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Antibacterial effects of the crude *Azadirachta indica* Neem bark extract on *Streptococcus sobrinus*

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Abstract *Azadirachta indica*, known as a Neem in the Indian sub-continent, is widely grown all over the tropics. The anti-bacterial activities of aqueous and acetonic extracts of Neem bark (NBE) were examined on agar plates by using *Streptococcus sobrinus*. In this study, the aqueous extract (5% w/v) showed no detectable antimicrobial activities on agar, the acetonic extract (5% w/v) at the same concentration, however, produced appreciable antimicrobial effects. The acetonic extract from the bark of Neem was bactericidal at concentrations ≤1% (w/v). The agar dilution test for minimum inhibitory concentration (MIC) of NBE was shown that the acetonic extract of Neem bark had an effect on the growth of *S. sobrinus* with MIC values of 0.05% (w/v). These results indicate that Neem bark constituents are considered to have the ability to suppress the growth of cariogenic bacteria.

Key words
Growth inhibition,
Neem bark extract,
Streptococcus sobrinus

Introduction

The evergreen tree, Neem, has been used as a traditional medicine for many centuries in India[1,2]. Earlier studies have indicated that stem bark of Neem contains some substances with strong anti-inflammatory activity[3]. Various preparations of Neem obtained from its different parts have been found to exert antibacterial, antimalarial, contraceptive and antiulcer activities[4-6]. Recently, Wolinsky *et al.*[7] have reported that the active components from a bark containing Neem stick have appeared to inhibit virulence factors of oral streptococci associated with dental plaque formation. From other studies, it has been noted that Neem can be regarded as a valuable plant source for the rationalization of its use in traditional medicine and for modern drug development[8]. Meanwhile, Neem's different parts such as the bark, leaves, seeds and flowers are widely used in traditional Indian medicine. Earlier reports have shown that dicotyledonous plants, their bark and pulp contain a tannin-like substance that inhibits bacterial growth[9]. The selection of bark from the Neem tree for the present study was based on a number of factors. It is most common in the Indian subcontinent region, and the bark is very easy to peel from the stem of the tree. It is also very cheap and reported to have antiplaque and many pharmacological properties[3,7]. Therefore, the stem bark of Neem was chosen as test material in this study to explore the hypothesis that it may contain active substances with anti-microbial properties. The purpose of the present study was to investigate the effects of aqueous and acetonic Neem bark extracts on the growth of *S. sobrinus*.

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Materials and methods

Preparation of crude extract

Extracts were obtained from the stem bark. The bark was peeled from the stem with a sharp knife and chopped into pieces, which was sun-dried and ground into powder using a blender (Matsuden MJ-700, Tokyo, Japan). The resulting powder was then stored at room temperature in a clean, air-tight and

wide mouth container.

Acetonic NBE

Ten grams of Neem bark powder was mixed with 100 m*l* of distilled acetone in a conical flask. The mixture was then magnetically stirred for 24 h at room temperature. The homogenate was vacuum filtered through filter paper (Toyo Roshi Co., Tokyo, Japan). The clarified filtrate was evaporated using a vacuum rotary evaporator at about 35°C. The residue was resolved in a little acetone. The resulting concentrated solution was vacuum pumped using a direct drive rotary vacuum pump (Hitachi Ltd., Tokyo, Japan) to remove the acetone solvent and to obtain extract powder. A 5% (w/v) solution of the resulting extract was then prepared in 40% (v/v) distilled acetone. The prepared solution was stored at −20°C until use.

Aqueous NBE

Ten grams of Neem bark powder was mixed with 100 m*l* of distilled water in a conical flask. The mixture was then magnetically stirred for 60 h at room temperature. The homogenate was then centrifuged at 5000 rpm for 10 minutes. The supernatant was vacuum-filtered against filter paper (Toyo Roshi Co., Tokyo, Japan). The clarified filtrate was divided into two halves. One half was dialyzed against distilled water and the other half was not dialyzed. Both the dialyzed and non-dialyzed materials were then lyophilized. The 5% (w/v) solution of both lyophilizates was then prepared in distilled water. The prepared solution was stored frozen at −20°C until use.

Bacterium and media

Streptococcus sobrinus K1R, serotype g, was used in the present study.

Tryptic soy (TS) agar (Difco Laboratories, Detroit, Mich.), as a nutrient medium and Tryptic soy broth (Difco) as a routine growth medium were used.

Tests for antimicrobial activity

A 6-hour culture of *S. sobrinus* K1R, ($OD_{660} = 0.34$), was inoculated into melted TS agar followed by pouring into the sterile plates. The ditches were then filled with different concentrations (1% w/v, 3% w/v, and 5% w/v) of Neem bark extract (NBE). For the control experiments, 40% (v/v) distilled acetone as the negative control and 0.2% chlorhexidine gluconate (Wako Pure Chemical Ltd., Tokyo, Japan) as the positive control were used. The plates were incubated under anaerobic condition (95% N_2 and 5% CO_2 gas) at 37°C for 48 h and examined for inhibition zones of the growth of the bacterium around the extracts, and the average diameters of the inhibition zones were reported.

The MIC was determined by using the agar dilution method[13]. Two m*l* of extracted solutions at the different concentrations (1% w/v to 0.078% w/v) was mixed with 18 m*l* of sterile molten Nutrient agar in the conical flask. Final concentrations of NBE in the Nutrient agar were 0.1% w/v to 0.0078% w/v respectively. The mixture was then magnetically stirred to obtain a homogenous mix. The 2 m*l* of

Table 1 Inhibition of the growth of *S. sobrinus* in the presence of varying percentages of Neem bark extract (NBE)

	Zone of inhibition (mm) with S.D.
Preparation tested	TS agar
Acetonic NBE	mean ± S.D.
5%	16 ± 1
3%	14 ± 0
1%	12 ± 0
Aqueous NBE	
5% (dialyzed)	0
5% (non-dialyzed)	0
Control	
40% acetone (negative control)	0
0.2% chlorhexidine (positive control)	18 ± 1.5

Values represent the mean ± S.D. obtained from three different experiments.

40% v/v acetone without added test materials was used as the routine control. The resulting mix was poured into the sterile plates and allowed to set.

A cell suspension of *S. sobrinus* (3.5×10^9 CFU/ml) was diluted with the sterile saline to become about 10^6 CFU/ml. Aliquots of the 10 μl bacterial suspension from this dilution (10^6 CFU/ml) were then spotted at four different places onto each concentration plate. For comparison, 10 μl was pipetted onto the control plate followed by anaerobic incubation at 37°C for 48 h (95% N_2 and 5% CO_2) and minimum inhibitory concentration was defined as the lowest concentration of extract at which no growth was observed within the area of inoculation.

For confirmation of the MIC, the following steps were continued. After incubation, a loop of sample was taken from each spot with a sterile platinum loop and streaked on Nutrient agar followed by incubation for 48 hours. The growth of bacteria forming visible colonies in these plates was observed.

Results

Table 1 shows the growth inhibition in the presence of varying percentages of NBE on TS agar. Acetonic Neem bark extract (NBE) produced the greatest antibacterial activity whereas *S. sobrinus* received no effect from the aqueous NBE both dialyzed and non-dialyzed on agar. In the control experiments, the 40% (v/v) distilled acetone was used as the negative control produced no visible zone of inhibition and 0.2% chlorhexidine gluconate used as the positive control exhibited the greatest zone of inhibition. The minimum inhibitory concentration (MIC) of acetonic NBE against *S. sobrinus* was 0.05% (w/v).

Discussion

Several plants serve as sources of therapeutic agents containing fluoride, antibiotics and other medicinal compounds[10,20]. The active substances isolated from these plants have been shown to possess potent antimicrobial, analgesics and anti-inflammatory activities[14–16,19]. Neem is used in traditional medicine as a source of many therapeutic agents in the Indian culture and grows well in the tropical countries. Its twigs provide a chewing stick and is widely used in the Indian sub-continent[10]. Very recently, it has been reported that the bark containing Neem chewing-stick has been shown to have plaque inhibitory substances[7]. In addition, earlier studies on Neem have shown that it contains an active substance with multiple medicinal properties[4–6,18]. Results from the present study have demonstrated that acetonic NBE by ditch plate method showed strong anti-bacterial activity against *S. sobrinus*. The Neem bark extract at 5% w/v concentration exhibited an appreciable zone of inhibition (16 mm on TS agar) compared to the positive control of 0.2% chlorhexidine (18 mm on TS agar). However, aqueous NBE even at a high concentration of 5% w/v did not show growth inhibitory effects on agar.

Mutans streptococci in particular, *Streptococcus mutans* and *S. sobrinus* are the causative agents for dental caries in man[11]. In another study, it has been observed that *S. sobrinus* is more acidogenic than *S. mutans*[12]. Since *S. sobrinus* is involved in dental plaque formation and subsequent incidence of dental caries, it was chosen as the test organism in our study. The MIC of acetonic NBE against *S. sobrinus* was found to be 0.05% w/v as determined by agar dilution method. Meanwhile, this MIC value was obtained from eight serial dilutions of NBE where the maximum was 0.1% w/v and minimum was 0.0078% w/v final concentration. In the agar dilution test, no growth was observed in the first and second dilution that is 0.1% w/v and 0.05% w/v concentration respectively. From our study it was observed that the acetone extract of Neem at low concentration of $\leq$1% w/v showed a bactericidal effect (data not shown). We have also assayed the three fractions of acetonic NBE to observe the growth inhibitory effect of the fractionated extract. The hexane and ethylacetate fractions exhibited surprising growth inhibitory effects on agar plates. However, the aqueous fraction of acetonic NBE still did not show antimicrobial effect (unpublished data). Though the aqueous NBE exhibited no detectable effect on growth, its active compound have been reported to have strong anti-inflammatory activity. Based on our experimental results, it is our speculation that extract compounds obtained from Neem bark using water not sufficient enough to show the growth inhibitory effect against *S. sobrinus*. On the contrary, sufficient extraction of active substance from plants (Neem) source using organic solvent, acetone, exhibited detectable growth inhibition. In a short, due to insufficient extract materials derived from Neem bark using water, aqueous NBE did not cause inhibition of growth against *S. sobrinus* on agar. However, further investigation will be needed to determine the efficacious

ability of the aqueous extract of Neem bark. We hope to report in the future the chemical nature of the active constituents of the NBE.

In conclusion, the extracts of Neem when used as a medicinal plant, could be useful for the growth inhibition of the cariogenic bacterium, *S. sobrinus*.

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