

Antimicrobial Efficacy of *Azadirachta indica* (Neem) Twigs Aqueous and Ethanol Extracts on Tooth Root Canals Biofilms

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ABSTRACT

Phytochemicals present in medicinal plants possess a wide range of properties including; antibacterial, anti-inflammatory and anti-carcinogenic activities. *Azadirachta indica* (Neem) twigs are used in many communities as toothbrushes and are said to prevent dental caries and gum disease. However the antimicrobial activity of neem in endodontics is unknown, hence the need to test its effects on root canal biofilms. Materials and methods: Twigs of *Azadirachta indica* were collected, dried and ground into coarse powder. Aqueous and 80 % ethanol extraction procedures were done. The mixtures were gravity filtered and the filtrates, sterile filtered through Nalgene[®] disposable filter unit with 0.45 µm pore size filter. The aqueous filtrates were freeze- dried while the ethanol filtrates were reduced under vacuum and the yields determined. Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of the extracts on pure strains of *Enterococcus faecalis* (ATCC 29212), *Streptococcus mutans* (ATCC 25175), *Staphylococcus aureus* (ATCC 25923), *Fusobacterium nucleatum* (ATCC 25586), *Lactobacillus acidophilus* (ATCC 4356) and *Candida albicans* (ATCC 24433) were determined using tenfold liquid micro dilution method. Growth was determined as optical density at 630 nm after incubation for 24 and 48 hrs at 37°C. Sodium hypochlorite (5.25%) and 2 % Chlorhexidine were used as positive controls. Analysis of variance was done. Results: The inhibitory effects of 50% weight /volume (w/v) aqueous extracts was; *C. albicans* (84.6%), *S. aureus* (100%) and *L. acidophilus* (80.9%) at 24 hrs incubation. At 48 hrs, the inhibitory effects of ethanol extracts (50% w/v) on *E. faecalis*, *S. mutans* and *F. nucleatum* was 96.2, 81.5% and 63.9 % respectively while that of aqueous extracts was 85.5%, 91.7% and 62.2% for *E. faecalis*, *S. aureus* and *C. albicans* respectively. The MIC was 50% w/v. Conclusion: Aqueous and ethanol extracts of *Azadirachta indica* showed potential to be a source of antimicrobial agent against tested root canal biofilms.

Key words: *Azadirachta indica*, aqueous, ethanol extracts, root canal biofilms.

INTRODUCTION

Microorganisms are involved in the development of caries which if untreated progresses to affect pulp. Dental pulp inflammation (pulpitis) is commonly caused by microorganisms¹ which access the pulp tissue through; carious lesions, periodontal disease, leakage of microbes, or their antigens around lateral canals, or leaking cavity margins of restorations and the blood stream². The microorganisms frequently detected in pulpal infections and reinfection includes; *E. faecalis*, *S. mutans*, *S. aureus*, *L. acidophilus*, *F. nucleatum* and *C. albicans*^{3,4,5}.

To eliminate these microorganisms the irrigants used in endodontics are required to have antiseptic effects, be

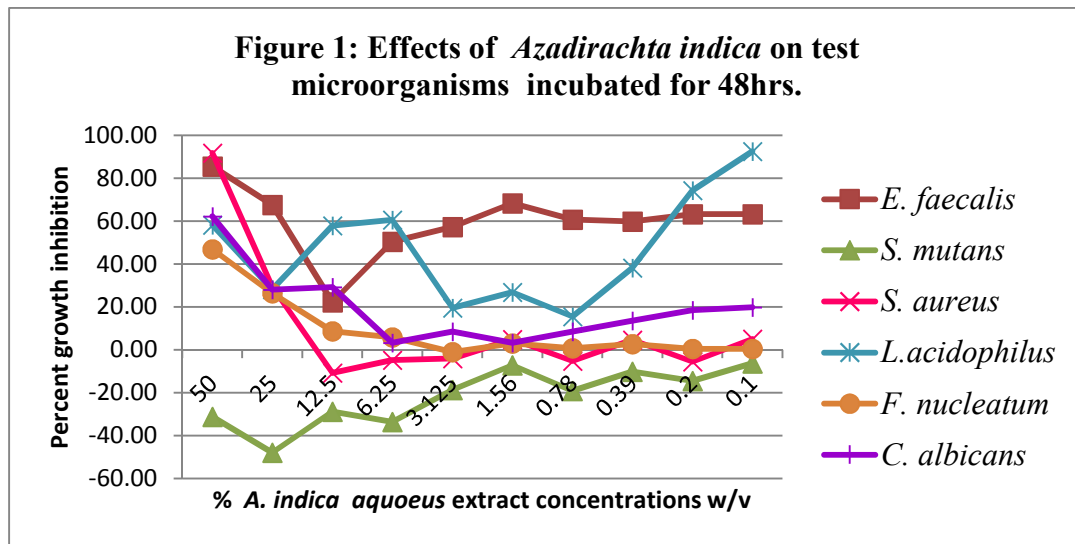
bacteriostatic, bactericidal and biocompatible with minimal peri-radicular tissue effect. Unfortunately most of the irrigants, used including 5.25 % Sodium hypochlorite (NaOCl), 2% Chlorhexidine gluconate (CHX) and 17 % Ethylene diaminetetraacetic acid (EDTA) do not meet all of these requirements^{6,7,8}. Alternative to these conventional agents are needed and medicinal plants analysis as a potential source of safe irrigants remains largely uninvestigated.

The Neem plant, *Azadirachta indica* is a member of the mahogany family Meliaceae⁹ and grows in tropical and semi-tropical parts but is currently grown in many countries throughout the world. In Kenya it is commonly

Table 1: The inhibitory effects of aqueous extracts of *Azadirachta indica* on test microorganism incubated for 24hrs at 37°C.

Percentage inhibitory effects of <i>Azadirachta indica</i> aqueous extract (µg/mL).									
Extracts Conc.	50	25	12.5	6.25	3.13	1.56	0.78	0.39	*NaOCl
<i>E. faecalis</i>	46.2	10.1	-6.7	-12.6	-4.2	14.3	5.9	-0.8	85.1
<i>S. mutans</i>	30.6	-68.6	-61.1	-38.6	-32.7	-22.9	-36.0	-37.8	45.0
<i>S. aureus</i>	100.2	45.8	-11.0	-5.8	4.0	5.4	-4.8	4.7	89.8
<i>L. acidophilus</i>	80.9	29.4	72.3	81.9	30.2	40.2	38.7	55.8	99.6
<i>C. albicans</i>	84.3	32.9	34.7	8.5	15.1	4.4	12.7	13.9	41.5

*5.25% Sodium hypochlorite positive control.



known as “Mwarubaini” meaning it can treat forty (many) diseases and is found in most parts of the country¹⁰. Almost every part of the tree has been used as a fungicide, antibacterial, antiviral agents and for dental treatments¹¹. The “Neem” twigs are used as toothbrushes and are said to prevent dental caries and gum disease^{12,13,14}.

Bohora *et al.*, (2010)¹⁵ and Nayak *et al.*, (2011)¹⁶ report that the “Neem” leaf extracts have statistically significant antimicrobial effect against *E. faecalis* and *C. albicans*. More recently Raja *et al.*, (2013)¹⁷ reported that Neem leaf and bark aqueous extracts exhibited high antimicrobial activity against selected microorganisms. It is notable that these studies are limited to leaf extracts.

The aim of the current study was to evaluate the antimicrobial efficacy of aqueous and ethanol extracts of *Azadirachta indica* (Neem) twigs on root canal biofilms associated with unfavorable root canal treatment outcome and reinfection.

MATERIALS AND METHODS

Ethical approval to undertake this study was obtained from Kenyatta National Hospital and University of Nairobi Research, Ethics and Research Committee.

Plant material

The twigs of *Azadirachta indica* were collected after identification by a taxonomist (S.T. K. Kabuitu). They were air dried under shade at room temperature to dehydrate them and then ground into a coarse powder and placed in pre-labeled polythene bags.

Extraction procedure

One hundred grams of *Azadirachta indica* coarse powder was weighed on Kerne PL2100-2, electronic precision and analytical balance (KERN & Sohn GmbH Balingen-Frommern, Germany) and added into a round bottom flask. The Seven hundred and fifty (750 ml) of sterile distilled water was added into the flask, stirred and left to soak for 3 days at room temperature. The second and third extraction was done in 500ml and 250 ml sterile distilled water respectively.

At the end of each soaking period, the mixtures were gravity filtration through Whatman No.1 (Whatman Ltd. England) filter paper to remove most of the plant materials. The filtrates were pooled and sterile filtered through Nalgene® disposable filter unit (Thermo Fisher Scientific, Inc. Waltham, MA USA.) containing 0.45 µm pore size filter. Aqueous filtrates (30 ml) were aliquoted into 50 ml Falcón® Centrifuge tubes and frozen at -20 °C before freeze drying at (Jouan LP-3) -50 °C and 0.3 mbars vacuum. The mass obtained was weighed and the percentage extract yield determined and stored at 4 °C until further analysis.

Ethanol extraction protocol was similar to the aqueous extraction except that, 80% ethanol was used and the filtrates were reduced and concentrated using rotary evaporator (Heidolph, Laborota 4000, Schwabach, Germany) at 150-200 rpm, 80° C under vacuum. The partially solid, sticky or wet extracts were placed into pre weighed, sterile airtight bottles and transferred to hot air

Table 2: Percentage inhibitory effects of *Azadirachta indica* ethanol extract ($\mu\text{g/mL}$).
Positive control 5.25% Sodium hypochlorite (NaOCl).

Extracts conc.	50	25	12.5	6.25	3.125	1.56	0.78	0.39	NaOCl
<i>E. faecalis</i>	96.18	26.65	12.99	-5.54	28.27	-17.00	-11.37	-5.35	57.37
<i>S. mutans</i>	81.53	14.57	19.58	13.45	12.79	13.24	18.1	12.59	93.42
<i>S. aureus</i>	-11.9	-15.19	-17.51	-14.67	-10.65	-0.89	-1.53	-1.01	91.09
<i>L. acidophilus</i>	-8.67	10.37	14.79	37.49	-45.3	-63.4	15.21	-13.5	96.1
<i>F. nucleatum</i>	63.91	14.16	8.62	6.43	4.95	5.48	5.71	4.13	58.11
<i>C. albicans</i>	8.82	21.11	4.63	-2.22	1.79	1.88	-0.83	8.24	65.5

Table 3: Shows Minimum Inhibitory Concentrations and Minimum bactericidal Concentrations

Microorganism	Aqueous extracts		Ethanol extracts			
	24hrs	48hrs	24hrs	48hrs		
	MIC	MBC	MIC	MIC	MBC	MIC
<i>E. faecalis</i>	50(46.2)	-	50(85.5)	0.1(87.7)	6.25*(104.6)	50(96.2)
<i>S. mutans</i>	-	-	-	50(71.9)	-	50(81.5)
<i>S. aureus</i>	50(100.2)	50*	50 (91.7)	-	-	-
<i>L.acidophilus</i>	50(80.9)	-	50 (58.1)	50(83.1)	-	-
<i>F. nucleatum</i>	-	-	-	-	-	50(63.9)
<i>C. albicans</i>	50(84.3)	-	50(62.2)	-	-	-

**A.indica* showed bactericidal effect on *E. faecalis* and *S. aureus*. In brackets, % inhibition values, MIC-Minimum Inhibitory Concentration, MBC- Minimum Bactericidal Concentration.

drying oven (Memmert GmbH & Co. KG, Germany), set at 40°C for drying for 72hrs.

Microorganisms

Pure strains of freeze dried cultures of *Enterococcus faecalis* (*E. faecalis*, ATCC® 29212™), *Streptococcus mutans* (*S. mutans* ATCC® 25175™), *Staphylococcus aureus* ssp. *aureus* (*S. aureus* ATCC® 25923™), *Fusobacterium nucleatum* (*F. nucleatum* ATCC® 25586™), *Lactobacillus acidophilus* (Moro) Hansen and Mocquot (*L. acidophilus* ATCC® 4356™) and *Candida albicans* (*C. albican* ATCC® 24433™) were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA).

Preparation of inoculum and storing

The stock cultures were maintained below -80 °C as recommended by ATCC for each microorganism. The frozen stocks of *E. faecalis* (ATCC 29212) and *S. mutans* (ATCC 25175), were grown in Brain Heart Infusion broth (BHI Oxoid) under 95% N₂ and 5%CO₂ (v/v), *F. nucleatum* (ATCC 25586) in Modified chopped meat medium in an anaerobic chamber (80% N₂, 10% CO₂ and 10% H₂), *S. aureus* strains (ATCC 25923) in Trypticase Soy, *L. acidophilus* (ATCC 4356), in Lactobacilli MRS broth (Oxoid) under 95% N₂ and 5% CO₂ (v/v) and *C. albicans* (ATCC 24433) in Sabouraud's agar in 5% carbon dioxide. All microorganisms were incubated at 37 °C and for each organism (primary subculture) and a second subculture (day 1 working culture) was made.

Microbial growth was determined visually by changes in turbidity according to 0.5 McFarland standards (1.5 X 10⁸ CFU/mL) at 24 hours for the bacteria and 48hrs for *C. albicans*. Strict observances of experimental protocols was undertaken to ensure purity of microorganisms.

Minimal Inhibitory Concentration

The freeze-dried *Azadirachta indica* aqueous extract (1g) was resuspended in 2 mL of sterile distilled water and another 1g of ethanol extract resuspended in the 20 % (v/v)

DMSO to make 50 % stock solutions. Sterile Microtiter plates 96U well with lids (8 by 12 matrix, Bioster.S.P.A, Italy), with retention capacity of more than 200 μl per well were used. 160 μl of sterile growth media was drawn with adjustable 20-200 μl micropipette (Eppendorffs® Research® Plus Hamburg, Germany) using 20 μl sterile disposable micropipette tips into the wells. Tenfold microdilution¹⁸ with modification was used. 20 μl of 50 % (w/v) extract solution was drawn, added to the first well, mixed properly and 20 μl solution drawn and added to the second well. This was repeated serially for all the wells and last 20 μl from the 10th well was discarded.

Each well was inoculated with 10 μl of bacterial suspension at a density of 1.5x10⁸ CFU/mL using 0.5-10 μl Gilson adjustable micropipette (Gilson Inc. Middleton, WI. USA). The 11th well contained broth and microorganisms and 12th broth plus extract for intra-experimental control. Baselines readings were obtained at 1 hour before incubation in Heratherm™ Compact Microbiological Incubators at 37°C for 24 and 48 hrs. Growth of microorganisms was read of as optical density using BioTek Elisa Photometer (BioTek Instruments, Inc. USA) at 630 nm. Sterile distilled water and 2% CHX were used as negative and positive controls respectively. All microorganisms were tested independently, each carried out in triplicate compared with the efficacy of 5.25% NaOCl and 17% EDTA liquid.

Minimum Inhibitory Concentrations were calculated based on the density of growth control and expressed as the lowest extract concentration that resulted in 80% growth reduction and while Minimum Bactericidal Concentrations was the lowest concentration of the plant extracts that did not yield any bacterial growth upon subculture compared to that of the extract free growth control.

The data was analyzed using the Statistical Package for Social Sciences (SPSS) 21.0 for Windows and Microsoft excel. Analytical tests and computations were carried out,

to calculate the mean, standard deviations, percent inhibitory effects and minimal inhibitory concentrations of the extracts, One way analysis of variance (ANOVA) was done and statistically significant level set at $P < 0.05$.

RESULTS

The inhibitory effects of aqueous extracts on test microorganisms are shown in table 1. The *A.indica* aqueous extracts showed 100.2% inhibition of *S. aureus*, 84.3% on *C. albicans*, and 80.9% on *L. acidophilus* at 50% w/v extract concentration which comparable to 85.1% obtained with 5.25 % sodium Hypochrite. *S. mutans* was the most resistant 30.6%, followed by *E. faecalis* 46.2% at 24hrs incubation (Table 1). It is notable that the effects reduced proportionately with increased dilution of the extracts. The inhibitory effects on *E. faecalis*, *S. mutans*, *L. acidophilus* and *C. albicans* were 85.5%, -31.2%, 58.1% and 62.2% respectively at 48hrs incubation (Fig.1). The differences are statistically significant at $p \leq 0.05$. The effects on *S. aureus* remained almost the same at the two incubation periods. The effects of most microorganisms showed resistance to 2% CHX except for *S.mutans* with 40.9 % inhibition.

For the ethanol extracts *S. aureus*, was most resistant (8.8%), followed by *C. albicans* (16.8%) and *E. faecalis* (45.1%) while *L. acidophilus* and *S. mutans* resulted 83.1% and 71.9% inhibition at 50% w/v. These results appear to be the reverse of aqueous extracts (Table 1) which may be due to the difference in phytochemical contents obtained by the two extract ants.

At 48 hrs incubation *E. faecalis*, *S. mutans* and *F. nucleatum* were the most inhibited by 50% w/v *A.indica* ethanol extracts in that order (Table 2). There were minimal effects with increased dilution of extracts. *S.aureus*, *L. acidophilus* and *C .albicans* were the most resistant with further dilution. The Minimum Inhibitory concentrations and Minimum bactericidal Concentrations for aqueous and ethanol extracts are presented in table 3.

DISCUSSION

In recent years there is an exponential growth in the field of herbal medicine because of their natural origin, availability, efficacy and safety in terms of fewer side effects¹⁹ compared to synthetic medications. Drug resistant microorganisms require use of bactericidal agents which are safe and easily available. Further investigation and focus on medicinal plants may provide these options.

In the current study, ethanol twig extracts at 50% w/v ($\mu\text{g/ml}$) showed statistically significant ($P \leq 0.05$) growth inhibition of *L. acidophilus* and *S. mutans* at 24 hrs and *E. faecalis*, *S. mutans* and *F. nucleatum* at 48hrs incubation. Further 50% w/v ($\mu\text{g/ml}$) concentration of aqueous extracts inhibited growth of *E. faecalis* and *S. aureus* with significant effects on *C. albicans*. These results also show that the inhibitory effects for *E. faecalis* increased with incubation time while that for *S. mutans*, *L. acidophilus* and *C. albicans* decreased at 48 hrs incubation period. This is important because of the duration medicaments are left in the root canal. An agent that is effective has to retain substantivity for a longer period of time. The decrease in

the inhibitory effects with time may suggest a bacteriostatic effect where the organism is reduced but not eliminated and is able to re grow with time. The desired bactericidal effects were observed only with *S. aureus* and *E. faecalis*.

The effects of 50% w/v aqueous and ethanol *A. indica* on some microorganism were similar with 5.25% Sodium hypochlorite, 2% Chlorhexidine and 17% EDTA. It is also notable from these results that all the microorganisms that are involved in advanced caries lesion and are associated with primary infection²⁰, chronic apical periodontitis^{3,21}, reinfection and unfavourable root canal treatment are significantly inhibited by either the aqueous or ethanol extracts. This means that the crude extracts can be tested and analysed further and purified with aim of developing them into a viable root canal irrigant or medicament. The results of this study have demonstrated reasonable antimicrobial activity of aqueous and ethanol extracts of twigs of *Azadirachta indica* on tested root canal biofilms.

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