ejpmr, 2022,9(5), 320-325

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

ANTIBACTERIAL EFFICACY OF *SPIRULINA PLATENSIS* AS COMPARED TO CHLORHEXIDINE AGAINST THE PERIODONTAL PATHOGENS, *PORPHYROMONAS GINGIVALIS* AND *TANNERELLA FORSYTHIA*. AN IN-VITRO STUDY

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Article Received on 07/03/2022 **Article Revised on 28/03/2022** **Article Accepted on 19/04/2022**

ABSTRACT

Spirulina is a cyanobacterium or a blue-green algae known to have a wide range of therapeutic properties. It has antimicrobial effects *against Streptococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella sp, Proteus sp,* and *Enterobacter sp*. Little has been researched on the beneficial effects of Spirulina on particular periodontopathic organisms. The objective of this study is to evaluate the antibacterial effects of Spirulina Platensis on two periodontopathic bacteria, *Porphyromonas gingivalis,* and *Tannerella forsythia,* and to compare its efficacy with that of the gold standard, Chlorhexidine.

KEYWORDS: Disc diffusion, Minimum Inhibitory concentration, *Pophyromonas gingivalis*, *Tannerella forsythia*, Spirulina.

INTRODUCTION

Dental plaque is a diverse community of micro-organisms found on the tooth surface as a biofilm, embedded in an extracellular matrix of polymers of host and microbial origin. Bacterial biofilms are gaining research impetus due to their deleterious impact on disease onset and persistence.[1] The dental biofilm is composed of predominantly anaerobic Gram-negative species, including the red complex, which appears later in biofilm development and comprises a consortium of three species, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. These three species cause Periodontitis, an inflammatory process leading to the loss of the supporting periodontium.[2,3] Antimicrobials and antibiotics constitute the recommended treatment however, over time, these species develop and exhibit antimicrobial resistance.[4] Herbal and algal extracts containing natural phytochemicals are gaining popularity as an alternative medical cure to combat such drawbacks.[5] *Spirulina platensis* (Oscillatoraceae family) is a spiral-shaped blue-green unicellular microalga that grows in fresh water, salt water, as well as in brackish bodies of water. Spirulina has been used as a source of food by humans for centuries.[6] Usage of *Spirulina platensis* prevents cancers, lowers blood cholesterol levels and decreases the nephrotoxicity of pharmaceuticals and other toxicities such as that from radiation[7] Spirulina is known to have antimicrobial effects against *Streptococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella sp, Proteus sp, and Enterobacter sp*., and they also inhibit the replication of several viruses such as Herpes simplex virus (HSV-1), human cytomegalo virus, measles virus and Influenza A virus.[8] In dentistry, Spirulina has shown a reduction in Probing pocket depth (PPD) and gain in Clinical Attachment Level (CAL) in chronic periodontitis patients when used in the form of in-situ gel along with scaling and root planning.[9] A recent animal study suggests that through its anti-inflammatory effect, Spirulina Maximus reduced the bone loss in periodontitis induced by *P.gingivalis*.[10] Spirulina has also been used in the management of oral submucous fibrosis[11] and leukoplakia[12] and the antibacterial activity was studied against various Gram-positive and Gram-negative organisms. This is a pioneer in-vitro study comparing the antibacterial effect of *Spirulina platensis* and Chlorhexidine on the periodontopathic bacteria, *Porphyromonas gingivalis* and *Tannerella forsythia.*

MATERIALS AND METHODS

The pure strains of anaerobic bacteria *Porphyromonas gingivalis* (ATCC33277) and *Tannerella forsythia* (ATCC43037) used in the study, were grown in HiMedia

(M210-500G) and brain heart infusion broth (500 g). Further, these pure strains were isolated by sub-culturing them in blood agar culture media. Minimum inhibitory concentration (MIC) and disc diffusion tests were performed in triplicates to assess the antibacterial activity. The antibacterial effect of *Spirulina platensis*, 0.2% Chlorhexidine gluconate and Normal saline were studied. The commercially available pure form of *Spirulina platensis* powder (Sorich Organics USDA Certified Organic Spirulina Powder) was used in the study. To prepare the ethanolic extract of *Spirulina platensis*, 10 mg of fine powder of Spirulina was weighed and extracted with 1ml ethanol. Three variants of stock solutions were prepared by weighing 10 mg of compounds of Ethanolic extract of spirulina, Chlorhexidine, Normal saline and further dissolving it in 1 ml of DMSO (Dimethyl Sulfoxide) individually.

Minimum Inhibitory Concentration Test (MIC): MIC was performed in Triplicate, which involved diluting each drug nine times in Thioglycolate broth. In the initial tube 20microliter (µl) of the drug was added into 380 µl of Thiogylcolate broth. For dilutions, 200 µl of Thiogylcolate broth was added into the next nine tubes separately. And from the initial tube 200 µl were transferred to the first tube containing 200 µl of Thiogylcolate broth. This was considered as 10:1 dilution. From the 10:1 diluted tube, 200 µl was transferred to the second tube to make 10:2 dilutions. This serial dilution was repeated up to 10:9 dilutions for each drug. From the maintained stock cultures of required organisms, 5µl was taken and added into 2 millllilitre (ml) of Thiogylcolate broth. In each serially diluted tube, 200 µl of above culture suspension was added. The tubes were incubated for 24 hours and observed for turbidity. Standard values for MIC was <0.215 µg/ml.[13]

Disc Diffusion Test: Visually adjusted turbidity with broth to equal a 0.5 McFarland turbidity standard was vortexed. The suspensions were standardised with a photometric device. Brain Heart Infusion agar at room temperature was used. Within 15 minutes of adjusting the inoculum to a McFarland 0.5 turbidity standard, a sterile cotton swab was dipped into the inoculum and rotated against the wall of the tube above the liquid to remove excess inoculum. The entire surface of the agar plate was swabbed three times by rotating the plates approximately 60° between streaking to ensure even distribution. The inoculated plate was allowed to stand for at least 3 minutes but not exceeding 15 minutes, before making wells. Wells were made in the plate by heating a hollow tube of 5 mm diameter and pressing it on the above inoculated Agar plate, and removing it immediately. Three wells were made on each plate. With the help of a micropipette, 50 µl was added to compare three compounds: 10 mg of Spirulina in 1ml DMSO, 0.2% chlorhexidine, and 0.9% normal saline. The plates were incubated within 15 minutes of compound application for 18-24 hours at 37° C in an incubator. The plates were assessed only if the growth lawns were confluent or nearly confluent.[13]

The diameter of the inhibition zone was measured to the nearest whole millimetre with the help of a Vernier Caliper. The standard values for disc diffusion were >34 mm.

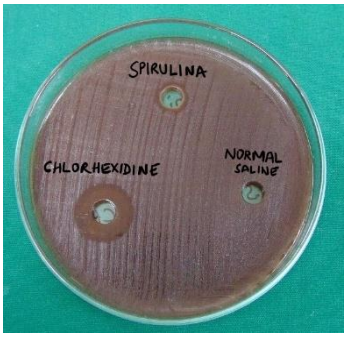

Fig 1

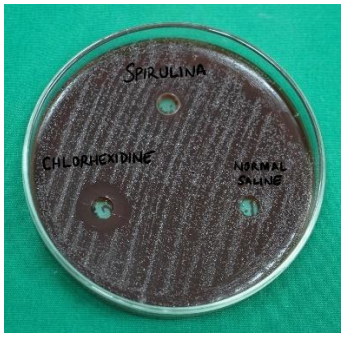

Fig 2

STATISTICAL ANALYSIS

Statistical analysis was done using SPSS version 20 (IBM SPSS statistics). Descriptive statistics of the explanatory and outcome variables were calculated by median and IQR (based on data distribution) for quantitative variables. The level of significance was set at 5%.

RESULTS

The results obtained from the MIC test and Disc diffusion test were statistically analyzed. The results of MIC for *Porphyromonas gingivalis* (Table 1) showed significant sensitivity to Spirulina extract and chlorhexidine for concentrations of 100 µg/ml, 50 µg/ml, 25 µg/ml, with a mean percentage of 33.3% respectively for both groups with a significant P-value of 0.011. At a concentration of 12.5 µg/ml, the chlorhexidine group showed 33.3% sensitivity, whereas the Spirulina group showed 22.2% sensitivity and 11.1% resistance; further, serial dilutions of Spirulina extract showed 0% sensitivity. Contrasting, the chlorhexidine group showed sensitivity to dilutions up to 0.2 µg/ml for 22.2% with a p-value of 0.076. The MIC of Spirulina for *Porphyromonas gingivalis* was found to be 12.5 µg/ml.

Table 2 shows the statistical results of the MIC of *Tannerella Forsythia*. The bacteria showed sensitivity towards spirulina extract at 100 µg/ml, 50 µg/ml, 25 µg/ml, 6.25 µg/ml, 3.12 µg/ml, 1.6 µg/ml with a mean percentage of 33.3% in each concentrations respectively with a P-value of 0.011. And, 0.8 µg/ml concentration of spirulina showed 11.1% sensitivity. All the serial dilution concentrations were sensitive to chlorhexidine. The MIC of Spirulina for *Tannerella Forsythia* was found to be 0.8 µg/ml. From the above test results of MIC, the Chlorhexidine group has shown better results than the Spirulina group against both the study organisms.

In Table 3, the results of the disc diffusion test are enumerated. *Porphyromonas gingivalis* showed a maximum disc diffusion diameter of 28mm for chlorhexidine and 13mm diameter for Spirulina. T forsythia showed a maximum disc diffusion diameter of 25mm in chlorhexidine and 12mm diameter in Spirulina. Thus, by comparing the two drugs, a statistically significant result is seen in terms of chlorhexidine and a lesser significant result in terms of Spirulina extract (Fig. 1, Fig. 2).

Table 1: Distribution of the Inhibition method at different concentrations for P gingivalis.

Concentration	P.gingivalis		Groups			Total	Chi-square value	p value
			CHX	NS	Spirulina			
100 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
50 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
25 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
12.5 µg/ml	R	Count	0	3	1	4	6.3	0.043*
		%	0.0%	33.3%	11.1%	44.4%		
	S	Count	3	0	2	5		
		%	33.3%	0.0%	22.2%	55.6%		
6.25 µg/ml	R	Count	0	3	3	6	9	0.011*
		%	0.0%	33.3%	33.3%	66.7%		
	S	Count	3	0	0	3		
		%	33.3%	0.0%	0.0%	33.3%		
3.12 µg/ml	R	Count	0	3	3	6	9	0.011*
		%	0.0%	33.3%	33.3%	66.7%		
	S	Count	3	0	0	3		
		%	33.3%	0.0%	0.0%	33.3%		
1.6 µg/ml	R	Count	0	3	3	6	9	0.011*
		%	0.0%	33.3%	33.3%	66.7%		
	S	Count	3	0	0	3		
		%	33.3%	0.0%	0.0%	33.3%		
0.8 µg/ml	R	Count	0	3	3	6	9	0.011*
		%	0.0%	33.3%	33.3%	66.7%		
	S	Count	3	0	0	3		
		%	33.3%	0.0%	0.0%	33.3%		
0.4 µg/ml	R	Count	0	3	3	6	9	0.011*
		%	0.0%	33.3%	33.3%	66.7%		
	S	Count	3	0	0	3		
		%	33.3%	0.0%	0.0%	33.3%		
0.2 µg/ml	R	Count	1	3	3	7	5.14	0.076
		%	11.1%	33.3%	33.3%	77.8%		
	S	Count	2	0	0	2		
		%	22.2%	0.0%	0.0%	22.2%		

*p value <0.05 =significant p value, R-Resistant, S-Sensitive, CHX-Chlorhexidine, NS-Normal saline.

Table 2: Distribution of the Inhibition method at different concentrations for T forsythia.

Concentration	Tannerella forsythia		Groups			Total	Chi-square value	p value
			CHX	NS	Spirulina			
100 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
50 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
25 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
12.5 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
6.25 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
3.12 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
1.6 µg/ml	R	Count	0	3	0	3	9	0.011*
		%	0.0%	33.3%	0.0%	33.3%		
	S	Count	3	0	3	6		
		%	33.3%	0.0%	33.3%	66.7%		
0.8 µg/ml	R	Count	0	3	2	5	6.3	0.043*
		%	0.0%	33.3%	22.2%	55.6%		
	S	Count	3	0	1	4		
		%	33.3%	0.0%	11.1%	44.4%		
0.4 µg/ml	R	Count	0	3	3	6	9	0.011*
		%	0.0%	33.3%	33.3%	66.7%		
	S	Count	3	0	0	3		
		%	33.3%	0.0%	0.0%	33.3%		
0.2 µg/ml	R	Count	0	3	3	6	9	0.011*
		%	0.0%	33.3%	33.3%	66.7%		
	S	Count	3	0	0	3		
		%	33.3%	0.0%	0.0%	33.3%		

* p value <0.05 =significant p value , R-Resistant, S-Sensitive, CHX-Chlorhexidine, NS-Normal saline.

Table 3: Distribution of the Zone of Inhibition using Disc Diffusion Method.

Micro organisms	Groups	N	Minimum	Maximum	Median	IQR
Porphyromonas gingivalis	CHX	3	25	28	25.00	-
	NS	3	0	0	-	-
	Spirulina	3	12	13	12.00	-
Tannerella forsythia	CHX	3	23	25	25.00	-
	NS	3	0	0	-	-
	Spirulina	3	8	12	10.00	-

CHX-Chlorhexidine, NS-Normal saline, IQR-Interquartile range

DISCUSSION

In the present study, the antibacterial effect of Spirulina on *Porphyromonas gingivalis* and *Tannerella forsythia* was studied, and its efficacy was compared with that of chlorhexidine. The results of our study showed that Spirulina exhibited antibacterial properties against both, *Porphyromonas gingivalis* and *Tannerella forsythia*. The results obtained in our study are as follows: The MIC of Spirulina was found to be 12.5µg/ml and 0.8µg/ml for *Porphyromonas gingivalis* and *Tannerella forsythia*, respectively. The Disc diffusion diameters of Spirulina were 13mm and 12mm for *Porphyromonas gingivalis* and *Tannerella forsythia*, respectively. Though in our study a positive result showing the antibacterial activity of Spirulina was obtained, it was not statistically significant in comparison to the antibacterial effect of chlorhexidine. Kaushik et al in an in-vitro study assessed the antibacterial activity of *Spirulina platensis* on Gram-positive *Staphylococcus aureus* and four Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Klebsiella pneumonia*. The MIC of methanolic extract of *Spirulina platensis* showed a potent antibacterial activity compared to other organic extracts of spirulina.[14] The antibacterial activity of algal extracts depends upon the type of solvent used for extraction.[15] In our study, a similar methanolic extract was used for the microbiological analysis. Furthermore, an in-vitro study by Kumar et al, who investigated the antimicrobial activity of methanolic extract of a blue-green alga, *against Proteus vulgaris, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger, Aspergillus* flavus, and *Rhizopus nigricans* using agar cup diffusion method, significant antimicrobial activity was seen.[16]

As a recent advancement, the incorporation of silver ions and silver-based compounds in Nanoparticles along with algal and plant extracts has proven to have a worthy therapeutic advantage. Furthermore, a recent in-vitro study by Sayed et al, where *Spirulina platensis* extract was used for the biosynthesis of silver nanoparticles (AgNPs) showed good antibacterial activity against the oral pathogens *Streptococcus mutans, Staphylococcus aureus*, and *Enterococcus faecalis*.[17] Similarly, Govarthanan et al studied the antibacterial efficacy of silver nanoparticles (AgNPs) synthesised with the extract of *Spirulina platensis* against *Staphylococcus* sp. and *Klebsiella* sp., and they obtained a result where an extensive reduction in the growth rate of these pathogens was noted.[18] The antibacterial activity of Spirulina could be attributed to γ-linolenic acid[19] as well as the synergetic effect of lauric and palmitoleic acid.[20] The antimicrobial activity of microalgae could be explained by the presence of cyclic peptides, alkaloids, and lipopolysaccharides.[21] The above-mentioned in-vitro studies on Spirulina Platensis showed potent antibacterial effects against various bacterial species causing oral disease as well as a systemic disease. Our in-vitro study is a pioneer study that compared the antibacterial activity of Spirulina platensis and Chlorhexidine on Red complex periodontal pathogens, *Porphyromonas gingivalis* and *Tannerella forsythia.*

Hence, further clinical trials have to be performed in-vivo in order to utilise Spirulina as a potent anti-bacterial agent to prevent and treat periodontal disease and other oral infections.

CONCLUSION

This in-vitro study has proven through MIC and Disc diffusion tests that, Spirulina has antibacterial properties against *Porphyromonas gingivalis* and *Tannerella forsythia*. However, Spirulina does not stand superior to chlorhexidine in its anti-bacterial effects on periodontopathic bacteria. More in-vitro and in-vivo studies are required to test the unique antibacterial properties of Spirulina against various periodontal pathogens.

LIMITATION

Our study was performed in-vitro, on only two of the Red complex periodontal pathogens. It is suggested that, further clinical trials are required to test the unique antibacterial properties of Spirulina against various periodontal pathogens.

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