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Anti-Oxidative Effects of Bioactive Compounds in Spirulina Microalgae and Bilberry

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Abstract

Spirulina and Bilberry are underexplored and underutilized in the food industry. Therefore, this research focuses on determining the antioxidative properties of Spirulina and Bilberry for future use in functional food product development. The objective was to determine the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) in Spirulina and Bilberry extracts (Aqueous and Ethanol extracts) and their antioxidative potential (2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Potential (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), and Nitric Oxide Radical Scavenging Ability (NORS)). Spirulina and Bilberry pure and combination samples [100% Spirulina (100S), 100% Bilberry (100B), 50% Spirulina + 50% Bilberry (50S + 50B), 75% Spirulina + 25% Bilberry (75S + 25B), & 25% Spirulina + 75% Bilberry (25S + 75B)], were extracted with aqueous (deionized water) and 80% ethanol solutions. Colorimetric antioxidant assays were used to determine total phenolics, total flavonoids, and their antioxidant potential. 80% ethanol Spirulina and Bilberry (pure and combination) extracts resulted in higher TFC, FRAP, and DPPH, whereas aqueous extracts had higher TPC, NORS, and TEAC, suggesting both hydrophilic and lipophilic bioactive compounds in Spirulina and Bilberry. Spirulina and Bilberry are two potential functional food ingredients for the food industry due to their antioxidative properties.

Keywords

Spirulina, Bilberry, Antioxidant, Functional Foods, Functional Food Ingredients, Total Phenolics, Total Flavonoids

1. Introduction

Oxidative stress, a phenomenon that precipitates the imbalance between the production and accumulation of reactive oxygen species (ROS) or free radicals in the

human body, has been associated with the development of various chronic diseases such as obesity, heart disease, diabetes, high blood pressure (hypertension), and cancer [1] [2]. Free-radical scavengers, better known as antioxidants, provide aid in the prevention of cellular damage done by free radicals. Examples of general free-radical scavengers include vitamin C, vitamin E, and glutathione [3]. Free radicals are simply molecules that are unstable due to a loss of an electron that is produced either from normal cell metabolisms, such as in the mitochondria, or from external sources, such as pollution, cigarette smoke, or radiation [1]. Antioxidants are referred to as free radical scavengers due to their role in stabilizing unstable radicals by donating an electron to their orbital structure, protecting the body from damage [4]. Antioxidant capacity assays are utilized to determine the antioxidant ability or capacity, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Potential (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), and Nitric Oxide Radical Scavenging (NORS). Moreover, when there is an overaccumulation of free radicals in the body, the overload may lead to cellular damage and the development of chronic diseases, such as cancer, diabetes, and obesity [5]. Fortunately, improving nutrition in the body by incorporating functional foods into the diet can aid in the reduction of reactive oxygen species, whereby preventing the development of chronic diseases [6].

Functional foods are those foods that provide the human body with benefits beyond their basic nutritive value, such as promoting proper health and development and aiding in reducing chronic diseases such as diabetes and heart disease [6]. The chemical components that make up functional foods are what give them their beneficial effect. Chemical components such as phenolics and flavonoids have been shown to provide immunity to the body by stimulating the activity of immune cells in the body, which can provide protection against cancer and viruses [7]. Common functional foods may include fruits, vegetables, algae, nuts, and legumes [8]. It is important for the food industry to constantly find new sources of functional foods to keep up with present food and medicinal/health trends.

Spirulina is a blue-green algae that is considered a "superfood" due to its high protein levels [9]. The algae were first used by the Aztecs in the post-classic period as an endurance booster and to treat various diseases [10]. Bilberry is a berry, similar to the American blueberry, that has been used for medicinal purposes since the Middle Ages [11]. Each food ingredient is high in nutrients and vitamins such as vitamin K, vitamin C, niacin phosphorous, and iron. With their high nutrients, vitamins, and mineral content, these two ingredients are unfortunately understudied and underutilized in the food industry.

The epidemic of chronic diseases is fast growing in adults in the United States. Previous research suggests that Spirulina and Bilberry both have health-promoting properties that have the potential to reduce or prevent the risk of chronic diseases by having antioxidant properties. These functional ingredients are underutilized, and utilizing them may have benefits to diversifying food products for adolescents. Therefore, identifying their antioxidative capabilities can better promote their use in the food industry.

2. Materials and Methods

2.1. Sample Preparation

Organic Spirulina powder was purchased from the company, Triquetra Health, and powdered Bilberry was purchased from the company, Nordic. Aqueous (deionized water) and ethanol (80% ethanol) solutions were used to perform the sample extracts, using 100ml of each solution per 5g of the Spirulina and Bilberry [pure (100%) and combinations (50%/50%, 75%/25%, 25%/75%)] samples. Both aqueous and ethanol extracts were placed into a Bransonic M5800H Ultrasound Sonic Bath for 1 hour and centrifuged at 1107 g force at 4˚C for 20 minutes. Following, the supernatants were filtered using Whatman filter paper, then evaporated using a rotary evaporator and reconstituted using 10ml of the specific solution. Afterwards, the samples were stored in a 4˚C cooler. The overall experimental layout can be seen in Figure 1.

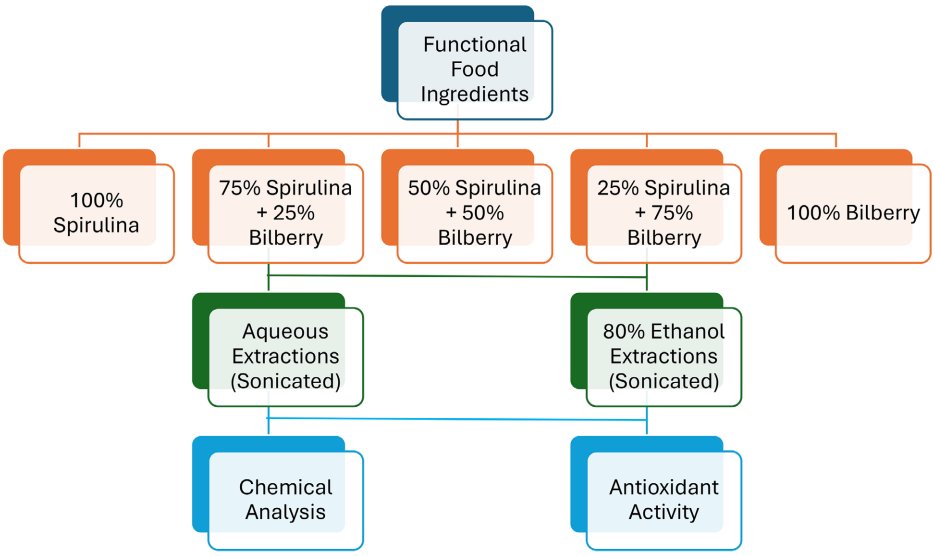


Figure 1. Overall experimental layout.

2.2. Chemical Analysis and Antioxidant Activities

The experimental layout for the chemical analysis and antioxidant activity of the Spirulina and Bilberry samples is shown below in Figure 2. The chemical assays included tests for Total Phenolic Content (TPC) and Total Flavonoid Content (TFC), while the antioxidant assays included the following assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Potential (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), and Nitric Oxide Radical Scavenging Ability (NORS).

2.2.1. Chemical Analysis Assays

The phytochemical content of the Spirulina and Bilberry extracts was determined utilizing the following assays: Total Phenolic Content (TPC) and Total Flavonoid (TFC).

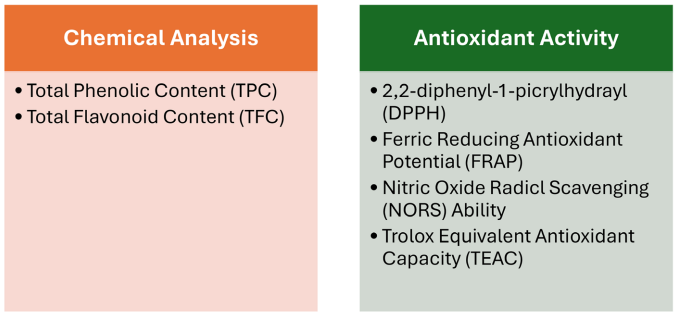


Figure 2. Chemical analysis and antioxidant activity assays.

1) Total Phenolic Content (TPC)

Phenolic compounds are phytochemicals that contain benzene rings with one or more hydroxyl substituents, that are mainly found in most plant tissue [12]. Total phenolic content in the Spirulina and Bilberry extracts was determined using an adaptation of the Folin-Ciocalteu method [13]. Using gallic acid as the standard, diluted extracts were combined with deionized water and Folin-Ciocalteu's reagent and incubated for 5 minutes. Afterwards, sodium bicarbonate was added to the samples and incubated for a total of 90 minutes. Following, the samples were read for their absorbance at 750 nm using a Synergy HTX microplate reader.

2) Total Flavonoid Content (TFC)

Flavonoids, a main group of phenolic compounds, are said to be a component in nutraceutical, medicinal, and pharmaceutical applications that have beneficial effects on the body [14]. Total flavonoid content was determined using catechin as the standard [15]. Diluted samples were incubated with deionized water and sodium nitrite for 5 minutes, and then incubated for an additional 5 minutes after adding 10% aluminum chloride to the mixture. Following, the samples were read at an absorbance of 510 nm after adding sodium hydroxide and an additional amount of deionized water.

2.2.2. Antioxidant Activity Assays

The antioxidant activity of the Spirulina and Bilberry extracts were tested using the following assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Potential (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), and Nitric Oxide Radical Scavenging Ability (NORS).

1) 2,2-diphenyl-1-picrylhydrazyl (DPPH)

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical scavenging assay that was used to evaluate the antioxidant potential of the Spirulina and Bilberry sample extracts [16]. Diluted samples were combined with the DPPH radical and read at an absorbance of 517 nm at 30-minute intervals for 90 minutes [17].

2) Ferric Reducing Antioxidant Potential (FRAP)

Ferric Reducing Antioxidant Potential (FRAP), also known as Ferric Reducing Ability of Plasma and Ferric Reducing Antioxidant Power, is a test that will assess antioxidative power of the samples used in this experiment. From an adaptation of Benzie & Strain, diluted samples were combined with deionized water, 10 mM

iron sulfate, and FRAP reagent [18]. The samples were then read at 593 nm.

3) Nitric Oxide Radical Scavenging Ability (NORS)

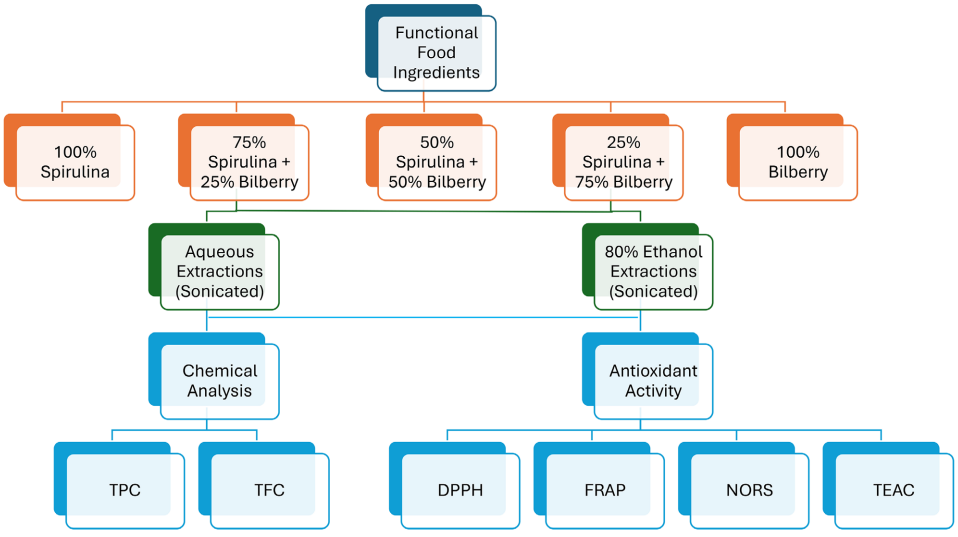
Nitric Oxide Radical Scavenging Ability (NORS) assay was used to determine the scavenging activity of the Spirulina and Bilberry extracts against the nitric oxide free radical [19]. Using Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dichloride) and ascorbic acid as the standard, diluted extracts were combined with the ascorbic acid and 10 mM sodium nitroprusside and incubated at 25˚C for 150 minutes. Afterwards, Griess reagent was added to the samples to be read at 546 nm [20].

4) Trolox Equivalent Antioxidative Capacity (TEAC)

Trolox Equivalent Antioxidative Capacity (TEAC) assay evaluates the capacity of radicals, such as ABTS (2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radicals, which can be scavenged by an antioxidative [21]. During the procedure, diluted samples were added with ethanol diluted ABTS radical stock solution (ABTS radical and potassium persulphate—0.7 ± 0.025 absorbance at 734 nm) and read at an absorbance of 734 nm at 1-minute intervals for 6 minutes [22].

2.3. Statistical Analysis

The experimental design for the objective is a 5 × 2 factorial, with 5 ingredient combinations (100% Spirulina, 100% Bilberry, 50% Spirulina + 50% Bilberry, 75% Spirulina + 25% Bilberry, and 25% Spirulina + 75% Bilberry) and 2 extraction solvents (aqueous and ethanol), which can be seen in **Figure 3**. All experiments were conducted in triplicates and the data were represented as means ± standard error of mean utilizing Proc Glimmix, a one-way ANOVA, and a Tukey's studentized range ($p \leq 0.05$) to determine significant differences in SAS 9.4 tool.



a. (TPC: Total Phenolic Content, TFC: Total Flavonoid Content, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, FRAP: Ferric Reducing Antioxidant Potential, TEAC: Trolox Equivalent Antioxidative Capacity, NORS: Nitric Oxide Radical Scavenging Ability).

Figure 3. Chemical analysis and antioxidative activity experimental design.

3. Results

All the assays conducted compared 100% Spirulina (100S) and 100% Bilberry (100B) to the following combination samples: 50% Spirulina + 50% Bilberry (50S + 50B), 75% Spirulina + 25% Bilberry (75S + 25B), and 25% Spirulina + 75% Bilberry (25S + 75B).

3.1. Total Phenolic Content (TPC)

Phenolic compounds are natural substances characterized by an aromatic group containing one or more hydroxyl groups, which are major contributors to the antioxidative capacity of fruits and vegetables [23] [24]. The TPC assay utilizes Folin-Ciocalteu reagent, which has an intense yellow color until it interacts with phenolic compounds. The phenolic proton dissociates, forming a phenolate ion, which reduces the Folin-Ciocalteu reagent from a yellow color to a blue color [23]. The phenolic content was read for its absorbance at 750 nm [13]. **Table 1** shows the total phenolic content (TPC) of Spirulina and Bilberry (pure and combination) utilizing ethanol (ET) and aqueous/water (AQ) extraction solvents.

Table 1. Total phenolic content (TPC) of Spirulina and Bilberry extracts (80% ethanol & aqueous).

Spirulina and Bilberry combinations	TPC (mg G.A.E./100g DW)	
	80% ethanol extracts	Aqueous extracts
100% Spirulina (100S)	434.27 ± 15.12^{ey}	1556.69 ± 237.94^{bx}
100% Bilberry (100B)	1987.57 ± 40.76^{ax}	1526.55 ± 118.88^{by}
50% Spirulina + 50% Bilberry (50S + 50B)	1403.77 ± 41.18^{cx}	1196.61 ± 46.70^{dy}
75% Spirulina + 25% Bilberry (75S + 25B)	1001.51 ± 49.60^{dy}	1400.00 ± 180.82^{cx}
25% Spirulina + 75% Bilberry (25S + 75B)	1777.40 ± 56.56^{by}	1900.94 ± 582.06^{ax}

a. G.A.E.: Gallic acid equivalent; DW: Dry weight. Means ± standard error (n = 3). Significant differences ($p \leq 0.05$) of Spirulina and Bilberry combinations, shown in columns, indicated by letters "abc". Significant differences ($p \leq 0.05$) of extraction solvents, shown in rows, indicated by letters "xy".

3.1.1. Comparing Total Phenolic Content of Spirulina (S) and Bilberry (B) Based on Pure and Combination Extracts

The TPC of 100S ET (434.27 ± 15.12 mg G.A.E./100g DW) was significantly ($p \leq 0.05$) lower compared to the various ET combinations and 100B ET extract (1987.57 ± 40.76 mg G.A.E./100g DW). The TPC of selected combinations ranged from a low of 1001.51 ± 49.60 mg G.A.E./100g DW (75S + 25B) to a high of 1777.40 ± 56.56 (25S + 75B). Bilberry and Spirulina combined at equal concentrations (50S + 50B) (1403.77 ± 41.18 mg G.A.E./100g DW) had a significantly higher ($p \leq 0.05$) TPC value compared to the 100S. The TPC of 100B ET (1987.57 ± 40.76 mg G.A.E./100g DW) was about 4.5 folds higher than 100S ET, which contributed to the higher TPC in the combination samples ranging from 2.5 to 4.5 folds higher than 100S ET alone.

TPC of AQ 100S extract (1556.69 ± 237.94 mg G.A.E./100g DW) was higher compared to the 100B (1526.55 ± 118.88). However, the highest TPC was found

in the AQ extracts of 25S + 75B samples (1900.94 ± 582.06). The lowest phenolic content was found in the combination samples, *i.e.*, 50S + 50B (1196.61 ± 46.70 mg G.A.E./100g DW), and 75S + 25B AQ extract (1185.31 ± 17.55 mg G.A.E./100g DW). Overall, with higher Bilberry concentrations, the TPC values were higher.

3.1.2. Comparing Total Phenolic Content of Spirulina and Bilberry Based on Solvent Extraction

The TPC of Spirulina and Bilberry, pure and combination samples, varied between each solvent (ET and AQ). The TPC (mg G.A.E./100g DW) of ET 100S was significantly ($p \leq 0.05$) lower than that of the AQ extract. However, 100B AQ (1526.55 ± 118.88 mg G.A.E./100g DW) sample presented a 23% lower TPC value compared to 100B ET extract (1987.57 ± 40.76 mg G.A.E./100g DW). Comparing between combinations, 75S + 25B and 25S + 75B had 28% and 6% higher TPC values in their AE extracts compared to AQ. However, for the 50S + 50B combination samples, ET had 14.76% higher TPC than AQ extracts. Overall, the AQ extracts showed higher TPC values compared to the ET Spirulina and Bilberry (pure and combination) extracts (except 100B ET), suggesting that phytochemicals in these functional ingredients may be more hydrophilic than lipophilic.

These results indicate that Spirulina and Bilberry (pure and combination) extracts had higher extractability of total phenolic contents in water compared to ET. Similar results were found in the Agustiar *et al.*, 2022 study where AQ Spirulina extracts (47.41 ± 3.78 mg of GAE·g-1 DW cell) were higher than the ET extracts (19.79 ± 2.62) [25]. However, these findings contradict the findings of Vrancheva *et al.*, 2020, as the Bilberry extracts showed higher total phenolics in the ET (70.98 ± 0.35) extracts rather than the AQ (65.04 ± 0.10) [26]. The results of the two studies indicate that Bilberry has a higher total phenolic content than Spirulina. The differences in phenolic content outcomes may be influenced by variety, soil, cultivation, and extraction methods.

3.1.3. Phenolics Present in Spirulina and Bilberry

Using high performance lipid chromatography (HPLC) analysis, Guldas *et al.*, 2020 discovered the following phenolic compounds present in Spirulina: acacetin (35.37 ± 0.91 μg/100g), pinocembrin (27.23 ± 0.23 μg/100g), sakuranetin (0.78 ± 0.09 μg/100g), luteolin (0.68 ± 0.09 μg/100g), kaempherol (0.53 ± 0.04 μg/100g), methylquercetin (0.47 ± 0.07 μg/100g), quercetin (0.26 ± 0.04 μg/100g), apigenin (0.25 ± 0.06 μg/100g), and gallic acid (0.03 ± 0.01 μg/100g) (Mean values ± SD (n = 3) [27]. The phenolic compounds present in Bilberry were determined in a study done by *et al.* include the following: Gallic acid derivative (569.00 ± 3.00 mg/100g DW), 5-caffeoylquinic acid (58.00 ± 6.00 mg/100g DW), caffeoyl hexoside (18.00 ± 6.00 mg/100g DW), quinic acid (8.00 ± 3.00 mg/100g DW), and gallic acid (0.83 ± 0.06 mg/100g DW) [28].

3.2. Total Flavonoid Content (TFC)

Flavonoids are major phenolic contents found in foods such as fruits and vegetables. These natural compounds are composed of two benzene rings with a hydroxyl group

(A and B rings) linked by carbon atoms [29]. The total flavonoid content (TFC) was determined utilizing the phytochemical, catechin, as the standard. The compounds were read at an absorbance of 510 nm [15]. The TFC of the Spirulina and Bilberry extracts are shown in Table 2.

Table 2. Total flavonoid content (TFC) of Spirulina and Bilberry extracts (80% ethanol & aqueous).

Spirulina and Bilberry combinations	TFC (mg C.E./100g DW)	
	80% ethanol extracts	Aqueous extracts
100% Spirulina (100S)	8.31 ± 1.56^{ey}	10.48 ± 2.47^{cx}
100% Bilberry (100B)	41.41 ± 3.53^{ax}	10.19 ± 1.21^{cy}
50% Spirulina + 50% Bilberry (50S + 50B)	17.44 ± 1.83^{cx}	15.68 ± 1.60^{ay}
75% Spirulina + 25% Bilberry (75S + 25B)	14.27 ± 0.91^{dx}	13.66 ± 4.65^{by}
25% Spirulina + 75% Bilberry (25S + 75B)	24.95 ± 2.36^{bx}	15.18 ± 0.73^{ay}

*C.E.: Catechin equivalent; DW: Dry weight. Means ± standard error (n = 3). Significant differences ($p \leq 0.05$) of Spirulina and Bilberry combinations, shown in columns, indicated by letters "abc". Significant differences ($p \leq 0.05$) of extraction solvents, shown in rows, indicated by letters "xy".

3.2.1. Comparing Total Flavonoid Content of Spirulina and Bilberry Based on Pure and Combination Extracts

The TFC of 100S ET extract (8.31 ± 1.56 mg C.E./100g DW) had the lowest flavonoid content compared to the 100B ET (41.41 ± 3.53 mg C.E./100g DW) and combination extracts (14.27 - 24.95 mg C.E./100g DW). Among combinations, 25S + 75B ET extract (24.95 ± 2.36 mg C.E./100g DW) had the highest TFC value compared to its counterparts.

Under polar extraction conditions, 100S AQ (10.48 ± 2.47 mg C.E./100g DW) extracts had a higher TFC value compared to 100B AQ (10.19 ± 1.21 mg C.E./100g DW). However, the highest TFC values were actually seen in the combination samples from 13.66 ± 4.65 (75S+25B) to 15.68 ± 1.60 (50S + 50B). A synergistic effect can be seen in all, with a 10.09% and 39.16% higher TFC with higher Bilberry concentrations (50S + 50B and 25S + 75B respectively) than 75S + 25B sample extracts.

3.2.2. Comparing Total Flavonoid Content of Spirulina and Bilberry Based on Solvent Extraction

The TFC of Spirulina and Bilberry extracts varied between different solvents. TFC of 100S AQ (10.48 ± 2.47 mg C.E./100g DW) TFC value was higher than that of the ET extract (8.31 ± 1.56 mg C.E./100g DW). However, 100B had significantly ($p \leq 0.05$) higher flavonoid content in its ET extract (41.41 ± 3.53 mg C.E./100g DW) than AQ (10.19 ± 1.21 mg C.E./100g DW) (almost 4 folds higher). For the combination samples, 50S + 50B AQ (15.68 ± 1.60 mg C.E./100g DW) was significantly ($p \leq 0.05$) lower than ET (17.44 ± 1.83). A significantly ($p \leq 0.05$) higher TFC was found in the 75S + 25B ET (14.27 ± 0.91 mg C.E./100g DW) compared to the AQ extract (13.66 ± 4.65 mg C.E./100g DW). Similar results were found between the 25S + 75B ET (24.95 ± 2.36 mg C.E./100g DW) and AQ (15.18 ± 0.73 mg C.E./100g DW) samples. Overall, in a nonpolar solution, ethanol, higher total

flavonoids were able to be extracted from the Spirulina and Bilberry (pure and combination) samples compared to water.

Using quercetin as the standard, results from the Agustiar *et al.*, 2022 supported the findings of this study as they found similar results that ethanolic extracts of Spirulina (25.28 ± 1.35) showed higher TFC compared to the water extracts (8.27 ± 2.36) [25]. Similar results were repeated by Vrancheva *et al.*, 2020 as ethanolic Bilberry extracts (34.96 ± 0.17) were greater than the AQ extracts (24.64 ± 0.06) [26].

3.2.3. Flavonoids Present in Spirulina and Bilberry

Flavonoids present in Bilberry include anthocyanins and flavonols. The anthocyanins found in a study done by Stanoeva *et al.*, 2017 includes: petunidin-3-O-glucoside (512.00 ± 0.60 mg/100g DW), delphinidin-3-O-glucoside (422.00 ± 25.00 mg/100g DW), delphinidin-3-O-galactoside (397.00 ± 12.00 mg/100g DW), malvidin-3-O-glucoside (371.00 ± 6.00 mg/100g DW), malvidin-3-O-galactoside (342.40 ± 0.06 mg/100g DW), and cyanidin-3-O-glucoside (314.00 ± 13.00 mg/100g DW) [28]. The flavonols present include quercetin-3-O-glucoside (38.00 ± 6.00 mg/100g DW), quercetin-3-O-rutinoside (rutin) (14.10 ± 0.06 mg/100g DW), laricitrin-3-O-galactoside (6.90 ± 0.01 mg/100g DW), and myricetin (3.30 ± 0.30 mg/100g DW) (Stanoeva *et al.*, 2017). Seghiri *et al.*, 2019 found the following flavonoids present in Spirulina: catechin (584.53 ± 29.22 mg/kg), rutin (0.93 ± 0.05 mg/kg), and quercetin (0.01 ± 0.05 mg/kg) [30].

3.3. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Antioxidative Activity

DPPH radical has an intense purple color due to its delocalization in its aromatic rings. During the process of the DPPH radical, the deep purple color turns into a pale yellow when the radical accepts an electron from the selected antioxidative, neutralizing the radical. An absorbance of 517nm was utilized in the radical scavenging assay [17]. **Table 3** shows the DPPH antioxidative activity of Spirulina and Bilberry (pure and combination) utilizing ethanol (ET) and aqueous/water (AQ) extraction solvents.

Table 3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) % inhibition by Spirulina and Bilberry extracts (80% ethanol & aqueous).

Spirulina and Bilberry combinations	DPPH % inhibition (μg/ml)	
	80% ethanol extracts	Aqueous extracts
100% Spirulina (100S)	66.93 ± 15.85^{bx}	19.98 ± 5.98^{cy}
100% Bilberry (100B)	95.01 ± 1.13^{ax}	59.71 ± 6.75^{ay}
50% Spirulina + 50% Bilberry (50S + 50B)	88.66 ± 8.07^{ax}	36.07 ± 3.45^{by}
75% Spirulina + 25% Bilberry (75S + 25B)	81.56 ± 11.64^{ax}	24.22 ± 2.14^{by}
25% Spirulina + 75% Bilberry (25S + 75B)	93.88 ± 2.59^{ax}	31.47± 2.42^{by}

a. Means ± standard error (n = 3)—80% Ethanol (n = 3). Significant differences ($p \leq 0.05$) of Spirulina and Bilberry combinations, shown in columns, indicated by letters "abc". Significant differences ($p \leq 0.05$) of extraction solvents, shown in rows, indicated by letters "xy".

3.3.1. Comparing DPPH% Inhibition by Spirulina and Bilberry Based on Pure and Combination Extracts

There were no significant ($p \leq 0.05$) differences in the inhibition of DPPH radical by ET extracts, 100B (95.01 ± 1.13) and combinations (50S + 50B: 88.66% ± 8.07, 75S + 25B: 81.56% ± 11.64 and 25S + 75B: 93.88% ± 2.59). However, there was a significant difference comparing 100S ET (66.93% ± 15.85) to its counterparts. Similarly, 100B AQ (59.71% ± 6.75) had a significantly ($p \leq 0.05$) higher inhibition compared to 100S (19.98% ± 5.98). There were no significant ($p \leq 0.05$) differences found in the % DPPH inhibition between the AQ extracts of three Spirulina and Bilberry combinations (50S+50B: 36.07% ± 3.45, 75S+25B: 24.22% ± 2.14, & 25S+75B: 31.47% ± 2.42).

3.3.2. Comparing DPPH% Inhibition by Spirulina and Bilberry Based on Solvent Extraction

DPPH inhibition was observed to be lower in the AQ extracts compared to ET extracts with the lowest inhibition (%) seen in 100S samples (19.98% - 59.71%). There was a 3.35-fold reduction of % DPPH inhibition by 100S AQ (19.98% ± 5.98) compared to its ET extract (66.93% ± 15.85), while between the ET (95.01% ± 1.13) and AQ extracts (59.71 ± 6.75) of 100B, there was only a difference of 1.59 folds. The ET combination extracts were significantly ($p \leq 0.05$) higher than its AQ counterparts, where ET ranged from 81.56% to 93.88%, and AQ 24.22% to 36.07%. Apart from the AQ 100S, 50S + 50B, 75S + 25B, and 25S + 75B, all other extracts had at least a 50% inhibition of DPPH which shows antioxidative activity of Spirulina and Bilberry samples (pure and combination). Similar results were found in a recent study utilizing water, 80% ethanol, 80% methanol, and 80% acetone, where the ethanolic extracts (55.89 ± 0.35) showed higher DPPH % inhibition compared to water (14.02 ± 0.38) [31].

Table 4. Ferric reducing antioxidative potential (FRAP) of Spirulina and Bilberry extracts (80% ethanol & aqueous).

Spirulina and Bilberry combinations	FRAP (mM Fe (II)/100g DW)	
	80% ethanol extracts	Aqueous extracts
100% Spirulina (100S)	31.85 ± 1.44^{dy}	47.07 ± 1.12^{cx}
100% Bilberry (100B)	229.48 ± 37.51^{ax}	113.51 ± 0.70^{ay}
50% Spirulina + 50% Bilberry (50S + 50B)	132.50 ± 10.96^{bx}	47.70 ± 18.91^{cy}
75% Spirulina + 25% Bilberry (75S + 25B)	82.57 ± 2.42^{cx}	69.69 ± 4.25^{bcy}
25% Spirulina + 75% Bilberry (25S + 75B)	176.32 ± 7.66^{abx}	95.72 ± 7.72^{aby}

*Fe (II)—Ferric iron; DW—Dry weight. Means ± standard error (n = 3). Significant differences ($p \leq 0.05$) of Spirulina and Bilberry combinations, shown in columns, indicated by letters "abc". Significant differences ($p \leq 0.05$) of extraction solvents, shown in rows, indicated by letters "xy".

3.4. Ferric Reducing Antioxidative Potential (FRAP)

Ferric reducing antioxidative potential (FRAP) assay is based upon the reduction of ferric-tripyridyl triazine $[FeIII(TPTZ)]^{3+}$ to ferrous complex $[FeII(TPTZ)]^{2+}$

caused by the present antioxidative, which develops a dark blue color [24]. The FRAP of the Spirulina and Bilberry extracts is shown in **Table 4**. Significant differences ($p \leq 0.05$) were found between the different sample combinations and extraction solvents.

3.4.1. Comparing FRAP of Spirulina and Bilberry Based on Pure and Combination Extracts

The 100B ET extract (229.48 ± 37.51 mM Fe (II)/100g DW) had a significantly ($p \leq 0.05$) higher FRAP compared to the 100S ET extract (31.85 ± 1.44 mM Fe (II)/100g DW). Comparing the combination samples, 25S + 75B ET (176.32 ± 7.66 mM Fe (II)/100g DW) had the highest FRAP value, followed by 50S + 50B ET (132.50 ± 10.96 mM Fe (II)/100g DW) and 75S + 25B ET (82.57 ± 2.42 mM Fe (II)/100g DW).

Similar results were found for the AQ extracts where 100B (113.51 ± 0.70 mM Fe (II)/100g DW) had a significantly ($p \leq 0.05$) higher FRAP in comparison to 100S (47.07 ± 1.12 mM Fe (II)/100g DW). The 25S+75B AQ extract (95.72 ± 7.72 mM Fe (II)/100g DW) showed significantly ($p \leq 0.05$) higher FRAP compared to the 50S + 50B (47.70 ± 18.91 mM Fe (II)/100g DW) and 75S + 25B (69.69 ± 4.25 mM Fe (II)/100g DW) extracts. FRAP of AQ 100S (47.07 ± 1.12 mM Fe (II)/100g DW) and 50S + 50B (47.70 ± 18.91 mM Fe (II)/100g DW) were similar; however, they were significantly ($p \leq 0.05$) lower compared to the other extracts (pure and combination). Overall, FRAP ranged from a low of 31.85 ± 1.44 to a high of 229.48 ± 37.51 in ET extracts, and 47.07 ± 1.12 to 113.51 ± 0.70 in AQ.

3.4.2. Comparing FRAP of Spirulina and Bilberry Based on Solvent Extraction

Significant differences were found within the Spirulina and Bilberry (pure and combination) ET and AQ extracts. FRAP of 100S AQ extract (47.07 ± 1.12 mM Fe (II)/100g DW) was significantly ($p \leq 0.05$) higher compared to the 100S ET (31.85 ± 1.44 mM Fe (II)/100g DW), while the 100B ET was significantly ($p \leq 0.05$) higher compared to the 100B AQ extract. The combination samples show a trend similar to the100B extracts where FRAP values were higher in the ET compared to the AQ extracts. Overall, samples higher in Bilberry and those extracted in 80% ET had the highest FRAP values and were significantly ($p \leq 0.05$) higher compared to the AQ.

3.5. Nitric Oxide Radical Scavenging (NORS) Ability

Nitric oxide (NO) is a free radical developed from the L-arginine in vascular endothelial cells. The nitric oxide radical scavenging (NORS) assay involves the interaction between nitric oxide radical with sodium nitroprusside. In the presence of an antioxidative, the formation of NO^{3-} and NO^{2-} will not occur [24]. The NORS ability of the Spirulina and Bilberry extracts are shown in **Table 5**. Significant differences ($p \leq 0.05$) were found between the different sample combinations and extraction solvents.

Table 5. Nitric oxide radical scavenging ability (NORS) of Spirulina and Bilberry extracts (80% ethanol & aqueous).

Spirulina and Bilberry combinations	NORS (mM NO/100g DW)	
	80% ethanol extracts	Aqueous extracts
100% Spirulina (100S)	13.03 ± 2.49^{cy}	21.61 ± 1.42^{ax}
100% Bilberry (100B)	28.41 ± 3.57^{ax}	11.35 ± 2.02^{by}
50% Spirulina + 50% Bilberry (50S + 50B)	14.42 ± 0.99^{cy}	20.48 ± 0.75^{ax}
75% Spirulina + 25% Bilberry (75S + 25B)	12.88 ± 1.50^{cy}	26.52 ± 8.85^{ax}
25% Spirulina + 75% Bilberry (25S + 75B)	21.08 ± 1.87^{bx}	20.95 ± 5.22^{ax}

a. *NO—Nitric oxide; DW—Dry weight. Means ± standard error (n = 3). Significant differences ($p \leq 0.05$) of Spirulina and Bilberry combinations, shown in columns, indicated by letters "abc". Significant differences ($p \leq 0.05$) of extraction solvents, shown in rows, indicated by letters "xy".

3.5.1. Comparing NORS of Spirulina and Bilberry Based on Pure and Combination Extracts

Within the ET extracts, 100B (28.41 ± 3.57 mM NO/100g DW) showed significantly ($p \leq 0.05$) higher NORS value compared to 100S (13.03 ± 2.49 mM NO/100g DW) and the combination samples. The highest NORS by the combination samples was seen by the 25S + 75B extracts (21.08 ± 1.87 mM NO/100g DW), and the lowest being 75S + 25B (12.88 ± 1.50 mM NO/100g DW), suggesting that the NORS values decreased with increase in the amount of Spirulina. An opposite trend was seen in AQ extracts, where 100B AQ (11.35 ± 2.02 mM NO/100g DW) had a significantly ($p \leq 0.05$) lower NORS value compared to 100S (21.61 ± 1.42 mM NO/100g DW) and the combination samples. NORS of combination samples ranged from a low of 20.48 ± 0.75 (50S + 50B) extract to a high of 26.52 ± 8.85 (75S + 25B).

3.5.2. Comparing NORS of Spirulina and Bilberry Based on Solvent Extraction

100S AQ extract (21.61 ± 1.42 mM NO/100g DW) had a significantly ($p \leq 0.05$) higher NORS value compared to ET extract (13.03 ± 2.49 mM NO/100g DW). However, the NORS value in the 100B ET (28.41 ± 3.57 mM NO/100g DW) was significantly ($p \leq 0.05$) higher than the 100B AQ extract (11.35 ± 2.02 mM NO/100g DW). AQ 50S + 50B (20.48 ± 0.75) and 75S + 25B (26.52 ± 8.85 mM NO/100g DW) had higher radical scavenging ability compared to the ET extracts (12.88 ± 1.50 - 14.42 ± 0.99 mM NO/100g DW), whereas the 25S + 75B combination scavenging ability was higher in the ET (21.08 ± 1.87 mM NO/100g DW) than AQ (20.95 ± 5.22 mM NO/100g DW); however, there were no significant differences between the two. Overall, extracts containing higher concentrations (%) of Spirulina content (AQ) and higher Bilberry (ET) had greater scavenging activity. 25S + 75B had a 1.46 to 1.64 higher NORS compared to the ET samples, except 100B ET. Similarly, the AQ 75S + 25B was 1.27 to 2.34-fold higher compared to the other AQ samples, suggesting a synergistic effect between Spirulina and Bilberry.

Among AQ, there were no significant differences in NORS among 100S (21.61

± 1.42 mM NO/100g DW), 50S + 50B (20.48 ± 0.75 mM NO/100g DW), 75S + 25B (26.52 ± 8.85 mM NO/100g DW), and 25S + 75B (20.95 ± 5.22 mM NO/100g DW); however, the lowest NORS was seen in 100B AQ (11.35 ± 2.02 mM NO/100g DW). Comparing the extracts (ET & AQ), all pure and combination samples were significantly ($p \leq 0.05$) different with the exception of 25S + 75B, where NORS of AQ and ET were not significantly different.

3.6. Trolox Equivalent Antioxidative Capacity (TEAC)

The Trolox Equivalent Antioxidative Capacity (TEAC) assay involves a blue-green ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) solution in its unstable form. In the presence of an antioxidative, the ABTS will accept an electron from the antioxidative, turning the blue-green color to a pale blue color [24]. The TEAC of the Spirulina and Bilberry extracts are shown in Table 6. Significant differences ($p \leq 0.05$) were found between the different sample combinations and extraction solvents.

Table 6. Trolox equivalent antioxidative capacity (TEAC) of Spirulina and Bilberry extracts (80% ethanol & aqueous).

Spirulina and Bilberry combinations	TEAC (MM T.E./100g DW)	
	80% ethanol extracts	Aqueous extracts
100% Spirulina (100S)	148.68 ± 13.22^{ax}	137.81 ± 5.05^{abx}
100% Bilberry (100B)	19.41 ± 0.16^{ey}	86.82 ± 9.12^{cx}
50% Spirulina + 50% Bilberry (50S + 50B)	66.35 ± 0.64^{cy}	114.59 ± 10.99^{bx}
75% Spirulina + 25% Bilberry (75S + 25B)	120.64 ± 8.11^{bx}	143.62 ± 2.57^{ax}
25% Spirulina + 75% Bilberry (25S + 75B)	38.76 ± 3.50^{dy}	104.20 ± 6.53^{bcx}

a. T.E.—Trolox equivalent; DW—Dry weight. Means ± standard error (n = 3). Significant differences ($p \leq 0.05$) of Spirulina and Bilberry combinations, shown in columns, indicated by letters "abc". Significant differences ($p \leq 0.05$) of extraction solvents, shown in rows, indicated by letters "xy".

3.6.1. Comparing TEAC of Spirulina and Bilberry Based on Pure and Combination Extracts

The TEAC of 100S (148.68 ± 13.22 MM T.E./100g DW) extract was significantly ($p \leq 0.05$) higher than the 100B (19.41 ± 0.16 MM T.E./100g DW) and combination samples in ET. 75S + 25B (120.64 ± 8.11 MM T.E./100g DW) extract had a significantly ($p \leq 0.05$) higher TEAC value within the combination samples, illustrating an increase in TEAC value when Spirulina increases in a nonpolar extraction solution. TEAC values of Spirulina in ET samples contributed to the majority of the antioxidative potential suggesting higher presence of lipophilic phytochemicals.

Similar results were seen within the AQ results of the pure and combination samples of Spirulina and Bilberry. 100S (137.81 ± 5.05 MM T.E./100g DW) had a significantly ($p \leq 0.05$) higher TEAC value than the 100B (86.82 ± 9.12 MM T.E./100g DW) extract. 25S + 75B (104.20 ± 6.53 MM T.E./100g DW) combination had a significantly ($p \leq 0.05$) lower TEAC value compared to the 75S + 25B

(143.62 ± 2.57 MM T.E./100g DW) sample.

3.6.2. Comparing TEAC of Spirulina and Bilberry Based on Solvent Extraction

100S ET extract (148.68 ± 13.22 MM T.E./100g DW) had a higher TEAC value than that of the AQ solution (137.81 ± 5.05 MM T.E./100g DW). However, 100B ET TEAC value (19.41 ± 0.16 MM T.E./100g DW) was significantly ($p \leq 0.05$) lower compared to the 100B AQ sample (86.82 ± 9.12 MM T.E./100g DW). For the combination samples of Spirulina and Bilberry, higher TEAC was seen in the AQ samples, suggesting that both Spirulina and Bilberry (pure and combination) have higher extractability of antioxidative phytochemicals in a polar solvent. Higher TEAC was observed in samples that had higher concentrations of Spirulina (AQ and ET solvents), indicating that Spirulina has higher antioxidative potential. The reason for this could be its high vitamin E content, whereas Bilberry has a higher vitamin C content. However, the correlation between vitamin E and vitamin C, explains why the Bilberry combination samples still had a fairly high TEAC in the AQ extract.

3.7. Correlation Between TPC, TFC, and Antioxidative Assays

Comparing the correlation between total phenolic, total flavonoid, and antioxidative assays is important to see if there were any relationships between the chemical and antioxidative assays between the two extraction solvents: water and ethanol.

3.7.1. Correlation Between TPC, TFC, and Antioxidative Assays in 80% Ethanol Extracts

Between TPC, TFC, and antioxidative assays in ethanol (ET) solvents, there was a positive correlation coefficient (≥0.90) between TPC and TFC (0.90), meaning that when TPC values increase, TFC values will increase as well. Positive correlations were also found between TPC and FRAP (0.99), TFC and FRAP (0.95), TFC and NORS (0.97), and NORS and FRAP (0.91). Overall, the majority correlation was found in TFC with antioxidative assays.

3.7.2. Correlation Between TPC, TFC, and Antioxidative Assays in Aqueous Extracts

Between TPC, TFC, and antioxidative assays in aqueous (AQ) solvents, there were no positive correlation coefficient (≥ 0.90) between assays, suggesting that in polar solvents, the bioactive compounds present in Spirulina and Bilberry did not correlate between chemical and antioxidative assays.

4. Discussion

Utilizing 80% ethanol and aqueous extracts in the chemical ana antioxidative assays resulted in different findings for each pure and combination sample of Spirulina and Bilberry. Findings indicated that 80% of ethanol extracts showed higher levels of total flavonoids, FRAP, and DPPH activities, while aqueous extracts exhibited higher levels of total phenolics, NORS, and TEAC. This suggests the

presence of both hydrophilic and lipophilic bioactive compounds in Spirulina and Bilberry extracts. Bilberry-rich extracts (100B and 25S + 75B) showed greater antioxidative activities, highlighting synergistic effects when combining Spirulina and Bilberry for antioxidative activities.

5. Conclusions

Spirulina and Bilberry are two ingredients that can be used and introduced into the food industry as potential functional food ingredients due to their antioxidative and anti-obesity properties. Spirulina, a blue-green algae known for its high protein content, has been deemed a "Food of the Future" due to its protein content ranging between 60% - 70% dry weight, and natural blue and green pigments, which gives it the potential to be used as an alternative protein or natural color additive in the food industry [32]. Bilberry, native to northern parts of the United States, Europe and Canada, is a dark berry commonly mistaken for blueberry. Previously used for medicinal purposes, such as in diabetes and scurvy, Bilberry has the potential to be used as a natural color additive in the food industry [33]. Bilberry, a dark berry, commonly mistaken for the well-known blueberry, is primarily native to Europe, as well as the northern parts of the United States. It is one of the richest natural sources of anthocyanins, and it has previously been used for many medicinal purposes [33]. Through limited research, studies suggest that the berry has many health benefits, such as improving vision due to its nutritional components and bioactive compounds, making it deemed the term a "superfood."

In the study, the phytochemical composition of Spirulina and Bilberry combinations was determined utilizing total phenolic and total flavonoid assays with ethanol and water extraction solvents. Antioxidative properties were evaluated using DPPH, FRAP, NORS, and TEAC assays. Findings indicated that both Spirulina and Bilberry, were pure and in combination samples. exhibited antioxidative activities due to their phenolic and flavonoid contents, which is the main contributor to a food ingredient possessing antioxidative capacity. The purpose of the combinations was to determine if there were any synergistic effects between both ingredients, which was determined in the study. Overall, the best combination samples were the 25%S + 75%B, suggesting that small amounts of Spirulina were able to increase the rich antioxidative properties of Bilberry, which shows great evidence for the use of Spirulina and Bilberry as functional food ingredients in the food industry.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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