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# An evaluation of the Antimicrobial Activity of Five Endodontic Sealers on three bacterial species. (In vitro study)

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## Abstract

To evaluate the antimicrobial action of five endodontic sealers after 24 hr and 48 hr.

The sealers studied were Canason, Acroseal, Epiphany, AH-Plus and MTA, while the microorganisms used *were Streptococcus viridans, Staphylococcus aureus* and *Enterococcus faecalis.* Agar diffusion method on Muller Hinton agar was employed; fourty five petriplates with 25 ml of Muller Hinton agar were inoculated with 0.1 ml of the experimental suspension. Five cavities , each one measuring 5 ml in diameter and 4 ml in depth , were made in each agar plate using cork poorer and then completely filled with the product to be tested .

Canason containing eugenol and formaldehyde proved to be the most effective against all microorganisms tested. This was followed by Epiphany, Acroseal and AH-Plus which showed antibacterial activity on all tested microorganisms higher than that of MTA which showed the least action on all tested microorganisms. No antimicrobial activity was seen after 48 hrs.

All the sealers evaluated in this study showed different inhibitory effect against all bacterial strains.

## Introduction

Root canal therapy is an invaluable measure to preserve teeth that would otherwise need to be extracted. With advancing technology, a better understanding of root canal anatomy, and improved materials, root canal therapy is achieving an increasingly high overall success rate [(1)]**.** However, bacteria inside the root canal system have a significant impact on this success rate. When a tooth is infected prior to treatment, the success of root canal therapy drops to 86%, which is a compromise from the 96% success rate of root canal treated teeth without apical periodontitis [(2)].

A few bacterial species, predominantly facultative anaerobes are responsible for causing apical periodontitis observed in root canal failure[(3)]. These microorganisms that have leaked into the canal after its obturation or from bacterial not eliminated during therapy [(4)]**.** Since removing all bacteria in the canal prior to obturation has proven to be difficult even after chemomechanical preparation [(5)]**.**

Bacteria may also persist within the dentinal tubules or lateral canals after

root canal obturation and may repopulate the former root canal [6].At the final stage of root canal obturation, the remaining bacteria should be destroyed to get successful results. Endodontic sealer has the ability to do this action as one of the requirements of good sealer cement as determined by Grossman [7]. Endodontic Sealer remains for a long time inside the root canal, and it may reach bacteria located in inaccessible regions of the root canal system [8].

Endodontic treatment can be aided by clarification of the antibacterial susceptibility of the pathogenic bacteria present inside the infected pulp, to these endodontic sealers which have different antibacterial activities against various microorganisms presents inside diseased pulp. These differences in antimicrobial activities are attributed to their chemical constituents and additives incorporated within the sealers. The most desirable chemical would be the one that combines maximum antibacterial effect with minimum toxicity. Therefore, one has to choose the one which combines antimicrobial effect with low toxic effect [9,10].

## Materials and method

Three standard bacterial strains obtained from the clinical laboratories of the medical city in Baghdad were used in this study which were *Streptococcus viridans*, which obtained an a chocolate agar media, *Staphylococcus aureus*, which obtained on a tryptone soya blood agar media and *Enterococcus faecalis*, which obtained on a tryptone soya blood agar media. The microorganisms were identified - in the central health laboratories-ministry of health in Baghdad by a combination of colonial pigmentation, colonial morphology, haemolysis on a tryptone soya blood agar, cell morphology (microscopic morphology) and biochemical tests. A total of 75 samples were used in this study which were divided into 5 groups consisted of 15 plate for each group ,15 plates inoculated with *Streptococcus viridans* containing 5 types of sealers as group 1,15 plates inoculated with *Staphylococcus aureus* containing 5 types of sealers as group 2, 15 plates inoculated with *Enterococcus faecalis* containing 5 types of sealers as group 3, 15 plates with 5 types of sealers without any bacteria as a negative control group and 15 plates with inoculums without any sealer as a positive control group.

The tests for the three types of bacteria (*Streptococcus viridian*, *Staphylococcus aureus* and *Enterococcus faecalis*) were done with Agar Diffusion method. Five sealers were used in this study which was Acroseal (Septodont), Canason (Voco), Epiphany (Pentron), AH-plus (Dentsply) and MTA (Angelus). 4 to 5 pure colonies of each bacterial strain were taken by a sterile loop. These colonies were inoculated in 10ml of BHI broth in a small screw cap tubes. Incubation of these tubes was done for 24 hour at 37 $^{o}$C. Turbid suspensions were noticed at the next day. 5 ml of a sterile 0.85% normal saline solution in screw cap tubes were prepared. Bacterial strains were individually inoculated into the tubes and the suspension was adjusted visually to match the turbidity of a McFarland 0.5 scale. This number of standard contains approximately $1.5 \times 10^{8}$/ml of bacterial cell density.

A 9 cm diameter plates with 25 ml of Mueller Hinton Agar media in each were prepared. A sterile spreader was used to inoculate the microorganisms from the prepared normal saline tubes inoculated with microorganisms which had been fit to 0.5 McFarland standard. With an adjustable micropipette 0.1 ml

of each bacterial suspension was added to the surface of the plates which were inoculated by spreading the suspension in three directions, and a final spreading was done over the outer rim of the plate. After that, the plates were allowed to dry for 3-5 minutes. Within 15 minutes, after inoculation of the plates, five wells measuring 4 mm in depth and 5 mm in diameter were made in each agar plate using cork poorer. Each was filled completely with the five types of sealers after being mixed according to the manufacturer's instructions. The plates were preincubated in culture media at environmental temperature for two hours before incubation to allow dissociation and diffusion of sealers. The plates were incubated at 37 $^{o}$C for 24 hours in the incubator [11]**.** The agar plates were examined for bacterial inhibition zones at the next day. With a scientific ruler (with accuracy of 0.5 mm) the diameter of these zones were measured by passing the ruler through the center of the wells. Inhibition zones were recorded at 24 and 48 hours for each sealer for each bacterial strain.

## Results

### Effect of the five sealers on Streptococcus viridans:

From **Fig 1** it's clear that Canason exhibited the highest mean of inhibition zone value (31.800), followed by Epiphany, Acroseal, AH-plus with values of (13.900), (13.800), (9.300) respectively. The least mean value of the antibacterial action of *Streptococcus viridans* was shown by MTA with a mean of (5.930). Also, it can be seen that there are no changes in the antibacterial action of all endodontic sealer after 48 hours**.**

### Effect of the five sealers on *Staphylococcus aureus*

From **Fig.3** it's clear that Canason exhibited the highest mean of inhibition zone value (33.800) followed by Epiphany with a mean of (16.50). AH- plus came after Epiphany in its antibacterial activity against *Staphylococcus aureus* with a mean of (11.870) and higher than Acroseal with a mean of (11.670). The least mean value of antibacterial activity against *Staphylococcus aureus* was shown by MTA with a mean value (11.530).

### Effect of the five sealers on Enterococcus faecalis

**Fig.5** shows that Canason had the highest antibacterial activity with the mean value of (34.00) while the lowest mean was shown by MTA (5.670). The other sealers where ranging in between as Epiphany, Acroseal and AH-plus with a mean values of (16.77), (10.13) and (8.70) respectively.

### Effect of each of the five sealers on the three bacterial strains.

Statistical analysis of data by using analysis of variance ANOVA was done which showed that there was a statistically high significant difference between each type of the five endodontic sealers in their antibacterial action against the three types of bacteria as shown in **table 1**.

## Discussion

Establishing the spectrum of activity of any antimicrobial agent is useful for improving the infection control process. In general, there are three in vitro techniques that have been used for this purpose – the dilution method which yields a quantitative result for the amount of antimicrobial agent that is needed; the agar diffusion method, which gives an inhibition zone around the well containing the agent and that could be related to its effect, and the direct exposure method, which

provides qualitative information about the substances. The method of measuring antimicrobial activity used here was to determine the size of the zone of bacterial growth inhibition around the specimen. This size of this zone will depend on at least two major factors. The first is the toxicity of the components of the material under study. The second is the diffusibility of any toxic factors released from the specimen. This diffusibility is a function of the hydrophilicity or hydrophobicity of the substances being released and the rate of which these substances are released from the matrix of the specimen under study [12].

However, great care was taken to keep the plates for 2 hrs at room temperature to allow the diffusion of the agents through the agar and then incubated at 37°c under appropriate gaseous condition [11]. In the present study different sealers showed varying effects on different bacteria. Based on aforementioned factors, Canason endodontic sealer had the highest mean value among the others in inhibiting *Streptococcus viridans* growth. The paraformaldehyde, which is incorporated in the cement, is a potent antibacterial agent with a low molecular weight and low surface tension. These properties determined the higher penetrability and spreading of this endodontic sealer [13]. It seems probable that release of formaldehyde from Canason was the source of its antibacterial activity. Formaldehyde is a phenolic compound which has a strong antibacterial activity in vitro[14]. Furthermore, Eugenol incorporated with Canason potentiated the high antibacterial action of it on *Streptococcus viridans*. Eugenol is a chemical essence of oil of clove, it has been used in endodontics primarly as anodyne and also as an antibacterial agent in higher concentration ($10^{-2}$-$10^{-3}$ mol $L^{-1}$) [15]. Eugenol is also lipophilic, affecting the lipid in cell membrane and increasing the cell membrane permeability so this may be another mechanisms by which eugenol could have antibacterial activity [16]. Canason was more effective in inhibiting the growth of *Staphylococcus aureus* than *Streptococcus viridans* since *Streptococcus viridans* is less sensitive to eugenol than *Staphylococcus aureus* [17]. Canason had too the highest level between other sealers in inhibition of *Enterococcus faecalis* growth for the same reasons mentioned before.

Epiphany sealer is a dual-curable resin composite containing a new redox catalyst, developed with a self-etching primer, and a new thermoplastic filled polymer (Resilon) in place of the gutta percha. Epiphany sealer came in the second stage in inhibition of bacterial growth for *Streptococcus viridans*, *Staphylococcus aureus* and *Enterococcus faecalis*. A possible explanation for the high antibacterial activity of this sealer could be that water diffusion leads to erosion of the composite resin material causing release of unreacted monomers [18]. Since epiphany is a dual curable methacrylate resin sealer and based on a mixture of bisphenol A-glycidyl methacrylate (BisGMA), urethane dimethacrylate (UDMA) and hydrophilic difunctional methacrylate, another reason for the high antibacterial activity of this sealer could be the residual monomers which were shown to be the main components released from cured dental composite materials [19] in addition to amine and epoxy resin components of the sealer [20]. The oxygen inhibition layer of the surface of any polymerizing resin leaves an uncured monomer layer which could be another reason [21]. Although epiphany and AH-plus are epoxy resin sealers Epiphany showed

more antibacterial activity than AH-plus especially against *Staphylococcus aureus and Enterococcus faecalis*. This may be due to the difference in solubility between the two sealers. **Versiani *et al.* (2006)** found that AH-plus solubility was within the normal range, whereas Epiphany showed higher value than the ANSI/ADA 2000 [22]. Also there was an extensive calcium release from Epiphany which has been shown to favor a more alkaline pH of the environment. This high calcium release by Epiphany sealer could be another reason to explain the high level of antibacterial activity [23].

Acroseal sealer which is a calcium hydroxide based endodontic sealer showed an antibacterial activity for all types of bacteria in different amount. This antibacterial activity is probably due to one of the following mechanisms. The first is the damage of the bacterial cytoplasmic membrane. As well as CaOH sealers depend on ionization that release hydroxyl ions causing an increase in pH, A pH > 9 may reversibly or irreversibly inactivate cellular membrane enzymes of the microorganisms, resulting in a loss of biological activity of the cytoplasmic membrane [24]. Or leading to the destruction of the phospholipids or nonsaturated fatty acids that result in a loss of cytoplasmic membrane integrity [25]. The second is protein denaturation. The alkalinezation provided by calcium hydroxide induces the breakdown of ionic bond that maintains the tertiary structure of protein. These change frequently result in the loss of the biological activity of the enzyme and disruption of the cellular metabolism. Structural proteins may also be damaged by hydroxyl ions [26]**.** The third is Damage to the DNA. Hydroxyl ions react with the bacterial DNA and induce the splitting of the strands. Genes are then lost. Consequently, DNA replication is inhibited and the cellular activity is disarranged. Free radicals may also induce lethal mutations [27]**.** It has been suggested that the ability of calcium hydroxide to absorb carbon dioxide may contribute to its antibacterial activity specially facultative and obligate anaerobic [28]**.** Another reason to inhibit bacterial growth may be because of the presence of amines in the epoxy base of Acroseal as a new calcium hydroxide based sealer [29]. Acroseal Calcium hydroxide sealer showed an antibacterial activity less than epiphany and Canason for several reasons. It may had been due to it's low diffusibility in agar and due to the buffering ability of the artificial media which reduced it's high pH and lowering it's antibacterial activity [30]**.** The low solubility and diffusibility of CaOH as well as the dentine buffering ability, may make it difficult to reach an increase in the pH of eliminating bacteria located within dentinal tubules the same as in agar diffusion method [31]. Acroseal, appears to have lower solubility than other calcium hydroxide sealers, probably because of it's epoxy resin component which may be another reason to its decreased antibacterial activity [29]**.** In the present study, the preincubation in culture medium at environmental temperature for 2 hrs before incubation allowed dissociation and diffusion of the sealer evaluated in agar medium for short period of time, and influenced the results of calcium hydroxide based sealer, thus providing evidence of antimicrobial activity [11]**.** The high significant difference of Acroseal antibacterial activity between *Streptococcus viridans* , *Staphylococcus aureus* and *Enterococcus faecalis* may be due to difference in the optimum pH for growth for each bacteria. Because *Streptococcus viridans* grows easily in neutral or slightly acid media (pH =

6.8-7.0), the inhibition caused by this material is more than that on *Staphylococcus aureus* which need an optimum pH for growth between 7 and 7.5 [32]. Low antibacterial activity of Acroseal sealer on *Enterococcus faecalis* may due to ability of this bacteria to tolerate very high pH values varying from 9 to 11 [33].

AH-plus sealer which is a new resin based sealer showed an antibacterial activity lower than that of Acroseal on all types of bacteria. This lower antibacterial activity could probably be due to its low contents of water-soluble toxic compounds such as formaldehyde and short sitting time which may induce milder antibacterial activity [34]. Or it could be due to minute amount of formaldehyde from the sealer or by the release of the amine and epoxy resin components of the sealer [20] since AH-plus sealer based on polymerization reaction of epoxy resin amines [35].

MTA sealer showed the least antibacterial action. This sealer is marketed in grey-coloured (GMTA) and white-coloured (WMTA) preparation; both are 75% Protland cement, 20% bismuth oxide and 5% gypsum by weight. In recent years, the use of the white-coloured preparation has become more popular. The main component of GMTA formula are tricalcium Oxide, tricalcium silicate, bismuth oxide, dicalcium oxide, Tricalcium aluminate, tetracalcium aluminoferrite and calcium sulphate dehydrate. The WMTA preparation, however, lack tetra calcium aluminoferrite [36]. MTA has a soluble fraction mainly composed of calcium hydroxide and the water in contact with MTA had a high alkaline pH, ranging from 11.94 to 11.99 [37]. It's therefore possible that MTA, releasing calcium hydroxide, possesses antimicrobial activity [38].

There is probably no absolute way of determining the effectiveness of any sealer via in vitro studies. The results of such antibacterial tests may not highly correlate with in vivo data, however, its' save to say that , if a test material consistently induces a strong antibacterial effect in the sensitivity tests, it is very likely also to exert antibacterial action in living tissue. The most desirable endodontic sealer would be one that combines maximal antibacterial effect with minimal toxicity. Therefore, one has to chose the one which combines a reasonably high antibacterial effect with a low toxic effect.

## Conclusion

Canason showed the highest antimicrobial activity against all microorganisms used in this study, while Acroseal , Epiphany and AH-Plus showed antibacterial activity lesser than that of Canason .The least antimicrobial activity was showed by MTA against all microorganisms used in this study. There were a high significant differences in the antibacterial activity of the five endodontic sealers on *Streptococcus viridans , Staphylococcus aureus* and *Enterococcus faecalis.* There were no significant differences in the antibacterial activity of the Acroseal , AH-Plus and MTA on *Staphylococcus aureus.* There was no difference in the zones of inhibition between the 24 and 48 hrs time period for all types of microorganisms used in this study. So it is advisable according to this result not to depend on the antimicrobial activity of the sealer alone in the treatment of infected root canal.

## References

1- Friedman S, Mor C. The success of endodontic therapy-healing and

functionality. CDA. Journal 2004, 32: 493-503.
2- Friedmans S, Abitbol S, Lawrence HP. Treatment outcome in endodontics: The Tonoto study. Phase 1: Enitial treatment. Journals of Endodontics 2003, 29: 787-93.
3- Molander Ak, Reit C, Dahlen G, Kvist T. Microbiological status of root-filled teeth with apial periodontitis. International Endodontic Journals 1998, 31: 1-7.
4- Siqueira Jf Jr, Rocas In, Oliveira JCM, Santos KRN. Molecular detection of Black-pigmented bacteria in infections of endodontic rogin. Journals of Endodontic 2001, 27(6): 563.
5- Bystram A, Sundqvist G. Bacteriologic Evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dental Research 1981, 89(8): 321.
6- Oquntabi BR. Dentine tubules infection and endodontic therapy implications. International Endodontic Journals 1994, 27: 218-222.
7- Grossman Li. Endodontic practice. 10th edition Philadephia. Lea Fabiger Company 1982.
8- Jose J, Siquera Jr, Milton DV. Influence of different vehicles on the antimicrobial effects of calcium hydroxide. Journals of endodotics 1998, 24: 663-665.
9- Sangberg L, Langeland K. Biologic effect of dental materials I. Toxicity of root canal sealer filling materials on Hella cells in vitro. Oral surgery 1973, 35: 407.
10- Orstavik D. Antibacterial properties of root canal sealers, cements and pastes. International Endodontic Journals 1981, 14: 125-133.
11- Sipert C. R, Hussne R. P, Nishiyama C. K, Torres S.A. In vitro antimicrobial activity of fill canal, Sealapex, Mineral trioxide aggregate, Protland cement and Endorez. International Endodontics Journals 2005, 38: 539-543.
12- Barry AL-Thorusberry C. Susceptibility test procedures: diffusion test procedure. American Society for Microbiology 1980, 46: 463-79.
13- Wayman B.F. A bacteriological and histological evaluation of 58 periradcular lesions. Journals of Endodontics 1992, 18: 152.
14- Ohara PK, Torabinejad M, Kettering JD. Antibacterial effects of various endodontic irrigants on selective anaerobic bacteria. Endodontic Dental Traumatolo. 1993, 9: 95-100.
15- Thomas P. A, Bhat KS, Katian KM. Antibacterial properties of dilute formcresol and eugenol and propulene glycol. Oral surgery 1980, 49: 166-170.
16- Söderberg TA. Effects of zinc oxide, rosin and acids and their combinations on bacterial growth and inflammatory cells. Scandinavian Journals of plastic reconstructive surgery and hand surgery 1990, 24 (S 22): 1-87.
17- Cox ST, John H, Hambree, Zames P, Mcknight. The bacteriological potential of various endodontic materials for primary teeth. Oral surgery Oral medicine oral pathology 1978, 45: 947-954.
18- Gopferich A. Mechanisms of polymer degradation and erosion. Biomaterials 1996, 17(14): 103.
19- Ruyter IE. Physical and Chemical aspects related to substances released from polymer materials in an aqueous environment. Advanced Dental Reasearch 1995, 9(7): 344.
20- Cohen BI, Pagnillo MK, Musikant BL, Deutsch AS. For maldehyde from endodontic materials. Oral Health 1998, 88(9): 37.
21- Peutzfeldt A. Resin Composities in dentistry: The monomer system, European Journal of Oral science 1997, 105: 97-116.
22- Versiani MA, Carvalho-Junior JR, Padilha MI, Lacey S, Pascon EA, Sousa-neto MD. A comparative study of physic chemical properties of AH-plus and Epiphany root canal sealant. International Endodontics Journals 2006, 39: 464-71.
23- Shipper G, Teixeiva FB, Arnold RR, Trope M. Periapial inflammation after coronal microbial inoculation of dog roots filled with gutta-percha or Resilon. Journals of Endodontics 2005, 31(6): 91.
24- Estera C, Sydney GB, Baumanun LL, Felippe O jr. Mechanisms of action of calcium and hydroxyl ions of calcium hydroxide on tissue and bacteria. Brazillian Dental journals 1995, 6: 85-90.
25- Rubin E, Farber JL, Patdogia. Rio de Janeiro. Interlivros, 1990: 2-30.
26- Voet D, Voet JG. 1995 Biochemisty, 2nd edition, New York. USA. John Wiley & Sons. Inc.
27- Imlay JA, Linn S 1988 DNA Damage and oxygen radical toxicity. Science 240. 20 1302-9.
28- Kontakiotis E, Nakou M, Georgopoulou M. 1995 In vitro study of the indirect action of calcium hydroxide on the anaerobic flora of the root canal. International Endodontics Journals 1995, 28(9): 285.
29- Eldeniz AU, Erdemir A, Kurtaglu F. Comparative evaluation of pH and

calcium ion release of Acroseal sealer with Apexit and Sealapex sealers. Oral surgery Oral medicine Oral pathology Oral radiology and Endodontics 2007 (in press).
30- Siqueira JF, Goucalves RB. Antibacterial activity of root-canal sealers against selected anaerobic bacteria. Journals of Endodontics 1996, 22: 79-80.
31- Siqueira JE, Jr, Lopes HP. Mcdansms of antimicrobial activity of calcium hydroxide. Acritical review. International Endodontics Journals 1999, 32(9): 361.
32- Svensater G, Larson UB, Grief EG, Cvikovitch DG, Hamilton IR. Acid tolerance response and survival by oral bacteria. Oral microbial Immunol 1997, 12(5): 266-73.
33- Gomes BPFA, Lilley JD, Drucker DB. Association of endodontic symptoms and signs with particular combinations of specific bacteria. International Endodontic Journals 1996, 29: 69-75.
34- Azar NG, Heidari M, Bahrami ZS, Sokri F. In vitro cytotoxicity of new epoxy root canal sealers. Journals of endodontics 2000, 26(5): 462.
35- Cohen BI, Pagnillo MK, Musikant BL, Deutsch AS. An in vitro study of the cytotoxicity of two root canal sealers. Journals of Endodontics 2000, 26(9):228.
36- AL-Hezaimi K, AL-Hamdan K, Naghshbandi J. Oglesby S, Simon JHS, Rotstein I. Effect of white coloured MTA in different concentrations on *Candida albicans* in vitro. Journals of Endodontics 2005, 31(6): 864.
37- Fridland M, Rosado R. MTA solubility and porosity with different water –to-powder ratios. Journals of Endodontics 2003, 29: 814-17.
38- Holland R, de Souza V, Nery MJ . Reaction of rat connective tissue to implanted dentin tube filled with MTA, protland cement or calcium hydroxide. Brazillian Dental Journals 2001, 12: 3-8.

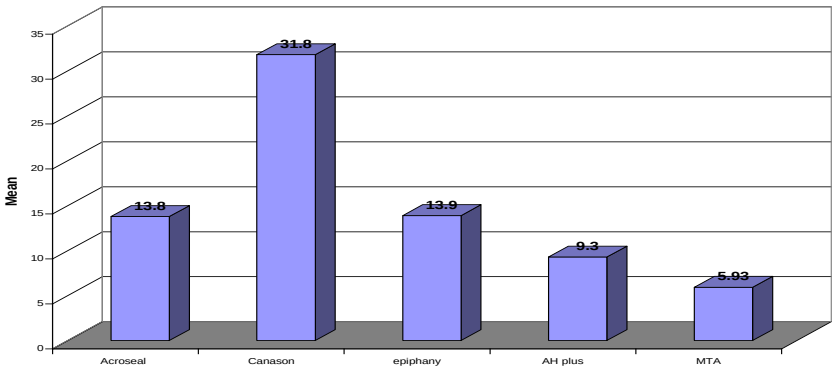


Fig. 1 Comparism between the mean of inhibition zones of endodontic sealers produced against *Streptococcus viridans* after 24 hours

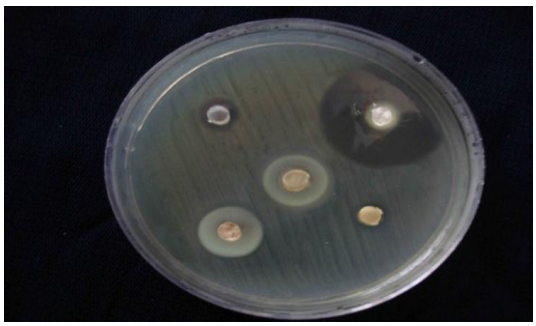

Fig.2 Agar diffusion method of Endodontic sealer on *Streptococcus viridans* on Mueller Hinton Agar media

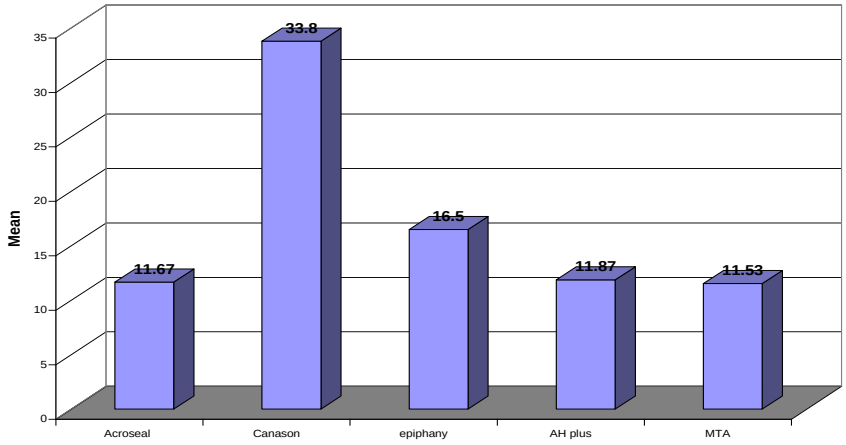


Fig. 3 Comparism between the mean of inhibition zones of endodontic sealers produced against *Staphylococcus aureus* after 24 hours

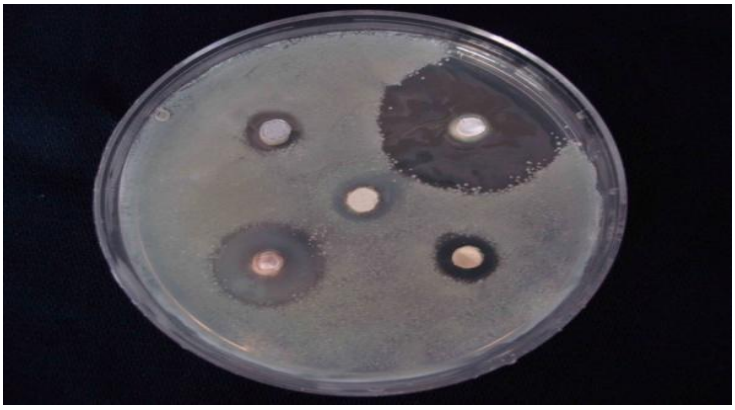

Fig. 4 Agar diffusion method of Endodontic sealer on *Staphylococcus aureus* on Mueller Hinton Agar media

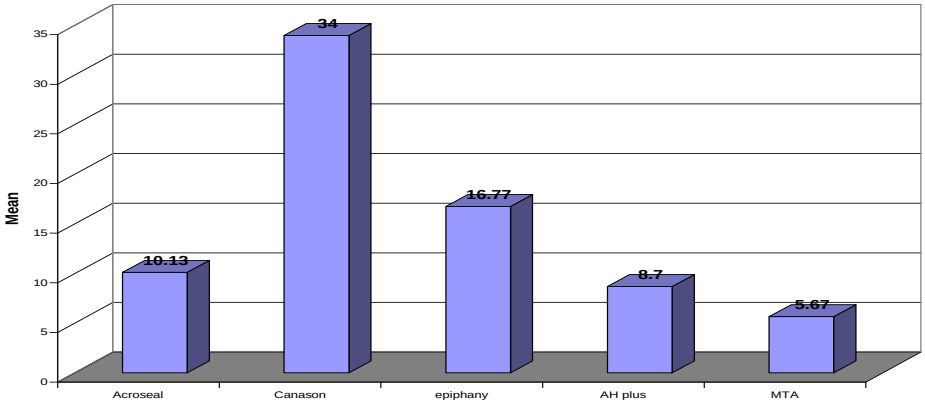


Fig. 5 Comparism between the mean of inhibition zones of endodontic sealers produced against *Enterococcus faecalis* after 24 hours

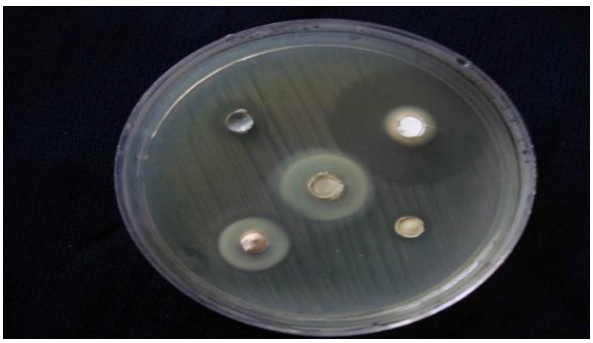

Fig. 6 Agar diffusion method of Endodontic sealer on *Enterococcus faecalis* on Mueller Hinton Agar media

Table 1 ANOVA test show the difference among the five endodontic sealers on the three types of bacteria

| Endodontic sealers | F-test | P-value | Sig |
|---|---|---|---|
| **Acroseal** | 87.32 | 0.000 | HS |
| **Canason** | 22.85 | 0.000 | HS |
| **Epiphany** | 47.83 | 0.000 | HS |
| **AH plus** | 91.25 | 0.000 | HS |
| **MTA** | 373.7 | 0.000 | HS |

*HS: Highly significant difference at level P < 0.001*