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RESEARCH ARTICLE

Phytochemicals from *Citrus Limon* Juice as Potential Antibacterial Agents

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Abstract:

Background:

Citrus limon (lemon) belongs to the Rutaceae family and has great therapeutic applications. The chemical ingredients of *C. limon* have been used in the formulation of several ethnic herbal medicines. The application of antibiotics has shown the development of drug resistance in antibacterial drugs. Due to the drug-resistant nature of microorganisms, there is an urgent need to develop a novel drug active against wildtype and MDR resistant strains of pathogens.

Aim:

The present study is an endeavor to characterize the juice of *C. limon* towards its total antioxidants potential activity (FRAP), DPPH and antibacterial efficacy.

Methods and Materials:

The antimicrobial activity was evaluated using different bacterial species such as *Salmonella typhi, Neisseria gonorrhoeae, Citrobacter species, Shigella flexneri* and *Staphylococcus epidermidis*.

Results:

The results of the present study indicated the antibacterial potential of *C. limon* fruit juice. Among the tested bacterial species, *Shigella flexneri* displayed maximum inhibition followed by the other microbes such as *Staphylococcus epidermidis, Citrobacter species* and *Salmonella typhi.*

Conclusion:

These findings may be utilized in the development of cost effective, safe and efficient novel drugs active against several pathogenic multi drug-resistant microorganisms.

Keywords: Antibacterial, Bacterial species, *Citrus limon*, Multi drugs resistance, Phytochemicals, Antimicrobial activity.

Article History	Received: November 07, 2019	Revised: December 11, 2019	Accepted: January 06, 2020

1. INTRODUCTION

Plants have been of great importance from ancient times for various reasons. Plants are known to act as a vital source of natural products which may be utilized in the synthesis of new drugs to protect human beings from different pathogenic infections [1]. Consumption of fruit juices plays a beneficial role in the maintenance of good health and prevention from several ailments. Citrus fruit juice is commonly consumed by humans due to its nutritional value and special flavor. The positive health benefits of juice have been ascribed in part to the presence of vitamin C (ascorbic acid) in plenty [2, 3]. Also, citrus fruits have been reported to encompass numerous bioactive compounds including phenolics, flavonoids, vitamins, and essential oils. These bioactive compounds are believed to contain plenty of phytochemicals exhibiting antioxidative, anti-inflammatory, antitumor, and antimicrobial properties [4 - 8]. In addition, lemon juice (both from the fresh fruit and juice concentrates) has been reported to contain a significant amount of citric and ascorbic acid content, which is responsible for its acidic behavior. Acid content of any solution is determined by its pH. Among the all citrus fruits, lemon is most acidic with pH 2.30. The acid value of fruits decreases as the fruit ages [9].

Bacterial species have the genetic ability to transmit and

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acquire resistance against drugs. Hence, the application of plant products may prove to be a viable option. In the present study, we have evaluated the efficacy of *C. limon* fruit juice against the five pathogenic microbes such as *Salmonella typhi* (*S. typhi*) or *Salmonella enterica typhi, which* is an obligate parasite that survives only in the human body and is susceptible to various antibiotics. This is a Gram-negative, facultative anaerobic bacterium belonging to the family Enterobacteriaceae. Genus *Salmonella* is a rod-shaped Gram-negative bacterium responsible for causing typhoid fever. Presently, there are over 2,500 identified serotypes of *Salmonella*. The bacterial species can be controlled and killed by pasteurization. They are sensitive to low pH (4.5 or below). The bacterial cell division is halted at an Aw of 0.94, in combination with a pH of 5.5 [10, 11].

Neisseria gonorrhoeae (*N. gonorrhoeae*), also known as *gonococci,* is a Gram-negative diplococcus bacterium. It was isolated by Albert Neisser. The species are facultatively intracellular and are found in pairs. It is responsible for causing the sexually transmitted disease called gonorrhea. Gonococcal infection is a major health problem; nearly sixty million cases are reported worldwide annually. The highest number of cases have been reported from developing countries including India. More than 300,000 cases are reported from United States in Centers for Disease Control and Prevention. The site of infection of gonococcus (exclusive human pathogen) is urogenital epithelia. However, in men and women urethra and the uterine cervix are the initial sites for gonococcal infection. It has been studied that this organism remains intracellularly located during infection in humans. Antibiotic treatment is provided initially for the gonococcal infection [12, 13].

*Citrobacter*a Gram-negative bacterium belongs to the family Enterobacteriaceae. Species of Citrobacter have a capacity to convert tryptophan to indole. The species *C. amalonaticus*, *C. koseri*, and *C. freundii* can use citrate as a sole carbon source. *Citrobacter* species are differentiated by their ability to build up uranium from phosphate complexes. These bacteria are found abundantly in the surroundings with soil sewage, fresh water and can also be found in human intestine. They do not cause serious diseases but can be a reason for a few infections like sepsis, meningitis and urinary tract infection. The bacteria have been reported to cause bacteremia, osteomyelitis, endophthalmitis, suppurative arthritis, endocarditis, and intra-abdominal infections, particu-larly in neonates and immune-compromised hosts [14, 15].

Shigella flexneri (*S. flexneri*) is a rod shaped, non-spore forming, non-motile bacterium. It causes shigellosis, an acute bloody diarrhea. *Shigella flexneri* is the most common cause of the endemic form of shigellosis, resulting into death of a large number of people. *Shigella flexneri* has become a major cause of health risk in both the under-developed and the developing countries. It was recognized by Shiga (1890) that one species of *Shigella* may cause bacillary dysentery. The bacterial species were isolated from a shigellosis patient in 1984 in China. Less number of organisms from 10 to 100 can produce disease. It is a Gram-negative bacterium; It initiates infection by interaction with cells and causes intense inflammation in the colonic and rectal epithelium. The mode of infection is direct contact with an infected person or with contaminated food and water [16].

Staphylococcus epidermidis (*S. epidermidis*) belongs to the species of genus *Staphylococcus* and it is a Gram-positive bacterium. *S. epidermidis is a* facultative anaerobic bacterium. It is present in normal human flora (skin and mucosal flora). The patients with weak immune system are more susceptible to infection or otherwise, it is known as less pathogenic. The infection caused by *S. epidermidis* is known as nosocomial infection because it is acquired by hospital equipment or health care facility. It colonizes on the skin surface of human beings. Additionally, the abundant prevalence of *S. epidermidis* is on the human skin. This high incidence is mainly due to the extraordinary capacity of *S. epidermidis* to stick to the surfaces of indwelling medical devices during device insertion and formation of multilayered agglomerations called biofilms [17, 18].

The problem of resistance of microorganisms to antimicrobial drugs is one of the world's current challenges. The advent of some new naturally bioactive components from plants or plant-based products has been of interest to many researchers. Hence, a great deal of attention has been paid to the antibacterial activities of *C. limon* juice as a potential and promising source of pharmaceutical agents. Due to the rapid increase of antibiotic resistance, plants that have been used as medicines over hundreds of years, offer as an obvious choice for study. It is interesting to determine whether their traditional uses are supported by actual pharmacological effects. On the other hand, plant-based antimicrobials are attractive as they are often devoid of many side effects associated with synthetic antimicrobials. The aim of this study is to detect presence of different phytochemicals in the juice of *C. limon*, and to evaluate its antibacterial activity against different bacterial isolates. The search for new antimicrobial compounds from natural sources is, thus, an ongoing process.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Mueller-Hinton Agar (MHA), Luria Bertani broth, miller and Ampicillin sodium injection (SAM-500) were purchased from SRL Pvt. Ltd., Himedia and Saralife, respectively. All other chemicals used were of analytical and molecular grade.

2.2. Collection of Plant Materials and Preparation of Extracts

C. limon was collected from Allahabad, India. The plant specimen was authenticated by Prof. A. Satyanarayan, Department of Botany, University of Allahabad. The *C. limon* fruit juice was freeze dried and stock solutions of 5 mg/ml and 10 mg/ml were prepared in DDW. In each assay, freshly prepared lemon juice was used.

2.3. Qualitative and Quantitative Determinations of Phytochemicals from *C. limon* Juice

The qualitative examination of phytochemicals from *C. limon* juice was carried out following the methods as described elsewhere with some required modification [19 - 21]. The phytochemicals analysis included the examination of alkaloids, flavonoids, phenols, quinines, terpenoids and carbohydrate. The quantitative examination of total phenolic contents was performed using Folin Ciocalteu's reagent according to the method of Singleton and Rossi (1965) and Madhavi *et al*. (2019) with some modifications [22, 23]. One ml extract was taken in a test tube and diluted with 10 ml of distilled water followed by the addition of Folin Ciocalteu's reagent (1:10) 1.5 ml into each test tube and incubation of reaction mixture at 25 ± 2°C for 5 min. To this, 4 ml of Na_2CO_3 (20%, w/v) was added to each of the test tubes. By the addition of distilled water, the final volume was adjusted to 25 ml and stirred. Gallic acid was used to prepare a standard curve and to quantify total phenolics in the preparation. The optical density of the blue colored complex was measured at 765 nm wavelength after 30 min.

The total flavonoids from *C. limon* juice were determined by using aluminum chloride colorimetric method [24, 25]. In brief, 0.5 ml of extract (10mg/ml) in methanol was separately mixed with 1.5ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The reaction mixture was incubated at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm. The quantity of flavonoids was assessed using gallic acid as a standard.

2.4. Preparation of Drug Stock Solution

Ampicillin was dissolved in an appropriate volume of water to get 5 mg/ml of stock solution. 5 µl was used in each standard well as a positive control.

2.5. Culture Media

The media employed for the study was solid Luria broth and MHA media.

2.6. Microbial Species

Five different clinical bacterial species *viz*. *S. typhi*, *N. gonorrhoeae*, *Citrobacter, S. flexneri* and *S. epidermis* were used. The bacterial species were incubated in Luria broth media at RT for 2hrs using shaker incubator at 37°C.

2.7. Sterilization of Materials

The petri dishes, pipette tips, pipettes, forcipes, loop, disc packed into metal canisters were appropriately sterilized in the hot air oven at 170°C for 1h at each occasion. The culture media was autoclaved at 12°C for 30 min. All the other required apparatuses were properly autoclaved before use.

2.8. Antibacterial Activity

The antimicrobial activity of different concentrations of *C. limon* juice was assayed using disc diffusion method (6 mm sterilized disc) placed on solidified media using sterile forcipe. Petri plates containing 20 ml MHA medium were seeded with bacterial species and spread homogeneously on each petri dish for solidification. Stock solutions of *C. limon* juice extract were dissolved in DDW to get required concentrations *i.e.* 100, 200, 300, 500, 700 and 1000µg/disc. Ampicillin (25µg in total volume of 5µl) and DDW (5µl) were used as positive and negative controls, respectively. The plates were then incubated at 37°C for 24h. The antimicrobials present in the plant extract were allowed to diffuse out into the medium and interact with the test organisms in the freshly seeded plate. The diameter of the zone of inhibition was measured in millimeter (mm) after 24 h in each experimental condition.

2.9. Statistical Analysis

All experiments were performed in triplicate in an independent manner. The data were expressed as mean ± SE of three replicates and values were analyzed statistically.

3. RESULTS

In order to characterize the presence of some phytochemicals in *C. limon* juice, the relevant qualitative tests were performed. The results showed that the *C. limon* juice extract was rich in antioxidant phytochemicals such as alkaloids, flavonoids, phenols, quinines, terpenoids and carbohydrates. The flavonoids are known to show antioxidant activity through scavenging or chelating mode of actions. Therefore, in the present investigation, the total flavonoid content present in the extract was estimated using aluminum chloride colorimetric method. In *C. lemon* fruit juice extract, the flavonoid content was found to be 19.5 ± 1.15 mg/g. The total phenolic content of *C. limon* juice was estimated to be 7.5 ± 0.56 mg/g.

A systematic examination of *in vitro* antibacterial activity of the aqueous preparation *C. limon* showed inhibitory effect on the bacterial growth. The results of antimicrobial activity of different concentrations of lemon fruit juice against the bacterial species using both the positive and negative controls are presented in Table **1**. The antibacterial efficacy of *C. limon* fruit juice was evaluated by using disc diffusion method as described in Materials and Methods. Among the tested bacterial species, *S. flexneri* displayed maximum zone of inhibition (15.2 ± 0.17 mm) at 1000 µg/disc concentration followed by *S. epidermidis* (13.4 ± 0.38 mm). At 700 µg/disc concentration, *C. species* and *S. typhi* exhibited zones of inhibition of 13.4 ± 0.32 mm and 9.7 ± 0.17 mm, respectively. The diameter of zone of clearance for standard drug, ampicillin, against different bacterial species used was found to be in the range from 14 mm to 16 mm which shows broad-spectrum range of the drug. However, *N. gonorrhoeae* was found to display resistance against *C. limon* fruit juice, but it showed sensitivity to ampicillin displaying a zone of inhibition of 14 ± 0.26 mm. The layout of this experiment on a LB agar plate is shown in Fig. (**1**).

4. DISCUSSION

C. limon is an important medicinal plant of the family Rutaceae. The alkaloids isolated from different parts of this plant such as flower, juice, leaves, peel stem, and root have been shown to display their antibacterial potential against clinically important pathogenic bacterial species [26]. Citrus flavonoids have a broad spectrum of biological activity includ-ing antibacterial, antifungal, anti-diabetic, anticancer and antiviral activities [27]. Limonoids obtained from *C. limon* are known to exhibit substantial antibacterial and antifungal activities.

Table 1. Effect of different concentrations of C. limon juice on some bacterial species.

Bacterial Species	Diameter of Zone of Inhibition (mm)							
	Blank; DDW	*C. lemon* Juice Concentrations (µg/disc)						Ampicillin 5 mg/disc
		100	200	300	500	700	1000	
S. typhi	-	6.0 ± 0.11	06 ± 0.11	7.8 ± 0.25	8.6 ± 0.20	9.7 ± 0.17	9.5 ± 0.35	15.6 ± 0.20
N. gonorrhoeae	-	-	-	-	-	-	-	14 ± 0.26
Citrobacter species	-	6.6 ± 0.44	7.0 ± 0.23	7.7 ± 0.17	9.7 ± 0.26	13.4 ± 0.32	12.6 ± 0.23	15 ± 0.17
S. flexneri	-	9.6 ± 0.26	11.4 ± 0.34	12.3 ± 0.34	11.6 ± 0.34	15.1 ± 0.20	15.2 ± 0.17	16.1 ± 0.14
S. epidermis	-	8.8 ± 0.38	6.8 ± 0.21	10.7 ± 0.46	10.7 ± 0.18	12.7 ± 0.21	13.4 ± 0.38	14 ± 0.11

All data represent mean ± SE of three independent experiments. '—' represents no inhibition zone observed for respective microorganisms.

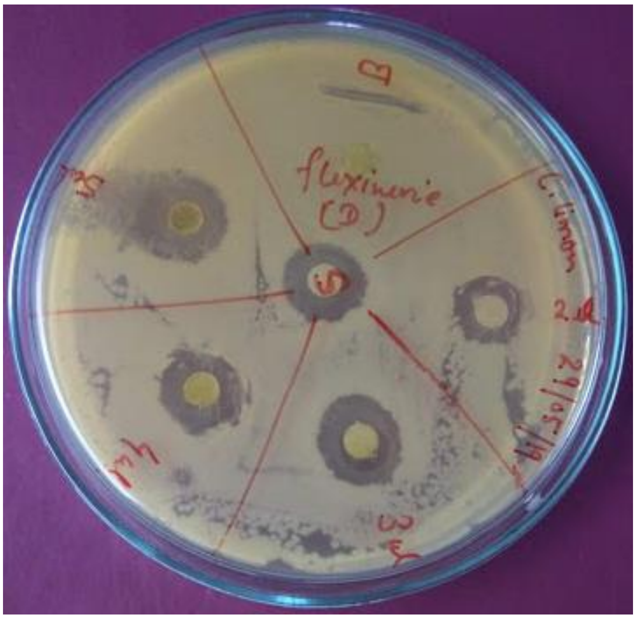

Fig. (1). One of the representative plates showing antimicrobial efficacy of *C. limon* fruit juice against *S. flexneri.* S (in centre) and B (on top) represents presence of standard drug *i.e.* ampicillin (25µg, positive control) and DDW (negative control), respectively. The sections in the plate marked as 2, 3, 4 and 5µl denote the concentrations of *C. limon* juice to be 100, 200, 300 and 500µg/disc, respectively.

The extracts of citrus fruits *e.g.* lemon, orange and grape fruit are among the most studied natural antimicrobials for food processing because of their strong inhibitory potential of the growth of bacteria [28]. The results reveal the presence of alkaloids, flavonoids, steroids, terpenoids, saponins, cardiac glycosides, and reducing sugars in the juice concentrates responsible for its potential against tested bacterial species. The phytochemicals from lemon have been reported to exhibit antibacterial activity. These phytochemicals are citral [29], carveol, carvone, citronellol, and citronellal [30], Linalool and linalyl acetate [31], α-pinene and β-pinene [32].

Earlier studies have demonstrated that some phyto-chemicals such as essential oils, protopine and corydaline alkaloids, lactons, polyacetylene, acyclic sesquiterpenes, hypericin and pseudohyperic present in the *C. limon* extract are effective in arresting the growth of various bacteria [33 - 35]. Ghasemi *et al*. (2009) have indicated that citrus varieties are considered as rich sources of secondary metabolites with the ability to produce a broad spectrum of biological activities [36]. Giuseppe *et al*. (2007) have reported the presence of limonoids in Citrus species, which could be considered responsible for activity against many clinically isolated bacterial strains [37]. Ekawati and Darmanto (2019) have demonstrated lemon juice as a potential antibacterial agent against diarrhea-causing pathogen [38].

The phenolic and flavonoid contents of any extract are responsible for its antioxidant behavior. Our findings suggest that *C. limon* fruit juice contains a significant amount of total phenols and flavonoids. Xi *et al*. (2017) have mentioned the total phenolic and flavonoid contents in two different cultivars such as pangdelusaningmeng and beijingningmeng of lemon; the values for total phenolics were 0.47 ± 0.01 and 0.38 ± 0.01; and for total flavonoids 0.44 ± 0.01 and 0.33 ± 0.00 µg/g, respectively [39].

In an *in vitro* anti-microbial study of *C. limon* fruit peels, the results similar to that of ours have been discussed (Kumar *et al*., 2011) using disc diffusion method. They have used extracts prepared in different solvents *e.g*. acetone, ethanol, ethyl acetate, petroleum ether, water and analyzed the phytochemical constituents present in each of them. The acetone extracts of *C. limon* peel have been found to exhibit significant antibacterial activities against *Escherichia coli, Bacillus subtilis, Klebsiella pneumonia, Salmonella typhi* and

Staphylococcus aureus. The *C. limon* fruit peel extracts have also been found effective against some gastrointestinal pathogens such as *Shigella* Spp. and *E. coli* strains [40]. The present investigation has revealed the antibacterial efficacy of *C. limon* juice against *Salmonella typhi, Neisseria gonorrhoeae, Citrobacter species, Shigella flexneri* and *Staphylococcus epidermidis*. Among the tested bacterial species, *Shigella flexneri* displayed maximum inhibition followed by the other microbes such as *Staphylococcus epidermidis, Citrobacter species* and *Salmonella typhi.*

The effects of natural and concentrated lemon juice, fresh and dehydrated lemon peel extracts, and essential oils, have been examined against *V. cholerae* O1 biotype Eltor serotype Inaba tox+ [41]. A complete inhibition for *V. cholerae was* recorded by using concentrated lemon juice and essential oils at all studied dilutions (diluted to 10^{-2}, and 2 x 10^{-3}) and different exposure periods (5, 15 and 30 min.). The extract of fresh and dehydrated lemon peel was found to moderately inhibit the expansion of *V. cholera.* It was moderately inhibited by using fresh and dehydrated lemon peel extracts. Lemon juice (freshly squeezed) displayed complete inhibition of *V. cholerae* after 5 min of exposure to 108 CFU/ml by using disc diffusion method.

In these experiments, the peel extract of *C. limon* was shade dried and powdered. This material was used to prepare extracts in cold water, hot water, acetone, ethyl acetate, methanol, and ethanol. These extracts have been evaluated for their antibacterial and antifungal activities against *Salmonella typhimurium, Trichophyton mentagrophytes, Micrococcus aureus, Pseudomonas aeruginosa, Microsporum canis* and *Candida albicans* using agar well diffusion method *in vitro*. Except the hexane extract, all the extracts of lemon registered their antimicrobial activities against the tested bacterial pathogens. The methanol and acetone extracts have been found to display maximum zone of inhibition *i.e.* 18 mm.

In addition, lemon juice has been found to inhibit the growth of several fungal species [42]. These workers have shown that the fungal pathogens demonstrated a zone of inhibition of 18 mm only in methanolic extract of *C. limon*. They have also studied the potential inhibitory effect of *C. limon* oil on lipophilic, yeast-like fungus *Malassezia furfur* by disc diffusion and micro-dilution methods. The diameter of inhibition zone was found to be 50 mm which were greater than inhibition zone of reference antibiotics such as for gentamycin 16.5 mm and for streptomycin 17 mm. The antibacterial activity of crude extracts (aqueous and ethanolic) of *C. limon* fruits has been determined against four wound isolates of pathogens. These isolates such as *Staphylococcus sp, Pseudomonas sp, Escherichia coli* and *Klebsiella sp* have been observed with inhibition zones 20, 18, 20 and 15 mm for aqueous extract and 15, 20, 11, and 10 mm for ethanolic extract, respectively, using *in vitro* disc diffusion method [42]. These microbes were found to be coupled with a variety of pathogenesis to the organisms. The abovementioned extracts might diminish the risk of microbes mediated pathogenesis after administration in humans.

CONCLUSION

The results of the present study demonstrate that aqueous extract of the fruit of *C. limon* contained the phytochemicals such as alkaloids, flavonoids, phenols, quinines, terpenoids and carbohydrate. The extract was found to possess promising antimicrobial activity against several bacterial species. It appears that the use of lemon extract as an antibacterial agent would be suitable for the development of cost-effective, safe and efficient novel drugs active against several pathogenic multidrug-resistant microorganisms in the future. It could be used as a natural antimicrobial and also represents a useful therapeutic supplement.

AUTHORS' CONTRIBUTIONS

NS, JJ, and PT performed the experiments and wrote the article prepared and assembled the Figures and Tables; BS had planned the experiments, reviewed, and critically organized the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

Research grant from UPCST-Lucknow, India.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

NS, JJ, PT and BS are grateful to the University Grant Commission, New Delhi for providing financial assistance in the forms of Research Fellowships to NS, JJ and PT. BS is grateful to UPCST-Lucknow for providing a research grant to BS. Authors acknowledge UGC-SAP and DST-FIST for support to the Department of Biochemistry, University of Allahabad, Allahabad, India. Authors are thankful to Prof. Gopal Nath and Dr. Reetika Singh for providing the bacterial strains.

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