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Biochemical and Genetic Characterization of 12 *Silybum marianum* Accessions Collected from Borg El-Arab, an Egyptian Habitat

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ABSTRACT

Silybum marianum, known as milk thistle (MT), a plant that grows throughout the Mediterranean, southern Europe and parts of the US, has been used for some 2000 years as both food and medicine. The aims of this study were to determine the morphological, biochemical (protein profile and isozyme) and molecular (RAPD- and ISSR-PCR) characteristics of MT and to identify and quantify its active ingredients. Twelve accessions of MT collected from Borg El-Arab, an Egyptian habitat located at the north/west coast, were tested. SDS-PAGE electrophoresis indicated that two accessions cultivated in location 3 had specific bands. Data analysis of isozyme banding patterns showed different migration rates for four isozymes (α- and β-esterase, peroxidase and acid phosphatase). These isozymes, belonging to different *Silybum* accessions found in the locations, may represent a degree of genetic variation. RAPD-PCR fingerprinting resulted in 83 amplicons (DNA bands), 33 of which were polymorphic. Furthermore, 10 accessions proved to have specific molecular markers. ISSR-PCR fingerprinting resulted in 40 amplicons, 16 of which were polymorphic. Six accessions-specific markers were found for MT accessions. The estimation of genetic distance based on SDS-PAGE, isozymes, RAPD-PCR and ISSR-PCR ranged from 85 to 95%.

Keywords: fingerprinting, medicinal plants, PCR, SDS-PAGE
Abbreviations: IRAP, inter-retrotransposon amplified polymorphism; **ISSR**, inter simple sequence repeat; **LDL**, low density lipoprotein; **MT**, milk thistle; **PAGE**; polyacrylamide gel electrophoresis; **PCR**, polymerase chain reaction; **RAPD**, random amplified polymorphic DNA; **SDS**, sodium dodecyl-sulphate

INTRODUCTION

Silybum marianum, a plant that grows throughout the Mediterranean, southern Europe and parts of the US, has been used for some 2000 years as both food and medicine. *Silybum* species are members of the family *Compositae/ Asteraceae* which has many folklore names, e.g., holy thistle, marian thistle, Mary thistle, milk thistle (MT), Our Lady's thistle, St. Marythistle, wild artichoke, or Mariendistel. The active chemical component of MT is silymarin, which is a combination of three flavonoids: 50% silybinin, 25% silychristin and 25% silydianin (Ball *et al*. 2005).

Silymarin is widely used as a hepatoprotectant and as a supportive therapy of liver disorders such as cirrhosis, hepatitis and fatty acid infiltration due to alcohol and toxic chemicals (Ball *et al*. 2005; Kren and Walterova 2005). Silymarin also shows beneficial effects in radiation injury to the membrane of liver cells (Ramadan *et al*. 2002). Silymarin is capable of protecting liver cells directly by stabilizing the membrane permeability by inhibiting lipid peroxidation and preventing liver glutathione depletion (Skottova *et al*. 2003). Studies have suggested that silymarin and its polyphenolic fraction could have beneficial effects on some atherosclerosis risk factors. Silymarin has a hypolipidemic effect (Soboleva *et al*. 2006) and preventive effect on low density lipoprotein (LDL) peroxidation *in vitro* (Locher *et al*. 1998). Silymarin also has protective effects against stress-induced gastric ulcers and induces recovery of pancreatic function after alloxan damage in rats (Soto *et al*. 2004). Silymarin has anticarcinogenic and cancer chemopreventive effects: a combination of silibin and mitoxantrone exhibited a pattern of synergy to inhibit cell growth and induce apoptosis in human prostate cancer (Flaig *et al*. 2007). While almost the entire plant can be used for food, it is the seeds that are used to make MT seed extract. The seeds are the only part of this plant that has silymarin. Also, 25% of seed weight is a fixed oil with a great potential as edible oil (Rizk *et al*. 1970).

Traditionally, diversity within and between populations was determined by assessing differences in morphology. However, application of biochemical and genetic techniques have an important potential to provide a new tool for the study of wild as well as domesticated species, as for example, in the investigation of evolution and migration of species from their gene pool centers (Morsy 2007).

Polyacrylamide gel electrophoresis (PAGE) is considered a rapid and accurate test to identify and characterize different species (Lioi *et al.* 1999). El-Shanshoury (2002) investigated seed protein by SDS-PAGE to study the genetic variability between 30 samples of *Lathyrus sativus* L. collected from different countries. The resulting profiles showed different patterns indicating the variability among accessions of different conditions. Isozymes have been used in genetics for defining systematic phylogentic relationships and to assess the genetic divergence between taxa (Bonnin *et al*. 1996; Yang *et al.* 1996). In this regard, protein and enzyme polymorphism has been successfully used to assess genetic variability in many plant species. Morsy (2007) demonstrated that a combined system (protein, isozyme and RAPD) could discriminate between populations of *Achillea fragrantissima* (Forssk.) SCH.BIP. Motlagh *et al.* (2006) developed an electrophoretic method for the separation and detection of polysaccharides, glycoproteins, and proteins

Received: 30 January, 2010. Accepted: 26 August, 2010.

Original Research Paper

Table 1 Code number, description, GPS number of MT accessions.

Accession	Description	GPS
1pA	1st location, Pink flowers and black seeds	N 30° 55' 462''
1pB	1st location, Pink flowers and variegated seeds	E 29° 35' 40''
1pC	1st location, Pink flowers and white seeds	
2wA	2nd location, White flowers and black seeds	N 30° 54' 702"
2wB	2nd location, White flowers and black variegated seeds	E 29° 33' 557"
2wC	2nd location, White flowers and White variegated seeds	
2wD	2nd location, White flowers and White seeds	
3hA	3rd location, Pink flowers and black seeds	N 30° 57' 834"
3hB	3rd location, Pink flowers and variegated seeds	E 29° 38' 141"
3hC	3rd location, Pink flowers and White seeds	
4pA	4th location, Pink flowers and black seeds	N 31° 06' 618"
CU	Semi-cultivated MT	E 29° 52' 700"

P = var. *Marianum*;
W = var. *albiflorum*

that constitute *Acacia* gum and other gums that used SDS-PAGE with 2-[*N*-morpholino] ethanesulfonic acid buffer and silver staining. The electrophoresis results showed the similarity in bands between species of *Acacia* gum.

The advent of molecular biology has provided another dimension to detect genetic polymorphism based on protein or DNA, facilitating many biological research approaches such as phylogenetic relationships, taxonomy and genetic diversity (Saghai *et al*. 1994). Polymorphisms scored using molecular techniques such as random amplified polymorphic DNA (RAPD) have allowed direct comparisons of variations in nucleotide sequences and have proved to be a potential tool in various type of genetic analysis (Moncketon and Jeffreyes 1993). Polymorphism may be defined as simultaneous occurrence within or between populations of multiple phenotypic forms of a trait attributable to the alleles of a single gene or the homologs of a single chromosome (Acquaah 1992). Many investigators employed RAPD-PCR to assay genetic fingerprints and diversity in plants such as *Artemisia* (Sangwan *et al*. 1999).

In recent years, microsatellites, or simple sequence repeats (SSRs), have been recognized as useful molecular markers in marker-assisted selection, the analysis of genetic diversity, population analysis, and other purposes in various species (Allender *et al.* 2007). SSRs are short (mostly 1-6 bp) tandem repeats of DNA sequences, which are not only very common but also hypervariable among the types of tandem repetitive DNA in the genomes of eukaryotes (van der Schoot *et al.* 2000). SSRs are inherited in a co-dominant manner, multiallelic variation, and can be analyzed using a convenient PCR-based method, which makes it easy to screen a large number of individuals. Thus, SSR markers are becoming a preferable molecular marker in crop breeding. A number of SSR markers have been developed and characterized from some economically important crops such as maize (Sharopova *et al.* 2002), rice, barley, cotton (Liu and Wendel 2001), and soybean (Qin *et al.* 2006). Inter-simple sequence repeat (ISSR) markers are DNA sequences delimited by two inverted SSRs composed of the same units and spaced out by less than around 4 kb.

ISSR analysis involves the PCR amplification of genomic DNA using a single primer that targets the repeat, with 1-3 bases that anchor the primer at the 3′ or 5′ end. Unlike SSR markers, ISSR-PCR does not require sequence information or prior genetic studies; thus, it provides a simple tool for developing molecular makers in plant genomes. So far, ISSR-PCR has provided highly reproducible results and generated abundant polymorphism in a number of crop species (Bornet *et al.* 2002). ISSR analysis does not require sequence information or prior genetic studies, and thus it provides a simple tool for developing molecular markers in plant genomes (Cui *et al*. 2008). ISSR-PCR was used for the same purpose in plants such as *Brassica* (Cui *et al*. 2008) and *Pelargonium* (De Wet *et al*. 2008).

Two different varieties have been described under *Silybum marianum*: S. *marianum* var. *marianum* with purple flowers and *S. marianum* var. *albiflora* with white flowers (Tachholm 1974). Both varieties are distributed widely and cultivated in the Mediterranean area and share the same ecological biosphere (Sadaqat *et al.* 1983). In Egypt, MT grows widely as a winter crop on canal banks and pastures while the plant cultivation in new reclaimed lands is more suitable for the plant growth traits and silymarin production (Hetz *et al.* 1995).

The aims of this study were to determine the morphological, biochemical (protein profile and isozyme) and molecular (RAPD- and ISSR-PCR) characteristics of MT and to identify and quantify its active ingredients. Twelve accessions of MT collected from Borg El-Arab were tested. To our knowledge, this is the first report on MT molecular characterization.

MATERIALS AND METHODS

Plant materials

Seeds of 12 accessions belonging to a couple of *S. marianum* varieties *viz*. *marianum* (with purple flowers) and *albiflora* (with white flowers) were used in this study. Eleven accessions were collected from 4 locations at different latitudes from Borg El-Arab area located 60 km north/west of Alexandria, Egypt (**Table 1**). Seeds from a cultivated plant were also obtained from the herbarium of the National Research Center, Giza, Egypt.

Biochemical analysis

1. Fixed oil content

Fixed oil was extracted with petroleum ether (40-60°C) from three replicates for each seed accession and determined according to AOCS (1970) using a Soxhlet apparatus.

2. Protein electrophoresis

Total protein was extracted out according to the protocol of Payne and Corfield (1979). One g of each accession's seeds was frozen with liquid nitrogen and ground with 4 mL water-soluble extraction buffer in a mortar. The extraction buffer was made up of 10% SDS (sodium dodecyl-sulphate) to denature the proteins, 0.1% β-mercaptoethanol to break the disulphide links between the peptidic chains of the protein, 20% glycerol to weigh down proteins at the bottom of the gel wells, Tris 1 M which confers pH 6.8 and a coloring agent (bromophenol blue) for protein detection. Samples were centrifuged at 8000 × *g* for 20 min at 4°C. Supernatants containing a water-soluble storage protein fraction were transferred to clean Eppendorf tubes, and then incubated in a drying oven at 100°C for 10 min to accelerate the denaturation of proteins. The supernatants were then deposited in a well of the electrophoresis gel (each well corresponding to each accession). SDS-PAGE was performed according to the method of Laemmli (1970). Bands were detected on gels using Coomassie brilliant blue stain then scanned using a Gel Doc-2000 Bio-Rad system.

3. Isozyme electrophoresis

MT seeds were soaked in distilled water for 24 h to allow maximum imbibition. Five seeds of each accession were used for the extraction of isozymes with 1 mL extraction buffer (100 mM Tris, pH 7.5, 0.1 mM EDTA and 0.05% Triton X-100). Each sample was vortexed for 15 sec and the homogenates were centrifuged at 8000 × *g* for 20 min at 4°C, then the resultant supernatants were used as enzyme sources for three isozymes namely esterase (Est; E.C. number) using two different substrates, α and β-naphthyl acetate; peroxidase (Px; E.C. number) and acid phosphatase (Acp; E.C. number). The supernatants were then separated in 8% native-PAGE according to Stegemann *et al.* (1985). After electrophoresis, the gels were individually stained according to the enzyme type using the appropriate substrate and chemical solution (Wendel and Weeden 1990) then incubated at 37°C in a dark room for complete staining. Gels were scanned using the Gel Doc-2000 Bio-Rad system.

DNA fingerprinting

1. Isolation of genomic DNA

DNA samples were extracted according to Junghans and Metzlatt (1990) with some modifications. Ten seeds of each accession were germinated in the laboratory to obtain MT leaves. Leaf tissue (30 mg) was first homogenized with liquid nitrogen in a mortar until a fine powder was obtained, then further thoroughly ground in a 700 µL extraction buffer (50 mM Tris-HCl pH 7.5; 50 mM EDTA; 100 mM NaCl, 0.5% SDS). The leaf extracts were transferred to clean tubes then distilled water was added up to 15 mL. The tubes were incubated at 4°C for 10 min. After spinning for 10 min at 12,000 × *g*, the supernatant was transferred into a new sterilized tube and an equal volume of phenol: chloroform: isoamyl (25: 24: 1, v/v) was added and mixed by repeated inversion. After this, centrifugation at 12,000 × *g* for 5 min was used to completely clear out the top aqueous phase. This upper phase was then transferred into a new sterilized tube; cold isopropanol was added, and then incubated for 15 min at 4°C to precipitate the DNA. The DNA precipitate was then collected by a brief spinning at 12,000 × *g* for 5 min. The resulting pellet was washed with 70% ethanol, dried for 30 min in air and resuspended in TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0). The samples were then incubated for 2 h at 37°C in the presence of RNase A (Sigma, 5 U/µL for each sample) to eliminate RNA. After spectrophotometric quantification of DNA, it was used in RAPD- and ISSR-PCR fingerprinting.

2. RAPD fingerprinting

Fifteen (10-mers) random primers were used in this study; however, only 10 gave reproducible results (**Table 2**). PCR reactions were conducted according to Williams *et al.* (1990). RAPD-PCR thermocycling was carried out on a Techne TC-312® (China) thermal cycler using the following thermoprofile: initial strand separation cycle at 94°C for 4 min, followed by 35 cycles including a denaturation step at 94°C for 45 sec, an annealing step at 37°C for 45 sec and a polymerization step at 72°C for 1.5 min with a final extension step at 72°C for 10 min. The PCR reaction mixture contained 10X reaction buffer (15 mM $MgCl_2$, 0.2 µM dNTPs, 0.2 µM primers, 0.25 U *Taq* DNA polymerase) and 20 ng DNA; the volume was completed with sterile distilled water up to 25 µL. For DNA band separation, approximately 15 µL amplified products was loaded on a 1.2% agarose gel. DNA patterns displayed on ethidium bromide-stained agarose gels were scanned and analyzed by EgyGene GelAnalyzer V3 software to determine relative mobility (RF), molecular size (MS) of fragments in base pairs, and the presence/absence of each fragment (Egygene 2010).

3. ISSR fingerprinting

A set of 15 primers were initially employed in ISSR analysis; however 8 of them produced reproducible ISSR profiles (**Table 3**). The PCR reaction was performed according to the above mentioned protocol in RAPD analysis. Approximately, 15 µL of the amplified ISSR products was loaded on a 1.2% agarose gel for band separation. DNA patterns displayed on ethidium bromide-stained agarose gels were scanned and analyzed by EgyGene GelAnalyzer V3 software to determine relative mobility (RF), molecular size (MS) of fragments in base pairs, and presence/absence of each fragment (Egygene 2010).

Table 2 List of RAPD 10-mer primers and their nucleotide sequences.

Primer name	Sequence (5′-3′)
L12	GGG CGG TAC T
L13	ACC GCC TGC T
L20	TGG TGG ACC A
I15	TCA TCC GAG G
X06	ACG CCA GAG G
Z05	TCC CAT GCT G

Table 3 List of ISSR primers names and their nucleotide sequences.

Primer name	Sequence (5′-3′)
HB08	GAGAGAGAGAGAGG
HB10	GAGAGAGAGAGACC
HB12	CACCACCACGC
HB13	ACG CCA GAG G
844B	TCC CAT GCT G
17898B	CACACACACACAGT

4. Phylogenetic tree

Variations in SDS-PAGE, isozymes, RAPD-PCR and ISSR-PCR bands were pooled together to generate a dendrogram using Dice equation as implemented in SPSS program V10.

RESULTS AND DISCUSSION

Fixed oil content of MT seeds

Seed oil composition constitutes another characteristic which contributes to the rich diversity of a plant genus. The fixed oils of plant seeds were classically classified as non-drying, semi-drying or drying oils. Drying oils are highly unsaturated oils that will polymerize when exposed to oxygen in air, usually in the presence of a catalyst (Akpuaka and Nwankwor 2000).

The highest fixed oil content (36%) was found in accession 1PA while the lowest content (9%) was in accession 2WA (**Fig. 1**). This variation in oil contents among MT accessions tested could probably be attributed to differences in environmental conditions.

Protein electrophoresis profiles

SDS-PAGE of total storage proteins was carried out on the 12 MT accessions to provide information concerning the type and biosynthesis of various protein fractions. Electrophoretic banding patterns of total proteins are shown in **Fig. 2**. The protein bands were revealed with different molecular weights ranging from 13.5 to 1.3 kDa. The resulting protein profile showed 6 polymorphic bands while the remaining bands were considered as consistent bands with different molecular weights. There was one unique band generated for accession CU with a molecular weight of 2.2 kDa. Protein and enzyme polymorphism has been successfully used to identify cultivars in various fruit and crop species, including *Zygophyllum* spp. (Nour El-Din *et al*. 2004), sunflower (Raymond *et al.* 1995), *Acacia* gum (Motlagh *et al*. 2006) and *Achillea fragrantissima* (forssk.) SCH.BIP (Morsy 2007).

Isozyme polymorphism

Isozymes merely represent different structural configurations of the same polypeptide chain of an enzyme (Weeden 1983). For this reason, three isozymes including Est using two different substrates, α and β-naphthyl acetate, Px and Acp were used to study the different gene/genes expression

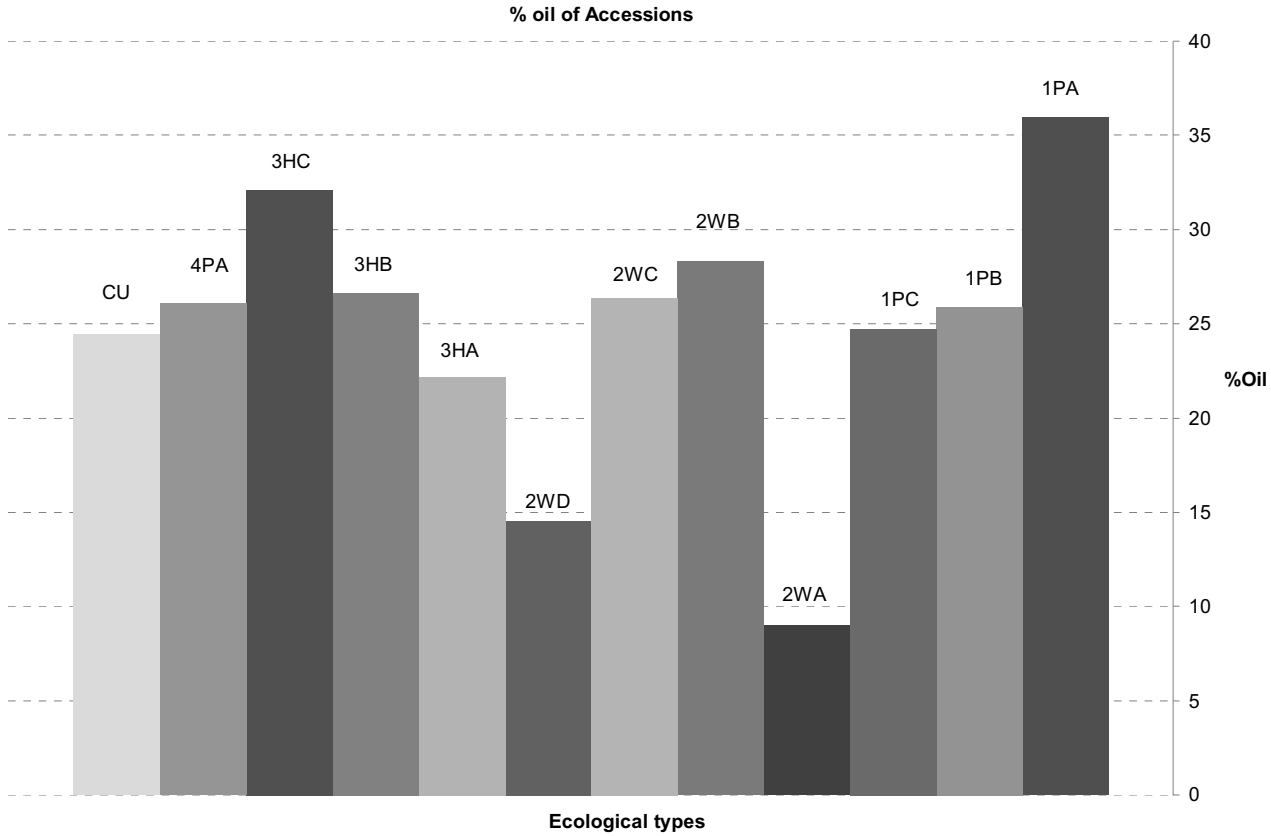

Fig. 1 Fixed oil content (%) of seeds of 12 accessions of *Silybum marianum*.

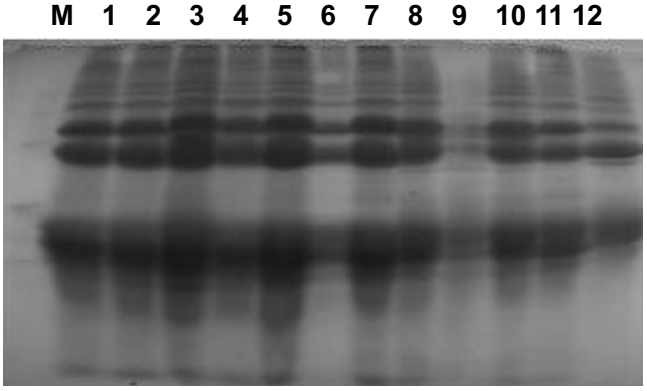

Fig. 2 Profile of monodimentional electrophoresis of total storage proteins of the 12 *Silybum marianum* accessions seeds.

for MT accessions.

The resolved esterase isozymes included many bands ranging from 4 to 6 depending on the accession. One esterase band was unique for α-Est in accession CU, while no unique bands were detected for β-Est in all accessions (**Fig. 3A, 3B**). Px banding patterns are illustrated in **Fig. 3C**: It was identified by one monomorphic banding pattern for all the studied accessions. **Fig. 3D** shows the banding pattern of Acp, which revealed a highly polymorphic banding pattern. There were 2 polymorphic bands; one of them presented in all accessions except accession 1PC which may be considered as a negative marker for this accession. Su and Su (1999) investigated isoperoxidases of 14 species of *Populus* spp. by PAGE: there were highly similar values among natural hybrid species. Isoenzyme analysis provided new information for plant classification at the biochemical level.

Molecular genetic analysis

1. RAPD analysis

The genetic variability among the 12 accessions of *S. marianum* based on RAPD relationships has been studied. Initial screening of 15 10-mer random primers resulted in 10 primers that produce informative and polymorphic products resolvable by agarose gel electrophoresis (**Fig. 4**). Eighty three DNA bands were amplified, 33 out of which were polymorphic. In general, the number of resolved DNA fragments for each MT accession ranged from 6 to 15, while the molecular size of those fragments ranged from 90 to 5250 bp. As shown in **Fig. 4A**, primer I15 showed 6 fragments with molecular sizes ranging from 990 to 215 bp. There were no polymorphic fragments, i.e. all fragments were monomorphic. However, primer L12 resulted in 15 fragments with molecular sizes ranging from 2500 to 90 bp. Five polymorphic fragments (33.3%) with corresponding molecular size of 2500, 225, 175, 150 and 90 bp was observed. All other fragments were monomorphic (**Fig. 4B**). Primer L13's pattern exhibited 7 fragments with molecular sizes ranging from 5250 to 400 bp. One polymorphic fragment (14.3%) with a corresponding molecular size of 5250 bp was observed. The other fragments were all monomorphic (**Fig. 4C**). Primer L20's pattern showed 7 fragments with molecular sizes ranging from 1600 to 250 bp. Three polymorphic fragments (57.1%) numbered 1, 4 and 6 with corresponding molecular sizes of 1500, 500 and 300 bp, respectively, were observed. Fragment 7 (257.3 bp) appeared only in accession 10 which may be considered as a positive marker (**Fig. 4D**). Primer X06's pattern resulted in 8 fragments with molecular sizes ranging from 2000 to 200 bp. Three polymorphic fragments (37.5%), number 3, 4 and 6, with corresponding molecular sizes of 1225, 1000 and 500 bp, respectively, were observed. The other 5 fragments were monomorphic (**Fig. 4E**). Primer Z05's pattern exhibited 7 fragments with molecular sizes ranging from 2000 to 300 bp (**Fig. 4F**). Three polymorphic fragments (42.9%)

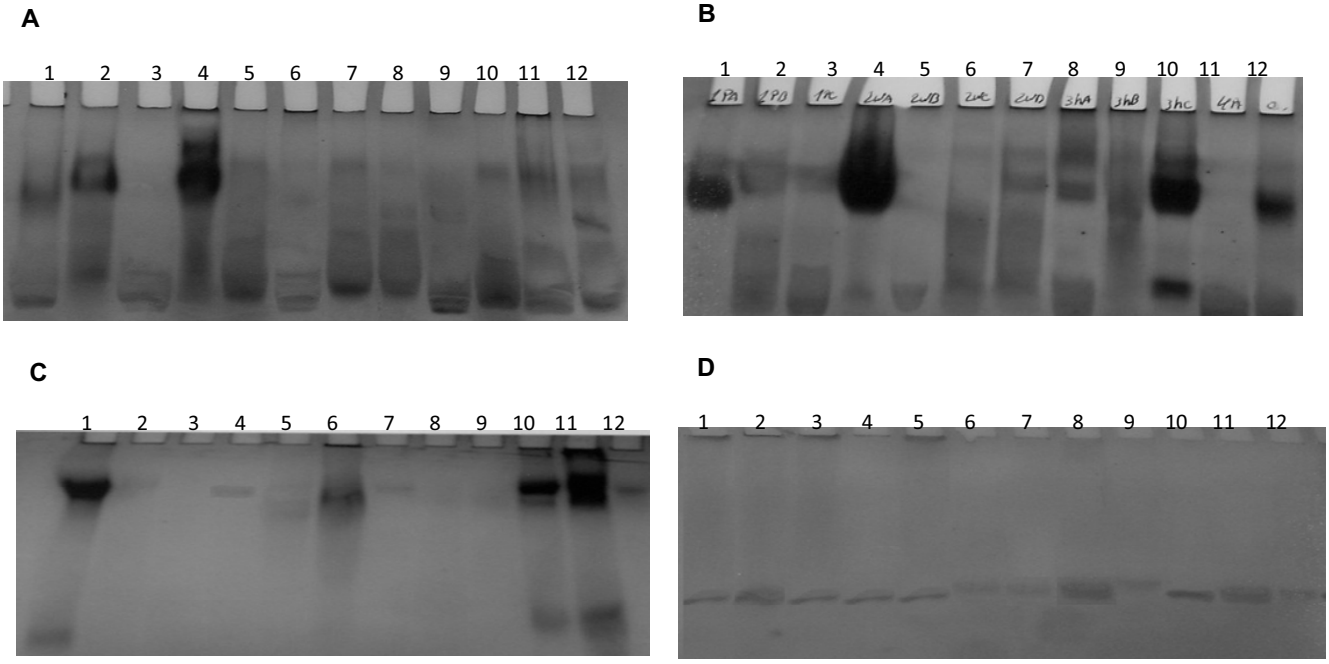

Fig. 3 Zymogram profiles among the 12 *Silybum* accessions. Esterase α-naphthyl acetate (**A**), esterase-β-naphthyl acetate (**B**), acid phosphatase (**C**), peroxidase (**D**).

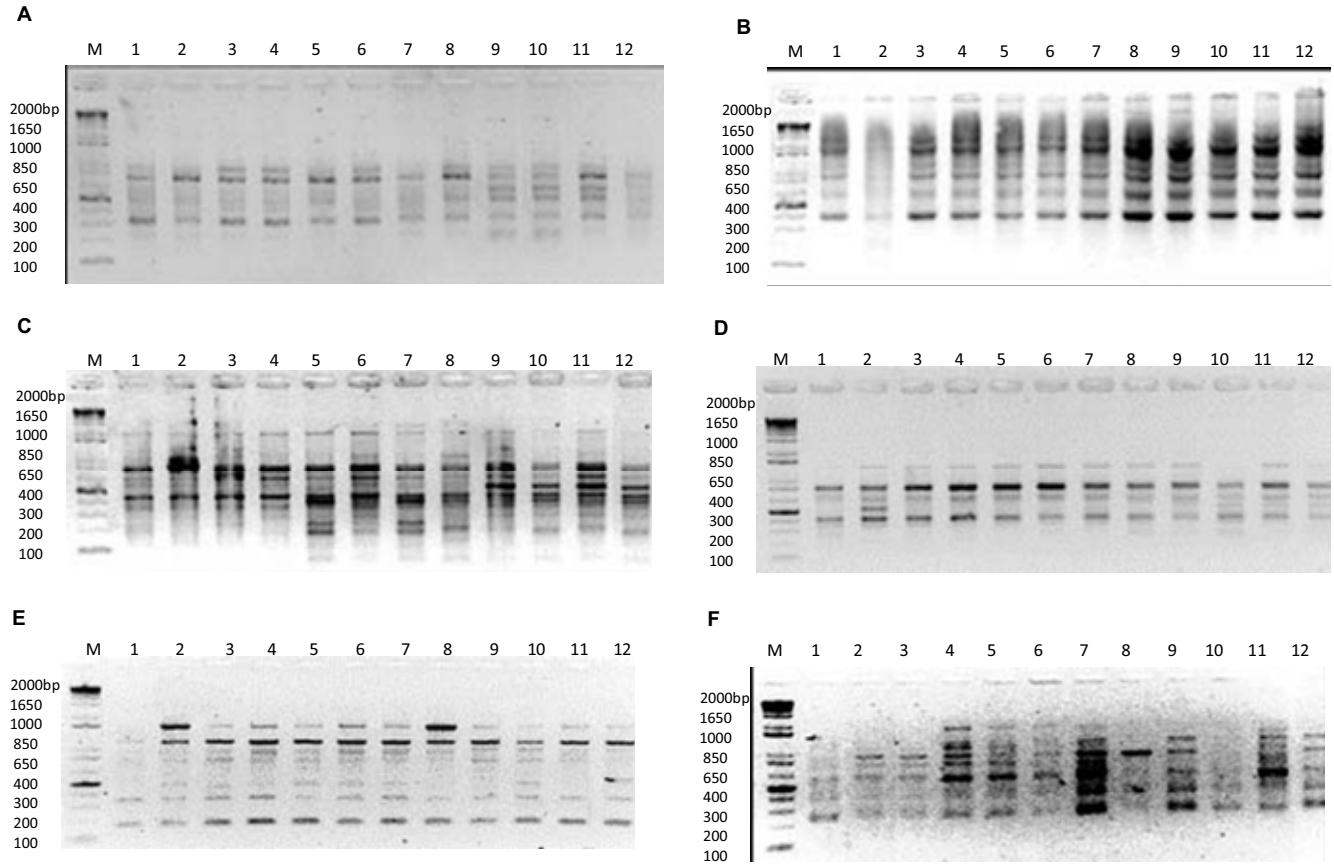

Fig. 4 RAPD-PCR polymorphism of DNA of in 12 *Silybum* accessions using 6 primers. Primers: I15 (**A**), L12 (**B**), L13 (**C**), L20 (**D**), X06 (**E**) and Z03 (**F**).

numbered 1, 2 and 4 with corresponding molecular sizes of 2000, 1500 and 1000 bp, respectively, were observed. The remaining 4 fragments were monomorphic.

2. ISSR analysis

The genetic variability among MT accessions based on ISSR analysis relationships has been performed. Initial screening of 15 primers resulted in 8 primers that produced informative and polymorphic products (**Fig. 5**). Forty PCR bands were amplified, 16 of which were polymorphic. The molecular size of amplified DNA fragments ranged from 135 to 2155 bp. Primer HB08 in ISSR analysis produced 8 bands with 3 polymorphic fragments (**Fig. 5A**). However, primer HB10 produced 7 products distributed among all accessions tested with molecular weights ranging from about 1000 to 250 bp (**Fig. 5B**). A total of 5 distinguishable DNA bands with different molecular weights were not present in all accessions. Also, the number of total bands varied between accessions where the lowest number of DNA

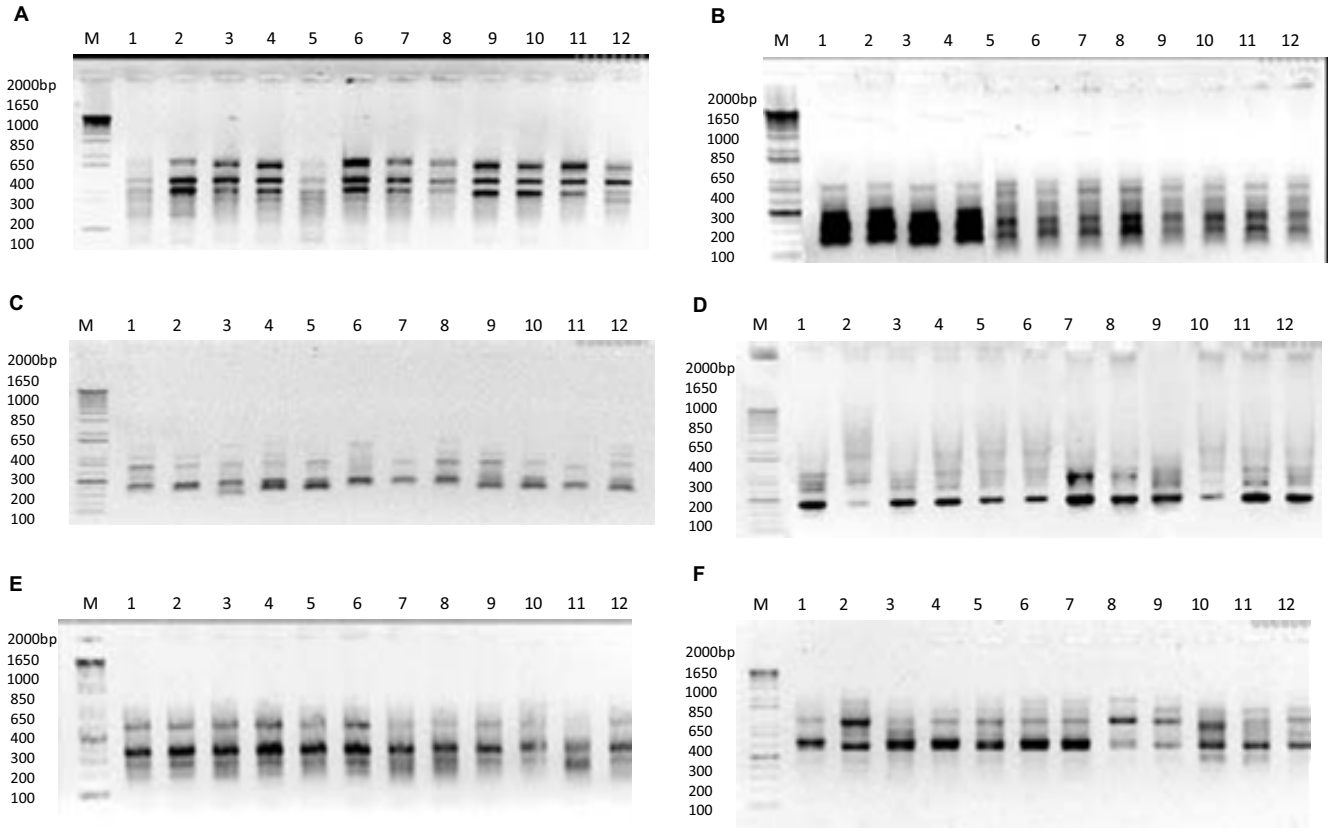

Fig. 5 DNA polymorphism generated by ISSR primers in 12 *Silybum* accessions using 5 primers. Primers: HB08 (**A**), HB10 (**B**), HB12 (**C**), HB13 (**D**), 844B (**E**) and 17989 (**F**).

bands was one band in accessions 2, 6, 7 and 11, while the highest number was 4 bands in accessions 4, 9 and 12. Three polymorphic fragment numbers 1, 4 and 5 were observed (**Fig. 5C**). As shown in **Fig. 5D**, all observed bands were polymorphic since they were present in some accessions and absent in others. It is interesting to note that accession 10 had 2 unique DNA bands of 1425 and 1000 bp, respectively. These unique bands could be used to distinguish these accession from others (**Fig. 5E**). Primer 17898B produced 4 fragments with molecular sizes ranging from 750-200 bp (**Fig. 5F**). No polymorphic bands among the studied MT accessions were identified.

Specific marker for *Silybum* accessions

The results of SDS-PAGE data confirmed that there were 3 negative markers for accession 3hB with molecular weight 13.5, 5 and 4.5 kDa. Also positive and negative markers at 3 and 1.3 kDa for accession CU were observed. As shown in **Table 4**, RAPD- and ISSR-PCR data analysis confirmed that there were 2 positive markers (150 and 300 bp) for accessions belonging to location 1, one positive marker (2000 bp) for accessions belong to location 2 (var. *albiflorum*) and one positive marker (5250 bp) for accessions belong to location 3 (unique phenotype).

Comparative class pattern analysis based on protein, isozyme, RAPD-PCR and ISSR-PCR (phylogenetic tree)

We noticed that it is not possible to differentiate between the populations tested based on one identification system alone. Therefore we applied the combined class pattern system to obtain a better resolution. As shown in **Fig. 6**, the dendrogram separated the 12 accessions into two main clusters, and then each cluster was separated into two sub-clus-

Table 4 Total amplified (TAF) and polymorphic fragments (PF), polymorphic percentage and the specific markers (SM) MT accessions using RAPDPCR and ISSR-PCR analysis according to geographical location.

Primer no.	Primer name	TAF	PF	P%	SM
RAPDs					
1	I15	6	0	0	-
2	L12	15	5	33.3	1 positive SM (146.33 bp) for locations 1, 2, 3
3	L13	7	1	14.3	1 positive SM (5244.22 bp) for locations 8, 9,10
4	L20	7	4	57.1	1 positive SM (257.35 bp) for location 10; 1 positive SM (306.33 bp) for locations 1, 2, 3
5	X06	8	3	37.5	1 negative SM (1229.7 bp) for location 1
6	Z05	7	3	42.9	1 positive SM (1969.98 bp) for locations 4,5,6,7
Total		50	16	32	
ISSRs					
1	HB08	8	3	37.5	1 negative SM (379.68 bp) for location 8
2	HB10	5	1	14.3	-
3	HB12	3	4	80	1 positive SM (494.64 bp) for location 1; 1 negative SM (412.84 bp) for location 11
4	HB13	5	2	40	1 negative SM (956.04 bp) for location 10
5	844B	3	5	50	2 positive SMs (1422.43, 1188.79 bp) for location 10
6	17898B	10	0	0	-
Total		34	15	44	

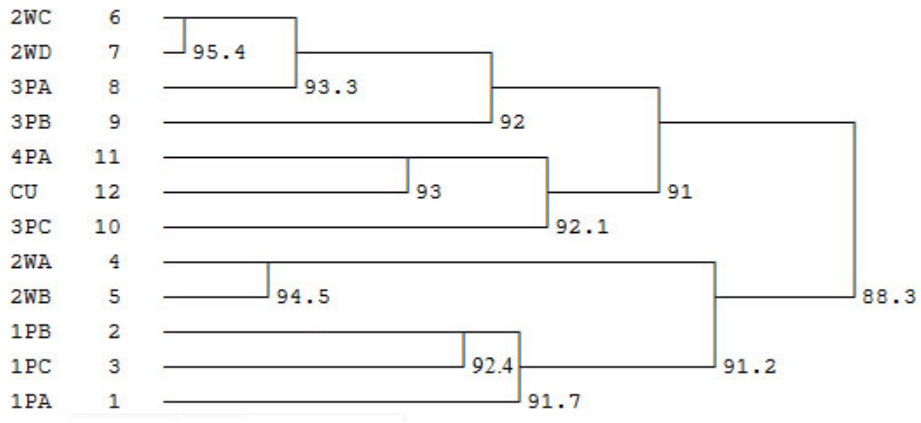

Fig. 6 Dendrogram demonstrated relationship among 12 accessions of *Silybum marianum* varieties based on protein, isozymes, RAPD-PCR and ISSR-PCR.

Table 5 Similarity matrix among MT accessions based on the combined data analysis.

	1PA	1PB	1PC	2WA	2WB	2WC	2WD	3PA	3PB	3PC	4PA
1PB	0.92										
1PC	0.92	0.92									
2WA	0.90	0.89	0.91								
2WB	0.90	0.89	0.90	0.94							
2WC	0.89	0.89	0.89	0.91	0.95						
2WD	0.89	0.89	0.90	0.90	0.90	0.95					
3PA	0.88	0.87	0.89	0.89	0.87	0.93	0.95				
3PB	0.85	0.85	0.86	0.88	0.88	0.91	0.92	0.93			
3PC	0.87	0.85	0.86	0.88	0.86	0.88	0.90	0.91	0.92		
4PA	0.87	0.88	0.90	0.88	0.90	0.90	0.92	0.90	0.92	0.92	
CU	0.86	0.86	0.88	0.90	0.89	0.89	0.92	0.91	0.91	0.91	0.93

ters. Accessions that belonged to the same variety and location were grouped together.

The closest relationship was scored between accessions 2WC and 2WD (**Table 5**). The similarity matrix indicated that the highest similarity (95%) was between accessions 1PA and 3PB while the lowest similarity (85%) was between accessions 1PB and 3PC.

El-Sherbeny (2004) reported that the combined class pattern for protein, isozymes and RAPD allowed a complete discrimination among all populations of 4 species of family *Labiatae*. Morsy (2007) characterized and identified 5 populations of *Achillea fragrantissima* on biochemical and molecular bases using SDS-PAGE, Native-PAGE and RAPD. It was concluded that the combined class pattern based on protein, isozyme and RAPD-PCR, allowed for the complete discrimination of all populations. It is evident from these observations that fingerprinting based on combined molecular analysis systems is superior to fingerprinting based on any individual analysis system.

Mansour *et al.* (2010) detected genomic variation within 10 tomato (*Lycopersicon esculentum* Mill.) cultivars using three different molecular marker systems, namely RAPD, ISSR and IRAP. Dendrograms constructed for the RAPD, ISSR and IRAP results individually and collectively reveal that similarity and clustering are highly dependent on the marker system used.

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