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Posted Date: 10 July 2023

doi: 10.20944/preprints202307.0569.v1

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Article

Metronidazole Potentiation by Panax Ginseng and Symphidium Officinale: A New Strategy for *P. gingivalis* Infection Control

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Abstract: *Background*: The important periodontal disease pathogen Porphyromonas gingivalis produces large biofilms that increase its pathogenicity. Finding natural antimicrobial agents is crucial because of the rise in antibiotic resistance. *Objective*: The purpose of this study was to determine if plant extracts such as Symphytum officinale (S) and Panax ginseng (G) were effective against *P. gingivalis* separately and in combination with a common antibiotic Metronidazole (F). *Materials and Procedures*: Six different dilutions were produced the plant extracts and antibiotic separately and in combination with G and F and S and F using two-fold serial dilution technique. To evaluate the effects of these substances biofilm inhibition experiments were conducted. Plaque samples were collected from periodontitis patients to isolate *P. gingivalis* and standard strain of *P. gingivalis* (ATCC 33277) were bought. Additionally, Acylated Homoserine Lactones (AHLs) detection was carried out to look for any activity that would interfere with quorum sensing. GraphPad prism was used for statistical analysis with p value < 0.05. *Results*: The combinations of Symphytum Officinale and Metronidazole (S+F) showed the maximum effectiveness in biofilm inhibition (98.7%), which was better than G+F (98.2%) with substantial variations in biofilm inhibition levels in different treatment regimes. Notably, the isolate from the patient was more active than the standard strain. Additionally, the plant extracts and their combinations at particular dilutions had notable inhibitory effects on the generation of AHL ($p < 0.05$). *Conclusion*: The study highlights the possibility of Symphytum Officinale and Panax Ginseng as effective treatments for *P. gingivalis* biofilm and AHLs, both on their own and in combination with Metronidazole. These organic substances may open the door to cutting-edge methods of treating periodontal disorders.

Keywords: Porphyromonas gingivalis; Symphytum Officinale; Panax Ginseng; Metronidazole; Biofilm Inhibition; Acylated Homoserine Lactones; Periodontal Diseases

1. Introduction

Globally, periodontal diseases pose a serious threat to public health because they affect a huge number of people worldwide and, if ignored, could result in tooth loss. One of the common oral bacteria present in the oral plaque of the patients with periodontitis (Schmidt et al.,2014) and known to be actively involved in the progression of gingivitis, is the Gram-negative anaerobic bacteria Porphyromonas gingivalis (*P. ginivalis*) [1]. On both soft and hard oral tissues, it is known to develop biofilms, an organised community of bacterial cells encased in a self-produced polymeric matrix [2]. These biofilms [3] are a characteristic of chronic periodontitis and provide resistance to host defense and common antimicrobial therapies, helping to enhance the pathogenicity of *P. gingivalis* [4]. A major concern to world health is antibiotic resistance, which has rendered many common antibiotics ineffective.

 doi:10.20944/preprints202307.0569.v1

The search for new antimicrobial drugs, particularly those derived from natural resources, has been sparked by this dilemma [5]. Due to their shown effectiveness against a variety of bacterial infections, chemicals originating from plants have attracted enormous attention in this context [6,7]. Comfrey, also known as Symphytum Officinale, and Panax Ginseng are two such promising herbs that have demonstrated exceptional antibacterial qualities in prior tests [1]. Furthermore, it is thought that the quorum sensing (QS) phenomenon in bacteria, a cell to cell communication activity, is crucial for biofilm formation and other virulence characteristics [8]. Acylated Homoserine Lactones (AHLs), signaling molecules found in many gram-negative bacteria, including *P. gingivalis*, are crucial for QS [9]. Therefore, preventing the synthesis of these molecules may be able to reduce bacterial pathogenicity and prevent the development of biofilms, making it a viable technique for treating chronic infections like periodontitis [5].

Allantoin, rosmarinic acid, and mucilage are just a few of the many bioactive substances found in Symphytum Officinale, a plant that has long been used to cure a variety of diseases. The anti-inflammatory, antibacterial, and wound healing abilities of these substances have been studied in the past. An aphrodisiac, adaptogen, and all-purpose cure-all, Panax ginseng is a well-known medicinal herb with East Asian origins [2]. The primary active ingredients in Panax ginseng are saponins called ginsenosides, which have strong antibacterial and anti-inflammatory activities [10,11]. The investigation of these natural substances for their capacity to thwart quorum sensing and biofilm formation processes may eventually result in the development of novel approaches for the periodontal disease therapy [9].

One of the main therapies for anaerobic bacterial infections, including those brought on by *P. gingivalis*, is the nitroimidazole antibiotic metronidazole. It causes harm to DNA and prevents bacteria from making proteins. The advent of antibiotic resistance and its associated negative consequences, however, have called into doubt its exclusive usage. Metronidazole may be more effective when combined with natural plant extracts, which would lower the dosage needed and the risk of adverse effects on human health [12]. Despite Symphytum Officinale and Panax Ginseng having well-known antibacterial capabilities, nothing is known about how these two substances interact with *P. gingivalis*, specifically how they affect biofilm inhibition and AHL generation. Furthermore, few research have examined the possible synergistic effects of using these extracts in conjunction with common antibiotics like metronidazole though most of the investigations have concentrated on individual plant extracts [13–15]. By examining the effects of Symphytum Officinale and Panax Ginseng on *P. gingivalis*, both separately and in combination with metronidazole, this study seeks to close this information gap. This study might make a substantial contribution to the creation of periodontal disease treatment plans [16,17].

A standard strain of *P. gingivalis* and a clinical isolate are utilized in this work in an effort to identify the extent of effectiveness of the proposed treatment [18]. In light of this, the current study sought to assess the antibacterial effectiveness of Panax ginseng and Symphytum Officinale extracts against *P. gingivalis*. The experiment was then expanded to look at how these extracts affected the growth of AHL and *P. gingivalis* biofilm when combined with metronidazole, a medication frequently used to treat periodontitis. The effects on a common strain of *P. gingivalis* and a patient-derived clinical isolate were compared, providing information on the possible therapeutic uses of these plant extracts.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

The main bacterial strain used in this investigation was the well-known laboratory strain *P. gingivalis* ATCC 33277 (American Type Culture Collection). Additionally, a clinical isolate of *P. gingivalis* was included to the study to assess the application of the research findings. The plaque samples were collected from periodontitis patients. The injection of Gracey-curette enabled the collection of subgingival plaque specimens. When the curette touched the bottom of the periodontal

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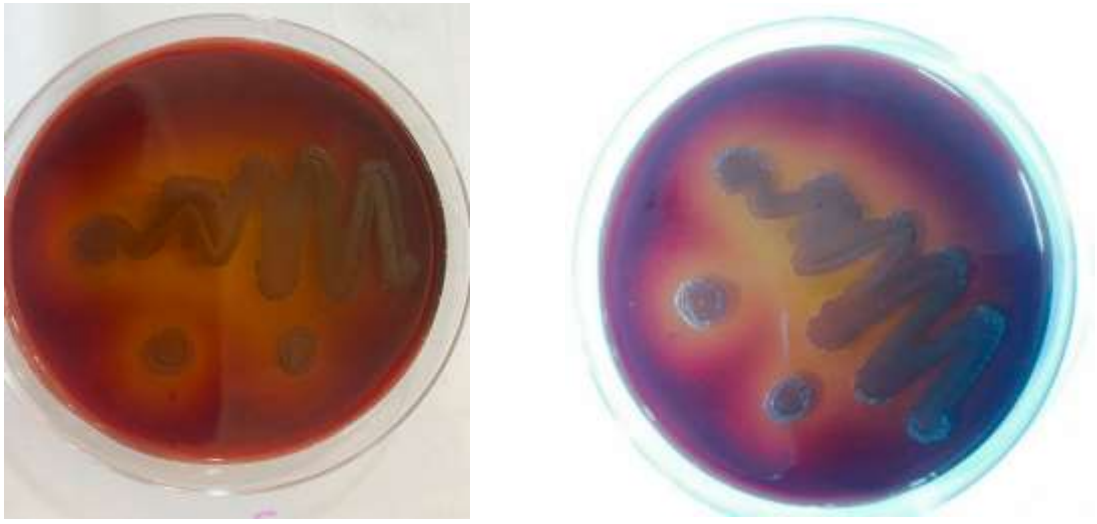
pocket without damaging the soft tissues, it collected a subgingival plaque. Following that, the plaque sample was immersed in sodium thioglycolate.

Both the standard strain and clinical isolate of *P. gingivalis* were grown anaerobically in brain heart infusion (BHI) [19] broth that had been treated with hemin and vitamin K while maintaining a 37°C temperature (Figure 1).



(a) (**b**)

Figure 1. *P. gingivalis* bacterial culture condition (a) clinical isolate (b) standard strain ATCC (33277)

2.2. Plant Extracts and Antibiotic Working Concentrations

Both Panax ginseng and Symphytum Officinale (comfrey) were purchased from reputable vendors (Rejuvica Health, USA). A suitable solvent liquid herbal extract contains no alcohol, no addative materials [20]. Aqueous extracts of Panax ginseng and comfrey were sterilized using millipore disposable filters (0.22 μm). The selected antibiotic, metronidazole, was purchased from a local pharmacy. Dimethyl sulfoxide (DMSO) was used to dissolve all substances in order to create workable concentrations. Experimental extracts include each of Symphytum Officinale and Metronidazole (S + F), followed by Symphytum Officinale alone, then the Panax Ginsengand and Metronidazole (G+ F), then the Panax Ginseng(G) alone, and finally the Metronidazole (F) alone. Working concentrations for Metronidazole, Panax Ginseng, and Symphytum Officinale were produced both alone and in combination. To enable a thorough investigation of each chemical's effects throughout a variety of doses, six dilutions (D1 to D6) were generated for each compound using a systematic two-fold serial dilution technique as shown in Table 1.

Table 1. Concentrations of plant extracts and Metronidazole

Dilution	G (mg/ml)	S (mg/ml)	F (mg/ml)	G+F (mg/ml)	S+F (mg/ml)
D1: first dilution	500	165	250	150	83
D2: second dilution	250	82.5	125	75	41.5
D3: third dilution	125	41.25	62.5	37.5	20.75
D4: fourth dilution	62.5	20.625	31.25	18.75	10.375
D5: fifth dilution	31.25	10.312	15.625	9.375	5.187
D6: sixth dilution	15.625	5.156	7.812	4.687	2.593

2.3. Biofilm Inhibition Assay

P. gingivalis was grown in a brain-heart infusion medium containing 10% blood and 0.2 mg/mL vitamin K. (Sigma-Aldrich, St. Louis, MO). Bacteria thrived in an anaerobic chamber with 5% H_2, 10% CO_2, and 85% N_2 at 37°C for 48 to 72 hours [21].

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Using a microtiter plate biofilm test, the potential of plant extracts and Metronidazole to prevent *P. gingivalis* from forming biofilms was evaluated. Both the standard strain and isolate were exposed to various test drug doses. The microplates were then incubated anaerobically at 37°C for 24 hours, and the amount of biofilm inhibition was measured using crystal violet staining. The variations in optical densities (ODs) were used to quantify the percentage of biofilm inhibition. The isolates were deemed as strong if the OD was more than 0.240, Moderate if the OD was between 0.120 and 0.240 and weak if the OD was less than 0.120 [22].

2.4. Detection of Acylated Homoserine Lactones (AHLs)

AHLs, a crucial component of *P. gingivalis*'s quorum sensing, were determined using a colorimetric technique [23]. At different dilutions, the effects of Symphytum Officinale, Panax Ginseng, and Metronidazole on the generation of AHLs by two *P. gingivalis* strains were observed in Figure 2.

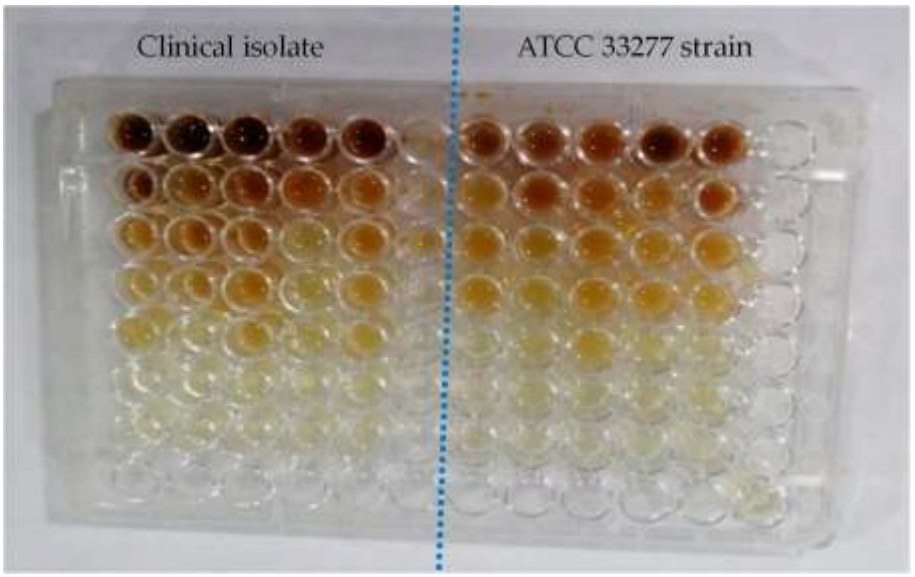

Figure Error! No text of specified style in document.**2.** Mean detection of Acylated Homoserine Lactones (AHLs) *P. gingivalis* (strain ATCC 33277 and bacterial isolated from patients)

2.5. Quorum Sensing Inhibition Assay

The potential of the plant extracts to inhibit quorum sensing was assessed by using a colorimetric method, which produces orange pigmentation in the presence of AHL, a quorum sensing molecule. The reporter strain was co-cultured with *P. gingivalis* in the presence and absence of plant extracts. The intensity of orange pigmentation was taken as a measure of AHL activity and was quantified spectrophotometrically. The isolates were deemed to produce weak or negative AHLs if the OD was less than 0.98 [24]. the negative control (-) without AHLs signal molecules and the positive control (+) with AHLs signal molecules.

2.6. Statistical Analysis

All statistical analyses were conducted with the assistance of the Statistical Package for Social Sciences (SPSS) version 24.0. All experiments were performed in triplicate, and the data were expressed as mean ± standard deviation. Statistical analyses were performed using one-way ANOVA, followed by Tukey's multiple comparison tests. P values < 0.05 were considered statistically significant.

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3. Results

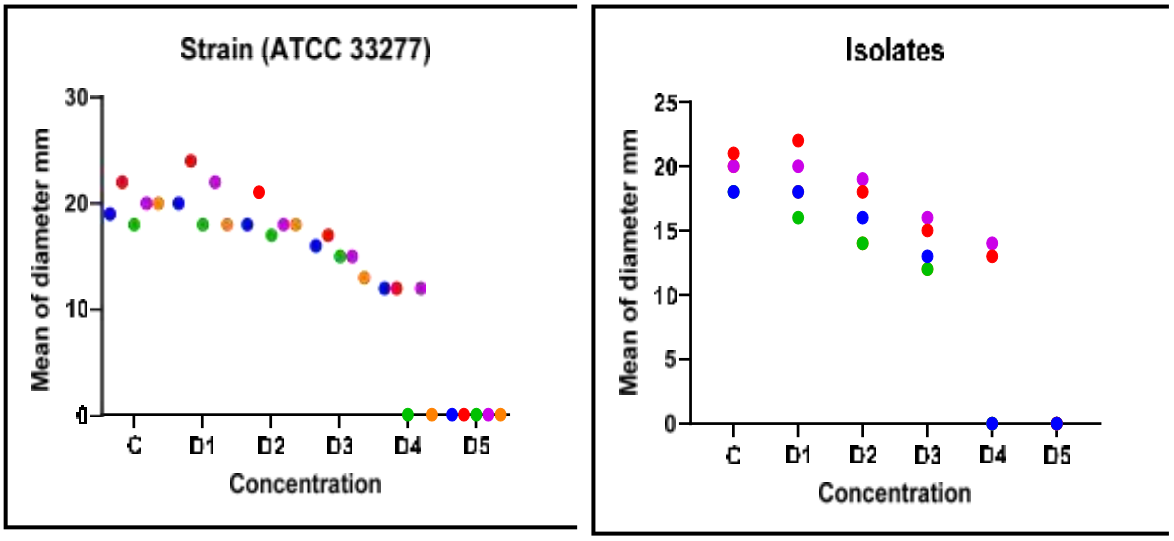
3.1. Antibacterial Activity of Plant Extracts and Metronidazole

The initial assessment was based on the antibacterial properties of Metronidazole, Panax Ginseng, and Symphytum Officinale (Comfrey). Antibacterial efficacy of the plants extracts and Metronidazole against the standard bacterial strain (*P. gingivalis* ATCC 33277) and the clinical isolate was evaluated separately and in combination. The outcomes showed a clear antibacterial activity, with diverse effects at various doses (Table 1) only D4 was nonsignificant for the strains ($p > 0.05$) (Figure 3). While the lowest concentration D5 did not show any antibacterial activity at all.

Table Error! No text of specified style in document.**2.** Antibacterial activities of plant extracts and Metronidazole

Microorganism	Concentration	Mean inhibition zone diameter, D (mm)					F-test	P-value
		G	G+F	S	S+F	F		
Strain (ATCC 33277)	C	20.5120	24.0125	18.6111	22.0367	20.4119	132.25	0.000
	D1	20.2476	22.5019	18.2110	20.8970	18.2778	129.63	0.005
	D2	18.3017	21.000	17.1556	18.0000	18.2100	141.11	0.003
	D3	16.1506	17.8642	15.0556	15.6410	13.6321	128.63	0.001
	D4	12.000	12.2590	0	12.3780	0	99.52	0.142
	D5	0	0	0	0	0	-	-
Isolate	C	18.3889	22.8690	18.5124	20.1056	20.1359	138.28	0.0025
	D1	18.2778	21.9810	16.2531	20.0245	18.0189	129.69	0.0015
	D2	16.5000	18.4587	14.4376	19.0849	14.5890	137.18	0.0032
	D3	13.6111	15.1398	12.1400	16.8941	12.3801	98.69	0.0015
	D4	18.3889	13.6782	0	14.7901	0	91.57	0.521
	D5	0	0	0	0	0	-	-

(D) dimension of zone inhibition, Panax Ginseng (G) and Symphytum Officinale (S), Metronidazole (F), Panax Ginseng + Metronidazole (G+F), Symphytum Officinale+ Metronidazole (S+F).



(a) (b)

Figure 3. Antibacterial inhibition zone diameters created by plant extracts and Metronidazole against *P. gingivalis* (a) strain ATCC 33277 and (b) clinical isolate.

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3.2. Biofilm Inhibition Rate of Bacterial Standard Strain and Isolates

Under the impact of several experimental extracts, biofilm inhibition rates of the standard and isolated bacterial strains were compared. The substantial antibacterial activity of the test compounds was shown by the considerable variation in biofilm inhibition across the various treatments ($p < 0.05$) (Table 2).

Table 2. Shapiro-Wilko tests on biofilm inhibition rates of bacterial standard strain and isolates

Concentration	Drugs and Extracts								
	G	G+F		S		S+F		F	
	P value	Statistics	P value	Statistics	P value	Statistics	P value	Statistics	P value
D1	0.015	0.0325	0.027	0.452	0.027	0.521	0.021	0.338	0.024
D2	0.040	0.363	0.029	0.421	0.033	0.401	0.018	0.396	0.029
D3	0.071	0.324	0.041	0.393	0.046	0.412	0.020	0.375	0.035
D4	0.013	0.401	0.031	0.401	0.048	0.393	0.041	0.410	0.066
D5	0.022	0.0400	0.033	0.421	0.042	0.410	0.068	0.482	0.075
D6	0.020	0.0389	0.030	0.406	0.049	0.423	0.058	0.398	0.058

(D) dimension of zone inhibition, Panax Ginseng (G) and Symphytum Officinale (S), Metronidazole (F), Panax Ginseng + Metronidazole (G+F), Symphytum Officinale+ Metronidazole (S+F).

3.3. Acylated Homoserine Lactones (AHLs) production

Finally, it was assessed how the plant extracts and the antibiotic metronidazole affected the creation of AHLs, an essential component of bacterial communication. As indicated in Table 3, the plant extracts and metronidazole significantly inhibited the formation of AHLs ($p < 0.05$).

Table 3. Influence of plant extracts and Metronidazole on AHLs production

Microorganism	Concentration	Mean of OD detection (Ahls)					F-test	P-value
		G	G+F	S	S+F	F		
Strain (ATCC 33277)	C	1.926	2.248	2.114	1.845	1.984	88.95	0.0014
	D1	0.953	0.887	0.925	0.914	0.987	41.24	0.0036
	D2	0.824	0.654	0.725	0.226	0.432	51.14	0.000
	D3	0.127	0.092	0.344	0.114	0.354	44.56	0.0025
	D4	0.017	0.001	0.065	0.023	0.098	39.23	0.0051
	D5	0.009	0.000	0.006	0.005	0.006	33.54	0.0052
Isolate	C	2.462	1.845	2.259	2.357	2.009	29.68	0.0041
	D1	0.997	0.854	0.965	0.967	0.995	36.05	0.002
	D2	0.895	0.115	0.912	0.854	0.927	41.98	0.0042
	D3	0.487	0.078	0.235	0.089	0.827	42.56	0.003
	D4	0.129	0.012	0.059	0.012	0.094	40.25	0.000
	D5	0.05	0.000	0.001	0.002	0.009	40.14	0.009

(D) dimension of zone inhibition, Panax Ginseng (G) and Symphytum Officinale (S), Metronidazole (F), Panax Ginseng + Metronidazole (G+F), Symphytum Officinale+ Metronidazole (S+F).

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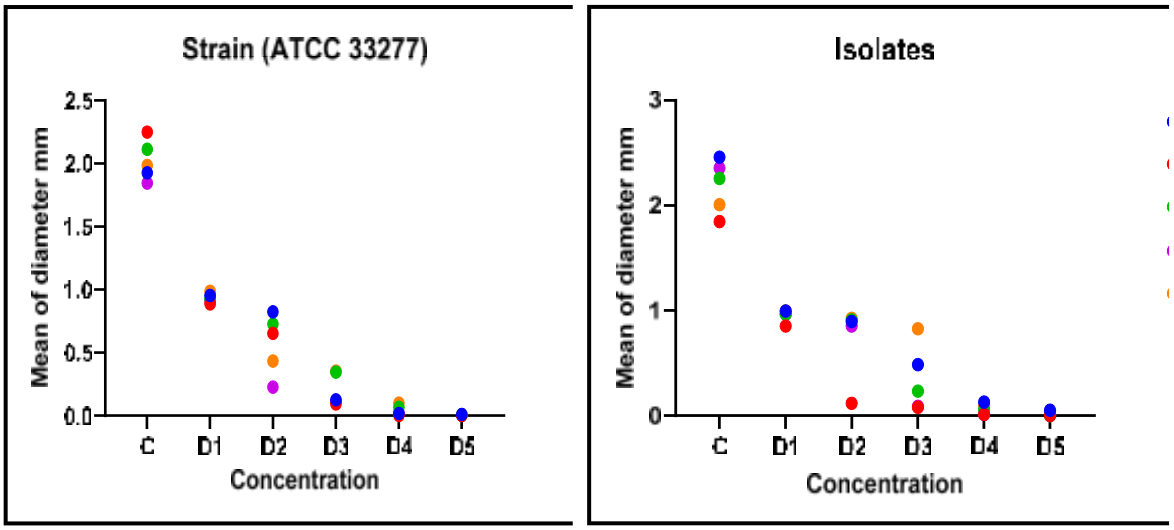
Overall, the findings strongly suggested that Metronidazole, Panax Ginseng, and Symphytum Officinale (Comfrey) have antibacterial and biofilm inhibitory effects, suggesting that they may be used to treat *P. gingivalis* infections. The fact was that these results remained true for both the common laboratory strain and clinical isolate indicating their significance in the actual clinical environment (Figure 4).



(a) (b)

Figure 4. Mean diameter for detecting Acylated Homoserine Lactones (AHLs) with P. gingivalis (a) strain ATCC 33277 and (b) clinical isolate

3.4. Dose-Dependent Effects of G, S, and F

The results demonstrated dose-dependent nature due to the antibacterial and biofilm inhibiting effects of different doses of G, S, and F. D1 dose produced the most effective results with respect to the values of extract combination. The results of this study will be extremely useful in determining the combinations and concentrations of each medication that will have the greatest therapeutic impact $p < 0.05$ (0.00014) (Table 4).

Table 4. Percentage of biofilm inhibition for bacterial strains used.

Microorganism	Concentration	Percentage of biofilm inhibition				
		G	G+F	S	S+F	F
Strain (ATCC 33277)	D1	98.1	98.2	98	98.7	97.8
	D2	96.1	97.8	96.9	98.3	97.4
	D3	95.2	96.5	95.2	96.4	94.6
	D4	94.2	95.3	94	96.1	95.1
	D5	69	80.7	78.1	85.5	69.1
	D6	44.3	45.3	54.8	55.8	51.9
Isolate	D1	97.2	99.2	97.4	98.2	97.2
	D2	96	98.7	96.1	98	96.1
	D3	94.2	97.2	92.9	97.3	94.8
	D4	93.9	92.8	91.3	92.1	92
	D5	69.9	71.7	68.6	79.1	70.3
	D6	43.2	41.2	37.1	34.2	67.8
X^2	3.254					
P value	0.00014					

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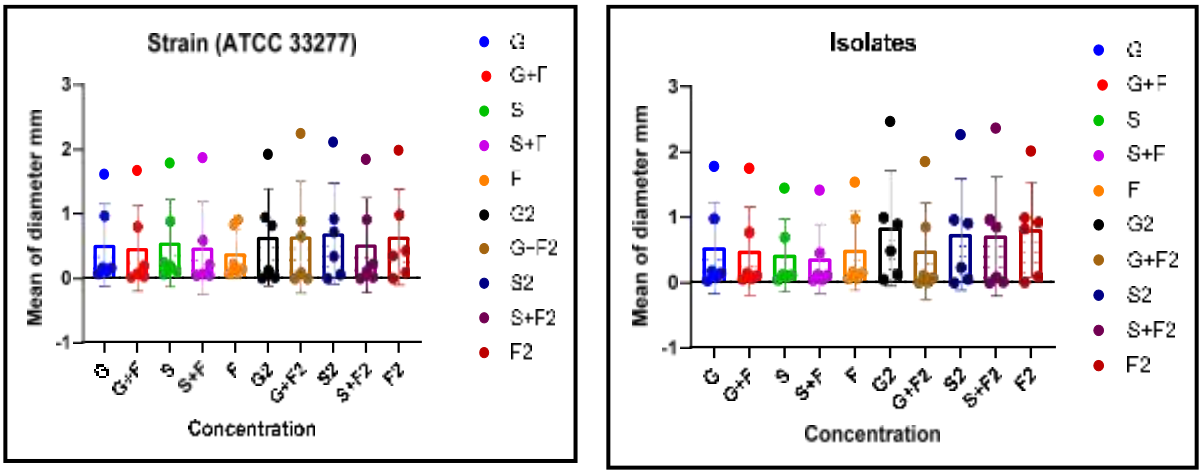
3.5. Correlation Between Biofilm Inhibition and AHL Production

This part presents the relationship, if any, between biofilm inhibition and AHL production in the clinical isolate and standard strain when G, S, and F were used. As indicated in Figure 5, the plant extracts and metronidazole significantly inhibited the formation of biofilm and AHLs ($p < 0.05$). This significant association, it could imply that the antibacterial and anti-biofilm actions of these medications are, at least in part, influenced by the disruption of bacterial quorum sensing mechanisms. Where G2, G+F2, S2, S+F2, F2 represent the extract doses that inhibited AHLS in strains and isolates, and there was a correlation ($p<0.05$) between them and biofilm inhibition.



(**a**) (**b**)

Figure 5. Relationship between biofilm inhibition and AHL production for (**a**) standard strain and (**b**) clinical isolate

4. Discussion

The current investigation focused on both a standard strain and clinically isolated bacteria to examine the inhibitory potential of Panax Ginseng (G), Symphytum Officinale (S), and Metronidazole (F) against *P. gingivalis*. The results support earlier research showing that *P. gingivalis* is very amenable to biofilm reduction through a variety of treatment approaches, both conventional and modern [25]. When exposed to the tested treatments, a substantial suppression of biofilm formation was seen in both the standard strain and the clinical isolate. These findings are consistent with previous research demonstrating the powerful antibacterial and anti-biofilm capabilities of G, S, and F [2].

Additionally, it appeared that the combination therapies (G+F, S+F) had a more prominent impact than the separate treatments, which may have been the result of a synergistic action by the two [12]. The generation of AHL, a crucial component of bacterial quorum sensing, was successfully reduced by the tested treatments in both the clinical isolate and the standard strain, according to the research. The capacity of *P. gingivalis* to coordinate group behaviour may be compromised by this restriction of AHL synthesis, which would reduce the pathogenic potential of the organism [9]. Intriguingly, the separated bacteria were more energetic and active than the conventional strain, corroborating other studies that claim clinical isolates may display unique characteristics [26]. However, the specific causes of this disparity are yet unknown and need more research. Depending on the treatment concentration, a sizable variance in the observed outcomes was noticed, pointing to a dose-response connection. This is in line with other studies showing that the concentration of antibacterial drugs frequently affects their effectiveness [27].

A possible strategy for reducing bacterial pathogenicity is to disrupt quorum sensing processes. It is possible to significantly lessen the pathogenic potential of bacteria like *P. gingivalis* by interfering with their coordination and communication. Bacterial quorum sensing, which is mediated by signal molecules like AHLs, has become a fascinating topic of research, especially in connection to bacterial

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pathogenicity and the development of durable biofilms [5]. Notably, the current findings confirm the biofilm-inhibitory effects found by providing strong evidence that G, S, and F can impair quorum sensing with *P. gingivalis* by preventing the generation of AHL. This capacity to interfere with the quorum sensing systems is consistent with other investigations that have noted the inhibitory effects of natural extracts on quorum sensing [28]. The complexity of bacterial behaviour in actual clinical settings is highlighted by the increased activity that was seen in clinical isolates compared to the reference strain. It serves as a reminder that bacterial pathogenicity and medication resistance can differ greatly amongst strains, depending on a number of variables including their genetic make-up and the environment from which they are separated [29].

The results showed a strong dose-dependent response for the inhibitory effects of G, S, and F. Other research investigating the antibacterial activity of natural extracts have shown that such a reaction is a typical characteristic of many antimicrobial compounds [1]. These dose-response correlations are essential for pinpointing the precise concentrations that can produce the best therapeutic outcomes while minimising any side effects. When considered as a whole, these results highlight the promising antibacterial and anti-biofilm activities of G, S, and F against *P. gingivalis*, suggesting potential for innovative treatments to avert or cure infections caused by bacteria that form biofilms.

This work adds to the expanding body of research that supports the use of natural extracts as alternatives or supplements to conventional antimicrobial treatments by confirming the ability of G, S, and F to disrupt these bacterial communication routes [27]. Additionally, the synergistic effects of combining therapies (G+F, S+F) provide new avenues for creating more powerful and comprehensive treatment plans. The combination of several antibacterial drugs frequently leads in increased antibacterial effectiveness, as shown by the findings and those of other researchers [30]. Therefore, it is important to investigate the ramifications of these synergistic effects, including any potential effects on lowering the probability of bacterial resistance.

The significant difference between the clinical isolate and standard strain of *P. gingivalis*' susceptibility to the tested therapies can be attributed to a number of variables. The strain's sensitivity to antimicrobial drugs can be changed by differences in genetic variants, mutations, or the capacity to acquire resistance genes [13]. Future research may focus on elucidating these elements and how they relate to *P. gingivalis* infection treatment efficacy[4].

The results of this investigation provide light on the efficacy of Metronidazole (F), Symphytum Officinale (S), and Panax Ginseng (G) as antibacterial and anti-biofilm agents against *P. gingivalis*. Further in vivo research is needed to verify these findings. The therapeutic potential and safety profile of these medicines might be further understood using animal models or human participants in clinical studies. While it was found in this study that G, S, and F can hinder quorum sensing in *P. gingivalis* by blocking the production of AHL, the fundamental mechanism underpinning this process are still not fully understood. The particular molecular processes by which these natural extracts and antibiotics disrupt quorum sensing pathways needs further investigation. This information will aid in the creation of more focused and successful therapy approaches.

This research is limited to providing enough number of samples and microbiological analyzing advance technique.

5. Conclusion

In conclusion, this study focused on both a standard strain and clinically isolated bacteria to examine the inhibitory potential of Panax Ginseng (G), Symphytum Officinale (S), and Metronidazole (F) against *P. gingivalis*. The findings confirmed that *P. gingivalis* was susceptible to several biofilm-reduction therapy modalities. The antibacterial and anti-biofilm properties of G, S, and F, the tested treatments showed a significant inhibition of biofilm formation against both the standard strain and the clinical isolate. Combination therapy (G+F, S+F) had a more pronounced effect than the individual therapies, possibly as a result of synergistic activity.

Author Contributions: Conceptualization, S. M. I., and A. S. A.; methodology, S. M. I., and A. S. A.; validation, S. M. I., A. S. A. and J. H.; formal analysis, S. M. I., A. S. A. and J. H.; investigation, S. M. I.; resources, S. M. I.;

data curation, S. M. I.; writing—original draft preparation, S. M. I., A. S. A. and J. H.; writing—review and editing, S. M. I., A. S. A. and J. H.; visualization, S. M. I.; supervision, A. S. A. and J. H.

Funding: This research was funded by University of Iraqi Ministry of Higher Education and Scientific Research for their financial assistance (Grant No. [Protocol-3]).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study (Ethical approval Reference number 382).

Data Availability Statement: Upon reasonable request, the data created and examined during the current study can be made available.

Acknowledgments: We appreciate the assistance and resources provided by the Department of Periodontics at the Baghdad College of Dentistry.

Conflicts of Interest: The authors declare no conflict of interest.

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