Analysis of antimicrobial activity of root canal sealers against endodontic pathogens using agar diffusion test (In vitro study)

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ABSTRACT

Background: Antibacterial action of root canal filling is an important factor for successful root canal treatment, so the aim of the study was to identify and to compare the antimicrobial effect of new sealer (GuttaFlow) to commonly used endodontic sealers (AH Plus, Apexit and EndoFill) against four endodontic microbes.

Materials and methods: Twenty patients aged (30-40) years with infected root canals were selected. Four types of microorganisms were isolated from root canals *(E faecalis, Staphylococcus aureus, E coli and Candida albicans*) and cultured on Mueller Hinton agar Petri-dishes. After identification and isolation of bacterial species, agar diffusion method was used to assess the antibacterial action of four contemporary endodontic sealers used in root canal obturation (AH Plus, Apexit, EndoFill and GuttafFlow). Four wells measuring (5mm depth and 4mm diameter) were created in each Petri dish and sealer was applied into them incubated overnight at 37 C° for bacterial species and 48 hr. at 37 C° for *Candida albicans* prior to determination of results. Zones of inhibition (no growth of bacteria) were examined around the wells containing sealer & diameters of the zones were measured in mm. The mean of inhibition zones for each group was measured and statistically analyzed among groups using ANOVA and between groups using LSD tests.

Results: There was a highly significant difference (P<0.001) among all the tested groups. EndoFill showed the maximum antibacterial action against tested microorganisms. GuttaFlow showed moderate to weak antimicrobial effect, Apexit had weak effect, while AH Plus had no antibacterial action.

Conclusion: All the tested materials except AH Plus had antibacterial efficacy against *E faecalis, Staphylococcus aureus,* E *coli and Candida albicans.* EndoFill had favorable results among tested sealers and *E faecalis was the most resistant bacteria*, but none of the materials totally inhibited microbial growth. Thus, endodontic treatment must be performed under aseptic conditions.

Keywords: Endodontic sealers, anaerobic bacteria, *Candida albicans*, Mueller Hinton agar and agar diffusion test. (J Bagh Coll Dentistry 2014; 26(3):27-34).

INTRODUCTION

Comprehensive and successful obturation of root canals is directly related to adequate removal of microorganisms and their by-products which can be done by mechanical root canal instrumentation, antibacterial irrigation and adequate filling of the root canal space [(1)]. However, these procedures do not completely sterilize the root canal system due to the anatomical complexities of many root canals, such as dentinal tubules, ramifications, deltas, and fins which cannot be sufficiently cleaned, even after meticulous mechanical procedures. Thus bacteria may penetrate into an obturated root canal within few days; persisting or re-infecting bacteria may induce or sustain apical periodontitis [(2)]. Facultative and strict anaerobic bacteria are the most common microorganisms of the endodontic microbiota and cause infections that stimulate periapical bone resorption and refractory to endodontic treatment.

The most resistant species in the oral cavity are facultative microorganisms such as *Enterococcus faecalis, Staphylococcus aureus* and even *Candida albicans* which could cause failure of root canal treatment. Therefore, endodontic filling materials should be antibacterial/antimicrobial and this can be done by adding anti-microbial agents to root canal sealers [(3)]. Today, numerous sealers are available based on various formulas, such as epoxy resin sealers, calcium-hydroxide-based materials, gutta purcha based sealer and zinc oxide eugenol (ZOE) cements with and without paraformaldehyde additions [(4)].

ZOE based sealers have some antimicrobial activity because of the diffusion property of zinc oxide and eugenol into the agar media [(5)].

Calcium hydroxide compounds are widely used because of their alkalinity that provides excellent bactericidal effect. Resin-based root canal filling materials have steadily gained popularity and are now accepted and used for anterior and posterior teeth. The bonding systems have improved sealing ability, which explains the resistance of some materials to bacterial penetration [(6)].

GuttaFlow is a contemporary endodontic material based on polyvinylsiloxane (polydimethyl siloxane) that consists of gutta-percha and injectable system [(7)].

The agar diffusion method has been widely used to test the antimicrobial activity of dental materials and medications; the advantage of this method is that it allows direct comparisons of root canal sealers against the test microorganisms,

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indicating which sealer has the potential to eliminate bacteria in the local microenvironment of the root canal system [8].

The objective of this study was to analyze in vitro the antimicrobial properties of new sealer which is (GuttaFlow) and compare it with three contemporary endodontic materials used as sealers in root canal obturation (AH Plus, Apexit and EndoFill) against different microorganisms.

MATERIALS AND METHODS

In this study, four contemporary endodontic materials were used as sealers in root canal obturation which are gutta purcha containing sealer (ROEKO GUTTAFLOW® 2 FAST.COLTENE/ Germany), resin based sealer (AH Plus. Dentsply/ Germany), calcium hydroxide sealer (Apexit. Voco / Germany) and ZOE based sealer (EndoFill. PD / Switzerland), table (1) and fig. (1).

Table 1: Types of sealers used and their Ingredients

Sealer	Ingredient	
AH Plus	**Paste A** Epoxy resin Calcium tungstate Zirconium oxide Aerosol Iron oxide Silica Iron oxide pigments	**Paste B** Adamantane amine N. N- Dibenzoyl 5-oxanonane TCD-Diamine Calcium tungstate Zirconium oxide Aerosil Silicone oil Silica
Apexit	**Paste A** Calcium hydroxide / Calcium oxide Hydrated collophonium Fillers and other auxiliary materials (highly dispersed silicon dioxide, phosphoric acid alkyl ester)	**Paste B** Disalicylate Bismuth hydroxide / Bismuth carbonate Fillers and other auxiliary materials (highly dispersed silicon dioxide, phosphoric acid alkyl ester)
GUTTAFLOW	**Paste A** Polydimethylsiloxane, silicone oil, zirconium oxide,	**Paste B** gutta-percha
EndoFill	Powder: Zinc oxide Hydrogenated resin Bismuth subcarbonate Barium sulfate Sodium borate	Liquid: Eugenol Sweet almond oil

Figure 1: Sealers used in the study

Bacterial strains

Three standard bacterial strains were used in the study which were, G+ve *Staphylococcus aureus* and *Enterococcus faecalis*, which were isolated and cultured on blood agar media, G-ve *E.coli* which was isolated and cultured on MacConkey agar media (Sisco research Inc. India), also one fungal strain *Candida albicans* which was isolated and cultured on a Sabouraud agar media (ThermomFisher scince Inc. UK).

The antibacterial effect of the tested materials was assessed using agar diffusion method.

Sterilization method

Sterilization of mouth mirror, kidney dishes and all clean glasses were conducted by dry air oven at 180 C° for 1 hour. Benches and floor of

the laboratory were disinfected by detol antiseptic solution [9].

Patient selection and isolation of bacteria

Twenty patients aged (30-40) years with infected root canals which were diagnosed clinically and radiographically and none of them had received any antibiotic treatment for three months.

Rubber dam was used for isolation of teeth before microbiological sampling and the teeth were disinfected with 10% povidone iodine solution to avoid contamination of working field.

Access opening was prepared after all caries were removed and coronal restorations using new fissure bur for each tooth. After confirmation of working length radiographically, each root was instrumented using new sterile barbed broaches and files, and then a sterile paper point was introduced inside canals and left for 1 min. and then removed and placed immediately into a transporting media to preserve bacteria from damage or death and microorganisms were isolated within 4 hrs [10].

Identification of bacterial species

Microorganisms were identified at microbiology department (Al-Nahrin Medical College). Based on colony morphology (size, shape, and color), selected colony for each bacteria was subcultured aerobically and anaerobically and those bacteria which failed to grow aerobically were identified to be anaerobes.

Biochemical tests were used to distinguish between G^{+ve} and G^{-ve} bacteria; Api 20E test (BioFire Diagnostics, Inc. USA) was used to identify G^{+ve} bacteria while Api strep (BioMérieux, France) test was used to identify G^{-ve} bacteria. Api candida (BioMérieux, France) was used to recognize fungal species (*Candida albicans*) [11].

Reactivation and subculturing of microorganisms

Brain Heart Infusion (BHI) broth (Biomark Company / India) was used for the reactivation of the bacterial species (*Entrococc fecalis*, *Staphylococcus aureus* and *E.coli* after isolation of each microorganism.

In order to standardize the final turbidity to the 0.5 standard of the McFarland scale, microorganisms were seeded in 20 × 10 mm sterile Petri dishes containing agar media supplemented with 5% blood using swabs saturated in the bacterial suspension and incubated at 37 Cº for 24hr.

Candida albicans was reactivated in Sabouraud agar broth and seeded in Petri dishes containing Sabouraud agar medium in the same way as described for the bacterial species and incubated at 37 Cº for 48hr [12].

Sample Grouping

A total of 50 plates containing agar media were divided into four test groups and one control group (10 plates for each group). Each type of microorganism was tested ten times:

Group I: 10 plates were inoculated with *Staphylococcus aureus* containing 4 types of sealers.

Group II: 10 plates were inoculated with *E. coli* containing 4 types of sealers.

Group III: 10 plates were inoculated with *Enterococcus faecalis* containing 4 types of sealers.

Group IV: 10 plates were inoculated with *Candida albicans* containing 4 types of sealers.

Group V: 10 plates with inoculums, without any sealer as a positive control group.

Plate's preparation

Petri dishes (20×10 mm) containing agar media were inoculated with bacterial suspension by using cotton tipped applicator using sterile swabs and 100 aliquots of each microbial suspension were spread on the Petri dishes. After dividing the Petri dish into four equal sections, a copper coil was used to create four wells (5mm in depth, 4mm diameter) on the Petri dishes and these wells were made at equal distance from each other. Sealers were mixed on sterile glass plates using sterile stainless steel spatula according to manufacturer instructions and placed immediately in the wells in concentration of 0.2 ml to have equal amount of sealer in each well. The positive control groups were streaked with bacteria but no root canal sealer was used, then plates were left for 2hr. at room temperature for diffusion of sealer and to ensure direct contact between sealer and microorganism. The plates were incubated aerobically at 37Cº for 24hr considering bacterial species and 48hr for fungal species [2, 13,14].

Sealer susceptibility test

These tests for the four types of microorganism (*Enterococcus faecalis, Staphylococcus aureus, E. coli* and *Candida albicans*) were done with agar diffusion method. The inhibitory zones were considered to be the shortest diameter from the outer margin of the well to the initial point of the microbial growth using a digital caliber with a resolution of 0.01 mm under reflects light [15] and the measurements

were recorded at 24 hours for each bacterial species and 28 hrs for *Candida albicans*. Experiments were repeated 10 times (n=10) and the mean of readings were recorded [16].

Figure 2: Types of agars used A- *Candida albicans* inoculated on Sabouraud agar B- *E coli* inoculated on MacConkey agar C- *E faecalis and Staphylococcus aureus* inoculated on blood agar

RESULTS

The mean, standard deviation, standard error, minimum reading and maximum calculations of the zones of inhibition of microbial growth in mm of each endodontic sealer have been summarized in table (2) and fig. (3). The inhibitory potential of each material was categorized as strong, moderate strong, moderate, weak, or non-inhibitory depending on the average size of the zones, table (3). All sealers showed zones of inhibition against microorganisms except for control and AH Plus groups which showed no inhibitory effect on all tested microorganisms. EndoFill produced the largest inhibitory zone followed by GuttaFlow, On the other hand; Apexit produced the smallest inhibitory zones against the tested microorganisms (by average values), fig.(4). In this study the results found that distilled water (control group) showed no inhibition of growth of tested microorganisms. Furthermore it appears that *E.faecalis* was the most resistant organism to the effect of the sealers in this experiment. EndoFill had the largest inhibitory zone on *S. aureus followed* by *E.coli then Candida albicans and E.faecalis*.

Table 2: Mean values of antimicrobial activity of root canal sealers against microorganisms

Group	Subgroups	Mean (mm)	Zone categories	SD ±	SE	Min.	Max.
Staphylococcus aureus	**AH Plus**	0	No	0	0	0	0
	Apexit	2.1	No	0.5	0.2	1.5	2.5
	EndoFill	19.4	Moderate Strong	0.5	0.3	19	20
	GuttaFlow	9.6	Moderate	0.5	0.1	9	10
	Control	0	No	0	0	0	0
E.coli	**AH Plus**	0	No	0	0	0	0
	Apexit	2.4	Weak	0.5	0.1	2	3
	EndoFill	9.4	Moderate	0.4	0.1	9	10
	GuttaFlow	5.6	Moderate	0.3	0.1	5	6
	Control	0	No	0	0	0	0
E.faecalis	**AH Plus**	0	No	0	0	0	0
	Apexit	0.6	No	0.4	0.1	0	1
	EndoFill	4.4	Weak	0.5	0.1	4	5
	GuttaFlow	2.4	Weak	0.3	0.1	2	3
	Control	0	No	0	0	0	0
Candida albicans	**AH Plus**	0	No	0	0	0	0
	Apexit	1.6	No	0.5	0.2	1	2
	EndoFill	6.6	Moderate	0.4	0.2	5	7
	GuttaFlow	3.4	Weak	0.3	0.1	3	4
	Control	0	No	0	0	0	0

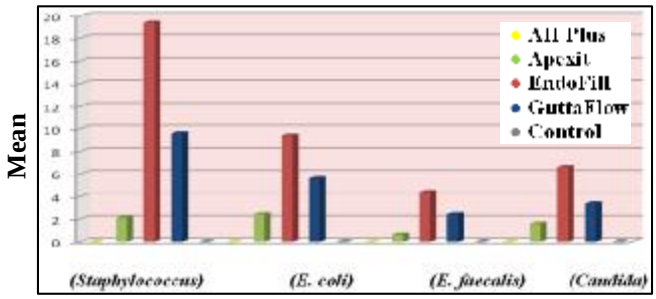

Figure 3: Bar chart showing differences between the mean of inhibition zones of endodontic sealers produced against tested microorganisms

Table 3: Inhibition categories according to the proportional distribution of the data set

Rank	Range of zone diameter (mm)
No	2
Weak	2.4-6.2
Moderate	6.3-10.3
Moderate strong	10.4-26.8
Strong	>26.8

(***S. aureus***) (***E.coli***) (***E. faecalis***) (***Candida albicans***)

Figure 4: Inhibition zones of the tested sealers

Statistical analysis of data by using analysis of variance (ANOVA) was done which showed that there was a statistically high significance difference (P< 0.001) between the four endodontic sealers in their antibacterial action against all tested microorganisms, table (4).

Table 4: ANOVA test to show the statistical difference of antimicrobial effect between endodontic sealers against microorganisms

Microorganism	F	P value	Sig.
Staphylococcus aureus	253	0.00	HS*
E.coli	450	0.00	HS*
E.faecalis	101	0.00	HS*
Candida albicans	214	0.00	HS*

***Highly significant at level P<0.001.**

When a significant difference was found, least significant difference (LSD**)** test was done to analyze the data to show the difference in susceptibility against microorganisms between different pairs of sealers, table (5, 6, 7 and 8).

These investigations had shown that there was a highly significant difference among each pair of sealers against all tested microorganisms:

1. AH Plus had a high significant difference P<0.001 compared to Apexit, EndoFill, and GuttaFlow, except for *E.faecalis* group in which there was significant difference P<0.05 between AH plus and Apexit.
2. Apexit showed highly significant difference P<0.001 compared to EndoFill, GuttaFlow and control, except for *E.faecalis* and *Candida* groups in which there was a significant difference P<0.05 between Apexit and GuttaFlow.
3. EndoFill showed high significant difference P<0.0001 compared to GuttaFlow and control.
4. GuttaFlow showed highly significant difference P<0.001 compared to control.

Table 5: LSD test to compare the antibacterial action between each pair of endodontic sealers against *Staphylococcus aureus*

Sealer	P value	Sig.
AH Plus vs. Apexit	0.00	HS*
AH Plus vs. EndoFill	0.00	HS*
AH Plus vs. GuttaFlow	0.00	HS*
Apexit vs. EndoFill	0.00	HS*
Apexit vs. GuttaFlow	0.00	HS*
Apexit vs. Control	0.00	HS*
EndoFill vs. GuttaFlow	0.00	HS*
EndoFill vs. Control	0.00	HS*
GuttaFlow vs. Control	0.00	HS*

***Highly significant at level P<0.001.**

Table 6: LSD to compare the antibacterial action between each pair of endodontic sealers against *E.coli*

Sealer	P value	Sig.
AH Plus vs. Apexit	0.00	HS*
AH Plus vs. EndoFill	0.00	HS*
AH Plus vs. GuttaFlow	0.00	HS*
Apexit vs. EndoFill	0.00	HS*
Apexit vs. GuttaFlow	0.01	HS*
Apexit vs. Control	0.00	HS*
EndoFill vs. GuttaFlow	0.00	HS*
EndoFill vs. Control	0.00	HS*
GuttaFlow vs. Control	0.00	HS*

***Highly significant at level P<0.001.**

Table 7: LSD to compare the antibacterial action between each pair of endodontic sealers against *E. faecalis*

Sealer	P value	Sig.
AH Plus vs. Apexit	0.04	S*
AH Plus vs. EndoFill	0.00	HS*
AH Plus vs. GuttaFlow	0.00	HS*
Apexit vs. EndoFill	0.00	HS*
Apexit vs. GuttaFlow	0.01	S*
Apexit vs. Control	0.04	S*
EndoFill vs. GuttaFlow	0.00	HS*
EndoFill vs. Control	0.00	HS*
GuttaFlow vs. Control	0.00	HS*

***Highly significant at level P<0.001. * Significant at level P<0.05.**

Table 8: LSD to compare the antibacterial action between each pair of sealers against *Candida*

Sealer	P value	Sig.
AH Plus s vs. Apexit	0.00	HS*
AH Plus vs. EndoFill	0.00	HS*
AH Plus vs. GuttaFlow	0.00	HS*
Apexit vs. EndoFill	0.00	HS*
Apexit vs. GuttaFlow	0.01	S*
Apexit vs. Control	0.00	HS*
EndoFill vs. GuttaFlow	0.00	HS*
EndoFill vs. Control	0.00	HS*
GuttaFlow vs. Control	0.00	HS*

*** Significant at level P<0.05 *Highly significant at level P<0.001.**

DISCUSSION

Successful root canal treatment not only means removal of microbial entity, but also preventing any future predilection of re-infection and using biocompatible sealing agent [17].

Chemo-mechanical debridement is more likely to eradicate the bacteria that adhere superficially to the root canal walls. However, bacteria that infect dentinal tubules and remain in undebrided parts of the root canal system may cause recurrent infection [18].

Hence the ideal objectives of the root canal treatment are not only the elimination of infection, but also preventing reinfection of the treated root canal system especially in clinical situations of persistent or recurrent infections.

Microorganisms that survive chemomechanical debridement must be killed by sealers with sustained antibacterial activity and excellent adhesion to dentin. However the antimicrobial components of the sealer do not have selective toxicity against microorganisms; they also exert toxic effects on host cells. **Hil et al and Huang et al** [19,20] proposed that the ideal root canal sealer must have both good antimicrobial activity and low toxic effects on surrounding periapical tissue.

In this study, agar diffusion test (ADT) was used. This method which is the most widely used method for the identification of which material that has an antimicrobial effect within the root canal system. The result of ADT are highly influenced by many variables such as the diffusion ability of the material across the medium, the selection of the agar medium and microorganisms, control and standardization of inoculation density, incubation and reading point of the zones of inhibition [6]. Antibacterial effect of four different types of root canal sealers was tested; GuttaFlow, a new gutta percha based material, the well described epoxy resin based AH Plus, EndoFill as a zinc oxide eugenol based sealer and calcium hydroxide sealer known as Apexit.

Anaerobic and facultative bacteria were chosen in the study because these types of microorganisms are usually minor constituents of primary infections, they have been found with higher frequency in cases of treatment failure. Microorganisms, such as *E. faecalis*, *S. aureus*, *Ecoli* and even *C. albicans* have been considered as the most resistant oral species and possible causes of failure of root canal treatment [21]. *E. faecalis* is Gram-positive facultative anaerobic cocci that are considered as a normal part of human intestinal flora. The high resistance of this type of bacteria to antibacterial action of the sealers used in this study could be explained by the ability of *Enterococci* to survive very harsh environments including extreme alkaline pH (9.6) and salt concentrations. They resist bile salts, detergents, heavy metals, ethanol, azide, and desiccation. They can grow in the range of 10 to 45°C and survive a temperature of 60°C for 30 min [22]. *E. coli* is Gram-negative, facultative anaerobic and non-sporulating, rod shaped cells.

While *Staphylococcus aureus* is facultative anaerobic Gram-positive coccal bacterium which is frequently found in the human respiratory tract . *Candida albicans* is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans [23]. The sealers evaluated in this study showed different inhibitory effects depending on the type of root canal sealers and bacterial species tested.

EndoFill Which is a zinc oxide eugenol based sealer had the maximum average zones of inhibition as compared to other tested sealers. Findings of this study agree with studies that found large inhibitory zones produced by sealers similar to EndoFill against microorganisms such as *S. aureus*, *C.albicans* and *E. faecalis* [13].

On the other hand, this material was the only effective sealer on the most resistant one (*E.faecalis*). The strong antibacterial effect of EndoFill may be related to the action of free eugenol liberated from the material which is a phenolic compound that is effective against mycotic cells in their vegetative [24].

AH Plus which is a new resin based sealer that showed absence of antimicrobial action against all tested species and this is in accordance with a previous study by **Andre et al** and **Estela et al** [25,26] who found AH Plus to be ineffective against *Enterococcus faecalis* and **Kapalan et al** [27] who found AH Plus to be ineffective against *Candida albicans.*

The low antimicrobial effect of AH Plus against tested species might be ascribed to the minimal amount of formaldehyde released over time. The elimination of formaldehyde release from AH Plus has made it an ineffective as antimicrobial sealant [28].

Apexit which is a calcium hydroxide based endodontic sealer that showed an antibacterial activity agaist *Staphylococcus aureus and E. coli*, but no effect against *E. Faecalis* and *Candida albicans.* Apexit was less effective than EndoFill and GuttaFlow, but more effective than AH Plus. **Zhang et al** [29] also showed poor antibacterial activity for Apexit in comparison to six other sealers against *E. Faecalis.* **Apexit** induce antimicrobial action by releasing hydroxide ions OH^- and increasing pH levels above 12.5 creating unfavorable change for microbial growth which alter the integrity of the cytoplasmic membrane leading to a saponification reaction. The absence of any significant effect on *Candida albicans* could lead to the conclusion that the release of hydroxyl ions is not sufficient to inhibit this yeast whose optimum growth pH is 5 [2].

GuttaFlow is a cold, flowable, self-curing obturation material that combines gutta-percha and sealer into one injectable system. This material contains gutta-percha in particle form combined with a polydimethylsiloxane [7].

GuttaFlow showed moderate inhibition on *E.coli* and *S. aureus* isolates while weak effect on *E.faecalis* and *Candida albicans*. The antibacterial activity of Gutta Flow may be attributed to the preservative (nanosilver) present in this type of sealer which causes oligodynamic effect, in which, metal ions (silver) combine with sulfur groups and denature the cellular proteins [7].

These results disagree with Ivan, **Ines et al** and **Lavanya et al** [5, 30] who found no or minimum effect of GuttaFlow on inhibition of microbes. The controversial results could be explained by variation in conditions of the experiments such as the amount of material used, bacterial inoculation, test method, incubation period and interval times.

As a conclusion; root canal sealers showed different inhibitory effects depending on their types and bacterial species tested. Root canal sealers containing eugenol proved to be most effective against the microorganisms in the root canal. Under the conditions of this in vitro study, EndoFill showed strong to moderate antimicrobial action against tested species while the new GuttaFlow filling material showed moderate to week effect in comparison with Apexit and AH Plus, indicating potentiality of EndoFill and GuttaFlow as an antibacterial agents. However, it is necessary to investigate other properties of the new material (GuttaFlow).

None of the sealers tested totally inhibited microbial growth. Thus, endodontic treatment must be performed under aseptic conditions, using powerful chemo-mechanical debridement, an intracanal dressing, adequate filling, and coronal restoration.

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