



Antimicrobial Efficacy of Novel Ethanolic Extract of *Morinda Citrifolia* Against *Enterococcus Faecalis* by Agar Well Diffusion and Broth Dilution Methods - An *In Vitro* Study

Eficácia de um novo antimicrobiano etanólico de extrato de *Moringa citrifolia* contra *Enterococcus Faecalis* por meio dos métodos de difusão em ágar e de diluição em caldo – um estudo *in vitro*

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ABSTRACT

Objective: The long-term success of root canal treatment is ultimately related to the effective debridement and disinfection of the root canal system. Hence, the irrigants play an important role in achieving the good penetrability and bactericidal activity. The present study was mainly aimed at evaluating the *in vitro* antimicrobial efficacy of Novel Ethanolic Extract of *Morinda Citrifolia* by agar well diffusion and broth dilution methods. **Material and methods:** The antibacterial effect of Ethanolic Extract of *Morinda Citrifolia* was investigated against *Enterococcus Faecalis* (*E. Faecalis*). Agar well diffusion and broth dilution methods were used to determine the Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) **Results:** The MIC of Ethanolic Extract of *Morinda Citrifolia* extract was found to be 12.5 mg/ml and the MBC was found to be 25 mg/ml **Conclusion:** Novel Ethanolic Extract of *Morinda Citrifolia* possess antimicrobial activity against *E. Faecalis*. But still, future studies are needed.

KEYWORDS

Ethanolic Extract of *Morinda Citrifolia*; Endodontics; Irrigant; Root Canal; Minimal Inhibitory Concentration; MBC.

RESUMO

Objetivo: O sucesso a longo prazo do tratamento de canais radiculares está intimamente relacionada com a eficácia do debridamento e desinfecção do Sistema do canal radicular. Consequentemente, os irrigantes têm um importante papel na capacidade de penetração e atividade bactericida. O presente estudo teve como objetivo principal a avaliação *in vitro* da eficácia antimicrobiana do novo extrato etanólico de *Morinda Citrifolia* por meio dos métodos de difusão em ágar ou de diluição em caldo. **Material e métodos:** O efeito antibacteriano do extrato etanólico de *Morinda Citrifolia* foi investigado contra *Enterococcus Faecalis* (*E. Faecalis*). Os métodos de diluição em ágar e de diluição em caldo foram usados para determinar a concentração inibitória mínima (MIC) e concentração bactericida mínima (MBC). **Resultados:** O MIC do extrato etanólico de *Morinda Citrifolia* foi obtido na concentração de 12.5mg/ml e a MBC foi de 25mg/ml **Conclusão:** O novo extrato etanólico de *Morinda Citrifolia* apresenta atividade antimicrobiana contra *E. Faecalis*. Porém, futuros estudos são necessários.

PALAVRAS-CHAVE

Extrato etanólico de *Morinda Citrifolia*; Endodontia; Irrigantes; Canal radicular; MIC; MBC.

INTRODUCTION

The long-term success of root canal treatment is ultimately related to the effective debridement and disinfection of the root canal system [1]. Chemo-mechanical preparation plays a vital role in achieving successful endodontic therapeutic outcomes. Sodium hypochlorite (NaOCl) is one of the most commonly used root canal irrigant because of its ability to show a broad-spectrum antimicrobial activity and its ability to dissolve the necrotic pulp tissue [2]. Even though it is the most potent irrigant, it has some adverse characteristics like its tissue toxicity, allergic potential and disagreeable taste. This prompted for the search for alternative irrigants [3].

Literature evidence has shown a wide range of natural plant extracts exhibit antimicrobial property and therapeutic benefits and can be used as root canal irrigants [3-8]. Morinda Citrifolia has been found as a first herbal alternative to sodium hypochlorite as a root canal irrigant. It has a broad range of therapeutic benefits as antibacterial, antiviral, antitumor, anti-helminthic, antifungal, analgesic, hypotensive, anti-inflammatory and immune enhancing effects [5-7]. The antibacterial activity is mainly by two antibacterial compounds L-aperuloside and alizarin [3].

Very few studies [9-13] have been reported on antibacterial activity of Morinda Citrifolia juice on Enterococcus Faecalis (E. Faecalis), the effect of Ethanolic Extract of Morinda Citrifolia has yet not clearly identified. The present study was mainly aimed at evaluating the invitro antimicrobial efficacy of Novel Ethanolic Extract of Morinda Citrifolia by agar well diffusion and Broth dilution methods.

MATERIALS AND METHODS:

The study was approved by institutional ethical committee. This in vitro study was two folds, we first identified and collected

the powder of plant material and then we evaluated the MIC and antimicrobial efficacy of the extract against E. faecalis [14-17]

Bacterial strains

Bacterial strains gram positive E. Faecalis ATCC 29212, was chosen based on their testing importance. The bacterial microorganisms were cultured on nutrient agar by using spread plate technique and were incubated for 24 hours at 37°C. The bacterial strains were grown in Mueller-Hinton agar (MHA) plates at 37°C the bacteria were grown in the nutrient broth at 37°C and maintained on nutrient agar slants at 4°C. The stock cultures were maintained at 4°C. Sterile spreader was used for inoculation of this organisms across respective media.

Ethanolic extract preparation

The powder of plant materials was initially defatted followed by 1000 ml of ethanol by using a Soxhlet extractor for 72 hrs at a temp not exceeding the boiling point of the solvent. The extract was filtered using Whatman filter paper (No. 1) and then concentrated in vacuum and dried at 45°C for ethanol removal, and the extracts were kept in sterile bottles under refrigerated conditions until use. The dry weight of the plant extracts was obtained by the solvent evaporation and used to determine concentration in mg/ml.

Determination of Minimal Inhibitory Concentration

The MIC for the test solutions were determined by broth dilution method according to the CLSI 2012 standard protocol [18] The cultures were then incubated and subsequently, serially diluted to reach the density of 2×10^4 cells per ml. Cell counting was done using haemocytometer. Two millilitres of MHA broth were dispensed in tubes, and 100 μ L of cell culture was inoculated in it. Then, 100 μ L of different concentration of Ethanolic Extract of Morinda Citrifolia (6.25, 12.5, 25.0, 50.0 and 100 mg/ml) was added to each tube. Growth control was run in parallel with every

experiment. All the experimental tubes were incubated in anaerobic jars for 48 h. After completion of incubation period, the optical density was measured at 600 nm. MIC was defined as the minimum concentration of the extract that caused 20% inhibition in growth of test microorganism. Each experiment was carried out in a triplicate set. The lowest concentration prior to colour change was considered as the MIC.

The percentage of bacterial inhibition was computed using the following equation:
 Percentage Inhibition = $\frac{\text{OD in control} - \text{OD in test}}{\text{OD in control}} \times 100$

Determination of Minimum Bacterial Concentration

The antimicrobial efficiency of Ethanolic Extract of Morinda Citrifolia was evaluated by the agar well diffusion method using MHA (Mueller-Hinton agar). The microorganism was then inoculated (0.25 mL) into molten MHA and poured into petri dishes. Wells of uniform diameter (6 mm) were then made on the solidified agar. The discs (6 mm in diameter) were impregnated with experimental test solution (25.0, 50.0 and 100 mg/mL) (100 µL/disc) placed on the inoculated agar. Vancomycin (30 mg/ml) used as positive reference for bacteria respectively. Disc without samples was used as a negative control. Antimicrobial activity was evaluated by measuring the inhibition zone and the negative control (solvent without plant extract) were placed separately in each well. Plates were then left at room temperature for 1 h to allow the solutions diffusion into the MHA, plates were then incubated at 37°C over night. Finally, the zones of inhibition were measured from the base of the plates and the experiments were performed in duplicate and repeated independently three times.

MBC value was determined by sub culturing the test dilution on to freshly prepared nutrient agar media. The plates were incubated further for 18-42 h at 37 °C. The highest dilution that yielded no single

bacterial colony, (i.e.) which showed no visible turbidity on the nutrient agar plates was taken as MBC.

Statistical analysis

For statistical analysis of data, multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the LSD test for post hoc analysis. Statistical significance was accepted at a level of $P < 0.05$. Data were analysed using SPSS (version 11).

RESULTS

Table 1 depicts the MIC. Each value is expressed as mean \pm SD ($n = 3$) and the results showed statistically significant difference as compared with negative control ($P < 0.001$) (figure 1). MIC of the test sample is 12.5 mg/ml.

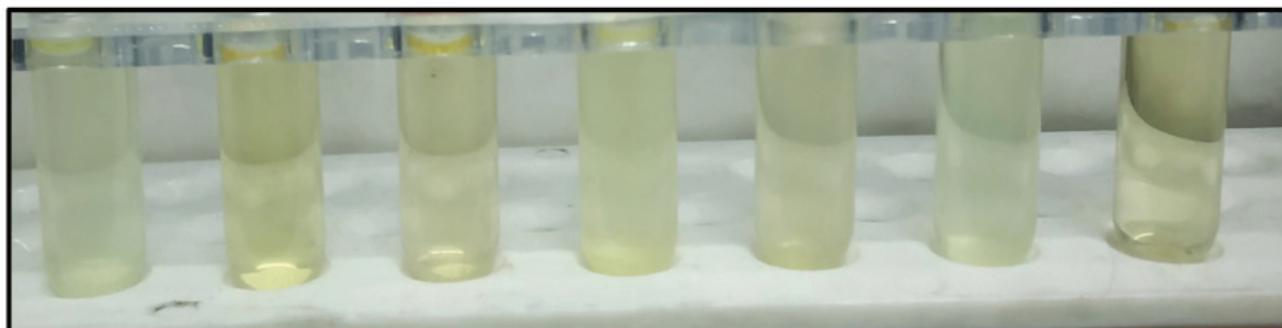
Minimum Bacterial Concentration

Table 2 depicts the zone of inhibition at different concentration. At the concentration of 25mg/ml of Ethanolic Extract of Morinda Citrifolia showed less than 0.15% of inoculum to grow on the surface medium (figure 1). NI means no inhibition zone. Each value is expressed as mean \pm SD ($n = 3$) and the results showed statistically significant difference as compared with negative control ($P < 0.05$). MBC of the test sample is 50 µg/ml

Table 1 - Minimal Inhibitory Concentration of Ethanolic Extract of Morinda Citrifolia

Plant extract	Conc (mg/ml)	<i>E. faecalis</i>
Morinda citrifolia extract	625	0.421 \pm 0.18
	125	0.385 \pm 0.09*
	25	0.359 \pm 0.15*
	50	0.175 \pm 0.14*
	100	0.106 \pm 0.11*
Positive control (µg)	30	0.079 \pm 0.03*
Negative control	-	0.482 \pm 0.03

Each value is expressed as mean \pm SD ($n = 3$). * $p < 0.01$ as compared with negative control.



NC MCE(6.25) MCE(12.5) MCE(25) MCE(50) MCE(100) PC

Figure 1 - Broth Dilution Method. PC - Positive control; NC- Negative control; MCE (6.25,12.5, 25, 50 and 100 mg/ml).

Table 2 - Zone of Inhibition at Different Concentrations of Ethanolic Extract of *Morinda Citrifolia*

Samples	Conc (mg/ml)	<i>E. faecalis</i> Zone of Inhibition (mm)
Negative control	-	NI
MCE	25	10.3 ± 0.05*
	50	13.1 ± 0.09*
	100	15.4 ± 1.3*
Vancomycin (µg)	30	17.5 ± 1.9*

NI means no inhibition zone. Each value is expressed as mean ± SD (n = 3). *p<0.001 as compared with negative control. MCE- *Morinda citrifolia* Extract

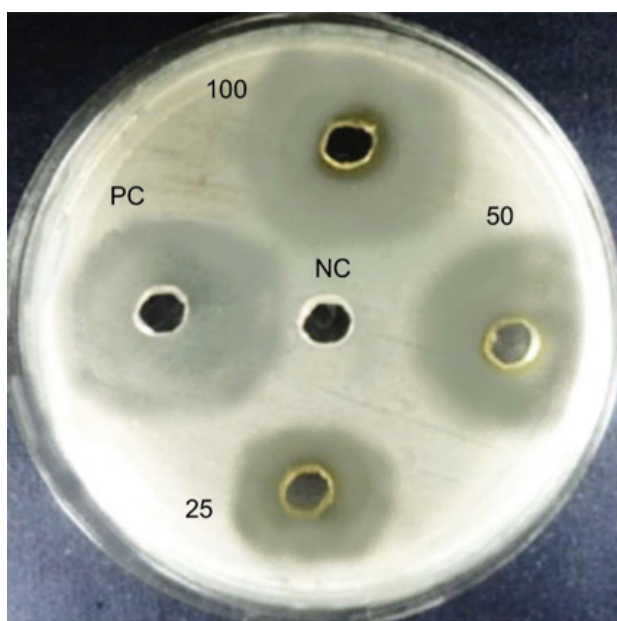


Figure 2 - Zone of Inhibition against *E. Faecalis*. PC - Positive control; NC- Negative control; MCE (25, 50 and 100 mg/ml)

DISCUSSION

Mechanical instrumentation alone cannot reach the anatomical complexities such as narrow isthmuses, accessory canals into the dentinal tubules. Hence, the irrigants play an important role in achieving the good penetrability and bactericidal activity to inhibit the microorganisms present in the biofilms and removal of smear layer, inactivate the endotoxins and dissolve the necrotic pulp remnants [19,20].

Vinoth et al., reported predominant antibacterial activity in the organic solvent as compared to water, which indicates that the active compounds responsible for the bactericidal activity are more soluble in the organic solvents [21]. The evaluated effectiveness of the plants was not due to one constituent, but due to the combined action of other chemical compounds involved in it. Bioactive compounds like alkaloids, flavonoids are the major compounds promoting antimicrobial activity [22].

The assessment was done only on *E. Faecalis* as it is a primary pathogen and resistant to the irrigants used for root canal disinfection. It is also shown that it was also isolated as resistant pathogen in secondary infections. Its prevalence is highest in cases of failed root canals and persistent apical periodontitis cases [23-26]. Hence the present study was mainly aimed to evaluate the activity of the extract on the specified pathogen.

Beside its antibacterial effect on *E. Faecalis*, previous reports have reported role of ethanolic extract of *Morinda Citrifolia* in inhibiting *Streptococcus Mutants* (MTCC497), *Streptococcus Mitis* (MTCC2696), *Streptococcus Aureus*, *Pseudomonas Aeruginosa*, *Bacillus Subtilis*, *E. Coli* [27,28]. This is due to presence of secondary metabolic phenolic compounds L-aperuloside and alizarin

The limitation of the present study was the tested pathogen was only the *E. Faecalis*. The data obtained is not sufficient to generalize the clinical situations. But still, it can be a pavement for the preliminary assessment of the bioactivity of the Novel Ethanolic compound assessed. Future studies have to be done using various compounds and pathogenic organisms to get a comprehensive idea.

CONCLUSION

Present study concludes that the Novel Ethanolic Extract of *Morinda Citrifolia* possess antimicrobial activity against *E. Faecalis*. But still, future studies have to be done assessing its activity on various pathogens to generalize the data obtained to the clinical situations.

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