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Comparison of Antimicrobial Activities of *Moringa oleifera* leaf, Propolis, 2% Chlorhexidine gluconate and MTAD on *E. faecalis*- An In-vitro Study.

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ABSTRACT

The aim of the study was to evaluate and compare antibacterial activities of two extracts of dry and fresh *Moringa oleifera* leaves prepared in ethanol, water soluble propolis, 2% chlorhexidine gluconate and MTAD against root canal pathogen *Enterococcus faecalis*. *Moringa oleifera* leaves, water soluble propolis, Biopure MTAD, 2% chlorhexidine gluconate, *Enterococcus faecalis* strain(ATCC 29212) and Mueller Hinton Agar were used. *Enterococcus faecalis* was cultured in the Mueller Hinton agar medium. Antimicrobial susceptibility test was done by agar diffusion method. Minimum Inhibitory Concentration (MIC) was determined using broth dilution method. MTAD showed maximum zone of inhibition followed by 2% chlorhexidine gluconate. The dry leaf extract showed slightly larger zone of inhibition than the fresh leaf extract but lesser than MTAD and chlorhexidine. Propolis, contrary to some other studies, showed no zone of inhibition. The recorded MIC values of both dry and fresh *Moringa* leaf extracts was 50%. Propolis showed no MIC values. 2% chlorhexidine and MTAD exhibited MIC values of 0.125% and 3.12% respectively. Results showed 2% chlorhexidine gluconate possesess lesser antimicrobial activity than MTAD but more than *Moringa oleifera* leaf extracts. *Moringa oleifera* showed lesser antimicrobial activity than MTAD and 2% chlorhexidine gluconate, but is an effective and cheaper substitute.

Keywords:- *Moringa oleifera*, Propolis, *E. faecalis*, MTAD

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INTRODUCTION

One of the primary objectives of endodontic therapy is microbial reduction or their elimination, to promote the normal healing and re-establishment of the health of periradicular tissues. Various studies have demonstrated that mechanical instrumentation cannot sufficiently disinfect root canals. Regardless of the use of stainless steel or nickel titanium instruments, irrigating solutions and intracanal medicaments are required to eradicate microorganisms over a period of time. A variety of chemicals have been promoted for this purpose [1]. An endodontic irrigant/ medicament should ideally exhibit powerful antimicrobial activity, disinfect the root canal space, and have no cytotoxic effects on periradicular tissues. Therefore an equally effective and safe irrigant/intracanal medicament is desirable [2].

Facultative organisms like *Enterococcus faecalis* (Gram positive bacteria) are considered by many to be the most resistant species, and one of the possible causes of root canal treatment failure [2]. It is a microorganism commonly detected in asymptomatic, persistent endodontic infections. *E. faecalis* is considerably resistant to the common intracanal medication with calcium hydroxide compared with most other microbes. The highly complex nature of the organism poses a great challenge for endodontists.

Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective antimicrobial agents, which led to the search of alternative antimicrobial drugs for the treatment of infectious diseases from natural sources. Literature has addressed many plants with potential source for new therapies in endodontics.

Moringa oleifera is the most widely cultivated species of the genus Moringa, which is the only genus in the family Moringaceae. It is a fast-growing, drought-resistant tree that is native to the southern foothills of the Himalayas in northwestern India, but widely cultivated in tropical and sub-tropical areas. It has an impressive range of medicinal uses with high nutritional value[3] and has been widely used for treating bacterial infection, fungal infection, anti inflammation, malnutrition and diarrhoea. One area in which there has been significant scientific research is the reported antibiotic activity of this tree.

Propolis is a natural resinous substance that honeybees collect from various plants and use in the hive to cover hive walls, fill cracks or gaps and embalm dead invaders. The chemical composition of propolis is very complex and varies depending on the local flora at the site of collection. At room temperature it is a sticky substance, but becomes hard and brittle at low temperature. It is composed of resin and balsams (50 -70%), essential oils and wax (30-50%), pollen(5-10%) and other constituents which are amino acids, minerals, Vitamins- A, B-complex, E and the highly active biochemical substance known as bioflavenoid (Vit P), phenols and aromatic compounds [4,5]. Flavenoids are well known plant compounds which have antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory proprieties. Propolis has found to be very effective against gram positive bacteria [6] especially against *Staphylococcus aureus* [7] and against gram negative bacteria especially Salmonella [8].

Bio-Pure MTAD antibacterial root canal cleanser is designed to chemically clean and disinfect the root canal system following endodontic instrumentation. Bio pure MTAD cleanser is a mixture of Tetracycline isomer (Doxycycline), Acid & Detergent. When used as directed, it is proven to exert a potent antimicrobial activity. The doxycycline present in MTAD has high binding affinity for dentin, allowing for a prolonged antibacterial effect [9].

Chlorhexidine gluconate (CHX) is a cationic biguanide that seems to act by adsorbing onto the cell wall of microorganism resulting in leakage of intracellular components. Furthermore, because of its cationic structure, chlorhexidine has the unique property of substantivity. At low concentration it is bacteriostatic and at a high concentration it is bactericidal. This substance adheres to the cell wall of Gram positive and negative bacteria, causing selective protein precipitation from the cell wall, cytoplasm coagulation and the breakdown of intracellular components [10]. This mechanism allows chlorhexidine to act as a bacteriostatic agent at low concentrations and a bactericidal agent at high concentrations [10-12].

MATERIALS AND METHODS

The present study was conducted at the Nitte University Centre for Science, Education and Research, Deralakatte and Department of Pharmacology, NGSM Institute of Pharmaceutical Sciences, Deralakatte, Mangalore, Karnataka. Water soluble propolis, fresh leaves of *Moringa oleifera*, strains of *Enterococcus faecalis* (ATCC 29212), Biopure MTAD, 2% chlorhexidine gluconate, Mueller Hinton Agar were used. Ethanol was used as the control.

Preparation of the *Moringa oleifera* leaf extracts

Fresh leaves of Moringa oleifera leaves of pharmaceutical grade, grown organically without use of pesticides were collected. Authentication of leaves was done at the Department of Pharmaceutics, NGSM institute of Pharmaceutical sciences.

Ethanol extracts of fresh and dry leaves

Fresh *Moringa oleifera* leaves were collected. 40g each of fresh and dried leaves were weighed out separately, crushed in a grinder and dipped in 200 ml ethanol in 2 conical flasks stoppered with rubber cork and left for 48 hours with occasional shaking. The extracts were filtered using sterile filter paper (Whattman's no.1) into clean glass containers with lid. The standard extracts obtained were stored in a refrigerator at 4^0 C for antibacterial activity test.

Water soluble propolis, Biopure MTAD, 2% chlorhexidine gluconate was obtained commercially.

Experimental

Grouping of experimental irrigants

Ethanolic extract of dried *Moringa oleifera* leaves, ethanolic extract of fresh *Moringa oleifera* leaves, water soluble propolis, 2% chlorhexidine gluconate, MTAD and ethanol(control) were grouped together on Mueller Hinton agar for evaluating the antimicrobial efficacy of each against *E. faecalis.* 4 such similar groups were formed with each of the above mentioned irrigants and the experiment was repeated 4 times to eliminate any procedural or observational errors.

Evaluation of antimicrobial efficacy

- **Test organism:** *Enterococcus faecalis* (ATCC 29212)
- **Media used:** Mueller Hinton Agar

Preparation of microbial suspension

The density of the selected organism was adjusted equal to that of 0.5 McFarland standard (1.5×10^8 CFU/ml) by inoculating them to nutrient broth. McFarland was used as a reference to adjust the turbidity of microbial suspension so that the number of microorganisms will be within given range.

Antimicrobial susceptibility test by Agar Well diffusion method

The antimicrobial properties of *Moringa oleifera* ethanol extract (both dry and fresh), commercially purchased water soluble propolis, MTAD and 2% chlorhexidine were tested according to the modified Kirby–Bauer method. *E. faecalis* ATCC (29212) was cultured on Mueller Hinton Agar (MHA). A single colony from the fresh culture was picked with a sterile loop and transferred into Mueller Hinton Broth (MHB). The broth was then incubated at 37°C for overnight. The densities of the organism suspensions were adjusted equal to the 0.5 McFarland standard. 6mm wells were punched on a dry Mueller Hinton agar medium. The cultures were plated on Mueller Hinton agar medium. The 20µl of the known concentrations of the extracts were then transferred into the punched wells. The seeded plates were incubated aerobically, for 18-24 hrs at 37^0C and zone of inhibition was measured using a millimeter scale. The zones of inhibition of the extracts were compared with the zone of inhibition exhibited by the MTAD and 2% chlorhexidine. The extract was said to be possessing antibacterial activity if it exhibited similar zone sizes as that of the MTAD and 2% chlorhexidine and the control-ethanol.

Determination of Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration where no visible turbidity is observed in the micro titre plates (bacteriostatic concentration). The Vollekova et al [13] modified by Usman et al(2007)[14] Broth dilution method was employed. Minimum Inhibitory Concentration (MIC)

was done according to the CLSI guidelines. A pure culture of *E. faecalis* was inoculated on Mueller Hinton Broth; the optical density of planktonic suspension of each culture was adjusted to $1.5X10^8$ cfu/ml (Mac Farland 0.5standard). A known concentration of the extracts was serially diluted to two folds in broth in sterile micro titre plate. After the extract agent was diluted, a volume of the standardized inoculum equal to the volume of the diluted antimicrobial agent was added to each dilution plate, bringing the microbial concentration to approximately 500,000 cells per milliliter. Two control plates were also included. One tube contained only the *E. faecalis* culture which served as the positive control and other tube contained undiluted extract which served as the negative control. The inoculated, serially diluted extract was incubated aerobically for 37^0 C for 18hrs. After incubation, the series of dilution plates were observed for microbial growth, indicated by turbidity and/or a pellet of microorganisms in the bottom of the vessel. The last tube in the dilution series that did not demonstrate growth corresponds with the minimum inhibitory concentration (MIC) of the antimicrobial agent. The MIC endpoint is the lowest concentration of the extract at which there is no visible growth in the micro titre plates. The tests were repeated in triplicates.

RESULTS

The evaluation of antimicrobial properties was done by measuring the diameters of zones of inhibition using Agar Well Diffusion Method and the MIC values of the experimental materials against *Enterococcus faecalis.* The results obtained were tabulated and represented in tables and graph.

Table 1 and Graph 1 facilitate comparison of means of diameters of zones of inhibition of experimented materials against *Enterococcus faecalis*: The ethanol extract of dry *Moringa oleifera* leaf showed a mean zone of inhibition of 13.5mm, whereas that of fresh *Moringa oleifera* leaf was found to be13.25mm. The commercially available water soluble Propolis did not show any zone of inhibition against *Enterococcus faecalis*. 2% Chlorhexidine showed a mean zone of inhibition of 18 mm while MTAD showed a mean zone of inhibition of 18.75mm. The positive control ethanol showed no zone of inhibition. Table 2 facilitates comparison of mean values of diameters of zones of inhibition of ethanol extracts of experimented materials against *Enterococcus faecalis* using one way ANOVA analysis which proved that there is a statistically significant difference between four groups, with highest in MTAD followed by 2% chlorhexidine followed by ethanol extract of dry leaf followed by ethanol extract of fresh leaves. Propolis did not show any antibacterial properties; therefore it was excluded from the statistical analysis.

Table 3 shows post hoc analysis using Tukey HSD (Honestly Significant Difference) Test, making multiple comparisons by pair wise comparison method, between values of diameters of zones of inhibition of experimental materials. All the groups were compared with each of the others by pair wise comparison method. The results obtained shows that the difference is mainly between the value of diameters of zone of inhibition showed by 2% chlorhexidine gluconate and the ethanol extracts as well as between that exhibited by MTAD and the ethanol extracts.MIC values of experimental materials against *Enterococcus faecalis* were tabulated in

Table 4. The MIC value of the ethanol extracts of dry and fresh Moringa leaves against *Enterococcus faecalis* was found to be 50%. The water soluble Propolis extract showed no MIC values for *Enterococcus faecalis*. MTAD showed MIC value of 3.12% while 2% chlorhexidine gluconate was found to exhibit an MIC value of 0.125% against Enterococcus faecalis.

Table 1: Comparison of values of diameters of zones of inhibition of ethanol extracts of dry and fresh Moringa leaves, commercially available water soluble Propolis, 2% Chlorhexidine gluconate and MTAD against Enterococcus faecalis, obtained using a millimeter scale

PRODUCT	Group A	Group B	Group C	Group D
Ethanol extract of dry Moringa oleifera leaf	12mm	13mm	14mm	15mm
Ethanol extract of fresh Moringa oleifera leaf	12 mm	12mm	14mm	15mm
Water soluble Propolis	-	-	-	-
2% Chlorhexidine gluconate	15mm	18mm	20mm	19mm
MTAD	16mm	19mm	18mm	22mm

Table 2: Comparison of mean values of diameters of zones of inhibition of ethanol extracts of dry and fresh Moringa leaves, 2% Chlorhexidine gluconate and MTAD against *Enterococcus faecalis* using one way ANOVA analysis

PRODUCT	N	Mean	Std. Deviation	Mean Square	F	Sig.
Ethanol extact of *dry Moringa oleifera* leaf	4	13.5	1.29099			
Ethanol extract of fresh *Moringa oleifera* leaf	4	13.25	1.5			
2%Chlorhexidine gluconate	4	18	2.16025			
MTAD	4	18.75	2.5	33.75	9.101	0.002
Total	16	15.875	3.11716			

Table 3: Post hoc analysis using Tukey HSD(Honestly Significant Difference) Test making multiple comparisons by pair wise comparison method between values of diameters of zones of inhibition of ethanol extracts of dry and fresh Moringa leaves, 2% Chlorhexidine gluconate and MTAD against *Enterococcus faecalis*

Multiple Comparisons				
Dependent Variable- VALUES Test method used -Tukey HSD(Honestly Significant Difference) Test				
(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.
MTAD	Ethanol extract of fresh *Moringa oleifera* leaf	5.50000	1.36168	.008
	Ethanol extact of dry *Moringa oleifera* leaf	5.25000	1.36168	.011
	2% Chlorhexidine gluconate	.75000	1.36168	.945
Ethanol extract of fresh *Moringa oleifera* leaf	Ethanol extact of dry *Moringa oleifera* leaf	-.25000	1.36168	.998
	2% Chlorhexidine gluconate	-4.75000	1.36168	.020
Ethanol extact of dry *Moringa oleifera* leaf	2% Chlorhexidine gluconate	-4.50000	1.36168	.028

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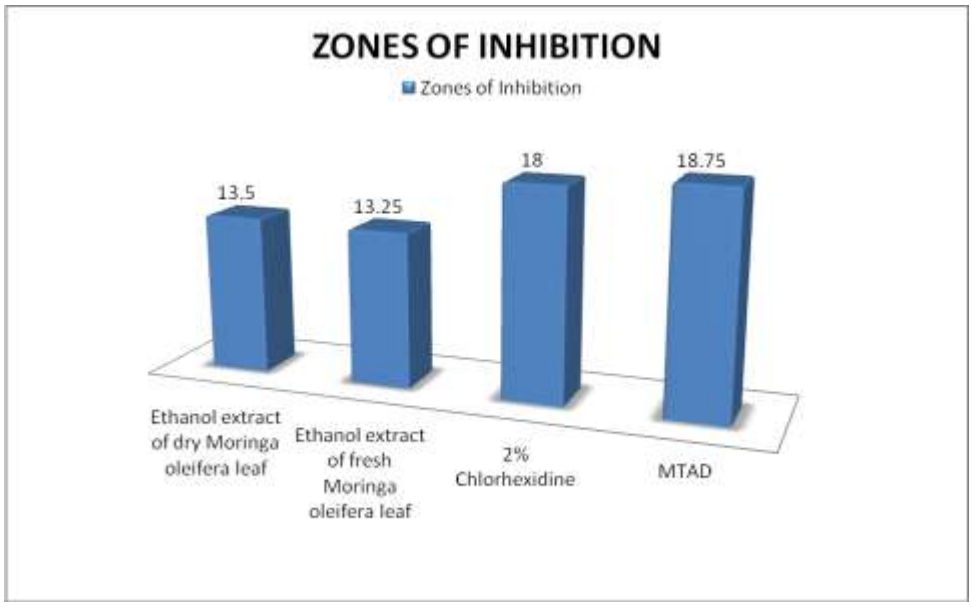
Table 4: MIC values of ethanol extracts of dry and fresh Moringa leaves, 2% chlorhexidine gluconate and MTAD against *Enterococcus faecalis*

PRODUCT	100%	50%	25%	12.5%	6.25%	3.12%	1.56%	0.78%	0.39%	0.19%
Ethanol extract of dry *M. oleifera* leaf	NG	NG	G	G	G	G	G	G	G	G
Ethanol extract of fresh *M. oleifera* leaf	NG	NG	G	G	G	G	G	G	G	G
MTAD	NG	NG	NG	NG	Ng	NG	G	G	G	G
2% Chlorhexidine	NG (2%)	NG (1%)	NG (.5%)	NG (.25%)	NG (.12%)	G (.O6%)	G (.03%)	G (.01%)	G -	G -

NG -- No Growth, G – Positive Growth of bacteria



Graph 1: Bar diagram representing mean values of diameters of zones of inhibition of ethanol extracts of dry and fresh *Moringa oleifera* leaf, 2% chlorhexidine gluconate and MTAD against *Enterococcus faecalis*

DISCUSSION

The causative role of microorganisms in the pathogenesis of pulp and periapical diseases has been well established and justifies the goal of elimination of bacteria as a critical step in root canal therapy [15]. However, complete elimination of bacteria is not always achieved in clinical practice due to the anatomical complexities of root canals and consequent limitations of access by instruments [16]. Facultative microorganisms such as *Enterococcus faecalis, Staphylococcus aureus,* etc, which are considered by many to be the most resistant species in the oral cavity, and one possible cause of root canal treatment failure [17], may persist in small numbers, which adversely affects the outcome of treatment.

This study entails the important antimicrobial activity of the *Moringa oleifera* leaf in inhibition of *E. faecalis* as a root canal pathogen. An examination of the phytochemicals of Moringa species reveals that this plant family is rich in compounds containing the simple sugar, rhamnose, and also in a fairly unique group of compounds called glucosinolates and isothiocyanates [18,19]. The *Moringa* plant is also rich in a number of vitamins and minerals as well as other more commonly recognized phytochemicals such as the carotenoids (including β-carotene or pro-vitamin A). Medicinal properties of the plant include, antitumour [20], antidiabetic [21], antinflammatory, antiulcer [22], antispasmodic [23], diuretic [24], antihypertensive [25], cholesterol lowering [26], antioxidant, antidiabetic, hepatoprotective [27], antibacterial and antifungal activities.

Another naturally obtained material with potential for antimicrobial use is propolis. It is a sticky resinous hive product used by bees. The propolis used in this study is commercially obtained and is manufactured under the commercial name 'Probee Propolis' and exhibits antimicrobial, antiviral and antioxidant functions. Propolis can cure inflammation, heart diseases, diabetes and cancer [28]. Several biological activities such as anticancer [29], antioxidant [30-32] anti-inflammatory [33], antibacterial [34], antifungal [35] and antiviral [36] activities have been reported in Propolis and its constituents. Propolis is non-toxic, yet reports of allergic reactions are not uncommon [5].

Chlorhexidine gluconate is a cationic biguanide that seems to act by adsorbing onto the cell wall of microorganism resulting in leakage of intracellular components. Furthermore, because of its cationic structure, chlorhexidine(CHX) has the unique property of substantivity. At low concentration it is bacteriostatic and at a high concentration it is bactericidal. A clinical study showed that a 2% CHX solution, used as a final irrigant, significantly decreased bacterial loads in root canals that had been irrigated with sodium hypochlorite during canal preparation [37]. It is better known for its excellent biocompatibility[9] than for its antimicrobial effectiveness [13].

MTAD: Biopure MTAD cleanser is a mixture of Tetracycline isomer, Acid & Detergent. When used as directed, it is proven to exert a potent antimicrobial activity. According to an in vitro study on the antimicrobial effect of Biopure MTAD on eight strains of *Enterococcus faecalis*, MTAD was effective in completely eliminating growth in seven of eight strains of *E. faecalis*. The MIC/MLC tests showed that MTAD inhibited most strains of *E. faecalis* growth when diluted 1:8192 times and killed most strains of *E. faecalis* when diluted 1:512 times [38].

Evaluation of antimicrobial efficacy: The antimicrobial susceptibility test was done by Well Diffusion method. All the data was collected and statistical analysis was done. One way ANOVA was used for comparison of the means and standard deviation. The results were tabulated accordingly.

The ethanol extract of dry *Moringa oleifera* leaf showed a mean zone of inhibition of 13.5mm, whereas the mean zone of inhibition exhibited by ethanol extract of fresh Moringa oleifera leaf was found to be13.25mm. The commercially available water soluble Propolis did

not show any zone of inhibition against *Enterococcus faecalis*. 2% chlorhexidine showed a mean zone of inhibition of 18mm while MTAD showed a mean zone of inhibition of 18.75mm.The positive control ethanol showed no zone of inhibition.

The results of this in vitro study showed that BioPure MTAD is a viable medicament against *E faecalis* in vitro. This was in accordance with the findings of Shabahang and Torbinejad [39] which showed the efficacy of BioPure MTAD against *E.faecalis*. Although 2% chlorhexidine gluconate showed less antimicrobial effect on E. faecalis than BioPure MTAD, it still had an observable effectiveness against this bacterium. This study supports previous reports of studies that conclude that 2% chlorhexidine demonstrated significant inhibition against *E. faecalis* [11].

The ethanolic extracts of *Moringa oleifera* leaf also produced a significant antimicrobial activity on the *Enterococcus faecalis* strain, hence proving to be an acceptably effective cheaper substitute to MTAD and 2% CHX. Earlier studies by others also confirmed antimicrobial activity of ethanol extract (1175 µg disc-1) of fresh *Moringa oleifera* leaves by exhibiting inhibitory effect against all the gram- negative bacteria tested along with employed gram – positive bacteria [40]. The antimicrobial efficacy of natural substances like Curcuma longa(turmeric) extracts, against *Enterococcus faecalis* have been proved through earlier studies.[41] However further in vivo and other studies to prove the efficacy of *Moringa oleifera* to be used as an irrigant/intracanal medicament are yet essential. Contrary to results reported by some authors, the water soluble propolis failed to exhibit any antimicrobial effect on *E. faecalis* [6-8].

Determination of Minimum Inhibitory Concentration (MIC):

MIC is defined as the lowest concentration where no visible turbidity is observed in the micro titre plates (bacteriostatic concentration). The MIC value of both the ethanol extracts of dry and fresh Moringa leaf against *Enterococcus faecalis* was found to be 50%. Water soluble Propolis extract showed no MIC values for Enterococcus faecalis. MTAD showed MIC value of 3.12% while 2% chlorhexidine was found to exhibit an MIC value of 0.125% against *Enterococcus faecalis*.

CONCLUSION

The present in vitro study was done to evaluate and compare the antimicrobial properties of ethanolic extracts of dry and fresh *Moringa oleifera* leaves, water soluble propolis, 2% chlorhexidine gluconate and MTAD, against the endodontic pathogen *Enterococcus faecalis.* Chlorhexidine and MTAD are known effective irrigants. Results regarding MTAD and CHX were in accordance with various former studies, MTAD showing maximum antibacterial activity followed by 2% chlorhexidine. Propolis however, unlike the reports stated in some other studies, fails to show any antimicrobial property against *E.faecalis*. *Moringa oleifera* showed lesser antimicrobial activity than MTAD and 2% chlorhexidine gluconate, but is an effective and cheaper substitute as an irrigant / medicament owing to its anti microbial properties. However, further in vivo studies and other studies are essential to prove its efficacy in usage for the same.

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