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Investigation of antibacterial activity of heated Kombucha beverages prepared with several herbal teas using response surface methodology

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Abstract
The composition and biological activities of kombucha beverage depends on type of herbal tea, concentration of sucrose and the fermentation time. This study aimed to investigate the effect of different preparation conditions on antibacterial activity of heated kombucha beverages by Response Surface Methodology (RSM). Four types of herbal teas, including black tea, green tea, lemon verbena and peppermint were prepared with three concentrations of sucrose (2, 5 and 8%) and inoculated with active kombucha culture. After 7, 14 and 21 days, beverages were heated by autoclaving and their antibacterial activity against four bacteria including *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus* was evaluated by well method. The RSM was used to investigate the effect of sucrose concentration, fermentation time and type of herbal tea on antibacterial activity of heated beverages. Increasing sucrose concentration had significant effect ($P<0.0001$) on antibacterial activity of heated beverages against all tested bacteria. Increasing of fermentation time had significant effect ($P< 0.0001$) on antibacterial activity of heated beverages against *E. coli* and *S. dysenteriae*. Type of herbal tea had significant effect on antibacterial activity against *S. aureus* and *S. dysenteriae*. The highest antibacterial activity against *E. coli* and *S. dysenteriae* was observed in beverages prepared with lemon verbena. Beverages prepared with green tea showed highest antibacterial activity against *S. aureus*. The highest antibacterial activity against *B. cereus* was observed in heated beverages prepared with black tea and peppermint. In general, the results showed significant antibacterial activity of heated kombucha beverages against the tested bacteria.

Keywords: Antimicrobial effect; Fermented beverage; Fermentation conditions; Sucrose concentration; Fermentation time

Introduction
Considering the disadvantages of consuming carbonated beverages with high sugar content, kombucha with its probiotic properties can be a good alternative. Kombucha is a sweet and sour drink prepared by fermenting tea and sugar by a symbiotic microbial consortium, which is mainly composed of acetic acid bacteria and yeasts, contains nutritious compounds that enhance the immune system and eliminate toxins. It also offers a new taste to the consumers (Jayabalan *et al.*, 2014; Velicanski *et al.*, 2007). Kombucha beverage consists the

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cellulose layer floating on the surface as well as fermented liquid. The cellulosic layer consists of a combination of yeasts and bacteria (Atiyeh and Duvnjak, 2003). The most abundant bacteria in the cellulose layer are acetic acid bacteria, especially Komagataeibacter, Gluconobacter, and Acetobacter species (Roos and Vuyst, 2018). *Schizosaccharomyces pombe, Saccharomycodes ludwigii, Kloeckera apiculata*, *Saccharomyces cerevisiae, Zygosaccharomyces bailii*, *Torulaspora delbrueckii*, *Brettanomyces bruxellensis* are the most abundant yeasts (Coton *et al.*, 2017). In addition, lactic acid bacteria (Lactobacillus and Lactococcus) comprise less than 1% of the microorganisms of this fermented beverage (Marsh *et al.*, 2014).

The microorganisms are embedded in a cellulose floating matrix that is produced by acetic acid bacteria and form a biofilm (Watawana *et al.*, 2016). *Acetobacter xylinum* is the most important species in the production of this cellulose matrix (Yamada *et al.*, 2012).

Besides, the Kombucha beverage contains various organic acids, enzymes and amino acids. Glucuronic acid, Gluconic acid, Lactic acid, Acetic acid, Malic acid, Tartaric acid, Carbonic acid, Butyric acid, Malonic acid, Oxalic acid and ethanol, 14 types of amino acids, water soluble vitamins, especially the family of vitamins B and C and some of the hydrolytic enzymes considered as the most important compounds in this beverage (Malbasa *et al.*, 2011).

Glucuronic acid, which is a vigorous compound of Kombucha tea, plays vital roles in body such as: resistance to diseases, detoxification of the body, transfer of hormones and other important substances, and as an intermediate agent in producing vitamin C. The reusability of this Kombucha culture is considered as one of its advantages because this Kombucha culture is frequently propagated in a favorable condition) (Teoh *et al.*, 2004; Zarowska *et al.*, 2001).

One of the biological activities attributed to this fermented beverage is its antibacterial effects. Various mechanisms have been proposed for this biological activity. Including the production of antimicrobial proteins and enzymes by microorganisms in kombucha, the acidic pH of this beverage, the high content of organic acids such as acetic acid (Sreeramulu *et al.*, 2000; Greenwalt *et al.*, 1998).

The metabolic concentration and composition of this beverage depends on several factors, including type of herbal tea, sucrose concentration and fermentation time and any change in the fermentation conditions might affect the final product and therefore the biological activities including antibacterial effects (Villarreal soto *et al.*, 2018; Wolfe and Dutton, 2015)

Usually black and green tea is used to prepare this beverage. In the present study, kombucha beverages were prepared with black tea, green tea, lemon verbena and peppermint, different concentrations of sucrose and at different fermentation times. The beverages were then heated by autoclaving and the effects of independent variables on the antibacterial activity of the heated fermented beverages were analyzed using by response surface methodology.

Materials and methods

Preparation of kombucha beverages

Leaves of lemon verbena (*Lippia citriodora*) prepared from research farm of Azadshahr Islamic Azad University (Iran), black tea (*Camellia sinensis*) and green tea (*Camellia sinensis*) were prepared from tea gardens of the Lahijan City in northern Iran. Peppermint (*Mentha piperita*) was purchased from medicinal herbs store. Plants were scientifically approved in the Botany Laboratory of Azadshahr Islamic Azad University. One g of the dried leaf of each plant was mixed with different level of sucrose (2, 5 and 8%) and boiled water to a total volume of 100 ml. It was then incubated at 70°C for 15 to 20 min for brewing. The mixture was filtrated through filter paper and the supernatant was inoculated with 25 g/l of actively growing Kombucha culture, sealed with sterilized gas, and kept for different storage days (7, 14 and 21 days) at room temperature. The fermented liquids were centrifuged at 5000 g for 15 min (Battikh *et al.*, 2012; (Valiyan *et al.*, 2020). The resulting supernatants were autoclaved at 121°C under 15 psi for 15 min. The autoclaved beverages were used to evaluate the antibacterial activity.

Preparation of Bacterial Strains

The strains of the tested bacteria were two Gram-negative bacteria of *Escherichia coli* (PTCC 1338) and *Shigella dysenteriae* (PTCC 1188), and two Gram-positive bacteria of *Staphylococcus aureus* (PTCC 112) and *Bacillus cereus* (PTCC 1154). They were purchased from the Iranian Research Organization for Science and Technology (IROST) in a lyophilized form. Then, they were recovered in BHI[1] medium (Merck) for 24 h at 37°C in the microbiology laboratory of the Azadshahr branch, Islamic Azad University. The 24-hour culture of each bacterium was inoculated into Nutrient Broth medium (Merck) and it was incubated at 37°C to obtain turbidity equal to 0.5 McFarland=1.5×10^8 CFU/ml (Clinical and Laboratory Standards Institute [CLSI], 2018).

Evaluation of antibacterial activity by well method

Antibacterial activity of heated fermented beverages was evaluated based on agar well diffusion method. For this purpose, a bacterial suspension equivalent to 0.5 McFarland (1.5×10^8 CFU/ml) was prepared from all bacterial strains and a uniform culture was prepared from this suspension on the surface of the Mueller Hinton Agar medium (Merck). Then, wells with a diameter of 8.2 mm were created by using a cork borer. The heated fermented beverages for testing were poured into the wells, and plates were incubated at 37^0c for 24 to 48 h. Following that, the sensitivity and resistance of the each tested bacteria was determined by measuring the diameter of inhibition zone around the wells (CLSI, 2018; Sreeamulu *et al.*, 2000).

Statistical design and data analysis

Response surface methodology (RSM) is a statistical technique that determines the relationship between one or more response variables with several independent variables. This technique builds and designs different tests and models and evaluates the interactions between different parameters and can identify and measure optimal conditions with minimum number of experiments. It also offers a suitable formula for the desired response (Wang and Liu, 2008).

The statistical analysis of the data was performed using the Design-Expert software (Version 10.0.0, Stat-ease Inc., Minneapolis, MN, USA). The experimental design of CCD is shown in Tables 1 and 2. The variables were coded according to Eq. (1):

$$x_i = (X_i - X_0)/\Delta X \qquad (1)$$

where x_i= (dimensionless) coded value of the variable X_i, X_0= the value of X_i at the center point, and ΔX= the step change. The behavior of the system was explained by the following second-degree polynomial equation:

$$Y=\beta_0 + \sum_{i=1}^{4}\beta_i x_i + \sum_{i=1}^{4}\beta_{ii} x^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij} x_i x_j \qquad (2)$$

where Y is response, β_0, β_i, β_{ii} and β_{ij} are constant coefficients and x_i represents the coded independent variables.

Additionally, the regression analysis and the coefficients were calculated. The fit of the regression model was checked by the adjusted coefficient of determination (R^2_{Adj}). The two-dimensional graphical representation of the system behavior called the response surface was used to describe the individual and cumulative effects of the variables as well as the mutual interactions between the variables on the dependent variable. A statistically significant and the suitable polynomial response surface models of each tested bacteria inhibition zone response variable for each tea were detected.

Results and discussion

A summary of different runs of experiments and their respective values is shown in Table 3. The runs contain 36 treatments related to

1 Brain Heart Infusion

different fermentation conditions, namely 3 levels of fermentation time (7, 14 and 21 days) and 3 levels of sucrose concentration (2, 5 and 8%) on antibacterial activity of four heated kombucha beverages based on black tea, green tea, lemon verbena and peppermint against four bacterial pathogens including *S. dysenteriae*, *E. coli*, *S. aureus* and *B. cereus*. The results of antibacterial activity are based on the diameter of inhibition zone and the mean of three replicates. In addition to these results, the predicted values of the prediction models obtained by the software are presented (Table 3).

Table 1- Code and levels of variables chosen for the trails

	Symbols		Levels*				
Factors	**coded**	**Uncoded**	**-α**	**-1**	**0**	**+1**	**+α**
Sucrose concentration (%)	x_1	X_1	2	2	5	8	8
Storage day	x_2	X_2	7	7	14	21	21
	x_3	X_3		1.30	1.90	2.50	

*$x_1 = (X_1\text{-}5)/3$; $x_2 = (X_2\text{-}14)/7$; α=1

Table 2- Levels of categorical factor (Nominal) chosen for the trails

Name	Type	Level	L1	L2	L3	L4
Kind of herbal tea	Nominal	4	Black tea	Green tea	Lemon tea	peppermint

Effect of independent variables on the antibacterial activity of heated kombucha beverages against *E. coli*

According to the analysis of variance, it was found that, except for the type of herbal tea, the variables of sucrose concentration and fermentation time had a significant effect (P<0.0001) on the antibacterial activity of heated kombucha beverages against *E. coli* (P<0.001). Also, the interaction effects of sucrose concentration with fermentation time and sucrose concentration with type of herbal tea at 99.9% and fermentation time with type of herbal tea at 99% had significant effects on the antibacterial activity of heated kombucha against this Gram-negative bacteria (Table 4).

Figure. 1 (a, b, c & d) shows the positive effect of the independent variables of the present study on increasing the diameter of inhibition zone of *E. coli* in all fermented beverages prepared with different herbal teas. With increasing sucrose concentration from 2 to 8% and fermentation time from 7 to 21 days, the antibacterial activity of heated fermented beverages increased (Figure 1). As can be seen in Figure. 1, this effect is particularly more evident in heated kombucha beverage prepared with peppermint and lemon verbena (Figure 1 c & d). This means that these beverages have shown greater antibacterial activity against *E. coli* than fermented beverages prepared from black and green tea.

Also the prediction models obtained by the software are presented in Table 4. In this models, R-sq is 0.78 which is a fairly good value and Adeq-Precision value is greater than 4, indicating the model's desirability, so the greater parameter (16.92) indicates the navigation of these models.

Effect of independent variables on the antibacterial activity of heated kombucha beverages against *S. dysentriae*

Analysis of variance of mean diameter of inhibition zone of *S. dysenteriae* in heated fermented beverages showed that all independent variables including sucrose concentration, fermentation time and type of herbal tea and their interactions had significant effect on the antibacterial activity of heated kombucha beverages (P<0.001).

Figure 2 (a, b, c & d) shows the positive effect of increasing sucrose concentration and storage time on increased antibacterial activity (increase in diameter of inhibition zone) of heated kombucha beverages against *S. dysenteriae*. This effect is particularly significant in beverages prepared with lemon verbena and peppermint and kombucha beverages prepared with lemon verbena in 8% sucrose and fermentation time of 21 days have the highest antibacterial activity against *S. dysenteriae* (Figure 2. c). The prediction models obtained by the software (Table 4) as for *S. dysentriae*, showes R-sq of 0.78 and Adeq-Precision of 16.1, which indicating the models desirability and indicates the navigation of these models (Table 4).

Table 3- Central composite design arrangement of process variable and actual values and predicted values of responses

treatment	Sucrose con. (%)	storage day	kind of tea†	Inhibition zone of bacteria (mm)*							
				E. coli		*S. dysenteriae*		*S. aureus*		*B. cereus*	
				Actual values	Predicted values	Actual values	Predicted values	Actual values	Predicted values	Actual values	Predicted values
1	2	7	BT	0.00	0.73	9.33	7.17	0.00	5.75	0.00	7.55
2	2	7	GT	0.00	4.95	0.00	6.98	10.67	10.11	14.33	13.28
3	2	7	LT	9.33	5.48	11.00	10.60	13.67	10.44	17.00	15.26
4	2	7	PT	8.33	4.50	13.00	10.52	8.33	4.35	8.67	5.88
5	2	14	BT	8.67	10.68	11.33	11.23	8.67	9.92	16.00	14.08
6	2	14	GT	15.00	13.16	22.00	15.75	12.67	11.46	23.33	18.54
7	2	14	LT	0.00	-0.19	9.00	10.60	8.67	5.00	8.67	3.70
8	2	14	PT	8.33	7.95	13.00	12.20	8.67	11.78	11.00	14.39
9	2	21	BT	10.00	12.40	14.00	17.61	12.00	14.53	15.00	21.33
10	2	21	GT	0.00	1.44	11.00	13.13	12.00	13.18	15.00	13.62
11	2	21	LT	0.00	1.27	10.00	8.39	12.67	13.43	12.00	13.85
12	2	21	PT	0.00	-2.59	9.67	7.45	11.00	9.65	11.67	10.33
13	5	7	BT	9.00	8.03	15.00	14.60	13.00	12.72	9.67	11.12
14	5	7	GT	8.67	9.82	8.67	10.75	13.67	14.18	11.33	13.82
15	5	7	LT	8.33	7.92	9.00	10.71	13.00	11.61	15.00	12.78
16	5	7	PT	8.67	6.19	15.33	12.78	16.00	14.31	10.67	8.11
17	5	14	BT	8.56	7.98	11.00	11.41	14.22	13.37	12.33	11.57
18	5	14	GT	9.00	9.99	11.00	10.69	13.67	15.62	13.67	14.73
19	5	14	LT	0.00	-0.47	8.67	7.78	11.00	7.73	13.00	8.27
20	5	14	PT	11.00	4.19	10.33	10.43	16.67	14.15	16.33	15.17
21	5	21	BT	0.00	5.17	17.00	16.88	11.67	16.54	14.33	18.31
22	5	21	GT	0.00	4.35	8.33	10.58	0.00	3.88	0.00	4.93
23	5	21	LT	12.67	10.97	17.33	14.12	13.67	11.51	15.67	14.31
24	5	21	PT	12.33	13.90	18.67	21.47	15.00	15.11	19.67	19.93
25	8	7	BT	0.00	0.72	9.33	10.10	0.00	2.09	0.00	1.09
26	8	7	GT	10.00	9.31	15.67	14.54	11.67	10.93	13.00	12.94
27	8	7	LT	16.33	14.19	23.33	22.78	18.00	15.73	24.00	21.05
28	8	7	PT	0.00	-3.27	11.67	9.32	9.33	5.32	11.00	5.92
29	8	14	BT	0.00	1.29	10.67	9.91	10.00	11.13	11.33	12.37
30	8	14	GT	0.00	2.15	13.00	14.30	11.67	12.91	13.00	15.07
31	8	14	LT	0.00	3.05	8.67	11.67	0.00	2.42	0.00	4.30
32	8	14	PT	11.33	9.56	13.00	13.16	9.33	9.44	15.00	13.23
33	8	21	BT	14.00	12.38	18.00	18.45	11.67	12.42	17.00	18.41
34	8	21	GT	0.00	0.93	9.67	10.75	0.00	1.57	0.00	2.18
35	8	21	LT	10.33	9.40	16.00	13.13	11.00	9.80	15.67	13.59
36	8	21	PT	14.00	14.18	19.33	19.32	16.00	13.99	23.33	21.25

†BT: Black tea, GT: Green tea, Lemon tea: LT, PT: Peppermint tea, *Data are means of 3 replicates

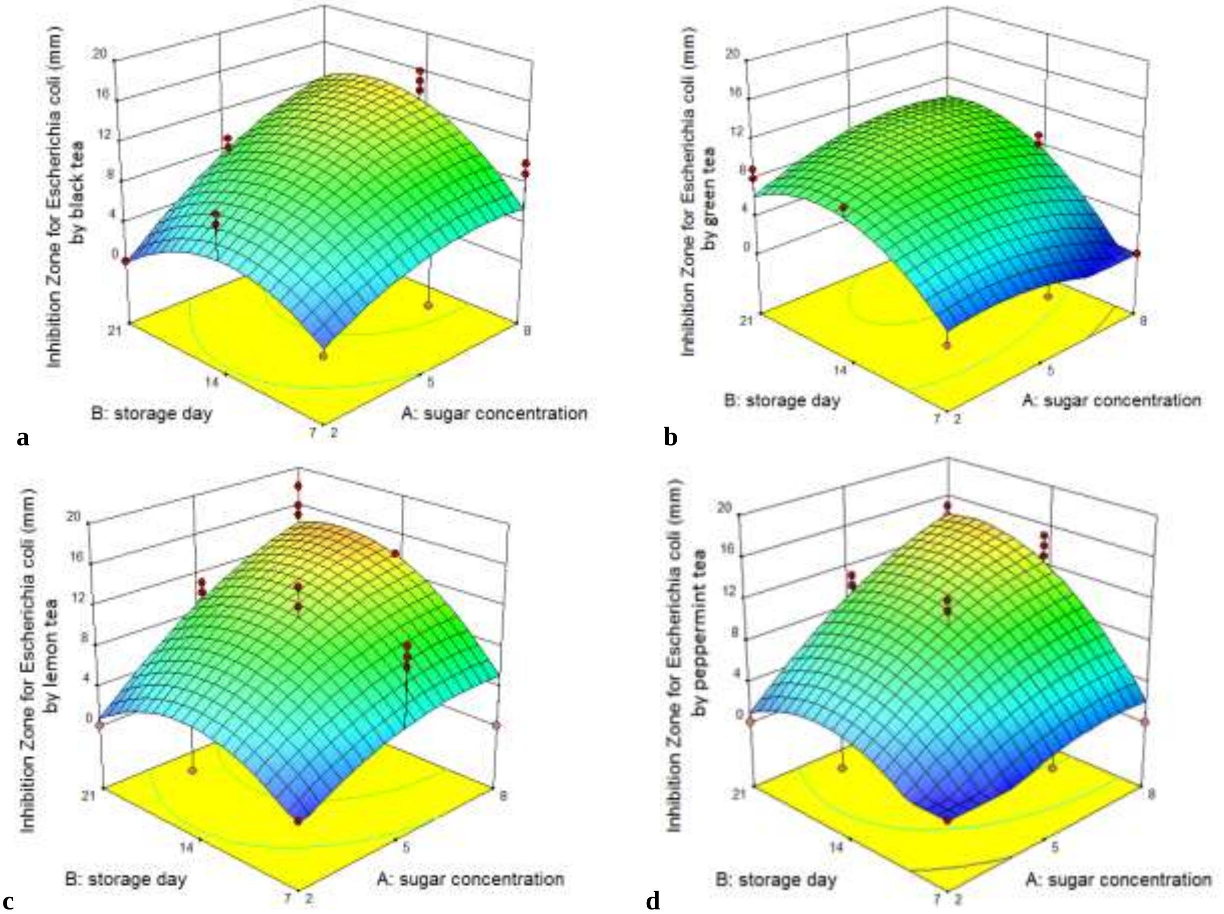

Fig. 1. Inhibition zone of *Escherichia coli* in heated Kombucha beverages (Prepared by black tea (a), green tea (b), lemon verbena (c) and by peppermint (d))

Effect of independent variables on the antibacterial activity of heated kombucha beverages against *S. aureus*

Analysis of variance of mean diameter of inhibition zone of *S. aureus* in heated beverages showed that among the independent variables, sucrose concentration and type of herbal tea had significant effect on antibacterial activity of kombucha beverages against *S. aureus* (P<0.001). In addition, in analysis of the interaction effects, sucrose concentration with fermentation time, sucrose concentration with herbal tea type and fermentation time with herbal tea type were significant at levels of 99%, 99.9% and 99%, respectively (Table 4). Among the heated kombucha beverages, beverage prepared with lemon verbena at 8% sucrose showed the highest antibacterial activity against *S. aureus* (Figure 3. c).

R-sq of 0.736, Adeq-Precision of 15.14 and the prediction models obtained by the software for *S. aureus*, indicate the desirability and the navigation of these models (Table 4).

Effect of independent variables on the antibacterial activity of heated kombucha beverages against *B. cereus*

In investigation of effective variables on antibacterial activity of heated fermented beverages against *B. cereus*, variance analysis results in Table 4 showed that among the independent variables, only sucrose concentration had significant effect on the antibacterial activity of heated beverages against *B. cereus* (P<0.001). Also, the interaction between sucrose concentration with fermentation time and sucrose concentration with herbal tea type had significant effect on this antibacterial activity (P<0.001) and with increasing sucrose concentration and fermentation time in all heated fermented beverages an increase in antibacterial activity (increase in diameter of inhibition zone) was evident (Figure 4. a, b, c & d). This effect is particularly significant in fermented beverages prepared with lemon verbena, peppermint and black tea.

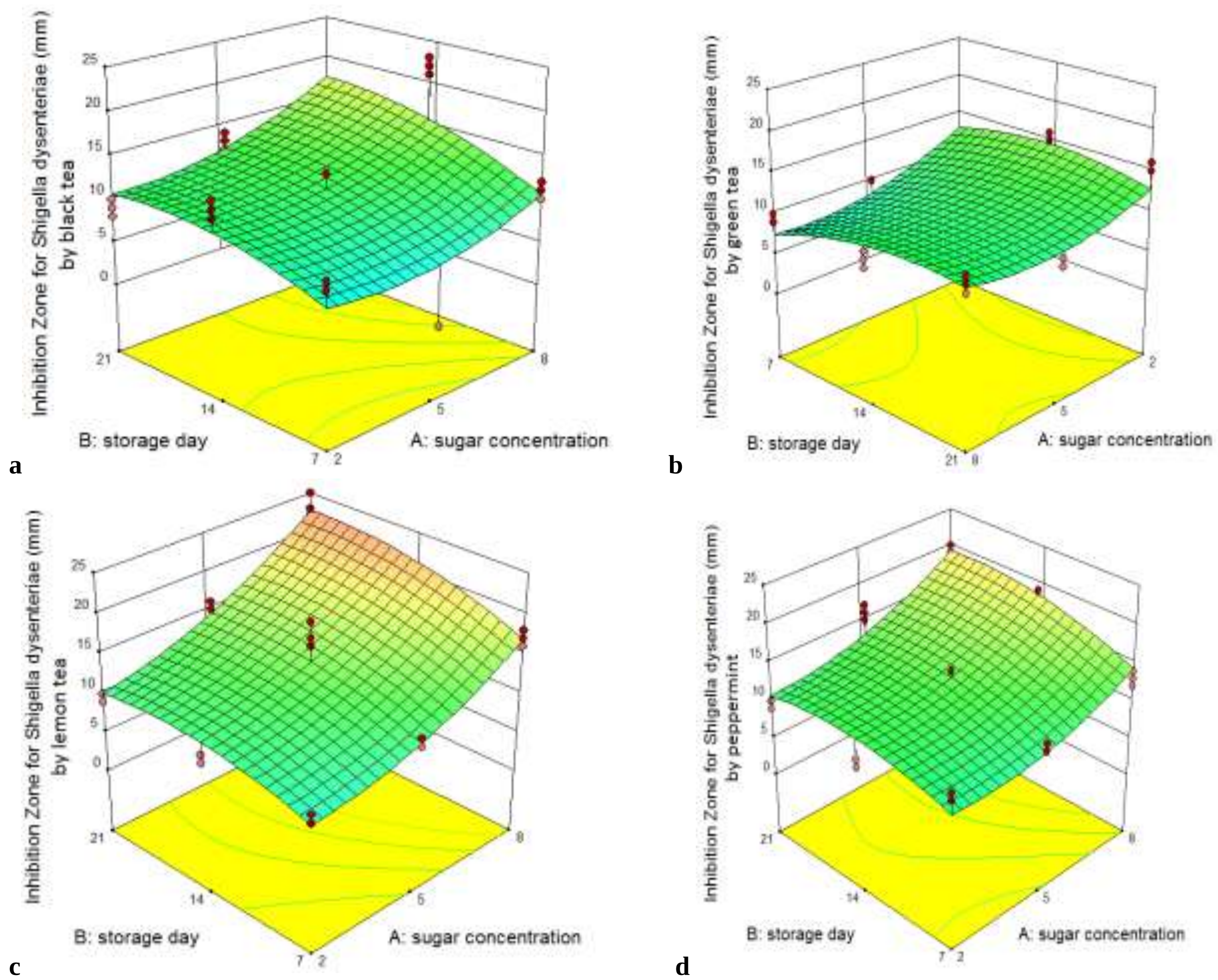

Fig. 2. Inhibition zone of *Shigella dysenteriae* in heated Kombucha beverages (Prepared by black tea (a), green tea (b), lemon verbena (c) and by peppermint (d))

The prediction models obtained by the software for *B. cereus* in Table 4, with R-sq of 0.732 and Adeq-Precision of 15.63 indicate the desirability and navigation of these models (Table 4).

In general, increasing sucrose concentration had significant effect on antibacterial activity of heated kombucha beverages against all tested bacteria and increased antibacterial activity of these beverages. Increasing of fermentation time had significant effect on antibacterial activity of heated beverages against *E. coli* and *S. dysentriae*. Also type of herbal tea had significant effect on antibacterial activity of heated kombucha beverages against *S. aureus* and *S. dysentriae*. Batikh et al. (2012) showed the antibacterial activity of various analogs of Kombucha after 21 days of fermentation. Moreover, a direct correlation between fermentation time with an increase in the production of acetic acid and organic acids and the diameter of the inhibition zone was observed in the study by Talawat et al. (2006).

Increased antibacterial activity with increasing fermentation time was also reported by Sreeramulu et al. (2000). This implies that antimicrobial components are microbial metabolites produced by the bacteria and yeast responsible for fermentation in the kombucha beverage and in the fermentation process.

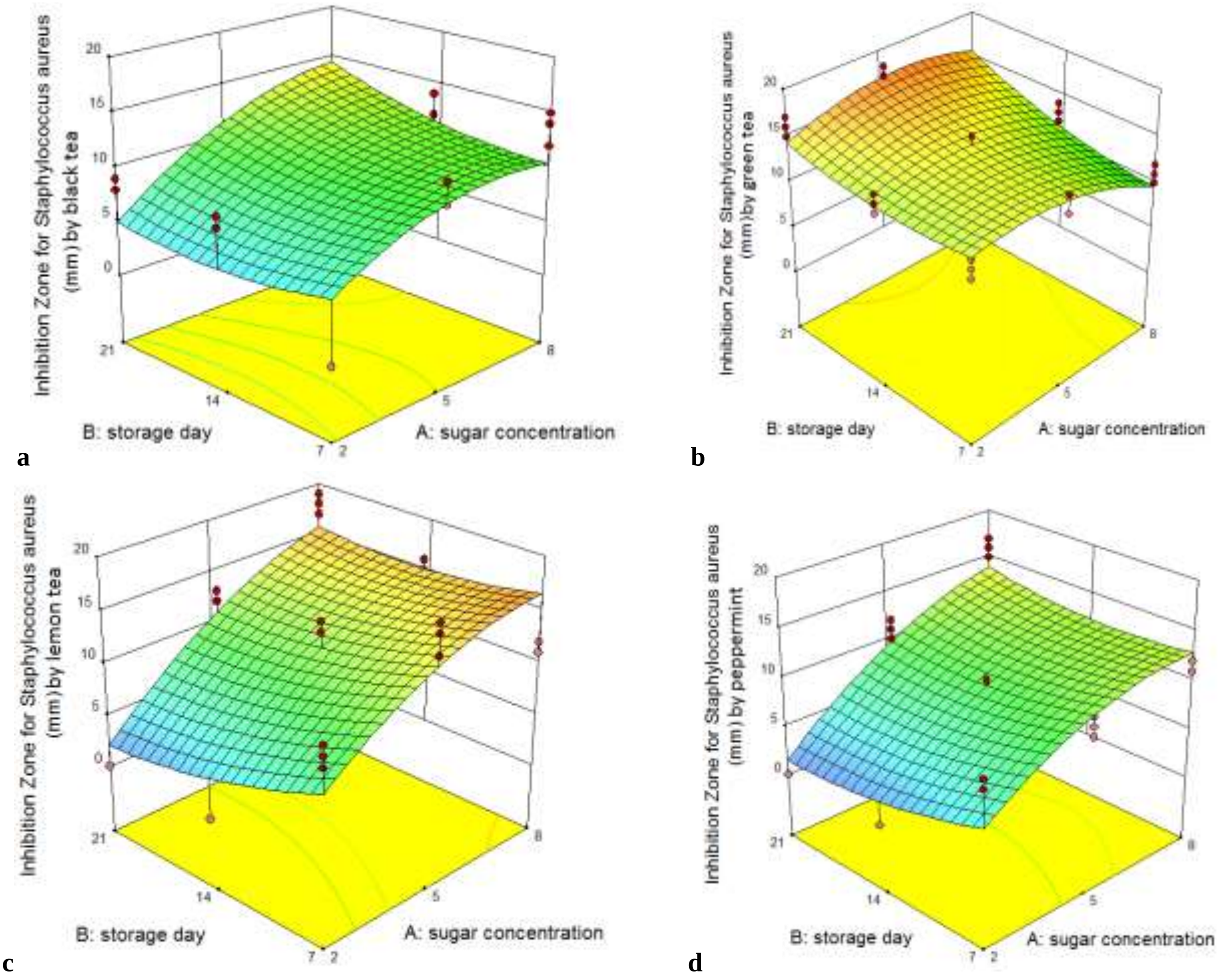

Fig. 3. Inhibition zone of *Staphylococcus aureus* in heated Kombucha beverages (Prepared by black tea (a), green tea (b), lemon verbena (c) and by peppermint (d))

Table 4- P-value and other parameters extracted from analysis of variance table

Source	*Escherichia coli*	*Shigella dysenteriae*	*Staphylococcus aureus*	*Bacillus cereus*
	p-value			
Model	2.09E-25***	2.722E-19***	4.293E-21***	7.675E-21***
A:sucrose concentration	1.68E-17***	1.751E-11***	1.254E-17***	3.551E-23***
B:storage day	1.96E-15***	1.802E-07***	0.796	0.892
C:kind of tea	0.197	1.035E-05***	1.081E-08***	0.6961
AB	4.02E-06***	0.020456642*	0.0028**	3.347E-06***
AC	4.63E-07***	4.839E-12***	1.546E-09***	9.111E-08***
BC	0.0083**	0.182145761	0.00154**	0.4029
A^2	0.0015**	0.0006231***	0.00048***	0.00958**
B^2	4.23E-11***	0.002975**	0.0683	0.72511
Lack of fit	8.369E-41***	2.743E-29***	1.844E-28***	2.484E-31***
R-Sq.	0.788907	0.71048	0.736382	0.732911
Adj R-Sq.	0.75713	0.666897	0.696698	0.692704
Pred R-Sq.	0.715423	0.617704	0.62945	0.625955
Adeq Precision	16.92704	16.10733	15.14196	15.63691

*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$

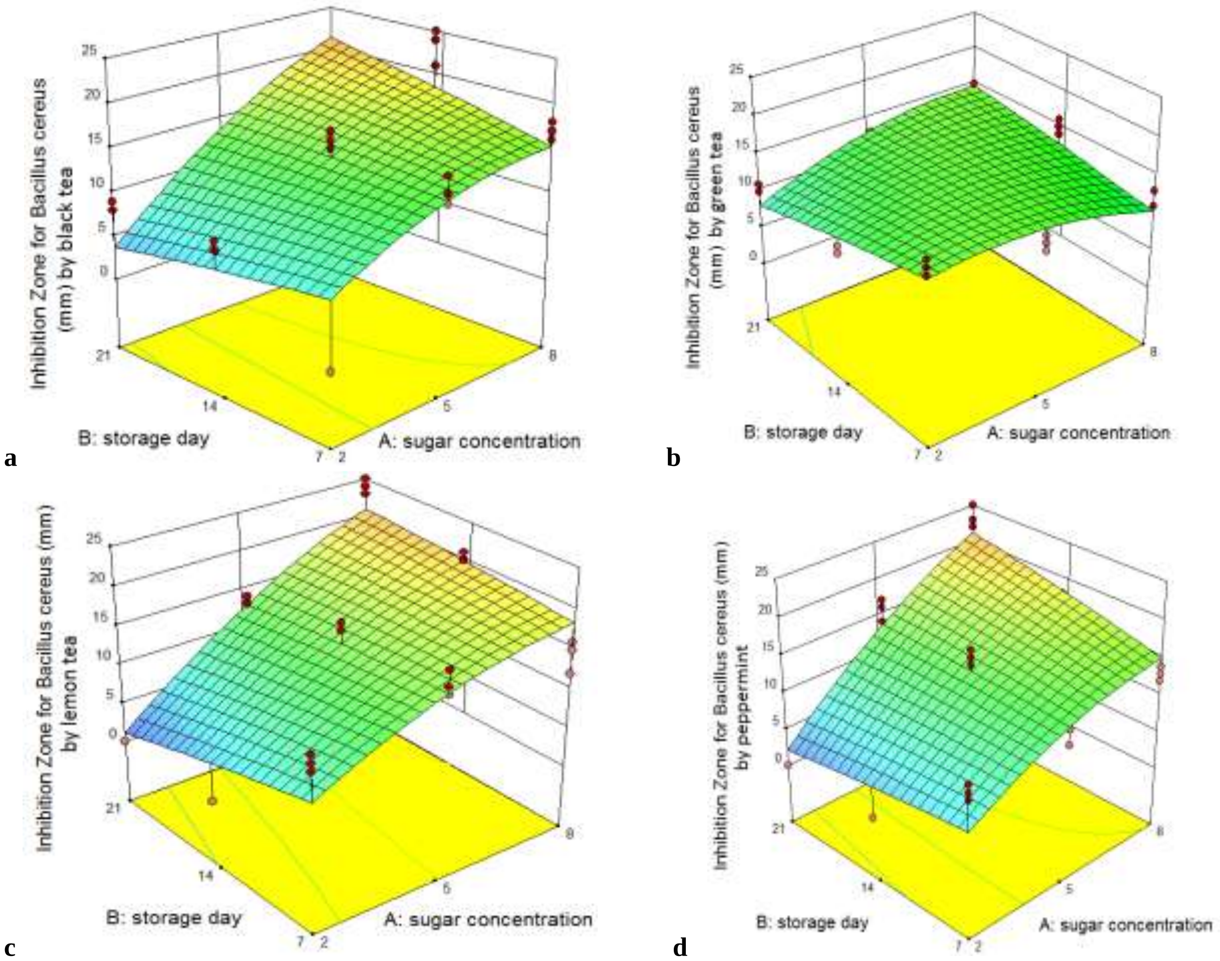

Fig. 4. Inhibition zone of *Bacillus cereus* in heated Kombucha beverages (Prepared by black tea (a), green tea (b), lemon verbena (c) and by peppermint (d))

Also this is related to the basis of production of this fermented beverage and its strong dependence on the amount of sucrose available. The sucrose in tea is metabolized to glucose and fructose by the microorganisms in kombucha. At first, Glucose was converted by yeasts into ethanol and carbon dioxide and then ethanol was changed into acetic acid and other organic acids by acetic acid bacteria (Antibacterial activity of these beverages are attributed to these compounds). Considering the need of microorganisms in kombucha for a carbon source (sucrose), it is concluded that the increase in antibacterial activity was associated with the increase in sugar concentration (Jayabalan *et al.*, 2014; Batikh *et al.*, 2011; Talawat *et al.*, 2006; Sreeramulu *et al.*, 2000; Haizhen *et al.*, 2008).

Since the fermented beverages studied were heated and the protein and enzyme compounds (Antibacterial activity is thought to be related to them) have been eliminated, is strengthened the relationship between antibacterial activity with organic acids and their dependence on sucrose consumption and organic acid production.

Prediction of fermentation conditions to achieve the best antibacterial activity of heated kombucha beverages

One of the capabilities of the surface response method is to search for optimal conditions for optimal response and to introduce the best formula and conditions for achieving optimal results with minimum number of tests. In this regard, the relationships presented in Table 5 represent the best predictive model for calculating the diameter of inhibition zone of the tested bacteria, which is specifically expressed for each herbal tea.

Table 5- Final equation for inhibition zone of bacteria for each herbal tea

Kind of bacteria	Kind of tea	Final Equation
E . coli	**black tea**	-15.0386+ 2.191358* A+2.160714* B+0.093254* A*B- 0.20525* A^2- 0.08617* B^2
	green tea	-14.2423+ 0.728395* A+ 2.565476* B+ 0.093254* A*B -0.20525* A^2- 0.08617* B^2
	lemon verbena	-17.5941+ 2.339506 B*A 2.311508* B+ 0.093254* A*B- 0.20525* A^2- 0.08617* B^2
	Peppermint	-21.8164+ 2.302469* A+ 2.525794* B+ 0.093254* A*B- 0.20525* A^2- 0.08617* B^2
S. dysenteriae	**black tea**	3.37345679- 1.841049383* A+ 1.096230159* B+ 0.04265873* A*B+ 0.211419753* A^2- 0.033446712* B^2
	green tea	14.26234568- 3.359567901* A+ 0.826388889* B+ 0.04265873* A*B+ 0.211419753* A^2- 0.033446712* B^2
	lemon verbena	2.651234568- 0.896604938* A+ 1.016865079* B+ 0.04265873* A*B+ 0.211419753* A^2- 0.033446712* B^2
	Peppermint	6.00308642- 1.581790123* A+ 0.953373016* B+ 0.04265873* A*B+ 0.211419753* A^2- 0.033446712* B^2
S. aureus	**black tea**	4.86882716+ 2.617283951* A- 0.755952381* B+ 0.057539683* A*B- 0.223765432* A^2+ 0.020975057* B^2
	green tea	14.09104938+ 1.24691358* A- 0.621031746* B+ 0.057539683* A*B- 0.223765432 * A^2 +0.020975057 * B^2
	lemon verbena	7.924382716+ 3.302469136* A- 1.10515873* B+ 0.057539683* A*B- 0.223765432* A^2+ 0.020975057* B^2
	Peppermint	4.979938272+ 3.098765432* A- 0.970238095* B+ 0.057539683* A*B- 0.223765432* A^2+ 0.020975057* B^2
B. cereus	**black tea**	4.472222222+ 2.541666667* A- 0.368055556* B+ 0.118055556* A*B- 0.208333333* A^2- 0.005102041* B^2
	green tea	15.0462963+ 0.708333333* A- 0.487103175* B+ 0.118055556* A*B- 0.208333333* A^2- 0.005102041* B^2
	lemon verbena	6.083333333+ 2.930555556* A- 0.606150794* B+ 0.118055556* A*B- 0.208333333* A^2- 0.005102041* B^2
	Peppermint	2.305555556+ 2.782407407* A- 0.360119048* B+ 0.118055556* A*B- 0.208333333* A^2- 0.005102041* B^2

Table 6 shows the highest antibacterial activity of heated kombucha beverages against each of the tested bacteria. The highest antibacterial activity against *S. dysentriae* with a diameter of the inhibition zone of 23 mm was observed in fermented beverage prepared with lemon verbena at the sucrose concentration of 8% and fermentation time of 20 days. Also, preparation of heated kombucha beverages based on peppermint and black tea created the highest antibacterial activity against *B. cereus*, (mean of diameter of inhibition zone of 21 mm) at the sucrose concentration of 8% and in 21 days of fermentation time. The best fermentation conditions for obtaining the highest antibacterial activity against *S. aureus*, with the highest diameter of the inhibition zone of 17 mm, was seen in the beverage prepared with green tea, the sucrose concentration of 5.5% and the fermentation time of 21 days. Finally, the highest antibacterial activity against *E. coli*, with the highest diameter of the inhibition zone of 15 mm, was observed in heated kombucha beverage prepared with lemon verbena, sucrose concentration of 8% and in 21 days of fermentation time. Other results obtained from Tables 6, and 3 indicate the higher resistance of *E. coli* to heated beverages, compared to the other tested bacteria. The resistance of *E. coli* to kombucha beverage has been reported by others (Battikh *et al.*, 2012).

Cell wall resistance of Gram-negative bacteria to inhibitors such as antimicrobial chemicals, herbal compounds, extracts, essential oils, antibiotics, etc. is related to lower permeability of the outer membrane of these bacteria and the presence of lipopolysaccharide in the cell wall as well as the periplasmic space of these bacteria, which restricts the entry of antimicrobial agents into the bacterial cell (Hayouni *et al.*, 2007; Russel, 1991; Burt., 2004). Although *S. dysenteriae* and *E. coli* are both Gram-negative and from the family Enterobacteriaceae, significant sensitivity of *S. dysentriae* to heated kombucha beverages was interesting. In studies of antibacterial activity of honey samples, this sensitivity was attributed to specific differences of each microorganism species to the antibacterial activity of honey sample (Tumin *et al.*, 2005; Ceyhan and Ugur, 2001; Taormina *et al.*, 2001; Nzeako and Hamdi, 2000).

Table 6- Individual preparation (fermentation conditions) for heated kombucha beverages in order to achieve the maximum inhibiton zone of each bacteria

Bacteria	Sucrose concentration (%)	Storage day	Kind of tea	inhibition zone (mm)
S. dysenteriae	8.0	20	lemon tea	23
B. cereus	8.0	21	black tea or peppermint	21
S. aureus	5.5	21	green tea	17
E. coli	8.0	18	lemon tea	15

In general, heated kombucha beverages showed significant antibacterial activity against the tested bacteria. It has also been reported in similar studies (Velicanski *et al.*, 2007, 2014; Battikh *et al.*, 2011; Sreeramulu *et al.* 2000).

In the study of the antimicrobial activity of various kombucha beverage analogues, showed that the antimicrobial activity of heated beverages remained stable by 56% decrease, and beverages prepared with lemon verbena (*L. citriodora*), peppermint (*M. piperita*) and fennel (*F. vulgare*) were among the heated beverages that showed the highest antimicrobial activity. Due to the sensitivity of proteins and enzymes to heat, they suggested that the antimicrobial activity of heated fermented beverages was not due to heat-sensitive compounds. (Battikh *et al.*, 2012).

The production of protein metabolites and enzymatic compounds of antimicrobial nature by microorganisms found in kombucha beverages along with the acidic pH and high content of acetic acid and other organic acids in these beverages are mechanisms thought for antimicrobial activity in these fermented beverages (Sreeramulu *et al.*, 2000; Greenwalt *et al.*, 1998). The type of herbal tea used in the preparation of kombucha beverages can also cause the antimicrobial activity of these beverages due to the presence of antimicrobial compounds in the plant's natural constituents.

Due to the high sensitivity of proteins and enzymes to the heat and activity of these compounds at high pH, the antibacterial activity of heated beverages cannot be attributed to the production and the presence of these compounds with the heat sensitive protein nature and the high content of acetic acid and other organic acids in the fermented beverages and the presence of the intrinsic antimicrobial compounds of the plants used can justify the antibacterial activity of the beverages.

Sreeramulu et al. (2000) studied the antimicrobial activity of the kombucha beverage, reported antibacterial activity against *E. coli*, *S. sonnei*, *S. typhimurium*, *S. enteritidis* and *C. jejuni*, even after heat treatment. Due to the presence of this antibacterial activity even in neutralized beverages, the researchers attributed the antibacterial activity of this fermented beverage to the presence of antimicrobial compounds other than acetic acid and protein compounds. Of course, numerous studies have also identified acetic acid as the dominant compound of the antimicrobial activity of kombucha beverages (Velicanski *et al.*, 2014; Steinkraus *et al.*, 1996; Cetojevic Simin *et al.*, 2008). Acetic acid and other organic acids can cause antimicrobial activity by acidifying the cytoplasm and by accumulating the separated acid anion into toxic amounts (Mani-Lopez *et al.*, 2012).

In addition to the acidic pH and high content of acetic acid and other organic acids, the presence of antibacterial compounds inherent in the used plant in preparation of beverages can be effective on this activity. For example, compounds such as phenylpropanoid with verbascoside (the most abundant compounds) and other compounds such as iridoid, verbenalin, along with flavonoids, luteolin, and aryenine, have been identified in the plant of lemon verbena (Bilia *et al.*, 2008). In the present study, the highest antibacterial activity against *E. coli* and *S. dysenteriae* was observed in heated kombucha beverages prepared with lemon verbena (at 8% sucrose concentration and fermentation time of 18 and 20 days) (Table 6). Heated kombucha beverage prepared with green tea (at 5.5% sucrose concentration and 21 days fermentation time) showed the highest antibacterial activity against *S. aureus* (Table 6). This effect may be due to natural compounds in green tea such as catechins including epigallocatechin galate, epigallocatechin, epicatechin gallate, epicatechin and galocatechin and other compounds (An *et al.*, 2004; Noormandi & Dabaghzadeh, 2015). In the study of the quality of kombucha beverages prepared with various herbal teas, from beverages prepared with the green tea was reported the best quality and the highest antibacterial activity against *S. aureus* and *E. coli* (Primiani *et al.*, 2018). Also increased antimicrobial activity of green tea has been reported due to epimerization of the tea catechins under the heating conditions (Kim *et al.*, 2007).

Also, the highest antibacterial activity against *B. cereus* was observed in two heated kombucha beverages, prepared with black tea and peppermint (at 8% sucrose concentration and 21 days fermentation time) (Table 6). In the study of the biochemical and antibacterial activities of the essential oil of peppermint leaves, the most important constituents of the essential oil of peppermint leaves collected from Iran were identified as, alpha terpinen, isomentone, trans carveol, betacariophyllene and piperidinone oxide and were confirmed the antibacterial activity of peppermint (*Mentha piperita*), against *E. coli* and *S. aureus* (Yadegarinia *et al.*, 2006).

Catechins are the most important constituents in tea to which the biological effects of tea are related to them. Catechins comprise at least four major phenolic compounds, including epigallocatechin, epicatechin gallate, epigallocatechin gallate, and epicatechin. The most common in green tea is epigallocatechin gallate, which accounts for about 50% of tea catechins. The compounds of tea catechins may change during fermentation and oxidation processes. For example, tea catechins changed to thearubigins and theaflavins during fermentation. Usually, green tea catechins have a content of about 13 to 30%, which in black tea is about 5% instead, the content of oxidized phenolic compounds is 25% in black tea. Since black tea undergoes extensive fermentation, its catechin composition differs from green tea. Therefore, greater amounts of natural tea catechins are usually preserved in green tea, which undergoes less fermentation. The mechanism of the effect of tea catechins on the antibacterial activity might due to the effect of these compounds on the cell membrane and cell wall of bacteria (Song and Seong, 2007).

The high affinity of tea catechins to the cell wall components of bacteria causes their antibacterial activity. Differences in susceptibility of *S. aureus* and Gram-negative bacteria to catechins are related to this binding (Yoda et al., 2004). This antibacterial activity is strengthened by the replacement of the gallate group of catechins (Stapleton *et al.*, 2004).

Antimicrobial effect of tea catechins against *B. cereus* at nanomolar levels even higher than tetracycline and vancomycin have been reported to promote the use of green tea in the treatment of food poisoning (Friedman *et al.*, 2006).

Conclusion

The results showed significant antibacterial activity of heated kombucha beverages against the gastrointestinal pathogenic bacteria. Increasing sucrose concentration and fermentation time increased the antibacterial activity of these beverages. The highest antibacterial activity against *E. coli* and *S. dysenteriae* was observed in the heated fermented beverage prepared with lemon verbena. Kombucha beverages prepared with green tea showed the highest antibacterial activity against *S. aureus* and the highest antibacterial activity against *B. cereus* was observed in two heated kombucha beverages prepared with black tea and peppermint. The antibacterial activity of heated beverages cannot be attributed to the production and presence of compounds with a heat-sensitive protein nature, and the high content of acetic acid and other organic acids in the beverages and the presence of the inherent antimicrobial compounds of the plants used may justify the antibacterial activity of the fermented beverages.

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Declaration of interest

All authors declare that they had no conflicts of interest.

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بررسی فعالیت ضدباکتریایی نوشیدنی‌های کامبوچا حرارت دیده، تهیه شده با چند دمنوش گیاهی به‌وسیله روش سطح پاسخ

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چکیده

ترکیبات و فعالیت‌های بیولوژیکی نوشیدنی کامبوچا به نوع دمنوش گیاهی، غلظت ساکارز و زمان تخمیر بستگی دارد. این مطالعه با هدف بررسی تأثیر شرایط مختلف آماده‌سازی بر فعالیت ضدباکتریایی نوشیدنی‌های حرارت دیده کامبوچا توسط روش سطح پاسخ (RSM) انجام شد. چهار نوع دمنوش گیاهی شامل چای سیاه، چای سبز، به لیمو و نعناع فلفلی با سه غلظت ۲، ۵ و ۸ درصد ساکارز تهیه شدند و با کشت فعال کامبوچا تلقیح گردیدند. پس از ۷، ۱۴ و ۲۱ روز، نوشیدنی‌ها اتوکلاو شدند و فعالیت ضدباکتریایی آنها علیه چهار باکتری شامل *اشریشیا کلی*، *شیگلا دیسانتری*، *استافیلوکوکوس اورئوس* و *باسیلوس سرئوس* با روش چاهک ارزیابی شد. برای بررسی اثر غلظت ساکارز، زمان تخمیر و نوع دمنوش گیاهی بر فعالیت ضدباکتریایی نوشیدنی‌های حرارت دیده از RSM استفاده شد. نتایج نشان داد که افزایش غلظت ساکارز اثر قابل توجهی بر فعالیت ضد باکتریایی نوشیدنی‌های حرارت دیده علیه همه باکتری‌های آزمایش شده داشت. افزایش زمان تخمیر تأثیر معناداری بر فعالیت ضدباکتریایی نوشیدنی‌های حرارت دیده علیه *اشریشیا کلی* و *شیگلا دیسانتری* داشت. نوع دمنوش گیاهی تأثیر قابل توجهی بر فعالیت ضدباکتریایی علیه *استافیلوکوکوس اورئوس* و *شیگلا دیسانتری* داشت. بیشترین فعالیت ضدباکتریایی علیه *اشریشیا کلی* و *شیگلا دیسانتری* در نوشیدنی‌های تهیه شده با به‌لیمو مشاهده شد. نوشیدنی‌های تهیه شده با چای سبز بیشترین فعالیت ضد باکتریایی را در برابر *استافیلوکوکوس اورئوس* نشان دادند. بیشترین فعالیت ضدباکتریایی علیه *باسیلوس سرئوس* در نوشیدنی‌های حرارت دیده تهیه شده با چای سیاه و نعناع مشاهده شد. به‌طور کلی، نتایج این تحقیق فعالیت ضدباکتریایی قابل توجه نوشیدنی‌های کامبوچا حرارت دیده را علیه باکتری‌های مورد آزمون نشان داد.

واژه‌های کلیدی: اثر ضدمیکروبی، نوشیدنی تخمیری، شرایط تخمیر، غلظت ساکارز، زمان تخمیر

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