Original Article

Detection of the Genotoxicity of *Gentiana* L. Extracts by Using RAPD-PCR and ISSR-PCR Techniques

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ABSTRACT

Backround: The RAPD- and ISSR-PCR techniques are offering an insight into the detecting the genotoxicity of *Gentiana* extracts. **Objective:** It is aimed with the present study, to detect the genotoxicity of methanol extracts of ten Turkish *Gentiana* L. taxa on germinated *Allium cepa* L. root tips. **Methods:** RAPD- and ISSR-PCR techniques were used for detection of genotoxicity of *Gentiana* extracts. **Results:** Four RAPD and three ISSR primers produced the reproducible polymorphic and monomorphic banding patterns among 10 RAPD and 10 ISSR primers for all DNA samples. It is not any serious alteration along with band intensity change, the disappearance of the bands, and appearance of the new bands in the band profiles amplified from the *Gentiana* extracts-treated genomic DNA sample of *A. cepa.* The most efficient results were obtained by RAPD-P9 and ISSR-1 primer among the seven productive primers. **Discussion:** It is not observed any variation in the RAPD- and ISSR-PCR band profiles in time and concentration-dependent manner. **Conclusion:** It is determined that the three different concentrations of *Gentiana* extracts did not interact with the *A. cepa* DNA.

Key words: *Gentiana* L., Genotoxicity, RAPD-PCR, ISSR-PCR.

INTRODUCTION

The genus *Gentiana* L. is a member of Gentianaceae which has world-wide distribution, but especially in temperate regions and about half the genera are grown in South America. The genus *Gentiana* L. is represented by 360 species all around the world.[1] In Turkey the genus *Gentiana* is represented by 12 species and 14 taxa.[2] *G. lutea* L., officinal species, roots contain the secoiridoid glucosides which are bitter tasting. The roots are used for the treatment of liver complaints, indigestion, gastric infections, anorexia, wound healing, hepatoprotective and anti-inflammatory agents in all of the world.[3,4] In Turkey, the roots are also used digestive system stimulant and appetizing, stimulate the red blood cells and against high fever, wound healing (external use of decoction).[5-9] Mostly the aerial parts and roots contain xanthones, free or glycosidic flavonoids, alkaloids, iridoids, secoiridoids and also triterpenoids.[4,10-12] The clastogenic agents cause lesions in the genetic material. The capacity of them called as genotoxicity. The damage in the DNA, mutations and chromosomal alterations are mainly evaluations of genotoxicity. Hence, the genotoxic agents possess the ability of alteration in DNA. The plant test system of *Allium cepa*, as a first screener of genotoxicity, is a model bioindicator. Intensely usages of medicinal plants for the treatment of diseases, it is required to investigate the toxicity potentials of them using *in vivo* test of *A. cepa* against to plant extracts due to their safe and efficient use. And so, the studies

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based on *A. cepa* are helping to prevent damages of human health.[13]

Germinated *Allium cepa* roots were treated with methanol extracts of nine Turkish *Gentiana* species and RAPD- and ISSR-PCR techniques were used to detect the possibly genotoxicity of extracts on *A. cepa* genomic DNA within the present study.

MATERIAL AND METHODS

Plant Materials

The plants were collected from different localities of Turkey and the list for them is given in Table 1. The voucher specimens are deposited in the Herbarium of Atatürk Horticultural Central Research Institute.

Extraction Procedure

Air dried herba of *Gentiana* species (20 g) were crushed; macareted with 500 mL MeOH using shaker incubator for 8 h (x3). The filtered extracts put together and concentrated using rotary evaporator at 40°C.

Genotoxicity Test Material and DNA Isolation

Germinated *Allium cepa* roots were tested at three concentrations (0.05, 0.1 and 0.5 mg/mL) of ten *Gentiana* methanol extracts and control group for 72 h and then grounded in liquid nitrogen. The DNA isolation was carried out by using the DNA purification kit (EurX GeneMATRIX Plant and Fungi DNA Purification Kit). The quantity and quality of the DNA were determined by using a Nanodrop Spectrophotometer (Shimadzu, Japan). DNA samples were diluted with sterile distilled water to 2 ng/µL.

PCR Analysis with RAPD and ISSR Primers

Gentiana extracts-treated *A. cepa* genome were amplified by PCR technique using RAPD and ISSR primers. RAPD-PCR amplifications were performed in 25 µL of reaction mixture containing of template DNA, 1×Taq polymerase buffer and 1 U of Taq polymerase (FIREPol, Solis BioDyne, Estonia), $MgCl_2$, dNTPs (dATP, dCTP, dGTP, and dTTP) and primer (Table 2, 3). Amplifications were carried out in Thermo Arktik Thermal Cycler (Thermo Scientific, USA) that was programmed at Table 4. PCR reactions were repeated twice to ensure reproducibility of amplified products. The PCR products were simultaneously separated on the same agarose gel (1.3%) containing ethidium bromide and photographed with the Gel Logic 212Pro imaging system (Carestream, USA). Molecular weights of PCR products were estimated using a 100 bp DNA Ladder and 100 bp plus DNA Ladder (Fermentas, USA).

Table 1: Collected *Gentiana* taxa and localities.

Plant Taxa	Collected from
G. asclepiadea L.	Mount Ilgaz/Kastamonu
G. cruciata L.	Mount Ilgaz/Kastamonu
G. olivieri Griseb.	Nizip/Gaziantep
G. pyrenaica L.	Zigana Passway/Gümüşhane
G. septemfida Pallas	Soğanlı Passway/Bayburt
G. boissieri Schott and Kotschy ex Boiss. (endemic)	Mount Bolkar/Niğde
G. gelida Bieb.	Çat to Erzurum Road/Erzurum
G. verna L. ssp. *balcanica* Pritchard	Uludağ/Bursa
G. verna L. ssp. *pontica* (Soltok.) Hayek	Soğanlı Passway/Bayburt
G. brachyphylla Vill. ssp. *favratii* (Rittener) Tutin	Mount Bolkar/Niğde

Table 2: RAPD-PCR contents and volumes.

Contents	Volume
dH_2O	11.8 µL
10 X TaqBuffer (Fermantas)	2.5 µL (1x)
25 mM $MgCl_2$ (Fermantas)	1.5 µL (1.5 mM)
2.5 mM dNTP (Fermantas)	2 µL (0.2 Mm)
2.5 µM Primer	4 µL (0.4 µM)
Templete DNA	3 µL
TaqPolimerase (Fermantas)	0.2 µL
Total	25 µL

Table 3: RAPD-PCR application procedure.

	Cycle(s)	Temperature (˚C)	Time
Initial Denaturation	1	95	4 min
Denaturation		94	55 sec
Annealing	45	30-34	60 sec
Extention		72	90 sec
Final Extention	1	72	7 min

Table 4: ISSR-PCR contents and volumes.

Contents	Volume
dH_2O	13.3 µL
10X TaqBuffer (Fermantas)	2.5 µL (1x)
25 mM $MgCl_2$ (Fermantas)	1.5 µL (1.5 mM)
2.5 mM dNTP (Fermantas)	2 µL (0.2 mM)
2.5 µM Primer	2,5 µL (0.25 µM)
Templete DNA	3 µL
TaqPolimerase (Fermantas)	0.2 µL
Total	25 µL

Table 5: ISSR-PCR application procedure.			
	Cycle(s)	**Temperature (˚C)**	**Time**
Initial Denaturation	1	95	4 min
Denaturation		94	45 sec
Annealing	45	49.2-60.5	45 sec
Extention		72	90 sec
Final Extention	1	72	7 min

Table 6: RAPD primers tested for amplification of *A. cepa* genomic DNA.			
Tested Primers	**Primer sequence (5'-3')**	**Tm (°C)**	**Appositeness**
OPC-04	**CCGCATCTAC**	**32**	**+**
OPC-07	**GTCCCGACGA**	**34**	**+**
P-9	**GGGAAGAGAG**	**32**	**+**
P-11	GGCCGATGAT	32	-
P-13	ACCGCCTTGT	32	-
P-14	CAGCACTGAC	32	-
P-16	TGGTGGCCTT	32	-
P-17	GTAGCACTCC	32	-
P-21	ACGGTGCCTG	34	-
OPD-09	**CTCTGGAGAC**	**32**	**+**

Amplification of ISSR fragments from *A. cepa* genomic DNA was performed in a total reaction volume of 25 µl containing of template DNA, 1X Taq polymerase reaction buffer, $MgCl_2$, dNTPs, primer and 1 U of Taq DNA polymerase (FIREPol, Solis BioDyne) (Table 4). Amplifications were performed in Thermo Arktik Thermal Cycler (Thermo Scientific). 4 min initial denaturation at 95°C and 1 cycle, 45 cycles of 45 sec. denaturation at 94°C, 50 sec annealing at 49.2-60.5°C (primer depended) for ISSR amplification, and a 90 sec extension at 72°C, followed by a final extension at 72°C for 7 min (Table 5). PCR-amplified fragments were separated on 1.3% agarose gel containing ethidium bromide. Gels were visualized under UV light and digitally photographed with Gel Logic 212Pro imaging system (Carestream). Molecular weights of ISSR-PCR products were estimated using 100 bp DNA Ladder and 100 bp plus DNA Ladder (Fermentas).

RAPD analysis was performed according to the method of Williams *et al.*[14] A set of 10 random 10-mer primers was purchased from Thermo Inc. (Burlington, MA, USA) and an initial primer screening was carried out for selection of suitable primers. After screening, four primers amplified clear, reproducible banding patterns were used for further analysis (Table 6).

ISSR analysis was performed according to the method of Wongsawad and Peerapompisal.[15] A set of 10 random 10-mer primers was purchased from Thermo Inc. (Burlington, MA, USA) and an initial primer screening was carried out for selection of suitable primers. Two primers amplified clear, reproducible banding patterns and used for further analysis (Table 7).

Gel photographs evaluated with the SynGene-Gene Tools (Cambridge, UK) software. The band profiles of primers and similarity matrix analysis were performed using SynGene-Gene Tools.

RESULTS AND DISCUSSION

The primers RAPD-OPC 04, RAPD-OPC 07, RAPD-OPD 09, RAPD-P9, ISSR-01 and ISSR-847 were amplified clear, reproducible banding patterns after the PCR analysis. The most reproducible banding patterns were obtained from RAPD-P9 (Figure 1) and ISSR-1 (Figure 2). The results were supported also by other primers (RAPD-OPC 04, RAPD-OPC 07, RAPD-OPD 09, and ISSR-847) but RAPD-OPC 04, RAPD-OPD 09, and ISSR-847 were the minority of reproducible banding patterns than the rest ones. The last three primers -RAPD-OPC 04, RAPD-OPD 09, ISSR-847 were not only amplified intraspecific species-specific, but also a lot of non-specific banding patterns, it is obvious that these primers are the least reproducible ones.

The band profiles of the RAPD-P9 (Figure 3) and ISSR 1 (Figure 4) were performed using SynGene-Gene Tools software. Due to the PCR samples were simul-

Table 7: ISSR primers tested for amplification of *A. cepa* genomic DNA.			
Tested Primers	**Primer sequence (5'-3')**	**Tm (°C)**	**Appositeness**
ISSR-01	**AGAGAGAGAGAGAGAGG**	**52.8**	**+**
ISSR-04	ACACACACACACACACC	52.8	-
ISSR-06	GAGAGAGAGAGAGAGAC	52.8	-
ISSR-10	GGGTGGGTTGGGGTG	58.8	-
ISSR-27	GTGCGTGCGTGCGTGC	59.4	-
ISSR-829	TCTCTCTCTCTCTCTCG	52.8	-
ISSR-847	**CACACACACACACACARC**	**53.7**	**+**
ISSR-861	ACCACCACCACCACCACC	60.5	-
ISSR-862	AGCAGCAGCAGCAGCAGC	60.5	-
ISSR-866	CTCCTCCTCCTCCTCCTC	60.5	-

taneously run on the same agarose gel, the gel image of samples after the 9-0.1 mg/mL sample (20. well) is darker. Therefore, some bands of the samples after the twentieth well are invisible.

Depend on the applied concentrations (0.05, 0.1 and 0.5 mg/mL) and ignorance of the non-specific banding patterns, the *Gentiana* extracts have not caused serious band changing within the intraspecific species-specific bands. For the RAPD-P9 primer, the highest concentration (0.5 mg/mL) of the banding patterns of 1th, 6th, 8th and 9th extracts (*G. pyrenaica*, *G. boissieri*, *G. septemfida* and *G. verna* ssp. *balcanica*) thinking the DNA damage due to the band changing. But the comparison to ISSR-1 primer amplification results, the species specific-banding patterns were preserved and there was no band changing for the mentioned extracts. Many studies report that the RAPD/ISSR PCR assays are more sensitive than classic tests such as comet, micronucleus assay due to detecting the temporary DNA changes. RAPD-PCR technique has been used successfully to detect various types of DNA change/damages/mutations in organisms induced by various types of contaminants,[16-20] pollutants.[21-23] Bajpai *et al.*[24] Bakry *et al.*[25] and Zietkiewicz *et al.*[26] used ISSR-PCR technique for investigation of genotoxicity. Along with these studies, it is declared that due to the interaction within contaminants/pollutants/plant extracts and DNA, detecting the changes in the number, position and intensity of DNA bands gave the genotoxicity through DNA damage. There are some studies detecting the genotoxic effects of plant extracts on plant agents. It was reported that changings occurred in RAPD profiles of *Zea mays* seeds treated with *Verbascum speciosum* extract included variation in band intensity, loss of bands and appearance of new bands compared with control.[27] According to another previous study,

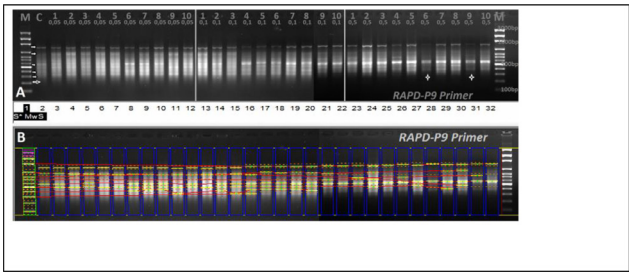

Figure 1A: Gel photographs of amplified PCR banding patterns of *A. cepa* root tip genome with RAPD-P9 primer. A- M: 100 bp DNA marker; C: Control group; 1-10: Methanol extracts of *G. pyrenaica*, *G. brachyphylla*, *G. olivieri*, *G. cruciata*, *G. asclepiadea*, *G. boissieri*, *G. gelida*, *G. septemfida*, *G. verna* ssp. *balcanica*, *G. verna* ssp. *pontica*; NC: Negative control; White filled arrow: Preserved band; Unfilled arrow: Non-specific band; Star: Band changing, B- Gel photographs evaluated with the SynGene-Gene Tools software, Yellow lines: preserved bands, Red lines: partial preserved bands. B. The primer gel photograph evaluated with the SynGene-Gene Tools File Version software.

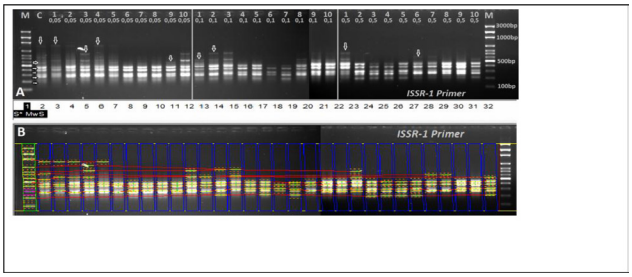

Figure 2: Gel photographs of amplified PCR banding patterns of *A. cepa* root tip genome with ISSR-1 primer. A- M: 100 bp DNA marker; C: Control group; 1-10: Methanol extracts of *G. pyrenaica*, *G. brachyphylla*, *G. olivieri*, *G. cruciata*, *G. asclepiadea*, *G. boissieri*, *G. gelida*, *G. septemfida*, *G. verna* ssp. *balcanica*, *G. verna* ssp. *pontica*; NC: Negative control; White filled arrow: Preserved band; Unfilled arrow: Non-specific band; Star: Band changing, B- Gel photographs evaluated with the SynGene-Gene Tools software, Yellow lines: preserved bands, Red lines: partial preserved bands. B. The primer gel photograph evaluated with the SynGene-Gene Tools File Version software.

Band No.	M	C	1 0.05	2 0.05	3 0.05	4 0.05	5 0.05	6 0.05	7 0.05	8 0.05	9 0.05	10 0.05	1 0.1	2 0.1	3 0.1	4 0.1	5 0.1	6 0.1	7 0.1	8 0.1	9 0.1	10 0.1	1 0.5	2 0.5	3 0.5	4 0.5	5 0.5	6 0.5	7 0.5	8 0.5	9 0.5	10 0.5
1	X																														
2	X																														
3	X																														
4		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X											
11	X															X															
12	X																														
13	X																														

Figure 3: RAPD-P9 primer band profiles obtained using SynGene-Gene Tools.

Band No.	M	C	1 0.05	2 0.05	3 0.05	4 0.05	5 0.05	6 0.05	7 0.05	8 0.05	9 0.05	10 0.05	1 0.1	2 0.1	3 0.1	4 0.1	5 0.1	6 0.1	7 0.1	8 0.1	9 0.1	10 0.1	1 0.5	2 0.5	3 0.5	4 0.5	5 0.5	6 0.5	7 0.5	8 0.5	9 0.5	10 0.5
1	X																														
2	X																														
3	X																														
4		X	X	X		X																									
5	X				X										X																
6	X				X							X		X									X								
7	X	X											X		X													X	X			
8	X	X	X	X	X							X											X								X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
12	X												X				X		X					X	X	X	X					X
13	X																														

Figure 4: ISSR-1 primer band profiles obtained using SynGene-Gene Tools.

some changes were reported to occur in RAPD profiles of germinated *Nepeta nuda* essential oil-treated *Z. mays* seeds.[28] Protein bands and RAPD analyses of *V. faba* treated with three *Dipterygium glaucum* extracts revealed some newly induced proteins and DNA fragments and other disappeared.[29] These studies support that RAPD/ISSR-PCR analysis could be applied as a suitable biomarker assay for the detection of genotoxic effects of plant extracts.

CONCLUSION

Evaluated RAPD and ISSR-PCR amplification patterns are determined that the three different concentrations of ten Turkish *Gentiana* taxa methanol extracts did not interact within the *A. cepa* DNA. The usage of RAPD and ISSR primers for detecting the plant extracts genotoxicity on plant test systems are gave preliminary but predictable and noteworthy outcomes.

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CONFLICT OF INTEREST

The authors declared that there is no conflict of interest in this research.

ABBREVIATIONS

DNA: Deoxyribonucleic acid; **RAPD-PCR:** Random Amplified Polymorphic DNA-Polymerase Chain Reaction; **ISSR-PCR:** Inter Simple Sequence Repeat- Polymerase Chain Reaction.

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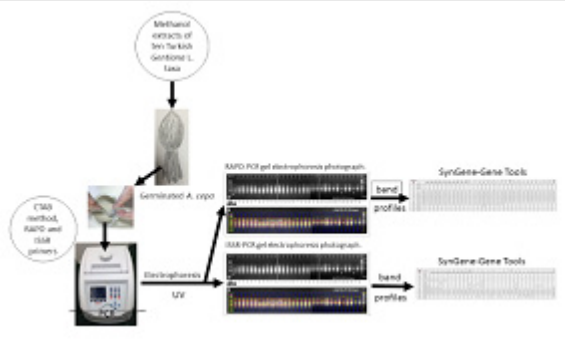
PICTORIAL ABSTRACT



SUMMARY

- Evaluation of the PCR amplification patterns, within the used primers, it is not detected any genotoxicity on *A. cepa* genome treated with *Gentiana* extracts.

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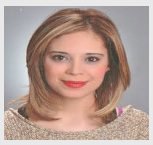

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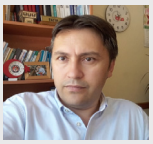

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