Genetic variation within Ushaar *(Calotropis procera Ait (Ait) f.)* genotypes using SDS-PAGE for protein and isozyme analysis

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ABSTRACT

Genetic relationships were evaluated among three genotypes of the Ushaar (*Calotropis procera Ait (Ait) f.*), collected from El Maghara, Shalateen and Sant Kathrin by employing SDS-PAGE for seed protein and peroxidase, polyphenyl oxidase and Alcohol dehydrogenase isozymes analysis. SDS-PAGE generated a total of 12 bands of which nearly 50 % were polymorphic. Two positive bands were detected in the Ushaar genotype from Shalateen at the molecular weights; 230.40 and 175.86 KDa. Moreover, two positive bands were found in the Ushaar genotype from Sant Kathrin at the molecular weights; 168.33 and 148.68 KDa. On the other hand, two negative bands were detected in the Ushaar genotype from Shalateen at the molecular weights; 82.06 and 76.86 KDa, respectively. Identifiable polymorphic bands were detected from peroxidase, polyphenyl oxidase and Alcohol dehydrogenase analysis. However, these polymorphic markers clearly distinguished between the three genotypes from El Maghara, Shalateen and Sant Kathrin regions. The Similarity indices and dendrogram for the genetic distances of the combination between the SDS-PAGE protein and the three isozymes revealed that the highest similarity was 42.90 % between the Ushaar genotypes from Sant Kathrin and Shalateen regions. Meanwhile, the lowest similarity index was between the Ushaar

genotypes from El Maghara and Shalateen regions (28.60 %). The dendrogram resulting from the combination of the two techniques separated the three Ushaar genotypes into one cluster (including El Maghara and Sant Kathrin genotypes) with the Shalateen genotype alone.

INTRODUCTION

Ushaar (*Calotropis procera Ait (Ait) f.*) is one of the important medicinal plants growing wild in different regions in Egypt. The plant as a whole is purgative and anthelmintic. The root bark is used to treat dysentery, as a diaphoretic and as an expectorant. The flowers are bitter and considered to be digestive, stomachic, tonic and to cure inflammation. The latex is supposed to be used as an abortifacient (**El-Fiki** *et al.***, 2007**). Family Asclepiadaceae composed of about 2,000 species of flowering herbaceous plants or shrubby climbers in more than 280 genera. Most family members have milky juice, pod like fruits, and tufted silky-haired seeds that drift on wind currents to new locations for sprouting such as *Calotropis procera (Ait.) Ait.f.*

Ushaar, also is considered as a poisonous shrubby plant from the Asclepiadaceae family that is found in parts of the world with dry, sandy, alkaline soils and warm climates such as Saudi Arabia. A juicy latex is present throughout the plant and is secreted when the plant is cut. Ushaar latex was used as a drastic purgative agent by Hindu physicians in Vedic times. This substance has a caustic effect on mucous membranes and may cause a local anesthetic effect when accidentally splashed into the eye. Significant ocular injury after intracorneal penetration of Ushaar latex has not been reported. *Calotropis procera (Ait)f.* commonly known, as 'Arka' is a popular medicinal plant found throughout the tropics of Asia and Africa and is used in many traditional systems of medicine (**Sharma and Sharma 1999**) as a purgative, anthelmintic, anticoagulant, anticancer as well as. tntipyretic, analgesic arid antimicrobial

(**Jain** *et al* **1996**) and is used also as an antiseptic for skin infection (**Fleurentin and Pelt 1982**).

Protein analysis (SDS-PAGE), officially sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein folding, post translational modifications and other factors).

Isozyme analysis (NATVE- PAGE) offers a raid and more reliable means for producing genetic profiles (Fingerprints) and elucidation of genetic relationships within and different taxa. These techniques are efficient tools for genetic, systematic and breeding studies. (**Mukhlesur** *et al.*, **(2004).**

The present work aimed to identify three Ushaar genotypes (*Calotropis procera (Ait.) Ait f.*) using biochemical genetic fingerprints (Protein and Isozymes electrophoresis).

MATERIALS AND METHODS

The present investigation has been carried out at Genetics and Cytology Unit, Genetic Resources Department, Desert Research Center (DRC), Cairo, Egypt. Three genotypes of Ushaar (*Calotropis procera Ait (Ait) f.*) were used in the study**.** Leafs and seeds from each plant were collected from their nature habitats, **(**Table 1).

Table (1): Names and origins of the three Ushaar genotypes.

Scientific Name	**Family**	**Genotype**	**Origin**
Calotropis procera Ait (Ait) f.	Asclepiadaceae	Ushaar Ushaar Ushaar	El Maghara, Shalateen Sant Kathrine

Plant materials: Three different genotypes of Ushaar (*Calotropis procera Ait (Ait) f.)* were collected from El Maghara, Shalateen and Sant Kathrin and subjected to SDS-PAGE for protein and isozyme analysis.

The molecular determination of seed storage proteins was achieved using one-dimensional SDS-polyacrylamide gel electrophoresis.

Finely ground meals from freeze-dried cotyledons (5 mg of meal/ml of buffer) were extracted overnight with constant agitation at 4°C using 0.2 M Tris-HCL buffer pH 6.8 containing 2 % (w/v) sodium dodycyl sulfate and subsequently centrifuged at 9000 g for 5 min. A portion of the clear supernatant was subsequently taken for analysis.

SDS-polyacrylamide-gel electrophoresis was performed according to the method of **Laemmli** (**1970**). SDS-denatured bovine serum albumin (66,000), oval albumin (45,000), glyceraldyhide-3 phosphate dehydrogenase (36.000), carbonic anhydrase (29.000), trypsinogen (24.000) and soybean trypsin inhibitor (20.000) were used for the calibration curve (**El-Fiky** *et al*., **2002**).

Isozyme assay: Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations among studied plant using three isozyme systems according to **Stegemann** *et al*.**, 1985**.

For Peroxidase, Polyphenyl Oxidase and Alcohol Dehydrogenase, analysis 500 mg fresh leaves were collected in icebox and homogenized by liquid N_2 and was added 100 µl of 0.2 M Phosphate buffer (pH 7.0 was adjusted by Potassium Phosphate, monobasic) and 10 µl of 2-Mercaptoethanol before centrifugation at 14000 rpm for 15 min at 4°C temperature to clear the cellular debris. The supernatant was recovered and used directly for electrophoresis or stored at a temperature of -20°e. All the extractions were performed at a temperature of approximately 4°C and icebox. Peroxidase loci were detected by 0.044 M Phosphate-0.028 M Citric acid buffer (pH 4.4-4.6) and 0.026% (w/v) O-dianisidine and 1 % H_20_2 in an incubator at 37°C temperature until the bands developed sufficiently to permit scoring. The bands were fixed by 7% acetic acid after staining.

Peroxidase isozymes were detected on the gel using guiacol and hydrogen peroxide according to **Show and Prasad (1970).**

Polyphenol oxidase isozymes were detected according to **Baaziz** *et al*.,

(1994), in which the gel was immersed in a solution containing 0.1 % 1-dihydroxyphenyl alanine solubilized in 0.05 phosphate buffer pH 7.5.

Alcohol Dehydrogenase isozymes were localized on the gel using Tris-HCL, NAD, ethanol, NBT and PMS according to **Wendel and Weeden (1990)**.

Relative band mobility was measured in relation to the dye front and indicated by Rf values.

1. Genetic distance relationships:

The banding patterns of the SDS-PAGE for protein and isozymes were scored and data were feed to the PC computer as 1 and 0 for the presence and absence of bands, respectively. Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products, were used to regenerate similarity coefficients, according to **Jaccard (1908)**. The similarity coefficients were then used to construct dendrograms, using the unweighted pair group method with arithmetic averages (UPGAMA) employing the SHAN (Sequantial, Agglomerative, Hierachical, and Nested clustering) from the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.1 Program (**Rohlf, 2000**).

RESULTS AND DISCUSSION

The protein banding patterns based on SDS-PAGE for the studied wild plant (*Calotropis procera Ait (Ait) f.*) from El Maghara, shalateen and Sant Catherin regions, Tables (1). The total number of bands is 12, 50 % of them are polymorphic bands with molecular weights ranging from 68.05 to 230.40 KDa. The maximum number of bands was 10 which found in the Ushaar genotype from Sant Kathrin. The minimum number of bands was 8 which found in the Ushaar genotype from El-Maghara and Shalateen regions. Results revealed that Ushaar genotype from Shalateen has two positive bands at the molecular weights; 230.40 and 175.86 KDa which may used as markers for this genotype.

Moreover, the obtained results showed that the Ushaar genotype from Sant Catherin has two positive bands at the molecular weights; 168.33 and 148.68 KDa as a marker bands for this genotype. On the other hand, the bands number 10 and 11 with the molecular weights of 82.06 and 76.86 KDa were found in the Ushaar genotype from Shalateen region. Generally, there are 6 common positive bands with molecular weight of 212.62, 190.10, 127.55, 95.08, 88.37 and 68.05 KDa, respectively.

These results were found in agreement with the findings of **Mukhlesur** *et al*.**, (2004)**, **Vladova** *et al*.**, (2004), Ramos** *et al*.**, (2006)** and **Nair and Keshavandran (2006)** who used protein SDS-PAGE to study the genetic variability and the genetic relationship among the different species (thirty cultivars of *Brassica rapa* , *Cucurbitaceae*, *Calotropis procera* and 93 germplasm accessions of *Gymnema Sylvester* R.Br) collected from different natural habitats. The resulted profiles showed different patterns indicating variability among accessions of different habitats.

The protein SDS-PAGE data showed that the genetic similarity indices ranged from 00.00 % to 66.70 % (Table 3). The closet relationship was detected between the Ushaar genotypes from El-Maghara and Sant Kathrin (66.70 %). On the other hand, the farest relationship of similarity matrices was detected between the Ushaar genotypes from Shalateen and Sant Kathrin (00.00 %), followed by El-Maghara and Shalateen (33.30 %). As indicated by the Fig. 2, the dendrogram based on the similarity matrices of protein SDS-PAGE banding patterns separated the three Ushaar genotypes into one cluster (including El-Maghara and Sant Kathrin genotypes) and Shalateen alone.

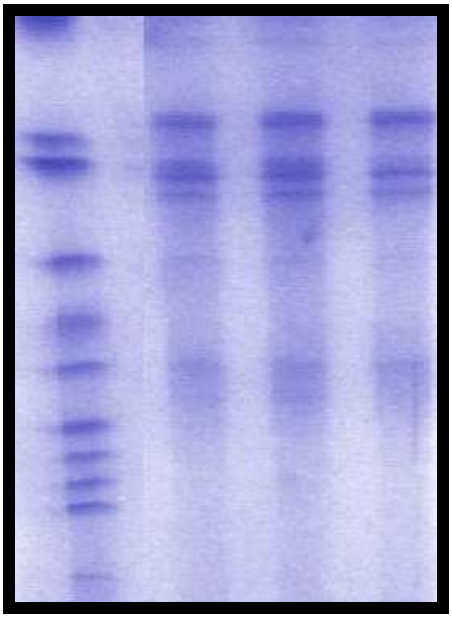


Fig. (1): SDS- PAGE protein banding patterns of *C. procera* Ait (Ait) f.

M: Marker.

1: Ushaar genotype from El Maghara**.**

2: Ushaar genotype from Shalateen

3: Ushaar genotype from Sant Catherin

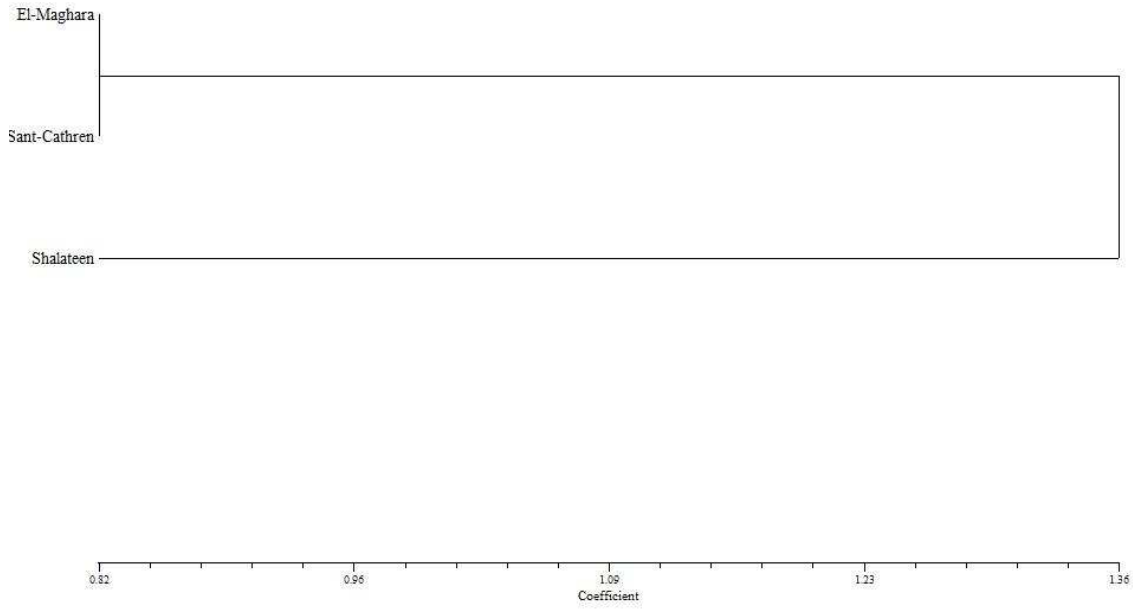
Table (2): Total protein banding patterns of the three Ushaar genotypes (*Calotropis procera* Ait (Ait) f.) as revealed by SDS- PAGE

Band No.	Molecular weight (KD_a)	Genotypes		
		Calotropis procera Ait (Ait) f.		
		El-Maghara	Shalateen	Sant Kathrine
1	230.40	0	1	0
2	212.62	1	1	1
3	190.10	1	1	1
4	175.86	0	1	0
5	168.33	0	0	1
6	148.-68	0	0	1
7	127.55	1	1	1
8	95.08	1	1	1
9	88.37	1	1	1
10	82.06	1	0	1
11	76.86	1	0	1
12	68.05	1	1	1
Total		8	8	10

Table (3): Similarity matrix among the three Ushaar genotypes based on SDS-PAGE protein data.

Genotype	El Maghara	Shalateen	Sant Kathrin
El Maghara	1		
Shalateen	0.333	1	
Sant Kathrin	0.667	0.000	1

Fig. 2: Dendrogram revealing the genetic distance among the three Ushaar genotypes using SDS-PAGE protein data.



In the present study, three isozyme systems (peroxidase**,** poly phenyl oxidase and alcohol Dehydrogenase) were investigated.

Peroxidase

The banding patterns of peroxidase isozyme are illustrated in Table (4) and Figure (3). A total of four bands are scored for the Ushaar genotypes. Results revealed that two negative bands were characterized in the Ushaar genotypes from El-Maghara as a marker bands at Rf 0.165 and 0.633, respectively.

Poly phenyl Oxidase

The banding patterns of poly phenyl Oxidase isozyme are found in Table (4) and Figure (4). A total of four bands are scored for the studied genotypes of Ushaar. The results showed that there were two characteristic positive bands (at Rf of 0.449 and 0.832**)** in the Ushaar genotypes from Shalateen**.** Two common bands were detected in three Ushaar genotypes at the Rf 0.511 and 0.523, respectively.

2.2. b. 3. Alcohole Dehydrogenase (ADH)

The banding patterns of Alcohol Dehydrogenase (ADH) isozyme are illustrated in Table (4) and Figure (5). A total of four bands are scored for the

studied genotypes of Ushaar. Alcohol Dehydrogenase isozyme showed polymorphism in a percentage of 75 %. The results revealed that the negative band at the Rf 0.198 is detected in the Ushaar genotype from El-Maghara. The band at the Rf. 0.225 is detected in both genotypes from El Maghara and Shalateen while being considered as a negative specific marker in the genotype from Sant Catherin. Moreover the band (at the Rf. 0.888) is detected in the Ushaar genotype from Sant Catherin only.

The isozyme systems data revealed that the genetic similarity indices ranged from 42.90 % to 28.60 % (Table 5). The closet relationship was detected between the Ushaar genotypes from Shalateen and Sant Catherin (42.90 %). On the other hand, the farest relationship of similarity matrices was detected between the Ushaar genotypes from El-Maghara and Shalateen and El-Maghara and Sant Catherin with the same percent (28.60 %), respectively. As showed by the Fig. 6, the dendrogram based on the similarity matrices of isozyme banding patterns classified the three Ushaar genotypes into one cluster (including El-Maghara and Shalateen genotypes) and Sant Kathrin alone.

Depending on the combination between the banding patterns of protein SDS-PAGE and isozyme data through using NTsyspc2 software, the similarity indices were 46.20 % between the Ushaar genotype from El Maghara and Sant Catherin regions, followed by 30.08 % between the Ushaar genotype from El-Maghara and Shalateen. The lowest similarity indices were detected between the Ushaar genotypes from Shalateen and Sant Katherin (23.10 %). The dendrogram resulted from the combination of the two techniques (Fig. 7) was revealed that only one cluster (including the Ushaar genotype from El Maghara and Sant Catherin regions) while the Ushaar genotype from Shalateen detected alone.

Table (4): Distribution of Peroxidase, Poly phenyl Oxidase and Alcohol Dehydrogenase Isozymes of three Ushaar genotypes according to their relative mobility.

Band No.	RF	Genotypes		
		Calotropis procera Ait (Ait) f.		
		El Maghara	Shalateen	Sant Kathrine
Peroxidase				
1	0.165	0	1^{++}	1^{+}
2	0.317	1^{+}	1^{++}	1^{-}
3	0.487	1^{+}	1^{-}	1^{-}
4	0.633	0	1^{++}	1^{+}
Total		2	4	4
Poly phenyl Oxidase				
1	0.449	0	1^{-}	0
2	0.511	1^{-}	1^{-}	1^{-}
3	0.523	1^{+}	1^{+}	1^{+}
4	0.832	0	1^{+}	0
Total		2	4	2
Alcohol Dehydrogenase				
1	0.198	0	1^{++}	1^{++}
2	0.225	1^{-}	1^{++}	0
3	0.339	1^{-}	1^{++}	1^{+}
4	0.888	0	0	1^{+}
Total		2	3	3

(-) Low density **(+)** Moderate Density **(++)** High Density

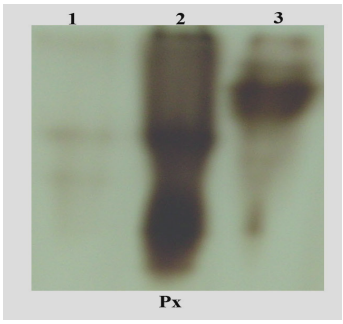


Fig (3): Zymogram of Peroxidase banding pattern for the three Ushaar genotypes from three different locations.

1: Ushaar genotype from El Maghara**.** **2:** Ushaar genotype from Shalateen

3: Ushaar genotype from Sant Catherin

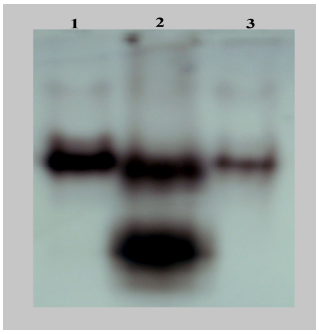


Fig (4): Zymogram of Poly phenyl Oxidase banding pattern for the three Ushaar genotypes from three different locations.

1: Ushaar genotype from El Maghara**.** **2:** Ushaar genotype from Shalateen

3: Ushaar genotype from Sant Catherin

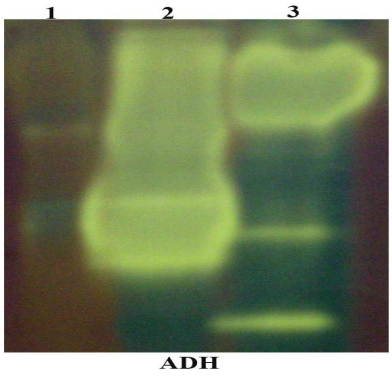


Fig (5): Zymogram of Alcohol Dehydrogenase banding pattern for the three Ushaar genotypes from three different locations.

1: Ushaar genotype from El Maghara**.** **2:** Ushaar genotype from Shalateen

3: Ushaar genotype from Sant Catherin

Table (5): Similarity matrix among the three Ushaar genotypes based on Isozymes data.

Genotype	El Maghara	Shalateen	Sant Kathrine
El Maghara	1		
Shalateen	0.286	1	
Sant Kathrine	0.286	0.429	1

Fig. 6: Dendrogram revealing the genetic distance for the three Ushaar genotypes using three enzyme systems analysis.

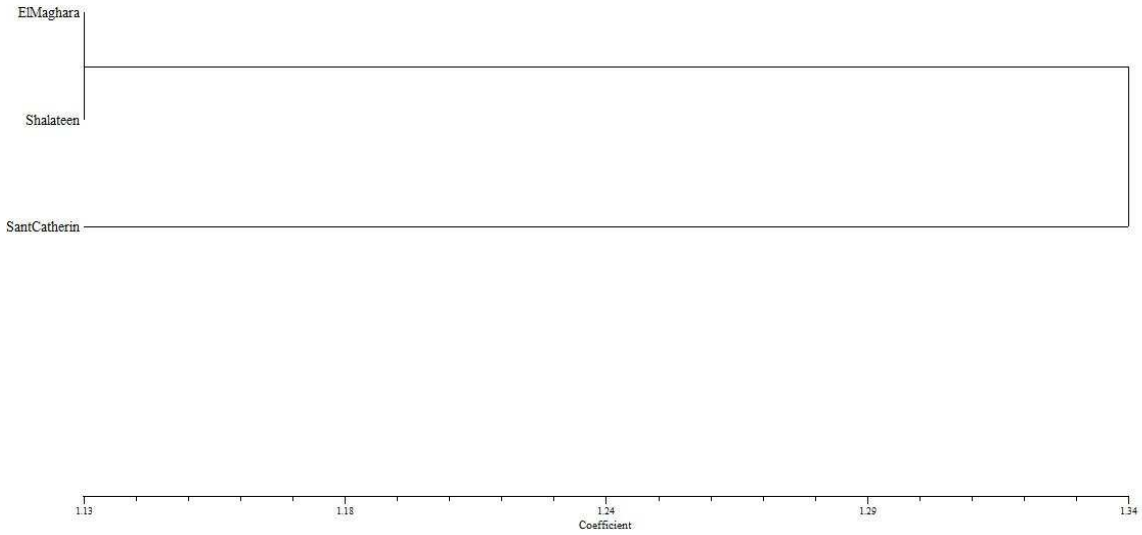


Fig. 7: Dendrogram showing the genetic distance between three Ushaar genotypes using SDS protein and isozymes analysis.

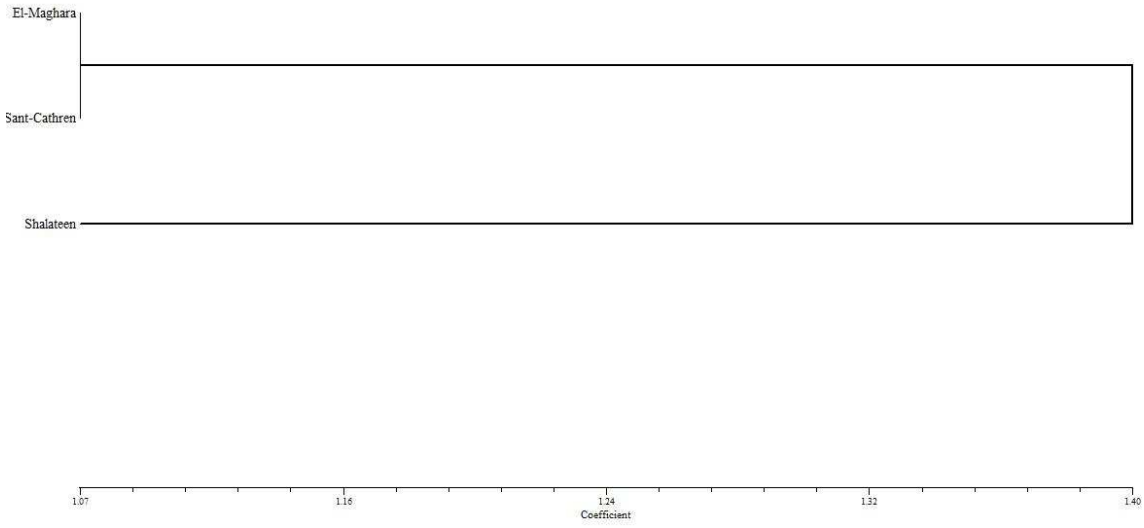


Table (6): Similarity matrix among the three Ushaar genotypes based on combination analysis of SDS-PAGE protein and isozymes data.

Genotype	El Maghara	Shalateen	Sant Kathrin
El Maghara	1		
Shalateen	0.308	1	
Sant Kathrin	0.462	0.231	1

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التباين الوراثى داخل التراكيب الوراثية لنبات العشار بإستخدام تحليل التفريد الكهربى للبروتين و المشابهات الانزيمية

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تم تحديد البصمة الوراثية لثلاثة تراكيب وراثية من ثلاثة مناطق مختلفة (المغارة، شلاتين و سانت كاترين) لنبات العشار *Calotropis procera Ait (Ait) f.*. تم أستخلاص البروتين و كذلك مشابهات ثلاثة إنزيمات (البيروكسيديز، البولى فينايل اوكسيديز و الكحول دى هيدروجينيز). و قد أستخدمت طريقة التفريد الكهربى بإستخدام ال PAGE لتحديد البصمة الوراثية لكل من البروتين و مشابهات الانزيمات. بالنسبة للتفريد الكهربى للبروتين، أظهرت النتائج أن عدد الحزم الكلية لنوع البروتين كانت اثنى عشر حزمة منها ستة حزم بنسبة ٥٠ % متباينة الصور. أوضحت النتائج ان التركيب الوراثى للعشار من شلاتين يحتوى على حزمتان موجبتان عند الاوزان الجزيئية ٢٣٠,٤٠ و ١٧٥,٨٦ كيلو دالتون. إضافة الى ذلك كانت هناك حزمتان موجبتان عند الاوزان الجزيئية ١٦٨,٣٣ و ١٤٨,٦٨ كيلو دالتون و ذلك فى التركيب الوراثى من منطقة سانت كاترين. كما كانت هناك حزمتان سالبتان عند الاوزان الجزيئية ٨٢,٠٦ و ٧٦,٨٦ كيلو دالتون و ذلك فى التركيب الوراثى من منطقة شلاتين.
بينت النتائج عند دراسة نشاط مشابهات الانزيمات فى الثلاثة تراكيب وراثية لنبات العشار ان هناك اختلافات فى بعض الحزم فى حالة كل من البيروكسيديز، البولى فينايل اوكسيديز و الكحول دى هيدروجينيز أمكن من خلالها تمييز هذه التراكيب الوراثية فى الثلاثة مناطق. بينت نتائج القرابة الوراثية والشجرة الوراثية بالنسبة للمسافات الوراثية من التحليل المشترك لكل من الSDS-PAGE و الIsozymes أن أعلى نسبة تشابه كانت ٤٢,٩٠ % بين التراكيب الوراثية لنبات العشارمن مناطق سانت كاترين و شلاتين. بينما كانت أقل نسبة تشابه ٢٨،٦٠ % بين التراكيب الوراثية من مناطق المغارة و شلاتين و المغارة و سانت كاترين. أوضحت نتائج الشجرة الوراثية الناتجة من التحليل المشترك بين الSDS-PAGE و الIsozymes حدوث انفصال الى عنقود واحد يضم كل من التراكيب الوراثية من مناطق المغارة و سانت كاترين و وجود التركيب الوراثى من منطقة شلاتين منفردا.