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Evaluation of the effect of environmental condition on metabolites and morphology of seeds and prediction of 4-hydroxy isoleucine and trigonelline as two therapeutic metabolites of Persian fenugreeks

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Abstract: *Trigonella foenum* is one of the oldest medicinal plants that grow in many parts of Iran with the diverse ecological situation. Employing this plant for treating diabetes and high cholesterol has a long history, because of some metabolites. Due to the habitat of fenugreek is a wide range of climatic conditions, it may have power to cope with climate variation. The main intention of this inquiry was to understand the effect of the environmental variables on this therapeutic plant features. It was also interesting for us to understand which environment variables are more impressive for enhancing of trigonelline and 4-hydroxy isoleucine content as the most important metabolites of this plant. For achieving this goal, environmental information and vegetal data were analyzed to discover the role of nature on the seed part of fenugreek life in 50 different regions of Iran. Canonical correspondence analysis (CCA) displayed that high content of metabolites and some morphological characteristics happened in high temperature and solar irradiation. Partial least squares regression (PLSR) and path analysis used to find the best predictors and direct and indirect effect of all variables on 4-hydroxy isoleucine and trigonelline. Ecological condition were the best predictors and had the highest direct and indirect impact on 4-hydroxy isoleucine. However, for trigonelline, the environment did not play a senior role. It seems that the reaction of components of fenugreek does not follow the same way. Studying on morphological, primary and secondary metabolites, and surrounding environment of fenugreek, helped us to have a more precise judgment about the life of this plant.

Keywords: fenugreek; medicinal plant; 4-hydroxy isoleucine; trigonelline; ecological condition; canonical correspondence analysis

1. Introduction

The legume (Leguminosae = Fabaceae) is a great family that has 750 genera and about 18000 species[1]. The legume seeds have valuable nutritionists with different chemical base[2]. Fenugreek is a member of this family as a medicinal plant that has a high amount of phenolic compound, flavonoid, alkaloid, and protein[3]. It also has unusual amino acid named 4-hydroxy isoleucine[4] and trigonelline as a saponin[5]. Fenugreek belongs to *Trigonella* genus that has around 260 species.

Some studies show chemical components and morphological traits of plants are more affected by the environmental condition than genetic variation[6,7]. Plant plasticity is an important subject to help plants to adopt the situation that they have to live in, survive, and produce another generation, to not remove from the pass of history[8]. The phenotypic plasticity defines as a power of the plants to change the direction of their way of growing, including morphological traits and functions, consisting of metabolites[6] in facing the different ecological condition.

This hypothesis is presented in some experimental study. Saudi researchers found that environment can affect chemical compositions of herbs[9]. They suggested that differences of fatty aides, moisture, and some mineral contents between seeds of some spices are due to the impact of environmental factors.

Since fenugreek has broad geographical habitats, from Mediterranean, Asia, Australia, Europe, Macaronesia, to North and South of Africa[5], it is predictable, that this plant for surviving in this different ecological condition needed to change its chemotypes content and morphological traits.

On the whole, medicinal plants have not attracted ecologist to study about the effect of natural habitats condition on their phenotypes as much as other usual plants, maybe because the therapeutic side of the medicinal plant is more important and more attractive for scientists [10].

As an undeniable fact, medicinal plant, amongst fenugreek, cannot escape from undesirable condition[11] like as many other common plants. Thus, it is undoubtful that environment condition in the regions that fenugreek, has been grown has a considerable effect on the content of chemical components[11]. Distribution of some species such as *T. foenum-graecum* L. includes a wide range of the universe. Thus they meet many different ecological requirements providing an opportunity to adopt themselves[12]. It is vivid that both morphological and metabolic traits have a role in reaction to climatic variation in three ways: adaptive evolution and phenotypic plasticity, or a combination of both them[13]. Based on our knowledge, there is no research investigates the morphological and metabolic changes of fenugreek seeds in their natural environment. Most studies searched on the effects of artificial stress on salinity[14][15][16], drought[17][18], and so on. However, this is the easiest way, because they control all variation except one that they want to examine it.

In contrast, phenotypic plasticity measurement is more difficult in natural habitats than controlled situations[13]. Though in some studies, the effect of environmental factors on the morphological traits was determined[13][19].

In a study, the geographical impact on morphological characteristics was investigated[20]. In Serbia, the effect of environmental features investigation of fenugreek anatomy presented air pollution can lead to the reduction of leaves thickness[21]. Amino acids composition of fenugreek was significantly different between regions with different ecological conditions. Seed yield of this medicinal plant was sensitive to modified date of sowing and different level of iragition[22].

Since fenugreek (*T. foenum-graecum* L.) is more attractive an applicable for scientist between all other species of *Trigonella* genus[4]; we chose that for investigating of the impact of environmental variables on its important metabolites and morphological traits.

We were interested in demonstrating whether there is a correlation between the climatic condition and phenotypic characteristics in fenugreek genotypes or not.

We also investigated the feasibility of determining a desirable prediction model for two valuable fenugreek metabolites using the PLSR technique include trigonelline and 4- hydroxy isoleucine. So, we used several analytical methods to evaluate fenugreek traits to address these questions.

First of all, using the correlation coefficient, we demonstrated the relationship of all phenotypic characteristics, including primary and secondary metabolites and morphological features.

After that, we used HCPC analysis (integration of hierarchical clustering on principal components) for understanding the variation and grouping data and determination of most critical variables in each group. To understand the relationship and effect of environmental factors on the fenugreek seeds, we invited the canonical correspondence analysis (CCA). Genetic advance and heritability helped us to the definition of heritance abilities of plants traits. Our result confirmed that the heritability of metabolites is less than morphological characteristics. We also, performed a PLSR approach to detect all vegetal and environmental features potential in prediction of two important metabolites of fenugreek (4-hydroxy isoleucine and trigonelline). Finally, we determined the direct and indirect effect of traits on these two metabolites by path analysis.

2. Results

2.1. The primary and secondary metabolites and morphological traits were significantly different between all regions

Analysis of variance displayed significant differences between all characters ($P < 0.001$). Average of all vegetal characteristics were shown in Excel S1. From all peaks of amino acids, only seven of them were selected based on their abundance. These seven amino acids included: aspartic acid, glutamic, valine, phenylalanine, leucine, isoleucine, and lysin. They also are the most abundant than other amino acids in fenugreek[5].

2.2. There was an association between some metabolites with some morphological features

The correlation coefficient (r^2) was estimated by the Pearson method based on the least-square mean. Some morphological characteristics and secondary metabolites were related to each other in both positive and negative ways (Figure 1).

Seed weight and seed width showed a significant positive correlation with the most metabolites including 4-hydroxy isoleucine as a therapeutic component, isoleucine, and leucine as primary metabolites, and trigonelline, antioxidant activity, phenol and flavonoid content as secondary metabolites. Some metabolites such as glutamic and lysin did not present any correlation with morphology of fenugreek.

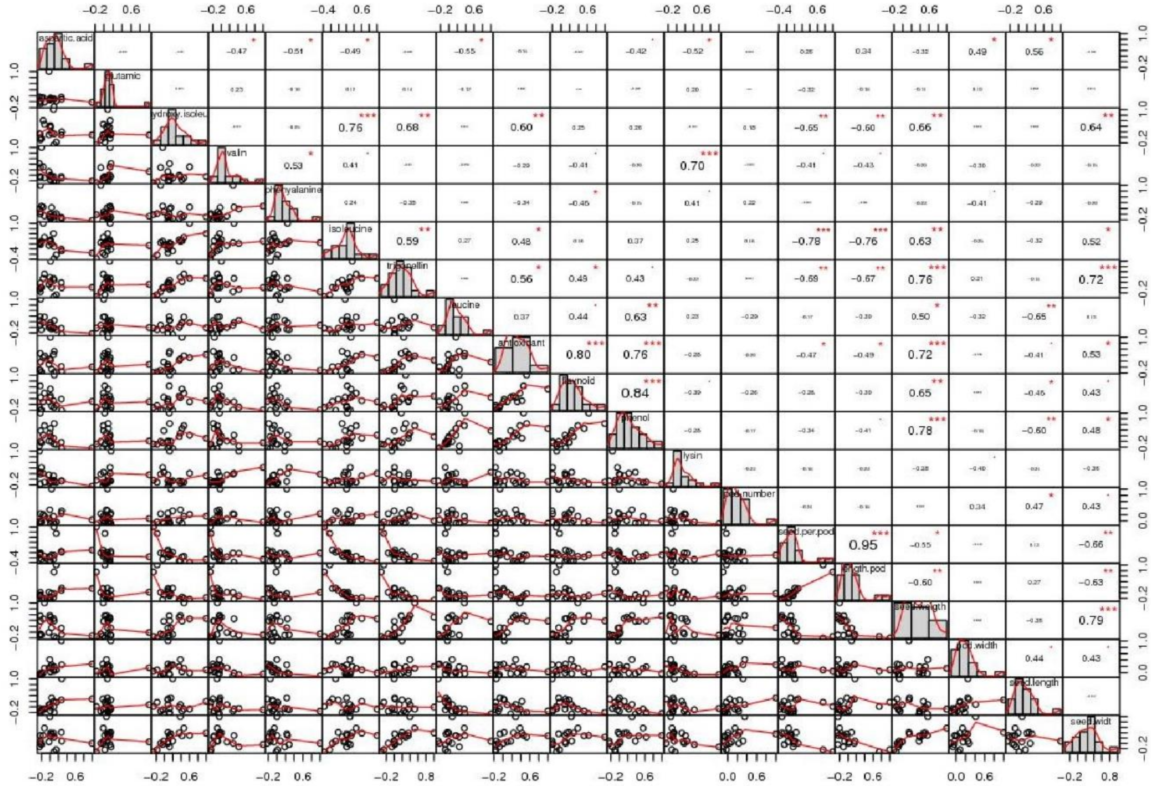

Figure 1. Correlation plot for19 phenotypic variables in fenugreek using Pearson's correlation coefficients (r). Significance codes: P-value (0 '***' ,0.001 '**' ,0.01 '*', 0.05 '.', 0.1 ' '). The distribution of variables is presented on the oblique. On the bottom of the oblique: the bivariate scatter plot with a coordinated line are shown. On the top of the oblique: the rate of the correlation and the significance level as stars

2.3. Hierarchical Clustering on Principal Components (HCPC) clustered regions in three groups and determined traits with the most variation

HCPC is a multivariant analysis that combined principal component and hierarchical clustering, and it is convenient when a dataset is multidimensional and includes multiple continuous variables. HCPC analysis permit to reduce the data dimension into less and most crucial information continuous data that lead to having more stable clustering. Thus, we preferred this method as a multivariant analysis for performing on this project dataset to recognize the variables with the most variance in related regions to them. In the first step of HCPC analysis, PCA was done with all samples and all variables. PCA results showed that the two first component described 33.6% of the variation among all 50 regions (Table 1, Figure S1).

Table 1. The eigenvalue, variances, and Cumulative variance percent retained by first two dimensions from HCPC analysis

Dim	Eigenvalue	Variance percent	Cumulative variance percent
Dim1	4.59	20.9	20.9
Dim2	2.80	12.7	33.61

These two axes presented 20.6% and 13% of the total variation, respectively. In dim1, 4-hydroxy isoleucine showed the most contribution (Figure 2). In the next step, seed weight, isoleucine, and seed width presented the high contribution to this dim, respectively. Phenol, seed length, and leucine were the pioneers of contribution to dim 2. Among the traits that had the most variance to each axis, we found all three kinds of variables (metabolic, morphological, and environmental). The percent of 20 top regions contribution is shown in figure 4.

In the second step of HCPC, Hierarchical clustering (HC) was done based on PCA results (Figure 3 & S2). All 50 regions were divided into three groups. The essential variables in each cluster were selected based on v.test (v. test ≥2) (Table 2). The first cluster had 18 members and mostly related to axis 3 (Figure 3). This cluster had three critical variables that their average is shown in table 1 and Excel S2. In this cluster nearest regions to the center of the cluster, includes Geshlage, Varish, BajiAbad, Boyinzahra, and Saveh.

Table 2. phenotypic traits, v. test, and *P-value*, and most important dim related to each cluster that defines the most variation in each of three groups from HCPC analysis

cluster	**variable**	**v. test**	**Mean in category**	**Mean in overall**	***P-value***	**Most important dim**
1	Seed per pod	2.59	11.83	10.52	0.0009	3
	Aspartic acid	2.53	4.13	3.03	0.001	
	Seed length	2.44	0.40	0.38	0.001	
2	Rain	3.182	14.07	9.87	0.001	2
	Valine	2.72	23.84	18.21	0.006	
	Phenylalanine	2.53	6.28	4.18	0.011	
	Lysin	2.08	0.44	0.39	0.037	
3	4-hydroxy isoleucine	3.93	87.86	46.16	0.000002	1
	Temperature	3.15	30.23	25.58	0.00002	
	Isoleucine	3.08	1.49	0.94	0.00006	
	Antioxidant	2.34	0.56	0.30	0.00009	
	Seed weight	3.84	14.07	11.95	0.0001	
	Solar irradiation	3.60	2047	1957.22	0.0006	
	Seed width	3.39	0.32	0.28	0.0001	
	Trigonelline	2.47	0.24	0.21	0.00004	

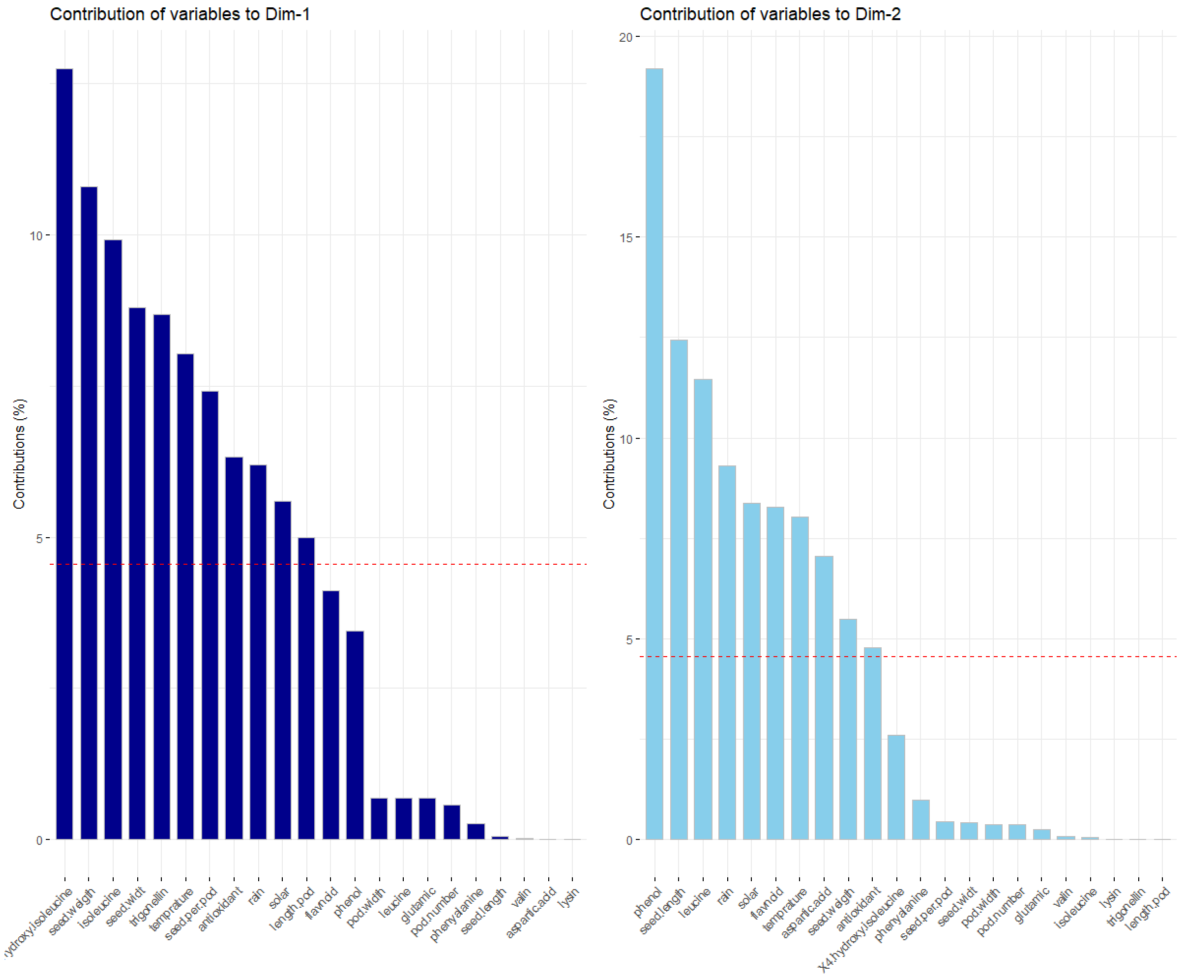

Figure 2. The bar plot of percent of variables contribution to dim one and two of HCPC result.

Red dash lines are the threshold.

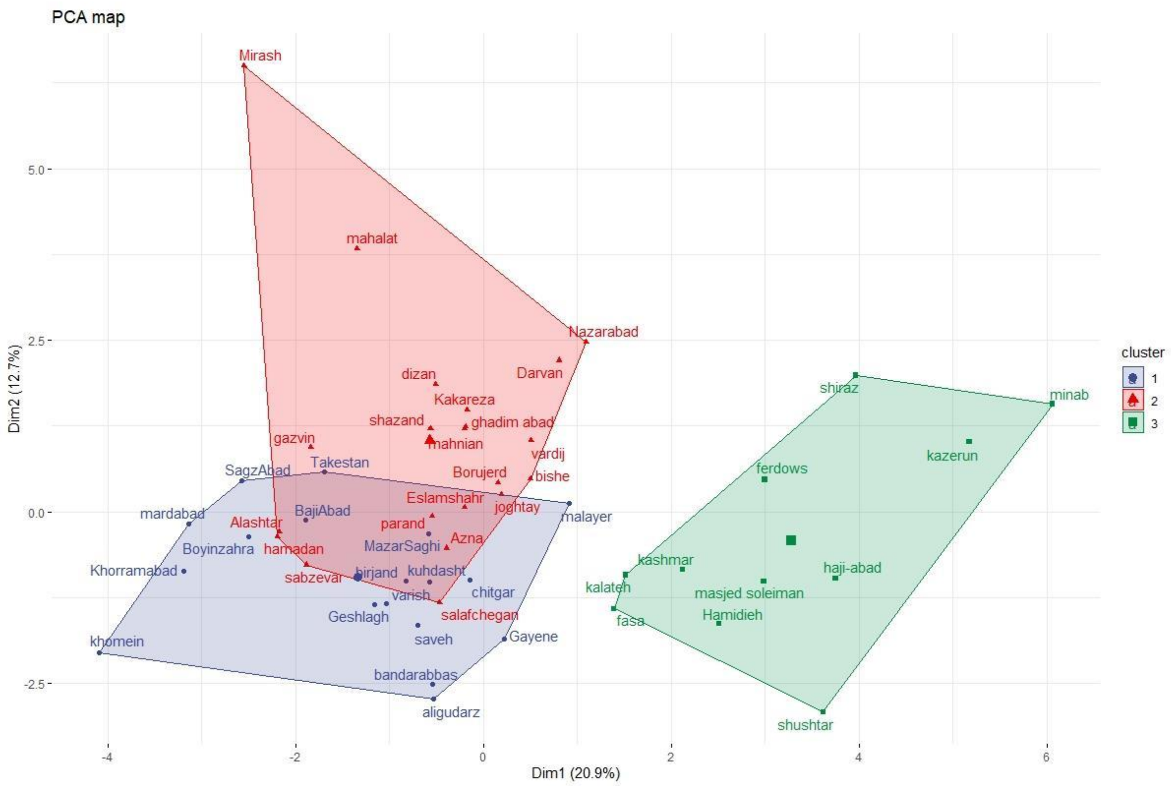

Figure 3. principal component map of regions coloured three clusters and their member's regions. Each coloured group presented a group based on HCPC analysis.

The second cluster also had 21 members, and dim two was significantly related to this cluster. The five top closest regions to this cluster center were consisted: Mahnian, Dizan, Parand, Bishe, and Joghtay. The average of most related traits to cluster 2 was higher than overall mean of these traits (Table2, Excel S2). The third cluster was associated with axes 1 and consist of 11 regions. Five areas include: Haji-abad, Ferdows, Kashmar, Masjed soleiman, and Fasa showed the least distance from the canter of cluster 3.

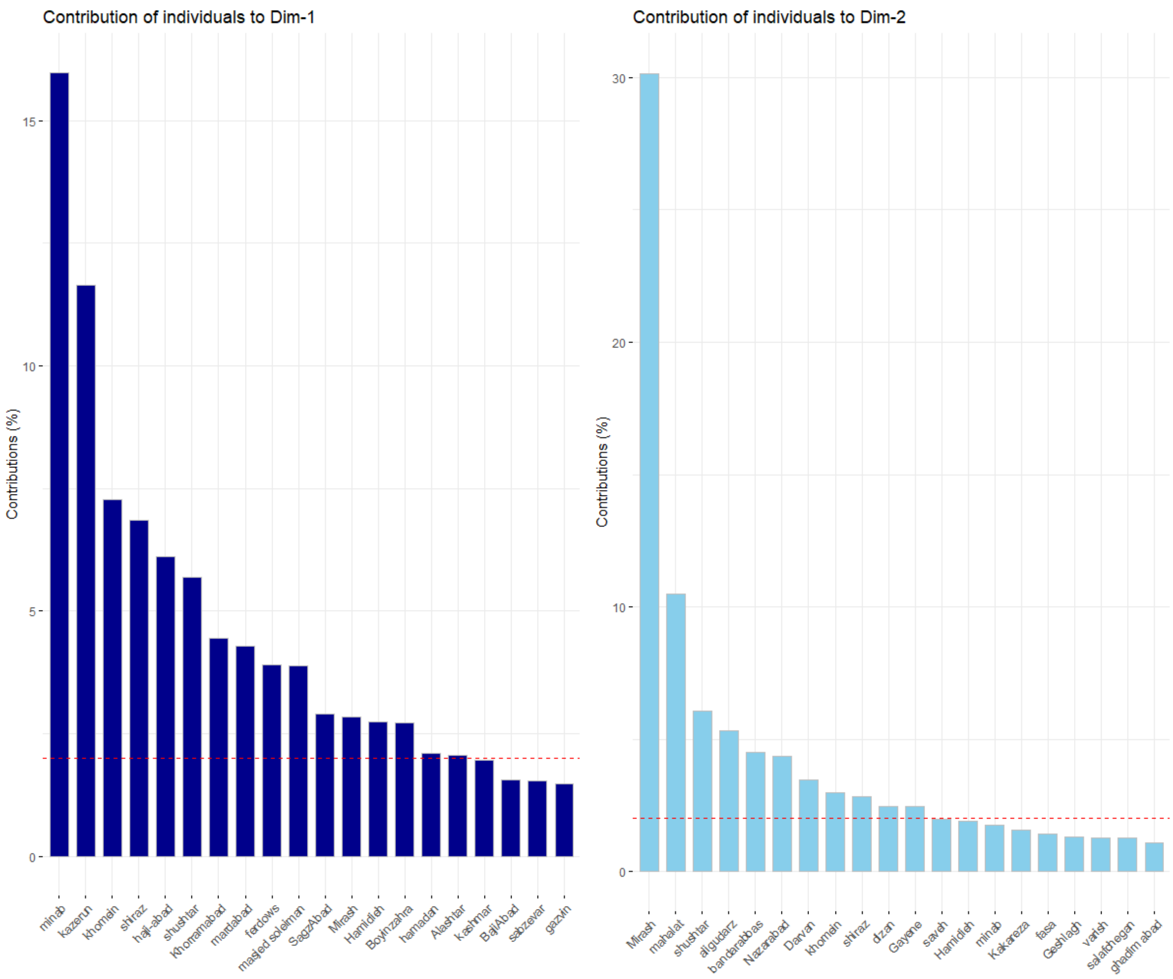

Figure 4. The bar plot of 20 top regions contribution to dim one and two of HCPC result. Red dash line represented the threshold.

2.3. Canonical corresponds analysis (CCA) displayed how each trait influenced by temperature, solar radiation, and rainfall

Environmental factors as solar radiation, precipitation, and the temperature have the power to impact on metabolites and morphology of plants in many species. We carried out CCA for identifying phenotypic variation related to the accrued ecological data. It was vital for us fundraising the desired condition for the biosynthesis of 4-hydroxy isoleucine and trigonelline as the most appreciated secondary component of fenugreek. So, we selected some regions of dim1 (top members (threshold ≥ 2)) and all members of cluster3, based on HCPC result, that trigonelline and 4-hydroxy isoleucine had the most variance and volume respectively, for investigation of the impress of ecological variation on these therapeutic secondary metabolites at first and in the next step on all other measured traits.

The result of the CCA analysis displayed that the first axis (CCA1) explained 87% of total inertia, and only 7% of the variation explanation was for the second axis (Table S3). CCA also displayed how each trait influenced by environmental variables (Figure S3, S4, Table S4).

In the first axis, rain played the most critical role. The leucine, seed per pod, and pod length presented the most coordination with CCA1. The 4-hydroxy isoleucine and trigonelline had negative coordination to this axis. The average rainfall of the most critical regions associated with this axis was much higher (17.81 mm) than all regions precipitation (9.85 mm). In this axis, the half value of solar irradiation (1841.80 kJ/m^2) and temperature (23.25˚) of the regions with most coordination with CCA1

(Figure S5, S6, Table S5) were lower than the overall mean (1957.22 kJ/m^2 and 25.58° respectively) (Excel S3).

The temperature and in the next step, solar irradiation had a positive association with CCA2. The isoleucine had the most coordination with CCA2. The 4-hydroxy isoleucine and trigonelline showed positive coordination with this axis. Phenol, flavonoid, and antioxidant showed a positive relationship with this dim (Figure S4, S5, Table S4).

The average of these two metabolites in the most coordinated regions to this dim, was higher than the total mean, especially 4-hydroxy isoleucine (Table S5, Excel S3). The average of temperature, solar radiation in the regions(27.04° and 1980.40 kj/m^2) that displayed most association with CCA2 (Figure S5, S6, Excel S3) were partly higher than the total average of them (25.58° and 1957.22 kJ/m^2).

2.4. Genetic advance and heritability displayed the different percent of heritance for metabolic and morphologic traits

Our results showed that the range of PCV was between 4.4-76% for phenol and aspartic acid content, respectively (Table 6). The leucine content had the biggest GCV (72%), and the lowest one was for phenol content (3.4%). The PCV was lower than GCV in glutamic, phenylalanine, isoleucine, leucine, and finally seed weight. The GCV was between 3.4 (phenol content) – 72% (leucine).

The heritance of total phenol, flavonoid, and antioxidant activity was medium (60, 50, and 53 respectively). The genetic advance was very divers between traits in a range of 3.9% for 4-hydroxyisoleucine to 97% for aspartic acid (Table 2). The 4-hydroxy isoleucine had low heritance, and genetic advance, that revealed that the environment has a vigorous influence on this branched-chain amino acid. The trigonelline, the other critical metabolites from fenugreek had moderate heritability (h^2 = 0.58), and genetic advance (GA = 29%) that reflect this component also is impressed by ecological variable but not as much as 4-hydroxy isoleucine.

Nonetheless, the GA of aspartic acid was high (GA = 97%) like as other amino acids (in a range of 52% for lysin to 96% for leucine).

Table 3. Estimation of PCV, GCV, h^2, and GA of phenotypic traits. The heritability and GA ≥90% are bold.

Trait	PCV%	GCV%	h2	GA%	Trait	PCV%	GCV%	h2	GA%
aspartic acid	76	60	0.62	**97**	Phenol	4.4	3.4	0.60	4
Glutamic	49	61	**0.91**	**91**	Lysin	28	28	**0.91**	52
4-hydroxyisoleucine	16	6	0.12	3.9	Pod number	46	45	**0.97**	**91**
Valine	33	33	**0.90**	61	100Seed weight	25	31	0.67	34
Phenylalanine	50	68	**0.90**	**92**	Pod length	18	18	**0.94**	34
Isoleucine	44	58	**0.92**	85	Pod width	32	31	**0.97**	63
Trigonelline	25	19	0.58	29	Seed length	7.6	6.7	0.77	12
Leucine	52	72	**0.90**	**96**	Seed width	9.6	9.1	0.89	17
Antioxidant	48	34	0.50	49	Seed per pod	26	24	**0.91**	48
Flavonoid	15	11	0.53	16					

2.5. Partial least squares regression (PLSR) represented that environmental variables can predict secondary metabolites

Partial least squares regression (PLSR) is a practical statistical analysis in natural science that used to modeling by connecting the predictors and dependent variables[23]. Since trigonelline and 4-hydroxy isoleucine are the most critical metabolites in fenugreek[5]; we chose them as responses variables (Y value) for PLSR analysis. All other variables include morphological traits, other metabolic traits, and environmental variables considered as latent variables (X values). In the first step, PLSR was performed with all variables to determination and elimination of useless predictors. The variable importance in the projection (VIP) was performed for selecting the relevant latent variables that have a practical impact on response[24]. The suitable threshold value for VIP was chosen more than 1 (VIP> 1) (table 5).

Table 5. Validation partial least square (PLS) regression models for trigonelline and 4-hydroxy isoleucine. Variable importance in the projection (VIP) more than one used to select relevant latent variables and root mean square errors (RMSEP) and R2 for cross-validation process in PLSR analysis. The VIP≥1 were bold

Traits	**Trigonelline**				**4-hydroxy isoleucine**			
	R^2	**RMSEP**	***P-value***	**VIP**	**R^2**	**RMSEP**	***P-value***	**VIP**
Aspartic acid	-0.12	0.052	0.886	0.018	-0.15	22.92	0.644	0.78
Glutamic	-0.07	0.050	0.486	0.03	-0.11	22.8	0.785	0.61
Valine	-0.085	0.050	0.903	0.007	-0.14	22.16	0.777	0.70
phenylalanine	-0.052	0.050	0.304	0.07	-0.067	21.87	0.655	0.24
Isoleucine	0.314	0.048	0.059	0.03	0.32	22.16	0.0008***	**1.74**
Leucine	-0.14	0.047	0.979	0.041	-0.197	22.30	0.939	0.031
Antioxidant	0.087	0.046	0.477	0.87	0.062	23.96	0.0385*	**1.20**
Flavonoid	-0.064	0.044	0.188	**1.04**	-0.066	24.68	0.86	0.31
Phenol	-0.087	0.043	0.288	0.47	-0.104	23.9	0.730	0.32
Lysin	-0.082	0.043	0.439	0.28	-0.112	23.40	0.390	0.33
pod number	-0.048	0.041	0.723	0.07	-0.083	22.77	0.216	0.41
seed per pod	-0.009	0.041	0.063.	**1.31**	0.025	21.72	0.261	0.87
pod length	-0.0511	0.041	0.131	**1.14**	-0.040	20.74	0.536	0.56
seed weight	0.130	0.040	0.037*	**1.88**	0.220	21.00	0.048*	**1.32**
pod width	-0.063	0.040	0.195	0.83	-0.066	21.28	0.894.	0.31
seed length	-0.037	0.040	0.599	0.040	0.080	20.11	0.145	0.75
seed width	0.061	0.040	0.033*	**1.38**	0.11	20.49	0.078	**1.13**
Solar	0.30	0.040	0.194	**1.32**	0.30	22.38	0.0002***	**1.76**
temperature	0.26	0.040	0.593	**1.02**	0.23	22.91	0.0006***	**1.77**
Rain	0.146	0.040	0.841	0.93	0.079	22.98	0.0045**	**1.74**

Significance. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

In the first step, the PLSR modeling by using all traits, the VIP of all latent variables were obtained. The more sensitive variables selected for both valuable metabolites according to their VIP (minimum VIP ≥1) (Table 5). Out of the 20 features used in the first round of PLSR analysis, only seven traits were most important for predicting of 4-hydroxy isoleucine and trigonelline (Table 5).

According to table 5, the highest VIP for trigonelline and 4-hydroxy isoleucine was seed weight and temperature, respectively. Seed weight was significant for 4-hydroxy isoleucine too, but not as much as for trigonelline. Climatic variables are more critical for 4-hydroxy isoleucine than trigonelline in prediction analysis. This result also confirmed that 4-hydroxy isoleucine is more sensitive to the environment, that we observed in the heritability analysis. Based on the significance of traits, just seed width and seed weight were selected for prediction modeling of trigonelline. For 4-hydroxy isoleucine, seed width was omitted because it was nonsense according to *P-value*.

By using just sensitive and significant traits for predicting model, the following equation simulated the 4-hydroxy isoleucine (Figure 5) and trigonelline (Figure 6) yields:

4-hydroxy isoleucine ~ -284.917+1.184***seed weight** +0.119***solar**+1.889***temprature**+0.150***rain**+12.466***isoleucine**+28.983***antioxidant**

Trigonelline ~ -0.159+0.0102***seed weigth**+0.056***seed width**

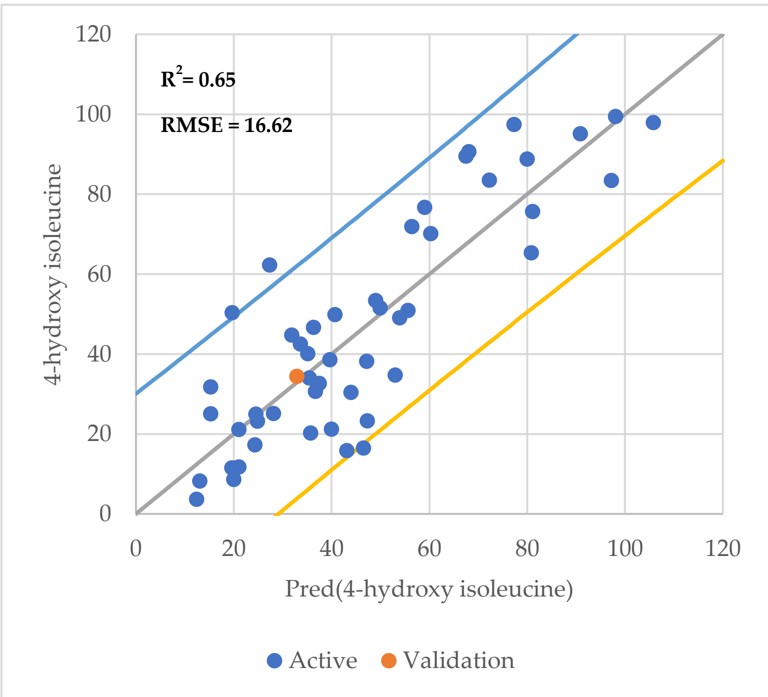

Figure 5. Observed and cross-validated predicted values of 4-hydroxy isoleucine. Each point shows one region. The prediction error (RMSEP) indicates the quality of the model in predicting the observed 4-hydroxy isoleucine yield, and the regression coefficient (R^2) indicates the fit between the predicted and observed 4-hydroxy isoleucine yield.

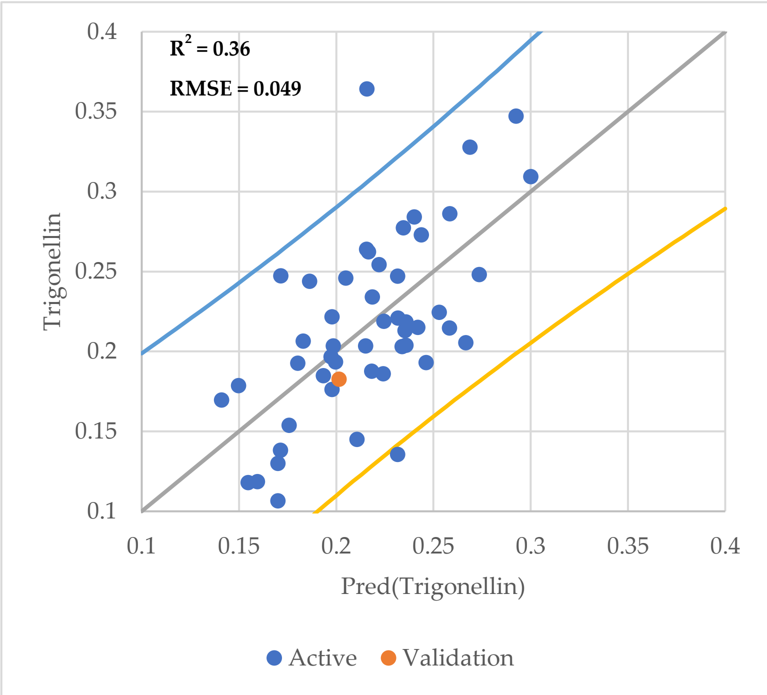

Figure 6. Observed and cross-validated predicted values of trigonelline. Each point shows one region. The prediction error (RMSEP) indicates the quality of the model in predicting the observed trigonelline yield, and the regression coefficient (R^2) indicates the fit between the predicted and observed trigonelline yield.

All two models for both metabolites were significant ($p<0.05$). The seed weight and sees width as a latent variable were the best factors for predicting of trigonelline yield. In 4-hydroxy isoleucine also four significances factors including solar, temperature, rain, isoleucine and antioxidant were the best parameters for predicting of this metabolite.

2.6. Path analysis revealed morphological and environmental factors had a direct and indirect effect on trigonelline and 4-hydroxy isoleucine, respectively.

Path analysis approach was introduced by Wright (1921) to determine the direct and indirect effect of variables on particular variable such as yield[25]. This approach may be of limited utility to a plant breeder, but it gives an insight into a complicated relationship among different characters in a biological system and provides information whether the observed correlation is due to the direct influence or through other variables. Thus, path analysis specifies the causes and also gives the relative importance of the characters[26].

accordance with the above-mentioned, we decided to find out the direct and indirect effect of all of morphological, metabolites, and climatic features on 4-hydroxy isoleucine and trigonelline by path analysis.

The pathway analysis of trigonelline (Excel S4) showed seed weight has the most direct effect, and flavonoid was in the next step. Phenol, leucine, and seed per pod were in following ranks indirect effect through seed weight on trigonelline, respectively. Since trigonelline has enhancer role in antioxidant activity may be the indirect efficacy of phenol would be logical[27]. On the other side, according to the correlation plot (Figure 1), Association between seed weight and these traits were a significantly positive and indirect effect of them also were positive. Thus, it is a good idea to use each of them for improvement of trigonelline yield.

The solar irradiation, temperature, isoleucine, and lysin showed the most positive direct effect on 4-hydroxy isoleucine, followed by antioxidant (Excel S5). Flavonoid displayed a negative impact on 4-hydroxy isoleucine yield. The temperature had the most indirect effect on this metabolite through solar irradiation. Solar irradiation via Isoleucine also was having an indirect impact on 4-hydroxy isoleucine. The antioxidant showed an indirect effect via phenol, flavonoid, and seed weight. The direct and indirect effects of seed weight on other features of seed was reported in several types of research[28][29]. The positive correlation between antioxidant and 4-hydroxy isoleucine may be due to the indirect efficacy of phenol and flavonoid. There was evidence that showed that 4-hydroxy isoleucine cause to increase antioxidant activity, especially in preventing diabetes and high cholestrol[27]. But it seems no evidence shows the path analysis of seed yield and metabolites. But we believe the correlation result of this study may confirm this effect (Figure 1).

The environmental condition had not considerable direct or indirect effect on trigonelline content. So, it seems that this metabolite is slightly more stable in compered with 4-hydroxy isoleucine and has more plasticity. Heritability percent of these two metabolites validate this result because the heritance of 4-hydroxy isoleucine is lower than trigonelline (Table 6).

Seed weight shows a direct effect and several indirect effects on both metabolites. These traits also had a positive correlation with them. It indicates that the more seed weight, the more metabolites indeed.

3. Discussion

The environment has the power to change of direction of the plant's life. Thus, we decided to investigate how these uncontrollable factors impact on the seeds of the fenugreek. We were interested in obtaining an overview of the relationship between primary and secondary metabolites, and seed morphology of this plant. On the other hand, since metabolites can impact on the anatomy of the plant,[30], we decided to calculate the correlation between metabolites and morphological traits. We also desired to achieve insight into the relationship between inside and outside of fenugreek seed. We believed the discovery of such associations is useful to exploit natural diversity and identification of related genes[31]. We found the association between 4-hydroxy isoleucine as a non-protein amino acid with antioxidant activity. It is maybe because this amino acid could raise the content of the antioxidant level in fenugreek seeds[32]. This metabolite also showed correlation with isoleucine, because isoleucine is a precursor for the biosynthesis of 4-hydroxy isoleucine[33]. Some amino acids showed a positive association with several metabolites. The hypothesis of the necessity of amino acids for metabolites proceses[34] approves this correlation. We believe since metabolites are mediators between the genome of the plant and the climatic variables, but not necessarily the final result of this interaction, the lack of correlation between some metabolites and some morphologies is reasonable[30]. Based on the correlation result, several morphological traits presented correlation with together. Such relation reported in some previous studies[35][36]. There is some evidence that verified the relationship between metabolite and anatomy of plants[37][30]. Breeders can use this connection to improve desired traits. In particular, morphology is more stable than in metabolites in changing ecology[31]. It is a good idea that morphological characteristics can be used to improve the metabolic traits, depending on their association.

Our results displayed that morphology of seeds is more affected by rainfall than solar irradiation and temperature. Fenugreeks that grown in regions with higher temperature and solar radiation had a bit smaller and lighter seed compared with others. But these features also had weak coordination with axis two. This result showed that all environmental factors affected seed properties, but the level of their impress is different. In another study high temperature during the period of reproductive growth lead to the decline of the reproductive growth period, failure in the number of crops and finally decrease the number of seeds and then the drop comes to seed yield[38][39]. Some papers showed the opposite results. For example, in a study, seed yield was decreased by lowering of solar radiation and temprature[40]. Other research also presented seed weight, number of pods, and number of seed per pod were more when the growing and maturity of fenugreek happened in more days at higher temperatures[41].

We found that phenol and flavonoid had a positive correlation with CCA1. Larsson et al. (1986)[42] as well as our study, observed solar irradiation reduced the amount of phenol. However, the opposite of this observation has been reported in research[30]. For instance, in a survey, solar radiation caused to increase of the biosynthesis of phenols and flavonoids as protective components as much as tree foliage[43].

4-hydroxy isoleucine and isoleucine showed positive coordination with CCA2. Isoleucine is the precursor of 4-hydroxy isoleucin[44], so, logically, both of them presented the same reaction to the same environmental variable.

Our research showed the content of trigonelline in the warmer region with higher solar radiation was more than in other areas. Some researchers suggested that alkaloids increase by increasing solar radiation, to protect plants cell against the production of reactive oxygen species (ROS) Some literature [45][30][46] such as trigonelline[5]. Trigonelline of coffee was more in sun-grown coffee trees compared to shade-grown trees[47].

This result approved this hypothesis that the favorable weather for the growing of fenugreek is the dry and cold temperature for the vegetative stage and dry and practically high temperature for seed production[22].

It is exciting that the results of this research showed that any of the three ecological variables did not have the same effect all over the metabolites and morphological traits. Even amino acids did not present a similar reaction to a different temperature, rain, and solar radiation. Even three branched-chain amino acids, including isoleucine, leucine, and valine[48], did not show the same reaction to a variety of nature. This finding was also observed in previous studies on other plants[6][49]. Heritability shows the potential of transfer of traits from one generation to next and helps breeders to choose the appreciate selection methods for the improvement of the desired features. However, without the estimation of genetic advance, broad-sense heritability may not be dependable[50]. Phenotypic coefficient variation (PCV) and genotypic coefficient variation (GCV) also give us a more distinct picture of the influence of traits from the surrounding environment on the heritage of variables. Thus, we calculated broad sense heritability coupled with the estimations of genetic advance and PCV and GCV for a more accurate assessment.

All of these four analyzes expanded our sight of the impact of the environment and genetics on the samples. The traits that have high GCV and low PCV are less affected by environmental condition[50]. A top percent of GCV take an excellent opportunity for the improvement of traits[50].

We found moderately high broad-sense heritability for phenol and antioxidant. This level of heritance was previously reported in some works of literature [51][52]. For instance, Tierno and Galarreta[51] said 94 and 88% of heritability for phenol and antioxidant activity in potato. But they did not consider GA and GCV. Therefore, judgment about the stability of their goodness for the breeding program is not honest. In red raspberry[53], they also observed a moderate heritability of total phenol, antioxidant activity, and flavonoid content. In general, phenolic content is mainly responsible for answering variation in the surrounding condition in plants. So, we suggest, although the inheritance of the phenolic compound is relatively high, however, their GA, and GCV is too low, because of their sensitivity to environmental condition. These three secondary metabolites have a primary role in plant protection and against oxidative stress in human disease[54] were related to each other in consider to correlation results (figure 2).

In many other plants, also, these compounds are associate in sum[54,55]. Based on our knowledge, no evidence presented a heritability of secondary metabolites in previous researches on fenugreek. Heritability of amino acids showed they are not stable in changing of ecological condition, and their responses are not the same. So for maintaining the quantity and quality of free amino acids and even all metabolites, breeders should try to produce a cultivar with least interaction with enviromnet[56].

In a study that was performed on the heritability of winter wheat metabolites, amino acids presented low or moderate heritability[49]. All studied morphological traits had high heritability. This high heritability and the range of heritability of morphological characters were much higher than metabolic characteristics. This high h^2 for morphological characteristics were reported in many previous pieces of research [57–60]. Since breeding for metabolites is harder than morphological features, because of their instability in even little change in the surrounding the environment, it is good suggestion using morphological trait with high h^2 and GA that are associated with desired metabolites.

PLSR method reduced the 20 latent variables into seven relatively and uncorrelated latent traits for each metabolite. Because of this perfect properties, PLSR is preferable when we have a large number of variables[23].

Environmental factors could predict 4-hydroxy isoleucine content, and its content is dependent on the condition of the surrounding environment. Path analysis also revealed that ecological variables could affect 4-hydroxy isoleucine, directly and indirectly. Comparing path analysis result with low heritance of this metabolites indicate 4-hydroxy isoleucine is sensitive to the condition of nature. In the opposite side, environmental factors did not have a considerable impact on trigonelline metabolite, either in PLSR nor in pathway analysis.

In a study that considered the impact of contaminant water irrigation on the metabolome and morphological trait of lettuce, by using PLSR method, they found some metabolites cause to disturbance of morphological characteristics under watering of the plant with contaminated water. They suggested that PLSR approach is helpful in comparison to the metabolic profile of different samples[61]. PLSR result in our study showed that the behavior of metabolites in facing of variation of their surrounding condition, is different from each other, and we cannot watch them just from one window. For example, trigonelline that is the critical saponin in fenugreek not depends on the environment as much as 4-hydroxy isoleucine that is a kind of non-protein amino acid.

4. Material and method

4.1. Plant seed material

To study the variation in metabolites and morphological traits of this plant under climatic variation, seeds of 50 genotypes were collected from 50 regions of Iran (Table S1, Figure 7[1]). The collected seeds stored in -20 freezer until their metabolites measured.

[1] - http://www.arcgis.com

Figure 7. The geographical locations of Trigonella foenum-graecum the seeds of 50 genotypes collected from different regions of Iran. Each of the purple points represents the sample collection location

4.2. Meteorological data collection

Climatic data (average rainfall and temperature) get from *Iran Meteorological Organization* (IRIMO). Solar irradiation (kJ/m2) data obtained from the Global Solar Atlas[2].

4.3. Seed morphometric characters

This project focused on the comprehending the relationship between phenotypic (morphological and metabolites) characteristic with the climatic condition. Therefore, some quantitative features, including pod number, seed per pod, pod width (mm), pod length (mm), 100 seed weight (mg), seed length (mm), seed width (mm), were recorded. These traits strongly associate with yeild[62]. All measurement was done in three replications and at least five biological replications (Excel S1).

4.4. Amino acids

The amount of 1000 ul of 80% ethanol (CAS #: 64-17-5) was added to the 100 mg of powdered seeds. Then the solution was shacked in 80° for 60 minutes. In the next step, samples were centrifuged for five minutes in 10000 rpm, and the supernatant was collected. Finally, supernatants evaporated by concentrator[63]. O-phthaldialdehyde (OPA) (CAS #: 643-79-8) used for derivation of free amino acids. Separation and detection of amino acids were determined by HPLC (*Knauer*, Berlin, Germany) and detection by fluorescence spectrophotometry with excitation absorbance at 340 nm and emission peak at 450 nm. The Chromgate software was used to peak integration. The OPA- derivate amnio

[2] - http://globalsolaratlas.info

acid were separated by Eurospher C18 column (250 mm×4.6 mm, with 5 µm particle size) stayed at 28°C. The OPA reagent prepared by mixing 0.05 gr OPA powder, 4500 ul methanol and 500 ul Borat buffer (2.85 gr Borax in 100 ml water 100°, pH= 9.5) and 2 ul 2-Mercaptoethanol. Solvent A included 80 ml Acetate sodium 0.05 molar and 20 ml methanol. Solvent B also was a mixture of 30 ml Acetate sodium 0.05 molar and 70 ml methanol. To inject to HPLC, 100 ul of extract and 50 ul of OPA reagent were mixed for 30 minutes. Then 20 ul of the mixture was injected and eluted at a flow rate of 1ml/min at 28°. The standard solution (CAS #: 781658-23-9) was used to determination of 4-hydroxy isoleucine. The absorbance excitation and emission picks of 4-hydroxy isoleucine were 330 and 440 nm, respectively[64].

4.5. Trigonelline

A volume of 100 mg of powdered seeds and 1ml methanol were mixed for 10 minutes by the vortex. In the next step, the mixture was sonicated (BANDELIN, SONOREX DIGITEC, Germany) for 30 minutes at room temperature. After supernatant transferred to another tube, the first step was repeated. This procedure was done according to Rongjie et al., 2010[65] method with some modification. Determination of trigonelline done by Agilent 1260 Infinity series HPLC (Agilent Technologies, Santa Clara, CA) equipment and Aminex column. The mobile phase consisted of the ratio of methanol: water, 95:5, respectively. The elution time and flow rates were 6 min and 1 ml/min, respectively. The absorbance of trigonelline detected at 263 nm. For detection of trigonelline, the standard solution (CAS #: 6138-41-6) was used. The OpenLab software was used to peak integration.

4.6. Phenol, flavonoid and antioxidants activity

The 100 mg of powdered seeds were sonicated by 80% methanol (CAS #: 67-56-1), for 1 hour in room temperature. Then samples were centrifuged in 5000 rpm for 15 minutes, and the upper phase moved to another tube and reached to 100 ml by using 80% methanol.

Measurement of total phenol content was done by Agbor and Vinson (2014)[66] method. Up to 100 ul of methanolic extract was diluted with 9 ml water and mixed with 1 ml of Folin-Ciocalteu reagent and shacked. After 10 minutes, 10 ml of w/v sodium carbonate aqueous solution was added to each tube. The diluted solution was stirred for 30 minutes. The absorbance was measured in 760 nm against the blank using a UV-Visible spectrophotometer — methanol used as blank. Gallic acid (CAS #: 149-91-7) was used as a standard for the calibration curve. Results were reported as gallic acid equivalents GAE mgGAE/100 gr dry seed.

Total flavonoid content was assayed by Kim et al. (2003)[67] protocol. A mixture of one ml of extract and 4 ml of distilled water and 300 ul of 0.3% $NaNo_2$ shacked for five minutes. Then 300 ul of 10% $AlCl_3$ and 200 ul of 1 M NaOH were added to the mixture. In the final step, 2.4 ml of distilled water was added to the mixture and thoroughly shacked. The 510 nm was select for determination of total flavonoid. Quercetin compound was used as a standard for quantifying of this compound. The results were reported as mgQE/100 gr dry seed.

To assay of antioxidant capacity of methanolic extract of fenugreek seeds, DPHH (2,2-diphenyl-1-picrylhydrazyl free radical) was done by Molyneux (2004)[68] method. A volume of 200 ul of methanolic extract was added to a mixture of 300 ul methanol and 2400 ul of DPPH solution (0.025 g/l) and shacked vigorously and incubated for 30 minutes in the darkroom. Then the absorbance and decrease in the color of the mixture were measured at 517 nm via UV/VIS Spectrometer (*CARY* 330 UV-Vis system). The control had all reagent except seed extract.

Antioxidant activity% = $absorbance\ of\ (control - extract) \big/ absorbane\ of\ control$

Control = initial absorbance

The following formula was used to report the quantification of all primary and secondary metabolites in mg per gr dry seed (Table S2):

X (mg/gr dry seed) = (VC)/W

where

X = amount of amino acids compounds in the extract

V = final content of the extract solution in ml

W = weight of the extract

C = concentration of standard compounds obtained from the calibration curve

4.7. Statistical analysis

To determine the relationship between fenugreeks primary and secondary metabolites and morphological features and environmental variables including temperature, rainfall, and solar irradiation, canonical corresponds analysis (CCA) was done by exposing of vegetal traits against ecological variables in *vegan*[69] package of R[70] software. Pearson correlation method was used to correlation analysis in the *corrplot*[71] package. The PCA and HCA analysis was done by using the HCPC method[72] to reduce the data set dimension for explaining the total variation and clustering of samples. The genotypic coefficients of variation (GCV) and phenotypic coefficients of variation (PCV), were estimated by using Hanson et al., (1956)[73] method. Genetic Advance (GA) as well was calculated by Johnson et al. (1955)[74]. All these genetic factors help us to make a more honest judgment about the interaction between nature and plant. The *XLSTAT* software did Partial least square regression (PLSR).

The partial least square regression(PLSR) model which determine the association of two sets of data using a linear multivariate model to predict plant properties of interest as a response, is increasingly used in many aspects of natural science[75]. The method of leave – one – out cross-validation was calculated to the determination of significant component numbers, and Root Mean Square Error of Prediction (RMSEP) for cross-validation process in PLSR analysis to prevent overfitting. Our purpose of this analysis was to predict the two most important metabolites of fenugreek, including 4-hydroxy isoleucine and trigonelline. In the final step, the path analysis was performed to recognize the direct and indirect effect of vegetal and environmental features on that two metabolites by the *agricolae*[76] package.

5. Conclusion

Fenugreek is an excellent medicinal plant in the treatment of some Incurable diseases like diabetes and high cholesterol. It is popular between many populations of the world, and it grows in a wide range of ecological condition. Thus, it is valuable to consider that this applicable and favorable spice likes to live and generates in which state to plan for the breeding purpose for increasing of unique and medical metabolites and related morphological variables. Results of this research showed that metabolites have less heritability than morphological features. Hence, plant breeders can effort on morphological traits that are impressive in expanding of desired metabolites. Plants have a complicated life, especially at the level of secondary metabolites. So, to get almost complete and comprehensive eyesight at them, we have to use a variety of methods.

Therefore, we used several multivariate analysis methods like HCPC, CCA, PLSR, and pathway methods for analyzing multidimensional traits. The results of each technique confirmed the results of the other methods. We also performed heritability, GA, GCV, and PCV for the considerate of genetic base and inheritance of characters, and also for more validation of our results. Along with these methods, we investigated the relationship of all features.

We considered the impact of climatic conditions on the appearance and inside of fenugreek seeds, and we tried to find and suggest a way to increase and improve the performance of two critical metabolites of fenugreek consist of 4-hydroxy isoleucine and trigonelline.

The biosynthesis of some valuable metabolites increases as a response in the face of changing of surrounding condition. Since these therapeutic components are biosynthesized in very low content in plants, so it would be a good suggestion that notice to the effect of the environmental situation for enhancing them can help to breeders to choose the more appropriate plan to the improvement of medical metabolites.

Supplementary Materials: The following are available online, Table S1: *Trigonella foenume L.* population sites include name, longitude, and latitude for 50 regions, Table S2: Equation of calibration curve and R2 value of measured primary and secondary metabolites by HPLC and spectrophotometer except for antioxidant activity, Figure S1: The association amongst measured variables and two first axis of PCA result. First and second dims explained 20.9% and 12.7% of the total variance, respectively. The color of vectors shows the rate of contribution of each variable to each dim, Figure S2: Dendrogram created by the hierarchical clustering based on HCPC result for studied regions, Table S3: The eigenvalue, constrained inertia and cumulative percent of first two CCA, Table S4: The principal coordination of canonical variables with two first axis of CCA analysis. Highest value and the lowest value displayed in green and red colors respectively in CCA1 and CCA2, Table S5: Principal coordinates of regions to each axis by all vegetal traits with CCA1 and CCA2. Highest value and the lowest value displayed in green and red colors, respectively. CCA1 and CCA2, Figure S3: CCA plot of the relationship between environmental and vegetal variables. Blue arrows showed environmental variables and green text displayed vegetal traits, Figure S4: Scatter plot of CCA that shows the coordinates of variables to each dimension, Figure S5: CCA ordination biplot of selected regions based on HCPC analysis, displaying environmental variables in blue arrows and each region in the black text, Figure S6: Scatter plot of CCA that shows the contribution of regions to each dim of CCA analysis, Excel S1: Average of all measured vegetal traits in fenugreek seed, Excel S2: The average of traits in each three clusters based on HCPC analysis, Excel S3: The average of traits in top five related regions to first two CCA, Excel S4: The direct and indirect effects of traits on trigonelline, Excel S5: The direct and indirect effects of traits on 4-hydroxy isoleucine.

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