International Journal of Enteric Pathogens

Int J Enteric Pathog. 2016 November;4(4):e37103 Original Article

http://enterpathog.com

doi 10.15171/ijep.2016.13

Evaluation of Antibacterial Activities of *Citrus limon, Citrus reticulata*, and *Citrus grandis* Against Pathogenic Bacteria

Sholeh Saeb[1,2], Mansour Amin[3,2*], Reza Seyfi Gooybari[4], Nasrin Aghel[4]

[1]Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3]Health Research Institute, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[4]Department of Pharmacology, School of Pharmacy, Ahvaz Jundishapour University of Medical Sciences, Ahvaz, Iran

***Corresponding Author:**
Mansour Amin; Health Research Institute, Infectious and Tropical Diseases Research Center, Department of Microbiology, School of Medicine , Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
Tel: +98-9163051096;
Fax: +98-6133332036;
Email: mnsamin@yahoo.com

Published Online November 15, 2016

Keywords: Antibacterial activity, E-test, *Citrus grandis, Citrus reticulata, Citrus limon*, Extract, Essential oil, pathogenic bacteria

Abstract

Background: Microorganisms resistant to most antibiotics are rapidly spreading, and there is an urgent and continuous need for novel antimicrobial compounds. The genus Citrus belongs to the family Rutaceae and has many biologically active secondary metabolites.

Objectives: The purpose of this study was to evaluate the antimicrobial activity of essential oil and extract of Lemon (*Citrus limon*), Mandarin (*Citrus reticulata*), and Pummelo (*Citrus grandis*) against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis*, and *Salmonella typhi*.

Materials and Methods: The fresh *Citrus* leaves were shade-dried and powdered. Then, their antimicrobial metabolites were extracted by 80% methanol, and a Clevenger-type apparatus was used for essential oil. Eight different concentrations of each leaf extract and essential oil were prepared. The antimicrobial susceptibility of Citrus leaf metabolites were assayed against 4 bacterial strains by agar disc diffusion and E-test method.

Results: In this study, minimum inhibitory concentrations (MICs) of different Citrus leaf extracts were determined against all four food-borne pathogens. The *C. grandis* leaf essential oil had potent antimicrobial activity against all four pathogens, and the *C. limon* leaf essential oil was effective on Gram positive bacteria. *Salmonella typhi* was resistant against these two leaves' essential oils.

Conclusion: The results showed that the extracts had no antimicrobial effect on tested bacteria. In this study, the antibacterial effect of essential oil of Citrus leaves on four strains of pathogenic microorganisms was confirmed. The *C. grandis* leaf essential oil had the most powerful antimicrobial properties, suggesting its potential application as natural preservative in foods or an effective medicine against different pathogenic microbes.

Received May 12, 2016; **Revised** August 28, 2016; **Accepted** October 8, 2016

Background

Nowadays, bacteria are considered as the main cause of morbidity and mortality in many developing countries, especially amongst children. Among the bacterial pathogens, *Escherichia coli*, *Salmonella* spp, and *Staphylococcus aureus* are most prevalent.[1] In the recent decades despite developments of different types of antibiotics by pharmacological industries, resistance to antibiotics has been increased significantly in many bacterial pathogens.

In general, bacteria have several genetic mechanisms to transmit and acquire resistance to antibiotics.[2-4] The antibacterial resistance in the bacterial populations is rapidly spreading; this is a serious threat to successful treatment of infectious diseases.[1,5] On the other hand, the side effects associated with the commercial antibiotics are frequently reported.[6] In this regard, there is an urgent and continuous need for finding and investigating novel antimicrobial compounds.

Most of the antibiotics have been developed from microorganisms. Moreover, the plant materials remain an important resource for finding the novel antimicrobial compounds.[1,7] There are many published reports on the antibacterial effectiveness of the traditional herbs against the gram-positive and gram-negative bacteria.[1] Microbial cells are negatively affected by plant-derived substances via various mechanisms of actions as these substances at-

tack the phospholipid bilayer of the cell membrane and disrupt enzymatic systems. The medicinal herbs have the bacteriostatic effects on the enzymatic activity associated with energy production, or they can cause denaturation of proteins, modifying cell wall permeability, or causing the loss of macromolecules. Therefore, it is difficult for the microorganisms to develop resistance against these medicinal herbs.[8]

The genus Citrus is one of the most effective herbs in traditional medicine that belongs to the family of Rutaceae. Citrus is the native genus of tropical and subtropical areas in Asia.[9] The members of this genus are characterized by many biologically active secondary metabolites such as flavonoids, limonoids, coumarins and furanocoumarins, sterols, volatile oils, organic acids, and alkaloids. Many Citrus species are recognized for their medicinal, physiological, and pharmacological activities including antimicrobial, antioxidant, anticancer, anti-inflammatory, and hypoglycaemic activities.[10] There has been an increasing interest in looking for the antimicrobial properties of the plant-derived extracts particularly the essential oils. Essential oils are rich sources of the biologically active compounds with antibacterial, antifungal, antiviral, insecticidal, and antioxidant properties.[5]

Objectives

The purpose of this study was to determine the antibacterial effects of leaf methanolic extract and essential oil of three species of Citrus against *Escherichia coli, Bacillus subtilis, S. aureus,* and *Salmonella typhi* by agar disc diffusion and E-test method.

Materials and Methods

Plant Collection and Identification

The fresh leaves of *Citrus grandis*, *Citrus reticulata,* and *Citrus limon* were collected in the Research Station of the Faculty of Agriculture (Shahid Chamran University of Ahvaz, Iran) in July 2015. The plants were identified phenotypically and confirmed for taxonomic identity by Dr. A. Eftekhari, Faculty of Agriculture, Shahid Chamran University. Voucher specimens were deposited at Faculty of Pharmacy, Ahvaz Jundishapur University of medical sciences, Iran.

Microbial Strains

The reference strains of four bacteria including *S. aureus* (PTCC 1112), *E. coli* (PTCC 1399), *B. subtilis* (PTCC 1156), and *S. typhi* (PTCC 1609) were obtained from Persian Type Culture Collection, Tehran, Iran. The strains were sub cultured for further use. The stock cultures were maintained on nutrient broth medium plus 10% glycerol at -80°C.

Extraction of Leaf Extract

The plants were shade-dried at room temperature for 72 hours and powdered in a blender. One hundred grams of each sample powder was weighed accurately and allowed to soak in 500 mL of 80% methanol (Merck, Germany). These extracts were incubated at ambient temperature for 24 hours. The extract was filtered through Whatman filter paper No. 3 and the solvent was evaporated under reduced pressure using vacuum evaporator at 40°C to dry. The extracts were transferred to glass vials and kept at 4°C before use.[11]

Preparation of Different Concentrations of Methanolic Extracts

Different plant extracts used in this study were dissolved in 80% methanol for evaluation of their antimicrobial susceptibility. The concentrations were as follows: *C. reticulata* 400 mg/mL, *C. limon* 150 mg/mL, and *C. grandis* 200 mg/mL.

Extraction of Leaf Essential Oil

The fresh leaves (50 g) of *C. grandis, C. reticulata,* and *C. limon* were separately subjected to hydro-distillation for 6 hours using a Clevenger-type apparatus. All essential oils were dried over anhydrous sodium sulphate and stored in brown vials in the refrigerator at 4°C for the next step of test.[10,11]

Preparation of Different Concentrations of Essential Oils

The stock solution of essential oils was prepared by dissolving 10 mg of leaf essential oils of three Citrus species in 10 mL of 80% methanol. Eight dilutions of these essential oils were made for determination of minimum inhibitory concentration (MIC). The concentrations of the *C. limon* leaf essential oil were prepared as follows: 160, 80, 40, 20, 10, 5, 2.5, and 1.25 mg/mL. The concentrations of the *C. reticulata* leaf essential oil were prepared as: 180, 90, 45, 22.5, 11.25, 5.75, 3, and 1.5 mg/mL and those of the *C. grandis* as: 120, 60, 30, 15, 7.5, 3.75, 2, and 1 mg/mL.[12]

Antimicrobial Susceptibility Test

Antibacterial activity was measured by disc diffusion method (Kirby-Bauer method). The paper disc diffusion (CLSI, 2015) was employed to determine the antibacterial activity of both essential oil and extract of the respective herbal preparations. All bacterial strains were grown separately in sterile Mueller Hinton agar (Merck, Germany) plate for 4 hours at 37°C. The turbidity of the inoculum was adjusted to 0.5 McFarland standard. The inoculum was spread over Mueller Hinton agar plate using a sterile cotton swab for obtaining a uniform microbial growth. The blank discs (Patan teb, Iran) were loaded with 10 µL of essential oils and extracts of three Citrus leaf species according to concentrations of the sterile condition. A blank disc was also loaded with 10 µL of 80% methanol as negative control. The plates were incubated for 24 hours at 37°C. After incubation, the diameters of the growth inhibition zones were measured.

Determination of Minimum Inhibitory Concentration

MIC was measured by modified E-test method.[12,13] All bacterial strains were grown separately in sterile Mueller Hinton Broth (Merck, Germany) tube for 4 hours at 37°C. The turbidity of the inoculum was adjusted to 0.5 McFarland standard. The inoculums were spread over the Mueller Hinton agar plate using a sterile cotton swab for obtaining a uniform microbial growth. The plates were dried at ambient temperature for 15 minutes before applying the discs. Eight sterile discs (6 mm in diameter) were kept on the agar surface in a line. Ten microliters of each dilution was separately impregnated with the disc. The diluted inoculum was incubated for 24 h at 37°C. The final concentrations of the essential oil of *C. limon* leaf were prepared as follows: 1600, 800, 400, 200, 100, 50, 25, and 12.5 µg/mL. The final concentrations of the essential oil of *C. reticulata* leaf were prepared as: 1800, 900, 450, 225, 112.5, 56.25, 28, and 14 µg/mL, and those of the essential oil of *C. grandis* leaf were prepared as: 1200, 600, 300, 150, 75, 37.5, 18.75, and 9.5 µg/mL. After incubation, the MICs were determined by detecting cut-off disc of the growth inhibition zones (Figure 1). This test was repeated for three times and the average results were reported.

Results

In this study, MICs of different Citrus leaf extracts were determined against four food-borne pathogens. The essential oils obtained from *C. grandis* leaf exhibited a significant antimicrobial activity against four tested pathogens. The essential oils extracted from leaves of *C. limon* and *C. reticulata* exhibited effective antimicrobial activities against *S. aureus, E. coli,* and *B. subtilis*. However, none of methanolic extracts derived from these three Citrus species showed antimicrobial activity against the tested bacteria. The *C. grandis* leaf essential oil had potent antimicrobial activity against all four pathogens, and the *C. limon* leaf essential oil was effective on Gram-positive bacteria. *Salmonella typhi* was resistant against two leaves' essential oils. The result of antibacterial activity of Citrus leaf essential oil is shown in Table 1.

Overall, the essential oils obtained from *C. grandis* leaf exhibited significantly higher activity against Gram negative bacteria like *E. coli* and *S. typhi* than those against Gram positive bacteria like *S. aureus* and *B. subtilis*. The essential oils extracted from leaf of *C. limon* showed significantly higher antibacterial activity against Gram positive bacteria such as *S. aureus* and *B. subtilis* than those against Gram negative bacteria like *E. coli* and *S. typhi*. The essential oils obtained from *C. limon* and *C. reticulata* had no activity against *S. typhi*.

Discussion

Essential oil and extract of plants have many potential applications and have been used for many thousands of years in food preservation, pharmaceuticals, alternative medicine, and natural therapies.[10,12] Essential oils are potential sources of novel antimicrobial compounds especially those against bacterial pathogens.[12] The volatile oils particularly essential oils of Citrus spp have shown bactericidal and fungicidal activities.[10] Citrus essential oils are a mixture of volatile compounds and are consisted mainly of monoterpene hydrocarbons. The studies have shown that monoterpenes exert damaging effects on microbial membrane.[13] An important characteristic of essential oils is their hydrophobicity, which enables them to divide the lipids of bacterial cell membrane and mitochondrial membrane, thereby disturbing the cell structure and making it more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions would lead to bacterial death. Gram-negative bacteria were more sensitive to the essential oils than gram-positive bacteria.[12]

Microbes are all around us and because of their resistance to many antibiotics, it has now become difficult to control them.[4] *Staphylococcus aureus* is a common cause of food poisoning in releasing enterotoxins into food. *Escherichia coli* are bacteria found in the environment, food, and intestines of both humans and animals. *Escherichia coli* cause serious food poisoning in their hosts. *Bacillus subtilis* is found in soil and in gastrointestinal tract of ruminants and humans. *Bacillus subtilis* presents some risk to humans. Typhoid fever is a potentially fatal multisystemic bacterial infection caused by *S. typhi*.

A large number of researchers have focused on the study of peel of Citrus fruits and their antimicrobial activities,[2,6,16-19] however our search of the literature revealed that there is a few studies about the antimicrobial activity of Citrus leaves. Therefore, the current study was done on antimicrobial activity of methanolic extract and hydro-distilled essential oil of citrus leaves.

In this study, MICs were determined for the antimicrobial activities of a few Citrus leaf extracts and essential oils and assayed against all four food-borne pathogens *(S. aureus, E. coli, B. subtilis, and S. typhi)*. Among them, the *C. grandis* leaf essential oil had potent antimicrobial activity against all four pathogens, and the *C. limon* leaf essential oil was the most effective agent against gram-positive bacteria. *S. typhi* was observed resistant against two leaves' essential oils. No antimicrobial activity was observed for the citrus leaf extracts against the selected bacterial strains. Leaf extracts might show antimicrobial activity against other bacterial strains or may show such activity

Table 1. The Minimum Inhibitory Concentration Values (µg/mL) of the Essential Oils of Citrus Leaves Against Tested Bacteria

Name of Plants	*Staphylococcus aureus*	*Escherichia coli*	*Bacillussubtilis*	*Salmonella typhi*
Citrus limon	200	400	200	-
Citrus reticulata	900	900	1800	-
Citrus grandis	900	150	600	300

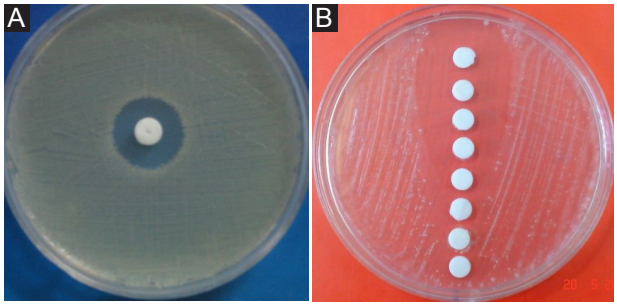

Figure 1. (A) Antimicrobial Susceptibility Assay of Essential Oil Obtained From *C. grandis* Leaf on *B. subtilis* by Disk Diffusion Method. (B) The MIC Value of the Essential Oil of *C. grandis* Leaf on *E. coli* by Modified E-test.

with further modifications in the extraction protocols.

Ekwenye and Edeha in 2010 evaluated the antibacterial activity of ethanolic and aqueous leaf extract of *Citrus sinensis* against bacterial strains like *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *S. aureus.* The antibacterial effect of aqueous extract showed a zone of inhibition on *E. coli*, which was 7 mm in diameter, while on the other organisms it showed little or no zones of inhibition ranging from 0-3 mm in diameter. The ethanolic extract also showed little zones of inhibition on the test organisms ranging from 1-3 mm in diameter. The MIC evaluated on the ethanolic and aqueous extracts using a 2-fold serial dilution showed no zones of inhibition.[20] A similar study was conducted by Hamdan et al in 2013 on the antimicrobial activity of the hydro-distilled essential oil of the fruit peel and the leaf of the Egyptian Cleopatra mandarin. The results revealed that all the tested essential oils had moderate antibacterial activities against bacteria including *S. aureus, E. coli, K. pneumonia,* and *P. aeruginosa*.[10] Adnan et al in his 2014 study observed that the methanolic extracts of leaves of *C. sinensis* and *Citrus paradisi* had high antitumor activities, whereas there was no antimicrobial activity against specified strains including *S. aureus, Micrococcus luteus, E. coli, Bordetella bronchiseptica, Salmonella typhimurium,* and *Enterobacter aerogens*.[21]

The *C. limon* leaf essential oil showed broad spectrum inhibitory against all gram-positive test bacteria. The higher antibacterial activity of the hydro-distillated essential oil from *C. lemon* leaf against gram-positive bacteria was related to Limonene content. There are higher amounts of Limonene in *C. limon* leaf oil (29.13%). The other compounds are Neral (12.72%), neryl acetate (8.53%), p-menth-1-en-7-al (4.63%), beta-pinene (6.35%), and nerol (4.42).[22] Therefore Limonene could be a potential component which contributed to the antibacterial activity of *C. limon*. These findings encourage the use of these oils as antimicrobial agents topically or internally from Citrus spp.

In the present study, *C. grandis* leaf essential oil had the most powerful antimicrobial properties and was found to be effective on both Gram positive and Gram negative organisms. Our present results demonstrated that the Citrus leaves could be very good source for the extraction of antimicrobial components. In addition, the resulting Citrus leaves' essential oils showed a wide spectrum of antimicrobial activities against tested bacteria, thereby suggesting their use as natural preservative in foods or an effective medicine against different pathogenic microbes. These results suggest that essential oils not only would be a natural alternative for chemicals in food preservation, but also could be used as a drug after proper pharmacological evaluation and clinical trials.

Authors' Contributions

Study design: NA. Experiment design, experiment conduct, data interpretation and manuscript edition: MA. Experiment performance: RSG. Data collection, data interpretation, grant application, and manuscript preparation: SS.

Conflict of Interest Disclosures

None.

Ethical Approval

The preliminary proposal of the work was approved by the University Institutional Review and Ethics Board (Ethic Committee code: IR.AJUMS.REC.1393.658).

Funding/Support

This study was financially supported by the Jundishapur University of Medical Sciences under the grant No. 744.

Acknowledgments

This work is part of a Pharm. D. thesis of Reza Seyfi gooybari, which has been approved in the Department of Microbiology of Ahvaz Jundishapur University of Medical Sciences. We acknowledge the assistance provided by the staff of the Laboratory of Microbiology.

References

1. Alavijeh PK, Alavijeh PK, Sharma D. A study of antimicrobial activity of few medicinal herbs. Asian J Plant Sci Res. 2012; 2(4):496-502.
2. Dhanavade MJ, Jalkute CB, Ghosh JS, Sonawane KD. Study antimicrobial activity of lemon (*Citrus lemon* L.) peel extract. Br J Pharmacol Toxicol. 2011;2(3):119-122.
3. Shinkafi SA, Ndanusa H. Antimicribial activity of *Citrus limon* on Acnevulgaris (PIMPLES). International Journal of Science Inventions Today. 2013;2(5):397-409.
4. Sarmah N, Kumari S. Compparative study of antibacterial activity of ripen and unripen Indigenous Citrus union of Assam, India. Int J Adv Res Technol. 2013;2(9):25- 31.
5. Owlia p, Saderi H, Rasooli I, Sefidkon F. Antimicribial characteristics of some herbal oils on *Pseudomonas aeruginosa* with special reference to their chemical compositions. Iran J Pharm Res. 2009;8(2):107-114.
6. Khushwaha A, Singh RP, Gupta V, Singh M. Antimicrobial properties of peel of Citrus fruits. Int J Univers Pharm Life Sci. 2012;2(2):24-38.
7. Dhiman A, Nanda A, Ahmad S, Narasimhan B. In Vitro antimicrobial status of methanolic extract of *Citrus sinensis* Linn. fruit peel. Chron Young Sci. 2012;3(3):204-208. doi:10.4103/2229-5186.99573.
8. Dostalova L, Detvanova L, Kalhotka L. Antimicrobial activity of aqueous herbal extracts. MENDELNET. 2014;2:403-406.
9. Madhuri S, Ashwini UH, Srilakshmi NS, Prashith Kekuda TR. Antimicribial activity of *Citrus sinensis* and *Citrus aurantium* peel extracts. J Pharm Sci Innov. 2014;3(4):366-368. doi:10.7897/2277-4572.034174.
10. Hamdan DI, Abdulla RH, Mohamed ME, El-Shazly AM. Chemical composition and biological activity of essential oils

of *Cleopatra mandarin* (*Citrus reshni*) cultivated in Egypt. J Pharmacogn Phytother. 2013;5(5):83-90. doi:10.4103/2229-5186.99573.
11. Hossaina MA, Shaha MD, Sanga SV, Sakarib M. Chemical composition and antibacterial properties of the essential oils and crude extracts of *Merremia borneensis*. Journal of King Saud University – Science. 2012;24(3):243-249. doi:10.1016/j.jksus.2011.03.006.
12. Amin M, Kalantar E, Mohammad-Saeid N, Ahsan B. Antibacterial effect and physicochemical properties of essential oil of *Zataria multiflora* Boiss. Asian Pacific Journal of Tropical Medicine. 2010;439-442. doi:10.1016/S1995-7645(10)60105-8.
13. Amin M, Montazeri EA, Mashhadizadeh MA, Sheikh AF. Characterization of shallot, anantimicrobial extract of allium ascalonicum. Pak J Med Sci. 2009;25(6):1-5.
14. Prabuseenivasan S, Jayakumar M, Ignacimuthu S. In vitro antibacterial activity of some plant essential oils. BMC Complement Altern Med. 2006;6.39. doi:10.1186/1472-6882-6-39.
15. Chanthaphon S, Chanthachum S, Chanthachum T. Antimicrobial activities of essential oils and crude extracts from tropical Citrus spp. against food-related microorganisms. Songklanakarin J Sci Technol. 2008;30(1):125-131.
16. Tao N, Gao Y, Liu Y, Ge F. Carotenoids from the Peel of Shatain Pummelo (Citrus grandis Osbeck) and its antimicrobial activity. American-Eurasian Journal of Agricultural Environmental Sciences. 2010;7(1):110-115.
17. Javed S, Javaid A, Mahmood Z, Javaid A, Nasim F. Biocidal activity of citrus peel essential oils against some food spoilage bacteria. J Med Plant Res. 2011;5(16):3697-3701.
18. Frassinetti S, Caltavuturo L, Cini M, Della Croce CM. Antibacterial and antioxidant activity of essential oils from Citrus spp. J Essent Oil Res. 2011;23:27-31. doi:10.1080/10412905.2011.9700427.
19. Lawal D, Bala JA, Aliyu SY, Huguma MA. Phytochemical screening and in vitro anti-bacterial studies of the ethanolic extract of *Citrus senensis* (Linn.) peel against some clinical bacterial isolates. Int J Innov Appl Stud. 2013;2(2):138-145.
20. Ekwenye U, Edeha OV. The antibactrial activity of crude leaf extract of *Citruc sinensis* (sweet orange). Int J Pharma Bio Sci. 2010;1(4):742-750.
21. Adnan M, Umer A, Ahmad I, Hayat K, Shakeel SN. Leaf extracts might show antimicrobial activity against other bacterial and fungal strains or with further modifications in the extraction protocols. Sains Malays. 2014;43(2):185-194.
22. Hamdan D, Ashour ML, Mulyaningsih S, El-Shazly A, Wink M. Chemical composition of the essential oils of variegated pink-fleshed lemon (Citrus x limon L. Burm. f.) and their anti-inflammatory and antimicrobial activities. Z Naturforsch C. 2013;68:275-284.