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ORIGINAL ARTICLE

Formulation of a root canal disinfectant solution based on essential oils of *Origanum vulgare* and *Rosmarinus officinalis* against enterococcus faecalis

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ABSTRACT

Background: *Enterococcus faecalis* is frequently associated with persistent endodontic infections and may resist conventional canal disinfection strategies. Essential oils have been proposed as natural antimicrobial candidates, but their interpretation must remain cautious until clinical comparators and safety data are available. **Objective:** This in vitro study evaluated the antibacterial activity of *Origanum vulgare* and *Rosmarinus officinalis* essential oils, alone and in combination, against a clinical isolate of *Enterococcus faecalis*, and assessed preliminary formulations intended for root canal disinfection research. **Materials and methods:** Essential oils were obtained by hydrodistillation from 300 g of each plant material. *Enterococcus faecalis* was isolated from an intracanal clinical sample collected from a tooth presenting odontogenic cellulitis and was identified by culture characteristics, Gram staining, catalase testing and API 20 STREP biochemical identification. Antibacterial activity was assessed by agar disk diffusion and broth microdilution. The inoculum was standardized to 0.5 McFarland. Vancomycin was used as an analytical positive control, whereas DMSO and/or Solubol served as negative controls. The interaction between both oils was interpreted using the fractional inhibitory concentration index (FICI). **Results:** *Origanum vulgare* showed greater antibacterial activity than *Rosmarinus officinalis*. The inhibition zone diameter was 30 mm for *Origanum vulgare* and 8 mm for *Rosmarinus officinalis*. MIC values were 2.78 mg/mL for *Origanum vulgare* and 11 mg/mL for *Rosmarinus officinalis*. The association of the two oils produced a FICI of 1.25, interpreted as an indifferent effect rather than synergy. *Origanum vulgare* formulated solutions showed inhibition diameters of 9-11 mm for the most concentrated formulations and MIC values ranging from 48 to 195 microL/mL; *Rosmarinus officinalis* formulated solutions did not show relevant antibacterial activity. **Conclusion***: Origanum vulgare* essential oil demonstrated promising in vitro activity against the tested clinical isolate of *Enterococcus faecalis*. However, the findings do not justify claiming replacement of sodium hypochlorite or chlorhexidine. The formulation should be presented as a preliminary candidate requiring chemical characterization, replicate-based statistical analysis, direct comparison with NaOCl and CHX, cytotoxicity testing, dentin/biofilm models and in vivo validation.

Keywords: *Origanum vulgare, Rosmarinus officinalis*, essential oils, *Enterococcus faecalis*, root canal disinfection, MIC, FICI.

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1. INTRODUCTION

Endodontic infection is a polymicrobial process involving microorganisms capable of colonizing the root canal system and maintaining periapical inflammation (1,2). Enterococcus faecalis is frequently discussed in persistent or recurrent endodontic infection because it may survive in nutrient-limited conditions, tolerate environmental stress and adhere to dentinal structures (1,2,9).

Sodium hypochlorite (NaOCl) and chlorhexidine (CHX) remain major endodontic disinfectants because of their antimicrobial properties (3,13). NaOCl is also valued for tissue dissolution (13). Nevertheless, these agents may present limitations, particularly tissue irritation, cytotoxicity at high concentrations, unpleasant clinical handling and incomplete activity in complex anatomical areas or biofilm conditions (3,13). These limitations justify the exploratory evaluation of alternative or adjunctive antimicrobial formulations (14,17).

Essential oils are volatile plant-derived products containing bioactive compounds with potential antimicrobial effects (7,8,18). *Origanum vulgare* is commonly associated with phenolic constituents such as carvacrol and thymol (7,10), whereas Rosmarinus officinalis contains compounds whose antimicrobial activity may vary according to chemotype, extraction conditions and assay design (5,12).

The aim of this study was to evaluate, under in vitro conditions, the activity of *Origanum vulgare* and *Rosmarinus officinalis* essential oils against *Enterococcus faecalis* isolated from an intracanal clinical sample, and to assess whether their individual or combined use could support the formulation of a candidate root canal disinfectant solution.

2. MATERIALS AND METHODS

Study design and ethical considerations

This was an exploratory in vitro laboratory study conducted in the biochemistry laboratory of the Faculty of Natural and Life Sciences, the LASNABIO laboratory of Abou Bekr Belkaid University of Tlemcen, and the Microbiology Department of CHU-Tlemcen. The tested bacterial material was an anonymized clinical Enterococcus faecalis isolate obtained from an intracanal sample collected during the management of a tooth presenting odontogenic cellulitis. No patient-identifying data are reported in the manuscript. The sampling procedure was performed under aseptic clinical conditions and the isolate was used for laboratory antimicrobial testing. Because the work used an anonymized bacterial isolate and did not report identifiable personal data, the study was handled as a non-interventional laboratory investigation; local institutional requirements for clinical sampling and confidentiality were respected.

Plant material and extraction of essential oils

Dried plant material of *Origanum vulgare* and *Rosmarinus officinalis* was obtained from a local herbalist in the Tlemcen market. For each plant, 300 g of material was subjected to hydrodistillation for 4 h using a Clevenger-type apparatus. The recovered essential oils were separated from the aqueous phase, transferred to tightly closed containers and preserved until testing. Extraction yield was calculated as: yield (%) = mass of extracted essential oil / mass of plant material x 100.

Physicochemical and organoleptic characterization

The appearance, odour and colour of each essential oil were recorded. Density was determined according to the principle of the ratio between the mass density of the essential oil and that of water at the same temperature. The refractive index was measured with a refractometer and adjusted to 20 degrees C when necessary.

Source, isolation and identification of Enterococcus faecalis

The bacterial strain was isolated from an intracanal sample collected from a tooth presenting odontogenic cellulitis. After tooth isolation and access cavity preparation, the intracanal sample was collected with a sterile K-file No. 10 without prior use of a disinfectant solution and under aseptic conditions. The sample was placed in a sterile vial containing 1 mL of physiological saline and rapidly transported to the Microbiology Department of CHU-Tlemcen for bacteriological analysis.

The sample was inoculated onto fresh blood agar, cooked blood agar, CHROMagar medium and a selective medium for Gram-negative bacilli; another portion was placed in buffered glucose broth for enrichment. Cultures were incubated at 37 degrees C for 24 h. Identification was based on macroscopic colony morphology, Gram staining, catalase testing and API 20 STREP biochemical identification. Before antimicrobial testing, the isolate was subcultured on nutrient agar and conserved at 4 degrees C.

Disk diffusion assay

Antibacterial activity was first assessed by the agar disk diffusion method on Mueller-Hinton agar, as commonly used for in vitro screening of antimicrobial activity (5,14). A bacterial suspension equivalent to 0.5 McFarland was prepared with a densitometer and

used to inoculate the agar surface. Sterile 6-mm Whatman paper disks were impregnated with *Origanum vulgare* essential oil, *Rosmarinus officinalis* essential oil, formulated solutions or controls. Vancomycin was used as an analytical positive control. DMSO and/or Solubol without essential oil served as negative controls. Plates were incubated for 24 h at 37 degrees C. Inhibition zone diameters were measured in millimetres; inhibition was considered relevant when the diameter exceeded the 6-mm disk diameter.

Broth microdilution and MIC determination

MIC values were determined using a 96-well microplate broth microdilution procedure. Serial two-fold dilutions were prepared in Mueller-Hinton broth. For essential oils, 25 microL of the tested solution were added to the first well and serially diluted; 70 microL of Mueller-Hinton broth and 5 microL of the bacterial suspension standardized to 0.5 McFarland were then added to each well. For the mixture assay, 12.5 microL from each essential oil stock solution were added before two-fold dilution. For formulated solutions, 25 microL of each preparation were added to the first well and serially diluted under the same principle. Growth control, solvent control and positive control wells were included. After incubation at 37 degrees C for 24 h, MIC was defined as the lowest concentration without visible microbial growth.

Combination assay and FICI interpretation

The interaction between *Origanum vulgare* and *Rosmarinus officinalis* essential oils was evaluated using the fractional inhibitory concentration index (FICI): FICI = FIC_Origanum + FIC_Rosmarinus, where FIC for each oil corresponds to the MIC of the oil in combination divided by the MIC of the same oil used alone. The interaction was interpreted as synergistic when FICI <= 0.5, additive when 0.5 < FICI <= 1, indifferent when 1 < FICI < 2 and antagonistic when FICI >= 2. In the present dataset, the FICI was 1.25 and was therefore interpreted as an indifferent interaction.

Preparation of disinfectant solutions

Because essential oils are hydrophobic, formulated solutions were prepared with Solubol as solubilizing agent according to the following ratio: 1 volume of essential oil + 4 volumes of Solubol, completed to 20 volumes with distilled water. Six *Origanum vulgare* formulations and five *Rosmarinus officinalis* formulations of 2.5 mL were prepared at different concentrations and then tested by disk diffusion and microdilution.

Statistical analysis and reproducibility statement

Data were tabulated descriptively using Microsoft Excel. The outcomes consisted of endpoint inhibition zone diameters and MIC values. Because the available experimental record did not include sufficient independent replicate-level numerical data for inferential comparison, no paramet ric or non-parametric statistical hypothesis test was performed. Results are therefore reported descriptively. This point was explicitly retained as a methodological limitation, and the conclusions were restricted to preliminary in vitro interpretation.

3. RESULTS

The experimental findings are summarized in two selected figures and four consolidated tables, presenting the source and identification of the clinical isolate, essential oil characterization, antibacterial activity, FICI interpretation and formulated solution results.

Table 1. Source of the clinical isolate, identification and experimental controls.

Component	Revised information reported in the manuscript
Source of bacterial strain	Clinical *Enterococcus faecalis* isolate obtained from an intracanal sample collected from a tooth presenting odontogenic cellulitis.
Sampling conditions	Tooth isolation and pulp access; sterile K-file No. 10; no disinfectant solution before sampling; aseptic conditions.
Transport	Sterile vial containing 1 mL physiological saline; rapid transfer to Microbiology Department, CHU-Tlemcen.
Culture and identification	Fresh blood agar, cooked blood agar, CHROMagar, selective medium and enrichment broth; incubation at 37 degrees C for 24 h; Gram-positive cocci, catalase-negative profile and API 20 STREP identification.
Inoculum standardization	0.5 McFarland using a densitometer.
Controls	Vancomycin as analytical positive control; DMSO and/or Solubol as solvent negative controls; growth control and sterility/medium control in microdilution.

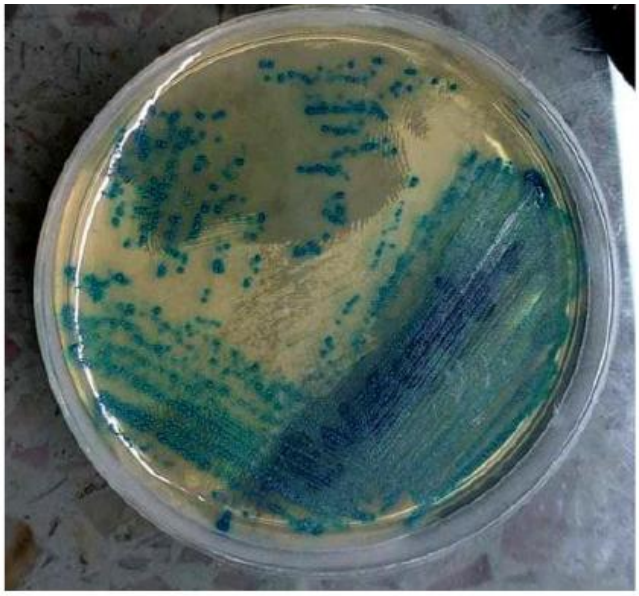

Figure 1. Colonies of Enterococcus faecalis on chromogenic medium after clinical intracanal sampling.

Table 2. Extraction yield and physicochemical characteristics of the essential oils.

Essential oil	Plant mass (g)	Extracted oil mass (g)	Yield (%)	Density	Refractive index	Organoleptic characteristics
Origanum vulgare	300	5.5952	1.86	0.89	1.464	Clear yellow, limpid liquid, strong aromatic odour
Rosmarinus officinalis	300	2.6346	0.87	0.88	1.498	Yellow oily liquid, strong odour

Table 3. Antibacterial activity of the essential oils against Enterococcus faecalis.

Agent	Disk diffusion inhibition zone (mm)	MIC	Interpretation
Origanum vulgare essential oil	30	2.78 mg/mL	Marked in vitro antibacterial activity.
Rosmarinus officinalis essential oil	8	11 mg/mL	Weak activity compared with Origanum vulgare.
Combination of both oils	Not applicable	FICI = 1.25	Indifferent interaction; no demonstrated synergy.
Vancomycin	18	0.02	Analytical positive control; not an endodontic irrigant comparator.
DMSO negative control	<=6	Not applicable	No relevant inhibition.

Note. Pure essential oil MICs are reported in mg/mL. Formulated solutions are reported separately in µL/mL. Units were separated to avoid the mg/mL versus microL/mL confusion noted by the reviewer.

Table 4. Antibacterial activity of formulated disinfectant solutions.

Formulation	Disk diffusion result	MIC result	Interpretation
Origanum vulgare solution 1	<=6 mm	Resistant/no MIC detected	No relevant activity.
Origanum vulgare solution 2	<=6 mm	Resistant/no MIC detected	No relevant activity.
Origanum vulgare solution 3	<=6 mm	Resistant/no MIC detected	No relevant activity.
Origanum vulgare solution 4	9 mm	48 *µL/mL*	Active formulation.
Origanum vulgare solution 5	10 mm	97 *µL/mL*	Active formulation.
Origanum vulgare solution 6	11 mm	195 *µL/mL*	Active formulation.
Rosmarinus officinalis solutions 1-5	<=6 mm	Resistant/no relevant MIC detected	No relevant activity under tested conditions.
Solubol control	<=6 mm	Resistant/no relevant MIC detected	Negative control.
Vancomycin solution	16 mm	0.02	Analytical positive control.

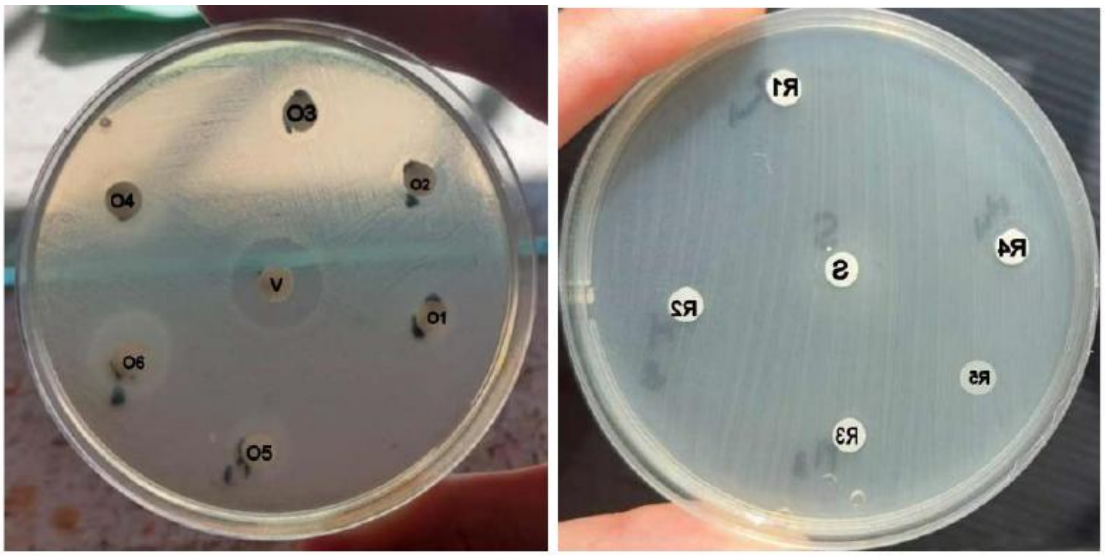

Figure 2. Representative disk diffusion assay of formulated essential-oil solutions against Enterococcus faecalis. Left: Origanum vulgare solutions; right: Rosmarinus officinalis solutions. Note. µL/mL is retained only for volumetric formulated solutions. It is not used interchangeably with mg/mL.

4. DISCUSSION

Principal findings

The present in vitro study showed that *Origanum vulgare* essential oil had a stronger antibacterial effect against the tested clinical isolate of *Enterococcus faecalis* than *Rosmarinus officinalis*. This finding is consistent with previous reports describing antimicrobial activity of *Origanum vulgare* preparations against oral and Gram-positive bacteria (10,16). This was observed both in the disk diffusion assay, where Origanum vulgare produced a 30-mm inhibition zone compared with 8 mm for *Rosmarinus officinalis*, and in MIC testing, where *Origanum vulgare* showed a lower MIC value.

Interpretation of the essential oil activity

The stronger activity of *Origanum vulgare* may be related to its phenolic constituents, particularly carvacrol and thymol, which are frequently associated with disruption of bacterial membrane integrity (7,10,18). In contrast, *Rosmarinus officinalis* showed limited activity in the present assay and should not be presented as equivalent to *Origanum vulgare* under the tested conditions (5,12).

Interpretation of the combination result

The previous manuscript created ambiguity by reporting an improvement in the combined MIC while concluding that there was no synergistic effect. The revised version clarifies this point by using the FICI interpretation, which is commonly used to assess interactions between antimicrobial agents or natural products (6,16). The FICI value was 1.25, which corresponds to an indifferent interaction. Therefore, the combination cannot be described as synergistic (6,16). This supports the decision not to retain the mixture as the main disinfectant formulation and to focus interpretation on Origanum vulgare.

Comparison with conventional disinfectants

Vancomycin was used only as an analytical positive control to confirm that the assay could detect inhibition of the tested Enterococcus faecalis isolate. It is not a conventional endodontic irrigant and does not replace comparison with clinically relevant agents (3,13). Therefore, the absence of direct NaOCl and CHX testing was added as a limitation. The present study does not demonstrate superiority over NaOCl or CHX and cannot support a definitive clinical substitution claim (3,13,17).

Limitations of the study

This study has several limitations. First, it was a preliminary in vitro investigation based on a clinical isolate and did not include dentin blocks, mature biofilm models, organic load simulation, tissue dissolution testing or in vivo conditions (17). Second, NaOCl and CHX were not included as direct clinical comparators, which limits clinical interpretation (3,13). Third, the chemical composition of the essential oils was not characterized by gas chromatography-mass spectrometry, although biological activity may vary according to chemotype, harvesting conditions and extraction parameters (7,12). Fourth, the available experimental record did not provide sufficient independent replicate-level data to support inferential statistics; therefore, results were interpreted descriptively. Fifth, cytotoxicity, tissue compatibility and interaction with dentin were not assessed. These limitations justify presenting *Origanum vulgare* as a promising candidate for further investigation rather than as a proven replacement for conventional root canal irrigants (17).

5. CONCLUSION

Origanum vulgare essential oil demonstrated notable in vitro antibacterial activity against a clinical isolate of Enterococcus faecalis obtained from an intracanal sample. *Rosmarinus officinalis* showed weaker activity, and the combination of the two oils showed an indifferent interaction according to FICI analysis (6,16). Formulated Origanum vulgare solutions showed antibacterial activity only at the most concentrated preparations. The revised interpretation supports further investigation of *Origanum vulgare*-based formulations as potential adjuncts in endodontic disinfection research. However, chemical characterization, standardized replicate-based testing, direct comparison with NaOCl and CHX, biofilm/dentin models, cytotoxicity assessment and in vivo studies are required before clinical recommendation (3,13,17).

Declarations

Ethics approval and consent to participate: The study used an anonymized clinical bacterial isolate obtained from an intracanal sample collected during clinical management of a tooth presenting odontogenic cellulitis. No patient-identifying information is reported. The work was conducted according to institutional clinical and laboratory procedures and confidentiality requirements.

Conflict of interest: The authors declare that they have no conflicts of interest related to this work.

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Data availability: The raw laboratory data supporting the findings are available from the corresponding author upon reasonable request.

Author contribution: All authors contributed to the conception, laboratory work, interpretation or drafting/revision of the manuscript and approved the final version for submission.

Competing interests: The authors declare that they have no competing interest.

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