

# EVALUATION OF PARSLEY EFFECT AGAINST GENTAMICIN GENOTOXICITY IN RATS USING RAPD ANALYSIS

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**G**entamicin is an aminoglycoside antibiotic, used to treat many types of bacterial infections, particularly those caused by Gram-negative organisms (Robert and Melanie, 2010). It is synthesized by Micromonospora, a genus of Gram-positive bacteria widely present in the environment (water and soil). Gentamicin works by binding the 30S subunit of the bacterial ribosome interrupting protein synthesis. The usual dosage of gentamicin is ranging from 2-5 mg/kg b.wt., however, in critically ill patients and dangerous infections, the dose could be increased up to 7.5-10 mg/kg (Pereira *et al.*, 2009). The use of gentamicin is associated with various toxic effects at its therapeutic doses, especially nephrotoxicity (Nourani *et al.*, 2006; Martinez-Salgado *et al.*, 2007; Balakumar *et al.*, 2010; Dehghani *et al.*, 2011). It is appeared to be a major cause of renal tubular tissue damage which is associated with the production of reactive oxygen species (Abdelaziz and Kandeel, 2011). This toxicity stills the major problem in clinical use (Robert and Melanie, 2010). Recent studies have shown that natural antioxidants obtained from different alternative systems of medicine display a wide range of biological activities. Various alternatives possessing

antioxidant properties were used in order to minimize gentamicin induced oxidative stress in animal models. Many plant extracts were reported to be effective in ameliorating organ toxicities (Upaganlawar *et al.*, 2006; Khan *et al.*, 2009; Abdel-Raheem *et al.*, 2010; Safa *et al.*, 2010).

Parsley is one of the major medicinal herbs. This herb is native to the Mediterranean region, belongs to the family of apiaceae of the genus *Petroselinum* and is known as *Petroselinum crispum*. The leaves and seeds of parsley are widely used traditionally as a food additive and herbal remedies for many ailments. In traditional medicine, it is used for treatment of digestive disorders, flatulence, insomnia, renal disorders, loss of appetite and as a diuretic. A large number of compounds have been isolated from parsley, including flavonoids, euginol, polyphenols, cineole, citronelol, coumarins and hydroxy coumarins (Shin *et al.*, 2010). It is considered as a good source of antioxidants, folic acid, vitamin C and vitamin A. The flavonoid apigenin is regarded as the active principle of parsley and is known to possess an anticarcinogenic (Birt *et al.*, 1997; Messina *et al.*, 2002), anti-inflammatory (Lee *et al.*, 1993) and

antimutagenic properties (Kuo *et al.*, 1992).

Random amplified polymorphic DNA (RAPD) is a PCR-based technique that has successfully been used in surveying genomic DNA for evidence of various types of DNA damage and mutational events in bacteria, plants, invertebrate and vertebrate animals (Atienzar *et al.*, 2002; Liu *et al.*, 2005; Cencki *et al.*, 2009). RAPD is the most widely used tool for assessment of the genetic variation due to a number of advantages (Atienzar *et al.*, 1999; Singh *et al.*, 1999). The assay of RAPD is suitable for any extracted DNA of sufficient quality, allows rapid analysis of a large number of samples. As arbitrary primers are used, specific details of DNA damage or the genome sequence in organisms are not needed. Furthermore, no radioactivity or enzymatic degradation of PCR products is required prior to analysis. Previous studies have shown that changes in DNA fingerprint (i.e. band patterns) observed reflect DNA alterations in genome from single base changes (point mutations) to complex chromosomal rearrangements (Atienzar *et al.*, 1999; 2002; Hagger *et al.*, 2005; Unyayar *et al.*, 2006) and that DNA fingerprinting offers a useful biomarker assay in assessment of genotoxicity (Savva, 1996 & 1998).

The present study was designed to evaluate the efficiency of parsley (leaves extract, fresh leaves, seeds and oil) in reducing the gentamicin genotoxic effects in

rat's liver and spleen using RAPD analysis.

## MATERIALS AND METHODS

### *Gentamicin*

Gentamicin was obtained from Nefrtary Company for Chemical and Drugs, Tanta, Egypt.

### *Plant materials*

Parsley (*Petroselinum crispum*) is the medicinal plant that utilized in this study. Four different forms of parsley were used to be tested for antigenotoxic activities. The applicable materials were parsley leaves extract, fresh leaves, seeds and oil. Leaves, seeds and oil were obtained from local shops of herbalists at Tanta, Egypt.

### *Preparation of leaves extract*

Leaves extract was carried out as practiced locally. One hundred gram of leaves were weighted and crushed in a mortar, the powder were soaked separately in 1 L of distilled water and boiled for 10-15 minutes. The extract was filtered separately with a 2.5 mm filter (WhatmanR no. 42) to remove the suspended particles and subsequently, the filtrate (the leaves extract) stored in the refrigerator at 4°C until use.

### *Experimental animals*

Thirty six adult male albino rats (Sprague-Dawely); *Rattus norvegicus* var. albus, weighting 130-150 g were pur-

chased from the Biological Products & Vaccines Holding Company, Helwan Farm, Cairo, Egypt. Rats were kept under the laboratory conditions of  $25\pm 5^{\circ}\text{C}$  and  $65\pm 5\%$  R. H. for two weeks as an acclimatization period. They were housed in metal wire cages (35x25x20 cm) and maintained on *ad libitum* diet and water. All animals received humane care in accordance with the protocol of National's Animal Care and Use Committee and Guidelines for the Care and Use of Experimental Animals and in accordance to Helsinki Declaration.

### ***Experimental Design***

After the acclimatization period, rats were randomly divided into six groups involved untreated group and treated groups. The untreated group (six rats) received the basal diet and considered as a negative control. The other groups (30 rats) were injected intraperitoneally with gentamicin at a dose of 10 mg/kg bwt. every 24 hr. for eight consecutive days according to Martínez-Salgado *et al.* (2007). After that, rats were divided into five groups of six rats each. One group of rats continued on the basal diet without any addition (gentamicin group) and was considered as a positive control. The remaining four groups received the different parsley forms for two weeks as follows: one group was fed on the basal diet and treated orally by stomach tube with 2 ml parsley leaves extract (Ex) whereas the other three groups were

fed on the basal diet supplemented with 10% of each of fresh parsley leaves (L), parsley seeds (S) and parsley oil (O).

At the end of the experimental period, all animals anesthetized with ether 24 h after the last treatment, and weighed. Furthermore, animals were scarified and the internal organs, liver and spleen, were weighed and maintained for DNA-RAPR analysis. The organs weights as well as animal body weights were used for the calculation of relative organ weight as follows: [organ weight (gm)/total body weight (gm)] x 100.

### ***Extraction and PCR amplification of DNA***

Genomic DNA from liver and spleen was extracted and purified using a conventional phenol/chloroform method as described by Tinwell *et al.* (1994). DNA profiles of liver and spleen were generated in RAPD reactions performed in a reaction volume of 25  $\mu\text{l}$ . The conditions of DNA amplification were optimized and followed the procedure of Williams *et al.* (1990) with some modifications. One of four decamer oligonucleotides: OP-A9 (5'-GGGTAACGCC-3'), OP-B5 (5'-TGCGCCCTTC-3'), OP-B7 (5'-GGTGACGCAG-3') and OP-B8 (5'-GTCCACACGG-3') (Bio Basic Inc, Canada) were used for each amplification. Each amplification reaction contained 40 ng template DNA, 0.8  $\mu\text{M}$  primer, 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTPs mix, 1 U Taq

DNA polymerase (ROVALAB, Germany) and 1 x reaction buffer ( $Mg_2^+$  free). The amplification was performed using a thermal cycler programmed for 1 cycle at 94°C for 5 min of denaturation, followed by 35 cycles consisting of 30 sec of denaturation at 94°C, 45 sec of annealing at 30°C and 1.5 min of extension at 72°C. An additional final extension step at 72°C for 2 min was included followed by hold at 4°C. PCR reaction products (12.5  $\mu$ L) were separated by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. A known DNA Ladder (50 bp DNA Ladder ready-to-use, Cat-no: 300003, GeneON) was run against the PCR products.

### **Data Analysis**

Obtained data were expressed as mean $\pm$ standard deviation (SD). Analysis of variance (ANOVA) was performed using the Statistical Package for Social Sciences (SPSS) software for Windows version 11.0. Genomic template stability (GTS) was calculated by following equation:  $GTS (\%) = (1 - a/n) \times 100$ ; since  $a$  is the number of polymorphic bands detected in each treated sample, and  $n$  is the number of total bands detected in the control. Polymorphism observed in RAPD profile included disappearance of a normal band and appearance of a new band in comparison to control RAPD profile (Luceri *et al.*, 2000; Atienzar *et al.*, 2002; Qari, 2010).

## **RESULTS AND DISCUSSION**

### ***Effects of parsley feeding on relative organ weight***

The mean values of relative weights of liver and spleen for rat groups fed on different parsley forms are presented in Table (1). No significant differences were found between the negative control rat group and all of the tested groups in relative liver weights. These results were in agreement with Awe and Banjoko (2013) who demonstrated that the liver weight was not affected after eight weeks of treatment with the leaf ethanol extract of *Petroselinum crispum*. Concerning the relative spleen weight, the positive control group recorded the highest significant decrease ( $0.33\pm 0.11\%$ ) than the other tested rat groups as well as the negative control group that recorded the highest value ( $0.45\pm 0.44\%$ ). All rat groups fed on parsley recorded a significant increase in relative spleen weights compared with the positive control group but it remains significantly decreased than negative control group. Only rat group fed on parsley oil did not differ significantly than negative control group. These results were in constant with the results of Abdelghany *et al.* (2012), who found that the ratio of spleen to total body weight was significantly smaller in the gentamicin nanoparticle treated cohort compared to the control and free gentamicin drug treatment groups after 48 hours.

***RAPD profile analysis***

RAPD profiles generated from DNA of rat's liver and spleen in the different groups were presented in Fig. (1). Four oligonucleotide primers were used for detection the changes in RAPD profiles of rat's liver and spleen DNA compared to the negative control group. Profiles generated by these primers revealed differences between negative control group and all other groups for both liver and spleen, with visible changes in the number and the intensity of amplified DNA fragments. Total number of bands as well as number of polymorphic bands were primer dependant and highly variable. For each primer, the amplified polymorphic bands were different with treatment groups.

Results of RAPD profile for liver DNA of rat groups fed on different forms of parsley were showed in Table (2). As can be seen, four random primers generated a total of twenty RAPD bands in negative control rats group. Band numbers are ranging from two bands for primer OP-B7 to eight bands for primer OP-B5 (Fig. 1). The four random primers tested gave specific and stable results, with apparent changes in the number and the intensity of amplified DNA bands. The positive control group gave variable bands as change intensity (increase/decrease) and polymorphic bands compared to the negative control group. An increase in band intensity was the major event arising in the patterns generated from liver DNA in positive control group. The results of RAPD profile for rat groups fed on different pars-

ley forms showed that the changes of bands intensity which were increased by gentamicin effect (positive control) in liver tissues were decreased in all groups fed on parsley except the group which fed on parsley seeds was increased. The increase in bands number and intensity was particularly obvious for rat's liver fed on parsley seeds. On the other hand, all parsley forms increased the disappearance of normal band compared to negative control group and the other groups, except the rat group fed on parsley seeds which decreased the disappeared bands to one band. These results indicate that parsley seed components may interaction with gentamicin causing toxic effect.

DNA variation which was induced in spleen cells of rat groups fed on different forms of parsley was showed in Table (3). A total of 28 bands were amplified by four primers in the negative control group. Primers OP-A9 and OP-B7 gave the lowest (one bands) and the highest (ten bands) number of RAPD products, respectively. The positive control group was clearly different from the negative control group. It was clearly reflected by changes in spleen RAPD profiles as variation in band intensity (both increases and decreases), disappearance of bands, and appearance of new bands. Concerning the rat groups fed on parsley, extra bands generally appeared for all parsley groups whereas three bands were only present in the positive control profiles. In addition, an increase in band intensity was the major event arising in the patterns generated by the positive control group. Such a result well agrees with

the effect of gentamicin on the patterns of liver DNA as mentioned previously. The increase in band intensity which induced by gentamicin was decreased with all parsley forms except the parsley leaves extract was increased (17 bands). The highest decrease in band intensity was appeared in rat group fed on parsley oil. These results were in agreement with the above results of relative spleen weight which indicated that parsley oil showed the best effect against gentamicin effect. On the other hand, the number of lost RAPD bands was much increased after seeds application, exhibiting six bands. These results confirm results of DNA-RAPD profiles of liver that parsley seeds may interaction with gentamicin causing more toxic effect than other forms of parsley.

#### ***Genomic template stability***

Changes in the RAPD pattern of rat's liver and spleen were expressed as decrease in genomic template stability (GTS) in relation to the pattern showed in the negative control group. The genomic template stability of spleen was nearly equal as liver for positive control group and both of them showed similar variation tendency (Tables 2 and 3). GTS was decreased to 80.00% and 82.00% for liver and spleen, respectively. The gentamicin effect (positive control) was ascribed to appearance of new bands and disappearance of normal bands, which tend to counterbalance each other in both liver and spleen RAPD profiles. In other words, the disappeared band was compensated by the

low frequency of newly appearing bands after gentamicin administration. The DNA variation; resulted from gentamicin in the liver and spleen, suggested that the amount of gentamicin reaching the liver or spleen could have been insufficient to produce an increase in the variations. In agreement with this suggestion, Tran Ba Huy and Deffrennes (1988) reported that the binding of gentamicin remained low and constant in the liver and spleen, regardless of the concentration of gentamicin added to the incubation medium. This suggested a nonspecific binding to these organs. It may also suggest a poor affinity of the binding sites of these organs for gentamicin.

Following application of the different parsley forms to rat groups injected with gentamicin, GTS values were changed to lower values for all parsley groups in comparison to positive control group, which indicated that GTS was significantly affected by parsley stress. Especially with application of parsley leaves extract for liver and fresh leaves for spleen, GTS values decreased to 50% and 46.43%, respectively. The obtained results showed that parsley leaves extract was the most effective against GTS value in liver, while the fresh leaves showed the most effect in spleen as compared to the other forms of parsley. Similar effect of reduction on GTS was reported for relative spleen weight with parsley application. Awe and Banjoko (2013) indicated that the leaf ethanol extract of *Petroselinum crispum* was hepatotoxic and nephrotoxic at continued oral doses equal to or more

than 1000 mg/kg, but no obvious toxicity when used at lower doses. In addition, Gazzani (1994) found that parsley showed weak antioxidant activity in groundnut oil under various heating conditions. On the other hand, obvious studies indicated that methanol extracts of parsley scavenge hydroxyl radical in addition to protecting against ascorbic acid-induced membrane oxidation (Fejes *et al.*, 2000). Furthermore, supplementation of diets with fresh parsley leaf can increase antioxidant capacity of rat plasma (Hempel *et al.*, 1999) and decrease oxidative stress in humans (Nielsen *et al.*, 1999). Campanella *et al.* (2003) reported that components of fresh parsley leaf scavenge superoxide anion *in vitro*.

The DNA variation detected by RAPD analysis reflect DNA alterations in genome ranged from single base changes (point mutations) to complex chromosomal rearrangements (Atienzar *et al.*, 1999 & 2002). The presence of the above types of DNA lesions and mutations may significantly affect the chemical reaction of PCR events (Bowditch, 1993). As suggested by Liu *et al.* (2005), appearance of new PCR products occurred because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural change or because some changes in DNA sequence have occurred due to mutations (resulting in new annealing events), and/or large deletions (bringing two pre-existing annealing sites closer), and/or homologous recombination (two sequences that match the sequence of the primer) (Atienzar *et al.*, 1999). Appar-

ent 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 *et al.*, 1999). On the other hand, modifications of band intensity and lost bands are likely to be due to one or a combination of the following events: (1) changes in oligonucleotide priming sites due mainly to genomic rearrangements and less likely to point mutations; (2) DNA damage in the primer binding sites; (3) interactions of DNA polymerase in test organism with damaged DNA.

These findings agree well with the observations of Du and Yang (1994), they observed that the activity and DNA synthesis were suppressed by the addition of gentamicin in suspended proximal tubules. Shugart and Theodorakis (1994) revealed that the genotoxic agents not only disrupt the integrity of the genome but also affect the expression of DNA directly or indirectly. These effects will lead to an increase in the incidence of different types of gene mutations and, on the long term; result in genetic variability of the exposed populations. Previous studies have also shown that changes in RAPD profiles induced by pollutants can be regarded as changes in genomic template stability (Atienzar *et al.*, 2000).

In conclusion, results indicated that genotoxic effect of gentamicin was induced mildly on rat's liver and spleen tissues at the dose studied. Meanwhile, the administration of rats with parsley revealed that parsley oil may be effective

form in reducing gentamicin genotoxic effect followed by parsley fresh leaves.

### SUMMARY

The present study was conducted to determine the protective effect of parsley (leaves extract, fresh leaves, seeds and oil) on rat injected intraperitoneally with gentamicin at a dose of 10 mg/kg b.wt. The toxic effects of gentamicin were assessed in term of the changes in relative organs weights in addition the genetic variation in DNA-RAPD profiles in both liver and spleen. Results showed that no significant differences were found between the negative control and all of tested groups for relative liver weights, while the group fed on parsley oil recorded the highest value of relative spleen weight which did not differ than negative control group. For RAPD profiles, the differences in RAPD patterns refer to band intensity, loss of normal bands and appearance of new bands as compared with the negative control group. All these differences were well represented in the patterns produced in gentamicin group as well as all rat groups fed on parsley. Changes in bands intensity which were major event increased by gentamicin effect, were decreased in all groups fed on parsley forms except the group fed on seeds (for liver DNA) and the group fed on parsley leaves extract (for spleen DNA) were increased. Parsley leaves and oil (for liver) and parsley oil (for spleen) exhibited the best effect in reducing the increase in band intensity.

All forms of parsley caused a decrease in the genomic template stability (%) values compared to gentamicin group. The major decrease was for leaves extract with regard to liver DNA and for seeds with regard to spleen DNA. Results of this study demonstrated the genotoxic effects of gentamicin on rat's liver and spleen. In addition, the results revealed that parsley oil may be the effective form in reducing gentamicin genotoxic effect followed by parsley fresh leaves.

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Table (1): Changes in relative organ weight of rats fed on different parsley forms; leaves extract (Ex), fresh leaves (L), seeds (S) and oil (O).

Rats groups	Relative liver weight (%)	Relative spleen weight (%)
Negative control	3.99 ± 0.53	0.45 ± 0.44 <sup>a</sup>
Positive control	3.66 ± 0.37	0.33 ± 0.11 <sup>b</sup>
Ex	3.11 ± 0.52	0.37 ± 0.09 <sup>ab</sup>
L	3.32 ± 0.30	0.35 ± 0.12 <sup>ab</sup>
S	3.41 ± 0.12	0.38 ± 0.10 <sup>ab</sup>
O	3.71 ± 0.14	0.39 ± 0.06 <sup>a</sup>

Means having the same superscript letter(s) in the same column are statistically insignificant ( $p < 0.05$ ).

Table (2): Changes in DNA-RAPD profile of rat's liver fed on different parsley forms; leaves extract (Ex), fresh leaves (L), seeds (S) and oil (O).

Primers	No. of bands in negative control	Positive control				Ex				L				S				O			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
OPA9	3	1	2	1	0	1	1	1	0	0	1	0	2	1	1	1	1	1	1	1	0
OPB5	8	0	0	8	0	1	0	6	0	1	0	3	4	1	0	3	1	1	0	4	0
OPB7	2	0	0	0	2	0	0	0	1	0	0	0	2	0	0	0	2	0	0	0	1
OPB8	7	1	0	0	0	0	7	0	0	0	7	0	0	4	0	7	0	0	6	0	0
Total	20	2	2	9	2	2	8	7	1	1	8	3	8	6	1	11	4	2	7	5	1
a+b		4				10				9				7				9			
GTS %		80.00				50.00				55.00				65.00				55.00			

a: appearance of new band, b: disappearance of normal band, c: increase in band intensity, d: decrease in band intensity, a+b: polymorphic bands, GTS: genomic template stability.

Table (3): Changes in DNA-RAPD profile of rat's spleen fed on different parsley forms; leaves extract (Ex), fresh leaves (L), seeds (S) and oil (O).

Primers	No. of bands in negative control	Positive control				Ex				L				S				O			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
OPA9	1	2	0	1	0	2	0	1	0	3	0	1	0	1	0	1	0	1	0	0	0
OPB5	9	1	1	7	0	2	2	7	0	5	2	1	3	0	3	3	1	2	2	2	1
OPB7	10	0	1	6	0	0	1	3	0	1	0	7	0	1	2	1	0	0	3	0	0
OPB8	8	0	0	0	1	6	0	6	1	4	0	3	1	6	1	7	0	4	0	3	1
Total	28	3	2	14	1	10	3	17	1	13	2	12	4	8	6	12	1	7	5	5	2
a+b		5				13				15				14				12			
GTS %		82.14				53.57				46.43				50.00				57.14			

a: appearance of new band, b: disappearance of normal band, c: increase in band intensity, d: decrease in band intensity, a+b: polymorphic bands, GTS: genomic template stability.

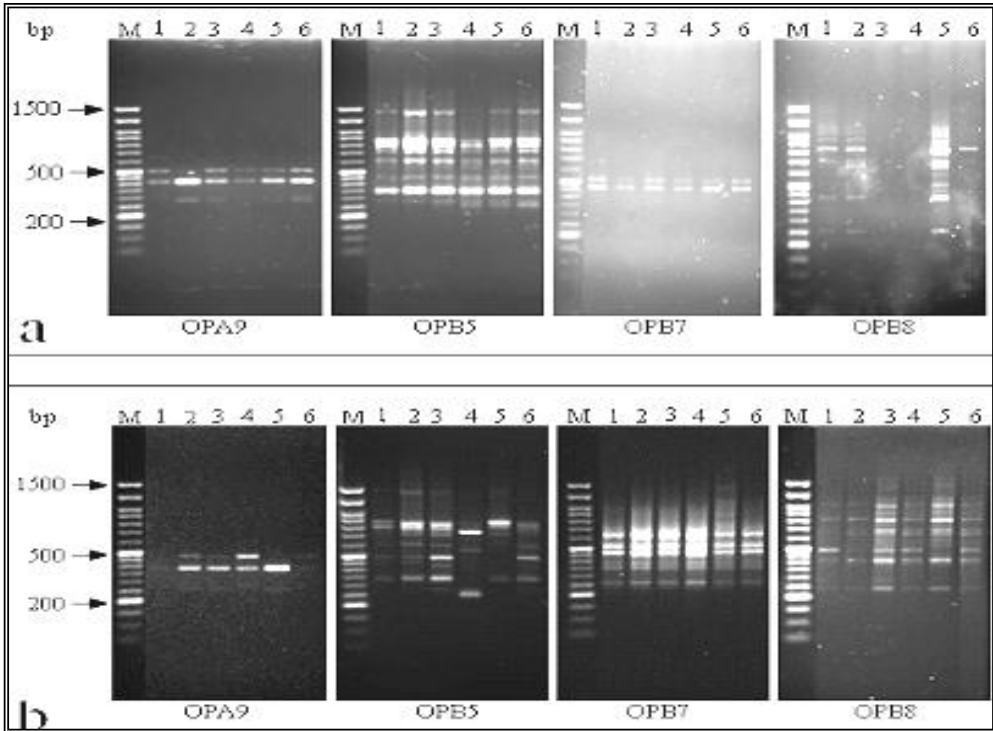


Fig. (1): RAPD profiles of genomic DNA from rat's liver (a) and spleen (b) fed on different parsley forms using primers OP-A9, OP-B5, OP-B7 and OP-B8. Lane M: DNA marker, lane 1: negative control, lane 2: positive control, lanes 3, 4, 5 and 6: rat groups fed on parsley leaves extract, fresh leaves, seeds and oil, respectively.