

Effect of Thymol Incorporation on Antibacterial Activity of the Locally Prepared Zinc Oxide-Guaiacol Root Canal Sealer: An in Vitro Study

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الخلاصة

الأهداف: تهدف الدراسة إلى إعداد صيغة جديدة عن طريق دمج بلورات الثايمول في مادة سداد قناة الجذر (أكسيد الزنك-غواياكول) المحضرة محليًا وتقييم النشاط المضاد للبكتيريا ومقارنة النتائج بسداد قناة الجذر القائم على (أكسيد الزنك الأوجينول). **المواد وطرائق العمل:** إضافة بلورات الثايمول بثلاث تراكيز مختلفة (2%، 5%، و 10%) لتحسين الفعالية المضادة للبكتيريا لسداد قناة الجذر (أكسيد الزنك - غواياكول): تم استخدام 40 صفيحة بتري من وسط أجار انتيروكوكس، كل صفيحة بها خمسة أبار. تم تقسيم أطباق بتري إلى أربع مجموعات حسب فترات حضانة (1 يوم، 2، 3، 7 أيام) لكل نوع، باستخدام اختبار انتشار الأجار مع 0.5 معايير مكفارلاند للعكارة وتحليلها إحصائيًا باستخدام الإحصاء الوصفي، واختبار انوفًا أحادي الاتجاه، واختبار دانكن. **النتائج:** مجموعات (أكسيد الزنك - غواياكول) المدمجة بالثايمول بالتركيزات المدروسة (2%، 5%، و 10%) تنتج مناطق تثبيط نمو بكتيرية استمرت في جميع فترات الحضانة عند (يوم 1، 2، 3، 7 أيام) وبفرق معنوي عند $p \leq 0.01$ مقارنة بمادة السداد القائمة على أكسيد الزنك الأوجينول. هناك انخفاض معنوي عند $p \leq 0.01$ في الفعالية المضادة للبكتيريا في جميع مجموعات الدمج في فترة حضانة 7 أيام، مجموعة دمج الثايمول 5% أنتجت أكبر مناطق تثبيط نمو بكتيرية لجميع فترات الحضانة. **الاستنتاجات:** خلصت هذه الدراسة إلى أن تركيبات السداد من (أكسيد الزنك - غواياكول) المحضرة مع ثلاثة تراكيز مدروسة من بلورات الثايمول لها فعالية مضادة للجراثيم أفضل من تلك الموجودة في سداد قناة جذر (أكسيد الزنك الأوجينول)، تركيبة الدمج 5% ثايمول هي الأفضل في الفعالية المضادة للبكتيريا من غيرها.

ABSTRACT

Aims: This study aims to prepare a new formula by thymol crystals incorporation into locally prepared zinc oxide-guaiacol root canal sealer and evaluating the antibacterial activity and comparing the results with zinc oxide eugenol-based root canal sealer. **Materials and Methods:** The addition of thymol crystals at three different concentrations (2%, 5%, and 10%) to improve the antibacterial activity of zinc oxide-guaiacol endodontic sealer was done: 40 Petri plates of Enterococcus agar media were used, each plate with five wells. Petri plates divided into four groups incubation intervals (1, 2, 3, and 7 days) for each type, using agar diffusion test (ADT) with 0.5 McFarland turbidity standards and statistically analyzed using descriptive statistics, a one-way ANOVA test, and Duncan's Multiple Range Test. **Results:** The thymol incorporated zinc oxide-guaiacol sealer groups at studied concentrations (2%, 5%, and 10%) produced bacterial growth inhibition zones persisted at all incubation time intervals at (1, 2, 3, and 7 days) and high significantly at $p \leq 0.01$ than that of zinc oxide eugenol based sealer. There is a significant reduction at $p \leq 0.01$ in antibacterial efficacy in all incorporation groups at 7 days incubation period, 5% thymol incorporation group with significant largest bacterial growth inhibition zones for all incubation periods. **Conclusions:** This study concluded that the prepared zinc oxide - guaiacol sealer formulas with three studied thymol crystals concentrations having better antibacterial efficacy than that of zinc oxide eugenol root canal sealer, 5% thymol incorporation formula is the best in antibacterial activity than others.

Keywords: Endodontic sealer, Zinc oxide eugenol, Agar diffusion test, Enterococcus faecalis.

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INTRODUCTION

The main aim of chemomechanical endodontic management is the elimination or severe reduction of microbe's existent in the root canal system ⁽¹⁾. Sealers are principally designated based on their sealing capacity, adhesive properties, biocompatibility, and antimicrobial ability ⁽²⁾.

The antimicrobial action of root canal sealers may help to eradicate residual microbes unaffected by chemomechanical planning of the root canal structure, stop the development of microbes, and help the healing procedure of apical and periapical tissues. Many studies have considered the agar diffusion test (ADT) is one of the most frequently used methods to estimate the antimicrobial activity of dental materials ⁽³⁾. Generally, zinc oxide-guaiacol endodontic sealer has setting time, film thickness, solubility, and flowability fulfilled the requirement of ANSI/ADA specification No. 57/2008 for dental root canal sealing materials. It has enhanced apical seal and biocompatibility related to that of ZOE, Its antibacterial activity against *E. faecalis* continued overall incubation times periods (1day, 2days, 3days, and 7days) but lower to that of ZOE based sealer. The antimicrobial action of zinc oxide-guaiacol endodontic sealer is produced through the main components of the material without introducing the definite antibacterial agent ⁽⁴⁾.

The locally prepared root canal sealer powder/liquid formula depends on the main reaction of zinc oxide and guaiacol as a base /acid

interaction with adding the other ingredients to improve materials properties: Zinc oxide 62%, natural rosin 20%, locally made egg-shell hydroxyapatite 2%, bismuth sub-carbonate 15%, zinc acetate 1%, guaiacol 85%, and olive oil 15% ⁽⁵⁾.

Today, the widespread of herbal or natural products due to their antimicrobial action, biocompatibility, lack of bacterial resistance, antioxidant, anti-inflammatory, easy obtainability, and low-cost properties ⁽¹⁾. Many natural happening antimicrobials have gained consideration among examiners due to their safety and nonhazardous status, such as thymol that displays the potential to obstruct the dental infection ⁽⁶⁾. The most frequently stated procedure of antibacterial action of thymol involves the disturbance of bacterial membrane effect in bacterial lysis and escape of intracellular substances causing in death ⁽⁷⁾. The thymol looks as the major active antibacterial ingredient of thyme oil, which employed its antimicrobial accomplishment ^(8,9).

Thymol (2-isopropyl-5-methylphenol) is a white crystal-like material that provides thyme its durable flavor, pleasurable aromatic perfume, and durable antiseptic features. It considers as a safe natural antibacterial agent with many dental, medical, and food industries ⁽¹⁰⁾.

The present study aimed to compare and evaluate the effect of (2%, 5%, and 10%) thymol crystals incorporation on the antibacterial activity of the locally prepared zinc oxide- guaiacol root canal sealer at different incubation periods

intervals (1 day, 2 days, 3 days, and 7 days) in presence of *Enterococcus faecalis*.

MATERIALS AND METHODS

1. Thymol incorporation

At the beginning, the thymol crystals (BDH, England) were grinded with an electrical grind-

er (Geepas, China), as well as all other elements of the original zinc oxide –guaiacol root canal sealer each one separately and to standardize particle size; the materials were sieved by using a sieve represented by double layers of muslin tissue ⁽¹¹⁾, then incorporated in zinc oxide- guaiacol endodontic sealer in three formulas (Table 1).

Table (1): The Thymol Crystals Standardized in Zinc Oxide- Guaiacol in Three Formulas.

First formula		Second formula		Third formula	
Powder	%	Powder	%	Powder	%
Zinc oxide	60%	Zinc oxide	57%	Zinc oxide	52%
Thymol crystals	2%	Thymol crystals	5%	Thymol crystals	10%
Natural rosin	20%	Natural rosin	20%	Natural rosin	20%
Hydroxyapatite	2%	Hydroxyapatite	2%	Hydroxyapatite	2%
Bismuth sub-carbonate	15%	Bismuth sub-carbonate	15%	Bismuth sub-carbonate	15%
Zinc acetate	1%	Zinc acetate	1%	Zinc acetate	1%
Liquid	%	Liquid	%	Liquid	%
Guaiacol oil	85%	Guaiacol oil	85%	Guaiacol oil	85%
Olive oil	15%	Olive oil	15%	Olive oil	15%

All test samples preparation was done at $23 \pm 2C^0$ and $50 \pm 5\%$ relative humidity determined by hygrometer (JUMBO DISPLAY HYGRO THERMOMETER, china). The powder/liquid ratio was (2:1) by volume, the period needed for mixing was (1minute) according to the pilot study. Zinc oxide eugenol endodontic sealer (tgsealer, PD/Switzerland) was used as a positive control group ⁽⁴⁾.

2. Initialization of Enterococcus selective media

Forty-two grams of agar *Enterococcus* media (HiMedia, India) were added to 1 liter of distilled water in a glass flask with continuous mixing by glass rod till totally melted in water. The mixture was heated up till initial blistering without over warming. The flask was detached from the heater, and the pH of the media was

identified by pH meter (pH 7). The media were left to come to be warm and emptied in one-use Petri dishes in a sterile condition in the hood with the existence of a gas burner. The plates were chilled and kept in the refrigerator (Concord, France) until used.

3. Bacterial isolation

The oral *Enterococcus faecalis* were insulated from the periapical lesion of a tooth, after indicated tooth isolation and access opening. Expand the root canal till file (size 25). Before any sterilization of the canal, a sterilized paper point of (size 20) was introduced cautiously into the canal for 1 minute. It was pulled cautiously and positioned into a sterilized vial having (5 ml) of sterilized brain heart infusion broth (Neogen culture media, UK) and vortexes to diffuse the sticking bacteria. It was then positioned at 37°C in an incubator for 30 minutes. All microbiological effort was completed in a laminar air current chamber (locally made, Iraq).

4. Subculturing of Bacteria

The suspension was marked on the superficial part of *Enterococcus's* selective media by a cotton swab. The only microorganism that matures on this media is *E. faecalis*. The *E. faecalis* seemed as reddish-pink colonies on the

superficial part of the media. Then incubation of the media anaerobically at 37°C by an anaerobic candle jar for 18 hours⁽¹²⁾.

5. Bacterial dilution

The brain heart infusion broth (Neogen culture media, UK) of 4.5 ml was organized in screw plugged vials to revitalize and dilute the bacteria to the desired dilution. Successive dilution was prepared by drawing 0.5 ml from the stock broth and supplementing to 4.5 ml of a freshly organized broth. The dilutions (1×10^{-3}) were completed until a detached colony in the Petri dish was shaped.

6. Antibacterial study

The antibacterial activity of trial sealer materials and ZOE based root canal sealers were experienced against *E. faecalis*⁽¹³⁾, by agar diffusion test (agar-well technique)⁽³⁾.

A pure sole *E. faecalis* colony was insulated from the identical cultured plate and inoculated into a sterilized screw plugged vial having 5 ml of brain heart infusion broth (BHI) and incubation of BHI broth at 37° C for 24 hours. From a 24-hours growing of the test microorganisms inoculation were adjusted to gain turbidity equal to the 0.5 McFarland standards scale (nearly 1.5×10^8 colony forming units /ml) (Figure 1).

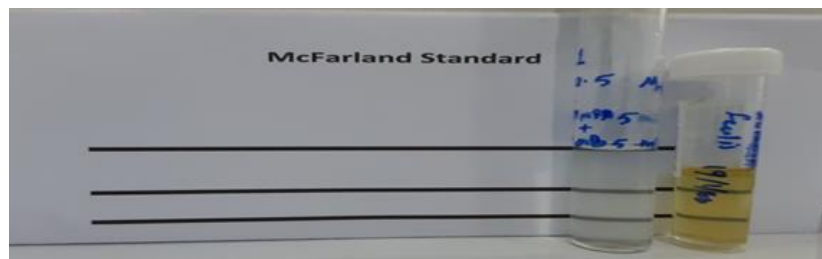


Figure (1): McFarland turbidity test.

In a pre-sterile test tube, 0.5 McFarland Standard was gained by adding 0.05 ml of 1.175% barium chloride ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) gradually and with constant excitement to 9.95 ml of 1% sulfuric acid. The suspended barium sulfate precipitate matches nearly 1.5×10^8 /ml of bacterial cell density. A total of 40 Petri plates of *Enterococcus* agar base media were utilized: Plates distributed randomly, into four test groups based on time breaks of incubation (1 day, 2 days, 3 days, and 7 days) with ten plates for each. Inoculation of bacteria was made by the application of sterilized cotton-tips (Ataco, China). Five wells of 6 mm dimension wide were perforated in each agar dish and packed with the freshly organized sealers. The inoculated dishes with sealers were saved at room temperature for 2 hours then incubation of

dishes at 37°C based on the incubation times.

Diameters of inhibition zone of microbial growing around wells having the sealer materials were evaluated with a digital caliper (Bosch, Germany) precise to the nearest 0.01 mm and noted after incubation time (Figure 2). Calculate the values of inhibition zone as in the following equation:

Inhibition zone = diameter of halo - diameter of the well

Statistical analysis was performed with the SPSS software, version 20.0 (IBM, USA). Descriptive statistics, One-way analysis of variance (ANOVA) and Duncan's Multiple Range Test were used for all comparisons. Significant differences were considered when $p \leq 0.01$.

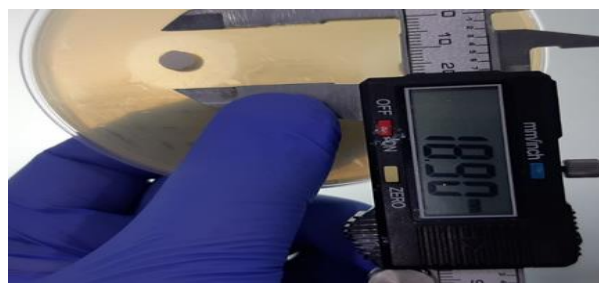


Figure (2): Evaluation Diameters of inhibition zone with a digital caliper.

RESULTS

The mean of inhibition zone diameters and standard deviations of tested sealers are demonstrated in Table (2). The result concluded that there is an increase in bacterial growth inhibi-

tion zones after thymol powder incorporations for all groups at all incubation periods (1 day, 2 days, 3 days, and 7 days) compared to that of zinc oxide- guaiacol root canal sealer.

Table (2): Mean of Inhibition Zone Diameters and Standard Deviations of Tested Sealers.

Time	Diameter of Inhibition Zones (mm)		
	Materials	Mean	Std. Deviation
24 hrs.	ZOE sealer	10.9750	.15448
	Zn-Gu sealer	10.1000	.37185
	Thymol 2%	13.0625	.19008
	Thymol 5%	14.6650	.57019
	Thymol 10%	12.2325	.53784
2days	ZOE sealer	11.3075	.17057
	Zn-Gu sealer	10.3975	.13137
	Thymol 2%	13.6075	.24185
	Thymol 5%	15.1575	.51990
	Thymol 10%	12.5100	.59042
3days	ZOE sealer	11.6775	.34383
	Zn-Gu sealer	10.5350	.24766
	Thymol 2%	13.9775	.13666
	Thymol 5%	15.7550	.04700
	Thymol 10%	12.9425	.50860
7days	ZOE sealer	11.4500	.35273
	Zn-Gu sealer	10.5650	.17516
	Thymol 2%	13.4175	.27059
	Thymol 5%	14.3500	.28936
	Thymol 10%	12.3850	.29877

Analysis of variance (ANOVA) and Duncan's Multiple Range Tests for zinc oxide eugenol, zinc oxide-guaiacol sealer before and after thymol crystals incorporation(2%, 5%, and 10%), are demonstrated in Table (3), and Figures (3,4). The statistical result revealed that

there is a statistically significant difference in the bacterial growth inhibition zone of tested sealers, and in between zinc oxide-guaiacol sealer groups before and after thymol powder incorporation in the studied concentrations. The 5% thymol incorporation groups in all

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incubation periods give the largest inhibition the 3rd day incubation period then declined at 7 zones. The antibacterial activity increased until days incubation time.

Table (3): Analysis of Variance (ANOVA) and Duncan's Multiple Range Tests for Antibacterial Activity of Tested Groups at Incubation Time Intervals.

Periods		Df	Mean square	F	Materials	Duncan Groups**
24 hrs.	Between Groups	4	38.006	232.501**	ZOE sealer	B
		55	.163		Zn-Gu sealer	A
	Within Groups				Thymol 2%	D
					Thymol 5%	E
	Total	59		Thymol 10%	C	
2 days	Between Groups	4	43.463	300.268**	ZOE sealer	B
		55	.145		Zn-Gu sealer	A
	Within Groups				Thymol 2%	D
					Thymol 5%	E
	Total	59		Thymol 10%	C	
3 days	Between Groups	4	49.115	534.882**	ZOE sealer	B
					Zn-Gu sealer	A
	Within Groups	55	.092		Thymol 2%	D
					Thymol 5%	E
	Total	59		Thymol 10%	C	
7 days	Between Groups	4	27.306	340.215**	ZOE sealer	B
					Zn-Gu sealer	A
	Within Groups	55	.080		Thymol 2%	D
					Thymol 5%	E
	Total	59		Thymol 10%	C	

**Different letters mean highly significant difference at $p \leq 0.01$.

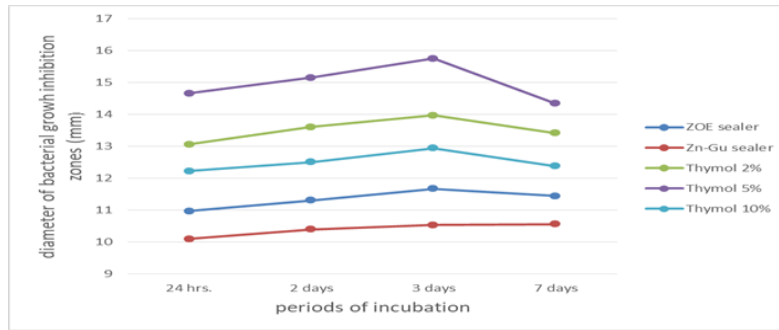


Figure (3): Bacterial growth inhibition zone for tested sealers at 24 hrs, 2 days, 3 days, and 7 days incubation periods.

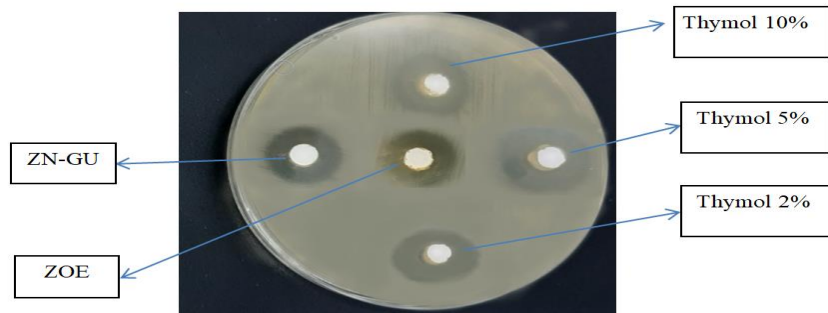


Figure (4): Bacterial growth inhibition zones around the tested materials.

DISCUSSION

The aims of root canal management are the eradication of infection from the root canal and inhibition of its reinfection through filling and sealing the space of the root canal, even though chemomechanical planning significantly diminishes the number of microbes in the root canal. About 40–60% of root canals stay positive for bacterial existence after this treatment. ⁽¹⁴⁾

Enterococcus faecalis is the microbe which is commonly correlated to the etiology of persistent periradicular lesions. It owns numerous virulence factors such as (gelatinase production, *Enterococcus* surface protein (Esp), aggregation substance (AS) and biofilm for-

mation) ⁽¹⁵⁾, that give to its ability to persist the effects of conventional root canal treatment. Also, this Gram-positive facultative anaerobe can attack dentinal tubules and bind to collagen. For these details, *E. faecalis* was the microbe designated in our research to assess the antimicrobial features of the endodontic materials experienced ⁽³⁾.

The agar diffusion test has been broadly used to examine the antimicrobial action of sealers and it is one of the most public and simple procedures. However, it has some restrictions such as the absence of standardization in an inoculum density, agar viscosity, plate storage situation, and dependence on the solu-

bility and diffusion properties of both the experiment material and media ⁽³⁾. Therefore, the plates were saved for two hours at room temperature (permit the diffusion) as proposed by Poggio ⁽³⁾.

The results of the present study revealed that all sealers showed antibacterial activity substantiated by the formation of growth inhibition zones against *E. faecalis* at all incubation time intervals. The antimicrobial ability of endodontic sealers is detected by its composition, which also affects its physical, chemical, and biological action ⁽¹⁶⁾.

Zinc oxide eugenol based sealer created significantly larger inhibitory zones at 1 day, 2 days, 3 days against *E. faecalis* as compared with zinc oxide-guaiacol sealer before incorporations which was in agreement to the similar inhibitory action of zinc oxide eugenol based sealers by Kothari and Langalia ⁽¹⁷⁾.

The greatest challenge in adjusting materials to modify their biological, antimicrobial, and/or mechanical features is gaining a product that offerings stability over time, has homogeneous integration of the additives, and can be easily manipulated ⁽¹⁶⁾. The integration of antimicrobial agents into endodontic root canal sealers raises this capability ⁽¹⁸⁾.

In this study, thymol crystals were incorporated as an antibacterial agent with three different concentrations (2%, 5%, and 10%) into zinc oxide-guaiacol sealer to improve antibacterial activity.

The incorporation of thymol crystals in three different concentrations gave bacterial growth inhibition zone larger than that of ZOE sealer (which possesses potent antibacterial activity) and zinc oxide-guaiacol sealer at 1day, 2days, and 3 days against *E. faecalis*. The sealer group with 5% thymol incorporation produced significantly largest inhibitory zones at 1day, 2days, 3 days, and 7 days against *E. faecalis*.

The thymol is the major active antibacterial ingredient of thyme oil, which employed its antimicrobial accomplishment. Studies on the mechanism of thymol's antibacterial activity show that its ability to participate in the lipid layer of the cell membrane raises the surface curvature. The hydrophilic part of the molecule cooperates with the polar portion of the membrane, whereas the hydrophobic benzene ring and aliphatic part chains sink into the inner portion of the biological membrane. This causes large modifications in membrane configuration by the weakening of the lipid layer, elasticity reduction, and fluidity rise. This procedure leads to improved permeability to potassium and hydrogen ions. It also disturbs the activity of internal membrane proteins like enzymes and receptors. Next integration into the cell membrane, thymol intermingles with its firm proteins through numerous non-specific processes, which lead to deviations in the modification and action of internal and membrane proteins. Therefore, the tension of cell membrane and weakening can be prompted by the

existence of thymol ⁽¹⁹⁾. Declining in antibacterial action after 10% thymol integration may be related to aggregation that occurs after mixing the ingredients of powder, this associated to the type of the thymol self-aggregating structure formed which depends on thermodynamic and geometric restrictions associated with the hydrophilic–hydrophobic balance within the molecule especially in high concentration. This aggregation decreases the available surface area leading to a decrease in the reactivity ⁽²⁰⁾.

CONCLUSIONS

The thymol crystals incorporation appears to produce better antibacterial activity against *E. faecalis* than that of zinc oxide eugenol and zinc oxide- guaiacol endodontic sealer at all incubation periods for studied concentrations. The 5% thymol crystals incorporation indicated for better antibacterial activity against *E. faecalis* with appropriate other properties.

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