Traditionally used herbal medicines with antibacterial effect on Aggregatibacter actinomycetemcomitans: Boswellia serrata and Nigella sativa

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Abstract:

Background: Since bacterial strains developed resistance against commonly used antibiotics and side effects became more serious, other alternatives have been postulated. There is an answer for this issue in ancient medicine. Many plants have been proved to provide antibacterial effect. In this study, Boswellia serrata (BS) and Nigella sativa (NS) were assessed to evaluate the antibacterial effect on Aggregatibacter actinomycetemcomitans (A.a) known as main pathogen of aggressive periodontitis. Materials and Methods: Broth microdilution method was used to obtain minimum inhibitory concentration (MIC) of crude extract of BS and NS. Furthermore, the logarithm of colony forming units grown in fresh brain heart infusion bacterial culture was assessed. Three groups including BS+ (containing only BS), NS+ (containing only NS), and BS-NS− (control group) were defined. For each group, the experiment was repeated 12 times. Results: MIC of BS and NS were 512 µg/mL and 128 µg/mL, respectively. No growth was observed in our negative control group. The mean ± standard deviation of logarithm of CFU/mL for BS, NS, and control group was 4.32 ± 0.3, 3.61 ± 0.3, and 5.57 ± 0.19, respectively. ANOVA test revealed significant difference (P values < 0.0001) of these groups which was later confirmed using the post hoc test of Tukey’s honest significant difference (all P < 0.0001). Conclusions: Both BS and NS are effective against A.a which should be taken into account as appropriate ingredient for oral hygiene products.

Key words: Aggregatibacter actinomycetemcomitans, aggressive periodontitis, Boswellia serrata, minimum inhibitory concentration, Nigella sativa

INTRODUCTION

Recently, the use of antibiotics has been restricted as bacterial strains developed resistance, and side effects became more serious.1 The one alternative for commonly used antibiotics is herbal medicine.2 Plants have been known for centuries to have antimicrobial effect against a wide spectrum of pathogens from bacteria to fungi and viruses.3,4 It has been reported that 35,000–70,000 plant species has been used as home remedies around the world.5 This huge number of medicinal plants has fueled recent studies to find extracts and active metabolites with antimicrobial properties.6–8

Since the specific plaque hypothesis has been introduced, it is believed that there are specific organisms to develop aggressive forms of periodontal disease. This idea became stronger as Aggregatibacter actinomycetemcomitans (A.a) was recognized in localized aggressive periodontitis.9 Although it has been shown that the periodontal infection is polymicrobial and contains hundreds of different bacterial species10–12 but the aggressive forms of the disease has been correlated to a specific Gram-negative anaerobic rods in the subgingival biofilm that colonize the periodontal crevice.11 A.a is a Gram-negative coccobacillus measuring about 0.4 ± 0.1 × 0.1 ± 0.4 micrometers in size12 with seven known serotypes.13 Each serotype may exhibit different virulence which is attributed to variations in the genome content and expression of the virulence gene.14 The most important virulence property of the bacteria is

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How to cite this article: Maraghehpour B, Khayamzadeh M, Najafi S, Kharazifard M. Traditionally used herbal medicines with antibacterial effect on Aggregatibacter actinomycetemcomitans: Boswellia serrata and Nigella sativa. J Indian Soc Periodontol 2016;20:603-7.
an expression of leukotoxin\textsuperscript{[15]} which can impair white blood cell function in different ways. Thus this ability is crucial for subsequent bacterial growth and stimulation of the host inflammatory response.\textsuperscript{[16]}

* Boswellia serrata* (BS) is known for its anti-inflammatory properties in folk medicine.\textsuperscript{[17]} It has been reported that boswellic acid, major constituents of the gum resin, is active against a large number of inflammatory diseases.\textsuperscript{[18]} In addition, recent studies have revealed the antibacterial effect of boswellic acid against different bacterial species.\textsuperscript{[19]}

* Nigella sativa* (NS) known as black cumen, have been used as food additive and herbal medicine for thousands of years in the Middle East, Far East and Asia.\textsuperscript{[20]} Conventionally, it has been used as carminative, diuretic, lactagogue and vermifuge and to treat fever, common cold, headache, asthma, rheumatic diseases and warts.\textsuperscript{[21-23]} Recent studies are indicative for possible antimicrobial, immune stimulant,\textsuperscript{[24]} and anti-inflammatory\textsuperscript{[25,26]} effects of NS oil and extracts.

In this study, we aimed to investigate antibacterial effect of BS and NS on A.a.

**MATERIALS AND METHODS**

**Preparation of extracts of medicinal plants**

The BS and NS were purchased from the commercially available products at local market of Tehran, Iran. The plants were poured in mortar and crushed in fine powder form with pestle. After 1 week of soaking the obtained powder in dimethyl sulfoxide (DMSO; Sigma-Aldrich, South Korea), the extract was filtered and centrifuged at 4000 rpm for 30 min at 4°C. The extract was concentrated in evaporator at 40°C and stored at 4°C for further analysis.

**Bacterial strains and culture conditions**

The pathogenic bacterial strain was obtained from Rayen Biotechnology Co. Ltd., Tehran, Iran. Cultures of lyophilized A.a ATCC 33384 were maintained on brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) and incubated in a microaerophilic atmosphere at 37°C and 5% CO2 for 48 h. For tests needed cultures on plates, cultures were first grown in supplemented BHI broth and then were transferred onto BHI agar (Merck, Darmstadt, Germany) plates. Fresh BHI bacterial culture, in the logarithmic growth phase, was adjusted to a concentration of 10\(^8\) CFU (colony-forming units)/ml as verified by colony counting.

**Minimum inhibitory concentration of Boswellia serrata and Nigella sativa**

The minimum inhibitory concentration (MIC) of BS and NS against A.a was assessed using broth microdilution method based on Clinical and Laboratory Standards Institute guidelines.\textsuperscript{[27]} Testing was done using 96well flat-bottomed microplate (Nunc, Denmark), with an assay volume of 200 µl/well. First, Mueller–Hinton broth (MHB) (Merck, Darmstadt, Germany) was added (95 µl) to each well. The BS (0.25–128 µg/ml mg/ml) and NS (100–300 mg/ml) extract transferred to wells and serially diluted 2-fold across the plate. The plates were then inoculated with a 20 µl/well of fresh MHB bacterial cultures, with a concentration of 10\(^8\) CFU/ml for A.a. The final bacterial cell concentration in the wells was 10\(^7\) CFU/ml. Then the microplates were incubated for 48 h at 37°C, under microaerophilic conditions. The MIC was defined as that concentration of the substance that will inhibit the visible growth of microorganism after 48 h of incubation. All tests were repeated at least 3 times.

**Antimicrobial effect of Boswellia serrata and Nigella sativa against Aggregatibacter actinomycetemcomitans**

To determine the antimicrobial effect of herbal medicines on A.a, samples were distributed to 5 groups as follow: (1) Group BS+ (treated with MIC of BS), (2) Group NS+ (treated only with MIC of NS), and (3) Group BS – NS– (negative control; no exposure to either BS or NS against). Tests were performed using previously described procedure. For each group, the experiment was repeated 12 times.

**Statistical analysis**

In terms of comparing means, one-way ANOVA was used to analyze the data which was followed by the post hoc test of Tukey's honest significant difference (HSD) used to assess the significance of all paired groups comparisons. All analyses were performed using Statistical Package of Social Science software (SPSS version 22; SPSS, Inc., Chicago, IL, USA), and the P < 0.05 was set as statistically significant.

**RESULTS**

The bacteriostatic effect of BS and NS was assessed using micro broth dilution, and it revealed that MIC of BS and NS were 512 µg/ml and 128 µg/ml, respectively. No growth was observed in our negative control group. We also investigated BS and NS effect on colony forming unit/ml. Table 1 shows the logarithm of CFU/ml in each round of experiment. The mean ± standard deviation of logarithm of CFU/ml for BS+, NS+, and BS-NS-group was 4.32 ± 0.36, 3.61 ± 0.3, and 5.57 ± 0.19, respectively. ANOVA test was used to assess the significance of observed difference and it showed P < 0.0001. To confirm ANOVA test result, the post hoc test of Tukey’s HSD was used [Table 2]. This test was also indicative for statistically significant difference in multiple comparison between study subgroups (all P values < 0.0001). Homogeneity of the mean logarithm of CFU was tested as well [Table 3]. It was shown that logarithm of CFU means of study groups are not homogeneity P value < 0.0001.

**Table 1: Logarithm of colony forming unit/ml in each round of experiment**

| Number of experiment | BS+ | NS+ | BS-NS- |
|----------------------|-----|-----|--------|
| 1                    | 4.28| 3.41| 5.44   |
| 2                    | 4.33| 3.62| 5.79   |
| 3                    | 4.74| 3.73| 5.90   |
| 4                    | 3.97| 5.05| 5.37   |
| 5                    | 3.68| 3.23| 5.34   |
| 6                    | 4.25| 3.51| 5.52   |
| 7                    | 4.77| 3.93| 5.69   |
| 8                    | 4.01| 3.86| 5.38   |
| 9                    | 4.57| 3.12| 5.66   |
| 10                   | 4.73| 3.99| 5.81   |
| 11                   | 4.59| 3.61| 5.61   |
| 12                   | 3.96| 3.33| 5.39   |
| Mean±SD              | 4.32±0.36| 3.61±0.3| 5.57±0.19 |

BS – Boswell serrate; NS – Nigella sativa; SD – Standard deviation
antimicrobial activity against *A. a* which showed to be similar to doxycycline with similar inhibition zones. They claimed Tulsi leaf as effective and affordable adjunct treatment in order to manage periodontal conditions. Inhibitory effect of citrus sinensis peel on *A. a* (33384), was assessed by Hussain et al.,[41] MIC of citrus sinensis (up to 25 mg/mL concentration) as if it was prepared in cold ethanol, cold water, hot ethanol and hot water extract was 13.2 mg/mL, 30 mg/mL, 12.2 mg/mL, and 28.9 mg/mL, respectively. They claimed that *A. a* was inhibited by citrus sinensis but at very high concentrations. There is a growing evidence about antimicrobial, anti-fungal and antioxidant activities of one of the commonly consumed beverage, camellia sinensis known as green tea.[34,42] Araghizadeh et al.,[43] reported that aqueous extract in concentrations of 6.25, 12.5, 25, and 50 mg/ml produces inhibition zones ranging from 10 to 38 mm with MIC of 6.25 mg/ml for *A. a* (ATCC 29523). Previous studies were suggestive that Chlorogenic acid and caffeic acid which can be found in green coffee bean extract inhibit the growth of some Gram-positive microorganisms such as *Staphylococcus aureus*, *Bacillus cereus*, and *Lactobacillus bulgaricus*. In a study by Bharath et al.,[44] they showed that this inhibition is not limited to Gram-positives as it had MIC of 0.2 µg/ml on *A. a* (ATCC 29523).

There are limited studies about antimicrobial effect of BS and NS on *A. a* but other microorganisms have been studied in previous literature. Allahghadri et al.,[47] investigated antimicrobial property of essential oil from *Cuminum cyminum* (other member of Apiaceae). *Escherichia coli* (ATCC 25922), *S. aureus* (ATCC 25923), *Streptococcus faecalis* (ATCC 33186) were all sensitive to various oil dilutions.

Javed et al.,[49] used agar-disc diffusion method to investigate if BS and NS exhibits stronger antibacterial effect when combined. It was shown that they have synergistic effect on *S. aureus*, *Pseudomonas* spp, Klebsiella pneumonia, *Salmonella* paratyphi, and *Enterococcus faecalis*.

Antimicrobial properties of main components of each BS and NS have been investigated in recent studies. In a study by Halawani et al.,[40] antibacterial effect of thymoquinone (TQ) and thymohydroquinone (THQ) against *E. coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Salmonella typhimurium*, *Salmonella enteritidis*, and *S. aureus* was tested. Interestingly, they found that 3 and 6 µg/mL of TQ were enough to inhibit and kill *S. aureus*. Although greater values of THQ were needed to inhibit or kill *S. aureus* the organism was sensitive irrespective to antibiotics susceptibility. They also reported that both TQ and THQ exhibited antibacterial effect on other Gram-positive and Gram-negative microorganisms but Gram-negative ones were less susceptible and MIC ranged between 200 and 1600 µg/ml in this group.

Bakathir et al.,[50] aimed to assess in vitro antibacterial effect of NS ground seeds as if it was prepared the same as human digestion mechanism. In this regard, they grounded NS and 0.75 mg of obtained powder was added to 2.5 cc D. W. A modified disc diffusion method was used for determination of the antibacterial activities. They found clear inhibition of NS with concentration of 300 mg/ml with D. W against *S. aureus*. An outstanding result was found in this study; NS seeds from different geographical regions had different growth inhibition.

**DISCUSSION**

In this study, we showed that both traditionally used herbal medicine, BS and NS, have antibacterial effect on *A. a* which has been proposed to be associated with aggressive forms of periodontal disease.

Although the exact pathogenesis of *A. a* is not fully understood the phenomenon of the inflammatory response in the gingival tissue is responsible for the gradual loss of dental collagen’s attachment to the alveolar bone.[28] This is the logic behind using BS and NS as they were known for their anti-inflammatory properties for centuries.[12] Furthermore, recent studies have proved this feature.[8,9]

The current treatment of periodontal diseases concentrates on removing the biofilm by a combination of mechanical treatments and systemic antibiotics. However, it has been shown to be unable to fully remove the bacterial biofilm.[29] In this regard, different strategies have been introduced; photodynamic therapy with either chemical photosensitizer or herbal ones,[30] adjunctive use of systematic antibiotics,[31] oxygen high-level laser therapy[32] and potential probiotic bacteria such as *Lactobacillus* spp, with biofilm degradation of ≥90% reduction[33] are amongst these treatments.

In our previously published study, we discussed the anti-bacterial effect of curcumin on *A. a* which showed to be reinforced as simultaneous photodynamic therapy was applied.[34] Recent studies have dedicated to investigate inhibitory properties of different herbal medicine on *A. a* activity. In a study by Kapadia et al.,[35] they evaluated the antimicrobial activity of banana peel (*Musa paradisiaca* L.) extract on *A. a* (ATCC 43718) using well agar diffusion method. They showed that *A. a* had 12 mm inhibition zone against an alcoholic extract. Ocimum sanctum Linn which is commonly known as Tulsi, has been used by many traditional medical practitioners to cure various diseases.[36] Recent studies have revealed antioxidant properties[37] and COX2 inhibition of this herb.[38] Mallikarjun et al.,[39] used 5% and 10% concentrations of ethanolic extract of Tulsi and demonstrated that it had

### Table 2: Multiple comparison of study subgroups

| Group (a) | Group (b) | Mean difference (a-b) | 95% CI | P     | Lower bound | Upper bound |
|----------|-----------|-----------------------|-------|-------|--------------|-------------|
| BS+      | BS-NS-    | -1.25                 | -1.54 | -0.95 | <0.001       |             |
| NS+      | BS-NS-    | -1.95                 | -2.25 | -1.66 | <0.001       |             |
| BS+      | NS+       | 0.7                   | 0.4   | 1     | <0.001       |             |

**BS** – Boswell serrate; **NS** – Nigella sativa; **CI** – Confidence interval

### Table 3: Comparison of subgroups in terms of mean homogeneity (the results of Tukey’s honest significant difference test)

| Group | n   | Subset for alpha=0.05 | 1   | 2   | 3   |
|-------|-----|-----------------------|-----|-----|-----|
| NS+   | 12  | 3.61                  | 4.32| 5.57| 1.000|
| BS+   | 12  | 4.32                  | 1.000| 1.000|
| BS-NS-| 12  | 5.57                  | 1.000| 1.000|

**BS** – Boswell serrate; **NS** – Nigella sativa
property against tested bacterium; as NS from Hadhramaut showed higher inhibition than NS from Ethiopia. Researchers believed that this inhibition feature is due to two active ingredient, TQ and melamin.

Acetyl-11-keto-b-boswellic acid (AKBA) is the most potent antibacterial component of boswellic acids obtained from the oleo gum resin of BS. It has been investigated for inhibitory effect on oral cavity pathogens in Raja et al.’s study. AKBA had MIC of 2 µg/ml against Streptococcus mutans ATCC 25175, Actinomyces viscosus ATCC 15987 and S. sanguinis ATCC 10556. In addition, it had MIC of 4 µg/ml against Enterococcus faecalis ATCC 29212, Enterococcus faecium ATCC 8042, Prevotella intermedia ATCC 25611 and Porphyromonas gingivalis ATCC 33277. They also reported that AKBA can produce post-antibiotic effect for 5.7 ± 0.1 h at 2 × MIC. Furthermore, biofilms generated by S. mutans and A. viscosus were inhibited by AKBA. They proposed that AKBA is of great value to be used in mouthwashes.

Our study faced few limitations which made it difficult to compare our results to previous studies. According to our literature search, it would be the first time that a study evaluated antibacterial effect of BS and NS on A.a. The exact comparison of our result to previous studies is not possible. Although it would not be irrational to conclude if BS and NS showed antibacterial effect on both oral Gram-positive and Gram-negative microorganisms so it might have antibacterial effect on A.a but it is needed to be proved by other trials.

Second, we used commercially available BS and NS in local market, and it has been proved that herbs from different geographical regions may have introduce a different amount of active components. This issue may limit further comparison between studies using aqueous or alcoholic extracts of BS and NS as researchers worldwide may use locally available herbs.

Third, we used crude extract of BS and NS which resulted in higher MIC in our study compared to studies using active ingredients even for different bacterium. This issue should be considered when different MICs are compared as if they might be the result of different preparation of BS and NS against A.a.

CONCLUSION

Herbal medicine of different family should be taken into account as an adjunct or even main component of oral hygiene agents especially those targeting A.a in patients with aggressive forms of periodontitis. In this regard, BS and NS are amongst the most compatible herbs.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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