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# Study of inheritance and environment on tropane alkaloids within *Hyoscyamus* species

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**Abstract**

In the present study, HPLC technique was applied to determine scopolamine and hyoscyamine contents in five *Hyoscyamus* species including *H.niger* L., *H.reticulatus* L., *H.pusillus* L., *H.arachnoideus* Pojark., and *H.kurdicus* Bornm., collected from different geographical origins of North West of Iran. The range of genetic similarity was obtained between 91.07 and 99.89 within *Hyoscyamus* accessions based on scopolamine and hyoscyamine alkaloids composition. Estimated heritability was high for both traits and ranged from 0.82 to 0.99. The existance of high GCV indicates the genetical control of tropane alkaloids which leads us to achivement of genetic improvement by selection of desirable plants for breeding. Our data also implies the positive assosiation between N, P, K, Ca & EC and alkaloids yield in plants which will be useful for production programs in the future.

**Keywords:** environment; hyoscyamine; HPLC; Iran; scopolamine.
**Abbreviations:** HPLC-high performance liquid chromatography; GCV-genotypic coefficient of variability; PCV-phenotypic coefficient of variability; CV-coefficient of variability; LOD-limit of detection; LOQ-limit of quantification; RSD-relative standard deviation; EC-electrical conductivity; UPGMA-unweighted pair group method arithmetic average.

## Introduction

The genus *Hyoscyamus* L. belongs to the tribe Hyoscyameae Miers of *Solanaceae* family with 18 species all over the world (Yousaf et al., 2008) and 13 species in Iran (Khatamsaz, 1998). *Hyoscyamus* species are rich sources of tropane alkaloids, mainly hyoscyamine and scopolamine, which are used for their mydriatic, antispasmodic, anti-cholinergic, analgesic and sedative properties (Supria, 1998). Because of pharmacological and toxicological importance of tropane alkaloids, determination of these alkaloids in medicinal plants with application of different analytical techniques has been major of concern (Drager, 2002; Kartal et al., 2003). Alkaloids are nitrogenous organic cyclic compounds, normally with basic properties and having physiological effects in animals or man (Sarker and Maruyama, 2002). Thin layer, gas and liquid chromatography provide good separation of the plant components but require purification of the extract prior to analysis, resulting in a long assay time for a large number of the samples. Gas and liquid chromatography have been used extensively, but because scopolamine is heat labile, gas chromatography is impractical for measuring both scopolamine and hyoscyamine simultaneously. However, high performance liquid chromatography (HPLC) is commonly used to measure alkaloid content in plants (Oksman-Caldentey, 2007). The synthetic production of these alkaloids is more expensive than their extraction from plant materials. Hence, they are currently extracted industrially from various *Solanaceous* plants (Hosseini et al., 2011). Biosynthesis of alkaloids, although controlled genetically, could be affected by different environmental factors such as light, high temperature, stress, and also soil fertilization (Poutaraud and Girardin, 2005; Chatterjee et al., 1988). In plants, mineral elements uptake is a complex process governed by numerous factors such as plant species, genotype, availability and mobility of the minerals in the soil, soil properties, such as, pH, organic matter, clay content and etc. (Radanovic et al., 2002; Poutaraud and Girardin, 2005). Numerous studies have shown the significant influence of mineral nutrition on alkaloid synthesis (Al-Humaid, 2004; Lata, 2007). Scopolamine is more valuable alkaloid with world demand of ten times more than hyoscyamine (Sevon et al., 2001). To capture this global demand, the best way is the production of scopolamine and hyoscyamine, by development of *Hyoscyamus* yielding accessions integrated with high tropane alkaloids. Before planning of any breeding program for genetic improvement, the assessment of genetic variation in germplasm is a necessary step (Hemant et al., 2006). However, a few studies have been done to determine the genetic diversity in *Hyoscyamus* species based on alkaloid profiles (Bahmanzadegan et al., 2009; Hosseini et al., 2011). The purpose of this study was to determine the genetic variation and isolate accessions with high genetic distance for hybridization in breeding programs. In addition, the correlation between environmental parameters and the tropane alkaloids contents in *Hyoscyamus* accessions was also evaluated.

## Results and Discussion

After extraction and purification of alkaloids from different parts of the accessions, levels of two tropane alkaloids, hyoscyamine and scopolamine, were measured. The retention time (RT) of scopolamine and hyoscyamine were 3.7 and 9.5 minutes, respectively (Figures 1 & 2). The linearity of the method was tested using the standard solutions of scopolamine and hyoscyamine (Table 1). The calibration curve of scopolamine was linear in the range of 0.005 to 0.5 mg/ml, with correlation coefficient of 0.9952. The linearity of hyoscyamine was from 0.005 to 0.5mg/ml, with correlation coefficient of 0.9965. The calibration curves were represented by linear equations of Y=12127 X - 387083 and Y=77271 X $-3\times10^{6}$ for scopolamine and hyoscyamine, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the equations LOD= 3.3× N/B and LOQ=10× N/B where N is standard deviation of peak area (n=3), taken as measure of noise and B is the slope of the corresponding calibration curve. The values of LOD and LOQ were 0.002 and 0.008 mg/L for scopolamine, 0.00008 and 0.00025 mg/L for hyoscyamine, respectively. The accuracy of method was evaluated by calculating the recovery of scopolamine and hyoscyamine by the standard addition method. The analyzed samples were spiked with extra concentration levels of 100 ppm from scopolamine and hyoscyamine with mixtures reanalyzed by the same method. Repeatability analysis was expressed as the Relative Standard Deviation (RSD) which consists of multiple measurements of a sample by the same analyst under the same analytical conditions. Results of HPLC analyses were presented in supplemental table S1 which compares the values of scopolamine and hyoscyamine in different organs of 24 accessions of *Hyoscyamus* collected from different geographical origins.

***Accessions variability***

Analysis of variance showed significant differences among some accessions for two tropane alkaloids, scopolamine and hyoscyamine ($p<0.05$) (Supplemental table S1). A large variation between mean values of accessions for each trait was also evident (Supplemental table S1). Of the two tropane alkaloids studied, scopolamine showed higher variability among the accessions. According to the results of this study, scopolamine was the predominant tropane alkaloid in almost all parts of population organs. Our results also indicated that the distribution of scopolamine and hyoscyamine was different in various parts of the *Hyoscyamus* accessions investigated. The scopolamine and hyoscyamine contents were highest in leaves, followed by stems, seeds and roots (Supplemental table S1, Figure 3). The tropane alkaloids (sum of scopolamine and hyoscyamine) are quite different according to the sites, from 0.15 mg/g d.w. for the root of *H.niger* (Salmas site) to 12.21 mg/g d.w. for the leaf of *H.niger* (Bazargan site). *H.niger* from Bazargan site (h19) had the highest tropane alkaloids (4.57 mg/g d.w.) and *H.niger* from Salmas site (h20) had the least tropane alkaloids (0.15 mg/g d.w.) among the studied *Hyoscyamus* roots. Scopolamine was the predominant tropane alkaloid in leaves of *H.niger* (region of Bazargan: 10.87 mg/g d.w.). The data also indicated the presence of high hyoscyamine content in the leaves of *H.niger* (region of Bazargan: 1.33 mg/g d.w.). Thus, content of tropane alkaloids (sum of scopolamine and hyoscyamine) was highest in region with soil nitrogen (0.27%), phosphorus (17.60 me/L), potassium (119 me/L), $Ca^{+2}$ (9.06 me/L), EC (0.54 mms/cm) & pH (7.24) amounts and altitude of 2300 m. The least scopolamine and hyoscyamine contents were observed in roots of *H.niger* (region of Salmas: 0.08 mg/g d.w.), *H.reticulatus* of Khalkhal and *H.niger* of Khoy (0.07mg/g d.w.), respectively. Shukla et al. (2006) investigated the alkaloid profile of 98 accessions of opium poppy (Papaveraceae) and found large variability among the accessions in all five alkaloids. Yadav et al. (2006) investigated the genetic parameters and correlation between the five alkaloids in a collection of 122 opium poppy accessions. They found large genetic variation in the content of all five alkaloids, but low genetic correlations between the five alkaloids (the strongest was $r$ = 0.37), indicating almost independent inheritance of the alkaloids and thus a high potential for genetic improvement by breeding. In contrast to our results, scopolamine was the only main alkaloid of two population's seeds, and hyoscyamine was the major alkaloid of almost all parts; especially roots in *Hyoscyamus arachnoideus* (Hosseini et al., 2011). Miraldi et al. (2001) found atropine as the main alkaloid of different plant parts at different stages of growth. These authors also reported that stems contain the highest level of tropane alkaloids (atropine and scopolamine). Chalabian and Majd (2004) also found the higher rate of hyoscyamine production than scopolamine in all plant organs collected from different phenological stages of *Hyoscyamus reticulatus*. In the research of Bahmanzadegan et al. (2009) scopolamine was predominant tropane alkaloid in *H.pusillus*, *H.niger* and *H.kurdicus*, while *H.reticulatus* contained a higher amount of hyoscyamine. Our results are in contradiction to their results, since, *H.reticulatus* and four other *Hyoscyamus* species contained more scopolamine than hyoscyamine. Our finding is in agreement with data reported by Supria (1998) on *H.niger* and *H.reticulatus.* Our obtained data also indicated that the distribution of tropane alkaloids was different in various organs of *Hyoscyamus* accessions investigated (Supplemental table S1& Figure 3). Bahmanzadegan et al.(2009) found that *H.pusillus* and *H.kurdicus* collected in full flowering stage, contained the most scopolamine in leaves among organs followed by roots and stems whereas, *H.niger* and *H.reticulatus*, collected at the end of flowering and beginning of fruit formation stage showed the highest amounts of scopolamine in seeds then followed by roots and stems. Plant leaves and fruits were the most valuable organs for alkaloid accumulation followed by stems, roots, and crowns of *Datura innoxia* Mill. plants (Al-Humaid, 2003). In terms of mean amount of hyoscyamine to scopolamine ratio, *H.pusillus* was the predominant species. This is due to high hyoscyamine conversion to scopolamine, making it suitable species for transportation of the hyoscyamine 6β-hydroxylase (H6H) enzyme. In vivo, H6H catalyzes the epoxidation of hyoscyamine to scopolamine (Hashimato and Yamada, 1987). This finding was in harmony with the report published by Bahmanzadegan et al. (2009) that *H.pusillus* was the predominant species in respect to hyoscyamine/scopolamine ratio. In respect to hyoscyamine/scopolamine ratio roots had highest amount of hyoscyamine to scopolamine. Hyoscyamine is synthesized in roots at the stage of fruiting, transported to aerial parts and converted to scopolamine. Miraldi et al. (2001) reported that the root is the principle location of alkaloid synthesis and secondary modifications of alkaloids occur in the aerial parts. They added that, at plant maturity, alkaloids are decreased in roots of adult plants. This study shows that alkaloids level in the roots were very low compared to other plant parts.

***Broad sense heritability***

Earlier studies showed the involvement of additive, dominance and epistatic effects in the inheritance of opium alkaloids (Hemant et al., 2006). To achieve the existing variability among the accessions for particular traits, different genetic parameters were estimated which are present in table 2. To assess the heritable portion of total variability, phenotypic variance ($\delta^2$p) was partitioned into genotypic ($\delta^2$g) and error variance ($\delta^2$e). Data clearly indicated that variability existed in the accessions, were mainly due to

**Table 1**. Linear regression equation and correlation coefficient for scopolamine and hyoscyamine (n=3).

| Compounds | Linear regression equation | Correlation coefficient | LOD (mg/L) | LOQ (mg/L) |
|---|---|---|---|---|
| Scopolamine HBr.3$H_2$o | Y=12127x-387083 | 0.9952 | 0.00200 | 0.00800 |
| Hyoscyamine | Y=77271x-3×$10^6$ | 0.9956 | 0.00008 | 0.00025 |

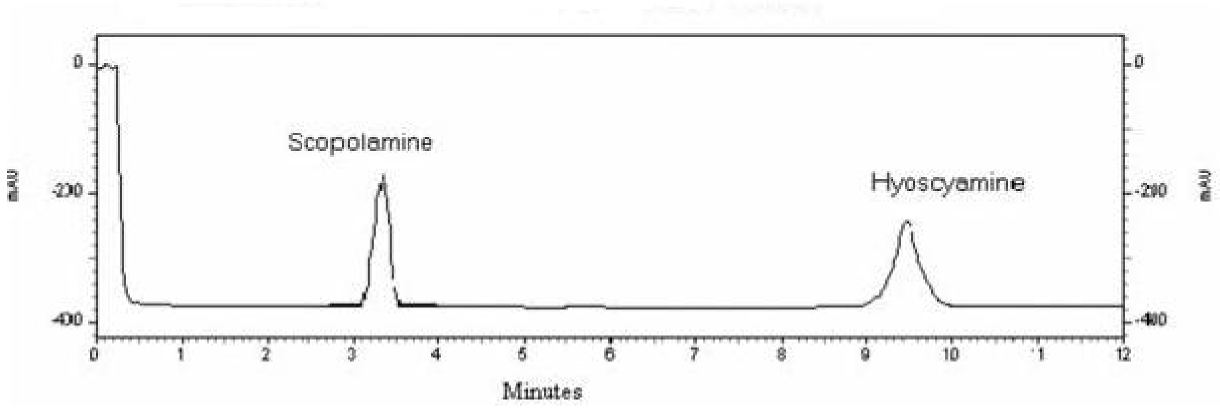


**Fig 1.** HPLC chromatograms of scopolamine and hyoscyamine standards.

genotypic variance as the error variance values are very less (Hemant et al., 2006).The coefficient values due to phenotypes and genotypes were high for scopolamine of leaf and scopolamine of seed, respectively. However, phenotypic coefficient of variation (PCV) was higher than those of genotypic coefficient of variation (GCV) for all traits of accessions, though the differences were very small since the variability in different traits is due to genotypic effects. Therefore, genetic improvement for these traits can easily be achieved by selection of promising plant types and also through crossing the desirable accessions among themselves followed by selection in segregating generations. The knowledge of heritability of a character is important as, it indicates the possibility and extent to which improvement is possible through selection (Robinson et al., 1949; Yadav et al., 2006). The heritability estimates were high for all the traits and ranged from 0.82 for scopolamine of leaf to 0.99 for scopolamine of seed and root, as well as, hyoscyamine of root and stem.

***Cluster analysis***

The results of cluster analysis based on UPGMA revealed three clusters at the similarity of 1.72 (Figure 5). The first main cluster (cluster I) can be divided into two sub clusters. The first sub- cluster consisted of h1, h4, h16, h7, h9, h24, h2, h5, h17, h8, h23, h12, h11, h20, h13, h15, h22 and h21. The h6, h14 and h10 were grouped in the second sub-cluster. The second main cluster (cluster II) manifested of genotypes labeled as h3 and h18 and the third main cluster (cluster III) comprised of h19. The *H.reticulatus* (h4) had the most similarity to *H.pusillus* h16).The two *H.reticulatus,* h9 and h24, collected from two different geographical regions, also showed highest similarity matrix (Figure 4). The least similarity percentage (91.07) was obtained between h11, h19 and h1 and the highest between h9 & h24 and h4 & h16 (99.89). Hence, amount of genetic variability in Iranian germplasm of *Hyoscyamus* is low. It was obvious that the genetic relationships among studied accessions did not have force tendency to associate with their geographical origins. Because this is the first report of the relationship between genotype and geographic origins of *Hyoscyamus* species we couldn't compare our results with other researchers' findings. The highest cophenetic correlation coefficient (r =0.87) was obtained between similarity data matrix and the cophenetic matrix, indicating a good fit between the dendrogram clusters and the similarity matrices.

***Results of Correlation coefficient***

Soil analyses showed variation within the 24 soils studied (Supplemental table S2). Roots of *Hyoscyamus* species were located at 15-25 cm depth in the ground and numerous thin roots grown down deeply into the soil. It seemed obvious to observe the best relations between tropane alkaloids and mineral elements in the 0-30 cm soil layer because of plant-root repartition. The highest coefficient of determination ($R^2$) was obtained in the 0-30 cm. For example $R^2$ for nitrogen in 0-30 cm of soil is 0.02, 0.01 in 30-60cm and 0.01in 60-90 cm. Investigation on the effect of environmental factors on scopolamine and hyoscyamine levels showed that as altitude increased, the level of the alkaloids in the studied accessions also increased. Investigation on hyoscyamine and scopolamine amounts of *Atropa belladonna* in different altitudes showed the significant effect of altitude on amounts of tropane alkaloids (Zarrate et al., 1997).Our results showed that the altitude had positive effect among environmental factors on tropane alkaloids contents. Correlation coefficient among tropane alkaloids, altitudes and soil properties are shown in table 3. In this study, the amount of soil nitrogen showed positive correlation with studied tropane alkaloids except scopolamine of stem (-0.21) (Table 3). High level of nitrogen caused induction of alkaloids level in *Hyoscyamus muticus* (Ahmed and Fahmi, 1949). Nitrogen fertilization was also found to increase the growth, yield and alkaloid content in *Datura innoxia* (Al-Humaid, 2003). In the study of Bensaddek et al. (2001), the effect of nitrate and ammonium concentrations was shown on growth and alkaloid accumulation of *Atropa belladonna* hairy roots. Hence, the importance of these elements in alkaloid biosynthesis is evident. In this regard, several studies indicated that a suitable amount of mineral nutrition enhances the

**Table 2.** Estimates of variance components and heritability in *Hyoscyamus* accessions.

| Traits | $\delta^2$g | $\delta^2$e | $\delta^2$p | heritability | GCV | PCV | CV (%) | F value |
|---|---|---|---|---|---|---|---|---|
| Scopolamine of stem | 2.01 | 0.05 | 2.06 | 0.97 | 1.07 | 1.09 | 110 | 118.83* |
| Hyoscyamine of stem | 0.04 | 8.48E-06 | 0.04 | 0.99 | 1.30 | 1.30 | 85 | 7715. 21* |
| Scopolamine of seed | 2.15 | 0.00 | 2.16 | 0.99 | 1.45 | 1.45 | 109 | 2320.79* |
| Hyoscyamine of seed | 0.04 | 0.00 | 0.05 | 0.87 | 0.94 | 1.00 | 99 | 21.08* |
| Scopolamine of leaf | 0.15 | 0.03 | 0.18 | 0.82 | 1.33 | 1.46 | 106 | 474.39* |
| Hyoscyamine of leaf | 0.15 | 1.35E-05 | 0.15 | 0.99 | 1.36 | 1.36 | 90 | 15679.48* |
| Scopolamine of root | 0.85 | 0.00 | 0.85 | 0.99 | 1.30 | 1.30 | 131 | 4218.50* |
| Hyoscyamine of root | 0.01 | 7.98E-05 | 0.03 | 0.99 | 0.86 | 0.86 | 87 | 1210.70* |

GCV: Genotypic coefficient of variability; PCV: Phenotypic coefficient of variability; CV: coefficient of variability *significant at p<0.05.

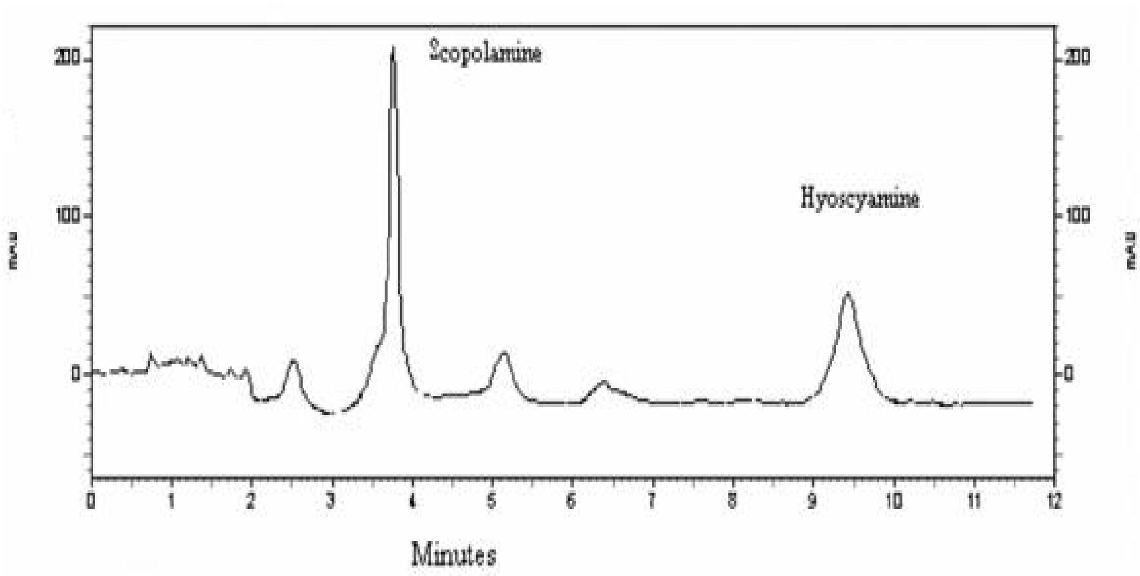


**Fig 2.** HPLC chromatograms of scopolamine and hyoscyamine in roots of *Hyoscyamus*niger from Sardasht region.

hyoscyamine and scopolamine synthesis in *Datura*, as a result of influencing the formation of amino acids converting to the tropane alkaloids (Pinnol et al., 1999). Phosphorus and tropane alkaloids levels of all organs showed positive correlation (Table 3). Elsheikh et al. (1982) reported the addition of phosphorus in soil will increase contents of alkaloids in *Hyosyamus muticus*. The amount of potassium showed positive correlation with alkaloids of all accessions (Table 3). $Ca^{+2}$ level showed the most significant positive correlation with studied tropane alkaloids of accessions ranging from 0.59 for scopolamine of stem to 0.93 for hyoscamine of leaf (Table 3). $Ca^{+2}$ activates the accumulation of tropane and indole alkaloid in *Hyoscyamus niger* and *Catharanthus roseus* whereas strongly inhibits isoquinoline alkaloid of *Papaver somniferum* (Poutaraud and Girardin, 2005). EC amount showed positive correlation with studied tropane alkaloids. The most correlation was observed with scopolamine of root and least with hyoscamine of leaf (Table 3). Poutaraud and Girardin, (2002) showed within pH range (6.7-8.2), major essential elements would expect to be well assimilated.

The soil pH level showed negative association with studied tropane alkaloids in accessions. We propose extensive laboratories studies under controlled conditions on the effects of nutrient elements on accumulation of tropane alkaloids in *Hyoscyamus* genotypes. We conclude that range of variation within *Hyoscyamus* accessions was extremly high based on scopolamine and hyosyamine alkaloids. The existance of high GCV indicates the genotypic control of these traits which leads us to achivement of genetic improvement for these two tropane alkaloids by selection of desirable plants for breeding in future. Our data also implies positive correlation between $Ca^{+2}$, N, P, K & EC and tropane alkaloids in most plant tissues. Besides, the negative assosiation between PH and tropane alkaloids was aslo evident which will be useful for production programs in the future.

**Table 3.** Correlation coefficient between pair wise traits: alkaloid content, altitude and soil properties in organs of *Hyoscyamus* accessions.

| Traits | Altitude | N | P | K | pH | EC | $Ca^{+2}$ |
|---|---|---|---|---|---|---|---|
| s stem | 0.49* | 0.17 | 0.27 | 0.21 | -0.20 | 0.15 | 0.89** |
| h stem | 0.21 | -0.21 | 0.19 | 0.13 | 0.07 | 0.13 | 0.59** |
| s+ h stem | 0.47* | 0.16 | 0.24 | 0.21 | -0.18 | 0.15 | 0.89** |
| s seed | 0.47* | 0.16 | 0.18 | 0.15 | -0.21 | 0.09 | 0.92** |
| h seed | 0.34 | 0.19 | 0.14 | 0.13 | -0.15 | 0.06 | 0.74** |
| s+ h seed | 0.45* | 0.17 | 0.18 | 0.15 | -0.20 | 0.09 | 0.91** |
| s leaf | 0.45* | 0.19 | 0.11 | 0.06 | - 0.24 | 0.01 | 0.93** |
| h leaf | 0.55** | 0.10 | 0.22 | 0.20 | -0.26 | 0.16 | 0.80** |
| s+ h leaf | 0.46* | 0.18 | 0.12 | 0.08 | -0.25 | 0.03 | 0.98** |
| s root | 0.49* | 0.20 | 0.16 | 0.13 | -0.15 | 0.08 | 0.90** |
| h root | 0.42* | 0.13 | 0.31 | 0.33 | -0.34 | 0.20 | 0.70** |
| s+ h root | 0.50* | 0.20 | 0.19 | 0.17 | -0.19 | 0.11 | 0.90** |

*significant at p<0.05, ** significant at p<0.01.

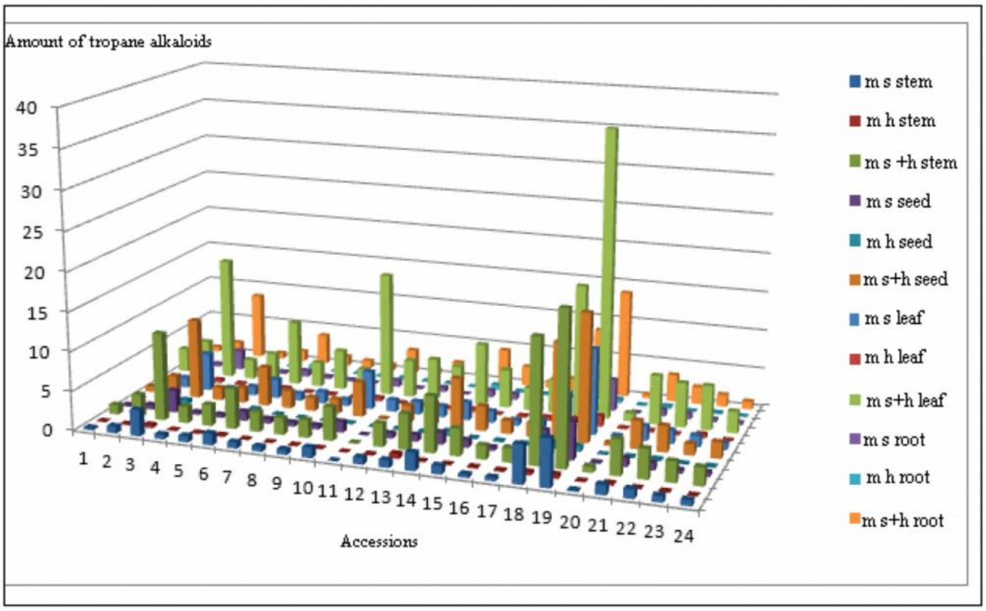


**Fig 3.** Amounts of tropane alkaloids in different organs of 24 accessions of *Hyoscyamus*. m s stem: mean of scopolamine of stem, m h stem: mean of hyoscyamine of stem, m s +h stem: mean of scopolamine + hyoscyamine of stem, m s seed: mean of scopolamine of seed, m h seed: mean of hyoscyamine of seed, m s+h seed: mean of scopolamine +hyoscyamine of seed, m s leaf: mean of scopolamine of leaf, m h leaf: mean of hyoscyamine of leaf, m s+h leaf: mean of scopolamine +hyoscyamine of leaf , m s root: mean of scopolamine of root, m h root: mean of hyoscyamine of root and m s+h root: mean of scopolamine +hyoscyamine of root. s: scopolamine, h: hyoscyamine. Numbers (1-24) are representative of codes (h1- h24) listed in supplemental table S1, respectively.

## Material and methods

### *Plant material*

Different organs including stem, leaf, root and seed of five *Hyoscyamus* species including *H.niger* L., *H.reticulatus* L.,*H.pusillus* L., *H.arachnoideus* Pojark., and *H.kurdicus* Bornm., were collected from natural habitats at 24 regions (Supplemental table S1& Figure 5). In each region, accessions were collected in a randomized complete block design with three replications. Experimental units in each block comprised of 2 lines of 6 m long. The data on 3 individuals in each block were recorded for alkaloids traits. Plants collected per site during end of flowering stage and beginning of fruit setting from 15 to 19 June 2010, depending on the sites. In this stage plants contain indehiscent capsules which seeds turn from green to brown. Alkaloids are stable during this period (Poutaraud and Girardin, 2002).

### *Chemicals*

L-hyoscyamine and scopolamine $HBr.3H_2o$ were purchased from Sigma-Aldrich. Acetonitrile, methanol and water were obtained from Merck which had HPLC grade.

### *Standard solutions*

A stock solution containing 500 ppm of scopolamine and hyoscyamine in methanol was prepared in a 10 ml volumetric flask. The standard solutions of scopolamine and hyoscyamine containing 5, 10, 20, 40, 50,100 and 300 ppm were obtained by serial dilution of the stock solution with methanol. Stock solution and working solutions were stored at 4˚c and brought to room temperature before use (Bahmanzadegan et al., 2009). The calibration graphs for standard samples were constructed by plotting the peak area of the alkaloids against their concentrations.

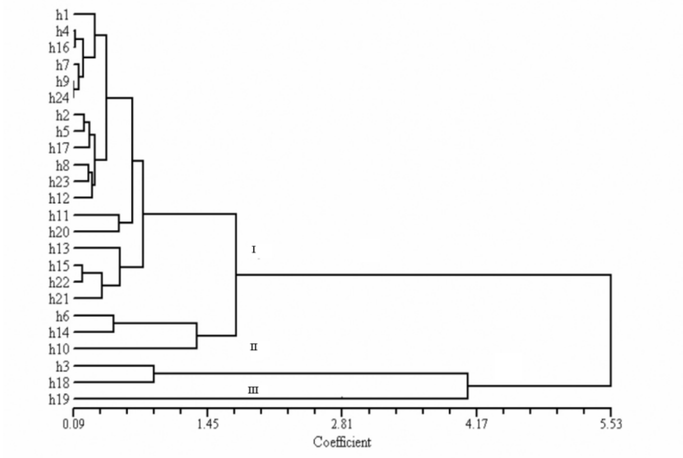


**Fig 4.** UPGMA dendrogram based on MANHAT coefficient among *Hyoscyamus* accessions studied.

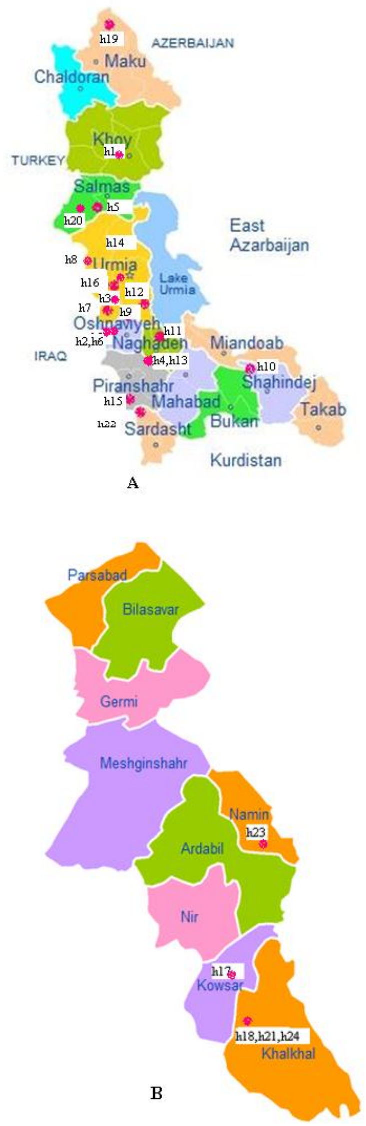


**Fig 5.** Sources and geographical localities of *Hyoscyamus* accessions tested. The codes on the map are representative of accessions collected from different locations. (A) West Azarbijan and (B) Ardabil provinces of Iran.

Linear calibration graphs were obtained with good correlation for standard solutions (Supplemental table S2).

***Apparatus and HPLC conditions***

HPLC analysis was carried out on a Well Chrome 2000, Knauer, Germany equipped with a Eurospher C18 column (dimension: 250×4.6mm) and UV detector (λ max: 210). Isocratic elution with a mixture of Water: Acetonitrile (65:35) at a flow rate of 0.5 ml/min was selected to achieve maximum separation and sensitivity.

***Alkaloid extraction***

Alkaloid was extracted from stem, leaf and root and seed materials (500 mg) based on the reported procedure by Kamada et al. (1986). The resulting extract was directly subjected to HPLC analysis.

***Estimating of broad sense heritability***

In order to assess the heritable portion of total variability, phenotypic variance ($\delta^2$p) was partitioned into genotypic ($\delta^2$g) and error variance ($\delta^2$e) (Table 2).

***Environmental factors analysis***

Three soil layer samples (0-30, 30-60 and 60-90 cm) of 24 sites were collected for chemical analyses (Supplemental table S2). Samples were dried at ambient air temperature and crushed to pass through a 2 mm sieve. Each soil sample was analyzed. Nitrogen (Kjeldahl method; Bremner, 1960), Phosphorus (Olsen and Sommers, 1982), Potassium (Flame photometer method; Jackson, 1973), EC (Bertsch and Bloom, 1996) and $Ca^{+2}$(Flame photometer method; Reeve, 1965) were assayed. Potentiometric measurement of pH was made on 1soil: 25$H_2O$ ratio (Bertsch and Bloom, 1996).

***Statistical analysis***

Each data was the mean of three replicates and means were considered as significant differences at $p<0.05$ level. Degree of correlation between tropane alkaloids contents and environmental data was analyzed using correlation coefficient analyses. The genetic distance was obtained based on MANHAT coefficient. Cluster analysis was performed on the distance matrix by un-weighted pair group method arithmetic average (UPGMA) method. The cophenetic correlation value (r) was estimated between similarity data matrix and the dendrogram to obtain correlation between the dendrogram clusters and the similarity matrices (Sneath and Sokal, 1973). Heritability estimates were calculated by the formulae used by Hemant et al., (2006). All statistical analyses were performed using NTSYS pc 2.02 (Rohlf, 1998) and SAS 9.1.3 (SAS Institute Inc, 2005) software.

***Measurement of environmental factors on alkaloids contents***

In order to determine the effect of environmental factors such as altitude, total nitrogen, available P, available K, $Ca^{+2}$, EC and pH on alkaloids contents canonical correlation analyses were done on original data that collected from field and laboratories studies.

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