



Antimicrobial activity of citric acid against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* as a sanitizer agent

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Abstract

In this study, it was aimed to investigate the antimicrobial activity and inhibition percentage of citric acid against *E. coli*, *S. aureus* and *C. albicans* as a sanitizer agent. It was researched that persistent/tolerant levels of microorganisms in the citric acid presence. Spectrophotometric Broth Microdilution Method and Surface Disinfection Test were used for antimicrobial activity and Inhibition percentage, respectively. Modified TDtest was used for persistent/tolerant levels of microorganisms. While MIC (Minimum Inhibitory Concentration) of citric acid on *E. coli* and *S. aureus* were 0.06 g/mL, it was 0.03 g/mL for *C. albicans*. It was applied the TDtest with citric acid solution and discriminate by tolerance level of *E. coli* than the other microorganisms at the end of the 48-h incubation. In a final test which was investigated the survival of *E. coli*, *S. aureus* and *C. albicans* exposure to citric acid by Surface Disinfection Test, there was no statistically significant difference between bacteria and yeast in terms of the number of inhibited cells. Although citric acid was rather active against all three microorganisms, the strongest and permanent antimicrobial effect was on *C. albicans*. Finally, citric acid should be used especially against anti-*Candida albicans* in permanent hygiene practices.

Keywords: Citric acid, *E. coli*, *S. aureus*, *C. albicans*, Persistent/tolerant, Sanitizer

Introduction

Citric acid known as tricarboxylic acid ($C_6H_8O_7$) is an preservative, acidulate, flavouring, emulsifier, sequestrate and buffering agent widely used across many industries such as food, beverage, pharmaceutical and cosmetic products (Thauer et al. 1988, Verhoff 2005). It is naturally found in Lime, Lemon, Raspberry, Tomato, Pineapple, strawberry, cranberry and was first crystallized from lemon juice and named by Scheele in Sweden in 1784 (Scheele 1784, Ciriminna et al. 2017). Colourless and odourless citric acid is slightly hygroscopic and highly soluble in water (62.07% at 25°C) and slightly hygroscopic (Dalman 1937). Its environmentally benign nature and quickly degrades in surface waters besides useful Physico-chemical structure make it ideally suited for food, cosmetic and pharmaceutical applications. The code of citric acid is E330 as a food ingredient in European Union (E331 and E332, respectively, for sodium and potassium citrate) and has been Generally Recognized as Safe (GRAS) in the US (Ciriminna et al. 2017). Citric acid's popularity has increased even more in household detergents and home hygiene because of restrictions on the use of phosphate in dishwasher detergents in the US (since 2010), EU (Ciriminna et al. 2017) and Turkey (Official Newspaper 2018). This situation has caused the citric acid market to take more place in the industrial field and will follow. It was shown that citric acid has an excellent antiviral disinfectant effect and reported to irreversibly disturb the virus's

structure. For this reason, it has been suggested that it can be used for permanent disinfection on the infected surfaces (Koromyslova et al. 2015).

It is industrially added to juices in the beverage industry and is widely used to increase the stability of concentrates. At the same time, thanks to its antioxidant and antimicrobial properties against foodborne pathogens such as *Escherichia coli*, *Salmonella*, *Listeria*, monocytogenes., it prevents spoilage and keeps the drink fresh (Rossle et al. 2009; Ciriminna et al. 2017). Besides, it is stated that will be needed more biotechnological products such as citric acid which do not have serious harm to nature and human, and which have useful powerful effects, in the future (Ciriminna et al. 2016).

Insufficient hygiene of food processing products and surfaces contributes to long-term surface trapping of pathogen microorganisms, cross-contamination and irreversible food contamination (Carpentier and Cerf 1993). Therefore, surfaces often are disinfected with many active chemicals, but most of the chemicals pose a concern for food safety as they leave residue and pose a threat to health (Akinyemi et al. 2006). Therefore, the importance of naturally originated antimicrobials is increasing day by day (Nural et al. 2018; Yabalak et al. 2020). Besides, microorganisms' gaining resistance against these chemicals over time makes more difficult achieve sufficient and permanent hygiene (Notermans et al. 1991).

In the present paper, it was first investigated the tolerance or persistence levels in *E. coli*, *S. aureus* and *C. albicans* exposed to the solution according to the modified Kirby–Bauer disk diffusion method besides evaluating the antimicrobial activity of citric acid as a sanitizer agent.

Materials and Methods

Materials and instruments

Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), Tryptic Soy Broth (TSB), Sabouraud Dextrose Broth (SDB), were supplied from Merck (Germany). *E. coli* (ATCC 25293), *S. aureus* and *C. albicans* were taken from Refik Saydam Hfzıssıhha Centre (Turkey). ELIZA spectrophotometer (MULTISKAN GO, Thermo Scientific) was used for antimicrobial measurements. Citric acid was purchased from Sigma.

Preparing the citric acid solution

To prepare the citric acid solution, 1 g citric acid was weighed and added to 30 mL of previously sterilized distilled water. It was then homogenized in the magnetic stirrer for 1 hour and stored at 4°C. The pH value of citric acid in water was determined using Ph meters.

Antimicrobial screening

The inoculums of *Escherichia coli* (ATCC 25293), *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 90028) were prepared in 4 mL TSB for bacteria and 4 mL SDB for yeast and incubated at 37°C, overnight. After 24 hours, the microorganism suspensions were adjusted to 0.5 McFarland Standard Turbidity and stored at +4 °C until experiments.

Broth Microdilution Method

The 50 µL of MHB medium were added into 96-well microtiter plates and two-fold serial dilutions of 50 µL citric acid solution were made x-axis along from 2nd to 10th columns and used columns 11 and 12 as the negative control (only MHB and microbe). Then, 5 µL cultures were inoculated on all wells except negative control. As positive controls, Ampicillin (AMP: 128 µg/mL) in bacteria and Fluconazole (FLC: 128 µg/mL) in yeast were used. Finally, all plates were incubated at 37°C for 24 hours and the growth (turbidity) was measured at 615 nm. MIC was determined as the lowest concentration where no visible turbidity was observed in the each in each row of the 96-well plate.

Modified TDtest (Tolerance Disc Test)

To evaluate tolerance or persistence levels in *E. coli*, *S.aureus* and *C. albicans* against citric acid was used by TDtest (Kirby–Bauer disk diffusion method modified). This method consists of two steps: First, the MIC values of citric acid were used for the well diffusion method. For this, the cultures at stationary phase were spread onto MHA plates and 6 mm diameter wells were drilled into the middle of them. The

50 μL (0.1g/mL) of citric acid was placed in the wells and incubated at 37°C for 24 hrs. Second: 50 μL glucose (10%) was placed in the well which discharged because of the diffusion of the citric acid solution into the agar. The alteration in the zone regions of the petri dishes re-incubated during 37°C for 24 hrs was measured and compared with the clear zone in the primary step. TDtest, which citric acid was replaced with the glucose, allows re-growth and detection of the surviving bacteria on the agar surface. According to the method, it is interpreted as susceptible strain if inhibition zone were found around the well after glucose addition and tolerant strain if colonies inside the clear zone after glucose

(Figure 1) (Gefen et al. 2017).

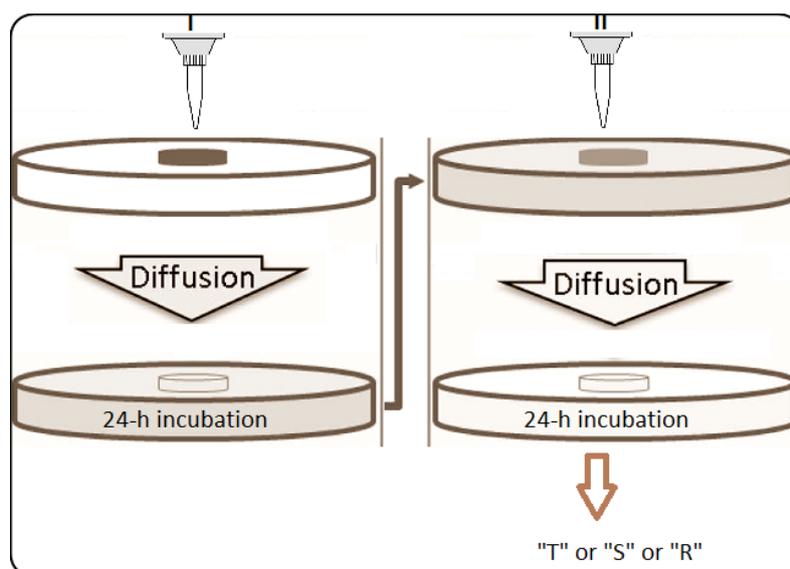


Fig 1. The diagram of the modified TDtest. Application of citric acid on the agar well (I), Replacing the citric acid with glucose solution (II). “T”, “S”, “R” Susceptible strain (S): no colonies in the inhibition zone around well after glucose addition. Tolerant strain (T): Colony observation in inhibition zone after glucose.

Surface Disinfection Test

Surface sanitizer tests were performed using the EN 13697:2015 standard “Chemical disinfectants and antiseptics. Quantitative non-porous surface test for the evaluation of microcidal activity of chemical disinfectants used in many industries such as food, cosmetic and agricultural. Test method and requirements without mechanical action”. The microcidal activity of citric acid as a sanitizer (C-SAN) was evaluated on glass lam. Lams were sterilized at 121°C for 15 min in an autoclave before each assay. Briefly, *E. coli* and *S.aureus* and *C. albicans* suspensions were diluted (ratio 1:1) with 0.3 and 3 g/l bovine serum albumin (BSA) to mimic dirty and clean working conditions (as in EN 13697:2015), respectively. Then 50 μl of resulting inocula (8 log CFU/ml) were spotted into sterile lams and dried at RT for 15 min. Later, 50 μl of 10% C-SAN was prepared on hard water as diluents according to EN 13697:2015 and 50 μl mixture was transferred to 96-ELISA plates by mixing with a pipette and 50 μl TTC (2,3,5- triphenyl tetrazolium chloride) was added. Plates were incubated at 37°C to be visualized by staining with TTC and after 4 hours, spectrophotometric measurements were made at 485 nm. Although there were no C-SAN agents studied in the negative control well, other conditions were the same.

For percent of inhibition was calculated according to the following formulae (1);

$$\text{Percent of Inhibition (\%)} = \left[1 - \frac{OD_{\text{test well}}}{OD_{\text{corresponding control well}}} \right] \times 100 \quad (1)$$

Statistical analysis

Statistical analyses and significance were measured by Tukey test in one way analysis of variance for MICs and IZ using SPSS 25. Differences were considered significant at $p \leq 0.05$.

Result and Discussion

Antimicrobial activity and response of microorganisms according to TDTest

The results showed that citric acid was effective against *E. coli*, *S. aureus* and *C. albicans* by broth microdilution and agar well diffusion method (Table 2). There was a statistically significant difference between bacteria and yeast in terms of MICs of citric acid. While MIC of citric acid on *E. coli* and *S. aureus* were 0.06 g/mL, it was 0.03 g/mL for *C. albicans*. In the present study, it was seen a statistically significant difference between inhibition zones at the end of the 24-h incubation ($p < 0.05$). The highest inhibition zone was found with 8.33 mm on *C. albicans* while the lowest IZ with 2.41 mm on *S. aureus* and also it was 4.56 on *E. coli* by well diffusion agar test ($p < 0.05$). Among bacteria, citric acid more affected *E. coli* from *S. aureus* ($p < 0.05$).

It was applied the TDtest with citric acid solution and discriminate by tolerance level (T) of *E. coli* than the other microorganisms at the end of the 48-h incubation (Table 2). At the end of the 48-h incubation, the inhibition zones were found as 8.30 mm, 2.07 mm, 4.27 mm for *C. albicans*, *S. aureus* and *E. coli*, respectively ($p < 0.05$).

Table 1. Minimal Inhibition Concentration and inhibition zone (mm) of citric acid against *E. coli*, *S. aureus* and *C.*

albicans. Res: Response of microorganisms in step 2 according to TDTest, S: Susceptible strain; T: Tolerant strain.

citric acid pH: 1.82			
	MIC (g/mL)		IZ (48 h)-Res
<i>E. coli</i> (Gram -)	0.06 ^a ±0.01	4.56 ^b ±0.03	4.27 ^b ±0.08-T
<i>S. aureus</i> (Gram +)	0.06 ^a ±0.02	2.41 ^a ±0.02	2.07 ^a ±0.02-S
<i>C. albicans</i>	0.03 ^b ±0.1	8.33 ^b ±0.01	8.30 ^b ±0.03-S

The average MICs were expressed with the standard deviation (\pm) and significance level (ANOVA, 25; 0.05, Tukey test). Values on the same column with different superscript letters differ statistically at the 0.05 level. The MICs of positive control: *E. coli* (16 μ g/mL), *S. aureus* (16 μ g/mL) and *C. albicans* (128 μ g/mL). The IZs of positive control: *E. coli* (10.1 mm), *S. aureus* (7.7 mm) and *C. albicans* (1.1 mm).

The solution had a strong and persistent antibacterial against *C. albicans* and *S. aureus*. The sensitivities of *C. albicans* and *S. aureus* can be presented as follows: *C. albicans* > *S. aureus*. Finally, it was determined that *E. coli* was tolerance to citric acid because of a few colonies in the inhibition region (Figure 2).

Evaluation of inhibition activity of C-SAN

Survival of *E. coli*, *S. aureus* and *C. albicans* were investigated by surface disinfection test exposure to C-SAN. The percentages of inhibition were used to assess the number of cells that died after 4 hours compared with negative control (live of 100% : 8 log). There was no statistically significant difference between bacteria and yeast in terms of the number of inhibited cells. In glass surface which mimicking dirty working conditions (3 g/mL BSA), while the inhibition order is presented: *S. aureus* (47.06%) > *E. coli* (41.33%) > *C. albicans* (36.23%); It is *C. albicans* (46.03%) > *S. aureus* (43.16%) > *E. coli* (35.3%) in clean working conditions (0.3 g/mL BSA) (Figure 3).

Discussion

In literature, there are studies in which MIC values of citric acid are determined against *E. coli*, *S. aureus* and *C. albicans*. For instance, Nagoba et al. (2008) found the MIC of citric acid to be 900 mg/ mL for *S. aureus* and 1500 mg/mL for *E. coli* (Nagoba et al. 2008).

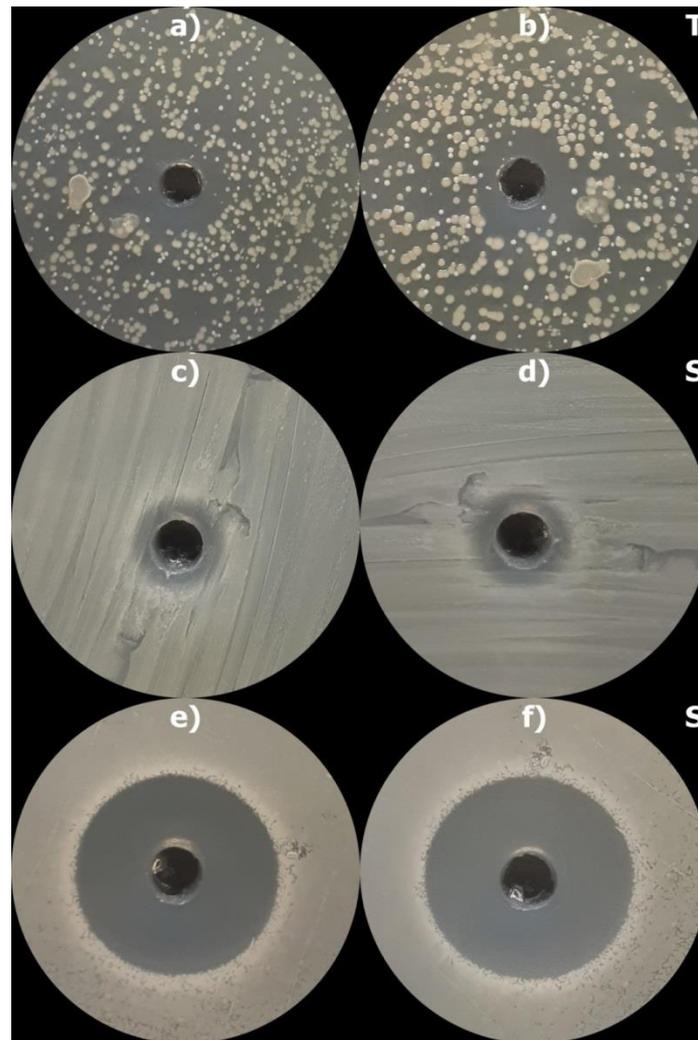


Fig 2. The images of tolerance and sensitivity levels of *E. coli* (a,b), *S. aureus* (c,d) and *C. albicans* (e,f) in exposure to citric acid. The first (a, c, e) and second (b, d, f) step of TDtest, S: Susceptible strain; T: Tolerant strain.

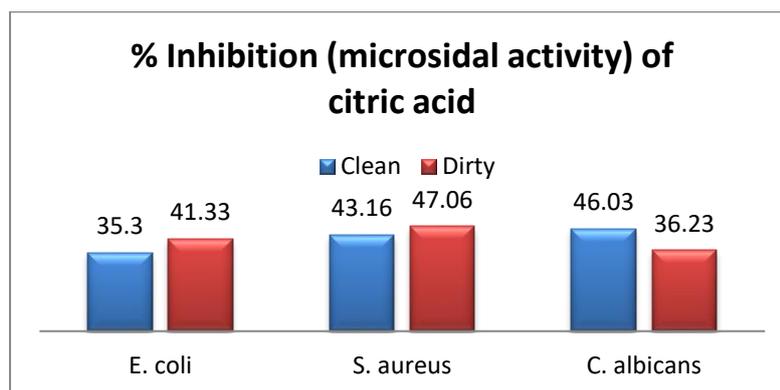


Fig 3. Microcidal activity (%) of citric acid against *E. coli*, *S. aureus* and *C. albicans* on a glass surface clean (0.3 g/mL BSA) and dirty (3 g/mL BSA) conditions at 4-h contact times according to EN 13697:2015. Values with same superscript letters don't differ statistically at the 0.05 level.

In a study which the antimicrobial activity of some organic acid including monolaurin citric acid, MIC was > 5 mg/ml against *Escherichia coli* (Skrivanova et al. 2006). Citric acid was active with MIC of 2-10 mg/mL against *C. albicans* and some fungi species (Shokri 2011). As also in this study (MICs: 0.03-0.06 g/mL), low MIC values of citric acid are due to being a low pH organic acid of acid. Generally,

organic acids inhibit microorganisms through a process which the acid pH within the cell induces breakdown and harm to enzymatic activity, proteins and DNA molecular structure or disturb the electron transport system thereby destroying the extracellular membrane (Mani-Lopez et al. 2012). Also, some organic acid such as citric acid, lactic acid, propionic acid, malic acid and tartaric acid have been shown to reduce microbial load on foods (Park et al. 2011).

The disc-diffusion test used in antimicrobial activities assesses only the concentration, namely the degree of resistance, at which the microorganism prevents proliferation. TDtest was also created with the technical development of this test. This method has made it possible to detect tolerant and persistent microorganisms by encouraging the growth of the surviving microorganisms in the inhibition zone after the antimicrobial agent has spread (Gefen et al. 2017). In literature, Kotkova et al., (2019) tried the TDTest for persistence or tolerance of *S. aureus* strain against 1 µg oxacillin and they showed that *S. aureus* was tolerant against this antibiotic and also this test was suitable for persistent/tolerant screening for clinic strains. However, in this study, *S. aureus* was permanent sensitive to citric acid and no colony was found even in the 48-h incubation. Especially, inhibition zone (8.33 mm) of citric acid against *Candida albicans* at the end of the 24-h incubation and the permanent inhibition (8.30 mm) observed although glucose was replaced with citric acid to encourage the reproduction of microorganism in the continuation of incubation, is remarkable. However, it was found *E. coli* to be tolerant compared to *S. aureus* and *C. albicans*. It can be suggested that this is related to the cell wall.

In surface disinfection test, the percentages of inhibition of *E. coli*, *S. aureus* and *C. albicans* exposure to C-SAN (pH: 1.82) were investigated and this rate changed between 35.3% and 47.06% at the end of the 4 hours. This indicates that almost half of the microorganisms are inhibited after 4 hours. No previous study has been carried out percent inhibition of this acid on microorganisms at 4-hours incubation. However, there are 24-hour inhibition studies and a few examples can be given. In a paper, citric acid (0.03%) did not inhibit the growth of *E. coli*, while it reduced the growth of *S. aureus* and the percentage of inhibition after 24 h incubation in nutrient broth were 33%, respectively (Abu-Ghazaleh 2013). In this study, the percentage of inhibition against *S. aureus* was below 50%, although the incubation time was shorter. This can indicate that inhibition of microorganisms occurs within the first few hours. No *Candida* inhibition studies were found.

These results show that citric acid has a strong antimicrobial effect and this effect is permanent for *S. aureus* and *C. albicans*. Hence it is concluded that citric acid application everywhere contaminated with pathogens can be generalized in case of detailed studies on human health.

Conclusion

In this study, the antimicrobial effect of citric acid on *E. coli*, *S. aureus* and *C. albicans* was investigated and it was checked whether this effect was permanent or tolerant according to TDTest method. In the study, the highest effect was on *C. albicans* and it was determined that *C. albicans* showed permanent sensitivity to citric acid. At the same time, the modified surface sanitizer test was measured at the 4th hour, when viable microorganisms can be stained with TTC dye, and at the end of this period, approximately half of the microorganisms were inhibited. As a result, it is a persistent antimicrobial agent, especially for *Candida* contamination, that can be used in place of hazardous chemicals that may be toxic to the human cell on all surfaces at risk of germ contamination.

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