Ethno Pharm Product: EPP 2024; 4 (1): 59-67

Reaserch Paper

Investigation of chemical and genetic diversity of Henna by using HPLC and ISSR marker

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Article information

Available online: Mar. 2024

Keywords:
genetic diversity
HPLC
ISSR markers
Lawsonia inermis

Abstract

Lawsonia inermis is utilized in the cosmetics industry and traditional medicine for the treatment of various ailments. Understanding the chemical and genetic diversity of this plant is essential for breeding purposes. This research investigated the chemical and genetic diversity of 12 distinct populations of *Lawsonia inermis* from different areas of Kerman province through the use of HPLC and ISSR markers. HPLC was employed to quantify the Lawson compound in the 12 populations, while three ISSR primers were utilized to evaluate genetic diversity. The findings showed notable diversity in Lawson content among samples collected from Jiroft, Shahdad, and Ghale Ganj, with the J5 sample (from Jiroft) displaying the highest value. Furthermore, the ISSR marker demonstrated that all populations could be grouped into three categories, with samples from the same region not necessarily clustering together. Although the marker could not differentiate populations based on Lawson content, it effectively distinguished them based on genetic diversity.

1.Introduction:

Henna, or *Lawsonia inermis*, is native to North Africa and Southeast Asia (Grieve, 2005) and has spread to regions including Egypt, Arab countries, Persian-speaking nations, India, Pakistan, Florida in the USA, China, and Sudan (Kokate 2001).

The Middle East has long utilized henna for dyeing hair and nails (Lauchli and Lautenschlager, 2001). The dyeing property of Henna is attributed to the presence of a substance called Lawson (Gallo et al., 2008).

Henna is a plant that encompasses a wide array of phytochemical compounds such as carbohydrates, glycosides, tannins, phenolic components, gums, mucilage, napthoquinone derivatives, terpenoids, sterols, aliphatic derivatives, xanthones, coumarin, fatty acids, amino acids, and other constituents (Chaudhary et al. 2010; Makhija et al. 2011).

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In addition to its use in the cosmetics industry, this plant is employed in traditional medicine to address various ailments such as inflammation (Ali et al.,1995), mycoses (Singh and Pandey, 1989), skin eruptions (Fernandez et al., 2002), headaches, and diseases of the digestive apparatus.

This plant is cultivated in the southern regions of Iran, such as Kerman, Hormozgan, and Sistan and Baloojestan Provinces (Amit S. Borade, 2011). Despite its greater significance in the pharmaceutical and cosmetics industries, its chemical diversity, particularly its valuable material Lawson, and genetic diversity, have not been thoroughly evaluated in Iran.

Genetic diversity forms the foundation for trait breeding (Huang et al., 2002; Stepien et al., 2007) and is crucial for the success of plant breeding and the development of new varieties (Marić et al., 2004). The examination of genetic and phenotype variation to identify groups with similar genotypes for conservation, evaluation, and utilization of genetic resources, as well as to analyze the diversity of germplasm before breeding, and to ascertain the unique and distinct genotypic and phenotypic structures of genotypes is indispensable (Franco et al., 2001).

Molecular markers are a reliable method for estimating genetic variation because they are not affected by the environment and there are a sufficient number of markers available to assess genetic diversity (Prasad et al., 2000). Various molecular markers, such as ISSR (Inter Simple Sequence Repeat), have been developed and used in studies to investigate the genetic diversity of medicinal plants (Boubaya et al., 2013; Shilpha et al., 2013; Peng et al., 2014; Kulhari et. al, 2015).

This study aimed to assess the genetic and chemical diversity of 12 different populations of Henna using ISSR and HPLC.

2.Material and Methods

2-1.Plant material and Lawson extraction

Twelve populations of *Lawsonia inermis* which collected from different region Kerman province (Table 1) were used in this study. Dried and powdered leaves of each sample (0.3 g) were mixed with methanol (1.5 mL) and sonicated in a model 3210E-MTH (Branson, Danbury, CT, USA) ultrasonic bath for 30 min (Putzbach *et al*., 2007). The mixture centrifuged with 13000 rpm during 2 min. The supernatant was filtered through Albet (Barcelona, Spain). Lawson measuring carried out using HPLC by method of Gallo (2008). After preparing of columns, first the Lawson standard injected, then prepared samples injected in room temperature condition during 8 minutes. The wavelength of 280 nm was used for identification of Lawson.

2-2.DNA extraction

Total genomic DNA was extracted from young leaves of plants according to the CTAB protocol (Boubaya *et al*., 2013) with minor modifications.

2-3.ISSR marker

Three ISSR primers were used (Table 2) according to Reddy *et al*. (2002). Polymerase Chain Reactions (PCRs) were carried out in a 25 µl volume, containing 2 units of *Taq* polymerase, 50 ng of genomic DNA template, 0.2 µM of primer, 2mµ of each dATP, dCTP, dGTP and dTTP, and 2.5 µl of 10X PCR reaction buffer. PCR product was separated in a 1% agarose gel and detected by the ethidium bromide staining method.

3.Data analysis:

Polymorphic ISSR fragments were scored as either present (1) or absent (0) across all populations. Only distinct, well-resolved fragments were scored.

The average Polymorphic Information Content (PIC) (Powell *et al*. (1996)), Effective number of

alleles (Hartl & Clark, 1989), Nei's gene diversity (Nei, 1973), Shannon's Information index (Shannon & Weaver, 1949) were calculated for ISSR marker system across all polymorphic assay units.

Binary matrix was used to estimate the genetic similarities between pairs, by employing Jaccard index. These similarity coefficients were used to construct dendrogram using the unweighted pair group method with arithmetic averages (UPGMA) employing the SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering) from the NTSYSPC (Numerical Taxonomy and Multivariate Analysis System), version 2.02 (Applied Biostatistics) program (Rohlf, 1990).

Table 1. Collection sits of Henna populations in Kerman province.

No	Code	Collection sits	No	Code	Collection sits
1	Bb	Bam	7	Sh4	Shahdad
2	Bd	Bam	8	J2	Jiroft
3	Gh1	Ghale Ganj	9	J5	Jiroft
4	Gh2	Ghale Ganj	10	Rod	Roodbar
5	Gh3	Ghale Ganj	11	R	Reigan
6	Sh1	Shahdad	12	K	Kahnooj

Table 2. ISSR primers used for genetic diversity of Henna populations

No.	Primer name	Primer sequence
1	807	AGAGAGAGAGAGAGAGT
2	825	ACACACACACACACACT
3	816	CACACACACACACACAT

4.Results

4-1.Chemical diversity

Chromatograms of standard Lawson (2-hydroxy-1,4-naphthoquinone) and leaf samples from different region of Kerman province obtained using the mobile gradient phase consisting of methanol for 4.5 minute run time (fig. 1 and 2).

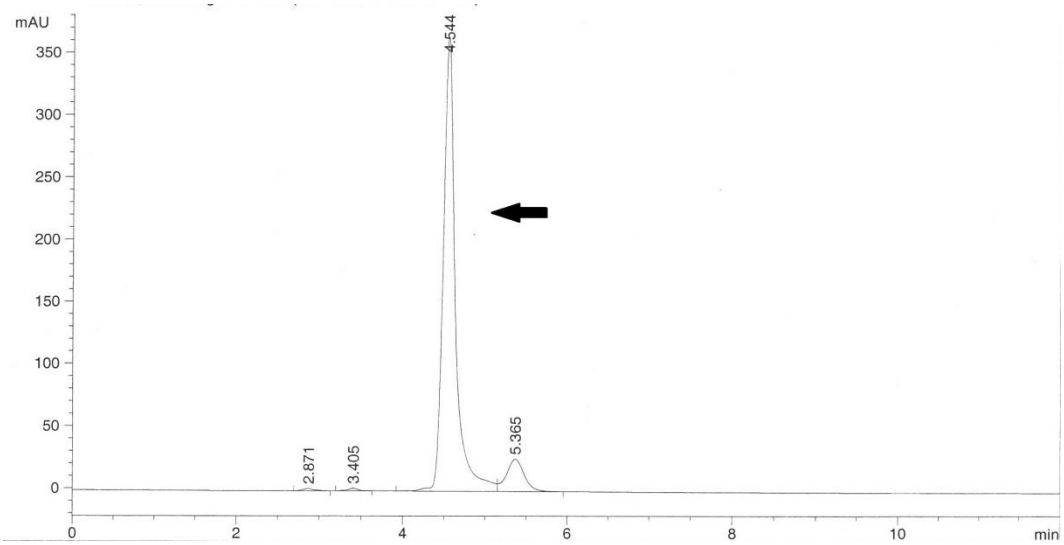

Fig. 1. HPLC chromatogram of standard Lawson.

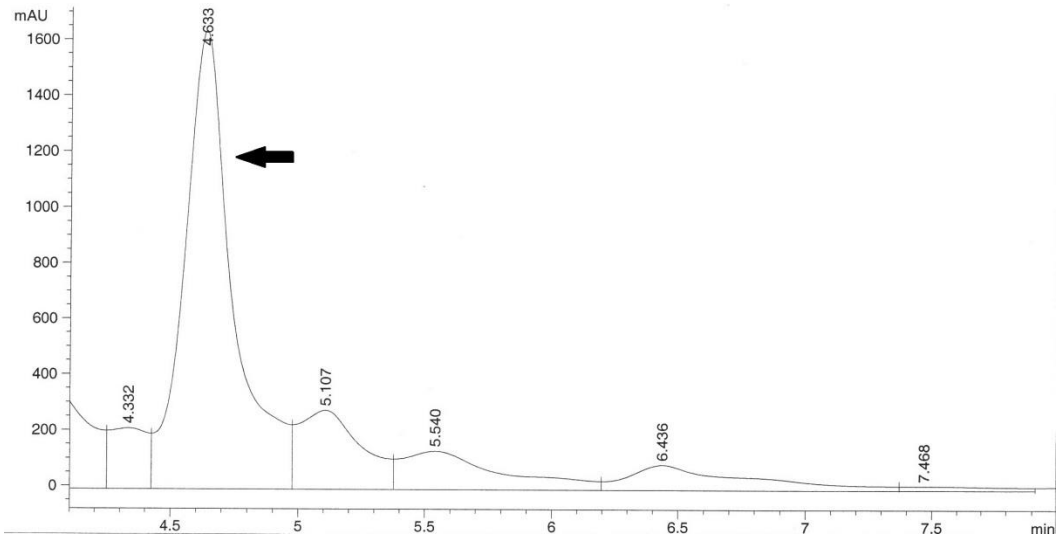

Fig. 2. HPLC chromatogram of Lawson from J5 sample.

The HPLC analysis of *Lawsonia inermis* representing the different region of Iran showed some interesting results. There is a lot of variation in the percentage of Lawson content among the different samples. Sample collected from the Jiroft (J5) showed the presence of high amount of Lawson (0.248 mg/gr dry matter), which was followed by Ghale Ganj sample (Gh3) (0.136 mg/gr dry matter) (table 3). The two samples collected from Shahdad (Sh1) and Bam (Bb) showed less percentage of Lawson that is, 0.039 and 0.040 mg/gr dry matter respectively.

Table 3. The Lawson value in different samples gathered from different region of Kerman province.

No.	Collection site	code	Lawson value (mg/gr dry matter)	No.	Collection site	code	Lawson value (mg/gr dry matter)
1	Bam	Bb	0.04	7	Shahdad	Sh4	0.124
2	Bam	Bd	0.05	8	Jiroft	J2	0.089
3	Ghale Gnj	Gh1	0.068	9	Jiroft	J5	0.248
4	Ghale Gnj	Gh2	0.085	10	Roodbar	Rod	0.077
5	Ghale Gnj	Gh3	0.136	11	Kahnooj	Kah	0.048
6	Shahdad	Sh1	0.039	12	Reigan	R1	0.048

4-2.Genetic diversity:

The three ISSR primer combinations generated a total of 52 scorable fragments ranging from 100 bp to 1 kb of which 36 (69.23%) were polymorphic across the 12 populations (table 4). On average, 12 polymorphic bands were amplified by each primer combination. The ISSR primer807 generated the highest (19 fragments) number of polymorphic bands and the lowest (6 fragments) were generated by primer816. These results confirm that ISSR is capable of detecting substantial numbers of polymorphic loci with a relatively small number of primers.

Across all populations the average PIC value (Powell et al., 1996), Effective number of alleles (Hartl & Clark, 1989), Nei's gene diversity (Nei, 1973), Shannon's Information index (Shannon & Weaver, 1949) were 0.285, 1.639, 0.241 and 0.535 respectively (table 5). Primer 816 had maximum value of PIC value, Effective number of alleles (Hartl & Clark, 1989), Nei's gene diversity (Nei, 1973), Shannon's Information index (Shannon & Weaver, 1949) and it means that this primer had important role in investigation of genetic diversity than other primers.

Estimates of genetic similarity of ISSR based on the 36 polymorphic markers between 12 populations of *Lawsonia inermis* ranged from 0 for Sh4 (Shahdad) and Bb (Bam), to 0.82 for J2 (Jiroft) and J5 (Jiroft).

The cluster analysis obtained with the UPGMA approach revealed three main groups (Fig. 3). The clusters 1, 2 and 3 consisted of four, seven and one populations, respectively, indicating that these populations are somewhat genetically diverse.

This grouping of data indicating possible existence of different varieties of *Lawsonia inermis* in Kerman province.

ISSR analysis also revealed differences in genotype banding patterns between different populations of *Lawsonia inermis* taken from one region (For instance, Sh1, Sh4 from Shahdad or Gh1, Gh2, Gh3 from Ghale Ganj) indicating a significant level of diversity in *Lawsonia inermis* germplasm grown in these regions (Fig. 2).

Table 4. Summary of primer names, polymorphic and monomorphic fragments of ISSR-data scored.

No	Primer name	Total number of bands	Number of polymorphic bands	Number of monomorphic bands	Percent of polymorphic bands
1	807	24	19	5	79.1%
2	825	18	11	7	61.1%
3	816	10	6	4	60%

Table 5. Analysis of banding parameters generated by ISSR assay for 12 populations of *Lawsonia inermis.*

No	Primer name	Effective number of alleles	Nei's gene diversity	Shannon's Information index	PIC
1	807	1.665	0.365	0.536	0.268
2	825	1.51	0.302	0.461	0.284
3	816	1.742	0.421	0.610	0.336
	means	1.639	0.362	0.535	0.296

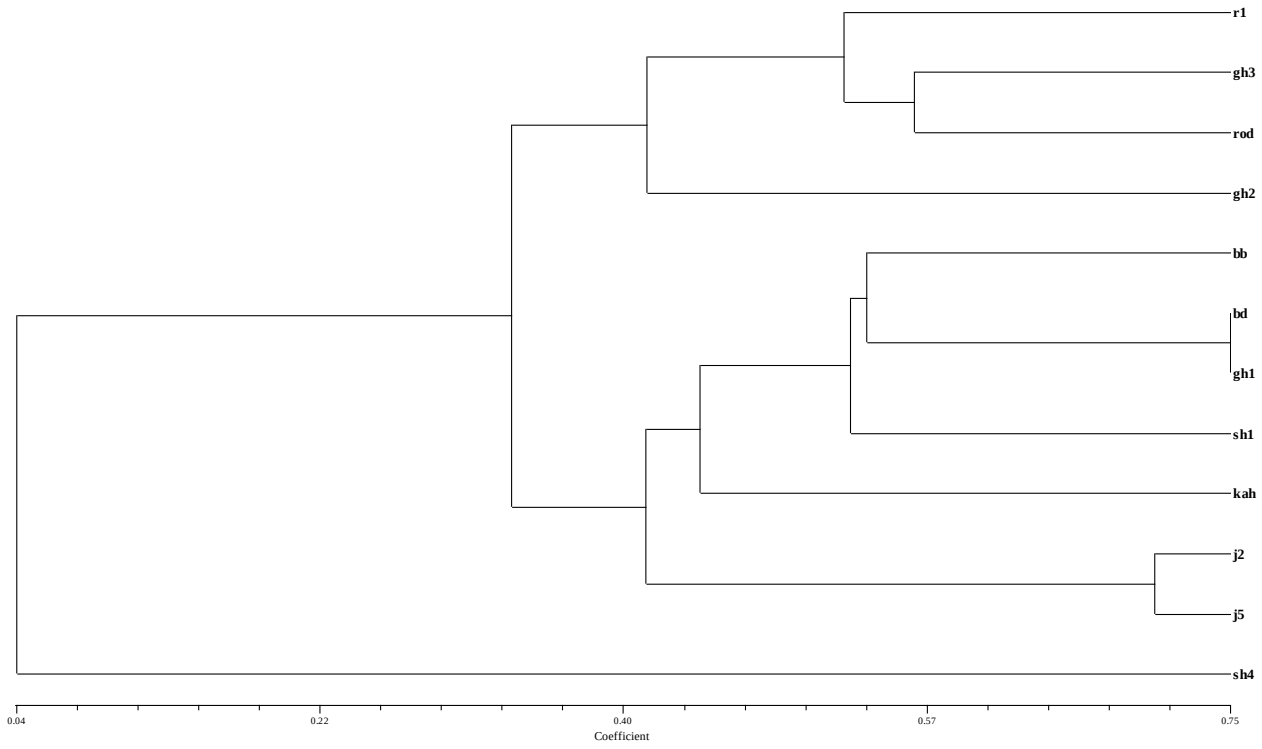

Fig. 3. Dendrogram showing the relationships among populations of *Lawsonia inermis* based on an analysis of 36 bands of ISSR using the Jaccard similarity coefficient and the UPGMA clustering method.

5.Discussion

Results showed that the samples collected from the Bam, Kahnooj, Roodbar and Reigan had minimum Lawson in compare of the rest of the sample and there is low diversity between the samples gathered from the Bam. But there is good diversity between the samples collected from the city of Jiroft, Shahdad and Ghale Ganj and the breeders can benefit from this diversity. Babula et al., (2005) showed that HPLC is useful for measuring of Naphtoquinones (Lawson Precursor) value in Henna plants. Gallo et al., (2008) indicated that HPLC is a useful method for detection of counterfeit samples of this plant for use in cosmetics. Hosseini et al. (2023) examined how harvest date affects bioactive compounds in Kahnooj, Bam, Jiroft, Qale gang, and Zehekalut genotypes. Their findings revealed that the harvest date significantly influenced secondary metabolites, including lawson, flavonoids, and tannins ($p<0.01$), as well as antioxidant activity. The interaction of harvest date and genotype showed that the Bam genotype exhibited the highest lawson level (90.56 µg/ml) in July, while the highest phenolic compounds were measured in the Kahnuj genotype in October (160.1 µg/g of gallic acid/DM). Additionally, a positive correlation was found between tannin, anthocyanin, and flavonoids with lawson, whereas phenol and antioxidant activity correlated negatively. Overall, regardless of harvest date, the Bam genotype yielded the greatest amounts of lawson, anthocyanin, and flavonoids, contrary to our study's findings.

The present method of HPLC has given the reliable quantification of Lawson from different samples, which can be used for the routine analytical work. This procedure can also be used for checking the status of the adulteration in marketed sample of *Lawsonia inermis* by comparing the Lawson contents. The samples that are having high percentage of Lawson can be exploited for commercial purpose and industrial utilization.

The similarity coefficient between samples in this study was between 0 to 0.82. Boubaya et al., (2013) showed that the similarity coefficient between 25 Henna germplasm based on ISSR marker ranged from 0.1 to 0.82. Cluster analysis showed that the samples of Bd (gathered from Bam) and Sh1 (gathered from Shahdad) has more similarity than others. Moreover the samples collected from the same region distributed in different groups for example Sh1 located in the second group and Sh4 in the third group but the samples gathered from Jiroft (J2 and J5) placed in the same group. Different studies showed that

Samples with lowest value of Lawson such as Bb, Bd, R1, Kah and Gh1 distributed in different groups and it means that this marker couldn't separate different samples based on Lawson value.

There is just one study about genetic diversity of Henna. Boubaya et al., (2013) investigated genetic diversity of 25 Henna germplasm by two ISSR primers. In their study the samples gathered from the same region distributed in the different groups like this study.

This study showed that ISSR marker can be useful for investigation of Henna genetic diversity.

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