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Method Validation and Assessment of Total Antioxidants, Polyphenols, Flavonoids, and Vitamin C in Cultivated Mulberry in Yemen

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Abstract

The nutritional quality of mulberries, particularly their antioxidant content, is vital for human health. Yemen produces mulberries in moderate quantities. This study validated the methods used to measure total antioxidant content (TAC), total polyphenols content (TPC), total flavonoid content (TFC), and vitamin C (Vit. C) in mulberry fruit samples Spectrophotometrically. The results demonstrated high repeatability, with relative standard deviation (% RSD) values ranging from 0.90 to 2.53 % for TAC, 0.65 to 1.87% for TPC, 1.55 to 3.48 % for TFC, and 0.64 to 2.64 % for vit. C. Additionally, the methods showed good linearity correlation coefficients (R^2) values of 0.9964, 0.9984, 0.9985, and 0.9987, high sensitivity, LOD values of 0.089, 0.188, 0.585, and 8.070 ppm, respectively; LOQ values of 0.296, 0.630, 2.854, and 26.900 ppm, respectively, and satisfactory accuracy (%R values of 89.66 to 102.78 % for TAC, 99.76 to 105.14% for TPC, 90.93 to 103.66% for TFC, and 92.80 to 98.21% for Vit. C). Analysis of Yemeni mulberry samples revealed TAC values ranging from 3792.00±0.018 to 6333.33±0.027 mg AAE/kg fw, TPC from 1431.50±0.025 to 3729.60±0.007 mg GAE/kg fw, TFC from 215.20±0.051 to 541.85±0.020 mg QE/kg fw, and Vit. C from 410.26±0.100 to 850.82±0.050 mg AAE/kg fw. This study confirms the methods' validity of antioxidants, which showed high sensitivity, accuracy, good linearity, and repeatability while testing Yemeni mulberry samples. The extracts exhibited high antioxidant activity, with potential applications in the prevention and treatment of oxidative stress-related diseases.

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Keywords:
Mulberry, Validation, Polyphenols, Flavonoids, Vitamin C

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1. INTRODUCTION

Free radical production from oxygen metabolism causes oxidative stress in the physiological system. Antioxidant-rich foods and vegetables can help reduce or avoid oxidative stress, which damages cells and is a contributing factor to conditions like diabetes, heart disease, and macular degeneration [1–9]. Both plants and animals are rich sources of naturally occurring antioxidants [7, 10]. Antioxidants are divided into two groups: fat-soluble and water-soluble. Water-soluble antioxidants, such as uric acid, glutathione, and ascorbic acid, mostly work in blood plasma and cell cytoplasm. As a catalyst, ascorbic acid removes reactive oxygen species (ROS) [7, 11].

Although glutathione has reversible oxidation-reduction capabilities and is a reducing agent, cell membranes are shielded from lipid peroxidation by fat-soluble antioxidants such as ubiquinol, carotenoids, and alpha-tocopherol. The practice of objectively demonstrating that a method continuously satisfies predetermined performance standards for its intended purpose is known as method validation. This phrase and validation are frequently used interchangeably [7].

The Moraceae family includes the black mulberry (*Morus nigra* L.), red mulberry, and white mulberry (*Morus alba* L.) [12]. The leaves of this fruit, which are

Figure 1. Yemeni Mulberry Trees and Mulberry Ripe Fruits

extensively distributed throughout Asia, North America, and Africa, are mostly used as a source of nutrition for silkworms (*Bombyx mori* L.) in several Asian nations. Several food items, for example, jams, ice creams, vinegar, juices, and wine, contain mulberry fruits [13].

Bioactive substances, such as phenolic compounds, flavonoids, anthocyanins, and organic acids, are abundant in black mulberries and provide several health advantages [14]. Phenolic acids (gallic, syringic, and neochlorogenic acids) and flavonoids (quercetin and rutin) are important components; they have anti-inflammatory, anti-cancer, anti-hyperglycemic, anti-hyperlipidemic, antioxidant, and neuroprotective qualities [14, 15].

Mulberry planting has been done in Yemen, especially in the Sana'a region (Figure 1). Numerous previous studies have extracted antioxidants from mulberries using several extraction methods, including solvent extraction [16], Solid-Liquid Extraction [17], Maceration assisted extraction. [18], Ultrasonic-assisted extraction (UAE) [18–21], ultrasonic irradiation [22], Microwave-assisted extraction [23–25].

Total phenolic compounds (TPC) by Folin-Ciocalteu (FC) method [26–28], 2,2-diphenyl-1-picrylhydrazyl DPPH [26–28], 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) [26, 29], ferric-reducing antioxidant power (FRAP) assay [27, 28, 30], total flavonoid contents (TFC) [26, 28, 30], and vitamin C [28, 31–34], are some of the methods used to determine the antioxidants in mulberry samples.

Antioxidant levels in mulberries grown in Sana'a, Yemen, have not been studied. Thus, this research aims to extract and determine the TAC, TPC, TFC, and vitamin C from Yemeni mulberries after validating their detection techniques.

2. MATERIALS AND METHODS

2.1. Spectrophotometer

The TAC, TPC, and TFC were analyzed using a double-beam UV-Vis Spectrophotometer specord200 (Analytik-jena, Germany).

2.2. Chemicals

All solvents and reagents used were of pure analytical grade: 99.7% ascorbic acid from (BDH, UK), 99.8% gallic acid from ACS, 99.9% quercetin from Pspark Scientific, and 96% ethanol (Scharlau, Spain). The deionized water (DIW) was purified using a Direct-Q3 (Millipore, Bedford, MA, USA) water purification system.

2.3. Preparation of Standards Solutions

Stock standard solutions (1000 and 5000 ppm) of ascorbic acid, gallic acid, and quercetin were prepared. The intermediate and the working solutions were prepared by diluting the solutions properly.

2.4. Mulberry Sample Collection and Homogenization

Different types of Yemeni mulberry samples were collected from the areas around Sana'a, Yemen, Ripe fruits of mulberry were sampled. After sampling, the fruits were immediately transported to the laboratory in plastic bags. Their length was about 2–3 cm, and their color was purplish-black, Figure 1. The samples were cleaned by washing using a tap and then DIW, sub-sampled and made ready for further processing, and stored below 4 ℃ in a refrigerator [35].

2.5. Fortification and Extraction of Mulberry Samples

Real samples were fortified with standard ascorbic acid, gallic acid, and quercetin solutions for TAC, TPC, and TFC analysis.

1 g of mulberry samples were mixed with 10 mL of ethanol: water (1:1 v/v), 0.2 g of NaCl was added. The samples were agitated for 10 minutes, centrifuged at 3500 rpm for five min., and stored below 4 ℃ in a refrigerator till be used for analysis.

1.0 g of homogenized mulberry was extracted using 30 mL of DIW and shaken for 15 mins. for vitamin C determination [36].

2.6. Determination of Antioxidants in Mulberry Samples

2.6.1. Determination of TAC

The concentration of TAC in the samples was correlated with the calibration curve of ascorbic acid according to the published methods [37–39], with some modifications. To determine TAC, 50 µL of mulberry extract was added to a 10 mL measuring flask, followed by 2 mL of acetate buffer (pH 4), 1.5 mL $FeCl_3$ (0.01M), and 1.5 mL of 2.2-dipyridyl solution (0.1%w/v). The solution was then made up to 10 mL with DIW, thoroughly mixed, and placed in a water bath at 50 °C for 30 minutes. The absorbance was then measured at 520 nm using a reagent as a blank. The results were expressed as milligrams of ascorbic acid equivalents per kilogram of fresh weight (mg AAE/kg fw).

2.6.2. Determination of TPC

Total polyphenol content determination with some adjustments, TPC was calculated using the Singleton et al. [40]. 200 μL of mulberry extract, 3.6 mL of DIW, and 0.4 mL of Folin–Ciocalteau reagent were added to a 10 mL measuring flask. The mixture was then agitated and allowed to sit for five minutes before adding 4 mL of a 7%w/v sodium carbonate solution. After adding 10 mL of DIW, the solution was incubated for 20 minutes at 50 °C in a water bath. TPC was measured against a reagent blank at λ_{max} 750 nm. A correlation was found between the TPC concentration and the gallic acid calibration curve. The findings were reported in milligrams of gallic acid equivalents per kilogram of fresh weight (mg GAE/kg fw).

2.6.3. Determination of TFC

Using the methodology outlined by Salomon et al. [41], the TFC was calculated. In a 10 mL measuring flask, 3 mL of mulberry extract and 2 mL of 2%w/v aluminum chloride were added. The volume was increased to 10 mL using a (2:1) ethanol: water solvent. Absorbance was measured at 430 nm after 10 minutes against a reagent blank. The analysis was done in triplicate, and the results were reported as milligrams of quercetin equivalents per kilogram of fresh weight (mg QE/kg fw).

2.6.4. Determination of Vit.C

Using redox titration, vitamin C was measured [36, 42]. After weighing 1 g of the sample into a 250 mL conical flask, 30 mL of distilled water was added, after 15 minutes of shaking, 2 mL of an indicator solution containing 0.5%w/v of starch was added. The sample solution was then titrated using an iodine solution of 0.005 mol. L^{-1}. The endpoint of the titration was determined to be the first enduring trace of a dark blue-black hue caused by the starch-iodide combination. The data were presented as milligrams of ascorbic acid equivalents per kilogram of fresh weight (mg AAE/kg fw), and the analysis was carried out in triplicate.

2.7. Validation of Antioxidants Analytical Methods

The method's linearity was determined by analyzing a series of spiked samples at different concentrations of antioxidant standards. The response was plotted versus the concentration of the analyses. The coefficients were calculated from the linear regression equation: y= mx ±b. Different concentrations of antioxidant standards were used to spike the mulberry samples in triplicate to carry out the accuracy and precision experiment. The precision of methods was evaluated using relative standard deviation (RSD, %). The recoveries (% R), LODs, and LOQs for each type of antioxidant standard (Ascorbic acid, Gallic acid, and Quercetin) which is used to determine TAC, TPC, TFC, and vitamin C, respectively, LOD, and LOQ were calculated from the slope of the calibration curve (S) of each type and the standard deviation (SD) of the blank mulberry sample according to the previous published papers [43–48].

3. RESULTS AND DISCUSSION

This study intends to establish the TAC, TPC, TFC, and vitamin C content of Yemeni mulberries following the validation of their detection methods.

3.1. Method Validation Results for TAC in Mulberry Samples

The TAC validation results for mulberry samples are summarized in (Table 1 and Figures 2), the obtained results showed that the curves of mulberry samples have good linearity over the range of concentration study, with correlation coefficients $R^{2=}$09964, the % RSD values ranged from 0.90-2.53%. The results reflected the high repeatability of the method. The recovery (%R) of the ascorbic acid ranged from 89.66–102.78%, which indicates the

Table 1. Method Validation Results for TAC in Mulberry Spiked Samples

Spiked Conc. of Ascorbic Acid (ppm)	Abs. of STD, (n=3)	Abs. of Un-spiked Sample	Ave. Abs. of Spiked Sample	Abs. Diff.	%R	SD	%RSD	LOD (ppm)	LOQ (ppm)
0.50	0.101	0.279	0.372	0.093	93.00	0.005	1.34	0.089	0.296
1.00	0.203	0.279	0.461	0.182	89.66	0.008	1.78		
1.50	0.270	0.279	0.525	0.246	90.99	0.005	0.90		
2.00	0.324	0.279	0.612	0.333	102.78	0.010	1.61		
3.00	0.541	0.279	0.798	0.519	96.00	0.020	2.53		

Conc.= Concentration, Abs.=Absorbance, Ave.=Average, STD=Standard, SD=Standard Deviation,

Diff.=Difference, %RSD=Relative Standard Deviation, LOD=Limit of Detection, LOQ=Limit of Quantification, %R=Recovery Percentage.

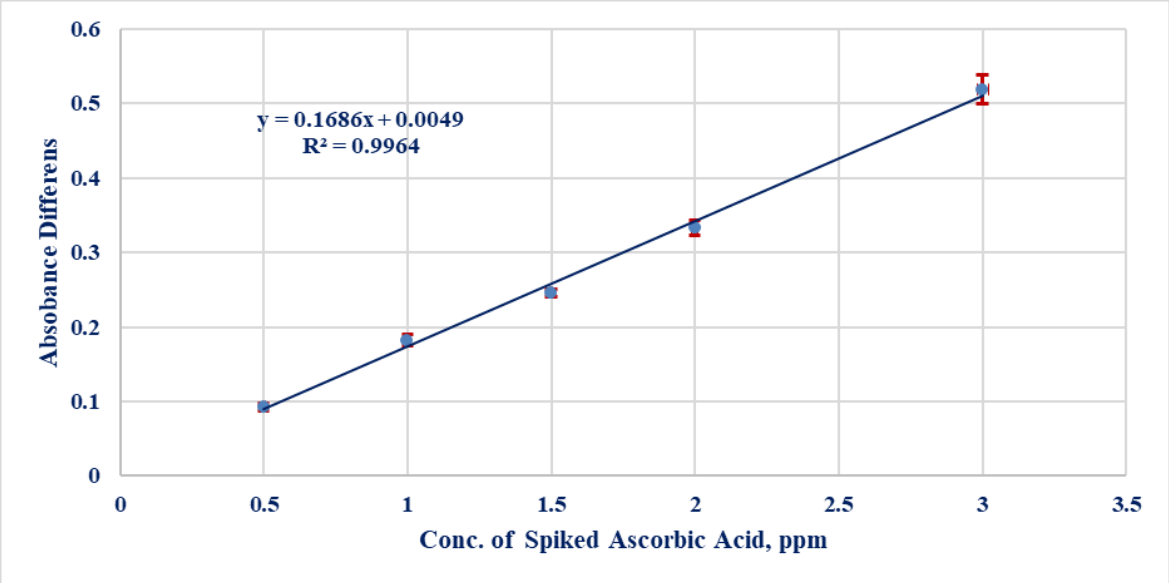

Figure 2. Calibration Curve of TAC of Spiked Mulberry Samples

proposed method has acceptable recovery for analysis of total antioxidants in mulberry samples. The LOD and LOQ values of mulberry samples are 0.089 and 0.296 ppm, respectively.

3.2. Method Validation Results for TPC in Mulberry Samples

The linearity results for the mulberry sample were summarized in (Table 2). The calibration curve was plotting the concentrations against the absorbance as shown in (Figure 3), the curve has a good linearity range, with a coefficient of determination R^2 of 0.9984. The relative standard deviation (%RSD) values ranged from 0.65 to 1.87%, reflecting the high precision of the analysis method. The recovery (%R) of gallic acid was in the range of 99.76- 105.14%. The computed LOD and LOQ values obtained 0.188 and 0.630 ppm, respectively.

3.3. Method Validation Results for TFC in Mulberry Samples

The calibration curve plotting the concentrations against the absorbance is shown in (Table 3 and Figure 4). The results showed that the curve of mulberry samples has good linearity over the range of concentration study, with correlation coefficients R^2 0.9985. The % RSD values ranged from 1.55%-3.48%. The results reflected the high repeatability of the analysis method. The accuracy was then calculated as the recovery %R, which ranged from 90.93-103.66%. The computed LOD and LOQ values obtained 0.585 and 2.854 ppm, respectively.

3.4. Method Validation Results for Vitamin C in Mulberry Samples

The calibration curve plotting the concentrations against the Volume Difference is shown in (Table 4 and Figure 5). The results showed that the curve of mulberry samples

Table 2. Method Validation Results of TPC in Spiked Mulberry Samples

Spiked Conc. of Gallic Acid (ppm)	Abs. of STD, (n=3)	Abs. of Un-spiked Sample	Ave. Abs. of Spiked Sample	Abs. Diff.	%R	SD	%RSD	LOD (ppm)	LOQ (ppm)
2.0	0.130	0.284	0.420	0.136	105.14	0.005	1.07	0.188	0.630
4.0	0.278	0.284	0.562	0.278	100.00	0.011	1.87		
6.0	0.412	0.284	0.695	0.411	99.76	0.007	1.01		
8.0	0.577	0.284	0.863	0.579	100.40	0.011	1.30		
10.0	0.704	0.284	0.988	0.704	100.09	0.006	0.65		

Conc.= Concentration, Abs.=Absorbance, Ave.=Average, STD=Standard, SD=Standard Deviation,

Diff.=Difference, %RSD=Relative Standard Deviation, LOD=Limit of Detection, LOQ=Limit of Quantification, %R=Recovery Percentage.

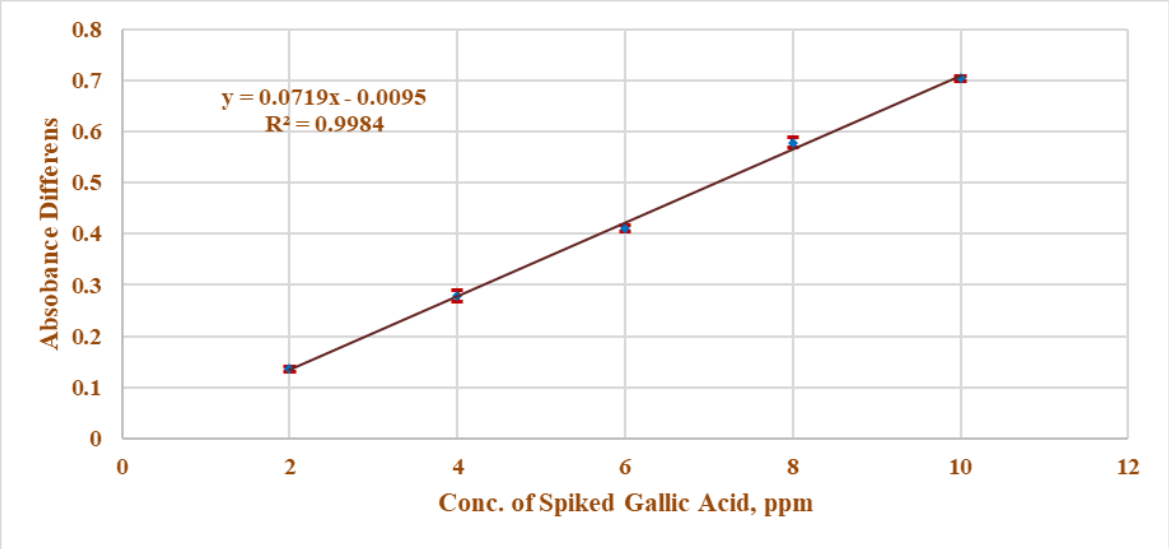

Figure 3. Calibration Curve of TPC of Spiked Mulberry Samples

Table 3. Method Validation Result for TFC in Spiked Mulberry Samples

Spiked Conc. of Quercetin (ppm)	Abs. of STD, (n=3)	Abs. of Un-spiked Sample	Ave. Abs. of Spiked Sample	Abs. Diff.	%R	SD	%RSD	LOD (ppm)	LOQ (ppm)
2.50	0.140	0.405	0.532	0.127	90.93	0.014	2.64	0.585	2.854
5.00	0.259	0.405	0.648	0.243	93.70	0.015	2.28		
7.50	0.389	0.405	0.774	0.369	94.78	0.027	3.48		
10.00	0.492	0.405	0.915	0.510	103.66	0.014	1.56		
15.00	0.749	0.405	1.138	0.733	97.82	0.018	1.55		

Conc.= Concentration, Abs.=Absorbance, Ave.=Average, STD=Standard, SD=Standard Deviation,

Diff.=Difference, %RSD=Relative Standard Deviation, LOD=Limit of Detection, LOQ=Limit of Quantification, %R=Recovery Percentage.

has good linearity over the range of concentration study, with correlation coefficients R^2 0.9987. The relative standard deviation (%RSD) values ranged from 0.64-2.64%, as shown in (Table 4). The results reflected the high repeatability of the analysis method. The accuracy was then calculated as the (recovery, %R) ranged from 92.80-98.21%, and the method sensitivity as LOD and LOQ values were 8.070 and 26.900 ppm.

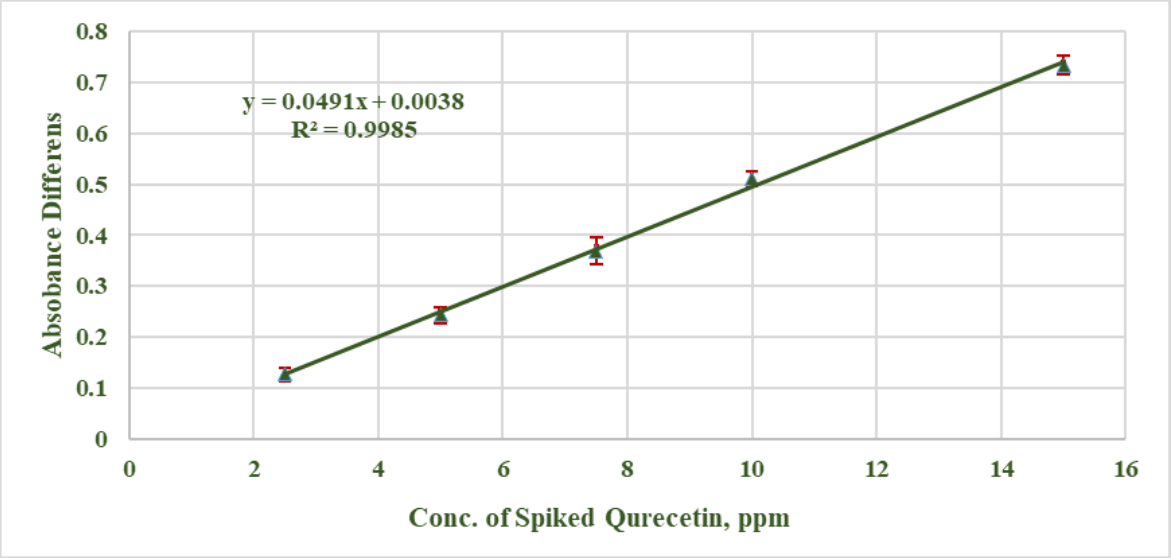

Figure 4. Calibration Curve of TFC of Spiked Mulberry Sample

Table 4. Method Validation Results for Vitamin C in Mulberry Spiked Samples

Spiked Conc. of Vit. C, ppm	Iodine Volume, mL (n=3)	Unspiked	Ave. Volume	Ave. Diff., n=3	%R	SD	% RSD	LOD (ppm)	LOQ (ppm)
12.50	0.417	2.2	2.59	0.39	92.80	0.064	2.49	8.070	26.900
25.00	0.733	2.2	2.89	0.69	94.55	0.050	1.73		
37.50	1.133	2.2	3.26	1.06	93.53	0.021	0.64		
50.00	1.317	2.2	3.49	1.29	97.72	0.036	1.03		
75.00	1.982	2.2	3.96	1.906	96.16	0.104	2.64		
100.00	2.607	2.2	4.76	2.56	98.21	0.040	0.85		

Conc.= Concentration, Abs.=Absorbance, Ave.=Average, STD=Standard, SD=Standard Deviation,

Diff.=Difference, %RSD=Relative Standard Deviation, LOD=Limit of Detection, LOQ=Limit of Quantification, %R=Recovery Percentage.

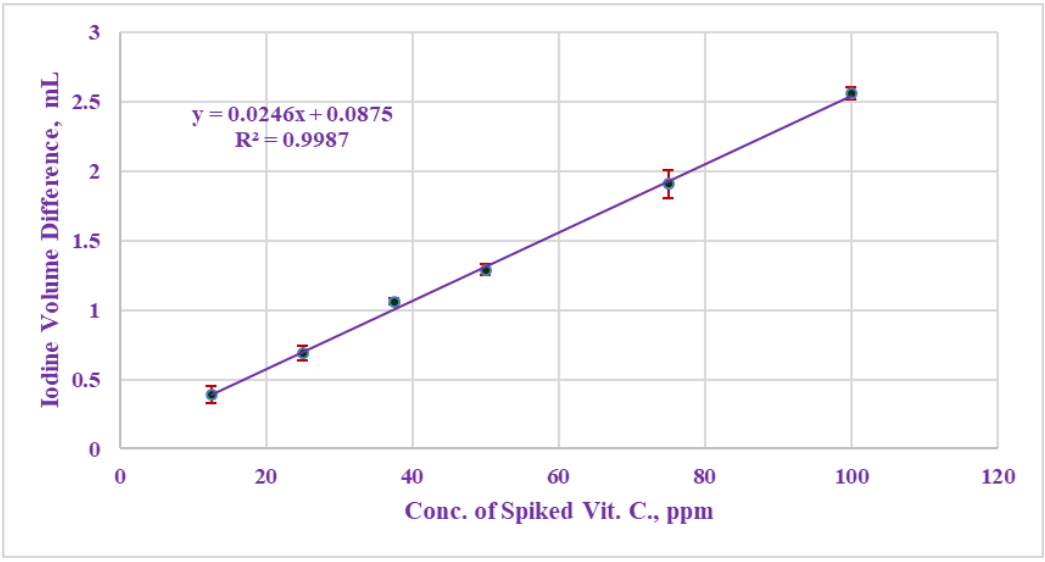

Figure 5. Calibration Curve of Spike Mulberry with Vitamin C

Table 5. Mulberry Samples Analysis Results of Antioxidants Results

Samples	TAA, mg AAE/kg fw	TPC, mg GAE/kg fw	TFC, mg Qu E/kg fw	Vit. C, mg/kg fw
Muleberry1	4247.79 ± 0.052	1431.50 ±0.025	288.46 ±0.001	754.72 ±0.071
Muleberry2	6333.33 ± 0.027	3729.60 ±0.007	292.05 ±0.256	680.47 ±0.161
Muleberry3	3792.00 ± 0.018	1780.22 ±0.010	236.24 ±0.002	410.26 ±0.100
Muleberry4	6116.54 ±0.018	3017.75 ±0.001	352.91 ±0.006	613.50 ±0.035
Muleberry5	5333.33 ± 0.060	2840.53 ±0.006	387.03 ±0.012	612.08 ±0.029
Muleberry6	5584.91 ±0.001	2962.94 ±0.001	541.85 ±0.020	850.82 ±0.050
Muleberry7	4746.03 ±0.021	2427.35 ±0.005	420.90 ±0.021	606.72 ±0.029
Muleberry8	5688.89 ±0.015	2946.30 ±0.010	215.20 ±0.051	623.43 ±0.076
Muleberry9	4934.80 ±0.020	2789.54 ±0.054	388.72 ±0.018	623.60 ±0.053
Muleberry10	6135.30 ±0.071	3234.02 ±0.076	467.19 ±0.046	833.20 ±0.068
n=3, fw= fresh weight				

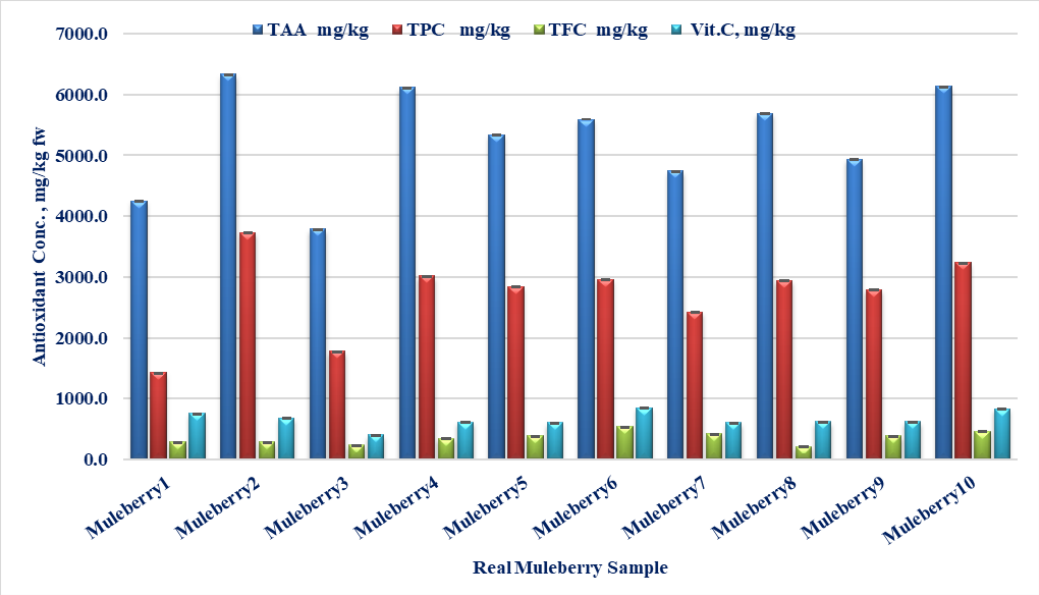

Figure 6. TAC, TPC, TFC, and Vit. C of Mulberry Real Samples Results

3.5. Real Samples Analysis Results

The validated methods were applied to the analysis of ten samples of mulberry cultivated in Yemen; the results are summarized in Table 5 and illustrated in Figure 6. TAC ranging from 3792.00±0.018- 6333.33±0.027 mg AAE/kg fw.

The total polyphenol content of mulberry samples ranged from 1431.50±0.025-3729.60±0.007 mg GAE/kg fw, as indicated in Table 5. Numerous studies were reported for TPC in mulberry samples, some of these studies had lower and higher values like the results obtained by (Skender et al. (2019) [33], Sona et al. (2022) [34], Carmine et al. 2019 [49], and Tian et al. (2025) [50], the results were (1951 - 2733 mg GAE/ kg fw), (1951 - 2733 mg GAE/ kg fw), equal to about (4855±7.1 mg GAE/kg fw), and (8420 to 15024 mg/kg), respectively.

The total flavonoid of mulberry samples varied between 215.20 ±0.051 and 541.85 ±0.020 mg QE/kg fw (Table 5). A study reported by Rong-Li MO et al. (2024) [30], showed that the amount of TFC was 250.75 mg QE/kg fw, which is lower than the values in this study.

While, Kolayli et al. (2024) [28], found the amount of TFC was reported to be 1643.3±6.12 mg QE/kg fw, which is higher than the values in this study. Flavonoids are among the main phenolic compounds in dietary extracts because of their strong antioxidant qualities and role as precursors for taste chemicals [51].

Vitamin C content mulberry samples were between 410.26 ±0.100 - 850.82 ±0.050 mg AAE/kg fw (Table 5). Skender et al. (2019) [33], showed that the amount of vitamin C ranged between 197.9±0.01 and 828.6± 0.05 mgAAE/kg fw. In contrast, 174.1 ± 0.35 – 283 ±1.10 mg/kg fw by Sona et al. (2022) [34], and 273.7±80.67 mg AAE/kg by Kolayli et al. (2024) [28], the values obtained in these studies were found to be lower than our study. Vitamin C reduces oxidative stress, fostering wound healing, and preserves a robust immune system. It is a vital nutrient for healthy skin because of its antioxidant qualities, shielding the body from harm brought on by free radicals (Bisma et al., 2021) [52].

The analysis results of mulberry samples demonstrate their excellent antioxidant properties. The TAC, TPC, TFC, and vitamin C levels in mulberry samples reflect their exceptional antioxidant qualities. These results are consistent with many other studies. Furthermore, the spectrophotometric measurements of TAC, TPC, TFC, and vitamin C, which demonstrated high repeatability, good linearity, and high sensitivity and accuracy in analyzing berry samples, are confirmed to be reliable by our study [53, 54].

4. CONCLUSION

This study explored a validated method for measuring antioxidants, including Total Antioxidant Content (TAC), Total Polyphenols Content (TPC), Total Flavonoids Content (TFC), and Vitamin C (vit. C) in mulberry samples commonly consumed in Yemen. Using a spectrophotometric assay, the method effectively extracted and quantified these antioxidants. The results demonstrate that the validated method is suitable for the determination of the antioxidants, with confirmed linearity, repeatability, and accuracy. The study highlights the potential of Yemeni mulberries as a rich source of bioactive compounds with potential health benefits, and provides new insights into the nutritional and pharmacological properties of these traditional fruits.

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