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Research Article

BOTANICAL STUDY, DNA FINGERPRINTING, TOTAL PROTEIN PROFILING, NUTRITIONAL VALUES AND CERTAIN PROXIMATES OF *V. ERVILIA* L.

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ABSTRACT

Viciaervilia L. Willd. (syn. *Erviumervilia* L.), Family Fabaceae, seeds are extensively usedas stock feedin several countries. The plant grows very rarely wild in Egypt. Macro- and micro-morphological characters of the seeds were presented for their identification in the entire or powdered forms.Plant material were fixed, freehand sectioned and stained with Safranin. DNA fingerprinting of the leaves gave a total of 32 different fragments.Seventeen bands were recorded in seed total protein banding profile with molecular weights ranged from 9 to175KDa. Leucin was the main essential amino acid (3.417 g/100 g seeds), while glutamic acid (5.856 g/100 g seeds) was the main non-essential amino acid using amino acid analyzer.Pharmacopoeial constants: moisture 8.4%, carbohydrates 26.76% and ash 3.68% were determined. Potassium and phosphorous occupied the highest positions (1518 and 1220 mg/100 g seeds respectively) among the macro elements, whereas the micro element Fe level was 77 mg/100 g seeds. The above data are valuable for identification and purification of *V. ervilia* L.

Keywords: *Vicia;* Macro- and micro-morphology*;* Nutritive-value; Amino acids; Macro and micro elements; Proximate analysis.

INTRODUCTION

Viciaervilia L. (≡ *Ervumervilia* L.) belongs to family Fabaceae. It is commonly known as bitter vetch, in Persian *gavdaneh*, in Arabic *kersannah*, in Spanish *yero*, in Greek *rovi*, and in Turkish *burçak*. It is an ancient grain legume crop of the Mediterranean region. The nutritional value of the seed for ruminant production has guaranteed the continued cultivation of *V. ervilia* in Morocco, Spain and Turkey. [1 and 2]

The wild strains of bitter vetch are present in areas that includes Anatolia and Northern Iraq, with an extension south along the Anti-Lebanon Mountains of Syria and Lebanon [3].It is very rare in the Egyptian flora [2] and is not found in the Egyptian market.

Due to the widespread use of *V. ervilia* L. seeds in the Arabian (Jordan and Saudia Arabia) markets, and the presence of closely related *Vicia* species, and the well-known importance of the seed as stock feed [4-7], it was necessary to introduce the seed to Egypt, confirm its identity and make a trial for its cultivation.

Many reports were traced in the available literature concerning the botanical characters of *V. ervilia* L. root, stem, leaf, aerial vegetative parts and flower [8-11]. A botanical study was traced on *V. ervilia* L. seed coat papillae using scanning electron microscopy [12]. Therefore a detailed study of the macro- and micromorphological features of the seeds was performed to aid in its identification and differentiation in the entire and powdered forms. Further identification was achieved by a DNA and total seed protein profiling. Certain pharmacopoeial constants, macro, microelements and amino acid content of the seeds were also determined.

MATERIALS AND METHODS

Material for botanical study

Samples of *V. ervilia* L. Willd. (≡*Erviumervilia* L.), family Fabaceae, seeds imported from Jordan in July 2010, were cultivated in the Experimental Station of Medicinal Plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza. Photos of the cultivated flowering aerial parts together with the root, pods, and seeds were sent to Kew Garden, England to confirm the identity. Voucher samples of the cultivated flowering fruiting plant(herbarium no. 14.4.2013.2) were deposited at the Museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University. Imported seeds were used for macro and micromorphological studies.

Material for molecular investigation

Samples of fresh leaves were obtained in spring 2011 for DNA fingerprinting. They were stored at -70°C, freeze-dried and ground to a fine powder prior to DNA isolation.

Buffers and agarose gel

Extraction buffer and agarose gel were prepared according to [13].

Primers: Five primers were used for randomly amplified polymorphic DNA (RAPD) analysis obtained from Operon Technologies Inc., Almeda, California, USA with the following sequence: A-10: 5'-GTGATCGCAG-3'; A-17: 5'-GACCGCTTGT-3'; A-18: 5'-AGGTGACCGT-3'; A-19: 5'-CAAACGTCGG-3'; G-18: 5'-GGCTCATGTG-3'.

Molecular weight marker: 100 bp ladder, Promega Corporation, Madisson, USA.

Apparatus: DNA thermocycler (Hybaid PCR Express) used for amplification of DNA, agarose gel electrophoresis tool (Biorad Wide Mini Sub Cell) used for separation of RAPD fragments according to size and ultraviolet Polaroid camera used for visualization of RAPD fragments.

Material for seed protein profiling

A- Solution for gel preparation:

1- Acrylamide/Bisacrylamide: 30% stock solution.

2- Stacking gel buffer: Tris-HCl solution (pH 6.6)

3- Separating gel: Tris-HCl solution (pH 8.8)

4- Running buffer: Tris base, glycine and sodium dodecyl sulphate in distilled water

5-Sample buffer: Tris borate solution (pH 8.2)

6-Staining solution: 0.1 g coomassie blue powder in a solution composed of 400 ml distilled water, 70 ml acetic acid, 200 ml ethanol and 60 ml trichloroacetic acid

7- Destaining solution: 15 ml ethanol, 50 ml glacial acetic acid and 300 ml distilled water

B- Protein molecular weight marker: A promega mixture (Heidelberg, Germany) containing nine proteins with the indicated molecular weights Mwt= 9, 13, 19, 26, 39, 52, 80, 125 and 175 KDa

Material for determination of certain pharmacopoeial constants and the macro and micro elements content

Imported seeds were used in this study. They were powdered and packed in dark-colored, tightly closed containers, kept in a cool place, for determination of certain pharmacopoeial constants[14] and macro and micro elements [15].

Method for botanical study

Seeds were separately imbedded in paraffin. Serial transverse sections, 10 -15 µm thick, were performed with a manual microtome and stained with Safranin and Fast Green [16]. Sections were mounted in synthetic balsam. Transverse sections and powder were examined by light microscope. Dimensions of different elements were determined using Reichert Austria Mikrometer 2 mm langGeteilt in 200 Teile C. Reichert Wien.

Methods for molecular investigations

DNA extraction and quantification, amplification of RAPD markers and analysis of RAPD data were performed according to [13]

Methods for protein extraction

Seed protein was analyzed using continuous polyacrylamide gel electrophoresis in vertical slab apparatus in the presence of sodium dodecyl sulfate (SDS-PAGE). A 0.1 g of the mature seed was powdered and mixed with 1 ml sample buffer. The slurry was centrifuged at 6000 rpm for 10 min. The supernatant was used immediately for electrophoresis.

Gel preparation

The gel used for separation was prepared and poured between the glass plates immediately after adding ammonium persulfate. After polymerization of the gel (11 cm), the stacking gel (3 cm) was poured into the glass plates and then comb was inserted to form sample wells.

Gel electrophoresis [17]

1-Sample preparation:
Protein extracts were diluted with sample buffer (1:3 v/v), then 500 µl of 10% SDS were added followed by 25 µl of mercatoethanol. They were then placed in a boiling water bath for 5 min and 5 µl of bromophenol was added as a tracking dye. Ten µl of the sample was loaded and 10 µl of marker protein mixture was used as standard.

2-Running conditions

Runs were carried out at a constant voltage of 200 Volt. Usual runs took approximately 120 min.

3-Gel staining and destaining
The chromatograms were stained with excess of coomassie brilliant blue stain R-250 for about one hr. After gel staining, the gel was transferred to destaining solution to remove excess stain.

Methods for determination of certain pharmacopoeial constants

The moisture, ash and crude fiber were determined in the seeds according to the Egyptian Pharmacopoeia [14].

Determination of the macro and micro elements

The percentage of sodium (Na), phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg) and iron (Fe) were determined in the seeds according to [15].

Methods for amino acids analysis

The total protein was extracted from the seeds using 50mM Tris-HCl PH (7.5). Acid hydrolysis of the protein was carried out [18]. One ml 6N HCI (HCI Suprapure® from Merck) was mixed with one mg protein in a hydrolysis tube. The solution was frozen in a mixture of dry ice/ethanol in a test tube. The tube was evacuated and sealed. The sealed tube was placed in an oven at 110 °C for 72 hours for hydrolysis, and then cooled down. The solution was centrifuged. The supernatant was evaporated at 40°C under vacuum in a rotary evaporator. The residue was then dissolved in a diluting buffer.

Apparatus and conditions

Eppendr of – Germany. LC3000 Amino acid analyzer

Conditions: flow rate: 0.2 ml/min, pressure of buffer from 0 to 50 bar, pressure of reagent from 0 to 150 bar, reaction temperature 123 °C

RESULTS AND DISCUSSION

Cultivation of *V. ervilia* L. imported seeds in Egypt gave an herb (Figure 1) that flowers in February and begins fruiting in March. The herb can grow to a height of 0.7 m and up to 0.9 m wide. Seeds were examined macro- and micro morphologically and were presented in (Figures 3-5). Dimensions in microns of the different elements were recorded (Table 1). Numerical values [19] of the leaves were recorded (Table 2).

Macro-morphology

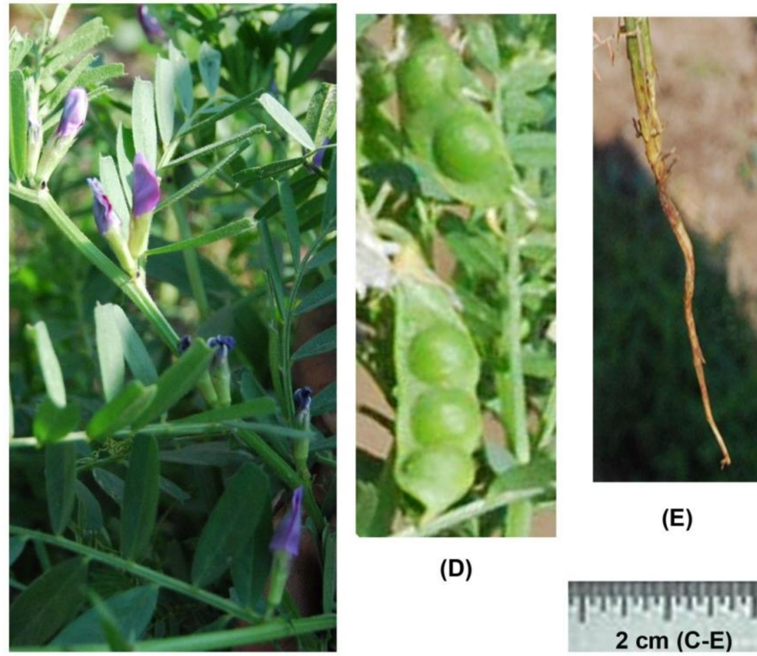
The seed: (Figure 2)

They are brown or creamy with darker brown or gray spots on the surface. The surface is glossy and glabrous. The seeds are angular or pyramidal in shape, measuring 2-5 mm w, 3-6 mm l and 2-4 mm thickness (Figure 2A, 2B). Weight of 100 seeds is 4.67g.

The hilum appears as dark scar beside the micropyle in a small depression on one of the seed corners. The seed is derived from anatropous ovule, with a creamy-orangeaccumbent embryo,with the radicle directed towards the micropyle. The seed consists of a seed coat and thin endosperm that surrounds a large, a well-developed central embryo. The embryo completely fills the seed and consists of two plano-convex cotyledons (Figure 2C). The seeds have a slight characteristic odor when crushed and a bitter taste.

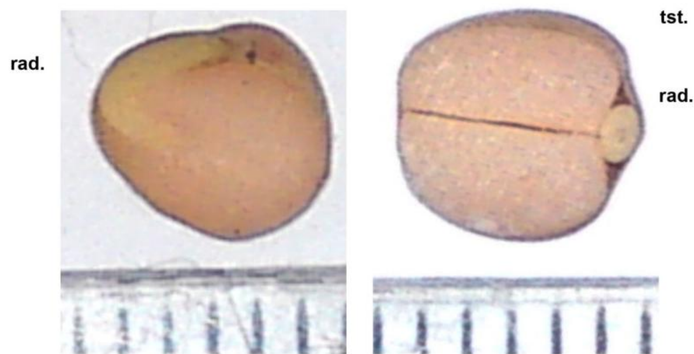
(A)

(B)



A. The herb, B-C. Flowering leafy branches, D. The pod, E. The root

Fig. 1:*V. ervilia* L. entire plant



A. Entire seeds, B. Longitudinal cut, C. Transverse cut. cot., cotyledon; h., hilum; rad., radicle; tst., testa.

Fig. 2: Macro-morphology of *V. ervilia* L. seed

Micro-morphology

The seed: (Figure3)

A transverse section in the seed coat (Figure 3A) is differentiated into palisade like epidermis and bearer hypodermis. The epidermis is formed of closely packed palisade-like cells which are radially elongated without intercellular spaces. The lumen is narrow and the walls are thick cellulosic lamellate. The epidermal cells are covered by thin cuticle and there is a light line that runs across this layer which is parallel to the seed surface very close to the cuticle.

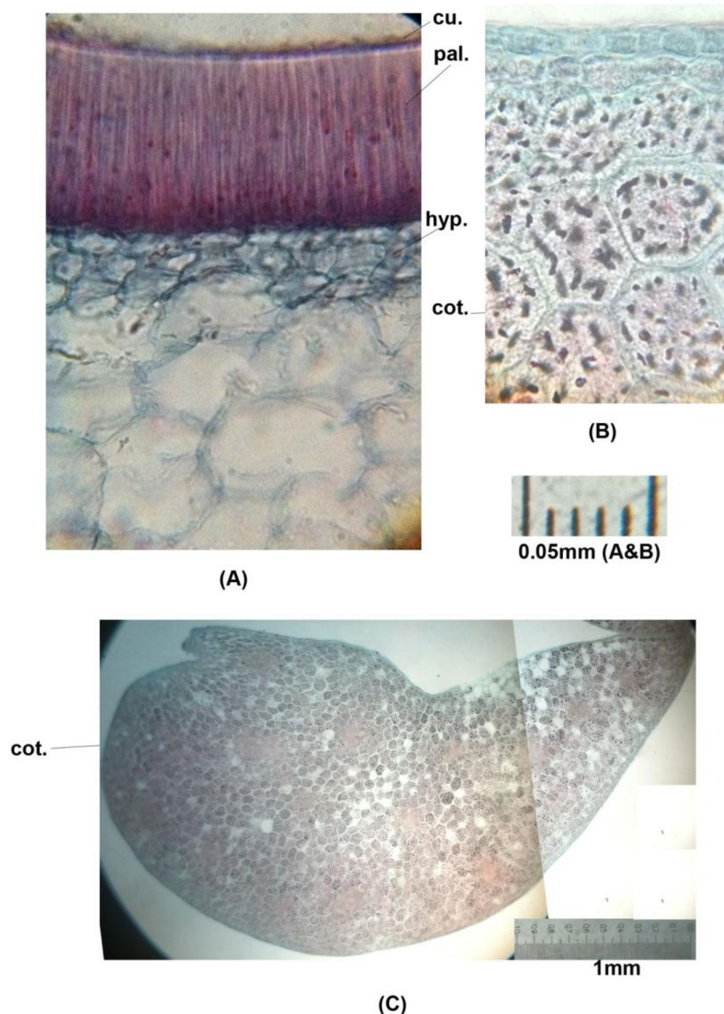
This is followed by a bearer layer (hypodermis), consisting of one row of radially elongated cells with considerably narrow intercellular spaces resembling the tibia bone (referred as bone-shaped cells or hourglass cells). The cell lumen is narrow in the middle parts and then wider at both extremities due to the uneven cellulosic thickening as shown in (Figure 3A). Following this bearer layer there is a narrow layer consisting of 2-5 rows of parenchymatous cells with thin cellulosic walls forming the endosperm.

The cotyledon (Figure 4B, C) is semicircular in shape. It is bounded by a typical epidermis of cubical shaped thin walled cells that are filled with aleurone grains and starch granules. The reminder of the cotyledon is largely composed of thin walled cellulosic parenchyma cells which are polyhedral in shape, radially elongated and filled with aleurone grains and starch granules. These cells are smaller and isodiametric near the cotyledon epidermis and larger and radially elongated towards the middle of the cotyledons.

The powdered seed: (Figure 4)

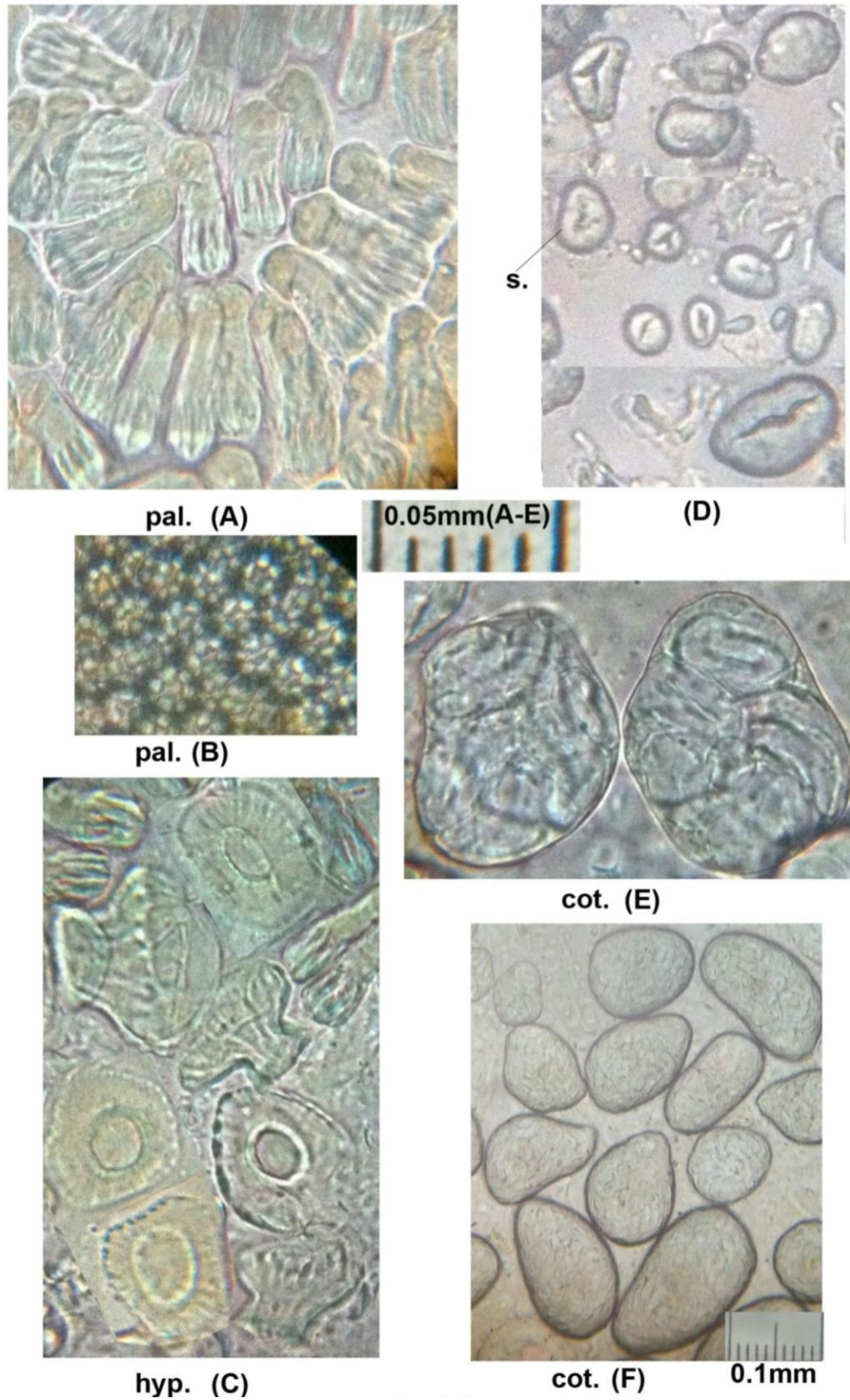
Powdered seeds are creamy orange in color with brown fragments, a characteristic odor and a bitter taste. It is microscopicallycharacterized by the presence of

1. Fragments of epidermal cells that are radially elongated (polygonal from surface view) with thick cellulosic lamellate walls, and covered with a smooth cuticle.
2. Fragments of the hypodermis with hour glass or the capital letter "I" shape with bar like thickening from surface and side view.
3. Simple large starch granules, mostly oval or angular in shape with central fissured hilum.
4. Fragments of cotyledon in which the cells are thin walled polyhedral parenchyma; some are isodiametric and others are elongated and filled with aleurone grains and starch granules.



A. Seed coat, B. First rows of the cotyledon, C. One cotyledon.cot., cotyledon; cu., cuticle; hyp., hypodermis; pal., palisade like epidermis.

Fig. 3: Transverse sections in*V. ervilia* L. seed



A.palisade like epidermis (side view), B.palisade like epidermis (surface view), C.hypodermis (side and surface views), D.starch granules, E.cotyledon, F.cotyledon (zoomed out).cot., cotyledon; hyp., hypodermis; pal., palisade like epidermis; s., starch.

Fig. 4: *V. ervilia* L. powdered seed

Table 1: Dimensions of different elements of *V. ervilia* L. seed

Parameter measured	**size (min-max) µm**		
	L	**W or D**	**H**
Palisade like epidermis		9, 13,16	34,50,78
Hypodermis		32,43,55	24,35,50
Cotyledon parenchyma	61,163,196	46,72,108	
Starch granules	8,27,41	7,17,25	

Analysis of RAPD data

The banding profile produced by five decamer primers used in RAPD analysis of *V. ervilia* L. is illustrated in Figure 5.

The RAPD electrophoretic profile of the DNA sample amplified with the five decamer primers showed distinguishable bands and generated 32 fragment patterns. The distribution of these bands is illustrated in Table 2.

The DNA was amplified using five decamer primers to reveal RAPD fragments. Each of the five primers successfully directed the amplification of a genom-specific fingerprint of DNA fragment; all amplifications were found to be prolific.

A total of 32 different fragments have been recorded, produced mainly by two of the five primers showing 9 bands by primers A-10 and G-18 ranging from 1.428 Kbp to 0.575 Kbp and from 1.315 Kbp to 0.314 Kbp respectively, while primers A-17 and A-19 produced only 4 bands.

Table 2: Molecular size in base pairs of amplified DNA fragments produced by five decamer primers in *V. ervilia* L.

	Primer				
	A10	**G18**	**A17**	**A18**	**A19**
Molecular size (bp)	1428	1315	1200	1390	1245
	1352	989	998	1245	989
	1245	902	793	1074	902
	1178	786	461	862	497
	1115	691		823	
	1017	575		698	
	919	445			
	717	377			
	575	314			
Total bands	9	9	4	6	4

A-10: 5'-GTGATCGCAG-3'; A-17: 5'-GACCGCTTGT-3'; A-18: 5'-AGGTGACCGT-3'; A-19: 5'-CAAACGTCGG-3'; b.p., base pair; G-18: 5'-GGCTCATGTG-3'.

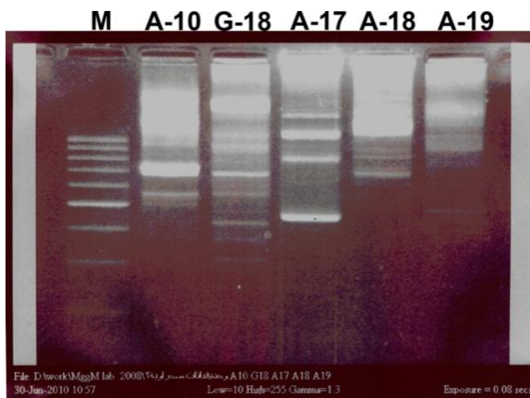


Fig. 5: The RAPD fingerprint of *V. ervilia* L. generated by the five decamer primers

A-10: 5'-GTGATCGCAG-3'; A-17: 5'-GACCGCTTGT-3'; A-18: 5'-AGGTGACCGT-3'; A-19: 5'-CAAACGTCGG-3'; b.p., base pair; G-18: 5'-GGCTCATGTG-3'; M: standard DNA molecular size marker.

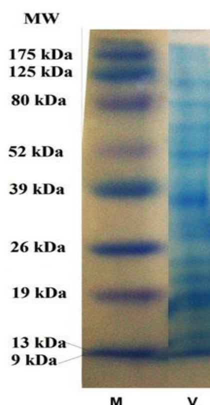


Fig. 6: Photograph of polyacrylamide gel illustrating electrophoretic band profiles of total protein extracted from *V. ervilia* L. seeds.

M, molecular weight marker; V, *Viciaervilia* L. protein extract

Total seed protein profiling

Total protein banding profile of *V. ervilia* L. seed is illustrated in Figure 6. Seventeen bands were recorded with molecular weights ranging from 9 to175KDa.*V. ervilia* L. seed can be clearly identifiable from its protein banding pattern and this is in accordance with several researches that have confirmed the usefulness of different SDS-PAGE procedures in plant taxonomic, evolutionary and genetic relationship studies [20-21].

Total protein profile of *V. ervilia* L. seed under investigation (Figure 6) has nearly equal amounts of three categories of polypeptide high, moderate and low molecular weight. This is in agreement with the findings of Valizadeh, 2001[22].

Pharmacopoeial constants and macro and micro elements

The moisture, carbohydrates and ash were determined in *V. ervilia* L. seed. The percentages were 8.4, 26.76 and 3.68 % respectively as shown in Table 3.

It is noteworthy to mention that crude fiber content of *V. ervilia* L under investigation is lower than thatestimated byTabatabaei and colleagues, 2000 [23] who estimated it by 7.75% in the same seed species grown in Iran. The estimated ash value in each sample was approximately the same, 3.68% in seeds under investigation and 3.97 % in Iranian sample(Table 4). These differences may be attributed to environmental conditions.

Evaluation of macro elements (Ca, Na, K and P); and microelement (Fe) revealed that potassium (K) occupied the highest position (1518 mg/100 g seeds) among the macroelements; followed by phosphorous (P) (1220 mg/100 g seeds) while calcium (Ca) was (206 mg/100 g seeds) and sodium (Na) was (56 mg/100 g seeds). Meanwhile, the microelement Iron (Fe) level was (77 mg/l00 g seeds). Detection of these elements in this seed adds to its veterinary dietary importance.

Moreover, a considerable amount of iron (77 mg/100 g seeds), the element which plays essential role in the treatment of iron deficiency anemia, the most common nutritional deficiency disorder in Egypt [24] was detected in *V. ervilia* L. seeds. Nutritional anemia occur when one or more of the nutrients that are necessary for red blood cells production are deficient; shortage of iron, vitamin B12 and folic acid are the most common causes of nutritional anemia [25].

Macro and micro elements in this study had important relation to the potential use of *V. ervilia*L. in medicine, if any. Calcium, magnesium and phosphorus contribute to prevention of osteoporosis and are needed for tooth formation and cell growth. Sodium and potassium, together with calcium are crucial for muscle

contractions. Besides, the two former elements help in maintenance of the normal heart rhythm and body water balance [26].

Elimination of anti-nutritional factors [27-28] of seeds is required before use for nutritional or medicinal purposes.

Table 3: Nutrients and macro and micro elements in *V. ervilia.* L. seeds

Nutrient	% (g/100g)	Elements	mg%
Moisture	8.4	P	1220
Total Carbohydrates	26.76	Ca	206
Fiber	5.38	K	1518
Ash	3.68	Na	56
		Fe	77

*Average of three determinations

Table 4: Comparison between certain proximates in the seed of *V. ervilia* L. under investigation and those in previous reports

	% "g/100g DM"			
Fiber	5.38	7.75	3.38	4.06
Ash	3.68	Nd	3.38	3.96
Crude Protein	Nd	22.21	26.56	28.52
Reference	seed under investigation	[23]	[7]	[29]
Geographical origin	Jordan	Iran	Iran	

Nd: not determined. DM: Dry matter

Amino acid content

Aminoacid profile is very close to soybean meal.Partial or complete substitution of soybean meal with bitter vetch grain (*V. ervilia* L.) did not affect nutrient intake or growth performance of Awassi lambs and resulted in a decrease in feed cost by more than 9% [6-7].

From table [5] it could be concluded that the total percentage of the amino acids is 31.606 g/100 g seeds. The main essential amino acid was leucine (3.417 g/100 g seeds). This was followed by phenylalanine (2.845 g/100 g seeds) and lysine (2.324 g/100 g seeds). The lowest percentage of the essential amino acids was methionine (0.236g/100g seeds) followed by threonine, isoleucine, valine and tyrosine (1.002 9/100 g seeds, 1.132 g/100 g seeds, 1.552 g/100 g seeds and 1.552 g/100 g seeds, respectively).

Glutamic acid (5.856 g/100 g seeds) was the main non-essential amino acid followed by aspartic acid and alanine (3.002 g/100 g seeds and 2.537 g/100 g seeds respectively). The lowest percentage of the non-essential amino acids was both glycine and proline (0.749 g/100 g seeds and 0.899 g/100 g seeds); followed by histidine, arginine and serine (1.37 g/100 g seeds, 1.507 g/100 g seeds and 1.626 g/100 g seeds respectively).

Leucine protects muscles and acts as fuel. It promotes healing of bones, skin, and muscle tissue, and is recommended for those recovering from surgery. It also lowers elevated blood sugar levels. Glutamic acid is important in the metabolism of sugars and fats and is used in treatment of ulcers [30]. Elimination of anti-nutritional factors [27-28] of seeds is required before use for nutritional or medicinal purposes.

The amino acid profile (Table 5) adds to chemo taxonomical value for the identification of the seeds of *V. ervilia* L.

Table 5: Percentage of amino acids in *V. ervilia* L. seeds

Amino Acid		g/100g	Daily dietary allowance of the amino acids (g /Kg of body weight) [31]		
			Infant	**Child (10 to 12 years)**	**Adult**
Essential amino acids	**Isoleucine**	1.132	0.083	0.028	0.012
	Leucine	3.417	0.135	0.042	0.016
	Lysine	2.324	0.099	0.044	0.012
	Methionine	0.236	0.049**	0.022**	0.010**
	Phenyl alanine	2.845	0.141***	0.022***	0.016***
	Threonine	1.002	0.068	0.028	0.008
	Tyrosine	1.552	0.021	0.004	0.003
	Valine	1.552	0.092	0.025	0.014
Non-essential amino acids	**Arginine**	1.507			
	Proline	0.899			
	Serine	1.626			
	Glutamic acid	5.856			
	Glycine	0.749			
	Alanine	2.537			
	Aspartic	3.002			
	Histidine	1.37			
Ammonium ion		4.448			
Total essential amino acids		14.06			
Total non-essential aminoacids		17.546			
Total determined amino acids		31.606			
Total determined amino acids and ammonia		36.054			

*Methionine+Cystine

**Phenyl alanine+Tyrosine

CONCLUSION

Botanical study appears to be an excellent method for identification of *V. ervilia* L. seeds. It is easy, rapid, sensitive, inexpensive and adaptable to simple laboratory conditions. The analysis of RAPD data can select the use of primersA-10 and G-18 for selective discrimination. The SDS-PAGE procedure using total protein samples is not suitable to detect the seed storage protein polymorphism within varieties or within populations of legumes. This is because all varieties or cultivars related to same species showed identical number of bands with similar mobility [22]. So our SDS-PAGE results gave fingerprint profile for the studied species, without being able to define the accession of *V. ervilia* L. Specific types of protein extracts (glutellins, albumins and isozymes) are required to differentiate between accessions of *V. ervilia* L., not the total protein extract [22]. Nutritional values and certain proximates determined can be used to confirm the identity of the plant.

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