Research Article **CODEN: AJPCFF** **ISSN: 2321 – 0915**

Asian Journal of Phytomedicine and Clinical Research

Journal home page: www.ajpcrjournal.com

https://doi.org/10.36673/AJPCR.2021.v09.i02.A10

COMPARATIVE EVALUATION OF CITRUS AURANTIFOLIA AND TOLUIDINE BLUE O AS A POTENTIAL PHOTOSENSITIZER AGAINST E-*FAECALIS*: AN *IN VITRO* STUDY

Jinet Joseph[1], Kavitarani Rudagi[*1], Zinnie Nanda[1]

[1*]Department of Conservative Dentistry and Endodontics, ACPM Dental College, Dhule, Maharashtra, India.

ABSTRACT

Objective(s): To compare and evaluate the Antimicrobial Efficacy of Different Light Activated Antimicrobial Agents (LAAAs) like citrus aurantifolia and Toluidine blue O against *E. Faecalis* within the root canal. **Materials and Methods:** 45 single rooted human permanent teeth, were used in this study. The teeth specimens were sectioned to obtain a standardized tooth length of 14 millimetre (mm). The root canals were cleaned and shaped using crown down technique with Protaper instruments (F3). *Enterococcus faecalis* (strain ATCC 29212) grown in tryptic soya broth (TSB) was inoculated in all the samples using a micropipette. Three experimental groups were formed of 15 samples each. Group I: Positive control. Group II (n=15): PDT with citrus aurantifolia oil. Group III (n=15): PDT with Toluidine Blue O. The optical fiber connected to diode laser was used to disinfect the canal. The shavings of the root canals were extracted using saline and 25 H-file, after which they were incubated in tryptic soya broth agar plates at 37ºC for 24hrs. The agar plates were analyzed for colony forming units (CFU) of Enterococccus faecalis in all the groups using a Digital colony counter and were subjected to statistical analysis using one way Analysis of Variance test (ANOVA). Pair-wise comparison was done using Post Hoc multiple comparison (Tukey) test. **Results:** The results of the study showed that, citrus aurantifolia showed antimicrobial effect when used as a photosensitizing agent. However TBO showed the best results followed essential oil of citrus aurantifolia against *E. Faecalis*. **Conclusions:** Within the limitations of the study, it was concluded that citrus aurantifolia has effective photosensitizing antimicrobial property and can be used as a potential LAAA.

KEYWORDS

Antimicrobial photodynamic therapy, Citrus aurantifolia, Photo activation and *E. faecalis*.

Author for Correspondence:

Kavitarani Rudagi,
Department of Conservative Dentistry and Endodontics,
ACPM Dental College, Dhule, Maharashtra, India.

Email: kavita.rudagi@gmail.com

INTRODUCTION

Disinfection of root canal system has always remained a challenge for the clinician in the field of endodontics, the microbial biofilm being the most important factor to mediate root canal infection. Root canal infections are polymicrobial and demonstrate high resistance to antimicrobial agents. To top the list *E. Faecalis* has gained popularity due to its presence in failed root canals, since they serve

as a niche area for microbes. Inspite of the conventional biomechanical preparation and irrigation protocols, the prepared root canal system fails to be completely free from residual bacteria[1].

Over the past decades, substantial efforts have been focused on developing alternate disinfection strategies that are specific to microbial cells. Light has been used as an antimicrobial agent since ancient times and one possible use is in Light Activated Disinfection (LAD), also called as Photodynamic therapy (PDT), which serves as a supplement to the existing protocols in root canal disinfection. LAD is based on the use of a light activated antimicrobial agent (LAAA), which has been one of the emerging novel approach[2,3].

When irradiated, excited photosensitizer (PC) reacts with substrate/ surrounding molecule to produce highly reactive singlet oxygen which induce irreversible damage to microorganisms. A photosensitizer when irradiated with light at a specific wavelength reacts with the surrounding molecular oxygen to produce highly reactive singlet oxygen that result in the cell lysis. Unlike antibiotics, LAD acts on multiple targets in a bacterial cell (such as membrane lipids, genomic DNA, proteins and enzymes) that reduce the chance of bacteria acquiring resistance to treatment[4]. Many *in vitro* and *in-vivo* studies have demonstrated the efficacy of a PDT to treat localised microbial infections. Various photosensitizers such as phenothiaziniums, cationic porphyrins, phthalocyanines, xanthenes (e.g. rose bengal), chlorins, bacteriochlorins, fullerenes, quantum dots have gained popularity due to their ability to inactivate both gram positive and gram negative bacteria[5,6]. Toluidine blue is a basic dye belonging to the family of phenothiazinium which is used mostly commonly as a photosensitizing antimicrobial agent and nuclear stain[7].

The growing interest in complementary medicine has drawn a great deal of attention in herbal medicine. Increasing resistance of microorganisms to antibiotics has opened new gates for herbal medicines with antimicrobial and medicinal properties. The use of PCs against multiresistant bacteria has led to the development of research in herbal field[8]. Recent studies have used curcuma long a which proves to be a potent photosensitizer. Along with it other herbs like Hypericum perforatum, hammelis virginiana, various species of citrus like lime (Citrus x aurantifolia), contain photosensitizing furanocoumarins are used as photosensitizers in dermatology, oncology, gynecology and urology and are therefore considered to be potential photosensitizers against root canal microbiota[9].

Previous study by E.F Nardini[8] *et al*, has shown citrus lemon to be a potential photosensitizer. Drawing a clue from this study, a literature review showed the presence of coumarin derivative which is responsible for the photosensitizing property in citrus aurantifolia which is used in this study. The present study investigated the antimicrobial effect of citrus aurantifolia as a potential photosensitizer in antimicrobial photodynamic therapy in endodontics against enterococcus faecalis.

MATERIAL AND METHODOLOGY

Collection of Teeth

Inclusion Criteria

45 single rooted human permanent teeth with Vertucci class I root canal configuration with fully formed apices, extracted for orthodontic and periodontal reasons were used in this study.

Exclusion Criteria

- Teeth with cracks
- Teeth with root caries
- Teeth with fractured root
- Teeth with dilacerations
- Teeth with root resorption

All selected teeth were stored in normal saline till the time of study.

Preparation of teeth specimen

The specimens were cleaned of superficial debris, calculus, tissue tags using ultrasonic scaler and then rinsed with normal saline. The teeth specimens were then sectioned with a diamond disc to obtain a standardized tooth length of 14 millimetre (mm) measured from the root apex for standardization. Working length was established using no. 10K file

until it was visible through apical foramen. The root canals were cleaned and shaped by keeping instruments 0.5mm short of apex. The instrumentation sequence consisted of Gates Glidden burs 4, 3 and 2 for a coronal 4 mm preparation, followed by using the crown down technique with Protaper instruments to an apical size of F3 protaper.

3ml of 17% EDTA was used as lubricant and 3ml of 5% sodium hypochorite (NaOCl) as irrigant between each instrument during the cleaning and shaping procedures. A final rinsing, aiming to remove smear layer and debris was performed using 3ml 17% EDTA solution for 3 minutes. Then, 3ml of 5% NaOCl was injected in the canal for 3 minutes to clean up any remnants. Final flush was done with normal saline[10,11]. The apical foramina were sealed with a glass ionomer restorative material. Teeth specimens were then autoclaved at 121^{o}C at 15 lbs pressure for 21 minutes.

INOCULATION OF *E. FAECALIS* IN TSB

Enterococcus faecalis (strain ATCC 29212) was grown in tryptic soya broth (TSB) for 24 hours in an incubator set at 37^{o}C. Turbidity of broth was adjusted to 1.0 × 109 colony forming units (CFU)/ml[12]. All samples (except group I) were inoculated with 10µl of the broth using a micropipette. They were then incubated in an incubator for 24 hours at 37^{o}C subsequently; the specimens were dipped in tubes containing normal saline to wash away loosely adherent bacteria. Root canals of the teeth were dried using sterile paper points. Three experimental groups were as follows: Group I (n=15): Teeth infected with *E. faecalis* with no PDT (positive control). Group II (n=15): PDT with citrus aurantifolia oil which was commercially available. (Blue Aroma).

Group III (n=15): PDT with Toluidine Blue O (C.I. 52040, Sigma Aldrich). 306mg of TBO was measured with the help of chemical balance. 100ml of distilled water was measured in a beaker. TBO powder was mixed in water and stirred well to get 0.01% aqueous solution[12]. The canals of specimens were filled to the level of the access cavity with this solution using a syringe and needle and they were incubated for 5 minutes. Then specimens were irradiated for 5 minutes.

Irradiation with laser

Light was applied using a plastic, flexible optical fiber with diameter of 600µm, which was connected to the diode laser at wave length of 670nm[13-16]. The fiber was initially placed 2mm short of the apex and moved gradually toward the middle and cervical third of the canal to impart thorough disinfection of the canal. These movements were repeated approximately 6 times per minute[17]. The root canals were again dried with sterile paper points.

Collection of sample

The specimens were filled with normal saline (NS) and gently filed in a circumferential way using size 25 H-files to working length. The contents of root canals were as pirated using a sterile syringe and serially diluted with NS. 100µl of each dilution was placed in culture plates containing Tryptic Soya Broth agar medium. The plates were incubated at 37^{o}C for 24 hours. The Tryptic Soya Broth Agar plates were analyzed for colony forming units (CFU) of Enter ococccus faecalis in all the groups using a Digital colony counter and tabulated.

Statistical Analysis

Data was analyzed using Statistical Package for Social Sciences version 20 (SPSS -20) statistical software. Growth of Enter ococccus faecalis in each group (CFU/ml) was presented as mean and standard deviation. Comparison of the mean difference in between all the groups was done using one way Analysis of Variance test (ANOVA). Pair-wise comparison was done using Post Hoc multiple comparison (Tukey) test. The mean difference was considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Table No.1 shows mean and standard deviation values of Colony Forming Units (CFU) of Enterococcus faecalis in each group. Comparing the antimicrobial effect of different LAAAs against *E. faecalis*, the lowest mean value of CFU was recorded with Toluidine Blue O (TBO) group (204.13), followed by Citrus aurantifolia group

(226.60) and then control group (597.80). The highest mean value of CFU was recorded with the control (saline) group (597.80). One way ANOVA test revealed that there were statistically highly significant differences among all the groups (p=0.001).

Table No.1: One way ANOVA test; *indicates significant difference at p≤0.05.

Table No.2 shows pair wise comparison of Colony forming units (CFU) between all 3 groups. Comparing the antimicrobial effect of different LAAAs against *E. faecalis*, between group I (positive control group) and group II (citrus aurantifolia), group II (226.60) showed significant reduction in *E. faecalis*.

On pair wise comparison between group II and group III (TBO), group III showed significant reduction in *E. faecalis* compared to group II.

Discussion

Endodontics is concerned with treatment of both primary and secondary infections of root canal. *E. faecalis* is the most commonly seen microorganism in either cases, especially in secondary infection of root canals[17].

E. faecalis has the ability to survive in extreme alkaline pH. It can invade and colonize in dentinal tubules to a depth >1000µm or even close to cementum which cannot be eliminated by irrigants alone and can survive a temperature of 600ºC[18]. This highly complex nature of the *E. faecalis* poses a great challenge for endodontists. Therefore, Enterococcus faecalis (ATCC 29212) was used in the present study. Combination of LAD along with mechanical debridement and antiseptic irrigation has shown to significantly reduce bacterial load and bacterial regrowth in infected root canals compared with either treatment alone[19,20]. It does not show toxicity against periapical tissues, effectively eliminates microorganisms organised in the biofilm structure and is not associated with the risk of resistance development, contrary to antibiotic therapy[21,22].

The result of this present study suggested that citrus aurantifolia and TBO showed significant difference in the antimicrobial activity against *E. Faecalis* Using low level laser at 650nm.

In this study citrus showed significant results compared to the control group against *E. Faecalis*. This was in accordance with a previous study in which they investigated the efficacy of photo activated Citrus Aurantifolia EO with natural light. It was observed that highest significant reduction of more than 99% was achieved by the Citrus group which were incubated in sunlight. Essential oil of Citrus Aurantifolia is a lipophilic product that interact with membrane proteins and enzymes, changing the cell permeability thereby causing cell death[23]. Antibacterial citral essential oil properties (i.e, in particular linalol, coumarine, furanocoumarins limonene and phenolic ingredients) are suggested to be related to both membrane and intra cellular-derived mechanisms. Free radical production may be augmented by light exposure and this may be expository to enhanced eradication of microorganisms[9]. This has been in accordance with the study by Fakrazad *et al*, (2015)[9] in which essential oil of citrus aurantifolia was shown to be an effective antifungal agent in eradication of C. albicans.

In this study, TBO showed significantly better results compared to citrus aurantifolia, the probable reasons for this can be Toluidine blue being an antimicrobial agent can cause 90-100% killing of *E. faecalis*[14,24,25], since they are known to have effective plasma membrane attacking photosensitizer, a great ability to form dimers or aggregates which are the principal species participating in photooxidation and the partition coefficient which is responsible for hydrophobicity, is high in TBO. This accounts for the greater ability of TB molecules to permeate and accumulate in the hydrophobic region of the cellular membrane and therefore have a greater photo bactericidal activity and so is able to penetrate into the plasma membrane and damage photolabile proteins which ultimately leads to the cell death in microorganisms[26,27]. This could be one possible reason for the reduced antimicrobial effect of citrus aurantifolia since it was oil based and therefore had

reduced permeability reducing its antibacterial effect against *E. faecalis*. Thus making way for further research using water soluble form of citrus aurantifolia in checking the antimicrobial photosensitizing efficacy against *E. Faecalis*.

Table No.1: Comparison of CFU

S.No	Groups	N	Mean	Std. Deviation	p value
1	Saline	15	597.80	12.376	0.001*
2	Citrus aurantifolia	15	226.60	19.041	
3	Toluidine Blue O	15	204.13	21.270	

Table No.2: Pair wise comparison

S.No	Groups	Mean CFU	Difference	p value
1	Saline	597.80	371.20	0.001*
2	Citrus aurantifolia	226.60		
3	Saline	597.80	393.667	0.001*
4	Toluidine Blue O	204.13		
5	Citrusauantifoa	226.60	22.467	0.004*
6	Toluidine Blue O	204.13		

Post HOC tukey test; * indicates significant difference at $p \leq 0.05$.

CONCLUSION

Within the limitations of the study, it was concluded that citrus aurantifolia has effective photosensitizing antimicrobial property and can be used as a potential LAAA.

ABBREVIATION

Photosensitizer (PC)
Light activated antimicrobial agent (LAAA)
Activated photodynamic therapy (aPDT)

ACKNOWLEDGEMENT

The authors wish to express their sincere gratitude to Department of Conservative Dentistry and Endodontics, ACPM Dental College, Dhule, Maharashtra, India for providing necessary facilities to carry out this research work.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

BIBLIOGRAPHY

1. Kishen A. Advanced therapeutic options for endodontic biofilms, *Endodontic Topics,* 22(1), 2010, 99-123.
2. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT), *The Journal of Antimicrobial Chemotherapy,* 42(1), 1998, 13-28.
3. Bhatti M, Mac Robert A, Meghji S, Henderson B, Wilson M. A study of the uptake of toluidine blue O by Porphyromonas gingivalis and the mechanism of lethal photosensitization, *Photochemistry and Photobiology,* 68(3), 1998, 370-376.
4. Kwiatkowski S, Knap B, Przystupski D, Saczko J, Kedzierska E, Knap-Czop K, Kulbacka J. Photodynamic therapy - mechanisms, photosensitizers and combinations, *Biomedicine and Pharmacotherapy,* 106(106), 2018, 1098-1107.
5. Abrahamse H, Hamblin M R. New photosensitizers for photodynamic therapy, *Biochemical Journal,* 473(4), 2016, 347-364.
6. Ge J, Lan M, Zhou B, Liu W, Guo L, Wang H, Jia Q, Niu G, Huang X, Zhou H, Meng X. A graphene quantum dot photodynamic therapy agent with high singlet oxygen

generation, *Nature Communications,* 5(1), 2014, 4596.
7. Wainwright M, Phoenix D A, Marland J, Wareing D R, Bolton F J. A study of photo bactericidal activity in the phenothiazinium seriesm, *Pathogens and Disease,* 19(1), 1997, 75-80.
8. Villacorta R B, Roque K E J, Tapang G A, Jacinto S D, Plant extracts as natural photosensitizers in photodynamic therapy: *In vitro* activity against human mammary adenocarcinoma MCF-7 cells, *Asian Pac. J. Trop. Biomed,* 7(4), 2017, 358-366.
9. Fekrazad R, Mir A P B, Barghi V G, Ghahfarokhi M S, Eradication of C. Albicans and T. Rubrum with Photo activated Indocyanine Green, Citrus aurantifolia essential oil and fluconazole, *Photodiagnosis and Photodynamic Therapy,* 12(2), 2015, 289-297.
10. Soukos N S, Chen P S, Morris J T, Ruggiero K, Abernethy A D, Som S, Foschi F, Doucette S, Bammann L L, Fontana C R, Doukas A G. Photodynamic therapy for endodontic disinfection, *Journal of Endodontics,* 32(10), 2006, 979-984.
11. Mohan D, Maruthingal S, Indira R, Divakar D D, Al Kheraif A A, Ramakrishnaiah R, Durgesh B H, Basavarajappa S, John J. Photo activated disinfection (PAD) of dental root canal system-An *ex-vivo* study, *Saudi Journal of Biological Sciences,* 23(1), 2016, 122-127.
12. George S, Kishen A. Influence of photosensitizer solvent on the mechanisms of photo activated killing of Enterococcus faecalis, *Photochemistry and Photobiology,* 84(3), 2008, 734-740.
13. Soukos N S, Chen P S, Morris J T, Ruggiero K, Abernethy A D, Som S, Foschi F, Doucette S, Bammann L L, Fontana C R, Doukas A G. Photodynamic therapy for endodontic disinfection, *Journal of Endodontics,* 32(10), 2006, 979-984.
14. Bonsor S J, Nichol R, Reid T M, Pearson G J. Microbiological evaluation of photo-activated disinfection in endodontics (an *in vivo* study), *B Den Jo,* 200(6), 2006, 337-341.
15. Kaiwar A, Usha H L, Meena N, Ashwini P, Murthy C S. The efficiency of root canal disinfection using a diode laser: *In vitro* study, *Indian J Dent Res,* 24(1), 2013, 14-18.
16. Souza L C, Brito P R, De Oliveira J C, Alves F R, Moreira E J, Sampaio Filho H R, Rocas I N, Siqueira J F Jr. Photodynamic therapy with two different photosensitizers as a supplement to instrumentation/irrigation procedures in promoting intracanal reduction of Enterococcus faecalis, *J Endod,* 36(2), 2010, 292-296.
17. Gajan E B, Aghazadeh M, Abashov R, Milani AS, Moosavi Z. Microbial flora of root canals of pulpally-infected teeth: Enterococcus faecalis a prevalent species, *Journal of Dental Research, Dental Clinics, Dental Prospects,* 3(1), 2009, 24-27.
18. Hedge V. Enterococcus faecalis; Clinical significance and treatment considerations, *Endodontology,* 21(2), 2009, 48-52.
19. Garcez A S, Ribeiro M S, Tegos G P, Nunez S C, Jorge A O, Hamblin M R. Antimicrobial photodynamic therapy combined with conventional endodontic treatment to eliminate root canal bio film infection, *Lasers in Surgery and Medicine,* 39(1), 2007, 59-66.
20. Ng R, Singh F, Papamanou D A, Song X, Patel C, Holewa C, Patel N, Klepac Ceraj V, Fontana C R, Kent R, Pagonis T C. Endodontic photodynamic therapy *ex vivo*, *Journal of Endo,* 37(2), 2011, 217-222.
21. Tanaka M, Kinoshita M, Yoshihara Y, Shinomiya N, Seki S, Nemoto K, Hirayama T, Dai T, Huang L, Hamblin M R, Morimoto Y. Optimal photosensitizers for photodynamic therapy of infections should kill bacteria but spare neutrophils, *Phochry and Photo,* 88(1), 2012, 227-232.
22. Prazmo E J, Godlewska R A, Mielczarek A B. Effectiveness of repeated photodynamic therapy in the elimination of intracanal Enterococcus faecalis biofilm: An *in vitro*

study, *Lasers in Medical Science,* 32(3), 2017, 655-661.
23. Ekawati E and Darmanto W. Lemon (Citrus Limon) juice has antibacterial potential against diarrhea-causing pathogen, *IOP Conference Series: Earth and Environmental Science,* 217(1), 2019, 012023.
24. Bonsor S J, Nichol R, Reid T M, Pearson G J. An alternative regimen for root canal disinfection, *British Dental Journal,* 201(2), 2006, 101-105.
25. Usacheva M N, Teichert M C, Biel M A. Comparison of the methylene blue and toluidine blue photobactericidal efficacy against gram-positive and gram-negative microorganisms, *Lasers in Surgery and Medicine,* 29(2), 2001, 165-173.
26. Fonseca M B, Junior P O, Pallota R C, Filho H F, Denardin O V, Rapoport A, Dedivitis R A, Veronezi J F, Genovese W J, Ricardo A L. Photodynamic therapy for root canals infected with Enterococcus faecalis, *Photomedicine and Laser Surgery,* 26(3), 2008, 209-213.
27. Nielsen H K, Garcia J, Vaeth M, Schlafer S. Comparison of riboflavin and toluidine blue O as photosensitizers for photo activated disinfection on endodontic and periodontal pathogens *in vitro*, *PloS One,* 10(10), 2015, e0140720.

Please cite this article in press as: Kavitarani Rudagi *et al.* Comparative evaluation of citrus aurantifolia and Toluidine blue o as a potential photosensitizer against E-faecalis: An *in vitro* study, *Asian Journal of Phytomedicine and Clinical Research,* 9(2), 2021, 62-68.