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A Novel Complementary Alternative Medicine: An In-Vitro Evaluation of Efficacy of Nigella sativa Extract as an Antibacterial Agent against Porphyromonas gingivalis

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ABSTRACT

Introduction: Periodontitis is an inflammatory disease which causes progressive destruction of the periodontium resulting in pocket formation, gingival recession and tooth loss. The overwhelming cost of periodontal treatment, unpleasant side effects of antibiotics, emergence of new bacterial strains and their resistance call for an alternative treatment modality which can address all these issues. Nigella sativa is an annual herbaceous plant. It is commonly known as black seed which has been used for more than 2000 years as a natural remedy. Numerous studies have confirmed the biological functions of black seed and demonstrated its anti-inflammatory, antimicrobial, antifungal and anticancer activity. **Objective:** To determine the frequency of Porphyromonas gingivalis in chronic periodontitis patients and evaluate the efficacy of black seed extract against it. **Methodology:** Porphyromonas gingivalis was isolated from subgingival plaque samples and identified up to species level. Ethanolic extract of black seed was screened for antibacterial activity by agar well diffusion and agar dilution method. A reference strain of Porphyromonas gingivalis ATCC 33277 was used as quality control. **Results:** All the tested strains of Porphyromonas gingivalis were sensitive to ethanolic extract of black seed at concentrations 25, 50, 75 and 100%. The extract had an MIC range of 2-4mg/ml. **Conclusion:** These results indicate that ethanolic extract of black seed has potent antibacterial activity against Porphyromonas gingivalis. So black seed extract can be used in periodontal therapy as an adjuvant to scaling.

Keywords: Periodontitis, Porphyromonas gingivalis, black seed, antibacterial activity.

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INTRODUCTION

Periodontitis is an inflammatory disease which destroys connective tissues and alveolar bone supporting the teeth and is initiated by microorganisms.[1] Periodontal infection affects about 10-15% of populations globally. It is characterized by severe damage to the periodontal apparatus, apical migration of the junctional epithelium and consequent alveolar bone loss.[2] It is one of the leading causes of tooth loss worldwide. A large number of local and systemic factors are involved in the pathogenesis of periodontitis. Among the local factors plaque accumulation and biofilm development are the primary causes.[3] Moreover; modern lifestyle factors like smoking and alcohol are also associated with periodontitis. Chronic periodontitis also occurs with severe systemic diseases, such as lung disorders, cardiovascular disorders, stroke, and diabetes mellitus.[4] Three bacterial species found in subginigval biofilm are most widely associated with chronic periodontitis; Treponema denticola, Porphyromonas gingivalis and Tannerella forsythia.[5]

Porphyromonas gingivalis is a non-spore forming, anaerobic rod-shaped bacterium which is non-motile and produces black pigment.[6]This organism has a number of virulence factors including fimbriae, degradative enzymes, lectin-type adhesions, lipopolysaccharides and outer capsule. LPS along with the cytokines cause resorption of alveolar bone and destroy periodontal tissue.[7] In addition, a group of cysteine proteases with trypsin like activity, known as gingipains, are associated with tissue damage and immune severance in periodontitis.[8]

Nigella sativa is a widely distributed seasonal plant that is a member of plant family Ranunculaceae under kingdom Plantae. It has been commonly used in the ancient cultures for culinary and medicinal purposes.[9] Black seed has been extensively studied for its therapeutic potential. It has been used for curing numerous medical conditions including hypertension, asthma, diabetes, inflammation,

eczema, fever, cough and influenza.[10] It has also been recommended for use on regular basis in Tibb-e-Nabwi.

This study was designed to determine the frequency of Porphyromonas gingivalis in chronic periodontitis patients and determine the antibacterial activity of black seed against Porphyromonas gingivalis so that it can be used as an adjuvant to periodontal therapy.

METHODOLOGY

Study design: Descriptive study.

Setting: Microbiology Department, University of Health Sciences, Lahore.

Period: September 2015 to September 2016.

Sample size: Total 73 cases were enrolled.

Inclusion criteria:[11]

1. Patients of both sexes between the age of 30 to 55 years
2. Periodontal pocket depth of 5 mm on two or more sites in a quadrant
3. Patients having atleast twenty teeth in oral cavity

Exclusion criteria:[1]

1. Patients with asthma, cardiac and renal diseases
2. Pregnancy
3. Patients receiving any periodontal treatment
4. Patients having received antibiotic therapy within the last three months
5. Patients with the history of alcoholism, smoking, chewing pan and betel nuts.

Sampling: After obtaining detailed patient history and evaluating the periodontal status two periodontal pockets were selected on each patient. After isolation and removal of plaque paper points were inserted deep into each pocket for 30 seconds and then placed in liquid transport medium without any delay.[12]

Microbiological Techniques: Samples were plated out onto anaerobic basal agar supplemented with 5% defibrinated sheep blood incubated in anaerobic jars at 35° C for 5 days. Gram negative rods forming black pigmented colonies were evaluated for fluorescence by long wave UV light test.[13] Lack of fluorescence differentiated Porphyromonas gingivalis from other bacteria as seen in Figure 1.

P. gingivalis was identified up to species level by Gram staining, colonial morphology, biochemical tests, fluorescence under long wave (366 nm) UV light and enzymatic activities using API 20A strips.

Preparation of Black Seed Extract

The extract was made and standardized at Applied Chemistry Research Centre, PCSIR Laboratories, Lahore. Nigella sativa seeds were dried and then crushed into a coarse powder using a grinder. This powder was soaked in absolute alcohol for 3 days with occasional stirring. The mixture was filtered by using Whatman Filter paper No. 1. The solvent was evaporated in vacuum with a rotary evaporator. The extract obtained was stored in amber bottles at 4°C.[14]

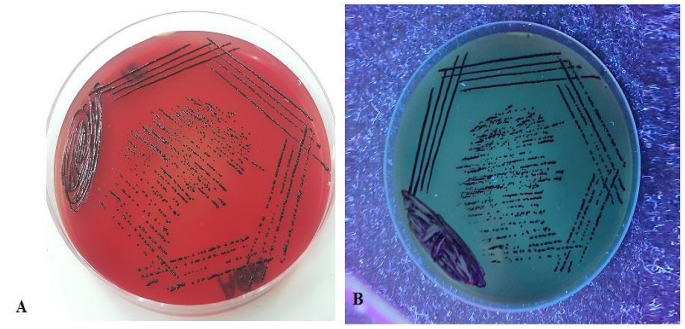

Figure 1: A. Dark pigmented colonies of Porphyromonas gingivalis, B. Non flourescent colonies of Porphyromonas gingivalis

Agar well diffusion technique

For preparing the inoculums, pure colonies were picked up from the plates and transferred to anaerobic basal broth. After adjusting the inoculum to a 0.5 MacFarland unit turbidity standard, agar plates were lawned in three directions. A sterile cork borer was heated and five wells (6 mm diameter) were punched out in the plates. Wells were filled with 75uL of different concentrations of the extract. The plates were incubated face upwards using anaerobic jars at 35°C for 48-72 hours.[15] Diameters of resulting inhibitory zones were measured in millimeters by using digital calipers (Sylvac Fowler ultra-cal II).

Determination of minimum inhibitory concentration:

Six hundred µl of the broth culture was put into the wells of the multi inoculator (Mast Diagnostic, UK). Agar plates containing different concentrations of the ethanolic extracts of black seed were inoculated with 31 isolates of P. gingivalis. These plates were kept in ananerobic jars for 48 hours at 35°C. MIC was determined by observing the plate containing lowest concentration of black seed extract inhibiting visible growth of isolates.[16]

Data analysis

The data were analyzed by using SPSS 18.0. Arithmetic mean of observations and standard deviation of mean values were calculated for quantitative variables. Qualitative variables were represented by graphs, frequencies and percentages.

RESULTS

Frequency of Porphyromonas gingivalis in chronic periodontitis patients is given in Figure 2. Data regarding the socioeconomic status, oral hygiene habits and bleeding on probing is summarized in Table 1. The results of agar well diffusion assay are shown in Table 2. Ten strains were picked at random and tested for their susceptibility to the extract.

In order to monitor quality control of the procedure P. gingivalis ATCC 33277 was also included in screening. No zone was produced by 95% ethanol which was used as negative control while metronidazole was used as positive control and produced a significant zone. On the basis of these results, MIC of the extract was performed by agar dilution method. Our MIC ranged from 2 mg/ml to 4 mg/ml, with MIC_{50} being > 2 mg/ml and MIC_{90} being 4 mg/ml.MIC range of black seed extract on 31 strains of P. gingivalis is shown in Table 3. At a concentration of 2 mg/ml, only 6.4 % isolates were inhibited, 100 % inhibition was observed at 4 mg/ml. Cumulative percentage of bacteria inhibited is explained by Figure 3.

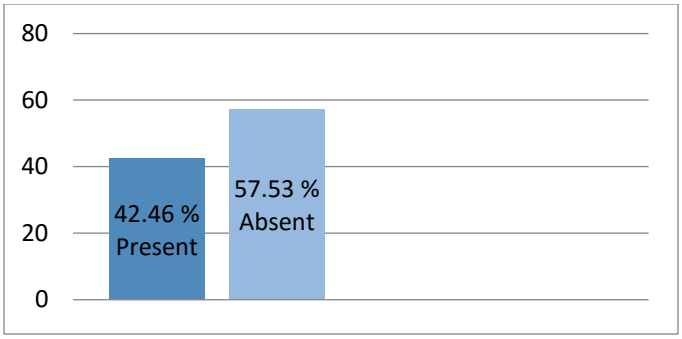

Figure 2: Frequency of Porphyromonas gingivalis in chronic periodontitis

Table 1: Demographic data and other factors associated with periodontitis

		Number	Percentage
Gender	Male	25	34.24%
	Female	48	65.7%
Socioeconomic status	Middle	14	19.17%
	Low	59	80.8%
Brushing	Never	15	20.5%
	Seldom	41	56.1%
	Daily	17	23.28%
Bleeding on probing	Present	55	75.3%
	Absent	18	24.6%

Table 2: Inhibitory effects of black seed extract on Porphyromonas gingivalis

STRAIN ID	Black seed extract (zones in mm)				Metronidazole disc (50 ug)
	25%		50%	75%	100%
	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD
51501	30.33±0.57	42±1	34.8±0.72	36.5±0.5	40.5±0.50
51504	30.2±0.87	44.83±1.25	34.8±0.72	37±0.0	41.33±1.15
51505	31.73±0.46	45.5±0.81	35.16±1.25	39.6±0.57	42±1
51512	28.93±0.90	47.83±0.76	30.83±0.76	35.13±1.02	43.16±1.25
51517	30.56±0.51	45.16±0.28	35.7±0.64	36.6±1.15	42±0.5
51527	30.2±0.87	45.5±0.5	33±0.8	37.33±0.46	43.9±1.35
51537	27.2±0.72	41.5±1.32	30.2±1.2	38.5±0.86	39.2±1.05
51542	32.3±0.3	44.5±0.5	34.8±0.72	37.2±1.05	40.33±1.52
51553	30.13±1.20	41.5±1.32	34.1±0.76	36.3±0.57	39.46±0.8
51565	31.8±0.72	40.16±0.76	36.4±0.50	36.5±0.86	40.6±1.03
ATCC 33277	31.4±0.50	44.83±1.25	33±0.8	35.13±1.02	39.2±1.05

Table 3: MIC of ethanolic extract of black seed on isolates using agar dilution assay

	MIC (mg/ml)			
	MIC Range	MIC 50	MIC 90	MIC 100
Porphyromonas gingivalis **n=31**	2-4	>2	4	4

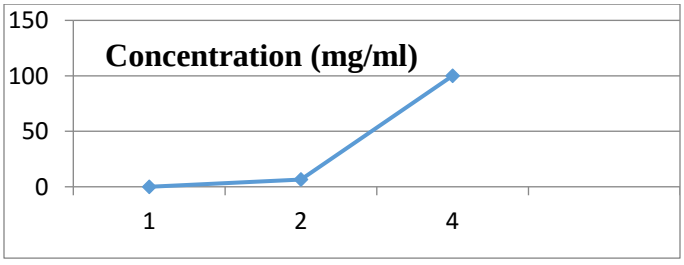

Figure 3: Cumulative percentage of Porphyromonas gingivalis inhibited (MIC) at different concentration of black seed extract

DISCUSSION

Periodontitis is a polymocrobial infection and is characterized by loss of connective tissue attachment and destruction of alveolar bone.[17] Success of periodontal therapy relies upon elimination or suppression of the subgingival bacteria. Thereby, antimicrobial agents attempt to directly reduce the pocket microflora when applied as an adjunct to mechanical debridement.[18] Porphyromonas gingivalis has been regarded as the main periodontopathogen involved in the onset and development of chronic periodontitis. Evidence from several researches has implicated that this specific bacteria plays a key role in the pathogenesis of chronic periodontitis.[19] In this study, subgingival biofilms of seventy three patients having chronic periodontitis were screened for the presence of P. gingivalis. 42.46% of the patients were detected to harbor P. gingivalis in their subgingival plaque samples. In the present study, associations between several factors with chronic periodontitis have been observed. Majority of the patients were women (62%). Hormonal changes and pregnancy in females can lead to decrease in mineral content of bone and teeth and thus, predisposes them to periodontitis.[20] 80.8% of the patients belonged to poor socioeconomic status. This is in accordance with Neuman et al. who identified a lower occupational status limiting the use of dental services.[21]On oral examination, 20.5% of the patients had poor oral hygiene. This suggests that periodontitis is associated with poor oral hygiene.

To the best of our knowledge, this is the first study in which black seed extract has been evaluated as an adjunct to conventional periodontal therapy in patients with chronic periodontitis. MIC of the extract was performed by agar dilution method. Our MIC ranged from 2 mg/ml to 4 mg/ml, with MIC_{50} being > 2 mg/ml and MIC_{90} being 4 mg/ml. At concentraion of 2 mg/ml, only 6.4 % isolates were inhibited, 100 % inhibition was observed at 4 mg/ml. A number of clinical researches have established that N. sativa extract has a strong efficacy against a number of Gram negative as well as Gram positive bacteria. In 2005, Nair et al., demonstrated that black seed extract had potent antimicrobial activity against all the strains of Listeria monocytogenes.[22] Another study also supports the antimicrobial activity of black seed extract against Gram negative bacteria where the zone of inhibition ranged from 18 to 32mm.[23] These results were in concordance with a study conducted by Khalid et al., in 2011. [24] In Pakistan, a study conducted by Hannan et al in 2008 indicated that N. sativa has antimicrobial activity against MRSA. Ethanolic extract of black seed inhibited the growth of all tested strains of MRSA and had MIC range of 0.2-0.5 mg/ml.[25]

Hence, we can conclude that within the limitations of the present study, patients with untreated moderate chronic periodontitis harbouring P. gingivalis in their subgingival biofilm may benefit from the topical administration of black seed extract in the form of toothpastes or mouthwashes. Ethanolic extract of black seed has potent antibacterial activity against P. gingivalis, however, this finding needs to be confirmed with further clinical trials.

CONCLUSION

In conclusion, black seed has antibacterial activity against P. gingivalis. Therefore, an addition of black seed to oral health-care products like toothpastes and mouthwashes may offer additional and complementary advantages in the maintenance of oral health and decreasing periodontal diseases. More studies should be carried out to explore the antimicrobial efficacy of black seed against subgingival flora associated with periodontitis.

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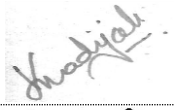
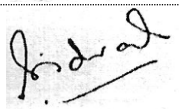
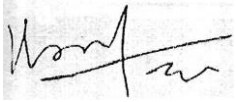
Dr. Khadijah Waris Lecturer, Department of Oral Pathology, Faisalabad Medical University, Faisalabad	Researcher, Data collector	
Dr. Sidrah Saleem Associate Professor, Head of Microbiology University of Health Sciences, Lahore	Research supervision	
Dr. Muhammad Usman Arshad Laboratory Manager of Microbiology, University of Health Sciences, Lahore	Practical work supervision	
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