International Journal of Biotechnology and Microbiology
www.biotechnologyjournals.com
Online ISSN: 2664-7680, Print ISSN: 2664-7672
Received: 10-03-2023, Accepted: 26-03-2023, Published: 11-04-2023
Volume 5, Issue 1, 2023, Page No. 39-42

Evalution of the antimicobial activity of citrus against haemophilus influenzae isolated from patients with respiratory tract infection as an alternative source of chemotherapy

Zena Hisham Al-Dabagh, Shakir Ghazi Gergees
Department of Biology, College of Science, University of Mosul, Mosul, Iraq

Abstract
Haemophilus influenzae is a Gram- negative coccobacillus which colonizes the upper respiratory tract and is responsible for many respiratory tract infections including otitis media, epiglottitis, meningitis, sinusitis, and pneumonia, particularly in children. *H. influenzae* has many virulence factors therefore is considered resistant to many drugs.The aim of this study was investigation of antibacterial activity of *Citrus limon, C. japonica* and black- dried lemon extracts against *H. influenzae* isolates. *H. influenzae* was isolated from different specimens including sputum, tonsils swabs, nasal swabs, otitis media swabs, and nasopharyngeal swabs. Specimens were grown on blood agar and chocolate agar. The specimen was identified using standard techniques, including biochemical tests, microscopic diagnosis, culture characteristics and PCR techniques.The effect of different concentrations of citrus was examined against *H. influenzae* strains. The results revealed the effectiveness and safety extracts of citrus against *H. influenzae* isolates that can be used to support the progress of alternative antimicrobial drugs. Lemon juice was most effective than black- dried lemon and *C. japonica* with an inhibition zone of 27 mm.

Keywords: *Haemophilus influenzae*, respiratory tract infections

Introduction
Haemophilus influenzae, a Gram-negative Coccobacilli, is frequently found as commensal in the human upper respiratory tract, particularly in children [1]. This bacterium can cause a variety of illnesses, from respiratory tract infections to invasive disease. *H. influenza* consists of six capsular serotypes (types a–f) and un-encapsulated types, often known as non-typeable *H. influenzae* (NTHi) [2]. This bacterium can cause many severe diseases, including pneumonia, epiglottitis, meningitis, and other infections in children and adults. *H. influenzae* has a number of mechanisms that make it resistant to antibiotic these mechanisms including: the ability to produce Beta-lactamase enzymes and by antibiotic-resistance mutations [3]. Citrus is considered a natural alternative that comforter the respiratory tract and has been used worldwide to cure such disease. Recently the world has turned its attention to the study of medicinal plants and citrus is one of these plants which has been found to have an inhibitory effect against many pathogens [4]. Citrus fruits belong to the family of Rutaceae which is classified in to 140 genera and 1,300 species including *Citrus limon* and *Citrus japonica* [5]. *Citrus limon* has many benefits including anticancer, anti-diarrheal, antibacterial, antifungal, antiviral insecticidal and antioxidant [22]. *C. limon* is the best source of nutrients and phytochemicals, including ascorbic acid, flavonoids, carotenoids and essential oils [6]. *Citrus japonica* or Kumquat is an orange oval citrus fruit about 2-3 cm in diameter, Kumquat has many benefits due to its content of unsaturated fatty acids which decreases blood cholesterol and can be utilized in preventing cardiovascular diseases. This fruit also has antioxidant, antimicrobial, antitumor and anti-inflammatory effects [7]. The present study aimed to investigate the antibacterial activity of citrus against *H. influenzae* and examine their benefits on respiratory tract infections.

Materials and methods
Isolation of *Haemophilus inflenzae*
The respiratory tract swabs were obtained from patients admitted to Mosul hospitals for the period between July and October of 2022. A total of 120 different samples were taken from individuals which includingZ: sputum, tonsils swabs, nasal swabs, otitis media swabs and nasopharyngeal swabs from both genders at ages ranging between (5-80) years.

Bacterial culture and initial diagnosis
The specimens were transported to the laboratory using a transport medium (chocolate brain heart infusion broth) and kept in a cooled box.Then the swabs were cultured on blood agar, gonococcal GC medium, MacConky agar, Levienthal's medium and chocolate agar. Plates were incubated at 37ºC for (18-24) hours with 5% CO_2. Suspected bacterial isolates that belonged to *H. influenzae* were subjected to Gram stain, oxidase test, and API-NH system (Biomereiux France). Nutritional requirements of *H. inflenzae* to both X and V factors were also tested.

Molecular detection of *Haemophilus influenzae*
Whole genomic DNA was extracted from suspected isolates of *H. influenzae* under study using the DNA extraction kit supplied by(Geneaid,USA). DNA was extracted according to the steps recommended by the company [8]. PCR assay was used to amplify the 1495bp fragment of the 16S rRNA gene,utilizing the primers F and R and the conditions were: initial denaturation at 95ºC for 3 minutes followed by 30 amplification cycles that included a denaturation step at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 1 minute. A final extension step was set at 72ºC [9]. PCR products were analyzed on 1% agarose gel, visualized using a transilluminator then the bands were purified by GFX PCR DNA and Gel band Purification Kit

and sent for sequencing at the Psomagene sequencing company (USA).

Extraction of black dry lemon

Black dried lemon was obtained from the local markets of Mosul city, Iraq and has been crushed roughly. Soxhlet was used in the extraction method by adding 200 gram of black dried lemon with 200 ml of 95% ethanol for 7 hours. The extraction solution was filtered, and then ethanol was evaporated using a rotary evaporator at 40ºC to produce the crude extract. The extract was kept in a sterile glass container at 4ºC until further used [17].

Extraction of kumquat

Kumquat fruits were obtained from the local market of Mosul, washed with distilled water and dried in an oven at 45ºC for two days. The fruits were crushed roughly and the same procedure above was used in the extraction of black dry lemon to obtain its crude extract. Extracts were then kept in sterile glass bottles at 4ºC until further used [20].

Preparation of lemon juice

Fresh *C. limon* was obtained from local market in Mosul city, washed with distilled water and cut in to two halves using a sterile knife to extract the lemon juice. The juice was extracted gently and aseptically and then filtered by a Whatman No.1 filter paper and kept at 4ºC until further used [19].

Preparation and sterilization of Alcoholic Extracts

The stock solution of the extracts were prepared by adding 1 gm of alcoholic extract from Kumquat and black dry lemon to 5ml of Dimethyl sulfoxide (DSMO) to obtain 200 mg/ml. Sterilization was done using a Millipore filter [5].

Antibacterial activity of citrus

The antibacterial activity of citrus (fresh lemon juice, kumquat, black dried lemon) against *H. influenzae* was done by using agar-well diffusion method as described elsewhere [5]. Four dilutions of the extracts were prepared (12.5, 25, 50, and 100 mg/ml) and tested against *H. influenza* isolates. Chocolate Muller-Hinton agar plates were cultured with a culture of *H. influenza* then wells of 6mm in diameter and 5 mm in depth were made using a sterile cork-borer. Approximately 20 µl of each dilution was transferred into the wells then plates were incubated at 37ºC for 24 hours with 5% CO_2 [6].

Antibiotics sensitivity test

Antibiotic sensitivity test was done for all 12 isolates of *H. influenzae* using disk diffusion method as described elsewhere [11]. Briefly, 0.1 ml from *H. influenza* culture (compared to 0.5 McFarland tube, 10 [6] cfu/ml) was spread on Chocolate Muller-Hinton agar medium plates. Eight antibiotic discs, Amoxiline (25mg), Erythromycin (10mg), Azithromycin (15), Bacitracin (10mg), Ciprofloxacin(10mg), Levofloxacin (5mg), Chloramphenicol(10mg) and Cefotaxime (30mg), were placed on the medium using a sterile forceps and plates were incubated at 37ºC for 24 hours with 5% CO_2 [21].

Results and discussion

Sample collection and identification

Among 120 swabs from respiratory tract infections in the city of Mosul-Iraq, 102(85%) showed positive cultures. It was found that only 12 cultures (10%) were approved to be *H. influenzae* using 16S rRNA gene sequencing. PCR products shown in figure 1 were sequenced and their sequences were blasted against their counterparts in NCBI and thereby identified as *H. influenzae*.

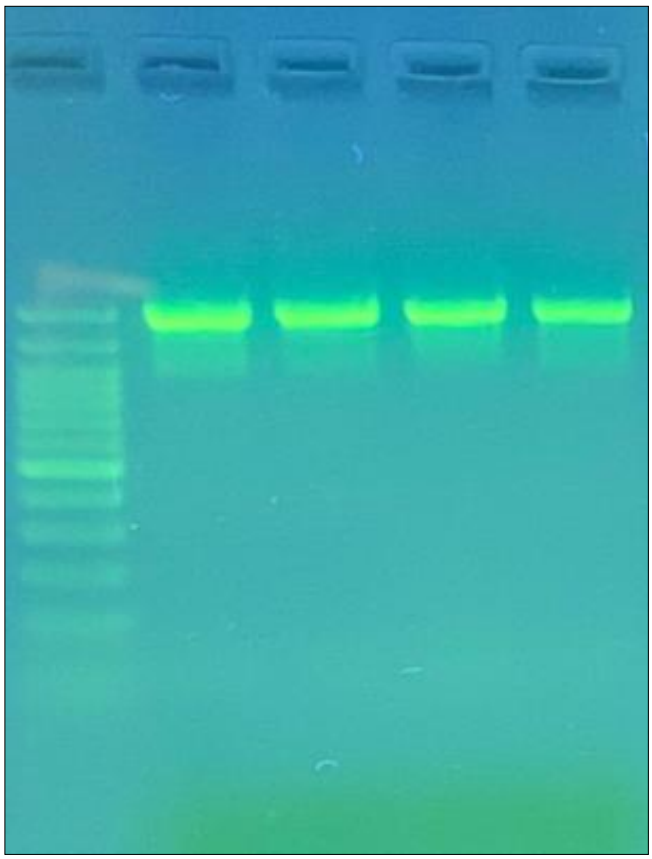

Fig 1: Amplification of 16S rRNA gene of selected samples of *H. influenzae***.**

Antibiotics susceptibility test

The ability of 12 *H. influenzae* isolates to resist eight antibiotics, Amoxicillin (25mg), Erythromycin (10mg), Azithromycin (15), Bacitracin (10), Ciprofloxacin(10), Levofloxacin (5mg), Chloramphenicol (10mg) and Cefotaxime (30) were tested (figure 5). The results showed that *H. influenzae* was resistant to many drugs especially amoxicillin(100%), chloramphenicol(83.3%), azithromycin(66.6%), and cefotaxime(75%) these results are in agreement with Zhou and coworkers as they found that the ratio of the resistance of bacteria to the antibiotic was 96% [12]. The current results also agreed with Amin as he showed that resistance of *H. influenzae* isolates to amoxicillin and erythromycin was 100% which is similar to the current results [13]. This resistance may be due to the ability of *H. influenzae* to produce β-lactamase enzymes which attack the β-lactam ring and cause alternation in penicillin- binding proteins (PBPs) so the antibiotic becomes useless [14]. Because of the inefficiency of such antibiotics our goal was to search for alternative agents that are safer and have no side effects on human health [15].

Table 1: Percentage of antibiotic resistance of *H. influenzae* to antibiotics understudy.

Antibiotics	Number and percentage of resistance isolates	Number and percentage of sensitive isolates
Levofloxacin	3(25)	9(75)
Cefotaxime	9(75)	3(25)
Chloramphenicol	10(83.3)	2(16.6)
Erythromycin	12(100)	0(0)
Amoxicillin	12(100)	0(0)
Bacitracin	12(100)	0(0)
Azithromycin	8(66.6)	4(33.3)
Ciprofloxacin	4(33.3)	8(66.6)

Antibacterial activity of citrus

More than 50% of drugs are from natural sources and plants account for more than 26 %of the total natural sources [16]. Citrus fruits have many benefits on human health due to their vitamin C (ascorbic acid) content in addition to their bioactive components [17]. Results from evaluating the activity of citrus showed that fresh lemon juice has the highest antibacterial activity against *H. influenzae* creating a high inhibition zone that reached 27 mm when using the concentration of 100mg/ml as shown in figure (2). Our results also showed that *C. japonica* or Kumquat extracts had antibacterial effects against *H. influenzae* with an inhibition zone that was around 25 mm when using the concentration of 100mg/ml as shown in figure (4).The black dry lemon extract showed less activity compared with lemon juice and Kumquat as shown in figure (3). The effectiveness of Black dry lemon extract is related to the antiplasmin property and antimitotic properies [18].The results shows that *Citrus limon* and *C. japonica* extracts could be helpful toovercome the resistance problem to antibiotic. Also this extracts have many phytochemical constituents including flavonoids, steroids, tannins, phenols and saponins. The presence of these components are responsible for the potent antibacterial activity of the extracts [22].

Table 2: Mean of inhibition zone in millimetres of four concentrations of Citrus extracts against *H. influenzae* isolates.

Type of Citrus	Citrus concentration in (mg/ml)			
	100	50	25	12.5
Black dry lemon	19	13	10	0
Fresh lemon juice	27	24	20	18
Kumquat	25	22	16	13

Fig 2: The antimicrobial effect of lemon juice extract on *H. influenzae.*

Fig 3: The antimicrobial effect of Black dried lemon extract on *H. influenzae.*

Fig 4: The antimicrobial effect of Citrus japonica extract on *H. influenzae.*

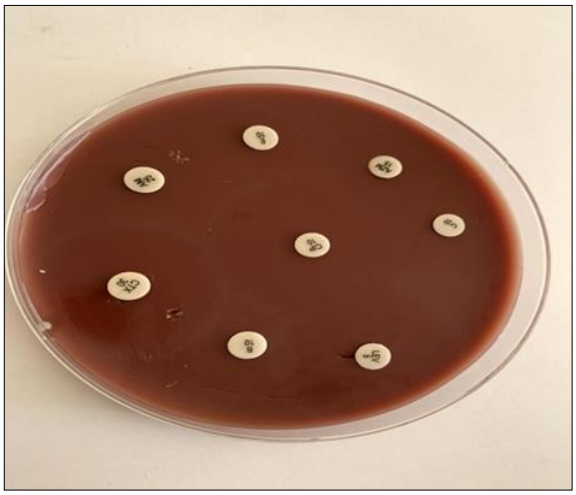

Fig 5: Antibiotic susceptibility of *H. influenzae* on chocolate muller Hinton agar to tested antibiotics understudy.

Conclusion and suggestions

Our current study showed that only 12 cultures (10%) from 120 swabs were approved to be *H. influenzae* using 16S rRNA gene sequencing.This rate of isolation in specimens taken from people with respiratory tract infections in the city of Mosul, *Haemophilus influenzae* showed high resistance to amoxicillin, chloramphenicol, azithromycin, and cefotaxime, Results showed that citrus extracts were safe and effective against *H. influenzae* isolates, which can be utilized to help the development of substitute antimicrobial medicines. With a 27 mm inhibitory zone, lemon juice outperformed black-dried lemon and *Citrus japonica.*

Acknowledgements

The authors want to thank the participating of Mosul hospitals.

References

1. Tabatabaei SR, Mohammadzadeh S, Zahraei SM, Mahmoudi S, Ghandchi G, Hoseini-Alfatemi SM, *et al*. Effect of Haemophilus influenzae type b vaccination on nasopharyngeal carriage rate in children, Tehran, 2019. BioMed Research International, 2021.
2. Fuji N, Pichichero M, Kaur R. Haemophilus influenzae Prevalence, Proportion of Capsulated Strains and Antibiotic Susceptibility During Colonization and Acute Otitis Media in Children, 2019-2020. The Pediatric Infectious Disease Journal,2021:40(9):792-796.
3. Abood DS, Nijris ON. Investigate the Spread of Haemophilus influenzae in Patients Infected with Upper Respiratory Tract Infection in Samarra city Anemia. Egyptian Academic Journal of Biological Sciences, G. Microbiology,2022:14(2):223-230.
4. Khan AA, Mahmood T, Siddiqui HH, Akhtar J. Phytochemical and pharmacological properties on Citrus limetta (Mosambi). Journal of Chemical and Pharmaceutical Research,2016:8(3):555-563.
5. Shakya A, Luitel B, Kumari P, Devkota R, Dahal PR, Chaudhary R. Comparative study of antibacterial activity of juice and peel extract of citrus fruits. Tribhuvan University Journal of Microbiology,2019:6:82-88.
6. Lin X, Cao S, Sun J, Lu D, Zhong B, Chun J. The chemical compositions, and antibacterial and antioxidant activities of four types of Citrus essential oils. Molecules,2021:26(11):3412.
7. Al-Saman MA, Abdella A, Mazrou KE, Tayel AA, Irmak S.Antimicrobial and antioxidant activities of different extracts of the peel of kumquat (Citrus japonica Thunb). Journal of Food Measurement and Characterization,2019:13(4):3221-3229.
8. Al-Buhilal JA, Rhumaid AK, Al-Tabtabai AMH, AL-Rubaey NKF. Molecular detection of Haemophilus influenzae isolated from eye swabs of patients with conjunctivitis in Hilla Province, Iraq. Journal of Applied Biology and Biotechnology,2022:10(2):1-7.
9. Abdulrazzaq R, Faisal R. Efficiency of Hichrome Enterococcus faecium Agar in the Isolation of Enterococcus spp. and other Associated Bacterial Genera from Water. Journal of Life and Bio Sciences Research,2022:3(01):01-06.
10. Balouiri M, Sadiki M, Ibnsouda SK. Methods for *in vitro* evaluating antimicrobial activity: A review. Journal of pharmaceutical analysis,2016:6(2):71-79.
11. Dawood HN, Zwain LA, Alqaraghuli HA. Bacteriological Profile and Antibiogram of Bacteria in Sputum Culture of Iraqi Patients: A retrospective study. Ibn AL-Haitham Journal For Pure and Applied Sciences,2021:34(3):1-9.
12. Zhou M, Fu P, Fang C, Shang S, Hua C, Jing C, *et al*. Antimicrobial resistance of Haemophilus influenzae isolates from pediatric hospitals in Mainland China: Report from the ISPED program, 2017-2019. Indian Journal of Medical Microbiology,2021:39(4):434-438.
13. Amin HM. Evaluation of certain immune criteria in secondary bacterial infections in people with COVID-19 in the city of Kirkuk. Msc Thesis, Pure Sciences/University of Kirkuk, 2022.
14. Carrera-Salinas A, González-Díaz A, Calatayud L, Mercado-Maza J, Puig C, Berbel D, *et al*. Epidemiology and population structure ofHaemophilus influenzae causing invasive disease. Microbial Genomics, 2021, 7(12).
15. Li XX, Xiao SZ, Gu FF, He WP, Ni YX, Han LZ. Molecular epidemiology and antimicrobial resistance of Haemophilus influenzae in adult patients in Shanghai, China. Frontiers in public health,2020:10:947051. doi: 10.3389/fpubh.2022.947051.
16. Alfarraj D, Manal M, Al K, Nadine MSM. Correlation of phenolic content and antibacterial activity of dried lime extracts against human pathogen. Biomedical Research,2018:29(16):3239-3242.
17. Younus AH, Naseer SM. The effect of antibiotics with citrus limon extract on local bacterial isolates. journal of the college of basic education,2015:21(89):61-72.
18. Marzoog TR, Naseer SM. The effect of antibiotics with citrus limon extract on local bacterial isolates. journal of the college of basic education,2016:22(94):13-24.
19. Saeb S, Amin M, Gooybari RS, Aghel N. Evaluation of antibacterial activities of Citrus limon, Citrus reticulata, and Citrus grandis against pathogenic bacteria. International Journal of Enteric Pathogens,2016:4(4):3-37103.
20. Harfouch RM, Janoudi H, Muhammad W, Hammami A, Chouman F. In Vitro Antibacterial Activity of Citrus limon Peel Extracts against Several Bacterial Strains. Journal of Chemical and Pharmaceutical Research,2019:11(7):48-51.
21. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 32th Edition CLSI Guideline M100-ED30. Wayne, PA: Clinical and Laboratory Standards Institute, 2022.
22. Ewansiha JU. Evaluation of Antibacterial Potency of Citrus Limon (Lemon) Juice Against Some Pathogenic Organisms as Alternative Source of Chemotherapy. European Journal of Biology and Biotechnology, 2020, 1(1).