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Spirulina and chlorella derived hard candies as functional food

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ABSTRACT

Hard candies with spirulina (*Arthrospira platensis*) and chlorella (*Chlorella vulgaris*) biomass were prepared. Their characterization include pH, moisture, hardness, color, total soluble solids, antioxidant, phenols, and flavonoid content. Safety tests were conducted on rats to examine protective effects of these candies against arsenate poisoning. Algal hard candies were slightly acidic, had low moisture content, and achieved optimal hardness. These candies contain 0.4 GAE mg/g phenols and 0.5 CAE mg/g flavonoids. Upon in-vivo analysis, algae candies increased acetyl cholinesterase, malondialdehyde, catalase, superoxide dismutase, and glutathione concentration in blood serum. Histopathology analysis revealed significant restoration in the liver, kidneys, and brain of Wistar rats treated with algal candies following arsenate poisoning. The phenolic and flavonoid content along with activation of antioxidative enzymes reveals radical scavenging activity of hard candies. Spirulina and chlorella candies may boost immune system, improving gut health, and enhancing antioxidant defense mechanisms and can be used as functional food.

1. Introduction

Functional foods are food products enriched or fortified with bioactive compounds to provide health benefits along with basic nutrition (Caroprese, Ciliberti, & Albenzio, 2015). Nutraceuticals refer to commodities derived from medicinal food, i.e., pills or capsules to provide health benefits. In recent years, consumption of functional foods is increased as awareness of the importance of a healthy diet continues to grow. Similarly, observed trends indicate that consumers favor natural foods over synthetic ones due to the harmful effects of artificial chemicals on the body (Roudbari, Barzegar, Sahari, & Gavlighi, 2024).

Confectionaries with visual attractiveness, texture, and taste have always remained popular among consumers of all ages. The confectionery industry holds major position in the global economy, while impacting the growing health-consciousness of consumers (Konar, Gunes, Palabiyik, & Toker, 2022). These trends have forced confectionary industry to produce products with low content of artificial additives and high levels of antioxidants, fibers, and proteins (Tarahi et al., 2023). Candies are the top-rated confectionery product consumed by all age groups, especially children. However, candies usually have low nutritional values, therefore confectionery industry is looking for a solution to develop candies with natural compounds with high nutritional value (Yadav, Kumari, Chauhan, & Verma, 2021). Currently, hard candies are made with sucrose, glucose syrup, acids, flavorings, and colorants. Adding functional ingredients to these can further enhance their nutritional value (Paternina, Moraes, Santos, de Morais, & Costa, 2022).

Owing to strong consumer appeal, numerous research projects are focused on developing antioxidant-rich functional candies. Souiy and fellows formulated hard candies with antiviral herbal extracts and Citrus lemon peel. Synthesized hard candies showed antioxidant properties with sensory acceptability (Souiy et al., 2023). In another study by Roundbari and fellows nutritional value of gummy candies was enhanced by incorporating pistachio extract (Roudbari et al., 2024).

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Confectioneries can be fortified with antioxidants, serving as functional foods. To date, only limited number of studies have documented the use of microalgae in confectionery. Agustini, Priadi, Hidhayati, Multri, and Rahman (2021) used Chroococcus Turgidus supplements in jelly candy formulation and reported enhanced proximate value of the jelly with antioxidant potential (Agustini et al., 2021). Candies prepared with white tea acquired phenolic content and antioxidant capacity (Mandura et al., 2020).

Microalgae has enormous potential to be used as a functional food due to its antioxidants, anticancer, and anti-inflammatory properties (Batista, Gouveia, Bandarra, Franco, & Raymundo, 2013). It also provides nutritional supplementation such as vitamins, proteins, phenols, sterols, polysaccharides, pigments, and long-chain polyunsaturated fatty acids. *Arthrospira platensis* and *Chlorella vulgaris* are among the most well-known microalgae, rich in vitamins, sterols, proteins, pigments, and long-chain polyunsaturated fatty acids. Both are valuable resources for the production of functional food products, owing to their numerous health benefits (Zhou et al., 2022). Spirulina has already been utilized as an additive in dairy products i.e. yogurt, cheese, fermented milk and kefir. The addition of spirulina increased protein concentration, along with growth promotion of lactic acid bacteria and enhanced nutritional profile (Atik, Gürbüz, Bölük, & Palabıyık, 2021; Çelekli, Alslibi, & Üseyin Bozkurt, 2019; Mazinani, Fadaei, & Khosravi-Darani, 2016; Varga, Szigeti, Kovács, Földes, & Buti, 2002). *Chlorella vulgaris* enriched cheese have higher protein, carbohydrates and fiber content (Mohamed, Abo-El-Khair, & Shalaby, 2013). *Arthrospira platensis* addition produced protein-rich maize snacks, with increased hardness, compactness and 11 % protein content (Lucas, Morais, Santos, & Costa, 2017). *Arthrospira platensis* supplemented biscuits resulted in flavor, nutritional composition, hardness and crispiness enhancement of the product (Gün, Çelekli, Bozkurt, & Kaya, 2022). In addition incorporation of *Arthrospira platensis* in white chocolate enriches its protein, lipid and mineral content (Özbal, Çelekli, Gün, & Bozkurt, 2022). *Arthrospira platensis* can be heated above its denaturation temperature in preparation of algae added marshmallow. *Arthrospira platensis* re-cools back to ambient temperature enhancing gelation which clearly indicates resistance of spirulina compounds while cooking in food products (Çelekli & Maraşlı, 2024). Addition of *Chlorella vulgaris* to gluten-free bread resulted in nutritional properties and antioxidant activity (Qazi, de Sousa, Nunes, & Raymundo, 2022). These reports clearly indicates both the algae (*Arthrospira platensis* and *Chlorella vulgaris*) have been used for production of functional foods.

As awareness grows for the role of a healthy diet in preventing acute and chronic diseases, people are increasingly seeking functional foods with high nutritional value. To enhance the nutritional content of candies, numerous studies are exploring the incorporation of bioactive compounds and plant extracts (Batista et al., 2013; Nuño et al., 2013). The present study focused on formulating spirulina and chlorella-based hard candies to leverage the antioxidant properties of microalgae. The microalgae were used in the formulation of functional hard candies, and their antioxidant potential was assessed through both in vivo and in vitro methods. For in vivo antioxidant evaluation, sodium arsenate poisoning was induced in rats to confirm the protective effects of these functional candies. Sodium arsenate is a growing global health concern due to its harmful effects on health, as it is widely present in soil, food, and water. It has been used in pesticides and insecticides, leading to its ingestion through food exposed to these chemicals. Sodium arsenate is a known carcinogen and inflammatory agent, triggering inflammation by generating reactive oxygen species within the body (Yousuf et al., 2023). *In-vivo* analysis of the current study focuses on the protective effects exerted by functional hard candies on sodium arsenate toxicity. This study is expected to benefit the confectionery industry by introducing functional hard candies made from natural ingredients with high antioxidant potential.

2. Material & methods

2.1. Materials

Algae powder (*Arthrospira platensis* and *Chlorella vulgaris*) were purchased from Nuturiga from Marin Biotechnology Products and Food (Aydin, Turkey). The nutritional content of algae biomass is stated in Table 1. All other chemicals such as sucrose, glucose syrup, citric acid, 2,2-diphenyl-1-picrylhydrazyl, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, folin–ciocalteau, sodium carbonate, gallic acid, catechin hydrate, methanol, ethanol, nitroblue tetrazolium, formazan, dithiobis 2-nitrobenzoic acid, hydrogen peroxide, butylated hydroxytoulene and formaldehyde were purchased from Sigma-Aldrich, St. Louis, MO.

2.2. Methods

The candies were prepared with 250 g sucrose and 325 g glucose syrup, mixed gently and poured into the vaccum batch cooker (Bosch, Germany). Mixture was cooked until the temperature reached 138 °C. Mixture was removed and cooled until it reached a temperature of 90–100 °C. The hard candy was molded with 4 % algae powder and 0.1 % citric acid. The molds were left for hardening and stored in ziplock butter paper lined polypropylene bags. Following hard candy formulations were prepared: 1. control hard candy without citric acid, 2. control hard candy with citric acid 3. *spirulina* candy with citric acid 4. *chlorella* candy with citric acid.

2.3. Physical analysis

For physical analysis, pH, moisture contents, hardness, total soluble solid contents and color analysis were performed.

2.3.1. pH analysis

pH was measured by pH meter Hanna using 945.27 official method (Horwitz & Latimer, 2005; AOAC International (2000), 2000) at 24–28 °C.

2.3.2. Moisture content

Moisture content of hard candies was measured by Karl Fisher (KF) (TitraLab KF1000 Series, HACH, UK) at 25°C with 2 component system. The analysis method (AOAC 991.02) was used. Each molded pastille product was put in blisters and kept in cool and dry place at room temperature (20–22 °C) until analysis (Lee, Durst, & Wrolstad, 2005).

2.3.3. Hardness analysis

The hardness of hard candy formulations was determined using Shore Hardness Tester type A. Durometereter was mounted in vertical position with metal indenter piercing the object. Samples are placed on plain surface and with the indenter hitting the surface. The hardness is measured in KgF. The analysis were conducted in triplicates (Mohamed & Aggag, 2003).

2.3.4. Total soluble solids content

Total soluble solids (expressed as g of total soluble solids contained

Table 1
Nutritional content of spirulina and chlorella biomass (Bito, Okumura, Fujishima, & Watanabe, 2020; Spínola, Costa, & Prates, 2022).

Sr. No.	*Spirulina*	g/100 g	*Chlorella*	g/100 g
1.	Protein	60–76	Protein	50–70
2.	Fats	7–15	Fats	7–20
3.	Carbohydrates	10–20	Carbohydrates	11–25
4.	Sugars	3–10	Sugars	5–25
5	Dietary Fibers	5–15	Dietary Fibers	5–20

in 100 g or °Brix) was measured using a refractometer (Atago Co. Ltd. RX-5000) with an accuracy of 0.01 °Brix. The Brix values were recorded immediately after cooking (Horwitz & Latimer, 2005; AOAC International, 2000).

2.3.5. Color analysis

The color of hard candies was measured using a bench top spectrophotometer (Data color 110TM, Lawrenceville, NJ, USA). The instrument was calibrated with white plate and the color was represented using L* (lightness), a* (redness), and b* (yellowness) color system. According to the method described by (Lee et al., 2005), mean values were determined from three replicate measurements.

Overall color difference (ΔE) is derived from ΔL*, Δa*, and Δb* values.

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

2.4. Chemical analysis

2.4.1. Determination of antioxidant capacity

Antioxidant analysis of the sample was conducted by DPPH method (Hangun-Balkir & McKenney, 2012). Trolox was used as standard with 1–0.325 mg/ml to construct the calibration curve ($r^2 = 0.998$) and results were expressed as TAE mg/g. Absorbance values were measured at 517 nm (UV/VIS Spectrophotometer Optizen Pop Nano Bio, Mecasys Co. LTD, Korea).

2.4.2. Determination of total phenolic content

Phenol content was measured by a modification of (Singleton, 1999). The 200 µl of sample was added to 1 ml of 1:10 dilution of Folin-Ciocalteu reagent. Subsequently, 5–8 mins later, 800 µl of Sodium carbonate (75 g/l) was added and incubated in the dark for 40 mins. Absorbance was measured at 765 nm via UV/VIS Spectrophotometer (Optizen Pop Nano Bio, Mecasys Co. LTD, Korea), and a standard calibration curve ($r^2 = 0.998$) was prepared using Gallic acid. The results were expressed as dry weight of gallic acid equivalent (GAE) mg/g.

2.4.3. Determination of total flavonoid content

Flavonoid content was measured by Mahboubi method (Mahboubi, Kazempour, & Nazar, 2013). Catechin was used as a standard and the absorbance was measured at 450 nm (UV/VIS Spectrophotometer Optizen Pop Nano Bio, Mecasys Co. LTD, Korea). A standard calibration curve ($r^2 = 0.998$) was prepared with Catechin. The flavonoid content was expressed as Catechin equivalent (CAE) mg/g.

2.5. In vivo trials

In-vivo trials were conducted with adult male Wistar rats, provided by Laboratory Animal House, NUST after the approval of Institutional Ethics Committee IRB No. 12-2022-ASAB-05/05.The ARRIVE Guidelines were followed for conducting experiments (Percie du Sert et al., 2020). The animals were 8–10 weeks old and acclimatized for 2 weeks on pelleted feed along with access to ad lib potable water under standard conditions [25 ± 2 °C temperature, 50 ± 15 % relative humidity and normal photoperiod (12 h light and dark cycle)].

Forty adult male Wistar rats, weighing between 180 and 200 g, were randomly allocated to ten groups with 4 animals in each group (n = 4). The distribution of rats is stated in Table 2.

The treatment continued for 50 days. The rats were further monitored for 48 h before their final sacrifice. Body weights of all the animals were recorded before the treatments and after the completion of the treatments. Blood sugar was measured at least once every week. Before sacrifice blood samples were collected from diethyl ether anesthetized rats heart in EDTA and serum vacutainers. Animals were sacrificed by cervical dislocation, vital organs were removed and collected in a formal saline solution (10 %) for histopathological studies (Ajiboye et al., 2024).

Table 2
Distribution of Wistar rats among experimental groups.

Sr. No.	Experimental group
1.	Group I served as control
2.	Group II animals were fed sodium arsenate [20 mg/kg body weight (BW)] dissolved in water.
3.	Group III were fed control hard candy without citric acid [5 mg/kg body weight (BW)].
4.	Group IV were fed control hard candy with 0.5 % citric acid [5 mg/kg body weight (BW)].
5.	Group V were fed *chlorella* candy [5 mg/kg body weight (BW)].
6.	Group VI were fed *spirulina* candy [5 mg/kg body weight (BW)].
7.	Group VII were fed control hard candy without citric acid [5 mg/kg body weight (BW)] along with sodium arsenate [20 mg/kg body weight (BW)].
8.	Group VIII were fed control hard candy with 0.5 % citric acid [5 mg/kg body weight (BW)] along with sodium arsenate [20 mg/kg body weight (BW)].
9.	Group IX were fed *chlorella* candy [5 mg/kg body weight (BW)] along with sodium arsenate [20 mg/kg body weight (BW)].
10.	Group X were fed *spirulina* candy [5 mg/kg body weight (BW)] along with sodium arsenate [20 mg/kg body weight (BW)].

2.5.1. Body weight measurement

The body weight of rats was recorded once every two weeks using a digital balance. The weight was recorded at the same time in the morning.

2.5.2. Blood sugar measurement

One Call® EZ II blood glucose monitoring system was used for the routine blood glucose testing. Blood sugar level was measured once every week. The blood glucose was first evaluated three hours before feeding at fasting. The second evaluation was conducted 3 h after feeding the animals. Blood samples for glucose testing of rats were collected by pricking the tails.

2.5.3. Hematological profile

A complete blood profile was performed to check red blood cells (RBCs), white blood cells (WBCs), platelets and hemoglobin (Hb) count. Haematology Analyzer was used to conduct the blood profiling of Wistar rats, following the manufacturer's guidelines (Sysmex XP-100, Kobe, Japan).

2.5.4. Hepatic & renal function test

Liver/Hepatic Function tests (LFTs) included Alkaline phosphatase (ALP), Alanine transaminase (ALT), Aspartate transaminase (AST), and Gamma-glutamyl transferase (GGT) levels, Albumin (ALB) and Bilirubin (BIL) levels. Renal function test (RFTs) were performed to check serum creatinine and urea levels. All measurements were performed according to the manufacturer's instructions using Chemistry Analyzer (CHEM-READER Smart-N SE250).

2.5.5. Biochemical analysis

Serum was used to measure levels of glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), acetylcholinesterase (AChE), and malondialdehyde (MDA). SOD levels are determined by nitro blue tetrazolium (NBT) formazan dye through optical density at 560 nm. GSH levels were determined by yellow color DNTB [50, 50 (dithiobis 2-nitrobenzoic acid)] and sulfhydryl groups by measuring absorbance at 412 nm. Catalase concentrations were measured using hydrogen peroxide. Malondialdehyde levels were measured using butylated hydroxytoluene with optical density measured at 240 nm and 532 nm. Acetylcholinesterase levels were measured using dithiobisnitrobenzoic acid at 480 and 660 nm. (Ahmed Amar, Eryilmaz, Demir, Aykan, & Demir, 2019; Geerts et al., 2005; Zahra, Patel, Dey, Pandey, & Mishra, 2021).

2.5.6. Histopathology

For histopathological analysis, animals were sacrificed by cervical dislocation. Vital organs were removed and collected in a formal saline solution (10 %). The 5 primary organs brain, lungs, heart, kidneys, and liver were perfused, fixed with 10 % formalin and embedded in paraffin. Sections in 3-μm thickness were placed on slides and applied for hematoxylin and eosin (H&E). Slides were then observed under the light microscope for the presence of any pathological changes.

2.6. Statistical analysis

Experiments were conducted in triplicates. Statistical analysis was performed via GraphPad Prism 9. Descriptive statistics included the mean and standard deviation. One-way ANOVA was performed for measuring variants.

3. Results & discussion

3.1. Physical analysis

The pH is one of the important quality parameters of hard candies as it influences candies' taste, color, moisture content, hardness, pigment stability, and sucrose inversion. Hard candies have weakly acidic pH due to the addition of citric acid. Acidic pH inhibits microbial growth along

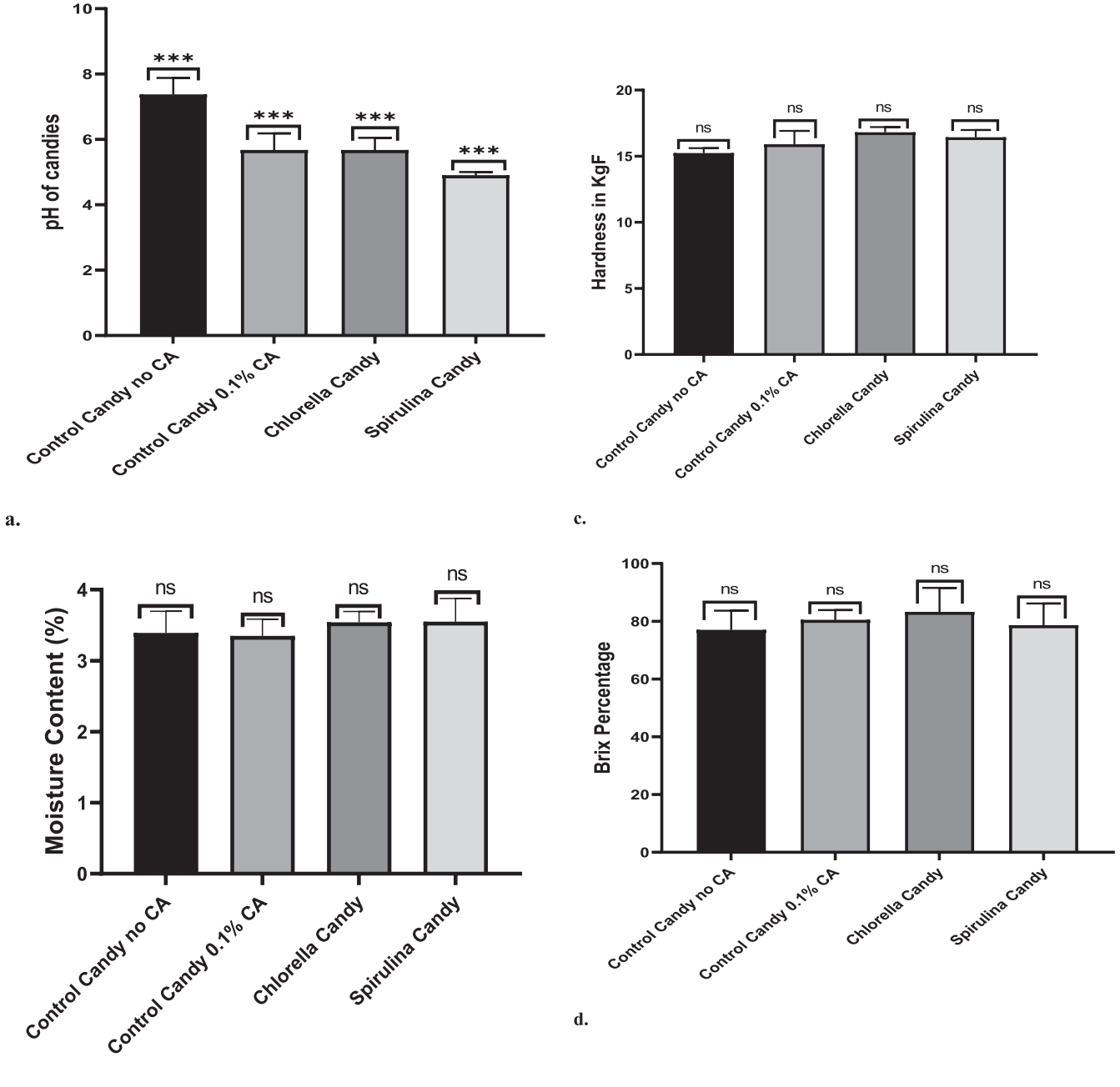
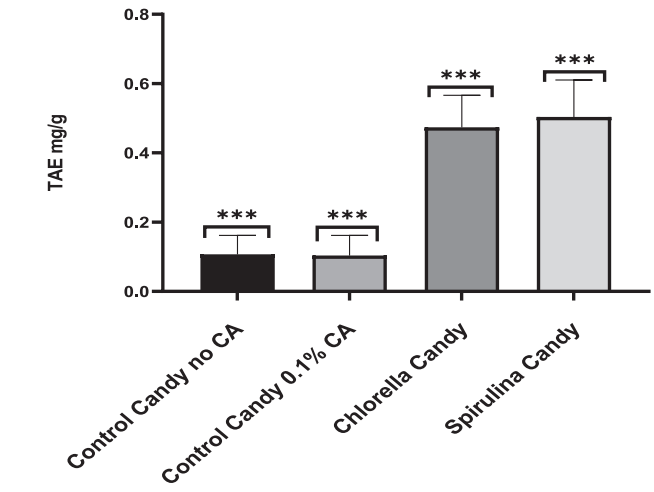


Fig. 1. Physical analysis of hard candies based on (a) pH, (b) Moisture, (c) Hardness, (d) Brix percentage, (e) Antioxidant content of candies (f) phenol content of candies, (g) flavonoid content of candies. The statistical significance was presented above bars as Ns indicates not significant for $p > 0.05$, *** indicates very highly significant $p < 0.001$, **** indicates extremely significant $p < 0.0001$. Control hard candy without citric acid is abbreviated as control hard candy no CA, while control hard candy with 0.1 % citric acid is abbreviated as control hard candy 0.1 % CA.



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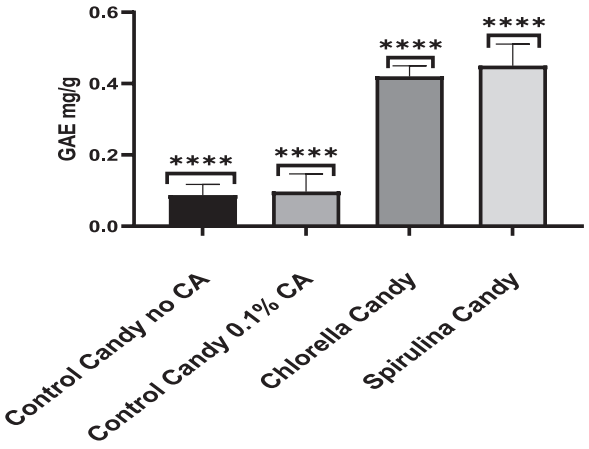
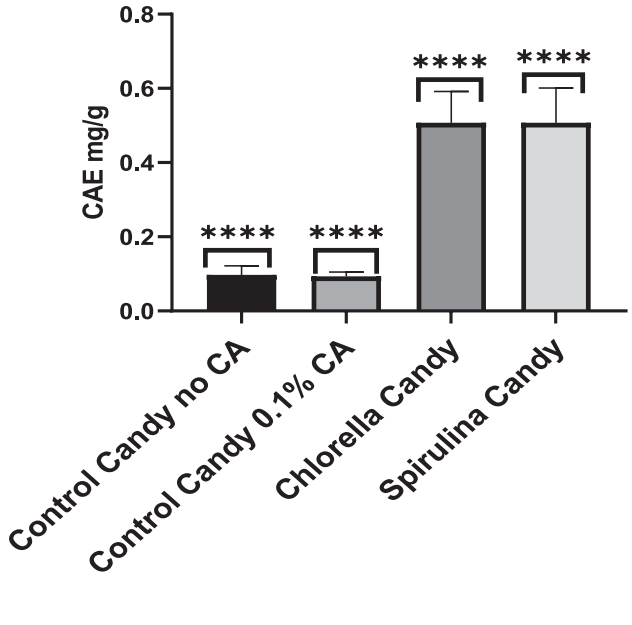


f.



g.

Fig. 1. (*continued*).

with increasing shelf life. The results of pH are compiled in Fig. 1a. The pH values of control candies without any citric acid were 7.37, while for candies with citric acid the pH was 5.67. The *Chlorella vulgaris* and *Arthrospira platensis* candies showed pH of 6.7 and 4.9 respectively. This falls under the optimum approved pH of 4–6. The critical pH for enamel health is 5.5 (Arafa, Filfilan, & Fansa, 2022; McIntyre, 1992). The inclusion of microalgae in candies increases the pH and reduces acidity significantly.

The moisture content and hardness of candies determined hard candy's final texture. The best candies have a hard texture with a glossy appearance. Moisture content in all samples are concluded in Fig. 1b. was 3.55–3.35. The addition of algae in candies did not significantly impact moisture absorption. Hardness results in Fig. 1c. revealed values between 15.2 and 16.4 kgF. These results are similar to those of candies prepared with medicinal plants such as *Cudrania tricuspidate* and *Zingiber Officinale* var. falling between the ranges of 15–18 kgF (Akib, Baane, & Fristiohady, 2016; Jeon, Oh, & Cho, 2021). Variation in hardness suggests the influence of external factors on the candies' firmness, since the variation was not significant. Both the hardness and moisture content results indicate that hard candies possess an amorphous structure with distinct crystalline elements.

The total soluble solid content of the algal hard candies was 76.9–83.1 as shown in Fig. 1. The Brix values of the hard candy samples were within the optimal range for hard candies. The ideal Brix values for hard candies are typically between 70 and 80 °Brix. (Hartel et al., 2018; Magwaza & Opara, 2015). The addition of algae biomass does not result in variation of Brix values. The dark color of confectionery products captures attention. (Baptista, Valentin, Saldaña, & Behrens, 2021). The color analysis of candies was compiled in Table 3. as Delta L*(brightness), a*(redness) and b*(yellowness). The L* values of candies ranged from 58.8 to 78.4. The a* values of candies ranged from −3.1 to-0.23. The b* values of candies ranged from 2.23 to 6.76. The change of color as expected was dark green. Addition of algae compared to control candies presents significant reduction in L*, a* and b*.

Table 3
Color analysis of candies Lightness (L*), redness (a*) yellowness (b*) with microalgae powder addition were analyzed.

	Candy Samples	L*	A*	B*
1.	Control hard candy no Citric acid	78.4 ± 4.95	−0.3 ± 0.08	3.03 ± 0.79
2.	Control hard candy 0.1 % Citric acid	76.67 ± 5.02	−0.23 ± 0.05	2.23 ± 0.05
3.	*Chlorella* Candy	58.87 ± 0.79	−2.2 ± 0.16	6.77 ± 0.189
4.	*Spirulina* Candy	63.63 ± 1.78	−3.1 ± 0.37	3.0 ± 0.45

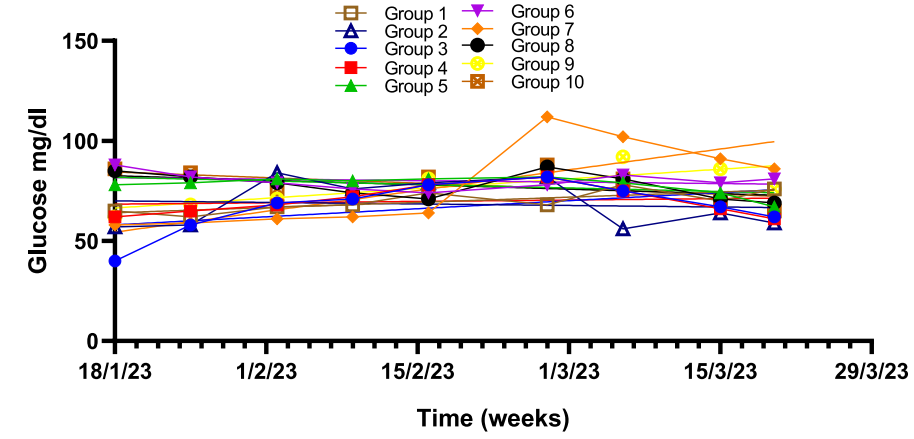
3.2. Chemical analysis

Phenolic compounds are recognized as natural antioxidants, making total phenol content and total flavonoid content useful indirect indicators of antioxidant potential. The phenolic and flavonoid content of both algae species *Arthrospira platensis* and *Chlorella vulgaris* are comparable.

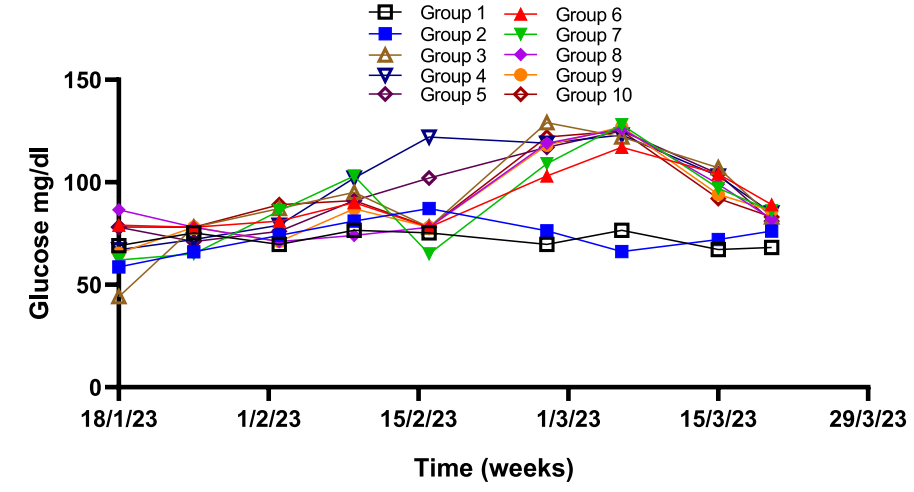
Results of antioxidant properties of hard candies were compiled in Fig. 1e. The formulated algae candies demonstrated an antioxidant content of 0.5 TAE mg/g, compared to 0.1 TAE mg/g in the control candies. The total phenol content in the control candies was measured at 0.09 GAE mg/g (as shown in Fig. 1f), while the algal candies exhibited a phenolic content of 0.4 GAE mg/g. In contrast, the phenolic content in Spirulina and Chlorella was reported to be 7.93 GAE mg/g and 8.70 GAE mg/g, respectively (Wu, Ho, Shieh, & Lu, 2005). The phenolic compounds in Spirulina are tannic acid, gallic acid, caffeic acid, salicylic acid e.t.c. (de Souza, Prietto, de Souza, & Furlong, 2015). Total flavonoid content of control candies was observed between 0.09 CAE mg/g. The flavonoid content in algae candies was reported as 0.5 CAE mg/g as indicated in Fig. 1g. Flavonoid content in Spirulina along with pigments, chlorophyll, carotenoids, and the phenolic contents also contribute to antioxidative properties (Kumar et al., 2022; Agustini, Suzery, Sutrisnanto, & Ma'ruf, 2015). The presence of flavonoids and phenols in the algal hard candies demonstrates the heat resistance of microalgae. Heat-sensitive materials are typically used as colorants or flavors due to the high-temperature cooking process involved in making hard candies (Ozel et al., 2024). However, the retention of algae-derived flavonoids and phenols in the hard candies confirms their resilience to these high temperatures. The absence of antioxidants, phenols, and flavonoids in the control candies further supports that the added antioxidative potential was solely due to the inclusion of algae.

3.3. In vivo safety assays

Safety assays were conducted in male Wistar rats. Weight gain was observed in rats that were given control candies and algal candies without sodium arsenate (Fig. 2c). In contrast, the lowest weight gain was recorded in groups 7, 8, 9, and 10, which were administered sodium arsenate and fed algal candies. These groups experienced weight gain during the initial weeks, followed by gradual weight loss in the later weeks, likely due to the effects of arsenate intake (Yadav et al., 2009). During fasting, the maximum blood glucose level reached 112 mg/dL after 38 days of treatment (Fig. 2a). After three hours of treatment, glucose levels peaked at 128 mg/dL on day 30 (Fig. 2b). By the end of the treatment, glucose levels stabilized at an average of 60–80 mg/dL. The highest glucose concentration was observed in the fourth week, which gradually decreased by the fifth week. These results indicate that sodium arsenate exposure is contributing to glucose tolerance in rats

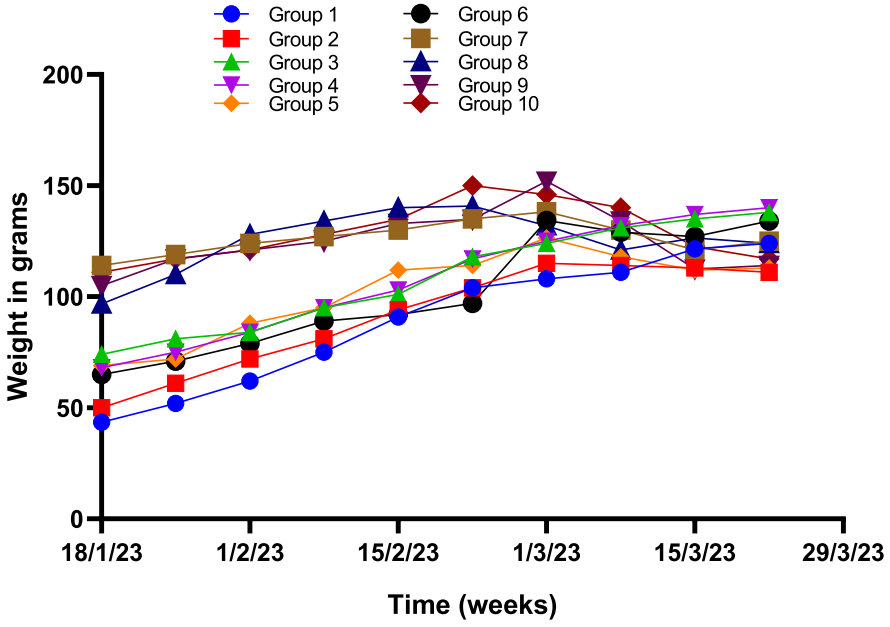


a.



b.

Fig. 2. Blood Glucose level and weight gain. (a) Blood glucose (mg/dl) in fasting. (b) Blood glucose (mg/dl) after 3 h, (c) Weight in grams.



c.

Fig. 2. (*continued*).

(Rezaei, Khodayar, Seydi, Soheila, & Parsi, 2017). Algae hard candies have reduced glucose tolerance in rats. Group 1 and 2 do not experience glucose spike while groups 3–10 experience higher glucose spike after 3 h of consumption. While the animals experience glucose tolerance after 4 weeks, the spike recedes with normal blood glucose level by the end of 6th week.

The complete blood count (CBC) is a commonly used test for detecting coagulopathies, infections, immunodeficiencies, hemorrhagic conditions, and both acute and chronic inflammations. In this study, all groups except group 2 displayed normal CBC profiles, as shown in Table 4. The reference ranges for key blood components are as follows: Mean white blood cells (WBCs): 3,600–14,500/μL, Mean red blood cells (RBCs): 3.8 × 10^6–8.5 × 10^6/μL, Mean hemoglobin (Hb): 10.4–16.5 g/dL, and Mean platelets: 500,000–148,000/μL (He et al., 2017). Group 2 exhibited the lowest mean RBC, hemoglobin, and platelet counts, along with the highest WBC count, indicating immune stress likely caused by sodium arsenate consumption. In contrast, results for groups 1 and 3–10 fell within the normal reference ranges, suggesting stable hematology in these groups.

Table 4
Variations in hematological profile of all 10 rat groups stated from G1 to G10.

Groups Reference Level	Mean WBCs (3600–14,500/μL)	Mean RBCs (3.8 × 10^6–8.5 × 10^6/μL)	Mean Hb (10.4–16.5 g/dL)	Mean Platelets (500,000–148,000/μL)
G1	6063.17 ± 0.25	7.86 ± 0.37	13.67 ± 0.15	123961.9854 ± 11547.01
G2	9484.81 ± 3.71	4.78 ± 0.58	9.7 ± 0.58	23961.9854 ± 11547.01
G3	7056.33 ± 0.47	8.88 ± 0.8	12.76 ± 0.19	1093968.688 ± 7710.6
G4	5852.38 ± 0.49	6.18 ± 0.42	11.52 ± 0.78	984975.2007 ± 6296.3
G5	3852.99 ± 1.25	7.21 ± 0.41	12.72 ± 0.75	1382613.304 ± 26474.58
G6	4396.97 ± 0.2	7.34 ± 0.31	14.96 ± 0.61	700846.4427 ± 34860.44
G7	4630.17 ± 0.21	7.99 ± 0.26	13.43 ± 0.49	877499.1959 ± 23103.54
G8	6789.55 ± 3.2	6.48 ± 0.57	11.26 ± 1.15	729352.2095 ± 14133.06
G9	3580.45 ± 2.6	7.25 ± 0.07	13.03 ± 0.25	658734.3083 ± 27130.98
G10	6779.52 ± 5.1	7.66 ± 0.24	12.75 ± 0.34	1142894.199 ± 117675

Liver and Kidney function tests were performed as these are the major organs involved in the metabolism and excretion of food therefore vulnerable to food-induced toxicity. The results are shown in Table 5. Doubling of alkaline phosphatase (ALP), bilirubin and a five times increase of alanine aminotransferase (ALT) is indicative of liver toxicity (Devarbhavi, 2012).

Since there is variation in normal levels from lab to lab, it was necessary to compare the values of each group with group 1. A significant difference was observed between the ALP of Group 1 and Group 2 highlighting the toxic effects of sodium arsenate on the liver. Notably the two groups consuming chlorella or spirulina candies with sodium arsenate exhibited a significant decrease in ALP and ALT levels in comparison to the positive control group which indicated the safety of these candies. However the effect of hard candy on Alkaline Phosphatase (ALP) was opposite, it increased the ALP levels which may be due to the high sugar intake provided by the candies. The ALT levels are reduced by control hard candy. The hard candy increases blood glucose levels however the sudden spikes causing high ALT and ALP typically associated with liver diseases were missing with algae candies. Algae candies consumed with sodium arsenate reduce ALT and ALP concentrations. This leads to the inference that algae candies have protective effects towards liver stress. Bilirubin levels do not present any change for any of these groups. Algae candies lowered the creatinine concentrations while urea concentrations were higher in animals with sodium arsenate and hard candy intake.

Sodium arsenate induces oxidative stress which was evaluated in blood serum samples of rats by quantifying antioxidant enzymes (Shahid et al., 2014). The results in Table 3 indicate that superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) concentrations increased in group 2, 7, 8, 9, 10 (feed on sodium arsenate) compared to group 1, 3, 4, 5, 6. Group 2 had the highest concentrations of SOD, CAT and GSH at

Table 5
Hepatic and renal function profile of all ten groups and Biochemical analysis for Acetylcholinesterase, malondialdehyde, superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) in rat blood. The Groups from group 1 to group 10 have been abbrev.

	ALT mg/dl	ALP mg/dl	Creatinine mg/dl	Bilirubbin mg/dl	Urea mg/dl
G1	249 ± 47.68	202 ± 29.8161	0.67 ± 0.15	0.7 ± 0.17	32 ± 5
G2	66.67 ± 28.290	327.33 ± 88.27	0.23 ± 0.15	0.77 ± 0.15	86.33 ± 13.2
G3	63.33 ± 33.36	463 ± 58.62	0.3 ± 0.1	0.6 ± 0.1	78.67 ± 7.02
G4	70.67 ± 5.69	727.67 ± 74.57	0.37 ± 0.06	0.47 ± 0.06	45.67 ± 2.52
G5	65.67 ± 11.02	624 ± 38	0.3 ± 0.0	0.57 ± 0.06	56.33 ± 20.43
G6	84 ± 14.18	575 ± 123	0.4 ± 0.1	0.47 ± 0.06	48.33 ± 5.5
G7	73.5 ± 20.5	176.5 ± 3.54	0.4 ± 0.1	0.5 ± 0.1	53.5 ± 6.4
G8	100 ± 18.4	735 ± 81.3	0.35 ± 0.07	0.55 ± 0.07	38 ± 0.0
G9	56 ± 12.7	389.5 ± 54.44	0.35 ± 0.07	0.5 ± 0.0	73 ± 16
G10	78 ± 15.77	325 ± 77.68	0.475 ± 0.13	0.5 ± 0.115	42.25 ± 4.5
	Acetyl cholinesterase ng/mL Range 2.5–4	**Malondialdehyde µmol/L**	**Superoxide Dismutase U/mL**	**Catalase U/mL**	**Glutathione mmol/L**
G1	2.35 ± 0.15	14.26 ± 0.22	102 ± 0.09	0.11 ± 0.06	0.15 ± 0.03
G2	6.8 ± 0.1	32.6 ± 0.27	154 ± 0.3	1.07 ± 0.09	1.02 ± 0.03
G3	2.3 ± 0.07	19.64 ± 0.27	112 ± 0.11	0.38 ± 0.03	0.44 ± 0.03
G4	2.2 ± 0.05	16.88 ± 0.14	110 ± 0.11	0.22 ± 0.1	0.43 ± 0.04
G5	3.33 ± 0.21	20.08 ± 0.24	94.99 ± 0.18	0.36 ± 0.03	0.42 ± 0.06
G6	2.71 ± 0.08	20.66 ± 0.20	95.48 ± 0.18	0.41 ± 0.06	0.48 ± 0.05
G7	3.14 ± 0.06	20.45 ± 0.18	145.07 ± 0.38	0.46 ± 0.06	0.87 ± 0.03
G8	3.4 ± 0.06	20.22 ± 0.14	141.73 ± 0.2	0.43 ± 0.05	0.54 ± 0.03
G9	3.27 ± 0.05	22.22 ± 0.13	141.87 ± 0.39	0.41 ± 0.03	0.57 ± 0.02
G10	3.7 ± 0.07	22.16 ± 0.05	141.4 ± 0.44	0.53 ± 0.04	0.56 ± 0.03

102 U/ml, 1.07 U/ml and 1.02 mmol/L respectively. The oxidative stress induced by sodium arsenate in group 2 is evident. Consumption of algal candy has reduced oxidative stress in rats as evidenced by results of group 7–10 with values in the range of 141–145 U/ml (SOD), 0.41–0.53 U/ml (CAT), 0.54–0.87 mmol/L (GSH). Acetylcholinesterase and malondialdehyde concentration were highly elevated in group 2 at 6.8 ng/ml and 32.6 µmol/L as indicated in Table 3. Acetylcholinesterase remained in the normal range of 2.5–4 ng/ml for all 9 groups. The control group contained 14.26 µmol/L while all other eight groups on hard candy diet contained 16.9–22.22 µmol/L of malondialdehyde. Results indicate that algal candies mitigate oxidative stress caused by sodium arsenate.

Histopathological examination is considered as gold-standard test to evaluate toxicity as serum markers usually increase late in the disease. Therefore, histopathology of all vital organs in the groups was compiled in Fig. 3.

Drug-induced liver injury is one of the manifestations of toxicity caused by, drugs, metabolites, and toxins (Fisher, Vuppalanchi, & Saxena, 2015; Ramachandran & Kakar, 2009). Upon histology, liver parenchyma shows lobular hexagonal anatomy with center of the hexagon made up of a central vein and hepatocytes radiating from it in a cord-like manner. It is usually manifested as acute or chronic hepatitis with or without acute or chronic cholestasis (Kleiner et al., 2013). All groups showed normal histological anatomy except for group two (Fig. 3. 1b) which showed infiltration of leukocytes with venous congestion suggesting early signs of liver inflammation. The hard candy groups with sodium arsenate i.e., Group VII, VIII, IX, and X also showed normal histology signifying the safety of these candies.

Since kidneys are also involved in excretion of various metabolites, it is also a common organ affected by them. Therefore to exclude nephrotoxicity, histopathological examination of kidney was performed. Nephrotoxicity usually manifests as glomerular disease or nephritis (Loh & Cohen, 2009). Glomerular disease upon histology is identified by the appearance of basement membrane vacuolization, retraction of glomerular tuft or podocyte proliferation. While nephritis is recognized by infiltration of leukocytes, tubular dilatation, loss of brush border, cytoplasmic vacuolization or prominent nuclei. Visualized histological sections of Fig. 3. 2b, 2l showed infiltration of leukocytes with patch hemorrhagic foci in both cortical and medullary slides indicating nephritis however glomerular membrane remained intact. All other groups in Fig. 3. 2a, c–k, m–t showed normal renal tissue.

The heart is a vital organ, and it is recommended to check cardiotoxicity in safety trials. Therefore, we performed histopathological analysis of cardiac tissue. Normal cardiac tissue shows tightly arranged mononucleated cardiomyocytes with striated appearance. None of the group showed any significant cardiotoxicity i.e., ischemic changes to cardiomyocytes which usually manifests as subendocardial necrosis, degeneration of muscle fascicles with or without hemorrhagic foci (Greaves, 1998). Though the positive control group in Fig. 3. 3b showed little loss of normal striated appearance.

Neurotoxicity is another major concern in safety analysis. Normal cerebrum on H & E staining shows a three layered anatomy with granular cell layer in dark blue, the molecular layer in pink with dispersed Purkinje cells, and the white matter underlying these folia. Toxicity to brain appear as neuronal loss, hemorrhage (Sharma et al., 2014). Few hemorrhagic foci and neuronal loss were observed in positive group in Fig. 3. 4b while all other groups showed normal histology. Normal histology in Fig. 3. 4a, c–j having both sodium arsenate and hard candy shows protective effect of these candies.

The lungs, a critical organ, can experience life-threatening complications if exposed to toxicity. Normal lungs largely appear as empty space due to alveoli. Bronchi on lungs showed pseudostratified epithelium in blue, with smooth muscle layer in pink (Leslie, 2009; Rossi, Erasmus, McAdams, Sporn, & Goodman, 2000). Damage to lung can manifest in form of variety of disease i.e. diffuse alveolar damage, bronchiolitis obliterans organizing pneumonia, nonspecific interstitial pneumonia, eosinophilic pneumonia, haemorrhage and veno-occlusive disease. Positive control Fig. 3. 5b showed high infiltration of leukocytes indicating interstitial pneumonitis. All other groups showed normal lung anatomy (Fig. 3. 5a, c–g,i) while group 8 and group 10 (Fig. 3. 5h,j) with sodium arsenate treatment had mild inflammatory changes which prove candies didn't exert strong protective effect on lungs however are safe to use without any risk of toxicity.

4. Conclusion

Current study concludes the safety of *spirulina* and *chlorella*-derived hard candies as a foods with antioxidative potential. Antioxidative

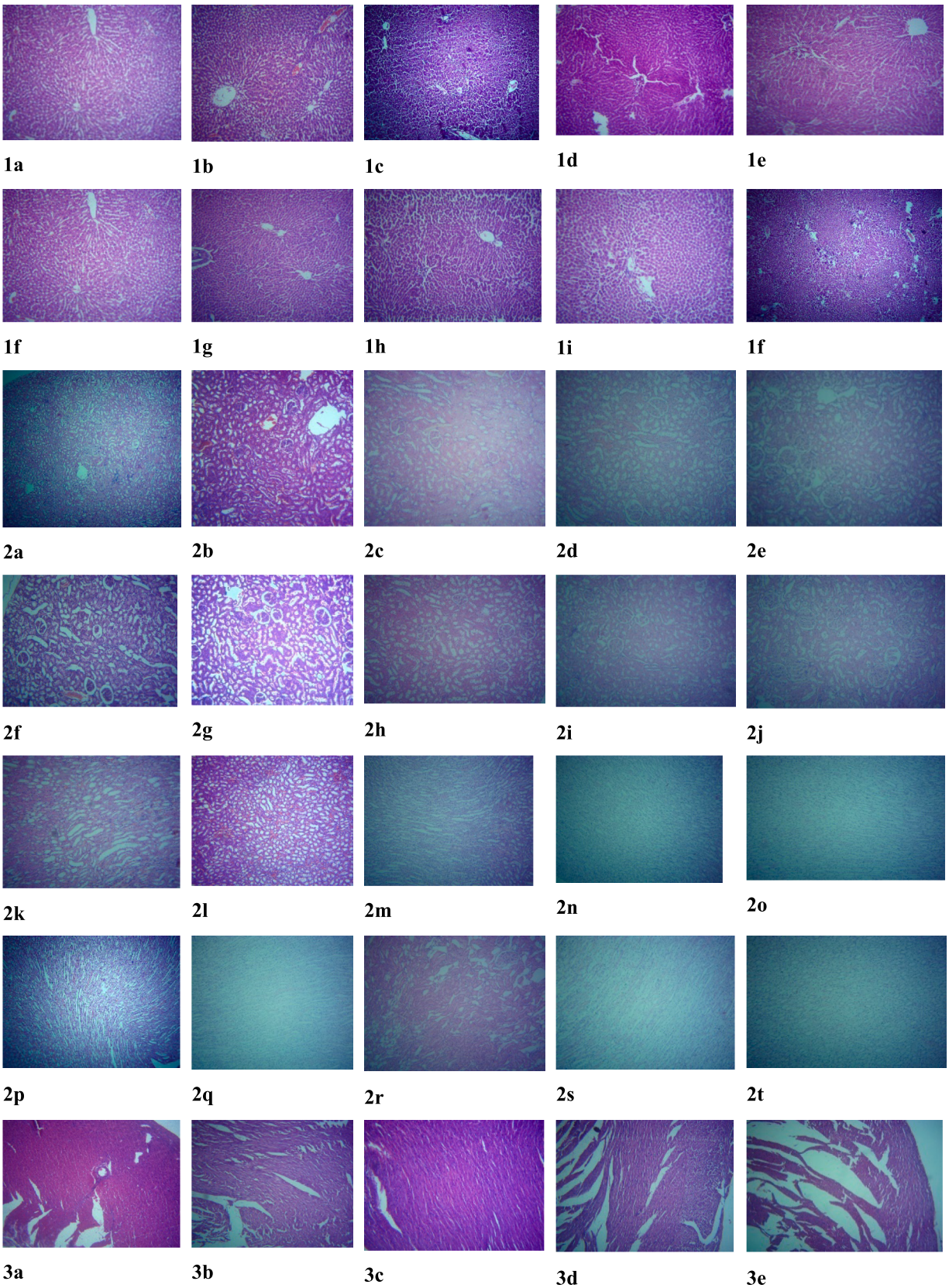


Fig. 3. Histopathological examination of tissues. The results are labeled with group numbers and tissues 91a–j) liver tissues, (2a–j) kidney cortex tissues, (2k–t) kidney medulla tissues, (3a–j) heart tissues, (4a–j) brain tissues and (5a–j) lung tissues. The ten groups are indicated in alphabetical order; a–j and k–t.

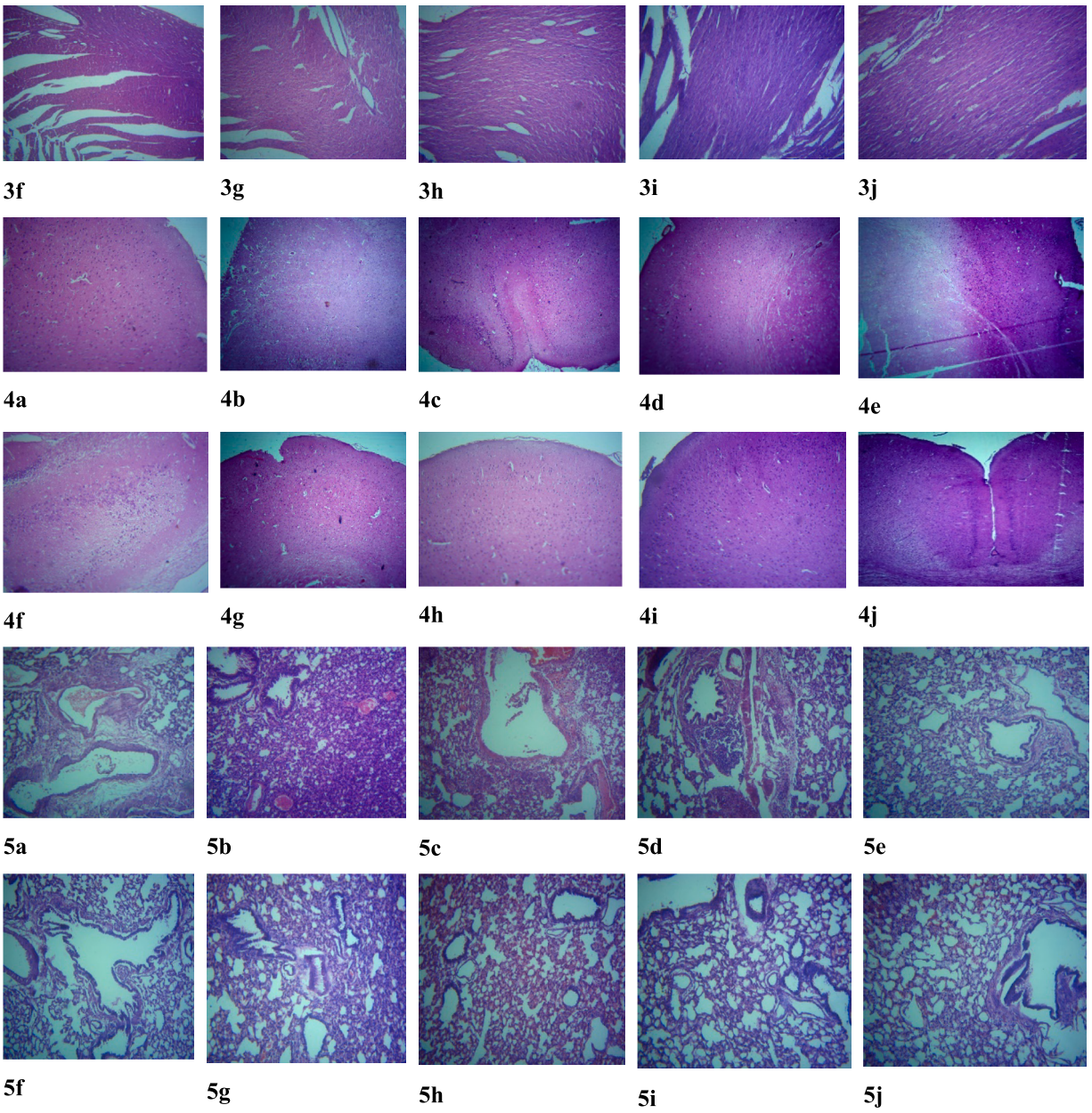


Fig. 3. (*continued*).

properties of algae mitigate stress induced by sodium arsenate along with protecting tissues from damage caused by arsenic toxicity. The antioxidative enzymes such as SOD, CAT and GSH were 141–145 U/ml, 0.41–0.53 U/ml, 0.54–0.87 mmol/L. These are higher in animals on diet with algae hard candies and sodium arsenate in comparison to animals, that are only fed algae hard candies. The animals with sodium arsenate and algae hard candy diet also had no tissue damge in histology while animals on arsenate diet had damaged tissues. Algae consumption triggered the activation of antioxidative enzymes. The incorporation of algae candies in diet has had positive results in animal studies. Future prospects include acceptance of algae foods and production along with the commercialization of algal-derived hard candies as functional foods. Awareness on environmental benefits of algae along with the adoption of algae-based products is required to use algal based products in food industry as functional foods. Overall, future prospects as a sustainable food source and nutraceutical ingredient of algae biomass are promising, however further research is required to fully explore the potential of chemical commodities and proteins as functional food commodities in food industry. This study is a step in the direction of further utilization of microalgae as food ingredient.

Ethics statement

All animal experiments were approved by the Institutional review committee of National University of science and technology.

CRediT authorship contribution statement

Shafia Maryam: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Mecit Halil Oztop:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Sarper Doğdu:** Resources, Methodology, Formal analysis. **Mehmet Ali Marangoz:** Validation, Formal analysis. **Zeshan Zeshan:** Methodology, Formal analysis. **M. Qasim Hayyat:** Project administration, Formal analysis. **Ramish Riaz:** Investigation, Formal analysis. **Muhammad Waqas Alam Chattha:** Formal analysis, Data curation.

Hussnain Ahmed Janjua: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data presented in this study are available on request from corresponding author.

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