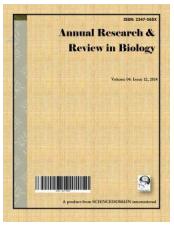

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Study on Citric Acid Production and Antibacterial Activity of Kombucha Green Tea Beverage during Production and Storage

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HP and FA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author FA managed the analyses of the study. Author SE managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to determine the amount of citric acid production and antibacterial activity of Kombucha green tea during its production and storage.
Study Design: Experimental study.
Place and Duration of Study: Department of Public Health, Maragheh University of Medical Sciences, between December 2016 and May 2017.
Methodology: The amount of citric acid at two temperatures of 20°C and 30°C was determined using the HPLC technique during 21 days. To survey the antibacterial effect of Kombucha on the growth of *Staphylococcus aureus*, *Salmonella typhimurium* and *Lactobacillus rhamnosus* bacteria, two processes of well and the disc were used.
Results: Production of citric acid undergone a change at 20°C from 5.92 on day 1 to approximately

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31.75 on day 21, and this difference was significant. Additionally, the amount of this organic acid at 30°C changed from 5.57 on day 1 to 15.43 on day 21. The amount of citric acid produced during storage at 20°C was significantly greater than that at 30°C *(p<0.05)*.
In the well method, for *Staphylococcus aureus* and *Salmonella typhimurium* at both temperatures of 20°C and 30°C, the diameter of the formed halo between different days was significantly different *(p<0.05)*. In the disc method, for *Staphylococcus aureus* at 20°C and 30°C the halo diameter in all experimental days were significantly greater *(p<0.05)* than day 1. For *Lactobacillus rhamnosus* bacteria, no halo was formed around the discs and wells.
Conclusion: By time increase, the pH decreased the amount of citric acid increased, and the halo diameter around the well and disk (in all positive cases) increased.

Keywords: Kombucha; green tea; citric acid; antibacterial activity.

1. INTRODUCTION

Functional foods, for example, probiotic, prebiotic and symbiotic foods and some other traditional beverages like Kombucha; are foods that offer health advantages beyond basic nourishment because of certain physiologically active components [1-4]. Kombucha is a fermented drink with a history of some thousand years in the East. This plain and almost low-cost beverage is a combination of green or black tea (*Camellia sinensis* L.) and sugar (glucose or sucrose), which is fermented using the Kombucha fungus changing its chemical composition [5,6].

Kombucha beverage has been claimed to be a dietary supplement that drinking it strengthens the body's immune system and prevents some disorders. Among the most valuable effects of Kombucha beverage, we can point out its regulating the physiologic activity of the gastrointestinal system and the glands, antibiotic characteristics, harmonizing the metabolism, relieving rheumatism, gout and hemorrhoids, helping maintain the pH (e.g. the acid-alkaline balance of the body), decreasing blood cholesterol, eliminating toxins and purifying the blood as well as having therapeutic effects on stress and diabetes [5,7,8].

Kombucha is a symbiotic growth of osmophilic yeast species and acetic acid bacteria (SCOBY) which have to be cultured in sugared tea. With each fermentation procedure, a new layer is formed on this plate which can be separated from the prior layer. This fungus is first placed as a thin sheet on the surface of tea and then becomes thickened. Thus far, diverse microorganisms have been isolated from the Kombucha fungus. The most popular bacteria in the culture belong to the bacterial genera *Gluconobacter* and *Acetobacter*. This layer is actually a symbiosis of bacteria (*Acetobacter xylinum*, *Acetobacter aceti* spp. xylinum, *Acetobacter xylinoides*, *Acetobacter pasteurianus*, *Corynebacterium glutamicum*) and yeasts (*Saccharomyces cerevisiae*, *Saccharomyces bisporus*, *Saccharomyces ludwigii*, *Zygosaccharomyces bailii*, *Schizosaccharomyces pombe*, *Candia krusei*, *Candida kefyer*, *Issatchenkia orientalis occidentalis*, *Pichia* sp., *Brettanomyces* sp., *Torulopsis* sp.) [5,7,8].

Many compounds have been isolated from Kombucha beverage, some of which are acetic acid, carbonic acid, folic acid, gluconic acid, glucuronic acid, lactic acid, oxalic acid, citric acid, malic acid, butyric acid, nucleic acid, ethanol, antibiotics, carbon dioxide, vitamin C and vitamins B including B1, B2, B6, and B12 [5,9].

Citric acid is a naturally found acid in all citrus fruits like limes, lemons, oranges. Apart from being present in fruits, citric acid is found in the human body (It is not produced via the body in great quantities and being a significant dietetic nutrient, it should be consumed throughout acidic foodstuffs) [10-13]. Citric acid has many nutritional properties and plays many significant roles in the body. Citric acid is alkaline and then, it can balance the acid levels in the body. It is also an antioxidant and can neutralize the harmful effects of free radicals, which are unstable compounds that activate the enlargement of neoplasmic tumors. This acid can also decrease the inflammation caused by tonsillitis. Gargling with a mixture of water and citric acid clears throat infection via killing the infection-causing microbes. Also, it's useful for skin care, skin color, mineral absorption, throat infections, and kidneys. Because citric acid demolishes bacteria, fungus, and viruses, it is employed in fungicides and disinfectants [5,14-20].

Green tea contains bioactive compounds that improve healthiness. It is loaded with polyphenols like flavonoids and catechins, which function as influential antioxidants. The antioxidants in green tea can lower the risk of different kinds of cancer. Green tea can destroy bacteria, and lowers the hazard of infection. Also, green tea may lower the risk of type II diabetes and cardiovascular disorders [7,21-25]. The beneficial properties of green tea may become more effective in an environment containing citric acid. The aim of this study was to determine the amount of citric acid production and antibacterial properties of Kombucha green tea drink during its production and storage.

2. MATERIALS AND METHODS

2.1 Preparing Kombucha Green Tea

In all trials, a fixed quantity of 10% sugar (sucrose) was employed. To prepare the Kombucha beverage, the required amount of sugar was added to one liter of boiling water in fully sterilized situations. Following the combination was boiled for 5 min, the pot was removed away from the flame and 10 g of green tea were added, then it was given time for tea to be entirely brewed. After separating the pulp, the Kombucha fungus sheet along with a cup of pre-prepared Kombucha juice was added to it. The blend was poured into a dish enveloped with sterile linen to let air penetrate the container for the fungus to breathe [26]. The Kombucha beverage was stored in a dark location at two temperature levels of 20°C and 30°C for 21 days.

2.2 Determination of pH

The pH value of Kombucha liquid samples was determined by means of an electronic pH meter ((AZ-8601, Taiwan) calibrated at pH 4 and 7.

2.3 Citric Acid Measurement Method

The quantity of citric acid production at two temperature levels of 20°C and 30°C was determined over an era of 21 days on days 0, 7, 14 and 21. Subsequent to completion of fermentation and centrifugation (at 1000 rpm for 3 minutes), the citric acid level was measured by means of the HPLC technique. In this process, the diluted sample (1 to 10) was put in the HPLC device following passing throughout Millipore filter (0.45 µ). Citric acid analysis was subsequently carried out via reverse phase HPLC (RP-HPLC). In the next step, 20 µl of the filtered sample was injected into a system equipped with a UV detector. The column analysis Nucleocil C-18 (4 mm ID × 250 mm, 5 µm) and single pump Bischoff HPLC system were used for the analysis. The mobile phase was 50 milli-mole of sodium dihydrogen phosphate with a pH of 2.58. The flow rate was adjusted to 1 ml/min and the column was placed at room temperature. Detection was conducted at 210 nm. The analysis of the recorded peaks was performed on the HPLC chart according to the standard citric acid storage time, and the concentrations were calculated from the standard curves multiplied by the dilution factor (mg/L) [27].

2.4 The Investigative Method of the Antibacterial Activity

To probe the antibacterial characteristic of Kombucha green tea, the effect of the supernatant of this liquid on the growth of *Staphylococcus aureus* (PTCC 1112), *Salmonella typhimurium* (PTCC 1709) and *Lactobacillus rhamnosus* (PTCC 1637) bacteria was investigated. Suspensions (100 µl) of target strain cultured for 18 h were spread on the plates uniformly. On days 0, 7, 14, and 21, samples of Kombucha liquid was applied to the surface of Müller-Hinton cultivation medium for *Staphylococcus aureus* and *Salmonella typhimurium* [6,26], and to the surface of the MRS cultivation medium for *Lactobacillus rhamnosus* [28,29] using the two processes of well and the disc.

On days 0, 7, 14, and 21, we took a certain quantity of Kombucha liquid and putted it in a rotary apparatus to concentrate the liquid (45°C for 30 min). Sterile supernatant was obtained by filtering the supernatant throughout a 0.2 µm sterile microfilter. Sterile samples (100 µl) were then transmitted into the wells (wells of 10 mm diameter were made with a hot, sterile *Pasteur* pipette) of agar plates inoculated with target strains. On the other hand, in the similar way, the immersed discs into the same sterile samples were located on the same target plates. First, the whole of plates were placed at 5°C for 1 h to create a pre-diffusion of Kombucha samples into the agar. The samples were then incubated at 37°C for 24 h and the halos formed around the wells and discs were finally measured [6,26,30]. Halo creation showed the antimicrobial activity of this liquid, where the enhancement in the diameter of the halo illustrated more antibacterial effect of Kombucha green tea drink. For the

rationale of control and comparison, unfermented green tea samples (sterile filtered) at the same concentration as that of Kombucha green tea were prepared for antimicrobial test. Standard discs of Gentamicin (10 UI) provided as positive antibiotic controls in accordance with CASFM 2005 principles [26].

2.5 Statistical Analysis

The investigation data acquired were analyzed by SPSS software version 21. The effect of time and the test temperature on the two examined response variables, i.e. citric acid concentration and antibacterial activity, were determined via Repeated Measures ANOVA and the results were reported as P-value.

3. RESULTS AND DISCUSSION

3.1 pH and Citric Acid Measurement

Results showed that the pH decreased slightly during the storage period from day 1 to day 21 at both temperatures of 20°C and 30°C (Table 1). During these days, the production of citric acid also undergone a change at 20°C from 5.92 on day 1 to approximately 31.75 on day 21, and this difference was significant. Additionally, the amount of this organic acid at 30°C changed from 5.57 on day 1 to 15.43 on day 21. The amount of citric acid produced during storage at 20°C was significantly greater than (approximately twice) that at 30°C ($p<0.05$) (Table 1).

Table 1. pH and citric acid concentration (mg/L) in kombucha in 20°C and 30°C

Day of incubation	Temperature	Citric acid (mg/L)	pH
1	20°C	5.92	2.72±0.01
	30°C	5.57	2.90±0.00
7	20°C	9.10	2.72±0.00
	30°C	6.10	2.85±0.00
14	20°C	22.45	2.71±0.00
	30°C	14.90	2.75±0.00
21	20°C	31.75	2.69±0.05
	30°C	15.43	2.79±0.00

3.2 Antibacterial Activity of Kombucha Green Tea

Inhibition zone of the unfermented green tea control (-) and Gentamicin (10UI) control (+) is shown in the Table 2. Inhibition zone measured in diameter around the disc for Gentamicin and around the well for unfermented green tea.

Table 2. Inhibition zone of the unfermented green tea control (-) and Gentamicin (10UI) control (+). Inhibition zone measured in diameter around the disc for Gentamicin and around the well for unfermented green tea

Bacterial strain	Unfermented green tea control (-)	Gentamicin (10UI) Control (+)
L. rhamnosus	0.0±0.0	0.0±0.0
S. typhimurium	0.0±0.0	23.0±0.0
S. aureus	0.0±0.0	22.0±0.0

Table 3 lists the diameter of the halo formed around the bacteria in millimeters on different days after incubation for the two well and disk methods at both 20°C and 30°C (mean ± Standard Deviation (SD)). In general, by time increase, the halo diameter around the well and disk increased in all positive cases. In the well method, for *Staphylococcus aureus* and *Salmonella typhimurium* at both temperatures of 20°C and 30°C, the diameter of the formed halo between different days was significantly different ($p<0.05$). In *Staphylococcus aureus* there was a significant difference between day 1 and day 7 ($p<0.001$) as well as day 1 and day 21 ($p<0.001$), and also between day 7 and day 21 ($p<0.001$) at 20°C. The diameter of the halo at 30°C for this bacterium was significantly different between day 1 and day 7 ($p<0.001$), day 14 ($p=0.012$) and day 21 ($p<0.001$) and also between day 7 and day 21 ($p<0.001$). For *Salmonella typhimurium* the diameter of halo in 20°C was significantly different between day 1 and day 7 ($p<0.001$), day 14 (0.007) and day 21 (0.023). For this bacterium at 30°C there was a significant difference between day 1 and day 7 ($p<0.001$), day 14 ($p=0.009$) and day 21 ($p=0.001$). The diameter was also significantly different between day 2 and 4 ($p=0.006$). In disk method for *Staphylococcus aureus* bacteria at 20°C there was a statistical significant difference between the diameter of halo in day 1 and 7 ($p=0.008$), day 14 ($p<0.001$) and day 21 ($p=0.015$), the difference between day 7 and day 14 was also significant ($p=0.049$). At the 30°C the diameter of halo at first day was statistically different with day 7 ($p=0.007$), day 14 ($p=0.005$) and day 21 ($p=0.005$). For *Salmonella typhimurium* at 20°C, the halo diameter difference between all two by two comparisons between days was significant ($p<0.001$ for day 1 and 7, $p=0.003$ for day 1 and 14, $p=0.011$ for day 1 and 21, $p=0.049$ for

Table 3. Antimicrobial activity of Kombucha green tea during incubation at 20°C and 30°C. Inhibition zone measured in diameter around the disc and well (Mean±SD)

Incubation day	Temperature	Disk method			Well method		
		L. rhamnosus	*S. typhimurium*	*S. aureus*	*L. rhamnosus*	*S. typhimurium*	*S. aureus*
1	20 ˚C	0.0±0.0	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0	0.0±0.0 [a]	0.0±0.0 [a]
	30 ˚C	0.0±0.0	0.0±0.0 [a]	0.0±0.0 [a]	0.0±0.0	0.0±0.0 [a]	0.0±0.0 [a]
7	20 ˚C	0.0±0.0	11.0±0.0 [b]	9.3±0.6 [b]	0.0±0.0	13.0±0.0 [b]	12.0±0.0 [b]
	30 ˚C	0.0±0.0	12.0±1.7 [b]	9.7±0.6 [b]	0.0±0.0	11.0±0.0 [b]	14.0±0.0 [b]
14	20 ˚C	0.0±0.0	14.7±0.6 [c]	13.0±0.0 [c]	0.0±0.0	19.3±1.1 [b]	19.3±1.1 [abc]
	30 ˚C	0.0±0.0	14.3±0.6 [b]	11.3±0.6 [b]	0.0±0.0	17.7±1.1 [bc]	14.7±1.1 [bc]
21	20 ˚C	0.0±0.0	15.3±1.1 [b]	13.3±1.1 [bc]	0.0±0.0	19.3±2.1 [b]	19.0±0.0 [c]
	30 ˚C	0.0±0.0	15.3±0.6 [b]	11.3±0.6 [b]	0.0±0.0	21.7±0.6 [c]	19.0±0.0 [c]

**Statistical significant difference between incubation days are calculated for each bacteria and each temperature separately and are indicated by different lowercase superscript letters (P<0.05). For S. typhimurium and S. aureus the diameter of halo in well method was statistically different in 20°C and 30°C (P=0.015 and P=0.002 respectively). In other cases there was not any statistical difference between incubation in 20°C and 30°C in diameter of halo (P>0.05)*

day 7 and 14) except for days 7 and 21, and 14 and 21 that had no significant difference ($p>0.05$). In *Salmonella typhimurium* at 30°C, there was only a significant difference between day 1 and the other days ($p=0.041$ in comparison with day 7 and $p=0.003$ in comparison with days 14 and 21), but there was no significant difference between other days ($p>0.05$).

For *Lactobacillus rhamnosus* bacteria, no halos were formed around the discs and wells on days 1, 7, 14, and 21, indicating the resistance of this bacterium to the anti-bacterial property of Kombucha green tea. It seems to be possible to use *Lactobacillus rhamnosus* and even other similar probiotic *Lactobacilli* accompany the Kombucha green tea beverage [31-33]. However, this requires more studies. Also, unfermented green tea (control group) did not show antimicrobial activity against test bacteria.

Kombucha tea has been studied via many investigators for its preventive activity on several pathogenic microorganisms. Kombucha liquid containing 33 g/L total organic acids has an antimicrobial efficiency against some harmful bacteria, for example, *Bacillus cereus*, *Salmonella typhimurium*, *Escherichia coli*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Campylobacter jejuni* [5,7]. Battikh et al. [34] accounted that Kombucha prepared from both green tea and black tea had antimicrobial potential in opposition to pathogenic microorganisms, and green tea showed the uppermost antimicrobial potential. On the other hand, green tea catechins have shown activity against both Gram-negative and Gram-positive pathogenic bacteria. In fact, Antimicrobial activity of Kombucha green tea is mainly attributed to the existence of organic acids and catechins [34]. Organic acids (particularly acetic acid and citric acid) and catechins are recognized to inhibit some of Gram-negative and Gram-positive microorganisms.

4. CONCLUSION

This study showed that the Kombucha green tea has an antibacterial activity against *Staphylococcus aureus* and *Salmonella typhimurium*, but not against *Lactobacillus rhamnosus*. Unfermented green tea (control group) did not show antibacterial activity against mentioned test bacteria. During the storage time of Kombucha green tea, by time increase, the pH decreased, the amount of citric acid increased and the halo diameter around the well and disk (in all positive cases) increased.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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