ISSN: 0975-833X

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

International Journal of Current Research
Vol. 15, Issue, 02, pp.23755-23759, February, 2023
DOI: https://doi.org/10.24941/ijcr.44845.02.2023

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

COMPARATIVE EVALUATION OF ANTIMICROBIAL EFFICACY OF AQUEOUS AND ETHANOLIC EXTRACT OF SALVADORA PERSICA WITH AND WITHOUT ULTRASONIC ACTIVATION- AN IN VITRO STUDY

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ARTICLE INFO

Article History:
Received 14th November, 2022
Received in revised form
17th December, 2022
Accepted 19th January, 2023
Published online 19th February, 2023

Key words:

NaOCL, Salvadora Persica, Irrigation, Ultrasonic activation, Antimicrobial Efficacy.

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ABSTRACT

Aim**: To* compare the antimicrobial efficacy of alcoholic and aqueous extracts of Salvadora persica with and without ultrasonic activation against E.Feacalis. ***Materials and Methods: The samples were irrigated as follows after preparation upto size F3: ***Group 1**:* Experimental combination of 5% NaOCl and 40% S. persica Aqueous extract(n=10),***Group 2**:* Experimental combination of 5% NaOCl and 40% S. persica Aqueous extract with ultrasonic activation (n=10), ***Group 3**:* Experimental combination of 5% NaOCl and 40% S. persica alcoholic extract (n=10),***Group 4**:* Experimental combination of 5% NaOCl and 40% S. persica alcoholic extract with ultrasonic activation (n=10). ***Statistical Analysis**:* Antimicrobial efficacy – Independent T test and post hoc Mann–Whitney test. ***Results:*** Results shows that in the non-activation group, alcoholic extract of Salvadora persica performed better than the aqueous extract. In activation groups both aqueous and alcoholic extracts were found to perform similarly. ***Conclusion:*** Both alcoholic and aqueous extracts of Salvadora persica in combination with NaOCl had antimicrobial activity against E. Faecalis.Ultrasonic activation resulted in better reduction of CFU's compared to non-activated groups for both aqueous and alcoholic extracts.

Citation: ***Dr. Jibin Karim V.A., Dr. Ranjini M.A., Dr. Swapna D.V., Dr. Ridyumna Garain, Dr. Vedavathi, B. and Dr. Parvathy, P.*** **2023.** "Proposition d'une filière de gestion des déchets solides ménagers dans une ville émergeante : cas de la ville d'Abomey-Calavi (Bénin)*". International Journal of Current Research*, 15, (02), 23755-23759

INTRODUCTION

Irrigation is importantfor success of root canal treatment. Irrigant has several functions, which may vary according to the chemical used (Basrani, 2012) Sodium hypochlorite is the main irrigating solution used to dissolve pulp tissue and kill microbes effectively. It is used in concentrations between 0.5-6% (Zehnder, 2006) To maximise the effectiveness of hypochlorite irrigation, the solution should be frequently refreshed and agitated. Ethylenediaminetetraacetic acid (EDTA) is another irrigant that must be as a final rinse to remove the smear layer. However, irrigating solutions show varying degrees of cytotoxicity and sodium hypochlorite may cause severe, immediate and long-lasting pain if it is expressed under pressure and then escapes through the apical foramen.

In theory, an optimal irrigating solution should have flushing action, dissolve organic and inorganic matter, good penetration within the root canal system,broad spectrum antimicrobial action,non-toxic to periapical tissue, non-allergenic, not react with other dental materials, not weaken dentin. Clearly, none of the presently available irrigating solutions can be regarded as optimal (Haapasalo, 2020). In recent years herbs are being extensively researcheddue to their high antimicrobial activity, biocompatibility, anti-inflammatory and anti-oxidant properties. The other advantages of herbal irrigants are low tissue toxicity, easy availability, cost effectiveness and limited microbial resistance (Balto, 2012). Salvadora persica or pilu, a commonly used agent in many herbal mouth rinse and toothpaste formulations, has got many properties like antimicrobial, anticariogenic, antioxidant and chelation actions (Nawal, 2007). Exploring the antimicrobial activity of S. persica is still in its early

stages, where crude and alcoholic extracts have shown general effectiveness against some pathogenic bacteria and fungi such as Bacillus subtilis, Escherichia coli, Lactobacillus brevis, Proteus vulgaris, Staphylococcus aureus, Streptococcus mutans, Lactobacillus acidophilus, Pseudomonas aeruginosa, Aspergillus niger, and Candida albicans (Abhary, 2016) E. faecalis**,** a facultative anaerobic Gram-positive bacterium, is the most common Enterococcus species cultured from nonhealing endodontic cases. Several invitro studies have proven the antimicrobial efficacy of that against Enterococcus faecalis (Balto, 2013). Several solvents have been tried for the extraction of Salvadora persica from its parts including hexane, methanol, ethanol etc. Studies have shown varying results with aqueous and alcoholic extract in terms of antimicrobial efficacy. The apical root canal imposes a special challenge to irrigation as the balance between safety and effectiveness is particularly important in this area. Different means of delivery are used for root canal irrigation, from traditional syringe-needle delivery to various machine-driven systems, including automatic pumps and sonic or ultrasonic energy. Hence this study was done to compare the antimicrobial efficacy of aqueous and ethanolic extract of salvadora persica with and without ultrasonic activation.

MATERIALS AND METHODS

For In vitro Antimicrobial Study

- 40% Salvadora persica alcoholic extract (Himalaya Drug Company, Bangalore)
- 40% Salvadora persica aqueous extract (Himalaya Drug Company, Bangalore)
- 5% sodium hypochlorite (Doer)
- Sterile saline solution
- 15 K file (Dentsply)
- Endomotor (Dentsply X Smart)
- Protaper rotary files (Dentsply)
- Irri safe ultrasonic tip (IrriSafe, Satelec, Acteon, France)
- Paper points (Dentsply)
- Micromotor straight hand piece (NSK, Japan)
- Diamond discs (Horico, Germany)
- Autoclave (Thermo fisher scientific)
- Brain heart infusion agar plates

STANDARDIZATION OF STUDY GROUP

40 Extracted single rooted premolars used for the study were cleaned of debris and adherent tissues and placed in fresh 0.5% Chloramine-T. All the teeth were decoronated with a diamond disc to obtain a standard tooth length of 15 mm. A size 15k was used to scout and establish patency till the tip of the file was seen at the apical foramen, from which 1 mm was subtracted to establish the working length. The samples were then instrumented with pro taper rotary files upto size F3 (apical size 30, 9% taper) to allow for adequate penetration of irrigant. Normal saline was used as working solution during instrumentation. The roots were then sterilized in an autoclave at 121 C and 15lb pressure and verified for sterility. A 24hr culture of *Enterococcus Faecalis* was grown in brain heart infusion broth with concentration of 1x1cells/ml. Each root canal was inoculated with 10µl of *E Faecalis* suspension using a sterile 1ml tuberculin syringe and incubated at 37^0c for 48 hrs

The samples were irrigated as follows:

Group 1: Experimental combination of 5% NaOCl and 40% S. persica Aqueous extract (n=10)

Group 2: Experimental combination of 5% NaOCl and 40% S. persica Aqueous extract with ultrasonic activation (n=10)

Group 3: Experimental combination of 5% NaOCl and 40% S. persica alcoholic extract (n=10)

Group 4: Experimental combination of 5% NaOCl and 40% S. persica alcoholic extract with ultrasonic activation (n=10). (The ultrasonic activation was carried out for 2 cycles of 30 s each for group 2 and 4 with Irrisafe ultrasonic file).

The canals were dried with paper points. Dentin Harvesting was done with Gates Glidden drills No. 4 and 5. The collected dentin scrapings were transferred into 1 ml sterile BHI broth and incubated at 37^OC for 24 h.

After the incubation period, the content of each tube was serially diluted, five times with 100 µL broth in 100 µL sterile saline. 50 µL from each tube was plated on BHI agar plates and incubated for 24 h. Colonies were counted and the total number of colony forming unit (CFU) per millilitre was calculated. CFU of *E. Faecalis* served as a measure of antibacterial activity. Data were statistically analysed.

STATISTICAL ANALYSIS: CFU counts were statistically analyzed using Independent T test and post hoc Mann–Whitney analysis.

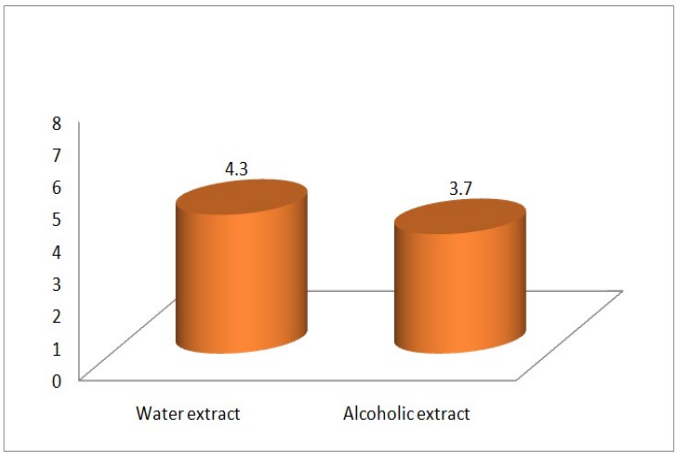
RESULTS

COMPARISON BETWEEN AQUEOUS EXTRACT AND ALCOHOLIC EXTRACT

Table 1. Result of Independent t test

Group	Mean	Std. Deviation	t-value	P-value
Aqueous extract	4.3	3.67	0.710	0.480
Alcoholic extract	3.7	3.88		

Since the P-value is greater than 0.05, accept the null hypothesis that there is no significance difference between water extract group and alcoholic extract group.



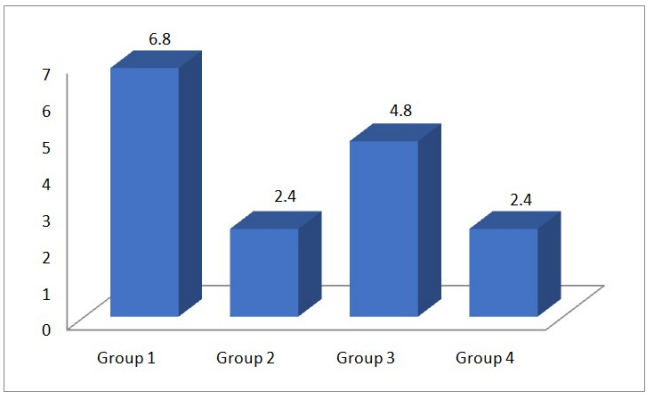
GROUPWISE COMPARISON

Table. Result of Mann-Whitney U Test

Pairs		Mean Rank Difference	P-value	Significance Difference
Group 1	Group 2	7.1	0.005	Present
Group 1	Group 3	3	0.230	Absent
Group 1	Group 4	7.1	0.005	Present
Group 2	Group 3	3.4	0.172	Absent
Group 2	Group 4	0	1	Absent
Group 3	Group 4	3.4	0.172	Absent

Group 1 possess significance difference between Group 2 and Group 4. P-values for every other pairs are greater than 0.05 which leads to the conclusion that there is no significance between other pairs.

Graph: Group Means



Results shows that in the non activation group, alcoholic extract of Salvadora persica performed better than the aqueous extract with mean CFU score of 6.8 and 4.8 respectively. In activation groups both aqueous and alcoholic extracts were found to perform similarly.

DISCUSSION

Debridement of the root canal by instrumentation and irrigation is considered the most important factor in the treatment of endodontic diseases. An ideal irrigant is one with tissue dissolving property, antibacterial activity and chelating actions. During and after instrumentation, the irrigants facilitate removal of microorganisms, tissue remnants and dentin chips from the root canal through a flushing mechanism and also remove the smear layer. All of these together contribute for the success of endodontic treatment. [1,3,9] Various compounds in aqueous solution have been used as root canal irrigants, such as sodium chloride (saline), sodium hypochlorite, iodine potassium iodide, chlorhexidine, MTAD, dequalinium acetate and EDTA (Hülsmann, 2003). NaOCl is considered as gold standard irrigant for endodontic treatment. It is the only irrigant with tissue dissolution action and good antimicrobial activity but lacks smear layer removal ability which necessitates the use of chelators. 17% EDTA as a final rinse removes the smear layer and opens up the dentinal tubules that aid in better root canal sealer penetration-thereby increasing the push out bond strength of the obturating materials (D'Arcangelo, 2007). However, these solutions have to be used consecutively andcannot be combined together, as they form an insoluble aggregate which may further block the root canals. So saline is usually advocated in between the use of these two irrigants. However, Studies have shown that 17% EDTA was highly erosive thereby affecting the tooth's fracture toughness and altered the Ca/PO4 ratio of root dentine that can affect bonding of resin sealer/cement (Mai, 2010). There is no single irrigating solution that alone sufficiently covers all the functions required from an irrigant. Therefore, the search for more biocompatible solution with minimal harmful effects on tooth structure and with paramount functioning as an irrigant still continues. The apical third of the root canal should be sufficiently enlarged to provide room for the irrigant to reach all the intricacies. Many techniques have been devised to improve the efficacy of irrigants such as increasing the concentration / contact time, temperature or by combination with other chemicals. Mechanical agitation techniques with the use of master cone gutta percha, endodontic files, sonic and ultrasonic devices have been popular and are easily dispensable with promising results (Virdee, 2018). But one has to be cautious to avoid extrusion of the irrigant and its undue sequelae. As already known, apical extrusion of either hypochlorite or EDTA results in severe toxic reactions in the periapical region. Various natural herbal products are being extensively researched for their antimicrobial property, biocompatibility, anti-inflammatory and anti-oxidant properties. Proposed advantages of using herbs could be their low tissue toxicity, easy availability, cost effectiveness and more importantly limited antimicrobial resistance. Salvadora persica is one such herbal extract that is studied extensively. Sticks of miswak from roots or shoots were used as toothbrush. Water, ethanol, methanol, ethyl acetate and acetic acid were used as solvents to extract the effective chemical compounds from Miswak sticks.[14]

Enterococcus faecalis (E. faecalis) is a gram positive facultative anaerobic bacterium. It was selected for the purpose of the present study because it is believed that it is one of the intra-canal bacteria which are most resistant to elimination by disinfecting agents (Siqueira, 2001). Chemical analysis of S. persica has demonstrated the presence of b-sitosterol and manisic acid, chlorides, organic compounds, such as pyrrole and piperidine derivatives, glycosides and flavonoids. It has a high ratio of alkaloids, such as salvadorine and trimethylamine, chlorides and fluorides, moderate concentrations of silica, sulfur, vitamin C, small quantities of tannins, saponins, flavonoids, and sterols. High amounts of sodium chloride and potassium chloride were also note. Sodium hypochlorite and Salvadora individually has antimicrobial activity against E faecalis as proven by Siqueira et al and Nawal A.k Al –Sabawi et al. (2000). Several studies have shown varying antimicrobial action with different solvents. Akpata and Akinrimisi found that alcoholic and water extracts of Salvadora persica inhibited the growth of Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa.[17] So in this study we compared the antimicrobial efficacy of Salvadora persica extracts in two different solvents of water and ethanol with and without ultrasonic activation.

The number of colony forming units (CFU's) of *E. Faecalis* served as a measure of antibacterial activity in this study. Results of this study showed that the mean colony forming units in the alcoholic extract groups of Salvadora persica was lower than that in aqueous extract groups. Although ultrasonic activation group of both the extracts resulted in similar performance of both solvents. Salvadora has antibacterial properties due to sulphur content and trimethylamine. An in vitro study on antimicrobial effect of alcoholic extract of Salvadora Persica (1%, 5%, 10%, 15%, and 20%), showed that all concentrations of Salvadora Persica extract had antimicrobial effect against aerobic and anaerobic bacteria recovered from teeth with necrotic pulps. The results showed that the alcoholic extract (Group 3) had a better antimicrobial efficacy than aqueous extract of Salvadora persica (Group 1) without PUI. Although there was difference in the mean CFU's this was not statistically significant. Sofrata et al. identified a volatile compound: benzyl isothiocyanate (BITC) in Salvadora persica extracts. In their study, the BITC exhibited rapid and strong bactericidal effect against Gram-negative bacteria but low effect on Gram-positive bacteria. The authors speculated that BITC must have penetrated through the outer bacterial membrane, interfered with the bacterial redox systems and thus hampered the ability of the bacterium to maintain its membrane potential (Sofrata, 2011) According to the study by M. Abhary et al,the solubility of antimicrobial agents in different polarity solvents had different effects on the growth of the bacterial flora. Antimicrobial agents with different polarities exist in S. persica chewing sticks and act against gram positive and negative bacteria. For example, the chloride concentration in the water extract was 11.5 times more than the ethanol extract, which suggests that the solubility of such chlorides requires high polarity solvents and indicates that high polar solutes are responsible for inhibiting the growth of some bacterial strains, such as L. acidophilus (gram +ve) and P. aeruginosa (gram −ve), which were more susceptible to the water extract than the ethanol or hexane extracts. However, ethanol extract was more effective against Strep. mutans (gram +ve), which suggests that there are selective semipolar antimicrobial agents in S. persica. This could be attributed to the differences in the antimicrobial efficacy of extracts from alcoholic and aqueous solvents in this study. The water and ethanol solvents have a polarity index of 9 and 5.2, respectively according to Snyder's polarity index. Metals and ions are expected to dissolve best in water, followed by ethanol (Abhary, 2015). The results of this study also showed that irrespective of the type of extract ultrasonic activation (Group 2 and 4) resulted in much lesser no of CFU's when compared to non-activation groups (Groups 1 and 3) and there was no statistically significant difference between the activation groups. The use of ethanol as an auxiliary method of conditioning intra radicular dentin has been proposed for controlling dentinal moisture and enhancing bond durability. This technique consists of applying ethanol before applying a two-step self-etching or a three-step etch-and-rinse adhesive system, so that the remaining water may

be replaced with ethanol. Monomers are hydrophobic, and may penetrate more deeply in ethanol-saturated than in water-saturated coronal dentin.[19] Improvements in adhesive technology have fostered attempts to incorporate adhesive dentistry in endodontics by introducing methacrylate-based sealers focusing on forming a single cohesive unit between the core material, sealing agent, and root canal dentin. Recently, acidic resin monomers were incorporated into these sealers to render them self-adhesive to dentin substrates, aiming to reduce the application time and errors that might occur during bonding steps. However, sealer adhesion to dentin may be affected by the moisture condition of the root canals before filling procedures. Thus, making smear-free dentin more wettable may improve sealer penetration.[20] Studies have shown that removal of the smear layer followed by a drying protocol using 70% isopropyl alcohol before canal obturation improved bond strength and penetration of the sealers into dentinal tubules of the root. Its also shown that application of 2% chlorhexidine or 100% ethanol may be an important clinical step that may be taken to enhance bond strength of fiber posts to intraradicular dentin, when dual resin cements are used. Both chlorhexidine and ethanol improved push-out bond strength to intraradicular dentin (Tudela, 2010).

During irrigation, radicular and coronal dentin are exposed to various chemical solutions to disinfect the endodontic space.[4] Thereby, this endodontic procedure may affect dentin surface and consequently their interactions with resin based materials.[15] Its well known that the oxidizing action of sodium hypochlorite leads to residual-free oxygen within the dentin matrix that may critically interfere with the initiation of the resin cements polymerization leading to poor bond strengths.[16] An additional side effect of sodium hypochlorite irrigation is the significant reduction in the microhardness of root canal dentin.[15, 17] This reduction demonstrates the direct effects of this chemical solution to dentin, which affect the adhesion and sealing ability of sealers to the softened chemical treated dentin surfaces (Kpata, 1977).

On the other hand, 0.2% chlorhexidine gluconate has harmless effect on the microhardness and roughness of root canal dentin, and showed highest bond strength when compared to sodium hypochlorite. The most common protocol to remove smear layer, before obturation, is the irrigation with 17% EDTA. Moreover is recognized that 17% EDTA is a chelating solution that may act as a reducing agent and has proven to restore the redox potential of the dentin and facilitate the polymerization of the resins. The results of our study showed that, either of the extracts of Salvadora persica extract had better antimicrobial efficacy and ultrasonic activation resulted in enhanced antimicrobial efficacy of both. So using an alcoholic preparation of Salvadora persica combined with Sodium hypochlorite clinically may not only provide antimicrobial efficacy, but also have proven smear layer removing ability and pulp tissue dissolution property. In addition it is of interest for further research to check if the usage of this experimental combination have any effect on bond strength of resin sealers and cements to the intraradicular dentin.

CONCLUSION

Within the limitations of this study, it can be concluded that:

- Both alcoholic and aqueous extracts of Salvadora persica in combination with NaOCl had antimicrobial activity against E. Faecalis.
- Alcoholic extract of Salvadora persica in combination with NaOCl had slightly better antimicrobial efficacy than aqueous extract. But this was not statistically significant. ($p > 0.05$)
- Ultrasonic activation resulted in better reduction of CFU's compared to non-activated groups for both aqueous and alcoholic extracts.
- There was statistically significant difference in CFU's between ultrasonically activated and non-activation groups of aqueous extract ($p<0.05$), but not for alcoholic extract.
- With both solvents, ultrasonic activation resulted in better reduction of CFU's. This was similar in both alcoholic and aqueous extract and was not statistically significant.

In current era of single visit endodontics, the irrigation protocols involving multiple irrigants and applications can not only increase the chair side time but also can result in inadequate disinfection. So, this experimental combination of NaOCl and Salvadora persica can overcome these drawbacks being a single irrigant. Alcoholic extract performed comparatively better antimicrobial efficacy than aqueous extract.The effect of this alcoholic extract on the bonding efficacy of resin sealers and cements may be of interest for further research.

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