ISSN: 0975-833X

*Available online at http://www.journalcra.com*

*International Journal of Current Research*
*Vol. 15, Issue, 10, pp.26199-26202, October, 2023*
*DOI: https://doi.org/10.24941/ijcr.46128.10.2023*

**INTERNATIONAL JOURNAL OF CURRENT RESEARCH**

## RESEARCH ARTICLE

# BIOCOMPATIBILITY ASSESSMENT OF OZONATED OLIVE OIL AND COMMON IRRIGATING AGENTS IN REGENERATIVE ENDODONTICS: A CYTOTOXICITY STUDY ON RED BLOOD CELLS

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**ARTICLE INFO**

*Article History:*
Received 15th July, 2023
Received in revised form
17th August, 2023
Accepted 25th September, 2023
Published online 31st October, 2023

*Key words:*

Regenerative Endodontics, Cytotoxicity, Irrigating Agents, Ozonated Oil.

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**ABSTRACT**

**Introduction:** The selection of appropriate treatment agents is critical in regenerative endodontics to ensure successful revascularization outcomes. Ozonated oil and water, known for their potent antibacterial and oxidation properties, offer a novel class of disinfectants. This study aims to evaluate the biocompatibility of ozonated olive oil and other commonly used irrigating solutions in regenerative endodontics, specifically focusing on their impact on red blood cells (RBCs). **Objective:** This research assesses the cytotoxic effects of ozonated olive oil and other irrigating solutions commonly employed in regenerative endodontics on red blood cells, aiming to identify potential alternatives with minimal adverse effects. **Methods:** Five test solution groups were established: Group I - 3% NaOCl, Group II - 1.5% NaOCl, Group III - 17% EDTA, Group IV - Ozonated olive oil, and Group V - Normal saline (control). Thirty-five test tubes containing diluted RBC suspensions were assigned randomly to these groups (seven per group). Each test tube received 100 microliters of the respective irrigant, followed by a 3-minute incubation and subsequent centrifugation. Hemoglobin (Hb%) concentration in the resulting supernatant was measured to estimate cytotoxicity. Statistical analyses employed Analysis of Variance and Tukey's Post Hoc analysis. **Results:** Among the test solutions, 3% NaOCl demonstrated the highest cytotoxicity, while normal saline exhibited the lowest, with mean Hb concentrations of 0.515 g/dl and 0.043 g/dl, respectively, due to hemolysis. Statistical analysis revealed significant differences among the various test groups ($p = 0.001$). Ozonated olive oil demonstrated cytotoxicity levels similar to saline, with no statistically significant difference between them ($p = 0.988$). **Conclusion:** In comparison to 3% NaOCl, 1.5% NaOCl, and 17% EDTA, ozonated olive oil displayed lower levels of cytotoxicity and released Hb concentration (0.086 g/dl). This suggests that ozonated olive oil holds promise as a potentially favorable adjunct irrigant for regenerative endodontic procedures, due to its reduced impact on red blood cells.



**Citation:** ***Lalitha Sravani Kanumalla, Ramyasaketha Narayanam, Chandrabhatla Srinivas Kumar and Jayalakshmi Pandranki.*** **2023.** "Biocompatibility Assessment of Ozonated Olive Oil and Common Irrigating Agents in Regenerative Endodontics: A Cytotoxicity Study on Red Blood Cells*". International Journal of Current Research*, 15, (10), 26199-26202.

# INTRODUCTION

Root canal treatment of immature young permanent teeth with open root apices presents a distinct and intricate clinical hurdle. Conventional apexification methods involving substances like calcium hydroxide, MTA, and Biodentine can impede further root growth, increasing the vulnerability of thin and delicate roots to fractures (1).

This issue highlights the demand for alternative approaches, such as Regenerative Endodontic Procedures (REPs), which have emerged from early investigations into the significance of blood clots in endodontics and the imperative role of pulp revascularization in fostering ongoing root development (2,3,4). The formation of a blood clot during REPs serves as a natural scaffold that supports the progressive development of dental pulp tissue. Additionally, the identification of mesenchymal stem cell types like Stem Cells of Apical Papilla (SCAP), along with their capability to differentiate into odontogenic-like cell lines, has significantly propelled the field of

regenerative endodontics.(5,6) Commonly recommended root canal irrigants for REPs include low-concentration sodium hypochlorite (1.5%) and ethylene diamine tetraacetic acid (17% EDTA), along with triple antibiotic paste as an intracanal medicament.(7) However, due to concerns about bacterial resistance and existing cytotoxic effects, there is a pressing need for biocompatible root canal irrigants that can rapidly disinfect root canals in the context of regenerative endodontics. Ozonated oil/water represents a cutting-edge class of disinfectants characterized by potent oxidation potential surpassing chlorine by 1.5 times. The antimicrobial efficacy of ozonated oil arises from the generation of oxidized radicals that induce alterations in cell membrane osmotic permeability, resulting in cellular damage.(8) This novel agent also possesses the capacity to stimulate angiogenesis and immune responses, making it a promising candidate for pulp revascularization.(9) However, the understanding of its impact on blood clot and red blood cells remains limited. Consequently, this study aims to assess the biocompatibility of various root canal irrigants utilized in Regenerative Endodontic Procedures (REPs), along with ozonated olive oil, by gauging their cytotoxic effects on red blood cells. The null hypothesis posits that ozonated olive oil yields cytotoxic effects akin to those of saline (control).

# MATERIALS AND METHODS

Human blood, sourced from a blood bank, was employed to extract red blood cells. The blood, initially possessing a hemoglobin content of 12.6 g/dl, underwent heparinization before centrifugation at 1000 rpm for 10 minutes. Plasma supernatant was discarded, and the obtained packed cell volume underwent double washing in Dulbecco's phosphate-buffered saline. Subsequently, the hematocrit of the resulting red blood cell suspension was adjusted to 45%.(10) A total of 35 test tubes each containing 2 ml of diluted red blood cells were prepared, divided into five distinct groups, comprising seven test tubes per group. The test solutions were categorized as follows:

Group I: 3% NaOCl
Group II: 1.5% NaOCl
Group III: 17% EDTA
Group IV: 0.9% Normal Saline
Group V: Ozonated Olive Oil

**Cytotoxic Analysis:** Each test material (100µl) was introduced to 2 ml of diluted red blood cells. The test tubes were then incubated at 37˚C for 3 minutes, followed by centrifugation at 1000 rpm for 10 minutes. On hemolysis(Fig. 1), the amount of released hemoglobin in the supernatant was quantified using the Cyano-haemoglobin method (Choi J *et al*., 2011) and measured photometrically at 540 nm. (11) The collected data was organized in a tabular format and subjected to statistical scrutiny using One-way ANOVA, followed by Tukey's Post-hoc analysis for conducting pairwise comparisons.

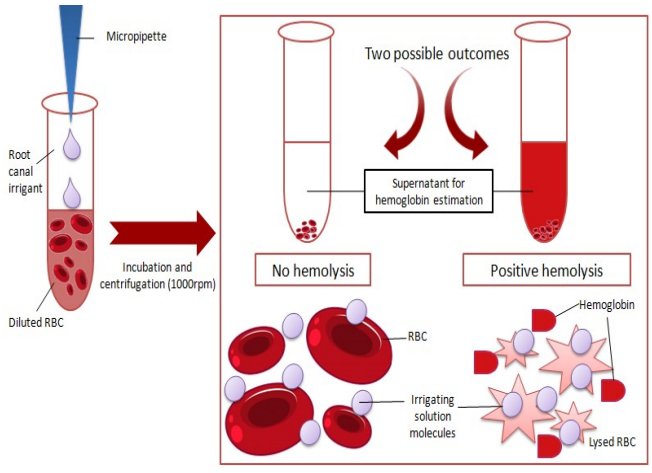


**Figure 1. Hemoglobin estimation after hemolysis**

# RESULTS

The study examined the cytotoxicity of various root canal irrigants, focusing on their impact on hemoglobin (Hb) concentration. Among the tested solutions, 17% EDTA exhibited the highest cytotoxicity, leading to a released Hb concentration of 0.514 g/dl in the supernatant liquid. Following closely was 3% Na OCl, which resulted in a released Hb concentration of 0.386 g/dl. Conversely, 1.5% NaOCl demonstrated relatively lower cytotoxicity, with a mean released Hb concentration of 0.229 g/dl, indicating its comparative biocompatibility compared to 3% NaOCl (Table 1 and Fig. 2).

**Table 1. Intergroup comparison between mean hemoglobin concentration values (g/dl)**

| S no | Group | Mean ± Std Dev | F ratio | p value |
|---|---|---|---|---|
| I. | 3% NaOCl | 0.514± 0.16762 | 26.923 | 0.001* |
| II. | 1.5% NaOCl | 0.229±0.09512 | | |
| III. | 17% EDTA | 0.386±0.10690 | | |
| IV. | Saline | 0.043± 0.05345 | | |
| V. | Ozonated Olive Oil | 0.086±0.03780 | | |

**p value <0.001 indicates high significance*

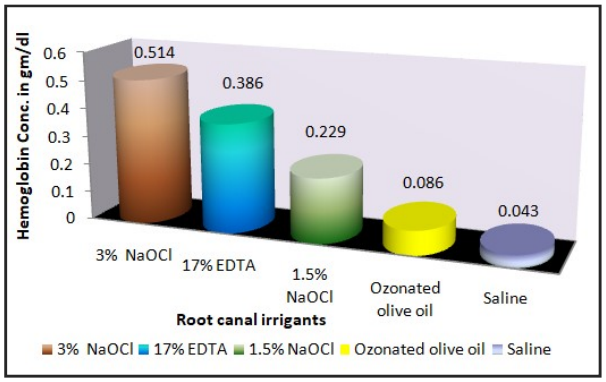


**Fig 2. Graph showing the intergroup of hemoglobin concentration in mg/dl between 3% NaOCl, 1.5% NaOCl, 17% EDTA solution and saline**

Distinctly lower levels of hemoglobin concentration were observed in ozonated olive oil (0.086 g/dl) and saline (0.043 g/dl). These findings strongly suggest that ozonated olive oil exhibits significantly reduced cytotoxicity when compared to the conventional root canal irrigants used for Regenerative Endodontic Procedures (REP). Utilizing a one-way ANOVA analysis, it was established that there exists a statistically highly significant difference among all the tested groups, indicated by an F ratio of 26.923 ($p < 0.05$). Specifically, a comparison between the mean Hb concentrations of ozonated olive oil and saline revealed a similar level of biocompatibility, with minimal cytotoxicity ($p > 0.05$). Similarly, no substantial difference was observed in cytotoxicity levels between 3% NaOCl and 17% EDTA ($p > 0.05$). Upon conducting pair-wise comparisons of Hb concentrations among the various groups, it became evident that there were significant differences in cytotoxicity levels ($p < 0.05$) across each group. (Table 2)

# DISCUSSION

Regenerative endodontic therapy (RET) emphasizes minimal or no instrumentation during procedures, underscoring the crucial roles of irrigation and intracanal dressings in achieving palpal cavity disinfection. The American Academy of Endodontics (AAE) guidelines of 2018 outlines the irrigation protocol for regenerative procedures. This protocol involves an initial application of 1.5% sodium hypochlorite (NaOCl), followed by subsequent treatments with saline or 17% ethylenediaminetetraacetic acid (EDTA) for 5 minutes each, per canal.

**Table 2. Post- Hoc statistical analysis for pair- wise comparison between saline and the test groups**

| Multiple Comparisons- Tukey's Posthoc analysis | | | | | | |
|---|---|---|---|---|---|---|
| **Groups** | **Groups** | **Mean Difference** | **Std. Error** | **Sig.** | **95% Confidence Interval** | |
| | | | | | **Lower Bound** | **Upper Bound** |
| Ozonated olive oil (O-O-O) | 17% EDTA | -0.30000 | 0.04124 | 0.000*** | -0.4355 | -0.1645 |
| | 1.5% NaOCl | -0.14286 | 0.04124 | 0.031** | -0.2783 | -0.0074 |
| | 3% NaOCl | -0.42857 | 0.04124 | 0.000*** | -0.5641 | -0.2931 |
| | Saline | 0.04286 | 0.04124 | 0.988* | -0.0926 | 0.1783 |
| 17% EDTA | 1.5% NaOCl | 0.15714 | 0.04124 | 0.011** | 0.0217 | 0.2926 |
| | 3% NaOCl | -0.12857 | 0.04124 | 0.077* | -0.2641 | 0.0069 |
| | Saline | 0.34286 | 0.04124 | 0.000*** | 0.2074 | 0.4783 |
| 1.5% NaOCl | 3% NaOCl | 0.28571 | 0.04124 | 0.000*** | 0.1502 | 0.4212 |
| | Saline | 0.18571 | 0.04124 | 0.001*** | 0.0502 | 0.3212 |
| 3% NaOCl | Saline | 0.47143 | 0.04124 | 0.000*** | 0.3359 | 0.6069 |

The mean difference is significant at the 0.05 level. ( **p>0.05 – Not significant; **p<0.05 – Significant; ***p<0.005 – Highly significant)*

The significance of these measures lies in the role they play in disinfecting the root canal, creating a microenvironment that facilitates the survival, proliferation, and differentiation of stem cells with minimal cytotoxicity (7). Research conducted by Heggers JP *et al* in 1991 delved into the bactericidal effects of different concentrations of NaOCl, revealing that it retains antibacterial efficacy at lower concentrations, such as 0.025%, without toxic effects.(12) However, at 0.25%, NaOCl demonstrated tissue toxicity. Notably, the clinically used concentrations of NaOCl have been found to compromise dentin-derived growth factors.(13) In contrast, EDTA, at 17%, promotes the release of biologically active growth factors embedded in the dentin matrix, which actively participate in regenerative processes like angiogenesis and stem cell activities.7,13 However, EDTA's effectiveness against E. faecalis has been questioned (12,14). Triple antibiotic paste (TAP) was initially recommended for RET, yet its long-lasting negative impact on stem cell survival has led to a shift in recommendations. High concentrations of TAP paste (>1 mg/ml) are no longer advised due to stem cell toxicity. Therefore, the effective disinfection of root canals while ensuring the favorable survival and function of stem cells remains a critical aspect of RET. The biocompatibility of endodontic materials encompasses various parameters, including cytotoxicity, mutagenicity, genotoxicity, histocompatibility, carcinogenicity, and microbial effects. As outlined by the International Organization for Standardization (ISO) 7405, cytotoxicity testing serves as a preliminary evaluation of new materials across different biological models.(15) Red blood cells (RBCs) serve as a sensitive model for evaluating intracellular content and hemoglobin, given their fragility and relevance in angiogenesis and pulp revascularization (16).

This study specifically investigated the hemolytic effects of commonly used irrigants in RET, including 1.5% NaOCl, 17% EDTA, and saline. The choice to include 3% NaOCl stemmed from observations that mature and old biofilms exhibit increased resistance to lower concentrations of NaOCl.(17) Surprisingly, the highest hemolytic effects were observed with 3% NaOCl, highlighting the concentration-dependent nature of NaOCl's cytotoxicity (figure 2). This process likely involves lysophospholipids porating the cell membrane, allowing for ion exchange and water influx, culminating in colloidal osmotic lysis (18). Moreover, 17% EDTA holds particular importance in the RET protocol for multiple reasons. It acts as a demineralizing agent, exposing the dentin matrix and releasing growth factors.(19,20) Conditioning dentin with EDTA facilitates adhesion, migration, and differentiation of dental pulp stem cells onto dentin. Its usage has also demonstrated increased stem cell survival expression and a partial reversal of NaOCl's deleterious effects (12,14). In the current research, it was observed that EDTA at a concentration of 17% resulted in higher levels of hemolysis compared to 3% NaOCl. Taweewattanapaisan P (2019) explained that residual EDTA within the root canal had a significant impact on the formation of blood clot fibrin. (21) The remaining EDTA reacted with calcium ions present in the blood, disrupting the blood clotting process. Despite its known anticoagulant properties, EDTA caused deformities in red blood cells and considerably reduced the density of the blood clot fibers. Therefore, thorough saline irrigation is necessary to eliminate any residual EDTA.

Furthermore, the inadvertent extrusion of EDTA into the periapical area could lead to vascular changes, activating inflammatory cells like neutrophils and macrophages, producing chemical mediators, and impairing cellular repair (22). To address these concerns, the study explored the potential of Ozonated Olive Oil (O-O-O). Over the past decade, ozone has gained recognition in the medical field for its antiviral, antifungal, and bactericidal properties, as well as its biocompatibility (8,9). Ozone's ability to combat microorganisms and support tissue healing has been harnessed in various applications, including post-extraction alveolitis and wound healing.(23) Studies have demonstrated the effectiveness of ozonated olive oil in skin and oral wound healing (8,9,23). Guinesi AS (2011) noted that while gaseous ozone has a short half-life, when combined with olive oil, it forms stable compounds that can be stored for months, obviating the need for an ozone generator (24). The interaction between ozone and olive oil leads to the creation of antibacterial and healing components that contribute to the efficacy of ozonated olive oil (25). The present study found that ozonated olive oil exhibited minimal cytotoxicity to red blood cells, comparable to saline, indicating its biocompatibility (24,25). Gornicki A and Guts Ze A (2000) suggested that ozone in lower concentrations could lead to cell membrane rigidization in erythrocytes, potentially due to modifications in membrane proteins. In terms of antimicrobial activity.(26) Wassef N and Fouad M (2019) found that ozonated olive oil was more effective against Enterococcus faecalis than 1% sodium hypochlorite, while Pratyusha M.V (2017) reported superior antibacterial effects against E. faecalis compared to 2% Chlorhexidine. (27,28) Moreover, Elshinawy MI (2018) discovered that ozonated olive oil exhibited minimal cytotoxicity against human fibroblasts (29). Apart from its lower cytotoxicity, ozonated olive oil also demonstrated the ability to inhibit biofilm formation, which could have implications for the prognosis of Regenerative Endodontic Procedures (REP). Valacchi *et al*. (2005) highlighted its potent inhibitory effect on mature biofilms. (25) The oily nature of ozonated olive oil might hinder the adhesion of root canal sealers to dentin, which is relevant as REP typically doesn't require such sealers. Given these attributes, the study suggests that ozonated olive oil has potential as an alternative natural irrigant in regenerative endodontic procedures. Nevertheless, the study had some limitations. It assessed the effects of irrigating solutions on red blood cell morphology and the interactions of ozonated oil with root dentin and other REP solutions. Further investigations are recommended to fully understand the synergistic effects of ozonated olive oil when combined with other agents commonly used in regenerative endodontics.

# CONCLUSION

In summary, taking into account the constraints of this in vitro study, the findings suggest that Ozonated olive oil exhibits a minimal impact on the hemolysis of red blood corpuscles when compared to 1.5% and 3% sodium hypochlorite as well as 17% EDTA. However, its effect is somewhat higher than that of saline, indicating its reasonable cytocompatibility. The observed differences among all the tested irrigants are statistically significant ($p < 0.001$). While this research proposes Ozonated olive oil as a potential substitute for traditional

root canal irrigants in regenerative endodontics, it's important to acknowledge the study's limitations. Further investigations focusing on the role of ozone as an irrigant are essential before its clinical applicability can be fully warranted. This includes delving into aspects such as its interaction with different tissue types and its overall impact within the complex environment of root canal treatments. Therefore, while promising, the practical viability of Ozonated olive oil as an irrigant in regenerative endodontics requires comprehensive examination through additional research.

# REFERENCES


1. Lin LM, Kahler B. A review of regenerative endodontics: current protocols and future directions. J Istanb Univ Fac Dent. 2017 Dec 2;51(3):41-51.
2. Murray PE, Garcia-Godoy F, Hargreaves KM. Regenerative endodontics: a review of current status and a call for action. J Endod. 2007 Apr;33(4):377-90.
3. Ostby BN. The role of the blood clot in endodontic therapy. An experimental histologic study. Acta OdontolScand1961;19(3-4):323-353.
4. Andreasen, JO, Andreasen FM and Andersson L. Textbook and color atlas of traumatic injuries to the teeth.3rd ed. Copenhagen, Denmark: Blackwell Munksgaard; 1993;216-256.
5. Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res. 2009 Sep;88(9):792-806.
6. Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Wang S, Shi S. Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS One. 2006 Dec 20;1(1):e79.
7. American Association of Endodontists. AAE clinical considerations for a regenerative procedure. 2018. Available at:https://www.aae.org/specialty/publications-research/research/regenerative-database/ Accessed March 3, 2021.
8. Tiwari S, Avinash A, Katiyar S, Iyer AA, Jain S. Dental applications of ozone therapy: A review of literature. The Saudi Journal for Dental Research. 2017 Jan 1;8(1):105-11.
9. Nogales CG, Ferrari PH, Kantorovich EO, Lage-Marques JL. Ozone therapy in medicine and dentistry. J Contemp Dent Pract. 2008 May 1;9(4):75-84.
10. Pashley EL, Birdsong NL, Bowman K, Pashley DH. Cytotoxic effects of NaOCl on vital tissue. J Endod. 1985 Dec;11(12):525-8.
11. Choi J, Reipa V, Hitchins VM, Goering PL, Malinauskas RA. Physicochemical characterization and in vitro hemolysis evaluation of silver nanoparticles. Toxicol Sci. 2011 Sep;123(1):133-43.
12. Heggers JP, Sazy JA, Stenberg BD, Strock LL, McCauley RL, Herndon DN, Robson MC. Bactericidal and wound-healing properties of sodium hypochlorite solutions: the 1991 Lindberg Award. J Burn Care Rehabil. 1991 Sep-Oct;12(5):420-4.
13. Zhao S, Sloan AJ, Murray PE, Lumley PJ, Smith AJ. Ultrastructural localisation of TGF-beta exposure in dentine by chemical treatment. Histochem J. 2000 Aug;32(8):489-94.
14. Torabinejad M, Shabahang S, Aprecio RM, Kettering JD. The antimicrobial effect of MTAD: an in vitro investigation. J Endod. 2003 Jun;29(6):400-3.
15. Calt S, Serper A. Smear layer removal by EGTA. J Endod. 2000 Aug;26(8):459-61.
16. ISO (1984) Biological evaluation of dental materials. International Organization for Standardization. Technical Report 7405.
17. ForoughReyhani M, Rezagholizadeh Y, Narimani MR, Rezagholizadeh L, Mazani M, Barhaghi MHS, Mahmoodzadeh Y. Antibacterial effect of different concentrations of sodium hypochlorite on Enterococcus faecalis biofilms in root canals. J Dent Res Dent Clin Dent Prospects. 2017;11(4):215-221.
18. Ionescu-Zanetti C, Wang LP, Di Carlo D, Hung P, Di Blas A, Hughey R, Lee LP. Alkaline hemolysis fragility is dependent on cell shape: results from a morphology tracker. Cytometry A. 2005 Jun;65(2):116-23.
19. Galler KM, Buchalla W, Hiller KA, Federlin M, Eidt A, Schiefersteiner M, Schmalz G. Influence of root canal disinfectants on growth factor release from dentin. J Endod. 2015 Mar;41(3):363-8.
20. Lin LM, Kahler B. A review of regenerative endodontics: current protocols and future directions. J Istanb Univ Fac Dent. 2017 Dec 2;51(3):41-51.
21. Taweewattanapaisan P, Jantarat J, Ounjai P, Janebodin K. The Effects of EDTA on Blood Clot in Regenerative Endodontic Procedures. J Endod. 2019 Mar;45(3):281-286.
22. Amaral KF, Rogero MM, Fock RA, Borelli P, Gavini G. Cytotoxicity analysis of EDTA and citric acid applied on murine resident macrophages culture. Int Endod J. 2007 May;40(5):338-43.
23. Bocci V. Ozonization of blood for the therapy of viral diseases and immunodeficiencies. A hypothesis. Med Hypotheses. 1992 Sep;39(1):30-4.
24. Guinesi AS, Andolfatto C, Bonetti Filho I, Cardoso AA, Passaretti Filho J, Farac RV. Ozonized oils: a qualitative and quantitative analysis. Braz Dent J. 2011;22(1):37-40.
25. Valacchi G, Fortino V, Bocci V. The dual action of ozone on the skin. Br J Dermatol. 2005 Dec;153(6):1096-1100.
26. Gornicki A, Gutsze A. In vitro effects of ozone on human erythrocyte membranes: an EPR study. Acta Biochim Pol. 2000;47(4):963-71.
27. Wassef N, Fouad M. Antibacterial effect of ozonated olive oil versus sodium hypochlorite against Enterococcus faecalis bacteria: In vitro study.Egyptian Dental Journal 2019; 65(4): 3271-3276.
28. Pratyusha M.V., Jayalakshmi, K.B., Prasannalatha N, Sujatha, I., Arul Selvan K and Zaharunnissa. Evaluation of antibacterial efficacy of ozonated olive oil and cold pressed neem oil against Enterococcus faecalis: an agar well diffusion study. International Journal of Current Research 2017; 9(5): 50711-50715.
29. Elshinawy MI, Al-Madboly LA, Ghoneim WM, El-Deeb NM. Synergistic Effect of Newly Introduced Root Canal Medicaments; Ozonated Olive Oil and Chitosan Nanoparticles, Against Persistent Endodontic Pathogens. Front Microbiol. 2018 3;9:1371.


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