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Elimination of *Enterococcus faecalis* in the root canals using ayurvedic extracts obtained from *Calotropis procera* : An *in vitro* study

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Abstract

The elimination of antimicrobial drugs resistant bacteria from root canals, such as *Enterococcus faecalis*, poses a challenge. These bacteria are predominantly isolated from failed endodontic cases and asymptomatic persistent periapical lesions. The aim of this study was to evaluate the elimination of *E. faecalis* from root canals using Ayurvedic extracts obtained from the leaves of *Calotropis procera*. Three forms of Ayurvedic extracts were prepared from the leaves of *C. procera*, namely Swarasa, Kwaatha and Hima. Initially, a disc diffusion test was conducted to assess the antimicrobial properties of these extracts against *E. faecalis*, comparing them with 2 routinely used endodontic irrigants. Seventy-two single-rooted mandibular premolars were instrumented up to the Protaper F2 rotary file and subjected to sterilization. The root canals were then cultured with *E. faecalis* to form a mature biofilm. All sample teeth were irrigated with the study irrigants and instrumented with the WaveOne GOLD large file (0.40/.08v). The debris collected in these files underwent testing for bacterial load using the colony-forming unit (CFU) test. Statistical analysis of the results was performed using One-way ANOVA and the Kruskal-Wallis test ($P<0.0001$). Ayurvedic extracts yielded significantly better results compared to control irrigants in the disc diffusion assay. However, all study irrigant groups performed efficiently against *E. faecalis* with no statistically significant difference observed using the CFU test in root canals. Ayurvedic extracts obtained from the leaves of *C. procera* were as effective as standard root canal irrigants (NaOCl and CHX) in eliminating *E. faecalis* from the root canals.

Keywords

Antimicrobial property; Ayurvedic extracts; *Calotropis procera* ; *Enterococcus faecalis* ; root canals

Introduction

Historically, India was considered the Golden Bird due to its wealth, culture and Ayurvedic medicine. This traditional Indian medicine, known as Ayurveda, was developed around 1500 BC and remains a living health tradition (1-3). Another traditional medicinal system, commonly referred to as the "science of life," is Ayurveda. The main classics of Ayurveda, such as Charaka Samhita, Sushruta Samhita and Ashtanga Hridaya of Vagbhata, provide

elaborate information on 700 herbal species with 6000 medicinal formulations (4).

One such plant mentioned in ancient Ayurvedic books is *Calotropis procera*. It is a woody weed found throughout the world. *C. procera* belongs to the family Asclepiadaceae and the subfamily Asclepiadeae (5). In English, it has various other names such as milkweed, swallowwort or Sodom apple. In Hindi, the plant is referred to as madar and in Sanskrit, as alarka (6). In traditional Ayurvedic medicine, various parts of the *C. procera* plant are used for treating asthma, tumors, diseases of the abdomen, eczema, leprosy, piles, ulcers, liver and spleen (7-9).

The use of herbs has been considered a feasible alternative to synthetic antimicrobials, which can contribute to the emergence of superbugs. Microorganisms hardly show any resistance to these phytocompounds, which act through nonspecific mechanisms of action (10). *Enterococcus faecalis* is commonly associated with periradicular asymptomatic persistent infections (11). Biofilm formed by *E. faecalis* makes these bacteria 1000 times more resistant to phagocytosis, destruction by antibodies and antibacterial agents (12).

This *in vitro* study aimed to test the null hypothesis that the Ayurvedic extracts obtained from *C. procera* will not be as effective as sodium hypochlorite (NaOCl) and chlorhexidine (CHX) in eliminating *E. faecalis*.

Materials and Methods

Chemicals, Micro-organism and Culture media

Sterile distilled water, RC Help (Prime Dental Products Pvt. Ltd, India), 2.5 % Sodium hypochlorite (NaOCl) [Vishal Dentocare Pvt Ltd, Gujarat, India], 2 % Chlorhexidine (CHX) [Amrit Chem. and Min.Ag.Mohali, India], 17 % Ethylenediaminetetraacetic acid (EDTA) [Prevest DenPro limited, India], 0.9 % Normal Saline (NS) [Medipolis Healthcare private limited, India] and nail varnish were used. The *E. faecalis* standard strain ATCC 29212 was employed. The culture media used included Brain-Heart Infusion broth (BHI) and Blood agar was purchased from HiMedia M210-100G, Mumbai, India.

Plant Material Collection

The *C. procera* plant specimen was identified at the Botanical Survey of India, Deccan Regional Centre in Hyderabad (BSI/DRC/2024-25/Identification/104). The voucher specimen was submitted to the parent institution for future reference. The leaves of *C. procera* were freshly plucked, rinsed with distilled water to clean them and left to shade dry for 20 days. Subsequently, the dried leaves were blended into a powder using an electric blender (Moulinex, France).

Preparation of Ayurvedic Extracts

Three forms of Ayurvedic extracts were prepared from leaves of *C. procera*: Swarasa (Fresh juice), Kwaatha (Decoction) and Hima (Cold aqueous extract). Swarasa was prepared by grinding 50 g of fresh leaves with 100 mL of sterile distilled water in an electric juicer (Moulinex, France). Kwaatha was obtained by boiling a mixture of 100 mL of water and 10 g of dried powdered leaves (DPL) at 100 °C for 10 min. For the preparation of Hima, 10 g of DPL was soaked in 100 mL of sterile distilled water for 24 h. All 3 Ayurvedic extracts obtained were subjected to filtration using Whatman filter paper no. 1.

Preparation of Teeth

A total of 72 human-extracted permanent, single-rooted, single-canal mandibular premolars (confirmed using radiographs) were collected after obtaining consent from the Institutional Ethical Committee (IHEC/SDC/PhD/ENDO-1617/19/007). Teeth with caries, root fractures and resorption were excluded from the study. The teeth were prepared according to the methodology outlined (13). A diamond disc was used to cut the teeth horizontally 14 mm from the apex to standardize the length of the teeth. A measurement 1 mm short of the apical foramen (13 mm) was considered the working length. ProTaper Universal rotary files (Denstply Maillefer, Ballaigues, Switzerland) up to F2 were used for canal instrumentation. RC Help (Prime Dental Products Pvt. Ltd, India) was the preferred lubricating agent. During instrumentation, the canals were rinsed with 2.5 % NaOCl. Post-instrumentation, the canals were rinsed with 1 mL 17 % EDTA, 5 mL NS and 1 mL of NaOCl respectively, for 3 min for smear layer removal. All canals were finally rinsed with a 5 mL NS solution. Two coats of nail varnish were applied to the external surface of the specimens. The teeth were placed into centrifuge tubes and autoclaved at 121 °C for 15 min.

Disc Diffusion Method

E. faecalis standard strains (ATCC 29212) were subcultured on blood agar plates in the Department of Microbiology and Immunology. From the subculture, a pure single colony was isolated and inoculated into Brain-Heart Infusion broth (BHI) [HiMedia M210-100G, Mumbai, India] until a turbidity of 0.5 McFarland standard (approximately 10^8 bacteria) was achieved. This broth culture was evenly swabbed over 13 blood agar plates to obtain a lawn culture. On each blood agar plate, 6sterile wafer discs with a diameter of 3 mm were placed. These discs were impregnated with 100 µL of Fresh Juice (Swarasa), Decoction (Kwaatha), Aqueous extract (Hima), 2.5 % NaOCl and 2 % CHX. One disc was left on each plate to act as a negative control. The blood agar plates were then incubated for 24 h at 37 °C.

E. faecalis inoculation into the Root Canals

A broth culture of *E. faecalis* standard strains (ATCC 29212) in 5 mL BHI broth with turbidity adjusted to 0.5 McFarland standard was prepared. The sterile root canals of the teeth were irrigated with 30 µL of the broth culture. Then, 2 mL of sterile BHI broth was added to each of the centrifuge tubes containing the sterile teeth. For mature biofilm formation, the centrifuge tubes were incubated for 4 weeks at a temperature of 37 °C. From each centrifuge tube, 1 mL of BHI broth was discarded and replenished with 1 mL of fresh sterile BHI broth at intervals of every 2 days.

Effectiveness of Ayurvedic Extracts

After 4 weeks of incubation, the growth of *E. faecalis* was confirmed by placing a paper point in the root canal to absorb BHI broth. Under sterile conditions, this paper point was vortexed in 1 mL of sterile NS for 1 min. A serial 10-fold dilution was performed to prepare a $1:10^2$ solution. Finally, 100 µL of the obtained solution was streaked on the blood agar plate for confirmation of *E. faecalis*. Six experimental groups were created by randomly dividing the specimens as follows [n=12]:

Fresh Juice

Decoction

Aqueous extract

Distilled water - negative control group

2 % Chlorhexidine - positive control group

2.5 % Sodium hypochlorite - positive control group

Root canal irrigation with 5 mL of different test solutions was performed in all the groups and specimens were left undisturbed for 5 min. Final flushing of the canals with 5 mL of NS was performed for 30 seconds to standardize the study groups. The canals were instrumented using WaveOne Gold 40/.08 files (Dentsply) after drying them with absorbent paper points. Under sterile conditions, the files were transferred into 1 mL NS-containing micro tubes and vortexed for 1 min. The obtained solution was then subjected to bacterial culture.

Statistical Analysis

Data were analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc., USA). The zone of inhibition of tested solutions obtained by the disc diffusion method was compared using a one-way analysis of variance test (ANOVA). The comparison of the number of CFUs obtained post-irrigation of study solutions in the root canals among the Six experimental groups was performed using the Kruskal-Wallis test.

Results

Disc Diffusion Assay

The comparison of zone of inhibition sizes among different groups (Swarasa, CHX, Kwaatha, NaOCl and Hima) was analyzed using a one-way analysis of variance (ANOVA) test. Assumptions of ANOVA include normality and homogeneity of variances. ANOVA is suitable for comparing means of multiple groups simultaneously, allowing for the assessment of differences in antimicrobial efficacy among various solutions. The ANOVA test revealed that the largest zone of inhibition was obtained with Swarasa/fresh juice, followed by 2 % CHX, Kwaatha/Decoction, 2.5 % NaOCl and lastly by Hima/Cold aqueous extract ($P<0.0001$) (Table 1).

Colony-Forming Unit (CFU) Test

The comparison of colony-forming units (CFUs) obtained post-irrigation of root canals among Ayurvedic leaf extracts and control solutions (NaOCl and CHX) was analyzed using the Kruskal-Wallis test. Similar to the disc diffusion assay, assumptions of normality and homogeneity of variances were assessed for the CFU data; thus, the Kruskal-Wallis test was performed. The reported p-values ($P<0.0001$) indicate that the observed differences in zone of inhibition sizes and CFUs among the tested solutions were statistically significant. The observation that Swarasa was highly effective and Hima was less effective among the 3 leaf extract forms indicates practical significance, which strengthens the interpretation of the statistical findings. Table 2 illustrates the CFUs obtained post-irrigation of the root canals inoculated with *E. faecalis* using Ayurvedic leaf extracts and control solutions.

Table 1. Comparison of anti-bacterial activity of fresh juice, decoction, 2 % CHX, 2.5 % NaOCl and aqueous extract against *E. faecalis* using disc diffusion method.

Study groups	N	Zone of inhibition (mm)		SD	p-Value
		Range	Mean		
Fresh Juice	13	14.68 to 20.02	18.01	1.42	
Decoction	13	12.63 to 16.34	13.96	1.15	
Aqueous Extract	13	10.12 to 13.23	11.49	0.99	<0.0001
2 % Chlorhexidine	13	14.31 to 18	16.12	1.22	
2.5 % Sodium hypochlorite	13	11.25 to 13.65	12.64	0.74	

Inference: *C. procera* fresh leaf juice showed the highest antimicrobial activity against *E. faecalis* compared to other experimental solutions.

Table 2. Comparison of colony-forming units (CFUs) obtained following irrigation of root canals, which were inoculated with *E. faecalis*, using 2 % Chlorhexidine, 2.5 % Sodium hypochlorite, fresh juice, decoction and aqueous extract.

Study Groups	N	CFUs		SD	p-value
		Range	Mean		
Distilled water	12	138 to 500	293.1	115.9	
2 % Chlorhexidine	12	0 to 4	0.8333	1.403	
2.5 % Sodium hypochlorite	12	0 to 6	1.5	2.111	
Fresh Juice	12	0 to 3	0.8333	1.03	<0.0001
Decoction	12	0 to 4	1.167	1.337	
Aqueous extract	12	6 to 15	11.5	2.78	

Inference: Different forms of *C. procera* leaf extracts were as effective as standard root canal irrigants in elimination of *E. faecalis* from the root canals.

Discussion

The incidence of *E. faecalis* has been noted to be 24 to 77 % in endodontically treated teeth with periapical lesions (14, 15). This high occurrence is attributed to various factors responsible for its survival in extreme conditions. It possesses virulence factors such as aggregation substance, cytolysin, lipoteichoic acid, lytic enzymes and pheromones (11). *E. faecalis* also exhibits host cell adherence, competition with other bacteria and alteration of host responses (11, 16). The biofilm formed by *E. faecalis* provides 1000 times more resistance to antimicrobials, antibodies and phagocytosis (17). It also has the ability to suppress lymphocyte action, resulting in persistent periapical infection and endodontic failure (18).

The use of herbal medicine like Ayurveda could be beneficial in combating microbial resistance, as phytocompounds possess nonspecific antimicrobial action (10). Ayurvedic medicine falls under the category of the oldest medical sciences, being practiced for over 5000 years (19). The Ayurvedic extracts can be prepared in 5 basic classical forms termed 'Pancavidhakasaaya'. These are: 'Swarasa', or fresh juice; 'Kalka', or fresh fine paste of plant parts; 'Kwaatha', or decoction; 'Faanta', or hot water infusion; and 'Hima', or cold water infusion. The Swarasa and Kalka are fresh plant materials directly used in treatment. Swarasa and Kwaatha are directly applied to patients after preparation from fresh plant material, whereas the forms of Ayurvedic extracts known as Kwaatha, Sheeta and Faanta are aqueous preparations made from dried plant parts (20).

Zehender proposed an irrigation regimen that included the use of NaOCl during instrumentation and CHX as one of the final flush irrigants (21). NaOCl, the most common endodontic irrigant, provides minor lubrication, bactericidal cytotoxicity and dissolution of organic material (22). Meanwhile, CHX offers the advantage of substantivity. Therefore, NaOCl and CHX were included as control groups in the present study.

The gas chromatography/mass spectrometry interpretation of crude extract obtained from *C. procera* leaves showed the presence of 15 components: 3-Eicosene, (3E)-3 -Icosene, Tetratriacontane, 1-Tridecene, 8-Pentadecanone, (1-Propyloctyl) Cyclohexane, 1-Heptadecene, 1-Nonadecene, Sulfurous acid, Di-n-octyl phthalate, 1-Tricosene, n-Tetratriacontane, 2-ethylhexyl isohexyl ester, 2,6,10,15-Tetramethylheptadecane and Docosane(23). The antibacterial property of Ayurvedic extracts obtained from *C. procera* leaves could be attributed to the synergistic effect of these phytochemicals or to a sole phytochemical component. The concentration of these components varies with the type of extract and affects the resultant antibacterial property.

To the best of our knowledge, no studies have been conducted to date to assess the antimicrobial efficacy of *C. procera* leaf extract against *E. faecalis* in root canals and compare it with NaOCl and CHX. In the present study, Swarasa and Kwaatha obtained from *C. procera* leaves were found to be as effective as the gold standard irrigants in eliminating *E. faecalis* when used as an irrigant in root canals. Therefore, the null hypothesis was rejected. The greater antibacterial effect of fresh juice extract could be attributed to the synergistic antimicrobial properties of chlorophyll and latex, which are lost when leaves are dried for preservation to be used in the future.

Conclusion and Future Prospects

When used as an irrigant in root canals, the Ayurvedic leaf extracts obtained from *C. procera* showed antimicrobial properties similar to NaOCl and CHX in eliminating *E. faecalis*. For classical Ayurveda to be adapted in modern endodontic practice, contemporary modifications are necessary and future prospects could include:

Conducting clinical trials to evaluate the effectiveness of Ayurvedic extracts *in vivo*, involving larger sample sizes, diverse patient populations and longer follow-up periods. Investigating the underlying mechanisms of action of Ayurvedic extracts on microbial biofilms, host-microbe interactions and tissue responses within the root canal system. Refining the formulation of Ayurvedic extracts to improve stability, bioavailability and antimicrobial potency while minimising cytotoxicity or adverse effects. Exploring synergistic interactions between Ayurvedic extracts and conventional endodontic irrigants or adjunctive therapies. Assessing the biocompatibility and tissue response of Ayurvedic extracts in animal models or *ex vivo* systems to evaluate their safety profile and potential for tissue regeneration within the root canal space.

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Authors' contributions

AH, UP were involved in designing the study, data collection and manuscript writing. SR and RS finalised, guided with necessary corrections and approved the manuscript.

Compliance with ethical standards

Conflict of interest: Authors deny having any conflict of interest.

Ethical issues: None.

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