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Investigation of the Effects of Different Levels f Hydroalcoholic Extract of Spirulina platensis on the Physicochemical, Sensory, and Antimicrobial Properties of a Functional Licorice Beverage

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ABSTRACT

The biological bioactive compounds and appropriate protein content of the microalga Spirulina platensis can provide a novel strategy for the development and maintenance of consumer health in the food and pharmaceutical sectors. Licorice (Glycyrrhiza glabra) has also been introduced as an effective natural oral medicinal plant and as a food additive. The present study aimed to investigate the effects of different levels of hydroalcoholic extract of Spirulina platensis on the physicochemical properties, sensory attributes, antimicrobial activity, and stability of a functional licorice beverage. The functional licorice beverages were prepared using inulin (0.5%), xanthan gum (0.15%), water (2000 mL), and licorice powder (1000 g). Subsequently, different levels of hydroalcoholic extract of Spirulina platensis (0.5, 1, and 1.5%) were added, and four treatments were prepared: control (T0), T1 (containing 0.5% hydroalcoholic extract of Spirulina platensis), T2 (containing 1% hydroalcoholic extract of Spirulina platensis), and T3 (containing 1.5% hydroalcoholic extract of Spirulina platensis). Finally, physicochemical properties, antioxidant activity, antimicrobial properties, and sensory characteristics of the samples were evaluated. The results showed that T3 had the highest pH value, and with increasing storage time, the pH of all samples decreased significantly ($P \leq 0.05$), while their acidity increased significantly ($P \leq 0.05$). T3 exhibited the highest viscosity (187 cP) compared to the other samples at day zero, and increasing the level of hydroalcoholic extract of Spirulina platensis significantly increased the viscosity of the samples ($P \leq 0.05$). The degree of phase separation (serum separation) of the functional beverages containing the hydroalcoholic extract of Spirulina platensis significantly decreased with increasing extract concentration ($P \leq 0.05$); however, with storage time, phase separation significantly increased on days 14 and 21 ($P \leq 0.05$). In color evaluation, the lowest lightness parameter (L*) was recorded in T3. At all storage times, increasing the level of hydroalcoholic extract of Spirulina platensis significantly decreased the color parameters lightness (L*), redness–greenness (a*), and yellowness–blueness (b*) ($P \leq 0.05$), and with increasing storage time, these color indices in T2 and T3 decreased significantly ($P \leq 0.05$). Evaluation of antimicrobial properties showed that Escherichia coli had the highest minimum inhibitory concentration, while Lactobacillus plantarum exhibited a significantly lower minimum bactericidal concentration than the other tested bacteria ($P \leq 0.05$). Sensory evaluation indicated that the sample containing 0.5% Spirulina platensis extract (T1) achieved the highest scores for odor, taste, and overall acceptance and was identified as the most suitable formulation. Based on the findings, the use of a low concentration of Spirulina platensis extract (0.5%) can improve nutritional and functional characteristics while maintaining desirable sensory quality of the licorice beverage and can be proposed as an appropriate option for the production of functional beverages.

1- Introduction

Food security is one of the major global challenges of the present century. Functional foods have expanded worldwide since 1993 with the aim of maintaining and promoting human health [1]. Increasing consumer awareness regarding the role of nutrition in health and well being has been the primary driving force behind the development of functional foods [2]. The consumption of functional foods provides health and wellness benefits to consumers without toxic or mutagenic effects [3]. Functional beverages are health promoting drinks that contain various nutrients such as ascorbic acid, tocopherols, β carotene, and phytochemicals in the human diet. In recent years, this category has emerged as one of the most important newly developed food products [4,5]. The market for functional beverages is expected to expand in the future due to the increasing prevalence of lifestyle related diseases such as diabetes and hypertension. Moreover, the use of natural ingredients in beverages can enhance consumer acceptance and preference [6–8]. Spirulina platensis (S. platensis) is an edible, multicellular, filamentous blue green microalga belonging to the cyanobacteria group [9]. This microalga is rich in essential macro and micronutrients and possesses therapeutic properties, effectively meeting a wide range of nutritional requirements [10]. The potential health benefits of Spirulina have attracted considerable attention mainly due to its chemical composition, including 58.16% protein, 24.75% carbohydrates, 4.61% lipids, essential amino acids (lysine, leucine, isoleucine, and valine), vitamins such as pantothenic acid, riboflavin, and vitamin E, essential fatty acids including linoleic acid, oleic acid, and γ linolenic acid, phytochemicals, and the pigment phycocyanin [11]. Spirulina is considered a valuable food source that has shown positive effects in the prevention and treatment of hypercholesterolemia, allergies, cancer, poisoning, cardiovascular diseases, and diabetes. Its therapeutic properties have also made Spirulina a promising option for individuals seeking natural therapies [12–14]. Spirulina extract (obtained using supercritical fluid extraction), as an accessible and safe antioxidant, can serve as an alternative to synthetic antioxidants and antimicrobial agents [15,16]. The potential applications of Spirulina as a food ingredient have been utilized to improve the health-promoting properties of products such as dietary supplements, beverages, fermented milks, cereals, bakery products, desserts, cakes, confectionery products, biscuits, snacks, soups, salad dressings, and dairy products including ice cream, yogurt, dairy-based beverages, and others [17–18-19]. Recently, the health-promoting, therapeutic, and nutritional aspects of Spirulina have been reviewed in scientific articles [11]. This microalga has been used as a protein supplement, a rich source of iron, and a natural colorant in many food products such as fermented whey-based sports beverages [20], functional fruit desserts and yogurt [21,22], cheese [23], fruit puree pastilles [24], ice cream [25], and functional beverages [26]. Licorice contains a diverse range of chemical compounds associated with human health. Therefore, it is consumed worldwide as a major source for meeting nutritional and medicinal needs [27,28]. Licorice (Glycyrrhiza glabra) belongs to the Fabaceae family, and the roots of this plant contain numerous bioactive compounds including coumarins, flavonoids, isoliquiritigenin, glabridin, glycyrrhizic acid, volatile oils, and phytosterols. Glycyrrhizin is recognized as the most important active compound in licorice root [29]. Licorice root also contains various sugars (up to 18%), flavonoids, sterols, amino acids, gum, starch, essential oils, and saponins. Its major saponin is glycyrrhizic acid or glycyrrhizin ($C_{42}H_{62}O_{16}$), which is composed of two glucuronic acid units and one molecule of glycyrrhetinic acid (aglycone) [30]. Glycyrrhizin salt, due to its

much higher sweetness compared to sugar (approximately 50 times sweeter), has wide applications in the food and pharmaceutical industries, and licorice root is commercially available in the form of powder, dry extract, and liquid extract [31]. Licorice root extract contains considerable amounts of glucose, sucrose, asparagine, albuminous substances, resin, and essential oil. The active compounds of this plant have numerous applications in the pharmaceutical, beverage, confectionery, and tobacco industries [32,33]. Furthermore, the consumption of licorice may affect the endocrine system, and some studies have reported a decrease in blood testosterone levels [34]. Other studies have shown that dried licorice root can increase the secretion of serotonin and prostaglandins in the stomach, thereby exerting anti-inflammatory and protective effects on the gastric mucosa [35]. Considering these properties and the high potential of licorice root, the present study aimed to develop a functional and health-promoting beverage by enriching the herbal extract of licorice with the hydroalcoholic extract of the microalga *Spirulina platensis*, which in addition to containing nutritional compounds, may exhibit beneficial biological effects on human health.

2-Materials& Methods

Preparation of Hydroalcoholic Algal Extract

Spirulina platensis algae were obtained from Nadian Kavoshgaran Pars Company (Iran). For the preparation of the hydroalcoholic extract, 200 g of dried algae was soaked in 80% ethanol at a ratio of 1:6 and maintained under static and protected conditions for 72 hours. After the extraction period, the solution was filtered using Whatman No. 1 (Extra Rapida) filter paper to remove solid particles. Subsequently, the solvent was separated from the extract using a rotary evaporator equipped with a vacuum pump. The obtained extract was then maintained at 50–55°C for concentration and drying. This method resulted in the production of a highly concentrated hydroalcoholic Spirulina extract with uniform quality, suitable for use in beverage formulations.

Preparation of Licorice Extract

The licorice roots obtained from Aria Pharmed Agro-Industry Company were initially examined visually, and their outer peel was removed. Subsequently, the inner woody part of the roots was milled into a uniform powder. Then, 2000 mL of distilled water was added to the powder, and the mixture was stored in a refrigerator for 24 hours to allow the extraction of soluble compounds. After the extraction period, the solution was heated indirectly until approximately 30% of its water content had evaporated. The solution was then cooled to 50°C, and xanthan gum was added at a concentration of 0.15%. Following a relative reduction in temperature, inulin was added at a concentration of 0.5%, and the mixture was thoroughly stirred until a uniform solution was obtained. Finally, the prepared extract was distributed into sterile bottles and stored [36]. This method resulted in the production of an extract with suitable concentration and uniform viscosity for use in beverage formulations.

Preparation of *Spirulina platensis* - Enriched Functional Licorice Beverage

The treatments were prepared by adding Spirulina extract at concentrations of 0.5%, 1%, and 1.5% to the licorice extract according to Table 1. Finally, the beverages were filled into 200 mL glass bottles and sealed. The beverages were pasteurized at 80°C using a water bath (Memmert, Germany), then cooled to 10°C and stored under refrigeration at 4°C until further analyses were performed.

Table 1. Formulations of licorice-based functional beverages enriched with *Spirulina platensi*

Sample	*Spirulina platensis* (%)	Licorice powder (g)	Inulin (%)	Xanthan gum (%)	Water (mL)
Control (T0)	–	1000	0.5	0.15	2000
Treatment 1 (T1)	0.5	1000	0.5	0.15	2000
Treatment 2 (T2)	1.0	1000	0.5	0.15	2000
Treatment 3 (T3)	1.5	1000	0.5	0.15	2000

Physicochemical Analysis of the Beverage

The pH of the samples was determined using a Martini pH meter (Italy). The acidity of the samples was measured according to Iranian National Standard No. 2852 and expressed as percentage of citric acid [37]. The viscosity of the samples was measured using a rotational viscometer (DV-I Prime, USA) equipped with spindle No. 64. The rotational speed was set at 100 rpm, and the test temperature was maintained at 25°C. The results were reported in centipoise (cP) [38]. The color of the samples was determined using a colorimeter (HunterLab, model Parma, 25 March 2005). The parameters L* (lightness: black to white), a* (red to green), and b* (yellow to blue) were recorded. The total color difference (ΔE) was calculated according to Equation (2) [39,40].To evaluate phase separation, identical 100 mL graduated cylinders were used. The samples were poured into the cylinders and sealed with parafilm. They were then stored at 4°C for 21 days. Phase separation was determined on days 0, 7, 14, and 21 by measuring the volume of the sedimented phase relative to the total sample volume and expressing it as a percentage [41,42]. To determine the free radical scavenging activity of antioxidants, various free radicals, including DPPH, peroxyl, hydroxyl, and superoxide radicals, were used.

Equation (1):

$$\text{Acidity (\%)} = \frac{V \times 100 \times 0/0067}{12/312}$$

V = volume of acid consumed

Equation (2): $\Delta E = [(L^* - L)^2 + (a^* - a)^2 + (b^* - b)^2]^{0.5}$

where:

- L^*= lightness parameter a^*= red/green color parameter b^*= yellow/blue color parameter

Preparation of Bacterial Suspension

The microorganisms used in this study included *Staphylococcus aureus* (PTCC 1112), *Escherichia coli* (PTCC 1270), and *Lactobacillus plantarum* (ATCC 1058). These bacterial strains were obtained from the Pasteur Institute of Iran and the Iranian Research Organization for Science and Technology. Each strain was cultured and maintained according to standard laboratory protocols, and prior to the experiments, they were grown in appropriate culture media until reaching the logarithmic phase of growth. For the preparation of microbial suspensions, each strain was first isolated from solid culture media and then inoculated into suitable liquid media (e.g., nutrient broth for Gram-positive and Gram-negative bacteria and MRS broth for Lactobacillus species). The cells were then uniformly suspended, and the final concentration of the suspension was adjusted

using optical density measurement at 600 nm (OD600) or by colony counting methods to ensure standardization for subsequent experiments. All procedures for preparing the microbial suspensions were carried out under aseptic conditions and at appropriate temperatures to prevent contamination and unexpected changes in microbial populations.

Broth Microdilution Assay for Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the hydroalcoholic extract of *Spirulina platensis* against the studied bacteria was determined using the broth microdilution method in sterile 96-well microplates [44]. The microplates consisted of 8 rows and 12 wells, with a capacity of 250 µL per well. Initially, 100 µL of nutrient broth medium was added to each well. Subsequently, 100 µL of the hydroalcoholic *Spirulina* extract was added to the first well of each row, and two-fold serial dilutions were performed from the first to the eleventh well. Accordingly, 100 µL was transferred from the first well to the second well, and this procedure was continued sequentially up to well number 11. After preparation of the serial dilutions, 10 µL of bacterial suspension (equivalent to 0.5 McFarland standard) was added to all wells except well number 11, which served as the negative control containing only culture medium and extract. Well number 12 in each row was considered the positive control, containing bacteria and dimethyl sulfoxide (DMSO), to evaluate bacterial growth. Following bacterial inoculation, the microplate was placed on a shaker for 30 seconds to ensure complete homogenization of the well contents. Optical density was measured at 620 nm using an ELISA reader at time zero. The microplate was then incubated at 37°C for 24 hours. After incubation, bacterial growth was evaluated visually as well as by measuring optical density using the ELISA reader. The MIC was defined as the lowest concentration of the extract at which no visible bacterial growth (absence of turbidity) was observed [44].

Determination of Minimum Bactericidal Concentration (MBC)

To determine the minimum bactericidal concentration (MBC), 10 µL aliquots were taken under completely sterile conditions and near a flame from the wells showing no turbidity in the minimum inhibitory concentration (MIC) assay (MIC concentration and higher) and inoculated onto blood agar culture medium. After inoculation, the microorganisms were incubated at 37°C for 24 hours.

Following the incubation period, the lowest concentration that resulted in the elimination of 99.9% of the bacterial population was considered as the minimum bactericidal concentration. All experimental procedures were performed in three independent replicates. This method enables differentiation between the inhibitory effect and the bactericidal effect of the extract and contributes to a more accurate determination of the antibacterial activity of the sample.

Sensory Evaluation

The sensory characteristics of the samples were evaluated by 13 trained panelists, including 9 women and 4 men aged between 30 and 40 years. The evaluation included the parameters of color, mouthfeel, taste, aroma, sweetness, consistency, and overall acceptability. A five-point hedonic scale was used for scoring the samples. In this scale, a score of 5 corresponded to “very good,” 4 to “good,” 3 to “moderate,” 2 to “poor,” and 1 to “very poor” [45]. Prior to the evaluation, the panelists received the necessary training to standardize the criteria for sensory judgment in order to reduce individual bias and ensure greater accuracy of the assessments.

Statistical Analysis

All experiments were performed in three independent replicates, and the data were expressed as mean ± standard deviation. One-way analysis of variance (One-Way ANOVA) was applied to determine significant differences among the samples. Mean comparisons were conducted using Duncan's Multiple Range Test. All statistical analyses were carried out using SPSS software version 19, and the level of statistical significance for all tests was considered at $P \leq 0.05$ (95% confidence level).

3- Results and Discussion

pH & Acidity

The results showed that acidity was significantly affected by both treatment type and storage time ($P \leq 0.05$). In all treatments, an increasing trend in acidity was observed from day 0 to day 21 of storage. On day 0, the highest acidity was observed in treatment 3 (approximately 0.29%), while the lowest value was recorded in the control treatment (approximately 0.20%). Treatments 1 and 2 showed intermediate values. The differences among treatments at this stage were statistically significant ($P \leq 0.05$). A similar trend was observed on days 7 and 14, and the order of treatments remained unchanged (Control < Treatment 1 < Treatment 2 < Treatment 3). However, the differences among some treatments gradually decreased over time, indicating that acidity values became closer toward the end of the storage period. On day 21, acidity reached its highest level in all treatments. Treatment 3 still showed the highest acidity (approximately 0.32%), whereas the control treatment exhibited the lowest value (approximately 0.25%). The gradual increase in acidity during storage is likely related to ongoing microbial activities and biochemical reactions leading to the production of organic acids. The higher acidity observed in treatment 3 may indicate more favorable conditions for the formation or release of acidic compounds in this treatment. The results also indicated that over time, the decrease in pH and the increase in acidity may be associated with the activity of protein and polysaccharide compounds present in *Spirulina* and its metabolites. These compounds may influence the acid–base balance of the system through hydrogen ion production, leading to gradual changes in pH. The buffering capacity of *Spirulina platensis* is mainly attributed to the presence of proteins, peptides, and amino acids in its composition, which can absorb hydrogen ions and moderate pH fluctuations. In other words, *Spirulina* with an alkaline pH of approximately 8.6–9.6 can counteract acidic food systems and slow down sudden decreases in pH. Therefore, it can function as a natural buffering agent in beverages and other food products. This buffering effect helps maintain controlled acidity changes and may prevent the body from using mineral reserves such as calcium from bones to neutralize excess acidity [46]. Previous studies have reported similar findings. Varga et al. (2012) demonstrated that *Spirulina* possesses an alkaline nature and high buffering capacity, which slows down pH reduction in the presence of acidic compounds [47]. In another study, evaluation of a spirulina-enriched orange pulp beverage showed that increasing the level of *Spirulina* resulted in a decrease in pH and an increase in acidity [48]. Similarly, research on functional kiwi and apple beverages containing *Spirulina* reported that increasing algae content was associated with reduced pH and increased acidity [49]. Overall, these findings indicate that *Spirulina platensis*, in addition to providing bioactive compounds and antioxidants, can act as a natural buffering agent in acidic food products and prevent rapid pH decline. This characteristic may play an important role in improving chemical stability, maintaining flavor and sensory quality of food products, and supporting bone health as well as the acid–base balance of the body.

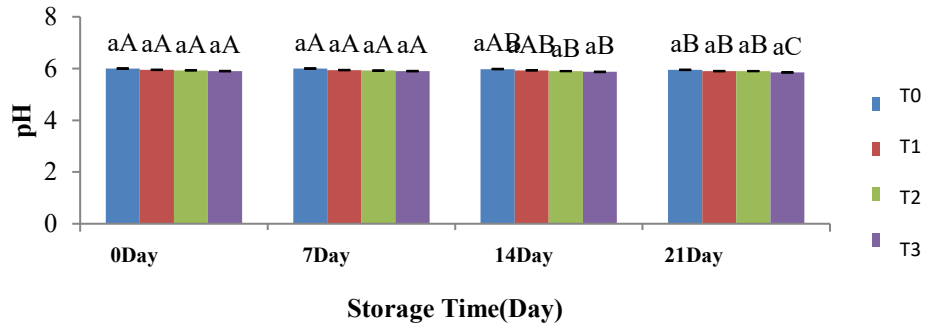


Fig1: pH of licorice drink containing hydroalcoholic extract of *Spirulina platensis* during storage period
Different lowercase letters indicate significant differences on each day and different uppercase letters indicate significant differences over time (p≤0.05).
T0 (control), T1 (0.5%), T2 (1%), and T3 (1.5%).

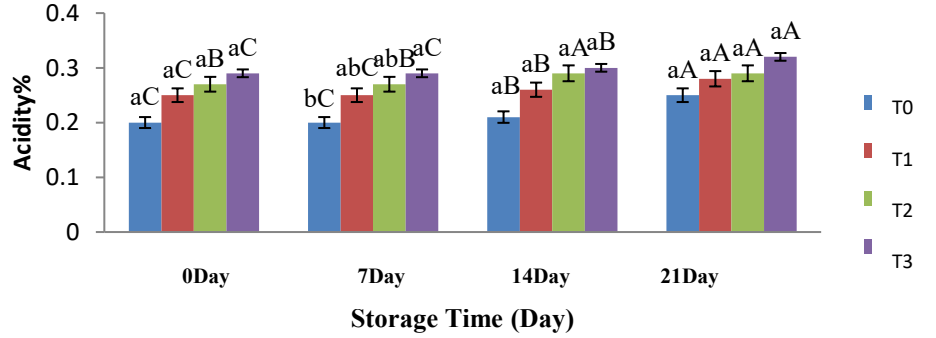


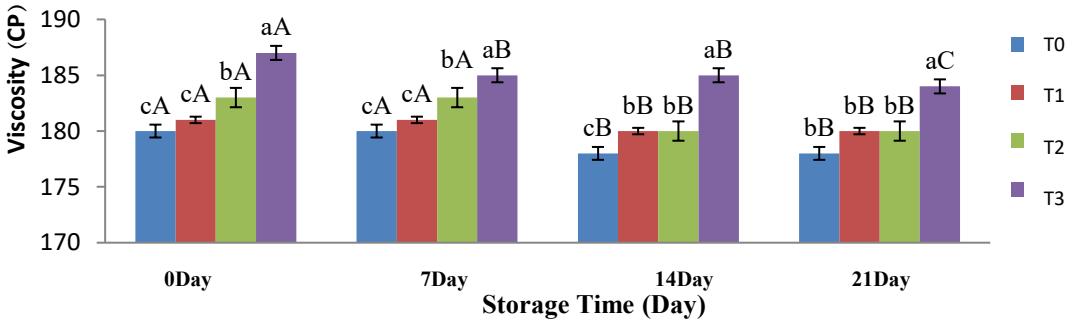
Fig2: Acidity of licorice drink containing hydroalcoholic extract of *Spirulina platensis* during storage period
Different lowercase letters indicate significant differences on each day and different uppercase letters indicate significant differences over time (p≤0.05).
T0 (control), T1 (0.5%), T2 (1%), and T3 (1.5%).

Viscosity

The results showed that both storage time and treatment had a significant effect on the viscosity of the samples ($P \leq 0.05$). In general, with increasing treatment level from the control to treatment 3, viscosity increased significantly. In all storage times, treatment 3 exhibited the highest viscosity, whereas the control treatment showed the lowest value.

On day 0, significant differences were observed among all treatments, and the order of the mean values was as follows: Control < Treatment 1 < Treatment 2 < Treatment 3 ($P \leq 0.05$). On day 7, although slight changes were observed compared with day 0, the overall trend remained unchanged and treatment 3 still had the highest viscosity. With continued storage until day 14, a significant decrease in viscosity was observed in the control, treatment 1, and treatment 2, while treatment 3 showed a smaller decrease and maintained greater

stability. On day 21, a similar trend was observed, where the lowest viscosity was recorded in the control treatment and the highest in treatment 3, and the differences among treatments remained statistically significant ($P \leq 0.05$). Evaluation of viscosity changes over time indicated that viscosity gradually decreased in all treatments during storage. However, the reduction was more pronounced in treatments with lower concentrations, whereas treatment 3 exhibited the smallest decline in viscosity, indicating greater structural stability during storage. The reduction in viscosity during storage may be attributed to the weakening of the network structure, reduction of intermolecular interactions, or enzymatic and microbial activities that may degrade the structural matrix of the product. In contrast, the higher viscosity and improved stability observed in treatment 3 suggest that this treatment likely strengthened the internal structure and enhanced the network cohesion of the system. The increase in viscosity is probably related to the higher dry matter content of *Spirulina* compared with licorice, as well as the high protein content and the presence of polysaccharide compounds such as soluble and insoluble fibers, which have the ability to absorb water and form three-dimensional networks. These networks increase friction between particles and reduce flowability, thereby increasing resistance to deformation and overall viscosity. Over time, the viscosity of all samples decreased significantly, which may be due to partial degradation of proteins, structural changes in polysaccharides, and reduced particle–particle interactions. *Spirulina* can absorb water and reduce the flowability of beverages, thereby increasing viscosity; however, physicochemical processes such as partial hydrolysis of polysaccharides or protein degradation during storage may gradually reduce viscosity. A significant increase in viscosity with the addition of *Spirulina* powder compared with the control sample was also observed, indicating the role of *Spirulina* bioactive compounds in improving the rheological properties of the beverage [50]. These findings are consistent with the report of Mohammadi et al. (2025), who observed a significant increase in viscosity in fruit beverages containing 2% *Spirulina* extract. Similarly, the results of Tafreshi et al. (2018) also confirmed an increase in viscosity in samples containing *Spirulina*. From a mechanistic perspective, *Spirulina* proteins can form hydrogel networks that trap water and increase water-holding capacity while reducing flowability. Polysaccharides also increase local friction and viscosity in the continuous phase, preventing particle sedimentation and phase separation and thereby improving the physical stability of the beverage. In addition, increased viscosity can reduce the migration rate of particles and improve the uniform distribution of soluble and suspended compounds, which plays an important role in the sensory quality and overall acceptance of the product. Overall, the addition of *Spirulina* to licorice beverage not only enhances the nutritional and antioxidant properties of the product but also improves its rheological behavior and physical stability, making it possible to develop high-quality functional beverages with improved shelf life.



Different lowercase letters indicate significant differences on each day and different uppercase letters indicate significant differences over time ($p \leq 0.05$) .T0 (control), T1 (0.5%), T2 (1%), and T3(1.5%)

Phase Separation

The results demonstrated that phase separation was significantly affected by both storage time and treatment concentration ($P \leq 0.05$). On

day 0, no phase separation was observed in any of the treatments, and no significant differences were found among the concentration levels ($P \geq 0.05$). However, with increasing storage time, the degree of phase separation increased significantly in all treatments ($P \leq 0.05$). The values began to increase from day 7 and reached their maximum levels on day 21, indicating a gradual reduction in the physical stability of the system during storage. Regarding the effect of concentration, increasing the treatment level significantly reduced phase separation at all storage times ($P \leq 0.05$). At each evaluation period, the treatment with the lowest concentration exhibited the highest degree of phase separation, whereas the treatment with the highest concentration showed the lowest amount. These differences became more pronounced on days 14 and 21, where the statistical distance among treatments increased considerably. Overall, the highest phase separation was observed in the lowest concentration treatment after the longest storage period (day 21), while increasing the treatment concentration significantly reduced the intensity of phase separation during storage. In beverages containing licorice extract, due to the relatively low pH and high acidity, the proteins present in the system tend to approach their isoelectric point. Under these conditions, the surface charge of protein particles decreases, reducing electrostatic repulsion among them. As a result, proteins become more prone to aggregation and sedimentation, leading to phase separation. This phenomenon is considered one of the major causes of instability in protein-containing beverages enriched with plant compounds. The improved stability observed with increasing *Spirulina* extract concentration may be attributed to the presence of polysaccharides, proteins, and phenolic compounds in this microalga. These compounds can enhance the viscosity of the continuous phase, improve particle cohesion, and contribute to the formation of stronger network structures within the beverage matrix, thereby preventing sedimentation and phase separation. Furthermore, some pigments and surface-active proteins present in *Spirulina*, such as phycocyanin, may act as natural emulsifying agents and increase system stability by reducing interfacial tension between phases. The presence of xanthan gum, as a high molecular weight anionic hydrocolloid, also contributed to increasing the viscosity of the continuous phase and promoting pseudoplastic behavior. Increased viscosity reduces the movement, sedimentation, or creaming rate of dispersed particles, thereby limiting phase separation. In addition, the formation of a weak three-dimensional network in the continuous phase can restrict particle mobility and improve physical stability. Similarly, inulin, as a soluble dietary fiber and gel-forming polysaccharide, may strengthen the system structure through increasing total soluble solids and promoting hydrogen bonding interactions. By enhancing viscosity and creating a fine structural network, inulin can reduce droplet coalescence and slow down the phase separation process. Moreover, its filling effect within the matrix can further improve the physical stability of the beverage. The results also indicated that homogenization prior to pasteurization had a significant effect on reducing phase separation and improving particle uniformity. Homogenization decreases particle size and improves the uniform distribution of the dispersed phase, thereby increasing particle interactions and enhancing colloidal stability. In addition, the combined effects of mechanical stirring and homogenization further improved physical stability and reduced the tendency of particles to sediment, which is in agreement with findings reported in studies on fermented dairy beverages such as Doogh and factors influencing their phase separation [42]. Overall, the findings suggest that the addition of *Spirulina* extract not only improves physical stability but also reduces phase separation and enhances particle uniformity, thereby playing an important role in

improving the quality and sensory acceptance of plant-based protein beverages.

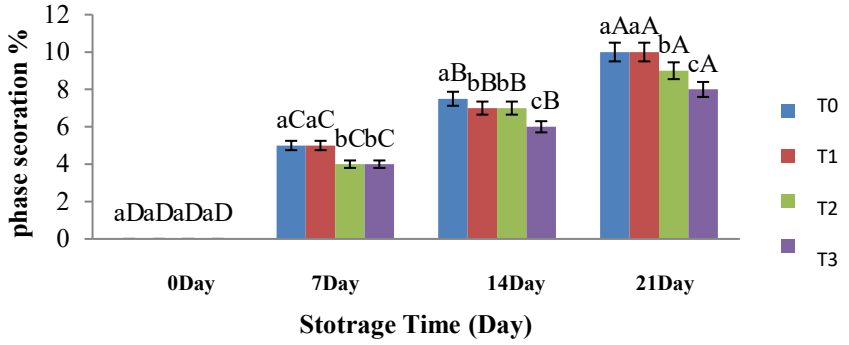


Fig4: Phase separation of licorice beverage containing hydroalcoholic extract of Spirulina platensis during the storage period
Different lowercase letters indicate significant differences on each day and different uppercase letters indicate significant differences over time ($p \leq 0.05$)
T0 (control), T1 (0.5%), T2 (1%), and T3(1.5%)

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The results of this study showed that the minimum inhibitory concentration (MIC) of the hydroalcoholic extract of *Spirulina* against *Escherichia coli* was significantly higher than that against *Staphylococcus aureus* and *Lactobacillus plantarum*. This finding indicates the relatively greater resistance of Gram-negative bacteria compared with Gram-positive bacteria to the active compounds present in the *Spirulina* extract.

On the other hand, the minimum bactericidal concentration (MBC) of the extract against *Lactobacillus plantarum* was reported to be significantly lower than that observed for the other two bacteria, indicating a higher sensitivity of this species to the antibacterial compounds of *Spirulina*. Antibiotic compounds present in cyanobacteria include fatty acids, bromophenols, peptides, polysaccharides, and alcohols. However, the precise chemical composition of many of these secondary metabolites has not yet been fully identified. Previous studies have shown that the antibacterial effects of cyanobacteria are often attributed to the presence of phenolic and low-molecular-weight peptide compounds that can interact with bacterial cell membranes, increase membrane permeability, and lead to leakage of intracellular contents. These effects are generally stronger in Gram-positive bacteria due to the simpler structure of their cell walls, which lack the outer lipopolysaccharide layer characteristic of Gram-negative bacteria [51]. In addition, the presence of phenolic compounds such as gallic acid, benzoic acid, chlorogenic acid, vanillic acid, caffeic acid, coumaric acid, and ferulic acid has been reported in *Spirulina*. These compounds, through their ability to inhibit free radicals and destabilize microbial cell membranes, exhibit dual antioxidant and antibacterial activities. Phenolic compounds may also exert inhibitory effects by denaturing proteins and suppressing essential bacterial enzymes. Moreover, the presence of bioactive polysaccharides in *Spirulina* may contribute to microbial growth control by stimulating the immune system and preventing bacterial adhesion to biological surfaces. Therefore, the use of *Spirulina* extract in food product formulations may serve as a natural alternative to chemical preservatives while improving shelf life and microbial safety.

Overall, the findings of this study indicate that *Spirulina*, as a sustainable and biocompatible source of antimicrobial compounds, has considerable potential for application in pharmaceutical and food industries [52].

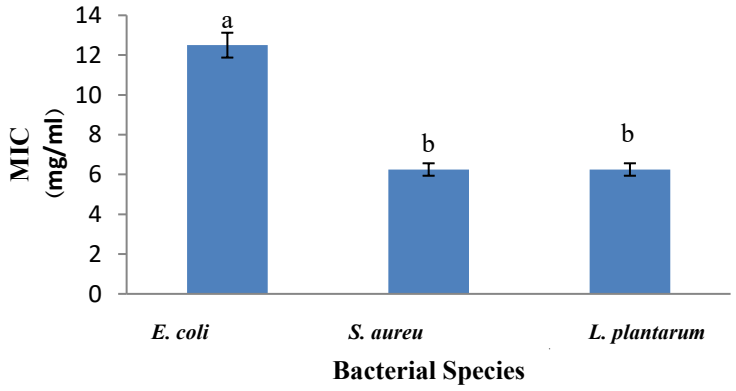


Fig6: Minimum bacterial inhibitory concentration (MIC) in hydroalcoholic extract of *Spirulina platensis*
Different lowercase letters indicate significant differences (p≤0.05)

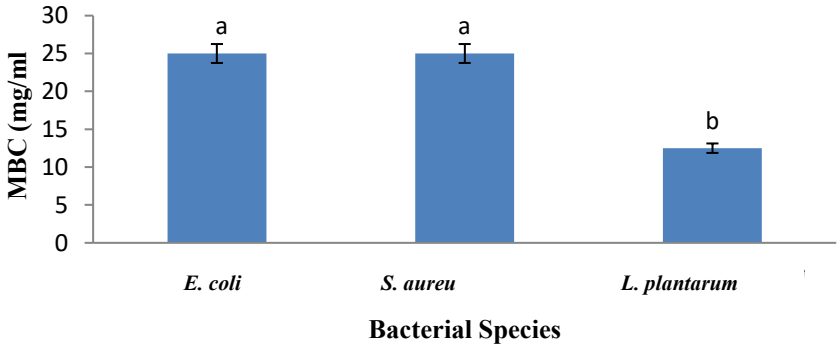


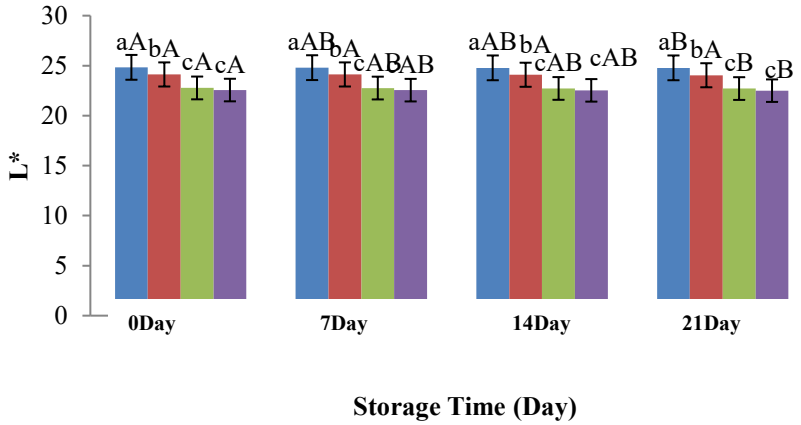
Fig7: Minimum bactericidal concentration (MBC) in hydroalcoholic extract of *Spirulina platesis*
Different lowercase letters indicate significant differences (p≤0.05)

Color Measurement

The results showed that at all storage intervals, increasing the concentration of hydroalcoholic *Spirulina* extract significantly decreased the lightness index (L*) of the samples ($P \leq 0.05$). In addition, over time, the L* values in treatments 3 and 4 also showed a significant decreasing trend. The reduction in the lightness index can be attributed to the presence of natural pigments in *Spirulina*, including chlorophyll, phycocyanin, and carotenoids, which become incorporated into the beverage structure and shift its color toward green and blue tones.

Studies have shown that the addition of *Spirulina* to various food products results in decreased values of L*, a*, and b*. In a study on functional fruit beverages, the presence of *Spirulina* algae caused a reduction in the color indices L*, a*, and b* compared with the

control sample [49]. Similarly, another study reported that the addition of *Spirulina* microalgae to kiwi-based fruit pastilles significantly decreased the L* and b* indices [24]. These changes have been attributed to the direct influence of the green-blue color of phycobiliproteins in *Spirulina*, as well as the strong light absorption of carotenoids at yellow and red wavelengths. Furthermore, results obtained from kiwi pastilles indicated that *Spirulina* was able to completely mask the yellow and brown colors produced by Maillard reactions and shift the product color toward blue. In the formulation of spaghetti enriched with *Spirulina* and *Chlorella* microalgae, the color parameter b* also exhibited a decreasing trend [53]. In a similar study on kiwi fruit leather enriched with *Spirulina*, increasing the concentration of this microalga caused a significant decrease in L* (lightness) and b* (yellowness) indices, while increasing the greenness of the samples (more negative a* values) compared with the control sample [54]. The results of the study by Salehifar et al. also showed that in the fortification of industrial cookies with *Spirulina platensis*, increasing the amount of microalgae led to a significant decrease in crumb lightness, which was attributed to the natural green color of *Spirulina*. Likewise, Khazaei-Pool et al. reported a reduction in the yellowness index in pastilles containing *Spirulina*, attributing this change to the presence of chlorophyll and phycocyanin pigments. In the same context, results obtained from pasta enriched with *Spirulina* also showed that the addition of this microalga produced a uniform green color in the final product [55]. Overall, the reduction in L* and b* indices in samples containing *Spirulina* can be attributed to the optical effects and absorption of higher wavelengths by photosynthetic pigments. Chlorophylls absorb light in the red and blue regions of the visible spectrum and reflect green light, resulting in decreased lightness and yellowness. In addition, the presence of phycocyanin and carotenoids, which possess color-stabilizing properties and resistance to oxidation, can not only produce a desirable green–blue color but also enhance color stability during product storage. These characteristics not only improve the nutritional value of the product but also represent an industrial advantage for producing natural products with stable color and without the need for artificial additives.



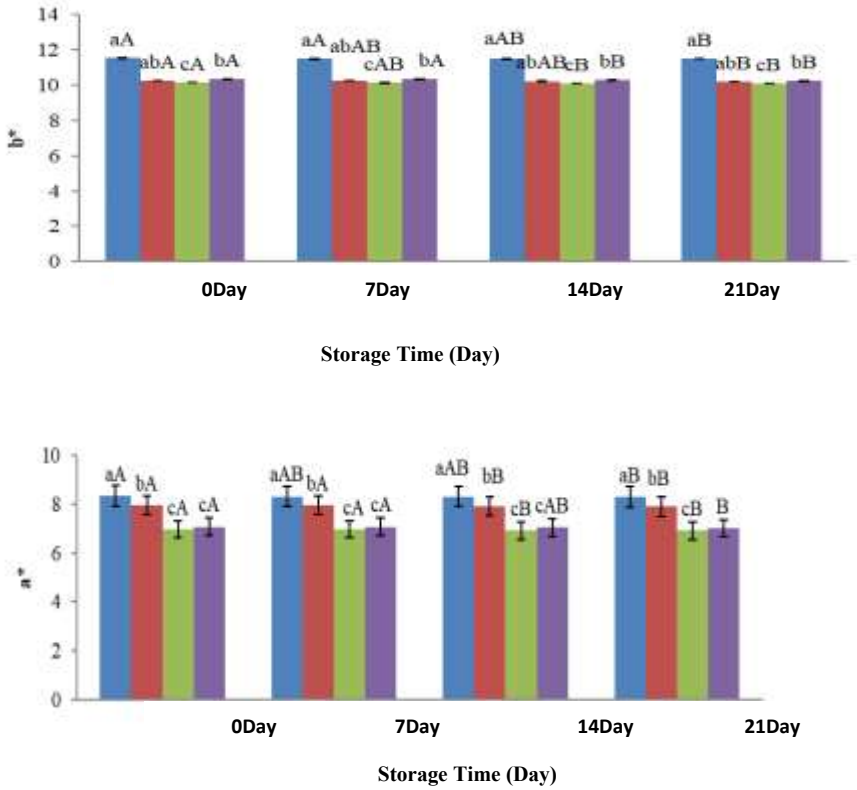


Fig8: Color index L^*, a^*, and b^* of licorice drink containing hydroalcoholic extract of *Spirulina platensis* during storage period
Different lowercase letters indicate significant differences on each day and different uppercase letters indicate significant differences over time ($p \leq 0.05$)
T0 (control), T1 (0.5%), T2 (1%), and T3 (1.5%)

Evaluation of Antioxidant Capacity

The antioxidant activity of the hydroalcoholic extract of *Spirulina* was evaluated at three different concentrations (500, 1000, and 1500 µg/mL) using the DPPH free radical scavenging assay. The results showed that increasing the extract concentration significantly enhanced the inhibition of free radicals. The highest DPPH radical scavenging activity was observed at the concentration of 1500 µg/mL (45.97%), while the lowest activity was recorded at 500 µg/mL (22.61%). This increasing trend indicates a direct relationship between extract concentration and antioxidant capacity. The enhancement of antioxidant activity at higher concentrations may be attributed to the greater presence of phenolic compounds, flavonoids, and organic acids such as gallic acid, ferulic acid, and caffeic acid, which neutralize free radicals through electron donation or acceptance mechanisms. In addition, natural pigments present in *Spirulina*, including phycocyanin and chlorophyll, play an important role in inhibiting oxidative reactions and reducing lipid peroxidation. These findings are consistent with previous studies reporting that the antioxidant activity of *Spirulina* extracts is concentration-dependent and significantly increases with higher levels of bioactive compounds, thereby enhancing the free radical scavenging capacity. Overall, it can be concluded that the hydroalcoholic extract of *Spirulina* possesses considerable antioxidant potential and may be used as a natural compound to improve the oxidative stability of food and pharmaceutical products. The ability of *Chlorella vulgaris* to inhibit

DPPH free radicals has also been reported by Adel-Karim et al. (2020) and Taghavi et al. (2019) [56,57]. In another study, the antioxidant properties of *Chlorella vulgaris* obtained through enzymatic hydrolysis demonstrated that proteins hydrolyzed by alcalase enzyme had favorable effects on the functional and antioxidant properties of the algae [58].

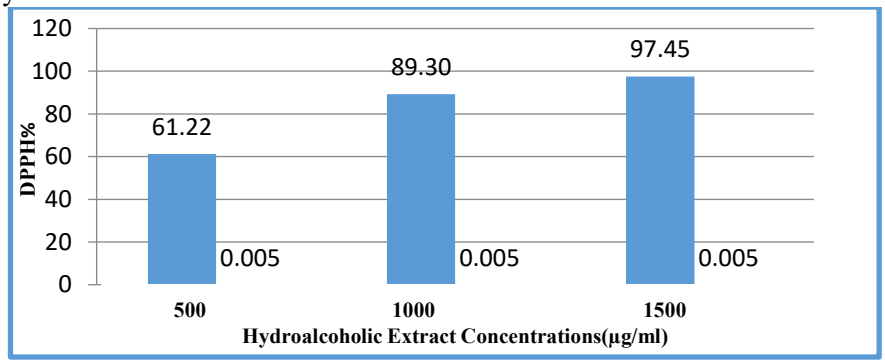


Fig9: Antioxidant activity of hydroalcoholic extract in three concentrations (500-1000-1500 µg/ml)

T1 (500 µg), T2 (1000µg), T3 (1500 µg)

Evaluation of Antioxidant Capacity (continued)

The antioxidant capacity of *Spirulina platensis* is likely attributed to the presence of bioactive compounds such as tocopherols and phycocyanins. In addition to their direct role in scavenging free radicals, these compounds may also act as synergistic agents that enhance overall antioxidant activity [21]. Among these compounds, the blue pigment phycocyanin is recognized as the principal phycobiliprotein present in all cyanobacteria. Numerous studies have demonstrated that phycocyanin can inhibit the growth of tumor cells through the induction of apoptotic pathways [59]. The mechanism underlying this effect involves the release of cytochrome c from mitochondria, degradation of poly (ADP-ribose) polymerase (PARP), and regulation of anti-apoptotic pathways, ultimately leading to programmed cell death (apoptosis). Furthermore, phycocyanin possesses antioxidant, anti-inflammatory, and neuroprotective properties and can function as an efficient antioxidant within the human biological system [60]. Animal studies have also shown that phycocyanin extracted from *Spirulina platensis* exhibits hepatoprotective effects. In one experiment, administration of this pigment prior to the induction of hepatotoxicity in mice resulted in reduced tissue damage and increased antioxidant capacity. This protective property has been attributed to the presence of the phycocyanobilin chromophore group within the structure of phycocyanin, which plays a key role in neutralizing free radicals [61].

In general, cyanobacteria are considered valuable sources of biochemical compounds due to their ability to produce diverse and biologically active natural products. Species such as *Anabaena*, *Nostoc*, *Spirulina*, and *Oscillatoria* are capable of synthesizing various secondary metabolites, including polysaccharides, peptides, and phenolic compounds, which exhibit notable antioxidant and antimicrobial activities. The presence of peptide synthetase enzymes in cyanobacteria leads to the biosynthesis of bioactive compounds such as microcystins. Moreover, species such as *Chlorella* spp. and *Scenedesmus quadricauda* are able to produce

polysaccharides that function as protective agents against oxidative stress and enhance cellular resistance to oxidative damage [62,63].

Sensory Evaluation

The results of the sensory evaluation showed that increasing the concentration of the hydroalcoholic extract of *Spirulina platensis* significantly decreased the flavor scores of the samples ($P \leq 0.05$).

The sensory evaluation results on day 0 demonstrated that increasing the concentration of *Spirulina* extract led to a reduction in the scores related to flavor, odor, and overall acceptability. The control sample obtained the highest score for all three attributes and showed no statistically significant difference compared with treatment 1, indicating the desirable preservation of sensory characteristics at low concentrations of *Spirulina* extract. In contrast, treatments 2 and especially 3 exhibited a considerable decrease in flavor and overall acceptability scores and showed a statistically significant difference compared with the control sample ($P \leq 0.05$). These findings indicate that although increasing the level of *Spirulina* may improve the functional properties of the beverage, high concentrations negatively affect consumer sensory acceptance (Figure 10). In the sensory evaluation conducted on day 7, increasing the concentration of *Spirulina* extract in the licorice beverage reduced the scores for odor, flavor, and overall acceptability. The control sample achieved the highest mean score in all three attributes and showed a statistically significant difference compared with the other treatments. Treatment 1 remained at a more desirable level than treatments 2 and 3 and exhibited overall acceptability close to that of the control sample, whereas treatments 2 and especially 3 showed a significant decline in sensory scores. These results suggest that during storage up to day 7, increasing the concentration of *Spirulina* extract negatively affected the sensory quality of the beverage, and treatment 1, containing the lower concentration of *Spirulina*, showed the best preservation of organoleptic properties. On day 7, increasing the concentration of *Spirulina* noticeably reduced the sensory quality of the beverage. Over time, samples containing higher concentrations, particularly treatment 3, exhibited a greater decline in flavor and overall acceptability compared with the control, which may be attributed to changes in phenolic compounds and their interactions with beverage components during storage (Figure 11). The sensory evaluation results on day 14 of storage showed significant differences among the treatments in terms of odor, taste, and overall acceptability ($P > 0.05$). The results on day 21 of storage also demonstrated statistically significant differences among the treatments regarding odor, flavor, and overall acceptability ($P > 0.05$). In all sensory parameters, the control treatment obtained the highest score significantly. These findings indicate that on day 21, the sensory quality of the control sample was better preserved than that of the different treatments, while the treated samples, particularly treatment 3, exhibited a significant reduction in odor, flavor, and consequently overall acceptability throughout the storage period. Although no statistically significant difference was observed between the control sample and the treatment containing 0.5% extract at all storage intervals, a substantial decrease in flavor scores was reported at higher concentrations. This reduction may be attributed to the presence of phenolic compounds and specific pigments of *Spirulina*, such as phycocyanin and chlorophyll, which may produce a characteristic algal flavor and odor. Similarly, the odor (aroma) scores of the samples significantly decreased with increasing levels of *Spirulina* extract, although no statistically significant difference was observed between the control sample and the 0.5% treatment. The lower odor acceptability at higher concentrations is likely due to the high

concentration of volatile algal compounds and altered sensory perception of the samples. The results related to overall acceptability also followed a similar trend, such that increasing the level of *Spirulina* extract significantly reduced overall acceptance ($P \leq 0.05$). However, no statistically significant difference was observed between the control sample and the treatment containing 0.5% extract, indicating the favorable acceptability of this level of extract in the beverage formulation. In a similar study, the effects of agar, guar, and *Spirulina* addition on kiwi puree-enriched fruit pastilles were investigated, and the results showed that their effects on overall acceptability were not significant. In fact, kiwi puree was able to effectively mask the flavor effects of *Spirulina*. Furthermore, that study reported that samples containing 0.25% and 0.5% *Spirulina* exhibited higher overall acceptability than the control sample [24]. Moshkenani et al. (2014) also reported that in probiotic doogh beverages containing *Spirulina* powder and mint, increasing the microalgae level decreased the sensory scores of the treatments [64]. Similarly, Salehifar et al., using a five-point hedonic test, stated that cookies enriched with 1% and 1.5% *Spirulina platensis* achieved the highest overall acceptability scores after the control sample, indicating the possibility of improving both nutritional and sensory characteristics of the product at low *Spirulina* concentrations. Other studies have also emphasized that some *Spirulina*-enriched products exhibited higher overall acceptability than the control samples. The presence of natural pigments such as phycocyanin, in addition to producing an attractive color, has increased consumer interest in East Asian countries such as China and Japan toward dairy products, gels, and pastilles containing *Spirulina* [53]. Based on the findings of similar studies, the use of compounds such as fruit purees and oligosaccharides as flavor-masking agents has been suggested to improve the sensory characteristics of *Spirulina*-containing products [23]. Overall, it can be concluded that the use of low levels of *Spirulina* extract (up to approximately 0.5%) can maintain high nutritional value while creating an appropriate balance between nutritional properties and sensory acceptability of the product.

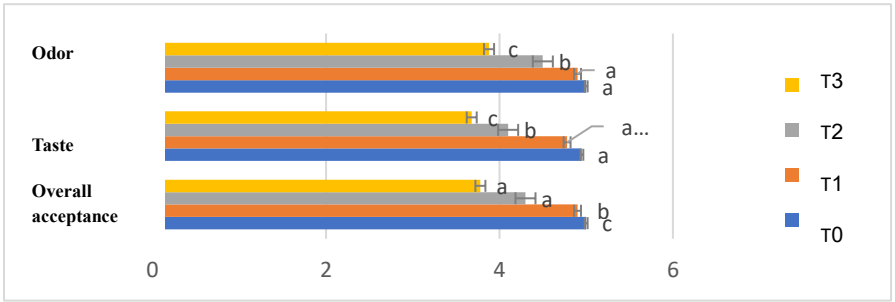


Fig10: Overall acceptance score, odor, and taste of licorice drink samples containing hydroalcoholic extract of *Spirulina platensis* on zero day
Different lowercase letters indicate significant differences on each day and different uppercase letters indicate significant differences over time ($p \leq 0.05$)

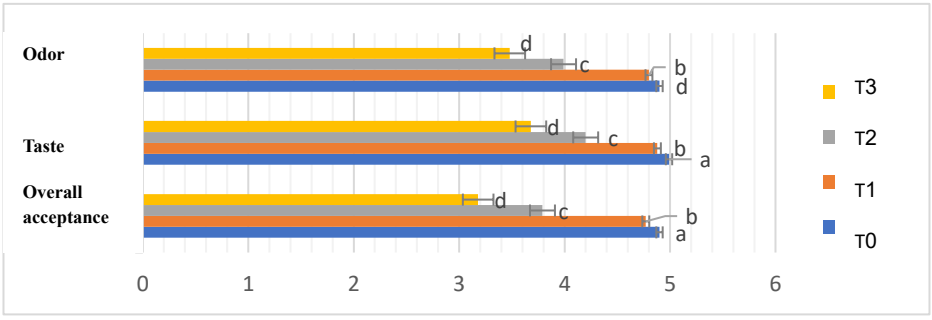


Fig11: Overall acceptance score, odor, and taste of licorice drink samples containing hydroalcoholic extract of *Spirulina platensis* on day 7. Different lowercase letters indicate significant differences on each day and different uppercase letters indicate significant differences over time (p≤0.05).

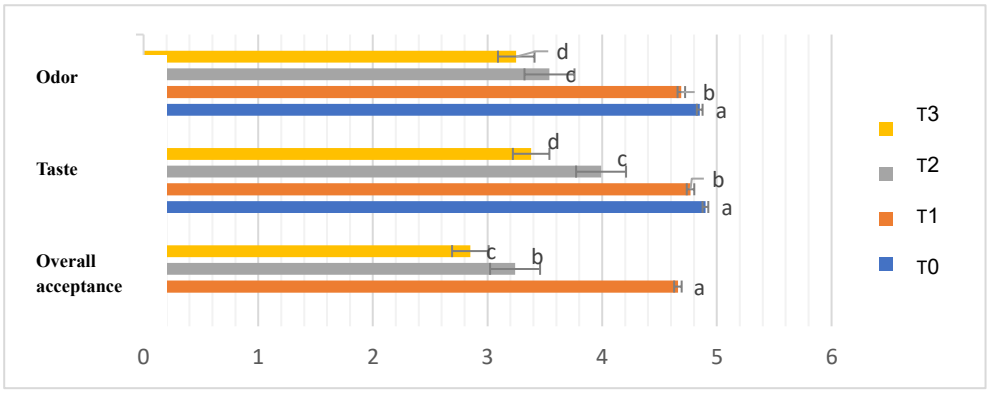


Fig12: Overall acceptance score, odor, and taste of licorice drink samples containing hydroalcoholic extract of *Spirulina platensis* on day 14

Different lowercase letters indicate significant differences on each day and different uppercase letters indicate significant differences over time (p≤0.05).

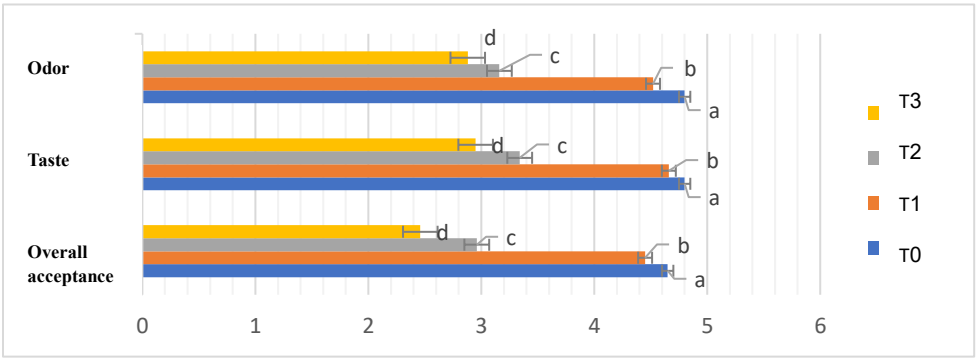


Fig13: Overall acceptance score, odor, and taste of licorice drink samples containing hydroalcoholic extract of *Spirulina platensis* on day21.

Different lowercase letters indicate significant differences on each day and different uppercase letters indicate significant differences over time (p≤0.05)

4- Conclusion

The results of this study showed that the addition of hydroalcoholic extract of *Spirulina platensis* to a functional beverage based on licorice extract had a significant effect on the physicochemical characteristics, stability, and biological activities of the product. Increasing the concentration of *Spirulina* led to a decrease in pH and an increase in acidity and viscosity. In general, the highest degree of

phase separation was observed at the lowest concentration and the longest storage time (day 21). At each storage interval, the treatment with the lowest concentration exhibited the highest level of phase separation. The minimum inhibitory concentration (MIC) of the hydroalcoholic extract of *Spirulina* against *Escherichia coli* was significantly higher than that observed for *Staphylococcus aureus* and *Lactobacillus plantarum*. Previous studies have also shown that the addition of *Spirulina* to various food products leads to a decrease in the color parameters L*, a*, and b*. The results of the DPPH assay indicated a significant increase in antioxidant activity with increasing extract concentration, with 45.97% free radical inhibition recorded at the concentration of 1500 µg/mL. This finding highlights the role of phenolic compounds, carotenoids, and chlorophyll degradation products in enhancing antioxidant capacity. In the sensory evaluation section, increasing the concentration of *Spirulina* resulted in decreased scores for flavor, odor, and overall acceptability. However, at the level of 0.5% extract, no statistically significant difference was observed compared with the control sample ($P \leq 0.05$), identifying this level as the most suitable concentration for enrichment. Overall, it can be concluded that the functional beverage containing 0.5% hydroalcoholic extract of *Spirulina platensis* exhibited the most optimal formulation in terms of physicochemical, sensory, and functional properties and may be considered a health-oriented beverage with potential applications in the food and pharmaceutical industries. Therefore, the use of a combination of licorice extract and *Spirulina platensis* in the production of functional beverages represents an effective step toward the development of natural, nutritious, stable products with potential therapeutic properties. It is recommended that future studies investigate the effects of these compounds on chemical stability, antioxidant activity under long-term storage conditions, and consumer acceptance at an industrial scale.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request, subject to copyright considerations.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

All research activities were carried out by the author.

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مقاله علمی_پژوهشی

بررسی تأثیر سطوح مختلف عصاره هیدروالکلی *اسپیرولینا پلاتنسیس* بر ویژگی‌های فیزیکوشیمیایی، حسی، ضدمیکروبی نوشیدنی فراسودمند شیرین‌بیان

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کلمات کلیدی:

***اسپیرولینا پلاتنسیس*،**

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نوشیدنی فراسودمند،

ویژگی های آنتی اکسیدانی،

ویژگی های میکروبی

چکیده

ترکیبات زیست‌فعال بیولوژیکی و محتوای مناسب پروتئینی در ریز جلبک *اسپیرولینا پلاتنسیس*، می تواند راهکاری نوین در توسعه و حفظ سلامتی مصرف کنندگان در حوزه غذا و دارو ایجاد کند. شیرین بیان نیز به عنوان یک داروی طبیعی خوراکی موثرو همچنین به عنوان افزودنی غذا معرفی شده است. پژوهش حاضر با هدف بررسی تأثیر سطوح مختلف عصاره هیدروالکلی *اسپیرولینا پلاتنسیس* بر ویژگی‌های فیزیکوشیمیایی، حسی، ضدمیکروبی و پایداری نوشیدنی فراسودمند شیرین‌بیان انجام شد.نوشیدنی های فراسودمند شیرین بیان به همراه این ترکیبات : اینولین(۰/۵ درصد) ،زانتان(۰/۱۵ درصد)، آب (۲۰۰۰سی سی) و پودر شیرین بیان ۱۰۰۰ گرم تهیه گردید. سپس سطوح مختلف عصاره هیدروالکلی *اسپیرولینا پلاتنسیس*(۰/۵-۱-۱/۵ درصد) به آن اضافه شد، تیمارها در ٤ گروه تیمار (شاهد)، تیمار۱ (حاوی ۰/۵ درصد عصاره هیدروالکلی *اسپیرولینا پلاتنسیس*)، تیمار۲ (حاوی ۱درصد عصاره هیدروالکلی *اسپیرولینا پلاتنسیس*) و تیمار ۳ (حاوی ۱/۵ درصد عصاره هیدروالکلی *اسپیرولینا پلاتنسیس*) آماده گردید. در نهایت خصوصیات فیزیکوشیمیایی، فعالیت آنتی اکسیدانی، ضد میکروبی و خصوصیات حسی آن ها مورد بررسی قرار گرفت. تیمار ۳ بیشترین میزان pH را دارا بوده است، با گذشت زمان میزان pH تمامی نمونه ها به طور معنی داری کاهش یافت ($P\leq ۰/۰۵$) ومیزان اسیدیته تمامی نمونه ها به طور معنی داری افزایش یافت($P\leq ۰/۰۵$). تیمار ۳ بیشترین میزان ویسکوزیته را با میزان ۱۸۷ سانتی پواز نسبت به سایر نمونه ها در زمان صفر نشان داد، با افزایش میزان عصاره هیدروالکلی اسپیرولینا، ویسکوزیته نمونه ها به طور معنی داری افزایش یافت($P\leq ۰/۰۵$). میزان دوفازی شدن(آب اندازی) ، نوشیدنی فراسودمند حاوی عصاره هیدروالکلی اسپیرولینا، با افزایش میزان درصد عصاره هیدروالکلی اسپیرولینا، به طور معنی داری کاهش یافت ($P\leq ۰/۰۵$) و از طرفی با گذشت زمان میزان دو فاز شدن نمونه ها در روزهای ١٤ ام و ٢١ ام به طور معنی داری افزایش یافت ($P\leq ۰/۰۵$). در ارزیابی رنگ کمترین میزان پارامتر روشنایی(L*) در تیمار ۳ ثبت شده است.در تمامی بازه های زمانی، با افزایش میزان عصاره هیدروالکلی اسپیرولینا، مولفه های رنگی روشنایی (L*)، قرمزی-سبزی(a*)،زرد-آبی(b*) نمونه ها به طور معنی داری کاهش یافت ($P\leq ۰/۰۵$) و از طرفی با گذشت زمان میزان شاخص های رنگی مذکور در تیمار ۳ و ۲ به طور معنی داری کاهش یافت ($P\leq ۰/۰۵$) ارزیابی ویژگی‌های ضدمیکروبی نشان داد که بالاترین حداقل غلظت بازدارندگی مربوط به باکتری *Escherichia coli* و پایین‌ترین حداقل غلظت کشندگی مربوط به *Lactobacillus plantarum* بود ($P\leq ۰/۰۵$). نتایج ارزیابی حسی بیانگر آن بود که نمونه حاوی ۰/۵ درصد عصاره اسپیرولینا تیمار۱ در شاخص‌های بو، طعم و پذیرش کلی، بالاترین امتیاز را به خود اختصاص داد و به‌عنوان مناسب‌ترین فرمولاسیون معرفی شد. بر اساس یافته‌ها، استفاده از غلظت پایین عصاره اسپیرولینا پلاتنسیس (۰/۵ درصد) می‌تواند ضمن بهبود ویژگی‌های تغذیه‌ای و عملکردی، کیفیت حسی مطلوب نوشیدنی شیرین‌بیان را حفظ نماید و به‌عنوان گزینه‌ای مناسب در تولید نوشیدنی‌های فراسودمند پیشنهاد می‌شود

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