Screening essential oils for their antimicrobial activities against the foodborne pathogenic bacteria Escherichia coli and Staphylococcus aureus

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The application of essential oils as antimicrobials is a current subject of research and a promising approach in terms of natural food preservation. Due to the diversity of EO producing plant genera and the inconsistent use of susceptibility testing methods, information on the antibacterial potency of many EO varieties is fragmentary. This study was performed to assess the minimal inhibitory concentrations (MIC) of 179 EO samples from 86 plant varieties, using a single method approach, excluding emulsifying agents. MICs were acquired in a broth micro-dilution assay, using a dispersion based approach to incorporate EOs in a concentration range of 6400 to 50 μg/ml. Staphylococcus aureus and Escherichia coli were used as model bacteria. At concentrations below 400 μg/ml S. aureus was inhibited by 30 E. coli by 12 EO varieties. Axadractha indica (50 μg/ml vs. S. aureus) and Lirsea cubeba (50 μg/ml vs. S. aureus, 200 μg/ml vs. E. coli) essential oils were identified as promising new antimicrobial EO candidates with significant antimicrobial activity against the two foodborne pathogenic bacteria.

1. Introduction

Investigating the antimicrobial activities of plant essential oils (EO) has concerned many scientific studies within the last two decades. Besides a few general screenings [1, 2, 3], most studies were focused on one type of essential oil only, mainly Thymus vulgaris, Origanum vulgare or Cinnamomum species. Significant activities of these and other EOs against certain foodborne pathogenic bacteria such as Escherichia coli, Listeria monocytogenes, and Salmonella typhimurium have been demonstrated [4, 5, 6]. Furthermore, there has been continuous work regarding the identification of single active compounds from well investigated EOs, such as thymol, carvacrol or eugenol and partial elucidation of their cellular mechanism of action [7, 8, 9]. Most work on antimicrobial EOs was inspired by the idea of identifying alternative preservative agents with an overall “green” and “natural” or “bio-based” character for modern food technology applications.

The findings published to date, are still very fragmentary regarding a wide variety of essential oils [10]. Some EOs are still untested or negative results remain unpublished. In addition, it is difficult to reliably compare results from literature data, due to the strong variance of the used antimicrobial susceptibility testing methods. This complicates the selection of the most suitable EO candidates for further antimicrobial research and applications. Additionally, there are some constraints for EOs concerning food application, especially their sensory impact. EO components are considered aroma compounds (e.g. Thymol, Citral, Limonene, α-/β-Pinene, etc.), meaning they generally induce sensory activity [10]. Regarding food application it is therefore recommended to choose an essential oil based on the sensory profile of the targeted food product [11]. EOs with low inhibitory concentrations are considered advantageous to achieve antimicrobial effects in the product without affecting the sensory properties. For this study we defined a critical minimal inhibitory concentration (MIC) of 400 μg/ml in vitro, to select EOs with the most promising suitability for future application trials.

This study was performed to extent the available information on antibacterial activities of plant essential oils, intending to normalize the discussion on the antibacterial activity of plant essential oils with quantitative data for a large number of EOs. The comparability of the results is maximized by the use of a reproducible, quantitative method for MIC determination, combining the advantages of a dispersion approach, as developed by Remmal, Bouchikhi [12] and Friedman, Henika [13], with the reliability of a standardized broth microdilution assay [14]. Emulsifying agents and organic solvents were excluded when possible, as several authors argued that such additives distort susceptibility tests [12, 15]. The trials included a wide variety of EOs, available from three

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different German essential oil retailers and was focused on the two foodborne pathogenic model bacteria: *Escherichia coli* and *Staphylococcus aureus*.

2. Materials and methods

2.1. Essential oils

Essential oils were provided by three different German essential oil manufacturers (Neumond GmbH, Raisting, Germany; Frey&Lau GmbH, Henstedt-Ulzburg, Germany; Dülberg Konzentrab GmbH, Hamburg, Germany). Overall, essential oils from 86 plant varieties were accessible, whereof 38 equal varieties where available in triplicate and 20 in duplicate, but from the different sources, respectively. Altogether 179 commercial oil samples were tested. The investigated samples did not contain additives or solvents and were confirmed to be natural by the manufacturers. Furthermore, EOs were considered sterile. EO samples were stored in resealable vials at 5 °C in the dark, but were allowed to adjust to room temperature prior to investigation. The samples were washed twice in sterile 1/4 Ringer’s solution. Prior to use, cells were washed twice in sterile 1/4 Ringer’s solution and vigorously shaken for 30 s. Oil containing stock dispersions turned slightly white and were stable towards phase separation for up to 48 h. Solutions were adjusted to 12.8 mg/ml in glass vials which were sealed with a sterile, gas-permeable seal (BREATHSel, Greiner bio-one, Frickenhausen, Germany). Turbidity measurement was performed after incubation in a microplate reader (Tecan, Männedorf, Switzerland) at 595 nm. The seals were removed and plates were shaken orbital for 30 s. Each well was measured at nine spots with 5 flashes per spot. Obtained spot-OD-values of each well were averaged as well-OD-values. Values of parallel wells were averaged afterwards and blank-OD-values were subtracted. The minimal inhibitory concentration (MIC) was defined as the lowest concentration tested which did not allow cell growth within 24 h at 37 °C.

2.2. Bacterial strains and growth conditions

The Gram-positive bacterium *Staphylococcus aureus* DSM 1104 and the Gram-negative bacterium *Escherichia coli* DSM 1103 were used as test organisms. The strains were obtained from the Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and are both recommended for antimicrobial susceptibility testing. Stock cultures were grown in 100 ml sterile tryptic Braunschweig, Germany) and are both recommended for antimicrobial organisms. The strains were obtained from the Leibniz Institute DSMZ.

2.3. Essential oil incorporation

Essential oil incorporation was optimized for the execution of a broth microdilution assay for antibacterial susceptibility testing [16]. To avoid the interfering influences of organic solvents or emulsifying agents, a dispersion approach, as first described by Remmal, Bouchikhi [17], was chosen. To increase their viscosity, Mueller-Hinton broth (MHB) (Merck-Millipore, Darmstadt, Germany) and deionized water were spiked with 0.15 % agarose (Merck-Millipore, Darmstadt, Germany) prior to sterilization. All media were adjusted to pH 7.0 ± 0.1. EO stock solutions were adjusted to 12.8 mg/ml in glass vials which were sealed and vigorously shaken for 30 s. Oil containing stock dispersions turned slightly white and were stable towards phase separation for up to 48 h. Air bubbles were driven out by slight vortexing. A small number of EOs was found to be unstable in dispersion, due to complex-formation and clouding. For these EOs dimethyl sulfoxide (DMSO) was used to aid oil incorporation. Final DMSO concentration was 4.4 % (v/v) and had no growth inhibitory effects. Oils incorporated in DMSO are marked separately in the results tables.

2.4. Broth microdilution assay

Antibacterial susceptibility testing was performed according the CLSI laboratory standard for broth microdilution assays [16]. Concentrations tested in the assays ranged from 6400 to 50 μg/ml in bisecting dilution steps. Assays were performed using sterile 96 well microplates (transparent, F-bottom) (Greiner bio-one, Frickenhausen, Germany). Each concentration was tested in triplicate and a single blank (broad with corresponding EO-concentration). Three oils were tested in parallel per plate (n = 3). Ultimately, each well contained 100 μl of EO dispersion. Inoculum was prepared by a hundredfold dilution of the adjusted cell suspension in double concentrated (2x) MHB. Each well was spiked with 100 μl inoculated 2xMHB resulting in a final cell count of 1.0*10⁸ cfu per well. Inoculation media were used immediately to avoid growth dependent shifts in the cell count. Growth, sterility and quality controls were analyzed for every culture, but on separate microplates. Chloramphenicol (2.0–0.008 μg/ml) served as control bacteriostatic to assess cell susceptibility for each batch culture (data not shown). Prior to incubation for 24 h at 37 °C, the inoculated plates were shaken orbital for 30 s on a plate mixer (Kisker, Steinfurt, Germany). To avoid loss of volatile essential oil and humidity during incubation each plate was sealed with a sterile, gas-permeable seal (BREATHSel, Greiner bio-one, Frickenhausen, Germany). Turbidity measurement was performed after incubation in a microplate reader (Tecan, Männedorf, Switzerland) at 595 nm. The seals were removed and plates were shaken orbital for 30 s. Each well was measured at nine spots with 5 flashes per spot. Obtained spot-OD-values of each well were averaged as well-OD-values. Values of parallel wells were averaged afterwards and blank-OD-values were subtracted. The minimal inhibitory concentration (MIC) was defined as the lowest concentration tested which did not allow cell growth within 24 h at 37 °C.

3. Results

The MIC values of 179 essential oils from 86 plant species against *E. coli* DSM 1103 and *S. aureus* DSM 1104, determined in a broth microdilution assay, are presented in Table 1. The results show growth inhibitory activities for the majority of the tested EO samples. Inhibition was generally stronger against the Gram-positive bacterium *S. aureus* than against Gram-negative *E. coli*. At or below the preliminary defined critical concentration of 400 μg/ml 46 EO samples from 30 plant genera inhibited *S. aureus*, whereas *E. coli* was only inhibited by 22 samples from 12 plant genera. EO varieties from different providers rarely revealed identical MIC values. Nonetheless, *Azadirachta indica*, *Backhousia citriodora*, *Cinnamomum cassia*, *Cinnamomum verum*, *Leptospermum scoparium*, *Litsea cubeba*, *Nardostachys jatamansi*, *Origanum vulgare*, *Pogostemon cablin*, *Sanfitalum album*, *Thymus syzigis* and *Vetiveria zizanoides* were found to be the most inhibitory EOs against *S. aureus* with MIC values of 50 μg/ml. Only four EOs, *Cinnamomum cassia*, *Cinnamomum verum*, *Origanum vulgare* and *Thymus syzigis* could exhibit inhibitory effect against *E. coli* at 50 μg/ml. But certain other EOs from *Backhousia citriodora*, *Cupressus sempervirens*, *Cymbopogon citratus*, *Cymbopogon martini*, *Cymbopogon nardus*, *Litsea cubeba*, *Origanum majorana*, *Origanum vulgare*, and *Syzgium aromaticum* still revealed promising activity with MICs between 100 and 400 μg/ml against *E. coli*.

Some EOs did not show any antibacterial activities. *Cananga odorata*, *Cupressus sempervirens*, *Daucus carota*, *Foeniculum vulgare*, *Juniperus communis*, *Pimpinella anisum* oils were available from each provider, but did not show any activity against *S. aureus* or *E. coli*, respectively. *Artemisia pallasii*, *Boswellia carteri*, *Matricaria chamomilla*, *Pinus mugo*, *Piper nigrum*, *Pogostemon cablin*, *Pinus sylvestris* and *Vetiveria zizanoides* only exhibited inhibitory activity against *S. aureus*. Growth of *E. coli* was not affected by these EOs. For *Pogostemon cablin* and *Vetiveria zizanoides* these findings were particularly distinct, as the three EO samples from the different providers revealed the same results for *E. coli*. Exclusive inhibitory potential against *E. coli* was only shown for samples from *Cinnamomum camphora* and *Citrus sinensis*.

4. Discussion

The number of scientific studies on the antimicrobial activity of plant
| Plant botanical name | Oil common name | Extracted plant part | MIC (μg/ml) S. aureus | MIC (μg/ml) E. coli |
|---------------------|----------------|----------------------|-----------------------|-----------------------|
|                     |                |                      | a         | b         | c         | a         | b         | c         |
| 1. Abies alba        | silver fir    | branches             | n. l.     | n. l.     |           |           |           |           |
| 2. Abies procera     | noble fir     | branches             | n. l.     | n. l.     |           |           |           |           |
| 3. Achillea millifolium | yarrow   | herb                 | 6400      | n. l.     |           |           |           |           |
| 4. Anethum graveolens | dill         | seeds + herb         | n. l.     | n. l.     |           |           |           |           |
| 5. Angelica archangelica | root     | garden angelica      | 400       | n. l.     | 1600      |           |           |           |
| 6. Anethum nobilis   | roman chamomile | blossom            | 200*      | n. l.     | n. l.     |           |           |           |
| 7. Artemisia dracunculus | leaves      | tarragon             | 6400      | n. l.     | n. l.     |           |           |           |
| 8. Artemisia pallens | davana       | herb                 | 6400      | n. l.     | n. l.     |           |           |           |
| 9. Asarabachus indica | neem         | seeds                | 50*       | 1600*     |           |           |           |           |
| 10. Backhousia caesia | lemon myrtle | herb                 | 50        | 200       |           |           |           |           |
| 11. Boswella carteri | olibanum     | resin                | 1600      | n. l.     | n. l.     |           |           |           |
| 12. Cananga odorata  | ylang-ylang  | blossom              | n. l.     | n. l.     | n. l.     |           |           |           |
| 13. Canaria humicola | elemi        | resin                | 400       | n. l.     | 6400      | n. l.     |           |           |
| 14. Carnus carvi     | caraway      | seeds                | 800       | n. l.     | 3200      | 6400      | 1600      |           |
| 15. Cedrus atlantica | atlas cedar  | wood                 | n. l.     |           |           |           |           |           |
| 16. Cinnamomum camphora | ravistara | leaves              | 3200      |           | 6400      |           |           |           |
| 17. Cinnamomum camphora | camphor    | branches             | n. l.     | n. l.     | 6400      |           |           |           |
| 18. Cinnamomum camphora | ho         | leaves               | 1600      |           | 800       |           |           |           |
| 19. Cinnamomum cassia | chinese cinnamon | branches       | 50        | 50        | 200       | 50        | 50        |           |
| 20. Cinnamomum verum | true cinnamon | bark                | 200       | 50        | 800       | 200       | 50        | 200       |
| 21. Citrus limon      | cistrose     | leaves + branches    | 400       |           |           |           |           |           |
| 22. Citrus aurantifolia | lime        | peel                 | 1600      | 800      | 3200      | 6400      | 800       | 6400      |
| 23. Citrus aurantium | bitter orange | peel               | 1600      | n. l.     | 3200      | 1600      | n. l.     | 1600      |
| 24. Citrus aurantium | neroli      | blossom              | 6400      | 800      | 3200      | 3200      | 800       | 6400      |

Table 1
Minimal inhibitory concentrations (μg/ml) of essential oils from three different manufacturers (a, b, c) against Staphylococcus aureus DSM 1104 and Escherichia coli DSM 1103.

...continued on next page...
essential oils has strongly grown over the last three decades. Due to the use of many different microbiological methods for susceptibility testing and different definitions of antimicrobial activity, the comparability of studies on essential oils is often critical. Many studies focus on selected EOs, providing insight into their activity against one or more microorganisms [4, 5, 7, 8, 18], but only few publications compress information on the EO variety tested. In comparison to other essential oils, providing insight into their activity against one or more microorganisms [4, 5, 7, 8, 18], but only few publications compress information on the EO variety tested. In comparison to other essential oils

| Plant botanical name | Oil common name | Extracted plant part | MIC (μg/ml) |
|----------------------|----------------|----------------------|------------|
| *S. aureus* | | | |
| | | | |
| *E. coli* | | | |
| | | | |
| 61 | Mentha piperita citrata | bergamot mint | 1600 |
| 62 | Mentha spicata | spearmint | 1600 |
| 63 | Myrtus communis | myrtle leaves + branches | 1600 |
| 64 | Nardostachys jatamansi | spikenard roots | 1600 |
| 65 | Ocimum basilicum | basil | 1600 |
| 66 | Origanum majorana | marjoram leaves | 1600 |
| 67 | Origanum vulgare | oregano leaves | 1600 |
| 68 | Pelargonium graveolens | rose geranium leaves | 1600 |
| 69 | Pinus sylvestris | scots pine needles | 1600 |
| 70 | Lavandula officinalis | lavender flowers | 1600 |
| 71 | Mentha spicata | spearmint leaves | 1600 |
| 72 | Pelargonium graveolens | rose geranium leaves | 1600 |
| 73 | Pelargonium graveolens | rose geranium leaves | 1600 |
| 74 | Pogostemon cablin | patchouli leaves | 1600 |

| Plant botanical name | Oil common name | Extracted plant part | MIC (μg/ml) |
|----------------------|----------------|----------------------|------------|
| *S. aureus* | | | |
| | | | |
| *E. coli* | | | |
| | | | |
| 75 | Pinus sylvestris | scots pine needles | 1600 |
| 76 | Rosmarinus officinalis | rosemary leaves | 1600 |
| 77 | Salvia lavandulifolia | salvia spanish leaves | 1600 |
| 78 | Salvia officinalis | salvia leaves | 1600 |
| 79 | Salvia sclarea | clary sage leaves | 1600 |
| 80 | Santalum album | sandalwood wood | 1600 |
| 81 | Syzygium aromaticum | clove buds | 1600 |
| 82 | Thymus mastichina | thyme leaves | 1600 |
| 83 | Thymus sylvestris | thyme linalool leaves | 1600 |
| 84 | Thymus sylvestris | thyme thymol leaves | 1600 |
| 85 | Vetiveria zizanoides | vetiver roots | 1600 |
| 86 | Zingiber officinale | ginger roots | 1600 |

a = Neunmond GmbH.  
b = Frey & Lau GmbH.  
c = Düllberg Konzentra GmbH.  
blank space = EO not available.  
n. I. = no inhibition.  
* = contains DMBO.

cubeba EO is produced in high amounts and cheaply available. Further research is necessary to identify its full antimicrobial spectrum and to optimize its potential. As the tested *Litsea cubeba* EOs tested in this study fell below the defined food application limit of 400 μg/ml they might be considered a promising candidate for food preservative applications, also due to its unique, refreshing aroma [25, 27]. The main active compound of *L. cubeba* EOs is the monoterpene Citral, which has been found to be positive in terms of sensory effects when used as an antimicrobial compound in food products [28].

*Nardostachys jatamansi* and *Azadirachta indica* might also become interesting regarding food preservation applications, as both plants are important in traditional Indian medicine and consequently have histories of safe use [29, 30]. *Nardostachys jatamansi* is dominated by different sesquiterpenes which, to our best knowledge, have not been investigated concerning antimicrobial activities yet [30]. The essential oil from *Azadirachta indica*, commonly known as neem-tree essential oil, mainly consists of the compounds Azadirachtin and Nimbin. The compounds are known to possess antimicrobial activity, but are predominately used as spermicides [29, 31].

By comparing the results of EOs from a single plant species, but from different manufacturers, it becomes evident that simple postulations regarding the antibacterial effect of a certain EO cannot be made easily. As shown in Table 1, most EO varieties revealed differing MIC values when purchased from another manufacturer. Possible reasons may be versatile, as chemical composition is affected by various external factors, such as geographic origin, environmental conditions, point of harvest or other processing dependent influences [32, 33, 34]. These findings are in line with the results from previous works [35, 36] and enhance the often
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requested need for chemical characterizations of antimicrobial EOs to identify the active compounds and their interdependencies [3]. Consequently, EO optimization and standardization regarding antimicrobial activity appears to be inevitable for application.

This study also revealed results which differ greatly from those reported by others. The lacking growth inhibition by Cananga odorata, Cupressus sempervirens, Juniperus communis or Pimpinella anisum may be due to the comparably low concentrations used. Authors, who found these oils to be inhibitory, used way higher concentrations, revealing MICs ranging from 12.5 mg/mL for Cupressus sempervirens EO against E. coli [37] to 40 μg/mL for Juniperus communis EO versus S. aureus [38]. In regard of future applications in food systems and the strong sensory impact of essential oils on food these EO varieties appear to be unsuitable. Given the focus of application it is apparent to select essential oils with very low MICs. As described before, we defined a critical MIC level of 400 μg/mL in order to identify EO varieties with a greater applicability concerning food preservation. In general it is recommended to couple food application studies with sensory profiling trials. Another peculiarity in this study is the fact, that none of the Foeniculum vulgare EOs showed any inhibitory activity. In previous studies fennel seed essential oil was found to be bactericidal at comparable concentrations between 20 μL and 80 μL/mL against E. coli and S. aureus by Dadalioglu and Evrendilek [39]. In this case clarification can only be achieved by chemical analysis of the respective oils which has not been performed as part of our investigations, due to the more broadened approach. On the other hand it was once more affirmed, that Daucus carota essential oils completely lack antimicrobial activity, as also stated by Hammer, Carson [3].

5. Conclusion

In summary, this study provides insight into the in vitro antibacterial activity of a wide variety of essential oils from many different plant genera against E. coli and S. aureus. The data contributes to the ongoing scientific investigation regarding the application of essential oils as natural food preservative agents. As the comparison of MICs from different studies is most often difficult, due to the use of varying quantitative or semi-quantitative methods, this study aimed to normalize the discussion by testing a wide variety of plant essential oils with a single, standardized quantitative method for MIC detection. After benchmarking EOs from thyme and oregano as the most active, EO varieties from Azadirachta indica and Litsea cubeba were identified as promising candidates concerning possible applicability in food.

Declarations

Author contribution statement

Julian Thielmann: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Pamina Kazman: Performed the experiments; Analyzed and interpreted the data.

Peter Muranyi: Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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