Antimicrobial activity of *Azadirachta indica* (neem) and *Salvadora persica* (miswak) extracts as endodontic irrigants

**Introduction**

Cytotoxicity and harmful effects of the gold standard endodontic irrigant used in clinical practice (NaOCl) have prompted researchers to look for alternatives that are easily available, non-toxic and have effective antimicrobial activity. It has been found that the extracts of certain natural plants could be used as effective endodontic irrigants. John et al. showed, in an in-vitro study, that natural products, such as propolis, *Acacia nilotica*, *Azadirachta indica* (neem) and *Aloe vera*, might have a promising role as root canal irrigants.

Neem is a tree well known in India and its neighbouring countries as one of the most common medicinal plants, having multiple properties and a wide spectrum of biological activity. It is also called Indian neem, Margosa tree or Indian lilac. It is an evergreen tree up to 24 m tall with a hardy and fast-growing stem. The leaves are divided into numerous leaflets resembling a full-grown leaf, which are auxiliary bunches and 1.5 to 2 cm long. It has green or yellow fruits with a seed in each and small white flowers.

Neem contains the active constituents sodium nimbinate, salannin, gedunin, nimbin, azadirachtin,
nimbidol, quercetin and nimbidin. Neem leaves contain carbohydrates, fibre, at least 10 amino acid proteins, calcium, carotenoids and fluoride\(^4\).

Biological properties and pharmacological actions of neem are known and well established, with extracts produced from different parts of the neem tree. Extracts may be obtained from its leaf, flowers, bark, roots, seed and oil\(^5,6\). Neem has various common biological properties: it is antiviral, antifungal, antimicrobial and antibacterial. It is also known to have antipyretic, anti-inflammatory, anti-tumour, analgesic, anti-helminthic, anti-cariogenic and anti-oxidant activities\(^4\).

In dentistry, \textit{A. indica} has been investigated and suggested for use as an alternative to chlorhexidine in cases of periodontal disorders due to its antimicrobial potential against oral microorganisms\(^7,8\).

The literature has shown that neem extract can be used as an endodontic irrigant due to its antimicrobial and therapeutic effects against root canal microorganisms\(^5\). The most important factor favouring its clinical application and its use as an endodontic irrigant is the biocompatibility to human periodontal ligament fibroblasts and, thus, it is not likely to cause the severe injury to patients that might occur with NaOCl accidents\(^9,10\). However, there is lack of documentation or data regarding neem research in endodontics.

The chewing stick miswak is prepared from the roots, twigs and stem of \textit{Salvadora persica} (Arak tree), and plays a role in the promotion of oral hygiene\(^11\). It was initially used by Babylonians around 7000 years ago\(^12\), followed by the Greek and Roman empires. It has also been used by Jewish, Egyptian as well as Japanese communities\(^13\). Nowadays, chewing sticks are widely used in Asia, Africa, South America and throughout the Middle East\(^12,14\).

\textit{S. persica} is considered to be a medicinal herbal plant\(^15\). It exhibits antibacterial effects on \textit{Streptococcus mutans}, which is the most significant microbe contributing to dental caries. This effect of \textit{S. persica} against cariogenic bacteria is due to its active components such as salvadorea and trimethylamine\(^16\).

As the value of miswak is in its mechanical cleaning action, the use of miswak has been reported to be effective against some periodontal pathogens and some other bacteria contributing to dental plaque development\(^17\). A comparative study aimed to assess plaque removal in miswak and toothbrush users and showed that miswak was as effective on plaque removal as tooth brushing\(^18\). Miswak has also been documented as an antifungal agent\(^14\).

Al-Lafi and Ababneh\(^19\) found that benzyl thiocyanate present in \textit{S. persica} inhibited the growth of \textit{S. mutans}, and suggested that \textit{S. persica} decreases the incidence of dental caries. Wolinsky and Sote\(^20\), by isolation of the active ingredient from \textit{S. persica}, found that the limonoid had a great antimicrobial activity against various Gram-positive and Gram-negative microorganisms.

The antimicrobial and cleaning effects of miswak have been attributed to various chemicals detected in its extracts and due to its high content of NaCl and KCl, as well as salvadorea, salvadorine, saponins, tannins, vitamin C, silica and resin, in addition to cyanogenic glycoside and benzyl isothiocyanate\(^21\).

The use of miswak as an endodontic irrigant was studied by comparing its effects with other currently used root canal irrigants. It was reported that miswak extract had a significant antimicrobial effect against both aerobic and anaerobic bacteria, with a maximum effect at a concentration of 15\%\(^22\). Various concentrations of miswak extract were tested, and an effective antimicrobial property was reported\(^21,23,24\). However, there is insufficient evidence to support the utilisation of this extract as an irrigant solution in endodontic practice.

The present study aimed to evaluate and compare the antimicrobial activity of \textit{A. indica} (neem) and \textit{S. persica} (miswak) extracts as endodontic irrigants against \textit{Enterococcus faecalis} in comparison with the standard (NaOCl) solution. The null hypothesis was that there is no significant difference in the antimicrobial activity between the tested extracts (neem and miswak) and the control group (saline).
Materials and methods

Ethical approval was sought from Umm Al-Qura University Faculty of Dentistry (UQUDENT) Institutional Review Board (IRB). No study activities were started until IRB approval was obtained.

Sample collection and preparation

Fifty intact, unrestored, non-caries mature human single-rooted extracted anterior and premolar teeth were selected and examined radiographically to ensure the presence of a single canal. Teeth were cleaned and stored in normal saline solution until use. Each tooth was decoronated using a diamond stone to standardise the length of roots of all teeth to approximately 14 ± 1 mm. The working length (WL) for each root was recorded using a size 15 K-file (Dentsply Maillefer, Ballaigues, Switzerland) up to 1-mm short of the apical foramen. Canal preparation for all samples was performed with the step-back technique using hand K-files up to ISO size 40 master apical file. During instrumentation, all canals were irrigated with 5 ml of 2.5% NaOCl solution using a disposable syringe with a 23-gauge needle. Following preparation of root canals, the apical foramina were sealed with light-cured composite resin. Subsequently, each root specimen was autoclaved to be sterilised in a glass tube containing 3 ml of brain heart infusion (BHI) broth for 20 minutes at 121°C, and incubated for 48 hours at 37°C.

Preparation of A. indica (neem) leaves extract

Mature fresh neem leaves were collected from the Arafat region in Makkah, and identification of the plant was performed by an expert. Leaves were washed with sterilised distilled water, then 100 g of leaves were added to 200 ml of absolute ethanol. The mixture was macerated for 10 to 15 minutes, and the extract was filtered using a muslin cloth for coarse residue. The extraction process was repeated using the coarse residue and 100 ml of ethanol. Both extracts were pooled together and filtered through Whatman quantitative filter paper, Grade 1 (Sigma Aldrich, Gillingham, UK). Alcohol was removed from the extract by placing it in an incubator at 37°C for a few days until the volume reached approximately 100 ml. The extract was kept ready and stored in an airtight container (Fig 1).

Preparation of S. persica (miswak) extract

Chewing sticks were cut with a sharp knife into small pieces, and allowed to dry at 37°C for 1 week. These pieces were ground to powder using a cereal blender. Ethanol (120 ml of 60% concentration) was added to 40 g of miswak powder in a sterile well-capped flask, left for 3 days at room temperature and then filtered using Whatman quantitative filter paper, Grade 1. The extract was incubated at 37°C until it became dry and was stored in sterile screw-capped vials until needed. Serial dilutions of the extract were prepared using sterilised Ringer's lactate by taking 1 g of the alcoholic extract of miswak and dissolving it in 2.5 ml of Ringer's lactate to give 100% concentration. Then 60%, 30% and 15% concentrations were prepared (Fig 1).

Root canal inoculation

E. faecalis ATCC 29212 strain was used in root canal inoculation. It was grown on blood agar at 37°C for 24 hours. BHI broth was inoculated with the bacterial strain and the culture was allowed to grow until its optical density was approximately 1.5 x 10⁸ colony forming units (CFU)/ml, by comparing its turbidity to a 0.5 McFarland standard spectrophotometrically. Sterile BHI broth (2 ml) was removed from the tubes and replaced with 2 ml of the bacterial suspension. The tubes were then closed and incubated at 37°C for 48 hours. The tubes were equally divided into five groups according to the solution used for irrigation, with 10 roots per group:

- Group A: normal saline (control group)
- Group B: 2.5% NaOCl solution
- Group C: neem leaves extract
- Group D: 15% miswak extract
- Group E: a combination of 1.25% NaOCl and neem leaves extract (used alternately).
Each root was removed from the test tube and rinsed thoroughly with sterile physiological saline. A volume of 5 ml of the tested solution was used for irrigation of each sample and was allowed to remain in the canal for 10 minutes. A final flush was performed using 4 ml of sterile physiological saline in each sample using a disposable syringe with a 23-gauge needle.

**Microbial sampling and bacterial counting**

Excess moisture from the canal was removed using a sterile paper point. The first microbiological sample was taken from each root canal lumen using a dry sterile standardised paper point size 40, which reached the full WL for 10 seconds to absorb the fluid. The paper point was kept in a sterile Eppendorf tube containing 1 ml of sterile saline solution, and vortexed for 30 seconds. Serial dilutions of each sample were prepared ($10^2$, $10^3$, $10^4$, $10^5$ and $10^6$) and used for the bacterial count. After that, aliquots were plated out on bile-esculin agar and incubated at 37°C for 2 days. Bacterial colonies for *E. faecalis* were counted and multiplied by its dilution factor to get the number of CFU/plate (Fig 2).
Statistical analysis

Data were analysed using SPSS software version 22 (IBM, Armonk, USA). The Kruskal–Wallis test was used to compare the antimicrobial activity between groups, and Mann–Whitney test was used to assess significance between pairs of groups.

Results

A total of 50 extracted single-rooted teeth were used in this study. Teeth were divided equally into five groups. Group E, which was irrigated with a combination of 1.25% NaOCl and neem leaves extract used alternately, showed the lowest mean value of CFU, and the highest mean value was obtained with the control group, as shown in Table 1.

Comparison of all groups using the Kruskal–Wallis test revealed that the difference in bacterial counts between groups was statistically significant ($P = 0.001$).

The Mann–Whitney test was used for two-group comparison. Statistically significant results were found between all groups and the control except for the miswak group ($P = 0.545$). When group B was compared with group C and group E, the differences were statistically insignificant ($P = 0.183$ and 0.543, respectively), but there was a statistically significant difference when compared with group D ($P = 0.001$). There was a statistically significant difference between group C and group D at ($P < 0.001$), but the difference was insignificant for group C compared with group E ($P = 0.085$). When group D was compared with the combination group the difference was statistically significant ($P < 0.001$).

Discussion

The present study was conducted to evaluate and compare the antimicrobial activity of different irrigating solutions against *E. faecalis* in human single-rooted extracted teeth by root canal inoculation, microbial sampling and bacterial counting.

Table 1  The mean ± standard deviation values of colony-forming units (CFU) in all studied groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of CFU</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (Saline)</td>
<td>$4.33 \times 10^6 \pm 13.24 \times 10^6$</td>
<td>0.001</td>
</tr>
<tr>
<td>Group B (NaOCl)</td>
<td>$0.001 \times 10^6 \pm 0.003 \times 10^6$</td>
<td></td>
</tr>
<tr>
<td>Group C (Neem)</td>
<td>$0.0005 \times 10^6 \pm 0.0004 \times 10^6$</td>
<td></td>
</tr>
<tr>
<td>Group D (Miswak)</td>
<td>$0.0045 \times 10^6 \pm 0.0041 \times 10^6$</td>
<td></td>
</tr>
<tr>
<td>Group E (NaOCl + Neem)</td>
<td>$0.00007 \times 10^6 \pm 0.000020 \times 10^6$</td>
<td></td>
</tr>
</tbody>
</table>

*E. faecalis* was selected as the test organism because it has been reported to be the most common bacterial species repeatedly associated with failed root canal therapy and canals with persistent infections$^{27,28}$. It is a facultative organism that can survive in an environment lethal to many other microorganisms and can resist a wide range of intracanal medicaments; therefore, it has been used frequently in endodontic research$^{29,30}$. *E. faecalis* ATCC 29212 strain was selected in the present study as this strain has been studied previously in a similar context$^{9,26,31-37}$.

The irrigating solutions used in the present study were: saline as control irrigant; 2.5% and 1.25% NaOCl. Neem leaf extract and 15% miswak extract were the test materials. NaOCl was selected as it is the gold standard irrigant, used in clinical practice at concentrations ranging from 0.5% to 5%$^{38,39}$. It is the most commonly used endodontic irrigant due to its ability to dissolve...
organic and necrotic tissues and its wide-spectrum antimicrobial activity. However, it has a cytotoxic effect that damages living tissues, and other disadvantages include unpleasant taste, foul smell, and its corrosion of metal objects. These disadvantages increase with concentration.

A concentration of 2.5% NaOCl was used in the present study to test its antimicrobial effect against *E. faecalis* as previous studies tested antimicrobial activity of higher concentrations of NaOCl (> 2.5%) and reported an apparent antimicrobial effect. To overcome possible side effects of high concentrations of NaOCl and to meet the beneficial properties of the solution, 1.25% of NaOCl was used alternately with neem leaf extract to enhance the antimicrobial efficacy of both solutions.

Neem leaf and miswak extracts were selected in the present study owing to their previously studied beneficial properties, such as their antimicrobial and therapeutic effects, which suggested them as endodontic irrigants. They are safe to use and not likely to cause severe injury to patients.

A wide range of methods and new technologies are available for extraction of medicinally active portions of plants with the use of selective solvents. One of the traditional and most commonly used methods is maceration. This involves soaking plant materials (coarse or powdered) in a closed container with a solvent such as ethanol at room temperature for a period of time. This process is intended to soften and break the plant cell walls to release the soluble phytochemicals; the mixture is then pressed or strained by filtration. In the present study, the extraction methods followed those used frequently in previous studies.

Several concentrations of miswak extract have been tested for antimicrobial effect. In the present study 15% alcoholic extract of miswak was prepared, as previous studies confirmed a significant antimicrobial effect at this concentration.

The methodology of this study (sample collection and preparation, root canal inoculation, microbial sampling and bacterial counting) followed that of Darrag, with some modifications. All irrigants were allowed to stay in the canals for 10 minutes according to Berber et al., who reported that NaOCl eliminated the strains of *E. faecalis* in 10 minutes’ contact time. Bile-esculin agar plates were used instead of BHI agar plates to facilitate identification and counting of the bacterial colonies as this is the selective medium for *E. faecalis*.

Culture-based techniques are widely available and have a broad-range nature to detect the presence of bacteria. These techniques allow identification of unexpected species, quantification of all major viable cultivable microorganisms in samples and determination of antimicrobial susceptibilities of isolates. However, some important limitations of culture methods make a comprehensive analysis of the endodontic microbiota difficult to achieve. Low sensitivity, or the possibility that remaining bacterial cells have been stressed and are in a viable but not cultivable state, are the main disadvantages.

Human single-rooted extracted teeth were used in the present study to simulate the real environment of root canal treatment, which is better than disc diffusion methods. The minimum instrumentation size needed to facilitate penetration of irrigants to the apical third of the root canal is a size 30 file. In the present study, canals were enlarged up to ISO size 40 K-file to ensure bacterial inoculation and penetration of irrigants to the apical third of root canals. Following root canal preparation, the apical foramina were sealed with light-cured composite resin to prevent bacterial microleakage.

Kruskal–Wallis and Mann–Whitney tests were used for statistical analysis as the data were not normally distributed. The Kruskal–Wallis test showed that the difference in mean values of bacterial counts for all groups was statistically significant. The results showed that none of the tested irrigants completely eliminated the tested organism in root canals but there was a reduction in bacterial counts between treatment groups in comparison with the control. The Mann–Whitney test showed that the difference in mean values of bacterial counts between all groups with the control was statistically significant, except between miswak and control groups.

The results of the present study are in accordance with those of Al-Obaida et al., who reported...
that there was no significant difference between 20% miswak extract in the growth of *E. faecalis* after 1 hour of exposure and the control group (saline). Conversely, other studies, like those of Al-Salman et al\textsuperscript{24} and Shingare and Chaugule\textsuperscript{21}, found a significant difference between miswak extract and the control group (saline). These studies used 10% water extract of miswak and 12.5% alcoholic extract of miswak, respectively.

This difference in results regarding the antimicrobial effect of miswak extract against *E. faecalis* might be related to the difference in methodology, concentration of miswak extract and the type of miswak extract, as some studies used alcoholic extract\textsuperscript{21,23} (as in the present study), while Al-Salman et al\textsuperscript{24} used the aqueous extract of miswak.

In the present study, 15% miswak extract showed an antimicrobial effect against *E. faecalis* but it was lower than that of neem leaf extract, NaOCl solution and the combination group. This finding is in agreement with the results reported by Shingare and Chaugule\textsuperscript{21}, who found that NaOCl had a higher antimicrobial effect than 12.5% miswak extract.

In the present study, the lowest mean value of CFU was noticed with group E (alternate use of 1.25% NaOCl and neem leaves extract) followed by group C (neem extract), group B (2.5% NaOCl solution) and lastly, group D (15% miswak extract). Even with a lower concentration of NaOCl, the combination of 1.25% NaOCl and neem leaves extract had a better antimicrobial effect than neem extract or 2.5% NaOCl alone. This means that the combination of NaOCl with herbs (neem) potentiated the antimicrobial efficacy of both irrigants in addition to reducing side effects of the chemical solution. This finding is in agreement with the results of a previous study in as far as the combination of 2.5% NaOCl and neem leaves extract had better antimicrobial properties than individual irrigants due to the synergistic action between herbal and chemical irrigants\textsuperscript{36}. The extract might disrupt the bacteria adhering to the root canal walls and aid in the permeation of NaOCl to produce its antibacterial action. This may explain the synergism between the two irrigants.

Ethanol has been used to reduce the surface tension of NaOCl, which helps increase spreading ability\textsuperscript{49}. In the present study, ethanol was an integral part of the leaf extract and could have increased the spreading ability of the neem drug within the canal. When used with NaOCl within the root canal (sequential irrigation), ethanol helped reduce the surface tension of NaOCl. This could further explain the synergism between NaOCl and neem\textsuperscript{36}.

Neem extract has undergone extensive pharmacological screening and been found to have several pharmacological activities due to the presence of active constituents, such as nimbidin, nimbin, nimbolide, gedunin, azadirachtin, mahmoodin, margolone and cyclictrisulphide, responsible for its antibacterial action\textsuperscript{50}. Its anti-adherence activity by altering bacterial adhesion and the ability of the organism to colonise has resulted in having the maximum reduction in adherence of *E. faecalis* to dentin\textsuperscript{51}. In the present study, neem leaf extract showed a pronounced antimicrobial efficacy against *E. faecalis* that was even higher than that of 2.5% NaOCl.

The results of the present study are in accordance with the results of previous studies that compared the antimicrobial effect of neem leaf extract with different concentrations of NaOCl similar to those used in this study (2%, 2.5% and 3%), and they found that NaOCl showed less antimicrobial effect than neem extract\textsuperscript{2,9,26,31}. Conversely, Jose et al\textsuperscript{52} reported that 2.5% NaOCl showed a higher effect against *E. faecalis* than neem leaf extract. Even with a higher concentration of NaOCl (5.25%), previous studies found that NaOCl had a lower antimicrobial effect than neem leaf extract\textsuperscript{32,35}, which means that *A. indica* (neem) is a viable irrigant against *E. faecalis*.

The limitations of this study lie in selecting *E. faecalis* as the tested organism, and support the need for further studies to test the efficacy of herbal extract against root canal biofilms. Also, pre-clinical and clinical trials are needed to evaluate antimicrobial properties of herbal extracts and their biocompatibility.
Conclusions

Under the tested conditions and within the limitations of this study, it can be concluded that neem leaf extract can be used as an endodontic irrigant with effective antimicrobial properties against *E. faecalis*, whereas miswak extract resulted in the lowest antimicrobial effect against *E. faecalis*. Furthermore, the combination of herbal and chemical agents can produce a synergistic antimicrobial effect.

Declaration

The authors deny any conflicts of interest related to this study.

References


