, 1-methylpentyl
10	19.881	1.00	3-Methyl-oxiran-2-yl)-methanol, Butanamide, 3, N-dihydroxy-
11	20.900	4.75	Ethanol, 2-bromo- N-(2-Methoxyethyl) alanine, Triethyl borate
12	21.708	1.32	2-O-Mesyl arabinose (3-Methyl-oxiran-2-yl)-methanol, Imidazolidine, 1,2,3-triacetoxy-5-nitroimino-
13	22.098	1.99	1H-Pyrimidine-2,4-dione, 5-fluoro-1-(2-hydroxyethoxymethyl)- Hexadecanol

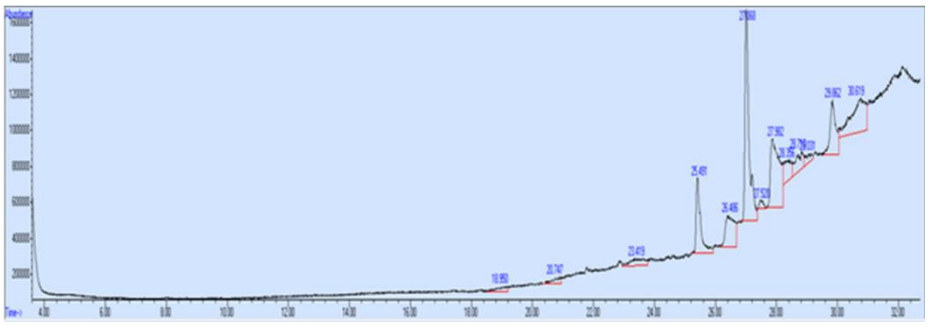

Figure 2: GC-MS chromatogram of Mo ethanolic Leaves extracts. Each extract was loaded as a 1 µl in a standard capillary column within the mass spectrometer at 100°C for 6 minutes, then gradually increased to 255 C at a rate of 10 C/min and maintained for 10 minutes. Helium was served as the carrier gas in flow rate of 1 ml/min. The mass spectrometer operated in the electron impact (EI) ionization mode with parameters set at 1500 V for the ion source temperature 245 C and 150 C for the MS quad temperature. Mass spectral data were collected using the GC-MSD Agilent Chem Station Software. Identification of the extracted components was based on the comparison of the obtained MS values with the collection of the National Institute of Standards and Technology (NIST).

Antimicrobial evaluation of antibiotics and Ethanolic Mo extract

The outcomes of the agar well diffusion test demonstrated that Mo leaves exhibited a dose-dependent antimicrobial effect across all examined concentrations. The smallest average inhibition zone measured 5 ±0.3 mm at a concentration of 25 mg/ml, while the largest inhibition zone, measuring 13±0.5 mm, was observed at a concentration of 100 mg/ml as showed in Figure 3A. In contrast, CHX 0.12% consistently exhibited the most substantial inhibition zone, measuring 18.2±0.2 mm, in comparison to Mo leaves. Nevertheless, no antibacterial activity was evident across concentrations of Augmentin (30 µg), while metronidazole (5 µg) showed 2 ±0.3 mm inhibition zone against *P. gingivalis* as showed in Table 2 and Figure 3B.

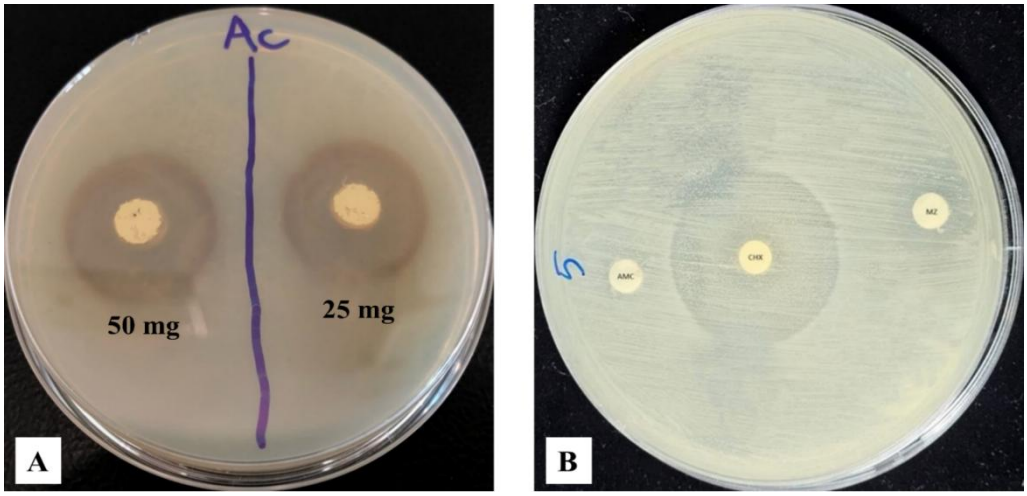

Figure 3: The inhibition zone around the antibiotics and extract was detected using Agar well and disk diffusion test. (A) Mo Leaves showed inhibition zone at concentrations 25 and 50 mg/ml to *P. gingivalis*; (B) There is no inhibition zone around 30 µg Augmentin (AMC) and 5 µg Metronidazole (MZ) disks which indicate no sensitivity to *P. gingivalis* while 0.12% Chlorohexidine (CHX) was sensitive. All plates were then anaerobically incubated for 48 hours at 36.5°C. The diameter of the inhibition zones was assessed using vernier caliper and recorded as the average width of the inhibition zone surrounding each well containing the extracts solutions

Table 2: Sensitivity of *P. gingivalis* to different concentrations of Mo ethanolic Leaves extract

Mo Ethanolic Leaves Extract/CHX/Antibiotics	Inhibition zone (mm)	Standard deviation	*P*-value
CHX (0.12%)	18	0.284* ^	
Leaves 100 mg	13	0.521* ^	
Leaves 75 mg	10	0.639* ^	0.000
Leaves 50 mg	10	0.258* ^	
Leaves 25 mg	5	0.329* ^	
Metronidazole (5 µg)	2	0.347* ^	
Augmentin (30 µg)	Resistant	Null	

CHX: Chlorhexidine; *Comparison to CHX by Kruskal, significance at $P< 0.05$. ^Comparison

As there were no and barely detectable antibacterial sensitivity of Augmentin and Metronidazole against *P. gingivalis* in disk diffusion method, only the MIC values of the ethanolic extract from Mo leaves against *P. gingivalis* were determined through serial macro dilutions, assessed by optical density (OD). The lowest concentration of Mo extract exhibiting antibacterial efficacy against *P. gingivalis* was 0.78 mg/ml (OD=0.025). Furthermore, the extract exhibited minimal bactericidal effect at concentration 1.55 mg/ml compared to CHX at concentration 0.06 mg/ml (OD=0.014).

Anti-biofilm assay of Mo leaf ethanolic extracts

The anti-biofilm influence of the ethanolic extract of Mo leaf revealed significant highest anti-biofilm activity at a concentration of 1.55 mg/ml (MBC value), which was equivalent to 0.06% CHX compared to other Mo leaf extract's concentrations (P <0.0001). This had a similar effect at a concentration of 0.78 mg/ml which represent the MIC value, followed by lowest anti-biofilm activity at 0.34 mg/ml, as shown in Figure 4. Interestingly, the anti-biofilm score of Mo leaf extract was increased with a decrease in extract concentrations. The experiment was conducted in triplicate at three different times.

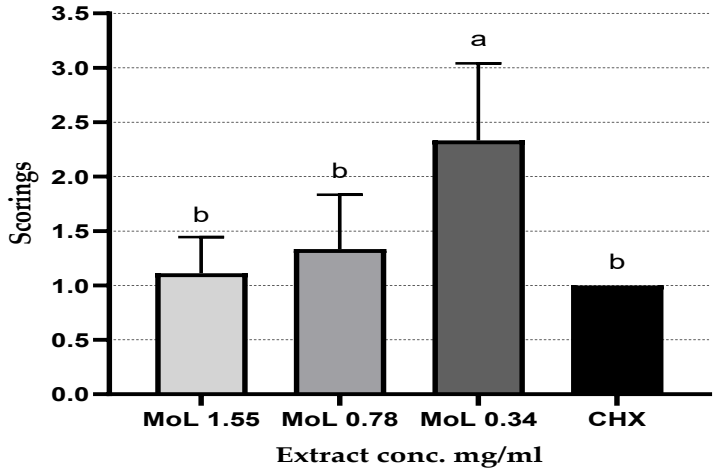

Figure 4: Anti-biofilm effect of Mo Leaf ethanolic extract concentrations compared to 0.06% chlorhexidine was detected using the tube adhesion method. Serial dilutions of the test tubes were poured and rinsed for 1 min with phosphate-buffered solution at pH 7.3, and left to dry. Staining with 0.1% crystal violet were conducted with all tubes for 1 min and rinsing with distilled water to remove the remaining stain. Antibiofilm activity was viewed in term of stain formation score. Highest antibiofilm effect was viewed with no/weak stain and vise versa. Score 1=absent or weak stain; 2=moderate stain; 3=strong. MoL: Mo leaf, CHX: chlorhexidine 0.06% used as a positive control. The experiment was conducted in triplicate at three different times. Significance at $P< 0.05$ and shared letter show non-significant difference.

Discussion

The main objective in treating periodontitis is to produce a healthy periodontal environment by promoting health-associated bacteria which results in an equilibrium between the host defense system and the oral miroflora [(26)]. Virulent pathogenic bacteria are commonly resid in deep periodontal lesions. These areas are effectively treated by traditional non-surgical and surgical treatments, with necessitating the use of adjunctive systemic anti-bacterial therapy [(4)]. *P. gingivalis*, recognized as a critical pathogen in periodontal health, is documented to exert a significant influence triggering the onset and advancement of periodontitis by switching the subgingival microflora from being symbiotic to dysbiotic one,

deteriorating the entire sub-gingival microbial ecosystems. Focusing on this pathogen could disrupt various pathways and interactions associated with the progression of periodontal disease, including direct bacterial interaction and manipulation or evasion of the host's immune response [1].

Sampling of subgingival biofilm using paper point was widely used for isolation and counting of subgingival microbiome compared to other methods include curette sampling. Although curette instrument has shown to harvest significantly more bacteria as it collects and penetrate the apical part of subgingival biofilm in both dental and periodontal tissue parts, the composition of different periodontal pathogens including *P. gingivalis* as collected by paper point was found to be similar [27], or even higher [28], compared to curette sampling, indicating that both are suitable for routine microbiological diagnostics. However, the invasive and traumatic nature of using curettes can exerts sense of patient discomfort compared to easy non-invasive use of paper point in subgingival biofilm sampling. For all these reasons, paper point technique was used for subgingival biofilm sampling.

Moringa oleifera L. Extraction

Maceration has been extensively employed over other methods because of its simplicity, cost-effectiveness, and compatibility with heat-sensitive compounds [16]. Ethanolic-based extraction of natural products considered effective due to its wide solubility spectrum as an uncharged solvent, it can extract a diverse range of compounds, encompassing both water-soluble hydrophilic constituents and fat-soluble lipophilic substances. Its antimicrobial activity helps minimize contamination hazards and maintain the integrity of the extracts. Ethanolic derivatives are comparatively safe and inexpensive, evaporating rapidly post-extraction to simplify concentration procedures [29]. Moringa oleifera leaf ethanolic extracts contain considerable significant concentrations of polyphenolic compounds and flavonoids, exerts potent antioxidant activity, and greater antibacterial effect against number of anaerobic bacteria that showed significant sensitivity towards the ethanolic compared to aqueous extract [30].

Analysis of Mo Leaves extract components

Among analytical platforms used in metabolomics, GC/MS is frequently preferred owing to its cost-efficiency, robust reproducibility, operational stability, and simplified data analysis. GC/MS considered as a crucial analytical tool for both qualitative and quantitative determination of contaminants and residues in food. It offers rapid and sensitive analysis, high peak resolution, and enables the identification of thermally stable and volatile and nonvolatile compounds [17]. For these characteristics, GC/MS was used to explore Mo Leaves extract components.

Determination of the antimicrobial sensitivity, MIC and MBC of Mo leave extract.

The bacterial sensitivity against Mo and the tested antibiotics was determined using disk-diffusion method. This method presents numerous advantages including simplicity, cost-effectiveness, capacity to evaluate a wide range of microorganisms and antimicrobial compounds, and the straightforward interpretation of outcomes [31]. The MIC of the ethanolic extract of Mo leaves was determined using a macro two-fold serial dilution. According to EUCAST and CLSI, the most reliable, precise, and recommended method to determine the MIC breakpoints of any antimicrobial agent against the pathogen of interest is the broth microdilution method. The two-fold serial dilution step provides more precise MIC values compared to methods with larger dilution factors (e.g., 10-fold dilutions), allowing for a finer resolution of the effective concentration [32]

Comparison with Synthetic Antibiotics

The utilization of plant-based derivatives is a longstanding therapeutic concept that used to be a common healthcare remedy in many developing countries where access to contemporary healthcare is limited. Moreover, the documented use of these medicinal plants in treating various health conditions, specifically oral and periodontal diseases, is noteworthy [10]. The diverse array of anti-inflammatory, antioxidant, antibacterial and other properties associated with herbal treatment suggests their potential as an alternative that offer stability, safety, and bioactive effects in lieu of synthetic medications. The use of commercial antibiotics including Augmentin and Metronidazole over several decades has resulted in increased bacterial resistance [33]. Augmentin and Metronidazole are commonly used as adjunctive treatment of periodontal diseases in specific clinical categories such as generalized periodontitis Stage III in young adults [4]. Due to the side effects and emerging of bacterial resistance towards antibiotics, it would be helpful to use alternative herbal medicinal approach to treat periodontal disease. While numerous *in vitro* and *in vivo* studies have explored the application of Mo extracts against both primary and secondary colonizers, scarcity of data concerning the antibacterial influences of these plant extracts; in particular, against anaerobic bacteria including *P. gingivalis* is established. Therefore, the objective of this research was to evaluate the antibacterial effects of Mo ethanolic leaves compared to Augmentin and Metronidazole against *P. gingivalis*. There were no reported sensitivity reactions of Augmentin and Metronidazole against all clinically isolated *P. gingivalis* strains which was agreed with the previous findings [34]. In this context, it is crucial to highlight that recent findings indicate *P. gingivalis* has the capability to transfer plasmid DNA, chromosomal DNA, or a combination of both. This capability could potentially serve as a functional system for the transfer of resistance determinants as well [35].

Role of Phytochemicals

MIC values of Mo ethanolic leaves extracts against *P. gingivalis* were determined through optical density (OD) measurements using serial macro dilutions. In the current study, it was observed that ethanolic Mo leaves extract exhibited antibacterial effects at an MIC of 0.78 mg/ml and MBC at 1.55 mg/ml. Moreover, Mo leaves have demonstrated increased components of Trans-11-octadecenoic acid, Oleic Acid (18.15), and 1H,3H-Quinoline-2,5-dione, 1-4-fluorophenyl (16.28%) as showed in Table 1. Each of these components has been shown to exert anti-bacterial effects against *P. gingivalis* in vitro [36]. The observed antibacterial effect can be attributed to the influence of phenolic ingredients and fatty acids, particularly oleic acid effect. These components have shown to induce alterations in phospholipid composition, causing destruction of the cell membrane, and ultimately leading to cell lysis [37]. Furthermore, it was discovered that oleic acid inhibits the synthesis of hemagglutinin, which may limit the adherence ability and subsequent colonization of *P. gingivalis* at the target site [38].

Biofilm Inhibition

A polymicrobial consortia merits to live in a biofilm over the planktonic state. Inspite of the advantages attributed to biofilm formation including bacterial synergism, antibiotic resistance, nutrient exchange, and neutralizing harmful molecules [39], Bacterial need for stable and firm colonization in this mode of living remains challenging. This mediated through firm adherence on the colonizing hard and soft surfaces, including teeth and soft tissues. Accordingly, the biological effect of the ethanolic extract of Mo leaf was investigated by evaluating the anti-biofilm activity of the ethanolic Mo L. leaf extracts using tube adhesion method. The same tubes following determining the MIC of these extracts were used. Highest anti-biofilm activity was observed with the MBC tube value =1.55 mg/ml, score=1 (1.11±0.33) followed by MIC tube value=0.78 mg/ml, very close to score=1 (1.33±0.5), while the minimum activity was related to

Mo leaf conc. =0.34 mg/ml, score=2 (2.33±0.7). The trend of these results was supported by our previous investigation using Mo leaf aqueous extract although using different extract solvent [13]. However, these anti-biofilm effects were shown to be increased with decreasing their concentrations. This may be attributed to the activity of the bioactive compounds that may be attenuated when decreasing their concentrations against bacterial adherence and survival, resulting in changing the whole biofilm formation and vise-versa.

Limitations and Future Work

One limitation of this study is that some biologically active components of Mo leaves may not be detected by GC-MS analysis. This could be brought by the probable chance of being not detected by the GC-MS itself due to its sensitivity of detection, although they were extracted or no chance to be extracted using the involved solvent. However, using other methods for phytochemical screening in future studies including high-performance liquid chromatography (HPLC) is suggested for detecting ingredients that can't decompose at high temperatures or subjected to vaporization.

Conclusion

Augmentin and Metronidazole exhibited no sensitivity against *P. gingivalis*, suggesting its resistance against these widely used medications in treating periodontitis. In contrast, Mo leaves ethanolic extract demonstrated a promising antibacterial effect against clinical *P. gingivalis* isolates. However, further characterizations of Mo leaves such as cytotoxicity test needs to be explored. These findings may provide valuable insights for developing herbal medicine-related products as alternatives to regional antibiotic overuse in the treatment of periodontal diseases. In addition, the potential to translate these derivatives in oral care formulations at preclinical and clinical levels would be greatly encouraged.

Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

FBT; study conception and design. AFM; data collection. FBT and AFM; statistical analysis and interpretation of results. FBT, AFM and OHS; Writing - review & editing. Supervision; FBT and OHS. All authors reviewed the results and approved the final version of the manuscript to be published.

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Informed consent

Informed consent was obtained from all individuals or their guardians included in this study.

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تحديد الفعالية المضادة للبكتيريا لحمض الأموكسيسيلين-كلافولانيك (أوغمنتين®) والميترونيدازول والمستخلص الإيثانولي من المورينجا أوليفيرا ل. ضد البكتيريا المعزولة سريريًا. (دراسة مختبرية)

فراس بشير هاشم الطويل، علي فيصل مظلوم، عمر حسن سليمان

المستخلص

الخلفية: يُستخدم حمض الأموكسيسيلين-كلافولانيك (أوغمنتين®)، وميترونيدازول، ومزيج الأموكسيل/مترو، والأزيثروميسين بشكل شائع كعلاج مساعد لالتهاب دواعم السن. وقد أظهرت مستخلصات أوراق المورينجا أوليفيرا (L) قدرتها على تثبيط نمو البكتيريا اللاهوائية الاختيارية، بما في ذلك المتصورة اللثوية، والإشريكية القولونية، والزائفة الزنجارية، والبواسير الشمعية، والسالمونيلا سوني، والمكورات العنقودية الذهبية، والبواسير الرقيقة، والسالمونيلا الصفراء، والسالمونيلا الحالة للدم، والبواسير ميجاتيريوم، بالإضافة إلى تثبيط نمو الأغشية الحيوية السنية. الأهداف: أُجريت هذه الدراسة لتحديد الفعالية المضادة للبكتيريا لحمض الأموكسيسيلين-كلافولانيك (أوغمنتين®)، والميترونيدازول، والمستخلص الإيثانولي لأوراق المورينجا ضد العزلة السريرية من المتصورة اللثوية. المواد والطرق: حُددت أدنى تركيزات تثبيط (MIC) وأدنى تركيزات قاتلة للبكتيريا (MBC) وحساسية بكتيريا *P. gingivalis* المعزولة سريريًا ضد مستخلصات أوراق المولبيدينوم الإيثانولية، والأوجمنتين، والميترونيدازول، باستخدام التخفيف التسلسلي المزدوج، وانتشار بئر الأجار، وانتشار القرص، على التوالي. استُخدمت تقنية كروماتوغرافيا الغاز-مطياف الكتلة (GC-MS) للتحليل الكيميائي النباتي لمستخلص أوراق المولبيدينوم باستخدام مذيب الإيثانول. النتائج: أظهرت بكتيريا *P. gingivalis* تثبيطًا لمستخلص أوراق المولبيدينوم، بينما أظهرت مقاومةً للأوجمنتين والميترونيدازول. وُجد أن أدنى تركيز تثبيطي (MIC) وأدنى تركيز قاتل للبكتيريا لمستخلص أوراق المولبيدينوم كانا 0.78 ملغم/مل و1.56 ملغم/مل ضد *P. gingivalis*، على التوالي. أظهر المستخلص أعلى تأثير مضاد للأغشية الحيوية عند أدنى تركيزين (1.55 ملغ/مل) و0.78 ملغ/مل، أي ما يعادل 0.06% من الكلورهيكسيدين، مقارنةً بأقل تركيز له (0.34 ملغ/مل)، وكان هذا النشاط متناسبًا عكسيًا مع تركيز المستخلص. الخلاصة: على الرغم من عدم ظهور أي حساسية تجاه بكتيريا *P. gingivalis* لدى كلٍّ من أوغمنتين وميترونيدازول، إلا أن التأثير المضاد للبكتيريا الواعد لمستخلص أوراق المولبيدينوم ضد هذه البكتيريا قد يشير إلى إمكانية استخدامه في تعزيز صحة الفم، مما يشير إلى إجراء المزيد من الدراسات الحيوية للتحقق من صحة مستخلصات المولبيدينوم كبدائل علاجية في علاج التهاب دواعم السن.