Heliyon 6 (2020) e03195

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Chemo-metric analysis of carotenoids, chlorophylls, and antioxidant activity of *Trifolium hybridum*

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ARTICLE INFO

Keywords:
Food technology
Food quality
Food analysis
Food composition
Food chemistry
Carotenoids
Trifolium
HPLC
Chemometrics

ABSTRACT

This work determined carotenoids and chlorophylls profiles, in addition, the antioxidant potential of *Trifolium hybridum* from different geographical regions. Carotenoids separation and identification were carried out using HPLC-DAD. Result revealed eight carotenoids and nine chlorophylls in all samples. The major carotenoids were 8-apo-carotenal, all-*E*-neoxanthin, phytofluene, all-*E*-flavoxanthin, all-*E*-lutein and all-*E*-β-carotene. The major chlorophylls were pheophytin *a*, pyropheophytin *a*, hydroxyl-pheophytin *a*', hydroxyl-pheophytin *a* chlorophyll *b* and its derivatives, chlorophyll *a*, hydroxyl-chlorophyll *a*, pheophytin *b*, and pyropheophytin *a*. The highest concentrations of carotenoid were in extracts from Dir samples (382.5 μg/g), and Buner sample (485.1 μg/g), followed by the Malakand (379.6 μg/g) and Swat (375.3 μg/g). The principal component analysis revealed significant correlation in carotenoids and chlorophylls. Significant variations in carotenoids, chlorophylls, total flavonoids, and total phenolic contents were observed among all selected samples. The carotenoid and chlorophylls profile of *Trifolium hybridum* described herein could be useful for food colourant development in food industries.

1. Introduction

Trifolium hybridum (English name: Alsike clover) is a glabrescent and perennial plant having vertically ascending stems belonging to the genus *Trifolium* (Genus clover) [1]. The alsike clover is a self-fertile plant with tri-foliate leaves and contains 16 to 20 flowers per plant. The medicinal properties of the *Trifolium* include anti-cancer, anti-obesity [2], anti-angiogenesis, and anti-oxidation [3], anti-bacterial [4] and used as a drug against eczema and rheumatism [5]. The two famous clovers grown in Pakistan are *Trifolium repens* and *Trifolium pratense*. Thes species are considered beneficial in curing of a sore throat, meningitis, pneumonia [6] and hepatotoxicity [7]. The herbs of the *Trifolium* genus are enriched with biologically active anti-oxidative materials [8, 9]. The antioxidants of this plant origin include phenolic compounds, carotenoids, folic acid, ascorbic acid, tocopherols, and tocotrienols [10]. Most of the studies on this plant had been focused on bioactive phenolic composition and medicinal properties [11, 12, 13]. However, recently chlorophylls or their derivatives have also shown to possess important health-promoting properties such as antioxidant, antimutagenic, chemopreventive, anti-inflammatory, prebiotic and anti-microbial properties [14].

Carotenoids are natural pigments comprises of eight isoprene units. There are more than seven hundred different carotenoids known so far. The most important and prominent is β-carotene [15]. Carotenoids are categorized in the two groups such as carotenes and xanthophylls. Both classes are soluble in organic solvents. The colour, taste, and acceptability of plant foods may be due to the carotenoids [16]. The carotenoids especially, lutein, zeaxanthin, lycopene, and β-carotene have been investigated to strengthen immunity by their antioxidant actions [17]. These carotenoids are industrially important compounds as nutraceuticals and functional foods. In plants, they are important for their specific responses to abiotic and biotic stresses [18]. For examples, recent studies showed that carotenoids were present in high amounts in marshy and moist areas as compared to dry areas [19]. It was confirmed that water resources and geographical locations are important parameters for carotenoids and chlorophylls [20]. The present study was carried out for the first time to evaluate the carotenoids profile, variation in carotenoids profile, as well as the antioxidant activity of *Trifolium hybridum* collected from different areas.

2. Materials and methods

2.1. Materials

Butylated hydroxytoluene (BHT), ethanol, lutein, chlorophyll *a*, methanol, β-carotene, 8-apo-carotenal, and 1,1-diphenyl-2

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https://doi.org/10.1016/j.heliyon.2020.e03195
Received 14 June 2019; Received in revised form 18 October 2019; Accepted 7 January 2020

picrylhydrazyl radical (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). Merck (Germany) delivered the acetone. Methyl tertiary butyl ether (MTBE, PubChem CID 104324) and ammonium acetate were bought from Daejung Chemicals (Daejung, South Korea). Deionized double distilled water was prepared using Daihan Labtech (South Korea). All other chemicals and reagents were of the analytical standard of high purity.

2.2. Sample collection

The fresh alsike clover plants of one variety only were collected from four different regions/districts of Malakand division, Khyber Pakhtunkhwa, Pakistan. At the time of sample collection, temperature, collection site, latitude and longitude of the respective area, the irrigation system and the herbarium status of the samples were recorded as shown in Table 1.

2.3. Sample preparation

Leaves of the clover were separated gently, stems and roots were discarded. Leaves were washed with tap water. The cleaned leaves were grounded with laboratory grinder into a paste. Then, the paste was used to prepare extract samples for HPLC analysis.

The extract of the sample was prepared using 2 g of grounded *Trifolium hybridum* leaves mixed with 10 mL of solvents. Three different solvents were used i.e., methanol (100%), absolute deionized distilled water (100%), and methanol-deionized distilled water (50:50 %). The samples were then shaken for 24 h, filtered using Whatman filter paper. The extract was used for measurement of TPC (total phenolic contents), TFC (total flavonoid contents), and DPPH radical scavenging activity (RSA).

2.4. Extraction of the carotenoids & chlorophylls

The method of Kimura, Rodriguez-Amaya [21] was used with some modifications for the extraction of carotenoids and chlorophylls. In short, 1 g of the sample paste (in triplicate) was taken and mixed with 10 mL of ice-cold acetone. The mixture was then shaken at 100 rpm using a shaker (Wittig Labortechnik, Germany) for 1 h. It is followed by the addition of 5 mL of ethanol (100%) into the mixture and shaken again for 30 min. Samples were filtered using Whatman filter paper. Extractions were continued until the complete discolouration of the samples was achieved. Solvents were evaporated and the residue was dissolved in 2 mL HPLC solvents and filtered into HPLC vials using a syringe filter with a pore size of 0.45 μm (Agilent PTFE syringe filter, Agilent Technologies, Germany).

2.5. HPLC-DAD analyses of carotenoids & chlorophylls

Reversed-phase HPLC-DAD (Agilent 1260 Infinity II) system was employed for analysis of the chlorophylls and carotenoids in the samples. The carotenoids and chlorophylls were separated using a C18 column (Agilent-Zorbax-Eclipse C18) with a pore size of 3.5 μm, maintained at a temperature of 25 °C. The HPLC system was coupled with DAD (diode array detector) for the detection of the separated compounds. The tertiary gradient phase system comprises of solvent A (deionized water and methanol with the ratio of 8:92, v/v) having ammonium acetate of 10 mM. Solvent B composition was deionized water with 0.01 mM ammonium acetate. Whereas, solvents C was absolute MTBE [22]. The mobile phase flow rate was 1 mL/min for the separation of 50 μL injection volume of the samples. At zero minute the solvents A, B, C gradient system initiated with a ratio of 80:18:2. The concentration of the solvents A, B, C reached the ratio of 80:12:8 respectively at 3 min. At 25 min, the ratio of the solvents was A 65%, B 5%, C 30%. The gradient at the end of the run was 60% A, 0% B, 40% C with post gradient elution of 10 min for achieving the initial gradients. The range of the recorded spectra was 200–750 nm. Utilizing Open-Lab CDS Chemstation (Agilent Technologies, Germany), the chromatograms were obtained at 450 and 650 nm. The identification and quantification of the carotenoids were based on the retention time and absorption spectra of the available standards. The carotenoids and chlorophylls were quantified with the help of peak areas against the standard calibration curves and expressed μg per g of fresh weight.

2.6. Principal component analysis

Principal component analysis (PCA) was carried out using XLSTAT software (Addinsoft, USA). All carotenoids and chlorophylls identified in each sample were included. The PCA biplot mapped the variables (individual identified carotenoids and chlorophylls) and samples (Swat, Buner, Lower Dir, and Malakand) through loadings and scores in dimensional spaces determined by principal components (PCs) with eigenvalues ≥ 1.0 [20].

2.7. Total flavonoids content

For the determination of the total flavonoid contents in the *Trifolium hybridum*, 0.5 mL of the $AlCl_3$ (2 %) was mixed with 0.5 mL of the extract. The resulted mixture was then incubating for 1 h. After incubation, the absorbance of each sample was measured at 420 nm using a spectrophotometer**.** Total flavonoid contents were calculated using rutin standard calibration and expressed as mg/g of quercetin equivalent.

2.8. Total phenolic compounds

Folin-Ciocalteu's (FC) reagent was used for the measurement of total phenolic contents (TPC) of the alsike clover extract as given in details recently [23]. Briefly, the sample extract (0.5 mL) was mixed with 2 mL of the FC reagent followed by the addition of 2 mL of sodium carbonate (7.5 %). The resultant mixture was incubated for 1 h and the absorbance was noted at 765 nm using Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan). The TPC was quantified using six points calibration curve of gallic acid in the range of 5–100 mg. Total phenolic contents were expressed as the mg of (GAE) gallic acid equivalents per 100 of the sample.

2.9. DPPH radical scavenging activity

The DPPH method was employed for the determination of the RSA in the different extracts of alsike clover using a method as reported recently [24]. The DPPH (0.1 mM) solution in methanol was employed. The samples extract RSA were measured in triplicate with DPPH and extract concentration and incubation for half an hour in dark. After incubation, the absorbance of each sample was measured at 515 nm using UV-1700 spectrophotometer (Shimadzu, Japan). The % RSA calculation was calculated from the absorbance of the samples and DPPH control.

Table 1. Characteristics and physical conditions of the sample collection of *Trifolium hybridum*.

Collection Site	District	Temperature (°C)	longitude	Latitude	Irrigation system
Jowar	Buner	19	72.302094	34.556093	Rain + tube well water
Ramora	Dir	15	72.066588	34.668511	Rain + River water
Shamozai	Swat	17	72.116489	34.684040	Rain + Tube well water
Batkhela	Malakand	21	72.149776	34.875037	Rain + River water

2.10. Data analyses

All analysis was performed in triplicate or otherwise mentioned. One-way analysis of variance (ANOVA) was performed on the obtained data. The post hoc test of the Holm–Sidak method at $\alpha = 0.05$ using XLSTAT software (Addinsoft, USA) macros for MS excel were also carried out.

3. Results and discussion

3.1. Identification of carotenoids & chlorophylls

Figure 1 shows the separation of seventeen carotenoids and chlorophylls, which were identified in each *Trifolium hybridum* sample with detail spectral characteristics given in Table 2. Peak 1 was identified as "8-apo-carotenal", which eluted at a retention time (RT) of 1.0 min, with maximum absorption spectra at 412 nm. It may be formed by the oxidative cleavage of the β-carotene double bond at position 7′, and 8′ double bond [25]. Compounds 2 and 3 were eluted at RT of 1.7 min and 2.3 min, respectively and identified as "all-*E*-neoxanthin" and "hydroxy chlorophyll *a*" with the λ max of 416, 440, 468 and 430, 618, and 662 nm, respectively. The neoxanthin was also reported by Livingston et al. [26] in yellow sweet clover and red clover. Compound 4 eluted at 3.2 min, identified as "phytofluene" with the absorption maxima of 329, 345, and 365 nm.

Similarly, compounds 5 and 6 were identified as "pheophytin *a*" and "pyropheophytin *a*". Both chlorophylls eluted at the retention time of 5.1, and 6.0 min, respectively. The pheophytin *a*, and pyropheophytin *a*, were identified from their absorption spectra of 410, 508, 538, 610, 666 nm and 408, 508, 540, 610, 668 nm, respectively. The pheophytin *a* was also previously reported in three clovers (*T. hybridum, T. pratense*, and *T. repens*) by Hynninen [27]. Peak 7 was "all-*E*-flavoxanthin" identified with λ max of 398, 422, and 448 nm, which was eluted at 9.4 min. Peak 8 and 9 were "hydroxy pheophytin *a*" and "all-*E*-lutein" with λ max 400, 506, 538, 666 nm and 424, 448, 472 nm, eluted at 10.9 and 14.7 min, respectively. The lutein; a xanthophyll carotenoid was also reported in the *Trifolium pratense* by Livingston et al. [26] and in *Trifolium repens* L. by Elgersma et al. [28]. Compound 10 and 11 were 9-cis isomers of lutein such as 9-*Z*-lutein and 9′-*Z*-lutein with the λ max of 325, 418, 442, 468 nm and 330, 418, 440, and 466, respectively. These compounds eluted at 17.1 and 17.8 min.

Similarly, at 23.0 min, compound 12 was identified as "chlorophyll *b*" with absorption spectra of 342, 464, 600, 648 nm, which was also reported by Yoo et al. [29] in *Trifolium repens* L.(white clover). With λ max of 342, 464, 600, 650 nm "13′-hydroxy-lactone chlorophyll *b*" was identified as compound 13, which was eluted at 23.7 min. Compound 14 and 15 were found as "chlorophyll *a*" and "pheophytin *b*", which were eluted at 25.5 min and 26.7 min, with the λ max of 432, 618, 664 nm and 436, 600, 654 nm, correspondingly. Chlorophyll *a* was previously reported in white clover by Yoo et al. [29]. Compound 16 and 17 were found to be "hydroxy pheophytin *a*" and "all-*E*-β-carotene" eluted at the retention time of 28.9 and 32.7 min, which was identified with the absorption spectra of 400, 505, 536, 610, 666 nm and 418, 452, 478 nm, respectively. All-*E*-carotene also reported in white clover by Elgersma et al. [28] and in *Trifolium patulum Tausch* and *Trifolium pratense* L. by Casacchia et al. [30]. These results showed that *Trifolium repens* is a good source of important carotenoids and chlorophylls.

3.2. Variations in carotenoids & chlorophylls

A total of seventeenth carotenoids and chlorophylls were identified in alsike clover leaves sample as shown in Table 3. This table indicates that 8-apo-carotenal was present in a higher amount (23.7 μg/g) in Malakand sample, followed by samples from Buner (16.9 μg/g) and Swat samples (16.3 μg/g). The 8-apo-carotenal (10.4 μg/g) was present in the lowest amount in Lower Dir. All-*E*-neoxanthin was present in higher amount in Swat samples (20.0 μg/g), followed by Lower Dir (9.7 μg/g) and a lower and similar amount (8.4 μg/g) in both Malakand and Buner samples. Similarly, the amount of hydroxyl-chlorophyll *a* was lowest in Lower Dir (4.0 μg/g), followed by Swat (6.5 μg/g), whereas the higher amount in Buner (13.8 μg/g), followed by Malakand (12.2 μg/g) samples. Phytofluene was present in higher amount in Buner samples (19.1 μg/g), followed by Malakand (11.2 μg/g) and Swat (10.3 μg/g), whereas the lowest amount in Lower Dir (6.4 μg/g). Pheophytin *a* was present in the lowest amount in Lower Dir (3.5 μg/g) samples, followed by Buner (9.5 μg/g). It was present in a considerable higher amount in Malakand (36.7 μg/g) and in Swat samples (11.9 μg/g). Similarly, pyropheophytin *a* was significantly higher in Lower Dir (26.9 μg/g). Its amount was lower in Buner (13.2 μg/g) and Malakand (12.0 μg/g) samples. It was present in the lowest amount in Swat (6.9 μg/g) samples. All-*E*-flavoxanthin was present in the highest amount in Swat (49.7 μg/g), followed by Lower Dir (46.8 μg/g) samples, whereas no significant difference in the amount of flavoxanthin was present in Malakand (40.5 μg/g) and Buner (41.5 μg/g) samples. The amount of hydroxyl-pheophytin *a* was significantly higher in Buner, followed by Swat and lowest in Lower Dir and Malakand samples. All-*E*-lutein was present in a significantly higher amount in Buner (243.8 μg/g), followed by Lower Dir (219.2 μg/g). It was present in the lowest amount in Swat (210.0 μg/g) and Malakand (208.9 μg/g) samples. The 9-*Z*-lutein was observed higher in Swat (16.7 μg/g), followed by Lower Dir (12.1 μg/g), Buner (12.3 μg/g), and Malakand (13.4 μg/g). The 9′-*Z*-lutein was present in higher amount in Lower Dir (25.8 μg/g), followed by Swat (21.2 μg/g) with the almost similar lower amount in both Buner (19.7 μg/g), and Malakand (19.0 μg/g) samples.

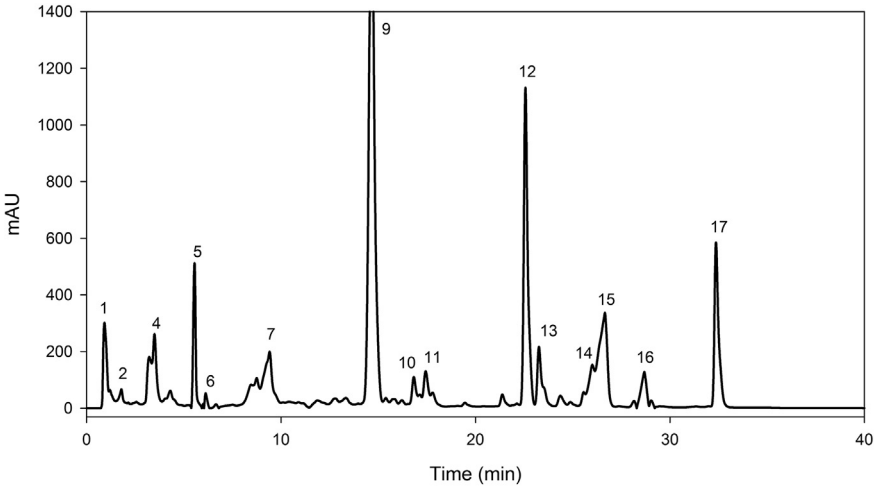

Figure 1. Representative HPLC-DAD chromatogram of *Trifolium hybridum* L at 450 nm (Malakand sample).

Table 2. Variations in the composition of carotenoids and chlorophylls in *Trifolium hybridum* from different areas.

Peak No	Rt (min)	Compound	Absorption Maxima (nm)	Carotenoids & Chlorophylls (μg/g)*			
				Lower Dir	Swat	Buner	Malakand
1	1.0	8-Apo-carotenal	412	10.4 ± 1.0a	16.3 ± 1.3b	16.9 ± 0.4b	23.7 ± 2.1c
2	1.7	All-*E*-neoxanthin	416, 440, 468	9.7 ± 1.1a	20.0 ± 0.1b	8.4 ± 1.3a	8.4 ± 0.8a
3	2.3	Hydroxy-chlorophyll *a*	430, 618, 662	4.0 ± 0.2a	6.5 ± 0.7b	13.8 ± 2.7c	12.2 ± 0.2c
4	3.2	Phytofluene	329, 345, 365	6.4 ± 0.5a	10.3 ± 0.5b	19.1 ± 0.9c	11.2 ± 0.6b
5	5.1	Pheophytin *a*	410, 508, 538, 610, 666	3.5 ± 0.2a	11.9 ± 0.4b	9.5 ± 0.4c	36.7 ± 4.0d
6	6.0	Pyropheophytin *a*	408, 508, 540, 610, 668	26.9 ± 2.4a	6.9 ± 0.5b	13.2 ± 0.6c	12.0 ± 1.4c
7	9.4	All-*E*-flavoxanthin	398, 422, 448	46.8 ± 5.0a	49.7 ± 3.3a	41.5 ± 0.1a	40.5 ± 3.2b
8	10.9	Hydroxy-pheophytin *a*	400, 506, 538, 666	7.3 ± 1.2a	21.6 ± 1.2b	28.4 ± 5.1c	3.4 ± 0.2d
9	14.7	All-*E*-lutein	424, 448, 472	219.2 ± 7.1a	210.0 ± 5.9a	243.8 ± 8.6b	208.9 ± 3.1c
10	17.1	9-*Z*-Lutein	325, 418, 442, 468	12.1 ± 1.2a	16.7 ± 2.2b	12.3 ± 1.6a	13.4 ± 0.6a
11	17.8	9′-*Z*-Lutein	330, 418, 440, 466	25.8 ± 0.8a	21.2 ± 0.8b	19.7 ± 1.4b	19.0 ± 3.7b
12	23.0	Chlorophyll *b*	342, 464, 600, 648	143.4 ± 2.9a	144.7 ± 6.2a	122.5 ± 6.0b	99.5 ± 4.7c
13	23.7	13′-Hydroxy-lactone chlorophyll *b*	342, 464, 600, 650	31.7 ± 3.2a	18.9 ± 0.6b	24.1 ± 3.3c	23.4 ± 1.5c
14	25.5	Chlorophyll *a*	432, 618, 664	33.3 ± 0.5a	78.1 ± 2.2b	44.1 ± 2.4c	24.0 ± 2.2d
15	26.7	Pheophytin *b*	436, 600, 654	41.4 ± 6.0a	10.1 ± 1.2b	16.3 ± 3.2c	67.8 ± 0.5d
16	28.9	Hydroxy pheophytin *a*'	400, 505, 536, 610, 666	25.0 ± 0.3a	27.1 ± 0.6b	42.8 ± 1.0c	34.6 ± 3.1d
17	32.7	All-*E*-β-carotene	418, 452, 478	52.1 ± 3.6a	35.4 ± 4.2b	123.4 ± 7.8c	50.2 ± 1.8a

* Data is mean with standard deviation of the triplicate readings. Different letters (a-d) in the same line indicate significant difference at $p < 0.05$ by One-way ANOVA, followed by the Holm-Sidak method of multiple comparisons.

Table 3. Variations in total flavonoid contents, total flavonoid contents and DPPH radical scavenging activity of the *Trifolium hybridium* of different areas and using different solvents.

Parameters	Extraction	Buner	Swat	Malakand	Lower Dir
Total Flavonoid Contents (mg/g)	Water (100 %)	1.89 ± 0.2a	86.0 ± 4.9b	222.6 ± 6.6c	8.87 ± 0.8d
	Methanol-Water (50:50)	3.94 ± 0.5a	122.9 ± 5.1b	72.2 ± 12.0c	8.12 ± 1.1d
	Methanol (100 %)	2.81 ± 0.1a	62.2 ± 4.2b	70.2 ± 6.9b	1.81 ± 0.2c
Total Phenolic Contents (mg/g)	Water (100 %)	130.6 ± 1.5a	125.0 ± 10.3a	118.4 ± 6.3b	111.4 ± 0.7b
	Methanol-Water (50:50)	101.0 ± 7.8a	105.8 ± 5.4a	105.3 ± 4.5a	104.7 ± 6.0a
	Methanol (100 %)	413.1 ± 21.3a	381.9 ± 22.5b	97.4 ± 5.7c	188.0 ± 13.1d
DPPH radical scavenging activity (%)	Water (100 %)	40.9 ± 3.7a	25.4 ± 3.3b	43.8 ± 5.2a	40.9 ± 3.7a
	Methanol-Water (50:50)	72.8 ± 1.9a	39.1 ± 5.8b	72.9 ± 1.9a	72.9 ± 1.9a
	Methanol (100 %)	69.2 ± 6.1a	69.1 ± 3.1a	73.7 ± 1.1a	69.2 ± 6.0a

Data is mean with the standard deviation of the triplicate readings. Different letters (a-d) in the same line indicate significant difference at $p < 0.05$ by One-way ANOVA, followed by the Holm-Sidak method of multiple comparisons.

Chlorophyll *b* was almost present in a similar higher amount in both Swat (143.4 μg/g) and Malakand (144.7 μg/g) samples as compared to Lower Dir and Buner samples. The 13′-hydroxy-lactone chlorophyll *b* amount was identified in each selected samples in order of Lower Dir (31.7 μg/g), Swat (18.9 μg/g), Buner (24.1 μg/g), and Malakand (23.4 μg/g). Chlorophyll *a* in Swat (78.1 μg/g) samples was higher than that of Dir (33.3 μg/g), Buner (44.1 μg/g), and Malakand (24.0 μg/g) samples. Pheophytin *b* was present in higher amount in Malakand (67.8 μg/g), followed by Lower Dir (41.4 μg/g) with the amount present in Swat (10.1 μg/g) and in Buner (16.3 μg/g) samples. The hydroxyl-pheophytin *a*' was present in high amount in the Buner (42.8 μg/g) and Malakand samples (34.6 μg/g), followed by Swat (27.1 μg/g) and Lower Dir (25.0 μg/g). All-*E*-β-carotene was higher in Buner (123.4 μg/g), followed by Lower Dir (52.1 μg/g) and Swat samples (50.2 μg/g), followed by Malakand (35.4 μg/g) samples having the lowest amount.

3.3. Principal component analysis

Figure 2 shows the biplot of the loadings and scores in the respective dimensional spaces. The PCs shows 89.37% variance in Malakand, Swat, Buner, and Lower Dir samples. The F1 variance was higher (74.56 %), followed by F2 (14.82 %). The generated score plot shows some homogeneity among certain variables. There was a high correlation among the Malakand and Lower Dir samples, whereas the correlation was highly significant in samples from Swat and Buner. The samples from all regions were highly correlated in terms of β-carotene, flavoxanthin, chlorophyll *b*, lutein, hydroxyl-pheophytin *a*', and chlorophyll *a* with squared cosine values of 0.969, 0.960, 0.991, 0.998, 0.628, and 0.814, respectively. A high amount of chlorophyll *b* and lutein shows that these compounds may be used as principal fingerprinting pigment and carotenoid in *Trifolium hybridum*. All other parameters show the lowest correlation among the samples. These results indicated that the same variety of *Trifolium* grown under different, but relatively similar area may be correlated in several chemical components, but also varied in several other parameters.

3.4. Total flavonoids contents

In the Buner samples, the total flavonoids contents were present in large amounts (3.94 mg/g) in methanol-water (50%) extract. The Buner samples were lower in TFC (1.89 mg/g) in aqueous extract, followed by the methanol extract 2.81 mg/g as shown in Table 3. Similarly, the Swat samples were found in the lowest amounts (62.2 mg/g) in methanol extract, followed by the aqueous (100%) extract (86.0 mg/g) and highest amount (122.9 mg/g) in MeOH-water extract.

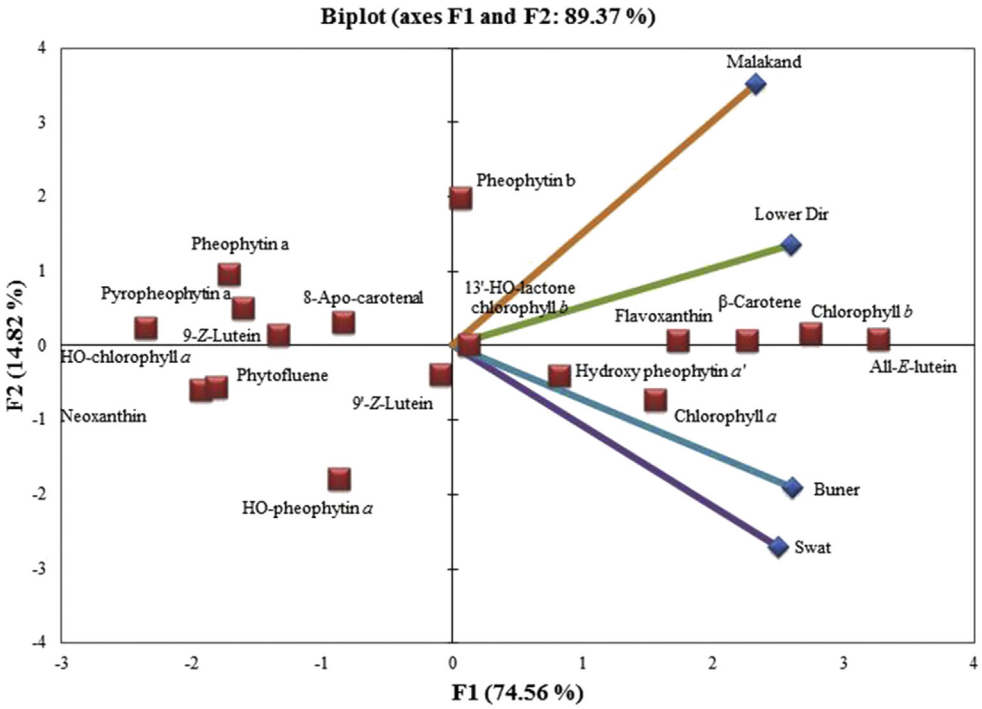

Figure 2. Biplot of the principal component analysis (PCA) applied to quantitative variables of all *Trifolium hybridum* samples.

In Malakand sample, TFC amount was investigated in a large amount (222.6 mg/g) in solvents extract and with lowest amount in MeOH-water and methanol extract as 70.2 mg/g and 72.2 mg/g respectively. In the same way, the TFC of Lower Dir sample was found in a low amount (1.81 mg/g) in methanolic extract and MeOH-water (50:50) solvent was measured with the second larger amount (8.12 mg/g). The TFC was higher (8.87 mg/g) in aqueous extract of the Lower Dir samples. The TFC of Swat and Malakand samples was higher than *Trifolium pratense* samples (26.61 mg/g) reported by Esmaeili et al. [31] and lower in Buner and Lower Dir samples. This may be due to the difference in the variety, climate, sample preparation and method of analysis. These results show that *Trifolium* samples collected from different areas show a different amount of TFC in different solvents.

3.5. Total phenolic contents

Table 3 shows that TPC in Buner samples of *Trifolium hybridum* was higher in the methanolic extract (413.1 mg/g), followed by aqueous extract (130.6 mg/g). The TPC was lower (101.0 mg/g) in the methanol-water (50:50) extract of Buner samples. Swat samples contained a large amount (381.9 mg/g) of TPC in the methanolic extract, followed by the aqueous samples (125.0 mg/g), whereas lowest amounts of TPC (105.8 mg/g) in MeOH-water (50:50) samples. Similarly, the TPC in Malakand samples shows lower amounts (97.4 mg/g) in methanol solvent, followed by the MeOH-water (105.3 mg/g). The TPC was found in the lower amounts in the MeOH-water extract of Lower Dir samples. Whereas, a higher amount (188.0 mg/g) in methanol extract, followed by aqueous with the amount of 111.4 mg/g. The TPC of all samples was higher than *Trifolium pratense* samples (26.61 mg/g) reported by Esmaeili et al. [31]. The high amount of phenolic contents in *Trifolium hybridum* may be beneficial by serving as antioxidants. Kolodziejczyk-Czepas et al. [32] reported that different *Trifolium* species-rich in phenolic compounds has anti-adhesive and anti-aggregative properties of platelets in the blood. Thus, *T. hybridum* species may also be a potential source of anti-platelets aggregative properties.

3.6. DPPH radical scavenging activity

Table 3 indicates that the DPPH radical scavenging activity of the Buner sample was higher in MeOH-water extract than aqueous and methanol extracts. The RSA of the Swat samples was lower (25.4%) in the aqueous extract as compared to MeOH-water (39.1%) and methanol (69.1%) extracts. Similarly, the Malakand samples were found to have higher radical scavenging activity in methanol (73.7%) as compared to MeOH-water (72.9%) and aqueous (43.8%) extract. The RSA of Lower Dir samples was higher (72.9%) in MeOH-water and methanol (69.2 %), followed by aqueous extract (40.9 All solvent extracts from each sample possessed lower RSA ability than the previous value (205.47%) reported by [31] in methanolic extract of *in-vivo* grown *Trifolium pratense*. These results suggest that aqueous extracts of all samples were lower in RSA values as compared to methanol-water and methanol extracts.

4. Conclusions

In conclusion, the present work revealed for the first time, the variation in carotenoids, chlorophylls and antioxidant activity of the *Trifolium hybridum* collected from the different locations of Malakand division, KPK, Pakistan. Seventeenth chlorophylls & carotenoids were identified in which the major carotenoids were all-*E*-neoxanthin, all-*E*-flavoxanthin, all-*E*-lutein, 9-*Z*-lutein, 9′-*Z*-lutein, phytofluene, and all-*E*-β-carotene. The major chlorophylls were chlorophyll *a* & pheophytin *a* and its derivatives, chlorophyll *b,* 13′-hydroxy-lactone chlorophyll *b* and pheophytin *b*. Considerable variations were observed in carotenoids, chlorophylls TPC, TFC and RSA values of each sample. The present results suggest that *Trifolium hybridum* was rich in important chlorophylls and carotenoids, phenolics and flavonoids contents, which could be used as a potential colourant in food industries.

Declarations

Author contribution statement

Alam Zeb: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Asif Hussain: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The facilities used were from project No. 2344, kindly supported by the Higher Education Commission of Pakistan is therefore highly appreciated. The authors declare no conflict of interest.

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