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RESEARCH ARTICLE

The effect of *Ruscus Hyracanus* extract on physicochemical, microbial and organoleptic properties of kefir

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Abstract: Kefir is a traditional functional fermented drink with acidic-alcoholic properties. In this study, the effect of *Ruscus hyracanus* extract (0, 0.25%, and 0.5%) on the *physicochemical, microbial,* and sensory properties of kefir during 20 days of storage was evaluated. Total phenolic content (TPC) and antioxidant activity (AO) were evaluated using Folin-Ciocalteu, and *2,2-diphenyl-1-picrylhydrazyl* (DPPH) techniques. The results revealed that the stability and viscosity of kefir samples containing *Ruscus hyracanus* extract were higher than control samples. Increasing the amounts of *Ruscus hyracanus* extract had a significant effect on the TPC, AO, and lactic acid (LAB) numbers of enriched kefir ($P<0.05$). The presence of *Ruscus hyracanus* extract had a significant effect on the L* values of kefir ($P<0.05$). In general, a kefir sample containing 0.5% *Ruscus hyracanus* extract was more acceptable in comparison with the other samples on the 20th day of storage.

Keywords: Antioxidant, Kefir, Lactic acid bacteria, Phenol, *Ruscus Hyracanu*

Introduction

Kefir is a fermented dairy product with acidic-alcoholic properties that is related to people living in the Caucasus mountains. The Kefir idiom has its origin from the word "kef" which means 'pleasant taste' in Turkish. Generally, kefir is produced from the acidic and alcoholic fermentation of cow's milk using kefir seeds as a starter. Kefir grains are slimy, elastic, cauliflower-shaped, and in different sizes that are composed of *Kluyveromyces*, *Saccharomyces*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Acetobacter* species (Spizzirri et al. 2022).

Fermented kefir usually contains ethanol, lactic acid, and CO_2 as major components and acetaldehyde, acetoin, and diacetyl as aromatic ingredients. Also, nutritious components such as amino acids, folic acid, vitamin K, vitamin B_1, and B_{12} increment over the fermentation period. Kefir generally contains 0.5% to 2% alcohol, a different amount of fat, with an acidic, slightly rough yeasty taste and pH of around 4.0 (Montanuci et al. 2012). These days, kefir is popular because of the presence of bioactive metabolites that confer health benefits. Lactose intolerance improvement, antimicrobial activity, antioxidant activity, anti-inflammatory effect, wound healing, antitumor activity, and immune system modulation are health-promoting properties that have been attributed to kefir consumption (Perna et al. 2019; Znamirowska et al. 2017).

Kefir fortified with plant extract rich in bioactive components can enhance neutraceutical benefits and promotes functional characteristics. Kim et al. (2017) reported beneficial effect of *Linum usitatissimum* (flaxseed) extract on the growth and viability of kefir-isolated Lactic acid bacteria. Similarly, Atalar (2019) presented that the presence of hazelnut milk in kefir improved bioactive properties, nutritional values and viability of LAB microorganisms. Also, Perna et al. (2019) stated that the total phenolic content (TPC) and antioxidant activity of donkey kefir increased in fortified samples with honey and *Rosmarium officinalis* essential oil.

The *Ruscus hyracanus* L is a rhizomatous, perennial, and treasureless shrub and is widely found in Mediterranean countries, north Africa, eastern Europe, southwest Asia, and different provinces of Iran (Shamalizade Baii et al. 2017). The *Ruscus hyracanus* extract contains flavonoids, phenolic, phytosterols, saponins, triterpenoids, and linalool compounds (Baharfar et al. 2016), which have high antimicrobial (especially antifungal) and antioxidant activities (Dehghan et al. 2016;

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Hadzifejzovic et al. 2013). The *Ruscus hyracanus* extract has diverse medicinal properties such as appetizing, diuretic, vasoconstrict, anti-laxative, antibleeding, anti-nephritis, and anti-infection properties (Dehghan et al. 2016).

Consumer demand for fortified kefir has increased due to benefit from its health-giving properties. The objective of the present study was to evaluate the effect

of *Ruscus hyracanus* extract addition on physicochemical properties and microbial quality of fortified kefir during storage.

Materials and Methods

Materials

Cow milk (2/5% fat) was purchased from Pegah company in Tehran (Iran). The DVS kefir starter culture in lyophilized form was supplied by Christian Hansen (Horsholm, Denmark). The *Ruscus hyracanus* leaf was purchased from the local market in Tehran, Iran. All chemicals used in this research provide from analytical grade and were prepared by Merck (Germany) company.

Preparation of *Ruscus hyracanus* extract and Kefir manufacture

For the *Ruscus hyracanus* extract preparation, the method of Ok and Jeong (2012) was applied with slight modifications. Briefly, *Ruscus hyracanus* samples were cut and put onto drying trays in a freeze-dryer (Biotron, Gyeonggi, Korea). Then, 10 g of ground *Ruscus hyracanus* mixed with 200 mL of distilled water and filtered through Whatman No. 1 filter paper. Next, the extract was centrifuged and supernatant was filtered using a 0.22 µm nylon filter. Finally, the *Ruscus hyracanus* extract was dried by freeze-dryer. Kefir was prepared according to Glibowski and Zielińska (2015) with some modifications. Briefly, *Ruscus hyracanus* powder (0, 0.25%, and 0.5% w/v) and DVS kefir culture was added at a level of 0.04 g into 4 L pasteurized and homogenized milk with 2.5% fat. The flasks were incubated for 24 h at 25 °C. Then, kefirs were mixed with a laboratory spoon and filled into 250 mL PET bottles. Kefir samples were stored at 4 °C for 20 days. Kefir samples were assessed on the 1st, 10th, and 20th days of storage and tests have been performed in triplicates.

Physicochemical assessments

The pH value of the sample was determined with a calibrated pH meter by directly putting the probe into kefir samples. Titrable acidity was determined by titratable the samples with 0.1 N NaOH (Dewi et al. 2020). The viscosity was assessed using a Brookfield Viscometer (RVDV-III, Ultra, USA) with LV3 spindle. The samples were poured into the measuring vessel and sheared from 1.0 to 500 (1/s) at 20°C (Liu and Lin, 2000). The centrifugal method described by Bensmira and Jiang (2012) was applied for the assessment of syneresis of kefir samples. To evaluate color parameters, L*, a*, and b* indexes in kefir samples, the Hunterlab instrument (UltraScanvis, US-Vis 1,310, USA) was applied. Lightness was determined between zero (black) to 100 (white), a* was evaluated from + 127 (red) to –128 (green), and b* was determined from + 127 (yellow) to –128 (blue) (Zonoubi and Goli, 2020). All tests were performed in three replications.

Total phenolic content and antioxidant activity measurement

At first, kefir samples were centrifuged at 7690 g for 20 min. Syringes filter with 0.45 µm (MSCA, Shanghai, China) was used for sample filtration. The total phenolic content (TPC) and antioxidant activity (AO) of kefir samples were determined according to the method of Bensmira and Jiang (2015) with some modifications. The TPC was evaluated by Folin-Ciocalteu method, and AO was evaluated based on the free radical reduction of 2,2-diphenyl-1-picrylhydrazyl and tests have been performed in triplicates.

Microbial analysis

For the enumeration of the *Lactic acid bacteria*, the dilutions of samples were plated in the MRS agar using the *Pour Plate method for counting the number of colony-forming units present in the liquid samples*. The plates were placed in a CO_2 incubator for 72 h at 37°C. The results were expressed as Log cfu/g (Vasheghani Farahani et al. 2022). Kefir samples were assessed on the 1st, 10th, and 20th days of storage and tests have been performed in triplicates.

Sensory analysis

Sensory evaluation was performed by 12 panelists (6 women and 6 men, aged 20-30) from graduate students of Tehran Azad University's Food Science and Technology Department (Tehran, Iran) who were trained about the properties of kefir products. Five-point hedonic scale ranging from 1 (dislike extremely) to 5 (like extremely) was applied by the panelist for sensory evaluation on the 20th days of storage (Rojas-Torres et al. 2021). All tests have been performed in triplicates.

Statistical analysis

Experiments were performed in triplicate, and the significant differences between means were analyzed using one-way ANOVA and LSD post hoc tests (SPSS, version 22, 2016). The nonparametric data were analyzed by applying the Kruskal-Wallis tests.

Results and Discussion

pH and titrable acidity measurements

The pH values and acidity of the different kefir samples during cold storage were reported in Fig. 1 (a, b). A decrease in pH goes in hand with an increase in the acidity during fermentation and

these changes were significant ($p<0.05$). However, statistical analysis cleared that the presence of *Ruscus hyracanus* extract decreased the pH of kefir samples insignificantly ($pÃ0.05$). The kefir sample containing 0.5% *Ruscus hyracanus* extract had the highest acidity on the 1st day, and the lowest acidity belonged to the control sample on the 1st day. The previous studies have stated the increased trend in acidity due to the lactic acid bacteria growth, fermentation of lactose and production of lactic acid, and other organic acids (Vasheghani Farahani, 2022). Aiello et al. (2020) reported a reduction in the pH of lentil-supplemented kefirs after 14 days of storage. Okur (2022) reported the increase in acidity in fortified kefir when olive leaf extract was used.

Viscosity and syneresis measurements

The viscosity of kefir samples was in the range of 4.017 to 6.387 (cP) which was higher than the 3 (cP) reported by Saygili et al. (2022) at 20°C. Kefir viscosity is influenced by milk physicochemical properties like dry matter, fat, protein concentration, pH, acidity, and temperature. Moreover, the assessment conditions of viscosity comprising shear rate, temperature, and spindle number are important (Haji Ghafarloo et al. 2019). As it is shown in Figure 2a, the addition of *Ruscus hyracanus* extract and the storage time caused significant increase in the viscosity of the kefir samples ($p<0.05$). The viscosity of kefir may be influenced by carbohydrates contained in *Ruscus hyracanus* extract and the water absorption properties of these components (Edziri et al. 2020). Moreover, the protein-protein interaction and links between the proteins rearranging due to the decrease in pH value during the storage period of kefir increased viscosity. Also, the viscosity might be raised due to the increment of the water- binding capacity of proteins after moving away from the isoelectric pH value (Bulut et al. 2021). As has been reported by Maleki et al. (2021), increasing the amount of free and microencapsulated extract of *Tragopogon collinus* caused a significant increment in the viscosity of probiotic yogurt. Also, Bulut et al. (2021) reported that the apparent viscosity of the fortified set-type yogurt with different plant extracts increased during storage.

The syneresis data for kefir samples are revealed in Figure 2b. Aqueous phase separation in fermented milk products occurs due to the accumulation of protein particles during storage and their deposition under gravity. Some other factors such as stabilizers, acidity, solids, and the type of milk and culture can be effective in the formation of the aqueous phase of fermented sweet drinks (Montanuci et al. 2012). In general, the addition of *Ruscus hyracanus* extract decreased the syneresis in kefir samples significantly ($p<0.05$), but the difference between treatments on the 1st day was not significant ($p>0.05$). Reduction in the syneresis level of *Ruscus hyracanus* extract in kefir samples was related to higher total solids and increased interactions between *Ruscus hyracanus* carbohydrate and protein particles. Also, *Ruscus hyracanus* extract contains higher phenolic components which react with casein and whey proteins and improve the kefir stability (Gomes et al. 2023). As has been reported by Znamirowska et al. (2017), adding garlic powder (1% w/v) to kefir reduced syneresis significantly ($p<0.05$). Similarly, Gomes et al.

Table 1: The effect of *Ruscus Hyracanus* extract on the color indexes (a) L* (b) b* and (c) a* of kefir during 20 days of storage [a,b,c]

Samples	L*		
Day	Day1	Day 10	Day 20
Control	88.33 ± 0.2^{Aa}	88.22 ± 0.06^{Aa}	85.17 ± 0.07^{Ab}
T_1	85.53 ± 0.7^{Ba}	85.84 ± 0.3^{Ba}	84.87 ± 0.4^{Bb}
T_2	85.53 ± 0.3^{Ba}	85.67 ± 0.05^{Ba}	84.2 ± 0.5^{Bb}
	b*		
Control	2.37 ± 0.1^{Aa}	2.33 ± 0.05^{Aa}	1.29 ± 0.3^{Ab}
T_1	2.67 ± 0.06^{ABa}	2.48 ± 0.09^{Aa}	1.43 ± 0.08^{Ab}
T_2	3.04 ± 0.04^{Aa}	2.96 ± 0.2^{Aa}	1.8 ± 0.05^{Ab}
	a*		
Control	0.456 ± 0.08^{Aa}	0.303 ± 0.3^{Ab}	-0.553 ± 0.1^{Ac}
T_1	0.826 ± 0.06^{Aa}	0.363 ± 0.05^{Aab}	-0.253 ± 0.4^{Ab}
T_2	1.28 ± 0.02^{Aa}	0.91 ± 0.1^{Aab}	0.273 ± 0.08^{Ab}

[a] Samples were included (Control (0% *Ruscus Hyracanus* extract), T_1 (0.25 *Ruscus Hyracanus* extract), and T_2 (0.5% *Ruscus Hyracanus* extract)

[b] Means within each column followed by different letters (A–D) show significant difference ($P<0.05$) between treatments at the same time

[c] Means within each row followed by different letters (a–b) show significant difference ($P<0.05$) at a treatment during storage period

(2023) reported that adding *Moringa oleifera* leaf extract to the yogurtsŒ§ matrix diminished the increased level of syneresis significantly ($p<0.05$).

Color analysis

The basis of colorimetry is the measurement of L*, a*, and b* indexes which indicate white to black, green to red, and blue to yellow respectively. Table 1 shows the effect of different concentrations of *Ruscus Hyracanus* extract and storage time on the investigated color parameters. By increasing the amount of *Ruscus hyracanus* extract, a significant decrease was observed in the brightness of kefir samples ($p<0.05$). Presumably, the presence of dark compounds in the *Ruscus hyracanus* extract could diminish the brightness of the product. According to Table 1, with the increase of storage time, the L* index of the kefir samples decreased significantly ($p<0.05$). The reduction of the L* index during storage may be related to increasing proteolysis and lipolysis reactions and changes in the structure of kefir protein and fat globules as well as color substances during the storage period. Following the trend as observed by Znamirowska et al. (2017), who reported L* value decreased in kefir in the presence of garlic powder (1% w/v).

Table 3 shows the effect of *Ruscus hyracanus* extract and storage time on the b*parameter. By increasing the amount of *Ruscus hyracanus* extract, no significant effect was observed in the yellowness of kefir samples. As results revealed the b* value was decreased significantly during storage ($p<0.05$). Presumably, the reduction in the intensity of the yellow color during storage was due to the degradation of yellow pigments of kefir samples. Contrary to these results Ardalanian and Fadaei (2018) reported all probiotic doogh samples containing ginseng extract exhibited higher b* values. Table 3 shows the effect of different percentages of *Ruscus hyracanus* extract and storage time on a* parameter. The results showed that only on the 1st day with the increase of *Ruscus hyracanus* extract to 0.5%, the a* parameter tended towards the red color, but on the 20th day with the increase of *Ruscus hyracanus* extract, this parameter tended towards the green color, which was not significant ($p>0.05$). Probably, the *Ruscus hyracanus* extract contains compounds producing red color, but these pigments are not stable over time and only increase the redness on the first day of storage. Similarly, Felfoula et al. (2017) observed regardless of the ginger addition intensity of the bovine milk increased.

Total phenolic content and Antioxidant activity

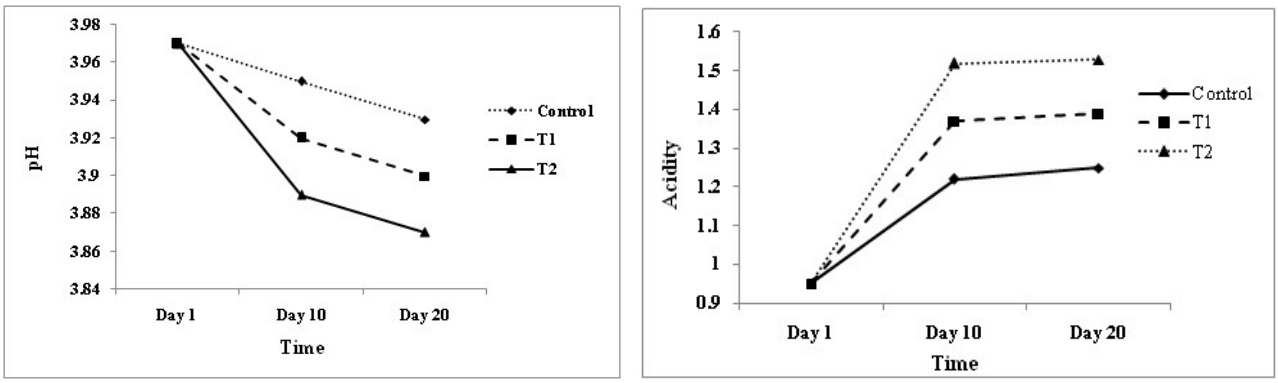

Fig. 1 The effect of *Ruscus Hyracanus* extract on pH (a) and acidity (b) of kefir during on 20th day of storage

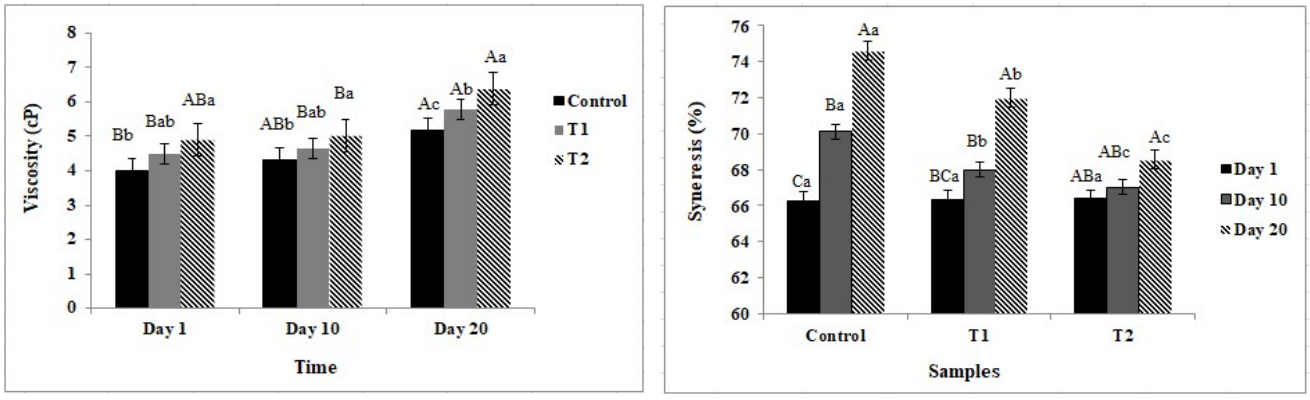

Fig. 2 The effect of *Ruscus Hyracanus* extract on the viscosity (a) and syneresis (%) (b) of kefir during 20 days of storage

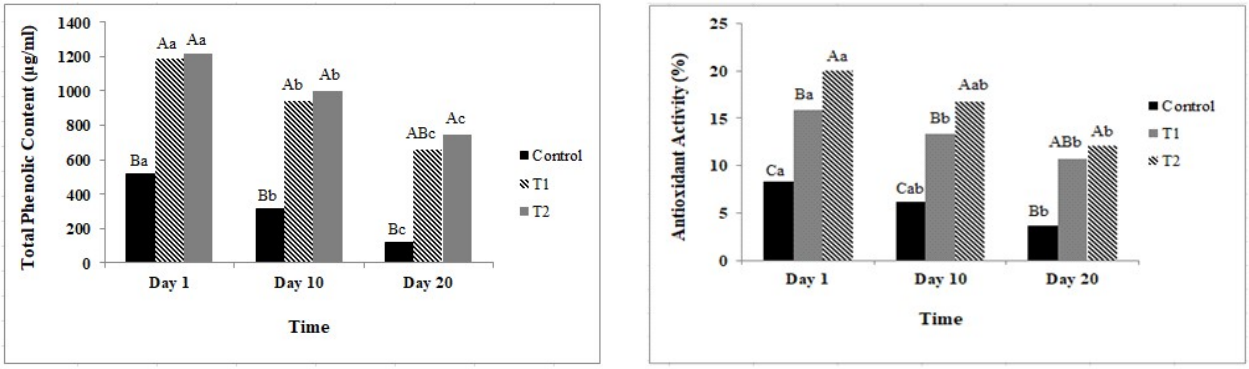

Fig. 3. The effect of *Ruscus Hyracanus* extract on total phenolic count (mg GAE/g) (a) and antioxidant activity (DPPH (%)) of kefir samples on 20th days of storage

Table 2. The effect of *Ruscus Hyracanus* extract on the Lactic Acid Bacteria (LAB) count of kefir during 20 days of storage [a,b,c]

Samples	Viable Lactic acid bacteria count (Log cfu g^{-1})		
Day	Day1	Day 10	Day 20
Control	7.2 ± 0.2^{ABa}	7.3 ± 0.08^{ABa}	7.36 ± 0.2^{Ba}
T_1	7.38 ± 0.09^{Aa}	7.43 ± 0.3^{Aa}	7.46 ± 0.04^{ABa}
T_2	7.44 ± 0.3^{Aa}	7.49 ± 0.07^{Aa}	7.53 ± 0.07^{Aa}

[a] Samples were included (Control (0% *Ruscus Hyracanus* extract), T_1 (0.25 *Ruscus Hyracanus* extract), and T_2 (0.5% *Ruscus Hyracanus* extract)

[b] Means within each column followed by different letters (A–D) show significant difference ($P<0.05$) between treatments at the same time

[c] Means within each row followed by different letters (a–b) show significant difference ($P<0.05$) at a treatment during storage period

The AO and TPC of fortified kefir in the presence of *Ruscus hyracanus* extract are presented in Fig. 3 (a, b). It can be observed that using *Ruscus hyracanus* extract in kefir increased the TPC and AO of kefir samples, and these increments were related to increasing in *Ruscus hyracanus* concentration. As presented in Fig. 3 (a, b), the incorporation of *Ruscus hyracanus* extract significantly enhanced the AO activity from 8.3 in the control sample to 20.1 in the kefir containing 0.5% *Ruscus hyracanus* extract. Antioxidants as a chemical ingredient could maintain the human body from progressive diseases by preventing oxidative damage through scavenging free radicals. *Ruscus hyracanus* extract as natural antioxidant could absorb free radicals and improve the antioxidant properties of kefir. The presence of flavonoid glycosides, p-coumaric acid, amides of hydroxycinnamic acids, and phenolic acids in Ruscus species led to increasing in AO activity of fortified kefir (Hadzifejzovic et al. 2013). Hadzifejzovic et al. (2013) found that the methanolic extract of *Ruscus aculeatus* L. and *Ruscus hypoglossum* revealed antioxidant activity and there is a relatively strong correlation between the total phenolic content and the antioxidant capacity of plants' extracts. Jakovljević et al. (2016) reported the antioxidant activity of Ruscus species and their potential for inhibiting lipid peroxidation. Moreover, the results showed that the AO activity decreases significantly in all fortified kefir treatments over storage. Presumably, the phenolic component in plant extract was changed through oxidation reactions during storage and decreased the AO activity. Similarly, Soliman and Nasser (2022) found that the AO activity reduced significantly in stirred yogurt samples with the increment in the storage period.

Changes in survivability of Lactic Acid Bacteria

Table 2 shows the effect of *Ruscus hyracanus* extract and the storage time on the variation of LAB *numbers* in kefir samples. The results showed that the presence of *Ruscus hyracanus* extract improve the viability of LAB significantly compare to the control samples ($p<0.05$). As seen in Table 2, the highest and lowest population of LAB was related to the sample containing 0.5% *Ruscus hyracanus* extract on the 20th day and the control sample on the 1st day, respectively. From these observations, it can be concluded that LAB used *non-digestible carbohydrates* for their growth and development, which caused their population to increase. According to the present study, some researchers investigated that *Ruscus hyracanus* extract increased LAB microorganisms in dairy drinks. The obtained results of LAB survivability were consistent with the acidity results. Ghosi Hoojaghan et al. (2022) reported that fennel extract could improve the growth of LAB in Iranian doogh. Similarly, Ardalanian and

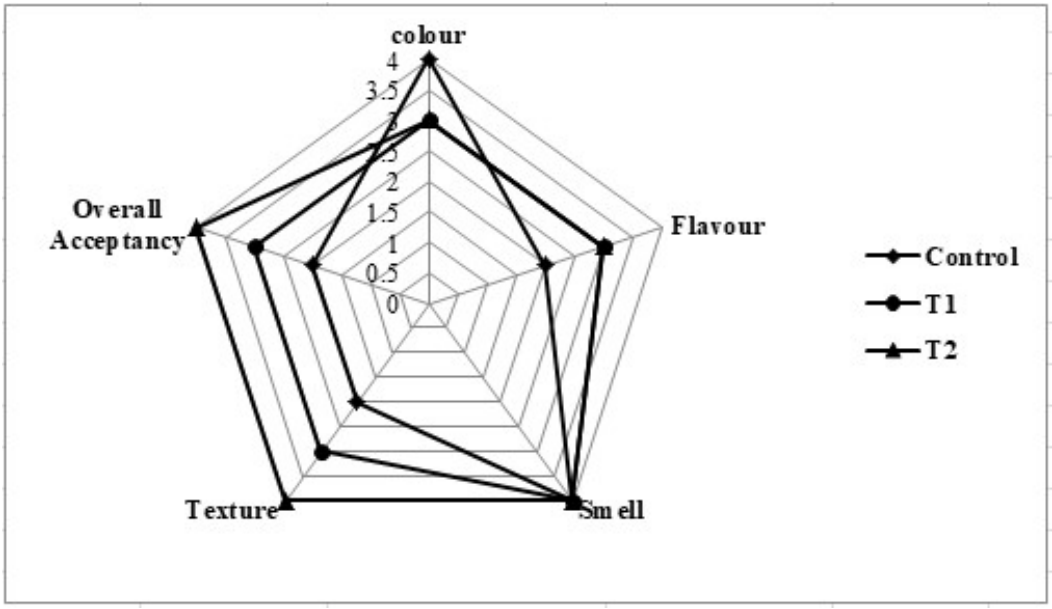

Fig.4. The effect of *Ruscus Hyracanus* extract on sensory properties of kefir samples on 20th days of storage

Fadaei (2018) reported that the viability of *Lactobacillus acidophilus* (La5) and *Bifidobacterium lactis* (Bb12) increased by the addition of Ginseng extract in the probiotic Doogh. According to Table 2, with increasing storage time, the population of LAB had insignificant changes ($p>0.05$). It was observed that the highest number was on the 20th day and the lowest was on the 1st day of storage. Contrary to these results Haji Ghafarloo et al. (2019) stated that the number of *Bifidobacterium Bifidum* decreased significantly during 30 days of storage of symbiotic doogh.

Sensory properties

Figure 4 reveals results of comparing the sensory properties of kefir reported by panelists on the 20th day of storage for color, flavor, smell, texture, and overall acceptability. Regarding the smell parameters, all samples revealed the same acceptance and had no significant difference on the 20th day. The T_1 treatment with 0.25% *Ruscus hyracanus* extract and T_2 treatment with 0.5% *Ruscus hyracanus* extracts had higher flavor scores compared to control samples. The presence of *Ruscus hyracanus* extract increased the flavor score significantly ($p<0.05$). This revealed that *Ruscus hyracanus* extract covers undesirable flavor created in the kefir over storage due to the presence of phenolic compounds in *Ruscus hyracanus* extract. Similarly, Haji Ghafarloo et al. (2019) stated that doogh samples with 0.25% ginger extract obtained higher flavor scores than the samples without extract. Regarding the color parameters, the highest color score was related to the control sample without *Ruscus hyracanus* extract ($p<0.05$). It seems that reducing brightness, yellowness, and redness in the treated kefir samples is effective factor in reducing the color score. Our findings are in agreement with the research of Ghosi Hoojaghan et al. (2022) who noted that the presence of fennel extract decreased the color score of treated doogh. As Figure 5 revealed adding *Ruscus hyracanus* extract increased the texture score significantly ($p<0.05$). The highest texture score was related to the T_2 sample while the control sample had the lowest texture score. Presumably, the distinction of panelists related to the changes in the viscosity characteristics of samples. Similarly, Glibowski and Zielińska (2015) stated the texture score between set-type kefirs with and without inulin did not differ. The overall acceptance score of kefir samples revealed that there was a significant difference between samples ($p<0.05$). The highest overall acceptance score belong to T_2 treatment with 0.5% *Ruscus hyracanus* extract and control samples had the lowest overall acceptance. Ardalanian and Fadaei (2018) reported an increase in the overall acceptance score of doogh enriched by ginger extract ($p<0.05$).

Conclusions

In this research, a new functional kefir by applying *Ruscus hyracanus* extract in kefir formulation was assessed. The results of this study showed a significant increment in the TPC and AO activity of kefir in the presence of *Ruscus hyracanus* extract. According to the results, adding *Ruscus hyracanus* extract at the level of 0.5% increased the number of LAB significantly. The kefir sample containing 0.5% *Ruscus hyracanus* extract had the highest number of LAB and overall acceptability score in treated samples. Generally, our results recommended the consumption of this enriched kefir as a functional product for progress in consumer health. Also, syneresis of enriched kefir decreased, kefir stability increased, and the viscosity of treated samples improved. Moreover, adding *Ruscus hyracanus* extract had a proper effect on the sensory properties of the kefir samples.

Acknowledgments

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