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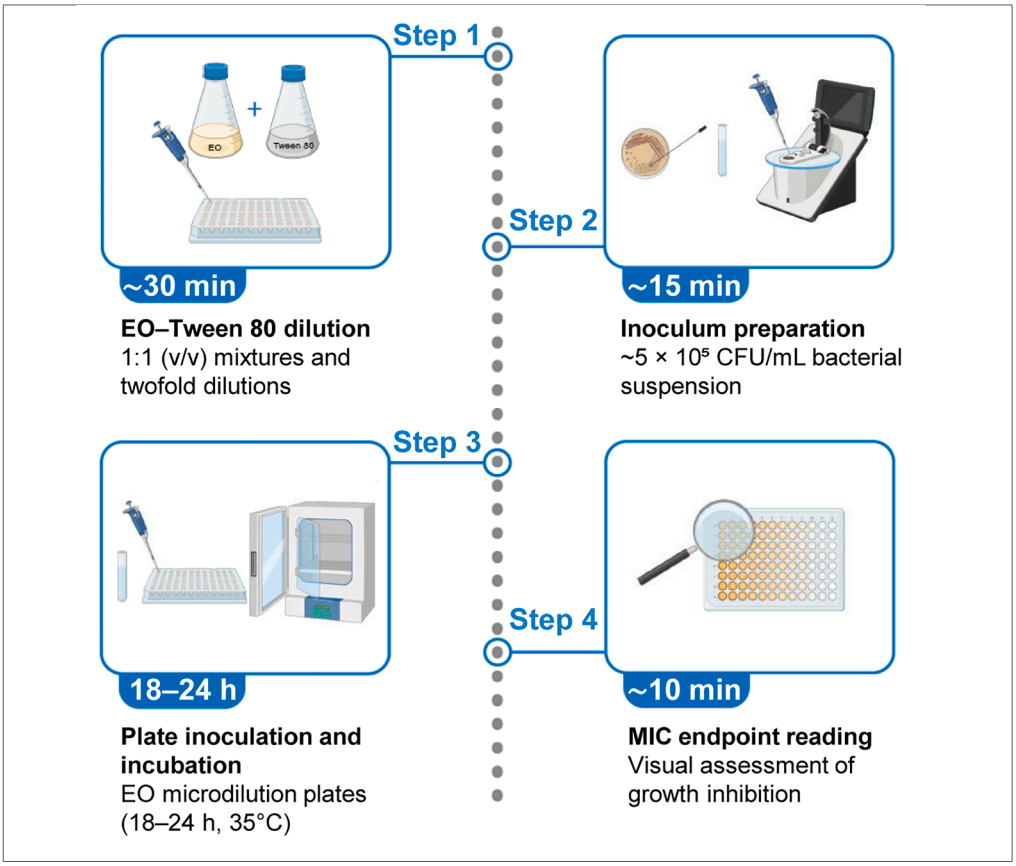
Protocol for determining minimum inhibitory concentrations of essential oils against bacterial pathogens using broth microdilution



Essential oils (EOs) are widely investigated for antimicrobial activity, yet minimum inhibitory concentrations (MICs) lack cross-study comparability due to the absence of a harmonized reference assay. Here, we present a protocol to determine MICs of EOs against aerobic bacterial pathogens using a broth microdilution technique. We describe steps for incorporating EO dispersion using Tween 80 and preparing a standardized bacterial inoculum. We detail procedures for establishing two-fold dilution series in 96-well plates and visually determining the MIC endpoint.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps for EUCAST-aligned broth microdilution for EO MIC determination

Instructions for MIC-based categorization of EO activity

Instructions for automated EO microdilution plate preparation and inoculation

Procedures for preparing pre-loaded EO microdilution plates for multicenter use

Di Vito et al., STAR Protocols 7, 104587
June 19, 2026
https://doi.org/10.1016/j.xpro.2026.104587

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Protocol for determining minimum inhibitory concentrations of essential oils against bacterial pathogens using broth microdilution

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https://doi.org/10.1016/j.xpro.2026.104587

SUMMARY

Essential oils (EOs) are widely investigated for antimicrobial activity, yet minimum inhibitory concentrations (MICs) lack cross-study comparability due to the absence of a harmonized reference assay. Here, we present a protocol to determine MICs of EOs against aerobic bacterial pathogens using a broth microdilution technique. We describe steps for incorporating EO dispersion using Tween 80 and preparing a standardized bacterial inoculum. We detail procedures for establishing two-fold dilution series in 96-well plates and visually determining the MIC endpoint.

BEFORE YOU BEGIN

Essential oils (EOs) are complex mixtures of volatile and hydrophobic plant-derived compounds widely investigated for antimicrobial activity.[1–3] Although chemical quality and specification ranges for many EOs are defined by ISO and European Pharmacopoeia monographs,[4–6] no harmonized microbiological reference framework exists for determining their antimicrobial activity. In contrast to conventional antibiotics—whose minimum inhibitory concentrations (MICs) are determined using standardized broth microdilution procedures aligned with ISO and implemented by organizations such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[7–9]—EO susceptibility testing remains highly heterogeneous.

Published in vitro assays differ in vehicle selection, dispersion strategy, inoculum preparation, incubation conditions, endpoint determination, and reporting units.[10–12] Combined with the intrinsic hydrophobicity, volatility, and optical interference of EOs, this variability leads to poor inter-laboratory comparability of MIC values and limits translational and regulatory integration.[13]

The protocol described below addresses these methodological inconsistencies by adapting the ISO/EUCAST broth microdilution framework to the specific physicochemical constraints of EOs. The protocol was developed and validated using fourteen pharmacopoeia- or ISO-listed essential oils,[14–27] which were tested against five ATCC reference strains and 250 clinical isolates

representing five clinically relevant bacterial species. The approach can be extended to additional EOs and aerobic bacterial pathogens following the same framework.

Innovation

This protocol introduces an EUCAST-aligned broth microdilution framework specifically adapted for EOs. The innovation lies not in the assay principle itself, but in the systematic integration of EO-specific physicochemical considerations into a standardized antimicrobial susceptibility testing workflow.

EO dispersion is standardized using polysorbate 80 (Tween 80) at experimentally validated non-toxic concentrations, with a fixed 1:1 EO-to-vehicle ratio to optimize micellar dispersion while minimizing optical interference. EO concentration ranges are predefined (typically 2.5–80 μL/mL) to balance antimicrobial activity detection with assay performance and endpoint readability. The protocol also incorporates standardized bacterial inoculum preparation aligned with ISO/EUCAST recommendations for aerobic bacteria.

In addition, defined stability criteria are established for frozen, pre-loaded EO microdilution plates, using an equivalence margin of ±1 twofold dilution relative to freshly prepared plates. Together, these elements provide a reproducible and harmonized structure for EO MIC determination and support inter-laboratory comparability and multicenter evaluation.

Conceptual considerations

EOs are immiscible in aqueous media and prone to phase separation, volatilization, and oxidative degradation. When directly added to broth, they may form unstable emulsions, surface films, or droplets that interfere with visual MIC interpretation. Surfactant-mediated dispersion (using the vehicle Tween 80) is therefore required to achieve homogeneous distribution without inhibiting bacterial growth.

Volatility and oxidative instability necessitate immediate sealing of microdilution plates with gas-impermeable adhesive films to minimize evaporation and prevent cross-contamination between wells. Because EO turbidity may mimic bacterial growth, visual endpoint interpretation requires comparison with uninoculated vehicle controls to distinguish microbial sedimentation from optical artifacts.

Institutional permissions

Clinical bacterial isolates used in this study were obtained from a local institutional biobank and handled in accordance with institutional guidelines and biosafety regulations. No identifiable human data or direct patient involvement was included in this protocol.

Bacterial inoculum preparation

Timing: ~1–2 h (excluding culture incubation, 18–24 h)

Use reference strains and clinical isolates maintained according to standard microbiological procedures. For method development and quality control, the following ATCC strains are recommended: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29211, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 700603.

1. Subculture bacterial strains/isolates on appropriate non-selective agar and incubate under aerobic conditions (18–24 h).
2. Select well-isolated bacterial colonies and suspend them in sterile saline.
3. Adjust turbidity to 0.5 McFarland using a McFarland standard or a calibrated densitometer or spectrophotometer.

4. Dilute the 0.5 McFarland suspension 1:100 in cation-adjusted Mueller–Hinton broth (CAMHB) to obtain a working suspension that yields approximately 5×10^5 CFU/mL in the test wells, in accordance with EUCAST recommendations.
5. Perform periodic viable counts by plating the 0.5 McFarland suspension and its 1:100 dilution to confirm inoculum accuracy (target range: 20–80 colonies per plate after incubation).

Note: Use suspensions within 15–30 min of preparation to minimize variations in cell density.

Essential oil stock and dispersion preparation

Timing: ~30–60 min

Use EOs of certified origin compliant with ISO standards or European Pharmacopoeia monographs. In this study, oils were obtained from a single commercial supplier (Pranarôm International S.A., Ghislenghien, Belgium) to ensure batch consistency. Chemical specifications, GC–MS profiles, and lot numbers for the oils used are provided in Table S1. Record batch number, declared density, and storage conditions for traceability.

6. Equilibrate essential oils to 20°C–25°C before use and mix gently to ensure homogeneity.
7. If mass-based reporting or comparison with conventional antimicrobials is required, determine EO density from supplier documentation or by gravimetric measurement.
8. Prepare a 1:1 (v/v) mixture of EO and polysorbate 80 (Tween 80) prior to dilution in cation-adjusted Mueller–Hinton broth (CAMHB).
9. Vortex thoroughly until a visually uniform dispersion is obtained.
10. Minimize exposure to light and air during handling and avoid repeated freeze–thaw cycles.

Note: The final concentration of Tween 80 in test plate wells should not exceed 80 μL/mL to avoid vehicle-associated growth inhibition.

Note: Confirm vehicle compatibility by including growth controls containing Tween 80 without EO before proceeding to plate setup.

Note: Prepare EO–Tween 80 mixtures immediately before dilution unless frozen pre-loaded EO plates are used.

Plate setup and optional pre-loading

Timing: ~45 min

Use sterile, flat-bottom 96-well microtiter plates suitable for broth microdilution.

11. Assign each EO to a dedicated twofold dilution series across one row or column of the plate.
12. Using the EO–Tween 80 mixture prepared above, perform twofold serial dilutions in cation-adjusted Mueller–Hinton broth (CAMHB) to obtain final EO concentrations typically ranging from 2.5 to 80 μL/mL, unless oil-specific dilution ranges have been predefined.
13. Include the following controls on each plate:
    a. Growth control (CAMHB + inoculum, no EO).
    b. Vehicle control (CAMHB + Tween 80 + inoculum, no EO).
    c. Sterility control (CAMHB only).
14. Seal plates immediately after loading using gas-impermeable adhesive films.

⚠ CRITICAL: Proper sealing is required to minimize EO volatilization and prevent cross-well contamination during incubation or storage.

15. For optional pre-loading, store sealed plates at −20°C until use.

Note: Validate EO stability prior to extended storage.

Note: MIC values obtained from frozen plates should remain within ±1 twofold dilution compared to freshly prepared plates. Oils exhibiting larger MIC shifts after storage should be tested using freshly prepared plates.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Staphylococcus aureus	ATCC	ATCC 29213
Enterococcus faecalis	ATCC	ATCC 29211
Escherichia coli	ATCC	ATCC 25922
Pseudomonas aeruginosa	ATCC	ATCC 27853
Klebsiella pneumoniae	ATCC	ATCC 700603
Clinical bacterial isolates (5 species; n = 250)	Local institutional biobank	See Table S3
Chemicals, peptides, and recombinant proteins		
Essential oils (14 types; see Table S1)	Pranarôm International	Lot numbers listed in Table S1
Polysorbate 80 (Tween 80)	Merck KGaA	P8074-500ML
Dimethyl sulfoxide (DMSO)	Merck KGaA	D2650-100ML
Cation-adjusted Mueller–Hinton broth (CAMHB)	Merck KGaA	90922-500G
Mueller–Hinton agar (MHA)	Merck KGaA	70191-500G
Physiological saline (0.9% NaCl)	bioMérieux	423719
Absolute ethanol	PanReac AppliChem	131086.1212
Critical commercial assays		
McFarland Standard 0.5	bioMérieux	423719
Software and algorithms		
epMotion 5070 MultiCon software	Eppendorf	5070000282
Other		
Densitometer	bioMérieux	21250
Vortex mixer	Scientific Industries	SI-0236
Gas-impermeable sealing film	Corning	UC-500
96-well U-bottom microplates	Corning	3599
epMotion 5075 Liquid Handling Workstation	Eppendorf	Model 5075
epT.I.P.S Motion Pipette Filter Tips (50 µL)	Eppendorf	0030015215
Sterile disposable pipette tips (10 µL)	Sial	SIAL-UTF10-10
Sterile disposable pipette tips (200 µL)	Sial	SIAL-UTF200-10
Sterile disposable pipette tips (1000 µL)	Sial	SIAL-UTF1000-10
Sterile 1.5 mL microcentrifuge tubes	Eppendorf	0030120086
Sterile 15 mL conical tubes	Corning	352096
Collection swab (cotton swab + wooden stick)	Wellkang Ltd	2122–0004
CO_2-free incubator	Thermo Fisher	50145516

MATERIALS AND EQUIPMENT

Essential oil–Tween 80 working solution (1:1, v/v)		
Component	Final concentration (% v/v)	Amount
Essential oil	16	0.24 mL
Tween 80	16	0.24 mL
CAMHB	68	1.02 mL
Total	100	1.50 mL

Note: Prepare solutions immediately before use. Each stock is sufficient to prepare ten 96-well plates.

Vehicle control (Tween 80 in CAMHB)		
Component	Final concentration (% v/v)	Amount
Tween 80	16	0.24 mL
CAMHB	84	1.26 mL
Total	100	1.50 mL

Note: Prepare solutions immediately before use. Each stock is sufficient to prepare ten 96-well plates.

⚠ CRITICAL: Tween 80 (polysorbate 80) may cause irritation upon contact; handle with appropriate personal protective equipment and avoid aerosol formation. Essential oils are volatile and may be irritant or flammable; handle in a well-ventilated area or fume hood and avoid direct contact and inhalation.

STEP-BY-STEP METHOD DETAILS

Optional calibration of turbidity-CFU correlation

Timing: 1 day (including incubation)

This section describes optional calibration of the relationship between bacterial turbidity and viable counts for reference strains.

1. Prepare a 0.5 McFarland suspension from a fresh (≤24 h) culture grown on Mueller–Hinton agar (MHA).
2. Measure optical density (OD_{600}) and record the value.
3. Prepare serial dilutions (1:100, followed by tenfold dilutions as needed).
4. Plate 100 µL of appropriate dilutions in triplicate on MHA and incubate at 35 ± 1°C for 18–24 h.
5. Count colonies on plates containing 100–400 CFU and calculate CFU/mL.
6. Confirm that a 0.5 McFarland suspension corresponds approximately to 1–2 × 10^8 CFU/mL for the strain tested.

Note: This calibration step is recommended during method implementation but is not required for routine EO testing once correlation has been established.

Preparation of bacterial inoculum for EO testing

Timing: 30–40 min (excluding culture incubation, 18–24 h)

This section describes preparation of the standardized inoculum used for essential oil MIC determination.

7. Subculture bacterial isolates on non-selective agar and incubate under aerobic conditions for 18–24 h.
8. Select well-isolated colonies and suspend them in sterile saline.
9. Adjust turbidity to 0.5 McFarland using a McFarland standard or a calibrated densitometer or spectrophotometer.
10. Dilute the 0.5 McFarland suspension 1:100 in cation-adjusted Mueller–Hinton broth (CAMHB).

Note: This results in a working suspension of approximately 1 × 10^6 CFU/mL.

Note: Use the working suspension within 30 min of preparation to prevent variations in cell density.

Preparation of EO 2× working solutions

Timing: ~30 min (for up to 14 EOs)

This section describes the preparation of essential oil (EO) working solutions at twice the highest intended final test concentration.

11. Prepare a 2× working solution of each EO corresponding to the highest final test concentration, using the EO–Tween 80 mixture prepared above and cation-adjusted Mueller–Hinton broth (CAMHB).
12. Label sterile 1.5 mL microcentrifuge tubes for each EO.
13. Prepare sufficient volume of each 2× solution to allow serial dilution across the assigned row or column of the 96-well plate.
14. Mix thoroughly by pipetting and vortex for 5–10 s to ensure homogeneity.

Note: The 2× concentration is required because an equal volume of bacterial inoculum will be added later, resulting in the desired final (1×) EO concentration in each well.

Loading of EO two-fold serial dilution into 96-well plates

Timing: 20–30 min per plate

This section describes preparation of EO dilution series in 96-well microtiter plates.

15. Add 50 μL of sterile CAMHB into each well designated for EO dilutions, except for the starting wells of each dilution series.
16. Dispense 100 μL of the corresponding 2× EO working solution into the designated starting wells.
17. Mix thoroughly by pipetting up and down.
18. Transfer 50 μL from the starting well into the adjacent well containing 50 μL CAMHB and mix.
19. Repeat sequential transfers to generate a twofold serial dilution across the assigned row or column.
20. After mixing the final dilution well, discard 50 μL to maintain equal volumes.

Note: At this stage, each well contains 50 μL of EO dilution at 2× the intended final test concentration.

21. Prepare control wells on the same plate as follows:
    a. Growth control: 50 μL CAMHB.
    b. Vehicle control: 50 μL CAMHB containing Tween 80 at the highest concentration tested.
    c. Sterility control: 100 μL CAMHB.

Note: Do not add bacterial inoculum at this stage.

Inoculation of EO microdilution plates

Timing: 5–10 min per plate

This section describes addition of the bacterial inoculum to plates containing EO dilutions.

22. Add 50 µL of the standardized bacterial suspension to each well containing EO dilutions and to growth and vehicle control wells. Do not inoculate sterility control wells.

Note: This results in a final volume of 100 µL per well, corresponding to a 1:1 mixture of EO dilution and inoculum.

⚠ CRITICAL: Dispense inoculum carefully to avoid splashing or cross-contamination between wells.

23. Seal the plate immediately with a gas-impermeable adhesive film and apply the lid.

⚠ CRITICAL: Proper sealing prevents evaporation and diffusion of volatile EO components.

24. Incubate plates at 35 ± 1°C under aerobic conditions for 18–24 h.

Incubation and EO MIC determination

Timing: 18–24 h incubation; 5–10 min reading

This section describes control validation and EO MIC endpoint reading.

25. Verify control wells after incubation:
    a. Visible growth in growth control wells.
    b. Comparable growth in vehicle control wells.
    c. No growth in sterility control wells.

Note: Repeat the assay if control criteria are not met.

26. Determine the MIC as the lowest EO concentration showing complete inhibition of visible growth.
27. In cases of ambiguous turbidity or film formation, compare with vehicle control wells and apply EUCAST visual reading principles.

Note: Report MIC values in µL/mL.

Optional: Automated preparation and inoculation of EO microdilution plates

If a programmable liquid-handling workstation is available, EO dilution loading and bacterial inoculation may be automated following the volumetric scheme described above and the standardized plate layout shown in Figure 1.

Program the instrument to dispense CAMHB, load 2× EO working solutions into starting wells, perform twofold serial dilutions with mixing cycles, and dispense bacterial inoculum into all designated wells except sterility control wells.

⚠ CRITICAL: Verify dispensing accuracy, tip depth, and mixing efficiency before running the program.

EXPECTED OUTCOMES

This protocol is expected to generate reproducible minimum inhibitory concentration (MIC) values for essential oils (EOs) against both bacterial reference strains and clinical isolates when inoculum preparation, EO dispersion, and plate handling are performed as described.

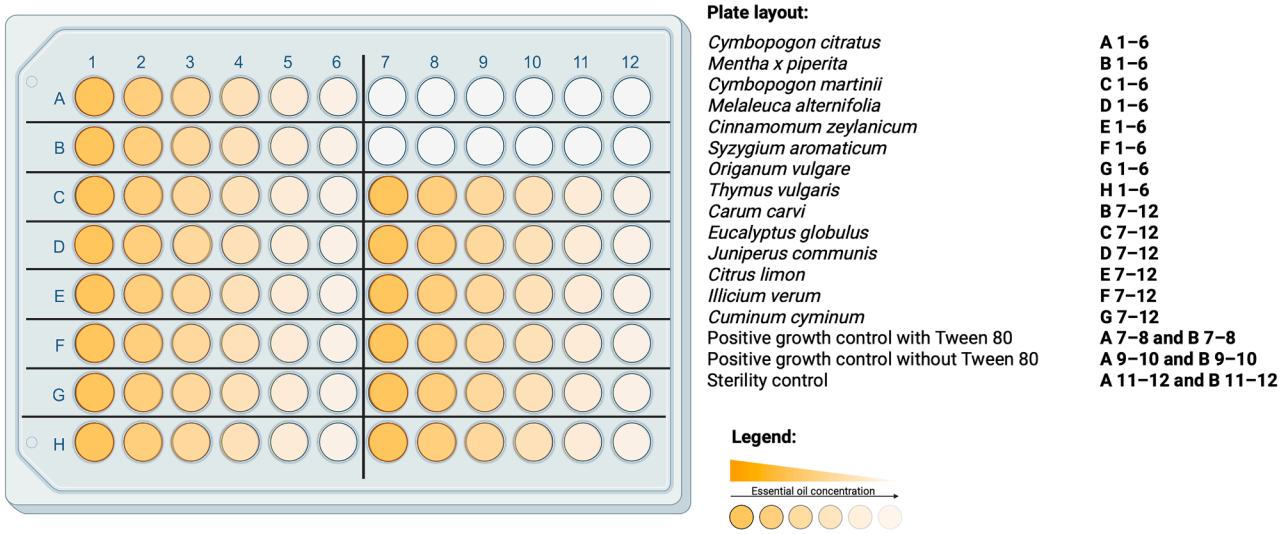

Figure 1. Standardized 96-well plate layout for essential oil MIC determination

Schematic representation of the 96-well plate layout used for essential oil (EO) minimum inhibitory concentration (MIC) testing. Columns 1–6 and 7–12 contain twofold serial dilutions of EOs at decreasing concentrations (yellow gradient). Positive growth controls with Tween 80 are positioned in wells A7–A8 and B7–B8, growth controls without Tween 80 in wells A9–A10 and B9–B10, and sterility controls in wells A11–A12 and B11–B12. This layout supports both manual and automated loading and enables reproducible MIC determination.

Using the standardized procedure, ATCC reference strains should yield a final inoculum of approximately 5×10^5 CFU/mL in each well, consistent with EUCAST recommendations. Viable counts from 0.5 McFarland suspensions and corresponding 1:100 dilutions typically result in 20–80 colonies per plate, with strain-specific CFU/mL values clustering within a narrow range (Table S2). Significant deviations from these values indicate suboptimal inoculum preparation, inaccurate dilution, or medium-related issues and warrant repetition of the assay.

Tween 80 supports homogeneous EO dispersion at final concentrations $\leq$80 μL/mL without inhibiting bacterial growth (Figure 2). In contrast, DMSO exhibits concentration-dependent toxicity at lower levels and may alter apparent MIC values. An EO:Tween 80 ratio of 1:1 (v/v) results in stable dispersions and reproducible MIC values, whereas higher EO:Tween 80 ratios increase phase separation and optical artifacts, leading to broader MIC variability (Figure 3).

Under optimized conditions, MIC values for the fourteen selected EOs fall within EO-specific and species-specific ranges. Highly active EOs, including *Origanum vulgare*, *Thymus vulgaris* CT thymol, and *Cinnamomum zeylanicum*, typically inhibit *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29211 at <5–10 μL/mL, whereas MICs for *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853 are often higher. Between-run variability is generally $\leq$1 twofold dilution; larger deviations suggest pipetting inaccuracies, incomplete EO–Tween 80 mixing, or inadequate plate sealing.

Application of the protocol to 250 clinical isolates representing five species (n = 50 per species) yields well-defined MIC distributions for each EO–species combination (Table S3), with isolates typically clustering into three MIC strata (<5, 5–20, and $\geq$40 μL/mL), enabling practical categorization of EO activity as high, intermediate, or low. For example, *Thymus vulgaris* CT thymol shows high activity against *E. coli* and *K. pneumoniae*, but limited activity against *P. aeruginosa*, whereas *Cinnamomum zeylanicum* demonstrates consistently high activity across species. In some EO–species combinations, MIC distributions are continuous rather than bimodal, suggesting variable susceptibility rather than discrete resistant subpopulations.

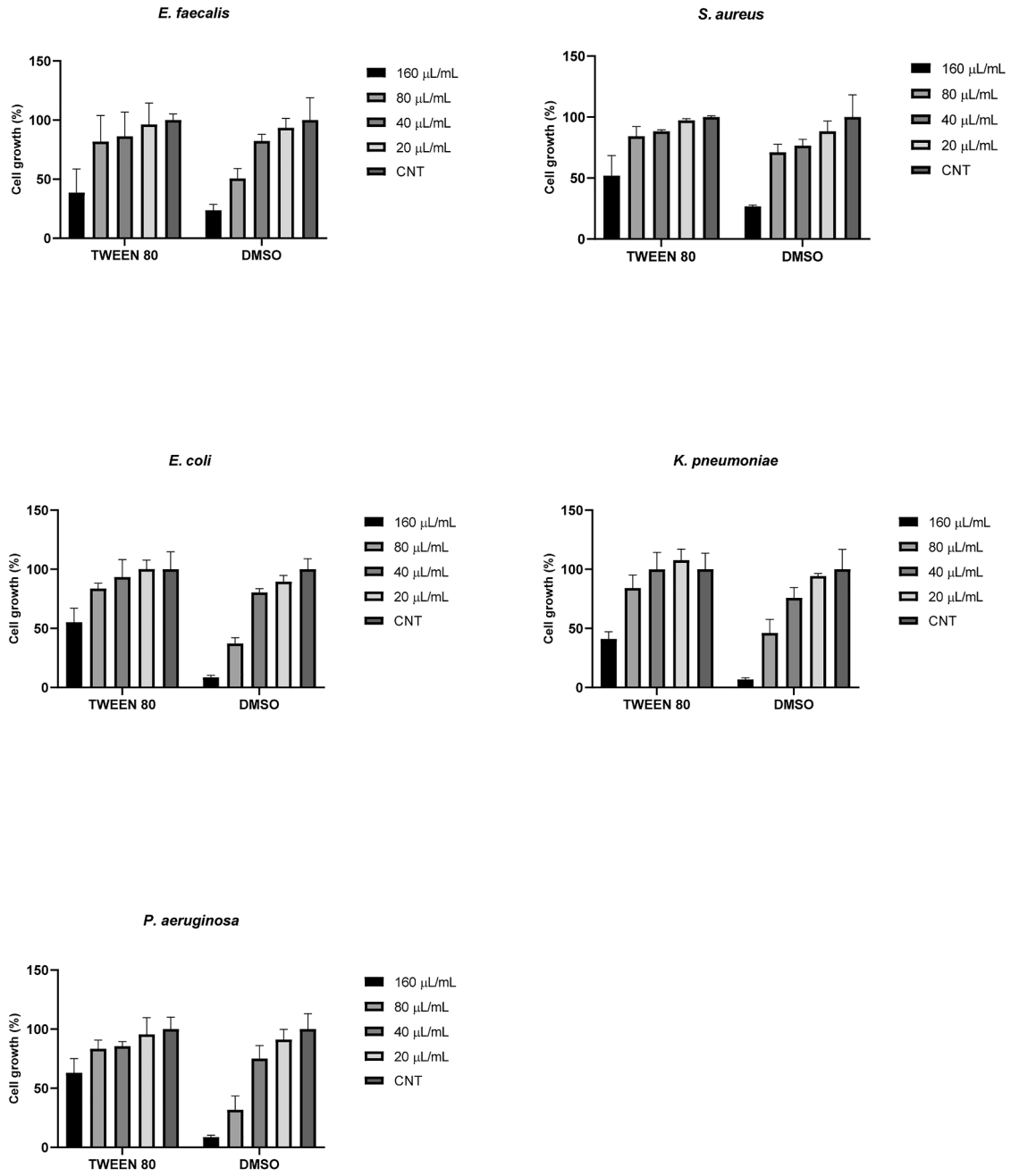

Figure 2. Comparison of vehicle effects of Tween 80 and DMSO on bacterial growth

Percentage growth of five ATCC reference strains exposed to increasing concentrations of Tween 80 or DMSO (20–160 µL/mL). Growth was normalized to the corresponding untreated control (CNT = 100%). Tween 80 maintained growth levels comparable to controls up to 80 µL/mL, whereas DMSO reduced bacterial growth at concentrations >20 µL/mL. Data represent mean ± SD of three independent experiments.

Pre-loaded EO plates stored at −20°C exhibit EO-specific stability profiles. Several oils retain MIC values within ±1 twofold dilution of freshly prepared plates for extended storage periods, supporting multicenter distribution. Other oils display MIC drift after prolonged storage unless vacuum sealing and protection from light are applied. Oils showing greater variability should therefore be tested using freshly prepared plates to avoid artifactual MIC elevation.

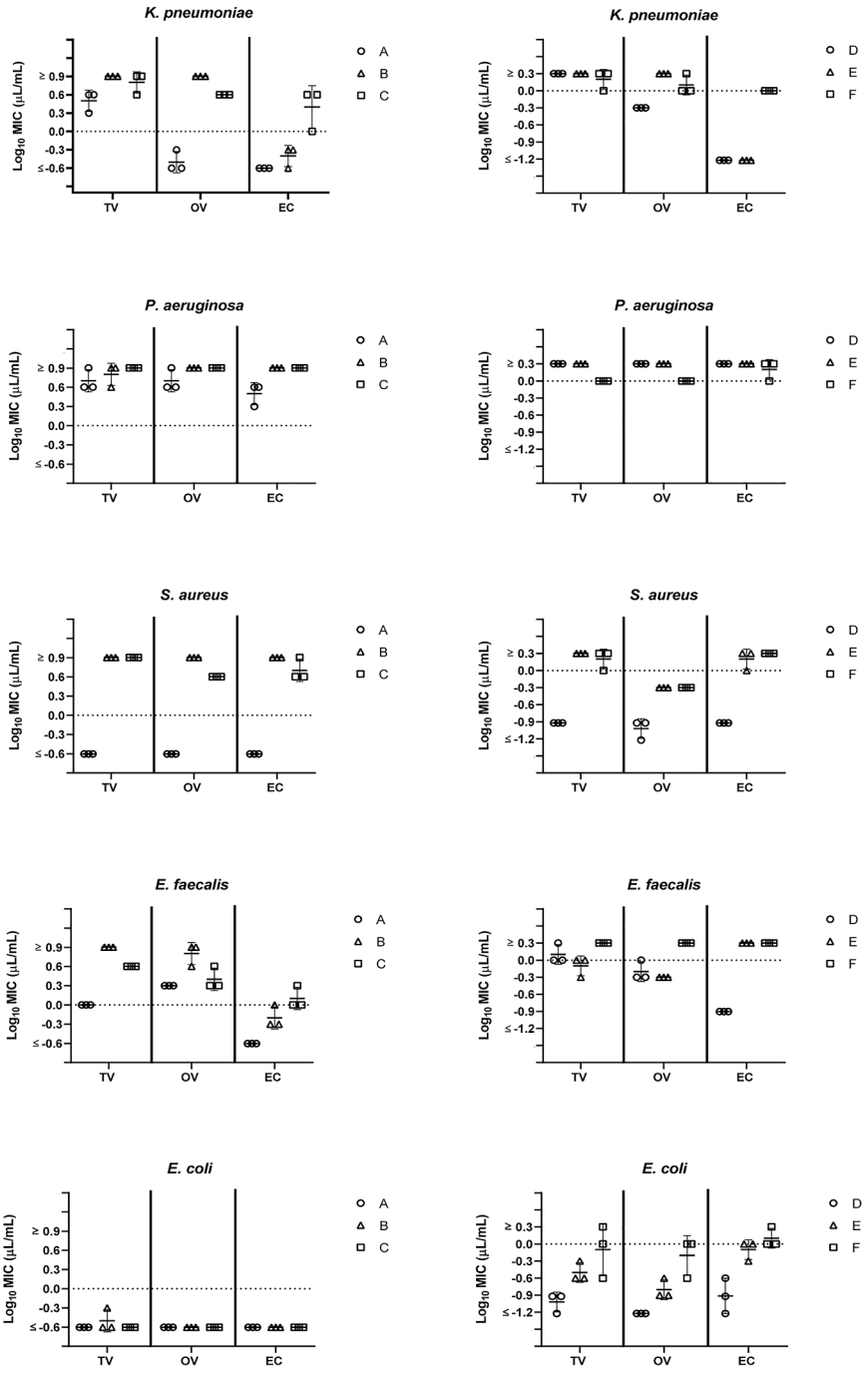

Figure 3. Effect of EO:Tween 80 ratio on MIC determination

MIC values obtained for three reference essential oils (*Thymus vulgaris* CT thymol (TV), *Origanum vulgare* (OV), and *Eugenia caryophyllata* (EC)) using different EO:Tween 80 ratios. Experiments were performed at fixed EO concentrations of 80 µL/mL (left panel) and 20 µL/mL (right panel). In the left panel: A, EO 80 µL/mL + Tween 80 80 µL/mL (1:1); B, EO 80 µL/mL + Tween 80 20 µL/mL (4:1); C, EO 80 µL/mL + Tween 80 10 µL/mL (8:1). In the right panel: D, EO 20 µL/mL + Tween 80 20 µL/mL (1:1); E, EO 20 µL/mL + Tween 80 5 µL/mL (4:1); F, EO 20 µL/mL + Tween 80 2.5 µL/mL (8:1). Symbols represent MIC values from three independent experiments.

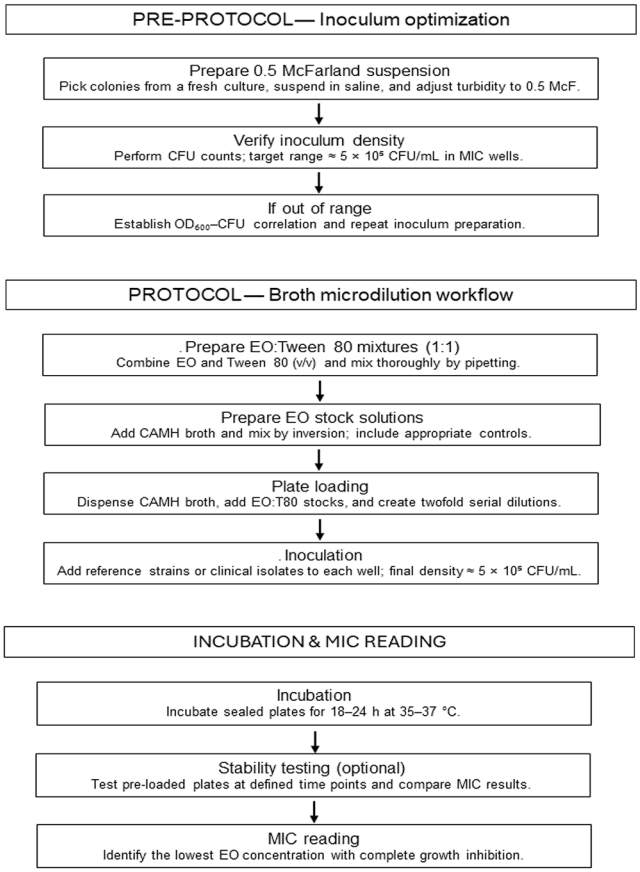

Figure 4. Workflow overview for essential oil MIC determination

Flowchart summarizing inoculum standardization, preparation of EO 2× working solutions, plate loading and inoculation, incubation, MIC determination, and optional stability testing of pre-loaded plates.

Overall, when implemented according to the workflow described, the protocol generates internally consistent MIC datasets suitable for inter-laboratory comparison and provides a framework for future establishment of epidemiological cut-off values (ECOFFs) for selected EOs.

The overall experimental workflow is summarized in Figure 4.

LIMITATIONS

Although this protocol provides a standardized framework for minimum inhibitory concentration (MIC) determination of essential oils (EOs), several limitations should be considered. First, EOs are chemically complex natural mixtures whose composition may vary according to chemotype, geographical origin, extraction method, and storage conditions. Even when compliant with ISO or European Pharmacopoeia specifications, batch-to-batch variability may influence antimicrobial potency and MIC values. Therefore, reproducibility across laboratories depends on the use of well-characterized and traceable EO batches.

Second, the protocol relies on surfactant-mediated dispersion using Tween 80 to achieve homogeneous EO distribution in aqueous broth. Although concentrations $\leq$80 μL/mL do not inhibit bacterial growth under the described conditions, minor variations in surfactant quality or concentration may influence dispersion stability and optical interpretation. In addition, very high EO concentrations may generate turbidity or surface films that complicate visual endpoint interpretation.

Third, the protocol is optimized for aerobic bacterial pathogens tested in cation-adjusted Mueller–Hinton broth (CAMHB) under EUCAST-aligned conditions. It may require adaptation for fastidious organisms, strict anaerobes, or non-standard growth media. Furthermore, because no established clinical breakpoints exist for EOs, MIC values generated using this approach support comparative and epidemiological analyses rather than direct therapeutic decision-making.

Finally, EO volatility and susceptibility to oxidative degradation may affect long-term stability of pre-loaded plates. Although several oils retain stable MIC values during frozen storage, others require strict protection from light and air or extemporaneous preparation to avoid artifactual MIC drift.

TROUBLESHOOTING

Problem 1

Droplets or phase separation are observed in EO stock solutions during preparation (Step 11).

Potential solution

Ensure that reagents are added in the correct order (Tween 80 → EO → CAMHB). Vortex thoroughly after addition and warm Tween 80 at ≤37°C for 2–5 min before pipetting to reduce viscosity. Avoid prolonged delays between mixing and plate loading.

Problem 2

Irregular twofold dilution series or concentration gradients are observed across wells (Steps 15–17).

Potential solution

Verify that pipette tips reach the bottom of each well during mixing. Increase mixing cycles if needed and aspirate/dispense slowly to avoid bubble formation. Ensure consistent pipetting volumes and replace tips between dilution series.

Problem 3

Poor growth is observed in positive control wells (Step 25).

Potential solution

Use fresh (≤24 h) colonies for inoculum preparation and confirm accurate adjustment to 0.5 McFarland. Verify incubation temperature and atmosphere and prepare fresh cation-adjusted Mueller–Hinton broth (CAMHB) if medium quality is uncertain.

Problem 4

Reduced growth occurs in vehicle control wells containing Tween 80 (Step 25).

Potential solution

Confirm that the final concentration of Tween 80 does not exceed validated limits (≤80 μL/mL). Prepare fresh vehicle controls and repeat plate loading if inhibition persists.

Problem 5

Cross-well inhibition or edge effects are observed after incubation (Steps 22–24).

Potential solution

Seal plates immediately after inoculation using a gas-impermeable adhesive film and apply the lid securely. Avoid reopening plates before endpoint reading to prevent diffusion of volatile EO components.

Problem 6

MIC endpoints are ambiguous due to haze or surface film formation (Steps 26–27).

Potential solution

Compare wells carefully with vehicle control wells to distinguish bacterial growth from sedimentation or optical artifacts. Gently tap the plate to redistribute contents and reassess visually. Repeat the assay if interpretation remains uncertain.

Problem 7

MIC values shift by more than one twofold dilution between independent runs (Step 26).

Potential solution

Standardize mixing time, inoculum preparation, and plate sealing procedures across experiments. Minimize EO exposure to air and limit freeze–thaw cycles. Use freshly prepared EO working solutions if variability persists.

Problem 8

Reduced EO activity is observed after frozen storage of pre-loaded plates (Step 23).

Potential solution

Vacuum-seal plates and protect them from light during storage at −20°C. Thaw plates at 20°C–25°C while sealed and proceed promptly with inoculation. For oils showing limited stability, prepare plates extemporaneously.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maurizio Sanguinetti (maurizio.sanguinetti@unicatt.it).

Technical contact

Requests for technical details regarding protocol implementation should be addressed to Maura Di Vito (maura.divito@unicatt.it).

Materials availability

This study did not generate new unique reagents. Essential oils were obtained from a commercial supplier as specified in the key resources table. Bacterial reference strains are available from ATCC. Clinical bacterial isolates are available upon reasonable request and in accordance with institutional and biosafety regulations.

Data and code availability

This study did not generate new datasets or custom code. All data supporting the findings of this protocol are included in the article and its supplemental information.

ACKNOWLEDGMENTS

This work was supported by Pranarôm Italia, which funded the academic research position (RTDA) of M.D.V. The funding body had no role in the study design, data collection, data analysis, data interpretation, or manuscript preparation. The abstract figure was created using BioRender.com.

AUTHOR CONTRIBUTIONS

Conceptualization, M.D.V., A.Z., F.B., M.S., and B.P.; methodology, M.D.V. and M.M.; investigation, M.D.M., D.C., and S.R.; data curation, M.D.V. and F.B.; writing – original draft, B.P.; writing – review and editing, all authors; supervision, M.S.; funding acquisition, M.D.V.

DECLARATION OF INTERESTS

M.D.V. is the recipient of academic funding from Pranarôm Italia. A.Z. is an employee of Pranarôm International S.A.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2026.104587.

REFERENCES

1. Ramsey, J.T., Shropshire, B.C., Nagy, T.R., Chambers, K.D., Li, Y., and Korach, K.S. (2020). Essential oils and health. Yale J. Biol. Med. *93*, 291–305.

2. de Sousa, D.P., Damasceno, R.O.S., Amorati, R., Elshabrawy, H.A., de Castro, R.D., Bezerra, D.P., Nunes, V.R.V., Gomes, R.C., and Lima, T.C. (2023). Essential oils: chemistry and pharmacological activities. Biomolecules *13*, 1144. https://doi.org/10.3390/biom13071144.

3. Khwaza, V., and Aderibigbe, B.A. (2025). Antibacterial activity of selected essential oil components and their derivatives: a review. Antibiotics *14*, 68. https://doi.org/10.3390/antibiotics14010068.

4. European Medicines Agency (2026). Microbiological aspects of herbal medicinal products and traditional herbal medicinal products—Scientific guideline. https://www.ema.europa.eu/en/human-regulatory-overview/research-development/scientific-guidelines/multidisciplinary-guidelines/herbal-medicinal-products-scientific-guidelines.

5. European Medicines Agency (2026). Herbal medicinal products—Human regulatory overview. https://www.ema.europa.eu/en.

6. Committee on Herbal Medicinal Products (HMPC) (2022). Guideline on quality of herbal medicinal products/traditional herbal medicinal products. EMA/HMPC/201116/2005 Rev. 3 (European Medicines Agency. https://www.google.com/url?sa=t&source=web&rct=j&opi=89978449&url=https://www.ema.europa.eu/en/documents/scientific-guideline/final-guideline-quality-herbal-medicinal-productstraditional-herbal-medicinal-products-revision-3_en.pdf&ved=2ahUKEwjPrKz6gb-RAxVdh_0HHbbREJ4QFnoECBkQAQ&usg=AOvVaw3ume3gBuGzxsjWxzRC3d1B.

7. European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2026). MIC determination of non-fastidious and fastidious organisms. https://www.eucast.org/bacteria/methodology-and-instructions/mic-determination/.

8. Clinical and Laboratory Standards Institute (2024). Performance Standards for Antimicrobial Susceptibility Testing, 34th ed. CLSI supplement M100 (CLSI).

9. International Organization for Standardization (2019). Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices—Part 1: Reference Method for the Determination of Minimum Inhibitory Concentration (MIC) (ISO).

10. Chen, S., Li, Z., Chitrakar, B., Gu, Z., Ban, X., Hong, Y., Cheng, L., and Li, C. (2024). Minimum inhibitory concentration (MIC) of essential oils against microorganisms: methods, function, accuracy factors and application. Food Rev. Int. *40*, 3445–3470. https://doi.org/10.1080/87559129.2024.2357612.

11. Hulankova, R. (2024). Methods for determination of antimicrobial activity of essential oils in vitro — a review. Plants *13*, 2784. https://doi.org/10.3390/plants13192784.

12. Vanegas, D., Abril-Novillo, A., Khachatryan, A., Jerves-Andrade, L., Peñaherrera, E., Cuzco, N., Wilches, I., Calle, J., and León-Tamariz, F. (2021). Validation of a method of broth microdilution for the determination of antibacterial activity of essential oils. BMC Res. Notes *14*, 439. https://doi.org/10.1186/s13104-021-05838-8.

13. Van de Vel, E., Sampers, I., and Raes, K. (2019). A review on influencing factors on the minimum inhibitory concentration of essential oils. Crit. Rev. Food Sci. Nutr. *59*, 357–378. https://doi.org/10.1080/10408398.2017.1371112.

14. International Organization for Standardization (2003). *Oil of Cumin Seed* (*Cuminum Cyminum* L.). ISO 9301:2003 (ISO).

15. International Organization for Standardization (1974). *Oil Of Lemongrass* (*Cymbopogon Citratus*). ISO 3217:1974 (ISO).

16. International Organization for Standardization (2021). *Oil of Palmarosa* (*Cymbopogon Martinii* (Roxburgh) W. Watson var. *motia*). ISO 4727:2021 (ISO).

17. International Organization for Standardization (2010). *Oil of Origanum vulgare* L. Subsp. *Hirtum*. ISO 13171:2010 (ISO).

18. European Pharmacopoeia (2008). *Caraway oil* (Carvi aetheroleum). Monograph 01/2008:1817 (Council of Europe).

19. European Pharmacopoeia (2011). *Cinnamon Bark Oil*, Ceylon. Monograph 04/2011:1501 (corrigé 11.6) (Council of Europe).

20. European Pharmacopoeia (2022). *Lemon oil* (Limonis aetheroleum). Monograph 04/2022:0620 (Council of Europe).

21. European Pharmacopoeia (2021). *Eucalyptus oil* (Eucalypti aetheroleum). Monograph 07/2021:0390 (Council of Europe).

22. European Pharmacopoeia (2008). *Clove oil* (Caryophylli floris aetheroleum). Monograph 01/2008:1091 (corrigé 7.6) (Council of Europe).

23. European Pharmacopoeia (2008). *Star Anise Oil* (Anisi Stellati Aetheroleum). Monograph 01/2008:2108 (Corrigé 7.0) (Council of Europe).

24. European Pharmacopoeia (2013). *Juniper oil* (Juniperi aetheroleum). Monograph 07/2013:1832 (corrigé 10.0) (Council of Europe).

25. European Pharmacopoeia (2008). *Tea tree oil* (Melaleucae aetheroleum). Monograph 01/2008:1837 (corrigé 7.0) (Council of Europe).

26. European Pharmacopoeia (2019). *Peppermint oil* (Menthae piperitae aetheroleum). Monograph 04/2019:0405 (Council of Europe).

27. European Pharmacopoeia (2012). *Thyme oil, thymol type* (Thymi typo thymolo aetheroleum). Monograph 01/2012:1374 (corrigé 10.0) (Council of Europe).