

Seasonal variation in yield, nutritive value, and antioxidant capacity of leaves of alfalfa plants grown in arid climate of Saudi Arabia

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ABSTRACT

In addition to its high nutritional value, alfalfa (*Medicago sativa* L.) has several health benefits including antioxidant, neuroprotective, antimicrobial, antiulcer, hypocholesterolemic, hypolipidemic, and estrogenic effects. We aimed to assess the nutritional value and antioxidant capacity of alfalfa leaves at different times of the year in arid regions. Alfalfa was harvested in autumn, winter, spring, and summer and fresh yield and leaf:stem ratio were measured. Leaves were collected to determine their nutritional value and antioxidant capacity using two different extracts *viz.*: absolute ethanol or distilled water. Fresh yield and leaf:stem ratio were highest in spring (20 t ha⁻¹ and 57.6%, respectively), followed equally by winter and autumn, and were lowest in summer (10.75 t ha⁻¹ and 51.6%, respectively). The autumn cut had the highest crude protein (33.52%) and crude fat content (3.79%) and the lowest content of crude fiber (13.95%) and acid detergent fiber (16.55%), which are good indicators of suitability for human nourishment. However, leaves collected in autumn were characterized by lower digestibility values (86.2%) compared to the other cuts. Antioxidant capacity of leaves harvested in autumns and extracted with either ethanol or distilled water was examined in terms of total polyphenols, total flavonoids, DPPH scavenging capacity, and reducing power. Ethanol extract showed higher antioxidant capacity in terms of total phenol and total flavonoid contents (6.41 and 4.88 mg gallic acid equivalents g⁻¹ DW, respectively). These results suggest that autumn is the most appropriate season to cut alfalfa leaves for human nutritional purposes.

Key words: Antioxidants, DPPH, flavonoids, food additives, nutrition, polyphenols.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a perennial herb belonging to the Fabaceae family. It is one of the most important forage crops globally and therefore, it is commonly known as the “queen of forages”. Recently, alfalfa has been proposed as an important source of protein for human nourishment. Due to its high protein content, alfalfa may be an alternative, inexpensive, and sustainable protein source to mitigate the extensive use of animal protein resulting from exponential global population growth (Morin et al., 2011; Hadidi et al., 2019). It is used in nutritional supplement products, as a garnish, and in the form of tablets and/or drinks to improve digestion (Stochmal and Oleszek, 2007). The green leaves of alfalfa plants are rich sources of protein (Firdaous et al., 2017). However, they have not been fully utilized in human nourishment until now. In general, phytochemical studies on green alfalfa leaves have shown that the major active compounds found in these leaves are vitamins, flavonoids, saponins, coumarins, phytoestrogens, amino acids, alkaloids, digestive enzymes, terpenes, and phytosterols (Bora and Sharma, 2011; Silva et al., 2016; Getachew et al., 2018). Alfalfa may be used for its

antioxidant, neuroprotective, antimicrobial, antiulcer, hypocholesterolemic, hypolipidemic, and estrogenic effects (Bora and Sharma, 2011; Al-Dosari, 2012; Bákonyi et al., 2020). Furthermore, it can potentially be used to treat heart disease, menopausal symptoms in women, cancer, diabetes, stroke, and atherosclerosis (Bora and Sharma, 2011; Rahman and Parvin, 2014; Sadeghi et al., 2016).

In animal feed, alfalfa is added to increase antioxidant activity of blood serum and liver. Such enhancement ability of alfalfa is attributed mainly to its contents of flavonoids and phenols (Erugur et al., 2018; Chen et al., 2020). Flavonoids extracted from alfalfa leaves were reported to have high antioxidant activity in terms of diphenylpicryl hydrazine (DPPH) scavenging and ABTS assay (Jing et al., 2015). Furthermore, alfalfa-derived flavonoids enhanced the *in vitro* activity of different antioxidant enzymes, e.g., catalase and superoxide dismutase (Ouyang et al., 2016). Similar to flavonoids, phenols are known as strong antioxidant molecules that enhance immunity and disease resistance via inhibiting the production of reactive oxygen species that oxidize proteins, lipids, and DNA causing oxidative stress (Silva et al., 2006; Al-Rimawi et al., 2016). However, studies of the nutritional value and antioxidant activity of alfalfa leaves and the impact of seasonal changes on them are lacking. Therefore, in the current study, we aimed to assess the nutritional value of alfalfa leaves in the four different seasons of the year, to identify the most suitable season to utilize alfalfa leaves grown in arid regions for human nourishment.

MATERIALS AND METHODS

Study design

Experiments were performed at the Agricultural Research Station in Dirab, Riyadh (24°250' N, 46°340' E; 400 m a.s.l.), Saudi Arabia, during the period from 2013 to 2015. A randomized complete block design was used in this study and the plants were randomly cultivated in blocks of 4 × 3 m. Plant cultivation was performed with three replicates. Climate data for the study years are shown in Figure 1.

Plant cultivation and sampling

Seeds of the local alfalfa (*Medicago sativa* L.) 'Alhassawy' were cultivated on 10 November 2013 for 2 yr (2013-2014 and 2014-2015). Seeding rate was 40 kg ha⁻¹ in 4 × 3 m plots with a spacing of 15 cm between lines. Irrigation and fertilization were applied following the standards of the study region. The first cut was performed after 3-mo of cultivation and further cuts were performed regularly, at the start of the flowering stage during the growth period. The cutting height was 5 cm and after cutting, fresh yield (t ha⁻¹) was estimated. Alfalfa plants were randomly sampled during each season and leaves were separated from stems and weighed to calculate the ratio of leaves to stems. A random leaf was chosen from each plot for further analysis.

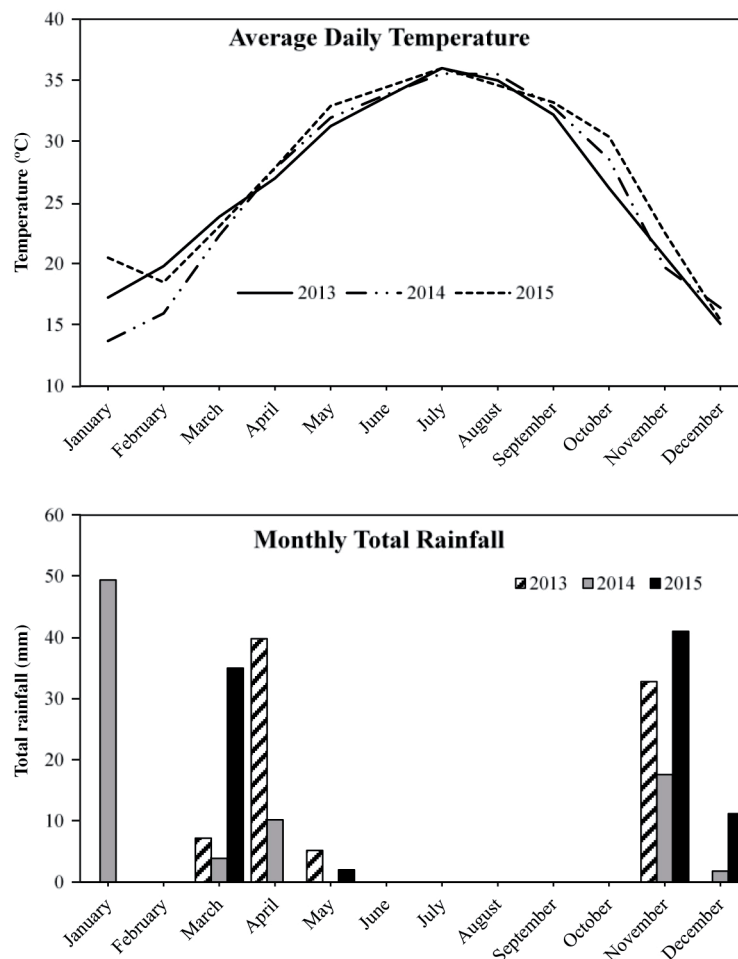
Nutritive value analysis

All chemical analyses were performed in the laboratories of the College of Food and Agricultural Sciences, King Saud University and Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten (VDLUFA) e.V., Speyer, Germany. Dry matter content was determined by weighing fresh alfalfa leaves (W1), oven-drying the leaves at 65 °C for 48 h, and then weighing them again (W2). DM percentage was then calculated. The dried leaves were ground into a fine powder and used for further analysis. Crude ash (CA) content was measured by combusting a known amount of leaves for 6 h at 550 °C. Crude protein (CP), fiber, neutral detergent fiber (NDF), acid detergent fiber (ADF), crude fat (CFA), and water-soluble carbohydrates (WSC) content were determined using a feed and forage analyzer (NIRSystems 5000, FOSS GmbH, Hamburg, Germany). Digestibility of alfalfa DM was also examined using the feed and forage analyzer as an indicator for the nutritive value at different seasons.

Total polyphenol content

Based on the nutritive values, the autumn sample was selected for further determination of total polyphenol, total flavonoid, diphenylpicryl hydrazine (DPPH) scavenging, and reduction power. For extraction, 1 g dried alfalfa powder was extracted with 20 mL of either absolute ethanol or distilled water using a shaker (200 rpm) at 25 °C for 1 h. Then the mixture was centrifuged at 6000 rpm for 10 min at room temperature. The supernatant was filtered using a Whatman filter paper number 2 and the obtained extract was used for further analyses.

Figure 1. Temperature and rainfall data in the study region during the years 2013-2015.



The total polyphenol content (TPC) was determined according to the method of Hayat (2020). In summary, 25 μL extract were mixed with 1500 μL water and then 125 μL reagent (undiluted Folin Ciocalteu) was added to the mixture. After 1 min incubation, 375 μL 20% sodium carbonate were added and the final volume of the mixture was made to 2500 μL by adding 475 μL water. Spectrophotometer (Jenway 6705 UV/Vis., Cole-Parmer, Staffordshire, UK) was used to determine absorbance at 760 nm after 30 min incubation time at room temperature. Gallic acid (gallic acid equivalents, GAE) was used as standard to express TPC per gram dry weight of the sample ($\text{mg GAE g}^{-1} \text{DW}$).

Total flavonoid content

The total flavonoid content (TFC) was determined according to the method of Hayat (2020). Briefly, extract (250 μL) was combined with 1000 μL water and then 75 μL each NaNO_2 and AlCl_3 were added. The mixture was incubated for 5 min at room temperature and then 500 μL 1 M NaOH and 600 μL water were added. Spectrophotometer was used to detect absorbance at 510 nm. Blank was prepared without extract. Total flavonoids were expressed as the mean \pm standard deviation (SD) mg catechin equivalents (CE) g^{-1} alfalfa powder as determined by a calibration curve prepared with an external standard.

DPPH scavenging

The free radical scavenging capacity of the extract was determined using diphenylpicryl hydrazine (DPPH) using the method described by Noreen et al. (2017) with some modifications. An aliquot of extract (130 μL) and 0.1 mM DPPH solution was mixed and incubated in the dark for 30 min and then absorbance was measured at 510 nm. Control was

prepared in the same manner, but ethanol was used instead of extract. Ethanol was used as a blank. The scavenging percentage was calculated by using the following equation:

$$\text{DPPH scavenging \%} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Reducing power

The ferric reducing power of the sample was determined according to the method of Hayat et al. (2010). In brief, 0.5 mL extract was mixed with 1.25 mL buffer (0.2 M, pH 6.6) and 1.25 mL potassium ferricyanide, then incubated for 20 min at 50 °C. After that, 1.25 mL trichloroacetic acid were added to the mixture and centrifuged at 3000×g for 10 min at room temperature. An aliquot (1.25 mL) was taken from the supernatant, to which 1.25 mL water and 0.25 mL ferric chloride were added. The absorbance was measured at 700 nm. Blank was prepared without extract.

Statistical analysis

Data obtained from each treatment (season) are reported as the average of 2 yr and three replicates each year ± SD. Means were compared using the least significant difference method, with a significance level of $P \leq 0.05$. Pearson correlation coefficients were used to assess the relationships between different studied nutritive values of alfalfa leaves. All statistical tests were performed using SPSS Statistics 20.0 software (IBM, Armonk, New York, USA).

RESULTS AND DISCUSSION

Fresh yield

The fresh yield of alfalfa varied according to the seasons of the year, with the highest value in spring (20 t ha⁻¹), followed by winter (14.43 t ha⁻¹) and autumn (13.58 t ha⁻¹), and then summer (10.75 t ha⁻¹) (Table 1). The mild climate in terms of temperature in spring (Figure 1) led to this increase in fresh yield (Sharratt et al., 1986), whereas the rise in summer temperatures (Figure 1) led to a decrease in fresh yield (Sharratt et al., 1987; Bitá and Gerats, 2013).

Leaf:Stem ratio

The ratio of leaves to stems was highest in spring (57.6%), then autumn (56.7%) and winter (56.1%), and was lowest in summer (51.6%) (Figure 2). This is due to the changes in weather during the seasons, which affects yield, quality, and ratio of leaves to stems and the growth of alfalfa plants (Brown and Tanner, 1983; Sanderson and Wedin, 1988).

Nutritive value

The correlations between different nutritive parameters of alfalfa leaves collected during different seasons were analyzed; Table 2 shows the Pearson's correlation coefficients between these parameters. Positive correlations were found between DM and crude ash (CA, $r = 0.853$; $P \leq 0.01$), DM and water-soluble carbohydrates (WSC, $r = 0.0717$, $P \leq 0.01$), ADF and digestibility (Dig, $r = 0.726$, $P \leq 0.01$), and neutral detergent fiber (NDF) and Dig ($r = 0.661$, $P \leq 0.05$). However, there were

Table 1. Changes in yield and nutritive value of alfalfa leaves collected during different seasons.

Season	Winter	Spring	Summer	Autumn
Yield, t ha ⁻¹	13.87 ± 0.10b	33.52 ± 0.15a	13.95 ± 0.12b	3.79 ± 0.01c
DM, %	27.45 ± 0.44a	22.60 ± 0.51c	24.63 ± 0.41b	21.38 ± 0.54c
CA, %	16.33 ± 0.37a	14.80 ± 0.54b	14.67 ± 0.26b	13.87 ± 0.10c
CP, %	31.98 ± 0.04c	32.57 ± 0.12b	32.93 ± 0.10b	33.52 ± 0.15a
CF, %	15.13 ± 0.04b	15.18 ± 0.05b	16.89 ± 0.21a	13.95 ± 0.12c
CFA, %	2.73 ± 0.02b	2.32 ± 0.03c	2.14 ± 0.02c	3.79 ± 0.01a
WSC, %	5.67 ± 0.02a	3.51 ± 0.18d	5.14 ± 0.04b	4.61 ± 0.14c
NDF, %	26.11 ± 0.17c	29.53 ± 0.18a	23.54 ± 0.19d	27.68 ± 0.15b
ADF, %	18.38 ± 0.11b	19.83 ± 0.17a	18.50 ± 0.12b	16.55 ± 0.08c
Dig, %	87.32 ± 0.12a	87.95 ± 0.05a	86.10 ± 0.03b	86.20 ± 0.27b

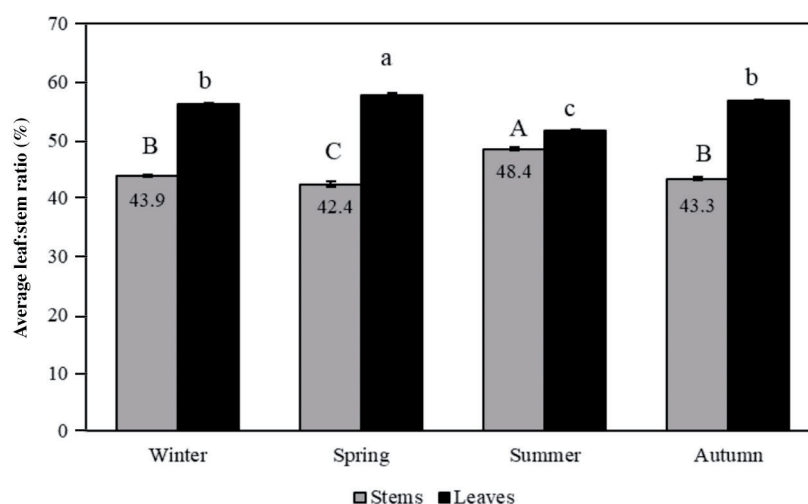
Values are the average of 2 yr (2013-2014 and 2014-2015), with three replicates each. Values are shown as means ± standard deviation (SD). Means in the same row followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

DM: Dry matter; CA: crude ash; CP: crude protein; CF: crude fiber; CFA: crude fat; WSC: water-soluble carbohydrates; NDF: neutral detergent fiber; ADF: acidic detergent fiber; Dig: digestibility.

multiple negative correlations. DM and crude protein (CP, $r = -0.798$, $P \leq 0.01$), CP and CA ($r = -0.893$, $P \leq 0.01$), crude fat (CFA) and crude fiber (CF, $r = -0.860$, $P \leq 0.01$), NDF and WSC ($r = -0.771$, $P \leq 0.01$) and CFA and acid detergent fiber (ADF) ($r = -0.863$, $P \leq 0.01$) were negatively correlated. Furthermore, there were negative correlations between CF and NDF ($r = -0.706$, $P \leq 0.05$), CP and ADF ($r = -0.625$, $P \leq 0.05$), and CP and Dig ($r = -0.659$, $P \leq 0.05$).

The data obtained in this study showed a significant difference in the amount of DM in alfalfa leaves in different seasons of the year (Table 1). DM reached its highest levels in winter (27.45%) and its lowest levels in autumn (21.38%). This may be mainly due to the climate conditions in Saudi Arabia, which is classified as an arid region, with higher rainfall percentages and lower temperatures during winter, which are favorable conditions for the growth of alfalfa plants. Similarly, there was significant difference between CA content in alfalfa leaves collected in winter, as compared to those collected in autumn (Table 1). However, CP content in alfalfa leaves was highest in autumn (Table 1) and lowest in winter (31.98%). Overall, CP percentages in the different seasons were higher than those reported in other studies. Protein content in alfalfa leaves has previously been reported to be 28% (Sommer and Sundrum, 2014). Milić et al. (2011) studied the chemical composition of different alfalfa cultivars and found that protein content ranged from 18.4% to 20.9% in all cultivars. They concluded that leaves of different cultivars accumulate more protein and minerals than stems. However, fiber content is lower in alfalfa leaves than in the stems. Furthermore, different alfalfa cultivars have been shown to have CP content ranging from 18.8% to 20.2% (Twidwell et al., 2002). In general, green leaves of alfalfa plants are considered to be a rich source of protein that has not yet been fully utilized (Firdaous et al., 2017).

Figure 2. Average leaf:stem ratio over 2 yr during all seasons, with three replicates each.



Different letters indicate significant differences in the percentage of leaves (lowercase letters) and stems (uppercase letters) between the seasons according to Duncan's multiple range test ($P \leq 0.05$).

Table 2. Pearson's correlation coefficients between different nutritive values of alfalfa leaves.

	DM	CA	CP	CF	CFA	WSC	NDF	ADF	Dig
DM	1								
CA	0.853**	1							
CP	-0.798**	-0.893**	1						
CF	0.421	0.191	-0.261	1					
CFA	-0.427	-0.381	0.540	-0.860**	1				
WSC	0.717**	0.502	-0.284	0.239	0.063	1			
NDF	-0.505	-0.181	0.023	-0.706*	0.311	-0.771**	1		
ADF	0.265	0.365	-0.625*	0.508	-0.863**	-0.405	0.194	1	
Dig	0.177	0.472	-0.659*	-0.168	-0.345	-0.454	0.661*	0.726**	1

DM: Dry matter; CA: crude ash; CP: crude protein; CF: crude fiber; CFA: crude fat; WSC: water-soluble carbohydrates; NDF: neutral detergent fiber; ADF: acidic detergent fiber; Dig: digestibility.
N = 12. * $P \leq 0.05$; ** $P \leq 0.01$.

In addition to CP content, ligneous cellulose content is one of the most important quality indicators of alfalfa leaves (Milić et al., 2011). Crude fiber content showed different values in the different seasons, except for winter and spring when the values did not differ significantly (Table 1). The highest crude fiber content was found in summer (16.89%) and the lowest was found in autumn (13.95%). Nevertheless, NDF content showed a high degree of variance between different seasons (Table 1). The highest NDF content was found in spring (29.53%), while the lowest NDF content was found in summer (23.54%). NDF content is a reliable indicator of the intake rate of alfalfa DM. Lower values of NDF content indicate higher quality, due to higher levels of nutrient uptake. However, ADF is a major indicator of potential production energy. Therefore, increased ADF content in alfalfa leaves indicates a potential decrease in energy and nutritive quality of the leaves (Katić et al., 2008). Our results showed that ADF content in alfalfa leaves reached the highest values in spring (19.83%, Table 1) and the lowest values in autumn (16.55%). The results obtained in this study showed that alfalfa leaves had the lowest fiber content (crude fiber and ADF) and the highest CP content in autumn. High protein content and low fiber content are indicators of high nutritive quality (Milić et al., 2011). Moreover, our results showed that there was a negative correlation between CP and crude fiber content in alfalfa leaves (Table 2).

Analysis of CFA content in alfalfa leaves showed a high degree of variance between the different seasons (Table 1). The highest CFA content was found in samples collected in autumn, while the lowest CFA content was found in samples collected in summer (3.79% and 2.14%, respectively). Low CFA content indicates high nutritive quality of alfalfa leaves (Katić et al., 2008). On the contrary, WSC content in alfalfa leaves reached the highest values during winter (5.67%) and decreased to the lowest values during spring (3.51%). These data showed significant differences in WSC content of alfalfa leaves in different seasons (Table 1).

Digestibility is one of the most important indicators of good nutritive value. There were significant differences among digestibility values of alfalfa leaves collected during different seasons of the year (Table 1). The highest digestibility values were found in spring (87.95%), whereas summer and autumn showed the lowest digestibility values, with nonsignificant differences between these two seasons. Sommer and Sundrum (2014) found that the digestibility of alfalfa leaves was 74%. Alfalfa leaves from spring growth have been shown to have higher digestibility than leaves from summer regrowth (Tremblay et al., 2002). High digestibility values in spring may be attributed to the fresh growth of alfalfa plants during this season. Mature alfalfa plants are generally characterized by low digestibility because of an increase in the amount of cell wall material in the stems and leaves (Tremblay et al., 2002). Leaves grown in summer are often more mature, with more lignified cell walls compared to leaves grown in spring (Sanderson and Wedin, 1988). Lignin inhibits the digestibility of polysaccharides and thus, higher lignin content in cell walls decreases the digestibility and nutritive quality of alfalfa leaves (Katić et al., 2008). It has been shown that alfalfa leaves with high DM content and digestibility have more nutritive value for cows and humans (Émile et al., 1997).

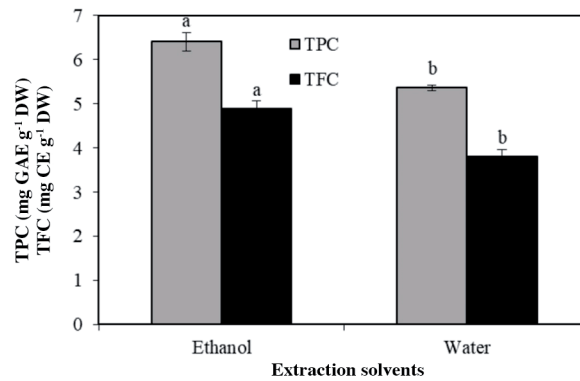
Effect of extraction solvents on the TPC and TFC of alfalfa

The ethanol extract contains higher amount of TPC and TFC compared with distilled water extract (Figure 3). The higher TPC and TFC were observed in the ethanol extract (6.41 and 4.88 mg GAE g⁻¹ DW, respectively). It is argued that methanol has the highest efficiency for extraction of flavonoids and phenols; however, ethanol is the safest extractor with the lowest residues. Such increase in TPC and TFC amounts in ethanol extracts may be attributed to the solvent polarity. The highest amounts of flavonoids were obtained via extraction with 70% ethanol in *Mentha longifolia* (Stanisavljević et al., 2012) and *Eriobotrya japonica* plants (Zhou et al., 2011). Many studies described that ethanol solvent can extract higher TPC and TFC of the plant samples compared with distilled water (Do et al., 2014). Our results agree with previous reports of total flavonoid contents in alfalfa leaves (Stochmal and Oleszek, 2007; Jing et al., 2015; Babakhani et al., 2017). Similarly, TPC contents revealed in our study are in accordance with the findings of previous reports (Babakhani et al., 2017; Chen et al., 2020).

DPPH scavenging capacity and reducing power of alfalfa

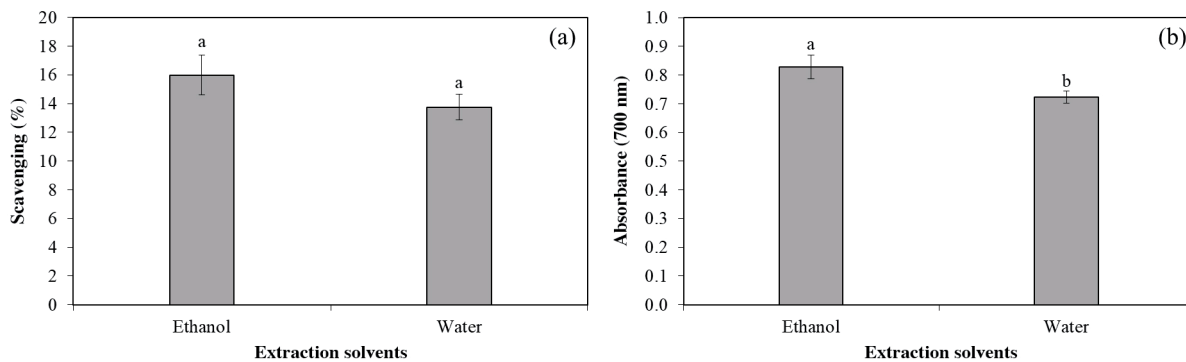
The effect of extraction solvents on DPPH scavenging capacity of alfalfa is shown in Figure 4a. In a similar way ethanol extract was significantly higher in DPPH scavenging capacity compared with water extraction. There was significant difference in DPPH scavenging capacity between ethanol extract (15.99%) and distilled water extract (13.75%). The ferric ion reducing power of ethanol and distilled water extraction of alfalfa is shown in Figure 4b. The reducing power of alfalfa

Figure 3. Effect of extraction solvents on the total polyphenol (TPC) and total flavonoid (TFC) contents of alfalfa.



Different letters indicate significant differences in the contents of TPC and TFC between the seasons according to Duncan's multiple range test ($P \leq 0.05$).
GAE: Gallic acid equivalents; CE: catechin equivalents; DW: dry weight.

Figure 4. Effect of extraction solvents on the DPPH scavenging capacity (a) and reducing power of alfalfa (b).



Different letters indicate significant according to Duncan's multiple range test ($P \leq 0.05$).

samples showed a similar trend as the DPPH scavenging. All the samples were tested at a concentration of 1 mg mL^{-1} and the ethanol extracted sample showed a non-significantly higher reducing power (0.828) than the distilled water extracted sample (0.724). The results of reducing power and DPPH scavenging echoed the trend of TPC and TFC of alfalfa samples which showed that the antioxidant capacity of the samples was in a part due to their flavonoid and phenolic contents. Oxidative stress adversely affects health as a result of oxidation of lipids, proteins, and even DNA leading to cell and tissue damage in addition to other malfunctional effects and diseases (Battino et al., 2020; Corteselli et al., 2020). Flavonoids and phenols may stimulate the antioxidant activity alleviating such adverse effects of oxidative stress. Indeed, flavonoids act as antioxidant molecules via scavenging oxygen free radicals and inhibiting the superoxide ion formation and thus indirectly inhibiting prooxidant enzymes and redox-sensitive genes and transcription factors (Battino et al., 2020; Chen et al., 2020). On the other hand, the antioxidant activity of polyphenols is attributed mainly to activation of antioxidant enzymes via providing the required hydrogen atom from their hydroxyl groups (Ebrahimzadeh et al., 2018).

CONCLUSIONS

There were significant differences in all the studied parameters between cuts of alfalfa collected in different seasons (autumn, winter, spring, and summer). Fresh yield and leaf:stem ratio were highest in spring, followed by winter and autumn (with nonsignificant differences), and were lowest in summer. The autumn cut showed the highest crude protein and crude fat content, whereas leaves cut in autumn had the lowest crude fiber and acid detergent fiber content. The results obtained in this study suggested that autumn was the best season to cut alfalfa leaves for nutritional purposes in arid areas.

However, alfalfa leaves cut in autumn were characterized by low digestibility values, which may affect their potential benefits. Further research is needed to confirm these results and identify the most suitable timing for cutting alfalfa leaves for nutritional purposes.

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