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Research Article

Variations of Carotenoids, Total Bioactive Contents, and Antioxidant Activity in Leaves of *Medicago polymorpha*

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Medicago polymorpha L. (MP) is a herbaceous plant commonly known as "bur clover." It is aboriginal to Central and Western Asia and extensively distributed around the world. This study determines the changes in carotenoids and antioxidant potential of different MP of different origins. The sample leaves were analyzed for identification and quantification of carotenoids using a reversed-phase HPLC system. The result showed nine carotenoids and pigments in each sample. The major carotenoid compounds were phytoene, phytofluene, neoxanthin and its isomer (9-*Z*-neoxanthin), violaxanthin, lutein along with their isomers (9-*Z*-lutein, 9′-*Z*-lutein), and all-*E*-*β*-carotene. The major pigments were 15-hydroxy-lactone chlorophyll *a*, pheophytin *a*, pheophytin *a* ′, pyropheophytin *a*, divinyl chlorophyll *a*, chlorophyll *b* ′, chlorophyll *b*, 13′-hydroxy-lactone chlorophyll *b*, and hydroxy pheophytin *a* ′. The carotenoids were detected in considerable amounts in the samples from Lower Dir (213 *μ*g/g), Swat (171 *μ*g/g), and Buner (157 *μ*g/g). Chlorophylls were higher in Lower Dir (203.4 *μ*g/g), Swat (184.0 *μ*g/g), and Buner (152.2 *μ*g/g) and significantly lower amounts in Malakand samples (141.7 *μ*g/g). The total carotenoids in Lower Dir (51.2%) were higher than in Swat (48.2%), Buner (50.8%), and Malakand samples (44.6%). The amounts of violaxanthin and lutein were significantly higher in Lower Dir (73.6%) samples, followed by Malakand (51.7%), whereas Buner and Swat samples showed the lowest levels. In conclusion, MP leaves are a good source of important carotenoids having potential antioxidant properties, which are highly correlated to the violaxanthin and lutein contents.

1. Introduction

Medicago polymorpha L. (Bur medic) is an effectively polymorphic species of the Medicago genus and Fabaceae family and comprises 87 different types of species [1]. The plant is extensively naturalized throughout the Mediterranean Basin and adjacent arid land [2, 3]. It is found in the Mediterranean climate region as a nitrogen-fixing, influentially palatable, and nutritious plant [4–6]. Unlike other medic ($x = 8$), Bur medic carries $x = 7$ basic genomic number [7]. Germinating in late autumn, its trifoliate leaves are formed before branches are produced from crowns and their flowers are mostly alone or in some members reach ten [8]. It boosts soil fertility, improves yield, and saves wedding costs, if, in relationship with winter weed control crops. A sustainable agricultural system, it has been incorporated into integrated pest management programs [9].

To improve memory efficiency, traditionally, *Medicago sativa* was used as an antioxidant and anti-inflammatory in central nervous system (CNS) disorders. Also, it is extensively utilized as a homeopathic and Ayurvedic medicine in a diverse range of CNS disorders [10]. Isoflavone synthase enzyme from model legume barrel medic was used to improve expression and solubility for structural studies [11]. Medic species were also subjected to introductory antianxiety screening studies [10]. In nature, more than 700 carotenoids have been detected and isolated [12]. Carotenoids are beneficial due to their chemical properties and perform a conserved function in all photosynthetic organisms. Accessory pigments like xanthophylls and

Table 1: The sample collection sites, districts, humidity, pressure, temperature, herbarium status, and irrigation system.

Collection site	District	Humidity (%)	Temperature (°C)	Herbarium status	Irrigation system
Sirsinai	Swat	18	18	Local field	Rain+well water
Batkhela	Malakand	21	19	Local field	Rain+river water
Peer Baba	Buner	33	14	Local field	Rain+well water
Chakdara	Dir	29	13	Local field	Rain+tube well water

oxygenated carotenes are the light-harvesting antennae of the chloroplasts, which are responsible for energy transformation to the chlorophylls [13]. The main function of nearly all carotenoids in nature is the conversion into beneficial vitamins and nutrients [14]. In addition, carotenoids are involved in pollination and pigmentation [15]. Additionally, carotenoids are helpful in the synthesis of plant hormones like strigolactones [16, 17]. However, there is a paucity of research on the carotenoids and chlorophyll content of MP. The current study's objective was to assess the carotenoid content and variation in carotenoid content found in samples taken from various locations within Pakistan's Malakand Division.

2. Materials and Methods

2.1. Materials. The analytical solvents involved in this study are BHT, methanol, ethanol, diphenyl-1-picrylhydrazyl (DPPH), methyl tertiary butyl ether, ammonium acetate, and acetone which were purchased from Merck (Karachi, Pakistan). A deionization system (Milli-Q) was used to prepare deionized double distilled water (Merck, Darmstadt, Germany). All other related solvents and reagents were of the highest purity.

2.2. Sample Collection. Healthful unwilted samples of *Medicago polymorpha* were collected from different localities of KPK, Pakistan. During sample collection, the irrigation system, herbarium status, temperature, pressure, humidity, and collection sites of the samples are documented in Table 1. The separated leaves were washed with tap water, kept in the shade for dehydration, and, at last, grinded into a paste. Then, leaves' paste was used for further analysis of extractions.

2.3. Extraction. Extraction of grinded MP leaves was carried out via the reported method with some modifications [18]. Firstly, ice-cold acetone (10 mL) was taken and added to the grinded leaf sample (1 g) and shaken for one hour at $1 \times g$. Next, 5 mL pure ethanol was mixed and shaken (Wittig Labor Technik, Wertheim, Germany) for half an hour. A Whatman filter was used to filter the mixture. The extraction was continued till the leaf's discoloration. Solvents were removed from the mixture under vacuum and at a temperature of 35–40°C. The residue was then mixed with two milliliters of HPLC solvent, and an Agilent syringe filter with a pore size of 0.45 μm was used to filter into the HPLC vials.

Several solvent extracts, including water, methanol-water, and methanol, were used to assess the total phenolic TPC, TFC, and RSA of different MP. For this purpose, 1 g sample was mixed with either water (100%), methanol-water (1 : 1, *v*/*v*), or methanol (100%) separately for 24 hours. The extract was filtered to remove the residue.

2.4. Analysis of Carotenoids. Agilent HPLC system (1260 Infinity, Agilent Technologies, Waldbronn, Germany) was equipped with a DAD detector, autosampler, degasser, and a quaternary pump. A rapid Agilent Zorbax Eclipse column (C18, 4.6 × 100 mm, 3.5 μm, 1260 Infinity, Agilent Technologies, Waldbronn, Germany) was employed and kept at 25°C. The three solvents, i.e., A (10 mM ammonium acetate, methanol, and deionized water), B (0.1 mM ammonium acetate in distilled water), and C (100% of MTBE), were utilized as gradient system [19]. The sample amount injected was 50 at a flow rate of 1 mL/min. Before initiation (0 min), the gradient system ABC was 80, 18, and 2%, respectively. After 25 min, the gradient was changed to 65, 5, and 30, and at 40 min, it reached 60, 0, and 40% with 10 min of postrun of the starting gradient.

The OpenLab ChemStation was utilized to record spectra and chromatograms across the wave range of 190 to 750 nm. The published literature [20] states that standard compounds, retention times, and absorption spectra were useful to identify and characterize carotenoids and pigments. The chromatogram's peak area served as the basis for the qualitative analysis of carotenoid compounds, which were then reported as milligrams per 100 g of fresh weight basis.

2.5. Total Phenolic Contents. Total phenolic contents (TPC) were determined in MP leaves using Folin-Ciocalteu (FC) reagent as previously stated in detail by Prior et al. [21]. Fresh FC reagent was used in these experiments. For TPC measurements, a spectrophotometer was used to measure the absorbances of the samples, standards, and blank. The unit of TPC values was mg of gallic acid equivalents (GAE)/100 g sample using gallic acid as a standard calibration curve.

2.6. Total Flavonoid Contents. The total flavonoid contents (TFC) of the MP samples were measured using 2% fresh $AlCl_3$ solution. The method starts with 0.5 mL extracts, and 0.5 mL of $AlCl_3$ was subsequently added and then incubated for 1 h. A spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 420 nm was used for the analysis of TFC in each sample. The TFCs were expressed as mg/g based on a fresh weight basis and a standard calibration curve of rutin.

2.7. DPPH Scavenging Activity. Using the DPPH assay, the radical scavenging activity (RSA) of MP was determined for each sample in triplicate [22]. In brief, DPPH solution (0.1 mM, 1.95 mL) was combined with the sample extract (50), and the mixture was incubated for 30 minutes in the

dark. Next, a Pharmaspec 1700 spectrophotometer (Shimadzu, Kyoto, Japan) was used to analyze the sample absorbance at 515 nm. The absorbance of the standard DPPH solution and the samples were used to compute the percent RSA.

2.8. Principal Component Analysis. The GraphPad Prism version 10.0.2 (GraphPad Software, Inc., California, USA) was used to conduct principal component analysis (PCA). In each sample, TPC, TFC, RSA, and detected carotenoids and chlorophylls were included. Using loadings and scores in dimensional spaces created by PCs with eigenvalues larger than 1, principal component analysis (PCA) biplots were used to map the variables (individually identifiable carotenoid and chlorophyll, TPC, TFC, and RSA) and samples (Swat, Malakand, Buner, and Lower Dir) (Kaisar rule).

2.9. Data Analyses. Every analysis was done in triplicate, unless otherwise specified. An analysis of variance (ANOVA) in one direction was performed on the gathered data. The Holm-Sidak method post hoc test was also carried out with GraphPad Prism version 10.0.2. (GraphPad Software, Inc., California, USA).

3. Results and Discussion

3.1. Carotenoid and Chlorophyll Composition. Plant carotenoids are composed of forty carbon isoprenoids with polyene chains which maintain up to fifteen conjugated bonds. Carotenoids are beneficial due to their chemical properties and perform a conserved function in all photosynthetic organisms [13]. Figure 1 shows 18 peaks of carotenoids and chlorophylls in each sample of *M. polymorpha* along with detailed characteristics (Table 2). These contain nine carotenoids and chlorophylls each. A 40-carbon symmetric molecule "phytoene" was identified with the absorption maxima (λ_{max}) of 286 nm. Compound 2 was identified as "phytofluene" with 329, 347, and 366 nm of absorption maxima. It was also identified by Ignasiak and Lesins [23] in *M. falcate* and *M. platycarpos*.

All-*E*-neoxanthin and 9-*Z*-neoxanthin were identified from λ_{max} of 416, 440, and 468 nm and 412, 436, and 464 nm, respectively. These compounds were also reported by Ignasiak and Lesins [24] in *M. ruthenica* and *M. cretacea*. Compound 4 was "15-hydroxy-lactone chlorophyll *a* $''$ having λ_{max} of 418, 614, and 654 nm. 15-Hydroxy-lactone chlorophyll *a* was found in a previous report by Matsubara et al. [25]. At peaks 5 and 6 with λ_{max} of 410, 508, 538,610, and 666 nm, and 408, 508, 538, 610, and 666 nm, two compounds, namely, pheophytin *a* and pheophytin *a* $'$, were identified.

Similarly, compounds 7 and 8 were "pyropheophytin *a*" and "divinyl chlorophyll *a*" with λ_{max} of 410, 610, 666, and 608 nm and 410, 608, and 666 nm, respectively. At absorption maxima of 416, 440, and 472 nm and 424, 448, and 472 nm, two important carotenoids, namely, all-*E*-violaxanthin and all-*E*-lutein, were eluted. Both were previously reported by Ignasiak and Lesins [23]. However, a significant amount of lutein was also reported in 14 medic species and considered 30-80% of the total carotenoid's contents. At peak 11 and peak-12, 9-*Z*-lutein and 9$'$-*Z*-lutein were detected as having characteristics absorption maxima (325, 418, 442, and 468 nm and 330, 418, 440, and 466 nm). These compounds were evaluated by Demmig-Adams [26] in different *Medicago* species.

Similarly, peak 13 was chlorophyll *b* $'$ and 14 was chlorophyll *b*. Chlorophyll *b* $'$ and chlorophyll *b* were also reported by Demmig-Adams [26] in *Medicago*. Peak 15 was 13$'$-hydroxy-lactone chlorophyll *b* having an absorption maxima of 462, 600, and 650 nm. This chlorophyll compound was also reported previously by Matsubara et al. [25]. Peak 16 and 17 were identified as "9-*Z*-neoxanthin" and "hydroxy pheophytin *a*" with λ_{max} of 412, 436, 464, and 408 and 504, 534, 610, and 666 nm. Peak 18 was all-*E*-β-carotene with the λ_{max} of 418, 452, 478 nm. β-Carotene was found the last compound and previously reported by Fernández-Marín et al. [27]. The amount of β-carotene along with other carotenoids can be enhanced with the application of potassium nitrate priming on different *Medicago* species [28].

3.2. Variations of Carotenoids and Chlorophylls. Table 2 demonstrates that the levels of phytoene in each sample did not significantly differ from one another. A higher phytofluene content was present in the samples from Swat (8.5 μg/g), Buner (5.7 μg/g), and Malakand (5.5 μg/g), whereas the lowest was present in Lower Dir (2.7 μg/g). The amount of phytoene production in plants can be affected significantly by genetic factors, light, and nitrogen reduction [29]. Thus, the difference in the location of plants could affect the production of phytoene. The levels of all-*E*-neoxanthin in Lower Dir, Swat, and Buner samples were not significantly different, whereas the greatest concentrations were found in Buner samples (14.5 μg/g). Neoxanthin has been found to protect plants and algae from light-induced stress [30]. The reason neoxanthin is important is because its lack may have an impact on the functional chlorophyll antenna size of photosystem-II, but not photosystem-I [31].

15-Hydroxy-lactone chlorophyll *a* was significantly greater in samples from Swat (32.4 μg/g), Malakand (23.4 μg/g), and Buner (19.0 μg/g) samples. Pheophytin *a* was present in significantly elevated amounts in Malakand samples (24.9 μg/g), while a lower amount was found in Buner (17.6 μg/g), Lower Dir (18.1 μg/g), and Swat (19.4 μg/g). There were significant variations in the amounts of pheophytin *a* $'$ in all samples. No significant variations in amounts of pyropheophytin in samples from Lower Dir and Swat, whereas the amounts were significantly lower in Buner and Malakand samples. Lower Dir had a notably greater concentration of divinyl chlorophyll *a* (33.0 μg/g), and there were no significant variations in the samples of Swat, Buner, and Malakand. Pheophytins are responsible for electron carriers in photosystem II; nevertheless, they also have a big impact on how ripe the berries become [32]. In the ear of BALB/c mice, pheophytin significantly reduced the inflammatory response brought on by 2-tetradecanoylphorbol-13-acetate, including the formation of edema [33].

All-*E*-violaxanthin was significantly higher in Swat (12.9 μg/g) and Buner (11.3 μg/g) samples, whereas Lower Dir samples displayed significantly smaller amounts

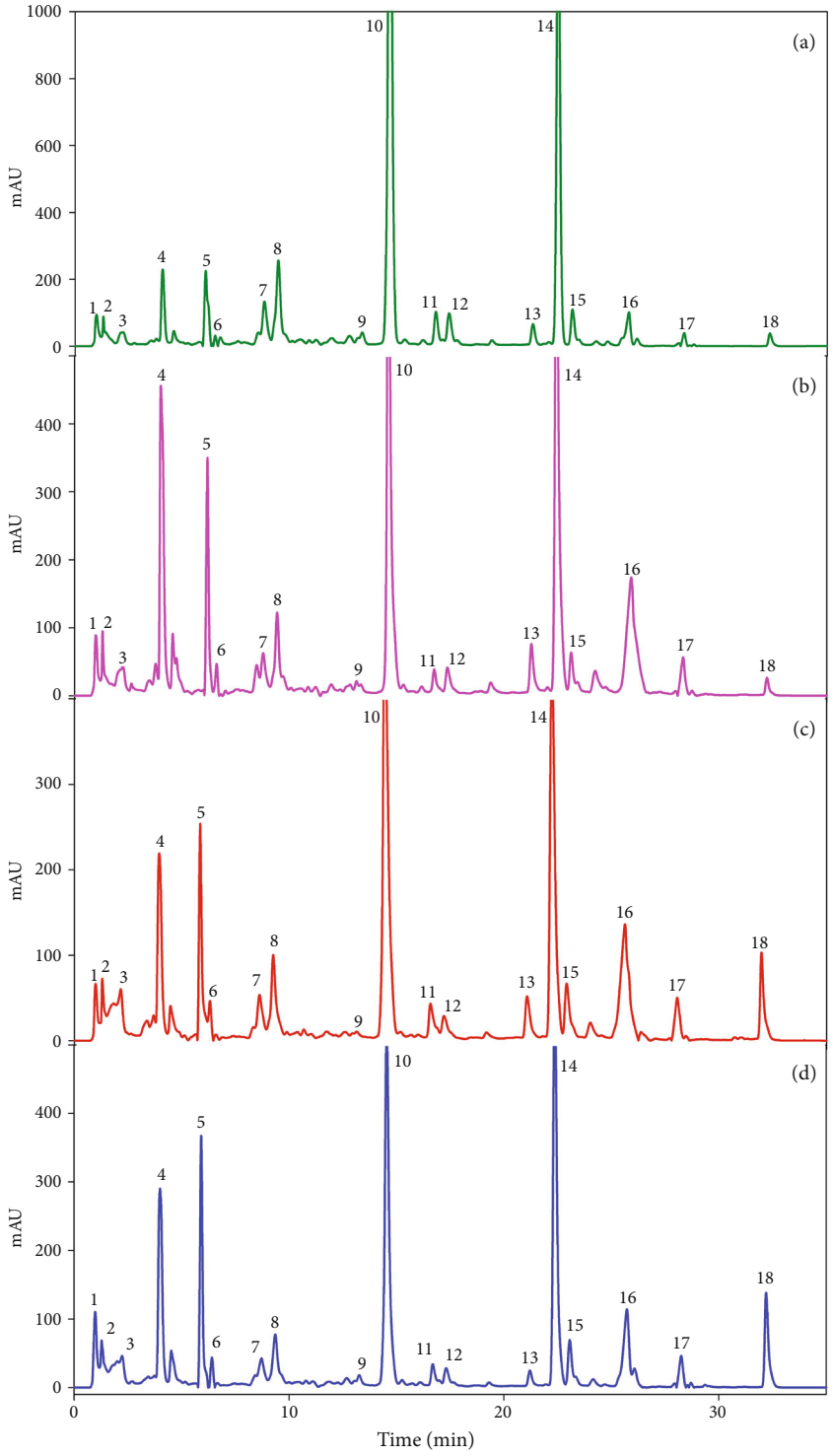

Figure 1: Representative HPLC-DAD chromatograms of leaves of *Medicago polymorpha* of the selected regions. (a) Lower Dir, (b) Swat, (c) Buner, and (d) Malakand samples.

(1.3 μg/g). In Dir samples, all-*E*-lutein was the most abundant (156 μg/g), while no significant variation was noted in samples of Swat and Buner. Similarly, the concentration of 9-*Z*-lutein in the samples of Lower Dir was 11.7 μg/g. There was no significant difference in the samples of Buner and Swat, whereas Malakand samples showed significantly lower amounts (6.0 μg/g). A similar trend was noted for 9′-*Z*-lutein.

Chlorophyll b' was found in higher amounts in Swat (12.7 μg/g), Buner (10.3 μg/g), Lower Dir (8.8 μg/g), and Malakand (6.5 μg/g) samples. The highest concentration of chlorophyll *b* was detected in Lower Dir (98.2 μg/g) and

Table 2: Variations in the composition of carotenoids and pigments in *Medicago polymorpha* from various regions.

Peak no.	Compound	Absorption maxima (nm)	Pigments and carotenoids (μg/g)*			
			Lower Dir	Swat	Buner	Malakand
1	Phytoene	286	5.9 ± 0.5a	6.2 ± 0.1a	5.7 ± 0.3a	7.0 ± 0.9b
2	Phytofluene	329, 347, 366	2.7 ± 0.5a	8.5 ± 0.9b	5.7 ± 0.1c	5.5 ± 1.2c
3	All-*E*-neoxanthin	416, 440, 468	7.0 ± 0.1a	7.0 ± 0.01a	14.5 ± 0.5b	4.1 ± 1.0c
4	15-Hydroxy-lactone chlorophyll *a*	418, 614, 654	14.3 ± 1.1a	32.4 ± 2.9b	19.0 ± 0.3c	23.2 ± 1.1d
5	Pheophytin *a*	410, 508, 538, 610, 666	18.1 ± 1.1a	19.4 ± 0.4a	17.6 ± 0.2a	24.9 ± 0.7b
6	Pheophytin *a* ′	408, 508, 538, 610, 666	4.0 ± 0.2a	8.1 ± 0.9b	6.2 ± 0.3c	1.5 ± 0.01d
7	Pyropheophytin *a*	410, 610, 666	14.0 ± 1.3a	13.7 ± 0.6a	11.8 ± 0.01b	8.5 ± 0.4c
8	Divinyl chlorophyll *a*	408, 608, 666	33.0 ± 1.7a	16.5 ± 2.6b	15.5 ± 0.2b	14.6 ± 1.4b
9	All-*E*-violaxanthin	416, 440, 472	1.3 ± 0.2a	12.9 ± 1.8b	11.3 ± 0.2b	6.8 ± 0.4c
10	All-*E*-lutein	424, 448, 472	156.1 ± 3.1a	66.6 ± 1.5b	63.0 ± 1.7b	52.2 ± 2.0c
11	9-*Z*-Lutein	325, 418, 442, 468	11.7 ± 0.1a	8.2 ± 0.2b	8.7 ± 0.2b	6.0 ± 0.7c
12	9′-*Z*-Lutein	330, 418, 440, 466	11.2 ± 0.7a	13.0 ± 0.5b	9.2 ± 0.3b	5.3 ± 1.0d
13	Chlorophyll *b* ′	462, 600, 648	8.8 ± 0.2a	12.7 ± 0.4b	10.3 ± 0.3c	6.5 ± 1.0d
14	Chlorophyll *b*	464, 600, 648	98.2 ± 1.0a	61.5 ± 2.7b	48.9 ± 0.3c	48.6 ± 2.2c
15	13′-Hydroxy-lactone chlorophyll *b*	462, 600, 650	7.6 ± 3.1a	10.4 ± 0.1b	11.5 ± 0.3b	6.7 ± 0.9a
16	9-*Z*-Neoxanthin	412, 436, 464	15.1 ± 0.5a	37.3 ± 1.5b	29.7 ± 0.4c	15.6 ± 0.3a
17	Hydroxy pheophytin *a* ′	408, 504, 534, 610, 666	5.4 ± 0.2a	9.3 ± 0.01b	11.4 ± 0.3c	7.2 ± 0.2d
18	All-*E*-β-carotene	418, 452, 478	2.8 ± 0.01a	11.9 ± 0.2b	9.4 ± 0.1c	11.6 ± 0.8b
Total amounts			417.2	355.6	309.4	255.8
Violaxanthin-lutein (%)			73.6	46.3	47.2	51.7
Total carotenoids (%)			51.2	48.2	50.8	44.6
Total chlorophylls (%)			48.7	51.7	49.1	55.3

*Values are means with standard deviation of triplicate readings. Different letters (a-d) in the same row represent significance among the samples at $p < 0.05$ at Holm-Sidak method.

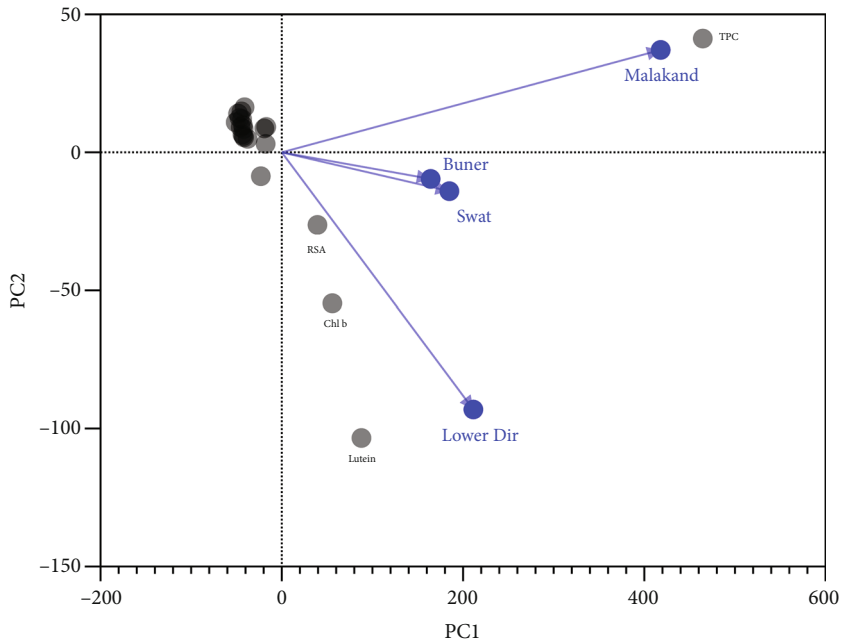

Figure 2: Biplot of the principal component analysis (PCA) used for quantitative data of different samples of *Medicago polymorpha*. The variables are quantitative individual carotenoids, TPC, TFC, and RSA values.

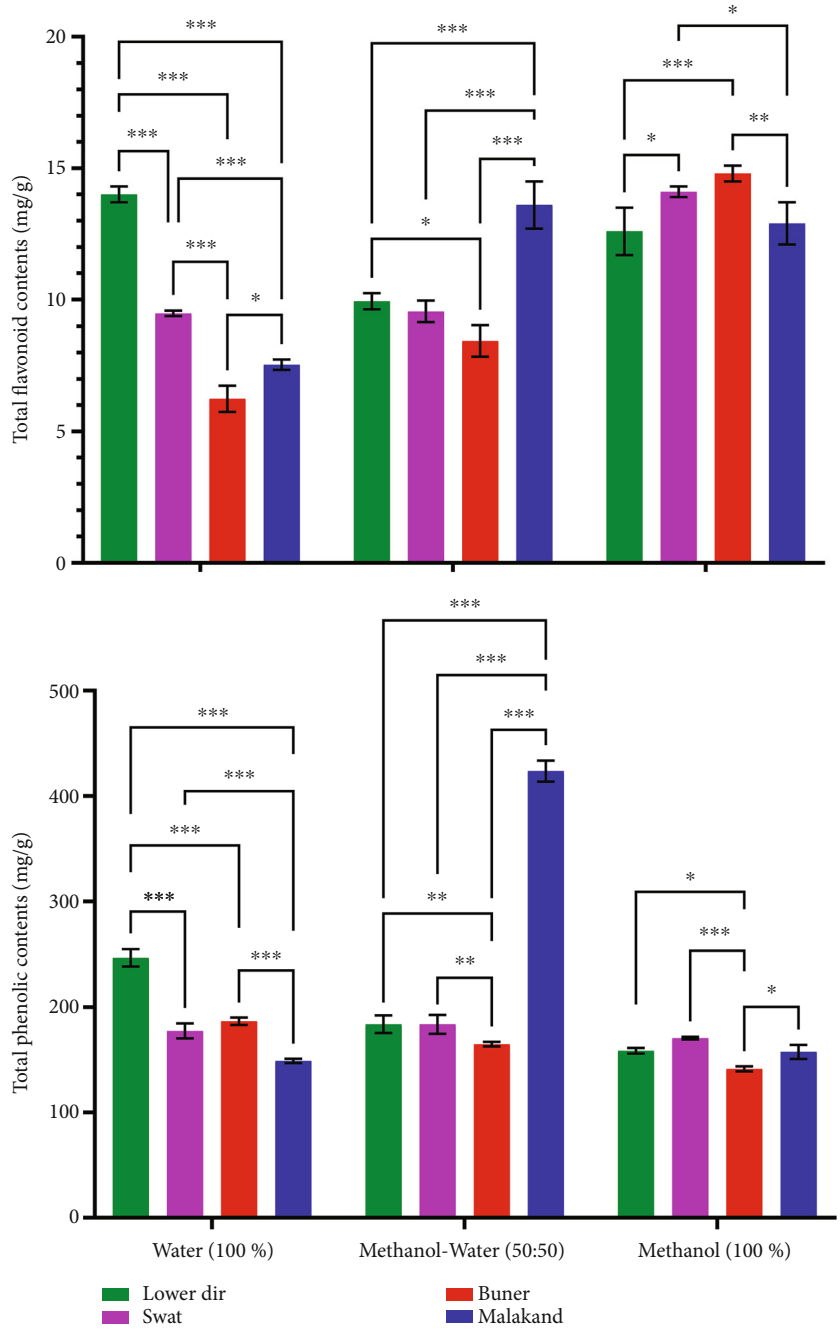

Figure 3: Total flavonoid and total phenolic contents of the different extracts of *Medicago polymorpha* of selected regions. Data are the mean of triplicates with standard deviation. $^{*}p = 0.01$, $^{**}p = 0.003$, $^{***}p < 0.001$, versus each other for the sample using Dunnett's multiple comparison tests.

Swat (61.5 μg/g) samples, while no significant difference was present in Buner and Malakand samples. There was no significant difference in the amounts of 13′-hydroxy-lactone chlorophyll *b* in the Lower Dir and Malakand samples, and the amounts were significantly higher in Swat and Buner samples. 9-*Z*-Neoxanthin has shown a higher amount in Swat (37.3 μg/g) than that of Buner (29.7 μg/g). Nevertheless, the amounts of 9-*Z*-neoxanthin in Lower Dir and Malakand samples did not differ significantly. The samples of Buner showed significantly higher amounts of hydroxy pheophytin *a* ′ (11.4 μg/g), followed by Swat samples; however, there was no significant variance between the Lower Dir and Malakand samples. All-*E*-β-carotene was significantly higher in both Swat and Malakand samples, whereas the lowest amounts (2.8 μg/g) were present in Lower Dir samples. All-*E*-β-carotene is a provitamin A carotenoid, and the proximal and middle intestines are organs where most of them are transformed [34]. Research from both epidemiological and molecular perspectives demonstrates that β-carotene and its vitamin A derivatives promote fat

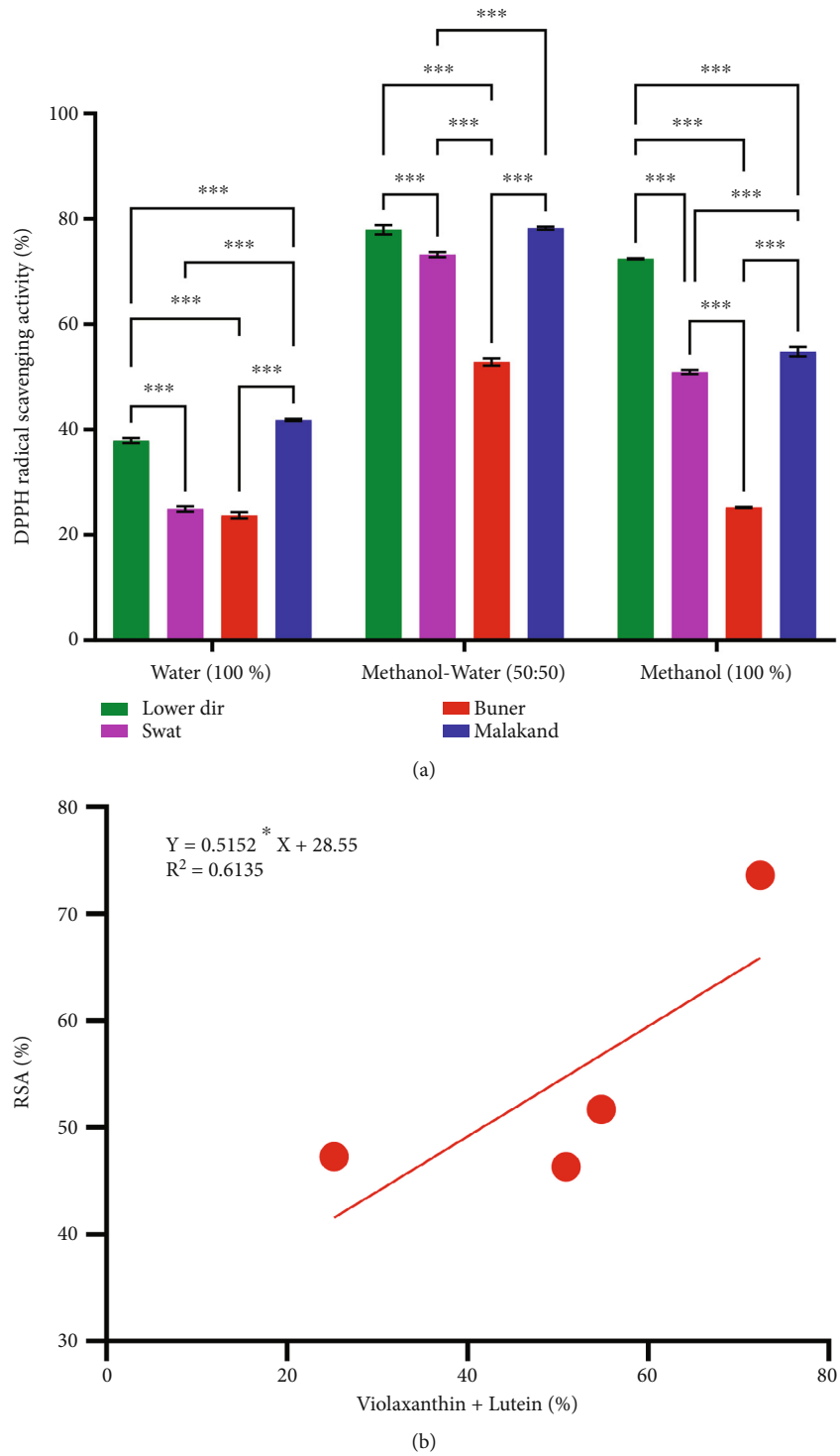

FIGURE 4: Changes in the DPPH radical scavenging activity of the different extracts of *Medicago polymorpha* of selected regions. (a) RSA and (b) linear correlation plot of RSA and violaxanthin and lutein. Data are the mean of triplicates with standard deviation. $p = 0.01$, $p = 0.003$, $^{***}p < 0.001$, versus each other for the sample using Dunnett's multiple comparison tests.

metabolism across several organs, hence mitigating the onset of obesity [35]. The total amounts of carotenoids in Lower Dir were 51.2%, Swat 48.2%, Buner 50.8%, and Malakand samples 44.6%. The amounts of violaxanthin and lutein were significantly higher in Lower Dir (73.6%) samples, followed by Malakand (51.7%), whereas Buner and Swat samples showed the lowest levels, i.e., 47.2 and 46.3%, respectively. The total contents of violaxanthin and lutein were lower (83.7-98.1%) than reported by Ignasiak and Lesins [24].

3.3. Principal Component Analysis. With the help of factors like the 18 carotenoids and chlorophylls that were detected in the samples from various districts and TPC, TFC, and RSA, the PCA was examined to determine any potential correlation in terms of correlation coefficient. The loadings and scores in the corresponding dimensional spaces are plotted in Figure 2. The samples' variance as represented by the major components was 96.31%. The F1 variance was greater (93.06%) than the F2 variance (6.5%). There was some homogeneity among various factors, according to the computed score plot. Chlorophyll b (Cb) and RSA indicated uniformity in the samples from Buner and Swat. In terms of all-*E*-lutein, chlorophyll *b*, and RSA, the samples from Swat and Buner contrasted favorably with those from Lower Dir. The correlation coefficients for all other substances were strong. *M. polymorpha*'s secondary metabolites' PCA revealed larger concentrations in mowed fields, indicating that PCA can be a useful tool for identifying quick discrimination alterations in mowed crops [36]. These findings imply that the PCA analysis may reveal considerable correlation and variance in several samples of *M. polymorpha* cultivated in various soil or water conditions depending on carotenoid and chlorophyll composition.

3.4. Total Flavonoid Contents. Figure 3 shows variation in total flavonoid contents (TFC) of *Medicago polymorpha* of different areas and different solvents. All extracts showed a considerable variation in TFC content between samples, except for the Lower Dir, Swat, and Buner methanol-water extracts. There was no significant difference in the amounts of TFC of Swat and Buner samples of methanol extracts. In Buner samples, the concentration of TFC was high (14.8 mg/g) in methanol (100%), then came methanol-water extract. However, the TFC was in low amounts in water extract. In Swat, in methanolic extract, the amount of TFC was higher (14.1 mg/g), whereas lower amounts (9.49 and 9.56 mg/g) of TFC were present both in methanol-water and water extracts. In Malakand samples, the TFC was seen in high amounts (13.6 mg/g) in the same ratio of methanol-water, succeeded by methanol (12.9 mg/g), while a smaller amount (7.54 mg/g) was detected in the water extract. In Lower Dir, the water extracts have the TFC values (14.0 mg/g) followed by methanol (12.6 mg/g), while lower amounts (9.94 mg/g) were observed in methanol-water extracts.

3.5. Total Phenolic Contents. Figure 3 shows the effects of different solvents and different areas on the total phenolic contents of MP leaves. The TPC values of water extracts varied significantly, except for the Swat and Buner samples. There were no significant variations in the TPC values of Lower Dir and Swat samples of methanol-water extracts. In the extracts of methanol, there were significant variations among the Lower Dir and Buner samples, Swat and Buner samples, and Buner and Malakand samples. Among all the samples, the Malakand sample was irrigated through the river Swat, which has a higher salt concentration than water wells. The result reported by Hernandez et al. [37] revealed that antioxidant activities increase with salinity. The variations in results may be due to the irrigation, variety, and climate.

3.6. Radical Scavenging Activity. Figure 4 shows the effects of different solvents and different areas on the radical scavenging activity (RSA) of MP leaves. There were significant variations among the samples' RSA of the water extracts. The highest RSA values were observed for Malakand samples, followed by Lower Dir samples. In methanol-water extracts, there were significant variations among the samples. The samples of Lower Dir and Malakand showed the highest values of RSA, followed by the Swat sample. In methanol extracts, significant variations were observed among the samples. The Lower Dir samples showed the highest values of % RSA followed by Malakand samples. Upon plotting the RSA values of the methanol extracts against the total amounts of violaxanthin and lutein (Figure 4), a significant linear correlation ($y = 0.5152x + 28.55$, $R^2 = 0.6135$) was observed suggesting that radical scavenging activity was based on the amounts of the specific carotenoids.

4. Conclusions

The changes in carotenoid pigments and antioxidant capacity of MP cultivated in various locales have been demonstrated for the first time in this study. Chromatography accurately separated, identified, and quantified nine carotenoids and pigments in all samples. Significant variations in the TFC, TPC, and RSA were observed among all samples. Chlorophylls were higher to Lower Dir > Swat > Buner > Malakand, and a reverse order was observed for carotenoid contents. The total amounts of violaxanthin and lutein were for the higher antioxidant activities of the samples.

Data Availability

Data are available by contacting the corresponding author (Dr. Alam Zeb).

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Alam Zeb made great contribution to the conceptualization, investigation, methodology, formal analysis, data curation, supervision, original draft preparation, and editing. Khalil Ahmad carried out formal analysis and data curation.

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