JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2022/31886
RESEARCH ARTICLE

Ascorbic Acid from *Citrus limon* (L.) Osbeck Fruit Source: A Promising Natural Agent for Caries Prevention and Management

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Abstract

Dental caries is an age-old oral disease afflicting humans irrespective of gender, age, socio-economical background, and geographical location. Despite extensive research being done worldwide to prevent dental caries, it is undoubtedly still a significant public health issue. *Streptococcus mutans,* a commensal bacteria of the oral microflora, is the primary etiological agent strongly linked to dental caries. In recent years, medicinal plant-based phytotherapy has drawn great attention towards managing various diseases as it is effective and safe. The aim of the investigation was to evaluate the antibacterial activity of fresh *Citrus limon* fruit juice against *S. mutans* and to quantify its ascorbic acid (Vitamin C) concentration. Additionally, to determine the MIC, MBC and growth curve assay of standard ascorbic acid against *S. mutans*. Fresh *C. limon* fruit juice demonstrated significant antimicrobial activity against the *S. mutans* reference strain (MTCC 497) by the agar well diffusion method. The ascorbic acid concentration of *C. limon* juice was found to be 0.194 % w/w by HPLC technique. The MIC and MBC values of standard ascorbic acid against *S. mutans* were found to be 12.5 mg/ml and 25 mg/ml, respectively. The growth curve assay demonstrated that ascorbic acid at a sub-MIC concentration of 6.25 mg/ml did not display any significant variations in the growth rate of *S. mutans*. To summarize, *C. limon* fruit juice is a potential source of natural antibacterial metabolites against *S. mutans*. Ascorbic acid, one of the chief components of *C. limon* fruit, is a promising agent for the development of anti-caries products. Further studies are warranted for developing novel formulations using ascorbic acid with other natural metabolites for the prevention and better management of caries.

Keywords: Antimicrobial Activity, Ascorbic Acid, *C. limon*, Dental Caries, HPLC, *S. mutans*

1. Introduction

Dental caries is the most common oral disease that affects people all over the world. *S. mutans,* which is most commonly isolated from cariogenic biofilms, plays an important role in caries initiation by producing extracellular polysaccharide substance (EPS) in the dental biofilm[1]. The major physiological properties of *S. mutans* that are responsible for tooth decay are regulated through quorum-sensing mechanisms, which mainly include acid and biofilm production along with acid tolerance[2,3].

The health care system is undoubtedly revolutionised with the discovery and usage of antibiotics, as millions of lives are saved, significantly averting major health complications; however, the excessive usage of antibiotics has consequently led to a dreadful impact on community health[4]. The antibiotic prescriptions in dentistry are for preventive and therapeutic measures[5]. Chlorhexidine is a gold

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Article Received on: 07.11.2022 Revised on: 11.11.2022 Accepted on: 15.11.2022

standard antiplaque agent that has been widely used for decades in a variety of oral health care products[6]. There is a lack of awareness and concern among the public about the overuse of chlorhexidine, which may lead to microbial resistance. To combat the problem of antibiotic resistance, researchers are continuously looking for new therapeutic strategies[7]. The notion that herbal medicines are safer than prescription drugs has gained traction in recent years, which has led to a sharp increase in the use of phyto-pharmaceuticals[8]. Herbal remedies can be used alongside or even in place of traditional dental therapy to provide effective antimicrobial control of dental plaque and thus caries prevention[9]. Natural remedies have been investigated as a prophylactic measure against dental caries since chemical compounds destroy both pathogenic and commensal organisms[10].

C. limon is one of the potential natural sources that is well known for a variety of health benefits[11]. *C. limon* has traditionally been used to treat fevers, chest pain, high blood pressure, sore throats, rheumatism, scurvy, toothaches, bleeding gums, and bad breath because it contains ascorbic acid, which aids in infection prevention[11,12]. An intriguing link has been discovered between low ascorbic acid levels in saliva and the progression of caries activity[13]. It has been found that ascorbic acid is equivalently effective when compared with chlorhexidine in preventing oral bacterial growth[14]. Naturally, vitamin C occurs in two isomeric forms: D-ascorbic acid occurs in a reduced form, while L-ascorbic acids are chemically active and occur in oxidised forms. These two types of vitamin C are interchangeable[15]. The determination of ascorbic acid can be done by various analytical methods. Volumetric methods are the conventional techniques in which titration is performed using oxidant solutions, namely dichlorophenol indophenol (DCPIP)[16,17], bromate[18], or potassium iodate[19]. Volumetric procedures can lack specificity, which restricts their application to samples devoid of additional reducing agents[20]. When it comes to selectivity and specificity, liquid chromatography is an effective method for determining the quantity of ascorbic acid[21-23]. HPLC, along with electrochemical detection, has proven to be a sensitive and selective approach for assessing ascorbic acid in foods and biological fluids[24-26]. Ascorbic acid is inexpensive, readily available, and has few or no negative side effects. Since ascorbic acid has proven antioxidant benefits and has been used as an adjuvant in cancer treatment, it is regularly given as a dietary supplement[27]. The aim of this study was to assess the antimicrobial activity of fresh *C. limon* fruit juice against *S. mutans* and quantify its ascorbic acid concentration. Furthermore, to determine the MIC, MBC and growth curve assay of standard ascorbic acid against *S. mutans*.

2. Materials and Methods

2.1 Collection and Identification of *C. limon* Fruit

The *C. limon* (L.) Osbeck fruits were collected from the Foundation for Revitalization of Local Health Traditions (FRLHT), Bengaluru, India. The identification and authentication of the fruit sample was done by Dr N M Ganesh Babu, Associate Professor, Head, Centre for Herbal Gardens, The University of Trans-Disciplinary Health Sciences and Technology, Bengaluru, India.

2.2 Fruit Juice Preparation of *C. limon*

The fresh *C. limon* fruits were rinsed with sterile distilled water, and surface sterilized using 70% alcohol, and aseptically cut into two halves. Aseptically, the juice was collected by squeezing the fruit. The juice was filtered using a Whatman membrane filter (0.45 µm) and immediately used without refrigeration[28-30].

2.3 Bacterial Culture Revival

S. mutans freeze dried culture (MTCC 497) was obtained from the Microbial Type Culture Collection, Chandigarh, India, and revived in Brain Heart Infusion (BHI) broth. To obtain isolated pure colonies, the broth culture was streaked on BHI agar, and the plates were anaerobically incubated for 48 hrs at 37°C.

2.4 Screening of Fresh *C. limon* Fruit Juice for Antibacterial Activity

The antimicrobial activity of fresh *C. limon* fruit juice was evaluated against *S. mutans* by the agar well diffusion method on BHI agar. The turbidity of *S. mutans* bacterial suspension was adjusted to obtain the 0.5 McFarland standard for performing an antimicrobial susceptibility screening test. The optimized bacterial inoculum was aseptically swabbed on BHI agar (1.7% agar) using sterile cotton swabs. Using a 7 mm sterile cork borer, wells in the agar medium were made. Approximately 100 µl of *C. limon* juice were aseptically transferred into the wells, and the plates were anaerobically incubated for 24 hrs at 37°C. The experiment was repeated three times to confirm the reproducibility and reliability of the results.

2.5 Quantification of Ascorbic Acid by High Performance Liquid Chromatography (HPLC)

The HPLC was carried out to determine the ascorbic acid present in *C. limon* juice extract. For the HPLC analysis of ascorbic acid, a Shimadzu prominence ISO equipped with quaternary pump and auto sampler was used.

2.5.1 Chromatographic Conditions

1.5 g of NaH_2PO_4 was dissolved and diluted to 1000 ml with water (double-distilled water for HPLC-Merck) in the volumetric flask. Further, the pH was adjusted to 2.5 pH using orthophosphoric acid (OPA) and filtered through 0.45 µm Whatman filter paper (Solvent A). Solvent B was methanol (HPLC grade), and the ratio of the mobile phase was 60:40 with respect to buffer and methanol, respectively. The chromatographic conditions were reverse phase chromatographic technique with isocratic elution technique using phenomenix; column: C18 250 X 4.6 mm, 5 µm; flow rate: 1.5 ml/min, column oven temperature:35°C, UV detector wavelength: 220 nm; injection volume: 20 µl.

2.5.2 Chromatographic Technique: Isocratic Elution Technique

Standard preparation: 10 mg of L-ascorbic acid was weighed in an analytical balance (Radwag AS 82/220. R2- Poland) dissolved in water and volume made up to 100 ml with water, further, sonication was carried for a period of 20 mins, then finally filtered and injected into the HPLC system sample preparation.

The *C. limon* fruit juice extract of about 1g was weighed in an analytical balance, dissolved, and diluted to 100 ml with water. Further, sonication was carried out for a period of 20 mins, then finally filtered and injected into the HPLC system. The sample was analysed at a UV detector wavelength of 220 nm by isocratic elution technique. The overall concentration of ascorbic acid in the fresh *C. limon* fruit juice was quantified.

2.6 Minimum Inhibition Concentration and Minimum Bactericidal Concentration

The microdilution method was employed to determine the MIC and MBC of ascorbic acid against *S. mutans* with minor modifications[31]. A bacterial inoculum in BHI broth was prepared, and the density of the broth culture was modified to the 0.5 McFarland standard, and for the broth dilution protocol, 1:100 dilution was prepared. The inoculated microtiter plates were incubated at 37°C in a candle jar, and after 24 hrs the MIC was recorded. A two-fold serial dilution of ascorbic acid solution was carried out, from 50 to 0.39 mg/ml. The lowest concentration of ascorbic acid that completely inhibited visible bacterial growth was considered as MIC. The test was performed in triplicates. The determination of MBC was carried out by subculturing 10 µl from MIC test dilutions on BHI agar and incubated for 24 hrs at 37°C. The lowest concentration that displayed no bacterial growth (not greater than five colonies) on an agar plate was considered as MBC.

2.7 Growth Curve Assay

The sub-MIC concentration effect of ascorbic acid on *S. mutans* growth was determined with a few modifications[32]. *S. mutans* overnight culture grown in BHI broth, supplemented with 6.25 mg/ml of ascorbic acid, was anaerobically incubated at 37°C to get an inoculum concentration of 1.5 X 10^4 CFU/ml. BHI broth lacking ascorbic acid was used as a control. The *S. mutans* growth rate was measured every hour up to 24 hrs by a UV-Vis spectrophotometer at 600 nm. The

experiment using control was carried out in triplicates. Using Student's t-test, the significance of ascorbic acid treatment on *S. mutans* growth was determined.

3. Results

The pure culture of *S. mutans* revived on BHI agar plate is represented in Figure 1. The antibacterial activity screening test results of fresh *C. limon* fruit juice demonstrated an average 29.67 mm zone of inhibition against *S. mutans*. Figure 2 depicts a representative image of the antibacterial activity of *C. limon* fruit juice.

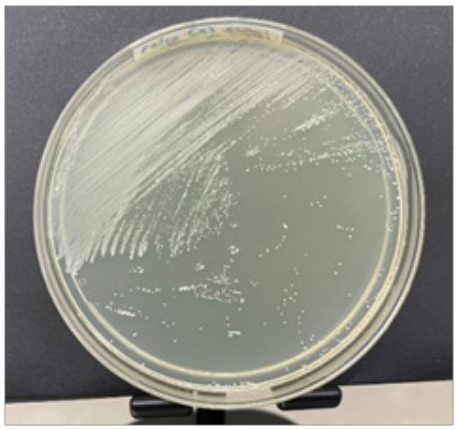


Figure 1. Revival of *S. mutans* (MTCC 497) on BHI agar.

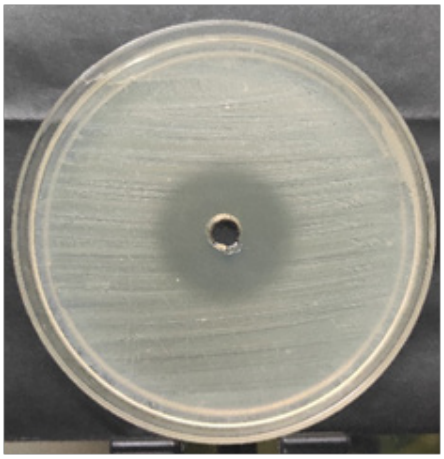
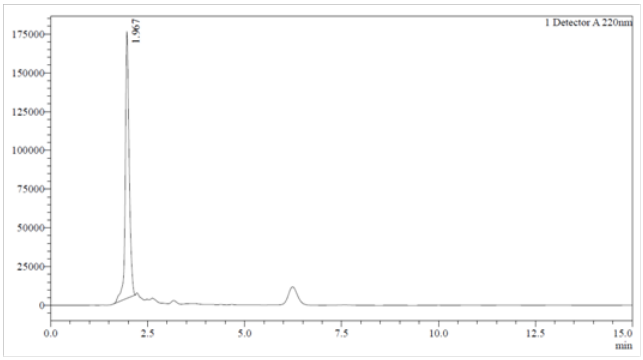


Figure 2. Antibacterial activity of fresh *C. limon* fruit juice against *S. mutans.*

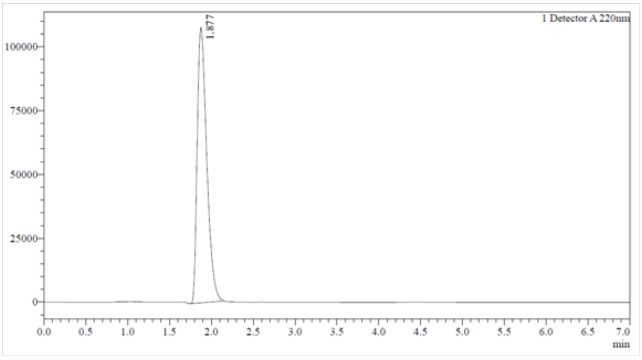
The retention time of the ascorbic acid in the chromatogram indicates nearly about 1.5 - 2.25 min in the sample and standard (Figure 3 and Figure 4). This confirms the presence of ascorbic acid in the *C. limon* juice extract. The data obtained from the HPLC analysis confirmed that an overall concentration of 0.194%w/w of ascorbic acid is present in the *C. limon* juice extract. This is equivalent to 194 mg/ 0.194g in 100g of crude extract.



Detector A 220nm

Peak#	Name	Ret. Time	Area	Area%	Tailing Factor	Theoretical Plates
1	Ascorbic acid	1.967	1208572	100.000	1.103	1786
Total			1208572	100.000		

Figure 3. The output of HPLC chromatogram of *C. limon* fruit juice extract sample.



Detector A 220nm

Peak#	Name	Ret. Time	Area	Area%	Tailing Factor	Theoretical Plates
1	Ascorbic acid	1.877	851656	100.000	1.512	1189
Total			851656	100.000		

Figure 4. The output of HPLC chromatogram of standard ascorbic acid.

The MIC of ascorbic acid was found to be 12.5 mg/ml against *S. mutans*. The wells in the microtiter plate that displayed no visible bacterial growth were subcultured on BHI agar. At a concentration of 25 mg/ml no bacterial growth was observed on the BHI agar plate, indicating the MBC of ascorbic acid.

The growth curve assay at a sub-MIC concentration of 6.25 mg/ml demonstrated that the growth pattern of *S. mutans* in the presence (treated sample) and absence (control sample) of ascorbic acid did not display a significant change. The Students t-Test for the paired two sample for Means is represented in Table 1. The

Table 1. Students t-Test (Paired two Samples for Means)

	Control	Treated
Mean	.65433	.63813
Std. Deviation	.462625	.459604
t	.122	
p value	.904	
Remarks	Sig. at 5% level	

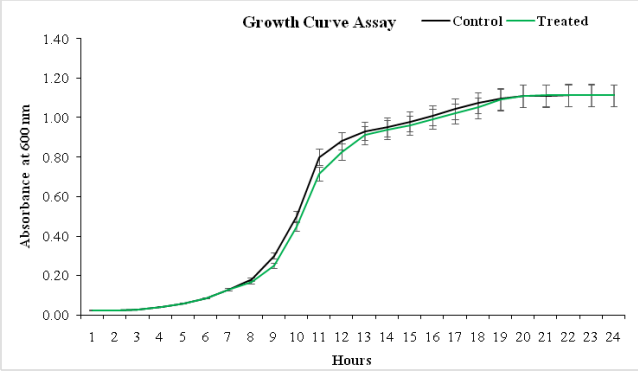


Figure 5. *S. mutans* growth curve assay in the absence and presence of ascorbic acid at sub-MIC concentration (6.25 mg/ml).

S. mutans growth curve pattern in the presence and absence of ascorbic acid is represented in Figure 5.

4. Discussion and Conclusion

The present study reports that *C. limon* fruit juice is a remarkable natural herbal candidate possessing antimicrobial activity against cariogenic *S. mutans*. To the best of our knowledge, this is the first study of its kind to correlate ascorbic acid in *C. limon* fruit juice, which may be one of the primary agents responsible for its antimicrobial activity against *S. mutans*, and to confirm the results using a standard ascorbic acid.

It is well documented that in fruits and vegetables organic acids like acetic, citric, succinic, sorbic, maleic, and tartaric acids are the chief constituents that contribute a vital role in reducing the pH, depression of inner pH of the organisms by un-dissociated acid molecule ionization or substrate transport system breakdown by altering the permeability of the cell membrane[33]. Earlier investigators have reported that antimicrobial property of *C. limon* fruit juice is attributed to the organic acids, vitamins, secondary metabolites and their respective interactions with each other[30]. The antimicrobial activity in *C. limon* fruit juice may be due to ascorbic acid, which may either act individually or through combinatorial activity with other metabolites in the fruit juice. Our study is in agreement with an earlier investigation stating that metabolites of *C. limon* fruit juice may interact with each other for its antimicrobial activity[30].

In our investigation, the MIC and MBC of ascorbic acid were 12.5 and 25 mg/ml respectively, against *S. mutans,* whereas in an earlier study, the average MIC and MBC were 9.38 and 10.16 mg/ml, respectively[34]. These MIC and MBC result variations may be due to the susceptibility pattern of the *S. mutans* strains used, the purity of ascorbic acid, and the experimental settings carried out in different laboratories. Ascorbic acid at a sub-inhibitory dose of 19.5–312.5 mg/ml demonstrated 100% anti-biofilm activity and also displayed downregulation of antibiotic resistance genes against *Pseudomonas aeruginosa*[35]. It is reported that in clinical setting, antibiotic therapy should be prescribed along with ascorbic acid for routine bacterial infection treatment[35]. In our study, ascorbic acid at a sub-MIC concentration (6.25 mg/ml) displayed no significant change in growth rate pattern and is in agreement with the results of Eydou *et al*,[34] reporting that there is no statistically significant difference in the growth rate pattern among the control and ascorbic acid treated at 1/2 sub-MIC (5.61 mg/ml) against *S. mutans*.

Because synthetic and natural ascorbic acid are chemically identical, there are no reported differences in their biological activities[36]. The study suggests that further formulation studies needs to be conducted using ascorbic acid as the primary component in conjunction with other metabolites of *C. limon* juice for the development of anti-caries products. The reason for ascorbic acid being considered as one of the chief components in formulation studies for the development of anti-caries products as it has been earlier proposed as one of the adjunct therapies to combat multi-drug resistance infections in humans and a potential agent to replace antibiotics, possessing immunomodulatory properties[37].

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