INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 21, No. 6, p. 76-82, 2022

RESEARCH PAPER OPEN ACCESS

Antibacterial activity against *S. agalactiae* of four essential oils of peppermint *(Mentha piperita* L.*)*, lemongrass *(Cymbopogon citratus)*, garlic *(Allium sativum)*, and green tea *(Camellia sinensis)*

Huyen Nguyen Thi Thuong[1], Thi-Hieu Tran[2], Phuong Nguyen Ngoc[2], Van-Thanh Vo*[1]

[1]*Department of Human and Animal Physiology, Biology Faculty, Ho Chi Minh City University of Education, 280 An Duong Vuong Str, Ward 4, District 5, Ho Chi Minh City, Vietnam*

[2]*Department of Biochemistry, Microbiology, and Genetics, Biology Faculty, Ho Chi Minh City University of Education, 280 An Duong Vuong Str, Ward 4, District 5, Ho Chi Minh City, Vietnam*

Key words: Antibacterial activity, Essential oils, Peppermint, Lemongrass, Garlic, Green tea, *S. agalactiae*.

http://dx.doi.org/10.12692/ijb/21.6.76-82 Article published on December 04, 2022

Abstract

The antibacterial activity test of four essential oils, including peppermint *(Mentha piperita)*, lemongrass *(Cymbopogon citratus)*, garlic *(Allium sativum)*, and green tea *(Camellia sinensis)* at 100% concentration, was conducted by agar well diffusion method. The results of peppermint EO showed the largest zone of inhibition (23.00±3.93 mm), and *S. agalactiae* was considered susceptible. The following result is lemongrass EO (19.50±2.66 mm) showed tested organism was intermediately inhibited but was nonsusceptible with garlic EO (ZOI=12.50±2.48 mm) and green tea EO (ZOI=9.00±2.19 mm). Then, the experiment was performed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The results recorded the MIC increasing gradually as follows: lemongrass EO (0.6%) < peppermint EO (0.75%) < garlic EO (1.75%) < green tea EO (4.5%). Similarly, the MBC value was lemon grass EO (1.0%) < peppermint EO (1.75%) < garlic EO (1.75%), and non-identified MBC of green tea EO. The difference in MIC/MBC along with ZOI between peppermint and lemongrass is believed to depend on the degree of diffusion of essential oil into agar medium and how the compounds affect bacterial cells.

* **Corresponding Author:** Van-Thanh Vo ✉ thanhvv@hcmue.edu.vn

Introduction

The disease is always mentioned as a head cause of production loss, leading to a significant loss for the world economy. China - a top country in aquaculture of Asia, directly lost disease outbreaks of around 205,000 tons, equivalent to 401 million USD in 2018 (FAO, 2020). Aquaculture's most common bacterial pathogens were reported, including *Aeromonas hydrophila*, *Edwardsiella tarda, Pseudomonas* sp., *Flexibacter columnar*, *Vibrio* sp., and *Streptococcus* sp. (Mishra *et al.*, 2017). Streptococcosis, caused by *Streptococcus* sp., is affecting many fish species and occurring in many countries in recent years. Among them, a hemorrhagic disease caused by *Streptococcus agalactiae* became the most common disease on tilapia farms (Alsaid *et al.*, 2010).

The use of antibiotics in the treatment of fish diseases has increased because of the expansion of intensive aquaculture production to meet the great demand of the population. However, improper and indiscriminate use of antibiotics causes many negative impacts on the environment and people and especially leads to the emergence of new antibiotic resistance pathogens (Schmidt *et al.*, 2000). Therefore, developing a new approach using plant extracts has gained more and more attention because of the potential for antibiotic replacement.

Source materials from plants are diverse, abundant, and available in nature. Plant extracts have long been used in traditional medicine for their medicinal properties in the treatment of fungi or bacteria diseases. Nowadays, plant extracts have gradually played an important role in aquaculture (Muniruzzaman and Chowdhury, 2004).

In particular, the essential oils of peppermint, lemongrass, garlic, and green tea have been shown to be resistant to several species of bacteria and fungi. However, the studies of these essential oils against *S. agalactiae* have been limited (Alsaid *et al.*, 2010; Singh *et al.*, 2011; Kamble *et al.*, 2014). In Vietnam, peppermint is a very popular flavoring. Lemongrass and garlic have become two common spices in Vietnamese cuisine, while green tea has been associated with Vietnamese traditions for centuries. For those reasons, the present study selected the essential oils of peppermint (*Mentha piperita* L.), lemongrass (*Cymbopogon citratus*), garlic *(Allium sativum)*, and green tea *(Camellia sinensis)* to test their antibacterial activity against *S. agalactiae* bacteria and to determine the minimum inhibitory concentration and minimum bacterial concentration.

Materials and methods

Essential oil sources

The present study used the commercial essential oils provided by Heber Vietnam Co., Ltd.

Bacteria inoculum preparation

The bacterial strain *S. agalactiae* was received from the Faculty of Microbiology, Ho Chi Minh City University of Agriculture and Forestry.

Bacteria were grown proliferatively in Tryptic Soy Broth (TSB) medium at 37°C for 24 hours to reach a density of 10^8 CFU/mL (corresponding to Mc Farhland 0.5) diluted 100 times with sodium chloride 0.85% to reach a density of 10^6 CFU/mL.

Antibacterial activity assay

Experiment on the inhibitory effect on S. agalactiae of four essential oils at 100% concentration

Peppermint essential oil (EO), lemongrass EO, garlic EO, and green tea EO was carried out by agar well diffusion method (Balouiri *et al.*, 2016). 20 mL of sterile TSB medium was poured into a petri dish for solidification. 100 µL of *S. agalactiae* bacteria at a concentration of 10^6 CFU/mL were spread evenly on a plate with sterile swabs. A hole with a diameter of 8 mm has been punched aseptically with a sterilized cork borer. 100 µL of essential oil was taken with a sterilized pipette and added to each well under sterile conditions. The well was filled with only 100 µL of sterile distilled water for the control dish. All dishes were incubated at 4°C for essential oil diffusion, then took them for incubation at 37°C for 24h. Each essential oil was performed on six dishes corresponding to 6 replications.

The antibacterial activity of essential oils was evaluated by measuring the diameter of the inhibition zone after incubation, and the data were interpreted using the CLSI standard (CLSI, 2021).

Preparation of concentration of essential oils and stock

In the present study, the eight concentrations of each essential oil were determined as follows:

Peppermint EO: includes concentrations ranging from 0.125-1.125 (% v/v) with the step of 0.125.

Lemongrass EO: includes concentrations ranging from 0.1–0.8 (% v/v) with the step of 0.1.

Garlic EO: includes concentrations ranging from 1.0–2.75 (% v/v) with the step of 0.25.

Green tea EO: includes concentrations ranging from 0.5–4.0 (% v/v) with the step of 0.5.

Each stock of each essential oil was made by diluting *X* L of EO with 2 L of 100% DMSO and *Y* L of TSB medium to obtain the concentration of essential oil in each stock was *X%* and *2% DMSO*. At final, *X%* were 2%, 2%, 5%, and 5%, corresponding to peppermint EO, lemongrass EO, garlic EO, and green tea EO.

Y = 100 L - *X* L of EOs - 2 L *DMSO*.

Resazurin 0.01% preparation

Resazurin 0.01% was prepared by dissolving 10 mg of resazurin powder in 100 mL of distilled water. The reagent solution was sterilized using a 0.22 (µm) filter and stored at 4°C for two weeks before use (Elshikh *et al.*, 2016).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The experiment was performed on a 96-well plate volume of 200 µL. Each essential oil was tested with three replicates. The amount of tested concentration from each stock was taken and mixed with sterile 2% DMSO (calculated using the formula $C_1.V_1=C_2.V_2$) to obtain the total volume of 100 µL. The negative control well contained only 200 µL of sterile sodium chloride (0.85%). The positive control well contained 100 µL of TSB broth. Then, 100 µL of *S. agalactiae* at a concentration of 10^6 CFU/mL was added to each well (except for the negative control well). The 96-well plate was incubated at 37°C. After 24 hours, 20 L of 0.01% resazurin was added to each well, incubated for another 2 to 4 hours, and read the results. Resazurin is a purple-blue solution; wells with active living cells will create resorufin, which is pink-colorless. MIC is determined as the lowest concentration at which the reagent color is not changed (Elshikh *et al.*, 2016).

The 50 µL solution was taken from each well of 3 concentrations (including MIC and the following two concentrations) to determine MBC, then spread on BHIA medium and incubated at 37°C. After 24 hours of culturing, check the growth of bacterial colonies on each agar plate. The MBC value was defined as the concentration at which no colonies appeared; each trial was replicated thrice.

Data analysis

Data obtained from the diameter of ZOI was subjected to descriptive statistical analysis. The significance of study data was calculated by ANOVA One-way and was performed as Mean±SD. Minitab 18 was used for statistical analysis.

Results and discussion

Antibacterial activity assay

The antibacterial ability of the essential oils is shown in Table 1. The antagonism level of *S. agalactiae* towards the essential oils is revealed at three levels. The tested organism exhibited susceptibility to peppermint EO, with a diameter of zone of inhibition (ZOI) was 23.00±3.93 mm. The result of lemongrass EO showed the intermediate level, with ZOI recorded as 19.50±2.66 mm. Finally, *S. agalactiae* was considered nonsusceptible to garlic EO and green tea EO, and ZOI were 12.50±2.48 mm and 9.00±2.19 mm, respectively.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Results of MIC and MBC of four EOs were recorded and exhibited in Table 2. In the MIC trial, the concentration increased as lemongrass EO (0.6%) <

peppermint EO (0.75%) < garlic EO (1.75%) < green tea EO (4.5%). Meanwhile, the highest MBC was 1.75% of peppermint EO and garlic EO, followed by 1.0% of lemongrass EO. The MBC of green tea EO could not be identified.

Several researchers have been focusing on using plant extracts against many common pathogens as an alternative to antibiotics (Citarasu, 2010; Subramani and Michael, 2017). The antibacterial activity of essential oils is attributed to the presence and content of various compounds with antibacterial properties. The present study did not focus on analyzing the antibacterial compounds of essential oils but on testing the antibacterial activity and investigating MIC and MBC against *S. agalactiae*.

Table 1. Zone of inhibition (mm) ±SD of peppermint EO, lemongrass EO, garlic EO, and green tea EO after 24h exposure to *S. agalactiae*.

Essential oils	ZOI (mm)	CLSI, 2021
Peppermint	23.00±3.93	susceptible
Lemongrass	19.50±2.66	intermediate
Garlic	12.50±2.48	nonsusceptible
Green tea	9.00±2.19	nonsusceptible

S. agalactiae exhibited susceptibility to peppermint EO when tested by agar-well diffusion method (ZOI=23.00±3.93 mm). The results can be compared with the result of Tyagi and Malik (2011) when the ZOI of peppermint EO against the gram-positive bacteria *B. subtilis* was recorded as 22 mm. MIC was defined as the lowest concentration that completely inhibits the growth of the tested organism (Balouiri *et al.*, 2016), and MBC was the lowest concentration of oil that leads to the death of 99.9% of inoculum (Burt, 2004). In the present study, *S. agalactiae* was inhibited at a minimum concentration of 0.75% (v/v) and was killed at a minimum concentration of 1.75%. Differences in the source of materials of the present study (using commercial essential oils, which extract method and the number of raw materials used was unidentified) create a limitation in comparing the results of our research with ones in the world. Therefore, the determination of MIC and MBC of the experiment aims to contribute some basic data for in *vivo* studies in the animal in the future. The antibacterial activity of peppermint is due to its chemical compounds, in which menthol (33–60%) and menthone (15–32%) are the two highest abundant and have been shown to have the main antibacterial role (McKay and Blumberg, 2006). Lemongrass (*Cymbopogon citratus*) is one of the essential ingredients in Asian cuisine because of its lemon flavor (Singh *et al.*, 2011). Besides, many studies have also demonstrated that lemongrass EO has antibacterial and antifungal properties (Ushimaru *et al.*, 2007). The present results showed that the tested organism had intermediate resistance to lemongrass, and the diameter of ZOI was smaller than peppermint (19.50±2.66 mm).

The study of Singh *et al.* (2011) showed that *S. agalactiae* was sensitive to lemongrass EO. Meanwhile, Adukwu *et al.* (2012) reported that the diameter of ZOI of lemongrass EO was higher than that of grapefruit, lime, and bergamot EO and proved that citral – the main component of this essential oil, plays a key antibacterial role with the similar diameter of lemongrass EO. The MIC of lemongrass EO was determined in the present study at a concentration of 0.6%, lower than that of peppermint EO despite its higher ZOI.

Similarly, the MBC of lemongrass was found to be 1.0%, lower than the MBC of peppermint, about 0.75%. The agar well diffusion method depends on the composition of the essential oil and medium used, resulting in the different diffusion levels of EO. Therefore, this method is not recommended in studies requiring specific concentrations. In general, this reason may explain the difference in inhibition zone diameters of the two essential oils mentioned above.

Table 2. Minimum inhibitory concentration (% v/v) and minimum bactericidal concentration (% v/v) for peppermint EO, lemongrass EO, garlic EO and green tea EO against *S. agalactiae*.

Essential oils	MIC	MBC
Peppermint	0.75	1.75
Lemongrass	0.6	1.0
Garlic	1.75	1.75
Green tea	4.5	-

Garlic is also a wonderful spice indispensable in the cuisine of many countries. In medicine, garlic has been shown to be resistant to several species of bacteria (including Gram-negative and Gram-positive) and fungi (Phay *et al.*, 1999; Hsieh *et al.*, 2001; Ward *et al.*, 2002). The present study found *S. agalactiae* was nonsusceptible with garlic EO, with the inhibition zone around the agar wel lbeing 12.50±2.48 mm. The resistance test against 17 common foodborne pathogens using aqueous garlic extracts of Durairaj *et al.* (2009) showed that the lowest diameter of ZOI was 22 mm and the highest value was 54 mm (at 100% concentration), both higher when compared to zone formed by common antibiotics. As mentioned earlier, the diffusion ability of essential oils into the agar can influence the test results, and it's associated with the chemical compounds. The MIC and MBC results of garlic EO were found to be similar, which was 1.75%. These results suggest that the antibacterial compounds in garlic EO may exhibit a more effective impact on direct and potentially cause bacterial cell death. The antibacterial activity of garlic extract is contributed most by allicin. Allicin interferes with RNA production and lipid synthesis. Once the production of messenger RNA is disrupted, it means that the amino acids and proteins necessary for cell division are prevented, resulting in the failure to grow and develop a bacterial population (Durairaj *et al.*, 2009).

Green tea has been widely consumed around the world for 2000 years and is the second most popular drink after water (Subramaniam *et al.*, 2012). Green tea extract posses tannins, phenols, alkaloids, and flavonoids, which contribute to this plant's antibacterial properties. Tannins inhibit bacterial growth by binding strongly to iron and impact glucosyl-transferase (GTF). Alkaloids interfere with cell division resulting in the growth of the organism. Flavonoids reduce the ability to utilize the environment through inhibition of adhesion and anti-glycosyl activity (Anita *et al.*, 2015). In the present study, testing for the antibacterial activity of green tea EO showed the lowest ZOI (9.00±2.19 mm) and was in the same resistance level as garlic EO – nonsusceptible. The test of Subramaniam *et al.* (2012) on *S. mutan* showed that the diameter of ZOI of green tea at 100% concentration was 5.37±1.02 mm, and there was no significant difference at 50% concentration.

On the other hand, Anita *et al.* (2015) reported that the value of ZOI of green tea EO increased gradually by increasing concentration, ranging from 8.33–12.67 mm of bacteria *L. acidophilus* and 10–18.33 mm of bacteria *S. mutan*. In addition, the minimum concentration that can inhibit the growth of bacteria was found at 4.5% (v/v) and was determined as the highest MIC value in this trial. The limitation of the antibacterial compounds in green tea oil used could explain this result. First, the origin of the materials used can be various, even though they are the same species. Second, the difference can come from the method applied in the extraction process. Different processes can yield different products with different concentrations of compounds. Results on the minimum bactericidal concentration of green tea EO have not been recorded because of the appearance of colonies at all investigated concentrations. Therefore, future experiments should use other methods for better results.

Conclusion

The results of the present study partly demonstrate that peppermint and lemongrass essential oils exhibited significant antibacterial activity at 100% concentrations, although lemongrass was a lower inhibitory effect. Meanwhile, garlic EO showed a smaller ZOI diameter. Still, the MIC and MBC values were similar to that of peppermint's MBC, which was thought to be related to the ability of allicin to the method applied. Although green tea has been mostly shown to have antibacterial effects, the current result seems to be the opposite of the results reported worldwide. Each experiment has its limitations. Lacking specific information on the origin, content, and extraction method of each plant used is the limitation of this present study. Even so, the study results are still worthy of recognition and use as a database for future *in vivo* experiments in the animal. The MIC values were ranked as lemongrass (0.6%) < peppermint (0.75%) < garlic (1.75%) < green tea (4.5%), and MBC 1.0%, 1.75%, 1.75%, and non-identified, respectively.

References

Adukwu EC, Allen SCH, Phillips CA. 2012. The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*. Journal of Applied Microbiology **113,** 1217–1227. https://doi.org/10.1111/j.1365-2672.2012.05418.x

Alsaid M, Daud H, Bejo SK, Abuseliana A. 2010. Antimicrobial activities of some culinary spice extracts against *Streptococcus agalactiae* and its prophylactic uses to prevent Streptococcal infection in Red Hybrid Tilapia (*Oreochromis* sp.). World Journal of Fish and Marine Sciences **2,** 532–538.

Anita P, Balan In, Ethiraj S, Madan Kumar P, Sivasamy S. 2015. In vitro antibacterial activity of *Camellia sinensis* extract against cariogenic microorganisms. Journal of Basic and Clinical Pharmacy **6,** 35. https://doi.org/10.4103/0976-0105.145777

Balouiri M, Sadiki M, Ibnsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis **6,** 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. International Journal of Food Microbiology **94,** 223–253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022

CLSI. 2021. *Performance standards for antimicrobial susceptibility testing*. Clinical and Laboratory Standards Institute.

Durairaj S, Srinivasan S, Lakshmanaperumalsamy P. 2009. *In vitro Antibacterial Activity and Stability of Garlic Extract at Different pH and Temperature*. Electronic Journal of Biology **5,** 5-10.

Elshikh M, Ahmed S, Funston S, Dunlop P, McGaw M, Marchant R, Banat IM. 2016. Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. Biotechnology Letters **38,** 1015–1019. https://doi.org/10.1007/s10529-016-2079-2

FAO. 2020. *The State of World Fisheries and Aquaculture 2020*. FAO - Fisheries and Aquaculture Department. https://doi.org/10.4060/ca9229en

Hsieh PC, Mau JL, Huang SH. 2001. Antimicrobial effect of various combinations of plant extracts. Food Microbiology **18,** 35–43. https://doi.org/10.1006/fmic.2000.0376

Kamble MT, Gallardo WG, Chavan BR, and Rusydi R. 2014. *Antimicrobial Activity of Bioactive Herbal Extracts Against Streptococcus agalactiae Biotype 2*. International Journal of Basis and Applied Biology **2,** 152-155.

McKay DL, Blumberg JB. 2006. A review of the bioactivity and potential health benefits of peppermint tea (*Mentha piperita* L.). Phytotherapy Research **20,** 619–633. https://doi.org/10.1002/ptr.1936

Mishra S, Rakesh D, Dhiman M, Choudhary P, Debbarma, Sahoo S, Barua A, Giri B, Ramesh R, Ananda K, Mishra C, Swain P. 2017. Present status of fish disease management in freshwater aquaculture in India: State-of- the-art-review. Journal of Aquac Fisheries **1,** 1-9.

Muniruzzaman M, Chowdhury BR. 2004. Sensitivity of fish pathogenic bacteria to various medicinal herbs. Bangladesh Journal of Veterinary Medicine **2,** 75–82.

Phay N, Higashiyama T, Tsuji M, Matsuura H, Fukushi Y, Yokota A, Tomita F. 1999. An antifungal compound from roots of Welsh onion. Phytochemistry **52,** 271–274. https://doi.org/10.1016/S0031-9422(99)00170-3

Schmidt AS, Bruun MS, Dalsgaard I, Pedersen K, Larsen JL. 2000. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four danish rainbow trout farms. Applied and Environmental Microbiology **66,** 4908–4915.

Singh BR, Singh V, Singh RK, Ebibeni N. 2011. Antimicrobial activity of lemongrass (*Cymbopogon citratus*) oil against microbes of environmental, clinical and food origin. International Research of Pharmacy and Pharmacology **1,** 228–236.

Subramaniam P, Maheshwar Reddy K, Eswara U. 2012. Effect of different types of tea on *Streptococcus mutans*: An in vitro study. Indian Journal of Dental Research **23,** 43. https://doi.org/10.4103/0970-9290.99037

Tyagi AK, Malik A. 2011. Antimicrobial potential and chemical composition of Mentha piperita oil in liquid and vapour phase against food spoiling microorganisms. Food Control **22,** 1707–1714. https://doi.org/10.1016/j.foodcont.2011.04.002

Ushimaru PI, Mariama TN, Luiz CD, Luciano B, Ary FJ. 2007. Antibacterial activity of medicinal plant extracts. Brazilian Journal of Microbiology **38,** 717–719. https://doi.org/10.1590/S1517-83822007000400024

Ward PML, Fasitsas S, Katz SE. 2002. Inhibition, resistance development, and increased antibiotic and antimicrobial resistance caused by nutraceuticals. Journal of Food Protection **65,** 528–533. https://doi.org/10.4315/0362-028X-65.3.528