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GENOTOXIC EFFECT OF PEGANUM HARMALA EXTRACTS ON THE GROWTH OF *VICIA FABA* L. AND DNA USING NUCLEAR MICROSATELLITES

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Abstract

Assessment of genotoxins-induced DNA damage and mutations at molecular level is important in ecogenotoxicology. Treatment with *Peganum harmala* crude extract can result in cytotoxic, carcinogenic and mutagenic effects. Mutagenesis is indicative of genetic instability and can be assayed using microsatellites or simple sequence repeats (SSRs). The application of SSR marker analysis using capillary electrophoresis (CE) apparatus showed differences in SSR profiles within the extract as well as between the two extracts. All the 12 SSR primer pairs used produced amplification products which showed 100% polymorphism. The total number of scoreable loci obtained with the 12 used primers were 112 alleles. Of the 112 alleles detected 12 alleles (10.7 %) were considered as zero alleles. Three alleles produced by various primers were detected at locus VfG_22 to 15 at locus VfG_693 and VfG_873, with an average of 9.3 bands/SSR. The discrimination power of each SSR locus was estimated by the PIC, which ranged from 0.47 for locus VfG_22 to 0.88 for locus VfG_693 with a mean of 0.70. About 83.3% of the 18 alleles observed in untreated samples using the 12 primers disappeared from almost all the treatments with water and ethanol extracts. Statistical analysis of morphometric traits showed significant differences between the two extracts in all studied traits except the percentage of germination and root dry weight which were non significant. However, on the other hand significant differences were observed between treatments and the control for all the traits studied.

Key words: SSR, Nuclear satellite, *Peganum harmala*, *Zygophyllaceae*, Capillary electrophoresis (CE).

Introduction

The use of plants, plant extracts or plant-derived chemicals to treat diseases is a therapeutic modality that has been explored for centuries (Shinwari *et al*., 2013; Mahmood *et al*., 2013, Muhammad *et al*., 2013). Over 40,000 species of tropical flowering plants are said to possess medicinal properties (Idu *et al*., 2008) and are currently in use for various medical conditions. The majority of the people of African descent patronize herbal or traditional medicine for their health needs. It is estimated that 70 - 80% of patients in Africa are treated by traditional healers and herbal practitioners (Diallo *et al*., 1996; Nyika, 2007).Yet, many reports reveal that drugs of plant origin are not free from toxic effects (Gilani *et al*., 2010). Hepatic failure and even death following ingestion of herbal medicine have been reported (Dickens *et al*., 1994). Several medicinal plants are mutagenic, clastogenic and carcinogenic (Nandi *et al*., 1998).

Peganum harmala L. (Zygophyllaceae) is a perennial herbaceous plant grows in different coastal and inland habitat types including sandy, rocky and gravel soils. The species is widely distributed in Middle East, Pakistan, India, Mongolia and China (Frison *et al*., 2008; Boulos, 2009; Saqib *et al*., 2011). In Egypt the plant populations are distributed mainly along the Mediterranean coastal strip, Eastern desert and Sinai (El-Bakatoushi, 2011). *Peganum harmala* is listed as one of the "worst weeds in the West" by the Centre for Invasive Plant Management (Anon., 2009).

Several studies have used the chromosome aberration and micronucleus assay to measure the genotoxic and cytotoxic effects of *P. harmala* extracts on root tip cells of *A. cepa*, *Zea mays* and *V. faba* plants (Abderrahman, 1997 & 1998; Mekki, 2014). Genetic-ecotoxicology or eco-genotoxicology, the effective evaluation and proper environmental monitoring of potentially genotoxic pollutants have been improved and will continue to be improved with the development of sensitive and selective methods to detect toxicant induced alterations in the genomes of a wide range of biota (Atienzar *et al*., 1999; Theodorakis *et al*., 2006). Recently, advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis in eco-genotoxicology. DNA based techniques (RFLP, RAPD, AFLP, SSR, ISSR and VNTR) are used to evaluate the variation at the DNA sequence level (Haq *et al*., 2013; Mehmood *et al*., 2013; Shinwari *et al*., 2014; Jamil *et al*., 2014).

Of the many molecular techniques available to researchers, microsatellites, or simple sequence repeats (SSRs), are becoming increasingly widespread. SSRs are tandemly repeated tracts of DNA composed of 1–6 base pair (bp) long units spread throughout the genome of bacteria, fungi, plants, animals and human (Turi *et al*., 2012). Compared to other markers, SSRs are more abundant, ubiquitous in presence and highly polymorphic. Thus, they are extremely useful for fine-scale genetic analysis (Gupta *et al*., 1996; T´oth *et al*., 2000). Furthermore, SSRs are likely to be one of the most reproducible techniques, especially when compared to RAPDs (Powell *et al*., 1996; Jones *et al*., 1997; Sultan *et al*., 2013). Because of these advantages, SSRs have already been used to study genotoxic effects in several species (Zienolddiny *et al*., 2000; Speit & Merk, 2002; Jin *et al*., 2003) In plant research, SSRs have been extensively used in taxonomic studies (Prasad *et al*., 2000; Rabbani *et al*., 2010), genetic mapping (Ma *et al*., 2004, Kylin 2010) and Kadam (2012) used SSRs in wheat plants as a methodology to assess genetic instability in the offspring plants exposed to radiation near the Chernobyl nuclear power plant and to screen drought tolerance gene type respectively. Abtahi (2013) used SSR technique to asses genetic variability

induced by gamma radiation in canola. However, the application of SSRs in higher plant bioassays for the detection of genomic DNA damage and/or mutational events (deletions, insertions, point mutations) as a consequence of exposure to genotoxic and cytotoxic agents remains unexplored.

Materials and Methods

Plant materials: Seeds of the medicinal plant *Peganum harmala* L. were collected from Wadi Gebal in Saint Katherine, Sainai, Egypt in the end of the summer of 2011. A voucher specimen is preserved in the herbarium of our Institute (Suez Canal University, Ismailia, Egypt).

Extract preparation: The aquatic and ethanolic crude extracts were obtained from the seeds according to Al-Mizrakchi (1998). The stock solutions of the two extracts (aqueous and ethanolic) as well as the different concentrations (12.5, 25, 50 and 100%) were prepared as described by Mekki (2014).

Treatments: Seeds of *Vicia faba* plants were pre-soaked in water for 24 h. and treated with the different concentrations (12.5, 25, 50 and 100%) of the two extracts for time periods 3, 6, 12, and 24 hr. One set of seeds was kept untreated to act as control. Then, three replications of each of 30-seeds were sown in the botanical garden of Suez Canal University in October 2012 using randomized complete block (RCB) statistical design. After 15 days from sowing, about 1 g of young and fresh leaves was picked up for genomic DNA extraction, the plants were left to raise the M2 generation. During the growing season (2012/2013), germination percentage, plant height, pod length, number of seeds per pod, 100 seed weight, fresh and dry weights of shoot and root, and the number of branches per plant were recorded using five randomly selected plants from each treatment.

Total genomic DNA extraction: Total genomic DNA was extracted according to the basic DNA extraction protocol of Dellaporta *et al*. (1983) with slight modifications by Porebski *et al*. (1997) and adapted to faba bean for obtaining good quality. A weight (0.2 g) from young leaves was ground in liquid nitrogen to fine powder and extracted using 10 ml preheated (65º C) cetylhexadecyl-trimethyl ammonium bromide (CTAB) extraction buffer [3% CTAB (w/v), 100 mM Tris- HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (w/v) PVP (Polyvinyl pyrrolidone)], then 1% (v/v) of β-mercaptoethanol (15 mM) with further grinding.. The mixture was incubated at 65º C for 60 min, followed by two extractions with chloroform/isoamyl alcohol (24:1). The nucleic acids were precipitated with cold isopropanol, and the pellet was dissolved in 1 mL TE 0.1X (Tris-EDTA) buffer (10 mM Tris-HCl, pH = 8 and 1 mM EDTA, pH = 8). Co-precipitated RNA was removed by digestion with RNAase A. 4 µl (10 mg/mL). The DNA was further purified by 300 µl phenol:chloroform: isoamyl alcohol (25:24:1), then left overnight at (-20º C) using 1/10 vol. from 2 M sodium acetate (pH = 8.0) and one volume of cold isopropanol alcohol. The precipitate was washed twice with 10 mM ammonium acetate in 76 % ethanol, and the pellet was dissolved in 0.1 XTE buffer. The purified total DNA was quantified by gel electrophoresis, and its quality was verified and concentrations by Nanodrop spectrophotometer model ND1000. DNA samples were then stored at 4ºC.

PCR (SSR) amplification and product electrophoresis: The twelve SSR primer pairs used for PCR amplification as described by Zeid *et al*. (2009). These primers were synthesized by Oligo Macrogen, Seoul, Korea. PCR reactions were performed according to published procedures by Zeid *et al*. (2009). Microsatellites amplification was performed in 25 µl volume contained 2µl (30 ng) of template DNA, 1µl (20 pmol) forward primer, 1µl (20 pmol) reverse primer, 12.5 µl Master Mix, and 8.5 µl PCR water. The amplification was carried out in a thermocycler (Eppendorf Mastercycler Gradient Eppendorf, Hamburg, Germany). After a first denaturation step at 95ºC for 4 min, the reaction went through 35 cycles at 95ºC for 45 sec., 58ºC for 1 min., 72ºC for 1 min. followed by a final extension step of 7 min at 72ºC. The analyses were repeated at least twice to assure the reproducibility of the results. PCR products were detected by electrophoresis on Multi-capillary electrophoresis QIAxcel (QIAGEN Germany) system for DNA fragmentation analysis.

Statistical analysis: The data of growth parameters were statistically analyzed using the analysis of variance techniques according to Gomez & Gomez (1984). In applying the F test, the error due to replicates was also determined. When the F value was found to be significant at 1% and 5% level of probability, the least significant difference (LSD) was calculated. The DNA fragments amplified by microsatellite primers were scored as present (1) or absent (0) and recorded with reference to the molecular weight markers. The number of polymorphic bands, percentage of polymorphism and polymorphic information content (PIC) were calculated for each primer combination. UPGMA dendrogram were constructed based on Dice similarity coefficient (Nei & Li, 1979) using NTSYS software version 2.2.

Results and Discussion

Morphological traits: The present study is designed to evaluate the genotoxic potential of aqueous and ethanolic extracts of *Peganum harmala* L. using *Vicia faba* plants bioassay. The statistical analysis for source of variance (ANOVA) showed high significant differences among treatments for all the traits under evaluation (Table 1). A cluster analysis based on the phenotypic distance matrix (Fig. 1) revealed at least five major groups (over all the 32 treatments and the control). Mean separation comparisons of the five groups for various traits indicated significant differences between the treatments in the different groups for all traits except the number of seeds/legume and germination percentage (Table 2). It may be explained by reduction of the means of all treatments for these traits. However, on the other hand significant differences were observed between treatments and the control for all the traits studied (data not shown).

Table 1. Source of variances and the mean square for all traits studied with the 32 treatments and the control.

Source of variance	D.F	Length of legume	Number of seeds/ Legume	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	Plant height/ cm	Number of branch	Germination percentage	Weight 100 seeds
Blocks	2	13.15	2.13	60	34.01	62.1	1.25	102.03	8.01	764.5	3.84
Treat	32	35.56**	3.04**	10900.4**	171.59**	793.4**	15.72**	699.68**	37.71**	2667.9**	312.41**
Error	64	9.93	1.01	71.12	24.97	30.61	0.87	84.62	19.54	219.1	1.79
Total	98										

Table 2. Means of agronomic traits for each group derived from cluster analysis of 32 treatments with control (Giza cultivar).

Treatments	Length of legume	Number of seeds/ legume	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	Plant height/ cm	Number of branch	Germination percentage	Weight 100 seeds
Group 1	14.46 b	4.64 a	175.45 c	29.92 ^{b}	37.65 b	6.54 b	83.02 ab	12.08 ^{ab}	34.18 a	134.58 b
Group 2	14.40 b	4.27 ^{a}	230.62 b	35.12 ab	56.27 a	8.93 a	98.52 a	14.18 a	35.38 a	131.22 bc
Group 3	17.35 a	4.65 ^{a}	270.00 a	36.15 a	57.20 a	7.15 b	89.35 a	11.30 ^{ab}	51.00 a	152.95 a
Group 4	15.12 ^{b}	4.40 a	119.96 d	20.79 c	29.70 bc	4.20 c	87.96 a	11.30 ^{ab}	36.42 a	126.08 c
Group 5	14.60 ^{b}	4.62 a	78.78 a	17.62 c	22.00 c	4.14 c	65.66 b	8.22 ^{b}	49.60 a	127.68 c

The untreated sample (control) was clustered with the treatment with 12.5% of aqueous extract for 3 hour in a separate group. This group (group 3) revealed the highest number of seeds per legume, weight of 100 seeds, germination percentage and Length of Legume. On the other hand, group 5 comprised 5 treatments possessing the lowest values of, number of branch per plant, root dry weight, root fresh weight, shoot dry weight and shoot fresh weight and the shortest in plant height. On average, water extract treatments revealed increase in the plant height and the number of branches per plant, increased, only after treatments for 3, 6, and 12 hours.

However, both of extraction methods treatments showed reduction in germination percentage and the weight of 100 seeds. But the number of seeds per legume and length of legume showed reduction only in the short period 6 and 12 hour. This result is in agreement with the previous study of Shao *et al*. (2012). They reported that the ethanol extract of *P. harmala* inhibited the root and shoot lengths of wheat and lettuce plants. Also, Mekki (2014) reported on the previous cytotoxic study of aqueous and ethanolic extract on root tip cells of *V. faba* that ethanolic extract of *P. harmala* was more cytotoxic and induced higher chromosomal aberrations, inhibition in total pigments and reduction in plant height than aqueous extract.

On average, we can conclude that treatments with both aqueous and ethanolic extractions revealed significant increase in the plant height and the number of branches per plant, significant reduction in germination percentage, the shoot and root fresh and dry weights as well as, the weight of 100 seeds. However, length of legume showed increases in all treatments with 12.5% aqueous extract and also with 25% and 100% except after the time duration of 24 h. However, the concentration 50% of aqueous extract caused inhibition in the length of legume. On the other hand, the ethanolic extract reduced the length of legume in all treatments except with 100% concentration for all the four time durations.

The values of the number of seeds per legume were enhanced in concentrations 12.5, 25 and 100% of aqueous extract after the durations of 6 and 12 hours and inhibited with the highest and the lowest concentrations (12.5% and 100%) and for all time durations at 50% concentration.

SSRs polymorphism and genetic diversity: Evaluation of the genotoxic effects of *P. harmala* extracts at the DNA level using *V. faba* bioassay were conducted using microsatellites or simple sequence repeats (SSRs). SSRs are composed of tandem repeats of short sequence motifs (1–6 bp) that are polymorphic, mainly in the number of tandem repeated units. Therefore, different chromosomal mutations as well as point mutations can be detected by this type of molecular marker.

In the present study, the application of SSR marker analysis was used for the determination of genetic stability of the SSRs in the treated samples of *V. faba* plants with different extracts and concentrations of *P. harmala* seeds. The treated samples of *V. faba* with different concentrations (12.5, 25, 50 and 100%) of either aqueous or ethanol extract of *P. harmala* seeds for different duration times (3, 6, 12, and 24 hours) with control were analyzed using 12 SSR primer pairs. The application of SSR marker analysis using Capillary Electrophoresis (CE) apparatus showed differences in

SSR profiles within and between the two extracts. All the 12 SSR primer pairs used produced amplification products which showed 100% polymorphism. This polymorphism indicates the induction of mutations on almost all the tandem repeat region due to the treatment with the genotoxic extract.

The total number of scoreable alleles were 112 alleles over all 12 loci used (Table 3). Of the 112 alleles detected, 12 alleles (10.7%) were considered as zero alleles. The number of alleles which produced by various primers ranged from 3 were detected at locus VfG_22 to 15 at locus VfG_693 and VfG_873 with an average of 9.3 bands/SSR (an example of gel image of the amplification pattern obtained with primer VfG_693 is shown in Fig. 2A, B). A larger size range of amplified bands was observed in these treatments as well as of control. These values are higher than the values reported in faba bean by Zeid *et al*. (2009). The discrimination power of each SSR locus was estimated by the PIC Values which ranged from 0.47 for locus VfG_22 to 0.88 for locus VfG_693 with a mean of 0.70 (Table 3). A reflection of allele diversity and frequency among all the treatments with aqueous and ethanolic showed variation from one locus to another. The genetic diversity of each SSR locus appeared to be associated with the number of alleles detected per locus. The higher of PIC value for the locus associated with, the higher number of alleles detected (Table 3). This observation is in agreement with what was reported by Yu *et al*. (2003) and Lapitan *et al*. (2007).

In 5 out of the 12 SSRs used, only one or two bands were present per treatment, suggesting the detection of a single locus (Viruel *et al*., 2005). The other seven primer pairs (vfG_33, vfG_93, vfG_283, vfG_413, vfG_444, vfG_693 and vfG_813) produced more bands than expected according to the diploid construction of this specie, probably due to the genomic rearrangements accumulated or due to the duplication of some loci in *V. faba* beans under effective treatments. No fixed alleles were shown within the treatments and the control over all the primer pairs used in this study, compared to the presence of a high number of new alleles (Tables 4, 5). This may indicate that a high variability is detected due to the genotoxic effect of the different treatments with *P. ha rmala* extracts, since *P. harmala* was listed as one of the "worst weeds in the West" by the Centre for Invasive Plant Management (CIPM, 2009). Mekki (2013) also reported on a great variations in mitotic indix (MI), the percentage of micronuclei and the percentage of chromosomal aberrations due to the treatment with *P. harmala* extracts. These effects might be also due to the presence of some heavy metals in the extract as stated in the studies of Al-Moaruf *et al*. (2004) and Haider *et al*. (2004) on the extracts of *Azadirachta indica*, *Mangifera indica*, *Cymbopogon citratus* and *Morinda lucida*.

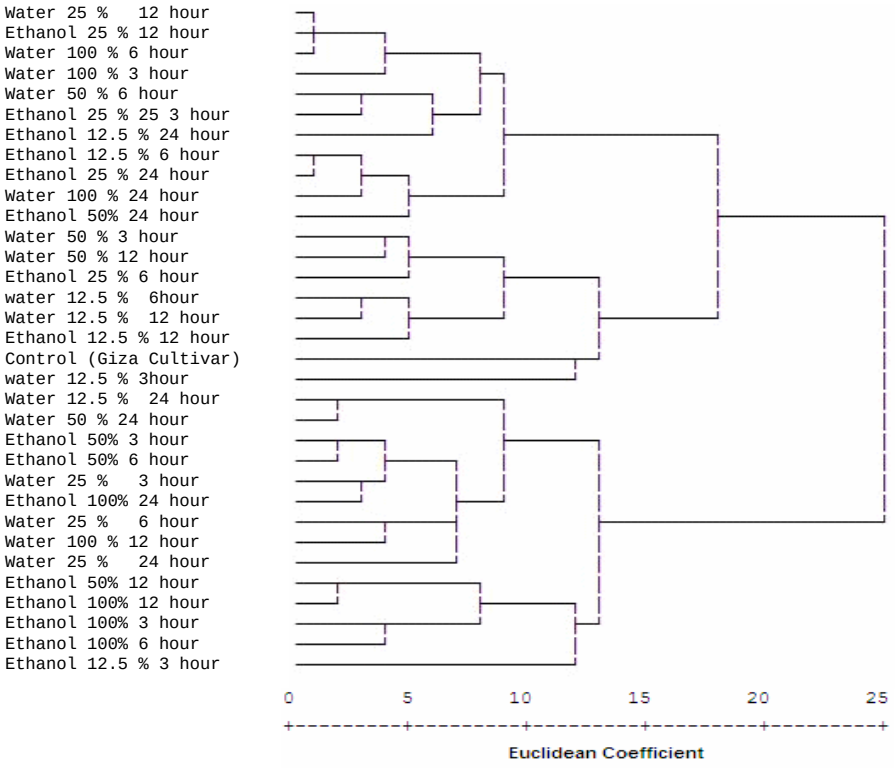

Fig. 1. Dendrograms of 32 treatments and its control (Giza cultivar) obtained by UPGMA cluster analysis based on morphological traits.

Table 3. SSR primers, number of fragments, percentage of polymorphism and polymorphism information content (PIC)in 32 treatments and Giza cultivar (control).

Primer	Repeat type & length	Annealing tem	PIC Values	Total number of alleles	Polymorphic alleles	Percentage of polymorphism
VfG_13	(AG)15	58	0.68	9	9	100%
VfG_22	(AG)15	58	0.47	3	3	100%
VfG_33	(AG)7 + 14	60	0.72	8	8	100%
VfG_193	(AG)9	58	0.62	11	11	100%
VfG_93	(AG)16	55	0.61	6	6	100%
VfG_283	(AG)19	50	0.65	8	8	100%
VfG_313	(AG)7	58	0.66	8	8	100%
VfG_344	(AG)12	50	0.76	8	8	100%
VfG_413	(AG)7 + 10	50	0.73	10	10	100%
VfG_444	(AG)12	57	0.82	11	11	100%
VfG_693	(AG)9	56	0.88	15	15	100%
VfG_873	(AG)10	58	0.86	15	15	100%

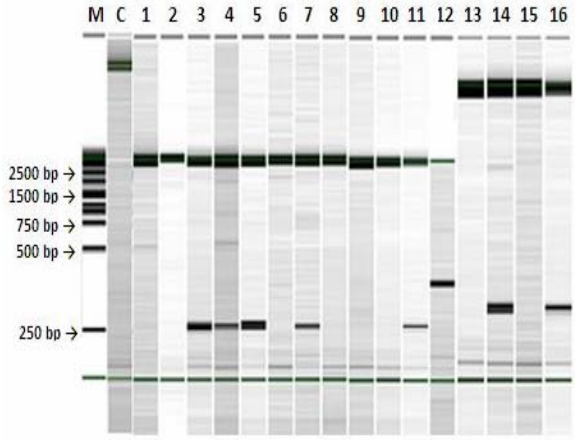

Fig. (2 -A): SSR profiles in the 16 treatments with water extract. M = DNA marker, C= untreated sample, 1-4 treated samples with 12.5 % of *P. harmala* water extract, 5-8 Treatments with 25%, 9-12 Treatments with 50% and 13-16 Treatments with 100%. Each concentration applied for 3,6,12 and 24 hours.

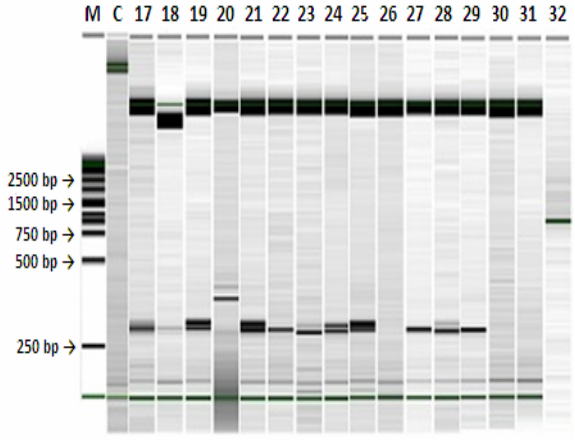

Fig. (2-B): SSR profiles in the 16 treatments with ethanol extract. M=DNA marker, C= untreated sample, 17-20 treated samples with 12.5 % of *P. harmala* ethanol extract, 21-24 Treatments with 25%, 25-28 Treatments with 50% and 29-32 Treatments with 100%. Each concentration applied for 3,6,12 and 24 hours.

The presence of the high polymorphism of DNA may also indicate important structural changes that can significantly affect the chemical reaction of PCR events (Bowditch *et al*., 1993). Appearance of new PCR products, occurred because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural changes. It may be also because some changes in DNA sequence have occurred due to mutations, and/or large deletions (bringing two pre-existing annealing sites closer) and/or due to homologous recombination, in which two sequences that match the sequence of the primer (Atienzar *et al*., 1999).

The number of new alleles produced by the 12 primers in each treatment with aqueous extract ranged from 2 to 21, with an average of 6.3 alleles per treatment. Out of the 16 treatments with aqueous extract, 5 treatments produced only 2 - 6 new alleles with the 12 primers (T1, 2, 4, 14, and 15). The other 11 treatments produced from 11 to 22 new alleles, the maximum number was observed in T7 (25% for 12h.) while the minimum number was detected in T6 (25% for 6 h.) (Table 4). On the other hand, the number of new alleles observed in the 16 samples treated with ethanol extract were found to range from 1 in treatments 6 and 15 to 24 new alleles in treatment 5. Treatments, T6, 9, 10, 14 and 15 produced only 1, 2, or 3 new alleles with the 12 primers. The other 11 Treatments produced higher numbers of new alleles ranged from 12 in treatment 1 to 24 alleles in treatment 5 (Table 5).

The appearance of extra bands occurred mostly with treatments for different loci, the apparent new bands may also be the results of genomic template instability related to the level of DNA damage, the efficiency of DNA repair and replication (Atienzar, 1999). Previous studies have shown that changes in DNA fingerprint (i.e. band patterns) observed reflect DNA alterations in genome from single base changes to complex chromosomal rearrangements (Atienzar *et al*., 1999; Atienzar *et al*., 2002) and that DNA fingerprinting offers a useful biomarker assay in assessment of genotoxicity (Savva 1996, 1998).

Table 4. The number of alleles (bands) in control and the molecular size (base pair bp) of the disappearance (a) and/or appearance (b) of bands for all primers in the *V. faba* plant samples treated with water extract of *P. harmala* seeds.

	Control	12.50%				25%			
		3h	6h	12h	24h	3h	6h	12h	24h
Primer 1	1	a 255 b 0	a 255 b 0	a 255 b 66	a 255 b 0	a 255 b 246	a 255 b 246,268	a 0 b 253, 290	a 255 b 246,274
Primer 2	1	a 46 b 0	a 46 b 0	a 46 b 36	a 46 b 0	a 46 b 0	a 46 b 0	a 46 b 0	a 46 b 0
Primer 3	3	a 41,59,192 b 0	a 41,59,192 b 0	a 41,59,192 b 157	a 41,59,192 b 0	a 41,59,192 b 47, 157	a 41,59,192 b 0	a 41,59, 192 b 157	a 41,59,192 b 157, 168
Primer 4	2	a 69, 273 b 0	a 69, 273 b 0	a 69, 273 b 206	a 69, 273 206	a 69, 273 b 0	a 69, 273 b 206	a 69, 273 b 0	a 69, 273 b 0
Primer 5	1	a 255 b 0	a 255 b 0	a 255 b 0	a 255 b 0	a 255 b 41, 167	a 255 b 0	a 255 b 41, 155	a 255 b 0
Primer 6	2	a 28, 59 b 0	a 28, 59 b 0	a 59 b 51	a 28, 59 b 0	a 28, 59 b 33,51,296	a 28, 59 b 0	a 28, 59 b 91,296	a 28, 59 b 0
Primer 7	2	a 24, 152 b 0	a 24, 152 b 0	a 24, 152 b 131	a 24, 152 b 0	a 24, 152 b 131	a 24, 152 b 0	a 24,152 b 24,131	a 24, 152 b 131
Primer 8	2	a 192,221 b 0	a 192,221 b 0	a 192,221 b 171,204	a 192,221 b 0	a 192, 221 b 171,204,	a 192,221 b 208	a 192,221 b 171, 204	a 192,221 b 171, 204
Primer 9	1	a 67 b 0	a 67 b 0	a 67 b 53,241	a 67 b 241	a 67 b 221,246	a 67 b 246	a 67 b 204, 246	a 67 b 204,246
Primer 10	1	a 195 b 0	a 195 b 0	a 195 b 43,278,287	a 195 b 43, 278	a 195 b 43, 278	a 195 b 43,281,287	a 195 b 43,278,287,312	a 195 b 43,273,287
Primer 11	1	a 51 b 75,255,263	a 51 b 75,264	a 51 b 81,264,275	a 51 b 330,357	a 51 b 75,264,270	a 51 b 75,264	a 51 b 44,70,255,270	a 51 b 75,258,270
Primer 12	1	a 228 b 0	a 28 b 0	a 228 b 54	a 228 b 0	a 228 b 248	a 228 b 248	a 228 b 248	a 228 b 248
Total	18	a(18) b(3)	a(18) b(2)	a(17) b(17)	a(18) b(6)	a(18) b(19)	a(18) b(11)	a(17) b(22)	a(18) b(16)
	Control	**50%**				**100%**			
		3h	**6h**	**12h**	**24h**	**3h**	**6h**	**12h**	**24h**
Primer 1	1	a 255 b 246	a 255 b 246	a 255 b 246	a 255 b 246	a 255 b 246	a 255 b 0	a 255 b 0	a 255 b 246,261
Primer 2	1	a 46 b 0	a 46 b 36	a 46 b 36	a 46 b 0	a 46 b 36	a 46 b 0	a 46 b 0	a 46 b 36
Primer 3	3	a 41,59,192 b 47, 168	a 41,59,192 b 0	a 41,59,192 b 157	a 41,59,192 b 157	a 41,59,192 b 32,47,157,168	a 41,59,192 b 0	a 41,59,192 b 0	a 41,59,192 b 157
Primer 4	2	a 69, 273 b 178	a 69, 273 b 206	a 69, 273 b 205	a 69, 273 b 206	a 69, 273 b 0	a 69, 273 b 0	a 69, 273 b 236, 267	a 69, 273 b 0
Primer 5	1	a 255 b 0	a 255 b 0	a 255 b 0	a 255 b 41, 167	a 255 b 167	a 255 b 0	a 255 b 0	a 255 b 41,134,167
Primer 6	2	a 28, 59 b 0	a 28, 59 b 91,251,296	a 28, 59 b 0	a 28, 59 b 0	a 28, 59 b 296	a 28, 59 b 0	a 28, 59 b 0	a 28, 59 b 0
Primer 7	2	a 24, 152 b 0	a 24, 152 b 131	a 24, 152 b 131	a 24,152 b 127	a 24, 152 b 464	a 24, 152 b 464	a 24, 152 b 0	a 24, 152 b 464
Primer 8	2	a 192,221 b 171, 204	a 192,221 b 171, 204	a 192,221 b 171, 204	a 192,221 b 171, 204	a 192,221 b 171, 204	a 192,221 b 0	a 192,221 b 0	a 192,221 b 171, 204
Primer 9	1	a 67 b 246	a 67 b 246	a 67 b 246	a 67 b 246	a 67 b 221, 241	a 67 b 0	a 67 b 0	a 67 b 53,226,246
Primer 10	1	a 195 b 43,278,287	a 195 b 278	a 195 b 273,287	a 195 b 43,278,287,312	a 195 b 43,278,287	a 195 b 0	a 195 b 0	a 195 b 43,273,287
Primer 11	1	a 51 b 44,75,264,270	a 51 b 70	a 51 b 75,255,273	a 51 b 75,255,275	a 51 b 75	a 51 b 75	a 51 b 75	a 51 b 0
Primer 12	1	a 228 b 202,220,248,279	a 228 b 46,248,260	a 228 b 248	a 228 b 248	a 228 b 244	a 228 b 0	a 228 b 0	a 228 b 202,241,266,285
Total	18	a(18) b(18)	a(18) b(15)	a(18) b(13)	a(18) b(17)	a(18) b(18)	a(18) b(2)	a(18) b(3)	a(18) b(20)

Table 5. The number of alleles (bands) in control and the molecular size (base pair bp) of the disappearance (a) and/or appearance (b) of bands for all primers in the *V. faba* plant samples treated with ethanol extract of *P. harmala* seeds.

	Control	12.50%				25%			
		3h	6h	12h	24h	3h	6h	12h	24h
Primer 1	1	a 255 b 0	a 255 b 246	a 255 b 246	a 255 b 246	a 255 b 246	a 255 b 0	a 255 b 66,250	a 255 b 66
Primer 2	1	a 46 b 36	a 46 b 36	a 46 b 36	a 46 b 36	a 46 b 36	a 46 b 0	a 46 b 36	a 46 b 0
Primer 3	3	a 41,59, 192 b 157	a 41,59, 192 b 157	a 41,59, 192 b 157, 168	a 41,59, 192 b 0	a 41,59, 192 b 47,157,168	a 41,59, 192 b 0	a 41,59, 192 b 157	a 41,59, 192 b 47
Primer 4	2	a 69,273 b 206	a 69,273 b 0	a 69,273 b 178, 206	a 69,273 b 178	a 69,273 b 182,206	a 69,273 b 0	a 69,273 b 0	a 69,273 b 39,59
Primer 5	1	a 255 b 41	a 255 b 41,167	a 255 b 41,134,167	a 255 b 0	a 255 b 167	a 255 b 0	a 255 b 167	a 255 b 0
Primer 6	2	a 28,59 b 33, 51	a 28,59 b 33, 51	a 28,59 b 51	a 59 b 51	a 28,59 b 33, 51	a 28,59 b 0	a 28,59 b 0	a 28,59 b 33, 51
Primer 7	2	a 24,152 b 131	a 24,152 b 131, 263	a 24, 152 b 131	a 24, 152 b 131	a 24,152 b 131	a 24, 152 b 0	a 24,152 b 131	a 24,152 b 131
Primer 8	2	a 192, 221 b 171	a 192,221 b 176,204	a 192,221 b 171, 204	a 192,221 b 54, 204	a 192, 221 b 176, 208	a 192,221 b 0	a 192,221 b 176, 208	a 192,221 b 54, 204
Primer 9	1	a 67 b 226, 246	a 67 b 221, 246	a 67 b 226, 246	a 67 b 246	a 67 b 226, 246	a 67 b 0	a 67 b 246	a 67 b 53,63,246
Primer 10	1	a 195 b 278, 287	a 195 b 273, 287	a 195 b 278,287	a 195 b 55,73,278,287	a 195 b 43,278,287,318	a 195 b 0	a 195 b 43, 278	a 195 b 43,73,278,318
Primer 11	1	a 51 b 0	a 51 b 0	a 51 b 260, 270	a 51 b 75,260,270	a 51 b 260, 275	a 51 b 75	a 51 b 270	a 51 b 44, 75,
Primer 12	1	a 228 b 0	a 228 b 256	a 228 b 54	a 228 b 54	a 228 b 213,238,256	a 228 b 0	a 228 b 54	a 228 b 54
Total	18	a(18) b(12)	a(18) b(16)	a(18) b(20)	a(17) b(16)	a(18) b(24)	a(18) b(1)	a(18) b(13)	a(18) b(19)
	Control	**50%**				**100%**			
		3h	**6h**	**12h**	**24h**	**3h**	**6h**	**12h**	**24h**
Primer 1	1	a 255 b 0	a 255 b 0	a 255 b 246,261	a 255 b 246	a 255 b 246	a 255 b 0	a 255 b 0	a 255 b 246
Primer 2	1	a 46 b 36	a 46 b 36	a 46 b 36	a 46 b 36	a 46 b 0	a 46 b 0	a 46 b 0	a 46 b 36
Primer 3	3	a 41,59, 192 b 0	a 41,59, 192 b 0	a 41,59, 192 b 157	a 41,59, 192 b 157	a 41,59, 192 b 47,157,168	a 41,59, 192 b 0	a 41,59, 192 b 0	a 41,59, 192 32,47,157
Primer 4	2	a 69,273 b 0	a 69,273 b 0	a 69,273 b 178,206	a 69,273 b 0	a 69,273 b 0	a 69,273 b 0	a 69,273 b 0	a 69,273 b 0
Primer 5	1	a 255 b 0	a 255 b 0	a 255 b 0	a 255 b 41	a 255 b 0	a 255 b 0	a 255 b 0	a 255 b 0
Primer 6	2	a 28,59 b 0	a 28,59 b 0	a 28,59 b 0	a 28,59 b 33	a 28,59 b 0	a 28,59 b 0	a 28,59 b 0	a 28,59 b 296
Primer 7	2	a 24, 152 b 0	a 24, 152 b 0	a 24,152 b 131	a 24, 152 b 0	a 24, 152 b 131	a 24, 152 b 0	a 24, 152 b 0	a 24, 152 b 131
Primer 8	2	a 192,221 b 0	a 192,221 b 0	a 192,221 b 171, 204	a 192,221 b 171, 204	a 192,221 b 171, 204	a 192,221 b 0	a 192,221 b 0	a 192,221 b 0
Primer 9	1	a 67 b 0	a 67 b 0	a 67 b 246	a 67 b 246	a 67 b 246	a 67 b 0	a 67 b 0	a 67 b 246, 269
Primer 10	1	a 195 b 0	a 195 b 0	a 195 b 43,278,287,312	a 195 b 278,287,312	a 195 b 43,273,287	a 195 b 0	a 195 b 0	a 195 b 43, 278
Primer 11	1	a 51 b 81	a 51 b 75	a 51 b 75, 270	a 51 b 127, 401	a 51 b 81	a 51 b 75,260,270	a 51 b 75	a 51 b 75,260,270
Primer 12	1	a 228 b 0	a 228 b 0	a 228 b 54,213,256,266	a 228 b 46,260	a 228 b 202,220,244,279	a 228 b 0	a 228 b 0	a 228 b 54,238,244
Total	18	a(18) b(2)	a(18) b(2)	a(18) b(20)	a(18) b(15)	a(18) b(16)	a(18) b(3)	a(18) b(1)	a(18) b(17)

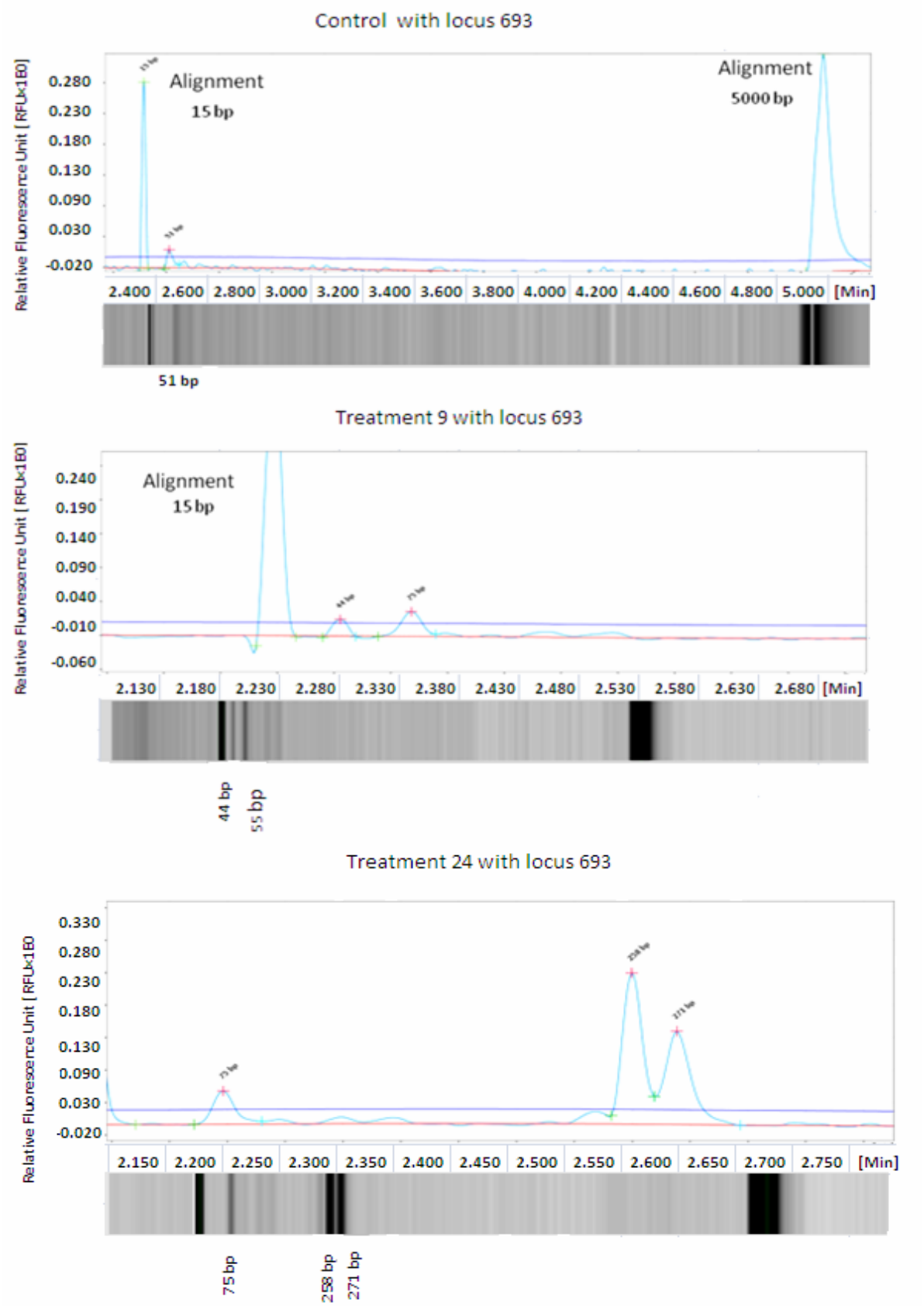

Fig. 3. Capillary electropherogram of SSRs products for control, water (9) and ethanol (24) treatment with locus 693.

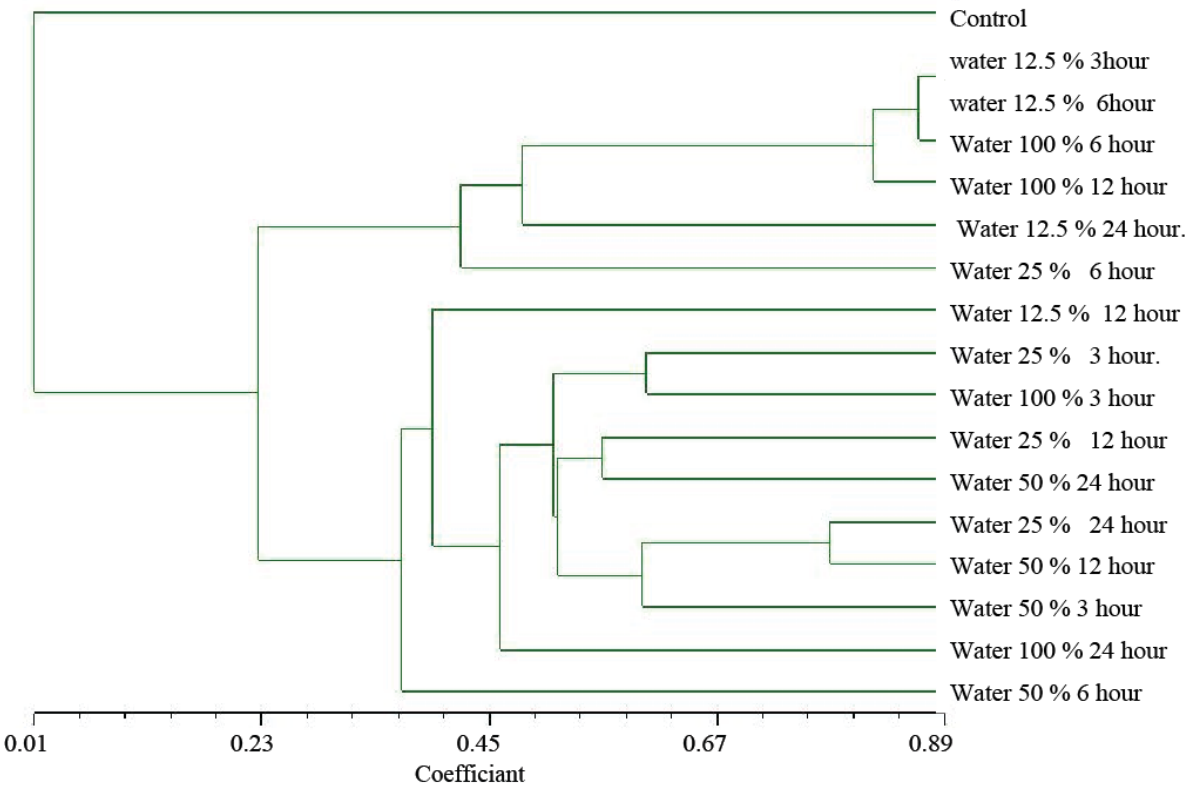

Fig. 4. UPGMA dendrogram of the Water treatments and control cultivar with 94 SSR fragments using the similarity matrix generated by Dice coefficient.

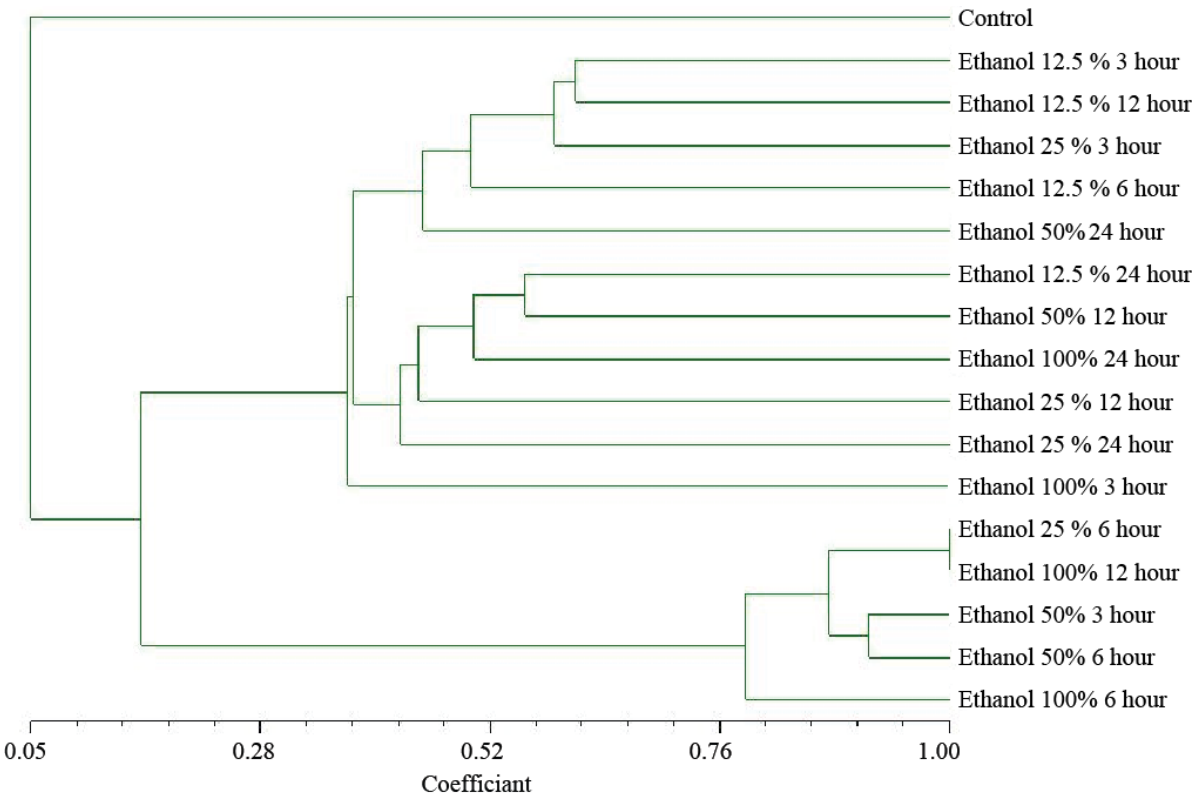

Fig. 5. UPGMA dendrogram of the Ethanol treatments and control cultivar with 91 SSR fragments using the similarity matrix generated by Dice coefficient.

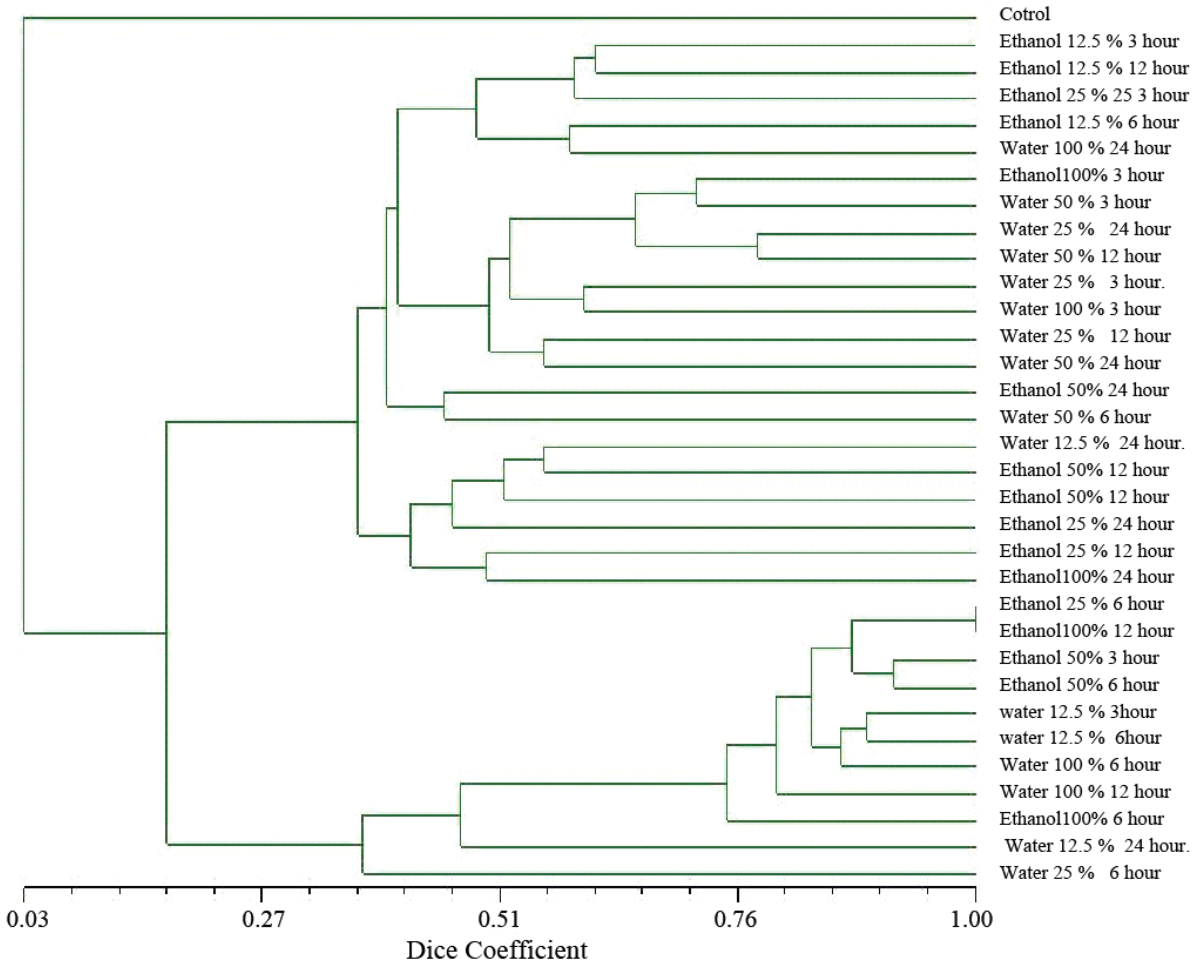

Fig. 6. UPGMA dendrogram of the 32 treatments and control cultivar with 112 SSR fragments using the similarity matrix generated by Dice coefficient.

About 83.3% of the 18 alleles observed in untreated samples using the 12 primers disappeared from almost all the treatments with water and ethanol extracts (an example is shown in Fig. 3) except 3 (16.7%) alleles observed in the samples treated with 12.5 and 25% water extract for 12 hr with primer VfG_13 and VfG_283 respectively, and in 12.5 % of ethanol extract after exposure to 24 hr with primer VfG_283.

The dendrogram generated using the Dice similarity coefficients and based on 12 SSR loci, grouped the 16 treatments with aqueous extract and control into 4 clusters, (Fig. 4). Control was clustered alone in group 1, the second and the third groups included all the treated samples except one sample, T10 (treated with the concentration 50% For 6 h.) which was grouped in a separate group (group 4). Group 2 included 6 treated samples (T1, 2, 4, 6, 14 and T15). Treatments 1, 2, showed maximum genetic similarity, these two treatments were exposed to the lowest concentration (12.5%) for the lower durations (3 and 6 h.) and grouped close to the control. However different situation was observed with T_{14} and T_{15} in which they were treated with the highest concentration 100% for 6 and 12h. Treatment 4 and 6 (both treatments with 12.5% for 24 and 25% for 6 h respectively). T4 showed two new rare alleles with size 330 and 357 bp generated by primer VfG_963 only in this treatment (Fig. 2A). Group 3 included all the other 9 treated samples (T3, 5, 7, 8, 9, 11, 12, 13 and 16). These samples in group 3 included samples from the four concentrations 12.5% for 12 h., 25% for 3, 12 and 24 h., 50% also for durations 3, 12, and 24h, 100% treatment for 3 and 24 hours. All the 9 samples generated new alleles ranged from 13 to 22 alleles.

The dendrogram for the samples treated with ethanol extract grouped the 16 treated samples and the control into 5 clusters (Fig. 5). Group 1 included control only separated from the treated samples. Group 2 included 5 treated samples, 3 (T1, 2, 3,) of them are treatments with the lowest concentration (12.5% for 3, 6 and 12h) and the fourth sample (T5) is a treatment with 25% for 3 h. but the other treatment T12 is the treatment with 50% for 24h.

Group 3 included 5 treated samples (T4, 7, 8, 11, 16), all of them are treatment for either 12 or 24 h. Group 4 has only one treatment (T13), the treatment with 100% for 3h. Group 5 included 5 treated samples (T6, 9, 10, 14 and 15). This group had 2 samples from 100% concentration and 2 samples from 50% concentration and one sample from 12.5 % concentration. All the five treatments were exposed for 3, 6 or 12 hours.

The combined dendrogram for the all 32 samples treated with aqueous and ethanolic extracts as well as the control are show in (Fig. 6). The combined dendrogram of the data of SSR grouped all the data (32 treated samples and control) into 6 main gropes. The Control was clearly separated alone in group 6. This indicates the occurrence of great variation in DNA level in all treatments by *P. harmala* extracts. While in the dendrogram based on the morphological characteristics, the control was clustered with the sample treated with the lowest concentration for the lower duration. This treatment with the lowest concentration of water extract for the lowest duration (12.5% for 3h.) has previously showed the minimum effects in the percentage of nuclear and chromosomal aberrations and mitotic index (Mekki, 2014).

In conclusion, our data support the view that the SSR analysis is a highly sensitive method for the detection of DNA damage induced by different mutagenic agents and toxic chemicals. Moreover, as the objective in this research was to establish the existence of DNA damage for hazard identification in risk assessment of *P. harmala* extract, the presence of polymorphism in the DNA ‘‘fingerprint” SSR profiles can provide sufficient evidence for identification of the genotoxic effect. Consequently, that SSR markers will become a useful tool in assessment genetic diversity induced by mutation. This result is agreement with that of (Kumar *et al*., 2011) who reached the same conclusion using ISSR markers. Nevertheless, distinct classification of treatments based on agro-morphological and SSR data in the present study implies that morphological and SSR markers reflect different aspects of genetic variation among treatments.

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