Effect of Tea Tree, Thymus Vulgaris and Nigella Sativa Oils on The Elimination of Enterococcus Faecalis (In Vitro Study)

Rafid J. Al-Badr, B.D.S. [1]
Hussain F. Al-Huwaizi, B.D.S., M.Sc., Ph.D.[2]

ABSTRACT

Background: The main goal of chemomechanical endodontic treatment is the reduction or elimination of microorganisms from root canal system. The intracanal medicaments were used to enhance the disinfection process. This study was conducted to evaluate the antibacterial effect of thymus vulgaris, tea tree essential oils and cold pressed black seed oil (BSO) against *E.faecalis*.

Materials and methods: *E.faecalis* was isolated from ten patients in need for endodontic treatment. The sensitivity of *E.faecalis* to the tested oils was evaluated in different concentrations in agar well diffusion method and compared with calcium hydroxide. The sensitivity of *E.faecalis* to vapor of the tested oils was also evaluated, in disk vaporization method using inverted agar plate and compared to tricresol formalin (TC) and camphorated monochlorophenol (CMCP).
The micro broth dilution method was used to evaluate the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the tested oils against *E.faecalis*. The presence of biologically active volatile components of two samples of BSO with different origins was evaluated by the use of high performance liquid chromatography (HPLC).

Results: All the tested oils exhibited antibacterial activity against *E.faecalis* in different concentrations with different levels in agar well diffusion and disk vaporization methods. The MBC was 2µl/mL, for thymus vulgaris oil and 32µl/mL for tea tree and Black seed oils. The vapor forming medicaments (TC and CMCP) induced effective antibacterial action but calcium hydroxide showed a low antibacterial action against *E.faecalis*. The active volatile components were present in one sample of BSO only (the Iraqi one).

Conclusion: The three oil extracts were active against *E.faecalis*, and the origin, condition of storage and method of extraction may affect the components of cold pressed black seed oil.

Keywords: Essential oils, E.faecalis, Intracanal medicaments, calcium hydroxide. **(J Bagh Coll Dentistry 2017; 29(1):55-62)**

INTRODUCTION

The main goal of chemomechanical endodontic treatment is the removal or severe reduction of microorganisms present in the root canal system. The complex anatomy of root canal system and the remaining bacteria in some areas like ramifications, accessory canals and dentinal tubules may prevent this goal to be achieved completely. Therefore, the use of intracanal medicaments was advocated to promote the elimination of remaining bacteria and their byproducts from the root canal system [1,2]. *E.faecalis* is the bacterial species related mostly to persistent endodontic infection causing failure endodontic treatment due to its resistance to some known intracanal medicaments
E.faecalis is capable to live within the root canal and resist difficult conditions like starvation and high pH.[3].

Because no intracanal medicaments traditionally used as dressing have all the required properties like inhibition and prevent recolonization of all bacteria in the root canal system, and some of medicaments irritate the periradicular area or with cytotoxic effects [4], so there is a trends toward finding new agents with good antibacterial activity and less or not irritate to the periapical area [5]. Herbal or natural derivatives have been used in dental and medical practice for long time and became even more popular today due to their antimicrobial activity, biocompatibility, lack of microbial resistance, anti-oxidant, anti-inflammatory, easy availability and low cost properties [6,]

MATERIALS AND METHODS

Patient selection and isolation of bacteria:

Ten patients diagnosed with pulpal necrosis with periapical changes indicated for endodontic treatment were included in this study. All the selected teeth were single rooted teeth. The age of the patient ranged from 25-45 years. The teeth were symptomatic and the diagnosis confirmed by radiographic examination that was done in

(1)Master Student, Conservative Department, College of Dentistry, University of Baghdad.
(2)Professor, Conservative Department, College of Dentistry, University of Baghdad.

diagnostic unit in a specialized dental center(Karbala).

The patients under antibiotic treatment and the teeth with severely destructive crown that interfere with placement of rubber dam were excluded.

The field was isolated by using rubber dam in a septic procedure was done by 10 % povidone iodine solution to the tooth and the field around it. The access cavity was prepared in two steps the first was the removal of all carious lesions and/or coronal restorations by a sterile carbide fissure bur, then disinfecting the field again. The second step was opening access cavity to the pulp chamber by using a low speed round carbide bur. New sterile files were introduced into the root canals to the end of the working length (confirmed by x-ray) to remove the content of root canals, followed by enlargement of the canals with minimal instrumentation to the size 20# without use of any irrigants. The canal was flooded with sterile saline solution and agitation vertically with file was done in order to form a suspension of bacteria from all the parts of the root canal[7]. Then two paper points were inserted to the full working length and kept for 60 seconds until the canal was dry. They were transferred directly and immediately to a tube of sterile transport media (AMIES)[8].The samples were transported to the laboratory within two hours for isolation and identification. [9]

Identification of micro-organism:

1- Morphological characteristic:Examination was done directly on blood and Pfizer agar plates, and according to the description cited by **Public Health England (2014).**[10]

2-Gram's stain:Slide of suspected bacteria was prepared and Gram's stain method was performed to identify the Gram's positive property of *E.faecalis.*[10]

3-Catalase production Test: Drop of 3% hydrogen peroxide immediately placed on microorganismson a sterile slide. The evolution of bubbles of gas indicated a positive test, no reaction (no bubbles) indicate catalase negative test.[10]

4-Analytic Profile Index (API): This test was done according to manufacturer's instructions using API 20 Strep identification kits a standardized system combining 20 biochemical tests. It enables species identification of most streptococci and enterococci, and those most common related organism.[11]

5-Mast strep test;Procedure was doneaccording to manufacturer's instructions by picking up 2-6 colonies of suspected *E.faecalis* from freshly growing overnight culture and suspended in 0.4 mL extraction enzyme then added one drop ofsuspension to one drop of latex reagent previously placed on the test card.[12]

Test the sensitivity of *E.faecalis* to different concentrations of Thyme oil (TO), tea tree oil (TTO), and black seed oil(BSO):

Agar well diffusion method:

Five different concentrations (75%-50%-25%-12.5%) beside 100% were prepared of the three experimental oils (TO, TTO oils4life/UK. And BSOAL-Emad co. Iraq) by diluting the oils with dimethyl sulfoxide (DMSO) (CDH Co., Ltd.India).The procedure was done by mixing 10% DMSO solution to experimental oils in 3:1, 1:1, 1:3 and 1:7(oil: 10%DMSO)[13].MHA(Himedia/India) media in petri dish was inoculated with 100µl of *E.faecalis* suspension that was prepared as 0.5 Mcfarland (turbidity standard).The inoculum was spread in all directions by mean of sterilized cotton swap. Four wells of equal size (6 mm in diameter) and 4 mm depth were prepared in each agar plate; two wells were filled with 100 µl of two different concentrations of the test oil. One well was filled with 10%DMSO solution as negative control, the last well filled with Ca $(OH)_2$.

Plates incubated aerobically for 18-24 hours at 37°C. Zone of inhibitions which is clear zone of no growth of the bacteria were measured across the diameter of each well by using a digital vernier caliper, no zone indicated a complete resistance of bacteria to the agents [14].

Test the sensitivity of *E.faecalis* to a vapor of TTO, TO, and BSO:

The inoculated petri dishes was sterilized and inverted, filter paper disk of 5 mm diameter was impregnated with 15 µl of each experimental oil concentrations, tricresol formalin and CMCP, then placed on the inner surface of the lid of the petri dish (one disk for each plate). The petri dish was wrapped by a laboratory parafilm to keep the vapor of the medicament inside.

Plates were incubated aerobically for 18-24 hour at 37°C. Zone of inhibition which is clear zone of no growth of the bacteria was measured by digital vernier caliper [15].

Determination of MIC of the tested oils:

Bacterial inoculation was prepared by direct colony suspension method to reach 0.5 McFarland that equal to $1*10^8$ bacterial cell/mLwith Mueller

Hinton broth (MHB)(Himedia/India).In the first well of microtiter (96-wells plate) 12.8 µl of the tested oil was placed and mixed with 184 µl MHB broth and 3.2 µl DMSO, so the final volume in the first well is 200µl.Then eight concentrations of oils was ranged 32-0.25µl/mL was prepared in next seven wells. Final concentration of DMSO ≤0.8% v/v [16].Then one well was added with 200µl of uninoculated media serve as negative control and other one with inoculated media serve as positive control.The last concentration that lacks visual turbidity matching the (-ve) control was considered as MIC,and the result confirmed with subculture of 10 µl from each well to determine if the inhibition was reversible or permanent.

Determination of MBC of the tested oils:

The final sub culture were done by taking 10 µl from each (well mixed) well and cultured on agar plates to identify the MBC.The MBC was the first concentration higher or equal to MIC with no growth on agar media. [17]

HPLC analysis of two types of BSO one of them made in Iraq and the other made in the United Kingdom for determination of active volatile components (thymoquinone):

Samples from the two BSO was analyzed by HPLC. The separation occurred on liquid chromatography Shimadzu LC-20AD, the eluted peaks were monitored by SPD-20A UV/VIS detector spectrophotometry. The active compounds of BSO separated on FLC (Fast liquid Chromatographic) column (100-10 C18), mobile phase utilized was composed of water: methanol: 2-propanol (50:45:5% v: v), UV monitoring was carried out at 254 nm for thymoquinone, dithymoquinone. Flow rate was 2 mL/min. The result was compared with standard figure [18].

RESULTS:

Identification of *E.faecalis:*

The tested colonies were identified as Gram positive, Catalase negative, grow well on blood agar as circular smooth elevated entire edged and the size of colony about 1-2mm, with no hemolysis. *E.faecalis* grow well on Pfizer agar with blackening around the colony. The result was confirmed by API 20 and Mastastrep latex co-agglutination test when visible aggregation of the latex particles, within few seconds of mixing of the bacteria with the specified reagent appeared.

Sensitivity of *E.faecalis* to different concentrations of TTO, TO, and BSO: Agar well diffusion method:

The diameter of inhibition zone was found to increase as the concentrations of the oils increases. Ten percent DMSO showed no zone of inhibition while $Ca(OH)_2$ showed small to moderate zones of inhibition compared to all the tested oils concentrations as shown in Table (1)**.** One way ANOVA was performedamong different concentrations of each oil as in table (1).

Least significant difference (LSD) test was used between different concentrations of tested oils and Ca(OH)2, as in table (2). It was found that a highly significant difference between $Ca(OH)_2$ and all agents except between $Ca(OH)_2$, TO 12.5% and TTO 12.5% there isno significant difference.

Test the sensitivity of *E.faecalis* to a vapor action of TTO, TO, and BSO:

The diameter of inhibition zonewas found to increase as the concentrations of the oils increases. Ten percent DMSO showed no zone of inhibition, T.C showed a large zone of inhibition larger than CMCP, TO, TTO except for BSO, T.C nearly equal to 50% BSO, and less than BSO 100% and BSO75%. CMCP showed a zone of inhibition more than TTO 100% and TO 75%, and less than T.C, TO100%, and BSO 100%, 75%, 50%. Not all tested concentrations give inhibition zone in vapor action method as in table (3).

One way ANOVA was performed among different concentrations of each oil as in table (3), and (LSD) test used between different concentrations of tested oils, T.C, and CMCP as in table (4). It showed a highly significant difference between T.C and all agents except between T.C and BSO 50% ,75% , were no significant difference, and for CMCP the table showed a highly significant difference with all tested concentrations except TTO100% there was no significant difference.

Determination of MIC and MBC of TO, TTO, BSO:

The MIC of TO was 1µl/mL, and MIC for BSO and TTO were 16 µl/mL. The MBC was 2 µl/mL for TO and 32 µl/mL for TTO, BSO.

HPLC analysis of two types of BSO one of them made in Iraq and the other made in United Kingdom for determination of active volatile components (thymoquinone):

The result showed exact match in retention time of thymoquinone between BSO Iraq and the reference chart also the result chart showed presence of thymol and dithymoquinone in BSO Iraq as in figure (1).The resulted chart of BSO UK show no

matching with any retention time of the reference one.

DISCUSSION

Biocompatibility, low toxicity, lack of microbial resistance, easy availability and low cost are the major advantages of using herbal alternatives in endodontic dentistry [19]. In this study the results of sensitivity test showed that TOwas able to inhibit the growth of *E.faecalis* at different concentrations. This finding was in coincidence with **silva *et al.*,(2013).**[20]

The diameter of inhibition zones increased as the concentration of thymus vulgaris essential oils increased from 12.5% to 100%. This result come in agreement with **Mith *et al.*, (2013)**[21] who studied the antimicrobial properties of TO using agar diffusion method against different types of microorganisms and the results supported the hypothesis that increasing concentration of TO would increase the inhibition of bacterial growth and TO possesses antimicrobial activity on different microorganisms.

TO of 25%, 50%, 75% and 100% concentrations showed larger inhibition zones than $Ca(OH)_2$, and statistically all these concentrations presented highly significant difference, which suggests that they have higher antimicrobial activity than $Ca(OH)_2$. There was no significant difference between TO 12.5% and Ca $(OH)_2$ and this finding presents a great promise to use TO as an alternative to Ca $(OH)_2$.

In this study TO showed powerful antibacterial activity with larger inhibition zone than TTO with statistically significance difference in all concentrations except 12.5% there was no significance difference. This result is in coincidence with another study on these two essential oils tested on another type of bacteria like erythromycin-resistant group A Streptococci [22].

In this study TO showed inhibition zone comparable to BSO, with larger zone in concentration 100% than BSO, but the inhibition zones of TO was smaller than the zone formed by BSO for other tested concentrations. This activity of TO may be due to its biological active constituents thymol and carvacrol, such compounds were reported to have an active effect on the bacterial cells membrane, which might destroy these microorganisms [22].

In the present study the results of sensitivity test showed that TTO was able to inhibit the growth of *E.faecalis* at different concentrations. This finding was in agreement with [23]. The antimicrobial activity of TTO on different microorganisms at different concentrations with increased activity when the concentrations increased were verified by many studies [24, 25].

In the present study TTO at 25%, 50%, 75% and 100% concentrations showed larger inhibition zones than Ca $(OH)_2$, and smaller inhibition zone than TO. Statistically all these concentrations showed highly significant difference, which suggests that they have higher antibacterial activity than $Ca(OH)_2$ and lower antibacterial activity than TO but there was no significant difference between TO 12.5%, TTO 12.5 and $Ca(OH)_2$. TTO showed smaller inhibition zone than BSO in all tested concentrations with highly significant difference.

The antibacterial activity of TTO may be due to its active constituent terpinen-4-ol, and /or other constituents like alpha terpinene, such components were reported as antibacterial agents when used alone or as constituents of TTO. The suggested mechanisms of action of TTO were inhibition of respiration of bacterial cell, loss of intracellular material and /or loss of membrane integrity and function [26].

Cold pressed Black seed oil was tested in this study in different concentrations and the result showed a promising antibacterial activity with large inhibition zones with statistically high significance difference when compared to Ca (OH) 2,TTO, 25% and 12.5% concentrations of TO, and there was no significance difference between BSO and 50% ,75%,100% TO.

These results were in coincidence with **Youssef *et al.*, (2013)**[27] who tested pure BSO in different concentrations on microorganisms isolated from surgical wounds infections and they found sensitivity of *E.faecalis* to BSO.

Majeed, (2006)[28] tested the activity of cold press black seed oil on common pathogenic bacteria related to root canal infection and the result showed good and comparable antibacterial activity to krezoform.

The antibacterial activity of BSO is related directly to its active constituent thymoquinone and its related compounds thymohydroquinone and thymol. These active volatile constituents are present in low percent in cold pressed oil and itshighly sensitive to light, heat, depends onsource of seed and extraction method [29].

In this study Calcium hydroxide (Ca $(OH)_2$ was found to have a minimal antibacterial activity in agar diffusion method which coincides with **Al-**

Huwaizi (2000)[30] and **Ballal *et al.*, (2007)**[31] who found that Ca $(OH)_2$ combined with distilled water had minimal antibacterial activity in the agar diffusion model.

The result of the present study disagrees with **Mehrvarzfar *et al.*, (2011)**[32] who found calcium hydroxide have superior antibacterial effect on *E.faecalis* than Bioglass 45S5 in direct exposure test.

Test the sensitivity of *E.faecalis* to a vapor action of TTO, TO, and BSO:

The antibacterial activity of CMCP was found to be inferior to TC and this result agrees with **Al-Huwaizi (2000)**[30]**,** who stated that the vapor action of TC was more effective than CMCP in eradication of microflora in necrotic root canals. The results of this study also agrees with **Silva *etal.*, (2012)**[33]**,** who found TC vapor effectively inhibited *E.faecalis* in vitro.

In the present study *E.faecalis* was sensitive to vapor action of 100%, 75% TO and 100% TTO and the result of inhibition zone was comparable to CMCP and smaller than TC and BSO.

The result of TO vapor action of this study was in agreement with **Khadir *et al*., (2013)**[34] who stated that *E.faecalis* was sensitive to vapor of one type of thymus genus. The result is also in agreement with **Dobre *et al*., (2011)**[15] who found that the vapor of TO has a good antimicrobial action against some types of gram positive and negative bacteria beside different types of fungi.

The antibacterial and antifungal activity of TTO vapor against many types of bacteria and fungi was examined also **(Inouye *et al*.,2006).**[35]

In the present study the BSO vapor action was promising. The inhibition zones of BSO 100%, 75% concentrations were greater than the biggest zone in this study that formed by TC, and the 50% concentration of BSO was comparable to TC with 30 mm zone of inhibition. This result complies with **Majeed, (2006)**[28] who stated that vapor of cold pressed black seed oil exhibit antibacterial action comparable to krezoform against different aerobic and anaerobic necrotic root canal bacteria.

Determination of MIC and MBC of TO, TTO and BSO:

The MIC of TO was found to be 1 µl/mL and the MBC of TO was 2 µl /mL. The result of MIC complies with **Sienkiewicz *etal.*, (2012)**[36], who found the MIC of TO against *E.faecalis* was 0.5 µl /mL for bacteria isolated from urine, 0.75 µl /mL for bacteria isolated from wound and 1.25 µl /mL for bacteria was isolated from bed sore. The MIC of TTO was found to be 16 µl /mL and the MBC was 32 µl/mL and this result is in agreement with **Rusenova and Parvanov, (2009)**[37]who found the MIC of TTO for standard *E.faecalis* was 1.0 % v/v.

This result disagree with some studies that may be due to different types and sources of bacteria, method used in the study of MIC, type of broth used and source of essential oil [17,38].

The MIC of BSO was 16 µl /mL and the MBC was 32 µl /mL. There is no available research concerned with MIC and MBC of the cold pressed black seed oil extract against *E.faecalis*.

HPLC analysis of two types of BSO one of them made in Iraq and the other made in United Kingdom for determination of active volatile components (thymoquinone):

In the present study the HPLC analysis of cold pressed black seed oil from Iraq showed matching in the area of retention time of volatile components (thymoquinone. Dithymoquinone and thymol) with the reference chart. The sample from cold pressed black seed oil manufactured in UK when analyzed showed no matching with any of the active volatile components in the reference chart.

The result of differences in biologically active volatile components between two samples may be due to the different origin of black seeds, age of the oil, methods of extraction and especially the condition of storage such as exposure to light and heat [18].

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Table 1: ANOVA, Mean, S.D of inhibition zone in millimeter of E.faecalis to different concentrations and different agents (Agar well diffusion methods).

Agents	No.	Mean	S.D	ANOVA
TO 100%	10	31.507	0.784	F=228.290 D.F=15 P=.000
75%	10	27.971	1.203	
50%	10	26.27	2.092	
25%	10	19.594	1.003	
12.5%	10	12.105	1.378	
TTO 100%	10	24.122	3.093	
75%	10	22.288	2.047	
50%	10	19.156	1.553	
25%	10	18.288	0.841	
12.5%	10	11.071	1.111	
BSO 100%	10	30.577	0.907	
75%	10	28.118	1.027	
50%	10	27.305	0.947	
25%	10	25.492	0.651	
12.5%	10	17.57	1.017	
$Ca(OH)_2$	10	10.975	0.607	
10%DMSO	10	0	0	

Table 2: LSD between Ca(OH)2 and each concentration of each oil.

Agents	Concentrations%	Mean Difference	P-value	Description
TO	100	-20.53	.000	HS
	75	-16.99	.000	HS
	50	-15.29	.000	HS
	25	-8.61	.000	HS
	12.5	-1.13	.076	NS
TTO	100	-13.14	.000	HS
	75	-11.31	.000	HS
	50	-8.18	.000	HS
	25	-7.31	.000	HS
	12.5	-0.09	.880	NS
BSO	100	-19.60	.000	HS
	75	-17.14	.000	HS
	50	-16.33	.000	HS
	25	-14.51	.000	HS
	12.5	-6.59	.000	HS

Table 3: ANOVA, Mean, S.D of *E.faecalis* inhibition zone in millimeter of different concentrations and different agents (vaporization method).				
Agents	**NO.**	**Mean**	**S.D**	**ANOVA**
T.C	10	30.867	0.584	
CMCP	10	13.779	1.248	F=514.863
TO 100%	10	18.529	0.904	
75%	10	11.319	1.009	D.F=7
TTO 100%	10	13.172	1.019	
BSO 100%	10	34.598	2.248	P=.000
75%	10	32.038	1.644	
50%	10	30.104	1.503	
10%DMSO	10	0	0	

Table 4: LSD among T.C, CMCP and each concentration of tested oils(vaporization)				
Control	**Agents**	**Mean Difference**	**P-value**	**Description**
T.C	TO100%	12.33	.000	HS
	75%	19.54	.000	HS
	TTO 100%	17.69	.000	HS
	BSO 100%	-3.73	.000	HS
	75%	-1.17	.058	NS
	50%	.76	.214	NS
CMCP	TO100%	-4.75	.000	HS
	75%	2.46	.000	HS
	TTO 100%	.60	.322	NS
	BSO100%	-20.81	.000	HS
	75%	-18.25	.000	HS
	50%	-16.32	.000	HS