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Original Article

The Correlation of Some Secondary Metabolites of Alfalfa (*Medicago sativa* L.) with Plant Organ and Harvest Time

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Abstract

Background: Alfalfa (*Medicago sativa* L.) is an important source of phytoestrogens. The abundance of alkaloids, phenols, flavonoids, and isoflavonoids has made this plant a rich source of these plant estrogens. Cultivation of alfalfa as a rich source of phytoestrogens for medicinal purposes has provided opportunities for alternative use of this forage.

Methods: The study was carried out as a hydroponic culture in a perlite-cocopeat compacted bed with three replications. Roots and shoots of alfalfa plants were sampled separately in two stages (the 30th and 60th days after sowing). Plant samples were extracted with methanol solvent, and total phenols, flavonoids, and isoflavonoids contents were measured by spectrophotometric colorimetric method.

Results: The data analysis showed a significant effect of plant organ and harvest time on the contents of total phenols, flavonoids, and isoflavonoids ($P \leq 0.05$). The maximum accumulation of these compounds was in the plant shoots, and with the increase of the harvest time, the content of these phytoestrogens increased. Spearman's correlation analysis showed the different effects of the plant organ on the correlation level of the mentioned metabolites, so the flavonoids of the roots and shoots showed the most positive correlation, while isoflavonoids did not show a significant correlation ($P \leq 0.05$).

Conclusions: The presence of the maximum contents of total phenols, flavonoids, and isoflavonoids in the shoots of alfalfa can be concluded that the distribution of secondary metabolites in plants, the same as the primary metabolites, is mainly dependent on the plant organ and tissue. Furthermore, the maximum content of these metabolites in the vegetative stage of alfalfa is due to the transition from the vegetative stage to the reproductive stage in these plants. Therefore, the late vegetative stage is the best phenological stage and the most suitable harvest time for medicinal applications.

Keywords: Flavonoid, Isoflavonoid, Phenol, Phytoestrogens, Roots, Shoots.

Introduction

Alfalfa (*Medicago sativa* L.) is a perennial herbaceous plant originates from Asia and its genus is one of the most extensive genera of the family Fabaceae, which has about 87 different species worldwide, and 15 species of annual and perennial grasses in Iran. Alfalfa is the oldest cultivated plant in the world. Since the distant past, this plant has been cultivated for soil improvement, livestock feeding, and medicinal uses [1, 2]. In traditional medicine, alfalfa medicinal plant is used to improve memory, treat kidney pain, cough and muscle pain, as rejuvenating, anti-diabetic, antioxidant, anti-inflammatory, anti-fungal, anti-asthmatic, anti-microbial, diuretic, lactating, and also is used to treat disorders of digestive and central nervous systems [1].

Alfalfa is an important source of phytoestrogens, due to the abundance of alkaloids, phenols, flavonoids, and isoflavonoids in this plant. Trigonelline, genistein, daidzein, coumestrol, formonontin, and biocanin A are the main phytoestrogens identified in alfalfa. It has been determined that phytoestrogens have estrogenic and, or anti-estrogenic effects on humans and animals, as well as androgenic and progesterone effects; due to the similarity of their chemical structure and molecular weight to 17-β-estradiol and their behavior as selective modulators of the estrogen receptor. They are potent inhibitors of bladder, uterus, prostate, breast, and kidney cancer cells. In addition to protection against cancers, cardiovascular diseases and osteoporosis, their potential anti-microbial and anti-diabetic effects have been proven [1, 3-9].

The presence of isoflavonoids is limited to the family Fabaceae. Epidemiological studies suggest that communities with high dietary intake of phytoestrogens have a lower risk of many diseases [10, 11].

Alfalfa isoflavones, similar to many plant compounds such as lignin, tannins, anthocyanins, and many phytoalexins, are produced from the phenylpropanoid pathway, which is catalyzed by key enzymes such as isoflavone synthase 1 (IFS1) and cytochromes P450 [12,13].

Some studies have reported changes in the content of alfalfa phytoestrogens according to the harvest time [7, 14]. It has been determined in several cultivars of *M. sativa* that the concentration and yield of phytoestrogens generally increased in spring harvests in the year after planting [14]. Also, some phytoestrogens of *M. sativa* were investigated in consecutive months; and the result showed that their amount were low in the early stages of plant growth [7]. Therefore, the growth stage and the method of harvesting are important factors in fodder quality. Different harvest times affect the plant's ability to re-grow [15].

So far, little research from Iran has been done on the effect of plant organs and different harvest times on the secondary metabolites of alfalfa, which is more important considering the high production potential of this plant in arid and semi-arid regions. Therefore, this study aimed to identify the best plant organ and phenological stage, regarding the highest amount of secondary metabolites.

Materials and methods

Test design

This experiment was carried out in a factorial completely randomized design with two factors, harvest time, and plant organ, on *M. sativa* cv. Yazdi with three replicates. Each experimental unit was an alfalfa plant in a perlite-cocopeat compacted bed as a hydroponic culture in a cylindrical pot with a diameter and height of 25 cm.

Planting

Plants were planted in the research greenhouse of Ferdowsi University of Mashhad in the agricultural year of 2015. First, the seeds of *M. sativa* cv. Yazdi were prepared by Pakan Bazr Esfahan Company, and their germination percentage was checked. Briefly, 100 alfalfa seeds were initially sterilized with 5% w/v sodium hypochlorite solution for one minute, and then were placed in each petri dish on paper (four petri dishes with a diameter of 20 cm), and 10 ml of distilled water was added. The dishes were placed in the dark at 25 °C for 72 hours. After three days, the seed germination percentage was calculated from Equation 1.

Germination percentage (%) = number of germinated seeds / total number of seeds × 100 (1)

According to the findings, the average seed germination percentage was 95.25%, which was considered suitable for planting and performing the experiment. To prepare the planting bed, perlite (medium grain) and cocopeat fibers were washed separately to remove any contamination, and sterilized in an autoclave (Vertical Autoclave, PECO Laboratory Equipments, Iran) (at 121 °C and 100 kPa for 15 minutes), and then the plastic cylindrical pots were filled with a perlite-cocopeat mixture ratio of 1:1 (v/v). Finally, the pots were washed several times, and the bed was compacted. After that, three alfalfa seeds were planted in each pot, and after the establishment of the plants, only one superior plant was kept in each pot.

Environmental conditions

Alfalfa plants were grown in a greenhouse environment with a relative humidity of 60±5% and light conditions of 16 hours of light with a light intensity of 150 μmol photons m^{-2} s^{-1} at 25±2 °C and eight hours of darkness at 20±2 °C.

Irrigation and feeding of plants

The plants were irrigated with distilled water (50 ml) every three days until the seeds germinated. After germination and until the 10-leaf stage (up to 4 weeks), they were irrigated with 150 ml of distilled water every three days, and 150 ml of Hoagland's nutrient solution (½ modified), every two weeks. From the 10-leaf stage onwards, the plants were irrigated weekly 150 ml of ½ modified Hoagland's nutrient solution, and after three days, irrigated with 250 ml of distilled water to remove the effects of accumulation of the elements in the pots.

Preparation of ½ modified Hoagland's nutrient solution

Hoagland's nutrient solutions were prepared using the method described by Hoagland and Arnon (1950) with some modifications [16]. Briefly, the stock solutions of macronutrients (KNO_3, $Ca(NO_3)_2$, KH_2PO_4, and $MgSO_4$), micronutrients (KCl, H_3BO_3, $MnSO_4$, Na_2Mo_4, $ZnSO_4$, and $CuSO_4$) and iron were made, and the complete Hoagland's nutrient solution was prepared. All stock and complete Hoagland's nutrient solutions were prepared with distilled water (the required amounts of each element to form stock solutions and the amount of stock solution required to form complete Hoagland's nutrient solution are listed in Table 1).

Thereafter, to prepare ½ modified Hoagland's nutrient solution, the complete Hoagland's nutrient solution was diluted by ½ with distilled water and used to irrigate the plants. The solutions were kept away from light.

Table 1: Preparation of complete Hoagland's nutrient solution

	Nutrient elements	The amount of nutrient elements to make 100 ml of stock solution*	The amount of stock solution to make 1000 ml of complete Hoagland's nutrient solution
Macro-nutrients stock solutions	KNO_3	10 g	5 ml
	$Ca(NO_3)_2$	23.615 g	5 ml
	KH_2PO_4	13.6 g	1 ml
	$MgSO_4$	24.65 g	1 ml
Micro-nutrients stock solution	KCl	0.1864 g	2 ml
	H_3BO_3	0.0733 g	
	$MnSO_4$	0.0169 g	
	Na_2Mo_4	0.004 g	
	$ZnSO_4$	0.0288 g	
	$CuSO_4$	0.0562 g	
Iron stock solution	1 g of $FeSO_4$ was heated in 500 ml of distilled water; then 1 g of NaEDTA was dissolved until a yellow solution was obtained.		1 ml

** Stock solutions of macronutrients were prepared for each element separately, and the stock solution of micronutrients was prepared for all elements as a unit. All stock and complete Hoagland's solutions were made with distilled water.*

Sampling

Sampling of alfalfa plants was done from the roots and shoots, separately, in two stages of the 30th and 60th days after sowing (DAS). The samples were dried in an oven (Oven model 5-1486, Memmert Co., Germany) at 40 °C for 72 hours.

Extraction

The extraction of dried plant samples of the roots and shoots was done using the method described by Ismail *et al.* (2010) with some modifications [17]. Briefly, 100 mg of dry powder of alfalfa roots/shoots was mixed with 2 ml of methanol (80% v/v) and swirled in an incubator shaker (WiseCube model WIS-20, Daihan Scientific Co., Korea) at 45 °C and 150 rpm for 45 minutes; then incubated in Sonicate (Sonicate model 2600S, Parsonic Co., Japan) at 50 °C and 40 kHz for 44 minutes, and then the extracts were filtered using filter paper (Whatman No. 1, England), concentrated and dried under a chemical hood (at 55 °C for about 4 hours), and kept at -20 °C for further analysis.

Dilution of extracts

Total phenols, flavonoids, and isoflavonoids assays were performed with the diluted extract (1 mg ml^{-1}) [17]. For this purpose, 2 mg of completely dried extract was dissolved in 2 ml of methanol (80% v/v) in the sonicate at 50 °C and 40 kHz for 40 minutes; in such a way that after every 20 minutes of sonication, it was vortexed (WiseMix model VM-10, Daihan Scientific Co., Korea) at 70 °C for two minutes.

Quantifying the total phenolic content

Total phenolic content (TPC) was determined based on the Folin-Ciocalteu colorimetric assay using gallic acid as a standard, and the method described by Ismail *et al.* (2010) with some modifications [17]. Briefly, 2.5 ml of Folin-Ciocalteu reagent (10% v/v with distilled water) and 2 ml of sodium carbonate (7.5% w/v with distilled water) were added to 0.1 ml of the diluted extract; so that the volume of the reaction solution became 4.6 ml. Each plant sample was measured in three technical replicates. After 30 minutes of incubation at 40 °C in the dark, the absorbance (average of three technical replicates) of the reaction solution was measured using a UV-Vis spectrophotometer (Jasco-LCD type-model 7800, Japan Spectroscopic Co., Japan) at 760 nm against the blank solution (distilled water), and the TPC was expressed from the standard curve as µg gallic acid equivalent per mg of dry extract.

Finally, statistical analysis was performed on the TPC of treatment samples relative to the control group, and the findings were reported based on the mean of three replicate experiments and the standard deviation (Mean±SD).

Standard curve of gallic acid

0.02 g of gallic acid was initially added to the volume of 50 ml with distilled water to make the 400 µg ml^{-1} stock solution, and then the concentrations of 0.032, 0.063, 0.094, 0.125, 0.188, 0.25, 0.313, 0.375, 0.438, 0.5, 0.563, 0.625, 0.782, 0.938, 1.094, and 1.25 ml of the stock solution were added to the volume of 2.5 ml with distilled water to gain the concentrations of 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, and 200 µg ml^{-1}, respectively.

Then, the absorbance of concentrations was measured in three technical replications, the same as before; and the standard curve of gallic acid was drawn based on the relationship between the concentration of the solutions and their corresponding average absorbance (three technical replications), using Microsoft Excel 2013 software, and the line equation to calculate the TPC of unknown samples was presented (Figure 1).

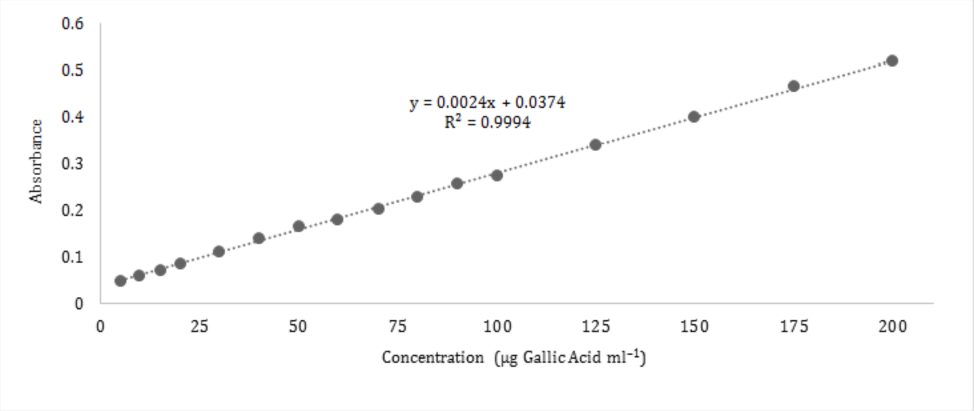


Figure 1 Gallic acid standard curve in spectrophotometric analysis

Quantifying the total flavonoid content

Total flavonoid content (TFC) was measured based on an aluminum chloride colorimetric assay using quercetin as a standard, and the method described by Chang *et al.* (2002) with some modifications [18]. Briefly, 1.5 ml of 80% v/v methanol, 0.1 ml of aluminum trichloride (10% w/v with distilled water), 0.1 ml of potassium acetate (1 M), and 2.8 ml of distilled water were added to 0.5 ml of the diluted extract; So that the volume of the reaction solution became 5 ml. Each plant sample was measured in three technical replicates. After 30 minutes of incubation at ambient temperature (25 °C), the absorbance (average of three technical replicates) of the reaction solution was measured using the UV-Vis spectrophotometer at 415 nm against the blank solution (similar to the reaction solution without extract and aluminum trichloride), and the TFC was expressed from the standard curve as µg quercetin equivalent per mg of dry extract. Finally, statistical analysis was performed on the TFC of treatment samples relative to the control group, and the findings were reported based on the mean of three replicate experiments and the standard deviation (Mean±SD).

Standard curve of quercetin

0.02 g of quercetin was initially added to the volume of 50 ml with methanol (80% v/v) to make a 400 µg ml^{-1} stock solution, and then the concentrations of 0.032, 0.063, 0.094, 0.125, 0.188, 0.25, 0.313, 0.375, 0.438, 0.5, 0.563, and 0.625 ml of the stock solution were added to the volume of 2.5 ml with methanol (80% v/v) to gain the concentrations of 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg ml^{-1}, respectively. After that, the absorbance of the concentrations was measured in three technical replications, the same as before; and the quercetin standard curve was drawn based on the relationship between the concentration of the solutions and their corresponding average absorbance (three technical replications), using Microsoft Excel 2013 software, and the line equation to calculate the TFC of unknown samples were presented (Figure 2).

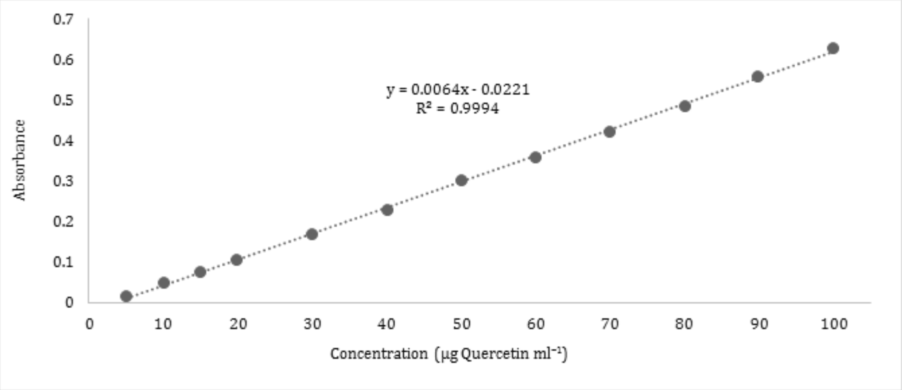


Figure 2 Quercetin standard curve in spectrophotometric analysis

Quantifying the total isoflavonoid content

Total isoflavonoid content (TIC) was measured based on an aluminum chloride colorimetric assay using genistin as a standard, and the method described by César *et al.* (2008) with some modifications [11]. Briefly, 0.125 ml of aluminum trichloride (2% w/v with methanol 80% v/v) and 2.375 ml of methanol (80% v/v) were added to 0.625 ml of the diluted extract; so that the volume of the reaction solution became 3.125 ml. Each plant sample was measured in three technical replicates. After incubating the samples with aluminum trichloride at ambient temperature (25 °C) for 10 minutes, the absorbance (average of three technical replicates) of the reaction solution was measured using the UV-Vis spectrophotometer at 382 nm against the blank solution (methanol 80% v/v), and the TIC was expressed from the standard curve as µg genistin equivalent per mg of dry extract. Finally, statistical analysis was performed on the TIC of treatment samples relative to the control group, and the findings were reported based on the mean of three replicate experiments and the standard deviation (Mean±SD).

Standard curve of genistin

3 mg of genistin was initially added to the volume of 100 ml with methanol (80% v/v) to make the 30 µg ml^{-1} stock solution, and then the concentrations of 0.08, 0.17, 0.34, 0.5, 0.67, 0.84, 1, 1.17, 1.34, 1.5, 1.67, 1.84, 2, 2.17, 2.34, 2.5, 2.67, 2.84, 3, 3.17, 3.34, 4.17, 5, 5.84, 6.67, 7.5, 8.34, and 9.17 ml of the stock solution were added to the volume of 10 ml with methanol (80% v/v) to gain the concentrations of 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, and 27.5 µg ml^{-1}, respectively. Next, the absorbance of the concentrations was measured in three technical replications, the same as before; and the genistin standard curve was drawn based on the relationship between the concentration of the solutions and their corresponding average absorbance (three technical replications), using Microsoft Excel 2013 software, and the line equation to calculate the TIC of unknown samples were presented (Figure 3).

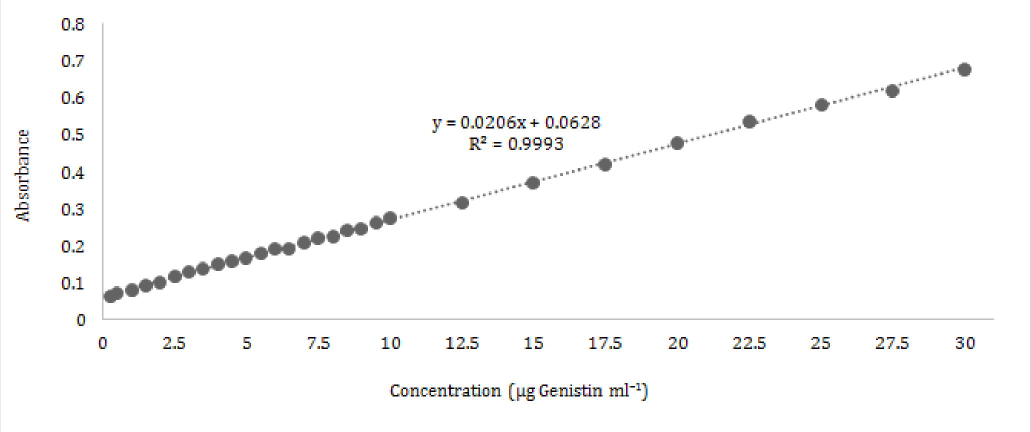


Figure 3 Genistin standard curve in spectrophotometric analysis

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20, Microsoft Excel 2013, and GraphPad Prism 8. Analysis of the variance of the experiment data was done by the two-way ANOVA test. The mean comparisons between the treatment groups were done by Duncan's multi-range test ($P \leq 0.05$), and the findings were reported based on the mean of three replications and standard deviation (Mean±SD). The data dispersion between independent and dependent variables was analyzed by linear regression, and the intensity and direction of the relationship between the measured attributes by Spearman's correlation coefficients (rs). Graphs were drawn with Microsoft Excel 2013 and GraphPad Prism 8.

Results

The contents of secondary metabolites of alfalfa roots and shoots

The TPC, TFC, and TIC of the roots and shoots of alfalfa were evaluated in the 30th and 60th DAS. The variance analysis of the data showed that the effect of plant organs on the content of all three groups of secondary metabolites, as well as the effect of harvesting time on the TPC and TIC was significant at $P \leq 0.01$. The effect of harvesting time on the TFC and also, the interaction effects of harvest time and plant organs on the TPC and TIC were significant at $P \leq 0.05$ while the interaction effect of harvest time and plant organ on the TFC was not significantly different (Table 2).

Table 2 Analysis of variance (mean squares) of the effects of plant organ and harvest time on the TPC, TFC, and TIC of alfalfa

Variation Resources	Degrees of Freedom	Mean squares		
		TPC	TFC	TIC
Plant organ	1	28415.93 **	141.09 **	22.93 **
Harvest time	1	6406.92 **	18.5 *	8.15 **
Plant organ × Harvest time	1	506.28 *	0.01 ns	2.25 *
Residual	8	93.48	1.94	0.22
Coefficient of Variation (%)	----	7.31	4.22	7.59

**, and ** indicate significance at P≤0.05 and P≤0.01, respectively, and ns indicates non-significant.*

The mean comparisons of the TPC, TFC, and TIC of the roots and shoots of alfalfa in the 30th and 60th DAS showed that the highest amount of these compounds was in the shoots on the 60th DAS (210.58, 37.68, and 8.81 µg gallic acid, quercetin, and genistin equivalent per mg of dry extract, respectively) and the lowest amount of these metabolites was in the roots on the 30th DAS (67.05, 28.33, and 4.39 µg gallic acid, quercetin, and genistin equivalent per mg of dry extract, respectively) which were significant ($P \leq 0.05$) (Table 3).

Table 3 Mean comparisons of the TPC, TFC, and TIC of the roots and shoots of alfalfa in the 30th and 60th DAS (as µg gallic acid, quercetin, and genistin equivalent per mg of dry extract, respectively)

Plant organ	Harvest time	Secondary metabolites contents*		
		TPC	TFC	TIC
Roots	30th	67.05±6.71 d	28.33±1.47 b	4.39±0.41 c
	60th	100.27±3.36 c	30.87±0.48 b	5.18±0.43 c
Shoots	30th	151.38±13.62 b	35.24±0.39 a	6.29±0.35 b
	60th	210.58±2.77 a	37.68±1.63 a	8.81±0.35 a

**The results are based on the mean of three replicate experiments (Mean±SD). In each column, means with at least one letter in common, are not significantly different by Duncan's multiple range test (P≤0.05).*

Also, the TPC, TFC, and TIC increased by 116.35%, 23.17%, and 57.83%, respectively, in the shoots compared to the roots at both harvest times; which indicates the maximum accumulation of these metabolites in the shoots of the plant (Figure 4). On the other hand, the total metabolites of the roots and shoots in each group of the phenols, flavonoids, and isoflavonoids increased by 42.32, 7.82, and 57.83%, respectively, in the 60th DAS relative to the 30th DAS, which indicates an increase in the mentioned metabolites during the phenology stages of the plant (Figure 4).

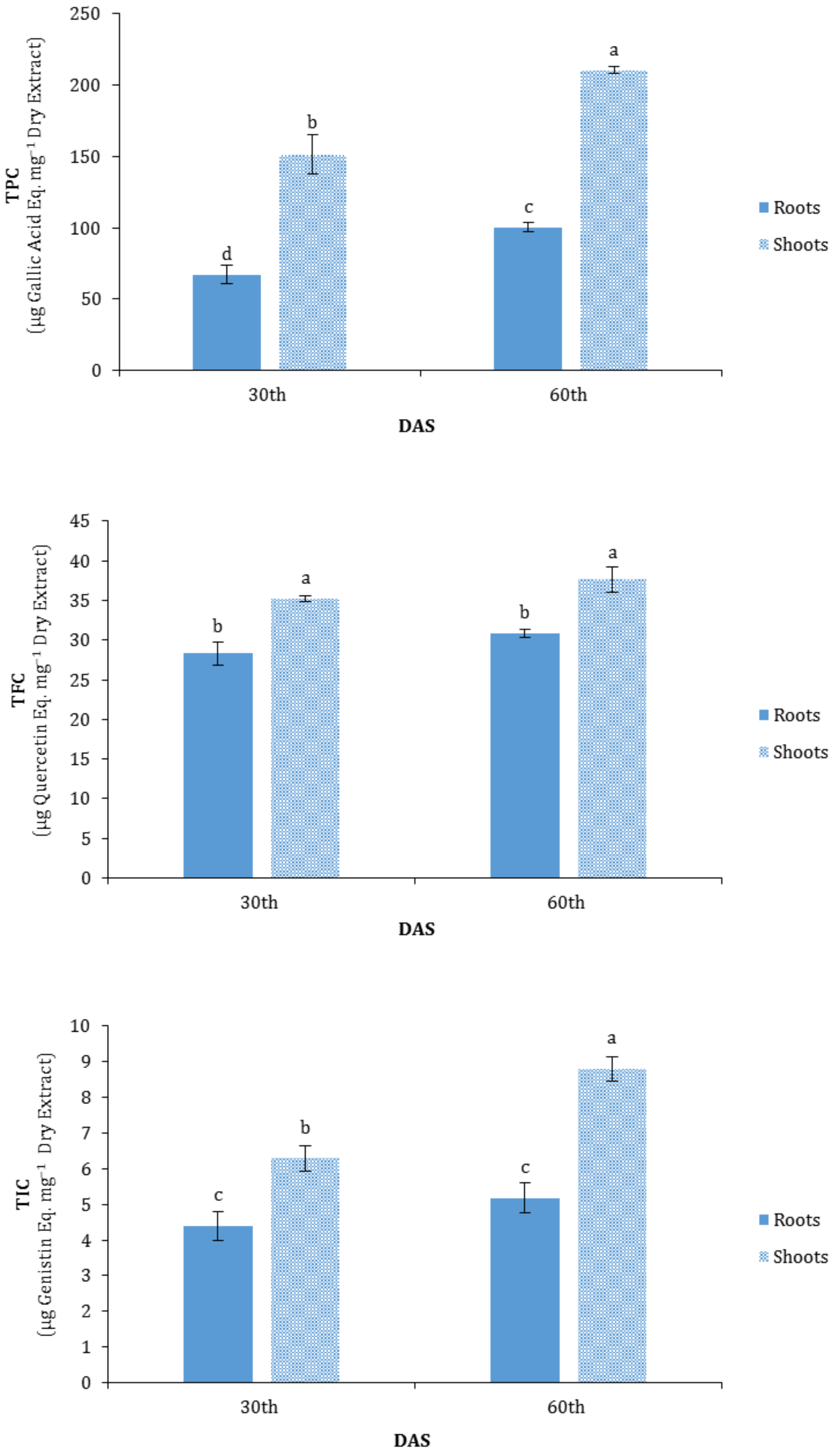


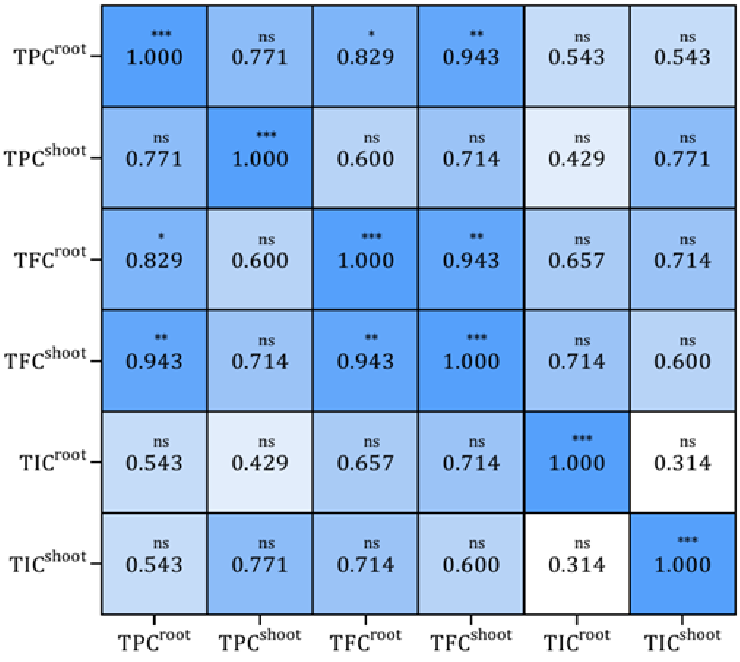
Figure 4 The TPC, TFC, and TIC of the roots and shoots of alfalfa in the 30th and 60th DAS (as µg gallic acid, quercetin, and genistin equivalent per mg of dry extract, respectively). The results are based on the mean of three replicate experiments. Means with at least one letter in common, are not significantly different by Duncan's multiple range test (*P*≤0.05) (Error bars = ±SD)

Correlation between the TPC, TFC, and TIC of alfalfa roots and shoots

The relationship between TPC, TFC, and TIC of alfalfa roots and shoots was investigated at both harvest times. For this purpose, first, the data dispersion was analyzed by linear regression model. Due to the non-significance ($P≤0.05$) of the slope of the regression lines and the inappropriateness of their determination coefficients (R^2), the linear regression model of the data was rejected (R^2 for TPC^{roots}, TPC^{shoots}, TFC^{roots}, TFC^{shoots}, TIC^{roots}, and TIC^{shoots} were 0.3894, 0.7269, 0.4668, 0.1847, 0.5435, and 0.9072, respectively). Therefore, to investigate the relationship between the mentioned metabolites, Spearman's correlation coefficients (rs) were calculated.

Spearman's correlation analysis indicated a positive and significant relationship ($P≤0.05$) between some secondary metabolites of the roots and shoots. By comparing the correlation coefficients, as shown in Figure 5, the highest positive correlations were observed between TFC^{roots}-TFC^{shoots}, TFC^{shoots}-TPC^{roots}, and TFC^{roots}-TPC^{roots} (according to the maximum rs: 0.933, 0.905, and 0.885, respectively), which all three of them were significant; but the other correlations were not significant ($P≤0.05$). This comparison showed the different effects of the plant organ on the correlation level of the mentioned metabolites. So, the flavonoids of the roots and shoots showed the most positive correlation while isoflavonoids did not show a significant correlation ($P≤0.05$). The correlation coefficients and their significance levels are demonstrated in Figure 5.

On the other hand, by comparing (absolute value) the slope of the regression lines of the secondary metabolites contents of the roots and shoots of alfalfa, it was found that the most significant effects of the plant organs were on the TPC^{shoots}, TPC^{roots}, TIC^{shoots}, TFC^{roots}, TFC^{shoots}, and TIC^{roots} (according to the maximum slope: 14.85, 7.23, 0.7332, 0.6637, 0.4291, and 0.2345, respectively).



	TPC^{root}	TPC^{shoot}	TFC^{root}	TFC^{shoot}	TIC^{root}	TIC^{shoot}
TPC^{root}	*** 1.000	ns 0.771	* 0.829	** 0.943	ns 0.543	ns 0.543
TPC^{shoot}	ns 0.771	*** 1.000	ns 0.600	ns 0.714	ns 0.429	ns 0.771
TFC^{root}	* 0.829	ns 0.600	*** 1.000	** 0.943	ns 0.657	ns 0.714
TFC^{shoot}	** 0.943	ns 0.714	** 0.943	*** 1.000	ns 0.714	ns 0.600
TIC^{root}	ns 0.543	ns 0.429	ns 0.657	ns 0.714	*** 1.000	ns 0.314
TIC^{shoot}	ns 0.543	ns 0.771	ns 0.714	ns 0.600	ns 0.314	*** 1.000

Figure 5 Comparing the correlation of the TPC, TFC, and TIC of the roots and shoots of alfalfa. The results are based on Spearman's correlation coefficients (rs) (*, **, and *** indicate significance at $P≤0.05$, $P≤0.01$, and $P≤0.001$, respectively, and ns indicates non-significant)

Discussion

Effect of plant organ on TPC, TFC, and TIC

Investigation of total phenols, flavonoids, and isoflavonoids contents of alfalfa roots and shoots showed that the maximum accumulation of these metabolites was in the plant's shoots. Data analysis showed a significant effect of the plant organs on the contents of the mentioned compounds ($P \leq 0.05$). Therefore, it was found that the type of plant organ can affect the contents of its secondary metabolites.

Other studies showed a significant effect of plant organs on the content of phenolic compounds in the studied plant species. In a study on *M. sativa*, the highest contents of phenols, flavonoids, and tannins were reported in leaves [19]. In a phytochemical study on several perennial plant species from the legume family, including *M. sativa*, *M. lupulina* L., *Trifolium pratense* L., *T. medium* L., *Onobrychis viciifolia* Scop., *Astragalus glycyphyllos* L., and *A. cicer* L., some isoflavonoid compounds, tannins, and triterpene saponins were identified and compared in the shoots (leaf, stem, and flower). It was found that in most of the mentioned species, the highest amount of these metabolites were in the order of leaves > stems > flowers, and it was stated that the plant organ affects the secondary metabolites [20].

In a study on different extracts of some tissues of *M. sativa*, was found that the methanolic extracts of the leaves contained the highest content of TPC, and the flowers contained the highest TFC, and the methanolic extract of the stem showed the lowest amount of these compounds [21]. It has been determined that the leaves *of Smilax campestris* Griseb. contained more TPC and TFC than the roots and rhizomes [22]. In another study, the highest amounts of phenolic and flavonoid compounds were reported in the shoots of the *Sophora flavescens* Aiton compared to the roots. [23] Similar findings have been reported in other researches on the purslane plant that most phenolic compounds were in purslane leaves [24, 25].

Studies have shown that leaves and flowers contain higher levels of secondary metabolites (such as phenolic acids and terpenoids) than stems and roots. In a study on different organs (root, stem, and leaf) of *M. sativa*, was shown that the maximum TPC and TFC and antioxidant activity of the methanol extract were in leaf > stem > root, respectively, and the TPC and TFC of leaves were more than twice that of roots [26]. In another study, the highest amount of phytoestrogens was observed in flowers among different organs of *M. sativa* and different stages of maturity [27]. Likewise, in another research, the TPC of different extracts of *M. sativa* flowers has been identified and compared, and the TPC of the methanolic extract was found to be 263.5 mg gallic acid equivalent per 100 g of dry extract. It was also found that the aqueous extract performed better than methanol and acetic acid to extract the phenolic metabolites [28].

In the study on the species of the genus *Hypericum* L., it was determined that the highest content of TPC was assigned to the leaves [29]. The highest amount of flavonoids was observed in the leaves of *H. pruinatum* Boiss. & Balansa [30]. Furthermore, the highest total phenolic and flavonoid content was reported in the leaves of *Melissa officinalis* L. [31]. A significant difference in TPC and TFC and antioxidant activity has been observed between different organs (fruit > flower > leaf > stem > leaf petiole) of *Sinopodophylum hexandrum* (Royle) T. S. Ying, shows a positive relationship between the TPC and the antioxidant activity of these organs [32]. The flowers of *Sambusus nigra* L. have the highest amount of polyphenols and phenolic acids, and the leaves and fruits of this plant are rich in flavonoids and anthocyanins. Also, different TPCs have been reported in the organs of *Crataegus pentagyna* Willd.; so, the maximum TPCs were in flowers, leaves, and fruits [32].

Plant organs have different functions and levels of exposure to environmental conditions that can affect the distribution of metabolites and their antioxidant capacity. The concentration of phenolic compounds in a plant is closely related to the type and function of the target organ [32]. According to the relationship between secondary metabolism and primary metabolism in plants, the presence of secondary metabolites is influenced by primary metabolism, and the structural precursors of these compounds are obtained from primary metabolites. Therefore, the distribution of secondary metabolites, similar to primary metabolites, is highly dependent on organs and tissues [33], and the mentioned studies also showed the maximum accumulation of phenolic compounds in the shoots of plants compared to the roots.

The effect of harvesting time on TPC, TFC, and TIC

Investigating the contents of secondary metabolites at the 30th and 60th DAS showed that the TPC, TFC, and TIC reached their highest level in the second stage (60th DAS). Data analysis showed the significant effect of harvesting time on the content of the mentioned compounds ($P \leq 0.05$) so that the content of these secondary metabolites increased as the harvest time increased.

In a study, *M. sativa* leaves were harvested in autumn, winter, spring, and summer, and their aqueous and alcoholic extracts were analyzed. The highest TPC and TFC, as well as antioxidant activity, were observed in the alcoholic extract of autumn leaves [34]. A comparative study of the TIC of different aqueous, aqueous-alcoholic, and alcoholic leaf extracts of seven species of alfalfa (*M. minima* (L.) Bartalini, *M. tornata* L., *M. truncatula* Gaertn., *M. rigidula* L., *M. scutelata* Mill., *M. segitalis* L., and *M. sativa*) has been done; and *M. sativa* TIC of all type of solvents was the lowest, but it did not mention the time and quality of harvesting [35]. In another study on several cultivars of *M. sativa*, it was found that these metabolites increased in the spring harvest of the year after planting [14]. It has been reported that the content of phenolics strongly depends on the growth conditions, degree of maturity, and maturity of alfalfa, and also the time of harvest affects the diversity and chemical composition and total isoflavonoid content [7]. Therefore, the change in phenol content and antioxidant capacity during different stages of plant growth is closely related to its metabolic and physiological changes [36].

In another study, it was reported that the content of flavonoids in the shoots of *M. sativa* changed in multiple harvests in a year. Therefore, the first harvest showed the highest amount of these compounds compared to the subsequent harvests, and it was stated that the TFC gradually decreases during the season [37]. It was determined in research on several cultivars of *M. sativa* at the initial flowering stage, that the concentration and yield of phytoestrogens generally increased in the spring harvest of the year after planting [14]. In another study, the highest amount of phytoestrogens was observed in flowers among different organs of *M. sativa* and different maturity stages [27]. In a study, it was reported that the content of phenolics is highly dependent on the growth conditions and maturity of alfalfa; and the harvest time affects the diversity and chemical composition as well as the total isoflavonoid content, and some phytoestrogens of *M. sativa* were low in consecutive months of harvest and the initial stages of plant growth [7].

In general, plant phenols show significant quantitative and qualitative changes not only at genetic levels (between and within species and clones), but also between different developmental and physiological stages. The assessment of seasonal and genetic changes in the content and activity of phenolic compounds allows choosing the best time to harvest the plant [32]. In addition to the seasonal fluctuations that occur during the growing season, the age of the plant can also be an important factor in the content of secondary metabolites [38].

Several genetic, ontogeny, morphogenetic, and environmental factors can affect the biosynthesis and accumulation of secondary metabolites [37, 39]. Due to the close relationship between secondary metabolism and primary metabolism in plants, the existence of secondary metabolites is influenced by the primary metabolism of the plant. Structural precursors of secondary metabolites are derived from primary metabolites. The main pathways of secondary metabolites in plants and their relationship with primary metabolism have been identified [33].

Conclusion

This experiment confirmed that the TPC, TFC, and TIC were significantly higher in the shoots than in the roots; their maximum amount in the shoots of alfalfa is proof of the synthesis and accumulation of these secondary metabolites, mainly in the shoots of the plant. According to the findings of the present research, it can be concluded that the distribution of secondary metabolites in plants, similar to primary metabolites, is highly dependent on organs and tissues, and the maximum accumulation of phenolic compounds is in the shoots of plants relative to the roots. On the other hand, the highest TPC, TFC, and TIC in the late vegetative stage of alfalfa is due to the transition from the vegetative stage to the reproductive stage in these plants and can be an important factor in determining the quality and yield of the product. Since the presence of alfalfa phytoestrogens is very important and beneficial to humans for their medicinal properties as well as fodder production of meat animals; the late vegetative stage is a valuable phase and the most suitable time for harvesting, because of the highest level phytoestrogens. Also, postponing the harvest time to the late vegetative stage, the plant will be able to store the necessary energy for the next re-growth after harvesting.

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