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Effect of *Hibiscus sabdariffa* and *Camellia synensis* extracts on microbial, antioxidant and sensory properties of ice cream

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Abstract Herbal extracts have been widely used by consumers for food fortification and medical purposes worldwide and are focused in traditional medicine in recent years. At this study, we aimed to examine the antimicrobial, antioxidant and sensory characteristics of ice creams fortified with different combinations of Hibiscus sabdariffa and Camellia synensis determined by Design of Experiments software. Levels of 10-100 and 40-400 mg/kg for Hibiscus sabdariffa and Camellia synensis extracts, respectively, were selected and experiments were conducted by central composite design. Generally, 13 runs were introduced by the software and followed in laboratory for analysis. Antimicrobial activity was studied against Escherichia coli and Staphylococcus aureus as indicators of gram negative and gram positive bacteria. Results showed that all combinations were active against both bacteria but Staphylococcus aureus was more sensitive than Escherichia coli. Importantly, Camellia synensis was more effective than Hibiscus sabdariffa in both antimicrobial and antioxidant experiments but sensory panelists selected the sample containing lowest concentrations of

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both extracts. However, frequent consumption of low-dose fortified dairies with *Hibiscus sabdariffa* and *Camellia synensis* could be helpful for consumers interested in functional foods.

Keywords Antimicrobial activity · Antioxidant activity · *Camellia synensis* · *Hibiscus sabdariffa* · Ice cream · Sensory attribute

Introduction

Addition of bioactive ingredients to foods and beverages helps in health promotion and has impact on prevention of some non-communicable diseases to some extent. Herbal extracts are interested in recent years for medical purposes because of their constituents affecting on human health. For instance, Camellia synensis and Hibiscus sabdariffa that in turn are publically named as green tea and sour tea, have been widely used as food ingredient other than infusion (Mozaffari-Khosravi et al. 2013). It was estimated that nearly 4.2% of all beverages consumed in the world (mainly in Asia) is related to green tea (Raman et al. 2019). Technically, the C. synensis leaves is not exposed to fermentation and therefore contains high level of natural antioxidants including catechins from which epigallocatechin gallate is the major constituent (Mathew 2015; Saeed et al. 2017), that accounted approximately 65% of all catechins (Raman et al. 2019). Phenolic compounds in green tea are responsible for stimulation of antioxidant enzymes, inactivation of free radicals by electron donation of OH groups attached to benzene and metals chelation. Moreover, green tea is effective in metabolism and growth inhibition of several bacteria (Alipoor et al. 2012; Saeed et al. 2017). In the study of Matan et al. (2015), green tea

extract had inhibitory impact on Listeria monocytogenes, Escherichia coli and Salmonella thyphymorium inoculated on dragon fruit. The authors reported that green tea at concentration of 5% could prolong the shelf life of contaminated fruits up to 15 days that was comparable to lower than 5 days for non-treated fruits. They reported that antioxidants (mainly epigallocatechin gallate) were responsible for antimicrobial efficacy (Matan et al. 2015). Green tea has also positive impact on tooth health by suppression of oral pathogenic bacteria. Golmohammadi (2018) found that green tea extract had slightly higher inhibitory impact on Streptococcus mutans than chlorhexidine 0.2%, probably due to the extract components' binding to microbial cell wall and interfering to its adherence to dental surface. However, the antimicrobial impact was not significant in healthy microorganisms. For example, green tea extract had no inhibitory effect on Lactobacillus rhamnosus in fermented pork after 3 weeks of fermentation at 16-18 °C and 6 months of storage at 4 °C which might be due to the fact that probiotics produce antioxidants through which can survive in the presence of catechins compared to pathogenic bacteria (Neffe-Skocinska et al. 2015). In addition, green tea is one of ingredients used in production of energy drink and the plant infusion is used in obese people for weight loss and lowering LDL cholesterol through noradrenaline-induced thermogenesis and suppression of the gene involved in fatty acid synthase production (Grumezescu and Holban 2019). Nonetheless, green tea is popular in Asian countries compared to H. sabdariffa extract (majorly prepared from dried calyces) that is popular in Europe and United States of America (FAO 2004). Several beneficial impacts have also been reviewed for H. sabdariffa extract. With regards, Riaz and Chopra (2018) reported anticancer (by interfering in enzymatic metabolism and apoptosis induction), serum lipid lowering (through lipase inactivation) and antimicrobial (as a result of cell wall and fimbriae complexation through which cell permeability and adhesion to biological surfaces are affected, respectively) activities in H. sabdariffa extract that directly related to phenolic compounds. In addition, promotion of mineral availability such as iron has been associated with H. sabdariffa extract because of its acidic nature and high ascorbic acid content (Riaz and Chopra 2018). Importantly, organic acids and derivatives have negative impact on lipogenesis by inhibition of enzymes in formation of acetyl CoA. In addition, H. sabdariffa is introduced as a dietary source of iron (Peter et al. 2014).

Considering the positive impact of both *C. synensis* and *H. sabdariffa*, researchers have examined their functional properties derived from different vehicles. With regards, Granato et al. (2018) reviewed the latest studies on health effects of herbal extracts added to dairy products. They

concluded that ice cream containing natural additives such as tea extract could be one of appropriate carriers of antioxidant and antimicrobial compounds especially in countries that ice cream is popular. Furthermore, another study reported that by increasing the temperature from 4 to 37 °C, anthocyanin level was reduced in *H. sabdariffa* extract that suggests the cold stored or frozen foods as better carrier for heat-sensitive antioxidant to achieve their high bioavailability (Sinela et al. 2017). For this reason, the objective of current study was to optimize the mixture of *C. synensis* and *H. sabdariffa* to be added to ice cream by measuring the antimicrobial, antioxidant and sensory characteristics. The study conducted based on the design prepared by Response Surface Methodology (central composite design method).

Materials and methods

Design of experiments

Design Expert Software Version 7.1.5 was used for treatments' designing. The technique of Response Surface Methodology and method of Central Composite Design were conducted. Two numeric variables of extracts concentration including *C. synensis* and *H. sabdariffa* at two levels of 40–400 and 10–100 mg/kg, respectively, were assigned. The levels were selected based on results of previous studies (Hutkova et al. 2016; Portillo-Torres et al. 2019). By considering alpha values, ranges of 1–109 and 4–436 were determined for *H. sabdariffa* and *C. synensis*. In total, 13 runs were achieved and followed in the laboratory for antioxidant and sensory experiments (Table 1).

Table 1
Total runs achieved by central composite design for combination of *Hibiscus sabdariffa* and *Camellia synensis*

Runs	Hibiscus sabdariffa	Camellia synensis
1	55	436
2	55	4
3	10	400
4	100	40
5	55	220
6	109	220
7	55	220
8	1	220
9	10	40
10	55	220
11	55	220
12	100	400
13	55	220

Furthermore, the third categoric variable of time (months 1–4) was added for microbial analysis which resulted in 52 runs. The control without extracts but containing both microorganisms was used for comparison to verify the effectiveness of extracts under freezing. The results were analyzed by ANOVA method and significance level was P = 0.05.

Extract preparation

C. synensis leaves and *H. sabdariffa* calyces were purchased from local market (Lahijan, Iran). For extraction, they were grinded mechanically to visually fine powder was achieved. Then, 20 g of powder was mixed with 200 ml ethanol (70% v/v) followed by shaking at room temperature for 72 h. The mixture was filtered by Whatman filter paper No. 2 and then rotary evaporated (Heidolph, Germany) for 2 h at 40 °C for solvent removal. The extracts were maintained in sealed bottles at 4 °C up to analysis (Gramza et al. 2004).

Sample preparation

Ice cream samples were prepared in Pak company (Tehran, Iran). The control was free of extract and the treatments contained extracts at ratios determined by central composite design. The extracts were added to the final formula of ice cream and mixed. Then, the products were packed in 1-kg boxes and transferred to freezing room.

Microbial analysis

Lyophilized bacteria including *Escherichia coli* PTCC 1860 and *Staphylococcus aureus* PTCC 1431 were purchased from Iranian Biological Resource Center and activated in the laboratory for analysis according to the supplier's direction. Then, serial dilutions were prepared up to 10^{-3} for experiments. Treatments and control were inoculated up to final concentration of 2×10^3 CFU/g. Microbial growth of treatments and control were enumerated each month for 120 days. Samples were stored under freezing condition until the end of analysis. For *E. coli* enumeration, suspensions were pour plated in Violet Red Bile Agar (Merck, Germany) and surface plate on Baird Parker Agar (Merck, Germany) was done for *S. aureus*. Inoculated plates were incubated at 37 °C for 24–48 h (Kanbakan et al. 2004).

Antioxidant analysis

This experiment was conducted based on inactivation of DPPH free radicals according to the methods of Borras-Linares et al. (2015) and Sharma et al. (2008) with some modifications. To avoid the interfering effect of turbidity in pure samples, different dilutions of ice cream in distilled water at concentrations of 300, 450, 600, 750 and 900 mg/l were prepared. Then, methanolic DPPH reagent in concentration of 100 μ M was prepared. Four ml of DPPH reagent was mixed with one ml of diluted ice cream and stored in darkness for 30 min. The absorbance was measured in spectrophotometer (Unico, UV-2100, USA) at 517 nm. A similar way was followed by methanol (96% v/ v) as blank. Among all dilutions of ice cream, the antioxidant activity was appropriately observed at the highest concentration (900 mg/l). Therefore, the results of this concentration were used for comparison of samples. DPPH inactivation was calculated according to Eq. (1):

DPPH (%) =
$$\frac{(A_{blank} - A_{treatment})}{A_{blank}}$$
 (1)

Sensory evaluation

This test was conducted at the end of study. Ice creams (free from microorganisms but contained extracts) were sensory evaluated by 30 untrained people through 5-point Hedonic method. They were requested to rank the samples from 1 to 5 for color, taste and overall acceptance. The rank 1 was the worst and 5 was the best selection of panelists. The means of ranks were compared by One-way ANOVA test followed by Duncan post hoc and differences were significant at $P \le 0.05$.

Results and discussion

Antimicrobial effect of *H. sabdariffa* and *C. synensis* extracts in ice cream

The results of ANOVA analysis for both *E. coli* and *S. aureus* are presented in Table 2. The models presented in Eqs. (2) and (3) are significant with non-significant lack of fit. R-squared and Adequate Precision that reflect the ratio of signal to noise are high enough (respect to the minimum of 4 as criteria) implying the fitness of model presented. The results showed that majority of variables and their interactions had significant impact on *E. coli* and *S. aureus* growth. The models identified as follows:

(2)

Table 2 Analysis of variance for H. sabdariffa and C. synensis extarcts' impact on microbial growth and antioxidant activity in ice cream

Source	Sum of Squares	df	Mean Square	F Value	p value Prob > F	
E. coli						
Model	16.41	14	1.17	15.00	< 0.0001	Significant
А	0.050	1	0.050	0.63	0.4311	
В	4.90	1	4.90	62.72	< 0.0001	
С	6.74	3	2.25	28.75	< 0.0001	
AB	1.28	1	1.28	16.36	0.0003	
AC	0.30	3	0.10	1.30	0.2893	
BC	1.56	3	0.52	6.65	0.0010	
A^2	0.69	1	0.69	8.86	0.0051	
B^2	0.76	1	0.76	9.77	0.0034	
Residual	2.89	37	0.078			
Lack of fit	1.73	21	0.082	1.13	0.4065	Not significant
Pure error	1.16	16	0.073			
Cor total	19.31	51				
R-squared		0.8502				
Adeq precision		13.903				
S. aureus						
Model	35.96	12	3	30.7	< 0.0001	Significant
А	0.11	1	0.11	1.16	0.2883	
В	1.83	1	1.83	18.74	0.0001	
С	30.17	3	10.06	103.03	< 0.0001	
AB	1.57	1	1.57	16.11	0.0003	
AC	1.19	3	0.4	4.06	0.0133	
BC	1.09	3	0.36	3.73	0.019	
Residual	3.81	39	0.098			
Lack of fit	2.25	23	0.098	1.01	0.5050	Not significant
Pure error	1.56	16	0.097			
Cor total	39.77	51				
R-squared		0.9043				
Antioxidant activity						
Model	0.14	5	0.029	4286.03	< 0.0001	Significant
А	6.656E-003	1	6.656E-003	984.73	< 0.0001	
В	0.12	1	0.12	17963.04	< 0.0001	
AB	9.025E-003	1	9.025E-003	1335.13	< 0.0001	
A^2	3.344E-004	1	3.344E-004	49.48	0.0002	
B^2	7.126E-003	1	7.126E-003	1054.14	< 0.0001	
Residual	4.732E-005	7	6.760E-006			
Lack of fit	4.732E-005	3	1.557E-005			Not significant
Purr error	0.000	4	0.000			
Cor total	0.14	12				
R-squared		0.9997				
Adequate precision		204.926				

A: H. sabdariffa; B: C. synensis; C: time

Sqrt (S. aureus + 0.10) =
$$+1.45 - 0.064 \text{ A} - 0.26 \text{ B}$$

- 0.21 C₁ + 1.28 C₂ - 0.37 C₃ + 0.31 AB
+ 9.790E - 003 AC₁ + 0.32 AC₂ - 0.26 AC₃
- 0.17 BC₁ - 0.21 BC₂ + 0.09 BC₃
(3)

The higher minus coefficient of green tea than sour tea (-0.42 vs. - 0.042 for E. coli; -0.26 vs. - 0.064 for S.aureus) relates to the strong antibacterial activity of C. synensis extract. It is verified in counter and 3D plots during storage time. According to the Fig. 1, except for month two, C. synensis was more effective in suppression of E. coli compared to H. sabdariffa within the ranges of concentration studied in the current work. In accordance, it is obvious in 3D plots that the slope of bacterial loss is sharper for green tea. Same results were observed for S. aureus (Fig. 2) so that the microorganism was totally suppressed at the end of month three. In agreement, it has been reviewed that herbal extract including green tea was more effective on gram positive bacteria such as S. aureus compared to no or limited inhibition of E. coli due to the protective role of outer membrane on gram negative bacteria (Siddiqui et al. 2016). In another study, C. synensis extract showed higher inhibition on S. aureus (MIC = 6.25 mg/ml, inhibition zone = 15 mm) than E. coli and Pseudomonas aeruginosa (MIC = 12.5 mg/ml for both, inhibition zone = 9 and 10 mm respectively) that were almost equally inhibited. In was reported that caffeine is the prominent component in green tea (48.21%) that shows antimicrobial activity by inhibition of protein and DNA synthesis (Gupta and Kumar 2017). Interestingly, authors also reported that green tea polyphenols are effective against antibiotic resistant S. aureus (Malongane et al. 2017). To the contrary, Abdallah (2016) showed that H. sabdariffa was significantly effective against gram positive and negative bacteria so that S. aureus was the most susceptible microorganism followed by gram negative bacteria including E. coli. Effectiveness of extract was also comparable to antibiotics in disc diffusion analysis. H. sabdariffa extract and concentrate had also inhibitory effect on Salmonella enterica, E. coli, and Listeria monocytogenes, respectively. Treatments were efficient enough in suppression of all bacteria and temperature (4-25 °C) did not have impressive impact on microorganisms particularly after 48 h (Jaroni and Ravishankar 2012). Similar results about gram-dependent susceptibility of bacteria were reported for H. sabdariffa extract (Bayram et al. 2015). On the other hand, efficient bacterial loss during 3-4 months in ice creams could be related to the effectiveness of methanol extraction that carries more functional groups than hydro-extraction process (Siddiqui et al. 2016). With regards, higher amount of alcohol extraction (6.8%

w/w) compared to water extraction (2.2% w/w) in *C.* synensis analysis was determined (Mathew 2015). Opletal et al. (2017) implied that presence of viscosity-enhancer compounds such as pectin and mucilage interfere in aqueous extraction.

Extract-pathogens interaction in ice cream offers the vehicle for probiotic fortification owing to approved results of no inhibition on favorable bacteria. Relevantly, encapsulated L. casei and Bifidobacterium lactis within chitosanalginate containing C. synensis were significantly protected in ice cream during three months of storage at -18 °C. The higher count of L. casei compared to control was maintained from first day until the end of study but B. lactis showed lower count in treatment groups relative to control up to the middle of storage time and then was better protected. The authors believed that differences in bacterial protection were probably due to their differences in oxygen demand not effectiveness of plant extract. They further concluded that C. synensis has protective impact on the probiotic bacteria probably due to its antioxidant activity and inclusion of selenium as growth promotor (Noori et al. 2017).

Antioxidant activity of *H. sabdariffa* and *C. synensis* extracts in ice cream

It can be seen in Table 2 that *C. synensis* is more efficient (coefficient of 0.13 vs. 0.031 for *C. synensis* and *H. sab-dariffa*, respectively). The model presented for antioxidant activity in treated ice cream was as follows:

Antioxidant =
$$1.89 + 0.031 \text{ A} + 0.13 \text{ B} + 0.048 \text{ AB}$$

+ $8.643\text{E} - 003 \text{ A}^2 + 0.04 \text{ B}^2$ (4)

In agreement, higher DPPH radical scavenging activity was reported for *C. synensis* than Roselle tea prepared from *H. sabdariffa* leaves (Mabel et al. 2017). In current study, high R-squared (0.9997) refers to the fitness of model. Although, the highest antioxidant activity was observed at maximum level of both extracts (Fig. 3).

Sanguigni et al. (2017) examined the antioxidant level in serum of male volunteers consumed ice cream fortified with cocoa powder, hazelnut and green tea as source of phenolic compounds. They found that serum antioxidant capacity increased up to 19-fold in treatment group compared to control consumed ice cream free of natural antioxidants. Therefore, oxidative stress reduced and nitric oxide increased in volunteers in the presence of polyphenols that were almost maintained at low temperature of ice cream.

Antioxidants of tea species are both polar and non-polar in nature and method of extraction determines the level of

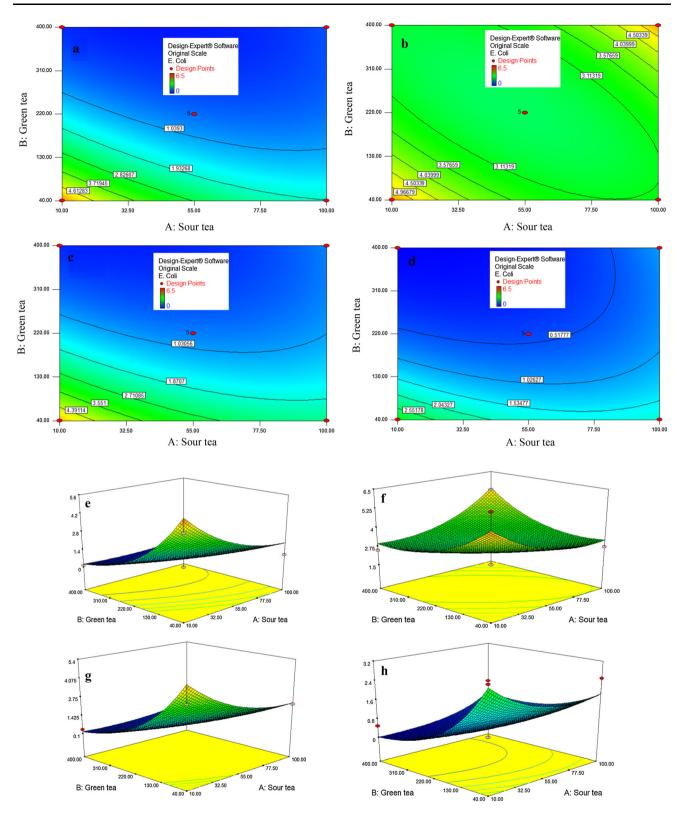


Fig. 1 Impact of H. sabdariffa and C. synensis extracts on E. coli growth during 4 months under freezing: a-d counter plots, e-h 3D plots

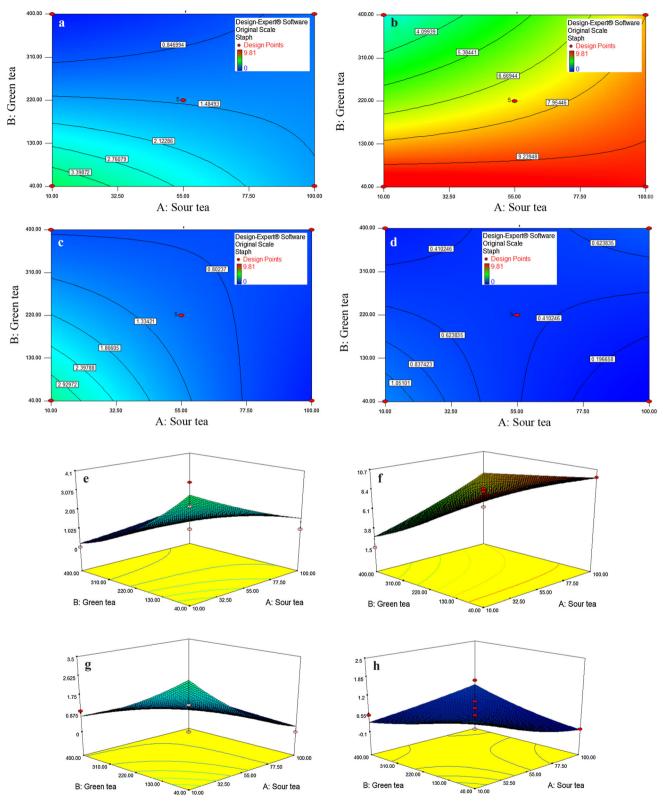


Fig. 2 Impact of H. sabdariffa and C. synensis extracts on S. aureus growth during 4 months under freezing: a-d counter plots, e-h 3D plots

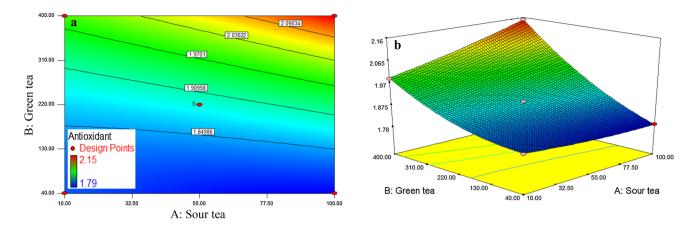


Fig. 3 Impact of C. synensis and H. sabdariffa extracts on antioxidant activity of ice cream in month four; a counter plot, b 3D plot

free radical scavenging. With regards, Nasrabadi et al. (2018) examined antioxidant capacity of *H. sabdariffa* seed powder and found higher free radical scavenging potential (DPPH = 94.15%) in non-aqueous extracts compared to aqueous counterpart (DPPH = 90.1%).

Antioxidant potency of herbal extracts has been studied and reviewed by several scientists. Preservation of lipids in various types of oils and meat products against oxidation by tea polyphenols was comparable to other natural and synthetic antioxidants such as tocopherol, ascorbyl palmitate, BHA and BHT (Grumezescu 2017). It has been approved that the antioxidant activity of green and sour tea is comparable to ascorbic acid as reference. In relevance, Mathew (2015) observed higher antioxidant potency for C. synensis compared to ascorbic acid in three analysis of lipid peroxidase, nitric oxide and superoxide radical scavenging that was due to the high content of antioxidants mentioned earlier having antimicrobial activity. For example, caffeine has significant role in antioxidant activity of green tea. In the study conducted on characterization of decaffeinated green tea was concluded that antioxidant level was significantly reduced after caffeine removal (Das et al. 2019). Moreover, higher antioxidant activity by DPPH method in H. sabdariffa extracts (IC50: 0.18-0.26 mg/ml) compared to ascorbic acid (IC50: 0.32 mg/ml) was observed (Kouakou et al. 2015). Okereke et al. (2015) reported that flavonoids are the highest constituent of H. sabdariffa calyces extract. Presence of some compounds named as co-pigments that protect the anthocyanin from nucleophilic attack of solvents have been investigated by authors. Maciel et al. (2018) found a higher color retention in H. sabdariffa aqueous extract at 60 °C than higher temperatures in the presence of added phytic and chlorogenic acids that originally exist in green and sour tea. The authors reported that the color was lower retained at higher temperatures because of both pigment and copigment thermal sensitivity. In comparison to control,

chlorogenic acid that is highly found in *H. sabdariffa* (Achir et al. 2019) had the highest impact (Maciel et al. 2018). In agreement, anthocyanin loss in *H. sabdariffa* extract was observed at 37 °C during long time of 40 days in the study of Achir et al. (2019) that might be due to the protective impact of high level of chlorogenic acid. However, phenolic compounds are heat sensitive and processing under mild temperatures favors on their better protection. Therefore, cold-stored or frozen foods such as ice cream are one of the best carriers for the fortification with natural bioactive compounds.

Sensory characterization of ice creams fortified by *H. sabdariffa* and *C. synensis* extracts

Microbial-free samples were tested by a group of sensory panellist at the end of month four in view of color, taste and

Table 3 Sensory scores of ice creams fortified with different combinations of *Hibiscus sabdariffa* and *Camellia synensis*

Runs	Color	Flavor	Overall acceptance
1	3.60 ± 0.81	1.50 ± 0.86	3.87 ± 0.82
2	4.03 ± 0.10	4.13 ± 0.82	4.73 ± 0.45
3	0.87 ± 0.68	1.44 ± 0.68	2.03 ± 0.81
4	3.90 ± 0.99	4.40 ± 0.67	4.23 ± 1.01
5	1.93 ± 0.91	1.80 ± 0.80	3.00 ± 0.69
6	2.93 ± 0.83	1.30 ± 0.70	3.87 ± 0.68
7	1.43 ± 0.82	3.87 ± 0.86	4.10 ± 0.76
8	1.37 ± 0.76	2.53 ± 0.86	2.33 ± 0.84
9	2.23 ± 0.77	4.43 ± 0.63	3.97 ± 0.72
10	1.97 ± 0.85	3.03 ± 0.76	3.90 ± 0.71
11	1.80 ± 0.76	4.03 ± 0.56	2.73 ± 0.87
12	3.00 ± 0.87	1.87 ± 0.63	3.07 ± 0.83
13	2.83 ± 0.75	1.97 ± 0.61	4.23 ± 0.94

overall acceptance. Results of sensory attributes are presented in Table 3. It can be seen that run number 2 was the best in color and overall acceptance that is related to the sample containing *H. sabdariffa* and *C. synensis* in the ratio of 55:4 mg/kg, and the sample corresponded to 10:40 ratio was significantly accepted in flavor. The higher flavor palatability of low concentration of extracts was probably due to the lower amount of bitter polyphenols (Malongane et al. 2017) and sourness of H. sabdariffa. Preciado-Saldaña et al. (2019) investigated the characteristics of H. sabdariffa and C. synensis mixed beverages and reported that besides adequate antioxidant content at all levels, the sensory score was significantly reduced by increasing the concentration of C. synensis extract (ratio of 7:3 for H. sabdariffa:C. synensis was the best). In their opinion, catechins and gallic acid was mainly responsible for unpalatability of C. synensis and anthocyanin of H. sabdariffa had impact on its better sensory acceptance. Compared to our results, it is likely that sourness is more accepted by the consumers in beverages than dairies. However, both extracts had desirable antimicrobial and antioxidant properties that could offer them as an alternative in formulation of new dairy products. Importantly, overconsumption of green tea may lead to intoxication of vital organs and can induce aluminum accumulation in human body (Saeed et al. 2017). It may be due to the fact that green tea is not subjected to fermentation and includes high amount of polyphenols as antioxidant. What is important is that antioxidant should be delivered adequately to neutralize pro-oxidant agents. Otherwise, they can pose pro-oxidant role in excessive levels and harm to body organs (Malongane et al. 2017). Although, it is unlikely that daily intake of green tea up to 150 mg per kg body weight has toxic effect in healthy consumers (Riaz and Chopra 2018). It would be less important for sour tea because its consumption as infusion or its intake as ingredient in other products is self-limited by most of consumers due to high sourness and hypotension effect.

Conclusion

Dairy products have been introduced as appropriate carrier of functional ingredients. In current study, we examined the functionality of *H. sabdariffa* and *C. synensis* in ice cream. As a result, high antimicrobial activity against *E. coli* and *S. aureus* were observed and high antioxidant capacity determined for both extracts. Although, *C. synensis* showed higher potential than *H. sabdariffa* in both examinations. Furthermore, *S. aureus* was more suppressed by two extracts compared to *E. coil*. In sensory evaluation, the best overall acceptability was observed for moderate and least amounts of *H. sabdariffa* and *C. synensis* (55 and 4 mg/kg) respectively. We concluded that fortification of dairy products with low to moderate concentrations of the extracts could help in promotion of health status in consumers.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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