Journal of Science-FAS-SEUSL (2021) 02(02) 65-76
ISSN:2738-2184

Antimicrobial activity of essential oils separated from selected plants of Sri Lankan Rutaceae.

Haroon M. Haniffa[1]*, R. Fathima Safana[1,2], and S. Kathirgamanathar[2]

[1]Department of Chemical Sciences, Faculty of Applied Sciences, South Eastern University of Sri Lanka

[2]Herbal Technology Section, MRDC, Industrial Technology Institute, Malabe, Sri Lanka

Abstract

The family Rutaceae has great economic importance for its numerous edible fruits and essential oils. The present study aimed to determine the antimicrobial activity of essential oils separated from four plants, namely, *Citrus aurantifolia, Citrus latifolia, Aegle marmelos,* and *Limonia acidissima,* of the family Rutaceae, and they were analyzed for the characterization of the different antimicrobial compounds through Gas Chromatography-Mass Spectrometry (GC-MS). The antibacterial activities of essential oils were determined against gram-positive (*Bacillus subtilis)* and gram-negative (*Escherichia coli)* bacteria, along with two fungal strains, *Aspergillus* spp. *and Trichoderma* spp. In this study, the values of the Minimum Inhibitory Concentration (MIC) of these four oils were found within the range of 1.25–5 (V/V%). Among the four oils, *C. latifolia* showed potential antibacterial properties with a zone of inhibition of 9.66 mm and 11.25 mm against *E. coli* and *B. subtilis,* along with an MIC value of 1.23 ± 0.005 and 2.4 ± 0.01 (v/v%), respectively. GC–MS analysis of essential oils of Rutaceae family plants revealed that *α*-citral, *β*-citral, D-limonene, β-myrcene, citronellal, linalool, 3-carene, geraniol, geranyl acetate, caryophyllene, terpinene, bicyclo [3.1.0] hexane, and 4-methylene-1-(1-methylethyl) were found as major compounds, and they could be responsible for the antimicrobial activity of the oils.

Keywords: Essential oil, Rutaceae, Antimicrobial activity, GC-MS analysis, MIC, *Bacillus subtilis, Escherichia coli*

1. Introduction

The indigenous systems of medicine in Sri Lanka consist of Ayurveda, Siddha, and Unani medicine [1]. Medicinal plants have been used to prepare medicines in these medical systems since ancient times for the treatment of various diseases in human beings [2, 3]. *C. aurantifolia, C. latifolia, A. marmelos,* and *L. acidissima*) contain aromatic plants and are thus expected to contain essential oils [4], which are frequently used as flavorings in a variety of goods, including

Corresponding author: haroonmh@seu.ac.lk

beverages, soaps, cosmetics, and household products. Their essential oils have also been frequently used in aromatherapy [5] and medical treatments due to their antimicrobial, antifungal, antibacterial, and antiparasitic properties [6-8]. The highest antimicrobial activity of the essential oil of lime leaf indicated that it contains geranial, limonene, neral, kariofilena, and citronellal. It is widely cultivated around the world; and almost all parts of the plant are used in traditional medicine as preserves, astringents, diuretics, insect repellents, antiseptics, and antimicrobials for treatment of gastrointestinal ailments, coughs, colds, sore throats, acne, asthma, chilblains, dull skin, flu, varicose veins, and also citral oil is extracted for use in perfumes [9]. The essential oil of *C. latifolia* enabled the identification of twenty-six volatile components. In the volatile extract, different groups of terpenoid compounds are present, such as hydrocarbons, alcohols, aldehydes, ketones, esters, and others [10]. The monoterpenes are dominant and are represented mainly by Limonene. Geranial and neral oxygenated monoterpenes are the most common oxygenated monoterpenes.Sesquiterpenes are mainly represented by β-caryophyllene[11] and *C. latifolia,* which contains a wide range of active ingredients. They are rich in vitamin C, flavonoids, acids, and volatile oils [12].They also contain coumarins such as bergapten, which sensitize the skin to sunlight [11, 13].

Various chemical constituents like alkaloids, coumarins, and steroids have been isolated and identified from *A.marmelos* oil, *i.e.*, α-phellandrene, α-pinene and δ-carene were major components identified and sesquiterpene hydrocarbons represented, *i.e.* γ-cadinene was the major component, and oxygenated compounds represented, *i.e.,* monoterpene alcohols, sesquiterpene alcohols, aldehyde, ester and *trans-2*-hydroxycinnmic acid [14]. Medicated oil prepared from the leaves of the plant not only helps to prevent colds, coughs and other respiratory ailments, but is also a good hair tonic when mixed with cumin seeds and massaged onto the scalp [15].

The essential oil from the *L. acidissima* leaves has been found to be rich in methyl chavicol, linalool, caryophyllene, cis-amethole, p-methoxy phenyl-2-propanone, elemicine-3,4-dimethoxy benzaldehyde, and alcohol. Light petroleum ether extract of the leaves afforded stigmasterol; the ether extract gave psorlerin and bergapten, while ethyl acetate extracts yielded orienthin, vitexin and saporarin. The essential oil is considered a substitute for anise and funnel oil [16].The *L. acidissima* leaves are good for vomiting, hiccoughs, and dysentery. The leaves are traditionally used in Ayurveda as antiemetic, aromatic, astringent, carminative, cardiotonic, expectorant, purgative, sudorifi, and useful in anorexia, bronchitis, calculus, and cardiac debility. Cough, diarrhea, gastropathy, hiccups [16]. Even though a lot of studies have been done in the world on the family of Rutaceae, there is very limited data available on the antimicrobial activity of Sri Lankan Rutaceae. Therefore, in this paper, we report the antimicrobial activity of essential oils extracted from four selected plants of the family Rutaceae, namely, *C. aurantifolia*, *C. latifolia*, *A. marmelos*, and *L. acidissima.*

2. Experimental Setup

2.1. *Collection of plant leaves*

The leaves of the selected plants (*C. aurantifolia, C. latifolia, A. marmelos, and L. acidissima or F. limonia)* were collected during the month of June 2019 from the Kalmunai area in the Ampara district of Sri Lanka. The plants were identified by Dr. Mrs. R. A. S. W. Ranasinghe, Deputy Director, National Botanical Garden, Peradeniya (reference no. NH/BOT/4/2020-37).

2.2. *Distillation of essential oil from plant leaves*

450 g of each plant's leaves were cut into small pieces and transferred to separate round-bottom flasks. The oil is distilled using the Clevenger apparatus for 6 hours. The resulting volatile oil was collected, then released from the air content by adding anhydrous sodium sulfate, separated the oil, and measured the volume using the scaled measuring cylinder. The oil was stored at 4 °C until used for the antimicrobial assay.

2.3. *Determination of Essential Oil Component*

Chemical components of essential oil were analyzed by Gas Chromatography-Mass Spectrometry as Thermo Scientific TRACE^{TM} 1300 Series GC operated with a split mode injector, Thermo Scientific AI/AS 1310 Series autosampler and Thermo Scientific^{TM} ISQ^{TM} Series GC-Single Quadrupole MS. The following were the specifications used for analysis. Column: TG Wax MS (Acid deactivated polyethylene glycol) 30 m, 0.25 mm i.d, 0.25 µm film thickness (Thermoscientific, USA). Temperature program: from 60 °C – 150 °C at 3 °C/min and from 150 °C – 240 °C at 7 °C/min. Injection temperature: 240 °C. Injection volume: 1.0 µL. Inlet pressure: 86.3 kPa. Carrier gas: He, flow rate: 1.000 mL/min. Injection mode: split (50:1). Mass interface temp: 250 °C; MS mode: EI detector voltage: 70 eV; mass range: 40 – 450; interval: 0.2 sec. Data handling was done through Xcalibur software. The relative amount of individual components of the total oil is expressed as a percentage peak area. The relative amount of individual components of the total oil is expressed as a percentage of the peak area relative to the total peak area. Qualitative identification of the different constituents was performed by comparison of their relative retention times and mass spectra with those of authentic reference compounds, or by retention indices (RI) and mass spectra. Compound identification was done by comparing the National Institute of Standards and Technology (NIST) library data of the mass spectra peaks with those reported in literature.

2.4. *Antimicrobial assay*

Antimicrobial activity of essential oils of plant leaves was determined on bacterial strains (*E coli* and *Bacillus* spp) and fungi (*Aspergillus* spp and *Trichoderma*) by agar diffusion method, using paper disc (6 mm diameter). All the experiments were conducted in triplicate. Each bacterial and fungal isolate was subcultured for purity before used.

Preparation of Bacterial / Fungi Inocula (0.5 MacFarland Standard)

Each isolated bacterial/fungal colony was taken separately onto a sterile cotton wool plug and smeared on the inner wall of a fertile universal bottle containing approximately 2 mL of salt water. The bottle was capped and vortexed (whirlpooled) for 5 s to uniformly suspend the bacterial or

fungal culture. The turbidity of the suspension was made similar to that of the 0.5 MacFarland standard by the addition of more micro living things or dilution with more normal salt water.

Disc Diffusion Method

A broth suspension with a turbidity alternative to the 0.5 McFarland standard was prepared from a pure culture of each of the test microbes. A Muller Hinton Agar (MHA) plate was infected with 1 mL of the broth suspension and the plate rotated to allow even spread of the substance. After removing the excess fluid, the plate is allowed to dry for 15 minutes at 37 °C.Each oil (5 µL) was impregnated into blank discs (6 mm diameter) and incubated at 37 °C for 24 hrs. For bacteria, MHA was used as a media, and Potato Dextrose Agar (PDA) was used as a media for fungi.

2.5. Determination of MIC (Minimum Inhibitory Concentration)

The Minimum Inhibitory Concentration of the oils was determined using the Brain Heart Infusion Broth dilution method [17]

2.6. Statistical analysis

The diameter of the inhibition zone and MIC resulting from replicates were expressed as mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance (ANOVA, P value < 0.05) using statistical software, DMRT.P system.

3. Results and discussion

3.1 GC-MS analysis

The yield percentage of essential oil of *C aurantifolia* and *C latifolia* leaves was 0.6% (v/w) and 0.2% (v/w), respectively, on a wet weight basis, while the yield percentage of *A. marmelos* leaves and *L. acidissima* was 0.8% (v/w) and 0.7% (v/w) on a wet weight basis, respectively.

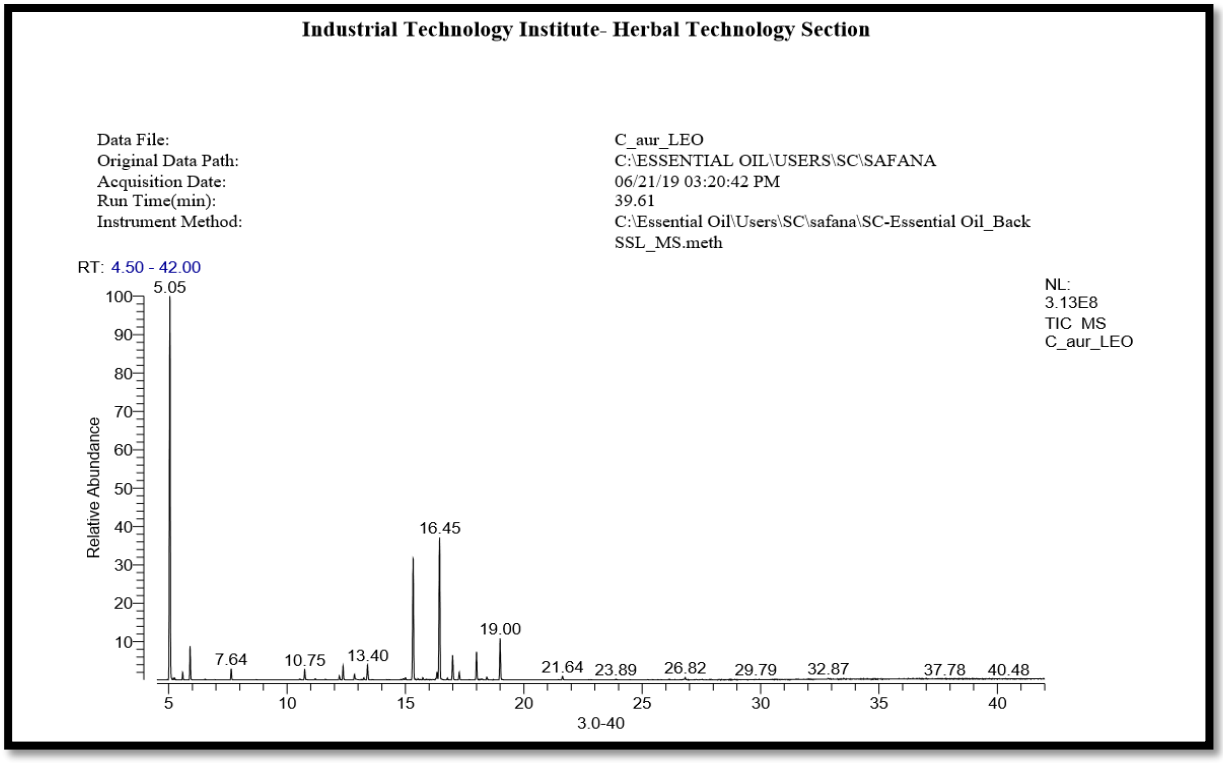


Figure 1.GC-MS Chromatogram of *Citrus aurantifolia* essential oil

The GC- MS results revealed that the essential oil of *C. aurantifolia* leaves has 50 chemical components, including D-limonene (41.13%), Cis-citral (14.75%), *α*-citral (17.20%), and geraniol (4.37%) as major compounds. The compounds can be classified into 6 groups, namely, monoterpene, monoterpene alcohol, monoterpene aldehyde, sesquiterpene, sesquiterpene alcohol, and others. (Fig.1)

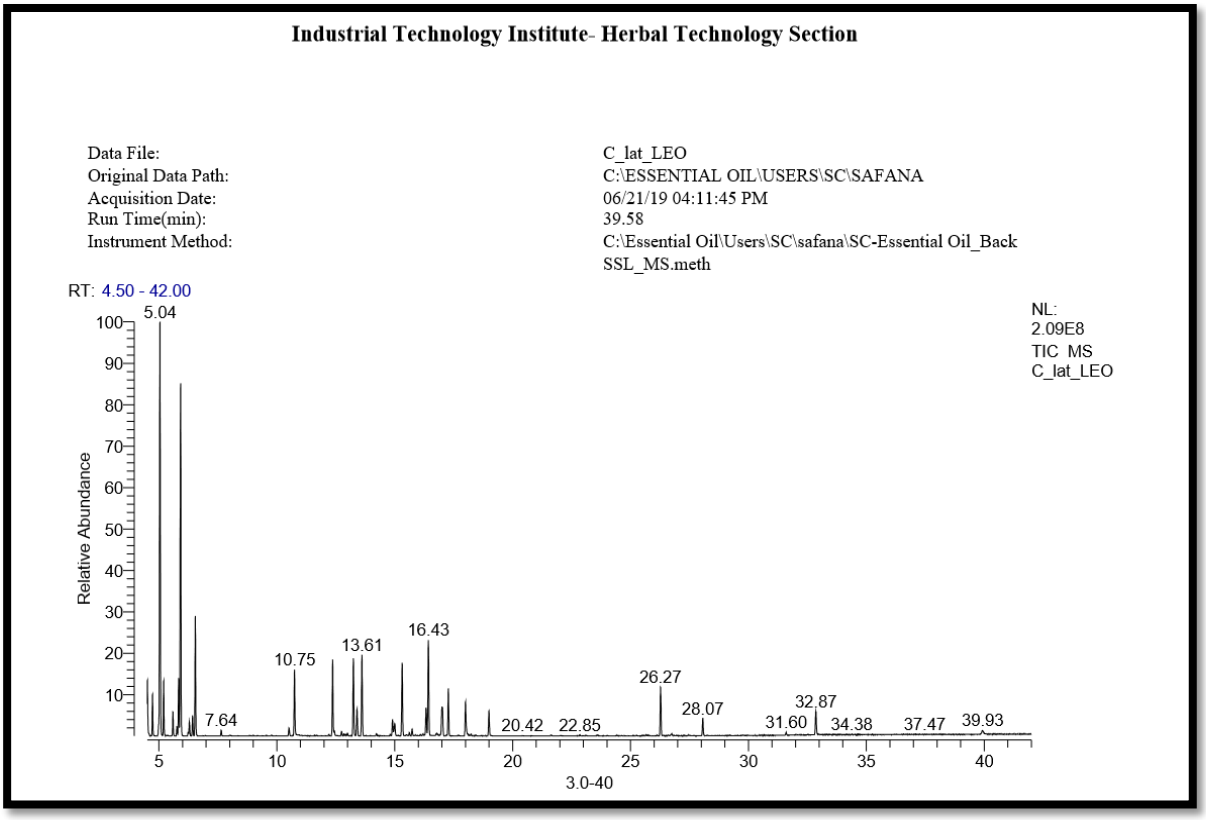


Figure-2. GC-MS Chromatogram of *Citrus latifolia* essential oil

Similarly, the GC-MS result shows that the essential oil of *C. latifolia* leaves has 50 chemical components including D-limonene (11.21%), Bicyclo [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-(22.28%), 3-carene (12.86%), α-myrcene (4.34%) as major compounds (Fig.2)

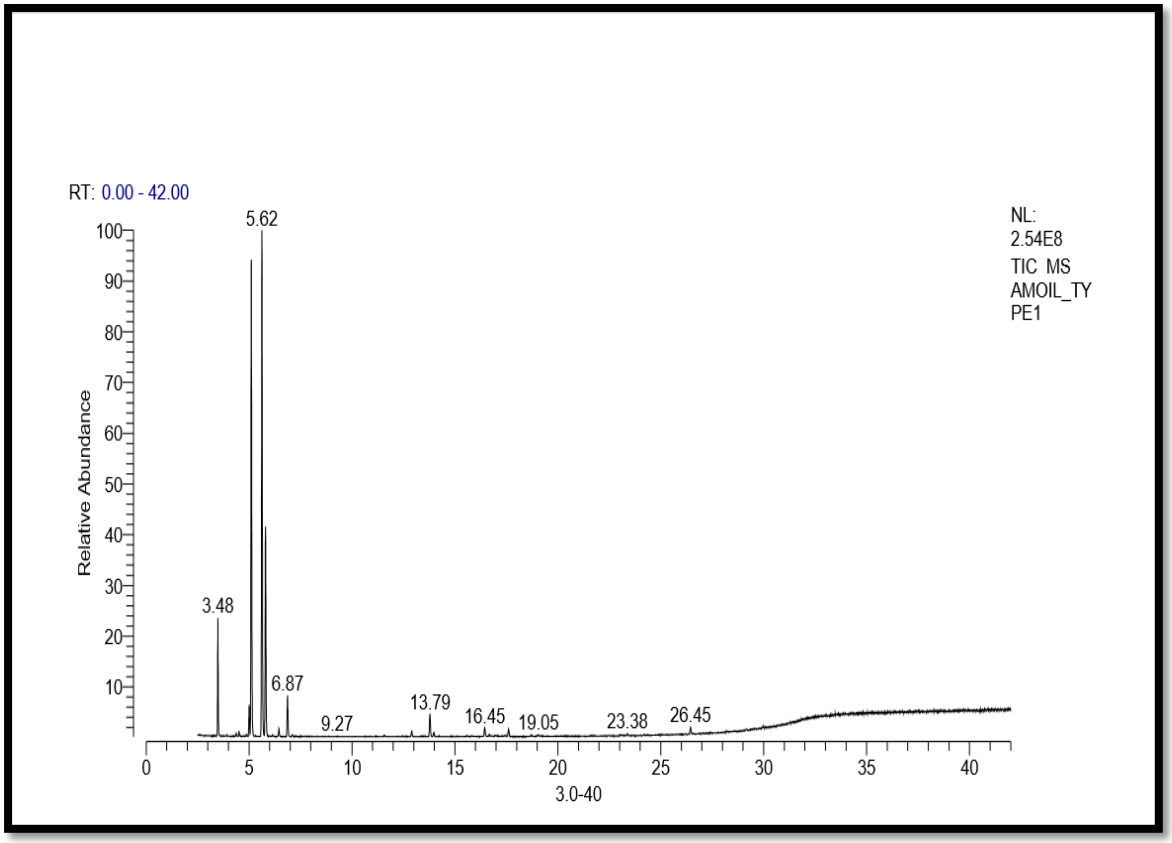


Figure 3. GC-MS Chromatogram of *Aegle marmelos* essential oil

Likewise, the GC- MS result revealed that the essential oil of *A. marmelos* leaves has 50 chemical components, including D-Limonene (5.62%), 3-Carene (6.35%), Bicyclo [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-(3.49%), β-Myrcene (1.9.%), and o-Cymene (6.87%) as major compounds.(Fig.3)

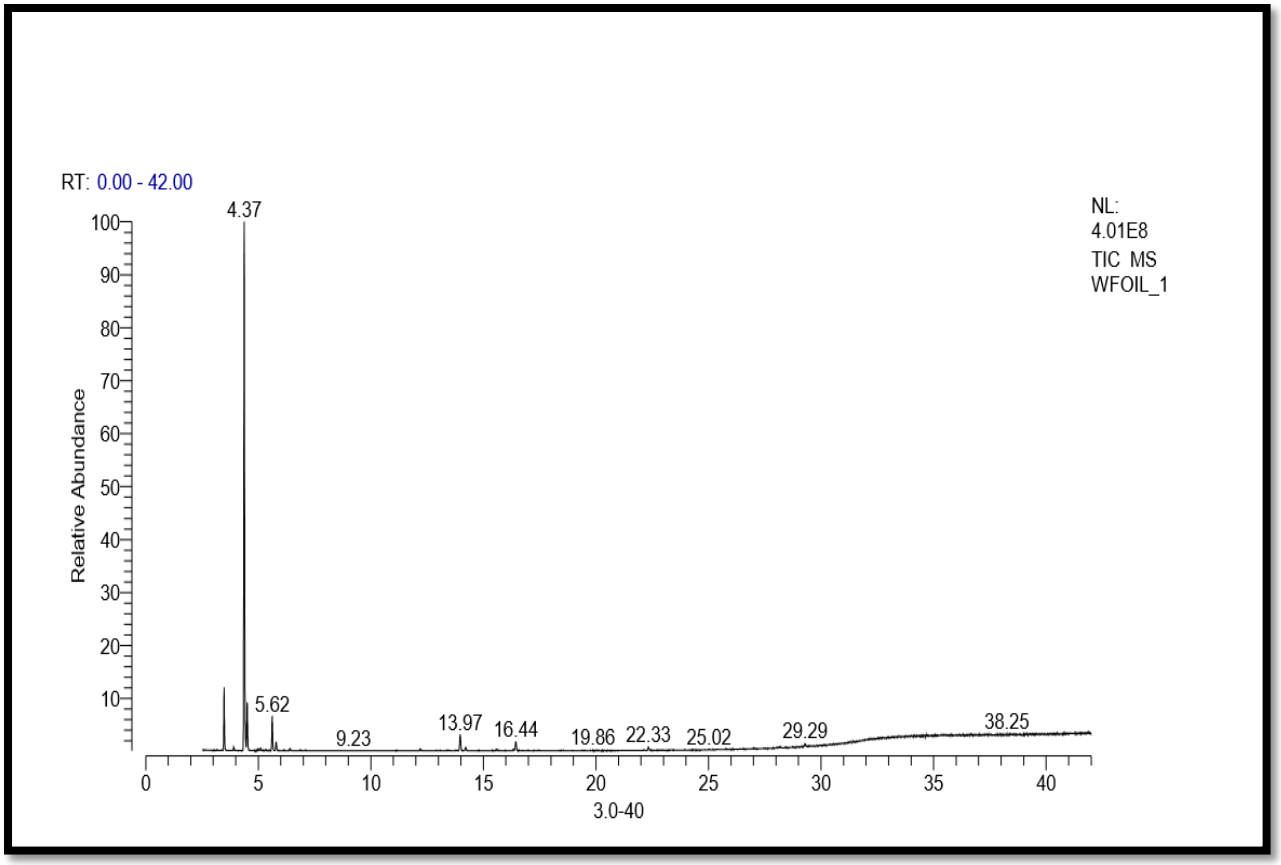


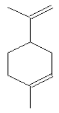
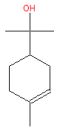
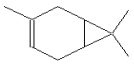
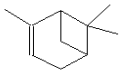
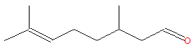
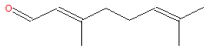
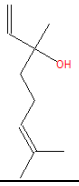
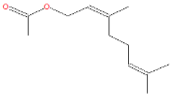
Figure 4. GC-MS Chromatogram of *Limonia acidissima* essential oil

The essential oil of *L. acidissima* leaves consists of 50 chemical components, including D-Limonene (5.62%), Bicyclo [3.1.1] heptane, 6,6-dimethyl-2-methylene-, (IS)-(4.37%), Bicyclo [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-(6.67%), and 1R-α-Pinene (3.48%) as major compounds. The compounds distinguished can be classified into 6 groups, namely, monoterpene, monoterpene alcohol, monoterpene aldehyde, sesquiterpene, and sesquiterpene alcohol in different percentages. (Fig.4).

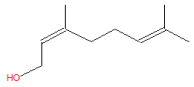
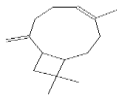
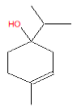
Table 1. GC-MS profile of essential oils of Rutacea family plants leaves

3.2 Antimicrobial activity of the essential oils

Essential oils, which are obtained from medicinal plants, have been used by mankind for a wide range of medicinal purposes [18]. Antimicrobial screening was carried out with 5 µL of the oil sample using the disc diffusion method against one gram-positive (*Bacillus subtilis)* and gram-negative (*Escherichia coli)* bacteria and two fungal strains, *Aspergillus* spp. *and Trichoderma* spp. Among the four oils, *C.latifolia* showed potential antibacterial properties against the tested bacteria. But these four oils were inactive against the fungal strains. In this study, the MIC values for these four oils were found within the range of 1.25 - 5 (V/V%) (Table 3). *C. latifolia* essential

Compounds	**Retention time**	**NIST No.**	**Structure**
Monoterpenes			
D-Limonene	5.03	365767	
α-Terpineol	15.74	186483	
3-Carene	4.27	77623	
o-Cymene	6.87	113988	
1R-α-Pinene	3.48	140985	
Acyclic Monoterpenoids			
R)- (+)-Citronellal	10.75	108479	
cis-Citral	15.33	290609	
Linalool	12.37	352637	
cis-Geranyl acetate	16.33	352640	

oil has Zones of Inhibition (ZOI) of 9.66 mm and 11.25 mm against *E. coli* and *Bacillus* spp., respectively (Table 2).The MIC value of *C. latifolia* oil is 1.25 (v/v%) against *E. coli* and 2.5

cis-Geraniol	18.00	352645	
Sesquiterpenes			
Caryophyllene	13.40	291486	
Oxygenated Monoterpenes			
.(-)-Terpinen-4-ol	13.61	68755	
α-Terpineol	15.74	186483	

(v/v%) for *B. subtilis* (Table 3). Similarly, the MIC value of *A. marmelos* oil is 2.5 (v/v%) against *E. coli* and 5 (v/v%) against *B. subtilis*. Therefore, this oil is less active for these pathogenic bacterial strains than *Citrus latifolia* oil. Ibrahim, *et al.* (2015) reported that *A. marmelos* oil has shown the lowest MIC value against *A. niger* at 50 µL/mL but in our study the MIC value of *A. marmelos* oil was 2.4 ± 0.1 (v/v%) against *E. coli* and 5.0 ± 0.25 (v/v%) for *Bacillus subtilis* with the Zone of Inhibition (ZOI) of 9.11 ± 0.5 mm and 10.3 ± 1.7 mm against *E. coli* and *B. subtilis*, respectively. The same authors further reported that the essential oil of *C. aurantifolia* leaf showed antibacterial activity against *B. subtilis*, with a minimum inhibitory concentration of 0.125% (v/v) and the same authors further reported that it did not show antifungal activity [4, 17]. The essential oil of *C. aurantifolia*, on the other hand, demonstrated antibacterial activity against the bacteria *B. subtilis*, with a minimum inhibitory concentration of 2.4 0.1 (v/v%), according to the findings of this study. According to Jazet et al. (2008) [11], essential oil of *C. latifolia* exhibited no antibacterial activity. However, the current study found essential oil of *C. latifolia* to have Zones of Inhibition (ZOI) of 9.6 mm and 11.2 mm (Table 2) against *E. coli* and *B.subtilis*, with MIC values of 1.23 0.05 and 2.4 0.1 (v/v%), respectively. (Table 3).

4. Conclusion

This study revealed that four essential oils separated from the Sri Lankan traditional medicinal plants, *C. aurantifolia, C. latifolia, A. marmelos,* and *L. acidissima* of the family Rutaceae, were found to have an antibacterial effect against *E. coli* and *B. subtili,* and particularly, for the first time, we report the antibacterial properties of essential oils from Sri Lankan medicinal plants. All four oils resulted in high ZOI against *Bacillus subtili* as *C. aurantifolia* (9.4 mm), *C. latifolia* (11.2

mm), *A. marmelos* (10.3 mm), and *L. acidissima* (10.9 mm). GC–MS analysis revealed that *α*-citral, *β*-citral, D-limonene, β-myrcene, citronellal, linalool, 3-carene, geraniol, geranyl acetate, caryophyllene, terpinene, bicyclo [3.1.0] hexane, and 4-methylene-1-(1-methylethyl) were found as major compounds and they could be responsible for the antimicrobial activity of the oils.

Table: 2. Diameter of Zone of inhibition (mm) essential oils of plant leaves

Essential oils	**Microorganism**	
	Escherichia coli	*Bacillus subtili*
Citrus aurantifolia	8.7 ± 0.3^{a}	9.4 ± 0.3^{a}
Citrus latifolia	9.6 ± 1.6^{a}	11.2 ± 0.3^{a}
Aegle marmelos	9.1 ± 0.5^{a}	10.3 ± 0.3^{a}
Limonia acidissima	7.7 ± 0.2^{a}	10.9 ± 0.3^{a}

Values are mean of replicates ± standard deviation at $P<0.05$

Table-3. Minimum Inhibitory Concentration MIC (v/v %) of essential oils of plant leaves

Essential oils	**Microorganism**	
	Escherichia coli	*Bacillus subtili*
Citrus aurantifolia	2.4 ± 0.1^{b}	1.2 ± 0.1^{b}
Citrus latifolia	1.2 ± 0.01^{b}	2.4 ± 0.16^{b}
Aegle marmelos	2.4 ± 0.1^{b}	5.0 ± 0.2^{b}
Limonia acidissima	1.2 ± 0.1^{b}	2.5 ± 0.1^{b}

Values are mean of replicates ± standard deviation at $P<0.05$

Acknowledgments

Kindly acknowledge the technical support obtained from the Industrial Technology Institute, Colombo and the Department of Chemical Sciences, Faculty of Applied Sciences, South Eastern University of Sri Lanka, Oluvil, Sri Lanka.

References

1. Organization, W.H., Traditional medicine in the WHO South-East Asia Region: review of progress 2014–2019. 2020.
2. Upadhyay, R., Essential oils: anti-microbial, antihelminthic, antiviral, anticancer and antiinsect properties. Journal of Applied Biosciences, 2010. **36**(1): p. 1-22.
3. Jones, M. and C. Liyanage, Traditional medicine and primary health care in Sri Lanka: Policy, perceptions, and practice. Asian Review of World Histories, 2018. **6**(1): p. 157-184.
4. Musdja, M.Y., A. Agusta, and A.M. Pariwidjayanti, Activity test and inhibitory mechanism of essential oil of lime leaf (citrus aurantifolia, swingle) against some of bacterial pathogens. 2017.

5. Ali, B., et al., Review article essential oils used in aromatherapy: a systemic review. Asian Packag J Trop Biomed. 2015; 5 (8): 601–11. This review enumerates the various oils used by people who do aromatherapy.
6. Lemes, R.S., et al., Chemical composition and antibacterial activity of essential oils from Citrus aurantifolia leaves and fruit peel against oral pathogenic bacteria. Anais da Academia Brasileira de Ciências, 2018. **90**: p. 1285-1292.
7. Chouhan, S., K. Sharma, and S. Guleria, Antimicrobial activity of some essential oils—present status and future perspectives. Medicines, 2017. **4**(3): p. 58.
8. Abers, M., et al., Antimicrobial activity of the volatile substances from essential oils. BMC complementary medicine and therapies, 2021. **21**(1): p. 1-14.
9. Sandoval-Montemayor, N.E., et al., Chemical composition of hexane extract of Citrus aurantifolia and anti-Mycobacterium tuberculosis activity of some of its constituents. Molecules, 2012. **17**(9): p. 11173-11184.
10. González-Mas, M.C., et al., Volatile compounds in citrus essential oils: A comprehensive review. Frontiers in plant science, 2019. **10**: p. 12.
11. Jazet, D.P., et al., Antiradical potential and antifungal activities of essential oils of the leaves of Citrus latifolia against Phaeoramularia angolensis. African Journal of Biotechnology, 2008. **7**(22).
12. Klimek-Szczykutowicz, M., A. Szopa, and H. Ekiert, Citrus limon (Lemon) phenomenon—a review of the chemistry, pharmacological properties, applications in the modern pharmaceutical, food, and cosmetics industries, and biotechnological studies. Plants, 2020. **9**(1): p. 119.
13. Md Othman, S.N.A., et al., Essential oils from the Malaysian Citrus (Rutaceae) medicinal plants. Medicines, 2016. **3**(2): p. 13.
14. Ibrahim, N.A., et al., Chemical composition, antimicrobial and antifungal activities of essential oils of the leaves of Aegle marmelos (L.) Correa growing in Egypt. Journal of Applied Pharmaceutical Science, 2015. **5**(2): p. 001-005.
15. Dutta, A., et al., Ethnological and Ethno-medicinal importance of Aegle marmelos (L.) Corr (Bael) among indigenous people of India. American Journal of Ethnomedicine, 2014. **1**(5): p. 290-312.
16. TRAVELLED, F.L.A.P.L., www. ijrap. net. International Journal of Research in Ayurveda & Pharmacy, 2010. **1**(1): p. 98-106.
17. Thileepan, T., V. Thevanesam, and S. Kathirgamanathar, Antimicrobial Activity of Seeds and Leaves of Myristica fragrans against Multi-resistant Microorganisms. J Agric Sci Technol A, 2017. **7**(5): p. 302-308.
18. de Oliveira, M.S., et al., Potential of medicinal use of essential oils from aromatic plants. Potential of essential oils, 2018: p. 1-21.