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Original Research

LC-ESI-QTOF-MS^2 Characterization of Phenolic Compounds in Different Lentil (*Lens culinaris* M.) Samples and Their Antioxidant Capacity

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Abstract

Background: Lentil (*Lens culinaris* M.) is a legume widely consumed worldwide. It is rich in bioactive compounds, including polyphenolic compounds that contribute to positive health benefits. **Methods**: This study aimed to determine the phenolic content and antioxidant activity of black, red, green, and brown whole lentils. Towards this end, the lentils' phenolic compounds were evaluated regarding their total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), total condensed tannin (TCT), total proanthocyanin content (TPAC), total anthocyanin content (TAC). For the antioxidant activity 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), hydroxyl radical scavenging activity (•OH-RSA), ferrous ion chelating activity (FICA), reducing power assay (RPA) and phosphomolybdate (PMA) assay were accessed. To identify individual phenolic compounds, liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS^2) was used. **Results**: The results showed that green lentils exhibited the highest TPC (0.96 mg gallic acid equivalents (GAE)/g) whereas red lentils presented the highest TFC (0.06 mg quercetin equivalents (QE)/g). Black lentils were noted with the highest TCT (0.03 mg catechin equivalents (CE)/g), TPAC (0.009 mg cyanidin chloride equivalents (CCE)/g), and TAC (3.32 mg/100 g) contents. While the greatest TTC (2.05 mg tannic acid equivalents (TAE)/g) was observed in the brown lentil. Regarding the total antioxidant capacity, red lentils (4.01 mg ascorbic acid equivalents (AAE)/g) presented the greatest activity, whereas the lowest was found in the brown samples (2.31 mg AAE/g). The LC-ESI-QTOF-MS^2 tentatively identified a total of 22 phenolic compounds, containing 6 phenolic acids, 13 flavonoids, 2 lignans, and 1 other polyphenol. The relationships among phenolic compounds by Venn Diagram showed a high number of overlapping compounds in brown and red lentils (6.7%), and a low number of overlapping compounds between the green, brown, and black lentils (2.6%). Flavonoids were the most abundant phenolic compound within the studied whole lentils, with the brown lentils being the richest in phenolic compounds, especially flavonoids. **Conclusions**: This study emphasized a comprehensive understanding of the antioxidant potential of lentils and disclosed the phenolic distribution across various lentil samples. This may increase interest in the development of functional food products, nutraceutical ingredients, and pharmaceutical applications with lentils.

Keywords: lentil; phenolic compound; flavonoid; antioxidant activity; saponin; LC-ESI-QTOF-MS^2

1. Introduction

Lentil (*Lens culinaris* Medikus) is an edible pulse, cultivated in 52 countries and recognized among the main cold season legume in the world [1]. Lentils are globally commercialized on a wild scale, and its leading producers are Canada, India, Australia, Turkey, Nepal, and the USA, accounting for over 80% of the world's total lentil production [2]. The cultivated lentil is a diploid, self-pollinating, and annual legume [3]. The taxonomy of *L. culinaris* presents four subspecies: subsp. *culinaris*; *orientalis*, *odemensis*, and *tomentosus* [3]. These pulses are also known as a significant source of dietary protein in developing nations [4]. Lentils exist as a spectrum of colors, including black, brown, green, red, orange or yellow, depending on the composition of the cultivar, seed coat, along with cotyledon [5]. In the past, lentil was named "poor man's meat", emerging in ancient Europe. Thereby, people have considered it a cheap and excellent substitute for animal protein for a long time, as it contains 24.63 g/100 g of protein, and is a potential overall source of nutrient for individuals with deficiencies in micronutrients [6,7]. Additionally, enriched phenolic compounds are also detected in various types of lentils [5].

Polyphenols are secondary compounds extensively distributed in the plant kingdom [8]. It is characterized by the presence of several phenolic groups, that is, aromatic rings with hydroxyl groups. Polyphenols can be

classified as several classes, i.e., hydroxycinnamic acids, hydroxybenzoic acids, anthocyanins, proanthocyanidins, flavonols, flavanols, flavones, flavanones, isoflavones, lignans, and stilbenes [9]. Due to the high antioxidant power of polyphenols, they can have different effects, including anti-inflammatory effects, in addition to affecting blood sugar through distinct mechanisms, such as inhibiting glucose absorption in the intestine and improving insulin resistance [10]. Furthermore, various potential mechanisms of polyphenols are responsible for certain disease prevention, including inhibition of bacterial replication enzymes, induction of apoptosis in tumor cells, and stimulation of cytokine production by monocytes/macrophages [11]. Additionally, people who follow diets rich in polyphenols have a low risk for a number of chronic diseases [12]. Thus, polyphenols exhibit strong health potential for the human body.

The high content of phytochemicals in pulse-based diets including polyphenols is related to health benefits [13]. Lentil presents a diverse phenolic profile in which phenolic acids, flavonoids, and lignans are the majority. Phenolic acids are phenols that own one carboxylic acid functional group, which is the major class of phenolic compounds [14,15]. They are usually classified as the derivatives of hydroxy-benzoic acid and hydroxycinnamic acid [14]. These compounds show strong antioxidant activity and have been studied for their potential against oxidative damage [16]. Flavonoids are polyphenolic secondary metabolites commonly linked with a cetone group [17]. These polyphenols stand for one of the major groups of phenols and they are low molecular weight compounds with a broad-spectrum occurrence [16]. Flavonoids are known for their disease preventive activities including antimicrobial, antioxidant, anti-inflammatory, and as inhibitory substances for various stages of tumor development [15,16]. Lignans are secondary metabolites that belong to the group of diphenolic compounds and present a dibenzylbutane skeleton [17]. Lignans are non-flavonoid compounds, that became to be broadly investigated [15]. They exhibit their potential health benefits through antimicrobial, and anticancer activities [15,16].

Lens culinaris have been used in traditional practices to reduce the prevalence of ailments such as obesity, diabetes, cancers, and cardiovascular diseases. The seed is rich in secondary metabolites and bioactive functional groups, including phytosterols, trypsin/protease inhibitors, lectins, defensins, dietary fibers, polyphenols, flavonoids, phytate, triterpenoids, and saponin [5]. Some phenolic compounds, such as quercetin and populins, which belong to the flavonoid group, were characterized in different lentils and their antioxidant potential was also reported [18]. Moreover, a greater level of flavan-3-ols, proanthocyanidins and some flavonols were noted in the seed coats of lentils [5]. Such active ingredients have potential advantages to be used in alternative medicines, to act as antioxidant, antibacterial, antifungal, antiviral, cardioprotective, anti-inflammatory, reno-protective, antidiabetic, anticancer, anti-obesity, hypolipidemic and chemo-preventive [5]. However, there have been few studies on red, green, brown, and black lentils in terms of phenolic compounds, most studies have compared lentils with other legumes in terms of phenolic compounds [19–23].

In-depth knowledge of the phenolic profile of different types of lentils has not been widely studied, leading to gaps that demand further research on the phenolic profile and antioxidant activity of these pulses. Therefore, this study aimed to carry out the characterization of the phenolic substances and the antioxidant potential of four different types of lentils including red, green, brown, and black lentils. Polyphenolic components were extracted from the lentil samples and studied regarding their TPC, TFC, TTC, TPAC, and TAC overall contents. For the antioxidant potential evaluation, DPPH, FRAP, ABTS, •OH-RSA, FICA, RPA, and PMA were tested. Further, the polyphenolic compounds were screened and characterized by LC-ESI-QTOF-MS^2. The present research will give more credible information on lentils' antioxidant and health-promoting characteristics to optimize their use in the food, supplement, and pharmaceutical industries.

2. Materials and Methods

2.1 Chemical and Regents

The majority of chemicals utilized for extraction, identification, and quantification were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and were of analytical grade, which contain potassium persulfate, aluminum chloride hexahydrate, Folin-Ciocalteu's phenol reagent, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), quercetin, vanillin, catechin, gallic acid monohydrate, and Trolox. The chemicals anhydrous sodium carbonate, 3-hydroxybenzoic acid, sodium acetate, sodium hydroxide, and potassium chloride were obtained from Chem-Supply Pty Ltd. (Adelaide, SA, Australia). Thermo Fisher Scientific Inc (Waltham, MA, USA) supplied acetic acid, ethanol, sulfuric acid, hydrochloric acid, n-butanol, hydrogen peroxide, trisodium phosphate and sodium carbonate. Polyvinylpolyrrolidone, Iron(II) sulfate heptahydrate, Iron(III) chloride, Iron(II) chloride, ethylenediaminetetraacetic acid, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate, tannic acid, escin, cyanidin chloride, ammonium molybdate, sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, potassium ferricyanide(III), and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) as well.

2.2 Sample Preparation

The four types of lentils utilized in this research (red, green, brown, and black) were purchased at Melbourne's local market. All the lentil samples were crushed into fine powders using a grinder (Breville Smart Grinder™ Pro, model BCG820BSSXL, Melbourne, VIC, Australia) and reserved in a dark location at room temperature to prevent exposure to light.

2.3 Extraction of Phenolic Compounds

The extraction process of phenolic compounds followed the study from [24] with slight alterations. Two grams of each lentil sample powder was thoroughly mixed with 70% ethanol (1:10, *w/w*) and homogenized using an Ultra-Turrax T25 Homogenizer (IKA, Staufen, Germany) at 10,000 rpm for 30 s. After that, the mixture was incubated in a ZWYR-240 incubator shaker (Labwit, Ashwood, VIC, Australia) at 120 rpm at 10 °C for 16 hours. Then, the extracts were centrifuged at 8000 rpm for 15 minutes at 4 °C (ROTINA380R, Hettich Refrigerated Centrifuge, Tuttlingen, Baden-Württemberg, Germany) and the supernatant was collected and frozen at –20 °C for subsequent analysis.

2.4 Estimation of Phenolic Compounds and Antioxidant Assays

2.4.1 Total Phenolic Content (TPC)

A modified version of the Folin-Ciocalteu technique was used to access the TPC of lentil samples [25]. Twenty-five microliters of lentil samples were added to 25 μL of Folin-Ciocalteu reagent solution (1:3 dilution with water) in a 96-well plate (Costar, Corning, NY, USA) containing 200 μL of Milli-Q water. The mixture was incubated for 5 minutes at 25 °C and then, 25 μL of sodium carbonate (10%, *w/v*) was added to the reaction mixture. It was held for 60 minutes at 25 °C in the dark. The absorbance at 765 nm was evaluated using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A gallic acid standard curve containing 0–200 μg/mL gallic acid in methanol was prepared. The TPC results were displayed as mg of equivalent gallic acid (GAE) gram of sample.

2.4.2 Total Flavonoid Content (TFC)

The TFC of different lentils was evaluated using the aluminum chloride method modified [26]. Eighty microliters of lentil samples were transferred to 80 μL of aluminum chloride solution (2%, *w/v*), followed by mixing 120 μL sodium acetate (50 g/L) in the 96-well plate, accompanied by a 150-minute incubation at 37 °C. At 440 nm, the absorbance was determined. The quercetin standard curve was constructed utilizing a 0–50 μg/mL quercetin methanolic solution. The TFC values were displayed as mg of quercetin equivalent (QE) per gram of sample.

2.4.3 Total Tannin Content (TTC)

The determination of TTC was measured using a polyvinylpolypyrrolidone (PVPP) method [27]. Total tannin constituents were measured in Eppendorf tubes by transferring 120 μL of the lentil extract, 180 μL of distilled water, 150 μL of Folin–Ciocalteu reagent (50% *v/v*), and 675 μL of sodium carbonate (20% *w/v*). The mixture was vortexed and stored at room temperature for 40 minutes in the dark. Centrifuge was then conducted and 200 μL of the supernatant was added to a 96-well plate. After that, the absorbance was read at 725 nm. Then, a second analysis was carried out to determine the phenolic compounds left after tannins were precipitated with PVPP. Fifty milligrams of PVPP were added, accompanied by the addition of 0.5 mL of water along with 375 μL of sample extract. The mixture was centrifuged at 8000 g for 10 minutes at 4 °C after being vortexed and maintained at 4 °C for 15 minutes. Using the same methods outlined above for total phenolic compounds, the supernatant was kept for Folin-Ciocalteu analysis.

To calculate the tannin content, the values of the first and second experiments were subtracted. A standard curve was prepared using a 0–250 μg/mL Tannic acid solution. The TTC results were displayed as mg of Tannic acid equivalents (TAE) per gram of sample.

2.4.4 Total Condensed Tannins (TCT)

The TCT was evaluated utilizing a vanillin-sulfuric acid method [28]. Twenty-five microliters of lentil samples were transferred to 150 μL of vanillin solution (4%, *w/v*), followed by the addition of twenty-five microliters of 32% sulfuric acid in a 96-well plate along with a 15-minute incubation under 25 °C. At 500 nm, the absorbance was determined. The standard curve was constructed using a 0–1000 μg/mL catechin. The TCT results were shown as mg of equivalent catechin (CE) per gram of sample.

2.4.5 Total Proanthocyanidin Content (TPAC)

The TPAC was measured using a modified method of [29], which relies on the acid-catalyzed oxidative cleavage of proanthocyanidins' C–C interflavanic bond in butanol-HCl. Reagent A was prepared by dissolving 35 milligrams of $FeSO_4 \cdot 7H_2O$ in 2.5 mL of concentrated HCl, then making a solution up to 50 mL with butanol. Briefly, 30 μL of each extract and 800 μL of reagent A were added in Eppendorf tubes and incubated at 95 °C for 50 minutes. After cooling down to room temperature, two hundred microliters of the mixture was transferred into a 96-well plate and read the absorbance at 550 nm. The standard curve was prepared using a 0–500 μg/mL cyanidin chloride solution. The TPAC results were shown as mg of cyanidin chloride equivalents (CCE) per gram of sample.

2.4.6 Total Anthocyanin Content (TAC)

The determination of TAC was performed according to the pH differential method developed by [30]. Four hundred microliters of the sample extract were placed into a cuvette, followed by the addition of 2.8 mL of pH 1.0 buffer (potassium chloride, 0.025 M). Another 400 μL of sample extract and 2.8 mL of pH 4.5 buffer (sodium acetate, 0.4 M) were added into a cuvette. Absorbance was read at 510 and 700 nm, separately. To calculate the absorbance the following equation was used: Abs = (A_{510nm} – A_{700nm}) $pH_{1.0}$ – (A_{510nm} – A_{700nm}) $pH_{4.5}$ and the molar extinction coefficient for cyanidin 3-glucoside was considered as 26,900. Results were displayed as mg of cyanidin 3-glucoside equivalents per 100 grams of sample.

2.4.7 2.2-diphenyl-1-picrylhydrazyl Assay (DPPH)

The DPPH activity was evaluated according to a modified version of [31]. In a 96-well plate, forty microliters μL of lentil samples were transferred to 260 μL of DPPH methanolic solution (0.1 mM) and incubated for 30 minutes at 25 °C. At 517 nm, the absorbance was determined. The standard curve was constructed using a 0–200 μg/mL Trolox. The DPPH radical scavenging activity was shown as mg of Trolox equivalents (TE) per gram of sample.

2.4.8 Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP assay was computed using a modified version of [32]. The FRAP technique assesses a material's capacity to convert Fe^{3+}-TPTZ (ferric-2,4,6-tripyridyl-s-triazine) to Fe^{2+}-TPTZ. The FRAP reagent was made by combining $FeCl_3$ solution (20 mM), TPTZ solution (10 mM), and sodium acetate solution (300 mM) in a volume ratio of 1:1:10. Then, twenty microliters of lentil samples were transferred to 280 μL of prepared FRAP solution in a 96-well plate, which was incubated at 37 °C for 10 minutes. At 593 nm, the absorbance was determined. The standard curve was prepared using a 0–200 μg/mL Trolox aqueous solution. The FRAP results were displayed as mg of Trolox equivalents (TE) per gram of sample.

2.4.9 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) Radical Scavenging Assay (ABTS)

The ABTS assay was evaluated using a modified form of the $ABTS^+$ radical cation decolorization test [33]. Before usage, ABTS cations were produced by combining 5 mL of ABTS solution (7 mmol/L) with 88 μL of potassium persulfate solution (140 mM) in a dark environment for sixteen hours. To reach an initial absorbance of 0.70 at 734 nm, the $ABTS^+$ solution was further diluted using analytical-grade ethanol. Then, 10 μL of lentil samples were combined with 290 μL of prepared $ABTS^+$ solution in a 96-well plate and placed in the dark condition for six minutes at 25 °C. Under 734 nm, the absorbance was read. Utilizing the calibration curve developed with a 0–500 μg/mL Trolox aqueous solution, the antioxidant capacity of lentil samples was evaluated. The ABTS results were displayed as mg of Trolox equivalents (TE) per gram of sample.

2.4.10 Hydroxyl Radical Scavenging Activity (•OH-RSA)

Modifications were made to the Fenton-type reaction approach [34] to determine •OH-RSA. In a 96-well plate, 50 μL of lentil samples were mixed with 50 μL $FeSO_4 \cdot 7H_2O$ (6 mM) and 50 μL H_2O_2 (30%, 6 mM) and incubated for ten minutes at 25 °C. Following incubation, fifty microliters of 3-hydroxybenzoic acid (6 mM) was transferred. At 510 nm, the absorbance was read. Utilizing a calibration curve produced with a 0–300 μg/mL Trolox solution, the antioxidant capacity of lentil samples was determined. The results of the •OH-RSA were displayed as mg of Trolox equivalents (TE) per gram of sample.

2.4.11 Ferrous Ion Chelating Activity (FICA)

FICA was determined by modifying the method of [35]. In a 96-well plate, 15 μL of lentil samples were added to fifty microliters of ferrozine (5 mM, with extra 1:6 dilution in water), fifty microliters of ferrous chloride (3 mM, with further addition of 1:15 dilution in water), and eighty-five microliters of water, followed by a 10-minute incubation under 25 °C. Under 562 nm, the absorbance was read. Utilizing a calibration curve produced with 0–50 μg/mL Ethylenediaminetetraacetic acid (EDTA), the antioxidant capacity of lentil samples was measured. The FICA results were shown as mg of EDTA per gram of sample.

2.4.12 Reducing Power Assay (RPA)

The RPA was identified by following the technique of [36] with some modifications. In a 96-well plate, 10 μL of lentil samples were added to 25 μL sodium phosphate buffer (0.2 M, pH 6.6) and 25 μL $K_3[Fe(CN)_6]$ and incubated for 20 minutes at 25 °C. Due to the addition of twenty-five microliters of a 10% TCA solution, eighty-five microliters of water, and 8.5 μL of $FeCl_3$, the reaction would be stopped. The incubation was sustained for fifteen minutes at 25 °C before being discarded. Under 750 nm, the absorbance was measured. Using the calibration curve constructed with 0–500 μg/mL Trolox solution, the antioxidant capacity of lentil samples was measured. The RPA results were shown as mg of Trolox equivalents (TE) per gram of sample.

2.4.13 Phosphomolybdate Assay (PMA)

The total antioxidant capacity was determined using a modified PMA assay [37]. Blending H_2SO_4 (0.6 M), Na_3PO_4 (28 mM), and ammonium molybdate (4 mM) produced the PMA reagent. In a 96-well plate, 40 μL of lentil samples were added to 260 μL of the produced dye and incubated for 90 minutes at 90 °C. The plate was then allowed to cool to ambient temperature for 10 minutes. The absorbance was read at 695 nm. The antioxidant capacity of lentil samples was determined via a calibration curve pro-

duced with 0–300 μg/mL ascorbic acid. The PMA values were shown as mg of ascorbic acid equivalents (AAE) per gram of sample.

2.5 Determination of Total Saponins (TSC)

The determination of saponins was conducted based on the vanillin-sulfuric acid method, modified by [38]. In brief, 10 μL of sample extract was transferred in 1.5 mL Eppendorf tubes and placed in the oven incubator at 60 °C until solvents were evaporated. Then, 200 μL of 4% vanillin (dissolved in ethanol) and 1000 μL of 72% sulfuric acid were added, followed by 15-min incubation under 60 °C. After cooling down to the ambient temperature, 0.25 mL of the mixture was transferred to a 96-well plate, followed by measuring the absorbance under 560 nm. The total saponin content of lentils was evaluated by using a calibration curve produced with 0–25 μg/mL Aescin. The TSC results were displayed as mg of Aescin equivalents (AE) per gram of sample.

2.6 LC-ESI-QTOF-MS^2 Characterization of Phenolic Compounds

Samples used for LC-ESI-QTOF-MS^2 analysis were extracted utilizing two different extraction methods. First, extracts were produced using 70% ethanol and mixed using an Ultra-Turrax T25 Homogenizer (IKA, Staufen, Germany) at 10,000 rpm for thirty seconds. Then the prepared extracts were placed in a ZWYR-240 incubator shaker (Labwit, Ashwood, VIC, Australia) at 120 rpm at 10 °C for 16 hours. Then, another extraction method was performed using ultrasonic for 5 minutes in an ice water bath with a cell disruptor (Branson, model Digital Sonifier 450) at an amplitude of 40%. After both extraction methods, extracts were centrifuged at 8000 rpm for 15 minutes at 4 °C (Hettich ROTINA 380R, Tuttlingen, Baden-Württemberg, Germany) and the supernatant was collected and frozen at –20 °C for subsequent analysis.

Phenolic characterization was made by following the method of [39] with some modifications and was conducted by Agilent 1200 series HPLC (Agilent Technologies, CA, USA) connected with an Agilent 6520 Accurate Mass Q-TOF LC-MS^2 (Agilent Technologies, Santa Clara, CA, USA). Compound Separation was carried out using a Synergi Hydro-RP 80 Å, LC Column (250 mm $\times$ 4.6 nm, 4 μm) (Phenomenex, Lane Cove, NSW, Australia) with Phenomenex C18 ODS (4.0 $\times$ 2.0 mm) guard column to protect the column. Mobile phase A was made by water/acetic acid (98:2, *v/v*), and mobile phase B was made by acetonitrile/water/acetic acid (100:99:1, *v/v/v*). The degassing process was performed under 25 °C for 15 min. The gradient program was conducted by a mixture of mobile phase A and B as follow: 90% A and 10% B (0 min); 75% A and 25% B (20 min); 65% A and 35% B (30 min); 60% A and 40% B (40 min); 45% A and 55% B (70 min); 20% A and 80% B (75 min); 100% B (77 min); 90% A and 10% B (85 min). The flow rate was set to be 0.8 mL/min and five-microliter was the sample injection volume. The peak was identified by positive and negative modes and nitrogen gas was used as a nebulizer and drying gas at 45 psi, with a flow rate of 0.5 mL/min. Capillary and nozzle voltage was placed at 3.5 kV and 500 V respectively, while the mass spectra were obtained in the range of 50–1300 amu with collision energy (10, 15, and 30 eV) for fragmentation. Data collection and assays were performed using Agilent LC-ESI-QTOF-MS^2 Mass Hunter Data Acquisition Software Version B.03.01 (Agilent Technologies, Santa Clara, CA, USA). Compounds identified by LC-ESI-QTOF-MS/MS that had library identification scores greater than 80 were selected for characterization and *m/z* verification.

2.7 Statistical Analysis

The polyphenol content and antioxidant assay data were reported as means $\pm$ standard deviation (SD), and studies were conducted in triplicate (n = 3). Minitab Statistical Software for Windows Version 18.0 was used to conduct a one-way analysis of variance (ANOVA) accompanied by Tukey's honestly significant differences (HSD) multiple rank test at a significance level of $p < 0.05$ (Minitab Inc., State College, PA, USA). The correlation between phenolic compounds and antioxidant activities was performed via XLSTAT-2019.1.3 (Addinsoft Inc. New York, NY, USA).

3. Results and Discussion

The antioxidant potential and the association between phenolic substances and antioxidant activity in the lentil samples were estimated following different assays. In addition, LC-ESI-QTOF-MS/MS was used as a tool to determine and characterize the phenolic compounds. The phenolic content, antioxidant activity, and saponin content results are listed in Table 1.

3.1 Phenolic Compounds and Saponin Estimation (TPC, TFC, TTC, TCT, TPAC, TAC, and TSC)

Phenolics are essential secondary metabolites widely found in nature. Most studies correlate phenolic compounds with their potential health benefits, including antioxidant capacity and other health-promoting properties [40]. According to a recent review of polyphenols in lentils [13], the most frequently found polyphenols in lentils comprise phenolic acids, flavonols, flavan-3-ol, proanthocyanidins, anthocyanidins, or condensed tannins, and anthocyanins. Therefore, this study intended to evaluate the TPC, TFC, TTC, TCT, TPAC, and TAC, besides the saponin estimation. The Folin-Ciocalteu method was utilized to quantify the total phenolic content of lentils (Table 1). There was no statistically significant difference in the results of red and brown whole lentils, which showed a value of 0.79 $\pm$ 0.02 mg GAE/g and 0.81 $\pm$ 0.12 mg GAE/g, separately. How-

Table 1. Assessment of the phenolic content, antioxidant capacity, and saponin content existing in different lentil samples.

Assays	BKL	GWL	RWL	BWL
TPC (mg GAE/g)	0.84 ± 0.03^{b}	0.96 ± 0.07^{a}	0.79 ± 0.02^{c}	0.81 ± 0.12^{c}
TFC (mg QE/g)	0.05 ± 0.01^{b}	0.02 ± 0.02^{c}	0.06 ± 0.01^{a}	0.04 ± 0.01^{b}
TTC (mg TAE/g)	1.56 ± 0.04^{b}	1.11 ± 0.03^{c}	1.03 ± 0.03^{d}	2.05 ± 0.04^{a}
TCT (mg CE/g)	0.03 ± 0.12^{a}	0.01 ± 0.24^{b}	-	0.02 ± 0.07^{a}
TPAC (mg CCE/g)	0.09 ± 0.01^{a}	-	-	0.01 ± 0.01^{b}
TAC (mg/100 g)	3.32 ± 0.01^{a}	2.78 ± 0.01^{c}	1.72 ± 0.01^{d}	3.04 ± 0.01^{b}
DPPH (mg TE/g)	4.32 ± 0.08^{b}	5.24 ± 0.02^{a}	4.65 ± 0.02^{b}	3.21 ± 0.08^{c}
FRAP (mg TE/g)	2.13 ± 0.12^{b}	3.38 ± 0.05^{a}	2.05 ± 0.09^{b}	2.09 ± 0.01^{b}
ABTS (mg TE/g)	6.47 ± 0.81^{c}	9.82 ± 0.71^{a}	8.06 ± 0.47^{b}	7.94 ± 0.12^{b}
•OH-RSA (mg TE/g)	3.14 ± 0.09^{c}	3.87 ± 0.13^{a}	2.49 ± 0.22^{d}	3.74 ± 0.02^{b}
FICA (mg EDTA/g)	0.19 ± 0.07^{a}	0.08 ± 0.01^{c}	0.11 ± 0.01^{b}	0.09 ± 0.01^{c}
RPA (mg TE/g)	2.10 ± 0.17^{a}	1.66 ± 0.05^{c}	1.96 ± 0.04^{b}	1.59 ± 0.05^{c}
PMA (mg AAE/g)	3.18 ± 0.21^{c}	3.39 ± 0.17^{b}	4.01 ± 0.31^{a}	2.31 ± 0.14^{d}
TSC (mg AE/g)	0.01 ± 0.01^{c}	0.02 ± 0.01^{b}	0.02 ± 0.01^{b}	0.03 ± 0.01^{a}

The results are displayed in mg equivalents per gram in terms of fresh weight and expressed as mean $\pm$ standard deviation (SD) (n = 3); the lettering (^{a,b,c,d}) indicated the significant difference ($p < 0.05$) utilizing one-way analysis of variance (ANOVA) and Tukey's HSD test. TPC, total phenolic content; TFC, total flavonoid content; TTC, total tannin content; TCT, total condensed tannins; TPAC, total proanthocyanidin content; TAC, total anthocyanin content; DPPH, 2,2′-diphenyl-1-picrylhydrazyl assay; FRAP, ferric reducing antioxidant power assay; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid assay; •OH-RSA, hydroxyl-radical scavenging activity; FICA, ferrous ion chelating activity; RPA, reducing power assay; PMA, phosphomolybdate assay; TSC, total saponin content. GAE, gallic acid equivalents; QE, quercetin equivalents; TAE, tannic acid equivalents; CE, catechin equivalents; CCE, cyanidin chloride equivalents; TE, Trolox equivalents; EDTA, ethylenediaminetetraacetic acid; AAE, ascorbic acid equivalents; AE, aescin equivalents. RWL, red whole lentil; GWL, green whole lentil; BWL, brown whole lentil; BKL, black whole lentil.

ever, the TPC value of green whole lentils was significantly different ($p < 0.01$) from the other three kinds of lentils and displayed the greatest total phenolic content (0.96 $\pm$ 0.07 mg GAE/g), accompanied by black whole lentils (0.84 $\pm$ 0.03 mg GAE/g). This outcome is in accordance with the results found by [41], that observed the highest TPC values in green lentils (737.32 mg/100 g dry weight (d.w.) in aqueous-organic extract) when compared to red lentils, chickpeas, and peas.

Flavonoids are considered the most significant polyphenol in human diets, and it is among the main phenolic compounds present in lentils [42,43]. As shown in Table 1, flavonoids in lentils ranged from 0.02 $\pm$ 0.02 mg QE/g to 0.06 $\pm$ 0.01 mg QE/g. This research revealed that red whole lentils had the greatest total flavonoid content (0.06 $\pm$ 0.01 mg QE/g), accompanied by black whole lentils (0.05 $\pm$ 0.01 mg QE/g), and brown whole lentils (0.04 $\pm$ 0.01 mg QE/g). Moreover, green whole lentils (0.02 $\pm$ 0.02 mg QE/g) displayed the lowest flavonoid content among samples. There was no significant statistical difference between black and brown whole lentils in terms of flavonoid concentration. However, the above results are challenged by [44], who examined 33 samples of cool-season legumes and found that lentils' TPC values varied from 4.86 to 9.6 mg GAE/g and that green lentil had a greater quantity of flavonoids. Variations in total phenolics and flavonoid concentration may be influenced by the solvent used in the extraction, origin, harvesting year, and storage conditions of lentils.

Tannins are complex phenolic compounds that are typically separated into two groups, hydrolysable and condensed tannins [42]. Total tannin content (TTC) was measured utilizing the polyvinylpolypyrrolidone (PVPP) method. This method is based on tannin complexation/precipitation, instead of proteins [27]. Assuming that the phenolics that bind to proteins are identical to those that bind to PVPP, this method separates tannins from non-tannins by using this solid matrix. According to Table 1, brown lentils showed the highest total tannins (2.05 $\pm$ 0.04 mg TAE/g), accompanied by black (1.56 $\pm$ 0.04 mg TAE/g), green (1.11 $\pm$ 0.03 mg TAE/g), and red (1.03 $\pm$ 0.03 mg TAE/g) lentils. Similarly, Menga *et al.* [45] discovered that the brown lentils (4.45 mg CE/g) showed higher values of TTC than the green lentils (2.92 mg CE/g). Irakli *et al.* [46] reported that the total tannin content ranged from 2.86 to 3.20 mg GAE/g for five different kinds of lentils. The PVPP method applied in our study cannot determine the presence or absence of certain types of tannins in a mixture, such as condensed or hydrolysable tannins, but could be seen as the measurement of total tannins [47].

Condensed tannins are among the main phenolic compounds present in legume seeds and are usually discovered in lentils among other pulses [43]. Total condensed tannin content (TCT) was shown as mg/g equivalents of catechin

(CE) per gram of material. As depicted in Table 1, three out of four lentils had total condensed tannins, namely black whole lentils (0.03 $\pm$ 0.12 mg CE/g), green whole lentils (0.01 $\pm$ 0.24 mg CE/g) and brown whole lentils (0.02 $\pm$ 0.07 mg CE/g). Nonetheless, no tannins were detected in whole red lentils. In contrast, [48] observed a total condensed tannin concentration of 0.012 to 0.014 mg/g fresh weight in red lentils extracted using an ethanolic solution. The variation in the condensed tannin content of red lentils might be attributed to the concentration of the extraction solvents used, as our study used 70% ethanolic extraction while [48] applied 80% ethanolic extraction. In addition, lentil origin type may have a role in the final results, since [48] applied red lentils from India while the Australian variety was used for the red lentil in our research.

Flavanols occur either in a monomeric form (catechins) or in a polymeric form (proanthocyanidins) [49]. The oligomers of catechin and epicatechin molecules known as proanthocyanidins are mainly found in lentils with colored seed coats [3]. The determination of TPAC was conducted according to the HCl-butanol method. This method can also be used to measure the condensed tannin contents in different samples [47]. This is because condensed tannins when heated in an acid alcohol solution can be degraded into anthocyanidins through an acid-catalyzed oxidation process, therefore condensed tannins are also known as proanthocyanidins [42]. Based on the results, black whole lentils had the greatest proanthocyanidins' level, demonstrating a value of 0.09 $\pm$ 0.01 mg CCE/g accompanied by brown lentils (0.01 $\pm$ 0.01 mg CCE/g). However, no proanthocyanidins were detected for green and red whole lentils. Compared with the TCT values, a similar tendency has appeared. [46] reported a mean TPAC value for five lentils with different genotypes of 7.93 mg procyanidin B_2 equivalents/g. Using different standard compounds to display the results leads to results that cannot be directly compared. In our study, the calibration curve was based on measurements of cyanidin chloride. In the prior study, the results were shown as catechin or procyanidin B_2 equivalents [45,46].

Anthocyanins are a broadly studied flavonoid subgroup that is present in different foods as pigments for the pink, red, purple, or cyan color of such foods [42]. The determination of TAC in four kinds of lentils was performed and displayed as mg of cyanidin 3-glucoside equivalents per 100 g of lentil samples. TAC values in lentils varied from 3.32 $\pm$ 0.01 to 1.72 $\pm$ 0.01 mg/100 g based on our findings. Black lentils had the greatest total anthocyanin content (3.32 $\pm$ 0.01 mg/100 g), accompanied by brown lentils (3.04 $\pm$ 0.01 mg/100 g), green lentils (2.78 $\pm$ 0.01 mg/100 g), and red lentils (1.72 $\pm$ 0.01 mg/100 g). A range of 13.67–15.99 mg/100 g of anthocyanins in mixed lentils was reported, according to the study by [50]. Anthocyanins, a class of water-soluble flavonoids, broadly exist in fruits as well as vegetables. Several factors may have an impact on the stability of anthocyanins, containing light, pH values, temperature, ascorbic acid, oxygen, and enzymes, leading to different results [51].

Saponins are amphiphilic glycosidic secondary metabolites produced by several plants that possess emulsifying and foaming properties [52]. Lentils are one of the main sources of saponins in the human diet [13]. According to our findings, the highest saponin level was displayed in brown lentils (0.03 $\pm$ 0.01 mg AE/g). No significant differences were shown between green and red whole lentils ($p > 0.05$), while black lentils possessed the lowest saponin content among all the lentil samples. The amount of saponin in lentils varies depending on the cultivar, location, soil type, weather, and season of harvest, extraction methods, as well as processing methods, such as soaking, cooking, and blanching [13,53]. The total amount of saponins in 44 different genotypes of lentils was estimated by [54], who observed a concentration from 1.70 to 3.50 mg/g. Pure ethanol used in ultrasonic dried lentil extracts had the highest total saponin content (1.063 mg/g), followed by ethanol: water (0.328 mg/g) and water extract (0.019 mg/g), according to the study of [55].

3.2 Antioxidant Activities (DPPH, FRAP, ABTS, RPA, •OH-RSA, FICA, and PMA)

Antioxidants play an essential part in the preservation of human health, preventing oxidative processes related to the deterioration of food quality, and in the treatment of diseases, due to their ability to delay, control and reduce oxidative stress [56]. Among the antioxidant assays, the DPPH, ABTS, •OH-RSA, and FICA have been applied to measure the electron transfer capacity of bioactive compounds related to the presence of polyphenols, while FRAP and RPA have been used to evaluate the ability of samples to donate electrons to reduce a Fe^{3+} [57]. Therefore, the antioxidant studies of lentil samples were carried out by testing DPPH, FRAP, ABTS, RPA, •OH-RSA, FICA, and PMA assays, which illustrated the potential to scavenge the free radical results from various kinds of lentils (Table 1). The radical scavenging activity of DPPH focuses on the hydrogen ability donation to eliminate free radicals [58]. As dispalyed in Table 1, the radical scavenging activity varied from 3.21 to 5.24 mg TE/g, with green whole lentils exhibiting the highest value (5.24 $\pm$ 0.02 mg TE/g) and brown lentils the lowest (3.21 $\pm$ 0.08 mg TE/g). No significant differences ($p > 0.05$) were shown between black and red whole lentils, noting values of 4.32 $\pm$ 0.08 and 4.65 $\pm$ 0.02 mg TE/g, respectively. This result was consistent with a previous study in which there were significant differences in DPPH between lentils, black soybeans, and soybean legumes ($p < 0.05$). Lentils exhibited the highest level of DPPH, with French green lentils (19.87 μmol TE/g) presenting the highest concentration among the lentil subgroups [44].

The reducing power of FRAP is on the basis of the reduction of ferric-tripyridyltriazine $[FeIII(TPTZ)]^{3+}$, to produce an intense blue-colored ferrous complex

Table 2. Pearson's correlation coefficients (r) between antioxidant assays.

Variables	TPC	TFC	TCT	DPPH	FRAP	ABTS	•OH-RSA	FICA	RPA
TFC	–0.897*								
TCT	–0.274	0.255							
DPPH	0.646	–0.278	–0.496						
FRAP	0.975**	–0.889*	–0.478	0.683					
ABTS	0.684	–0.729	–0.847	0.499	0.825*				
•OH-RSA	0.660	–0.904**	0.050	–0.144	0.611	0.463			
FICA	–0.289	0.518	0.820*	–0.05	–0.467	–0.867*	–0.412		
RPA	–0.354	0.703	0.442	0.247	–0.448	–0.702	–0.763	0.870*	
PMA	0.050	0.45	–0.494	0.793	0.134	0.163	–0.703	0.093	0.550

*Significant correlation with $p \leq 0.05$ and **Significant correlation with $p \leq 0.01$.

$[FeII(TPTZ)]^{2+}$ [59]. The ferric reducing antioxidant power in lentils was between 2.05 to 3.38 mg TE/g. The greatest antioxidant capacity was discovered in green lentils with 3.38 $\pm$ 0.05 mg TE/g. While red whole lentils had the lowest FRAP value, of 2.05 $\pm$ 0.09 TE/g. This conclusion is comparable to the findings by [41], who observed the greatest FRAP in green lentils (140.32 μmol/g d.w. in aqueous-organic extract) when compared to red lentils, beans, soybeans, and chickpeas. FRAP values for black and brown lentils showed no significant difference ($p >$ 0.05). Similarly, there were no significant differences between Brewer's lentils (12.02 $\pm$ 0.33 mmol Fe^{2+} equivalents/100 g) and Red Chief lentils (11.37 $\pm$ 0.66 mmol Fe^{2+} equivalents/100 g) in terms of the study of [44].

The radical scavenging activity of ABTS is based on the measurement of the electron transfer capacity of the antioxidant evaluated [60]. In the ABTS analysis, a variation from 6.47 $\pm$ 0.81 to 9.82 $\pm$ 0.71 mg TE/g was observed among all the evaluated lentils. Green whole lentils had the highest ABTS value (9.82 $\pm$ 0.71 mg TE/g) among the lentils, followed by the red lentils (8.06 $\pm$ 0.47 mg TE/g), whereas the black lentils showed the lowest radical scavenging activity of 6.47 $\pm$ 0.81 mg TE/g. The ABTS content of Pardina lentils (green lentils, 14.8 μmol TEAC g^{-1}) was greater than that of Crimson lentils (red lentils, 14.0 μmol TEAC g^{-1}), according to prior research [18], which is consistent with our results. Moreover, there were statistically significant variations in ABTS levels between red and brown lentils ($p < 0.05$).

In the •OH-RSA analyses, hydroxyl radicals (•OH) are formed due to the existence of Fe^{2+} ion and hydrogen peroxide via the Fenton reaction [61]. While FICA assay measure the antioxidant potential by the chelating ability of ferrous ion [59]. Whereas RPA causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form in the existence of reducers (i.e., antioxidants) [36].

In •OH-RSA, FICA, and RPA assay, there were no significant differences between green and brown whole lentils. Meanwhile, in •OH-RSA assay, green whole lentils had the strongest antioxidant potential (3.87 $\pm$ 0.13 mg TE/g), accompanied by the brown whole lentils (3.74 $\pm$ 0.02 mg TE/g), black whole lentils (3.14 $\pm$ 0.09 mg TE/g), and red whole lentils (2.49 $\pm$ 0.22 mg TE/g), respectively. However, red whole lentils showed highly significant antioxidant activity compared to the green and brown lentils in the FICA and RPA assays. Besides, the FICA and RPA values followed the same trend, with black lentils performing the best, followed by red, green, and brown lentils. To our knowledge, this is the first time that the antioxidant potential of different lentils has been analyzed by •OH-RSA, FICA, and RPA, and limited data are available for comparison.

PMA is often used to measure the TAC of liquid food extracts based on an electron transfer mechanism. This test uses phenolic chemicals to convert molybdenum (VI) to molybdenum (V). The total antioxidant capacity of lentils ranged from 2.31 $\pm$ 0.14 to 4.01 $\pm$ 0.31 mg AAE/g, with red whole lentils having the highest PMA value at 4.01 $\pm$ 0.31 mg AAE/g, accompanied by green whole lentils with 3.39 $\pm$ 0.17 mg AAE/g, black whole lentils with 3.18 $\pm$ 0.21 mg AAE/g, and brown whole lentils with 2.31 $\pm$ 0.14 mg AAE/g. Further, significant differences were observed among all different types of lentils ($p < 0.05$). This result parallels the findings from [62], in which beans with red or black pigmentation exhibited greater PMA concentrations. The amount of flavonol glycosides, anthocyanins, and condensed tannins (proanthocyanidins) determines the color of lentil seed coats [62].

3.3 Correlations of Phenolic Contents and Antioxidant Activities

It is generally recognized that the antioxidant activity of plants is related to their phenolic composition [44]. Some recent studies suggest this correlation, such as the study by [63] and [64].

Pearson's correlation test established a relationship between phenolic levels and antioxidant tests (Table 2). FRAP showed the strongest association with TPC in the Person Correlation study (r = 0.975; $p < 0.01$). Meanwhile, Pearson's correlation coefficient r = –0.897 ($p <$ 0.05) demonstrated a strong negative relation between total phenolic content and total flavonoid content. The signifi-

Table 3. Characterization of phenolic compounds in various kinds of lentil samples by LC-ESI-QTOF-MS^2.

No.	Proposed compounds	Molecular formula	RT (min)	Ionization (ESI$^+$/ESI$^-$)	Molecular weight	Theoretical (*m/z*)	Observed (*m/z*)	Error (ppm)	MS^2 production	Sample
Phenolic acid										
Hydroxybenzoic acids										
1	Ellagic acid	$C_{14}H_6O_8$	8.012	$[M–H]^-$	302.0036	300.9963	300.9962	–0.3	284, 229, 201	BWL
Hydroxycinnamic acids										
2	Ferulic acid	$C_{10}H_{10}O_4$	4.099	$[M–H]^-$	194.0575	193.0502	193.0500	–1.0	178, 149, 134	*BWL
3	Caffeic acid	$C_9H_8O_4$	4.790	$[M–H]^-$	180.0405	179.0332	179.0331	–0.6	143, 133	BWL
4	Ferulic acid 4-*O*-glucoside	$C_{16}H_{20}O_9$	5.029	$[M–H]^-$	356.1117	355.1044	355.1041	–0.8	193	BWL
5	Caffeoyl glucose	$C_{15}H_{18}O_9$	47.840	$[M–H]^-$	342.0949	341.0876	341.0878	0.6	179, 161	*BKL, GWL, RWL
Hydroxyphenylpropanoic acids										
6	Dihydrocaffeic acid 3-*O*-glucuronide	$C_{15}H_{18}O_{10}$	4.499	$[M–H]^-$	358.0897	357.0824	357.0818	–1.7	181	RSL
Flavonoids										
Flavanols										
7	Procyanidin dimer B7	$C_{30}H_{26}O_{12}$	4.288	$[M–H]^-$	578.1379	577.1306	577.1302	–0.7	451	RWL, BWL, GWL
8	Procyanidin trimer C1	$C_{45}H_{38}O_{18}$	4.310	$[M–H]^-$	866.2056	865.1983	865.1985	0.2	739, 713, 695	*GWL, BWL
9	4”-*O*-Methylepigallocatechin 3-*O*-gallate	$C_{23}H_{20}O_{11}$	4.916	$[M–H]^-$	472.0989	471.0916	471.0917	0.2	169, 319	BWL
Flavanones										
10	Neohesperidin	$C_{28}H_{34}O_{15}$	4.485	$[M+H]^+$	610.1895	611.1968	611.1969	0.2	593, 465, 449, 303	GWL
11	Sakuranetin	$C_{16}H_{14}O_5$	5.750	$[M+H]^+$	286.0841	287.0914	287.0920	2.1	269, 203, 175	BWL
Flavonols										
12	Quercetin 3-*O*-rutinoside	$C_{27}H_{30}O_{16}$	4.069	$[M–H]^-$	610.1556	609.1483	609.1495	2.0	301	BWL
13	Myricetin 3-*O*-rhamnoside	$C_{21}H_{20}O_{12}$	4.234	$[M–H]^-$	464.0983	463.0910	463.0909	–0.2	317	RWL
14	Quercetin 3-*O*-glucosyl-xyloside	$C_{26}H_{28}O_{16}$	4.293	**$[M–H]^-$	596.1412	595.1339	595.1343	0.7	265, 138, 116	*GWL, BWL
15	Quercetin 3-*O*-rhamnoside	$C_{21}H_{20}O_{11}$	4.350	$[M–H]^-$	448.1047	447.0974	447.0972	–0.4	447, 287	*GWL, BKL, RWL
16	Quercetin 3-*O*-xyloside	$C_{20}H_{18}O_{11}$	4.406	$[M–H]^-$	434.0859	433.0786	433.0788	0.5	301	*GWL, BKL, BWL
Dihydroflavonols										
17	Dihydroquercetin	$C_{15}H_{12}O_7$	5.448	$[M–H]^-$	304.0609	303.0536	303.0539	1.0	285, 275, 151	RWL
Anthocyanins										
18	Cyanidin 3-*O*-(6′-*p*-coumaroyl-glucoside)	$C_{30}H_{27}O_{13}$	4.714	**$[M–H]^-$	595.1446	594.1373	594.1394	3.5	287	*BWL
Isoflavonoids										
19	Dalbergin	$C_{16}H_{12}O_4$	5.087	$[M–H]^-$	268.0734	267.0661	267.0665	1.5	252, 224, 180	GWL
Other polyphenols										
Alkylmethoxyphenols										
20	4-Vinylsyringol	$C_{15}H_{14}O_3$	5.066	$[M+H]^+$	242.0946	243.1019	243.1026	2.9	225, 211, 197	RWL
Lignans										
21	Secoisolariciresinol-sesquilignan	$C_{30}H_{38}O_{10}$	45.717	$[M–H]^-$	558.2503	557.2430	557.2422	–1.4	539, 521, 509, 361	BWL
22	Deoxyschisandrin	$C_{24}H_{32}O_6$	55.151	$[M–H]^-$	416.2218	415.2145	415.2159	3.4	402, 347, 361, 301	*RWL, BWL

*Compound was scanned in more than one lentil samples, Compound was scanned in more than one lentil samples, data presented in this table are from lentil samples. **Compounds were detected in both negative $[M-H]^-$ and positive $[M+H]^+$ mode of ionization whereas only single mode data was presented. As displayed in the table, sample lentils are mentioned in abbreviated form. RWL, red whole lentil; GWL, green whole lentil; BWL, brown whole lentil; BKL, black whole lentil.

cant association between FRAP and TPC implies that the phenolic content of lentil extract is primarily responsible for its antioxidant properties. This outcome is comparable to the study of [44]. It was discovered that the connection between ABTS and FRAP was strongly correlated ($p < 0.05$) (r = 0.825). By scavenging ABTS radicals, ABTS determines the hydrogen-donating and chain-breaking ability of antioxidants. Correlations showed that the ability to scavenge free radicals was determined by the polyphenol content of the samples, while antioxidants with strong hydrogen-donating ability to scavenge radicals were also effective in improving antioxidant and anti-free radical capacity and contributed significantly to the total antioxidant capacity of lentils. Saharan *et al.* [65] examined the link between the phenolic content of several types of beans and their corresponding antioxidant activities, reporting that a significant correlation ($p < 0.01$) was obtained among total phenolic, flavonoid contents with radical scavenging activity (maximum in pigeon pea; i.e., $r^2 = 0.955$ and $r^2 = 0.976$, separately).

Furthermore, substantial negative associations were found between •OH-RSA and TFC (r = –0.904; $p < 0.01$), ABTS and FICA (r = –0.867; $p < 0.05$), as well as between FRAP and TFC (r = –0.889, $p < 0.05$). Moreover, a strong positive association existed between FICA and RPA (r = 0.870, $p < 0.05$). Few studies have previously used •OH-RSA, FICA, and RPA to establish the antioxidant potential of lentils. To our knowledge, this is the first time that •OH-RSA, FICA, and RPA studies have been done on various lentil samples, and comparative data are scarce. Several investigations using RPA and •OH-RSA methods concluded that antioxidant activity is positively correlated with phenolic content, which is not in agreement with our study [66–68].

3.4 LC-ESI-QTOF-MS^2 Characterization of Phenolic Compounds from Different Lentil Samples

LC-ESI-QTOF-MS^2 analysis has been widely utilized to identify phenolic compounds from several plant-based samples [67,69,70]. In this research, LC-ESI-QTOF-MS^2 was used to evaluate the phenolic components in ethanolic and ultrasonic extracts of lentils. On the basis of retention time (RT), mass to charge (*m/z*) values, and MS^2 spectra in negative and positive ionization modes ($[M - H]^-$/$[M + H]^+$), the phenolic compounds in four lentil samples were identified and characterized utilizing Agilent LC-MS/MS MassHunter Qualitative Software and Personal Compound Database and Library (PCDL) (Table 3). By combining negative and positive modes, more compounds present in the lentils with broader chemical diversity would be identified and characterized. Compounds having a PCDL score over 80 and a mass error less than ± 5 ppm were chosen for further MS^2 identification and *m/z* characterization and verification purposes. More information about the total ion chromatograms of the lentil samples can be found at supplementary materials (**Supplementary Fig. 1**).

According to the literature, lentils are an important dietary source of extractable polyphenols, such as phenolic acids, condensed tannins (proanthocyanidins), anthocyanidins, flavan-3-ols, flavanols, flavones, flavanones, and stilbenes [6]. In this study, 22 phenolic compounds, comprising 6 phenolic acids, 13 flavonoids, 2 lignans, and 1 other polyphenol, were tentatively characterized by LC-ESI-QTOF-MS^2 and are summarized in Table 3. Polyphenols, containing flavonoids, phenolic acids, and stilbenes, have considerable antioxidant activity and are indicated to help control free radicals arising from oxidative stress. Other bioactive properties including antimicrobial, anti-inflammatory, antineoplastic, estrogenic, hepatoprotective, and hypolipidemic activities have also been reported [71].

3.4.1 Phenolic Acids

Phenolic acids are simple phenolics and their subclasses are hydroxybenzoic acid derivatives and hydroxycinnamic acid derivatives [6]. In this research six phenolic acids were identified in each of the four lentil samples. Hydroxybenzoic acids (1), hydroxycinnamic acids (4), and hydroxyphenylpropanoic acids (1) were tentatively characterized in various lentil samples.

3.4.1.1 Hydroxybenzoic Acids and Hydroxycinnamic Acids. One type of hydroxybenzoic acid along with four kinds of hydroxycinnamic acids was identified in selected lentil samples. Compound 1 with $[M - H]^-$ *m/z* at 300.9963 was only detected from brown whole lentils and characterized as ellagic acid based on the product ion at 284 *m/z*, 229 *m/z*, and 201 *m/z*. Ellagic acid was also present in the spices from Australia in terms of previous research [57]. Three hydroxycinnamic acid derivatives (Compound 2, 3, 4) were only found in the brown whole lentils. These three compounds were tentatively characterized in negative mode, containing ferulic acid (Compound 2), caffeic acid (Compound 3), and ferulic acid 4-*O*-glucoside (Compound 4) with $[M - H]^-$ at *m/z* 193.0502, 179.0332, and 355.1044, respectively. And ferulic acid was confirmed by the characteristic ions at *m/z* 178, *m/z* 149, and *m/z* 134 due to the loss of CH_3 (15 Da), CO_2 (44 Da), and CH_3 with CO_2 (59 Da) [72], was also identified in bitter cumin by [73]. Caffeic acid was identified with MS^2 spectrum by the characteristic ions of *m/z* 143 (loss of two molecules of water, 36 Da) and *m/z* 133 (loss of HCOOH, 46 Da) [74], which was also detected in black spices and pepper [75,76]. Moreover, ferulic acid (Compound 2) and caffeic acid (Compound 3) both were previously found in 6 different varieties of lentils, namely CDC green land, CDC invincible, 3493-6, CDCSB-2, maxim, and black lentils, according to the study of [77]. The loss of $C_7H_{13}O_5$ (177 Da) and $C_7H_{10}O_7$ (206 Da) from the precursor ion of ferulic acid 4-*O*-glucoside produced the fragment peaks (*m/z* 178 and *m/z* 149) [78], which was also found in hops and juniper berries [24]. Caffeoyl glucose (Compound 5), identified based on $[M - H]^-$, was

found in black whole lentils, red whole lentils, and green whole lentils. The molecular ion of caffeoyl glucose (*m/z* 341.0878) produced the major fragment ion at *m/z* 179, corresponding to the loss of glucoside (162 Da) from the product ion [57]. The exsitence of caffeoyl glucose in Australian grown apples was also previously reported [79].

3.4.1.2 Hydroxyphenylpropanoic Acids. Compound 6 was tentatively characterized as dihydrocaffeic acid 3-*O*-glucuronide, and only found in black whole lentil based on $[M - H]^-$ *m/z* at 357.0818 in negative ionization mode, which identification was additionally aided by the MS^2 spectrum. The identity of dihydrocaffeic acid 3-*O*-glucuronide was verified by the product ions at *m/z* 181 [M – H – 176], related to the neutral loss of hexuronyl moiety (glucuronyl moiety), which was also found in Australian grown berries, according to the previous research [80].

3.4.2 Flavonoids

Flavonoids are characterized by a C6–C3–C6 backbone structure. Its classification includes distinct subgroups of flavan-3-ols, flavones, flavanols, flavanones, anthocyanidins, and isoflavones, and oligomers such as proanthocyanins [6]. A total of 13 flavonoids were discovered and characterized in all four lentil samples. In this study, we tentatively characterized 6 subclasses in different lentil samples, including flavanols (3), flavanones (2), flavonols (5), dihydroflavonols (1), anthocyanins (1), and isoflavonoids (1).

3.4.2.1 Flavanols. Three flavanols (Compound 7, Compound 8, and Compound 9) were tentatively identified according to specific criteria: precursor and product ions, all of which in negative ionization mode in this study. Procyanidin dimer B7 (Compound 7, *m/z* 577.1302, RT = 4.288 min) was identified in red whole lentil, green whole lentil, and brown whole lentil samples, procyanidin trimer C1 (Compound 8, *m/z* 865.1985) was found in green whole lentil and brown whole lentil. 4”-*O*-Methylepigallocatechin 3-*O*-gallate (Compound 9, *m/z* 471.0917) was only characterized in brown whole lentils. The precursor ions of procyanidin dimer B7 produced the product ions at *m/z* 451; the precursor ions of procyanidin trimer C1 produced the product ions at *m/z* 739, *m/z* 713, and *m/z* 695, indicating the expected loss of heterocyclic ring fission (HRF) reaction (126 Da), retro-Diels-Alder (RDA) (152 Da) and H_2O [81]; the precursor ions of 4”-*O*-methylepigallocatechin 3-*O*-gallate produced the product ions at *m/z* 169 and *m/z* 319. Previous research also mentioned the presence of procyanidin trimer C1 (Compound 8) in six different lentil samples [77].

3.4.2.2 Flavanones. Neohesperidin (Compound 10 with $[M + H]^+$ at *m/z* 611.1969, RT = 4.485 min) was presented in green lentils. The MS^2 spectrum of neohesperidin displayed the product ions at *m/z* 593, *m/z* 465, *m/z* 449, and *m/z* 303. The presence of neohesperidin in grapefruit and lime peel was also previously reported by [82]. Compound 11 (Sakuranetin) displayed the $[M + H]^+$ *m/z* at 281.092, RT = 5.75 min, and was observed in brown whole lentil, and confirmed by the product ions at *m/z* 269, *m/z* 203, and *m/z* 175. Sakuranetin is one of the most distinctive natural products of the plant, as previously reported in sweet cherries [83].

3.4.2.3 Flavonols. Compounds 12, 14, 15, and 16 were tentatively identified the presence in red, green, brown, and black lentils, including quercetin 3-*O*-rutinoside (Compound 12) with $[M - H]^-$ at *m/z* 609.1495, quercetin 3-*O*-glucosyl-xyloside (Compound 14) with $[M - H]^-$ at *m/z* 595.1343, quercetin 3-*O*-rhamnoside (Compound 15) with $[M - H]^-$ at *m/z* 447.0972 and quercetin 3-*O*-xyloside (Compound 16) with $[M - H]^-$ at *m/z* 433.0788, respectively. The presence of quercetin 3-*O*-rutinoside in jelly palm and leaves of fishtail palm were discovered in previous studies [84,85]. Quercetin 3-*O*-glucosyl-xyloside could be detected in both ionization modes, identified based on the fragment peaks at *m/z* 265, *m/z* 138, and *m/z* 116, present in mango peel by product from Australia according to [86]. Meanwhile, myricetin 3-*O*-rhamnoside was dispalyed with the molecular formula $C_{21}H_{20}O_{12}$ and the precursor ion $[M - H]^-$ at *m/z* 463.0909 (Compound 13). The MS^2 spectrum of myricetin 3-*O*-rhamnoside showed the product ions at *m/z* 317, indicating the presence of a desoxyhexose sugar [87]. Myricetin 3-*O*-rhamnoside was also discovered in black spices and tobacco [76,88]. Quercetin 3-*O*-rutinoside and quercetin 3-*O*-xyloside were detected in both brown whole and black whole lentil samples; quercetin 3-*O*-glucosyl-xyloside and quercetin 3-*O*-xyloside were identified in brown and green whole lentils; quercetin 3-*O*-rhamnoside was characterized in both red and green whole lentils.

3.4.2.4 Dihydroflavonols and Anthocyanins. Dihydroquercetin ($[M - H]^-$ ion at *m/z* 303.0539 was considered as Compound 17 found in red lentil, yielding product ions at *m/z* 275 [M – H – CO], *m/z* 285 [M – H – H_2O], and *m/z* 151 [M – H – RDA cleavage] [89], was one of the highly specific polyphenols in pears [90]. By contrast to cyanidin 3-*O*-(6′-p-coumaroyl-glucoside), dihydroquercetin was only detected in the red whole lentils. Cyanidin 3-*O*-(6′-p-coumaroyl-glucoside) (Compound 18 with $[M - H]^-$ at *m/z* 594.1394, RT = 4.714 min) was the only anthocyanins present in brown whole lentil. The MS^2 spectrum of cyanidin 3-*O*-(6′-p-coumaroyl-glucoside) showed the product ions at *m/z* 287. The existence of cyanidin 3-*O*-(6′-p-coumaroyl-glucoside) in blackberry fruit and kiwifruit was also previously reported [66,91]. Both compounds (Compound 17 & 18) have been found before in the black lentil samples according to the study of [92].

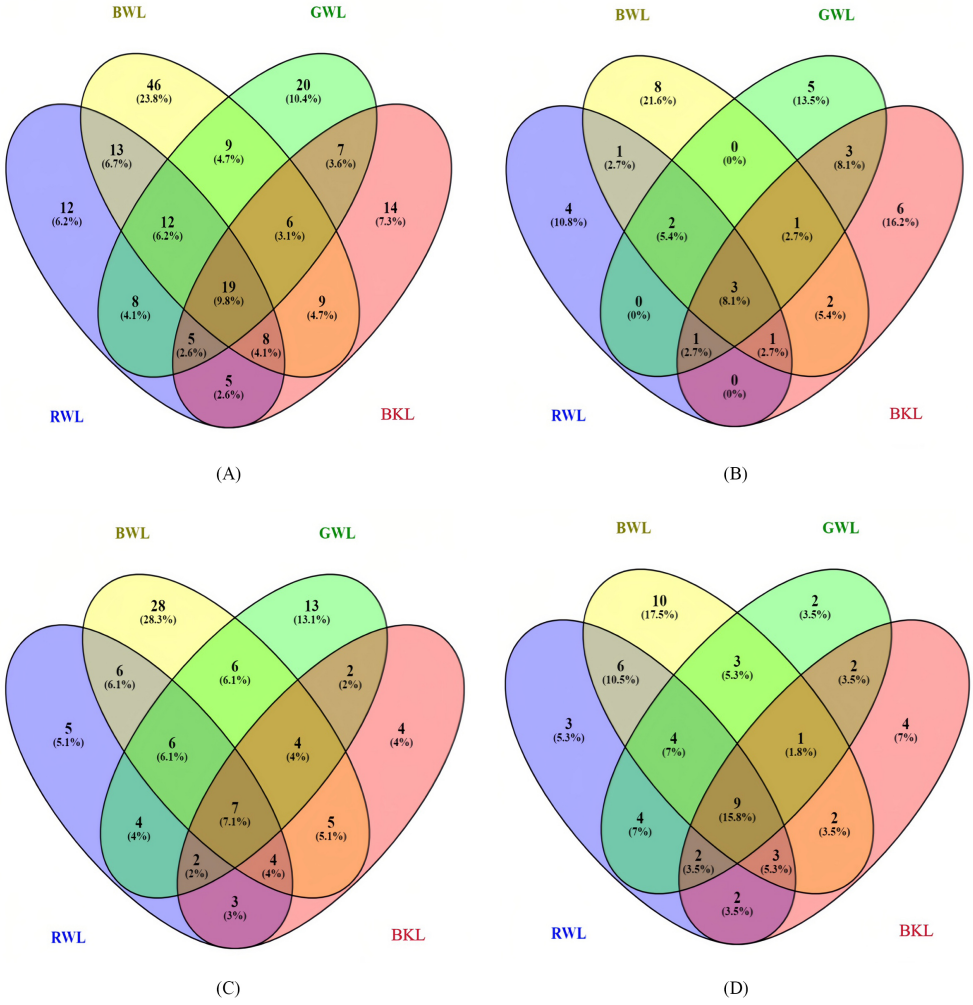

Fig. 1. Venn diagram of phenolic compounds exist in four lentil samples (red, green, brown, and black whole lentils). (A) displays the relations of total phenolic compounds present in different lentil samples. (B) displays the relations of phenolic acids in present in different lentil samples. (C) displays the relations of flavonoids present in different lentil samples. (D) displays the relations of other phenolic compounds present in different lentil samples. As shown in the graph, sample lentils are mentioned in abbreviated form. RWL, red whole lentil; GWL, green whole lentil; BWL, brown whole lentil; BKL, black whole lentil.

3.4.2.5 Isoflavonoids. Compound 19, with the molecular formula $C_{16}H_{12}O_4$ and possessing the precursor ion $[M - H]^-$ at *m/z* 267.0665 in the negative ionization mode in green whole lentil, was characterized as dalbergin. Dalbergin produced fragments at *m/z* 252, *m/z* 224, and *m/z* 180, due to the loss of CH_3 (15 Da), C_2H_3O (43 Da), $C_3H_3O_3$ (87 Da) from the precursor ion, separately [93]. To our knowledge, this is the first time that isoflavonoid derivatives have been identified and characterized in lentils.

3.4.3 Other Polyphenols

One other polyphenol was detected and characterized in the red lentil sample, classified as alkylmethoxyphenols.

Alkylmethoxyphenols. Compound 20 was identified as 4-vinylsyringol according to the precursor ion $[M + H]^+$ *m/z* 243.1026 and was only found in the red lentil sample. The identity of 4-vinylsyringol was confirmed by the product ions at *m/z* 225, *m/z* 211, and *m/z* 197, respectively. 4-vinylsyringol was also present in the giant reed (*Arundo Donax* L.) and edible lotus (*Nelumbo nucifera* G.), in terms of the previous research [70,94].

3.4.4 Lignans

Lignans are represented by two phenylpropane units connected by a C6-C3 bond between the central atoms of the respective side chains [95]. A total of 2 lignans were detected and characterized in two out of four lentils.

Compound 21 with $[M - H]^-$ *m/z* at 557.2422, RT = 45.717 min was only discovered from brown whole lentil and characterized as secoisolariciresinol-sesquilignan in terms of the product ion at *m/z* 539, *m/z* 521, *m/z* 509 and *m/z* 361, corresponding to the loss of CO_2 (44 Da) from precursor ion. Secoisolariciresinol-sesquilignan was

identified as the dominant lignans present in flaxseeds according to the previous study [96]. The presence of secoisolariciresinol-sesquilignan in spices from Australia and palm fruits was also noted [57,97]. Deoxyschisandrin (Compound 22, *m/z* 415.2159) was identified in brown and red lentil samples in negative mode, previously characterized in schisandra [98]. The molecular ions of deoxyschisandrin produced the product ions at *m/z* 402, *m/z* 347, *m/z* 361, and *m/z* 301, corresponding to the loss of CH_3 (15 Da), C_5H_{10} (70 Da), C_4H_8 (56 Da) and $C_7H_{16}O$ (152 Da) from the precursor ion [99]. Both secoisolariciresinol-sesquilignan and deoxyschisandrin could be found in the brown whole lentils, whereas deoxyschisandrin was only detected in the red whole lentils.

3.5 Distribution of Phenolic Compounds—Venn Diagram

Lentils possess a large diversity of phenolic compounds, which vary across varieties. Hence, researchers have developed a strong interest in the distribution of phenolic chemicals in lentils. The distribution of phenolic compounds in lentils, identified in various hues such as BWL (yellow), GWL (green), BKL (red), and RWL (blue), is shown using Venn diagrams (Fig. 1). In terms of the Venn diagram of total phenolic compounds, there are 46 (23.8%), 20 (10.4%), 14 (7.3%), and 12 (6.2%) distinct compounds in brown, green, black, and red whole lentils, respectively. Nineteen (9.8%) compounds were shared by all four lentil samples. The highest number of overlapping total phenolic compounds in BWL and RWL was 13 (6.7%), while the lowest number of overlapping total phenolics was found in red, green, and black lentils (2.6%). In the majority of overlapped compounds and all unique compounds, lentil samples have more flavonoids than phenolic acids. The greatest concentrations of distinct phenolic acids and flavonoids were still shown in brown whole lentils, 21.6%, and 28.3%, respectively. No common overlapping phenolic acids exist between red and black lentils, red and green lentils, and brown and green lentils, which is a considerable divergence, while flavonoids in this area overlapping by 3 (3%), 4 (4%), and 6 (6.1%), respectively. Additionally, three phenolic acids and seven flavonoids were found in each of the four lentils. Unique polyphenols were detected in brown whole lentils (17.5%), green whole lentils (3.5%), black whole lentils (7%) and red whole lentils but not in other polyphenols (5.3%). Four lentil samples had a total of nine (15.8%) polyphenols on average. The highest number of overlapping compounds was six in brown along with red lentils, while the lowest number was three in green, brown, along with black whole lentils. Differences in phenolic composition require further studies to investigate the effects of specific phenolics.

4. Conclusions

Conclusively, antioxidant assays and LC-ESI-QTOF-MS^2 were successfully conducted to determine, identify, and characterize the antioxidant potential and phenolic compounds in four different kinds of lentils. Among them, green lentils had larger quantities of total phenolic compounds than other varieties, whereas red lentils contained higher concentrations of flavonoids, and black lentils indicated higher amounts of condensed tannins. In addition, green lentils exhibited higher antioxidant activities for ABTS, DPPH, FRAP, along with •OH-RSA assays, while black lentils had greater antioxidant activities in terms of FICA and RPA assays. Besides, red lentils were noted with the highest total antioxidant capacity (4.01 mg AAE/g), while brown lentils showed the lowest (2.31 mg AAE/g). The LC-ESI-QTOF-MS^2 technique effectively separated and characterized the phenolic compounds in lentil samples, and a total of 22 phenolic compounds were tentatively identified. Among the discovered phenolic compounds, flavonoids were found the most dominant in various lentils, contributing to certain health potential for human body. *In vitro* digestibility and bioavailability studies should be accessed in the following research to reinforce the commercialization of lentils with therapeutic effects as functional ingredients that can be further applied in the food and pharmaceutical industries.

Availability of Data and Materials

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

Author Contributions

MX and ML designed the research study. MX and ML performed the research. ML and TSPdS conducted the data analysis. MX and ML wrote the manuscript (original draft). TSPdS, CB, FD and HARS wrote the manuscript (review and editing). HARS supervised and administrated the project. HARS carried out funding acquisition. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2803044.

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